VOLUNTARY BINGE-LIKE ETHANOL CONSUMPTION SITE-SPECIFICALLY INCREASES C-FOS IMMUNOEXPRESSION IN MALE C57BL6/J MICE.

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ABSTRACT

Nathan William Burnham: Voluntary binge-like ethanol consumption site-specifically increases c-Fos immunoexpression in male C57BL6/J mice. (Under the direction of Todd E. Thiele)

Forced-ethanol exposure, via intraperitoneal injection (i.p.) or gavage, is commonly used in combination with c-Fos quantification to assess binge-like neuronal activity. Voluntary binge-like ethanol consumption-induced neuronal activity is less understood. Using the "drinking-in-the-dark" (DID) paradigm, we assessed the effects of binge-like ethanol consumption on c-Fos immunoreactivity (IR) in brain regions implicated in ethanol intake and compared this to i.p. ethanol-induced c-Fos. To this end, male C57BL/6J mice were divided into four groups: DID-EtOH (20% v/v), DID-water, i.p.-EtOH (1.5 g/kg), or i.p.-saline (15 mL/kg) and experienced one 4-day DID cycle or a single i.p. injection, respectively, prior to sacrifice. Relative to controls, EtOH groups significantly increased c-Fos IR in several brain regions implicated in neurobiological responses to ethanol. Differences in DID-EtOH and i.p.-EtOH c-Fos were not observed across brain regions examined. These results provide insight into the brain regions that modulate binge-like ethanol intake stemming from DID procedures.

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LIST OF ABBREVIATIONS

A2	A2 region of the NTS
AcbC	Nucleus accumbens core
ANOVA	Analysis of Variance
AP	Anterior/posterior
BEC	Blood ethanol concentration
BLA	Basolateral Amygdala
BNST	Bed Nucleus of the Stria Terminalis
CeA	Central Amygdala
CeMPV	Medial Posterioventral portion of the Central Amygdala
CRF	Corticotropin-Releasing Factor
DID	Drinking-in-the-Dark paradigm
DV	Dorsal/Ventral
dBNST	Dorsal Bed Nucleus of the Stria Terminalis
EtOH	Ethanol
EW	Edinger-Westphal Nucleus
Fig	Figure
g/kg	Gram per kilogram
i.p.	Intraperitoneal
IR	Immunoreactivity
ITF	Inducible Transcription Factor
LC	Locus Coeruleus
LH	Lateral Hypothalamus

LPBn	Lateral Parabrachial Nucleus
µl	microliter
mg/dl	Milligram per deciliter
ML	Medial/lateral
mPVN	Magnocellular Paraventricular Nuclei
mRNA	Messenger Ribonucleic Acid
NPY	Neuropeptide-Y
NTS	Nucleus of the Solitary Tract
PFA	Paraformaldehyde
PVA	Paraventricular Nucleus of the Thalamus, anterior
PVN	Paraventricular Nucleus of the Hypothalamus
PVT	Paraventricular Nucleus of the Thalamus
TH	Tyrosine Hydroxylase
TRPV1	Transient Receptor Potential Channel Vanilloid receptor 1
vBNST	Ventral Bed Nucleus of the Stria Terminalis
VTA	Ventral Tegmental Area
v/v	Volume/volume

CHAPTER 1: INTRODUCTION

The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines a binge episode as a 2 hour period in which an individual consumes enough alcohol to elicit blood ethanol concentrations (BECs) exceeding 0.08% (80 mg/dL) (NIAAA, 2004). Previous estimates suggest binge ethanol drinking is the primary route of alcohol consumption for US individuals under the age of 21, though the majority (70%) of US binge episodes are attributable to individuals over the age of 26 (Naimi et al., 2003). Unsurprisingly, researchers have linked regular binge drinking to numerous short-term consequences, such as accidental injury (Gmel et al., 2006) and long-term consequences including heart disease, high blood pressure, and type-2 diabetes (Fan et al., 2008). Furthermore, individuals who binge drink early in life are at greater risk of developing alcohol dependence later in life (Miller et al., 2007, Hingson et al., 2005, Rubinsky et al., 2010). Accordingly, identification of neurochemical pathways that modulate binge drinking is of utmost priority as such knowledge could prove beneficial in determining pharmaceutical treatments for binge-behavior prevention.

Animal Models of Binge Ethanol Induction

Many existing models of binge drinking in rodents involve forced exposure techniques, such as intragastric gavage and intraperitoneal administration, though these methods inherently fail to model voluntary consumption observed in humans. Recently, researchers have adopted a preclinical model of voluntary binge-like ethanol consumption

termed “drinking-in-the-dark” (DID), a 4-day paradigm involving C57BL/6J mice that has been demonstrated to promote high levels of consumption and reliably generate BECs exceeding 80 mg/dL (Rhodes et al., 2005, Rhodes et al., 2007, Thiele and Navarro, 2014). Using this paradigm, researchers have shown that ethanol recruits a variety of neurochemical systems (Sprow and Thiele, 2012). Furthermore, several brain regions have been implicated in modulating binge-like ethanol intake. For example, in response to binge-like ethanol drinking CRF and NPY levels are increased or decreased, respectively, in the bed nucleus of the stria terminalis (BNST) (Pleil et al., 2015), 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).

C-Fos as a Measure of Forced-Binge Ethanol-Induced Activity

Numerous studies have measured neuronal activation following ethanol administration via quantification of inducible transcription factors (ITFs), such as c-Fos. Assessment 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 intraperitoneal administration techniques. For example, 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 intraperitoneally (i.p.) increases c-Fos IR in the nucleus of the solitary tract (NTS) (Thiele et al., 2000), Edinger-Westphal

nucleus (EW) (Turek and Ryabinin, 2005), and the paraventricular nucleus of the hypothalamus (PVN), CeA, dBNST, and EW (Knapp et al., 2001).

C-Fos Induction Following Voluntary Ethanol Intake

Assessment of c-Fos induction following voluntary ethanol consumption in limited-access consumption or operant paradigms (Bachtell et al., 1999, Weitemier et al., 2001, Sharpe et al., 2005) or chronic consumption in two-bottle choice paradigms (Li et al., 2010, Sajja and Rahman, 2013) indicate that voluntary consumption can region-specifically increase c-Fos expression. One notable previous study examined c-Fos IR following limited-access consumption in mice that generated BECs that exceeded binge-like levels (i.e., greater than 80 mg/dL). Mice drank ~2.9 g/kg of ethanol from a 10% ethanol/10% sucrose solution and achieved BECs of ~250 mg/dL over a 30 minute test that began 2.5 hours into the dark cycle (Ryabinin et al., 2003). However, c-Fos IR resulting from binge-like ethanol drinking stemming specifically from DID procedures has not been examined. 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, including noradrenergic brainstem structures, extended amygdaloid structures [BNST, CeA, & basolateral amygdala (BLA)], the LH, and the EW. To this end, c-Fos synthesized in response to voluntary binge-like ethanol consumption was compared to voluntary water consumption, as well as forced binge-like ethanol exposure via i.p. injection of ethanol. Finally, we assessed tyrosine hydroxylase (TH)/c-Fos co-expression in the LC and A2 nucleus of the NTS 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.

CHAPTER 2: METHODS

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 hr reverse light/dark cycle with lights off at 0830 hr. 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.

Surgery and Recovery

Prior to surgery, mice were weighed and anesthetized using a ketamine (66.7 mg/kg; Henry Schein, Dublin, OH) and xylazine (6.67 mg/kg) cocktail administered by i.p. injection. Mice received 0.1 mL 1% Lidocaine HCl (Hospira, Inc., Lake Forest, IL) subcutaneously above the skull. Mice received unilateral injections of green and red Retrobeads (0.2 – 0.3 µL/site; Lumafluor, Durham, NC) aimed at ipsilateral BNST (AP +0.15, ML -1.00, DV -4.8; no angle) and VTA (AP -3.1, ML -0.50, DV -4.6; no angle) structures via Hamilton syringe. Following surgery conclusion, animals received topical analgesic cream containing Lidocaine and Prilocaine (25mg/g of each; Akorn Pharmaceuticals, Gurnee, IL). Subjects recovered for two weeks before undergoing testing. However, the size and spread of RetroBead injection sites proved highly variable across animals, complicating quantification

and interpretation of pathway involvement. Accordingly, RetroBead labeling analyses were excluded.

Voluntary consumption: "Drinking-in-the-Dark" Procedure

A four-day drinking-in-the-dark (DID) paradigm was used. Briefly, animal weights were collected 30 minutes prior to homecage water bottle removal. Beginning three hours 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) or tap water (n = 15/group). 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 immediately after ethanol access and analyzed via an alcohol analyzer (Analox Instruments, Lunenburg, MA) to assess BECs. Animals were then perfused with 4% paraformaldehyde.

Forced-binge Episode Induction

The remaining mice (n=20/50) were randomly divided into two groups of 10, with one group receiving a single i.p. injection of 0.9% saline (15 mL/kg) and the other receiving a 1.5 g/kg dose of ethanol (13% v/v; 15 mL/kg). For this experiment, animals were not habituated to injection procedures to avoid potential sensitization of stress responses associated with repeated handling and injection. Like the mice used in DID procedures, these mice were single-housed from arrival until sacrifice. On injection day, mice were removed from home cages, injected with either saline or ethanol at the time corresponding to DID onset for animals in the voluntary consumption group, and then placed back into home cages

for two hours. Mice receiving i.p. injections were perfused two hours post-injection, mimicking the duration of ethanol exposure mice received in the DID cohort.

Immunohistochemistry

Mice were transcardially perfused with phosphate buffered saline (PBS) for 3 minutes followed immediately by 4% paraformaldehyde (PFA) for 7 minutes using a Masterflex L/S perfusion pump (catalogue #7200-12, Cole-Parmer, Vernon Hills, IL). Brains were collected and postfixed in approximately 15 mL 4% PFA for 24 h. Next, brains were cryoprotected in approximately 15 mL PBS solution containing 20% sucrose and 0.01% sodium azide (catalogue #S2002, Sigma-Aldrich, St. Louis, MO). Brains were sectioned into 40 µm slices via vibratome (model VT1000 S, Leica Biosystems, Buffalo Grove, IL). Tissue was placed in cryopreserve and stored in a -20° freezer for later retrieval.

Sections underwent three 30 s rinses prior to overnight incubation in rabbit anti-cFos (catalogue #sc-52, lot# D0512, Santa Cruz Biotechnology, Dallas, TX), diluted 1:20,000 in PBS with 0.25% Triton-X (Fisher Scientific, Waltham, MA) and 0.01% sodium azide. Following two 30 s rinses, sections incubated for 30 min in biotinylated donkey anti-rabbit secondary (1:1k; code #711-065-152, Jackson Immunoresearch, West Grove, PA) diluted 1:1000 in PBS. Sections underwent four additional rinses before incubating for 1 h in Vectastain ABC kit (Vector Laboratories, Burlingame, CA) diluted 1:1000 in PBS. Sections rinsed twice before exposure to PBS containing 0.05% diaminobenzidine tetrahydrochloride hydrate (DAB; catalogue #D5637, Sigma-Aldrich, St. Louis, MO), 0.05% ammonium nickel (II) sulfate hexahydrate (catalogue #A-1827, Sigma-Aldrich), and 0.01% hydrogen peroxide. The stain was terminated via rinses in PBS containing 0.01% sodium azide. Next, were placed into a 1:20,000 dilution of sheep anti-tyrosine hydroxylase (TH; catalogue #AB1542,

EMD Millipore, Billerica, MA). TH staining procedures were identical to c-Fos staining with the exceptions that the secondary step utilized donkey anti-sheep (1:1k; code #713-065-147, Jackson Immunoresearch) and nickel was omitted during the DAB step.

Data Analyses

Among the 15 mice receiving ethanol in the DID procedure, 8 (53%) exceeded the criteria for a binge episode (80 mg/dl) with a mean consumption ethanol consumption of 3.98 ± 0.47 g/kg and BEC of 121.63 ± 27.57 mg/dl. The 7 mice who failed to binge (consumption: 3.00 ± 0.77 g/kg; BEC: 40.23 ± 24.04 mg/d) were excluded from further analyses as our objective was to assess c-Fos expression in response to binge-like levels of ethanol consumption. Failed immunostaining missing or partial sections further limited the number of animals in subsets of analyses. Counts represent the average of bilateral IR within 2 – 3 tissue slices per brain region for each animal. Brain region identification was based upon coordinates indicated in The Mouse Brain in Stereotaxic Coordinates atlas (Franklin and Paxinos, 1997). Slice were matched to images in the atlas corresponding to AP position relative to bregma (dBNST & vBNST: +0.38, +0.26, +0.14; CeA & BLA: -1.06, -1.34, -1.58; PVA (paraventricular nucleus of the thalamus, anterior): -0.22, -0.46, -0.58; PVT (paraventricular nucleus of the thalamus): -1.22, -1.46, -1.82; EW: -3.4, -3.64, -3.8; LH (lateral hypothalamus): -1.22, -1.34, -1.46; LC: -5.34, -5.4, -5.52; lPBn: -5.2, -5.34; A2: -7.32, -7.48, -7.56).

Brain regions from one animal (mouse 1) in the DID-water cohort were photomicrographed and encircled in Adobe Illustrator (San Jose, CA) based upon boundaries established in the brain atlas at each level of AP listed above. To maintain consistent boundary identification of brain regions across animals, experimenter-encircled boundaries

from mouse 1 were overlayed with all other mice. Differences in IR within a given brain region were assessed via 2 (route of administration) x 2 (solution) multivariate ANOVA, and t-tests were used for planned comparisons. All counts were performed by an individual blind to experimental conditions and are labeled as mean ± standard error. Analyses were performed in SPSS (SPSS Inc., Chicago, IL) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

CHAPTER 3: RESULTS

Ethanol consumption and BECs

With DID procedures mice (n = 8) drank 3.98 ± 0.47 g/kg of ethanol and had BECs of 121.63 ± 27.57 mg/dl. While tail blood samples were not collected prior to perfusion for i.p.-injected mice used to assess c-Fos IR, a follow-up study with male C57BL/6J mice (n = 9) confirmed that an i.p. injection of a 1.5 g/kg dose of ethanol (13% v/v) produced BECs 2-h post injection (100.23 ± 44.54 mg/dl) that were similar to BECs achieved by binge drinking mice. A t-test comparing BECs between DID and i.p. injected mice failed to achieve significance [$t(15)=1.172$, $p=0.26$].

Ethanol-induced c-Fos IR

Fig. 1A shows the average number of c-Fos positive nuclei per brain region following binge-like ethanol drinking (DID) versus water consumption, and **Fig. 1B** shows the average number of c-Fos-positive nuclei per brain region following a single i.p. injection of a 1.5 g/kg dose of ethanol dose versus the group given i.p. injection of saline. Photomicrographs from brain regions exhibiting significant increases of ethanol-induced c-Fos IR for the DID and i.p. injection procedures are presented in **Figs. 2 and 3**, respectively. **Table 1** shows the statistical results from a 2 (route of administration) x 2 (solution) multivariate ANOVA that assessed differences in IR within in the brain regions examined. There we several significant main effects of route of administration, reflecting great overall c-Fos IR associated with the i.p. injection procedure in the BLA (DID = 36.83 $\pm$ 5.73; i.p. = 62.89 $\pm$ 4.18), EW (DID =

17.83 ± 2.18; i.p. = 25.63 ± 1.59), lPBn (DID = 24.58 ± 10.56; i.p. = 69.93 ± 7.71), and A2 (DID = 5.75 ± 3.39; i.p. = 25.36 ± 2.47), and overall great c-Fos IR associated with DID procedure in the LH (DID = 76.75 ± 5.43; i.p. = 50.43 ± 3.96) and LC (DID = 8.42 ± 1.13; i.p. = 5.02 ± 0.82). There were also several significant main effects of solution, reflecting great overall c-Fos IR associated with ethanol exposure relative to vehicle in the dBNST (ethanol = 69.55 ± 5.40; vehicle = 36.38 ± 4.95), vBNST (ethanol = 47.69 ± 3.00; vehicle = 28.03 ± 2.75), CeA (ethanol = 41.39 ± 5.54; vehicle = 23.19 ± 5.08), BLA (ethanol = 62.53 ± 5.23; vehicle = 37.20 ± 4.79), LH (ethanol = 73.08 ± 4.96; vehicle = 54.10 ± 4.55), EW (ethanol = 34.25 ± 1.99; vehicle = 9.22 ± 1.83), LC (ethanol = 9.92 ± 1.03; vehicle = 3.52 ± 0.95), and A2 (ethanol = 24.31 ± 3.09; vehicle = 6.8 ± 2.83). Interestingly, there were no significant interaction effects between the route of administration and solution main effects, suggesting that increased c-Fos IR stemming from ethanol exposure did not significantly depend on the route of administration.

Planned comparisons of ethanol-induced c-Fos IR in brain regions examined

Because we were interested in making direct comparisons of c-Fos IR between ethanol and vehicle groups we performed plan comparisons at each brain region examined with DID procedures (**Fig. 1A**) and i.p. injection procedures (**Fig. 1B**). T-tests showed that, relative to water controls, voluntary binge-like ethanol drinking significantly increased c-Fos labeling in dBNST [$t(11) = 4.11$, $p = 0.002$], vBNST [$t(11) = 2.22$, $p=0.048$], CeA [$t(9) = 5.45$, $p = 0.0004$], PVT [$t(12) = 2.76$, $p = 0.017$], LH [$t(6) = 1.59$, $p = 0.032$], EW [$t(14) = 8.25$, $p < 0.0001$], LC [$t(15) = 3.30$, $p = 0.005$], and the A2 [$t(12) = 31.48$, $p < 0.0001$]. No significant differences were detected in the BLA [$t(9) = 0.90$, $p = 0.39$], PVA [$t(12) = 0.50$, $p = 0.62$], or lPBn [$t(6) = 1.71$, $p = 0.138$]. Double immunohistochemistry indicated that

approximately 35-40% of A2 TH-positive neurons co-expressed c-Fos in DID binge animals, whereas approximately 3-5% of TH-positive A2 neurons co-expressed c-Fos in water drinking controls. Additionally, approximately 40-50% of LC TH-positive cells co-expressed c-Fos in DID binge animals, whereas approximately 5-10% of TH-positive LC neurons co-expressed c-Fos in water drinking controls.

T-tests indicated that an ethanol injection produced significant increases of c-Fos IR, relative to saline-injected controls, in the dBNST [$t(13) = 4.88$, $p < 0.001$], vBNST [$t(13) = 4.20$, $p = 0.001$], CeA [$t(12) = 3.74$, $p = 0.003$], BLA [$t(12) = 2.98$, $p = 0.011$], PVT [$t(13) = 3.33$, $p = 0.005$], LH [$(11) = 3.31$, $p = 0.007$], EW [$t(15) = 10.90$, $p < 0.001$], LC [$t(15) = 4.15$, $p < 0.001$], and the A2 [$t(15) = 5.81$, $p < 0.001$]. No significant changes in c-Fos IR were detected in the PVA [$t(13) = 1.77$, $p = 0.100$] or the lPBn [$t(11) = 1.58$, $p = 0.142$]. Approximately 60-70% of TH-positive A2 neurons co-expressed c-Fos in ethanol-injected animals. Conversely, nearly 15% of TH-positive A2 neurons co-expressed c-Fos in saline-injected animals. Similarly, about 60-70% of TH-positive LC neurons co-expressed c-Fos in ethanol-injected mice, whereas about 10-20% of TH-positive LC neurons co-expressed c-Fos in saline-injected mice.

CHAPTER 4: DISCUSSION

Importantly, voluntary binge-like ethanol consumption and i.p. injection of a 1.5 g/kg dose of ethanol were associated with similar BECs allowed us to make meaningful comparisons of c-Fos expression across brain regions and conditions. When brains were collected immediately after ethanol drinking on day 4 of the DID test, elevated c-Fos IR was observed in mice that voluntarily binged relative to the water drinking control group. Specifically, mice that voluntarily binged displayed elevated c-Fos IR in areas of the extended amygdala (dBNST, vBNST, CeA), a major relay center from midbrain to cortex in the PVT (Kelley et al., 2005), EW, LC, and the A2 region of the NTS (Fig. 1A). Interestingly, these same brain regions showed ethanol-induced c-Fos IR in mice given forced i.p. ethanol injections, though there was additionally a significant elevation of c-Fos IR in the BLA of ethanol-injected mice (Fig. 1B), which was not evident in mice engaging in binge-like ethanol consumption. Importantly, the absence of significant interaction effects between the route of administration and solution main effects suggests that increased c-Fos IR stemming from ethanol exposure did not significantly depend on the route of administration.

There are caveats that need to be addressed. First, though BECs at sacrifice did not differ between the different route of administration procedures, the onset of peak BEC likely differed between procedures. Further, the DID procedure was associated with 4-days of ethanol exposure while i.p. injection of ethanol occurred once. Though we cannot completely

rule out the potential influence of these procedural differences on our c-Fos IR data, these concerns are somewhat tempered by the observation that ethanol-induced c-Fos IR did not differ significantly between routes of administration. An additional concern is that mice underwent cranial surgery for placement of Retrobeads into the BNST and VTA. While we cannot rule out the possibility that prior surgery may have influence subsequent effects of ethanol on c-Fos IR, many studies that use DID procedures involve prior surgery before consumption studies, and thus our study is particularly informative for studies the incorporate surgery.

In 4 of the brain regions examined (the BLA, EW, lPBn, and A2) overall c-Fos IR was greater following i.p. relative to DID procedures. The transient receptor potential channel vanilloid receptor 1 (TRPV1) is a cation channel responsible for detection and transduction of noxious chemical and thermal stimuli (Ellingson et al., 2009, Caterina et al., 1997, Szallasi and Blumberg, 2007). TRPV1 is found in the peritoneum, where i.p. injections of capsaicin stimulate TRPV1 and subsequent abdominal nociception (Trevisan et al., 2013). It is possible that activation of the TRPV1 system by injection of ethanol and saline contributed to the increased c-Fos IR in the BLA, EW, lPBn and A2. Consistent with this idea, intraperitoneally-administered hypertonic saline has been found to increase c-Fos protein (Ceccatelli et al., 1989) and mRNA (Sharp et al., 1991) in several brain regions. Additionally, potential induction of stress stemming from pain and/or the involuntary nature of i.p. injection could have also played a role. However, that fact that we observed overall greater levels of c-Fos IR in the LH and LC with DID, relative to i.p., procedures, and nodifferences in c-Fos IR on other regions examined, suggest that potential contributions of pain and/or stress to i.p. injection-induced c-Fos IR are brain-region specific.

Previous studies have shown that voluntary ethanol consumption in limited access sessions, wherein water-restricted C57BL6/J mice were allotted 30 min to consume either 10% ethanol (2.5 hr into dark cycle; Sharpe et al., 2005) or 10% ethanol/10% sucrose (Bachtell et al., 1999) solutions, increased c-Fos IR in the nucleus accumbens core (AcbC), the medial posterioventral portion of the central nucleus of the amygdala (CeMPV; Bachtell et al., 1999), and the EW (Bachtell et al., 1999, Sharpe et al., 2005, Weitemier et al., 2001). Similarly, we found that a single binge-like drinking cycle with DID procedures increased c-Fos IR in the CeA and EW, though our results indicate a DID-elicited binge-like episode can increase c-Fos IR in regions, such as the BNST and PVT, which were not detected with 30 min limited access procedures (Sharpe et al., 2005). In combination with chronic voluntary ethanol consumption using 24-h, two-bottle choice procedures, where ethanol consumption increased FosB presence in the AcbC, dorsolateral striatum, orbitofrontal cortex (Li et al., 2010), and ventral striatum (Sajja and Rahman, 2013), our results expand our understanding of how voluntary ethanol consumption, and more specifically, voluntary binge-like ethanol consumption, recruits that activity of various brain regions.

Interestingly, in a study with C57BL/6J mice that used a 10% ethanol/10% sucrose solution which promoted binge-like drinking (~2.9 g/kg of ethanol) and associated BECs (~250 mg/dL) over a 30 minute test, there was no evidence of increased c-Fos IR in areas of the amygdala and reduced c-Fos IR in the LH relative to water dining mice, both observations that are inconsistent with our findings. Similar to our results though, they did find increased c-Fos IR in the EW (Ryabinin et al., 2003). Numerous procedure differences between the studies described above reinforcing the idea that ethanol-induced c-Fos IR is

procedure specific and highlight the novelty of the present work which reveals neuronal activity specific to DID procedures.

In assessing TH and c-Fos co-expression in noradrenergic stimuli (A2 and LC), we found that voluntary water consumption produced near-zero co-expression in both structures. Conversely, the percentage of co-expression was elevated among animals that binge drank ethanol (A2: 35-40%; LC: 40-50%). I.p. ethanol injections yielded a slightly greater co-expression pattern (A2: 60-70%; LC: 60-70%) than saline-injected animals. These expression patterns are in line with previously reported findings in male rats, where i.p. ethanol injections elicited ~35-60% and ~65-75% co-expression in the LC and A2, respectively (Thiele et al., 2000). The LC innervates numerous brain regions including the orbitofrontal cortex, medial prefrontal cortex, anterior cingulate, lateral hypothalamus, amygdala, and ventral tegmental area, among others (Chandler et al., 2014, Foote et al., 1983), while the A2 innervates the BNST, accumbens shell, lateral hypothalamus, and ventral tegmental area, among others (Delfs et al., 2000, Mejias-Aponte et al., 2009, Smith and Aston-Jones, 2008). Given the high levels of TH/c-Fos co-expression in A2 and LC, as well as increased c-Fos in regions innervated by the A2 and/or LC (lateral hypothalamus, BNST, amygdala), noradrenergic circuitry may play an important role in voluntary ethanol consumption. Accordingly, our lab is currently investigating possible involvement of noradrenergic circuitry in voluntary binge-like ethanol consumption.

An examination of our c-Fos IR data indicates that in many brain regions c-Fos IR was relatively high under baseline water drinking and saline injection conditions. An inherent aspect to the DID procedure is that behavioral assessment is performed during the dark cycle, when mice are more active and consume the majority of their daily food and water. In rats,

others have demonstrated that c-Fos mRNA expression oscillates depending on time of day, with increased c-Fos activity occurring at night and weak activity during rest hours (Grassi-Zucconi et al., 1993). Moreover, light/dark exposure can site-specifically influence c-Fos IR in the rat brain, with the "lights-on" phase increasing c-Fos presence in the suprachiasmatic nuclei (Aronin et al., 1990) and the "lights-off" phase increasing c-Fos IR in various regions including the PVT, magnocellular paraventricular nuclei (mPVN), and dorsomedial nuclei (Choi et al., 1998). Interestingly, in reviewing the forced binge-induced c-Fos literature, this work was almost exclusively conducted in the lights-on portion of rodent light cycles. Thus, higher-than-anticipated basal c-Fos IR in the control conditions of our studies may potentially be a product of collecting measures during the dark cycle.

Finally, we must note that our procedures were performed in males exclusively. Researchers have shown that adult female C57BL/6J mice voluntarily consume significantly more ethanol (g/kg) than male counterparts in intermittent 24-h access paradigms (Hwa et al., 2011), under conditions of food or water deprivation (Middaugh and Kelley, 1999), and in two-bottle choice paradigms with 2-h limited access sessions (Vetter-O'Hagen et al., 2009) or continuous access (Yoneyama et al., 2008). Thus, future studies should investigate potential sex differences in voluntary binge-induced neuronal activation.

Taken together, our results indicate that a single 4-day binge-like ethanol drinking session with DID procedures region-specifically activated c-Fos IR in brain areas implicated in neurobiological responses to ethanol and ethanol drinking. Binge-like ethanol drinking mice and mice given i.p. injection of ethanol displayed similar BECs, increasing our ability to make comparisons of c-Fos IR between the different ethanol exposure paradigms. DID and i.p. injection procedures increased c-Fos IR in similar regions, and the absence of significant

interaction effects between the route of administration and solution main effects suggest that increased c-Fos IR stemming from ethanol exposure did not significantly depend on the route of administration. This work provides insight for future studies aimed at identifying the brain regions and circuitry involved in modulation of binge-like ethanol drinking, particularly those employing DID procedures.

Table 1: Influence of Route of Administration, Solution, or Interaction of Route of Administration and Solution on c-Fos IR Across Brain Regions.

Source	Brain Region Quantified	df	F	P-Value
Route of Administration (DID or i.p.)	dBNST	1,8	2.077	0.188
	vBNST	1,8	0.396	0.547
	CeA	1,8	0.428	0.531
	BLA	1,8	13.469	**0.006**
	PVA	1,8	0.543	0.482
	PVT	1,8	0.405	0.542
	LH	1,8	15.312	**0.004**
	EW	1,8	8.322	**0.020**
	LC	1,8	5.874	**0.042**
	lPBn	1,8	12.026	**0.008**
	A2	1,8	21.851	**0.002**
Solution (Vehicle or Ethanol Binge)	dBNST	1,8	20.495	**0.002**
	vBNST	1,8	23.328	**0.001**
	CeA	1,8	5.847	**0.042**
	BLA	1,8	12.725	**0.007**
	PVA	1,8	1.564	0.246
	PVT	1,8	5.058	0.055
	LH	1,8	7.967	**0.022**
	EW	1,8	85.694	**< 0.001**
	LC	1,8	20.796	**0.002**
	lPBn	1,8	0.472	0.512
	A2	1,8	17.421	**0.003**
Interaction	dBNST	1,8	1.738	0.224
	vBNST	1,8	0.285	0.608
	CeA	1,8	1.172	0.310
	BLA	1,8	1.38	0.274
	PVA	1,8	0.346	0.573
	PVT	1,8	0.631	0.450
	LH	1,8	0.268	0.619
	EW	1,8	0.018	0.895
	LC	1,8	1.584	0.244
	lPBn	1,8	0.58	0.816
	A2	1,8	2.05	0.190

Bolded p-values indicate significance ($p < 0.05$). Regions analyzed were dBNST, dorsal bed nucleus of the stria terminalis; vBNST, ventral bed nucleus of the stria terminalis; CeA, central amygdala; BLA, basolateral amygdala; PVA, anterior paraventricular nucleus of the thalamus; PVT, paraventricular nucleus of the thalamus; LH, lateral hypothalamus; EW,

Edinger-Westphal nucleus; LC, locus coeruleus; lPBn, lateral parabrachial nucleus; A2, A2 nucleus of the nucleus tractus solitarus (NTS).

Figure 1: C-Fos immunoexpression following voluntary and forced binge episodes. A) Voluntary binge-like ethanol consumption (20% ethanol during 2 hr DID test day) significantly increases c-Fos immunoexpression in the dBNST, vBNST, CeA, PVT, LH, EW, LC, and A2 region of the NTS relative to water-consuming controls. B) 1.5 g/kg ethanol (13% v/v) delivered IP significantly increased c-Fos immunoexpression in dBNST, vBNST, CeA, BLA, PVT, LH, EW, LC and the A2 region of the NTS relative to saline-injected controls. All values are mean ± SEM. # signifies $p < 0.01$; * signifies $p < 0.05$.

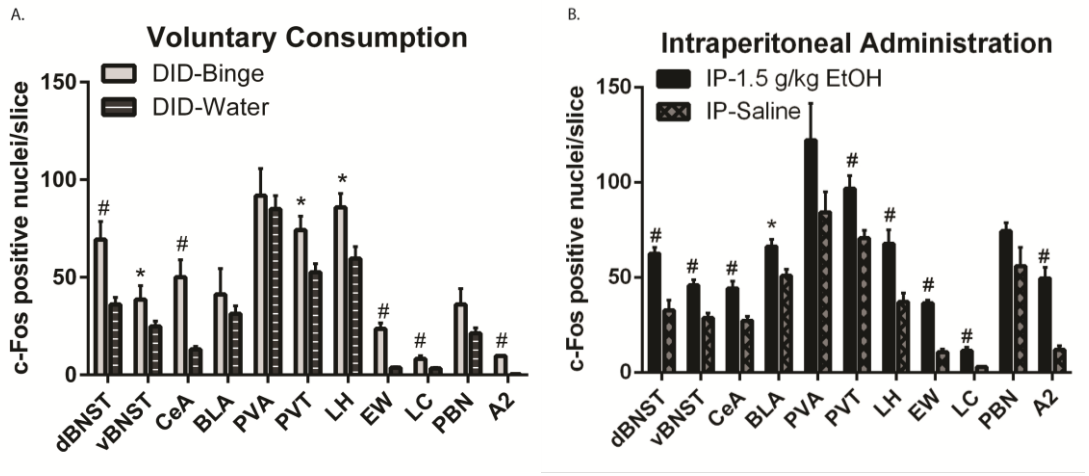


Figure 2: Representative photomicrographs from brain regions where significant c-Fos differences were observed between animals voluntarily binging or consuming water. A) dorsal bed nucleus of the stria terminals (dBNST); B) Ventral bed nucleus of the stria terminalis (vBNST); C) Central amygdala (CeA); D) Paraventricular nucleus of the thalamus (PVT); E) Lateral hypothalamus (LH); F) Edinger-Westphal nucleus (EW); G) Locus coeruleus (LC); H) A2 region of the NTS. In A-F, outlined areas represent approximate regions of c-Fos IR quantification. A-F imaged at 20x whereas G-H imaged at 40x, with scale bars denoting 0.1 mm. Representative c-Fos and TH co-expression indicated by black arrowheads.

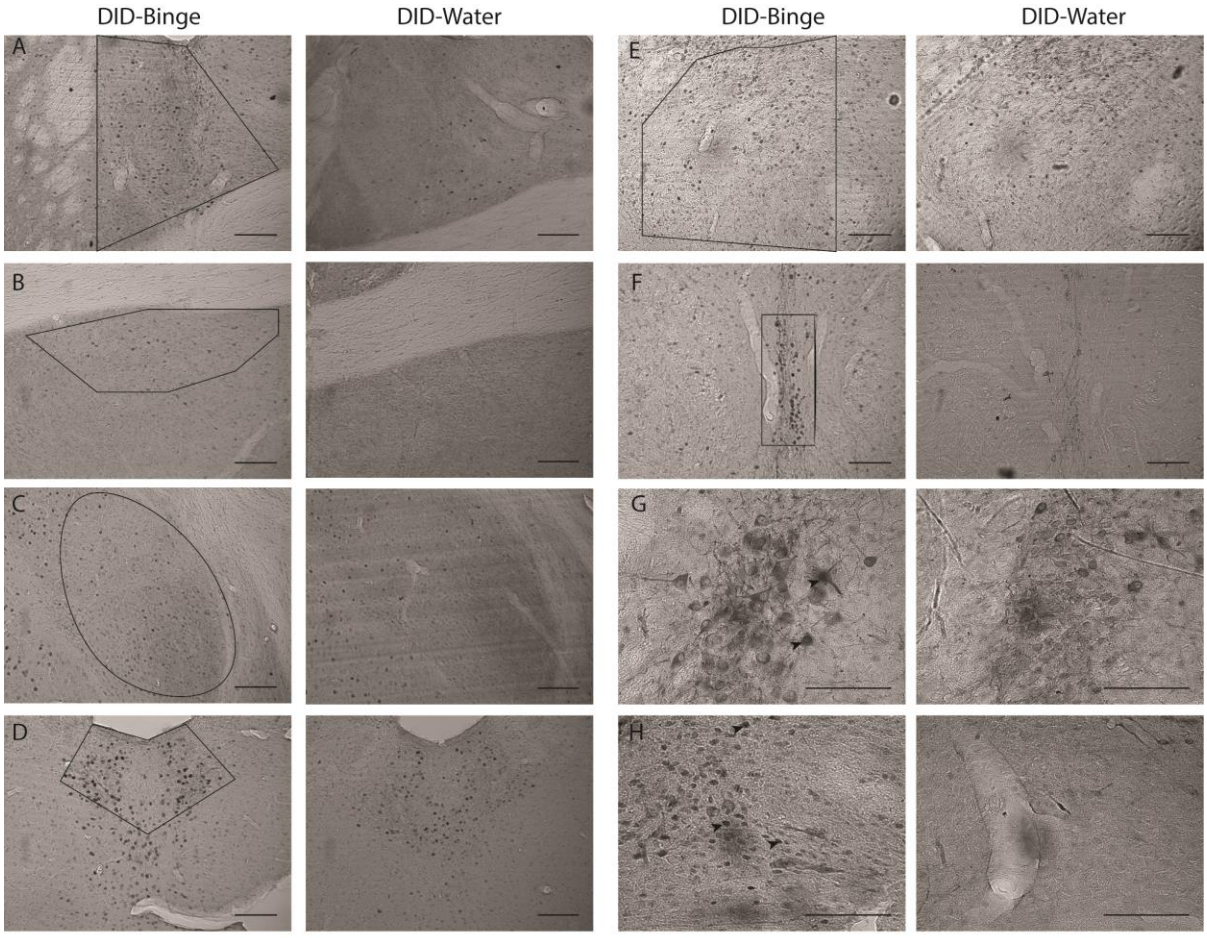
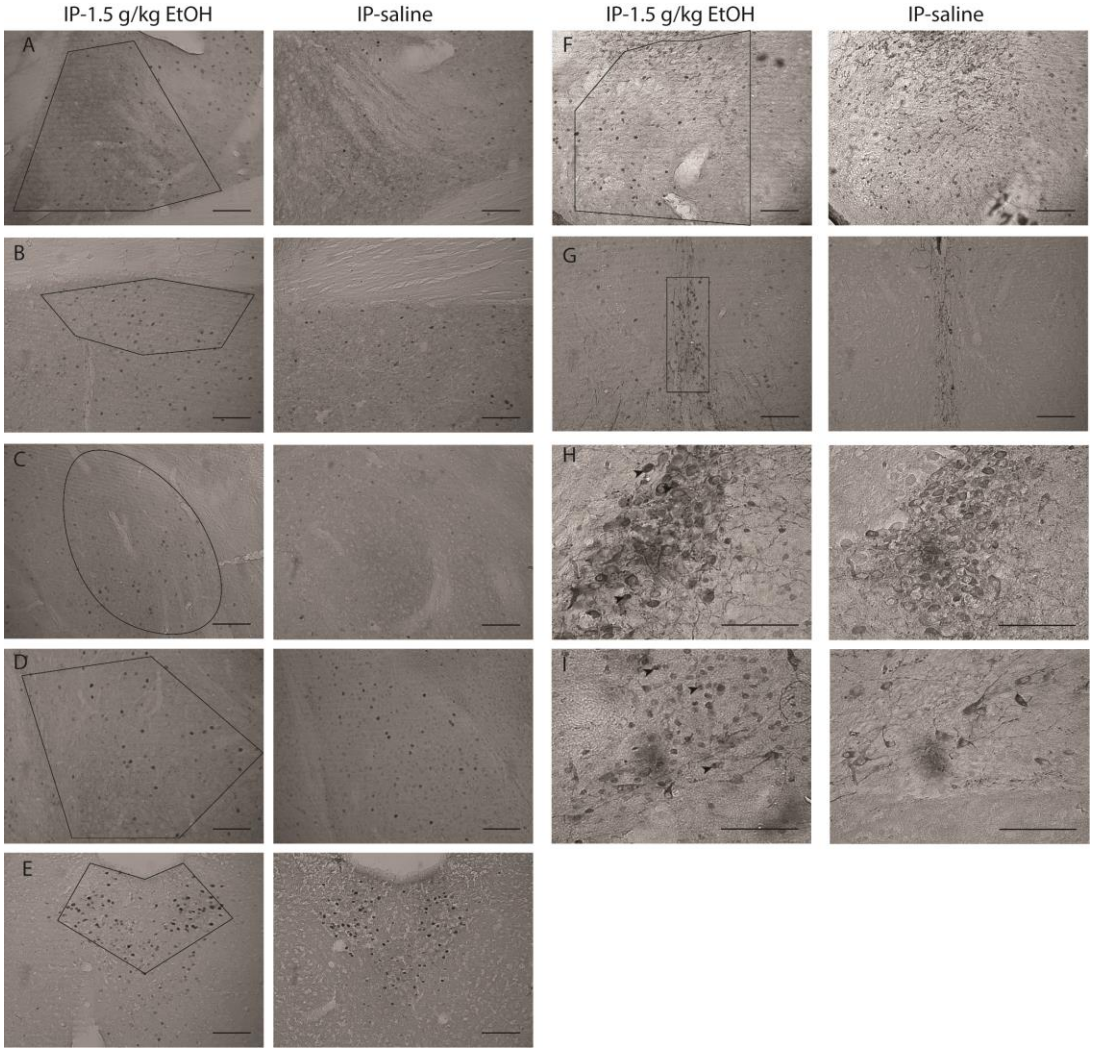


Figure 3: Representative photomicrographs from brain regions where significant c-Fos differences were observed in animals receiving a forced binge (i.p. ethanol) or saline injection. A) dorsal bed nucleus of the stria terminals (dBNST); B) Ventral bed nucleus of the stria terminalis (vBNST); C) Central amygdala (CeA); D) Basolateral amygdala (BLA); E) Paraventricular nucleus of the thalamus (PVT); F) Lateral hypothalamus (LH); G) Edinger-Westphal nucleus (EW); H) Locus coeruleus (LC); I) A2 region of the NTS. In A-G, outlined areas represent approximate regions of c-Fos IR quantification. A-G imaged at 20x whereas H-I imaged at 40x, with scale bars denoting 0.1 mm. Representative c-Fos and TH co-expression indicated by black arrowheads.



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