Activation of the kappa opioid receptor / dynorphin system alters stress and threat responding during acute withdrawal from intermittent alcohol drinking in male mice.

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Highlights

- Intermittent alcohol drinking changed stress reactions in mice.
- KOR antagonist norBNI altered stress responses in alcohol drinkers.
- Alcohol and swim stress increased c-Fos immunoreactivity in BNST dynorphin neurons.
- Swim stress enhanced excitatory drive onto BNST dynorphin cells in alcohol mice.
- BNST dynorphin knockdown affected some stress behavior, but not alcohol drinking.

Abstract

The dynorphin/kappa opioid receptor (KOR) system in the brain regulates both stressful experiences and negative, aversive states during withdrawal from drugs of abuse. We explored the role of this system during acute withdrawal from long-term alcohol drinking, focusing on the bed nucleus of the stria terminalis (BNST), a region strongly implicated in alcohol-withdrawal induced alterations of behavior. Male C57BL/6J mice were subjected to repeated forced swim tests, home cage exposure to a predator odor, and a visual threat after eight weeks intermittent access to alcohol or water. Systemic injection of KOR antagonist norBNI reversed alcohol-related differences in immobility time during the second swim test and reduced burying behavior in response to predator odor, but did not affect behavioral response to visual threat. In dynorphin-GFP reporter mice, c-Fos immunoreactivity was increased in dynorphin-containing neurons after repeated swim stress and alcohol drinking. Using dynorphin-GFP mice, there was enhanced spontaneous excitatory synaptic drive onto dynorphin neurons in the BNST after alcohol-drinking mice underwent forced swim stress. Finally, knockdown of dynorphin in the BNST using a viral shRNA affected swim stress behavior and responses to TMT in alcohol drinkers and controls, but did not affect alcohol drinking. These studies confirm BNST dynorphin recruitment during acute withdrawal as playing a key role in altered behavioral responses to stressful stimuli.

Key Words

Intermittent access to alcohol, forced swim stress, TMT, predator odor, looming disc, norBNI, dynorphin, kappa opioid receptor, c-Fos, synaptic transmission, electrophysiology, mice.

Abbreviations

Alcohol use disorder (AUD), alcohol (EtOH), water (H_2O), preprodynorphin (Pdyn), trimethylthiazoline (TMT), forced swim stress (SS), non-stress (NS), kappa opioid receptor (KOR), baseline (BL), norbinaltorphimine (norBNI), bed nucleus of the stria terminalis (BNST), spontaneous excitatory postsynaptic current (sEPSC), spontaneous inhibitory postsynaptic current (sIPSC), artificial cerebral spinal fluid (ACSF), phosphate buffer solution (PBS), tyramine signal amplification (TSA).

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1. Introduction

The endogenous dynorphin/kappa opioid receptor (KOR) system is a regulator of stress responses and anxiety-like behavior (Bruchas, Land, and Chavkin 2010). Dynorphin/KOR signaling has also been heavily studied for treating negative symptomology observed in alcohol dependence (Walker et al. 2012; Kharkhanis et al. 2017). Rats rendered alcohol dependent with ethanol vapor show anxiety-like behavior during acute withdrawal, including decreased time spent in the open arm of the elevated plus maze, increased 22 kHz ultrasonic vocalizations, and increased marble burying, all of which can be blocked with the KOR antagonist norbinaltorphimine (norBNI, Valdez and Harshberger 2012, Berger et al. 2013, Rose et al. 2016).

The mechanisms of how the dynorphin/KOR system contributes to alcohol-stress interactions remain to be clarified. The bed nucleus of the stria terminalis (BNST) contains both dynorphin and KOR (Le Merrer et al. 2009, Poulin et al. 2009) and has been broadly implicated in stress effects on drug intake. Stressors such as forced swim stress can increase KOR phosphorylation in the BNST, a marker of dynorphin release (Land et al. 2009, Bruchas et al. 2007). KORs in the BNST also appear to mediate interactions between stress and alcohol drinking, as BNST norBNI attenuates stress-induced alcohol seeking (Lê et al. 2018) and reduces alcohol withdrawal-induced 22-kHz ultrasonic vocalizations (Erikson et al. 2018). Our laboratory and others have shown that neuropeptide circuits in the BNST are critical for anxiety-like behavior, stress-related behavior, and stress behaviors affected by alcohol drinking (Bruchas et al. 2009, Crowley et al. 2016, Giardino et al. 2018, Hwa et al. 2020).

Given the prominent role of BNST dynorphin/KOR in alcohol withdrawal and stress, our group recently investigated the role of BNST KOR signaling in altered stress responses to predator odor during protracted withdrawal from intermittent alcohol drinking (Hwa et al. 2020). We showed that adult male mice with a history of chronic alcohol intake exhibited abnormal stress reactions to the synthetic, fox-derived predator odor trimethylthiazoline (TMT), which could be reversed with intra-BNST norBNI and floxed deletion of prodynorphin (Pdyn) in the BNST. The

current set of experiments focus on behavioral and electrophysiological characterization of BNST Pdyn neuronal activity during acute withdrawal. Specifically, this set of studies explores alcohol-induced KOR regulation of behavioral responses to repeated forced swim stress, exposure to TMT predator odor, and an overhead looming disc threat. Next, we investigated if alcohol and forced swim stress or TMT interact to enhance c-Fos expression, an immediate early gene marker for general cell activation, in Pdyn neurons in the BNST using a Pdyn-GFP reporter line (Al-Hasani et al. 2015, Hwa et al. 2020). Using whole cell patch clamp electrophysiology, intrinsic excitability and basal synaptic transmission properties were measured in $BNST^{Pdyn}$ cells after repeated forced swim and alcohol or water. Lastly, we used a viral Pdyn-shRNA (Cohen et al. 2014) to knockdown Pdyn expression specifically in the BNST to probe the impact on performance in repeated forced swimming and TMT exposure. Taken together, these findings highlight BNST KOR/dynorphin signaling as a key mediator of alcohol withdrawal-induced disruption of behavior.

2. Materials and Methods

2.1. Animals

We used adult male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) for behavioral pharmacology experiments with norBNI and Pdyn shRNA. For histology and electrophysiology studies, we generated a Pdyn-GFP reporter line by crossing *preprodynorphin*-IRES-Cre mice (*Pdyn*$^{IRES\text{-}Cre}$, Al-Hasani et al. 2015, Bloodgood et al. 2020, Hwa et al. 2020) and Rosa26-flox-stop-L10a-EGFP reporter mice (EGFP-*L10a*) to visualize Pdyn-expressing fluorescent cells. These mice were bred in the UNC facilities. Mice were singly housed in polycarbonate cages (GM500, Tecniplast, Italy) on a 12:12-h reversed dark-light cycle with lights off at 7:00am. Mice had *ad libitum* rodent chow (Prolab Isopro RMH 3000, LabDiet, St. Louis, MO) and H_2O. The UNC School of Medicine Institutional Animal Care and Use Committee approved

all protocols. Experiments were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

2.2. Intermittent EtOH Drinking

Mice were given two-bottle choice intermittent access to a 20% (w/v) EtOH solution and water for eight weeks (Hwa et al. 2011, Hwa et al. 2020). Drinking tubes positioned through customized cage lids consisted of conical centrifuge tubes (Catalog No. 05-502-10B; ThermoFisher) with rubber stopper (size 5.5, Ancare) and sippers (OT-101, 3.5 inch, Ancare). Bottles were weighed before and after 24 hour EtOH access on Mondays, Wednesdays, and Fridays. Fluid loss due to handling was averaged each week from a dummy cage and was subtracted from each mouse's daily consumption. Mice were weighed to calculate daily EtOH intake in grams/kilogram (g/kg). Daily EtOH preference was defined as the ratio of EtOH fluid consumed divided by the total fluid (EtOH plus H_2O) consumed.

2.3. Forced Swim Stress

After 8 weeks of EtOH drinking, mice were exposed to two episodes of swim stress (SS) according to the protocol described by Anderson, Lopez, and Becker (2016). Mice were placed in 22-24°C water in a 20.32(w) x 26.67(h) cm acrylic cylinder for 10 min 4 hr before EtOH access. After each swim test, mice were dried and a heat lamp was kept over the home cage. There was one drinking day in between the double swim trial days (e.g. Monday and Friday swim stress preceding EtOH access on Monday, Wednesday, and Friday; **Fig 1A**). Latency to immobility and duration of immobility were recorded for both trials.

2.4. TMT Predator Odor

After repeated swim tests, mice were tested for their behavioral responses to the predator odor trimethylthiazoline (TMT) during acute, 6-8 hour, withdrawal from EtOH drinking [**Fig 1A**]. We used home cage exposure to TMT for studying a variety of stress reactions as previously reported (Hwa et al. 2019, Hwa et al. 2020). Testing occurred under 15-20 lux dim lighting conditions in a separate, ventilated room with a fume hood. After a 10 min habituation to a cotton tip applicator placed in the corner of the home cage (baseline, BL), 2.5 µl TMT (BioSRQ) was applied to the cotton tip followed by 10 min of behavioral testing. We have previously characterized that C57BL/5J male mice display a repertoire of stress-related and exploratory behaviors during the home cage TMT test, such as burying, freezing, grooming, rearing, stretch-attend, and walking (Hwa et al. 2020). Among these behaviors, burying represents a canonical stress or defensive-like behavioral state in rodents (Pinel and Treit 1978, De Boer and Koolhaas 2003), which we have replicated in EtOH-naïve controls (Hwa et al. 2019, Hwa et al. 2020). Durations of seconds 1) burying, 2) in contact with the TMT, and 3) in the far corners of the cage, were recorded and quantified with Ethovision XT13 (Noldus, The Netherlands). Percent change from the baseline was quantified for time spent in contact with the object and spent in the far corners, and raw increase in burying behavior in sec was measured from the pre-trial baseline.

2.5. Looming disc

After TMT exposure, mice were tested for reactions to an overhead looming disc during 6-8 hr withdrawal from intermittent EtOH [**Fig 1A**]. Based off of the protocol described by Yilmaz and Meister (2013), we used a custom open field chamber (48 x 48 x 30 cm) with three matte walls and one transparent wall for observation. One strip of red infrared LED lights provided ambient light in the test box, and an infrared camera (Basler GigE) recorded testing. A wide computer monitor (HP 22es, 48 cm) spanned across the test chamber to display the overhead looming disc animation. A triangular prism-shaped protective hut (12 x 20 cm) was positioned in

the back corner during habituation and testing. Mice were placed into the test chamber for 10 min habituation to the arena, the protective hut, and the overhead monitor displaying a grey background (RGB 128, 128, 128). The looming disc animation was triggered when the mouse moved across the center of the arena after the 10 min habituation. The animation consisted of a 0.5 sec solid black circle rapidly increasing in size, repeated 15 times. In reaction to the looming disc, freezing behavior (s), maximum velocity (cm/s) of the dart, and the escape distance (cm) were recorded and quantified by Ethovision XT13.

2.6. NorBNI testing

Five mg/kg norBNI (Cat no. 0347, Tocris) was administered i.p., 1ml/100g, 16 hr before the second forced swim test to both EtOH and H_2O mice. We have previously reported this time point for norBNI pretreatment to minimized handling stress prior to the stress reactivity testing and allowed for KOR antagonism (Hwa et al. 2020) instead of non-specific mu opioid antagonism that occurs initially post-injection (Endoh et al. 1992). Importantly, norBNI is known for its ultra-long duration of action (Munro et al. 2012). Since published reports have shown behavioral effects of norBNI for up to 86 days post-injection (Potter et al. 2011), it is likely that norBNI was on board during the second forced swim test, the TMT test, and the looming disc assay.

2.7. C-Fos immunohistochemistry

Separate groups of Pdyn-GFP mice were used to measure c-Fos in the dorsal BNST after EtOH drinking and stress [**Fig 2A**]. Ninety min after either the second forced swim test or TMT exposure, mice were deeply anesthetized with Avertin before intracardiac perfusion with chilled phosphate buffered saline (PBS) and 4% paraformaldehyde in PBS. Brains were extracted, cryoprotected, and then sliced at 45 µm on a Leica 1200S vibratome. Coronal sections from Pdyn-

GFP mice were processed for c-Fos immunofluorescence to visualize co-localization of c-Fos expression in Pdyn-containing neurons.

The immunofluorescence protocol for c-Fos was performed according to previous studies (Hwa et al. 2019; Hwa et al. 2020) using tyramine signal amplification (TSA). Primary incubation occurred at 4°C for 48 hr in blocking buffer (0.3% Triton X-100 and 1% bovine serum albumin) containing a rabbit anti-*c-Fos* antibody (1:3000, ABE457, Millipore, Bellerica, MA). Secondary incubation consisted of goat anti-rabbit horse radish peroxidase-conjugated IgG (1:200, NEF812001EA, PerkinElmer, Waltham, MA) for 30 min followed by fluorescent tagging with Cy3-tyramide (1:50, PerkinElmer, Waltham, MA) for 10 min according to the TSA kit instructions. Slices were then affixed to slides with Vectashield mounting medium containing DAPI and coverslipped.

Coronal sections containing the BNST were imaged on a Zeiss 800 laser scanning confocal microscope (Carl Zeiss, Germany). For c-Fos immunofluorescence quantification within Pdyn neurons, Pdyn-GFP cells were chosen as the parent classifier, and c-Fos Cy3-labeled cells were set as the child classifier using the Image Analysis Wizard in Zen Blue software. This automatic segmentation ensured that counting settings for Pdyn-GFP cells, c-Fos cells, and c-Fos/Pdyn-GFP cells were consistent across images. Four coronal brain sections containing both right and left BNST were analyzed, and counts were averaged for each animal.

2.8. Slice electrophysiology

After long-term EtOH or H_2O drinking, Pdyn-GFP mice were deeply anesthetized with isoflurane 90 min following the second swim and were rapidly decapitated. Coronal brain sections containing the BNST were collected according to standard laboratory protocols (Pati et al. 2019). A Leica VT 1200s vibratome (Leica Biosystems, IL) cut 300 µM thick coronal slices in ice-cold, oxygenated sucrose solution containing in mM: 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose and 26 $NaHCO_3$ saturated with 95% O_2/5% CO_2. Slices were incubated at 32-35°C for at least one hour in normal artificial cerebral spinal fluid (ACSF) that contained in

mM: 124 NaCl, 4.4 KCl, 1 NaH_2PO_4, 1.2 $MgSO_4$, 10 D-glucose, 2 $CaCl_2$, and 26 $NaHCO_3$, saturated with 95% O_2/5% CO_2. Neurons in the dorsal BNST were identified using infrared differential interference contrast. Pdyn-GFP-containing cells were selected based on visualization using a 470 nm LED and GFP filter. Signals were acquired using an Axon Multiclamp 700B (Molecular Devices, Sunnyvale, CA). Data were sampled at 10 kHz, low-pass filtered at 3 kHz, and analyzed in pClamp 10.8 (Molecular Devices, Sunnyvale, CA). Access resistance was continuously monitored and changes greater than 20% from the initial value were excluded from data analyses. Two-four cells were recorded from each animal per set of experiments.

For neuronal excitability experiments, recordings were performed in current clamp mode using a K-gluconate based internal solution (in mM: 135 K-gluconate, 5 NaCl, 2 $MgCl_2$, 0.6 EGTA, 4 Na_2ATP, 0.4 Na_2GTP, and 10 HEPES, pH adjusted to 7.3 using KOH and volume adjusted to 285–290 mOsm). After allowing $BNST^{Pdyn}$ cells to stabilize, experiments were performed to determine rheobase (the minimum amount of current required to elicit an action potential) and action potential threshold. Next, spike frequency was assessed in a V-I plot, in which the number of action potentials fired were recorded in response to depolarizing current steps of 10 pA each ranging from 0-200 pA). After intrinsic excitability experiments were performed at resting membrane potential, current was injected to hold the cells at -70 mV and protocols were repeated.

Spontaneous synaptic transmission was assessed in voltage clamp mode using a cesium methanesulfonate-based intracellular solution (in mM: 135 cesium methanesulfonate, 10 KCl, 1 $MgCl_2$, 0.2 EGTA, 4 MgATP, 0.3 Na_2GTP, 20 phosphocreatine, pH 7.3, 285-290 mOsm with 1mg/ml lidocaine n-ethyl bromide). $BNST^{Pdyn}$ cells were first held at -55mV for monitoring spontaneous excitatory postsynaptic currents (sEPSCs), and, second held at +10mV to assess spontaneous inhibitory postsynaptic currents (sIPSCs).

2.9. BNST Pdyn knockdown

C57BL/6J mice were injected with recombinant adeno-associated virus (AAV) vectors encoding a Pdyn-specific short hairpin RNA (Pdyn-shRNA) under the control of a mU6 promotor and eGFP under the control of the CMV promotor (Cohen et al. 2014) into the BNST to knockdown Pdyn. Controls were injected with AAV encoding a scrambled shRNA (Pdyn-scr). We validated that this was effective in reducing Pdyn expression in mouse brain (data not shown). Mice were prepared for stereotaxic surgery under 3-4% isoflurane in oxygen anesthesia for induction followed by 1-2% isoflurane for maintenance. While placed on a body temperature regulation pad in the stereotaxic frame (Kopf Instruments, Tujunga, CA), the scalp was shaved and sterilized with 70% EtOH and betadine for the midline incision. Bilateral burr holes were drilled above the BNST at the coordinates AP +0.30 mm, ML ± 0.95 mm, DV -4.35 mm from bregma. 0.3 µl Pdyn-shRNA or Pdyn-scr was injected at a rate of 0.1 µl/min using a 1 µl Neuros Hamilton syringe (Hamilton, Reno, NV). Mice recovered for one week before starting EtOH drinking.

For placement verification, mice were deeply anesthetized with Avertin before intracardiac perfusion with chilled phosphate buffered saline (PBS) and 4% paraformaldehyde in PBS. Brains were extracted, cryoprotected, and then sliced at 45 µm on a Leica 1200S vibratome. Coronal sections containing the BNST were mounted onto slides and imaged on a Zeiss 800 laser scanning confocal microscope. Viral expression was confirmed and shown in **Fig 4B**.

2.10. Statistical Analyses

Statistical tests were analyzed with GraphPad Prism 8 (La Jolla, CA, USA). Mixed model ANOVA was used to analyze immobility time and latency to immobility across Trials (Swim I and Swim II) between Drinking groups (H_2O and EtOH). For TMT testing with norBNI or Pdyn-shRNA knockdown, two-way ANOVA was run to analyze contact with TMT, time spent in the far corners, and burying with Drug/Virus (no injection and norBNI; Pdyn-scr and Pdyn-shRNA) and Drinking as factors. Two-way ANOVA were similarly run for effects of norBNI on looming disc responses such as freezing, maximum velocity, and escape distance. For immunohistochemistry, c-Fos,

Pdyn-eGFP, and c-Fos/Pdyn co-localization were analyzed with two-way ANOVA with Stress (non-stress, NS, and swim stress, SS, or TMT) and Drinking as factors. Excitability variables such as resting membrane potential, rheobase, and action potential threshold were also analyzed with two-way ANOVA with Stress and Drinking as factors. A three-way mixed model ANOVA was used to analyze firing activity in VI plots with Stress, Drinking, and Current as factors. Four cells were not assessed for properties at -70 mV. Two-way ANOVA was used to compare synaptic transmission elements such as sE/IPSC frequency and amplitude. For effects of BNST Pdyn-shRNA on immobility time, a mixed model ANOVA was performed with Virus, Drinking, and Trial as factors. Sidak multiple comparisons tests were performed after significant main effects were found. Alpha was set to 0.05.

3. Results

3.1. Kappa opioid receptor regulation of alcohol-related responses to repeated swim test and predator odor

After a long-term history of voluntary EtOH drinking, mice were tested in three different stress and threat-related assays compared to single-housed, H_2O-drinking controls [**Fig 1A**]. Adult male C57BL/6J mice escalated their daily alcohol consumption (g/kg) and alcohol preference on the intermittent, two-bottle choice schedule for 8 weeks [**Fig 1B-C**].

In the repeated forced swim test, H_2O-drinking controls increased their immobility time from the first to the second swim, suggestive of learned stress coping responses, whereas EtOH drinkers did not [**Fig 1D**; Main effect of Trial $F(1,15)=5.466$, $p=0.0337$; Main effect of Drinking $F(1,15)=22.61$, $p=0.003$; Swim I H_2O vs Swim II H_2O $t(15)=4.494$, $p=0.0009$; Swim II H_2O vs Swim II EtOH $t(30)=2.933$, $p=0.0127$]. In a separate group of mice, norBNI administration caused an increase in immobility time in the EtOH mice, implying a norBNI rescue of EtOH-impaired responding [**Fig 1E**; Main effect of Drinking $F(1,13)=36.60$, $p<0.001$; Swim I H_2O vs Swim II H_2O $t(13)=4.170$, $p=0.0022$; Swim I EtOH vs Swim II EtOH $t(13)=4.398$, $p=0.0014$]. Latency to

immobility decreased from the first to the second swim [**Fig 1F**; Main effect of Trial $F(1,15)=41.79$, $p<0.001$; Swim I H_2O vs Swim II H_2O $t(15)=4.489$, $p=0.0009$; Swim I EtOH vs Swim II EtOH $t(15)=4.663$, $p=0.0006$]; however, EtOH history was not a factor. Latency to immobility was not affected by norBNI [**Fig 1G**; Main effect of Trial $F(1,26)=59.29$, $p<0.001$; Swim I H_2O vs Swim II H_2O $t(26)=4.887$, $p<0.0001$; Swim I EtOH vs Swim II EtOH $t(26)=6.047$, $p<0.0001$].

Next, mice were tested for their responses to TMT predator odor in the home cage. While TMT avoidance was maintained in H_2O controls compared to non-odor baseline, norBNI decreased high levels of TMT contact in the EtOH mice [**Fig 1H**, Main effect of Drinking $F(1,30)=5.950$, $p=0.0209$; Main effect of Drug $F(1,30)=5.438$, $p=0.0266$; no injection H_2O vs no injection EtOH $t(30)=2.933$, $p=0.0377$; no injection EtOH vs norBNI EtOH $t(30)=2.919$, $p=0.0390$]. Contact with the novel object during the no-odor baseline revealed an interaction between the factors, but post-hoc differences were not significant [**Fig 1H left inset**, Drinking x Drug Interaction $F(1,30)=6.577$, $p=0.0156$]. During the test, norBNI reduced time spent contacting the TMT in EtOH mice [**Fig 1H right inset**, Main effect of Drinking $F(1,30)=4.255$, $p=0.0479$; Main effect of Drug $F(1,30)=5.530$, $p=0.0255$; no injection EtOH vs norBNI EtOH $t(30)=1.697$, $p=0.049$]. NorBNI additionally increased percent change from baseline time spent in the far corners in EtOH mice [**Fig 1I**, Main effect of Drinking $F(1,30)=8.880$, $p=0.0057$; no injection EtOH vs norBNI EtOH $t(30)=2.954$, $p=0.0357$]. There were no EtOH or norBNI effects on raw time spent in the far corners in either the baseline period or the TMT test [**Fig 1I left and right inset**]. TMT-induced burying as a function of baseline increased with norBNI treatment in EtOH mice [**Fig 1J**, Main effect of Drinking $F(1,30)=10.38$, $p=0.0031$; no injection EtOH vs norBNI EtOH $t(30)=3.491$, $p=0.0090$]. Minimal burying activity during the baseline was different for norBNI-injected H_2O mice and non-injected EtOH mice [**Fig 1J left inset**, Main effect of Drinking $F(1,30)=8.811$, $p=0.0058$; norBNI H_2O vs no injection EtOH $t(30)=3.192$, $p=0.0197$]. Burying behavior in response to TMT was reduced in EtOH mice, which was restored in EtOH mice by norBNI pretreatment [**Fig 1J right inset**, Main effect of Drinking $F(1,30)=12.06$, $p=0.0016$; Main effect of Drug $F(1,30)=5.950$,

p=0.0208; no injection H_2O vs no injection EtOH t(30)=3.281, p=0.0156; no injection EtOH vs norBNI EtOH t(30)=3.932, p=0.0028].

Third, mice were assessed for reactions to the overhead looming disc. EtOH mice spent less time than controls freezing upon looming disc presentation, but norBNI did not affect these reactions in either group [**Fig 1K**, Main effect of Drinking F(1,30)=9.659, p=0.0041]. Further, EtOH mice had a heightened maximum velocity (cm/s) of the escape dart to the protective hut, but norBNI did not alter this response [**Fig 1L**, Main effect of Drinking F(1,30)=18.53, p=0.0002; norBNI H_2O vs norBNI EtOH t(30)=3.538, p=0.0080]. The escape distance (cm) was overall farther for EtOH mice compared to H_2O mice, but again, norBNI treatment was not a factor [**Fig 1M**, Main effect of Drinking F(1,30)=4.215, p=0.0489].

3.2. The bed nucleus of stria terminalis as a site of stress-alcohol interaction

Coronal brain slices containing the dorsal BNST were processed for c-Fos immunohistochemistry after H_2O or EtOH drinking and repeated forced swim stress (SS) or non-stress (NS) controls [**Fig 2A**]. Adult male Pdyn-GFP reporter mice were given intermittent access to EtOH or H_2O for 8 weeks [**Fig 2B-C**]. We measured c-Fos, Pdyn-GFP, and co-localization of c-Fos in Pdyn-GFP cells [**Fig 2D-E**]. EtOH mice that underwent SS had increased BNST c-Fos compared to non-stressed H_2O and EtOH mice [**Fig 2F**, Main effect of Stress F(1,17)=10.08, p=0.0055; NS H_2O vs SS EtOH t(17)=3.230, p=0.0291; NS EtOH vs SS EtOH t(17)=3.116, p=0.0372]. Two episodes of swim stress increased BNST Pdyn-GFP levels [**Fig 2G**, Main effect of Stress F(1,17)=6.911, p=0.0176]. There was an interaction between EtOH and SS for BNST c-Fos and Pdyn co-localization, where EtOH SS mice had more co-localized cells than the other groups [**Fig 2H**, Interaction F(1,17)=5.535, p=0.0309; NS H_2O vs SS EtOH t(17)=5.182, p=0.0004; NS EtOH vs SS EtOH t(17)=4.784, p=0.0010; SS H_2O vs SS EtOH t(17)=3.487, p=0.0168].

Both Pdyn-GFP EtOH and H_2O mice showed increases in c-Fos in response to TMT exposure [**Fig 2I**, Main effect of Stress F(1,20)=124.8, p<0.0001; NS H_2O vs TMT H_2O

t(20)=7.445, p<0.0001; NS EtOH vs TMT EtOH t(20)=8.495, p<0.0001]. TMT and EtOH history did not affect Pdyn-GFP [**Fig 2J**]. TMT increased c-Fos and Pdyn co-localized cells in EtOH drinkers compared to non-stressed EtOH mice [**Fig 2K**, Main effect of Stress F(1,20)=21.84, p=0.0001; NS H_2O vs TMT EtOH t(20)=3.976, p=0.0039; NS EtOH vs TMT EtOH t(20)=4.031, p=0.0039]. There was a trend that H_2O mice increased c-Fos Pdyn co-localized cells after TMT compared to non-stressed H_2O mice, and this was not different from EtOH mice exposed to TMT [**Fig 2K**, NS H_2O vs TMT H_2O t(20)=2.712, p=0.0526].

3.3. Enhanced excitatory transmission onto Pdyn neurons in the BNST after alcohol and swim stress

Intrinsic excitability of $BNST^{Pdyn}$ cells was assessed in H_2O and EtOH Pdyn-GFP mice exposed to repeated forced swim [**Fig 3A**]. The resting membrane potential was lower in SS EtOH mice compared to SS H_2O mice [**Fig 3B**, Main effect of Drinking F(1,43)=12.98, p=0.0008; SS H_2O vs SS EtOH t(43)=2.829, p=0.0417]. Mice that underwent SS had a reduced rheobase compared to non-stressed mice when cells were held at -70 mV [**Fig 3C**, Main effect of Stress F(1,39)=5.933, p=0.0195]. SS EtOH mice had a reduced action potential threshold compared to non-stressed EtOH mice when cells were held at -70 mV [**Fig 3D**, Main effect of Stress F(1,39)=4.153, p=0.0484; NS EtOH vs SS EtOH t(39)=3.068, p=0.0232]. Spike frequency experiments were next conducted across the four groups [**Fig 3E**]. When cells were clamped at -70 mV, there was an interaction between current, stress and EtOH in addition to an effect of current on spiking activity [**Fig 3F**, Interaction Current x Stress x Drinking F(20,540)=2.447, p=0.0005; Main effect of Current F(20,540)=75.35; p<0.0001; no significant post-hoc tests].

Basal synaptic activity of $BNST^{Pdyn}$ neurons was further recorded by measurement of sEPSC and sIPSC [**Fig 3G**]. SS EtOH mice displayed higher sEPSC frequency compared to all other groups [**Fig 3H**, Interaction F(1,42)=26.07, p<0.0001; NS H_2O vs SS EtOH t(42)=3.741, p=0.0033; NS EtOH vs SS EtOH t(42)=5.890, p<0.0001; SS H_2O vs SS EtOH t(42)=4.821,

p=0.0001]. There was no effect of EtOH and SS on sIPSC frequency [**Fig 3I**]. Since experiments were performed in the same cell, the sEPSC/sIPSC ratio was greater in SS EtOH mice compared to non-stress H_2O and EtOH mice, and SS H_2O mice, as well [**Fig 3J**, Interaction F(1,42)=14.79, p=0.0004; Main effect of Stress F(1,42)=16.91, p=0.0002; NS H_2O vs SS EtOH t(42)=4.306, p<0.0001; NS EtOH vs SS EtOH t(42)=5.649, p<0.0001; SS H_2O vs SS EtOH t(42)=3.842, p=0.0004]. Stress increased sEPSC amplitude, particularly in the EtOH mice [**Fig 3K**, Main effect of Stress F(1,42)=8.464, p=0.0058; NS EtOH vs SS EtOH t(42)=3.059, p=0.0229]. There were no effects of stress and EtOH on sIPSC amplitude [**Fig 3L**].

3.4. Knockdown of BNST Pdyn affects stress responses

An AAV encoding Pdyn-shRNA or Pdyn-scrambled was injected into the BNST [**Fig 4A-B**]. EtOH intake (g/kg/24hr) across 8 weeks was not altered by the knockdown in the BNST [**Fig 4C**]. Pdyn-shRNA did not affect daily EtOH preference either [**Fig 4D**]. When tested in the repeated forced swim test, H_2O Pdyn-scr control mice increased their immobility duration in the second swim, and EtOH Pdyn-scr controls did not, replicating the initial experiments [**Fig 4E**, Effect of Trial F(1,27)=5.215, p=0.0305; Effect of Trial x Virus F(1,27)=4.497, p=0.0433; H_2O Pdyn-scr Swim I vs H_2O Pdyn-scr Swim II t(27)=3.500 p=0.0448]. Both H_2O Pdyn-shRNA mice and EtOH Pdyn-shRNA mice did not increase their immobility time.

TMT responses were transformed as a function of baseline values, to investigate if Pdyn-shRNA-injected mice displayed any differences during the habituation period to the novel object in the home cage. EtOH mice showed increased contact with the TMT as percent change from baseline [**Fig 4F**, Main effect of Drinking F(1,27)=9.096, p=0.0055]. EtOH Pdyn-shRNA mice specifically had increased contact versus H_2O Pdyn-scr mice [t(27)=2.877, p=0.0466]. There were no group differences during the baseline on contact with the novel object [**Fig 4F left inset**]. In response to TMT, there was a trend towards a main effect of virus on avoidance, where Pdyn-shRNA-injected animals generally showed greater contact with the TMT compared to Pdyn-scr

controls [**Fig 4F right inset**, Main effect of Virus F(1,27)=3.772, p=0.0626]. Percent change from baseline time spent in the far corners was generally affected by EtOH and virus [**Fig 4G**, Main effect of Drinking F(1,27)=8.428, p=0.0073; Main effect of Virus F(1,27)=5.097, p=0.0323]. In particular, the EtOH Pdyn-shRNA mice spent reduced time in the far corners as a function of baseline compared to the H_2O Pdyn-scr mice [t(27)=3.714, p=0.0056]. There were no baseline differences in far corners time [**Fig 4G left inset**]. With TMT, Pdyn-shRNA mice had reduced time spent in the far corners of the home cage compared to Pdyn-scr mice [**Fig 4G right inset**, Main effect of Virus F(1,27)=5.108, p=0.0321]. EtOH history and virus injection also generally affected increases in burying in response to TMT [**Fig 4H**, Main effect of Drinking F(1,27)=7.309, p=0.0117; Main effect of Virus F(1,27)=4.611, p=0.0409]. Post-hoc comparisons indicated the H_2O Pdyn-scr group showed increased burying compared to baseline versus the EtOH Pdyn-scr group [t(27)=3.372, p=0.0136]. There were no group differences in burying during the baseline period [**Fig 4H left inset**]. EtOH mice showed reduced time burying compared to H_2O mice, but there were no significant post-hoc differences in virus groups [**Fig 4H right inset**, Main effect of Drinking F(1,27)=4.241, p=0.0492].

4. Discussion

4.1. Key findings

This series of experiments sought to determine the role of $BNST^{Pdyn}$ engagement in early withdrawal from long-term EtOH drinking as a follow-up to our recent investigation of $BNST^{Pdyn}$ in protracted abstinence (Hwa et al. 2020). Here, we found that KOR antagonist norBNI altered EtOH-induced disruptions in a repeated forced swim test and exposure to predator odor, but did not change reactions to a looming disc. Next, we identified an interaction between repeated forced swim stress and EtOH drinking through enhanced co-localization of c-Fos in Pdyn-containing neurons in the BNST. Since TMT stress showed less of a synergistic recruitment as swim stress did, we further examined intrinsic excitability and basal synaptic transmission in $BNST^{Pdyn}$ neurons

after swim stress and EtOH drinking. Forced swim stress generally made BNST^{Pdyn} cells more excitable to action potential spiking when cells were equalized at the same voltage. Swim stress also increased synaptic drive onto BNST^{Pdyn} neurons in EtOH mice, which was distinct from stressed H_2O mice. Finally, using a Pdyn-shRNA virus, we showed BNST^{Pdyn} knockdown affected swim stress and TMT behaviors in both EtOH- and H_2O-drinking animals, suggesting EtOH-specific contributions may originate from other sources.

4.2. Stress phenotypes during acute withdrawal from EtOH

Alcohol driven reductions in response to stress have been well-studied in humans (Sher 1987), so we aimed to determine how alcohol consumption could alter stress- and threat-related phenotypes in male C57BL/6J mice. In the forced swim test, EtOH and H_2O mice showed no differences in immobility duration or latency to immobility in the forced swim test after eight weeks intermittent EtOH drinking. Others have also reported no differences in first swim test immobility between controls and EtOH mice after chronic intermittent EtOH vapor or ip EtOH injections at various withdrawal time points (Ribeiro-Carvalho et al. 2011, Bray et al. 2017, Maldonado-Devincci et al. 2016). There has been a resurgence in behavioral interpretation of the rodent forced swim test; besides anti-depressive-like behavior, immobility can also be interpreted as stress coping behavior, especially during repeated trials (Commons et al. 2017, Molendijk and De Kloet 2019). In agreement with this literature, we interpret the subsequent increase in immobility time during the second swim trial as a learned adaptive state, to conserve energy resources by staying immobile, which was evident in H_2O controls but not in EtOH drinkers. This bears some superficial similarity to the immobility observed in H_2O controls in response to the overhead looming disc. In contrast, EtOH mice exhibited much less immobility in favor of immediate darting behavior, suggestive of hyperresponsivity and differential assessment of the overhead threat. These reactions were accompanied by a longer escape path, which we additionally consider maladaptive in reaction to descending threat.

We also tested mice in their home cage for avoidance and burying responses to the predator odor TMT. EtOH mice generally showed increased contact with the TMT and reduced burying, which we have shown persists into protracted abstinence (Hwa et al. 2020). Defensive burying is a prominent stress coping strategy when rodents are confronted with noxious stimuli (De Boer and Koolhaas 2003), suggesting altered coping strategy. Taken together, these results suggest a history of EtOH drinking may alter stress and threat responses. Many others have observed EtOH withdrawal impairments in anxiety-like behavior in classical tests such as: deficits in social interactions (File et al. 1989, Lowery-Gionta et al. 2015, Marcinkiewcz et al. 2015), elevated plus maze (Lal et al. 1991; Wilson et al. 1998), elevated zero maze and light-dark test (Kliethermes et al. 2004) and novelty-induced hypophagia (Pang et al. 2013, Sidhu et al. 2018). In addition, it was found that intermittent EtOH consumption led to increased sensitivity to social defeat stress (Nenning et al. 2020). These ‘affective disturbances’ during withdrawal (Holleran et al. 2016) may be also caused by slowed learning or low cognitive flexibility after intermittent EtOH exposure (Sey et al. 2019). Future studies should investigate stress-related cognitive impairment or behavioral flexibility in more formal tasks of decision making.

Comparing our TMT-evoked stress reactions to others, some have reported increased digging activity in 10-day withdrawal from chronic intermittent EtOH vapor (Sidhu et al. 2018), but this discrepancy may be explained by differential performance in the familiar home cage in response to TMT versus spontaneous digging activity in a novel cage. The Bains group, however, found decreased home cage digging after acute footshock stress (Füzesi et al. 2016), which is similar to the current results in EtOH-exposed mice after TMT. Our findings in the looming disc assay compliment the increased defensive responses to the bottle brush defined as increased escaping, which may be related to irritability or aggression found during withdrawal (Sidhu et al. 2018, Hwa et al. 2015). Since EtOH mice displayed both hypo- and hyper-responsivity phenotypes to different stimuli, these tests have differing levels of threat uncertainty. Central amygdala inputs to the BNST mediate sustained responses to temporally uncertain threats

(Walker and Davis 2008). It has been shown in humans that EtOH dampens stress responses to threat of uncertain intensity (Bradford et al. 2013). Because heightened reactivity to uncertain threat positively associated with problem drinking, and is present in current and remitted AUD groups, reactivity to uncertain threat may be an endophenotype for AUD, in addition to altered stress responses (Gorka et al. 2016, Gorka and Shankman 2017).

4.3. Utility of KOR antagonists for stress management related to alcohol

These experiments confirm a role for BNST Pdyn/KOR in stress responses during withdrawal from heavy drinking. The immunohistological results found increases in BNST c-Fos after both TMT and repeated forced swim stress. Though we have previously identified a synergistic increase of c-Fos in Pdyn cells between TMT and EtOH during protracted withdrawal (Hwa et al. 2020), we only found a co-localization interaction at the acute time point after repeated forced swim. This endogenous stress system has been known as a crucial mediator of repeated stress (McLaughlin et al. 2003). Using norBNI to block KOR, we observed an increase in immobility during the second forced swim in EtOH mice, suggesting excessive KOR signaling led to aberrant performance in this task. In contrast, BNST Pdyn-shRNA did not rescue the EtOH effect on 2nd swim immobility. However, this is difficult to interpret, as the same manipulation in H_2O mice decreased second trial immobility. This suggests that BNST dynorphin release may be required for adaptive responses in this assay, and that there may be an optimal, inverted U-shaped range of Pdyn signaling required. Similar to our findings, a report from the Carlezon lab also found that systemic norBNI reduced immobility during the second, repeated swim in non-EtOH-exposed rats (Mague et al. 2003). These corroborating data suggest a role for BNST Pdyn in repeated stress, distinct from EtOH history. As for the role of EtOH history, there is precedence for increased immobility during acute withdrawal from EtOH liquid diet in rats, which could be reversed by 20 mg/kg norBNI (Jarman et al. 2018). While our findings differ in the repeated trial

and direction of the EtOH effect, these studies share the commonality that norBNI reversed the EtOH-induced phenotype.

Aside from repeated stress, BNST KOR have been heavily implicated in models of ethanol dependence. For example, intra-BNST KOR antagonism reduces EtOH binge-like drinking in the dark (Haun et al. 2020) and stress-induced reinstatement for alcohol seeking (Lê et al. 2018). During withdrawal from chronic intermittent EtOH vapor, BNST KOR antagonism reduces negative affective behavior (Erikson et al. 2018). We have previously shown intra-BNST norBNI and BNST Pdyn floxed deletion increases avoidance of TMT in EtOH mice during protracted withdrawal (Hwa et al. 2020). So while systemic norBNI may share those effects on some stress behaviors, BNST knockdown via the shRNA may not have eliminated the contribution of Pdyn to affect behavior as robustly perhaps due to multiple sources of Pdyn driving behavioral changes.

Again, discrimination of the stressor or threat may contribute to differences in the role of BNST KOR (Fellinger et al. bioRxiv). Systemic norBNI did not affect reactions to the looming disc either in EtOH or H_2O drinkers. However, there is precedence for intra-BNST microinjections of norBNI not affecting anxiety-like behavior in the open field test (Ahrens et al. 2018), which bears some similarity to responses in the looming disc assay. It is possible that immediate behavioral responses to threat are regulated not by KOR, but instead via CRH in the PVN (Kim et al. 2019, Daviu et al. 2020).

4.4. Glutamatergic plasticity of BNST Pdyn cells

In our exploration of BNST Pdyn physiology after repeated forced swim stress and EtOH history, we found mild differences in intrinsic excitability and robust, interactive effects in synaptic transmission. Specifically, stressed mice exhibited reduced rheobase values, indicating shorter latencies to fire an action potential. Related, stressed EtOH mice had lower thresholds for firing action potentials. While we could not determine individual group differences, $BNST^{Pdyn}$ cells of manipulated groups generally fired more action potentials compared to non-stressed H_2O

controls. Taken together, these findings suggest repeated swim stress makes BNST^{Pdyn} cells more excitable. Others have found that ventral BNST somatostatin cells have increased rheobase, reduced AP threshold, and reduced number of action potentials during protracted abstinence from EtOH, suggestive of GABAergic disinhibition (Dao et al. 2020). Our lab recently has shown that withdrawal from chronic intermittent EtOH increases neuronal excitability of BNST CRF neurons, which overlap with Pdyn neurons in the dBNST (Pati et al. 2020). The interconnectedness of the BNST, and efferent projections make the BNST prime for long-term synaptic modulations by exogenous stressors and EtOH history (Ch'ng et al. 2018).

It has been known for some time that EtOH withdrawal affects BNST glutamatergic signaling (Kash et al. 2009, McElligott et al. 2010, Wills et al. 2012). The current synaptic transmission results suggest a metaplastic shift in glutamate transmission onto BNST^{Pdyn} neurons after EtOH and repeated forced swim. Again, our lab and others have identified increased sEPSC frequency related to EtOH in BNST CRF circuitry (Centanni et al. 2019, Pati et al. 2020). In addition to CRF, stress peptides like Pdyn and neurotensin modulate inhibition of BNST neurons during stress states (Normandeau et al. 2018). In the current studies examining BNST^{Pdyn}, it is likely that stress uncovers persistent alterations in BNST plasticity during withdrawal, as was recently shown in female mice after restraint stress during withdrawal from adolescent intermittent EtOH (Kasten et al. 2020). Withdrawal-related disruptions in novelty-induced hypophagia were blocked by an mGluR5 antagonist, again revealed only after restraint stress (Kasten et al. 2020). BNST mGluR5 signaling through Homer2 and ERK may be one possible downstream mechanism controlling adaptive responses regulating alcohol drinking (Campbell et al. 2019). Regarding plasticity through AMPA/NMDA, we have shown a TMT-induced increase in frontal inputs to BNST^{Pdyn} neurons at a later time point (Hwa et al. 2020). Future studies should identify the target inputs responsible for this increased glutamatergic drive in the Pdyn/KOR system after acute withdrawal from drinking and swim stress, a one-two punch in EtOH dependence (Kissler et al. 2014).

4.5. Limitations

The present experiments have limitations. In the behavioral pharmacology studies, we acknowledge there is no vehicle injection group. As we were exploring subtle differences in stress behavior, we employed a non-injected, baseline behavioral strategy instead. We have previously determined no behavioral differences between non-injected mice and saline-injected mice during protracted withdrawal, so we believe results would be similar at the acute time point (Hwa et al. 2020). Concerns for norBNI not being on board during stress testing are mitigated by the fact that systemic norBNI lasts for months post-injection (Potter et al. 2011).

Another consideration worth discussing is the repeated stress tests. It is plausible that the repeated forced swim tests affected the TMT behavioral tests. For reduction of animal numbers, we did not have a TMT only group of mice in the Pdyn-shRNA experiment. Therefore, it is difficult to interpret the EtOH-stress effects given the H_2O Pdyn-shRNA group showed mildly reduced avoidance with TMT predator odor. In this case, the constitutive knockdown may have overpowered otherwise quiescent behavioral effects. Considering this complexity, there may be an inverted U-shape relationship for adaptive responses to repeated swim stress and TMT. Additionally, this study only includes male mice. Given the BNST is a sexually dimorphic brain region, ongoing studies are looking at this stress-EtOH relationship in female animals. Female mice drink more EtOH per body weight than male mice, so we will explore the role of $BNST^{Pdyn}$ signaling in female stress behavior in a future set of studies.

Considering the synaptic transmission results, it was surprising that repeated forced swim stress in H_2O controls did not show altered synaptic drive compared to non-stressed mice. Our findings suggest that a history of EtOH drinking is required to observe differences in stress-induced signaling in the BNST, which stands in contrast to a single episode of forced swim affecting KOR function in the basolateral amygdala in adolescent rats (Varlinskaya et al. 2020). Unpublished data from our lab indicate that a single forced swim episode may affect sIPSC

frequency, adding to the complexity and episodic nature of the dynamic engagement of $BNST^{Pdyn}$. It is likely that a single swim session would engage the accumbens Pdyn/KOR system, so future studies can contrast synaptic transmission in other Pdyn-rich brain regions of affect.

4.6. Conclusions

These findings supplement our understanding that the BNST is an integrative structure for affective behavior related to substance abuse. The interaction of stress and EtOH drinking is complex, but the Pdyn/KOR system is a definite molecular mediator engaged within cycles of bingeing, withdrawal, and relapse. These data point to KOR antagonists as potential stress resilience medications for alcohol use disorder (Chavkin 2018).

5. References

Al-Hasani, R., McCall, J. G., Shin, G., Gomez, A. M., Schmitz, G. P., Bernardi, J. M., ... & Krashes, M. J. (2015). Distinct subpopulations of nucleus accumbens dynorphin neurons drive aversion and reward. *Neuron*, *87*(5), 1063-1077.

Anderson, R. I., Lopez, M. F., & Becker, H. C. (2016). Forced swim stress increases ethanol consumption in C57BL/6J mice with a history of chronic intermittent ethanol exposure. *Psychopharmacology*, *233*(11), 2035-2043.

Berger, A. L., Williams, A. M., McGinnis, M. M., & Walker, B. M. (2013). Affective cue-induced escalation of alcohol self-administration and increased 22-kHz ultrasonic vocalizations during alcohol withdrawal: role of kappa-opioid receptors. *Neuropsychopharmacology*, *38*(4), 647-654.

Bloodgood, D. W., Hardaway, J. A., Stanhope, C. M., Pati, D., Pina, M. M., Neira, S., ... & Kash, T. L. (2020). Kappa opioid receptor and dynorphin signaling in the central amygdala regulates alcohol intake. *Molecular psychiatry*, 1-13.

Bradford, D. E., Shapiro, B. L., & Curtin, J. J. (2013). How bad could it be? Alcohol dampens stress responses to threat of uncertain intensity. *Psychological science*, *24*(12), 2541-2549.

Bray, J. G., Roberts, A. J., & Gruol, D. L. (2017). Transgenic mice with increased astrocyte expression of CCL2 show altered behavioral effects of alcohol. *Neuroscience*, *354*, 88-100.

Bruchas, M. R., Land, B. B., & Chavkin, C. (2010). The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. *Brain research*, *1314*, 44-55.

Bruchas, M. R., Land, B. B., Aita, M., Xu, M., Barot, S. K., Li, S., & Chavkin, C. (2007). Stress-induced p38 mitogen-activated protein kinase activation mediates κ-opioid-dependent dysphoria. *Journal of Neuroscience*, *27*(43), 11614-11623.

Bruchas, M. R., Land, B. B., Lemos, J. C., & Chavkin, C. (2009). CRF1-R activation of the dynorphin/kappa opioid system in the mouse basolateral amygdala mediates anxiety-like behavior. *PloS one*, *4*(12), e8528.

Campbell, R. R., Domingo, R. D., Williams, A. R., Wroten, M. G., McGregor, H. A., Waltermire, R. S., ... & Quadir, S. G. (2019). Increased alcohol-drinking induced by manipulations of mGlu5 phosphorylation within the bed nucleus of the stria terminalis. *Journal of Neuroscience*, *39*(14), 2745-2761.

Centanni, S. W., Morris, B. D., Luchsinger, J. R., Bedse, G., Fetterly, T. L., Patel, S., & Winder, D. G. (2019). Endocannabinoid control of the insular-bed nucleus of the stria terminalis circuit regulates negative affective behavior associated with alcohol abstinence. *Neuropsychopharmacology*, *44*(3), 526-537.

Chavkin, C. (2018). Kappa-opioid antagonists as stress resilience medications for the treatment of alcohol use disorders. *Neuropsychopharmacology*, *43*(9), 1803-1804.

Ch'ng, S., Fu, J., Brown, R. M., McDougall, S. J., & Lawrence, A. J. (2018). The intersection of stress and reward: BNST modulation of aversive and appetitive states. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *87*, 108-125.

Cohen, A., Whitfield, T. W., Kreifeldt, M., Koebel, P., Kieffer, B. L., Contet, C., ... & Koob, G. F. (2014). Virus-mediated shRNA knockdown of prodynorphin in the rat nucleus accumbens attenuates depression-like behavior and cocaine locomotor sensitization. *PLoS One*, *9*(5), e97216.

Commons, K. G., Cholanians, A. B., Babb, J. A., & Ehlinger, D. G. (2017). The rodent forced swim test measures stress-coping strategy, not depression-like behavior. *ACS chemical neuroscience*, *8*(5), 955-960.

Crowley, N. A., Bloodgood, D. W., Hardaway, J. A., Kendra, A. M., McCall, J. G., Al-Hasani, R., ... & Lowell, B. B. (2016). Dynorphin controls the gain of an amygdalar anxiety circuit. *Cell reports*, *14*(12), 2774-2783.

Dao, N. C., Suresh Nair, M., Magee, S. N., Moyer, J. B., Sendao, V., Brockway, D. F., & Crowley, N. A. (2020). Forced Abstinence from Alcohol Induces Sex-Specific Depression-like Behavioral and Neural Adaptations in Somatostatin Neurons in Cortical and Amygdalar Regions. *Frontiers in Behavioral Neuroscience*, *14*, 86.

Daviu, N., Füzesi, T., Rosenegger, D. G., Rasiah, N. P., Sterley, T. L., Peringod, G., & Bains, J. S. (2020). Paraventricular nucleus CRH neurons encode stress controllability and regulate defensive behavior selection. *Nature Neuroscience*, *23*(3), 398-410.

De Boer, S. F., & Koolhaas, J. M. (2003). Defensive burying in rodents: ethology, neurobiology and psychopharmacology. *European journal of pharmacology*, *463*(1-3), 145-161.

Endoh, T., Matsuura, H., Tanaka, C., & Nagase, H. (1992). Nor-binaltorphimine: a potent and selective kappa-opioid receptor antagonist with long-lasting activity in vivo. *Archives internationales de pharmacodynamie et de therapie*, *316*, 30-42.

Erikson, C. M., Wei, G., & Walker, B. M. (2018). Maladaptive behavioral regulation in alcohol dependence: Role of kappa-opioid receptors in the bed nucleus of the stria terminalis. *Neuropharmacology*, *140*, 162-173.

Fellinger, L., Jo, Y. S., Hunker, A. C., Soden, M. E., & Zweifel, L. S. (2020). A midbrain dynorphin circuit promotes threat generalization. *bioRxiv*.

File, S. E., Baldwin, H. A., & Hitchcott, P. K. (1989). Flumazenil but not nitrendipine reverses the increased anxiety during ethanol withdrawal in the rat. *Psychopharmacology*, *98*(2), 262-264.

Füzesi, T., Daviu, N., Cusulin, J. I. W., Bonin, R. P., & Bains, J. S. (2016). Hypothalamic CRH neurons orchestrate complex behaviours after stress. *Nature communications*, *7*, 11937.

Giardino, W. J., Eban-Rothschild, A., Christoffel, D. J., Li, S. B., Malenka, R. C., & de Lecea, L. (2018). Parallel circuits from the bed nuclei of stria terminalis to the lateral hypothalamus drive opposing emotional states. *Nature neuroscience*, *21*(8), 1084-1095.

Gorka, S. M., & Shankman, S. A. (2017). Preliminary evidence that reactivity to uncertain threat is an endophenotype for alcohol use disorder. *Drug and alcohol dependence*, *180*, 265-271.

Gorka, S. M., Lieberman, L., Phan, K. L., & Shankman, S. A. (2016). Association between problematic alcohol use and reactivity to uncertain threat in two independent samples. *Drug and Alcohol Dependence*, *164*, 89-96.

Haun, H. L., Griffin, W. C., Lopez, M. F., & Becker, H. C. (2020). Kappa opioid receptors in the bed nucleus of the stria terminalis regulate binge-like alcohol consumption in male and female mice. *Neuropharmacology*, *167*, 107984.

Holleran, K. M., Wilson, H. H., Fetterly, T. L., Bluett, R. J., Centanni, S. W., Gilfarb, R. A., ... & Winder, D. G. (2016). Ketamine and MAG lipase inhibitor-dependent reversal of evolving depressive-like

behavior during forced abstinence from alcohol drinking. *Neuropsychopharmacology*, *41*(8), 2062-2071.

Hwa, L. S., Chu, A., Levinson, S. A., Kayyali, T. M., DeBold, J. F., & Miczek, K. A. (2011). Persistent escalation of alcohol drinking in C57BL/6J mice with intermittent access to 20% ethanol. *Alcoholism: Clinical and Experimental Research*, *35*(11), 1938-1947.

Hwa, L. S., Nathanson, A. J., Shimamoto, A., Tayeh, J. K., Wilens, A. R., Holly, E. N., ... & Miczek, K. A. (2015). Aggression and increased glutamate in the mPFC during withdrawal from intermittent alcohol in outbred mice. *Psychopharmacology*, *232*(16), 2889-2902.

Hwa, L. S., Neira, S., Flanigan, M. E., Stanhope, C. M., Pina, M. M., Pati, D., ... & Boyt, K. (2020). Alcohol drinking alters stress response to predator odor via BNST kappa opioid receptor signaling in male mice. *Elife*, *9*, e59709.

Hwa, L. S., Neira, S., Pina, M. M., Pati, D., Calloway, R., & Kash, T. L. (2019). Predator odor increases avoidance and glutamatergic synaptic transmission in the prelimbic cortex via corticotropin-releasing factor receptor 1 signaling. *Neuropsychopharmacology*, *44*(4), 766-775.

Jarman, S. K., Haney, A. M., & Valdez, G. R. (2018). Kappa opioid regulation of depressive-like behavior during acute withdrawal and protracted abstinence from ethanol. *PloS one*, *13*(9), e0205016.

Karkhanis, A., Holleran, K. M., & Jones, S. R. (2017). Dynorphin/kappa opioid receptor signaling in preclinical models of alcohol, drug, and food addiction. In *International Review of Neurobiology* (Vol. 136, pp. 53-88). Academic Press.

Kash, T. L., Baucum, A. J., Conrad, K. L., Colbran, R. J., & Winder, D. G. (2009). Alcohol exposure alters NMDAR function in the bed nucleus of the stria terminalis. *Neuropsychopharmacology*, *34*(11), 2420-2429.

Kasten, C. R., Carzoli, K. L., Sharfman, N. M., Henderson, T., Holmgren, E. B., Lerner, M. R., ... & Wills, T. A. (2020). Adolescent alcohol exposure produces sex differences in negative affect-like behavior and group I mGluR BNST plasticity. *Neuropsychopharmacology*, 1-10.

Kim, J. S., Han, S. Y., & Iremonger, K. J. (2019). Stress experience and hormone feedback tune distinct components of hypothalamic CRH neuron activity. *Nature communications*, *10*(1), 1-15.

Kissler, J. L., Sirohi, S., Reis, D. J., Jansen, H. T., Quock, R. M., Smith, D. G., & Walker, B. M. (2014). The one-two punch of alcoholism: role of central amygdala dynorphins/kappa-opioid receptors. *Biological psychiatry*, *75*(10), 774-782.

Kliethermes, C. L., Cronise, K., & Crabbe, J. C. (2004). Anxiety-like behavior in mice in two apparatuses during withdrawal from chronic ethanol vapor inhalation. *Alcoholism: Clinical and Experimental Research*, *28*(7), 1012-1019.

Lal, H., Prather, P. L., & Rezazadeh, S. M. (1991). Anxiogenic behavior in rats during acute and protracted ethanol withdrawal: reversal by buspirone. *Alcohol*, *8*(6), 467-471.

Land, B. B., Bruchas, M. R., Schattauer, S., Giardino, W. J., Aita, M., Messinger, D., ... & Chavkin, C. (2009). Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. *Proceedings of the National Academy of Sciences*, *106*(45), 19168-19173.

Le Merrer, J., Becker, J. A., Befort, K., & Kieffer, B. L. (2009). Reward processing by the opioid system in the brain. *Physiological reviews*, *89*(4), 1379-1412.

Lê, A. D., Funk, D., Coen, K., Tamadon, S., & Shaham, Y. (2018). Role of κ-opioid receptors in the bed nucleus of stria terminalis in reinstatement of alcohol seeking. *Neuropsychopharmacology*, *43*(4), 838-850.

Lowery-Gionta, E. G., Marcinkiewcz, C. A., & Kash, T. L. (2015). Functional alterations in the dorsal raphe nucleus following acute and chronic ethanol exposure. *Neuropsychopharmacology*, *40*(3), 590-600.

Mague, S. D., Pliakas, A. M., Todtenkopf, M. S., Tomasiewicz, H. C., Zhang, Y., Stevens, W. C., ... & Carlezon, W. A. (2003). Antidepressant-like effects of κ-opioid receptor antagonists in the forced swim test in rats. *Journal of Pharmacology and Experimental Therapeutics*, *305*(1), 323-330.

Maldonado-Devincci, A. M., Kampov-Polevoi, A., McKinley, R. E., Morrow, D. H., O'Buckley, T. K., & Morrow, A. L. (2016). Chronic intermittent ethanol exposure alters stress effects on (3α, 5α)-3-hydroxy-pregnan-20-one (3α, 5α-THP) immunolabeling of amygdala neurons in C57BL/6J mice. *Frontiers in Cellular Neuroscience*, *10*, 40.

Marcinkiewcz, C. A., Dorrier, C. E., Lopez, A. J., & Kash, T. L. (2015). Ethanol induced adaptations in 5-HT2c receptor signaling in the bed nucleus of the stria terminalis: implications for anxiety during ethanol withdrawal. *Neuropharmacology*, *89*, 157-167.

McElligott, Z. A., Klug, J. R., Nobis, W. P., Patel, S., Grueter, B. A., Kash, T. L., & Winder, D. G. (2010). Distinct forms of Gq-receptor-dependent plasticity of excitatory transmission in the BNST are differentially affected by stress. *Proceedings of the National Academy of Sciences*, *107*(5), 2271-2276.

McLaughlin, J. P., Marton-Popovici, M., & Chavkin, C. (2003). κ opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *Journal of Neuroscience*, *23*(13), 5674-5683.

Molendijk, M. L., & de Kloet, E. R. (2019). Coping with the forced swim stressor: current state-of-the-art. *Behavioural Brain Research*, *364*, 1-10.

Munro, T. A., Berry, L. M., Van't Veer, A., Béguin, C., Carroll, F. I., Zhao, Z., ... & Cohen, B. M. (2012). Long-acting κ opioid antagonists nor-BNI, GNTI and JDTic: pharmacokinetics in mice and lipophilicity. *BMC pharmacology*, *12*(1), 5.

Nennig, S. E., Fulenwider, H. D., Eskew, J. E., Whiting, K. E., Cotton, M. R., McGinty, G. E., ... & Schank, J. R. (2020). Intermittent ethanol access increases sensitivity to social defeat stress. *Alcoholism: Clinical and Experimental Research*, *44*(3), 600-610.

Normandeau, C. P., Suárez, M. T., Sarret, P., McElligott, Z. A., & Dumont, E. C. (2018). Neurotensin and dynorphin Bi-Directionally modulate CeA inhibition of oval BNST neurons in male mice. *Neuropharmacology*, *143*, 113-121.

Pang, T. Y., Renoir, T., Du, X., Lawrence, A. J., & Hannan, A. J. (2013). Depression-related behaviours displayed by female C57BL/6J mice during abstinence from chronic ethanol consumption are rescued by wheel-running. *European Journal of Neuroscience*, *37*(11), 1803-1810.

Pati, D., Marcinkiewcz, C. A., DiBerto, J. F., Cogan, E. S., McElligott, Z. A., & Kash, T. L. (2020). Chronic intermittent ethanol exposure dysregulates a GABAergic microcircuit in the bed nucleus of the stria terminalis. *Neuropharmacology*, *168*, 107759.

Pati, D., Pina, M. M., & Kash, T. L. (2019). Ethanol-induced conditioned place preference and aversion differentially alter plasticity in the bed nucleus of stria terminalis. *Neuropsychopharmacology*, *44*(11), 1843-1854.

Pinel, J. P., & Treit, D. (1978). Burying as a defensive response in rats. *Journal of Comparative and Physiological Psychology*, *92*(4), 708.

Potter, D. N., Damez-Werno, D., Carlezon Jr, W. A., Cohen, B. M., & Chartoff, E. H. (2011). Repeated exposure to the κ-opioid receptor agonist salvinorin A modulates extracellular signal-regulated kinase and reward sensitivity. *Biological psychiatry*, *70*(8), 744-753.

Poulin, J. F., Arbour, D., Laforest, S., & Drolet, G. (2009). Neuroanatomical characterization of endogenous opioids in the bed nucleus of the stria terminalis. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *33*(8), 1356-1365.

Ribeiro-Carvalho, A., Lima, C. S., Nunes-Freitas, A. L., Filgueiras, C. C., Manhães, A. C., & Abreu-Villaça, Y. (2011). Exposure to nicotine and ethanol in adolescent mice: effects on depressive-like behavior during exposure and withdrawal. *Behavioural brain research*, *221*(1), 282-289.

Rose, J. H., Karkhanis, A. N., Chen, R., Gioia, D., Lopez, M. F., Becker, H. C., ... & Jones, S. R. (2016). Supersensitive kappa opioid receptors promotes ethanol withdrawal-related behaviors and reduce dopamine signaling in the nucleus accumbens. *International Journal of Neuropsychopharmacology*, *19*(5).

Sey, N. Y., Gómez-A, A., Madayag, A. C., Boettiger, C. A., & Robinson, D. L. (2019). Adolescent intermittent ethanol impairs behavioral flexibility in a rat foraging task in adulthood. *Behavioural brain research*, *373*, 112085.

Sher, K. J. (1987). Stress response dampening. *Psychological theories of drinking and alcoholism*, 227-271.

Sidhu, H., Kreifeldt, M., & Contet, C. (2018). Affective disturbances during withdrawal from chronic intermittent ethanol inhalation in C57BL/6J and DBA/2J male mice. *Alcoholism: Clinical and Experimental Research*, *42*(7), 1281-1290.

Valdez, G. R., & Harshberger, E. (2012). Kappa opioid regulation of anxiety-like behavior during acute ethanol withdrawal. *Pharmacology Biochemistry and Behavior*, *102*(1), 44-47.

Varlinskaya, E. I., Johnson, J. M., Przybysz, K. R., Deak, T., & Diaz, M. R. (2020). Adolescent forced swim stress increases social anxiety-like behaviors and alters kappa opioid receptor function in the basolateral amygdala of male rats. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *98*, 109812.

Walker, B. M., Valdez, G. R., McLaughlin, J. P., & Bakalkin, G. (2012). Targeting dynorphin/kappa opioid receptor systems to treat alcohol abuse and dependence. *Alcohol*, *46*(4), 359-370.

Walker, D. L., & Davis, M. (2008). Role of the extended amygdala in short-duration versus sustained fear: a tribute to Dr. Lennart Heimer. *Brain Structure and Function*, *213*(1-2), 29-42.

Wills, T. A., Klug, J. R., Silberman, Y., Baucum, A. J., Weitlauf, C., Colbran, R. J., ... & Winder, D. G. (2012). GluN2B subunit deletion reveals key role in acute and chronic ethanol sensitivity of glutamate synapses in bed nucleus of the stria terminalis. *Proceedings of the National Academy of Sciences*, *109*(5), E278-E287.

Wilson, J., Watson, W. P., & Little, H. J. (1998). CCKB antagonists protect against anxiety-related behaviour produced by ethanol withdrawal, measured using the elevated plus maze. *Psychopharmacology*, *137*(2), 120-131.

Yilmaz, M., & Meister, M. (2013). Rapid innate defensive responses of mice to looming visual stimuli. *Current Biology*, *23*(20), 2011-2015.

Figure Legends

Figure 1 | Kappa opioid receptor regulation of alcohol-related responses to repeated swim test and predator odor. A| Experimental timeline of male C57BL/6J mice given two-bottle choice, intermittent EtOH for 8 weeks before stress testing in the repeated swim test, exposure to TMT predator odor, and an overhead looming disc. Long-lasting KOR antagonist norBNI was injected 16 hr before the second swim test. **B|** EtOH consumption (g/kg/24h) and **C|** EtOH preference ratio / 24 h across 8 weeks. **D|** Duration of immobility (s) across two forced swim trials in H_2O-drinking (blue) and EtOH-drinking (gold) mice. Median ± range are displayed. **E|** Immobility (s) across repeated forced swim trials in norBNI-pretreated H_2O (light blue) and EtOH (peach) mice. **F|** Latency to immobility (s) across swim trials and **G|** after norBNI treatment. In a home cage TMT exposure, percent change from baseline **H|** contact time with the TMT (s), **I|** time spent in the far corners (s), and **J|** burying duration (s) in non-injected mice (circles) and norBNI-injected mice (squares). Mean ± SEM are displayed. Inset graphs are the raw durations (s) of behaviors during the 10 min baseline with the object (left panels) and during the 10 min TMT test (right panels). In reaction to the looming disc, **K|** freezing behavior (s), **L|** maximum velocity (cm/s) of the dart, and **M|** escape distance (cm) to the hut were assessed in H_2O (blue) and EtOH (gold) mice. *$p<0.05$. **$p<0.01$.

Figure 2 | The bed nucleus of stria terminalis as a site of stress-alcohol interaction. A| Timeline of c-Fos immunohistochemistry experiments in Pdyn-GFP reporter mice. A coronal section of the bilateral bed nucleus of the stria terminalis (BNST) outlined in dotted lines. **B|** EtOH consumption (g/kg/24h) and **C|** EtOH preference ratio / 24 h across 8 weeks. **D|** Sample of Cy-3 labeled c-Fos puncta (pseudocolored red), Pdyn-eGFP (green), and co-localization (yellow) indicated by white arrows. **E|** Representative images of c-Fos immunostaining in Pdyn-eGFP reporter mice after H_2O (top left), EtOH (top right), H_2O + repeated forced swim stress (SS, bottom left), and EtOH + SS (bottom right). The dorsal BNST is outlined in white dotted lines. lat sep =

lateral septum, cp = caudate putamen, ac = anterior commissure. The white scale bar indicates 100 µm. The white solid rectangle locates the image from panel. Quantification of **F**│ c-Fos immunostaining (mm^2), **G**│ Pdyn-eGFP neurons (mm^2), and **H**│ co-localization of c-Fos in Pdyn-eGFP cells (mm^2) after repeated swim stress (SS) or non-stress (NS) controls after a history of intermittent EtOH (gold) or H_2O (blue). Measurement of **I**│ c-Fos immunostaining (mm^2), **J**│ Pdyn-eGFP neurons (mm^2), and **K**│ co-localization of c-Fos in Pdyn-eGFP cells (mm^2) after TMT predator odor exposure (TMT) or non-stress (NS) controls. *p<0.05. **p<0.01.

Figure 3 │ Enhanced excitatory transmission onto Pdyn neurons in the BNST after alcohol and swim stress. A│ Whole cell patch clamp recordings performed in Pdyn-GFP reporter mice to measure excitability and synaptic transmission in BNST Pdyn cells. **B**│ Resting membrane potential (mV) in H_2O (blue) and EtOH (gold) mice after repeated swim stress (SS) and non-stress (NS) controls. **C**│ Rheobase (pA), the minimum current needed to elicit an action potential, clamped at -70 mV. **D**│ Action potential firing threshold at -70 mV (mV). **E**│ Representative traces of action potential firing during a VI plot. **F**│ Frequency of action potentials fired across different current steps at -70 mV in H_2O (blue open circles), EtOH (gold open squares), H_2O + SS (blue filled circles), and EtOH + SS (gold filled squares) mice. **G**│ Sample traces of spontaneous excitatory postsynaptic currents (sEPSC, above four traces) and spontaneous inhibitory postsynaptic currents (sIPSC, below four traces) in H_2O (blue) and EtOH (gold) mice after non-stress (NS, left four traces) or repeated swim stress (SS, right four traces). Scale bar indicates 50 pA and 100 ms. **H**│ sEPSC frequency (Hz). **I**│ sIPSC frequency (Hz). **J**│ sEPSC/sIPSC ratio. **K**│ sEPSC amplitude (pA). **L**│ sIPSC amplitude (pA). *p<0.05. **p<0.01.

Figure 4 │ Knockdown of BNST Pdyn affects stress responses. A│ Experimental timeline of stress testing after BNST Pdyn-shRNA knockdown in C57BL/6J mice. **B**│ Pdyn-shRNA-GFP or Pdyn-scrambled-GFP (scr) was injected into the BNST of C57BL/6J mice. Representative image

of Pdyn-shRNA-GFP (green) localized to the dorsal BNST. lat sep = lateral septum, cp = caudate putamen, ac = anterior commissure. The white scale bar indicates 100 µm. Injection sites across coronal sections. **C**│ EtOH intake (g/kg/24h) and **D**│ daily EtOH preference / 24 h in BNST Pdyn-scr (gold) and Pdyn-shRNA (peach) mice. **E**│ Immobility time (s) during first and second forced swim trials. Median ± range are displayed. Percent change from baseline values for **F**│ TMT contact (s), **G**│ time spent in the far corners (s), and **H**│ burying behavior (s) in mice injected with Pdyn-scr (circles) or Pdyn-shRNA (squares). Inset graphs are the raw durations (s) of behaviors during the 10 min baseline with the object (left panels) and during the 10 min TMT test (right panels). *$p<0.05$.

Figure 1

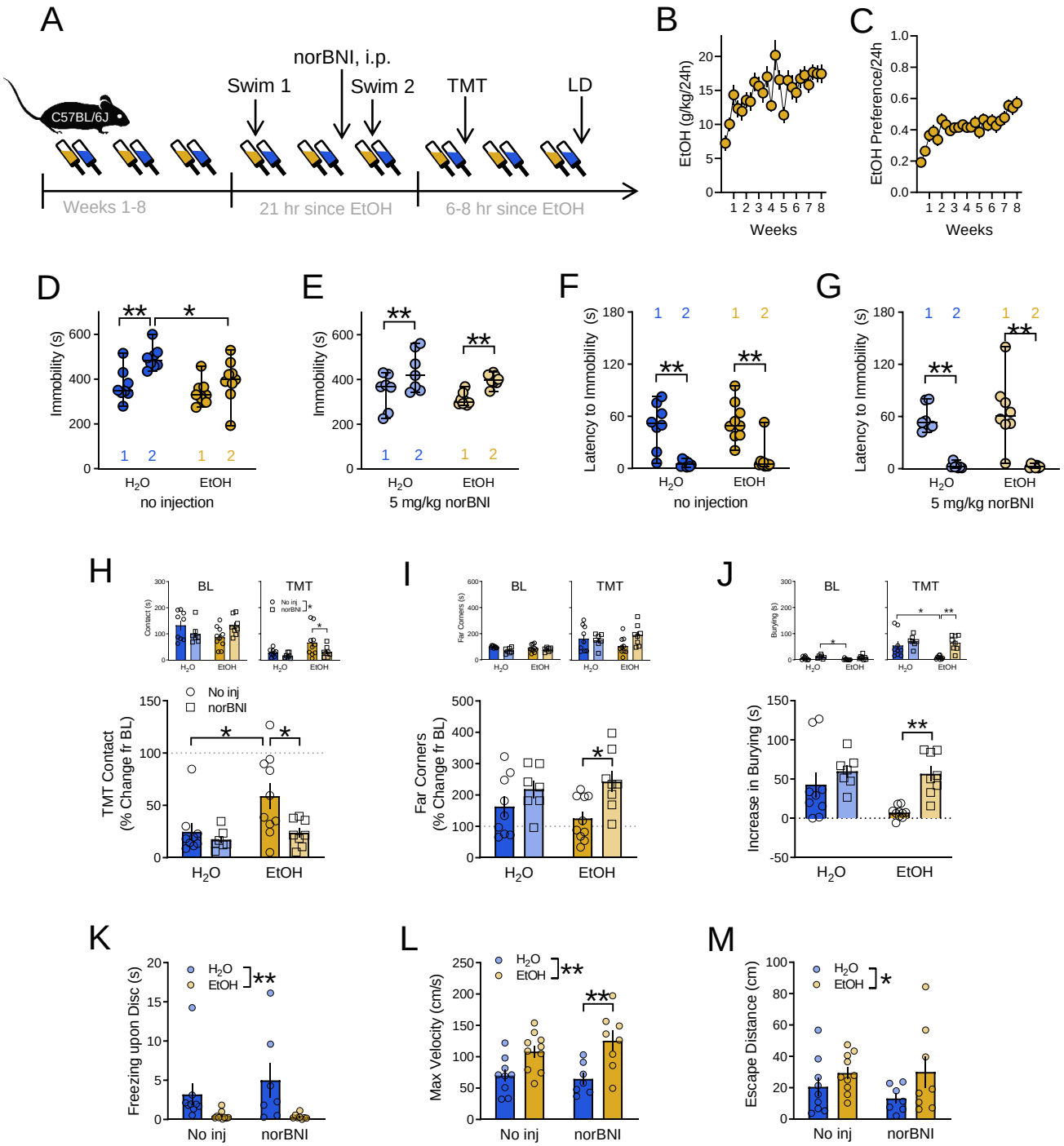

A
C57BL/6J
norBNI, i.p.
Swim 1
Swim 2
TMT
LD
Weeks 1-8
21 hr since EtOH
6-8 hr since EtOH
B
EtOH (g/kg/24h)
Weeks
C
EtOH Preference/24h
Weeks
D
Immobility (s)
H2O
EtOH
no injection
E
Immobility (s)
H2O
EtOH
5 mg/kg norBNI
F
Latency to Immobility (s)
H2O
EtOH
no injection
G
Latency to Immobility (s)
H2O
EtOH
5 mg/kg norBNI
H
BL
TMT
No inj
norBNI
TMT Contact (% Change fr BL)
H2O
EtOH
I
BL
TMT
Far Corners (% Change fr BL)
H2O
EtOH
J
BL
TMT
Increase in Burying (s)
H2O
EtOH
K
Freezing upon Disc (s)
H2O
EtOH
No inj
norBNI
L
Max Velocity (cm/s)
H2O
EtOH
No inj
norBNI
M
Escape Distance (cm)
H2O
EtOH
No inj
norBNI

Figure 2

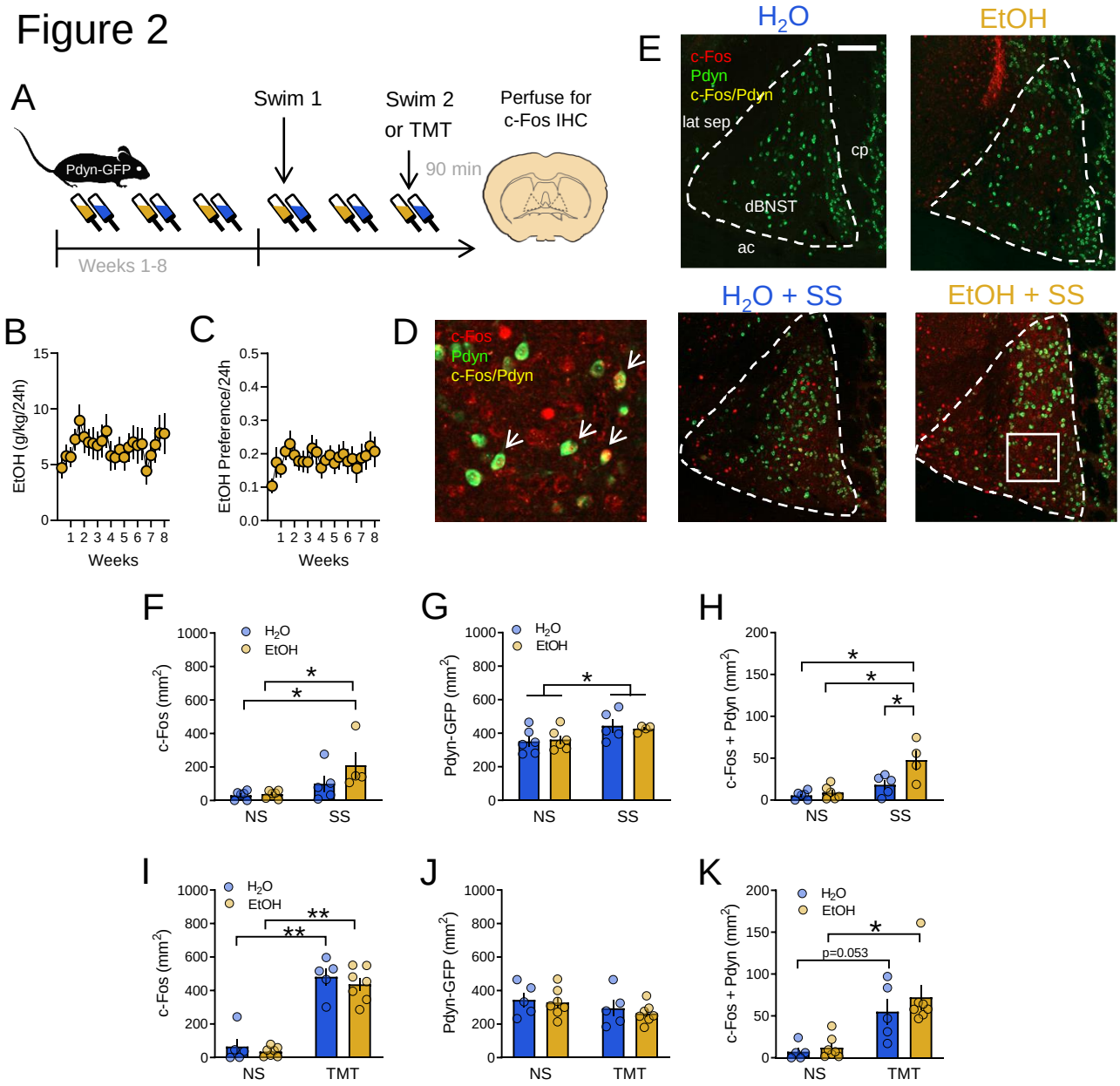

A
Pdyn-GFP
Swim 1
Swim 2
or TMT
90 min
Perfuse for
c-Fos IHC
Weeks 1-8
B
EtOH (g/kg/24h)
Weeks
C
EtOH Preference/24h
Weeks
D
c-Fos
Pdyn
c-Fos/Pdyn
E
H2O
EtOH
c-Fos
Pdyn
c-Fos/Pdyn
lat sep
cp
dBNST
ac
H2O + SS
EtOH + SS
F
c-Fos (mm2)
H2O
EtOH
NS
SS
G
Pdyn-GFP (mm2)
H
c-Fos + Pdyn (mm2)
I
TMT
J
K
p=0.053

Figure 3

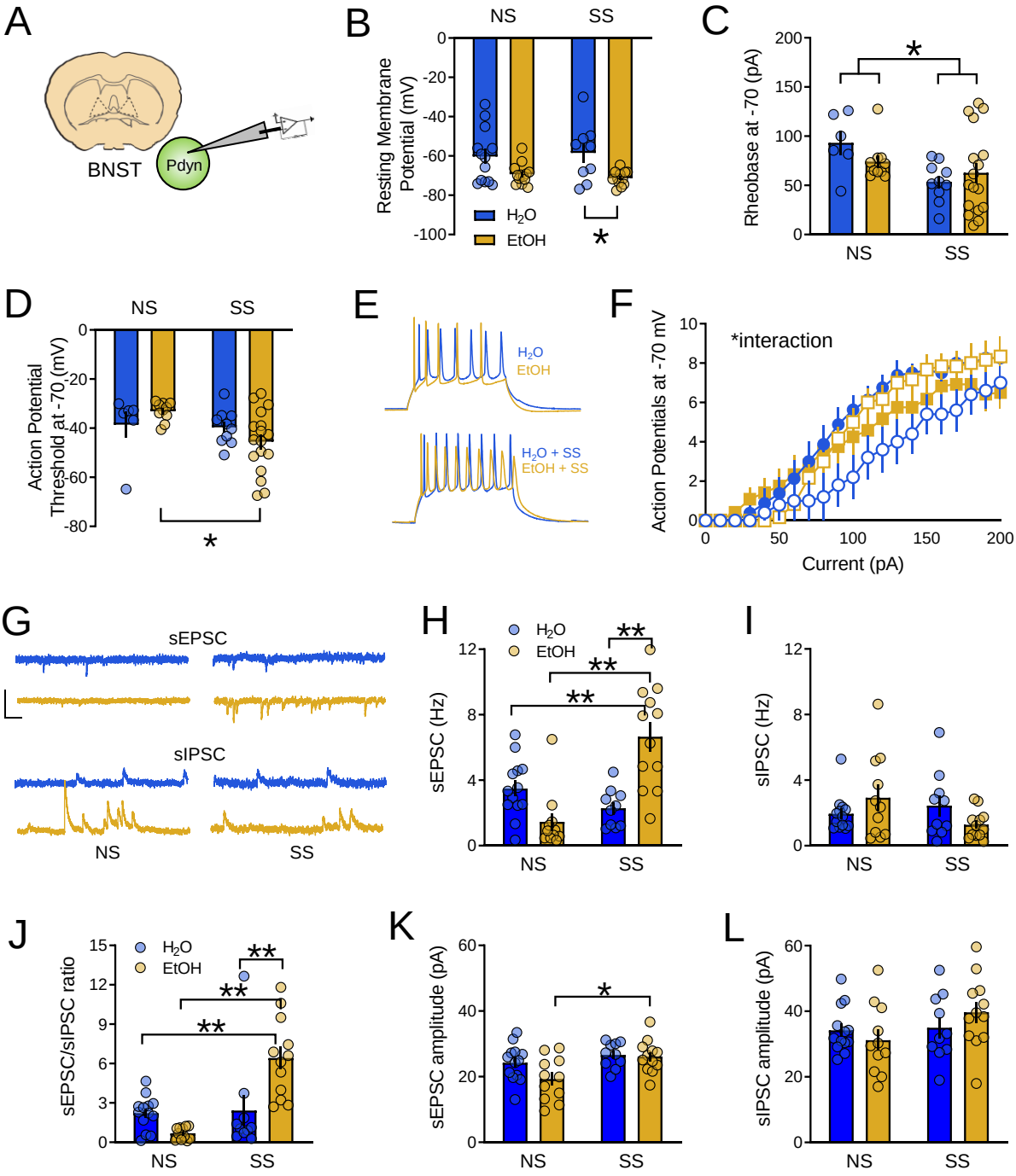
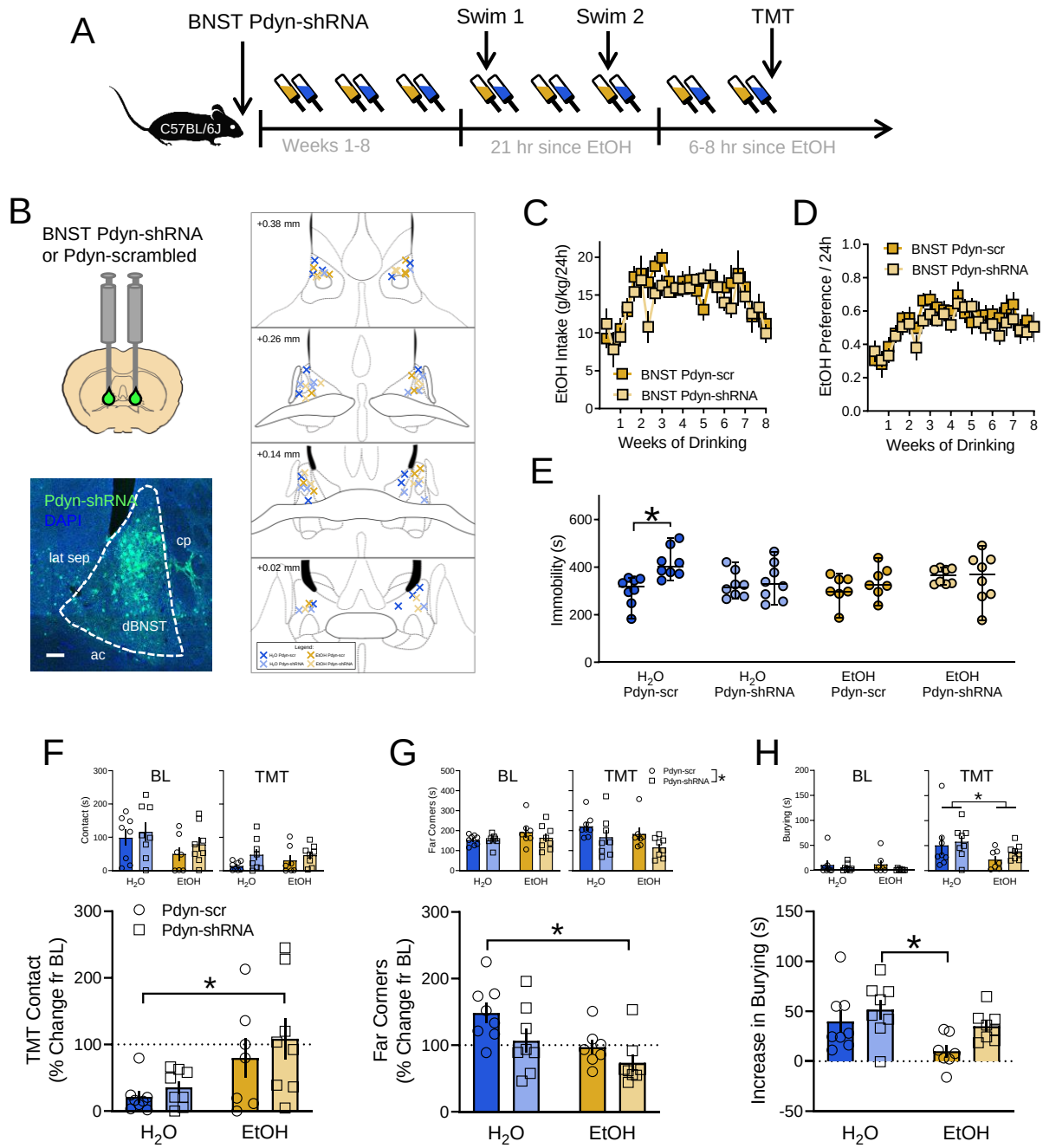

A
BNST
Pdyn
B
NS
SS
Resting Membrane Potential (mV)
0
-20
-40
-60
-80
-100
H2O
EtOH
*
C
Rheobase at -70 (pA)
200
150
100
50
0
*
NS
SS
D
NS
SS
Action Potential Threshold at -70 (mV)
0
-20
-40
-60
-80
*
E
H2O
EtOH
H2O + SS
EtOH + SS
F
Action Potentials at -70 mV
10
8
6
4
2
0
*interaction
0
50
100
150
200
Current (pA)
G
sEPSC
sIPSC
NS
SS
H
H2O
EtOH
sEPSC (Hz)
12
8
4
0
**
**
**
NS
SS
I
sIPSC (Hz)
12
8
4
0
NS
SS
J
H2O
EtOH
sEPSC/sIPSC ratio
15
12
9
6
3
0
**
**
**
NS
SS
K
sEPSC amplitude (pA)
60
40
20
0
*
NS
SS
L
sIPSC amplitude (pA)
60
40
20
0
NS
SS

Figure 4


A
BNST Pdyn-shRNA
Swim 1
Swim 2
TMT
C57BL/6J
Weeks 1-8
21 hr since EtOH
6-8 hr since EtOH
B
BNST Pdyn-shRNA
or Pdyn-scrambled
Pdyn-shRNA
DAPI
cp
lat sep
dBNST
ac
+0.38 mm
+0.26 mm
+0.14 mm
+0.02 mm
C
EtOH Intake (g/kg/24h)
BNST Pdyn-scr
BNST Pdyn-shRNA
Weeks of Drinking
D
EtOH Preference / 24h
BNST Pdyn-scr
BNST Pdyn-shRNA
Weeks of Drinking
E
Immobility (s)
H2O Pdyn-scr
H2O Pdyn-shRNA
EtOH Pdyn-scr
EtOH Pdyn-shRNA
F
BL
TMT
Contact (s)
TMT Contact (% Change fr BL)
Pdyn-scr
Pdyn-shRNA
H2O
EtOH
G
BL
TMT
Far Corners (s)
Far Corners (% Change fr BL)
H
BL
TMT
Burying (s)
Increase in Burying (s)