Dopamine D2 receptors in the bed nucleus of the stria terminalis modulate alcohol-related behaviors

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Abstract

Dysregulation of the dopamine (DA) system is a hallmark of substance use disorders, including alcohol use disorder (AUD). Of the DA receptor subtypes, the DA D2 receptors (D2Rs) play a key role in the reinforcing effects of alcohol. D2Rs are expressed in numerous brain regions associated with the regulation of appetitive behaviors. One such region is the bed nucleus of the stria terminalis (BNST), which has been linked to the development and maintenance of AUD. Recently, we identified alcohol withdrawal-related neuroadaptations in the periaqueductal gray/ dorsal raphe to BNST DA circuit in male mice. However, the role of D2R-expressing BNST neurons in voluntary alcohol consumption is not well characterized. In this study, we used a CRISPR-Cas9-based viral approach, to selectively reduce the expression of D2Rs in BNST GABA neurons (BNST*vgat Drd2*) and interrogated the impact on alcohol-related behaviors. In male mice, reduced BNST*vgat Drd2* expression potentiated the stimulatory effects of alcohol and increased voluntary consumption of 20% w/v alcohol in a two-bottle choice intermittent access paradigm. This effect was not specific to alcohol, as BNST*vgat Drd2* knockdown also increased sucrose intake in male mice. Interestingly, reduction in BNST*vgat Drd2* expression in female mice did

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not alter alcohol-related behaviors but lowered the threshold for mechanical pain sensitivity. Collectively, our findings suggest a role for postsynaptic BNST D2Rs in the modulation of sex-specific behavioral responses to alcohol and sucrose.

Keywords

Bed nucleus of the stria terminalis (BNST); Alcohol; Dopamine; D2 receptor; Pain

Introduction

Alcohol use disorder (AUD) is the most prevalent substance use disorder worldwide. According to a recent study, the rate of alcohol-related deaths increased to ~25 % during the first year of the COVID-19 pandemic [1]. AUD is characterized by repeated cycles of drinking and withdrawal accompanied by neuroadaptations in brain regions involved in motivated behaviors [2]. Decades of research have implicated several neurotransmitter systems in the pathophysiology of AUD including the mesocorticolimbic dopamine (DA) system [3,4]. Alcohol increases the firing rate of ventral tegmental area (VTA) DA neurons [5] along with an increase in dopamine release in several brain regions, such as the nucleus accumbens (NAc) [6,7] and striatum [8]. However, repeated cycles of withdrawal from chronic alcohol result in reduced DA release, and this hypodopaminergic state is hypothesized to drive craving [9–11].

DA acts on two main subfamilies of receptors, D1 and D2. These subfamilies can be further divided into subtypes, with the D1-like subfamily including dopamine D1 and D5 receptors and the D2-like subfamily including dopamine D2, D3, and D4 receptors [12]. Several lines of evidence from both human and animal studies suggest the importance of D2 receptors in AUD. Reduced levels of D2Rs in limbic areas have been observed in both AUD patients [13–17] and in alcohol-preferring rodents [18–20]. However, findings from preclinical studies in animal models have been inconsistent. For example, while pharmacological blockade of D2-like receptors in NAc reduced ethanol seeking and drinking [21,22], overexpression of striatal D2Rs also reduced ethanol self-administration and preference [23].

One possible reason for these confounding data is that D2Rs have differential effects on alcohol consumption in a cell-type and region-specific manner. However, to date, most studies have focused on VTA DA neurons and their striatal projections. The bed nucleus of the stria terminalis (BNST) is an important hub interconnecting stress and reward circuits [24–26] and receives dopaminergic inputs from several midbrain regions, including VTA, and ventrolateral periaqueductal gray/dorsal raphe (vlPAG/DR) [27–29]. Additionally, the vast majority of neurons in the dorsal BNST (dBNST) are GABAergic in phenotype [30] and send projections to areas implicated in AUD, including the VTA [31–33]. Similar to the NAc, alcohol dose-dependently increased extracellular BNST DA concentrations [34]. Further, the existence of D2Rs in BNST has been characterized using a combination of slice physiology [29,35–37], *in situ* hybridization [37–39] and receptor autoradiography [40] approaches. Prior studies have implicated BNST D2Rs in the modulation of fear generalization [38] and impulsivity [41]. However, the role of BNST D2Rs in alcohol

consumption remains unclear. Here, we used a CRISPR-Cas9-based viral approach to selectively knock down D2Rs from BNST GABA neurons and explored the role of BNST D2Rs in alcohol-related behaviors in both male and female mice.

Materials and methods

Mice

Dopamine transporter expressing (DAT-cre) mice [42] were bred and housed at the University of Washington, Seattle. Vesicular GABA transporter expressing (VGAT-ires-Cre) mice were generated as previously described [43], were bred in-house, and maintained on a C57BL6/J background. All behavioral experiments were performed on adult male (N = 33) and female mice (N = 41), aged 8–16 weeks at the start of the procedures, in accordance with the NIH guidelines for animal research and with the approval of the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. For all behavioral experiments, except the drinking studies, mice were maintained on a standard 12 h cycle (lights on at 07:00) in a temperature-controlled colony with ad libitum access to food and water. For the drinking experiments, mice were transferred to a reverse light cycle (light off at 07:00) and allowed to acclimate for a week before the start of the experiments.

Design of Drd2 CRISPR construct

A single guide RNA (sgRNA) targeting *Drd2* was designed as described before (Star Protocols; [44]). Primers for sgDrd2 were: forward CACCGAGAACTGGAGCCGGCCCTTCA and reverse AAACT-GAAGGGCCGGCTCCAGTTCTC. AAV1-FLEX-SaCas9-U6-sgRosa26 was previously published [44]. AAVs for CRISPR constructs were purified as described [44]. For sequencing validation, AAV1-FLEX-SaCas9-sgDrd2 was injected into the VTA of DAT-Cre mice along with AAV1-FLEX-EGFP-KASH. Four weeks following injection the VTA was micro-dissected, nuclei were isolated and fluorescently sorted as described in [44]. The sequence flanking the sgRNA target was amplified by PCR with the following primers: initial forward: TCTCAGCTCTGCTAGCTC and initial reverse: GCA-TAACCAGTGTGGCCA; final forward: ACTCCTGCTCACTCCTG and final reverse: CTTCTCTCTGGATACAGC. The final amplicon was deep sequencing using Amplicon-EZ (Genewiz).

Surgical procedure

Adult mice were anesthetized with isoflurane (2 %–3 %) in oxygen (0.8–1 l/min) and then secured on a stereotaxic frame (Model 1900, Kopf Instruments, CA) for intracranial viral infusions. To minimize postoperative discomfort, meloxicam (5 mg/kg SC) was administered at the time of the surgery and for two additional days. The BNST (from bregma in mm: AP +0.14 mm, ML ±0.90 mm, DV −4.25 mm) was targeted using standard coordinates from Paxinos & Franklin. Microinjections were performed with a 1 μl Neuros Hamilton syringe (Hamilton, Reno, NV) controlled by a micro-infusion pump (WPI, FL) at a rate of 100 nl/min. For behavioral experiments, AAV1-CMV-FLEX-saCas9-U6-sgDrd2 (plasmid gift from Dr. Larry Zweifel, packaged at UNC vector core; 1.3×10^{12} vg/mL) was mixed with AAV1-CAG-FLEX-EGFP-WPRE (Addgene; Cat # 51,502; 1×10^{13} vg/mL)

in a 4:1 ratio. 300 nl of this mixture was infused bilaterally in the BNST of VGAT-cre mice. The control mice received a mixture of AAV1-CMV-FLEX-saCas9-U6-sgRosa26 (plasmid gift from Dr. Larry Zweifel, packaged at UNC vector core; 3.8×10^{12} vg/mL) and the cre-dependent GFP virus. For the validation of the CRISPR-Cas9-mediated downregulation of *Drd2* expression, a separate cohort of mice was infused with the viral mixture in a 3:1 ratio. After infusion, injectors were left in place for 5 min to allow for viral diffusion. All surgeries were conducted using aseptic techniques in a sterile environment. Mice were closely monitored and allowed to recover before starting experiments.

Behavioral assays

~3–4 weeks post-surgery, age-matched mice were randomly assigned to experimental groups. One week before the first behavioral test, mice were singly housed and maintained on an Isopro RMH 3000 (LabDiet, St. Louis, MO, USA) diet, which has been shown to produce high levels of alcohol consumption [45]. On the day of the tests, animals were transported to the behavioral room and allowed to acclimate to the room for ~45–60 min. The experimental timeline is shown in Fig. 2. All mice went through the same sequence of behavioral tests. All behavioral testing except the drinking study, was carried out during the light cycle, and there was at least 48 h between test sessions. Male and female mice were tested on different days except for the ethanol-induced locomotor assay. The experimenter was blind to the genotype of the animals throughout behavioral experiments and data analysis.

Open field

The open field arena consisted of an apparatus made of white Plexiglas ($50 \times 50 \times 25$ cm). Mice were placed into the corner of the arena and allowed to freely explore for 10 min. The center of the open field was defined as the central 25 % of the arena. Light levels were ~200 lux. Sessions were video recorded and analyzed using the EthoVision software (Noldus Information Technologies, Leesburg, VA, USA).

Mechanical sensitivity

The Von Frey test was used to measure mechanical nociception, as described before [37]. Briefly, mice were confined to Plexiglas boxes on an elevated metal wire surface ($90 \times 20 \times 30$ cm) and habituated to the behavioral apparatus for a minimum of 60 min. Nylon monofilaments of forces ranging from 0.008 to 2 g (g) were applied to the hind paws using the simplified up-down method (SUDO) [46]. Starting with a mid-range force (0.16 g), the filament was applied to the mid-plantar surface of the hind paw for ten trials, then repeated with ascending or descending forces depending on the number of paw withdrawals. Withdrawal thresholds were defined as the minimum force (g) filament that elicits a withdrawal reflex for 50 % of the trials.

Thermal sensitivity

Thermal sensitivity was measured using the Hargreaves test [47] as described before [37]. Mice were placed in Plexiglas boxes on an elevated glass surface and habituated to the behavioral apparatus for at least 60 min. The beam intensity was set on the IITC Plantar

Analgesia Meter (IITC Life Science, Woodland Hills, CA). The mid-plantar surface of each hind paw was then exposed to a series of heat trials with 10 min inter-trial intervals. Four trials were conducted with radiant heat exposures sequentially alternating between left and right paws. Paw withdrawal latency was recorded. A cutoff time of 25 s was set to prevent excessive tissue damage.

Acoustic startle

The acoustic startle test was performed with the SR-LAB Startle Response System (SD Instruments). The system consisted of a sound-attenuating isolation cabinet with dimensions 38 cm × 36 cm × 46 cm containing a small plastic cylindrical enclosure with dimensions 4 cm (diameter) × 13 cm (length). Mice were placed in the plastic enclosure and allowed to acclimate for 5 min. After acclimation, mice were presented with the first of the startle stimulus trials. Ten stimuli of each intensity level (0, 90, 105, and 120 dB) were presented in a pseudo-random order (the constraint being that each intensity occurs within each block of four trials) with an intertrial interval of 30–50 s. Stimuli were delivered for 40 ms in each trial and the startle response was measured for 200 ms following stimulus delivery. Startle amplitude was defined as the largest peak 100 ms after the onset of the startle stimulus. For each animal, startle amplitudes for each stimulus level were averaged across the ten trials.

Ethanol-induced locomotion

Ethanol-induced locomotor activity was measured using a SuperFlex open field system (Omnitech Electronics, Accuscan, Columbus, OH), and a proprietary Fusion v6.5 software was used to track beam breaks to determine the total distance traveled during 30 min testing sessions (Fig. 5A). The open field arena measured 60 cm × 60 cm × 40 cm and the light levels were~25–30 lx. To reduce injection stress and novelty effects, mice were injected intraperitoneally (IP) with saline and were habituated to the locomotor boxes on days 1 and 2. On days 3 and 4, mice were placed in the locomotor boxes right after IP injection of saline or ethanol (2 g/kg) in a counterbalanced, crossover design. Ethanol (95 %) was prepared 20 % v/v in a solution of 0.9 % saline and administered intraperitoneally at a dose of 2 g/kg in a 12.5 mL/kg volume. Distance traveled (cm) was measured in 5 min bins for 30 min during the two testing sessions.

2-Bottle choice intermittent access to ethanol (2-BC IA)

After the completion of the last behavioral experiment, mice were transferred to the reverse light cycle and were habituated to two 50 ml water bottles. One week later, both male and female mice underwent 6 weeks of 2-BC IA (Fig. 6A), as described before [48]. Briefly, ~3 h into the dark cycle, mice were given free access to both water and 20% w/v ethanol bottles in their home cage for 24 h. Drinking sessions occurred three times per week (Mon, Wed, Fri). Bottles were weighed at the start and end of each session. At all other times, mice were given access to water alone. Ethanol and water bottle positions were alternated each session to account for any inherent side bias. This weekly access schedule was repeated for 6 weeks for a total of 18 sessions. To account for the fluid lost due to passive drip, ethanol, and water bottles were placed in an empty cage and weighed during each drinking session. Drip values were then calculated for each solution and total consumption was normalized to these values. The water mice had access to water alone. All mice were weighed every week

and ethanol intake was calculated as g/kg/24 h. 20% w/v ethanol was prepared fresh each week from 190-proof ethanol mixed with tap water.

Voluntary sucrose consumption

In one cohort of mice, 5 days after the final IA session, both water and ethanol mice were given free access to both water and 0.5% w/v sucrose bottles in their home cage for 2 h a day, 3 h into the dark cycle, for 5 days. Sucrose and water bottle positions were alternated each session to account for any inherent side bias. The drinking values for the last three sessions were averaged and compared.

Histology

All mice used for behavioral experiments were anesthetized with tribromoethanol (Avertin, 1 ml, IP) and transcardially perfused with ice-cold 0.01 M phosphate-buffered saline (PBS) followed by 4 % paraformaldehyde (PFA) in PBS. Brains were extracted and stored in 4 % PFA for 24 h and then moved to 30 % sucrose/PBS at 4 °C until the brains sank. 45 μm coronal sections were collected using a Leica VT1000S vibratome (Leica Biosystems; Deer Park, IL, USA) and then stored in PBS with 0.02 % sodium azide (Sigma Aldrich). Sections were mounted on slides, allowed to dry, coverslipped with VectaShield Hardset Mounting Medium with DAPI (Vector Labs, Newark, CA, USA), and stored in the dark at 4 °C. Keyence BZ-X800 All-in-one Fluorescence microscope (Keyence; Itasca, IL, USA) was used to image GFP expression to verify viral injection sites (Fig. 1E-F).

Fluorescent in situ hybridization (FISH)

To validate *Drd2* knockdown, mice were anesthetized with isoflurane, rapidly decapitated, and brains were collected and immediately frozen on dry ice and stored in a — 80 °C freezer. 12 μm thick coronal sections of BNST were obtained using a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany), and directly mounted onto Super-frost Plus slides (Fisher Scientific, Hampton, NH), and stored at −80 °C. RNAscope was performed according to the manufacturer's instructions (Advanced Cell Diagnostics, Newark, CA). To fluorescently label *Drd2* and VGAT mRNA in the BNST, slices were preprocessed with 4 % PFA and protease reagent, incubated with target probes for mouse *Drd2* and VGAT, then fluorescently labeled with probes targeting the corresponding channels (VGAT 488; Drd2 647; Advanced Cell Diagnostics, Newark, CA). The processed slides were then coverslipped using Pro-Long Gold Antifade Mountant with DAPI.

Confocal microscopy

Images were captured using a Zeiss 800 upright confocal microscope (Hooker Imaging Core, UNC–Chapel Hill). For quantification of D2R knockdown, tiled and serial z-stack images were obtained through a 20x objective (2 μm optical slice thickness), and converted to maximum projection intensity images using Zen blue software (2 slices/animal). Analysis was performed using subcellular particle detection in QuPath [49]. Cells were considered positive if they contained   5 puncta of *Drd2* mRNA.

Data and statistical analysis

All statistical analysis was performed using GraphPad Prism v.9 (La Jolla, CA, USA). Group comparisons were made using either a standard unpaired Student's *t*-test or two/three-way ANOVA depending on the number of independent and within-subjects variables in a dataset. Following significant interactions or main effects, planned post hoc analyses were performed and corrected for multiplicity. Male and female data were analyzed disaggregated as well as with a factorial design with sex as a covariate. If data violated assumptions of normality a Mann-Whitney U test was used for analysis. Data are expressed as mean ± SEM unless otherwise stated. P-values   0.05 were considered significant. A total of 4 mice were excluded from the analysis due to no AAV expression within the BNST.

Results

CRISPR-Cas9-mediated reduction in Drd2 expression

To define the contribution of BNST D2 receptors in alcohol-related behaviors, we used a CRISPR-Cas9 system to conditionally knock down D2R gene expression from BNST^{vgat} neurons (BNST^{vgat} sg*Drd2*) in male and female mice. We packaged a plasmid for cre-dependent expression of Staphylococcus aureus Cas9 (SaCas9) plus a single guide RNA (sgRNA) targeted to the gene encoding *Drd2* (sgDrd2) in an adeno-associated virus (AAV) vector (Fig. 1A) [44]. Mutagenesis was confirmed by targeted deep sequencing of the genomic region flanking the sgRNA (Supplementary to Fig. 1). FACS and Sanger sequencing from the VTA showed ~80 % mutagenesis in GFP+ cells (dopamine neurons) and none in GFP- cells, supporting the cre-dependence of this vector (data not shown). In a separate cohort, we injected sgDrd2 construct or a control virus (sgRosa26) bilaterally into the BNST of VGAT-cre mice (N = 5–6 mice/sex/group). An additional cre-dependent virus that drove the expression of GFP was co-administered to visualize the injection site. After waiting for at least 4 weeks to ensure sufficient viral expression we confirmed the mutagenesis of *Drd2* by performing fluorescent *in situ* hybridization (Fig. 1B-C). Quantification of *Drd2* mRNA expression in dBNST revealed a significant reduction in BNST^{vgat} sg*Drd2* mice compared to control animals with no effect of sex on *Drd2* expression (Fig. 1D; main effect of virus [$F_{(1,17)}$=15.7; p_{virus}=0.0010], but no effect of sex [$F_{(1,17)}$=0.06; p_{sex}=0.81] or sex x virus interaction [$F_{(1,17)}$=0.56; $p_{interaction}$=0.466]). When normalized to average *Drd2*$^+$ neurons in the control group, CRISPR knockout resulted in ~50 % reduction in *Drd2*$^+$ mRNA expression (t_9 = 4.78; p = 0.001; one sample t-test). In mice used for behavioral testing, we confirmed viral infection in BNST using fluorescence microscopy to visualize GFP expression (Fig. 1E-F).

Effect of BNST^{vgat} Drd2 knockdown on avoidance behavior and pain sensitivity

After waiting for 4 weeks to ensure sufficient expression of the virus, all mice went through a battery of behavioral assays to assess the impact of the reduction in BNST^{vgat} *Drd2* on avoidance-like behavior, pain sensitivity, and alcohol-related behaviors (Fig. 2). To assess the effect of knockdown on avoidance-like behavior, mice were placed in an open field arena and the total distance traveled and the time spent in the center zone was calculated. Selective knockdown of BNST^{vgat} *Drd2* (N = 15–16 male and 19–20 female mice per group) did not affect total distance traveled (Fig. 3A). A two-way ANOVA revealed a

significant main effect of sex [$F_{(1,66)}$=8.723; p_{sex} =0.004] but no main effect of virus [$F_{(1,66)}$=0.627; p_{virus}=0.431] or sex x virus interaction [$F_{(1,66)}$=0.0003; $p_{interaction}$=0.987]. Post-hoc comparisons showed a trend for increased locomotion in females [t_{66} = 2.13, $p_{corrected}$ = 0.072 for control mice and t_{66} = 2.05, $p_{corrected}$ = 0.087; Sidak's test]. Reduction in BNST^{vgat} *Drd2* expression did not alter the time spent in the center zone in both sexes (Fig. 3B; [$F_{(1,66)}$=0.048; p_{virus} =0.827]; [$F_{(1,66)}$=0.676; p_{sex} =0.414]; [$F_{(1,66)}$=0.322; $p_{interaction}$ =0.572]).

Next, we asked whether a region and cell-type-specific reduction in *Drd2* gene expression could modulate pain sensitivity. The Hargreaves test was used to measure thermal sensitivity. We found no significant effect of knockdown on paw withdrawal latency in both sexes (Fig. 3C; [$F_{(1,66)}$=0.165; p_{virus} =0.686]; [$F_{(1,66)}$=0.39; p_{sex} =0.535]; [$F_{(1,66)}$=0.286; $p_{interaction}$ =0.594]). To assess the impact of *Drd2* knockdown on mechanical sensitivity, we used the Von Frey assay. Interestingly, when analyzed separately, BNST^{vgat} sg*Drd2* female mice showed a significant reduction in withdrawal threshold (Fig. 3E; [U = 114.5; p = 0.032]). This effect was absent in male mice (Fig. 3D; p = 0.608). A two-way ANOVA with sex and virus as between-subject factors (Fig. 3F) showed a significant main effect of sex [$F_{(1,66)}$=6.038; p_{sex} =0.017] and a non-significant trend for knockdown effect [$F_{(1,66)}$=2.94; p_{virus}=0.091], but no sex x virus interaction [$F_{(1,66)}$=0.837; $p_{interaction}$=0.364]. Importantly, post-hoc analyses showed a significant difference in mechanical sensitivity in BNST^{vgat} sg*Drd2* females compared to BNST^{vgat} sg*Drd2* males [U = 66.5, $p_{corrected}$ = 0.013; Holm-Sidak test].

Reduction in BNST^{vgat} Drd2 expression does not alter startle response

To characterize the role of BNST D2Rs in regulating arousal-like behavior, mice were tested for acoustic startle responses (Fig. 4). Male (N = 15–16/group) and female mice (N = 19–20/group) were presented with four different levels of sound intensities in a randomized manner and the startle amplitudes were recorded. Sound intensities ranging from 0 to 120 dB resulted in increased startle responses in both males (Fig. 4A; [$F_{(1.7,48.17)}$=44.26; p_{dB}<0.001]) and females (Fig. 4B; [$F_{(1.71,63.27)}$=53.32; p_{dB}<0.001]) as revealed by repeated-measures two-way ANOVA with Geisser-Greenhouse correction. However, there was no main effect of *Drd2* knockdown ([$F_{(1,29)}$=0.104; p_{virus}=0.75 for males]; [$F_{(1,37)}$=0.186; p_{virus}=0.67 for females]) or dB x virus interaction ([$F_{(3,87)}$=0.294; $p_{interaction}$=0.83 for males]; [$F_{(3111)}$=1.91; $p_{interaction}$=0.133 for females]) in both sexes. Comparing the startle amplitude at 120 dB (Fig. 4C) revealed a main effect of sex [$F_{(1,66)}$=12.5; p_{sex}=0.0007], but no effect of virus [$F_{(1,66)}$=0.047; p_{virus}=0.83] or sex x virus interaction [$F_{(1,66)}$=1.08; $p_{interaction}$=0.303]. Post-hoc analyses with Holm-Sidak correction indicated an overall reduced startle response in females compared to males ([U = 86, $p_{corrected}$ = 0.036; for controls]; [U = 77, $p_{corrected}$ = 0.036; for BNST^{vgat} sg*Drd2*]).

BNST knockdown of Drd2 expression enhances stimulatory effects of ethanol in a sex-specific manner

Global knockout of D2 receptors in mice enhances ethanol-induced locomotion [50] in a familiar environment. Therefore, we wanted to evaluate whether knockdown of *Drd2* gene from BNST^{vgat} neurons altered the stimulatory effects of acute ethanol (Fig. 5).

After habituation to the locomotor boxes, male ($N = 14–15$/group) and female ($N = 19$/group) mice were challenged with saline and 2 g/kg of ethanol in a counterbalanced, within-subjects design, and locomotion was quantified using infrared beam breaks (Fig. 5A). Consistent with the literature, ethanol produced transient, stimulatory effects that peaked within the first 5 min (Fig. 5B-C) [51,52]. When both sexes were analyzed separately, a three-way RM-ANOVA revealed a main effect of time ([$F_{(5135)}=60.54$; $p_{time}<0.0001$ for males]; [$F_{(5180)}=220.5$; $p_{time}<0.0001$ for females]), ethanol ([$F_{(1,27)}=26.07$; $p_{etoh}<0.001$ for males]; [$F_{(1, 36)}=15.31$; $p_{etoh}=0.004$ for females]) and a time x ethanol interaction ([$F_{(5135)}=26.91$; $p_{interaction}<0.0001$ for males]; [$F_{(5180)}=108.4$; $p_{interaction}<0.0001$ for females]). Interestingly, only male mice had a significant effect of virus ([$F_{(1,27)}=5.59$; $p_{virus}=0.026$ for males]; [$F_{(1,36)}=0.004$; $p_{virus}=0.947$ for females]). A two-way RM-ANOVA in male mice comparing the distance traveled during the first 5 min resulted in heightened ethanol-induced stimulation in BNST^{vgat} sg*Drd2* mice (Fig. 5D). Specifically, there was a main effect of both virus [$F_{(1, 27)}=4.83$; $p_{virus}=0.037$] and ethanol [$F_{(1,27)}=68.54$; $p_{etoh}<0.001$], but no interaction effects [$F_{(1,27)}=2.08$; $p_{interaction}=0.16$]. Post-hoc analyses with Holm-Sidak correction indicated a significant increase in locomotion following ethanol injection when compared to saline ([$t_{27}=4.92$, $p_{corrected}<0.0001$; for controls]; [$t_{27}=6.76$, $p_{corrected}<0.0001$; for BNST^{vgat} sg*Drd2*]) with the magnitude of response being significantly larger in BNST^{vgat} sg*Drd2* male mice [$t_{54}=2.63$, $p_{corrected} = 0.022$]. In female mice (Fig. 5E), there was a main effect of ethanol [$F_{(1,36)}=221.6$; $p_{etoh}<0.001$], but no effect of virus [$F_{(1,36)}=1.01$; $p_{virus}=0.322$] or a virus x etoh interaction [$F_{(1,36)}=1.66$; $p_{interaction}=0.205$]. Similar to male mice, post-hoc analyses indicated a significant increase in locomotion following ethanol injection when compared to saline ([$t_{36}=11.44$, $p_{corrected}<0.0001$; for controls]; [$t_{36}=9.62$, $p_{corrected}<0.0001$; for BNST^{vgat} sg*Drd2*]; Holm-Sidak correction). When compared with sex as a covariate (Fig. 5F), a 3-way RM-ANOVA revealed a main effect of ethanol [$F_{(1,63)}=257.1$; $p_{etoh}<0.0001$], sex [$F_{(1,63)}=20.53$; $p_{sex}<0.0001$] and significant interactions between ethanol x sex [$F_{(1,63)}=13.24$; $p_{interaction}=0.0006$] and sex x virus [$F_{(1,63)}=5.01$; $p_{interaction}=0.029$]. Additionally, there was a statistical trend toward a 3-way interaction of sex, virus, and ethanol [$F_{(1,63)}=3.74$; $p_{interaction}=0.058$]. Consistent with known sex differences [53], on average, control female mice showed higher ethanol-induced stimulation when compared to male mice ([$t_{126}=6.24$, $p_{corrected}<0.0001$]; Holm-Sidak's correction). Compared to saline, ethanol injection resulted in increased locomotion in both sexes across groups (all $p < 0.0001$).

CRISPR knockdown of BNST^{vgat} Drd2 increases alcohol and sucrose intake in male mice

One week after the completion of ethanol-induced locomotion assay, both male ($N = 8$/group) and female ($N = 6–8$/group) mice were randomly assigned to either ethanol or water group. Mice in the ethanol group underwent 6 weeks of voluntary alcohol consumption in a two-bottle choice intermittent access to ethanol paradigm (Fig. 6A). Knockdown of BNST^{vgat} *Drd2* increased alcohol intake in males (Fig. 6B; [$F_{(1,14)}=6.33$; $p_{virus}=0.025$]; [$F_{(3.61,50.48)}=7.89$; $p_{weeks}<0.001$]; [$F_{(5,70)}=0.61$; $p_{interaction}=0.693$]) but not females (Fig. 6C; [$F_{(2.92,35.09)}=6.64$; $p_{weeks}=0.001$]; [$F_{(1,12)}=0.17$; $p_{virus}=0.688$]; [$F_{(5,60)}=0.45$; $p_{interaction}=0.813$]), as revealed by a two-way RM ANOVA with Geisser-Greenhouse correction. Consistent with the literature, when compared between sexes, female mice generally consumed more alcohol than males (Fig. 6D; [$F_{(1,26)}=36.28$; $p_{sex}<0.0001$])

[54]. Specifically, post-hoc analyses revealed female mice in both groups consumed more alcohol than their male counterparts ($t_{26}=5.68$, $p_{corrected}<0.0001$; for controls]; [$t_{26}=2.95$, $p_{corrected}=0.013$; for BNST^{vgat} sg*Drd2*]; Holm-Sidak's correction). ([$t_{26}=5.68$, $p_{corrected}<0.0001$]; Holm-Sidak correction). While not significant, there was a trend toward a significant interaction between sex and virus ([$F_{(1,26)}=2.87$; $p_{interaction}=0.102$]; [$F_{(1,26)}=0.93$; $p_{virus}=0.344$]).

Male BNST^{vgat} sg*Drd2* mice also had a significantly higher cumulative alcohol intake (Fig. 6G; [$F_{(1,14)}=5.71$; $p_{virus}=0.032$]; [$F_{(1.25,17.47)}=576.6$; $p_{weeks}<0.001$]; [$F_{(5,70)}=4.91$; $p_{interaction}=0.0007$]) and a non-significant trend toward increased ethanol preference (Fig. 6E; [$F_{(1,14)}=2.86$; $p_{virus}=0.113$]; [$F_{(3.28,45.87)}=21.85$; $p_{weeks}<0.001$]; [$F_{(5,70)}=0.537$; $p_{interaction}=0.748$]). There was no effect of knockdown on average fluid intake in males ($t_{14}=0.74$; $p = 0.474$; data not shown). In female mice, there was a main effect of time on both cumulative intake (Fig. 6H; [$F_{(1.15,13.74)}=408.3$; $p_{weeks}<0.001$]) and ethanol preference (Fig. 6F; [$F_{(3.18,38.12)}=10.79$; $p_{weeks}<0.001$]), but no effect of either knockout ([$F_{(1,12)}=0.14$; $p_{virus}=0.71$; for cumulative intake]; [$F_{(1,12)}=0.0005$; $p_{virus}=0.98$; for ethanol preference]) or time x virus interaction ([$F_{(5,60)}=0.14$; $p_{interaction}=0.983$; for cumulative intake]; [$F_{(5,60)}=0.53$; $p_{interaction}=0.752$; for ethanol preference]. Similar to male mice, D2R knockdown did not affect the mean fluid intake ($t_{12}=0.88$; $p = 0.399$; data not shown).

To determine the selectivity of BNST^{vgat} *Drd2* knockdown on alcohol consumption, in one cohort, mice ($N = 6$/group for males and $N = 8$/group for females) were given 2 h access to a bottle of 0.5% w/v sucrose in addition to water five days after the last IA session (Fig. 7). Mice in both the ethanol and the water groups were given access to sucrose and drinking data was averaged over the last three days. Similar to effects on alcohol drinking, conditional knockdown of BNST^{vgat} *Drd2* in the BNST significantly increased sucrose intake in males (Fig. 7A; [$t_{10}=3.53$; $p = 0.006$]) but not females (Fig. 7B; [$t_{14}=1.17$; $p = 0.261$]). A two-way ANOVA revealed a main effect of sex [$F_{(1,24)}=25.57$; $p_{sex}<0.0001$] but not virus [$F_{(1,24)}=1.48$; $p_{virus}=0.235$] on sucrose intake (Fig. 7C) along with a significant interaction between sex and knockdown effects [$F_{(1,24)}=8.69$; $p_{interaction}=0.007$]. Post-hoc analyses showed female controls consumed more sucrose than male controls ([$t_{24}=5.66$; $p_{corrected}<0.0001$ for controls]; [$t_{24}=1.49$; $p_{corrected}=0.275$ for KOs]) but only male BNST^{vgat} sg*Drd2* mice consumed more compared to male controls ([$t_{24}=2.75$; $p_{corrected}=0.022$ for males]; [$t_{24}=1.32$; $p_{corrected}=0.358$ for females]).

BNST^{vgat} sg*Drd2* male mice also had a higher sucrose preference (Fig. 7D; [$t_{10}=3.45$; $p = 0.006$]) which was absent in female mice (Fig. 7E; [$t_{14}=0.35$; $p = 0.73$]). A two-way ANOVA revealed a main effect of virus [$F_{(1,24)}=10.36$; $p_{virus}=0.004$] but not sex [$F_{(1,24)}=2.09$; $p_{sex}=0.161$] on sucrose preference (Fig. 7F) along with a significant interaction between sex and knockdown effects [$F_{(1,24)}=7.90$; $p_{interaction}=0.01$]. Post-hoc analyses showed female controls had higher sucrose preference compared to male controls ([$t_{24}=3.01$; $p_{corrected}=0.012$ for controls]; [$t_{24}=0.97$; $p_{corrected}=0.57$ for BNST^{vgat} sg*Drd2*]), but only male BNST^{vgat} sg*Drd2* mice displayed higher sucrose preference over their male counterparts ([$t_{24}=3.99$; $p_{corrected}=0.001$ for males]; [$t_{24}=0.31$; $p_{corrected}=0.942$ for females]).

Discussion

In our current study, we used a combinatorial genetic and CRISPR/SaCas9-based approach to probe the contributions of BNST dopamine D2 receptors in alcohol consumption and other relevant behaviors in male and female mice. Reduction of D2R gene expression in BNST GABA neurons resulted in distinct sex-specific behavioral changes. Our results add to the growing body of literature supporting the interpretation that D2Rs are critical for the rewarding and locomotor properties of alcohol.

BNST^{vgat} D2Rs and pain sensitivity

Preclinical studies have highlighted the role of BNST in the modulation of pain-related affect [55–58,]. In a study by Hagiwara et al., direct injection of a D1 receptor antagonist in the BNST enhanced formalin-induced hyperalgesia only in female rats [56], supporting a role for BNST DA signaling in pain. Prior work from our group identified a novel vlPAG/DR to dBNST DA circuit that modulates pain responses in a sex-specific manner [29,37]. We demonstrated that activation of vlPAG/DR dopaminergic projections to the BNST resulted in reduced pain sensitivity in male mice whereas in female mice it led to increased locomotion in a salient context. We also found sex-specific differences in BNST D2R mRNA expression with lower D2R mRNA in female mice [37]. Surprisingly, in the present study, knockdown of BNST^{vgat} *Drd2* did not alter mechanical or thermal nociception in male mice but increased mechanical nociceptive sensitivity in female mice. In contrast to our previous study, in our evaluation of the CRISPR vector efficacy in knocking down expression of *Drd2* we did not see any sex differences in *Drd2* expression. This lack of difference may be due to differences in the strain (C57BL/6 J vs Vgat-cre) or age of animals (6–8 weeks vs. 14–18 weeks). It is notable that despite this lack of difference in *Drd2* expression and lack of difference in the efficacy of knockdown, we still see sex-specific effects on pain. The reason for this difference is unclear but is supportive of our previous results that there is sex-specific control of pain behavior relating to the BNST.

BNST^{vgat} D2Rs and ethanol-induced locomotion

In rodents, IP injection of ethanol results in a transient increase in locomotion in a dose-dependent manner [51,52,50]. Enhanced sensitivity to the stimulatory effects of ethanol and resilience to the sedative properties are known to be associated with vulnerability for AUD [59, 60]. In our present study, we observed heightened ethanol-induced stimulation in BNST^{vgat} knockdown male mice. Our findings are consistent with prior work evaluating the effects of D2R deletion on ethanol locomotion. For example, in a study by Palmer et al., mice with global deletion of D2Rs showed enhanced ethanol-induced stimulation when tested in a familiar environment [50]. Similarly, using a transgenic approach, Bocarsly et al., found mice with selective deletion of D2Rs on striatal medium spiny neurons had a similar phenotype [61]. Interestingly, unlike the other two studies, we found a sex-specific effect since D2R knockdown did not alter ethanol stimulation in female mice. Collectively, our results suggest a causal link between BNST^{vgat} *Drd2* downregulation and ethanol-induced stimulation. A limitation of this data is that while disaggregated sex data showed a significant effect of ethanol on locomotion in males, this effect was lost when sex was

incorporated as a variable. Additional work is needed to examine whether BNST^{vgat} *Drd2* knockdown also provides resilience to the sedative properties of acute ethanol.

BNST^{vgat} D2Rs and voluntary alcohol intake

Though equivocal, it has been suggested that low levels of D2R expression are a predisposing factor for AUD [13,16,17]. Thus, one might predict that reducing D2R activity either through pharmacological or genetic approaches would enhance ethanol consumption. Consistent with this idea, here we report that genetic suppression of BNST^{vgat} D2Rs increased voluntary alcohol drinking in male mice in a two-bottle choice intermittent access paradigm. D2R knockdown also resulted in increased sucrose drinking in male mice suggesting a role for BNST D2Rs in the general consumption of palatable reward. Interestingly, neither alcohol nor sucrose intake was affected in females, hinting toward a plausible sexually dimorphic regulation of drinking behaviors by BNST D2Rs. However, our findings differ from prior work involving pharmacological and genetic manipulations of D2Rs. For example, previous studies using D2R-deficient mice showed a reduction in ethanol preference [62], ethanol-induced conditional place preference [63] as well as a lack of operant ethanol self-administration [64]. Similarly, results from studies using pharmacological methods have been inconclusive. In some studies, D2-like antagonists attenuated ethanol intake in rats and mice [65] while in another study, D2-like antagonists increased alcohol drinking [66,67]. Specific to BNST, prior work from Eiler II et al., demonstrated pharmacological blockade of D1-like receptors but not D2-like receptors in the BNST reduced ethanol and sucrose self-administration in male and female alcohol-preferring P rats [68]. This discrepancy with earlier work could be due to a multitude of reasons including lack of specificity, differences in animal models, and differences in experimental protocols.

Conclusions and functional implications

Using a combination of techniques, we and others have established the role of BNST in the maintenance of AUD. For example, alcohol withdrawal drives neuroplastic changes in BNST [69–72] and chemo-genetic manipulation of specific subpopulations of neurons alters binge-like drinking [73,74]. Alcohol is also associated with sexually dimorphic responses within the BNST [75,76]. In the current study, by combining transgenic mice with a CRISPR-based loss of function approach, we were able to dissect the contributions of D2R-expressed on BNST GABA neurons in alcohol-related behaviors. Our findings further implicate BNST in alcohol misuse by providing evidence for a causal link between BNST D2Rs and alcohol-related behaviors. Notably, though our findings suggest BNST^{vgat} D2Rs modulate alcohol and sucrose intake selectively in males, we cannot exclude the possibility that a lack of effect in females could be related to higher variability in female drinking data or variability in the viral expression and spread.

Previously, we and others have shown the presence of mostly non-overlapping D1 and D2 receptor-expressing neurons, primarily within the lateral part of dBNST [38,39,37]. Therefore, we cannot exclude the possibility of compensatory adaptations in BNST D1R function to overcome the lower levels of *Drd2*. However, we do not have data to support this possibility. Recent work from the Alvarez lab identified an upregulation of striatal

D1R function in response to selective deletion of D2Rs from medium spiny neurons as the driving force for escalated drinking and heightened ethanol stimulation [61]. Future studies would be required to examine how the D1 and D2 systems work together within the BNST to modulate alcohol-relevant behaviors. Beyond this, it is also important to consider how D2R expression may overlap with known markers in the BNST, and how deletion of D2R may drive changes in some of these markers. Interestingly, a previous study found, using a D2R-eGFP mouse, that the oval nucleus of the BNST contains a high proportion of D2R-containing neurons and that this population is enriched in PKC-delta expression [38]. This suggests that suppression of *Drd2* expression could lead to a more robust engagement of PKC-delta neurons in the BNST. These neurons have not been directly evaluated for a role in alcohol drinking and pain, but this is a potentially exciting future direction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Data availability

Data will be made available on request.

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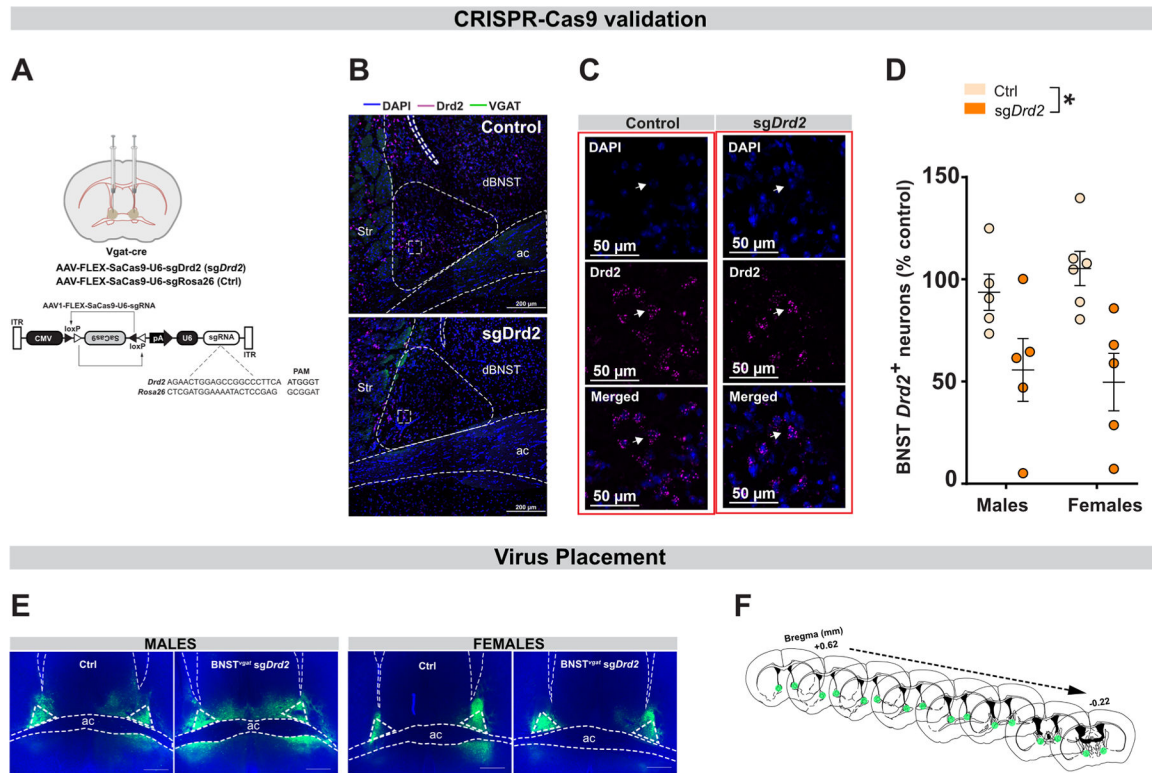
Fig. 1.

Validation of CRISPR-Cas9-mediated knockdown of BNST^{vgat} *Drd2* gene expression. (A)
Top: Surgical schematic of bilateral virus infusion in the BNST of VGAT-cre male and
female mice. Bottom: Detailed schematic of *Drd2* (sg*Drd2*) and Rosa26 (control) CRISPR
construct provided by the Zweifel lab. (B) Representative images of *in situ* hybridization for
D2 (cyan) and VGAT (green) mRNA from control (top) and sg*Drd2* (bottom) in the mouse
BNST. Scale bar=200 μm. (C) Representative images at higher magnification showing *Drd2*
(cyan) labeling inside neurons labeled with DAPI (blue) from control (left) and sg*Drd2*
(right) mice. Scale bar=50 μm. (D) Compared to controls, sg*Drd2* mice showed a significant
reduction in *Drd2* mRNA expression in the dBNST. (E) Representative viral infections in
BNST in males (left) and females (right), as indexed by GFP expression. Scale bar=500 μm.
(F) Representative map of viral spread from +0.62 mm to −0.22 mm (from bregma). ac:
anterior commissure; str: striatum. Data expressed as Mean ± SEM. *$p < 0.05$.

Experimental Timeline
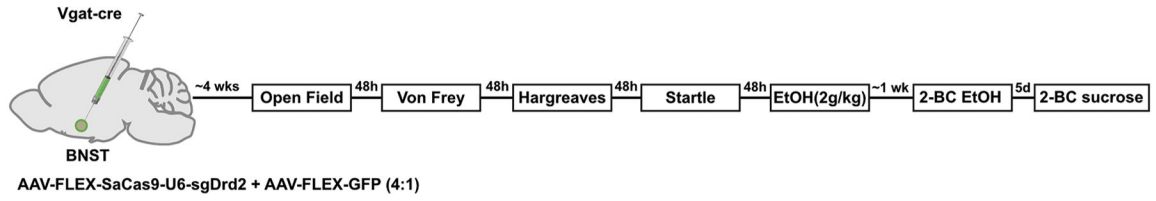
Fig. 2.

Experimental Timeline. Schematics of the experimental timeline showing the sequence followed for behavioral assays.
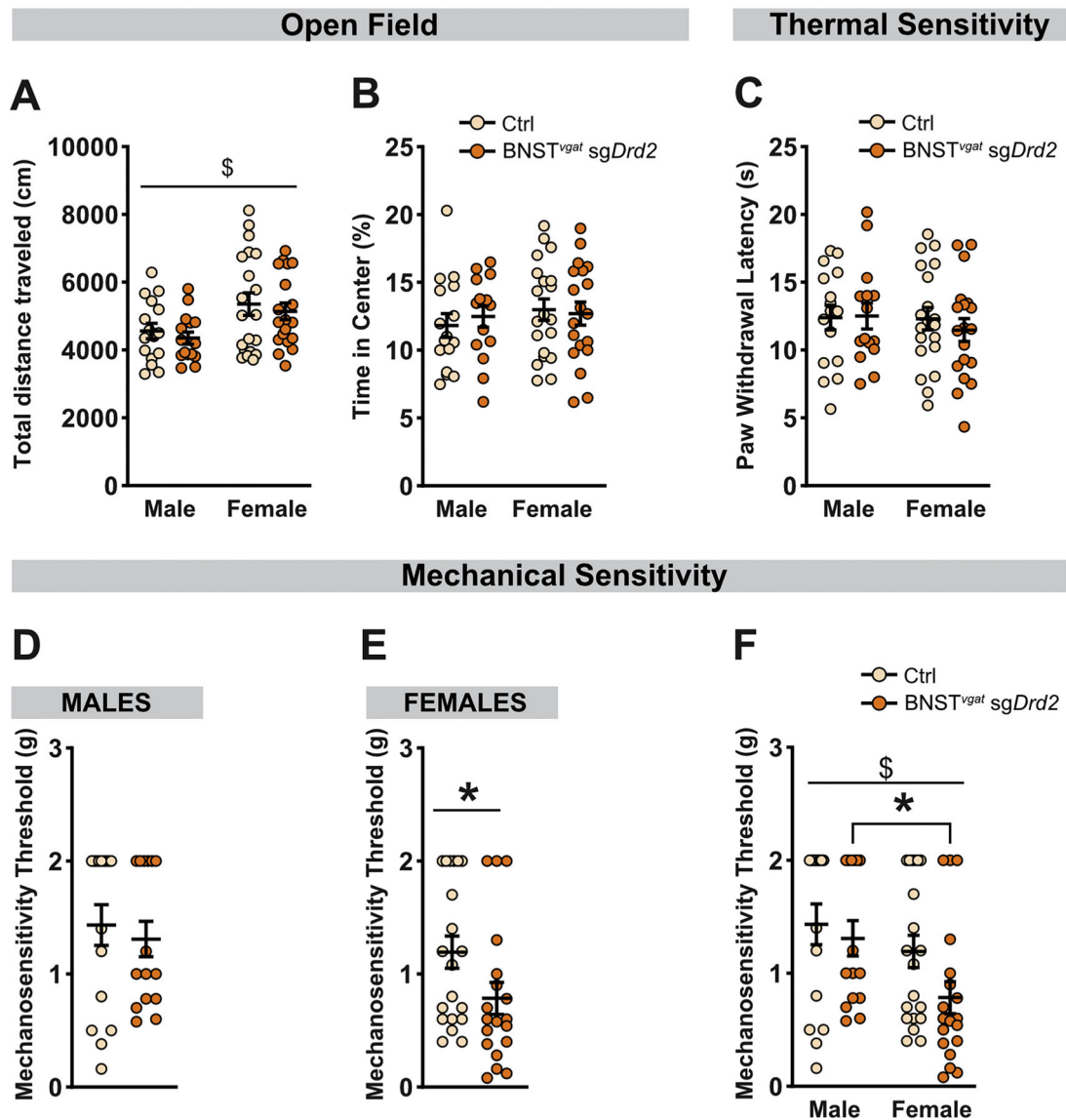
Fig. 3.

Impact of BNST^{vgat} *Drd2* knockdown on avoidance-like behavior and pain sensitivity. Avoidance-like behavior was assessed in an open field assay. No differences were observed either in the total distance traveled (cm) (A) or percentage time spent in the center of the open field arena (B) following knockdown. Hargreaves test was used to assess thermal sensitivity and average paw withdrawal latencies were compared (C). In both sexes, reduction in *Drd2* had no impact on withdrawal latency. Von Frey test was used to assess the contribution of BNST D2Rs in mechanical sensitivity (D-F). Paw withdrawal threshold was defined as the minimum force (g) filament that elicits a withdrawal reflex for 50 % of the trials. (D) No effect of knockdown on withdrawal threshold in male mice. (E) BNST^{vgat} sg*Drd2* female mice had a significantly lower withdrawal threshold compared to female controls. (F) There was a main effect of sex on mechanical pain threshold and a non-significant trend for a main effect of knockdown. Post-hoc analysis revealed a significantly
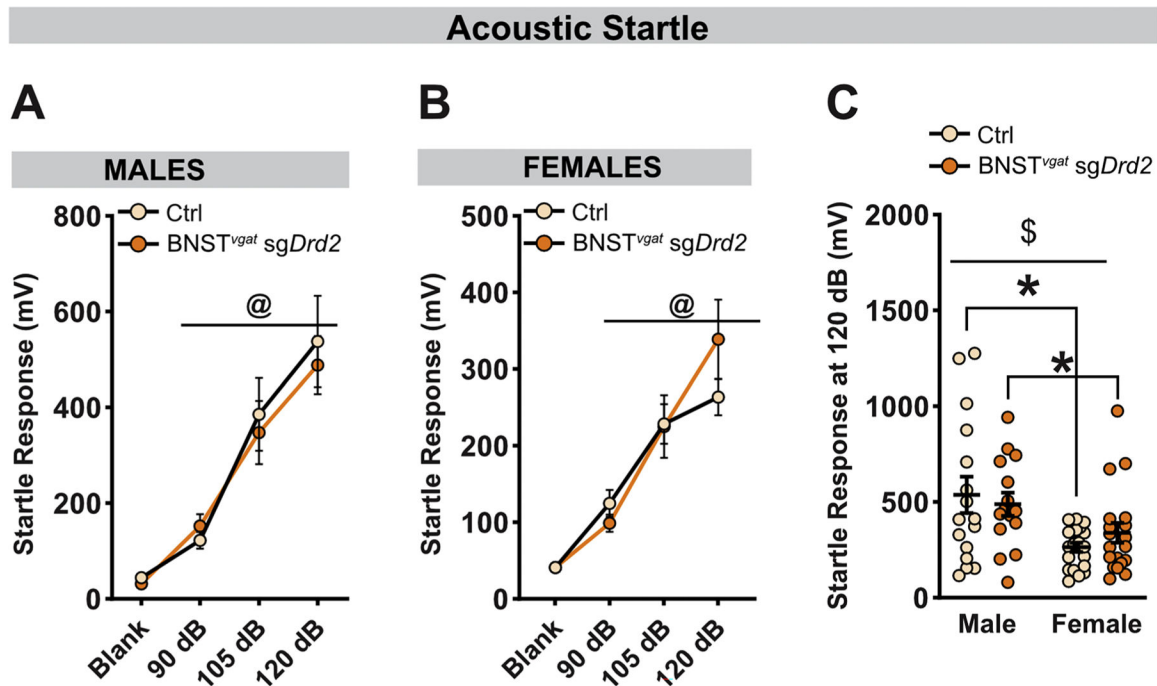
lower threshold in BNST^{vgat} sg$Drd2$ females compared to BNST^{vgat} sg$Drd2$ males. Data expressed as Mean ± SEM.*$p < 0.05$; $ main effect of sex.

Fig. 4.

Downregulation of BNST^{vgat} *Drd2* expression does not affect acoustic startle response. Average startle amplitudes (10 trials/dB) were plotted in response to increasing sound intensities. No effect of reduced *Drd2* expression on startle amplitude in males (A) and females (B). (C) Comparison of startle response at 120 dB between male and female mice. Overall, female mice displayed lower amplitude compared to male mice. No differences were observed between BNST^{vgat} sg*Drd2* and control mice. Data expressed as Mean ± SEM.*$p < 0.05$; @ main effect of decibel; $ main effect of sex.
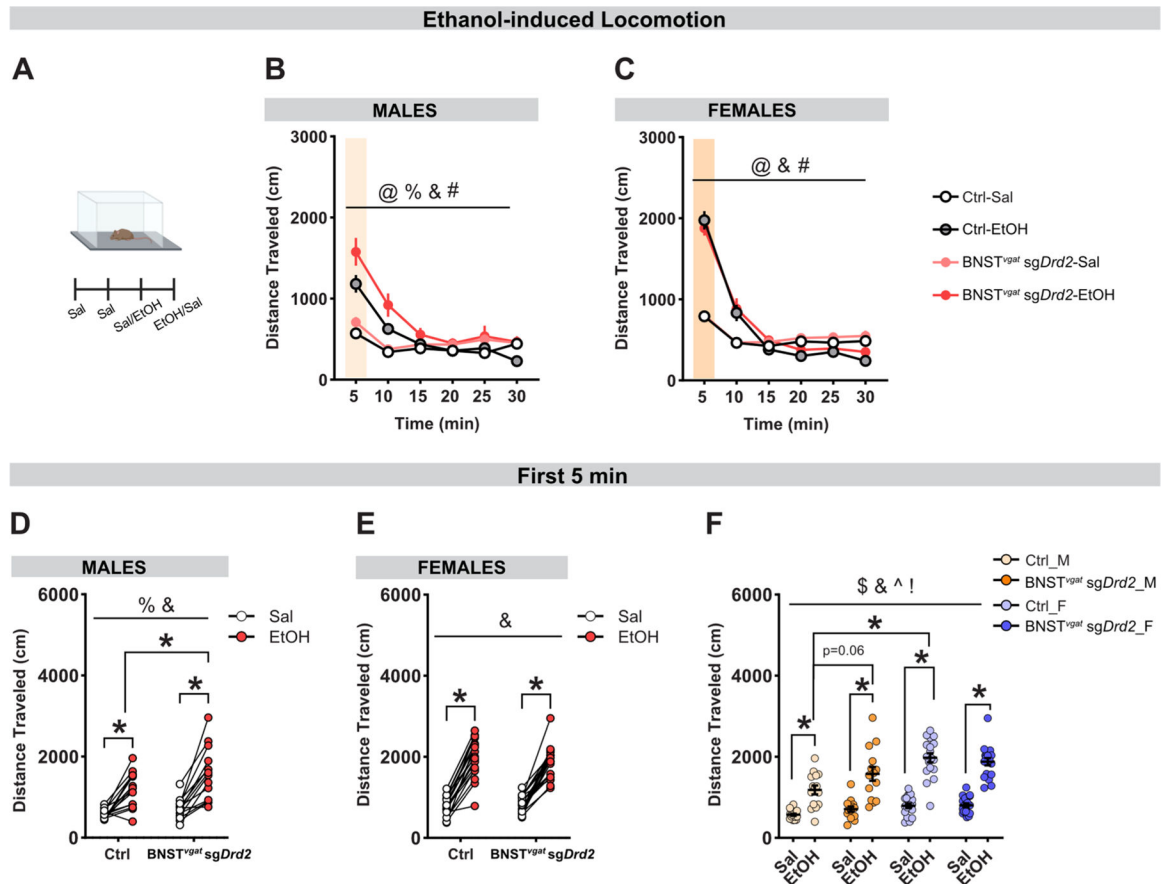
Fig. 5.

Reduction in BNST^{vgat} *Drd2* expression promotes ethanol-induced locomotion in male mice. (A) Schematic diagram representing ethanol-induced locomotor assay. Mice were habituated to the locomotor boxes for two days followed by IP injection of saline and 2 g/kg ethanol in a counter-balanced, crossover design. Total distance traveled (cm) was plotted in 5 min bins for 30 min. 2 g/kg ethanol injection resulted in a transient increase in locomotion in both sexes (B-C). Knockdown of *Drd2* gene altered the total distance traveled in a sex-dependent manner (D-F). Average distance traveled in the first 5 min post-drug injection was compared across groups. In both sexes, ethanol IP resulted in increased locomotion compared to saline. Compared to control males, BNST^{vgat} sg*Drd2* males had a stronger stimulatory response to ethanol (D). There was no effect of knockdown on ethanol-induced locomotion in female mice (E). (F) Female control mice traveled greater distances following ethanol injection compared to male control mice. Data expressed as Mean ± SEM. *$p <$ 0.05; @ main effect of time;% main effect of virus; & main effect of ethanol; $ main effect of sex; # ethanol x time interaction; ^ ethanol x sex interaction; ! sex x virus interaction; ethanol x sex x virus interaction $p = 0.058$.
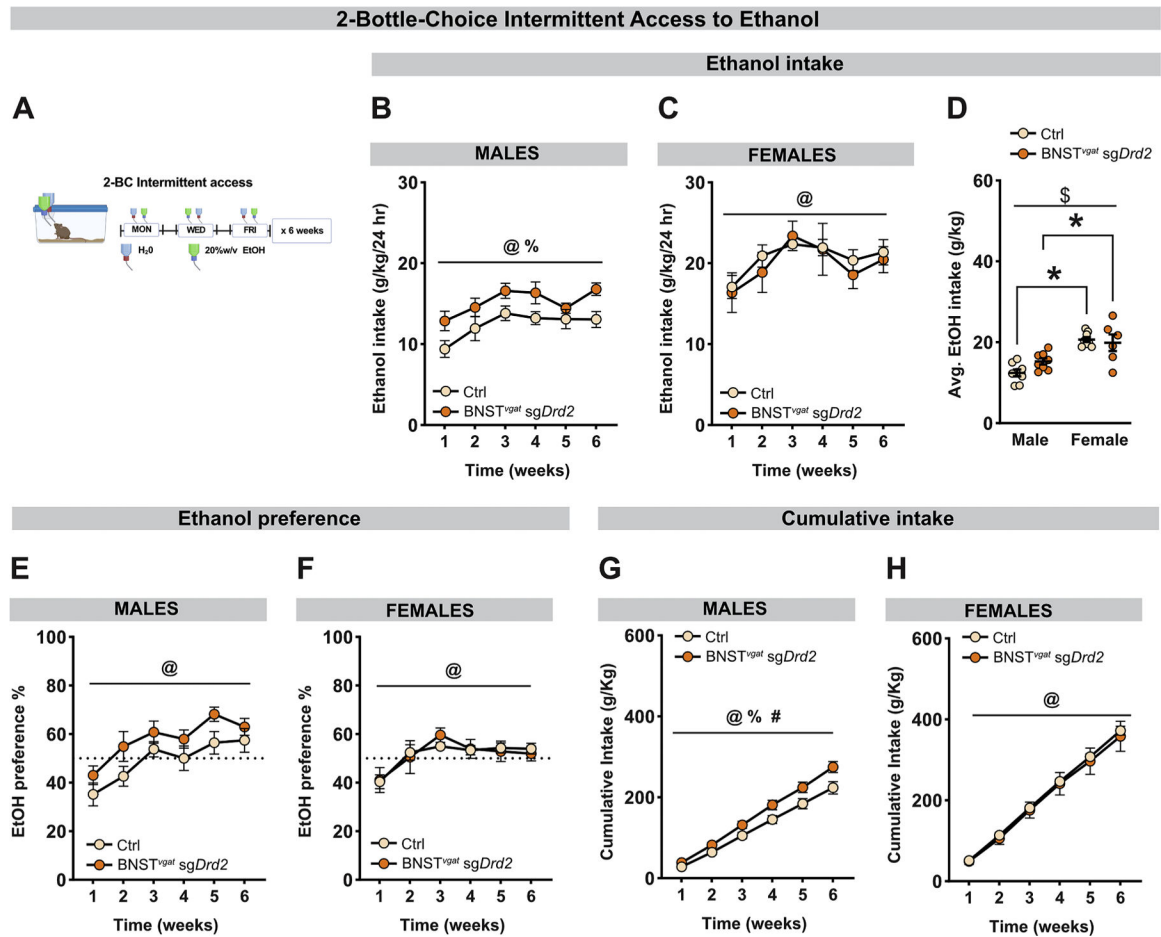
Fig. 6.

Knockdown of BNST*vgat Drd2* increases alcohol consumption in males. (A) Schematic diagram representing two-bottle choice intermittent access to 20% w/v ethanol in mice for 6 weeks. Average weekly alcohol intake (g/kg/24 h) in male (B) and female (C) mice for 6 consecutive weeks. Reduction in *Drd2* expression increased alcohol intake in males but not females. (D) Averages of weekly intake compared between sexes. Female mice consumed more alcohol compared to male mice and there was a trend toward a sex x virus interaction ($p = 0.10$). (E-F) Downregulation of BNST*vgat Drd2* did not affect ethanol preference in both sexes, although there was a non-significant trend in increased ethanol preference in male mice ($p = 0.11$). (G) Cumulative alcohol intake plotted over 6 consecutive weeks. BNST*vgat* sg*Drd2* males had a higher cumulative intake than controls. (H) No effect of knockdown on cumulative intake in females. Data expressed as Mean ± SEM.*$p < 0.05$; @ main effect of time;% main effect of virus; $ main effect of sex; # virus x time interaction.
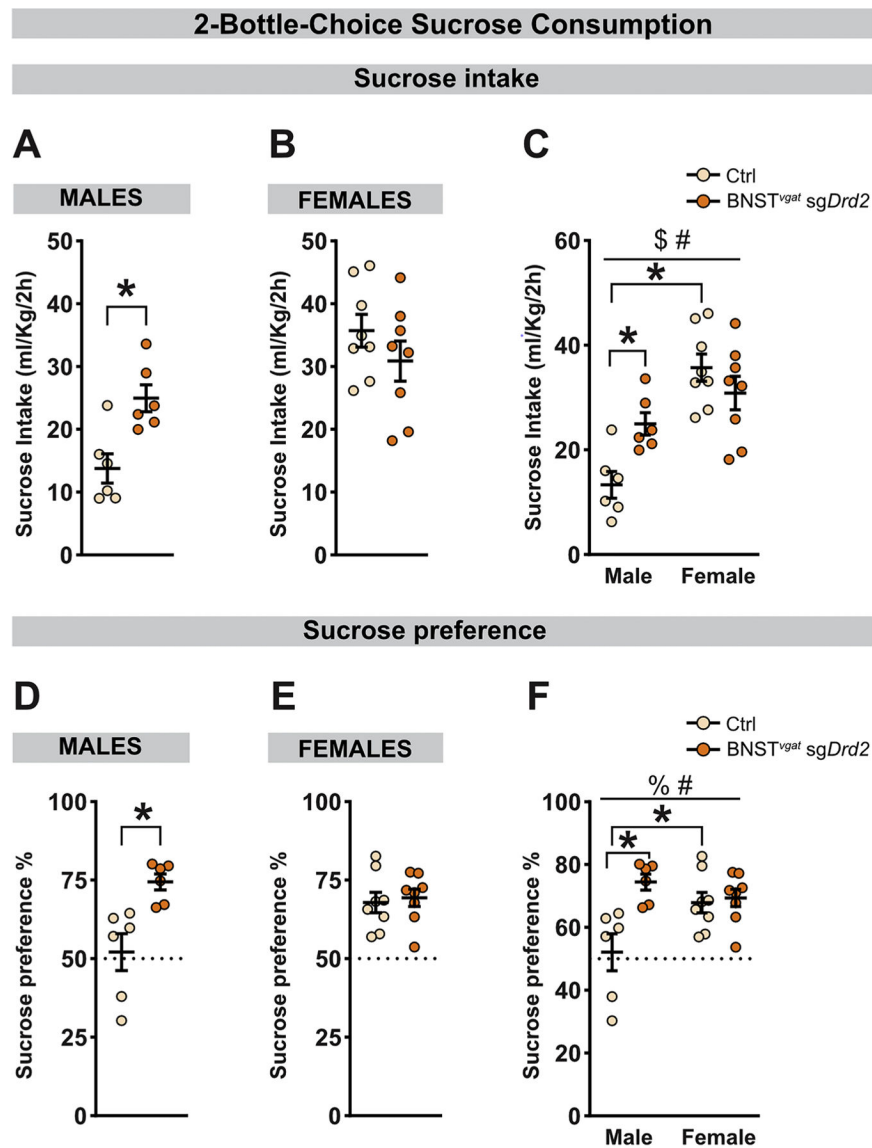
Fig. 7.

BNST^{vgat} *Drd2* knockdown increases sucrose intake in a sex-dependent manner. Average sucrose intake (ml/kg/2h) in male (A) and female (B) mice. Knockdown of BNST^{vgat} *Drd2* increased sucrose intake selectively in male mice. (C) Averages of sucrose intake compared between sexes. Female controls consumed more sucrose compared to male controls. (D-F) *Drd2* Knockdown also increased sucrose preference in males but not females. Female control mice had higher sucrose preference compared to male controls. There was no effect of virus in female mice. Data expressed as Mean ± SEM.*$p < 0.05$;% main effect of virus; $ main effect of sex; # virus x sex interaction.