GABA Release From Central Amygdala Neurotensin Neurons Differentially Modulates Reward and Consummatory Behavior in Male and Female Mice

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Abstract

The central nucleus of the amygdala is known to play key roles in alcohol use and affect. Neurotensin neurons in the central nucleus of the amygdala have been shown to regulate alcohol drinking in male mice. However, little is known about which neurotransmitters released by these specific cells drive alcohol consumption or whether these cells drive alcohol consumption in female mice. Here we show that knockdown of GABA release from central amygdala neurotensin neurons using a vGAT-shRNA-based AAV strategy reduces alcohol drinking in male, but not female, mice. This manipulation did not impact avoidance behavioral assays, except in a fasted novelty-suppressed feeding test, in which vGAT shRNA mice of both sexes demonstrated increased latency to feed on a familiar high-value food reward. These data show a role for GABA release from central amygdala neurotensin neurons in modulating consumption of rewarding substances in different motivational states.

Introduction

The central nucleus of the amygdala (CeA) is an integration center known to respond to stressful stimuli, regulate consummatory behavior, and become dysregulated in alcohol use disorder (AUD)[1–4]. One population of neurons in this region expresses neurotensin (Nts), a 13 amino-acid neuropeptide broadly known to regulate pain, metabolism, consummatory behavior, and reward among other processes[5–12]. Seminal early studies from T.K. Li and colleagues demonstrated that the neurotensin system could modulate several alcohol-associated behaviors[13]. The Choi lab additionally showed that global knockouts of both the Nts receptor 1 and 2 (NtsR1 and NtsR2 respectively) affected drinking and alcohol-related behaviors [14,15]. These features, along with a human study elucidating a correlation between NtsR1 gene polymorphisms and alcohol dependence, point to the Nts system as an excellent candidate to explore future AUD therapies[16]. Additionally, alcohol use and Nts have a unique relationship in terms of neuropeptide systems and substance use. The role of Nts in energy balance further complicates its relationship to alcohol consumption, as alcohol is both caloric and psychoactive. On this note, our previous work has found that optogenetic stimulation of CeA Nts neuron (CeA^{Nts}) terminals in the parabrachial nucleus (PBN) drives alcohol drinking while suppressing chow consumption when given the choice between consuming either[7]. When given the choice between consuming other, non-alcoholic rewarding fluids (sucrose, saccharin solutions) or consuming chow, the animals did not alter their chow consumption[7]. Furthermore, we found ablation of CeA^{Nts} neurons using a caspase AAV strategy significantly reduced alcohol drinking in male mice during a 2-bottle choice, intermittent access (IA) alcohol drinking paradigm[7]. Although these data establish a significant role for CeA^{Nts} neurons in alcohol consumption, questions remain regarding which neurotransmitters released by these neurons drive this behavior, and whether these effects are present in female mice.

In order to address these questions, we used this same caspase AAV strategy as well as a vGAT shRNA AAV strategy in order to compare behaviors between Nts-Cre male and female mice with CeA^{Nts} neurons ablated (CeA^{Nts}::caspase) and those with GABA release from these cells significantly reduced via the vGAT shRNA (CeA^{Nts}::vGAT-shRNA). This caspase virus has been validated in previous work from our group using fluorescent *in situ* hybridization and immunohistochemistry, which together showed a significant decrease in Nts neurons in the CeA after transduction of the virus[7]. Here, we use optogenetics and electrophysiology to show that vGAT shRNA significantly reduced GABA release from CeA^{Nts} neurons in Nts-Cre male and female mice, and altered postsynaptic $GABA_A$ receptor effects in male mice. We also show that

CeA^{Nts}::vGAT-shRNA male mice drink significantly less alcohol compared to eYFP controls (CeA^{Nts}::eYFP mice), akin to our previously published caspase data[7], while female alcohol consumption remained unchanged between CeA^{Nts}::vGAT-shRNA, CeA^{Nts}::caspase, and control females. We also employ a battery of assays to assess avoidance behavior between CeA^{Nts}::caspase, CeA^{Nts}::vGAT-shRNA mice, or control mice. We found that there were no differences in avoidance behavior among these groups, except in the novelty-suppressed feeding test. Surprisingly, in this test, CeA^{Nts}::vGAT-shRNA mice show a significantly increased latency to feed on a familiar, rewarding food (Froot Loops) when compared to both CeA^{Nts}::caspase mice and CeA^{Nts}::eYFP controls. This effect, in contrast to the alcohol drinking results, is present in mice of both sexes, while alcohol drinking is only reduced in males. Finally, we investigated pain and mechanosensory behaviors as CeA neurons have been implicated in such behaviors in previous studies[17,18]. We find that CeA^{Nts}::vGAT-shRNA mice show increased thermal nociception in a Hargreaves assay, while cold and mechanical sensitivity are not different in all groups. These data show a sex-dependent role for GABA release from central amygdala neurotensin neurons in modulating consumption of rewarding substances in different motivational states.

Materials and Methods

Animals

All studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and only after approval from the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Adult mice 8 weeks and older were used for all experiments. All studies made use of Nts-IRES-Cre mice[7,19] partially backcrossed to a C57BL/6J background (Jackson Laboratories). Animals were singly housed and kept on a reverse 12-hour light cycle with lights off at 7AM and had access to food and water *ad libitum*, except during the novelty suppressed feeding test.

Viruses and Stereotaxic Surgery

Mice were anesthetized using 1-3% inhaled isoflurane and mounted in a stereotaxic frame (Kopf Instruments). An incision was made using a scalpel blade before drilling the skull prior to AAV injection at the coordinates (from Bregma in mm) Anterior/Posterior: -1.15, Medial/Lateral: +/- 2.95, Dorsal/Ventral: -4.75. Viruses (from Stanford and UNC vector cores) were injected using a Hamilton Neuros syringe at 100nL/minute until the desired volume was infused (300nL per hemisphere for single virus animals, 400nL per hemisphere for mixtures of viruses). Viruses used for electrophysiological recordings include AAV5-Ef1α-doublefloxed-hChR2(H134R)-mCherry-WPRE (all animals) co-injected with AAV8-hSyn-FLEX-GFP-shvGAT in experimental animals to evaluate vGAT shRNA function. Mice used in drinking experiments and behavioral assays were injected with AAV5-Ef1α-DIO-eYFP (control) or AAV8-hSyn-FLEX-GFP-shvGAT or AAV5-hSyn-eYFP (for viral targeting evaluation after behavioral assays) plus AAV5-Ef1α-FLEX-taCasp3-TEVp to ablate Nts neurons. Mice were allowed to recover for at least 4 weeks before beginning experiments.

2 bottle choice drinking paradigm

In this paradigm, mice were kept on a reverse 12 hour light cycle with lights turning off at 7AM. Mice are allowed access to two water bottles in their home cage. One of these bottles is swapped for an alcohol bottle at 10AM and mice are allowed to drink as much of either substance as they'd

like until 10AM the next day. Bottles are measured by weight at the start and end of each drinking day. The alcohol concentration (weight/volume) increases throughout the study from 3% to 6% to 10% (3 days at each concentration). Mice are then maintained at 20% on a MWF schedule once they reach this concentration.

Open Field Assay

Mice were allowed to freely explore a 42cm x 42cm open field. Locomotor and place data were collected using Fusion software (Omnitech).

Elevated Plus Maze Assay

Mice were placed in the center of an elevated plus maze and allowed to explore the maze. Time spent in the closed arms, open arms, and center of the maze were tracked using Ethovision XT 14 (Noldus).

Light-Dark Box Assay

Mice were placed in the dark side of the apparatus (Med Associates) and allowed to freely explore for 15 minutes. Time spent in the light side and entries to the light side of the apparatus were tracked using Ethovision XT 14 (Noldus).

Novelty-Suppressed Feeding Test (NSFT)

On day 1 of the test, mice are exposed to a rewarding food in their home cage (Froot Loop™). On day 2, food is removed from the cage for 24 hours. On day 3, mice are placed in the left near corner of a 50cm x 50cm open field with a Froot Loop placed in the center. Latency to begin feeding was tracked using Ethovision XT 14 (Noldus). Upon the start of feeding, the test was stopped. The test was concluded after 10 minutes if the mouse did not feed. Mice were then allowed access to Froot Loops in their home cage for 10 minutes after the test. Any mouse that did not feed in the home cage in the 10 minute window following the test was excluded. Home cage feeding was observed following the NSFT. All mice included in the study consumed Froot Loop in the homecage feeding assay (n=1 excluded). In a majority of animals, the amount of Froot Loops consumed in the homecage was measured by weight after 10 minutes.

Novel Object Recognition Test

Novel Object Recognition Test was done based on the short habituation protocol outlined in Leger et al.[20] On day 1, mice were placed in a clean, empty 17cm x 35.5cm mouse cage with no bedding to habituate for 10 minutes. Objects (cube, cone, or cylinder of same color and height) were 3D printed (formlabs Pegasus) to be used for the remainder of the study. Objects were cleaned with 70% ethanol and allowed to dry completely before being used in another session. On day 2, two identical, randomly assigned objects were placed 5cm away from the short walls along the midline of the clean cage. Mice were placed in the center of the long wall on the near side of the cage and allowed to freely explore for up to 10 minutes. Once 20 seconds of total object interaction was reached (tracked by stopwatch), the day 2 session was stopped. Mice that did not interact with objects for 20 seconds in the 10 minute window were excluded. On day 3, a novel object was randomly assigned to each mouse, as well as a side (left or right, decided randomly) for the novel object to be placed. Mice were placed in the center of the long wall on the near side of the cage and allowed to freely explore for 10 minutes. Time spent interacting with objects were measured by a separate, blinded experimenter using two stop watches by observing

the video of the day 3 session. Mice that did not interact with objects for 20 seconds in the 10 minute window were excluded.

Electrophysiology

Mice were anesthetized with isoflurane, decapitated, and their brains were rapidly extracted. Brains were submersed in oxygenated, ice-cold high-sucrose low Na^+ artificial cerebral spinal fluid (aCSF; in mM: 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$) and sliced at 250 μM thickness with a Leica VT1000S vibratome. Coronal slices containing the central amygdala were placed in a holding chamber containing standard aCSF (in mM: 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$; osmolarity ~300 mOsm) and heated to 32°C for at least 1 hour prior to recording. Slices were transferred to the recording chamber, where aCSF containing kynurenic acid (3 mM; glutamate receptor antagonist) was heated (30°C) and perfused over the slice at a rate of 2 mL/minute. All voltage clamp recordings were completed in non-fluorescent CeA^{NTS} negative neurons using a cesium-chloride internal solution (in mM: 130 $CsCl_2$, 38 EGTA, 10 HEPES, 2 ATP, 2 GTP, 1 QX-314; pH ~7.35, osmolarity ~285 mOsm). Neurons were held at -80 mV for spontaneous IPSC recordings. For optically-evoked IPSC recordings, CeA^{NTS} terminals were stimulated for 5 ms with a 470 nM LED (CoolLED) while recording from CeA^{NTS} negative neurons held at -70 mV. Cells that did not elicit optical IPSCs at any laser intensity were excluded from analysis (n=3 cells total; Control: n=2 cells, vGAT shRNA: n=1 cell).

Perfusions and Microscopy

After concluding behavioral studies, mice were perfused using .01M phosphate buffered saline and then 4% paraformaldehyde. Brains were extracted and allowed to fix in 4% paraformaldehyde solution for 16-24 hours before being placed in 30% sucrose for at least 24 hours. Brains were then sliced at 40 microns on a Leica CM 3050S cryostat before being mounted on Fisherbrand slides with a DAPI counterstain. Images confirming viral targeting were collected on a Keyence microscope. Any mice showing a lack of viral expression were excluded from results (n=3).

von Frey

The von Frey test assesses mechanical hypersensitivity and was performed as previously described[21]. Mice were habituated for at least 1 hour in plexiglass boxes on an elevated metal wire rack. Nylon filaments of forces increasing from .008g to 2g were applied to the hind paws using the up-down method[22]. Starting with 0.16g, the filament was applied to the hind paw for ten trials, then repeated with ascending or descending forces depending on paw withdrawals. Paw withdrawal threshold was defined as the minimum force filament that elicits a paw withdrawal for half or more of the trials.

Cold Plantar Assay

The cold plantar assay was performed as previously described to assess cold sensitivity[23]. Mice were placed in plexiglass boxes on an elevated glass surface and habituated for at least 60 minutes. Dry ice was pounded into a powder using a hammer. The tip was cut off of a 3mL syringe and a cut into the side of the syringe was made lengthwise along the syringe to allow carbon dioxide to escape the syringe. Dry ice was then packed into the syringe. A solid pellet was made by pressing the syringe against a hard surface and pushing the syringe plunger down. This pellet was then applied to the bottom of the glass surface under the hind paw of each mouse (3

stimulations per hindpaw, each stimulation separated by at least 15 minutes). Paw withdrawal times were averaged for each mouse.

Hargreaves

The Hargreaves test to assess thermal nociception was performed as previously described[24]. Mice were placed in plexiglass boxes on an elevated glass surface and habituated for at least 60 minutes. The beam intensity was set on the IITC Plantar Analgesia meter (IITC Life Science). Both hind paws of each mouse were exposed to a series of four stimulations, with 10 minutes between each stimulation, alternating left and right paws. Paw withdrawal latency was recorded and averaged for each animal. 25 seconds was used as a cutoff time to prevent serious injury.

Conditioned Place Preference

This assay, based on study design by Cunningham et al.[25], makes use of a custom apparatus that takes advantage of SuperFlex Open Field boxes from Omnitech. Fusion software (Omnitech) is used to track mouse positioning throughout the experiment. The apparatus has a dividing wall in the middle with a small doorway in the center. On each side of the dividing wall are two contexts. One side has a circular grate floor and horizontal white and black bars on the walls. The other side has a bar grate floor and vertical black and white bars on the walls. In the pretest, mice are injected with 12.5mL/kg saline and are then allowed to freely explore the arena for 30 minutes with the doorway open. Innate side preference is determined by measuring time spent in each side using Fusion software. Each side/context is then paired with saline or ethanol. This study used a biased design in which the side on which mice spent the least amount of time in the pretest is paired with ethanol. Mice then undergo two 5-minute conditioning sessions per day for six days with each session separated by at least 4 hours. In the afternoon session (2PM), mice are injected with a 20% (weight/volume) ethanol solution (i.p. 2g/kg) and placed in one side with the doorway closed, while in the morning (10AM) they are injected with an equivalent volume of 0.9% saline and placed in the other side with the doorway closed. In the post test, mice are allowed to freely explore the arena with the doorway open again. Change in time spent on the ethanol-paired side is measured using Fusion software.

Conditioned Place Aversion

This assay, based on study design by Cunningham et al.[25] makes use of a custom apparatus that takes advantage of SuperFlex Open Field boxes from Omnitech. Fusion software (Omnitech) is used to track mouse positioning throughout the experiment. The apparatus has a dividing wall in the middle with a small doorway in the center. On each side of the dividing wall are two contexts. One side has a circular grate floor and horizontal white and black bars on the walls. The other side has a bar grate floor and vertical black and white bars on the walls. In the pretest, mice are allowed to freely explore the arena for 30 minutes with the doorway open. After the pretest, mice are injected with 12.5mL/kg saline as soon as they are taken out of the apparatus. Innate side preference is determined by measuring time spent in each side using Fusion software. Each side/context is then paired with saline or ethanol. This study used a biased design in which the side on which mice spent the least amount of time in the pretest is paired with saline. Mice then undergo two 5-minute conditioning sessions per day for six days with each session separated by at least 4 hours. In the afternoon session (2PM), mice are placed in the assigned ethanol side with the doorway closed for five minutes. Following this conditioning session, mice are injected with a 20% (weight/volume) ethanol solution (i.p. 2g/kg) as soon as they are removed from the apparatus. In the morning (10AM) they are placed in the other side with the doorway closed for

five minutes. Mice are then injected with an equivalent volume of 0.9% saline as soon as they are removed from the apparatus. In the post test, mice are allowed to freely explore the arena for 30 minutes with the doorway open again. Change in time spent on the ethanol-paired side is measured using Fusion software.

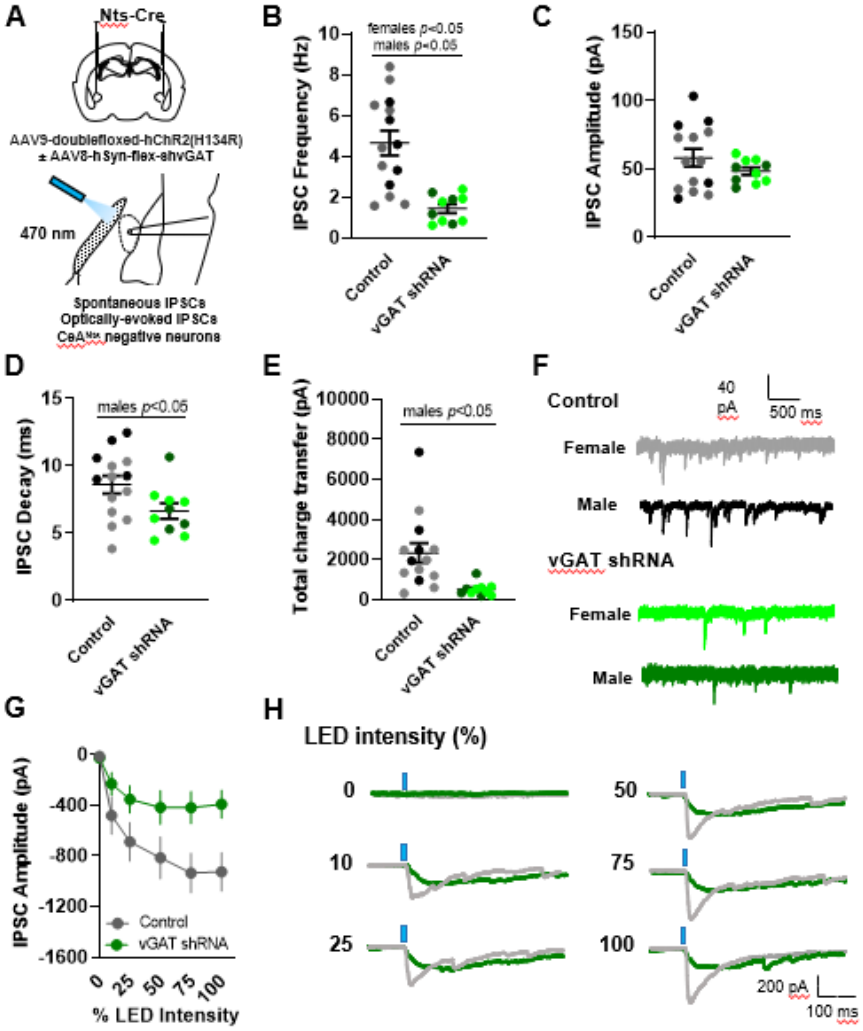
Data analysis and statistics section

Graphpad Prism 10 was used for statistical analyses. Direct sex comparisons were made in all cases, except alcohol drinking. Electrophysiology experiments were analyzed either by 2- or 3-way ANOVA. If a main effect was present, Sidak's multiple comparisons test was used. For all behavioral data, a 2-Way ANOVA was used to analyze results. If a main effect was present, Tukey's multiple comparisons test was used to analyze multiple comparisons. For total liquid drinking data, a 2-Way ANOVA was used to account for virus and sex. For drinking data only showing one sex (due to known drinking differences between the sexes, a repeated measures 2-Way ANOVA was used as well, but this time to account for the variables time/concentration and virus group. For drinking data, Sidak's multiple comparisons tests were used. For all data $\alpha \leq .05$.

Results

vGAT shRNA reduces GABA release from central amygdala neurotensin neurons in ex-vivo *slice electrophysiology*

In our prior published electrophysiology experiments we found that all tested non-Nts positive cells in the CeA received a direct projection from CeA^{Nts} neurons[7]. This suggests a strong contribution from this population to intra-CeA signaling. To explore this contribution, we decided to probe how manipulation of GABA vesicular packaging in CeA^{Nts} neurons would affect CeA inhibition. We injected a virus encoding cre-dependent channelrhodopsin with (experimental) or without (control) another virus encoding cre-dependent vGAT shRNA into the CeA of male and female Nts-Cre mice. We performed whole-cell patch clamp electrophysiology and recorded spontaneous and optically-evoked inhibitory postsynaptic currents (IPSCs) from non-fluorescent CeA^{Nts} negative neurons (Fig. 1A). We found that vGAT

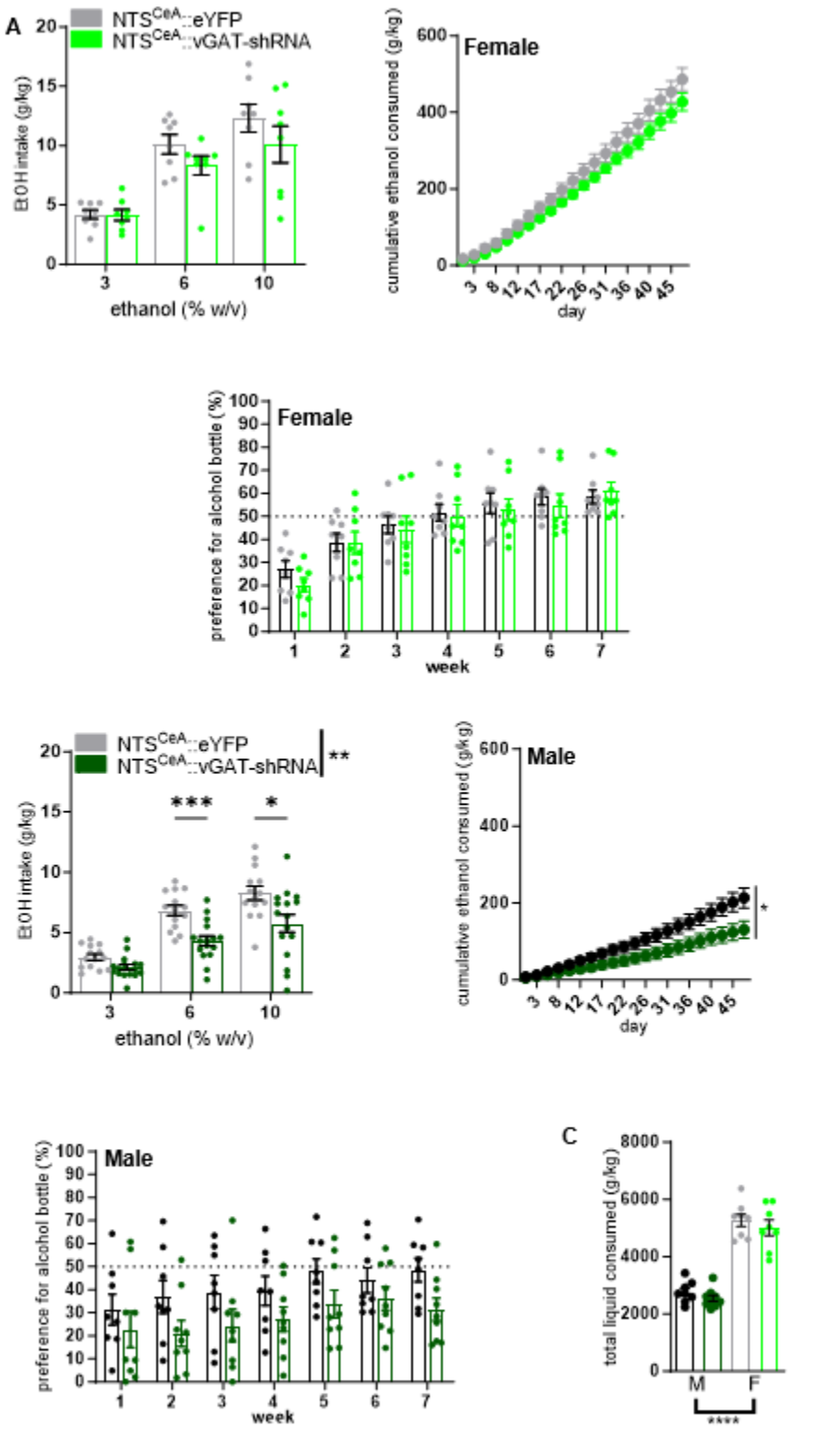


Electrophysiolgoical validation of vGAT shRNA-induced reduction of GABA release from CeA^{Nts} neurons (A) Schematic illustrating experimental design, spontaneous and optically-evoked IPSC electrophysiological recordings from CeA^{Nts} negative neurons in male (control: black, vGAT shRNA: dark green) and female (control: gray, vGAT shNRA: lime green) Nts-Cre mice injected with cre-dependent channelrhodopsin (control), or cre-dependent channelrhodospin and cre-dependent vGAT shRNA **(B)** vGAT shRNA mice have reduced IPSC frequency and **(C)** no difference in IPSC amplitude compared to controls **(D)** Male vGAT shRNA mice show a significant decrease in IPSC decay time, with no effect in female vGAT shRNA mice **(E)** Male vGAT shRNA mice show a significant decrease in total charge transfer, with no effect in female vGAT shRNA mice **(F)** Representative traces of spontaneous IPSCs **(G)** vGAT shRNA attenuates optically-evoked IPSCs elicited from CeA^{Nts} neuron terminals **(H)** Representative traces of optically-evoked IPSCs across a range of laser intensities (0, 10, 25, 50, 75, 100%).

shRNA significantly reduced the IPSC frequency in males and females (Fig. 1B; female control: n= 9 cells from 5 mice, male control: n= 5 cells from 4 mice, female vGAT: n= 6 cells from 2 mice, male vGAT shRNA: n=4 cells from 2 mice; 2-way ANOVA; main effect of vGAT shRNA: $F_{(1,20)}$=15.58, p=0.0008, no main effect of sex: $F_{(1,20)}$=0.00002, p=0.9966, no interaction $F_{(1,20)}$=0.01269, p=0.9114). Sidak's multiple comparisons test shows significant reductions in IPSC frequency in females (t=3.285, p=0.0074) and males (t=2.438, p=0.0479). These data suggest that vGAT knockdown attenuates presynaptic GABA release onto non-Nts neurons in the CeA in both sexes. Knockdown of vGAT did not significantly impact IPSC amplitude (Fig. 1C; 2-way ANOVA; no main effect of vGAT shRNA: $F_{(1,20)}$=2.242, p=0.1499, no main effect of sex: $F_{(1,20)}$=0.3864, p=0.5412, no interaction: $F_{(1,20)}$=1.416, p=0.2480). Notably, we found sex-dependent effects of vGAT shRNA on the IPSC decay kinetics (Fig. 1D; 2-way ANOVA; main effect of virus: $F_{(1,20)}$=7.933, p=0.0107, main effect of sex: $F_{(1,20)}$=5.716, p=0.0268, no significant interaction: $F_{(1,20)}$=2.059, p=0.1668). The post-hoc test showed that the main effect of sex was driven by the males, where we found a significant decrease in the IPSC decay time in the vGAT shRNA mice relative to the controls (Sidak's multiple comparisons test; females t=1.118, p=0.4769; males t=2.703, p=0.0272).

In order to further explore these orthogonal findings, and because these findings suggest that vGAT shRNA may promote postsynaptic changes in the male mice, we multiplied the frequency of the events by the area under the curve to determine the total charge transfer. Here, we found that the vGAT shRNA reduced the total charge transfer (Fig. 1E; 2-way ANOVA; main effect of vGAT shRNA : $F_{(1,20)}$=11.72, p=0.0027; no main effect of sex: $F_{(1,20)}$=1.761 p=0.1995, no interaction: $F_{(1,20)}$=1.212, p=0.2840). Post-hoc analysis revealed these effects were again driven by the male mice (Sidak's multiple comparisons test; females t=1.879, p=0.1441; males t=2.877, p=0.0186)). These data suggest that vGAT shRNA similarly modifies presynaptic spontaneous inhibition independent of sex; however, this manipulation appears to differentially affect postsynaptic GABAergic transmission in males and females.

Next, we recorded optically-evoked IPSCs across a range of LED intensities (0, 10, 25, 50, 75, 100%) to confirm that CeA^{Nts} terminals were the source of vGAT shRNA-induced deficits in presynaptic GABA release. We found that vGAT shRNA blunted optically-evoked IPSCs elicited from CeA^{Nts} terminals compared to controls (Fig. 1G; control: n=5 cells from 2 female mice, n=7 cells from 4 male mice; vGAT shRNA: n=3 cells from 1 female mouse, n=5 cells from 2 male mice; repeated measures mixed-effects 3-way ANOVA; main effect of LED intensity $F_{(5,73)}$=23.66, p<0.0001, trending main effect of vGAT shRNA: $F_{(1,15)}$=4.320, p=0.053, no main effect of sex: $F_{(1,15)}$=0.1519, laser intensity X vGAT shRNA interaction: $F_{(5,73)}$=4.269, p=0.0018). Collectively, these findings provide physiological evidence that vGAT shRNA disrupts GABA release from CeA^{Nts} neurons in male and female mice.



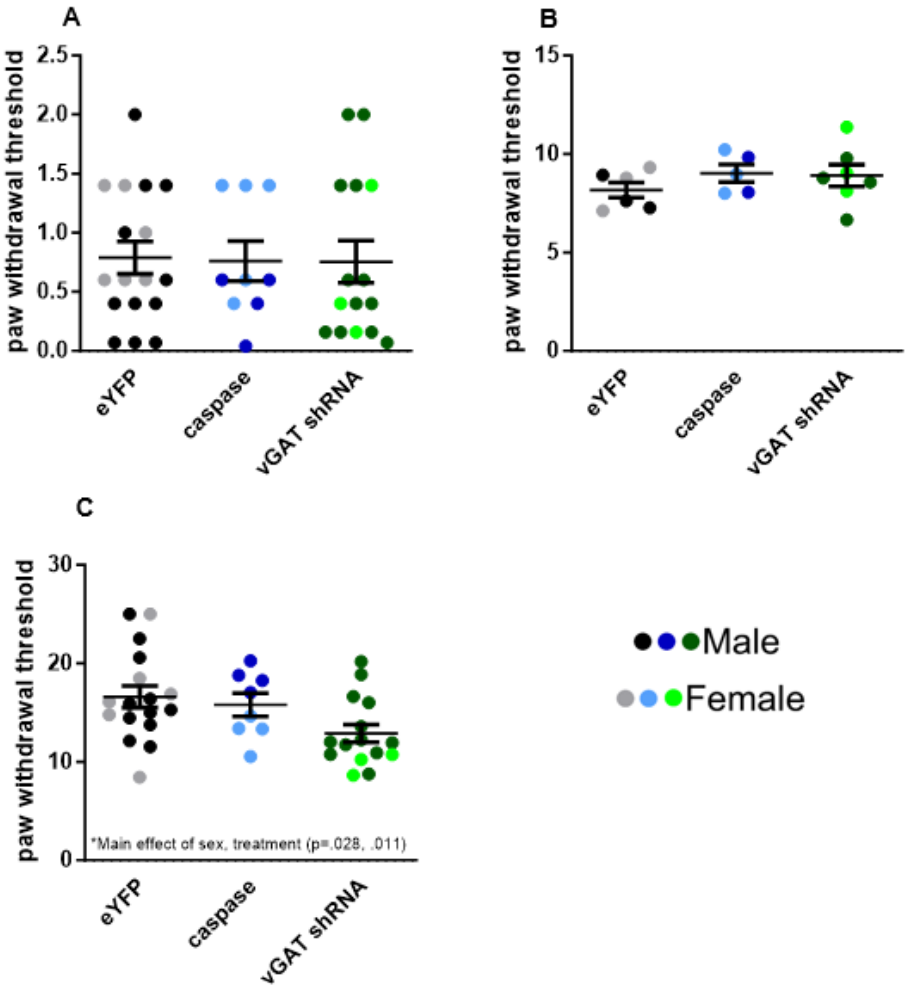
Knockdown of *vGAT* in NTS^{CeA} neurons decreases alcohol drinking in an IA paradigm in male mice. (A) Alcohol consumption was not significantly different between female CeA^{Nts}::caspase (n=8) and CeA^{Nts}::vGAT-shRNA (n=8) mice across 7 weeks of IA drinking. **(B)** Male CeA^{Nts}::vGAT-shRNA mice (n=9) consume less alcohol than CeA^{Nts}::eYFP mice (n=8) in an IA paradigm and demonstrate a trend of reduced preference . **(C)** Total liquid consumed throughout the study

Knockdown of GABA release in central amygdala neurotensin neurons reduces alcohol drinking in male mice

To determine the role of GABA release from CeA^{Nts} neurons in the modulation of alcohol consumption, we used a 2-bottle choice IA drinking paradigm to assess alcohol intake. In female mice (CeA^{Nts}::vGAT-shRNA n=8, CeA^{Nts}::eYFP n=8) we found no significant difference in drinking at lower ethanol concentrations (3, 6, 10%, 2-Way ANOVA. Time x treatment $F_{(2,20)}$=1.008, p=.3780). We followed this with intermittent access 2-bottle choice drinking over 7 weeks. We found in cumulative alcohol drinking (Time x treatment $F_{(20,280)}$=1.669, p=.038, lack of main effect of virus p=.1271), alcohol preference (Fig. 2A; 2-Way ANOVA. Time x treatment $F_{(6,84)}$=.6872, p=.6604), or total liquid consumed across the study (Fig. 2C; 2-Way ANOVA, main effect of sex p<.0001, no treatment effects). These results are similar to the same experiments performed in female CeA^{Nts}::caspase

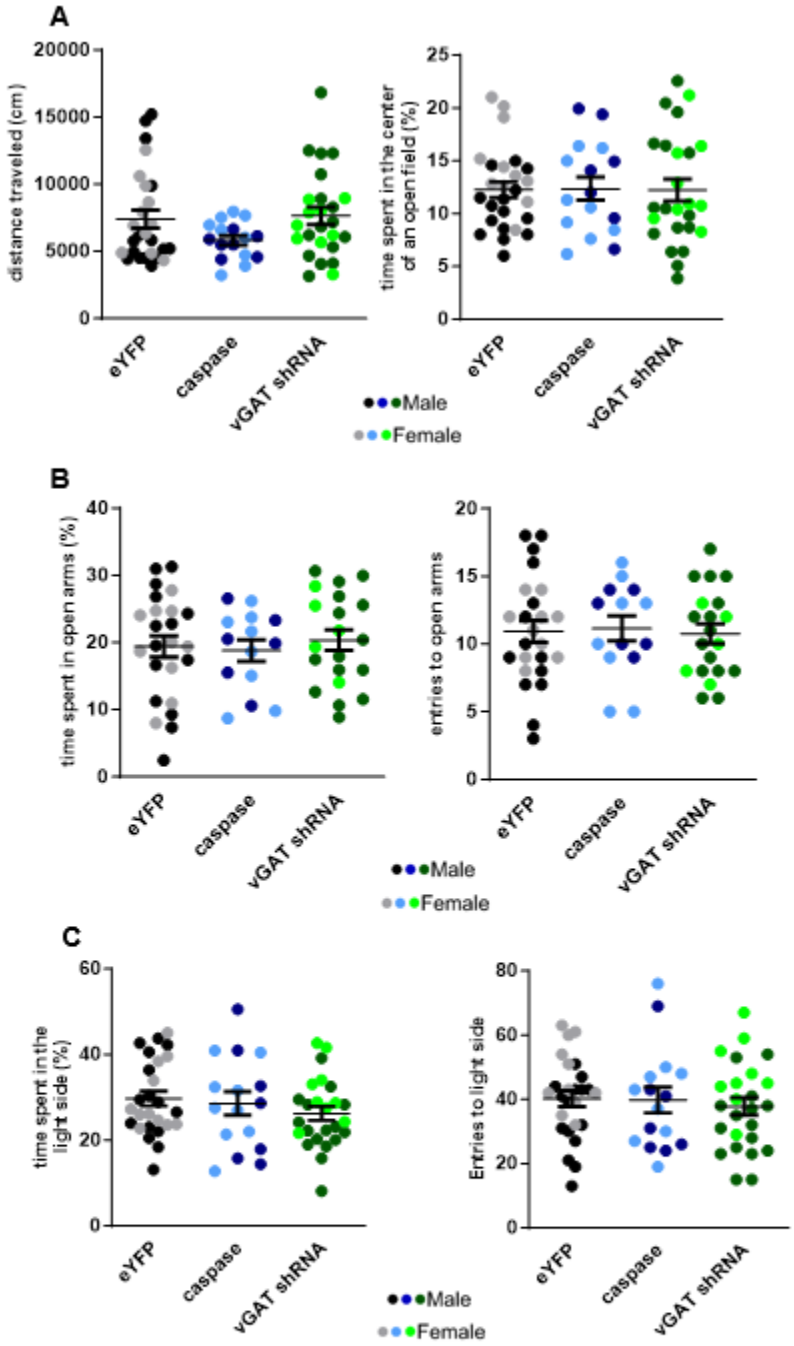
animals (Fig. S1), where we found no difference between CeA^{Nts}::caspase and CeA^{Nts}::eYFP female mice (2-Way ANOVA. Time x treatment $F_{(20,440)}$=.6354, p=.8865). In contrast to female mice, male CeA^{Nts}::vGAT-shRNA animals (n=9) showed a significant reduction in cumulative alcohol drinking (2-Way ANOVA. Time x treatment interaction $F_{(20,300)}$=5.079, p<0.0001; main effect of virus $F_{(1,15)}$=4.541, p=0.0500), alcohol drinking at lower concentrations (2-Way ANOVA, main effect of treatment $F_{(1,28)}$=12.76, p=.0013. Sidak's multiple comparisons tests showed significant differences between treatment groups at 6% ethanol p=.0007, as well as at 10% ethanol p=.0358) but showed no difference in total liquid consumed when compared to CeA^{Nts}::eYFP (n=8) animals (Fig. 2B, 2C). These results mirrored our findings in male CeA^{Nts}::caspase mice[7].

Knockdown of GABA release in central amygdala neurotensin neurons increases thermal nociception



vGAT knockdown in CeA^{Nts} neurons increases thermal sensitivity without altering other mechanosensory behaviors (A) Results from von Frey assay show no differences in baseline mechanical hypersensitivity between groups regardless of sex. **(B)** Results from the cold plantar assay show no differences in cold hypersensitivity between groups regardless of sex. **(C)** Results from the Hargreaves assay show main effects of sex (p<.028) and treatment (p=.011) in thermal hypersensitivity.

Because of the well-established role of the CeA in pain behaviors[17,18], we evaluated mechanical, heat nociception and cold sensitivity across all groups and sexes. There were no differences between groups in mechanical hypersensitivity in the von Frey assay (Fig. 3A; CeA^{Nts}::eYFP male n=11, female n=6; CeA^{Nts}::vGAT-shRNA male n=12, female n=3; CeA^{Nts}::caspase male n=4, female n=5; 2-Way ANOVA. Sex x treatment $F_{(2,35)}$=0.900, p=.4158). In the Hargreaves assay (Fig. 3C; CeA^{Nts}::eYFP male n=11, female n=6; CeA^{Nts}::vGAT-shRNA male n=12, female n=3; CeA^{Nts}::caspase male n=4, female n=4), we observed a significant increase in thermal nociception (2-Way ANOVA. Main effect of sex p<.028, main effect of virus



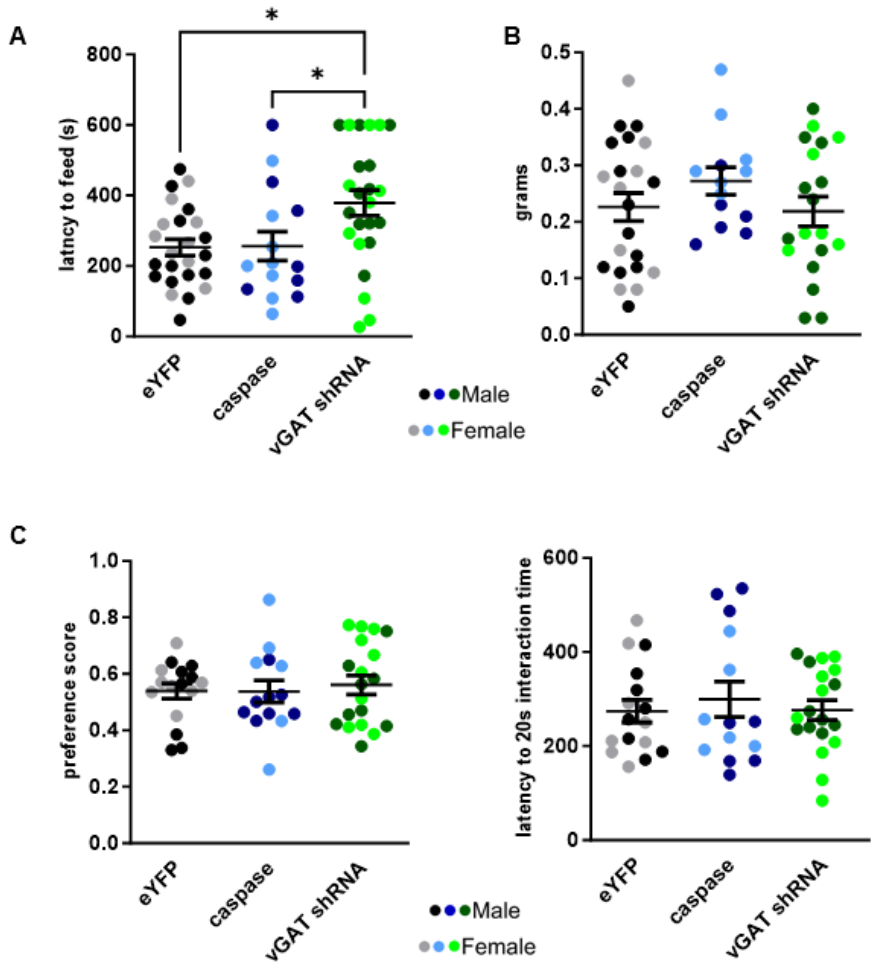
vGAT knockdown in CeA^{Nts} neurons or ablation of these neurons does not modulate avoidance behavior (A) Locomotor and time spent in the center of an open field show no differences in locomotion or avoidance behavior between groups. Females demonstrated significantly more distance moved than males as was expected (p=.0011). **(B)** Time spent in open arms and entries to open arms of an elevated plus maze over five minutes show no differences between groups regardless of sex. **(C)** There were no differences between groups with regard to time spent in the light side or entries to the light side of a light-dark box over fifteen minutes.

p=.011, but no significant post-hoc tests). To follow up on this difference driven by heat stimulus, we used the cold plantar assay[23] to measure cold sensitivity (Fig. 3B). We found that cold sensitivity was not different across virus groups and sexes (Fig. 3B; CeA^{Nts}::eYFP male n=3, female n=3; CeA^{Nts}::vGAT-shRNA male n=2, female n=3; CeA^{Nts}::caspase male n=4, female n=3; 2-Way ANOVA. Sex x treatment $F_{(2,12)}$=0.2136, p=.8107).

Knockdown of GABA release in central amygdala neurotensin neurons does not alter avoidance behavior

The CeA is well known to modulate avoidance/negative affective behaviors, which are relevant to substance use and consummatory behavior. Our previous study did not find differences in avoidance behavior in CeA^{Nts}::caspase male animals as compared to controls [7]. To investigate locomotor and avoidance behaviors in the vGAT shRNA and caspase animals of both sex, we used the open field (Fig. 4A), elevated plus maze (Fig. 4B), and light-dark box (Fig. 4C) assays. We found no significant group differences in locomotor behavior in the open field (CeA^{Nts}::eYFP male n=15, female n=11; CeA^{Nts}::vGAT-shRNA male n=16, female n=9; CeA^{Nts}::caspase male n=7, female n=9. 2-Way ANOVA. Sex x treatment $F_{(2,61)}$=0.5668, p=.5703), time spent in the

center of the open field (same n as locomotor data, 2-Way ANOVA. Sex x treatment $F_{(2,61)}$=2.963, p=.0592), time spent in the open arms or entries to the open arms of the elevated plus maze (CeA^{Nts}::eYFP male n=14, female n=10; CeA^{Nts}::vGAT-shRNA male n=15, female n=5; CeA^{Nts}::caspase male n=6, female n=8. Open arm time: 2-Way ANOVA. Sex x treatment $F_{(2,52)}$=0.1555, p=.8563. Open arm entries: 2-Way ANOVA. Sex x treatment $F_{(2,52)}$=0.1908, p=.8269), or in time spent in the light side and entries to the light side of the light-dark box (CeA^{Nts}::eYFP male n=15, female n=11; CeA^{Nts}::vGAT-shRNA male n=16, female n=9; CeA^{Nts}::caspase male n=7, female n=9. Time spent in light: 2-Way ANOVA. Sex x treatment $F_{(2,61)}$=1.394, p=.2558. Light entries: 2-Way ANOVA. Sex x treatment $F_{(2,61)}$=0.9226, p=.4030).



vGAT knockdown in CeA^{Nts} neurons increases latency to feed in the novelty suppressed feeding test without altering novel object preference (A) CeA^{Nts}::vGAT-shRNA mice showed an increased latency to feed compared to both CeA^{Nts}::caspase mice (p=.0448) and CeA^{Nts}::eYFP controls (p=.0167) in the novelty-suppressed feeding test (main effect of treatment p=.0101). **(B)** Post test consumption assessment from a subset of mice from Figure 5A show no differences between groups regardless of sex. **(C)** There were no differences in novel object preference or latency to interact with objects in the novel object recognition test.

Knockdown of GABA release in central amygdala neurotensin neurons modulates novelty-suppressed feeding but not novel object preference

Both the CeA and Nts signaling have roles in consummatory behaviors[5,6] and object recognition[26]. To explore a possible role for the CeA^{Nts} population in these biological processes, we used two novelty based assays: novelty object preference and NSFT. We found no differences between groups or sexes in novel object preference or in latency to reach the 20-second object interaction criteria (Fig. 5C; CeA^{Nts}::eYFP male n=8, female n=8; CeA^{Nts}::vGAT-shRNA male n=9, female n=10; CeA^{Nts}::caspase male n=8, female n=6) (Preference: 2-Way ANOVA. Sex x treatment $F_{(2,43)}$=0.06122, p=.9407. Latency: 2-Way ANOVA. Sex x treatment $F_{(2,43)}$=0.09053, p=.9136). However, in the novelty-suppressed feeding test (Fig. 5A), we observed a significant

difference in latency to feed on a familiar, rewarding food (2-Way ANOVA, main effect of virus p=.0101: main effect of sex $F_{(1,58)}$=0.6727, p=0.4155; interaction $F_{(2,58)}$ =0.8568, p=0.4298). CeA^{Nts}::vGAT-shRNA mice (male n=13, female n=11) showed a significantly longer latency to feed when compared to both CeA^{Nts}::caspase (male n=7, female n=8) mice (Tukey's multiple comparisons test p=.0448) and CeA^{Nts}::eYFP (male n=14, female n=10) controls (Tukey's multiple comparisons test p=.0167). Consumption of food in the home cage in a 10-minute window after the test was confirmed in all animals, and amount of food consumed was measured (Fig. 5B) in a subset of these animals (CeA^{Nts}::eYFP male n=13, female n=9; CeA^{Nts}::vGAT-shRNA male n=12, female n=7; CeA^{Nts}::caspase male n=6, female n=7). No differences were observed in total food consumed in the 10-minute window after the test. (2-Way ANOVA. Sex x treatment $F_{(2,38)}$=2.749, p=.0767) These data highlight that GABAergic signaling in CeA^{Nts} neurons does not play a role in novel object recognition but does contribute to the motivations to seek rewarding foods in a novel context.

Discussion

The CeA modulates substance use and avoidance behavior in mice, making it an important brain region to study in the context of developing future AUD therapies. While there have been substantial advances made to characterize signaling within the CeA, there remains a lack of understanding of the cell types and neurotransmitters that modulate such behaviors. We investigated the role of CeA^{Nts} neurons in a myriad of behaviors ranging from avoidance behavior, pain, and alcohol consumption to reward and aversion. We used both a selective caspase lesioning strategy and a GABAergic knockdown to probe the mechanisms responsible for these behaviors in both male and female mice. Here we demonstrate that GABA release from Nts-expressing neurons in the CeA contributes to the consumption of alcohol and the motivation to seek rewarding foods in certain contexts in a sex-dependent manner.

Physiological profiling of vGAT knockdown in CeA^{Nts} neurons

We have previously demonstrated that CeA^{Nts} cells signal within the CeA, and project to distal targets like the BNST and PBN [7]. Although this vGAT shRNA virus has been used to knock down GABAergic signaling in the CeA of corticotropin releasing factor (CRF-cre) rats [27], we wanted to validate whether this strategy would result in a reduction in GABAergic tone within the CeA of Nts-cre mice. We found that the vGAT knockdown strategy reduced both spontaneous GABAergic transmission within the CeA and light-evoked GABA release from CeA^{Nts} neurons. Our previous study, and our present sIPSC and optogenetic data indicate that the CeA^{Nts} neurons play a substantial role in regulating the internal GABAergic tone within the CeA of both male and female mice. Surprisingly, the vGAT knockdown resulted in a difference in the sIPSC decay kinetics and the total charge transfer of male mice in the CeA. These findings suggest that there is either a dynamic change in the post-synaptic receptor composition following the reduction in GABA tone, or that the loss of synaptic input onto a particular synapse can result in an overall change in the $GABA_A$ receptor stoichiometry on a given CeA neuron. We believe that these sex differences could explain some of the distinct behavioral outputs we observed, and we will discuss further below. Moreover, these data suggest that changes in presynaptic input may differentially regulate postsynaptic receptor stoichiometry in a sex-dependent fashion. Future studies, however, need to investigate whether similar physiological differences manifest in other regions that received CeA^{Nts} projections. In total, these data demonstrate that the GABAergic knockdown strategy results in a relevant physiological manipulation to the CeA system as a whole and gives us physiological context to interpret our subsequent behavioral experiments.

CeA^{Nts} neurons and reward as it relates to consumption

Neurotensin was first isolated from bovine hypothalamus in the 1970s[28], and was identified as a peptide involved in reward in the 1980s.[29] While perhaps primarily associated with aversion, the CeA is known to also impact reward and positive valence[30]. Furthermore, the CeA is now understood to be involved in both positive and negative alcohol reinforcement[31].

Nts in the CeA has been implicated in reward, with Nts administration to the CeA inducing a conditioned place preference[5,6,32]. We saw no differences between CeA^{Nts}::vGAT-shRNA mice, CeA^{Nts}::caspase mice, or controls in both sexes in alcohol conditioned place preference and conditioned place aversion assays (Fig. S2). These data suggest that GABA release from CeA^{Nts} neurons does not modulate reward or aversive ethanol learning in conditioned place assays. However, we do show in our previous work that ablation of CeA^{Nts} neurons reduces voluntary alcohol drinking in males, but not in females as we show here (Fig. S1). Meanwhile, knocking down GABA release from these neurons while leaving them otherwise intact results in a similar behavioral outcome, but the drive to initiate consumption of rewarding foods in the novelty-suppressed feeding test is blunted in both sexes (without alteration in homecage posttest consumption, Fig. 5A). This sex difference in rewarding consumption may be due to the fact that alcohol is a liquid and Froot Loops are a solid food. We previously reported that optogenetic stimulation of CeA^{Nts} neurons that project to the PBN in male mice drives drinking of rewarding fluids including alcohol, sucrose solution, and saccharin solution[7]. Interestingly, this optical stimulation did not enhance the consumption of Froot Loops in the homecage, and reduced solid food consumption in an alcohol/food choice situation[7]. Given this data along with the reduction in alcohol consumption in males after ablation of these neurons, it seems that GABA release from these neurons drives nonspecific consumption of rewarding substances (at least in the contexts discussed in this paper), while some other variable may modulate fluid consumption specifically. The difference could also be the result of differing GABA kinetics as shown in the electrophysiology experiments done here. These data point to a need for further studies of the CeA^{Nts} population in order to fully understand this discrepancy. Such studies should involve knockdown of Nts itself or perhaps knockdown of other signaling molecules in this population, which include dynorphin, somatostatin, and PKCΔ[7]. Other factors to consider in this context include the 24 hours of food deprivation leading up to the experiment, the fact that this food is palatable and not regular chow, and lack of alcohol drinking experience. Other studies have also observed that optogenetically inhibiting a similar population of neurons (Crh-Cre) under water deprivation enhanced fluid consumption, highlighting that motivational state is something that may alter behavioral responding[2]. Each of these factors could modulate neuronal function in this region and could lead to different behavioral outcomes.

CeA^{Nts} neurons and sex differences

A number of recent studies have examined sex-differences in excitatory and inhibitory signaling in the CeA[33–36]. The present study we contributes to this literature by demonstrating that a reduction in GABAergic tone from CeA^{Nts} neurons alters the postsynaptic profile of $GABA_A$ channels in a sexually divergent manner. We found that loss of presynaptic GABAergic tone increases the rate of decay in male mice, which may be due to an increase in the number of postsynaptic $GABA_A$ receptors containing the benzodiazepine-sensitive α1 receptor subunit[37–39]. Future studies exploring this possibility will be very valuable to the field. The sex difference in drinking following modulation of the CeA^{Nts} neurons could also be due to a compensatory

mechanism being engaged in female mice. The Nts gene contains an estrogen response element, meaning that the Nts system in females may have differential plasticity as compared to male mice[40]. This also further highlights the need to investigate Nts release from this population and its role in reward and consumption. Additionally, female mice consume significantly more fluid than do male mice, as the preference for alcohol was the same in our control animals (2-way ANOVA, main effect of sex $F_{(1,14)}$=1.366, p=0.26,2 Fig. 2C). This further complicates interpretation of these results and perhaps demonstrates a need to analyze these sexes separately and interpret them independently when investigating fluid consumption in general.

CeA^{Nts} neurons and sensory-driven behavior

Pain can be a driver of negative affect, which can potentiate misuse of substances such as alcohol[41,42]. The CeA is known to be a region involved in the regulation of pain and related behaviors[17,18,43–45]. Therefore, we investigated pain and sensory behaviors across all groups and sexes as these behaviors could be relevant in the context of alcohol use. We did not observe differences in mechanical or cold sensitivity between groups or sexes (Fig. 3A, 3B). We did, however, observe that vGAT knockdown increased thermal nociception (Fig. 3C). We were surprised to only see differences in one of these three assays. There are some instances where manipulations impact some modalities of pain-induced hypersensitivity but not others due to the limited battery of assays we used[18]. We did not enhance pain responses with inflammatory or other types of pain mediators in any assay. Given that the CeA responds to stressful stimuli, stimuli that do not qualify as “particularly noxious” may not produce a response robust enough to see differences between experimental groups whose manipulations take place in the CeA. Furthermore, it is interesting to note, that in both the Hargreaves assay and the NSFT assay, we saw differences only in the vGAT shRNA groups and not the caspase lesion groups. We assumed that the vGAT knockdown was an incomplete recapitulation of the caspase lesion, but our data suggest that plasticity within the circuit as a result of the vGAT knock down may have a more complex effect than the lesion strategy. It is worth noting these differences as our field moves towards complex strategies to manipulate neurons. Our data add to the findings that not all manipulations are as simple as turning a circuit on or off.

Conclusions

Although it is known that the CeA modulates avoidance and both positive and negative alcohol reinforcement, knowledge of the genetic, molecular, and signaling factors in this region that modulate such behaviors is lacking. Using a caspase lesioning strategy and GABAergic knockdown strategy, we investigated the role of GABA release from CeA^{Nts} in both male and female mice. We studied the role of CeA^{Nts} neurons in avoidance behavior, pain, reward and aversion learning, and consumption. Here, we show that GABA release from CeA^{Nts} neurons contributes to consumption of alcohol and the motivation to seek rewarding foods in specific contexts in a sex-dependent fashion.

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Author Contributions

GBG, MLTS, ZAM designed the research. MLTS did surgeries and drinking studies. GBG did surgeries and behavioral assays. KMB did pain and mechanosensory studies. SES did electrophysiological studies. GBG, DZ, LAW, AAP perfused mice and used microscopy to validate viral targeting and function. DZ was blinded and scored novel object recognition test videos. GBG and ZAM wrote the paper with contributions from MLTS and SES.

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Competing Interests

ZAM is subcontracted by Epicypher on a project unrelated to this work DA057749.

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Figure legends

Figure 1. Electrophysiolgoical validation of vGAT shRNA-induced reduction of GABA release from CeA^{Nts} neurons (A) Schematic illustrating experimental design, spontaneous and optically-evoked IPSC electrophysiological recordings from CeA^{Nts} negative neurons in male (control: black, vGAT shRNA: dark green) and female (control: gray, vGAT shNRA: lime green) Nts-Cre mice injected with cre-dependent channelrhodopsin (control), or cre-dependent channelrhodospin and cre-dependent vGAT shRNA (B) vGAT shRNA mice have reduced IPSC frequency and (C) no difference in IPSC amplitude compared to controls (D) Male vGAT shRNA mice show a significant decrease in IPSC decay time, with no effect in female vGAT shRNA mice (E) Male vGAT shRNA mice show a significant decrease in total charge transfer, with no effect in female vGAT shRNA mice (F) Representative traces of spontaneous IPSCs (G) vGAT shRNA attenuates optically-evoked IPSCs elicited from CeA^{Nts} neuron terminals (H) Representative traces of optically-evoked IPSCs across a range of laser intensities (0, 10, 25, 50, 75, 100%).

Figure 2. Knockdown of *vGAT* in NTS^{CeA} neurons decreases alcohol drinking in an IA paradigm in male mice. (A) Alcohol consumption was not significantly different between female

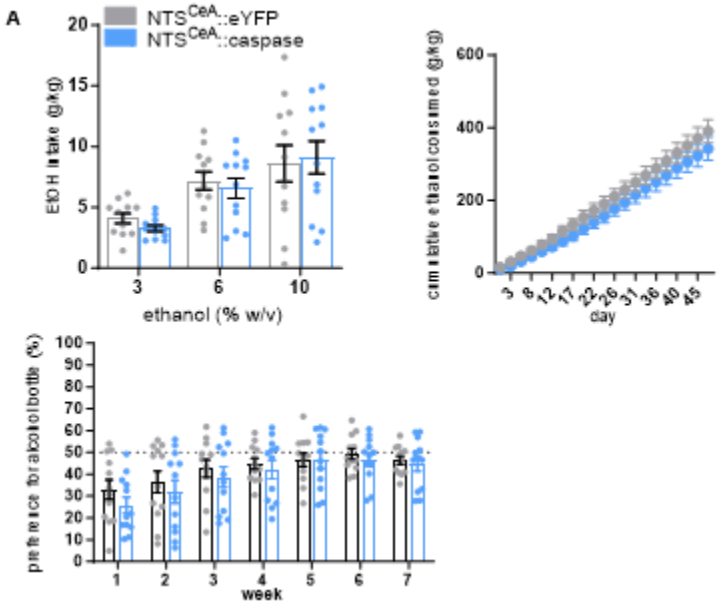
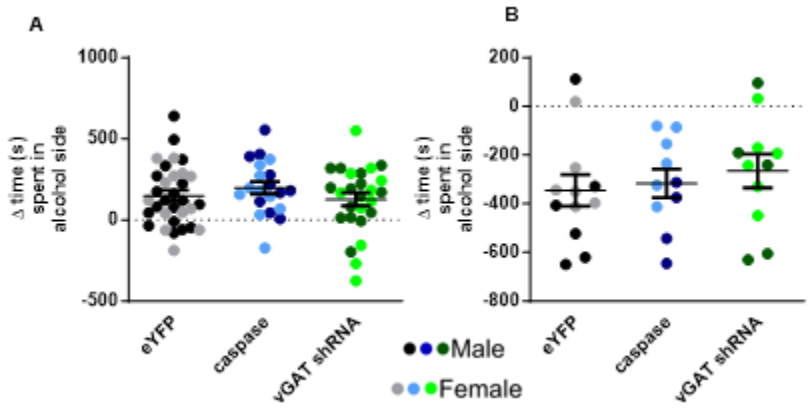
CeA^{Nts}::caspase (n=8) and CeA^{Nts}::vGAT-shRNA (n=8) mice across 7 weeks of IA drinking. (B) Male CeA^{Nts}::vGAT-shRNA mice (n=9) consume less alcohol than CeA^{Nts}::eYFP mice (n=8) in an IA paradigm and demonstrate a trend of reduced preference . (C) Total liquid consumed throughout the study

Figure 3. vGAT knockdown in CeA^{Nts} neurons increases thermal sensitivity without altering other mechanosensory behaviors (A) Results from von Frey assay show no differences in baseline mechanical hypersensitivity between groups regardless of sex. (B) Results from the cold plantar assay show no differences in cold hypersensitivity between groups regardless of sex. (C) Results from the Hargreaves assay show main effects of sex ($p<.028$) and treatment ($p=.011$) in thermal hypersensitivity.

Figure 4. vGAT knockdown in CeA^{Nts} neurons or ablation of these neurons does not modulate avoidance behavior (A) Locomotor and time spent in the center of an open field show no differences in locomotion or avoidance behavior between groups. Females demonstrated significantly more distance moved than males as was expected ($p=.0011$). (B) Time spent in open arms and entries to open arms of an elevated plus maze over five minutes show no differences between groups regardless of sex. (C) There were no differences between groups with regard to time spent in the light side or entries to the light side of a light-dark box over fifteen minutes.

Figure 5. vGAT knockdown in CeA^{Nts} neurons increases latency to feed in the novelty suppressed feeding test without altering novel object preference (A) CeA^{Nts}::vGAT-shRNA mice showed an increased latency to feed compared to both CeA^{Nts}::caspase mice ($p=.0448$) and CeA^{Nts}::eYFP controls ($p=.0167$) in the novelty-suppressed feeding test (main effect of treatment $p=.0101$). (B) Post test consumption assessment from a subset of mice from Figure 5A show no differences between groups regardless of sex. (C) There were no differences in novel object preference or latency to interact with objects in the novel object recognition test.

Supplementary figure 1. Caspase ablation of CeA^{Nts} neurons does not change alcohol drinking in female mice (A) Alcohol consumption and preference were not significantly different (2-Way ANOVA) between female CeA^{Nts}::caspase (n=12) and CeA^{Nts}::eYFP (n=12) mice across 7 weeks of IA drinking.



Supplementary figure 2. Neither vGAT knockdown in CeA^{Nts} neurons nor ablation of these neurons changes associative alcohol reward learning in conditioned place assays (A) There were no differences between groups or sexes in time spent on the ethanol-paired side of the apparatus during the conditioned place preference assay (CeA^{Nts}::eYFP male n=19, female n=13; CeA^{Nts}::vGAT shRNA male n=15, female n=11; CeA^{Nts}::caspase male n=15, female n=10. 2-Way ANOVA sex x treatment $F_{(2,72)}$=.1331, p=.8756). (B) There were no differences between groups in time spent on the ethanol-paired side of the apparatus during the conditioned place aversion assay (CeA^{Nts}::eYFP male n=6, female n=6; CeA^{Nts}::vGAT shRNA male n=5, female n=6; CeA^{Nts}::caspase male n=4, female n=6. 2-Way ANOVA sex x treatment $F_{(2,27)}$=.4544, p=.6396. Main effect of sex p=.0495).