Chemogenetic activation of mesoaccumbal Gamma-Aminobutyric Acid projections selectively tunes responses to predictive cues when reward value is unexpectedly decreased

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ABSTRACT

Background: Mesolimbic circuits regulate the attribution of motivational significance to incentive cues that predict reward, yet this network also plays a key role in adapting reward-seeking behavior when the contingencies linked to a cue unexpectedly change. Here we asked whether mesoaccumbal gamma-aminobutyric acid (GABA) projections enhance advantageous responding to incentive cues of unpredicted reward value, and whether these effects were distinct from global activation of ventral tegmental area (VTA) GABA circuits.

Methods: We used a viral targeting system to selectively activate mesoaccumbal GABA projections during a novel cue-dependent free-operant Value Shifting (VS) task, in which the volume of a sucrose reward associated with a predictive cue is unexpectedly altered, at the beginning and throughout the session. We compared the results with global activation of VTA GABA neurons, which will activate local inhibitory circuits and long loop projections.

Results: We found that activation of mesoaccumbal GABA projections decreases responding to incentive cues associated with smaller than expected rewards. Moreover, this tuning of behavioral responses was specific to cues associated with smaller than expected rewards, and did not impact responses to larger rewards, or measures more closely related to the reward itself. This effect was in marked contrast to activating all VTA GABA neurons, which resulted in a uniform decrease in responding to incentive cues irrespective of changes in the size of the reward.

Conclusions: Targeted activation of mesoaccumbal GABA neurons facilitate more advantageous reward-seeking behaviors. This suggests that these projections may play a very specific role in associative learning processes.

INTRODUCTION

Altering the motivational properties of a predictive cue when the value of a reward unexpectedly changes is a critical neurobehavioral component of survival. The mesolimbic system has been heavily implicated in incentive motivation processes, as well as in adapting behavior when reward contingencies unexpectedly change (1-6). While dopamine (DA) neurotransmission from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) is fundamental to both these processes, γ-aminobutyric acid (GABA) neurons are also present in the VTA(7-9). Yet the role of these neurons in regulating reward-seeking behavior is not well understood. While the VTA contains primarily GABA interneurons, there is a subpopulation of $VTA_{(GABA)}$ neurons that project to the NAc ($VTA_{(GABA)}$→NAc, (10-12)). Approximately one third of the total VTA neurons that project to the NAc are GABAergic (13). Optogenetic stimulation of all $VTA_{(GABA)}$ neurons, including interneurons, decreases anticipatory licking in response to an odor predicting a reward during a classical conditioning task(14). Combined with electrophysiology data demonstrating that activation of $VTA_{(GABA)}$ neurons inhibits dopamine neuronal firing during reward expectancy, others have suggested that $VTA_{(GABA)}$ regulation of dopamine neurotransmission contributes to encoding reward prediction error during associative learning processes (14, 15). However, the contribution of $VTA_{(GABA)}$ projections to the NAc in mediating these processes was not evaluated in these studies, and its role remains largely unknown. One study demonstrated that activation of $VTA_{(GABA)}$ terminals in the NAc enhances discrimination between a cue predictive of an aversive stimulus (i.e. foot shock) from a non-paired cue via GABAergic inhibition of cholinergic interneurons (CINs) (16). Yet there is scant evidence $VTA_{(GABA)}$→NAc projections play any role in responding to reward predictive cues. Indeed, we and others have recently reported that directly activating $VTA_{(GABA)}$ terminals in the

NAc does not impact sucrose consumption or cue-induced anticipatory licking in mice, nor does it regulate operant responding to reward predictive cues in rats (17, 18).

The primary target of $VTA_{(GABA)}$→NAc projections are tonically active cholinergic interneurons (CINs)(16). CINs "pause" when learning new cue-reward associations (16) and are active when reward-seeking is disadvantageous (e.g. during satiety, (19, 20) . It is therefore possible that $VTA_{(GABA)}$→NAc projections may not have a strong influence on reward-seeking in animals with well-established reward associations, but are engaged when establishing or adapting to new associations and contingencies. Here we test the hypothesis that $VTA_{(GABA)}$→NAc neurotransmission specifically facilitates adapting advantageous behavioral responses when the reward value associated with predictive cues abruptly changes. To test this, we chemogenetically activated mesoaccumbal GABA projections selectively during a novel cue-dependent operant task in rats, in which the magnitude of a natural reward (i.e. sucrose) associated with a predictive cue is unexpectedly altered (Value Shifting, VS). We compared these effects to global activation of $VTA_{(GABA)}$, which will activate GABA interneurons and projections throughout limbic and cortical brain regions.

METHODS AND MATERIALS

Subjects

Long Evans rats (Envigo, Indianapolis, IN) weighing between 290-320 grams were individually housed in 12h/12h light/dark, with lights on at 3AM. Experiments were conducted at 9AM. Food and water were available *ad libitum*. All procedures complied with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Care and Use Committee at the University at Buffalo.

Adeno-associated virus (AAV)

We use a combinatorial viral method to target specific neuronal subtypes including dopamine (21-23) and GABA neurons (18). Briefly, EF1α-DIO-hM3D-mCherry-AAV2/10 was co-infused with GAD1-Cre-AAV2/10 into the VTA (n=13; Figure 1A). The expression of the excitatory DREADD hM3D is dependent upon Cre recombinase to reorient the transgene into a translatable position in relation to the EF1a promoter. The GAD1-Cre-AAV2/10 construct provides Cre only in neurons that express glutamate decarboxylase 1. When co-infused they result in hM3D expression in GAD1+ GABAergic neurons (18). It should be noted that the population of dopamine neurons that also make GABA do so using a biochemical pathway independent of GAD1, and these neurons appear to be GAD1 negative(24). A separate cohort of rats (n=9) was co-infused with GAD1-CRE-AAV2/10 and a CMV-DIO-tdTomato-AAV2/10 to serve as a DREADD free controls.

Stereotaxic Virus Injection Surgery

Rats were bilaterally infused with 0.5 μl of virus combination (0.17 μl of GAD1-Cre and 0.33 μl of DIO-hM3D-mCherry AAV) into the VTA (in mm from bregma: AP -5.6, ML ±1.0, DV -8.5) over 2 min using a Nanoject III programmable injector (Drummond Scientific Company, Broomal, PA), followed by implantation with double 26-G guide cannulae (PlasticsOne, Roanoke, VA) above the NAc. Target coordinates for the cannula were (in mm from bregma): AP, +1.5; ML, ±1.1; DV, −5.5 (18). The guide cannula hub was fixed to the skull with acrylic dental cement and secured with stainless steel bone screws. Obturators were inserted into the cannulae to prevent occlusions and plastic dust caps secured them into place.

Behavioral Apparatus

Operant chambers

Med-Associates operant chambers (Georgia, VT) in sound-attenuating cubicles were equipped with two illuminated nosepoke ports on either side of a liquid receptacle (i.e. reward cup) equipped with an infrared entry detector. A white houselight and tone generator speaker were located on the opposite wall. Each chamber was also equipped with a pump to deliver 10% sucrose solution to the receptacle. During IC and VS sessions, a 10 ml syringe was used to deliver ~15 µl/s 10% sucrose solution, while a 30 ml syringe was used during free drinking sessions to deliver ~45 µl/s 10% sucrose solution. A dim white house light and speaker were located on the wall opposite the nosepokes and reward cup.

Operant Training

Incentive cue (IC) task

Rats were trained on the Incentive Cue (IC) task (Figure IC), which was modified from others described elsewhere (18, 25-27) and is described in detail in the Supplementary Materials. In the initial IC training task, the rats respond during a distinct 8-second audiovisual cue to obtain a ~64 µl sucrose reward, and importantly this volume was kept constant throughout the 1-hr session.

Value Shifting (VS) Task

Once rats reached criterion performance in the IC task, we probed rats with the Value Shifting (VS) task to assess the effect of altering the reward unexpectedly. In this task, diagrammed in Figure 1D (right panel), the IC predicting reward availability remained unchanged throughout all sessions. However, we divided the 1-hr session into three 20-min blocks where a successful

response to the IC resulted in the delivery of one of three fixed volumes of sucrose (16 µl, 48 µl or 128 µl), which was achieved by changing the length of time the pump and associated CS+ was on (1s, 3s, 8s). There were ~35 ICs in each block, and every IC in that 20-min block received the same designated volume of sucrose per successful response. The order of the three 20-min blocks, and the corresponding reward sizes, were randomized by the computer during the 1 hr session. All possible order permutations were equally represented in the VS probe tests (Figure 1D (bottom panel). Rats were maintained on the standard IC task (60 µl for 1-hr, Figure 1D, left) between VS task probes, with at least 2 days between probes and treatments.

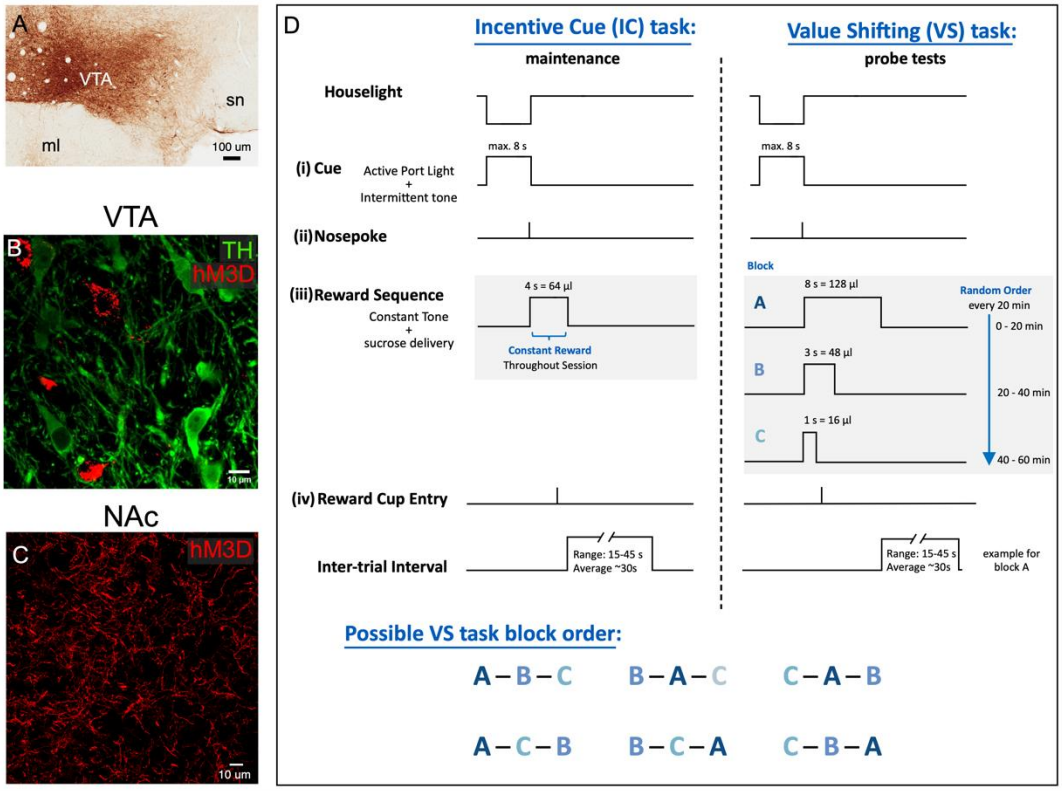

Figure 1 ***Viral targeting of activating DREADDs to VTA GABA neurons in the incentive cue and value shifting task.*** **(A)** Extent of virus expression within the VTA (scale bar 100 µm). Expression of hM3D mCherry DREADD expression in **(B)** VTA neuron bodies and (**C**) NAc terminals (red) compared to those expressing tyrosine hydroxylase (TH, green). **(D)** Schematics depicting the events in the incentive cue task the rats were trained and maintained on (left panel), the VS probe test (right panel), and the potential order of reward blocks in any given probe session (bottom panel). ml, medial mammillary nucleus, SN, substantia nigra.

Free drinking and locomotor activity

Rats expressing the DREADD in $VTA_{(GABA)}$ neurons were tested in a free drinking session in the operant chambers (n=8), while another cohort (n=5) were tested in open field chambers for locomotion as described previously (18), and in detail in the Supplementary Methods.

Drugs and treatment

To selectively activate mesoaccumbal GABA projections in the NAc, rats implanted with cannula were injected with 0.3 µl of 0.95 mM clozapine *N*-oxide (CNO, National Institutes of Drug Abuse, Baltimore, MD) dissolved in saline (28) at a rate of 0.15 µl/min, or an equal volume of vehicle. The microinjection cannulae (33-G) extended 2.0 mm beyond the ventral tip of the guide cannulae (final DV coordinate 7.5 mm below skull surface). The rats were gently restrained, the injectors inserted into the guides, and allowed to remain in place for 1 min before bilaterally infusion. After the infusion, the injection cannulae were allowed to remain in place for 1 min before removing. Each rat was placed in the operant chamber for testing 15 min after the microinfusion. For global activation of $VTA_{(GABA)}$, which include both VTA interneurons and projection neurons to the NAc, CNO was dissolved in 0.9% sterile saline and administered 0.3 mg/kg intraperitoneally (i.p.) 30 minutes prior to the start of the behavioral session. During the study, microinjection tests preceded systemic tests, and within each pair of treatments the order of CNO and saline injections were randomized. To exclude a potential confound from CNO conversion to clozapine on behavioral responses, a separate cohort of DREADD-free rats (n=6) were infused with clozapine (CZP, Enzo Life Sciences, Inc., Farmingdale, NY) dissolved with

0.9% sterile saline and 0.3mg/kg administered i.p. 30 min prior to a VS session. These sessions were counterbalanced with systemic saline vehicle and CNO treatments.

Histological verification, immunohistochemistry, and visualization

Histological verification of virus and cannula placements were conducted for all subjects using immunohistochemistry, as detailed in the Supplementary methods, and described elsewhere (18, 21, 29).

DATA ANALYSIS AND STATISTICAL ANALYSIS

For operant behavior, our primary metrics were the ***response ratio*** of responding to the IC predicting availability of sucrose (#nosepokes resulting in a reward/#IC presented), the ***latency to nosepoke*** in response to the IC ($Time_{nosepoke}$ - $Time_{IC}$), and the ***latency to enter the reward cup*** ($Time_{cup\ entry}$ – $Time_{nosepoke}$) during the VS probe trials. We adopted a sequential strategy of analysis for these experiments. To assess the effects of CNO or vehicle treatment on both activating DREADD (hM3D) and tdTomato rats, we first directly compared the overall average response ratio and response latencies during each reward size. To determine if the rats were adapting their response behavior during the VS probe trials, we then compared the response ratio and response latencies during the first 5 and last 5 minutes of each reward block. Lastly, we determined the response ratio for the first twelve trials after the rats experienced the unexpected reduction in sucrose volume to the lowest volume (i.e. trials 2-13) and last dozen ICs. The first trial was excluded as the rats were not aware of a change in sucrose volume until after they emitted a response. We also examined a number of secondary metrics to assess whether the treatment caused behavioral changes in unconstrained responses. These include ***accuracy*** (#total

active nosepokes/#reward**), *active nosepokes per IC*** (#total active nosepokes/#IC), ***reward cup entries per reward*** (#total cup entries/#rewards earned), and ***total inactive nosepokes***.

Control behavior metrics consisted of total volume consumed for free drinking sessions, and distance traveled for locomotion; further details are in the Supplementary Methods.

All data are presented at means ± SEM. Statistical comparisons were conducted using t-tests, one-way repeated-measures (RM) ANOVA, Two-way or Three-way mixed effects model analysis, followed by a Holms-Sidak correction for pairwise comparisons using GraphPad Prism 8. For clarity, only significant effects and interactions are reported. The level of significance was set to $\alpha=0.05$.

RESULTS

VIRAL TARGETING VTA$_{(GABA)}$ NEURONS

Our combinatorial viral system has been previously verified to drive transgene expression in VTA$_{(GABA)}$ neurons, to produce optically induced GABAergic inhibitory currents when channelrhodopsin-2 is expressed, and to functionally inhibit nearby dopamine neuronal firing when activating DREADDs are expressed and CNO is applied (18). Here we demonstrate consistent spread of the hM3D-mCherry DREADD through the VTA after infusion of 0.5 µl of combined viruses (Figure 1A.), that the DREADD expression is restricted to cell bodies not expressing tyrosine hydroxylase (TH), a marker for dopamine neurons (Figure 1B.), and that expression is found in terminals in the NAc (Figure 1C).

ACTIVATION OF MESOACCUMBAL GABA PROJECTIONS DECREASES REWARD-SEEKING WHEN REWARD SIZE IS UNEXPECTEDLY DECREASED

To assess how selective activation of mesoaccumbal GABA projections regulates reward seeking behaviors during unpredicted changes in reward contingencies, we first examined the response ratio during the VS probe task (Figure 2). When comparing the overall response ratio during each unexpected reward size across all treatments and groups, the response ratio was significantly decreased when the reward size was 16 µl (Figure 2A). Three-way Mixed Effects analysis showed fixed effects of reward volume $F_{2,40}$=23.56, $p<0.0001$, and hM3D vs tdTomato virus type $F_{1,40}$=5.362, p=0.0258, and a hM3D vs tdTomato x CNO vs VEH treatment interaction $F_{1,40}$=, p=0.0038. Importantly, the effects on response ratio performance during 16 µl was specific to rats expressing hM3D, and were not present with either vehicle or in DREADD free control subjects. In comparing response ratio in the first and last 5 minutes of each reward bin,

we found that a significant decrease only in the last 5 minutes of the 16 µl reward block compared to vehicle (Figure 2B). Three-way Mixed Effects analysis showed fixed effects of reward volume $F_{2,24}$=7.016, p=0.004, CNO vs VEH $F_{1,12}$=15.79, p=0.0018, and reward volume x within bin $F_{2,24}$=13.49, p=0.0001, CNO vs VEH x within bin $F_{1,12}$=9.335, p=0.01, and reward volume x CNO vs VEH x within bin $F_{2,24}$=6.081, p=0.0073 interactions. Statistical analyses showed that tdTomato controls were not sensitive to CNO between the first and last 5 minutes of any reward volume. However, they remained sensitive to unexpected changes in reward volume overall (Three-way Mixed Effects analysis, fixed effects of reward volume $F_{2,18}$=4.020 p=0.036). Moreover, tdTomato rats reduced their responding to the cue when comparing the beginning and end of the 16 µl reward block (Reward volume x within bin interaction $F_{2,18}$=5.222 p=0.0163.)

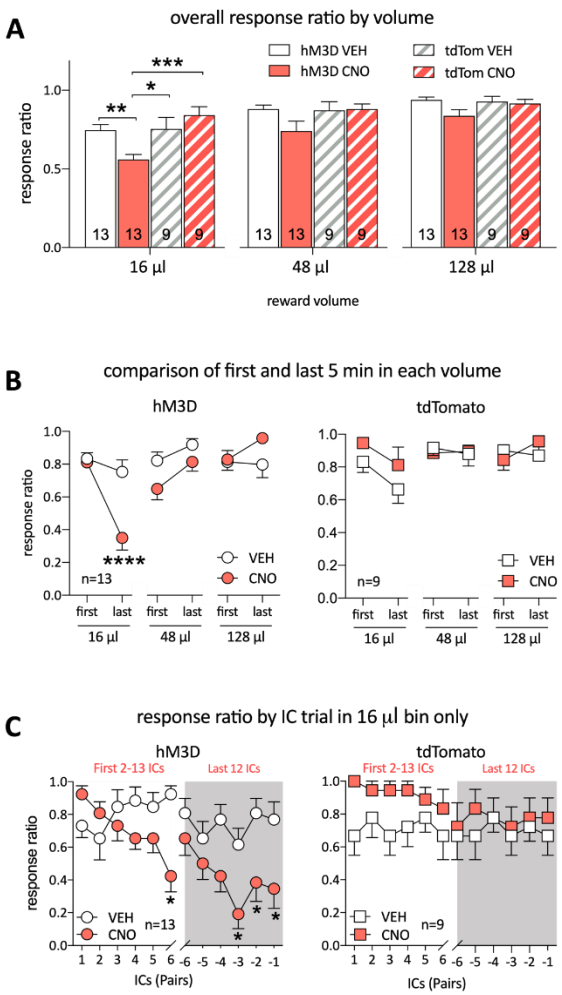

Figure 2 ***Activation of VTA$_{(GABA)}$ terminals in the NAc reduces responses to reward predictive cues reinforced by lower than expected rewards.*** All data are shown as mean±SEM. (**A**) The overall response ratio significantly decreased after intra-NAc CNO in rats expressing hM3D compared to vehicle, but not in rats expressing tdTomato, when the reward size was 16 µl. **(B)** Within the first and last 5 minutes of each reward size, intra-NAc injections of CNO only reduced IC performance in hM3D rats at the end of the 16 µl bin. **(C)** Responses to individual successive pairs of ICs during the 16 µl bin highlights an adaptive response only in CNO treated hM3D rats after experiencing a change in reward contingency. Asterisks represent significant differences between CNO and VEH determined by a Holms-Sidak post-hoc test (*p<0.05, **p<0.01, ***p<0.005 ****p<0.001)

Further analysis demonstrated that dynamic changes in response ratio occurred on a cue by cue basis during the 16 µl reward block only after intra-accumbal CNO treatment in DREADD-expressing rats (Figure 2C, Two-way Mixed Effects analysis, fixed effects of cue pair $F_{11,132}$=4.743 p<0.0001, CNO vs VEH $F_{1,12}$=14.88 p=0.0023, and a cue pair x CNO vs VEH $F_{11,132}$=3.325 p=0.0006 interaction). This effect was not observed in tdTomato controls.

We next examined whether activation of $VTA_{(GABA)} \rightarrow NAc$ projections influences the vigor of responding to the cue and to collect the reward in the VS probe task (Figure 3). Rats changed their latency to nosepoke to the IC relative to the reward size, and there was an overall effect of CNO treatment compared to vehicle control. However, there were no overall effects on hM3D vs. tdTomato virus type, and no significant interactions between reward size, hM3D vs tdTomato virus type, and CNO vs VEH treatment, (Figure 3A. Three-way Mixed Effects analysis, fixed effects of reward volume $F_{2,40}$=26.64, p<0.0001, and CNO vs VEH $F_{1,20}$=5.347, p=0.0315). Within the first and last 5 minutes of each reward bin, we found that that nosepoke latency was significantly increased after CNO compared to vehicle only in the last 5 minutes of the 16 µl reward block (Figure 3B). Three-way Mixed Effects analysis demonstrated fixed effects of reward volume $F_{2,24}$=9.766, p=0.0008, CNO vs VEH $F_{1,12}$=8.911, p=0.0114, and a reward volume x within bin $F_{2,24}$=8.093, p=0.0021 interaction). Although there was an overall difference in nosepoke latency depending on the reward size in the tdTomato controls (Three-way Mixed Effects analysis, fixed effects of reward volume $F_{2,16}$=6.973 p=0.0066) and the nosepoke latency at the beginning and end of each reward block differed according the reward size (reward volume x within bin interaction, $F_{2,16}$=4.670 p=0.0253), the CNO treatment had no effect. We found no significant differences in the latency to enter the reward cup to consume reward after a successful nosepoke response to an IC, either as a function of reward size, virus type, or CNO vs. VEH treatment (Figure 3C). Further, there were no differences in reward cup latencies between the first and last 5 minutes of each reward bin across all of the variables tested (Figure 3D).

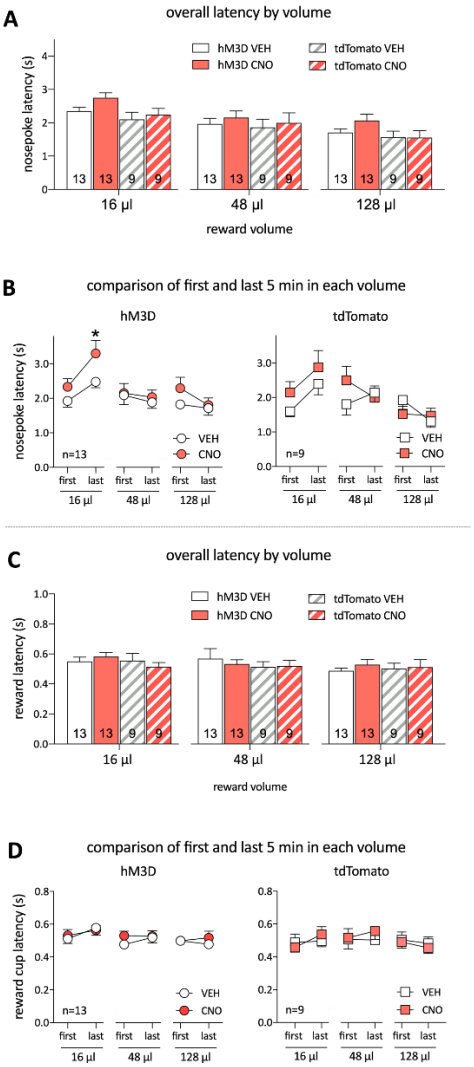

Figure 3 ***Activation of VTA$_{(GABA)}$ terminals in the NAc increases latency to respond to cues that predict lower than expected reward, but not latency to collect the reward.*** All data are shown as mean±SEM. (**A**) Overall latency to nosepoke increased when the reward size was reduced to 16 µl, but there were no significant differences within each reward size. **(B)** Within the first and last 5 minutes of each reward size, intra-NAc injections of CNO only increased the latency to nosepoke to the IC in hM3D rats at the end of the 16 µl bin. **(C)** No effect of intra-NAc treatment was seen in latencies to enter the reward cup after responding to the IC. **(D)** No within-reward size effects on latency to enter the reward cup were seen after intra-NAc treatment. Asterisks represent significant differences between CNO and VEH determined by a Holms-Sidak post-hoc test (*$p<0.05$)

Finally, we examined how mesoaccumbal GABA activation during the VS probe task influenced unreinforced responses, specifically accuracy, total active nosepokes per IC, reward cup entries per reward, and responses in the inactive nosepoke (Figure 4). While three of the

metrics of unconstrained behavior during the VS task showed a significant effect of reward size (Three-way Mixed Effects analysis, fixed effects of reward volume, 4A. Accuracy: $F_{2,40}$= 4.663, p= 0.0151, 4B. active nosepokes per IC $F_{2, 40}$ = 35.94, p< 0.0001, and 4C. reward cup entries per reward $F_{2, 40}$=10.39, p=0.0002), demonstrating that rats were adapting their responses as reward sizes changed, there were no effects of hM3D vs. tdTomato virus type, or CNO vs. VEH treatment in any group. There were no significant effects observed in inactive nosepoke responses.

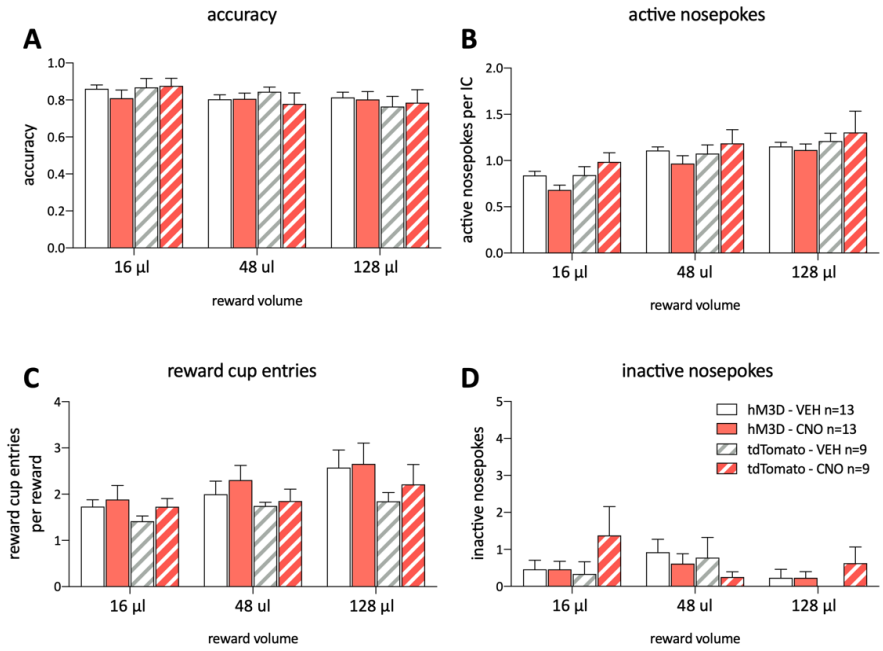

Figure 4 ***Activation of VTA$_{(GABA)}$ terminals in the NAc have no effect on the four possible response outcomes in the VS task.*** All data are shown as mean±SEM. There were no significant effects of intra-NAc treatment on accuracy (the number of active nosepokes per reward obtained) (**A**), active nosepokes per IC presented **(B)**, reward consummatory behavior as measured by reward cup entries per reward earned **(C)**, or changes in discriminating between the active and inactive nosepoke **(D)**.

GLOBAL ACTIVATION OF VTA$_{(GABA)}$ NEURONS DECREASED REWARD-SEEKING REGARDLESS OF CHANGES IN REWARD SIZES

In contrast to targeted activation of the mesoaccumbal GABA projections, global activation of VTA$_{(GABA)}$ neurons using systemic CNO, including local interneurons and projections to limbic and cortical regions, uniformly reduced responding to reward predictive incentive cues during the VS task (Figure 5). The response ratio was significantly decreased across all reward sizes after systemic CNO treatment (Figure5A. Three-way Mixed Effects analysis, fixed effects of reward volume $F_{2,40}$=16.40, p<0.0001, and hM3D vs tdTomato $F_{1,40}$=11.14, p=0.0018, CNO vs VEH $F_{1,20}$=28.47, p<0.0001, and a hM3D vs tdTomato x CNO vs VEH interaction $F_{1,40}$=18.36, p=0.0001). The effects of systemic CNO was limited to rats expressing hM3D targeted to VTA$_{(GABA)}$ neurons. Global activation decreased the response ratio within both the first and last 5 minutes across most reward bins compared to vehicle treatment (Figure 5B. Three-way Mixed Effects analysis, fixed effects of reward volume $F_{2,24}$=6.148, p=0.007, within bin $F_{1,12}$=88.41, p<0.0001, and a reward volume x within bin $F_{2,24}$=5.826, p=0.0087 interaction). Within the tdTomato controls we again observed a modest proportional change in response ratio with reward volume (Three-way Mixed Effects analysis, fixed effects of reward volume $F_{2,16}$=7.827, p=0.0043), but also observed a reward volume x treatment x within bin interaction ($F_{2,16}$=6.173, p=0.0103). However, no clear trend was apparent and comparing individual responses between CNO and vehicle revealed no significant differences.

When conducting a cue by cue analysis of the response ratio performance in the 16 µl reward block, we found that global VTA$_{(GABA)}$ activation produced a uniform reduction in response ratio from the beginning of the block (Figure 5C, Two-way Mixed Effects analysis, fixed effects of cue pair $F_{11,132}$=3.115 p=0.0009 and VEH vs CNO $F_{1,12}$=43.11 p<0.0001). In tdTomato controls,

there was no effect of CNO treatment, although there was an overall modest decrease in responding on a cue by cue basis in the 16 µl reward block (Two-way Mixed Effects analysis, fixed effects of cue pair $F_{11,88}$=1.932 p=0.0457),

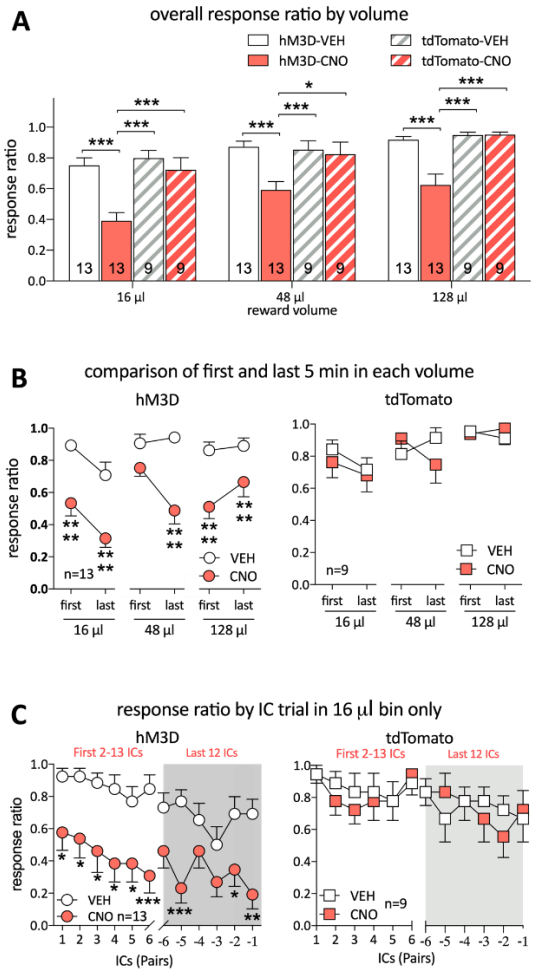

Figure 5 ***Global activation of VTA$_{(GABA)}$ neurons reduces responding to reward predictive cues regardless of reward size.*** All data are shown as mean±SEM. (**A**) Overall response ratio significantly decreased after systemic CNO in rats expressing hM3D compared to vehicle and tdTomato rats across all reward sizes. **(B)** Within the first and last 5 minutes of each reward size, systemic CNO treatment reduced IC performance in hM3D rats at both the beginning and end of the 16 µl and 128 µl reward bin. While CNO also reduced response ratios at the beginning and end of the 48 µl bin, it was only significant at the end. No differences between treatments were seen in tdTomato controls. **(C)** Responses to individual successive pairs of ICs during the 16 µl bin highlights a uniform decrease in responding to cues in CNO treated hM3D rats. Asterisks represent significant differences determined by a Holms-Sidak post-hoc test (*p<0.05, **p<0.01, ***p<0.005 ****p<0.001)

In examining the effects of global $VTA_{(GABA)}$ activation on other VS probe task metrics, we observed an overall increase in nosepoke latencies in all reward blocks in hM3D rats treated with systemic CNO (Figure 6A. Three-way Mixed Effects analysis, fixed effects of reward volume $F_{2,40}$=26.78, p<0.0001, hM3D vs tdTomato $F_{1,40}$=8.026 p=0.0072, CNO vs VEH treatment $F_{1,20}$=6.994, p=0.0155, and a virus x treatment interaction $F_{1,20}$=7.884 p=0.0077). The nosepoke latency was increased in hM3D, CNO treated rats compared to vehicle, between the first and last 5 minutes of each reward block (Figure 6B. fixed effects of reward volume $F_{2,24}$=9.226, p=0.001, CNO vs VEH $F_{1,12}$=11.65, p= 0.0051), although post-hoc tests revealed that this effect was not consistently statistically significant across all reward sizes. No effects were seen in tdTomato controls. Likewise, after global $VTA_{(GABA)}$ activation, rats showed an increase in latency to enter the reward cup after a successful response regardless of reward size (Figure 6C. Fixed effects of hM3D vs tdTomato $F_{1,40}$=6.808 p=0.0127, CNO vs VEH $F_{1,20}$=13.39, p=0.0016, and a hM3D vs tdTomato x CNO vs VEH interaction $F_{1,40}$=21.89 p<0.0001). Examining changes in the reward cup latency between the first and last 5 minutes of each reward bin showed that the latency increased regardless of whether it was at the beginning or end of the reward bin, and occurred during all reward sizes compared to vehicle (Figure 6D. Fixed effects of treatment $F_{1,12}$=31.30, p=0.0001).

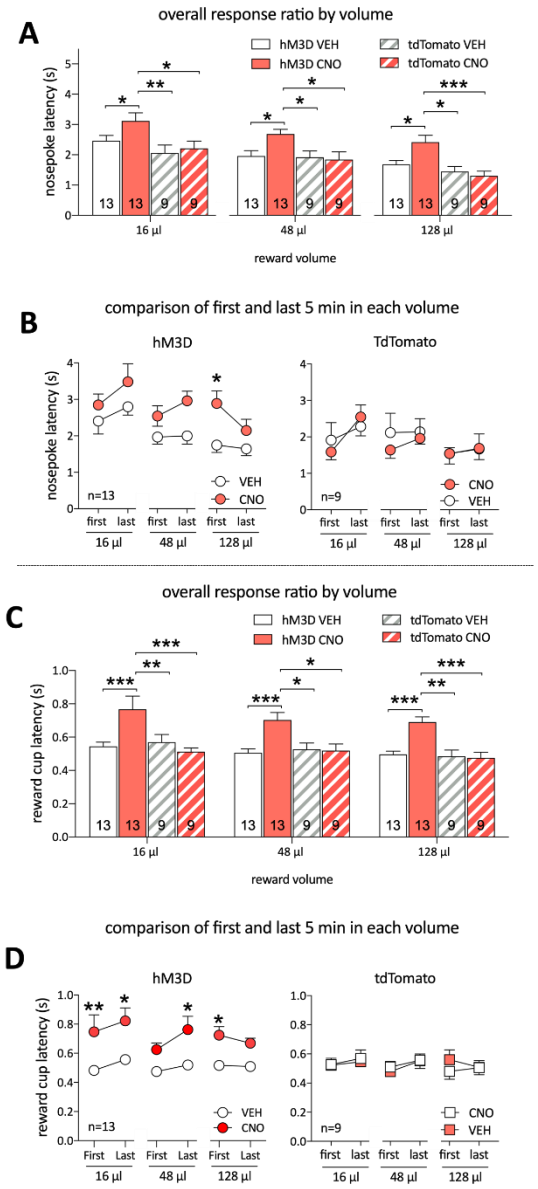

Figure 6 ***Global activation of VTA$_{(GABA)}$ neurons increases the latencies to respond to cues and collect the reward irrespective of reward size.*** All data are shown as mean±SEM. (**A**) Overall latency to nosepoke increased with all reward sizes after CNO pretreatment compared to controls. **(B)** Within the first and last 5 minutes of each reward size, VTA$_{(GABA)}$ activation increased nosepoke latencies overall in both the first and last 5 minutes of each reward bin, although most of this could be attributed to the first 5 min during the 128 µl reward block. No changes were seen in tdTomato controls. **(C)** Latencies to enter the reward cup significantly increased in hM3D rats given systemic CNO across all reward sizes compared to controls **(D)**. Latencies to enter the reward cup were significantly greater in the first 5 min of the 16 and 128 µl reward blocks, and the last 5 minutes of the 48 µl bin. No within-reward block effects on latency to reward were seen in tdTomato controls. Asterisks represent significant differences determined by a Holms-Sidak post-hoc test (*p<0.05, **p<0.01, ***p<0.005).

Finally, we examined how global $VTA_{(GABA)}$ activation mesoaccumbal during the VS probe task influenced accuracy, total active nosepokes per IC, reward cup entries per reward, and responses in the inactive nosepoke. Similar to $VTA_{(GABA)}$→NAc projection activation, rats were adapted their responses as reward sizes changed, except for responses in the inactive nosepoke (Three-way Mixed Effects analysis, fixed effects of reward volume, Figure 7A. Accuracy: $F_{2,40}$= 3.594, p= 0.0367, Figure 7B. active nosepokes per IC $F=_{2,\ 40}$ = 12.48, p< 0.0001, and Figure 7C. reward cup entries per reward $F_{2,\ 40}$=4.049, p=0.025). However, in contrast to targeted $VTA_{(GABA)}$→NAc projections activation, global $VTA_{(GABA)}$ activation resulted in a decrease in active nosepokes per IC after treatment across in all reward blocks (Fixed effects of hM3D vs tdTomato $F_{1,40}$=8.002, p=0.0073, CNO vs VEH $F_{1,20}$=27.61, p<0.0001, and a significant hM3D vs tdTomato x CNO vs VEH, $F_{1,40}$ p=12.26 p=0.0012).

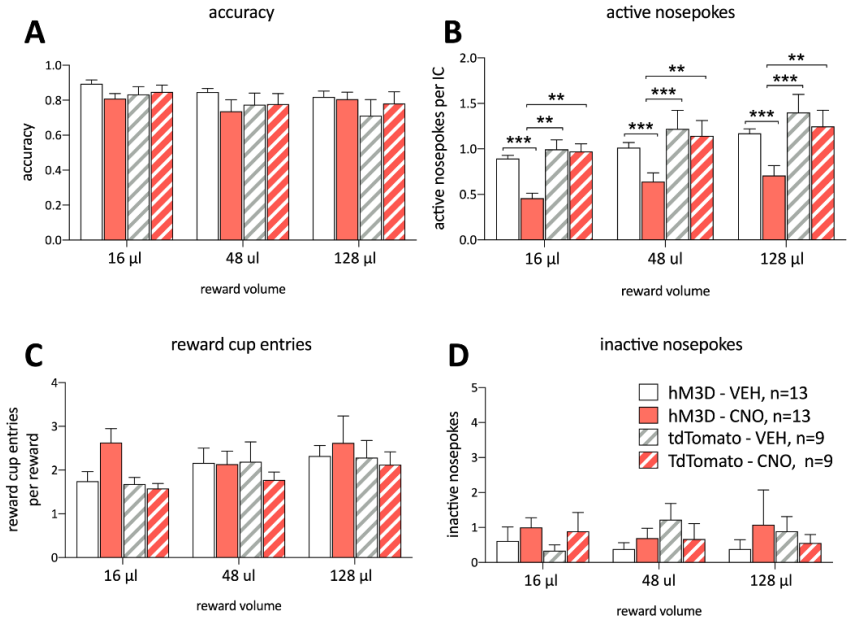

Figure 7 ***Systemic activation of $VTA_{(GABA)}$ neurons selectively impacts measures of responding to the IC in the VS task.*** All data are shown as mean±SEM. There were no significant effects of CNO treatment on accuracy (the number of active nosepokes per reward) (**A**), while there was a decrease in active nosepoke per IC as a result of treatment across all reward sizes compared to controls **(B)**. There were no effects of VTA-GABA activation on reward cup entries per reward earned **(C)**, or changes in the number of inactive nosepokes **(D)**.

MESOACCUMBAL GABA ACTIVATION DOES NOT ALTER SUCROSE PALATABILITY OR LOCOMOTOR ACTIVITY.

To determine if the effects we observe may be contributable to the primary reinforcing effects of sucrose, we assessed free drinking consumption of sucrose. Under these conditions, rats will drink in 2-min a similar amount of sucrose obtained during the entire 1-hr VS task challenge at baseline or after saline. These data indicate that the palatability of sucrose remained unchanged after mesoaccumbal $VTA_{(GABA)}$ activation (Fig. 8A), and do not account for the specific changes in VS task performance within the 16 µl reward bin seen in our earlier experiments. As well, targeted mesoaccumbal activation did not impact the total distance traveled in an open field compared to saline vehicle pretreatment (Fig. 8B), indicating that the primary results in the VS task were not a result from an inhibition in locomotor activity.

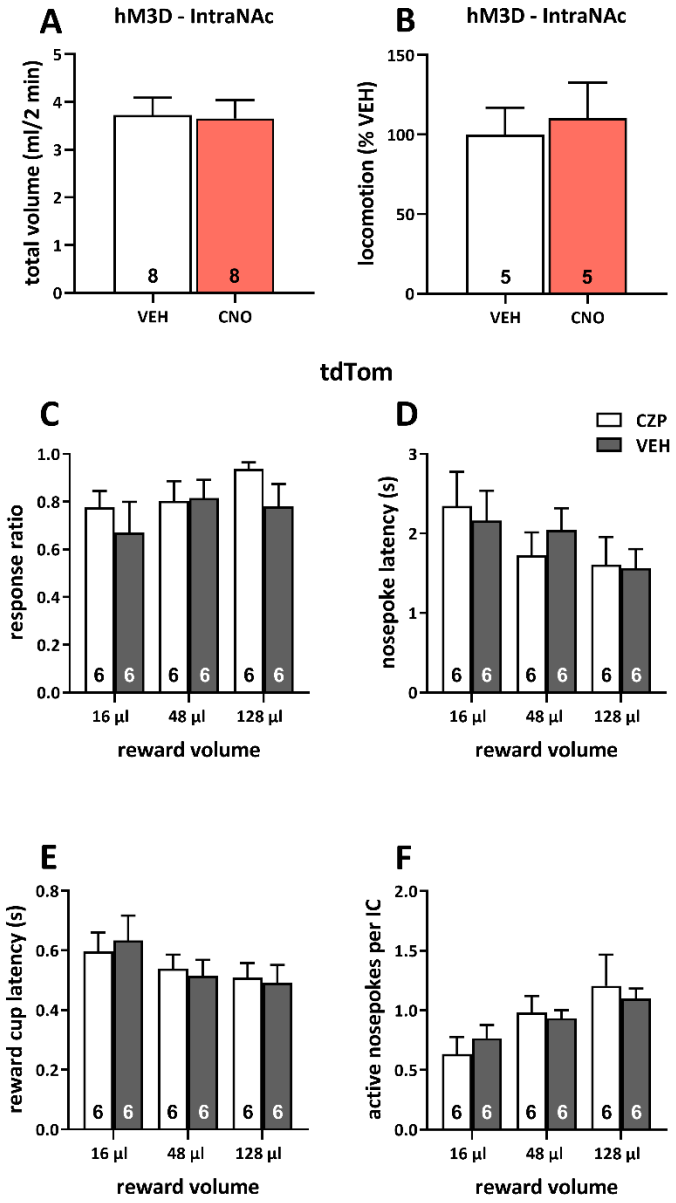

Figure 8 ***Targeted mesoaccumbal activation does not alter sucrose consumption or locomotor behavior, nor does systemic CZP impact VS task performance in DREADD-free controls.*** Data presented as mean ± SEM. **(A)** The total volume of sucrose (ml) obtained during the first 2 min of a free-drinking session produced similar level of consumption allowed during the entire VS session. However, intraNAc CNO pretreatment did not alter the total volume consumed during free-drinking conditions **(B).** Distance traveled (normalized as the percent of saline vehicle control) during a 1-hr open-field locomotor test also did not statistically differ between vehicle and CNO pretreatment. Rats injected with a tdTomato sham virus into the VTA did not show a behavioral effect after systemic pretreatment with 0.3 mg/kg i.p. CZP in the VS task metrics of **(C)** response ratio, **(D)** nosepoke latency, **(E)** reward cup latency, or **(F)** active nosepoke per IC, four of the VS task metrics that showed an effect after pretreatment with CNO in hM3D+ rats.

Due to recent evidence that CNO is converted to CZP in vivo (30), we also determined if any of the effects of systemic CNO on VS task performance could be attributed to activity at receptors other than hM3D. We found that an equivalent systemic dose of CZP (0.3 mg/kg, i.p.), had no impact on the four of the metrics of the VS task that showed an effect after systemic pretreatment with CNO in hM3D+ rats (Fig.8C-F). In agreement with other studies on operant responding (31-34), metabolism to CZP at the dose of CNO used in this study (0.3 mg/kg, i.p.) are not attributable to the primary results in our experiments.

DISCUSSION

This study was undertaken to test the hypothesis that $VTA_{(GABA)}$ projections to the NAc specifically facilitate adapting behavioral responses in the face of changing reward contingencies. We achieved this by selectively activating mesoaccumbal GABA terminals using DREADDs, while unexpectedly changing the volume of a sucrose reward associated with a reward-predictive cue. We found three main novel findings, first, that activation of mesoaccumbal GABA projections selectively tunes behavioral responses to a cue when the reward is smaller than expected. Secondly, the decrease in responding induced by $VTA_{(GABA)}$→NAc projection activation occurred with experience, and became more pronounced over the 20-min bin. Third, that this tuning was specific to responding to the incentive cue associated with the reward, and did not impact reward consumption. In contrast, global activation of all $VTA_{(GABA)}$ neurons, which would affect GABA projections to different regions as well as GABA interneurons, attenuated responding regardless of reward contingency, decreased responding to the cue irrespective of experience with the new reward contingency, and attenuated reward consumption.

Reward seeking is a multifaceted process, and engages multiple reward-related systems and neural circuits (10, 35-37). Several different reward processes have been ascribed to mesolimbic circuitry, including those underlying associative learning, which evaluate the outcome of a goal-directed action against an established, learned value (14, 15). For example, negative reward prediction is a type of behavioral learning that occurs when an earned reward is worse than predicted (38), resulting in a subsequent decrease in seeking that reward. We found in rats well trained to expect a fixed volume of reward (64 µl), that during Value Shifting probe tests, where the sucrose reward is unpredictably decreased from that obtained during training sessions (64 µl), or in relation to larger rewards obtained in earlier reward blocks (48 or 128 ul) within the session, that mesoaccumbal $VTA_{(GABA)}$ activation selectively decreased the response ratio and increased the nosepoke latency to a cue suddenly predicting the much smaller volume of sucrose reward (16 ul). Further, a central component of a learned process is that the changed reward outcome must be experienced before an alteration in reward-seeking behavior can occur. Indeed, our results demonstrate that rats initially responded to the cue predicting the smallest reward at levels identical to controls. Yet as rats gained more experience with the new reward contingency with subsequent cue presentations, activation of $VTA_{(GABA)}$→NAc projections facilitated a decrease in responding to the less than expected reward. In addition, the latency to respond to the cue predicting the smallest reward increased at the end of the bin, compared to saline vehicle controls. Together, this suggests that activation of $VTA_{(GABA)}$→NAc projections selectively facilitated a progressive decrease in the choice to respond to the cue over time, along with a decrease in the vigor of the response to the cue (39). These effects were limited to behaviors specific to the cue itself, as there were no effects on the latency to enter the reward cup to obtain a reward, or other task measures (e.g. accuracy, reward cup entries, inactive

nosepokes). Interestingly, the effects of mesoaccumbal GABA projections were specific to the smallest reward, and a comparable increase in either choice or vigor of response was not seen with an unpredicted larger than expected reward. While it is possible that $VTA_{(GABA)}$→NAc projections specifically contribute to forming new stimulus-outcome associations, we cannot preclude the possibility the rats reached a performance ceiling during this instrumental task.

To our knowledge, this is the first demonstration that $VTA_{(GABA)}$→NAc projections can alter cue processing for rewards. Indeed, we and others have generally shown that selectively activating this pathway does not alter cue-induced reward seeking (17, 18). However, one study demonstrated that this pathway enhances discrimination of cues predicting an aversive stimulus(16) . In that study, mice exhibited generalized freezing to two distinct tones, but optogenetic activation of $VTA_{(GABA)}$ terminals in the NAc during conditioning later resulted in the mice freezing less to the non-predictive tone, while freezing was maintained for the tone predicting the foot shock (16). In this way, the non-predictive tone was marked as less motivationally relevant. The authors determined this effect to occur through selective disinhibition or "pausing" of local CINs in the NAc. Future studies examining whether our results also occur through disinhibition of accumbal CINs will be useful in determining if this is a common pathway for facilitating learning new cue-reinforcement contingencies regardless of valence.

The results in this study substantially expand our earlier work, in which we observed no change in cue-induced responding after activating $VTA_{(GABA)}$ projections to the NAc in the standard IC task, where reward size was kept constant (18). The Value Shifting task has different reward contingencies and is likely engaging distinct reward-seeking processes than that of the standard IC task. The Value Shifting challenge differs notably from the IC task in that the reward

sizes are randomly assigned to the same predictive cue within the session. To prevent rats from becoming over-trained on this Value Shifting task and to preserve the novelty of the changed reward contingencies, these sessions were conducted sparingly and always after several days of IC task sessions. A significant aspect of our data is that activation of $VTA_{(GABA)} \rightarrow NAc$ projections alter responding in the Value Shifting challenge even though the rats are very well-trained, and continue to run in the IC task on intervening days. Thus, our subjects have a strong pre-established cue-reward expectancy established by prior training in the IC task. Interestingly, after global activation of $VTA_{(GABA)}$ neurons, we are able to show a decrease in responding, increase in nosepoke and reward latencies, and an increase in the active nosepokes per IC during the Value Shifting challenge, irrespective of the reward size. This supports our earlier findings that global $VTA_{(GABA)}$ activation preferentially inhibits the incentive salience of the reward-predictive cues (18) possibly through $VTA_{(GABA)}$ interneuron inhibition of dopamine neurons, or activity of $VTA_{(GABA)}$ projections to other brain regions. During the Value Shifting probe, changes in performance after systemic activation are present at the beginning of each reward bin. This is a defining characteristic of incentive motivational processes in that changes in reward-seeking behavior occur before experience with the reward itself (2). While our previous study found a decrease in accuracy in responding to the cue after systemic $VTA_{(GABA)}$ activation, as quantified by the number of active nosepokes per reward, this trend did not persist in this follow-up experiment. However, we had previously concluded that the change in accuracy seen in the IC task was the result of a drop in responding to the cue as opposed to behavioral disinhibition leading to inappropriate responding to the cue, and this can be clearly seen during the VS task when quantified by the number of active nosepokes per cue.

Similar to our previous work, we found that the behavioral effects we report here as a result of targeted $VTA_{(GABA)}$→NAc activation are not a result of a change in the generalized palatability of sucrose, as there were no effects on sucrose consumption under free-drinking conditions. Similarly, there were no effects of $VTA_{(GABA)}$→NAc activation on generalized locomotion, suggesting that the decreases in responding to cue associated with a lower than expected reward did not result from a suppression of locomotor activity. Indeed, our data demonstrate that when the rats do respond in the other reward bins, they do so with similar speed, and are thus capable of emitting faster responses (Figure 3). In addition, since it has recently been shown that high doses of CNO are converted to CZP in vivo (30), all of our behavioral metrics for both intracranial CNO and systemic administration of CNO included comparisons with DREADD-free controls. We also determined that systemic CZP in DREADD-free rats did not alter the behavioral metrics of the VS probe, demonstrating that there is no confound from the potential metabolism of CNO to CZP.

In summary, our data demonstrate that targeted activation of $VTA_{(GABA)}$ projections to the NAc preferentially regulates associative learning related to new stimulus-outcomes, possibly underlying negative reward prediction processes. This specific behavioral consequence in a robust operant model is distinct from global $VTA_{(GABA)}$ activation, which appears to preferentially regulate the attribution of incentive salience to reward predictive cues, regardless of the value of the subsequent reward. Further studies will be needed to determine the precise local circuitry within the NAc that contribute to these reward-seeking behaviors.

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