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Dopamine in the Dorsal Bed Nucleus of Stria Terminalis signals Pavlovian sign-tracking and reward violations

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Acknowledgements: This work was supported by a McKnight Memory and Cognitive Disorders Award (McKnight Foundation; DC), and a National Institute on Drug Abuse (NIDA) grant R01DA043533 (DC), and the Department of Anatomy and Neurobiology at the University of Maryland, School of Medicine. The funders had no role in the study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

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Abstract

Midbrain and striatal dopamine signals have been extremely well characterized over the past several decades, yet novel dopamine signals and functions in reward learning and motivation continue to emerge. A similar characterization of real-time sub-second dopamine signals in areas outside of the striatum has been limited. Recent advances in fluorescent sensor technology and fiber photometry permit measurement of dopamine binding correlates, which can divulge basic functions of dopamine signaling in non-striatal dopamine terminal regions, like the dorsal bed nucleus of the stria terminalis (dBNST). The dBNST receives dense dopaminergic input from several regions including the ventral tegmental area, ventral periacqueductal gray, and substantia nigra. Here, we record fluorescent $GRAB_{DA}$ signals in the dBNST during a Pavlovian lever autoshaping task that has established individual differences in cue-evoked striatal dopamine signals in sign- and goal-tracking rats. We observe greater Pavlovian cue-evoked dBNST $GRAB_{DA}$ signals in sign-tracking (ST) compared to goal-tracking/intermediate (GT/INT) rats. We find the magnitude of cue-evoked dBNST $GRAB_{DA}$ signals decrease immediately following reinforcer-specific satiety. When we deliver unexpected reward or omit expected reward, we find that dBNST dopamine signals encode bidirectional reward prediction errors in GT/INT rats, but only positive prediction errors in ST rats. Since sign- and goal-tracking approach strategies are associated with distinct drug relapse vulnerabilities, we examined the effects of experimenter-administered and self-administered fentanyl on dBNST dopamine associative encoding. Systemic fentanyl injections do not disrupt dBNST cue discrimination but generally potentiate dBNST dopamine signals. Fentanyl self-administration experience is sufficient to reverse reward seeking from food to fentanyl and dBNST dopamine signaling discriminates fentanyl-associated active vs. inactive lever pressing under extinction conditions. These results reveal multiple dBNST dopamine correlates of learning and motivation that depend on the Pavlovian approach strategy employed.

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Introduction

Survival depends on learning to associate environmental cues with food or other natural rewards. Individual differences in learning and motivational processes support the acquisition, expression and updating of cue-reward associations. Recent evidence suggests that distinct learning strategies are predictive of dysregulated motivation for drug associated cues/conditioned stimuli (CS) (Chang et al., 2022; Martin et al., 2022; Pitchers et al., 2017; Saunders et al., 2013). Midbrain and striatal dopamine signals are broadly implicated in a diverse array of learning and motivational processes, including CS-reward associations underscoring the importance of dopamine (DA) in adaptive behavior that promotes survival (Langdon et al., 2018; Lee et al., 2022; Nasser et al., 2017). Yet considerably less is known about the role of DA signals in areas outside of the striatum during adaptive and maladaptive cue-reward learning. Recent advances in fluorescent sensor technology and fiber photometry permit measurement of DA binding correlates (Labouesse et al., 2020). These new techniques can reveal understudied functions of DA signaling in non-striatal DA terminal regions like the dorsal bed nucleus of the stria terminalis (dBNST), an extended amygdala nucleus, that is critical for dysregulated CS-triggered opioid relapse (Gyawali et al. 2020). Here, we characterize basic dBNST DA correlates by recording fluorescent $GRAB_{DA}$ signals during a Pavlovian task that distinguishes two distinct relapse vulnerability phenotypes.

Recent studies identify unique learning strategies that predict heightened CS-triggered relapse vulnerability (Chang et al., 2022; Martin et al., 2022; Pitchers et al., 2017; Saunders et al., 2013). In particular, a simple Pavlovian Lever Autoshaping task distinguishes two extreme tracking phenotypes: 1) sign-tracking (ST) rats that approach and vigorously engage with the reward predictive lever cue, even though cue interaction is not necessary to obtain food reward and 2) goal-tracking rats that interact with the foodcup during cue presentation where food reward is delivered after lever retraction (Boakes R.A, 1977; Flagel et al., 2007; Hearst & Jenkins, 1974;

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Meyer et al., 2012). Sign-tracking rats show heightened CS-triggered drug relapse vulnerability compared to goal-trackers. A third group called intermediates approach both the food cup and lever at similar levels, and their relapse vulnerability is like that of goal-tracking rats (Saunders & Robinson, 2010). Fast scan cyclic voltammetry recording of real-time dopamine indicated that sign-, but not goal-tracking, evokes increases in phasic fluctuations in DA in the nucleus accumbens (NAc) during CS presentation (Flagel et al., 2011). NAc DA is necessary for both the expression of sign-tracking and for sign-trackers heightened CS-triggered drug relapse, but not for goal-tracking or their relapse behavior (Saunders et al., 2013). Given the critical role of dBNST in CS-triggered relapse, we aimed to determine whether there are similar individual differences in dBNST DA signaling in sign- and goal-tracking rats using the Pavlovian Lever Autoshaping task (Buffalari & See, 2011; Gyawali et al., 2020; Silberman & Winder, 2013).

Midbrain dopamine neuron activity strengthens cue-outcome associations by serving as a bidirectional prediction error signal where, unexpected reward delivery increases and omitted reward decreases dopamine neuron firing relative to expected reward (Montague et al., 1996; Schultz, 2015; Schultz et al., 1997). Over the course of learning, the phasic dopamine activity transfers from the unconditioned stimulus (US) to the CS (Montague et al., 1996; Schultz, 2015; Schultz et al., 1997). In the NAc, transfer of dopamine signals from US to the CS occurs only in ST rats (Flagel et al., 2011; Flagel & Robinson, 2017), and NAc DA antagonism reduces sign-tracking but not goal-tracking behaviors (Saunders & Robinson, 2012). Together, these studies support the Pavlovian lever autoshaping task (and sign-tracking) as a reliable framework for studying dopamine's role in regions of the brain critically involved in cue-motivated natural and drug reward seeking behaviors.

The dBNST receives dense dopaminergic input from several midbrain regions including the ventral tegmental area, ventral periacqueductal gray, and to a much lesser extent, the substantia nigra (Hasue and Shammah-Lagnado 2002, Meloni et al. 2006). dBNST dopamine is

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associated with a variety of reward-motivated behaviors. dBNST dopamine release is increased during intra-oral sucrose infusion and in response to cues that predict intracranial self-stimulation of the medial forebrain bundle (Lin et al., 2020; Park et al., 2012, 2013). Further, dopamine antagonist injections in the dBNST reduce responding for sucrose in a binge eating paradigm (Maracle et al., 2019). All major drugs of abuse, including opioids, increase tonic extracellular dopamine in the BNST, and a dBNST dopamine antagonism reduces cocaine self-administration and ethanol seeking (Carboni et al., 2000; Eiler et al., 2003; Epping-Jordan et al., 1998). Despite these studies implicating dBNST dopamine in motivated behaviors, a comprehensive characterization of endogenous dBNST dopamine dynamics in cue-induced behaviors is lacking. To address this, we used a dopamine sensor $GRAB_{DA}$ in combination with fiber photometry to examine basic properties of the dBNST dopamine signals; their role during lever autoshaping, reward violations, outcome specific-satiety, systemic fentanyl administration, and during fentanyl-seeking after fentanyl self-administration (Sun et al., 2018).

Materials and Methods

Subjects: We used 8 weeks old male and female Sprague Dawley rats (Charles River, n = 42) weighing >250g before surgery. After surgery, we individually housed the rats and maintained them under a reversed 12:12 h light/dark cycle (lights off at 9 AM). We performed all experiments in accordance with the “Guide for the care and use of laboratory animals” (8th edition, 2011, US National Research Council) and the University of Maryland Institutional Animal Care and Use Committee approved all experimental procedures. We excluded rats because of lack of viral expression, incorrect fiber optic placements, or lack of significant $GRAB_{DA}$ signal during behavioral event compared to baseline (n = 26) or failure of catheter patency (n = 2).

Virus and fiber optic implantation surgery: We anesthetized 9-week-old rats with isoflurane (4.5% induction, 2-3% maintenance) and placed them in a stereotaxic frame. We maintained stable body temperature with a heating pad and administered pre-operative analgesic carprofen (5 mg/kg,

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s.c) and lidocaine (10mg/mL at the site of incision). We made a scalp incision and drilled a hole above left dBNST AP = 0.0 or -0.1 from bregma, ML = +3.5, DV = - 6.75 or -6.8 at 16° from midline for viral injection and DV = -6.6 or -6.7 relative to skull for fiber implantation. In addition, we also drilled 3 holes anterior and posterior to attach anchor screws. We lowered 5μL Hamilton syringe unilaterally into the dBNST and injected AAV9.hsyn.DA4.4.eyfp ($1.14x10^{14}$ GC/mL; WZ Biosciences) via a micropump at a volume of 0.7 - 1μL over 10 minutes. We implanted the fiber optic (ThorLabs CFMC54L10, 400 μm, 0.50 NA, 10 mm) 0.1 mm or 0.15 mm above the virus injection site. We anchored the fiber optic to the skull using dental cement (Metabond and Denmat) and jeweler screws. We handled the rats at least three times a week after surgery before start of the behavioral and photometry sessions.

Catheterization surgery: We implanted (n = 5) rats with intravenous catheters as previously described in Gyawali et al., 2020, Martin et al., 2020 after the completion of testing in Pavlovian Lever Autoshaping. In brief, we made an incision in rats' back which was used to insert Silastic tubing (Dow Silicones Corp) embedded within the 22-gauge stainless steel cannula (Plastics One). We inserted the other end of the tube into the right jugular vein. We flushed rats daily with 0.05mL Taurolidine-Citrate (TCS; Access Technologies) catheter lock solution to promote catheter patency. We also injected intravenous 0.1mL of methohexital sodium (1mg) periodically to test catheter patency and removed rats from the study if they did not display a sudden, reversible loss of muscle tone.

Apparatus: We conducted behavioral experiments in operant chambers housed in sound attenuating cabinets (Med Associates). Each chamber had one white house light that was illuminated during the entire session. On the opposite wall, two retractable levers (CS+ and CS-, right or left location counterbalanced) were located on either side of the foodcup. The foodcup was attached to a programmed pellet dispenser that delivered 45mg training pellets (Testdiet, 5TUL, protein 20.6%, fat 12.7%, carbohydrate 66.7%). During intermittent access fentanyl self-

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administration sessions, a red light in place of the white house light, served as a discriminative stimulus. We used 20 ml syringes containing fentanyl solutions attached to a syringe pump to deliver the drug through the back mount catheter controlled by the Med PC software (Med Associates).

Pavlovian Lever Autoshaping (PLA): We conducted all training sessions during the dark phase. Schematic of our behavioral design can be found in Figure 1A. Five weeks after viral injection surgery, we maintained rats at 90% of *ad libitum* body weight and during all behavioral sessions unless noted otherwise. Prior to the PLA training, we exposed rats to 25 magazine training trials divided into three sessions to acclimatize rats to the operant box and fiber optic cables. The three sessions consisted of 7,8, and 10 trials respectively in which two food pellets (US) were delivered, 0.5s apart using a variable interval (VI) 60s (50-70s) schedule. After magazine training sessions, we trained rats in five 46-minute PLA sessions. Each session consisted of 25 reinforced (CS+) and 25 non-reinforced (CS-) lever presentation trials on a mean VI 45s (35-55s) schedule (Fig. 1B). Each CS+ trial consisted of the insertion and retraction of a lever for 10s followed by delivery of two food pellets, 0.5s apart. CS- trials consisted of insertion/retraction of another lever, but no US delivery. We recorded food-cup and lever approach during the ten seconds CS interaction and calculated a Pavlovian Conditioned Approach (PCA) score (Meyer et al., 2012). We use PCA score as a comprehensive measure of individual differences in PLA that account for contact, latency, and probability differences. We used each rat's Days 4 and 5 average PCA score to determine whether they are sign-trackers (avg PCA score $\geq$ 0.5, ST) or goal-trackers/intermediates (avg PCA score $<$ 0.5, GT/INT).

Reward Prediction Error (RPE) Probe sessions: After five PLA sessions, we gave rats (n = 13) one session in which we violated rats' reward expectations to probe for reward prediction error signaling. During this session, only CS+ lever was presented, and rats received 48 trials divided into three different trial types presented in pseudorandom order. In the 'expected reward'

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condition, we gave 24 reinforced CS+ → US trials (50% of total trials). In the 'unexpected reward' condition, we delivered two food pellets (US) randomly during the intertrial interval period without the predictive CS+ (12 trials, 25% of total trials). Finally, in the 'unexpected reward omission' condition, we delivered the CS+, but omitted the US (12 trials, 25% of total trials) (Patriarchi et al., 2018).

Satiety test: After the RPE session, we trained a subset of rats (n = 11) in PLA for two more days when rats were either sated on food pellets or hungry. In the first day, we gave half the rats 30g of the training food pellets in a ramekin for 30 minutes (pellet sated condition) in their home cage after rats had completed 25 out of 50 trials. For the other half of the rats, we gave empty ramekins in their home cage (control condition). After 30 minutes, we placed the rats back into the operant chamber where they completed the remaining 25 trials in PLA. The next day, we gave training pellets to rats that received empty ramekins on the first day and vice versa. We ran the chow satiety test in a subset of rats (n = 7) using the same experimental design as pellet satiety test but replaced the food pellets in the ramekins with homecage chow instead.

Fentanyl i.p injections: We injected 5μg/kg i.p fentanyl (Cayman Chemical) or vehicle in rats (n = 4) 5 minutes before PLA sessions. We selected this dose based on pilot experiments. In two counterbalanced PLA sessions, we gave the rats either i.p injection of fentanyl or saline.

Intermittent access fentanyl self-administration: The intermittent fentanyl self-administration schedule was similar to that described in Fragale et al., 2021. In brief, we gave rats (n = 5) access to fentanyl in 5 min bins separated by 25 min of drug unavailable periods in a total of 95-minute sessions for 10 days. At the start of each 5 min bin, we gave rats an initial 2 second priming infusion of 0.5μg fentanyl combined with a 5 second compound light and tone cue to indicate availability of the drug. During the drug available period, an active lever press resulted in 2 seconds infusion of 0.5μg fentanyl while inactive lever presses were recorded but did not result in drug infusion or cue presentation. The drug delivery was accompanied by a 2-second

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compound cue above the active lever. There was no timeout following drug infusions beyond the length of the infusions (2s). For the self-administration sessions, each rat's PLA CS+ lever was designated as the inactive lever and CS- lever was designated as the active lever. During non-availability 25 min periods, we retracted the levers and turned off the red light. We cycled through 4 drug availability/no-availability periods giving rats 20 minutes of total access to fentanyl in each 95-minute session. We gave rats *ad libitum* chow in this part of the study.

Extinction probe test: The extinction probe tests occurred on Day 1 and Day 15 after the end of self-administration sessions. The behavioral setup was identical to self-administration sessions except both active and inactive levers were extended for the entire session. We recorded BNST $GRAB_{DA}$ signals while rats responded on the levers in a 20-minute session. A press on the active lever would result in 2s presentation of tone and light cue but no fentanyl infusion while a press on the inactive lever had no consequences.

Fiber Photometry: We used LEDs (ThorLabs) to deliver 465 nm (wavelength to excite $GRAB_{DA}$) and 405 nm (isosbestic control) and measure dopamine activity. The isosbestic signal is used as a control for fiber bleaching and motion artifacts as it is subtracted from the 465 nm signal during analysis. We sinusoidally modulated the intensity of the 465 nm and 405 nm light at 210 and 337Hz respectively and connected the LEDs to a four-port fluorescence mini cube (Doric Lenses). The combined LED output passed through a fiber optic cable (1 m long; 400 μm core; 0.48 NA; Doric Lenses) which was connected to the implanted fiber optics with sleeves. We maintained the light intensity at the tip of the fiber optic cable at 10-15 μW across behavioral sessions. We collected the $GRAB_{DA}$ and isosbestic control channel emission using the same fiber optic cable and focused the emission light onto a photoreceiver (Newport). We low pass filtered and digitized the emission light at 3Hz and 5 KHz respectively by a digital processor controlled by Synapse software suite (RZ5P, Tucker Davis Technologies (TDT)). We time-stamped the behavioral

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events including lever insertion/retraction, lever press, food cup entry etc. by sending them as TTL (transistor-transistor logic) pulses to Synapse software.

Histology: After all behavioral testing, we deeply anesthetized rats with isoflurane and transcardially perfused them with 200 mL of 0.1 M PBS followed by 400 mL of 4% paraformaldehyde (PFA) in distilled H_2O. We quickly removed the brains and post-fixed them in 4% PFA for at least two hours before we transferred them to 30% sucrose in PBS for 48 hours at 4°C. We subsequently froze the brains using dry ice and stored them in −20°C until sectioning. We collected 50 µm coronal sections containing BNST on a cryostat (Leica Microsystems) and preserved them in a cryopreservant. We mounted the sections on slides and coverslipped them with Vectashield mounting medium with DAPI (Vector Laboratories). We verified fiber optic placements and viral expression in the dBNST using anatomical boundaries defined by Paxinos and Watson (Paxinos & Watson, 2006) under a confocal microscope. A representative example and summary of $GRAB_{DA}$ expression and fiber placements are shown in Fig. 1C.

Photometry Analysis: We analyzed the signals using custom-written MATLAB (Mathworks) scripts. We calculated ΔF/F (z score) by smoothing signals from the isosbestic control channel (Lerner et al., 2015; Root et al., 2020). We regressed the isosbestic signal onto the $GRAB_{DA}$-dependent signal to create a fitted isosbestic signal by using the linear model generated during the regression. We then calculated z scores by subtracting the fitted isosbestic signal from $GRAB_{DA}$-dependent signal and dividing by the fitted isosbestic signal. This resulted in $GRAB_{DA}$ signal devoid of artifacts created by photobleaching, fiber bending, or movements. We collected z scores in the behavioral window of interest defined as 5s before cue onset to 10s after pellet delivery. We quantified area under the curve (AUC) in the 2s following cue onset and pellet delivery and independently calculated these parameters for CS+ and CS- trials. In all dopamine signal analyses, unless otherwise noted, we subtract CS- signal from the CS+ signal. We defined significant transients in our behavioral window if the peak amplitude was ≥ 2 z-score (p=0.05)

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above baseline (5s prior to cue onset) and excluded recordings that did not meet this criterion. We also removed trials where the patch cord disconnected from further signal processing. For extinction probe test, we extracted significant transients resulting from active and inactive presses after baselining to the first second of 2s prior to the lever press.

Statistical Analysis: We analyzed the data using SPSS, Graphpad Prism, and Matlab. We used mixed design repeated measures ANOVAs to analyze PLA behavioral and $GRAB_{DA}$ signal data. Whenever ANOVAs revealed significant interactions between groups, we ran t-tests with Bonferroni corrections for multiple comparisons to guard against Type I error. We define dependent measures, within/between-subject factors, and report significant effects and interactions in the corresponding results section.

Results

BNST $GRAB_{DA}$ signals respond to cues and reward in a Pavlovian learning task

We sought to determine if BNST $GRAB_{DA}$ signals correlate with individual differences in approach to Pavlovian cues (Experiment timeline in Fig. 1A). First, we trained rats in PLA for five days (Fig. 1B) to examine the acquisition of lever- and food cup-directed behaviors across training in sign and goal tracking/intermediate rats. Representative and histological inventory of $GRAB_{DA}$ expression from these rats is shown in Fig. 1C. We analyzed the behavioral PCA score using a mixed ANOVA with between subject factors of Tracking (ST, GT/INT) and within subject factors of Session (Day 1, Day 5; Fig. 1D). ST rats show greater PCA score on Day 5 compared to GT/INTs (Fig. 1D, PCA score: Session: $F_{(1,14)} = 67.3$, $p<0.001$, Session x Tracking: $F_{(1,14)} = 15.04$, $p=0.002$, Tracking: $F_{(1,14)} = 11.59$, $p=0.004$; post-hoc, Day 5 ST vs. GT/INT presses: $t_{14} = 4.92$, $p<0.001$). Next, to confirm rats could discriminate the reinforced and non-reinforced lever cues, we examined the difference between CS+ and CS- presses (Δ presses) and pokes (Δ pokes) using a mixed ANOVA with between subject factors of Tracking (ST, GT/INT) and within subject factors of Session (Day 1, Day 5, Fig. 1E). ST rats show better discrimination (Δ presses) for lever

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directed behavior (Δ presses) on Day 5 compared to GT/INTs (Session: $F_{(1,14)} = 35.75$, $p<0.001$, Session x Tracking: $F_{(1,14)} = 11.66$, $p<0.001$, Tracking: $F_{(1,14)} = 17.81$, $p=0.001$; post-hoc, Day 5 ST vs. GT/INT presses: $t_{14} = 3.93$, $p=0.002$). In contrast, GT/INTs show better discrimination for food cup directed behavior (Δ pokes) on Day 5 compared to STs during the CS (Fig. 1F: Session x Tracking: $F_{(1,14)} = 4.90$, $p=0.044$, Tracking: $F_{(1,14)} = 15.17$, $p=0.002$; post-hoc, Day 5 ST vs. GT/INT pokes: $t_{14} = -3.92$, $p=0.002$).

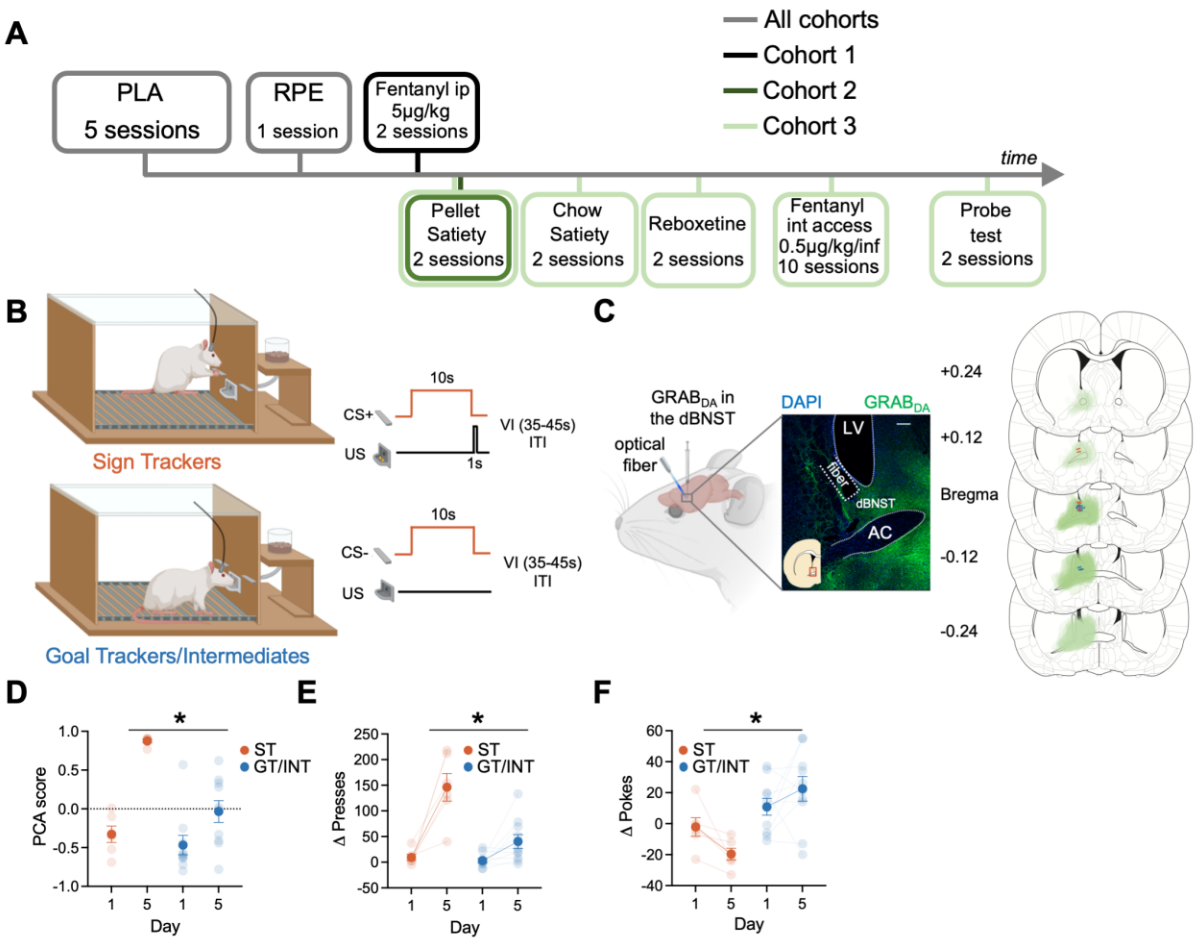

Figure 1: Individual differences emerge during Pavlovian Lever Autoshaping (PLA). **(A)** Experimental timeline. We trained all rats for five daily reinforced PLA sessions to determine their tracking groups followed by a single reward prediction error (RPE) session. We injected the first cohort of rats with i.p fentanyl in PLA and tested the second and the third cohort of rats on 2 counterbalanced PLA pellet satiety sessions. We tested the third cohort of rats on 2 counterbalanced PLA chow satiety and with reboxetine i.p. injection sessions. We then catheterized and trained these rats on intermittent access fentanyl self-administration for 10 sessions. Finally, we tested these rats' cue-induced responding on two extinction sessions. **(B)** PLA sessions consisted of presentation of 10s of cue (either conditioned stimulus, CS+ or CS- lever, pseudorandom order with an intertrial interval (ITI) varying (variable interval (VI)) between 35 and 45s)

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followed by lever retraction and delivery of two food pellets in the foodcup. Some rats (Sign Trackers, STs) engage with the cue while others (Goal trackers, GTs) wait for the food pellets in the food cup during cue period. Others display both lever and food cup behaviors (Intermediates, INTs) **(C)** Left: representative expression of $GRAB_{DA}$ construct and fiber placement in dorsal Bed Nucleus of Stria Terminalis (dBNST). White scale bar: 250μm. Right: The extent of $GRAB_{DA}$ expression and fiber placement across five coronal planes with anterior distance from bregma (millimeters) in the dBNST in STs (orange) and GT/INTs (blue). Drawings are adapted from Paxinos and Watson (2007). **(D)** Average PCA scores for STs and GT/INTs on Day 1 and Day 5 of PLA. **(E)** Average Δ Presses (CS+) – (CS-) on Day 1 and Day 5. **(F)** Average Δ Pokes (CS+) – (CS-) on Day 1 and Day 5. Data are mean±SEM.

To investigate the endogenous dBNST dopamine activity across PLA training, we used fiber photometry to monitor the fluorescent activity of the genetically encoded dopamine sensor, $GRAB_{DA}$ (Sun, Zeng et al. 2018). We see evidence of associative encoding during PLA (Fig. 2). Both lever insertion and retraction/reward delivery increased dBNST $GRAB_{DA}$ signals in ST and GT/INT rats (representative heat map and population average traces on Day 1 and Day 5 for STs in Fig. 2A and GT/INTs in Fig. 2B). To determine whether ST and GT/INT rats show differences in cue-evoked dopamine signals across acquisition of PLA, we compared the strength of CS+ onset (Δ lever extension area under curve (AUC) = (CS+) – (CS-) AUC; 2s after CS onset) signals between Day 1 and Day 5 using a mixed ANOVA with between subject factors of Tracking (ST, GT/INT) and within subject factor of Session (Day 1, Day 5). While CS+ onset-evoked $GRAB_{DA}$ signals increased across conditioning for both ST and GT/INT (Fig. 2C, Session: $F_{(1,14)} = 19.69$, p=0.001) the magnitude of the CS+ signal increase differed between tracking groups (Session x Tracking: $F_{(1,14)} = 5.99$, p=0.028, Tracking: $F_{(1,14)} = 10.35$, p=0.006). Post hoc analyses revealed greater cue-evoked dBNST $GRAB_{DA}$ signal in ST compared to GT/INT on Day 5, which was not evident on Day 1 (Day 1: $t_{14} = 0.17$, p=0.87; Day 5: $t_{14} = 2.93$, p=0.011). Next, we asked whether $GRAB_{DA}$ signals correlated with the tracking phenotype. We observed a positive correlation between Day 5 CS onset $GRAB_{DA}$ signals and Day 5 PLA score (Fig. 2D; $R^2 = 0.41$, p=0.009) but not Day 1 CS onset $GRAB_{DA}$ signals and Day 1 PLA score (Fig. S1A; $R^2 = 0.21$, p=0.09).

Next, we examined tracking differences in the sustained $GRAB_{DA}$ signal between STs and GT/INTs throughout the duration of the CS, during which STs and GT/INTs show differences in

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lever and foodcup directed behaviors. We compared Day 1 vs. Day 5 CS+ maintained (Δ cue-period AUC = (CS+) – (CS-) AUC during the full 10s CS lever insertion period) $GRAB_{DA}$ signaling. CS+ maintained $GRAB_{DA}$ signals increased across conditioning for both ST and GT/INT (Fig. 2E, Session: $F_{(1,13)} = 11.45$, p=0.005, Session x Tracking: $F_{(1,13)} = 3.07$, p=0.1, Tracking: $F_{(1,13)} = 16.5$, p=0.001). Like cue onset, we saw a strong positive correlation between Day 5 $GRAB_{DA}$ signals during CS interaction and Day 5 PLA score (Fig. 2F, $R^2 = 0.49$, p=0.004) but not Day 1 $GRAB_{DA}$ signals and Day 1 PLA score (Fig. S1B, $R^2 = 0.08$, p=0.3) suggesting that as rats display ST behavior, there's an increase in sustained $GRAB_{DA}$ signal.

Prior work shows that NAc dopamine shifts from US to CS after conditioning in STs but not GTs (Flagel et al., 2011). Since we observed differences in CS evoked BNST $GRAB_{DA}$ signals between STs and GT/INTs, we wanted to determine if there was similar tracking specificity in the US to CS shift for BNST $GRAB_{DA}$ signals. We quantified the relative CS/US dynamics across conditioning using a difference score (Δ cue-reward AUC = (CS+) – (US) AUC for the 2s after CS+ onset and reward delivery) and compared between Day 1 and Day 5. We used a mixed ANOVA with between subject factors of Tracking (ST, GT/INT) and within subject factor of Session (Day 1, Day 5). The relative CS/US dynamics across PLA differed by tracking group (Fig. 2G, Session: $F_{(1,14)} = 4.79$, p=0.046, Session x Tracking: $F_{(1,14)} = 8.9$, p=0.01). We found no tracking group differences in the (CS+) – (US) difference score on Day 1 but by Day 5, the CS/US difference score was greater in STs compared to GT/INTs (ST vs. GT/INT, Day 1: $t_{14} = -1.6$, p=0.13; ST vs GT/INT, Day 5: $t_{14} = 2.43$, p=0.029). While the correlation between (CS+) – (US) $GRAB_{DA}$ signal and Day 5 PCA scores was marginal (Fig. 2H, $R^2 = 0.22$, p=0.06) there was no relationship between these measures on Day 1 (Fig. S1C, $R^2 = 0.025$, p=0.56). Overall, these data indicate sign-tracking specific dBNST $GRAB_{DA}$ signals increase to Pavlovian cue onset and during cue-maintained sign-tracking behaviors, and back propagate from the reward to cue onset across conditioning.

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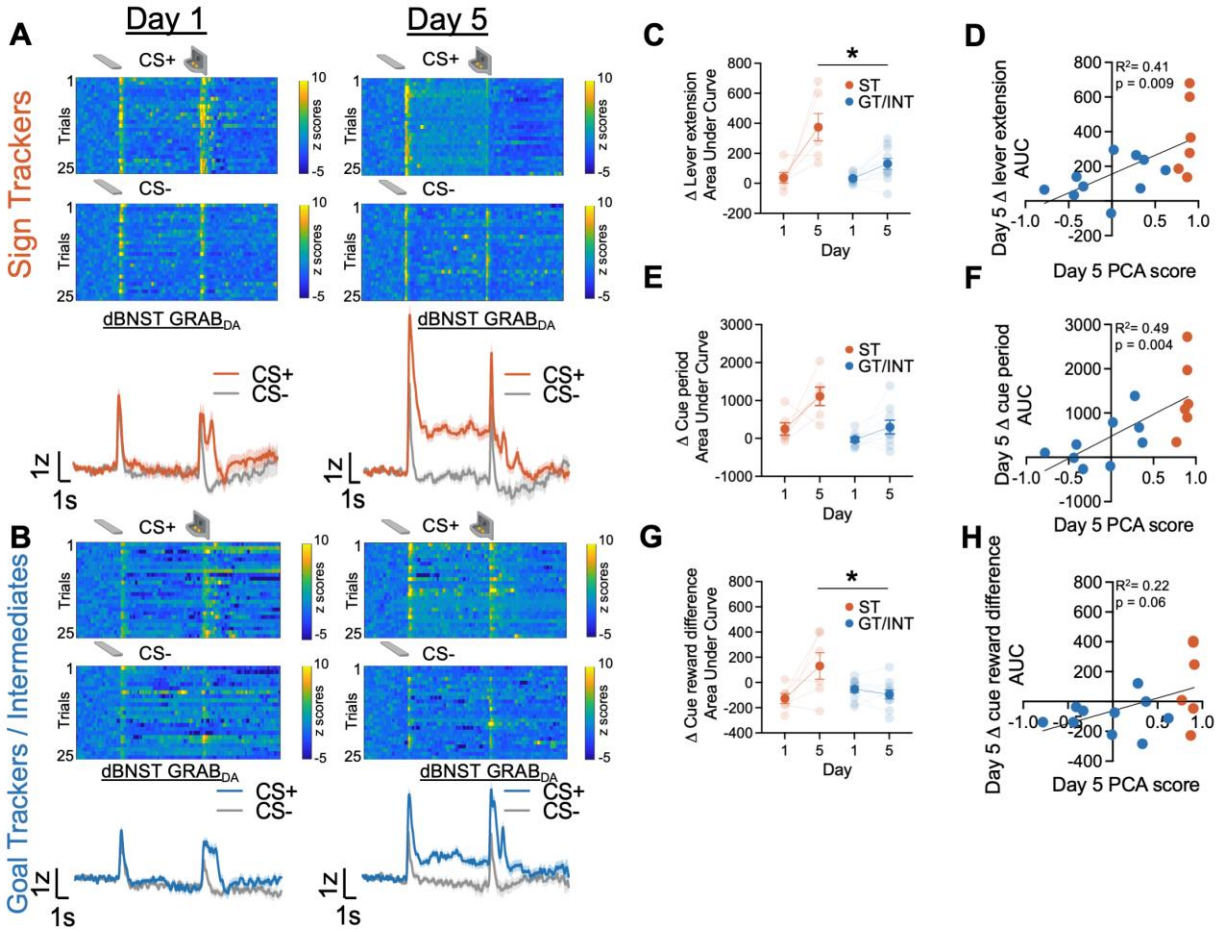

Figure 2: Dorsal bed-nucleus of stria terminalis (dBNST) GRAB$_{DA}$ signals during PLA between STs and GT/INTs. Representative heat maps illustrating GRAB$_{DA}$ signal changes (z-scores) during CS+ and CS- presentations on Day 1 (top left) and Day 5 (top right) and trial-averaged GRAB$_{DA}$ signal change (z-scored ΔF/F) during CS+ and CS- presentations on Day 1 (bottom left) and Day 5 (bottom right) in **A)** STs and **B)** GT/INTs. **C)** Trial averaged quantification Δ lever extension ((CS+) – (CS-); 2s) GRAB$_{DA}$ area under (AUC) between STs and GT/INTs. **D)** Correlation between Day 5 PCA scores and Day 5 Δ lever extension AUC. **E)** Trial averaged quantification of Δ cue period ((CS+) – (CS-); 10s) in AUC during cue period (10s) between STs and GT/INTs. **F)** Correlation between Day 5 PCA scores and Day 5 Δ cue period AUC. **G)** Trial averaged quantification of Δ cue-reward (CS+) – (US), 2s) in AUC between STs and GT/INTs. **H)** Correlation between Day 5 PCA scores and Day 5 change in Δ cue-reward AUC. Data are mean±SEM. *p<0.05

BNST dopamine encodes reward prediction error

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After five Pavlovian autoshaping sessions, we conducted a Reward Prediction Error (RPE) session in which we randomly intermixed expected food reward trials and unexpected food reward delivery with omission trials. Expected reward (Expected) trials are identical to those delivered during training, with a 10s CS+ lever insertion followed by retraction and food reward delivery. Unexpected reward (Positive) trials consist of randomly delivered food reward that is not signaled by a cue. Unexpected omission (Negative) trials consist of 10 s CS+ lever insertion and retraction, but no food reward is delivered. During these sessions we monitored BNST $GRAB_{DA}$ signals to examine whether dopamine signals track errors in reward prediction (representative heat map and population average traces for each trial type in Fig. 3A-C; Schultz et al., 1997).

First, to determine whether BNST $GRAB_{DA}$ signals encode bidirectional reward prediction error, we compare signals on expected, positive and negative trials. Notably, because lever retraction occurs simultaneously with reward delivery, and sign- and goal-trackers may be in different locations at this time, we examine the signals during the six seconds after reward delivery or omission, which captures the period corresponding to violations in reward expectations (Fig. 3D). We performed a repeated measures ANOVA including Trial Type (Expected, Positive, Negative) and Bin (three 2 s bins) as a factor. We observed a difference between the three trial types in the bins following reward delivery/omission (Fig. 3D, Bin: $F_{(2,72)} = 13.65$, $p<0.001$, Bin x Trial Type: $F_{(4,72)} = 13.99$, $p<0.001$, Trial Type: $F_{(2,36)} = 3.49$, $p=0.041$). Post hocs confirm that in the second 2-second bin after reward delivery/omission, BNST $GRAB_{DA}$ signals differed from one another for all three trial types, Expected vs. Positive (population traces in Fig. 3E; $p=0.013$), Expected vs. Negative (population traces in Fig. 3F; $p=0.043$) and Positive vs. Negative ($p=0.0004$).

Then to determine whether there are tracking differences in dBNST RPE signals, we separately analyzed RPE data for STs and GT/INTs (population traces for STs and GT/INTs for all three trial types in Fig. 3G-H). We performed a mixed ANOVA including factors of Tracking (ST, GT/INT) and Trial Type (Positive, Negative) for the 3 bins after reward delivery. We observed a difference

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between ST and GT/INT positive and negative trials in bins 8,9, and 10 average z score (Fig. 3G-H insets, Trial Type: $F_{(1,11)} = 6.81$, p=0.024, Trial Type x Tracking: $F_{(1,11)} = 5.58$, p=0.038, Tracking: $F_{(1,11)} = 0.46$, p=0.51). Post hoc analyses revealed that only in GT/INT rats did BNST $GRAB_{DA}$ signals differ significantly for positive and negative trial types (p=0.015). Together, this suggests that there are individual differences in dBNST $GRAB_{DA}$ RPE signals and RPE-evoked reward seeking behaviors.

We collected behavioral data during RPE sessions and examined pre-trial food cup checking rate (response/10s prior to CS onset/reward delivery) on the trial after a reward violation, during which prior studies establish invigoration of conditioned responses and orienting (Holland & Gallagher, 1993, Calu et al., 2010, Roesch et al., 2010). Rats increase their pre-trial foodcup checking on trials after a reward violation (Fig. 3I). We performed repeated measures ANOVA including factors of Trial Type (Expected, Positive, Negative) and Tracking (ST,GT/INT). While ST rats increase their pre-trial food cup checking after both positive and negative trials, GT/INTs only increase their pre-trial food cup checking following negative trials (Fig. 3I, Trial Type: $F_{(2,22)} = 10.9$, p=0.001, Trial Type x Tracking: $F_{(2,22)} = 4.39$, p=0.025, Tracking: $F_{(1,11)} = 1.77$, p=0.21). These data indicate that STs and GT/INTs use different reward seeking behavioral strategies following violation of reward expectation.

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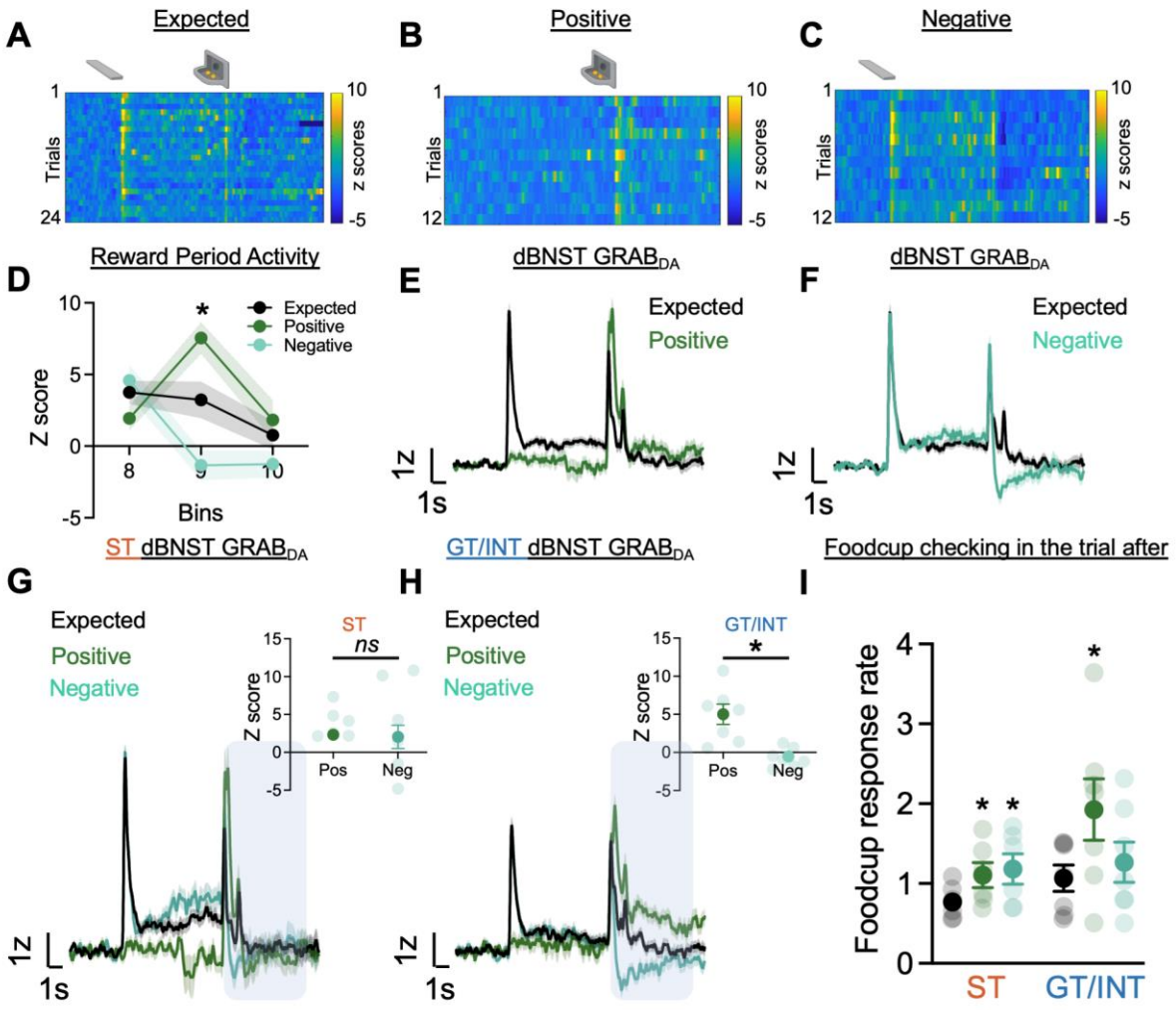

Figure 3: Individual differences in reward prediction error (RPE) A-C) Representative heat maps during Expected, Positive (unexpected reward) and Negative (unexpected omission) reward trials. **D)** Average binned z-scores (2s bins) during Expected, Positive and Negative trials 6s post reward delivery (bins 8-10). Trial-averaged GRAB$_{DA}$ signal change (z-scored ΔF/F) during **E)** Expected vs. Positive trials and **F)** Expected vs Negative trials. Trial-averaged GRAB$_{DA}$ signal change (z-scored ΔF/F) during all three trials and average positive and negative trial z scores in bins 8-10 (inset) in **G)** STs and **H)** GT/INTs. **I)** Average foodcup checking response rate (responses/10s) during 10s pre-trial period on trial after expected, positive, and negative trials in STs vs GT/INTs. Data are mean±SEM, *p<0.05.

Reinforcer-specific but not general satiety attenuates cue-triggered GRAB$_{DA}$ signal

Following the RPE session, we determined whether motivational state alters CS-evoked GRAB$_{DA}$ signals during PLA. After rats completed 25 trials of PLA along with the GRAB$_{DA}$ recordings, we sated them on the training pellets presented in ceramic ramekin in the homecage or presented a

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sham condition in which an empty ramekin was placed in the homecage for 30 minutes. Immediately after, we recorded $GRAB_{DA}$ signals during the remaining 25 trials of PLA sessions. First, we compared Δ presses and Δ pokes ((CS+) – (CS-)) between hungry and sated or hungry and sham sated conditions using two-way ANOVA with factors of State (Hungry, Sate) and Condition (Real, Sham). The number of presses differed based on the satiety condition compared to hungry condition (State x Condition: $F_{(1,20)} = 9.65$, p=0.006). Post doc analysis revealed that rats sated on training pellets decreased lever presses predictive of food pellet reward (Fig. 4A left, hungry vs sated presses: $t_{10} = 3.02$, p=0.013; hungry vs sham sated presses: $t_{10} = -1.51$, p=0.16). In contrast, the number of pokes generally but not differentially increased during sated and sham sated condition compared to hungry condition (Fig. 4A right, State: $F_{(1,20)} = 6.73$, p=0.017, State x Condition: $F_{(1,20)} = 3.72$, p=0.068). Similarly, we examined cue-evoked $GRAB_{DA}$ signal ((CS+) – (CS-); 2s after cue onset) between hungry and sated or hungry and sham sated conditions using ANOVA with factors of State (Hungry, Sate) and Condition (Real, Sham). The differential change in lever presses was associated with difference in cue-evoked $GRAB_{DA}$ signal during sated and sham sated condition compared to hungry condition (State x Condition: $F_{(1,20)} = 6.68$, p=0.018). Post doc analysis revealed that rats sated on pellets show a decrease in cue evoked $GRAB_{DA}$ signals but not in sham sated conditions (Fig. 4E left, hungry vs sated: $t_{10} = 2.71$, p=0.022; hungry vs sham sated: $t_{10} = -0.95$, p=0.35). While we observed a decrease in cue-triggered dopamine signals in sated conditions, there was no change in reward consumption related dopamine signals in both sated and sham sated conditions (Fig. 4E right, F's < 0.52, p's > 0.05). These results further bolster our finding that BNST $GRAB_{DA}$ is involved in associative cue-outcome learning and may reflect a signal that is inhibited in a reduced motivational state.

Next, we examined whether the reduction in cue evoked $GRAB_{DA}$ signal is specific to the training pellet or whether it is sensitive to a general satiety state by sating rats on homecage chow. We conducted similar analysis as pellet satiety. When we sated rats on chow, the number of presses

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differed based on the satiety condition compared to hungry condition (State x Condition: $F_{(1,12)}$ = 5.86, p=0.032), however, there was no change in cue-evoked $GRAB_{DA}$ signals (Fig. 4B,D,F, F's < 1.8, p's > 0.05). Similarly, the number of pokes also differed based on the satiety condition compared to hungry condition (State x Condition: $F_{(1,12)}$ = 9.61, p=0.009). Post hoc analysis revealed that rats decreased their poking when sham sated compared to hungry (t_6 = 2.87, p=0.03). This is presumably due to a concurrent non-significant increase in lever presses (sham sate presses: t_6 = -1.92, p=0.1). But this decrease in foodcup pokes was not accompanied with a change in reward consumption evoked $GRAB_{DA}$ signal (F's < 1.3, p's > 0.05). These results suggest that when rats are sated on the outcome associated with the Pavlovian cue, there is an attenuation in $GRAB_{DA}$ signals while a general satiety doesn't attenuate cue responding or $GRAB_{DA}$ signals.

In the current and following sections, we describe general role of BNST $GRAB_{DA}$ signals but do not report tracking differences due to low statistical power.

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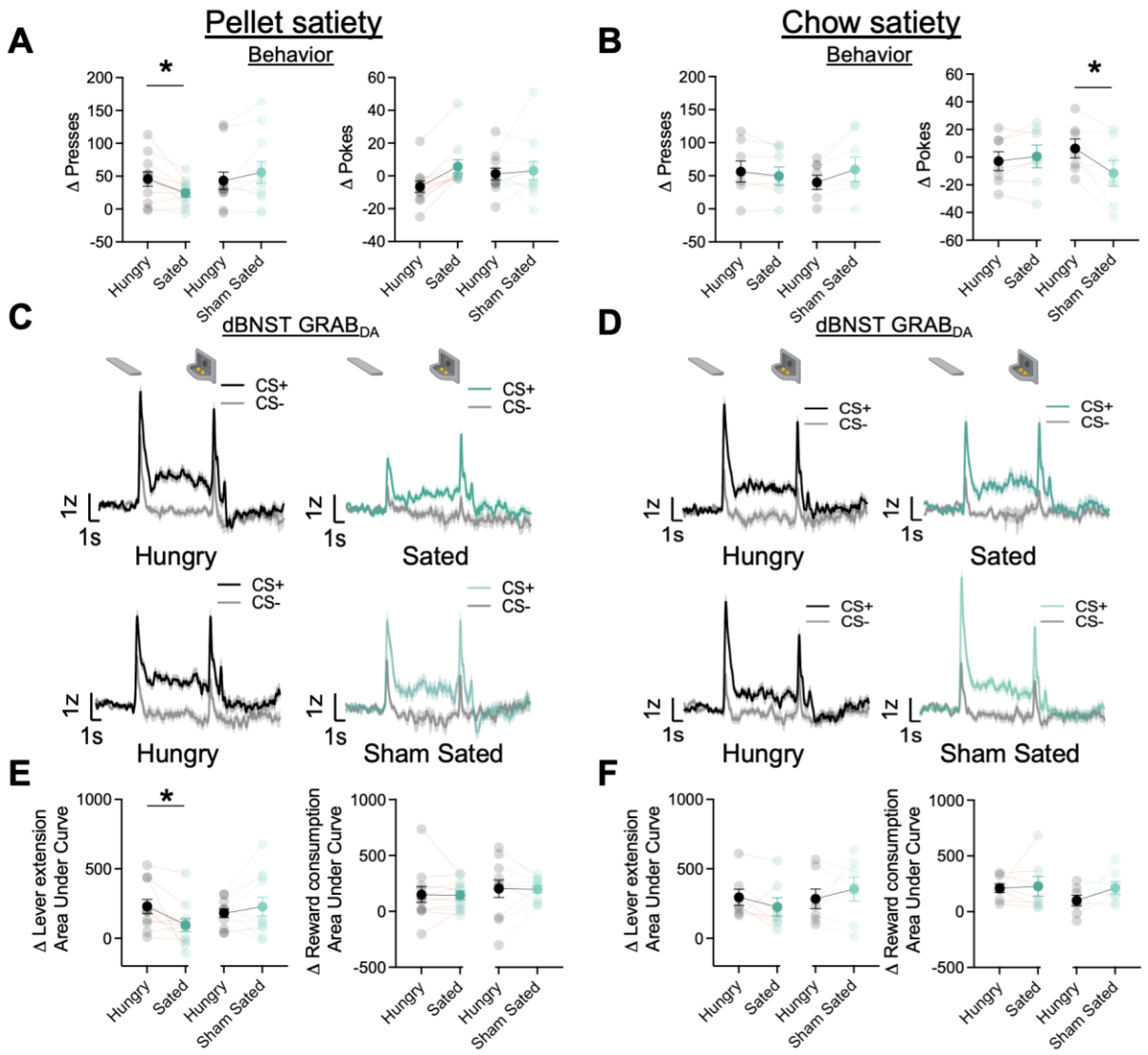

Figure 4: dBNST GRAB$_{DA}$ signals attenuate after reinforcer-specific but not general satiety. **A)** Average Δ Presses (CS+) – (CS-) (left) and average Δ pokes (CS+) – (CS-) (right) when rats were either sated on training food pellets in ramekin or sham-sated (ramekin only). **B)** Average Δ Presses (CS+) – (CS-) (left) and average Δ pokes (CS+) – (CS-) (right) when rats were either sated or sham-sated on homecage chow. **C)** Trial-averaged GRAB$_{DA}$ signal change (z-scored ΔF/F) during CS+ and CS- presentations when rats were hungry versus sated (top) and when rats were hungry versus sham-sated (bottom) on food pellets and **D)** on homecage chow. **E)** Trial average quantification of change (CS+) – (CS-) in area under GRAB$_{DA}$ z-scored curve (AUC) during lever extension (2s) (left) and reward consumption (right) between food pellet sated and sham sated and **F)** between homecage chow sated and sham sated conditions. Data are mean±SEM, *p<0.05.

Systemic fentanyl administration boosts GRAB$_{DA}$ signals to reward related cues

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Several studies show that there is an increase in tonic and phasic dopamine release in the NAc following administration of drugs of abuse (see Willuhn et al., 2010 for review). Microdialysis studies establish that several classes of drugs of abuse, including opioids, increase tonic DA in the BNST (Carboni et al., 2000). We sought to determine if there are phasic increases in task-related BNST $GRAB_{DA}$ signals following systemic injections of synthetic μ-opioid agonist, fentanyl. We recorded $GRAB_{DA}$ signals during PLA after i.p injection of 5μg/kg fentanyl (population average traces for saline and ip fentanyl injection in Fig. S2A). We observed main effects of Treatment (vehicle, fentanyl) and CS (CS+,CS-), but the interaction was not significant, indicating that cue discrimination is maintained with systemic fentanyl injections, which generally potentiate DA signaling in the dBNST (Fig. S2B, CS: $F_{(1,6)} = 24.42$, p=0.003, Treatment: $F_{(1,6)} = 7.16$, p=0.037, CS x Treatment ($F_{(1,6)} = 2.8$, p=0.15).

Fentanyl self-administration-associated cues enhance phasic $GRAB_{DA}$ signals in extinction

Next, we aimed to determine whether $GRAB_{DA}$ signals respond to cues associated with self-administered fentanyl. First, we trained rats to associate discrete CS (tone and light) with fentanyl self-administration during an intermittent access fentanyl self-administration for 10 sessions. We use intermittent access schedule of reinforcement, as this schedule leads to greater progressive ratio, reinstatement, and incubation effects than continuous access (Fragale et al., 2021; Kawa et al., 2016; Nicolas et al., 2019). As the sessions progressed, rats consumed more fentanyl and were exposed to more cue pairings (Fig. 5A, Session $F_{(9,36)} = 8.82$, p<0.001). Rats started out pressing more on the inactive lever as this was previously their PLA CS+ lever. However, after 4 sessions, rats switched their responding and pressed more on the active lever and discriminated the fentanyl-paired active lever compared to inactive lever (Fig. 5B, Session: $F_{(9,72)} = 2.89$, p=0.006, Session x Lever: $F_{(9,72)} = 5.97$, p<0.001, Lever: $F_{(1,8)} = 6.74$, p=0.032).

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After fentanyl self-administration sessions, we examined whether $GRAB_{DA}$ signaling was greater for the fentanyl-associated active lever (+cue) compared to the inactive lever press. Population $GRAB_{DA}$ traces during active and inactive lever presses on Day 1 and Day 15 extinction tests are shown in Fig. 5C. Across two extinction sessions, the dBNST $GRAB_{DA}$ signal discriminated active vs. inactive lever presses (Fig. 5C,D, main effect of Lever: $F_{(1,8)} = 8.20$, $p=0.021$). Qualitatively, the discrimination appears to improve across forced abstinence, however the difference in dBNST $GRAB_{DA}$ signaling that lever discrimination between sessions was marginal (Session (Day 1, 15) x Lever (active, inactive): $F_{(1,8)} = 4.40$, $p=0.069$).

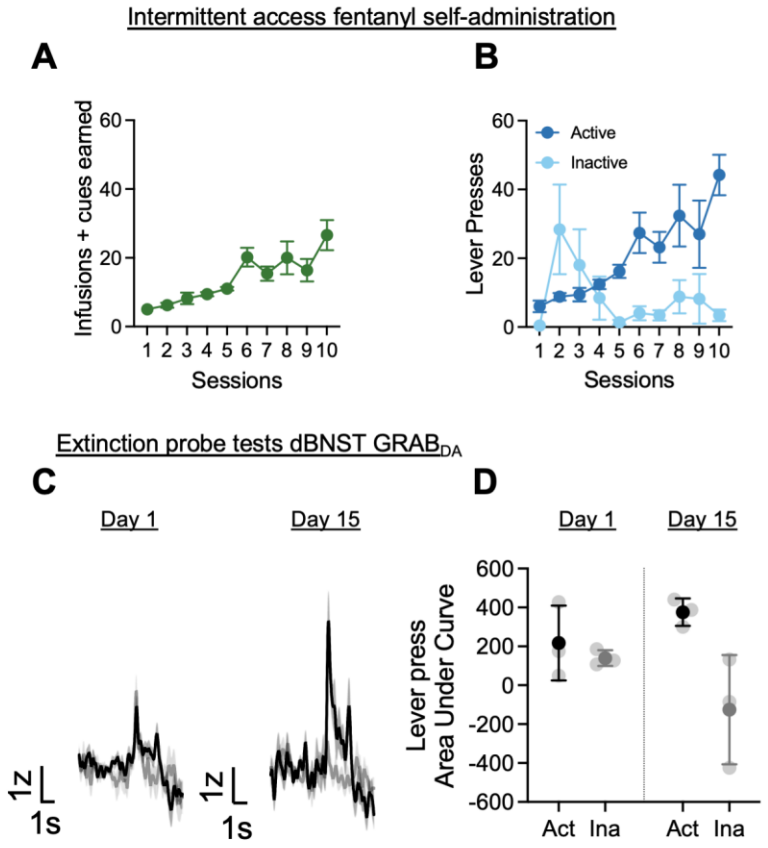

Figure 5: Fentanyl self-administration associated cues enhances dBNST $GRAB_{DA}$ signals. A) Fentanyl infusions + cues earned and **B)** active versus inactive lever presses across the 10 daily intermittent access fentanyl self-administration sessions. **C)** Averaged $GRAB_{DA}$ signal change (z-scored $\Delta F/F$) during active and inactive lever presses in extinction probe tests Day 1 and Day 15 and their **D)** area under curve quantification (2s). Data are mean±SEM, *p<0.05. Act: Active Lever, Ina: Inactive Lever.

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dBNST $GRAB_{DA}$ signals during PLA are specific to dopamine

Even though the $GRAB_{DA}$ construct we used is 15 fold more sensitive to dopamine than norepinephrine, BNST norepinephrine plays an important role in motivated behaviors and dBNST receives noradrenergic input (Egli et al., 2005; Flavin & Winder, 2013; Sun et al., 2020). To validate that the signals we recorded during PLA were dopaminergic and not noradrenergic, we injected a norepinephrine reuptake inhibitor Reboxetine (1mg/kg) 30 minutes prior to PLA. Norepinephrine levels in the brain remain elevated at this dose for up to 3 hours peaking at ~20 minutes after injection (Page & Lucki, 2002). We found that Reboxetine injection did not increase BNST $GRAB_{DA}$ signal to lever extension or reward consumption (Fig. S3C, Epoch: $F_{(1,12)} = 3.82$, $p=0.074$, Epoch x Treatment: $F_{(1,12)} = 0.20$, $p=0.66$, Treatment: $F_{(1,12)} = 0.21$, $p=0.66$) compared to saline injection. Further, there is no difference in cue-interaction period between reboxetine and saline injected conditions ($t_6 = 1.14$, $p=0.3$). These data confirm that the signals we recorded during PLA are not sensitive to noradrenergic reuptake inhibition and are most likely due to fluctuations in DA signaling in the BNST.

Sex as a biological variable:

We use both male and female rats and have analyzed our photometry data from Pavlovian autoshaping, RPE, satiety and fentanyl test sessions using Sex instead of Tracking as a factor. We observed no main effects of Sex or interaction between Sex and any other factor.

Discussion

Using fluorescent dopamine sensor, $GRAB_{DA}$, we characterized phasic dBNST dopamine signals during a range of appetitive Pavlovian and instrumental conditions including lever autoshaping, reward violations, specific satiety, and cue-induced fentanyl-seeking. We found that dBNST dopamine signals are enhanced in STs compared to GT/INTs during cue presentation and shift from reward to cue across conditioning in STs but not in GT/INTs. Further, dBNST dopamine signals encode bidirectional reward prediction error and are greater in GT/INTs

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compared to STs following reward violations. Additionally, dBNST dopamine signals decrease to cue when rats are sated on food pellets associated with the cue but not when sated on homecage chow. Systemic fentanyl injections do not disrupt dBNST cue discrimination but generally potentiates dBNST dopamine signals. Fentanyl self-administration experience is sufficient to reverse reward seeking and dBNST dopamine signaling discriminates fentanyl-associated active vs. inactive lever pressing under extinction conditions.

Pharmacological studies establish that dopamine signaling in the dBNST maintains responding for sucrose and ethanol rewards and regulates the reinforcing properties of cocaine (Epping-Jordan et al., 1998, Eiler et al., 2003). Microdialysis and voltammetry studies show that natural and drug rewards, including opioids, increase tonic and phasic DA in the BNST (Carboni et al., 2000, Park et al., 2012, Park et al., 2013). Although dBNST dopamine is important for a variety of appetitive motivated behaviors, little is known about cue-evoked dopamine signaling and its role in cue-triggered motivation. A recent study showed BNST $GRAB_{DA}$ signals associated with both cues and rewards (Lin et al., 2020). Our data extend these findings by showing individual differences in CS- and US-evoked BNST dopamine signaling during Pavlovian conditioning. We also demonstrate that CS-evoked BNST DA signals are state dependent and outcome-specific.

Consistent with prior studies, we observed individual differences in sign- and goal-tracking behaviors elicited by the CS (Boakes R.A, 1977; Hearst & Jenkins, 1974; Nasser et al., 2015; Robinson et al., 2014). Accompanied with this behavioral variation, we observed tracking differences in $GRAB_{DA}$ signals to CS onset and differences in dopamine signal transfer from US to CS, both of which were stronger in sign-tracking compared to goal-tracking and intermediate rats. We observed a relationship between CS maintained $GRAB_{DA}$ signal and PCA scores, indicating sign-tracking approach and interaction with the lever cue is associated with heightened dBNST $GRAB_{DA}$ signaling. These findings for the dBNST dopamine signal are consistent with prior tracking differences in nucleus accumbens (NAc) dopamine signals during Pavlovian lever

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autoshaping (Flagel et al., 2011). We also find that only in ST rats did $GRAB_{DA}$ signals adhere to Sutton & Barto (2018) reinforcement learning algorithm, which states that after learning, reward-evoked signals are temporally transferred back to antecedent cues predicting reward delivery (Nasser et al., 2017; Sutton & Barto, 2018). Consistent with this, we observed an increase in sustained $GRAB_{DA}$ signal during the entire 10s CS interaction period on Day 5 of PLA training compared to Day 1. Sustained BNST $GRAB_{DA}$ signals during cue interaction period could reflect a number of processes, including 1) ongoing lever interaction, 2) the incentive value gain of the CS, 3) the strength of CS-US association, and/or 4) the back propagating US to CS signal. Our results suggest that dopamine signaling differences between STs and GTs is not just limited to NAc and could be present across a distributed network receiving dopaminergic projections.

To adapt to environmental changes and learn about future rewards, dopaminergic neurons calculate reward prediction errors (RPE) (Nasser et al., 2017; Schultz et al., 1997; Watabe-Uchida et al., 2017). Here, we examined if BNST $GRAB_{DA}$ signals encode RPE and whether there are individual differences in dBNST $GRAB_{DA}$ signals and behavioral strategies following violations of reward expectations. We found that dBNST $GRAB_{DA}$ signals follow the classical bidirectional prediction error signal such that the signals increased following unexpected reward delivery and decreased following unexpected reward omission. Consistent with attention for learning theories and empirical studies, we observed that rats increase their food cup checking behavior on a trial after a positive or negative reward violation (Calu et al., 2010; Pearce & Hall, 1980; Roesch et al., 2010). Such excitatory behavioral responses (more checking for both increases and decreases in reward) before the trial are evidence for an incremental attentional processes, which reflect enhanced attention to environmental predictors for the purpose of increasing the rate of learning for either excitatory or inhibitory associations (Pearce & Hall, 1980, Holland & Gallagher, 1993, Roesch et al., 2007, Calu et al., 2010, Roesch et al., 2010). Sign-tracking rats increase foodcup checking on trials after both unexpected reward delivery and omission, whereas GT/INTs increase

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foodcup checking only after reward omission. Behaviorally this suggests GT/INT rats may be more sensitive to negative reward violations than positive, which is consistent with their sensitivity to outcome devaluation and their insensitivity to conditioned reinforcement (Robinson & Flagel, 2009, Morrison et al., 2015, Nasser et al., 2015, Smedley & Smith, 2018). dBNST $GRAB_{DA}$ signals in GT/INTs were greater for positive compared to negative reward violations, which leads to the prediction that this signal difference may be necessary for enhancing attention specifically when expected outcomes are omitted. In ST rats, dBNST $GRAB_{DA}$ signals were no greater for positive compared to negative reward violations, which was not consistent with their increased foodcup checking on trials after both reward violation types. While limited to draw conclusions from this without direct manipulations of the dopamine system, perhaps dBNST dopamine signaling is not contributing to this attention-related behavioral effect in ST rats. Alternatively, signal differences at other timepoints outside of reward violation itself, may contribute to ST sensitivity to positive and negative reward violations in this task. Notably, compared to GT/INT rats, ST rats show greater cue-maintained dBNST $GRAB_{DA}$ signals during lever interaction, which qualitatively increase or decrease for a brief time after lever retraction relative to the end of the CS period. The possibility that these relative differences in signal before and after lever retraction contribute to error processing in sign-trackers is worth future exploration, particularly with causal role manipulations of the BNST dopamine signal.

What could be the source of dopamine in the dBNST during RPE? BNST receives heavy dopaminergic afferents from the A10 Ventral Tegmental Area (VTA) and A10dc ventral periaqueductal grey/dorsal raphe (vPAG/DR) dopaminergic cell groups, and to a lesser extent from the substantia nigra pars compacta and the retrorubral nucleus (Daniel & Rainnie, 2016; Hasue & Shammah-Lagnado, 2002; Melchior et al., 2021; Meloni et al., 2006; Vranjkovic et al., 2017). While VTA and SNc dopamine neurons classically encode bidirectional reward prediction error signals, vPAG dopamine and its projections unidirectionally encode rewarding and aversive

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outcomes, suggesting salient event detection (Berg et al., 2014; García-García et al., 2017; Lin et al., 2020; Nasser et al., 2017; Schultz, 1997; Walker et al., 2020; Watabe-Uchida et al., 2017). Different aspects of the dBNST DA signaling we observed leads us to postulate both dopamine projections may be contributing. For the bidirectional RPE we observed in dBNST, we predict that VTA dopaminergic projections are the source of dopamine during reward violations. In contrast, for the greater CS signaling in ST compared to GT/INT rats may reflect salient features of the CS that support the attracting and reinforcing properties of cues in sign-tracking rats, which may also be supported by vPAG/DR→BNST dopamine. The current findings inform future projection specific studies to determine specific facets of behavior supported by dopaminergic signaling in the BNST.

The BNST is a highly sexually dimorphic brain region (Shah et al., 2004, Hisasue et al., 2010, Tsuneoka et al., 2017), highlighting the necessity of studying both sexes to fully understand the contribution of BNST DA to motivated behavior. A previous rat study did not observe sex differences or ovarian hormone effects in sign- and goal-tracking behaviors, although we and others see evidence for increased propensity for females to sign-track (Pitchers et al., 2015, Madayag et al., 2017, Kochli et al., 2020). We used both male and female rats in the present study and analyzed our photometry data from Pavlovian autoshaping, RPE, satiety and cue-induced seeking test sessions using Sex instead of Tracking as a factor. While we observed no sex effects here, prior studies establish BNST-mediated sex differences in opioid withdrawal (Luster et al., 2020). This finding is relevant for the extension of the present findings, as we and other find strong relapse effects for opioid cues in both sexes of sign- and goal-trackers (Chang et al., 2022, Martin et al., 2022). While there is limited evidence for sex differences in incubation of fentanyl seeking (a form of relapse), we find this effect to be dependent on dBNST CRFR1 receptor signaling (Reiner et al., 2019, Gyawali et al., 2020, Reiner et al., 2020). Drug-induced synaptic plasticity in the dBNST requires both dopamine and CRF and molecular and

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electrophysiology studies suggest that DA increases CRF release in the dBNST (Day et al., 2002, Kash et al., 2008). Given the known role for sex differences in CRF-induced relapse and opioid withdrawal, it is critical to include both sexes when studying BNST DA and CRF systems (Buffalari et al., 2012, Luster et al., 2020).

To our surprise we found evidence for outcome-specific state dependent BNST $GRAB_{DA}$ signaling. Consistent with our prior studies, we found that rats decreased their lever responding only when they were sated on food pellets specifically associated with the lever cue, but not when sated on homecage chow (Keefer et al., 2020, Kochli et al. 2020). Similarly, we observed decreased cue-evoked BNST $GRAB_{DA}$ when rats were sated on food pellets but not when they were sated on chow. All rats ate all their pellets during these reinforced sessions, and we did not see any change in $GRAB_{DA}$ signals during reward consumption when sated on either food pellets or chow. Prior studies report a similar decrease in cue-evoked dopamine signals in the basolateral amygdala and dopaminergic neuron activity in the DRN during satiety (Cho et al., 2021; Lutas et al., 2019). Based on these studies that manipulated state using hunger or satiety, we expected dopamine signals to generally decrease to cues both when sated on chow or training pellets, but we found BNST dopamine signals only decreased when sated on the training pellet associated with the cue. However, other studies find evidence for sensory specific signaling in dopamine function and signaling (Sharpe et al., 2017, Takahashi et al., 2017). This suggests BNST DA signals may carry sensory specific information that is critical for higher order learning processes (Burke et al., 2007, Burke et al., 2008, Malvaez et al., 2015, Lichtenberg et al., 2017, Sharpe et al., 2017, Takahashi et al., 2017, Malvaez et al., 2019, Keefer et al., 2021, Lichtenberg et al., 2021, Sias et al., 2021).

Previous studies show elevated BNST dopamine, dopamine induced plasticity, and dopamine mediated seeking behavior during and after drug administration (Carboni et al., 2000; Eiler et al., 2003; Epping-Jordan et al., 1998; Kash et al., 2008; Krawczyk et al., 2013; Krawczyk

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et al., 2011a; Krawczyk et al., 2011b; Melchior et al., 2021; Stamatakis et al., 2014). We extend these findings by reporting that systemic fentanyl injections do not disrupt dBNST cue discrimination and generally potentiate dBNST dopamine signals. We find that fentanyl self-administration experience is sufficient to reverse reward seeking and that dBNST dopamine signaling discriminates fentanyl-associated active vs. inactive lever pressing under extinction conditions. Due to attrition across this study, our power to detect differences in task-specific events during systemic fentanyl and after fentanyl self-administration was limited. The present study supports the need for future work aimed at fully characterizing drug-induced changes to dBNST DA cue and reward encoding during natural and opioid reward seeking.

Dopamine projections to the BNST are concentrated in the dBNST and synapse specifically onto the CRFergic neurons (Meloni et al., 2006; Phelix et al., 1994). Molecular and electrophysiology studies suggest that dopamine increases local CRF release in the dBNST and drug-induced synaptic plasticity in the dBNST requires both dopamine and CRF (Day et al., 2002; Kash et al., 2008; Silberman et al., 2013). These anatomical and *ex vivo* physiology studies suggest dopamine and CRF are critically interacting to drive reward and stress-related behaviors. Indeed, our prior work indicates that CRF receptor activation in the dBNST is necessary for CS-triggered opioid relapse (Gyawali et al., 2020). Further, dBNST dopamine receptor activation decreases blood corticosterone levels in mice suggesting that an increased dopamine response in the dBNST could serve as an anxiolytic signal, which could promote continued drug seeking (Daniel & Rainnie, 2016; Kash et al., 2008; Melchior et al., 2021; Meloni et al., 2006).

The present findings add substantially to the role of dBNST dopamine in motivated behaviors, providing a comprehensive characterization of endogenous dBNST dopamine dynamics in cue-induced behaviors under a number of different natural and drug reward conditions. The fluorescent dopamine sensor $GRAB_{DA}$ is an extremely useful tool for studying

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real-time BNST DA dynamics in the context of motivated behaviors (Lin et al. 2020, Sun et al. 2020).

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Ethics Statement

The animal study was reviewed and approved by University of Maryland, School of Medicine Institutional Animal Care and Use Committee.

Author Contributions

DC conceived, developed and supervised the project. UG and DAM acquired and analyzed the data. UG and DC designed the experiments, interpreted the data, and wrote the manuscript. All authors contributed to manuscript revision and approved the submitted version.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

We thank Jessie Feng for technical assistance and thank the Animal Care Facility for colony maintenance. We thank Asaf Keller and the Department of Anatomy and Neurobiology for sharing photometry equipment.

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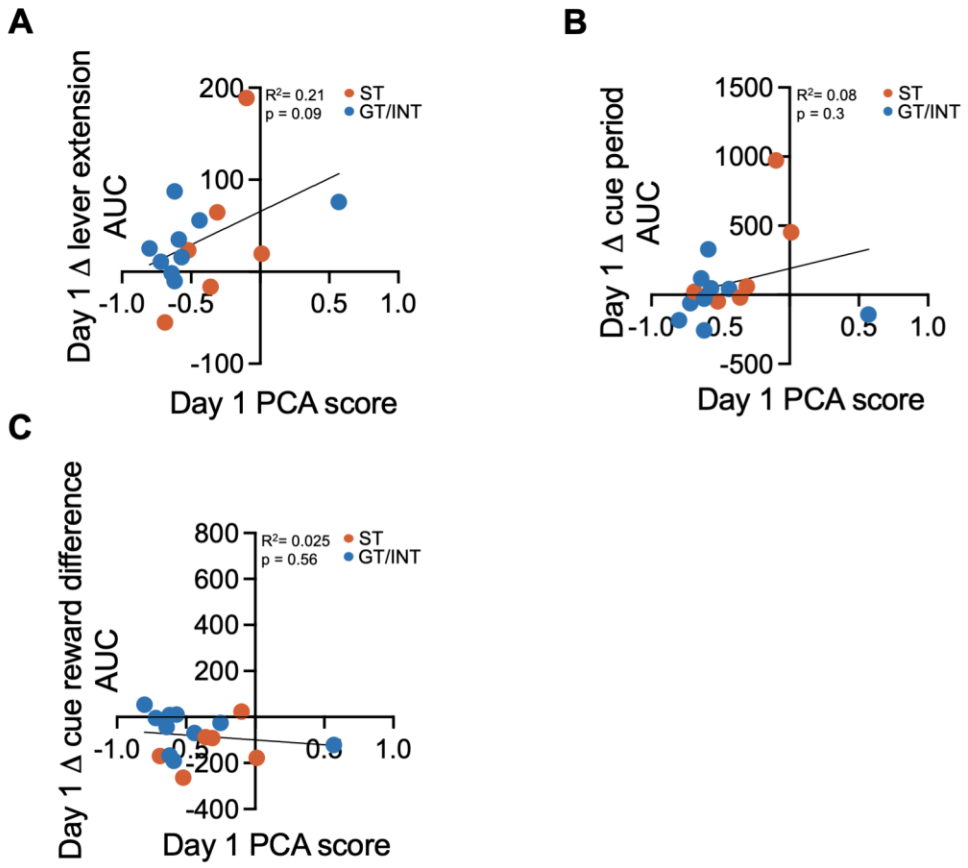

Figure S1: Correlation between **A)** Day 1 PCA scores and Day 1 Δ lever extension AUC. **B)** Day 1 PCA scores and Day 1 Δ cue period AUC and **C)** Day 1 PCA scores and Day 1 Δ cue-reward difference AUC.

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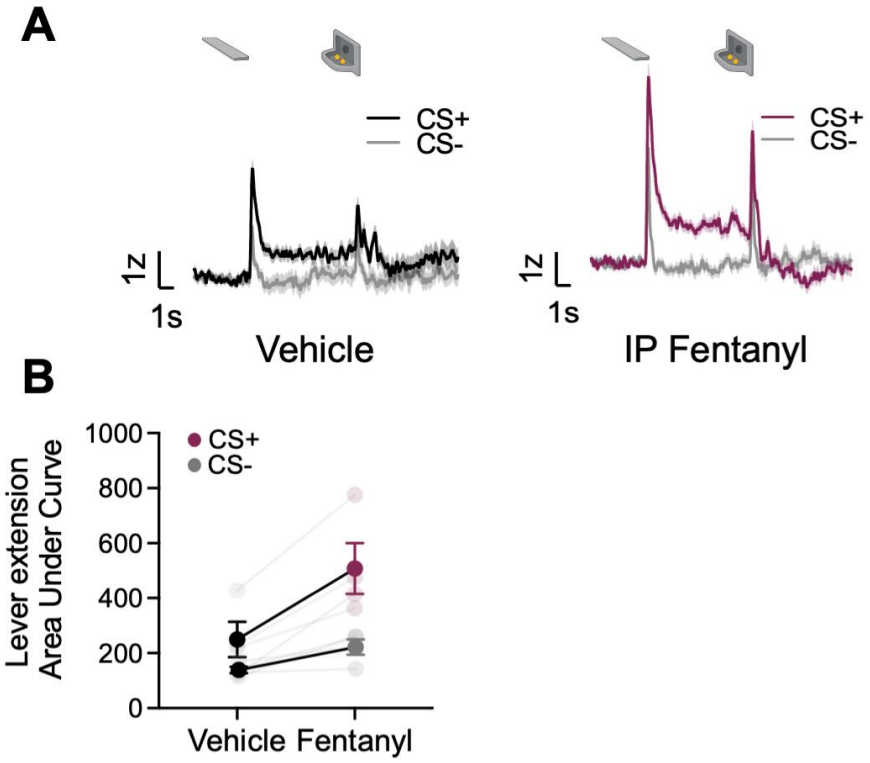

Figure S2: A) Trial-averaged $GRAB_{DA}$ signal change (z-scored ΔF/F) when rats were injected with vehicle (left) or fentanyl (right) during PLA **C)** Trial average quantification of area under $GRAB_{DA}$ z-scored curve (AUC) during CS+ and CS- lever extension (2s) between vehicle and fentanyl conditions. Data are mean±SEM, *p<0.05.

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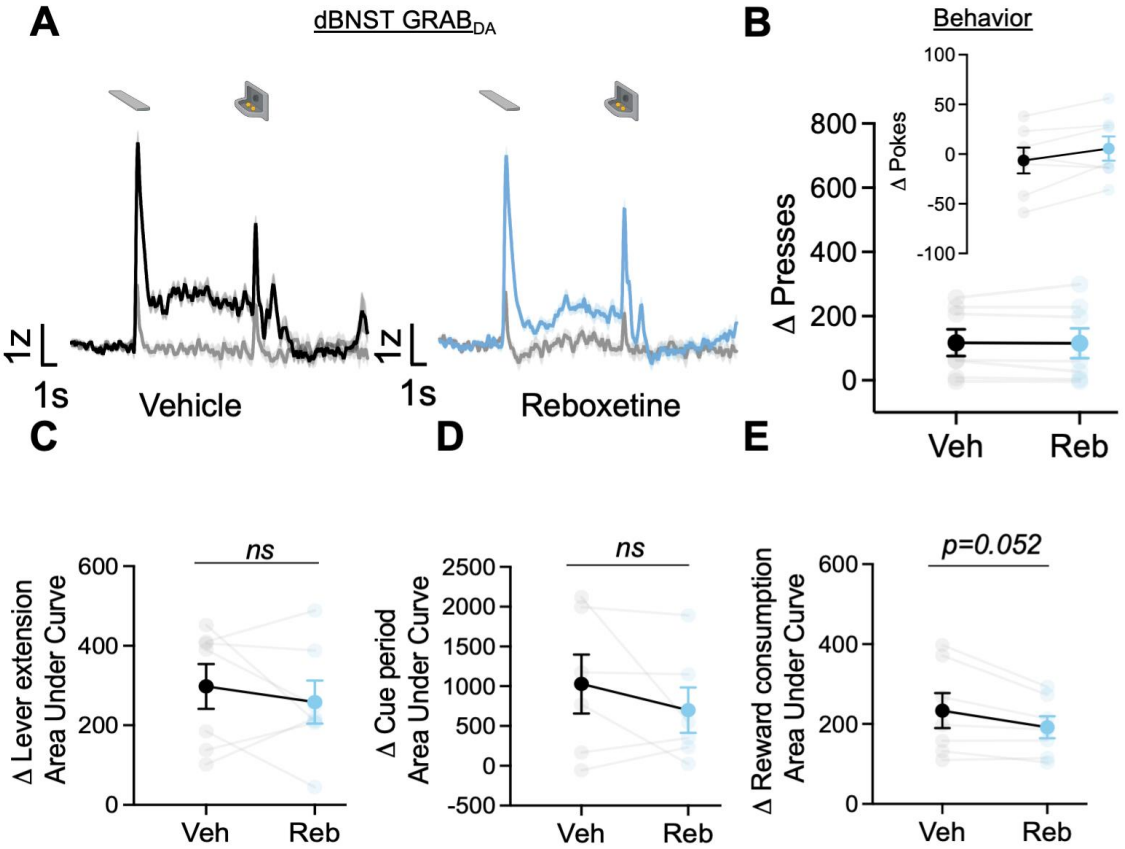

Figure S3: A) Trial-averaged GRAB$_{DA}$ signal change (z-scored ΔF/F) and **B)** Average Δ Presses (CS+) – (CS-) and average Δ pokes (CS+) – (CS-) (inset) when rats were injected with vehicle or Reboxetine during PLA. Trial average quantification of change (CS+) – (CS-) in area under GRAB$_{DA}$ z-scored curve (AUC) during **C)** lever extension (2s), **D)** cue period (10s) and **E)** reward consumption between vehicle and reboxetine conditions. Data are mean±SEM. Veh = Vehicle, Reb = Reboxetine.