Behavioral/Cognitive

Decreased Ventral Tegmental Area CB1R Signaling Reduces Sign Tracking and Shifts Cue–Outcome Dynamics in Rat Nucleus Accumbens

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Sign-tracking (ST) rats show enhanced cue sensitivity before drug experience that predicts greater discrete cue-induced drug seeking compared with goal-tracking or intermediate rats. Cue-evoked dopamine in the nucleus accumbens (NAc) is a neurobiological signature of sign-tracking behaviors. Here, we examine a critical regulator of the dopamine system, endocannabinoids, which bind the cannabinoid receptor-1 (CB1R) in the ventral tegmental area (VTA) to control cue-evoked striatal dopamine levels. We use cell type-specific optogenetics, intra-VTA pharmacology, and fiber photometry to test the hypothesis that VTA CB1R receptor signaling regulates NAc dopamine levels to control sign tracking. We trained male and female rats in a Pavlovian lever autoshaping (PLA) task to determine their tracking groups before testing the effect of VTA → NAc dopamine inhibition. We found that this circuit is critical for mediating the vigor of the ST response. Upstream of this circuit, intra-VTA infusions of rimonabant, a CB1R inverse agonist, during PLA decrease lever and increase food cup approach in sign-trackers. Using fiber photometry to measure fluorescent signals from a dopamine sensor, $GRAB_{DA}$ (AAV9-hSyn-DA2m), we tested the effects of intra-VTA rimonabant on NAc dopamine dynamics during autoshaping in female rats. We found that intra-VTA rimonabant decreased sign-tracking behaviors, which was associated with increases in NAc shell, but not core, dopamine levels during reward delivery [unconditioned stimulus (US)]. Our results suggest that CB1R signaling in the VTA influences the balance between the conditioned stimulus-evoked and US-evoked dopamine responses in the NAc shell and biases behavioral responding to cues in sign-tracking rats.

Key words: **dopamine; endocannabinoids; optogenetics; Pavlovian; pharmacology; photometry**

Significance Statement

Substance use disorder (SUD) is a chronically relapsing psychological disorder that affects a subset of individuals who engage in drug use. Recent research suggests that there are individual behavioral and neurobiological differences before drug experience that predict SUD and relapse vulnerabilities. Here, we investigate how midbrain endocannabinoids regulate a brain pathway that is exclusively involved in driving cue-motivated behaviors of sign-tracking rats. This work contributes to our mechanistic understanding of individual vulnerabilities to cue-triggered natural reward seeking that have relevance for drug-motivated behaviors.

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Introduction

Cues that reliably predict outcomes in the environment powerfully regulate behavior across conditioning. In addition to their predictive value, some cues gain enhanced motivational value that can lead to, or be associated with, maladaptive behavior. Training in a Pavlovian lever autoshaping (PLA) task reveals distinct conditioned responding phenotypes: sign-tracking (ST) rats that engage predominantly with an insertable lever cue show enhanced discrete cue-induced relapse after cocaine experience. This stands in contrast to goal-tracking (GT) and intermediate (INT) rats that engage more with the food cup and show lower levels of discrete cue-induced relapse after cocaine experience

(Hearst and Jenkins, 1974; Tomie, 1996; Flagel et al., 2007; Meyer et al., 2012). Prior studies establish a role for nucleus accumbens (NAc) dopamine (DA) and endocannabinoid signaling in driving sign tracking (Flagel et al., 2011; Bacharach et al., 2018). Here we investigate the extent to which midbrain endocannabinoid signaling and downstream nucleus accumbens DA dynamics interact to drive sign-tracking behaviors.

Cue-evoked dopamine release in the NAc core distinguishes ST from GT phenotypes (Flagel et al., 2011). While pharmacological studies indicate that dopamine signaling drives the acquisition and expression of both sign-tracking and goal-tracking behaviors (Danna and Elmer, 2010; Lopez et al., 2015; Fraser et al., 2016), subsecond cue-evoked NAc core dopamine is strongly correlated with and necessary for sign-tracking behavior, but not for goal-tracking behavior (Flagel et al., 2011; Saunders and Robinson, 2012; Clark et al., 2013; Fraser and Janak, 2017). Uncovering factors controlling this tracking-related difference in striatal dopamine signaling is crucial to understanding the neurobiological mechanisms driving individual differences in motivation toward reward-predictive cues.

Because of their role in regulating the dopamine system, we hypothesize that midbrain endocannabinoid signaling drives differences in cue-evoked striatal dopamine in sign-tracking and goal-tracking phenotypes. Endocannabinoids are critical regulators of the dopamine system and blocking cannabinoid receptor-1 (CB1R) decreases striatal dopamine release (Cheer et al., 2000, 2004; Lupica et al., 2004; Lupica and Riegel, 2005; Cheer et al., 2007; Oleson et al., 2012; Wenzel et al., 2018). The ventral tegmental area (VTA) provides dense dopaminergic projections to the NAc, which are regulated by endocannabinoids (eCBs) acting at presynaptic CB1R to influence dopamine neuron firing and striatal dopamine release (Cheer et al., 2004; Lupica and Riegel, 2005). Blocking CB1R signaling decreases natural and drug reward self-administration, cue-induced reinstatement, and sign tracking (McLaughlin et al., 2003; De Vries and Schoffelmeer, 2005; McLaughlin et al., 2006; Justinova et al., 2008; de Bruin et al., 2011; Oleson et al., 2012; Schindler et al., 2016; Bacharach et al., 2018). Blocking CB1R signaling in the VTA decreases both cue-evoked NAc dopamine and associated reward seeking (Oleson et al., 2012; Wenzel et al., 2018). We previously demonstrated that systemically blocking CB1R receptors decreases the enhanced attractive and reinforcing properties of lever cues in sign-tracking rats (Bacharach et al., 2018). We posit that the locus of CB1R effects on sign tracking is via VTA CB1R suppression of NAc dopamine release.

We hypothesize that CB1R receptor signaling in the VTA regulates cue-evoked NAc dopamine levels to control sign tracking in rats. To test this, we use an optogenetic approach to examine the role of VTA → NAc dopamine projections in the expression of a Pavlovian conditioned approach (PavCA) in ST, GT, and INT rats. We use intracranial pharmacology to examine the effect of intra-VTA infusion of the CB1R reverse agonist rimonabant (Rimo) on the Pavlovian conditioned approach. Finally, combining intracranial pharmacology with fiber photometry, we determine the extent to which VTA rimonabant affects cue-evoked and reward-evoked NAc core and shell dopamine dynamics during Pavlovian conditioned approach. Altogether, we conclude from our data that CB1R signaling in the VTA maintains the conditioning-dependent behavioral and NAc DA bias toward cues in sign-tracking rats.

Materials and Methods

Experimental subjects

We used female (n = 52) and male (n = 6) transgenic TH::Cre Sprague Dawley rats (Envigo; run in four cohorts) for the optogenetics experiment, female (n = 22) Long–Evans rats (Charles River Laboratories; run in two cohorts) for the pharmacology experiment, and female (n = 32) Long–Evans rats (Charles River Laboratories; run in three cohorts) for the photometry experiments. All rats weighed between 215 and 350 g at experimental onset. All rats were single housed and maintained on a 12 h light/dark cycle (zeitgeber time 0 at 7:30 A.M.). All rats had *ad libitum* access to standard laboratory chow and tap water before food deprivation to 90% of their baseline weight, which was maintained for all experimental phases. Chow was provided after daily behavioral sessions. All procedures were performed in accordance with the National Research Council *Guide for the Care and Use of Laboratory Animals* (eighth edition) and were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Surgical procedures

For all surgeries, we anesthetized rats with 3–5% isoflurane (VetOne) and gave a subcutaneous injection of the analgesic carprofen (5 mg/kg). Before the first skull incision, we gave rats a subdermal injection of 10 mg/ml lidocaine at the incision site. After lowering cannula or fiber optics into place, we secured them to the skull using jeweler's screws and dental cement (Dentsply Caulk, Dentsply). All coordinates given are the distance from bregma according to the Paxinos and Watson (2006) rat brain atlas.

Optogenetics. We infused 500 nl of the Cre-dependent inhibitory chloride pump halorhodopsin (Halo; AAV5-ef1a-DIO-eNphr3.0-eYFP; UNC Vector Core, Chapel Hill, NC) or the control virus AAV5-ef1a-DIO-eYFP (UNC Vector Core) bilaterally in to the VTA [coordinates from bregma: anteroposterior (AP), −5.4 mm; mediolateral (ML), ±2.15 mm 10° angle; dorsoventral (DV), −8.2 mm] at a rate of 100 nl/min using a microinfusion pump (UltraMicroPump III, World Precision Instruments) and a 10 µl syringe (Hamilton). The needle tip was left in place for 5 min after infusion, raised 0.1 mm, and left another 5 min before the final raising. We then implanted two 200-µm-core, 0.67 numerical aperture (NA) fiber optics with ceramic zirconia ferrules (Prizmatix, Holon, Israel) targeting the NAc core, bilaterally (coordinates from bregma: AP, +1.8 mm; ML, +2.15 mm 6° angle; DV, −6.6 mm). Fiber optics were secured with DenMat then a thin layer of dental cement. For two of four cohorts, we trained and tested rats in Pavlovian lever autoshaping 6–8 weeks after surgery. In two of four cohorts, we gave rats [Halo, n = 14; enhanced yellow fluorescent protein (eYFP), n = 19] 3 d of training before surgically injecting virus and implanting fiber optics, followed by a 6 week recovery/viral expression period, then a fourth training session before testing.

Pharmacology. We implanted guide cannulae (23 ga; Plastics One) bilaterally into the VTA at a 10° angle (coordinates from bregma: AP, −5.4 mm; ML, ±2.2 mm; DV, −7.33 mm). Cannulae were secured with jeweler's screws and dental cement. At the end of surgery, we inserted into guide cannulae the dummy cannulae, which were kept in the guide cannula and which were only removed during infusion habituation and infusion test procedures. Animals were given 2–3 weeks for recovery before testing.

Fiber photometry. Guide cannulae were implanted in the VTA that were identical to those used in pharmacology experiments. In addition, we infused 1 µl of AAV9-hSyn-DA2m ($GRAB_{DA}$) into the nucleus accumbens unilaterally at a 6° angle (coordinates from bregma: AP, +1.8 mm; ML, +2.15 mm; DV, −6.6 mm). Following virus infusion, we implanted one 400-µm-core, 0.67 NA fiber optic (ThorLabs) in the NAc, 0.1 mm dorsal to the virus injection coordinates. Fiber optics were first cemented in place with Metabond (Parkell), then with Denmat. The entire headcap was covered in a thin layer of dental cement. Rats were given 4 weeks for recovery before testing.

Histology

At the end of experiments, we deeply anesthetized rats with isoflurane and transcardially perfused them with 100 ml of 0.1 M sodium PBS, followed by 400 ml of 4% paraformaldehyde (PFA) in PBS. We removed brains and postfixed them in 4% PFA for 2 h before we transferred them to 30% sucrose in PBS for 48–72 h at 4°C. We subsequently froze brains and stored them at −20°C until sectioning. Coronal sections (50 μm)

containing NAc and VTA were collected using a cryostat (Leica Microsystems).

To verify cannula placements, we stained brain sections with cresyl violet and coverslipped with Permount (Thermo Fisher Scientific). To verify viral expression and fiber placements, we mounted and coverslipped all brain sections with Vectashield DAPI (Vector Laboratories) and the mounting medium Mowiol (Sigma-Aldrich). We used a spinning disk confocal microscope (model SP8, Leica) to verify viral expression and fiber optic/cannula placement. For optogenetic experiments, rats were excluded from analysis if cell body labeling of Halo or eYFP was observed outside of the VTA (in SNc), if there was no terminal expression in the NAc core, and/or fiber optics were not targeting the NAc core. For pharmacology experiments, rats were excluded if cannulae were not targeting the VTA. For photometry experiments, rats were excluded if there was no virus expression below the fiber optic in the NAc, or if the conditioned stimulus (CS)-evoked dopamine signal was <2 z scores above baseline during the vehicle (Veh) testing day.

Behavioral apparatus

Experiments were conducted in individual sound-isolated standard experimental chambers (25 × 27 × 30 cm; Med Associates). For Pavlovian lever autoshaping, each chamber had one red house light (6 W) located at the top of a wall that was illuminated for the duration of each session. During PLA, the opposite wall in the chamber had a recessed food cup (with photobeam detectors) located 2 cm above the floor grid. The food cup was attached to a programmed pellet dispenser that delivered 45 mg food pellets (5TUL purified rodent tablet, catalog #1811155, TestDiet; protein, 20.6%; fat, 12.7%; carbohydrate, 66.7%). One retractable lever was positioned on either side of the food cup counterbalanced, 6 cm above the floor.

Optogenetics. Each Med Associates chamber was equipped with a green LED (525 nm; 300 mW; Prizmatix) to deliver light to the NAc core. A transistor–transistor logic (TTL) pulse was generated by MedPC software 1 s before lever insertion and was sent to an minicontroller (Arduino), which drove an LED for 11 s total, (terminating with the retraction of the lever). The complete light path is MED TTL → Arduino → LED → Patch cord (1000 μm core; Prizmatix) → commutator (Prizmatix) → bifurcated (2 × 500 μm core; Prizmatix) fiber optic patch cord → two implanted fiber optics ferrules targeting the NAc core. A ceramic sleeve covered in black heat shrink tightly joined the patch cord and fiber optic and prevented light loss. We used a light meter (model PM100D, ThorLabs) to calibrate light output in each box before and after each session.

Fiber photometry. We used LEDs (ThorLabs) to deliver 465 nm light to measure $GRAB_{DA}$ fluorescence signals and 405 nm light as an isosbestic control. The two wavelengths of light were sinusoidally modulated at 210 and 337 Hz respectively. The LEDs connected to a 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) which was connected to implanted fiber optics (400 μm core; Thor Labs). We maintained the light intensity at the tip of the fiber optic cable at 10–15 μW across behavioral sessions. LED light collected from the $GRAB_{DA}$ and isosbestic channels was focused onto a femtowatt photoreceiver (Newport). We low-pass filtered and digitized the emission light at 3 Hz and 5 kHz, respectively, by a digital processor controlled by Synapse software suite (RZ5P, Tucker Davis Technologies). We time stamped the behavioral events including lever insertion, pellet delivery, lever press, and food cup entry through TTL pulses in Synapse software.

Training in Pavlovian lever autoshaping

Across all experiments, we gave rats a single 38 min magazine training session during which one food pellet was delivered into the food cup on a variable-interval (VI) 90 s schedule (60–120 s) for 25 trials. We trained rats in four or five daily PLA sessions, which consisted of 25 reinforced lever conditioned stimulus (CS+) presentations occurring on a VI 90 s schedule (60–120 s). CS+ trials consisted of the insertion of a retractable lever for 10 s, after which the lever was retracted and two food pellets were delivered to the food cup regardless of whether a lever or food cup response was made. For two-lever PLA experiments, on the opposite side of the wall from the CS+, we included a CS– lever for which the extension and retraction had no programmed consequences. Sessions consisted of 25 CS+ pairings and 25 CS– trials with a 90 s VI schedule between rewarded trials. For experiments in the unpaired condition, lever extension/retraction did not produce food pellets. Instead, two food pellets were delivered pseudorandomly during the intertrial interval (ITI; at least 20 s after/20 s before lever retraction/extension, respectively).

Measurements and difference scores

Behavioral measurements were collected during the 20 s pre-CS period (ITI), 10 s CS period, and the 5 s post-CS reward delivery period. An automated measurement of the latency to first contact of the lever and/or food cup during the cue for each trial was recorded. On trials in which a contact did not occur, a latency of 10 s was recorded. For each session, the lever or food cup probabilities were calculated by determining the number of trials in which the lever or food cup response was made, divided by the total number of trials in the session.

We used a PavCA analysis (Meyer et al., 2012) to determine sign-tracking, goal-tracking, and intermediate groups. The PavCA score quantifies the difference between lever-directed and food cup-directed behaviors, and ranges from −1.0 to +1.0. The PavCA score of an individual rat is the average of three difference score measures (each ranging from −1.0 to 1.0) including the following: (1) preference score, (2) latency score, and (3) probability score. The preference score is the total number of lever presses during the CS, minus the total number of food cup responses during the CS, divided by the sum of these two measures. The latency score is the session-averaged latency to make a food cup response during the CS, minus the session averaged latency to lever press during the CS, divided by the duration of the CS (10 s). The probability score is the probability of lever press minus the probability of food cup response observed across the session. ST PavCA scores range from +0.33 to +1.00, INT PavCA scores range from +0.32 to −0.32, and GT PavCA scores range from −0.33 to −1.00.

Testing in Pavlovian lever autoshaping

Optogenetics. We habituated rats to the optogenetic patch cable after day 4 of training. On subsequent sessions, rats were tethered to the patch cable during the session. During the OFF test, rats were tethered but no light was delivered. For the ON test, light was delivered to the NAc at 4–6 mW from the fiber tip. During the ON test, LED light delivery began 1 s before lever extension and remained on for the duration of the 10 s lever cue (11 s total). Lever retraction, termination of the LED light, and delivery of food occurred simultaneously at the end of each of the 25 trials.

Pharmacology. We habituated rats to handling and infusion procedures throughout training. After the last training day, we inserted injectors into the cannulae and the infusion pump was turned on, but nothing was infused. Before test sessions in PLA, we gave each rat an infusion of rimonabant or vehicle in two separate counterbalanced test sessions that occurred 48 h apart. We removed dummy cannulae and inserted 30 ga injector cannulae (Plastics One) extending 1.0 mm beyond the end of the guide cannulae. We connected each injector cannula using polyethylene-50 tubing, which was attached to a 5 μl syringe (Hamilton) that was placed in an infusion pump (CMA Syringe Pump 4004, Harvard Apparatus). We infused rimonabant (2 μg/μl) or vehicle bilaterally into the VTA at a rate of 0.25 μl/min for a total of 2 min or 0.5 μl total volume per hemisphere. We kept the injectors in place for an additional minute before slowly removing them and replacing dummy cannulae for behavioral testing. We tested rats in PLA 10 min after the completion of the infusion.

Drug solutions were prepared immediately before each test session. Rimonabant [5-(4-chlorophenyl)−1-(2,4-dichloro-phenyl)−4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide; catalog #SR141716A, National Institute on Drug Abuse Drug Supply Program] was dissolved in a 1:1:18 solution of ethyl alcohol (Sigma-Aldrich), emulphor (Alkamuls EL-620, Solvay Chemicals), and saline (Hospira) and sonicated for 15 min. The vehicle solution consisted of the 1:1:18 solution of ethyl alcohol, Emulphor, and saline.

Photometry. We habituated rats to patch cables during magazine training and performed recordings during all of the training and testing phases. Testing and drug infusions procedures were identical to pharmacology experiments.

Data and statistical analyses

Behavioral data were analyzed using SPSS statistical software (IBM) with mixed-design repeated-measures ANOVA. Significant main and interaction effects ($p < 0.05$) were followed by *post hoc* within-subject, repeated-measures ANOVA or *t* tests. For significant *post hoc t* tests, we report Cohen's *d* effect size.

Optogenetics. For training data, we used mixed repeated-measures ANOVA including the within-subject factor of Session (4), and the between-subject factors of Tracking (GT, INT, ST) and Virus (Halo, eYFP). For test data, we used mixed repeated-measures ANOVA including the within-subject factor of Light (OFF, ON), and between-subject factors of Tracking (GT, INT, ST) and Virus (Halo, eYFP). In cases with a significant Treatment × Tracking interaction, we then probed individually Response × Treatment interactions within each tracking group.

Pharmacology. For PLA training data, we used mixed repeated-measures ANOVA of lever and food cup measures (contact, latency, and probability), using between-subject factors of Tracking group (ST, INT) and the within-subject factor of Session to analyze lever-directed and food cup-directed behaviors. For test data, we used mixed repeated-measures ANOVA including within-subject factor of Treatment (Veh, Rimo) and between-subject factors of Tracking (INT, ST). In cases with a significant Treatment × Tracking interaction, we then probed individually Response × Treatment interactions within each tracking group.

Photometry. For training data, we used mixed repeated-measures ANOVA including within-subject factor of Session (5). For test data, we used mixed repeated-measures ANOVA including within-subject factors of Treatment (Veh, Rimo) and Epoch (Cue, Reward).

Photometry signal analysis. To calculate $\Delta F/F$, a least-squares linear fit was applied to the 405 nm signal. $\Delta F/F$ = (490 nm signal − fitted 405 nm signal)/fitted 405 nm signal. To calculate *z*-scores on each trial, we took the average of the $\Delta F/F$ signal over a 10 s baseline period before the CS insertion and divided the total trial measurements by that average. All 25 trials per session for each animal were averaged in to one average per animal, which was used for subsequent analysis. To screen for a reliable photometry signal, we defined significant transients in a behavioral window (5 s post-lever insertion) as having a maximum CS-evoked peak amplitude ≥ 2 *z*-score ($p = 0.05$) above baseline. We excluded rats whose vehicle day CS-evoked signals did not meet this criterion. Peak height was calculated using the "findpeaks" function in MATLAB. We took the maximum peak height above $z = 0$ in the 2.5 s following the event of interest [either CS (lever extension) or US (lever retraction and pellet delivery)]. In case there were no peaks above $z = 0$, the peak height measure was recorded as 0 for that subject. The latency at which the maximum peak occurred was used for the peak latency calculation. We used the "trapz" function in MATLAB to examine area under the curve for 5 s postevent. For training data in Figure 5, of $n = 22$ rats, $n = 4$ were only recorded on day 1 and day 5. Thus, data seen in Figure 5*C*, inset, are from $n = 18$ animals, while data seen in Figure 5, *C* and *D*, are from all $n = 22$ animals. When examining differences in signal between the NAc core and shell seen in Figure 5, *E* and *F*, we analyzed data from $n = 20$ of 22 rats because $n = 2$ fiber placements were exactly on the core/shell border.

Data availability

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

Results

Individual differences in the acquisition of conditioned approach in a Pavlovian lever autoshaping task

We first examined the role of the VTA → NAc dopamine projection in regulating individual differences in Pavlovian approach. We infused a Cre-dependent control virus (AAV5-ef1a-DIO-eYFP) or a Cre-dependent Halo (AAV5-ef1a-DIO-eNphr3.0-eYFP) into the VTA of TH::Cre rats and implanted bilateral optical fibers targeting the NAc core (Fig. 1*A*,*B*). After a recovery period, we trained rats (Halo, $n = 31$; eYFP, $n = 27$) in four PLA sessions where the extension and retraction of a lever for 10 s predicted the delivery of food (Fig. 2*A*). We classified rats as sign-trackers (ST group), goal-trackers (GT group), or intermediates (INT group) based on the PavCA score, which reflects the tendency of an animal to approach the lever relative to the food cup. Over acquisition, we observed main effects of Session ($F_{(3,156)} = 34.921$, $p < 0.001$) and Tracking ($F_{(2,52)} = 67.568$, $p < 0.001$), and a Session × Tracking interaction ($F_{(6,156)} = 24.923$, $p < 0.001$), confirming individual differences in conditioned approach (Fig. 2*B*). The main effects and interactions of all behavioral measures collected in PLA are presented in Table 1. There were no main effects or interactions with Virus (maximum $F = 3.061$; minimum $p = 0.086$), demonstrating that the virus infused had no effect on the acquisition of behaviors that characterize sign, goal, and intermediate rats.

VTA-NAc dopamine terminal inhibition selectively reduces approach in sign-trackers

Prior pharmacology studies establish a role for NAc core dopamine in specifically driving sign tracking (Saunders and Robinson, 2012; Clark et al., 2013; Fraser et al., 2016; Fraser and Janak, 2017). Here we test the role of VTA-NAc core projections by inhibiting VTA dopaminergic axons projecting to the NAc core during the 10 s lever cue in sign-tracking, goal-tracking, and intermediate groups. We gave two test sessions; one in which the rats were tethered to the patch cable but light was not delivered (OFF condition), and a second session in which rats were tethered and LED light was delivered to the NAc core (ON condition; Fig. 2*A*).

We examined the effect of dopamine inhibition on the number of lever contacts in the ST group (Fig. 2*C*) and found a main effect of Light ($F_{(1,14)} = 8.735$, $p = 0.010$) and a Light × Virus interaction ($F_{(1,14)} = 67.602$, $p = 0.015$). This interaction was driven by a decrease in pressing by the Halo group ($t_{(9)} = 3.884$, $p = 0.004$, Cohen's $d = 0.71$) but not the eYFP group ($t_{(5)} = 0.277$, $p = 0.793$). We additionally examined food cup contacts and found no significant main effects or interactions [maximum $F = 3.35$, minimum $p = 0.089$ (main effect (ME) light); Fig. 2*D*]. In addition to lever and food cup contacts, we analyzed the latency and probability to lever and food cup contact and found no significant interactions with virus (maximum, $F = 2.07$; minimum, $p = 0.172$). Collectively, these results demonstrate that dopamine inhibition in ST rats does not affect the latency or probability to make a response, but selectively reduces the vigor with which the animals engage with and press the lever. Consistent with pharmacological manipulations in the NAc core (Saunders and Robinson, 2012; Clark et al., 2013; Fraser and Janak, 2017), inhibition of the VTA → NAc dopamine projection is necessary for the vigor of lever responding in the sign-tracking rats.

We next examined the effect of dopamine terminal inhibition in INT rats. Although the INT rats had substantial levels of lever pressing by the end of training, there was no Light × Virus interaction ($F_{(1,23)} = 0.208$, $p = 0.653$; Fig. 2*E*). Poking behavior remained unaffected by dopamine inhibition as well. Although there was a main effect of Light ($F_{(1,23)} = 19.504$, $p < 0.001$), there was no Light × Virus interaction ($F_{(1,14)} = 0.848$, $p = 0.367$; Fig. 2*F*). Intermediate rats display a similar number of

both lever-directed and food cup-directed behaviors, and our results demonstrate that NAc dopamine inhibition does not affect the levels of either response. The preferred response of GT rats is food cup-directed behavior. There was no significant interaction with Light × Virus ($F_{(1,15)}$ = 0.125, p = 0.729; Fig. 2*G*). We found a main effect of Light when examining pressing behavior ($F_{(1,15)}$ = 5.547, p = 0.033; Fig. 2*H*), but no Light × Virus interaction ($F_{(1,15)}$ = 0.922, p = 0.352).

We performed an analysis of continuous data showing the relationship between PavCA score and the difference in lever contacts between treatments (ΔLever contacts = contacts light ON – contacts light OFF). We found a significant negative correlation (R^2 = 0.3452, p = 0.0005; Fig. 2*I*), indicating the greater the PavCA score (ST), the larger the decrement in lever presses induced by dopamine inhibition. We did not see a significant correlation in the eYFP control group (R^2 = 0.03746, p = 0.33; Fig. 2*J*), nor did we observe any relationship between PavCA score and difference in food cup responses between treatments (R^2 = 0.0012, p = 0.8554; eYFP: R^2 = 0.0014, p = 0.853; data not shown).

To further confirm that inhibiting VTA dopaminergic axons in the NAc was specific to lever pressing of the ST, we analyzed data only from the rats expressing halorhodopsin using between-subjects factors of Tracking (ST, INT, GT) and within-subjects factors of Light (OFF, ON) and Response (press, poke). We found a three-way interaction ($F_{(2,28)}$ = 7.98, p = 0.002) that was driven by a decrease in pressing by the sign-tracking rats. This analysis further demonstrates that VTA dopamine axon inhibition specifically reduces lever pressing in sign-tracking rats.

In conclusion, inhibiting dopaminergic terminals in the NAc specifically reduced the amount of lever pressing in ST animals and had no effect on the responding in INT or GT rats. Here we have demonstrated this VTA → NAc pathway is important for regulating sign-tracking behavior, we next manipulate CB1R in the VTA where we predict CB1R regulates cue-evoked NAc DA levels during sign tracking.

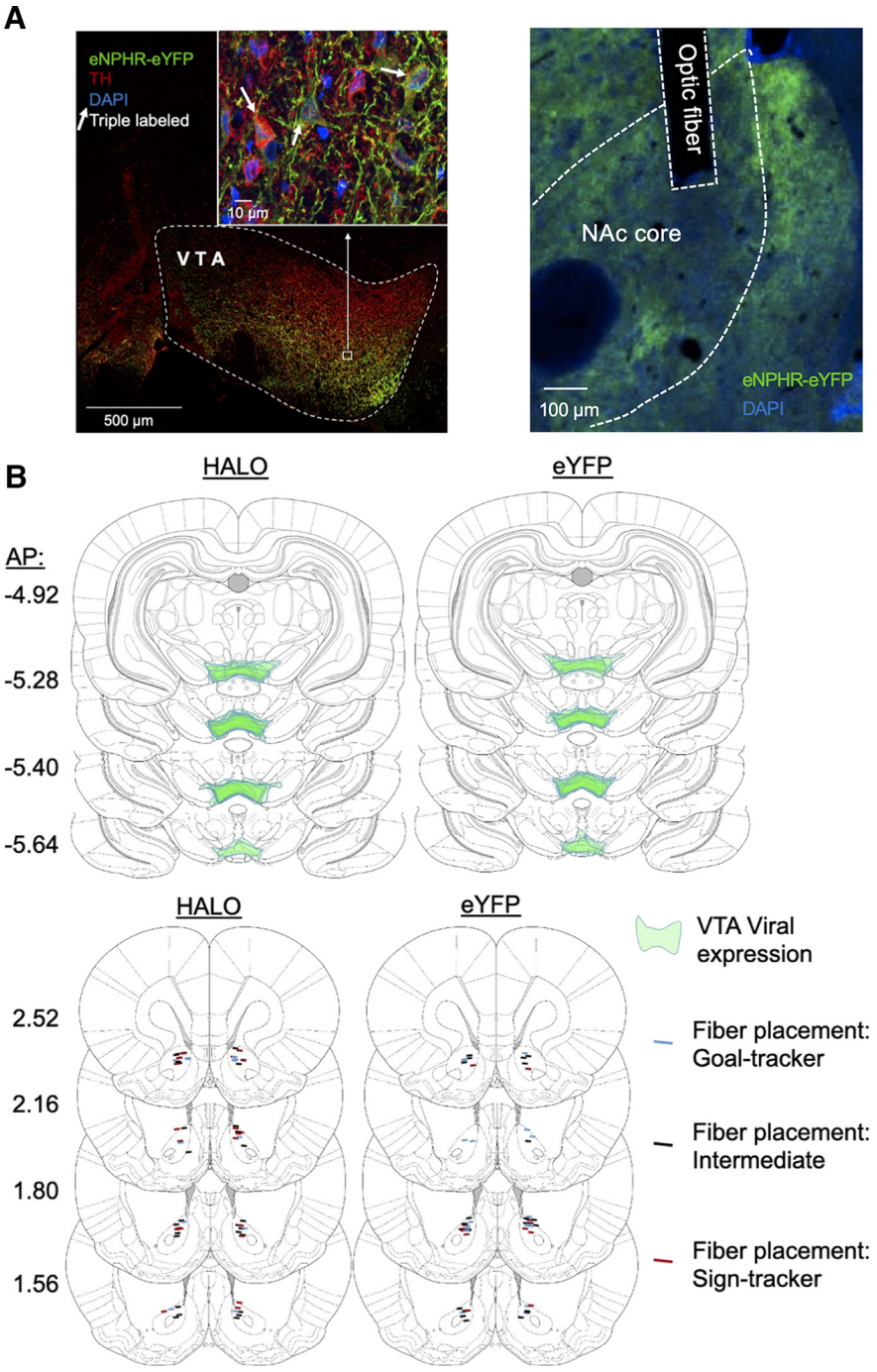

Figure 1. Histologic verification of viral and optogenetic fiber placement. ***A***, Left, Representative VTA transduction of halorhodopsin (green). Staining for tyrosine hydryoxylase (TH) is in red; staining for DAPI is in blue. White arrows depict triple overlap. Right, Representative image halorhodopsin terminal expression (green) and DAPI (blue) in the nucleus accumbens. White dotted line indicates boundary of NAc core. ***B***, Top, The extent of viral transduction (in mm) of halorhodopsin and eYFP in the VTA. Bottom, Bilateral optogenetic fiber optic placement in the NAc across the three tracking groups.

Intra-VTA CB1R inhibition reduces sign tracking

Given the specific effects of VTA → NAc core dopamine inhibition in sign-tracking rats, we next examine how VTA CB1Rs regulate cue attraction in sign-trackers. As a comparison group, we include INT rats who also display lever-directed behaviors, but do not require NAc dopamine to execute these behaviors (Fig. 2*E*,*F*). All rats were implanted with cannulae targeting the VTA (Fig. 3*A*). First, we trained 19 rats (ST, 11; INT, 8) for five PLA sessions to determine tracking groups. For the PavCA score, we observe main effects of Session ($F_{(4,68)}$ = 21.98, p < 0.001) and Tracking ($F_{(1,17)}$ = 22.77, p < 0.001) and a Session × Tracking interaction ($F_{(4,68)}$ = 3.45, p = 0.013; Fig. 3*B*). On the fifth training session, INT rats showed similar levels of lever-directed and food cup-directed behaviors (PavCA = 0.13), whereas ST rats showed predominantly lever-directed behaviors (PavCA = 0.62), further confirming that these two groups of rats show different patterns of

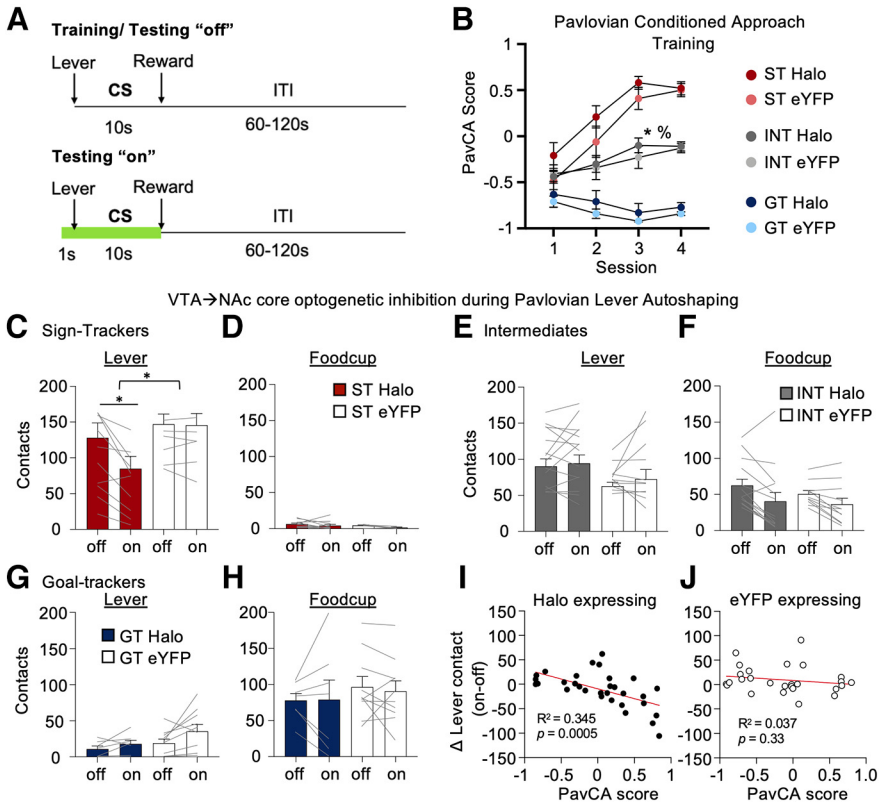

Figure 2. VTA-NAc dopamine terminal inhibition selectively reduces approach in sign-trackers. ***A***, After training in PLA, rats were given test sessions with light turned Off (top) or On (bottom), where light was delivered to the NAc core during the 10 s cue period. ***B***, The mean ± SEM PavCA scores for ST, GT, and INT rats that show individual differences in conditioned responding in PLA task. *Main effect of Session; %Significant Session × Tracking interaction. ***C***, ***D***, Terminal inhibition of dopamine axons in the NAc significantly reduces the amount of lever contacts (***C***) and has no effect on food cup contacts in sign-trackers (***D***). *Curved bracket indicates significant Light × Virus interaction; *straight bracket indicates significant *post hoc* within-subject *t* test. ***E***, ***F***, No significant effects of terminal inhibition in INT rats. ***G***, ***H***, No significant effects of terminal inhibition in GT rats. ***I***, Correlation between the change in lever-pressing behavior between light conditions as a function of the PavCA scores of Halo-expressing rats. ***J***, Correlation between the change in lever-pressing behavior between light conditions and PavCA scores of eYFP-expressing rats.

conditioned responding in response to Pavlovian reward-predictive cues by the end of training. We present main effects and interactions of all PLA training measures in Table 2.

Before the sixth and seventh reinforced PLA sessions, we gave rats counterbalanced intra-VTA infusions of rimonabant or vehicle. Based on previous findings (Bacharach et al., 2018), we predicted that intra-VTA rimonabant would reduce measures of sign-tracking behavior. Indeed, we found that blocking VTA CB1R decreased the PavCA score selectively for ST, but not INT, rats (Fig. 3*C*). We found a main effect of Tracking ($F_{(1,17)} = 16.94$, $p = 0.001$) and a Treatment × Tracking interaction ($F_{(1,17)} = 10.67$, $p = 0.005$). This interaction was driven by a significant decrease in PavCA in the sign-trackers ($t_{(10)} = 3.272$, $p = 0.008$, Cohen's $d = 0.78$) and a nonsignificant increase in PavCA in INT rats ($t_{(7)} = -1.544$, $p = 0.167$, Cohen's $d = 0.43$).

Of the three scores that comprise the PavCA index, the probability score was most affected by intra-VTA rimonabant injections (Fig. 3*D*). We found a main effect of Tracking ($F_{(1,17)} = 19.63$, $p < 0.001$) and a Treatment × Tracking interaction ($F_{(1,17)} = 24.40$, $p < 0.001$). Compared with vehicle, ST rats showed a significant decrease ($t_{(10)} = 4.509$, $p = 0.001$, Cohen's $d = 0.76$), whereas INT rats showed a significant increase ($t_{(7)} = -2.646$, $p = 0.033$, Cohen's $d = 0.61$) in the probability score when VTA CB1R signaling was decreased. To further understand the change in probability score, we compared the probability to poke versus the probability to press across treatment conditions within each tracking group (Fig. 3*E*). Sign-trackers showed a main effect of Response ($F_{(1,10)} = 48.31$, $p < 0.001$) and a Treatment × Response interaction ($F_{(1,10)} = 20.332$ $p = 0.001$), suggesting that rimonabant differentially affected pressing versus poking behavior. Sign-trackers showed a significant reduction in the probability to lever press ($t_{(10)} = 4.734$, $p < 0.001$, Cohen's $d = 0.58$) and an increase in the probability to poke, though the latter was not significant ($t_{(10)} = -1.573$, $p = 0.147$, Cohen's $d = 0.44$). Intermediates showed a main effect of Treatment ($F_{(1,7)} = 15.16$, $p = 0.006$) and Treatment × Response interaction ($F_{(1,7)} = 7.00$, $p = 0.033$). Intermediates showed a reduction in the probability to press ($t_{(7)} = 2.950$, $p = 0.021$, Cohen's $d = 0.91$) and a strong reduction in the probability to poke ($t_{(7)} = 4.194$, $p = 0.004$, Cohen's $d = 1.49$). At first pass, these data suggest that CB1R blockade reduces all approach behaviors in intermediate rats, but shifts sign-tracking rats away from lever directed and toward food cup-directed behaviors.

As a measure of general task engagement and motivation to consume a reward, we measured the latency to collect the pellet once it was delivered for each trial (Fig. 3*F*). We found a main effect of Treatment ($F_{(1,17)} = 12.65$, $p = 0.002$) and a Treatment × Tracking interaction ($F_{(1,17)} = 8.73$, $p = 0.009$). Rimonabant had no effect on the latency to collect the pellet in ST rats ($t_{(10)} = -0.686$, $p = 0.51$, Cohen's $d = 0.13$), suggesting that the changes in the lever and food cup approach of the ST rat reported above were not because of decreased task engagement. In contrast, intra-VTA rimonabant increased the latency to collect the pellet in INT rats ($t_{(7)} = -3.212$, $p = 0.015$, Cohen's $d = 1.21$), suggesting that their motivation to consume the pellets may have in part contributed to an overall reduced lever and food cup approach during the cue. Regardless of the latency to collect the pellet, all rats tested still ate 100% of the pellets delivered during the task, confirming that there were no deficits in consummatory behavior arising from VTA CB1R manipulation.

To further examine this possibility, we next determined whether rimonabant decreased overall task engagement or motivation to consume food reward. We examined total behavior (lever contacts plus food cup contacts) during testing and found a main effect of Treatment ($F_{(1,17)} = 19.16$, $p < 0.001$) and a Treatment × Tracking interaction ($F_{(1,17)} = 6.82$, $p = 0.018$; Fig. 3*G*). This interaction was driven by a significant decrease in contacts in INT rats ($t_{(7)} = 4.741$, $p = 0.002$, Cohen's $d = 1.22$). These data indicate that intra-VTA rimonabant injections did not affect overall levels of conditioned responding in ST rats, but this treatment blunted behavior overall in INT rats. We further probed this interaction by investigating lever versus food cup contacts in each tracking group (Fig. 3*H*). Similar to the probability data, ST rats showed a main effect of Response ($F_{(1,10)} = 24.732$, $p = 0.001$)

Table 1. Main effects and interactions of all behavioral measures collected in PLA

		Lever						Food cup					
		Contact		Latency		Probability		Contact		Latency		Probability	
Factor	df	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Session	(3,156)	59.965	<0.001	33.276	<0.001	35.963	<0.001	3.962	0.009	3.185	0.026	2.427	0.068
Tracking	(2,52)	43.241	<0.001	38.632	<0.001	80.645	<0.001	12.628	<0.001	22.588	<0.001	25.657	<0.001
Virus	(1,52)	1.537	0.221	0.125	0.726	0.875	0.354	0.383	0.539	3.198	0.08	3.831	0.056
Session × Tracking	(6,156)	23.015	<0.001	10.522	<0.001	10.116	<0.001	14.473	<0.001	13.420	<0.01	13.967	<0.01
Session × Virus	(3,156)	1.163	0.326	2.009	0.115	0.762	0.517	0.805	0.493	1.399	0.245	1.343	0.263
Tracking × Virus	(2,52)	0.299	0.743	0.236	0.791	0.182	0.834	1.081	0.347	1.694	0.194	2.306	0.110
Session × Tracking × Virus	(6156)	0.887	0.506	1.070	0.383	1.078	0.378	0.791	0.578	0.484	0.820	0.470	0.830

and a Treatment × Response interaction ($F_{(1,10)} = 10.661$, $p = 0.0009$). This interaction was driven by a decrease in lever contacts ($t_{(10)} = 3.167$, $p = 0.01$, Cohen's $d = 0.50$) and an increase in food cup pokes ($t_{(10)} = -1.273$, $p = 0.116$, Cohen's $d = 0.44$). In contrast, INT rats showed a reduction in both responses (main effect of Treatment: $F_{(1,7)} = 22.473$, $p < 0.002$). In summary, ST had similar amounts of total approach behavior in which a reduction in lever-directed behavior was compensated for by an increase in food cup-directed behavior. INT rats still engaged in both types of responding, but both responses were decreased.

As a control, we determined whether locomotor activity changed with intra-VTA rimonabant injections. We gave an open-field test to a subset of ST and INT rats and found no differences in locomotor activity when rats were given intra-VTA vehicle or rimonabant infusions (mean ± SEM distance traveled in meters: Veh = 19.65 ± 1.44; Rimo = 18.48 ± 0.86). There were no main effects of Tracking, Treatment, or a Tracking × Treatment interaction (maximum $F = 1.147$; minimum $p = 0.307$).

Together, CB1R inhibition in the VTA causes divergent behavioral response profiles across ST and INT rats. Rimonabant in the VTA caused a decrease in all appetitive motivated behaviors measured in the INT rats. In contrast, rimonabant only decreased behavior directed toward the lever cue in ST rats, while increasing the amount of cue-induced food cup behaviors. Thus, CB1R receptor blockade decreases sign-tracking behavior leading to more balanced levels of lever-directed and food cup-directed behaviors.

CS-evoked dopamine is specific to rewarded lever

To gain further mechanistic insight into the effect of rimonabant on sign-tracking behavior, we measured $GRAB_{DA}$ signals in the NAc in the presence and absence of CB1R inhibition. Because we used photometric measurement with a fluorescent dopamine sensor, we first validated that NAc $GRAB_{DA}$ signals comply with basic observations that NAc DA signals are specific to a learned association between a CS and food reward US. We trained a group of fiber-implanted, NAc $GRAB_{DA}$-expressing rats (Fig. 4*A*) in a two-lever Pavlovian autoshaping procedure. Here, a CS+ lever predicted food reward (US) and a separate CS– lever predicted no reward. Using a within-subject design, we examined total behavior (lever and food cup contact) during the CS+ versus CS– periods across training (Fig. 4*B*) and found a main effect of Cue ($F_{(1,5)} = 11.939$, $p = 0.018$) and a Cue × Session interaction ($F_{(4,20)} = 11.325$, $p < 0.001$). Rats showed more approach during the CS+, compared with the CS– presentations on day 5 of training ($t_{(5)} = 3.598$, $p = 0.016$, Cohen's $d = 1.88$). This confirms that by day 5 of training, the rats could behaviorally discriminate between a reinforced and a nonreinforced cue. On day 5 of training, we found that the CS-evoked DA peak height was greater for the CS+ lever than the CS– ($t_{(5)} = 2.947$, $p = 0.032$, Cohen's $d = 0.79$; Fig. 4*C*,*D*). Next, as a between-subject validation of associative NAc DA signaling, we trained a separate group of fiber-implanted, NAc $GRAB_{DA}$-expressing rats ($n = 4$) rats in an unpaired control condition in which we delivered the same number of food pellets, but lever extension was delivered pseudorandomly during the ITI period and was never explicitly paired with pellet delivery. Unpaired rats displayed low levels of lever and food cup approach during lever extension periods throughout training (Fig. 4*E*). Rats did not develop conditioned responses to either the lever or food cup (day 1 vs day 5 ($t_{(3)} = 1.023$, $p = 0.382$; Fig. 4*E*, inset left). Despite not showing a conditioned lever or food cup approach, rats were still engaged in the task and collected the pellet faster on day 5 than day 1 of training ($t_{(3)} = 3.696$, $p = 0.034$, Cohen's $d = 2.56$; Fig. 4*E*, inset right). In this unpaired condition, we observed very low levels of lever-associated NAc $GRAB_{DA}$ signals, which diminished, though not significantly, as training progressed (day 1 vs day 5: $t_{(3)} = 2.425$, $p = 0.119$; Fig. 4*F*). In the unpaired rats, NAc $GRAB_{DA}$ signals to the CS on day 1 likely represent an intrinsic stimulus salience or novelty signal (Mirenowicz and Schultz, 1994; Horvitz et al., 1997), which diminishes over time as rats become familiar with the lever extension and retraction. There was a reliable NAc $GRAB_{DA}$ signal to the US food delivery (Fig. 4*G*), which remained unchanged from day 1 to day 5 of training ($t_{(3)} = -1.594$, $p = 0.209$; Fig. 4*G*, inset), confirming the unpredictable delivery of the US in unpaired rats. Our results indicate that the NAc $GRAB_{DA}$ signal mimics what is observed using voltammetry and that increases in dopamine to the cue occur to a greater degree for a food-reinforced cue relative to a nonreinforced or unpaired cue.

CS-evoked dopamine increases with sign-tracking behavior across Pavlovian training

Next, in separate group of 22 rats (Fig. 5*A*) we measured NAc $GRAB_{DA}$ signals as they acquired a sign-tracking response (PavCA: main effect of Session: $F_{(4,84)} = 33.630$, $p < 0.001$; Fig. 5*B*). Further, when examining lever and food cup contacts, ST rats showed main effects of Session ($F_{(4,84)} = 5.229$, $p < 0.001$) and Response ($F_{(1,84)} = 48.689$, $p = 0.003$), and a Session × Response interaction ($F_{(4,84)} = 19.172$, $p < 0.001$), where lever contact was significantly greater than food cup contact on day 2 to day 5 of training (p values < 0.0001; Fig. 5*B*, inset).

Voltammetry studies establish that ST, but not GT, show a transfer of DA transients from the US to CS over Pavlovian training (Flagel et al., 2011; Clark et al., 2013; Saddoris et al., 2016), which is interpreted as a signal supporting the

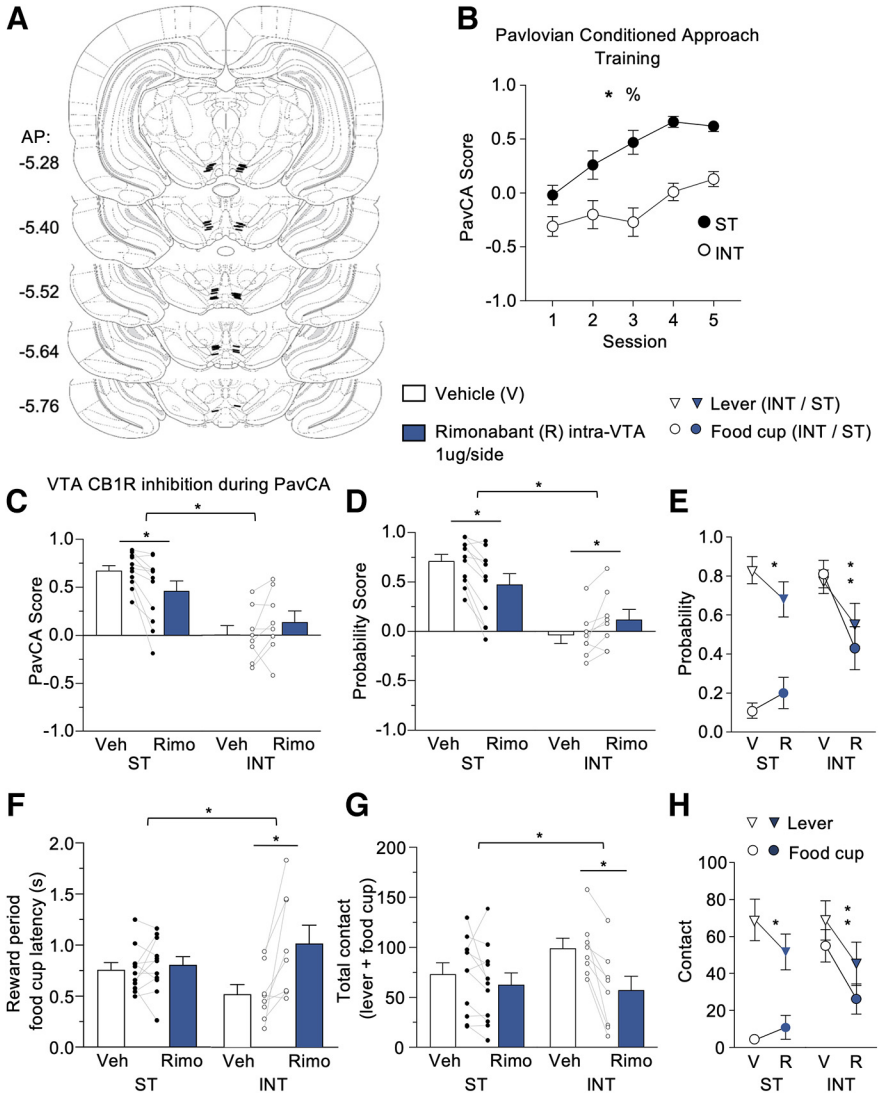

Figure 3. Intra-VTA CB1R receptor inhibition reduces sign tracking. ***A***, Coronal sections (in mm) depicting the location of VTA injector tips for rimonabant infusion. ***B***, The mean ± SEM PavCA scores of ST and INT that acquire individual differences in conditioned responding in PLA task. *Main effect of Session; %Significant Session × Tracking interaction. ***C***, Rimonabant significantly decreases PavCA in ST, but not in INT. *Curved bracket indicates significant Tracking × Treatment interaction; *straight bracket indicates significant effect of treatment. ***D***, ST rats show a significant reduction in the probability score, and INT rats show a significant increase in probability score. *Curved bracket indicates significant Tracking × Treatment interaction; *straight bracket indicates significant effect of Treatment. ***E***, In ST rats, the probability of pressing is significantly reduced, while that of poking is increased. In INT rats, both pressing and poking is significantly reduced. *Significant effect of Treatment. ***F***, Latency to collect the pellet after each trial is not changed in ST rats but is significantly increased in INT rats. ***G***, Total behavior (presses plus pokes) was unaffected by rimonabant in ST rats but was decreased in INT rats. ***H***, In ST rats, the number of lever presses is significantly reduced while poking is increased. In INT rats, both pressing and poking is significantly reduced.

enhanced motivational value of the CS in ST rats. We examined the NAc $GRAB_{DA}$ signal across training (Fig. 5*C*, inset) and found main effects of Session ($F_{(4,68)} = 5.044$, $p = 0.001$) and Epoch (CS, US; $F_{(1,17)} = 36.414$, $p < 0.001$) and a Session × Epoch interaction ($F_{(4,68)} = 19.022$, $p < 0.001$). The CS-evoked NAc $GRAB_{DA}$ signal was greater than US-evoked $GRAB_{DA}$ signal on day 2 to day 5 of training (p values < 0.0007). These data reflect the classic signature of a dopamine response to transfer from the US to the CS across conditioning (Schultz et al., 1997; Day et al., 2007).

We next compared the relationship of CS-evoked and US-evoked $GRAB_{DA}$ signals for each rat across day 1 and day 5. We calculated the peak height difference score (peak height: (CS – US)/CS) on day 1 versus day 5 (Fig. 5*D*). We found that there was a significant increase in this score on day 5 compared with day 1 of training ($t_{(21)} = -5.771$, $p < 0.001$, Cohen's $d = 1.53$), demonstrating that $GRAB_{DA}$ signals to the US were transferred to the CS in ST rats over the course of training.

Because of known differences in dopamine release and dynamics in NAc core versus shell (Zahm, 1999; Cacciapaglia et al., 2012; Saddoris et al., 2013; West and Carelli, 2016), we examine region-specific NAc $GRAB_{DA}$ signals across conditioning (Fig. 5*E*,*F*). We examined the area under the curve between two time epochs [Cue (CS), 5 s post-lever insertion; Reward (US), 5 s post-lever retraction], which reveals the main effect of Epoch (CS > US: $F_{(1,18)} = 12.97$, $p = 0.002$) and an Epoch × Region interaction ($F_{(1,18)} = 12.76$, $p = 0.002$; Fig. 5*G*), which is driven by greater NAc core CS versus US responses ($t_{(11)} = 5.220$, $p < 0.001$, Cohen's $d = 1.26$) and greater US $GRAB_{DA}$ responses in NAc shell than NAc core ($t_{(18)} = 2.184$, $p = 0.042$, Cohen's $d = 0.94$). These results confirm that cue-evoked NAc $GRAB_{DA}$ signal increases across conditioning in sign-tracking rats, consistent with prior reports (Day et al., 2007; Flagel et al., 2011; Clark et al., 2013; Saddoris et al., 2016). We also provide evidence that dopamine is released differentially in the core versus shell during Pavlovian lever autoshaping training.

Intra-VTA rimonabant primarily affects dopamine encoding of outcomes rather than cues

To understand how VTA CB1R inhibition affects DA signals in sign-tracking rats, we measured $GRAB_{DA}$ signals in the NAc in the presence and absence of VTA rimonabant infusions. We used 13 ST rats in our analysis. Nine rats were excluded from test analysis because of off-target cannula placement in the VTA infusion site (n = 4), excessive damage at the VTA infusion site (n = 2), or a loss of signal during testing (n = 3). Similar to our prior behavioral findings, we found that intra-VTA rimonabant significantly decreased the PavCA score of ST rats ($t_{(12)} = 3.476$, $p = 0.005$, Cohen's $d = 0.82$; Fig. 6*A*). The decrease in PavCA was accompanied by similar reductions in the preference, latency, and probability scores (vehicle vs rimonabant, p values = 0.019, 0.005, and 0.002, respectively).

Because of known differences in dopamine release and dynamics in NAc core versus shell in training and consistent with prior reports (Zahm, 1999; Cacciapaglia et al., 2012; Saddoris et al., 2013; West and Carelli, 2016), we include NAc Subregion as a statistical factor in addition to Epoch and Treatment (Rimo, Veh). We first examined the area under the curve during the two epochs and found a significant NAc Subregion × Epoch × Treatment interaction ($F_{(1,11)} = 5.311$, $p = 0.042$; Fig. 6*B*). To

Table 2. Main effects and interactions of all PLA training measures

		Lever						Food cup					
		Contact		Latency		Probability		Contact		Latency		Probability	
Effect	df	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Session	(4,68)	9.649	<0.001	22.185	<0.001	18.602	<0.001	7.644	<0.001	3.610	0.01	4.146	0.005
Tracking	(1,17)	0.763	0.395	0.475	0.500	2.052	0.170	17.49	<0.001	27.433	<0.001	25.23	<0.001
Session × Tracking	(4,68)	1.645	0.173	2.681	0.039	3.673	0.009	9.811	<0.001	9.049	<0.001	11.458	<0.001

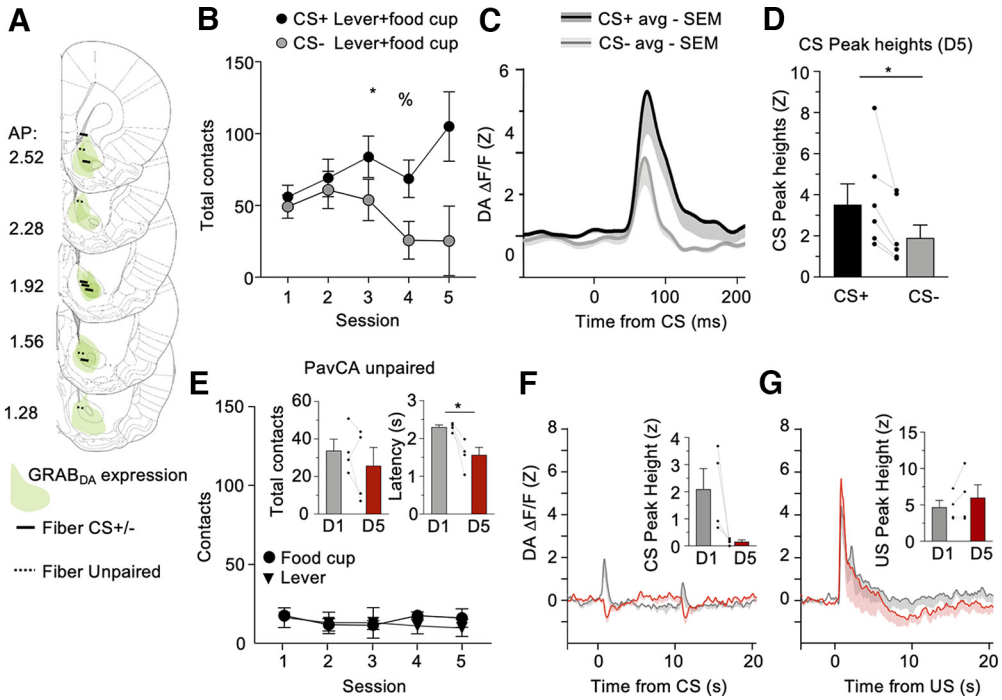

Figure 4. CS-evoked dopamine is specific to the rewarded lever. ***A***, Histologic placements of photometry fibers in the NAc. Two separate groups of rats were used for training in two-lever PLA or Unpaired PLA. Six rats were run in the two-lever program, and their fiber placements are indicated by the solid black line. Four rats were run in the unpaired program, and placements were indicated with a dashed line. ***B***, Rats show behavioral discrimination (presses plus pokes) between the CS+ and CS– lever. *Main effect of Session; %significant Session × Response interaction. ***C***, ***D***, Mean +/– SEM shading of GRABDA signal (DA ΔF/F (Z)). The CS-evoked $GRAB_{DA}$ signal is significantly higher to the rewarded than the unrewarded lever by day 5 of training. *Main effect of Stimulus. ***E***, Rats in the unpaired condition display low amounts of behavior toward both the lever or food cup. Left inset, Total behavior does not increase from day 1 to day 5, suggesting that no conditioned behavior has emerged. Right inset, Latency to collect the pellet is reduced over training, suggesting that rats are still engaged and food motivated during the task. *Main effect of Session. ***F***, $GRAB_{DA}$ responses to the lever on day 1 versus day 5 of training: a CS-evoked excitatory response to the lever is not established. ***G***, $GRAB_{DA}$ signal to pellet delivery remains high across training.

follow up, we analyzed the total $GRAB_{DA}$ signal during the US period and found a main effect of Treatment ($F_{(1,11)} = 7.919$, $p = 0.017$), and a Treatment × Subregion interaction ($F_{(1,11)} = 9.996$, $p = 0.009$). This interaction was driven by increases in $GRAB_{DA}$ signals specifically in the NAc shell during the US reward period ($t_{(5)} = -3.257$, $p = 0.023$, Cohen's $d = 0.66$). We present NAc subregion-specific traces and heatmaps in Figure 6, *C* and *D*.

Consistent with this, US peak heights were also increased with rimonabant treatment (main effect of Treatment: $F_{(1,11)} = 10.228$, $p = 0.008$) and varied by NAc subregion (Treatment × Subregion interaction ($F_{(1,11)} = 5.77$, $p = 0.035$; Fig. 6*E*). This was driven by increases in US peak height in the NAc shell ($t_{(5)} = -2.735$, $p = 0.041$, Cohen's $d = 0.78$). Last, we examined dopamine dynamics in response to the CS. On a population level, the CS peak height was not affected by intra-VTA rimonabant treatment, and we observed a large amount of variability in CS peaks across NAc (Fig. 6*F*). Placements of VTA infusions and NAc recording sites are shown in Figure 6, *G* and *H*.

Discussion

Here, we demonstrate that optogenetic inhibition of VTA-NAc core dopamine projections reduces lever approach, specifically in sign-tracking rats. While intermediate rats also display lever approach, VTA-NAc core DA terminal inhibition did not affect this behavior, nor did it affect the preferred food cup response in goal-tracking rats. Hence, VTA → NAc core dopamine release during the cue period is necessary for the enhanced cue attraction specifically seen in sign-tracking rats. Having confirmed the importance of VTA-NAc core DA projections in ST rats, we next examined whether disrupting VTA CB1R signaling similarly decreased lever approach in sign-tracking rats. We compared the effects of intra-VTA rimonabant injections in ST and intermediate rats because both display the lever-directed approach but differently engage VTA-NAc core. We found that intra-VTA infusions of the CB1R inverse agonist rimonabant resulted in different response profiles between ST and INT rats. In INT rats, decreasing CB1R signaling decreased all appetitive motivated behaviors that we measured. Both lever and food

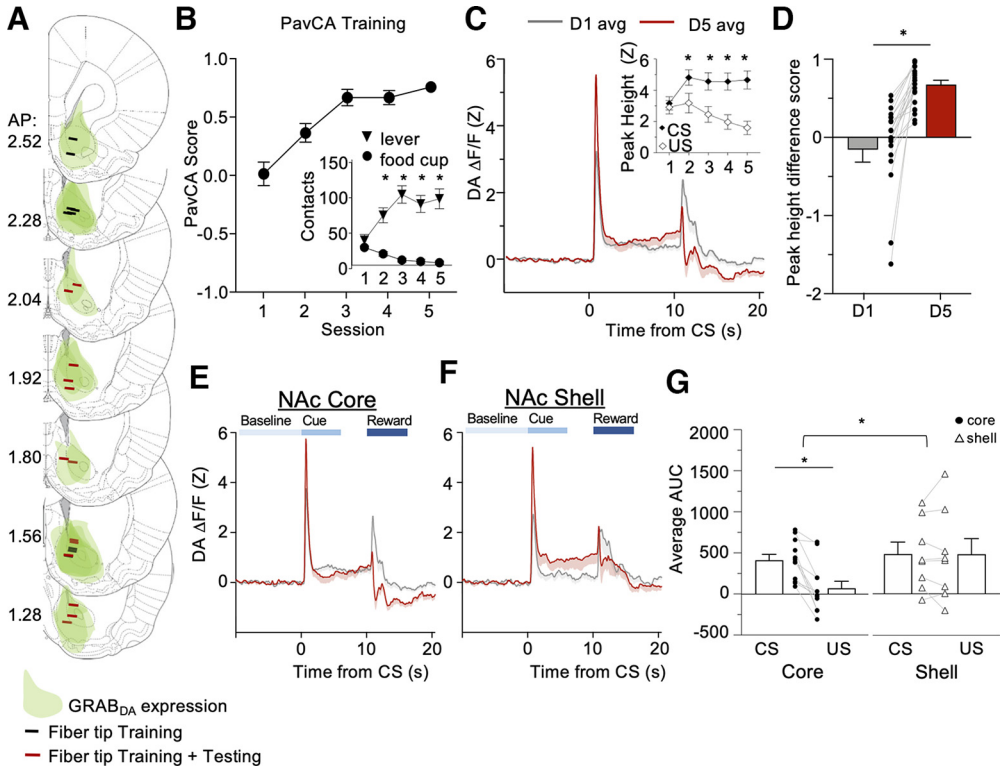

Figure 5. CS-evoked dopamine increases with sign-tracking behavior across Pavlovian training. ***A***, Placements of fiber optics where the GRAB$_{DA}$ signal was recorded. Black and red fiber placements were used in the analysis of training data, whereas only red fibers were used in the analysis of Test data. ***B***, The mean ± SEM PavCA scores of sign-trackers that show a significantly higher number of presses than pokes throughout training (inset). *Significant within-subjects repeated-measures *t* test comparing presses to pokes. ***C***, Mean GRAB$_{DA}$ signal of 22 rats on day 1 versus day 5 of PLA. ***C***, inset, The peak height of CS-evoked dopamine is significantly higher than that for US dopamine on days 2–5 of training. Data from 18 rats; see Materials and Methods. *Significant within-subjects repeated measures *t* test comparing CS versus US peak heights. ***D***, We calculated the peak height difference score [(CS peak height − US peak height)/CS peak height)] for each animal. A positive score indicates that the CS peak is larger than the US peak. There was a significant change in this relationship over training. *Main effect of Session. ***E***, Mean +/− SEM GRAB$_{DA}$ signal of $n = 12$ rats recorded from the NAc core of day 1 (gray) versus day 5 (red) of training. ***F***, Mean +/− SEM GRAB$_{DA}$ signal of $n = 10$ rats recorded from the NAc shell of day 1 (gray) versus day 5 (red) of training. ***G***, Area under the curve (average of day 1 and day 5) analysis showing differences in region (core vs shell) and time epoch (CS vs US) *Curved bracket indicates significant Subregion × Epoch interaction; *straight bracket indicates significant effect of Epoch.

cup responses decreased, and the latency to collect the reward increased. In ST rats, intra-VTA rimonabant selectively reduced lever approach while increasing food cup approach, shifting the rats away from sign tracking (lever directed) and toward goal tracking (food cup behaviors). These results suggest that intact VTA CB1R signaling biases behavior toward the lever and away from the food cup in ST rats. We predicted that the VTA rimonabant-induced decrease in cue attraction would be associated with disrupted cue-triggered dopamine signals downstream in the NAc. To our surprise, we did not observe effects of VTA CB1R inhibition on CS-evoked NAc DA, but rather on US-evoked DA, which was driven by increased US DA signaling in the NAc shell.

We did not observe effects of VTA CB1R inhibition on CS-evoked NAc DA signaling, which contrasts with previous reports demonstrating that CB1 receptor inhibition decreases dopamine release in the NAc as well as cue-motivated behaviors (Cheer et al., 2004; Oleson et al., 2012; Wenzel et al., 2018). Key differences in study approach and design may explain the discrepancy in findings. First, we use fiber photometry to measure fluorescent dopamine sensor signals, while prior studies used voltammetry to measure dopamine concentrations. Another key difference between our study and others is that the present photometry study used exclusively female rats while these prior studies used males. We used female rats for several reasons. First, 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). Second, in our systemic rimonabant study, we observed greater CB1R manipulation effect sizes in females compared with males on the attracting and reinforcing properties of cues (Bacharach et al., 2018). Consistent with these behavioral and pharmacological findings, a recent study shows higher conditioned responding in females compared with males (Lefner et al., 2022). Females in that study showed lower US-evoked NAc DA responses compared with males during Pavlovian conditioning. This latter result has relevance for the present findings, which point toward a role for VTA CB1R regulation of behavioral and dopaminergic cue bias observed in female rats. Future studies designed to probe sex differences would be needed to determine whether the present findings are sex specific.

Here we examine effects of VTA CB1R signaling on NAc dopamine dynamics during Pavlovian behavior, whereas prior work examining such effects used instrumental tasks. Importantly, prior instrumental studies used short, fixed intertrial intervals (10–20 s), which has implications for CB1R receptor involvement in dopamine neuron activity. Dopamine is intimately involved in interval timing (Buhusi and Meck, 2005; Mikhael and Gershman, 2019). Oleson et al. (2012) found that there was larger cue-induced dopamine release in a fixed-interval setting, which was affected more by rimonabant versus a variable interval task. Here we used a Pavlovian lever autoshaping task with a much longer 90 s variable intertrial interval. This long and unpredictable interval between trials may limit our ability to see rimonabant

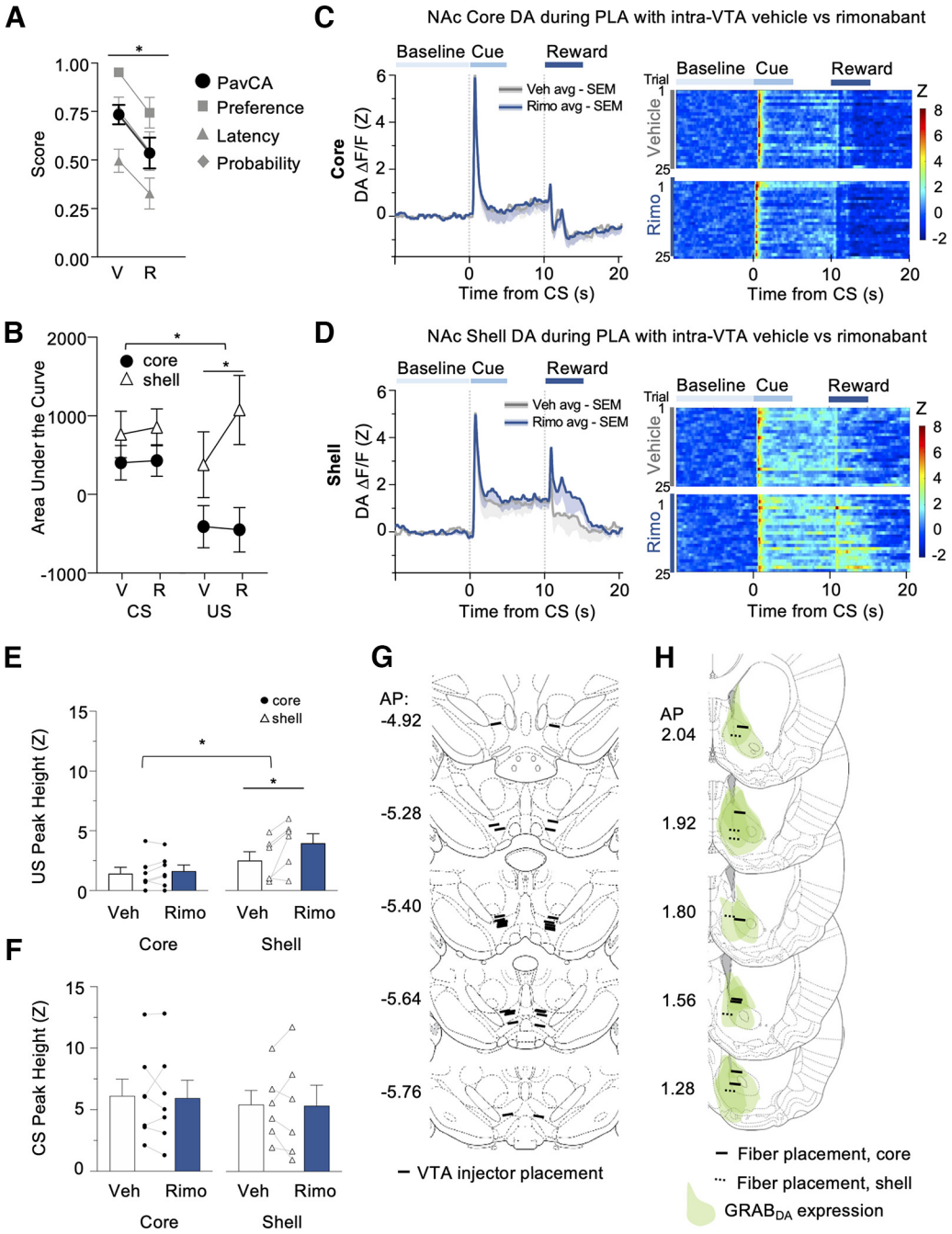

Figure 6. Intra-VTA rimonabant primarily affects dopamine encoding of outcomes rather than cues. ***A***, Rimonabant significantly reduces PavCA scores in ST rats, as well as preference, latency, and probability scores. *Main effect of Session. ***B***, Area under the curve for the 5 s CS period versus the 5 s US period in animals recorded in NAc core versus shell under vehicle or rimonabant conditions. *Curved bracket indicates significant Epoch × Subregion × Treatment interaction; *straight bracket indicates significant effect of Treatment. ***C***, Left, Mean +/− SEM shading of $GRAB_{DA}$ signal in the NAc core in vehicle (gray) versus rimonabant (blue) conditions. Right, Trial by trial heatmap of average $GRAB_{DA}$ signal in NAc core over vehicle and rimonabant conditions. ***D***, Left, Mean +/− SEM shading of the $GRAB_{DA}$ signal in the NAc shell in vehicle (gray) versus rimonabant (blue) conditions. ***D***, Right, Trial-by-trial heatmap of the average $GRAB_{DA}$ signal in NAc shell over vehicle and rimonabant conditions. ***E***, US peak height analysis showing differences in region (core vs shell) and treatment (core vs shell). *Curved bracket indicates significant Subregion × Treatment interaction; *straight bracket indicates significant effect of Treatment. ***F***, Rimonabant does not change the CS-induced peak height in core or shell. ***G***, Site of injector tip placement in the VTA for rimonabant infusion. ***H***, $GRAB_{DA}$ expression and photometry recording location in NAc. Solid lines indicate recording location for NAc core and dotted lines indicate the location of NAc shell fibers.

effects on CS-evoked NAc DA. Future studies would be necessary for determining whether differences in CB1R inhibition of CS-evoked DA are because of Pavlovian versus instrumental task parameters or intertrial interval variations.

We found evidence that manipulating VTA CB1R signaling influences dopamine release during the US/reward period, which varies by NAc subregion. There are anatomical and functional differences between the core and shell (Jones et al., 1996; Zahm, 1999). In cued-instrumental and Pavlovian settings, NAc core dopamine release is associated with the predictive value of a cue (Roitman et al., 2004; Cacciapaglia et al., 2012; Hart et al., 2014; Saddoris et al., 2015; Stelly et al., 2021). In addition to the predictive value of this dopamine signal, core dopamine also carries incentive motivational value (Berridge, 1996; Flagel et al., 2011;

Saunders and Robinson, 2012; Saunders et al., 2013). The NAc shell DA is more heavily involved in representing contextual elements associated with reward seeking, tracking reinforcer value, and representing reward-guided motivation (Bossert et al., 2007, 2012; Cacciapaglia et al., 2012; Cruz et al., 2014; West and Carelli, 2016; Valyear et al., 2020). Additionally, NAc shell dopamine release is less temporally restricted to cue onset, and differential dopamine dynamics are observed during cue onset, the entire cue period, and the US period (Saddoris et al., 2015, 2016). We find that VTA CB1R blockade increases NAc shell dopamine signaling during the US period and is associated with increased food cup exploration, which may give rise to changes in US processing and/or reinforcer value representation.

Our results using VTA CB1R inhibition during Pavlovian conditioning are consistent with a substantial instrumental literature showing that CB1R signaling maintains both cued food-seeking and drug-seeking behaviors (McLaughlin et al., 2003; De Vries and Schoffelmeer, 2005; Ward and Dykstra, 2005; Economidou et al., 2006; Salamone et al., 2007; Ward et al., 2007; Justinova et al., 2008; de Bruin et al., 2011; Oleson et al., 2012; Schindler et al., 2016). Consistently, sign-trackers and intermediates show decreases in lever approach when we disrupt VTA CB1R signaling. However, in intermediate rats this attenuating effect was not specific to the lever, as food cup responding and task engagement were generally reduced, consistent with prior rimonabant manipulations (McLaughlin et al., 2003; Ward and Dykstra, 2005; McLaughlin et al., 2006; Salamone et al., 2007; Ward et al., 2007; Oleson et al., 2012). However, this was not the case in sign-trackers that decreased lever approach but showed no differences in overall task engagement and tended to increase food cup responding when VTA CB1R signaling was disrupted. Thus, blocking VTA CB1R signaling rebalanced lever-directed and food cup-directed behavior, such that a decrease in lever approach was compensated for by an increase in food cup approach to result in a significant decrease in the PavCA score. The eCB system is also involved in maintaining Pavlovian cue–reward associations. Systemic blockade of CB1R signaling disrupts cue-driven approach behaviors in Pavlovian tasks (Bacharach et al., 2018; Gheidi et al., 2020). The present findings using region-specific manipulation of CB1R provide evidence that the VTA is a site of endocannabinoid action to promote sign tracking and confirm our prior work showing that systemic rimonabant dose-dependently reduces sign tracking and the attracting and reinforcing properties of the lever cue (Bacharach et al., 2018).

Our photometry and optogenetic data from the NAc core corroborate these findings in that NAc core is necessary for cue approach in sign-trackers, and we see strong cue-induced dopamine under both vehicle and rimonabant condition. Prior pharmacology and voltammetry tracking studies have established NAc core DA as necessary for sign tracking (Flagel et al., 2011; Saunders and Robinson, 2012; Clark et al., 2013; Fraser and Janak, 2017). Consistent with manipulations of NAc dopamine receptors, we show that optogenetic inhibition of VTA-NAc core dopamine projections during lever presentation similarly reduces lever approach in ST rats, but not in intermediate rats that also display lever approach.

We find that both NAc core DA inhibition and VTA CB1R inhibition decrease sign tracking. We observe consistent effects of VTA CB1R inhibition on PavCA scores across our pharmacology experiment (Fig. 3) when rats are untethered, and our combined pharmacology and photometry experiment (Fig. 6) when rats are tethered. This suggests that this technical difference of tethering rats, which does moderately slow responding, did not interfere with VTA treatment effects on behavior. We do not observe NAc region-specific differences in effects of VTA CB1R inhibition on cue-evoked DA signaling, but we do observe differences in CS relative to US DA signaling NAc shell.

Together, our results suggest that both VTA CB1R signaling and NAc core DA support the maximal expression of sign-tracking behavior. Disrupting VTA CB1R signaling rebalances behavior away from sign tracking and toward goal tracking. Further, the intra-VTA rimonabant-induced shift in behavior of ST rats is related to increases in US-evoked NAc shell dopamine signaling, indicating that VTA CB1R receptors are involved in maintaining NAc representations of the CS–US relationship. Our results suggest that CB1R signaling in the VTA maintains the conditioning-dependent behavioral and NAc DA bias toward cues relative to outcomes in sign-tracking rats, potentially by downregulating reward-related NAc shell DA signaling. Future causal role studies are needed to test the necessity of NAc shell DA for mediating the balance between CS-directed and US-directed behaviors in lever autoshaping.

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