

Endocannabinoids facilitate reward engagement through retrograde gain control

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Neuromodulatory signalling is poised to serve as a neural mechanism for gain control, acting as a crucial tuning factor to influence neuronal activity by dynamically shaping excitatory and inhibitory fast neurotransmission. The endocannabinoid (eCB) signalling system, the most widely expressed neuromodulatory system in the mammalian brain, has been demonstrated to filter excitatory and inhibitory inputs through retrograde, presynaptic action in vitro and ex vivo^{1–9}. However, whether eCBs exert retrograde gain control to ultimately facilitate motivated behaviours in freely moving mammals has not been established. Here, using a suite of in vivo physiological, imaging, genetic and machine learning-based approaches, we uncover a fundamental role for the dynamic release of eCBs in controlling behavioural engagement during reward seeking through a genetically and anatomically defined thalamostriatal circuit.

Reward-seeking behaviours are evolutionarily conserved and necessary for survival, and are disrupted in both substance-use disorder and numerous psychiatric diseases^{10–14}. The nucleus accumbens (NAc) is a key nodal mesocorticolimbic structure that mediates action selection as well as reward seeking^{15–19}. The NAc incorporates input from numerous excitatory, inhibitory and neuromodulatory projections to ultimately integrate affective information and translate this information into motivated behavioural outputs^{20–22}. However, we lack complete understanding of critical fundamental mechanisms that govern reward seeking, specifically how diverse sources of excitatory input to the NAc are integrated and filtered by distinct neuromodulators to shape discrete behavioural outputs. For example, the NAc receives glutamatergic input from over half a dozen brain structures, yet it is unclear why such diverse sources of this single neurotransmitter exist and how each input is ultimately filtered in real time into useful information downstream by local neuromodulatory signals^{23–25}.

Brain slice electrophysiology studies have shown that the eCB signalling system dynamically tunes both excitatory and modulatory input into the NAc^{26–30}. In the central nervous system, this signalling system mainly involves activation of cannabinoid 1 receptors (CB₁R) by the eCBs anandamide and 2-arachidonylglycerol (2-AG), and represents the most widely expressed neuromodulatory system in the mammalian brain^{1–5}. Unlike conventional neurotransmitters released by presynaptic vesicular mechanisms, lipophilic eCBs are produced on-demand by postsynaptic neurons and primarily function retrogradely to activate presynaptic CB₁Rs and attenuate neurotransmitter release^{6–9}. However, the lipophilic nature of these modulators, as well as the diverse neuronal subtypes and specific stimuli that drive their production, have made the investigation of their in vivo function in motivated behaviours intractable. Furthermore, the mechanistic link

between neural activity-dependent eCB release, modulation of afferent input and how specific behavioural features are shaped by this retrograde action in vivo remains to be established.

Here we discovered specific behavioural parameters necessary to evoke transient eCB release in the NAc in vivo during freely moving motivated behaviour. Using a complementary suite of approaches, our results indicate that eCB production and release from a genetically defined medium spiny NAc neuron population is specifically time locked to behavioural engagement, and that these eCBs drive reward seeking through retrograde action at presynaptic terminals arising from the anterior paraventricular thalamus (aPVT), a key nodal structure for the regulation of motivated behaviours^{31,32}. This dynamic and fundamental retrograde signalling mechanism acting through CB₁Rs dynamically shapes the vigour of engagement in reward-seeking behaviours through its discrete suppression of efferent glutamatergic input into the NAc.

eCBs are released in the NAc during reward consumption

To determine how eCB dynamics in the NAc facilitate reward seeking and consumption, we injected the medial NAc shell of adult mice with an AAV (AAV1-hSyn-GRAB_{eCB2.0}) to drive the neuronal expression of the fluorescent eCB biosensor, GRAB_{eCB2.0}, and implanted photometry fibres dorsal to the injection site³³ (Fig. 1a and Extended Data Fig. 1a,b). Mice were then placed in a Pavlovian reward-conditioning assay and trained to associate a cue light with the protrusion of a sipper containing a 10% sucrose solution across 5 days. On day 1 of training, consumption of the sucrose solution elicited a pronounced increase in the GRAB_{eCB2.0} signal, indicative of local eCB release time locked to consummatory events (Fig. 1b). However, on day 5 of conditioning, the onset of the

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increase in the GRAB_{eCB2.0} signal shifted earlier to the time of cue onset, and rose to approximately 50% of the maximal response before sipper delivery. This suggests that eCB release is not solely tied to reward consumption, but may sculpt reward-seeking behaviours or be involved in cue encoding (Fig. 1c–e). Accordingly, in a reward-omission test in which the sipper was not delivered on 50% of cue trials, the GRAB_{eCB2.0} signal still increased despite a lack of reward delivery (Extended Data Fig. 1r–v). Blocking 2-AG biosynthesis with the diacylglycerol lipase inhibitor DO34 strongly attenuated the reward-induced increase in the GRAB_{eCB2.0} signal, whereas inhibition of anandamide hydrolysis had no effect on the signal, suggesting that 2-AG is mediating this response (Fig. 1f–i and Extended Data Fig. 1c,e,g–i,l). To determine whether eCB release in the NAc is either selective for stimulus valence or unique to reward-seeking behaviour, we exposed the same cohorts of mice to a Pavlovian fear-conditioning assay. We found that shock exposure also elicited an increase in the GRAB_{eCB2.0} signal, showing that eCB production in the NAc is similarly elicited by aversive stimuli (Extended Data Fig. 1w,x,z). However, during fear retrieval (extinction day 1), exposure to the shock-predictive tone did not elicit such response (Extended Data Fig. 1y,aa). Together, these results indicate that both rewarding and aversive stimuli can elicit robust eCB release within the NAc (Extended Data Fig. 1bb).

One major function of 2-AG activation of presynaptic CB₁R is to decrease the probability of fast transmitter release, thus we next identified neuronal projections to the NAc that express CB₁R (Fig. 1j,k). We injected a retrograde virus to drive the expression of Cre recombinase (AAV2-retro-hSyn-Cre) into the NAc of flox-stop Ai14 Td-Tomato reporter mice, and identified five principal sources of excitatory inputs: the medial prefrontal cortex, aPVT, posterior PVT (pPVT), basolateral amygdala (BLA) and ventral hippocampus (Fig. 1l,n and Extended Data Fig. 2a–c). We then used a semi-quantitative *in situ* hybridization approach, RNAscope, to examine the colocalization of *cre* mRNA transcript derived from our retrograde viral approach (NAc projectors) and *Cn1r* mRNA transcript overlap. Overall, the aPVT had the largest percentage of neurons projecting to the NAc, as well as the largest percentage of neurons projecting to the NAc that co-expressed CB₁R (Fig. 1l,n and Extended Data Fig. 2a–c).

Recent studies have emphasized that functional differences exist along the anteroposterior axis of the PVT, yet few genetic markers have been identified to isolate and/or manipulate neurons located in the aPVT^{24,25,34–38}. We analysed the Allen Brain Atlas database and published PVT RNA sequencing results and found that the expression of neuropeptide neurotensin (NTS) is selectively enriched in the aPVT compared with the pPVT³⁹. We corroborated these initial findings with RNAscope, confirming selective expression of NTS in the aPVT. We also found that in the aPVT, NTS neurons have strong co-expression of CB₁R (83.9 ± 5% overlap; Fig. 1n,o). Using channelrhodopsin-2 (ChR2)-assisted circuit mapping⁴⁰, we found that these neurons send strong excitatory projections to the NAc as well as the bed nucleus of the stria terminalis, central amygdala and zona incerta (Extended Data Fig. 2p–bb).

To determine how aPVT input to the NAc is regulated by CB₁R signalling, we performed whole-cell patch-clamp electrophysiological recordings. NTS-Cre mice were crossed with a D1R–tdTomato reporter mice, and their progeny were injected with AAV5-DIO-ChR2-eYFP in the aPVT to facilitate recordings of optically evoked excitatory postsynaptic currents from either D1R⁺ or D1R[−] neurons (the latter being purportedly D2R⁺; see Methods; Extended Data Fig. 2l–o) in the medial NAc shell (Fig. 1p). Whole-cell recordings of optically evoked input–output curves demonstrated that there is a significant bias in excitatory input onto D1R⁺ neurons compared with D1R[−] (Extended Data Fig. 2d). SR141716 (10 μM) inverted this bias, resulting in a larger excitatory response observed in D1R[−] than D1R⁺, suggesting tonic eCB shunting of excitatory input onto D1R[−] neurons (Extended Data Fig. 2e). In agreement with the involvement of 2-AG in this response, the intrinsic excitatory bias onto D1R⁺ neurons was eliminated by augmenting 2-AG levels by inhibiting

its degradation by monoacylglycerol lipase with its selective inhibitor, JZ184 (1 μM; Extended Data Fig. 2f,h–k). To determine whether phasic 2-AG signalling is involved, we recorded depolarization-induced suppression of excitation (DSE), a form of short-term synaptic plasticity known to be mediated by retrograde 2-AG signalling^{6,9}. We found that DSE was selectively expressed at aPVT–NAc D1R[−] synapses (Fig. 1q,r). These results indicate that both tonic and phasic 2-AG signalling are primarily expressed at aPVT–NAc D1R[−] (putatively D2R⁺) synapses, and that tonic eCB signalling at these synapses drives the observed excitatory bias onto D1R⁺ neurons (Fig. 1s). Together, these *in vivo* and *in vitro* results demonstrate that salient rewarding and aversive stimuli both evoke robust 2-AG release, which acts to modulate extrinsic excitatory input to the NAc in a retrograde CB₁R-dependent manner.

aPVT–NAc activity is negatively correlated with reward seeking

To discern whether eCB signalling facilitates reward-seeking behaviours through modulation of extrinsic excitatory input, we first determined the behavioural role of the aPVT–NAc circuit. We injected NTS-Cre mice with AAV-DJ-GCaMP6s in the aPVT followed by fibre-optic implantation in the medial NAc shell to measure calcium dynamics of aPVT^{NTS}–NAc terminals during Pavlovian reward conditioning. We observed significant differences in both the GCaMP6s signal and the reward-seeking behaviours across conditioning as mice learned task parameters (Fig. 2a–j and Extended Data Fig. 3a–e). Using 1,080-px 20-Hz videos of behaviour, we quantified reward seeking as the percentage of time poking, pawing or sniffing at the sipper port (Methods; Fig. 2d and Supplementary Videos 1–4). On day 1 of conditioning, mice only spent roughly one-quarter of their time (24.1%) engaging with the sipper port during the cue window before sipper protrusion (0–6 s), as they had not yet learned to associate the cue with the rewarding outcome. However, by day 5 of conditioning, mice spent two-fifths (38.6%) of the time engaging with the sipper port during the cue window, in a display of learned expectation of reward delivery (Fig. 2d,f–g). Conversely, on day 1 of conditioning in the post-sipper time window (26–46 s), mice spent almost one-third (31.8%) of the time engaging with the sipper port following sipper retraction, as they had not yet learned the associated time-out period (inter-trial interval, ITI) following sipper retraction. However, by day 5 of conditioning, this behaviour had significantly diminished to less than one-fifth (19.0%) of the time, as the mouse learned that the sipper will not be extended again until the next cue delivery epoch (Fig. 2d,f,g and Supplementary Video 2). Calcium transients were isolated during these key epochs including in the cue period before sipper protrusion (0–6 s) and in the period immediately following cue cessation and sipper retraction (26–46 s). In the pre-sipper time window (0–6 s), the excitation observed on day 1 transitioned to inhibition on day 5, and vice versa in the post-sipper time window (26–46 s; Fig. 2e). We found that whether quantifying either the pre-sipper or post-sipper time window, the activity of the aPVT^{NTS}–NAc presynaptic terminals was significantly and negatively correlated with an animal's relative epochs of sipper port engagement (Fig. 2f–j). This result indicates that the activity of these projections could be signalling engagement and disengagement from reward seeking.

A control reward-omission experiment demonstrated that this inhibition was not dependent on reward consumption, as significant inhibition of these presynaptic terminals was also observed during omission trials (Extended Data Fig. 3l–o). Furthermore, analysis of ignored cue trials on day 5 of conditioning demonstrated a lack of inhibitory effects, suggesting that the inhibition is probably not being driven by attribution of incentive salience to the cue (Extended Data Fig. 3p). Finally, to determine whether aPVT^{NTS}–NAc presynaptic activity was simply reflective of locomotion (inhibited during decrease, excited during increase), we found that in a fixed ratio 1 (FR1) and progressive ratio (PR)

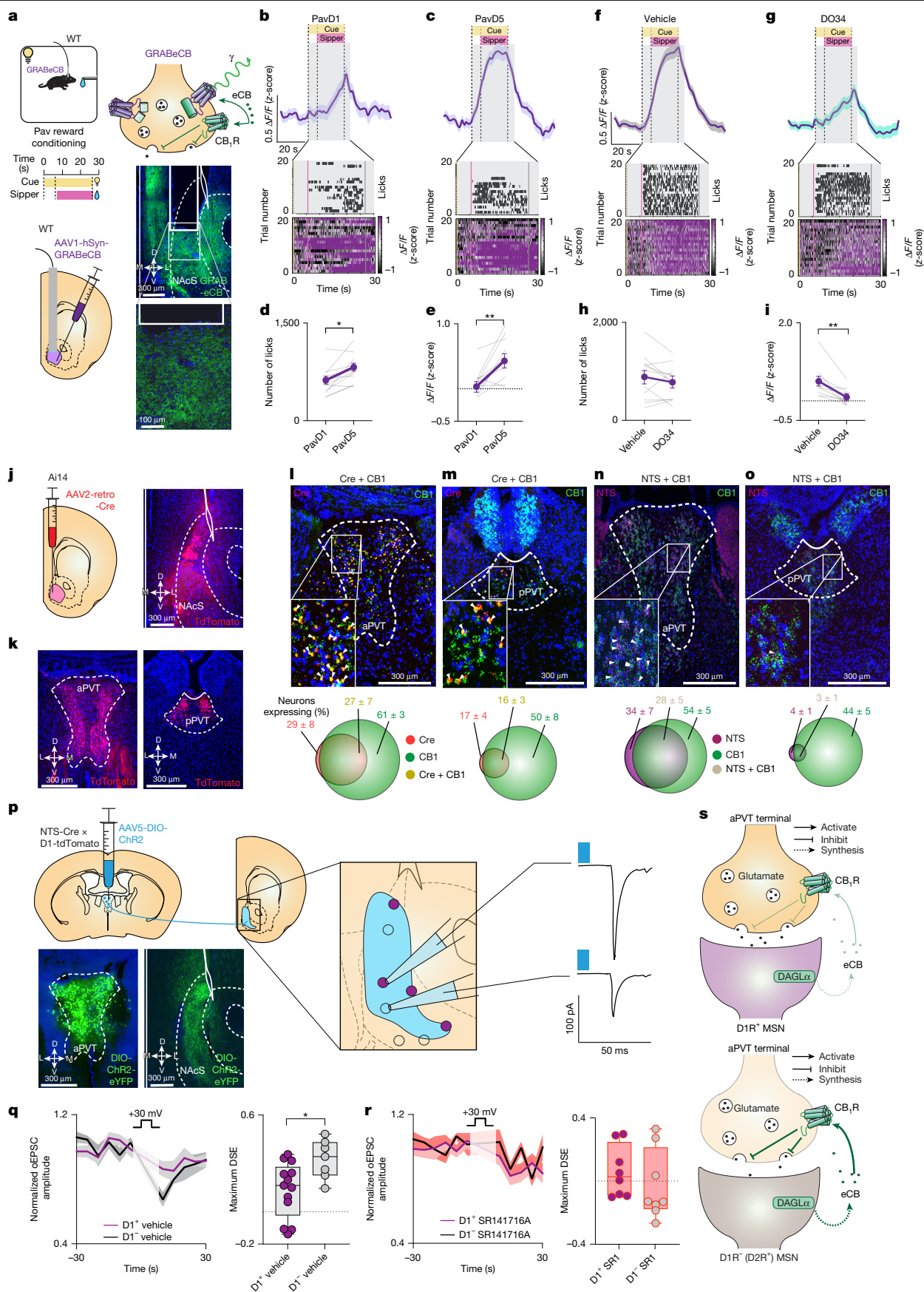


Fig. 1 | See next page for caption.

Fig. 1 | eCBs are released in the NAc during reward consumption and inhibit glutamate release from aPVT terminals. **a**, Schematic of fibre photometry recordings of GRAB_{eCB2.0} in the NAc during Pavlovian (Pav) reward conditioning. D, dorsal; L, lateral; M, medial; V, ventral; WT, wild type. **b,c**, Photometry trace with representative lick raster and heatmap of the GRAB_{eCB2.0} signal aligned to cue onset on day 1 (D1; **b**) and day 5 (D5; **c**) of conditioning. **d,e**, Comparison of the number of licks per session ($P = 0.0468$; **d**) and the average GRAB_{eCB2.0} signal (**e**) from 0 to 26 s ($P = 0.0027$) between day 1 and day 5 ($N = 11$). **f,g**, Trace with representative lick raster and heatmap of the GRAB_{eCB2.0} signal aligned to cue onset after treatment with vehicle (**f**) or DO34 (**g**). **h,i**, Comparison of the number of licks per session ($P = 0.2514$; **h**) and average photometry z-score (**i**) from 0 to 26 s ($P = 0.0011$) between vehicle and DO34 ($N = 11$). **j**, Schematic of retrograde-tracing and RNAscope studies in Ai14 mice. NAcS, NAc shell. **k**, Epifluorescent images of TdTomato-positive NAc-projecting neurons in the aPVT and pPVT. **l,m**, RNAscope image and quantification of *cre* and *Cbl1r*

transcript levels in the aPVT (**l**) and pPVT (**m**) ($N = 4$ and 3 , respectively). **n,o**, RNAscope image and quantification of *Nts* and *Cbl1r* transcript levels in the aPVT (**n**) and pPVT (**o**) ($N = 3$ and 3 , respectively). Arrowheads denote transcript colocalization. **p**, Schematic for whole-cell voltage-clamp recordings of optically evoked excitatory postsynaptic currents from NAc D1R⁺ and D1R⁻ neurons. **q,r**, Recordings of depolarization-induced suppression of excitation in vehicle-treated (**q**; D1R⁺ $n = 13$ and D1R⁻ $n = 7$; $P = 0.0184$) and SR141716A-treated (**r**; D1R⁺ $n = 8$ and D1R⁻ $n = 8$; $P = 0.3763$) slices. The horizontal dotted lines indicate zero. **s**, Schematic depicting enhanced retrograde eCB signalling at aPVT^{NTS}-NAc D1⁻ synapses compared with aPVT^{NTS}-NAc D1⁺ synapses. The error bars represent $\pm$ s.e.m. (**d–i**) or minimum to maximum values (**q,r**); the boxplots represent the interquartile range with the line at median; N represents the number of mice and n represents the number of neurons. P values are reported from a two-tailed Student's paired t -test (**d,e,h,i**) and two-tailed unpaired t -test (**q,r**); * $P < 0.05$ and ** $P < 0.01$.

operant task, aPVT^{NTS}-NAc presynaptic activity is still inhibited during reward-seeking behaviours that require an active motor sequence (nose poke; Extended Data Fig. 3q–u). This result is further corroborated by evidence collected in open-field activity, in which we observed no calcium dynamics that time locked to locomotor initiation events or rearing bouts (Extended Data Fig. 3v–x).

To determine whether the activity of aPVT^{NTS}-NAc terminals is only coincident with engagement in reward-seeking behaviour or conserved for other behaviours, we trained mice in an aversive Pavlovian fear-conditioning and fear-extinction task (Fig. 2k and Extended Data Fig. 4a–g). During fear conditioning, we observed a transient (approximately 5 s) peak in the GCaMP6s signal time locked to foot shock (Extended Data Fig. 4c,d). On day 1 of fear extinction (recall), we measured an inhibition of aPVT^{NTS}-NAc terminals, similar to what we observed during engagement in reward-seeking behaviours, suggesting that the aPVT^{NTS}-NAc pathway does not encode stimulus valence (Fig. 2l). When the GCaMP6s signal was aligned to the end of freezing bouts (disengagement) on extinction day 1, there was also a concomitant increase in fluorescence (Extended Data Fig. 4e,f). As mice underwent extinction training, the inhibitory signal broadly decreased, and their degree of engagement in defensive freezing behaviours was likewise negatively correlated with the activity of aPVT^{NTS}-NAc presynaptic terminals (Fig. 2l–o and Extended Data Fig. 4g). Collectively, these results indicate that aPVT^{NTS}-NAc activity is negatively correlated with behavioural engagement in both reward seeking and defensive freezing.

We then sought to establish whether there is a causal role for aPVT^{NTS}-NAc in behavioural engagement by selectively bidirectionally photomanipulating neuronal activity of the projections (Fig. 2p) during discrete epochs of reward seeking. Mice were injected with either AAV5-DIO-ChR2-eYFP, AAV5-DIO-PPO-eYFP (an inhibitory G_i-coupled opsin) or AAV5-DIO-YFP as control, in the aPVT with bilateral fibre-optic implants placed above the medial NAc shell. Following Pavlovian reward conditioning, mice injected with ChR2 or eYFP received counterbalanced days of closed-loop photo-stimulation, in which every lick resulted in a 2-s burst of 20-Hz blue-light photo-stimulation delivered to aPVT^{NTS}-NAc terminals. Stimulation of aPVT^{NTS}-NAc terminals reduced licking behaviour, either using closed-loop photo-stimulation (Fig. 2q) or constant 20-Hz photo-stimulation throughout the duration of the cue (Extended Data Fig. 5a,b). Conversely, 10-Hz photo-inhibition of aPVT terminals (PPO) from 5 s before sipper retraction to 20 s after increased engagement with the sipper port during this epoch (Fig. 2r). To test whether activation of the aPVT^{NTS}-NAc is necessary and sufficient for conditioned freezing behaviour, we either selectively photo-activated or inhibited aPVT^{NTS}-NAc terminals using ChR2 or PPO, respectively, throughout the cue period (20 Hz 20 s and 10 Hz 20 s, respectively) during fear retrieval. Photo-activation of the aPVT terminals was sufficient to reduce conditioned freezing, whereas photo-inhibition resulted in a trend towards increased freezing, probably due to a ceiling effect on the expression of freezing behaviour during recall (Extended Data Fig. 4h,i).

Neither activation nor inhibition of the aPVT^{NTS}-NAc pathway was innately reinforcing or aversive having no effect in either intracranial self-stimulation of real-time place preference (Extended Data Fig. 5c–h). Overall, these findings establish the causal role of aPVT^{NTS}-NAc projections in dynamic tuning of behavioural engagement in both appetitive and aversive behaviours (Fig. 2s).

Retrograde eCBs facilitate engagement in reward seeking

Our electrophysiology results established that 2-AG inhibits aPVT^{NTS}-NAc terminals by activating CB₁R, and our in vivo calcium recordings indicate broad presynaptic inhibition of aPVT^{NTS}-NAc terminals during epochs of behavioural engagement. Thus, we next determined whether this in vivo inhibition was mediated by endogenous release of 2-AG acting at CB₁R. Using mice expressing a DIO-GCaMP6s in aPVT^{NTS}-NAc terminals, we found that the CB₁R antagonist SR141716 (10 mg kg⁻¹; intraperitoneal) robustly reduced the inhibition of these terminals in vivo, as well as significantly reduced sucrose consumption events (Extended Data Fig. 6a–d).

We subsequently used a Cre-dependent CRISPR–Cas9 viral approach to selectively delete the gene encoding CB₁R from aPVT neurons to establish whether 2-AG–CB₁R inhibition of aPVT^{NTS}-NAc terminals was necessary for engagement in reward-seeking behaviours^{41,42} (Fig. 3a and Extended Data Fig. 6b–n). Fluorescent in situ hybridization showed that the sgRNA targeting *Cnr1* (sg*Cnr1*) virus elicited an approximately 47% reduction in the number of aPVT neurons co-expressing NTS and CB₁R (that is, 30.4% versus 16.2%) compared with sgRNA targeting *Rosa26* (sg*Rosa*) controls (Extended Data Fig. 6f–n). To assess how deletion of the gene encoding aPVT CB₁R influenced aPVT^{NTS}-NAc axon terminal activity, as well as engagement in reward-seeking behaviours, we co-injected these viruses (*Rosa*-targeting or *Cnr1*-targeting sgRNA) with AAV5-DIO-GCaMP6s in the aPVT, and implanted a fibre optic above the medial NAc shell (Fig. 3a and Extended Data Fig. 7a–h). Although deletion of the gene encoding CB₁R in aPVT^{NTS}-NAc neurons did not alter the total number of licks during ad libitum access (Extended Data Fig. 7f), it significantly reduced the length and timing of licking bouts (Extended Data Fig. 7g). This result suggests that 2-AG–CB₁R signalling at aPVT^{NTS}-NAc terminals modulates the innate temporal patterns of reward engagement, without impacting satiety-related behavioural measures.

We next examined the mechanism mediating reward-engagement-induced inhibition of aPVT^{NTS}-NAc axon terminals as well as the behavioural consequences of CB₁R removal from aPVT neurons. We treated GCaMP6s and sg*Cnr1*-expressing mice or GCaMP6s and sg*Rosa*-expressing mice with the diacylglycerol lipase inhibitor (to block 2-AG production), DO34 (20 mg kg⁻¹) or a vehicle control. In vehicle-treated animals, deletion of the gene encoding CB₁R from aPVT neurons significantly attenuated the engagement-induced inhibition of GCaMP6s fluorescence,

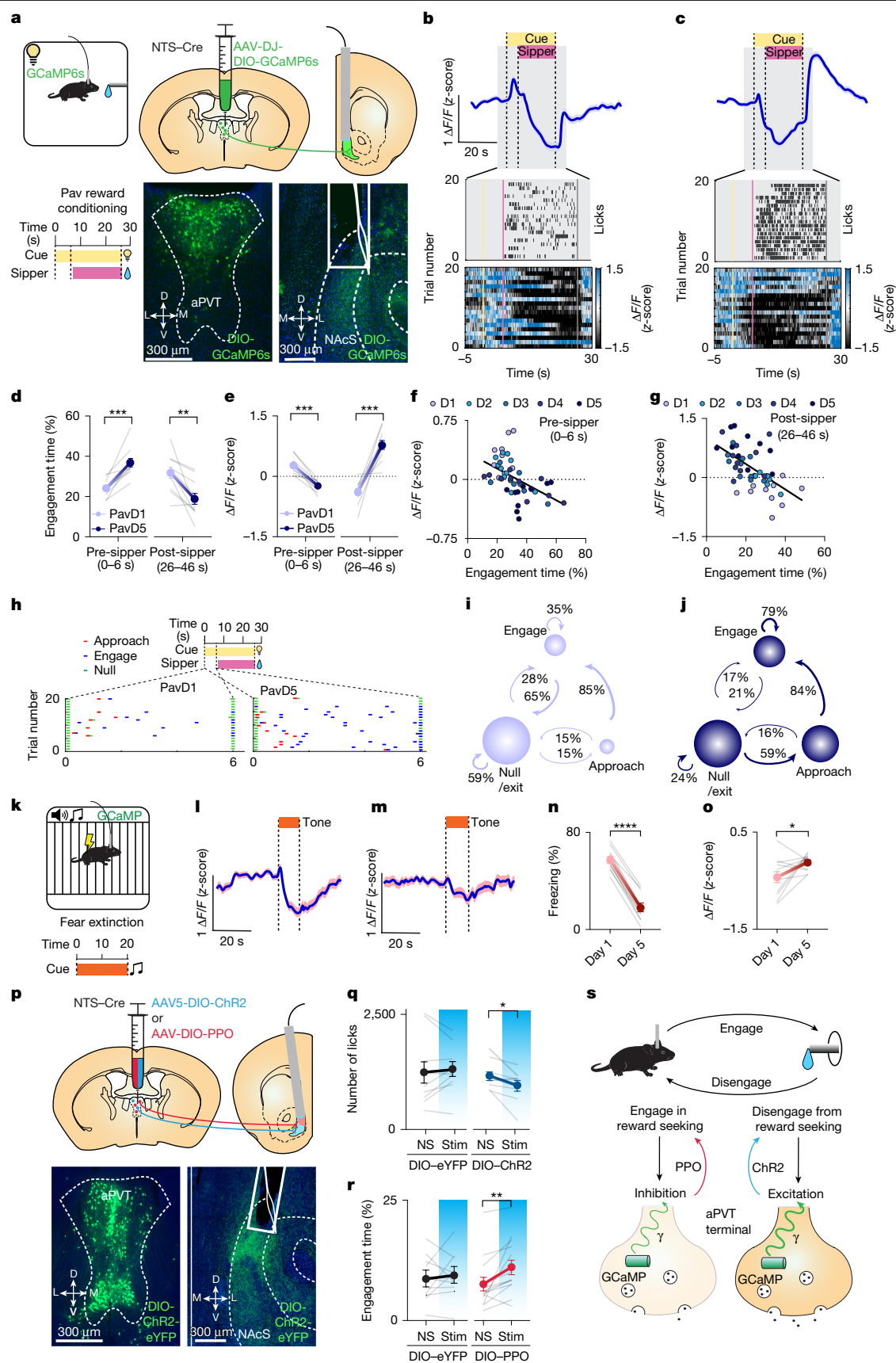


Fig. 2 | See next page for caption.

Fig. 2 | aPVT–NAC presynaptic activity is negatively correlated with engagement in reward-seeking behaviours. **a**, Schematic of fibre photometry recordings of GCaMP6s-expressing aPVT^{NTS}–Nac terminals. **b,c**, Photometry trace with representative lick raster and heatmap of the cue-aligned GCaMP6s signal during day 1 (**b**) and day 5 (**c**) of Pavlovian reward conditioning ($N = 10$). **d**, Comparison between day 1 and day 5 of sipper port engagement time during the pre-sipper ($P = 0.0002$) and post-sipper ($P = 0.0039$) time window ($N = 10$). **e**, Comparison between day 1 and day 5 of the average GCaMP6s signal during pre-sipper (0–6 s, $P = 0.0001$) and post-sipper (26–46 s, $P = 0.0002$) time window ($N = 10$). **f**, Correlation between pre-sipper extension engagement time and average photometry z-score across 5 days of conditioning ($R^2 = 0.2553$, $P = 0.0002$, $N = 10$). **g**, Correlation between post-sipper retraction engagement time and the average GCaMP6s signal across 5 days of conditioning ($R^2 = 0.3299$, $P = 1.59 \times 10^{-5}$, $N = 10$). The horizontal dotted lines indicate zero. **h**, Raster plot showing timepoints of initiation in the sipper approach, sipper engagement and null behaviours during the cue period before sipper extension. **i,j**, Transition diagrams showing probabilities of transitions between approach, engage and null/exit behaviours on day 1 (**i**) and day 5 (**j**) of conditioning during the

pre-sipper time window, with the size of circles corresponding to the total number of events. **k**, Schematic of the Pavlovian fear conditioning or extinction. **l,m**, Photometry trace of the GCaMP6s signal during fear extinction on day 1 (**l**) and day 5 (**m**). **n**, Quantification of cue-evoked freezing on day 1 and day 5 of extinction ($P = 3.45 \times 10^{-8}$, $N = 11$). **o**, Quantification of the GCaMP6s signal on day 1 and day 5 of extinction ($P = 0.0318$, $N = 11$). **p**, Schematic of optogenetic manipulation of aPVT^{NTS}–Nac terminals using DIO–ChR2 and DIO–PPO. **q**, Number of licks during no stimulation (NS) or closed-loop 20-Hz optogenetic stimulation (stim) of aPVT^{NTS}–Nac terminals in DIO–ChR2-expressing ($P = 0.0300$, $N = 9$) and DIO–eYFP-expressing ($P = 0.6323$, $N = 9$) animals. **r**, Number of licks during no stimulation or sipper retraction-aligned 10-Hz optogenetic inhibition of aPVT^{NTS}–Nac terminals in DIO–PPO-expressing ($P = 0.0090$, $N = 13$) and DIO–eYFP-expressing ($P = 0.7608$, $N = 11$) animals. **s**, Summary schematic depicting behavioural engagement-induced inhibition of aPVT^{NTS}–Nac terminals. All error bars represent $\pm$ s.e.m.; N represents the number of mice. P values are reported from a Student's two-tailed paired t -test (**d,e,n,o,q,r**) and simple linear regression (**f,g**): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

and significantly reduced the time spent engaging with the sipper port throughout the cue period (Fig. 3b,c,f,g and Extended Data Fig. 7h,i). This change was not simply due to a reduction in the number of engagement events, as the absence of engagement-related presynaptic inhibition was still observed in sg*Cnr1*-injected mice when specifically isolating trials where animals consumed sucrose (Extended Data Fig. 7i). In DO34-treated animals, deletion of the gene encoding CB₁R had no effect either on the aPVT^{NTS}–Nac GCaMP6 signal or the degree of engagement in reward seeking (Fig. 3d–g and Extended Data Fig. 7i,j). Furthermore, DO34 reversed engagement-induced inhibition of aPVT^{NTS}–Nac terminals in sg*Rosa* mice and reduced sipper engagement, whereas had no effect on the GCaMP6s signal or sipper engagement in sg*Cnr1* animals (Fig. 3f,g). The mutual occlusion effects between sgRNA targeting *Cnr1* knockdown and DO34 treatment provide corroborating evidence that 2-AG–CB₁R signalling facilitates engagement in reward seeking through inhibition of aPVT^{NTS}–Nac axon terminals. These results also suggest that eCB signalling represents a key endogenous G_i–GPCR mechanism acting in a feedforward retrograde manner to suppress excitatory input into the ventral striatum during reward seeking (Fig. 3f,g).

We next assessed whether CRISPR knockdown of aPVT CB₁R impaired engagement in defensive freezing behaviours in a Pavlovian fear conditioning–extinction paradigm (Fig. 3h,i and Extended Data Fig. 7k–n). During fear conditioning, deletion of the gene encoding CB₁R from aPVT^{NTS} neurons neither affected the activity of aPVT^{NTS}–Nac terminals nor freezing behaviours. Similarly, during fear recall, this manipulation did not attenuate freezing-induced inhibition of aPVT^{NTS}–Nac terminals or freezing behaviours (Fig. 3h,i and Extended Data Fig. 7k–n). These data suggest that although inhibition of aPVT^{NTS}–Nac terminals represents a common feature of engagement in both reward-seeking and freezing behaviours, 2-AG–CB₁R signalling at aPVT^{NTS}–Nac terminals is necessary for engagement in reward seeking but not for defensive freezing, suggesting a unique undefined mechanism driving aversion-related inhibition.

To test the specificity of CB₁R signalling at aPVT^{NTS}–Nac terminals for regulating engagement in reward-seeking behaviours, we performed a similar set of experiments in which we deleted *Cbl1r* from BLA^{VGLUT1}–Nac terminals using GCaMP6s fibre photometry (Extended Data Fig. 7o–aa). In contrast to deletion of the gene encoding CB₁R from aPVT^{NTS} neurons, post-hoc-corrected multiple comparisons revealed no significant effects of *Cbl1r* deletion on either engagement in reward-seeking or BLA^{VGLUT1}–Nac terminal activity (Extended Data Fig. 7y–aa). However, examination of the overall effects of genotype and treatment revealed significant main effects of both *Cbl1r* knockout as well as DO34 treatment on both engagement in reward-seeking and BLA^{VGLUT1}–Nac terminal activity. These results suggest that CB₁R on

BLA^{VGLUT1}–Nac projections may have some role in regulating reward seeking, and future studies should examine whether there are unique aspects of behavioural engagement in reward seeking differentiating the effects of CB₁R on aPVT^{NTS} neurons versus BLA^{VGLUT1} neurons. Deletion of *Cbl1r* from BLA^{VGLUT1} neurons had no effect on freezing behaviours or BLA^{VGLUT1}–Nac terminal activity (Extended Data Fig. 8a–i).

To determine whether 2-AG binds to presynaptic CB₁R on aPVT terminals in the Nac, we injected mice with AAV1-hSyn-GRAB_{eCB2.0} or the control virus mutant-GRAB_{eCB2.0} (GRAB_{eCB2.0Mut}; which is not activated by 2-AG) into the aPVT and implanted a fibre optic above the medial Nac shell (Fig. 3j). We detected a progressive increase in the reward-engagement-induced GRAB_{eCB2.0} signal from day 1 to day 5, and no change in the GRAB_{eCB2.0Mut} signal (Fig. 3k–n). Compared with GRAB_{eCB2.0Mut} controls, we detected a significant increase in the GRAB_{eCB2.0} signal on day 5 of reward conditioning. We then tested whether 2-AG binds to aPVT terminals following exposure to aversive stimuli in a Pavlovian fear conditioning–extinction paradigm. Similar to our results with local Nac expression of GRAB_{eCB2.0} (Fig. 1), foot shock elicited a robust increase in the GRAB_{eCB2.0} signal, whereas the shock-predictive cue elicited no change in the GRAB_{eCB2.0} signal during fear recall (Extended Data Fig. 8j–l).

Finally, we sought to identify an in vivo correlate to the DSE phenomenon (neural depolarization induced production of eCBs, demonstrated in Fig. 1) within the ventral striatum. Specifically, we examined whether we could evoke 2-AG production and release through photostimulation of Nac neurons in vivo. Our electrophysiological results indicated that most of the phasic 2-AG release in the Nac is mediated via D2R neurons, which have been shown to co-express the opioid peptide enkephalin with 95% overlap with D2R expression^{17,43}. Thus, we injected proenkephalin–Cre (Penk–Cre) mice with the red-shifted channelrhodopsin AAV5-DIO-ChRimson–tdTomato in the medial Nac shell and GRAB_{eCB2.0} in the aPVT along with an implanted fibre optic in the Nac for tandem optogenetics and fibre photometry recordings of the aPVT–Nac GRAB_{eCB2.0} signal (Fig. 3o). Photo-activation of Nac Penk neurons (20 Hz, 10 s) was sufficient to elicit a significant increase in the GRAB_{eCB2.0} signal, indicating in vivo-evoked release of 2-AG (Fig. 3p). Overall, these findings demonstrate that in vivo release of 2-AG in the Nac functionally tunes the degree of engagement in reward-seeking behaviour through time-locked gain control of aPVT axon terminal activity (Fig. 3q).

Cell-type-specific modulation of reward seeking by eCBs

Our evidence supports the role of 2-AG–CB₁R signalling in filtering excitatory aPVT^{NTS} input to the Nac to control reward seeking, and that Nac^{D2R–Penk} neurons produce 2-AG. However, the necessity of activity-dependent 2-AG release from Nac^{D2R–Penk} neurons for driving

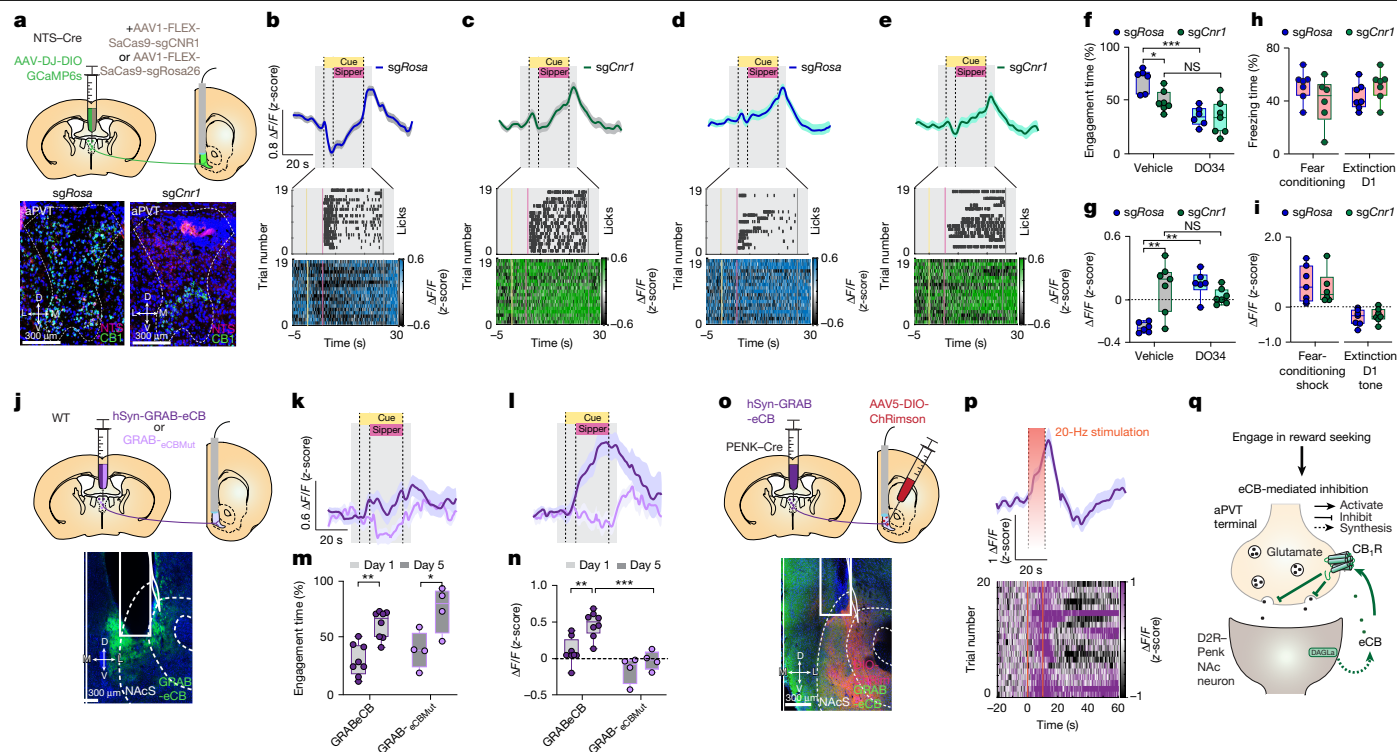


Fig. 3 | eCB inhibition of aPVT terminals drives engagement in reward-seeking behaviours. **a**, Schematic of CRISPR–Cas9 deletion of the gene encoding CB₁R from aPVT NTS neurons and photometry recordings of aPVT^{NTS}–NAC terminals during Pavlovian reward conditioning. **b–e**, Photometry trace with representative lick raster and heatmap of the GCaMP6s signal from vehicle-treated (**b,c**) and DO34-treated (**d,e**) sgRosa-injected (*N* = 6) and sgCnr1-injected (*N* = 7) mice. **f**, Comparison of the percentage of sipper port engagement time showing reduced engagement in vehicle-treated sgCnr1 animals (*P* = 0.0280, *N* = 7) and DO34-treated sgRosa animals (*P* = 0.0003, *N* = 6) compared with vehicle-treated sgRosa controls (main effects: treatment *P* < 0.0001, genotype *P* = 0.0357, interaction *P* = 0.0627). **g**, Comparison of the average GCaMP6s signal from 0 to 26 s showing blunted inhibition of aPVT terminals in vehicle-treated sgCnr1 animals (*P* = 0.0005, *N* = 7) and DO34-treated sgRosa animals (*P* = 0.0004, *N* = 6) compared with vehicle-treated sgRosa controls (main effects: treatment *P* = 0.0093, genotype *P* = 0.0313, interaction *P* = 0.0002). **h**, Comparison of freezing behaviour between sgRosa-injected (*N* = 7) and sgCnr1-injected (*N* = 7) animals during fear conditioning (*P* = 0.1880) and day 1 of fear extinction (*P* = 0.1789). **i**, Comparison of the GCaMP6s signal between sgRosa-injected (*N* = 7) and sgCnr1-injected (*N* = 7) animals during fear conditioning (0–5 s post-shock, *P* = 0.4953) and day 1 of fear extinction (0–20 s post-tone, *P* = 0.7392). **j**, Schematic of photometry

recordings of GRAB_{eCB2.0} or GRAB_{eCBmut} on aPVT–NAC terminals during Pavlovian reward conditioning. **k,l**, Photometry trace of the GRAB_{eCB2.0} (*N* = 8) or GRAB_{eCBmut} (*N* = 4) signal on day 1 (**k**) and day 5 (**l**) of reward conditioning. **m**, Comparison of the percentage of sipper port engagement time showing enhanced engagement time on day 5 in both GRAB_{eCB2.0} (*P* = 0.0024, *N* = 8) and GRAB_{eCBmut} (*P* = 0.0120, *N* = 4) compared with their respective day 1 values (main effects: sensor *P* = 0.1023, day *P* < 0.0001, interaction *P* = 0.7257). **n**, Comparison of the average GCaMP6s signal from 0 to 26 s showing an enhanced GRAB_{eCB2.0} signal at day 5 of reward conditioning compared with GRAB_{eCB2.0} day 1 (*P* = 0.0017, *N* = 8) and GRAB_{eCBmut} day 5 (*P* = 0.0009, *N* = 4; main effects: sensor *P* < 0.0001, day *P* = 0.0039, interaction *P* = 0.1241). The horizontal dotted lines indicate zero. **o**, Schematic of simultaneous optogenetic activation of NAc Penk neurons and photometry recordings of aPVT–NAC GRAB_{eCB2.0} (*N* = 4). **p**, Trace and representative heatmap showing GRAB_{eCB2.0} dynamics time locked to 10-s 20-Hz stimulation of NAc Penk neurons. **q**, Summary schematic depicting reward-engagement-induced retrograde eCB inhibition at aPVT–NAC^{Penk} synapses. All error bars represent minimum to maximum values; the boxplots represent the interquartile range with a line at median; *N* represents the number of mice. *P* values are reported from two-way ANOVA with Sidak post-hoc correction (**f–i,m,n**): not significant (NS), **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

engagement in reward seeking remained a critical unresolved question. To test this hypothesis, mice performed Pavlovian reward conditioning in a custom-built linear track behavioural corridor, which allowed for multi-plane video recordings of animal behaviour. To garner unbiased quantifiable parameters and features of engagement in reward-seeking behaviours, we leveraged a custom SLEAP pose estimation algorithm to identify and track specific locations on the body of the mouse (that is, points of interest) during reward conditioning⁴⁴ (Fig. 4a). A supervised machine learning algorithm was subsequently used to identify frame-by-frame instances of sipper port engagement, sipper port disengagement, walking, rearing and grooming (Fig. 4b–g).

Functional studies have garnered conflicting reports regarding the role of NAc^{D2R}–Penk neurons in reward seeking and reinforcement^{25,45–47}. This could be due in part to the fact that these studies that used optogenetics to manipulate this population targeted all Penk neurons in the NAc, which represents 40–50% of the entire NAc neuron population, and the recent evidence of the diversity of encoding features of these

neurons^{48,49}. To gain further specificity and precision, we devised an anterograde trans-synaptic labelling strategy that allowed us to specifically capture NAc Penk neurons that received input from the aPVT. We leveraged a recently developed AAV1 virus (AAV-trans-DIO-FLP) that shows strong anterograde trans-synaptic infectivity to drive expression of ChRimson in Penk neurons that receive aPVT input^{50,51} (Fig. 4h). Following the characterization of its labelling (Extended Data Fig. 9a), we co-injected Penk–Cre mice with AAV-trans-DIO-FLP into the aPVT combined with AAV-fDIO-ChRimson into the NAc shell, followed by fibre-optic implantation (Extended Data Fig. 9b).

To specifically manipulate the activity of aPVT–NAC^{Penk} neurons during reward seeking, we utilized real-time SLEAP tracking and supervised classification of behaviours to detect sipper port engagement (see Methods). We subsequently generated a closed-loop optogenetic stimulation paradigm within the SLEAP architecture in which behavioural engagement signatures elicited 20-Hz optogenetic stimulation of aPVT–NAC^{Penk} neurons. We found that this stimulation selectively

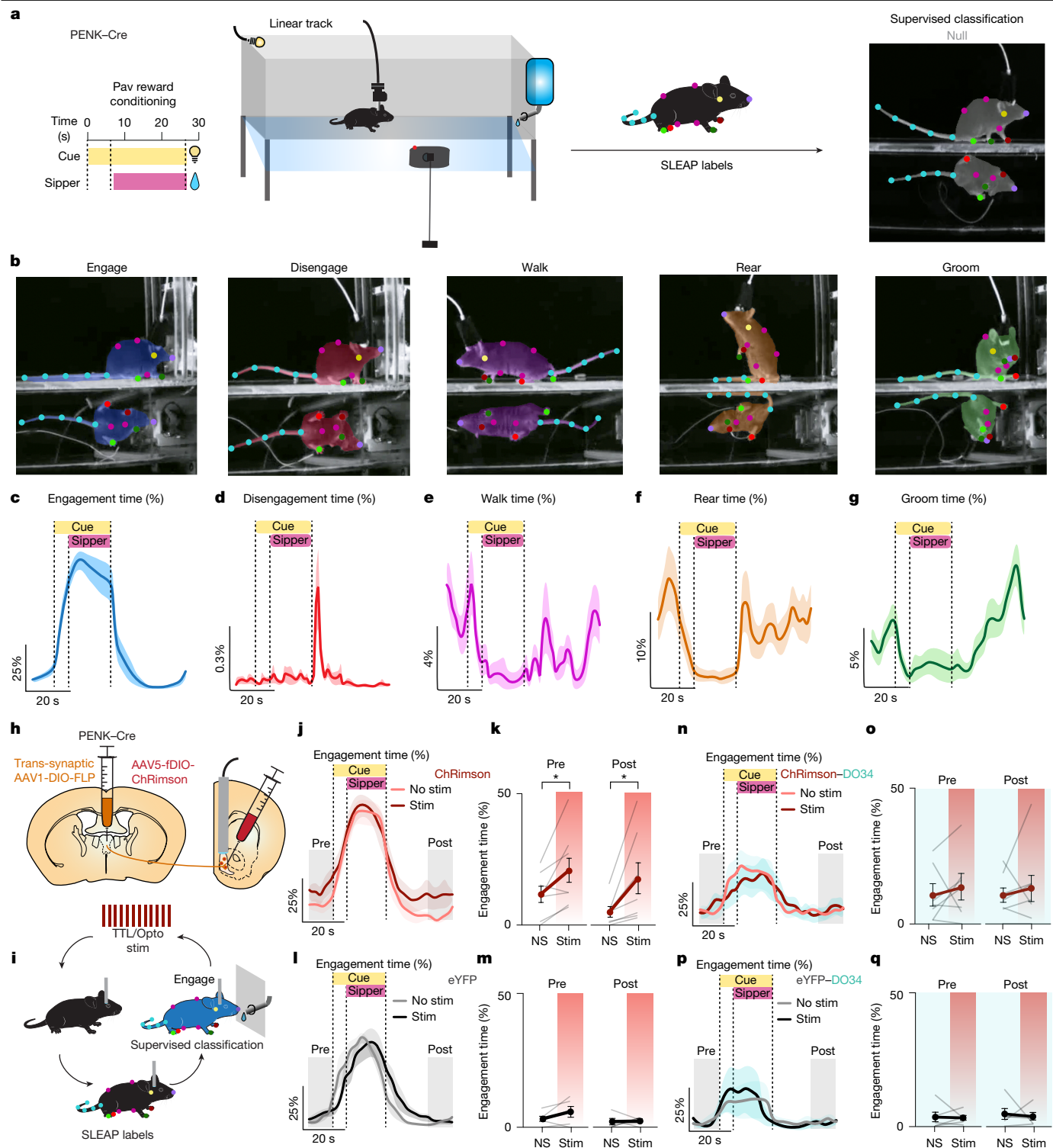


Fig. 4 | aPVT-NAc^{Penk} neurons facilitate engagement in reward seeking in an eCB-dependent manner. **a**, Schematic of Pavlovian reward conditioning in the linear track and subsequent SLEAP labelling and supervised classification of behaviour. **b**, Example frames of five classified behaviours: sipper port engagement, disengagement, walk, rear and groom. **c–g**, Cue-aligned traces of the percentage of time engaging with sipper port (**c**), disengaging from sipper port (**d**), walking (**e**), rearing (**f**) and grooming (**g**) in trained mice ($N = 12$). **h**, Schematic of trans-synaptic capture of NAc^{Penk} neurons that receive aPVT input for subsequent optogenetic stimulation of aPVT-NAc^{Penk} neurons. Opto, optogenetic; TTL, transistor-transistor logic. **j**, Percentage of time engaging with sipper port on counterbalanced ChRimson stimulation to

no stimulation days ($N = 7$). **k**, Quantification of the percentage of sipper port engagement in the pre-cue (–10 to 0 s, $P = 0.0483$, $N = 7$) and post-cue (50–60 s, $P = 0.0422$, $N = 7$) ITI periods. **l, m**, Same as panels **j, k**, respectively, for eYFP control mice ($P = 0.9205$ and $P = 0.7546$, $N = 5$). **n**, Percentage of time engaging with sipper port on counterbalanced ChRimson stimulation to no stimulation days in mice treated with 25 mg kg^{–1} DO34. **o**, Quantification of the percentage of sipper port engagement in the pre-cue (–10 to 0 s, $P = 0.4465$, $N = 7$) and post-cue (50–60 s, $P = 0.5292$) ITI periods in mice treated with 25 mg kg^{–1} DO34. **p, q**, Same as panels **n, o**, respectively, for eYFP control mice ($P = 0.8803$ and $P = 0.7369$, $N = 5$). All error bars represent $\pm$ s.e.m.; N represents the number of mice. P values are reported from a Student's two-tailed paired t -test (**k, m, o, q**): $*P < 0.05$.

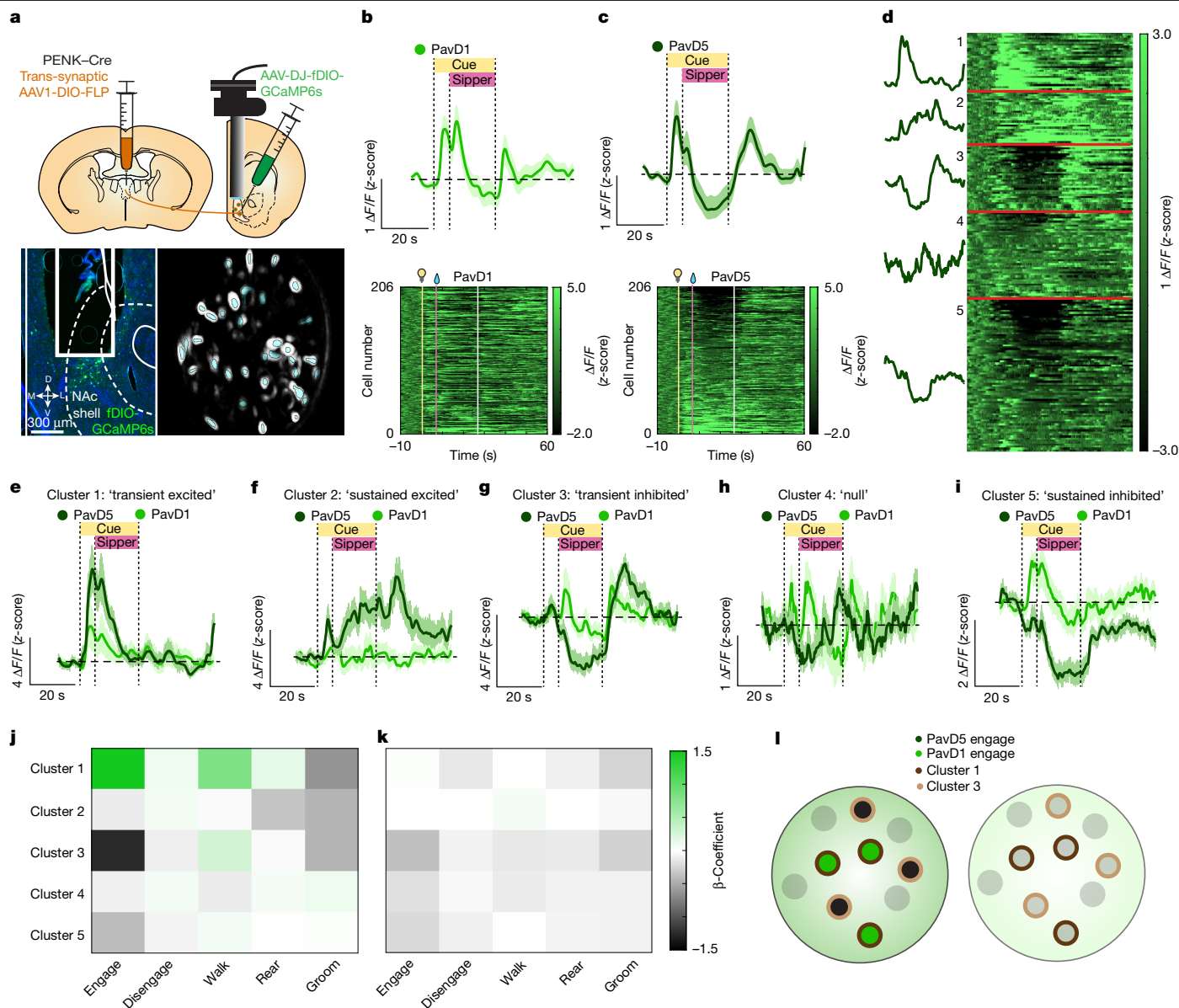


Fig. 5 | Encoding of engagement in reward seeking by aPVT-NAc^{Penk} ensembles. **a**, Schematic of trans-synaptic capture of NAc^{Penk} neurons that receive aPVT input for subsequent one-photon recordings of GCaMP6s of aPVT-NAc^{Penk} neurons, and GRIN lens placement and representative maximum-projection image of aPVT-NAc^{Penk} neurons. **b, c**, Average cue-aligned trace and heatmaps for 206 matched neurons from day 1 (**b**) and day 5 (**c**) of Pavlovian reward conditioning ($N = 10$). **d**, Hierarchical clustering revealed five distinct clusters of aPVT-NAc^{Penk} neurons based on the cue-aligned calcium signal on PavD5. **e–i**, Traces of five unique cue-aligned clusters of aPVT-NAc^{Penk} neurons

increased engagement in reward seeking during ITI periods preceding and following cue and sipper delivery (Fig. 4j–m and Extended Data Fig. 9c–f). These effects were specific to the ITI periods and did not increase engagement during sipper delivery, suggesting that closed-loop stimulation selectively reinforces engagement in reward seeking while not directly affecting consummatory behaviours. To further corroborate the specificity of the SLEAP-based closed-loop optogenetic effect, we tested an additional closed-loop experiment in which stimulation was instead triggered only during walk events (Extended Data Fig. 9g). This stimulation during walk epochs had no effect on engagement in reward seeking (Extended Data Fig. 9h–k). Finally, we assessed the necessity of eCB release from NAc^{D2R-Penk} neurons for the closed-loop optogenetically induced increase in engagement of

on day 5 of conditioning, as well as their neuron-matched traces and heatmaps from day 1 on conditioning. The horizontal dashed lines indicate zero. **j**, Heat plot of β -coefficients of a generalized linear model trained on each PavD5 behaviour throughout the 70-s window and fit to the activity of each cluster during the corresponding time window. **k**, Same as in panel **j**, but for PavD1 behaviours. **l**, Graphic summarizing results of the generalized linear model, depicting positive encoding of engagement in reward-seeking behaviours by aPVT-NAc^{Penk} cluster 2 and negative encoding by aPVT-NAc^{Penk} cluster 3.

reward seeking. To test this, we pre-treated mice with 20 mg kg⁻¹ DO34 to block production of 2-AG (Fig. 4n–q). This pretreatment completely abolished the effect of NAc^{D2R-Penk} ChRimson stimulation on increasing sipper port engagement. Overall, these studies further establish that activity-dependent production of 2-AG from aPVT-NAc^{Penk} neurons facilitates engagement in reward seeking.

Encoding of reward seeking by aPVT-NAc^{Penk} ensembles

Trans-synaptic labelling allowed us a singular ability to manipulate a specific population of NAc^{Penk} neurons; however, it has been established that this population is not homogenous in its dynamics during Pavlovian reward conditioning^{48,49}. As we had determined that

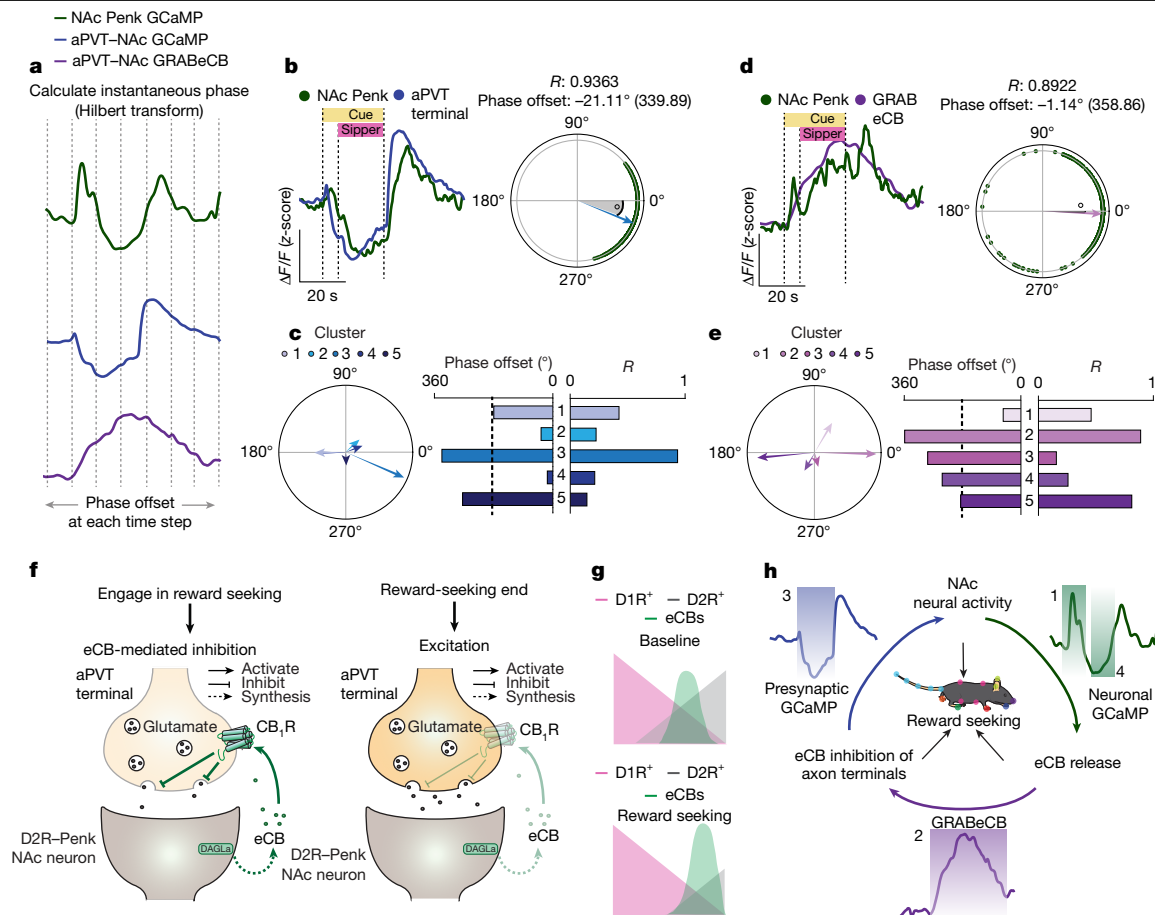


Fig. 6 | Entrainment of aPVT-NAc^{Penk} neuron activity to eCB release and aPVT-NAc terminal activity. **a**, Schematic depicting alignment of aPVT-NAc^{Penk} GCaMP, aPVT^{NTS} terminal GCaMP6 and aPVT terminal GRAB_{eCB2.0} signals. **b**, Overlay of the aPVT-NAc^{Penk} cluster 3 GCaMP6s signal with the aPVT^{NTS} terminal GCaMP6s signal from day 5 and corresponding Rayleigh plot for circular uniformity. **c**, Summary plot of Hilbert and Rayleigh analyses depicting Pearson R and phase-offset values for the entrainment of each cluster to the aPVT^{NTS} terminal GCaMP6 signal. **d**, Overlay of the aPVT-NAc^{Penk} cluster 2 GCaMP6s signal with the aPVT terminal GRAB_{eCB2.0} signal from day 5, and the corresponding Rayleigh plot for circular uniformity. **e**, Summary plot

of Hilbert and Rayleigh analyses depicting Pearson R and phase-offset values for the entrainment of each cluster to the aPVT terminal GRAB_{eCB2.0} signal. **f**, Summary schematic depicting reward-seeking-induced eCB production by aPVT-NAc^{Penk} neurons and subsequent retrograde action at aPVT terminals, and excitation of aPVT terminals upon disengagement. **g**, Summary schematic depicting relative aPVT input bias onto NAc neurons at baseline and during reward seeking. **h**, Summary schematic depicting the reciprocal relationship between aPVT-NAc^{Penk} neuron activity (1), eCB release and presynaptic binding (2), eCB tuning of excitatory input from the aPVT (3) and entrainment of aPVT-NAc^{Penk} neurons to aPVT terminal activity (4).

reward-seeking-induced 2-AG release occurs in an activity-dependent manner, we next assessed how engagement in reward seeking is encoded by the activity of discrete ensembles of NAc Penk neurons. To understand how these behaviours were encoded at the neural level, we co-injected Penk-Cre mice with AAV-trans-DIO-FLP into the aPVT combined with AAV-DJ-fDIO-GCaMP6s into the medial NAc shell to induce expression of GCaMP only in NA neurons that specifically receive aPVT efferent input. We then implanted a 0.6-mm GRIN lens over the medial NAc shell (Fig. 5a and Extended Data Fig. 9l). Using the spatial footprints of the neurons in the field of view, we tracked and matched 206 aPVT-NAc^{Penk} neurons between day 1 and day 5 of Pavlovian reward conditioning.

To examine the functional heterogeneity of these neurons, we used principal component analysis-based dimensionality reduction followed by hierarchical clustering to partition the neurons into functional clusters^{52,53} (Fig. 5b,c). These analyses were done on the z-scored fluorescence activity of the cue-aligned calcium trace on day 5 of conditioning, and neurons were subsequently matched to day 1 activity profiles. We found that the neurons were roughly partitioned into one of five total clusters: (1) transient excited, (2) sustained excited, (3) transient inhibited, (4) null, and (5) sustained inhibited (see Methods; Fig. 5d–i and Extended Data Fig. 9m,n). To substantiate cluster identities, a support

vector machine was trained on PavD5 principal component coordinates and cluster labels, which demonstrated accurate decoding of cluster identity for unshuffled day 5 data but not for day 5 shuffled or day 1 shuffled–unshuffled (Extended Data Fig. 9o).

Simultaneous capture of one-photon recordings of aPVT-NAc^{Penk} GCaMP6s activity and high-resolution linear track videos for SLEAP classification enabled us to determine how engagement in reward-seeking behaviours is encoded by changes in the activity of this neural population. Thus, we used a generalized linear model fit of the calcium activity of the five clusters, trained on z-scored traces (derived from frame-by-frame instances) of the five aforementioned behaviours throughout the 70-s window used for analysis of our cue-aligned one-photon imaging data (10 s before to 60 s after the cue; Extended Data Fig. 10). Of all behaviours across both days of Pavlovian conditioning, we identified 2 β -coefficients with an absolute value > 1 , belonging to the fit of PavD5 engagement to cue cluster 1 (transient excited; $\beta = 1.483$) and cue cluster 3 (transient inhibited; $\beta = -1.2476$; Fig. 5j,k and Extended Data Fig. 10a–p). In this case (identity link), these coefficients represent the change in value of the response variable (cluster activity) given a 1 unit change in the predictor (behaviour). Together, these results indicate that engagement in reward-seeking behaviours is at least partially encoded by the activity of discrete aPVT-NAc^{Penk} ensembles (Fig. 5l).

Entrainment of somatic and terminal activity to eCBs

We found that the activity of specific clusters of aPVT–NAC^{Penk} neurons resembled either aPVT^{NTS}–NAC terminal activity or aPVT terminal GRAB_{eCB2.0} signal. To assess whether these signals were entrained to each other, we time aligned the signals and used a Hilbert analysis to decompose constituent waveforms and a subsequent Rayleigh test for circular uniformity^{54,55} (Fig. 6a and Extended Data Fig. 10q–z). These analyses provide us with the phase offset between time-varying signals and have been previously used to correlate single-unit neural activity to local field potentials⁵⁶. When the clusters were aligned to aPVT axon terminal activity, we found the highest degree of entrainment ($R = 0.9363$) between the transient inhibited cluster (3) and the aPVT terminals with a phase offset of -21.11° , suggesting that the activity of the cluster lags behind the activity of the axon terminal (Fig. 6b,c). When the cluster activity was aligned with the GRAB_{eCB2.0} signal, we found the greatest degree of entrainment within the sustained excited cluster (2; $R = 0.8922$) with a phase offset of -1.14° , suggesting that the GRAB_{eCB2.0} and aPVT–NAC^{Penk} cluster 2 signals probably emerge contemporaneously (Fig. 6d,e). These findings indicate that specific ensembles of aPVT–NAC^{Penk} neurons are entrained to aPVT^{NTS}–NAC axon terminal activity in tandem with the kinetics of localized retrograde 2-AG release. Furthermore, this result indicates that discrete postsynaptic neuronal ensembles can communicate via specific retrograde neuromodulation mechanisms such as eCBs to filter incoming excitatory drive onto striatal medium spiny neurons, dynamically impacting engagement in reward-seeking behaviours.

Discussion

The behavioural role of endogenous eCB signalling on distinct neural circuits as it relates to more complex behaviours such as reward seeking has been difficult to probe owing to its widespread expression and a lack of molecular tools to assess eCB dynamics in vivo^{2,57,58}. Here we used a series of complementary methods to uncover a specific and dynamic role for the eCB 2-AG release in driving engagement in reward-seeking behaviours. In particular, we isolated a previously undefined characteristic of eCB action at presynaptic accumbal CB₁Rs in a directed temporally discrete control of reward engagement. This retrograde gain reduction via eCBs acts as a fundamental negative-feedback mechanism through which excitatory drive onto D2R–Penk medium spiny neurons from thalamic inputs is tuned. Although it has been known that eCBs can reduce excitatory transmission^{6,9} ex vivo, establishing an in vivo correlate of DSE and demonstrating its causal role in facilitating reward seeking expands our understanding of how presynaptic G_i-coupled GPCRs act as powerful facilitators of learned and motivated behaviours^{59,60}.

Although multiple excitatory projections to the NAC express CB₁R, we detected the highest density in aPVT projectors that express the neuropeptide NTS. Electrophysiological characterization of this pathway revealed that these inputs are regulated by both tonic and phasic 2-AG produced by D2R–Penk neurons. In vivo, the activity of these projections was negatively correlated with engagement in both reward-seeking behaviours and conditioned freezing. However, CRISPR–Cas deletion of *Cbl1r* from aPVT NTS neurons was only sufficient to attenuate engagement in reward-seeking behaviours (and concomitant aPVT terminal inhibition), leaving engagement in aversive behaviours intact. Given that the activity-dependent production of 2-AG was primarily evoked from D2R–Penk neurons, we used a trans-synaptic labelling strategy to show that aPVT–NAC^{Penk} neurons promote engagement in reward seeking in a 2-AG-dependent manner and that these neurons encode engagement in reward-seeking behaviours.

Although work has demonstrated the feasibility of one-photon and two-photon recordings of NAC neurons in a genetically specific

manner, to our knowledge, no studies to date have recorded from NAC neurons with respect to both afferent connectivity and genetic identity⁴⁹. The trans-synaptic approach that we used provided us the means to image from NAC^{Penk} neurons receiving direct monosynaptic input from the aPVT, allowing us unprecedented granularity, which is uniquely important in the NAC given the well-characterized functional diversity of NAC neurons^{48,49}. By utilizing novel pose estimation and machine learning algorithms to garner quantitative metrics of behavioural states and transitions, we demonstrated that the activity of specific clusters of aPVT–NAC^{Penk} neurons specifically encode engagement in reward seeking. Together with signal processing analyses (Hilbert transform and Rayleigh test), we have reported that unique clusters of aPVT–NAC^{Penk} neurons are entrained either to eCB release or aPVT terminal activity.

Here we have reported a fundamental retrograde neuromodulatory mechanism that involves localized ventral striatal 2-AG production in engagement in reward-seeking behaviours through gain control of an anatomically and genetically defined thalamostriatal circuit (Fig. 6f,g). These results establish a causal link between single-cell neural activity (aPVT–NAC^{Penk}), neuromodulator release (2-AG) and G_i–GPCR-mediated tuning of excitatory projections (aPVT^{NTS}–NAC) to reveal how eCBs dynamically regulate complex behaviours such as engagement in reward seeking (Fig. 6h). These results provide a framework for understanding neuromodulatory substrates in vivo, while also establishing potentially unique endophenotypes implicated in the pathophysiology of mood and substance-use disorders.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-026-10967-w>.

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Methods

Animals

Adult (16–35 g, 12–52 weeks) male and female WT, NTS–Cre, Penk–Cre and Ai14 mice were group housed, given ad libitum access to food and water, and maintained on a 12 h light–dark cycle (lights off at 09:00, lights on at 21:00), with an ambient temperature of 70–73 °F and humidity of approximately 40%. Mice were bred in a barrier facility and transferred to the holding facility at least 1 week before the start of surgical interventions or experiments. Littermate-matched controls were used in manipulation studies (optogenetics, CRISPR and behavioural pharmacology). All animals were drug and test naive, unless otherwise noted. For all reward assays, mice were water restricted to approximately 90% of baseline body weight for at least 5 days before behavioural testing. If body weight dipped below approximately 85% of baseline weight, mice were removed from water restriction. Statistical comparisons did not detect any sex differences, and therefore male and female mice were combined for all of the data presented in this article. All animals were monitored daily by either facility staff or experimenter throughout the duration of the study. All procedures were approved by the Animal Care and Use Committee at the University of Washington, and conformed to US National Institutes of Health guidelines.

Drugs

For all behavioural experiments, drugs were injected intraperitoneally and prepared in a 17:1:1 vehicle consisting of saline, ethanol, kolliphor and DMSO, respectively. Drugs were administered at the following concentrations: 10 mg kg⁻¹ SR141716A (Sigma), 20 mg kg⁻¹ DO34 (Sigma), 5 mg kg⁻¹ JZL184 (Sigma) and 3 mg kg⁻¹ URB597 (Sigma). SR141716A, JZL184 and URB597 were injected 90 min before behavioural testing, and DO34 was injected 120 min before behavioural testing.

Stereotaxic surgeries

Mice were anaesthetized in an induction chamber (1–4% isoflurane) and placed into a stereotaxic frame (model 1900, Kopf Instruments) where they were mainlined at 1%–2% isoflurane. For all viral injections, a Hamilton Neuros syringe was used, and virus was infused at a rate of 50–100 nl min⁻¹. For retrograde-tracing studies, 150 nl of AAV2-retro-DIO-Cre was injected into the medial NAc shell of Ai14 mice at two dorsoventral coordinates per hemisphere (mm, relative to bregma): anteroposterior +1.7, dorsoventral –4.4 and –4.1, and mediolateral +0.65. For ex vivo electrophysiology studies, NTS–Cre mice were injected with 500 nl of AAV5-DIO-ChR2-eYFP in the aPVT (14° mediolateral angle, with final coordinates of anteroposterior –0.4, dorsoventral –3.75 and mediolateral 0). For NAc fibre photometry experiments, 500 nl of AAV1-hSyn-GRAB_{ecb2.0} was injected into the right medial NAc shell (anteroposterior +1.7, dorsoventral –4.2 and mediolateral +0.65), and 400-µm fibre optic with a stainless steel ferule (Doric) was implanted at the same coordinates. All implants were secured using Metabond (Parkell). For terminal aPVT fibre photometry experiments, AAV5-DIO-GCaMP6s (Addgene) or AAV1-hSyn-GRAB_{ecb2.0} was injected into the aPVT of NTS–Cre or WT mice respectively at a 14° angle, with final coordinates of anteroposterior –0.4, dorsoventral –3.75 and mediolateral 0, with a fibre optic implanted in the NAc shell (same coordinates as above). For CRISPR or photometry experiments, AAV-DJ-DIO-GCaMP6s was mixed in a 2:1 ratio with AAV1-FLEX-sgCnr1-SaCas9 or AAV1-FLEX-sgRosa26-SaCas9 and then injected into the aPVT of NTS–Cre mice or the BLA (anteroposterior –1.25, mediolateral ±3.15 and dorsoventral –5.1) of VGLUT1–Cre mice. These viruses were single-vector CRISPR–Cas9 constructs, which carried a guide RNA directed against a sequence in the first coding exon of *cnr1* (sgCnr1) or a guide RNA directed against the *Rosa26* locus to control for double-stranded breaks. Three weeks after viral injection, a 400-µm fibre optic was implanted in the right medial NAc shell at anteroposterior +1.7, dorsoventral –4.2 and mediolateral ±0.65.

For optogenetics experiments, 500 nl of AAV5-DIO-ChR2-eYFP or AAV5-DIO-PPO-eYFP (Addgene) was injected into the aPVT of NTS–Cre mice. Three weeks after injection, bilateral 200-µm fibre optics were implanted over the medial NAc shell at a 10° angle (final coordinates of anteroposterior +1.7, dorsoventral –4.0 and mediolateral ±0.65). For recording aPVT terminal GRAB_{ecb2.0} with concomitant optogenetic activation of NAc PENK neurons, PENK–Cre mice were injected with 500 nl of AAV1-hSyn-GRAB_{ecb2.0} in the aPVT and 500 nl of AAV5-DIO-ChRimson in the NAc, with a fibre optic implanted above (same coordinates as above). For closed-loop optogenetic studies in the linear track, 500 nl of AAV1-trans-DIO-FLP was injected into the aPVT of PENK–Cre mice. Three weeks after this injection, 500 nl of AAV5-fDIO-ChRimson was injected into the medial NAc shell (anteroposterior +1.7, dorsoventral –4.2 and mediolateral ±0.65) with 200-µm fibre optics implanted above the injection site (anteroposterior +1.7, dorsoventral –4.0 and mediolateral ±0.65). For one-photon imaging experiments, 500 nl of AAV1-trans-DIO-FLP was injected into the aPVT of PENK–Cre mice. Three weeks later, 250 nl of AAV5-fDIO-GCaMP6s was injected in the right medial NAc shell at two dorsoventral coordinates per hemisphere: anteroposterior 1.7, dorsoventral 4.4 and 4.1, and mediolateral 0.65. During the same surgery, a 600-µm cuffed GRIN lens (Inscopix) was implanted at coordinates of anteroposterior 1.7, dorsoventral 4.15 and mediolateral 0.65. Three weeks after viral injection and lens implantation, we attached a baseplate to an Inscopix miniscope to identify the optimal imaging plane. The baseplate was subsequently attached to the implant with Metabond. All mice were given at least 3 weeks of recovery time post-surgery before experimentation.

Tissue processing

For all in vivo optogenetics, fibre photometry or one-photon imaging experiments, mice were transcardially perfused with a 4% paraformaldehyde solution in phosphate-buffered saline (PBS). Heads were subsequently removed and were immediately placed into conical tubes containing the same solution and allowed to set for 24–72 h. After this time, fibre-optic implants were removed along with the remainder of the skull, and brains were put into a cryoprotectant 30% w/v sucrose solution in PBS. Once brains had sunken to the bottom of the tubes (typically around 72 h), brains were mounted on cutting blocks using OCT (Sakura Finetek) and sliced in 40-µm sections on a Leica CM1900 cryostat at –18 °C. Slices were then placed into PBS and either immediately mounted on SuperFrost Plus slides (Fisher) or stored for up to 3 days. Mice with misplaced fibre-optic implants were excluded from the study.

In situ hybridization using RNAscope

For quantification of mRNA transcripts using RNAscope (ACD Bio), mice were briefly anaesthetized using isoflurane and subsequently rapidly decapitated. Brains were immediately removed from the skull and were placed into a beaker containing 2-methylbutane that had been sitting on dry ice for more than 20 min. After 30–60 s of submerging, brains were removed and placed into conical tubes in a –80 °C freezer for long-term storage. Before sectioning, brains were placed in the cryostat compartment for more than 30 min to allow them to come up to temperature. Following mounting using OCT, brains were sliced at 16 µm and directly mounted onto SuperFrost Plus slides. Slides were stored at –80 °C.

Fluorescence in situ hybridization was performed according to the RNAscope 2.0 Fluorescent Multiple Kit User Manual for Fresh Frozen Tissue (Advanced Cell Diagnostics). In brief, slides were fixed in 40% paraformaldehyde and then serially dehydrated using increasing concentrations of ethanol (50%, 75%, 100% and 100%). Slices were then treated with protease IV and allowed to incubate in a 40 °C hybridization oven for 30 min. Following serial rinses in a wash buffer, slices were incubated with probes directed against Cre (312281-C3), CB1 (457341) or NTS (420441-C2) for 2 h. Samples subsequently went through sequential

amplification steps, and then application of opal dyes corresponding to each channel (TSA Vivid Fluorophore 520 (PN 323271), TSA Vivid Fluorophore 570 (PN 323272) and TSA Vivid Fluorophore 650 (PN 323273)). Slides were counterstained with DAPI, and coverslips were mounted with Vectashield Hard Set mounting medium (Vector Laboratories).

Image acquisition and analysis

For histological verification of fibre-optic or lens placement, images were obtained on a Leica DM6B epifluorescent microscope at $\times 10$ or $\times 20$ magnification. For RNAscope analysis, images were collected on an Olympus Fluoview 3000 at $\times 20$ or $\times 40$ magnification. Z-stacked images were taken with optical section thickness of 2–3 μm . Images were subsequently analysed using either HALO or QuPath software. For either analysis, brain region boundaries were drawn in accordance with the Allen Brain Institute mouse reference atlas. For HALO, DAPI-stained nuclei were used to mark cells, and cell boundaries were set to approximately 8- μm radius from the nucleus centre. Thresholds were set for detection in each channel, and this threshold was applied to all analysed images in a given cohort. To account for background noise, a neuron had to have more than two transcripts to be classified as positive for a specific marker. For QuPath analysis, DAPI was similarly used to define neurons and cell boundaries were set to an approximately 8- μm radius from the nucleus centre. Individual channels were set via the classifier module, and these classifier settings were held consistent for all images within a cohort. The classifiers were set by individually sorting through each cell automatically registered by the QuPath software and identifying the first ‘false-positive’ neuron, using this as the threshold cut-off for subsequent identification of positive neurons.

Electrophysiology

Coronal brain slices were prepared at 250 μm on a vibrating Leica VT1000S microtome, and transcardially perfused with ice-cold and oxygenated cutting solution consisting of: 93 mM *N*-Methyl-D-glucamine, 2.5 mM KCl, 20 mM HEPES, 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 mM NaH_2PO_4 , 0.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 25 mM glucose, 3 mM Na^+ -pyruvate, 5 mM Na^+ -ascorbate and 5 mM *N*-acetylcysteine. Following collection of coronal sections, the brain slices were transferred to a 34 °C chamber containing oxygenated cutting solution for a 10-min recovery period. Slices were then transferred to a holding chamber consisting of 92 mM NaCl, 2.5 mM KCl, 20 mM HEPES, 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 mM NaH_2PO_4 , 30 mM NaHCO_3 , 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 25 mM glucose, 3 mM Na -pyruvate, 5 mM Na -ascorbate and 5 mM *N*-acetylcysteine, and were allowed to recover for 30 min or longer. For recording, slices were perfused with oxygenated artificial cerebrospinal fluid (ACSF; 31–33 °C; 300–303 milliosmols) consisting of: 113 mM NaCl, 2.5 mM KCl, 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 mM $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mM NaH_2PO_4 , 26 mM NaHCO_3 , 20 mM glucose, 3 mM Na^+ -pyruvate and 1 mM Na^+ -ascorbate, at a flow rate of 2–3 ml min^{-1} . Stocks of 50 mM SR1 or 5 mM JZL184 were made in DMSO and diluted 1:5,000 in ACSF or HEPES for a final concentration of 10 μM and 1 μM , respectively. Bovine serum albumin (0.05% w/v; Sigma-Aldrich) was added to ACSF or HEPES to help the drugs from precipitating out of solution. For all drug experiments, slices were incubated in a HEPES bath containing the drugs for more than 30 min before transferring over to the slice rigs for recording.

NAC neurons were initially voltage clamped in whole-cell configuration using borosilicate glass pipettes (2–4 M Ω) filled with internal solution containing: 125 mM K^+ -gluconate, 4 mM NaCl, 10 mM HEPES, 4 mM MgATP, 0.3 mM Na-GTP and 10 mM Na-phosphocreatine (pH 7.30–7.35). The patch pipette also included 50 μM picrotoxin to block GABA_A currents. D1⁺ medium spiny neurons (MSNs) were identified by tdTomato fluorescence. For all D1⁺ neurons, multiple electrophysiological metrics were recorded to ensure that we were specifically recording from MSNs. This included capacitance, membrane resistance, resting membrane potential, sag current, afterhyperpolarization current and evoked action potential firing. Multiple publications have

identified that these features differ between MSNs and interneurons, and any neurons that had electrophysiological features of interneurons were excluded from the dataset^{61–64} (Extended Data Fig. 2). Sag current was recorded following a 1-s 100-pA hyperpolarizing current, and the amplitude of the deflection below the stable baseline was calculated. For afterhyperpolarization current, we analysed the amplitude of the inwards current immediately following a train of action potentials induced by a 500-pA current step. Action potential firing was monitored during 40 consecutive successive sweeps of currents starting at –100 pA and increasing by 20 pA each time. Following break-in to the cell, we waited 3 min or longer to allow for exchange of internal solution and stabilization of membrane properties. Neurons with an access resistance of more than 30 M Ω or that exhibited greater than a 20% change in access resistance during the recording were not included in our datasets.

Ex vivo optogenetics

For electrophysiological interrogation of the aPVT^{NTS}–NAC circuit, mice were injected with 500 μl of AAV5-DIO-ChR2(H134R)-eYFP into the aPVT. Three to five weeks of viral expression was allowed before euthanizing the mice. For all experiments using SR141716A or JZL184, corresponding vehicle recordings were obtained from the same mice. For optogenetic recordings of input–output curves, we used a Thorlabs LEDD1B T-Cube driver and obtained separate recordings of 470-nm wavelength stimulations at seven output levels corresponding to 5.7, 2.7, 1.6, 1, 0.5, 0.2 and 0.1 mW of LED intensity. Paired-pulse ratio (PPR) recordings of optically evoked excitatory postsynaptic currents (oEPSCs) were obtained in voltage clamp with an inter-stimulus interval of 50 ms. PPR is reported as a ratio between the amplitude of the second oEPSC divided by the first. Recordings of DSE were obtained following a 10-s voltage step to +30 mV. A baseline of ten oEPSCs were taken before the depolarizing step, and all data are plotted as an oEPSC amplitude normalized to the baseline period. A light exposure time of 2 ms was used for all optogenetic experiments.

Fibre photometry

Fibre photometry recordings were obtained throughout the duration of all behavioural tests presented in this article. Before recordings, a fibre-optic cable was attached to the fibre-optic implant using a plastic or ceramic ferrule sleeve (ZR2.5, Doric). For all photometry recordings, we used a Tucker–Davis Technologies RZ10x processor. A 531-Hz sinusoidal 470-nm LED light (Lx465) was used to excite GCaMP6s and evoke Ca^{2+} -dependent emission. A 211-Hz sinusoidal 405-nm LED light (Lx405) was used as the Ca^{2+} -independent isosbestic control emission. For GRAB_{ecb2.0} recordings, we used a 490-nm LED for GRAB_{ecb2.0} excitation and a 435-nm LED as an isosbestic control, as previous studies have indicated that the peak excitation wavelength for this sensor is slightly red-shifted³³. LED intensity was measured at the tip of the cable with a dummy implant attached and adjusted to 30 μW before each day of recording. GCaMP6s fluorescence travelled through the same optic fibre before being bandpass filtered ($525 \pm 25 \text{ nm}$; FMC4, Doric), transduced by a femtowatt silicon photoreceiver (2151, Newport) and recorded by a real-time processor (RZ10, TDT). The envelopes of the 531-Hz and 211-Hz signals were extracted in real time by the TDT program Synapse at a sampling rate of 1,017.25 Hz. For ChrimsonR stimulation experiments, a 635-nm laser was used with a custom filter cube (Doric) at 5–7 mW of intensity to deliver 20-Hz pulsed light through the same fibre-optic cable used for photometry recordings.

Photometry analysis

Custom MATLAB scripts were developed for analysing fibre photometry data in the context of mouse behaviour and can be accessed via GitHub. The isosbestic 405/435-nm excitation control signal was scaled to the 470/490-nm excitation signal, then this refitted 405/435-nm signal was subtracted from the 470/490-nm excitation signal to remove

movement artefacts from intracellular Ca^{2+} -dependent GCaMP6s or GRAB_{ecB2.0} fluorescence. Baseline drift was evident in the signal due to slow photobleaching artefacts, particularly during the first several minutes of each hour-long recording session. A double exponential curve was fit to the raw trace and subtracted to correct for baseline drift. After baseline correction, $\Delta F/F$ was calculated by subtracting the median baseline subtracted signal from the baseline subtracted signal and dividing by the median raw signal. All photometry data presented in this article are z-scored to a 10–20-s window before cue onset.

Behavioural apparatuses and analysis

Pavlovian reward conditioning. Mice were habituated to a Med Associates operant conditioning box (MED-307W-D2) for 2–3 days (30 min per session), during which a sipper containing a 10% sucrose solution was extended into the chamber. Mice were subsequently trained in Pavlovian reward conditioning, in which the houselight was illuminated 6 s before sipper extension, which lasted for 20 s, followed by sipper retraction and houselight shut-off. Licks were recorded via a contact lickometer and registered in MedPC. The cue–sipper pairing was presented in 20 pseudorandom trials with ITIs of 30, 60, 90 and 120 s. For all photometry experiments, a 1,080-px webcam was mounted above the chamber for subsequent mouse behaviour analysis. ‘Approach’ behaviours were classified as nose or head entrances into a 3 inch by 3 inch square area around the sipper port, and ‘exits’ were classified as instances of leaving this area. For engagement in reward-seeking behaviours, we analysed the percentage of time the mouse spent pawing, sniffing, licking or poking at the reward port in three defined time windows: 0–6 s (cue period, pre-sipper), 0–26 s (entire cue period) or 26–46 s (post-cue–sipper time period). ‘Null’ behaviours were classified as any non-engagement, approach or exit behaviour lasting more than 1 s in duration. These behaviours were analysed in a blinded manner. For creation of the transition diagrams, ‘null’ and ‘exit’ behaviours were grouped for visualization purposes. The same Pavlovian protocol (identical cue–reward pairings, ITI and sucrose concentration) was used for conditioning in the linear track (see below).

Operant condition. Using the same apparatus for Pavlovian reward conditioning, we trained mice on FR1, FR3 and PR schedules of reinforcement. Animals nose-poked into illuminated nose-poke ports, randomly designated as ‘active’ or ‘inactive’. Following a second delay, an active nosepoke resulted in cue light presentation and subsequent protrusion of the sipper, which stayed protruded for 20 s. In a FR1 schedule of reinforcement, one nosepoke elicited sipper protrusion. Following 3 days of FR1 training, mice were trained in a FR3 schedule, which necessitated three consecutive active poke to elicit sipper protrusion. Finally, in the PR task, the number of nosepokes required to elicit sipper protrusion escalated exponentially as previously described⁶³.

Pavlovian fear conditioning and extinction. In separate Med Associates chambers (NIR-022MD), mice were trained in Pavlovian fear conditioning. The chamber consisted of a conductive grid floor (29.53 × 23.5 cm) illuminated by an infrared light. Mice were conditioned to ten 20-kHz tones lasting 10 s, co-terminating with a 2 s 0.5-mA shock. Tones were presented in a pseudorandom pattern. For fear extinction, mice were exposed to 20 20-Hz tones lasting 20 s for 5 consecutive days. Freezing behaviours were calculated manually by an experimenter blinded to animal group and genotype.

In vivo optogenetics

For all experiments using Chr2, we used a 20-Hz stimulation frequency with a power output between 6 and 8 mW (measured at fibre tip). For all experiments using PPO, we used a 10-Hz stimulation frequency with a power output between 8 and 10 mW.

Pavlovian reward conditioning. For the reward-conditioning experiments, mice were exposed to the identical training regimen as described above. For modulation of reward-seeking behaviours, we used two separate stimulations paradigms for Chr2. On counter-balanced days, mice either received 20-Hz stimulation throughout the duration of the cue period (26 s) or received closed-loop 20-Hz stimulation in which each lick triggered (via TTL) a 2-s stimulation pulse. For PPO, we drove 10-Hz inhibition via a TTL pulse, which began the stimulation pattern 5 s before houselight shut-off and continued for 20 s after houselight shut-off.

Pavlovian fear conditioning. For the reward-conditioning experiments, mice were exposed to the identical training regimen as described above. Mice received optical stimulation or inhibition on day 1 of extinction (fear recall day). For Chr2, mice received 20-Hz stimulation time locked to cue presentation (20 s). For PPO, mice received 10-Hz inhibition time locked to cue presentation (20 s).

Operant self-stimulation. Using the operant boxes described above, mice were trained in an FR1 schedule of reinforcement. One nosepoke either elicited a 2-s 20-Hz stimulation (Chr2) or a 10-Hz inhibition (PPO) pulse.

Linear track recordings

For high-resolution point-of-interest and kinematic behavioural analysis, a linear track behaviour corridor was utilized to capture dual perspectives of animal positions and behaviour (bottom-up and side profile). The chamber was modified from the original published by Machado et al.⁶⁴ with a mirror positioned at a 45° angle below a clear Plexi-glass floor. A single high-frame rate camera was used to capture minute, quick animal actions during experimental windows (Basler ace camera: acA800-150 μm). The linear track was illuminated with four infrared floodlights and two supportive infrared lights positioned around the chamber to brighten all angles of animal positions. All videos were recorded at 60 frames per second. The camera was operated via a custom Python script, which allowed for manipulation of session length, exposure and capture rate (GitHub), outputting compiled mp4 videos for SLEAP analysis. The maximum session length was set to 40 min, although most recordings were stopped before this point, coinciding with the end of the behavioural assay.

Machine learning behavioural tracking

Tracking and pose estimation of videos within the linear track was performed using the SLEAP platform, an open-source computer vision system utilizing a custom convolutional neural network designed for variational animal behaviour tracking⁴⁴. A custom, in-house tracking algorithm was developed for our linear track chamber using a subset of 1,767 frames from 313 videos of adult (6 weeks to 6 months of age) male (51%) and female (49%) mice in different lighting conditions to ensure model robustness and versatility. This model was utilized to track 29 selected points of interest across all frames of all behavioural videos and were then exported as .h5 files for subsequent analysis. These 29 points were selected to represent the status of the animal from the bottom-up and side profiles in a single-animal model (for example, nose-side profile and tail-base-bottom profile). Of the total training frames, 50% were randomly selected by SLEAP and 50% were hand-picked for valuable frames to improve model accuracy. Final model precision and recall were 97.15% and 98.74%, respectively. An analysis package was used to export the positional data and calculate features for respective classification models.

Supervised analysis of standard behaviours

Identification of core behavioural responses within the linear track was performed with an automated supervised machine learning algorithm (random forest classifier) to identify frame-by-frame instances of

Article

walking, rearing, grooming and laying on belly. This model was trained on 1.4 million hand-scored frames of naturalistic behaviour within the linear track from a dataset of 45 videos consisting of adult (6 weeks to 6 months of age) female (51%) and male (49%) mice. Videos of animals were treated with either vehicle or some drug to expand the model versatility across different behavioural modifications. The model was trained on 1.35 million frames, and a separated test set revealed a final precision and recall of 98.3% and 97.7%, respectively. Further code was used to isolate behavioural event timestamps from the supervised model to align neural data when applicable.

Engagement analysis

To evaluate mouse engagement with a sipper port, we built a pipeline that processes data from multiple experimental sessions. This pipeline takes three key inputs: (1) SLEAP joint output files in H5 format, containing joint coordinates of shape (2, 30, n), where n is the number of frames; (2) session names to be processed (for example, PENKtrans_M2_D5); and (3) sipper port locations as pixel coordinates in an array of shape (2, 2). Our preprocessing stage involves three key steps. First, we detached metadata from the joint files to isolate pose data. Next, we extracted the side head position, specifically the neck and nose coordinates, which are crucial for our analysis. Finally, we converted head and sipper locations from pixel coordinates to centimetres, allowing for standardized spatial analysis across different experimental setups.

Our analysis focused on three main aspects of mouse behaviour:

- (1) For distance calculation, we computed the Euclidean distance between the mouse's nose and the sipper port for each frame, and utilized NumPy's `linalg.norm` function; any length greater than the width of the box was clipped to the width value.
- (2) For head direction determination, we compared the x value of the neck position against the x values of the body positions. The body positions were averaged and then checked whether the x position of the neck was greater than the body average. If it was, we labelled the frame as facing right.
- (3) For turning identification, we identified turning behaviour based on the sign changes in the difference in distances. We only identified significant turns where the change in direction was greater than 1.5 cm. We also only analysed every second instead of the whole series to filter out noise.

To predict periods of engagement with the sipper port, we used a multi-step filtering process:

- (1) For naive engagement identification, we initially classified frames as engaged if the nose was within 2 cm of the sipper port.
- (2) For anatomical feasibility filter, we removed frames where the nose was more than 3 cm away from the neck, as these represent anatomically infeasible postures.
- (3) For direction filter, we excluded frames where the mouse was not facing the sipper, based on our head direction calculation.
- (4) For stability filter, we removed frames where the neck was more than 4 cm from the sipper, indicating unstable nose position.
- (5) For temporal coherence filter, we filtered out engagement periods shorter than 1 s, with a 0.5-s tolerance for brief disengagements, to focus on sustained engagement behaviours.

Closed-loop optogenetic stimulation

Setup.

- (1) Using a static frame capture, the coordinates of the side and bottom views of the sipper were determined.
- (2) A conversion factor for pixels to centimetres was obtained from the static frame by marking the lower left and lower right corners of the linear track. The factor was calculated by taking the length of the linear track in centimetres divided by the length of the linear track in pixels

Real-time tracking and analysis.

- (1) For collection of animal joints, key body positions (for example, neck, nose and truck) were determined using the aforementioned SLEAP pipeline.
- (2) For frame-level engagement detection, using the joints, engagement was detected as previously described. These data are pushed into a sliding window to allow for continuous detection and parameter adjustment.
- (3) For thresholded engagement detection, a TTL pulse was sent to the Arduino control box only if the number of engagements in the sliding window was equal to the threshold value.
- (4) For patterned optical stimulation, the TTL pulse triggered a 1-s 20-Hz pulse of 635-nm laser stimulation (approximately 6 mW), followed by a 1-s timeout period.

Post-hoc analyses.

- (1) For behavioural analysis, the collected joints were smoothed to account for missing or invalid values, and then fed into the aforementioned random forest classifier to identify basic behaviours (that is, walk, rear, groom and null). Joints were subsequently analysed in the aforementioned engagement processing pipeline.
- (2) For alignment to cue, binned behaviours (1-s bins) were subsequently aligned to the cue.

Configurations. For our use, we used an analysis window of 16 frames with a threshold of 4 frames with a 2-s block period. We used the default engagement detection setting, which we defined as the side and bottom view of the nose being 2 cm, and the side view of the neck being 3 cm from the sipper. We also decided that for joints to be valid, the side view of the nose needed to be 4 cm or less away from the side view of the nose, and the bottom view of the nose needed to be 4 cm or less away from the bottom view of the body centre.

One-photon imaging

At least 1 week after baseplate attachment, mice were habituated to a Inscopix nVista 2.0 miniscope for 30–35 min for at least 3 days in the linear track, during which mice also had access to sipper containing a 10% sucrose solution. During the habituation phase, optimal field of view, focus, gain and LED power were determined visually by assessing the activity of putative neurons in the field of view using the Inscopix data collection GUI. Gain values ranged between 2 and 6, and LED power was set between 20% and 50% of transmission range. Once determined, these parameters were held static for the remainder of the testing regimen.

Following this habituation period, mice were trained in Pavlovian reward conditioning as described above. Mice were scruffed and attached to the miniscope, and then immediately placed in the linear track. The Inscopix recordings were triggered directly by the MedPC program via a TTL pulse to ensure synchronization of event timestamps and calcium activity. The MedPC program was started 10 s after starting the linear track camera, and videos were synced post-hoc via visible behavioural events (cue on, sipper protraction and lick, among others). Animal rotations were compensated for by an automatic motorized commutator (Inscopix).

Calcium imaging analysis

Initial processing. All preprocessing was done using the Inscopix data processing software. Videos were initially spatially downsampled by 4 $\times$, followed by application of a bandpass filter (0.005–0.5 μm^{-1}). Lateral motion was corrected using the built-in motion correction feature in the Inscopix data processing software. Motion corrected tiffs were subsequently exported for cell segmentation and further analysis. Using custom MATLAB scripts (see GitHub), the motion-corrected tiff video was then processed using the constrained non-negative matrix

factorization approach, which has been optimized to isolate signals from individual putative neurons from microendoscopic imaging⁶⁵. Putative neurons were then manually sorted and registered using previously established criteria⁶⁶. For all analyses presented in this article, the non-denoised, non-deconvoluted traces were used. For each cell, the raw fluorescence trace was z-scored to a 10-s baseline period preceding the behavioural or task-related features presented in each figure.

Cell matching. For matching of cells across days, we used the CellReg GUI as detailed in Sheintuch et al.⁶⁷. In brief, maximum projection spatial footprints were extracted from the exported tiff files using a custom MATLAB code. Cell registration occurred in five subsequent steps:

- (1) Spatial footprints from day 1 and day 5 of Pavlovian reward conditioning were loaded into the CellReg GUI.
- (2) Field-of-view alignment was performed using rigid alignment, incorporating translations and rotations, with a maximal rotation of 30°.
- (3) Probabilistic modelling was run with a maximum distance of 12 μm .
- (4) Initial cell registration was performed using spatial correlations.
- (5) Final cell registration incorporated the probabilistic model from step 3 as well as the spatial correlations from step 4.

Principal component analysis and hierarchical clustering. For all Pavlovian reward conditioning imaging data presented in this article, cells were sorted based on their day 5 activity and then back-matched to day 1 using custom MATLAB codes (GitHub). For hierarchical clustering, a principal component analysis was initially performed based on the activity of the neurons on day 5 of Pavlovian reward conditioning over a 1-min window coinciding with cue onset for cue-associated activity and a 10-s window for engagement-associated activity. Using built-in MATLAB Linkage function, an agglomerative hierarchical clustering algorithm was applied to the principal component analysis of the day 5 activity. We set an a priori threshold that to be classified as a cluster, the number of neurons in said cluster must comprise more than 10% of the total population of recorded neurons (that is, more than 20 of 206 neurons). As agglomerative hierarchical clustering starts with n clusters = n cells, we began by setting the cluster number equal to our a priori threshold (that is, 10 clusters for 10% in each, assuming equal distribution among clusters). We then sequentially decreased cluster size by 1 until our a priori criteria were met.

Cluster validation with support vector machine

Input data. Principal component analysis and hierarchical clustering (deterministic) were done using MATLAB on both day 5 and day 1 data, and then export principal component coordinates and cluster labels. The cluster labels were based on the day 5 data, and the corresponding cell in day 1 got the same cluster label. All data have been exported as an Excel file.

Data preprocessing. The data and cluster labels were loaded and then split 80:20 into training and testing data, respectively. The 80% represent the training data and the remaining 20% is the testing data. Then, the data were then scaled by fitting sklearn's standard scaler to the training data and then applying it to the testing data.

Training the support vector machine. Used sklearn's Support Vector Classifier to perform multiclass classification using one versus one approach. Using 80% of the training data, we first performed cross-validation using stratified k -fold and random search. The cross-validation seem to suggest that the linear kernel performed best across different numbers of principal components. We then used the identified hyperparameters.

Classifying individual neuron activity. The fitted model was used to predict the classes for the testing data, as well as entirety of day 1 data. The F1 score and accuracy are reported.

Testing against pseudo-random data. The support vector machine was then fit to shuffled day 5 data (each time step values were shuffled), and then fit to day 5 data with shuffled cluster labels. In each case, NumPy's permutation was used. Again, accuracy and the F1 score were used to evaluate performance of the model.

Generalized linear model

Inputs. To analyse relationships between neural activity and behaviour during cue periods, we developed a pipeline that processes and aligns multiple behavioural measurements. This pipeline requires four inputs: (1) SLEAP joint output files in H5 format, containing joint coordinates of shape $(2, 30, n)$, where n is the number of frames; (2) engagement periods as timestamp pairs in Excel format; (3) lick events as timestamp pairs in Excel format; and (4) cue-period timestamps in Excel format.

Data preprocessing. Our preprocessing involves three main stages. First, we converted all behavioural events into binary time series, where 1 indicates the presence of a behaviour and 0 indicates its absence. Second, we extracted additional behavioural states (walk, rear, groom and rest) using a pre-trained random forest classifier on the SLEAP joint data. Finally, we identified disengagement events, defined as the first frame following each engagement period.

Behaviour alignment. We aligned all behavioural time series to cue onset, extracting data from 10 s pre-cue to 34 s post-cue. For each session, we aggregated behaviour across trials by summing the binary arrays rather than averaging them. This summation approach created a behavioural density measure that better corresponds to our calcium imaging data structure, which represents aggregate neural activity across the session rather than trial-level responses. Using density measures instead of trial-averaged behaviour ensured that our predictor variables (behavioural measures) maintained the same temporal structure as our response variables (calcium signals). These density measures were then z-scored to standardize their scale across sessions and behaviours.

Model fitting. We used two levels of generalized linear model analysis:

- (1) For cluster-level modelling, we fit generalized linear models using the normalized behavioural densities as predictors and mean cluster activity as the response variable. We used a Gaussian distribution with an identity link function.
- (2) For neuron-level modelling, individual neuron activity served as the response variable, with the same model structure as the cluster-level analysis.

For both analyses, we extract and store model coefficients (β), their associated P values and model intercepts. As we used the identity link function, the β -coefficients directly represent the strength and direction of the relationship between each behaviour and neural activity.

Model validation. We used residual analysis to verify model assumptions, we saw a relatively low mean residual value and standard deviation.

Phase analysis

Inputs. The analysis required at least two time series that were able to be loaded as NumPy arrays; they should have the same sampling rate and length. The analysis assumes that each element corresponds to each other. In our case, the time series was exported from MATLAB after preprocessing and linear interpolation to ensure sampling rate and length match. No further data preprocessing was necessary.

Extracting instantaneous phase. The instantaneous phase of each time series was extracted using the Hilbert transform, creating an analytic signal, which is a complex-valued function that extends a real-valued function to the complex plane. The instantaneous phase was then

Article

obtained by taking the angle of this analytic signal. SciPy signal's Hilbert function in conjunction with NumPy was used to extract the phases.

Calculating phase difference. The phase difference between the two signals was computed by subtracting the instantaneous phase of one signal from the other. This difference was then wrapped to the range $[-\pi, \pi]$ to ensure that it fell within a single cycle.

Computing R and theta mean. To quantify the consistency of the phase difference across time, we use circular statistics. Specifically:

- (1) For R (mean resultant length), this measure represents the concentration of phase differences around the mean. It is calculated by:
 - (a) Converting each phase difference to a unit vector on the complex plane (using cosine for the real part and sine for the imaginary part).
 - (b) Taking the mean of these vectors.
 - (c) Calculating the magnitude of this mean vector.
- (2) For theta mean (mean phase difference), this is the average angle of the phase differences. It is calculated by:
 - (a) Taking the arctangent of the ratio of the mean sine to the mean cosine of the phase differences.

R ranged from 0 to 1, where 0 indicates uniformly distributed phases and 1 indicates perfectly consistent phase differences.

Rayleigh plot. The analysis results were then plotted in a polar graph, where each phase difference was plotted along with an arrow representing the R and mean theta values.

Statistical analysis

Unless otherwise noted, all summary data are expressed as mean $\pm$ s.e.m. or mean $\pm$ maximum variance. Statistical significance was taken as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$, as determined by the Student's *t*-test (paired and unpaired), linear regression, one-way analysis of variance (ANOVA) and two-way repeated-measures ANOVA, followed by Holm-Sidak post-hoc tests as appropriate. For all datasets presented in this article, the Grubbs outlier test was performed individually on each group, with outliers removed for $\alpha < 0.05$. If a metric (for example, engagement) from a subject for a particular experiment exceeded the outlier threshold, other metrics (for example, $\Delta F/F$) from that same subject from the same experiment were excluded from the dataset. Statistical analyses were performed in GraphPad Prism 9.0 or 10.0. Animals were pseudo-randomly assigned groups as designated by an a priori power analysis and sex balancing. Behavioural scoring was conducted by a researcher in a blinded manner.

List of excluded data

- Electrophysiology recording of PPR of D1R⁺ neurons with SR1 cell 12: Grubbs outlier test.
- Electrophysiology recording of PPR of D1R⁺ neurons with vehicle cell 12: Grubbs outlier test.
- Electrophysiology recording of PPR of D1R⁺ neurons with JZL cell 9: Grubbs outlier test.
- Electrophysiology recording of the maximum amplitude of D1R⁺ neurons with JZL cell 8: Grubbs outlier test.
- Electrophysiology recording of the maximum amplitude of D1R⁺ neurons with SR1 cell 1: Grubbs outlier test.
- In vivo optogenetics Pavlovian reward-conditioning Chr2 subject 4: never licked.
- In vivo optogenetics fear recall Chr2 subject 2: fibre lost.
- aPVT sgRosa CRISPR vehicle Pavlovian subject 4: Grubbs outlier test.

- aPVT sgCnr1 CRISPR fear-conditioning subject 7: fibre lost.
- aPVT–Nac GcAMP Pavlovian reward-conditioning day 2 engagement subject 10: video did not capture.
- BLA sgRosa vehicle Pavlovian subject 1: Grubbs outlier test.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data associated with this project have been uploaded to Figshare (https://figshare.com/authors/David_Marcus/23255861).

Code availability

All code associated with this project has been uploaded to Figshare (https://figshare.com/authors/David_Marcus/23255861).

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Author contributions D.J.M. and M.R.B. conceptualized the study. D.J.M., M.R.B., B.B.L., L.S.Z., Y.L., N.S., A.E.E. and G.C. formed the methodology. D.J.M., G.C., E.F.S., R.O., S.H., B.A.W., S.J.K., A.A.P. and A.S. conducted the investigation. D.J.M., G.C., S.C.P., A.E.E. and E.A. performed the data analysis. D.J.M. and M.R.B. performed the visualization. D.J.M., B.B.L. and M.R.B. acquired funding. D.J.M. and M.R.B. wrote the original draft of the manuscript. D.J.M., M.R.B., B.B.L. and N.S. reviewed and edited the manuscript.

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Competing interests N.S., B.B.L. and M.R.B. are co-founders and members of the Scientific Advisory Board of Biosyft, LLC, and A.E.E. is the Chief Executive Officer of Biosyft, LLC. Biosyft is developing and commercializing applications related to some of the technologies described in this manuscript. While the specific findings reported here are not directly aligned with Biosyft's current commercial activities, given the related nature of the technology and its potential applications, the authors disclose these relationships. The other authors declare no competing interests.

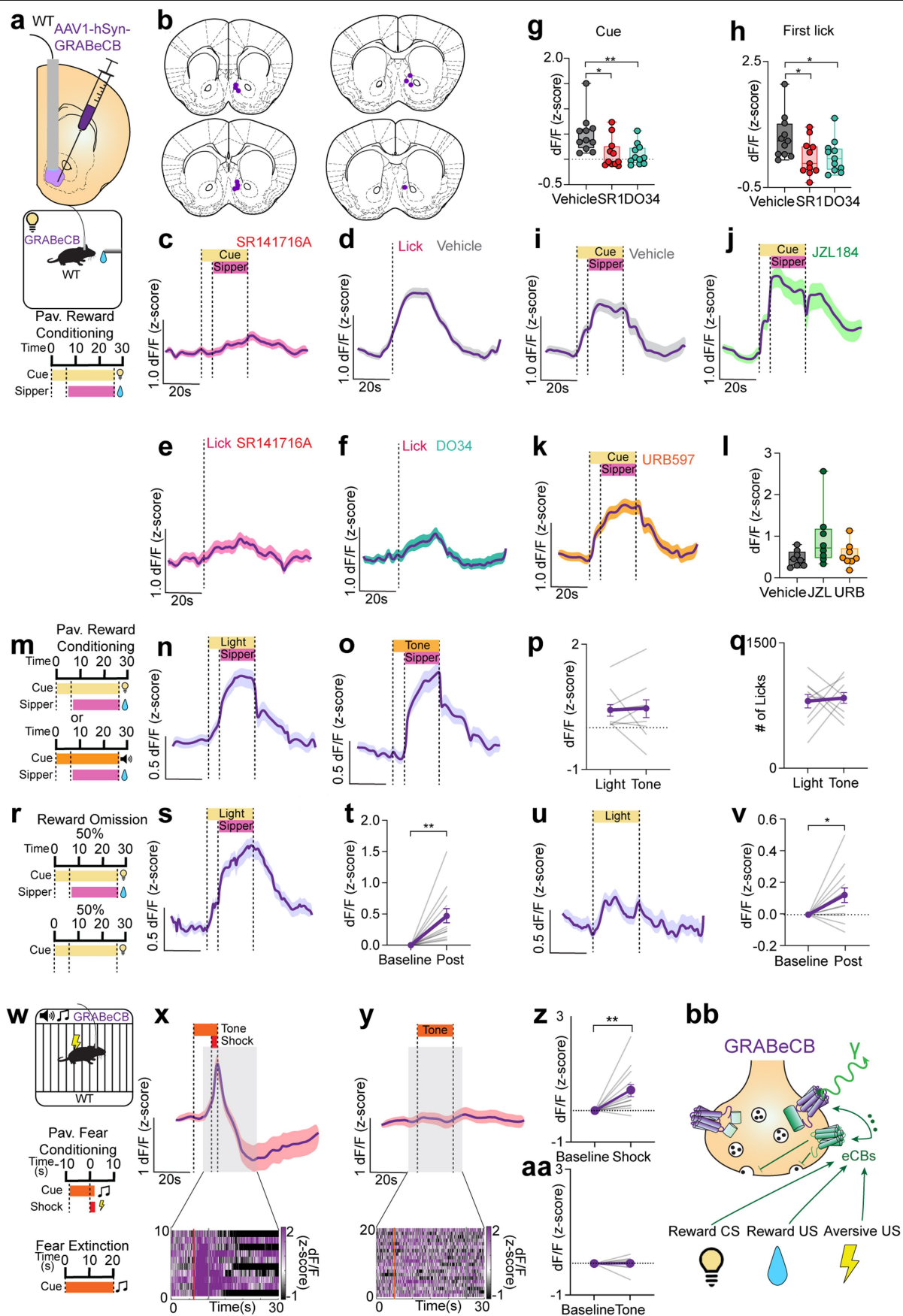
Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41586-026-10967-w>.

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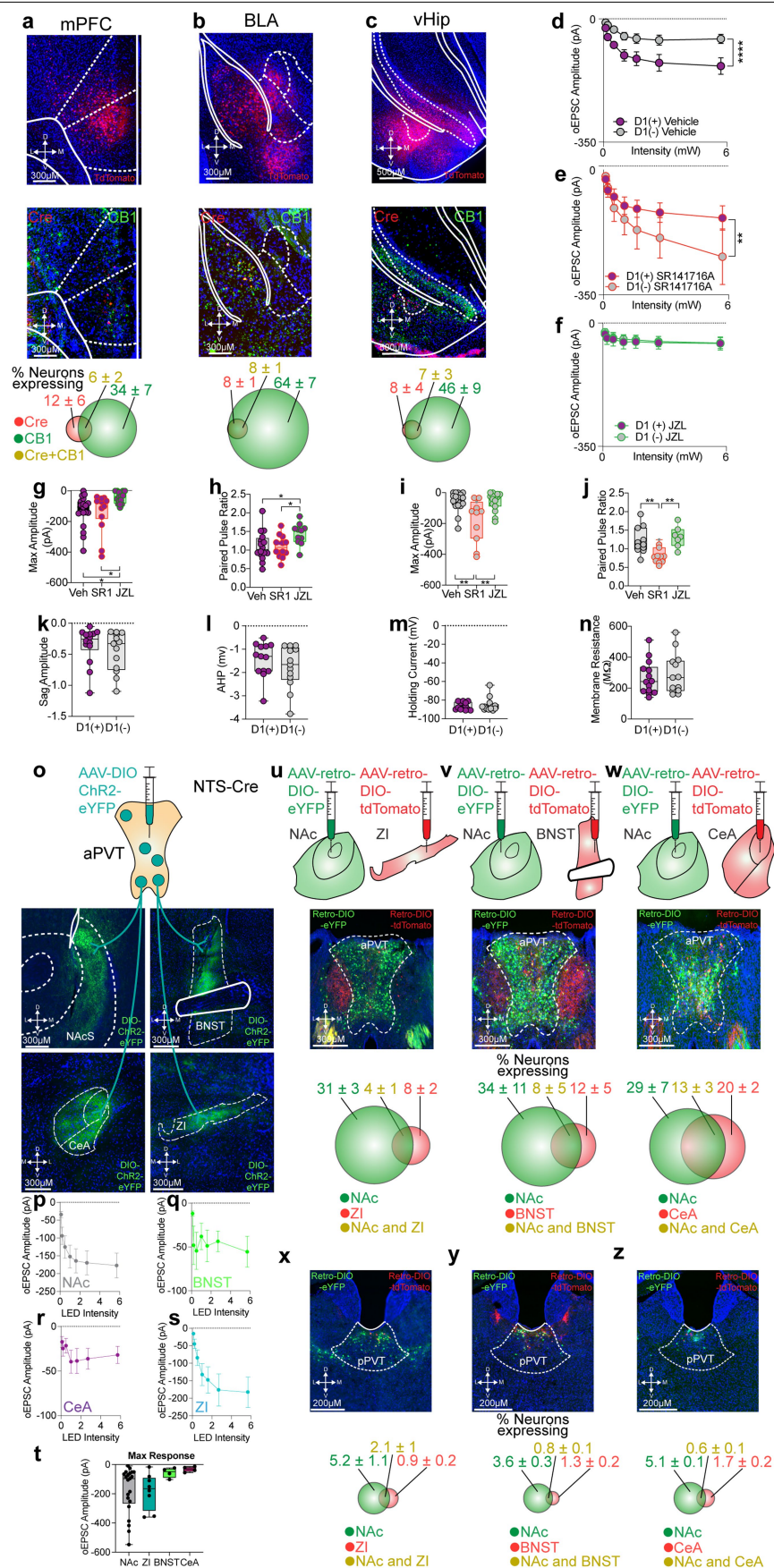
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Extended Data Fig. 1 | See next page for caption.

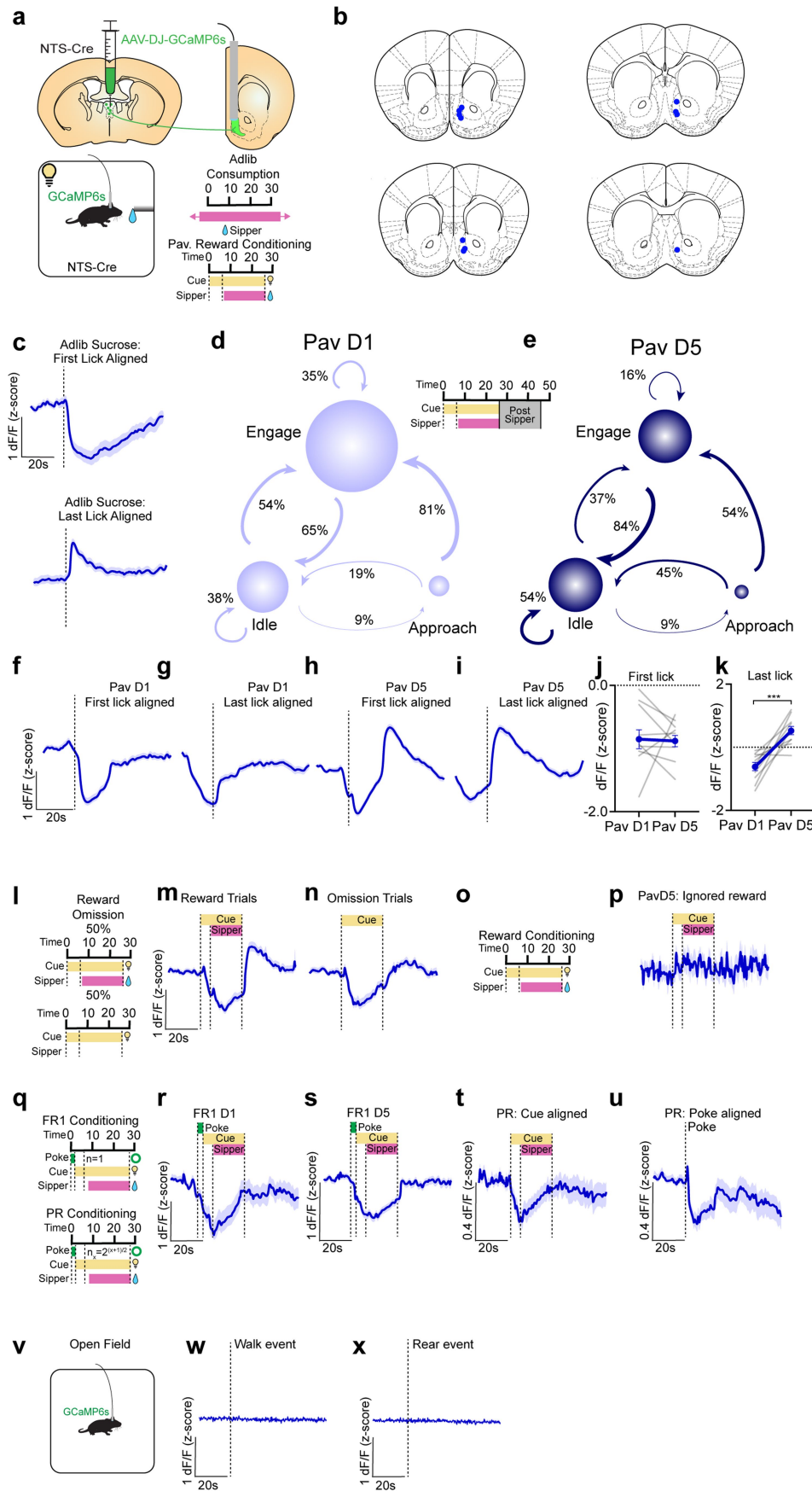
Extended Data Fig. 1 | 2-AG is released in the NAc following rewarding stimuli. (a) Schematic for fiber photometry recordings of GRAB_{eCB2.0} in the NAc during Pavlovian reward conditioning. (b) Fiber placements for GRAB_{eCB2.0} cohort displayed in Fig. 1. (c) Photometry trace of cue-aligned GRAB_{eCB2.0} signal in SR141716A (10 mg/kg) treated animals (N = 11). (d-f) Photometry trace of lick-aligned GRAB_{eCB2.0} signal in vehicle, SR141716A (10 mg/kg), and DO34 (20 mg/kg) treated animals (N = 11). (g) Comparison of cue-aligned GRAB_{eCB2.0} signal (0-26 s post cue) showing attenuation of GRAB_{eCB2.0} signal (0-20 s post lick) in SR141716A (p = 0.0131) and DO34 (p = 0.0079) treated animals compared to vehicle (N = 11). (h) Comparison of lick-aligned (0-20 s) GRAB_{eCB2.0} signal in SR141716A (p = 0.0343) and DO34 (p = 0.0469) treated animals compared to vehicle (N = 11). (i-k) Photometry traces of cue-aligned GRAB_{eCB2.0} signal in vehicle, JZL184 (5 mg/kg), and URB597 3 mg/kg treated animals (N = 8). (l) Comparison of cue-aligned (0-26 s) GRAB_{eCB2.0} signal in JZL184 (p = 0.0914) and URB597 (p = 0.6983) treated animals compared to vehicle (N = 8) (m) Schematic for Pavlovian reward conditioning with different cue modalities (n,o) Photometry traces of light and tone cue aligned GRAB_{eCB2.0} signal (N = 7).

(p) Comparison of light and tone aligned GRAB_{eCB2.0} signal (N = 7). (q) Comparison of number of licks in light and tone Pavlovian reward conditioning sessions (N = 7). (r) Schematic for reward omission assay. (s,t) Photometry trace and quantification of cue-aligned (0-26 s) GRAB_{eCB2.0} signal during rewarded trials in the reward omission assay (N = 11). (u,v) Photometry trace and quantification of cue-aligned (0-26 s) GRAB_{eCB2.0} signal during non-rewarded trials in the reward omission assay (N = 11). (w) Schematic for Pavlovian fear conditioning/extinction. (x,y) Photometry trace of GRAB_{eCB2.0} signal during Pavlovian fear conditioning and extinction with representative heatmaps (N = 11). (z,aa) Comparison of GRAB_{eCB2.0} signal during foot shock (0-5 s, p = 0.0049) and cued recall (0-20 s, p = 0.8987) (N = 11). (bb) Summary schematic of elicited eCB release in the NAc. Error bars represent $\pm$ SEM (p,q,t,v,z,aa), or min/max values (g,h,i,l); box plots represent interquartile range with line at median; N represents number of mice. p values reported from one-way ANOVA (g,h,i,l) and two-tailed paired t test (p,q,t,v,z,aa). *p < 0.05, **p < 0.01.



Extended Data Fig. 2 | Histological analysis of CB₁R expressing inputs to the NAc and histological/electrophysiological characterization of excitatory aPVT^{NTS} projections. (a-c) (Top) TdTomato positive NAc projecting neurons in the medial prefrontal cortex (mPFC) Basolateral Amygdala (BLA) and ventral Hippocampus (vHip) and (bottom) RNAscope image and quantification of Cre and CB₁R transcript levels in the mPFC BLA and vHip (N = 3, 3, 3). (d-f) Optically evoked input-output curve from D₁R+ and D₁R- neurons in vehicle (D₁R+ n = 19, D₁R- n = 20, $7.36 \cdot 10^{-5}$), SR141617A (D₁R+ n = 14, D₁R- n = 12, p = 0.0050), and JZL184 (D₁R+ n = 17, D₁R- n = 15, p = 0.9698) treated slices. (g,h) 1 μ M JZL184 treatment significantly reduces the maximum oEPSC amplitude (p = 0.0193) and increases the Paired Pulse Ratio (p = 0.0355) in D₁(+) neurons compared to vehicle treated slices (Veh: n = 19, SR141617A: n = 14, JZL184: n = 16). (i,j) 1 μ M SR141617A significantly increases the maximum oEPSC amplitude (p = 0.0016) and reduces the Paired Pulse Ratio (p = 0.0092) in D₁(-) neurons (Veh: n = 20, SR141617A: n = 11, JZL184: n = 15). (k-n) Electrophysiological comparison of sag

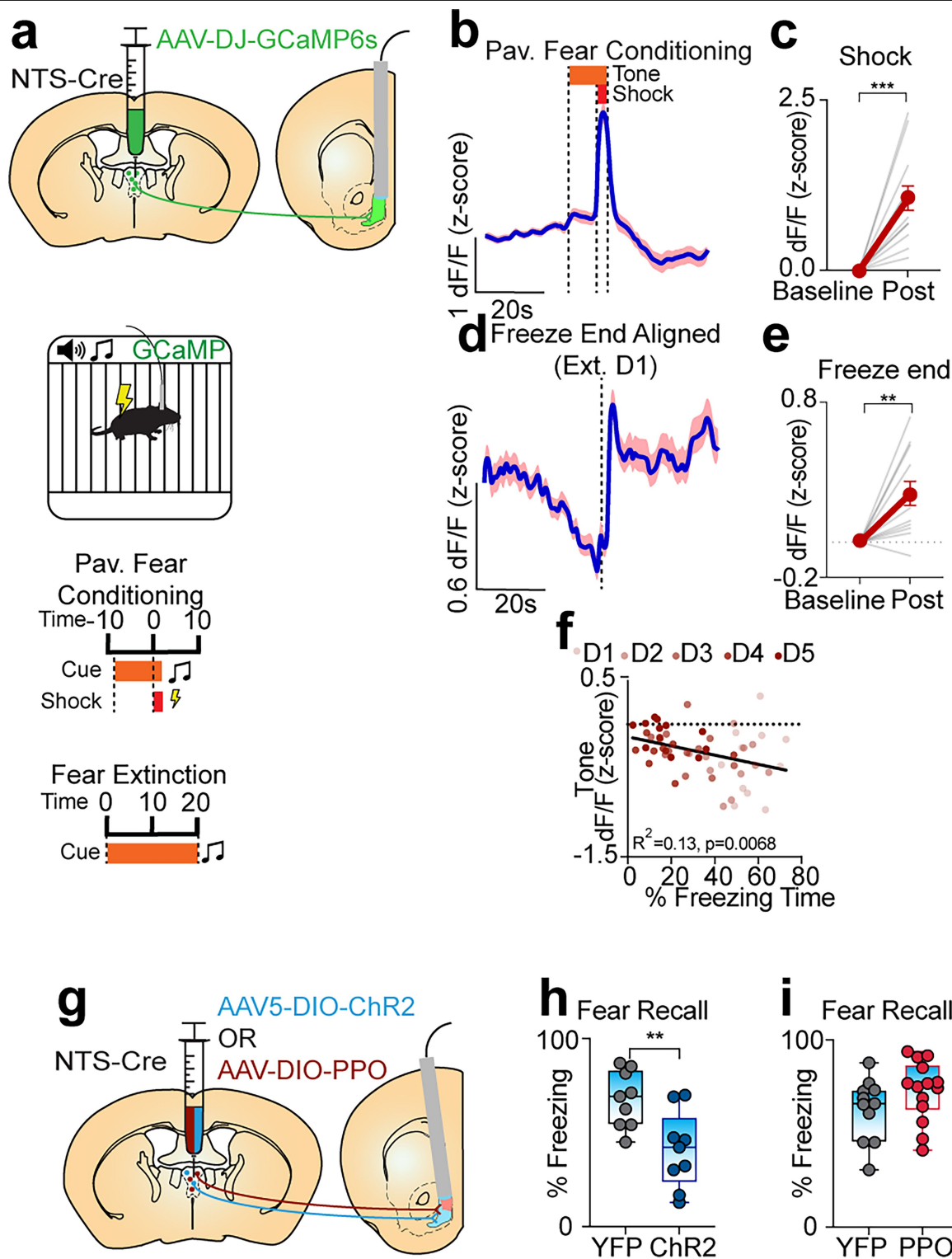
current amplitude (p = 0.3871) after hyperpolarization voltage (p = 0.3404), holding current (p = 0.6077), and membrane resistance (p = 0.6166) between D₁(+) (n = 14) and D₁(-) (n = 13) neurons. (o) Schematic for electrophysiological characterization of aPVT output to NAc, BNST, CeA, and ZI. (p-s) Optically evoked input/output curves for aPVT input to the NAc (n = 21), ZI (n = 8) BNST (n = 4), and CeA (n = 4). (t) Comparison of maximal aPVT evoked oEPSC in the NAc, ZI, BNST, and CeA. (u-w) Schematics, representative images, and quantification of collateralization of aPVT projections to the NAc and the ZI, BNST, and CeA, respectively (n = 3, 3, 3). (x-z) Representative images and quantification of collateralization of pPVT projections to the NAc and the ZI, BNST, and CeA, respectively. Error bars represent $\pm$ SEM (d-f, p-s), or min/max values (g-n, t); box plots represent interquartile range with line at median; n represent number of neurons. p values reported from two-tailed unpaired t-test (k-n), one-way ANOVA (g-j), and two-way ANOVA with Holm-Sidak post-hoc correction (d-f). *p < 0.05, **p < 0.01, ****p < 0.0001.



Extended Data Fig. 3 | See next page for caption.

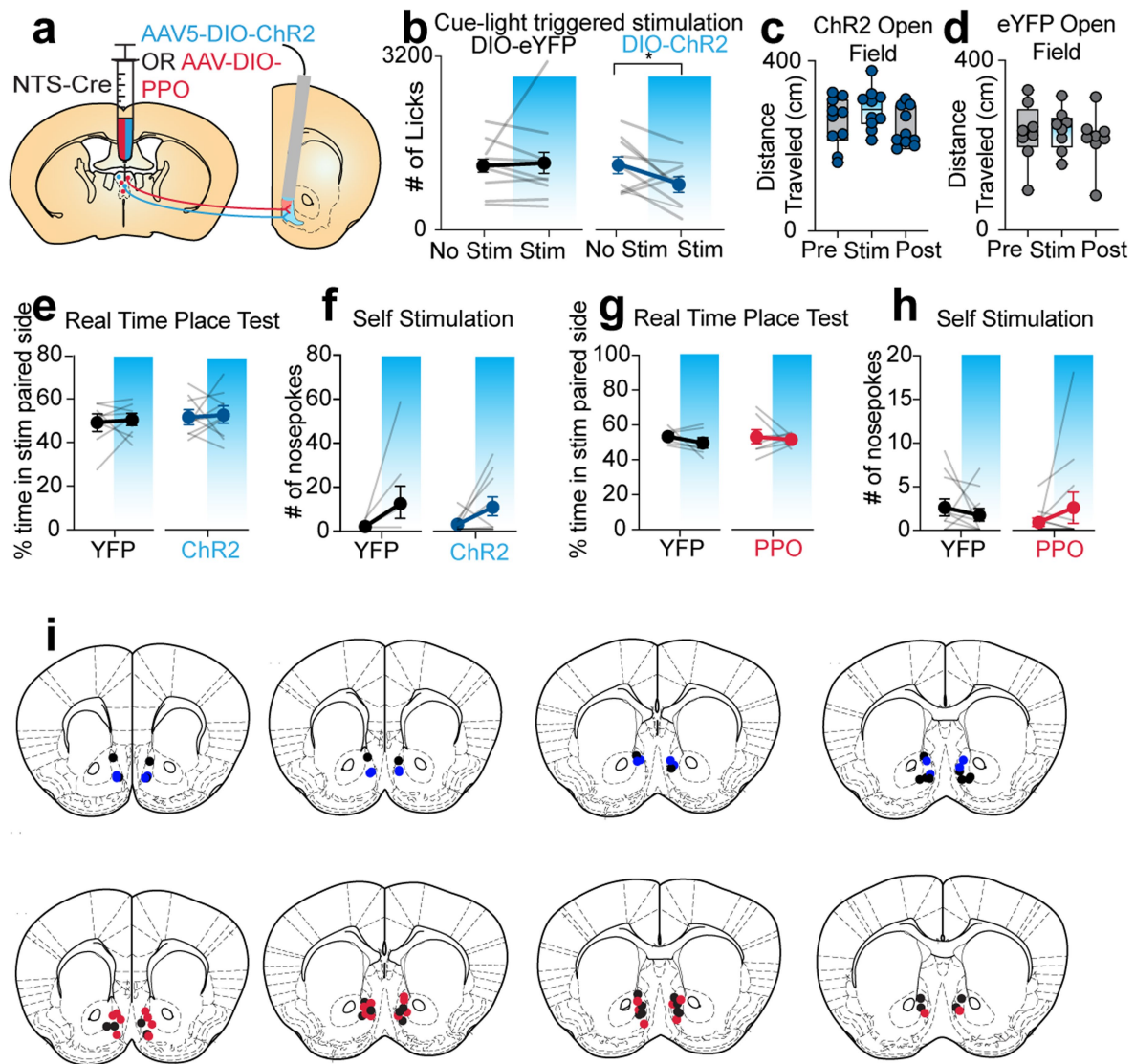
Extended Data Fig. 3 | Inhibition of aPVT^{NTS}-NAC terminals is not dependent on reward receipt and is not associated with locomotor activity. (a) Schematic for fiber photometry recordings from GCaMP6s expressing aPVT^{NTS}-NAC terminals during ad libitum sucrose consumption and Pavlovian reward conditioning. (b) Fiber placements for aPVT^{NTS}-NAC GCaMP cohort displayed in Fig. 2. (c) Photometry trace of GCaMP6s signal aligned to first and last licks in a licking bout during ad libitum consumption (N = 10). (d,e) Transition diagram showing probabilities of transitions between approach, engage, and null/exit behaviors on day 1 and day 5 of conditioning during the post-sipper time window, with size of circles corresponding to number of events. (f,g) Photometry traces of GCaMP6s signal aligned to first lick and last lick on day 1 of reward conditioning. (h,i) Photometry traces of GCaMP6s signal aligned to first lick and last lick on day 5 of reward conditioning. (j,k) Quantification of first lick (0-20 s, $p = 0.8736$, N = 10) and last lick (0-20 s, $p = 0.0004$, N = 10) aligned GCaMP6s signals on day 1 and day 5 of reward conditioning. (l) Schematic for fiber photometry recordings from GCaMP6s expressing aPVT^{NTS}-NAC terminals during reward omission. (m,n) Photometry traces of GCaMP6s signal

during rewarded and non-rewarded trials during the reward omission test, demonstrating aPVT^{NTS}-NAC terminal inhibition in the absence of reward receipt. (o) Schematic for fiber photometry recordings from GCaMP6s expressing aPVT^{NTS}-NAC terminals during reward conditioning. (p) Photometry trace of GCaMP6s signal on day 5 of reward conditioning during ignored cue/reward trials (no reward engagement), demonstrating a lack of inhibition when the cue/reward is ignored. (q) Schematic for fiber photometry recordings from GCaMP6s expressing aPVT^{NTS}-NAC terminals during Fixed Ratio 1 (FR1) training and Progressive Ratio (PR) training. (r,s) Photometry traces of GCaMP6s signal aligned to cue on day 1 and day 3 of FR1 conditioning, demonstrating inhibition during active operant responding for reward. (t,u) Photometry traces of GCaMP6s signal aligned to cue and nose poke during PR conditioning. (v) Schematic for fiber photometry recordings of GCaMP6s from aPVT^{NTS}-NAC terminals during the open field assay. (w,x) Photometry traces of GCaMP6s signal aligned to walk and rear events during the open field assay. All error bars represent $\pm$ SEM; n represent number of neurons. p values reported from two-tailed paired t-test (j,k). *** $p < 0.001$.



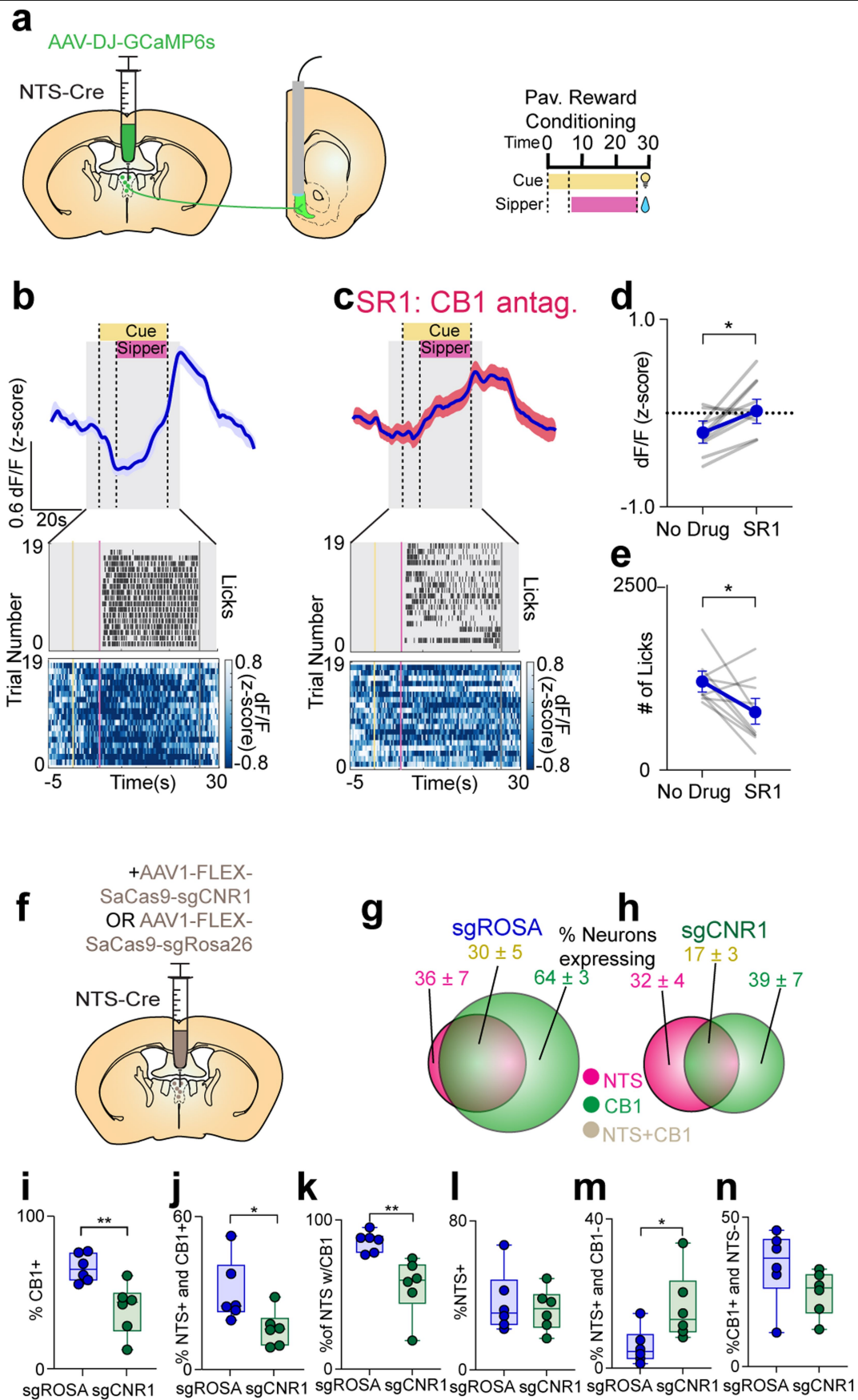
Extended Data Fig. 4 | aPVT^{NTS}-Nac terminal activity is negatively correlated with engagement in defensive freezing behaviors. (a) Schematic for fiber photometry recordings from GCaMP6s expressing aPVT^{NTS}-Nac terminals during Pavlovian fear conditioning and extinction. (b) Photometry trace of GCaMP6s signal during Pavlovian fear conditioning. (c) Quantification of GCaMP6s signal during foot shock (0-5 s, N = 11, p = 0.0002). (d) Photometry trace of GCaMP6s signal aligned to freeze end on extinction day 1. (e) Quantification of GCaMP6s signal aligned to freeze end (0-20 s, N = 11, p = 0.0024). (f) Correlation between freezing time and tone-aligned photometry Z-score (0-20 s) across 5 days of extinction (N = 11, R² = 0.13,

p = 0.0068). (g) Schematic for optogenetic manipulation of aPVT-Nac terminals using DIO-ChR2 and DIO-PPO. (h) 20hz optical stimulation ChR2 (N = 9) expressing aPVT^{NTS}-Nac terminals during the 20 second cue presentation decreases freezing time compared to eYFP (N = 9) controls (p = 0.0053). (i) 10hz optical stimulation of PPO (N = 14) expression aPVT^{NTS}-Nac terminals does not alter freezing behavior compared to eYFP (N = 11) controls (p = 0.1470). Error bars represent $\pm$ SEM (c,e), or min/max values (h,i); box plots represent interquartile range with line at median; N represent number of mice. p values reported from paired two-tailed t-test (c,e), unpaired two-tailed t-test (h,i) and simple linear regression (f). **p < 0.01, ***p < 0.001.



Extended Data Fig. 5 | Neither activation nor inhibition of aPVT^{NTS}-NAC terminals alters locomotion, drives real-time place preference (RTPP), or drive intracranial self-stimulation (ICSS). (a) Schematic for optogenetic manipulation of aPVT^{NTS}-NAC terminals using DIO-ChR2 and DIO-PPO. (b) 20hz photo-stimulation of ChR2 expressing aPVT^{NTS}-NAC terminals during cue presentation reduces engagement in consummatory behaviors (ChR2: N = 9 p = 0.0427, eYFP: N = 9, p = 0.8190). (c,d) Neither 20hz photo-stimulation (2 mins off, 2 mins on, 2 mins off) of ChR2 expressing aPVT^{NTS}-NAC terminals (N = 10, p = 0.3655) nor eYFP expressing aPVT^{NTS}-NAC terminals (N = 8, p = 0.8947) alters locomotor behavior. (e) 20hz photo-activation of aPVT^{NTS}-NAC terminals does not support RTTP (ChR2: N = 10 p = 0.8141, eYFP:

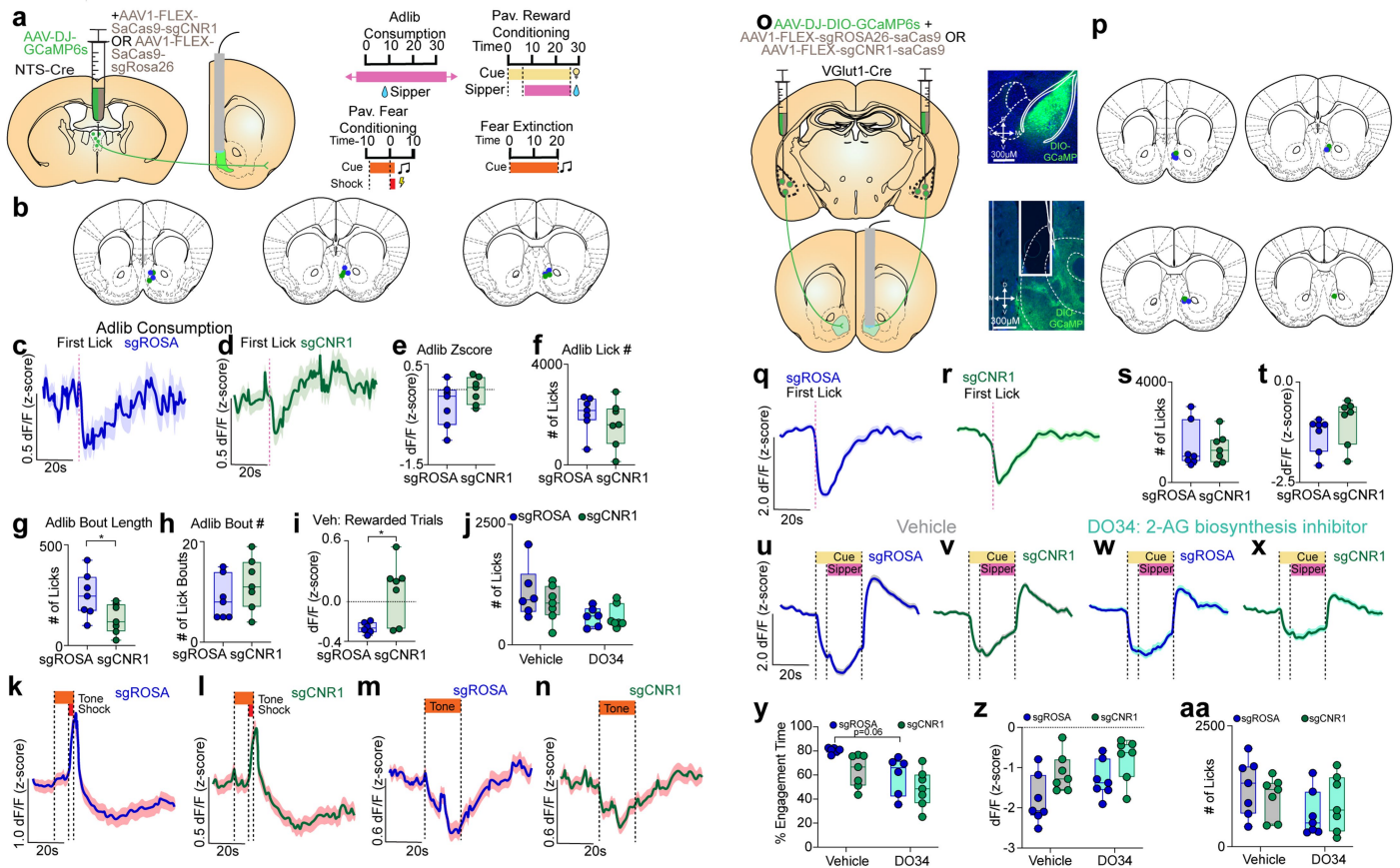
N = 9 p = 0.7191). (f) 20hz photo-activation (2 second burst per nose poke) of aPVT^{NTS}-NAC terminals does not support ICSS (ChR2: N = 10 p = 0.0968, eYFP: N = 8, p = 0.1670). (g) 10hz photo-inhibition of aPVT^{NTS}-NAC terminals does not support RTTP (PPO: N = 7 p = 0.7492, eYFP: N = 6 p = 0.3216). (h) 10hz photo-inhibition of aPVT^{NTS}-NAC terminals does not support ICSS (PPO: N = 13 p = 0.2565, eYFP: N = 11 p = 0.4527). (i) Fiber placements for optogenetics cohort displayed in Fig. 2. Error bars represent $\pm$ SEM (b,e-h), or min/max values (c,d); box plots represent interquartile range with line at median; N represent number of mice. p values reported from paired two-tailed t-test (b,e-h), and one-way ANOVA (c,d). *p < 0.05.



Extended Data Fig. 6 | See next page for caption.

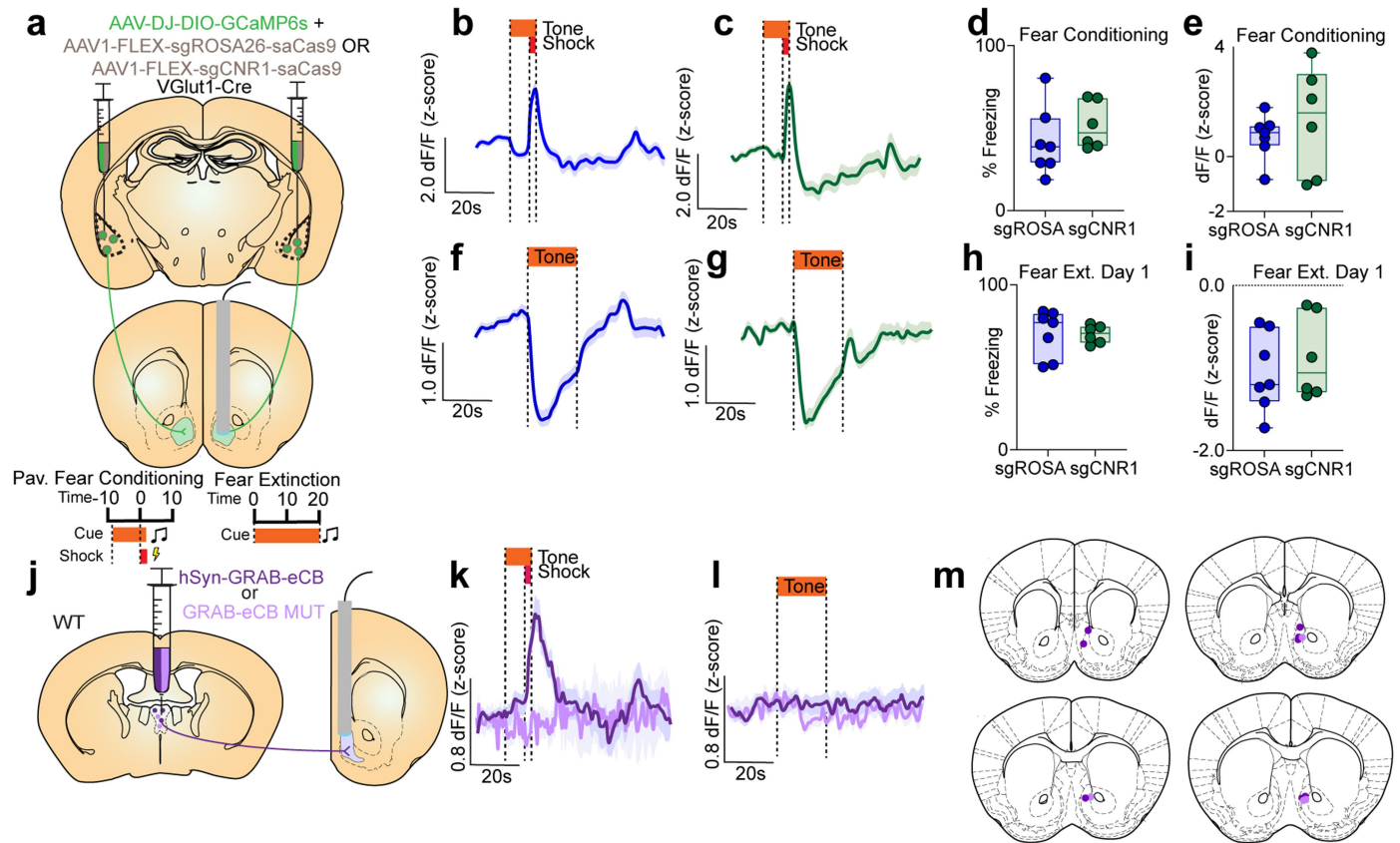
Extended Data Fig. 6 | Effect of CB₁R antagonism on inhibition of aPVT^{NTS}. NAc terminals and characterization of AAV1-FLEX-sgCNR1 deletion of CB₁R from aPVT NTS neurons. (a) Schematic for fiber photometry recordings from GCaMP6s expressing aPVT^{NTS}. NAc terminals during Pavlovian reward conditioning. (b,c) Photometry trace with representative lick raster and heatmap of cue-aligned GCaMP6s signal in control mice or mice treated with 10 mg/kg SR141716A (N = 10). (d,e) Effect SR141716A on cue-aligned photometry Z-score (p = 0.0064) and total number of licks (p = 0.0270) throughout the session (N = 10). (f,g,h) Diagram showing % of neurons expressing NTS, CB₁R, or NTS and CB₁R in the aPVT from NTS-Cre mice injected with sgROSA (N = 6) or

sgCNR1 (N = 6). (i) Quantification of % of total neurons with CB₁R transcript (p = 0.0064). (j) % of NTS neurons CB₁R transcript (p = 0.0333). (k) % of NTS neurons expressing CB₁R (p = 0.0055). (l) % of total neurons expressing NTS (p = 0.6507). (m) % of total neurons that are NTS positive and CB₁R negative (p = 0.0354). (n) % of total neurons that are CB₁R positive and NTS negative (p = 0.1512). Error bars represent $\pm$ SEM (d,e), or min/max values (i-n); box plots represent interquartile range with line at median; N represent number of mice. p values reported from paired (d,e) and unpaired (i-n) two-tailed t-test. *p < 0.05, **p < 0.01, ***p < 0.001.



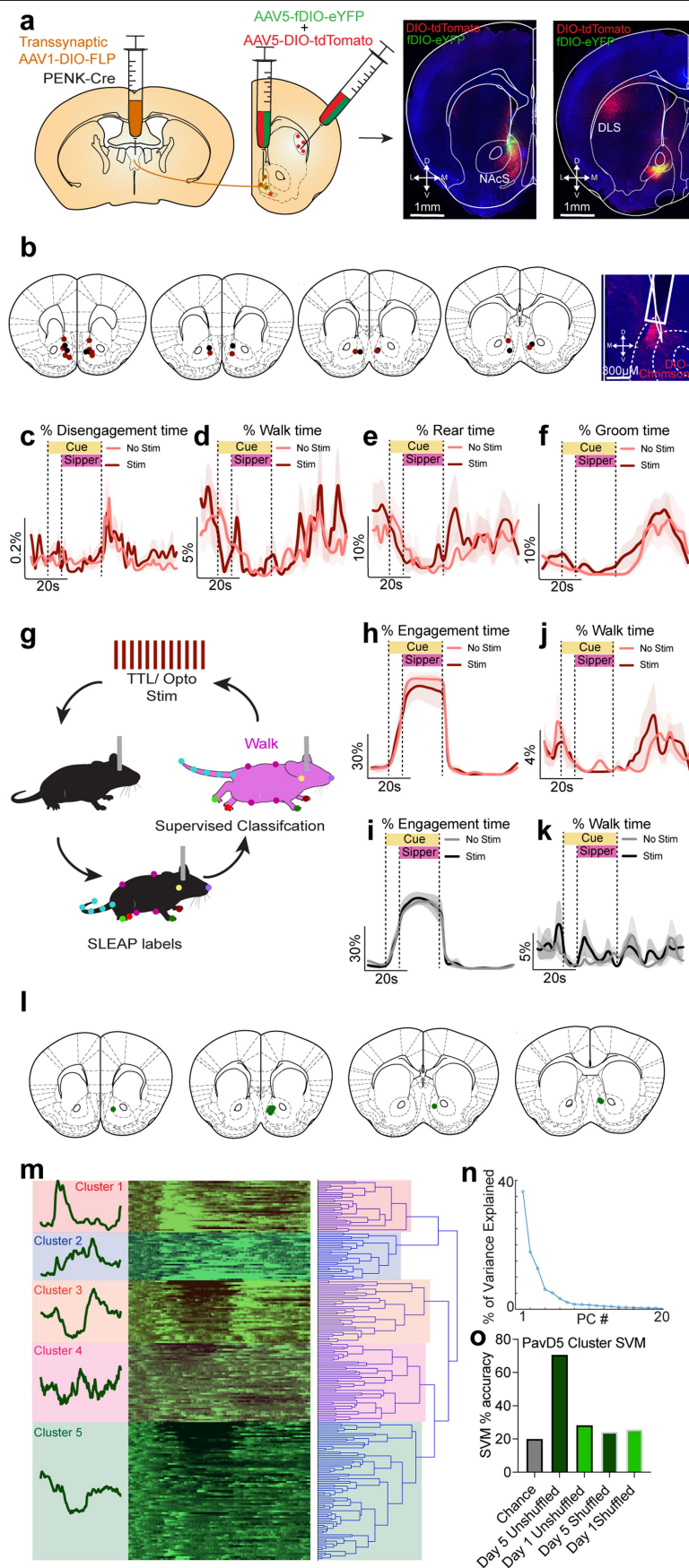
Extended Data Fig. 7 | Effect of AAV1-FLEX-sgCNR1 deletion of CB₁R from aPVT NTS neurons and BLA Vglut1 neurons on reward consumption and Pavlovian reward conditioning. **(a)** Schematic for CB₁R deletion from the aPVT and photometry recordings of GCaMP6s aPVT^{NTS}-NAc terminals during adlib sucrose consumption and Pavlovian reward conditioning. **(b)** Fiber placements for aPVT CB₁R deletion/aPVT^{NTS}-NAc cohort in Fig. 3. **(c,d)** Photometry traces of GCaMP6s signal aligned first lick during adlib sucrose consumption from sgROSA (N = 7) and sgCNR1 (N = 7) injected mice. **(e)** Comparison of first lick-aligned (0-20 s) GCaMP6s signal between sgROSA (N = 7) and sgCNR1 (N = 7) injected mice ($p = 0.1741$) during adlib sucrose consumption. **(f-h)** Comparison of total lick number ($p = 0.3705$), bout length ($p = 0.0393$), and bout number ($p = 0.2828$), between sgROSA (N = 7) and sgCNR1 (N = 7) injected mice during adlib sucrose consumption. **(i)** Comparison GCaMP6s signal (0-20 s) during rewarded trials in vehicle treated sgROSA (N = 6) and sgCNR1 (N = 7) injected mice during Pavlovian reward conditioning ($p = 0.0373$). **(j)** Comparison of # of licks showing a main effect of DO34 treatment on reducing sucrose consumption (Main effects: Treatment $p = 0.0025$, genotype $p = 0.0146$, interaction $p = 0.5607$). **(k,l)** Photometry traces of GCaMP6s signal aligned to footshock during Pavlovian fear conditioning. **(m,n)** Photometry traces of GCaMP6s signal aligned to tone presentation of day 1 of fear extinction. **(o)** Schematic for CB₁R deletion from the BLA and photometry

recordings of GCaMP6s BLA^{Vglut}-NAc terminals during adlib sucrose consumption and Pavlovian reward conditioning. **(p)** Fiber placements for BLA CB₁R deletion/BLA^{Vglut}-NAc photometry. **(q,r)** Photometry traces of GCaMP6s signal during adlib sucrose consumption from sgROSA (n = 7) and sgCNR1 (n = 7) mice. **(s)** # of licks during adlib sucrose consumption (sgROSA N = 7, sgCNR1 N = 7). **(t)** Comparison of lick-aligned GCaMP6s signal during adlib sucrose consumption. **(u-x)** Photometry traces of GCaMP6s signal from vehicle and DO34 treated sgROSA (N = 7) and sgCNR1 (N = 7) mice. **(y)** Comparison of engagement time demonstrating a main effect of both treatment and genotype (Main effects: Treatment $p = 0.0025$, genotype $p = 0.0146$, interaction $p = 0.5607$, (sgROSA N = 7, sgCNR1 N = 7)). **(z)** Comparison of GCaMP6s signal (0-26 s post-cue) demonstrating a main effect of both treatment and genotype (Main effects: Treatment $p = 0.0395$, genotype $p = 0.0066$, interaction $p = 0.6757$, (sgROSA N = 7, sgCNR1 N = 7)). **(aa)** Comparison of total lick number demonstrating a trend toward a main effect of treatment (Main effects: Treatment $p = 0.0109$, genotype $p = 0.4333$, interaction $p = 0.4333$, (sgROSA N = 7, sgCNR1 N = 7)). All error bars represent min/max values; box plots represent interquartile range with line at median; N represent number of mice. p values reported from unpaired two-tailed t-test (**e-i, s, t**) or Two-way ANOVA (**j, y-aa**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Extended Data Fig. 8 | AAV1-FLEX-sgCNR1 deletion of CB₁R from aPVT NTS neurons does not affect fear conditioning or extinction and foot shock induced eCB release and binding to aPVT terminals in the NAc. (a) Schematic for CB₁R deletion from the BLA and photometry recordings of GCaMP6s BLA^{VGlut}-NAc terminals during Pavlovian fear conditioning and extinction. (b,c) Photometry traces of GCaMP6s signal aligned to foot shock from sgROSA (N = 7) and sgCNR1 (N = 7) injected mice during Pavlovian fear conditioning. (d,e) Comparison of freezing behavior ($p = 0.1880$) and GCaMP6s signal (0-5 s, $p = 0.4953$) between sgROSA (N = 7) and sgCNR1 (N = 7) injected animals during fear conditioning. (f,g) Photometry traces of GCaMP6s signal aligned to cue presentation from sgROSA (N = 7) and sgCNR1 (N = 7) injected mice during fear

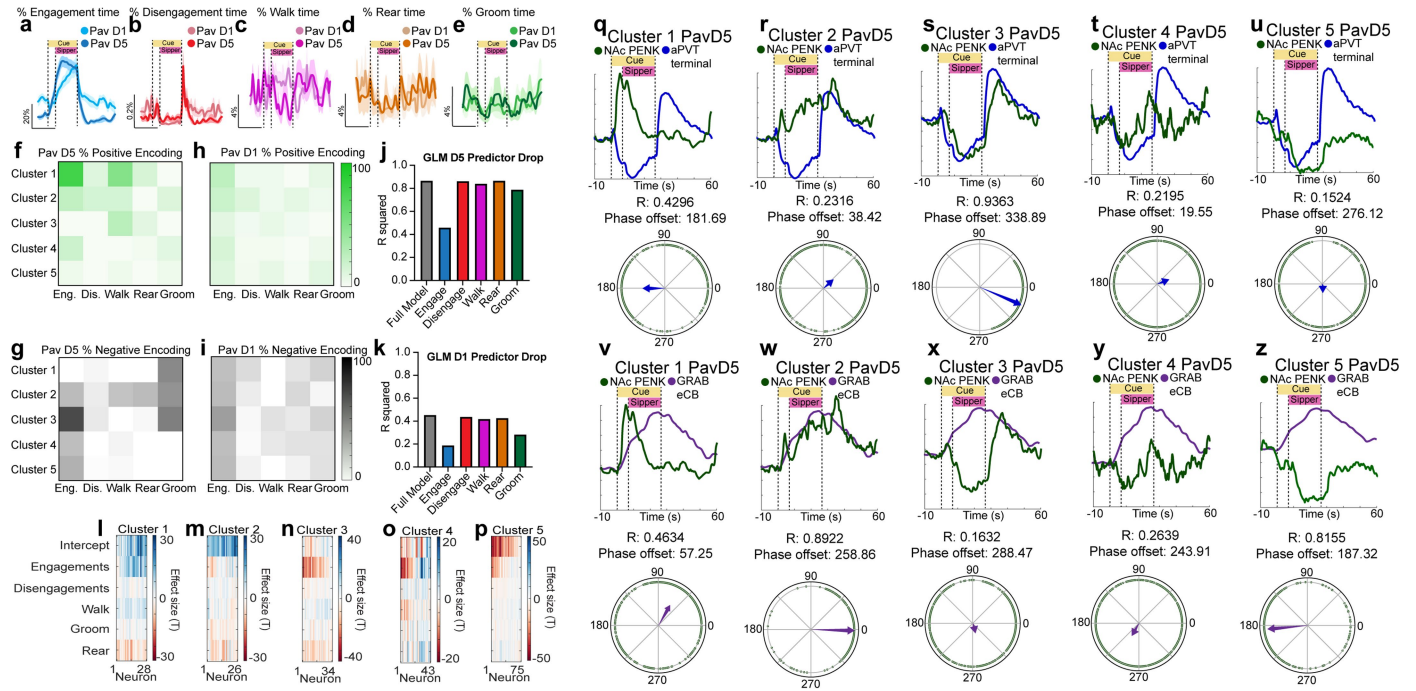
extinction. (h,i) Comparison of freezing behavior ($p = 0.9355$) and GCaMP6s signal (0-20 s, $p = 0.5312$) from sgROSA (N = 7) and sgCNR1 (N = 7) injected mice. (j) Schematic for photometry recordings of GRAB_{eCB2.0} or GRAB_{eCBMUT} during fear conditioning and fear extinction. (k) Photometry traces of GRAB_{eCB2.0} (n = 8) or GRAB_{eCBMUT} (n = 4) signal during fear conditioning. (l) Photometry trace of GRAB_{eCB2.0} (n = 8) or GRAB_{eCBMUT} (n = 4) signal during fear extinction. (m) Fiber placements for GRABeCB mice used in j-m and Fig. 4j-n. All error bars represent min/max values; box plots represent interquartile range with line at median; N represent number of mice. p values reported from unpaired two-tailed t-test (d,e,h,i).



Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | Characterization of transsynaptic labeling, specificity of closed-loop optogenetic stimulation, and cluster classification. (a) Schematic for validation of transsynaptic labeling approach. Transsynaptic AAV1-DIO-FLP was injected into the aPVT, AAV5-DIO-tdTomato was injected into the dorsolateral striatum (DLS), and a cocktail of AAV5-DIO-tdTomato and AAV5-fDIO-eYFP was injected in the NAc of Penk-Cre mice. The DLS, which expresses Penk but does not receive aPVT input, showed expression of AAV5-DIO-tdTomato but not AAV5-DIO-eYFP. The NAc, which expresses Penk and does receive aPVT input, showed expression of both AAV5-DIO-tdTomato and AAV5-DIO-eYFP. (b) Fiber placements for transsynaptic optogenetics cohort in Fig. 4 and representative image. (c-f) Closed-loop optogenetic stimulation during sipper port engagement

does not affect % time disengaging, walking, rearing, or grooming. (g) Schematic for closed-loop optogenetic activation of aPVT-NAc^{Penk} neurons during walk events. (h,i) Effect of walk elicited closed-loop activation of aPVT-NAc^{Penk} neurons on % engagement time in ChRimson (N = 7) and eYFP (N = 5) expressing animals. (j,k) Effect of walk elicited closed-loop activation of aPVT-NAc^{Penk} neurons on % walking time in ChRimson (N = 7) and eYFP (N = 5) expressing animals. (l) Lens placements for transsynaptic 1-photon imaging cohort in Fig. 5. (m) Dendrogram plot of hierarchical clustering of 206 tracked aPVT-NAc^{Penk} neurons based on Principal Component Analysis (PCA) of the activity of each neuron in the 60 second window following cue-onset. (n) Percent of variance explained by the top 20 PCs. (o) Support Vector Machine decoding of cluster identity, trained on PavD5 cluster activity.



Extended Data Fig. 10 | Validation of generalized linear model and Hilbert and Rayleigh analysis of cluster entrainment to aPVT^{NTS}-Nac GCaMP6s and aPVT-Nac GRABeCB_{2.0} signal. (a-e) Cue-aligned traces of % time engaging with sipper port, disengaging from sipper port, walking, rearing, and grooming on PavD1 and PavD5 (N = 10) from GRIN lens implanted animals. (f-g) Heatmap of % of neurons within each cluster encoding each behavioral state on PavD5, with a β coefficient threshold of ± 0.5 . (h,i) Heatmap of % of neurons within each cluster encoding each behavioral state on PavD1, with a β coefficient

threshold of ± 0.5 . (j,k) Effect of individually dropping each predictor on model accuracy for PavD5 and PavD1. (l-p) Contribution of effect sizes for each neuron in each cluster to the generalized linear model (T values). (q-u) Overlay of Pavlovian reward conditioning day 5 aPVT-Nac^{Penk} cluster GCaMP6s traces with aPVT^{NTS}-Nac terminal GCaMP6s traces and Rayleigh plots from cluster 1, 2, 3, 4, and 5. (v-z) Overlay of Pavlovian reward conditioning day 5 aPVT-Nac^{Penk} cluster GCaMP6s traces with aPVT-Nac terminal GRABeCB_{2.0} traces and Rayleigh plots from cluster 1, 2, 3, 4, and 5.

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Data exclusions The Grubbs outlier test was performed individually a single time on each dataset. If an animal's measurements were excluded from one dataset (e.g. Photometry Z-score), associated data from that same animal in the same experiment were also excluded (e.g. engagement time).

Replication All data in the manuscript were comprised from at least two cohorts of mice per experiment to ensure repeatability.

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