Nucleus Accumbens Local Circuit for Cue-Dependent Aversive Learning

Andrew Belilos[1], Cortez Gray[2], Christie Sanders[1], Christopher Richie[1], Ayesha Sengupta[1], Holly Hake[1], T. Chase Francis[2]

Affiliations:

1. Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, Maryland, USA
2. Department of Drug Discovery and Biomedical Sciences, College of Pharmacy, University of South Carolina, Columbia, South Carolina, USA

Abstract

Response to threatening environmental stimuli requires holistic detection and encoding of important environmental features that dictate threat. Animals need to recognize the likelihood that an environmental stimulus predicts threat and respond to these salient aversive stimuli appropriately. The nucleus accumbens is uniquely positioned to process this salient, aversive information and promote motivated output, through plasticity on the major projection neurons in the brain area. Here, we uncover a nucleus accumbens core local circuit whereby excitatory plasticity facilitates learning and recall of discrete aversive cues. We demonstrate that nucleus accumbens substance P release and long-term excitatory plasticity on dopamine 2 receptor expressing projection neurons is required for learning about aversion-associated cues. Additionally, we found learning and recall were dependent on different projection-neuron subtypes. Our work demonstrates a critical role for Nucleus Accumbens substance P in cue-dependent aversive learning.

Introduction

Aversive stimuli are some of the most salient stimuli as animals need to recognize threats to survive. Consequently, aversive stimuli elicit a rapid learning about and response to cues that predict aversive outcomes. Canonically, the major projection neurons of the Nucleus Accumbens (NAc) are studied in terms of motivational salience, a cognitive process that facilitates approach or avoidance in response to a stimulus and is often described in terms of the action it elicits. The NAc responds to salient cues in humans [1], is required for aversive learning [2,3], which is cue-dependent in the NAc core [3–5], suggesting the NAc may facilitate learning about these events to promote motivated response. It is likely consolidation and subsequent recall of these events that requires long-term plasticity to facilitate persistent memories and these NAc mechanisms to enable recognition of threat have yet to be determined.

Within the NAc, projection medium spiny neuron (MSN) subtypes are thought to promote opposing outcomes, with dopamine 1 (D1) receptor MSN activity driving rewarding and positive outcomes, and dopamine 2 (D2) receptor MSN activity driving aversive outcomes, suppressed reward, and punishment [6–15]. This understanding has been updated as it is now known MSN subtype activity participates in more complex and pivotal roles in guiding learned behavior. For instance, NAc core D2-MSNs have been shown to be required for discrimination [16] and reversal learning [17,18]. It is also known both MSN subtypes are active in response to reward [19–21]. However, modulation of activity of these subtypes during learning or shaping has the capability of altering response to a stimulus, where D2-MSNs produce an aversive response or blunt reward when activated [6–15]. This suggests that enhancing activity, such as through potentiation of excitatory

transmission on D2-MSNs, during learning, may provide a means of exacerbating learning about aversion associated stimuli.

We recently uncovered a mechanism in which glutamatergic long-term excitatory potentiation (LTP) is elicited on D2-MSNs exclusively by activity of D1-MSNs [22] and required the activation of cholinergic interneurons by release of the neuropeptide substance P. Both acetylcholine and substance P are released by salient stimuli including drugs of abuse and aversive stimuli [23] and these neuromodulators contribute to many of the same behaviors as activation of D2-MSNs. Additionally, phasic activity changes of cholinergic neurons in response to cue-dependent associative outcomes have been observed in primates [24,25] and rodents [26]. Like D2-MSN neuronal activity, striatal acetylcholine is necessary for behavioral flexibility [27], discrimination [28], reversal learning [29], and aids in the expression of cue-motivated behavior [30]. These similarities, and the fact that this circuit promotes plasticity position this circuit as a likely candidate for signaling aversion-related outcomes. Therefore, we hypothesized this substance P-acetylcholine local circuit is necessary for supporting aversive learning through plasticity on D2-MSNs.

In this paper, we aimed to determine neurobiological mechanisms which underpin NAc core dependent aversive learning. Using pharmacology and gene knockout technology, we found substance P receptors in cholinergic neurons were required for cue-dependent aversive learning. These findings were corroborated through enhanced D1-MSN activity and acetylcholine release in response to aversive foot shocks and disrupting this circuit at any synaptic and cellular node diminished learning about cues. Our work provides a new perspective on how MSN subtypes interact to facilitate learning about aversive cues.

Results

Neurokinin 1 receptor signaling is required for cue-dependent aversive learning

Since substance P is released by strongly salient stimuli and are involved in many of the same aversive outcomes as D2-MSNs, we first tested whether substance P release and binding to its primary receptor, the neurokinin 1 (NK1) receptor was necessary for aversive learning. A Pavlovian fear conditioning model was used as a model of aversive learning, where freezing was the main measure of learning (**Fig. 1a**). To determine if substance P was required for associative aversive learning, mice were injected with L-733,060 prior to fear conditioning. NK1 receptor antagonism suppressed cue-dependent, but not context dependent freezing (**Fig. 1b**), without affecting locomotion (**Extended Data Fig. 1**). These results suggest substance P is required for cue-dependent aversive learning and recall.

The NK1 receptor is found throughout the brain, including in regions known to be associated with cue-dependent fear conditioning. In the NAc, the NK1 receptor (encoded by the *Tacr1* gene) is found in all choline acetyltransferase (ChAT) expressing cholinergic cells. Therefore, we used a clustered regularly interspaces short palindromic repeats (CRISPR) Cas9 system, generated an AAV expressing a guide RNA for the *Tacr1* gene (*Tacr1*-gRNA), and injected it in the NAc core of ChAT-Cre/Cas9 mice, which express Cas9 exclusively ChAT cells. RNA scope *in situ* hybridization was used to assess the efficacy of the knockout. Since we were unable to separate ChAT cells expressing or not expressing the *Tacr1* guide RNAs (see Methods), all Chat expressing cells in the GFP expression NAc core region were quantified for *Tacr1* expression. A significant reduction in the *Tacr1* transcript in *Chat* positive cells was observed (**Fig 1c**). Additionally, a functional and near complete inhibition of substance P-induced increase in ChAT firing was observed in mice expressing Cas9 and the *Tacr1*-gRNA (**Fig. 1d**). Next, we tested the functional knockout in fear conditioning behavior. NK1 receptor knockout suppressed cue-dependent, but not context-dependent freezing (**Fig. 1e**), suggesting the cue-dependent reduction in freezing due to NK1 receptor antagonism was indeed an effect selective

for the NAc core. Overall, these experiments indicate substance P is required for cue-dependent recall of aversive cues via the acetylcholine rebound.

D1-MSNs are required for cue-dependent fear conditioning

The primary source of substance P in the striatum is D1-MSNs which exclusively express and release the peptide and we have demonstrated activation of D1-MSNs releases substance P [22]. Since substance P signaling is required for aversive learning and substance P is released in the striatum in response to salient aversive stimuli [23], we predicted D1-MSN activity would increase in response to salient, aversive stimuli. To selectively assay activity of D1-MSNs, Dyn-Cre mice were injected with AAV-DIO-GCaMP7f and implanted with fibers in the NAc core for assessment of *in vivo* activity with fiber photometry. We first acquired signals in response to random interval, unpredictable foot shocks. D1-MSN calcium activity scaled with the magnitude of foot shock (**Fig. 2a-b**). Next, we assayed activity during conditioning and cue recall. During conditioning, foot shock but not the cue increased D1-MSN calcium activity (**Fig. 2c**). D1-MSN calcium activity was not increased by the cue during cue recall (**Fig. 2d**), despite an increase in freezing (**Extended Data Fig. 2a**), indicating D1-MSNs may be necessary for cue-dependent conditioning but not recall.

To test the necessity of D1-MSN activity during cue-dependent aversive learning, we used a temporally selective optogenetic strategy to inhibit D1-MSNs during cue-dependent behavior. Mice were injected with AAV-DIO-eNpHR3.0 and implanted optogenetic fibers in the NAc core of Dyn-Cre mice (**Fig. 2e**). Optogenetic inhibition of D1-MSNs did not affect locomotion (**Extended Data Fig. 2b**). D1-MSNs were inhibited selectively for 10 sec during the cue and shock during conditioning. D1-MSN inhibition during the cue and shock in conditioning diminished cue recall but not context recall (**Fig. 2f**). In another group of mice, we selectively inhibited D1-MSNs during every other 10 sec cue to determine if inhibition reversibly inhibited cue-dependent freezing. No change in behavior was observed from D1-MSN inhibition (**Fig. 2g**). Altogether, these results indicate D1-MSN activity contributes to cue-dependent learning, but not recall.

Substance P signaling is required for shock-driven acetylcholine release

Substance P is released from D1-MSNs in response to salient stimuli [23,31] and has a role in aversion [32,33]. Additionally, substance P promotes increased activity of ChAT neurons via the NK1 receptor [22,34]. In addition, previous studies have indicated ChAT neuron exhibit phasic activity patterns in response to aversive stimuli *in vivo* [25] . Therefore, it was predicted substance P would cause ChAT activity and acetylcholine release *in vivo*. An adeno-associated virus (AAV) containing the GPCR-activation-based sensor for acetylcholine (GRAB-ACh3.0) [35] was injected into the NAc core of mice and expressed in neurons (**Fig. 3a**). To evaluate acetylcholine binding *in vivo*, the virus was injected, a fiber was implanted in the NAc core, and fluorescent transients were observed with fiber photometry. In an open field chamber, mice were injected with the broad muscarinic antagonist scopolamine, which has been observed to block acetylcholine binding to the GRAB-ACh3.0 sensor [35]. Scopolamine significantly diminished the magnitude of the signal (**Fig. 3b**). *Ex vivo* slice studies demonstrated bath application of low (1 µM) and high (100 µM) concentrations of acetylcholine significantly increase bulk fluorescence intensity in a concentration dependent manner (**Fig. 3c**). These results verify that the GRAB-ACh3.0 sensor causes fluorescence through acetylcholine release and binding to the GRAB-Ach3.0 receptor sensor.

Next, to determine if acetylcholine is released by aversive stimuli in a substance P-dependent manner, foot shocks (10 total) were given at random, unpredictable intervals in behavior naïve mice while recording GRAB-ACh3.0 fluorescent activity in the NAc core. Shock alone produced a decrease in response followed by an increase in release (**Fig. 3d-e**). Similar to D1-MSN calcium activity, increasing the intensity of the shock increased the amount of acetylcholine release (**Fig. 3d-f**). Unlike D1-MSNs, this acetylcholine release habituated to the

shock over repeated presentations. Antagonism of the NK1 receptor with L-733,060 attenuated the increase in acetylcholine release and did not diminish habituation to the shock (**Fig. 3g-h**). These results suggest aversive stimuli increase acetylcholine release via substance P and acetylcholine release scales with the intensity of an aversive stimulus.

Substance P promotes plasticity on D2-MSNs during cue-dependent aversive learning

Substance P causes long-term potentiation (LTP) of excitatory transmission on D2-MSNs through enhanced acetylcholine release and activation of the muscarinic 1 receptor signaling pathway [22]. This mechanism would provide a cell-type specific mechanism for enhancing activity of D2-MSNs to either promote aversive outcomes or to facilitate learning about aversive stimuli. To first determine if aversive conditioning and subsequent acetylcholine release acts through muscarinic 1 receptors on D2-MSNs, mice were implanted with cannulas in the NAc core and infused with the muscarinic 1 receptor-like antagonist pirenzepine (4 ng/µL, bilateral) prior to conditioning or prior to cue retrieval. Compared to saline infused controls, cue recall was suppressed when pirenzepine was infused prior to conditioning (**Fig. 4a**), but not prior to cue recall (**Fig. 4b**).

To determine if muscarinic 1 receptor plasticity occurred on D2-MSNs in a substance P-dependent manner, we conditioned mice to a cue after injection with saline or L-733,060 and conducted whole-cell electrophysiology recordings 1 day after cue recall. Substance P-dependent LTP on D2-MSNs is primarily induced through insertion of GluR2-lacking AMPA receptors [22]. These GluR2-lacking AMPA receptors are sensitive to intracellular peptides at positive membrane potentials and excitatory synaptic currents at positive potentials show inward rectification when these receptors have been inserted in the synaptic membrane. Therefore, we assessed inward rectification of electrically evoked excitatory post-synaptic currents (EPSCs), which would be suggestive of increased extracellular membrane-bound GluR2-lacking AMPA receptors. Mice that received conditioning (Cue + Shock) displayed significant rectification at +40 mW as compared to non-conditioned mice (Cue only) (**Fig. 4c**). Additionally, mice injected with L-733,060 displayed no difference in rectification compared to non-conditioned mice, suggesting that suppressing substance P signaling was responsible for LTP observed on D2-MSNs (**Fig. 4c**). As a secondary measure, we compared sensitivity of electrically evoked currents on D2-MSNs from conditioned and non-conditioned mice to the selective GluR2-lacking AMPA receptor antagonist 1-naphthyl acetyl spermine trihydrochloride (NASPM) (100 µM). NASPM significantly diminished currents in conditioned mice relative to non-conditioned mice, verifying the presence of more GluR2-lacking AMPA receptors (**Fig. 4d**). Furthermore, amplitude, but not frequency of spontaneous excitatory post-synaptic currents (sEPSCs) was increased on D2-MSNs (**Fig. 4e**). Shock only mice or context only mice displayed no changes in inward rectification on D2-MSNs (**Extended Data Fig. 3**). D1-MSNs did not display inward rectification from any groups assayed (**Fig. 4f, Extended Data Fig. 3**). Results indicate NAc core muscarinic-1 receptor signaling is necessary for cue-dependent aversive learning and signatures of substance P-dependent LTP are expressed following cued aversive learning.

D2-MSNs activity is necessary for recall of aversive-conditioned cues

Next, to compare the dynamics of D2-MSN activity to D1-MSN activity during fear conditioning, A2a-Cre mice were injected with AAV-DIO-GCaMP7f and implanted with fibers for photometry (**Fig. 5a**) and subjected to 10 unpredictable foot shocks at various foot shock intensities. D2-MSNs responded to all foot shock intensities (**Fig. 5b**). In contrast to D1-MSNs, D2-MSN calcium activity did not increase with increasing foot shock intensity (**Fig. 5c**) and responses rapidly attenuated after the first foot shock (**Fig. 5d**). Similar to D1-MSNs, D2-MSNs responded to the first foot shock during conditioning, but not the cue (**Fig. 5e**). These results may

suggest that D1- and D2-MSNs play different roles in the acquisition of aversive conditioned stimuli.

If D2-MSNs are critically involved in encoding salient, aversive stimuli, potentiation of excitatory pathways to D2-MSNs should increase the chances of D2-MSN activation and enhance overall activity in relation to the cue. Additionally, this activity should be diminished by suppressing learning. To assess how D2-MSNs were active during recall of the context or cue, we used a latent inhibition procedure where pre-exposure to a conditioned stimulus diminishes associative learning [36]. A2a-Cre/tdTomato mice were used to histologically identify D2-MSNs (tdTomato positive) and putative D1-MSNs (tdTomato negative). Mice were subjected to 40 cues in the conditioning context prior to foot shock conditioning. Mice that received pre-conditioning to the cue froze significantly less to the cue (**Extended Data Fig. 4a**). We sacrificed half of the mice 1.5 hrs after the context on day 2 and the remaining mice after cue recall on day 3 to examine for cell-type specific expression of C-fos, a marker of neuronal activity. C-fos was significantly lower in NAc core D2-MSNs in mice receiving pre-conditioning to the cue compared to mice that were not pre-exposed (**Extended Data Fig. 4b**). C-fos expression in D2-MSNs strongly and positively correlated with freezing levels (**Extended Data Fig. 4c**). No differences in C-fos expression were observed in putative D1-MSNs following context or cue recall (**Extended Data Fig. 4d**) and, unlike D2-MSNs, no correlation was observed between C-fos expression in D1-MSNs and freezing (**Extended Data Fig. 4e**). In addition, C-fos expression in shell D2-MSNs (**Extended Data Fig. 4f**) or putative D1-MSNs (**Extended Figure 4g**) was not different across groups in both contextual and cue recall. These results suggest NAc core D2-MSN activity may play an active role recall of salient, aversive information and only D2-MSN activity may be necessary for cue recall.

To verify this activity in real time and *in vivo*, A2a-cre mice were injected with AAV-DIO-GCaMP7f in the NAc core and implanted with fibers for fiber photometry. A2a-cre mice displayed significant freezing to the cue compared to a novel context (**Extended Data Fig. 5a**). Unlike D1-MSNs, D2-MSNs were activated during cue recall (**Fig. 6a-c**) in response to the associated aversive cue and this signal did not habituate over repeated plays of the cue (**Fig. 6b, Extended Data Fig. 5b**).

To determine if D2-MSN activity was required for recall of associated cues, AAV-DIO-eNpHR3.0 was injected, and fibers were implanted into the NAc of A2a-cre mice for selective optogenetic inhibition of D2-MSNs (**Fig. 6d**). D2-MSNs were inhibited during the 10 second cue on every other cue. D2-MSN inhibition reversibly inhibited freezing to the cue (**Fig. 6e**). This inhibition was not dependent on locomotor effects (**Extended Data Fig. 5c**). Taken together, associative aversive stimuli promote activity of D2-MSNs in response to associative cues and this response is required expression of cue-dependent aversive learning.

Discussion

Our work has demonstrated a di-synaptic local circuit within the NAc core that is critical for processing aversive cues, with necessary roles for NAc core D1-MSN and D2-MSN activity at different stages of learning and recall (**Fig. 6f**). We have shown that D1-MSN activity and substance P is required for cue-dependent aversive conditioning but is not required for recall of conditioned cues. Next, we demonstrated that *in vivo* acetylcholine release by an aversive stimulus was facilitated by substance P. We then found activity of D1-MSNs was necessary for conditioning cues to aversive stimuli and activity of D2-MSNs, but not D1-MSNs, was necessary for the recall. Lastly, we showed substance P plasticity, through an acetylcholine-release, muscarinic receptor mechanism drives D2-MSN plasticity [22] and activity in response to the cue after conditioning. Overall, our results provide evidence for a Nucleus Accumbens local circuit involved in learning about aversive cues.

This work is consistent with previous studies showing MSNs respond to different aspects of associative reward conditions, where D1-MSNs respond to outcomes and D2-MSN activity

responds to associative cues [21]. This would further support the hypothesis that the NAc core supports learning about salient cues [1]. Furthermore, the duration of MSN subtype responses is temporally different in both rewarding [21] and aversive-associated stimuli. D1-MSNs rapidly respond to the non-associative, salient stimuli and D2-MSNs tend to display extended activity following associative cue presentation. It is hypothesized D2-MSNs may be actively engaged in either retaining expression of the learned association or suppressing additional associations. More work will be needed to test the temporal nature of D1-MSN and D2-MSN activity in response to associative conditioning and learning.

We provide a new role for D1-MSNs and the neuropeptide substance P in mediating aversive learning. D1-MSNs are thought to facilitate reward; however, the data here and data from others [19,20,37] suggest aversive events activate D1-MSNs and this activity participates in associative learning through the release of peptides. While acetylcholine may act as a learning signal facilitating the salience of associative stimuli, we have discovered substance P works upstream to promote ChAT activation, acetylcholine release, and plasticity on D2-MSNs. This plasticity facilitates information transfer from excitatory brain regions to the NAc core. As the effect of substance P and acetylcholine plasticity is cue-dependent but not context-dependent, it is likely that extensive excitatory projections arising from limbic regions including the basolateral amygdala [38,39] may facilitate this D2-MSN activity during cue recall. Future experiments will need to assess what inputs undergo substance P, acetylcholine-dependent potentiation.

Our work shows acetylcholine release plays a significant role in aversive learning and its release is likely driven by substance P/NK1 receptor signaling. This could be done through varying the upstream activity of D1-MSNs, subsequent substance P release, and the activation of ChAT neurons. Our previous work has shown that the activity of ChAT cells can be graded by varying concentration of substance P [22]. We demonstrated D1-MSN activity increases with foot shock intensity, likely leading to increasing substance P release, and this might be a method of grading acetylcholine release and learning or response to an aversive stimulus. Other factors are likely contributing to this affect as well. We cannot rule out the role of dopamine input via D2 receptors[40], midbrain GABA input[41], thalamic input[42], intrinsic inhibition[43] or other mechanisms in generation of these cue-dependent outcomes. In fact, substance P and ChAT acetylcholine is known to promote dopamine release [44–47]. Dopamine release could accompany the increase in acetylcholine observed in response to novelty[48] or initial phasic transients of foot shock. Therefore, selective plasticity could be occurring due to coordinated acetylcholine and dopamine release during learning.

Despite a non-habituating D1-MSN calcium signal following repeated foot shocks, the observed acetylcholine signal habituated to repeated foot shocks. After binding of substance P to the NK1 receptor and activation, rapid beta-arrestin-mediated endocytosis occurs [49,50] and attenuates additional responses to substance P for 5-15 min [22,34,49]. At this point, NK1 receptor activation of ChAT cells would be less sensitive to substance P, thereby decreasing the size of the signal. Therefore, since we observed the acetylcholine release to be NK1 receptor dependent, we predict the habituation of the signal from shock 1 to shock 10 is due to NK1 receptor internalization. This, along with the NK1 receptor antagonist attenuation of the acetylcholine signal, indicates the increase in acetylcholine release is most likely due to release of substance P and activation of the NK1 receptor on ChAT neurons. In addition, the D2-MSN foot shock signal is also rapidly attenuated and much smaller after repeated shocks. Mice only required two cue shock pairings for associating, perhaps indicating the plasticity on D2-MSNs occurs quickly, within the first few associations. This may further suggest that particularly salient stimuli can produce this plasticity rapidly.

Overall, we demonstrate substance P signaling and plasticity in the NAc core is critical for learning about salient, aversive cues. We uncovered a new role of D1-MSN local communication to facilitate acetylcholine plasticity on D2-MSNs that is relevant to cue-dependent aversive learning. Enhanced processing of salient stimuli, through this substance P/D2-MSN mechanism

could lead to exacerbated response to aversive stimuli and could enhance susceptibility to aversive stimuli that cause stress or anxiety disorders. Therefore, targeting these neurobiological mechanisms may be useful in therapies aiming to treat anxiety and mood disorders.

Methods

Animals

Male and female mice 10-15 weeks were used in all studies. For GRAB-ACh3.0 virus, cannula infusion experiments, and systemic drug injections without imaging or electrophysiology, C57Bl6/J mice (JAX: 000664) were used. For optogenetic inhibition experiments either B6;129S-Pdyntm1.1(cre)Mjkr/LowlJ (Dyn-Cre; JAX: 027958) or Tg(Adora2a-cre)KG139Gsat/Mmucd (A2a-Cre; MMRRC: 031168-UCD). For CRISPR-Cas9 experiments, B6;129-Gt(ROSA)26Sortm1(CAG-cas9*,-EGFP)Fezh/J (LSL-Cas9; JAX: 024857) mice were crossed with B6;129S6-Chattm2(cre)Lowl/J (ChAT-Cre) mice for conditional expression of Cas9 in ChAT-Cre cells. All transgenic animals were bred in house. Mice were fed *ad libitum* and were housed in a reverse light/dark (12/12 hr). Experimental procedures were approved and conducted in accordance with NIDA-IRP ACUC and University of South Carolina IACUC and NIH guidelines.

Surgical methods

Mice used in fluorescence imaging, optogenetic, or CRISPR knockdown experiments were anesthetized with ketamine/xylazine for intracranial AAV injection, hair was removed, scalps were cleaned with ethanol and betadine, and lidocaine was used prior to scalp incision. Body temperature was maintained with an electric heating pad. Mice received bilateral stereotactic injections (0.3-0.5 µL) of either AAV9-hsyn-GRAB-ACh3.0 (Addgene; plasmid# 121922; packaged by Vigene Biosciences)[35], AAV9-syn-FLEX-jGCaMP7f (Addgene; 104492-AAV9), AAV5-EF1a-DIO-eNpHR3.0-eYFP (Addgene; 26966-AAV5), or AAV1-mU6-Tac1r-gRNA1::EF1a-NucEnv-EGFP::hU6-Tacr1-gRNA2 in the NAc core (created and packaged by NIDA GEVVC Core) (A/P: 1.6, Lat: 1.6, D/V: -4.4, 10° angle). For photometry or optogenetics, mice were then implanted with either a 400 µm photometry fiber (Doric) or 200 µm core optogenetic fiber fixed with head screws and dental cement. Following surgery, mice were injected with warm saline to replenish fluids and given carprofen (5 mg/kg) for pain relief during recovery.

In vivo fiber photometry, *ex vivo* imaging, and *in vivo* optogenetics

In vivo fiber photometry was used to assess cell-type specific calcium activity (GCaMP7f) or acetylcholine release (GRAB-ACh3.0) using a Tucker-Davis Technologies LUX RZ10 fiber photometry system. Signals were tested and mice were habituated for 3-5 days prior behavioral recordings. For all recordings, sensors were excited at 470 nm to acquire the fluorescent emission signal and the isosbestic point for GFP at 405 nm as a normalization control. Excitation signals were generated by two light-emitting diodes and emission signals were collimated through a Doric fluorescent mini cube before being processed and digitized using the Synapse software suite. Analysis was performed offline using a custom Python script which extracted fluorescent signals, smoothed the signal with a linear fit to the 405 nm signal and applying the regression to the 470 nm signal, calculated $\Delta F/F$, aligned signals based on a timestamp, extracted fluorescent signals around events, and normalized signals (z-score) based on the 10 seconds prior to an event. Area Under the Curve (AUC) was assessed to find mean changes in calcium or acetylcholine activity.

For *ex vivo* imaging of GRAB-ACh3.0, slices were prepared as discussed in the electrophysiology methods section. Acetylcholine (1 or 10 µM) was bath applied after a 2 min baseline. Videos were acquired with a 40x objective at a 10 Hz frame rate using QCapturePro7 and analyzed offline with ImageJ. A subregion 100x100 µm devoid of fiber tracts in the NAc core was chosen in each image and analyzed for fluorescence intensity over time. Fluorescence traces were flattened using a linear regression fit and normalized (z-score) to the 1 min prior to acetylcholine bath application.

For optogenetic inhibition experiments, mice were habituated to patch cables 5 days prior to behavior. Percent transmission was calculated during creation of optogenetic fibers and 594 nm light was delivered at 3-5 mW from a DPSS laser (OptoEngine LLC). Optogenetic inhibition was delivered during the onset of the cue and one second after the offset of the cue for all conditioning cues and on every other cue during cue recall.

Behavior

Pavlovian fear conditioning was conducted over a 3 day period (**Fig. 1a**) in Med Associates Fear conditioning chambers contained in sound attenuating cabinets. Prior to each phase of fear conditioning, mice were habituated in a separate room for at least 1 hour in the lighting condition of the test. Conditioning was conducted in white light with vanilla scent (10% vanilla extract in ethanol) chamber with a grid floor. Following a 2 min baseline, 2 white-noise (80 dB) cues co-terminating in a 0.6 mA foot shock separated by a 30 sec interval. The next day mice were exposed to the conditioning context for 5 min. On the third day, mice were placed in a new context where floors were covered with white, opaque plexiglass, a curved white plexiglass insert, no light (red light in behavior room), and vinegar scent (1% acetic acid in water). After a 2 min baseline, 5-6 cues at 30 sec intervals were played back. In all tests, freezing was measured using VideoFreeze (Med Associates), which has been thoroughly validated in relation to hand scoring [51]. Freezing thresholds were set at least 30, 0.033 sec frames of frozen motion and motion indices below 6 for mice without fiber or optogenetic tethers and below 500 for mice with optical tethers. For mice exposed to foot shock only, after a 2 min baseline each mouse was exposed to an unpredictable foot shock set to random intervals at least 30 sec between each shock. Mice received one intensity of foot shock per day, and each subsequent day were subjected to increasing foot shock intensities or decreasing foot shock intensities to control for previous foot shock intensities from interfering with the signal for any subsequent foot shock.

Electrophysiology

Whole-cell electrophysiological recordings were done one day following cue recall. Mice were anesthetized with Euthasol, brains were extracted, and cut (300 µM) in ice cold N-Methyl D-Glucamine (NMDG) cutting artificial cerebrospinal fluid (ACSF) solution (in mM): 92 NMDG, 20 HEPES, 25 glucose, 30 sodium bicarbonate, 1.2 sodium phosphate, 2.5 potassium chloride, 5 sodium ascorbate, 3 sodium pyruvate, 2 thiourea, 10 magnesium, 0.5 calcium chloride, osmolarity 305-310 mOsm, pH 7.34. Immediately after slicing, slices were placed in warm (32°C) NMDG solution for 2-5 min, then transferred to HEPES holding solution at room temperature which was the same as NMDG solution except NMDG was replaced with 92 mM sodium chloride and contained 1 mM magnesium chloride and 2 mM calcium chloride. Recording began 1 hr after brain slicing was complete and was performed at 33°C. Slices were perfused at a rate of 2 mL/min with ACSF (in mM): 125 sodium chloride, 26 sodium bicarbonate, and 11 glucose, 2.5 potassium chloride, 1.25 sodium phosphate, 2.4 calcium chloride, 1 magnesium chloride, osmolarity 305-310 mOsm, pH 7.34. To block inhibitory currents, synaptic recordings were done holding cells at -70 mV in the presence of picrotoxin (100 µM) and with a cesium methanesulfonate internal

solution (in mM): 117 cesium methanesulfanate, 20 HEPES, 2.8 sodium chloride, 4 magnesium ATP, 0.3 sodium GTP, 0.4 EGTA, pH 7.30, osmolarity 280-287 mOsm. For IV curves, spermine (100 mM) was added to the pipette solution. NASPM (100 µM) was used to isolate currents from inserted GluR2-lacking AMPA receptors. For cell-attached ChAT recordings, ACSF was used as the internal solution.

Whole-cell currents or cell attached currents were obtained with borosilicate glass pipettes (whole cell: 1.7-3.0 MΩ; cell attached: 3.0-4.0 MΩ) and signals were amplified and digitized using a Multiclamp 700B amplifier (4kHz low-pass filter for synaptic, 1kHz for cell-attached or spontaneous EPSCs) and Digidata 1322 digitizer (10 kHz), respectively (Molecular Devices). Synaptic currents were evoked with a concentric bipolar stimulating electrode (FHC) and evoked currents were stabilized for 5-10 min prior to data acquisition. Series resistance drifts of more than 20% led to exclusion of the cell from analysis. D1- and D2-MSNs were identified under the visual guidance of a BX61 Olympus microscope and Tdtomato fluorescence. Cholinergic neurons were identified by cell size (40-60 µm diameters) and high baseline firing rates.

CRISPR-Cas9 for *Tacr1* knockout

The SpCas9 guide RNA seed sequences within the first exon of mouse *TACR1* were identified and scored using the Guide Design Tool at CRISPR.mit.edu. Two gRNAs (gRNA 1 A55: CCGTATAGGCGGCTGCCCAA, and gRNA 2 B108: TTCCGTGGTGGGCAACGTAG) were chosen based on their likelihood for functioning as a “nickase-compatible” pair. These sequences were inserted into plasmids already containing RNA pol III promoters and SpCas9 gRNA scaffold sequences. (gifts from Charles Gersbach; Addgene plasmid # 53187; http://n2t.net/addgene:53187; RRID:Addgene_53187, Addgene plasmid # 53188; http://n2t.net/addgene:53188; RRID:Addgene_53188). Then each intact gRNA expression cassette was amplified by PCR and both were inserted into an AAV expression vector that contained a EGFP-KASH reporter (pOTTC1553, Addgene #131682), resulting in plasmid pOTTC1642 (Addgene #195018). All cloning reactions used PCR amplification and ligation-independent cloning (In-Fusion, Takara Inc.). Reactions involving non-AAV plasmids were transformed into Stellar competent cells (Takara Inc.) and those involving AAV plasmids were transformed into NEB Stable competent cells (New England Biolabs).

The pOTTC1642 viral vector was packaged by triple transfection along with pHelper and rep/cap plasmids (serotype 1) and purified by affinity chromatography on GE AVB columns [52]. Purified vectors were titered by droplet digital PCR with an assay that recognizes the EF1a reporter. Titered vectors were tested for GFP expression by transduction into rat primary cortical neurons prior to *in vivo* use.

Immunohistochemistry and *in situ* hybridization

Expression of GRAB-ACh3.0 or eNpHR3.0-eYFP was identified by histological staining for GFP. Briefly, brains slices (40 µm) from perfused (4% paraformaldehyde) brains were washed with 1X PBS, blocked with 1X PBS buffer containing 0.01% Triton X, 10% Normal Donkey Serum and incubated with a primary anti-body for GFP made in chicken (Aves lab, cat#: GFP-1020). Slices were washed and incubated with a secondary anti-chicken antibody made in donkey conjugated to Alexa Fluor 488 (Jackson immuno, cat#: 703-545-155), stained with DAPI and mounted on slides. All images were acquired on a Fluoview 1000 BX51WI confocal microscope (Olympus).

In situ hybridization was performed on fresh frozen 16 µm slices according to the ACDBio specifications as described previously [22]. EGFP-KASH maintained its fluorescence through the protocol. The probes used were as follows: *Chat* (Mm-Chat-C1 (nucleotide target region 1090-

1952), GenBank #: NM_009891.2); *Tacr1* (Mm-Tacr1-C3 (nucleotide target region 845-1775), GenBank #: NM_009313.5). All images were taken within a week of the protocol and image intensities were maintained across slices and slides. Quantification was done in the NAc core, where EGFP-KASH expression was found. Due to the cre-independent expression of EGFP-KASH and expression of GFP in ChAT cells, we were unable to specifically identify cells with or without expression and, therefore, all *Chat* positive cells in the expression region were analyzed. RNA was quantified using ImageJ by selecting 10 individual pixel values for every image and averaging to find the intensity values of a single RNA puncta. Intensity values of *Tacr1* in ChAT positive cells were assessed by selecting perinuclear RNA, quantifying intensity, and dividing the total intensity by the intensity of a single puncta value to calculate total RNA puncta. A total of 4 slices from each animal was taken and averaged. Slice averages are represented as individual points.

Statistical Analysis

Detailed statistics can be found in **Supplementary Table 1**. Data was analyzed and statistics were calculated using Graphpad Prism. For two-sample data, two-sided, un-paired or paired *t*-tests were used. For more than 2 variables over repeated time points one-way or two-way repeated measure (RM) ANOVAs were used followed by post-hoc tests using Sidak's multiple comparison test. In most cases with an interaction, main effects were reported. For electrophysiology experiments, all are represented as N/n (Mice/cells). Animal exclusions for behavioral experiments were made only when viral expression was absent unilaterally or bilaterally following histological staining.

Acknowledgements

We would like to thank Dr. Geoffrey Schoenbaum for his assistance in conceptualization of the latent inhibition experiments. This work was supported by that National Institutes of Mental Health R00MH123673, University of South Carolina College of Pharmacy, and the National Institute on Drug Abuse Intramural Research Program. Construction and packaging of the AAV vector expressing mTACR1 gRNAs was performed by the National Institute on Drug Abuse Genetic Engineering and Viral Vector Core, RRID:SCR_022969.

Author contributions

Conceptualization, T.C.F.; Methodology and Investigation, A.B., C.G., C.S., C.R., A.S., H.H., and T.C.F.; Writing, T.C.F.; Review and Editing, A.B., C.G., A.S., C.R., and T.C.F.; Supervision, T.C.F.

Competing Interests

The authors declare no competing interests and have nothing to disclose.

References

1. Zaehle, T. *et al.* Nucleus Accumbens Activity Dissociates Different Forms of Salience: Evidence from Human Intracranial Recordings. *The Journal of Neuroscience* **33**, 8764 LP – 8771 (2013).

2. Schoenbaum, G. & Setlow, B. Lesions of Nucleus Accumbens Disrupt Learning about Aversive Outcomes. *Journal of Neuroscience* **23**, 9833–9841 (2003).

3. Dutta, S., Beaver, J., Halcomb, C. J. & Jasnow, A. M. Dissociable roles of the nucleus accumbens core and shell subregions in the expression and extinction of conditioned fear. *Neurobiol Stress* **15**, 100365 (2021).

4. Wendler, E. *et al.* Neurobiology of Learning and Memory The roles of the nucleus accumbens core , dorsomedial striatum , and dorsolateral striatum in learning : Performance and extinction of Pavlovian fear-conditioned responses and instrumental avoidance responses. *Neurobiol Learn Mem* **109**, 27–36 (2014).

5. Ray, M. H., Russ, A. N., Walker, R. A. & McDannald, M. A. The Nucleus Accumbens Core is Necessary to Scale Fear to Degree of Threat. *The Journal of Neuroscience* **40**, 4750 LP – 4760 (2020).

6. Francis, T. C. & Lobo, M. K. Emerging Role for Nucleus Accumbens Medium Spiny Neuron Subtypes in Depression. *Biol Psychiatry* **81**, 645–653 (2017).

7. Lee, H. J. *et al.* Activation of Direct and Indirect Pathway Medium Spiny Neurons Drives Distinct Brain-wide Responses. *Neuron* **91**, 412–424 (2016).

8. Kravitz, A. V, Tye, L. D. & Kreitzer, A. C. Distinct roles for direct and indirect pathway striatal neurons in reinforcement. *Nat Neurosci* **15**, 816–818 (2012).

9. Francis, T. C. *et al.* Nucleus accumbens medium spiny neuron subtypes mediate depression-related outcomes to social defeat stress. *Biol Psychiatry* **77**, 212–222 (2015).

10. Smith, R. J., Lobo, M. K., Spencer, S. & Kalivas, P. W. Cocaine-induced adaptations in D1 and D2 accumbens projection neurons (a dichotomy not necessarily synonymous with direct and indirect pathways). *Curr Opin Neurobiol* **23**, 546–552 (2013).

11. Lobo, M. K. *et al.* Cell type-specific loss of BDNF signaling mimics optogenetic control of cocaine reward. *Science* **330**, 385–390 (2010).

12. Ferguson, S. M., Phillips, P. E., Roth, B. L., Wess, J. & Neumaier, J. F. Direct-pathway striatal neurons regulate the retention of decision-making strategies. *J Neurosci* **33**, 11668–11676 (2013).

13. Tai, L. H., Lee, A. M., Benavidez, N., Bonci, A. & Wilbrecht, L. Transient stimulation of distinct subpopulations of striatal neurons mimics changes in action value. *Nat Neurosci* **15**, 1281–1289 (2012).

14. Bock, R. *et al.* Strengthening the accumbal indirect pathway promotes resilience to compulsive cocaine use. *Nat Neurosci* **16**, 632–638 (2013).

15. Calipari, E. S. *et al.* In vivo imaging identifies temporal signature of D1 and D2 medium spiny neurons in cocaine reward. *Proc Natl Acad Sci U S A* **113**, 2726–2731 (2016).

16. Iino, Y. *et al.* Dopamine D2 receptors in discrimination learning and spine enlargement. *Nature* **579**, 555–560 (2020).

17. MacPherson, T. *et al.* Nucleus accumbens dopamine D2-receptor expressing neurons control behavioral flexibility in a place discrimination task in the IntelliCage. *Learning and Memory* **23**, 359–364 (2016).

18. Macpherson, T., Kim, J. Y. & Hikida, T. Nucleus Accumbens Core Dopamine D2 Receptor-Expressing Neurons Control Reversal Learning but Not Set-Shifting in Behavioral Flexibility in Male Mice. *Front Neurosci* **16**, 885380 (2022).

19. Soares-Cunha, C. *et al.* Nucleus Accumbens Microcircuit Underlying D2-MSN-Driven Increase in Motivation. *eNeuro* **5**, ENEURO.0386-18.2018 (2018).

20. Soares-Cunha, C. *et al.* Activation of D2 dopamine receptor-expressing neurons in the nucleus accumbens increases motivation. *Nat Commun* **7**, 11829 (2016).

21. Owesson-White, C. *et al.* Cue-Evoked Dopamine Release Rapidly Modulates D2 Neurons in the Nucleus Accumbens During Motivated Behavior. *J Neurosci* **36**, 6011–6021 (2016).

22. Francis, T. C., Yano, H., Demarest, T. G., Shen, H. & Bonci, A. High-Frequency Activation of Nucleus Accumbens D1-MSNs Drives Excitatory Potentiation on D2-MSNs. *Neuron* **103**, (2019).

23. Commons, K. G. Neuronal pathways linking substance P to drug addiction and stress. *Brain Res* **1314**, 175–182 (2010).

24. Aosaki, T. *et al.* Responses of tonically active neurons in the primate's striatum undergo systematic changes during behavioral sensorimotor conditioning. *J Neurosci* **14**, 3969–3984 (1994).

25. Ravel, S., Legallet, E. & Apicella, P. Responses of Tonically Active Neurons in the Monkey Striatum Discriminate between Motivationally Opposing Stimuli. *The Journal of Neuroscience* **23**, 8489 LP – 8497 (2003).

26. Schulz, J. M. *et al.* Enhanced high-frequency membrane potential fluctuations control spike output in striatal fast-spiking interneurones in vivo. *J Physiol* **589**, 4365–81 (2011).

27. Aoki, S., Liu, A. W., Zucca, A., Zucca, S. & Wickens, J. R. Role of Striatal Cholinergic Interneurons in Set-Shifting in the Rat. *The Journal of Neuroscience* **35**, 9424 LP – 9431 (2015).

28. Okada, K. *et al.* Enhanced flexibility of place discrimination learning by targeting striatal cholinergic interneurons. *Nat Commun* **5**, 3778 (2014).

29. Ragozzino, M. E., Mohler, E. G., Prior, M., Palencia, C. A. & Rozman, S. Acetylcholine activity in selective striatal regions supports behavioral flexibility. *Neurobiol Learn Mem* **91**, 13–22 (2009).

30. Collins, A. L. *et al.* Nucleus Accumbens Cholinergic Interneurons Oppose Cue-Motivated Behavior. *Biol Psychiatry* **86**, 388–396 (2019).

31. Sandweiss, A. J. & Vanderah, T. W. The pharmacology of neurokinin receptors in addiction: prospects for therapy. *Subst Abuse Rehabil* **6**, 93–102 (2015).

32. Tomaz, C. & Nogueira, P. J. C. Facilitation of memory by peripheral administration of substance P. *Behavioural Brain Research* **83**, 143–145 (1997).

33. Ebner, K. & Singewald, N. The role of substance P in stress and anxiety responses. *Amino Acids* **31**, 251–272 (2006).

34. Aosaki, T. & Kawaguchi, Y. Actions of substance P on rat neostriatal neurons in vitro. *The Journal of neuroscience* **16**, 5141–5153 (1996).

35. Jing, M. *et al.* An optimized acetylcholine sensor for monitoring in vivo cholinergic activity. *Nat Methods* **17**, 1139–1146 (2020).

36. Lubow, R. E. Latent inhibition. *Psychol Bull* **79**, 398 (1973).

37. Pignatelli, M. *et al.* Cooperative synaptic and intrinsic plasticity in a disynaptic limbic circuit drive stress-induced anhedonia and passive coping in mice. *Mol Psychiatry* **26**, 1860–1879 (2021).

38. Britt, J. P. *et al.* Synaptic and behavioral profile of multiple glutamatergic inputs to the nucleus accumbens. *Neuron* **76**, 790–803 (2012).

39. Gale, G. D. *et al.* Role of the Basolateral Amygdala in the Storage of Fear Memories across the Adult Lifetime of Rats. *The Journal of Neuroscience* **24**, 3810 LP – 3815 (2004).

40. Gallo, E. F. *et al.* Dopamine D2 receptors modulate the cholinergic pause and inhibitory learning. *Mol Psychiatry* **27**, 1502–1514 (2022).

41. Al-Hasani, R. *et al.* Ventral tegmental area GABAergic inhibition of cholinergic interneurons in the ventral nucleus accumbens shell promotes reward reinforcement. *Nat Neurosci* **24**, 1414–1428 (2021).

42. Matsumoto, N., Minamimoto, T., Graybiel, A. M. & Kimura, M. Neurons in the thalamic CM-Pf complex supply striatal neurons with information about behaviorally significant sensory events. *J Neurophysiol* **85**, 960–976 (2001).

43. Zhang, Y.-F., Reynolds, J. N. J. & Cragg, S. J. Pauses in Cholinergic Interneuron Activity Are Driven by Excitatory Input and Delayed Rectification, with Dopamine Modulation. *Neuron* **98**, 918-925.e3 (2018).

44. Guzman, R. G. & Kendrick, K. M. Effect of substance P on acetylcholine and dopamine release in the rat striatum: a microdialysis study. *Brain Res* **622**, 147–154 (1993).

45. Brimblecombe, K. R. & Cragg, S. J. Substance P Weights Striatal Dopamine Transmission Differently within the Striosome-Matrix Axis. *Journal of Neuroscience* **35**, 9017–9023 (2015).

46. Cachope, R. *et al.* Selective activation of cholinergic interneurons enhances accumbal phasic dopamine release: setting the tone for reward processing. *Cell Rep* **2**, 33–41 (2012).

47. Threlfell, S. *et al.* Striatal dopamine release is triggered by synchronized activity in cholinergic interneurons. *Neuron* **75**, 58–64 (2012).

48. Kutlu, M. G. *et al.* Dopamine signaling in the nucleus accumbens core mediates latent inhibition. *Nat Neurosci* **25**, 1071–1081 (2022).

49. McConalogue, K., Corvera, C. U., Gamp, P. D., Grady, E. F. & Bunnett, N. W. Desensitization of the neurokinin-1 receptor (NK1-R) in neurons: effects of substance P on the distribution of NK1-R, Galphaq/11, G-protein receptor kinase-2/3, and beta-arrestin-1/2. *Mol Biol Cell* **9**, 2305–2324 (1998).

50. Bowden, J. J. *et al.* Direct observation of substance P-induced internalization of neurokinin 1 (NK1) receptors at sites of inflammation. *Proceedings of the National Academy of Sciences* **91**, 8964–8968 (1994).

51. Anagnostaras, S. *et al.* Automated Assessment of Pavlovian Conditioned Freezing and Shock Reactivity in Mice Using the Video Freeze System . *Frontiers in Behavioral Neuroscience* vol. 4 Preprint at (2010).

52. Howard, D. B. & Harvey, B. K. Assaying the Stability and Inactivation of AAV Serotype 1 Vectors. *Hum Gene Ther Methods* **28**, 39–48 (2017).

Figures

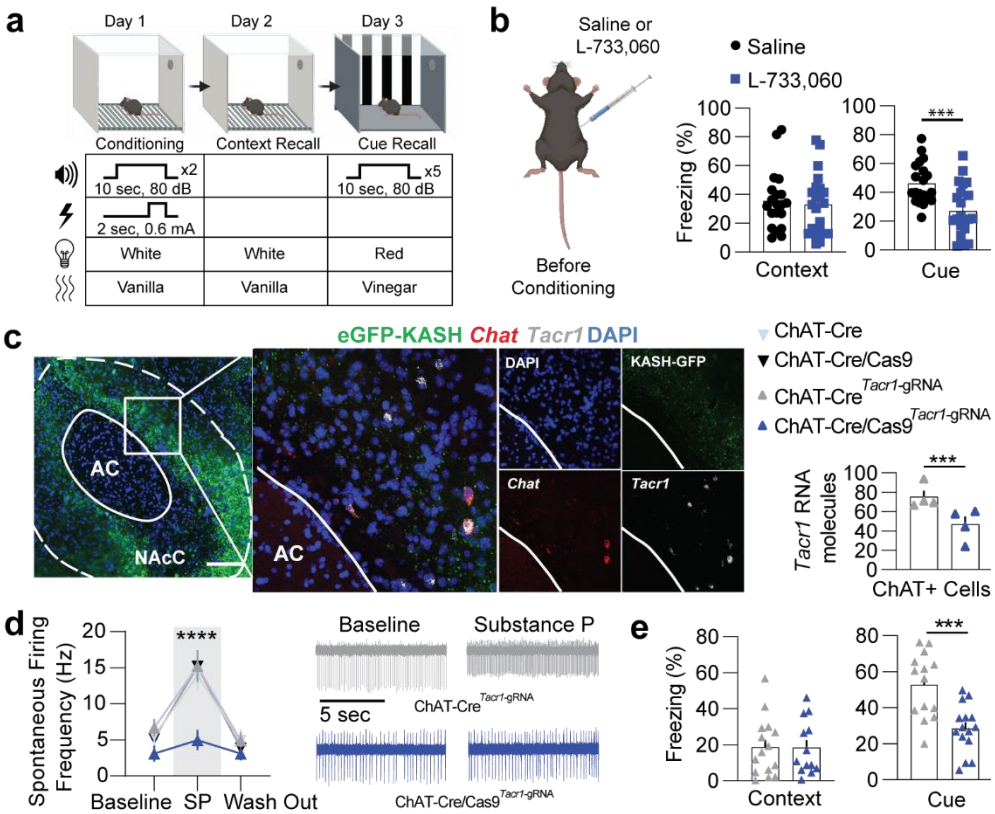

Figure 1. NAc substance P signaling is required for cue-dependent aversive learning. (a) Fear conditioning paradigm for aversive learning. **(b)** Injection of the NK1 receptor antagonist L-733,060 (10 mg/kg) or saline was given prior to conditioning. NK1 receptor antagonist suppressed freezing in response to the cue during cue recall ($p < 0.001$) and no difference in freezing was observed in the context ($p > 0.05$) (n = 12, 10 mice). **(c)** Fluorescent image of EGFP-KASH expression in the NAc core. Scale bar 100 µm. Insert: Expression of EGFP-KASH (green), *Chat* RNA (red), *Tacr1* RNA (grey/white), and DAPI (blue). CRISPR-Cas9 mediated knockout of *Tacr1* decreased measured *Tacr1* RNA puncta in *Chat* RNA positive cells ($p < 0.001$) (n = 4 mice per group). **(d)** Graph and representative traces showing Cas9 and *Tacr1*-gRNA was required to suppress substance P(SP)-dependent activation of ChAT cells (Interaction $p<0.0001$; post-hoc Baseline vs. SP ChAT-Cre/Cas9^{Tacr1} $p > 0.05$, Mice/cells(N/n) = 4/10; Baseline vs. SP ChAT-Cre^{Tacr1}, ChAT-Cre/Cas9, or ChAT-Cre $p < 0.0001$) (N/n = 4/9, 4/8, 3/7). **(e)** *Tacr1* knockout decreased freezing to the cue ($p < 0.001$), but not the conditioning context ($p > 0.05$) (n = 12, 12 mice). For detailed statistics see **Supplementary Table 1**.

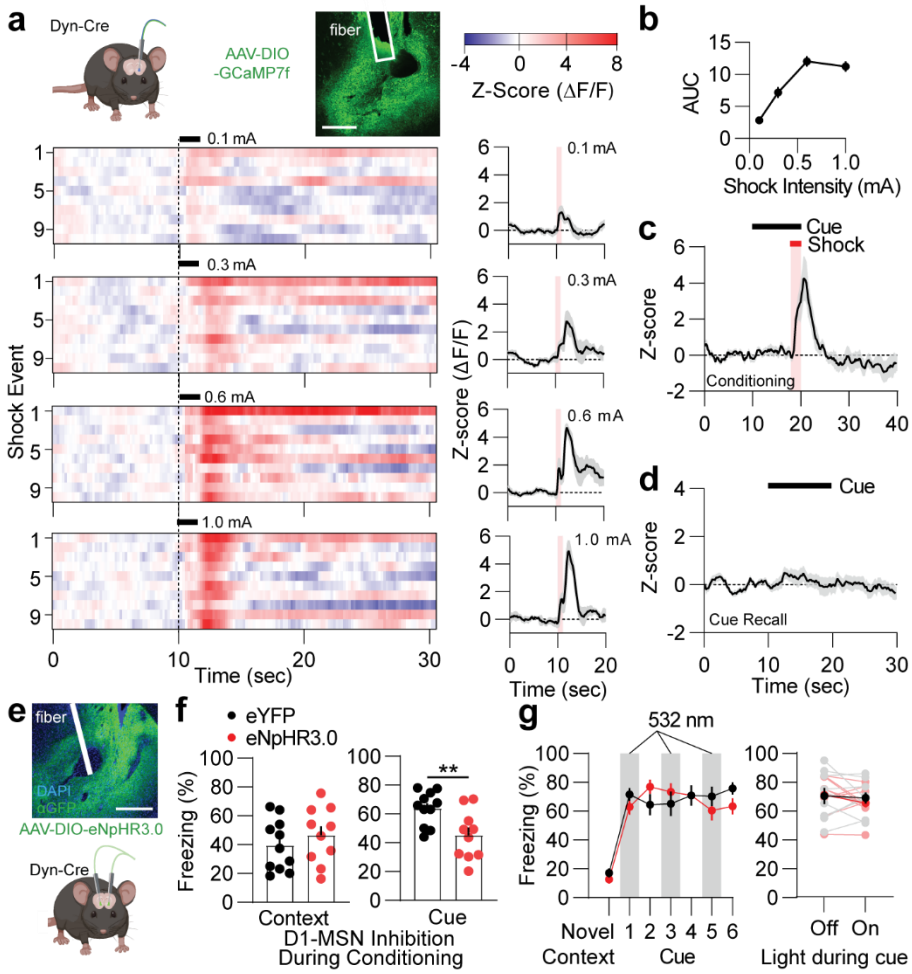

Figure 2. D1-MSN activity is required for cue-dependent conditioning. (a) A cartoon of a Dyn-Cre mouse injected with the calcium indicator GCaMP7f and a representative image of its expression in the NAc core with a fiber track. Scale bar 200 µm. Heat maps and time plots representing average z-scores of D1-MSN calcium-driven fluorescence over increasing foot shock intensity (0.1-1.0 mA). **(b)** AUC increased with shock intensity ($p < 0.01$) (n = 5 mice). **(c)** Averaged z-score D1-MSN calcium activity during fear conditioning. **(d)** Average z-score D1-MSN calcium activity in response to the condition cue during cue recall. **(e)** Expression of eNpHR3.0-eYFP, fiber track for optogenetic inhibition of the NAc core, and cartoon displaying bilateral implant and expression of the optogenetic construct in mice. **(f)** Light inhibition of D1-MSNs containing eNpHR3.0 decreased freezing to the cue ($p < 0.01$), but not the conditioning context ($p > 0.05$) (n = 11, 10 mice). **(g)** Inhibition of D1-MSNs during cue recall had no effect on freezing (Laser off vs. on, $p > 0.05$) (n = 9, 10 mice). For detailed statistics see **Supplementary Table 1**.

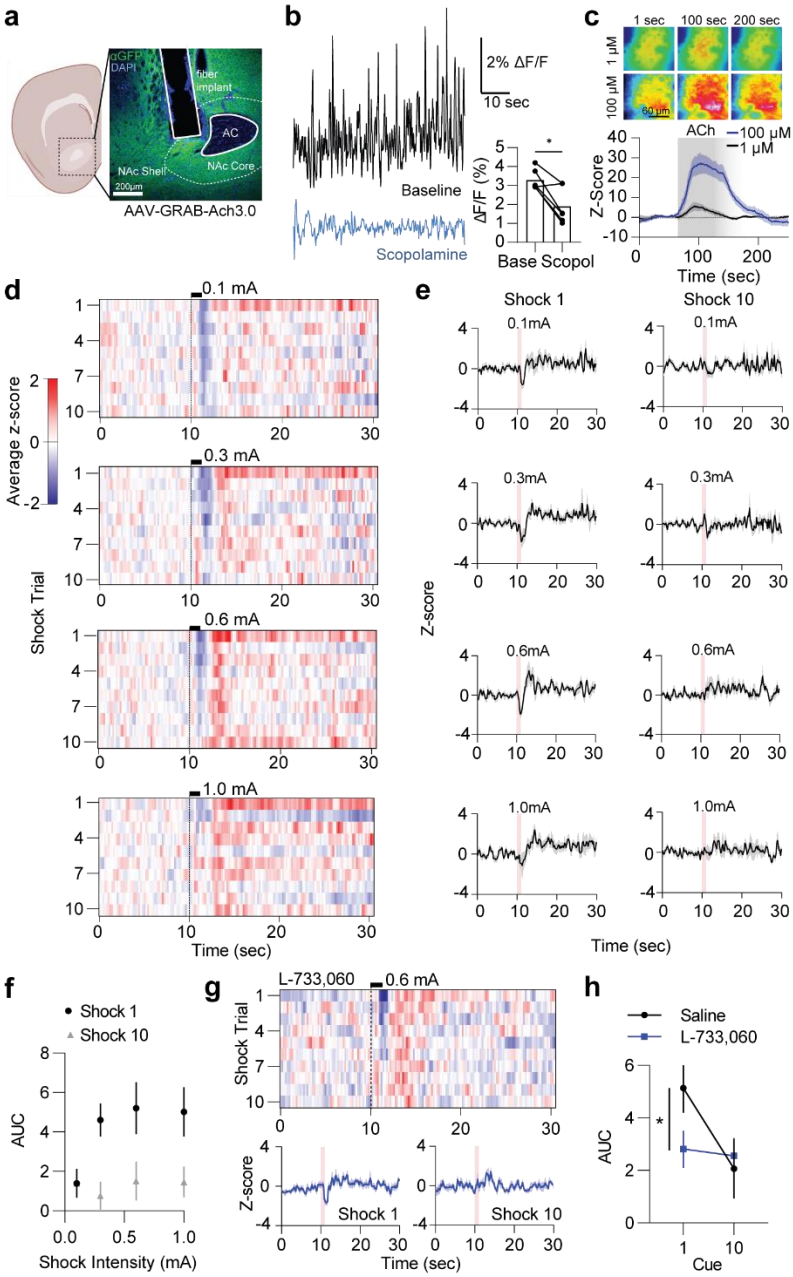

Figure 3. Acetylcholine release during shock is substance P-dependent. (a) Representative image of GRAB-ACh3.0 expression in the NAc core. **(b)** Intraperitoneal injection of scopolamine (1 mg/kg) significantly decreases the in vivo acetylcholine signal from GRAB-ACh3.0 in the NAc core ($p < 0.05$, n = 6 mice). **(c)** Bath application of acetylcholine significantly enhances bulk fluorescence of the GRAB-ACh3.0 sensor in the NAc. **(d)** Heatmaps and **(e)** time courses of fluorescent signals due to acetylcholine release from 10 random foot shocks at intensities of 0.1-1.0 mA amplitudes. Time courses are taken from average z-scores from shock 1 and shock 10. **(f)** AUC measurement of acetylcholine release at varying shock intensities for shock 1 and shock 10. Acetylcholine released varied across both AUC and shock intensity (Cue 1 vs. Cue 10, $p < 0.05$) (n = 9 mice). **(g-h)** NK1 receptor antagonism suppressed AUC of the acetylcholine signal varied across both drug treatment (AUC Saline vs. L-733,060 during Cue 1, $p < 0.05$) and shock presentation (Cue 1 vs. Cue 10, $p < 0.05$) (n = 5, 6 mice). For detailed statistics see **Supplementary Table 1.**

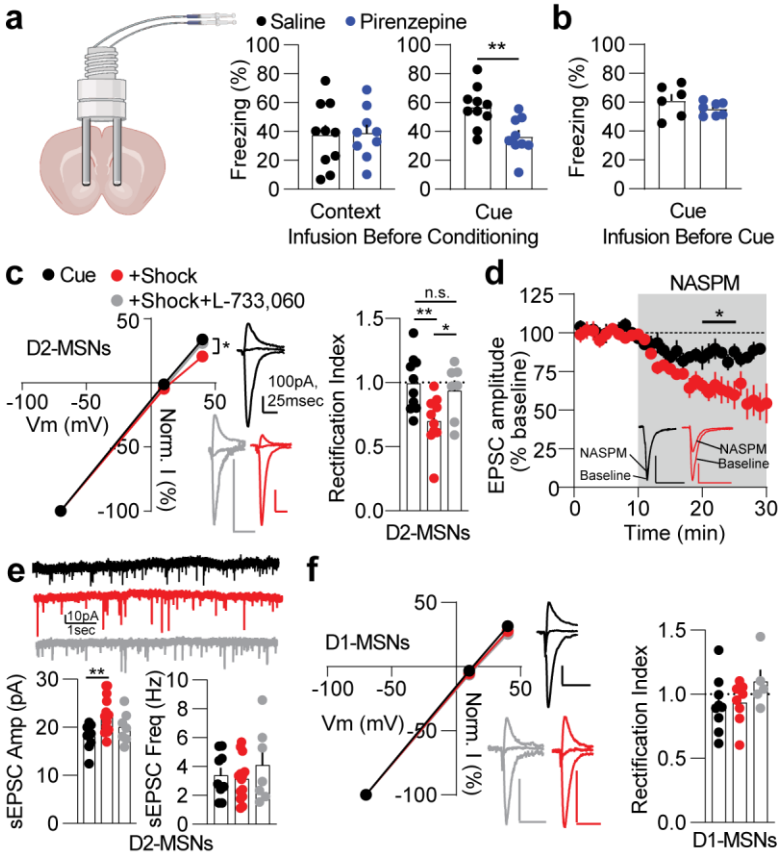

Figure 4. Muscarinic signaling and substance P plasticity is required for cue-dependent aversive learning. (a) Selective infusion of the muscarinic 1 receptor antagonist pirenzepine (bilateral) prior to conditioning suppressed cue-dependent ($p < 0.01$) but not conditioning context dependent freezing ($p > 0.05$) (n = 10, 9). **(b)** Infusion of pirenzepine prior to cue recall had no effect on freezing ($p > 0.05$) (n = 6, 7). **(c)** Cue/shock conditioning enhanced inward rectification of the I-V curve generated from electrically evoke excitatory currents on D2-MSNs (Interaction $p < 0.001$) from the cue/shock group compared to cue only (post-hoc $p < 0.05$) and cue/shock with L-733,060 (post-hoc $p < 0.05$). Rectification index indicated more rectification for D2-MSNs from cue/shock mice (Interaction, $p < 0.01$) compared to cue only (post-hoc, $p < 0.01$) and cue/shock with L-733,060 (post-hoc, $p < 0.05$) (N/n (Mice/cells) = 5/10, 5/10, 4/7). **(d)** NASPM suppressed currents more from D2-MSNs from cue/shock mice compared to cue only mice (25-30 min average, $p < 0.05$) (N/n = 4/6, 4/5). **(e)** Spontaneous excitatory post-synaptic current (sEPSC) amplitude ($p < 0.05$), but not frequency ($p > 0.05$) is increased in D2-MSNs following fear conditioning (N/n = 6/14, 5/9, 4/7). **(f)** D1-MSNs do not show inward rectification across conditions ($p > 0.05$) (N/n = 6/8, 4/9, 4/5). For detailed statistics see **Supplementary Table 1**.

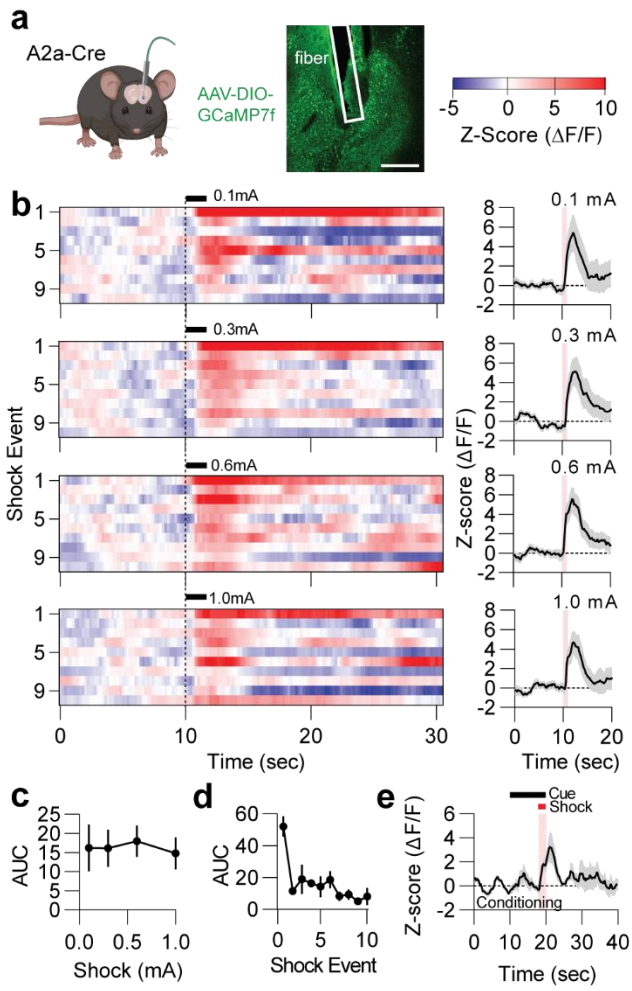

Figure 5. D2-MSNs do not scale with shock intensity. (a) A cartoon of an A2a-Cre mouse injected with the calcium indicator GCaMP7f and a representative image of its expression in the NAc core with a fiber track. Scale bar 200 µm. **(b)** Heat maps and time plots representing average z-scores of D2-MSN calcium-driven fluorescence over increasing foot shock intensity (0.1-1.0 mA). **(c)** AUC of an average of all 10 foot shocks indicated was no change in response across foot shock intensity ($p > 0.05$). **(d)** AUC rapidly habituated after the first 0.6 mA foot shock ($p < 0.0001$) (n = 10 mice). **(e)** D2-MSNs responded to the first conditioning shock but not the cue. For detailed statistics see **Supplementary Table 1**.

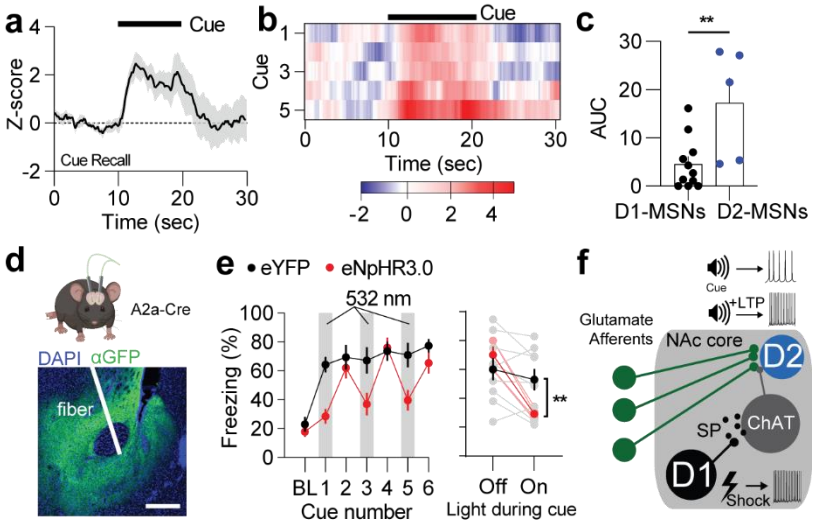

Figure 6. D2-MSNs are required for cue recall. (a) Time plot and **(b)** heat map showing D2-MSN calcium fluorescent signals increased in response to recalled cues. **(c)** D2-MSN AUC in response to D1-MSNs was larger in response to the recalled cue ($p < 0.01$) (n = 11, 5 mice). **(d)** eNpHR3.0-eYFP expression in D2-MSNs within the NAc core and cartoon depicting bilateral inhibition of the NAc core. **(e)** Inhibition of D2-MSNs reversibly suppressed freezing to recalled cues (Over cues $p < 0.01$; Laser off vs on $p < 0.0001$; post-hoc laser on eYFP vs. eNpHR3.0 $p < 0.05$) (n = 9, 6 mice). **(f)** Model for the mechanism of aversive cue learning. Shock activates D1-MSNs releasing substance P and activating ChAT neurons. ChAT neurons release acetylcholine selectively during cue/shock pairings and promotes glutamatergic long-term potentiation (LTP) on D2-MSNs rendering them more responsive to the cue. For detailed statistics see **Supplementary Table 1**.