Divergent projections of the paraventricular nucleus of the thalamus mediate the selection of reactive and proactive defensive behaviors

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Divergent projections of the paraventricular nucleus of the thalamus mediate the selection of reactive and proactive defensive behaviors

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ABSTRACT

The appropriate selection of reactive and proactive defensive behaviors amid fearful situations is essential for survival. Studies in both rodents and primates have shown that reactive defensive responses depend on the activity of the central nucleus of the amygdala (CeA) whereas proactive ones primarily rely on the nucleus accumbens (NAc). However, the mechanisms underlying flexible switching between CeA-driven (reactive) and NAc-driven (proactive) defensive responses, remain unknown. Here, using a behavioral task in which mice must trade a reactive defensive strategy (i.e. freezing) for an instrumental one (i.e. active avoidance) to avoid punishment, we discovered that the paraventricular nucleus of the thalamus (PVT) mediates the selection of defensive behaviors through its interaction with the CeA and the NAc. *In vivo* calcium imaging using fiber photometry showed that unlike the PVT–CeA pathway which drives conditioned freezing responses, the PVT–NAc pathway is inhibited during freezing and instead signals active avoidance events. In addition, optogenetic manipulations of these circuits revealed that activity in the PVT–CeA or PVT–NAc pathway biases behavior toward the selection of reactive or proactive defensive responses, respectively. Our collective findings provide direct evidence that the PVT, a structure increasingly considered as a potential site for guiding behavioral decisions amid motivational conflicts, mediates flexible switching between opposing defensive behaviors.

INTRODUCTION

The use of Pavlovian conditioning paradigms has allowed scientists to achieve substantial knowledge of the neuronal circuits and cellular processes underlying fear conditioning and its associated Pavlovian fear reactions (e.g., freezing, flight)¹⁻⁴. In contrast, the mechanisms controlling instrumental (proactive) defensive responses such as active avoidance and those that guide the selection of reactive and proactive defensive behaviors, are far less studied⁵⁻⁸. This is surprising considering that in nature animals readily engage and switch between both types of defensive strategies depending on threat imminence⁸⁻¹⁰.

While studies on the neurobiology of defensive behaviors have pointed to the contributions of both cortical and subcortical networks to the expression of specific defensive behaviors^{8,10}, it is generally recognized that Pavlovian reactions such as freezing and conditioned flight are driven by genetically-defined neuronal subpopulations of the CeA^{4,11,12} and that proactive defensive behaviors largely depend on the NAc¹³⁻¹⁵. Interestingly, reports from the last decade suggest that these two regions of the brain likely compete for the control of defensive behaviors^{13,16}. Specifically, these studies demonstrated that while lesions and pharmacological manipulations of the CeA attenuate freezing and promote active avoidance behavior^{16,17}, inactivation of the NAc decreases avoidance but enhances freezing^{13,14}. Despite these seminal observations, the mechanisms dictating the balance of the competition between these two regions and behavioral outcomes remain unknown.

A potential candidate for mediating the selection of these two types of defensive behaviors is the basolateral amygdala (BLA) which sends projections to both the NAc

and the CeA¹⁸. Indeed, while BLA projections to the CeA are known to mediate conditioned freezing responses¹⁹, their projections to the NAc have been shown to support active avoidance behavior¹⁴. However, studies show that the necessity of the BLA for the expression of conditioned freezing and avoidance behaviors decreases with the passage of time²⁰⁻²², suggesting that other brain regions likely contribute to arbitrating between these two defensive strategies. We predicted that the posterior portion of the PVT (pPVT) could be implicated in this process, considering its strong innervation of the CeA and the NAc^{23,24} as well as its documented role in mediating conditioned freezing responses, particularly at remote timepoints^{12,22}. Consistent with this prediction, here we show that the pPVT drives active avoidance through its projections to the NAc. Moreover, we demonstrate that divergent projections from the pPVT to the NAc and CeA mediate the selection of active avoidance and freezing behavior, respectively. These findings highly the existence of a previously unrecognized switch for the selection of reactive and proactive defensive behaviors in the midline thalamus.

RESULTS

pPVT^{D2R} neurons are inhibited during freezing

The posterior PVT (pPVT), is a stress-sensitive region of the thalamus that sends robust, largely non-overlapping projections to the CeA and the NAc (Extended Data Fig. 1)²⁵. Unlike primates and carnivores, the rodent thalamus is largely devoid of GABAergic neurons²⁶. Thus, projection neurons of the PVT are predominantly

89 glutamatergic²⁷. Although activation of CeA-projecting pPVT neurons is thought to be
90 necessary for the retrieval of conditioned fear memory and its accompanying of freezing
91 behavior^{12,22}, the NAc is the main target of most pPVT neurons (Extended Data Fig.
92 1h)²⁵. Surprisingly, the contribution of pPVT–NAc neurons to fear-related behaviors is
93 currently unknown. To investigate this, we first monitored the activity of dopamine D2
94 receptor expressing neurons of the pPVT (pPVT^{D2R}) – a marker of stress-sensitive PVT
95 neurons, the majority of which project to the NAc (Extended Data Fig. 1g-j)²⁸⁻³⁰ – during
96 fear conditioning (Extended Data Fig. 2, 3, 4). Towards this goal, we expressed the
97 genetically encoded calcium sensor GCaMP6s in pPVT^{D2R} neurons of *Drd2*-Cre mice
98 (Extended Data Fig. 2a). Next, following a habituation session (Day 0), on Day 1 mice
99 were fear conditioned to an auditory cue (conditioned stimulus; CS) that co-terminated
100 with a footshock (unconditioned stimulus; US), and on Day 2 a fear memory retrieval
101 test was performed (Extended Data Fig. 2b). On both days, bulk changes in GCaMP6s
102 fluorescence in pPVT^{D2R} neurons were monitored using fiber photometry. We reasoned
103 that since most pPVT^{D2R} neurons project to the NAc, bulk GCaMP6s fluorescence
104 gathered from this neuronal population with fiber photometry would likely be dominated
105 by the activity of PVT–NAc neurons. Consistent with previous reports, pPVT^{D2R} neurons
106 were readily activated by the US during the conditioning session on Day 1^{28,29}
107 (Extended Data Fig. 2g, j, n). Notably, we also observed the emergence of small but
108 consistent CS-evoked GCaMP6s responses in late conditioning trials, suggesting that
109 the activity of these neurons is modulated by learning²² (Extended Data Fig. 2l, m). On
110 Day 2, fear memory retrieval was associated with an average decrease in GCaMP6s
111 fluorescence upon CS presentation (Extended Data Fig. 2h, k). These inhibitory

GCaMP6s responses were variable and apparent in many but not all CS presentations (Extended Data Fig. 3a), suggesting that they could be tied to behavior. To explore this possibility, we classified all retrieval trials into three groups based on the percentage of freezing displayed and quantified the average CS-evoked response for each trial category (Extended Data Fig. 3a-d) (See Methods). As predicted, the average change in GCaMP6s fluorescence for each trial category varied as a function of freezing behavior, with the highest freezing scores giving rise to the most robust inhibitory responses (Extended Data Fig. 3a-d). Notably, we did not observe any effect of retrieval trial number on the GCaMP6s signal (Extended Data Fig. 4). These results indicate the existence of an inverse relationship between freezing behavior and pPVT^{D2R} neuron activity. Consistent with this, pairwise comparisons of CS-evoked movement and GCaMP6s fluorescence uncovered a modest but positive correlation between these two parameters (Extended Data Fig. 3c). Importantly, no such correlation was observed on imaging data gathered during habituation, prior to fear conditioning (Extended Data Fig. 3e, f). As such, our collective results demonstrate that conditioned freezing is associated with decreased activity among pPVT^{D2R} neurons.

pPVT^{D2R} neurons signal active avoidance

The emergence of a positive correlation between the calcium signal of pPVT^{D2R} neurons and movement following fear conditioning, likely reflects an overall link between the activity of this neuronal population and the selection of active coping strategies during fearful situations. To formally assess this possibility, we trained mice in a two-way signaled active avoidance task (2AA) where performing a specific action – moving to the neighboring compartment of a shuttle box – upon presentation of the CS,

enables mice to avoid a footshock (US) (Fig. 1b). In well-trained mice, CS-related responses typically involve trading reactive (freezing) responses in favor of proactive (instrumental) behavior (Fig. 1c)⁶. Still, animals engage in both freezing and avoidance behavior on a trial-by-trial basis (Extended Data Fig. 5c, d). As such, this task provides an opportunity for assessing neuronal activity while animals transition between reactive and instrumental fear-related behavioral responses within single test sessions.

Consistent with previous reports, mice displayed prominent freezing behavior to the CS in early sessions of the 2AA task⁶(Fig. 1c) (Extended Data Fig. 5a). However, additional training led to progressive decreases in CS-evoked freezing and a concomitant increase in active avoidance responses (Fig. 1c) (Extended Data Fig. 5a). After three sessions of 2AA training (Days 1-3), pPVT^{D2R} neurons were imaged using fiber photometry during two additional training sessions (Days 4 and 5) (Fig. 1c). Imaging trials were subsequently divided into two behaviorally relevant categories: trials in which mice avoided the US (avoidance) and trials in which they failed to do so (failure). Notably, during failure trials, mice reliably shuttled to the adjacent compartment in response to the US (Supplementary Movie 1). The data for avoidance and failure trials are summarized in Figure 1 and Extended Data Figure 5 (Fig. 1d-h) (Extended Data Fig. 5b-d). Classifying trials in this manner revealed important differences in the activity of pPVT^{D2R} neurons during avoidance and failure trials (Supplementary Movie 1). First, while CS presentations elicited robust increases in GCaMP6s fluorescence in pPVT^{D2R} neurons during avoidance trials, failures were mostly associated with strong decreases in the activity of these neurons (Fig. 1g). In addition, presentation of the US during failure trials was associated with robust activation of pPVT^{D2R} neurons, consistent with

our fear conditioning data as well as with previous reports (Fig. 1d, e)^{28,29}. Interestingly, whereas avoidance trials were by definition accompanied by instrumental responses, mice spent significantly more time freezing in response to the CS during failure trials (Fig. 1f). This suggests that the differential dynamics of calcium signals of pPVT^{D2R} neurons observed across these trial categories could be related to behavior. To investigate this possibility, we analyzed calcium transients time-locked to discrete task and behavioral events such as CS onset, maximum velocity and freezing behavior (Fig. 1h). These analyses revealed that while CS onset, maximum velocity and escape behavior (shuttling in response to the CS or US onset) were all associated with increases in GCaMP6s fluorescence, freezing behavior was accompanied by attenuated pPVT^{D2R} neuronal activity for both avoidance and failure trials (Fig. 1h). Thus, while mean signal variations more accurately distinguish avoidance and failure trials (Fig. 1f, g), discrete fluctuations in calcium transients largely answers to moment-to-moment variation in behavior and not trial type (Fig. 1h). It is interesting that rises in neuronal activity were correlated with maximum velocity particularly for avoidance trials (Fig. 1h). This result indicates a potential relationship between pPVT^{D2R} neuron activity and vigor, such as that necessary to avoid or escape an impending threat. Collectively, our findings support the idea that, at the population level, pPVT^{D2R} neurons signal instrumental defensive behaviors and are suppressed during freezing.

To investigate whether activation of pPVT^{D2R} neurons is required for active avoidance, we expressed in these neurons the inhibitory opsin eNpHR3.0 (Halorhodopsin; Halo) and trained mice in the 2AA task as described above (Fig. 1i) (Extended Data Fig. 6). After 2AA training, mice were subjected to three test sessions,

Test Days 1-3 (Fig. 1j-l). On Test Day 1 (light off), both control and Halo-expressing mice displayed robust avoidance behavior (Fig. 1j, k). On Test Day 2 (light on), light stimulation during CS presentations significantly attenuated the avoidance rate of Halo-expressing but not control mice (Fig. 1j, k). This effect was accompanied by a significant increase in the latency to avoid and an increase in CS-evoked freezing behavior (Fig. 1k, l). These parameters, partially recovered on Test Day 3 (light off) (Fig. 1j-l). Importantly, restricting optogenetic inhibition of pPVT^{D2R} neurons to the inter-trial interval (ITI) did not significantly alter behavior (Extended Data Fig. 6k-t). Similarly, we did not observe any light stimulation-induced effect on locomotion in an open field arena (Extended Data Fig. 6j). In summary, these findings show that, at the population level, pPVT^{D2R} neurons are required for active avoidance behavior and are inhibited during freezing.

pPVT–NAc projections drive avoidance and antagonize freezing

While most pPVT^{D2R} neurons project to the NAc (Extended Data Fig. 1)²⁹, it is unclear whether pPVT^{D2R}–NAc projections are modulated by passive and/or instrumental responding in the 2AA task. To address this question, we expressed the genetically encoded calcium indicator GCaMP7s in pPVT^{D2R} neurons and implanted an optical fiber unilaterally in the NAc to measure the activity of pPVT^{D2R}–NAc projections using fiber photometry (Fig. 2a-d) (Extended Data Fig. 7a). We trained GCaMP7s-expressing mice in the 2AA task, classified individual test trials as either avoidance or failure as described above, and analyzed the corresponding change in fluorescent signal associated with presentations of the CS (Fig. 2e-i) (Extended Data Fig. 7b). To ensure sufficient numbers of avoidance and failure trials during imaging sessions, mice

were only trained for three days before imaging started (Fig. 2d) (Extended Data Fig. 7a). These analyses revealed that pPVT^{D2R}–NAc projections were rapidly engaged following CS presentations (Fig. 2i) and were robustly activated during avoidance and escape episodes (Fig. 2i) irrespective of trial type. In contrast, CS-evoked freezing was associated with a reduction in GCaMP7s fluorescence (Fig. 2i). These calcium dynamics largely resembled those observed in pPVT^{D2R} neurons (Fig. 1d-h) (Extended Data Fig. 5b). Together, these findings demonstrate that pPVT^{D2R}–NAc projections signal active avoidance and are suppressed during freezing behavior.

It is important to highlight that, unlike calcium responses recorded from the cell bodies of pPVT^{D2R} neurons, for which increases were readily observed during shuttling events (Fig. 1d), increases in GCaMP7 fluorescence in pPVT^{D2R}–NAc terminals appeared to emerge mostly at the conclusion of avoidance behavior (CS offset) (Fig. 2e, f). While alternative interpretations to this discrepancy are considered in the Discussion section of this manuscript, one potential explanation is that calcium transients accompanying avoidance behavior could result in weaker fluorescent changes at pPVT^{D2R}–NAc axon terminals, thereby limiting our ability to accurately detect these with fiber photometry. To circumvent this limitation, we employed a viral vector strategy that allowed us to record the cell bodies of NAc-projecting neurons directly in the pPVT (Extended Data Fig. 8a, b). As with our axon terminal imaging experiment, mice were first trained in the 2AA task for three sessions and then fiber photometry imaging from the cell bodies of pPVT–NAc neurons was performed during two additional sessions (Extended Data Fig. 8c, d). Notably, our results largely resembled those obtained from pPVT^{D2R} neurons (Extended Data Fig. 8e-j). In

particular, we noticed that while pPVT–NAc neurons were strongly recruited during avoidance trials, they were mostly suppressed during failures (Extended Data Fig. 8i). Moreover, pPVT–NAc neurons signaled active behavioral responses and were instead inhibited during freezing (Extended Data Fig. 8e, j) (Supplementary Movie 2). Together, these findings demonstrate that pPVT projections to the NAc predominantly signal active defensive responses.

Next, to investigate whether activation of pPVT–NAc projections is critical for active avoidance, we optogenetically inhibited pPVT^{D2R}–NAc projections bilaterally using Halo in a cohort of mice displaying robust avoidance behavior following training (>70% initial avoidance rate; See Methods) (Fig. 3a-c). Similar to the experiment described above, following 2AA training (Fig. 3d-f) (Extended Data Fig. 9a) mice were subjected to three test sessions (Test Days 1-3), and on Test Day 2 light stimulation was delivered through optical fibers to silence pPVT^{D2R}–NAc communication during CS presentations. Unlike control subjects, light stimulation significantly decreased avoidance rate and increased the latency to avoid (Fig. 3g, h). In addition, a concomitant increase in CS-evoked freezing was observed on Test Day 2 (Fig. 3i). Importantly, pairwise comparison of light-evoked changes in freezing and avoidance behavior across test sessions revealed an inverse relationship between these two parameters (Extended Data Fig. 9c). Finally, restricting optogenetic inhibition of pPVT–NAc projections to the ITI did not significantly alter behavior (Extended Data Fig. 10l-o). Similarly, as with pPVT^{D2R} neurons, we did not observe any light stimulation-induced effect on locomotion (Extended Data Fig. 10p). Together, these results demonstrate that silencing pPVT^{D2R}–NAc communication biases defensive behavioral responses towards

reactive coping strategies. Notably, this effect is independent of the post-training avoidance rate, because similar observations were made in a cohort of mice in which the initial avoidance rate was markedly lower (Extended Data Fig. 10a-k). Moreover, our observation that optogenetic inhibition of pPVT–NAc projections on Test Day 2 lead to lasting behavioral effects on Test Day 3, indicates a potential role for this projection in the formation of persistent associations between fear cues and specific coping strategies.

pPVT–CeA projections signal failure to avoid

As described above, pPVT^{D2R} neurons that project to the NAc are mostly distinct from those that project to the CeA (Extended Data Fig. 1)²⁵. However, these anatomical differences do not necessarily implicate that functional distinctions exist between the two efferent pathways. Thus, to investigate how pPVT^{D2R}–CeA projections are modulated in the 2AA task, we expressed GCaMP7s in pPVT^{D2R} neurons of Drd2-Cre mice and implanted an optical fiber unilaterally in the CeA to measure the activity of pPVT^{D2R}–CeA projections with fiber photometry (Extended Data Fig. 11a-d). These experiments showed that unlike pPVT^{D2R}–NAc terminals, pPVT^{D2R}–CeA projections are not positively modulated by avoidance (Extended Data Fig. 11e-j). Surprisingly, CS-evoked freezing behavior were not associated with significant changes in GCaMP7s fluorescence (Extended Data Fig. 11j). In contrast, pPVT^{D2R}–CeA neurons were predominantly active during failure trials (Extended Data Fig. 11g, j), suggesting a link between engagement of pPVT^{D2R}–CeA projections and failure to avoid. Notably, previous studies have linked pPVT–CeA communication to the expression of conditioned fear – where Pavlovian reactions dominate behavior^{12,22}. Altogether, these

findings suggest the existence of functional differences between pPVT^{D2R}–CeA and pPVT^{D2R}–NAc projections, with the activity of pPVT^{D2R}–CeA projections increasing during failures (when freezing dominates [Extended Data Fig. 11g]).

pPVT–CeA projections inhibit active avoidance

Previous reports demonstrated that active avoidance is under negative control by the CeA, which controls Pavlovian reactions^{16,17}. Specifically, these studies showed that while blocking protein synthesis in the CeA promotes active avoidance¹⁷ lesions of this region of the amygdala can unmask avoidance behavior in animals that initially fail to avoid after 2AA training¹⁶. A major conclusion drawn from these studies is that CeA-driven Pavlovian reactions compete with and thereby prevent the expression of instrumental defensive behaviors. Because pPVT–CeA communication has been previously shown to support conditioned freezing behavior^{12,22}, we hypothesized that attenuating pPVT–CeA projections could boost active avoidance. Consistent with this prediction, optogenetically silencing pPVT–CeA projections using Halo diminished freezing behavior and promoted avoidance behavior (Extended Data Fig. 12a-k). Notably, this manipulation did not impact behavior when light stimulation was restricted to the ITI period (Extended Data Fig. 12l-o). Together, these results suggest that silencing pPVT–CeA communication biases behavior away from reactive defensive behavior and in favor of proactive ones.

Remarkably, Halo-mediated silencing of pPVT–CeA projections also promoted active avoidance behavior in a subset of mice that failed to achieve >30% avoidance rate after the third 2AA session (See Methods) (Fig. 4) (Extended Data Fig. 13). Consistent with previous manipulations, the increase in avoidance behavior was

accompanied by a reduction in the latency to avoid and CS-evoked freezing (Fig. 4h, i). Moreover, pairwise comparisons revealed that consistent with the idea that in the 2AA task animals trade Pavlovian and instrumental defensive strategies, increases in active avoidance induced by silencing pPVT–CeA projections were linked to a reduction in freezing behavior (Extended Data Fig. 13c). Altogether, these results suggest that silencing pPVT–CeA communication biases behavior away from freezing and in favor of avoidance.

Our collective observations indicate that task-related activity within the pPVT–NAc or the pPVT–CeA pathway biases defensive behavior towards active or passive responses, respectively. To investigate whether artificial stimulation of either pathway can generate behavioral bias, we expressed the red-shifted channelrhodopsin-2 variant ChrimsonR in pPVT^{D2R} neurons and implanted optical fibers bilaterally over the NAc or the CeA. Consistent with our model, we observed that while optogenetic stimulation of pPVT–NAc terminals potentiated, stimulation of pPVT–CeA projections diminished avoidance behavior (Extended Data Fig. 14). These results lend further support to our conclusion that divergent projections of the pPVT mediate the selection of opposing defensive behaviors.

pPVT projections to the NAc and the CeA flexibly control the selection of defensive behaviors

A recurrent theme in our reported observations is the idea the pPVT projections to the NAc and the CeA can bias the selection of reactive and proactive defensive behaviors. But whether these projections flexibly control defensive behavior is unclear. To assess this, we selectively silenced pPVT projections to the NAc and the CeA in the

same subjects across two individual test sessions (Fig. 5) (Extended Data Fig. 15).
Notably, we found that while silencing pPVT–NAc projections impaired avoidance
behavior (consistent with the above results), inhibition of pPVT–CeA terminals the
following day fully restored active avoidance (Fig. 5e, f). Interestingly, an opposing but
similarly dynamic modulation of freezing behavior was observed across sessions (Fig.
5g) (Extended Data Fig. 15d). In conclusion, these data demonstrate that the pPVT can
flexibly bias defensive behaviors via the downstream circuitry described in the present
study. Future studies should aim at identifying the local circuit dynamics and upstream
mechanisms that guide the selection of defensive behaviors. As a proxy to this, using
monosynaptic rabies tracing, we identified regions of the mouse brain that preferentially
innervate pPVT–CeA or pPVT–NAc neurons (Extended Data Fig. 16).

DISCUSSION

In the present study, we have identified the pPVT as a key brain structure that
mediates the selection of competing defensive strategies, namely Pavlovian reactions
and instrumental behavior. These findings add to a growing body of literature that
places the PVT as an important regulator of emotional and motivational processes^{31,32}.
Importantly, we uncovered that the anatomical and functional segregation of PVT
projections to the CeA and the NAc underscores its role in biasing behavioral selection.
Collectively, these findings expand on previous studies linking PVT function to reactive
defensive responses^{12,22,28,33}, provide a circuit mechanism by which opposing defensive
strategies are leveraged⁸, and support the notion that the PVT is critical for the
orchestration of adaptive behavioral responses^{32,34,35}.

Recent literature shows that the pPVT is predominantly sensitive to aversive stimuli^{28,29}. In turn, the pPVT appears to orchestrate adaptive responses that allow animals to cope with ongoing demands (Penzo and Gao, in press). Consistent with this view and in addition to its role in driving Pavlovian defensive reactions, the pPVT is now recognized to promote goal-directed instrumental responses including food seeking, drug seeking and maternal behaviors³⁶⁻⁴⁰. Importantly, some of these studies have directly implicated NAc projections of the pPVT as guiding instrumental behaviors during aversive states³⁷⁻⁴². In light of these recent reports, our finding that the pPVT–NAc pathway also regulates instrumental defensive behaviors suggests that these projections may generally promote goal-directed behaviors. From this perspective, the pPVT–CeA pathway could support behavioral strategies that favor Pavlovian responding irrespective of whether responses are reward oriented or defensive in nature (Penzo and Gao, in press). Accordingly, it was recently proposed that pPVT–CeA projections drive morphine-induced conditioned place preference likely through Pavlovian incentive motivation⁴³.

The pPVT is not the only brain region that innervates both the NAc and the CeA. Indeed, the BLA and the insular cortex also send divergent projection to these structures and these projections have been implicated in emotional and motivational processes^{14,19,44-46}. Particularly relevant to the present study is the notion that the BLA has been shown to contribute to both conditioned freezing and active avoidance behavior via its projections to the CeA and the NAc, respectively. This leads to the question of what the differential contributions of the BLA and the PVT are to these opposing defensive behaviors. One possibility discussed earlier in this manuscript is

that the BLA's contribution to conditioned reactive and proactive defensive behaviors decreases over time²⁰⁻²². As such the PVT may increasingly become the dominant pathway by which freezing and active avoidance behaviors are regulated. Alternatively, and possibly in addition to this time dependent role, the PVT may serve to contextualize aversive memories by integrating these with information about internal state (owing to its strong innervation by the hypothalamus and the brainstem). Consistent with this idea, the PVT has been recently proposed to arbitrate amid motivational conflicts³². Future studies should aim to identify the precise mechanisms by which the PVT regulates behavior and how these differ from those of other regions known to regulate the same behaviors.

Emergent studies support the notion that the neuronal circuits of the PVT are functionally diverse³¹. Indeed, we recently identified two major classes of PVT neurons that can be distinguished on the basis of genetic, anatomic (connectional) and functional differences²⁹. Specifically, it was suggested that PVT neuronal subtypes could be identified due to their expression, or lack thereof, of the *Drd2* gene (and D2R protein). Functionally, compared to D2R negative PVT neurons which at the population level appear to be modulated by stimulus salience, D2R-expressing neurons are primarily sensitive to aversive stimuli^{28,29}. Our results expand on this recent report by showing further anatomical and functional heterogeneity among aversive-sensitive pPVT^{D2R} neurons. These findings should encourage future studies to classify PVT neurons based on the intersection of genetic markers and projection target³¹. Similar intersectional approaches have led to the discovery of functional segregation in other brain areas⁴⁷⁻⁵¹.

The observation that optogenetic inhibition of pPVT–NAc projections leads to lasting impairments in active avoidance behavior, is reminiscent of a previous report demonstrating that silencing pPVT–CeA projections produces similar lasting effects in freezing behavior during fear memory retrieval²². In that study, the authors concluded that the persistent reduction in freezing behavior indicated an overall role for this pathway in controlling the maintenance of fear memories. Our findings are at odd with this interpretation. Specifically, we found that while silencing pPVT–NAc projections produces a lasting impairment in active avoidance, the same manipulation led to a concomitant increment in CS-evoked freezing. As such, our findings do not support a conclusion in which persistent reductions in avoidance behavior resulting from pPVT–NAc silencing are due to deficits in fear memory maintenance. Instead, in light of this previous report and our collective findings, we propose that the activation of pPVT projections to the NAc and the CeA links fear (CS/US) associations to the selection of specific defensive strategies. As such, our findings help remodel current views on the role that the PVT plays in the formation and maintenance of aversive memories. Subsequent studies in this topic should aim at identifying the local and upstream circuit mechanisms that promote behavioral bias.

A major focus of the present study is to uncover the neural circuit mechanisms by which animals switch between two opposing defensive strategies, Pavlovian freezing, and active avoidance. In nature, animals typically engage in these different behavioral strategies as a function of threat imminence⁸⁻¹⁰. From this perspective, active avoidance is thought to have evolved as a way to deal with distant threats, whereas reactive behaviors mostly emerge during imminent threats¹⁰. Within this framework the idea that

the 2AA task yields a subset of animals that fail to engage in avoidance behavior (low avoiders), seems to suggest that this task might be ineffective for studying ethologically relevant defensive behaviors. One potential explanation to the variability in behavioral outcome, could be that some animals view the threat (US) as more imminent than others, and thus display freezing behavior when avoidance is possible. Consistent with this notion, a recent study demonstrated that reducing threat imminence by increasing the duration of the CS virtually eliminates low avoiders⁵². Thus, whereas here we have effectively exploited individual differences in animal performance to extract meaningful information of the mechanisms that underlie behavioral selection, identifying ethologically relevant behavioral models that allow for animals to engage in both proactive and reactive defensive strategies as a function of threat imminence would be of tremendous value to studies on the neurobiology of defensive behaviors.

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FIGURE LEGENDS

Figure 1. pPVT^{D2R} neurons control active avoidance and are inhibited during freezing.

a, Left: Representative image from a mouse expressing GCaMP6s in pPVT^{D2R} neurons of Drd2-Cre mice and implanted with an optical fiber in the pPVT. Right: Optical fiber placements (n = 5 mice). **b**, Schematic of the 2AA task. **c**, Avoidance Rate (black) and freezing time during the CS (red) across training (Days 1-3) and test sessions (Days 4-5). Fiber photometry imaging sessions are depicted by the blue lines. **d**, Representative imaging traces from a sample subject. Individual imaging traces for avoidance (blue) and failure (red) trials. Black square pulses depict the duration of each CS presentation. Yellow and red backgrounds show the maximal duration of CS and US presentation, respectively. **e**, Top: Heatmaps showing individual calcium responses for all avoidance

567 and failure trials recorded. Bottom: Average calcium signal and CS duration of the upper
568 panels (Avoidance trials, blue; failure trials, red; CS duration, black). **f**, Freezing time
569 during the CS (top) in avoidance trials is significantly less than failure trials, while the
570 peak (middle) and mean (bottom) of calcium signals during the CS is significantly larger
571 in avoidance trials. Avoidance, $n = 118$ Trials; Failure, $n = 122$ Trials; two-tailed
572 Student's t -test. $***P < 0.001$. **g**, Quantification of calcium signal during pre-CS, CS and
573 post-CS for avoidance (blue) and failure trials (red). AUC, One-way ANOVA followed by
574 two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance: $n =$
575 118 Trials; $F(2, 351) = 75.89$; group comparisons, pre-CS vs CS $***P < 0.001$, pre-CS vs
576 post-CS $*P = 0.038$, CS vs post-CS $***P < 0.001$. Failure: $n = 122$ Trials; $F(2,$
577 $363) = 66.54$; group comparisons, $***P < 0.001$. **h**, Left: Averaged calcium responses
578 during CS onset, CS maximal velocity, escape initiation, escape and CS freezing
579 epochs for all avoidance (blue) and failure (red) trials. Right: quantification of calcium
580 signal in each epoch duration. AUC, two-tailed Student's t -test. CS: Avoidance, $n = 118$
581 Events; Failure, $n = 122$ Events; $P = 0.74$. Max. Velocity: Avoidance, $n = 117$ Events;
582 Failure, $n = 122$ Events; $*P = 0.024$. Escape initiate: Avoidance, $n = 113$ Events; Failure,
583 $n = 115$ Events; $***P < 0.001$. Escape: Avoidance, $n = 118$ Events; Failure, $n = 122$
584 Events; $**P = 0.0064$. Freezing: Avoidance, $n = 92$ Events; Failure, $n = 157$ Events; $P =$
585 0.98 . **i**, Schematic of the viral vector strategy for optogenetic silencing of pPVT^{D2R}
586 neurons. **j-l**, Left: Optogenetic inhibition of pPVT^{D2R} neurons significantly decreases
587 avoidance rate (**j**), while increases latency to avoid (**k**) and freezing time during the CS
588 (**l**). Right: Normalization of the Day 1 in each group. Ctl, $n = 8$ mice; Halo, $n = 9$ mice,
589 two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger

and Yekutieli. Avoidance Rate: $F(2, 30) = 5.48$; group comparisons, Halo, Day 1 vs Day 2 $***P < 0.001$, Day 1 vs Day 3 $P = 0.06$. Latency to avoid: $F(2, 30) = 2.48$; group comparisons, Halo, Day 1 vs Day 2 $**P = 0.0037$. CS freezing: $F(2, 30) = 0.19$; group comparisons, Halo, Day 1 vs Day 2 $*P = 0.04$. Optogenetic inhibition sessions are depicted by the yellow background. Data shown as mean $\pm$ s.e.m.

Figure 2. pPVT^{D2R}–NAc axon terminals signal active avoidance.

a, Schematic of the viral vector strategy and optical fiber placement used for fiber photometry imaging of pPVT^{D2R}–NAc axon terminals. **b**, Representative images from a mouse expressing GCaMP7s in pPVT^{D2R} neurons and implanted with an optical fiber in the NAc. **c**, Optical fiber placements ($n = 4$ mice). **d**, Avoidance Rate (black) and freezing time during the CS (red) across all training and test sessions. Fiber photometry imaging sessions are depicted by the blue lines. **e**, Representative imaging traces from a sample subject. Individual imaging traces for avoidance (blue) and failure (red) trials. Black square pulses depict the duration of each CS presentation. Yellow and red backgrounds show the maximal duration of CS and US presentation, respectively. **f**, Top: Heatmaps showing individual calcium responses for all avoidance and failure trials. Bottom: Average calcium signal and CS duration of the upper panels (Avoidance trials, blue; failure trials, red; CS duration, black). **g**, Freezing time during the CS (top) in avoidance trials is significantly lower compared to failure trials, while the peak (middle) and mean (bottom) values of the calcium signal during the CS are similar for both groups. Two-tailed Student's t -test. Avoidance, $n = 80$ Trials. Failure, $n = 130$ Trials. Freezing, $***P < 0.001$. Peak Signal, $P = 0.14$. Mean Signal, $P = 0.75$. **h**, Quantification

of calcium signal during pre-CS, CS and post-CS periods for avoidance (blue) and failure (red) trials. AUC, One-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance: $n = 80$ Trials, $F(2, 237) = 4.37$; group comparisons, pre-CS vs CS $**P = 0.0065$, pre-CS vs post-CS $P = 0.67$, CS vs post-CS $*P = 0.021$. Failure: $n = 130$ Trials, $F(2, 387) = 121.2$; group comparisons, pre-CS vs CS $***P < 0.001$, pre-CS vs post-CS $P = 0.072$, CS vs post-CS $***P < 0.001$. **i**, Left: Averaged calcium responses during CS onset, CS maximal velocity, escape initiation, escape and CS freezing epochs for all avoidance (blue) and failure (red) trials. Right: Quantification of calcium signal in each epoch duration. AUC, two-tailed Student's t -test. CS: Avoidance, $n = 80$ Events; Failure, $n = 130$ Events; $P = 0.17$. Max. Velocity: Avoidance, $n = 80$ Events; Failure, $n = 129$ Events; $***P < 0.001$. Escape initiate: Avoidance, $n = 79$ Events; Failure, $n = 123$ Events; $P = 0.74$. Escape: Avoidance, $n = 80$ Events; Failure, $n = 130$ Events; $P = 0.78$. Freezing: Avoidance, $n = 76$ Events; Failure, $n = 175$ Events; $P = 0.78$. Data shown as mean $\pm$ s.e.m.

Figure 3. Optogenetic inhibition of pPVT^{D2R}–NAc axon terminals decreases active avoidance and increases freezing.

a, Schematic of the viral vector strategy and optical fiber placement for optogenetic silencing of pPVT^{D2R}–NAc axon terminals in the 2AA task. **b**, Representative images from a mouse expressing Halo-mCherry in pPVT^{D2R} neurons (left) and implanted with optical fibers in the NAc (right). **c**, Optical fiber placements ($n = 8$ mice per group). **d-f**, Avoidance rate (d), latency to avoid (e) and freezing time during the CS (f) across all training sessions for both Ctl (black) and Halo (red) groups. **g-i**, Left: Optogenetic

inhibition of pPVT^{D2R}–NAc axon terminals persistently decreases avoidance rate (g), while increases latency to avoid (h) and freezing time during the CS (i). Right: Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: $F(2, 28) = 18.04$; group comparisons, Halo, Day 1 vs Day 2 $***P < 0.001$, Day 1 vs Day 3 $***P < 0.001$. Latency to avoid: $F(2, 28) = 5.63$; group comparisons, Ctl, Day 1 vs Day 3 $*P = 0.028$; Halo, Day 1 vs Day 2 $***P < 0.001$, Day 1 vs Day 3 $***P < 0.001$. CS Freezing: $F(2, 28) = 1.45$; group comparisons, Halo, Day 1 vs Day 2 $*P = 0.049$, Day 1 vs Day 3 $**P = 0.0092$. Optogenetic inhibition session is depicted by the yellow background. Data shown as mean $\pm$ s.e.m.

Figure 4. Optogenetic inhibition pPVT^{D2R}–CeA axon terminals increases active avoidance and reduces freezing.

a, Schematic of the viral vector strategy and optical fiber placement used for optogenetic silencing of pPVT^{D2R}–CeA axon terminals in the 2AA task. **b**, Representative images from a mouse expressing Halo-mCherry in pPVT^{D2R} neurons and implanted with optical fibers in the CeA. **c**, Optical fiber placements ($n = 9$ mice per group). **d-f**, Avoidance rate (d), latency to avoid (e) and freezing time during the CS (f) across all training days in both Ctl (black) and Halo (red) groups. **g-i**, Top: Optogenetic inhibition of pPVT^{D2R}–CeA axon terminals gradually increases avoidance rate (g) and reduces the latency to avoid (h) and freezing time during the CS (i). Bottom: Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: $F(5,$

80) = 9.05; group comparisons, Halo, Day 1 vs Day 4 *** $P < 0.001$, Day 1 vs Day 5 *** $P < 0.001$, Day 1 vs Day 6 *** $P < 0.001$, Day 2 vs Day 4 * $P = 0.015$, Day 2 vs Day 5 *** $P < 0.001$, Day 2 vs Day 6 *** $P < 0.001$, Day 3 vs Day 4 * $P = 0.015$, Day 3 vs Day 5 *** $P < 0.001$, Day 3 vs Day 6 *** $P < 0.001$, Day 4 vs Day 6 *** $P < 0.001$, Day 5 vs Day 6 * $P = 0.012$. Latency to avoid: $F(5, 80) = 4.52$; group comparison, Halo, Day 1 vs Day 4 * $P = 0.011$, Day 1 vs Day 5 *** $P < 0.001$, Day 1 vs Day 6 *** $P < 0.001$, Day 2 vs Day 5 * $P = 0.043$, Day 2 vs Day 6 *** $P < 0.001$, Day 3 vs Day 5 ** $P = 0.0085$, Day 3 vs Day 6 *** $P < 0.001$, Day 4 vs Day 6 ** $P = 0.0027$. CS Freezing: $F(5, 80) = 1.17$; group comparisons, Halo, Day 3 vs Day 6 * $P = 0.012$, Day 4 vs Day 6 ** $P = 0.007$, Day 5 vs Day 6 * $P = 0.018$. Optogenetic inhibition sessions are depicted by the yellow background. Data presented mean $\pm$ s.e.m.

Figure 5. pPVT^{D2R} projections to the NAc and the CeA flexibly bias the selection of defensive behaviors.

a, Schematic of the viral vector strategy and optical fiber placement for optogenetic silencing of pPVT^{D2R}–NAc or pPVT^{D2R}–CeA axon terminals in the same subject. **b-d**, Avoidance rate (b), latency to avoid (c) and freezing time during the CS (d) across all training sessions for both Ctl (black) and Halo (red) groups. **e-g**, Top: Optogenetic inhibition of pPVT^{D2R}–NAc axon terminals (Day 2) decreases avoidance rate (e), increases latency to avoid (f) and freezing time during the CS (g), while following optogenetic inhibition of pPVT^{D2R}–CeA axon terminals (Day 3) reverses these changes. Bottom: Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: $F(3,$

42) = 6.09; group comparisons, Halo, Day 1 vs Day 2 $**P = 0.0017$, Day 2 vs Day 3 $**P = 0.0072$, Day 2 vs Day 4 $**P = 0.0078$. Latency to avoid: $F(3, 42) = 5.56$; group comparisons, Halo, Day 1 vs Day 2 $**P = 0.0029$, Day 2 vs Day 3 $*P = 0.012$, Day 2 vs Day 4 $*P = 0.02$. CS Freezing: $F(3, 42) = 0.74$; group comparisons, Halo, Day 1 vs Day 2 $P = 0.086$, Day 1 vs Day 4 $*P = 0.013$, Day 2 vs Day 3 $*P = 0.043$. Optogenetic inhibition sessions are depicted by the yellow background. Data shown as mean $\pm$ s.e.m.

Extended Data Figure 1. pPVT^{D2R} projections to NAc and CeA are partially segregated.

a, Schematic of the viral vector strategy for anterograde tracing pPVT projections. **b**, Representative images showing Halo-mCherry expression in neurons of the pPVT (left) and axon terminals within the NAc (middle) and the CeA (right). **c**, Schematic of the viral vector strategy for anterograde tracing pPVT^{D2R} projections. **d**, Representative images showing ChR2-YFP expression in pPVT^{D2R} neurons (left) and axon terminals within the NAc (middle) and the CeA (right). **e**, Schematic of the retrograde tracing strategy used for labeling CeA- and NAc-projecting pPVT neurons. **f**, Representative images showing the targets for dual-color CTB injections, green into the NAc (left) and red into the CeA (right). **g**, Representative images showing the retrograde labeled NAc-projecting cells (green) and CeA-projecting cells (red) are predominantly present in the D2R-labeled (grey) pPVT region. **h**, Quantification of the density of CeA- and NAc-projecting neurons in the pPVT. NAc-projectors (NAc+), 1180 ± 163.36 ; CeA-projectors (CeA+), 732.2 ± 57.97 ; $n = 5$ mice; two-tailed Student's t -test, $*P = 0.032$. **i**, Most of NAc-projecting or

CeA-projecting pPVT cells express D2R protein. D2R+NAc+/NAc+, $99.21 \pm 0.22\%$; D2R+CeA+/CeA+, $99.68 \pm 0.26\%$; two-tailed Student's *t*-test, $P = 0.2$. **j**, Quantification of the percentage of double-projecting cells in pPVT. NAc+CeA+/NAc+, $16.71 \pm 0.96\%$; NAc+CeA+/CeA+, $27.18 \pm 3.39\%$; two-tailed Student's *t*-test, $*P = 0.018$. Data shown as mean $\pm$ s.e.m.

Extended Data Figure 2. pPVT^{D2R} neuron activity during fear conditioning and retrieval.

a, Left: Schematic of AAV9-hSyn-Flex-GCaMP6s injection and optical fiber implantation into the pPVT of Drd2-Cre mice used for fiber photometry. Right: Optical fiber placements ($n = 6$ mice). **b**, Experimental paradigm: Mice were conditioned with five presentations of an auditory CS (30 s) that co-terminated with a 0.5 mA foot shock (2 s; US) in a context A after experiencing four presentations of unpaired CS in habituation session. During retrieval session, eight unpaired CS presentations were delivered 24 h following conditioning in a context B. **c-e**, Summary plot of freezing behavior during the habituation (c), conditioning (d) and retrieval (e) sessions. **f-h**, Average calcium responses during the habituation (f), conditioning (g) and retrieval (h) sessions. CS and US durations are depicted by the yellow and red backgrounds under the trace, respectively. **i-k**, Quantification of calcium signal during pre-CS, CS, post-CS periods of habituation (i), conditioning (j) and retrieval (k) sessions. AUC, One-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Habituation: $n = 20$ Trials; $F(2, 57) = 0.1$; group comparisons, pre-CS vs CS $P = 0.76$, pre-CS vs post-CS $P = 0.89$, CS vs post-CS $P = 0.66$. Conditioning: $n = 30$ Trials; $F(2,$

87) = 2.17; group comparisons, pre-CS vs CS $P = 0.067$, pre-CS vs post-CS $P = 0.083$, CS vs post-CS $P = 0.92$. Retrieval: $n = 48$ Trials; $F(2, 141) = 7.7$; group comparisons, pre-CS vs CS $***P < 0.001$, pre-CS vs post-CS $P = 0.85$, CS vs post-CS $**P = 0.0012$. **l**, Average calcium responses during early (Trials 1-3; left) and late (Trials 4-5; right) conditioning trials. CS and US durations are depicted by the yellow and red backgrounds under the traces, respectively. **m**, Quantification of calcium signal during the first 5 s following the onset of CS during conditioning sessions. Calcium signal significantly increased in the late trials compared with the early ones. AUC, Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, $n = 12$ Trials; $F(4, 112) = 1.49$. Group comparisons: Early, 1 s vs 4 s $P = 0.054$, 1 s vs 5 s $*P = 0.021$; Late, 1 s vs 2 s $P = 0.058$, 1 s vs 3 s $**P = 0.0043$, 1 s vs 4 s $***P < 0.001$, 1 s vs 5 s $***P < 0.001$, 2 s vs 4 s $P = 0.07$, 2 s vs 5 s $**P = 0.0035$, 3 s vs 5 s $*P = 0.049$. **n**, Calcium signal during US presentation in the late trials is higher than the early trials ($n = 6$ mice; two-tailed paired Student's t -test, $P = 0.054$). **o**, Top: Heatmaps showing calcium responses of Conditioning Trials 1-5 from individual subjects, respectively. CS duration is depicted by the yellow line above the heatmap. Bottom: Average calcium responses of the top panels. CS and US durations are depicted by the yellow and red backgrounds under the traces, respectively. Data shown as mean $\pm$ s.e.m.

Extended Data Figure 3. The activity of pPVT^{D2R} neurons is positively correlated with movement during the CS following fear memory retrieval.

750 **a**, Left: Heatmap showing calcium responses (dF/F in Z-score) of individual trials during
 751 retrieval session after aligned by freezing percentage during the CS. CS duration is
 752 depicted by the yellow line above the heatmap. CS onset is depicted by the black dash
 753 line. Right: Freezing percentage during the CS for individual trials. $n = 6$ mice, 8 retrieval
 754 trials per mouse. **b**, Left: Linear regression analysis of the area under curve (AUC) of
 755 calcium signal and freezing percentage during the CS for each retrieval trial ($P = 0.007$,
 756 $R^2 = 0.15$). Based on the level of freezing percentage during the CS, all retrieval trials
 757 are divided into three groups: Low freezing (L; Freezing < 40%; $n = 20$ trials), moderate
 758 freezing (M; $40\% \leq \text{Freezing} \leq 60\%$; $n = 16$ trials) and high freezing (H; Freezing > 60%;
 759 $n = 12$ trials). Right: Comparison of average calcium signal during the CS for the three
 760 groups. AUC, $F(2, 45) = 3.3$, one-way ANOVA followed by two-stage linear step-up
 761 procedure of Benjamini, Krieger and Yekutieli. Group comparisons, L vs H, $*P = 0.013$.
 762 **c**, Top: Average of calcium responses for each group. CS duration is depicted by the
 763 yellow background under the trace. Middle: Average movement index for each group.
 764 Bottom: Linear regression analysis of average calcium signal and movement index
 765 during the CS for each group. The coefficient for high freezing trials (f, $R^2 = 0.44$) is
 766 larger than the ones for low freezing (d, $R^2 = 0.16$) or moderate freezing (e, $R^2 = 0.23$).
 767 **d**, Comparison of the calcium signal (left) and movement index (right) during pre-CS,
 768 CS and post-CS for the three groups. AUC, two-way ANOVA followed by two-stage
 769 linear step-up procedure of Benjamini, Krieger and Yekutieli. Calcium signal: $F(4,$
 770 $90) = 3.37$; group comparisons, M, pre-CS vs CS $*P = 0.018$, CS vs post-CS $*P = 0.01$;
 771 H, pre-CS vs CS $***P < 0.001$, CS vs post-CS $***P < 0.001$. Movement index: $F(4,$
 772 $90) = 3.6$; group comparisons, L, pre-CS vs CS $*P = 0.012$; M, pre-CS vs CS $*P = 0.023$;

H, pre-CS vs CS $***P < 0.001$, CS vs post-CS $***P < 0.001$. **e**, Average movement index in the habituation session. **f**, Linear regression analysis shows poor correlation of average calcium signal and movement index during the CS for the habituation session ($P = 0.0028$, $R^2 = 0.012$). **g**, Individual subjects contributing to each freezing category (L, low freezing; M, moderate freezing; H, high freezing) in the retrieval session. Data shown as mean $\pm$ s.e.m.

Extended Data Figure 4. The individual trial activity of pPVT^{D2R} neurons during fear retrieval session.

a, Top: Calcium responses of Retrieval Trials 1-8 from individual subjects, respectively. CS duration is depicted by the yellow line above the heatmap. Bottom: Average calcium responses of the top panels. CS duration is depicted by the yellow background under the trace. **b**, Quantification of calcium signal during pre-CS, CS, post-CS periods of the Retrieval Trials 1-8. AUC, One-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. $n = 6$ subjects per trial; Trial 1, $F(2, 10) = 1.36$; Trial 2, $F(2, 10) = 4.55$, group comparisons, pre-CS vs CS $*P = 0.018$, CS vs post-CS $*P = 0.04$; Trial 3, $F(2, 10) = 0.092$; Trial 4, $F(2, 10) = 1.55$; Trial 5, $F(2, 10) = 0.74$; Trial 6, $F(2, 10) = 2.83$; Trial 7, $F(2, 10) = 4.94$, group comparisons, pre-CS vs CS $*P = 0.024$, CS vs post-CS $*P = 0.02$; Trial 8, $F(2, 10) = 2.71$. No significant change among other group comparison. **c**, Linear regression analysis of average calcium signal and movement index from the Retrieval Trials 1-8, respectively.

Extended Data Figure 5. The activity of pPVT^{D2R} neurons in the 2AA task, related to Figure 1.

a, Latency to avoid (black) and freezing time during ITI (red) across the days. Fiber photometry imaging sessions are depicted by the blue lines. **b**, Left: Linear regression analysis of peak calcium signal and freezing time during the CS for avoidance (blue; A; $R^2 = 0.069$, $P = 0.0043$) and failure trials (red; F; $R^2 = 0.15$, $P < 0.001$). Right: Linear regression analysis of average calcium signal and freezing time during the CS for avoidance (blue; A; $R^2 = 0.17$, $P < 0.001$) and failure trials (red; F; $R^2 = 0.24$, $P < 0.001$). **c**, Quantification of the latency to freezing after CS onset for avoidance and failure trials. Left: Counts of the freezing latency. Right: cumulative probability plots for the Left panel. Avoidance, $n = 56$ Events; Failure, $n = 109$ Events. **d**, Quantification of the latency to escape after CS onset for avoidance and failure trials. Left: Counts of the escape latency. Right: cumulative probability plots for the Left panel. Avoidance, $n = 118$; Failure, $n = 122$ Trials. Data shown as mean $\pm$ s.e.m.

Extended Data Figure 6. Optogenetic inhibition of pPVT^{D2R} neurons in the 2AA task.

a, Representative image from a mouse expressing Halo-mCherry and implanted with an optical fiber in pPVT^{D2R} neurons. **b**, Optical fiber placements (Ctl, $n = 8$ mice; Halo, $n = 9$ mice). **c**, Schematic of the 2AA task. **d-g**, Avoidance rate (d), latency to avoid (e) and freezing time during the CS (f) and the ITI (g) across the training days for both Ctl (black) and Halo (red) groups. **h**, Left: Quantification of the freezing time during the ITI. Right: Normalization of the Day 1 in each group. ITI Freezing in s, $F(2, 30) = 0.36$, two-

way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and
 Yekutieli. No significant change among each group comparison. Optogenetic inhibition
 sessions are depicted by the yellow background. **i**, The changes in freezing behavior
 across test sessions are plotted as a function of changes in avoidance behavior. Day 2
 vs Day 1: Halo, $P = 0.23$, $R^2 = 0.2$; Ctl, $P = 0.71$, $R^2 = 0.025$. Day 3 vs Day 1: Halo, $P =$
 0.065 , $R^2 = 0.41$; Ctl, $P = 0.27$, $R^2 = 0.2$. Day 3 vs Day 2: Halo, $P = 0.013$, $R^2 = 0.61$; Ctl,
 $P = 0.67$, $R^2 = 0.033$. **j**. Free moving distance in the open field. Ctl, $n = 8$ mice; Halo, $n =$
 6 mice. Optogenetic inhibition duration is depicted by the yellow background. **k**,
 Schematic of the viral vector strategy and optical fiber placement used for optogenetic
 silencing of pPVT^{D2R} neurons in the 2AA task. **l**, Optical fiber placements (Ctl, $n = 6$
 mice; Halo, $n = 7$). **m-p**, Avoidance rate (m), latency to avoid (n) and freezing time
 during the CS (o) and the ITI (p) across all training days in both Ctl (black) and Halo
 (red) groups. **q-t**, Left: Optogenetic inhibition of pPVT^{D2R} neurons during the ITI has little
 effect on avoidance rate (q), the latency to avoid (r) and freezing time during the CS (s)
 and the ITI (t). Right: Normalization of the Day 1 in each group. Two-way ANOVA
 followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.
 Avoidance rate, $F(5, 80) = 9.05$; latency to avoid, $F(5, 80) = 4.52$; CS Freezing, $F(5,$
 $80) = 1.17$; ITI Freezing, $F(5, 80) = 0.44$; no significant change among each group
 comparison. Optogenetic inhibition sessions are depicted by the yellow background.
 Data shown as mean $\pm$ s.e.m.

Extended Data Figure 7. Fiber photometry imaging of GCaMP7s fluorescence
from the NAc terminals of pPVT^{D2R} neurons in the 2AA task, related to Figure 2.

a, Quantification of the latency to avoid (black) and freezing time during ITI (red) across the days. Fiber photometry imaging sessions are depicted by the blue lines. **b**, Left: Linear regression analysis of peak calcium signal and freezing time during the CS of avoidance (blue) and failure trials (red) (avoidance trials, $P = 0.19$, $R^2 = 0.022$; failure trials, $P = 0.11$, $R^2 = 0.02$). Right: Linear regression analysis of the average calcium signal and freezing time during the CS of avoidance (blue) and failure (red) trials (avoidance trials, $P = 0.012$, $R^2 = 0.079$; failure trials, $P = 0.093$, $R^2 = 0.022$). Data shown as mean $\pm$ s.e.m.

Extended Data Figure 8. NAc-projecting neurons of the pPVT signal active avoidance.

a, Schematic of the viral vector strategy and optical fiber placement used for fiber photometry imaging of NAc-projectors in pPVT. **b**, Optical fiber placements ($n = 5$ mice). **c**, Avoidance Rate (black) and freezing time during the CS (red) across all training and test sessions. Fiber photometry imaging sessions are depicted by the blue lines. **d**, Quantification of the latency to avoid (black) and freezing time during ITI (red) across the training and test sessions. Fiber photometry imaging sessions are depicted by the blue lines. **e**, Representative imaging traces from a sample subject. Individual imaging traces for avoidance (blue) and failure (red) trials. Black square pulses depict the duration of each CS presentation. Yellow and red backgrounds show the maximal duration of CS and US presentation, respectively. **f**, Top: Heatmaps showing individual calcium responses for all avoidance and failure trials. Bottom: Average calcium signal and CS duration of the upper panels (Avoidance trials, blue; failure trials, red; CS

864 duration, black). **g**, Freezing time during the CS in avoidance trials is significantly lower
 865 compared to failure trials (top), while the peak (middle) and mean (bottom) values of the
 866 calcium signal during the CS in avoidance trials are much higher than failure trials. Two-
 867 tailed Student's *t*-test. Avoidance, *n* = 85 Trials. Failure, *n* = 125 Trials, ****P* < 0.001. **h**,
 868 Top: Linear regression analysis of peak calcium signal and freezing time during the CS
 869 of avoidance (blue) and failure trials (red) (avoidance trials, *P* = 0.67, *R*² = 0.002; failure
 870 trials, *P* = 0.55, *R*² = 0.003). Bottom: Linear regression analysis of the average calcium
 871 signal and freezing time during the CS of avoidance (blue) and failure (red) trials
 872 (avoidance trials, *P* = 0.17, *R*² = 0.02; failure trials, *P* = 0.47, *R*² = 0.004). **i**,
 873 Quantification of calcium signal during pre-CS, CS and post-CS periods for avoidance
 874 (blue) and failure (red) trials. AUC, One-way ANOVA followed by two-stage linear step-
 875 up procedure of Benjamini, Krieger and Yekutieli. Avoidance: *n* = 85 Trials, *F*(2,
 876 252) = 45.22; group comparisons, pre-CS vs CS ****P* < 0.0001, pre-CS vs post-CS *P* =
 877 0.11, CS vs post-CS ****P* < 0.0001. Failure: *n* = 125 Trials, *F*(2, 372) = 81.81; group
 878 comparisons, ****P* < 0.001. **j**, Left: Averaged calcium responses during CS onset, CS
 879 maximal velocity, escape initiation, escape and CS freezing epochs for all avoidance
 880 (blue) and failure (red) trials. Right: Quantification of calcium signal in each epoch
 881 duration. AUC, two-tailed Student's *t*-test. CS: Avoidance, *n* = 85 Events; Failure, *n* =
 882 125 Events; *P* = 0.052. Max. Velocity: Avoidance, *n* = 85 Events; Failure, *n* = 125
 883 Events; *P* = 0.65. Escape initiate: Avoidance, *n* = 82 Events; Failure, *n* = 116 Events;
 884 ****P* < 0.001. Escape: Avoidance, *n* = 85 Events; Failure, *n* = 125 Events; ****P* < 0.001.
 885 Freezing: Avoidance, *n* = 76 Events; Failure, *n* = 169 Events; *P* = 0.82. Data shown as
 886 mean ± s.e.m.

Extended Data Figure 9. Optogenetic inhibition of pPVT^{D2R}–NAc axon terminals in the 2AA task, related to Figure 3.

a, Freezing time during the ITI across all training sessions for both Ctl (black) and Halo (red) groups. **b**, Left: Optogenetic inhibition of pPVT^{D2R}–NAc axon terminals has little effect on freezing time during the the ITI. Right: Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. $F(2, 28) = 0.149$. Optogenetic inhibition session is depicted by the yellow background. **c**, The changes in freezing behavior across test sessions are plotted as a function of changes in avoidance behavior. Day 2 vs Day 1: Halo, $P = 0.036$, $R^2 = 0.55$; Ctl, $P = 0.82$, $R^2 = 0.0096$. Day 3 vs Day 1: Halo, $P = 0.0022$, $R^2 = 0.81$; Ctl, $P = 0.036$, $R^2 = 0.55$. Day 3 vs Day 2: Halo, $P = 0.56$, $R^2 = 0.058$; Ctl, $P = 0.0038$, $R^2 = 0.78$. Data shown as mean $\pm$ s.e.m.

Extended Data Figure 10. Optogenetic inhibition of pPVT–NAc axon terminals in the 2AA task.

a, Schematic of the viral vector strategy and optical fiber placement for optogenetic silencing of pPVT–NAc axon terminals in the 2AA task. **b**, Optical fiber placements (Ctl, $n = 13$ mice; Halo, $n = 11$ mice). **c-f**, Avoidance rate (c), latency to avoid (d) and freezing time during the CS (e) and ITI (f) across all training sessions for both Ctl (black) and Halo (red) groups. **g-j**, Left: Optogenetic inhibition of pPVT–NAc axon terminals decreases avoidance rate (g), while increases latency to avoid (h) and freezing time during the CS (i), not the ITI (j). Right: Normalization of the Day 1 in each group. Two-

way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: $F(2, 44) = 4.89$; group comparisons, Halo, Day 1 vs Day 2 $*P = 0.013$, Day 1 vs Day 3 $P = 0.062$. Latency to avoid: $F(2, 44) = 2.88$; group comparisons, Halo, Day 1 vs Day 2 $*P < 0.024$, Day 1 vs Day 3 $P = 0.08$. CS Freezing: $F(2, 44) = 1.1$; group comparisons, Halo, Day 1 vs Day 2 $P = 0.065$. ITI Freezing: $F(2, 44) = 0.46$; no significant change among each group comparison. Optogenetic inhibition session is depicted by the yellow background. **k**, The changes in freezing behavior across test sessions are plotted as a function of changes in avoidance behavior. Day 2 vs Day 1: Halo, $P = 0.0017$, $R^2 = 0.68$; Ctl, $P = 0.36$, $R^2 = 0.075$. Day 3 vs Day 1: Halo, $P = 0.12$, $R^2 = 0.25$; Ctl, $P = 0.36$, $R^2 = 0.076$. Day 3 vs Day 2: Halo, $P = 0.12$, $R^2 = 0.25$; Ctl, $P = 0.067$, $R^2 = 0.27$. **l-o**, Left: Optogenetic inhibition of pPVT–NAc axon terminals during the ITI has little effect on avoidance rate (**l**), the latency to avoid (**m**) and freezing time during the CS (**n**) and the ITI (**o**). Right: Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate, $F(2, 18) = 0.16$; latency to avoid, $F(2, 18) = 0.22$; CS Freezing, $F(2, 18) = 0.037$; ITI Freezing, $F(2, 18) = 0.48$; no significant change among each group comparison. Optogenetic inhibition sessions are depicted by the yellow background. **p**. Free moving distance in the open field. Ctl, $n = 9$ mice; Halo, $n = 8$ mice. Optogenetic inhibition duration is depicted by the yellow background. Data shown as mean $\pm$ s.e.m.

Extended Data Figure 11. Fiber photometry imaging of GCaMP7s fluorescence from the CeA terminals of pPVT^{D2R} neurons in the 2AA task.

933 **a**, Schematic of the viral vector strategy and optical fiber placement. **b**, Representative
934 images from a mouse expressing GCaMP7s in pPVT^{D2R} neurons (left) and implanted
935 with an optical fiber in the CeA (middle). Optical fiber placements (right, $n = 5$ mice). **c**,
936 Avoidance Rate (black) and freezing time during the CS (red) across all training and test
937 sessions. Fiber photometry imaging sessions are depicted by the blue lines. **d**,
938 Quantification of the latency to avoid (black) and freezing time during ITI (red) across
939 the sessions. Fiber photometry imaging sessions are depicted by the blue lines. **e**,
940 Representative imaging traces from a sample subject. Individual imaging traces for
941 avoidance (blue) and failure (red) trials. Black square pulses depict the duration of each
942 CS presentation. Yellow and red backgrounds show the maximal duration of CS and US
943 presentation, respectively. **f**, Top: Heatmaps showing individual calcium responses for
944 all avoidance and failure trials. Bottom: Average calcium signal and CS duration of the
945 upper panels (Avoidance trials, blue; failure trials, red; CS duration, black). **g**, Freezing
946 time (top) and the peak values of the calcium signal (middle) during the CS in avoidance
947 trials are significantly lower compared to failure trials, while the mean values of the
948 calcium signal during the CS are similar for both groups (bottom). Two-tailed Student's
949 t -test. Avoidance, $n = 235$ Trials. Failure, $n = 65$ Trials. Freezing, $***P < 0.001$. Peak
950 Signal, $*P = 0.026$. Mean signal, $P = 0.63$. **h**, Top: Linear regression analysis of peak
951 calcium signal and freezing time during the CS of avoidance (blue) and failure trials
952 (red) (avoidance trials, $P = 0.35$, $R^2 = 0.004$; failure trials, $P = 0.12$, $R^2 = 0.04$). Bottom:
953 Linear regression analysis of the average calcium signal and freezing time during the
954 CS of avoidance (blue) and failure (red) trials (avoidance trials, $P = 0.73$, $R^2 = 0.0005$;
955 failure trials, $P = 0.082$, $R^2 = 0.05$). **i**, Quantification of calcium signal during pre-CS, CS

and post-CS periods of avoidance (blue) and failure (red) trials. AUC, One-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance, $n = 235$ Trials, $F(2, 702) = 1$; Failure, $n = 65$ Trials, $F(2, 192) = 0.025$; no significant change among each group comparison. **j**, Left: Averaged calcium responses during CS onset, CS maximal velocity, escape initiation, escape and CS freezing epochs in all avoidance (blue) and failure (red) trials. Right: Quantification of calcium signal in each epoch duration. AUC, two-tailed Student's t -test. CS: Avoidance, $n = 235$ Events; Failure, $n = 65$ Events; $P < 0.001$. Max. Velocity: Avoidance, $n = 234$ Events; Failure, $n = 62$ Events; $P = 0.57$. Escape initiate: Avoidance, $n = 173$ Events; Failure, $n = 101$ Events; $P = 0.14$. Escape: Avoidance, $n = 234$ Events; Failure, $n = 65$ Events; $P = 0.42$. Freezing: Avoidance, $n = 100$ Events; Failure, $n = 116$ Events; $P = 0.39$. Data shown as mean $\pm$ s.e.m.

Extended Data Figure 12. Optogenetic inhibition of pPVT–CeA axon terminals in the 2AA task.

a, Schematic of the viral vector strategy and optical fiber placement for optogenetic silencing of pPVT–CeA axon terminals in the 2AA task. **b**, Optical fiber placements (Ctl, $n = 11$ mice; Halo, $n = 12$ mice). **c–f**, Avoidance rate (c), latency to avoid (d) and freezing time during the CS (e) and ITI (f) across all training sessions for both Ctl (black) and Halo (red) groups. **g–j**, Left: Optogenetic inhibition of pPVT–CeA axon terminals increases avoidance rate (g), while decreases latency to avoid (h) and freezing time during the CS (i) and the ITI (j). Right: Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and

979 Yekutieli. Avoidance rate: $F(2, 42) = 3.27$; group comparisons, Ctl, Day 1 vs Day 3 $*P =$
 980 0.021 , Day 2 vs Day 3 $*P = 0.046$; Halo, Day 1 vs Day 2 $**P = 0.0061$, Day 1 vs Day 3
 981 $*P = 0.024$. Latency to avoid: $F(2, 42) = 5.35$; group comparisons, Ctl, Day 1 vs Day 3
 982 $*P = 0.041$; Halo, Day 1 vs Day 2 $**P = 0.0049$, Day 1 vs Day 3 $*P = 0.028$. CS
 983 Freezing: $F(2, 42) = 2.77$; group comparisons, Halo, Day 1 vs Day 2 $**P = 0.0078$. ITI
 984 Freezing: $F(2, 42) = 1.67$; group comparisons, Halo, Day 1 vs Day 2 $*P = 0.038$.
 985 Optogenetic inhibition sessions are depicted by the yellow background. **k**, The changes
 986 in freezing behavior across test sessions are plotted as a function of changes in
 987 avoidance behavior. Day 2 vs Day 1: Halo, $P = 0.0036$, $R^2 = 0.59$; Ctl, $P = 0.82$, $R^2 =$
 988 0.0061 . Day 3 vs Day 1: Halo, $P = 0.28$, $R^2 = 0.12$; Ctl, $P = 0.068$, $R^2 = 0.44$. Day 3 vs
 989 Day 2: Halo, $P = 0.12$, $R^2 = 0.22$; Ctl, $P = 0.12$, $R^2 = 0.24$. **l-o**, Left: Optogenetic
 990 inhibition of pPVT–CeA axon terminals during the ITI has little effect on avoidance rate
 991 (**l**), the latency to avoid (**m**) and freezing time during the CS (**n**) and the ITI (**o**). Right:
 992 Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage
 993 linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate, $F(2$
 994 $, 28) = 0.0052$; latency to avoid, $F(2, 28) = 0.22$; CS Freezing, $F(2, 28) = 0.61$; ITI
 995 Freezing, $F(2, 28) = 0.086$; no significant change among each group comparison.
 996 Optogenetic inhibition sessions are depicted by the yellow background. **p**. Free moving
 997 distance in the open field. Ctl, $n = 8$ mice; Halo, $n = 9$ mice. Optogenetic inhibition
 998 duration is depicted by the yellow background.
 999 Data shown as mean $\pm$ s.e.m.

Extended Data Figure 13. Optogenetic inhibition of pPVT^{D2R}–CeA axon terminals in the 2AA task, related to Figure 4.

a, Freezing time during the ITI across all training days in both Ctl (black) and Halo (red) groups. **b**, Left: Optogenetic inhibition of pPVT^{D2R}–CeA axon terminals gradually reduces freezing time during the the ITI. Right: Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. $F(5, 80) = 0.44$; group comparisons, Ctl, Day 1 vs Day 6 $***P < 0.001$, Day 2 vs Day 6 $*P = 0.011$, Day 3 vs Day 6 $**P = 0.0074$, Day 4 vs Day 6 $*P = 0.048$; Halo, Day 1 vs Day 6 $*P = 0.017$. Optogenetic inhibition sessions are depicted by the yellow background. **c**, The changes in freezing behavior between Test Day 1 and other test sessions are plotted as a function of changes in avoidance behavior. Day 2 vs Day 1: Halo, $P = 0.26$, $R^2 = 0.27$; Ctl, $P = 0.81$, $R^2 = 0.0092$. Day 3 vs Day 1: Halo, $P = 0.48$, $R^2 = 0.073$; Ctl, $P = 0.038$, $R^2 = 0.48$. Day 4 vs Day 1: Halo, $P = 0.0084$, $R^2 = 0.65$; Ctl, $P = 0.031$, $R^2 = 0.51$. Day 5 vs Day 1: Halo, $P = 0.031$, $R^2 = 0.51$; Ctl, $P = 0.21$, $R^2 = 0.21$. Day 6 vs Day 1: Halo, $P = 0.032$, $R^2 = 0.5$; Ctl, $P = 0.042$, $R^2 = 0.47$. Data presented mean $\pm$ s.e.m.

Extended Data Figure 14. Optogenetic stimulation of pPVT^{D2R}–NAc or pPVT^{D2R}–CeA axon terminals in the 2AA task.

a, Schematic of the viral vector strategy and optical fiber placement for optogenetic stimulating of pPVT^{D2R}–NAc axon terminals in the 2AA task. **b**, Optical fiber placements (Ctl, $n = 8$ mice; ChR, $n = 12$ mice). **c-f**, Avoidance rate (c), latency to avoid (d) and freezing time during the CS (e) and ITI (f) across all training sessions for both Ctl (black)

1024 and ChR (red) groups. **g-j**, Top: Optogenetic stimulation of pPVT^{D2R}–NAc axon
1025 terminals increases avoidance rate (g), while decreases latency to avoid (h); freezing
1026 time during the CS (i) and the ITI (j) has no change. Bottom: Normalization of the Day 1
1027 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of
1028 Benjamini, Krieger and Yekutieli. Avoidance rate: $F(2, 36) = 6.21$; group comparisons,
1029 ChR, Day 1 vs Day 2 $**P = 0.0039$, Day 1 vs Day 3 $*P = 0.048$. Latency to avoid: $F(2,$
1030 $36) = 3.34$; group comparisons, ChR, Day 1 vs Day 2 $*P = 0.031$, Day 1 vs Day 3 $P =$
1031 0.06 . CS Freezing: $F(2, 36) = 0.16$; ITI Freezing: $F(2, 36) = 0.21$; no significant change
1032 among each group comparison. Optogenetic stimulation sessions are depicted by the
1033 yellow background. **k**, The changes in freezing behavior across test sessions are
1034 plotted as a function of changes in avoidance behavior. Day 2 vs Day 1: Halo, $P = 0.2$,
1035 $R^2 = 0.16$; Ctl, $P = 0.36$, $R^2 = 0.14$. Day 3 vs Day 1: Halo, $P = 0.072$, $R^2 = 0.28$; Ctl, $P =$
1036 0.22 , $R^2 = 0.24$. Day 3 vs Day 2: Halo, $P = 0.85$, $R^2 = 0.0039$; Ctl, $P = 0.31$, $R^2 = 0.17$. **l**,
1037 Schematic of the viral vector strategy and optical fiber placement for optogenetic
1038 stimulating of pPVT^{D2R}–CeA axon terminals in the 2AA task. **m**, Optical fiber
1039 placements (Ctl, $n = 7$ mice; ChR, $n = 10$ mice). **n-q**, Avoidance rate (n), latency to
1040 avoid (o) and freezing time during the CS (p) and ITI (q) across all training sessions for
1041 both Ctl (black) and ChR (red) groups. **r-u**, Top: Optogenetic stimulation of pPVT^{D2R}–
1042 CeA axon terminals decreases avoidance rate (r), while increases latency to avoid (s)
1043 and freezing time during the CS (t); freezing time during the ITI (u) has no significant
1044 change. Bottom: Normalization of the Day 1 in each group. Two-way ANOVA followed
1045 by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance
1046 rate: $F(2, 30) = 5.43$; group comparisons, Ctl, Day 1 vs Day 2 $*P = 0.022$; ChR, Day 1 vs

Day 2 $*P = 0.04$. Latency to avoid: $F(2, 30) = 1.89$; group comparisons, Ctl, Day 1 vs Day 2 $*P = 0.03$; ChR, Day 1 vs Day 2 $**P = 0.0025$. CS Freezing: $F(2, 30) = 1.01$; ChR, Day 2 vs Day 3 $**P = 0.0099$. ITI Freezing: $F(2, 30) = 0.66$; no significant change among each group comparison. Optogenetic stimulation sessions are depicted by the yellow background. **v**, The changes in freezing behavior across test sessions are plotted as a function of changes in avoidance behavior. Day 2 vs Day 1: Halo, $P = 0.035$, $R^2 = 0.44$; Ctl, $P = 0.32$, $R^2 = 0.2$. Day 3 vs Day 1: Halo, $P = 0.036$, $R^2 = 0.44$; Ctl, $P = 0.079$, $R^2 = 0.49$. Day 3 vs Day 2: Halo, $P = 0.88$, $R^2 = 0.003$; Ctl, $P = 0.19$, $R^2 = 0.32$. Data shown as mean $\pm$ s.e.m.

Extended Data Figure 15. Optogenetic inhibition of pPVT^{D2R}–NAc or pPVT^{D2R}–CeA axon terminals in the 2AA task, related to Figure 5.

a, Optical fiber placements ($n = 8$ mice per group). **b**, Freezing time during the ITI across all training sessions for both Ctl (black) and Halo (red) groups. **c**, Left: Optogenetic inhibition of pPVT^{D2R}–NAc axon terminals (Day 2) decreases freezing time during the ITI, while following optogenetic inhibition of pPVT^{D2R}–CeA axon terminals (Day 3) reverses these changes. Right: Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. $F(3, 42) = 1.08$; Halo, Day 1 vs Day 2 $*P = 0.032$. Optogenetic inhibition sessions are depicted by the yellow background. **d**, The changes in freezing behavior across test sessions are plotted as a function of changes in avoidance behavior. Day 2 vs Day 1: Halo, $P = 0.0047$, $R^2 = 0.76$; Ctl, $P = 0.11$, $R^2 = 0.37$. Day 3 vs Day 1: Halo, $P = 0.25$, $R^2 = 0.21$; Ctl, $P = 0.0044$, $R^2 = 0.77$. Day 4 vs Day 1: Halo, $P = 0.83$, $R^2 = 0.0084$; Ctl, $P = 0.81$, $R^2 = 0.01$. Day 3 vs Day 2: Halo, $P = 0.022$, $R^2 = 0.61$; Ctl, $P =$

0.064, $R^2 = 0.46$. Day 4 vs Day 2: Halo, $P = 0.019$, $R^2 = 0.63$; Ctl, $P = 0.99$, $R^2 < 0.001$.
Day 4 vs Day 3: Halo, $P = 0.32$, $R^2 = 0.16$; Ctl, $P = 0.27$, $R^2 = 0.2$. Data shown as mean
 $\pm$ s.e.m.

Extended Data Figure 16. Monosynaptic inputs of NAc- and CeA-projecting neurons of the pPVT.

a, Schematic of the viral vector strategy to trace the inputs to NAc-projectors or CeA-projectors in pPVT. **b**, Representative images showing the rabies starter cells (Rabies-GFP and TVA-mCherry double-labelled cells) in pPVT neurons. **c**, Quantification of monosynaptic inputs to NAc-projectors or CeA-projectors in pPVT. NAc-projectors, $n = 3$ mice; CeA-projectors, $n = 2$ mice. To normalize retrogradely labeled (GFP+) cells between subjects, a connectivity index for each brain region was computed by dividing the number of retrogradely labeled cells by the number of starter cells (See Methods). The target selectivity was observed for many inputs including the medial prefrontal cortex (CeA-projectors), the agranular insular cortex (NAc-projectors), the ventromedial hypothalamus (CeA-projectors) and the substantia innominata (NAc-projectors).

METHODS

Mice

All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by the National Institute of Mental Health (NIMH) Animal Care and Use Committee. Mice used in this study were group housed under a 12-h light-dark cycle (6 a.m. to 6 p.m. light), with food and water available *ad libitum*. After

surgery, mice were singly housed. Drd2-Cre mice were obtained from GENSAT (founder line ER44). In addition, we used C57BL/6NJ strain mice (The Jackson Laboratory). Both male and female mice 8–20 weeks of age were used for all experiments. Animals were randomly allocated to the different experimental conditions reported in this study.

Viral vectors

AAV9-hSyn-Flex-GCaMP6s-WPRE-SV40 was produced by the Vector Core of the University of Pennsylvania. AAV9-EF1 α -DIO-eNpHR3.0-mCherry, AAV2-CaMKII2 α -eNpHR3.0-mCherry, AAV2-CaMKII2 α -mCherry, AAV2-EF1 α -DIO-hChR2(H134R)-YFP-WPRE and AAV2-EF1 α -DIO-mCherry were produced by the Vector Core of the University of North Carolina. AAV5-hSyn-FLEX-ChrimsonR-tdTomato (Addgene plasmid 62723), AAV9-CAG-FLEX-tdTomato (Addgene plasmid # 28306) and AAV9-Syn-Flex-jGCaMP7s-WPRE (Addgene plasmid # 104491) were purchased from Addgene. AAV2(retro)-CAG-iCre (Addgene plasmid # 81070) was produced by Vector Biolabs. AAV9-EF1 α -FLEX-TVA-mCherry (Addgene plasmid # 38044) and AAV9-CAG-FLEX-RG (Addgene plasmid # 38043) were produced by Vigene Biosciences, Inc. EnvA-SAD- Δ G-eGFP (Addgene plasmid # 32635) was produced by the Viral Vector Core of the Salk Institute for Biological Studies. All viral vectors were stored in aliquots at -80°C until use.

Stereotaxic surgery

All viral injections were performed using previously described procedures¹² and an AngleTwo stereotaxic device (Leica Biosystems) at the following stereotaxic

1118 coordinates: pPVT, -1.60 mm from bregma, 0.06 mm lateral from midline, and -3.30 mm
1119 vertical from cortical surface, 6.12° angle for both fiber photometry and optogenetics;
1120 NAc, 1.70 mm from bregma, 0.60 mm lateral from midline, and -4.60 mm vertical from
1121 cortical surface, 9.99° angle for optogenetics; CeA, -1.42 mm from bregma, 2.90 mm
1122 lateral from midline, and -4.80 mm vertical from cortical surface. For fiber photometry
1123 and optogenetic experiments, an optical fiber (400 µm for photometry, Doric Lenses;
1124 200 µm for optogenetics, Thorlabs) was implanted over the target immediately after viral
1125 injections (coordinates: pPVT, -1.60 mm from bregma, 0.05 mm lateral from midline,
1126 and -3.00 mm vertical from cortical surface, 6.12° angle; NAc, 1.70 mm from bregma,
1127 0.60 mm lateral from midline, and -4.30 mm vertical from cortical surface, 9.99° angle
1128 for optogenetics; CeA, -1.42 mm from bregma, 2.88 mm lateral from midline, and -4.50
1129 mm vertical from cortical surface) and cemented using Metabond Cement System
1130 (Parkell) and Jet Brand dental acrylic (Lang Dental Manufacturing). Following all
1131 surgical procedures, animals were returned to their home cages and placed on a
1132 heating pad for 24 h postsurgical recovery and monitoring. Animals received
1133 subcutaneous injections with Metacam (meloxicam, 1-2 mg/kg) for analgesia and anti-
1134 inflammatory purposes. For targeting pPVT, all AAVs were injected at a total volume of
1135 approximately 1 µL and were allowed 4 weeks for maximal expression. For targeting
1136 NAc, AAV2-retro-Cre virus was injected at a total volume of approximately 0.5 µL. For
1137 retrograde tracing of NAc-projecting and CeA-projecting pPVT cells, CTB-647 and CTB-
1138 555 (1.0% in PBS; ThermoFisher Scientific) were injected into the NAc (0.5 µL) and
1139 CeA (0.3 µL), respectively and allowed 4 days for retrograde transport. Mice without
1140 correct targeting of optical fibers, tracers or vectors were excluded from this study.

Fiber photometry

Fiber photometry was performed as previously described^{28,29}. Briefly, after a four-week post-surgery recovery period, mice were allowed to habituate to the fiber patch cord in their home cage for approximately 5 min before each behavior test. Real-time population-level GCaMP fluorescence and isosbestic autofluorescence signals were excited by the fiber photometry system (Doric Lenses) using two sinusoidally modulated 473 nm (211 Hz) and 405 nm (531 Hz) LEDs (DC4100, ThorLabs). Both LEDs were combined via a commercial Mini-cube fiber photometry apparatus (Doric Lenses) into a fiber patch-cord (400 μ m core, 0.48 NA) connected to the brain implant in each mouse. The light intensity at the interface between the fiber tip and the animal was adjusted from 10 to 20 μ W (but was constant throughout each test session for each mouse). GCaMP and autofluorescence signals were collected through the same fiber and focused onto two separate photoreceivers (2151, Newport Corporation). An RZ5P fiber photometry acquisition system with Synapse software (Tucker-Davis Technologies) collected and saved real-time demodulated emission signals and behavior relevant TTL inputs of used for data analysis. For each trial, GCaMP signals ($F_{473\text{ nm}}$) were compared with autofluorescence signals ($F_{405\text{ nm}}$) to control for movement and bleaching artefacts. Signal data was de-trended by first applying a least-squares linear fit to produce $F_{\text{fitted } 405\text{ nm}}$, and dF/F was calculated as $(F_{473\text{ nm}} - F_{\text{fitted } 405\text{ nm}})/F_{\text{fitted } 405\text{ nm}}$. All GCaMP signal data is presented as the z-score of the dF/F from baseline (pre-CS) segments. Changes in fluorescence following behaviorally relevant events were quantified by measuring the area under the dF/F curve.

1164

1165 **Fear conditioning**

1166 Prior to training, mice were handled and habituated to the fiber patch cord in the
1167 conditioning context. Mice were fear conditioned in a Mouse Test Cage (18 cm x 18 cm
1168 x 30 cm) equipped with an electrifiable floor connected to a shock generator (H13-15;
1169 Coulbourn instruments) and placed inside a sound attenuated cabinet (H10-24A;
1170 Coulbourn Instruments). The auditory fear conditioning procedure was carried in two
1171 separate contexts: a Context A (conditioning) and a Context B (retrieval). These
1172 contexts differed in the appearance of the walls and the texture of the floors used. In
1173 addition, for habituation and conditioning sessions, the test cage (Context A) was wiped
1174 clean with 70% ethanol in between subjects. Similarly, before each retrieval session, the
1175 test cage (Context B) was wiped clean with 0.5% acetic acid and equipped with a fruity
1176 scent to facilitate context discrimination. The cabinet was illuminated at all times and the
1177 subject's behavior was captured with a USB camera (ACT-VP-02; Coulbourn
1178 Instruments) at 3.7 Hz during each session. FreezeFrame software (ActiMetrics) was
1179 used to control the delivery of both 4-kHz, 75-dB tones (30 s; CS) and 0.6-mA
1180 footshocks (2 s; US). Both GCaMP signal and TTLs of CS and US presentations were
1181 monitored with the RZ5P fiber photometry acquisition system.

1182 For habituation, on day 0 a 120-s pre-stimulus interval (PSI) was followed by 4 trials of
1183 CS presentations with pseudorandom 30-60-s inter-trial interval (ITI) in Context A. On
1184 day 1, fear conditioning was conducted in Context A. The 120 s of PSI was followed by
1185 5 trials of CS presentations that co-terminated with the US, with pseudorandom 30-60-s
1186 ITI. On day 2, a fear memory retrieval session was conducted in Context B. A 120 s PSI

was followed by 8 trials of unreinforced CS presentations with pseudorandom 30-120-s ITI. Freezing behavior was recorded and analyzed with FreezeFrame. For the retrieval session, the freezing data of all the trials were linearly aligned from maximum to minimum (Figure 1d, right panel). Based on the distribution, all trials were divided into three groups: Low freezing (L; Freezing < 40%; n = 20 trials), moderate freezing (M; 40% ≤ Freezing ≤ 60%; n = 16 trials) and high freezing (H; Freezing > 60%; n = 12 trials). For all trials recorded, we calculated the area under the curve (AUC) for both calcium signal and movement.

Two-way signaled active avoidance (2AA)

Mice were trained on the two-way signaled active avoidance as previously described¹⁶. Briefly, the behavioral apparatus consisted of a custom-built shuttle box (18 cm x 36 cm x 30 cm) that contained two identical chambers separated by a hurdle (17.5 cm x 6 cm). The hurdle projected 3 cm above the floor and allowed mice easy access to both chambers. The floor consisted of electrifiable metal rods (H10-11M-TC-SF, Coulbourn Instruments) and was connected to a shock generator (H13-15; Coulbourn instruments). Before each subject was trained/tested, the shuttle box was wiped clean with 70% ethanol. The mouse's behavior was captured with a USB camera during each session. ANY-maze software (Stoelting) was used to control the delivery of 4-kHz, 75-dB tones (CS), 0.5-mA footshocks (US) and the TTL output for optogenetic stimulation. A speaker located on the top of the shuttle box (50 cm high) was used to deliver the CS. Subjects' movement and TTLs of CS, US and optogenetic stimulation were recorded by ANY-maze.

1210

1211 After a 5-min habituation period, mice were trained with daily sessions of 2AA, each
1212 consisting of 30 presentations of the CS (lasting up to 15 s each). Trials in which
1213 subjects failed to shuttle to the adjacent chamber prior to the termination of the CS
1214 resulted in the presentation of the US (lasting up to 15 s each) until subjects escaped to
1215 the opposite chamber (failure trials). For trials in which subjects shuttled to the opposite
1216 chamber during the CS, the CS was abruptly terminated, and the delivery of the US
1217 prevented altogether (avoidance trials). Avoidance rate was calculated as the
1218 percentage of the number of avoidance trials over the total number of trials.

1219

1220 For fiber photometry experiments in which the cell bodies of pPVT^{D2R} neurons were
1221 imaged (Figure 1), mice were subjected to five 2AA sessions (1 session/day) and the
1222 GCaMP signal was collected on Days 4 and 5 as described above. For optogenetic
1223 experiment of pPVT^{D2R} cell body (Figure 1), mice were initially trained for 5 days. If a
1224 subject did not reach 30% of avoidance rate by Day 5, it was not used in further test
1225 sessions. Test sessions for optogenetic manipulations consisted of CS presentations
1226 (no US). In the second test session, subjects received light stimulation with a yellow
1227 light source (Ce:YAG + LED Driver; Doric Lenses) during each CS presentation (light
1228 onset was 5 s prior to the onset of the CS and culminated 5 s after the offset of the CS).
1229 The light intensity at the interface between the fiber tip and the mouse was ~10 mW. For
1230 experiments in which fiber photometry imaging of pPVT^{D2R} terminals and pPVT^{NAc}
1231 neurons was conducted (Figure 2, Extended Data Figure 5 and Extended Data Figure
1232 7), mice were initially trained for 3 days. GCaMP signal was collected across two

subsequent training or test sessions (referred to as Days 4-5 or test sessions) with the RZ5P fiber photometry acquisition system. For experiments involving optogenetic inhibition of pPVT^{D2R}-NAc terminals (Figure 3), mice were initially trained for 3 days. If a subject did not reach 70% of avoidance rate by Day 3, it was not used in further testing sessions. As described above, these test sessions involved presentations of the CS alone (no US) and the subjects received light stimulation paired to CS presentations during the second test session. For experiments in which we optogenetically inhibited pPVT^{D2R}-CeA terminals (Figure 4), we selectively screened for poor avoiders as follows: mice were initially trained for 3 days, and if a subject reached over 30% of avoidance rate by Day 3, it was not used in further test sessions. As these are essentially mice that failed to learn active avoidance, subsequent test sessions involved presentations of both the CS and the US while subjects received light stimulation paired to the CS from the second to the sixth test sessions. For experiments involving optogenetic manipulation of pPVT terminals (Extended Data Figure 6, Extended Data Figure 8 and Extended Data Figure 9), mice were initially trained for 3 days and tested with another 3 days subsequently. These test sessions involved both CS and US presentations and the subjects received light stimulation paired to CS presentations during the second test session. For experiments in which we optogenetically manipulated pPVT or its circuits during ITIs (Extended Data Figure 4, Extended Data Figure 6 and Extended Data Figure 8), mice were initially trained for 3 days and tested with another 3 days subsequently. These test sessions involved both CS and US presentations and the subjects received light stimulation paired to ITIs during the second test session. For experiments to optogenetically inhibit pPVT-NAc and pPVT-

CeA terminals within the same subjects (Figure 5), mice were initially trained for 3 days and tested with another 4 days subsequently. These test sessions involved both CS and US presentations and the subjects received light stimulation paired to CS presentations through NAc-implanted opto fibers during the second test session and CeA-implanted opto fibers during the third test session. All optogenetic experiments involved light stimulation in both mice expressing the inhibitory opsin eNpHR3.0 or excitatory opsin ChrimsonR in pPVT neurons, as well as in control mice that expressed a fluorescent reporter instead (mCherry or tdTomato).

Data analysis for two-way signaled active avoidance

The moment-to-moment position of each mouse was determined by using an overhead camera (30 frames/s) in combination with automated tracking and trial control system (ANY-maze). We performed, post-hoc position tracking of the animal's nose and body center from video in the tracking software TopScan (CleverSys). CS and US times from ANY-maze and raw video tracking position values from TopScan were exported and analysis was performed with custom routines in the R statistical computing environment (R core Team 2019; R Foundation).

Missing positions up to 10 successive frames were linearly interpolated with custom routines in R; in the very rare cases that over 10 successive frames were missing the entire video was re-tracked with optimized tracking parameters. For imaging sessions, video tracking and ANY-maze TTL pulse timestamps were zero corrected to align behavioral and calcium signal timestamps. Next, calcium signals and/or position frames

during US and CS were flagged by matching the relevant timestamps to TTL pulse times from ANY-maze, and the frame-by-frame distance traveled for the nose and body center was calculated for the tracking data. In order to minimize the effects of noise in the tracking data, we calculated the 40% quantile of the frame-by-frame distance traveled by the animal's nose and body center for each session; in all cases, this yielded a distance value of 0 or 1 mm. This quantile value served as a movement threshold, i.e. an inter-frame distance traveled less than or equal to the quantile value was considered non-movement. We then created a binary vector and frames with coincident immobility of the nose and center body were set to 1. Changepoint analysis⁵³ (R package version 2.2.2, URL:<https://CRAN.R-project.org/package=changepoint>), with a minimum segment length of 30 video frames, was then applied to this vector. This approach allowed us to statistically determine when transitions to (and from) coincident periods of non-movement of nose and body occurred which were used as a proxy for freezing behavior. Next, each sustained bout of non-movement was isolated, and we probed whether there was any movement that lasted for ≥ 5 consecutive video frames. If such movement did occur, we truncated the bout of immobility at the start of movement. Finally, immobility bouts with a duration ≥ 1 s were considered freezing. To ensure robustness of our method, we compared statistically derived event flags against manually scored videos in a subset of behavioral sessions.

We isolated freezing events (see Freeze detection section above) which occurred during the CS as CS freezing and those occurred during ITI as ITI freezing. CS or ITI freezing were averaged within session and then within each group and plotted as mean

1302 $\pm$ s.e.m. For each avoidance trial we flagged the video frame where animals first
1303 crossed the barrier and then calculated the time interval between this event and the
1304 onset of the cue, named Latency to avoid. Latency to avoid was averaged within
1305 session and then within each group and plotted as mean $\pm$ s.e.m.

1306 As previously described (see Fiber photometry session), GCaMP data was normalized
1307 as dF/F . Next, we used the behavioral flags calculated from the video tracking to create
1308 average peri-event time histograms (PETH) time locked to the onset of the behavior
1309 events of interest, including CS onset (CS), highest movement velocity during the CS
1310 (Max. Velocity), escape or avoidance movement onset (Escape Initiate), escape or
1311 avoidance moment (Escape) and freezing onset during the CS (Freezing). Then, using
1312 the 10 s before each CS onset as a baseline for each trial, we calculated Z-scores from
1313 the normalized calcium signals ((Normalized signal - Mean of normalized baseline
1314 signal)/ Standard deviation of normalized baseline signal)). All trials in each session
1315 were separated into avoidance and failure trials as described above. For each trial type,
1316 the Z-score from 10 s before to 30 s after CS onset was plotted in heatmaps for all trials
1317 in all test sessions. The mean of all recorded activity for each trial type was plotted
1318 below the corresponding heatmap. We isolated the calcium signal from 2 s before to 2 s
1319 after the onset of each behavior event from avoidance and failure trials separately.

1320 Using the 2 s before the onset as a baseline for each event, we calculated Z-scores
1321 again. Then, we calculated AUC of the Z-score from 2 s after the onset of each behavior
1322 event. Lastly, we plotted the mean of all signal transitions and AUC for each event type
1323 from each trial type. All 2AA photometric signals and behavioral performance were
1324 analyzed blind.

1325

1326 **Optogenetic inhibition of pPVT neurons and projections in an open field arena**

1327 After habituating to the fiber patch cord in home cage for at least 10 min, mice were
1328 placed in a square enclosure (50 × 50cm) to roam freely for 6 min. Mice received 2 min
1329 of light stimulation 2 min after the beginning of the test (min 2-4) The light intensity at
1330 the interface between the fiber tip and the mouse was ~10 mW. The cumulative
1331 distance traveled in the open field apparatus was tracked, recorded, and quantified
1332 using ANY-maze behavioral tracking software.

1333

1334 **Monosynaptic tracing of inputs to NAc-projecting or CeA-projecting neurons of**
1335 **the PVT**

1336 To limit monosynaptic rabies tracing to NAc-projecting or CeA-projecting neurons of the
1337 PVT, AAV2(retro)-CAG-iCre was bilaterally injected into the NAc (0.5 µl per injection) or
1338 CeA (0.3 µl per injection) of C57BL/6NJ mice. Within the same surgical procedure, a
1339 virus mixture of AAV9-EF1a-FLEX-TVA-mCherry and AAV9-CAG-FLEX-RG at a 1:1
1340 ratio was injected into the pPVT (1.5 µl), followed by an injection of the pseudotyped
1341 rabies virus EnvA-SAD-ΔG-eGFP (1.5 µl) in the same location of pPVT two weeks later.
1342 This viral strategy results in the ability of G-deficient rabies virus to only infect TVA-
1343 receptor expressing NAc- or CeA-projecting neurons. One week after the rabies virus
1344 injection, mouse brain tissues were collected and subjected to analysis.
1345 Brain sections were scanned by an AxioScan (Carl Zeiss) with a 5x objective. Cells
1346 were detected and quantified on NeuroInfo (MBF Bioscience). First, histological images
1347 were manually registered onto the Allen Brain Atlas (2017) on NeuroInfo. Next, linear

and non-linear transforms were applied to optimize fit. Cell coordinates were interpolated onto the registered images to generate region-specific cell counts. Starter cell count was generated by quantifying number of mCherry and GFP double positive pPVT cells. Finally, we generated a connectivity index by normalizing the fraction of retrogradely labeled (GFP⁺) cells for a given brain region, to the number of starter cells. Connectivity indices for each region were averaged within groups (CeA-projecting and NAc-projecting). Regions neighboring the pPVT (midline and medial thalamic nuclei) were excluded from our analyses due to the possibility of artifacts related to the injection site. All monosynaptic rabies tracing data were analyzed by a blind experimenter.

Histology and immunofluorescence

Animals were deeply anesthetized with euthanasia solution (Vet One) and transcardially perfused with PBS (pH 7.4, 4 °C), followed by paraformaldehyde solution (PFA, 4% in PBS, 4 °C). After extraction, brains were post-fixed in 4% PFA at 4 °C for a minimum of 2 h, and subsequently cryoprotected by transferring to a 30% PBS-buffered sucrose solution until brains were saturated (for over 24 h). Coronal brain sections (50 µm) were cut using a freezing microtome (SM 2010R, Leica). For immunofluorescence staining, Brain sections were incubated in PBS (pH 7.4) with 10% normal goat serum (NGS) and 0.1% Triton X-100 (Sigma Aldrich) for 1 h, and then incubated using the following antibody (overnight, at 4 °C): anti-D2R (1:300, mouse, Frontier Institute, D2R-Rb-Af960, validated for both species and application by producer). After four washing steps, Alexa-Fluor-conjugated secondary antibodies were used (Molecular Probes, 1:500, for 2 h at room temperature). All washes and incubations were done over a shaker at low speed.

Finally, sections were subsequently mounted onto glass slides and a coverslip was added using ProLong Diamond antifade (Thermo Fisher Scientific). Images were taken using an LSM 780 laser-scanning confocal microscope (Carl Zeiss). Image analysis and cell counting were performed using ImageJ software (Fiji, version 1.52p). Optical fiber placements for all mice included in this study are presented in Figure 1a, Figure 2c, Figure 3c, Figure 4c, Figure 5b, Extended Data Figure 4a and 4k, Extended Data Figure 5b, Extended Data Figure 6b, Extended Data Figure 7b, Extended Data Figure 8b and Extended Data Figure 9b and 9m.

Statistical analysis

All data were plotted and analyzed with OriginPro 2018 (OriginLab) and GraphPad Prism (Version 8.0.1; GraphPad Software). All data are presented as mean $\pm$ s.e.m. There were no assumptions or corrections made before data analysis. Differences between two groups were tested with a two-tailed Student's *t*-test, where $P < 0.05$ was considered significant. Differences among multiple groups were examined with analysis of variance (ANOVA, one-way and two-way repeated measures) followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, where $P < 0.05$ was considered significant. No statistical methods were used to predetermine sample size. For tracing experiments, the sample size is 2-5 mice. For fiber photometry experiments, the sample size is 4-6 mice. For optogenetic experiments, the sample size is 6-13 mice. All experiments were replicated at least once. All experiments were randomized, and investigators were blinded to allocation during experiments.

1394 **Life Sciences Reporting Summary**

1395 Additional information on experimental design and materials used in our study is
1396 available in the Life Sciences Reporting Summary.

1397

1398 **Data availability**

1399 All the data that support the findings presented in this study are available from the
1400 corresponding author upon reasonable request.

1401

1402 **Code availability**

1403 R code used to analyze active avoidance behavior and photometric signal is available
1404 from the corresponding author upon reasonable request.

1405

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1414

1415 **AUTHOR CONTRIBUTIONS**

1416 J.M. performed all experiments. M.K. assisted with histological procedures and
1417 analyzed the monosynaptic rabies tracing data. B.S.B. assisted with the monosynaptic
1418 rabies tracing experiments. J.d.H. developed custom tools for analyzing behavior and
1419 calcium signals. J.M. and J.d.H analyzed the data. Y.C. contributed to funding
1420 acquisition and writing. J.M. and M.A.P. designed the study, interpreted results, and
1421 wrote the paper.

1422

Figures

Figure 1

Figure 1

pPVT2R neurons control active avoidance and are inhibited during freezing. **a**, Left: Representative image from a mouse expressing GCaMP6s in pPVT2R neurons of Drd2-Cre mice and implanted with an optical fiber in the pPVT. Right: Optical fiber placements (n = 5 mice). **b**, Schematic of the 2AA task. **c**, Avoidance Rate (black) and freezing time during the CS (red) across training (Days 1-3) and test sessions (Days 4-5). Fiber photometry imaging sessions are depicted by the blue lines. **d**, Representative imaging traces from a sample subject. Individual imaging traces for avoidance and failure trials recorded. Bottom: Average calcium signal and CS duration of the upper panels (Avoidance trials, blue; failure trials, red; CS duration, black). **e**, Heatmaps of Z-score for Avoidance Trials and Failure Trials. **f**, Z-score for Avoidance Trials and Failure Trials. **g**, AUC for Avoidance Trials and Failure Trials. **h**, Z-score and AUC for various behavioral metrics: CS, Max. Velocity, Escape Initiate, Escape, and Freezing. **i**, Schematic of the AAV-ef1α-DIO-Halo-mCherry or AAV-ef1α-DIO-mCherry injection into the PVT of Drd2-Cre mice. **j**, Avoidance Rate (%) and Normalization for Test sessions (Days 1-3). **k**, Latency to avoid (s) and Normalization for Test sessions (Days 1-3). **l**, CS Freezing (s) and Normalization for Test sessions (Days 1-3).

trials, while the peak (middle) and mean (bottom) of calcium signals during the CS is significantly larger in avoidance trials. Avoidance, $n = 118$ Trials; Failure, $n = 122$ Trials; two-tailed Student's t-test. $***P < 0.001$. g, Quantification of calcium signal during pre-CS, CS and post-CS for avoidance (blue) and failure trials (red). AUC, One-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance: $n = 118$ Trials; $F(2, 351) = 75.89$; group comparisons, pre-CS vs CS $***P < 0.001$, pre-CS vs post-CS $*P = 0.038$, CS vs post-CS $***P < 0.001$. Failure: $n = 122$ Trials; $F(2, 363) = 66.54$; group comparisons, $***P < 0.001$. h, Left: Averaged calcium responses during CS onset, CS maximal velocity, escape initiation, escape and CS freezing epochs for all avoidance (blue) and failure (red) trials. Right: quantification of calcium signal in each epoch duration. AUC, two-tailed Student's t-test. CS: Avoidance, $n = 118$ Events; Failure, $n = 122$ Events; $P = 0.74$. Max. Velocity: Avoidance, $n = 117$ Events; Failure, $n = 122$ Events; $*P = 0.024$. Escape initiate: Avoidance, $n = 113$ Events; Failure, $n = 115$ Events; $***P < 0.001$. Escape: Avoidance, $n = 118$ Events; Failure, $n = 122$ Events; $**P = 0.0064$. Freezing: Avoidance, $n = 92$ Events; Failure, $n = 157$ Events; $P = 0.98$. i, Schematic of the viral vector strategy for optogenetic silencing of pPVTD2R neurons. j-l, Left: Optogenetic inhibition of pPVTD2R neurons significantly decreases avoidance rate (j), while increases latency to avoid (k) and freezing time during the CS (l). Right: Normalization of the Day 1 in each group. Ctl, $n = 8$ mice; Halo, $n = 9$ mice, two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance Rate: $F(2, 30) = 5.48$; group comparisons, Halo, Day 1 vs Day 2 $***P < 0.001$, Day 1 vs Day 3 $P = 0.06$. Latency to avoid: $F(2, 30) = 2.48$; group comparisons, Halo, Day 1 vs Day 2 $**P = 0.0037$. CS freezing: $F(2, 30) = 0.19$; group comparisons, Halo, Day 1 vs Day 2 $*P = 0.04$. Optogenetic inhibition sessions are depicted by the yellow background. Data shown as mean $\pm$ s.e.m.

Figure 2 **Figure 2**

pPVTD2R–Nac axon terminals signal active avoidance. **a**, Schematic of the viral vector strategy and optical fiber placement used for fiber photometry imaging of pPVTD2R–Nac axon terminals. **b**, Representative images from a mouse expressing GCaMP7s in pPVTD2R neurons and implanted with an optical fiber in the NAc. **c**, Optical fiber placements (n = 4 mice). **d**, Avoidance Rate (black) and freezing time during the CS (red) across all training and test sessions. Fiber photometry imaging sessions are depicted by the blue lines. **e**, Representative imaging traces from a sample subject. Individual imaging traces for avoidance (blue) and failure (red) trials. Black square pulses depict the duration of each CS presentation. Yellow and red backgrounds show the maximal duration of CS and US presentation, respectively. **f**, Top: Heatmaps showing individual calcium responses for all avoidance and failure trials. Bottom: Average calcium signal and CS duration of the upper panels (Avoidance trials, blue; failure trials, red; CS duration, black). **g**, Freezing time during the CS (top) in avoidance trials is significantly lower compared to failure trials, while the peak (middle) and mean (bottom) values of the calcium signal during

the CS are similar for both groups. Two-tailed Student's t-test. Avoidance, n = 80 Trials. Failure, n = 130 Trials. Freezing, ***P < 0.001. Peak Signal, P = 0.14. Mean Signal, P = 0.75. h, Quantification of calcium signal during pre-CS, CS and post-CS periods for avoidance (blue) and failure (red) trials. AUC, One-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance: n = 80 Trials, F(2, 237) = 4.37; group comparisons, pre-CS vs CS **P = 0.0065, pre-CS vs post-CS P = 0.67, CS vs post-CS *P = 0.021. Failure: n = 130 Trials, F(2, 387) = 121.2; group comparisons, pre-CS vs CS ***P < 0.001, pre-CS vs post-CS P = 0.072, CS vs post-CS ***P < 0.001. i, Left: Averaged calcium responses during CS onset, CS maximal velocity, escape initiation, escape and CS freezing epochs for all avoidance (blue) and failure (red) trials. Right: Quantification of calcium signal in each epoch duration. AUC, two-tailed Student's t-test. CS: Avoidance, n = 80 Events; Failure, n = 130 Events; P = 0.17. Max. Velocity: Avoidance, n = 80 Events; Failure, n = 129 Events; ***P < 0.001. Escape initiate: Avoidance, n = 79 Events; Failure, n = 123 Events; P = 0.74. Escape: Avoidance, n = 80 Events; Failure, n = 130 Events; P = 0.78. Freezing: Avoidance, n = 76 Events; Failure, n = 175 Events; P = 0.78. Data shown as mean ± s.e.m.

Figure 3

Figure 3

Optogenetic inhibition of pPVTD2R–NAc axon terminals decreases active avoidance and increases freezing. a, Schematic of the viral vector strategy and optical fiber placement for optogenetic silencing of pPVTD2R–NAc axon terminals in the 2AA task. b, Representative images from a mouse expressing Halo-mCherry in pPVTD2R neurons (left) and implanted with optical fibers in the NAc (right). c, Optical fiber placements (n = 8 mice per group). d-f, Avoidance rate (d), latency to avoid (e) and freezing time during the CS (f) across all training sessions for both Ctl (black) and Halo (red) groups. g-i, Left: Optogenetic inhibition of pPVTD2R–NAc axon terminals persistently decreases avoidance rate (g), while increases latency to avoid (h) and freezing time during the CS (i). Right: Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: $F(2, 28) = 18.04$; group comparisons, Halo, Day 1 vs Day 2 $***P < 0.001$, Day 1 vs Day 3 $***P < 0.001$. Latency to avoid: $F(2, 28) = 5.63$; group comparisons, Ctl, Day 1 vs Day 3 $*P = 0.028$; Halo, Day 1 vs Day 2 $***P < 0.001$, Day 1 vs Day 3 $***P < 0.001$. CS Freezing: $F(2, 28) = 1.45$; group comparisons, Halo, Day 1 vs Day 2 $*P = 0.049$, Day 1 vs Day 3 $**P = 0.0092$. Optogenetic inhibition session is depicted by the yellow background. Data shown as mean $\pm$ s.e.m.

Figure 4

Figure 4

Optogenetic inhibition pPVTD2R–CeA axon terminals increases active avoidance and reduces freezing. a, Schematic of the viral vector strategy and optical fiber placement used for optogenetic silencing of pPVTD2R–CeA axon terminals in the 2AA task. b, Representative images from a mouse expressing Halo-mCherry in pPVTD2R neurons and implanted with optical fibers in the CeA. c, Optical fiber placements (n = 9 mice per group). d-f, Avoidance rate (d), latency to avoid (e) and freezing time during the CS (f) across

all training days in both Ctl (black) and Halo (red) groups. g-i, Top: Optogenetic inhibition of pPVTD2R–CeA axon terminals gradually increases avoidance rate (g) and reduces the latency to avoid (h) and freezing time during the CS (i). Bottom: Normalization of the Day 1 in each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: $F(5, 80) = 9.05$; group comparisons, Halo, Day 1 vs Day 4 $***P < 0.001$, Day 1 vs Day 5 $***P < 0.001$, Day 1 vs Day 6 $***P < 0.001$, Day 2 vs Day 4 $*P = 0.015$, Day 2 vs Day 5 $***P < 0.001$, Day 2 vs Day 6 $***P < 0.001$, Day 3 vs Day 4 $*P = 0.015$, Day 3 vs Day 5 $***P < 0.001$, Day 3 vs Day 6 $***P < 0.001$, Day 4 vs Day 6 $***P < 0.001$, Day 5 vs Day 6 $*P = 0.012$. Latency to avoid: $F(5, 80) = 4.52$; group comparison, Halo, Day 1 vs Day 4 $*P = 0.011$, Day 1 vs Day 5 $***P < 0.001$, Day 1 vs Day 6 $***P < 0.001$, Day 2 vs Day 5 $*P = 0.043$, Day 2 vs Day 6 $***P < 0.001$, Day 3 vs Day 5 $**P = 0.0085$, Day 3 vs Day 6 $***P < 0.001$, Day 4 vs Day 6 $**P = 0.0027$. CS Freezing: $F(5, 80) = 1.17$; group comparisons, Halo, Day 3 vs Day 6 $*P = 0.012$, Day 4 vs Day 6 $**P = 0.007$, Day 5 vs Day 6 $*P = 0.018$. Optogenetic inhibition sessions are depicted by the yellow background. Data presented mean $\pm$ s.e.m.

Figure 5

Figure 5

pPVTD2R projections to the NAc and the CeA flexibly bias the selection of defensive behaviors. a, Schematic of the viral vector strategy and optical fiber placement for optogenetic silencing of pPVTD2R–NAc or pPVTD2R–CeA axon terminals in the same subject. b-d, Avoidance rate (b), latency to avoid (c) and freezing time during the CS (d) across all training sessions for both Ctl (black) and Halo (red) groups. e-g, Top: Optogenetic inhibition of pPVTD2R–NAc axon terminals (Day 2) decreases avoidance rate (e), increases latency to avoid (f) and freezing time during the CS (g), while following optogenetic inhibition of pPVTD2R–CeA axon terminals (Day 3) reverses these changes. Bottom: Normalization of the Day 1 in each group. Two-way ANOVA followed by two stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: $F(3, 42) = 6.09$; group comparisons, Halo, Day 1 vs Day 2 $**P = 0.0017$, Day 2 vs Day 3 $**P = 0.0072$, Day 2 vs Day 4 $**P = 0.0078$. Latency to avoid: $F(3, 42) = 5.56$; group comparisons, Halo, Day 1 vs Day 2 $**P = 0.0029$, Day 2 vs Day 3 $*P = 0.012$, Day 2 vs Day 4 $*P = 0.02$. CS Freezing: $F(3, 42) = 0.74$; group comparisons, Halo, Day 1 vs Day 2 $P = 0.086$, Day 1 vs Day 4 $*P = 0.013$, Day 2 vs Day 3 $*P = 0.043$. Optogenetic inhibition sessions are depicted by the yellow background. Data shown as mean $\pm$ s.e.m.

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