Divergent projections of the paraventricular nucleus of the thalamus mediate the selection of passive and active defensive behaviors

Jun Ma¹, Johann du Hoffmann²,³, Morgan Kindel¹, B. Sofia Beas¹, Yogita Chudasama⁴,⁵ and Mario A. Penzo¹

The appropriate selection of passive and active defensive behaviors in threatening situations is essential for survival. Previous studies have shown that passive defensive responses depend on activity of the central nucleus of the amygdala (CeA), whereas active ones primarily rely on the nucleus accumbens (NAc). However, the mechanisms underlying flexible switching between these two types of responses remain unknown. Here we show in mice that the paraventricular thalamus (PVT) mediates the selection of defensive behaviors through its interaction with the CeA and the NAc. We show that the PVT–CeA pathway drives conditioned freezing responses, whereas the PVT–NAc pathway is inhibited during freezing and, instead, signals active avoidance events. Optogenetic manipulations revealed that activity in the PVT–CeA or PVT–NAc pathway biases behavior toward the selection of passive or active defensive responses, respectively. These findings provide evidence that the PVT mediates flexible switching between opposing defensive behaviors.

The use of Pavlovian conditioning paradigms has enabled scientists to obtain substantial knowledge of the neuronal circuits and cellular processes underlying fear conditioning and its associated behavioral Pavlovian fear responses (for example, freezing and flight)¹–⁴. In contrast, the mechanisms controlling instrumental defensive responses, such as active avoidance and those that guide the selection of passive and active 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 imminence⁹–¹².

Although studies on the neurobiology of defensive behaviors have pointed to the contributions of both cortical and subcortical networks to the expression of specific defensive behaviors⁹–¹⁰, it is generally recognized that Pavlovian reactions, such as freezing and conditioned flight, are driven by genetically defined neuronal subpopulations of the CeA¹¹,¹² and that active 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 behaviors¹³,¹⁶. Specifically, these studies demonstrated that, although lesions and pharmacological manipulations of the CeA attenuate freezing and promote active avoidance behavior¹⁷,¹⁸, inactivation of the NAc decreases avoidance but enhances freezing¹⁹,²⁰. 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), because it sends projections to both the NAc and the CeA²¹. Indeed, BLA projections to the CeA are known to mediate conditioned freezing responses²², whereas BLA projections to the NAc have been shown to support active avoidance behavior²³. However, the necessity of the BLA for the expression of conditioned freezing and avoidance behaviors decreases with the passage of time²⁴–²², 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 NAc²³,²⁴ as well as its documented role in mediating conditioned freezing responses, particularly at remote time points²⁵–²⁷. 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 highlight the existence of a previously unrecognized switch for the selection of passive and active defensive behaviors in the midline thalamus.

**Results**

**pPVT²²R neurons are inhibited during freezing.** The pPVT is a stress-sensitive region of the thalamus that sends robust, largely non-overlapping projections to the CeA and the NAc (Fig. 1a–d)²⁵. Unlike primates and carnivores, the rodent thalamus is largely devoid of GABAergic neurons²⁶. Thus, projection neurons of the PVT are predominantly glutamatergic²⁷. Although activation of CeA-projecting pPVT neurons is thought to be necessary for the retrieval of conditioned fear memory and its accompanying freezing behavior¹²,²², the NAc is the main target of most pPVT neurons (Fig. 1e–j)²⁸. Surprisingly, the contribution of pPVT–NAc neurons to fear-related behaviors is currently unknown. To investigate this, we first monitored the activity of dopamine D2 receptor expressing neurons of the pPVT (pPVT²²R)—a marker of stress-sensitive PVT neurons, most of which project to the NAc (Fig. 1g–j)²⁹,³⁰—during
fear conditioning (Extended Data Figs. 1 and 2). Toward this goal, we expressed the genetically encoded calcium sensor GCaMP6s in pPVTD2R neurons of Drd2-Cre mice (Extended Data Fig. 1a). Next, after a habituation session (Day 0), on Day 1 mice were fear conditioned to an auditory cue (conditioned stimulus (CS)) that co-terminated with a footshock (unconditioned stimulus (US)), and on Day 2 a fear memory retrieval test was performed (Extended Data Fig. 1b–e). On both days, bulk changes in GCaMP6s fluorescence in pPVTD2R neurons were monitored using fiber photometry. We reasoned that, because most pPVTD2R neurons project to the NAc, bulk GCaMP6s fluorescence gathered from this neuronal population with fiber photometry would likely be dominated by the activity of PVT–NAc neurons. Consistent with previous reports, pPVTD2R neurons were readily activated by the US during the conditioning session on Day 1 (refs. 28,29) (Extended Data Fig. 1g,j,n,o). Notably, we also observed the emergence of small but consistent CS-evoked GCaMP6s responses in late conditioning trials, suggesting that the activity of these neurons is modulated by learning22.
activity of this neuronal population and the selection of active cop-
movement after fear conditioning might reflect a link between the
failure trials (Supplementary Video 1). First, CS presentation elic-
during avoidance trials, whereas, in failure trials, it mostly induced
robust increases in GCaMP6s fluorescence in pPVTD2R neurons,
consistent with our fear conditioning data as well as with previous reports (Fig. 2d,e)28.

Importantly, whereas mice engaged in instrumental responses dur-
ing avoidance trials (by definition), they spent significantly more
time freezing in response to the CS during failure trials compared to
avoidance trials (Fig. 2f). This suggests that the differential dynam-
ics of calcium signals of pPVTD2R neurons observed across these trial
categories could be related to behavior. To investigate this possibil-
ity, we analyzed calcium transients time-locked to discrete task and
behavioral events, such as CS onset, maximum velocity and freezing
behavior (Fig. 2h). These analyses revealed that, although CS onset,
maximum velocity and escape behavior (shutting in response to
the CS or US onset) were all associated with increases in GCaMP6s
fluorescence, freezing behavior was accompanied by attenuated
pPVTD2R neuronal activity for both avoidance and failure trials
(Fig. 2h). Thus, although mean signal variations more accurately
distinguish avoidance and failure trials (Fig. 2f,g), discrete fluctua-
tions in calcium transients largely reflect moment-to-moment
variation in behavior rather than trial type (Fig. 2h).

To investigate whether activation of pPVTD2R 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. 2i and Extended Data Fig. 4a–j).
After 2AA training, mice were subjected to three test sessions: Test
Days 1–3 (Fig. 2j–l). On Test Day 1 (light off), both control and
Halo-expressing mice displayed robust avoidance behavior (Fig. 2j,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. 2j,k). This effect was accompanied by a signif-
icant increase in the latency to avoid and an increase in CS-evoked
freezing behavior (Fig. 2k,l). These parameters partially recovered
on Test Day 3 (light off) (Fig. 2j–l). Notably, restricting optogenetic
inhibition of pPVTD2R neurons to the inter-trial interval (ITI) did
not significantly alter behavior (Extended Data Fig. 4k–t). Similarly,
we did not observe any light stimulation-induced effect on locomo-
ton in an open field arena (Extended Data Fig. 4i). In summary,
these findings show that, at the population level, pPVTD2R
neurons are required for active avoidance behavior and are inhibited
during freezing.

**pPVTD2R neurons signal active avoidance.** The emergence of a posi-
tive correlation between the calcium signal of pPVTD2R neurons and
movement after fear conditioning might reflect a link between the
activity of this neuronal population and the selection of active cop-
sembling during fearful situations. To formally assess this possibil-
ity, after expressing GCaMP6s in pPVTD2R neurons of Drl2-Cre
mice (Fig. 2a), we trained mice in a two-way signaled active avoid-
ance (2AA) task where performing a specific action—moving to
the neighboring compartment of a shuttle box—upon presenta-
tion of the CS, enables mice to avoid a footshock (US) (Fig. 2b).
Mice that are well-trained on this task typically forgo passive (freez-
ing) responses to CS presentation in favor of active (avoidance)
responses (Fig. 2c and Extended Data Fig. 3). Nevertheless, on
a trial-by-trial basis, they do engage both freezing and avoid-
ance behavior upon CS presentation (Extended Data Fig. 3c,d).
As such, this task provides an opportunity for assessing neuronal
activity while animals transition between passive and instrumen-
tal 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 task (Fig. 2c and
Extended Data Fig. 3a). However, additional training led to progres-
sive decreases in CS-evoked freezing and a concomitant increase
in active avoidance responses (Fig. 2c and Extended Data Fig. 3a).
After three sessions of 2AA training (Days 1–3), pPVTD2R neurons
were imaged using fiber photometry during two additional training
sessions (Days 4 and 5) (Fig. 2c). 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
Video 1). The data for avoidance and failure trials are summarized in
Fig. 2 and Extended Data Fig. 3 (Fig. 2d–h and Extended Data
Fig. 3b–d). Classifying trials in this manner revealed important dif-
fences in the activity of pPVTD2R neurons during avoidance and
failure trials (Supplementary Video 1). First, CS presentation elic-
it robust increases in GCaMP6s fluorescence in pPVTD2R neurons
during avoidance trials, whereas, in failure trials, it mostly induced
strong decreases in the activity of these neurons (Fig. 2g). In addi-
tion, presentation of the US during failure trials was associated with
robust activation of pPVTD2R neurons, consistent with our fear
conditioning data as well as with previous reports (Fig. 2d,e)28.

**pPVT–NAc projections drive avoidance and antagonize freezing.**
Although most pPVTD2R neurons project to the NAc (Fig. 1d), it is
unclear whether pPVTD2R–NAc projections are modulated by passive
and/or active responding in the 2AA task. To address this question,
we expressed the genetically encoded calcium indicator GCaMP7s
in pPVTD2R neurons and implanted an optical fiber unilaterally in
the NAc to measure the activity of pPVTD2R–NAc projections using
fiber photometry (Fig. 3a–e). We trained GCaMP7s-expressing
mice in the 2AA task, classified individual test trials as either avoid-
ance or failure as described above and analyzed the correspond-
ing change in fluorescent signal associated with presentations of
the CS (Fig. 3f–k). To ensure sufficient numbers of avoidance and
failure trials during imaging sessions, mice were trained for only 3
days before imaging started (Fig. 3d, e). These analyses revealed
that pPVTD2R–NAc projections were rapidly engaged after CS presenta-
tion and were robustly activated during both avoidance and escape
episodes irrespective of trial type (Fig. 3k). In contrast, CS-evoked
freezing was associated with a reduction in GCaMP7s fluores-
cence in this projection (Fig. 3k). These calcium dynamics largely
resembled those observed in pPVTD2R neurons (Fig. 2d–h
and Extended Data Fig. 3b). Together, these findings demonstrate

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pPVTD2R–NAc projections signal active avoidance and are inhibited during freezing behavior.

It is important to highlight that, unlike calcium responses recorded from the cell bodies of pPVTD2R neurons, for which increases were readily observed during shuttling events (Fig. 2d), increases in GCaMP7 fluorescence in pPVTD2R–NAc terminals appeared to emerge mostly at the conclusion of avoidance behavior (CS offset) (Fig. 3f*g). One potential explanation to this finding is that calcium transients accompanying avoidance behavior could result in weaker fluorescent changes at pPVTD2R–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 pPVVT (Fig. 4a*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 pPVVT–NAc neurons was performed during two additional sessions (Fig. 4c*d). Notably, our results largely...

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**Fig. 2 | pPVTD2R neurons control active avoidance and are inhibited during freezing behavior.**

- **a.** Representative image of GCaMP6s expression and fiber placements (n=5 mice).
- **b.** Schematic of the 2AA task.
- **c.** Behavior performance during 2AA.
- **d.** Representative imaging traces for avoidance and failure trials.
- **e.** Top, heat maps of calcium responses. Bottom, average calcium signal and CS (black line).
- **f.** Freezing, Peak and Mean Signal during the CS for each trial type. Avoidance, n=118 Trials; Failure, n=122 Trials; two-tailed Student’s t-test. Freezing, ***P = 2.04 x 10–6; Peak Signal, ***P = 1.19 x 10–10; Mean Signal, ***P = 2.78 x 10–15.
- **g.** Quantification of calcium signal for data in e. AUC, one-way ANOVA followed by two-stage step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance: n=118 Trials; F2.30 = 75.89; group comparisons, pre-CS versus CS ***P < 0.001, pre-CS versus post-CS ***P = 0.038, CS versus post-CS ***P < 0.001. Failure: n=122 Trials; F2.30 = 66.54. ***P < 0.001. **h.** Left, average responses for different events. Right, quantification of signal for each event on the left. 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 for the silencing of pPVTD2R neurons.
- **j-I.** Avoidance rate (j), latency to avoid (k) and freezing during the CS (l). Behavior data normalized to Day 1 are included. 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: F2.30 = 5.48; group comparisons, Halo, Day 1 versus Day 2 ***P < 0.001, Day 1 versus Day 3 P = 0.06. Latency to avoid: F2.30 = 2.48; group comparisons, Halo, Day 1 versus Day 2 **P = 0.0037. CS freezing: F2.30 = 0.19; group comparisons, Halo, Day 1 versus Day 2 *P = 0.04. All data in the figure are shown as mean ± s.e.m.
resembled those obtained from pPVTD2R neurons (Fig. 4e–j). In particular, we noticed that, although pPVTD2R–NAc neurons were strongly recruited during avoidance trials, they were mostly suppressed during failures (Fig. 4i). Moreover, pPVTD2R–NAc neurons signaled active behavioral responses and were, instead, inhibited during freezing (Fig. 4j and Supplementary Video 2). Together, these findings demonstrate that pPVTD2R projections to the NAc predominantly signal active defensive responses. Next, to investigate whether activation of pPVTD2R–NAc projections is critical for active avoidance, we optogenetically inhibited pPVTD2R–NAc projections bilaterally using Halo in a cohort of mice displaying robust avoidance behavior after training (>70% initial avoidance rate; Methods and Fig. 5a–c). Similarly to the experiment described above, after 2AA training (Fig. 5d–g) 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 pPVTD2R–NAc communication during CS presentations. Unlike in control subjects, light stimulation in Halo-expressing mice significantly decreased avoidance rate and increased the latency to avoid (Fig. 5h,i). In addition, these mice showed a concomitant increase in CS-evoked freezing on Test Day 2 (Fig. 5j,k). Notably, pairwise comparison of light-evoked changes in freezing and avoidance behavior across test sessions revealed an inverse relationship between these two parameters (Fig. 5l). Together, these results demonstrate that silencing pPVTD2R–NAc communication biases defensive behavioral responses toward passive 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. 5a–k). Moreover, our observation that optogenetic inhibition of pPVTD2R–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. Finally, restricting optogenetic inhibition of pPVTD2R–NAc projections to the ITI did not significantly alter behavior (Extended Data Fig. 5l–o). Similarly, as with pPVTD2R neurons, we did not observe any light stimulation-induced effect on locomotion (Extended Data Fig. 5p).

**pPVTD2R–CeA projections signal failure to avoid.** As described above, pPVTD2R neurons that project to the NAc are mostly distinct from those that project to the CeA (Fig. 1). However, these anatomical differences do not necessarily imply that functional distinctions exist between the two efferent pathways. Thus, to investigate how pPVTD2R–CeA projections are modulated in the 2AA task, we expressed GCaMP7s in pPVTD2R neurons of Drd2-Cre mice and implanted an optical fiber unilaterally in the CeA to measure the activity of pPVTD2R–CeA projections with fiber photometry (Fig. 6a–d).
These experiments showed that, unlike pPVTD2R–NAc terminals, pPVTD2R–CeA projections are not positively modulated by avoidance (Fig. 6c–j). Surprisingly, CS-evoked freezing behavior was not associated with significant changes in GCaMP7s fluorescence (Fig. 6i). In contrast, pPVTD2R–CeA neurons were predominantly active during failure trials (Fig. 6g,j), suggesting a link between engagement of pPVTD2R–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—over instrumental defensive strategies, increases in active avoidance and failure (Extended Data Fig. 6l–o). Together, these results suggest that silencing pPVT–CeA communication biases behavior away from passive defensive behavior and in favor of active ones. Similarly, we did not observe any light stimulation-induced effect on locomotion (Extended Data Fig. 6p).

Of note, 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 (Methods, Fig. 7 and Extended Data Fig. 7). Consistent with previous manipulations, the increase in avoidance behavior was accompanied by a reduction in the latency to avoid and a reduction in CS-evoked freezing (Fig. 7h,i). Moreover, pairwise comparisons revealed that, consistent with the idea that in the 2AA task animals switch between 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. 7c). 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
**Fig. 5** | 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, Fiber placements (n = 8 mice per group). d–g, Avoidance rate (d), latency to avoid (e), freezing time during the CS (f) and ITI freezing (g) across all training sessions for both Ctl and Halo groups. h–k, Left, Optogenetic inhibition of pPVTD2R–NAc axon terminals persistently decreases avoidance rate (h), whereas increases latency to avoid (i) and freezing time during the CS (j), but has little effect on freezing time during the the ITI (k). Right, normalization to Day 1 for each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: F2,28 = 18.04; group comparisons, Halo, Day 1 versus Day 2 ***P < 0.001, Day 1 versus Day 3 ***P < 0.001. Latency to avoid: F2,28 = 5.63; group comparisons, Ctl, Day 1 versus Day 3 *P = 0.028; Halo, Day 1 versus Day 2 ***P < 0.001, Day 1 versus Day 3 ***P < 0.001. CS freezing: F2,28 = 1.45; group comparisons, Halo, Day 1 versus Day 2 *P = 0.049, Day 1 versus Day 3 **P = 0.0092. ITI freezing: F2,28 = 0.149. I, Linear regression of the changes in freezing behavior across test sessions as a function of changes in avoidance behavior. All data in the figure are shown as mean ± s.e.m.
behavior toward 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 neurons and implanted optical fibers bilaterally over the NAc or the CeA. Consistent with our model, we observed that, although optogenetic stimulation of pPVT–NAc terminals potentiated avoidance behavior, stimulation of pPVT–CeA projections reduced it (Extended Data Fig. 8). These results lend further support to our conclusion that divergent projections of the pPVT mediate the selection of opposing defensive behaviors.

pPVT flexibly control the selection of defensive behaviors. A recurrent theme in our reported observations is the idea that the pPVT projections to the NAc and the CeA can bias the selection of passive and active 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 test sessions (Fig. 8 and Extended Data Fig. 9). Notably, we found that, although silencing pPVT–NAc projections impaired avoidance behavior (consistent with the above results), inhibition of pPVT–CeA terminals the next day fully restored active avoidance (Fig. 8e,f). Interestingly, an opposing but similarly dynamic modulation of freezing behavior was observed across sessions (Fig. 8g and Extended Data Fig. 9d).

In conclusion, these data demonstrate that the pPVT can flexibly bias defensive behaviors via projections to NAc and CeA. Future studies should aim at identifying the local circuit dynamics and upstream mechanisms that guide or determine which type of defensive response is selected. As a start, using monosynaptic rabies tracing, we identified regions of the mouse brain that preferentially innervate pPVT–CeA or pPVT–NAc neurons (Extended Data Fig. 10).

**Discussion**

In this study, we identified the pPVT as a key brain structure that mediates the selection of competing defensive strategies, namely Pavlovian freezing 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. Notably, 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 passive defensive responses.

Recent literature shows that the pPVT is predominantly sensitive to aversive stimuli and support the notion that the PVT is critical for the orchestration of adaptive behavioral responses.
adaptive responses that allow animals to cope with ongoing demands. 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 behaviors. Importantly, some of these studies have directly implicated NAc projections of the pPVT as guiding instrumental behaviors during aversive states. In light of these recent reports, our finding that the pPVT–NAc pathway also regulates instrumental defensive behaviors suggests that these projections might 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. Accordingly, it was recently proposed that pPVT–CeA

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**Fig. 7** | Optogenetic inhibition pPVT<sup>Drd2</sup>–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<sup>Drd2</sup>–CeA axon terminals. **b**, Representative images from a mouse expressing Halo-mCherry in pPVT<sup>Drd2</sup> neurons and implanted with optical fibers in the CeA. **c**, Fiber placements (<i>n</i> = 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 and Halo groups. **g–i**, Top, avoidance rate (**g**), latency to avoid (**h**) and freezing time during the CS (**i**) during optogenetic inhibition of pPVT<sup>Drd2</sup>–CeA axon terminals. Bottom, normalization to Day 1 for each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: <i>F</i><sub>6,80</sub> = 9.05; group comparisons, Halo, Day 1 versus Day 4 ***<i>P</i> < 0.001, Day 1 versus Day 5 ***<i>P</i> < 0.001, Day 1 versus Day 6 ***<i>P</i> < 0.001, Day 2 versus Day 4 *<i>P</i> = 0.015, Day 2 versus Day 5 **<i>P</i> < 0.001, Day 2 versus Day 6 ***<i>P</i> < 0.001, Day 3 versus Day 4 *<i>P</i> = 0.015, Day 3 versus Day 5 ***<i>P</i> = 0.0005, Day 3 versus Day 6 ***<i>P</i> < 0.001, Day 4 versus Day 6 ***<i>P</i> < 0.001, Day 5 versus Day 6 ***<i>P</i> = 0.012. Latency to avoid: <i>F</i><sub>6,80</sub> = 4.52; group comparison, Halo, Day 1 versus Day 4 *<i>P</i> = 0.011, Day 1 versus Day 5 ***<i>P</i> < 0.001, Day 1 versus Day 6 ***<i>P</i> < 0.001, Day 2 versus Day 5 **<i>P</i> < 0.001, Day 2 versus Day 6 ***<i>P</i> < 0.001, Day 3 versus Day 4 *<i>P</i> = 0.015, Day 3 versus Day 5 ***<i>P</i> = 0.0005, Day 3 versus Day 6 ***<i>P</i> < 0.001, Day 4 versus Day 6 ***<i>P</i> < 0.001, Day 5 versus Day 6 ***<i>P</i> = 0.012, Day 4 versus Day 6 ***<i>P</i> = 0.007, Day 5 versus Day 6 *<i>P</i> = 0.018. All data in the figure are shown as mean ± s.e.m.
projections drive morphine-induced conditioned place preference likely through Pavlovian incentive motivation.

The PVT 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. Particularly relevant to the present study is the notion that the BLA’s contribution to conditioned passive 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 passive and active defensive behaviors decreases over time. As such, the PVT might 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 might 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 conflicts. 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 diverse. Indeed, we recently identified two major classes of PVT neurons that can be distinguished on the basis of genetic, anatomic (connectional) and functional differences. Specifically, it was suggested that PVT neuronal subtypes could be identified by 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. Our results expand on this recent report by showing further anatomical and functional heterogeneity among aversive-sensitive pPVT neurons. It is important to note that non-D2R-expressing PVT neurons also project to the NAc and the amygdala. However, the contributions of these parallel projections (and how they differ to those of D2R-expressing neurons) to defensive behaviors remain unaddressed. Our findings should encourage future studies to classify...
PVT neurons based on the intersection of genetic markers and projection target, an approach that has led to the discovery of functional segregation in other brain areas. In addition, implementing imaging techniques with cellular resolution should facilitate the identification of functionally distinct PVT cell types that might have remained undetected in our experiments using bulk imaging approaches from projection-defined neurons.

The observation that optogenetic inhibition of pPVT–NAc projections leads to lasting reductions 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 retrieval. 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 odds with this interpretation. Specifically, we found that, although silencing of pPVT–NAc projections reduced active avoidance, it led to a concomitant increase 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 was to uncover the neural circuit mechanisms by which animals switch between two opposing defensive behaviors: freezing and active avoidance. In nature, animals typically engage in these different defensive behaviors as a function of threat imminence. From this perspective, active avoidance is thought to have evolved as a way to deal with temporally and spatially distant threats (pre-encounter), whereas freezing behavior and other reactive responses, such as flight, mostly emerge during highly imminent threats (post-encounter and circa-strike). Within this framework, the idea that the 2AA task yields a subset of animals that fail to engage in avoidance behavior (low avoiders) might seem to suggest that this task is not suitable for studying ethologically relevant defensive strategies. However, a potential explanation for the variability in behavioral outcome observed with the current task, particularly the low avoider phenotype, could be that, due to the short duration of the CS (15 s), some animals view the threat (US) as highly imminent 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 avoiders. This study underscores the notion that parameters of the 2AA task (including CS and ITI duration) can be optimized to mimic naturalistic differences in threat imminence. In the current study, we exploited individual differences in animal performance that are harnessed by standard 2AA protocols to obtain information about the mechanisms that mediate switches in defensive behavior strategy. We predict that the circuit mechanisms described in our study for biasing the selection of opposing defensive behaviors mirror those engaged when animals switch defensive strategies as a function of threat imminence. Developing ethologically relevant behavioral models in which animals engage in both pre-encounter (active) and post-encounter reactive defensive modes (for example, freezing) with varying degrees of threat imminence would allow for further exploring the neurobiology of defensive behavior selection. Of note, ethologically relevant behavioral tasks have been recently developed to assess transitions between post-encounter and circa-strike elicited defense.

Online content
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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 maintained in a colony housed under a 12:12 h light/dark cycle (6:00–18:00 h), at temperatures of 70–74°F and 40–65% humidity, with food and water available ad libitum. Before surgery, mice were singly housed. Dr2-Cre mice were obtained from GenSAT (founder line ER44). In addition, we used C57BL/6J 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.

Fiber photometry. Mice were allowed to habituate to the fiber patch cord in their home cage for approximately 5 min before each behavior test. GCaMP fluorescence and movement and bleaching artifacts. Signal data were de-trended by first applying a least-squares linear fit to produce \( F_{ \text{raw}} \) and \( \text{dI}/\text{dF} \) was calculated as:

\[
(\text{dI}/\text{dF})_{\text{baseline}} = \frac{F_{\text{max}} - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} 
\]

All GCaMP signal data are presented as the \( z \)-score of the \( \text{dI}/\text{dF} \) from baseline (pre-CS) segments.

Data analysis for 2AA. We performed post hoc position tracking of the animal’s nose and body center from video in the 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 scripts in the R statistical computing environment (R Core Team 2019, R Foundation).

Missing positions up to ten successive frames were linearly interpolated with custom routines in R. 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. 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 0.2–0.5 mm. By subtracting this value from the quantile value, a movement threshold—that is, 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 analysis (R package version 2.2.2 [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 ≥2 s. A non-movement bout of ≥2 s was 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) that occurred during the CS as CS freezing and those that occurred during ITI as ITI freezing. For each trial, we calculated the time interval between the moment of movement crossing the hurdle and the CS onset, named latency to avoid. CS, ITI freezing or latency to avoid were averaged within session and then within each group and plotted as mean ± s.e.m.

For fiber photometry, GCaMP data were normalized as ΔF/ΔF₀. Next, we used the behavioral flags calculated from the video tracking to create average peri-event time histograms (PETHs) time-locked to the onset of the behavior events of interest, including CS onset (CS), highest movement velocity during the CS (Max. Velocity), escape or avoidance movement onset (Escape Initiate), escape or avoidance moment (Escape) and freezing onset during the CS (Freezing). All trials in each session were separated into avoidance and failure trials as described above. For each trial type, the z-score from 10 s before to 30 s after CS onset was plotted in heat maps for all trials in test sessions. The mean of all recorded activity for each trial type was plotted below the corresponding heat map. We isolated the calcium signal from 2 s before to 2 s after the onset of each behavior event from avoidance and failure trials separately. Using the z-score from 1 s before onset as a baseline for each event, we calculated z-scores and area under the curve (AUC) of the z-score from 2 s before to 2 s after the onset of each behavior event. Lastly, we plotted the mean of signal transitions and AUC for each event type from trial type. All 2A2 photometric signals and behavioral performance were analyzed blinded.

Optogenetic inhibition of pPVT neurons and projections in an open field arena. After habituating to the fiber patch cord in the home cage for 10 min, mice were placed in a square enclosure (50 cm × 50 cm × 30 cm) to roam freely for 6 min. Mice received 2 min of light stimulation 2 min after the beginning of the test (min 2–4). The light intensity at the interface between the fiber tip and the mouse was ~10 mW. The cumulative distance traveled in the open field apparatus was recorded, tracked and quantified using ANY-maze behavioral tracking software.

Monosynaptic tracing of inputs to NAc-projecting or CeA-projecting neurons of the PVT. To limit monosynaptic rabies tracing to NAc-projecting or CeA-projecting neurons of the PVT, AAV2 (retro)-CAG-G-Cre was bilaterally injected into the NAc (0.5 μl per injection) or CeA (0.3 μl per injection) of C57BL/6NJ mice. Within the same surgical procedure, a virus mixture of AAV9-EF1a-FLEX-TVA-mCherry and AAV9-CAG-FLEX-RG at a 1:1 ratio was injected into the pPVT (1.5 μl), followed by an injection of the pseudotyped rabies virus EnvA-SAD-G-D6-GFP (1.5 μl) in the same location of pPVT 2 weeks later. Mice were killed and subjected to analysis 1 week later. Brain sections were scanned by an Axiostar (Carl Zeiss) with a 0 μm objective. Cells were detected and quantified on Neurolino (MBF Bioscience). Starter cell center was generated by quantifying number of mCherry and GFP double-positive pPV T cells. 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 pPV T (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 blinded experimenter.

Histology and immunofluorescence. Animals were deeply anesthetized with euthanasia solution (Vertone) and transcardially perfused with PBS (pH 7.4, 4°C), followed by paraformaldehyde (PFA) solution (4% in PBS, 4°C). After extraction, brains were post-fixed in 4% PFA at 4°C for a minimum of 24 h and subsequently cryoprotected by transferring to a 30% PBS-buffered sucrose solution until brain tissues 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 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, rabbit, Frontier Institute, D2RRb-A960). After washing, Alexa Fluor 488-conjugated secondary antibodies (1:500, goat anti-mouse, Molecular Probes A-11001). Finally, sections were subsequently mounted onto glass slides for imaging (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 Figs 2a, 3c, 4b, 5c, 6b and 7c and Extended Data Figs 1a, 4b,j, 5b, 6b, 8m and 9a.

Statistics and reproducibility. All data were plotted and analyzed with OriginPro version 2016 and version 2018 (OriginLab) and GraphPad Prism (version 8.0.1, GraphPad Software). All data are presented as mean ± 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; 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; P < 0.05 was considered significant. The sample sizes used in our study, such as the numbers of animals, are typically the same or exceed those estimated by power analysis (power = 0.80, α = 0.05). 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 4–13 mice. All experiments were replicated at least once, and similar results were obtained. All experiments were randomized, and investigators were blinded to allocation during experiments. Data distribution was assumed to be normal, but this was not formally tested.

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

Data availability. All the data that support the findings presented in this study are available from the corresponding author upon reasonable request. Source data are provided with this paper and are publicly available at the following repository: https://github.com/Penzolab/Source-Data-07092021.git.

Code availability. R code used to analyze active avoidance behavior and photometric signal is available at the following repository: https://github.com/Penzolab/Data-analysis-of-Two-way-active-avoidance-task.git.

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

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Correspondence and requests for materials should be addressed to M.A.P.

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Extended Data Fig. 1 | pPV'T2R neuron activity during fear conditioning and retrieval. a, Representative image of GCaMP6s expression in pPV'T2R neurons and optical fiber placements (n = 6 mice). b, Experimental paradigm. c–e, 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. i–k, Quantification of calcium signal during 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. Conditioning: n = 30 trials; F(2, 87) = 2.17. Retrieval: n = 48 trials; F(2, 141) = 7.7; group comparisons, pre-CS vs CS ***P = 0.0006, 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. m, Quantification of calcium signal during the first 5 s following the onset of CS during conditioning sessions. 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 3 s **P = 0.0043, 1 s vs 4 s ***P < 0.0003, 1 s vs 5 s **P < 0.001, 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. Bottom: Average calcium responses of the top panels. All data in figure shown as mean ± s.e.m.
Extended Data Fig. 2 | The activity of pPVTD2 neurons is positively correlated with movement during the CS following fear memory retrieval.

a, Calcium responses of individual retrieval trials aligned by percentage CS freezing (See Methods) (right). n = 6 mice, 8 trials per mouse. b, Left: Linear regression of CS calcium signal and freezing percentage for each trial. Right: Average CS calcium signal for each group (L, n = 20 trials; M, n = 16 trials; H, n = 12 trials). AUC, F(2, 45) = 3.3, one-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Group comparisons, L vs H, *P = 0.013. c, Average calcium responses (top), average movement index (middle) and linear regression of average calcium signal and movement index during the CS (bottom) for each group. d, Comparison of calcium signal (left) and movement index (right) for each group (L, n = 20 trials; M, n = 16 trials; H, n = 12 trials). AUC, two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Calcium signal: F(4, 90) = 3.37; movement index: F(4, 90) = 3.6; for group comparisons ***P < 0.001, *P < 0.05. e-f, Average movement index (e) and linear regression of average calcium signal and movement index during the CS (f) for the habituation session. g, Individual subjects contributing to each group. h, Calcium responses of Trials 1-8 and average in bottom panels. i, Quantification of calcium signal during the Trials 1-8. AUC, One-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. n = 6; Trial 1, F(2, 10) = 1.36; Trial 2, F(2, 10) = 4.55; 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; Trial 8, F(2, 10) = 2.71; for group comparisons *P < 0.05. j, Linear regression of average calcium signal and movement index from Trials 1-8. All data in figure shown as mean ± s.e.m.
Extended Data Fig. 3 | The activity of pVT\textsuperscript{D2R} neurons in the 2AA task, related to Fig. 2.  

a, Latency to avoid and freezing time during the ITI across days (n = 5 mice).  

b, Left: Linear regression of peak calcium signal and freezing time during the CS for avoidance (blue; A; R\textsuperscript{2} = 0.069, P = 0.0043) and failure trials (red; F; R\textsuperscript{2} = 0.15, P < 0.001). Right: Linear regression of average calcium signal and freezing time during the CS for avoidance (blue; A; R\textsuperscript{2} = 0.17, P < 0.001) and failure trials (red; F; R\textsuperscript{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. All data in figure shown as mean ± s.e.m.
Extended Data Fig. 4 | Optogenetic inhibition of pPV^D2R neurons in the 2AA task. a, Representative image from a mouse expressing Halo-mCherry in pPV^D2R neurons and implanted with an optical fiber. b, 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 training days for each group. h, Left: Freezing time during the ITI. Right: Normalization to Day 1 for each group. ItI freezing in s, two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. F(2, 30) = 0.36, Non-significant. i, Linear regression of the changes in freezing behavior across test sessions as a function of changes in avoidance behavior. j, Moving distance in the open field. Ctl, n = 8 mice; Halo, n = 5 mice. k, Schematic of the viral vector strategy and optical fiber placement used for optogenetic silencing of pPV^D2R neurons in the 2AA task. l, 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 training days in both Ctl and Halo groups. q-t, Left: Effect of optogenetic inhibition of pPV^D2R neurons during the ITI on avoidance rate (q), the latency to avoid (r) and freezing time during the CS (s) and the ITI (t). Right: Normalization to Day 1 for 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; non-significant change among each group comparison. All data in figure shown as mean ± s.e.m.
Extended Data Fig. 5 | 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,** 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 training sessions for each group. **g–j,** Left: Avoidance rate (g), latency to avoid (h), freezing time during the CS (i) and ITI (j) during optogenetic inhibition of pPVt–NAc axon terminals. Right: Normalization to Day 1 for 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. Latency to avoid: F(2, 44) = 2.88; group comparisons, Halo, Day 1 vs Day 2 *P = 0.024. CS freezing: F(2, 44) = 1.1. ITI freezing: F(2, 44) = 0.46; non-significant change among other group comparison. **k,** Linear regression of the changes in freezing behavior across test sessions as a function of changes in avoidance behavior. **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 to Day 1 for each group. Ctl, n = 7 mice; Halo, n = 4 mice. 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, Halo, Day 2 vs Day 3 *P = 0.022; non-significant change among other group comparison. **p,** Moving distance in the open field. Ctl, n = 9 mice; Halo, n = 8 mice. All data in figure shown as mean ± s.e.m.
Extended Data Fig. 6 | 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, 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 training sessions for each group. g–j, Left: Avoidance rate (g), latency to avoid (h) and freezing time during the CS (i) and the ITI (j) of optogenetic inhibition of pPVt–CeA axon terminals. Right: Normalization to Day 1 for each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: F(2, 42) = 3.27. Latency to avoid: F(2, 42) = 5.35. CS freezing: F(2, 42) = 2.77. ITI freezing: F(2, 42) = 1.67. For group comparisons **P < 0.01, *P < 0.05. k, Linear regression of the changes in freezing behavior across test sessions as a function of changes in avoidance behavior. l–o, Left: Avoidance rate (l), the latency to avoid (m) and freezing time during the CS (n) and the ITI (o) during optogenetic inhibition of pPVt–CeA axon terminals during the ITI. Right: Normalization to Day 1 for each group. Ctl, n = 6 mice; Halo, n = 10 mice. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate, F(2, 28) = 0.0052; latency to avoid, F(2, 28) = 0.22; CS freezing, F(2, 28) = 0.61; ITI freezing, F(2, 28) = 0.086; non-significant change among each group comparison. p, Moving distance in the open field. Ctl, n = 8 mice; Halo, n = 9 mice. All data in figure shown as mean ± s.e.m.
Extended Data Fig. 7 | Optogenetic inhibition of pPV\textsuperscript{T2A}\_CeA axon terminals in the 2AA task, related to Fig. 7. **a**, Freezing time during the ITI across training days in both Ctl and Halo groups. n = 9 mice per group. **b**, Left: Optogenetic inhibition of pPV\textsuperscript{T2A}\_CeA axon terminals gradually reduces freezing time during the ITI. Right: Normalization to Day 1 for 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 \(* * * P = 0.0006\), 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 \(* P = 0.017\). **c**, Linear regression of the changes in freezing behavior between Test Day 1 and other test sessions as a function of changes in avoidance behavior. All data in figure shown as mean ± s.e.m.
Extended Data Fig. 8 | Optogenetic stimulation of pPVtD2R–NAc or pPVtD2R–CeA axon terminals in the 2AA task. a, Schematic of the viral vector strategy and optical fiber placement for optogenetic stimulation of pPVtD2R–NAc axon terminals in the 2AA task. b, 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 training sessions for each group. g–j, Top: Avoidance rate (g), latency to avoid (h) and freezing time during the CS (i) and the ITI (j) during optogenetic stimulation of pPVtD2R–NAc axon terminals. Bottom: Normalization to Day 1 for each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: F(2, 36) = 6.21. Latency to avoid: F(2, 36) = 3.34. CS freezing: F(2, 36) = 0.16; ITI freezing: F(2, 36) = 0.21. For group comparisons **P < 0.01, *P < 0.05. k, Linear regression of the changes in freezing behavior across test sessions as a function of changes in avoidance behavior. l, Schematic of the viral vector strategy and optical fiber placement for optogenetic stimulating of pPVtD2R–CeA axon terminals in the 2AA task. m, Fiber placements (Ctl, n = 7 mice; ChR, n = 10 mice). n–q, Avoidance rate (n), latency to avoid (o) and freezing time during the CS (p) and ITI (q) across training sessions for both groups. r–u, Top: Avoidance rate (r), latency to avoid (s) and freezing time during the CS (t) and the ITI (u) during optogenetic stimulation of pPVtD2R–CeA axon terminals. Bottom: Normalization to Day 1 for each group. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Avoidance rate: F(2, 30) = 5.43. Latency to avoid: F(2, 30) = 1.89. CS freezing: F(2, 30) = 0.88; ITI freezing: F(2, 30) = 0.66. For group comparisons **P < 0.01, *P < 0.05. v, Linear regression of the changes in freezing behavior across test sessions as a function of changes in avoidance behavior. All data in figure shown as mean ± s.e.m.
Extended Data Fig. 9 | Optogenetic inhibition of pPV'T2R–NAc or pPV'T2R–CeA axon terminals in the 2AA task, related to Fig. 8.  

**a**, Optical fiber placements (n = 8 mice per group).  
**b**, Freezing time during the ITI across training sessions for both Ctl and Halo groups.  
**c**, Left: Effect of optogenetic inhibition of pPV'T2R–NAc axon terminals (Day 2) and pPV'T2R–CeA axon terminals (Day 3) on ITI freezing. Right: Normalization to Day 1 for 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.  
**d**, Linear regression of the changes in freezing behavior across test sessions as a function of changes in avoidance behavior. All data in figure shown as mean ± s.e.m.
Extended Data Fig. 10 | 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).
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐  The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

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☐  A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

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☐  Give P values as exact values whenever suitable.

☐  For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐  For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐  Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  Fiber photometry data were obtained using Synapse version 92 (Tucker-Davis Technologies). Fear conditioning data were collected through FreezeFrame 4 (Coulbourn Instruments). 2-way signaled active avoidance behavior was controlled and acquired using ANY-maze version 5 (Stoelting).

Data analysis  All data were analyzed using Origin Pro version 2016 & version 2018 (OriginLab Corp.) and GraphPad Prism version 8.0.1 (GraphPad Software). Image analysis and cell counting were performed using ImageJ software (Fiji, version 1.52p). TopScan software (CleverSys) was used for video tracking of 2-way signaled active avoidance behavioral data. R Studio was used to analyze photometric signals. We used the R package version 2.2.2 (URL:https://CRAN.R-project.org/package=changepoint). R code used to analyze active avoidance behavior and photometric signal is available at the following repository: https://github.com/Penzolab/Data-analysis-of-Two-way-active-avoidance-task.git.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All the data that support the findings presented in this study are available from the corresponding author upon reasonable request. Source Data are provided with this paper, and are publicly available at the following repository: https://github.com/Penzolab/Source-Data-07092021.git.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
The sample sizes used in our study, such as the numbers of animals, are typically the same or exceed those estimated by power analysis (power = 0.80, \( \alpha = 0.05 \)).

**Data exclusions**
Mice without correct targeting of optical fibers, tracers or vectors were excluded from this study. For monosynaptic rabies tracing experiments, 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.

**Replication**
All experiments were replicated at least once, and similar results were obtained.

**Randomization**
Animals were randomly allocated to the different experimental conditions reported in this study.

**Blinding**
Investigators were blinded to allocation during experiments. All 2AA photometric signals and behavioral performance were analyzed blind. All monosynaptic rabies tracing data were analyzed by a blind experimenter.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

**Materials & experimental systems**

- n/a
- [x] Antibodies
- [x] Eukaryotic cell lines
- [x] Palaeontology and archaeology
- [x] Animals and other organisms
- [x] Human research participants
- [x] Clinical data
- [x] Dual use research of concern

**Methods**

- n/a
- [x] ChIP-seq
- [x] Flow cytometry
- [x] MRI-based neuroimaging

Antibodies

**Antibodies used**

- anti-D2R (1:300, rabbit, Frontier Institute, D2R-Rb-Af960); Alexa-Fluor-488 conjugated secondary antibody (1:500, goat anti-mouse, Molecular Probes, A-11001).

**Validation**

The anti-D2R antibody was validated for both species and application by the producer (Frontier Institute). Antibody specificity was checked by the detection of single protein bands by immunoblot and aborted immunohistochemical signal by using antigens to pre-absorb antibodies.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**

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. Mice used in this study were group housed under a 12-h light-dark cycle (6 a.m. to 6 p.m. light), at temperature of 70–74°F and 40–65% humidity, with food and water available ad libitum.

**Wild animals**

No wild animals were used in this study.

**Field-collected samples**

No field-collected samples were used in this study.
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.

Note that full information on the approval of the study protocol must also be provided in the manuscript.