Neuroscience

Tachykinin1-expressing neurons in the parasubthalamic nucleus control active avoidance learning

Reviewed Preprint
v1 • December 18, 2024
Not revised

Ruining Hu, Nannan Wu, Tong Liu, Liuting Zou, Songjie Lv, Xiao Huang, Rongfeng K Hu

Department of Psychological Medicine, Zhongshan Hospital, Institute for Translational Brain Research, State Key Laboratory of Medical Neurobiology, MOE Frontiers Center for Brain Science, MOE Innovative Center for New Drug Development of Immune Inflammatory Diseases, Fudan University, Shanghai, China • Institute for Translational Brain Research, Fudan University, Shanghai, China • Department of Psychological Medicine, Zhongshan Hospital, Fudan University, Shanghai, China

https://en.wikipedia.org/wiki/Open_access
Copyright information

eLife Assessment

This **useful** work identifies a key role for Tachykinin-1 parasubthalamic neurons in avoidance learning. At present, the evidence for the conclusions regarding fiber photometry, viral transfection, reporting of behavioral outcomes, and pathway-specificity is **incomplete**. This work will be of interest to neuroscientists studying neural mechanisms for avoidance and aversion.

https://doi.org/10.7554/eLife.104643.1.sa4

Abstract

Summary

Active avoidance is a type of instrumental behavior that requires an organism actively to engage in specific actions to avoid or escape from a potentially aversive stimulus and is crucial for the survival and well-being of organisms. It requires a widely distributed, hard-wired neural circuits spanning multiple brain regions, including the amygdala and thalamus. However, less is known about whether and how the hypothalamus encodes and controls active avoidance learning. Here we identify a previously unknown role for the parasubthalamic nucleus (PSTN), located in the lateral subdivision of the posterior hypothalamus, in the encoding and control of active avoidance learning. Fiber photometry calcium imaging shows that the activity of tachykinin1-expressing PSTN ($PSTN^{Tac1}$) neurons progressively increases during this learning. Cell-type specific ablation and optogenetic inhibition of $PSTN^{Tac1}$ neurons attenuates active avoidance learning, whereas optogenetic activation of these cells promotes this learning via a negative motivational drive. Moreover, the PSTN mediates this learning differentially through its downstream targets. Together, this study identifies the PSTN as a new member of the neural networks involved in active avoidance learning and offers us potential implications for therapeutic interventions targeting anxiety disorders and other conditions involving maladaptive avoidance learning.

Introduction

Aversive learning is a type of learning that occurs when a neutral stimulus becomes associated with a negative or aversive outcome[1–5] and plays a vital role for the survival of animals across various species[1–4,6]. It involves two fundamental types of conditioning: Pavlovian conditioning and instrumental conditioning[1–4]. The underlying neuronal circuits associated with aversive learning are complex and have been extensively studied in the context of fear conditioning, particularly through Pavlovian conditioning paradigms[7,8]. However, the neural mechanisms underlying instrumental defensive behaviors, such as active avoidance, are not completely understood. Indeed, several interconnected brain regions, such as the paraventricular thalamic nucleus (PVT)[9], the lateral habenula (LHb)[10], the amygdala[11–14], the prefrontal cortex (PFC)[6,15] and the periaqueductal gray (PAG)[16], have been recently identified to involve active avoidance behavior, greatly expanding our understanding of the neural circuit mechanisms that underlie this behavior. In contrast, both traditional lesion and pharmacological experiments have found that the hypothalamus plays essential roles in regulating active avoidance[17–21], but less is known about what hypothalamic neuronal types and their projections are involved in the regulation of this behavior and how these neurons and circuits are dynamically modulated by learning.

The parasubthalamic nucleus (PSTN), located in the lateral subdivision of the posterior hypothalamus[22,23], is composed of mostly glutamatergic neurons, some of which express Tachykinin 1(*Tac1*), adenylate cyclase-activating polypeptide (*Adcyap1*) as well as corticotropin-releasing factor (*Crh*). Based on recordings and manipulations of these genetically labeled subpopulations of the PSTN, this brain region has recently been implicated in the regulation of feeding behaviors[24–27], appetite suppression[28,29], alcohol consumption[30–33], arousal[34], and fear-associated behaviors[26,35,36]. In addition, the PSTN has been found to promote conditioned taste aversion[22,37,38] and place avoidance behaviors[26,28,35,36]. For instance, optogenetic activation of glutamatergic neurons or PACAP-expressing neurons or Tac1-expressing neurons in the PSTN promotes real-time place aversion[26,28,39,40], suggesting that activation of the PSTN could generate a negative emotional state. Moreover, previous work has demonstrated that the PSTN sends outputs to the PVT[29,41], an important brain node implicated in active avoidance learning [9,42], a process that can be prompted by negative motivational drives and be modified through experience [3,4]. These results led us to hypothesize that the PSTN might be an attractive candidate brain node for the regulation of active avoidance learning. However, to date, no study has been directly examined the exact functional role of and *in vivo* neuronal responses of the PSTN as well as its associated circuits in active avoidance learning.

Results

Neural responses of PSTN^{Tac1} neurons during active avoidance learning

Based on recent studies supporting that *Tac1* gene represents the most useful genetic marker to label a subpopulation of PSTN glutamatergic neurons [22,24,27,29,30,32,33,39], we thus selected the Tac1-Cre transgenic mouse line for subsequent experiments. In this mouse line, Cre expression is under the control of the promoter for Tachykinin1(*Tac1*), allowing us to specially monitor and manipulate Tac1-labeled neurons' activity. We first used fiber photometry, an approach that combines fiber optics and photometry to measure bulk neural dynamics in live animals[43], to monitor the response of Tac1-expressing cells in the PSTN (PSTN^{Tac1}) from male

mice by direct foot shock. To achieve this goal, we unilaterally injected AAV expressing Cre-dependent fluorescent calcium indicator GCaMP7f in the PSTN of Tac1-IRES-Cre mice and in the same surgery installed a fiber optic above the viral injection site (**Figure 1A**). We observed a significant fluorescence increase in GCaMP7f-expressing mice rather than GFP-expressing controls while undergoing foot shock (Figure S1A and B). Post hoc analysis suggested that GCaMP7f expression was largely restricted within the PSTN (**Figure 1B**).

Next, to examine the *in vivo* neuronal responses of the PSTN in active avoidance learning, we used a modified two-way active avoidance learning task (2AA; **Figure 1C and D**), based on previous work[9]. In our study, the 2AA task included a 7-day training procedure (100 trials/day) (**Figure 1C**). During this experiment, a test mouse experienced a tone (Conditioned stimulus, CS), and must shuttle to the other side of chamber in a given response window to prevent an aversive foot shock (Unconditioned stimulus, US) (**Figure 1D**). The performance of active avoidance learning was calculated by the percentage of cue-guided successful shuttles over total trials (Avoidance rate) and the duration from trial onset to crossing compartments (Latency). Over the course of training, mice significantly increased their successful shuttling rates to avoid foot shock occurrence and decreased the latency to cross compartments (**Figure 1E**). Remarkably, the avoidance rate could reach and stay up to 70% across day 2 - day 7 (**Figures 1E** and S1H), suggesting that our modified 2AA training system is highly effective to being applied to investigate active avoidance learning. To further examine the avoidance performance on day 1, we sequentially and averagely divided 100-trial training into five sessions. We found that animals progressively improve their shuttling performance spinning from 22.5±5.7% to 70.0±8.3% (**Figure 1F**). Moreover, on day 7, mice could exhibit a stable behavioral performance (>70%) across sessions (**Figure 1G**).

Based on animals' behavioral performance (**Figure 1E-G**), we then focused on analyzing calcium fluorescent transients on days 1 and 7. On day 1, we observed a significant increase in CS-evoked calcium fluorescent signals in late trials relative to those in early trials (**Figure 1H-K**), strongly indicating that active avoidance learning is capable of reshaping the activity of these cells. Next, to examine the neuronal dynamics for both avoidance trials and failure trials from well-trained mice on day 7, we pooled all those calcium imaging data and analyzed the response patterns for avoidance trials and failure trials, respectively. We observed a significant increase in CS-evoked calcium fluorescent signals in avoidance trials compared to those in failure trials (**Figure 1L-O**), indicating that well-learned mice apply a higher CS-evoked response in guiding avoidance behavior. In contrast, although our control mice also exhibited a considerable behavioral performance in 2AA task (Figure S1C), they had no bulk changes in fluorescence throughout the training (**Figure 1D-G**). Moreover, consistent with the behavioral performance during 2AA (**Figures 1E** and S1H, I), a very similar neuronal response pattern for those GCaMP-expressing male mice was observed on days 2-7 (Figure S1H-J). Finally, we also examined the response patterns of $PSTN^{Tac1}$ neurons of female mice during 2AA. We analyzed behavioral performance and neuronal dynamics in female GCaMP-expressing mice on day 1, and found similar neuronal response patterns between early trials and late trials, compared to those in male mice, regardless of the behavioral performance (Figure S2). These data illustrate that the activity of $PSTN^{Tac1}$ neurons in both sexes of mice can be shaped in a similar manner on the early stage of 2AA task.

Together, our study modified a new 2AA training system that can effectively and quickly evaluate the animals' avoidance performance. Using this system, we provided the first *in vivo* calcium response evidence for modulation of $PSTN^{Tac1}$ neurons' activity by learning in both sexes.

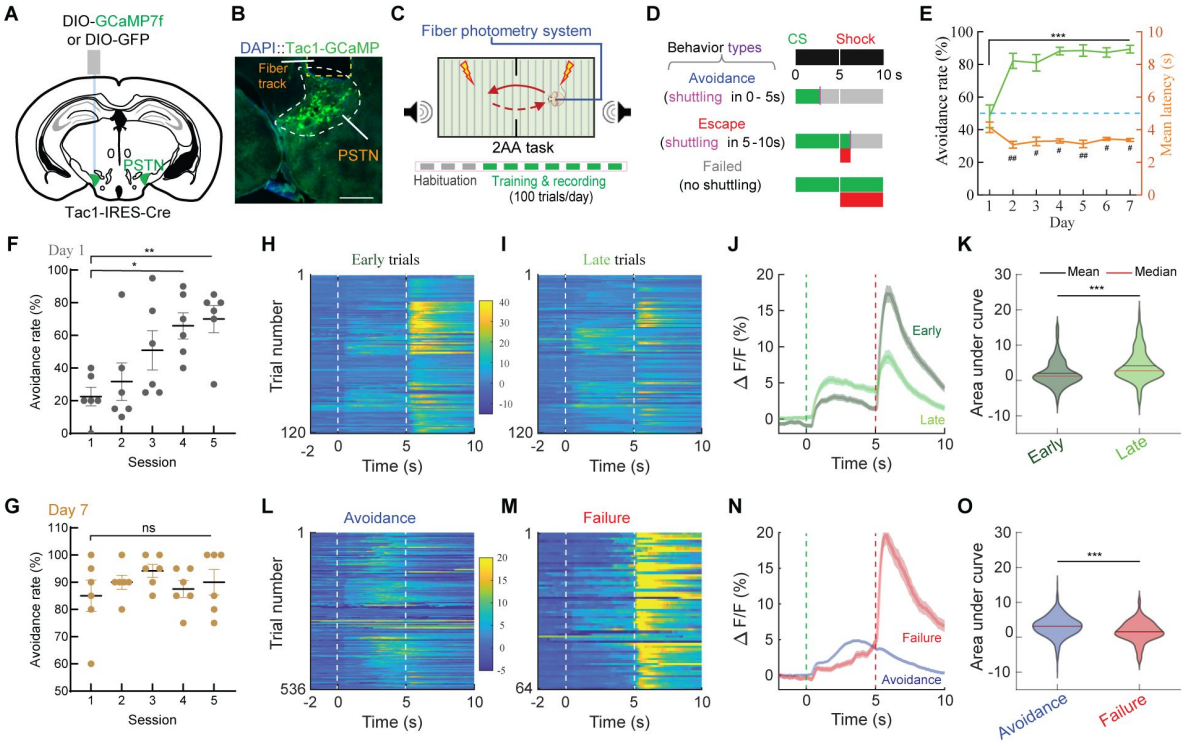

Figure 1.

Neural dynamics of PSTN^{Tac1} neurons during active avoidance learning.

(A) Schematic of Cre-dependent GCaMP7f injection into the PSTN of Tac1-IRES-Cre mice.
(B) Example image showing GCaMP7f expression and fiber location in the PSTN. Yellow dashed line shows fiber track; white dashed line delineates the PSTN. Scale bar, 200 μm.
(C) Schematic of two-chamber active avoidance (2AA) task (upper) and the training pipeline (bottom). Red arrow lines refer to shuttle direction; Gray boxes and green boxes refer to 3-day habituation and 7-day training and recording, respectively.
(D) Behavioral protocol and definition of different behavioral readouts. In 2AA, a test mouse experienced a tone (Conditioned stimulus, CS) followed by an aversive foot shock (Unconditioned stimulus, US). Each trial has a constant 10-second-long time window and the trial-trial interval is ~ 15–30 s. According to the shuttle onset in a given trial, it can be divided into three types: an avoidance trial (shuttle within 0–5 s and no shock), an escape trial (shuttle within 5–10 s and a short foot shock) and a failed trial (no shuttle and a maximum of shock). Green-filled boxes, CS duration; red-filled boxes, shock duration; purple lines, shuttle onset.
(E) Time course and line graph illustrating average avoidance rate and mean latency along 7-day training procedure. One-way repeated-measures ANOVA with Bonferroni post-hoc correction (For avoidance rate: ***P < 0.001; For mean latency: $^{\#}$P < 0.05, $^{\#\#}$P < 0.01).
(F, G) Scatterplots illustrating average avoidance rate across five sessions (20 trials/session) on day 1 (F) and on day 7 (G). One-way repeated-measures ANOVA with Bonferroni post-hoc correction (*P < 0.05 and **P < 0.01).
(H, I) Heatmaps for pooling normalized fluorescence signals from the first 20 trials (early trials, H) and the last 20 trials (late trials, I) of each mouse. Time window for each trial is from 2 s before CS to 10 s after CS. Scale bar refers to % ΔF/F.
(J) The peri-stimulus time histogram (PSTH) plots for all early trials and late trials during the time window.
(K) Violin plots for the area under curve (AUC) of the average responses of early trials and late trials during CS. Two-tailed paired t test (***P < 0.001).
(L, M) Heatmaps for pooling normalized fluorescence signals from avoidance trials (L) and failure trials (M) of each mouse. Time window for each trial is from 2 s before CS to 10 s after CS. Scale bar refers to % ΔF/F.
(N) The PSTH plots for all avoidance trials and failure trials during the time window. O, Violin plots for the AUC of the average responses of avoidance trials and failure trials during CS. Two-tailed unpaired t test (***P < 0.001).
In (E-O), n = 6 mice; in (E-K), n = 20 early trials and 20 late trials each animal; in (G), n = 5 sessions from 100 trials each animal; and in (L-O), n = 536 avoidance trials and n = 64 failure trials. In (E, F, G, J, K, N and O), data was shown as mean ± SEM; in (K, O), shown as median. For detailed statistics information, see Supplementary Table 1.

PSTN^{Tac1} neurons are required for mediating active avoidance learning

We next asked whether the activity of these cells is necessary to control active avoidance learning. To this goal, we conducted cell-specific ablation of PSTN^{Tac1} neurons by bilaterally injecting AAVs expressing Cre-dependent caspase-3 (or EGFP as a control) into the PSTN of Tac1-IRES-Cre mice (**Figure 2A**). Post hoc histological analysis demonstrated that ablation remarkably reduced the number of PSTN^{Tac1} neurons relative to that of control mice (**Figure 2B-C**). The majority of control mice displayed robust avoidance behavior in 2AA task with high avoidance rates and low latencies to trial onset (**Figure 2D-I**), but all ablated mice showed significantly attenuated active avoidance behaviors throughout the training (**Figure 2F-I**), suggesting that PSTN^{Tac1} neurons are required for active avoidance learning. We then tested whether PSTN^{Tac1} neurons' activity is indispensable for the maintenance of learned active avoidance behavior (Figure S3). We screened a cohort of Tac1-IRES-Cre mice displaying robust avoidance performance (with an average avoidance rate > 70%) after 5-day 2AA training. These selected mice were then bilaterally injected with Cre-dependent caspase-3 (or EGFP as a control) into the PSTN. We found that ablated mice exhibited no differences in behavioral performance compared to those in control mice on post-test day 1 (Figure S3E, F). On post-test day 2, ablated mice and control mice had no significant difference in avoidance rate but had a small difference in the latencies to trial onset (Figure S3E, F). The results illustrated that ablation of PSTN^{Tac1} neurons largely had no effect on the learned active avoidance behavior. Collectively, these findings suggest that PSTN^{Tac1} neurons are required for active avoidance during the learning stage rather than post-learning stage.

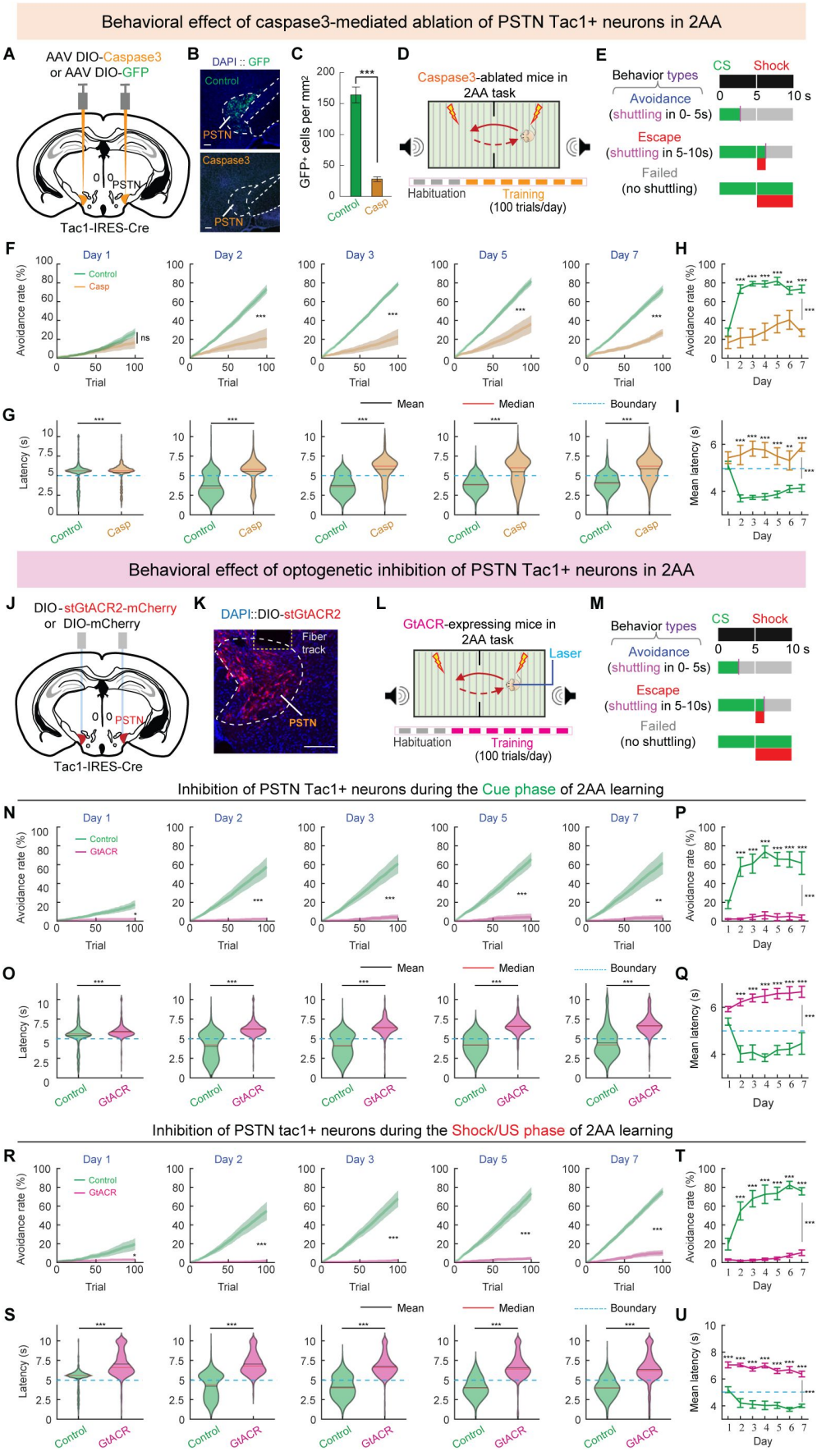
Behavioral effect of caspase3-mediated ablation of PSTN Tac1+ neurons in 2AA
AAV DIO-Caspase3
or AAV DIO-GFP
Tac1-IRES-Cre
PSTN
DAPI :: GFP
Control
Caspase3
GFP+ cells per mm2
Control
Casp
Caspase3-ablated mice in
2AA task
Habituation
Training
(100 trials/day)
Behavior types
CS
Shock
Avoidance
(shuttling in 0- 5s)
Escape
(shuttling in 5-10s)
Failed
(no shuttling)
Day 1
Day 2
Day 3
Day 5
Day 7
Avoidance rate (%)
Trial
Day
Mean
Median
Boundary
Latency (s)
Mean latency (s)
Behavioral effect of optogenetic inhibition of PSTN Tac1+ neurons in 2AA
DIO-stGtACR2-mCherry
or DIO-mCherry
DAPI::DIO-stGtACR2
Fiber
track
GtACR-expressing mice in
2AA task
Laser
Inhibition of PSTN Tac1+ neurons during the Cue phase of 2AA learning
GtACR
Inhibition of PSTN tac1+ neurons during the Shock/US phase of 2AA learning

Figure 2.

Necessity of PSTN^{Tac1} neurons mediating active avoidance learning.

(A) Schematic of Cre-dependent caspase3 injection into the PSTN of Tac1-IRES-Cre mice.

(B) Example images showing caspase injection (bottom) effectively ablated PSTN Tac1^{+} neurons whereas control GFP injection (upper) did not. Scale bar, 200 μm.

(C) Bar graph illustrating caspase ablation significantly reduced the number of GFP^{+} cells in the PSTN compared to the controls. Two-tailed Mann Whitney test (***P < 0.001).

(D) Schematic of two-chamber active avoidance task and training protocol. Gray boxes and orange boxes refer to 3-day habituation and 7-day training, respectively.

(E) Behavioral protocol and definition of different behavioral readouts.

(F, G) Average cumulative distribution of avoidance rates (F) and violin plots of latencies (G) for Caspase-ablated mice and EGFP-expressing controls on days 1-3, 5 and 7 of 2AA task. Two-way repeated-measures ANOVA with Bonferroni post-hoc correction in (F) (ns = not significant; ***P < 0.001) and two-tailed unpaired t test in (G) (***P < 0.001).

(H, I) Line graphs of average avoidance rate (H) and mean latency (I) for caspase3-experessing mice and GFP-expressing controls along 7-day training. Two-way repeated-measures ANOVA with Bonferroni post-hoc correction (**P < 0.01 and ***P < 0.001).

(J) Schematic showing viral injection and fiber implantation for optogenetic inhibition of PSTN Tac1^{+} neurons.

(K) Example image of GtACR expression and fiber location in the PSTN. Yellow dashed line shows fiber track; white dashed line delineates the PSTN. Scale bar, 200 μm.

(L) Schematic of two-chamber active avoidance task and training protocol. Gray boxes and purple boxes refer to 3-day habituation and 7-day training and inhibition, respectively.

(M) Behavioral protocol and definition of different behavioral readouts.

(N, O) Average cumulative distribution of avoidance rates (N) and violin plots of latencies (O) for GtACR-expressing mice and mCherry-expressing controls during CS inhibition on days 1-3, 5 and 7. Two-way repeated-measures ANOVA with Bonferroni post-hoc correction in (N) (*P < 0.05, **P < 0.01 and ***P < 0.001) and two-tailed unpaired t test in (O) (***P < 0.001).

(P, Q) Line graphs of average avoidance rate (P) and mean latency (Q) for GtACR-expressing mice and mCherry-expressing controls along 7-day training. Two-way repeated-measures ANOVA with Bonferroni post-hoc correction (***P < 0.001).

(R, S) Average cumulative distribution of avoidance rates (R) and violin plots of latencies (S) for GtACR-expressing mice and mCherry-expressing controls during US inhibition on days 1-3, 5 and 7. Two-way repeated-measures ANOVA with Bonferroni post-hoc correction in (R) (*P < 0.05 and ***P < 0.001) and two-tailed unpaired t test in (s) (***P < 0.001).

(T, U) Line graphs of average avoidance rate (T) and latency (U) for GtACR-expressing mice and mCherry-expressing controls along 7-day training. Two-way repeated-measures ANOVA with Bonferroni post-hoc correction (***P < 0.001).

In (F-I), n = 8 control mice and 8 Caspase-3 ablated mice; in (N-Q), n = 9 control mice and 7 GtACR-expressing mice; in (R-U), n = 8 control mice and 8 GtACR-expressing mice. In (F-I, N-Q, and R-U), data was shown as mean ± SEM; in (G, O and S), shown as median. For detailed statistics information, see Supplementary Table 1.

To further investigate the exact role of activities of PSTN^{Tac1} neurons during CS or US in controlling active avoidance behavior, we optogenetically suppressed the activity of these neurons bilaterally using a Cre-dependent silencing opsin GtACR2 (**Figure 2J-M**). We first examined the necessity of activity of these neurons during CS by delivering 5 s-long continuous photoinhibition during that phage while mice were performing in the 2AA task. Our results demonstrated that photoinhibition significantly attenuated active avoidance behavior in GtACR2-expressing mice, but not mCherry-expressing controls (**Figure 2N-Q**). Similarly, we then performed the experiment for optogenetic inhibition of PSTN^{Tac1} neurons during US. The results showed a similar behavioral effect with that in photoinhibition during CS (**Figure 2R-U**). Together, both ablation and photoinhibition experiments revealed that PSTN^{Tac1} neurons permit active avoidance learning.

Optogenetic activation of PSTN^{Tac1} neurons promotes active avoidance learning and encodes aversive memory

Because our calcium imaging results found that the activity of PSTN^{Tac1} neurons progressively increases during 2AA learning (**Figure 1H-K**), we thus hypothesized that activation of these neurons during CS is sufficient to promote active avoidance behavior. To this end, we bilaterally injected a Cre-dependent activating opsin ChR2 into the PSTN of male Tac1-IRES-Cre mice (**Figure 3A**, B). 5 s pulsed light stimulation for activation of PSTN^{Tac1} neurons was delivered once a trial starts in the 2AA task (**Figure 3C**, D). Our results showed that optogenetic activation of these cells during CS significantly shortened the shuttle latencies and increased avoidance rates (**Figure 3E-H**), suggesting that activation of PSTN^{Tac1} neurons in male mice can promote active avoidance learning. Furthermore, we also examined behavioral effect by activation of these neurons in female mice, and found that optogenetic activation of PSTN^{Tac1} neurons in female mice significantly reduced the mean latencies to cross the other side (Figure S4) and showed an increased trend in their avoidance rates (Figure S4). This difference in avoidance rates between sexes may lie in greater individual variations in behavioral performance in females. Altogether, despite some sex differences in behavioral performance, optogenetic activation of PSTN^{Tac1} neurons in both sexes of mice has a promoting effect on animal's active avoidance behavior.

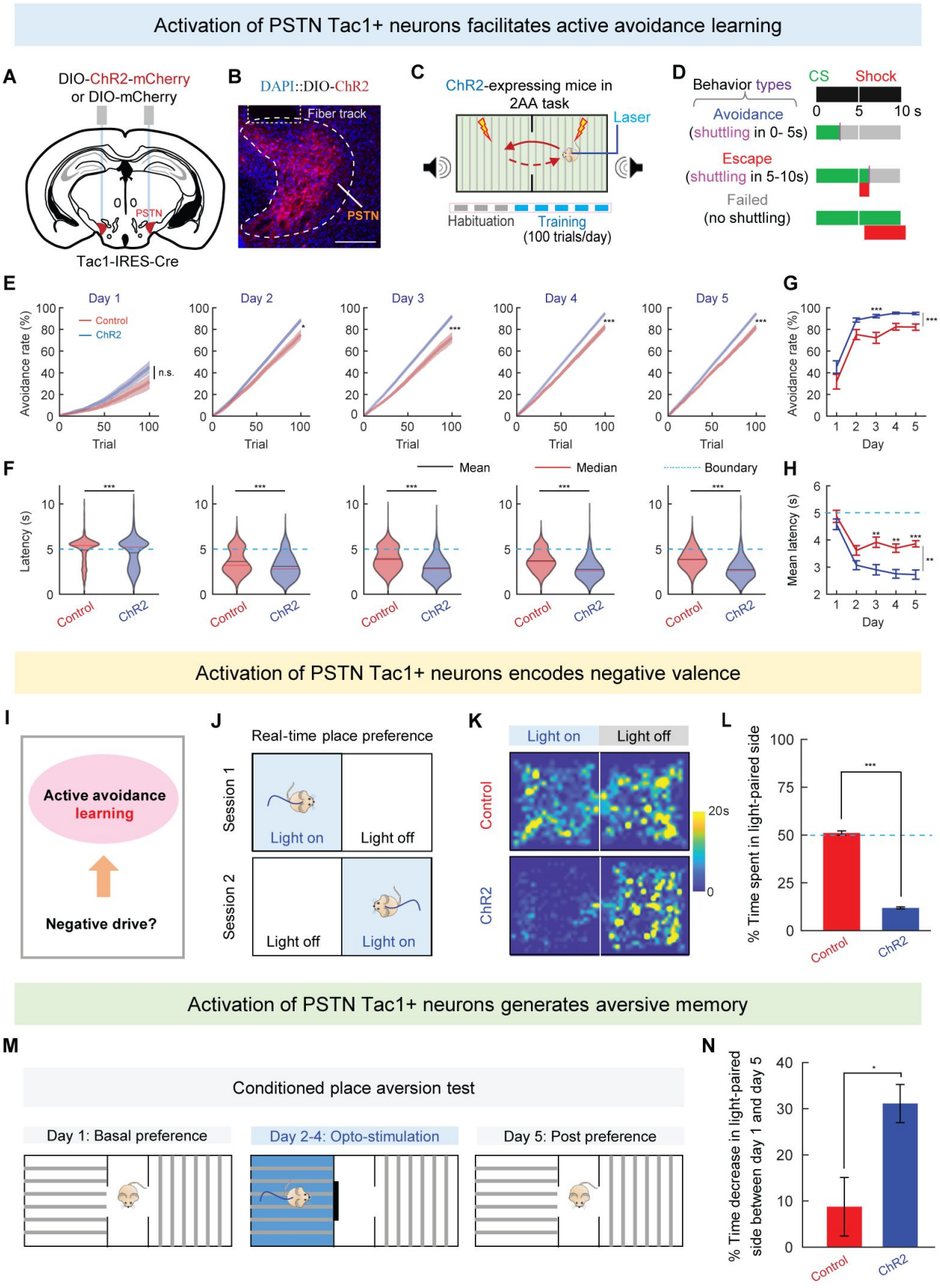
Activation of PSTN Tac1+ neurons facilitates active avoidance learning
A
DIO-ChR2-mCherry
or DIO-mCherry
PSTN
Tac1-IRES-Cre
B
DAPI::DIO-ChR2
Fiber track
PSTN
C
ChR2-expressing mice in
2AA task
Laser
Habituation
Training
(100 trials/day)
D
Behavior types
CS
Shock
0
5
10 s
Avoidance
(shuttling in 0- 5s)
Escape
(shuttling in 5-10s)
Failed
(no shuttling)
E
Day 1
Day 2
Day 3
Day 4
Day 5
Control
ChR2
Avoidance rate (%)
n.s.
Trial
G
Avoidance rate (%)
Day
F
Mean
Median
Boundary
Latency (s)
Control
ChR2
H
Mean latency (s)
Day
Activation of PSTN Tac1+ neurons encodes negative valence
I
Active avoidance
learning
Negative drive?
J
Real-time place preference
Session 1
Session 2
Light on
Light off
K
Light on
Light off
Control
ChR2
20s
0
L
% Time spent in light-paired side
Control
ChR2
Activation of PSTN Tac1+ neurons generates aversive memory
M
Conditioned place aversion test
Day 1: Basal preference
Day 2-4: Opto-stimulation
Day 5: Post preference
N
% Time decrease in light-paired
side between day 1 and day 5
Control
ChR2

Figure 3.

Sufficiency of PSTN^{Tac1} neurons mediating active avoidance learning.

(A) Schematic showing viral injection and fiber implantation for optogenetic activation of PSTN Tac1^{+} neurons.

(B) Example image of injection site and ChR2 expression in the PSTN of Tac1-IRES-Cre animals. Yellow dashed line shows fiber track; white dashed line delineates the PSTN. Scale bar, 200 μm.

(C) Schematic of two-chamber active avoidance task and training pipeline. Gray boxes and blue boxes refer to 3-day habituation and 5-day training, respectively.

(D) Behavioral protocol and definition of different behavioral readouts.

(E, F) Average cumulative distribution of avoidance rates (E) and violin plots of latencies (F) for ChR2-experessing mice and mCherry-expressing controls on each day. Two-way repeated-measures with Bonferroni post-hoc correction in (E) (ns = not significant; *P < 0.05 and ***P < 0.001) and two-tailed unpaired t test in (F) (***P < 0.001).

(G, H) Line graphs of average avoidance rate (G) and mean latency (H) for ChR2-experessing mice and mCherry-expressing controls along 5-day training. Two-way repeated-measures ANOVA with Bonferroni post-hoc correction (**P < 0.01 and ***P < 0.001).

(I) Schematic of experiment model.

(J) Schematic showing real-time place preference assay (RTPP). Light blue area indicates the chamber paired with light stimulation when the animal enters.

(K) Representative heatmaps showing locomotion trajectories of control (top) and ChR2-experessing (bottom) mice in the RTPP test.

(L) ChR2-expressing animals displayed a significant decrease in time spent in stimulation-coupled chamber compared to mCherry-expressing controls. Two-tailed Mann-Whitney test (***P < 0.001).

(M) Schematic of conditioned place aversion (CPA) test and experimental procedures.

(N) During post preference test on day 5, ChR2-expressing animals exhibited a remarkable reduction in time spent in preferred side on day 1 compared to mCherry-expressing controls, suggesting that activation of MeA GABAergic neurons could induce a long-term aversive memory for stimulation-coupled chamber. Two-tailed Mann-Whitney test (*P < 0.05).

In (E-H), n = 8 control mice and 14 ChR2-expressing mice; in (L), n = 9 control mice and 9 ChR2-expressing mice; in (N), n = 11 control mice and 8 ChR2-expressing mice. In (E-H, L, and N), data was shown as mean ± SEM; in (F), shown as median. For detailed statistics information, see Supplementary Table 1.

As known, learning is highly dependent on individuals' motivational drive[1,2,12,44,45]. Consistent with a recent study[40], we used a real-time place preference assay and confirmed that animals expressing ChR2 into PSTN^{Tac1} neurons developed a consistent avoidance for the chamber coupled with light stimulation compared to the controls (**Figure 3I-L**), suggesting that PSTN^{Tac1} neurons encode negative motivational drive in promoting this behavior. To exclude the possibility of generating this aversive effect by sparse cells of PSTN neighboring areas, we conducted additional characterizations of behavioral effects of activation of sparsely Tac1-

expressing neurons in two adjacent areas of PSTN, demonstrating no differences between light-coupled sides and its counter-balanced sides (Figure S5). These results indicate that PSTN^{Tac1} neurons, but not its adjacent Tac1-expressing neurons, encode negative motivational drive in promoting this behavior.

To further test whether optogenetic activation of PSTN^{Tac1} neurons could generate aversive memory, we applied a five-day conditioned place aversion assay, in which includes pre-testing baseline phase (day 1), stimulation phase (days 2-4) and post-testing phase (day 5) (**Figure 3M**). We found that optogenetic activation of these neurons from ChR2-expressing mice could significantly reduce the percentage of time spent on the stimulation-paired side between pre-testing baseline phase and post-testing phase (**Figure 3N**), which is divergent from a recent study[40].

Downstream targets of the PSTN mediate active avoidance learning

We next sought to dissect what the downstream circuitry of the PSTN is involved in mediating active avoidance learning. Indeed, a recent study has mapped and functionally characterized the whole-brain downstream targets of cell-type specific neurons in the PSTN in feeding behavior[29] and the identified projections are largely consistent with those by traditional anterograde tracer experiment[23]. Here we used a viral-based anterograde tracing approach to confirm major projections from the PSTN (Figure S6), including the PVT/intermediodorsal nuclei of the dorsal thalamus (IMD) complex, the central nucleus of the amygdala (CeA), and the parabrachial nucleus (PBN). Among its projection targets, the PVT has been recently found to involve associative learning and defensive behaviors[9,42]. Therefore, we speculated that the PSTN-to-PVT/IMD pathway is an attractive candidate for mediation of active avoidance learning. To fully characterize the circuit dynamics and functional role of this circuit, we used axon-localized GCaMP6s based fiber photometry and optogenetics-assisted circuit mapping and manipulations (**Figure 4A-D**). Similar to the findings from the soma, the PSTN-to-PVT/IMD circuit signals aversive information on active avoidance behavior and is modulated by learning (**Figure 4E-H**). Moreover, optogenetic manipulations confirmed that this circuit is both necessary and sufficient to mediate this learning (**Figure 4I-P**). Together, the PVT/IMD is an important downstream target of the PSTN in the regulation of active avoidance learning, adding the PSTN as a new member to the established neural network that underlies this behavior.

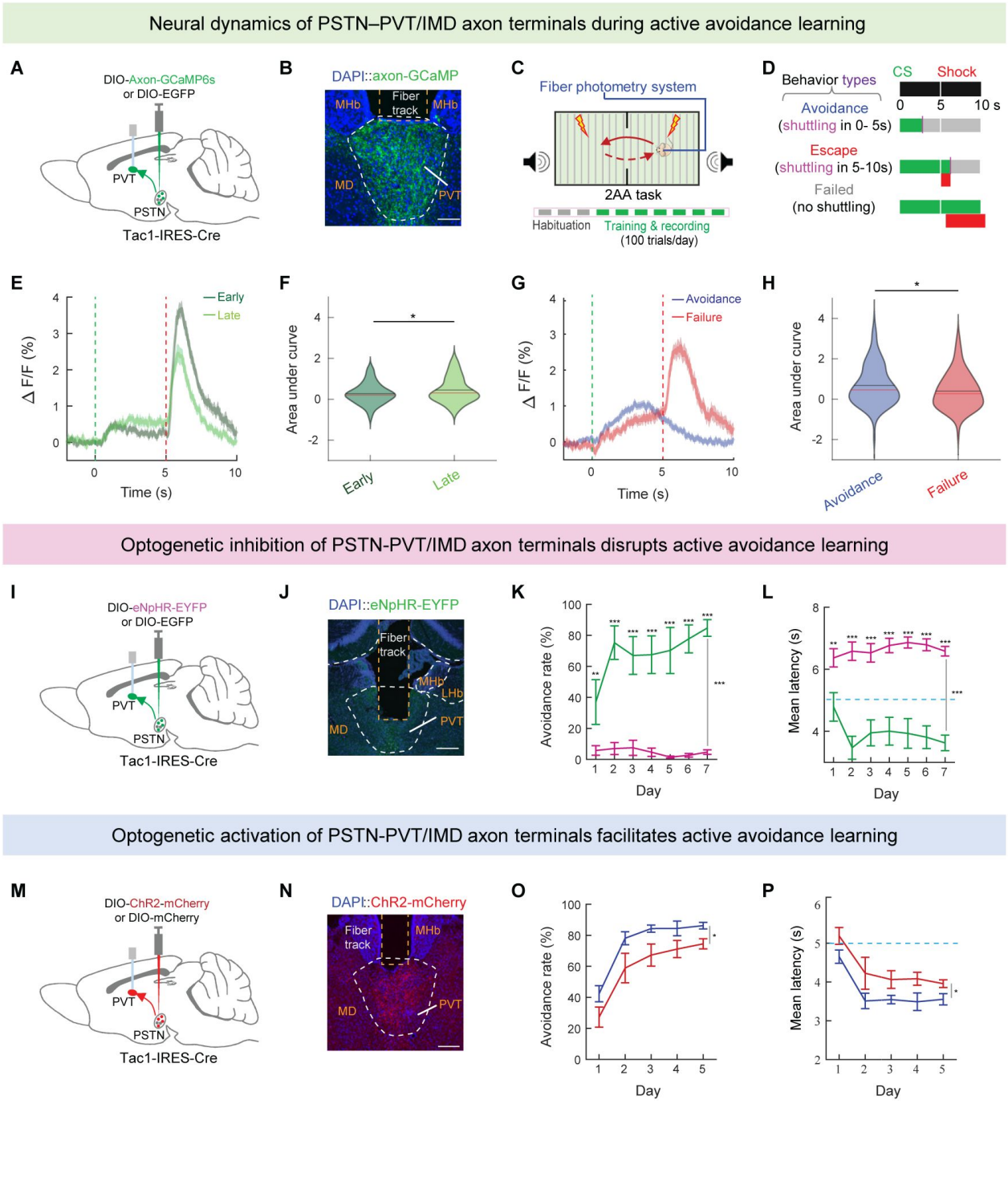
Neural dynamics of PSTN–PVT/IMD axon terminals during active avoidance learning
A
DIO-Axon-GCaMP6s
or DIO-EGFP
PVT
PSTN
Tac1-IRES-Cre
B
DAPI::axon-GCaMP
Fiber track
MHb
MD
PVT
C
Fiber photometry system
2AA task
Habituation
Training & recording
(100 trials/day)
D
Behavior types
CS
Shock
0
5
10 s
Avoidance
(shuttling in 0- 5s)
Escape
(shuttling in 5-10s)
Failed
(no shuttling)
E
Early
Late
Δ F/F (%)
Time (s)
F
Area under curve
Early
Late
G
Avoidance
Failure
Δ F/F (%)
Time (s)
H
Area under curve
Avoidance
Failure
Optogenetic inhibition of PSTN-PVT/IMD axon terminals disrupts active avoidance learning
I
DIO-eNpHR-EYFP
or DIO-EGFP
PVT
PSTN
Tac1-IRES-Cre
J
DAPI::eNpHR-EYFP
Fiber track
MHb
LHb
MD
PVT
K
Avoidance rate (%)
Day
L
Mean latency (s)
Day
Optogenetic activation of PSTN-PVT/IMD axon terminals facilitates active avoidance learning
M
DIO-ChR2-mCherry
or DIO-mCherry
PVT
PSTN
Tac1-IRES-Cre
N
DAPI::ChR2-mCherry
Fiber track
MHb
MD
PVT
O
Avoidance rate (%)
Day
P
Mean latency (s)
Day

Figure 4

The PSTN projects to the PVT/IMD to mediate active avoidance learning.

(A) Schematic of Cre-dependent axon GCaMP6s injection into the PSTN and fiber placement in the PVT.

(B) Example image showing axon-GCaMP6s expression and fiber location in the PVT. Orange dashed line indicates fiber location and red dashed line delineates the PVT. Axon-GCaMP signals were strengthened by immunostaining with anti-GFP. Scale bar, 100 μm.

(C) Schematic of two-chamber active avoidance (2AA) task (upper) and the training pipeline (bottom).

(D) Behavioral protocol and definition of different behavioral readouts.

(E) PSTH plots for all early trials and late trials during the time window. Time window for each trial is from 2 s before CS to 10 s after CS.

(F) Violin plots for AUC of average responses of early trials and late trials during CS. Two-tailed paired t test (*P < 0.001).

(G) PSTH plots for all avoidance trials and failure trials during the time window. Time window for each trial is from 2 s before CS to 10 s after CS.

(H) Violin plots for AUC of average responses of avoidance trials and failure trials during CS. Two-tailed unpaired t test (*P < 0.001).

(I) Schematic showing eNpHR injection and fiber implantation for optogenetic inhibition of PSTN-PVT circuit.

(J) Example image of eNpHR expression in axon terminals in the PVT. Orange dashed line indicates fiber location and white dashed line delineates the PVT. Scale bar, 200 μm.

(K, L) Line graphs of average avoidance rate (K) and latency (L) for eNpHR-expressing mice and mCherry-expressing controls along 7-day training. Two-way repeated-measures ANOVA with Bonferroni post-hoc correction (**P<0.01 and ***P < 0.001).

(M) Schematic showing viral injection and fiber implantation for optogenetic activation of PSTN-PVT circuit.

(N) Example image of axon terminal expression of ChR2 in the PVT. Orange dashed line indicates fiber location and red dashed line delineates the PVT. axon terminal mCherry signals were strengthened by immunostaining with anti-mCherry. Scale bar, 200 μm.

(O, P) Line graphs of average avoidance rate (O) and mean latency (P) for ChR2-experessing mice and mCherry-expressing controls along 5-day training. Two-way repeated-measures ANOVA with Bonferroni post-hoc correction (*P < 0.05).

In (E-H), n = 3 axon-GCaMP-expressing mice; in (G, H), n = 213 avoidance trials and n = 87 failure trials; in (K, L), n = 4 control mice and 8 eNpHR-expressing mice; in (O, P), n = 8 control mice and 9 ChR2-expressing mice. In (G-J, K, L, O, P), shown as data was shown as mean ± SEM; in (F, H), shown as median. For detailed statistics information, see Supplementary Table 1.

In addition to the PVT, the PSTN also heavily projects the PBN and CeA [22] (Figure S6). These brain areas have been implicated in aversive learning[22,26,46]. To further characterize functional roles of the PSTN→PBN circuit and the PSTN→CeA circuit in mediating active avoidance learning, we first optogenetically activated these circuits during the CS of 2AA task (Figure S7A and F). We observed no significant differences in mean avoidance rates and mean latencies to cross the other side across 5-day training between ChR2 groups and control groups (Figure S7A-E). Furthermore, we found that optogenetic inhibition of these circuits remarkably attenuates animals' behavioral performance during 2AA task (Figure S7F-J). These results demonstrate that the PSTN→PBN circuit and the PSTN→CeA circuits are required for active avoidance learning.

Discussion

Although a growing body of literature has largely expanded our knowledge of neural mechanisms underlying active avoidance learning[3,5,9,14–16,42], the hypothalamus receives less attention in the regulation of this behavior. The PSTN, a small nucleus located on the lateral edge of the posterior hypothalamus, has just been identified less than two decades and is still a relatively understudied brain region[22,23]. Despite the current research on the functional roles for the PSTN in the regulation of feeding behaviors [24–27], appetite suppression[28,29], alcohol consumption[30–33], as well as encoding of aversive experiences[26,28,35,36], it has not been directly studied its role in the encoding and control of active avoidance learning. In the present study, through a series of well-designed experiments using animal models, we for the first time established the PSTN as a crucial brain node that encodes and controls active avoidance learning differentially via its downstream projections. This work further addresses the long-standing question of what neural substrates underlie learning within the mammalian brain.

Firstly, we identify the hypothalamic PSTN^{Tac1} neurons as novel neural substrates important for active avoidance learning. Despite recent studies indicating that the PSTN is also associated with aversion[22,37,38], it still remains elusive about its involvement in active avoidance learning. Here, our ablation and optogenetic silencing experiments clearly demonstrated that PSTN^{Tac1} neurons are required for active avoidance learning (**Figures 2** and S3), providing a first causal link these cells in the PSTN to active avoidance learning. In addition, we find that optogenetic activation of these cells is capable of promoting active avoidance learning (**Figures 3** and S6). These results jointly highlight the necessity and sufficiency of Tac1-expressing cells in the PSTN in controlling active avoidance learning, adding the PSTN a new member of neural networks that control this learning[9,10,15]. Interestingly, recent studies have also demonstrated that the PSTN is involved in fear-related avoidance behaviors[26,35]. Together, these findings indicate that the PSTN involves both the regulation of avoidance behavior and its learning. Furthermore, our calcium imaging results demonstrated that punishment-predictive cues significantly enhance PSTN^{Tac1} neurons' activity during the late phase of training (**Figures 1** and S2), similar with previous findings from other brain regions, such as the lateral habenula[10] and the PVT[9]. This result further supports the PSTN as a cellular substrate necessary for active avoidance learning.

Secondly, we find that PSTN^{Tac1} neurons could promote the generation of aversive memory. As known, learning is driven by either appetitive or negative motivational drives[4]. Both traditional tracer-based and viral-based whole-brain circuit mapping experiments revealed that the PSTN heavily connects to brain structures that involves the regulation of emotions and motivation [22,23,27,29,34]. In our study, activation of PSTN^{Tac1} neurons can induce a strong aversive emotional drive, similar with the findings of previous studies on activation of other subpopulations of this brain region[26,28]. Remarkably, we further found that activation of these neurons can promote the generation of aversive memory (**Figure 3N**). This line of data further supports the role of Tac1-expressing neurons in the PSTN in active avoidance learning. On the contrary, a recent study by Serra et al. has shown that optogenetic activation of these cells in the PSTN does not promote conditioned place aversion while activation of Pitx2-expressing

neurons in the subthalamic nucleus (STN), adjacent to the PSTN, mediates aversive learning[40]. Based on our calcium imaging and functional experiments (**Figures 1**-**3**), our study supports that PSTN^{Tac1} neurons play important roles in learning and memory, at least in active avoidance learning. This divergence is possibly due to the use of different conditioning parameters. Interestingly, both studies have found that PSTN^{Tac1} neurons can induce a strong aversive emotional drive[40], strongly suggesting that the PSTN belongs to an important brain node within neuronal circuits of motivation. Moreover, the findings by Serra et al. [40] and our study show that both PSTN and its neighboring STN are newly identified members of neural networks controlling aversive learning, suggesting that these two brain areas function similarly in motivation and learning. In addition to *Tac1*, the PSTN also contains several other molecularly distinct cell groups (i.e., *Adcyap1* and *Crh*). Thus, examining how/whether other subtypes of the PSTN are involved in active avoidance learning represents an important future research direction of this field.

Finally, we reveal differential regulation of active avoidance learning by downstream projections of the PSTN. Despite some recent studies indicating that the PSTN projects to multiple brain regions to regulate diverse behaviors, such as feeding related behaviors[29] and arousal[34], it has not been directly examined the functional roles and synaptic dynamics of its downstream circuitries. Similar with previous findings [9,10], we observed that activity of axonal terminals of the PSTN in the PVT/IMD is gradually increased over learning. This observation resembles the expression of an LTP-like process at the PSTN-to-PVT/IMD synapses [10] to facilitate avoidance behavior. In addition, our functional manipulation results demonstrated that the PSTN-to-PVT circuit is both necessary and sufficient for active avoidance learning. This greatly expands our knowledge of PVT neurocircuitry in this learning[9,42]. Fascinatingly, a previous work has also identified a lateral hypothalamus-to-lateral habenula pathway involved in this learning[10]. These findings suggest the existence of two parallel neural circuits within the hypothalamus-to-thalamus circuitries for the regulation of active avoidance learning. Surprisingly, unlike the PSTN-to-PVT/IMD circuit, optogenetic activation of the PSTN→PBN circuit and the PSTN→CeA circuits did not promote active avoidance learning. Overall, these findings demonstrate that the PSTN mediates active avoidance learning possibly via different downstream projections, which is distinct from the regulation of appetite suppression by these circuits in a similar way[29]. In addition to downstream projections selected in this study, the PSTN also heavily projects to several other brain areas associated with motivational or motor components of aversive learning[22,23,29], such as the PAG, substantia innominate (SI), and bed nucleus of the stria terminalis (BNST). Therefore, an in-depth functional study of these uncharacterized downstream circuits of the PSTN is needed.

In summary, we identified the PSTN as a key brain node for encoding and control of active avoidance learning. Importantly, instrumental defensive behaviors like active avoidance can be modified through experience and learning, resulting in that the neural circuits underlying these behaviors exhibit remarkable plasticity. Therefore, more studies are needed to fully understand the extent of the involvement of PSTN and its circuitry in active avoidance learning and neuropsychiatric disorders, as well as its potential as a therapeutic target[6].

Materials and methods

Mice

Tac1-IRES-Cre mice (8-12 weeks old) were purchased from Shanghai Model Organisms Center (Catalog # NM-KI-200083). Tac1-IRES-Cre mice were crossed to C57BL/6J mice (purchased from GemPharmatech) to produce heterozygous animals (Tac1-IRES-Cre/+, 8-16 weeks old and both sexes) for stereotaxic surgery and behavioral experiments. Animals were grouped housed in 12 h

light-dark cycle (9 p.m. - 9 a.m. light), with food and water available ad libitum. Animal care and use were strictly followed according to the institutional guidelines and governmental regulations of China. Experiments were performed exactly as approved by the IACUC at Fudan University.

Viruses

AAV9-EF1α-DIO-EGFP (Catalog #BC211231-0015-9), AAV9-CAG-FLEX-jGCaMP7f (Catalog #BC210625-0207-9), AAV9-EF1a-DIO-Axon-GCaMP6s (Catalog #BC210909-0197-9) and AAV9-EF1α-DIO-taCasp3-T2A-TEVP (Catalog #BC210909-0130-9) were purchased from Brain Case. AAV2/9-hSyn-DIO-stGtACR2-mCherry-WPRE-pA (Catalog #S0981-9-H50), AAV2/2-hEF1α-DIO-hChR2(H134R)-mCherry-WPRE-pA (Catalog #S0170-2-H50), AAV2/9-hEF1α-DIO-mCherry-WPRE-pA (Catalog #S0197-9-H50), AAV2/9-hEF1α-DIO-eNpHR3.0-mGFP-WPRE-pA (Catalog #S0178-9-H50) and AAV2/9-hSyn-DIO-EYFP-WPRE-pA (Catalog #S0276-9-H50) were purchased from Taitool Bioscience.

Stereotaxic surgeries

Tac1-IRES-Cre/+, were anesthetized with isoflurane and mounted on a stereotaxic device (RWD Life Science). Both sexes of mice were used in the experiments. Injections were carried out using a pulled, fine glass capillary (RWD Life Science).

For fiber photometry calcium imaging, Tac1-IRES-Cre/+mice were injected with 50-100 nL AAV9-CAG-FLEX-jGCaMP7f into the PSTN (ML ±1.15 mm, AP -2.40 ~ -2.45 mm, DV - 4.90 mm from bregma). An optic fiber (200 um core diameter, Inper) was then placed in the virus injection site in the PSTN.

For Caspase-mediated ablation of PSTN $Tac1^+$ neurons, Tac1-IRES-Cre mice were injected bilaterally with 100 nL AAV9-EF1α-DIO-taCasp3-T2A-TEVP and 50 nL AAV9-EF1α-DIO-EGFP into the PSTN (ML ±1.15 mm, AP -2.40 ~ -2.45 mm, DV - 4.90 mm from bregma).

For optogenetic activation or inhibition of PSTN $Tac1^+$ neuron, Tac1-IRES-Cre mice were injected bilaterally with 100 nL AAV2/9-hSyn-DIO-stGtACR2-mCherry-WPRE-pA into the PSTN for inhibition or 100 nL AAV2/2-hEF1α-DIO-hChR2(H134R)-mCherry-WPRE-pA into the PSTN for activation (PSTN: ML ±1.15 mm, AP -2.40 ~ -2.45 mm, DV - 4.90 mm from bregma). An optic fiber (200 um core diameter, Inper) was then placed 0.4-0.5 mm above the virus injection site in the PSTN.

For optogenetic activation of $Tac1^+$ neuron in two PSTN adjacent areas (defined as P1 and P2), Tac1-IRES-Cre mice were injected bilaterally with 50 nL AAV2/2-hEF1α-DIO-hChR2(H134R)-mCherry-WPRE-pA into the P1 or P2 for activation (P1: ML ±0.75 mm, AP -2.40 ~ -2.45 mm, DV - 4.00 mm from bregma; P2: ML ±1.70 mm, AP -2.40 ~ -2.45 mm, DV – 5.60 mm from bregma). An optic fiber (200 um core diameter, Inper) was then placed 0.4-0.5 mm above the virus injection site in the P1 or P2.

For fiber photometry calcium imaging of the axonal terminals in the PVT from the PSTN, Tac1-IRES-Cre/+mice were injected with 100 nL AAV9-EF1a-DIO-Axon-GCaMP6s into the PSTN (ML ±1.15 mm, AP -2.40 ~ -2.45 mm, DV - 4.90 mm from bregma). An optic fiber (200 um core diameter, Inper) was then placed in the PVT (ML 0 mm, AP -1.07 ~ -1.43 mm, DV – 3.05 mm from bregma).

For optogenetic activation or inhibition of the downstream pathways (PSNT-PVT, PSNT-PBN, and PSNT-CeA), Tac1-IRES-Cre mice were injected bilaterally with 100 nL AAV2/9-hEF1α-DIO-eNpHR3.0-EYFP-WPRE-pA into the PSTN for inhibition or 100 nL AAV2/2-hEF1α-DIO-hChR2(H134R)-mCherry-WPRE-pA into the PSTN for activation (PSTN: ML ±1.15 mm, AP -2.40 ~ - 2.45 mm, DV - 4.90 mm from bregma). An optic fiber (200 um core diameter, Inper) was then placed in the PVT (ML 0 mm, AP -1.50 ~ -1.60 mm, DV - 2.95 mm from bregma) or in the PBN (ML ±1.25 mm, AP -5.50 mm, DV -3.20 mm from bregma) or in the CeA (ML ± 2.95 mm, AP - 1.40 mm, DV - 4.20 mm from bregma).

For control experiments, the animals with the same genetic background were injected with EGFP-expressing or mCherry-expressing AAVs.

Behavioral assays

Automated system for quantitative analysis of two-chamber active avoidance task

In this study, we adopted and modified a widely used two-way active avoidance learning task (2AA; **Fig. 1c**), based on previous work[9]. The apparatus (L x W x H: 30 cm x 15 cm x 50 cm) was divided into two compartments (**Fig. 1c**). Electric foot shock (0.8 mA) was delivered by an Arduino-controlled Shocker (Beijing Xeye S&T Co., Ltd). Automated system for 2AA task was built on Matlab program (MathWorks). A top view of camera was used to obtain mouse real-time locations. During 2AA task, a test mouse experienced a tone (Conditioned stimulus, CS) and must shuttle to the other side of chamber in a given response window to prevent an aversive foot shock (Unconditioned stimulus, US). Each animal had a training of 100 trials each day for all 2AA task throughout the study. Each trial has a constant 10-second-long time window and the trial-trial interval is ~ 15 s – 30 s. Once a trial starts, a sound cue is generated. If the test mouse crossed the other side of chamber within 5 seconds (defined as an avoidance trial), the sound cue was terminated immediately and no foot-shock was delivered. If the test mouse crossed the other side of chamber within 5 s – 10 s relative to trial onset (defined as an escape trial), the sound cue was terminated immediately and foot-shock was delivered 5 s after trial onset and terminated immediately when the test mouse successfully crossed compartments. If the test mouse failed to cross the chamber throughout the trial (defined as a failure trial), it experienced a maximum of shock duration (5 s) and the cue sound lasts 10 s. We examined the performance of active avoidance learning by computing the percentage of cue-guided successful shuttles over total trials and the duration from trial onset to crossing compartments (defined as the latency).

Caspase-3 ablation experiments

For examination of behavioral effect by caspase-3-mediated ablation before training (**Figure 2D-I**), the performance of active avoidance learning was measured described above. Caspase-3-expressing and EGFP-expressing mice were trained in 2AA task ~3.5 weeks after viral injection. For examination of behavioral effect by caspase-3-mediated ablation after training (Figure S3), we first screened a cohort of Tac1-IRES-Cre mice displaying robust avoidance performance (with an average avoidance rate > 70%) after 5-day 2AA training. Those selected mice were then bilaterally injected with Cre-dependent caspase-3 (or EGFP as a control) into the PSTN. After viral expression, the mice were undergone a 2-day post-testing of 2AA task.

GtACR inhibition experiments

GtACR inhibition experiments (**Figure 2J-U**) were performed 2AA task described above and divided in two stimulation modes (during CS phase and US phase). To further dissect the exact role of activities of PSTN Tac1+ neurons during CS or US in controlling active avoidance behavior, optogenetic inhibition was performed during CS (continuous 5s stimulation and final blue laser output powers of 5-10 mW mm^{-2}) or during US (continuous 5s stimulation and final output powers of 5-10 mW mm^{-2}) from different group of test animals for each trial across the whole 7-day training. Prior to each experiment, a ferrule patch cord was coupled to the ferrule fiber implanted in the mouse using a zirconia split sleeve (Inper). An Arduino microcontroller board and a custom MATLAB program were used to control the initiation and termination of the test and record the behavioral data.

ChR2 activation experiments

ChR2 activation in the 2AA task

ChR2 activation experiments for males (**Figure 3**) and females (Figure S4) were performed 2AA task described above. To further dissect the exact role of activities of PSTN Tac1+ neurons during CS in controlling active avoidance behavior, optogenetic activation was performed during CS (time window: 0-5s) for each trial across a 5-day training. The stimulation parameters were as follows: frequency: 20 Hz, pulse duration: 20 ms, and laser power: ~2.5 mW mm^{-2}. Prior to each experiment, a ferrule patch cord was coupled to the ferrule fiber implanted in the mouse using a zirconia split sleeve (Inper). An Arduino microcontroller board and a custom MATLAB program were used to control the initiation and termination of the test.

Real time place preference test (RTPP)

ChR2-expressing or mCherry-expressing male mice were introduced into a two-chamber apparatus (60 cm x 30 cm x 30 cm for each chamber) and were allowed to freely move between the two chambers based on our recent paper[51]. Briefly, each test consisted of two consecutive 15-min sessions. In the first session, mice were allowed to freely explore the two compartments for 15 minutes, during which entering into one of the two chambers triggered optogenetic stimulation (ChR2: 473 nm, 20 ms, 20 Hz for soma stimulation, 2.5 mW mm^{-2}, for up to 20-s duration). Exiting the stimulated chamber immediately terminated the light stimulation. During the second session, the opposite chamber was paired with light stimulation. An Arduino microcontroller board and a customized MATLAB program were used to control laser pulses, based on real-time tracking of mouse locations. We calculated and compared three parameters between ChR2 group and control group: time spent in the light-paired side, distances travelled in the light-paired side, and the number of visits to the light-paired side. In addition, to exclude the possibility of generating this aversive effect by sparse cells of PSTN neighboring areas, we conducted the RTPP for additional characterizations of behavioral effects of activation of sparsely Tac1-expressing neurons in two adjacent areas of PSTN (Figure S5).

Conditioned place aversion test (CPA)

To study the effect of optogenetic activation of PSTN Tac1+ neurons on generating a long-term aversive memory, we used a widely used conditioned place aversion (CPA) test. The CPA test includes three phases: pre-conditioning, conditioning, and post-conditioning. Briefly, ChR2-expressing or mCherry-expressing mice were introduced into a three-chamber apparatus to allow them to acclimate to the testing environment for 30 minutes on three days before starting the experiment. The apparatus typically consists of three interconnected chambers (the chamber on two sides: 30 cm x 30 cm x 30 cm; the middle chamber: 15 cm x 30 cm x 30 cm), each with distinct visual and tactile cues. The middle chamber is neutral and serves as a control, while the other two chambers are designated as conditioning chambers. During the pre-conditioning phase (on day 1), animals were initially placed in the middle chamber to freely explore all three chamber for 30 minutes and then the basal preference for each side was calculated for subsequent experiments. During the conditioning phase (on days 2-4), optogenetic stimulation with a cycle of 1 second light on (ChR2: 473 nm, 20 ms, 20 Hz for soma stimulation, 2.5 mW mm^{-2}) and 4 second light off was consistently paired with one specific compartment that a given animal preferred to explore on day 1. During the post-conditioning phase (on day 5), animals were allowed to freely explore both chambers without any aversive stimulus present. We assessed the aversive response by calculating the percentage of time changes spent in the preferred side during pre-conditioning phase between day 1 and day 5.

Optogenetic manipulations of the downstream pathways in the 2AA task

ChR2 activation of the downstream circuit experiments (**Figure 4** for the PSTN-PVT; Figure S7) for the PSTN-to-PBN circuit and the PSTN-to-CeA circuit) was performed during the CS phase of 2AA task, but with the stimulation parameters (Frequency: 50 Hz, Pulse duration: 5 ms, and laser power: ~2.5 mW mm^{-2}). NpHR inhibition of the downstream circuit experiments (**Figure 4** for the PSTN-to-PVT circuit; Figure S7 for the PSTN-to-PBN circuit and the PSTN-to-CeA circuit) were also conducted during the CS phase of 2AA task, but with the stimulation parameters (continuous 5s stimulation and final yellow laser output powers of 5-10 mW mm^{-2}).

Fiber photometry experiments and data analysis

Photometry experiments were performed as previously described [47]. Fluorescence signals were acquired with a fiber photometry system (ThinkerTech). The LED power was adjusted at the tip of the optical fiber to 30–50 μW to minimize bleaching. Behaviors were recorded by a video camera. To monitor the response of Tac1-expressing cells in the PSTN (PSTNTac1) by direct foot shock, the test mouse received 10 times of 1s random aversive foot shock stimulus, with a 30~90 s of trial-interval. To record neuronal responses of PSTN $Tac1^+$ neurons during 2AA task, subject animals were allowed to freely behave in a shuttle-box chamber and the fluorescent signals were recorded simultaneously. Photometry data were analyzed using custom MATLAB programs. To measure dynamics of fluorescence intensity immediately before and after the onset of specific stimuli (e.g., sound and shock), (F−F0)/F0 (ΔF/F) was calculated, where F0 was the baseline fluorescence signal averaged over a 1 s window between 5 s and 4 s (**Figures 1**, **4E-H**, and S1 and 2), prior to stimulus onset. The analysis time window was from 2 s before CS to 10 s after CS. ΔF/F values were presented as mean with an SEM envelope. To measure neural activity (i.e., fluorescence changes of GCaMP7f or axon-GCaMP6s) of PSTN $Tac1^+$ neurons or axon terminals in the PVT between early trials and late trials (pooled first 20 trials or last 20 trials of each animal on day 1) or between avoidance trials and failure trials (on day 7), we calculated the AUC per second between 0 s and 5 s from the CS onset.

Histology and Imaging

Animals used in the study were sacrificed 4-6 weeks post-injection and perfused with 4% PFA. The brains were dissected out and fixed in 4% PFA for two additional hours at room temperature, rinsed with 3x PBS, and then put into 30% sucrose solution overnight at 4°C. To visualize viral expression and fiber placement, 60 μm sections were cut on a cryostat (RWD, China). For improving the fluorescent signals of PSTN terminals in the PVT, we conducted immunostaining experiments, largely based on our previous study[48]. Briefly, free-floating sections containing PSTN terminals in the PVT were washed in PBS 3x 5 minutes, followed by 0.5 hour blocking in 10% normal donkey serum (NDS). Primary antibodies (rabbit anti-GFP for axon-GCaMP sections or rabbit anti-mCherry for ChR2 sections; Abcam, 1:1000) were diluted in 0.5% PBS/Triton X-100 and 10% NDS and incubated overnight at 4°C. Sections were then washed with PBS 3x 5 minutes and incubated with secondary antibodies (goad anti-rabbit 488 or 555; Abcam, 1:1000) 2 hours at room temperature. Sections were then washed with PBS three times and mounted onto Superfrost Plus slides, dried > 40 minutes at room temperature, and coverslipped in 50% glycerol containing DAPI (1 ug/mL). Images were acquired using a confocal microscope (OLYMPUS FV3000) with 10x or 20x objective lens. Quantification of ablated cells (**Figures 2B** and S3B) and measurements of ChR2 and eNpHR special expression patterns were performed by using ImageJ software. For optogenetic experiments, we only selected data from those mice, with >90% of the infected areas within the PSTN and the optic fiber being exactly hit in the PSTN.

Data analysis and statistics

No statistical methods were applied to pre-determine sample sizes, but our sample sizes were selected based on previous experience from related research and literature. Animals were randomly assigned to control and manipulation groups. Data collection and analysis were not performed blind to the conditions of experiments. Figures were plotted using Prism version 10 (GraphPad) or MATLAB 2023a (MathWorks). Data is reported as violin plots, or mean ± SEM plots. Statistical methods used in this study include two-way repeated measures ANOVA, one-way repeated measures ANOVA, Kruskal-Wallis test, two-sided Mann-Whitney test, two-sided unpaired t test, and two-sided paired t test (see Supplementary Table 1). The data met the assumptions of the statistical tests used. Normality of the data was tested using the Kolmogorov-Smirnov test. Statistically significant differences were established at *P < 0.05, **P < 0.01 and ***P < 0.001; ns indicates not significant. All statistic tests are summarized in the Supplementary Table 1.

Acknowledgements

The authors are supported by the National Science and Technology Innovation 2030 Major Projects of China (STI2030-Major Projects-2022ZD0207300), National Natural Science Foundation of China (No: 82301395), and Fudan University (No: JIF2641002). We also thank Dr. Weizhe Hong from UCLA for insightful advice at initial stage of this study.

Additional information

Author contributions

Conceptualization, R.K.H. and X.H.; formal analysis, R.K.H. and R.H.; investigation, R.H., N.W., T.L., L.Z., S.L.; writing – review & editing, R.K.H. and R.H.; supervision, R.K.H.; funding acquisition, R.K.H.

Declaration of Interests

The authors declare no competing interests.

Data and code availability

A subset of the data and the codes used or generated in this study are available at a Zenodo repository (*https://zenodo.org/records/12747284*). Or directly request data and codes by contacting the corresponding author (hurongfeng@fudan.edu.cn).

References

1. Yau J.O.Y., McNally G.P (2019) **Rules for aversive learning and decision-making** *Curr Opin Behav Sci* **26**:1–8 https://doi.org/10.1016/j.cobeha.2018.08.006

2. Likhtik E., Johansen J.P (2019) **Neuromodulation in circuits of aversive emotional learning** *Nature Neuroscience* **22**:1586–1597

3. Ozawa T., Johansen J.P (2018) **Learning rules for aversive associative memory formation** *Curr Opin Neurobiol* **49**:148–157 https://doi.org/10.1016/j.conb.2018.02.010

4. Fanselow M.S., Poulos A.M (2005) **The neuroscience of mammalian associative learning** *Annu Rev Psychol* **56**:207–234 https://doi.org/10.1146/annurev.psych.56.091103.070213

5. Malezieux M., Klein A.S., Gogolla N (2023) **Neural Circuits for Emotion** *Annu Rev Neurosci* **46**:211–231 https://doi.org/10.1146/annurev-neuro-111020-103314

6. Kredlow M.A., Fenster R.J., Laurent E.S., Ressler K.J., Phelps E.A (2022) **Prefrontal cortex, amygdala, and threat processing: implications for PTSD** *Neuropsychopharmacol* **47**:247–259 https://doi.org/10.1038/s41386-021-01155-7

7. Pardi M.B., Vogenstahl J., Dalmay T., Spanò T., Pu D.L., Naumann L.B., Kretschmer F., Sprekeler H., Letzkus J.J (2020) **A thalamocortical top-down circuit for associative memory** *Science* **370**:844–848 https://doi.org/10.1126/science.abc2399

8. Mobbs D (2018) **The ethological deconstruction of fear(s)** *Curr Opin Behav Sci* **24**:32–37 https://doi.org/10.1016/j.cobeha.2018.02.008

9. Ma J., du Hoffmann J., Kindel M., Beas B.S., Chudasama Y., Penzo M.A. (2021) **Divergent projections of the paraventricular nucleus of the thalamus mediate the selection of passive and active defensive behaviors** *Nat Neurosci* **24**:1429–1440 https://doi.org/10.1038/s41593-021-00912-7

10. Trusel M., Nuno-Perez A., Lecca S., Harada H., Lalive A.L., Congiu M., Takemoto K., Takahashi T., Ferraguti F., Mameli M (2019) **Punishment-Predictive Cues Guide Avoidance through Potentiation of Hypothalamus-to-Habenula Synapses** *Neuron* **102** https://doi.org/10.1016/j.neuron.2019.01.025

11. Corrigan B.W., Gulli R.A., Doucet G., Roussy M., Luna R., Pradeepan K.S., Sachs A.J., Martinez-Trujillo J.C (2022) **Distinct neural codes in primate hippocampus and lateral prefrontal cortex during associative learning in virtual environments** *Neuron* **110**:2155–2269 https://doi.org/10.1016/j.neuron.2022.04.016

12. Steinberg E.E. *et al.* (2020) **Amygdala-Midbrain Connections Modulate Appetitive and Aversive Learning** *Neuron* **106**:1026–1043 https://doi.org/10.1016/j.neuron.2020.03.016

13. Morse A.K., Leung B.K., Heath E., Bertran-Gonzalez J., Pepin E., Chieng B.C., Balleine B.W., Laurent V (2020) **Basolateral Amygdala Drives a GPCR-Mediated Striatal Memory Necessary for Predictive Learning to Influence Choice** *Neuron* **106**:855–869 https://doi.org/10.1016/j.neuron.2020.03.007

14. Fadok J.P. *et al.* (2017) **A competitive inhibitory circuit for selection of active and passive fear responses** *Nature* **542**:96–100 https://doi.org/10.1038/nature21047

15. Kajs B.L., Loewke A.C., Dorsch J.M., Vinson L.T., Gunaydin L.A (2022) **Divergent encoding of active avoidance behavior in corticostriatal and corticolimbic projections** *Sci Rep-Uk* **12** https://doi.org/10.1038/s41598-022-14930-3

16. Tovote P. *et al.* (2016) **Midbrain circuits for defensive behaviour** *Nature* **534**:206–212 https://doi.org/10.1038/nature17996

17. Saha S., Datta S (2005) **Two-way active avoidance training-specific increases in phosphorylated cAMP response element-binding protein in the dorsal hippocampus, amygdala, and hypothalamus** *Eur J Neurosci* **21**:3403–3414 https://doi.org/10.1111/j.1460-9568.2005.04166.x

18. Asdourian D., Dark J.G., Chiodo L., Papich P.S (1977) **Active-Avoidance in Rats with Unilateral Hypothalamic and Optic-Nerve Lesions** *Physiol Behav* **19**:209–212 https://doi.org/10.1016/0031-9384(77)90329-8

19. Asdourian D., Dark J., Chiodo L., Papich P (1976) **Active-Avoidance Performance of Rats with Lateral Hypothalamic and Optic-Nerve Lesions** *B Psychonomic Soc* **8**:262–262

20. Smith G.P., Levin B.E., Ervin G.N (1975) **Loss of Active Avoidance Responding after Lateral Hypothalamic Injections of 6-Hydroxydopamine** *Brain Res* **88**:483–498 https://doi.org/10.1016/0006-8993(75)90659-9

21. Levin B.E., Smith G.P (1972) **Impaired Learning of Active Avoidance Response in Rats after Lateral Hypothalamic Injection of 6-Hydroxydopamine** *Neurology* **22**

22. Shah T., Dunning J.L., Contet C (2022) **At the heart of the interoception network: Influence of the parasubthalamic nucleus on autonomic functions and motivated behaviors** *Neuropharmacology* **204** https://doi.org/10.1016/j.neuropharm.2021.108906

23. Goto M., Swanson L.W (2004) **Axonal projections from the parasubthalamic nucleus** *J Comp Neurol* **469**:581–607 https://doi.org/10.1002/cne.11036

24. Dunning J.L., Krull C., Kreifeldt M., Angelo M., Ramakrishnan C., Deisseroth K., C., C (2023) **The parasubthalamic nucleus refeeding ensemble delays feeding initiation** *BioRxiv* https://doi.org/10.1101/2023.01.28.525750

25. Sanchez M.R., Wang Y.S., Cho T.F.Y.I., Schnapp W.B., Schmit M., Fang C.H., Cai H.J (2022) **Dissecting a disynaptic central amygdala-parasubthalamic nucleus neural circuit that mediates cholecystokinin-induced eating suppression** *Mol Metab* **58** https://doi.org/10.1016/j.molmet.2022.101443

26. Nagashima T., Tohyama S., Mikami K., Nagase M., Morishima M., Kasai A., Hashimoto H., Watabe A.M (2022) **Parabrachial-to-parasubthalamic nucleus pathway mediates fear-induced suppression of feeding in male mice** *Nat Commun* **13**

27. Barbier M., Chometton S., Pautrat A., Miguet-Alfonsi C., Datiche F., Gascuel J., Fellmann D., Peterschmitt Y., Coizet V., Risold P.Y (2020) **A basal ganglia-like cortical-amygdalar-hypothalamic network mediates feeding behavior** *P Natl Acad Sci USA* **117**:15967–15976 https://doi.org/10.1073/pnas.2004914117

28. Zhang T., Perkins M.H., Chang H., Han W.F., de Araujo I.E. (2022) **An inter-organ neural circuit for appetite suppression** *Cell* **185**:2478–2494 https://doi.org/10.1016/j.cell.2022.05.007

29. Kim J.H. *et al.* (2022) **A discrete parasubthalamic nucleus subpopulation plays a critical role in appetite suppression** *Elife* **11** https://doi.org/10.7554/eLife.75470

30. Dunning J.L., Kreifeldt M., Okhuarobo A., Lopez C., Krull C., Angelo M., Mehta M., Shah T., Contet C (2023) **Differential Control of Alcohol Consumption by Neuronal Subpopulations of the Mouse Parasubthalamic Nucleus** *Alcohol* **109**:88–88

31. Dunning J.L., Kreifeldt M., Okhuarobo A., Macedo G.C., Lopez C., Krull C., Angelo M., Zhu S., Shah T., Contet C (2022) **Differential Control of Alcohol Consumption by Neuronal Subpopulations of the Mouse Parasubthalamic Nucleus** *Alcohol Clin Exp Res* **46**:135–135

32. Okhuarobo A., Kreifeldt M., de Macedo G.C., Igbe I., Contet C. (2020) **Chemogenetic Activation of Parasubthalamic nucleus Corticotropin-Releasing Factor Neurons recapitulates Alcohol Withdrawal-Related Behavioral Symptomatology** *Faseb J* **34** https://doi.org/10.1096/fasebj.2020.34.s1.09210

33. Contet C (2020) **Early Life Adversity Accelerates Alcohol Intake Escalation in Mice: Sex Specificity and Potential Role of Parasubthalamic Corticotropin Releasing Factor** *Neuropsychopharmacol* **45**:39–40

34. Guo H. *et al.* (2023) **Parasubthalamic calretinin neurons modulate wakefulness associated with exploration in male mice** *Nat Commun* **14** https://doi.org/10.1038/s41467-023-37797-y

35. Wang H. *et al.* (2024) **A molecularly defined amygdala-independent tetra-synaptic forebrain-to-hindbrain pathway for odor-driven innate fear and anxiety** *Nat Neurosci* **27** https://doi.org/10.1038/s41593-023-01562-7

36. Liu C., Lee C.Y., Asher G., Cao L.Q., Terakoshi Y., Cao P., Kobayakawa R., Kobayakawa K., Sakurai K., Liu Q.H (2021) **Posterior subthalamic nucleus (PSTh) mediates innate fear-associated hypothermia in mice** *Nat Commun* **12** https://doi.org/10.1038/s41467-021-22914-6

37. Chometton S., Pedron S., Peterschmitt Y., Van Waes V., Fellmann D., Risold P.Y. (2016) **A premammillary lateral hypothalamic nuclear complex responds to hedonic but not aversive tastes in the male rat** *Brain Struct Funct* **221**:2183–2208 https://doi.org/10.1007/s00429-015-1038-3

38. Caulliez R., Meile M.J., Nicolaidis S (1996) **A lateral hypothalamic D1 dopaminergic mechanism in conditioned taste aversion** *Brain Res* **729**:234–245 https://doi.org/10.1016/0006-8993(96)00426-X

39. Wang F. *et al.* (2023) **A parabrachial to hypothalamic pathway mediates defensive behavior** *Elife* **12** https://doi.org/10.7554/eLife.85450

40. Serra G.P., Guillaumin A., Vlcek B., Delgado-Zabalza L., Ricci A., Rubino E., Dumas S., Baufreton J., Georges F., Wallén-Mackenzie A (2023) **A role for the subthalamic nucleus in aversive learning** *Cell Rep* **42** https://doi.org/10.1016/j.celrep.2023.113328

41. Zhang X.B., van den Pol A.N. (2017) **Rapid binge-like eating and body weight gain driven by zona incerta GABA neuron activation** *Science* **356**:853–858 https://doi.org/10.1126/science.aam7100

42. Zhu Y.J., Nachtrab G., Keyes P.C., Allen W.E., Luo L.Q., Chen X.K (2018) **Dynamic salience processing in paraventricular thalamus gates associative learning** *Science* **362**:423–429 https://doi.org/10.1126/science.aat0481

43. Gunaydin L.A. *et al.* (2014) **Natural Neural Projection Dynamics Underlying Social Behavior** *Cell* **157**:1535–1551 https://doi.org/10.1016/j.cell.2014.05.017

44. Campese V.D., Kim I.T., Kurpas B., Branigan L., Draus C., LeDoux J.E (2020) **Motivational factors underlying aversive Pavlovian-instrumental transfer** *Learn Memory* **27**:477–482 https://doi.org/10.1101/lm.052316.120

45. Vander Weele C.M. *et al.* (2018) **Dopamine enhances signal-to-noise ratio in cortical-brainstem encoding of aversive stimuli** *Nature* **563**:397–401 https://doi.org/10.1038/s41586-018-0682-1

46. Campos C.A., Bowen A.J., Roman C.W., Palmiter R.D (2018) **Encoding of danger by parabrachial CGRP neurons** *Nature* **555**:617–622 https://doi.org/10.1038/nature25511

47. Hu R.K., Zuo Y.N., Ly T., Wang J., Meera P., Wu Y.E., Hong W.Z (2021) **An amygdala-to-hypothalamus circuit for social reward** *Nat Neurosci* **24**:831–842 https://doi.org/10.1038/s41593-021-00828-2

48. Chen P.B., Hu R.F.K., Wu Y.E., Pan L., Huang S., Micevych P.E., Hong W.Z (2019) **Sexually Dimorphic Control of Parenting Behavior by the Medial Amygdala** *Cell* **176** https://doi.org/10.1016/j.cell.2019.01.024

Author information

Ruining Hu*

Department of Psychological Medicine, Zhongshan Hospital, Institute for Translational Brain Research, State Key Laboratory of Medical Neurobiology, MOE Frontiers Center for Brain Science, MOE Innovative Center for New Drug Development of Immune Inflammatory Diseases, Fudan University, Shanghai, China

*These authors contributed equally.

Nannan Wu*

Department of Psychological Medicine, Zhongshan Hospital, Institute for Translational Brain Research, State Key Laboratory of Medical Neurobiology, MOE Frontiers Center for Brain Science, MOE Innovative Center for New Drug Development of Immune Inflammatory Diseases, Fudan University, Shanghai, China

*These authors contributed equally.

Tong Liu*

Department of Psychological Medicine, Zhongshan Hospital, Institute for Translational Brain Research, State Key Laboratory of Medical Neurobiology, MOE Frontiers Center for Brain

Science, MOE Innovative Center for New Drug Development of Immune Inflammatory Diseases, Fudan University, Shanghai, China

*These authors contributed equally.

Liuting Zou
Department of Psychological Medicine, Zhongshan Hospital, Institute for Translational Brain Research, State Key Laboratory of Medical Neurobiology, MOE Frontiers Center for Brain Science, MOE Innovative Center for New Drug Development of Immune Inflammatory Diseases, Fudan University, Shanghai, China

Songjie Lv
Institute for Translational Brain Research, Fudan University, Shanghai, China

Xiao Huang
Department of Psychological Medicine, Zhongshan Hospital, Fudan University, Shanghai, China

For correspondence: huang.xiao@zs-hospital.sh.cn

Rongfeng K Hu
Department of Psychological Medicine, Zhongshan Hospital, Institute for Translational Brain Research, State Key Laboratory of Medical Neurobiology, MOE Frontiers Center for Brain Science, MOE Innovative Center for New Drug Development of Immune Inflammatory Diseases, Fudan University, Shanghai, China
ORCID iD: 0009-0000-0164-6215

For correspondence: hurongfeng@fudan.edu.cn

Editors

Reviewing Editor
Michael McDannald
Boston College, Chestnut Hill, United States of America

Senior Editor
Kate Wassum
University of California, Los Angeles, Los Angeles, United States of America

Reviewer #1 (Public review):

This study is focused on a population of neurons in the mouse parasubthalamic nucleus (pSTN) that express Tackhykinin1 (Tac1). This gene has been used before to target pSTN for functional circuit studies because it is fairly selective for pSTN in this region, though it targets only a subset of pSTN neurons. Prior work has shown that activity in these neurons can impact motivated behaviors, including feeding and drinking behaviors, and that their activity is associated with aversion or avoidance behaviors. While not breaking much new ground, this study adds to that work by making use of a 2-way active avoidance assay, where a CS predicts a US (footshock), that the mice can escape. Using fiber photometry the authors show convincing evidence that Tac1 neurons in pSTN increase their activity in response to a US footshock, and that after some pairings the neurons will start responding to the CS too, though to a lesser extent than the US. Their most important data shows that either ablation or

optogenetic inhibition of these cells can hugely block the active avoidance (escape) behavior, suggesting these neurons are key for the performance of this task, which they interpret as key for learning the task (but see more below). They show that optogenetic stimulation is aversive in a real-time place assay, and when paired with footshock can enhance active avoidance behavior. Finally, they show that Tac1 pSTN axons in PVT recapitulate these effects while showing that axons in CEA or PBN may only recapitulate some of these effects (more below). Overall I think the data is solid and shows that the activity of Tac1 pSTN neurons in the 2 way active avoidance task is causally related to avoidance behavior in the direction that would be predicted by recent literature. However, I think the authors overstate the conclusions in the title, abstract, and text. I do not think the data make a strong case for a role for these cells in learning, at least in any classical sense, as used in the title and abstract and elsewhere. Also the statement in the abstract that the pSTN mediates its effects 'differentially' through its downstream targets is not convincingly supported by data.

Major concerns:

(1) The authors infer that the activity in the Tac1 pSTN neurons is necessary for aversive or avoidance 'learning'. But this is not well defined, what exactly does that mean and what types of evidence would support or falsify such a hypothesis? Moreover, the authors show convincingly, and in line with prior reports, that these cells are activated by aversive stimuli (here footshock), and that activation of these cells is sufficient to induce avoidance behavior. Because manipulation of these cells can serve as a primary negative reinforcer, it becomes even more challenging and important to explain how experiments that manipulate these cells while measuring behavior/performance can discriminate between changes in: (1) primary aversion, (2) motivation to avoid, (3) associative learning, or (4) memory/retrieval. The authors seem to favor #3, but they don't make a clear case for this point of view or else what they mean by 'avoidance learning'. In my opinion, the data do not well discriminate between possibilities 1 through 3. The authors should clarify their logic and temper their conclusions throughout.

(2) Abstract line 37 is not well supported. The authors focus mostly on pSTN projections to PVT and show that the measurements or manipulation of these axons recapitulates the effects seen with pSTN cell bodies. The authors do fewer studies of axons in CeA and PBN, but do find that they can recapitulate the effects with opsin inhibition, but detect no effects with opsin stimulation. However, the lack of effect with opsin stimulation in Figure S7a-e proves very little on its own. It could be technical, due to inadequate expression or functional efficacy. It is not supported by histological and functional evidence that the manipulation was effective. Overall I can only conclude that the projections to these regions might be very similar (based on the inhibition data), or might be a little different. The data are thus inadequate to support the authors' claim that the pSTN mediates learning differentially through its downstream targets.

Other concerns:

(3) Line 93 is not adequately supported by data in Figure 1b. Additional data is needed that shows expression across cases, including any spread that may be visible when zooming out from pSTN. Additional methods are needed to indicate what exclusion criteria were applied and how many mice were excluded. These data could help support the statement on line 93 that expression was largely restricted within pSTN.

(4) From the results and methods it is not clear where the GFP signal would come from in the mice expressing Casp3 for the ablation studies. It is therefore not clear if the absence of GFP should be taken as evidence of cell loss. For example, it is not clear if multiple vectors were used, if volumes and titers were carefully matched between control groups, or if competition/occlusion between AAVs could be ruled out. It is also not clear how this was quantified, that is how many sections/subjects and how counting was done. It is not clear how

long was waited between the AAV infusion, behavior, and euthanasia, perhaps especially important for the ablation done after avoidance learning occurred.

(5) The authors should consider showing individual measurements and not just mean/sem wherever feasible, for example, to support the statement on line 141 that 'all ablated mice showed...'.

(6) S3 is an important control for interpreting data in Figure 2d-i. Something similar is needed to support the inferences made in 2j-u. The very strong effect showing a lack of active avoidance in response to CS or the US when pSTN Tac1 neurons are inhibited during CS or during US suggests that something gross may be going on, such as a gross motor or sensory response that supersedes the effect of footshock. The authors do not comment on whether there are any gross behavioral responses to the inhibition, but an experiment as in S3 is needed, for example, to show that behavior is intact during pSTN inhibition if delivered after the mice already learned to associate CS with US.

(7) The authors use 100 shocks of 0.8 mA for 7 days. I think this is quite strong and in the pSTN inhibition experiments it seems to be functionally 'inescapable' and could thus produce behaviors similar to 'learned helplessness'. Can the authors consider whether this might contribute to the striking findings they observed in their opsin inhibition assays?

(8) The description of the experiment in S5 is inadequate. What are the adjacent areas? Where do the authors see spread? The use of the word 'case' in figure S5 implies an individual case, but the legend says 5 mice were used for 'case 1' and 3 mice were used for 'case 2'. The use of the word 'off-target in the figure implies that the expression was of the intended target. But the text of results and methods implies it was intentional targeting of unnamed and unshown adjacent regions. This should be clarified.

(9) The authors suggest the CPA study is divergent from Serra et al 2023. Though I think this could be due to how the conditioning was done, it would be helpful for the authors to include less processed data. This would aid in possible interpretations for any divergences across studies. Can the authors include raw data (in seconds of time spent) in each compartment for each group across baseline and test days?

https://doi.org/10.7554/eLife.104643.1.sa3

Reviewer #2 (Public review):

Summary:

The manuscript by Hu et. al presents a clearly-designed examination of the role of tachykinin1-expressing neurons in the parasubthalamic nucleus of the lateral posterior hypothalamus (PTSN) in active avoidance learning. These glutamatergic neurons have previously been implicated in responding to negative stimuli. This manuscript expands the current understanding of PTSNTac1 neurons in learned responses to threats by showing their role in encoding and mediating the active avoidance response. The authors first use bulk fiber photometry imaging to show the encoding of the active avoidance procedure, followed by cell-type specific manipulations of PTSNTac1 neurons during active avoidance. Finally, they show that encoding and mediation of active avoidance in a downstream target of PTSNTac1 neurons, the PVT/intermediodorsal nuclei of the dorsal thalamus (IMD), has the same effect as what was discovered in the cell body. This contrasts other output regions of the PTSN, such as the PBN and CeA, which were not found to promote active avoidance learning. The experiments presented were well-designed to support the conclusions of the authors, however, the manuscript is missing several key control experiments and supplemental information to support their main findings.

Strengths:

The manuscript provides information on a brain region and downstream target that mediates active avoidance learning. The manuscript provides valuable information via necessity and sufficiency experiments to show the role of the population of interest (PTSNTac1 neurons) in active avoidance learning. The authors also performed most behavior experiments in male and female mice, with adequate power to address potential sex differences in the control of active avoidance by PTSNTac1 neurons. Finally, the manuscript provides valuable information about the specificity of the PTSNTac1 downstream target in regulating active avoidance learning, identifying the PVT/intermediodorsal nuclei of the dorsal thalamus as the key target and ruling out the PBN and CeA.

Weaknesses:

However, several main conclusions of the paper must be interpreted carefully due to missing or inadequate control experiments and histological verification.

(1) Inadequate presentation of viral localization. The authors state that expression was "largely restricted within PSTN" however there is no quantification of the amount of viral expression beyond the target region. Given that Tac1 is expressed in neighboring regions, it is critical to show the viral expression and fiber implant location data for all animals included in the figures. Furthermore, criteria for inclusion and exclusion based on mistargeting should be delineated. This should also be clearly outlined for the experiments in Figure S5, where "behavioral effects of activation of sparsely Tac1-expressing neurons in two adjacent areas of PSTN" was tested but the location of viral expression in those cases is unclear.

(2) Lack of motion artifact correction with isosbestic signal for GCamp recordings. It is appreciated that the authors included a separate EGFP-expressing group to compare to the GCamp-expressing group, however, additional explanation is required for the methods used to analyze the raw fluorescent signal. Namely, were fluorescent signals isosbestic-corrected prior to calculating ΔF/F? If no isosbestic signal was used to correct motion artifacts within a recording session, additional explanation is needed to explain how this was addressed. The lack of motion artifacts in the EGFP signal in a separate cohort is inadequate to answer this caveat as motion artifacts are within-animal.

(3) Missing control experiment demonstrating intact locomotor performance in caspase ablation experiments. The authors use caspase ablation of PTSNTac1 neurons prior to active avoidance learning to appraise the necessity of this cell population. However, a control experiment showing intact locomotor ability in ablated mice was not performed.

(4) Missing control experiment demonstrating [lack of] valence with PTSN silencing manipulations. The authors performed a real-time and conditioned place preference experiments for ChR2-expressing mice (Fig 3M) and found stimulation to be negatively-valenced and generate an aversive memory, respectively. Absent this control experiment with silencing, an alternative conclusion remains possible that optogenetic silencing via GtACR2 created nonspecific location preferences in the active avoidance apparatus, confounding the interpretation of those results.

(5) Incomplete analysis of sex differences. Data in female mice is conspicuously missing from inhibition experiments. The rationale for exclusion from this dataset would be useful for the interpretation of the other noted sex differences.

https://doi.org/10.7554/eLife.104643.1.sa2

Reviewer #3 (Public review):

Summary:

This study by Hu et al. examined the role of tachykinin1 (Tac1)-expressing neurons in the para subthalamic nucleus (PSTH) in active avoidance of electric shocks. Bulk recording of PSTH Tac1 neurons or axons of these neurons in PVT showed activation of a shock-predicting tone and shock itself. Ablation of these neurons or optogenetic manipulation of these neurons or their projection to PVT suggests the causality of this pathway with the learning of active avoidance.

Strengths:

This work found an understudied pathway potentially important for active avoidance of electric shocks. Experiments were thoroughly done and the presentation is clear. The amount of discussion and references are appropriate.

Weaknesses:

Critical control experiments are missing for most experiments, and statistical tests are not clear or not appropriate in most parts. Details are shown below.

(1) There are some control experiments missing. Notably, optogenetic manipulation is not verified in any experiments. It is important to verify whether neural activation with optogenetic activation is at the physiological level or supra-physiological level, and whether optogenetic inhibition does not cause unwanted activity patterns such as rebound activation at the critical time window.

(2) Neural ablation with caspase was confirmed by GFP expression. However, from the present description, a different virus to express EITHER caspase or GFP was injected, and then the numbers of GFP-expressing neurons were compared. It is not clear how this can detect ablation.

(3) In many places, statistical approaches are not clear from the present figures, figure legends, and Methods. It seems that most statistics were performed by pooling trials, but it is not described, or multiple "n" are described. For example, it is explicitly mentioned in Figure 4H, "n = 3 mice, n = 213 avoidance trials and n = 87 failure trials". The authors should not pool trials, but should perform across-animal tests in this and other figures, and "n" for statistical tests should be clearly described in each plot.

(4) It is also unclear how the test types were selected. For example, in Figure 1K and O with similar datasets, one is examined by a paired test and the other is by an unpaired test. Since each animal has both early vs late trials, and avoidance vs failure trials, paired tests across animals should be performed for both.

(5) It is also strange to show violin plots for only 6 animals. They should instead show each dot for each animal, connected with a line to show consistent increases of activity in late vs early trials and avoidance vs failure trials.

(6) To tell specificity in avoidance learning, it is better to show escape in the current trials with optogenetic manipulation.

(7) For place aversion, % time decrease across days was tested. It is better to show the original number before normalization, as well.

(8) For anatomical results in Figure S6, it is important to show images with lower magnification, too.

(9) Inactivation of either pathway from PSTH to PBN or to CeA also inhibits active avoidance, but the authors conclude that these effects are "partial" compared to the inactivation of PSTH to PVT. It is not clear how the effects were compared since the effects of PSTH-CeA inactivation are quite strong, comparable to PSTH-PVT inactivation by eye. They should quantify the effects to conclude the difference.

(10) Supplementary table 1: as mentioned above, n for statistical tests should be clearer.

https://doi.org/10.7554/eLife.104643.1.sa1

Author response:

> ***Reviewer #1 (Public review):***
>
> *This study is focused on a population of neurons in the mouse parasubthalamic nucleus (pSTN) that express Tackhykinin1 (Tac1). This gene has been used before to target pSTN for functional circuit studies because it is fairly selective for pSTN in this region, though it targets only a subset of pSTN neurons. Prior work has shown that activity in these neurons can impact motivated behaviors, including feeding and drinking behaviors, and that their activity is associated with aversion or avoidance behaviors. While not breaking much new ground, this study adds to that work by making use of a 2-way active avoidance assay, where a CS predicts a US (footshock), that the mice can escape. Using fiber photometry, the authors show convincing evidence that Tac1 neurons in pSTN increase their activity in response to a US footshock, and that after some pairings the neurons will start responding to the CS too, though to a lesser extent than the US. Their most important data shows that either ablation or optogenetic inhibition of these cells can hugely block the active avoidance (escape) behavior, suggesting these neurons are key for the performance of this task, which they interpret as key for learning the task (but see more below). They show that optogenetic stimulation is aversive in a real-time place assay, and when paired with footshock can enhance active avoidance behavior. Finally, they show that Tac1 pSTN axons in PVT recapitulate these effects while showing that axons in CEA or PBN may only recapitulate some of these effects (more below). Overall I think the data is solid and shows that the activity of Tac1 pSTN neurons in the 2 way active avoidance task is causally related to avoidance behavior in the direction that would be predicted by recent literature. However, I think the authors overstate the conclusions in the title, abstract, and text. I do not think the data make a strong case for a role for these cells in learning, at least in any classical sense, as used in the title and abstract and elsewhere. Also, the statement in the abstract that the pSTN mediates its effects 'differentially' through its downstream targets is not convincingly supported by data.*

We are very pleased that Reviewer 1 thought our data is solid.

> *Major concerns:*
>
> *(1) The authors infer that the activity in the Tac1 pSTN neurons is necessary for aversive or avoidance 'learning'. But this is not well defined, what exactly does that mean and what types of evidence would support or falsify such a hypothesis? Moreover, the authors show convincingly, and in line with prior reports, that these cells are activated by aversive stimuli (here footshock), and that activation of these cells is sufficient to induce avoidance behavior. Because manipulation of these cells can serve as a primary negative reinforcer, it becomes even more challenging and important to explain how experiments that manipulate these cells while measuring behavior/performance can discriminate between changes in: (1) primary aversion, (2) motivation to avoid, (3) associative*

> *learning, or (4) memory/retrieval. The authors seem to favor #3, but they don't make a clear case for this point of view or else what they mean by 'avoidance learning'. In my opinion, the data do not well discriminate between possibilities 1 through 3. The authors should clarify their logic and temper their conclusions throughout.*

Thank you Reviewer 1 for providing us insightful suggestions. Based on our fiber photometry data that the activities of PSTN Tac1+ neurons show a significant increase in CS-evoked calcium fluorescent signals in late trials relative to those in early trials (Figure 1H-K) and our optogenetic inhibition experiments during CS (Figure 2N-Q), these results illustrate that the activities of PSTN Tac1+ neurons are modulated by learning and are required for active avoidance learning. Moreover, PSTN Tac1+ neurons are activated by footshock and activation of these cells is sufficient to induce avoidance behavior. These findings demonstrate that PSTN Tac1+ neurons encode aversive information. Together, our current data support that PSTN Tac1+ neurons encode both aversive event and its predicting cue. We will clarify our conclusions in the revised manuscript.

> *(2) Abstract line 37 is not well supported. The authors focus mostly on pSTN projections to PVT and show that the measurements or manipulation of these axons recapitulates the effects seen with pSTN cell bodies. The authors do fewer studies of axons in CeA and PBN, but do find that they can recapitulate the effects with opsin inhibition, but detect no effects with opsin stimulation. However, the lack of effect with opsin stimulation in Figure S7a-e proves very little on its own. It could be technical, due to inadequate expression or functional efficacy. It is not supported by histological and functional evidence that the manipulation was effective. Overall, I can only conclude that the projections to these regions might be very similar (based on the inhibition data), or might be a little different. The data are thus inadequate to support the authors' claim that the pSTN mediates learning differentially through its downstream targets.*

In the revised version of manuscript, we will provide more histological and functional evidence for the PSTN-to-CeA and PSTN-to-PBN circuits to support our conclusion on the functional roles of these downstream targets. Similar with our anterograde experiment that the PSTN densely projects to CeA and PBN (Figure S6), optogenetic activation and inhibition experiments showed dense axonal terminals in the CeA and PBN from the PSTN and this line of data will be included in the revised manuscript. In addition, we will further examine these circuits by investigating the functional roles of CeA-projecting or PBN-Projecting PSTN neurons during 2-way active avoidance task.

> *Other concerns:*
>
> *(3) Line 93 is not adequately supported by data in Figure 1b. Additional data is needed that shows expression across cases, including any spread that may be visible when zooming out from pSTN. Additional methods are needed to indicate what exclusion criteria were applied and how many mice were excluded. These data could help support the statement on line 93 that expression was largely restricted within pSTN.*

In the revised version of manuscript, we will provide larger example images containing pSTN and its adjacent areas to demonstrate that the viral expression is well restricted into this brain area. Moreover, we will provide detailed information on the exclusion criteria and the number of mice excluded in the Method section.

> *(4) From the results and methods it is not clear where the GFP signal would come from in the mice expressing Casp3 for the ablation studies. It is therefore not clear if the absence of GFP should be taken as evidence of cell loss. For example, it is not clear if multiple vectors were used, if volumes and titers were carefully matched between control groups,*

> *or if competition/occlusion between AAVs could be ruled out. It is also not clear how this was quantified, that is how many sections/subjects and how counting was done. It is not clear how long was waited between the AAV infusion, behavior, and euthanasia, perhaps especially important for the ablation done after avoidance learning occurred.*

I totally agree with Reviewer 1's concerns. We will perform immunohistochemistry or in situ hybridization for Tachykinin-1 itself and then measure colocalization of GFP with Tachykinin-1 inside and outside of the PTSN, and the degree of absence of Tachykinin-1 in Casp mice. In addition, we will provide more detailed experimental information in the revised manuscript.

> *(5) The authors should consider showing individual measurements and not just mean/sem wherever feasible, for example, to support the statement on line 141 that 'all ablated mice showed...'.*

Thank you Reviewer 1 for this suggestion. We will re-plot the data as individual measurements in the revised manuscript.

> *(6) S3 is an important control for interpreting data in Figure 2d-i. Something similar is needed to support the inferences made in 2j-u. The very strong effect showing a lack of active avoidance in response to CS or the US when pSTN Tac1 neurons are inhibited during CS or during US suggests that something gross may be going on, such as a gross motor or sensory response that supersedes the effect of footshock. The authors do not comment on whether there are any gross behavioral responses to the inhibition, but an experiment as in S3 is needed, for example, to show that behavior is intact during pSTN inhibition if delivered after the mice already learned to associate CS with US.*

Thank you Reviewer 1 for this insightful suggestion. During the review process, we have performed this line of experiment as in Figure S3. We measured the behavioral responses during pSTN optogenetic inhibition after the mice already learned to associate CS with US and found most GtACR-expressing mice showed unaffected avoidance learning. This data will be included in the revised manuscript.

> *(7) The authors use 100 shocks of 0.8 mA for 7 days. I think this is quite strong and in the pSTN inhibition experiments it seems to be functionally 'inescapable' and could thus produce behaviors similar to 'learned helplessness'. Can the authors consider whether this might contribute to the striking findings they observed in their opsin inhibition assays?*

I agree with the Reviewer 1's comment on the string findings in the optogenetic inhibition results. Indeed, based on the results on days 1 and 2, optogenetic inhibition of PSTN tac1+ neurons has significantly blocked GtACR-expressing animals' behavioral performance during 2-way active avoidance task. To examine whether the effect by optogenetic inhibition of these neurons could possibly decline with prolonged training, we conducted additional 5-day training. We will discuss and add this comment in the revised manuscript.

> *(8) The description of the experiment in S5 is inadequate. What are the adjacent areas? Where do the authors see spread? The use of the word 'case' in figure S5 implies an individual case, but the legend says 5 mice were used for 'case 1' and 3 mice were used for 'case 2'. The use of the word 'off-target in the figure implies that the expression was of the intended target. But the text of results and methods implies it was intentional targeting of unnamed and unshown adjacent regions. This should be clarified.*

We will add histological images and clarify these comments in the revised manuscript. The purpose of this experiment is to illustrate that even slightly spreading ChR2 viruses into Tac1+ neurons of the adjacent areas of the PSTN did not result in behavioral changes and this will indirectly support the main behavioral function caused by the PSTN tac1+ neurons rather than its neighboring areas. Because Tac1+ neurons outside the PSTN are sparsely expressed, it is quite difficult to completely restrict the viral expression in the PSTN from the anterior to the posterior. Thus, we will provide detailed information on the exclusion criteria and the number of mice excluded in the Method section.

> *(9) The authors suggest the CPA study is divergent from Serra et al 2023. Though I think this could be due to how the conditioning was done, it would be helpful for the authors to include less processed data. This would aid in possible interpretations for any divergences across studies. Can the authors include raw data (in seconds of time spent) in each compartment for each group across baseline and test days?*

We will follow Reviewer 1's suggestion to include raw data (in seconds of time spent) in each compartment for each group across baseline and test days in the revised manuscript.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The manuscript by Hu et. al presents a clearly-designed examination of the role of tachykinin1-expressing neurons in the parasubthalamic nucleus of the lateral posterior hypothalamus (PTSN) in active avoidance learning. These glutamatergic neurons have previously been implicated in responding to negative stimuli. This manuscript expands the current understanding of PTSNTac1 neurons in learned responses to threats by showing their role in encoding and mediating the active avoidance response. The authors first use bulk fiber photometry imaging to show the encoding of the active avoidance procedure, followed by cell-type specific manipulations of PTSNTac1 neurons during active avoidance. Finally, they show that encoding and mediation of active avoidance in a downstream target of PTSNTac1 neurons, the PVT/intermediodorsal nuclei of the dorsal thalamus (IMD), has the same effect as what was discovered in the cell body. This contrasts other output regions of the PTSN, such as the PBN and CeA, which were not found to promote active avoidance learning. The experiments presented were well-designed to support the conclusions of the authors, however, the manuscript is missing several key control experiments and supplemental information to support their main findings.*
>
> *Strengths:*
>
> *The manuscript provides information on a brain region and downstream target that mediates active avoidance learning. The manuscript provides valuable information via necessity and sufficiency experiments to show the role of the population of interest (PTSNTac1 neurons) in active avoidance learning. The authors also performed most behavior experiments in male and female mice, with adequate power to address potential sex differences in the control of active avoidance by PTSNTac1 neurons. Finally, the manuscript provides valuable information about the specificity of the PTSNTac1 downstream target in regulating active avoidance learning, identifying the PVT/intermediodorsal nuclei of the dorsal thalamus as the key target and ruling out the PBN and CeA.*

We highly appreciate that Reviewer 2 thought that our experiments presented were well-designed to support the conclusions and provided valuable information in several aspects.

> *Weaknesses:*
>
> *However, several main conclusions of the paper must be interpreted carefully due to missing or inadequate control experiments and histological verification.*
>
> *(1) Inadequate presentation of viral localization. The authors state that expression was "largely restricted within PSTN" however there is no quantification of the amount of viral expression beyond the target region. Given that Tac1 is expressed in neighboring regions, it is critical to show the viral expression and fiber implant location data for all animals included in the figures. Furthermore, criteria for inclusion and exclusion based on mistargeting should be delineated. This should also be clearly outlined for the experiments in Figure S5, where "behavioral effects of activation of sparsely Tac1-expressing neurons in two adjacent areas of PSTN" was tested but the location of viral expression in those cases is unclear.*

Similar with questions 3 and 8 of Reviewer 1. We will provide the viral expression and fiber implant location data for all animals included in the figures and histological images in Figure S5 in the revised manuscript. Moreover, we will provide detailed information on the exclusion criteria and the number of mice excluded in the Method section.

> 1. *Lack of motion artifact correction with isosbestic signal for GCamp recordings. It is appreciated that the authors included a separate EGFP-expressing group to compare to the GCamp-expressing group, however, additional explanation is required for the methods used to analyze the raw fluorescent signal. Namely, were fluorescent signals isosbestic-corrected prior to calculating ΔF/F? If no isosbestic signal was used to correct motion artifacts within a recording session, additional explanation is needed to explain how this was addressed. The lack of motion artifacts in the EGFP signal in a separate cohort is inadequate to answer this caveat as motion artifacts are within-animal.*

We will follow Reviewer 2's suggestion and perform isosbestic-correction for fluorescent signals prior to calculating ΔF/F. We will re-plot related figures and add this information in the revised manuscript.

> *(3) Missing control experiment demonstrating intact locomotor performance in caspase ablation experiments. The authors use caspase ablation of PTSNTac1 neurons prior to active avoidance learning to appraise the necessity of this cell population. However, a control experiment showing intact locomotor ability in ablated mice was not performed.*

We will follow Reviewer 2's suggestion to perform a control experiment showing intact locomotor ability in caspase 3-ablated mice and will include this data in the revised manuscript.

> *(4) Missing control experiment demonstrating [lack of] valence with PTSN silencing manipulations. The authors performed a real-time and conditioned place preference experiments for ChR2-expressing mice (Fig 3M) and found stimulation to be negatively-valenced and generate an aversive memory, respectively. Absent this control experiment with silencing, an alternative conclusion remains possible that optogenetic silencing via GtACR2 created nonspecific location preferences in the active avoidance apparatus, confounding the interpretation of those results.*

Thank you Reviewer 2 for this useful suggestion. We will examine the valence with PTSN silencing manipulations by using a RTPP test and add this data in the revised manuscript.

> *(5) Incomplete analysis of sex differences. Data in female mice is conspicuously missing from inhibition experiments. The rationale for exclusion from this dataset would be useful for the interpretation of the other noted sex differences.*

Thank you Reviewer 2 for this useful suggestion. During the review process, we have performed ablation and inhibition experiments in females, demonstrating similar behavioral effects as those in males. We will add these data in the revised manuscript.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *This study by Hu et al. examined the role of tachykinin1 (Tac1)-expressing neurons in the para subthalamic nucleus (PSTH) in active avoidance of electric shocks. Bulk recording of PSTH Tac1 neurons or axons of these neurons in PVT showed activation of a shock-predicting tone and shock itself. Ablation of these neurons or optogenetic manipulation of these neurons or their projection to PVT suggests the causality of this pathway with the learning of active avoidance.*
>
> *Strengths:*
>
> *This work found an understudied pathway potentially important for active avoidance of electric shocks. Experiments were thoroughly done and the presentation is clear. The amount of discussion and references are appropriate.*

We are very pleased to have Reviewer 3's positive comments on the manuscript.

> *Weaknesses:*
>
> *Critical control experiments are missing for most experiments, and statistical tests are not clear or not appropriate in most parts. Details are shown below.*
>
> *(1) There are some control experiments missing. Notably, optogenetic manipulation is not verified in any experiments. It is important to verify whether neural activation with optogenetic activation is at the physiological level or supra-physiological level, and whether optogenetic inhibition does not cause unwanted activity patterns such as rebound activation at the critical time window.*

Thank you Reviewer 3 for this useful suggestion. We will perform in vitro slice recording experiments to verify optogenetic manipulations and add this line of evidence in the revised manuscript.

> *(2) Neural ablation with caspase was confirmed by GFP expression. However, from the present description, a different virus to express EITHER caspase or GFP was injected, and then the numbers of GFP-expressing neurons were compared. It is not clear how this can detect ablation.*

Similar with question 4 of Reviewer 1. We will perform immunohistochemistry or in situ hybridization for Tachykinin-1 itself and then measure colocalization of GFP with Tachykinin-1 inside and outside of the PTSN, and the degree of absence of Tachykinin-1 in Casp-ablated mice. In addition, we will provide more detailed experimental information in the revised manuscript.

> *(3) In many places, statistical approaches are not clear from the present figures, figure legends, and Methods. It seems that most statistics were performed by pooling trials, but*

> *it is not described, or multiple "n" are described. For example, it is explicitly mentioned in Figure 4H, "n = 3 mice, n = 213 avoidance trials and n = 87 failure trials". The authors should not pool trials, but should perform across-animal tests in this and other figures, and "n" for should be clearly described in each plot.*

We have provided all statistical information in the Supplementary Table 1. In the revised manuscript, we will perform across-animal tests, re-plot new figures and provide clear statistical information.

> *(4) It is also unclear how the test types were selected. For example, in Figure 1K and O with similar datasets, one is examined by a paired test and the other is by an unpaired test. Since each animal has both early vs late trials, and avoidance vs failure trials, paired tests across animals should be performed for both.*

Following Reviewer 3's suggestion, we will perform across-animal tests. In the first version of our manuscript, for fiber photometry experiments, we pooled trial data of each animal and performed statistics tests across trials. Because avoidance and failure trials were different, we thus selected an unpaired test for this kind of dataset.

> *(5) It is also strange to show violin plots for only 6 animals. They should instead show each dot for each animal, connected with a line to show consistent increases of activity in late vs early trials and avoidance vs failure trials.*

Similar with question 4 of Reviewer 3, we pooled trial data of each animal and performed statistics tests across trials. We will perform across-animal tests and re-plot figures by connecting with a line to show consistent increases of activity in late vs early trials and avoidance vs failure trials for each animal.

> *(6) To tell specificity in avoidance learning, it is better to show escape in the current trials with optogenetic manipulation.*

Thank you Reviewer 3 for this useful suggestion. We will follow this suggestion and add this analysis in the revised manuscript.

> *(7) For place aversion, % time decrease across days was tested. It is better to show the original number before normalization, as well.*

Similar with question 9 of Reviewer 1, we will show the original number before normalization in the revised manuscript.

> *(8) For anatomical results in Figure S6, it is important to show images with lower magnification, too.*

We will follow this suggestion and provide histological images with lower magnification in the revised manuscript.

> *(9) Inactivation of either pathway from PSTH to PBN or to CeA also inhibits active avoidance, but the authors conclude that these effects are "partial" compared to the inactivation of PSTH to PVT. It is not clear how the effects were compared since the effects of PSTH-CeA inactivation are quite strong, comparable to PSTH-PVT inactivation by eye. They should quantify the effects to conclude the difference.*

We will quantify the effects of different downstream targets of the PSTN to make a precise conclusion.

(10) Supplementary table 1: as mentioned above, n for statistical tests should be clearer.

As mentioned above, we will perform across-animal tests and provide clear statistical information in the figure legends and supplementary table 1.

https://doi.org/10.7554/eLife.104643.1.sa0