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Repeated activation of preoptic area-recipient neurons in posterior paraventricular nucleus mediates chronic heat-induced negative emotional valence and hyperarousal states

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eLife Assessment

This **important** study identifies one way in which episodic heat exposure can result in negative changes in motivated and affective behaviors. This work positively expands the field of thermoregulation. The data were collected using a myriad of next-generation approaches, including extensive behavior testing, thermal monitoring, electrophysiology, circuit mapping, and manipulations. There is **convincing** evidence that neurons of the paraventricular thalamus change plastically over three weeks of episodic heat stimulation this affects behavioral outputs such as social interactions and anxiety-related behavior. Conclusions regarding the specificity of the POA-pPVT pathway compared to other inputs to the PVT in the control of observed effects would benefit from further validation. The study will be of interest to behavioral neuroscientists, climate/environmental biologists, and pre-clinical neuropsychiatrists.

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Abstract

Mental and behavioral disorders are associated with extended period of hot weather as found in heatwaves, but the underlying neural circuit mechanism is poorly known. The posterior paraventricular thalamus (pPVT) is a hub for emotional processing and receives inputs from the hypothalamic preoptic area (POA), the well-recognized thermoregulation center. The present study was designed to explore whether chronic heat exposure leads to aberrant activities in POA recipient pPVT neurons and subsequent changes in emotional states. By devising an air heating paradigm mimicking the condition of heatwaves and utilizing emotion-related behavioral tests, viral track tracing, *in vivo* calcium recordings, optogenetic manipulations and electrophysiological recordings, we found that chronic heat exposure for 3 weeks led to negative emotional valence and hyperarousal states in mice. The

pPVT neurons receive monosynaptic excitatory and inhibitory innervations from the POA. These neurons exhibited a persistent increase in neural activity following chronic heat exposure, which was essential for chronic heat-induced emotional changes. Notably, these neurons were also prone to display stronger neuronal activities associated with anxiety responses to stressful situations. Furthermore, we observed saturated neuroplasticity in the POA-pPVT excitatory pathway after chronic heat exposure that occluded further potentiation. Taken together, long-term aberration in the POA to pPVT pathway offers a neurobiological mechanism of emotional and behavioral changes seen in extended periods of hot weather like heatwaves.

Introduction

Against a backdrop of global warming, heatwaves that are characterized by abnormally hot weather for extended periods have become more frequent and intense. This is highlighted by that the July 2023 was the hottest month ever recorded by human (Tollefson, 2023). Traditional views support that repetitive heat exposure and the related heat acclimation have some beneficial effects in allowing the animals and humans to gain heat tolerance, such as strengthening the cardiovascular system, reducing energy metabolism and weight (Murray KO, et al., 2022; Podstawski R, et al., 2021; Périard et al., 2016; Fausnacht DW, et al., 2021). However, many studies have also established a correlation between heatwaves and impaired physical health leading to increased mortality (Barriopedro et al., 2011; Garcia-Herrera et al., 2010; Mitchell et al., Mora et al., 2017; 2016; Robine et al., 2008). In addition, elevated temperatures could impact mental health by triggering feelings of anger, stress, aggression, and depression (Beecher et al., 2016). In fact, epidemiological research conducted across various regions also extensively demonstrated a positive association between chronic heat exposure and higher hospital admissions for mental and behavioral disorders (Amr et al., 2012; Basu et al., 2018; Dominiak et al., 2015; Hansen et al., 2008; Lee et al., 2002; Mulder et al., 1990; Trang et al., 2015; Whitney et al., 1999). Nevertheless, our understanding of how the brain regulates emotional changes following prolonged heat exposure remains limited.

Current understanding about heat-induced emotional changes mainly focuses on the impact of heat stress on the hypothalamic-pituitary-adrenal (HPA) axis, which leads to abnormal plasma concentrations of the hormone cortisol and neurotransmitters such as 5-hydroxytryptamine (5-HT), noradrenaline and adrenaline, all of which play a significant role in modulating emotional states (McMorris et al., 2006; Wang et al., 2015). However, how heat affects emotions from the perspective of neural circuits has not been substantiated. Moreover, the relationship between chronic heat exposure and emotional changes is likely to be more complex. Multifaceted factors, such as the levels of physiological stress (Sampath et al., 2023), changes of neuroendocrine system (Podstawski et al., 2021), sleep disturbances (Altena et al., 2022), adaptation process (Oppermann et al., 2021), even the age and individual health status (Kenny et al., 2010; Malmquist et al., 2022), could interact in a various way to directly and indirectly contribute to emotional changes in response to chronic heat exposure. Thus, gaining insights into the circuitry mechanisms behind emotional changes caused by chronic heat exposure is crucial for a more comprehensive understanding of the neurobiological foundations of emotional regulation.

Emerging evidence has suggested that the paraventricular thalamus (PVT) serves as an important integrative node that detects aversive sensory and homeostatic challenges and regulates emotional responses and adaptive behaviors. As such, PVT neurons receive inputs from diverse nuclei in the hypothalamus, midbrain, and hindbrain, and has been implicated in a variety of behavioral responses including arousal (Ren et al., 2018), pain (Jurik et al., 2015), anxiety (Heilbronner et al., 2004; Li et al., 2010; Spencer et al., 2004), fear and fear memory (Do-Monte et al., 2015; Penzo et al., 2021). Therefore, PVT is now considered as an essential component of the emotional processing system in the brain (Penzo et al., 2021).

Among the various inputs from different brain regions to the PVT, the hypothalamus preoptic area (POA) is a well-known thermoregulatory center of the brain (Yu et al., 2016; Zhang et al., 2011; Zhao et al., 2016). Anatomically, the posterior PVT (pPVT) has been reported to receive projections from POA warm-sensitive neurons (Tan et al., 2016). Here, we hypothesize that the direct projection from POA to pPVT contributes to chronic heat-induced emotional disturbances. Based on the murine model, we dissected the synaptic connection from POA to pPVT, tracked its activities and interrogated its involvement in behavioral responses of the animals under acute and chronic heat exposure paradigms. Our results uncovered long-term aberration in the activity of this pathway and pPVT neurons mediating chronic heat-induced changes in emotional and hyperarousal states, providing a neurobiological basis of how chronic heat affects mental states.

Results

Chronic heat produces negative emotional valence and hyperarousal states but not depression-like behaviors in mice

c57BL/6 mice were exposed to chronic heat by putting them into a pre-heated (38 ± 2°C) chamber daily for 21 days with free access of food and water (details in Materials and Methods). In our preliminary experiments, various durations of daily heat exposure were tested. We settled to 90 minutes due to its robustness in inducing emotional state-related behavioral changes without causing the collapse of the animals. Throughout the entire process of chronic heat exposure, we monitored the physiological states of mice daily. We did not observe abnormal changes in body temperature the day after chronic heat exposure (**Figure 1**-figure supplement 1A and B) but affected normal gain in body weight (**Figure 1**-figure supplement 1C and D) and reduced food consumption (**Figure 1**-figure supplement 1E and F), compared with control mice. On the other hand, mice subjected to chronic heat displayed a higher prevalence of stress responses in various behavioral tests. Specifically, in the elevated plus maze test, chronic heat-exposed mice spent less time in the open arms (**Figure 1B and C**), indicating increased stress levels. Moreover, in the three-chamber test, these mice displayed reduced interests in exploring the unfamiliar male mouse compared to the inanimate object (**Figure 1D and E**), suggesting decreased sociability. Similarly, during the female encounter test, these mice performed less engagement with the unfamiliar female mouse compared with the unfamiliar male mouse (**Figure 1F and G**), indicating decreased innate motivation. Interestingly, the chronic heat-exposed mice displayed decreased latencies for the first attack but increased attack durations in the resident-intruder test (**Figure 1H and I**), indicating elevated aggression levels.

We also observed that after chronic heat exposure, the mice displayed heightened alertness, suggesting a state of hyperarousal. To confirm this, we quantified the acoustic startle response of the animals by digital video capture of their rapid movements (Pantoni et al., 2020). Chronic heat-exposed mice displayed amplified acoustic startle response characterized by increased body fluctuations within 200 ms of the delivery of a 105 dB sound stimulus (**Figure 1J-M** and Figure 1-video supplement 1). However, these mice did not exhibit clear hyper-locomotion during the three-chamber test, the female encounter test, or the open field test (**Figure 1**-figure supplement 1G-I), suggesting increased agitation in mice. Collectively, our data demonstrate that chronic heat exposure triggers increased stress responses as well as increased arousal in mice.

The fact that the observed changes in emotional and arousal states are the results of long-term rather than short-term heat exposure is supported by the lack of effect measured one day following one-time acute heat exposure for 90 mins (**Figure 1**-figure supplement 2). Furthermore, we did not observe signs of depression in chronic heat-exposed mice, as there were no significant increases in immobile time during forced swimming (**Figure 1N**) and the tail

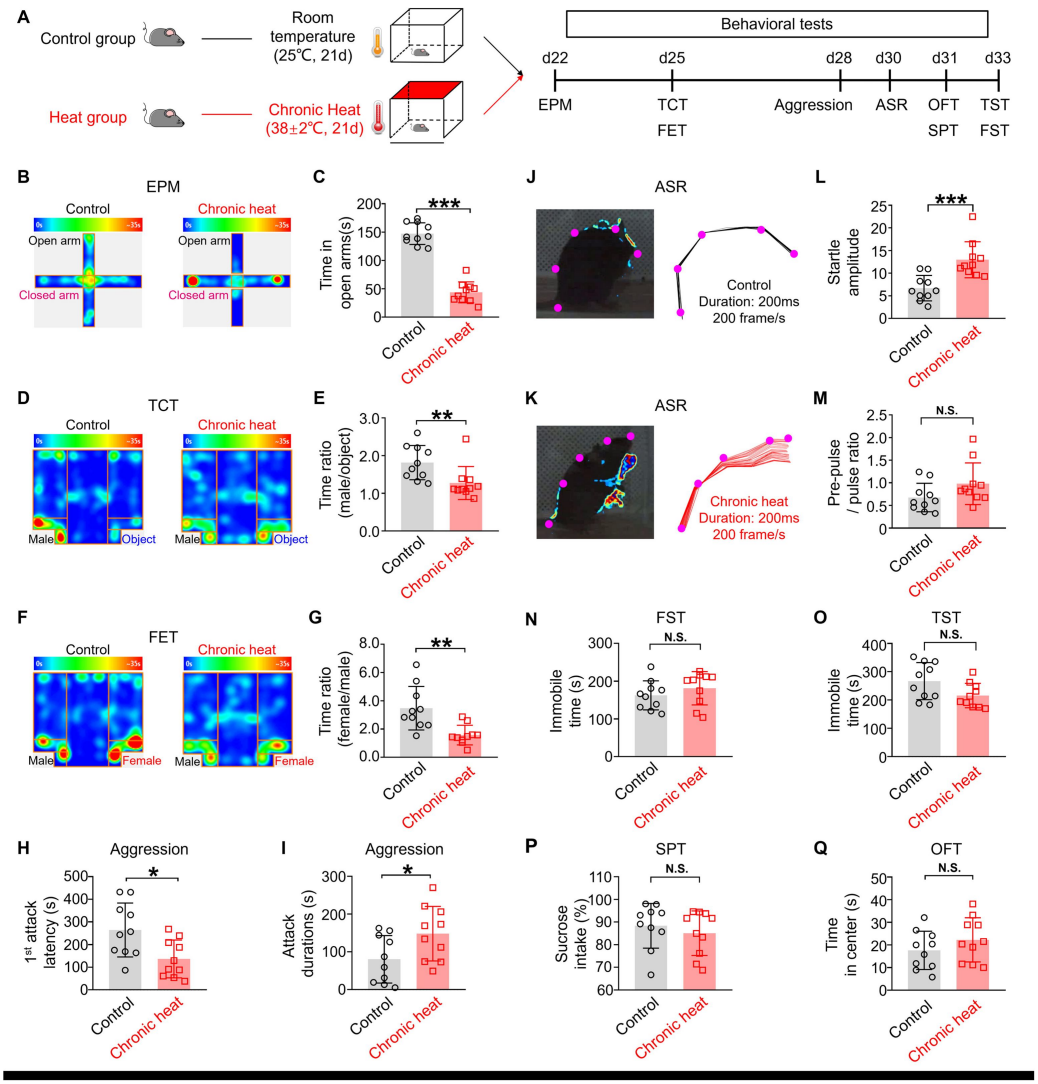


Figure 1.

Chronic heat exposure produces negative emotional valence and hyperarousal states but not depression-like behaviors.

(A) Experimental schematics. Mice (n=10 in each group) were divided into Control and Heat groups and conducted with chronic exposure to room temperature and heat conditions, respectively, followed by behavioral tests. **(B, C)** The heatmap of representative tracking trace examples in elevated plus maze (EPM) test and the time spent in the open arms of EPM (Mann-Whitney unpaired two-tailed U test; U=0, ***p<0.001). **(D, E)** The heatmap of representative tracking trace examples in three-chamber test (TCT) and the interaction time with an unfamiliar male mouse relative to the inanimate object in TCT (Mann-Whitney unpaired two-tailed U test; U=13, ***p=0.0039). **(F, G)** The heatmap of representative tracking trace examples in female encounter test (FET) and the time surrounding the unfamiliar female mouse compared to an unfamiliar male mouse in FET (Mann-Whitney unpaired two-tailed U test; U=10, **p=0.0015). **(H, I)** The 1st-time attack latency (Mann-Whitney unpaired two-tailed U test; U=21, *p=0.0288) and the attack durations (Mann-Whitney unpaired two-tailed U test; U=23, *p=0.0433) in the aggression test. **(J, K)** Visualized and representative acoustic startle response example (left panel) and corresponding labeled body parts' skeletons (shown as purple dots) (right panel) in acoustic startle response (ASR) test from Control and Chronic Heat group.**(L, M)** The startle amplitude (Mann-Whitney unpaired two-tailed U test; U=6, ***p=0.0003) and the pre-pulse / pulse ratio (Mann-Whitney unpaired two-tailed U test; U=26, p=0.0753) in ASR test. **(N-Q)** The immobile time in the forced swim test (FST) (Mann-Whitney unpaired two-tailed U test; U=35, p=0.2799) and in the tail suspension test (TST) (Mann-Whitney unpaired two-tailed U test; U=26, p=0.0753; The percentage of sucrose intake in the sucrose preference test (SPT) (Mann-Whitney unpaired two-tailed U test; U=49.5, p=0.4887); The time spent in the center of the open field test (OFT) (Mann-Whitney unpaired two-tailed U test; U=35.5, p=0.2888). *p<0.05, **p<0.01, ***p<0.001, N.S.: not significant.

suspension tests (**Figure 1O**), no obvious decrease in sucrose intake (**Figure 1P**), and no sign of decreased exploration as there was no change in the time spent by these animals in the center zone of the open field (**Figure 1Q**).

Involvement of the hypothalamic preoptic area to posterior paraventricular thalamus projection

To explore the involvement of POA and pPVT in chronic heat exposure induced behavioral changes, we first studied the effect of heat treatment *per se* on their activities via c-Fos staining. We found that acute heat treatment (for 90 mins) significantly increased c-Fos expressions of POA and pPVT neurons (**Figure 2**-figure supplement 1A-C), supporting not only the notion that the POA serves as a thermoregulation center (Morrison et al., 2011) but also the recruitment of pPVT following heat treatment. Next, to dissect the anatomical relationship between POA and pPVT especially in the context of heat exposure, we injected viruses (AAV1-hSyn-cre, AAV-FLEX-TVA-mCherry, AAV-FLEX-RG and subsequently SAD△G (EnvA) virus) of a monosynaptic retrograde tracing rabies virus system (details in Materials and Methods) into pPVT, which resulted in clear expression of retrogradely-labeled neurons within POA, its upstream area (**Figure 2A-C**). Moreover, a significant overlap (84 ± 5%, 3 mice) was observed between the retrogradely-labeled and c-Fos expressed neurons in POA following heat exposure (**Figure 2D**). Furthermore, almost all the POA recipient pPVT neurons identified by a Cre-dependent anterograde labelling strategy (93 ± 2%, 3 mice) were strongly activated by heat exposure (**Figure 2**-figure supplement 2). Together, our results indicate that most of the pPVT-projecting POA neurons were neurons that respond to heat treatment which would then recruit their downstream neurons in pPVT by exerting a net excitatory influence.

To functionally characterize the synaptic connection between POA and pPVT, we administered AAV9-hSyn-ChR2-mCherry into the POA for optogenetic manipulation of its neurons and their terminals in pPVT. By patch-clamp recordings in brain slices prepared from these animals, we first confirmed robust firing of ChR2-mCherry-expressing neurons in POA upon light stimulation at various frequencies (**Figure 2E**). When we applied 473 nm light stimulation to POA terminals in pPVT, in some neurons, a clear inhibitory outward postsynaptic current was recorded in pPVT neurons held at +10 mV, which was sensitive to picrotoxin (**Figure 2F**). In some other neurons, an inward excitatory postsynaptic current sensitive to CNQX was recorded when the neurons were held at –70 mV (**Figure 2G**). Quantification of the presence of these light-evoked postsynaptic currents revealed the presence of both excitatory and inhibitory currents in 34.9% of neurons recorded, only inhibitory currents in 52.4% of neurons and only excitatory currents in 12.7% (**Figure 2H**). Since these currents could be completely blocked by tetrodotoxin (TTX) but restored with the addition of 4-aminopyridine (4-AP), it indicated the monosynaptic connection, (an example shown in **Figure 2I-K**). Collectively, our findings demonstrate that heat responsive POA neurons project directly to pPVT via both excitatory and inhibitory innervations.

Activity changes of POA recipient pPVT neurons throughout chronic heat exposure

We speculated that repeated activation of POA recipient pPVT neurons underline the emotional valence changes observed following chronic heat exposure. To gain support for this hypothesis, we first monitored the activity changes of these neurons during the course of chronic heat exposure by *in vivo* fibre photometry of calcium signals (**Figure 3A and B**). We injected AAV1-hSyn-Cre-EGFP into POA and Cre-dependent AAV9-hSyn-Flex-jGCaMP8F into pPVT and implanted optical fibers into pPVT neurons after enough expression of GCaMP. The fibre photometry recordings revealed fluctuations in signals composed of spontaneous events (details in Materials and Methods) superimposed on the baseline. The detected spontaneous calcium events presumably represent brief trains or bursts of neuronal firing (Ali et al., 2020). We first confirmed that the signals were stable for extended period (at least 21 days) in a group of mice not undergoing any

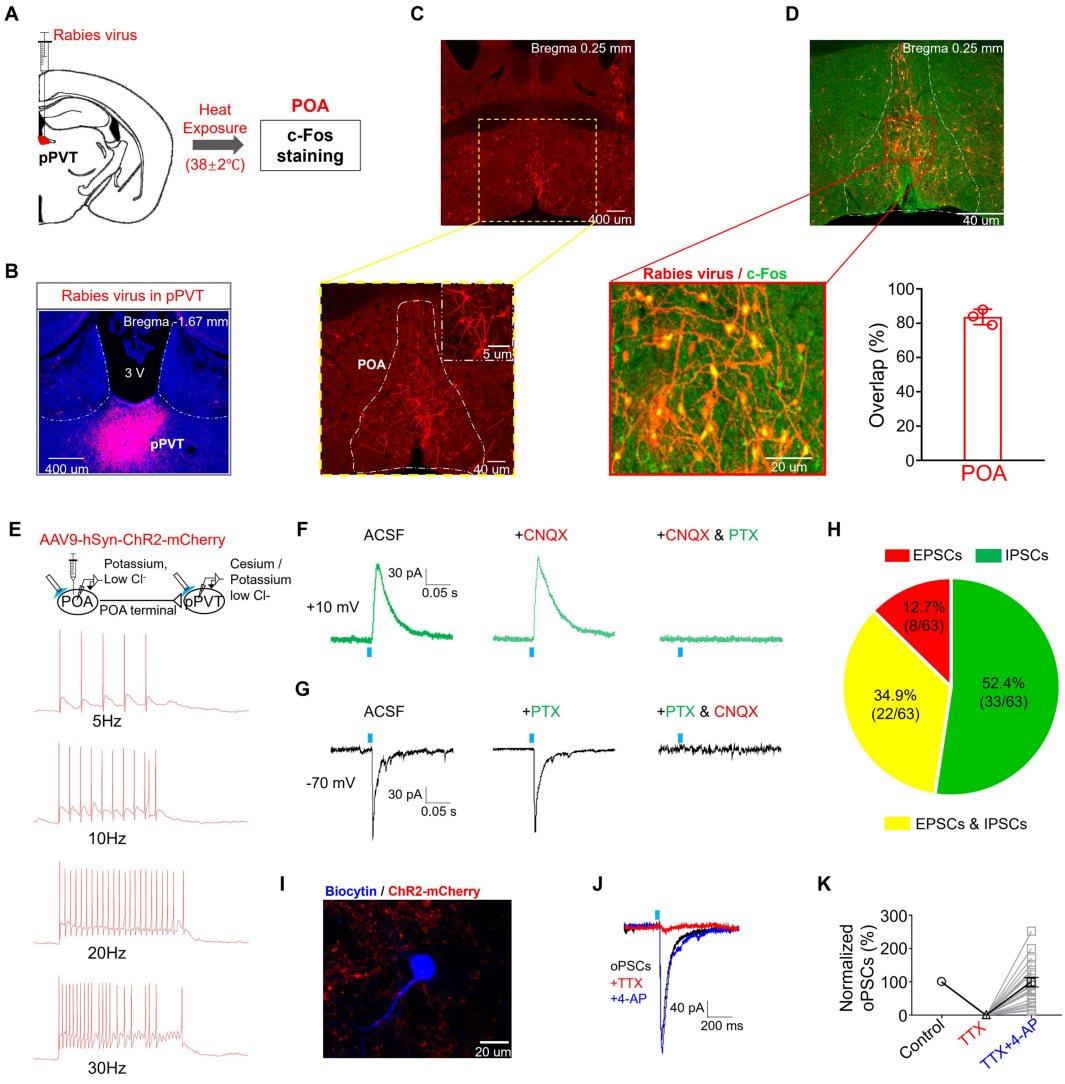


Figure 2.

Involvement of the hypothalamic preoptic area to posterior paraventricular thalamus projections.

(A) The strategy of virus injection followed by heat exposure-induced c-Fos staining (n=3 mice). **(B)** The representative microphotograph showed the expression of rabies virus in the pPVT regions. **(C)** Rabies virus retro-labeled POA neurons. Upper: magnification: x4, scale bar: 400 μm; Lower: magnification: x10, scale bar: 40 μm; The top right corner of the lower picture: magnification: x60, scale bar: 5 μm. **(D)** The representative microphotograph and quantification showed that most of pPVT rabies virus retro-labeled POA neurons co-stained with heat exposure-induced c-Fos. Upper: magnification: x10, scale bar: 40 μm; Lower and left: magnification: x20, scale bar: 20 μm. **(E)** The strategy of virus injection (n=5 mice) and patch-clamp recording was performed on POA expressing ChR2-mCherry neurons and pPVT neurons using potassium and cesium, low chloride internal solutions, respectively. Representative traces showed that POA expressing ChR2-mCherry neurons exhibited robust firing in response to optical stimulation at different frequencies (n=15 neurons from 5 mice). **(F, G)** Representative traces showed that blue light stimulation evoked either excitatory postsynaptic current (EPSC) which could be blocked by cyanquixaline (CNQX, 10 μM) or inhibitory postsynaptic current (IPSC) which could be blocked by picrotoxin (PTX, 100 nM). **(H)** Pie chart showed the projection types recorded on pPVT neurons (n=63 neurons from 10 mice). **(I)** The representative recorded pPVT neuron was visualized by biocytin staining and was found being surrounded by POA expressing ChR2-mCherry terminals. Magnification: x60, scale bar: 20 μm. **(J, K)** The representative trace showed that the application of tetrodotoxin (TTX) eliminated the oPSC held at −70 mV while the addition of 4-amino-pyridine (4-AP, 1mM) recovered it and the quantification (n=24 neurons from 10 mice).

treatment (Figure 3-figure supplement 1). As illustrated in **Figure 3C**, prior to subjecting the mice to chronic heat exposure, we recorded the neuronal activities of POA recipient pPVT neurons for each mouse as the pre-heat control. We then recorded the calcium events one day after acute heat exposure on day 1 and after chronic heat exposure on day 21. Interestingly, when compared with the pre-heat condition of day 1, both the frequency and amplitude during heat exposure on day 1 and day 21 were significantly increased (**Figure 3D and F**). However, such increases in calcium events disappeared on the day following heat exposure on day 1 indicating its transient nature (**Figure 3E**). In contrast, after 21 days of heat exposure, such increases in the frequency of calcium events persisted (**Figure 3G**). It is worth noting that there were no significant differences in both the frequency and amplitude of calcium events during heat exposure on day 1 and day 21 (**Figure 3H**).

Based on the increased neuronal activities of POA recipient pPVT neurons after chronic heat exposure, we investigated the impact of activating the pathway from the POA to pPVT on emotional valence and arousal levels of mice (Figure 3-supplement figure 3A). Utilizing the real-time place preference behavioral paradigm, we noticed that when the blue light was turned on, mice that had already gone through the habituation process quickly entered the chamber with the light turned off (Figure 3-supplement figure 2B and C). This finding suggests that the activation of the POA to pPVT circuit induces an aversive emotional valence. Notably, optogenetic activation did not affect the movement activity of the mice during the experiment (Figure 3-supplement figure 2D). And we did not observe any change in core body temperature when stimulating the POA-pPVT circuit. On the other hand, as pupil size is widely recognized as the gold standard for reflecting arousal levels in rodent models (Privitera et al., 2020), we further studied the effect of optogenetic activation of POA excitatory terminals in pPVT in mice under head fixation. The photos (Figure 3-supplement figure 2E) and video (Figure 3-video supplement 1) clearly demonstrated the enlargement of both the pupil (Figure 3-supplement figure 2F and G) and eye sizes (Figure 3-supplement figure 2H and I) of the mice during light stimulation. Collectively, our results demonstrate that POA recipient pPVT neurons exhibited heightened activities after chronic heat exposure. Moreover, the activation of the POA to pPVT circuit is associated with negative emotional states and higher levels of arousal.

POA recipient pPVT neurons are sufficient and necessary for chronic heat exposure-induced negative emotional valence and hyperarousal states in mice

To confirm the direct relationship between repeated activation of POA recipient pPVT neurons and the negative emotional and hyperarousal states in mice, we conducted chronic activation of POA excitatory terminals within pPVT by injecting CaMKII-promotor encoded ChR2 into the POA and implanting an optical fiber in pPVT. We applied a chronic activation protocol, described by Sidor et al. (Sidor et al., 2014), which involved a cycle of 2 minutes on and 2 minutes off, for a total of 20 minutes per day, over a period of up to 21 days. Following chronic optogenetic activation, we observed several behavioral changes in mice that were similar to those seen in mice exposed to chronic heat. Notably, in the elevated plus maze test, mice subjected to chronic optogenetic activation spent less time in the open arms (**Figure 4B and C**), representing increased stress levels. In the three-chamber test, these mice displayed reduced interests in exploring the unfamiliar male mouse compared to an inanimate object (**Figure 4D and E**), suggesting decreased sociability. During the female encounter test, these mice engaged less with the unfamiliar female mouse compared with the unfamiliar male mouse (**Figure 4F and G**), indicating decreased innate motivation. In the resident-intruder aggression test, the chronic heat-exposed mice exhibited both the decreased latencies in initiating the first attack and increased attack durations (**Figure 4H and I**), suggesting elevated aggression levels. Additionally, compared to the control group, mice after chronic optogenetic activation exhibited more pronounced body fluctuations when captured by a high-resolution camera in response to a 105 dB

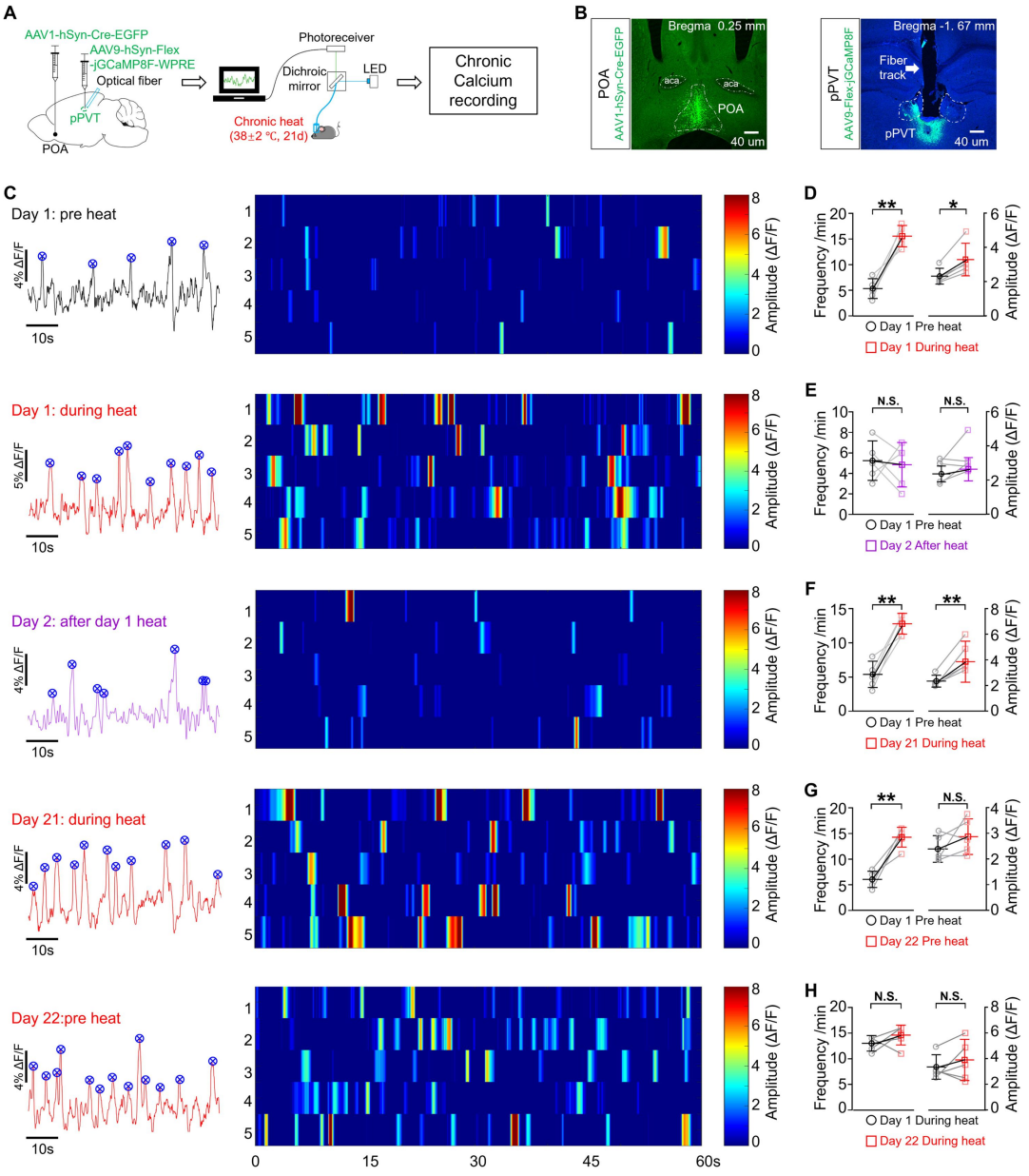
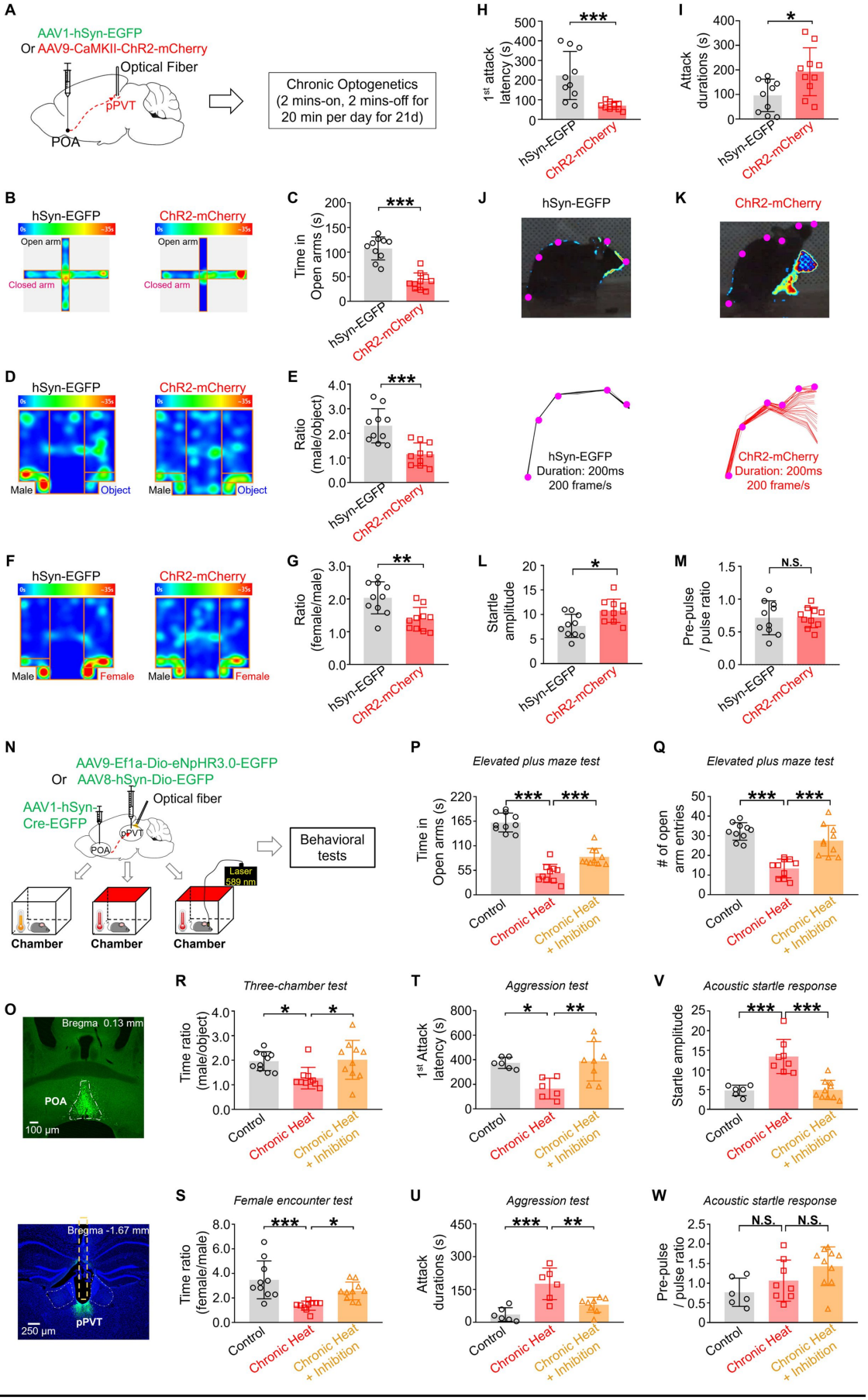


Figure 3.

Activity changes of POA recipient pPVT neurons throughout chronic heat exposure.

(A) Experimental schematics. Mice (n=5) were stereotaxically injected with Cre-dependent GCaMP into the pPVT, followed by the implantation of optical fiber and chronic calcium recording. **(B)** Representative microphotographs showed the virus expression in the POA and pPVT regions, scale bar: all 40 μm. **(C)** From the top to the bottom: the representative calcium events (on the left panel) and the calcium events from different mice (on the right panel) on different days. Each vertical stripe in the heatmap represents one calcium event for each mouse. Statistical analysis of the frequency and amplitude of POA recipient pPVT neurons' calcium events compared for **(D)** Pre and During heat exposure on day 1 (Paired, parametric, two tailed t-test; Frequency (*t=6.278, df=4, P=0.0033*); Amplitude (*t=4.344, df=4, *p=0.0122*)); **(E)** Pre heat on day 1 and After heat on day 2 (Paired, parametric, two tailed t-test; Frequency (*t=0.2787, df=4, p=0.7943*); Amplitude (*t=1.726, df=4, p=0.1595*)); **(F)** Pre heat on day 1 and During heat exposure on day 21 (Paired, parametric, two tailed t-test; Frequency (*t=6.124, df=4, **p=0.0036*); Amplitude (*t=4.704, df=4, **p=0.0093*)); **(G)** Pre heat on day 1 and after Chronic heat on day 22 (Paired, parametric, two tailed t-test; Frequency (*t=6.216, df=4, **p=0.0034*); Amplitude (*t=1.36, df=4, p=0.2454*)); **(H)** During heat exposure on day 1 and day 21 (Paired, parametric, two tailed t-test; Frequency (*t=1.242, df=4, p=0.2821*); Amplitude (*t=0.9424, df=4, p=0.3993*)). * $p<0.05$, ** $p<0.01$, N.S.: not significant.

sound stimulus, indicating the heightened arousal levels (**Figure 4J-M**). Our results directly demonstrated that chronic optogenetic activation of POA excitatory terminals within pPVT circuit could induce negative emotional and hyperarousal states in mice.


A
AAV1-hSyn-EGFP
Or AAV9-CaMKII-ChR2-mCherry
Optical Fiber
pPVT
POA
Chronic Optogenetics
(2 mins-on, 2 mins-off for
20 min per day for 21d)
H
1st attack latency (s)
hSyn-EGFP
ChR2-mCherry
I
Attack durations (s)
B
hSyn-EGFP
ChR2-mCherry
Open arm
Closed arm
C
Time in Open arms (s)
J
hSyn-EGFP
K
ChR2-mCherry
D
Male
Object
E
Ratio (male/object)
hSyn-EGFP
Duration: 200ms
200 frame/s
ChR2-mCherry
Duration: 200ms
200 frame/s
F
Female
G
Ratio (female/male)
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Startle amplitude
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Pre-pulse / pulse ratio
N.S.
N
AAV9-Ef1a-Dio-eNpHR3.0-EGFP
Or AAV8-hSyn-Dio-EGFP
AAV1-hSyn-Cre-EGFP
Optical fiber
Laser 589 nm
Behavioral tests
Chamber
P
Elevated plus maze test
Time in Open arms (s)
Control
Chronic Heat
Chronic Heat + Inhibition
Q
# of open arm entries
O
Bregma 0.13 mm
POA
100 μm
R
Three-chamber test
Time ratio (male/object)
T
Aggression test
1st Attack latency (s)
V
Acoustic startle response
Startle amplitude
Bregma -1.67 mm
pPVT
250 μm
S
Female encounter test
Time ratio (female/male)
U
Attack durations (s)
W
Pre-pulse / pulse ratio

Figure 4.

POA recipient pPVT neurons are sufficient and necessary for chronic heat exposure-induced negative emotional valence and hyperarousal states in mice.

(A) Experimental schematics. Mice (n=10 in each group) were stereotaxically injected with either AAV1-hSyn-EGFP or AAV9-CaMKII-ChR2-mCherry into the POA, followed by the implantation of optical fiber into pPVT, chronic optogenetic activation, and behavioral tests. **(B, C)** The heatmap of representative tracking trace examples in EPM test and the time spent in the open arms (Mann-Whitney unpaired two-tailed U test; U=2, ***p<0.001). **(D, E)** The heatmap of representative tracking trace examples in TCT and the interaction time with an unfamiliar male mouse relative to the inanimate object (Mann-Whitney unpaired two-tailed U test, U=8; ***p=0.0007). **(F, G)** The heatmap of representative tracking trace examples in FET and the time surrounding the unfamiliar female mouse compared to an unfamiliar male mouse (Mann-Whitney unpaired two-tailed U test; U=13, **p=0.0039). **(H, I)** The 1st-time attack latency (Mann-Whitney unpaired two-tailed U test; U=8, p=0.007) and the attack durations (Mann-Whitney unpaired two-tailed U test; U=19, *p=0.0185) in the aggression test. **(J, K)** Visualized acoustic startle response examples (upper panel) and corresponding labeled body parts' skeletons (lower panel) from hSyn-EGFP group and ChR2-mCherry group. **(L, M)** The startle amplitude (Mann-Whitney unpaired two-tailed U test; U=17, *p=0.0115) and the pre-pulse / pulse ratio (Mann-Whitney unpaired two-tailed U test; U=48, p=0.9118) in ASR test. **(N)** Experimental schematics. Mice (n ≥ 6 in each group) were stereotaxically injected with AAV1-hSyn-Cre-EGFP in the POA and either AAV9-Ef1a-Dio-eNpHR3.0-EGFP or AAV8-hSyn-Dio-EGFP into the pPVT, followed by the implantation of optical fiber and behavioral tests. **(O)** Representative microphotographs showed the expression of AAV1-hSyn-Cre-EGFP and AAV9-hSyn-Dio-eNpHR3.0 within the POA and pPVT, respectively. Magnification: x4, scale bar: 100 μm (upper panel) and 250 μm (lower panel). **(P, Q)** The time (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 27)=83.03, ***p<0.001; Control vs. Chronic heat, ***p<0.001; Chronic heat vs. Chronic heat + inhibition, ***p<0.001) and the entry numbers (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 20.92)=27.96, ***p<0.001; Control vs. Chronic heat, ***p<0.001; Chronic heat vs. Chronic heat + inhibition, ***p<0.001) into the open arms of EPM. **(R)** The time spent with an unfamiliar male mouse compared to an inanimate object in TCT (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 27)=5.381, *p=0.0108; Control vs. Chronic heat, *p=0.0292; Chronic heat vs. Chronic heat + Inhibition, *p=0.0175). **(S)** The ratio of time with an unfamiliar female mouse compared to an unfamiliar male mouse in FET (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 27)=10.94, ***p=0.0003; Control vs. Chronic Heat, ***p=0.0002; Chronic Heat vs. Chronic Heat + Inhibition, *p=0.0335). **(T, U)** The 1st attack latency (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 17)=7.426, *p=0.0048; Control vs. Chronic Heat, *p=0.0157; Chronic Heat vs. Chronic Heat + Inhibition, *p=0.0063) and the attack durations (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 17)=13.38, ***p=0.0003; Control vs. Chronic Heat, ***p=0.0003; Chronic Heat vs. Chronic Heat + Inhibition, **p=0.0051) in the aggression test. **(V, W)** The startle amplitude (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 21)=21.03, ****p<0.0001; Control vs. Chronic Heat, ***p<0.001; Chronic Heat vs. Chronic Heat + Inhibition, ***p<0.001) and the pre-pulse / pulse ratio (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 21)=3.802, *p=0.039; Control vs. Chronic Heat, p=0.2616; Chronic Heat vs. Chronic Heat + Inhibition, p=0.2246) in ASR test. *p<0.05, **p<0.01, ***p<0.001, N.S.: not significant.

To establish the necessity of POA recipient pPVT neurons for chronic heat exposure-induced emotional changes, we optogenetically inhibited the POA recipient pPVT neurons during chronic heat exposure by employing a cycle stimulation strategy of 3 minutes ON followed by 3 minutes OFF. Only mice with sufficient virus infection and accurate fiber implantation were included in the data analysis (**Figure 4O and P**). The optogenetic inhibition prevented chronic heat-induced behavioral changes, including anxiety levels (**Figure 4Q and R**). sociability (**Figure 4S**), innate motivation (**Figure 4T**), aggression levels (**Figure 4U and V**) and arousal state (**Figure 4W and X**). These findings further supported the essential role of POA recipient pPVT neurons in mediating chronic heat exposure-induced negative emotional and hyperarousal states.

Chronically activated POA recipient pPVT neurons exhibit exaggerated response to stressful situations

To gain further insight into the roles of POA recipient pPVT neurons in stress-related behaviors, we elucidated the relationship of their activities with specific behaviors indicative of emotional transitions and hyperarousal states. In the elevated plus maze test in which the chronic heat-exposed mice spent less time in the open arms (**Figure 1B and C**), there were also reduced number of entries into the open arms (**Figure 5B**). More detailed analysis revealed that, compared with untreated mice, these animals more frequently paused at the center of the maze and then returned to the closed arms (**Figure 5C**). Furthermore, mice after chronic heat exposure exhibited a higher chance of running, rather than walking, back to the closed arms (**Figure 5D**) and their running speed significantly increased compared to their pre-heat condition (**Figure 5E**), presumably reflecting a state of increased anxiety. Interestingly, POA recipient pPVT neurons consistently exhibited a peak in activity when they were about to run back to the closed arm, while chronic heat-exposed mice exhibited a significantly larger peak (**Figure 5F-H**). Notably, no comparable increases of activities in the POA recipient pPVT neurons were observed when chronic heat-exposed mice paused at the center and finally walked out to the open arms (Figure 5-supplement figure 1A-C) or walked to the closed arms (Figure 5-supplement figure 1D-F). Similar phenomena were found with respect to the transitions from the open arm to the closed arm, not only in terms of number of transitions (**Figure 5I and J**), walking and running behavior (**Figure 5K**), and speed of running (**Figure 5L**), but also a larger calcium peak response aligned with the running behavior (**Figure 5M-O**).

In the chronic heat-treated mice, we also observed a context-dependent change in behavior reminiscent of the hyperarousal states when we introduced them back into the heat chamber. The mice exhibited apparent locomotion hyperactivity (**Figure 5P**), as evidenced by a notable increase in motion speeds exceeding 400 mm/s (**Figure 5Q**) and a higher frequency of fast running (**Figure 5S**). A clear time correlation was found between the heightened calcium activities in POA recipient pPVT neurons and the main component of locomotion hyperactivity, namely fast running under this condition (**Figure 5T-V**). In contrast, when being placed into a different chamber not associated with heat exposure, no obvious changes were observed among these chronic heat-exposed mice in their total distance traveled (Figure 5-supplement figure 1G), motion speed (Figure 5-supplement figure 1H), number of running exceeding 400 mm/s (Figure 5-supplement figure 1I), the frequency of fast running (Figure 5-supplement figure 1J), or the neuronal activities (Figure 5-supplement figure 1K-M). Collectively, our data suggest the heightened or exaggerated activities of POA recipient pPVT neurons after chronic heat exposure could serve as a driving force for behaviors manifesting negative emotional and hyperarousal states in response to stressful conditions.

Increased pre– and post-synaptic excitability of pPVT neurons but saturated circuitry neuroplasticity capacity following chronic heat exposure

To explore the potential mechanisms underlying the heightened activities of POA recipient pPVT neurons after chronic heat exposure, we conducted *in vitro* slice recordings on pPVT neurons obtained from mice exposed to room temperature, acute heat, and chronic heat, respectively (**Figure 6A and B**). Both acute and chronic heat exposure significantly increased the amplitude of miniature inhibitory postsynaptic currents (mIPSCs) in pPVT neurons but there was no difference in mIPSCs amplitude between the acute and chronic heat groups (**Figure 6C**). On the other hand, we observed a significant increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs) specially in pPVT neurons after chronic but not acute heat exposure (**Figure 6F**). These results suggest that while heat exposure could modulate both inhibitory and excitatory synaptic inputs onto pPVT neurons, there was a differential increase in

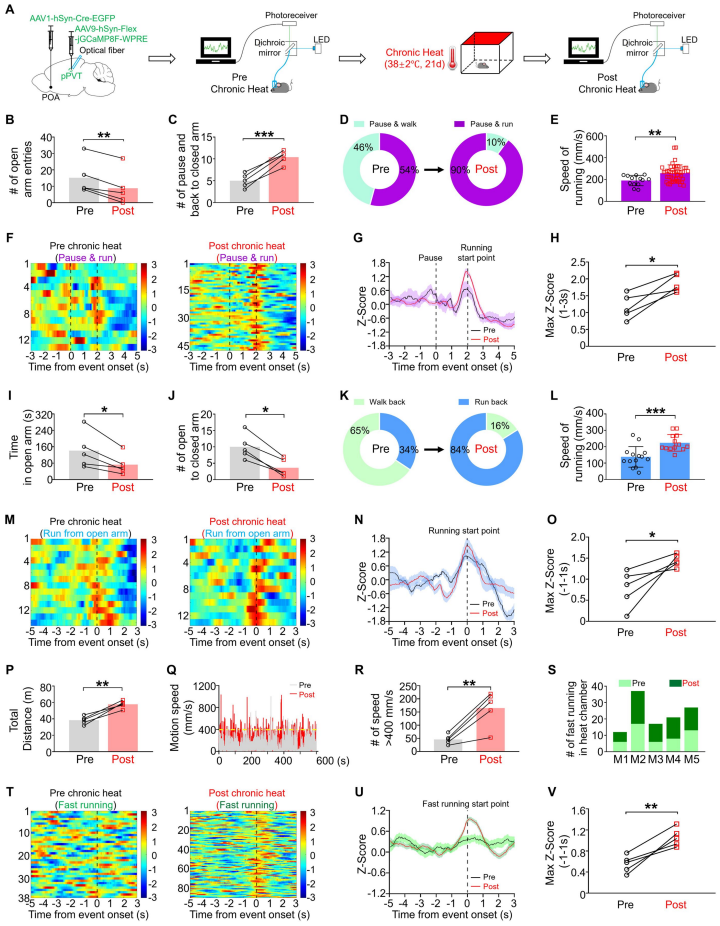

Figure 5.

Chronically activated POA recipient pPVT neurons exhibited exaggerated response to stressful situations.

(A) Experimental schematics. Mice (n=5) were stereotaxically injected with AAV1-hSyn-Cre-EGFP in the POA and AAV9-hSyn-Flex-jGCaMP8F-WPRE in the pPVT, followed by the implantation of optical fiber and calcium recording. **(B)** The number of entries into the open arms of EPM (Paired, parametric, two tailed t-test; t=6.901, df=4, ***p*=0.0023). **(C)** The number of instances where the mice paused at the center area followed by back to closed arms of EPM (Paired, parametric, two tailed t-test; t=9, df=4, ****p*=0.0008). **(D)** Pie chart showed the changes of the percentage of pause and then running back to closed arms. **(E)** The changes of running speed in EPM (Mann-Whitney unpaired two-tailed U test; U=139, ***p*=0.0028). **(F-H)** Heatmaps showed the calcium activities of POA recipient pPVT neurons when mice performed the pause-and-run-back-to-closed-arms behavior for pre (n=13 trials from 5 mice) and post conditions (n=47 trials from 5 mice), Z-Score calcium signals, and statistical comparison (Paired, parametric, two tailed t-test; t=4.387, df=4, *p=0.0118). **(I)** The time spent in open arms of EPM (Paired, parametric, two tailed t-test; t=3.649, df=4, **p*=0.0218). **(J)** The total number of the behavioral event: back from open to closed arms (Paired, parametric, two tailed t-test; t=6.901, df=4, ***p*=0.0023). **(K)** Pie chart showed the changes of the number of running episodes from open to closed arms for pre and post conditions. **(L)** The changes of running speed in EPM (Mann-Whitney unpaired two-tailed U test; U=23, ****p*=0.0005). **(M-O)** Heatmaps showed the calcium activities of POA recipient pPVT neurons when mice performed fast running from open to closed arms for pre (n=14 trials from 5 mice) and post conditions (n=13 trials from 5 mice), Z-Score calcium signals, and statistical comparison (Paired, parametric, two tailed t-test; t=3.087, df=4, *p=0.0367). **(P)** The total distances of mice travelled in the chamber previously subjected to chronic heat (Paired, parametric, two tailed t-test; t=6.876, df=4, ***p*=0.0023). **(Q)** The changes of motion speed for pre and post conditions from one representative mouse. **(R)** The number of instances with motion speed exceeding 400 mm/s (Paired, parametric, two tailed t-test; t=5.100, df=4, ****p*=0.007). **(S)** The number of fast running episodes for pre and post conditions. **(T-V)** Heatmaps showed the calcium activities of POA recipient pPVT neurons when the mice performed fast running in the previous chronic heat-exposed chamber (n=90 trials from 5 mice) compared to pre-heat condition (n=38 trials from 5 mice), Z-Score calcium signals, and statistical comparison (Paired, parametric, two tailed t-test; t=5.456, df=4, **p=0.0055). **p*<0.05, ***p*<0.01, ****p*<0.001.

presynaptic excitability of the excitatory pathway after chronic heat exposure but an increase in postsynaptic response to inhibitory input. Furthermore, when we examined the excitability of pPVT neurons by injecting a sequence of inward currents up to 100 pA to pPVT neurons, pPVT neurons from chronic heat-exposed mice exhibited significant increases in the number of action potentials from 30 to 70 pA (**Figure 6G and H**). Further analysis unveiled that these neurons exhibited a reduced rheobase for action potential generation (**Figure 6I**). However, no apparent alterations were observed in the other parameters (Figure 6-supplement figure 1).

The increase in presynaptic excitability of the POA to pPVT excitatory pathway suggested plastic changes induced by the chronic heat treatment. While it would be difficult to follow the excitability of this pathway in vivo during the chronic heat treatment, we sought to examine the synaptic plasticity capacity of this pathway before and after chronic heat treatment to shed light on its involvement. We injected CaMKII-promotor encoded ChR2 into the POA of mice and divided them into two groups: a control group exposed to room temperature and a chronic heat group. After 21 days of heat exposure, sagittal slices that largely preserved the POA to pPVT pathway were prepared from both the control and chronic heat groups to induce long-term potentiation (LTP) (**Figure 6J**). Local optogenetic stimulation vertically (470 nm) through the objective was applied to elicit light-evoked EPSCs in POA recipient pPVT neurons and baseline of 10 minutes was recorded. High frequency stimulation (HFS) is an effective induction protocol for eliciting LTP at excitatory synapses (Zhu et al., 2016; Grover et al., 2009; Hwong et al., 2008). Therefore, by delivering blue light at various frequencies (10, 30, 50, 70, 90 Hz) to activate the POA excitatory terminals within pPVT, we developed an optogenetic HFS protocol (HFS_{opto}: light pulses at 30 Hz, repeated 3 times at 20 s interval) effective in eliciting potentiated EPSCs on POA recipient pPVT neurons after baseline recording. HFS_{opto} protocol could successfully induce LTP at POA-pPVT synapses. The amplitude of light-evoked EPSCs in the control group following HFS_{opto}, at the onset, showed an obvious increase and gradually became stable potentiation, lasting at least 30 min (150 % ± 18 of baseline, 14 neurons from 5 mice) (**Figure 6K and L**). However, when we applied the same protocol to the POA recipient pPVT neurons of mice after chronic heat exposure, typically no LTP could be induced (**Figure 6K-M**), suggesting that the pathway in these mice were already saturated during chronic heat exposure.

Taken together, our findings suggest enhanced excitatory inputs to pPVT neurons and increased membrane excitability of these neurons may underlie the behavioral changes observed in mice following chronic heat exposure.

Discussion

In this study, we aimed to uncover the neurobiological basis of how extended periods of high temperature such as heatwaves impact on emotional processing in the mammalian brain. We demonstrated that chronic heat exposure led to negative emotional valence and hyperarousal states in mice, but not depression-like behavior. We discovered that hypothalamic POA recipient pPVT neurons in the thalamus mediate such emotional state changes via increased baseline activities and excitability in response to stressful conditions. Presumably, this is attributed to that repeated activation of the POA to pPVT circuit modulates both pre– and post-synaptic processes leading to their long-term changes.

Different from utilizing heating panels (Venkatachalam et al., 2007) to investigate heat-induced thermoregulation, our study employed an air heating strategy. This choice was motivated by the aim of replicating the realistic conditions induced by heatwaves. To ensure the well-being of the animals and prevent unnecessary physiological harm, we maintained temperatures around 38°C, as temperatures exceeding 40°C can lead to adverse effects on the body (Deuis et al., 2017). It is noted that our study differs from many previous studies that applied less high temperature but continuously over several days and weeks, which could have beneficial effects related to

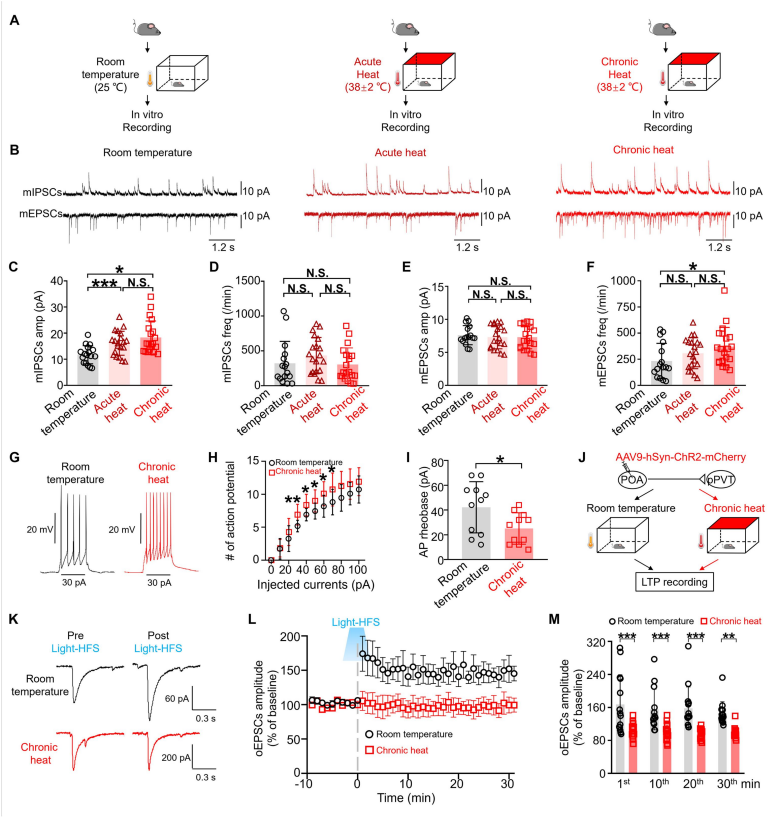

Figure 6.

Increased pre– and post-synaptic excitability of pPVT neurons but saturated circuitry neuroplasticity capacity following chronic heat exposure.

(A) Experimental schematics. *In vitro* brain slice recording was performed on mice from three groups (n=16 neurons from 3 mice in the Room temperature group, n=20 neurons from 4 mice in the Acute heat group, and n=20 neurons from 4 mice in the Chronic heat group). **(B)** Representative traces of miniature postsynaptic currents from three groups. Duration: 12 s. Scale bar: 10 pA. **(C, D)** The changes of mIPSCs' amplitude (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 54)=8.226, ***p=0.0008, Room temperature vs. Acute heat, ***p=0.0005; Room temperature vs. Chronic heat, *p=0.0268) and frequency (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 53)=1.346, p=0.269, Room temperature vs. Acute heat, p=0.4327; Room temperature vs. Chronic heat, p=0.9787) of pPVT neurons. **(E, F)** The changes of mEPSCs' amplitude (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 52.98)=0.1995, p=0.8198, Room temperature vs. Acute heat, p=0.8433; Room temperature vs. Chronic heat, p=0.8433) and frequency (One-way repeated measures ANOVA with Tukey post-hoc test; F(2, 50.1)=2.981, p=0.0598, Room temperature vs. Acute heat, *p=0.2931*; Room temperature vs. Chronic heat, **p*=0.0473*) of pPVT neurons. **(G)** The representative traces of action potential of pPVT neurons upon 100 pA current injection. **(H)** The changes of excitability of pPVT neurons when different currents were injected to the patched pPVT neurons (n=11 neurons from 3 mice in each groups; Mann-Whitney unpaired two-tailed U test; 30 pA: U=22.5, **p=0.0091; 40 pA: U=31, *p=0.048; 50 pA: U=29.5, *p=0.0412; 60 pA: U=25, *p=0.0167; 70 pA: U=29.5, *p=0.0396). **(I)** The changes of rheobase of action potential (Mann-Whitney unpaired two-tailed U test; U=28.5, **p=0.0345*). **(J)** Experimental schematics. Mice (n=5 mice in each group) stereotaxically injected with AAV9-CaMKII-ChR2-mCherry into the POA were then divided into the Room temperature and Chronic heat groups. Sagittal slices of mice were prepared for LTP induction and recording. **(K)** The representative traces showed pPVT neurons from mice exposed to room temperature and chronic heat exhibited different amplitude of oEPSCs after blue light-mediated high frequency stimulation. **(L)** The LTP induction and recording of POA to pPVT pathway from slices of mice exposed to room temperature and chronic heat conditions after blue light stimulation at 30 Hz (n=14 neurons from control group and 18 neurons from chronic heat group). **(M)** Statistical comparison of the amplitude of oEPSCs at different time points (Two-way repeated measures ANOVA with Sidak post-hoc test; Interaction: F(3, 120)=0.3486, p=0.7902; Optical stimulation main effect: F(3, 120)=76.2, ***p<0.001; Time points effect: F(3, 120)=0.8215, p=0.4844; 1st min: Room temperature vs. Chronic heat: ***p<0.001; 10th min: Room temperature vs. Chronic heat: p=0.0001; 20th min: Room temperature vs. Chronic heat: ***p<0.001; 30th min: Room temperature vs. Chronic heat: **p=0.0024). *p<0.05, **p<0.01, ***p<0.001, N.S.: not significant.

cardiovascular fitness, energy metabolism and even cognitive functions (Murray et al., 2022). Our findings revealed that the protocol of chronic heat exposure at 90 min per day for 21 days did not impact the thermoregulatory function in mice. In the various emotional state-related behavioral tests we conducted, including the open field, three-chamber, and female encounter test, we did not observe locomotion hyperactivity in mice. However, a distinct acoustic startle response was noted following chronic heat exposure. This may initially appear contradictory, as hyperarousal states are typically associated with hyperlocomotion (Zhang et al., 2020). However, POA recipient pPVT neurons exhibited heightened responses to stressful and aversive situations, which suggested that chronic heat exposure might primarily induce a state of increased susceptibility towards anxiety and hyperarousal rather than persistent hyperarousal states in mice.

There are a number of studies revealing the connections from the POA to pPVT. Some previous reports have already shown that both excitatory and inhibitory neurons within POA contribute to thermoregulatory function (Morrison et al., 2011). Augustine et al. on the other hand demonstrated that POA neurons expressing nNOS send both excitatory and inhibitory projections to PVT, with an emphasis on their roles in thirst regulation (Augustine et al., 2018). In contrast, Allen et al. (Allen et al., 2017) previously described those thirst-related POA neurons sending projections to PVT mediated reinforcing behavior, highlighting their independence from POA heat-responsive neurons. The findings of the present study enriched our understanding that heat-responsive neurons in the POA could send both excitatory and inhibitory projections to pPVT, and uncovered the role of excitatory projections from POA to pPVT in chronic heat exposure-induced emotional changes. While the role of the excitatory inputs in mediating the negative emotional valence and hyperarousal state is unveiled in this study, the role of the inhibitory inputs awaits further investigation.

Notably, we observed that POA recipient pPVT neurons exhibited higher baseline activities and displayed heightened response to stress or aversive stimuli following chronic heat exposure. Consistently, a recent study also found that long-term heat exposure rendered POA neurons to become tonically active (Ambroziak et al., 2024). Interestingly, prior studies have reported that PVT neurons exhibited stronger activation when mice were subjected to novel stimuli after repeated restraint stress (Vertes et al., 2015; Bhatnagar et al., 1998; Heydendael et al., 2011). This also effectively supports our theory of heightened susceptibility to anxiety and our results provide a rationale for this widely observed phenomenon. Meanwhile, the specific neuronal signals correlated with actions indicative of heightened anxiety such as fast running to closed arms and fast running in chronic-heat exposed chamber were not induced by the motion *per se*. Our recording was conducted when mice were spontaneously behaving without any pre-set cues, excluding the influence of salient stimuli on the activity of POA recipient pPVT neurons (Penzo et al., 2021).

In addition to the enhanced excitatory connections from POA to pPVT after chronic heat exposure, we observed an increased amplitude of inhibitory postsynaptic response after both acute and chronic heat exposure. Increased inhibition could act to compensate for the initial over-excitation at critical time, to ensure that neurons briefly activated by stress can be effectively and prevent the occurrence of excessive stress-related behaviors (Perica et al., 2023). pPVT neurons are anatomically innervated by strong inhibitory projections from different brian nuclei (Penzo et al., 2021). Acute stress-mediated disinhibition of pPVT neurons, as reported by Beas et al. (Beas et al., 2018), can rapidly induce an anxiety-like state in mice. Other studies also showed that inhibition of activated neurons induced by stressful stimuli is effective to avoid abnormal affective behavioral performances (Cruz et al., 2024; Ghasemi et al., 2022). Our results showed that long-term optogenetic inhibition of POA recipient pPVT neurons during chronic heat exposure can effectively prevent changes of emotional and hyperarousal states in mice. Therefore, the increase in mIPSC amplitude observed under both acute and chronic heat conditions in this study suggests the potential presence of negative feedback regulation in response to the over-excitation.

However, under chronic heat exposure, further persistent significant increase in excitatory synaptic transmission may surpass this effect, leading to a final net activation in the pPVT neurons. However, the potential mechanisms by which increased excitatory synaptic transmission and enhanced postsynaptic inhibitory responses interact to achieve activation or inhibition of neurons themselves still require further study.

Current hypothesis regarding the mechanism of pPVT in mediating anxiety-like behavior primarily focused on the dopamine-mediated disinhibition of hypothalamic inhibitory terminals within pPVT during acute stress (Beas et al., 2018). Our study distinctly revealed that the enhanced excitatory inputs to pPVT and increased excitability following chronic heat exposure are responsible for the ensuing negative emotional and hyperarousal states. Interestingly, different from previous studies that reported a decrease in the amplitude of mIPSC in pPVT-expressing dopamine receptor 2 neurons 24 hours after acute restraint stress (Beas et al., 2018), our slice recording from pPVT neurons 24 hours after acute heat exposure exhibited an increase in mIPSCs amplitude. The reasons behind these differences could be attributed to the treatment applied and the specific neuronal types under investigation. Both the enhanced presynaptic excitability and increased intrinsic postsynaptic excitability following chronic heat exposure suggested the involvement of long-term neuroplasticity. The fact that LTP of the POA to pPVT pathway was no longer inducible after chronic heat exposure strongly suggested that this pathway was potentiated during the heat treatment, which accounted for the increased excitability of POA terminals to pPVT and their increased neuronal activities. Overall, both the enhanced excitatory inputs to pPVT and increased excitability may play an indispensable role in chronic heat exposure-induced emotional changes in mice.

There are certain limitations in the scope of the present study. First, our study was conducted solely on male mice. Further investigation is required to determine whether chronic heat exposure can elicit similar behavioral phenotypes in female mice. Also, the pPVT is known to express various peptide receptors (Curtis et al., 2021). However, the specific roles of these peptide receptors in chronic heat exposure-induced emotional changes remain unexplored. Some possible mechanisms may contribute to heightened activities of POA recipient pPVT neurons, such as the increased sensitization, the increased activity of hypothalamus-pituitary-adrenal axis, the transition from chronic adaptation to pathological condition (Herman, 2013), and even the chronic effect of dopamine-induced disinhibition of inhibitory terminals within pPVT, all of which required further investigations. Moreover, POA and pPVT were both previously reported to be activated by stimuli other than heat (Penzo et al., 2021; Zhang et al., 2021). Additional studies are needed to explore the specificity of POA recipient pPVT neurons on chronic heat-induced emotional changes.

In conclusion, our findings provide compelling evidence that the heightened activities of POA recipient pPVT neurons are critically involved in chronic heat-exposure-mediated negative emotional valence and hyperarousal states, and render mice to enter anxiety-like state more readily, as summarized in **Figure 7**. These results shed a new light on the mechanisms underlying heatwaves-induced emotional changes.

Materials and methods

Animals

Adult c57BL/6 male mice (25-45g) were used in this study. Animals were bred and maintained by the Laboratory Animal Service Centre of The Chinese University of Hong Kong (CUHK). All experiments were performed following the CUHK guideline approved by the Animal Experimentations and Ethics Committee.

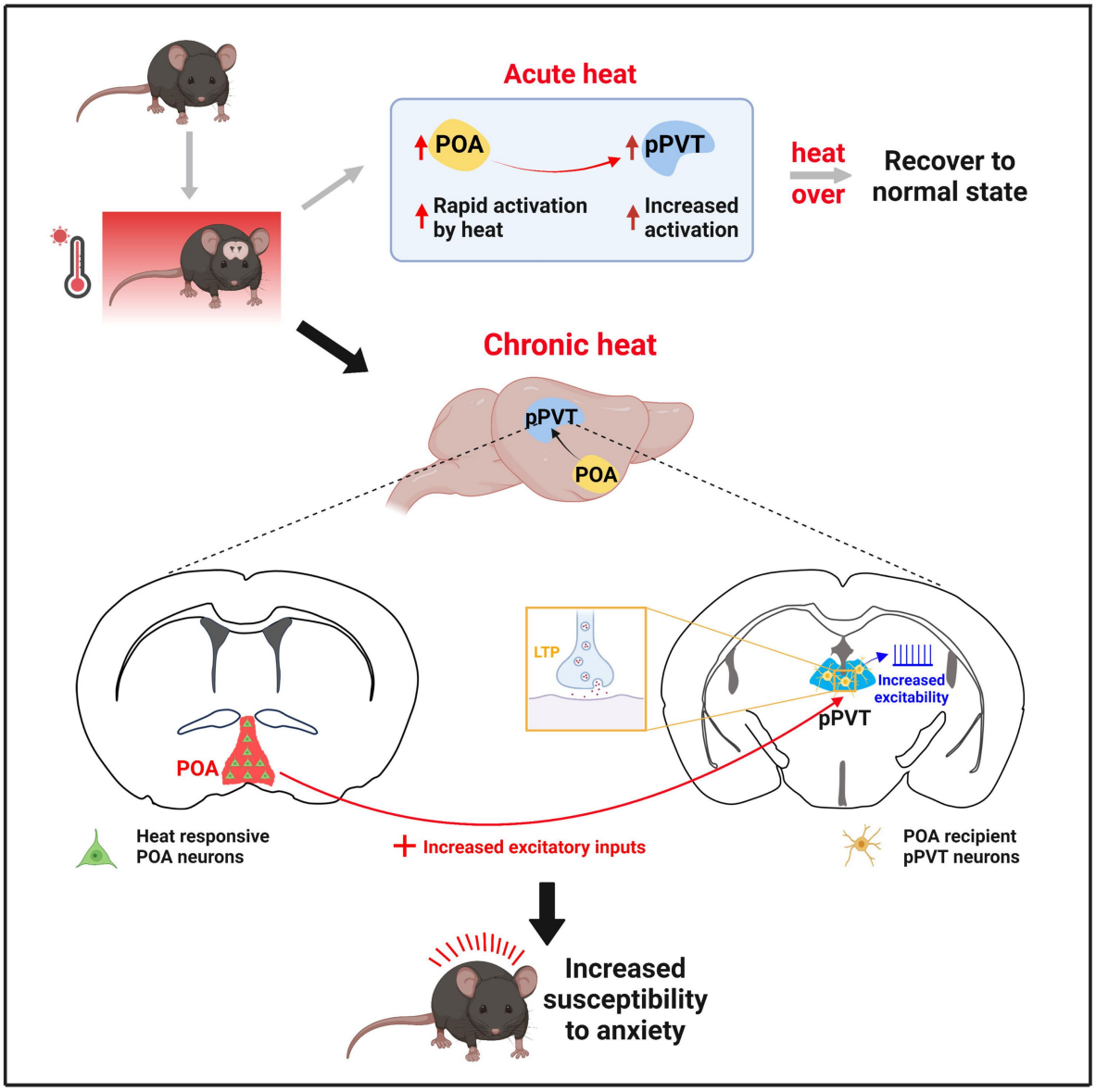

Figure 7.

A working model of neural circuit mechanisms underlying POA recipient pPVT neurons-mediated chronic heat exposure-induced negative emotional valence and hyperarousal states.

Different from acute heat exposure, chronic heat exposure-induced enhancement in excitatory inputs to pPVT and saturated neuroplasticity contributed to the increased membrane excitability underlie the heightened activities of POA recipient pPVT neurons, rendering mice become more susceptible to stressful situations manifested as negative emotional valence and hyperarousal states.

Stereotaxic surgery and viruses

Mice were anesthetized with ketamine and xylazine and placed gently in a stereotaxic frame (Narashige, Tokyo). A Hamilton syringe (33-gauge) filled with AAV viruses was placed into target brain areas according to the corresponding coordinates: POA (+0.62 mm A/P, 0 mm M/L, 5.83 mm D/V), pPVT (−1.47 mm A/P, 0 mm M/L, 3.13 mm D/V) from brain skull. 0.10-0.35 μl viruses were injected at 10 nl/min speed. After injection, the needle was left in the targeted brain area for an additional 10 minutes before retraction. The following viruses were used: AAV1-hSyn-Cre (Addgene, retrograde tracing), AAV1-hSyn-Cre-EGFP (Addgene, optogenetics, photometry recording), Rabies virus (BrainVTA, retrograde tracing), AAV9-hSyn-ChR2-EGFP (Addgene, functional connectivity), AAV9-hSyn-Flex-jGCaMP8F-WPRE (Addgene, photometry recording), AAV9-CaMKII-ChR2-mCherry (Addgene, optogenetic activation), AAV9-hSyn-Dio-eNpHR3.0-EGFP (Addgene, optogenetic inhibition), AAV9-hSyn-Dio-EGFP (Addgene, control experiments). All AAV virus titers were > 5 x 10^{12} particles per ml.

Protocols for chronic heat exposure

The temperature (38 ± 2 J) was adopted based on other reports (Bridges et al., 2012; Cassuto, 1968; Cure, 1989; Jin et a., 2011; Rousset et al., 1984). A home-made chamber was constructed for chronic heat exposure (80 cm [L] × 40 cm [W] × 60 cm [H]) which was composed of two built-in components (heating system and an environmental monitoring sensor). For the heating system, a heater with (1) adjustable buttons that could control the heating power and (2) a “dual heating” setting that was able to stop heating when the temperature exceeded 40 J in the chamber and restarted heating when the temperature in the chamber decreased to lower than 36 J. After the air temperature of the chamber was gradually heated to the targeted temperature ranges and was monitored to be stable for at least 30 minutes, mice would be gently put into the chamber for 90 minutes of heat exposure per day for 21 days. During heat exposure, mice were freely accessible to drinking water and consuming food. The time point within each day to start heat exposure was random. For the environmental monitoring sensor, it was used to monitor the chamber temperature value and humidity level (Peng He Dian Zi, Shanghai, China). And the behavioral tests were conducted the following day after 21 days’ heat exposure.

The method for measuring the body temperature of mice was based on a previous report (Kawakami et al., 2018). Briefly, prior to exposing them to heat, the mice were gently held by their tails to expose their lower abdomen. An infrared thermometer sensor was positioned beneath the lower abdomen and the temperature was measured. This measurement process was repeated three times for each mouse, and the stable values obtained were then averaged to determine the specific mouse’s body temperature.

Behavioral experiments

A variety of emotion-related behavioral tests were conducted on mice after acute and chronic heat exposure.

Elevated plus maze test. The self-made elevated plus maze (EPM) apparatus was made up of two open arms (25 cm [L] × 5 cm [W] × 0.5 cm [H]) and two closed arms (25 cm [L] × 5 cm [W] × 16 cm [H]) arranged in a “plus” shape and elevated 50 cm above the floor. The mice were gently placed at the junction of the open and closed arms while facing toward one of the open arms to initiate the test. Mice were allowed for a 6-min exploration, and their behavior was videotaped and then analyzed and quantified by the ANY-Maze tracking software (Version 4.7, Stoelting CO).

Three-chamber test & Female encounter test. The chambers for both the three-chamber test and female encounter test were self-made by using an open-field arena (50 cm [L] x 40 cm [W] x 30 cm [H]) with two identical transparent chambers (40 cm [L] x 1 cm [W] x 20 cm [H]) with holes in the

middle surface, the stranger male and female mouse were handled for 3 min and then habituated in a wire cage placed in the 3-chamber apparatus for 5-10 min for four consecutive days. The tested mouse was habituated to the 3-chamber apparatus for 10 min with all chambers unobstructed when the handling was over. The tested mouse was then guided to the center chamber when the habituation was over, and bidirectional exits were blocked. A wired cup (of size 0.5 cm [L] x 0.5 cm [W], which prevents large body contact and mating behavior but only induces innate sex-motivated exploratory movement around the cup) with a stranger mouse and an empty cup was introduced into the other two chambers, respectively, and then all chambers were opened for a sociability test. For the female encounter test, a wired cup with a strange male mouse and a wired cup with a strange female mouse was introduced into the left and right chambers, respectively, and all chambers were then opened for a motivation test. Their behavior was videotaped and then analyzed and quantified by the ANY-Maze tracking software (Version 4.7, Stoelting CO).

Resident-intruder aggression test. Mice were fed in a single cage, in which a female mouse, separated by a wire mesh screen, was put in the corner of each cage for seven days to facilitate each tested mouse to build their territory. After chronic heat exposure, the unfamiliar intruder mouse with a color label on the back was introduced to the cage of each tested mouse, and the video was recorded for 15 minutes. Each tested mouse's first attack latency and total attack durations were calculated and counted manually and double-blind by another lab mate.

Acoustic startle response test. The acoustic startle response (ASR) test was conducted based on a recent study (Pantoni et al., 2020). In our study, a sound-proof box was purchased, in which a trumpet, a high-speed camera (200 frames per second, Plexon), and a box made from acrylic plate (10 cm [L] x 5 cm [W] x 25 cm [H]) were prepared. Mice exposed to sound at 105 dB measured by a decibel meter exhibited obvious startle response while 75 dB evoked no response. 105 dB was thus set as the sound decibel of pulse-only stimulation with 0.2 s duration, while 75 dB was set as pre-pulse stimulation with 0.2 s duration. Before the ASR test, mice were placed into an acrylic box for three days of environmental habituation and then for the test. Pre-pulse inhibition (PPI) test started with at least 20 min baseline to make the tested mice quiet and immobile, and then the habituation phase, which consisted of 10 pulses, each 20 s apart, and finally presented with a PPI phase, in which 20 trials: 5 pulse-only trials and 15 pre-pulse & pulse trials were delivered. In the pre-pulse & pulse trials, the pre-pulse was presented about 500 ms before the pulse stimulus. The mice's startle response was captured by a high-speed camera and then analyzed with the machine learning method: DeepLabCut. The brief details were as follows: the mice's body parts were manually labeled and labeled data were input into the google co-lab for the machine learning process until the learning error was lower than 0.5. The successful training data were exported as a .csv file. The startle amplitude of mice was calculated as the average value of all the labeled body parts' vibration amplitude within 200 ms after the sound was broadcast. Each trial was manually checked, and startle response affected by the mice's random moving would not be collected for further analysis. The startle response was visualized with custom MATLAB scripts.

The formula for calculating the PPI ratio was:

PPI ratio = amplitude of pre-pulse & pulse trial / amplitude of pulse-only trial.

Open field test (OFT). Mice were gently placed in the center of the OFT chamber (60 cm [L] × 60 cm [W] × 40 cm [H]) and allowed for free exploration for 10 mins. Mice's behaviors were analyzed and quantified by the ANY-Maze tracking software (Version 4.7, Stoelting CO).

Forced swim test (FST). The apparatus of the forced swim test was a cylindrical tank that was 30 cm high and 20 cm in diameter. And the water was 15 cm tall at room temperature (23-25℃). Tested mice were gently put into water, and the video was recorded for 6 min. Mice's immobile

time in the water was analyzed and quantified by the ANY-Maze tracking software (Version 4.7, Stoelting CO).

Sucrose preference test (SPT). Mice from different groups were habituated for two days for two bottles (one is a sucrose solution bottle, the other is a water bottle, with left and right random placement) in their home cages. After habituation, the sucrose preference of mice from both groups was tested. The intake volume of water and 2% sucrose solution was measured within 2 hours. Sucrose preference was determined as the ratio of the intake volume of sucrose solution to total water consumption.

Tail-suspension test (TST). The tested mice were gently grabbed with the tail and invertedly suspended in front of the recording camera by fixing their tail to the adhesive tape for 6 minutes of recording. Mice immobile time during the suspension was analyzed and quantified by the ANY-Maze tracking software (Version 4.7, Stoelting CO).

Immunohistochemistry

Immunostaining for c-Fos was conducted on four distinct groups of mice: those exposed to acute heat, chronic heat, those injected with rabies virus into the pPVT and exposed to heat, and those with virus-labelled POA recipient pPVT neurons and exposed to heat.

Retrograde tracing. The rabies virus system was adopted for retrograde tracing, as previously reported (Watabe-Uchida et al., 2012). On day 1, 50 nl of AAV1-hSyn-cre, 50nl of AAV-FLEX-TVA-mCherry, and 50 nl of AAV-FLEX-RG were injected into pPVT. Subsequently, 80 nl of SAD △ G virus was injected into pPVT on day 10. Mice were then allowed seven days for full expression before being sacrificed.

Tissue preparation. For mice from different groups, brain samples were prepared as follows: 40 to 60 minutes after exposure to heat, mice were anesthetized with i.p. injection of a ketamine-xylazine cocktail and transcardially perfused with 1X phosphate-buffered saline (PBS, Invitrogen) and then 4% paraformaldehyde (PFA, Sigma-Aldrich). The brain was then extracted and postfixed overnight in 4% PFA and finally dehydrated with 30% glucose solution for 48 h. The well-prepared brain samples were embedded in OCT (ThermoFisher) and sliced into 30-μm coronal sections by a cryostat (Leica) and stored in 1X PBS before immunohistochemistry.

Immunofluorescent staining. Brain sections were blocked in 5% normal goat serum (ThermoFisher) in PBS with 0.3% Triton X-100 (Sigma Aldrich) for 40 min and then incubated with primary rabbit anti-c-Fos antibody (1:2000, Cell Signaling Technology) at 4°C overnight. After several thorough washing pieces in PBS, the sections were incubated with goat anti-rabbit IgG-Alexa Fluor 488 secondary antibody (1:1000, Invitrogen) in a blocking solution for 2 hours. After that, the sections were rinsed in PBS again and finally mounted onto glass slides. Microscopic images were taken under a confocal laser scanning microscope (C1, Nikon).

Neuronal counting. As for the statistical analysis of c-Fos positive neurons, profile counting based on the rectangular square drawn in each picture was performed on image J. For every counted brain area, c-Fos positive neurons on three consecutive brain slices were selected and measured. And the value averaged from profile counting on three consecutive brain slices was regarded as the c-Fos positive neuronal number of specific brain nuclei for one mouse.

Optogenetic experiments

For activation: 100 nL of AAV9-CaMKII-ChR2-mCherry was injected into POA, optical fibers in pPVT. For inhibition: 100 nL of AAV1-hSyn-Cre-EGFP was injected into POA, 200 nL of AAV9-hSyn-Dio-eNpHR3.0-EGFP or AAV9-hSyn-Dio-EGFP in pPVT.

Real-time place preference test. Mice were placed into a two-chamber box for 10 minutes of habituation to make sure that mice had no preference for either side of the chamber. Another 10 minutes' recording was started. The left chamber was set as the light ON compartment while the other was the light OFF chamber. Once mice entered light ON chamber, 473-nm light with 10 ms, 10 Hz (unless otherwise indicated, Newdoon Technology) was delivered via an optic cable (200-μm core, 0.37 NA, Doric Lens) while the light was turned off when mice entered light OFF chamber. Laser power was 5 mW measured at the tip of the fiber, which was implanted 0.1-0.2 mm above the targeted nucleus.

Elevated plus maze test, three-chamber test, and female encounter test. To explore the effect of optogenetic activation of POA excitatory terminals within pPVT on the levels of anxiety, sociability, and motivation in mice, mice in these three behavioral tests were recorded with 3 minutes as the baseline, followed by another 3 minutes' delivery of 473-nm light stimulation via an optic cable (200-μm core, 0.37 NA, Doric Lens).

Measurement of pupil size. To directly reflect the effect of POA excitatory terminals within pPVT on hyperarousal levels, mice were head-fixed and 473-nm light was delivered with 10 Hz, 10 ms at the mode of 10 s light off, 10 s light on, and 10 s light off to measure the changes of pupil size. And pupil size of mice during the whole process was synchronously captured through a high-speed camera at 30 frames per second. All pictures were finally integrated into video format through the FFmpeg program. The changes in pupil size among established videos were further analyzed by the DeepLabCut method (Mathis et al., 2018) for precise tracking and plotting. The time point for statistical analysis of normalized pupil size was at 5s for Pre, 15s for Light, and 25s for Post, respectively.

Chronic optogenetic stimulation. Mice are subjected to 473-nm light stimulation with 10 ms, 10 Hz (unless otherwise indicated, Newdoon Technology) via an optic cable (200-μm core, 0.37 NA, Doric Lens) 2 mins-on, 2 mins-off for 20 min per day for up to 21 days (Sidor et al., 2014). Laser power does not exceed 5 mW measured at the tip of the fiber.

Optogenetic inhibition. Mice exposed to chronic heat were synchronously illuminated with a 589-nm light laser (Newdoon Technology) to inhibit POA recipient pPVT neurons specifically. The stimulus was 20 mW with a cyclical mode consisting of 3 minutes' light continuous on and 3 minutes' light off, which was conducted for 21 days only during the heat exposure period (90 minutes per day) for each mouse. The emotion-related behaviors and hyperarousal states were measured as formerly mentioned and analyzed by Any-maze software.

Fiber photometry

100 nL of AAV1-hSyn-Cre-EGFP in POA, 200 nL of AAV9-hSyn-Flex-jGCaMP8F-WPRE in pPVT, optical fibers in pPVT. Calcium signals were recorded for 1 minute for each mouse before, during heat exposure on day 1, the second day after heat exposure on 1st day, and after chronic heat exposure on day 22. The frequency and amplitude of calcium events were collected for data analysis.

Before and after chronic heat exposure, the performance of each mouse in the elevated plus maze and the chronic heat-exposed chamber was in combination with calcium recording for 10 minutes, respectively. Targeting of event-related time points and calculation of speed were achieved through the SLEAP machine learning method (Pereira et al., 2022).

Fiber photometry data were collected with a TDT system at a sampling frequency of 1017 Hz. The LED power at the tip of the patch cord was less than 20 μW. The 405– and 465-signals were simultaneously recorded. The isosbestic 405 nm control signal was filtered using a polyfit regression to limit the influence of fluorescence decay during the session. And the fitted control signal was then subtracted from the 465-signal to remove artifacts from the intracellular calcium-dependent GCaMP fluorescence. The calculation for the dynamics of fluorescence in chronic

calcium recording was performed using the formula △ F/F=(F_{465}-F_{405}) / F405. As corroborated by previous studies (Shao et al., 2022; Xia et al., 2021), a calcium signal wave exceeding μ + 3σ was regarded as a fluorescence transient. Here, μ and σ respectively denote the average and the standard deviation of the baseline signal of each mouse. The baseline signal for each mouse was randomly selected, with a duration ranging from 5 to 10 seconds. As for the activity changes of POA recipient pPVT neurons during emotional transitions and hyperarousal states, the △F/F was then analyzed using a z-Score relative to the mean and standard deviation of the session. Data were visualized and analyzed with custom MATLAB scripts.

Electrophysiological recordings

Brain slice preparation and whole-cell recording. As for the confirmation of functional connectivity, coronal mice brain slices were prepared as follows. Mice quickly anesthetized by isoflurane were then transcardially perfused with pH7.4 NMDG artificial cerebrospinal fluid (aCSF) in which contains (mM): NMDG 92, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 20, HEPES 10, Glucose 25, Na-ascorbate 5, thiourea 2, Na-pyruvate 3, $MgSO_4$ 10, $CaCl_2$ 0.5, N-acetyl-L-cysteine 12. Once completion of perfusion, mice's brain was quickly and smoothly extracted and placed into cutting NMDG solution for frozen 30 s. And coronal sections at 270-μm were prepared with a vibratome (Campden 5100MZ-PLUS Vibrotome) and then transferred to a chamber with 34c:°C NMDG aCSF for the first 15 minutes of recovery. And subsequently, brain slices were further gently transferred into another chamber holding aCSF at room temperature containing HEPES, pH 7.4, which contains (mM): NaCl 92, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 20, HEPES 10, Glucose 25, Na-ascorbate 5, thiourea 2, Na-pyruvate 3, MgSO4 10, $CaCl_2$ 0.5, N-acetyl-L-cysteine 12. After 40 to 60 minutes of recovery, the brain slices were placed into a recording chamber with normal aCSF, pH 7.4, which contained (mM): NaCl 125, KCl 2.5, Glucose 11, $NaHCO_3$ 26, NaH_2PO_4 1.25, $CaCl_2$ 2, and $MgCl_2$ 2 for neuronal recording.

Functional connectivity. To record optically evoked postsynaptic currents, we used an internal solution containing (mM): K-gluconate 130, KCl 10, HEPES 10, EGTA 1, $MgCl_2$ 2, Na_2-ATP 2, Na_3-GTP 0.4. Light pulses at 470 nm were delivered through a light stimulator (Polygon400 DSI-E-0470-0590-NK1 Dynamic Spatial Illuminator) to activate POA ChR2-EGFP-expressing terminals while patched pPVT neurons were held at –70mV. The neuron with access resistance <20 MΩ and leak current <100 pA was included for data collection.

Measurements of synaptic activity and intrinsic properties. To measure the changes in synaptic transmission of pPVT neurons after chronic heat exposure and acute heat exposure, coronal mice's brain slices were prepared as mentioned above. The internal solution for both miniature inhibitory (sIPSCs, held at +10 mV) and excitatory (sEPSCs, held at –70 mV) post synaptic currents recording was Cesium-gluconate based solution with pH 7.3-7.4 which contains (mM): CsMSF 130, HEPES 10, EGTA 1, $MgCl_2$-$6H_2O$ 2, Na_2ATP 2, NaGTP 0.4, QX-314-Cl 5, TEA-OH 5. And Tetrodotoxin (0.5 mM) was present for both miniature EPSCs (mEPSCs) and miniature IPSCs (mIPSCs) recording. Once completion of recording, brain slices were fixed overnight with 4% PFA and the correct position of recorded pPVT neurons was identified by staining with streptavidin-conjugated Alex 405 (ThermoFisher). For measuring the firing frequency under current clamp recording, steady state current was injected in +10 pA increments from 0 to 100 pA. All action potential properties and excitability recordings in Figure S8 were performed in the presence of 10 μM CNQX and 100 μM picrotoxin. As mentioned in the previous paper (Knowland et al., 2017), the firing rate evoked by current injections and the half-width were calculated in Clampfit software (Molecular Devices) by using peak detection function and full-width at half max amplitude, respectively. The threshold was measured as the change in the voltage from rest at which the slope =20 V/s. Membrane resistance was calculated from the change in voltage elicited after a 50 ms 5 mV hyperpolarizing step from –70 mV (the last 10 ms of the step from baseline was taken as △V). Capacitance and membrane time constant were calculated by Clampfit during the first minute after breaking in to cell.

Long-term potentiation. To measure the neuroplasticity of the POA to pPVT pathway, two batches of mice with enough expression of CaMKII-ChR2-mCherry within the POA were prepared: One serving as the control group and the other as the chronic heat group. Sagittal brain slices from the mice were prepared, and a low-chloride internal solution was used to record pPVT neurons. EPSCs evoked by focal optogenetic stimulation through the objective (approximately 2-3 mm above the surface of brain slice) in a top-down vertical direction were elicited using a Polygon400 Multiwavelength Dynamic Patterned Illuminator (Mightex). A circular area (roughly 500 μm diameter) covering the recorded cell soma was illuminated with brief pulses of blue light (470 nm, duration: 2 ms). LTP induced by optogenetic stimulation of pPVT neurons was elicited by HFS consisting of three episodes of 30 Hz blue light pulses at 20 s intervals.

All above signals were collected using a MultiClamp 700B amplifier controlled by Clampfix 10.4 software via a Digital data 1550 interface (Molecular Devices). Electrical signals were filtered at 3 kHz, digitized at 10 kHz, and further analyzed using Clampfit 10.7 (Molecular Devices).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0. Values were shown as meanc:±c:standard error of the mean (SEM). Student's t-test (paired or unpaired, parametric or nonparametric), one-way ANOVA with Tukey post-hoc test, or two-way ANOVA followed by Sidak post-hoc test, were conducted for statistical analysis. The value for statistical significance was pc:<c:0.05. For each experiment, the detailed statistics are described in the corresponding figure legend.

Data availability

All data are contained in the main text, extended data or the supplementary materials are available from the author upon reasonable request. The source data underlying **Figs 1C**, E, G-I, L, M-Q, 2H, K, 3D-H, 4C, E, G-I, L, M, P-W, 5B-E, H-L, O-S, V, 6C-F, H, I, L, M and **Figure 1**-figure supplement 1A-I, **Figure 1**-figure supplement 2B, D, F, G, H, K, L, **Figure 2**-figure supplement 1C, **Figure 3**-figure supplement 1C, D, **Figure 3**-figure supplement 2C, D, G, I, **Figure 5**-figure supplement 1C, F, G-J, M, **Figure 6**-figure supplement 1A-F are provided as a Source Data file.

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Additional information

Author contributions

Y Ke and WH Yung perceived the project, designed the experiments, and analyzed the data. ZP Cao designed the experiments, collected, and analyzed the data. All authors discussed the findings and contributed to the writing of the manuscript.

Ethics

All experimental procedures were approved by the Animal Experimentations and Ethics Committee (AEEC number: 18-232-MIS-5-C) at the Chinese University of Hong Kong (Animal license number: (23-120) in DH/HT&A/8/2/1 Pt.45). All surgery was performed under deep surgical anesthesia and every effort was made to minimize animal suffering.

Additional files

Supplemental information

Figure 1-video supplement 1.

Figure 3-video supplement 1.

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Reviewer #1 (Public review):

Summary:

The manuscript by Cao et al. examines an important but understudied question of how chronic exposure to heat drives changes in affective and social behaviors. It has long been known that temperature can be a potent driver of behaviors and can lead to anxiety and aggression. However, the neural circuitry that mediates these changes is not known. Cao et al. take on this question by integrating optical tools of systems neuroscience to record and manipulate bulk activity in neural circuits, in combination with a creative battery of behavior assays. They demonstrate that chronic daily exposure to heat leads to changes in anxiety, locomotion, social approach, and aggression. They identify a circuit from preoptic area (POA) to posterior paraventricular thalamus (pPVT) in mediating these behavior changes. The POA-PVT circuit increases activity during heat exposure. Further, manipulation

of this circuit can drive affective and social behavioral phenotypes even in the absence of heat exposure. Moreover, silencing this circuit during heat exposure prevents the development of negative phenotypes. Overall the manuscript makes an important contribution to the understudied area of how ambient temperature shapes motivated behaviors.

Strengths

The use of state-of-the-art systems neuroscience tools (in vivo optogenetics and fiber photometry, slice electrophysiology), chronic temperature-controlled experiments, and a rigorous battery of behavioral assays to determine affective phenotypes. The optogenetic gain of function of affective phenotypes in the absence of heat, and loss of function in the presence of heat are very convincing manipulation data. Overall a significant contribution to the circuit-level instantiation of temperature induced changes in motivated behavior, and creative experiments.

Weaknesses

The authors have fully addressed all of my questions and concerns, with the exception of one comment. They mention that they did carry out measurements of core body temperature as a control during optogenetic experiments and did not see any effects. However, I could only find this reported in the text but could not find the data in the main or supplementary figures.

https://doi.org/10.7554/eLife.101302.2.sa3

Reviewer #2 (Public review):

Summary:

The study by Cao et al. highlights an interesting and important aspect of heat- and thermal biology: the effect of repetitive, long-term heat exposure and its impact on brain function. Even though peripheral, sensory temperature sensors and afferent neuronal pathways conveying acute temperature information to the CNS have been well established, it is largely unknown how persistent, long-term temperature stimuli interact with and shape CNS function, and how these thermally-induced CNS alterations modulate efferent pathways to change physiology and behavior. This study is therefore not only novel but, given global warming, also timely.

The authors provide compelling evidence that neurons of the paraventricular thalamus change plastically over three weeks of episodic heat stimulation and they convincingly show that these changes affect behavioral outputs such as social interactions, and anxiety related behaviors.

Strengths:

• It is impressive that the assessed behaviors can be (i) recruited by optogenetic fiber activation and (ii) inhibited by optogenetic fiber inhibition when mice are exposed to heat. Technically, when/how long is the fiber inhibition performed? It says in the text "3 min on and 3 min off". Is this only during the 20 minutes heat stimulation or also at other times?
• It is interesting that the frequency of activity in pPVT neurons, as assessed by fiber photometry, stays increased after long-term heat exposure (day 22) when mice are back at normal room temperature. This appears similar to a previous study that found long-term heat exposure to transform POA neurons plastically to become tonically active (https://www.biorxiv.org/content/10.1101/2024.08.06.606929v1). Interestingly, the POA neurons that become tonically active by persistent heat exposure described in the above study are largely

excitatory and thus these could drive the activity of the pPVT neurons analyzed in this study. How can it be reconciled that the majority of the inputs from the POA are found to be largely inhibitory (Fig. 2H)? Is it possible that this result stems from the fact that non-selective POA-to-pPVT projections are labelled by the approach used in this study and not only those pathways activated by heat? These points would be nice to discuss.
• It is very interesting that no LTP can be induced after chronic heat exposure (Fig. K-M); the authors suggest that "the pathway in these mice were already saturated" (line 375). Could this hypothesis be tested in slices by employing a protocol to extinguish pre-existing (chronic heat exposure-induced) LTP? This would provide further strength to the findings/suggestion that an important synaptic plasticity mechanism is at play that conveys behavioral changes upon chronic heat stimulation.
• It is interesting that long-term heat does not increase parameters associated with depression (Fig. 1N-Q), how is it with acute heat stress, are those depression parameters increased acutely? It would be interesting to learn if "depression indicators" increase acutely but then adapt (as a consequence of heat acclimation) or if they are not changed at all and are also low during acute heat exposure.

https://doi.org/10.7554/eLife.101302.2.sa2

Reviewer #3 (Public review):

In this study, Cao et al. explore the neural mechanisms by which chronic heat exposure induces negative valence and hyperarousal in mice, focusing on the role of the posterior paraventricular nucleus (pPVT) neurons that receive projections from the preoptic area (POA). The authors show that chronic heat exposure leads to heightened activity of the POA projection-receiving pPVT neurons, potentially contributing to behavioral changes such as increased anxiety level and reduced sociability, along with heightened startle responses. In addition, using electrophysiological methods, the authors suggest that increased membrane excitability of pPVT neurons may underlie these behavioral changes. The use of a variety of behavioral assays enhances the robustness of their claim. Moreover, while previous research on thermoregulation has predominantly focused on physiological responses to thermal stress, this study adds a unique and valuable perspective by exploring how thermal stress impacts affective states and behaviors, thereby broadening the field of thermoregulation.

While the manuscript has been revised and some efforts have been made to address the reviewers' concerns, the majority of the issues raised remain insufficiently resolved. Therefore, the reviewer has highlighted key major points that the authors should address to strengthen the manuscript's conclusions.

Major points
The manuscript highlights the increased activity in pPVT neurons receiving projections from the POA (Figure 3) and shows that these neurons are necessary for heat-induced behavioral changes (Figures 4N-W). However, it remains unclear whether the POA-to-pPVT projection itself plays a critical role. Since pPVT recipient neurons can receive inputs from various brain regions, the role of the POA input in driving these effects needs to be validated more explicitly.
(1) To establish this, the authors should conduct experiments directly inhibiting the POA-to-pPVT projection and demonstrate whether the increased activity in pPVT neurons due to chronic heat exposure is abolished when the POA is blocked.
(2) Alternatively, the authors could use anterograde labeling from the POA and specifically target recipient neurons in the pPVT to confirm that the observed excitatory inputs originate from the POA (related to Figure 6).
(3) If these experiments are not feasible, the authors should consider toning down the emphasis on the POA's role throughout the manuscript and discussing this limitation

explicitly. The term "POA recipient pPVT neurons" should be used consistently to avoid misleading implications that the POA-to-pPVT excitatory projection is definitively established as the key pathway.
a) For example, in lines 368-369, the phrase "The increase in presynaptic excitability of the POA to pPVT excitatory pathway" represents a logical jump, as the data only support the "differential increase in presynaptic excitability of the excitatory pathway" (as described in lines 358-359) without specifically confirming the POA-to-pPVT pathway.
b) Similarly, in lines 442-446, the statement "the role of excitatory projections from POA to pPVT in chronic heat exposure-induced emotional changes" should be revised to "the role of excitatory projection recipient pPVT in chronic heat~," as the data do not provide direct evidence that heat-responsive POA neurons projecting to pPVT mediate these effects. Such revisions would improve clarity and ensure that the conclusions remain aligned with the presented data.

https://doi.org/10.7554/eLife.101302.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The manuscript by Cao et al. examines an important but understudied question of how chronic exposure to heat drives changes in affective and social behaviors. It has long been known that temperature can be a potent driver of behaviors and can lead to anxiety and aggression. However, the neural circuitry that mediates these changes is not known. Cao et al. take on this question by integrating optical tools of systems neuroscience to record and manipulate bulk activity in neural circuits, in combination with a creative battery of behavior assays. They demonstrate that chronic daily exposure to heat leads to changes in anxiety, locomotion, social approach, and aggression. They identify a circuit from the preoptic area (POA) to the posterior paraventricular thalamus (pPVT) in mediating these behavior changes. The POA-PVT circuit increases activity during heat exposure. Further, manipulation of this circuit can drive affective and social behavioral phenotypes even in the absence of heat exposure. Moreover, silencing this circuit during heat exposure prevents the development of negative phenotypes. Overall the manuscript makes an important contribution to the understudied area of how ambient temperature shapes motivated behaviors.*
>
> *Strengths:*
>
> *The use of state-of-the-art systems neuroscience tools (in vivo optogenetics and fiber photometry, slice electrophysiology), chronic temperature-controlled experiments, and a rigorous battery of behavioral assays to determine affective phenotypes. The optogenetic gain of function of affective phenotypes in the absence of heat, and loss of function in the presence of heat are very convincing manipulation data. Overall a significant contribution to the circuit-level instantiation of temperature-induced changes in motivated behavior, and creative experiments.*
>
> *Weaknesses：*
>
> *(1) There is no quantification of cFos/rabies overlap shown in Figure 2, and no report of whether the POA-PVT circuit has a higher percentage of Fos+ cells than the general POA*

> *population. Similarly, there is no quantification of cFos in POA recipient PVT cells for Figure 2 Supplement 2.*

Thanks for the comment. The quantification results of c-Fos signal have been provided in the main text and figures.

> *(2) The authors do not address whether stimulation of POA-PVT also increases core body temperature in Figure 3 or its relevant supplements. This seems like an important phenotype to make note of and could be addressed with a thermal camera or telemetry.*

Thanks for raising this point. We did indeed monitor the core body temperature during stimulation of POA-PVT pathway, but we did not observe any significant changes. We have included this finding in the revised manuscript.

> *(3) In Figure 3G: is Day 1 vs Day 22 "pre-heat" significant? The statistics are not shown, but this would be the most conclusive comparison to show that POA-PVT cells develop persistent activity after chronic heat exposure, which is one of the main claims the authors make in the text. This analysis is necessary in order to make the claim of persistent circuit activity after chronic heat exposure.*

Figure 3G does compare the Day 1 preheat to Day22 preheat, and the difference was significant. The wording has been corrected to avoid confusion. Also, we have modified Figure 3D to 3H in our revised manuscript to improve the clarity of these plots.

> *(4) In Figure 4, the control virus (AAV1-EYFP) is a different serotype and reporter than the ChR2 virus (AAV9-ChR2-mCherry). This discrepancy could lead to somewhat different baseline behaviors.*

Thanks for bringing out this issue. We acknowledge that using AA1-EGFP (a different serotype and reporter compared to the AAV9-ChR2-mCherry) as our control virus is not ideal. But based on our own prior experiments, we observed no significant differences in baseline behaviors between animals injected with AAV1 and AAV9 EYFP as well as control mice without virus injection. Therefore, we believe that the baseline behaviors of the animals were unaffected.

> *(5) In Figure 5G, N for the photometry data: the authors assess the maximum z-score as a measure of the strength of calcium response, however the area under the curve (AUC) is a more robust and useful readout than the maximum z score for this. Maximum z-score can simply identify brief peaks in amplitude, but the overall area under the curve seems quite similar, especially for Figure 5N.*

Thanks for the comment. We agree with the reviewer that the area under the curve (AUC) is an alternative readout for measurement of the strength of calcium response. However, the reason why we chose the maximum z-score is based on the observation that we found POA recipient pPVT neurons after chronic heat treatment exhibited a higher calcium peak corresponding to certain behavioral performances when compared to pre-heat conditions. We thus applied the maximum z-score as a representative way to describe the neuronal activity changes of mice during certain behaviors before and after chronic heat treatment. The other consideration is that we want to reflect that POA recipient pPVT neurons become more sensitive and easier to be activated after chronic heat exposure under the same stressful situations compared to control mice. The maximum z score represented by peak in combination with particular behavioral performances is considered more suitable to highlight our findings in this study.

> *(6) For Fig 5V: the authors run the statistics on behavior bouts pooled from many animals, but it is better to do this analysis as an animal average, not by compiling bouts. Compiling bouts over-inflates the power and can yield significant p values that would not exist if the analysis were carried out with each animal as an n of 1.*

Thanks for the comment and suggestion. We had tried both methods and the statistical results were similar. As suggested, we have updated Fig 5V, as well as Fig. 5H and 5O by comparing animal average in our revised manuscript.

> *(7) In general this is an excellent analysis of circuit function but leaves out the question of whether there may be other inputs to pPVT that also mediate the same behavioral effect. Future experiments that use activity-dependent Fos-TRAP labeling in combination with rabies can identify other inputs to heat-sensitive pPVT cells, which may have convergent or divergent functions compared to the POA inputs.*

Thanks for the valuable suggestion, which would enhance the conclusion. We will consider adopting this approach in future investigations into this question.

> ***Reviewer #2 (Public review):***
>
> *Summary*
>
> *The study by Cao et al. highlights an interesting and important aspect of heat- and thermal biology: the effect of repetitive, long-term heat exposure and its impact on brain function.*
>
> *Even though peripheral, sensory temperature sensors and afferent neuronal pathways conveying acute temperature information to the CNS have been well established, it is largely unknown how persistent, long-term temperature stimuli interact with and shape CNS function, and how these thermally-induced CNS alterations modulate efferent pathways to change physiology and behavior. This study is therefore not only novel but, given global warming, also timely.*
>
> *The authors provide compelling evidence that neurons of the paraventricular thalamus change plastically over three weeks of episodic heat stimulation and they convincingly show that these changes affect behavioral outputs such as social interactions, and anxiety-related behaviors.*
>
> *Strengths*
>
> *(1) It is impressive that the assessed behaviors can be (i) recruited by optogenetic fiber activation and (ii) inhibited by optogenetic fiber inhibition when mice are exposed to heat. Technically, when/how long is the fiber inhibition performed? It says in the text "3 min on and 3 min off". Is this only during the 20-minute heat stimulation or also at other times?*

Thanks for pointing out the need for clarification. Our optogenetic inhibition had been conducted for 21 days during the heat exposure period (90 mins) for each mouse. And to avoid the light-induced heating effect, we applied the cyclical mode of 3 minutes' light on and 3 minutes' light off only during the process of heat exposure but not other time. The detailed description has been supplemented in the Method part of our revised manuscript.

> *(2) It is interesting that the frequency of activity in pPVT neurons, as assessed by fiber photometry, stays increased after long-term heat exposure (day 22) when mice are back at normal room temperature. This appears similar to a previous study that found long-*

> *term heat exposure to transform POA neurons plastically to become tonically active (https://www.biorxiv.org/content/10.1101/2024.08.06.606929v1). Interestingly, the POA neurons that become tonically active by persistent heat exposure described in the above study are largely excitatory, and thus these could drive the activity of the pPVT neurons analyzed in this study.*

Thanks for pointing out this study that suggests similar plasticity of POA neurons under long-term heat exposure serving a different purpose. We have included this information in our discussion as well.

> *(3) How can it be reconciled that the majority of the inputs from the POA are found to be largely inhibitory (Fig. 2H)? Is it possible that this result stems from the fact that non-selective POA-to-pPVT projections are labelled by the approach used in this study and not only those pathways activated by heat? These points would be nice to discuss.*

Thanks for raising these important questions. Although it is not our primary focus, we are aware of the substantial inhibitory inputs from POA to pPVT which suggests an important function. However, we do not think that this pathway, which would exert an opposite effect on POA-recipient pPVT neurons compared to the excitatory input, contributes to the long-term effect of chronic heat exposure. This is due to the increased, rather than decreased, excitability of the neurons. There is a possibility that this inhibitory input serves as a short-term inhibitory control for other purpose. Further work is needed to fully address this question.

> *(4) It is very interesting that no LTP can be induced after chronic heat exposure (Figures K-M); the authors suggest that "the pathway in these mice were already saturated" (line 375). Could this hypothesis be tested in slices by employing a protocol to extinguish pre-existing (chronic heat exposure-induced) LTP? This would provide further strength to the findings/suggestion that an important synaptic plasticity mechanism is at play that conveys behavioral changes upon chronic heat stimulation.*

We agree with the reviewer that the results of the suggested experiment would further strengthen our hypothesis. We will try to confirm this in future studies.

> *(5) It is interesting that long-term heat does not increase parameters associated with depression (Figure 1N-Q), how is it with acute heat stress, are those depression parameters increased acutely? It would be interesting to learn if "depression indicators" increase acutely but then adapt (as a consequence of heat acclimation) or if they are not changed at all and are also low during acute heat exposure.*

Based on our observations, we did not find increased depression parameters after acute heat stress in our experiments (data not shown), which was consistent with other two previous studies (Beas et al., 2018; Zhang et al., 2021). It appears that acute heat stress is more associated with anxiety-like behavior and may not be sufficient to induce depression-like phenotypes in rodents, aligning with our observation during experiments.

Beas BS, Wright BJ, Skirzewski M, Leng Y, Hyun JH, Koita O, Ringelberg N, Kwon HB, Buonanno A, Penzo MA (2018) The locus coeruleus drives disinhibition in the midline thalamus via a dopaminergic mechanism Nat Neurosci 21:963-973.

Zhang GW, Shen L, Tao C, Jung AH, Peng B, Li Z, Zhang LI, Whit Tao HZ (2021) Medial preoptic area antagonistically mediates stress-induced anxiety and parental behavior Nat Neurosci 24:516-528.

> *Weaknesses/suggestions for improvement.*

> *(1) The introduction and general tenet of the study is, to us, a bit too one-sided/biased: generally, repetitive heat exposure --heat acclimation-- paradigms are known to not only be detrimental to animals and humans but also convey beneficial effects in allowing the animals and humans to gain heat tolerance (by strengthening the cardiovascular system, reducing energy metabolism and weight, etc.).*

Thanks for the suggestion. We have modified the introduction in our revised manuscript to make it more balanced.

> *(2) The point is well taken that these authors here want to correlate their model (90 minutes of heat exposure per day) to heat waves. Nevertheless, and to more fully appreciate the entire biology of repetitive/chronic/persistent heat exposure (heat acclimation), it would be helpful to the general readership if the authors would also include these other aspects in their introduction (and/or discussion) and compare their 90-minute heat exposure paradigm to other heat acclimation paradigms. For example, many past studies (using mice or rats)m have used more subtle temperatures but permanently (and not only for 90 minutes) stimulated them over several days and weeks (for example see PMID: 35413138). This can have several beneficial effects related to cardiovascular fitness, energy metabolism, and other aspects. In this regard: 38{degree sign}C used in this study is a very high temperature for mice, in particular when they are placed there without acclimating slowly to this temperature but are directly placed there from normal ambient temperatures (22{degree sign}C-24{degree sign}C) which is cold/coolish for mice. Since the accuracy of temperature measurement is given as +/- 2{degree sign}C, it could also be 40{degree sign}C -- this temperature, 40{degree sign}C, non-heat acclimated C57bl/6 mice will not survive for long.*
>
> *The authors could consider discussing that this very strong, short episodic heat-stress model used here in this study may emphasize detrimental effects of heat, while more subtle long-term persistent exposure may be able to make animals adapt to heat, become more tolerant, and perhaps even prevent the detrimental cognitive effects observed in this study (which would be interesting to assess in a follow-up study).*

Thanks for pointing out the important aspect regarding the different heat exposure paradigms and their potential impacts. We have incorporated these points into both the Introduction and Discussion sections of the revised manuscript.

> *(3) Line 140: It would help to be clear in the text that the behaviors are measured 1 day after the acute heat exposure - this is mentioned in the legend to the figure, but we believe it is important to stress this point also in the text. Similarly, this is also relevant for chronic heat stimulation: it needs to be made very clear that the behavior is measured 1 day after the last heat stimulus. If the behaviors had been measured during the heat stimulus, the results would likely be very different.*

Thanks for the suggestion, and we have clarified the procedure in the revised manuscript.

> *(4) Figure 2 D and Figure 2- Figure Supplement 1: since there is quite some baseline cFos activity in the pPVT region we believe it is important to include some control (room temperature) mice with anterograde labelling; in our view, it is difficult/not possible to conclude, based on Fig 2 supplement 2C, that nearly 100% of the cfos positive cells are contacted by POA fibre terminals (line 168). By eye there are several green cells that don't have any red label on (or next to) them; additionally, even if there is a little bit of red signal next to a green cell: this is not definitive proof that this is a synaptic contact. It is therefore advisable to revisit the quantification and also revisit the interpretation/wording about synaptic contacts.*

> *In relation to the above: Figure 2h suggests that all neurons are connected (the majority receiving inhibitory inputs), is this really the case, is there not a single neuron out of the 63 recorded pPVT neurons that does not receive direct synaptic input from the POA?*

Thanks for the comments. For Figure 2-figure supplement 1, the baseline c-Fos activity in pPVT were indeed measured from mouse under room temperature. Observed activity may be attributed to the diverse functions that the pPVT is responsible for. Compared to the heat-exposed group, we observed significant increases in c-Fos signals, suggesting the effect of heat exposure.

For Figure 2-figure supplement 2, through targeted injection of AAV1-Cre into the POA, we achieved selective expression of Cre-dependent ChR2-mCherry in pPVT neurons receiving POA inputs. Following heat exposure, we observed substantial colocalization between heat-induced c-Fos expression (green signal) and ChR2-mCherry-labeled neurons (red signal) in the pPVT. This extensive overlap indicates that POA-recipient pPVT neurons are predominantly heat-responsive and likely mediate the behavioral alterations induced by chronic heat exposure. We have validated these signals and included updated quantification in our revised manuscript.

For Fig 2H, we specifically patched those neurons that were surrounded by red fluorescence under the microscope, ensuring that the patched neurons had a high likelihood of being innervated from POA. This is why all 63 recorded pPVT neurons were found to receive direct synaptic input from the POA.

> *(5) It would be nice to characterize the POA population that connects to the pPVT, it is possible/likely that not only warm-responsive POA neurons connect to that region but also others. The current POA-to-pPVT optogenetic fibre stimulations (Figure 4) are not selective for preoptic warm responsive neurons; since the POA subserves many different functions, this optogenetic strategy will likely activate other pathways. The referees acknowledge that molecular analysis of the POA population would be a major undertaking. Instead, this could be acknowledged in the discussion, for example in a section like "limitation of this study".*

Thanks for the suggestion. We have supplemented this part in our revised manuscript.

> *(6) Figure 3a the strategy to express Gcamp in a Cre-dependent manner: it seems that the Gcamp8f signal would be polluted by EGFP (coming from the Cre virus injected into the POA): The excitation peak for both is close to 490nm and emission spectra/peaks of GCaMP8f (510-520 nm) and EGFP (507-510 nm) are also highly overlapping. We presume that the high background (EGFP) fluorescence signal would preclude sensitive calcium detection via Gcamp8f, how did the authors tackle this problem?*

Thank you for pointing out this issue. We acknowledge that we included AAV1-EGFP when recording the GCaMP8F signal to assist in the post-verification of the accuracy of the injection site. But we also collected recording data from mice with AAV1-Cre without EGFP injected into POA and Cre-dependent GCaMP8F in pPVT, albert in a smaller number. We did not observe any obvious differences in the change in calcium signal between these two virus strategies, suggesting that the sensitivity of the GCaMP signals was not significantly affected by the increased baseline fluorescence due to EGFP.

> *(7) How did the authors perform the social interaction test (Figures 1F, G)? Was the intruder mouse male or female? If it was a male mouse would the interaction with the female mouse be a form of mating behavior? If so, the interpretation of the results*

> *(Figures 1F, G) could be "episodic heat exposure over the course of 3 weeks reduces mating behavior".*

Thanks for the comment. For this female encounter test, we strictly followed the protocol by Ago Y, et al., (2015). During this test, both the strange male and female mice were placed into a wired cup (which is made up of mental wire entanglement and the size for each hole is 0.5 cm [L] x 0.5 cm [W]), which successfully prevented large body contact and the mating behavior but only innate sex-motivated moving around the cup. We have supplemented the details in the method part of our revised manuscript.

Ago Y, Hasebe S, Nishiyama S, Oka S, Onaka Y, Hashimoto H, Takuma K, Matsuda T (2015) The Female Encounter Test: A Novel Method for Evaluating Reward-Seeking Behavior or Motivation in Mice Int J Neuropsychopharmacol 18: pyv062.

> ***Reviewer #3 (Public review):***
>
> *In this study, Cao et al. explore the neural mechanisms by which chronic heat exposure induces negative valence and hyperarousal in mice, focusing on the role of the posterior paraventricular nucleus (pPVT) neurons that receive projections from the preoptic area (POA). The authors show that chronic heat exposure leads to heightened activity of the POA projection-receiving pPVT neurons, potentially contributing to behavioral changes such as increased anxiety level and reduced sociability, along with heightened startle responses. In addition, using electrophysiological methods, the authors suggest that increased membrane excitability of pPVT neurons may underlie these behavioral changes. The use of a variety of behavioral assays enhances the robustness of their claim. Moreover, while previous research on thermoregulation has predominantly focused on physiological responses to thermal stress, this study adds a unique and valuable perspective by exploring how thermal stress impacts affective states and behaviors, thereby broadening the field of thermoregulation. However, a few points warrant further consideration to enhance the clarity and impact of the findings.*
>
> *(1) The authors claim that behavior changes induced by chronic heat exposure are mediated by the POA-pPVT circuit. However, it remains unclear whether these changes are unique to heat exposure or if this circuit represents a more general response to chronic stress. It would be valuable to include control experiments with other forms of chronic stress, such as chronic pain, social defeat, or restraint stress, to determine if the observed changes in the POA-pPVT circuit are indeed specific to thermal stress or indicative of a more universal stress response mechanism.*

We also share similar considerations as the reviewer and indeed have conducted experiments to explore this possibility. Our findings suggest that the POA-pPVT pathway may also mediate behavioral changes induced by other chronic stress, e.g. chronic restraint stress. Nevertheless, given the well-known prominent role of POA neurons in heat perception, we do believe that the POA-pPVT has a specialized role in mediating chronic heat induced changes. The role of this pathway in other stress-related responses will need a more comprehensive study in the future.

> *(2) The authors use the term "negative emotion and hyperarousal" to interpret behavioral changes induced by chronic heat (consistently throughout the manuscript, including the title and lines 33-34). However, the term "emotion" is broad and inherently difficult to quantify, as it encompasses various factors, including both valence and arousal (Tye, 2018; Barrett, L. F. 1999; Schachter, S. 1962). Therefore, the reviewer suggests the authors use a more precise term to describe these behaviors, such as valence. Additionally, in lines 117 and 137-139, replacing "emotion" with "stress*

> *responses," a term that aligns more closely with the physiological observations, would provide greater specificity and clarity in interpreting the findings.*

Thanks for the suggestion. We have modified the description of “emotion” to “emotional valence” in various places throughout the revised manuscript.

> *(3) Related to the role of POA input to pPVT,*
>
> *a) The authors showed increased activity in pPVT neurons that receive projections from the POA (Figure 3), and these neurons are necessary for heat-induced behavioral changes (Figures 4N-W). However, is the POA input to the pPVT circuit truly critical? Since recipient pPVT neurons can receive inputs from various brain regions, the reviewer suggests that experiments directly inhibiting the POA-to-pPVT projection itself are needed to confirm the role of POA input. Alternatively, the authors could show that the increased activity of pPVT neurons due to chronic heat exposure is not observed when the POA is blocked. If these experiments are not feasible, the reviewer suggests that the authors consider toning down the emphasis on the role of the POA throughout the manuscript and discuss this as a limitation.*
> *b) In the electrophysiology experiments shown in Figures 6A-I, the authors conducted in vitro slice recordings on pPVT neurons. However, the interpretation of these results (e.g., "The increase in presynaptic excitability of the POA to pPVT excitatory pathway suggested plastic changes induced by the chronic heat treatment.", lines 349-350) appears to be an overclaim. It is difficult to conclude that the increased excitability of pPVT neurons due to heat exposure is specifically caused by inputs from the POA. To clarify this, the reviewer suggests the authors conduct experiments targeting recipient neurons in the pPVT, with anterograde labeling from the POA to validate the source of excitatory inputs.*

For point (a), we acknowledge that pPVT neurons receiving POA inputs may also receive projections from other brain regions. While these additional inputs warrant investigation, they fall beyond the scope of our current study and represent promising directions for future research. Notably, compared to other well-characterized regions such as the amygdala and ventral hippocampus, the pPVT receives particularly robust projections from hypothalamic nuclei (Beas et al., 2018). Our optogenetic inhibition of POA-recipient pPVT neurons during chronic heat exposure effectively prevented the influence of POA excitatory projections on pPVT neurons. Furthermore, selective optogenetic activation of POA excitatory terminals within the pPVT was sufficient to induce similar behavioral abnormalities in mice, strongly supporting the causal role of POA inputs in mediating chronic heat exposure-induced behavioral alterations.

Beas BS, Wright BJ, Skirzewski M, Leng Y, Hyun JH, Koita O, Ringelberg N, Kwon HB, Buonanno A, Penzo MA (2018) The locus coeruleus drives disinhibition in the midline thalamus via a dopaminergic mechanism Nat Neurosci 21:963-973.

Regarding point (b), we acknowledge certain limitations in our in vitro patch-clamp recordings when attributing increased pPVT neuronal excitability to enhanced presynaptic POA inputs. Nevertheless, our brain slice recordings clearly demonstrated heightened excitability of pPVT neurons following chronic heat exposure. This finding was further corroborated by our in vivo fiber photometry recordings specifically targeting POA-recipient pPVT neurons, which confirmed that the increased pPVT neuronal activity was indeed modulated by POA inputs. The causal relationship was strengthened by our observation that optogenetic activation of POA excitatory terminals within the pPVT reproduced behavioral abnormalities similar to those observed in chronic heat-exposed mice. Additionally, our inability to induce circuit-specific LTP in the POA-pPVT pathway suggests that these synapses were already potentiated and saturated, reflecting enhanced excitatory inputs from the POA to pPVT. Collectively, these findings support our conclusion that increased excitatory

projections from the POA to pPVT likely represent a key mechanism underlying chronic heat exposure-induced behavioral alterations in mice.

> *(4) The authors focus on the excitatory connection between the POA and pPVT (e.g., "Together, our results indicate that most of the pPVT-projecting POA neurons responded to heat treatment, which would then recruit their downstream neurons in the pPVT by exerting a net excitatory influence.", lines 169-171). However, are the POA neurons projecting to the pPVT indeed excitatory? This is surprising, considering i) the electrophysiological data shown in Figures 2E-K that inhibitory current was recorded in 52.4% of pPVT neurons by stimulation of POA terminal, and ii) POA projection neurons involved in modulating thermoregulatory responses to other brain regions are primarily GABAergic (Tan et al., 2016; Morrison and Nakamura, 2019). The reviewer suggests showing whether the heat-responsive POA neurons projecting to the pPVT are indeed excitatory (This could be achieved by retrogradely labeling POA neurons that project to the pPVT and conducting fluorescence in situ hybridization (FISH) assays against Slc32a1, Slc17a6, and Fos to label neurons activated by warmth). Alternatively, demonstrate, at least, that pPVT-projecting POA neurons are a distinct population from the GABAergic POA neurons that project to thermoregulatory regions such as DMH or rRPa. This would clarify how the POA-pPVT circuit integrates with the previously established thermoregulatory pathways.*

Thanks for the comment and suggestion. We acknowledge that there are both excitatory and inhibitory projections from POA to pPVT. Although it is not our primary focus, we are aware of the substantial inhibitory inputs from POA to pPVT which suggests an important function. However, we do not think that this pathway, which would exert an opposite effect on POA-recipient pPVT neurons compared to the excitatory input, contributes to the long-term effect of chronic heat exposure. This is due to the increased, rather than decreased, excitability of the neurons. There is a possibility that this inhibitory input serves as a short-term inhibitory control for other purpose. Further work is needed to fully address this question.

> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations for the authors):***
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> *I have a number of suggested minor edits that would improve the readability and interpretation of figures for the reader. In many figures, there are places where it is unclear what is being tested, and making minor changes would make the manuscript flow more easily for the reader:*
>
> *(1) The authors could add additional details about the behavior paradigms in the Figures, especially Figure 1. How long was the chronic heat exposure for? At what temperature? What is the length of time between the end of heat exposure and the start of behaviors? What was the schedule of testing for EPM and social behaviors? Was it all on the same day or on different days? These details will make it easier for the reader to understand the behavior tests.*

We have revised our experimental scheme, especially Figure 1, and added more detailed descriptions in the method section. The modifications have also been applied to the other figures.

> *(2) In Figures 1J and 1K, it is a bit unclear what is being shown in the right panel, since there are no axes or labels to interpret what is being plotted.*

We have added body kinetics (purple dot) in the left panel of Figure 1J and 1K to align with the right panels, and we have updated our descriptions in the figure legend.

> *(3) In general, Figure 1 would benefit from more headers/labels or schematics to demonstrate what is being tested (for example, it's unclear that forced swim, tail suspension, open field, aggression, sucrose preference, or acoustic startle are being studied unless the reader looks at the figure legend in depth. Simple schematics or titles for each panel would help.*

We have added the abbreviated titles for each panel of Figure 1 to help readers to better understand what was being tested.

> *(4) Figure 2A would benefit from edits to the schematic so that it is clear that heat exposure is being done before the animal is sacrificed and cFos is stained.*

We have revised the text to clarify that heat exposure occurred before the animal was sacrificed and c-Fos was stained.

> *(5) Figure 2D: would help if the quantification of overlap of cFos and rabies was shown in the figure in addition to reporting it in the text (84%).*

We have added quantification in Figure 2D.

> *(6) The supplemental data in Figure 2 - Supplemental Figure 1 showing increased Fos in PVT and POA after heat exposure would actually help if it was in main Figure 2 so that the reader can more clearly see the rationale for choosing the POA-PVT circuit. But this is a matter of preference and up to the author where they want to show this data.*

Thanks for the suggestion. But considering the layout and space, we will prefer to retain this part in Figure 2-supplemental figure 1.

> *(7) Figure 3 would benefit from a behavior schematic illustrating the time course of the experiment and what the heat exposure protocol is for each day (how many minutes heat 'on' vs 'off', the temperature of heat, etc). Also, what is different about day 22 that makes it chronic heat vs day 21? Currently, it is a bit hard to understand the protocol.*

We have added the temperature and time of chronic heat exposure in the schematic of Figure 3. The “day 22” represented the time point after chronic heat exposure. And we measured the calcium activity of POA recipient pPVT neurons on day 22 to compare with day 1 to demonstrate that the activity changes of POA recipient pPVT neurons after chronic heat exposure.

> *(8) Figure 3D, it is unclear what the difference is between the Day 1 data on the left and Day 1 data on the right. Same with Figure 3H, unclear what the difference is between the left and the right.*

The left panel and right panel reflect different parameters: frequency /min (left) and amplitude (△F/F) for Figure 3D-3H. By doing this, we want to reflect the dynamic activity changes of POA recipient pPVT neurons throughout chronic heat exposure process. Now, all figures in panel 3D to 3H have been revised to make them clearer in meaning.

> *(9) Figure 4A would benefit from schematics showing the stimulation protocol for chronic optogenetics (how many days? Frequency? Duration of time? Etc)*

We have added detailed schematics in our Figure 4A.

> ***Reviewer #2 (Recommendations for the authors)***
>
> *(1) It is interesting that social behavior appears to be reduced upon long-term heat exposure but not after acute heat exposure. Interaction of animals, such as huddling, can be used by animals as a form of behavioral thermoregulation in cold environments and heat may drive animals apart to allow for better heat dissipation. The social interaction measured here is not huddling (because, I assume, the animals are separated by a divider?) but is this form of behavior measured here related to huddling/"social thermoregulation"? This could be discussed.*

Our behavioral tests were performed at room temperature. Even though huddling is a type of social behavior, based on our observation, the tested mouse was actively revolving around the mental cap, suggesting this type of behavior is not related to huddling/social thermoregulation type of social behavior.

> *(2) Line 113: The statement "Chronic treatment did not change body temperature" should be clarified/rephrased because 90 minutes of 38 degrees centigrade exposure to heat will increase the body temperature of mice. It would be helpful if the authors made clear that they measure body temperature before the heat stimulus (and not during the heat stimulus), which is now only obvious if one digs into the methods section.*

We have revised the text and clarified that body temperature was measured before the heat stimulus in the revised manuscript.

> *(3) Figure 1J and K: for the non-experts, these graphs are difficult to interpret, some more explanation is needed (what exactly is measured ?). We believe that the term "arousal" may not be justified in this context because the authors have not measured sleep patterns (EEG and EMG) to show that the mice arouse from a sleep (or sleep-like) stage; the authors may consider changing the terminology, e.g. something along the lines of "agitation" or "activity".*

We have further elaborated the meaning of Figure 1J and K in our revised manuscript. The acoustic startle response is a well-recognized behavioral parameter reflecting arousal levels in rodent model. The more agitation in response to stimulus, the higher the arousal levels in mice. We have used the term “agitation” to describe mice’s performance in the acoustic startle response test.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) The authors suggest in the introduction of the manuscript that the HPA axis and other multifaceted factors may influence emotional changes caused by heat stress (lines 63-78). However, there are no experiments or discussions on how the POA-pPVT circuit interacts with these factors. In line with the study's proposed direction in the introduction section, it would be valuable to explore, or at least discuss, whether and how the POA-pPVT circuit interacts with the HPA axis or other neural circuits known to regulate emotional and stress responses. Alternatively, the reviewer suggests revising the content of the introduction to align with the focus of the study.*

Although POA is known to possibly interact with the HPA axis via its connection with the paraventricular nucleus of the hypothalamus, there is hardly any evidence for the pPVT. Thus, we prefer not to speculate this question, which remains open, in our current manuscript.

> *(2) In Figure 5, the authors report that pPVT neurons that receive projections from the POA exhibited increased responses to stressful situations following chronic heat exposure. However, considering the long pre- and post-recording time gap of approximately three weeks, the additional expression of GCaMP protein over time could potentially account for the increased signal. Therefore, the reviewer recommends including a control group without heat exposure to rule out this possibility.*

We have included Figure 3-figure supplement 1 in our manuscript to exclude the effect of expression of GCaMP protein over time on the recording of calcium signal.

> *(3) Related to Figure 2, a) Please include quantification data of the overlap between retrogradely labeled and c-Fos-expressing POA neurons, which can be presented as a bar graph in Figure 2. This would be beneficial for readers to estimate how many warm-activated POA neurons connected to the pPVT are actively engaged under these conditions.*

In the revised manuscript, we have included the quantification analysis in Figure 2.

> *b) The images in Figure 2 - Figure Supplement 1 seem to degrade in quality when magnified, making it difficult to discern finer details. Higher-resolution images would greatly improve the clarity and help in accurately visualizing the c-Fos expression patterns in the POA and pPVT regions.*

We have changed our images of Figure 2-figure supplement 1 to higher-resolution in the revised manuscript.

> *c) The c-Fos images in Figure 2D and Figure 2 - Figure Supplement 2C appear unusual in that the c-Fos signal seems to fill the entire cell, whereas c-Fos protein is localized to the nucleus. Could the authors clarify whether this image accurately represents c-Fos staining or if there might be an issue with the staining or imaging process?*

We are confident that the green signals in both Figure 2D and Figure 2-figure supplement 2C, which did not occupy the whole cell body, have already accurately reflected the c-Fos and that they were nucleus staining. We have updated the amplified picture in Figure 2D.

> *d) In Supplemental Figure 2B, the square marking the region of interest should be clearly explained in the figure legend to ensure that readers can fully understand the context and focus of the image.*

We have further modified our figure legend in Figure 2-figure supplement 1 in our revised manuscript.

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