nature portfolio

Peer Review File

A parabrachial-hypothalamic parallel circuit governs cold defense in mice

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this study, Yang and colleagues used interdisciplinary techniques to illustrate the involvement of the lateral Parabrachial (LPB)- dorsomedial hypothalamus (DMH) in cold defense response for thermoregulation in mice, which complements previous findings that LPB to the preoptic area (POA) in regulating body temperature in mice. Specifically, aided by cre-dependent AAV tracing and c-Fos staining, the authors identified LPB Vglut-2 neurons projecting to POA and DMH are both responses to cold/warm exposure. Next, they used in vivo fiber photometry for neuronal calcium activities and observed LPB Vglut2-to-DMH pathways are robustly activated during gradients of cooling exposures. By employing a series of neuronal activity manipulations, they revealed that LPB→POA/DMH pathways form parallel roles in cold defense. Additionally, optogenetic activation shows LPB Vglut2→DMH pathway induces strong hyperthermia. Moreover, by selectively blocking the synaptic outputs of the downstream neurons using cell type-specific expression of TeNT, they found cold-defense responses depend on both Vglut2 and Vgat-expressing neurons in the DMH but not POA Vglut2-neurons. Notably, blocking both LPB→POA/DMH pathways exhibited a much stronger impairment in cold defense, suggesting a cumulative or additive effect of the two pathways. Activation of the LPB Vglut2→DMH pathway rapidly relieves hypothermia, increases iBAT thermogenesis, and suppresses body weight gain. Using retro-TRAP-seq, they identify DMH-projecting LPB SST neurons as cold-activated neurons. Finally, they showed LPB SST→DMH pathway increases Tcore via iBAT thermogenesis and is required for cold defense. Overall, this comprehensive study revealed an important central mechanism in understanding how neuronal circuits affect thermoregulation. The design of this study is straightforward, and the results are novel, exciting, and convincing. I have the following comments for the authors that will hopefully help them to improve the paper.

Major comments:

Their data shows that LPB neurons were activated in responding to cold exposure, and ~60% project to POA or DMH, and the authors claim that the two projecting pathways work in parallel in cold defense. I wondered if other parallel pathways work in concert with the POA and DMH projections in cold defense. This should either be tested or discussed.

It is convincing that the authors had shown multiple experiments that the activation LPB Vglut2-DMH pathway induces a strong cold defense response. In figure 4e-g the authors showed that DMH terminal activation could overcome LPB Vglut-2 soma hM4Di inhibition. Can the author show that chemogenetic inhibition can prevent the possible backpropagation of action potentials induced by the chemogenetic stimulation at the terminals? Moreover, I wonder if LPB Vglut-2 soma hM4Di inhibition would decrease Tcore? Although Figure 4g may argue against it, it is unclear why saline injection will cause an apparent slight decrease in the core temperature, similar to the CNO injection condition.

Several points need to be clarified for the optogenetic activation experiments. It is unclear what frequency and intensity are used in most paradigms. Have the authors evaluated the best photoactivation frequency in LPB-POA/DMH pathways? In Figure 5, what is the rationale for using 12 mw, 10 Hz 10 ms, 30-min per 2-h, 4 loops per day photoactivation? How robust could the LPB-DMH neuron follow this manipulation?

It is convincing that LPB SST-DMH plays an important function in cold defense. I would suggest that the author verify the colonization of SST and Vlut2 within in LPB region since SST are robust GABAergic neuron makers in most cases. If they do not totally overlap with Vglut2, are they GABAergic? Moreover, this may be beyond the scope of the current study. I wonder if SST, as a neuropeptide, is involved in the LPB SST-DMH pathway to modulate temperature regulation.

In figure 7, the author used SST-cre cross with LepR-cre or ChAT-cre mouse. Is it possible that DMH also has SST neurons?

Reviewer #2 (Remarks to the Author):

In this manuscript, the author characterized a new LPB□DMH pathway that is important for cold defensive behaviors. The battery of viral tools used in this manuscript is very impressive and the author provided solid data to support the LPB□DMH pathway in cold defense. However, results from this manuscript also raised some puzzling but important questions. These questions need to be address before we can put this new discovery in the context of our understanding of the central pathway for thermoregulation.

1. Why both LPB□DMH and LPB□MPO pathways are required for cold defensive behaviors? What is relationship between neurons in DMH and MPO that received inputs from LPB?

2. Similarly, why both DMHvglut2 vs. DMHVgat are required for cold induced defensive behavior?

3. In Fig.5F, why body weight no longer increase after light stimulation start in control mice? could it be the stress caused by long term light stimulation? why not using chemogenetic stimulation which could be less stressful?

4. Because LPBSST neurons also project to MPO? What is the percentage of LPBSST neurons□MPO neurons are Fos+ after cold stimulus?

5. There are so many markers for the DMH projecting LPBVglut2 neurons? are these neurons co-label same neurons or they label different population of neurons? this question is important because LPBSST neurons only represent less than 20% of Fos+ neurons after cold stimulus.

6. Are LPB is the only input to drive cold response in the DMH?

Minor point:

1. Because the limited retrograde efficiency of retrograde tracing virus, the conclusion of '20% projected to both regions' is likely underestimate.

2. Using terminal fiber photometry to study the kinetics of temperature response is not very meaningful, as they could be strongly modulate by varies autoreceptors located in the terminal.

Reviewer #3 (Remarks to the Author):

In this paper, the authors examine the hypothesis that cold responsive neurons in the lateral parbrachial nucleus (LPB) project to the dorsomedial nucleus of the hypothalamus, where they activate cold-defense pathways. I typically begin a review with a summary of all of the experiments that were done, however, that is nearly impossible for this paper. It is extremely long (over 7,000 words) and gives the results of over 40 experiments, many of which are very complicated. In most cases, there is no n given (in others where it is given, it is often very low numbers, such as 3 animals), and in no case is there given sufficient detail on injection placement (some of these injections have to miss their targets, but there is no information on how many total animals were done, how they picked the small number of animals they present, or what the anatomical controls, i.e., missed injections, showed), controls are not done for many key experiments, and it is rare to find any statistical analysis.

Having said that, I think this is an interesting story, which would be of interest to many scientists who work on thermoregulation. But it is impossible to evaluate the work critically in its current state because so much of the necessary information on the rigor of the experiments is missing. I would strongly encourage the authors, to include complete information for each experiment. This would include the power analysis that should have been done before the studies were started indicating the number of animals that should be in each group; the actual numbers of animals used in each experiment; how they chose which ones to present in the paper; details about the actual results in the animals that are included and the ones that are not included, as well as controls; how they did the statistics; and what the statistical findings were. This is particularly important for experiments involving stereotaxic injections, some of which will miss their intended target. How were those cases identified? They should be analyzed by someone who does not know the physiological results. The ones that hit the target should be analyzed separately from those that missed the target, which then serve as anatomical controls. But you need to present heat maps showing the actual injection placements in both sets of animals.

Responses to Reviewers

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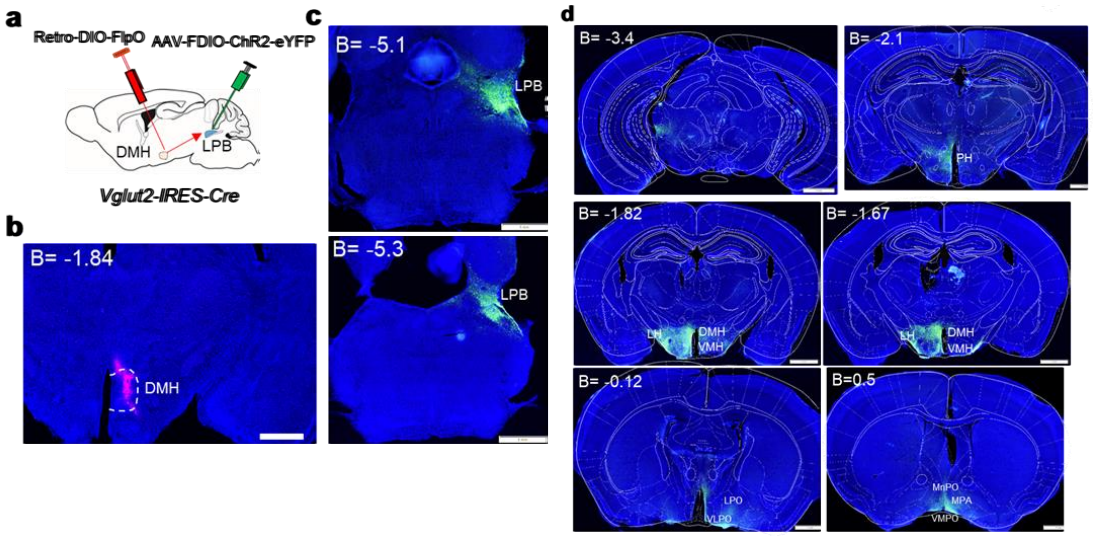
In this study, Yang and colleagues used interdisciplinary techniques to illustrate the involvement of the lateral Parabrachial (LPB)- dorsomedial hypothalamus (DMH) in cold defense response for thermoregulation in mice, which complements previous findings that LPB to the preoptic area (POA) in regulating body temperature in mice. Specifically, aided by cre-dependent AAV tracing and c-Fos staining, the authors identified LPB Vglut-2 neurons projecting to POA and DMH are both responses to cold/warm exposure. Next, they used in vivo fiber photometry for neuronal calcium activities and observed LPB Vglut2-to-DMH pathways are robustly activated during gradients of cooling exposures. By employing a series of neuronal activity manipulations, they revealed that LPB→POA/DMH pathways form parallel roles in cold defense. Additionally, optogenetic activation shows LPB ^{Vglut2}→DMH pathway induces strong hyperthermia. Moreover, by selectively blocking the synaptic outputs of the downstream neurons using cell type-specific expression of TeNT, they found cold-defense responses depend on both Vglut2 and Vgat-expressing neurons in the DMH but not POA ^{Vglut2}-neurons. Notably, blocking both LPB→POA/DMH pathways exhibited a much stronger impairment in cold defense, suggesting a cumulative or additive effect of the two pathways. Activation of the LPB ^{Vglut2}→DMH pathway rapidly relieves hypothermia, increases iBAT thermogenesis, and suppresses body weight gain. Using retro-TRAP-seq, they identify DMH-projecting LPB SST neurons as cold-activated neurons. Finally, they showed LPB SST→DMH pathway increases T_{core} via iBAT thermogenesis and is required for cold defense. Overall, this comprehensive study revealed an important central mechanism in understanding how neuronal circuits affect thermoregulation. The design of this study is straightforward, and the results are novel, exciting, and convincing. I have the following comments for the authors that will hopefully help them to improve the paper.

R: Thanks for the appreciation of our efforts in the dissection of the cold-defense circuit.

Major comments:

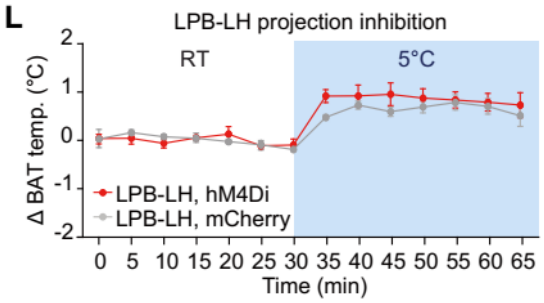
Their data shows that LPB neurons were activated in responding to cold exposure, and ~60% project to POA or DMH, and the authors claim that the two projecting pathways work in parallel in cold defense. I wondered if other parallel pathways work in concert with the POA and DMH projections in cold defense. This should either be tested or discussed.

R: We appreciate the attention given to this matter. The DMH-projecting LPB^{Vglut2} neurons were found to project to several brain regions, including the LH and VMH, as shown in **Extended Data Fig. 3**. Nevertheless, the LPB→LH projection was demonstrated to be dispensable for cold-induced thermogenesis, as evidenced by **Cited Fig. 1L** (*1*), Furthermore, although LPB neurons projecting to the VMH were predominantly located in the LPBc region, as depicted in **Cited Fig. 2b, c**. (*2*), they were not co-localized with the LPBel neurons that were activated by cold stimuli. Thus, we have included a discussion in **lines 606-613**, noting that "it is noteworthy that the LPB neurons projecting to the POA/DMH region also have projections to other brain regions involved in thermoregulation, such as the LH and VMH (**Extended Data Fig. 3**). However, the LPB→LH projection was not found to be necessary for cold-induced thermogenesis (*1*), and the VMH-projecting LPB neurons were not responsive to cold stimuli (*2, 3*). Further investigation is necessary to ascertain the existence of parallel pathways that operate in conjunction with the LPB→POA/DMH projections.".

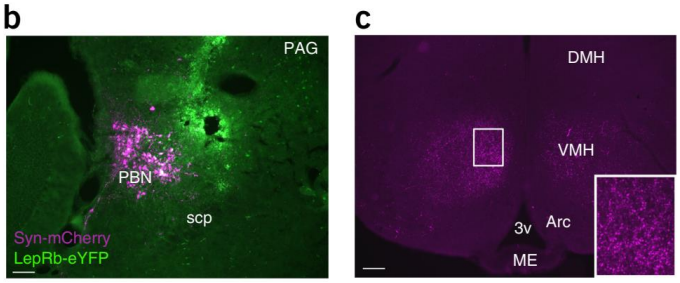


Extended Data Fig. 3 | Projection pattern of DMH-projecting LPB^{Vglut2} neurons throughout the brain. (a) Scheme for mapping the axonal projections of DMH-projecting LPB^{Vglut2} neurons. For labeling DMH-projecting LPB^{Vglut2} neurons, retrograde AAVs carrying Cre-dependent FlpO were injected in the DMH, which drove the expression of FlpO-dependent ChR2-eYFP in the

LPB. A red tracer (CTB647) was co-injected into the DMH to indicate the injection sites. (b) Representative image showing the injection sites in the DMH viewed by red tracer (CTB647). (c) Representative images showing ChR2-eYFP expression in DMH-projecting LPB^{Vglut2} neurons in the LPB. (d) Representative images are showing ChR2-eYFP expression in axonal terminals at various brain sites.



Cited Fig. 1 | (L) LPB-LH projection inhibition had no effect on iBAT temperature during cold challenge. Cited from (*1*).

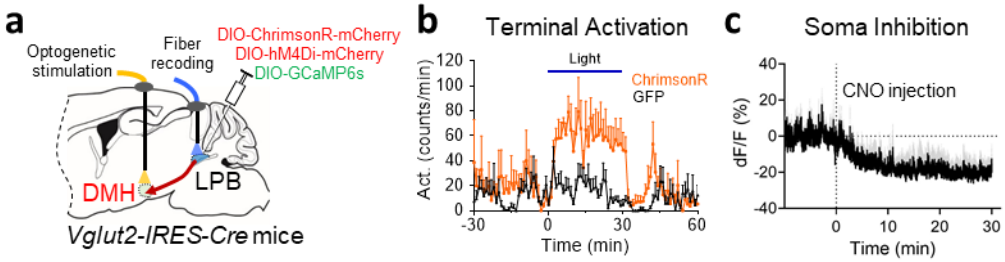


Cited Fig. 2 | (b) LPB lepR positive neurons mainly distributed in LPBc and send projections to VMH (c). Cited from (*2*).

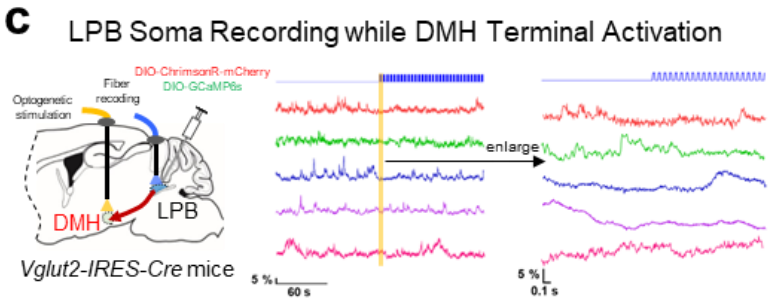
It is convincing that the authors had shown multiple experiments that the activation LPB^{Vglut2}-DMH pathway induces a strong cold defense response. In figure 4e-g the authors showed that DMH terminal activation could overcome LPB $^{Vglut-2}$ soma hM4Di inhibition. Can the author show that chemogenetic inhibition can prevent the possible backpropagation of action potentials induced by the chemogenetic (optogenetic?) stimulation at the terminals?

R: We acknowledge the potential concern of backpropagation of action potentials in optogenetic experiments. However, the limited literature on this topic suggests that this issue may be overestimated. To address this concern, it would be ideal for us to conduct electrophysiological recordings of backpropagation of action potentials in LPB brain slices. As simultaneous activation of DMH terminals and recording of backpropagation of action

potentials in LPB brain slices is technically challenging due to the long distance between LPB and DMH, we directly recorded calcium activity in vivo. We used red-shift opto-tools ChrimsonR to estimate whether photoactivated DMH terminals were strong enough to evoke calcium activity in LPB somata. Co-expression of GCaMP6s, DREADD-Gi, and ChrimsonR was done in the LPB, followed by the activation of DMH terminals using a 589-nm laser (**Reviewer only Fig. 1a**). Ca^{2+} activity was recorded in the LPB, and we confirmed that DMH terminal photostimulation could induce hyperactivity, and DREADD-Gi inhibition could substantially reduce LPB somatic neural calcium activity (**Reviewer only Fig. 1b-c**). However, we did not observe any calcium signal changes in LPB somata due to DMH terminal photoactivation (in short or long terms) (**Extended Data Fig. 7c**). Thus, we conclude that backpropagated action potentials are too weak to be detected by in vivo calcium signals. Additionally, we argue that if any undetected backpropagation of action potentials exists, it should be blocked by DREADD-Gi inhibition since Gi inhibition has a larger impact on LPB neural activity than that of DMH terminal photoactivation.



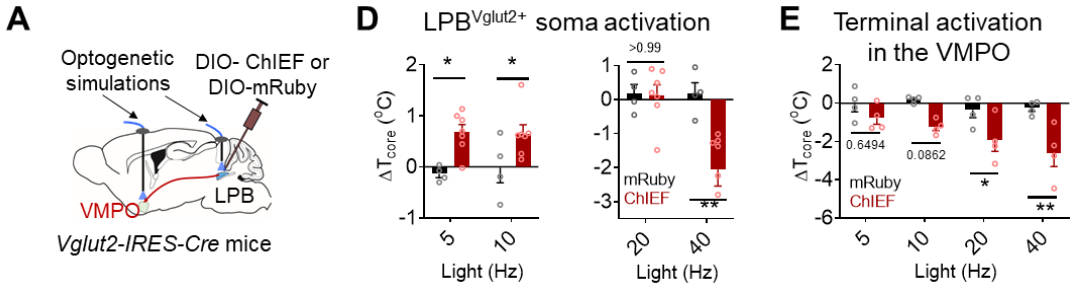
Reviewer only Fig. 1 | (a) Simultaneously expressing the GCaMP6s, DREADD-Gi and ChrimsonR in the LPB, then the DMH terminals were simultaneously activated using a 589nm laser and Ca^{2+} activity was recorded in the LPB; (b) Photoactivation of DMH terminal with ChrimsonR result in hyperactivity. (c) LPB neurons' calcium activity was inhibited by hM4Di after CNO injection.



Extended Data Fig. 7 | (c) Scheme for simultaneously expressing the GCaMP6s and ChrimsonR in the LPB, then the DMH terminals were simultaneously activated using a 589nm laser and Ca^{2+} activity was recorded in the LPB (left panel). No obvious calcium signal changes were recorded in LPB after activating DMH's terminals (n = 5 mice). The blue pulse line indicates light stimulation (right panel). Light pattern: 589 nm, 6 mW, 10 Hz, 10 ms, 2-s on followed by 2-s off, with the cycles repeating for 10 min.

Moreover, I wonder if LPB $^{Vglut-2}$ soma hM4Di inhibition would decrease T_{core}?

R: In brief, we did not find that acute inhibition of LPB^{Vglut2} soma with hM4Di changed T_{core}. As previously reported (**Cited Fig. 3**) (*3*), broad activation of LPB^{Vglut2} soma can lead to hypothermia or hyperthermia, depending on the stimulation frequency. Therefore, it is not surprising that broad inhibition of LPB^{Vglut2} neurons did not alter T_{core}, as there are mixed hypothermic and hyperthermic effects in this region. Our results show that the acute increase of T_{core} after Sal/CNO injection was induced by stress, as indicated in the baseline data before injection (**Fig. 4g**).



Cited Fig. 3 | The change of T_{core} after photoactivation of LPB^{Vglut2} soma (D) and VMPO terminal (E) with different frequencies. Light pattern: 473 nm, 6 mW, 5/10/20/40 Hz, 10 ms, 2-s on followed by 2-s off, with the cycles repeating for 30 min.

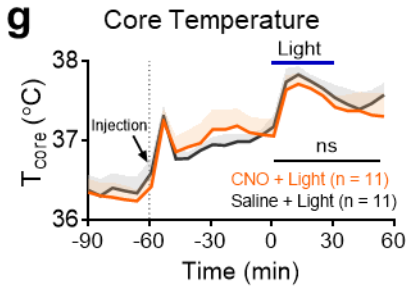


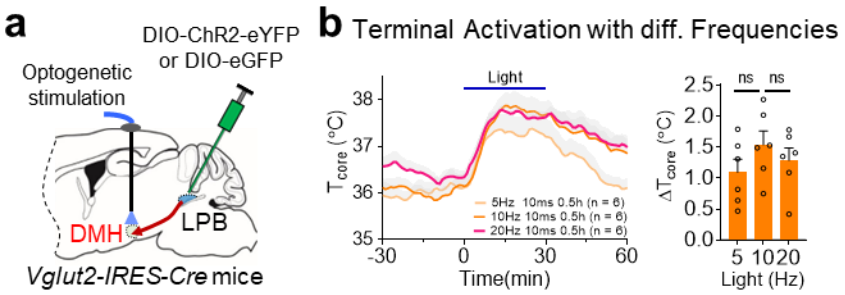
Fig. 4 | (g) Changes in T_{core} after activating the LPB^{Vglut2}→DMH projection while blocking LPB neurons (n = 11 mice). CNO was injected at -60 min to silence neurons as indicated (i.p., 10 mg/kg) and saline was used as the control.

Although Figure 4g may argue against it, it is unclear why saline injection will cause an apparent slight decrease in the core temperature, similar to the CNO injection condition.

R: As previously mentioned (**Fig. 4g**), the observed phenomenon involves the restoration of normothermia following an acute increase in T_{core} induced by Sal/CNO injection-induced stress and is not a hypothermia response.

Several points need to be clarified for the optogenetic activation experiments. It is unclear what frequency and intensity are used in most paradigms. Have the authors evaluated the best photoactivation frequency in LPB-POA/DMH pathways?

R: We have provided information regarding the frequency and intensity of optogenetic activation experiments in the figure legend (6 or 12mW 10 Hz, 10 ms, 2-s on followed by 2-s off, with the cycles repeating for 30 min). We did evaluate the photoactivation frequency for both pathways and published the data for stimulation of the LPB-POA pathway (*3*). We did observe that the hypothermia induced by terminal activation of the LPB→POA pathway increased as the photoactivation frequency increased (**Cited Fig. 3)** (*3*). In the case of the LPB→DMH pathway, we found that hyperthermia induced by 10-Hz photoactivation was greater than that induced by 5 Hz and 20 Hz, although this difference was not statistically significant. We have now added this data to **Extended Data Fig. 7a, b**.



Extended Data Fig. 7 | (a-b) The change of T_{core} after photoactivation of LPB^{Vglut2} DMH terminal with different frequencies. Light pattern: 473 nm, 12 mW, 5/10/20 Hz, 10 ms, 2-s on followed by 2-s off, with the cycles repeating for 30 min.

In Figure 5, what is the rationale for using 12 mw, 10 Hz 10 ms, 30-min per 2-h, 4 loops per day photoactivation? How robust could the LPB-DMH neuron follow this manipulation?

R: In our study, experiments were primarily conducted during the active phase of the mice (9:00 am- 9:00 pm). Each mouse required fiber attachment and equipment setup, which typically took 1-2 hours. Therefore, the available time for photoactivation was limited to 9-10 hours. In addition, to ensure adequate recovery time from the previous photoactivation (which lasted 1-1.5 hours), each photoactivation session was limited to 30 minutes. Thus, we could only perform a maximum of 4 photoactivation sessions per day. As illustrated in **Fig. 5e**, the LPB-DMH neurons exhibited a robust response to optogenetic manipulation, resulting in a 1-1.5°C increase in T_{core}. We have included this data in our figures for reference.

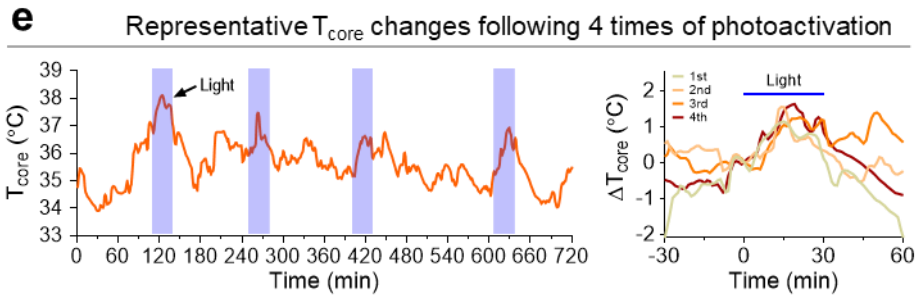
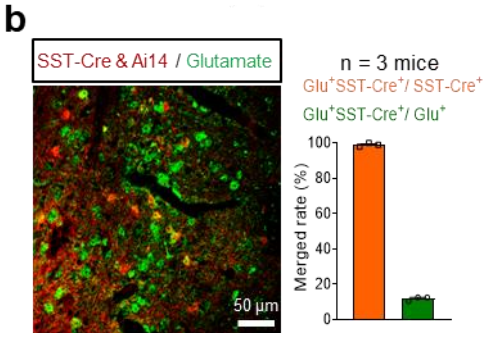


Fig. 5 | (e) The change of T_{core} after 4 times photoactivation (left panel) of LPB^{Vglut2} DMH terminal and the comparison (right panel) of T_{core} change for each photoactivation.

It is convincing that LPB^{SST}-DMH plays an important function in cold defense. I would suggest that the author verify the colonization of SST and Vglut2 within in LPB region since SST are robust GABAergic neuron makers in most cases. If they do not totally overlap with Vglut2, are they GABAergic? Moreover, this may be beyond the scope of the current study. I wonder if SST, as a neuropeptide, is involved in the LPB SST-DMH pathway to modulate temperature regulation.

R: In response to the first question, we're glad that we have the same interest as the reviewer. The relevant data had already been included in original **Extended Data Fig. 8b**. Our analysis has confirmed that the LPB^{SST} neurons are primarily glutamatergic, as confirmed by glutamate staining. This finding is consistent with previous studies(*3-6*) reporting that the vast majority of neurons (>98%) in LPB are also glutamatergic, as evidenced by the sequencing of the SST gene from DMH-projecting LPB^{Vglut2} neurons (**Fig. 6a-c**). As SST is primarily used as a GABAergic neuron marker in the cortex, it is worth noting that in other regions such as the hypothalamus, SST neurons are capable of exhibiting both glutamatergic and GABAergic phenotypes (*7*). Therefore, we did not present this data in the main figure.



Extended Data Fig. 8 | (b) Overlap between SST-IRES-Cre labeled neurons (Tdt^{+}) in the LPB and the following immuno-positive neurons and glutamate. SST-IRES-Cre mice were crossed with Ai14 (Rosa-CAG-LSL-tdTomato-WPRE) to label SST-IRES-Cre in the LPB.

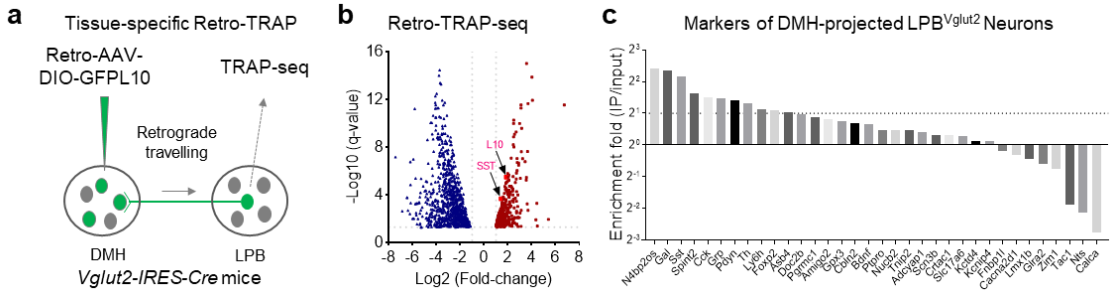


Fig. 6 | (a) Projection-specific transcriptomic analysis (retro-TRAP), where GFP-tagged translational ribosomes from DMH-projecting LPB^{Vglut2} neurons were immunoprecipitated and associated mRNAs were sequenced. Retrograde tracing virus carrying Cre-dependent GFP-tagged ribosomal protein L10 (AAV-Retro-hEF1a-FLEX-GFPL10) was injected into the DMH, which traveled to the LPB and expressed GFPL10 after recombination by Vglut2-IRES-Cre. SST, somatostatin. (b) Volcano plots (q value versus log2 fold change) for LPB mRNAs after retro-TRAP sequencing. (c) Retro-TRAP fold enrichment (IP/Input) for PB-expressed genes downloaded from the Allen Institute.

In regard to the second question, it is worth noting that the SST peptide was administered into the brain over 50 years ago, and numerous studies (*8-11*) have since reported that this injection can induce hyperthermia, particularly when targeting the DMH. It would be interesting to explore the potential role of endogenous SST peptide in thermoregulation in future research.

In figure 7, the author used SST-cre cross with LepR-cre or ChAT-cre mouse. Is it possible that DMH also has SST neurons?

R: We would like to acknowledge that the data referred to by the reviewer has indeed been included in **Fig. 7q** of our manuscript. In addition, we have provided clarification in **lines 519-521** of our manuscript by stating that "since SST-Cre is also expressed in the DMH (**Fig. 7q, left panel**).". Furthermore, our statement "blocking DMH$^{ChAT+SST}$ neurons had no effect" serves to exclude the role of DMH^{SST} neurons in this manipulation.

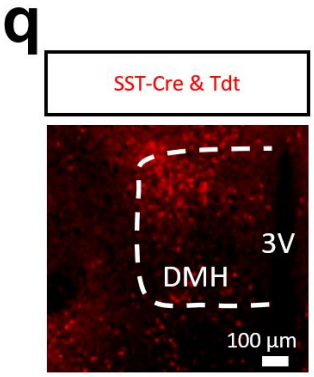


Fig. 7 | (q) Representative SST-Cre & Tdt expression in the DMH.

Reviewer #2 (Remarks to the Author):

In this manuscript, the author characterized a new LPB→DMH pathway that is important for cold defensive behaviors. The battery of viral tools used in this manuscript is very impressive and the author provided solid data to support the LPB→DMH pathway in cold defense. However, results from this manuscript also raised some puzzling but important questions. These questions need to be addressed before we can put this new discovery in the context of our understanding of the central pathway for thermoregulation.

1. Why both LPB→DMH and LPB→MPO pathways are required for cold defensive behaviors? What is the relationship between neurons in DMH and MPO that received inputs from LPB?

R: For the first question why two parallel pathways are required in cold defenses. As we wrote in the discussion section: "The evolution of parallel neural circuits in cold defense not only enables resilience to hypothermia but also provides a scalable, robust, and efficient network in heat production when both pathways are recruited." (**lines 618-621**). In line with this hypothesis, we have presented data to show that activation of the LPB→DMH pathway is powerful enough to reverse cold-induced hypothermia (**Fig. 4p-r**). Furthermore, we have shown that both pathways are additively or synergistically required to boost cold defense (**Fig. 3g-k**).

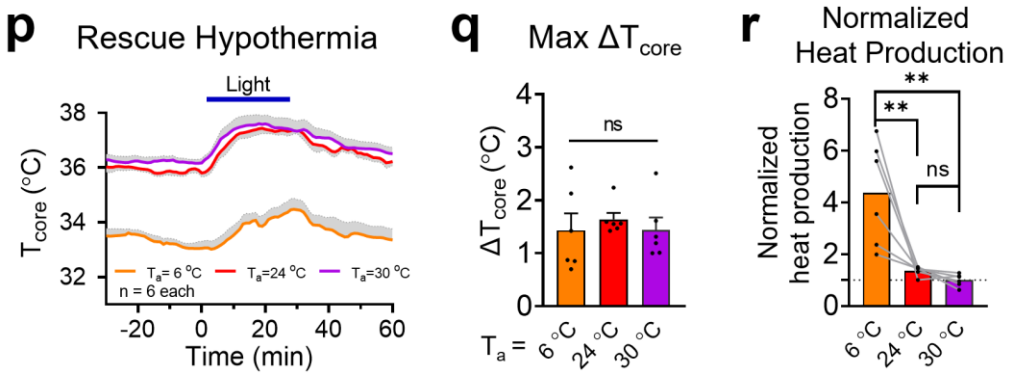


Fig. 4 | (p-q) Changes in T_{core} after photoactivation of the LPB^{Vglut2}→DMH projection under different T_a (6, 24, 30°C; n = 6 mice each) (p). The maximum ΔT_{core} during photoactivation was quantified in (q). (r) Normalized heat production of mice after photoactivation of the LPB^{Vglut2}→DMH projection under different Ta as indicated. Heat production was normalized by the formula (T_{core} -T_a) / ΔT_{core} as reported.

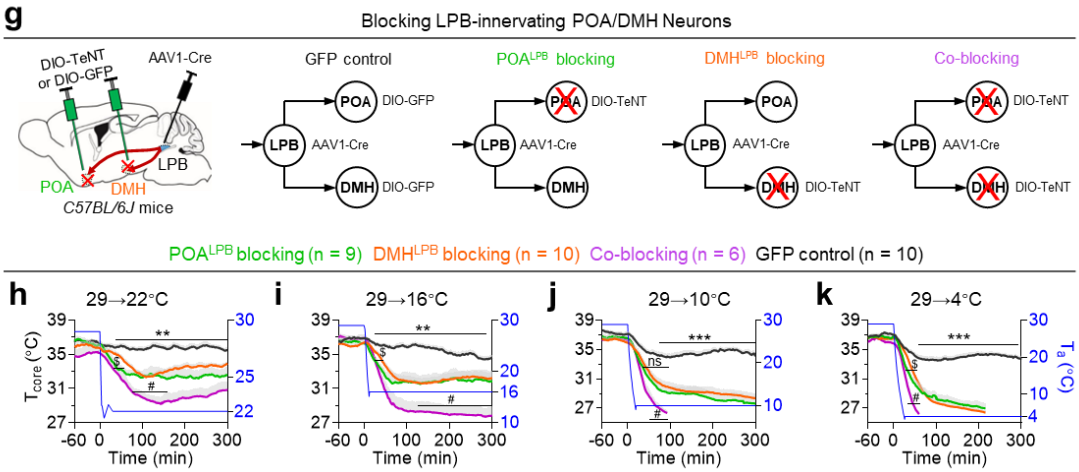
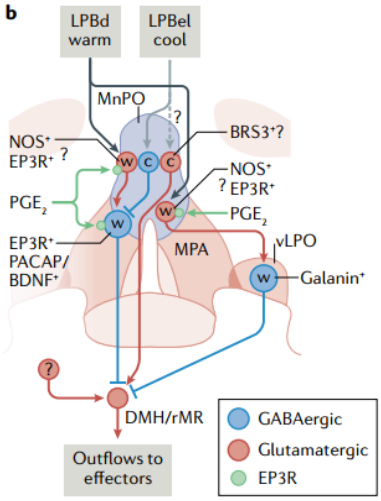


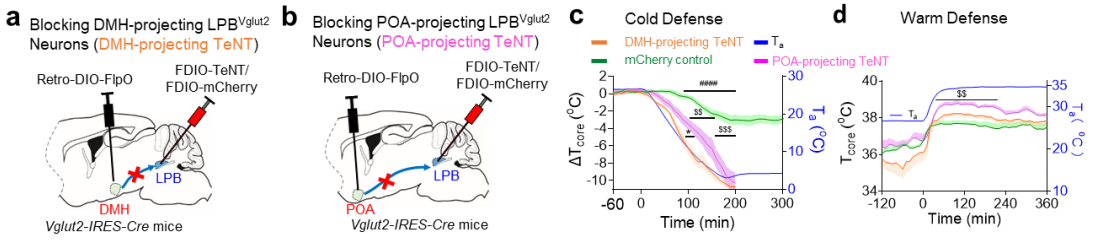
Fig. 3 | (g) Blocking LPB-innervating POA/DMH neurons or both using TeNT. The detailed scheme is shown in Extended Data Fig. 5a. Briefly, anterograde transsynaptic Cre carried by AAV1 (AAV1-hSyn-Cre) was injected in the LPB to drive expression of Cre-dependent TeNT injected in either the POA (POA^{LPB} blocking), or the DMH (DMH^{LPB} blocking), or both (co-blocking). Cre-dependent GFP co-injected in both the POA and DMH was used as the control (GFP control). (h-k) T_{core} changes in response to a series of cold exposures, namely 29→22ºC (h), 29→16ºC (i), 29→10ºC (j), and 29→4ºC (k), after blocking POA^{LPB}, DMH^{LPB}, or both types of neurons (n = 10 mice for GFP and DMH^{LPB} blocking group; n = 9 mice for POA^{LPB} blocking group; n = 6 mice for the co-blocking group). *, DMH^{LPB} blocking vs. GFP; $, DMH^{LPB} vs. POA^{LPB} blocking; #, POA^{LPB} vs. co-blocking.

For the second question regarding the relationship between neurons in DMH and MnPO/VMPO that received inputs from LPB. As summarized by Kazuhiro (*12*) (**Cited Fig. 4**), LPB-innervating MnPO/VMPO neurons contain separate subgroups of neurons for either cold or warm defenses and therefore are responsible for both cold and warm defenses. In contrast, LPB-innervating DMH neurons are only responsible for cold defense. We also verified this conclusion by blocking MnPO/VMPO-projecting or DMH-projecting LPB^{Vglut2} neurons (**Reviewer only Fig. 2a-b**). Both blockings impaired cold defenses but only blocking MnPO/VMPO-projecting LPB^{Vglut2} neurons impaired warm defense (**Reviewer only Fig. 2c-d**). Taken together, LPB-innervating MnPO/VMPO and DMH neurons function in parallel in cold defense. In contrast, according to previous numerous literature (*12-14*), DMH neurons, including

LPB-innervating DMH neurons, are expected to act downstream of LPB-innervating MnPO/VMPO neurons to form a feed-forward pathway in warm defense.



Cited Fig. 4 | (b) A model of the POA local circuit that controls effector responses to thermal and infection stresses. Cutaneous warm-sensory inputs from the dorsal part of the lateral parabrachial nucleus (LPBd) activate glutamatergic interneurons in the MnPO (blue-shaded area), which then activate GABAergic projection neurons in the MPA, the MnPO and the ventral part of the lateral preoptic area (vLPO) (red shaded areas). These projection neurons inhibit excitatory neurons in the dorsomedial hypothalamus (DMH) and rostral medullary raphe region (rMR) that otherwise drive cold-defensive responses. (Cited from (*12*))

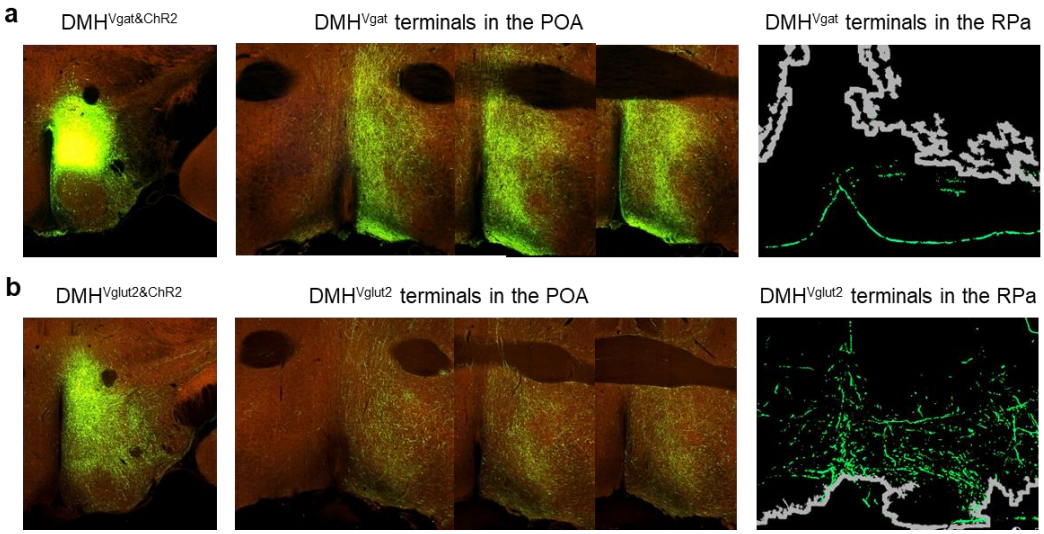


Reviewer only Fig. 2 | (a) Scheme for blocking DMH-projecting LPB^{Vglut2} neurons. For blocking DMH-projecting LPB^{Vglut2} neurons, retrograde AAVs carrying Cre-dependent FlpO were injected in the DMH, which drove the expression of FlpO-dependent TeNT in the LPB. (b) Scheme for blocking POA-projecting LPB^{Vglut2} neurons. For blocking POA-projecting LPB^{Vglut2} neurons, retrograde AAVs carrying Cre-dependent FlpO were injected in the POA, which drove the expression of FlpO-dependent TeNT in the LPB. (c-d) Changes in T_{core} after blocking of DMH-projecting LPB^{Vglut2} or POA-projecting LPB^{Vglut2}

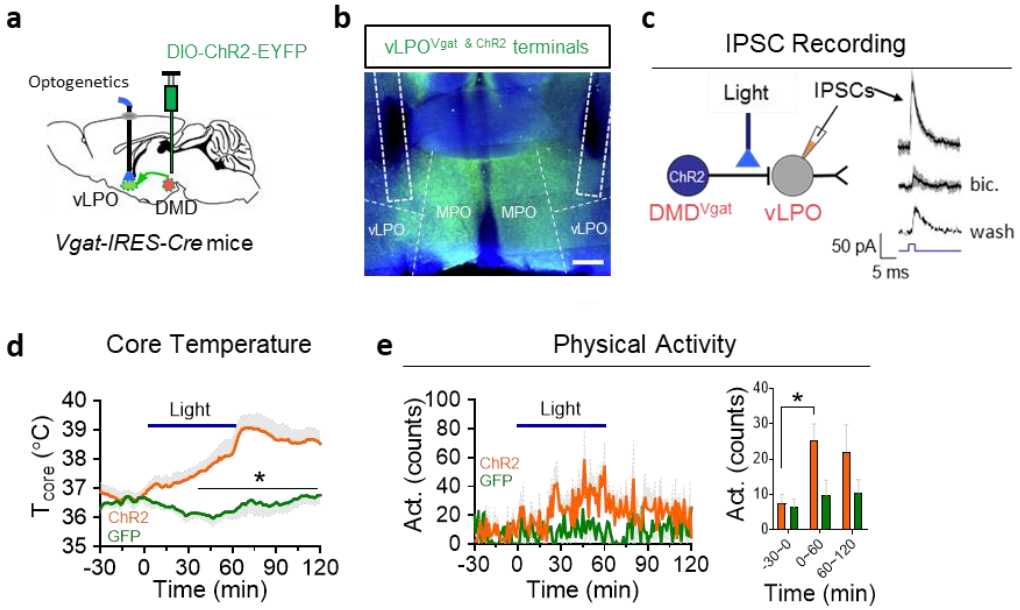
neurons during the cold (c) or warm (d) challenge. #, mCherry vs DMH-projecting TeNT; $, mCherry vs POA-projecting TeNT; *, DMH-projecting TeNT vs POA-projecting TeNT.

2. Similarly, why both DMH^{Vglut2} vs. DMH^{Vgat} are required for cold induced defensive behavior?

R: In our previous publication in *PNAS* (*15*), we clearly demonstrated that both DMH^{Vglut2} vs. DMH^{Vgat} are important for cold defense since they both could bidirectionally control T_{core} upon activation/inhibition. By comparing the whole-brain projection data of $DMH^{Vglut2/Vgat}$ neurons from the Allen Brain Institute, we found that DMH^{Vgat} neurons mainly project to the POA with few projections to RPa (rMR), while DMH^{Vglut2} neurons send significantly more projections to RPa than DMH^{Vgat} neurons (**Cited Fig. 5**). Therefore, we proposed that DMH^{Vglut2} neurons primarily provide excitatory input to premotor neurons in the RPa to stimulate thermogenesis, while DMH^{Vgat} neurons may mainly suppress POA hypothermic neurons to inhibit heat loss. Working together, these two neuron types could maximize heat production to defend against cold. In line with the evidence, our preliminary data suggest that the projection from the DMH^{Vgat} to the vLPO indeed could increase T_{core} (**Reviewer only Fig. 3**)



Cited Fig. 5 | The terminal distribution of DMH^{Vgat} (a) and DMH^{Vglut2} neurons (b) in the POA and RPa (downloaded from Allen brain institute).



Reviewer only Fig. 3 | (a) Scheme for photoactivation of neural terminals of DMH^{Vgat} neurons in the vLPO using ChR2. (b) Representative expression of ChR2 terminals from DMH^{Vgat} neurons in the vLPO. (c) ChR2-expressing terminals were activated with a 2-ms blue light to elicit inhibitory postsynaptic currents (IPSCs) in vLPO neurons. IPSCs were blocked by bicuculline (bic.) and recovered partially after washing. Shadowed areas were SD. (d-e) Changes of T_{core} (d), and physical activity (e) after neural terminals of DMH^{Vgat} neurons in the vLPO (ChR2, n = 6 mice; GFP, n = 4). Light pattern: 473 nm, 6 mW, 20 Hz, 10 ms, 2-s on 2-s off, 60 min.

3. In Fig.5F, why body weight no longer increases after light stimulation start in control mice? could it be the stress caused by long term light stimulation? why not using chemogenetic stimulation which could be less stressful?

R: We noticed that the stress caused by fiber attachment during long-term light stimulation might curb the body weight gains. Therefore, we wrote in **line 412-415**: “Although the photostimulation procedure itself appeared to curb weight gains in control mice, photoactivation of the LPB^{Vglut2}→DMH projection further reduced body weight without affecting cumulative food intake (**Fig. 5f-h**).”. As suggested by this reviewer, the chemogenetics could be suitable for long-term stimulation in soma stimulations. However, using chemogenetic to activate the terminal requires repetitive injection of CNO to the DMH through a cannula, which might also cause stress. Therefore, we did not adopt the chemogenetic approaches. We hope this reviewer would agree with us and understand the limitations of both approaches.

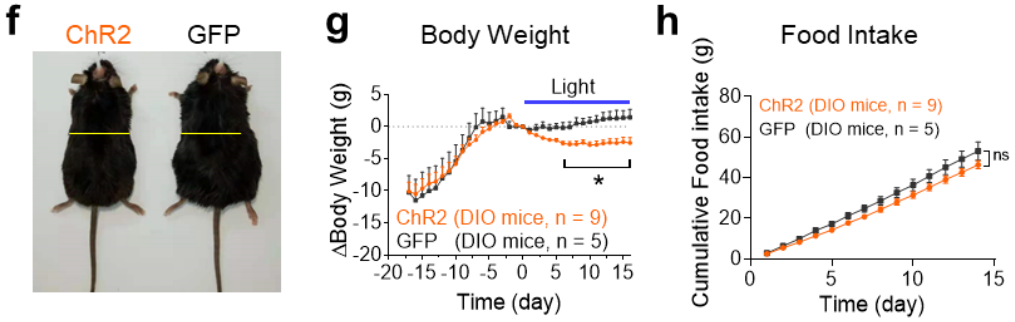
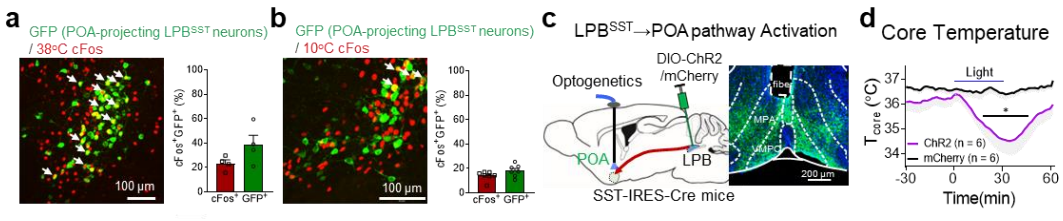


Fig. 5 | (f) Mice after two weeks of photoactivation (left, ChR2 group; right, GFP group). (g-h) Changes in body weight (g) and cumulative food intake (h) during two weeks of photoactivation (ChR2, n = 9 mice; GFP, n = 5 mice, a total of 10 mice for ChR2 and 6 mice for GFP were injected, 1 mouse for ChR2 and 1 mouse for GFP were excluded from the final analysis due to death). The light pattern is shown in (d).

4. Because LPB^{SST} neurons also project to MPO? What is the percentage of LPB^{SST} neurons→MPO neurons are Fos^{+} after cold stimulus?

R: Thanks for this careful notice. As shown below, about 20 percent of POA-projecting LPB^{SST} neurons are Fos^{+} after cold stimulus (**Extended Data Fig. 9b**). In contrast, more (~40%, **Extended Data Fig. 9a**) POA-projecting LPB^{SST} neurons are activated after a heat stimulus. Nevertheless, we photoactivated the LPB^{SST}→POA projections and found a hypothermia phenotype (**Extended Data Fig. 9d**). These data resembled the photoactivation phenotypes seen after bulk activation of LPB^{Vglut2}→POA projections, which causes hypothermia only. We added this data to **Extended Data Fig. 9a-d**.



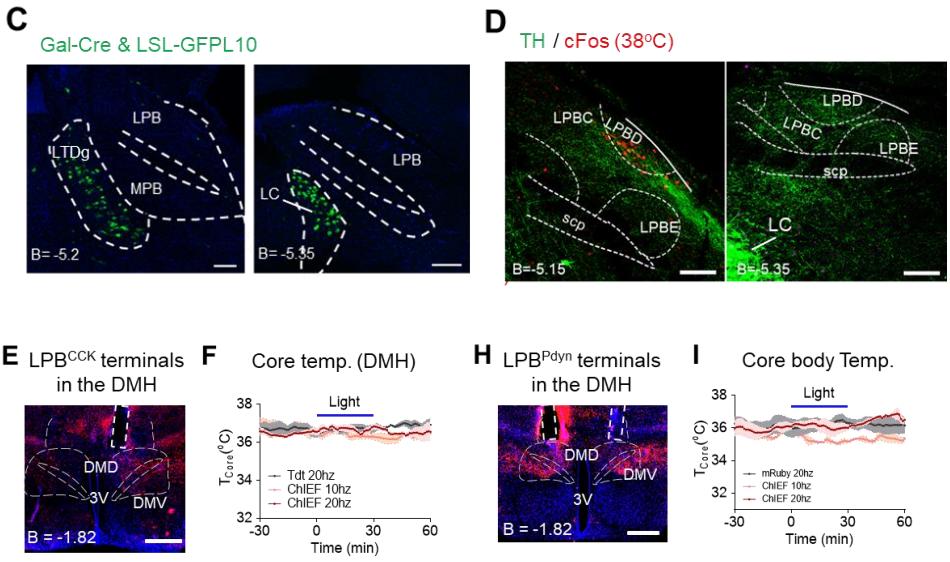
Extended Data Fig. 9 | (a-b) Overlap between POA-projecting LPB^{SST} neurons and heat-induced cFos (a) or cold-induced cFos (b) (n = 3 mice each). To label POA-projecting LPB^{SST} neurons, we injected retrograde AAVs carrying Cre-dependent GFPL10 (AAV-Retro-CAG-Flex-GFPL10) in the VMPO of SST-IRES-Cre mice, which drove the expression of GFPL10 in the LPB. Merged

cells were indicated by white arrows. (c) Design to activate the LPB^{SST}→POA projection via photostimulating of $LPB^{SST\ \&\ ChR2}$ terminals in the VMPO. The representative expression of ChR2-eYFP in the POA is shown in right. (d) Changes in T_{core} after photoactivation of the LPB^{SST}→POA projection. (n = 6 mice). Light pattern: 473 nm, 6 mW, 10 Hz, 10 ms, 2-s on followed by 2-s off, with the cycles repeating for 30 min.

5. There are so many markers for the DMH-projecting LPB^{Vglut2} neurons? are these neurons co-label same neurons or they label different population of neurons? this question is important because LPB^{SST} neurons only represent less than 20% of Fos^+ neurons after cold stimulus.

R: We thank this reviewer for noticing many "markers" for the DMH-projecting LPB^{Vglut2} neurons from our Retro-TRAP sequencing data. We actually studied several of them, including n4bp2os, Gal, SST, TH, CCK, and Pdyn. Since a Cre strain was not available to label n4bp2os+ neurons, we did not further investigate this marker. We reported previously that there were few Gal-Cre^+ and TH^+ neurons in the LPB (**Cited Fig. 6C, D**) (*3*). Instead, we found a cluster of Gal-Cre^+ neurons in the laterodorsal tegmental nucleus and the locus coeruleus (**Cited Fig. 6C**), and a cluster of TH^+ neurons in the locus coeruleus (**Cited Fig. 6D**). Their enrichment might presumably be due to tissue contamination from nearby areas. For CCK and Pdyn, we reported before that there were no changes in T_{core} after activation of $LPB^{Pdyn/CCK}$ terminals in the DMH (**Cited Fig. 6 E, F and H, I**) (*3*).

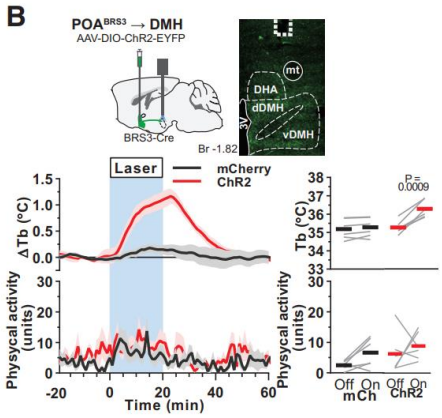
Nevertheless, we agree with the reviewer that there should exist other important markers for cold defense in the LPB-DMH pathway. Therefore, we wrote in **line 633-638**: "We showed that the LPB^{SST}→DMH^{LepR} pathway governs iBAT thermogenesis, suggesting a genetically defined projection controls specific cold defense activities. We reasonably speculate that other cold defense activities, including heart rate and muscle shivering, are also controlled by genetically defined neural projections.". Significant effort was needed to identify other genetic markers for cold defense in the LPB-DMH pathway.



Cited Fig. 6 | (C) GFP expression of Galanin-Cre & LSL-GFPL10 mice in the LPB, LDTg and LC. (D) The staining of warm-induced cFos and TH (tyrosine hydroxylase) in the LPB showed nearly no TH^+ soma in the LPB. (E) Expression of ChIEF from LPB^{CCK} neural terminals in the DMH. (F) Changes of T_{core} after photoactivation of LPB^{CCK} neural terminals in the DMH. (H) Expression of ChIEF from LPB^{Pdyn} neural terminals in the DMH. (I) Changes of T_{core} after photoactivation of LPB^{Pdyn} neural terminals in the DMH.

6. Are LPB is the only input to drive cold response in the DMH?

R: There are other inputs to the DMH besides the LPB. The POA has long been considered the primary upstream region for driving cold responses (*12, 13, 16*). Recent studies have identified POA^{BRS3} neurons that can drive cold response through the DMH (**Cited Fig. 7B**) (*17*). Additionally, the DP/DTT provides input to the DMH to drive thermogenic response during psychological stress (**Cited Fig. 8F**) (*18*). However, further studies are required to investigate whether the DP/DTT can also be activated by cold stress. Whether these different inputs act on the same or different DMH neural subtypes is unclear. Yet, based on the tracing studies, DMH^{BRS3} received much more input from the POA than from the LPB (**Cited Fig. 9**, (*14*)). Thus, it is reasonable to speculate that different inputs may act on slightly different DMH neural subtypes to promote thermogenesis.



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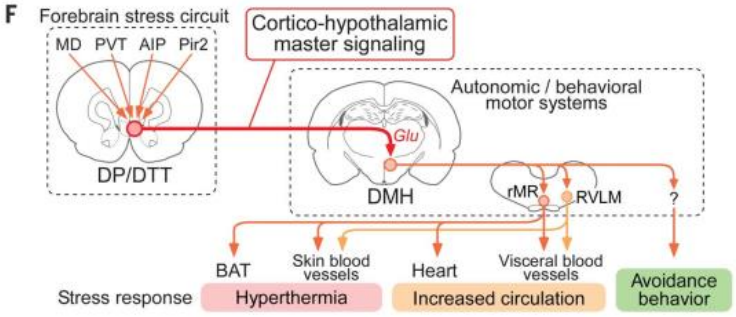
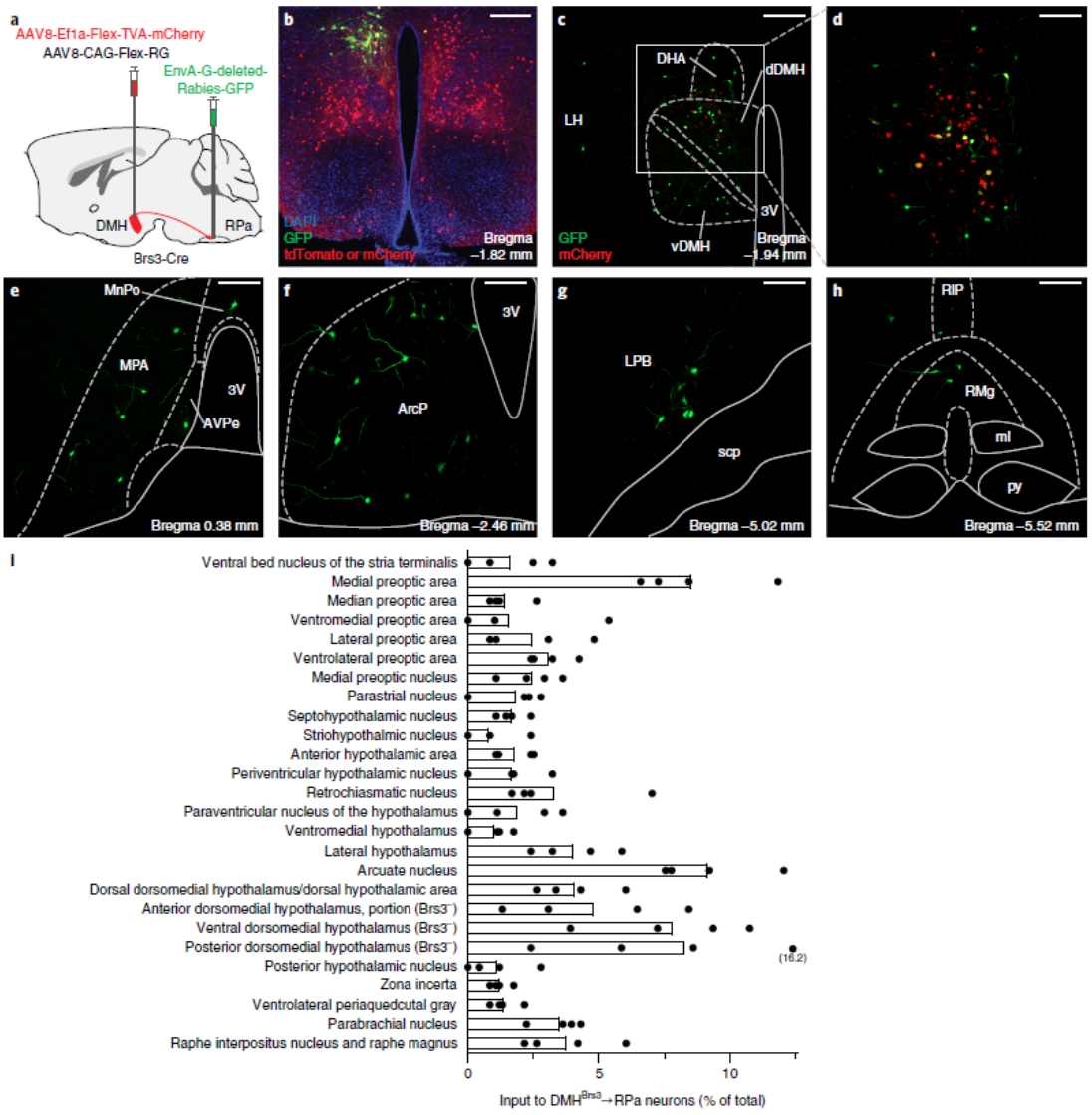
440 **Cited Fig. 7** | (B) Optogenetic stimulation of POA^{brs3}→DMH axons increases
441 T_{core}.



442

443 **Cited Fig. 8** | (F) The DP/DTT integrates signals from multiple forebrain regions
444 processing stress and emotion and then provides a glutamatergic (Glu) master
445 signal to the DMH to excite neuronal groups controlling different effectors.



Cited Fig. 9 | DMH^{Brs3}→RPa neurons receive input from POA and other nuclei. (a) Schematic of projection-specific rabies tracing. b, Brs3-Cre; Ai14 mice injected with AAV-DIO-TVA-mCherry (TVA, avian tumor virus receptor A) in the DMH and EnvA-G-deleted-Rabies-GFP in the RPa, showing the dDMH–DHA localization of DMH^{Brs3}→ RPa neurons. (c–i) Brs3-Cre mice were injected with Flex-TVA-mCherry and Flex-RG (RG, rabies glycoprotein) viruses in the DMH and EnvA-G-deleted-Rabies-GFP in the RPa. (c,d) DMH showing DMH^{Brs3}→ RPa starter neurons expressing TVA-mCherry and GFP. (d) Higher magnification of inset. (e–h) Examples of regions with higher numbers of input neurons, which express only GFP. (i) Areas with input neurons to DMH^{Brs3}→ RPa neurons, percentage of total.

Minor point:

1. Because the limited retrograde efficiency of retrograde tracing virus, the conclusion of '20% projected to both regions' is likely underestimate.

R: We express our gratitude to the reviewer for highlighting the limitations of our study with regard to retrograde efficiency, which we acknowledge. Accordingly, we have added a statement in **lines 1151-1152**: "It is noteworthy that this percentage might be underestimated due to the limited retrograde efficiency."

2. Using terminal fiber photometry to study the kinetics of temperature response is not very meaningful, as they could be strongly modulated by varies autoreceptors located in the terminal.

R: We appreciate the reviewer's valuable information. We would also like to note that we used retrograde labeling with GCaMP6s to record projection-specific soma calcium responses (**Fig. 2p-t**), which provides complementary data to the terminal recording data.

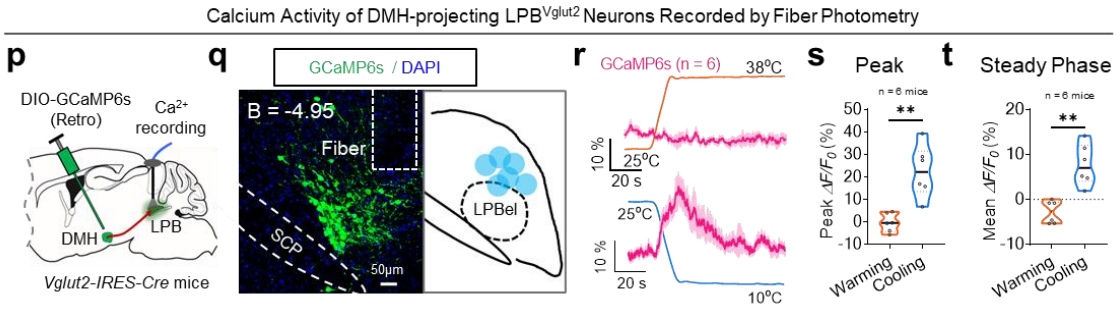


Fig. 2 | (p-q) Recording from DMH-projecting LPB^{Vglut2} neurons (p) and representative expression of GCaMP6s (left) and summary of fiber tracts (shown as blue dots, right) (q). Retrograde traveling AAV-Retro-hSyn-Flex-GCaMP6s were injected in the DMH of Vglut2-Cre mice, which traveled to the LPB to drive GCaMP6s expression in the soma of LPB^{Vglut2} neurons. (r) Calcium dynamics of DMH-projecting LPB^{Vglut2} neurons in response to floor warming (25 →38°C) or cooling (25→10°C) (n = 6 mice). (s) Peak $\Delta F/F_0$ values during floor warming or cooling (n = 6 mice, average $\Delta F/F_0$ of 5 trials per mouse). (t) Mean $\Delta F/F_0$ values during the steady phase after floor warming or cooling (n = 6 mice, average $\Delta F/F_0$ of 5 trials per mouse).

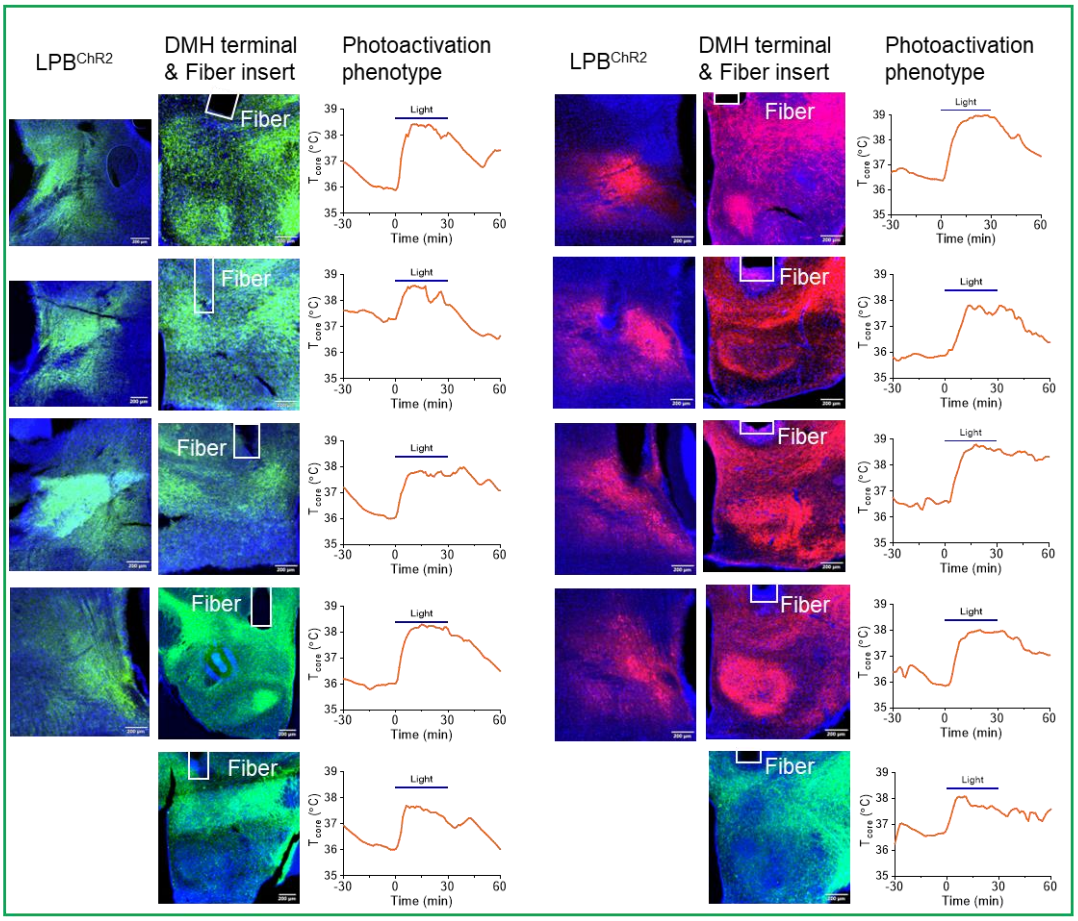
Reviewer #3 (Remarks to the Author):

In this paper, the authors examine the hypothesis that cold responsive neurons in the lateral parabrachial nucleus (LPB) project to the dorsomedial nucleus of the hypothalamus, where they activate cold-defense pathways. I typically begin a review with a summary of all of the experiments that were done, however, that is nearly impossible for this paper. It is extremely long (over 7,000 words) and gives the results of over 40 experiments, many of which are very complicated. In most cases, there is no n given (in others where it is given, it is often very low numbers, such as 3 animals), and in no case is there given sufficient detail on injection placement (some of these injections have to miss their targets, but there is no information on how many total animals were done, how they picked the small number of animals they present, or what the anatomical controls, i.e., missed injections, showed), controls are not done for many key experiments, and it is rare to find any statistical analysis.

R: We appreciate the reviewer for bringing up these points here and I believed these points have been brought up by this reviewer when we submitted to another journal. We actually have made significant efforts to reduce the complexity of the manuscript before the submission here. Now, we have made additional efforts to improve the clarity of the manuscript and included the technical and statistical details mentioned above.

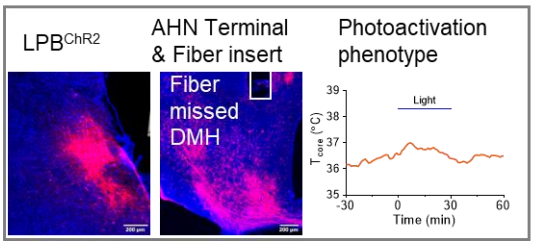
We have listed numbers in the figure or plotted data in dots to show numbers and had previously summarized **all numbers and detailed statistics** in **Extended Data Table 2**. Now, we clearly marked the numbers on the figures and described the statistics clearly in the figure legends as well. We used a minimum of n = 6 mice for behavioral testing, n = 3 mice for staining analysis, and n = 4 for EMG recording. After behavioral tests were finished, mice were perfused to check the virus expression and fiber insertion. Data from mice that showed little or no viral expression or had a fiber insertion that missed the target (often 0-20%) were excluded from the analysis (please see **Extended Data Fig. 6**, **Reviewer only Fig. 4** and methods **lines 69-71** for more information).

a



b

One mouse that missed target of photoactivation



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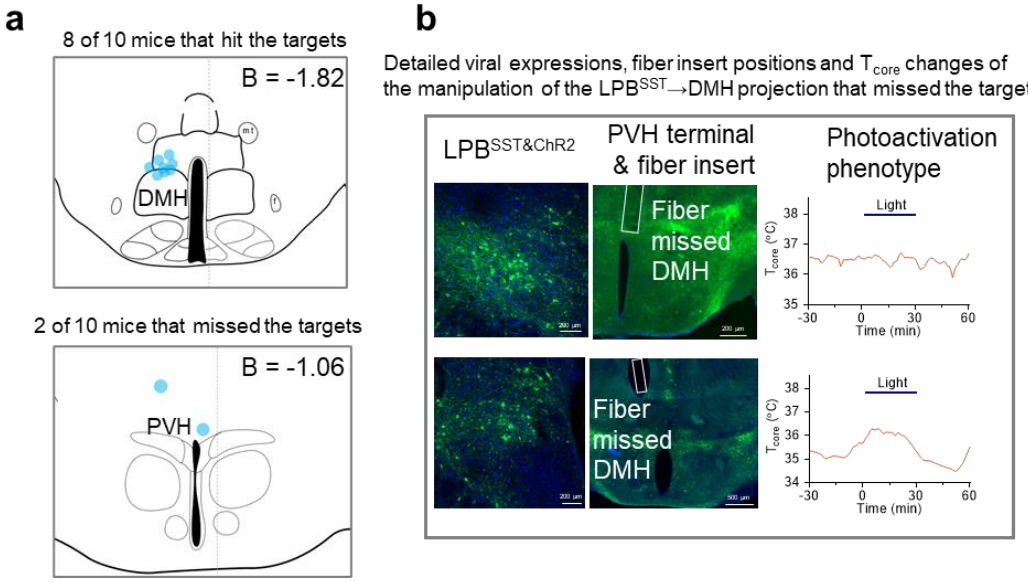
520 **Extended Data Fig. 6** | (a) Representative images demonstrating the
521 expression of ChR2 in LPB^{Vglut2} neurons, fiber insert positions and resulting
522 T_{core} changes after photoactivation LPB^{Vglut2}→DMH that hit the target. (b) Data
523 from one mouse exhibiting missed fiber insert position and T_{core} changes
524 following photoactivation. AHN, anterior hypothalamic nucleus.

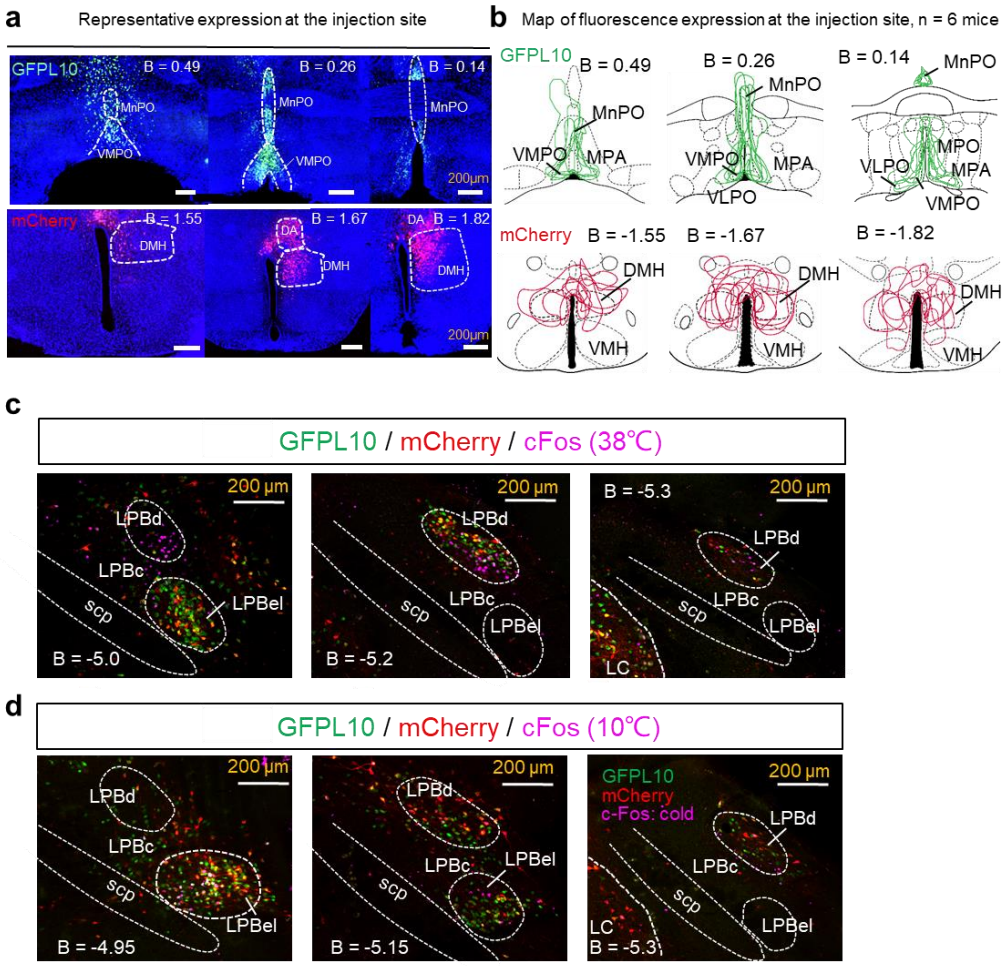


Reviewer only Fig. 4 | (a) Summary of fiber tracts that hit the target and miss target (shown as blue dots) of LPB^{SST}→DMH. (b) Data from two mice exhibiting miss fiber insert positions and T_{core} changes following photoactivation. PVH, Paraventricular hypothalamic nucleus.

For viral expression, besides showing representative virus expression images and optical fiber locations, we included the anatomical map of viral expression and optical fiber location maps (**Fig. 1d, 2b, 2q, 4b, 7b,** and so on). For example, we provided the anatomy map of retrograde tracers injected in the POA and the DMH (**Extended Data Fig. 2a**). We also provided the expression map of these retrograde tracers in the LPB (**Extended Data Fig. 2b**) and Heatmaps of TeNT expression and fiber tracts (shown as green dots) at different Bregma sites from key experimental mice (**Extended Data Fig. 5b and Extended Data Fig. 7n**).

As for the concern of control experiments, we indeed provided proper controls for all the experiments. The design of this study has been recognized by reviewers #1 and #2. “The design of this study is straightforward, and the results are novel, exciting, and convincing” by reviewer #1 and “The battery of viral tools used in this manuscript is very impressive and the author provided solid data to …” by reviewer #2.

Together, we thank this reviewer for witnessing the improvement of this manuscript and hope this reviewer would appreciate our efforts made to address the issues.



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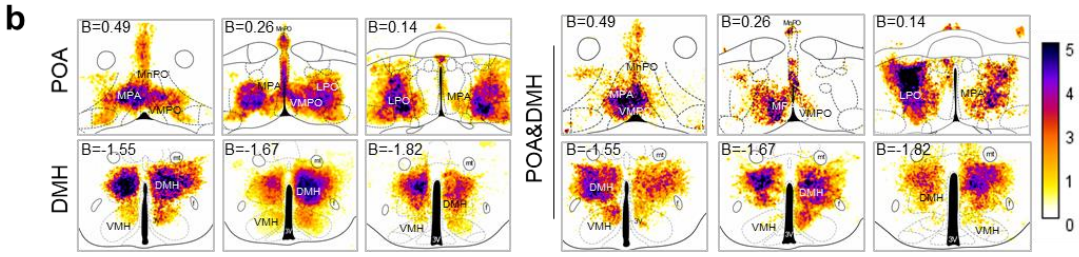
549 **Extended Data Fig. 2 |** Mapping the collateral projections of LPB^{Vglut2}
550 neurons to the POA (MnPO & VMPO) and the DMH.



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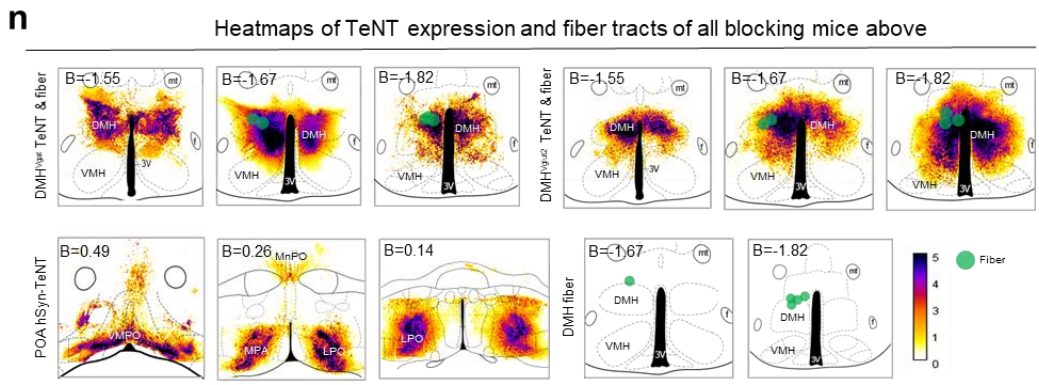
552 **Extended Data Fig. 5 |** (b) Heatmaps of TeNT expression after blocking LPB-
553 innervating POA or DMH neurons.



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555 **Extended Data Fig. 7 |** (n) Heatmaps of TeNT expression and fiber tract ends
556 (shown as green dots) at different Bregma sites from all experimental mice.
557 DMH^{Vglut2} blocking, n = 9 mice; DMH^{Vgat} blocking, n = 7 mice; POA blocking, n
558 = 7 mice. The relative scale for the expression intensity (measured by
559 fluorescence intensity) was shown on the right.

560

561 Having said that, I think this is an interesting story, which would be of interest
562 to many scientists who work on thermoregulation. But it is impossible to
563 evaluate the work critically in its current state because so much of the
564 necessary information on the rigor of the experiments is missing. I would
565 strongly encourage the authors, to include complete information for each
566 experiment.

567 R: We express our gratitude for the reviewer's interest in our work. We have
568 taken the reviewer's feedback into careful consideration and have made
569 significant modifications to the manuscript, including adding detailed
570 descriptions of experiments in legends, the methods and results sections.
571 Specifically, we have incorporated additional details on how we recorded body
572 temperature and BAT temperatures, provided proper descriptions of controls,
573 and included maps of injection sites. Moreover, based on feedback from other
574 reviewers during the previous review process, we have reorganized the
575 manuscript and eliminated extraneous details to enhance its readability.

576

577 This would include the power analysis that should have been done before the
578 studies were started indicating the number of animals that should be in each
579 group; the actual numbers of animals used in each experiment; how they chose
580 which ones to present in the paper; details about the actual results in the

animals that are included and the ones that are not included, as well as controls; how they did the statistics; and what the statistical findings were. This is particularly important for experiments involving stereotaxic injections, some of which will miss their intended target. How were those cases identified? They should be analyzed by someone who does not know the physiological results. The ones that hit the target should be analyzed separately from those that missed the target, which then serve as anatomical controls. But you need to present heat maps showing the actual injection placements in both sets of animals.

R: Again, thank you for bringing up these concerns once again. We would like to reiterate that we have taken the necessary steps to address these issues by including the relevant data and statistics in the manuscript. Specifically, we have listed the numbers in the figures and presented the data in dots to provide clarity. We have also provided a comprehensive summary of **all numbers and detailed statistics** in **Extended Data Table 2** and highlighted the figures' details and statistics in the figure legends. Additionally, we ensured that the experiments were conducted with a minimum of n = 6 mice for behavioral testing, n = 3 mice for staining analysis, and n = 4 for EMG recording. After the completion of behavioral tests, we conducted perfusions to check for virus expression and fiber insertion, and data from mice that showed little or no viral expression or had a fiber insertion that missed the target (often 0-20%) were excluded from the analysis (please see **Extended Data Fig. 6**, **Reviewer only Fig. 4** and methods **lines 69-71** for more information).

References

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have carefully addressed the critiques I commented on in the first round of reviews. This comprehensive study shows that the PBN-to-DMH work in parallel with PBN-to-POA in regulating body temperature. A large amount of data are presented. I don't have any further criticism that I want to raise. Congratulations on accomplishing such an excellent study.

Reviewer #2 (Remarks to the Author):

We appreciate the authors carefully addressed many of our concerns. However, several important questions have not been answered.

In our major point 1, I asked 'What is the relationship between neurons in DMH and MPO that received inputs from LPB?'. The authors said that 'DMH neurons, including

LPB-innervating DMH neurons, are expected to act downstream of LPB

innervating MnPO/VMPO neurons to form a feed-forward pathway in warm defense.'. It will be great for the authors to provide some experimental results to support their claim.

In our major point 2, I asked 'why both DMHVglut2 vs. DMHVgat are required for cold induced defensive behavior?' The authors again proposed a possibility 'we proposed that DMHVglut2 neurons primarily provide excitatory input to premotor neurons in the RPa to stimulate thermogenesis, while DMHVgat neurons may mainly suppress POA hypothermic neurons to inhibit heat loss.' but without data to support this hypothesis. Is DMHVglut2 neurons to RPa really stimulate thermogenesis?

In our major point 5, I like to see the sequencing results been validated with RNAscope staining to determining what is the best marker for the DMH-projecting LPBVglut2 neurons that is important for cold.

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R: We appreciate the recognition from this reviewer.

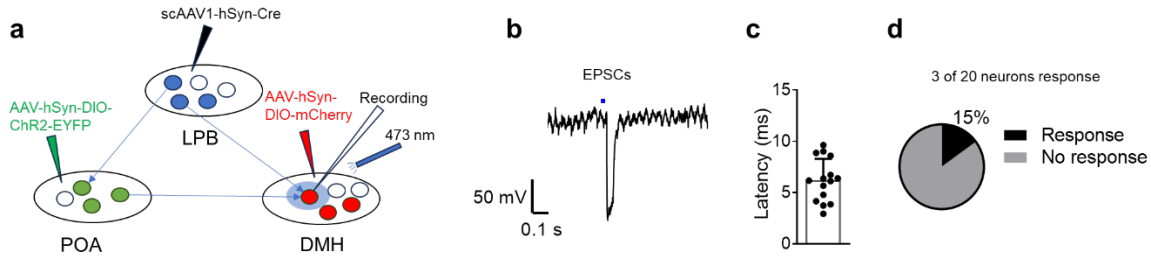
Reviewer #2 (Remarks to the Author):

We appreciate the authors carefully addressed many of our concerns. However, several important questions have not been answered.

In our major point 1, I asked 'What is the relationship between neurons in DMH and MPO that received inputs from LPB?'. The authors said that 'DMH neurons, including LPB-innervating DMH neurons, are expected to act downstream of LPB innervating MnPO/VMPO neurons to form a feed-forward pathway in warm defense.'. It will be great for the authors to provide some experimental results to support their claim.

R: In an effort to substantiate our claims, we undertook patch-clamp electrophysiology tests. In order to ascertain whether there is LPB innervating POA neurons direct innervate LPB innervating DMH neurons, we injected AAV1-hSyn-Cre into the LPB, and AAVs carrying Cre-dependent Channelrhodopsin-2 (AAV9-hSyn-DIO-ChR2-EYFP) into the POA and Cre-dependent mCherry (AAV9-DIO-mCherry) into the DMH of C57BL/6J mice. Light-induced excitatory postsynaptic currents (EPSCs) of DMH neurons were recorded through patch-clamp while photostimulating ChR2-expressing neural terminals in the DMH projected from LPB innervating POA neurons **(Reviewer only Fig. 1a)**. Light stimulations faithfully induced EPSCs, which was found within the range of a monosynaptic connection **(Reviewer only Fig. 1b-c)**. Around 15% of the recorded DMH neurons (3 of 20 neurons selected at random) exhibited EPSCs in response to light stimulations **(Reviewer only Fig. 1d)**. These data show that the LPB-innervating DMH neurons receive inputs from the LPB-innervating POA neurons, suggesting that LPB-innervating DMH neurons might act downstream of LPB-innervating POA neurons to form a feed-forward pathway.

We did not directly test whether this pathway is involved in feed-forward warm defense since it requires tons of evidence to resolve the complications. Originally, there are many evidences showing a GABAergic POA-DMH projection to inhibit thermogenesis (*1-3*). At the same time, more evidences point out that glutamatergic POA-DMH projection may help to reduce T_{core}. For example, most BDNF neurons are glutamatergic and projects to DMH (*1*, *4*). Glutamatergic QRFP neurons rely on DMH neurons to induce torpor (*5*). Therefore, given these complications, although we believe that LPB-innervating DMH neurons might act downstream of LPB-innervating POA neurons to control warm defense, it is beyond the scope of the current study. We hope the reviewer will understand the situation. Therefore, we did not include the data in the manuscript and would like to perform more careful analysis on this issue in the future.

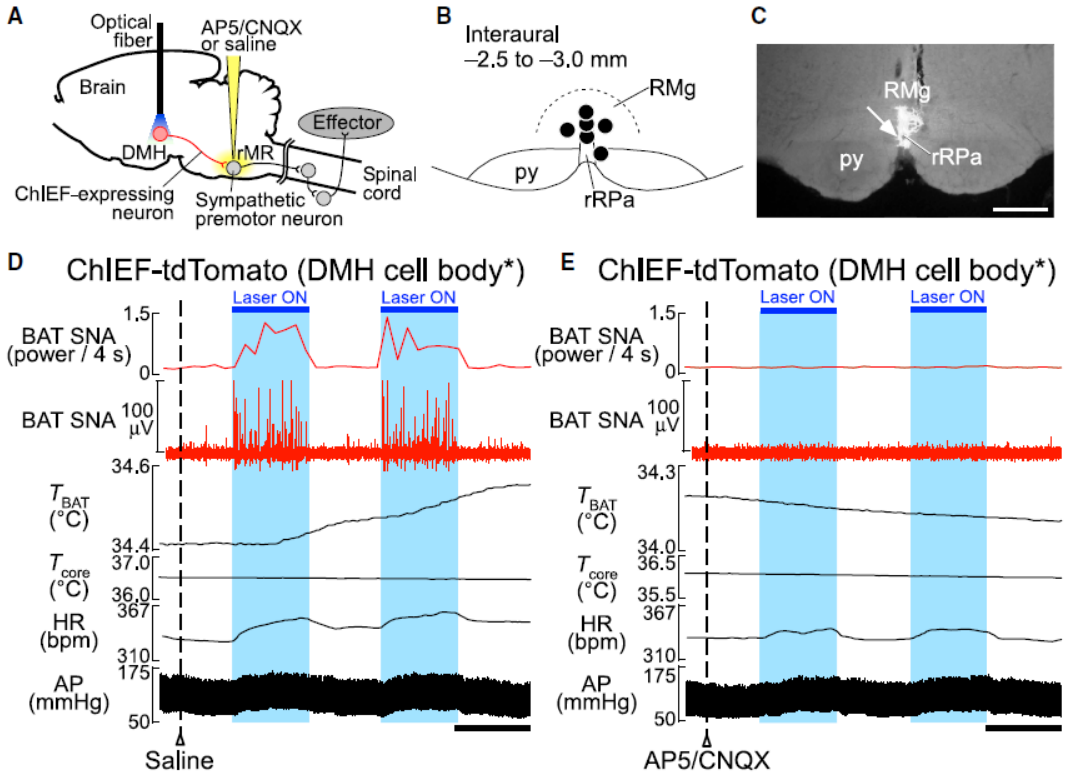


Reviewer only Fig. 1 | (a) Scheme for patch clamp recording LPB innervating DMH neurons after activating the terminal of LPB innervating POA neurons. To do so, we simultaneously injected AAV1-hSyn-Cre into the LPB, and AAVs carrying Cre-dependent Channelrhodopsin-2 (AAV9-hSyn-DIO-ChR2-EYFP) into the POA and Cre-dependent mCherry (AAV9-DIO-mCherry) into the DMH in C57BL/6J mice. (b) Induction of EPSCs in DMH neurons by light stimulation of neural terminals in the DMH projected from LPB innervating POA neurons (c) The mean latency of the induced EPSC after photoactivation of responsive neurons. (n = 20 trials from 3 neurons). (d) The EPSC response rate of DMH neurons to light stimulation. A total of 3 responsive neurons out of 20 randomly recorded DMH neurons from 3 mice.

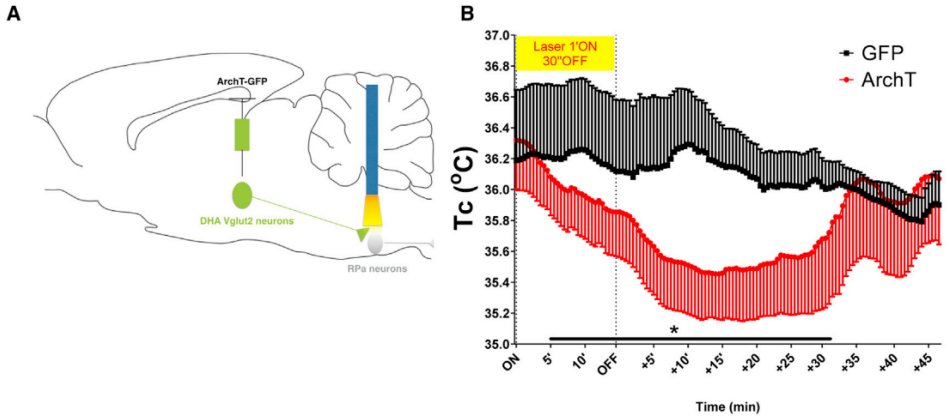
In our major point 2, I asked 'why both DMH^{Vglut2} vs. DMH^{Vgat} are required for cold induced defensive behavior?' The authors again proposed a possibility 'we proposed that DMH^{Vglut2} neurons primarily provide excitatory input to premotor neurons in the RPa to stimulate thermogenesis, while DMH^{Vgat} neurons may mainly suppress POA hypothermic neurons to inhibit heat loss.' but without data to support this hypothesis. Is DMH^{Vglut2} neurons to RPa really stimulate thermogenesis?

R: The glutamatergic DMH–rMR/RPa projection in promoting thermogenesis has been shown before in rats and mice repetitively. For example, Kataoka et al previously determined glutamatergic DMH–rMR projection drives BAT thermogenesis in rat (*6*). As cited figures shown below, activation of cell bodies in the DMH by **optogenetics** and following a saline nanoinjection into the rMR (**Cited Fig. 1B** and **1C**) elicited increases in BAT SNA and T_{BAT} (**Cited Fig. 1D**). In contrast, following a subsequent nanoinjection of glutamate receptor antagonist AP5/CNQX into the rMR (**Cited Fig. 1B** and **1C**), neither BAT SNA nor T_{BAT} was increased by activation of DMH neurons (**Cited Fig. 1E**).

Machado et al also identified DHA^{Vglut2}-RPa pathway mediate BAT thermogenesis in mice (*7*). They found optogenetic inhibition of the axon terminals of ArchT-GFP expressing DMH^{Vglut2} neurons in the RPa caused a significant reduction of baseline Tc (**Cited Fig. 2A** and **2B**).

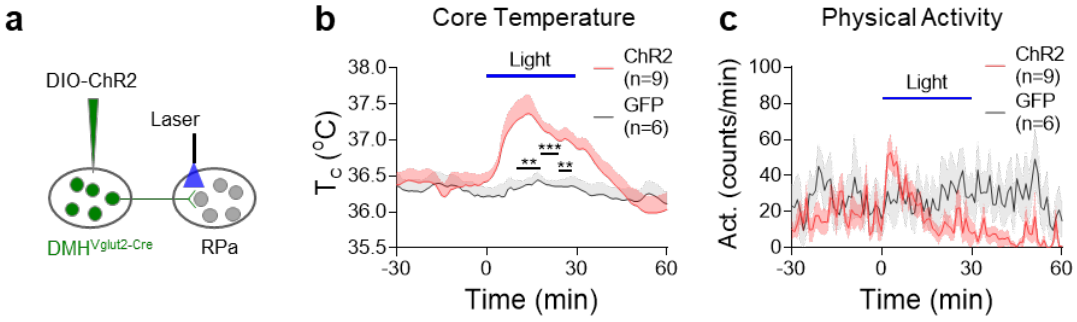


Cited Fig. 1 | (A) In vivo experiment to examine the effect of antagonizing glutamate receptors in the rMR on physiological responses to photostimulation of DMH neurons (DMH cell body*). (B and C) Nanoinjection sites in the rMR are mapped in (B). Each circle indicates a site of saline and AP5/CNQX injections made at the same location in each rat. The effect of saline was always tested first. Each injection site was labeled with fluorescent microspheres (arrow in [C]). Scale bar, 500 mm. (D and E) Effect of illumination of ChIEF-tdTomato-expressing cells in the DMH on BAT thermogenic and cardiovascular activities following saline (D) or AP5/ CNQX injection (E) into the rMR. Results from the same rat are shown. Horizontal bars, 30 s.



Cited Fig. 2 | (A) A schematic figure of the protocol for inhibition of DHA VGLUT2+ fibers in the RPa using AAV-DIO-ArchT-GFP. (B) Tc measurement in Vglut2-IRES-cre mice injected in the DHA bilaterally with AAV-DIO-ArchT-GFP (n = 5) or controls injected with GFP (n = 6) during optogenetic inhibition of DHA^{Vglut2} terminals in the RPa (35.73 ^{o}C ± 0.02^{o}C ArchT versus 36.15 ^{o}C ± 0.008 ^{o}C GFP controls during first 30 min after initiation of laser stimulation; Mann-Whitney test; ± SEM; *p < 0.0001). The laser inhibition causes an ~1 ^{o}C fall in Tc at maximum, which is about 10 min after the termination of the laser inhibition.

Nevertheless, we directly determined whether activation of the glutamatergic DMH-RPa projection would promote thermogenesis in mice. We expressed Cre-dependent ChR2 in DMH of the Vglut2-ires-Cre mice and activated the axon terminals of DMH^{Vglut2} neurons in the RPa by blue laser (**Extended Data Fig. 10a**). Compared with the GFP control mice, activation of the terminals of ChR2-expressing DMH^{Vglut2} neurons in the RPa significantly increased T_{core}, yet it only slightly increased the physical activity at the beginning of laser stimulation (**Extended Data Fig. 10b,c**). Taken together, the DMH^{Vglut2}-RPa projection is sufficiently to increase thermogenesis in mice. We added these data in **Extended Data Fig. 10a-c** and modified manuscript accordingly (lines 533-535).



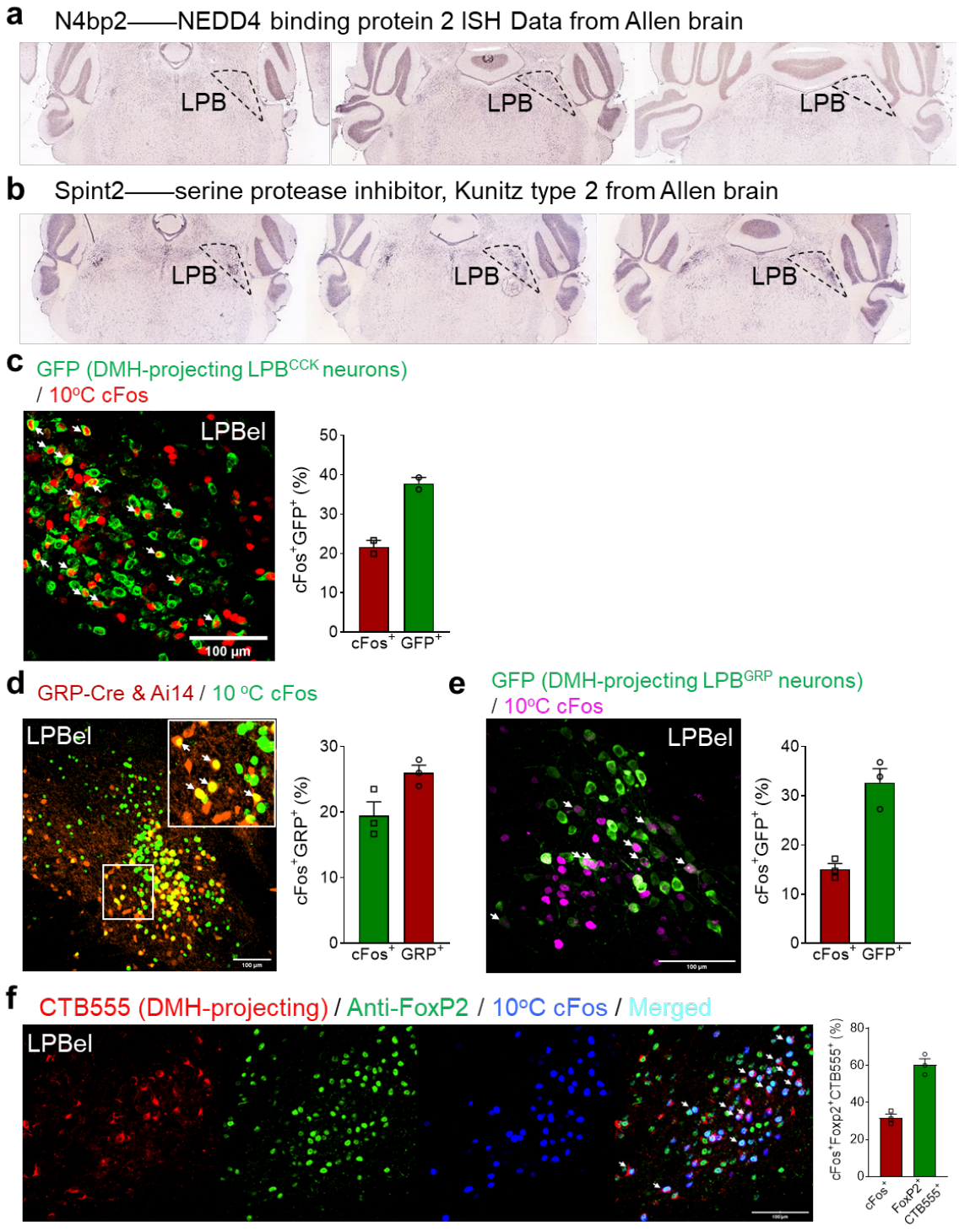
Extended Data Fig. 10 | (a) Activating the DMH^{Vglut2}→RPa projection via optogenetic activation of $DMH^{Vglut2\ \&\ ChR2}$ neural terminals in the RPa. AAVs carrying Cre-dependent ChR2 (AAV9-hEF1a-DIO-hChR2-EYFP) were injected into the DMH of Vglut2-IRES-Cre mice. An optical fiber was implanted above the RPa and used for optogenetic activation

of neural terminals. AAV9-hSyn-Flex-GFP was used as the control. (b-c) Changes of T_{core} (b) and physical activity (c) after photoactivation of $DMH^{Vglut2\ \&\ ChR2}$ neural terminals in the RPa (ChR2, n = 9 mice; GFP, n = 6 mice). Light pattern: 473 nm, 12 mW, 10 Hz, 10 ms, 2-s on followed by 2-s off, with the cycles repeating for 30 min.

In our major point 5, I like to see the sequencing results been validated with RNAscope staining to determining what is the best marker for the DMH-projecting LPB^{Vglut2} neurons that is important for cold.

R: Thanks for the reviewer's interest and curiosity, we now present more data as this reviewer suggested. As we previously stated, there were few Gal-Cre^{+} and TH^{+} neurons in the LPB. Thus, we validated the others: N4bp2, Spint2, CCK, GRP and FoxP2 (in the order of enrichment score). However, N4bp2 was not specifically expressed in the LPB according to Allen database (**Reviewer only Fig. 2a**). Spint2 appeared to enrich in the LPB according to Allen database (**Reviewer only Fig. 2b**). Yet, no antibodies or RNAscope probes available currently. The newly synthesized probe would take 2-3 months to synthesize and it may take even longer to ship from US to China. Therefore, we prioritized the validation of CCK, GRP, and FoxP2.

These results are shown below: about 38% of DMH-projecting LPB^{CCK} neurons were sensitive to cold exposure, accounting for 20% of cold-activated LPB neurons (**Reviewer only Fig. 2c**). And nearly 30% of DMH-projecting LPB^{GRP} neurons were sensitive to cold exposure, accounting for 15% of cold-activated LPB neurons (**Reviewer only Fig. 2d,e**). About 60% of DMH-projecting LPB^{FoxP2} neurons (shown as $FoxP2^{+}CTB555^{+}$ (DMH-projecting)) were sensitive to cold exposure, accounting for 32% of cold-activated LPB neurons (**Reviewer only Fig. 2f**). Therefore, FoxP2 might be another good marker for the DMH-projecting LPB^{Vglut2} neurons. However, due to the lack of FoxP2-Cre mice, we have not been able to test the functionality of the LPB^{FoxP2}-DMH projection at this time. Thus, we didn't present these data, and would like to further test it in the future.



Reviewer only Fig. 2 | (a-b) ISH data of N4bp2 (a) and Spint2 (b) from Allen brain. (c) Overlap between DMH-projecting LPB^{CCK} neurons and cold-induced cFos (n = 2 mice). To label DMH-projecting LPB^{CCK} neurons, we injected retrograde AAVs carrying Cre-dependent GFPL10 (AAV-Retro-CAG-Flex-GFPL10) in the DMH of CCK-IRES-Cre mice, which drove the expression of GFPL10 in the LPB. (d) Overlap between LPB^{GRP}

neurons (labeled with GRP-Cre & Ai14) and cold-induced cFos (n = 3 mice). (e) Overlap between DMH-projecting LPB^{GRP} neurons and cold-induced cFos (n = 3 mice). To label DMH-projecting LPB^{GRP} neurons, we injected retrograde AAVs carrying Cre-dependent GFPL10 (AAV-Retro-CAG-Flex-GFPL10) in the DMH of GRP-IRES-Cre mice, which drove the expression of GFPL10 in the LPB. (e) Overlap between DMH-projecting LPB^{FoxP2} neurons and cold-induced cFos (n = 3 mice). To label DMH-projecting LPB^{FoxP2} neurons, we injected retrograde CTB555 in the DMH of C57 mice, then stained the FoxP2 in the LPB. Merged cells were indicated by white arrows. Scale bar, 100μm. LPBel, lateral parabrachial nucleus, external and lateral part.

Reference:

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5. T. M. Takahashi *et al.*, A discrete neuronal circuit induces a hibernation-like state in rodents. *Nature*, (2020).
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7. N. L. S. Machado *et al.*, A Glutamatergic Hypothalamomedullary Circuit Mediates Thermogenesis, but Not Heat Conservation, during Stress-Induced Hyperthermia. *Current biology : CB* **28**, 2291-2301 e2295 (2018).

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors successfully addressed my remaining concerns with new data and analysis. I agree with the authors on not including some of the preliminary results in this current manuscript, but I hope these new results could potentially lead to exciting discoveries in near future.

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The authors successfully addressed my remaining concerns with new data and analysis. I agree with the authors on not including some of the preliminary results in this current manuscript, but I hope these new results could potentially lead to exciting discoveries in near future.

R: We are grateful for this reviewer’s recognition and happy to do something that could lead to new exciting discoveries in near future.