OUTLINING THE CENTRAL NERVOUS CIRCUITS UNDERLYING THERMOREGULATORY INVERSION IN THE RAT

Allegato 1: PROGRAMMA DI RICERCA

A. Summary and specific aims

CNS thermoregulatory networks maintain body core temperature ($T_{\text{core}}$) through a balance of cold and heat defensive responses. Thus, skin cooling elicits increased heat retention through cutaneous vasoconstriction (CVC) and heat production via sympathetic neural activation of brown adipose tissue (BAT) and shivering. Conversely, skin or core warming elicits inhibition of CVC, BAT thermogenesis, and shivering. However, under certain conditions, skin temperature changes can produce a paradoxical thermoregulatory response that we have termed thermoregulatory inversion (TI). TI is characterized by a paradoxical inhibition of thermogenesis in response to cold exposure and stimulation of thermogenesis during exposure to warm ambient temperature ($T_{\text{amb}}$). This inverted response is seen in hibernation and torpor, and may also occur during rapid eye movement (REM) sleep.

The neural basis for thermoregulatory inversion is only beginning to be studied. In normal thermoregulation, the pontine parabrachial nucleus (PBN) relays thermal information from the skin to the preoptic area of the hypothalamus (POA). POA modulates the activity of neurons in the dorsomedial hypothalamus (DMH), which in turn projects to the premotor output node in rostral nucleus raphe pallidus (rRPa). Effectors in rRPa project to the spinal cord and control thermogenesis through modulation of BAT and shivering thermogenesis and CVC. However, whether these same circuits are engaged in TI has not been determined. The overarching goal of the proposed studies is to elucidate the neural circuits through which thermal afferent input is “switched” to produce an inverted thermoregulatory response.

I recently demonstrated TI, in collaboration with my colleagues at Oregon Health and Science University (OHSU) and at University of Pittsburgh, using two experimental approaches in rats: blockade of POA output to DMH by a transection rostral to DMH (pre-DMH tranx), and central administration of an adenosine A1A receptor (A1AR) agonist, known to inhibit POA function. Both approaches point towards the POA as determining whether the response to warming or cooling is “normal” or paradoxical. Our central hypothesis is that blockade of the POA output unmasks a novel, short reflex loop that directly links PBN to the DMH and mediates the paradoxical thermoregulatory responses.

We will use a combination of in vivo neurophysiological approaches in anesthetized rats and anatomical studies to unveil the neural substrates underlying the paradoxical thermoregulatory response in TI. We will dissect the relevant thermoregulatory outputs involved in the regulation of TI.

Specific Aim of the Project: Test the hypothesis that exclusion of POA function is required to trigger TI and that DMH and rRPa are important outputs in the circuitry underlying TI. I recently demonstrated TI in anesthetized rats. In this one-year project, we will directly test the role of POA, extend those findings to test other thermoeffector responses (shivering and CVC), and the role of DMH and rRPa in these responses.

Specific Aim 2: Test the hypothesis that the thermosensory input triggering TI is relayed via PBN directly to DMH. We will determine whether neurons in the PBN are projecting directly to DMH and are involved in the regulation of BAT thermogenesis during TI.

Although apparently “paradoxical,” TI has physiological value in specific states, particularly hibernation. The proposed studies will start to investigate the neural mechanisms of TI to understand the pathways linking thermosensory input to this inverted output. Understanding these pathways and, possibly, in the near future their neurotransmitters creates potential new approaches for induction of stable, safe, and reversible therapeutic hypothermia that could block uncontrollable fever, reduce metabolism in ischemic injuries such as stroke or brain trauma, or induce a torpor-like therapeutic state.

B. Background and Significance

Research into the neural circuit mechanisms underlying thermoregulation has unveiled networks that control the neural outflow to thermoeffector organs. This network has evolved in mammals to maintain a homeostatic core temperature...
(T_{\text{CORE}})^2 by activating brown adipose tissue (BAT) and shivering thermogenesis during exposure to low ambient temperature (T_{\text{Amb}}), and by inhibiting thermogenesis in warm T_{\text{Amb}}^{2,13-17}. As outlined in Fig. 1A, cold and warm signals from the skin are transmitted via glutamatergic second-order neurons in the dorsal horn to the parabrachial nuclei (PBN). Cold-responsive neurons in the external lateral PBN (elPBN) and warm-responsive neurons in the dorsolateral PBN (dlPBN) relay thermosensory signals to the preoptic area of the hypothalamus (POA), including the median preoptic area (MnPO) and the medial preoptic area (MPA). The interaction of these cutaneous thermal inputs, with poorly understood thermodingulatory microcircuity in the POA, regulates the balance of inhibitory and excitatory POA outputs to thermogenesis-promoting neurons in the dorsomedial hypothalamus (DMH), that in turn excite thermogenic premotor neurons in the medullary rostral raphe pallidus nucleus (rRPa). In a cold T_{\text{Amb}}, excitation of DMH neurons prevails and thermogenesis is augmented. Conversely, in the warm, net suppression of DMH eliminates the excitatory drive to premotor neurons in the rRPa and thermogenesis ceases.

There are, however, several conditions (hibernation, torpor^{6,18}, REM sleep^{4,5}) in which these “standard” thermodingulatory responses to protect T_{\text{CORE}} are superseded by “thermodingulatory inversion (TI)”^{3}, in which cold exposure causes inhibition of thermogenesis and warm exposure stimulates thermogenesis. The neural circuits through which thermal afferent input is “switched” to produce an inverted thermodingulatory response have not been delineated.

The proposed studies take advantage of an experimental approach to inducing TI that I have developed in the rat, a non-hibernating rodent^{3,19}. Following complete medial brain transection between the POA and the DMH (pre-DMH transX), warm exposure produces a large increase in BAT thermogenesis whereas skin cooling produced nearly complete inhibition of BAT sympathetic nerve activity (SNA, Fig. 2)^3.

Pre-DMH transX disrupts the neural connections from POA to DMH; these finding point to the POA as playing a pivotal role in the “normal” response to warming, and suggest that elimination (or blockade) of POA from the thermodingulatory circuit leads to the paradoxical response. In support of this hypothesis, our preliminary results suggest that focal inhibition of POA using the GABA_A receptor agonist muscimol produces TI (Fig. 3). This preliminary observation will be examined in Expt. 1.1a. We will also extend my recent studies of TI^{13,15} beyond BAT thermogenesis to include shivering, another significant source of thermogenesis, and CVC, which contributes to heat retention (Expt. 1.2). Both Pre-DMH transX and/or the direct POA inhibition approaches will be used to induce TI.

Together, these models will establish TI as a unique and stable paradigm in the rat, and will allow us to begin to delineate the circuitry mediating the paradoxical thermodingulatory responses observed in TI. Like in normal thermodingulation some preliminary findings of mine suggest that DMH mediates the activation of BAT thermogenesis in TI. Expt. 1.3 will then test the hypothesis that DMH and rRPa are the output nodes of the inverted thermodingulatory responses, as they are for normal responses.

The POA is the main relay of thermal information under normal thermodingulation^{13,16,20}, and its inhibition (Fig. 3) or exclusion^2 (Fig. 2) seems to be sufficient to induce TI. We therefore hypothesize that inverted
control of thermogenesis is mediated by a previously unrecognized thermoregulatory pathway by which PBN neurons directly control the activity of DMH neurons after integrating signals from skin thermal afferents. This hypothesis, tested in experiments 2.1a and 2.1b is supported by some preliminary data of mine (not shown) revealing functional pathways connecting both the ePBN and the dIPBN to DMH.

Significance

TI may represent the fundamental mechanism by which certain mammals lower their TCORE to conserve energy in situations of metabolic challenge, such as hibernation, yet our studies in the rat\(^2\) indicate that this mechanism is also present in non-torpid mammals. Understanding TI and the neuronal pathways that mediate it, could provide new approaches to inducing a stable, safe, and reversible hypothermic state that could be used therapeutically to block uncontrollable fever, reduce metabolism in ischemic injuries such as stroke or brain trauma, or induce a therapeutic hypometabolic, torpor-like state in humans. Understanding of TI mechanisms could also inform management of postoperative shivering in patients maintained in a normothermic ambient, an example of a TI state possibly triggered by anesthesia\(^3\).

C. Innovation

Although the circuitry mediating normal thermoregulation is increasingly well understood, the neural mechanisms underlying TI have not been explored. We propose a novel circuit that is unmasked by exclusion of the influence of POA. Moreover, while a projection from PBN to DMH has been suggested\(^21,22\) and in one case documented\(^23\) using bulk tracing approaches, there was no evidence that this link was relevant to thermoregulation and it was never demonstrated in either landmark studies of thermosensory information between the PBN and POA\(^14,15\) or in the recent study of the molecular anatomy of the PBN\(^24,25\).

D. General Methods

Most of the proposed methods are well established by the PI and can be found in published work\(^3,19,26,27\). To ensure scientific rigor, animals will be assigned randomly to groups, and all experiments performed blinded to treatment where feasible.

In vivo physiologic studies in anesthetized rats. In rats (250-300 g) under isoflurane anesthesia, the femoral vein and artery will be respectively cannulated for drug delivery (iv) and for arterial pressure (AP) recording. Heart rate (HR) will be derived from the AP signal, while recordings of paw temperature (T\textsubscript{PAW}) will provide an indication of changes in cutaneous blood flow (increased T\textsubscript{PAW} indicates reduced cutaneous vasoconstriction (CVC). T\textsubscript{CORE}, T\textsubscript{PAW}, and T\textsubscript{BAT} will be recorded. A tracheotomy will be performed for artificial respiration. For BAT SNA recordings, rats will be transitioned to urethane/chloralose anesthesia, ventilated with O\textsubscript{2}-enriched air, and paralyzed with curare. Surgery will be performed to simultaneously record postganglionic SNA from a nerve bundle innervating the right interscapular BAT pad and T\textsubscript{BAT} from the left interscapular BAT pad. For recordings of shivering EMGs, rats will be maintained on isoflurane during surgery and then transitioned to Inactin anesthesia that, unlike urethane, supports the shivering response. Bipolar EMG recording electrodes will be inserted into the masseter, nuchal, and gastrocnemius muscles. Stereotaxically positioned micropipettes will be used for pneumatic (Picospritzer system) nanoinjection (60-120 nl) of drugs and to perform unit recording. Changes in BAT SNA, AP, T\textsubscript{CORE}, T\textsubscript{PAW}, and T\textsubscript{BAT} in response to the drug delivered will be measured. Injection sites will be labelled by injecting fluorescent beads at the end of drug nanoinjections. Rats will be euthanized and perfused with 4% paraformaldehyde (PFA) and brains processed for assessment of injection and recording sites.

Neuroanatomic studies. The retrograde tracers Cholera toxin B subunit (CTb) will be nanoinjected in brain regions of interest. After appropriate post-inoculation time (1 weeks) to permit retrograde transport rats will be anesthetized and perfused with PFA. Brains will be removed, sliced (40µm section) and immunohistochemistry processed to label neurons containing CTb.
Data analyses. Anatomical assessment of drugs injection sites will be performed post-mortem. Changes in physiologic variables will be analyzed by comparing the effects obtained following injection of drugs or skin warming/cooling with baseline values or responses to vehicle injections, using a t-test analysis or two-way ANOVA coupled with appropriate post-hoc tests.

Number of animals. For physiologic studies, sample sizes needed to obtain a power of 0.90 with \( \alpha = 0.05 \) were determined using G*Power software (V 3.1.9.2) and effect size and variance based on our recent publications\(^3\), and preliminary data. Group size of 6 was found to be sufficient for all physiologic variables, and will be used for all studies where these are the dependent variable of interest.

Limitation of proposed techniques. Pre-DMH transX could be considered a crude approach to inducing TI, as it disrupts both ascending and descending pathways involved in thermoregulation as well as other physiological systems. There are also potential inflammatory and hemorrhagic effects. Nevertheless, we are experienced with this procedure and have taken all precautions to minimize these confounds as described in our recent publication\(^3\). In addition, pre-DMH transX allows us to obtain a reliable, long-lasting TI, needed in some proposed experiments, and we will use focal pharmacological inactivation of POA in some experiments, which will provide converging support of the basic phenomena. Moreover, although perhaps more refined, the relatively short-lasting effects of pharmacological manipulations are not well suited for some of the proposed experimental procedures that require a prolonged and stable TI. However, we believe that definition of the core TI network, as proposed in this application, will provide more precise methods to induce this state.

E. Experimental Approach

Specific Aim 1: Test the hypothesis that DMH and rRPa are important outputs in the circuitry underlying TI.

Expt 1.1 Test the effects of POA exclusion on multiple mechanisms of thermoregulation.

Expt 1.1a Determine whether focal inhibition of POA induces TI. Results from pre-DMH transX and central CHA administration point towards the POA as playing a pivotal role in the mechanisms underlying TI\(^3\). We will test this hypothesis directly using focal inactivation of POA (Fig. 3). Rats will be prepared as described under General Methods. Baseline BAT SNA responses to a series of skin cooling and rewarming will be used to assess normal thermoregulatory responses. \( T_{\text{CORE}} \) and \( T_{\text{SKIN}} \) will then be decreased below 37°C, to activate BAT SNA and thermogenesis and the GABA\(_A\) receptor agonist muscimol (0.2 mM/100nl) or vehicle (aCSF) will be injected bilaterally in POA, first in MPA and later in MnPO, to inhibit all thermoregulatory neurons present in these areas. The skin cooling and rewarming sequence will then be repeated to verify the occurrence of a TI state. If this experiment is successful, focal inhibition of POA will also be used as an alternative to pre-DMH transection in Expts. 1.2 and 1.3. 2 groups, 6 animals/group.

Expt 1.2 Extend measures of TI to cutaneous vasoconstriction (CVC) and shivering thermogenesis. I have already demonstrated the effects of TI induced by pre-DMH transX on BAT SNA\(^3\); however, it remains to be determined whether vasomotion and shivering also respond to changes in thermosensory input in an inverted way under the TI paradigm. Expt. 1.2 will examine these thermoeffectors by pre-DMH transX to sever axons projecting from POA to DMH: pre-DMH transX will produce long-lasting TI in anesthetized animals. Studies will be performed as in my published work using BAT SNA and thermogenesis\(^3\), and in addition neck electromyogram (EMG) will be used as a measure of shivering and paw temperature as an index of CVC.

Expt 1.2a Test the effects of pre-DMH transX on shivering and CVC. Rats will be prepared as described under General Methods. Baseline EMG and CVC responses to a series of skin cooling and rewarming will be used to assess normal thermoregulatory responses. \( T_{\text{CORE}} \) and \( T_{\text{SKIN}} \) will then be increased above 37 °C, and a bilateral transection just rostral to DMH, as in our recent publication\(^3\), will be performed at increasing depths (-8, -9, -10 mm from brain surface). The skin cooling and warming will be used to test the occurrence of TI at each cut level. This sequential cutting will allow us to determine if there are differences in the responses due to the transection of distinct brain regions at each level. Sequential cutting will also allow us to determine the effect of “sham” surgery (dorsal cut) and full transection of connections from POA to DMH. 1 group, 6 animals.

Expected outcomes and interpretation for Expts. 1.1 and 1.2. We expect, as suggested by my preliminary data (Fig. 3), that inactivation of POA will induce TI. We also expect that both approaches to inducing TI will engage CVC and shivering thermogenesis. If not, it would suggest that different thermoeffectors are uniquely and differentially influenced in TI. This would be an interesting dissociation and worth pursuing.
Expt. 1.3. **Test the role of DMH and rRPa in TI.** In contrast to normal thermoregulatory responses, skin warming during TI evokes activation of BAT SNA and BAT thermogenesis (Fig. 2, right). To understand the neural mechanisms leading to this “inverted” response, we sought to determine the source of the descending excitatory drive to the spinal BAT sympathetic preganglionic neurons that control thermogenesis. Under a TI state induced by the exclusion of thermal POA function, the obvious candidates for excitatory drive to spinal BAT sympathetic preganglionic neurons are the premotor neurons in the rRPa and its DMH excitatory input (Fig. 1A), whose activity is required for all BAT and shivering thermogenic responses studied to date.\(^{1,13-16}\)

**Expt. 1.3a. Does warming-induced thermogenesis in TI require glutamatergic input to the DMH and rRPa in anesthetized animals?** BAT SNA will be recorded in anesthetized rats. Baseline normal thermoregulatory responses to a series of skin warming and cooling will be determined. With \(T_{\text{core}}\) and \(T_{\text{skin}}\) maintained at \(\sim 37°C\) (BAT SNA inhibited), a bilateral complete transection will be performed to initiate warm-evoked thermogenesis that characterizes TI. Saline (60 nl, vehicle control), or AP5/CNQX (a mixture of AMPA and NMDA receptor antagonists, 5 mM each, 60 nl) will be delivered into DMH or rRPa to determine if blockade of local glutamatergic transmission is sufficient to abolish increased BAT SNA and thermogenesis. 4 groups, 6 animals/group.

**Expt. 1.3b. Is cooling-induced inhibition of thermogenesis in TI mediated by GABAergic inhibition of DMH in anesthetized animals?** BAT SNA will be recorded in anesthetized rats. Baseline normal thermoregulatory responses to a series of skin warming and cooling will be determined. With \(T_{\text{core}}\) and \(T_{\text{skin}}\) kept below \(\sim 37°C\) (BAT SNA active), bilateral transection will be performed to initiate the cold-evoked thermogenesis that characterizes TI. Saline (60 nl, vehicle control) or the GABAA receptor antagonist bicuculline (60 nl, 0.5 mM) will be delivered into DMH or rRPa to determine if blockade of local GABAergic transmission is sufficient to abolish cold-induced inhibition of BAT SNA and thermogenesis. 4 groups, 6 animals/group.

**Expected outcome and interpretation for Expt. 1.3.** A common denominator of the approaches used to induce TI is inhibition or exclusion of POA thermoregulatory neurons. Neuronal pathways caudal to the POA must therefore mediate thermoregulatory responses in TI. Some preliminary data of mine support an essential role of the well-recognized thermogenesis-promoting neurons in DMH in driving the warm-evoked BAT and shivering thermogenesis observed in TI. Nevertheless, should our experiments fail to support a role for DMH or rRPa, we will inactivate other regions caudal to POA (e.g., ventromedial hypothalamic nucleus, arcuate nucleus, periaqueductal grey) to identify the source of the excitatory drive to thermogenesis.

**Specific Aim 2: Test the hypothesis that thermosensory input triggering TI is relayed via the PBN directly to DMH.**

Our preliminary data (not shown) point to DMH as critical for paradoxical thermogenesis in TI, suggesting that the same efferent pathway (DMH to rRPa to spinal preganglionic neurons) is recruited under normal cold-defense conditions and to produce paradoxical thermogenesis in TI. If so, activity of DMH neurons must be modulated by an alternative thermal afferent input in the absence of a relay through POA. Since the PBN is the primary thermosensory integration center, and since thermal afferent information influences thermogenesis in both normal and inverted states, it is reasonable to hypothesize that a direct projection from PBN conveys thermal afferent input to DMH in TI (Fig. 1B). Experiments under **Aim 2** will test this hypothesis that a novel pathway PBN to DMH is necessary to modulate the skin evoked BATSNA responses during thermoregulatory inversion.

**Expt. 2.1. Role of PBN neurons in mediating thermogenesis activation after skin warming in TI.**

**Expt. 2.1a. Determine whether PBN neurons are projecting to DMH?** Rats, will be injected with retrograde tracers CTb in DMH. One week later rats will be sacrificed ad potine brain slices at PBN level will be collected and processed for immunostaining against CTb. We expect to find a direct projection from PBN to DMH. 1 groups, 6 animals.

**Expt. 2.1b: Do glutamatergic inputs to PBN contribute to warm-evoked increase in BAT SNA and thermogenesis?** Since PBN is the primary target of thermosensory pathways arising in the dorsal horn and thermogenesis in TI is still under the control of cutaneous warm and cold stimuli, one would predict that blocking transmission through PBN should interfere with the warm-evoked activation and the cold-evoked inhibition of thermogenesis. In **Expt. 2.1d**, we will test the hypothesis that blocking excitatory, glutamatergic inputs to PBN will attenuate or block the warm evoked increase of BAT SNA and thermogenesis. A series of skin cooling and rewarming will be used to evoke normal thermoregulatory responses in anesthetized rats. \(T_{\text{core}}\) and \(T_{\text{skin}}\) will then be increased above 37.5°C and TI induced by pre-DMH transX or muscimol injection in POA, leading to increased BAT SNA and thermogenesis. At that point, saline (60 nl) or AP5-CNQX (mix of AMPA and NMDA antagonists, 60 nl, 1 mM) will be nanoinjected in the PBN to determine if blockade of glutamatergic transmission to PBN is sufficient to abolish the
warm-evoked increase in BAT SNA and thermogenesis. If not, it would suggest that glutamate is not the primary driver of PBN activity, and we will use the GABA_A agonist muscimol to determine whether PBN is a necessary relay of thermosensory input in TI. 4 groups, 6 animals/group.

**Expected outcome and interpretation for Aim 2**
The proposed experiments will characterize a novel thermoregulatory PBN→DMH pathway and if successful will demonstrate the essential role of PBN in redirecting skin thermal information directly to DMH for the modulation of BAT SNA during TI.

**Literature Cited**


OUTLINING THE CENTRAL NERVOUS CIRCUITS UNDERLYING THERMOREGULATORY INVERSION IN THE RAT

Allegato 2: PROGRAMMA DI ATTIVITA’ DELL’ASSEGNISTA

The activity of the postdoc will consist in:

i) microsurgery in anesthetized rats including:
   - cannulation of femoral artery and vein
   - tracheotomy for artificial ventilation
   - dissection for the recording of brown adipose tissue (BAT) sympathetic nerve activity (SNA)
   - cranial surgery for brain transection and drugs microinjection procedures
   - implantation of electrodes for the recording of EMG and probes for the acquisition of skin core and BAT temperature

ii) anatomical experiments using retrograde tracing (CTb) and immunohistochemistry techniques

iii) acquisition, analysis and statistical assessment of the data

The methods in the proposal are well established by the PI as demonstrated by the published works1-4. The researcher will have constant interaction with the PI who will train the researcher to execute and analyze the data for all the experiments lineup in the proposal. The researcher will also participate to journal clubs in which he will have the opportunity to train is speaker’s ability by presenting and discuss the results of the proposal with other faculty in the department.

A single person can run three to four physiological experiment/week. For all the experiments, including a 10% failure rate, we will need a total of 108 rats and 9 months estimated maximum time for the execution of the project (see also table 1). Two months are required for the analysis of the data. Some of these experiments and data analysis, will be executed by the PI of the project as a training section for the researcher. This will contribute to reduce the work days of the researcher making this project completely suited to be executed in one year.

Table 1

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References