Characterization of thermoregulatory efferents to the paraventricular nucleus of the rat hypothalamus

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Neuroscience from The College of William and Mary

By

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Abstract

The paraventricular nucleus of the hypothalamus (PVN) is the gatekeeper to temperature-modulating hormones such as vasopressin. Indeed, it is in theory a key target region for thermoregulatory efferents of the preoptic area of the anterior hypothalamus (POAH). Although several retrograde labeling studies have identified a connection between the POAH and PVN, none have directly characterized the thermoregulatory nature of this interaction. We will identify the phenotype of these connections by injecting retrograde-labeling nanoprobes into the PVN of live tissue slices and quantified labeled POAH somas. These probes are designed for selective uptake by glutamate-releasing axon terminals, after which they diffuse rapidly to the neuron soma. Our results indicate that glutamatergic neurons do project from the POAH to the PVN. This technique will allow for further electrophysiologic studies to identify the thermosensitivity of neurons with a known connectivity and phenotype.
Introduction

Does the POAH interact directly with the PVN? If so, how? To address these questions, we must first lay out the theoretical foundations for central thermoregulation, the PVN’s major roles in normal biological function, and what evidence exists of a functional relationship between the thermoregulatory POAH and the PVN.

1. Central Thermoregulation and the Hypothalamus

As endotherms, mammals maintain a carefully regulated core temperature of ~37°C. Although this temperature can vary slightly across individuals, time of day, and body states, the mammalian body works continuously to maintain a set-point temperature via metabolic, physiological, and behavioral mechanisms (Hammel, 1968). This set-point is both determined and maintained by the neurons in the POAH, which serves as the central nucleus in the hypothalamus for maintaining thermoregulatory homeostasis. Despite being a subject of research for the better part of a century, the mechanisms by which the POAH maintains thermal homeostasis continue to be elucidated.

The POAH was originally identified by researchers of the mid-20th century, who implanted thermodes directly into live animal brain tissue and observed the heat loss and heat retention responses that could be evoked with direct cooling or warming of various regions of interest. These studies discovered several regions of the brain stem and spinal cord that generate thermoregulatory responses in the periphery. Of particular interest was the POAH, which exhibited both exceptional sensitivity to subtle changes in temperature and a clear influence on lower thermoregulatory effector areas (Boulant, 2006).
Located in the rostral hypothalamus, the POAH receives information on peripheral temperature from cutaneous temperature-sensitive sensory neurons, which convey temperature information to the brain through the lateral spinothalamic tract. As shown by the thermode-implant studies, the POAH can also respond to changes in central temperature. If cooled below 37°C, the POAH induces mechanisms that both conserve heat and create it, ranging from cutaneous vasoconstriction to shivering (Griffin, 2004). Warming of the POAH above 37°C can lead to cutaneous vasodilation, sweating, and other heat loss mechanisms (Boulant, 2006). This is a result of the properties of the POAH neurons themselves, some of which display an inherent thermosensitivity (Boulant, 2000). Overall, the POAH acts as the thermostat of the brain, collecting temperature data and inducing the required physiological and behavioral changes to maintain homeostasis.

2. The POAH Synaptic Network

An organism’s temperature set-point is both created and maintained by POAH neurons, their inherent properties, and the synaptic network they form. These neurons can be classified most broadly into silent and tonically active neurons, with the former showing no activity in vitro and the latter continuing to fire spontaneous action potentials ex vivo. Amongst the spontaneously active neurons, there are three further classes of POAH neurons: warm-sensitive, cold-sensitive, and temperature-insensitive neurons. Approximately 70% of POAH neurons are temperature-insensitive, showing little change in firing rate (FR) during increases or decreases in temperature. Warm-sensitive neurons, which compose ~20% of the POAH population, increase their FR with rising temperature by a minimum of 0.8 impulses/sec/°C (Boulant, 2006; Imbery,
Less than 5% of the population is the elusive cold-sensitive neuron, which increases FR in response to decreases in POAH temperature. Cold-sensitive neurons are not inherently thermosensitive, but instead receive inhibition from local warm-sensitive POAH neurons. At lower temperatures, the tonically-firing cold-sensitive neurons are disinhibited, and therefore exhibit a higher FR (Boulant, 2000).

In 1965, H.T. Hammel used these phenotypes to create a theoretical thermoregulatory circuit (Fig. I.1). Hammel proposed that each neuron subtype played a particular role in thermoregulation. Temperature-insensitive neurons, which respond neither to temperature changes in the brain itself nor to peripheral temperature changes, provide steady tonic inputs, serving as a reference point. Warm-sensitive neurons receive input from peripheral temperature receptors and combine this information with their own inherent thermosensitivity. Thus these temperature-sensitive neurons can serve as both sensors and integrators of temperature input (Boulant, 2006). By Hammel’s model, the competitive inputs of these temperature-insensitive and temperature-sensitive neurons are what create the temperature set-point. A change in the FR of one cell type can override the other. For instance, if the warm-sensitive neurons were to begin increasing their FR in response to a rise in central temperature, their excitatory influence on warm-effector neurons would override the tonic inhibition provided by temperature-insensitive neurons. Warm-effector neurons would then increase their own FRs and induce heat loss pathways (Boulant, 2006).
Figure I.1 Hammel's Model for Thermoregulation.

Warm-sensitive (“W”) neurons receive excitatory input from dorsal horn spinal neurons (“SP”) and convey this information accordingly to warm-effector (“w”) and cold-effector (“c”) neurons, which go on to induce heat loss or heat production. Temperature-insensitive neurons (“I”) receive no peripheral input, instead providing a tonic input to warm- and cold-effector neurons. Dotted lines indicate the frequency of FR input from warm-sensitive and temperature-insensitive neurons, with (+) indicating an excitatory input and (-) an inhibitory input. Adapted from Boulant, 2006.
Subsequent electrophysiological and anatomical studies have supported Hammel’s model (Boulant, 2000; Boulant, 2006). Anatomically, biocytin staining of thermosensitivity-classified neurons revealed that while temperature-insensitive neurons orient their dendritic projections parallel to the third ventricle, warm-sensitive neurons orient their dendritic projections perpendicular to it, aligning their dendrites to connect with afferent temperature pathways passing through the periventricular area and medial forebrain bundle (Fig II.2; Griffin, 2004). This lends credence to the theory that warm-sensitive neurons monitor both central and peripheral temperature, and that temperature insensitive neurons do not collect peripheral temperature information. Furthermore, warm-sensitive neurons have been shown to respond not only to incoming peripheral temperature information but also to osmotic changes, cardiovascular changes, and even emotional stressors, expanding their role as coordinators of temperature control and peripheral information (Oka, 2001; Hori, 1986).
Figure I.2 – Camera lucida drawing of warm-sensitive and temperature-insensitive neurons.

Neurons’ thermosensitivity phenotype was identified via intracellular electrophysiological recording. Neurons were then filled with biocytin stain which was subsequently silver-enhanced. On the left are temperature-insensitive neurons, which orient their dendrites parallel to the third ventricle and do not react to central or peripheral temperature. On the right are warm-sensitive neurons, which orient their dendrites perpendicular to the third ventricle and appear to integrate central and peripheral temperature information. Adapted from Griffin, 2004.
3. Thermoregulatory Efferents of the POAH

In the event of a significant change in temperature, the POAH must induce the behavioral and physiological responses necessary to return core temperature to the set-point. It provokes these responses through several descending pathways.

During a drop in body temperature, organisms pursue heat retention and production mechanisms. Rats can produce heat via non-shivering thermogenesis, in which stores of brown adipose tissue (BAT) are metabolized (Nagashima, 2000). Caudal lesions between the POAH and the dorsomedial hypothalamus (DMH) were shown to induce BAT thermogenesis, and a 2007 study by Nakamura et. al. showed that injection of the inhibitory neurotransmitter GABA into the POAH was sufficient to create BAT thermogenesis as well, suggesting the POAH provides tonic inhibition to the non-shivering thermogenesis pathway.

When body temperature rises, organisms pursue heat loss mechanisms. While humans sweat to reduce heat via evaporation, rats can accomplish the same task by licking their fur. The salivary secretion required for this behavior can be induced by warming the POAH itself. When warmed, the POAH’s parasympathetic projections to the paraventricular nucleus and lateral hypothalamus become activated. These hypothalamic nuclei go on to innervate the medulla oblongata, which stimulates salivation secretion from the submandibular and sublingual glands (Nagashima, 2000).

Through these and many other mechanisms, the POAH can initiate changes in body temperature. Other thermoregulatory efferents of the POAH range from shivering induction via midbrain reticulospinal neurons to skin vasodilation via premotor neurons of the medulla oblongata (Nagashima, 2000).
4. The Paraventricular Nucleus

As alluded to in the case of the salivating rats, the paraventricular nucleus of the hypothalamus (PVN) is known to play a role in thermoregulation. So named due to its proximity to the third ventricle, the PVN serves as the brain’s neuroendocrine control center (Figure I.3). It gates the release of dozens of circulatory hormones ranging from vasopressin to follicle-stimulating hormone via its connections with both the anterior and posterior portions of the pituitary gland (Sawchenko, 1983).

The PVN is divided by cell type into two distinct regions: the magnocellular and parvocellular subnuclei. Magnocellular neurons, which earn their name from their large cell bodies, project to the posterior pituitary gland, where they release the hormones oxytocin and vasopressin directly into the bloodstream (Prabha, 2010). Classically represented as the water conservation hormone, vasopressin constricts peripheral blood vessels and stimulates water reabsorption at the kidney. Oxytocin has been most closely tied with childbirth, initiating milk let-down and uterine contractions (Kuzmiski, 2010).

The smaller parvocellular neurons of the PVN project in part to the median eminence, where they release peptides into the hypothalamo-pituitary portal system. These peptides travel to the anterior pituitary, where they stimulate the release of a bevy of hormones – ranging from follicle-stimulating hormone to thyroid-stimulating hormone – from pituitary neuroendocrine cells. Sawchenko and Swanson estimated that the parvocellular regions of the PVN express and respond to over thirty neurotransmitters and neurohormones (Sawchenko, 1983). Parvocellular cells of the PVN also project to several downstream effectors including the locus coeruleus, parabrachial nuclei, and the thoraco-lumbar region of the spinal cord. These connections allow
parvocellular PVN neurons to modulate sympathetic respiratory, behavioral, visceral and cardiac responses (Prabha, 2010).

Neurotransmitters in the PVN include norepinephrine, serotonin, and the ubiquitous glutamate (Sawchenko, 1983). As in the majority of the brain, glutamate – an excitatory neurotransmitter - and its inhibitory counterpart GABA are both present in the PVN. In magnocellular cells, metabotropic glutamate receptors have proven important in the long-term changes required for oxytocin release (Kuzmiski, 2010). Under basal conditions PVN neurons remain largely inactive, unlike their tonically-firing POAH neighbors. This has been tied to a high amount of GABAergic input to the PVN. Blocking GABA$_A$ receptors with the antagonist bicuculline leads to increased excitation of the PVN; however, blocking ionotropic excitatory amino acid receptors with the nonselective antagonist kynurenic acid silenced that response. This suggests that the majority of PVN cells have a sub-threshold resting membrane potential. Under basal conditions, they remain inhibited by GABAergic input; however, even if GABA$_A$ receptors are blocked, an excitatory neurotransmitter such as glutamate is required to garner a response (Prabha, 2010). Thus, the PVN appears to be under the control of a carefully-maintained balance of excitatory and inhibitory inputs. Known inputs include the anterior perifornical area and parastrial nucleus, but many of these inputs remain unknown (Elmquist, 1997).

As the brain’s major hormone control center, the PVN is responsible for enacting changes in both behavior and autonomic systems such as heart rate, blood pressure and breathing. The former is most generally controlled by the limbic system and the hypothalamus; the latter, by the hypothalamus and lower brain stem nuclei. The PVN is also a central part of the hypothalamic-pituitary axis, the “stress circuit” of the brain, and as such plays a role in the bodily changes that result from perception of environmental stressors. As a player in the sympathetic nervous
system, the PVN receives dense noradrenergic and adrenergic inputs from the locus coeruleus, ventral medulla and nucleus tractus solitarius (NTS) (Sawchenko, 1983). Known nearby afferents include the suprachiasmatic nucleus, which is intimately tied with circadian rhythms; the lateral hypothalamus, involved in feeding and drinking behavior; and the anterior hypothalamus – including its preoptic area (Sawchenko, 1983). A less well-defined input is angiotensin II, a circulatory hormone that increases during periods of dehydration or hemorrhage. When angiotensin II is high in the bloodstream, the PVN releases vasopressin; however, the means by which angiotensin II signals the PVN is not well known, as it neither crosses the blood-brain barrier nor enters the cerebrospinal fluid of the third ventricle (Prabha, 2010).

The PVN plays a role in everything the hypothalamus is known for: homeostatic control of fluid levels, metabolism, and behavior. With most of these properties affecting temperature, does the thermoregulatory POAH interact directly with the PVN?
5. Fever: The POAH and PVN

Fever provides a useful perspective for an examination of the relationship between the POAH and PVN, as both nuclei are involved in the febrile response. Fever itself is a hyperthermic state that most commonly results from pathogenic infection. Although the debate over whether fever is an adaptive or maladaptive trait continues, prior research has indicated that fever can both improve white blood cell function and inhibit growth of certain microbes. As such, fever is an indelible component of the immune system’s response to an acute infection (Elmquist, 1997).

Traditionally, cytokines were thought to be the main instigator of fever (Blatteis, 2007). Pathogen markers such as the bacterial cell wall component lipopolysaccharide (LPS) cause the release of cytokines like interleukin-1 into the bloodstream by white blood cells and other major immune system players (Imbery, 2008). These cytokines were classically thought to enter the brain at the organum vasculosum laminae terminalis (OVLT), which lies in close proximity to the POAH. The blood-brain barrier is particularly permeable at the OVLT, allowing larger molecules to enter. OVLT neurons respond to these immune system cues by releasing prostaglandin-E2 (PGE$_2$) into the POAH (Boulant, 2000). PGE$_2$ has been shown to both inhibit warm-sensitive neurons and excite temperature-insensitive neurons, which leads to a suppression of heat loss mechanisms and an increase in heat production. The animal begins retaining and producing heat at a higher temperature than 37°C, raising body temperature (Boulant, 2006).

Recent research has shown that the OVLT-POAH pathway is in fact the slower of two mechanisms, as it depends upon both the production of cytokines and the transcription and translation of the PGE$_2$-producing enzyme cyclooxygenase-2 (COX-2). Tumor necrosis factor (TNF)-$\alpha$ is the first cytokine to circulate in the bloodstream after an intravenous LPS injection,
yet it does not appear in the bloodstream until 30 minutes post-injection. Temperature increases within 10-12 minutes of LPS injection, before a significant amount of cytokines enter the bloodstream (Blatteis, 2005). Recent research has shifted focus to the liver, which also shows a response to peripheral LPS. Liver cells react to LPS by creating C5a, a compound that provokes Kupffer cells to activate the hepatic vagal nerve bundle by releasing PGE₂. In this case, PGE₂ is catalyzed by the membrane-associated enzyme PI-PLC rather than COX-2, resulting in faster PGE₂ production and release (Blatteis, 2007). This nerve has been shown to ultimately stimulate the release of norepinephrine in the POAH via the NTS, leading to a much faster rise in temperature than that provided by the slower COX-2 dependent OVLT pathway (Imbery, 2008). As such, it has been proposed that fever is initiated by one system – most likely, the hepatic vagal nerve – and then sustained by the eventual rise in circulating cytokines and the OVLT response that results. The NTS’s ventral noradrenergic bundle, which may receive input from the liver via the hepatic vagal nerve, releases norepinephrine (NE) into the POAH. NE inhibits warm-sensitive neurons and provokes the early temperature rise. Later, the increase in cytokines that has long been correlated with fever may take part in initiating a secondary fever phase by activating the OVLT, the hepatic vagal nerve, cerebrovascular endothelial cells, or some combination of the three (Blatteis, 2005).

Once the POAH has received an infection signal and begins the thermostat shift, theories suggest that the PVN enacts many of the autonomic and endocrine changes necessary for a temperature rise. Physiologically, the PVN has been shown to become active during fever (Saper, 1998). PVN neurons controlling the autonomic preganglionic neurons essential for the shifts in blood flow that are necessary for fever are particularly active during an LPS-induced fever. These neurons constrict peripheral blood vessels, driving cutaneous blood to deep vascular
beds to increase core body temperature (Elmquist, 1996; Scammell, 1996). When temperature exceeds the POAH’s set-point, the PVN reduces mesenteric blood flow. This is part of the general response to hyperthermia, in which blood is shunted from deeper vascular beds to the skin where heat can dissipate, leading to the well-known flush of fever (Chen, 2010). When the body is in the first stages of cultivating a fever, the PVN performs the opposite task: constricting peripheral blood vessels to pull cutaneous blood to the body’s core, raising core temperature (Scammell, 1996). This in combination with lesion studies and the activation of PVN during thermoregulatory responses provides strong evidence for a connection between the POAH and PVN.
Figure I.3. Anatomical orientation of PVN and POAH.

Nissl-stained coronal whole-brain slice from a *rattus norvegicus* adult. The densely populated triangular region at the top of the third ventricle is the paraventricular nucleus (PVN); immediately ventral to the PVN is the medial portion of the preoptic area (POAH), which is bordered laterally by the anterior hypothalamus and lateral hypothalamus. Adapted from Brain Maps.
6. Anatomical Evidence for POAH-PVN Interaction

As shown in Figure I.3, the POAH and PVN lie in close proximity to one another anatomically, providing an ideal target for retrograde labeling studies. In 1997, Saper and Elmquist observed LPS-activated projections from the ventromedial preoptic area (VMPO) to the PVN (Elmquist, 1997). Sawchenko’s 1983 survey identified “light” projections from the POAH to the PVN, with “more substantial” input rising from the medial POAH to the parvocellular division of the PVN (Sawchenko, 1983). Moga and Saper, whom used Fluorogold retrograde tracing, identified dense labeling in the medial preoptic after LPS activation (Moga, 1994). As far back as 1981, scientists concluded that there was “general agreement” that major hypothalamic inputs to the PVN included the anterior hypothalamus, periventricular preoptic area, medial preoptic area, and lateral hypothalamus (Silverman, 1981).

The nature of this interaction, however, remains unknown. A large percentage of POAH neurons are inhibitory, and the high tonic GABAergic inhibition of the PVN might initially suggest that the POAH may be one of the many inhibitory afferents of the PVN (Nakamura, 2008). However, the PVN shows a differential activation during the fever response, with parvocellular regions showing activity during the early phase, and magnocellular regions during the later phase. Release of vasopressin within the CNS by the PVN plays a potential role in stifling fever, rather than propagating it (Saper, 1998; Hallbeck, 2001). The anterior periventricular area of the hypothalamus provides GABAergic input to the PVN; the VMPO, meanwhile, provides inhibitory inputs to the anterior periventricular area. As such, Elmquist suggested that the POA may disinhibit the PVN by inhibiting the inhibitory periventricular area. However, as previously discussed, disinhibition alone is not enough to increase the firing rate of PVN neurons; excitatory input is required (Elmquist, 1997; Prabha, 2010). This evidence
suggests that there may be a combination of direct glutamatergic excitation of the PVN and indirect GABAergic inhibition of inhibitory nuclei projecting to the PVN.

7. Specific Aim and Hypothesis

As the PVN has been identified in several thermoregulatory mechanisms, the nature of the interaction between the POAH and the PVN is of central importance to a proper understanding of the brain’s thermoregulation network. The POAH has been shown to inhibit inhibitory nuclei projecting to the PVN. It has also been shown to provide direct input to the PVN itself. These parallel projections suggest that the POAH may indirectly disinhibit the PVN while also providing direct excitatory input. To identify any excitatory (i.e., glutamatergic) input from the POAH to the PVN, retrograde-labeling nanoparticles conjugated with a polyclonal vesicular glutamate transporter type-2 (VGLUT-2) antibody will be injected into the PVN. This technique will result in labeling of glutamate-releasing neurons of the POAH.
Methods

1. Treatment and Preparation

Tissue slices were prepared from male Sprague-Dawley rats (Harlan, 100-150 g). The animals were housed in standard conditions and provided food and water ad libitum. As per procedures approved by the Animal Care and Use Committee of the College of William and Mary, rats were anesthetized with isofluorene and sacrificed by decapitation. Brain tissue isolated into a tissue block containing the hypothalamus was sectioned into 500 µm slices of sagittal or coronal orientation. The slices were then placed in a submersion recording chamber and perfused with a continuous supply of artificial cerebrospinal fluid (aCSF), which contained: 124 mM NaCl, 26 mM NaHCO3, 10 mM glucose, 5 mM KCl, 2.4 mM CaCl2, 1.3 mM MgSO4, and 1.24 mM KH2PO4. The aCSF, aerated with a 95% O2 – 5% CO2 mix, dripped at 1-2 ml/min via gravity flow into the recording chamber, which had a volume of 2 ml. See Fig III.1 for a schematic of the recording chamber.

Antibody-conjugated gold nanoparticles (AuNPs) served as the retrograde labeler. The AuNPs were composed of a dielectric silica core surrounded by a thin gold coating (diameter = 5 nm; Nanopartz, Inc.). These gold nanoparticles were conjugated with a polyclonal antibody for VGLUT-2 (Millipore) and polyethyleneimine (PEI, Nanopartz, Inc.), and had a peak reflectance frequency of 524 nm (Fig. III.2 (a)).

Probe solution consisting of AuNPs in bovine serum albumin suspension was backfilled into glass microelectrodes pulled to a tip diameter of ~2-5 µm and injected into the PVN with a nitrogen puffer system. There, the VGLUT-2 antibody conjugated to the AuNPs’ surfaces facilitated specific uptake of the AuNPs into glutamate-releasing axon terminals.
AuNP transport relies on the transient exposure of VGLUT to the synaptic cleft. VGLUT is located on the membrane of synaptic vesicles within the axon terminal. Utilizing a chemical gradient maintained by ATP-driven H+-pumps, VGLUTs transport glutamate from the cytoplasm into the vesicular lumen to prepare the vesicle for quantal release of glutamate into the synaptic cleft (Shigeri, 2004). When glutamate is released into the synaptic cleft the synaptic vesicle membrane fuses with the presynaptic membrane, temporarily exposing the membrane-bound VGLUT to the synaptic cleft (Jung, 2006). The AuNPs were conjugated with a polyclonal antibody to VGLUT-2, the primary VGLUT isoform expressed in the hypothalamus (Santos, 2009). As synaptic vesicles release glutamate into the synapse, this antibody can bind to the exposed VGLUT-2, allowing the nanoparticles to hitchhike into newly forming synaptic vesicles and thereby be transported into the presynaptic terminal (Figure III.2 (b); Jung, 2006).

Once encapsulated in a synaptic vesicle, the cationic PEI conjugate utilizes the ‘proton sponge effect’ to induce an influx of water into the vesicular lumen. This hydrosaturation eventually lyses the vesicle and leaves the nanoparticles free to diffuse to the soma (Figure III.2 (c); Bergen 2008). Theoretically this diffusion is passive; however, the rapid uptake – with probes often visualized in somas dozens of µm away as quickly as 2 hours post-injection – might suggest that the AuNPs are assisted by some form of axonal transport mechanism, possibly by remaining within vesicles being transported to the soma for degradation (Bergen, 2008; Mendoza, 2010).

Following injection, the AuNPs were allowed to diffuse through the tissue for 6 hours (h). Treated slices were then removed from the recording chamber and placed in a 10% formalin solution at 12°C.
Figure III.1 Diagram of recording chamber.

aCSF was oxygenated with 95% O2 and 5% CO2 within the reservoir. It was then heated by the thermoelectric assembly (d) en route to the inner bath (a) within which the tissue was placed.
Figure III.2 AuNP mechanism of action.

(A) 5 nm gold nanoparticles are conjugated with polyclonal VGLUT-2 antibodies (‘y’-shape) and PEI (jagged line). (B) At the presynaptic terminal of glutamate-releasing neurons, VGLUT-2, ordinarily present on synaptic vesicles within the cytoplasm, is temporarily exposed as synaptic vesicles fuse with the presynaptic membrane. The VGLUT-2 antibodies attach here, allowing the nanoparticles to ‘hitchhike’ along into newly-forming synaptic vesicles. (C) Within the synaptic vesicle, PEI draws in water via the proton-sponge effect. The vesicle lyses, allowing the AuNPs to diffuse to the soma. By this mechanism, AuNPs injected into the PVN can enter any neuron releasing glutamate in the injection area. Adapted from Mendoza, 2010.
The 500 µm thick tissues were soaked in a 30% paraformaldehyde/sucrose solution prior to being sectioned into 50 µm slices with a freezing microtome (Leica SM-2000R). These slices were then transferred to a phosphate-buffered saline solution. The slices were immediately mounted on slides and left to dry at room temperature for a minimum of 12 h.

For the giemsa staining process, slides were first placed in a 50:50 chloroform-alcohol solution for 3 h at RT. The slides were then rehydrated by submersion for 5 minutes each in 300 mL of: 95.5% reagent alcohol, 70% reagent alcohol, and distilled water. This was followed by submersion in a phosphate buffer monobasic solution for 5 minutes and 4 minutes in Giemsa stain warmed to 60°C. Immersion in 95.5% reagent alcohol for 2 minutes removed excess stain; slides were then stored in 300 ml of xylene (Sigma; >98.5%) for 12 h. Coverslips were applied with Permount and given 12 h to set prior to microscopy.

2. Data Collection

Using a standard upright light microscope (Olympus CX41), AuNPs were visualized in dark field illumination and confirmed to be within a cell soma by observing the Giemsa-stained somas and probes under bright field illumination (Figure III.3). Upon confirmation, the labeled neurons were marked in relation to the injection site on a map adapted from a rat brain atlas (Paxinos, 1998). The map used was adjusted based on the tissue slice anatomy to account for slight variations in slicing; all coronal slices fell within the domains of the -0.92, -1.30, or -1.40 mm bregma maps (adapted from figures 22, 23, and 24 of Paxinos, 1998). Sagittal maps were at 1.1 mm bregma (Figure 105, Pellegrino, 1979). Counters were blinded to whether the tissue was
a control or experimental injection, and all labeled cells were verified a second time under a 100x oil immersion lens.

3. Statistics

Significance of results was determined by a non-parametric Mann-Whitney U Test, based on the total labeled POAH neurons observed for each 500 µm tissue slice. PVN-injected coronal slices were compared to PVN-injected slices of sagittal orientation and non-PVN-injected slices. To assess the probe distribution per 50 µm slice, a secondary statistical analysis was performed via a parametric independent samples t-test.
Figure III.3 Neuronal soma labeled with AuNPs.

An AuNP-labeled soma visualized under bright field (top panel), dark field (middle panel) and a composite image of bright and dark field (bottom panel). Adapted from Mendoza, 2010.
Results

Eighteen 500 µm tissue slices were processed and counted. The number of 50 µm slices preserved from each 500 µm tissue varied; on average, five 50 µm slices were obtained from each.

Injections of VGLUT-2-conjugated AuNPs into the PVN served as the experimental group, with both coronal (n=13) and sagittal (n=2) oriented slices produced. As an *in vitro* experiment, different axonal pathways are severed when tissue is sectioned on different anatomical planes; as such, the sagittal oriented slices were used to assess any potential advantage to one orientation or the other. These results are summarized in Figure IV.1.

Control groups included non-PVN injections of VGLUT-2-conjugated probes (n=2) and non-injected tissue (n=1). Figure IV.2 compares labeled POAH cells observed in PVN vs. non-PVN injected tissue. Average labeled POAH cells per injection type and slice orientation are summarized in Table IV.1. Figures IV.3, 4, and 5 show typical distribution of probes in a coronal PVN-injected slice, a sagittal PVN-injected slice, and a coronal non-PVN-injected slice. Statistical results are summarized in Tables IV.2 and IV.3.
Fig IV.1. POAH labeled cells by slice orientation.

Coronal and sagittal injections showed a similar rate of POAH labeling, with an average of 4 labeled POAH cells per coronal 500-µm slice and an average of 5 labeled POAH cells per sagittal 500 µm slice.
Fig IV.2 POAH labeled cells by injection site.

AuNPs were injected into either the PVN or a non-PVN region (one injection was higher, in the median PO; one injection was lower, in the AHA). Less POAH cells were observed when the injection site was not the PVN.
Fig IV.3 A coronal PVN injection map.

PVN injection resulted in labeling of the POA, AHA, and LH, as well as local PVN neurons and an average of 2.85 labeled cells per 500 µm slice in miscellaneous areas. This tissue was approximated to be -1.40 mm bregma and 7.60 mm interaural. Adapted from Paxinos, 1998.
Sagittal injections showed labeling of the AHA, POA, and LH, as well as an average of 3.5 labeled cells in miscellaneous areas. Adapted from Pellegrino, 1979.
Fig IV.5 A coronal non-PVN injection.

Injection far above the PVN showed nonspecific labeling, both ipsi- and contralateral. This slice is approximated to be at -1.30 mm bregma, 7.70 mm interaural. Adapted from Paxinos, 1998.
### Table IV.2 Mann-Whitney U test results.

<table>
<thead>
<tr>
<th>Injection Location</th>
<th>U</th>
<th>P (two-tailed)</th>
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<tr>
<td>Non-PVN injections</td>
<td>26.0</td>
<td><strong>0.01904762</strong></td>
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<tr>
<td>Sagittal</td>
<td>16.0</td>
<td>0.685714</td>
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<tr>
<td>No injection</td>
<td>13.0</td>
<td>0.1428572</td>
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</tbody>
</table>

Significant p values at $\alpha = .05$ are bolded. This is based on total observed labeled POAH neurons for each 500 µm tissue slice.

### Table IV.3 Independent samples t-test results.

<table>
<thead>
<tr>
<th>Injection Location, slice orientation</th>
<th>n</th>
<th>Average labeled POAH cells</th>
<th>Standard deviation</th>
<th>p-value in comparison with PVN, coronal</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVN, Coronal</td>
<td>71</td>
<td>0.7324</td>
<td>0.6752</td>
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<td>PVN, Sagittal</td>
<td>9</td>
<td>1.1111</td>
<td>0.9280</td>
<td>0.13583</td>
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<tr>
<td>Non-PVN, Coronal</td>
<td>6</td>
<td>0.1667</td>
<td>0.4083</td>
<td><strong>0.04232</strong></td>
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<tr>
<td>No injection, Coronal</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td><strong>0.00462</strong></td>
</tr>
</tbody>
</table>

Significant p values at $\alpha = .05$ are bolded. This is based on observed labeled POAH neurons per 50 µm slice.
The total data from each treated 500 µm-thick tissue showed a significant increase in labeled POAH neurons for PVN-injected tissue over non-PVN injected tissue (Table IV.2). When each 50 µm slice was statistically compared, a significant difference was again achieved between PVN and non-PVN injections (Table IV.3). Neither the Mann-Whitney U tests comparing the complete 500 µm tissues nor the independent samples t-test comparing each 50 µm slice revealed a significant difference in labeled POAH neurons between sagittal and coronal tissue, suggesting that significant numbers of axonal pathways were not severed in one anatomical orientation versus another.

Other regions within which labeled neurons were commonly observed included the anterior hypothalamus (average of 2.46 cells per PVN-injected coronal 500 µm tissue), the PVN (average of 2.8 neurons per PVN-injected coronal 500 µm tissue) and, more sparsely, the lateral hypothalamus (average of .692 cells per PVN-injected coronal 500 µm tissue). Interestingly, some labeled neurons were occasionally found contralateral to the injection site in coronal tissue. However, ipsilateral labeling remained significantly more common, with a total average of 12.6 ipsilateral labeled neurons compared to 0.62 contralateral neurons per PVN-injected coronal 500 µm tissue slice.

Overall, our findings suggest that glutamatergic projections do exist from the POAH to the PVN. No significant difference was detected between sagittal and coronal slices.
Discussion

The PVN and Thermoregulation

Our findings suggest that the PVN does receive glutamatergic input from the thermoregulatory POAH. As the PVN exerts control over key autonomic preganglionic neurons and temperature-modulating hormones such as vasopressin, this connection has long been suspected. Although connections have been identified in retrograde tracing attempts, the phenotype of PVN-projecting POAH neurons had not been previously investigated. This study provides the first direct evidence of a glutamatergic component of thermoregulatory efferents to the PVN.

As the gatekeeper to the autonomic and endocrine aspects of thermoregulation, the PVN shows a strong involvement in heat loss, reducing mesenteric blood flow to move blood towards vasodilated peripheral vessels such as the rat tail artery and increasing salivation to promote fur-licking behavior in rats (Hallbeck, 2001; Nagashima, 2000; Chen, 2010). The PVN also plays a role in heat retention and fever propagation. Both parvocellular and magnocellular PVN cells show activation during fever; lesions of the PVN result in a reduced febrile response to both intracerebroventricular PGE₂ injection and intraperitoneal LPS injection (Saper, 1998; Horn, 1994).

Direct electrical stimulation of the PVN can be antipyrogenic (Hallbeck, 2001). This may be due to projections of vasopressinergic cells to the ventral septal area (Matsunaga, 2000). When released in the ventral septal area, vasopressin can inhibit the febrile response while also increasing the risk of febrile seizure (Richmond, 2003). However, oxytocin and vasopressin have both been implicated as essential in the peripheral blood volume changes required for the acute phase febrile response, and do in fact show increased plasma levels after intravenous and
intraperitoneal administration of LPS. This suggests vasopressin may play disparate roles in the febrile response between the periphery and the central nervous system (Matsunaga, 2000; Saper, 1992).

Evidence of PVN control of thermoregulatory responses in conjunction with the identification of direct inputs from the POAH to the PVN suggests that effective, direct communication with the PVN is crucial for a fast response to deviations from the temperature set-point. While the PVN is able to disinhibit the PVN by inhibiting the anterior periventricular area, neurons of the PVN require excitatory input that the POAH could provide with its direct projection. This study has shown that such a direct communication does in fact exist between thermoregulatory POAH neurons and the PVN, and proven that labeling of this pathway with VGLUT-2 antibody-conjugated AuNPs is indeed possible.

**Methodology**

This project represents one of the first attempts to use the AuNPs for phylogenetic classification and connectivity assessment of an unknown circuit. The first and only other study to use AuNPs for targeted retrograde tracing of neurons, conducted by Mendoza et al. in 2010, focused largely on testing the efficacy of these newly-developed AuNPs, and demonstrated impressively selective results. Probes lacking VGLUT-2 antibody showed a significant decrease in efficacy; “naked” probes lacking both the VGLUT-2 and PEI conjugates showed worse efficacy still. Although it is possible that some nanoparticles are capable of passively diffusing into the axon terminal, probes lacking in VGLUT-2 were far less successful at reaching the soma (Mendoza, 2010).
In the first VGLUT-2 AuNP study, as many as 60 labeled cells were observed per 50 µm slice. This study saw far lower numbers, with a maximum of 7 labeled cells observed in a 50 µm slice. However, the POAH shows dense input to the dorsomedial hypothalamus, while previous retrograde tracing studies have suggested that the POAH provides only light input to the PVN (Sawchenko, 1983). Similarly, a study identifying VGLUT-2 expressing cells showed only sporadic labeling in the medial preoptic area (Ziegler, 2002). Thus, the reduced numbers of labeled cells may be the result of a combination of less cells being available to label and the highly selective nature of the probes themselves.

Although the AuNPs’ selectivity may result in reduced labeling, it also makes them highly advantageous for electrophysiology studies. This is, in fact, the reason this application was developed. AuNPs exhibit a low biotoxicity and a fast, specific uptake, allowing them to be visualized in living in vitro tissue. Other metallic tracers such as Fluorogold and cholera toxin B-gold provide neither the high specificity nor the low biotoxicity required for this application. Moreover, they cannot be visualized in the six to twelve hour window within which in vitro tissue can survive (Mendoza, 2010).

Anatomical observations – labeling of the lateral hypothalamus, PVN, and contralateral hypothalamic nuclei

Labeled cells were observed in the lateral hypothalamus (LH) and the paraventricular nucleus itself in PVN-injected tissue. The LH does show input to the medial parvocellular cells of the PVN (Silverman, 1981). LH neurons also express VGLUT-2 mRNA, confirming that a proportion of the LH population releases glutamate as a neurotransmitter (Ziegler, 2002). Local glutamate-GABA circuitry plays a large role in PVN control, suggesting that some nearby glutamate interneurons may exist (Herman, 2002). This, too, has been backed up with evidence
of VGLUT-2 mRNA expression in some magnocellular and parvocellular PVN neurons (Ziegler, 2002). This data suggests that the number of LH and PVN labeled cells observed is not extraordinary.

The third ventricle serves as a large obstacle between the PVN and contralateral hypothalamic nuclei, bringing into question the observed labeled cells in contralateral nuclei. However, the suprachiasmatic nucleus has shown significant projections to the contralateral portion of the PVN, and it is located beneath the ventral side of the third ventricle (Vrang, 1997). As such, contralateral labeling is not unprecedented, although it is presumably quite rare.

**Future aims and conclusion**

With POAH projections to the PVN classified, this work provides the foundations for several promising projects. In the more immediate future, it would be of use to pursue an in-depth study of potential GABAergic input to the PVN region and compare it to the results obtained here. An AuNP conjugated with vesicular GABA transporter (VGAT) has been synthesized and its efficacy tested, and could easily be utilized for this project. Moga observed dense labeling in the medial preoptic in a floregold retrograde tracing study; this, in combination with our modest results, suggests that there may be non-glutamatergic input from the preoptic to the PVN (Moga, 1994).

A particularly exciting endeavor will be to electrophysiologically examine PVN-projecting glutamatergic POAH cells and characterize them as warm-sensitive, temperature-insensitive, or silent neurons. A microscope capable of visualizing the probes in living *in vitro* slices will allow for targeted intracellular recordings of AuNP-labeled POAH cells. Preliminary targeted recordings have already proven successful, as seen in figure V.1. Intracellular recording
allow the AuNPs to truly shine: their low biotoxicity and fast uptake make them ideal for live recording of cells with known projections and a known phenotype. More importantly, they can be visualized without the dangers of a fluorescent probe, which heats up when fluoresced and could alter the neuron’s physiology or even damage the neuron. As AuNPs reflect rather than fluoresce, they do not run that risk (Mendoza, 2010). Following recording, the morphology of these neurons can then be studied in detail, and perhaps compared to the morphological observations that classified warm-sensitive neurons as medial-lateral and temperature-insensitive neurons as dorsal-ventral (Griffin, 1994).

This project sought to characterize efferent projections from the POAH to the PVN. Using a unique retrograde-labeling conjugated gold nanoparticle capable of specifically targeting glutamatergic neurons, glutamatergic projections from the POAH to the PVN were identified. This technique can now be used to record from PVN-projecting POAH neurons and classified for their thermosensitivity, providing the next step in characterizing the finely-tuned neural circuitry behind thermoregulation.
Figure V.1. Labeled neuron filled with lucifer yellow.

While an intracellular recording was taken of this neuron, lucifer yellow diffused from the electrode into the cell. Fluorescence microscopy revealed the soma (outlined in white) as well as some dendritic projections (top). A dark cluster of AuNPs is visible in a composite image of bright field with fluorescence (bottom, arrow).
References


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