5-2008

The Effects of Cirazoline and Clonidine on the Firing Rates of Thermally Classified Neurons in the Anterior Hypothalamus of the Rat

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THE EFFECTS OF CIRAZOLINE AND CLONIDINE ON THE FIRING RATES OF THERMALLY CLASSIFIED NEURONS IN THE ANTERIOR HYPOTHALAMUS OF THE RAT

An Honors Thesis Presented to

The Faculty of the Department of Biology

The College of William and Mary

In Partial Fulfillment

Of the Requirements for the Degree of

Bachelor of Science in Biology with Honors

Accepted for Honors:

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Dr. Carey K. Bagdassarian: __________________________

Andrew P. Speidell

2008
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Abstract

Previous studies have proved that during a lipopolysaccharide (LPS) challenge, norepinephrine (NE) levels in the preoptic area of the mammalian hypothalamus (PO/AH) rise and are correlated with an increase in core body temperature. Whole animal studies have revealed that selective activation of the $\alpha_1$- and $\alpha_2$-adrenergic receptors (ARs) can, respectively, induce a hyperthermic or hypothermic thermoregulatory response. Therefore, we hypothesize that in accordance with established models of neural thermoregulation, firing rate responses to the $\alpha_1$-AR agonist Cirazoline and the $\alpha_2$-AR agonist Clonidine should differ with respect to thermosensitivity of the neuron. To characterize these responses, single-unit recordings of neurons in rat hypothalamic tissue preparation were made. Neurons were classified as either warm sensitive or temperature insensitive through manipulations in local temperature. This was followed by treatment of the neuron with either Cirazoline or Clonidine. As hypothesized, the majority of insensitive neurons increased their firing rate when exposed to Cirazoline and decreased their activity when introduced to Clonidine. When warm sensitive neurons were treated with Cirazoline, all responded with a decrease in activity, while most increased their firing rate during treatment with Clonidine. These AR responses, in accordance with current neural thermoregulatory models, would produce the same hypothermic or hyperthermic effects on core body temperature as seen with in vivo studies.
INTRODUCTION

1. The Role of the Hypothalamus in Thermoregulation

The hypothalamus is the main regulator of the autonomic function of various systems within the mammalian organism. Most notably, the hypothalamus directs the regulation of osmotic and metabolic systems and lies at the top of the neural echelon which controls these autonomic functions. More specifically, neurons located in the preoptic area (POA) of the anterior hypothalamus function to control body temperature. A subset of POA neurons, the anterior hypothalamic nucleus (PO/AH), has been demonstrated to be the most important for temperature regulation (Boulant, 1991 and Boulant, 2000).

Afferent information from both cutaneous and body core thermoreceptors is integrated within the PO/AH neurons. This population of neurons projects to parts of the hypothalamus and can influence body temperature inhibition or excitation of these neurons (Boulant 1980). When PO/AH neurons were cooled locally in vivo through use of electrodes, responses involved with heat production were seen. In the same way, warming of these neurons in vivo resulted in heat loss responses.

The mammalian body can respond to warming and cooling both behaviorally and physiologically. Behavioral heat loss methods include activities such as seeking shade and licking of the skin. Physiological heat loss mechanisms consist of increases in evaporative heat loss through sweating and panting, and cutaneous vasodilation. Heat production behavioral responses can involve changing the amount of body surface exposed to sunlight among other actions. Physiologically, the animal can retain heat through shivering or through non-shivering thermogenesis. Non-shivering thermogenesis
is a collection of mechanisms that include an increased metabolism of brown adipose
tissue, vasoconstriction, and piloerection. PO/AH neurons have demonstrated the ability
to override heat production and heat loss responses brought on by normal peripheral
thermoreception (Boulant, 1991), which confirms the position of PO/AH neurons atop
the neural thermoregulatory hierarchy.

2. Differing Thermosensitivity of Hypothalamic Neurons

Previous studies have identified two major subpopulations of neurons within the
PO/AH. These two groups differ both physiologically through sensitivity to local
temperature and morphologically through differences in the orientation of their dendritic
projections (Griffin et al. 2001). Warm sensitive neurons respond to increased
temperature in their local environment by increasing firing rate, and conversely, respond
to decreased local temperature by decreasing their firing rate. Warm sensitive neurons
account for approximately 25% of neurons in the PO/AH. The majority (approximately
65%) of neurons in the PO/AH are designated temperature insensitive neurons. In
contrast to warm sensitive cells, insensitive neurons respond with little or no change in
their firing rate in reaction to a change in local temperature. (Kelso, 1982 and Griffin,
1996)

The remaining 10% of neurons are classified as cold sensitive neurons. This
small subset of neurons respond with an increased firing rate to decreasing local
temperatures and a decreased firing rate to increasing local temperatures. Two previous
studies demonstrated that by either cutting synaptic input to these cells, or by using an
antagonist to block synaptic activity, the cold sensitivity of the neuron could be
essentially neutralized. This observation demonstrates that the cold sensitivity is driven by synaptic input and is not an intrinsic characteristic of the neuron, as these cells appear temperature insensitive after synaptic blockade. In contrast, application of the same antagonist to warm sensitive and temperature insensitive neurons did not block their respective responses. (Boulant and Dean, 1986; Boulant 1998; and Kelso, 1982)

To assess the thermosensitivity of a neuron, the slope of the neuron’s response to local temperature must be analyzed. Through the use of single-unit recording equipment, the firing rate of the neuron can be plotted as a function of its local temperature. Cells exhibiting warm sensitivity have a thermosensitivity of 0.8 impulses·s\(^{-1}\)·ºC\(^{-1}\) or greater. Temperature insensitive neurons respond with a thermosensitivity of 0.79 impulses·s\(^{-1}\)·ºC\(^{-1}\) or less. The benchmark of 0.8 impulses·s\(^{-1}\)·ºC\(^{-1}\) was established through several different studies (Boulant and Hardy, 1974). This figure is significant because below a thermosensitivity of 0.8 impulses·s\(^{-1}\)·ºC\(^{-1}\), neurons fail to show a synchronized integrated response to both peripheral and local temperature information. Warm sensitive neurons are unique in the fact that they are able to integrate afferent information from thermoreceptors in the skin and spinal cord. In comparison to warm sensitive neurons, temperature insensitive neurons do not demonstrate the ability to integrate both central and peripheral sensory information. (Boulant & Bignall, 1973; Boulant & Dean, 1986)

In addition to differing in their thermosensitivity, warm sensitive and insensitive cells show a marked difference in their respective morphologies. Recent studies have demonstrated that within their location in the PO/AH, warm sensitive cells tend to orient their dendrites in the medial to lateral direction (Griffin, 2001). This observation suggests the ability of warm sensitive neurons to receive and integrate input from
ascending information from the skin and spinal cord. A few warm sensitive neurons have been found to extend their dendrites into the nearby third ventricle. It has been proposed that the cerebrospinal fluid in the third ventricle may include important compounds essential to thermoregulation, such as exogenous and endogenous pyrogens. Conversely, temperature insensitive cells extend their dendrites in the rostral to caudal direction and parallel to the third ventricle. Insensitive neurons do not appear to receive afferent information from spinal or cutaneous thermoreceptors. Figure 1 shows the contrasting morphologies of temperature insensitive and warm sensitive neurons within the PO/AH. The opposing morphologies for warm and temperature insensitive neurons suggest distinct roles within the thermoregulatory network. (Griffin et al, 2001)
3. Hammel’s Model of Hypothalamic Thermoregulation

An enduring model for temperature regulation proposes that thermoregulation is achieved through a network of warm sensitive and temperature insensitive neurons that ultimately synapse with effector neurons. These effector neurons, in turn, project to other hypothalamic centers and bring about either heat retention or heat loss responses. (Hammel, 1965) In this model, effector neurons combine the non-thermal activation of insensitive neurons with the central and afferent information of warm sensitive neurons. When synapsing on effector cells, temperature insensitive cells and warm sensitive cells act antagonistically. Thus, when the activity of both sets of neurons is approximately equal, the summation of inhibitory and excitatory activity will cancel out and a set point of temperature will be established. Figure 2 is a modification of the model proposed by H.T. Hammel to explain the neural basis of thermoregulation.

Figure 2: Hammel’s Model of Neural Thermoregulation.

W indicates a warm sensitive neuron, while I indicates a temperature insensitive neuron. The heat loss effector neuron and the heat production effector neuron are designated w and c respectively. OC indicates the optic chiasm and MB refers to the mammilary body within the hypothalamus. Note the synaptic input to warm sensitive cells, but not to temperature insensitive cells. Solid lines indicate the firing rate of each neuron and dotted lines represent the frequency of excitatory (+) and inhibitory (-) synaptic input. (Adapted from Boulant, 2006)
4. *The Neuronal Basis of Thermosensitivity*

In Hammel’s model, the two populations of cells must be able to respond in different physiological manners to differences in temperature. Previous electrophysiological studies have identified several ways in which this differential temperature response of PO/AH neurons may be achieved.

The difference between the two responses was at first believed to be the result of temperature on the resting membrane potential of warm sensitive neurons. Various studies have shown that while temperature does not affect resting membrane potential, it does have an affect on smaller potentials that are present immediately before the larger action potentials (Kobayashi, 1993). Upon closer examination, these depolarizing prepotentials were present in both temperature insensitive and warm sensitive neurons, yet only the prepotentials in the warm sensitive cells were affected by local temperature fluctuations (Griffin, 1995). As temperature rises, the prepotential of the warm sensitive neuron grows, and as a result, less excitatory potentials are needed to reach the action potential threshold. Thus, the neuron’s firing rate will increase if local temperature increases. At lower temperatures, the prepotential grows smaller, achieving the opposite effect with respect to firing rate (Griffin et al, 1996). In figure 3 below, it is clear that at higher temperatures, the prepotential of warm sensitive cells rises faster than that of temperature insensitive cells, giving warm sensitive cells shorter interspike intervals.

Two studies have attempted to explain exactly how temperature causes the fluctuation in the amplitude of the depolarizing prepotential. The potassium A current, a current which prevents firing of an action potential during hyperpolarization appears to shut down at higher temperatures, leading to shorter interspike intervals. In contrast to
warm sensitive cells, temperature insensitive cells use the metabolically driven sodium potassium pump to offset the deactivation of the outward potassium A current to maintain a steady firing rate regardless of temperature. (Curras & Boulant 1989 and Griffin et al, 1996).

Figure 3: The effect of temperature on the electrical properties of thermally classified neurons.

All figures show a whole-cell recording of neurons in the PO/AH. A and C show recordings that were taken over approximately one second at three different temperatures. The firing rate of the warm sensitive neuron (C) is higher at warmer temperatures due to the faster rise of the prepotential at those temperatures. Note the absence of a faster prepotential in the temperature insensitive cell (A). B and D illustrate the shortening of the interspike interval through superimposed pre- and action potentials at three different temperatures. In the warm sensitive cell (D), the interspike interval shortens as temperature rises, giving the neuron a faster firing rate. The same effect is absent in the temperature insensitive cell (B). (Adapted from Boulant, 1997)
5. Adrenergic Receptors in the Central Nervous System

This study is especially interested in the coordination of thermoregulation with the immune system to fight infection. This temporary increase in core body temperature is known as a fever and has been shown to be modulated in the same pathways that normal regulation of body temperature is controlled. A fever is beneficial to the mammal during infection in several different ways. A rise in body temperature increases the metabolic rate of lymphocytes and therefore increases the rate at which they can manufacture proteins and other factors to clear the invading pathogens. In addition, a shift in body temperature usually slows bacterial replication, as they are no longer in their optimal temperature for growth. The hyperthermia experienced during a fever is mediated by norepinephrine (NE), a neurotransmitter in the mammalian CNS.

Norepinephrine is the endocrine signal produced and secreted into circulation by the adrenal glands on the anterior region of the mammalian kidneys. This compound can act through the endocrine system, or can be manufactured and released presynaptically to work as a neurotransmitter in the CNS. The biosynthesis of NE involves modification of the amino acid tyrosine to produce the CNS neurotransmitter dopamine. Enzymes in the adrenal medulla, most notably dopamine β-hydroxylase, modify dopamine by substitution of a hydroxyl group to produce the norepinephrine molecule.

Various studies have identified several types of adrenergic receptors (adrenoreceptors, AR) in the CNS. The two classes of receptors, denoted α- and β-adrenoreceptors have several different responses in various cell types when binding their endogenous ligands. The alpha class of adrenergic receptors has two recognized subtypes, denoted α₁ and α₂, both of which can selectively bind norepinephrine and
epinephrine. Most importantly, alpha adrenoreceptors have been shown to be expressed on thermosensitive cells in the POAH (Mallick et al, 2001)

Both subtypes of alpha ARs work through G-coupled protein receptor mechanisms, yet each subtype has a unique mode of action through which its second messenger systems work. When a ligand binds to the $\alpha_1$-AR, the $G_q$ subunit of the membrane associated heterotrimeric G-protein disassociates. This subunit directly stimulates the phospholipase C enzyme. Phospholipase C has a key role in several second messenger systems, and has been demonstrated to affect ion conductance in some cell types. The $\alpha_2$-AR is also a G-coupled receptor, but in contrast to the $\alpha_1$-AR, a different subunit of the heterotrimeric G-protein ($G_i$) renders adenylate cyclase inactive. Ultimately, inactivation of this enzyme leads to a decrease in intracellular cAMP and calcium. This messenger system can also function to open or close ion channels in cells. Although it has been demonstrated that these two messenger systems can affect ion channel conductivity, there has not yet been research into how the response to ligands differs with respect to thermosensitivity. The compounds Cirazoline and Clonidine have been identified as selective agonists to the $\alpha_1$ and $\alpha_2$ adrenergic receptors, respectively. Although $\beta$-adrenoreceptors have been identified in the CNS, they are within the scope of this study.

6. LPS Infection Signaling

Lipopolysaccharide (LPS), an exogenous pyrogen present on the cell walls of gram-negative bacteria, is a compound known to elicit a febrile (fever-producing) response in mammals. Bacterial infection and corresponding elevated levels of LPS,
initiate heat production through the hypothalamo-pituitary-adrenal axis (HPA). When peripheral immune cells encounter LPS, they release interleukin (IL)-1, IL-6, and TNF-α. These factors work in part to co-stimulate other cells of the immune system and prime them for their respective roles. The endogenous cytokine interleukin 1 in particular is the most potent endogenous pyrogen known to activate the HPA axis (Dunn, 2000).

Both IL-1 and IL-6 have been demonstrated to stimulate CRH (corticotropin-releasing hormone) production from CRH neurons in the hypothalamic paraventricular nucleus (Tilders et al, 1998). CRH then stimulates the production of adrenocorticotropic hormone (ACTH) in cells located in the anterior lobe of the pituitary gland. In turn, ACTH acts to stimulate cells in the adrenal cortex to synthesize corticosteroids, which have a wide range of effects in several different mammalian systems. Specifically, corticosteroids are known to act locally to upregulate a particular enzyme in the adrenal cortex, dopamine β-hydroxylase, which produces norepinephrine (Wong, 1980). A recent study confirmed that an LPS challenge to the immune system directly results in sustained elevated levels of NE in the plasma via activation of the HPA axis (Wieczorek, 2006).

In order for NE to produce a rise in body temperature in response to the bacterial endotoxin LPS, NE must bind to adrenergic receptors present on neurons located in the PO/AH. Recent research has revealed that NE can leak into the prepotic area via the organum vasculosum of the lamina terminalis (OVLT) and that local production of NE is also stimulated through afferents to the hypothalamus. According to several studies, peripheral macrophages (especially Kupffer cells present in the liver) respond to LPS in the plasma during the initial stages of infection by releasing prostaglandin E2 (PGE₂) into
the serum and initiating an inflammatory response. Kupffer cells also activate the complement system, part of which acts to locally up-regulate the PGE$_2$-synthesizing enzyme COX-2 (Li, 2005 and Blatteis, 2006). Interleukin and prostanoid receptors present on the hepatic vagal nerve branch are stimulated during an LPS challenge, and studies have concluded that it is this nerve that carries the afferent signal to the brain (Mohan-Kumar, 2000). Entering the brain, the signal is first received by the nucleus tractus solitarius, and then is carried into the preoptic area via the noradrenergic bundle, stimulating local NE production (Phillis & Tebecis, 1967; Blatteis, 2007; and Feleder et al, 2007). Thus, a response to an endotoxin is initiated through both humoral and neural pathways.

7. PGE$_2$ and the Production of Fever

Experiments with whole animals have revealed that microdialysis of NE directly into the preoptic area generate a hyperthermic response in core body temperature. In addition, injections of NE agonists into the preoptic area elicit responses specific to the type of adrenoreceptor for which the agonist is selective. Cirazoline, an $\alpha_1$-AR agonist, when microdialyzed into the PO/AH, produces a immediate rise in core body temperature. When the $\alpha_2$-AR agonist Clonidine is administered in the preoptic area, a slight drop in core temperature is observed, followed by a sustained increase in temperature well above the physiological norm. When Cirazoline and Clonidine are injected simultaneously, and thus simulating the effects of NE, the early phases of each agonist’s effect are negated and only the later phase temperature rise in observed. (Quan & Blatteis, 1989 and Feleder et al, 2004)
Upon closer examination, the concentration of key fever-regulating molecule PGE\(_2\) appears to vary in concentration when NE or its agonists are injected. More specifically, a rise in PGE\(_2\) levels was observed only when the \(\alpha_2\)-AR was bound by either NE or its agonist Clonidine, and was not affected by Cirazoline. The \(\alpha_1\)-AR response is therefore responsible for the quick rise in body temperature, while the \(\alpha_2\)-AR response is responsible for a slight hypothermia and for stimulating local PGE\(_2\) production within both astrocytes and thermosensitive neurons. (Feleder et al, 2004)

PGE\(_2\) is known to directly affect the firing rate of thermally classified cells in the PO/AH to produce a new set point hyperthermic to the normal physiological temperature. PGE\(_2\) increases the firing rate of temperature insensitive cells while simultaneously decreasing the activity of warm sensitive cells. Since PGE\(_2\) is degraded and removed from the preoptic area much more slowly than is NE, and since it is believed that PGE\(_2\) is thought to be the main fever regulating molecule in the mammal, it is thought that the effects of PGE\(_2\) work to outlast those of NE on PO/AH neurons. Thus, the two PGE-2 mediated changes in input to effector neurons, an increase in activity of insensitive neurons and a decreased firing rate of warm sensitive neurons, when applied to Hammel’s model, are understood to produce a prolonged hyperthermia in rats. (Blatteis and Sehic, 1997 & Ranels and Griffin, 2003).

The effect of NE and AR agonists on the core body temperature in whole animals has been well classified. However, the binding of \(\alpha_1\)- and \(\alpha_2\)-AR agonists to their respective receptors and its effect on firing rate of thermally classified neurons still remains largely uninvestigated. This study seeks to determine the effects of Cirazoline and Clonidine on the firing rate of individual warm sensitive and temperature insensitive
neurons in vitro and how the differential responses to these agonists, in accordance with Hammel’s model, may have elicited core temperature changes seen as in whole animal experiments.
METHODS

Tissue slices from the anterior hypothalamus were prepared from male Sprague-Dawley rats (Harlan; 100-150 grams in weight). Rats were cared for under standard conditions and given food and water *ad libitum*. No sooner than 15 minutes prior to each recording session, a rat was anesthetized using isoflurane (Webster Veterinary) and promptly decapitated. The procedure was approved by the Animal Care and Use Committee of the College of William and Mary. The whole of the brain inside the skull was removed and a tissue block containing the hypothalamus was prepared by removal of the non-hypothalamic sections of the brain using a standard razor blade. Several 400μm thick slices containing the hypothalamus were prepared using a microtome (FHC Inc.) in either the sagittal or the coronal plane. The slices were allowed to equilibrate in a tissue chamber for approximately 30 minutes prior to recording.

While the recording sessions were underway, the tissue slices were continually perfused with a pyrogen-free artificial cerebrospinal fluid. The nutrient medium was prepared no more than an hour before recording and contained (in mM): 124 NaCl, 26 NaHCO₃, 10 D-(+)-glucose, 5 KCl, 2.4 CaCl₂, 1.3 MgSO₄ and 1.24 KH₂PO₄. The ACSF was kept at room temperature until use. The medium was oxygenated (95% O₂–5% CO₂) and warmed to a constant temperate of approximately 36.0 degrees Celsius by a thermoelectric assembly. The chamber received the nutrient medium at 1–1.5 ml·min⁻¹ and the temperature of the medium was continuously monitored by a thermocouple placed directly below the recorded slice.

Single-unit extracellular recordings were made from neurons in the PO/AH using 1.5 mm microelectrodes with a tip diameter of no greater than 1 μm. The microelectrode
was filled with 3M NaCl solution. An Xcell-3 Microelectrode Amplifier (FHC Inc.) was used to make the recordings, which were digitally stored for later analysis. After a 3:1 signal-to-noise ratio of the neuron’s activity had been isolated and the neuron appeared to be stable for at least 3 minutes, the recording session was initiated. The input voltage to the thermoelectric assembly was varied to obtain a temperature approximately 2°C above and below 36°C to determine the effect of local temperature on neuronal activity. The neuron’s thermosensitivity (impulses s\(^{-1}\)·°C\(^{-1}\)) was characterized by plotting the firing rate of the cell as a function of temperature. The linear coefficient of regression (m) was then used to determine neuronal thermosensitivity in the Sigmaplot program. In accordance with previous studies (Boulant and Hardy, 1974), a neuron was warm sensitive if its regression coefficient was greater than or equal to 0.8 impulses s\(^{-1}\)·°C\(^{-1}\).

After the thermosensitivity of the neuron had been characterized through a temperature swing, the cell’s response to either Cirazoline or Clonidine was tested. When a stable baseline temperature had been achieved (~36°C) for at least one minute, the perfusion medium was switched from ACSF to ACSF containing either Cirazoline (1-100μm) or Clonidine (1-100μm) (Sigma-Aldrich Chemical Co.). The new medium perfused the tissue slices for 5 minutes or until a minute after a significant response. After response to the agonist had been recorded, the medium was switched back to ACSF and the neuron was allowed to recover for a control period of at least 20 minutes. After the recording session had ended, one-minute samples of stable firing rate activity were digitized at 60 Hz for comparison (pClamp Software, Axon Instruments). The samples were collected during baseline condition (just prior to perfusion with an agonist), at the end of perfusion with an agonist or during the most significant response, and during the
control period if the cell recovered. From these firing rate samples, a mean and a standard error were calculated (Sigmaplot Software, SPSS Inc.). A response to an agonist was determined by comparison to baseline levels, using a standard T-test (P ≤ 0.05).

During each recording session, electrodes were placed within the PO/AH, using stereoscopic visualization (Imbery et. al. 2008). This region was not difficult to visualize in either type of tissue section. Once the recording session had ended, the location of the electrode was visually confirmed. After each recording, the location and depth of the electrode was noted on a section diagram adapted from an atlas of the rat brain (Paxinos and Watson, 1998). At the end of a recording session, tissue slices were removed from the chamber, fixed in a 2% formalin solution, and sectioned to a thickness of 40 - 50 μm. Sections were then mounted on gelatin-coated slides and stained with a giemsa counter stain to identify specific hypothalamic areas, so that the location of each recording within the PO/AH could be reconfirmed. Only neurons in the PO/AH were used in the present study.
RESULTS

Recordings were made from 93 neurons in the PO/AH. The thermosensitivities and firing rate responses of these neurons were analyzed. Of the 49 cells treated with Cirazoline, 37 (75.6%) were classified as temperature insensitive, with the remaining 12 neurons (24.5%) exhibiting warm sensitivity. The other 44 cells were treated with Clonidine during the recording session. 38 (86.3%) of these neurons were classified as temperature insensitive, while 6 (13.6%) exhibited warm-sensitivity. Table 1 summarizes the thermosensitivity of neurons recorded in this study. There was not found to be any pattern in location within the PO/AH of recorded neurons with respect to thermosensitivity. (Figure 4)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>N</th>
<th>Criteria</th>
<th>Mean +/- S.E.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insensitive</td>
<td>75</td>
<td>&lt;0.8</td>
<td>0.15 +/- 0.03</td>
<td>-0.60-0.77</td>
</tr>
<tr>
<td>Warm</td>
<td>18</td>
<td>≥0.8</td>
<td>1.48 +/- 0.22</td>
<td>0.80-4.69</td>
</tr>
</tbody>
</table>

Table 1: Thermosensitivity of POAH Neurons

Thermosensitivity (impulses·s⁻¹·ºC⁻¹)

There appeared to be a clear correlation in the directional response of the neuron’s firing rate with respect to thermosensitivity. This response was different depending on which agonist the neurons were exposed to. Of the temperature insensitive cells responding to the presence of Cirazoline, all experienced a rise in activity above their baseline firing rate. In the same way, warm sensitive cells that were exposed to Cirazoline during the recording session all decreased their firing rates during treatment with the agonist.
Figure 4: The recording locations of single neuron activity in response to temperature and AR agonist. Section diagrams are shown in the coronal plane and ordered from rostral to caudal, beginning with the upper left section and moving clockwise. Distance from bregma: A = −0.3 mm; B = −0.8 mm; C = −0.92 mm; D = −1.4 mm. Sections were adapted from an atlas of the rat brain (Paxinos and Watson, 1998). Colors indicate treatment with either Cirazoline (red) or Clonidine (blue). Red circles = insensitive neurons which showed a significant increase in firing rate, red squares = insensitive neurons that did not show a change in firing rate, red triangles = warm sensitive neurons which showed a significant decrease in firing rate. Blue circles = insensitive neurons which showed a significant decrease in firing rate, blue triangles = warm sensitive neurons which showed a significant increase in firing rate, blue squares = insensitive neurons which did not show a change in firing rate after treatment with Clonidine, blue diamond = Warm sensitive neurons that did not show a significant change in firing rate. 3V, third ventricle; ac, anterior commissure; AHA, anterior hypothalamic area; AVPe, anteroventral periventricular nucleus; BST, bed nucleus stria terminalis; fx, fornix; LH, lateral hypothalamus; LPO, lateral preoptic area; MnPO, median preoptic nucleus; MPO, medial preoptic nucleus; MPA, medial preoptic area; mfb, median forebrain bundle; ox, optic chiasm; Pa, paraventricular nucleus; Pe periventricular nucleus; PS, parastral nucleus; Re, reunions thalamic nucleus; Sch, suprachiasmatic nucleus; SM, stria medullaris of thalamus; SO, supraoptic nucleus; StHy, striohypothalamic nuc.; VLPO, ventrolateral preoptic area; VMPO, ventromedial preoptic area.
The direction of the firing rate response was found to be the opposite of those neurons exposed to Cirazoline when neurons were treated with the $\alpha_2$-AR agonist Clonidine. As a population, the vast majority (91.4%) of temperature insensitive cells which responded experienced a decrease in activity when treated with Clonidine. In contrast, most (83.3%) of those warm sensitive cells which responded to Clonidine showed an increase in firing rate during treatment with the $\alpha_2$-AR agonist. Although the direction of response was strongly influenced by thermosensitivity, there appeared to be no correlation of the magnitude of peak response with treatment of Cirazoline ($r^2 = 0.01$) nor with Clonidine ($r^2 = 0.03$). Figures 5A and 5B show the responses (as measured by percent change in firing rate) of recorded cells during the one minute treatment period.

![Figure 5A: The firing rate responses of PO/AH neurons to Cirazoline.](image)

The percent change in firing rate for all neurons ($N = 49$) in response to Cirazoline is plotted against thermosensitivity. Circles = insensitive neurons which showed a significant increase in firing rate, triangles = warm sensitive neurons which showed a significant decrease in firing rate, and squares = temperature insensitive neurons which did not show a significant change in firing rate. Dotted line indicates 0% change in firing rate. The solid diagonal line represents a regression fit to the entire set of data ($r^2 = 0.01$).
Of the neurons treated with Cirazoline, 5 (10.2%) cells did not show a significant change in firing rates from baseline levels. A non-responsive neuron was defined to be a cell that did not increase or decrease its firing rate by more than 15% during treatment with an AR agonist. I suspect that the medium with Cirazoline could have been too dilute to elicit any change in firing rate in these cases. As expected, of the cells perfused with higher concentrations of the agonist (10 or 100μM), virtually all showed a significant change in firing rate. Of the cells exposed to Clonidine, 3 (6.8%) neurons

Figure 5B: The firing rate responses of PO/AH neurons to Clonidine.
The percent change in firing rate for all neurons (N = 44) in response to Clonidine is plotted against thermosensitivity. Circles = insensitive neurons which showed a significant decrease in firing rate, squares = insensitive neurons which failed to show a significant change in firing rate, diamonds = insensitive neurons which showed a significant increase in firing rate. Triangles = warm sensitive neurons which showed a significant increase in firing rate, inverted triangles = warm sensitive neurons which did not show a significant change in firing rate. Dotted line indicates 0% change in firing rate. The solid diagonal line represents a regression fit to the entire data set ($r^2 = 0.03$).
failed to show a considerable change in activity. Table 2 summarizes the directional responses of neurons to either Cirazoline or Clonidine with respect to thermosensitivity.

Table 2: Effects of Cirazoline and Clonidine on the Firing Rate Activity of PO/AH Neurons

<table>
<thead>
<tr>
<th>Thermosensitivity</th>
<th>Agonist Type</th>
<th>N</th>
<th>Increase</th>
<th>Decrease</th>
<th>No Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inensitive</td>
<td>Cirazoline</td>
<td>37</td>
<td>32</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Warm</td>
<td>Cirazoline</td>
<td>12</td>
<td>0</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Inensitive</td>
<td>Clonidine</td>
<td>38</td>
<td>3</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td>Warm</td>
<td>Clonidine</td>
<td>6</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 6 shows the neuronal activity of a PO/AH temperature insensitive neuron during a fluctuation in local temperature and during exposure to Cirazoline. As the temperature was varied (~4°C), the neuron showed little change in activity ($m = 0.03$). However, when the medium was switched to one containing Cirazoline, the neuron’s activity rose from a baseline mean of 2.1 imp·s$^{-1}$ to an average of 14.7 imp·s$^{-1}$. After the medium had been switched back to one without Cirazoline, activity returned approximately to baseline levels.

The activity of a PO/AH warm sensitive neuron during a fluctuation in temperature and subsequent perfusion with a Cirazoline-containing medium is shown in Figure 7. The neuron showed a clear correlation in firing rate with respect to temperature ($m = 2.41$). In comparison to mean activity during a baseline period (3.3 imp·s$^{-1}$), the neuron decreased its activity to an average firing rate of 0.2 imp·s$^{-1}$. After Cirazoline had been cleared from the medium, the firing rate of this particular neuron remained low.
Figure 6: The effects of temperature and Cirazoline on the firing rate activity of a PO/AH temperature insensitive neuron.
A shows the firing rate of this neuron during changes in temperature and Cirazoline (10 μM; indicated by the solid bar above the graph). In B, firing rate (60 Hz) is plotted as a function of temperature. A linear regression is indicated by the solid line. In C, 1 min segments of firing rate activity are plotted as individual bar graphs, just before perfusion with Cirazoline (Baseline; 2.1 ± 0.1), during the peak of the response (Cirazoline; 14.7 ± 0.7), and several minutes after Cirazoline perfusion had stopped (Washout; 0.9 ± 0.1). For plots in C, error bars may be difficult to visualize.
Figure 8 illustrates the response of a PO/AH temperature insensitive neuron to treatment with the $\alpha_2$-AR agonist Clonidine. As with other recorded cells, the neuron showed little change in firing rate as temperature was varied ($m = -0.01$). When the neuron was introduced to a medium containing Clonidine, the neuron’s activity decreased from a baseline mean of 1.28 imp·s$^{-1}$ to an average of 0.63 imp·s$^{-1}$. Approximately 12 minutes after the end of perfusion with Clonidine, the neuron recovered to near-baseline
levels of activity. In some temperature insensitive neurons exposed to Clonidine, the neuron experienced a slight raise in firing rate towards the end of the recording session.

Figure 8: The effects of temperature and Clonidine on the firing rate activity of a POAH temperature insensitive neuron.

A shows the firing rate of this neuron during changes in temperature and Clonidine (1 μM; indicated by the solid bar above the graph). In B, firing rate (60 Hz) is plotted as a function of temperature. A linear regression is indicated by the solid line. In C, 1 min segments of firing rate activity are plotted as individual bar graphs, just before perfusion with Clonidine (Baseline; 1.28 ± 0.08), during the peak of the response (Clonidine; 0.63 ± 0.04), and several minutes after Clonidine perfusion had stopped (Washout; 1.19 ± 0.07). For plots in C, error bars may be difficult to visualize.
The activity of a PO/AH warm sensitive neuron during a fluctuation in temperature and subsequent perfusion with a Clonidine-containing medium is illustrated in Figure 9. The neuron’s firing rate showed a strong relationship with local temperature \((m = 0.82)\), confirming its warm sensitivity. In comparison to mean baseline levels of activity \((2.02 \text{ imp·s}^{-1})\), the cell raised its average firing rate to \(3.49 \text{ imp·s}^{-1}\) during the treatment period. After recovery to baseline levels of activity following removal of
Clonidine from the medium, some warm sensitive cells reduced their firing rate towards the end of the recording session. Table 3 provides a summary of the changes in firing rates experienced by recorded neurons with respect to thermosensitivity and type of agonist.

Table 3: Effects of Cirazoline and Clonidine on the firing rates of POAH neurons

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Agonist Type</th>
<th>N</th>
<th>Baseline (impulses·s(^{-1}) +/- S.E.)</th>
<th>Treatment (impulses·s(^{-1}) +/- S.E.)</th>
<th>Washout (impulses·s(^{-1}) +/- S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insensitive</td>
<td>Cirazoline</td>
<td>37</td>
<td>3.41 +/- 0.38</td>
<td>10.49 +/- 1.40*</td>
<td>2.68 +/- 0.84</td>
</tr>
<tr>
<td>Warm</td>
<td>Cirazoline</td>
<td>12</td>
<td>3.71 +/- 2.06</td>
<td>0.44 +/- 0.19*</td>
<td>0.41 +/- 0.00</td>
</tr>
<tr>
<td>Insensitive</td>
<td>Clonidine</td>
<td>38</td>
<td>3.38 +/- 0.41</td>
<td>2.05 +/- 0.32*</td>
<td>2.18 +/- 0.54</td>
</tr>
<tr>
<td>Warm</td>
<td>Clonidine</td>
<td>6</td>
<td>3.44 +/- 1.03</td>
<td>6.14 +/- 1.16*</td>
<td>2.01 +/- 0.56</td>
</tr>
</tbody>
</table>

*Significantly different from Baseline Firing Rate (Paired t-test \( p < 0.05 \))
DISCUSSION

This study confirms that neurons involved in the thermoregulatory network respond to norepinephrine through the $\alpha_1$- and $\alpha_2$-ARs. According to Hammel’s model, the change in activity of these neurons \textit{in vivo} brings about changes in core body temperature. More significantly, it is important to note that the directional change in firing rate of these neurons, when placed into Hammel’s model, would produce the core body temperature changes seen in the previous \textit{in vivo} studies. Activation of the $\alpha_1$-AR, in general, increased the activity of temperature insensitive neurons while simultaneously decreasing the firing rate of warm sensitive neurons. Ultimately, these two changes would work, through effector neurons, to elicit a hyperthermia \textit{in vivo}. Conversely, activation of the $\alpha_2$-AR decreased the activity of temperature insensitive neurons while increasing that of warm sensitive neurons. In opposition to the $\alpha_1$-AR, these two changes in activity would work to elicit a hypothermia as seen during the early phase of whole animal studies (Feleder et al, 2004)

A few neurons in the study did not respond with a significant change in firing rate. I defined a neuron to have an insignificant change in activity when the cell increased or decreased its firing rate by less than 15\% in either direction from baseline levels of activity. A lack of response to the AR agonist could be due to the fact that the neuron may have not expressed the appropriate adrenoreceptor on its membrane. As stated previously, the concentration of AR agonist in the ACSF medium may have been to dilute to elicit any change in firing rate.

In many of the neurons that were exposed to the $\alpha_2$-AR agonist Clonidine, a gradual shift in firing rate was seen at the end of the recording session. This response
was very delayed and was seen, on average, approximately 25 minutes after perfusion with the Clonidine-containing medium had ceased. This response was most likely a result of the production of PGE$_2$. The observation that this response was only seen in cells treated with the $\alpha_2$-AR agonist Clonidine lends support to this proposal. Furthermore, monitoring PGE$_2$ levels while selectively activating each ARs revealed that PGE$_2$ was manufactured only when the $\alpha_2$-AR was bound. Figure 10 shows a proposed model of stimulated PGE$_2$ synthesis via activation of the $\alpha_2$-AR. (Feleder et al, 2004)

Figure 10: Proposed mechanism for the interaction of NE and PGE$_2$ in the mammalian PO/AH
Presynaptic norepinephrine stimulates prostaglandin E$_2$ synthesis via the COX-2 enzyme in both the post synaptic warm sensitive neuron and in nearby astrocytic processes. PGE$_2$ acts to inhibit further production of additional NE through its receptors (EP$_3,4$) in the presynaptic cell. The combined effects of adrenergic and prostanoid receptors work to produce the early and late phase changes in activity in the postsynaptic neuron. (Adapted from Feleder, 2004)

It is important to note that in the mammalian organism, there exists no agonist that can exclusively bind one subtype of adrenoreceptor. Thus, when norepinephrine acts to stimulate neurons in the PO/AH, both $\alpha_1$- and $\alpha_2$-ARs are activated simultaneously. The role of norepinephrine in a thermoregulatory response must then encompass both
adrenoreceptors. As stated previously, in the early phase of the thermogenic response to an LPS challenge, the hyperthermic response of the $\alpha_1$-AR and the hypothermic response of the $\alpha_2$-AR work antagonistically to maintain a relatively stable core body temperature. It is thought that early hypothermic action of the $\alpha_2$-AR works to negate the quick hyperthermic response brought about by activation of the $\alpha_1$-AR and simultaneously activates production of PGE$_2$. Thus, in the later phase of a febrile response, it is thought that the longer lasting action of PGE$_2$ on PO/AH neurons drives a sustained hyperthermic response. Studies involving inhibition of the PGE$_2$ synthesizing enzyme COX-2 reveal that the late phase rise in core body temperature can indeed be attenuated, if not eliminated, by the absence of PGE$_2$. (Feleder et al, 2004 & Ranels and Griffin, 2003)

The latency of response in generation a thermoregulatory response *in vivo* was found to be much longer than the time needed to elicit a firing rate change in PO/AH neurons in this study. I propose two different explanations for the discrepancy in response time. Initially, the larger delay in response seen in whole animals was believed to be the result of the time needed to elicit physiological and behavioral changes. Indeed, firing rate changes could take effect *in vivo* immediately after AR binding, yet effector neurons would need greater time still to cause physiological changes that are dependent on manufacture and transport of hormones. A second possibility that can explain the delay in response between the two studies is suggested by my *in vitro* study. In the recording chamber, the medium was allowed to perfuse through each 400$\mu$m slice, while this perfusion may not happen nearly as quick in the whole animal brain. Thus, agonists were able to come in contact with their adrenoreceptors within seconds of their introduction into the medium *in vitro*.
There remain many questions concerning activation of adrenoreceptors in the PO/AH, especially their interaction with PGE$_2$. The roles of other COX isoforms have yet to be elucidated, although evidence exists that they may also share a large part in the febrile response generated by the PO/AH (Oshima, 1991). In addition, we still do not yet know how temperature insensitive cells and warm sensitive cells are able to respond in different manners to the same adrenoreceptor agonist. Whole cell recordings involving perfusion of these AR-agonists may be able to explain the intracellular mechanisms underlying this differential response, especially if they involve changes in ion channel conductance.
CONCLUSION

Regulation of body temperature is perhaps the most important role of the hypothalamus in the mammalian organism. Core temperature must remain within a narrow range for optimal functioning of enzymes. Variations of even a few degrees Celsius can prove fatal to organisms. The thermoregulatory network of the PO/AH must then be capable of eliciting both large-scale temperature change as well as causing finer adjustments in body temperature.

Furthermore, the mammalian nervous, immune, and endocrine systems must work closely together in order to initiate a response to infection. Generation of a fever depends critically on the interaction of these systems. In addition, there must be a quick and precise means to modulate responses to changes in the external and internal environment of the organism. Arguably, the most important factor in this immune response is the quick and sustained rise in body temperature. Actions of peripheral immune cells work to generate an electrical signal that ultimately results in NE release in the PO/AH of the hypothalamus. It is now known that release of NE is the quick means by which the mammal elicits changes in thermoregulatory neurons. More importantly, activation of the $\alpha_2$-AR by NE results in the sustained hyperthermic response known as a fever through PGE$_2$. Failure to elicit this change in temperature during an infection can be the difference between life and death of the organism. Therefore, in a broader sense, norepinephrine plays the most important role in the thermoregulatory response to infection in mammals.
REFERENCES


ACKNOWLEDGEMENTS

I would like to thank Dr. John D. Griffin for giving me the opportunities to achieve all that I have in the four years spent under his mentorship. Furthermore, I would like to thank the other members of the Griffin lab for their help, encouragement, and entertainment during my work as an undergraduate at the College. Finally, I am grateful to my committee members, Dr. Paul Heideman, Dr. Ashley Haines, and Dr. Carey Bagdassarian for their time and helpful comments.

This project was funded by the National Science Foundation: IBM-9983624, the National Institutes of Health: NS053794, and in part by a Howard Hughes Medical Institute grant through the Undergraduate Science Program to the College of William and Mary.