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The effect of increased cAMP on the firing rate of thermosensitive neurons in the preoptic and anterior regions of the hypothalamus

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by

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Abstract

Previous studies of the mechanisms of fever genesis have suggested that the prostanoid prostaglandin E2 (PGE_2) plays a key role. PGE_2 has been correlated with changing the firing rates of thermosensitive neurons within the preoptic and anterior regions of the hypothalamus (POAH), increasing the firing rates of temperature insensitive neurons while inhibiting warm sensitive neurons. PGE_2 has four receptor subtypes, two of which (EP3 and EP4) have been implicated by most studies to be involved in febrogenesis. EP3 and EP4 receptors work by respectively decreasing or increasing the intracellular cAMP concentrations, suggesting an important role for cAMP dependent kinases in PGE_2's modulation of the firing rate of thermosensitive neurons. We hypothesized that the activation of these cAMP dependent kinases would increase the firing rates of thermosensitive neurons. To test this hypothesis, single-unit recordings were made in a hypothalamic tissue preparation of neurons located in the POAH. The neurons recorded were classified as temperature insensitive or warm sensitive and then treated with 1μM sp-cAMP, an activator of cAMP dependent kinases. The majority of temperature insensitive neurons responded with a decrease in firing rate, which poses some interesting questions regarding the distribution and role of PGE_2 receptors in the fever generation process.

Introduction

1. Thermoregulation and the Hypothalamus

The mammalian hypothalamus maintains a state of equilibrium throughout the body. This homeostasis includes the regulation of osmotic balance, glucose levels, and the maintenance of core body-temperature around a set-point of about 37°C. While the hypothalamus works in concert with other structures within the brain stem and spinal cord, the higher-order regulation of temperature occurs within the preoptic and anterior regions of the hypothalamus (POAH) (Boulant, 2000).

Neurons within the POAH receive and integrate information from various internal sources to determine core body temperature. These neurons are a part of an integrative network that includes receptors on the skin that detect external temperature, as well as more local receptors within the brain stem and brain itself that provide information on local temperature changes. This information is taken together by specialized neurons within the hypothalamus and if the information indicates that temperature is too high or too low, these neurons can induce physiological and behavioral changes to return the body to its set-point. To induce heat loss, the mammal may experience sweating, panting, cutaneous vasodilatation, and may indulge in such behaviors as licking, wetting the skin, or moving into a cooler location. To induce heat gains, physiological actions include shivering, non-shivering thermogenesis, utilizing brown adipose tissue and vasoconstriction. The mammal may also seek out warmer environments or huddle in order to increase body temperature (Boulant, 2006). These effects were seen in experiments where animals were placed in hot or cold environments, as well as when

thermodes placed on the hypothalamus were filled with hot or cold water (Boulant, 1963).

2. Neuronal Thermosensitivity

Within the POAH, there have been found to be three types of neurons that respond differently to changes in temperature, as well as exhibiting differences in morphology (Boulant, 1998; Griffin, 2001). Neurons exhibiting warm sensitivity increase their firing rate when the local or environmental temperature increases, and decrease their firing rate in response to lowered local or environmental temperatures. Temperature insensitive neurons exhibit little or no change in firing rate when the local or environmental temperature is increased or decreased. Several studies have been done to determine the population of these neurons in the POAH. They all report very similar results- approximately 60% of POAH neurons are temperature insensitive and approximately 30% exhibit warm sensitivity (Boulant, 2006).

The remaining ~10% belong to the third type of thermosensitive neuron. Cold sensitive neurons respond to increased local or environmental temperature with a decrease in firing rate, and to decreased local or environmental temperature with an increase in firing rate. However, it is suggested that these neurons may not be inherently cold sensitive, and that this response may be due to the inputs received by these neurons. In one study, thermosensitive neurons were subjected to synaptic blockade and tested to see the effect on their thermosensitivity. Those neurons classified as cold sensitive lost this classification when subjected to a synaptic blockade, while neither warm sensitive nor temperature insensitive neurons lost their thermosensitivity (Boulant and Kelso, 1982).

Cold sensitive neurons are not the only ones to be affected by synaptic input. Intracellular recordings of POAH neurons have found that some are driven by excitatory postsynaptic potentials (EPSPs). These neurons appear to be thermosensitive; however, this thermosensitivity is driven by the increase in EPSPs found with this neuron population during a temperature increase. These neurons have short dendrites that are consistent with the fact that they receive only local information (Griffin, Saper & Boulant; 2001)

The thermosensitivity of a neuron is characterized by the slope of the frequency-temperature curve. When a neuron undergoes a change in temperature, its firing rate is plotted against the temperatures to create a thermoresponsive curve (Boulant and Bignall, 1973). The slope of this curve is called the thermosensitivity coefficient. A neuron is classified as warm sensitive if it has a thermosensitivity coefficient of at least 0.8 impulses per second per degree Celsius. This coefficient has been found to be the lowest slope at which a warm-sensitive neuron responds simultaneously with the change in temperature (Boulant and Dean, 1986). These warm sensitive neurons respond to changes in temperature peripherally as well as locally, suggesting that they are the neurons that integrate temperature information coming from the skin and spinal cord (Boulant and Bignall, 1973). This makes them crucial in the regulation of body temperature, as they are the ones receiving both outside and local information about the body's state.

It has also been found that there are also morphological differences between temperature insensitive and warm sensitive neurons within the POAH. Thermosensitive neurons in the POAH around the third ventricle were stained and then traced to determine

their dendritic formation. Temperature insensitive neurons were found to have dendrites that branched rostrally, ventrally, dorsally and caudally. The dendrites of the warm sensitive neurons branched medially, and laterally. The spinohypothalamic tract, which carries temperature information, enters the hypothalamus in the medial and lateral direction. Other nuclei tracts that can carry temperature information also end medially and laterally. Some warm sensitive neurons also appeared to stretch their dendrites towards the third ventricle. Cytokines, pyrogens, and other molecules implicated in the generation of fever can found within the cerebrospinal fluid. The orientation of warm sensitive neurons towards these sources of outside temperature information supports their role as integrators of both local and environmental temperature information (Griffin, Saper & Boulant; 2001).

3. Hammel's Model of Thermoregulation and the Physiology of Thermosensitivity

In 1965, Hammel proposed a model by which warm sensitive and temperature insensitive neurons work together to determine set point as well as alter that set point (Figure I.1 Boulant, 2006). Warm sensitive neurons receive information from outside the hypothalamus, where spinal cord neurons receive excitatory input from warm receptors and inhibitory input from cold receptors. Along with temperature insensitive neurons, these warm sensitive cells form an integrative network with effector neurons. While many of these effector neurons are also located within the POAH, some can be found in other locations of the hypothalamus, creating a diffuse network of thermoregulation. Some effector neurons are labeled as being heat loss effectors. These receive excitatory input from warm sensitive neurons and inhibitory input from temperature insensitive neurons. When the warm sensitive cells receive excitatory input from the spinal neurons,

they excite the heat loss effector neurons which, as their name suggest, induce physiological and behavioral heat loss actions.

Other effector neurons are heat retention neurons, which receive inhibitory inputs from warm sensitive cells and excitatory inputs from temperature insensitive cells. When the warm sensitive cells are over inhibited by the spinal neurons due to cold receptor activation, these effector neurons induce physiological and behavioral heat retention actions. The body's temperature set-point is the point at which the warm and insensitive inputs balance each other out. This has been determined experimentally to be around 37°C (Boulant, 2006).

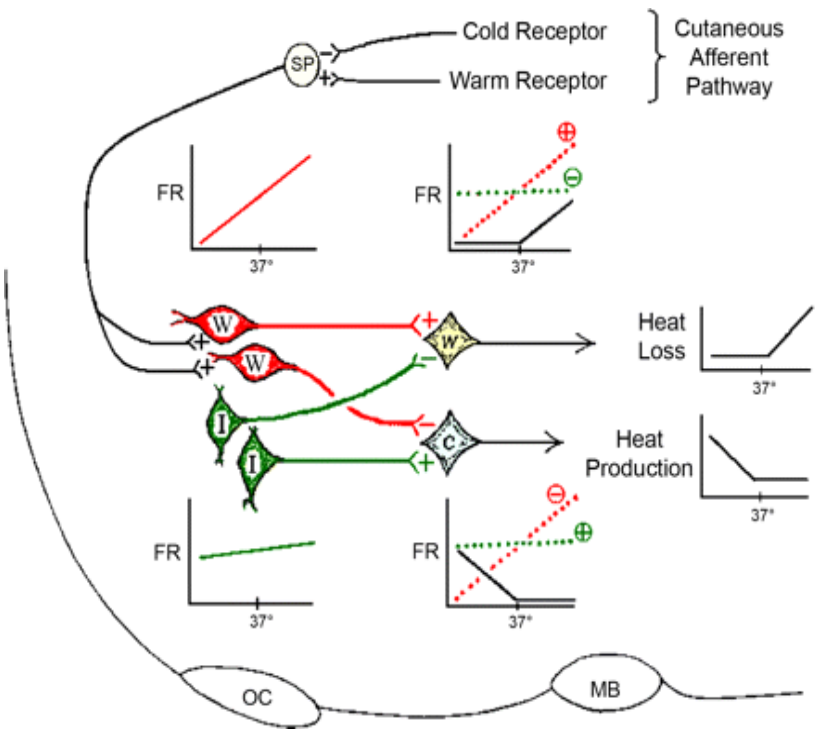


Figure I.1: Hammel's Model for Neural Thermoregulation.

A dorsal horn spinal neuron (SP) receives temperature information from receptors on the skin and synapses on a warm sensitive (W) neuron. When excited, the warm sensitive neurons stimulate warm effector neurons (w) which induce heat loss mechanisms. Conversely, inhibition of the warm sensitive neurons increases the excitation that temperature insensitive neurons (I) have on cold effector neurons (c). Cold effector neurons induce heat production mechanisms. OC indicates the optic chiasm; MB is the mammilary body.
(Adapted from Boulant, 2006)

With a working model of thermoregulation, research began into the cellular mechanisms that give warm sensitive or insensitive neurons their particular properties. In order for them to work within Hammel's model, they must somehow respond physiologically to temperature locally and, in the case of warm sensitive cells, to environmental temperature information.

One proposed mechanism was that warm sensitive cells undergo a change in resting membrane potential when the temperature rises, thus making them more likely to experience an action potential as well as changing the firing rate (Kobayashi and Takahashi, 1993). However, several intracellular studies that examined this possibility found that cold, warm, and insensitive cells showed no change in resting membrane potential at different temperatures (Griffin, 1995; Curras, 1991). However, temperature change was found to effect small depolarizing prepotentials that preceded the action potential (Figure I.2 Boulant, 1997). In warm sensitive neurons, the rate of rise in the prepotential was increased. When this occurs, it allows for the time between two action potentials, also called the interspike interval, to decrease. Thus, as the temperature increases, warm sensitive cells are able to decrease the time between their action potentials and increase their firing rates. This effect is not seen in insensitive or cold sensitive neurons.

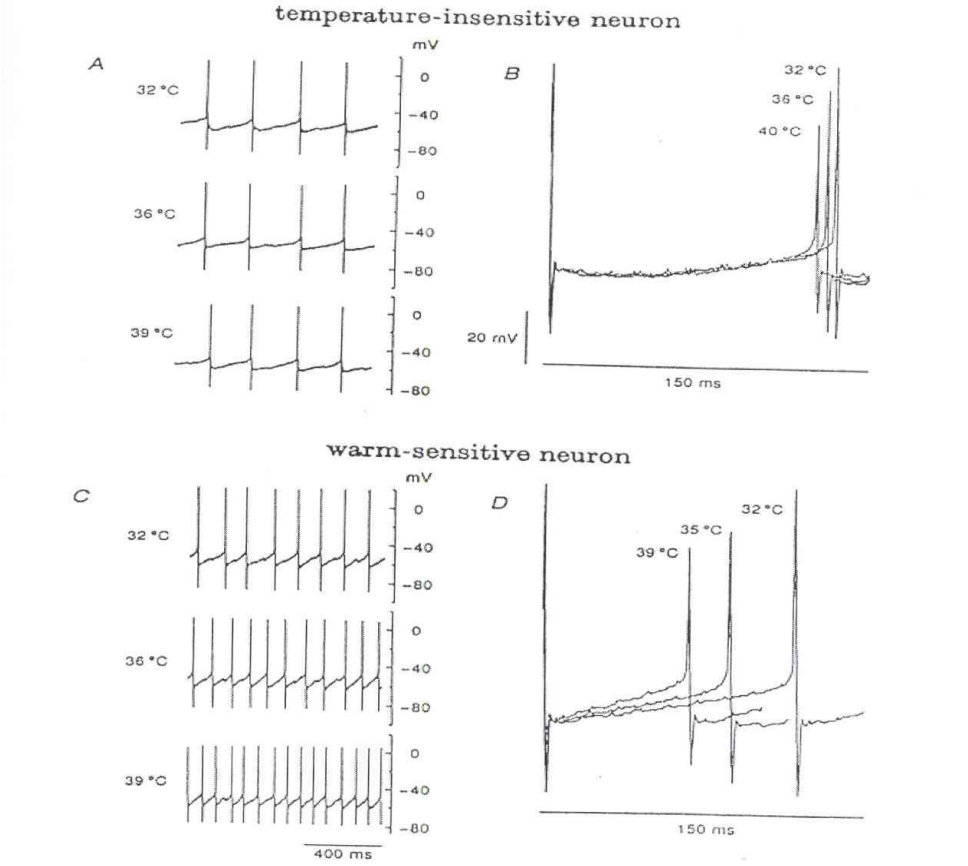


Figure I.2: The effect of temperature on the electrical properties of thermosensitive neurons. Figures A and B come from the recording of a temperature insensitive neuron in the POAH; C and D are of a warm sensitive neuron in the same area. The recordings in A and C were done at one second intervals at three different temperatures in order to show thermodependent differences. Note that at higher temperatures, the warm sensitive neuron undergoes an increase in firing rate due to a quicker prepotential at higher temperatures, something not seen in temperature insensitive cells. B and D superimpose the recordings to show the significant differences in the depolarizing prepotentials. The interspike interval is shorter in the warm sensitive neuron at higher temperature, which is not found in the temperature insensitive neuron.
(Adapted from Boulant, 1997)

Ion channels have also been implicated in determining the thermosensitivity of preoptic neurons. A study by Wechselberger et al. (2006) created models of hypothalamic neurons where the numbers of tandem pore potassium leak channels (specifically TASK-1, TREK and TRAAK) were altered to determine the effect on the neuron's thermosensitivity. These channels respond to temperature changes and play a role in the maintenance of hyperpolarized resting potentials. It was found that changing the expression of TASK-1 channels could change a neuron's thermosensitivity. Neurons where TASK-1 was the only type of potassium leak channel and neurons that had a relatively low number of these channels exhibited warm sensitivity. When the number of channels expressed was doubled, the neuron was found to be temperature insensitive. When the channel number was doubled again "silent neurons," neurons which fire only when electrically stimulated (as opposed to the spontaneously firing neurons which are the majority in the POAH), were created. When TREK and TRAAK channels were added into a model of a warm sensitive neuron, the neuron became temperature insensitive. Neuron expressing only TREK/TRAAK channels showed cold sensitivity. This study suggests that the number and type of potassium leak channels present in a POAH neuron may have an important effect on its thermosensitivity.

Potassium A currents may also play a role in determining the thermosensitivity of a hypothalamic neuron. These currents are involved in determining the length of the interspike interval. A study by Griffin et al. (1996) found that the activation of the potassium A currents is temperature dependent. At high temperatures, the activation of these currents increases the depolarization of the prepotential, shortening the interspike interval and increasing firing rate. Conversely, cooler temperatures increase inactivation

of the currents which lengthens the interspike interval and lowers firing rate. However, since these currents have been found in both warm sensitive and temperature insensitive neurons, there must be some other mechanism by which a neuron can remain insensitive to temperature changes.

For temperature insensitive neurons, the key factor seems to be the sodium-potassium pump. This pump helps maintain resting membrane potential, and seems to become more hyperpolarized as temperature warms (Boulant, 1998). This counterbalances the increased depolarizing prepotentials that warming induces. A study by Curras and Boulant in 1989 found that when temperature insensitive cells were treated with ouabain, which blocks the sodium-potassium pump, they became warm sensitive. Pyrogens and other substances that induce a fever may be able to block this pump and therefore change the mechanics of the thermoregulatory system (Boulant, 1998).

Finally, endogenous substances can play a role in the thermosensitivity of neurons. Pyrogens and anti-pyretics can interact with the sodium-potassium pump and other cellular mechanisms and alter the network's ability to modulate the body's set-point (Boulant, 1998). Drugs that increase cAMP within the cell have been found to increase thermosensitivity in warm sensitive and some temperature insensitive neurons (Griffin, 1990). cAMP appears to also increase the rate of depolarization for the prepotential, further decreasing the interspike interval and increasing the firing rate (Boulant, 1998). This suggests a role for cAMP in the thermosensitivity of neurons in the POAH.

4. Prostaglandin E2 and Fever

A fever is when the immune system's reaction to an infection causes the body's set-point is shifted upwards. Hammel's model predicts that a fever occurs when the warm-

sensitive cells are inhibited, and in kind the heat loss effector neurons. This allows for the heat production effectors to initiate heat production behaviors and cause a rise in the body's set-point (Blatteis, 2007). A fever is most often an immune response intended to help fight infection. The molecule that has been implicated in signaling the presence of infection and initiating the fever response is prostaglandin E2 (Scammel et. al., 1996).

Prostaglandins, along with thromboxanes, are members of the prostanoid family. The prostanoids are cyclooxegenase metabolites of arachidonic acids and are involved in many disease processes (Breyer et. al., 2001). Prostanoids are synthesized within the tissue that they affect (and are thus found throughout the entire body) and act locally on G-coupled receptors (Breyer et. al., 2001). Prostaglandin E2 is one of the most important molecule produced through this mechanism.

It was originally thought that pyrogenic cytokines produced by the phagocytes that attack invading bacteria were what directly induced the fever mechanism (Blatteis, 2006; Saper, 1996). However, these cytokines are large proteins that cannot cross the blood-brain barrier quickly enough to induce the rapid rise in temperature that we usually see with fever (Saper, 1996). It is now thought that these cytokines in turn induce the release of PGE_2, which being lipophilic, has the ability to cross the blood-brain barrier much more quickly. There are two other ways that cytokines can signal the production of a fever response. The first is through a "leaky" section of the blood-brain barrier called the organum vasculosum laminae terminalis, which would allow the larger proteins to move through and signal the direct production of PGE_2. The second is through cytokines stimulating the vagal nerve (possibly within the liver) which would then carry a neural

signal to the hypothalamus to being the production of PGE_2 (Blatteis, 2007; Scammel et. al., 1996).

Several studies have been done within the past 15 years to discover where exactly PGE_2 is having its fever inducing effects. In 1996, Scammel et. al. used Fos activation immunohistochemistry to track the fever-inducing effects of PGE_2. When the $PGE_2$was injected into the POAH, it was found to have fever-inducing effects in the ventromedial preoptic area (VMPO) as well as surrounding areas that are also involved in autonomic function. This effect is similar to the one produced by the injection of lipopolysaccharide complex (LPS). LPS is a component of gram-negative bacteria walls that stimulates the production of the pyrogenic cytokines that are believed to induce the production of PGE_2 (Blatteis, 2007).

The VMPO forms two distinct efferent pathways to nearby areas involved in homeostasis. The inhibitory pathway to the anterior perifornical region (APFx) is the larger of the two. This area forms an inhibitory connection with the pariventricular nucleus (PVN) as well as containing a population of warm sensitive neurons. When PGE_2 is administered to the VMPO the inhibitory effect on the APFx increases, which in turn decreases the inhibition on the PVN. The PVN controls some autonomic behaviors associated with heat production; the removal of inhibition to this area could allow these behaviors to occur and generate a fever. The VMPO also has a smaller pathway that connects to the PVN. It is believed that the warm sensitive neurons of the VMPO make an inhibitory connection to the PVN. These warm sensitive cells have been found to decrease their firing rate in the presence of PGE_2, which would remove the inhibitory

input to the PVN and once again allow the generation of a fever (Ranels and Griffin, 2003)

5. Prostaglandin E2 Receptor Subtypes

PGE_2 has four receptor subtypes, EP1-4. EP1 receptors, found in smooth muscle tracts and also believed to play some role in fever generation, are coupled to a calcium-based second messenger system that is coupled with protein kinase C. The EP2 receptors are modulated through a G-protein coupled receptor that seems to be modulated by concentrations of intracellular cAMP and the actions of protein kinase A. The EP4 receptor has a similar configuration as the EP2, with an excitatory G-protein modulated by cAMP, but exhibits some differences in relation to ligand binding. EP3 receptors have three different forms caused by alternative splicing. These isoforms can bind to either of two G-proteins, Gi and Gs. Gi causes an inhibition of adenylate cyclase, while Gs stimulates it. Thus depending on which isoform is expressed in the tissue, activation of the EP3 receptor can cause either an increase or decrease in intracellular cAMP concentrations (Negishi, 1995).

All four EP receptor subtypes are found to be expressed across the febrogenic area of the hypothalamus. However, when treated with LPS to induce Fos-immunoreactive responses, only EP1, 3 and 4 receptors responded. The c-fos gene is expressed in cells that have been activated and are likely to become expressed. Almost 80% of these neurons had activated EP4 mRNA. The activation of receptor mRNA during the treatment to LPS suggests the receptor's involvement in the fever-generating mechanism. This suggests that EP4 plays a significant role in the generation of a fever in response to infection. Although EP3 receptors were found to be widely distributed across the POAH,

only 10% of the Fos-immunoreactive neurons were found to have EP3 mRNA. However, the c-fos gene is activated by high levels of intracellular calcium or cAMP, so it is possible that many of the EP3 receptors in the POAH display the isoform that causes a decrease in cAMP production (Oka, 2000).

Further studies have been done to try to specify the role that each EP receptor plays in the febrile response. A study by Oka et. al. (2003) treated rats with a new generation of EP receptor agonists. This study found that EP1 and EP3 receptor agonists caused a significant increase in temperature, while EP4 agonists caused a significant decrease. However while these drugs have a binding receptor preference, there still is some cross-binding effects, particularly between the EP1 and EP3 agonists. It also does not allow us to differentiate between the EP3 subtypes involved in the generation of the febrile response.

Knockout studies have also been used to ascertain which EP receptors are critical to fever generation. In a study by Ushikubi et. al. (1998) mice were bred without one of the four EP receptors. When administered PGE_2, the mice without EP1, 2 and 4 receptors exhibited febrile responses, while those without EP3 responded the same as when treated with the vehicle. These mice also showed no response to interleukin (a pyrogen) that induced fever in the EP1 and 2 knockouts (the EP4 did not survive long enough to be used in subsequent tests). Finally, when injected with LPS in order to model a fever in response to infection, the EP3 knockouts were the only subjects to show no significant response. This confirms that EP3 receptors play some important role in the generation of a fever.

Looking at these studies, it is difficult to determine a full picture of the role of EP receptors in the febrile response. Fos-immunoreactive studies have implicated EP4 as being most important to fever generation. Agonist and knockout studies have pointed more towards EP3. However, each of these has their problems. Fos-immunoreactive studies leave out the EP3 receptors modulated by a decrease in intracellular cAMP. The agonists all have some cross-binding effects, and also cannot tell us much about which isoforms are being stimulated. Knockout mice by definition do not have the same integrative network that is seen in the wild type. These limitations further complicate the process of identifying the EP receptors and mechanisms involved in fever generation.

6. cAMP and Prostaglandin E2

While experimental evidence has shown an important relationship between the POAH, PGE2 and cAMP, the mechanistic details of this relationship are still unknown. The relationship between PGE_2 and cAMP has been studied in the context of other physiological processes, particularly the perception of pain.

PGE_2 acts as a pro-inflammatory prostaglandin and sensitizes neurons to inflammatory agents such as capsaicin. This sensitization occurs through an increase in the amount of intracellular cAMP, which in turn activates protein kinase A (PKA). The activation of PKA increases the sensitivity of the capsaicin receptor and increases the whole-cell current, which could possibly lead to longer or larger depolarization, increasing the neuron's excitability (Lopshire and Nicol, 1998). In another study, the increase in cAMP increased sensitization through the outward potassium current. The sensitizing actions of PGE_2 were completely blocked when co-administered with a drug

that inactivates PKA (Evans et. al., 1999). In addition, a study by Southall and Vasko (2001) found that the receptors involved in the sensitization of these sensory neurons were both the EP3C and EP4 receptor subtypes. These studies show that the modulation of cAMP levels within a cell expressing the PGE_2 receptor subtypes can have a significant effect on the electrophysiological properties of the neuron. It is easy to relate these results to the idea that cAMP levels could have an effect on the firing rate of thermosensitive neurons and thus the thermoregulatory system as a whole. The study by Southall and Vasko also shows that more than one receptor subtype can be involved in such a mechanism. This could explain the disagreement over the involvement of EP3 versus EP4 receptors in fever generation.

In the last few years studies have been undertaken to determine the exact role that cAMP plays in the process of fever generation. One study looked at the change in cAMP levels in the POAH upon the generation of a fever cause by PGE_2 or LPS injection directly into the hypothalamus of whole animals. They also studied the effect that cAMP agonists and antagonists had on the genesis of PGE_2 fever. A significant decrease in cAMP levels was found in a PGE_2 induced fever; however this decrease was seen across the entire POAH area, rather than in individual cells where the intracellular changes that affect firing rate are taking place. In addition, no significant change was seen in an LPS fever, or upon the administration of cAMP antagonists. The study then suggested that there may be a synergistic relationship between cAMP and another molecule in order to produce a fever (Steiner et. al, 2002).

One of the molecules suggested to work in concert with cAMP is cyclic guanosine monophosphate (cGMP). A recent study by Wright et. al. (2008) investigated the effect

of cGMP on the thermosensitivity and firing rate of POAH neurons, as well as looking at the distribution in the hypothalamus of cGMP, channels dependent on cGMP for activation and molecules important to cGMP synthesis. cGMP, channels requiring cGMP for activation and associated molecules were found to be present in neurons in the POAH. The study also found that a majority of both temperature insensitive and warm sensitive neurons exhibited a decrease in firing rate when treated with a cGMP agonist. The data also suggested that cGMP increases the thermosensitivity of warm sensitive neurons, both inside and outside the POAH. However, changes in firing rate or thermosensitivity in the presence of cGMP was not correlated with the presence of cGMP synthesizing molecules or channels requiring the presence of cGMP for activation (Wright et. al, 2008). While cGMP may play a significant role in fever genesis, it is not likely to be the only molecule central to the process. Despite this research, there has been very little work done on the effect of cAMP on single neurons within the VMPO. As cAMP plays a crucial role in the genesis of PGE_2 mediated fever, its effect at a neuronal level can help enlighten us as to the physiological mechanism of PGE_2 fever generation.

This study characterizes the changes in firing rate of thermosensitive neurons in the POAH with respect to temperature and sp-cAMP, a cAMP agonist, using *in vitro* tissue slice preparations and single-unit extracellular recording techniques. Our specific aim is to study the response of thermosensitive neurons in the POAH of the hypothalamus to treatment with sp-cAMP, a cAMP agonist. We hypothesize that in the presence of an increased level of intracellular cAMP due to the use of the agonist sp-cAMP, both warm sensitive and temperature insensitive neurons will demonstrate an increase in firing rate.

Methods

I. Preparation

Single-unit extracellular recordings were made from hypothalamic neurons in rat brain tissue slices. To prepare these slices, male Sprague Dawley rats (weighing 100-150 g) were used. Rats were housed under standard conditions (23°C, 12h:12h light:dark cycle, lights on at 8am). Before each recording, a rat was anesthetized using isofluorene and then sacrificed by decapitated, according to the procedures approved by the Animal Care and Use Committee of the College of William and Mary. Brain tissue was then isolated and prepared into a tissue block containing the hypothalamus. Tissue blocks were then sectioned into two or three 400μm coronal or sagittal slices containing the POAH.

Slices were placed in a recording chamber that was constantly perfused with pyrogen-free artificial cerebrospinal fluid (aCSF) containing (mM): 124 NaCl, 26$NaHCO_3$, 10 glucose, 5 KCl, 2.4$CaCl_2$, 1. 3$MgSO_4$ and 1.24KH_2PO_4. This was oxygenated with 95% O_2-5% CO_2 and was allowed to flow at about 1 ml min^{-1} (Kelso et. al 1983). A thermoelectrode was used to heat the aCSF to approximately 36°C, and a smaller thermocouple was placed below the tissue slices in the chamber in order to monitor the temperature constantly.

II. Data Collection and Analysis

Extracellular recordings were made using glass sharp tipped electrodes with a tip diameter of less than 1μm and filled with a 3M NaCl solution. Recordings were made with an Xcell-3 Microelectrode Amplifier (FHC Inc.). A temperature swing of 2-3°C

above the baseline 36°C was made once a cell had been isolated, determined to have a signal to noise ratio of at least 3:1, and had been firing stably for several minutes. To determine the neuronal sensitivity (impulses $s^{-1}{\circ}C^{-1}$), firing rate was plotted as a function of temperature. The slope of the regression line was the thermosensitivity coefficient (m). If the cell had a coefficient of at least 0.8 impulses $s^{-1}{\circ}C^{-1}$ it was determined to be warm sensitive, while any others were classified as temperature insensitive (Boulant 1986). All recordings were made from POAH neurons, an area which can be visualized in coronal and sagittal slices.

Once the neuron's thermosensitivity was classified, the neuron's response to sp-cAMP (sp-Adenosine 3',5'-cyclic monophosphate triethylamine, Sigma Chemical Co) was tested. After temperature swing, a baseline temperature of about 36°C was established for at least one minute in order to determine the baseline firing rate. Following acquisition of this baseline, the cell was switched from a perfusing medium of aCSF to a 1μM sp-cAMP medium. Treatment period lasted for 10 minutes. Following treatment, a wash period of at least twenty minutes was observed to determine if the cell returned to its baseline firing rate. Location of the electrode was then determined stereoscopically and recorded. After recording, one minute firing rate samples from the recording were digitized (60 Hz) using Clampfit Software (Axon Instruments) for comparison. One sample was taken from the baseline before the perfusion of sp-cAMP, a second towards the end of the treatment period, and a third from the end of the wash period after treatment. A mean and standard error was calculated for each of these sample periods, and then the response to sp-cAMP was measured (Sigmaplot Software) versus the baseline data using a standard T-test ($P \leq .05$).

Results

Recordings were made from 25 neurons within the POAH. During these recordings, their thermosensitivity and firing rate response to sp-cAMP were established. Of the recorded neurons 24 (96.2%) were found to be insensitive to temperature changes, and 1 (3.8%) was found to be warm sensitive. No pattern of location of the warm or temperature insensitive neurons was established. Table II.1 summarizes the thermosensitivity of the neurons recorded. Figure II.1 shows the location of the recorded neurons in the POAH.

Table II.1: Thermosensitivity of POAH Neurons

			Thermosensitivity (impulses·s^{-1}·$^oC^{-1}$)	
Cell Type	***N***	**Criteria**	**Mean +/- S.E.**	**Range**
Insensitive	24	<0.8	-.011 +/- .21	-0.72-0.65
Warm	1	≥0.8	1.26	N/A

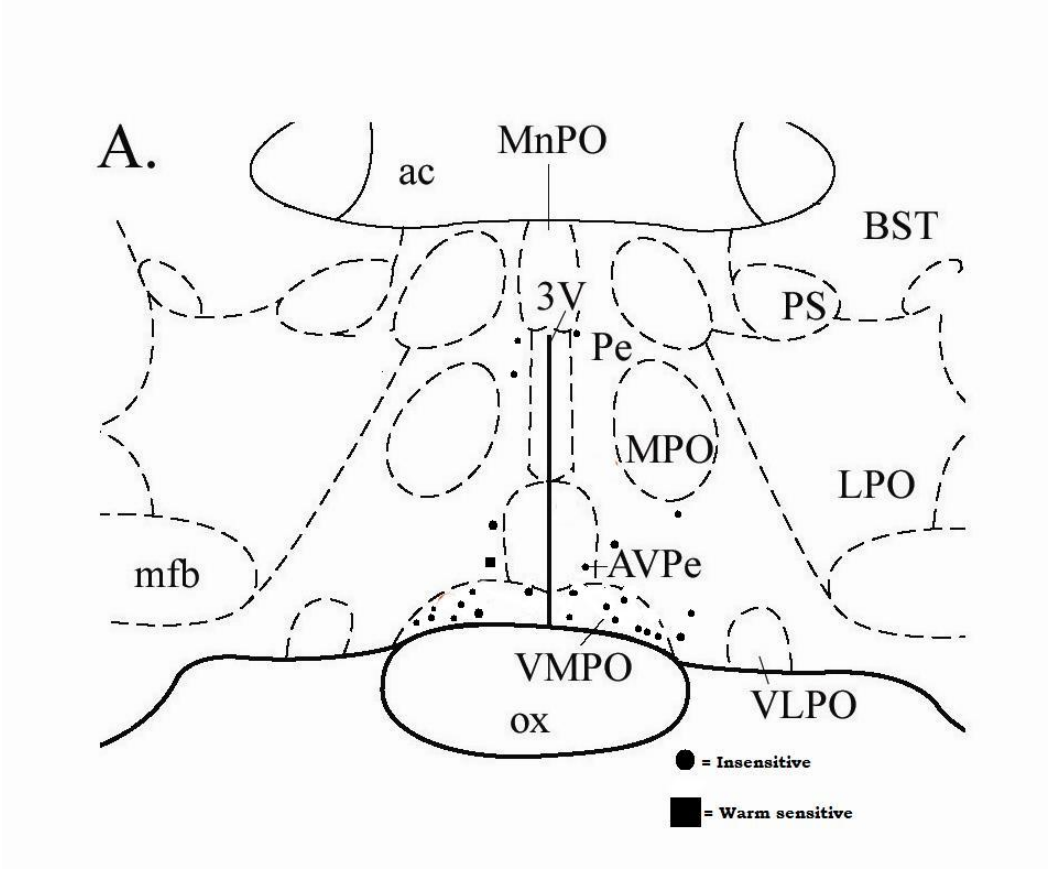


Figure II.1 Neuronal Locations of recorded POAH neurons

This coronal diagram shows the location of each recorded neuron within the POAH.

Insensitive neuron = ●, Warm sensitive neuron = ■

The temperature insensitive neurons overall showed a lower baseline firing rate (average 1.26 ± .037 imp/s) than the warm sensitive neuron (6.29±.111 imp/s). Both the temperature insensitive and warm sensitive neuron showed an overall decrease in their firing rate upon treatment with sp-cAMP; however this decrease was not found to be significant. Significant responses were seen only in individual temperature insensitive neurons. Table II.2 summarizes the firing rate responses of the recorded POAH neurons to treatment.

Table II.2: Effect of sp-cAMP on the Firing Rate Activity of POAH Neurons

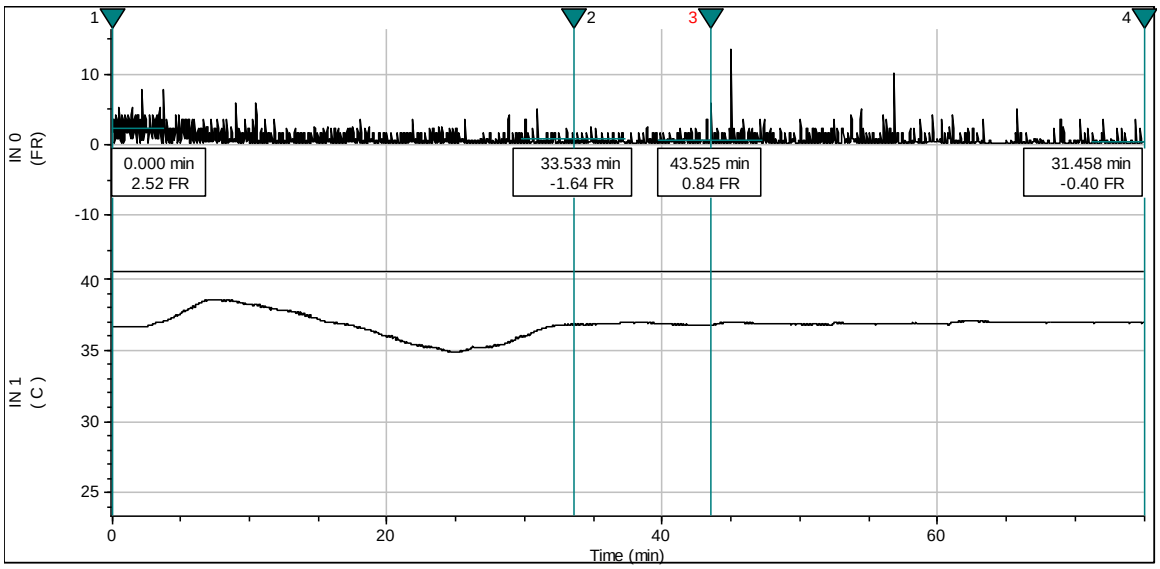
Thermosensitivity	*N*	Increase	Decrease	No Response
Insensitive	24	5	7	12
Warm	1	0	0	1

Of the 25 neurons which were recorded, 12 (48%) showed responses to treatment with sp-cAMP. The remaining neurons showed no significant response to treatment. Since all cAMP is a second messenger used in many cell processes other than thermoregulation, it would be difficult to believe that a neuron would not respond to treatment with an agonist. This lack of response is most likely due to the drug not fully penetrating the tissue or being washed out than the neuron's inability to respond to cAMP.

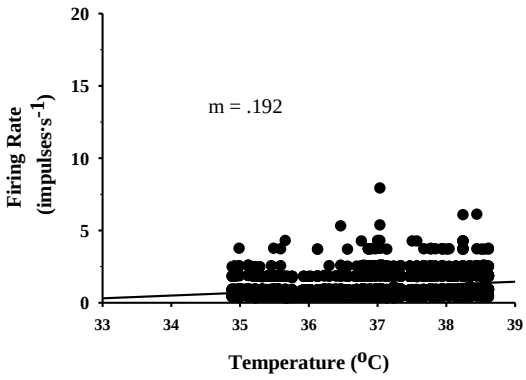
Out of the 24 temperature insensitive neurons, 12 (50%) responded to sp-cAMP. 7 of these neurons (a majority) showed a decrease in firing rate in response to treatment, while 5 others showed an increase in firing rate. The remaining 12 neurons (50%) showed no significant change in firing rate in response to sp-cAMP. The neuron in

Figure II.3 had a baseline firing rate of .64 imp/s and decreased to .47 imp/s during treatment. This neuron recovered during washout, increasing its firing rate to .80 imp/s. The neuron in Figure II.4 had a baseline firing rate of 1.50 imp/s, increased during treatment to 1.89 imp/s, and decreased during washout to .91 imp/s.

A.



B.



C.

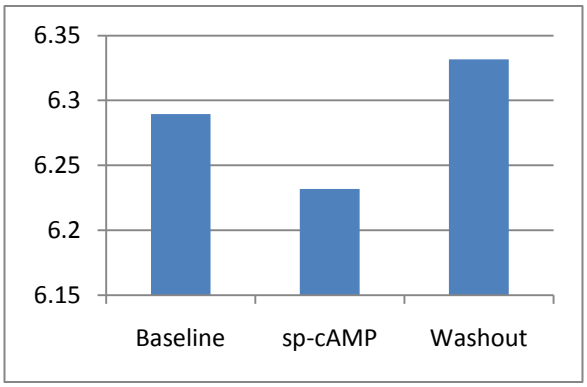
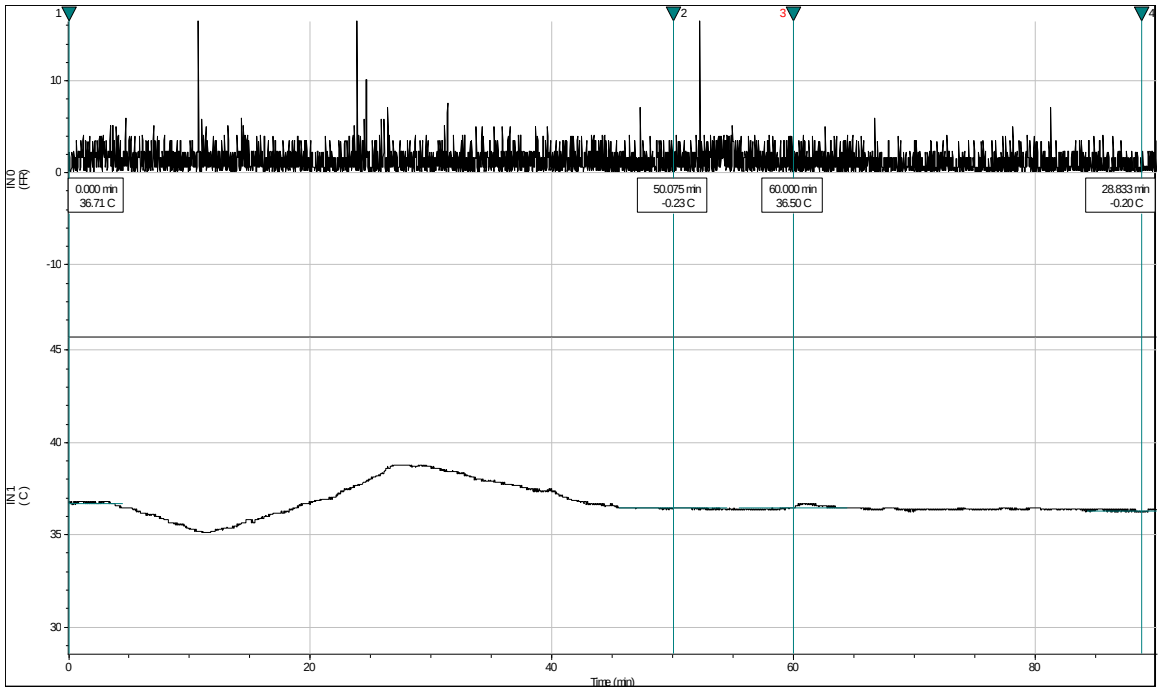


Figure II.3 The effects of temperature and sp-cAMP on the firing rate of a POAH temperature insensitive neuron

A shows the firing rate of this neuron during changes in temperature and during treatment with sp-cAMP (treatment time was between cursors 2 and 3). In **B** firing rate is plotted as a function of temperature. In **C** one minute samples of firing rate activity are plotted as a bar graph. These samples come from the minute just before perfusion, from the peak of the response, and at the end of the control washout period.

A



B. **C.**

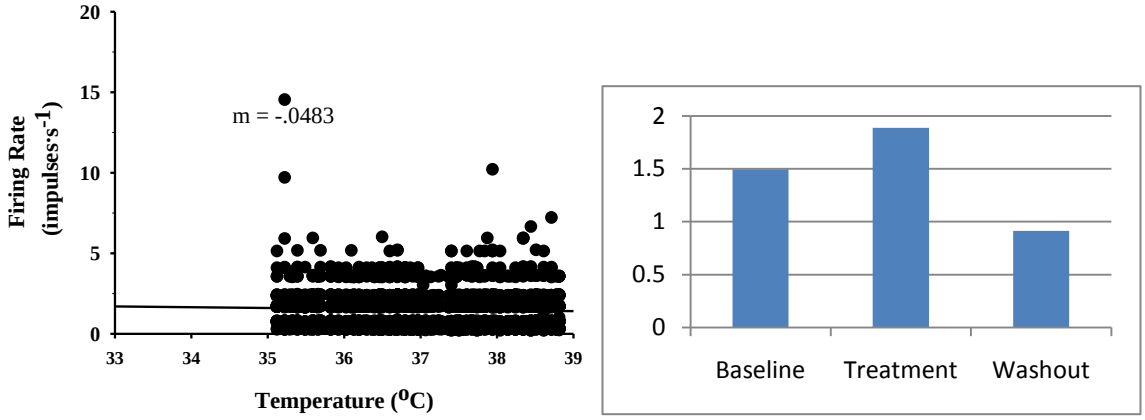
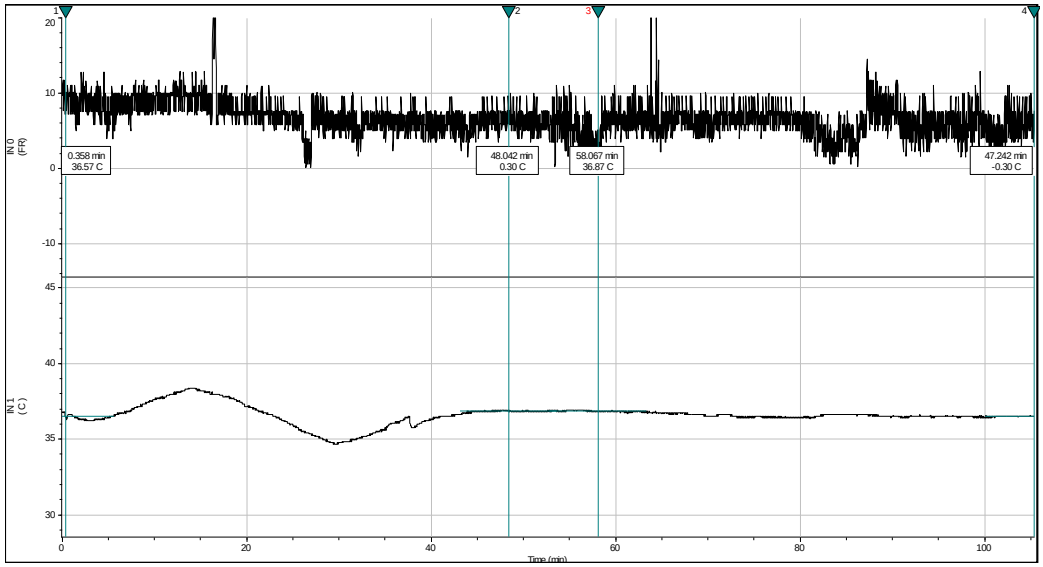


Figure II.4 The effects of temperature and sp-cAMP on the firing rate of a POAH temperature insensitive neuron

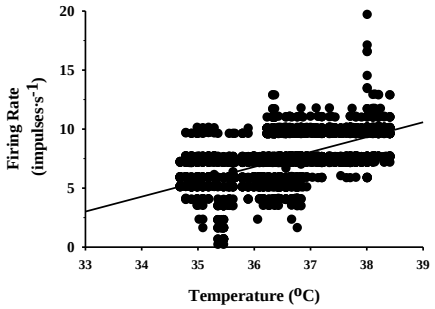
A shows the firing rate of this neuron during changes in temperature and during treatment with sp-cAMP (treatment time was between cursors 2 and 3). In **B** firing rate is plotted as a function of temperature. In **C** one minute samples of firing rate activity are plotted as a bar graph. These samples come from the minute just before perfusion, from the peak of the response, and at the end of the control washout period

Only one warm sensitive neuron was recorded. This neuron (Figure II.5) showed no significant change in firing rate in response to sp-cAMP treatment. This neuron had a baseline firing rate of 6.29±.111 imp/s which decreased insignificantly to 6.23±.110 imp/s upon treatment, and recovered to 6.33±.139 imp/s during the washout period.

A.



B.



C.

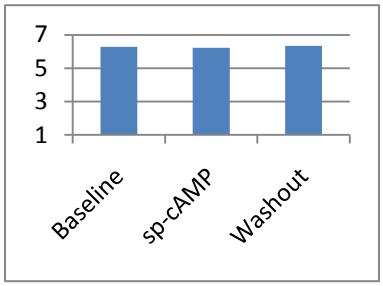


Figure II.4 The effects of temperature and sp-cAMP on the firing rate of a POAH warm sensitive neuron

A shows the firing rate of this neuron during changes in temperature and during treatment with sp-cAMP (treatment time was between cursors 2 and 3). In **B** firing rate is plotted as a function of temperature. In **C** one minute samples of firing rate activity are plotted as a bar graph. These samples come from the minute just before perfusion, from the peak of the response, and at the end of the control washout period.

Discussion

cAMP and Fever

Approximately half of the neurons recorded in this study showed a significant response to treatment with sp-cAMP. The majority of responding temperature insensitive neurons decreased their firing rate, with a large minority increasing their firing rate as had been predicted in our hypothesis. The responses of the POAH neurons to increased levels of cAMP in this study are similar to the ones seen in a previous study performed by Elizabeth Thompson in 2003. In this previous project 34 insensitive neurons were recorded, and 24 (70.6%) showed a response to sp-cAMP. 13 (54.2%) of these cells significantly increased their firing rate in response to the increase in cAMP. The other 11 (45.8%) responding neurons decreased their firing rates when treated. 10 warm sensitive neurons were recorded, of which 8 (80%) responded to sp-cAMP. 5 (62.5%) increased their firing in response to treatment, and 3 (37.5%) exhibited a decreased firing rate. When the two studies are looked at together, the results show that half of temperature insensitive neurons increase their firing rate when treated with a cAMP agonist, while the other half decreases their firing rate. The responsiveness of these neurons implies that cAMP has a significant role in the responsiveness of thermosensitive neurons in the POAH, and thus to the thermoregulatory network at large.

cAMP responsiveness has an important link to the mechanism by which PGE_2 stimulates a change in firing rate in neurons. Previous studies have shown that PGE_2 decreases the firing rate of warm sensitive neurons by decreasing the rate of rise of the prepotential and thereby decreasing the interspike interval (Ranels and Griffin, 2003). This prepotential has been linked to the outward potassium A current; inhibition of the

current at higher temperatures has been found to cause an increase in the depolarization of the prepotentials in warm sensitive neurons. PGE_2 may be causing the decrease in firing rate by decreasing inhibition of the potassium A current and thus decreasing prepotential polarization (Boulant, 1998).

Drugs that increase the production of cAMP increase the thermosensitivity of warm sensitive neurons, in addition to increasing the depolarization of the prepotential and increasing firing rate (Boulant, 1998). From this we can easily imagine a mechanism by which cAMP could cause the decreases in firing rate that have been seen during treatment with PGE_2. If levels of cAMP were to be decreased, this could conceivably decrease the inhibition of the potassium A current. This would in turn lead to the decrease in the depolarization of the prepotentials and the lowered firing rates seen in warm sensitive neurons treated with PGE_2.

The warm sensitive neuron recorded in this study was not found to have a significant response to sp-cAMP. However in the earlier study by Thompson, the majority of warm sensitive neurons showed an increase in firing rate in response to the increased levels of cAMP. This is the opposite of what we see in warm-sensitive cells treated with PGE_2, where there is ostensibly a decrease in cAMP leading to their decreased firing rate (Ranels and Griffin, 2003).

PGE_2 has the opposite effect on temperature insensitive neurons, significantly increasing their firing rate (Ranels and Griffin, 2003). This was seen in a large minority of the insensitive cells recorded in this study. This increase cannot necessarily be attributed to changes in the potassium A current, as studies have found that the net conductance does not change for temperature insensitive neurons. However, the actions

of the sodium-potassium pump do seem to have an effect on the firing rate and thermosensitivity of temperature insensitive neurons. The increase in intracellular cAMP may have an inhibiting effect on the Na-K pump, which has been known to increase firing rate (Boulant, 1998; Curras and Boulant, 1989).

The majority of the temperature insensitive neurons that showed a response to sp-cAMP decreased their firing rate, the opposite of what was expected. This suggests that there may be a diversity of EP receptors being expressed in the neurons of the VMPO. Previous studies have found that both EP4 and EP3 receptor subtypes play some sort of role in the process of febrogenesis. However, it has been difficult to tease out exactly what role each plays. The EP3 receptor alone has three isoforms that can either increase or decrease intracellular cAMP levels. Temperature insensitive neurons may not universally express the same PGE_2 receptor subtype, or even the same receptor isoform.

The expression of more than one receptor subtype would lend complexity and the possibility of a more fine-tuned response to PGE_2. Not every fever is the same- they can be caused by any number of different pyrogens, last from hours to days, and be extremely mild or dangerously high. Having neurons express different receptor subtypes allows them to cause different types of fever based on factors such as what neuronal group is being stimulated, how much PGE2 is present in the system, and which subtypes are being activated. This fits much better with the actual presentation of fever that we see *in vivo*. Rather than trying to determine which one subtype is being expressed within each kind of neuron, it may be more beneficial to look into how several subtypes interact to create different kinds of fevers.

To this end, one interesting line of future research is into the afferent pathways that the VMPO makes with the anterior perifornical region (APFx) and the pariventricular nucleus (PVN). Previous studies have indicated that temperature insensitive neurons form an inhibitory pathway to the APFx (which in turn connects to the PVN) and that warm sensitive neurons have a more direct inhibitory pathway to the PVN. Fever is generated when increased insensitive firing rates increase inhibition on the APFx and decrease inhibition on the PVN. However this study has demonstrated that not all temperature insensitive neurons respond to cAMP (and thus PGE_2) with an increase in firing rate. The question is then which pathway the temperature insensitive neurons that decrease their firing rate have a connection to. It would be interesting to use recently developed techniques to determine whether these neurons have connections to the APFx like the other temperature insensitive cells, or if they are part of the smaller inhibitory pathway directly to the PVN.

cAMP and Other Thermoregulatory Processes

Fever is not the only thermoregulatory response that the body experiences. As cAMP appears to be an important part of febrogenesis, so it can be imagined to be important in other thermoregulatory processes. Two of the most studied processes are heat stroke and hot flashes.

Heat stroke not only involves a dramatic change in thermoregulation, but also involves the process of osmoregulation which is also controlled by the hypothalamus. When an animal becomes dehydrated, particularly if it is in a warmer than usual environment, it may experience a rapid rise in body temperature. Thermosensitive neurons of the medial preoptic nucleus (MPO) in the hypothalamus have been found to

respond to changes in osmotic state, that is changes in the ratio of fluid to dissolved substances in the fluid (Nakashima et al, 1985). When these neurons were exposed to a hyperosmotic environment (as would be found in a dehydrated animal) warm sensitive neurons underwent a decrease in firing rate, while temperature insensitive neurons increased their firing rate.

This is the same response that we see when POAH neurons are exposed to PGE_2 and body temperature raises. The change of firing rate leading to a rise in body temperature is a fairly basic mechanism, and so we can imagine that many of the same processes seen in fever would be seen in heat stroke. As we have seen that cAMP is important in the generation of a fever, it may be that the same processes are important to the mechanisms underlying heat stroke.

Hot flashes are transitory rises in body temperature that are most often seen in women undergoing menopause, although men who have had their prostates removed or other forms of medical castration have also been reported to experience them. One of the peptides believed to play role is calcitonin gene-related peptide (CGRP), which has been found to increase during hot flashes (Wyon et. al, 2000). Warm sensitive neurons have been found to decrease firing rate during treatment with CGRP, while temperature insensitive neurons increase their firing rate (Braasch et al, 2008). As this is once again the same effect that we see with PGE2 mediated temperature increases, it is possible that cAMP is a modulating molecule for this process.

There are several future directions for this project. One would be to do intracellular recordings of single neurons to confirm the significant effects of cAMP on firing rate of thermosensitive neurons. Another would be to determine exactly which

EP3 receptor subtypes are involved in febrogenesis using recently developed antagonists. The final project would be to use recently developed techniques to trace the pathways connecting the APFx and PVN and the VMPO and figure out exactly which type of thermosensitive neurons connect to these areas and use EP receptor antagonists to determine which receptor types they express. These experiments would allow us to fill in some of the gaps in the process of fever generation.

Conclusion

The hypothalamus is necessary for many homeostatic processes, not the least of which is the regulation of body temperature. Thermoregulation works on an extremely fine scale; a small change in one bodily process can cause a large thermoregulatory reaction. Any large scale change in temperature affects not only the brain but almost every other system in the body.

The brain exists walks a fine line in terms of temperature. A change of more than one degree in the brain leads to extensive damage and death. Thus, the ability to finely and quickly regulate body temperature is vital for the survival of any animal. Animals must be able to rapidly sense changes in local and environmental temperature in order to ensure the body is able to respond before it becomes damaged beyond repair. The ability to integrate local and environmental temperature information has been found to be located within the preoptic area of the hypothalamus in mammals. Change in body temperature is linked intimately with changes in firing rate of the thermosensitive neuron populations found within the POAH. In this study, it has been suggested that levels of intracellular cAMP plays an important role in the mechanism by which firing rates

change in response to substances signaling the presence of an infection, leading to the generation of a fever. Thus, cAMP plays a role not only in changing the firing rate of a single neuron, but also in maintaining the delicate balance of the body's core temperature and ensuring survival.

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