2005

The Effects of Calcitonin Gene-Related Peptide on the Neurons of the Preoptic Anterior Hypothalamus: A Mechanism of a Hot Flash

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THE EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE ON THE NEURONS OF THE PREOPTIC ANTERIOR HYPOTHALAMUS

A Mechanism of a Hot Flash

A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Science

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by

Daniel Cameron Braasch

2005

APPROVAL SHEET

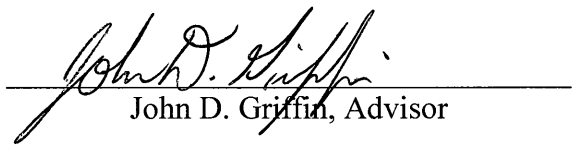
This thesis is submitted in partial fulfillment of

the requirements for the degree of

Master of Science

Daniel Cameron Braasch

Approved by the Committee, June 2005



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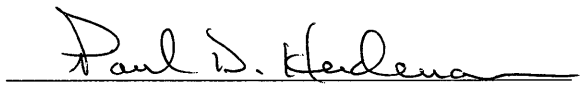

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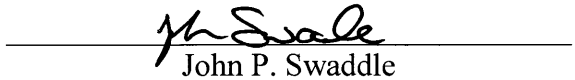

John P. Swaddle

DEDICATION

There are several people that have supported and helped me throughout my life. I would like to thank all of those who continuously challenged and encouraged me during my academic and personal life. I would especially like to thank my family and close friends for their assistance and encouragement. I dedicate this work to my parents, Harold and Myra Braasch, to whom I am forever indebted for the support and love that they have given me during my entire life.

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ACKNOWLEDGEMENTS

I would like to thank Dr. John D. Griffin for all his guidance and support throughout this work and my time at the College of William and Mary. Thanks to my thesis committee members, Paul D. Heideman and John P. Swaddle, for their help with my project and this thesis. Finally, I would also like to thank all those who were a part of Dr. Griffin's Lab for their help during my project.

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LIST OF ABBREVIATIONS

AP:	Action Potential
AHP:	After Hyperpolarizing Potential
aCSF:	Artificial Cerebral Spinal Fluid
CGRP:	Calcitonin Gene-Related Peptide
DMH:	Dorsal Medial Hypothalamus
EPSP:	Excitatory Post-Synaptic Potential
IPSP:	Inhibitory Post-Synaptic Potential
PO/AH:	Preoptic Anterior Hypothalamus
PGE_2:	Prostaglandin E_2
S.E.:	Standard Error
VMH:	Ventral Medial Hypothalamus
VMPO:	Ventral Medial Preoptic

ABSTRACT

The mammalian body maintains a steady internal temperature of approximately 37°C. A specific region of the brain, the preoptic and anterior hypothalamus (PO/AH), was found to be vitally important to thermoregulation. Isolated recordings from neurons in the PO/AH classified 60% of the neurons as temperature insensitive (≤ 0.79 impulses$\bullet s^{-1}\bullet °C^{-1}$ and ≥ -0.6 impulses$\bullet s^{-1}\bullet °C^{-1}$), 35% as warm sensitive neurons (≥ 0.8 impulses$\bullet s^{-1}\bullet °C^{-1}$) and the remaining 5% of neurons are cold sensitive neurons (≤ -0.6 impulses$\bullet s^{-1}\bullet °C^{-1}$).

For women following the onset of menopause and men during treatment of prostate cancer, a "hot flash" can become a frequent occurrence. This transient hyperthermic shift in temperature has been linked to the hormone, calcitonin gene-related peptide (CGRP), which acts peripherally to increase vasodilatation and centrally to increase sympathetic activation, including metabolic heat production.

The present study investigated the effects of calcitonin gene-related peptide on the firing rate of temperature insensitive and temperature sensitive neurons in preoptic anterior hypothalamus (PO/AH). It was hypothesized that CGRP will increase the firing rate of temperature insensitive neurons and decrease the firing rate of warm sensitive neurons. In reference to an enduring model of temperature regulation, the CGRP dependent changes in firing rate will result in a hyperthermic shift in the hypothalamic set point temperature.

Using a tissue slice preparation, I recorded the extracellular and intracellular activity of PO/AH neurons from the adult male rat, in response to temperature and CGRP (10 μM). In both recording methods, the majority of warm sensitive neurons responded to the micro-drop application of CGRP with a significant decrease in firing rate. While CGRP did not affect all temperature insensitive neurons, the majority of responsive neurons showed an increase in firing rate. Furthermore, intracellular recordings revealed that a decrease in the pre-potential rate of rise occurred in responsive warm sensitive neurons while responsive temperature insensitive neurons showed an increase in the threshold potential for an action potential. The results of both studies suggest that warm sensitive and temperature insensitive neurons in the PO/AH may play critical and contrasting roles in producing these transient hyperthermic shifts in body temperature.

THE EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE ON THE NEURONS OF THE PREOPTIC ANTERIOR HYPOTHALAMUS: A MECHANISM OF A HOT FLASH

CHAPTER I

INTRODUCTION

The mammalian body maintains a steady internal temperature of approximately 37°C. Regulation of temperature is vital to enabling proper bodily reactions and functions. Without an efficient regulation mechanism, organs and systems will malfunction, possibly leading to lethal effects. Therefore, body temperature must be maintained despite changing internal and external environmental conditions.

There are several mechanisms that are employed to maintain a stable temperature (Boulant, 1981). When temperature deviates from the norm, mechanisms are triggered to return the body to a normothermic level. To warm the body in a cool environment shivering and nonshivering mechanisms are triggered to produce heat. Shivering is the rhythmic contractions of skeletal muscles, while nonshivering mechanisms include such actions as increases in metabolism and activation of sympathetic signals, to breakdown brown adipose tissue. Both of which result in heat production. The circulatory system and vasomotor activity are also involved in maintenance of body temperature by properly distributing heat throughout the body. In cold environments, vasoconstriction in the periphery restricts heat to the core of the body so vital metabolic processes can continue. Conversely, in warm environments, circulation can distribute heat to the surfaces of the body where it can be readily lost to the environment. Additionally thermoregulation

occurs through alterations in behavior. In cool settings an organism will "huddle" with other organisms and/or hold extremities close to the body to minimize heat loss. In warm settings an organism will seek cooler environments (i.e. shade) and/or spread out its extremities to maximize heat loss (Boulant, 1991).

1. Thermoregulation and the Hypothalamus

Studies throughout the mid 1900s investigated the origins of thermoregulatory responses reviewed by Boulant, 1991. Lesion and direct stimulation experiments demonstrated that when particular regions of the brain were inactivated, a decrease or complete loss of thermoregulatory responses resulted. A specific region of the brain, the preoptic and anterior hypothalamus (PO/AH), was found to be vitally important to the production of thermoregulatory responses when the temperature of the periphery or core (i.e. spinal cord) was changed. In addition, thermoregulatory responses were seen when the temperature of the hypothalamus alone was changed. As a result, neurons in the PO/AH demonstrated the ability to produce thermoregulatory responses when peripheral, core and hypothalamic temperature were independently or collectively changed. The ability of neurons in the PO/AH to integrate temperature changes from various regions and produce appropriate responses led to the PO/AH being labeled as the "central thermostat" for the body (Hammel, 1965, Boulant, 1991).

2. Neurons of the Hypothalamus

Isolated recordings from neurons in the PO/AH led to the characterization of two thermally classified groups of neurons: temperature insensitive and temperature sensitive

neurons. Approximately 60% of PO/AH neurons are temperature insensitive, while the remaining 40% show a clear response to changes in the PO/AH temperature. Neurons are classified as temperature insensitive if there is little change in firing rate (≤ 0.79 impulses$\bullet s^{-1}\bullet °C^{-1}$ and ≥ -0.6 impulses$\bullet sec^{-1}\bullet °C^{-1}$) when temperature of the PO/AH is changed 2-3°C above and below normal physiological temperature (approximately 37°C). In contrast, neurons are said to be temperature sensitive if they exhibit a change in firing rate when the PO/AH temperature deviates from normothermic temperature (Figure 1). Furthermore, temperature sensitive neurons can be divided into two categories: warm sensitive and cold sensitive neurons. Warm sensitive neurons comprise 35% of the overall PO/AH neuron population while cold sensitive neurons make up the remaining 5%. Warm sensitive neurons show an increase in firing rate when temperature is increased (≥ 0.8 impulses$\bullet s^{-1}\bullet °C^{-1}$). Conversely, cold sensitive neurons decrease firing rate when temperature is increased(≤ -0.6 impulses$\bullet sec^{-1}\bullet °C^{-1}$). Further evidence has also shown that cold sensitivity is not an inherent property, and that the cold sensitivity of PO/AH neurons occurs as a result of synaptic inputs from nearby warm sensitive and temperature insensitive neurons; a concept that will be explained later in the introduction (Curras and Boulant, 1989).

The criterion for classifying a neuron as warm sensitive has been a long debated topic. Originally, the criteria for warm sensitivity was a change in firing rate of at least 0.8 impulses$\bullet s^{-1}\bullet °C^{-1}$ in response to a change in local brain temperature. This original criterion is important because it distinguished PO/AH neurons that not only responded to changes in hypothalamic temperature but were also shown to respond to peripheral thermal stimulation (Boulant and Hardy, 1974). This criterion was further supported by

FIGURE 1.1

INTRACELLULAR RECORDING FROM PO/AH NEURONS

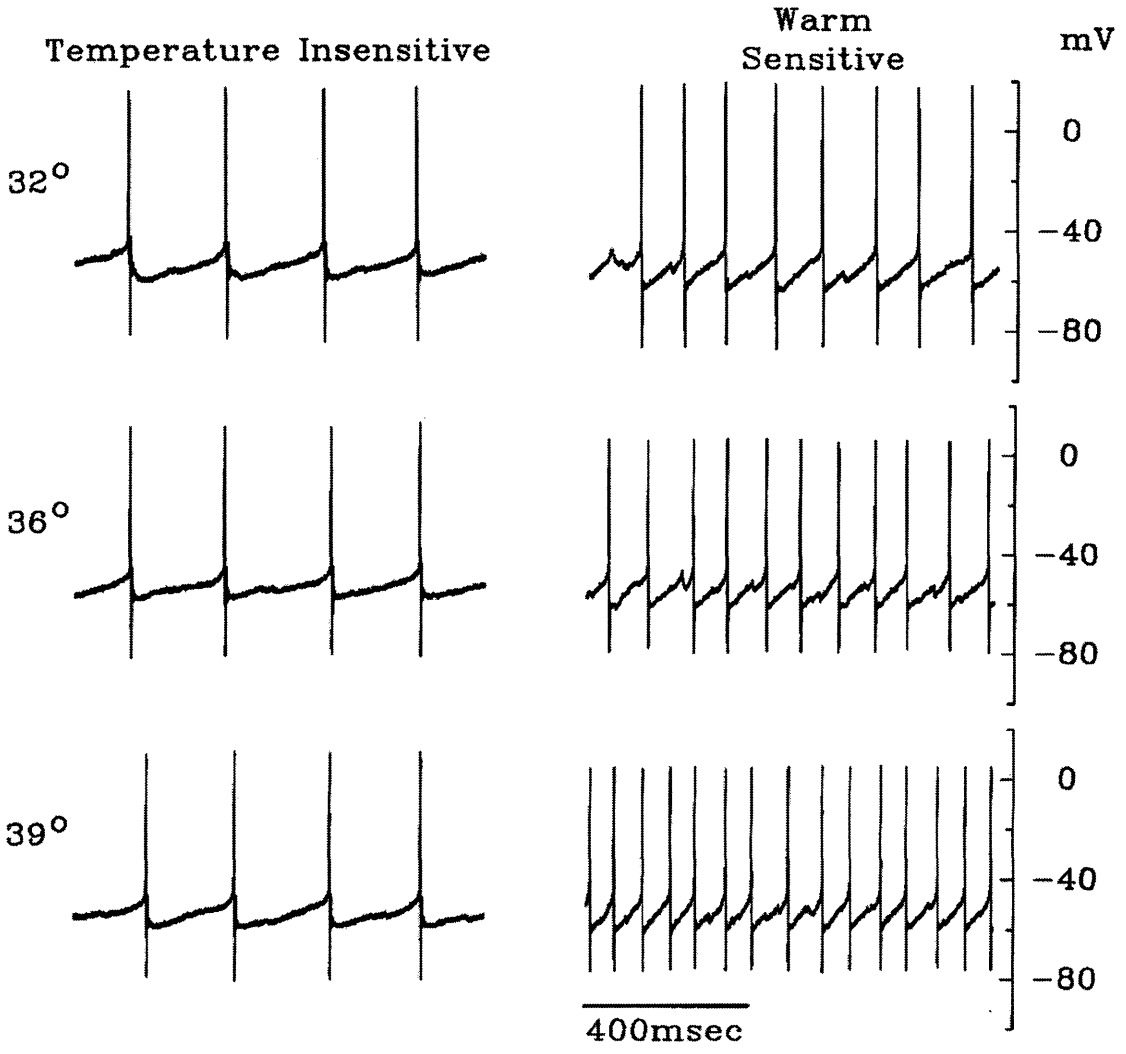

Figure 1.1. The figure shows the effect of temperature on the firing rates of a temperature insensitive (left) and warm sensitive (right) neuron.

recent work showing that warm sensitive neurons ($\geq$ 0.8 impulses$\bullet s^{-1}\bullet °C^{-1}$) have a distinct dendritic morphology with a lateral/medial orientation. In contrast, temperature insensitive neurons extend their dendrites in rostral and caudal directions (Griffin et. al., 2001).

3. Neuronal Thermosensitivity: Intracellular Mechanism

At the cellular level, warm sensitive and temperature insensitive neurons show a hyperpolarizing potential after each action potential followed by a pre-potential that leads to the next action potential. The difference between these neurons occurs when temperature is changed from normal physiological levels. At higher temperatures, temperature insensitive neurons maintain a constant inter-spike interval while warm sensitive neurons show a shorter pre-potential (Figure 2). In other words, the slope of the pre-potential is increased in warm sensitive neurons during heating, and as a result, the time interval between action potentials is decreased (Boulant, 1998). The pre-potential is at least in part, determined by an efflux of potassium ions called, the potassium A current (I_A current). At increased temperatures the I_A of warm sensitive neurons inactivates faster, allowing for a quicker depolarization to threshold, shorter pre-potential interval, and higher firing rate (Griffin et. al., 1996).

As temperature is increased there is also an increase in conductance through a TTX-sensitive Na^+ channel of both temperature insensitive and warm sensitive neurons. However it has recently been shown that the increase in the Na^+ conductance does not

FIGURE 1.2

INTRACELLULAR RECORDING FROM PO/AH NEURONS

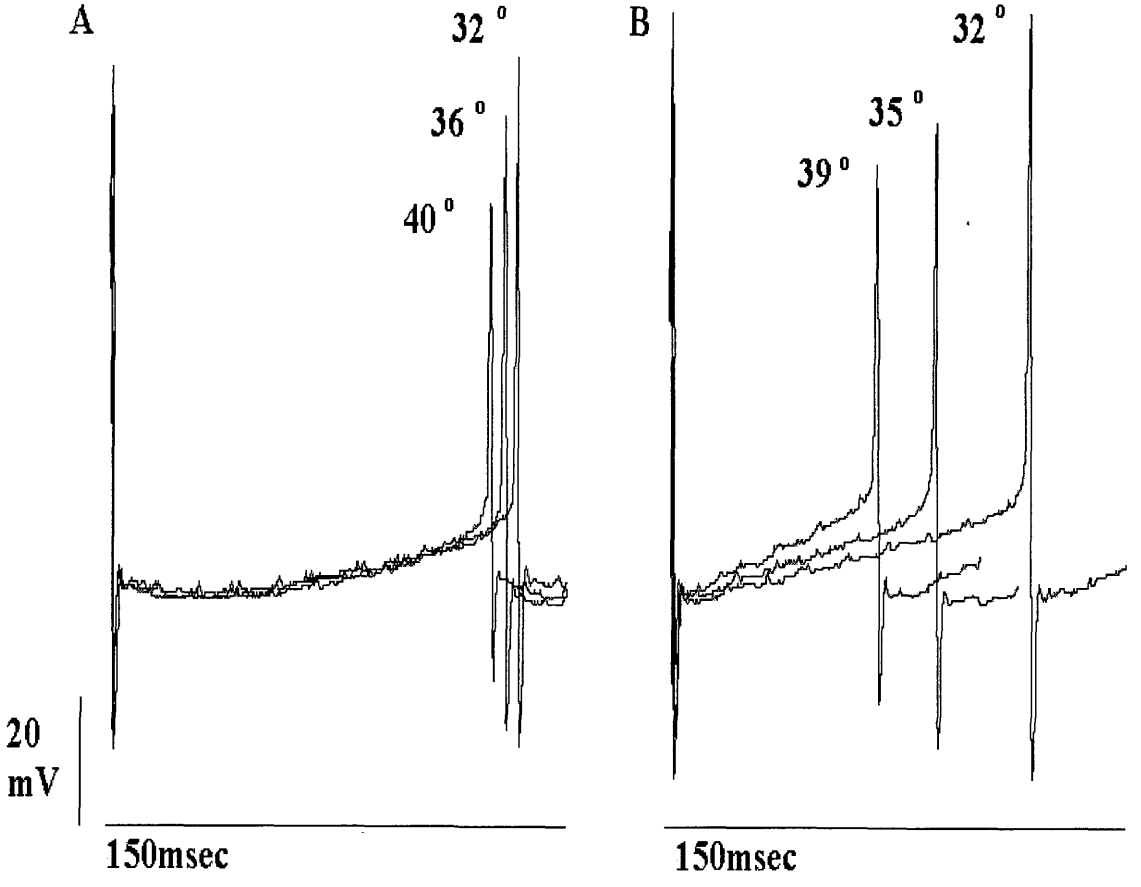

Figure 1.2. The figure shows the effect of temperature on action potentials from a temperature insensitive (left) and a warm sensitive (right) neuron.

cause a depolarization of warm sensitive neurons, which could lead to increased firing rate (Zhao and Boulant, 2005).

4. Set Point Temperature

As previously mentioned, under normal conditions, the body holds a constant internal temperature of approximately 37°C. When the core temperature is raised above this temperature, heat loss responses are triggered to reduce body temperature back to its optimal level. When the core temperature drops, heat production and retention occurs in order to raise body temperature. This "set point" response is determined by the integration of afferent signals and interactions of neurons within PO/AH. A model proposed by Hammel (1965) shows that interactions of warm sensitive neurons and temperature insensitive neurons in the PO/AH determine this set point. As shown in Figure 3, warm sensitive neurons positively synapse on warm effector neurons, while temperature insensitive neurons inhibit these same neurons. As the firing rate of warm sensitive neurons increases with an increase in core or peripheral temperature, the firing rate of the warm sensitive neurons equals the firing rate of the temperature insensitive neurons. At this temperature the excitation on effecter neurons from warm sensitive neurons equals the inhibition from temperature insensitive neurons. Above the set point temperature the warm effector neuron will receive a greater amount of stimulation resulting in a higher firing rate and a triggering of heat loss responses. The opposite situation occurs on cold effector neurons, where thermosensitive neurons inhibit cold effectors and temperature insensitive neurons stimulate cold effectors. When core or peripheral temperature drops below the set point temperature, there will be more

FIGURE 1.3

HAMMEL'S MODEL

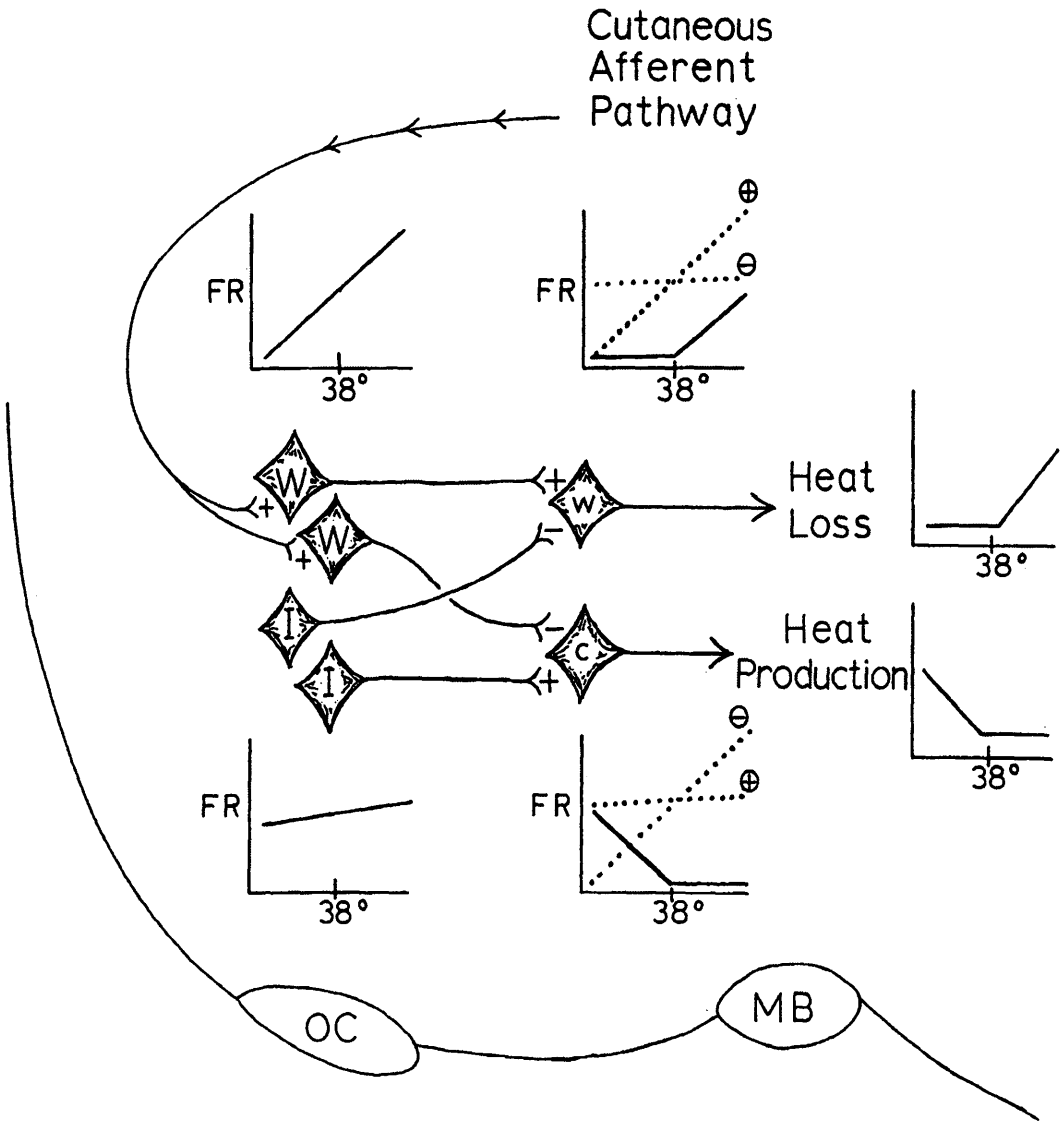

Figure 1.3. The model, proposed by Hammel (1965) and modified by Boulant (1991), shows set point regulation of body temperature by anterior hypothalamic neurons. OC, optic chiasm; MB, mammillary body; W, warm sensitive neurons; I, temperature insensitive neurons; w, warm effector neurons which control heat loss; c, cold effector neurons which control heat production. Solid lines indicate the firing rate of each neuron while dotted lines indicate excitatory (+) and inhibitory (-) synaptic input.

excitatory than inhibitory input to these neurons, and they will activate heat retention and heat production responses (Hammel, 1965, Boulant, 1991).

7. Effects on PO/AH Neurons and Fever

The activity of PO/AH neurons, mainly warm sensitive neurons, can be influenced by a variety of factors in their local environment besides temperature, such as glucose levels, testosterone, estrogen and osmotic balance (Silva and Boulant, 1986, Boulant and Silva, 1988). For example, a fever is defined as a rise in body temperature of 1-4°C in response to an immune challenge, such as the introduction of infectious microorganisms to the body. Through a cascade of factors, a signal is directed to the brain (Blatteis et. al., 1998). It has been shown that specific cytokines cause the production of prostaglandin E_2 (PGE_2) in the brain and that PGE_2 affects neuronal activity in a specific region of the PO/AH, the ventral medial preoptic (VMPO). Introduction of PGE_2 to this region through the OVLT or stimulation of afferent pathways causes a significant rise in temperature (> 1°C) compared to other regions of the hypothalamus.

During a fever, PGE_2 acts on the neurons of the PO/AH to raise the temperature of the body to a new set point for an extended period of time. The body will produce thermoregulatory responses based on deviations from the new, higher temperature. This shift in set point may result from two possible changes in the firing rate of PO/AH neurons. Hammel's model would suggest that an increase in the firing rates of temperature insensitive neurons or a decrease in the firing rates of warm sensitive neurons, would result in the new, higher set point temperature. Recent studies from our

lab have shown that in VMPO, the presence of PGE_2 causes warm sensitive neurons to decrease their firing rates while temperature insensitive neurons are unaffected or show an increase in firing rate (Ranels and Griffin, 2003, Ranels and Griffin, 2005).

6. Hot Flashes

In contrast to long-term fevers, hot flashes are defined as transient increases in body temperature of 0.2 to 0.4°C, which last from minutes to 2-3 hours (Macleay et al., 2003). For a long period of time, hot flashes were considered to affect only women in post-menopause (~65%). Recent studies have now shown that men who receive prostate cancer treatments, such as anti-androgen therapy, may also experience hot flashes that tend to last for a longer period of time than hot flashes experienced by women. The clinical diagnosis of a hot flash includes a warming of the neck and face, reddening of the skin, sweating, increased metabolic rate and more severe symptoms such as loss of breath and feeling faint. In addition, hot flashes have several side effects such as decreased sleep quality, depression and increased fatigue (Finck et al., 1998, Stein et al., 2000). Until recently, there was little data on the mechanism for this transient hyperthermic response. Recently, it was discovered that there is a rise in the serum level of the hormone, calcitonin gene-related peptide (CGRP) during a hot flash (Spetz et al., 2001; Wyon et. al., 1998). The rise in CGRP during a hot flash has been speculated to be involved in a mechanism that would lead to the increased body temperature observed during a hot flash.

FIGURE 1.4

THE PRODCUTION OF AN IDEAL FEVER

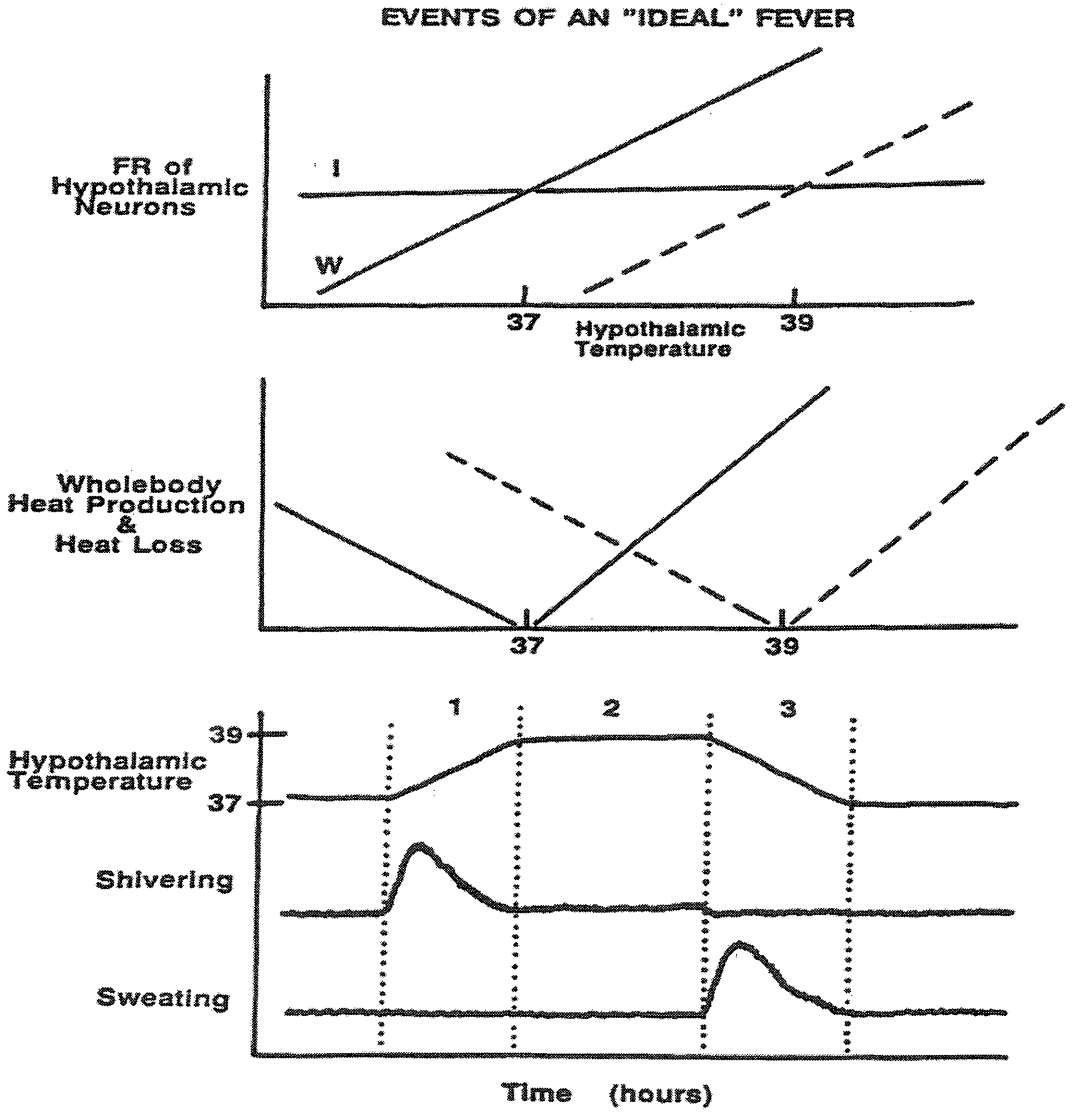

Figure 1.4. The top figure shows that a decrease in the activity of warm sensitive neurons will cause a hyperthermic shift in hypothalamic temperature to a new, higher set point temperature (39°C). The middle figure shows the resulting change in heat production and heat loss. The bottom figure shows shivering and sweating activities in response to an increase in hypothalamic temperature.

7. CGRP

An investigation to characterize the presence of CGRP in the brain indicated that the rodent PO/AH shows a clear sexual dimorphism in the expressi with females showing a higher level of expression than males. Furthermore, the low level of CGRP seen in males is a direct result of the suppressive effects of testosterone on the expression of CGRP (Herbison et. al., 1995). Upon castration of male rats and ovariectomization of female rats there is an increase in the level of cellular CGRP mRNA expression in the PO/AH compared to normal males and females. Furthermore, injection of CGRP into surgically or medically castrated male rats and ovariectomized female rats resulted in an increase in skin temperature, a result which was blocked by CGRP antagonists (Yuzurihara et. al., 2003, Noguchi et. al, 2002, Kobayoshi et al, 1995, Kobayoshi et al, 1999). Direct injection of CGRP into the dorsal medial hypothalamus (DMH) and the ventromedial hypothalamus (VMH) resulted in an increase in skin temperature and oxygen consumption, indicating an increase in metabolic rate, which is known to occur during increases in body temperature (Kobayoshi et al, 1999) This suggests that CGRP may play a role in the control of thermoregulatory responses through a direct effect on the activity of neurons in the hypothalamus.

8. CGRP Receptors: CRLR and RAMP

In addition to increased levels of CGRP, receptors for the hormone have been found in the PO/AH of rats (Henke et al., 1985). This receptor is composed of two subunits: calcitonin-receptor-like receptor (CRLR) and receptor-activity-modifying proteins (RAMPs). RAMP1 is a transmembrane protein that activates its G-protein

coupled partner, CRLR. Binding of CGRP to the extracellular domain of the RAMP1/CRLR complex activates a G-protein pathway that causes an increased production of cAMP (Leuthauser et. al., 2000). The mechanism by which increases in cAMP may affect neuronal activity in neurons of the PO/AH has yet to be determined. However, the effects of CGRP have been studied in other tissue types. CGRP acts on smooth muscle cells through a cAMP-dependent activation of protein kinase A (PKA) that opens ATP-sensitive K^+ channels (Quayle et. al., 1994). CGRP has also been shown to act on cortical neurons to increase cAMP levels and suppress delayed K^+ rectifier currents, potassium A currents (I_A) and Ca^{2+} conductance (Zona et. al., 1991). Within the PO/AH, changes in either the potassium, sodium or calcium conductance may lead to changes in neuronal activity and, thus, produce a change in thermoregulatory control.

9. Hypotheses, Specific Aims and Predictions

Hypothesis #1: Calcitonin gene-related peptide acts on preoptic anterior hypothalamic neurons leading to a decrease in the firing rates of warm sensitive neurons and an increase or no effect on temperature insensitive neurons.

Specific Aim #1: To determine the effects of calcitonin gene-related peptide on the firing rates of preoptic anterior hypothalamic neurons of male rats.

Prediction #1: Calcitonin gene-related peptide will decrease the firing rates of preoptic anterior hypothalamic warm sensitive neurons and increase the firing rates of temperature

insensitive neurons that will lead to a hyperthermic shift in hypothalamic set point temperature.

Hypothesis #2: Calcitonin gene-related peptide acts on preoptic anterior hypothalamic neurons to alter their cellular properties, leading to a decrease in the firing ratse of warm sensitive neurons and an increase in the firing rates of temperature insensitive neurons.

Specific Aim #2: To determine the effects of calcitonin gene-related peptide on the cellular properties of preoptic anterior hypothalamic neurons of male rats.

Prediction #2: Calcitonin gene-related peptide will decrease the prepotential rate of rise of preoptic anterior hypothalamic warm sensitive neurons and increase in the prepotential rate of rise of temperature insensitive neurons resulting in a decrease in firing rate of warm sensitive neurons and an increase in firing rate of warm sensitive neurons.

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Chapter II

The Effects of Calcitonin Gene-Related Peptide on the Firing Rates of Temperature Sensitive and Temperature Insensitive Neurons of the Rat Hypothalamus.

Abstract

"Hot flashes" can become a frequent occurrence for women following the onset of menopause and for men during the treatment of prostate cancer. This transient hyperthermic shift in temperature has been linked to the endogenous hormone, calcitonin gene-related peptide (CGRP), which acts peripherally to increase vasodilatation and centrally to increase sympathetic activation, including metabolic heat production. Recent studies have demonstrated that these centrally mediated responses may result from CGRP dependent changes in the activity of thermoregulatory neurons. Using a tissue slice preparation, we recorded the extracellular single-unit activity of anterior hypothalamic neurons from the adult male rat, in response to temperature and CGRP (10 μM). Based on the slope of firing rate as a function of temperature, neurons were classified as either warm sensitive ($\geq$ 0.8 impulses$\bullet s^{-1}\bullet {}^{o}C^{-1}$) or temperature insensitive ($\leq$ 0.79 impulses$\bullet s^{-1}\bullet {}^{o}C^{-1}$). All warm sensitive neurons responded to the micro-drop application of CGRP with a significant decrease in firing rate. While CGRP did not affect the majority of temperature insensitive neurons, responsive neurons showed increases in firing rate. This suggests that both warm sensitive and temperature insensitive neurons in the anterior

hypothalamus may play critical and contrasting roles in producing a transient hyperthermic shift in body temperature.

1. Introduction

The preoptic/anterior hypothalamus (PO/AH) has been shown to be a main thermoregulatory control region of the body for integrating and responding to changes in peripheral, core and hypothalamic temperature. The PO/AH is composed of temperature insensitive and temperature sensitive neurons. Temperature insensitive neurons, approximately 60% of the PO/AH neurons, show little variation in firing rate when hypothalamic temperature is changed from a normal temperature of 36°C. Of the remaining PO/AH neurons, 35% are classified as inherently warm sensitive, demonstrating an increase in firing rate when temperature is increased and a decrease in firing rate when temperature is decreased (Boulant, 1991).

An enduring model for temperature regulation indicates that a set point temperature is created from the interactions of temperature insensitive and temperature sensitive neurons of the PO/AH (Hammel, 1965). An alteration in peripheral, core and/or hypothalamic temperature from normothermic levels results in changes of firing rate that lead to an activation of thermoregulatory responses that return body temperature to its set point temperature. Studies have shown that a variety of substances can shift the set point temperature by altering the firing rates of the PO/AH neurons. For example, a mechanism for the prolonged hyperthermic shift in body temperature seen during a LPS fever can be explained by the alteration of the firing rates of neurons in ventral medial

preoptic (VMPO) due to prostaglandin E_2 (PGE_2) (Ranels and Griffin, 2003, Ranels and Griffin, 2005)

A hot flash is characterized as an increase in vasodilatation of the head and neck region, as well as increased sympathetic activation and metabolic rate resulting in an overall hyperthermic shift in core body temperature (Freedman, 1998). The transient hyperthermic shift in body temperature can range from 0.2°C to 0.4°C and last from 30 seconds to 3 hours (Macleay et al., 2003). Until recently hot flashes were thought to occur only in post-menopausal women. There is clear evidence that men who have undergone anti-androgen treatment for prostate cancer also experience hot flashes, which have more sustained periods of hyperthermia.

This transient hyperthermic shift in body temperature has been linked to an increase in the serum level of calcitonin gene-related peptide (CGRP), a known vasodilator (Wyon et. al, 1998, Spetz et. al, 2001). Studies have shown that intravenous injection of CGRP into castrated male rats and ovariectomized female rats results in increased skin temperature and sympathetic activation (Yuzurihara et al, 2003, Kobayashi et al., 1995, Kobayashi et. al, 1999). Injections of CGRP into the dorsal medial hypothalamus (DMH) and the ventral medial hypothalamus (VMH) caused increased oxygen consumption, heart rate and body temperature in rats (Kobayashi et. al, 1999). This work shows that the actions of CGRP are both peripheral and centrally mediated.

Centrally, CGRP has been found in the cell bodies of neurons in the supraoptic nucleus, paraventricular nucleus and infundibular nucleus of the human hypothalamus (Takahashi et al, 1989). In addition, the level of CGRP mRNA expression within PO/AH neurons been shown to increase after castration of male rats and ovarianectimization of

female rats (Herbison et. al., 1995). Distinct binding sites for CGRP are distributed throughout the rat brains including hypothalamus (Henke, 1985). Furthermore, an increase in CGRP receptors occurs during elevation of temperature due to CGRP application in rats (Noguchi et. al, 2002).

The exact mechanism for the hyperthermic shift in body temperature (i.e. hot flash) is unknown. The specific affect of CGRP on the activity of individual neurons of the hypothalamus has yet to be determined. The specific goal of this study was to investigate the affects of CGRP on thermally classified neurons of the PO/AH in a tissue slice. We hypothesized that an alteration of neuronal firing rates in response to CGRP would result in a hyperthermic shift in body's setpoint temperature.

2. Materials and Methods

Anterior hypothalamic tissue slices were prepared from male Sprague-Dawley rats (100-150 g in weight) that were housed under standard conditions (23°C, 12:12-h light:dark cycle, with lights on at 8:00 a.m.) and given food and water at lib. Prior to each recording session, a rat was anesthetized with isoflourine and sacrificed by quick decapitation according to the Animal Care and Use policies of the College of William and Mary. After the brain was removed, a tissue block containing the PO/AH was placed in a vibrotome where three coronal tissue slices of 400 μm thickness were removed. The tissue slices were immediately placed in an interface perfusion chamber for two hours prior to recording. Throughout the experiment the tissue was continuously bathed with artificial cerebral spinal fluid (aCSF; 124 NaCl, 26 $NaHCO_3$, 10 glucose, 5 KCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$, and 1.24 KH_2PO_4). The aCSF medium was maintained at a stable

temperature of 36°C as measured by a temperature gauge under the tissue and was oxygenated with 95%O_2/5% CO_2 to maintain a constant pH throughout the experiment.

The technique of extracellular single unit recording was employed to measure the firing rates of PO/AH neurons. All recordings were made using Xcell-3 Microelectrode Amplifier (FHC Inc.) and stored on VHS tape for later analysis. Using a stereoscopic microscope, glass microelectrodes with tip diameters of less than 1μm and filled with 3 M NaCl were placed in the PO/AH region of a tissue slice. All recordings in this study had to show a signal:noise ratio of at least a 3:1 and have a stable firing rate at 36°C for 2-3 minutes prior to changing temperature. After an isolated neuron met the criteria stated above, temperature was changed 2-3°C (at a rate of 1°C per minute) above and below 36°C by changing the voltage of a thermoelectric assembly through which the aCSF flowed before entering the recording chamber. The thermosensitivity of a neuron was determined by plotting firing rate as a function of temperature and determining the slope of the regression coefficient (*m*) of the plot. In accordance with previous studies, a neuron was considered warm sensitive if the regression coefficient was ≥ 0.8 impulses$\cdot s^{-1}\cdot °C^{-1}$ and cold sensitive if the regression coefficient is ≤ -0.6 impulses$\cdot sec^{-1}\cdot °C^{-1}$ (Boulant and Hardy, 1974, Griffin et al., 2001). All other neurons were classified as temperature insensitive.

Once neuronal thermosensitivity was determined, and a stable firing rate at 36°C was achieved, CGRP (10 μM) was applied by micro-drop technique and changes in firing rate were measured. Recordings were held for at least 10 minutes after firing rate returned to baseline levels. One-minute segments (60 Hz) of firing rate activity were measured for comparison before CGRP application (baseline), during maximum response

(CGRP) and after firing rate had returned to pre-treatment levels (washout). Firing rate segments were digitized (pClamp Software, Axon Instrument) and the mean firing rate and standard error was calculated. A Student's Standard T-test ($P \leq 0.05$) was used to determine the significance of firing rate changes in response to CGRP application. Also, to be classified as a response to CGRP a neuron must show a 15% change in firing rate. After each recording session, tissue slices were fixed in 4% formalin solution for at least 24 hours then placed in 30% sucrose solution for 2 hours in preparation for additional sectioning. Tissue was sectioned into 40 μm sections, mounted onto gel-coated slides and a giemsa staining procedure was used to identify hypothalamic nuclear groups and visually confirm the location of each recorded neuron within the PO/AH.

3. Results

Thermosensitivity of PO/AH Neurons

In this study, extracellular single unit recordings characterized the thermosensitivity of 45 PO/AH neurons. Of the recorded PO/AH neurons, 38 neurons did not show significant changes in firing rate in response to temperature (≤ 0.79 impulses$\bullet$sec$^{-1}\bullet$°C^{-1}) and were classified as temperature insensitive. The average thermosensitivity of temperature insensitive neurons was 0.18 impulses$\bullet$sec$^{-1}\bullet$°C^{-1}. The remaining 7 neurons showed a significant increase in firing rate as temperature was increased (≥ 0.80 impulses$\bullet$sec$^{-1}\bullet$°C^{-1}) and had an average thermosensitivity of 1.20 impulses$\bullet$sec$^{-1}\bullet$°C^{-1}.

Effects of CGRP on PO/AH Neurons

Once thermosensitivity was determined, CGRP was applied by micro-drop technique and changes in firing rate were measured. A significant response to CGRP was considered as a change in firing rate of 15% above or below the baseline firing rate. Table 1 outlines the specific firing rates for baseline, CGRP and washout periods of temperature insensitive and warm sensitive neurons.

A majority (21 of 38) of temperature insensitive neurons showed a significant increase in firing rate from baseline levels. Average neuronal firing rate increased from 3.37±0.43 impulses•s^{-1} before treatment to 4.95±0.53 impulses•s^{-1} in response to CGRP (washout firing rate = 4.04±0.56 impulses•s^{-1}). Figure 1 shows a temperature insensitive neuron that increased its firing rate approximately two minutes after exposure to CGRP. After a period of sustained increase, the firing rate returned to baseline levels. Although not all neurons fully returned to baseline levels, all responsive temperature insensitive neurons did show a trend of returning to baseline levels after a period of increased firing rate.

The remaining 17 temperature insensitive neurons did not show a significant change in firing rate in response to CGRP. The firing rate was 4.16±0.25 impulses•s^{-1} before exposure to CGRP and 4.39±0.26 impulses•s^{-1} during treatment with CGRP. Figure 2 shows a temperature insensitive neuron that has no change in firing rate in response to repeated applications of CGRP. All warm sensitive neurons (n = 7) showed a significant decrease in firing rate after CGRP application. Their average firing rate decreased from a baseline level of 8.18±0.82 impulses•s^{-1} to 3.34±0.55 impulses•s^{-1} in response to CGRP application (washout firing rate = 8.12±0.54 impulses•s^{-1}). Figure 3

TABLE 2.1

EFFECT OF CGRP ON THE FIRING RATE OF PO/AH NEURONS

Thermosensitivity (impulses•s^{-1}•°C^{-1})	*N*	Firing rate activity (impulses•s^{-1}±S.E.)		
		Baseline	**CGRP**	**Washout**
Temperature Insensitive ≤ 0.79				
No Change	17	4.16±0.25	4.39±0.26	3.63±0.35
Increase	21	3.45±0.48	5.08±0.60*	4.15±0.59
Warm Sensitive ≥ 0.80				
Decrease	7	8.18±0.82	3.34±0.55*	8.12±0.54

*Significantly different from baseline firing rate (Paired T-test P<0.05)

FIGURE 2.1

THE EFFECTS OF CGRP APPLICATION ON THE FIRING RATE OF A TEMPERATURE INSENSITIVE NEURON

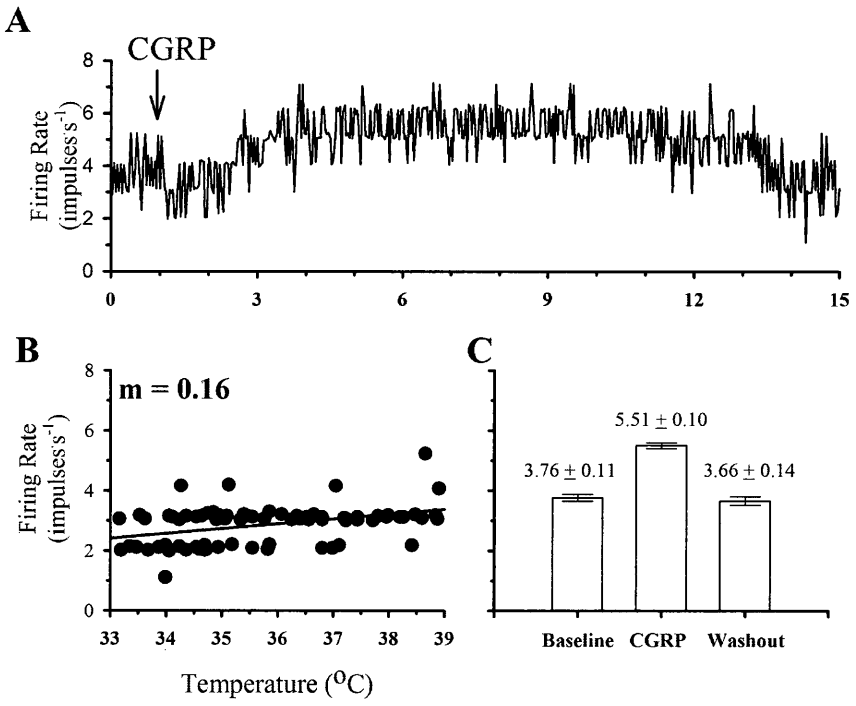

Figure 2.1. **A** shows the changes in firing rate due to CGRP application over time (minutes). The arrow indicates the time of CGRP application. **B** shows a plot of firing rate as a function of temperature with a regression line of slope = 0.16 impulses$\bullet s^{-1} \bullet {}^{o}C^{-1}$. **C** shows the average firing rates and standard errors for one minute samples of firing rate from baseline (3.76±0.11 impulses$\bullet s^{-1}$), CGRP (5.51±0.10 impulses$\bullet s^{-1}$) and washout (3.66±0.14 impulses$\bullet s^{-1}$) periods.

FIGURE 2.2

THE EFFECT OF CGRP APPLICATION ON THE FIRING RATE OF A TEMPERATURE INSENSITIVE NEURON

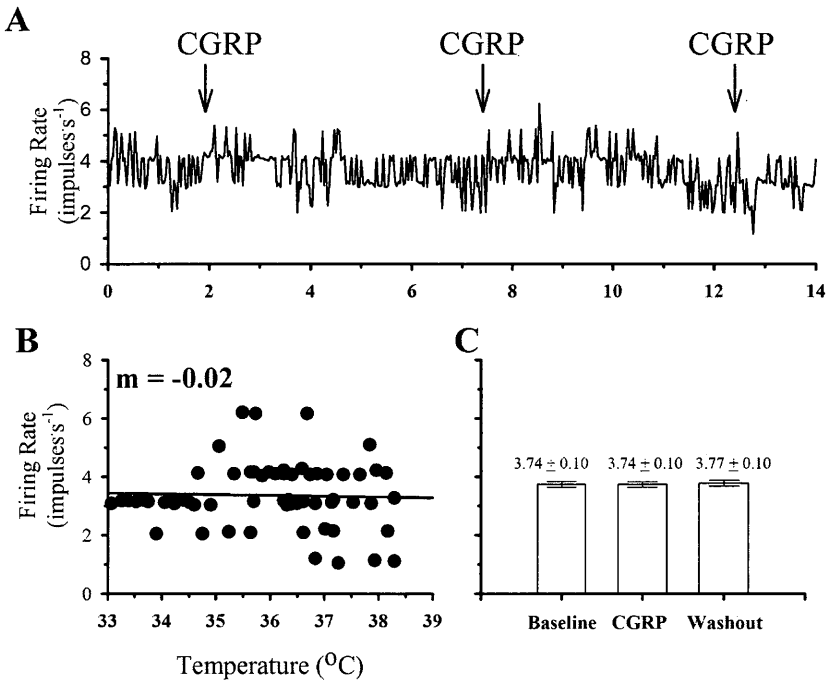

Figure 2.2. **A** shows that there are no changes in firing rate activity due to CGRP application over time (minutes). The arrows indicate the times of CGRP application. **B** shows a plot of firing rate as a function of temperature with a regression line of slope = – 0.02 impulses sec^{-1} $^{o}C^{-1}$. **C** shows the average firing rates and standard errors for one minute samples of firing rate from baseline (3.7±0.10 impulses•s^{-1}), CGRP (3.74±0.10 impulses•s^{-1}) and washout (3.77±0.10 impulses•s^{-1}) periods.

FIGURE 2.3

THE EFFECT OF CGRP APPLICATION ON THE FIRING RATE OF A TEMPERATURE SENSITIVE NEURON

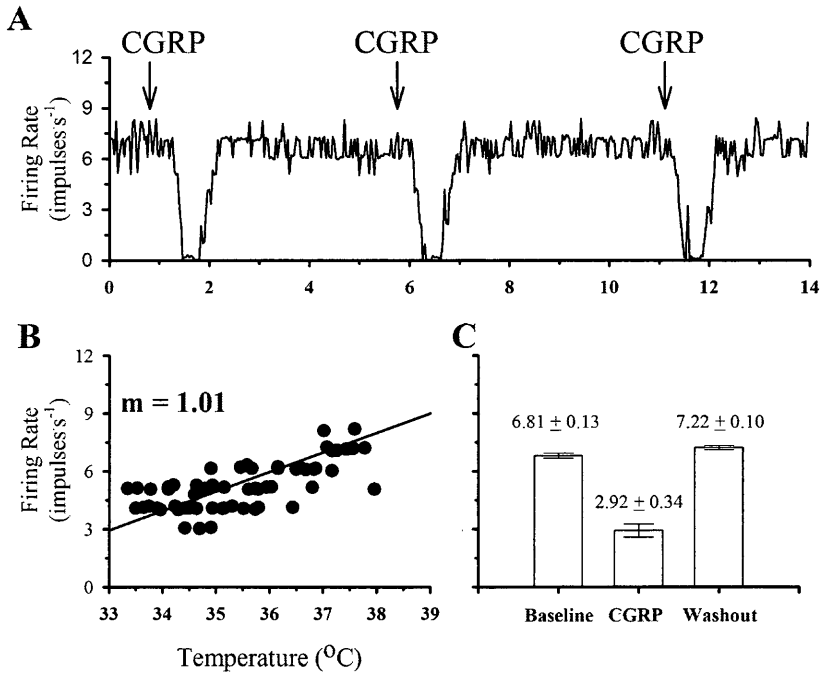

Figure 2.3. **A** shows the decreases in firing rate activity due to CGRP application over time (minutes). The arrows indicate the times of CGRP application. **B** shows a plot of firing rate as a function of temperature with a regression line of slope = 1.01 impulses sec^{-1} $^{o}C^{-1}$. **C** shows the average firing rates and standard errors for one minute samples of firing rate from baseline (6.81±0.13 impulses•s^{-1}), CGRP (2.92±0.34 impulses•s^{-1}) and washout (7.22±0.10 impulses•s^{-1}).

FIGURE 2.4

THE EFFECT OF CGRP ON THE FIRING RATES OF PO/AH NEURONS

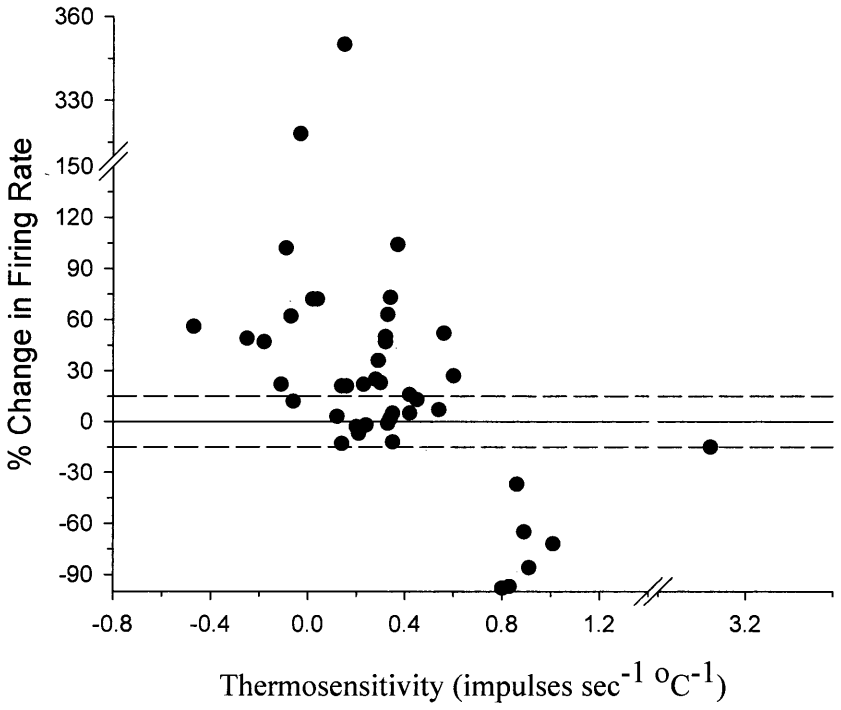

Figure 2.4. The effect of CGRP on the firing rates of PO/AH neurons. The percent change in firing rate activity in response to CGRP for each recorded neuron is plotted versus thermosensitivity (impulses•s^{-1}•$^{o}C^{-1}$). The dashed line indicated 0% change in firing rate activity. The dotted line indicates a 15% change in firing rate activity.

FIGURE 2.5

CORONAL DIAGRAMS THROUGH THE ANTERIOR HYPOTHALAMUS OF THE RAT BRAIN

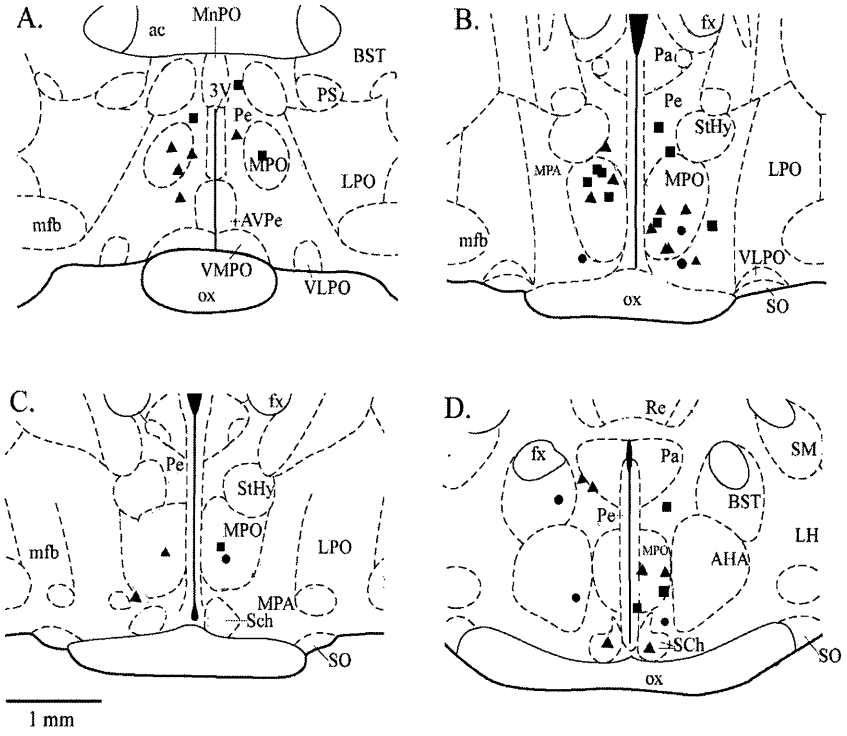

Figure 2.5. Diagrams start with the most anterior section (A) and progress to the most posterior section (D). The position of each recorded neuron is marked, non-responsive temperature insensitive neurons = ■, responsive temperature sensitive neurons = ▲ and warm sensitive neurons = ●. 3V, third ventricle; ox, optic chiasm; ac, anterior commisure; Pa, paraventricular nucleus; AHA, anterior hypothalamic area; Pe, periventricular hypothalamic nucleus; AVPe, anteroventral periventricular nucleus; PS, parastial nucleus; BST, bed nucleus lamina terminalis; Re, reunions thalamic nucleus; fx, fornix; SCh, suprachiasmatic nucleus; LH, lateral hypothalamic area; SM, nucleus of the stria medullaris; LPO, lateral preoptic area; SO, supraoptic nucleus; mfb, medial forebrain bundle; StHy, striohypothalamic nucleus; MnPO, median preoptic nucleus; VLPO, vontrolateral preoptic nucleus; MPA, medial preoptic area; VMPO, ventromedial preoptic nucleus; MPO, medial preoptic nucleus.

shows a warm sensitive neuron with a thermosensitivity of 1.10 impulses•sec^{-1}•$^{o}C^{-1}$ whose firing rates decreased after CGRP application. The decrease in firing rate due to CGRP and subsequent recovery to baseline firing rate is seen in all three treatments. The decrease in firing rate occurs within one minute of exposure to CGRP and returns to baseline firing rate levels approximately one minute after treatment.

Neuronal Locations

The locations of all recorded neurons are shown in Figure 5. Responsive and non-responsive neurons were not located in any specific region of the PO/AH.

4. Discussion

The endogenous mediator CGRP, has been linked to the hyperthermic shifts in body temperature that are seen in hot flashes that occur in both postmenopausal women and men who have been treated for prostate cancer. This hyperthermia can last up to 3 hours in males, indicating that CGRP effects are more than a simple peripheral vasodilatation (Macleay et al., 2003). Instead, CGRP effects include increased sympathetic activation, leading to increased metabolism and a rise in metabolic heat productions (Wyon et. al, 1998, Spetz et. al, 2001). Furthermore, studies have shown the presence of receptors for CGRP in the hypothalamus, which suggests a centrally mediated effect of CGRP on thermoregulatory neurons within the hypothalamus (Henke et al., 1985).

The PO/AH has been shown to be at the top of a hierarchy of thermoregulatory control regions throughout the central nervous system. This region is composed of two types of neurons, temperature insensitive and temperature sensitive neurons. Warm sensitive neurons, a specific type of temperature sensitive neurons, have been shown to receive and integrate input from peripheral and core body thermoreceptors, in addition to having the ability to respond to changes in PO/AH temperature (Boulant, 1991). A model proposed by Hammel (1965) indicates that interaction between the temperature insensitive neurons and warm sensitive neurons of the PO/AH creates a specific set point temperature. It can be hypothesized that alterations to the firing rates of either temperature insensitive neurons or warm sensitive neurons would result in a hypothermic or hyperthermic shift in set point temperature.

The criterion for the thermosensitivity of PO/AH neurons continues to be a debated topic within the literature. The functional criteria for warm sensitivity of ≥ 0.8 impulses$\bullet s^{-1}\bullet °C^{-1}$ and ≤ 0.79 impulses$\bullet s^{-1}\bullet °C^{-1}$ for temperature insensitive neurons have been used in past studies. These criteria have been used in past studies because it was shown that there were functional and morphological distinctions between neurons with these classes of neurons. Warm sensitive neurons have a lateral/medial orientation while temperature insensitive neurons extend their dendrites in rostal and caudal directions. Also, the frequency of synaptic input on hypothalamic neurons does not change with temperature suggesting that temperature insensitive neurons provide a majority of the connections within the PO/AH that help to establish the specific set point (Boulant, 1991, Griffin et al., 2001).

In this study, when the criteria for warm sensitivity stated above is used, there is a distinct and selective effect of CGRP on the neurons of the PO/AH. Temperature insensitive neurons show an increased firing rate response to CGRP while warm sensitive neurons show the contrasting response of decreased firing rates. In reference to the model for set point temperature proposed by Hammel these firing rate responses provide a mechanism for a hyperthermic shift in body temperature. The increased firing rates of temperature insensitive and/or decreased firing rates of warm sensitive neurons and the excitatory and inhibitory inputs on effector neurons would shift the set point to a higher temperature thus creating a new temperature for the body regulate around.

Acknowledgements

This Research was supported by NSF grant NBN-9983624, the Howard Hughes Medical Institute and the Borgenicht Foundation Grant. We would like to thank Erin M. Deegan, Eleanor R. Grimm, and Bethany Schaffer for their assistance in recording sessions and histological processing of neural tissue.

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Chapter III

The Effects of Calcitonin Gene-Related Peptide on the Cellular Properties of Temperature Sensitive and Temperature Insensitive Neurons of the Rat Hypothalamus.

Abstract

A hyperthermic shift in body temperature is one characteristic of hot flashes that occur in women following the onset of menopause and in men during the treatment of prostate cancer. This transient hyperthermic shift in temperature has been linked to the endogenous hormone, calcitonin gene-related peptide (CGRP), which acts peripherally to increase vasodilatation and centrally to increase sympathetic activation, including metabolic heat production. Previous extracellular single-unit recordings have demonstrated that these centrally mediated responses may result from CGRP dependent changes in the activity of thermoregulatory neurons in the preoptic anterior hypothalamus (PO/AH). Using a tissue slice preparation, I recorded the intracellular activity of PO/AH neurons from the adult male rat, in response to temperature and CGRP (10 μM). Based on the slope of firing rate as a function of temperature, neurons were classified as either warm sensitive ($m \geq 0.8$ impulses$\bullet s^{-1}{}^{o}\bullet C^{-1}$) or temperature insensitive (≤ 0.79 impulses$\bullet s^{-1}\bullet{}^{o}C^{-1}$). A majority warm sensitive neurons in this study responded to the micro-drop application CGRP with a significant decrease in firing rate. While CGRP did not affect the all temperature insensitive neurons, a majority of responsive neurons showed increases in firing rate. The opposing responses of warm sensitive and temperature

insensitive neurons were a result of two different mechanisms. A decrease in the prepotential rate of raise was seen in all responsive warm sensitive neurons, while a change in the threshold level was seen in responsive temperature insensitive neurons. Resting membrane potential, synaptic input and other action potential characteristics did not significantly contribute to changes in firing rate due to CGRP. This suggests that both warm sensitive and temperature insensitive neurons in the PO/AH may play critical and contrasting roles in producing these transient hyperthermic shifts in body temperature.

1. Introduction

An enduring model of the hypothalamus indicates that a set point temperature for the body is created by an interaction of temperature insensitive neurons and temperature sensitive neurons (Hammel 1965, Boulant 1991). Changes in the peripheral, core or hypothalamic temperature that results in a deviation from the established set point temperature, triggers a host of physiological responses. These responses include reactions such as shivering and non-shivering thermogenesis, sweating and vasodilatation that aim to return the body to its set point temperature. Within the hypothalamus, temperature insensitive neurons compose 60% of the neurons, while temperature sensitive neurons compose the remaining 40%, with 35% being classified as inherently warm sensitive and 5% as cold sensitive. Warm sensitive neurons integrate peripheral and core body temperatures while temperature insensitive neurons are believed to form connections within the hypothalamus, providing a steady level of background activity (Boulant, 1981, Griffin et al., 2001)

Temperature insensitive neurons do not show a significant change in firing rate (impulses s^{-1}) due to temperature. Conversely, temperature sensitive neurons show a significant change in firing rate, as temperature is change. Warm sensitive neurons show an increase in firing rate as temperature is increased and a decrease in firing rate as temperature is decreased below the physiological set point. Several studies set the criteria of temperature insensitivity as a thermosensitivity of ≤ 0.79 impulses$\bullet s^{-1}\bullet {}^{o}C^{-1}$ while, the criteria for warm sensitivity is ≥ 0.8 impulses$\bullet s^{-1}\bullet {}^{o}C^{-1}$ (Boulant, 1981). The criterion of ≥ 0.8 impulses$\bullet s^{-1}\bullet {}^{o}C^{-1}$ is considered a functional criterion because these neurons show responses to external stimulation. Furthermore, temperature insensitive and warm sensitive neurons show differences in their dentritic morphology. Temperature insensitive neurons and warm sensitive neurons have rostral/caudal and medial/lateral dendritic projections respectively (Griffin et. al, 2001). The mechanism for warm sensitivity has been shown to be due, at least in part, to increased inactivation of the potassium A current (I_A) as temperature increases and not a depolarization in resting membrane potential (Griffin et. al, 1996, Zhao and Boulant, 2005).

The transient increase in body temperature observed during a "hot flash," occurs in approximately 75% of women in menopause and 25% of men who have undergone anti-androgen treatments for prostate cancer. A hot flash is clinically characterized as a flushing of the face and neck with a transient increase in body temperature. The hyperthermic shift in body temperature has been linked to an increase in the level of a known vasodilator calcitonin gene-related peptide (CGRP) in the bloodstream (Spetz et al, 2001, Wyon et al., 1998). It has also been shown that intravenous injection of CGRP into rats causes an increase in thermoregulatory mechanisms such as respiratory rate and

skin temperature (Noguchi et al., 2002, Kobayashi et al., 1995, Kobayashi et al., 1999, Yuzurihara et al., 2003). Other studies have also shown that injection of CGRP into the dorsal medial hypothalamus (DMH) and the ventral medial hypothalamus (VMH) of rats results in increases in body temperature (Kobayashi et al., 1999). Additionally, the presence of a CGRP heterodimer receptor (calcitonin receptor-like-receptor, CRLR and receptor-activity-modifying-protein, RAMP) has been identified within the rat hypothalamus (Henke et al., 1985). In other tissues, RAMP has been shown to activate the production of cAMP, which results in changes in ion channel activity (Leuthauser et. al., 2000, Quayle et. al., 1994, Zona et. al., 1991).

The aim of this study was to investigate the cellular effects of CGRP on the PO/AH neurons. It is hypothesized that temperature insensitive neurons will increase and/or have no change in firing rate in response to CGRP while warm sensitive neurons will decrease their firing rates. We further propose that the change in activity of either insensitive neurons or warm sensitive neurons will be due to a change in specific cellular properties of responsive neurons.

2. Materials and Methods

Experiments to record the firing rate of individual PO/AH neurons were carried out in an isolated tissue slice preparation. Prior to each experiment, a male Sprague-Dawley rat was anesthetized and quickly decapitated using procedures currently approved by The College of William and Mary Animal Care and Use Committee. The brain of a rat was removed, and three coronal tissue slices, 400 μm in thickness, were sectioned using a vibratome and immediately transferred to an interface recording

chamber where they were perfused with artificial cerebral spinal fluid (aCSF) composed of (mM): 124 NaCl, 26 $NaHCO_3$, 10 glucose, 5 KCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$, and 1.24 KH_2PO_4. The aCSF medium maintained at a stable temperature of 36°C and was oxygenated with 95%O_2/5% CO_2 to maintain a constant pH.

The intracellular technique of whole cell patch clamping was used to record cellular activity of individual neurons in the PO/AH. Glass microelectrodes with tips of 2 μm were filled with a solution of (mM): 130 K-gluconate, 10 EGTA, 2 ATP, 1 MgCl2, 1 CaCl, having a pH of 7.2 and an osmolarity of 295 mOsmols. An oscilloscope was used to display electrical activity while a digital VCR recorded action potentials, resting membrane potential, tissue temperature and integrated firing rate. An acceptable recording had to be maintained for at least 20 minutes and have action potential peaks passing through zero millivolts. A subtraction of 12 mV was taken from every recording due to the liquid junction potential previously described by Griffin and Boulant (1995).

Using a stereoscopic microscope, the recording electrode was placed in the PO/AH region of a tissue slice. Once a single neuron was isolated, a gigahom seal was formed between the electrode tip and soma, and an intracellular recording was established by rupturing the cell membrane with suction. After a stable recording was kept for 2-3 minutes, the temperature of the recording chamber was altered 2-3°C above and below 36°C using a thermoelectric peltier. The thermosensitivity of a neuron was determined by analyzing firing rate as a function of temperature. Neurons were classified as warm sensitive if they showed a regression coefficient of ≥ 0.8 impulses$\bullet s^{-1}\bullet °C^{-1}$ and cold sensitive if the regression coefficient is ≤ -0.6 impulses$\bullet sec^{-1}\bullet °C^{-1}$ (Boulant and

Hardy, 1974, Griffin et al., 2001). All other neurons were classified as temperature insensitive.

Once a neuron's theremosensitivity was determined, CGRP (10 μM) was applied by a micro-drop technique, and changes in firing rate were recorded. One minute segments (60 Hz) were taken to compare firing rate before CGRP application (baseline), during maximum response (CGRP) and after firing rate had returned to pre-treatment levels (washout). A Student's Standard T-test ($P \leq 0.05$) was used to determine the significance of firing rate changes in response to CGRP application. Also, to be classified as a response to CGRP a neuron must show a 15% change in firing rate. Furthermore, membrane potential was constantly recorded (Griffin et al., 1995) to determine if there were any CGRP dependent changes in membrane potential. For each neuron, pCLAMP software (Axon Instrument) was used to create an average of 10 to 15 action potentials during baseline, CGRP and washout periods. Measurements were taken on the averaged action potentials to determine amplitude (resting membrane potential to peak), duration (at half amplitude), and presence or absence of after hyperpolarizing potential (AHP). Threshold potential of each averaged action potential was measured following the protocol presented in Burgoon and Boulant (1996). The depolarizing rate of rise was determined by measuring change in resting membrane potential over the 15-25 ms preceding threshold (Griffin et. al. 1996).

All synaptic potentials were counted and classified as either excitatory post-synaptic potentials (EPSP) or inhibitory post-synaptic potentials (IPSP). Post-synaptic potentials are considered rapid changes in membrane potential of at least 1 mV greater than background noise. Intervals of 20 seconds of data were taken at 34°C, 36°C, and

38°C as well as before treatment with CGRP, during maximum response and during washout. During each interval, the number of IPSPs and EPSPs were counted to determine the effects of temperature and CGRP on post-synaptic potentials frequency (Griffin et al, 2001).

After each recording session, tissue slices were fixed in 4% formalin solution for at least 24 hours then placed in 30% sucrose solution for 2 hours in preparation for additional sectioning. Tissue was sectioned into 40 μm sections, mounted onto gel-coated slides and a giemsa staining procedure was used to identify hypothalamic nuclear groups and visually confirm the location of each recorded neuron within the PO/AH.

3. Results

Thermosensitivity of PO/AH Neurons

The thermosensitivity of 23 PO/AH neurons was determined through the intracellular recording technique of whole cell patch clamping. In this study, there were 19 neurons that had firing rates of ≤ 0.79 impulses$\bullet s^{-1}\bullet {}^{o}C^{-1}$ and were thus classified as temperature insensitive. Temperature insensitive neurons had an average thermosensitivity of 0.26 impulses$\bullet s^{-1}\bullet {}^{o}C^{-1}$. The remaining 4 neurons displayed a thermosensitivity of ≥ 0.8 impulses$\bullet s^{-1}\bullet {}^{o}C^{-1}$ and were classified as warm sensitive. The average firing rate for warm sensitive neurons was 1.62 impulses$\bullet s^{-1}\bullet {}^{o}C^{-1}$.

Response of PO/AH Neurons to CGRP

The effects of CGRP on firing rate were studied for all 23 neurons. Of the 19 temperature insensitive neurons, 9 (47%) displayed an increase in firing rate in response to CGRP application. The firing rate of the responsive, temperature insensitive neurons,

TABLE 3.1

THE EFFECT OF CGRP ON THE FIRING RATE OF PO/AH NEURONS

Thermosensitivity (impulses•s^{-1}•°C^{-1})	N	Firing Rate (impulses•s^{-1}±S.E.) Baseline	CGRP	Washout
Temperature Insensitive ≤0.79	19	4.50±0.13	5.14±0.13*	4.56±0.13
No Change	8	4.40±0.13	4.56±0.13	3.68±0.12
Increase	9	4.73±0.12	6.20±0.15*	5.85±0.14
Decrease	2	3.88±0.15	2.71±0.10	2.29±0.11
Warm Sensitive ≥0.8	5	10.23±0.20	8.24±0.18	9.86±0.17
Decrease	4	10.27±0.21	7.79±0.19*	9.96±0.17

***Significantly different from Baseline Firing Rate (Paired T-test $p < 0.05$)**

increased from 4.73±0.12 impulses•s^{-1} to 6.20±0.15 impulses•s^{-1} (washout = 5.85±0.14 impulses•s^{-1}). Eight of the remaining temperature insensitive neurons showed no significant change in firing rate in response to CGRP application. All warm sensitive neurons (n = 4) showed a decrease in firing rate during CGRP application. The firing rates of warm sensitive neurons decreased from 10.27±0.21 impulses•s^{-1} to 7.79±0.19 impulses•s^{-1} during CGRP application (washout = 9.96±0.17 impulses•s^{-1}).

Electrical Properties

The average resting membrane potential for all neurons was -50.43 mV. Temperature insensitive neurons had a resting membrane potential of -51.47 mV while the resting membrane potential of warm sensitive neurons was -47.25 mV. As shown in Table 2, no neurons displayed a significant change in resting membrane potential due to CGRP application. Temperature insensitive neurons that showed a significant increase in firing rate had a baseline resting membrane potential of -52.41 mV which did not change in response to CGRP (-50.54 mV). Warm sensitive neurons that showed a decrease in firing rate in response to CGRP had a resting membrane potential of -44.80 mV before CGRP and -44.36 mV during response to CGRP. Overall, the resting membrane potential of all neurons consistently decreased throughout the recording.

The action potential amplitude and duration did not significantly change in response to CGRP (data not shown). There was a progressive decrease in action potential amplitude and duration over time that can be contributed to washout into the electrode and not an effect of CGRP on the neuron.

The prepotential (mV/ms) is the steady depolarization in resting membrane potential that leads to an action potential. While temperature insensitive neurons did not show a significant change in prepotential, responsive warm sensitive neurons had a decrease in their prepotential from 0.3735 mV/ms to 0.3482 mV/ms in response to CGRP. Figure 3 and 4 show a warm sensitive neuron with a decreased prepotential which results in a decrease firing rate during CGRP exposure.

The threshold potentials for all recorded neurons are shown in Table 4. Threshold potentials did not significantly change for warm sensitive neurons or non-responsive temperature insensitive neurons. Conversely, temperature insensitive neurons that increased their firing rates in response to CGRP application, showed a decrease in threshold from -30.67 mV at baseline levels to -29.62 mV during response to CGRP.

Synaptic input to all the recorded neurons was analyzed throughout each recording. A majority of the synaptic input was inhibitory and the input frequency did not change with temperature. As shown in Table 5, there was no significant change in the frequency of inhibitory or excitatory synaptic potentials between baseline and CGRP periods of temperature insensitive neurons. For warm sensitive neurons, the frequency of inhibitory synaptic input did not change during response to CGRP but the frequency of excitatory input did show a significant increase during response to CGRP.

TABLE 3.2

THE EFFECT OF CGRP ON THE RESTING MEMBRANE POTENTIAL PO/AH NEURONS

		Resting Membrane Potential (mV±S.E.)		
Thermosensitivity (impulses•s^{-1}•$^{o}C^{-1}$)	N	Baseline	CGRP	Washout
Temperature Insensitive				
≤0.79	19	-51.47±0.09	-50.04±0.07	-46.30±0.06
Warm Sensitive				
≥0.8	5	-44.80±0.10	-44.36±0.11	-41.03±0.12

TABLE 3.3

THE EFFECT OF CGRP ON THE PREPOTENTIAL OF PO/AH NEURONS

		Prepotential (mV/ms)		
Thermosensitivity (impulses•s^{-1}•$^{o}C^{-1}$)	N	Baseline	CGRP	Washout
Temperature Insensitive				
≤0.79	19	0.33	0.30	0.30
Warm Sensitive				
≥0.8	4	0.38	0.33	0.38

TABLE 3.4

THE EFFECT OF CGRP ON THE THRESHOLD POTENTIAL OF PO/AH NEURONS

Thermosensitivity (impulses•s^{-1}•$^{o}C^{-1}$)	N	Threshold (mV) Baseline	CGRP	Washout
Temperature Insensitive				
≤0.79	19	-30.99	-30.51	-30.15
No Change	8	-30.67	-30.99	-30.37
Increase	9	-30.67	-29.62	-29.54
Decrease	2	-33.49	-32.85	-32.22
Warm Sensitive				
≥0.8	3	-25.05	-26.44	-25.14

TABLE 3.5.

THE EFFECT OF CGRP ON INHIBITORY AND EXCITATORY POSTSYNAPTIC POTENTIALS

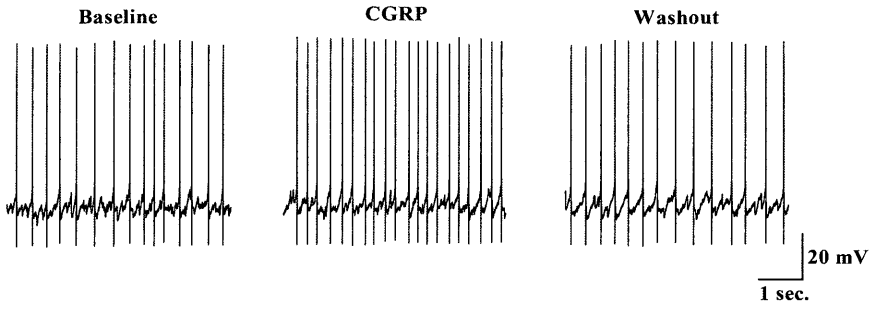
Thermosensitivity (impulses•s^{-1}•°C^{-1})	N	IPSP•s^{-1}			EPSP•s^{-1}		
		Baseline	CGRP	Washout	Baseline	CGRP	Washout
Temperature Insensitive							
≤0.79	19	3.62	3.76	4.06	1.71	1.52	1.54
Warm Sensitive							
≥0.8	4	4.34	6.20	4.98	1.9	2.97*	2.39

*Significantly different than baseline EPSP•s^{-1}

FIGURE 3.1

THE EFFECT OF CGRP ON A PO/AH TEMPERATURE INSENSITIVE NEURON

A.



B.

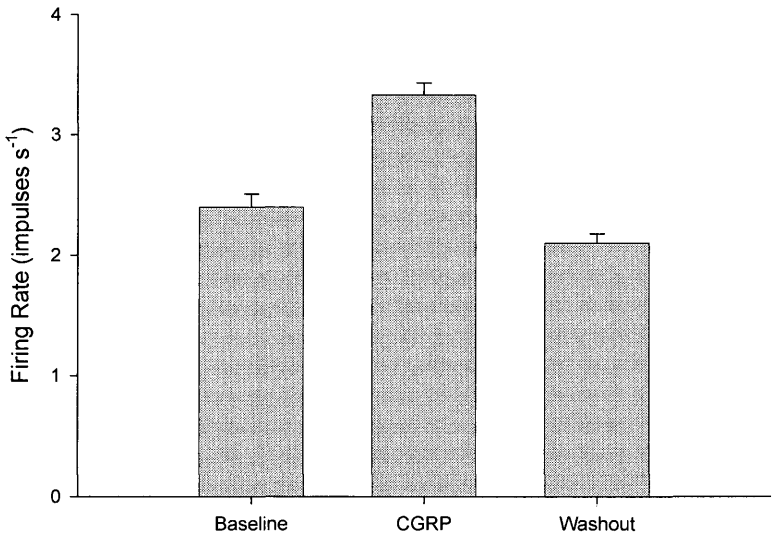


Figure 3.1. Panel A shows an increase in firing rate in response to application of CGRP (10 μm). The changes in average firing rate ± standard error from baseline (2.40±0.11 impulses•s^{-1}) to CGRP (3.33±0.10 impulses•s^{-1}) are shown in B (Washout = 2.10±0.08 impulses•s^{-1}).

FIGURE 3.2

ACTION POTENTIAL TRACES FROM A CGRP RESPONSIVE TEMPERATURE INSENSITIVE NEURON

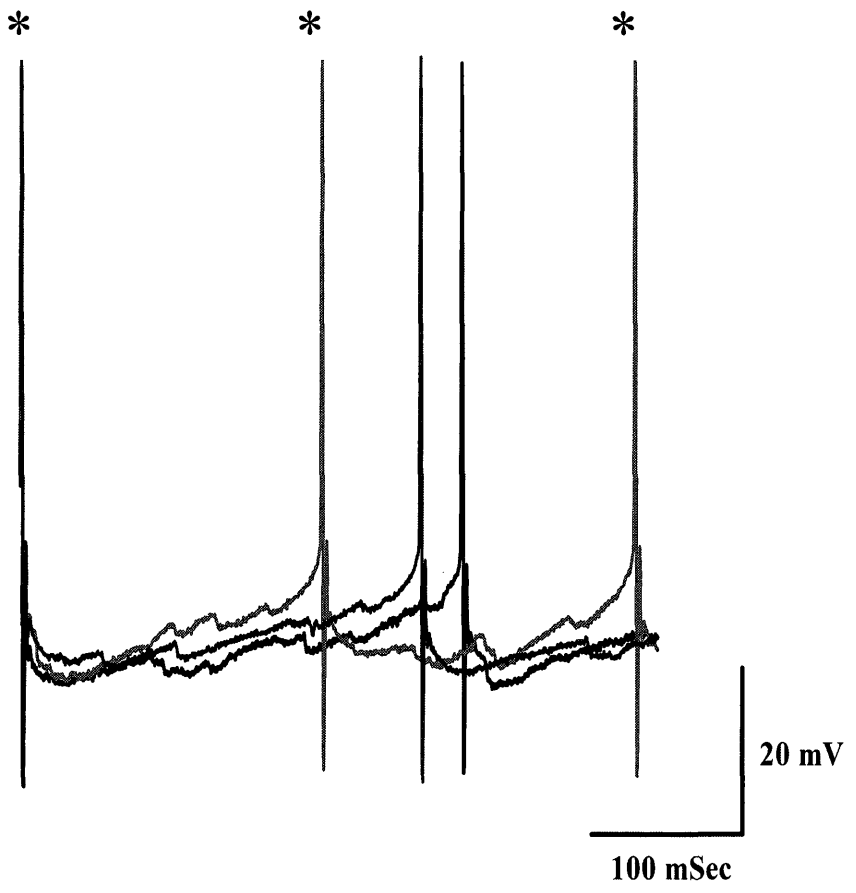
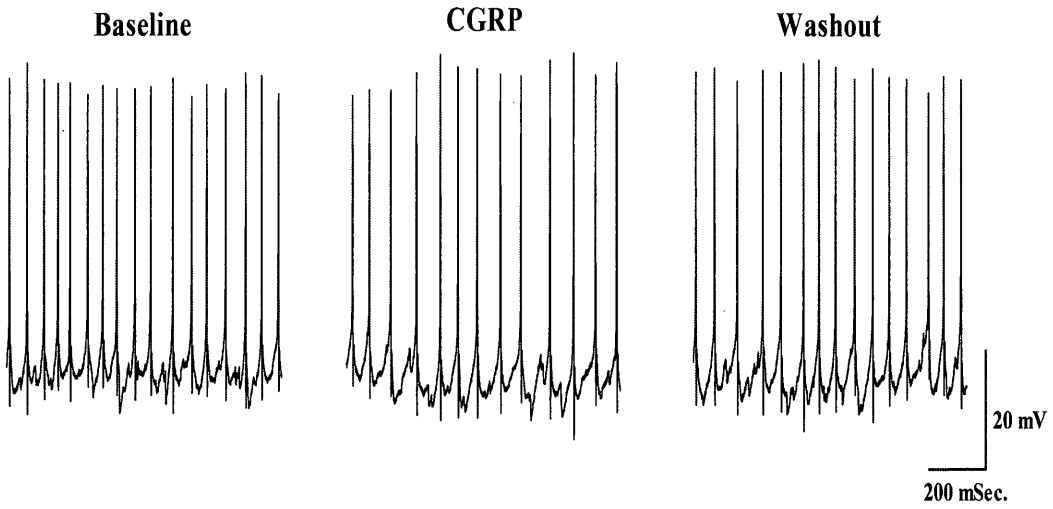


Figure 3.2. Action potentials from baseline, CGRP response (action potentials marked with asterisks), and washout are superimposed to show increases in threshold and firing rate during response to CGRP .

FIGURE 3.3

THE EFFECT OF CGRP ON A PO/AH WARM SENSITIVE NEURON

A.



B.

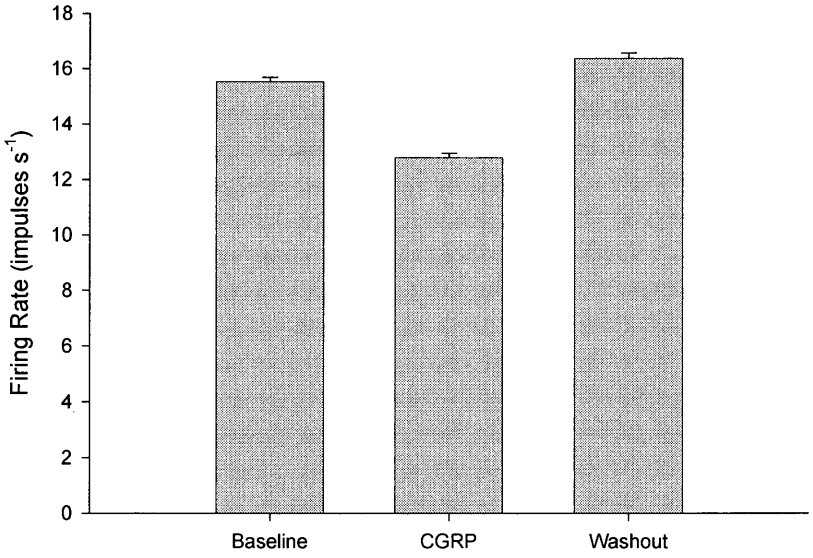


Figure 3.3. Panel A shows a decrease in firing rate from a baseline level of to in response to application of CGRP (10 μm). The changes in average firing rate ± standard error from baseline (15.53±0.16 impulses•s^{-1}) to CGRP (12.79±0.17 impulses•s^{-1}) are shown in B (Washout = 16.38±0.20 impulses•s^{-1}).

FIGURE 3.4

THE EFFECT OF CGRP ON ACTION POTENTIAL PREPOTENTIAL AND INTER-SPIKE INTERVAL OF A WARM SENSITIVE PO/AH NEURON

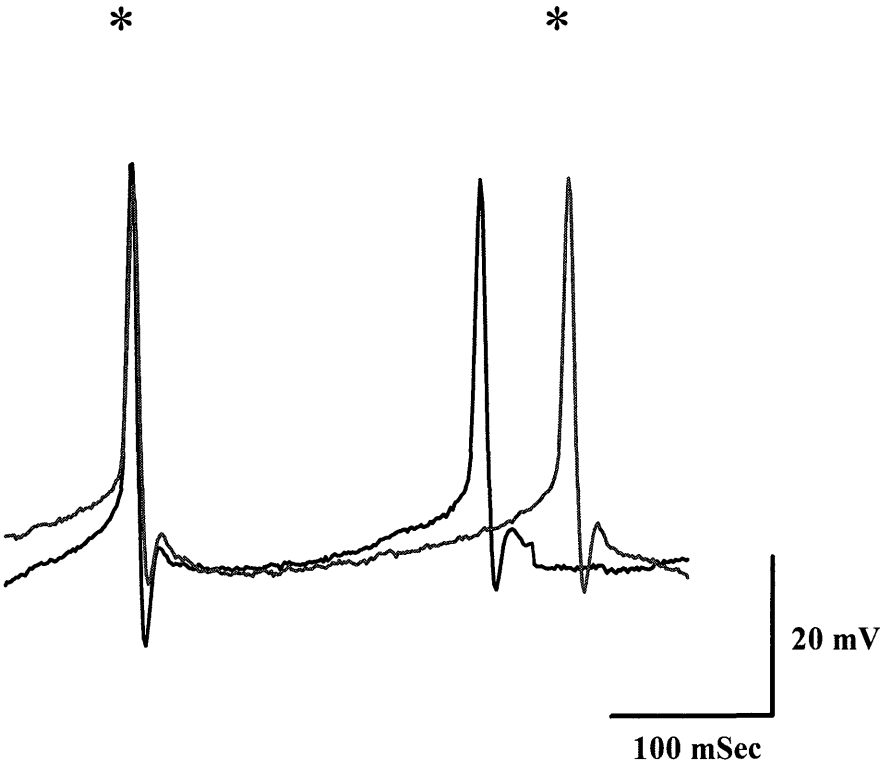


Figure 3.4. Action potential traces for baseline and CGRP (action potentials marked with asterisks) traces showing the decreased pre-potential and increased inter-spike interval in the CGRP responsive action potentials.

FIGURE 3.5

INTRACELLULAR WHOLE CELL RECORDINGS FROM PO/AH NEURONS

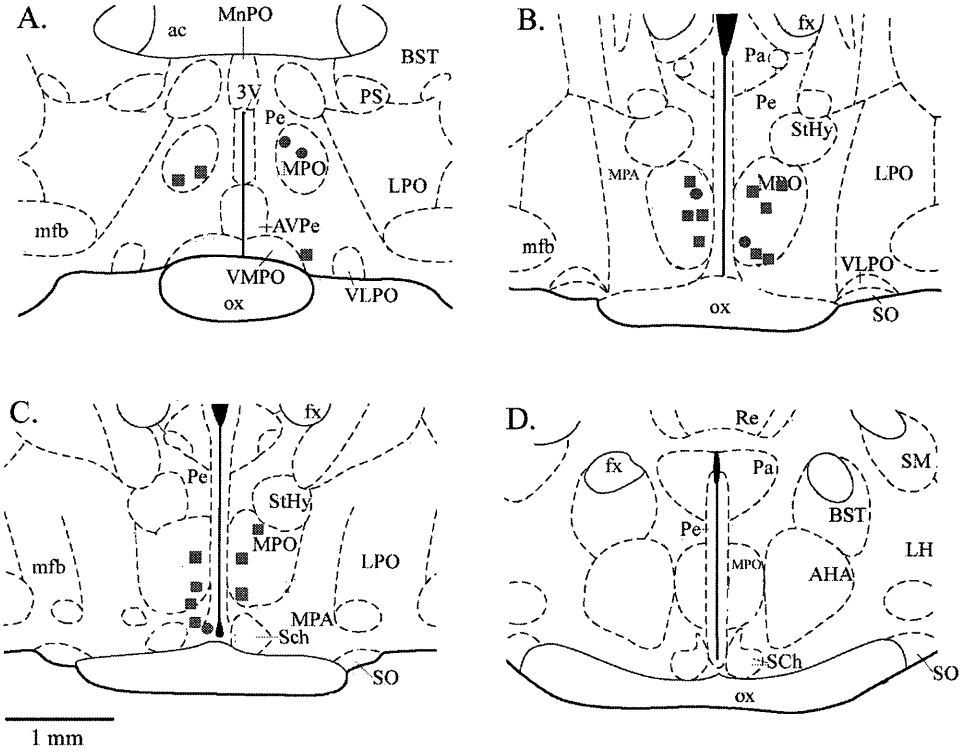


Figure 3.5. Coronal maps of the rat PO/AH showing the location of recorded all neurons. A shows the most anterior section of 400 μm while D is the most posterior. Temperature insensitive neurons = ■. Warm sensitive neurons = ●. 3V, third ventricle; ox, optic chiasm; ac, anterior commisure; Pa, paraventricular nucleus; AHA, anterior hypothalamic area; Pe, periventricular hypothalamic nucleus; AVPe, anteroventral periventricular nucleus; PS, parastial nucleus; BST, bed nucleus lamina terminalis; Re, reunions thalamic nucleus; fx, fornix; SCh, suprachiasmatic nucleus; LH, lateral hypothalamic area; SM, nucleus of the stria medullaris; LPO, lateral preoptic area; SO, supraoptic nucleus; mfb, medial forebrain bundle; StHy, striohypothalamic nucleus; MnPO, median preoptic nucleus; VLPO, vontrolateral preoptic nucleus; MPA, medial preoptic area; VMPO, ventromedial preoptic nucleus; MPO, medial preoptic nucleus.

4. Discussion

Neurons in the PO/AH have been shown to integrate peripheral, core and local temperature in order to establish a set-point temperature for the body (Boulant, 1981). An enduring model for set-point temperature proposed by Hammel suggests that temperature insensitive and warm sensitive neurons have opposing synaptic input on effector neurons. The temperature at which these opposing synaptic activities converge creates the body's set point temperature. Increases in bodily temperature results in the activation of heat loss responses, while decreases in temperature elicit heat production and retention responses. It has been shown that a hyperthermic shift in the set point temperature can occur as a result of changes in the firing rate of either temperature insensitive or warm sensitive neurons (Ranels and Griffin, 2003, Ranels and Griffin, 2005).

A previous extracellular study showed that a hyperthermic shift could result from the application of CGRP, a hormone known to increase in the blood during a hot flash and have vasodialatory effects (Spetz et al, 2001, Wyon et al., 1998). The study presented in Chapter 2 showed that a possible mechanism for the hyperthermic shift in temperature was an increase in the firing rate of temperature insensitive neurons and a decrease in the firing rate of warm sensitive neurons. The current study used the intracellular recording technique of whole cell patch clamping to study the cellular effects of CGRP on neurons of the PO/AH. The results indicate that CGRP caused similar firing rate responses for both temperature insensitive and warm sensitive neurons. The major difference between the previous extracellular study and the current intracellular study was the magnitude of firing rate responses with the intracellular study showing smaller

changes in firing rate. The most reasonable explanation for decreased responses to CGRP during intracellular recording is the effect of cellular washout into the glass electrode during a recording.

In accordance with the extracellular study, temperature insensitive neurons showed an increase or no change in firing rate in response to CGRP while warm sensitive neurons showed a decrease in firing rate. The changes in firing rate for both temperature insensitive and warm sensitive neurons were not a result of changes in resting membrane potential, action potential amplitude or duration. Furthermore, temperature insensitive neurons showed no change in the frequency of synaptic input in response to CGRP application. Warm sensitive neurons did have an increase in the frequency of inhibitory and excitatory synaptic input. Despite this increase in synaptic frequency, this is not considered a mechanism for changes in firing rate due to CGRP because both inhibitory and excitatory inputs increased, leading to no net effect on a neuron.

The changes in firing rate that were seen in temperature insensitive and warm sensitive neurons in response to CGRP can be explained by two different mechanisms. Responsive warm sensitive neurons, which decrease their firing rates in response to CGRP, show a decreased prepotential rate of rise. This resulted in a longer inter-spike intervals and decreased firing rates. A study by Griffin et. al (1996) showed that changes in the prepotential result in firing rate changes of warm sensitive neurons in the PO/AH. Furthermore, the change in prepotential was in part a result of decreases in the potassium A current (I_A). In warm sensitive neurons, an increased in activation of the I_A at higher temperatures explains the increases in firing rate at higher PO/AH temperatures. The

exact ionic conductance in warm sensitive neurons that is altered by CGRP was not determined but further studies should focus on the potassium A current.

Temperature insensitive neurons responded to CGRP with an increase in firing rate activity and an increase in threshold potential. The exact mechanism by which increases in threshold leads to increased firing rate activity has yet to be determined. Further studies investigating the specific ion channels that are affected will allow for a better explanation of the effects of CGRP on responsive temperature insensitive neurons. Overall, the responses of PO/AH neurons to CGRP indicate a hyperthermic shift in body temperature that can be explained by the increased firing rates of temperature insensitive neurons and the decreased firing rates of warm sensitive neurons.

Acknowledgements

This Research was supported by NSF grant NBN-9983624, the Howard Hughes Medical Institute and the Borgenicht Foundation Grant. We would like to thank Bethany Schaffer and Andy Speidell for their assistance in this work.

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Chapter IV

Conclusions

The studies presented in the previous chapters employed two electrophysiological techniques to investigate PO/AH thermoregulatory neurons and their responses to the hormone, calcitonin gene-related peptide (CGRP). The less invasive technique of extracellular single unit recording was first used to characterize changes in firing rates of PO/AH neurons in response to temperature and CGRP. A majority of temperature insensitive neurons did not show a significant change in firing rate in response to temperature. However, those that did respond had an increased firing rate as temperature was increased and a decrease in firing rate as temperature was decreased. After neuronal thermosensitivity was determined, CGRP (10 μM) was applied micro drop technique to determine the effects of CGRP, if any, on the thermally classified neurons of the PO/AH. Temperature insensitive neurons that responded to CGRP showed an increase in firing rate while warm sensitive neurons decrease their firing rate. In reference to the current model for set point temperature in the hypothalamus, the results seen in the extracellular study would indicate a hyperthermic shift in body temperature in response to CGRP.

Studies have shown that several homeostatic substances such as testosterone, estrogen and glucose levels can affect the firing rate of PO/AH neurons. Some of the temperature insensitive and temperature sensitive neurons showed a response to one or multiple factors, while many neurons showed no response to a factor(s) (Silva et. al,

1984, Boulant et. al, 1986). In the present studies presented in Chapter 2 and Chapter 3, CGRP produced a variety of firing rate changes on PO/AH neurons. It can be speculated that the temperature insensitive neurons that increased their firing rate and the warm sensitive neurons that decreased their firing rate in response to CGRP are the specific neurons responsible for the hyperthermic shift in body temperature seen during a hot flash. The temperature insensitive neurons that showed no change in firing rate in response to CGRP are most likely still involved in set point temperature regulation. Furthermore, it is possible that the neurons that did not respond to CGRP are involved in regulating other homeostatic conditions within the body.

The current project progressed to a second electrophysiological technique of whole cell patch clamping. The goals of the study were to determine if this technique could be used to study the effects of CGRP on PO/AH neurons and if so, study the cellular affects that lead to firing rate changes. The results showed that whole cell patch clamping was a satisfactory technique but the main problem was a decrease in the magnitude of responses to CGRP, most likely due to washout into the glass electrode. Further studies should use a perforated patch technique to prevent to prevent washout from occurring and increase the magnitude of responses to CGRP. Despite the problem of wash out during whole cell patch clamp recordings, the cellular responses to CGRP which drive alterations in firing rates were able to be studied. It was found that two different mechanisms were responsible for the two responses seen in PO/AH neurons. Temperature insensitive neurons that increased in firing rate showed an increase in threshold potential for an action potential. The exact ion channels and mechanisms that are responsible for the increased threshold levels are yet to be determined. Conversely,

warm sensitive neurons showed a decrease in firing rate activity, which was a result of decreased prepotential rate of rise. Previous studies by Griffin et. al (1996) have showed that warm sensitive neurons increase their firing rate in the hyperthermic range due to an increased inactivation of the potassium A current. Further studies should evaluate the role of the potassium A current in creating a decreased firing rate responses to CGRP in warm sensitive neurons.

Previous studies have shown that after binding to its receptor, CGRP activates a second messenger system that causes an increase in the production of cAMP (Leuthauser et. al, 2000). While CGRP has not been shown to increase cAMP levels in hypothalamic neurons, in Figure 4.1, I hypothesize that CGRP could bind to the RAMP1 and CRLR receptor sub-units on both temperature insensitive and temperature sensitive neurons of the PO/AH and result in an increase in cAMP production. An increase in cAMP levels due to CGRP activating its receptor may increase production of protein kinase A (PKA) and result in phosphorylation of ion channel(s). Since my data suggests that CGRP is altering the conductances of two or three different ion channels, it is plausible that PKA is phosphorylating a voltage sensitive Ca^{2+} or Na^{+} channel in temperature insensitive neurons. Conversely, Figure 4.2 shows a warm sensitive neuron where PKA may be activating a mediator that decreases the inactivation rate of the potassium A current by CREB phosphorylation. Decreasing the inactivation of the potassium A current would increase the K^{+} conductance leading to a decrease in prepotential rate of rise and a lower firing rate.

The establishment and regulation of hypothalamic set point temperature is based on an enduring neural network model, proposed by Hammel in 1965. Based on

Hammel's model the investigations in Chapters 2 and 3 explain how CGRP causes a hyperthermic shift in the hypothalamic set point temperature. Shown in Figure 4.3 is a new model for hypothalamic set point temperature regulation that includes CGRP and its effects on set point temperature. The new model shows four populations of neurons that are involved in set point temperature regulation. Of the four populations, one population of the warm sensitive neurons responds to CGRP by decreasing its firing rate and thus decreasing its synaptic input to effector neurons. Another population of temperature insensitive neurons respond to CGRP by increasing their firing rates and as a result, increasing their synaptic input to effector neurons. Overall, during periods of increased CGRP concentrations within the hypothalamus there is a change in the firing rates of two populations of spontaneously active neurons that leads to a hyperthermic shift in hypothalamic set point temperature.

FIGURE 4.1

INTRACELLULAR EFFECTS OF CGRP ON A TEMPREATURE INSENSITIVE PO/AH NEURON

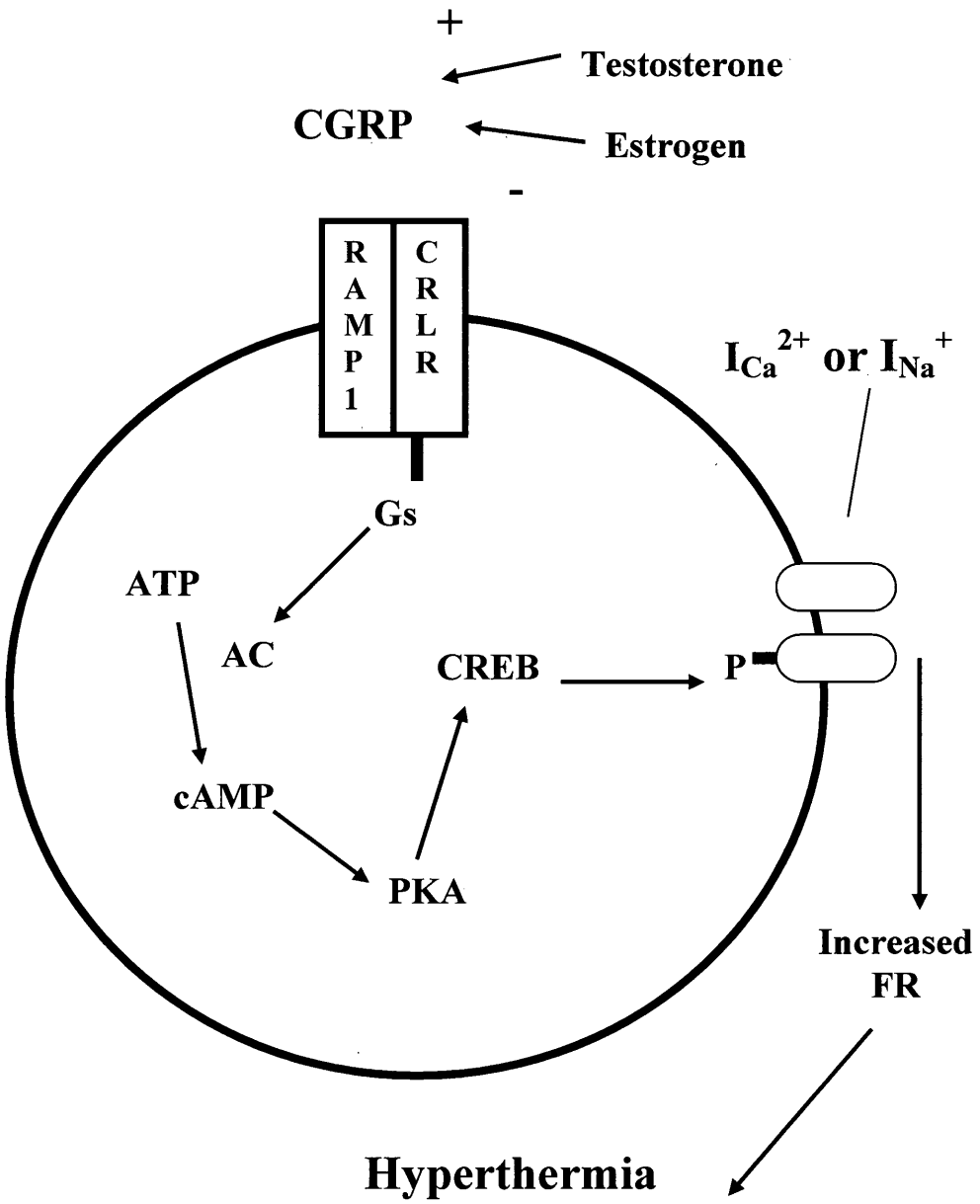

Figure 4.1. Schematic shows a possible pathway that would be activated by CGRP binding to a temperature insensitive neuron leading to an alteration of a specific ion channel and a change in firing rate.

FIGURE 4.2

INTRACELLULAR EFFECTS OF CGRP ON A WARM SENSITIVEV PO/AH NEURON

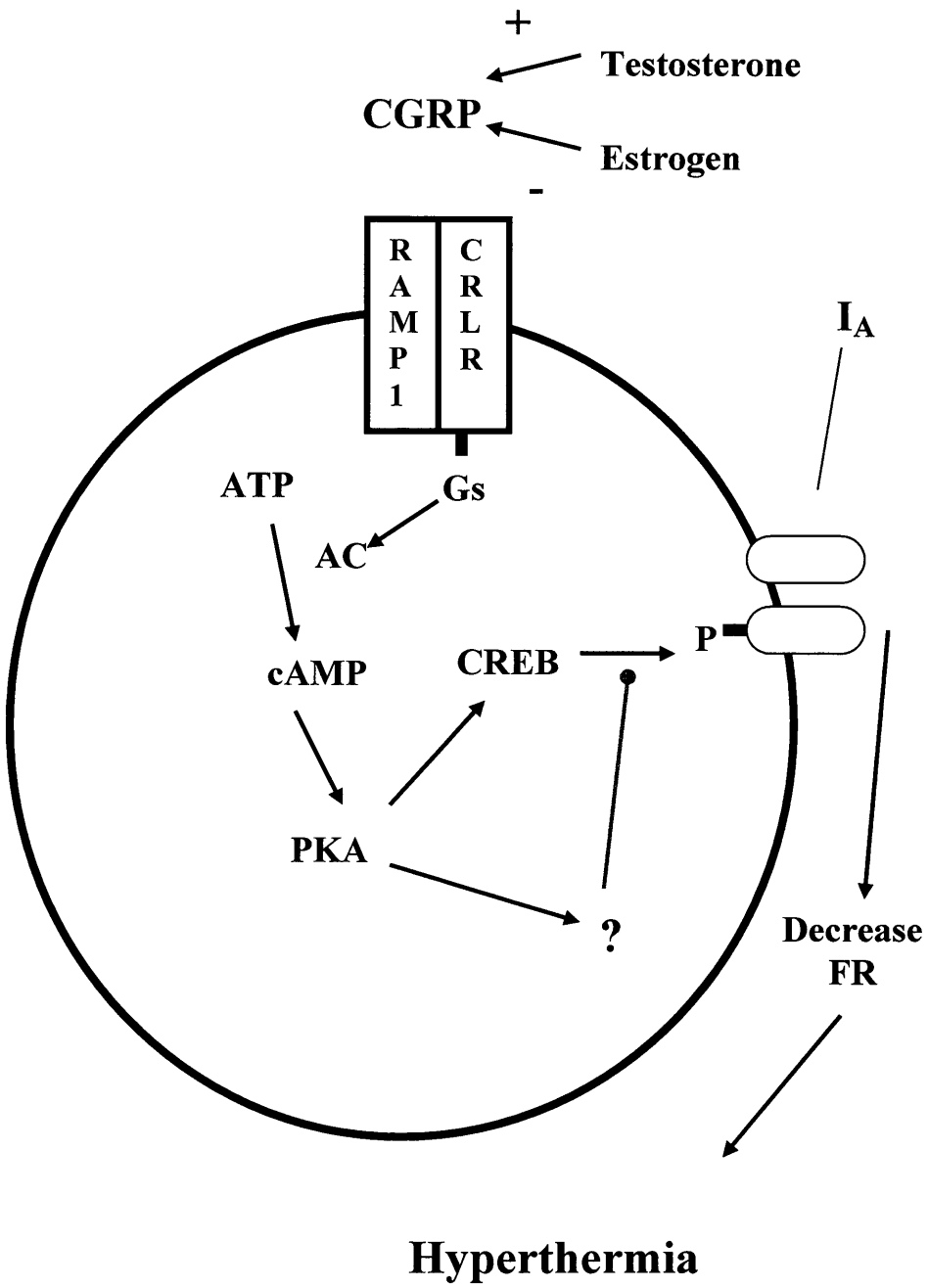

Figure 4.2. Schematic shows a possible pathway that would be activated by CGRP binding to a warm sensitive neuron leading to an alteration of a specific ion channel and a change in firing rate.

FIGURE 4.3

HAMMEL'S MODEL:
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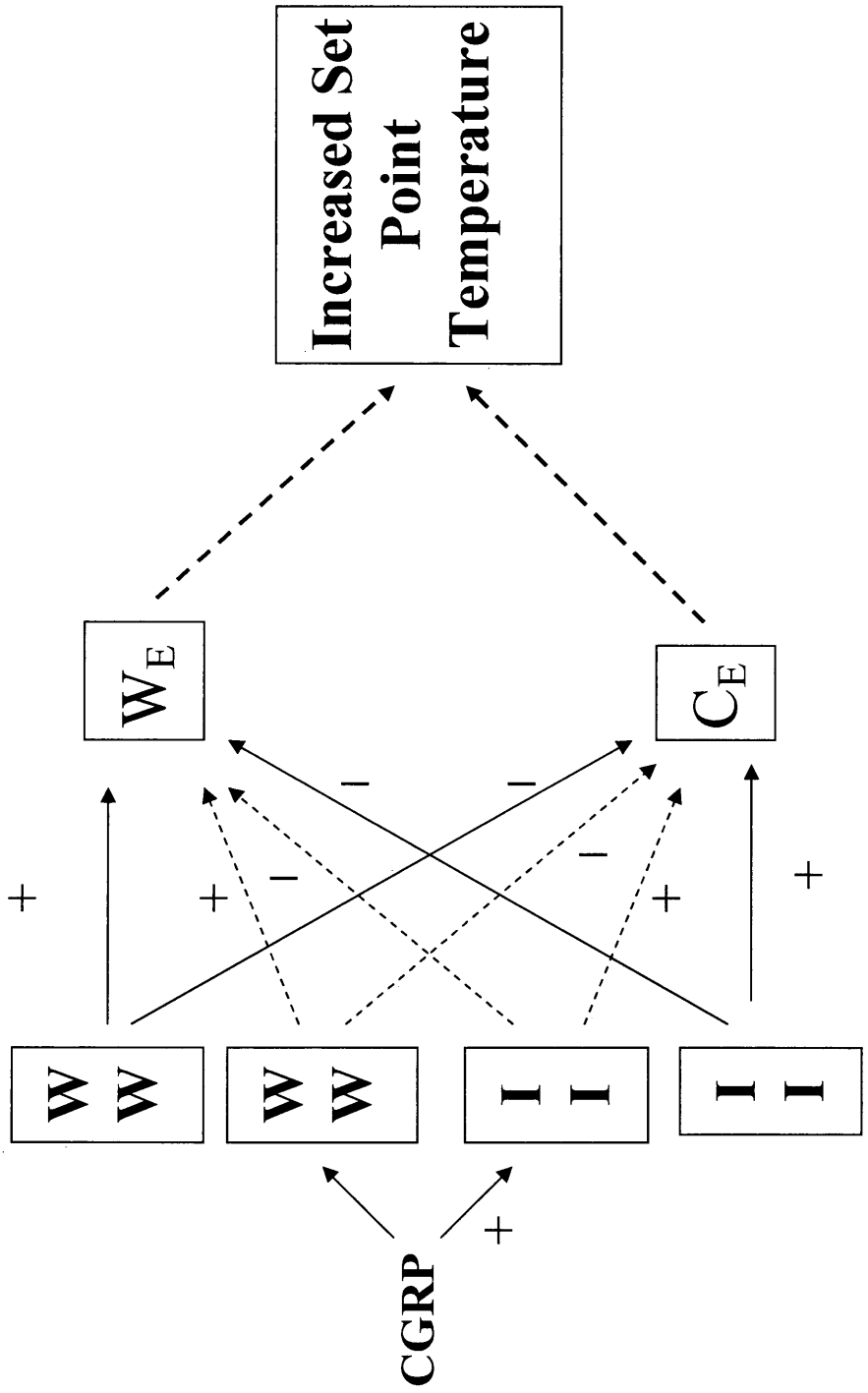

Future Directions

As previously described, a future project should begin to focus on determining the specific ion channels that are involved in creating responses CGRP in temperature insensitive and warm sensitive neurons of the PO/AH. By using current clamp studies, specific ions channels (i.e. I_A, Ca^{2+}, Na^{+}) could be identified as possible sites to be blocked by drugs to prevent the hyperthermic state seen in a hot flash.

The intracellular second messenger system, which leads to an alteration of channel activity, is unknown. Previous studies have shown that CGRP binding to smooth muscle cells opens ATP-sensitive K^{+} channels through a cAMP-dependent pathway (Quayle et. al., 1994). Also, CGRP acts on cortical neurons to increase cAMP levels and suppress delayed K^{+} rectifier currents, potassium A currents (I_A) and Ca^{2+} conductance (Zona et. al., 1991). A future study could use a cAMP blocker to determine if responses to CGRP could be blocked by an inhibition of a cAMP pathway.

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VITA

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