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Energy Balance Effects of Central Melanocortin, Cocaine and Amphetamine Related Transcript and Leptin: Moving Outside of the Hypothalamic Box.

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Abstract
Due to increasing rates of obesity and its comorbidities, there is tremendous interest in the central nervous system (CNS) control of energy balance. This basic science interest is guided in part by the need to develop effective drugs for the overweight and the obese patient. Despite a large literature, our understanding of the circuits and neurochemical receptors that mediate energy balance is still limited. The studies described here address this limitation by defining neural circuits that mediate melanocortin´s effects on energy balance. Disruption in CNS melanocortin receptor (MCR) signaling is the single largest monogenic cause of human obesity and also produces severe hyperphagia and reduced energy expenditure in rodents. Forebrain ventricular application of MCR agonists, triggers sympathetically mediated expenditure responses that have been attributed to signaling at hypothalamic structures. However, caudal flow of the injected ligands in CSF makes them available to extrahypothalamic sites. Given the widespread distribution of MCRs it is impossible to define which MCR-bearing neurons - among them several hypothalamic and hindbrain nuclei- contribute to the observed effects. Here we characterized the respective contributions of the hypothalamic and caudal hindbrain MCRs to energetic and intake control with ventricular (3rd and 4th v) as well as selective parnechymal MCR agonist delivery. Results demonstrate that thermogenic, cardiovascular and anorexic responses of similar size and duration can be obtained by stimulation of several hypothalamic and hindbrain MCR populations. Using an antagonist treatment we evaluated the endogenous hindbrain MCR contributions to the intake and thermogenic responses driven by leptin (a hormone produced by the adipose tissue) and an exposure to palatable, high-energy diet. Results indicated that hindbrain MCRs are required for mediation of anorexic and thermogenic effects of hindbrain leptin and for limiting overeating induced by palatable high-energy diet. The data presented here confirm the hypothesis that the melanocortin system's contribution to food intake and energy expenditure is distributed across spatially distinct regions of the brain. Taken together results demonstrate the presence of an independent hindbrain MCR-driven energy balance circuitry that responds to peripheral inputs (e.g. leptin) and physiological challenges (e.g. high-energy diet) similarly to what has been earlier ascribed to the hypothalamic MCR populations.

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ENERGY BALANCE EFFECTS OF CENTRAL MELANOCORTIN, COCAINE AND AMPHETAMINE RELATED TRANSCRIPT AND LEPTIN: MOVING OUTSIDE OF THE HYPOTHALAMIC BOX.

Karolina Patrycja Skibicka

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ABSTRACT

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KAROLINA PATRYCJA SKIBICKA

ADVISOR: HARVEY J GRILL

Due to increasing rates of obesity and its comorbidities, there is tremendous interest in the central nervous system (CNS) control of energy balance. This basic science interest is guided in part by the need to develop effective drugs for the overweight and the obese patient. Despite a large literature, our understanding of the circuits and neurochemical receptors that mediate energy balance is still limited. The studies described here address this limitation by defining neural circuits that mediate melanocortin´s effects on energy balance. Disruption in CNS melanocortin receptor (MCR) signaling is the single largest monogenic cause of human obesity and also produces severe hyperphagia and reduced energy expenditure in rodents. Forebrain ventricular application of MCR agonists, triggers sympathetically mediated expenditure responses that have been attributed to signaling at hypothalamic structures. However, caudal flow of the injected ligands in CSF makes them available to extrahypothalamic sites. Given the widespread distribution of MCRs it is impossible to define which MCR-bearing neurons - among them several hypothalamic and hindbrain nuclei- contribute to the observed effects. Here we characterized the respective contributions of the hypothalamic and caudal hindbrain MCRs to energetic and intake control with ventricular (3^{rd} and 4^{th} v) as well as selective parnechymal MCR agonist delivery. Results demonstrate that thermogenic, cardiovascular and anorexic responses of similar size

and duration can be obtained by stimulation of several hypothalamic and hindbrain MCR populations. Using an antagonist treatment we evaluated the endogenous hindbrain MCR contributions to the intake and thermogenic responses driven by leptin (a hormone produced by the adipose tissue) and an exposure to palatable, high-energy diet. Results indicated that hindbrain MCRs are required for mediation of anorexic and thermogenic effects of hindbrain leptin and for limiting overeating induced by palatable high-energy diet. The data presented here confirm the hypothesis that the melanocortin system's contribution to food intake and energy expenditure is distributed across spatially distinct regions of the brain. Taken together results demonstrate the presence of an independent hindbrain MCR-driven energy balance circuitry that responds to peripheral inputs (e.g. leptin) and physiological challenges (e.g. high-energy diet) similarly to what has been earlier ascribed to the hypothalamic MCR populations.

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CHAPTER 1: GENERAL INTRODUCTION

I. Energy Balance Control

According to the center for disease control, about 65% of Americans are either overweight or obese (Hedley et al., 2004). In the last decade obesity and comorbid pathologies including type 2 diabetes, hypertension, and cardiovascular disease have been dramatically increasing, becoming a great burden not only on the health care system but also on economy. The desire to prevent these pathologies has sparked an interest in defining the mechanisms of neural control of energy balance. Energy balance is achieved through the coordinated control of two systems, one controlling energy intake (feeding) and the other energy expenditure (limitation or facilitation of the use of available energy resources). Changes in feeding patterns have been most commonly linked to increases in body weight. Increases in the consumption of fats (particularly saturated fats), and simple carbohydrates, have contributed to the increases in the prevalence of obesity. Chronic overconsumption of calorically dense meals causes a disruption in homeostatic mechanisms of body weight control, often progressively leading to an allostatic level of energy balance that defends the increased body weight even after the intake has been reduced. This 'hard-wired' defense of body weight has been attributed to the ineffectiveness of many weight reduction diets. This brings attention to the second essential component of energy balance: energy expenditure (EE). Reduction in EE also contributes to the obesity epidemic. Therefore treatments targeting both energy intake and EE will be more effective then dieting alone. Clearly more attention needs to be focused on the EE side of the energy balance equation.

EE in mammals can be divided in to three main components: basal metabolism, adaptive thermogenesis and non-resting energy expenditure (physical activity). Adaptive thermogenesis is a response induced by challenging environmental stimuli such as cold, food consumption and pathogenic stimuli including infection and stress (Dulloo, 2002; Dulloo et al., 2004). Thermogenic responses are tuned to these different environmental stimuli. Relevant environmental and internal stimuli are processed and integrated, at a number of CNS sites and if need arises, thermogenic effectors are stimulated via the sympathetic nervous system (SNS). A number of sympathetic pre-motor nuclei drive thermogenic effectors. SNS control of brown adipose tissue (BAT) has the largest thermogenic capacity in rodents (Cannon and Nedergaard, 2004).

Various peptide systems contribute to both sides of energy balance control. The major contributors can be organized into two classes: anabolic and catabolic. On the catabolic side, central melanocortin system and leptin are of fundamental importance. In addition, cocaine and amphetamine related peptide (CART) has recently emerged as a potential counterpart of melanocortin in the energy balance effects. The work in this dissertation concerns the contribution of all three peptides to the control of feeding behavior and various aspects of energy expenditure.

II. Central Melanocortin System

a. General

Exogenous stimulation of the melanocortin system decreases food intake and increases EE; it is therefore, a potential target for treatment of obesity. While the melanocortin influence on feeding is under extensive scrutiny, much less is known about the melanocortin circuitry contributing to EE. The principal goals of the work proposed

below are to elucidate the central melanocortin circuits that contribute to EE control, to distinguish potentially separate contributions of melanocortin receptors (MCRs) in hypothalamus/forebrain and caudal brainstem, and to distinguish their engagement under different physiological conditions.

The central melanocortin system is unique since it is composed of not only neurons that produce the endogenous agonists for MC3R and MC4R, the two central receptors, but also of neurons that produce an endogenous antagonist (Cone, 2005). The endogenous agonists, α-MSH, β-MSH and γ-MSH, are products of the proopiomelanocortin (POMC) gene neurons which are located only in two regions of the brain: hypothalamic arcuate nucleus (ARC), and the nucleus of the solitary tract (NTS) within the caudal brainstem. Neurons producing the antagonist (AgRP) are present in the ARC (Broberger et al., 1998; Bagnol et al., 1999; Haskell-Luevano et al., 1999). The MC4Rs on the other hand, are widely distributed across the neuraxis, from the forebrain to the caudal brainstem (Mountjoy et al., 1994; Kishi et al., 2003). MC3R mRNA is much less distributed than MC4R, with most abundant expression sites in hypothalamic areas participating in thermogenesis and cardiovascular function. High MC3R expression has been identified in the ARC on POMC positive neurons and on terminal fields of these neurons, suggesting a possible role of MC3R as an autoreceptor in this area (Roselli-Rehfuss et al., 1993; Marks et al., 2006). None of the MC3R positive cells were found in the caudal brainstem regions such as NTS, pointing to diversity of MCR expression among different levels of the melanocortin system (Roselli-Rehfuss et al., 1993).

b. Food intake

The central melanocortin system is already recognized as an important contributor to feeding control in the mammalian energy balance equation. Disruption in CNS melanocortin activity is the single largest monogenic cause of human obesity. Polymorphisms and mutations of POMC gene as well as mutations of MC4R receptor have been linked to human obesity (Comuzzie et al., 1997; Krude et al., 1998; Hinney et al., 1999; Hixson et al., 1999). Similarly a deletion of MC4R or POMC gene, or overexpression of AgRP in mice, causes a pronounced obese phenotype (Klebig et al., 1995; Huszar et al., 1997; Wolff et al., 1999; Yaswen et al., 1999). Most pharmacological studies to date have focused on the intake effects of melanocortins. MC agonist and antagonist, administered directly into the rodent brain, cause a large decrease and a large long lasting increase in food intake respectively. However, an alteration in food intake following a disruption of melanocortin signaling is not the only factor contributing to obesity. MC4R KO mice for example have significantly altered EE, independent of the food intake effects, with 20% decrease in resting EE as measured by oxygen consumption, compared to weight matched controls (Ste Marie et al., 2000).

For the energy balance field many functions are localized to hypothalamic neurons and circuits. This is certainly true for the analysis of feeding control. Also for EE, research has focused on the sensory, integrative and motor output control mechanisms seated within hypothalamic nuclei and related forebrain structures. Unfortunately this focus has neglected potentially, and in some cases demonstrably, critical contributions of extrahypothalamic structures particularly those in caudal brainstem structures. For feeding function, the contribution of the caudal brainstem melanocortin system is highlighted in previous work of our laboratory. Emphasis on distributed control was

provided by the demonstration that local stimulation of MCRs in anatomically distinct regions (lateral and 4th ventricle injections) gives rise to an anorexic response (to melanocortin agonists) and orexigenic response (to antagonists) (Grill et al., 1998). The case was strengthened by the demonstration that the same response was obtained with parenchymal injections in the dorsal medulla at doses well below threshold for the ventricular effect (Williams et al., 2000a). This is not to discount the forebrain contribution. Indeed responses from the third ventricle were observed even when the caudal flow of CSF was blocked with a cerebral aqueduct plug [unpublished observations, for method see (Faulconbridge et al., 2005)]. Here we will apply a similar strategy to explore the melanocortin contribution to EE.

c. Thermoregulation

Anatomical analysis of CNS networks controlling SNS outflow to BAT, with pseudorabies virus injections into the intercapsular BAT, show convergence of the retrogradely labeled structures and known distribution of MC4Rs and MC neuron projections. As expected from literature on hypothalamic involvement in energy balance, BAT projections were traced with transsynaptically transported label to many hypothalamic sites including medial preoptic area of the hypothalamus (MPOA), as well as ventromedial and dorsomedial hypothalamus (Bamshad et al., 1999; Oldfield et al., 2002; Morrison, 2004). Importantly, all of the labeled hypothalamic nuclei receive melanocortin projections and express MCR (Jacobowitz and O'Donohue, 1978; Palkovits et al., 1987; Joseph and Michael, 1988; Sim and Joseph, 1994; Fan et al., 2005; Voss-Andreae et al., 2007). However the overlapping melanocortin projections with BAT retrograde tracing were also found in extrahypothalamic sites, particularly in caudal brainstem nuclei including: parabrachial nucleus (PBN) and raphe pallidus (RP), with

many BAT and melanocortin projections converging at the intermediolateral column, the site of BAT preganglionic sympathetic neurons (Voss-Andreae et al., 2007). In addition, BAT projections could also be traced to ARC and NTS, the only two CNS sites containing POMC neurons (Voss-Andreae et al., 2007). Such points of overlap may suggest that both hypothalamic and caudal brainstem divisions of the MC system may modulate BAT activity. A level of complexity is added by the interaction between the brainstem and hypothalamic melanocortin neuron signaling, made possible by the dual innervation of given structures (including NTS, PBN, ARC, PVN) by both POMC neuron centers [for review see (Fan et al., 2007)].

Pharmacological stimulation of central MCRs, with MTII (selective for MC3R and MC4R), in rats and wild type mice increases oxygen consumption (Fan et al., 1997; Zhang et al., 2004). Hypothalamic MCR stimulation via MTII injections to third ventricle of free feeding rats during the dark cycle increase EE as reflected in increases in core temperature (Murphy et al., 2000). MTII administration to the 3rd ventricle also dose-dependently increased sympathoexcitation to BAT, the effect was abolished by coadministration of MC3R/4R antagonist (SHU 9119) (Haynes et al., 1999). Chronic AgRP (MC3R/4R antagonist) infusion to the third ventricle decreases BAT UCP-1 (uncoupling protein associated with increased thermogenesis in BAT) expression independent of its orexigenic effects (Small et al., 2003). Much less is known about caudal brainstem MCR-mediated control of BAT thermogenesis. Forebrain but also caudal brainstem MCR stimulation via semi-chronic (3 injections over 36 hours) MTII administration to third and fourth ventricles leads to increased UCP-1 expression, which was shown to be dependent on sympathetic mediation as the denervation of BAT abolished the response (Williams et al., 2003). Taken together, pharmacological stimulation experiments suggest that both caudal brainstem and hypothalamic MCRs

have the potential to drive sympathetically mediated BAT-dependent thermogenic response. Therefore we will give both receptor populations equal attention while examining the hypothesis that anatomically distributed MCR expressing regions contributes to activation of thermogenic responses.

d. Cardiovascular regulation

Anatomical and pharmacological evidence points to a prominent role of melanocortins in cardiovascular function. Patients with dysfunctional MCRs have lower blood pressure and heart rate compared to weight matched controls, despite their obesity (Greenfield et al., 2009). Both parameters are also increased when MCR agonist is given to normal subjects (Greenfield et al., 2009). Central MCR agonist application produces profound and long lasting increases in blood pressure and heart rate in rats (D Ramaekers, 2002). Those effects are mediated by SNS excitation of the heart (Kuo et al., 2004). Conversely, blockade of central MCRs decreases both blood pressure and heart rate (Kuo et al., 2003). Both effects are independent of the feeding effects of melanocortins as they persist in pair fed rats (Kuo et al., 2003). The dissociation of the feeding and cardiovascular effects of MCR stimulation is further strengthened by the fact that obesity induced by high fat feeding attenuates the anorexic effect of MCR agonist, however the tachycardic effects persist (Silva et al., 2006). This dissociation is of critical importance as it suggests that MCR signaling underlies the hypertension and tachycadia observed in obese patients (without MCR mutations). Despite the broad distribution of MCRs in areas associated with cardiovascular control the literature has ascribed the action of central (lateral or 3rd ventricular) MCR agonist and antagonist application to activation of MCRs in hypothalamic nuclei (Kuo et al., 2003). In fact, ventricular drug application potentially allows for stimulation of not only the

hypothalamic MCRs but also the hindbrain MCRs, as the drug flows caudally with CSF. Thus, it is not at all clear which of the MCR expressing nuclei contribute to the ventricular effect. The specific role of hindbrain MCR in those effects remained largely unexplored. Therefore here we specifically address the role of hindbrain MCRs in the cardiovascular regulation.

e. Physical activity

A decrease in spontaneous locomotor activity can be a contributing factor to the pathogenesis of obesity and fat pad deposition (Castaneda et al., 2005). While little is known about the neural basis of this response, available evidence implicates many of the neuropeptides associated with food intake in the control of physical activity. Central (ventricular) MTII stimulation increases and endogenous antagonist – AgRP application decreases spontaneous locomotor activity in rats (Hwa et al., 2001; Tang-Christensen et al., 2004; Koo et al., 2008). Similarly, as for the thermic and cardiovascular effects of melanocortins, the neuroanatomical substrate of this MCR function is unclear. Our studies will therefore evaluate the role of hindbrain, as well as several forebrain/hypothalamic MCR-expressing nuclei in physical activity control.

III. Central Effects of Leptin

Leptin, a circulating hormone produced by the white adipose tissue, like melanocortin, is anorexic and thermogenic when applied centrally (Scarpace et al., 1997; van Dijk et al., 1999; Rahmouni et al., 2002). POMC neurons and MCRs are an important downstream mediator of the energy balance effects of basal forebrain-directed, third ventricular (3rd v) leptin delivery (Seeley et al., 1997; Satoh et al., 1998; Haynes et al., 1999). This perspective is supported by experiments that show that: leptin

signaling in these neurons increases POMC gene expression (Schwartz et al., 1997; Elmquist, 2001) and pretreatment with MCR antagonist attenuates the anorexic effects as well as some of the SNS- mediated energetic effects of 3rd v leptin delivery (Seeley et al., 1997; Haynes et al., 1999). Similarly, as described above for the melanocortin system, the central actions of leptin have been ascribed to the hypothalamic leptin receptor (ObRb) populations, without much attention given to the two hindbrain ObRb expressing nuclei (NTS, PBN). While evidence exists that part of the anorexia induced by leptin is mediated by NTS ObRb (Huo et al., 2007), the energetic effects of selective hindbrain ObRb stimulation have not been examined. Here we examine a role of hindbrain ObRb stimulation in the thermoregulation, cardiovascular functions and activity regulation and evaluate whether hindbrain MCRs contribute to the energy balance effects triggered by selective hindbrain leptin stimulation.

IV. Cocaine and amphetamine regulated transcript

In arcuate hypothalamic neurons CART is co-expressed with POMC. Increase in melanocortin signaling produces hypophagia, hyperglycemia, hyperthermia, tachycardia and increased activity (Fan et al., 2000; Gutierrez-Juarez et al., 2004; Skibicka and Grill, 2008a). Leptin upregulates and food deprivation downregulates hypothalamic expression of POMC as well as CART (Kristensen et al., 1998). These functional changes and the co-expression of CART with POMC, led to the suggestion that CART peptides have a catabolic function – with treatment producing reductions in food intake and increases in energy expenditure. The fact that 3rd and 4th v CART injection produces anorexia and arcuate and paraventricular nucleus CART delivery increases UCP-1 expression in interscapular brown adipose tissue is consistent with that notion (Wang et al., 2000; Kong et al., 2003). However, the EE effects of CART have been insufficiently

investigated, as those investigations have not gone beyond measuring UCP-1, which is a useful correlate of BAT activity, although not an accurate measurement of BAT thermogenesis. Here we investigate whether the EE function of CART parallels that of melanocortin at both the hindbrain and forebrain level of the neuraxis.

V. **Hindbrain competence (Chronic decerebrate preparation)**

We may demonstrate effects of MCR agonist and antagonist in hindbrain on thermogenesis. Such results, as argued, would stand alongside similar demonstrations for isolated stimulation of forebrain structures, making part of the case for anatomically distributed controls for thermogenesis most generally, and specifically for the melanocortin contribution. These outcomes, however, would not speak to the integrative substrates downstream of the MCRs that may be critical for expression and coordination of thermogenic responses. The hindbrain may contain receptor "triggers" for responses, but the hypothalamus may perform essential operations entailing both ascending and descending pathways. The sufficiency of the hindbrain substrates for mediation of responses observed in the whole animal is addressed below in proposed experiments with the utility of the chronic decerebrate (CD) rat preparation. Our laboratory has developed this preparation over many years, and applied it successfully to evaluate the integrative potential of the hindbrain. This work has focused on the control of ingestive behavior and to a lesser extent, endocrine function. It has been shown, for example, that the CD behaves as its neurologically intact control with respect to discriminative response to gustatory stimulation and modulation of meal size by such prototypical treatment as nutrient preloads, sham feeding, food deprivation and systemic administration of CCK and central administration of anorexic agents such as

urocortin-1 [for review see (Grill and Norgren, 1978; Grill and Kaplan, 2002). For feeding function, it can be concluded (from the intact like function in CD) that despite the many deficits of the CD rat (e.g. blindness, anosmia) complete circuits, from sensory inputs to integrative processing and effector commend, are contained within the hindbrain. We propose to determine whether a similar judgment can be derived with respect to thermoregulatory control. At some level, the feeding related work offers some encouragement, because of the coordinated control of effectors on both sides of energy balance; and because many of the same structures in the brainstem are implicated in the control of sympathetic outflow to thermogenic effectors (e.g. NTS, PBN, VLM) are also critical players in control of ingestive behaviors. The story that emerges from the proposed work, however, may not conform to principles of parsimony. A failure of the CD to display normal like responses would point to critical contributions of forebrain substrates, or at least to necessary neural communication that will add to our appreciation of the neuroanatomically distributed system for the control of EE.

VI. Overview of dissertation

The previous literature clearly establishes that administration of melanocortin to forebrain ventricles or directly to the hypothalamic nuclei results in decreased food intake and increased EE but the location of MCRs relevant to these responses had not been throughly explored. Given the presence of MCR in the hindbrain, we hypothesized that the thermogenic, tachycardic and activity responses to melanocortin may be triggered independently by MCR stimulation in the forebrain and separately in the hindbran. Our hypothesis is strenghtened by previous work clearly showing a forebrain independent, hindbrain trigger for MCR stimulation induced hypophagia. In Chapter 2

we find support for this hypothesis by showing that selective hindbrain MCR stimulation produces a pronounced and long-lasting hyperthermia for the BAT and body core, as well as tachycardia and hypophagia. To establish the independence of the neurociruitry mediating those obtained responses we evaluate them in a CD model. Obtaining similar responses in a CD rat as those seen in an intact rat would indicate that the circuitry downstream of the MCR stimulation required for the production of an energetic effect is endemic to the hindbrain. Subsequently we deliver melanocortin agonist (MTII) directly to the hindbrain parenchyma (caudal raphe) at doses below the threshold for the ventricles and obtain feeding and energetic responses, affirming a role for the hindbrain in the energy balance effects of exogenous MCR stimulation.

While Chapter 2 shall provide solid support for a hindbrain site of MCR action, it would not specify which of the several MCR expressing populations within the hindbrain underlie the responses obtained with fourth ventricular stimulation. Considering that several hindbrain MCR expressing nuclei (NTS, RVLM, and PBN) have well established connections to sympathetic and feeding behavior output we hypothesize that all of them contribute to MCR energy balance effects. We also predict that a similar range of energy balance effects could be obtained with MCR stimulation in the hypothalamic nuclei (PVN, RCh), as they are too well connected to the relevant output systems. We evaluate those predictions in Chapter 3 where we provide selective MCR stimulation to each of those nuclei and measure food intake, core temperature, heart rate and activity. If our predictions are correct we would have shown that melanocortinergic control of various parameters of energy balance is beyond doubt distributed across the whole neuraxis.

In Chapter 4 we begin to evaluate the peripheral inputs to the hindbrain melanocortin system. That a peripheral adipostat hormone, leptin, activates the hypothalamic melanocortin producing neurons and MCRs is well established. We examine whether a similar relationship exists for hindbrain selective leptin application. We first establish that hindbrain effects of leptin on energy expenditure are comparable to those obtained with hypothalamic/forebrain application. Next, we evaluate the effects of selective hindbrain MCR blockade on the hindbrain thermogenic, tachycardic and anorexic effects of leptin.

In Chapter 5 we continue the evaluation of peripheral inputs that require downstream hindbrain MCR activation. In Chapter 4 we assessed the hindbrain specific leptin-MCR interaction. Here we examine an interaction of hindbrain MCRs with the endogenous input emerging from high-fat feeding on food intake and energy expenditure. The studies reported here are the first to identify a contribution of endogenous activity at hindbrain MCRs to thermoregulation and cardiovascular function.

In Chapter 6, a similar strategy to that assessing hindbrain MCR effects is applied to examine the intake, blood glucose regulation, core temperature, heart rate and activity effects of hindbrain CART peptide application. In spite of the close association in the literature of CART with melanocortins and its assumed catabolic function, a surprising opposing effect of CART on temperature is obtained – a pronounced and long lasting hypothermia. Subsequently the neurocircuitry underlying this unexpected effect is characterized, to show that, unlike melanocortinergic hyperthermia, the CART hypothermia is mediated by forebrain processing and that both

the anorexia and hypothermia of hindbrain CART are mediated by hindbrain glucagon-like-peptide-1 receptors.

A general discussion of the results obtained across experiments is presented in Chapter 7.

CHAPTER 2.

ENERGETIC RESPONSES ARE TRIGGERED BY CAUDAL BRAINSTEM MELANOCORTIN RECEPTOR STIMULATION AND MEDIATED BY LOCAL SYMPATHETIC EFFECTOR CIRCUITS[1]

ABSTRACT

The central melanocortin system is a critical contributor to energy balance control. Melanocortin receptors (MC-Rs) are widely distributed throughout forebrain and caudal brainstem nuclei. In order to assess the contribution of hindbrain MC-Rs to the control of energy expenditure, the MC3/4R agonist MTII was delivered to either the 4th ventricle or medullary raphe of neurologically intact rats and chronic decerebrate rats and interscapular brown adipose tissue (IBAT) temperature (T_{IBAT}), core temperature (T_C), heart rate (HR) and spontaneous activity were recorded. Fourth ventricular MTII (0.1, 1.0 nmol) significantly increased T_{IBAT}, T_C and HR in intact rats (T_C: +0.33 ± 0.08, +0.41 ± 0.09 ^oC; HR: +40.84 ± 7.29, +69.04 ± 6.83 BPM) and in chronic decerebrates (T_C: +1.39 ± 0.67, +1.52 ± 0.37 ^oC; HR: +83.21 ± 19.2, +107.38 ± 17.65 BPM). Response magnitude was greater in chronic decerebrate rats than in neurologically intact rats. T_{IBAT}, T_C and HR were significantly increased after 10 pmol MTII delivery to the medullary raphe of intact rats, and here too, the response magnitude was greater in decerebrate rats. The hyperthermia, IBAT thermogenesis, and tachycardia observed in chronic decerebrate rats following 4th ventricular and hindbrain parenchymal MTII injections supports the hypothesis that hindbrain MC-R stimulation engages endemic circuits that link sympathetic outflows to thermogenic and cardiac effectors and that

[1] These results were partially reported at the 2006 North American Association for the Study of Obesity Annual Meeting, in Boston MA, and appeared in Endocrinology, 149(7):3605-16, 2008. This work was supported by Systems and Integrative Biology and Behavioral Neuroscience Training Grants, and NIH grant DK-21397.

forebrain processing and forebrain-caudal brainstem communication is not required for response production.

INTRODUCTION

Melanocortin ligands and receptors are an essential component of the central nervous system (CNS) control of energy balance. Mutations of the genes for the melanocortin 4 receptor (MC4-R) or the ligand precursor, pro-opiomelanocortin (POMC) are associated with severe obesity in humans (Yeo et al., 1998; Krude et al., 2003; Dubern et al., 2007). In rodent models, agonist stimulation (pharmacologic or genetic) of MC4-R potently reduces food intake and body weight, while antagonism of the MC4-R results in hyperphagia and obesity (Huszar et al., 1997; Grill et al., 1998; Yaswen et al., 1999). While MC-R (MC4-R, MC3-R) treatment-induced changes in body weight are typically ascribed to changes in food intake, other data suggest that MC-R effects on body weight may also be mediated by alterations in energy expenditure. For example, increased energy expenditure follows peripheral or forebrain ventricular application of MC-R agonists (Haynes et al., 1999; Murphy et al., 2000; Nordheim et al., 2006), while reduced expenditure results from MC-R antagonist treatment or targeted deletion of the MC4-R (Ste Marie et al., 2000; Voss-Andreae et al., 2007). Despite a wide anatomical distribution of MC4-R (Kishi et al., 2003), the field has emphasized hypothalamic contributions to MC4-R mediated energetic effects [e.g. (Harrold et al., 1999; Williams et al., 2000b)] but has not thoroughly characterized the contribution of extra-hypothalamic MC4-Rs or the downstream neural circuitry mediating MC4-R-induced energy balance effects.

This paper addresses whether MC-R bearing neurons in the caudal brainstem contribute to energy expenditure responses and whether the sympathetic output circuits

mediating the energetic effects involve processing by both caudal brainstem and forebrain structures. A useful strategy for highlighting the candidate MC-R bearing neurons comes from studies that examine MC4-R expression in the sympathetic pre-motor neurons controlling interscapular brown adipose tissue (IBAT) temperature. Notable among the identified neurons are the MC4-R expressing neurons in the hypothalamic paraventricular nucleus (PVN), the dorsomedial hypothalamic nucleus (DMH) and lateral hypothalamic area (LH) that are retrogradely labeled with pseudorabies-virus injections into IBAT, a key thermogenic effector in rodents (Cano et al., 2003a; Voss-Andreae et al., 2007). While MC-R bearing hypothalamic neurons, especially those of the PVN, provide a focus for many studies addressing the mediation of MC-R effects on energy expenditure (Harrold et al., 1999; Williams et al., 2000b), MC4-Rs are also expressed extra-hypothalamically in several caudal brainstem nuclei that are linked to the control of IBAT and cardiac responses. Caudal brainstem neurons, including those of the nucleus tractus solitarius (NTS), medullary raphe (raphe pallidus [RPa], raphe obscurus, raphe magnus), parabrachial nucleus, and rostroventrolateral medulla (RVLM) express MC4-R and are retrogradely labeled by IBAT pseudorabies virus injection (Voss-Andreae et al., 2007). A critical role for RPa neurons in the control of IBAT thermogenesis, heart rate and sympathetic outflows is well established (Rathner et al., 2001; Blessing, 2003; Cao and Morrison, 2003; Morrison, 2003; Horiuchi et al., 2004; Morrison, 2004; McAllen et al., 2006; Fan et al., 2007). Neurons expressing MC4-R in the RPa and RVLM are also associated with cardiovascular efferent control by other investigators (Coleman and Dampney, 1995; Samuels et al., 2002; Mountjoy et al., 2003).

To examine the contribution of MC-R-bearing caudal brainstem neurons to energy expenditure control and to determine whether processing endemic to the hindbrain (in the absence of forebrain processing) is required for MC-R mediated autonomic response production, MC-R agonist-induced energetic responses of neurologically intact rats were compared to those of rats whose caudal brainstem was neurally isolated from the forebrain via complete supracollicular transection. IBAT temperature, core temperature, heart rate, and spontaneous activity were monitored in response to hindbrain (4^{th}) ventricular, medullary raphe parenchymal and systemic (i.p.) injection of melanotan II (MTII), a ligand of the MC4-R and MC3-R. Results establish a role for the hindbrain MC-Rs in the control of energy expenditure and show that endemic caudal brainstem circuits are sufficient for hindbrain-generated response production and that hypothalamic processing and hypothalamic-forebrain communication is not necessary.

METHODS

Subjects

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 300–400 g at the time of surgery and housed individually in plastic bins under a 12:12-h light-dark cycle (8:00 am lights on), participated in the five experiments described below. Pelleted food (Purina 5001; St. Louis, MO) and water were available *ad libitum* unless otherwise noted. All procedures conformed to the institutional standards of animal care and use (University of Pennsylvania).

Surgery

Rats were anesthetized with ketamine (90 mg/kg), xylazine (2.7 mg/kg), and acepromazine (0.64 mg/kg) delivered intramuscularly.

4th intracerebroventricular and medullary raphe cannula. Rats in *Experiments 1,3,4,5 and 6* received a fourth-intracerebroventricular (4th i.c.v.) guide cannula (Plastics One, 22-G) with its tip stereotaxically positioned 2.0 mm above the 4th ventricle (coordinates: on the midline, 2.5 mm anterior to the occipital suture, and 4.5 mm ventral to the dura, with injector aimed 6.5 mm ventral from dura). Rats in *Experiments 4,5 and 6* also underwent a decerebration surgery. Rats in *Experiment 3A* received a guide cannula aimed at the medullary raphe (coordinates: on the midline, 3 mm posterior to lambda, and 7.4 mm ventral to the dura, with injector aimed 9.4 mm ventral to dura). Medullary raphe injections for the rats in *Experiments 3B and 6* utilized the 4th i.c.v guide cannula (above) with injectors positioned 5 mm below the guide cannula aimed at the medullary raphe (injector aimed 9.5 mm ventral to dura). Cannulas were attached to the skull with dental acrylic and jeweler's screws and closed with an obturator.

Decerebration surgery. Supracollicular decerebration was performed in two hemi-transection stages separated by at least 1 wk, as previously described (Grill and Norgren, 1978). Decerebrate rats received 4th i.c.v. cannulas during the second hemisection surgery. Pair-fed neurologically intact control rats were also anesthetized on two occasions and implanted with 4th i.c.v. cannulas during the second surgery. Rats recovered for at least 1 wk before the experiment started. The completeness of the intended transection was verified histologically after the experiment. Only rats with a histologically verified complete transection were included in the data analyses.

Telemetric transponder surgery: Telemetric transponders (HRC 4000 Mini-Mitter, VitalView, OR) for recording core temperature (Tc), heart rate (HR) and spontaneous physical activity (SPA) were inserted into the abdominal cavity, with the leads positioned subcutaneously and secured to the chest muscles on either side of the heart with sutures. In *Experiment 3* animals received a smaller telemetric transponder for recording IBAT temperature (T_{IBAT}) and SPA (G2, VitalView, OR). The skin overlying the IBAT pad was opened and the transponder positioned on the right side of IBAT, avoiding the midline vessels and nerves, and secured with sutures to the overlying muscle. In *Experiments 1, 4* and *6,* separate groups of animals were implanted with IPTT-300 (Bio Medic Data Systems, ID) transponders that measured only the T_{IBAT}.

Experimental procedure

Cannula position verification. At least 7 days after surgery, 4th i.c.v. cannula placement was assessed by measurement of the sympathoadrenal mediated glycemic response to 5-thio-D-glucose [210 μg in 2 μl of artificial cerebral spinal fluid (aCSF)] (Ritter et al., 1981). A post-injection elevation of at least 100% of baseline plasma glucose level was required for subject inclusion. The medullary raphe placement was determined histologically after the experiment with injection of pontamine sky blue at the 100 nL volume used in the experiments.

Habituation training. Prior to the start of experimental testing, rats were acclimated to handling and to injections used in a given experiment [4th i.c.v., parenchymal, intraperitoneal (i.p.)]

Food intake and body weight monitoring: Food was removed at injection time (early in the light cycle, between hours 9.30 and 11:00 am) and returned 8 h later, late in the light phase. Thereby, food was not available during the period of energetic response measurement. Food intake and body weight measurements were performed 24 h after the injection of drug. Given this design, all noted differences in food intake reflect longer latency effects of MTII (from hour 8 to hour 24-post injection). For *ad libitum* feeding rats, food was always available during the dark cycle and a minimum of 48 h was allotted between experimental testing for all animals.

Experiment 1: Effects of stimulating caudal brainstem MC-Rs via 4th i.c.v. MTII injection on energy expenditure. Neurologically intact rats (n=11) received 4th i.c.v. injections early in the light cycle. Three conditions were run in a counterbalanced fashion across separate days with at least 2 days between conditions. Responses were examined following a control condition with 4th i.c.v. vehicle (1 µl aCSF) and two doses of MTII: 0.1 nmol and 1.0 nmol (dose selection based on (Haynes et al., 1999; Murphy et al., 2000)). HR, T_C, and SPA were continuously monitored for 8 h at 5-min intervals (Tc and SPA) or 30-sec intervals (HR) in rats with implanted HRC-4000 transponders. T_{IBAT} was monitored every hour for 7 h in *Experiment 1*.

Experiment 2: Effects of systemic MC-R ligand injection on energy expenditure. This experiment was designed to determine whether any of the energy balance effects seen with 4th i.c.v. MTII injection could be attributed to actions on peripheral MC-Rs via drug efflux from the brain. All other features of the design were identical to *Experiment 1* except that vehicle (0.2 ml saline) and MTII (1.0 nmol in 0.2 ml saline) were injected i.p. (n=12).

Experiment 3: Effects of stimulating medullary raphe MC4-Rs via intraparenchymal MTII injection on energy expenditure. All rats received two counterbalanced conditions (100 nL injections of aCSF or MTII 10 pmol) separated by at least 2 days. Pilot studies determined that 5 or 10 pmol of MTII delivered 4th i.c.v. were without effect on Tc – data not shown. A: In one set of rats (n=8), T_{IBAT} and SPA were monitored with G2 transponders every 5 minutes for 8 h. B: In a second set of rats (n=12), HR, Tc, and SPA were monitored with HRC 4000 transponders for 8 h at 5-min intervals (Tc, SPA) and 30s intervals (HR).

Experiment 4: Effects of stimulating caudal brainstem MC-Rs via 4th i.c.v. injection on IBAT temperature in chronic decerebrate rats and intact control rats. Diet maintenance: Chronic decerebrate rats (CD) do not spontaneously ingest food (Grill and Norgren, 1978); therefore they were maintained with four daily gastric intubations of 9 ml of a liquid diet (AIN 76A rodent diet, Research Diets, New Brunswick, NJ). This maintenance regime provides 79 kcal/d and adequate hydration; rats gain weight on this regime. Feedings were separated by intervals of at least 2 h. CD and gavage-fed (GF) intact control rats were maintained on this feeding paradigm except as noted below during experimental testing. T_C of CD rats is more variable than that of GF control rats. Rectal temperature was measured at each gavage feeding and rats were cooled or heated if T_C was below 34.0 or above 38.5^{o}C (except during experimental testing). *Test days*: Experimental design was identical to that of *Experiment 1*, with the exception of the dose. All rats (CDs: n=10, GF: n=9) were tested under control condition: 4th i.c.v. vehicle (1 µl aCSF) and 1.0 nmol MTII.

Experiment 5: Effects of stimulating caudal brainstem MC-Rs via 4th i.c.v. MTII injection on core temperature, heart rate and activity in chronic decerebrate and in

intact rats with and without oral food access. Diet maintenance: CD (n=6) and GF control (n=11) rats were maintained as in *Experiment 4*. A third group, meal-fed intact rats (n=11), was included. These rats had oral access to the same diet that was intubated in the other two groups. Meal-fed rats were presented with 9 mL of liquid diet at the same times that the other groups received the diet by gavage. In all cases the 9 mL aliquot was consumed within 5-10 minutes. The feeding maintenance condition of the intact rats in the GF and meal-fed oral access groups were subsequently reversed in order to allow within-subject comparison. Rats were re-tested with MTII and vehicle injections after two weeks on a given feeding maintenance regime. *Test days:* Experimental design was identical to that of *Experiment 1*.

Experiment 6: Effects of stimulating medullary raphe MC-Rs via intraparenchymal MTII injection on energy expenditure in chronic decerebrate and gavage fed intact rats. CD (n=4) and GF intact controls (n=5) rats received medullary raphe injections early in the light cycle. The design was identical to that of *Experiment 3*. T_{IBAT} temperature was recorded every hour for 7 h.

Statistical analysis. All energy expenditure parameters were analyzed by ANOVAs on 5 or 6 h post-injection averages and followed by post-hoc t-tests and Tukey's honestly significant difference test as appropriate. Twenty-four h food intake and body weight were analyzed by ANOVA followed by post-hoc t-tests and Tukey's honestly significant difference test as appropriate. All statistical analysis was conducted using Statistica software (Tulsa. Oklahoma). Differences were considered significant at $P<0.05$.

RESULTS

Experiment 1: Core temperature: Figure 2.1A shows that 4th i.c.v. injection of each dose of MTII increased T_C for the 6 h post injection period in neurologically intact rats. A one-way ANOVA examining treatment effects on average post-injection T_C values revealed a significant drug treatment effect [$F(2, 20) = 8.12$, $P < 0.005$]. Post hoc analysis revealed a significant effect of both MTII doses on T_C (0.1 nmol; $P < 0.05$, 1.0 nmol: $P < 0.005$). IBAT temperature: Figure 2.1D shows that MTII significantly increased T_{IBAT} [$F(2, 16) = 8.39$, $P < 0.005$]. Both MTII doses significantly elevated T_{IBAT} (0.1 nmol; $P < 0.005$, 1.0 nmol: $P < 0.05$). A short latency increase in all measurements apparent in the vehicle condition reflects the animals' arousal associated with the injection procedure. For the 1.0 nmol MTII dose however, some rats did not show the transient elevation in T_C or T_{IBAT} giving rise to the impression that temperature parameters declined to this dose. This initial response was however variable and not statistically significant. Heart rate: Figure 2.1B shows that MTII dose-dependently increased HR, compared with saline control. A one-way ANOVA of average HR values for the 6 h period post-injection yielded a significant drug effect [$F(2, 20) = 33.33$, $P < 0.0001$]; post hoc analysis showed a significant effect of both doses of MTII on HR (0.1 nmol; $P < 0.0005$, 1.0 nmol: $P < 0.0001$). Spontaneous locomotor activity was not significantly increased by 4th i.c.v. MTII [$F(2, 20) = 2.45$, $P = 0.11$] (Figure 2.1C). Food intake and body weight: Both MTII doses significantly decreased 24 h food intake [$F(2, 20) = 17.28$, $P < 0.0001$] and body weight [$F(2, 20) = 17.75$, $P < 0.0001$] (Figure 2.1E-F).

Experiment 2. Peripheral delivery (i.p.) of the higher dose of MTII (1.0 nmol) was without effect on all of the measured energetic and food intake parameters (Figure 2.2).

Experiment 3: A, *T_{IBAT} group:* Figure 2.3E is a reconstruction of the injection sites for the 8 animals tested. Microscopic analyses of the dye injection revealed that 7 rats had placements within the medullary raphe. Figure 2.3A shows that average T_{IBAT} was significantly increased ($P < 0.005$) after 10 pmol MTII injection. Average SPA was also increased ($P < 0.05$) (Figure 2.3B). No significant changes were observed in 24 h food intake or 24 h body weight of these rats (Figure 2.3C-D). B, *Core temperature and heart rate group*: Figure 2.4F reconstructs the injection sites of a second group of animals and reveals that 9 rats had medullary raphe placements. Figure 2.4A-E displays the physiological response of the 9 rats with confirmed medullary raphe placements and shows that MTII injection significantly increased T_C and HR ($P < 0.05$ and $P< 0.0001$). These rats also showed a decrease in 24 h food intake following MTII, while SPA and 24 h body weight did not change significantly. Comparison of the injection sites in Figures 2.3E and 2.4F revealed that on average, the medullary raphe placements of the first group were caudal to those of the second group. These placement differences may account for the between group differences in SPA and food intake responses.

Experiment 4: Figure 2.5A shows that MTII (1.0 nmol) produced a large and long duration elevation in T_{IBAT} in CD rats. Two-way ANOVA (neurological preparation and drug treatment as main variables) revealed a significant interaction of drug and neurological preparation [$F(1,17) = 33.85$, $P < 0.0001$]. Post hoc test showed that there was a significant effect of MTII on CD T_{IBAT} ($P\leq 0.0001$), but surprisingly no effect on GF control rats ($P = 0.212$) (see Figure 2.7D). The absence of an energy expenditure effect in GF intact rats contrasted with the potent energetic effects of MTII observed in the chow-fed intact rats of *Experiment 1* and suggested that oral access to food in intact animals could be a relevant variable in the MTII driven sympathetic responses. This

finding encouraged inclusions of a second dose of MTII (0.1 nmol) and the addition of an oral food access meal-fed control group that was in *Experiment 5*.

Experiment 5: For chronic decerebrate rats, 4th i.c.v. administration of MTII significantly increased T_C, HR and SPA [T_C: $F(2,10) = 4.97$; $P < 0.05$, HR: $F(2, 10) = 19.99$; $P < 0.0005$, SPA: $F(2, 10) = 5.81$; $P < 0.05$] (Figure 2.5B-D). In meal-fed control rats with oral access to liquid diet, MTII treatment also significantly increased T_C and HR [T_C: $F(2,20) = 5.10$; $P < 0.05$, $F(2, 20) = 22.26$; $P < 0.0001$] (Figure 2.7A-B). Consistent with the T_{IBAT} results for GF intact rats in *Experiment 4*, there was no overall significant effect of MTII on T_C or SPA in GF controls (Figure 2.7A, C). While T_{IBAT} was increased under baseline conditions in GF rats as compared to intact chow fed controls, which could potentially contribute to lack of effect of MTII, the T_C of GF and chow fed rats after vehicle treatment was not significantly different, yet MTII effect was still not present in GF rats pointing to other mechanisms behind the lack of effect then increased baseline temperature. Although significant tachycardia was noted in these MTII treated GF rats [GF: $F(2, 20) = 11.16$; $P < 0.0005$] it was attenuated compared to the CDs or the meal-fed oral access controls (Figure 2.7B). Twenty-four h body weight was significantly decreased in the meal-fed rats with oral food access [$F(2, 20) = 5.3$; $P < 0.05$] following MTII delivery. Interestingly, body weight was also reduced by MTII in GF controls, [$F(2, 20) = 6.3$; $P < 0.05$] (Figure 2.8). Post hoc tests revealed that this effect is produced by the highest dose of MTII in both groups. CD body weight declined after both MTII doses; however that effect was not statistically significant. All groups had identical energy intake, indicating that the MTII effect on energy expenditure provided the basis for the observed suppression in body weight. It should be noted that the values for Tc,

T_{IBAT} and HR measured at baseline were the same for decerebrate and intact control rats.

Experiment 6: Reconstruction of the parenchymal placements revealed that the injection sites in 3 rats were located within the medullary raphe (Figure 2.6B). The average T_{IBAT} in CD rats with the confirmed medullary raphe placements was significantly increased ($P < 0.05$) after 10 pmol MTII injection (Figure 2.6A). No MTII-driven T_{IBAT} effect was observed in GF controls (Figure 2.7E).

DISCUSSION

We show that MC-R-bearing caudal brainstem neurons and their local caudal brainstem projections to sympathetic effectors play a critical and previously unrecognized role in the central melanocortin system's contribution to energy expenditure control. Long lasting and robust increases in thermogenesis and cardiovascular activity were observed after hindbrain ventricular delivery of MTII to neurologically intact control and to CD rats. Further support for the caudal brainstem MC-R site of action comes from the parenchymal injection results where IBAT thermogenesis and tachycardia were triggered by picomolar MTII stimulation of the medullary raphe MC4-Rs. These data are the first to demonstrate a functional effect of a ventricle sub-threshold MC-R agonist dose delivered to the MC4-R bearing neurons of the medullary raphe (Mountjoy et al., 1994; Kishi et al., 2003). The hyperthermia, IBAT thermogenesis and tachycardia observed in CD rats following 4th i.c.v. and parenchymal MTII injections supports the hypothesis that hindbrain MC-R stimulation engages endemic circuits that link autonomic outflows to thermogenic and cardiac effectors in the absence of forebrain processing or forebrain-caudal brainstem communication.

The qualitative profile of results for CD and for chow-fed control rats was similar; increases in energetic and cardiovascular parameters were observed in both groups after hindbrain MC-R stimulation. Quantitatively, however, the magnitudes of the mean energetic responses were two- (HR) to four-fold (temperature) greater in CD rats than in intact rats after 4th i.c.v. MTII application (Table 1). Quantitative differences between neurological groups were also observed with application of 10 pmol of MTII to the medullary raphe. Here, intact chow-fed rats increased IBAT temperature by ~0.5^{o}C on average. By contrast, the same dose of MTII increased IBAT temperature of a CD rat by ~3.0^{o}C (Figure 2.3A and 2.6A). There are several interpretations for these quantitative differences between decerebrate and intact rats. Endogenous agonist for the caudal brainstem MC-Rs originates from two anatomically disparate sources; one in the hypothalamic arcuate (ARC) nucleus (Knigge et al., 1981) and the other in the hindbrain NTS commissural nucleus (Joseph et al., 1983). Transection of the descending projections from ARC POMC neurons, could eliminate a significant percentage of the endogenous agonist for a given caudal brainstem nucleus. This could result in a compensatory increase in expression of MC-Rs in hindbrain nuclei. Upregulation of MC-Rs in response to decreased agonist availability has been reported (Harrold et al., 1999). While projections from both ARC POMC and NTS POMC neurons terminate in the caudal brainstem, it is not clear what the source[s] of endogenous agonist is for each of the individual MC-R expressing hindbrain nuclei. For each nucleus it is possible that the agonist is supplied entirely by NTS POMC neurons, ARC POMC neurons or some combination of the two (Jacobowitz and O'Donohue, 1978). Sim et al (Sim, 1994) showed that ARC POMC projections innervate midline caudal brainstem nuclei whereas lateral caudal brainstem regions receive projections from NTS POMC neurons; in some

cases, structures received terminal fields from both sources. In collaboration with H.R. Berthoud we have begun to examine tissues from decerebrated rats to quantify the percentage of alpha-melanocyte stimulating hormone (MSH) fibers that originate in ARC POMC neurons and project to various hindbrain MC-R bearing nuclei. For the NTS, we recently showed that ~70% of the MSH fibers terminating in all subregions of the NTS, originate in ARC POMC neurons (Berthoud HR, 2008). Additional work is needed to determine the source of MSH ligand for RPa, RVLM and other relevant hindbrain nuclei and whether eliminating a major source of endogenous ligand (ARC POMC neurons) increases exogenous agonist binding and receptor expression in caudal brainstem nuclei.

The activation of MC-Rs is also influenced by the endogenous antagonist – agouti-related protein (AgRP) – that arises in ARC neurons (Ollmann et al., 1997). It is generally thought that antagonist-containing neurons project most heavily to forebrain areas (Bagnol et al., 1999; Haskell-Luevano et al., 1999). However, hindbrain projections have also been reported (Broberger et al., 1998). The transection of descending AgRP projections may provide another explanation for the greater response to MTII observed in CD. The elimination of AgRP projections to hindbrain neurons that also receive MSH fibers (regardless of their origin) may result in greater MC-R activation and potentially contribute to the observed effects. A different type of interpretation for the greater response magnitude in CD rats relates to the role of forebrain-hypothalamic nuclei in mediating fine-grained response control. For example, heart rate is increased in response to skin cooling, and contributes to the dispersion of the heat produced by sympathetic activation of brown adipose tissue. For intact rats, there is a significant correlation between heart rate and ambient temperature over the 4°

to 23° C range. By contrast, for decerebrate rats, the heart rate response to an intermediate cold temperature is as robust as that observed with the coldest temperature, indicating a loss of fine-grained control in the absence of connections with the forebrain (Grill et al., 2005). A similar explanation for the greater MTII-stimulated responses of the CD rats could apply in the current data.

The responses of gavage-fed intact rats differed qualitatively from those of chow-fed intact rats and oral-fed rats (Table 1). The variability of these responses was great, however, yielding trends but no significant differences between the intact rat subgroups. Nonetheless, the pattern of these between-group differences is clear, with lesser response magnitude for the gavage-fed intact group than for the two intact groups with oral access to food. This outcome suggests a role for oral stimulation in the observed responses. Saito et al (Saito et al., 1989) demonstrated that oropharyngeal stimulation contributes to IBAT thermogenesis. Gavage feeding reduced oropharyngeal stimulation and decreased IBAT norepinephrine turnover relative to that seen in oral-fed rats. Our studies are consistent with the view that gavage feeding decreases the magnitude of sympathetically mediated energy expenditure output. Our data place components of the melanocortin system within the SNS output circuitry that is altered by oral exposure to food. Two provisos are worth noting. First, CD rats responded robustly to MC-R agonist, yet they were gavage fed. This suggests that the inhibitory effect of bypassing the mouth on MTII-driven energetic responses is forebrain mediated. Second, even though the energetic response magnitude of gavage-fed intact rats was attenuated, the animals still exhibited significant weight loss after the drug treatment (Figure 2.8). This result underscores the contribution of MC-R induced energy expenditure to body weight

control, as the food intake of these rats was matched to that in the vehicle condition such that weight loss could not be attributed to the anorectic effect of the treatment.

The energetic effects obtained from selective stimulation of the MC4-R-bearing neurons of the medullary raphe and with 4th ventricular agonist delivery, highlight the role of hindbrain MC-R-bearing neurons in the control of sympathetic, thermic and cardiac responses. That said, there is also a role for hypothalamic-forebrain processing in melanocortin mediated energetic effects. Many reciprocal neural projections exist between hypothalamic nuclei (especially PVN, ARC and the lateral hypothalamic area) and hindbrain nuclei. In fact, the literature on sympathetic outflows and energy expenditure control emphasizes a role for these hypothalamic nuclei [for review see (Richard, 2007)]. The neural circuitry underlying the expression of thermic and cardiac responses to hindbrain MC-R stimulation may well involve descending hypothalamic projections. We have already discussed that ARC POMC neurons project to a variety of sympathetic pre-motor targets in the hindbrain and therefore it is appropriate to consider that the application of MC-R agonist to MC4-R hindbrain neurons mimics, at least in part, the effects of endogenous ligand input from ARC. In addition, non-melanocortinergic projections from hypothalamic nuclei may also play a role in the control of MC-R-driven energetic response. MC-R-bearing PVN neurons receive ligand from ARC and project to the caudal brainstem ((Horvath and Diano, 2004)). At the same time, the results from the decerebrated rats make clear that forebrain processing and forebrain-brainstem communication are not required for caudal brainstem stimulated responses and that downstream circuits endemic to the caudal brainstem mediate the observed responses. The current data are consistent with those from previous studies from our lab (Williams et al., 2003) that show that the increase in UCP-

1 gene expression driven by 4th ventricular MTII can be mediated by circuitry intrinsic to the caudal brainstem and spinal cord.

The increased energetic response to 4th ventricular application of MTII in chow-fed intact rats reported here is consistent with results from an earlier report by Zheng et al (Zheng et al., 2005). However, responses in that study were of lesser magnitude and duration than those observed here with MTII doses within a similar range. In the Zheng et al study, rats had access to food during energetic response recordings and recordings took place during the dark/active phase. In our study, rats were tested in the light phase in the absence of food. These paradigmatic differences appear to explain the observed differences in responses. We observed that rats stimulated with MTII in the light phase without access to food have greater response magnitude and duration than when the same rats are examined in the dark phase with access to food (unpublished observations).

We showed that hindbrain MC-Rs contribute to the energy expenditure observed with central melanocortinergic stimulation. This result would seem consistent with the recent perspective of Balthasar et al (Balthasar et al., 2005). These investigators selectively expressed MC4-Rs in neurons of the PVN and amygdala in mice otherwise lacking the receptor. They found that this selective MC4-R expression reversed the hyperphagia seen in the MC4-R knockout mouse but did not increase the reduced energy expenditure profile of the knockout. Balthasar et al (Balthasar et al., 2005) suggest that the energy intake and energy expenditure effects of the central melanocortin system are controlled by anatomically distinct portions of the system. While we would agree that the melanocortin contribution to energy balance control is

distributed across different brain regions, our data and those of others [e.g. see (Grill and Kaplan, 2002; Taylor et al., 2007)] do not support the view that feeding function is uniquely associated with basal forebrain elements of the melanocortin system and that energetic function is controlled uniquely by more caudal elements of the system. In our view, stimulation of MC-Rs in a variety of central sites, including the hypothalamus and caudal brainstem, reduces feeding and increases energetic response. For example, the present findings show that in addition to the energetic effects of 4th ventricular MC-R agonist delivery, food intake was also significantly reduced. Previous work shows that hindbrain parenchymal application of pmol doses of MTII or the synthetic MC3/4R antagonist SHU-9119 results in a respective decrease or increase in food intake (Williams et al., 2000a). The idea that stimulation of anatomically disparate receptors can drive the same functional effect is however, not new. We and others have already shown that the feeding effect of central injection of leptin, urocortin, ghrelin, neuropeptideY, fenfluramine and norepinephrine is observed with basal forebrain, as well caudal brainstem ligand application (Grill and Kaplan, 2002; Faulconbridge et al., 2005; Taylor et al., 2007). It seems then that such a similarity in the functional output resulting from stimulation of anatomically distinct sites represents a degree of redundancy in the melanocortin system. While this suggests common outputs, it is likely that there are differences in the pattern of input received by MC-R bearing nuclei in different regions of the brain. Both energy intake and expenditure change in response to long term exposure to calorie dense diets (Bachman et al., 2002; Landsberg, 2006) and also in response to pathogens. The melanocortin system plays a role in mediating the energetic effects triggered by these diverse physiological conditions (Butler et al., 2001; Voss-Andreae et al., 2007). It is possible that responses resulting from these distinct challenges are processed at different levels of the brain, making the receptors

divergent based on input, but still producing the same functional output – decrease in intake and increase in expenditure.

MC-Rs are expressed on peripheral organs, like the heart (Mountjoy et al., 2003) and adrenal medulla (Mountjoy and Wild, 1998), implicated in metabolic and cardiovascular activity. Peripheral application of high doses of MC-4R agonist increases heart rate, blood pressure, IBAT temperature, as well as locomotor activity (Nordheim et al., 2006). Therefore, some part of the energetic effects obtained after central application of MC-R agonists to the brain may arise from an action on peripheral receptors. To evaluate this possibility we applied the highest dose of the agonist used centrally to the periphery and showed no effect on any measured energy expenditure parameter. While this result does not eliminate the potential role of the peripheral MC-Rs in energy expenditure, it confirms that energetic effects observed in our study are induced by stimulation of central and not peripheral receptors.

We showed that hindbrain targeted MC-R stimulation and medullary raphe MC4-R agonist injection increases energetic responses including hyperthermia, IBAT thermogenesis and tachycardia. The source of endogenous agonist for caudal brainstem MC-Rs arises from both local (NTS) and forebrain (ARC) sources. It is still unclear to what extent each nucleus contributes to the endogenous agonist supply of the specific MC-R bearing nuclei of the caudal brainstem mediated energetic responses under normal conditions. Therefore, the neural circuitry underlying the expression of thermic and cardiac responses to hindbrain MC-R stimulation may well involve descending hypothalamic projections. That the same pattern of response was observed in decerebrate and intact rats shows however, that the output circuitry responsible for the

observed effects lies within the hindbrain and does not require forebrain processing or forebrain-caudal brainstem communication. The results of this study suggest future investigations designed to determine the range of factors and environmental conditions (e.g. diet, pathogens, cold exposure) that engage the previously underappreciated caudal brainstem portion of melanocortin system as it participates in the control of energy expenditure.

ACKNOWLEDGMENTS

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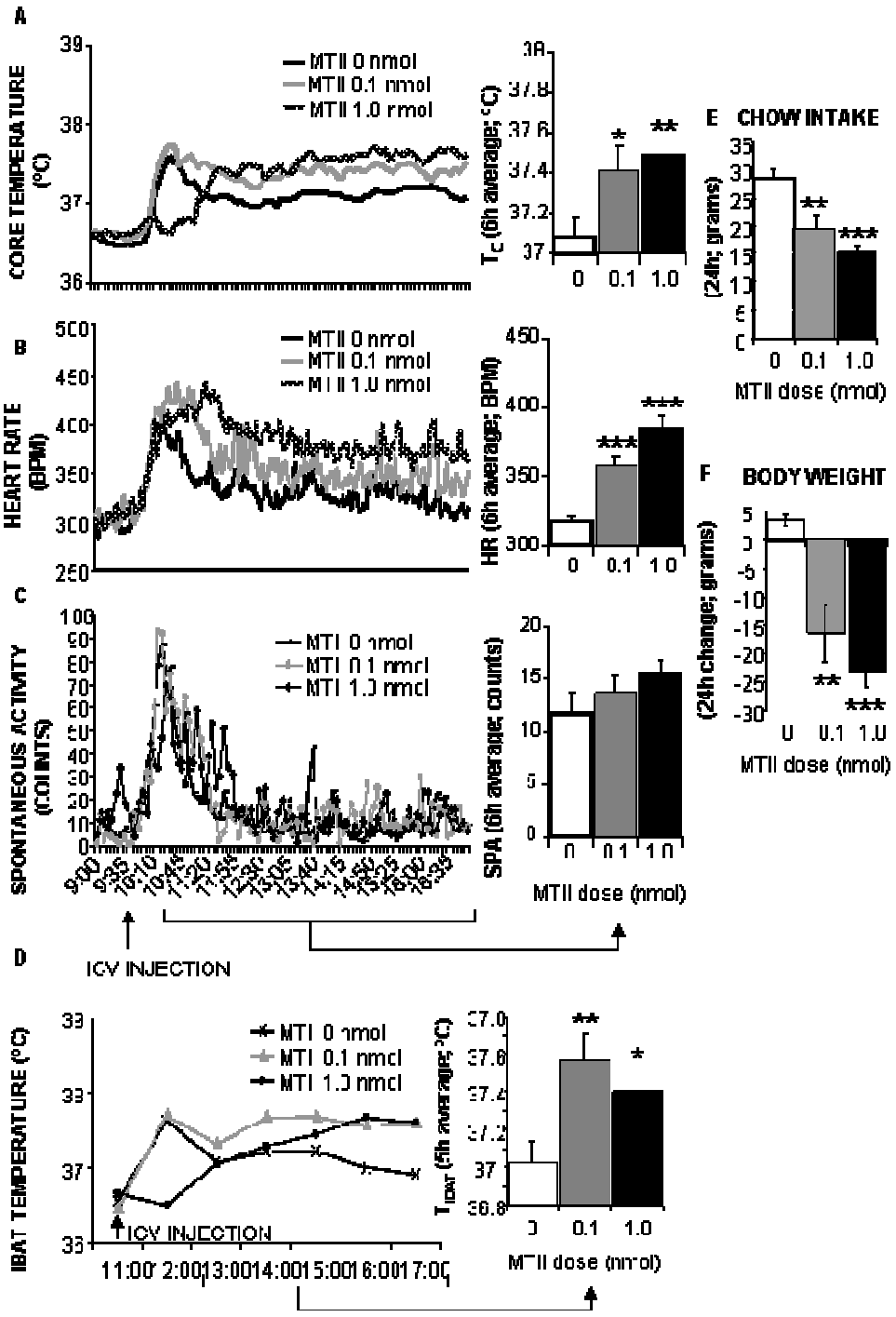

Figure 2.1: Effect of caudal brainstem melanocortin receptor stimulation with 4th ventricular MTII on (A) core temperature, (B) heart rate, (C) spontaneous activity and interscapular brown adipose tissue temperature (T_{IBAT}) (D) in chow fed neurologically intact rats.

Line graphs represent across-rat average parameter measurements through the recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. The histograms next to line graphs provide 6 h or 5 h (T_{IBAT}) post-injection averages + SEM for each parameter at each dose. Effect of 4th ventricular MTII injection on 24 h food intake (E) and 24 h change in body weigh (F) in chow fed intact rats. Food was made available to animals 8 h after injections and through the 12 h period of dark cycle. Histograms represent means + SEM. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

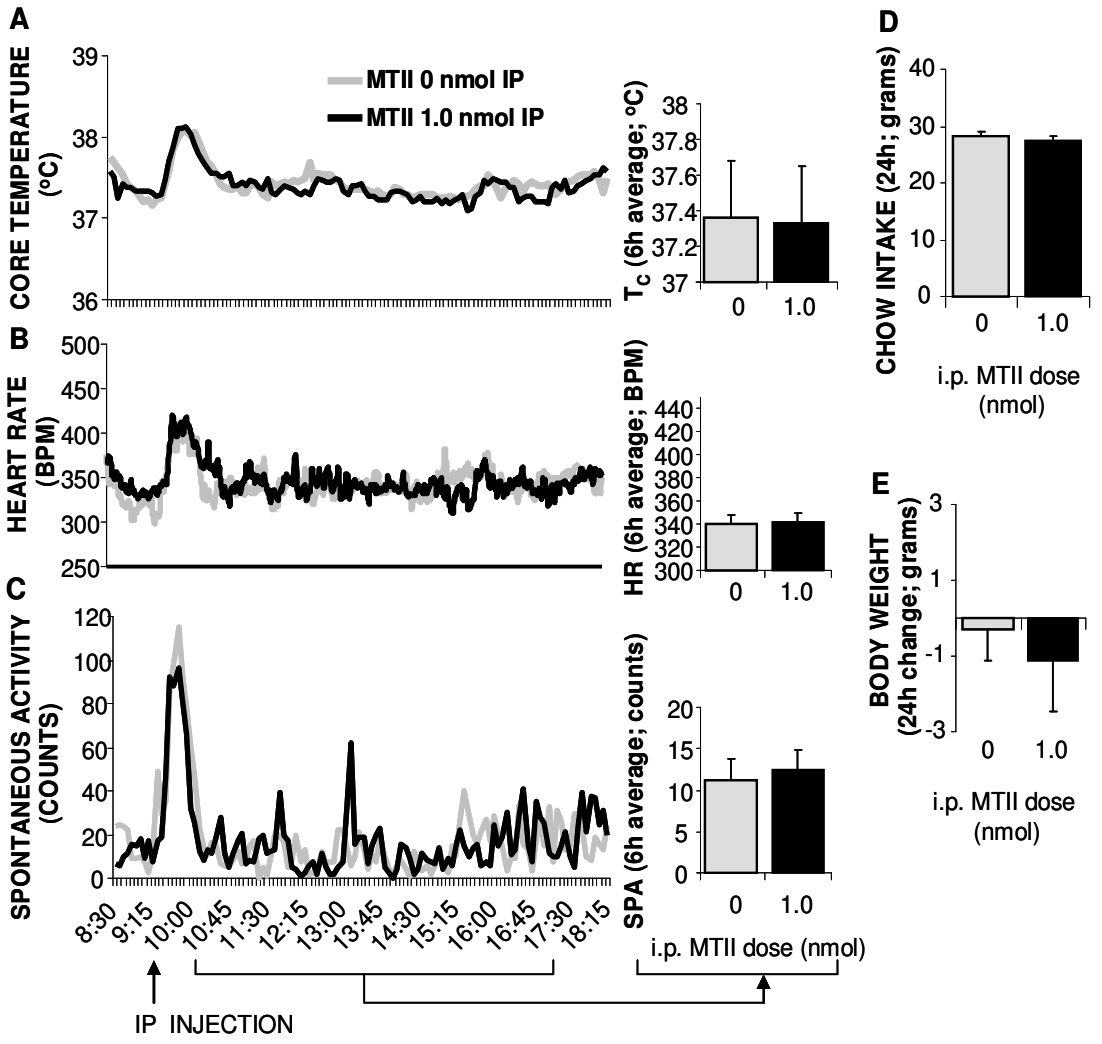

Figure 2.2: Effect of peripheral (i.p.) MTII treatment on (A) core temperature, (B) heart rate, (C) spontaneous activity, (D) 24 h food intake and (E) 24 h change in body weigh in chow fed intact rats. Line graphs represent across-rat average parameter measurements through the 8 h recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. Histograms represent means + SEM.

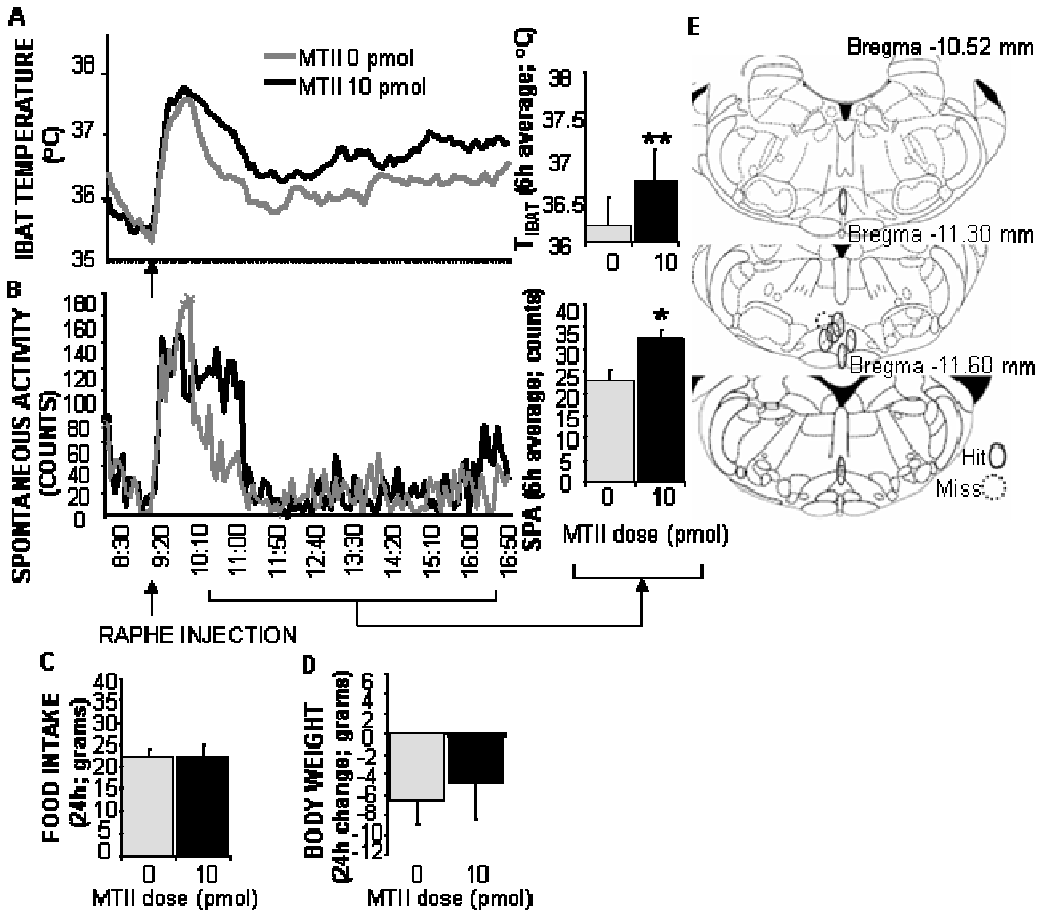

Figure 2.3: Effect of stimulation of medullary raphe MC4-Rs via parenchymal injection of 10 pmol MTII on (A) interscapular brown adipose tissue temperature (T_{IBAT}), (B) spontaneous activity, (C) 24 h food intake and (D) change in body weight. Line graphs represent across-rat average parameter measurements through the 8 h recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. Histograms represent means + SEM. **P* < 0.05, ***P* < 0.005. (E) Reconstruction of injection sites based on microscopic analysis of dye injection at the same volume (100 nL) as the melanocortin agonist. Microscopic analysis revealed that 7 of 8 rats had placements within the medullary raphe (raphe pallidus, raphe obscurus, raphe magnus*). S**olid line ovals* indicate instances where the dye injection placement was within the medullary raphe (positive placements). *Dotted line circles* represent negative placements (injection sites that were judged to be outside of medullary raphe). Placements shown were between -10.52 and -11.60 mm from bregma. (Paxinos, 1998)

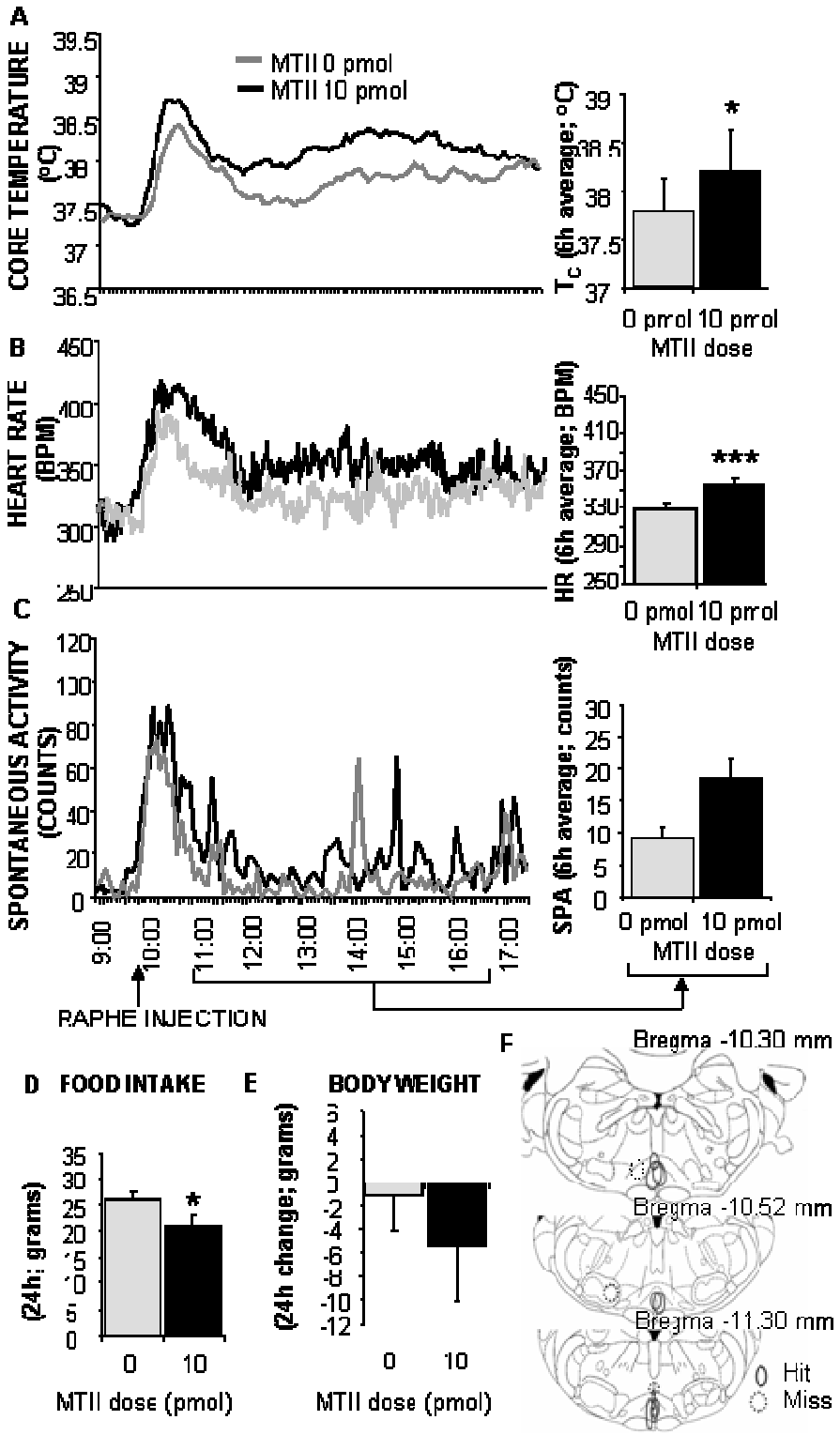

Figure 2.4: Effect of 10 pmol MTII stimulation of medullary raphe MC4-Rs on (A) core temperature, (B) heart rate, (C) spontaneous activity, (D) 24 h food intake and (E) 24 h change in body weigh. Line graphs represent across-rat average parameter measurements

through the 8 h recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. Histograms in a-c represent 6 h means + SEM. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$. (F) Reconstruction of injection sites based on microscopic analysis of dye injection at the same volume (100 nL) as the melanocortin agonist. Microscopic analysis revealed that 9 of 12 rats had placements within the medullary raphe. S*olid line ovals* mark the area of the dye injection for each rat with confirmed medullary raphe placement. *Dotted line circles* represent injection sites that were judged to be outside of medullary raphe. Placements shown were between -10.30 and -11.30 mm from Bregma. (Paxinos, 1998)

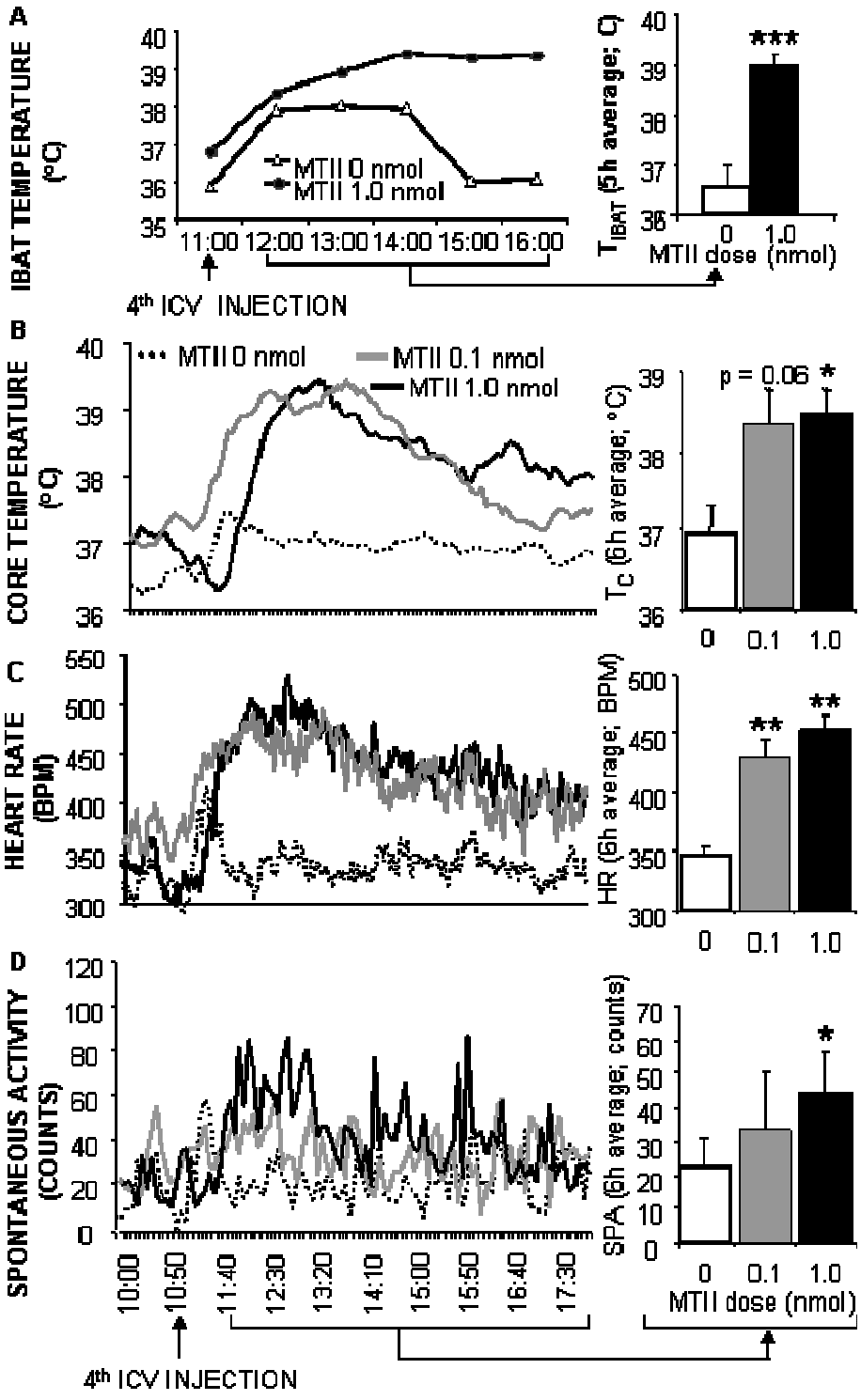

Figure 2.5: Effect of 4th ventricle MTII injection in chronic decerebrate rats on (A) interscapular brown adipose tissue temperature (T_{IBAT}), (B) core temperature, (C) heart rate and

(D) spontaneous activity in chronic decerebrate rats. Line graphs represent across-rat average parameter measurements through the recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. Histograms represent 6 h or 5 h (T_{IBAT}) means + SEM. $^{*}P < 0.05$, $^{**}P < 0.005$.

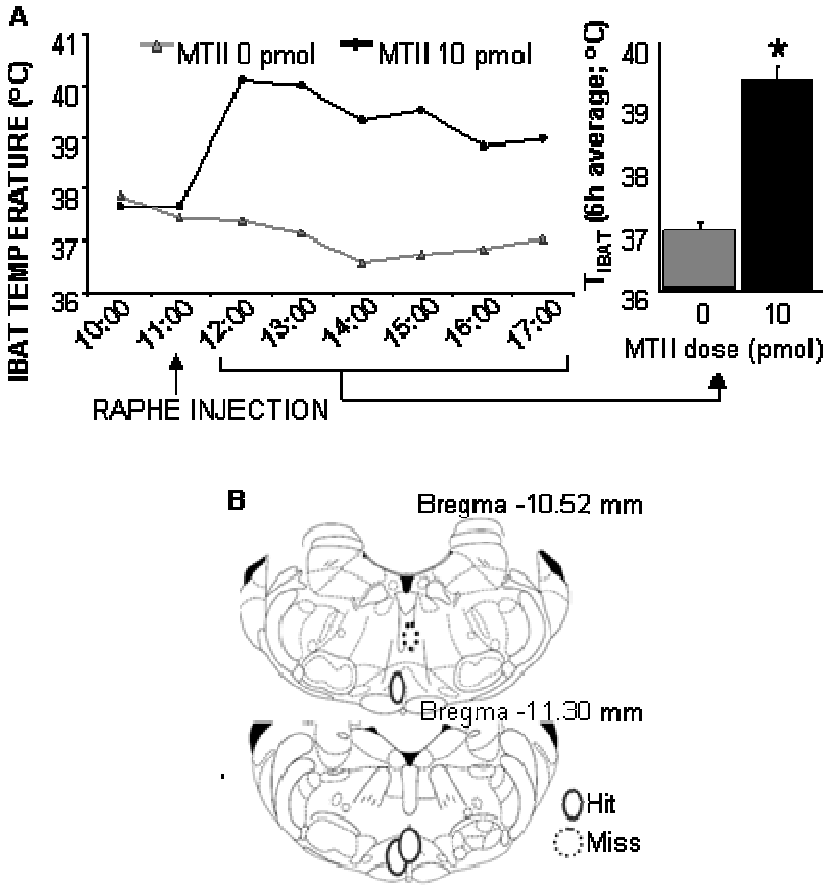

Figure 2.6: Effect of medullary raphe MC4-R stimulation with 10 pmol MTII in chronic decerebrate rats on (A) interscapular brown adipose tissue temperature (T_{IBAT}). Line graph represents across-rat average parameter measurements through the 8 h recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. Histogram represents 6 h means + SEM. *P < 0.05. (B) Reconstruction of injection sites based on microscopic analysis of dye injection at the same volume (100 nL) as the melanocortin agonist. Microscopic analysis revealed that 3 of 4 rats had placements within the medullary raphe. The dye injection placement for each animal is represented by *solid line ovals*. *Dotted line circles* represent negative placements. Placements shown were between -10.52 and -11.30 mm from Bregma. (Paxinos, 1998)

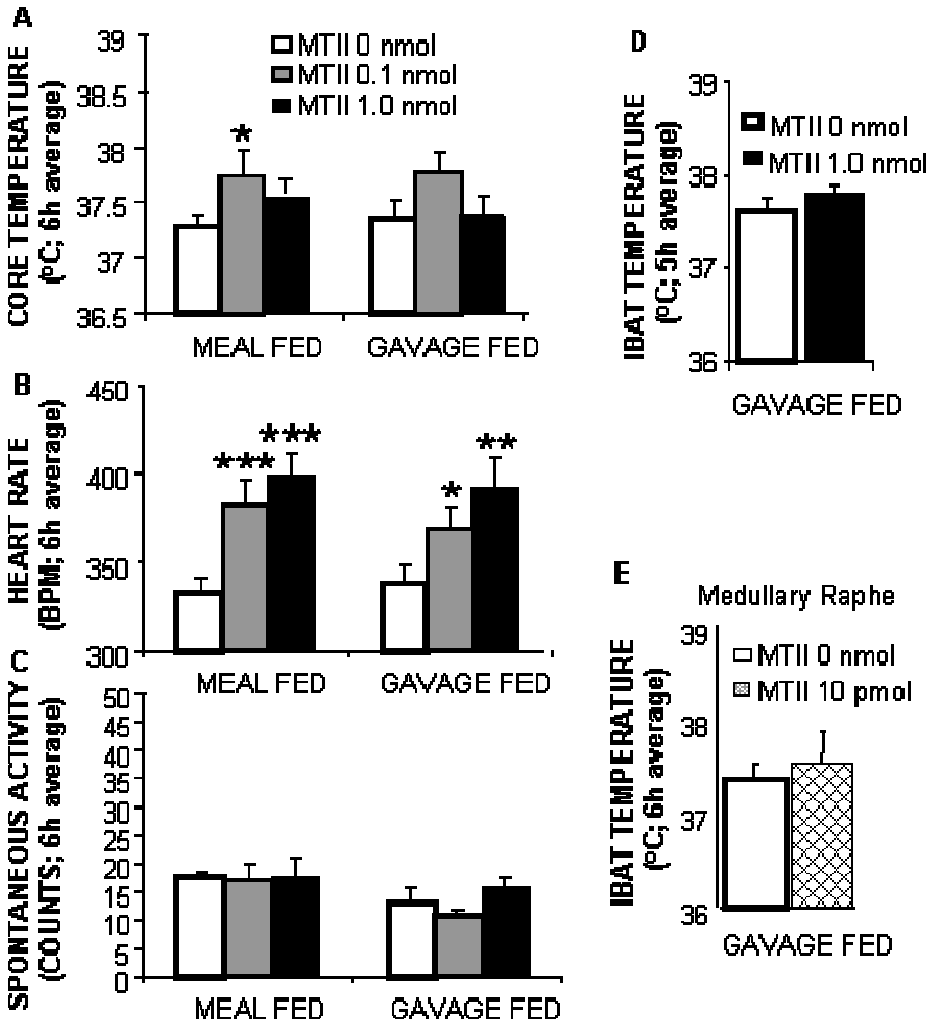

Figure 2.7: Effect of caudal brainstem MC-R stimulation with 4th i.c.v. delivered MTII in gavage-fed and meal-fed neurologically intact control rats on (A) core temperature, (B) heart rate (C) spontaneous activity and (D) interscapular brown adipose tissue temperature (T_{IBAT}). Effect of medullary raphe MTII delivery to gavage-fed rats on T_{IBAT} (E). Histograms represent means + SEM. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

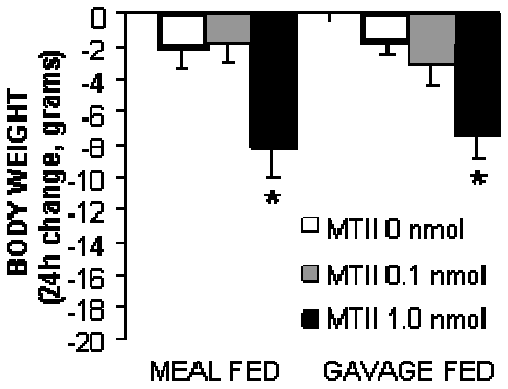

Figure 2.8: Effect of 4th i.c.v. MTII treatment on 24 h body weight change of gavage-fed and meal-fed neurologically intact rats. *Note*: all animals had identical food intake (either delivered by gavage or consumed in entirety during a meal), therefore all noted changes in body weight are a result of treatment induced changes in energy expenditure. Histograms represent means + SEM. *$P < 0.05$.

CHAPTER 3

HYPOTHALAMIC AND HINDBRAIN MELANOCORTIN RECEPTORS CONTRIBUTE TO THE FEEDING, THERMOGENIC AND CARDIOVASCULAR ACTION OF MELANOCORTINS[2]

ABSTRACT

Forebrain Forebrain ventricular delivery of melanocortin receptor (MC3/4R) agonist (MTII) increases energy expenditure (EE) and decreases food intake (FI). As forebrain ventricular delivery provides ligand to various anatomically distributed MC3/4R-bearing nuclei it is unclear which of the receptor subpopulations contributes to the feeding suppression and to the sympathetic-thermogenic effects observed. The literature indicates that re-expression of MC4R in the paraventricular nucleus (PVN) affects the feeding but not the energetic phenotype of the MC4R knockout; suggesting that divergent MC4R populations mediate EE (hindbrain) and FI (hypothalamus) effects of stimulation. Not consistent with this view are data indicating that PVN sympathetic pre-motor neurons express MC4Rs and that feeding effects are induced from hindbrain MC4R sites. Therefore we hypothesize an opposing perspective: that stimulation of anatomically diverse MC3/4R-bearing nuclei triggers energetic as well as feeding effects. To test this hypothesis, ventricle subthreshold doses of MTII (5 and 10pmol) were applied in separate experiments to six hindbrain and hypothalamic sites; core temperature (Tc), heart rate (HR), spontaneous activity (SPA) and FI were measured in

[2] These results were reported at the 2008 meeting of Society for Ingestive Behavior, in Paris, France. This work was supported by NIH grants DK-21397 and NRSA NS-059254.

behaving rats. Nucleus tractus solitarius and PVN stimulation increased Tc, HR, and SPA and decreased FI. Rostral ventrolateral medulla, parabrachial nucleus and retrochiasmatic area stimulation increased Tc, HR, but not SPA, and decreased FI. The response profile differed to some extent for each nucleus tested, suggesting differential output circuitries for the measured parameters. Data are consistent with the view that energetic and feeding responses are not controlled by regionally divergent MC3/4Rs and can be elicited from multiple, anatomically distributed MC3/4R populations.

INTRODUCTION

Humans with melanocortin receptor-4 receptor (MC4R) mutation are hyperphagic and obese (Yeo et al., 1998; Ma et al., 2004). They also exhibit lower energy expenditure (Cai et al., 2006) and decreased cardiovascular parameters (heart rate and blood pressure)(Greenfield et al., 2009). MC4R knockout mice show a similar energy balance profile. These results, and others, support the view that the central melanocortin system is an important element of the CNS circuits controlling food intake and energy expenditure. Ventricular pharmacologic stimulation of central melanocortin receptors (MCRs; MC3 and MC4Rs) increases energy expenditure and cardiovascular parameters, and decreases food intake(Grill et al., 1998; Murphy et al., 2000; Skibicka and Grill, 2008a). It is unclear however which of the anatomically distributed MCR subpopulations contribute to the food intake and to the sympathetic-energetic effects observed.

Despite the broad anatomic distribution of CNS MCRs, especially MC4R, MCR-expressing neurons in the hypothalamus are viewed as the principal site of action for the

food intake as well as for the energy expenditure effects of melanocortin ligand stimulation. This view, especially pertaining to food intake, is supported by data indicating that 3rd ventricular injections of MC3/4 R agonist (MTII) as well as intra-parenchymal injections into the paraventricular hypothalamic nucleus (PVN) reduces food intake (Murphy et al., 2000; Wirth et al., 2001). Additional support for this anatomical perspective comes from the results showing that re-expression of MC4Rs in the PVN (and in some other amygdala and hypothalamic neurons) in obese MC4R knockout mice reverses their hyperphagia to a level of food intake seen in wild-type control mice(Balthasar et al., 2005).

The obesity of these selective MC4R-expressing mice is not, however, fully reversed as their energy expenditure (measured by oxygen consumption) is not affected by the treatment, in contrast to their feeding. This result is surprising since it is clear that MC4R expressing parvocellular PVN neurons are sympathetic pre-motor neurons whose output contributes to the control of brown adipose tissue (BAT) thermogenesis (Cano et al., 2003b; Voss-Andreae et al., 2007; Song et al., 2008). This contrasting pattern of energy balance effects led to a new hypothesis about the control of energy balance function exerted by anatomically distinct MCRs – that hypothalamic/PVN MC4R expressing neurons contribute to control of food intake and MC4R expressing sites elsewhere, likely in the caudal brainstem, contribute to the energy expenditure effects of melanocortins.

Consistent with this organizational perspective several caudal brainstem nuclei contain sympathetic pre-motor neurons that express MC4R and contribute to the control of BAT thermogenesis including the raphe pallidus, rostral ventrolateral medulla

(RVLM), nucleus of the solitary tract (NTS) and the pontine parabrachial nucleus (PBN) (Voss-Andreae et al., 2007). Hindbrain ventricular or raphe pallidus injection of the MC3/4R agonist MTII increases energy expenditure (elevating core and BAT temperature) and heart rate (Skibicka and Grill, 2008a) (this combination appears to facilitate heat distribution and increases in uncoupling protein-1 expression in BAT tissue (Williams et al., 2003). While these caudal brainstem MCR-driven energetic effects are consistent with the idea of hypothalamic-caudal brainstem divergence of function for feeding versus energy expenditure other published findings are not. Application of picomolar doses of MC3/4R agonist (MTII) and antagonist (SHU-9119) to the NTS results respectively in robust food intake inhibition or hyperphagia (Williams et al., 2000a; Zheng et al., 2005; Li et al., 2007)); low-dose agonist stimulation of raphe pallidus inhibits feeding (Skibicka and Grill, 2008a). These findings are inconsistent with the notion of that the feeding effects of CNS MCR stimulation are localized to hypothalamic MCR stimulation. Rather, these results appear to indicate that the energy balance effects of melanocortins are anatomically distributed and not regionally segregated.

To better distinguish between the applicability of these two competing views of the relationship between MCR location and the control of energy intake and sympathetic-energy expenditure responses, investigation of responses obtained by stimulating a variety of individual MC4R expressing nuclei at the level of the hypothalamus and the caudal brainstem are required. Here we pursue such studies. Using ventricle subthreshold, picomolar doses of the MC3/4R agonist MTII experiments assessed the energy balance response profiles observed with direct parenchymal stimulation of six different MC4R bearing nuclei, two within hypothalamus [PVN,

Retrochiasmatic area (RCh) and anterior hypothalamic area] and three within the caudal brainstem (NTS, RVLM and PBN). For each nucleus examined MCR expression and connections to sympathetic outflow have been affirmed. The three caudal brainstem sites are anatomically segregated such that stimulation of one would not indirectly stimulate the other. Given that two hypothalamic sites are relatively close to each other a third site, the anterior hypothalamic area (AHA), was included as an anatomical control for the spread of injected ligand.

Stimulation of all but one of the sites tested led to an increase in core temperature and heart rate, a decrease in food intake and body weight, and in some cases increases in activity. These results are consistent with the hypothesis that energy balance effects of melanocortins are distributed across many anatomically distinct sites within the CNS.

METHODS:

Subjects

Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 300–400 g at surgery and housed individually in plastic bins maintained on a 12-h light, 12-h dark cycle (0800 h lights on), participated in the four experiments described below. Pelleted food (Purina 5001; St. Louis, MO) and water were available *ad libitum* unless otherwise noted. All procedures conformed to the institutional standards of animal care and use (University of Pennsylvania).

Surgery

Rats were anesthetized with ketamine (90 mg/kg), xylazine (2.7 mg/kg), and acepromazine (0.64 mg/kg) delivered im.

Cannula implantation.

All rats in received a guide cannula (22 gauge; Plastics One, Inc., Roanoke, VA) with its tip stereotaxically positioned 1.0 (AHA) or 2.0 mm above the target parenchymal site. Following coordinates were used: 1) NTS: 0.7mm from the midline, on the occipital suture, and 6.9mm ventral to the skull, with injector aimed 8.9mm ventral from skull. 2) RVLM: 2mm from the midline, 1.8mm rostral to occipital suture, and -7.9mm ventral to the skull, with injector aimed 10.9mm ventral to skull. 3) PBN: 2.0mm lateral to midline, 9.5-mm posterior to bregma, and 4.5-mm ventral to the dura, with injector aimed 6.5-mm ventral to dura. 4) PVN: 0.5mm lateral to midline, 1.8-mm posterior to bregma, and 5.7-mm ventral to the dura, with injector aimed 7.7-mm ventral to dura 5) AHA: 0.5mm lateral to midline, 1.8-mm posterior to bregma, and 7.7-mm ventral to the dura, with injector aimed 8.7-mm ventral to dura 6) RCh: 0.5mm lateral to midline, 1.8-mm posterior to bregma, and 7.7-mm ventral to the dura, with injector aimed 9.7-mm ventral to dura. Cannulas were attached to the skull with dental acrylic and jeweler's screws, and closed with an obturator. Cannula placement was verified histologically after the experiment by examining the anatomical placement of an injection of pontamine sky blue (100 nl volume matched drug delivery in the experiments) made at sacrifice. Only rats whose dye injection site was found within the intended parenchymal site were included in the physiological data analysis. For the NTS group an additional functional verification was applied. The placement was assessed 7 days after the surgery by measurement of the sympathoadrenal-mediated glycemic response to an injection of 5-

thio-D-glucose [21 μg in 100nl artificial cerebral spinal fluid (aCSF)] (Ritter et al., 1981). A post-injection elevation in baseline plasma glucose level of at least 100% was required for subject inclusion using this verification criterion.

Telemetric transponder surgery.

Under anesthesia telemetric transponders (HRC 4000 VitalView; Mini Mitter/Respironics, Bend, OR) for recording core temperature (T_C), heart rate (HR), and spontaneous physical activity (SPA) were inserted into the abdominal cavity, with the leads positioned subcutaneously and secured to the chest muscles on either side of the heart with sutures as described in (Skibicka and Grill, 2008a).

Experimental procedures.

Habituation training.

Before the start of experimental testing, rats were habituated to the handling and injection procedures to be used during formal testing.

Testing days.

All rats received vehicle injections (100 nl aCSF) counterbalanced with one (RCh, AHA; 10pmol MTII) or two doses (NTS, RVLM, PBN, PVN; 5 and 10 pmol MTII) of MTII. Core temperature, heart rate and spontaneous activity were recorded telemetrically for 1h before injections and for the 8h following injections; the frequency of measurement was every 5 min (T_C, SPA) or 1 min (HR). Food was removed just prior to injection time (early in the light cycle, between 0930 and 1000 h) and returned 8 h later, late in the light phase. Thereby, food was not available during the period of energetic-cardiovascular response measurement. Food intake and body weight measurements were performed 24 h after the injection of drug. Given this design, all noted differences

in food intake reflect longer latency effects of MTII (from 8–24 h after injection). For *ad libitum*-feeding rats, food was always available during the dark cycle. A minimum of a 48 h period intervened between experimental testing.

Statistical analysis:

Core temperature, HR and SPA were analyzed by ANOVA on post-injection 6 h average values of each parameter and followed by student's t-test or Tukey test as appropriate. Twenty four hour food intake and body weight were analyzed by ANOVA and followed by t-test or Tukey test as appropriate.

RESULTS

NTS: Microscopic assessment of the brains of rats in this group indicated that center of the dye injection was located within the NTS at the level of 4th ventricle; dye also reached the DMX in some cases. Figure 3.1 shows that MTII injection significantly increased Tc (5 pmol: $P < 0.05$; 10 pmol: $P < 0.0005$), HR (5 pmol: $P < 0.0005$; 10 pmol: $P < 0.0005$) as well as spontaneous activity (10 pmol: $P < 0.05$). Overnight food intake and body weight were reduced by 10 pmol MTII treatment ($P < 0.0005$, $P < 0.0005$ respectively). The 5 pmol MTII dose did not yield significant changes in activity, food intake or body weight.

RVLM: Microscopic analysis indicated that center of dye injection for these rats were located within the RVLM, with small amounts of ink in some cases reaching near the nucleus ambiguous. Figure 3.2 shows that MTII stimulation significantly increased Tc (5 pmol: $P < 0.05$; 10 pmol: $P < 0.05$), HR (5 pmol: $P < 0.05$; 10 pmol: $P < 0.0005$). No

significant spontaneous activity changes were noted after MTII application to the RVLM. Overnight food intake and body weight were reduced by 5 pmol MTII treatment (5 pmol: $P < 0.005$), (5 pmol: $P < 0.05$), respectively.

PBN: The dye injection for this group was centered at the external lateral nucleus of PBN with only trace amounts of ink reaching other PBN nuclei. Figure 3.3 shows that MTII stimulation significantly increased Tc (5 pmol: $P = 0.13$; 10 pmol: $P < 0.05$), HR (5 pmol: $P < 0.0005$; 10 pmol: $P < 0.0005$) but not spontaneous activity. Overnight food intake and body weight were reduced by the MTII treatment (5 pmol: $P < 0.005$; 10 pmol: $P < 0.005$), (5 pmol: $P = 0.055$; 10 pmol: $P < 0.05$), respectively.

PVN: The dye injection for rats in this group was centered at the dorsal PVN. Figure 3.4 shows that MTII stimulation significantly increased Tc (5 pmol: $P=0.23$; 10 pmol: $P < 0.05$), HR (5 pmol: $P < 0.0005$; 10 pmol: $P < 0.0005$) and spontaneous activity (5 pmol: $P < 0.0005$; 10 pmol: $P < 0.0005$). Overnight food intake and body weight were reduced by the MTII treatment (5 pmol: $P = 0.076$; 10 pmol: $P < 0.05$), (5 pmol: $P < 0.05$; 10 pmol: $P < 0.05$), respectively.

RCh: Microscopic evaluation determined that in all rats in this group the dye injection was centered in the RCh. Figure 3.5 shows that MTII stimulation significantly increased Tc (10 pmol: $P < 0.01$) and HR (10 pmol: $P < 0.0005$) but had no effect on spontaneous activity (10 pmol: $P= 0.42$). Overnight food intake and body weight were reduced by the MTII treatment (10 pmol: $P < 0.01$), (10 pmol: $P < 0.005$), respectively.

AHA: In order to show that the doses and volume chosen were selective for the target nuclei tested this site was selected as an anatomical control placement for the relative adjacency of PVN and RCh targets. MTII injected into the AHA did not produce any significant changes in any of the measured parameters (Figure 3.6). The location of the AHA directly adjacent to the two hypothalamic targets that yielded a positive effect (just dorsally to RCh and just ventrally to PVN) and the lack of observed effects confirm that this target served as a negative control area.

DISCUSSION

Each of the sites targeted in these studies - PBN, NTS, RVLM, PVN and RCh - was selected for its expression of MC4Rs and its established connection to the sympathetic outflow (Mountjoy et al., 1994; Mountjoy and Wild, 1998; Voss-Andreae et al., 2007; Song et al., 2008). This phenotypic profile provided the foundation for the hypothesis that direct melanocortinergic stimulation of these different hypothalamic and hindbrain neurons could drive energetic and cardiovascular responses. The results obtained are consistent with that hypothesis - direct administration of low, picomolar doses of MC3/4R agonist into these areas triggered hyperthermia, tachycardia and in some cases hyperactivity. Also noteworthy, stimulation of these sites induced anorexia and body weight loss. The lack of an effect on any measured parameter from melanocortinergic stimulation of the AHA site, intermediate between the PVN and RCh sites, supports the interpretation that the observed effects arise from ligand application to the targeted neurons. To our knowledge these are the first reports of the effects of direct melanocortinergic stimulation in PBN and RCh, and the first assessment of the energetic effects of PVN and RVLM MCR stimulation in a non-anesthetized rat. Collectively, the data presented indicate that the energy intake and the energy

expenditure effects of CNS MCR stimulation are mediated by an anatomically distributed network of MCR-bearing neurons. These data are not consistent with an anatomical organization of MCR-stimulated energy balance response where the control of feeding and energetic-sympathetic responses is regionally segregated.

Hyperthermia

Stimulation of MCR expressing neurons in NTS, PBN, RCh and PVN increased core temperature significantly (RVLM and hyperthermia is discussed later in the paragraph). BAT thermogenesis driven by sympathetic outflows is a likely contributor to the observed hyperthermia as both MC4R expression and neural connections to BAT (retrograde viral tracing from BAT) are present in these nuclei (Voss-Andreae et al., 2007). BAT temperature and UCP-1 gene expression was not measured in the current study, however, 4th ventricular delivery of MTII gives rise to a profile of response that includes hyperthermia, elevations in BAT temperature (Skibicka and Grill, 2008a) and in BAT UCP-1 mRNA (Williams et al., 2003; Song et al., 2008). Direct MTII injection into the caudal raphe (10 pmol, lowest effective dose) increases BAT temperature (Skibicka and Grill, 2008a). Elevation in BAT temperature is also observed after PVN MTII stimulation (50 pmol, lowest effective dose) in the hamster (Song et al., 2008). This study is the first to show thermal effects of direct PBN melanocortin stimulation. A contribution of PBN to the neural control of thermoregulation has been suggested by the work of others. Electrical stimulation of PBN produces BAT hyperthermia and increases oxygen consumption and bilateral PBN lesion disrupts the thermogenic response triggered by environmental cooling (Kobayashi and Osaka, 2003). Lateral PBN neurons are activated by skin cooling and may influence BAT thermogenesis via their rostral projections to neurons of the hypothalamic medial preoptic area (Nakamura and

Morrison, 2007). The contribution of PBN output to BAT thermogenesis could also involve descending PBN projections (Hermann et al., 1997) to the raphe pallidus, a region critical for the neural control of BAT thermogenesis (Morrison, 2003). While not a focus of study for its effects on temperature, the connection between RCh neurons and BAT (viral-tracing studies) and the MC4R expression in these neurons (Oldfield et al., 2002) suggests that the hyperthermia triggered by MCR stimulation of RCh involves BAT thermogenesis. Other mechanisms, may also contribute to the observed hyperthermia. Neither skin temperature nor vascular resistance changes were measured here and as such vasoconstriction remains a possible mechanism contributing to the increasing core temperature response to central by melanocortinergic stimulation. In fact, studies employing neuroexcitation (chemical and electrical) of the RVLM show that thermoregulatory effects are associated with changes in sympathetic activity to vasculature (specifically tail vein) rather than to changes in BAT thermogenesis (Morrison, 1999; Ootsuka and McAllen, 2005). Here, we showed that MCR stimulation of RVLM increased core temperature. Therefore it will be important to consider whether a given effect of MCR stimulation– e.g., hyperthermia – can arise from distinct MC4R-bearing nuclei via separable and distinct physiologic effectors. We noted above that the hyperthermic effect observed for NTS stimulation may result from a BAT-mediated output, whereas the hyperthermic effect following RVLM stimulation may be mediated by SNS control of the vasculature. Consistent with this view is data showing that SNS output to certain tissues can be differential with potentially different output circuitries reaching different tissues (Morrison, 1999; Rathner and McAllen, 1999; Morrison, 2001a, b).

Tachycardia

MTII stimulation of both hindbrain and hypothalamic nuclei elicited a marked and long-lasting tachycardia. The 5pmol MTII dose was effective in triggering this response from PVN, PBN, RVLM and NTS sites. The same dose was of varying effectiveness in inducing thermogenesis possibly implicating a higher sensitivity of the tachycardic response. All MTII stimulated tachycardic responsive areas in this study are directly or indirectly connected to autonomic outflow to the heart (Ter Horst et al., 1996; Ter Horst and Postema, 1997). A role for RVLM in cardiovascular control is well established; sympathoexitatory RVLM neurons project directly to intermediolateral cell column (IML) (Brown and Guyenet, 1985; Guyenet et al., 1996). MCR stimulated tachycardia from RVLM is likely mediated by the increased sympathetic nerve activity. Support for this interpretation comes from a study showing that RVLM injection of ACTH (with affinity for MC1-5R; greatest affinity for MC2R) in anesthetized rats increases sympathetic nerve activity, blood pressure and HR (Kawabe et al., 2006). The greater magnitude (2-3 fold larger) and duration (10 times longer; 20 min vs. 6h) of the HR effect in our study is likely attributable to the greater affinity of the agonist used (MTII vs. ACTH) and the behavioral state of rats. The tachycardic effect we report for NTS stimulation is not consistent with the direction of the effect – bradycardia – reported for ACTH injection into NTS at the area postrema level of anesthetized rats (Brown et al., 2006). The same group of investigators however showed that injections of SHU 9119 (MC3/4R antagonist) also produces bradycardia in anesthetized rats, potentially blocking the effects of endogenously released melanocortin agonist. A role for the PBN in cardiovascular regulation is well established. Electrical or chemical stimulation of PBN elicits tachycardia via RVLM projections and subsequent SNS pre-motor neuron activation (Mraovitch et al., 1982; Miura and Takayama, 1991; Agarwal

and Calaresu, 1993). Melanocortinergic stimulation of the PBN induced a small magnitude but statistically significant tachycardia; this is the first report of a direct effect on HR of melanocortins in PBN.

Spontaneous locomotor activity

Activity can be a contributing factor to the pathogenesis of obesity. Decreases in spontaneous activity are strongly correlated with obesity development and fat pad deposition (Castaneda et al., 2005). While little is known about the neural basis of this response, available evidence implicates many of the neuropeptides associated with food intake in the control of physical activity. Central (ventricular) MTII stimulation increases and antagonist (AgRP) application decreases spontaneous locomotor activity in rats (Hwa et al., 2001; Tang-Christensen et al., 2004; Koo et al., 2008). Our study suggests that MCR-bearing neurons of the PVN and PBN contribute to this effect of MTII, as low, picomolar doses of MTII in these nuclei increased physical activity.

Food intake

The NTS, RCh, PVN, and PBN are among the nuclei most prominently implicated in the neural control of food intake. The anorexia shown here after MTII delivery to NTS and PVN is consistent with the results of other publications (Giraudo et al., 1998; Williams et al., 2000a; Wirth et al., 2001). The anorexia induced by picomolar MTII dose delivered to PBN and RCh is the first such report. The size of the anorexic effect from these sites (~5g over the18 h measurement) is comparable to that observed after PVN and NTS stimulation. It is important to note that the experimental design employed here, however, only captures food intake effects of a longer term nature (6 h-24 h post drug injections). It is entirely possible that the magnitude and temporal

pattern of the effect on food intake would differ across these sites if food had been available and intake measured during the initial post-injection period (1-6 h post drug).

The energy balance effects reported here arise from the *exogenous* stimulation of a number of anatomically disparate sites and lead naturally to a consideration of the *endogenous* melanocortin system and its role in normal physiology. One important but unresolved question is the source of the endogenous agonist for the hypothalamic and hindbrain sites investigated. Two populations of POMC neurons, one in hypothalamic ARC and the other in hindbrain NTS, are the sources of endogenous α-melanocyte stimulating hormone (α-MSH). Some MCR-bearing nuclei are innervated by one of those populations but others might have dual innervation. Until recently this problem has not been adequately explored. Berthoud et al. (Berthoud HR, 2008) showed that MC4R-bearing NTS neurons receive a majority (~70%) of their α-MSH input from the hypothalamic arcuate POMC population but some (~ 30%) originate locally from the NTS POMC neurons (Berthoud HR, 2008). This result is consistent with a suggestion by Palkowitz et al (Palkovits et al., 1987) that hypothalamic α-MSH fibers might provide a majority of the innervation for the midline hindbrain nuclei like NTS and medullary raphe whereas the more lateral MCR expressing hindbrain regions (e.g. RVLM) might receive a greater percentage of their innervation from NTS α-MSH fibers. The PVN and RCh receive projections from ARC POMC neurons (Joseph and Michael, 1988) but NTS α-MSH projections to these areas have not been extensively studied and cannot be ruled out (see (Sim, 1994; Sim and Joseph, 1994). The PBN receives input from the commissural NTS (Sim, 1994; Sim and Joseph, 1994) and from the ARC (Joseph and Michael, 1988) however more remains to be done in determining the neurochemical phenotype of the PBN-projecting ARC and NTS neurons. Given that ARC and NTS

POMC neurons might receive a different set of inputs, the MCR-bearing target nuclei that receive projections from both POMC sources would logically integrate a wider range of information.

It remains to be determined how the melanocortinergic tone (i.e. activation of POMC neurons leading to α-MSH ligand release onto target nuclei) may be altered under specific environmental changes e.g. fasting, high-fat diet maintenance, cold challenge, infection etc. One approach to addressing these questions would be to block endogenous melanocortin signaling in a given nucleus through the use of site-specific receptor antagonist or via viral knockdown of a given melanocortin receptor. Garza et al (Garza et al., 2008) used RNAi technology to show that MC4R signaling in PVN is necessary for the regulation of food intake and body weight regulation under unique circumstances (energy expenditure not measured). The hyperphagia and obesity was observed only when rats were maintained on high-fat food; PVN MC4R knockdown rats maintained on chow were normophagic. The results suggest that the high-fat feeding alters endogenous melanocortinergic tone where normal MC4R function keeps high-fat induced hyperphagia in check.

It will be useful to determine the neurochemical phenotype of the MTII-stimulated neurons. Such detail could provide insight into the downstream pathways and help to better define the neurocircuitry mediating melanocortinergic effects. For example, in considering MCR function for NTS neurons it has become apparent that some MC4Rs are located presynaptically on vagal afferent terminals indicating that melanocortin signals are likely interacting with the vagal transmission to influence the excitability of NTS recipient neurons (Wan et al., 2008). This modulation could indicate

a potential mechanism for the anorexic effect of NTS MTII stimulation where vagal signals arising from e.g. gastric distention or CCK stimulation could be enhanced by MC4R activation leading to a more potent decrease of food intake than that induced by vagal activation alone (Wan et al., 2008). In the PVN, Mountjoy et al.(Mountjoy et al., 1994) report that high concentrations of the MC4R mRNA are found in both the parvocellular and magnocellular neurons, indicating the potential of central (e.g. sympathetic) as well as neuroendocrine mediation of MC4R stimulation effects in this nucleus. PVN thyrotropin-releasing hormone (TRH) expressing neurons are densely innervated by melanocortin fibers and central intake, thermogenic and cardiovascular effects of TRH are similar to those of MC4R stimulation (Lechan and Fekete, 2006) making this neuropeptide a potential mediator of some of the observed effects of melanocortin signaling. Oxytocin is also expressed in the parvocellular PVN neurons that influence BAT function (Oldfield et al., 2002), however interaction of melanocortin with oxytocin has been mainly indicated at the magnocellular division of PVN (Caquineau et al., 2006), leaving mediation of effects by oxytocin expressing neurons unclear. In PBN MC4R are expressed on neurons that are activated (expression of c-fos mRNA) by lipopolysaccharide or lithium chloride treatment and potentially project from the external lateral PBN to the amygdala, relaying to the forebrain information of relevance to certain aspects of sickness behavior (Paues et al., 2006).

The current data are consistent with the hypothesis that different aspects of energy balance control can be elicited by melanocortin ligand stimulation of a single MCR-bearing nucleus. It remains to be determined, however, whether the same neurons within each nucleus relay signals pertaining to all relevant parameters, e.g., intake,

thermogenesis and tachycardia or whether different sub-populations of neurons relay information pertaining to each output parameter separately.

Our results show that there is a certain degree of redundancy in the organization of the central melanocortin system, since the same responses can be obtained by stimulation of anatomically disparate MCR-expressing neurons. This idea of redundancy (Grill and Kaplan, 2002) is strengthened by the fact that rats with electrolytic PVN lesions show melanocortin-induced anorexia identical to that of controls (Dube et al., 2006) indicating that other MCR populations can be utilized when one is rendered dysfunctional. Although focal PVN MCR stimulation (as shown in the current data) induced energy balance effects it is not necessary for the physiological MCR output as shown in the PVN lesion result just noted. Redundancy of function can have interesting implications for our understanding of the CNS control of energy balance. For example characterizing changes in MCR expression in one area of the brain is no longer sufficient for indicating a physiological change in behavior, given the presence of other brain regions that could still mediate the MCR effect with the end result of unaltered physiological outcome (Munzberg et al., 2004; Munzberg et al., 2005; Skibicka and Grill, 2009b). Future studies should therefore benefit from extending their focus to a more distributed melanocortin system when evaluating control of energy balance.

Acknowledgements

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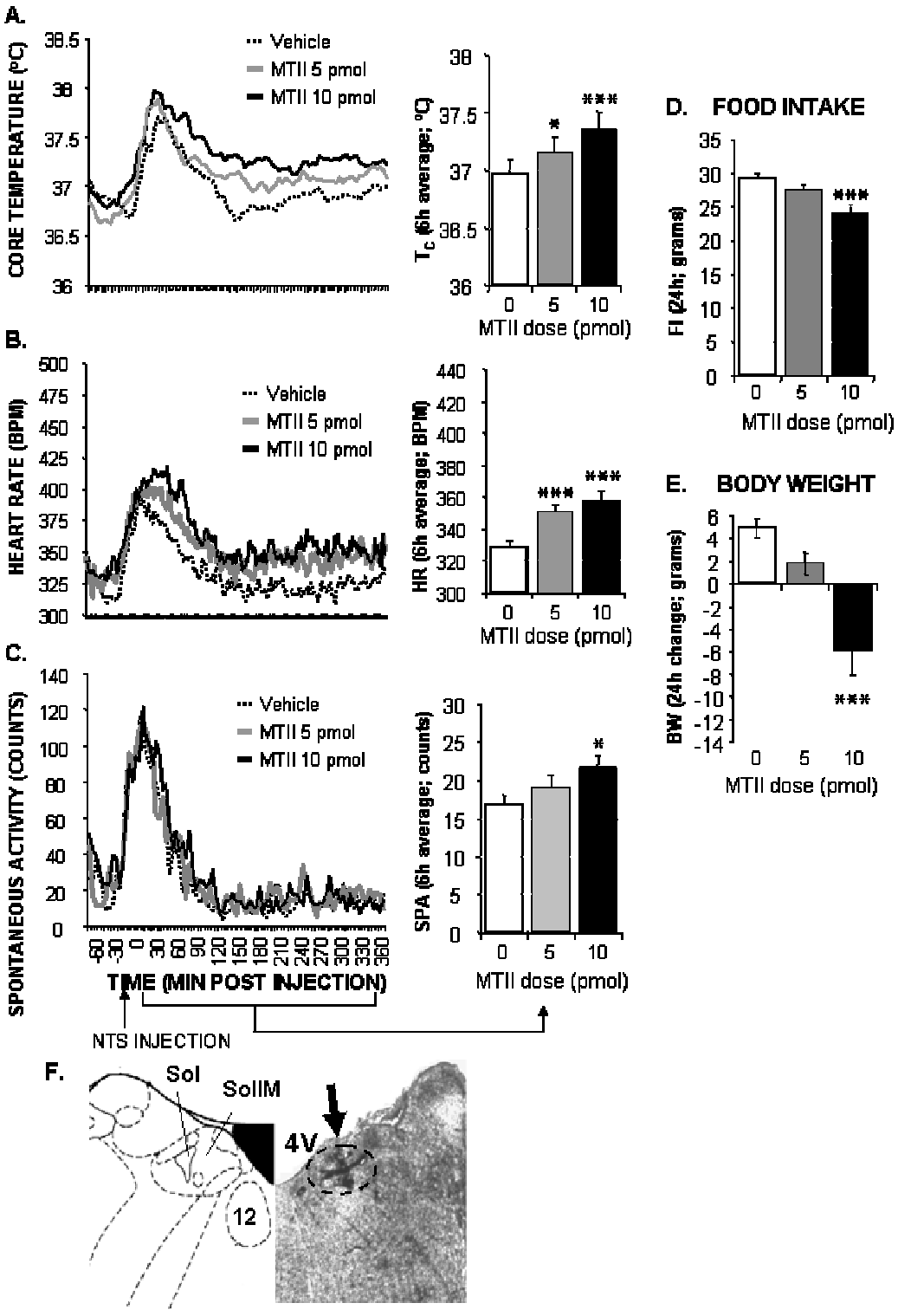

Figure 3.1: Effect of MTII stimulation of nucleus of the solitary tract (NTS) MCRs on (A) core temperature (T_C), (B) heart rate (HR), (C) spontaneous activity in rats. Line graphs

represent across-rat average parameter measurements through the recording period. The bracketed time period on the line graph x-axis indicates the periods used in the adjacent histograms, which provide 6 h post-injection averages + SEM for each parameter at each dose. Effect of NTS MTII injection on 24 h food intake (D) and 24 h change in body weigh (E) in awake, non-anesthetized rats. Food was made available to animals 6 h after injections and through the 12 h period of the dark cycle. Histograms represent means + SEM. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$. (F) Representative NTS injection site (indicated by the arrow). 4V, fourth ventricle; Sol, nucleus of the solitary tract; SolIM, intermedial nucleus of the solitary tract. All following figures presented in this chapter will have a similar format; the principal variable is the site of parenchymal site of injection of MTII.

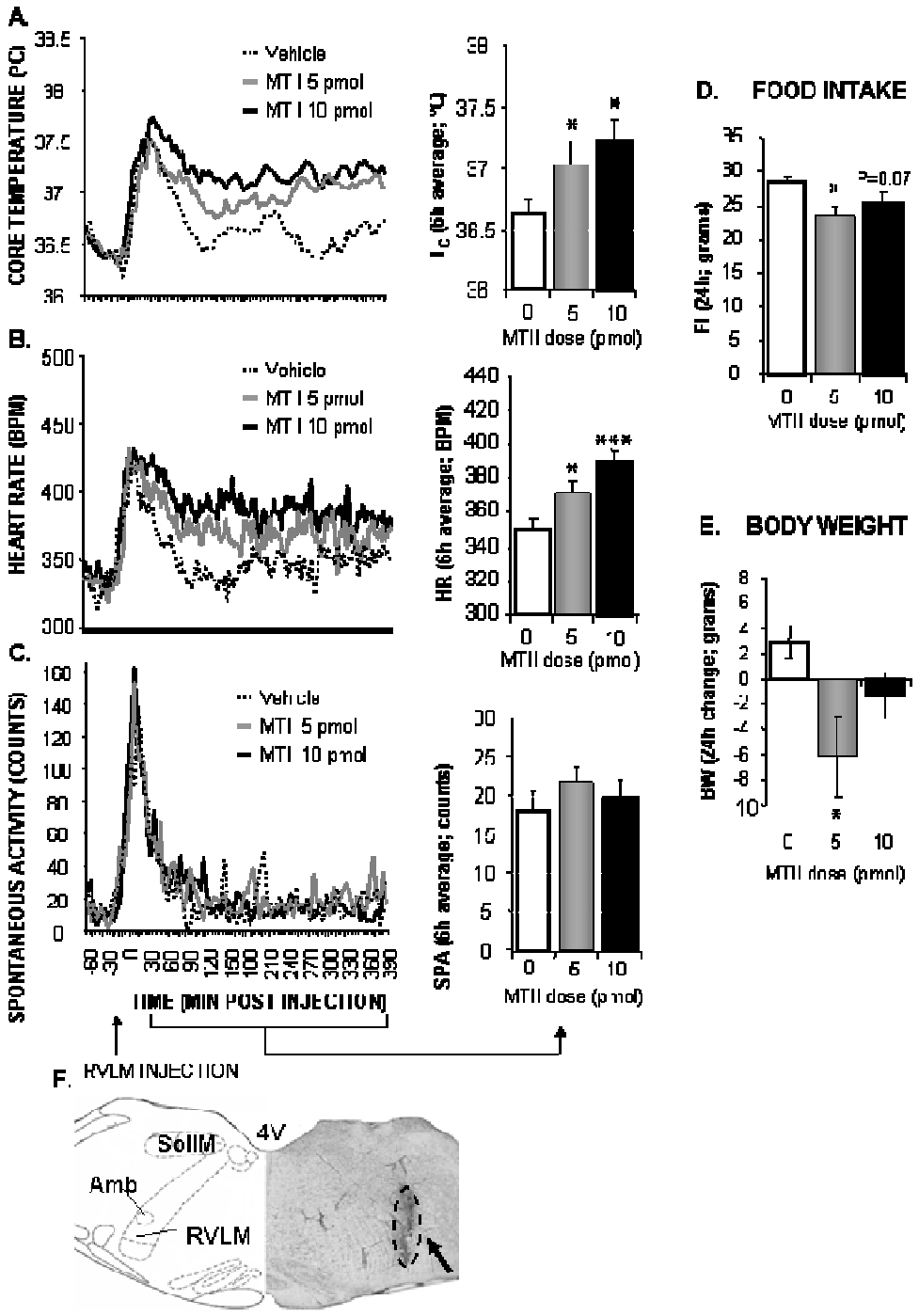

Figure 3.2: Effect of MTII stimulation of rostral ventrolateral medulla (RVLM) MCRs on T_C (A), HR (B), spontaneous activity (C), 24-h food intake (D), and 24-h change in body weight (E). (F) Representative RVLM injection site (indicated by the arrow). 4V, fourth ventricle; SolIM, intermedial nucleus of the solitary tract; Amb, nucleus ambiguus.

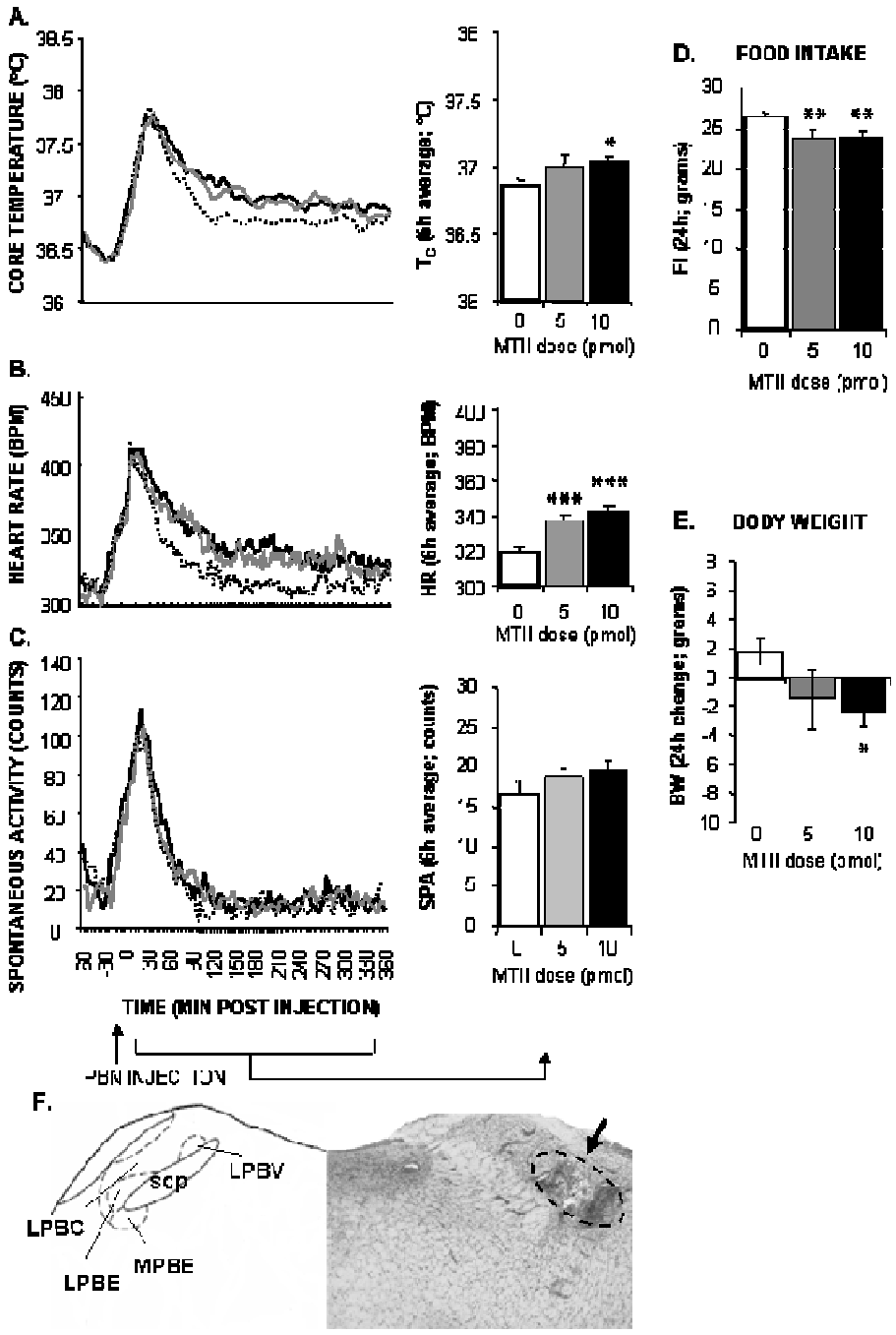

Figure 3.3: Effect of MTII stimulation of parabrachial nucleus (PBN) MCRs on T_C (A), HR (B), spontaneous activity (C), 24-h food intake (D), and 24-h change in body weight (E). (F) Representative PBN injection site (indicated by the arrow). LPBV, lateral parabrachial nucleus ventral; LPBE, lateral parabrachial nucleus external; LPBC, lateral parabrachial nucleus central; MPBE, medial parabrachial nucleus external; scp, superior cerebellar peduncle.

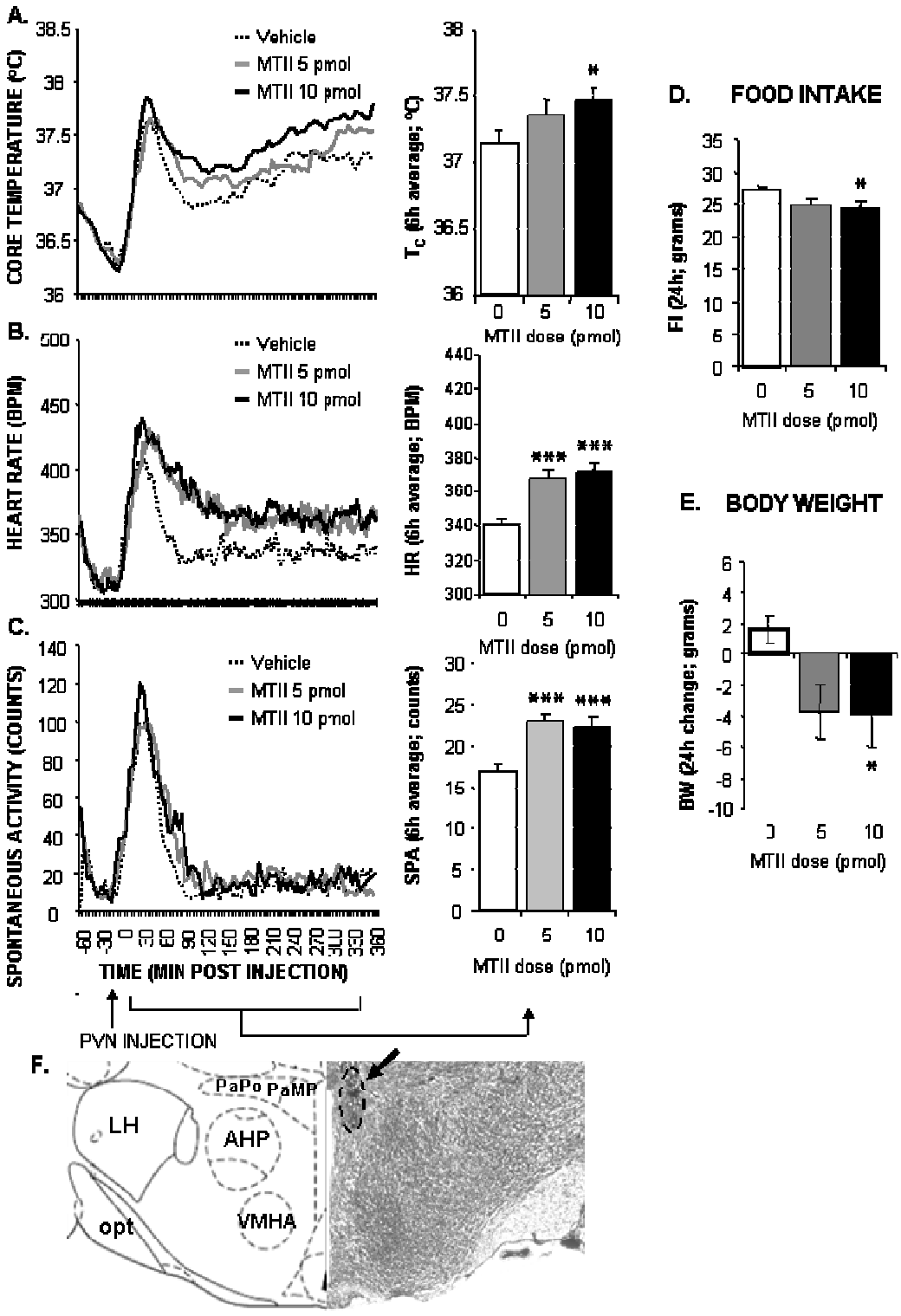

Figure 3.4: Effect of MTII stimulation of paraventricular nucleus (PVN) MCRs on T_C (A), HR (B), spontaneous activity (C), 24-h food intake (D), and 24-h change in body weight

(E). (F) Representative PVN injection site (indicated by the arrow). PaPo, paraventricular nucleus posterior part; PaMP, paraventricular nucleus medial parvicellular; LH, lateral hypothalamus; AHA, anterior hypothalamic area; VMHA, ventromedial hypothalamus anterior; opt, optic tract.

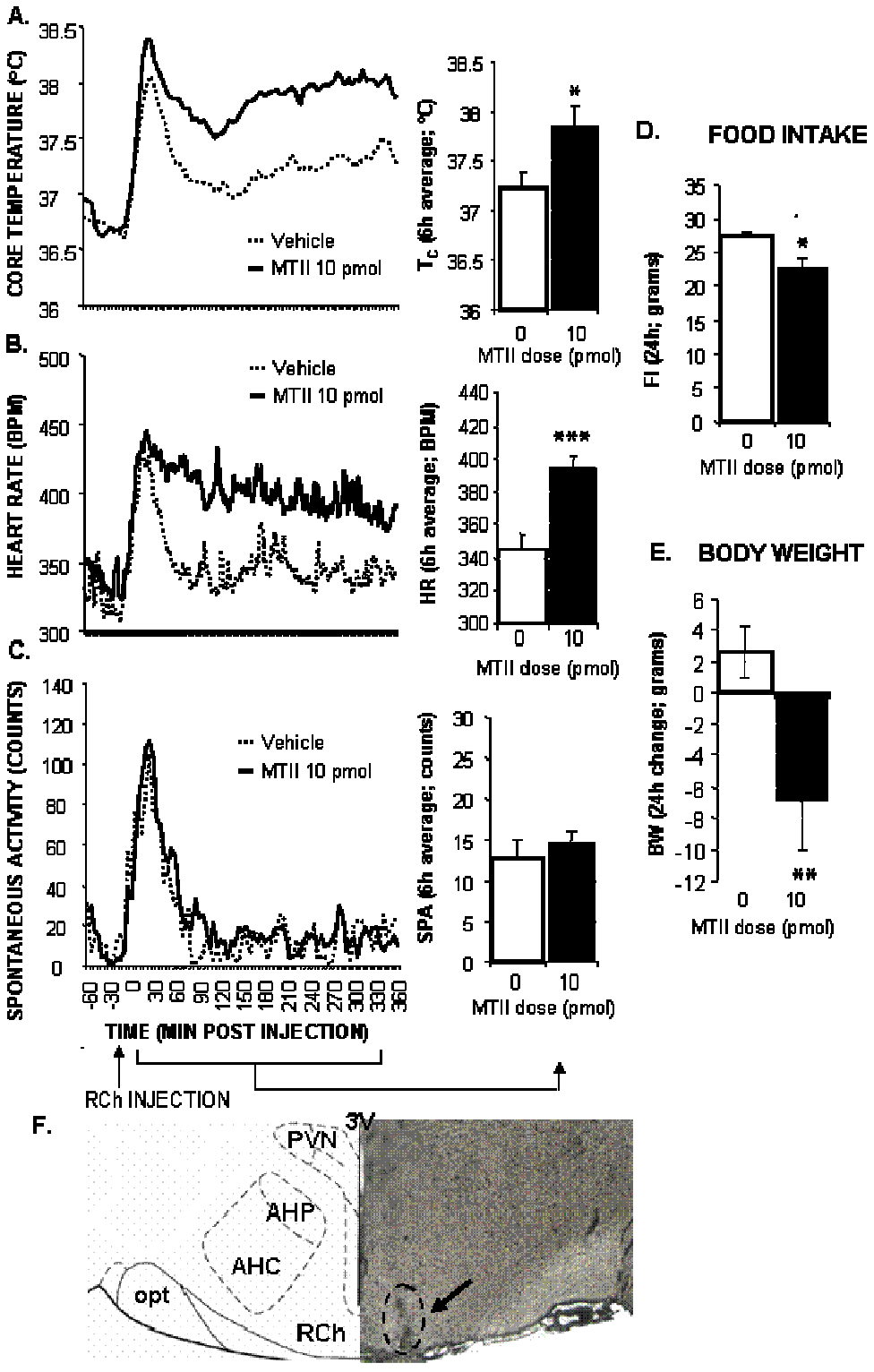

Figure 3.5: Effect of MTII stimulation of retrochiasmatic area (RCh) MCRs on T_C (A), HR (B), spontaneous activity (C), 24-h food intake (D), and 24-h change in body weight (E).). (F)

Representative PVN injection site (indicated by the arrow). AHC, anterior hypothalamic area central; AHP, anterior hypothalamic area posterior; opt, optic tract; 3V, third ventricle.

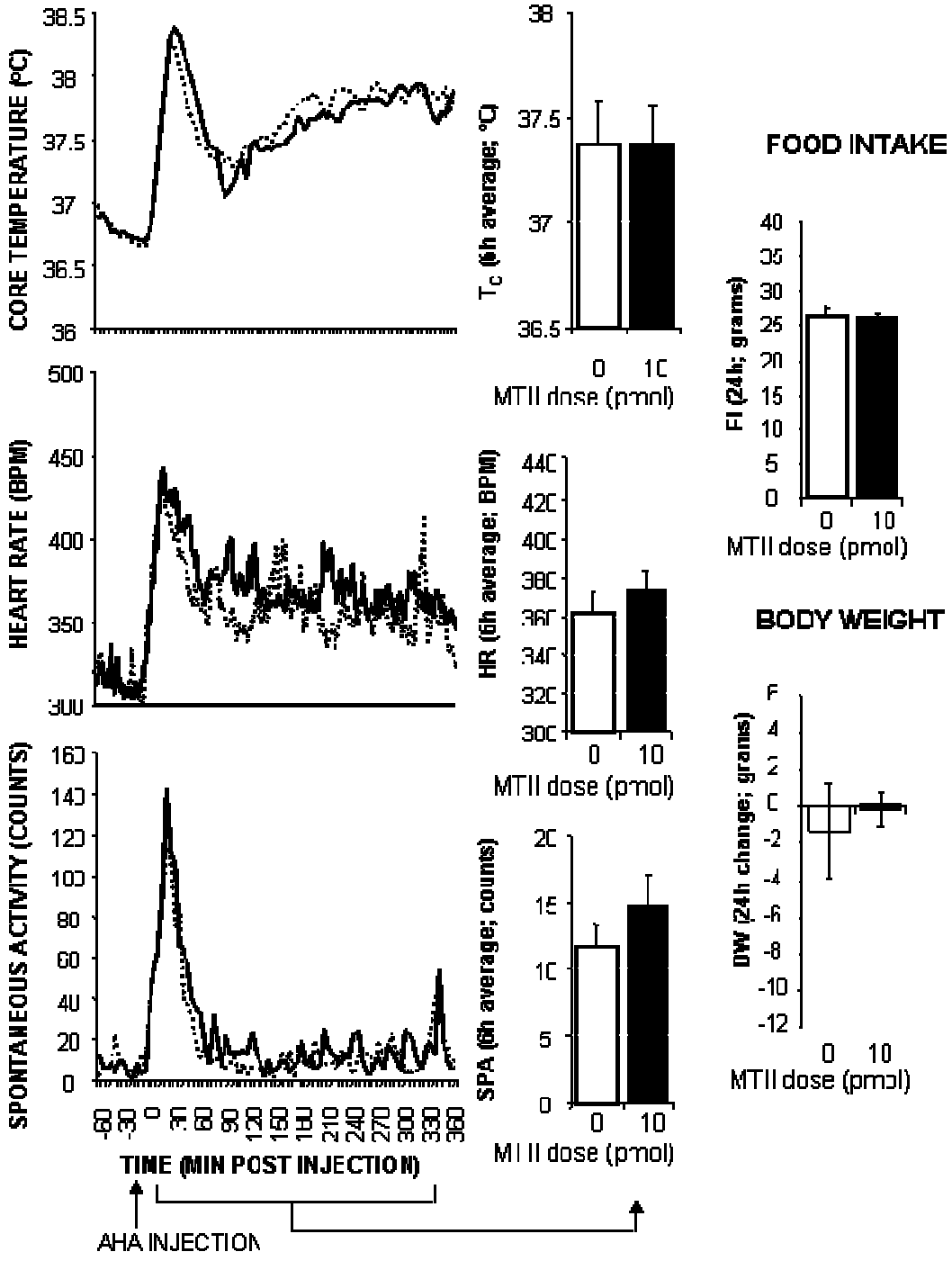

Figure 3.6: Effect of MTII stimulation of anterior hypothalamic area (AHA) MCRs on T_C (A), HR (B), spontaneous activity (C), 24-h food intake (D), and 24-h change in body weight (E).

CHAPTER 4

HINDBRAIN LEPTIN STIMULATION INDUCES ANOREXIA AND HYPERTHERMIA MEDIATED BY HINDBRAIN MELANOCORTIN[3] RECEPTORS

ABSTRACT

Of the CNS receptors that could mediate the energy balance effects of leptin those of the hypothalamic arcuate nucleus receive the greatest attention. Melanocortin receptors (MCRs) contribute to the feeding and energetic effects of hypothalamically delivered leptin. Energy balance effects of leptin are also mediated by extra-hypothalamic neurons including the hindbrain nucleus tractus solitarius. Hindbrain leptin receptors play a role in leptin's anorectic effects but their contribution to its energetic effects and their functional interaction with melanocortin systems within the hindbrain remains unexplored. Here, rats implanted with telemetric devices for recording energetic/cardiovascular responses were examined to determine whether: 1) hindbrain (4^{th} ventricular; v) leptin receptor stimulation triggers energetic and cardiovascular effects, 2) these effects are altered by a 6 week high-fat diet maintenance, and 3) hindbrain MCRs mediate the thermogenic, cardiovascular and anorexic effects of hindbrain leptin delivery. Results show that hindbrain leptin receptor stimulation produced long-lasting (>6h) increases in core temperature and heart rate and also decreased food intake and body weight. These responses were not altered by high-fat maintenance; in contrast to what has been reported for forebrain leptin delivery. Fourth v pretreatment with MCR antagonist SHU 9119 completely abolished the hyperthermia,

[3] These results were partially reported at the 2008 Keystone Meeting, in Banff, Canda and appeared in Endocrinology, 150(4):1705-11, 2009. This work was supported by NIH grants DK-21397 and NRSA NS-059254.

anorexia and body weight loss seen with hindbrain-directed leptin but had no effects of its own. These data highlight a role for hindbrain leptin receptors in the initiation of energetic and anorexic responses and show that MCRs are part of the downstream mediation of hindbrain leptin-induced energy balance effects, paralleling effects observed for hypothalamic leptin receptors.

INTRODUCTION

Disruption in central nervous system (CNS) leptin signaling results in severe obesity (Pelleymounter et al., 1995). Exogenous leptin delivery reverses the hyperphagia and reductions in activity, metabolism, and body temperature that contribute to the obesity observed in ob/ob mice (Halaas et al., 1995; Pelleymounter et al., 1995; Halaas and Friedman, 1997). Central leptin receptors mediate these energy balance effects (Elmquist et al., 1997). Although leptin receptors (Ob-Rbs) are anatomically distributed across nuclei in the basal forebrain, midbrain and hindbrain of relevance to energy balance control (Elmquist et al., 1998), the literature is dominated by a focus on the role of ObRb-bearing arcuate hypothalamic neurons. Recent experiments establish contributions to the control of energy intake from leptin signaling in the ventromedial hypothalamus (Dhillon et al., 2006), ventral tegmental area (Hommel et al., 2006) and nucleus tractus solitarius (NTS) (Huo et al., 2007). The neural mediation of leptin's energy expenditure effects receives considerably less attention than its energy intake effects (Hermann et al., 2006), yet the focus here too is on hypothalamic leptin signaling (Rahmouni and Morgan, 2007). This paper addresses the contribution of caudal brainstem leptin receptors in mediating the energetic effects of leptin.

The energy balance effects of basal forebrain-directed, third ventricular (3rd v) leptin delivery are mediated, in part, by downstream effects on hypothalamic proopiomelanocortin (POMC) neurons and melanocortin receptors (MC-Rs) (Seeley et al., 1997; Satoh et al., 1998; Haynes et al., 1999). This perspective is supported by experiments that show that: leptin signaling in these neurons increases POMC gene expression (Schwartz et al., 1997; Elmquist, 2001) and pretreatment with MC-R antagonist attenuates the anorexic effects, as well as some of the sympathetically-mediated energetic effects of 3rd v leptin delivery (Seeley et al., 1997; Haynes et al., 1999). Therefore, a second aim of this paper was to examine whether caudal brainstem MC-Rs and NTS POMC neurons contribute to the energy balance effects triggered by leptin signaling in the caudal brainstem.

High-fat (HF) diet feeding in rodents and humans results in obesity, hyperleptinemia, resistance to the effects of exogenous leptin and insulin delivery and glucose intolerance (Gleason et al., 2007; Myers et al., 2008). Leptin resistance is characterized by a decreased sensitivity to the anorexic and body weight effects of leptin (Myers et al., 2008). As noted above, there is a developing consensus that the effect of leptin on energy balance is mediated by several brain nuclei. By contrast, the neuroanatomical basis of leptin resistance is less clear. Leptin resistance may result from alterations in leptin signaling across several CNS sites or by contrast may be attributable to specific populations of Ob-Rb expressing neurons in the CNS (Munzberg et al., 2004; Enriori et al., 2007). A variety of experimental evidence is consistent in revealing that forebrain ventricular delivery of leptin results in diminished hypophagia in rats maintained on HF diet than in chow-maintained controls (Widdowson et al., 1997; Tulipano et al., 2004; Fam et al., 2007). While it is generally assumed that

hypothalamic Ob-Rb expressing neurons are the principal target of forebrain ventricular delivery, it is unclear whether other ObRb-expressing nuclei accessed by this injection also contribute to the reduction in response magnitude observed in HF-diet maintained rodents. Unexamined, are the effects of HF-diet maintenance on the hypophagia to hindbrain ventricular delivery of leptin. It also remains to be determined whether some, but possibly not all, responses induced by central leptin delivery are attenuated by HF-diet maintenance as sympathetic nervous system (SNS) activation is observed in leptin-treated animals that are otherwise leptin resistant (Rahmouni et al., 2002; Rahmouni et al., 2008). For these reasons we pursue the functional consequences of high-fat diet maintenance on the energy intake, as well as energy expenditure responses triggered by hindbrain-delivered, 4th ventricle leptin.

Collectively, the results described here establish a role for hindbrain Ob-Rb in mediating elevations in core temperature and heart rate. These energetic and cardiovascular responses, as well as anorexic and body weight responses triggered by hindbrain leptin delivery were found to be unaltered in rats fed high-fat diet. In addition, our studies provide support for the hypothesis that hindbrain MC-Rs are downstream mediators of hindbrain leptin-induced energetic and anorexic responses.

METHODS

Subjects: Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 300–400 g at the time of surgery, were housed individually in plastic bins, and maintained under a 12:12-h (8.00am lights on) light-dark cycle. Pelleted food (Purina 5001; St. Louis, MO) and water were available *ad libitum* unless otherwise noted. All

procedures conformed to the institutional standards of animal care and use (University of Pennsylvania).

Surgery: Rats were anesthetized with ketamine (90 mg/kg), xylazine (2.7 mg/kg), and acepromazine (0.64 mg/kg) delivered intramuscularly.

4th intracerebroventricular cannulation. All rats received a fourth-intracerebroventricular (4th v) guide cannula (Plastics One, 22-G) with its tip stereotaxically positioned 2.0 mm above the 4th v (coordinates: on the midline, 2.5 mm anterior to the occipital suture, and 4.5 mm ventral to the dura, with injector aimed 6.5 mm ventral from dura). Cannulas were attached to the skull with dental acrylic and jeweler's screws and closed with an obturator.

Telemetric transponder surgery: At the time of ventricular surgery, telemetric transponders (HRC 4000 Mini-Mitter, VitalView, OR) for recording core temperature (Tc), heart rate (HR) and spontaneous physical activity (SPA) were inserted into the abdominal cavity, external leads were positioned subcutaneously, and secured to the muscles on either side of the heart with sutures [as previously described (Skibicka and Grill, 2008b)].

Experimental procedure

Cannula position verification. At least 7 days after surgery, 4th v cannula placement was assessed by measurement of the sympathoadrenal mediated hyperglycemic response to the cytoglucopenia induced by 5-thio-D-glucose [210 μg in 2 μl of artificial cerebral

spinal fluid (aCSF)] (Ritter et al., 1981). A post-injection elevation of at least 100% of baseline glycemia was required for subject inclusion.

Habituation training. Prior to the start of experimental testing, rats were acclimated to handling, 4th v injections, and gastric gavages for oral glucose tolerance tests (OGTT) testing.

Food intake and body weight monitoring: Food was removed 1 h before treatment (1 h into the light cycle) and returned 8 h later, late in the light phase. Thereby, food was not available during the period of energetic response measurement as feeding can affect these responses. Food intake and body weight measurements were made 24 h after the injection of drug. Food was available during the dark cycle and a minimum of 48 h was allotted between experimental testing.

Experiment 1*: Effects of 4th v leptin delivery on energy expenditure and food intake in chow-fed and high fat-fed rats.*

Feeding maintenance: Rats were randomly assigned to standard chow (n=8) or HF diet [(Research Diets #D12266B, New Brunswick, NJ, USA; 4.47 kcal/g with 21% of the metabolizable energy content as protein, 31% as fat and 48% as carbohydrate), (n=16)] maintenance one week after surgery. All testing began after five weeks of maintenance on the respective diets. Food intake and body weight of all rats were monitored daily. The physiological alterations induced by HF-diet maintenance are well established and include hyperleptinemia-leptin resistance and hyperinsulinemia-insulin resistance [see for review (Gleason et al., 2007; Myers et al., 2008)]. The OGTT was administered before HF exposure and 1, 4 and 5 weeks after diet maintenance to provide one correlate of the physiological effects of HF diet maintenance. Food was withheld for a 6-h period

(with access to water) during the first half of the light cycle; an oral glucose load (2 g/kg) was delivered to each rat by gavage. Blood glucose was measured before gavage and at 30, 60, 90, 120 and 180 min post-glucose loading by collecting a drop of tail blood and placing it in a standard glucometer strip (Accucheck, Roche Diagnostics, Indianapolis, IN).

Experimental testing: All rats received 4th v injections early in the light cycle. Due to the caudal flow of the cerebral spinal fluid, low volume injections through the 4th v cannula under specific experimental conditions provide selective stimulation of hindbrain CNS regions without affecting hypothalamic and other forebrain regions as shown by: (Flynn and Grill, 1985; Kinzig et al., 2002; Blevins et al., 2004; Fan et al., 2004). Three conditions were run in a counterbalanced fashion across separate days. Energetic responses were examined in chow- and HF-fed rats following a control condition with 4th v vehicle (1 µl sodium bicarbonate) and two doses of leptin: 3 and 10 µg [dose selection based on (Widdowson et al., 1997; Levin and Dunn-Meynell, 2002; Tulipano et al., 2004)]. HR, T_C, and SPA were continuously monitored for 8 h at 5-min intervals (Tc and SPA) or 30-sec intervals (HR) in rats with implanted HRC-4000 transponders.

***Experiment 2*:** *Effect of hindbrain- delivered MC-R antagonist on the energy balance effects of leptin delivered to hindbrain.* All rats (n=9) received 4th v injections early in the light cycle. All conditions were separated by 2 days and run in a counterbalanced fashion. Responses were examined after four conditions as follows: 1) control condition with 4th v vehicle [1 µl of aCSF (vehicle for MC3/4-R antagonist - SHU 9119) with 1 µl of sodium bicarbonate (vehicle for leptin)], 2) aCSF +3 µg leptin, 3) 0.1 nmol SHU 9119 + sodium bicarbonate and 4) SHU 9119 with leptin. SHU 9119/vehicle was injected 20 minutes before leptin/vehicle treatment. SHU 9119 dose was selected from results of a

pilot study that tested the efficacy of SHU doses in blocking the energy balance effects of 4th v MC-R agonist (MTII; Phoenix Pharmaceutical, St Joseph, MO) without producing any independent effects alone. HR, T_C, and SPA were continuously monitored as in *Experiment 1*.

Statistical analysis. All energy expenditure parameters were analyzed by one or two-way ANOVAs on 6 h post-injection averages and followed by post-hoc t-tests and Tukey's honestly significant difference test as appropriate. Twenty-four h food intake and body weight were analyzed by ANOVA followed by post-hoc t-tests and Tukey's honestly significant difference test as appropriate. All statistical analysis was conducted using Statistica software (Tulsa. Oklahoma). Differences were considered significant at $P<0.05$.

RESULTS:

Experiment 1. Stimulation of hindbrain Ob-Rb increases energy expenditure and decreases food intake in chow- and high-fat fed rats:

Chow- fed group: Core temperature: Figure 4.1a shows that hindbrain-delivered leptin injection increased T_C for the 6 h post-injection period. A one-way ANOVA examining treatment effects on average post-injection T_C values revealed a significant drug treatment effect [$F(2, 10) = 13.89$, $P < 0.005$]. Post hoc analysis showed a significant effect of both leptin doses on T_C (3 μg; $P < 0.005$, 10 μg: $P < 0.005$). Heart rate: Figure 4.1b shows that leptin increased HR, compared with control treatment. A one-way ANOVA of average HR values for the 6 h post-injection period yielded a significant drug

effect [F (2, 10) = 6.40, P < 0.05); post hoc analysis showed a significant effect of the lower dose of leptin on HR (3 μg; P < 0.05, 10 μg: P < 0.323). Spontaneous physical activity was not significantly increased by leptin treatment [F (2, 10) = 1.79, P =0.216; Figure 4.1c]. Food intake and body weight: Fourth ventricle injection of leptin significantly decreased 24 h food intake [F (2, 14) = 6.883, P < 0.01; post hoc: 3 μg; P < 0.28, 10 μg: P < 0.05] and body weight [F (2, 14) = 4.155, P < 0.05; post hoc: 3 μg; P < 0.82, 10 μg: P < 0.05] (Figure 4.2a & b).

High-fat fed group: Oral glucose tolerance tests were performed prior to HF-diet exposure [area under the curve ± SE: 315.4 ± 15.8 chow-fed (CH) vs. 320.3 ± 7.4 high fat (HF)] and after 1 week (CH: 312.6 ± 16.2 vs. HF: 312.9 ± 5.5), 4 weeks (CH: 313.1 ± 8.9 vs. HF: 348.6 ± 6.5± 8.9) and 5 weeks (CH: 316.7 ± 8.0 vs. HF: 361.7 ± 6.4) on HF diet maintenance in comparison to chow-fed control rats. High-fat fed animals showed impairment in glucose clearance relative to chow-fed control rats after 4 weeks (P < 0.005) and 5 weeks (P < 0.0005) of high fat exposure. All rats on HF diet showed reduced glucose clearance after 5 weeks on HF diet; therefore all were included in the energy expenditure analysis. Core temperature: Figure 4.3a shows that 4th v injection of each dose of leptin increased T_C for the 6 h post injection period. A one-way ANOVA examining treatment effects on average post-injection T_C values yielded a significant drug treatment effect [F (2,28) = 7.00, P < 0.005]. Post hoc analysis revealed a significant effect of both leptin doses on T_C (3 μg; P < 0.01, 10 μg: P < 0.01). Heart rate: Figure 4.3b shows that leptin increased HR, compared with the aCSF vehicle treatment. A one-way ANOVA of average HR values for the 6 h period post-injection yielded a significant drug effect [F (2,28) = 5.47, P < 0.01]; post hoc analysis showed a significant effect of both doses of leptin on HR (3 μg; P < 0.05, 10 μg: P < 0.05). Spontaneous

physical activity was not significantly increased [F (2, 28) = 0.029, P = 0.972] by leptin treatment (Figure 4.3c). Food intake and body weight: Both leptin doses significantly decreased 24 h food intake [F (2, 30) = 18.79, P < 0.0001; post hoc: 3 μg; P < 0.0005, 10 μg: P < 0.0005] and body weight [F (2, 30) = 13.75, P < 0.0001; post hoc: 3 μg; P < 0.0005, 10 μg: P < 0.0005] (Figure 4.4a & b). Between-group comparisons (chow-fed vs. high-fat fed) were made for all parameters. No significant differences in T_C, HR, SPA and food intake of chow- and high-fat fed groups were found for any doses. Body weight responses were significantly different at the 0 μg and 10 μg doses, but when responses were expressed as change from vehicle no significant differences were found.

Experiment 2: Hindbrain- delivered MC-R antagonist blocks the energy balance effects of hindbrain leptin delivery. Hindbrain pretreatment with a MC-R antagonist reversed the effects of 4th v leptin treatment on energetic/cardiovascular responses, food intake and body weight, but was without effects on its own (Figures 4.5 & 6). Core temperature: Figure 4.5a shows that the leptin-induced increase in T_C for the 6 h post injection period was reversed by pretreatment with the MC-R antagonist SHU 9119. A two-way ANOVA examining the interaction of leptin and SHU 9119 on average post-injection T_C values revealed a significant interaction [F (1, 8) = 9.25, P < 0.05]. Post hoc analysis revealed a significant effect of only leptin alone on T_C (3 ug; P < 0.05) compared to vehicle. The leptin + SHU 9119 condition was not significantly different from vehicle (P = 0.88). Heart rate: Figure 4.5b shows that there was a trend for leptin to increase HR although the effect was not significant compared with the control treatment. A one-way ANOVA of average HR values for the 6 h post-injection period yielded a significant drug effect [F (3, 24) = 3.714, P < 0.05]; post hoc analysis showed a trend for leptin to increase HR (3 μg; P < 0.078), but SHU 9119 pretreatment attenuated the effect of

leptin on HR (P = 0.999). A two-way ANOVA of average HR values for the 6 h post-injection period examining the interaction of leptin and SHU 9119 did not reach significance [F (1, 8) = 2.61, P = 0.14]; Spontaneous physical activity was not significantly affected by leptin, SHU 9119, or their combination. There was no significant interaction [F (1, 8) = 2.06, P = 0.19] (Figure 4.5c). Food intake and body weight: Hindbrain-directed leptin delivery significantly decreased 24 h food intake. Two-way ANOVA revealed significant interaction of leptin and MC-R antagonist [F (1, 8) = 7.83, P < 0.05]; post hoc Tukey tests revealed that leptin decreased food intake (P < 0.005), pretreatment with SHU 9119 blocked the effects of leptin [leptin + SHU 9119: P = 0.97] (Figure 6a). Body weight was also significantly decreased by leptin treatment alone, pretreatment with SHU 9119 reversed this effect [F (1, 8) = 11.55, P < 0.05; post hoc Tukey test for leptin alone: P < 0.05, and for leptin SHU 9119: P = 0.79] (Figure 4.6b). SHU9119 application itself had no effect on any of the parameters measured.

DISCUSSION

The data gathered indicate that hindbrain leptin receptors contribute to the energy balance effects of CNS leptin delivery. These are the first data to show that stimulation of hindbrain leptin receptors drives thermogenic and tachycardic responses. The hypophagia and body weight reduction also observed with hindbrain leptin delivery confirm our earlier work in lean chow-fed rats (Grill and Kaplan). The present study also shows that hindbrain leptin delivery in high-fat diet maintained rats with reduced glucose clearance triggers anorexic and energetic responses of the same magnitude as those observed in chow-fed controls. These data indicate that alterations in hindbrain Ob-Rbs signaling do not contribute to the physiology of leptin resistance.

A role for hypothalamic melanocortin gene expression and MC-Rs in the downstream effects of hypothalamic leptin signaling is well established (Seeley et al., 1997; Haynes et al., 1999). Our experiments address a parallel but neglected question – whether hindbrain melanocortin signaling is downstream of the functional effects of hindbrain leptin signaling. We show that hindbrain delivery of the MC3/4-R antagonist SHU-9119 blocked the food intake, body weight and energetic responses triggered by 4th v leptin. This pattern of results is parallel with data generated for forebrain ventricular delivery using a similar strategy (Seeley et al., 1997; Satoh et al., 1998; Haynes et al., 1999). Systemic leptin treatment stimulates Ob-Rs on ARC POMC neurons, induces pSTAT3 in arcuate POMC neurons and increases POMC gene expression. These effects are mediated by direct action of leptin on these POMC neurons. Some controversy surrounds whether NTS POMC neurons express leptin receptors in mouse models (Ellacott et al., 2006; Huo et al., 2006). Whether the effects observed here are mediated by leptin receptor expression in rat NTS POMC neurons or leptin receptors on other neurons that in turn affect NTS POMC gene expression and melanocortin ligand release remains to be investigated.

Obesity induced by multi-week HF-diet maintenance did not reduce the magnitude of either the anorexic or energetic-sympathetic responses elicited by hindbrain leptin administration. These functional results are consistent with the findings of Munzberg et al (Munzberg et al., 2004) who showed that multi-week, HF diet maintenance did not reduce Ob-Rb signaling (pSTAT3 immunohistochemistry) in NTS neurons or in the neurons of the ventromedial, dorsomedial or premammilary hypothalamus [see also (Ladyman and Grattan, 2004)]. Of the brain regions sampled in their study, an attenuation of leptin signaling with diet-induced obesity was observed

only in the hypothalamic ARC nucleus; this reduced ARC-leptin signaling was detected as early as 6 days after the start of HF diet feeding (Munzberg et al., 2004). While we have not examined forebrain-directed leptin responses, others investigators have established that lateral ventricular leptin delivery (doses overlapping with those used here) in rats made obese with HF feeding (Widdowson et al., 1997; Tulipano et al., 2004) or in obese aged rats (Shek and Scarpace, 2000) result in markedly attenuated food intake and body weight effects of leptin. These examples of attenuated functional effects of leptin delivered to the forebrain ventricles of rats with some degree of leptin resistance contrast with what we observed in similarly maintained rats receiving 4th v. leptin. These contrasting data sets suggest that specific CNS Ob-Rb expressing regions respond differently to the obesity resulting from high-fat diet maintenance and also that lack of alterations in NTS leptin signaling (Munzberg et al., 2004) correlate with preserved effects on feeding and energy expenditure. Hindbrain Ob-Rbs, due to their lack of molecular and functional leptin resistance, might therefore account for the preserved SNS activation observed after peripheral leptin injections in animals otherwise leptin resistant (Rahmouni et al., 2002; Rahmouni et al., 2008). Ob-Rbs in the hindbrain might also contribute to partial anorexic responses observed after lateral ventricle leptin application, since the drug can diffuse with the caudal CSF flow and stimulate ObRb-expressing hindbrain neurons.

Our studies focus attention on the functional effects of hindbrain leptin receptors and, in addition, identify a downstream mediator of these effects, POMC neurons and hindbrain melanocortin receptors. Furthermore, our data along with findings by Munzberg et al (Munzberg et al., 2004) suggest that this hindbrain projection pathway may, unlike its hypothalamic counterpart, be less subject to the effect of the reduction in

leptin signaling observed with diet-induced obesity. The details of the mediating neural circuitry however still remain to be delineated. Neurons of the NTS and parabrachial nucleus (PBN) express OB-Rbs (Elmquist et al., 1998) and contribute to the control of feeding behavior and to sympathetic outflows (Grill and Kaplan, 2001; Cano et al., 2003a). We have recently conduced studies employing targeted leptin delivery to NTS as well as PBN are to resolve this issue. Direct application of leptin into the NTS resulted in an energetic a feeding profile of responses similar to that obtaiend with 4th v leptin delivery. Thus it is likely that the energetic and part of the anorexic responses observed here reflect NTS Ob-Rb stimulation (Figures 4.7-4.8). Parabriachial Ob-Rb stimulation on the other hand did not alter body temperature or heart rate, however produced hyperactivity, anorexia and body weight loss (Figures 4.9-4.11). These are the first data indicating a role for PBN Ob-Rb in energy balance control. Results from our current studies do not exclude the possibility that projections to forebrain neurons are required for response production. Little is known about the neurochemical phenotype and anatomic projections of Ob-Rb-expressing hindbrain neurons. NTS neurons are known to project to hypothalamic neurons including those in ARC and paraventricular hypothalamus. NTS neurons may project to POMC-expressing ARC neurons, that in turn, project back to the NTS to provide for the release of MC ligand in the NTS from a hypothalamic origin (Palkovits et al., 1987; Berthoud HR, 2008). The requirement for forebrain-hindbrain neural communication for leptin-induced increases in energy expenditure might be suggested by a study of Harris et al (ref). Harris et al. show that peripheral leptin stimulation in chronic decerebrate rats, that lack neural forebrain-hindbrain communication, does not increase energy expenditure; in fact a decrease in oxygen consumption in these rats is noted under certain experimental conditions. On the other hand in its simplest form, the mediating circuitry may be placed entirely

within hindbrain with circulating leptin stimulating hindbrain Ob-Rbs, which could in turn activate hindbrain POMC and MC-Rs, yielding behavioral and autonomic responses. We have already shown (Skibicka and Grill, 2008a) that the circuitry required for melanocortin receptor stimulation-induced hyperthermia and tachycardia is contained entirely within the hindbrain. Future experiments are needed to determine whether the neural link from hindbrain leptin to hindbrain melanocortin receptors is contained entirely within the hindbrain or whether the connection is mediated by circuitry involving forebrain neurons.

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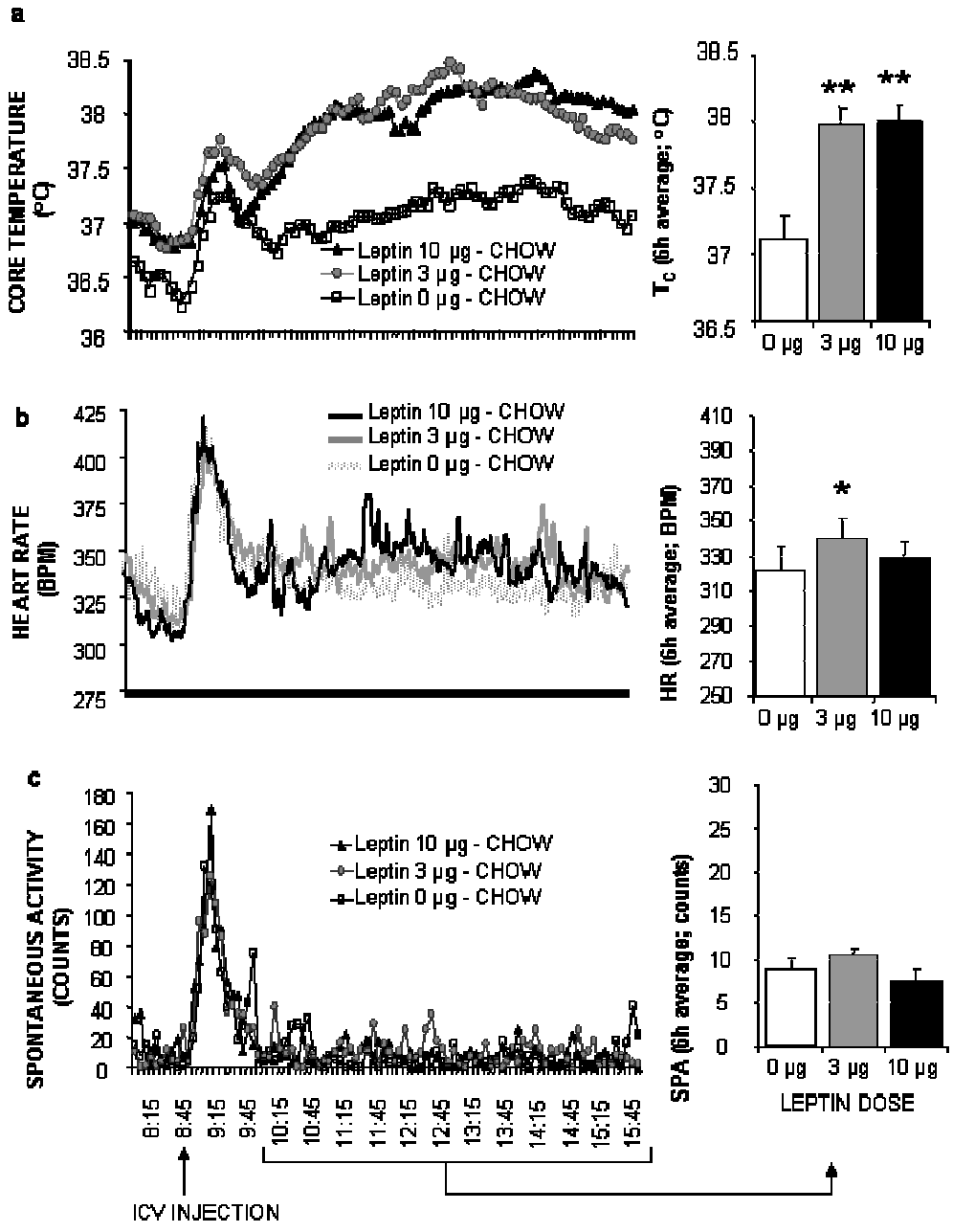

Figure 4.1: Effect of caudal brainstem leptin receptor stimulation (1 µl, 4th v. delivery) on (a) core temperature, (b) heart rate and (c) spontaneous activity **in chow fed rats**. Line graphs represent across-rat average parameter measurements through the 8 h-recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. The histograms provide 6 h post-injection averages + SEM for each parameter at each dose. *P < 0.05, **P < 0.005, n=6.

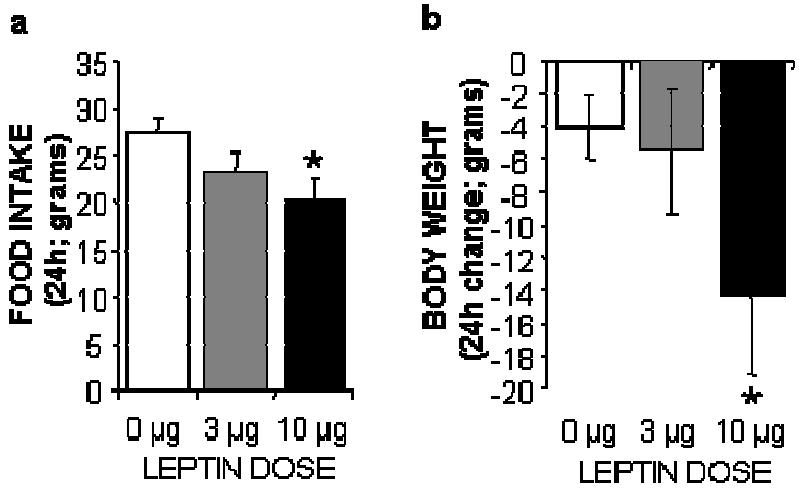

Figure 4.2: Effect of caudal brainstem leptin receptor stimulation (1 µl, 4th v. delivery) on (a) food intake and (b) body weight **in chow fed rats**. The histograms provide average values ± SEM. *P < 0.05, **P < 0.005, n=8.

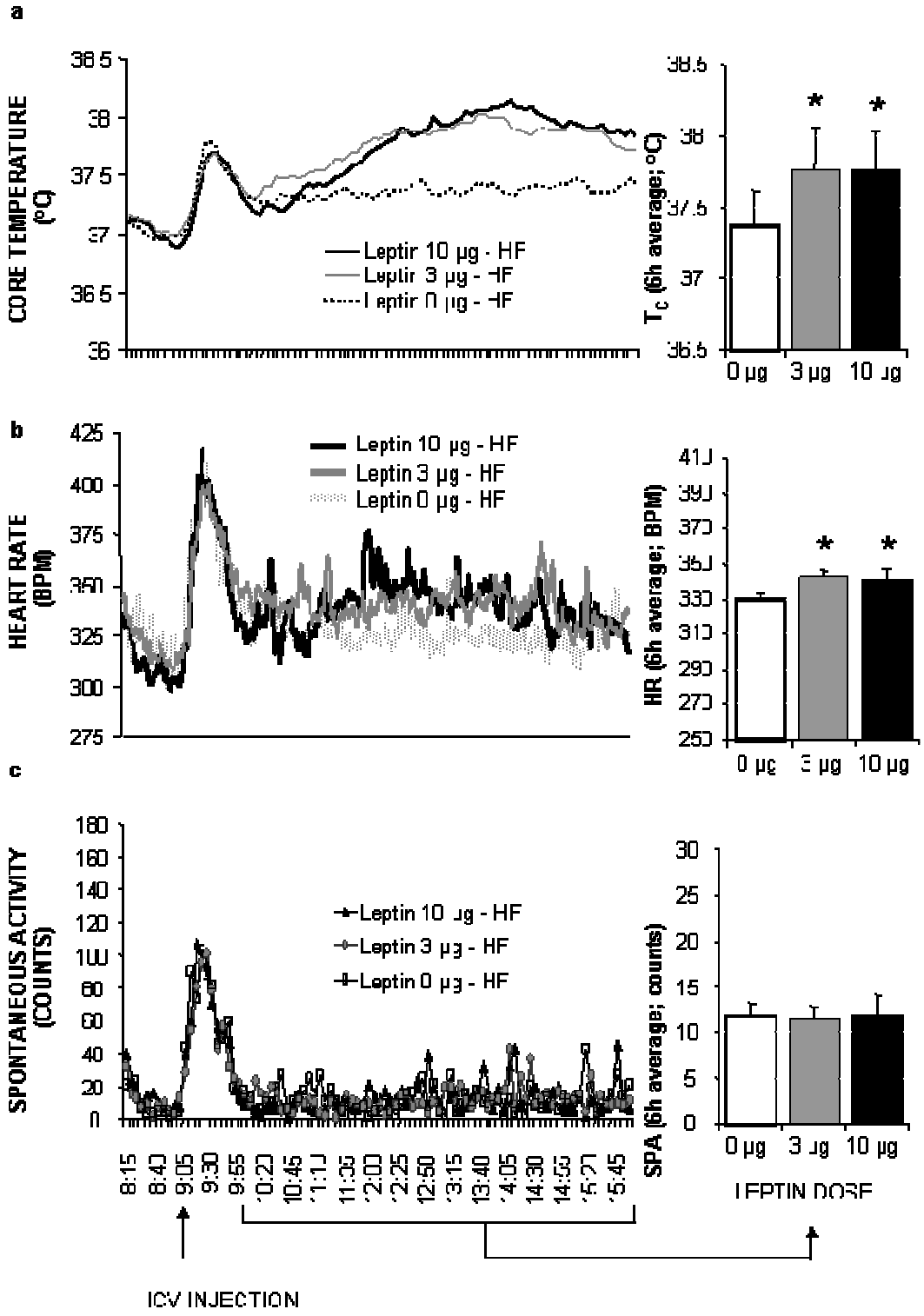

Figure 4.3: Effect of caudal brainstem leptin receptor stimulation (1 µl, 4th v. delivery) on (a) core temperature, (b) heart rate and (c) spontaneous activity **in high-fat fed rats**. Line

graphs represent across-rat average parameter measurements through the 8 h recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. The histograms provide 6 h post-injection averages + SEM for each parameter at each dose. *P < 0.05, ***P < 0.0005, n=15.

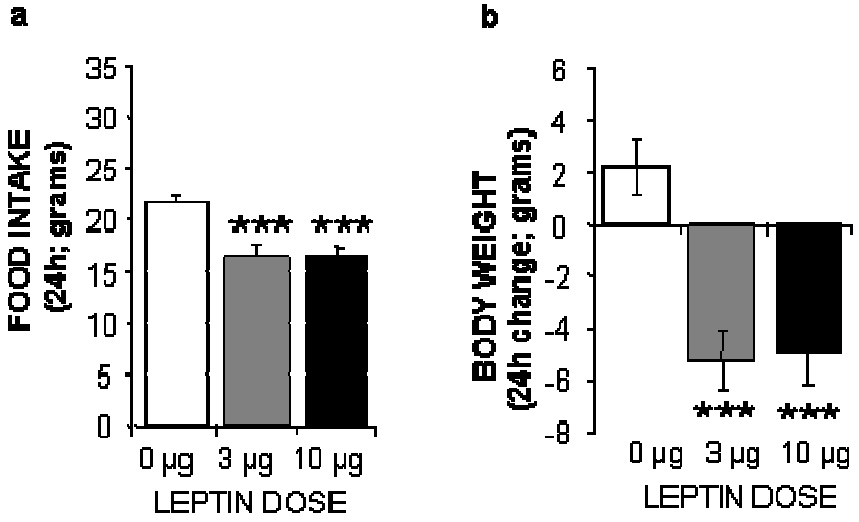

Figure 4.4: Effect of caudal brainstem leptin receptor stimulation (1 µl, 4th v. delivery) on (a) food intake and (b) body **in high-fat fed rats**. The histograms provide average values ±SEM. *P < 0.05, ***P < 0.0005, n=16.

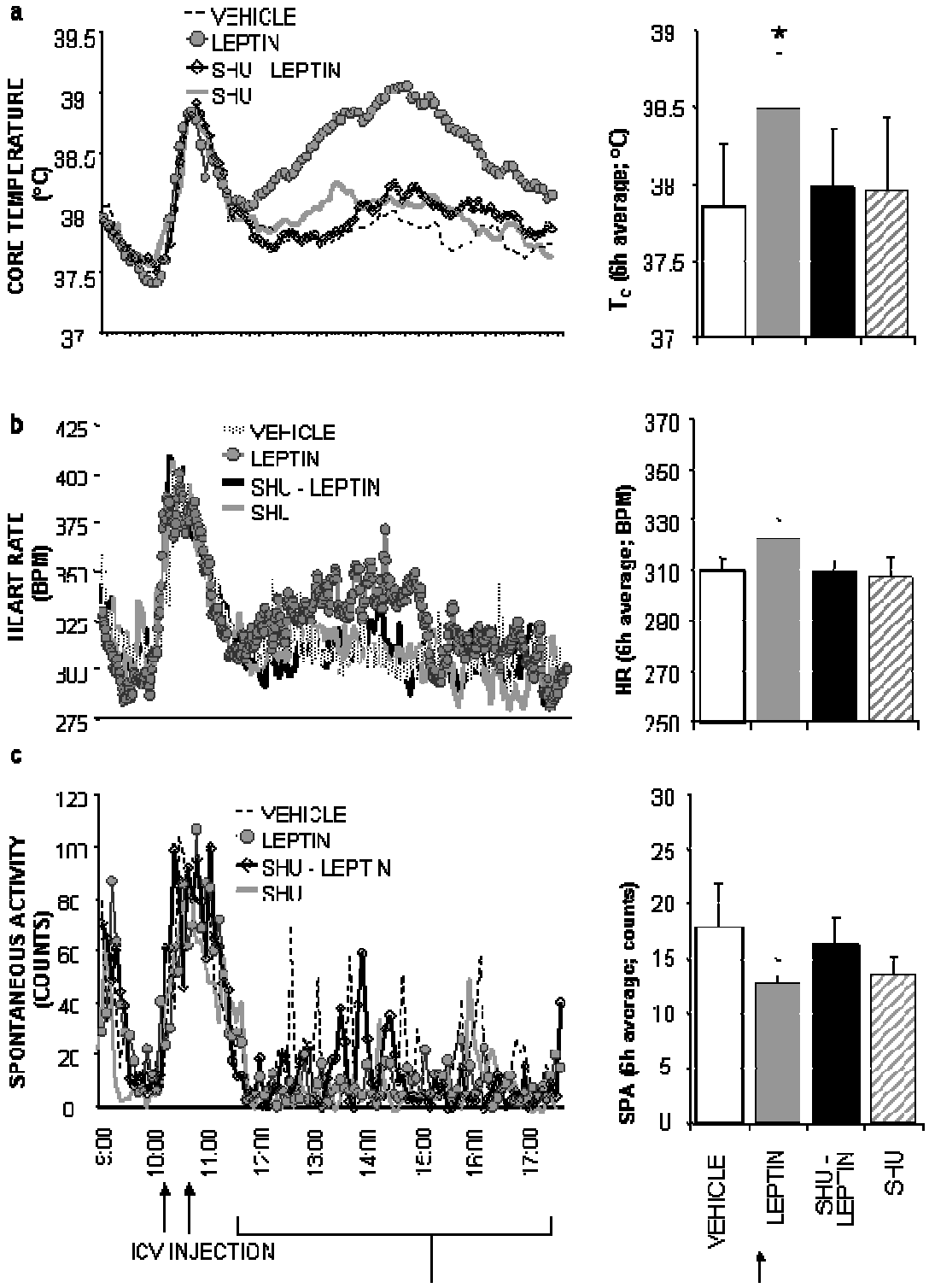

Figure 4.5: Effect of caudal brainstem melanocortin receptor blockade (1 µl, SHU 9119, 4th v. delivery) **on caudal brainstem leptin receptor stimulation** (1 µl, 4th v.

delivery) on (a) core temperature, (b) heart rate and (c) spontaneous activity in chow fed rats. Line graphs represent across-rat average parameter measurements through the 8 h recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. The histograms provide 6 h post-injection averages + SEM for each parameter at each dose. *P < 0.05, **P < 0.005, n=9.

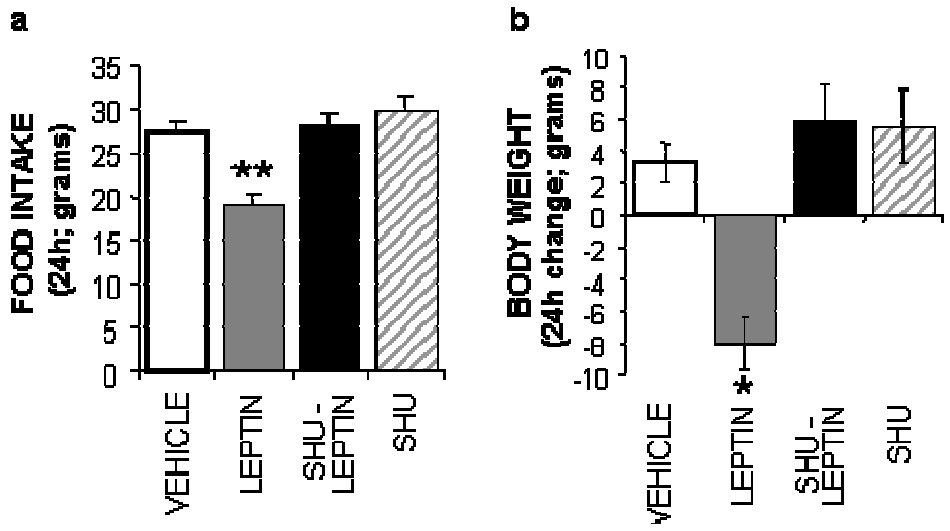

Figure 4.6: Effect of caudal brainstem melanocortin receptor blockade (1 μl, SHU 9119, 4th v. delivery) **on caudal brainstem leptin receptor stimulation** (1 μl, 4th v. delivery) on (a) food intake and (b) body in chow fed rats. The histograms provide average values ±SEM. *P < 0.05, **P < 0.005, n=9.

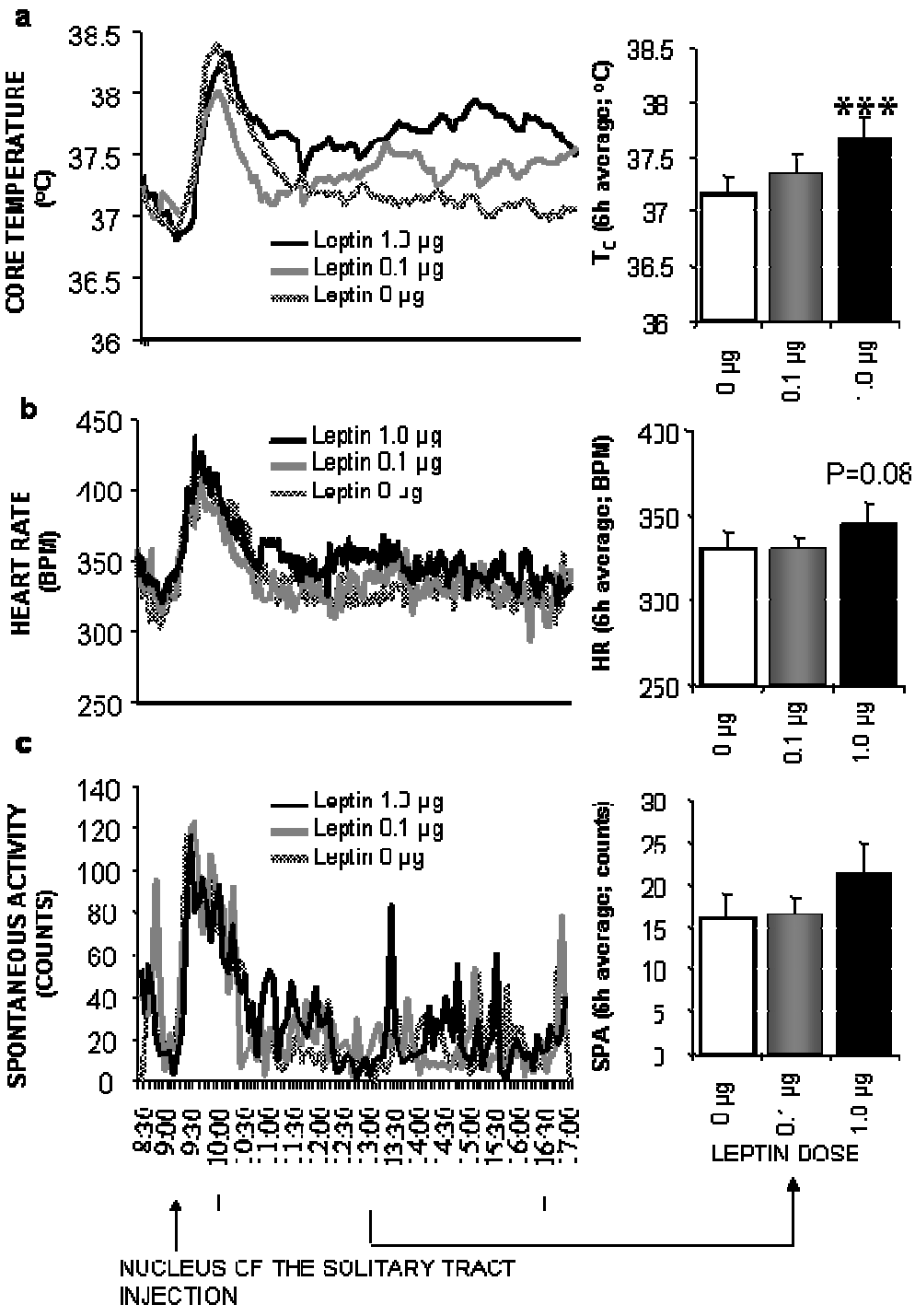

Figure 4.7: Effect of nucleus of the solitary tract (NTS) leptin receptor stimulation (0.1 µl, unilateral NTS parenchyma delivery) on (a) core temperature, (b) heart rate and (c) spontaneous activity **in chow fed rats**. For details on surgery, coordinates, placement

confirmation, injection strategy and statistical analysis see Methods in Chapter 3. Line graphs represent across-rat average parameter measurements through the 8 h-recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. The histograms provide 6 h post-injection averages + SEM for each parameter at each dose. ***P < 0.0005, n=9.

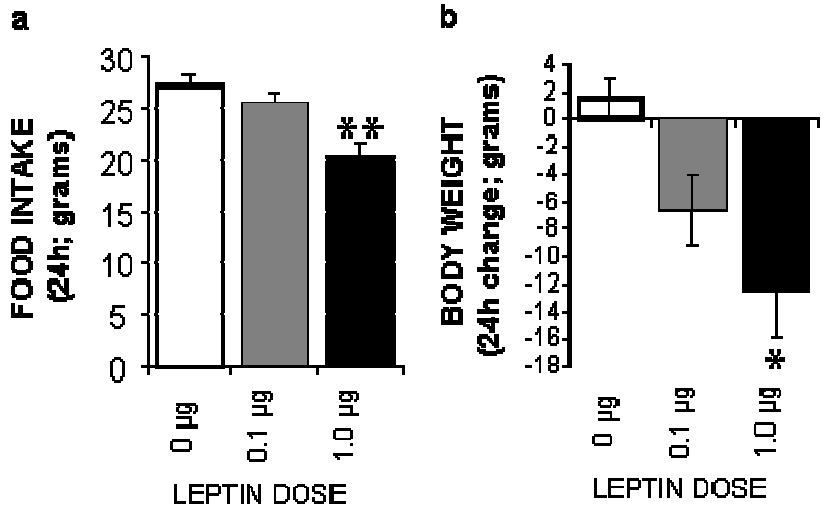

Figure 4.8: Effect of nucleus of the solitary tract (NTS) leptin receptor stimulation (0.1 µl, unilateral NTS parenchyma delivery) on (a) food intake and (b) body weight **in chow fed rats**. The histograms provide average values ± SEM. *P < 0.05, **P < 0.005, n=9.

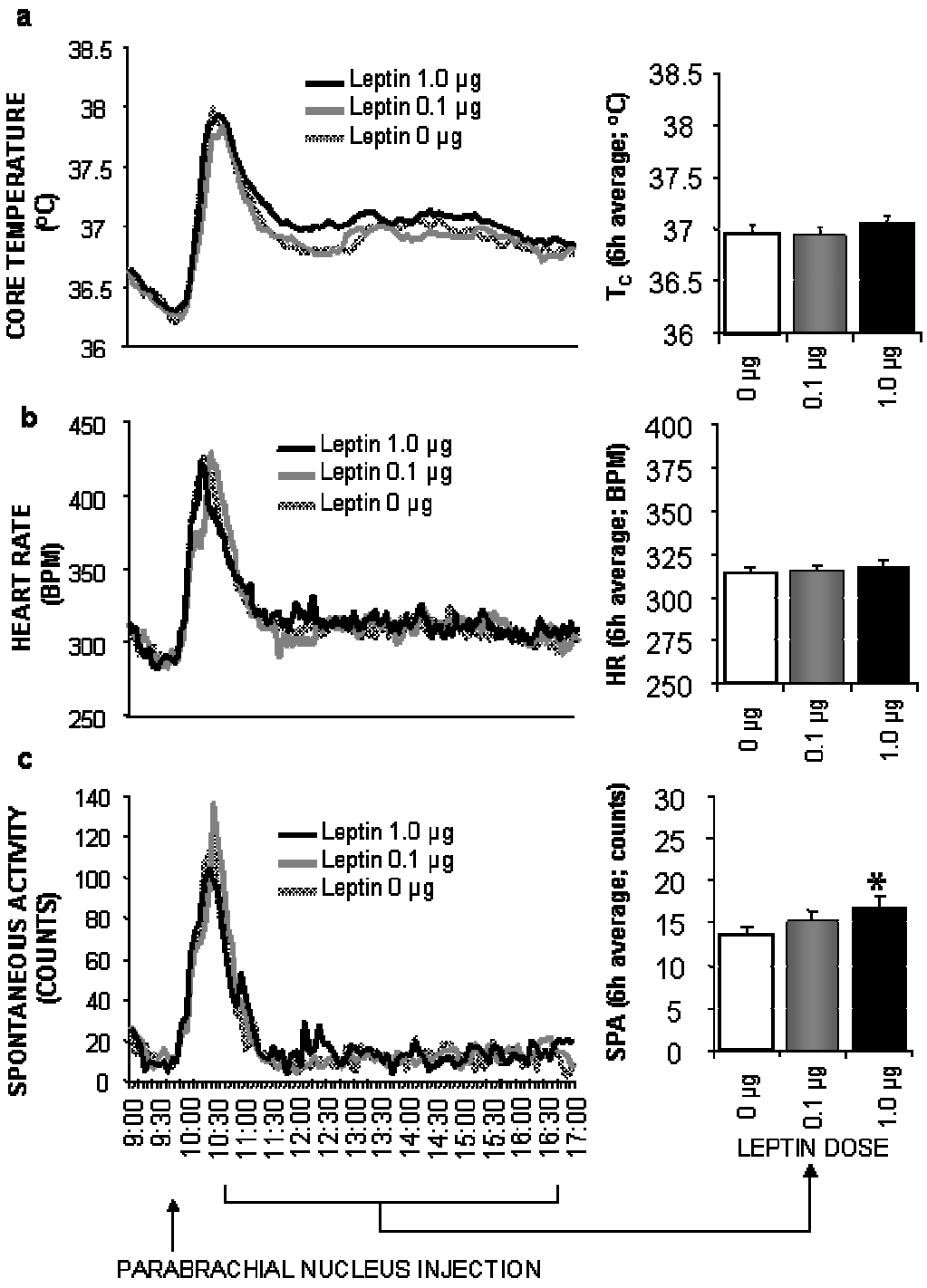

Figure 4.9: Effect of parabrachial nucleus (PBN) leptin receptor stimulation (0.1 µl, unilateral PBN parenchyma delivery) on (a) core temperature, (b) heart rate and (c) spontaneous activity **in chow fed rats**. For details on surgery, coordinates, placement confirmation,

injection strategy and statistical analysis see Methods in Chapter 3. Line graphs represent across-rat average parameter measurements through the 8 h-recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. The histograms provide 6 h post-injection averages + SEM for each parameter at each dose. *P < 0.05, n=29.

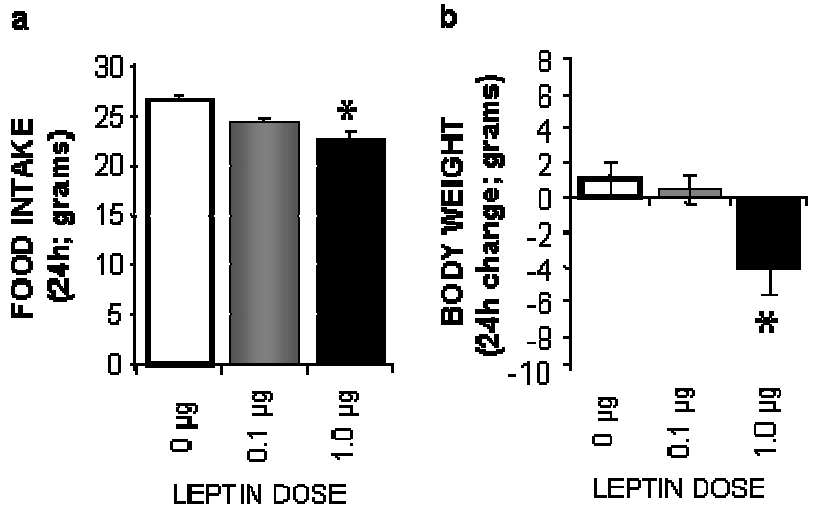

Figure 4.10: Effect of parabrachial nucleus (PBN) leptin receptor stimulation (0.1 µl, unilateral PBN parenchyma delivery) on (a) food intake and (b) body weight **in chow fed rats**. The histograms provide average values ± SEM. *P < 0.05, n=29.

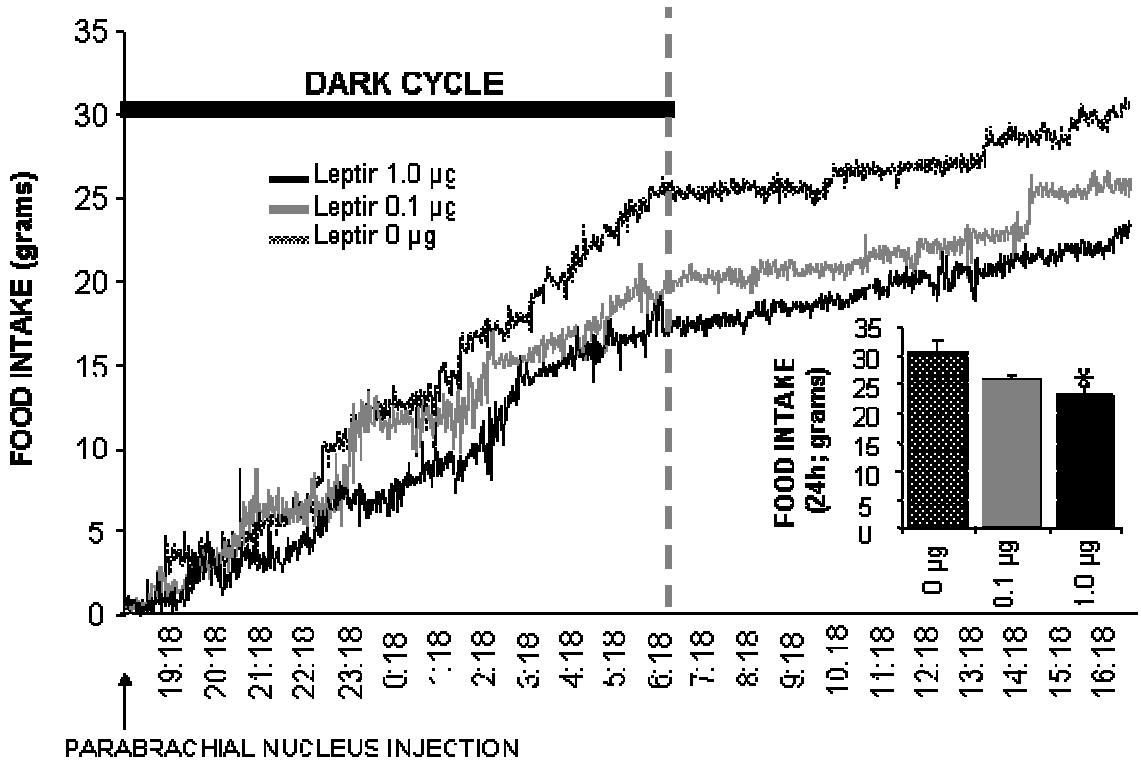

Figure 4.11. Effect of parabrachial nucleus (PBN) leptin receptor stimulation (0.1 µl, unilateral PBN parenchyma delivery) on the minute by minute food intake **in chow fed rats**. This experiment was performed in order to evaluate the novel anorexic effect of leptin in PBN that was initially discovered in the previous study (Figure 4.10). To focus on the intake effects, energy expenditure was not recorded here and unlike in the previous study standard chow (maintenance diet) was available to the rats immediately after injection. Injections took place 20 minutes before lights-out, as indicated by the arrow. X-axis indicates time of day. Intake was recorder every minute for a period of 24 hours. The histogram provides average 24h intake values ± SEM from the graph above. *P < 0.05, n=8.

CHAPTER 5

HINDBRAIN MELANOCORTIN RECEPTOR BLOCKADE EFFECTS ON HIGH-FAT DIET INTAKE AND BASELINE ENERGY EXPENDITURE

ABSTRACT

Palatable, high-fat diets induce hyperphagia that promotes positive energy balance.. The central melanocortin system is implicated in the compensatory adjustments of food intake and energy expenditure that are necessary to restore energy balance. Melanocortinergic contributions to energy balance regulation involve hypothalamic as well as hindbrain nuclei. Hypothalamic elements of this system are implicated in the compensatory response to high-fat feeding, but also in the maintenance of energy expenditure under baseline chow maintenance conditions. The contribution of hindbrain melanocortin receptors (MCRs) to both of those energy balance control responses is unexplored. Both questions are addressed here. To assess the role of endogenous hindbrain MCR activity to the control of energy balance under normal chow maintenance and under the energetic challenge posed by high energy diet maintenance feeding, cardiovascular, thermogenic and activity responses were examined in rats injected 4th ventricular (v) with the MC3/4 antagonist SHU 9119 or its vehicle. Hindbrain MCR blockade produced hypothermia and bradycardia without any changes in activity in chow-fed rats at baseline. The same effects on energetic-cardiovascular responses were observed in rats maintained on medium-fat diet. In contrast, diet maintenance differentially affected the feeding response to hindbrain MCR antagonism. 4th v SHU application increased high or medium-fat food intake and body weight but was without effect in rats maintained on chow. The response size observed in high-fat fed rats with 4th v delivery did not differ from that obtained with 3rd v MCR antagonist

injections. These data indicate that endogenous hindbrain MCR activity contributes to baseline temperature and heart rate maintenance and is required for limiting the hyperphagia and weight gain induced by high-fat feeding, as rats fed a high-fat diet are more sensitive to hyperphagic effects of hindbrain MCR blockade than chow fed controls.

INTRODUCTION

Palatable, energy-dense diets are over-consumed by humans and rodent models and the resulting hyperphagia is a primary determinant of the current obesity epidemic (Rothwell and Stock, 1979). The hyperphagia triggered by exposure to these can be viewed as a challenge on the energy balance. Restoring energy balance is an active process mediated by CNS circuits that involves curbing the hyperphagia and increasing energy expenditure (Rothwell and Stock, 1979). The central melanocortin system is an essential contributor to energy homeostasis under baseline, chow maintenance, conditions as indicated by severe hyperphagia, decrease in energy expenditure, and weight gain observed in mice or rats with dysfunctional melanocortin signaling (Huszar et al., 1997; Adage et al., 2001). Melanocortin signaling appears to also contribute to the compensatory adjustments in energy balance that are triggered by high-energy diet maintenance. MCRs are expressed in several CNS areas critical to energy balance control (Kishi et al., 2003). Despite accumulating evidence that both hypothalamic and extra-hypothalamic MCRs contribute to energy balance control (Grill et al., 1998; Skibicka and Grill, 2008a, 2009a), a role for extra-hypothalamic MCRs in the maintenance of baseline energy expenditure and in the energetic challenge posed by energy-dense diet maintenance has not been explored.

The coordinated control of energy intake and expenditure exerted by the POMC product α-melanocyte stimulating hormone (αMSH) and the responsiveness of POMC neurons to the metabolic signals, e.g., circulating fuels and metabolic hormones that are altered by high energy diet overconsumption (Schwartz et al., 1997; Benoit et al., 2002; Ibrahim et al., 2003; Jo et al., 2009) suggest that the central melanocortin system is well positioned to make energy balance adjustments in response to alterations in energy intake. A role for the melanocortin system in coordinating the energetic responses to high energy diet maintenance is suggested by results from MC4R KO mouse studies (Butler et al., 2001). These mice, hyperphagic on chow maintenance, significantly increase (by 50%) their food intake when exposed to a moderate fat-diet (Butler et al., 2001). Body weight, already greater than wild type controls on chow, increases further when MC4R-deficient mice are placed on moderate-fat diet. These data suggest that functional central MCR signaling is a critical element in curbing intake when rodents are maintained on high-energy diets. What is unaddressed by these results is a determination of which of the various melanocortin expressing nuclei contribute to these energy balance adjustments. The anorexic effects of central melanocortin signaling are often ascribed to the MCR-bearing hypothalamic nuclei, especially the paraventricular nucleus (PVN) (Balthasar et al., 2005; Taylor et al., 2007; Garza et al., 2008). A role for PVN MC4R signaling in the response to the high-energy diet maintenance challenge was highlighted recently in rats with targeted MC4R knock down in the PVN. These rats, unlike their wild-type controls showed differential response to diet. They gained more weight when maintained on high-fat diet but not when fed standard, low-fat chow (Garza et al., 2008).

MC4Rs are widely expressed anatomically and accumulating evidence suggests that the energy balance function exerted by CNS MCR signaling is anatomically distributed with contributions from both hypothalamic and extrahypothalamic MCRs (Grill et al., 1998; Skibicka and Grill, 2008a, 2009a). Recent data support a role for multiple hindbrain nuclei in the feeding and energetic-cardiovascular effect of MCR agonist stimulation (Grill et al., 1998; Skibicka and Grill, 2008a, 2009a). Reduced feeding, thermogenic and cardiovascular responses were triggered by discrete melanocortinergic stimulation of several hindbrain nuclei including nucleus of the solitary tract (NTS), parabrachial nucleus (PBN) and caudal raphe (Skibicka and Grill, 2009a). Endogenous contribution of hindbrain MCRs in chow fed rats was indicated by a hyperphagic effect of hindbrain MCR blockade with an MC3/4R antagonist (SHU 9119) (Grill et al., 1998; Williams et al., 2000a). In the current study we investigate whether pharmacological blockade of endogenous activity of hindbrain MCR disrupts (a) the intake response to the high-energy diet challenge and (b) to energy expenditure control under chow fed baseline conditions. These results are also compared to responses obtained by forebrain (3rd v) MCR blockade.

Diet-induced thermogenesis (DIT) describes the increase in sympathetic nervous system (SNS) activity to several organs including heart and brown adipose tissue (Dulloo, 2002) and the resulting increase in expenditure of energy driven by the sequential overconsumption of palatable, high-energy diets (Rothwell and Stock, 1979). POMC neurons are implicated in the energy expenditure adjustment to caloric over-consumption and a variety of data show that hypothalamic and hindbrain sympathetic premotor neurons co-express MCRs (Voss-Andreae et al., 2007; Song et al., 2008). MC4R KO mice, in contrast to their wild type controls, fail to increase oxygen

consumption in response to high-energy diet consumption (Butler et al., 2001). While experiments needed to resolve which of the anatomically distributed MC4R expressing nuclei contribute to this effect have not been undertaken, the field focuses on contributions from MCR-bearing hypothalamic nuclei. The current experiments characterize the contribution of endogenous hindbrain MCR signalling to energy balance control (measured parameters include: feeding, thermogenesis, cardiovascular changes and activity) under baseline, chow intake conditions and under the energetic challenge posed by high-energy diet maintenance. This is the first study to explore the endogenous contribution of hindbrain MCRs to energy expenditure in both the chow and high-energy diet feeding environment.

METHODS:

Subjects

Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 300–400g (10-12 weeks old) at surgery and housed individually in plastic bins under a 12-h light, 12-h dark cycle (0800 h lights on), participated in the five experiments described below. Pelleted food (Purina 5001; St. Louis, MO) and water were available *ad libitum* unless otherwise noted. All procedures conformed to the institutional standards of animal care and use committee (University of Pennsylvania).

Surgery

Rats were anesthetized with ketamine (90 mg/kg), xylazine (2.7 mg/kg), and acepromazine (0.64 mg/kg) delivered im.

Fourth and third intracerebroventricular (v.) cannula implantation.

Rats in experiments 1 and 2 received a fourth v. guide cannula (22 gauge; Plastics One, Inc., Roanoke, VA) with its tip stereotaxically positioned 2.0 mm above the fourth ventricle (coordinates: on the midline, 2.5-mm anterior to the occipital suture, and 4.5-mm ventral to the dura, with injector aimed 6.5-mm ventral from dura). Drug injections to the 4th v. at volumes used in our experiments have been shown to be confined to the hindbrain regions without stimulation of regions rostral to the 4th v. (Flynn and Grill, 1985; Blevins et al., 2004; Fan et al., 2004; Hayes et al., 2008b). A second group of rats in experiment 2 received a third v. guide cannula (coordinates: on the midline, 2-mm posterior to bregma, and 5.5-mm ventral to dura mater, with injector aimed 7.5-mm ventral to dura). Cannulas were attached to the skull with dental acrylic and jeweler's screws, and closed with an obturator as previously described (Skibicka and Grill, 2008a, 2009b).

Telemetric transponder surgery.

Telemetric transponders (HRC 4000 VitalView; Mini Mitter/Respironics, Bend, OR) for recording core temperature (T_C), heart rate (HR), and spontaneous physical activity (SPA) were inserted into the abdominal cavity, with the leads positioned sc and secured to the chest muscles on either side of the heart with sutures.

Experimental procedures

Cannula position verification.

At least 7 d after surgery, all brain cannula placements were assessed by measurement of the sympathoadrenal-mediated glycemic response to central injection of 5-thio-D-glucose [210 μg in 2 μl artificial cerebral spinal fluid (aCSF; Harvard Apparatus) for both

3rd and 4th v.] (Ritter et al., 1981). A post-injection elevation of at least 100% of baseline plasma glucose level was required for subject inclusion.

Habituation training.

Before the start of experimental testing, rats were acclimated to the handling and ventricular injection procedures used in a given experiment.

Experiment 1: We compared two groups of rats. One was maintained on the moderate-fat (MF) diet (Research Diets, 31% fat content); the other on regular chow (Figure 5.1A). Rats were weighed daily starting 2 days before MF diet exposure for 12 days. Both groups were tested with SHU 9119 (0.2nmol; Sigma) and vehicle in a two condition counterbalanced design at week 1 (days 4 and 7) on the diet (Figure 5.1B). MCR antagonist testing was planned for the first week of fat diet exposure as all prior published data established the strongest connection between feeding and energetic responses of MCR blockade in acute responses to fat (Butler et al., 2001; Voss-Andreae et al., 2007). All rats received injections 2 hours into light cycle. Food was removed at the time of injections (early in the light cycle) and returned 8 h later, late in the light phase. Thus, in this design food was not available during the period of energetic response measurement. Food intake and body weight measurements were made 24 h after the injection of drug. Given this design, all noted differences in food intake reflect longer latency effects of SHU (i.e. intake from 8–24 h after injection). For *ad libitum*-feeding rats, food was always available during the dark cycle.

Experiment 2: All rats (n=12 4th v, n=12 3rd v) were tested with 0.1nmol of SHU counterbalanced with vehicle while still maintained on chow. At least 72h were allowed to pass between injection days. The dose of SHU was chosen to not produce energetic

and feeding effects when applied alone on chow. At least 7 days were allowed to pass from the initial SHU test before the high-fat (HF) diet exposure. They were then switched to HF diet for 72h (Research Diets, 64% fat content); during that time all rats received daily injections (at 0, 24, 48h of diet exposure) of either SHU 0.1nmol (n=6 4th v, n=6 3rd v) or vehicle (n=6 4th v, n=6 3rd v). This paradigm was altered to capture possible immediate contribution of melancortin system to HF challenge based on results of a recent publication (Voss-Andreae et al., 2007) in which MC-R blockade attenuated brown adipose tissue activity induced by 48h HF diet exposure in mice. Food was available *ad libitum* at all times during this paradigm, with the exception of day 2 (where animals were deprived only in the light cycle in order to obtain energetic recordings without the possible confounding effects of food intake). Energy expenditure parameters were measured from 0h-72h during the high fat diet exposure. Food intake and body weight were measured at 24, 48 and 72h as illustrated in Figure 5.1C.

Statistical analysis:

Core temperature, HR and SPA were analyzed by ANOVA on post-injection 8 h average values of each parameter and followed by student's t-test or Tukey test as appropriate. Twenty four hour food intake and body weight were analyzed by ANOVA and followed by t-test or Tukey test as appropriate. Two way ANOVA was applied in experiment 1 (diet, drug) and three way ANOVA in experiment 2 (ventricle, drug, time), those were followed up by 1-way ANOVAS or post-hoc tests as appropriate. All statistical analysis was conducted using STATISTICA software (StatSoft, Inc., Tulsa, OK). Differences were considered significant at $P<0.05$.

RESULTS:

Experiment 1: Rats consumed 50% more kcal on the first 24h of MF exposure, during the next 8 days MF consumption was increased by 20-30% on average as compared to the chow control group (Figure 5.2A). There were trends to increase body weight staring day 5 of MF exposure, however not significant (Figure 5.2B). Two-way ANOVA revealed a significant diet-drug interaction for food intake ($P<0.05$) after 4th v. MC-R blockade. Fourth v. MC-R blockade with 0.2nmol SHU did not significantly alter chow intake, however intake in MF-fed rats was significantly increased ($P< 0.005$). All rats (MF-fed rats and chow-fed controls) exhibited mild but significant hypothermia ($P< 0.005$) and tachycardia ($P< 0.0005$) under SHU condition with no significant changes in activity in response to SHU delivery to 4th v. There were no significant diet-drug interactions on any of the energy expenditure parameters measured.

Experiment 2: A dose of MC-R antagonist without effect on any of the measured parameters was desired for a elucidating a specific effect of MC-R blockade on rats exposed to HF diet. SHU pretest on chow revealed that the 0.1nmol dose chosen for this study had no significant effect on any of the parameters measured while the rats were maintained on chow. After the rats were switched to HF diet however SHU application significantly increased HF food intake. Three-way ANOVA (ventricle, drug, time) revealed a significant effect of drug on food intake ($P< 0.005$) and no interaction of drug with ventricle (Figures 5.4A&C). Three-way ANOVA also indicated a significant effect of drug on body weight ($P< 0.05$) and no interaction of drug with ventricle (Figures 5.4B&D). There was no significant effect of drug and no significant drug-ventricle interaction on any of the energy expenditure parameters in the dark cycle. (Figures 5.5&6). In the light cycle there was no significant effect of drug and no drug-ventricle

interaction on heart rate or activity. High-fat fed rats that received SHU however had significantly increased core temperature ($p<0.05$). There was no drug-ventricle interaction.

DISCUSSION

Hindbrain MCR antagonism significantly decreased core temperature and heart rate, suggesting that endogenous hindbrain MCR activity and "melanocortinergic tone" contributes to the maintenance of body temperature and cardiovascular tone under chow fed, baseline conditions. The bradycardia and reduced core temperature were also induced by hindbrain MCR blockade in high-energy diet maintained rats, indicating that the contribution of hindbrain MCRs to energy expenditure is not altered by high-fat challenge. Diet maintenance was a factor in the feeding response to MCR antagonism for hindbrain as well as forebrain ventricular delivery of SHU-9119. Rats maintained on high-energy diets (either moderate or high-fat) were hyperphagic and gained weight in response to MCR antagonism while chow-fed rats were unaffected. These results suggest that increased endogenous melanocortinergic tone contributes to the compensatory reduction of high-energy diet consumption in response to chronic diet exposure.

Forebrain ventricular MCR antagonist treatment or MC4R knockout profoundly disrupts endogenous melanocortin signaling under baseline, chow diet maintenance and results in pronounced hyperphagia, hypothermia and decreased activity (Adage et al., 2001; Butler et al., 2001). The global nature of these procedures makes it impossible to distinguish between the contributions of anatomically distinct MCR populations to the

measured responses. The current study is the first to selectively implicate hindbrain MCRs in baseline energy expenditure maintenance by showing that hindbrain MCR antagonism triggers a long-lasting hypothermia and bradycardia. These data complement our experiments showing that agonist stimulation of hindbrain MCRs triggers responses of the opposite valence - hyperthermia and tachycardia (Skibicka and Grill, 2008a, 2009a).

As noted earlier MCR-bearing sympathetic pre-motor neurons are found in several hindbrain nuclei whose activity contributes to thermogenic and cardiovascular function (Skibicka and Grill, 2008a, 2009a). Additional experiments will be required to define which of these hindbrain MCR bearing neurons contributed to the energetic and cardiovascular effects observed here in response to 4^{th} v antagonist delivery. It is likely that differences between the inputs and outputs of a given structure might make its contributions more relevant to the responses observed under chow maintenance than for those observed in response to high-energy diet maintenance.

There are data that suggest that endogenous melanocortin signaling is affected by diet macronutrient content but the direction of these effects varies across studies. Stimulation and blockade of MCRs appears to selectively affect intake of diets high in fat, rather then those with increased carbohydrate or protein content (Hagan et al., 2001; Samama et al., 2003). Other experiments examine whether the anorexic or thermogenic responses to MCR stimulation (via forebrain application of MC3/4 agonist – MTII or αMSH) is affected by high-energy diet exposure. Results of those studies are highly variable ranging from indications that feeding responses to MCR agonist are enhanced (Hansen et al., 2001), unaltered (Li et al., 2004) or decreased (Clegg et al., 2003) in

high-fat fed rats. In other studies it was observed that the decrease in food intake induced by involuntary overfeeding of high-fat diet for 16 days was associated with an increased expression of hypothalamic POMC mRNA (Hagan et al., 1999). This high-fat induced decrease in intake is abolished by low dose 3rd v SHU application (Hagan et al., 1999). The role of PVN MCR signaling on palatable liquid diet intake was highlighted by results showing that PVN antagonist injection (agouti related protein; AgRP) potently increased intake (Taylor et al., 2007). More recently PVN MCR adenoviral vector knockdown increased high-fat intake without altering chow intake. Similar findings are reported with chronic interference with MCR signaling in the lateral hypothalamus (Kas et al., 2004). The current results with 3rd v SHU-9119 application are consistent with a diet selective hyperphagic effect (present in high-fat and absent in chow fed rats).

These studies conclude that hypothalamic MCRs are the principal site of action in mediating the observed effects. Not considered in those studies but the focus of the current experiments is a determination of whether MCR expressing neurons in the hindbrain contributes to the observed effects (Grill et al., 1998; Williams et al., 2000a; Zheng et al., 2005; Skibicka and Grill, 2009a). Hindbrain SHU 9119 application (4th v and direct NTS delivery) induced a pronounced chow hyperphagia similar in size and duration to that induced by forebrain drug application (Grill et al., 1998; Williams et al., 2000a). The failure to consider a role for hindbrain MCR expressing nuclei in studies of high-energy diet induced CNS changes may result from the prevailing view that hindbrain circuits are relevant only to short-term feeding control (Cone, 2005). Current data highlight the contribution of the hindbrain MCRs to the longer term consequences of high-energy diet maintenance. The dose of the drug chosen in the current study was not effective on baseline chow intake, but selectively affected moderate and high-fat

intake. This is the first set of data showing an increased sensitivity to MCR blockade in the hindbrain in rats fed a high-fat diet, as compared to those maintained on chow. Several populations of neurons in the hindbrain express MCRs and contribute to food intake. Two most prominent sites are the nucleus of the solitary tract and the parabrachial nucleus (Williams et al., 2000a; Skibicka and Grill, 2009a). Both sites are implicated in taste and palatability processing, whether blockade of MCR activity alters the ability to sense the high caloric content of the diet, process the taste or perhaps increases the palatability of the diet remains to be resolved.

The MCR antagonist used here targets both MCRs expressed in the brain – MC3 and MC4R. It is possible that for the 3rd v study blockade of both receptors contributes to the hyperphagic response selective to high-energy diet fed rats. However the fact that only MC4R, not MC3R knockout mice show and exacerbated hyperphagia and weight gain on high-fat diets provides a counterpoint to a possible MC3R contribution (Butler et al., 2000; Butler and Cone, 2002). Previous reports indicate that only expression of MC4R and not MC3R is altered by exposure to energy-dense diets (Harrold et al., 1999). Even a stronger case for a primary MC4R effect in the hindbrain can be made as several reports indicate no MC3R expression in the hindbrain (Roselli-Rehfuss et al., 1993).

The current data indicate that moderate and high-fat diet exposure engages the hypothalamic and hindbrain endogenous melanocortin system, what remains unknown is what signals, induced by fat feeding, directly engage POMC neurons. Leptin and insulin are peripheral circulating factors that increase with high-energy diet maintenance, and activate POMC neurons to potentially mediate fat diet activation of the central melanocortin system (Schwartz et al., 1997; Seeley et al., 1997; Satoh et al.,

1998; Benoit et al., 2002; Rahmouni et al., 2003; Skibicka and Grill, 2009b). Melanocortin receptor stimulation is the major downstream pathway within the CNS to propagate leptin mediated anorexia as well as energy expenditure (Schwartz et al., 1997; Seeley et al., 1997; Satoh et al., 1998; Skibicka and Grill, 2009b). Insulin-mediated anorexia as well as sympatho-excitation utilizes the melanocortin pathway (Benoit et al., 2002; Rahmouni et al., 2003). Since both leptin and insulin receptors are expressed on ARC POMC neurons and can stimulate common intracellular signals it is also possible that the integration of the two signals is required for POMC neuron activation. Relevant to the hindbrain MCR selective high-energy intake response is that many of the aforementioned signals also reach the hindbrain (NTS) POMC neurons. Hindbrain POMC neurons are responsive to leptin treatment and blockade of hindbrain MCRs abolishes the anorexic and energetic responses to hindbrain leptin stimulation. Insulin receptors are also expressed in the hindbrain (Folli et al., 1994). Studies are needed to resolve whether hindbrain leptin and/or insulin stimulation is upstream of the dietary increase in activation of hindbrain MCRs. Another interesting possibility is that leptinergic, insulinergic or other stimulation of hypothalamic POMC neurons and subsequent projections to hindbrain MCR expressing populations leads to curbing of the high-fat diet intake reported here.

No changes in thermogenesis and activity in rats challenged with moderate or high fat diet were observed. Previous reports showed for e.g. increased oxygen consumption after moderate-fat (25%) exposure already at the first 24h period in mice (Butler et al., 2001), however the same group reports that BAT SNS activity was not altered during that period, which would be consistent with lack of body temperature changes in our study. It is therefore possible that other tissues then BAT are involved in

the SNS DIT response (like muscle) as oxygen consumption is a more global measure of energy usage and reflects cumulative metabolic activity of all tissues. On the other hand another report shows an increase in molecular activity of BAT (via UCP-1 mRNA expression) in mice fed high fat (60%) diet (Voss-Andreae et al., 2007), which is suggestive of BAT activation. However direct measurement of BAT temperature would be necessary to assert translation of increased UCP-1 mRNA levels to increased thermogenesis. In fact Li et al (Li et al., 2004) report increased UCP-1 levels in rats fed a high-fat diet without any simultaneous changes in oxygen consumption. This could reflect a potential disassociation of molecular and physiological parameters of energy expenditure. Core body temperature is under tight central regulation. Therefore another possibility might be that while BAT thermogenic activity might increase after high-fat exposure, a change in core temperature is not seen as other counter-hyperthermic measures are engaged by the organism in order to maintain the core temperature at a constant level.

Together the findings presented here bring attention to the endogenous contribution of the hindbrain MCRs to a variety of energetic parameters and feeding responses. Future studies should resolve how this newly outlined contribution is integrated into the current energy balance control model, to determine if it is integrated with the hypothalamic circuitry or does it function independently and parallel to the hypothalamus energy balance controlling circuits.

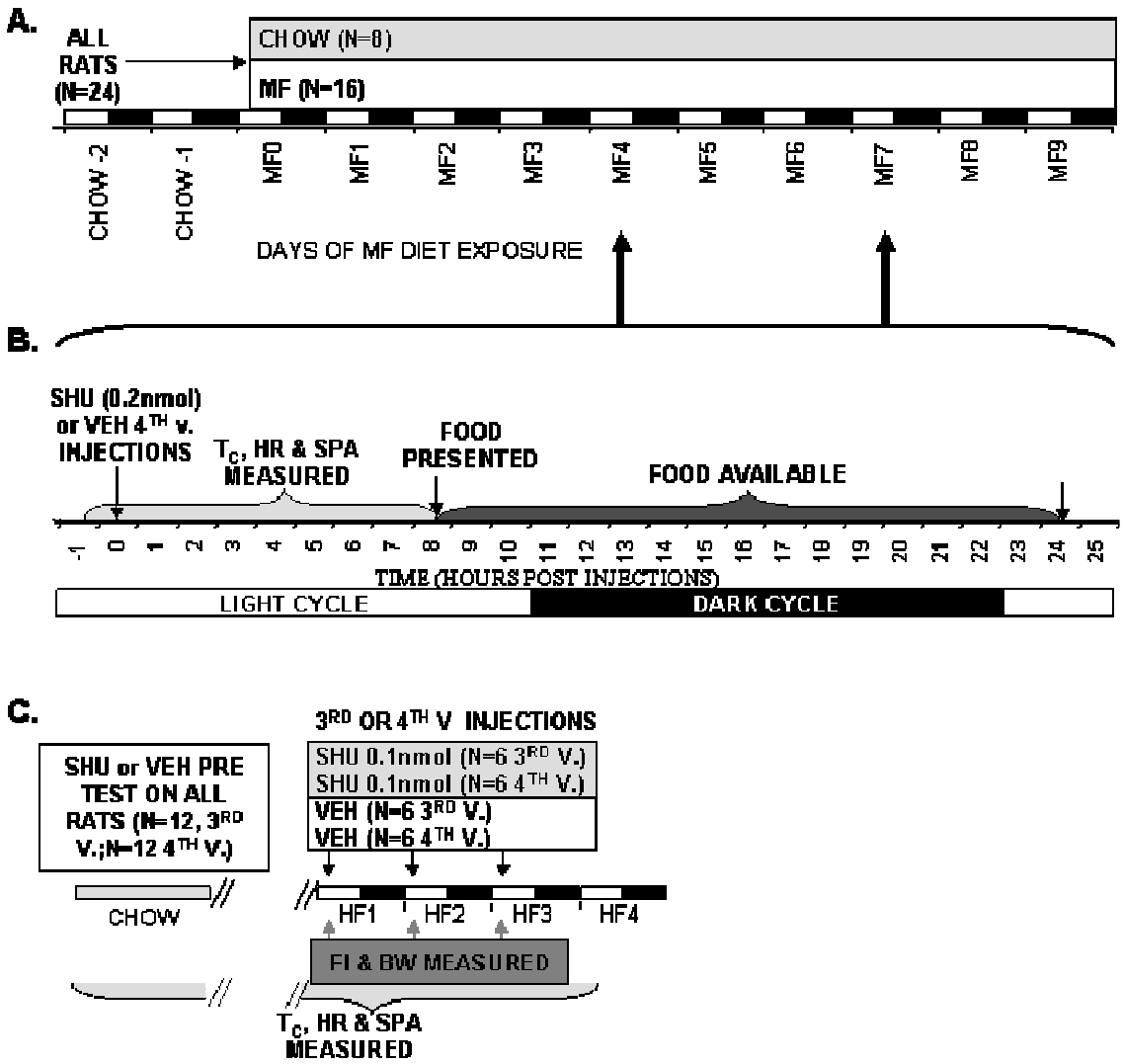

Figure 5.1: Experimental paradigm utilized for measurements of food intake (FI), body weight (BW), core temperature (T_C), heart rate (HR) and spontaneous activity (SPA) in experiment 1 (A); experimental days 4 and 7 of experiment 1 (B); in experiment 2 (C). Black bars represent periods of lights out, white bars indicate lights on. Moderate fat (MF), high fat (HF), ventricle (V).

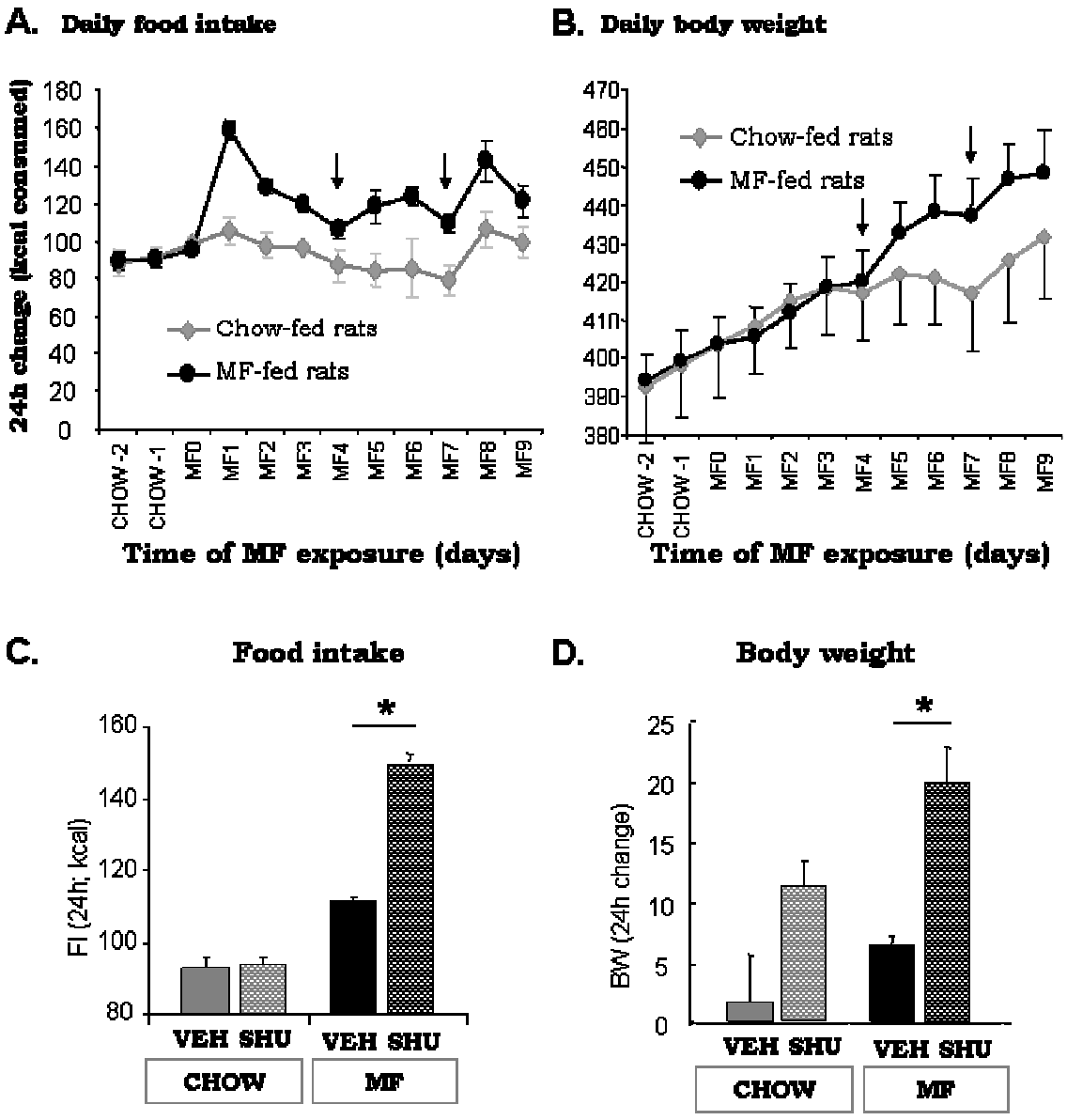

Figure 5.2: Enhanced hindbrain melanocortinergic tone after moderate-fat diet exposure (experiment 1). A. Daily average caloric intake of chow and moderate-fat fed rats B. Daily average body weight. Arrows represent 4th ventricle injection days. C. Hyperphagia and D. body weight gain after hindbrain MCR blockade with 0.2nmol SHU 9119 are selective for moderate-fat fed rats. Moderate fat (MF). * $P<0.05$

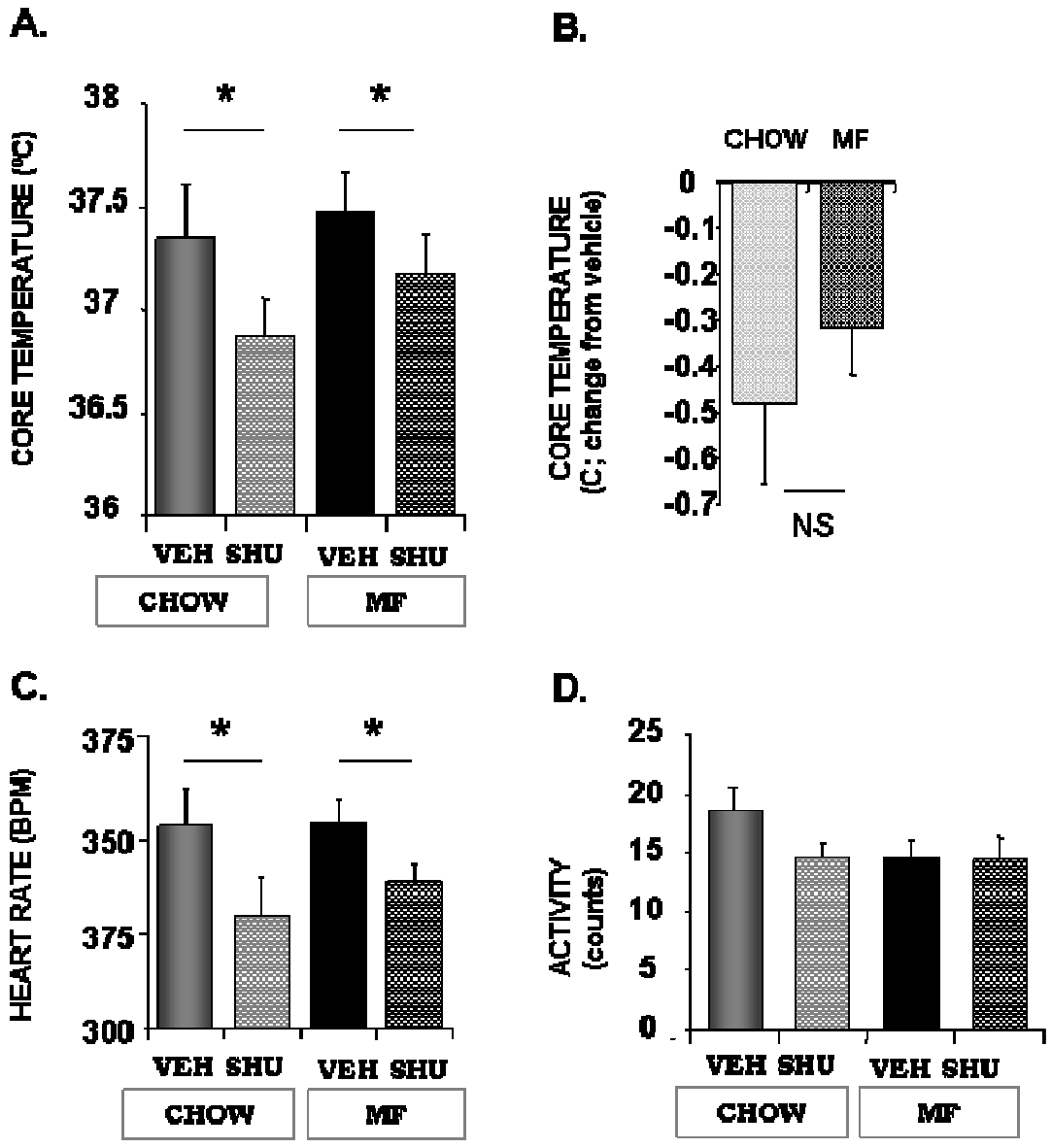

Figure 5.3: Energetic effects of blockade of hindbrain MCRs with SHU 9119 on A. core temperature B. Core temperature change relative to respective vehicle C. heart rate D. spontaneous activity in rats fed chow (baseline conditions) and moderate fat food. Histograms represent 8h average, with no food available during the period of energetic measurement. Moderate fat (MF). * P<0.05

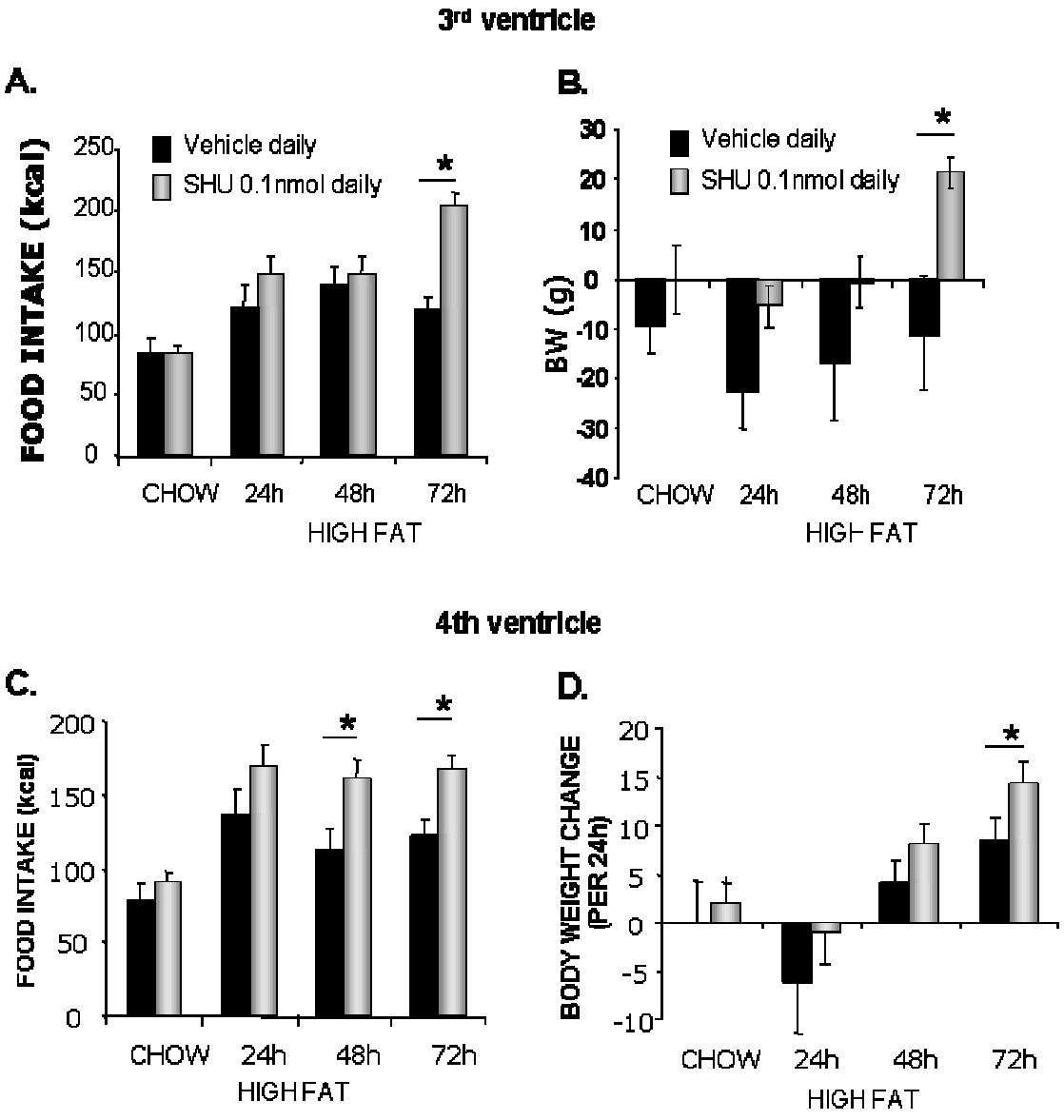

Figure 5.4: Enhanced hindbrain melanocortinergic tone after high-fat diet exposure – comparison of 3rd and 4th v studies. Hyperphagia and body weight gain selective for high-fat fed rats after MCR blockade with 0.1nmol SHU 9119 in the forebrain (3rd ventricle injection) A-B and hindbrain (4th ventricle injection) C-D. *P<0.05

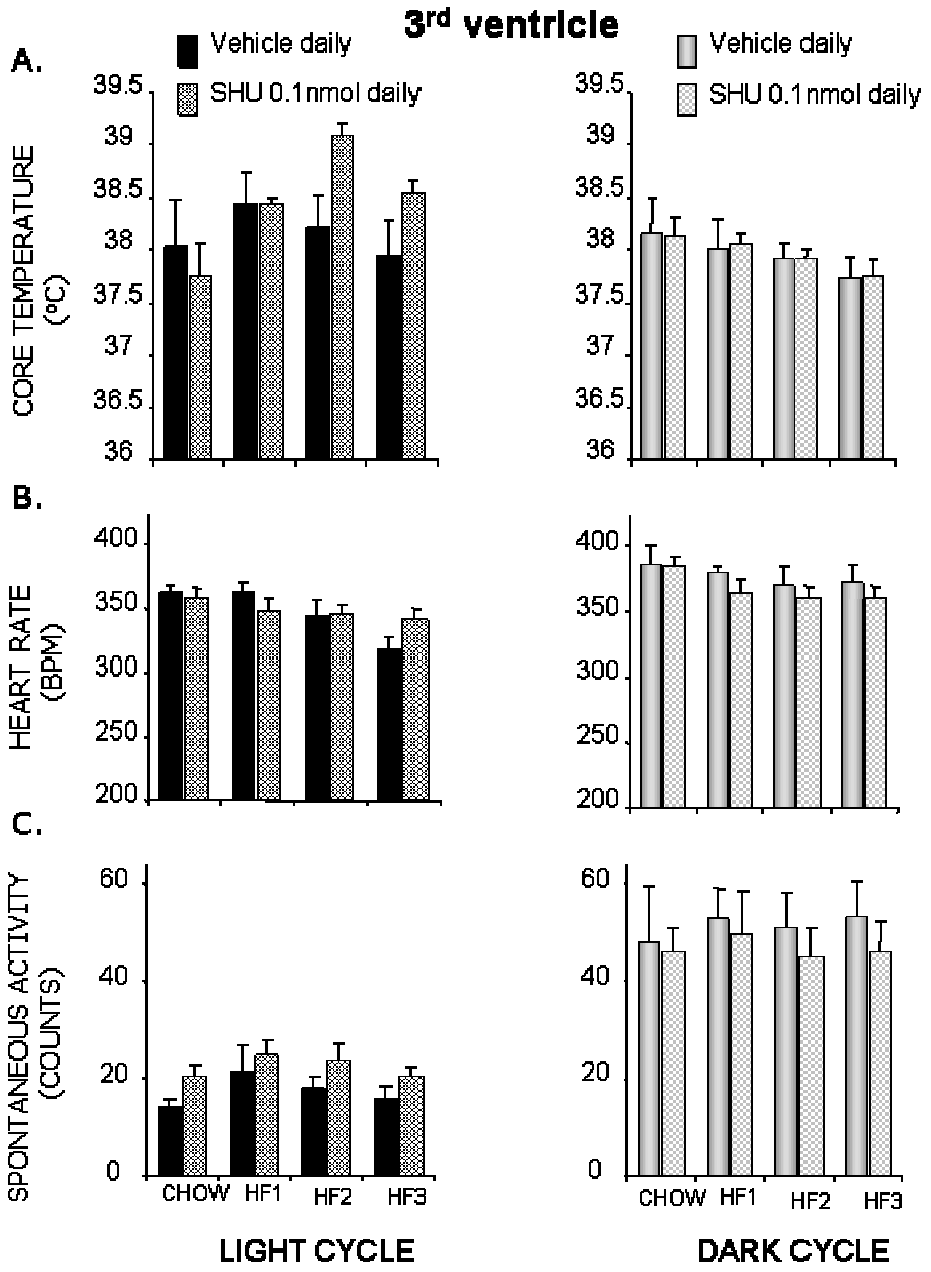

Figure 5.5: Energetic effects of blockade of hindbrain MCRs with daily 3rd ventricular application of a sub-threshold (in control chow fed condition) dose of SHU 9119 (0.1 nmol) in rats fed high-fat diet on A. core temperature B. heart rate C. spontaneous activity in the light (left) and dark (right) cycle. Histograms represent 8h average, with no food available during the period of energetic measurement.

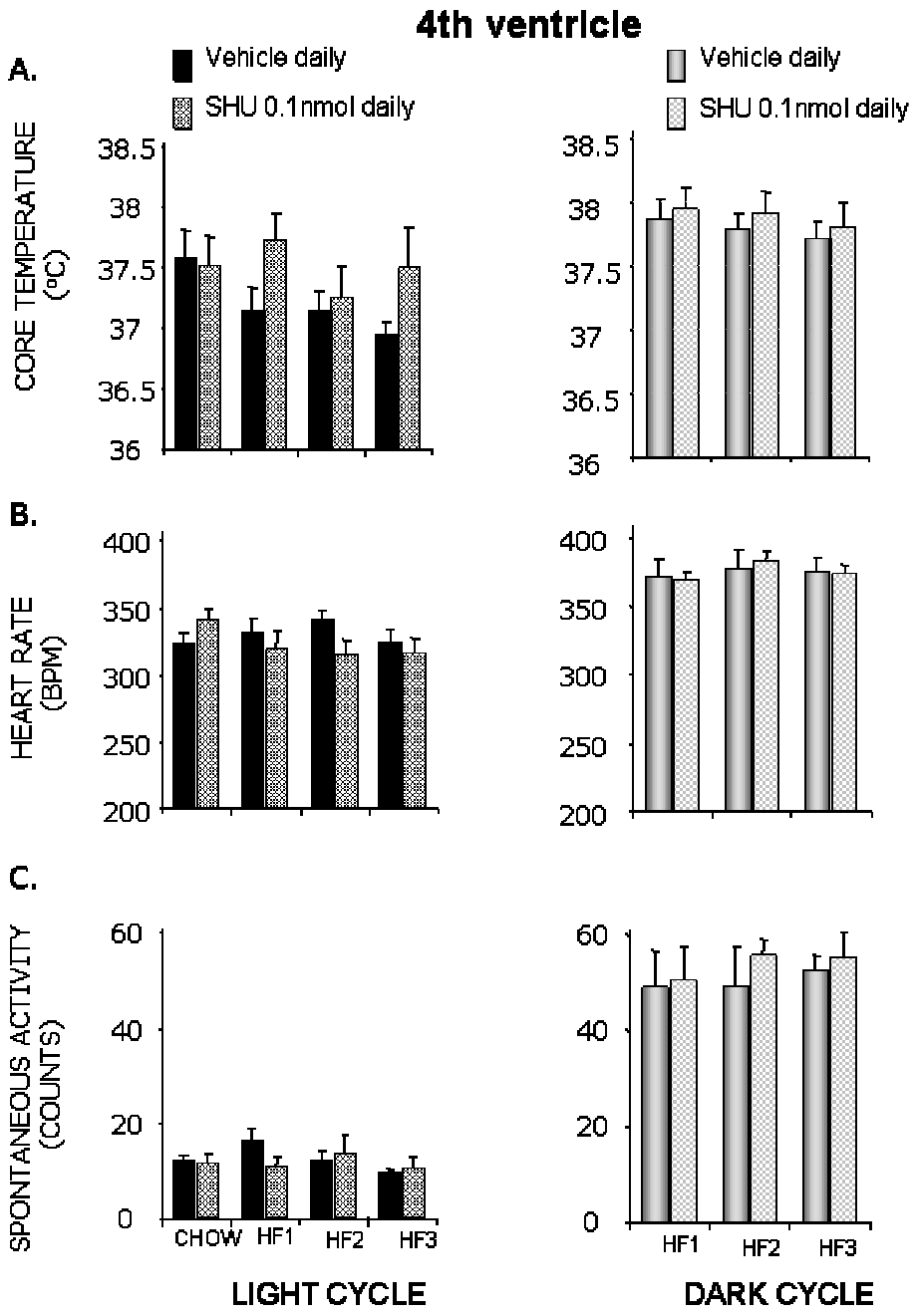

Figure 5.6: Energetic effects of blockade of hindbrain MCRs with daily 4th ventricular application of a sub-threshold (in control chow fed condition) dose of SHU 9119 (0.1 nmol) in rats fed high-fat diet on A. core temperature B. heart rate C. spontaneous activity in the light (left) and dark (right) cycle. Histograms represent 8h average, with no food available during the period of energetic measurement.

CHAPTER 6

HINDBRAIN CART INDUCES HYPOTHERMIA MEDIATED BY GLP-1 RECEPTORS[4]

ABSTRACT

Cocaine- and amphetamine-regulated transcript peptides (CART) are widely distributed throughout the neuraxis, including regions associated with energy balance. CART's classification as a catabolic neuropeptide is based on its inhibitory effects on feeding, co-expression with arcuate nucleus POMC neurons, and on limited analysis of its energy expenditure effects. Here we investigate whether: 1) caudal brainstem delivery of CART produces energetic, cardiovascular and glycemic effects, 2) forebrain – caudal brainstem neural communication is required for those effects and 3) GLP-1 receptors (GLP-1R) contribute to the mediation of CART-induced effects. Core temperature (Tc), heart rate (HR), activity and blood glucose were measured in rats injected 4th v. with CART (0.1, 1.0 and 2.0μg). Food was withheld during physiologic recording and returned for overnight measurement of intake and body weight. CART induced a long-lasting (> 6h); hypothermia; a 1.5°C and 1.6°C drop in T_C for the 1.0 and 2.0μg doses. Hindbrain CART application reduced food intake and body weight and increased blood glucose levels; no change in HR or activity was observed. Supracollicular decerebration eliminated the hypothermic response observed in intact rats to hindbrain ventricular CART, suggesting that forebrain processing is required for hypothermia. Pretreatment with the GLP-1R antagonist (exendin-9-39) in control rats attenuated CART hypothermia and

[4]These results were partially reported at the 2007 Obesity Society Meeting, in New Orleans, LA and appeared in Journal of Neuroscience, 2009. This work was supported by NIH grants DK-21397 and NRSA NS-059254

hypophagia, indicating that GLP-1R activation contributes to hypothermic and hypophagic effects of hindbrain CART while CART-induced hyperglycemia was not altered by GLP-1R blockade. Data reveal a novel function of CART in temperature regulation and open possibilities for future studies on the clinical potential of the hypothermic effect.

INTRODUCTION

Cocaine-amphetamine-regulated transcript (CART) neuropeptides were initially localized to the striatum and nucleus accumbens; regions associated with reward, motivation and addiction (Douglass and Daoud, 1996). Later CART synthesis was described in regions associated with energy balance control including the arcuate and paraventricular hypothalamus, nucleus tractus solitarius (NTS) and vagal afferent neurons (Koylu et al., 1997; Koylu et al., 1998; Broberger, 1999; Broberger et al., 1999; Vrang et al., 1999b; Vrang et al., 1999a). In arcuate hypothalamic neurons, CART is co-expressed with proopiomelanocortin (POMC) whose cleavage product, α-MSH, is a major catabolic contributor to energy balance (Butler and Cone, 2002; Cone, 2005). Leptin up-regulates and food deprivation down-regulates hypothalamic expression of both POMC and CART (Kristensen et al., 1998). Melanocortin receptor stimulation produces hypophagia, hyperglycemia, hyperthermia, tachycardia and increased activity (Fan et al., 2000; Gutierrez-Juarez et al., 2004; Skibicka and Grill, 2008a). Other studies reveal that forebrain ventricular CART delivery decreases food intake and in conjunction with the findings just described lead to the idea that CNS CART effects on energy balance are *catabolic* in nature (Kristensen et al., 1998; Kuhar et al., 2000; Rogge et al., 2008). The energy expenditure effects of CART are however, insufficiently

investigated. The experiments reported here contribute to a developing perspective that the functional effects of CNS CART signaling on energy balance are not consistent with a catabolic role of $CART_{55\text{-}102}$, as they revealed a hypothermic effect whose mediating neural pathway is described.

Central CART administration (forebrain ventricle) suppresses and blockade of CNS CART signaling with ventricular antiserum delivery increases food intake (Kristensen et al., 1998). Direct hypothalamic CART administration, however, elicits hyperphagia which contrasts with the hypophagia observed with ventricular delivery (Abbott et al., 2001; Smith et al., 2008). This disparity between the direction of effect for ventricular vs. direct hypothalamic CART administration suggests that the anorectic action of CART is mediated by extra-hypothalamic sites. In fact, several studies show that hindbrain ventricular (4^{th} ventricle) delivery of CART produces anorexia (Aja et al., 2001b; Zheng et al., 2001; Aja et al., 2002; Zheng et al., 2002).

While an indirect measure, the increases in brown adipose UCP-1 expression in response to arcuate and paraventricular nucleus CART delivery (Wang et al., 2000; Kong et al., 2003) indicates a role of hypothalamic CART in thermogenesis and catabolic action. However, the energetic and cardiovascular effects of hindbrain CART stimulation are largely unexplored (Hwang et al., 2004). CART is detected in several hindbrain regions associated with energy balance/thermoregulation including NTS, parabrachial nucleus (PBN), raphe pallidus, and rostral ventral lateral medulla (Dun et al., 2000; Dun et al., 2001). For this reason our experiments examined the energetic and cardiovascular effects produced by hindbrain (4^{th} v. and NTS) $CART_{55\text{-}102}$ delivery.

Hindbrain glucagon-like peptide-1 receptors (GLP-1R) mediate $CART_{55-102}$ -induced hypophagia and neuronal activation (Fos-Li) observed in multiple hindbrain sites (Aja et al., 2006). Some of these Fos-Li expressing nuclei are also relevant to thermoregulation. Therefore our experiments also assessed whether hindbrain GLP-1R contribute the energy expenditure effects of hindbrain $CART_{55-102}$ stimulation.

METHODS

Subjects

Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 300–400g (10-12 weeks old) at surgery and housed individually in plastic bins under a 12-h light, 12-h dark cycle (0800 h lights on), participated in the five experiments described below. Pelleted food (Purina 5001; St. Louis, MO) and water were available *ad libitum* unless otherwise noted. All procedures conformed to the institutional standards of animal care and use committee (University of Pennsylvania).

Surgery

Rats were anesthetized with ketamine (90 mg/kg), xylazine (2.7 mg/kg), and acepromazine (0.64 mg/kg) delivered im.

Fourth and third intracerebroventricular (v.) and NTS intraparenchymal cannula implantation.

Rats in experiments 1, 3 and 4 received a fourth v. guide cannula (22 gauge; Plastics One, Inc., Roanoke, VA) with its tip stereotaxically positioned 2.0 mm above the fourth ventricle (coordinates: on the midline, 2.5-mm anterior to the occipital suture, and 4.5-mm ventral to the dura, with injector aimed 6.5-mm ventral from dura). Rats in

experiment 3 also underwent a decerebration surgery. Drug injections to the 4th v. at volumes used in our experiments have been shown to be confined to the hindbrain regions without stimulation of regions rostral to the 4th v. (Flynn and Grill, 1985; Blevins et al., 2004; Fan et al., 2004; Hayes et al., 2008b). A second group of rats in experiment 4 received a third v. guide cannula (coordinates: on the midline, 2-mm posterior to bregma, and 5.5-mm ventral to dura mater, with injector aimed 7.5-mm ventral to dura). Rats in Experiment 5 received a cannula positioned 2.0-mm above the medial NTS (coordinates: 0.5-mm from the midline, on occipital suture, 5.9-mm ventral to skull; placement as we previously described in Hayes et al 2009). Cannulas were attached to the skull with dental acrylic and jeweler's screws, and closed with an obturator as previously described (Fan et al., 2000; Gutierrez-Juarez et al., 2004; Skibicka and Grill, 2008a).

Decerebration surgery.

Supracollicular decerebration was performed in two hemi-transection stages separated by at least 1 wk, as previously described (Grill and Norgren, 1978). Decerebrate rats received 4th v. cannulas and telemetric transponders (described below) during the second hemisection surgery. Pair-fed (gavage-fed) neurologically intact control rats were also anesthetized on two occasions and implanted with fourth v. cannulas and telemetric transponders during the second surgery. Rats recovered for at least 1 wk before the experiment started. The completeness of the intended transection was verified histologically after the experiment. Only rats with a histologically verified complete transection were included in the data analyses.

Telemetric transponder surgery.

Telemetric transponders (HRC 4000 VitalView; Mini Mitter/Respironics, Bend, OR) for

recording core temperature (T_C), heart rate (HR), and spontaneous physical activity (SPA) were inserted into the abdominal cavity, with the leads positioned sc and secured to the chest muscles on either side of the heart with sutures.

Experimental procedures

Cannula position verification.

At least 7 d after surgery, all brain cannula placements were assessed by measurement of the sympathoadrenal-mediated glycemic response to central injection of 5-thio-D-glucose [210 μg in 2 μl artificial cerebral spinal fluid (aCSF; Harvard Apparatus) for 3rd and 4th v. and 21 μg in 0.1 μl for NTS] (Ritter et al., 1981). A post-injection elevation of at least 100% of baseline plasma glucose level was required for subject inclusion.

Habituation training.

Before the start of experimental testing, rats were acclimated to the handling and injection procedures used in a given experiment.

Food intake and body weight monitoring.

As described in Figure 6.1, food was removed at the time of injections (early in the light cycle) and returned 8 h later, late in the light phase. Thus, food was not available during the period of energetic/sympathetic response measurement. Food intake and body weight measurements were made 24 h after the injection of drug. Given this design, all noted differences in food intake reflect longer latency effects of CART (i.e. intake from 8–24 h after injection). For *ad libitum*-feeding rats, food was always available during the dark cycle, and a minimum of 48 h was allotted between experimental testing for all animals.

Blood glucose response measurements: Blood glucose was measured before ventricular injections and at 30, 60, 120 and 240 min post-injection by collecting a drop of tail blood and placing it in a standard glucometer (Accucheck, Roche Diagnostics, Indianapolis, IN). Food (chow) was not available during the blood glucose measurements; it was returned immediately after the 240 min measurement and measured 24h after drug injection.

Experiment 1

To determine the energy expenditure and food intake effects of hindbrain $CART_{55\text{-}102}$ stimulation rats (n=23) received fourth v. vehicle injections (1 µl aCSF) counterbalanced with one dose of CART ($CART_{55\text{-}102}$, American Peptide Company, Inc., Sunnyvale, CA): 0.1 µg (19 pmol, n=4), 1.0 µg (190 pmol, n=9), 2.0 µg (380 pmol, n=10). $CART_{55\text{-}102}$ was chosen for this study as this peptide fragment has been previously shown to have physiological effects when applied to the hindbrain (Aja et al., 2001b; Aja et al., 2001a; Zheng et al., 2001; Aja et al., 2002; Zheng et al., 2002; Aja et al., 2006) and also effects on thermogenesis when applied to PVN (Wang et al., 2000). Core temperature, HR and SPA were recorded telemetrically for 1h before injections and 8h following injections every 5 min (T_C, SPA) or 30s (HR). Food intake and body weight were recorded 24h post injections. To assess whether hindbrain CART induced effects on glycemia changes in blood glucose were measured after 4th v. application of CART (2.0 µg) in a separate group of rats (n=12).

Experiment 2

All rats (n=11) received 2.0 µg of CART or vehicle (saline) intraperitoneally (ip). The purpose of this experiment was to test whether any of the effects of CART seen with the

central treatment can be ascribed to diffusion of the peptide out of CNS and stimulation of receptors in the periphery. Energy expenditure and food intake measurements were made as described in Experiment 1.

Experiment 3

To begin to define the neurocircuitry mediating the energy expenditure effects of hindbrain CART delivery the chronic decerebrate (CD) rat model was utilized. A lack of effect in CD rats compared to intact controls would indicate the requirement for forebrain neurocircuitry for mediation of a given effect. CD rats were maintained on a liquid diet (AIN 76A rodent diet; Research Diets, New Brunswick, NJ; 9ml, gavage-fed 4 times daily) as they do not feed spontaneously. This maintenance regime provides 79 kcal/d and adequate hydration; rats gain weight on this regime. Feedings were separated by intervals of at least 2 h. CD and gavage-fed intact control rats were maintained on this feeding paradigm except during experimental testing when animals were only fed three times: once 2 h before experiments commenced and twice after the experimental testing. Core temperature of CD rats is more variable than that of control rats. Rectal temperature was measured at each gavage feeding, and rats were cooled or heated if T_C was less than 34.0 or above 38.5°C (except during experimental testing). Rats received two doses of CART (1.0 and 2.0 μg) and vehicle in a counterbalanced design over three separate testing days (separated by a minimum of 48 h). Core temperature, HR and SPA were recorded telemetrically for 1h before injections and 8 h following injections.

Experiment 4

To determine whether the CART-induced changes in energy expenditure are mediated by GLP-1Rs all rats (n=9: 3rd v, n=7: 4th v.) received four of the following ventricular

injections counterbalanced over four testing days and separated by at least 48 h: 1) aCSF; 2) CART 1.0 μg; 3) CART 1.0 μg + Exendin-9 100 μg (Exendin 9-39, American Peptide Company, Inc., Sunnyvale, CA); 4) Exendin-9 100 μg. Exendin-9 dose selection based on (Aja et al., 2006). Core temperature, HR and SPA were recorded telemetrically for 1 h before injections and 8 h following injections. To determine if the CART-induced changes in blood glucose and food intake are mediated by GLP-1Rs, in a separate group of rats (n=10), blood glucose levels, 24 h food intake and body weight change were measured after 1) aCSF; 2) CART 2.0 μg; 3) CART 2.0 μg + Exendin-9 100 μg; 4) Exendin-9 100 μg. Some effects of CART (e.g. gastric emptying) are mediated by corticotrophin-releasing factor receptors (CRF-Rs) and not GLP-1Rs (Smedh and Moran, 2003) indicating that CART exerts it's CNS action through at least two separable neuropeptide pathways. To determine if the CART-induced changes in blood glucose and food intake are mediated by CRF-Rs, in a separate group of rats (n=8), blood glucose levels, were measured after 1) aCSF; 2) CART 2.0 μg; 3) CART 2.0 μg + CRF antagonist 38 μg (α -helical CRF-(9-41); Sigma-Aldrich) 4) CRF -(9-41). CRF-(9-41) dose selection was based on (Smedh and Moran, 2003). This dose was effective in blocking CART induced inhibition of gastric emptying, it was also shown to have no effects on CART hypophagia (Smedh and Moran, 2003).

Experiment 5

NTS is innervated by CART fibers and is also a site of thermoregulatory and intake control(Koylu et al., 1997; Koylu et al., 1998; Broberger, 1999; Broberger et al., 1999; Vrang et al., 1999b; Vrang et al., 1999a). To examine whether the NTS is the hindbrain locus for the energy expenditure and food intake effects of CART observed following 4th v. application all rats (n=12) received unilateral vehicle injections (0.1μl aCSF)

counterbalanced with one dose of CART: 0.1 μg into the parenchyma of the NTS. Energy expenditure, food intake and body weight were recorded as in experiment 1.

Statistical analysis:

Core temperature, HR and SPA were analyzed by ANOVA on post-injection 6 h average values of each parameter and followed by student's t-test or Tukey test as appropriate. Twenty four hour food intake and body weight were analyzed by ANOVA and followed by t-test or Tukey test as appropriate. All statistical analysis was conducted using STATISTICA software (StatSoft, Inc., Tulsa, OK). Differences were considered significant at $P<0.05$.

RESULTS

Experiment 1

Core temperature: Fourth ventricular CART injections produced large and long-lasting hypothermic responses, with an maximum drop of 0.7°C, 1.5°C and 1.6°C in T_C and 6h average effect size of -0.2°C ($P=0.902$), -1.1°C ($P<0.0005$), -1.3°C ($P<0.0005$) for the 0.1, 1.0 and 2.0μg doses respectively (Figure 6.2a). Heart rate: Although heart rate decreased slightly in some rats, this effect was not consistent across all rats and no significant changes were noted for any of CART doses used (Figure 6.2b). Spontaneous activity: No significant changes in spontaneous activity were observed (Figure 6.2c). Blood glucose was significantly increased after 2μg CART injection at 30 ($P<0.05$), 60 ($P<0.01$) and 120 ($P<0.05$) minutes after injection, or as area under the curve (AUC; $P<0.05$) (Figure 6.3a). Food intake was decreased by the 1.0 ($P=0.058$) and 2.0 μg ($P<0.05$) CART doses (food available 8-24 h post injection), (Figure 6.3b). Body weight

was also significantly decreased by the 1.0 (P<0.05) and 2.0μg (P<0.05) CART (Figure 6.3c).

Experiment 2

Peripheral application of CART had no effect on food intake, body weight and any of the measured energy expenditure parameters (Figure 6.4).

Experiment 3

Gavage-fed control rats: Core temperature was significantly decreased by 4th v. CART application at both the 1.0 (P<0.05) and 2.0 μg (P<0.0005) doses. CD rats: There was no significant hypothermia noted after CART application in this group. (Figure 6.5). To extract any possible short-term temperature effects that might have been present in the CD rats, data were reanalyzed this set of data (on both CD and gavage-fed control rats) by a two-way ANOVA looking at the interaction of time (30min periods) and drug. This analysis yielded a significant effect of drug (p=0.005), time (p<0.0005), and a significant interaction (p<0.0005) in gavage-controls. However there were no significant effects in CDs [drug (p=0.41), time (p=0.20), and drug-time interaction (p=0.74)].

Experiment 4

Significant hypothermia was noted when 1.0 μg of CART was applied alone in the 3rd (P<0.05) or 4th v. (P<0.05). This CART-induced hypothermic response was abolished following hindbrain-delivery of the GLP-1R antagonist Exendin-9 [100 μg; P=0.97 from aCSF, Tc]. Exendin-9 application alone was without effect [3rd v: P=0.72; 4th v.: p=0.99; Figure 6.6]. Consistently with results from Experiment 1 CART at 2.0 μg produced a

significant hyperglycemic response when delivered to the 4th v. (P<0.005); this effect was not altered by the Exendin-9 treatment (P<0.005 from aCSF). Fourth ventricular CART delivery (2.0 μg) significantly reduced chow intake (P<0.001); pretreatment with the GLP-1R antagonist attenuated this effect (P=0.21 from aCSF). Similarly the reduction in 24 h body weight produced by CART 4th v. (P<0.005) was attenuated by Exendin-9 pretreatment (P=0.69; Figure 6.7 a-d). In a separate group of rats the effect of CRF-R blockade on CART induced hyperglycemia was evaluated. CART at 2.0 μg produced a significant hyperglycemic response when delivered to the 4th v (P<0.01); this effect was not altered by the CRF-(9-41) treatment (P<0.05 from vehicle; Figure 6.7e-f). There was no effect of the CRF-(9-41) alone (P=0.99 from aCSF).

Experiment 5

Intraparenchymal injection of CART at a dose that was ineffective when applied in the 4th v. (0.1μg) significantly (P<0.05) decreased T_C when applied to the medial NTS parenchyma (Figure 6.8a). Only rats with confirmed NTS placement were included in the data analysis (representative injection site is shown in Figure 6.8b). No significant changes were observed in HR, SPA, food intake, and body weight.

DISCUSSION

Our experiments addressed the hypothesis that CART peptide's effects on energy balance cannot be characterized as simply catabolic in nature. CART peptide was applied to the 4th ventricle (hindbrain-directed), the 3rd ventricle (hypothalamus-directed), and directly to the NTS parenchyma and its effects on energetic (T_C and activity), cardiovascular (HR), food intake, body weight, and plasma glucose parameters

were measured. Our data show that CNS delivery of CART results in a hypothermia. We discuss the hypothermic effect first, the GLP-1R mediation of this response next, and then review the other effects observed.

These are the first data to show that hindbrain $CART_{55-102}$ delivery induced a pronounced and long-lasting *hypothermic* response (a 1.5°C and 1.6°C decrease in T_C for the 1.0 and 2.0 μg doses relative to vehicle treatment; >6h duration). Injections of CART into the forebrain ventricle (providing access of drug to forebrain and hindbrain sites due to the caudal flow of CSF), hindbrain ventricle, and hindbrain parenchyma (intra-NTS) were each effective in decreasing T_C. The potent and long duration of hypothermia triggered by injections selective to the hindbrain ventricle or NTS parenchyma, that bypass forebrain, indicated that CART responsive neurons located within the hindbrain and at least partially within the NTS mediated the hypothermic response. Additional experiments evaluating hypothalamic or forebrain parenchymal sites are needed to directly exclude the possibility of forebrain contribution to the hypothermic response. However, the few published studies that examine the energy expenditure effect of hypothalamic (ARC; PVN) CART injection show responses (increases in brown adipose tissue (BAT) UCP-1) that are typically associated with a *hyperthermic* action (Wang et al., 2000; Kong et al., 2003). That the thermic response to hypothalamic CART injection is opposite in direction to the current findings strengthens the hypothesis that the hypothermia was mediated by hindbrain CART responsive neurons.

We recently showed (Hayes et al., 2008a) that stimulation of hindbrain GLP-1R induces hypothermia similar in duration and size to that produced by CART in the present study. Current data establish that GLP-1 neurons and hindbrain GLP-1Rs are

downstream mediators of the effect of $CART_{55-102}$ on Tc as the hypothermic effect of CART was eliminated by blockade of hindbrain GLP-1Rs with Exendin 9-39. It is interesting to note that the hypothermic effect of hindbrain GLP-1R stimulation does not require intact forebrain-hindbrain communication as the effect was unaltered by supracollicular decerebration (Hayes et al., 2008a). By contrast, we showed here that the hypothermic effect of CART requires neural connections between the forebrain and hindbrain and forebrain processing is required, as the hypothermia was not present in rats with chronic supracollicular transsection. Therefore the circuitry mediating the hypothermic effect of CART might involve hindbrain neurons responsive to CART that project to the forebrain. The identity of those forebrain-projecting neurons is not known, however our data showing hypothermia in response to NTS CART stimulation suggests that at least part of CART's effects on temperature is mediated by potential CART receptors located in or near the NTS. NTS neurons project heavily in a rostral fashion (e.g. PBN) to nuclei that relay peripheral thermal information to thermoregulatory neurons in the hypothalamic preoptic area (Nakamura and Morrison, 2008). The forebrain-relayed information would be subsequently transmitted back to the hindbrain resulting in putative GLP-1 release from the NTS proglucagon expressing neurons and subsequent hindbrain GLP-1R stimulation. Once activated, the output pathway following hindbrain GLP-1R activation inducing hypothermia is contained within the hindbrain (Hayes et al., 2008a). Several nuclei expressing GLP-1Rs within the hindbrain contain sympathetic premotor neurons (Yamamoto et al., 2002). It is possible that inhibition of those neurons results in the observed hypothermia. Experiments evaluating hindbrain CART and GLP-1 effects on SNS neurons and BAT activity are needed to evaluate this possibility. Another potential mechanism contributing to hypothermia is SNS-mediated vasodilatation. A role of other neurotransmitters (e.g.

serotonin and dopamine) that are associated with hypothermic responses (Cryan et al., 2000; Catalina et al., 2002) in this pathway is possible and remains to be investigated.

While the *hypothermic* response to hindbrain $CART_{55-102}$ peptide stimulation contrasts with the *hyperthermic* effect of hindbrain melanocortin receptor stimulation, the anorexia and acute hyperglycemic effects of CART injection are similar to those observed with melanocortin delivery (Fan et al., 2000). The anorexic effect of hindbrain CART stimulation observed here is consistent with several studies (Kristensen et al., 1998; Rogge et al., 2008). Furthermore, Aja et al. (Aja et al., 2006) show that GLP-1Rs are downstream of the anorexia triggered by hindbrain CART as measured by alterations in palatable diet (Ensure®) intake. Our studies confirm this GLP-1R mediation of anorexic effects of CART and extend the conclusion to effects on consumption of standard chow (food of lesser palatability). We show, for the first time, that acute $CART_{55-102}$ stimulation increased blood glucose levels. This is consistent with the direction of acute effect of CNS melanocortin stimulation on blood glucose (Fan et al., 2000). Unlike CART's hypothermic and anorexic effect, the hyperglycemic effect was not mediated by hindbrain GLP-1Rs as blockade of hindbrain GLP-1Rs did not alter CART-induced hyperglycemia. Other effects of CART (e.g. gastric emptying) are mediated by CRF-Rs (Smedh and Moran, 2003), but not GLP-1Rs. Here we show that CART-induced hyperglycemia does not require hindbrain CRF-R mediation, indicating that a different mechanism might govern the hyperglycemic effect of CART.

CART peptides are not only widely present in the CNS but also detected in some areas relevant to energy balance outside of the CNS including the adrenal medulla, gut, and pancreas (Thim et al., 1998; Jensen et al., 1999; Thim et al., 1999). In order to

evaluate the potential contribution of CART stimulation outside of the CNS to energetic and anorexic effects of CART rats were injected ip with the highest dose of CART applied in the ventricle. Peripheral $CART_{55\text{-}102}$ application did not have any effects on any of the measured parameters, suggesting that effects seen after central CART application do not result from stimulation of potential receptors accessed by ip injection.

Our current data, along with those of others [see (Kuhar et al., 2000) for review], suggest that CNS CART peptide plays a role in several anatomically separable circuits. CART stimulation within the cortical and mesolimbic regions elicits reward and motivation responses (Jaworski and Jones, 2006). CART stimulation in the mesolimbic nucleus accumbens produces anorexia (Yang et al., 2005). Hypothalamic CART injection has varied effects on food intake, but appears to increase energy expenditure (UCP1 activity) (Wang et al., 2000; Kong et al., 2003). In hypothalamic explants CART suppresses release of MSH and induces agouti-related protein release. In addition, while CART and POMC are co-expressed in arcuate hypothalamus, CART is also co-expressed with the orexigenic peptide melanin-concentrating hormone in lateral hypothalamic neurons (Broberger, 1999). Hindbrain CART application decreases food intake, gastric emptying (Smedh and Moran, 2003), and as shown here core body temperature. Further evaluation of the complexity of CART's functional effects and the mediating neurocircuitry awaits the discovery of CART receptors.

The observed hypothermia was not associated with other anabolic indicators. Our measurements included several parameters that are associated with energy expenditure and sympathetic control (T_C, activity, HR). Melanocortin treatment for example alters all three parameters in the catabolic direction consistent with increased

energy expenditure and sympathetic activation (Fan et al., 2000; Gutierrez-Juarez et al., 2004; Skibicka and Grill, 2008a) . CART injection triggered a different pattern of response – hypothermia without alteration in activity and HR. If CART hypothermia is not a part of a coordinated pattern of energy balance effects, then what is the physiological utility of potent and sustained hypothermic response? Several processes have been associated with a marked hypothermia. Conditioned taste aversion (CTA) is often accompanied by a hypothermic response. Some studies (Aja et al., 2002) suggest that CART plays a role in CTA response. LiCl, a common inducer of CTAs, produces hypothermia via α1-adrenergic receptor (Amaro et al., 1996). It has been proposed that hypothermia might enhance CTA formation by extending the associative interval between presentation of taste conditioned stimuli and the unconditioned aversive effects of LiCl or other agents (Hinderliter et al., 2002; Hinderliter et al., 2004). Therefore the elicited CART hypothermia might be adaptive in facilitating CTA formation. Another process enhanced by hypothermia is neuroprotection (Sahuquillo and Vilalta, 2007). Neuronal survival after conditions of decreased oxygen supply (e.g. ischemia) is often increased if core temperature is decreased after the ischemic episode; this phenomenon is observed in rat models of ischemia and is utilized as a treatment strategy in ischemic patients (see (Hoesch and Geocadin, 2007) and (Nagel et al., 2008) for review). CART is associated with a neuroprotective effect in ischemia (Xu et al., 2006; Jia et al., 2008). Induction of hypothermia could be a part of the mechanism by which CART exerts a neuroprotective effect. Other experiments are needed to evaluate this hypothesis and whether the CART-induced hypothermia is physiological in nature. Our results indicate that the hypothermia induced by CART is long lasting and shows little tolerance upon repeated exposure (data not shown), two properties highly sought after in potential hypothermia-inducing neuroprotective treatments.

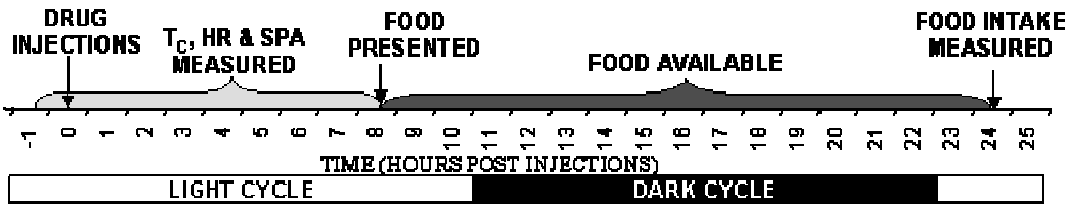

Figure 6.1: Experimental paradigm utilized for measurements of core temperature (Tc), heart rate (HR), spontaneous activity (SPA) and food intake.

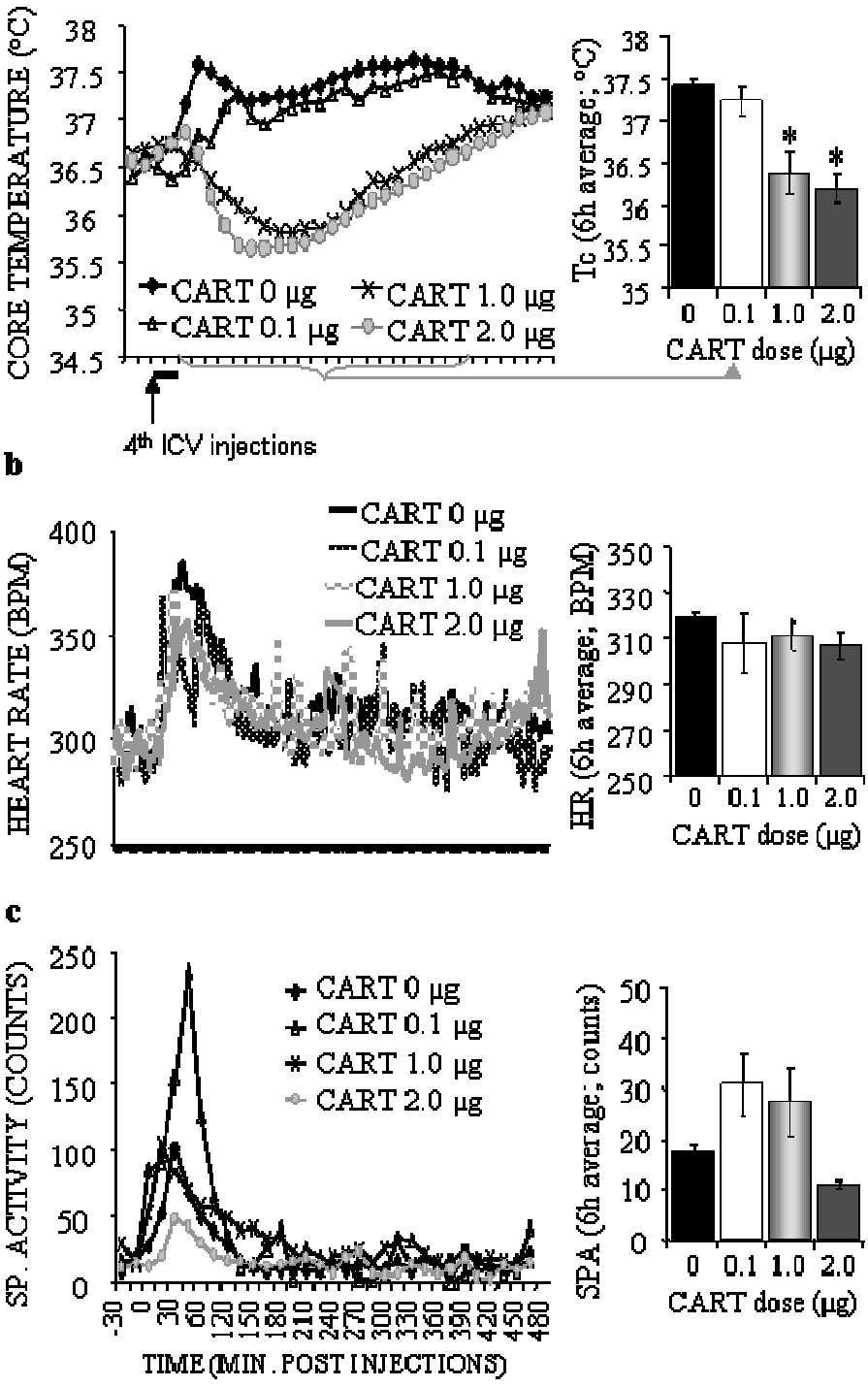

Figure 6.2: Effect of hindbrain CART stimulation (4th v. injection) on (a) core temperature (Tc), (b) heart rate (HR) and (c) spontaneous activity (SPA). Line graphs represent

across-rat average parameter measurements through the 8h recording period. The bracketed time period on the line graph x-axis indicates the periods used in the histograms. The histograms provide 6h post-injection averages + SEM for each parameter at each dose. *$P < 0.05$ from vehicle.

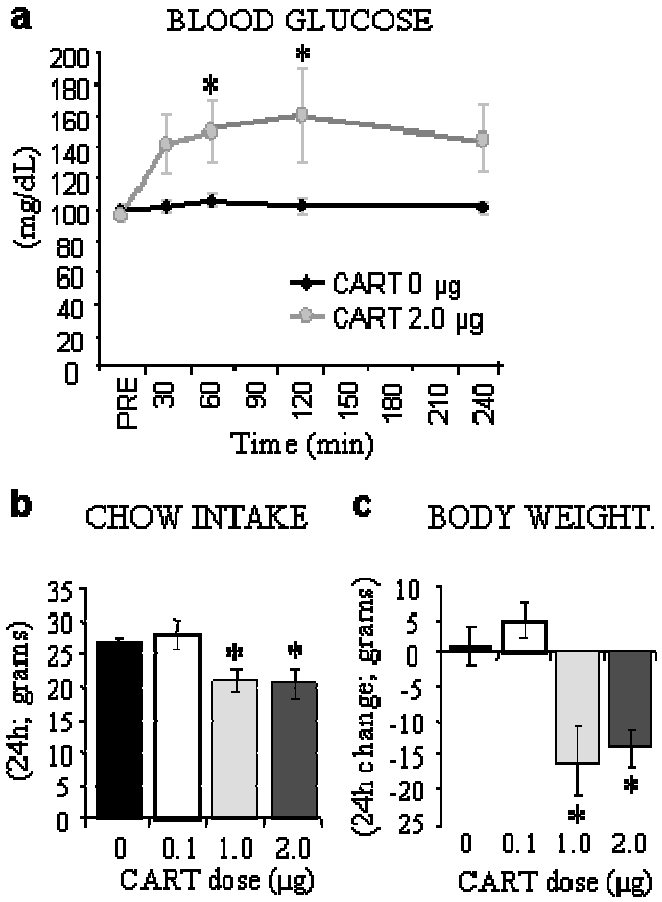

Figure 6.3: Effect of hindbrain CART stimulation (4th v. injection) on (a) blood glucose levels (b) 24h food intake (c) 24h body weight. The histograms provide averages + SEM for each parameter at each dose. *$P < 0.05$ from vehicle.

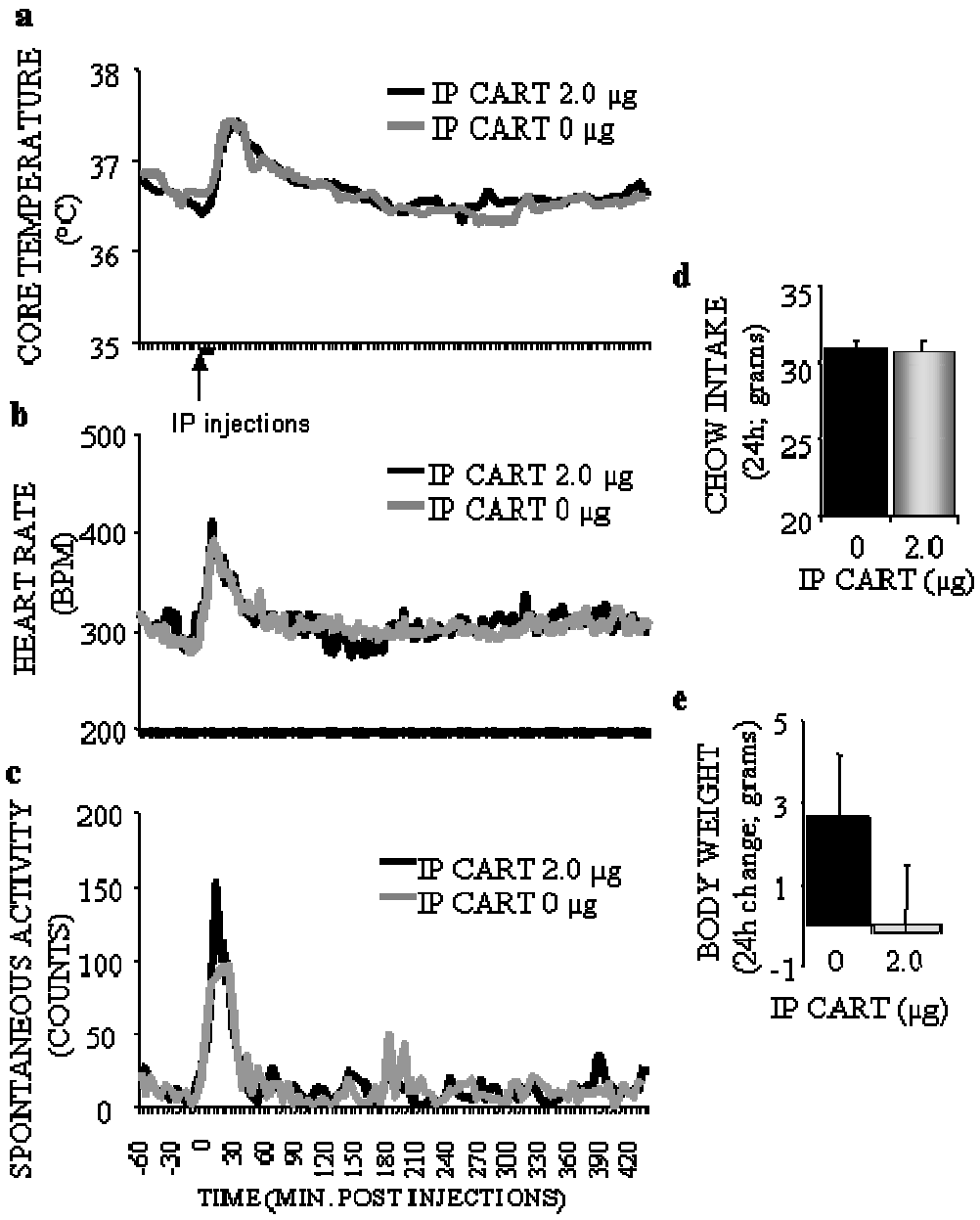

Figure 6.4: Effect of peripheral CART stimulation (ip delivery) on (a) core temperature, (b) heart rate and (c) spontaneous activity (d) 24h food intake (e) 24h body weight. The histograms provide averages + SEM.

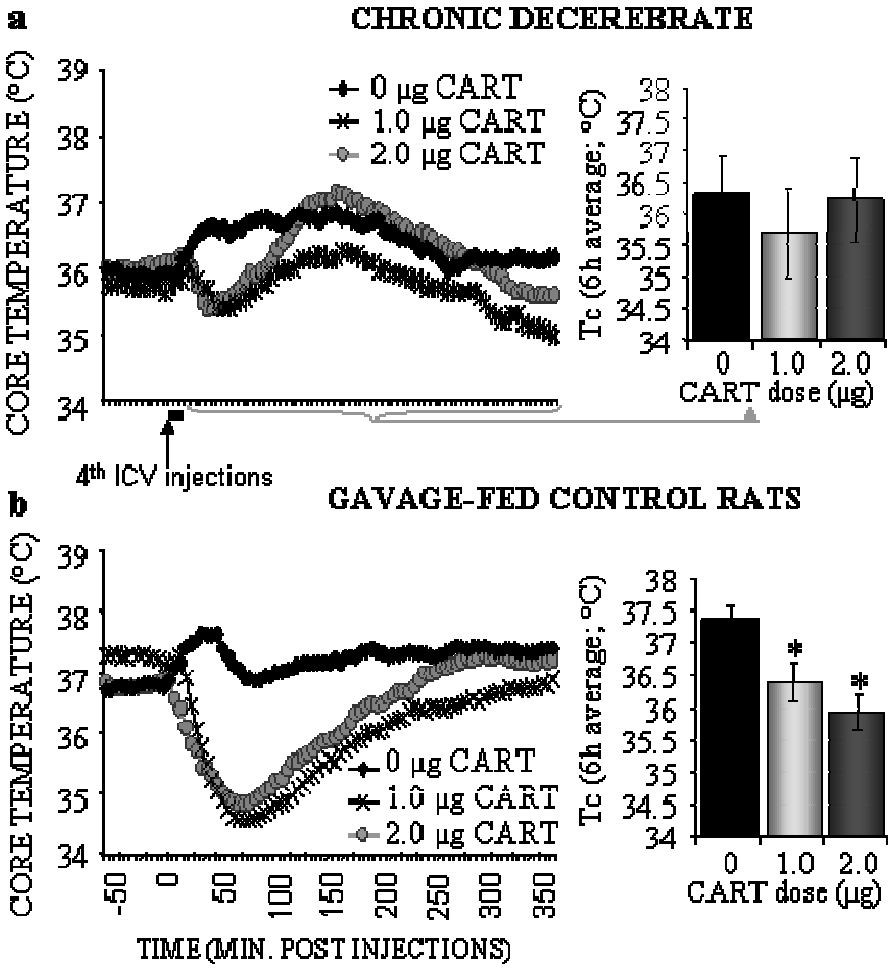

Figure 6.5: Effect of hindbrain CART stimulation (4th v. injection) on core temperature (Tc) in (a) **chronic decerebrate** and (b) **gavage-fed rats**. Line graphs represent across-rat average parameter measurements through the 8h recording period. The histograms provide 6h post-injection averages + SEM for each parameter at each dose. *$P < 0.05$ from respective vehicle.

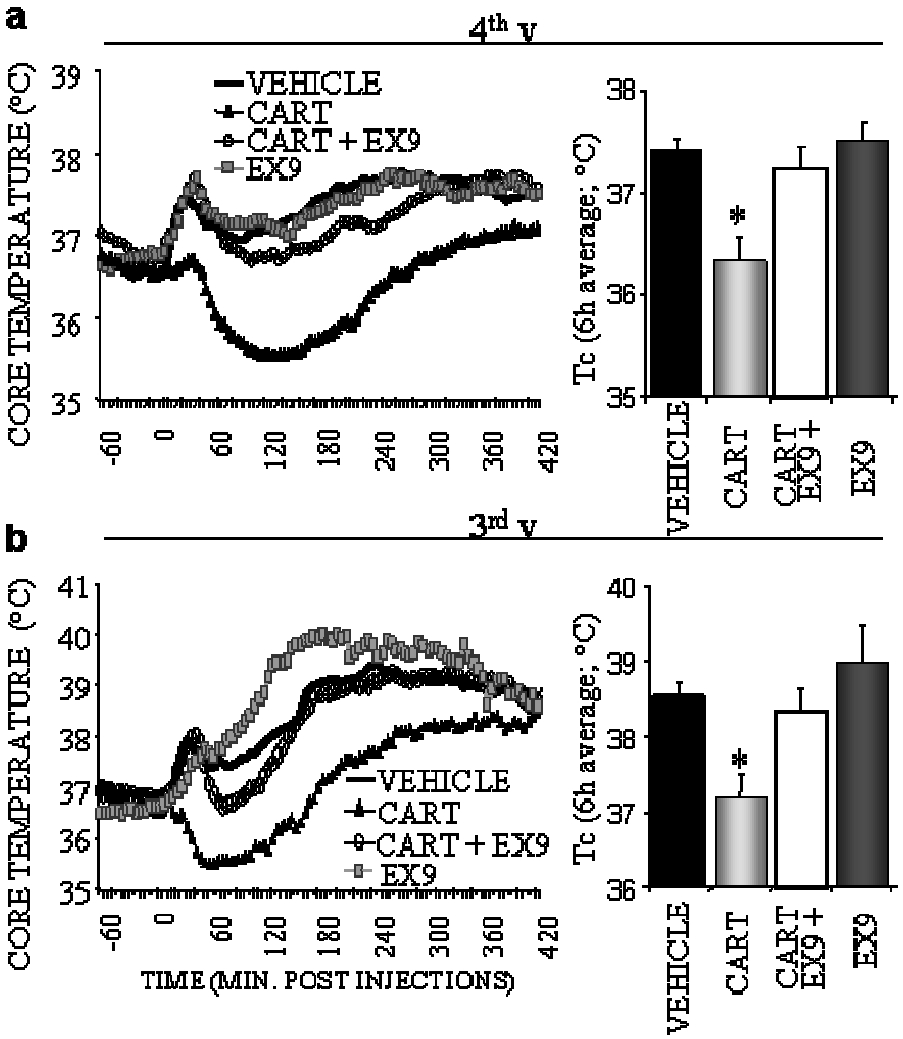

Figure 6.6: Effect of GLP-1R blockade by Exendin 9-39 (100 µg) on the CART-induced (1 µg) hypothermia with drugs delivered to the (a) 4th v. (b) 3rd v. Line graphs represent across-rat average parameter measurements through the 8h recording period. The histograms provide 6h post-injection averages + SEM for each parameter at each dose. *P < 0.05 from vehicle.

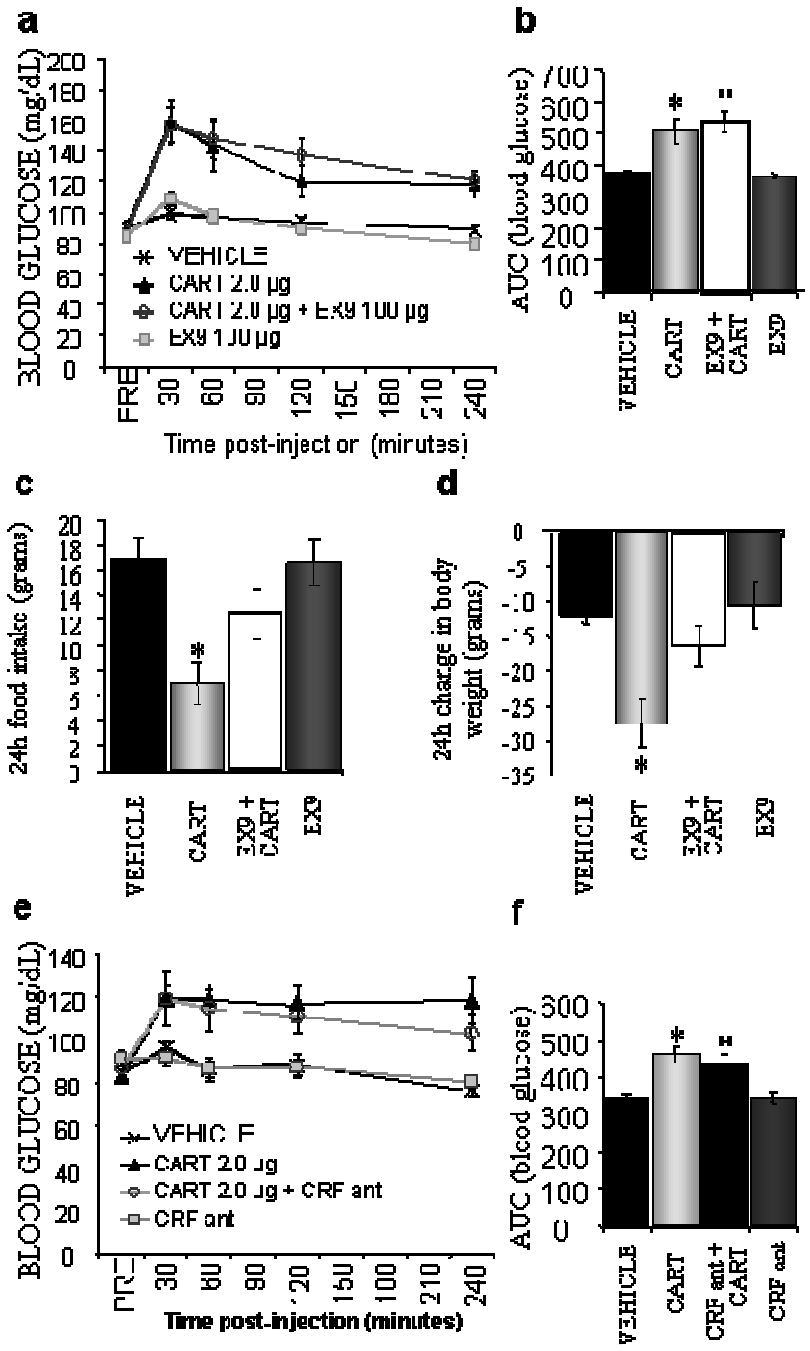

Figure 6.7: Effect of GLP-1R blockade by Exendin 9-39 (100 µg) on 4th v. 2 µg CART-induced (a-b) hyperglycemia (c) hypophagia (d) 24h body weight, (e-f) effect of CRF receptor blockade by (CRF ant; 38 µg of α -helical CRF-(9-41)) on 4th v. CART induced hyperglycemia. The histograms provide post-injection averages + SEM. Area under the curve (AUC) *P < 0.05 from vehicle.

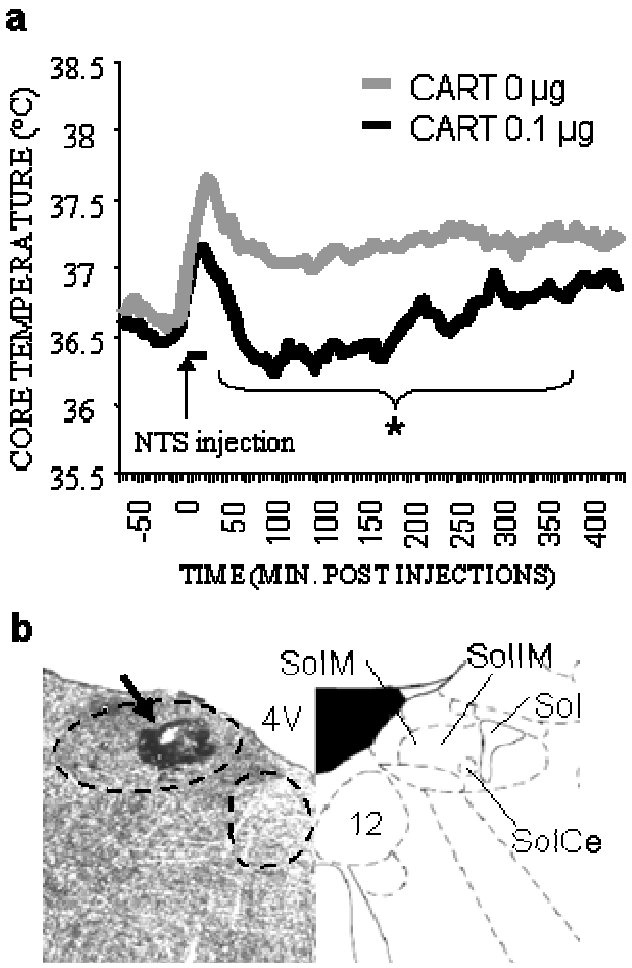

Figure 6.8: Effect NTS delivery of CART on core temperature (T_C). (a) line graph represents across-rat average parameter measurements through the 8h recording period. The bracket outlines 6h post-injection period. *$P < 0.05$ from vehicle. (b) representative NTS injection site (indicated by the arrow). 4V: 4th ventricle, Sol: nucleus of the solitary tract, SolM: medial nucleus of the solitary tract, SolIM: intermedial nucleus of the solitary tract, SolCe: central nucleus of the solitary tract.

CHAPTER 7

GENERAL CONCLUSIONS

DRUG	SITE OF APPLICATION	PHYSIOLOGICAL EFFECT					CHRONIC DECEREBRATE EFFECT	INTERACTIONS
		T_C	HR	SPA	FI	BW		
MTII (Ch. 2&3)	4th v	↑	↑	↔	↓	↓	T_C↑,HR↑, SPA↑, (FI N/A)	▪ All effects blocked by MC3/4R antagonist (SHU 9119)
	RVLM	↑	↑	↔	↓#	↓#	N/A	
	PBN	↑	↑	↔	↓	↓		
	RCh	↑	↑	↔	↓	↓		
	RAPHE	↑	↑	↑#	↓#	↔	T_{BAT}↑ as in intact	
	NTS	↑	↑	↑	↓	↓		
	PVN	↑	↑	↑	↓	↓		
	AHA	↔	↔	↔	↔	↔		
SHU 9119 (Ch. 5)	4th v	↓	↓	↔	↑	↑	N/A	▪ T_C and HR not altered by HF feeding, but increased FI & BW effect, suggesting enhanced hindbrain MSH tone with HF exposure
Leptin (Ch. 4)	4th v	↑	↑	↔	↓	↓	N/A	▪ 4th v leptin T_C,HR, FI & BW effects mediated by hindbrain MCRs as 4th v SHU 9119 attenuates those effects ▪ All 4th v effects not altered by high-fat diet feeding
	NTS	↑	↑#	↔	↓	↓		
	PBN	↔	↔	↑	↓	↓		
CART (Ch. 6)	3rd v	↓	↔	↔	↓	↓	N/A	▪ T_C , FI & BW effects mediated by hindbrain GLP-1Rs (their blockade attenuates 4th v CART effects)
	4th v	↓	↔	↔	↓	↓	(T_C,HR, SPA)↔ (FI N/A)	
	NTS	↓	↔	↔	↔	↔	N/A	
GLP-1 (Ch. 6)	4th v	↓	↑	↔	↓	↓	T_C↑↓,HR↑, SPA↓, FI↓	

Table 7.1: Summary of collected data. ↑ - increase, ↓ decrease, ↔ - no change in a measured parameter, # - weak effect (borderline significant or non consistent between experiments), N/A – not applicable, Ch - chapter. For a list of abbreviations please refer to appendix 1.

The experiments described in this dissertation challenge the hypothalamus centered focus on energy balance control that dominates the literature and instead make clear that CNS control of energy balance is broadly distributed across the neuraxis. Strong support is provided for a critical contribution of hindbrain melanocortin and leptin signaling to energy balance control. The role of the hindbrain neural processing to energy balance regulation is highlighted in several complementary ways. Results presented in Chapter 2 clearly indicate that feeding, energetic and cardiovascular responses can be obtained by hindbrain melanocortin receptor (MCR) stimulation. Further, caudal brainstem processing was found to be sufficient for mediation of the energetic and cardiovascular responses, as the same direction and duration of effects were observed in a chronic decerebrate rat model as those produced in a neurologically intact rat. These findings indicate that the mediating circuitry, that engages sympathetic outputs following hindbrain MCR stimulation, is contained entirely within the hindbrain. These are the first data to demonstrate the sufficiency of hindbrain MCR in initiating energy expenditure responses. Chapter 3 introduced another level of anatomical analysis outlining the individual energy balance response profiles from several discrete hindbrain MCR-expressing populations. Here, the role of several hypothalamic nuclei is also investigated, highlighting the fact that the goal of the work was not to shift the energy balance control model to the hindbrain but to create a balanced, anatomically distributed perspective. Data presented in Chapter 4 are consistent with the presence of a hindbrain specific leptin-melanocortin circuit, possibly parallel to the one that has been well established in the forebrain. The data presented herein have begun to approach the issue of inputs integrated by the hindbrain MCR population. This concept was advanced in Chapter 5, where endogenous hindbrain MCR

activity was shown to be necessary for the adaptive feeding responses elicited by chronic ingestion of a high-fat maintenance diet. Chapter 6 provides data on hindbrain-directed cocaine and amphetamine related transcript (CART)-mediated energy balance effects. Due to coexpression of CART peptide with the proopiomelanocortin (POMC) peptide in the hypothalamic POMC neuron population, the functional effects of CART on energy balance have been often linked to those of melanocortins. This idea was strengthened by the fact that both hypothalamic and hindbrain CART stimulation, similarly to MCRs stimulation, resulted in anorexia (Kristensen et al., 1998; Zheng et al., 2002). Here however, we show that unlike the hyperthermic effect of CART in the hypothalamus, application of CART to the hindbrain produces a large and sustained hypothermia. These data thus provide caution to general characterizations of neuropeptide effects in the hypothalamus to the hindbrain and emphasize the need for direct hindbrain testing.

With respect to the melanocortin system, our studies clearly provide support for an independent role of hindbrain MCRs in mediating energy balance effects, independent of neural communication with the forebrain. Those data speak to a hindbrain location of receptors and downstream mediating pathways for the melanocortinergic effects on energy balance. Future studies are needed to identify the endogenous source of hindbrain MCR activation that accounts for the energy balance effects observed here by exogenous hindbrain MCR ligand application. To affirm a completely independent hindbrain circuit two more questions, pertaining to events upstream of MCR activation, should be answered: 1) a source for endogenous ligand for the hindbrain MCRs would have to be located in the hindbrain (NTS POMC populations), and 2) this source would have to be driven by hindbrain selective inputs. In the simplest form, there could be two parallel independent circuits, one in the

forebrain and the other in the hindbrain. The first circuit would consist of a forebrain loop: peripheral inputs directly stimulating ARC POMC neurons that project to hypothalamic/forebrain MCR expressing nuclei that subsequently engage the sympathetic and anorexic output (by direct projections to spinal cords IML, or through hindbrain relays); and a hindbrain loop with peripheral inputs directly stimulating NTS POMC neurons projecting to hindbrain MCR expressing areas leading to sympathetic and anorexic action. This simplistic model however, was already challenged by several publications indicating that ARC POMC neurons send their projections caudally to the midline hindbrain MCR expressing nuclei, in addition to their multiple forebrain projections. In fact we have recently showed (in collaboration with HR Berthoud; Berthoud HR et al., 2008) that nearly 70% of MSH fibers in the NTS are of hypothalamic origin. Thus, NTS POMC neuron projections are likely a minority in the midline hindbrain nuclei, however they may play a major role in stimulation of the more laterally distributed MCR populations in the hindbrain like the RVLM (Sim, 1994). An additional level of hindbrain-forebrain melanocortin interaction is added by the fact that some nuclei, such as the PBN, are likely innervated by both POMC sources (Joseph and Michael, 1988; Sim, 1994) and indicate that in addition to two simple, parallel and separable forebrain and hindbrain circuits there are other MCR-driven circuits that contain necessary elements in both forebrain and hindbrain regions.

Inputs to the melanocortin agonist-producing POMC neurons from peripheral signals, conveying information about the metabolic status, are a fundamental element in the current model of CNS energy balance control. As is the case for existing MCR literature, here too the focus has mostly been on the inputs to the hypothalamic ARC POMC neurons. Data presented here (Chapters 2 and 3), however, clearly support the

need to extend the evaluation of inputs to the hindbrain NTS POMC populations. Studies in Chapters 4 and 5 have partly begun to evaluate the sources of inputs to the two CNS POMC populations. The hindbrain leptin-melanocortin study presented in Chapter 4 is highly suggestive of an independent hindbrain circuit, as both the input (hindbrain-directed leptin effects) and the output (MCR stimulation and downstream energetic effects) required to elicit a thermic and anorexic responses are located within hindbrain nuclei. To place this circuitry entirely within the hindbrain one more link, one from leptin receptor activation to NTS POMC activation, has to be characterized and localized to the hindbrain. Final confirmation of a circuit endemic to the hindbrain, bypassing any forebrain projection would be indicated if the same hindbrain leptin-melanocortin interaction was shown in a chronic decerebrate rat, where all neural forebrain-hindbrain connections are severed. This would indicate that all necessary components from receptors of peripheral inputs, through integratory components to output are contained in a forebrain-independent, hindbrain circuit.

Leptin is just one of many inputs potentially activating POMC neurons, resulting in release of the MSH ligand and activation of MCRs. It is still unclear which physiological or pathophysiological factors regulate the activity of the ARC and NTS POMC populations, and whether these populations are activated / inhibited together or separately. In the work presented here, we focus on view of brain organization that can be called distributed control in different contexts (high-fat diet, leptin) under which thermogenic, cardiovascular and feeding responses could be observed. At both hindbrain and forebrain, MCR signaling is involved in generation of feeding responses to these presented challenges. The location of POMC neurons required for this feeding response remains an open question. Interestingly the energetic effects of melanocortins

were not altered under high-fat conditions, indicating perhaps that some inputs (here arising from high-fat diet maintenance) regulate the feeding and energetic effects of melanocortins differentially.

In future studies it will be important to incorporate different testing paradigms, as the mediating substrates, and pathways that ultimately converge on sympathetic and feeding control centers, are likely to vary to some extent given different sensory components (e.g. diet vs. cold vs. systemic pathogen introduction). Fever, for example, is a centrally mediated response that can be induced by a systemic infection with blood-borne pathogens like bacteria (Saper, 1998; Zhang et al., 2000). Peripheral administration of lipopolysacharide (LPS), a piece of gram-negative bacterial cell wall that induces an immune system response, is a common model for infection-induced fever. Although during a fever, core temperature increases above its normally tolerated range, the response is arguably under feedback control; i.e. the temperature set range is increased. Brown adipose tissue (BAT) in rodents is the primary effector contributing to fever (Cannon and Nedergaard, 2004). CNS pathways involved in the thermogenic response to LPS have been partially elucidated by immunohistological analysis (Fos-like-immunoreactivity). Areas of CNS showing c-Fos induction include brainstem regions, such as the NTS and VLM, as well as hypothalamic regions, such as the PVN and MPOA (Zhang et al., 2000). Interestingly, melanocortin neurons and their receptors are found in these structures.

Like all homeothermic animals, rats apply thermoregulatory mechanisms to defend body temperature in response to challenging ambient temperatures. Thus, adaptive thermogenic responses are reliably driven by cold temperatures. In rodents,

shivering thermogenesis is recruited first. Melanocortin contribution to acute cold thermogenesis in MCR knock-out mice has been recently indicated (Voss-Andreae et al., 2007). We explicitly addressed the potential MC contribution to cold thermogenesis, at forebrain and hindbrain levels of the neuraxis, but were not able to show any significant effects of MCR blockade on maintenance of body temperature in the cold. While these data might suggest that MCR system is not essential for cold and fever responses in a rat (contrary to what has been shown in a mouse), they do not rule out contribution of this system to those responses. Several factors might have contributed here to mask the MCR contribution, considering the redundancy in the control of thermogenesis in those complex responses. The environment and energy state of the rats before the experiment could interact to bypass the requirement for MCR activation during our studies. This idea has been evaluated recently in mice that lack UCP-1, an uncoupling protein utilized in BAT to generate heat (Feldmann et al., 2009). Initial studies of diet-induced-thermogenesis in these UCP-1 KO mice have not been successful at showing any thermogenic dysfunction. However, a careful follow-up study has uncovered their deficit in mustering diet-induced thermogenesis but only when these mice were maintained at thermoneutrality (without thermal stress) (Feldmann et al., 2009). The constant thermal stress likely engaged other thermoregulatory mechanisms, which were then easily utilized for diet-induced thermogenesis despite the lack of UCP-1. Those findings underscore the importance of careful consideration of all environmental variables when conducting thermic studies. It is important to note that most experiments are conducted at a “room temperature”, comfortable for the experimenter, however several degrees below the rodent thermoneutral zone. It is entirely possible that alterations in the maintenance temperature also influenced the outcome of our above mentioned studies evaluating the MCR contribution to cold induced thermogenesis. Thus negative data

obtained may not be entirely conclusive and further studies availing of different paradigms are warranted.

The lack of effect of acute pharmacological studies in the non-feeding focused paradigms described above along with the strong nutritional/metabolic status component of the so far characterized inputs to the melanocortin system leads to another important question: do the melanocortin/leptin peptides play a primary role in non-feeding induced thermogenesis (cold, pathogen etc.), or do they play a permissive role, integrating the energy status into an equation requiring loss/usage of energy? The first would be supported if evidence of direct influence of signals associated with each of those challenges reaching POMC neurons (e.g. PGE or IL receptors) was found directly on POMC neurons. Even under this situation, their activity could be gated by other input they receive. Such input raising PGE2 levels should increase activity of POMC neurons leading to increased thermogenesis. This increase in POMC activity could be tampered by a simultaneous drop in leptin signaling, indicating insufficient energy stores resulting in a lower fever then one that would have been permitted in conditions of sufficient energy stores. Conversely, if leptin or other input to POMC neurons would signal excessive energy stores, it is possible that fever could be larger. Consistent with this hypothesis is the fact that obese rats have exaggerated fever and anorexia responses to interleukin 1b (Plata-Salaman et al., 1998).

Based on the weight of evidence presented here, it is clear that both the melanocortin system and its leptinergic inputs controlling for energy balance regulation are anatomically distributed within the CNS. What is the utility of this distributed model? Stimulation of either the NTS or ARC POMC neuron populations can lead to

simultaneous release of ligand onto many neuroanatomically divergent MCR populations, which may again converge, to induce similar output responses. This divergence might on the surface seem redundant, since final output parameters are likely similar, however one advantage of this system design is the gained ability of the output to be modulated on many levels. Each nucleus in which MCR expressing neurons are stimulated presumably receives many other unique inputs. Each of these separate MCR-expressing neurons now has the ability to modulate the final energetic outcome response. Therefore, the final outcome can now be finely modulated, filtered and integrated with other available energy status information that is being communicated to the CNS through the neuroendocrine system. Thus, the putative simultaneous release of neuronal ligands onto various brain regions could allow for fast and effective influence on many circuits instantaneously.

Appendix 1.

List of commonly used abbreviations:

5-TG – 5 thio-glucose

aCSF – artificial cerebrospinal fluid

AgRP – Agouti related protein

AHA – anterior hypothalamic area

ARC – arcuate nucleus of the hypothalamus

BAT – brown adipose tissue

BW – body weight

CART – cocaine and amphetamine related transcript

CD – chronic decerebrate

CNS – central nervous system

CSF – cerebrospinal fluid

EE – energy expenditure

FI – food intake

GLP-1 – glucagon like peptide 1

HF – high fat

HR – heart rate

I.C.V. - intracerebroventricular

IML – interomedial lateral column of the spinal cord

LPS – lipopolysacharide (a piece of gram negative bacterial cell wall)

MCR – melanocortin receptor

MF – moderate fat

MPOA – medial preoptic area of the hypothalamus

MSH – melanocyte stimulating hormone, an MCR agonist

MTII – melanotan II (exogenous MC3/4R agonist)

NTS – nucleus of the solitary tract

Ob-Rb – leptin receptors

POMC – proopiomelanocortin (a precursor for MSH)

PBN – parabrachial nucleus

PVN – paraventricular nucleus of the hypothalamus

RPa – raphe pallidus

RVLM – rostroventral medulla

RCh – retrochiasmatic area of the hypothalamus

SHU 9119 – and exogenous MC3/4R antagonist

SPA – spontaneous activity

Tc – core temperature

UCP-1 – uncoupling protein 1

V. - ventricular

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