The Effect of Stage of Lactation on the Response of Postpartum Rats to the Administration of Anorectic and Orexigenic Peptides

Radek Eric Budin

A Thesis
in the Department of
Psychology

Presented in Partial Fulfillment of the Requirements
For the Degree of Doctorate of Philosophy (Psychology) at
Concordia University
Montreal, Quebec, Canada

April 2016

CONCORDIA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

This is to certify that the thesis prepared

By: Radek Eric Budin

Entitled: The Effect of Stage of Lactation on the Response of Postpartum Rats to the Administration of Anorectic and Orexigenic Peptides

and submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy (Psychology)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

_____ Chair

_____ External Examiner

_____ External to Program

_____ Examiner

_____ Examiner

_____ Thesis Supervisor

Approved by: _____
Psychology Graduate Program Director

Date: _____
Dean, Faculty of Arts & Science

ABSTRACT

The effect of stage of lactation on the response of postpartum rats to the administration of anorectic and orexigenic peptides

Radek Eric Budin, Ph.D.

Concordia University, 2016

This dissertation investigated the response of female rats in varying reproductive states to the administration of anorexigenic and orexigenic hormones and how behavioural and neural responses to such compounds might be influenced by reproductive state. The first set of experiments examined cycling, early or late stage lactation rats after treatment with various anorexigenics and measured food intake, body, and litter weight. The ability of central leptin administration to induce the phosphorylation of STAT3 in hypothalamic nuclei was also compared in these groups. Central leptin administration reduced food intake and body weight in all animals tested in a dose dependent way, however this effect was attenuated for food intake in early lactation. Litters of dams receiving the highest dose of leptin weighed less than those receiving the lowest dose or saline. Chronic peripheral leptin administration had no effect on any measures in lactating rats. Central administration of 1μg leptin produced increased PSTAT3-IR in ARC and the VMH across all groups tested, with trend in the PVN. MTII resulted in a dose dependent suppression of food intake and body weight across all reproductive states, with the higher dose being more pronounced. MTII administration at both doses reduced litter growth. The second chapter investigated intracerebroventricular (ICV) administration of ghrelin (0, 0.25 μg, 0.5 μg, 1 μg) and its effects on food intake in cycling and mid-lactation females. In the second experiment the food intake of cycling rats following the ICV administration of ghrelin (0.25 μg or 1 μg) was compared to rats in early and late lactation. These same reproductive states were compared with regard to food intake following administration of a ghrelin antagonist (JMV 3002, 0.4 μg). Central ghrelin administration resulted in a dose dependent increase in food

intake in cycling rats while rats in mid-lactation showed a peak effect at the lowest dose of ghrelin administered. Cycling, early, and late lactating all responded to ghrelin with increased food intake. Ghrelin antagonist administration produced greater food intake reductions in late lactation compared to cycling or early lactation rats. Findings are discussed in relation to other neuropeptides and energy balance during lactation.

ACKNOWLEDGEMENTS

I would not be writing this acknowledgement section today if it were not for the endless patience and support of my mentor, Barbara Woodside. Thank you for the many years of encouragement and wisdom, both personally and academically. You were as close to a mother as I could wish for while being away from my own. Your nurturance on that front often included delicious meals as well! You always made the time for me despite the many important things going on in your life (including retirement!); I cannot express how grateful I am for that. I would also like to extend my thanks to Jim Jans who has been there for the ride as well, your humor always came at the right time. This same appreciation also goes out to Alison Fleming and Gary Kramer. Thank you for planting the seeds of curiosity in my young mind when it came to science and for your encouragement and support when it came to continuing my academic journey after leaving the home that you created for all the students in your lab. Thank you for the kindness and support that came from the Woodside lab while I was there, Claudia Frate and Sharon Ladyman for help with both data collection and training on the techniques that I employed to complete this piece of work. Thanks to Kim Breux, Shirley Black, and Isabelle Bouvier for their assistance as well. I am very grateful to Wayne Brake, who has also supported me throughout my time in graduate school, from chairing my Masters Defence, to reading my comprehensive paper, and finally by agreeing to be on my Ph.D. Committee. This appreciation also extends to Uri Shalev, who has also been there for me through my Masters as a committee member and for being kind enough to take the time to do this again for the present purpose. My appreciation also goes out to Dave Grattan and Richard Courtemanche for examining my thesis and for the thoughtful feedback on my work. Thanks to Jim Pfaus for being there to represent Dave during my defence and for providing the comedic relief when I needed it the most. I also want to thank all of my clinical supervisors (Virginia Penhune, Mark Ellenbogan, Stephanie Pantel, Wendy Wood, Hallie Frank, John Arrowood, Percy Wright, Faye Doyle, and Sylvain Roy) who have been there throughout the course of my training and beyond. They have helped me develop the skills that I hope to use to help improve the lives of others. I am eternally thankful for the friendships that I have developed over the course of this journey; this includes

Emis Akbari, Alex Akbari, Nassim Tabri, Leah Enns and Corrina Elliot. You all made my experience throughout graduate school fantastic, and I will always look back upon my memories of all of you fondly. You have not only been friends, also huge sources of support in my academic/clinical pursuits. I cannot forget to thank my family, very importantly the Prajzas, who took me into their home when I arrived back to Toronto and treated me as a son/brother, despite being a distant cousin. I also want to thank my father and mother who have been a constant source of love and encouragement over the years. Thanks also to my siblings, who have always been there when I needed to get away from the stresses and strains that came with this process. Finally I would like to thank Sarah Pumphery for her technical assistance with formatting.

TABLE OF CONTENTS

Page

LIST OF FIGURES

GENERAL INTRODUCTION **Page**

CHAPTER 1: The Effect of Stage of Lactation on the Response of Postpartum Rats to the Administration of Anorectic Peptides

CHAPTER 2: The Effect of Central Administration of a Ghrelin and a Ghrelin Antagonist on the Behavioural Responses of Postpartum Rats Across Lactation

LIST OF ABBREVIATIONS

ABC	avidin: biotinylated enzyme complex
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
AP	anteroposterior
AgRP	agouti-related peptide
α-MSH	alpha-melanocyte-stimulating hormone
ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus
AVP	arginine-vasopressin
BBB	blood brain barrier
BNST	bed nucleus of the stria terminalis
C	celsius
CART	cocaine and amphetamine regulated transcript
CCK	cholecystokinin
CNS	central nervous system
CORT	corticosterone
CRF	corticotropin-releasing factor
CRH	corticotropin-releasing hormone
CSF	cerebral spinal fluid
DA	dopamine
dH2O	distilled water
DMH	dorsomedial hypothalamus
fMRI	functional magnetic resonance imaging
FSH	follicle stimulating hormone
g	grams
GABA	gamma-aminobutyric acid

GHS-R	growth hormone secretagogue receptor
GHSR-1 a	growth hormone secretagogue receptor 1 a
GnRH	gonadotropin releasing hormone
hr	hour
HPA	hypothalamic-pituitary-adrenal axis
ICV	intracerebroventricular
IP	intraperitoneal
JAK/STAT	janus kinase/signal transducers and activators of transcription
LaH	lateral hypothalamus
LH	lutenizing hormone
LS	lateral septum
LSD	least significant difference
M	mean
MC	melanin-concentrating
MCH	melanin-concentrating hormone
ME	milk ejection
mg	milligram
MJ	megajoules
ml	millilitre
ML	medial-lateral
MPOA	medial preoptic area
mRNA	messenger ribonucleic acid
NAC	nucleus accumbans
NaOH	sodium hydroxide
NGS	normal goat serum
nmol	nanomole
NPY	neuropeptide Y
NTS	nucleus tractus solitaries

ObRa	short leptin receptor
ObRb	long leptin receptor
ObRe	soluble leptin receptor
OT	oxytocin
PBS	phosphate buffered saline
pg	picogram
ph	potential of hydrogen
POMC	proopiomelanocortin
pp	postpartum
PSTAT3	phosphorylated signal transducer and activator of transcript 3
PSTAT3-IR	phosphorylated signal transducer and activator of transcript 3
immoreactive	
PSTAT5	phosphorylated signal transducer and activator of transcript 5
PVN	paraventricular nucleus
PI3K	phosphoinositide 3-kinase
RT-PCR	reverse transcription polymerase chain reaction
SE	standard error
SEM	standard error mean
STAT3	signal transducer and activator of transcript 3
TNF-a	tumor necrosis factor alpha
µg	microgram
µl	microliter
µm	micrometers
V	ventral
VMH	ventromedial hypothalamus
VTA	ventral tegmental area

GENERAL INTRODUCTION

The notion of the adult mammalian brain being an unmodifiable and static functional division of the body was a misconception that dominated the emerging field of neuroscience in the 1500s and persisted for four centuries. The doctrine held that only in early development was the brain plastic, and afterwards changes in the central nervous system (CNS) went in the direction of deterioration; with the notions that brain injury or early deficits were permanent states that could not be reversed (Doidge, 2007). William James challenged this idea in 1890 when he first described plasticity as an alteration of the nervous system that corresponded with the formation of habits (Blanco, 2014). However it was not until the second half of the 20th century that this prevailing view of the static brain was overthrown with emerging evidence of brain re-organization occurring in later life (Rees, 2010). Today the concept is readily accepted and scientific literature is filled with examples of ways in which the brain exhibits this plasticity far beyond early developmental years. The purpose of this dissertation is to examine how the CNS changes in response to various physiological demands. Specifically, it reviews and investigates how the rat's energy balance system alters in order to address the energetic challenge of lactation.

Pregnancy and motherhood are crucial periods of nervous system malleability. When the adaptations in the maternal brain unfold smoothly without manipulation or interruption, they generally allow alterations in physiology and behaviour that ensure the survival of the progeny. The importance of this plasticity is reflected in the fact that its absence can result in withdrawal from the offspring, failure to provide essential aspects of care, or even, in certain species, infanticide (Fleming, Miceli, & Moretto, 1983).

Some of the neurochemical adaptations to motherhood begin during pregnancy, but in rodents the most dramatic behavioural changes occur close to the time of parturition. Aversion to pups, typical of nullparious rats, dissipates in the last days of pregnancy when nest building behaviour begins to emerge (Quinones-Jenab, Batel, Schlussman, Ho, & Kreek, 1997). At the time of birth, mothering begins with the licking of the pups as they emerge from the birth canal, along with retrieving and grouping them to the nest (Fleming, 1986). It is from this time until

approximately 16 to 18 days postnatally that the mother engages in the full repertoire of actions that are collectively known as maternal behaviour. These comprise of licking and grooming, retrieval of young to the nest and adopting a crouching/nursing posture over the litter (Fleming & Rosenblatt, 1974). Licking of the anogenital region is particularly important since pups are not capable of elimination without this stimulation during early life. In exchange the mother recoups water and electrolytes that she loses through nursing by consuming her offspring's urine (between 16-40ml/day) (Gubernik & Alberts, 1983; Gubernik & Alberts, 1985). Retrieval of pups to the nest area enables the dam to better protect her offspring from predators, regulate their temperature, and provide nourishment to the rapidly growing litter. Once grouped in the nest, the dam crouches over the litter ensuring the transfer of body heat to them since pups have limited thermoregulatory ability for some time (Farrell & Alberts, 2007). This also allows the pups to attach to the teats of the mother, thereby stimulating milk production and letdown.

Prior to milk letdown, pups need to attach to the nipples, as stimulation elicits the adoption of a reflexive high crouch by the dam, followed by milk letdown after approximately 10 minutes (Lincoln, Hill, & Wakerley, 1973; Lincoln & Wakerley, 1975; Russell, 1980). During nursing, the dam exhibits rigidly extended legs and holds an upright posture distinguished by a high dorsal arch and cessation of all other behaviour (Fleming & Rosenblatt, 1974).

Milk production begins prior to birth and serves as the primary source of nourishment for the pups during the first two weeks of life (Jeffers, 1935). Beginning at about 9 days of age, during the moments that they are apart from the dam, pups begin to sample solid food in their surroundings (Hall & Bryan, 1980). Interestingly, the time that the dam spends away from the litter during the first two weeks is driven by the temperature and size of the litter such that a greater number of pups and increased temperature in the nest leads to reduced contact between dam and offspring (Jans & Woodside, 1987). Thus, the amount of time that the dam spends nursing her young is not necessarily correlated with the energy demand of the litter. In fact, rat dams spend less time with their litters at the time of peak milk production (Day 14-15 postpartum (pp)) than they do early in lactation when the energy requirements of the litter are less (Cole, & Carlson, 1938; Grota & Ader, 1969; Ota & Yokoyama, 1967). Between 18 and 21

days of age, pups begin consuming solid food more regularly, while the duration of nursing bouts begin a steady decline starting on pp day 17 (Thiels, Alberts, & Cramer, 1990). This reduced reliance on the dam for nutrients continues until most nursing stops at approximately 5 weeks postpartum (Cramer, Thiels, & Alberts, 1990; Pfister, Cramer, & Blass, 1986). However in the case that pre-weanling pups are present along with rats beyond this age, nursing can continue up until day 70 pp (Pfister, Cramer, & Blass, 1986).

The pup directed behaviours described above are accompanied by other behaviours such as maternal aggression and hyperphagia that also serve to promote offspring survival. Aggression toward an intruder to the nest site is robust in the initial stages of lactation, and begins to decline at around two weeks pp (Mayer, Reisbick, Siegel, & Rosenblatt, 1987). It has been suggested that since virgin rats sensitized for maternal behaviour through repeated exposure to pups fail to exhibit maternal aggression (Erskine, Barfield, & Goldman, 1978), physiological changes unique to the parturient animal mediate this behaviour. Consistent with this idea, Mayer and colleagues (1990) found that administration of hormones in a pattern that mimics the profile of gestation elicits aggressive maternal behaviour in an intruder paradigm (Mayer, Monroy, & Rosenblatt, 1990).

Another highly salient change in the behaviour of the reproducing female are alterations in her feeding pattern. In order to support the energy expenditure required by the growth of her offspring, maternal food intake surges impressively beginning around the middle of gestation (up to 20 to 30% more than nulliparous rats), and increases even further across lactation, rising to up to 300% more than cycling rats by the time of peak milk production (Morrison, 1956; Strubbe & Gorissen, 1980).

The maintenance of higher than virgin levels of food intake in pp is driven, in part, by the energy drain of lactation, however eliminating milk production and letdown whilst maintaining suckling stimulation still results in moderate hyperphagia as observed in a study where galactophores were severed (Woodside, Abizaid, & Walker, 2000). Because suckling stimulation is sufficient to stimulate the continued release of lactation hormones (Chen & Smith, 2003), these data suggest a role for these hormones in the altered ingestive behaviour of lactating

rats. In contrast, although the rapid onset of pup-directed behaviours that occurs at parturition is mediated by hormones, its maintenance is largely hormonally independent and is maintained by a wide array of sensory signals that the mother receives from her offspring in the form of odours, touch, and vocalizations (Rosenblatt, 1967).

Pups are not just a source of relevant stimulation for their mothers; there is considerable evidence that they are also potent reinforcing stimuli. If separated from their young, dams will go to great lengths to approach and maintain contact with them. Mother rats will cross through electrified water, learn to navigate mazes, and even bar press hundreds of times over extended periods to gain proximity to their litter. They also show a conditioned place preference for an area previously associated with pup contact, highlighting the reinforcing properties the offspring have to their mother (Moss, 1924; Simons, 1924; Wilsoncroft, 1968). Dams even exhibit a preference for pups over cocaine within the first eight days pp (Mattson, Williams, Rosenblatt, & Morrell, 2001).

Some researchers have attempted to get to the heart (or more accurately, brain) of this highly motivated behaviour with the use of functional magnetic resonance imaging (fMRI). During nursing, ventral stimulation from a litter resulted in significant activity increase in the mesocorticolimbic system; areas implicated in mediating reward through the action of dopamine (DA). These areas have been shown to exhibit increased activity when virgin rats were given an ICV injection of cocaine. Interestingly a similar dose of cocaine in lactating rats led to a decrease in activity in the mesolimbic system, further supporting findings that ventral stimulation during nursing could be more reinforcing than cocaine administration early in the postnatal period (Ferris et al., 2005).

Numerous studies have demonstrated a role for DA acting in specific brain nuclei in modulating reinforcing aspects of mother litter interactions (see Numan & Stolzenberg, 2009). For example, microinfusions of a DA antagonist into the nucleus accumbens (NAC) result in dose dependent inhibition of pup retrieval (Keer & Stern, 1999), while a reduction in pup licking was seen when a DA antagonist was infused into the dorsal medial striatum (Keer & Stern, 1999). Lesions of the amygdala and the medial preoptic area (MPOA) serve to suppress operant

responding for pups, while destruction of the amygdala, the MPOA, and the NAC decrease crouching, licking and retrieving behaviours in the home cage (Lee, Clancy, & Fleming, 1999). Additional work has demonstrated that lesions of the ventral tegmental area (VTA) as well as destruction of connections between the MPOA and VTA had an impact on the oral aspects of care taking such as nest building and retrieval (Numan & Smith, 1984). Collectively, these studies highlight the importance that the MPOA, amygdala, and mesolimbic DA system has in regulating normative maternal behaviour (Numan, 2006).

Hormonal Changes Associated with Gestation, Parturition and Lactation

The behavioural and physiological adaptations to pregnancy and lactation are associated with alterations in circulating hormone levels that act both in the periphery and the brain to modify maternal physiology and behaviour. Vaginocervical stimulation received during mating elicits a twice daily surge in prolactin from the lactotrophs of the anterior pituitary that persists until approximately mid-gestation (Amenomori, Chen, & Meites, 1970). At this point, the placenta is sufficiently developed to secrete placental lactogen, which inhibits pituitary prolactin release by acting on prolactin receptors in both the pituitary and hypothalamus (Riddle, Bates, & Dykshorn, 1933; Tonkowicz, Roberston, & Voogt, 1983; Voogt, Soares, Robertson, & Arbogast, 1996). Placental lactogen production decreases just before birth and pituitary prolactin increases rapidly (Chan, Robertson, & Friesen, 1978). The bidaily surges of prolactin seen in the first half of pregnancy maintain the corpora lutea of gestation which secrete progesterone. Circulating progesterone levels show a significant rise during early gestation followed by a three day drop in serum concentration around gestation day 5, after which a dramatic increase in serum concentrations occur in the following 10 days, until they finally fall to below pre-pregnancy levels near birth (Pepe & Rothchild, 1974). In contrast, estradiol continues to maintain a low and steady concentration until it dramatically increases beginning at around gestation day 18 (Bridges, 1984). Progesterone, estradiol, prolactin, and glucocorticoids act in concert to encourage the growth of the mammary gland (Imagawa, Yang, Guzman, & Nandi, 1994), and to prime the dam for pup-directed behaviour. The decline in progesterone just prior to birth is necessary both for the initiation of parturition and to stimulate the onset of maternal behaviour.

Indeed, Bridges was able to induce maternal behaviour in virgin animals that are typically neophobic to newborns by administering a regimen of hormonal implants comprising estrogen and progesterone, followed by progesterone removal (Bridges, 1984). Subsequently he and his colleagues showed that this effect was dependent on the ability of this steroid regiment to induce an increase in prolactin release (Bridges 1985, also see Bridges, 2015; Mann & Bridges, 2001 for review).

Lactation is also associated with a unique hormonal profile. After a rapid decline just prior to parturition, circulating progesterone levels rise slowly to become significantly higher than nulliparous rats after peaking at approximately day 12 pp (Grota & Eik-Nes, 1967),and gradually declines to levels observed in virgins by day 20 pp (Woodside, 1991). On the other hand, basal serum corticosterone (CORT) concentrations are higher in lactating than in non-lactating females throughout the pp period (Fisher, Patchev, Hellbach, Hassan, & Almeida, 1995; Lightman, 1992). Estrogen levels soon after parturition are very low but slowly increase until reaching concentrations typically observed during estrus in non-lactating rats and from this time almost triples in concentration three weeks after birth (Smith & Neill, 1977).

Prolactin levels also decline after parturition but, in keeping with its role as the major lactogenic hormone in the rat (Nagasawa & Yanai, 1972), are rapidly increased after the onset of pup suckling, which induces a pulsatile pattern of 1 pulse/6 to 15 minutes (Grosvenor, Shyr, Goodman, & Mena, 1986; Higuchi et al., 1983). Removal of offspring from the mother results in both a rapid decrease in prolactin concentrations and suppression of milk production (Grosvenor, Mena, & Whitworth, 1979), while rats nursing large litters tend to have higher circulating prolactin concentrations (Mattheij, Gruisen, & Swarts, 1979; Ota & Yokayama, 1967). Although prolactin secretion is primarily elicited by nipple attachment, ventral stimulation provided by the litter has been found to stimulate prolactin release even when the dam's nipples had been excised (Moltz, Levin, & Leon, 1969). Furthermore, studies have found that odour and ultrasonic vocalizations from pups facilitates prolactin secretion in early and mid-lactation, although this effect is inhibitory on day 21 pp (Grosvenor, Mena, & Whitworth, 1977). Prolactin release is typically inhibited by DA secreted from tuberoinfundibular DA neurons in the arcuate nucleus

(ARC) that are themselves stimulated by prolactin. This negative feedback system is altered during lactation so that prolactin is no longer as effective at stimulating DA release onto the lactotropes of the anterior pituitary (Anderson et al., 2006; Anderson et al., 2006). Prolactin levels decrease towards weaning and can be only be partially restored by replacing older pups with a younger litter suggesting that the decline in prolactin is a function of both changes in suckling stimulation from the pups and alterations in the mother.

In addition to its role in milk production, prolactin has also been implicated in multiple aspects of maternal behaviour and care. As noted above, it plays an essential role in stimulating the onset of maternal behaviour and as described below it contributes to both the hormonal and behavioural hyporesponsiveness to stress seen in lactating rats. In addition, prolactin has been implicated in the hyperphagia of lactation.

Circulating levels of the neuropeptides oxytocin (OT) and arginine vasopressin also rise at the end of pregnancy. Centrally, OT is produced within the magnocellular cells of the hypothalamic paraventricular (PVN) and supraoptic nuclei. These cells then project to the posterior pituitary and the hormone is subsequently secreted into the blood stream (Higuchi et al., 1983). This system shows considerable plasticity. Around the time of birth the astrocytes that typically separate the cell bodies of the magnocellular neurons, retract their processes thereby allowing increased contact between these cells. This structural change ultimately enhances synaptic and extrasynaptic communication between magnocellular neurons and promotes both the birth process and the delivery of the mother's milk to her young (Oliet & Bonfardin, 2010). These changes seem to be reversible since intercellular contact is restored to virgin levels following the nursing period (Thedosis, Chapman, & Montagnese, 1986). The release of OT during parturition stimulates contraction of the uterus, which helps expel the pups from the birth canal. OT also binds to its receptors on myoepithelial cells within the mammary gland, stimulating alveoli contraction. This serves to release milk into the ducts and its subsequent expulsion from the nipple (Bealer, Armstrong, & Crowley, 2010; Brunton & Russell, 2008; Uvnas-Moberg & Eriksson, 1996).

Like prolactin, OT release is facilitated by suckling stimulation which is crucial in

eliciting the bolus release of OT required for milk letdown (Freund-Mercier & Richard, 1984; Higuchi, Uchide, Honda, & Negoro, 1985; Meyer, Freund-Mercier, Guerne, & Richard, 1987). Studies examining the response of OT neurons during nursing have revealed that the suckling induced release of OT and the milk ejection (ME) that follows occurs within 10 - 30 minutes of teat stimulation. Subsequent MEs occur at intervals between 3.6 - 6.6 minutes after the initial, with gaps in delivery being driven by a mammary gland refractory period (Jans & Woodside, 1987; Lincoln & Paisley, 1982). Any disturbance of mother and litter can disrupt the timing of MEs (Lincoln & Paisley, 1982).

As with prolactin, not only does OT play a role in delivery and the feeding of the dam's offspring, but it is also involved in other aspects of maternal behaviour. For example, infusion of OT and vasopressin into the ventricles has been found to encourage the rapid development of a full repertoire of maternal behaviour in virgins when primed with estrogen (Pedersen, Ascher, Monroe, & Prange, 1982). Destroying the PVN during mid-pregnancy disrupts the onset of maternal behaviour, although once pup care is established, such lesions are without effect (Insel & Harbaugh, 1989). Furthermore an OT antagonist infused into the shell portion of the NAC has been shown to delay the onset of maternal behaviour in dams following a period of isolation from pups (D'Cunha, King, Fleming, & Levy, 2011). Finally work by Champagne and her colleagues (2001) revealed that higher OT receptor levels in the hypothalamus of dams correlated with higher licking of their offspring (Champagne, Diorio, Sharma, & Meaney, 2001).

OT and vasopressin have also been implicated in maternal aggression. One study found increased binding of OT in the MPOA and the bed nucleus of the stria terminalis (BNST) was strongly and positively correlated with aggressiveness around the time of birth, and increased aggression during the first week of lactation was related to its action on the lateral septum (LS) (Bosch et al., 2010). Aggressive behaviour was also strongly associated with vasopressin binding in both the central amygdala and the PVN at parturition (Caughey et al., 2011).

Changes in the hormonal status of lactating rats are not limited to hormones implicated in maternal behaviour, milk production and delivery. Baseline CORT has been found to be chronically increased in lactating rats (as a result of suckling); while it's natural daily rhythm of

secretion typically is absent (Stern & Voogt, 1974; Tachi, Tomogane, & Yokoyama, 1981). In contrast, however, there is a down regulation in the ability of stressors (e.g. noise stress, predator odours, intruder paradigms, forced swim test) (Dechamps, Woodside, & Walker, 2003; Stern, Goldman, & Levine, 1973; Walker, Mitchell, & Woodside, 1995; Windle, Shanks, Lightman, & Ingram, 1997) to induce a hormonal response in lactating rats. Typically, the PVN of the hypothalamus produces and releases corticotropin-releasing hormone (CRH) and arginine-vasopression (AVP) in response to stress. These act on the anterior pituitary to stimulate the release of adrenocorticotropic hormone (ACTH) into the general circulation with CRH being a stronger ACTH secretagogue than AVP (see Rivier & Rivest, 1991). ACTH in turn acts at the adrenal cortex to release CORT. This cascade of hormonal release allows the animal to react physiologically and behaviourally to threats in its environment. CORT eventually feeds back to inhibit further release of CRH and ACTH. It has been proposed that these effects are the result of both a decrease in stimulatory inputs to the stress axis, and reductions in corticotripin-releasing factor (CRF) mRNA expression accompanied by increases vasopressin mRNA expression in the PVN resulting in a decrease in reduction in ability to stimulate ACTH release (see Rivier & Rivest, 1991). Interestingly, in early lactation the presence of the litter has a strong impact in the hormonal response to some stressors suggesting that stress appraisal plays a key role in at least some aspects of stress hyporesponsiveness at this stage of lactation (Walker et al., 2004).

The behavioural as well as the hormonal responses to stressors are changed in lactating rats. Perhaps one of the most striking differences between the virgin female rat and that of a rat that has experienced motherhood is their response to neonates. When presented with foster pups, nulliparous rats generally react initially with avoidance behaviours. For example, virgin rats have been observed to bury foster young in nesting material, or stay away from the cage area in which they have been placed, in some cases they will kill and even cannibalize these newborn conspecifics (Fleming & Luebke, 1981). Furthermore, when compared to virgins, dams exhibit less fear in situations that typically provoke timid behaviour, for example, by spending more time and activity in the center of an open field and reduced latency to emerge into a lit arena (Fleming & Luebke, 1981).

There are a number of hypotheses about the functional significance of the reduction in both the hormonal and behavioural responses to stressors during lactation. Work by Klampfl (2013) revealed that CRF placed into the PVN of lactating rats reduced maternal behaviours and aggressiveness, suggesting that hypothalamic-pituitary-adrenal (HPA) axis hypo-responsiveness during this reproductive state promotes the care and defence of the pups (Klampfl, Neumann, & Bosch, 2013). A similar role in the potentiation of maternal aggression has been suggested for the decrease in anxiety behaviour (see Gammie, 2005). It has also been suggested that the reduction in the hormonal response to stress serves both to limit the exposure of the pups to rapid increases in glucocorticoids which would reach them via the milk, and to conserve energy for use in lactation by avoiding the effects of high levels of these hormones on glucose metabolism (See Lightman, 1992).

Another change in hormonal state that serves to conserve energy for the current litter is a change in activity of the reproductive axis. Some rodents, including rats, show a pp estrus a few hours after birth during which conception can occur (Connor & Davis, 1980). If successful mating occurs then implantation of the ensuing blastocysts are delayed such that the maximal growth rate of the litter in utero does not coincide with peak milk production. If mating does not occur, the dam enters into a period of lactational infertility (Connor & Davis, 1980). The unique hormonal profile of this reproductive state ensures the suppression of ovarian follicles and a reduction of estradiol leading to a prolonged and continual state of diestrus, the duration of which depends on litter size and energy availability (Taya & Greenwald, 1982; Woodside, 1991; Woodside & Popeski, 1999). The primary cause of this state is a decrease in gonadotropin releasing hormone (GnRH) release following birth (Smith & Neill, 1977) thereby reducing the frequency and amplitude of luteinizing hormone (LH) pulses. Follicle stimulating hormone (FSH) concentrations are also transiently reduced but normal levels are restored within a few days pp (McNeilly, 1980). A major influence on GnRH release is kisspeptin (Liu, Brown, Herbison, & Grattan, 2014) and both kisspeptin mRNA expression in the ARC, AVPV as well as the response of kisspeptin positive neurons in the AVPV to electrical stimulation are changed in lactation (Ladyman & Woodside, 2014; Liu, et al., 2014). The factors influencing the

anovulatory state of lactation change as a function of stage of lactation such that in early pp suckling stimulation, independent of its effect on prolactin, suppresses the reproductive axis whereas later in lactation the high levels of prolactin induced by suckling inhibit LH and FSH (Smith & Neill, 1977). Removing the suckling stimulus returns the mother to a fertile state within two days (Hansen, Sodersten, & Eneroth, 1983).

Mechanisms of Energy Balance

Like all mammalian young, rat pups depend upon milk from their mothers for survival. The energy required to meet these demands comes from a number of sources: females gain fat during pregnancy that subsequently is used to provide energy for milk production (Vernon & Pond, 1997), there is a decrease in energy expenditure in brown fat thermogenesis (Trayhurn, 1989) and there is an increase in the absorptive capacity of the gut (Cripps & Williams, 1975). Most obviously, however, it also comes from dramatic changes in maternal ingestive behaviour. Lactating rats increase their food intake by as much as 300% over cycling rats, with this being dependant on the number of pups nursed. They also change their patterns of diet selection and when they eat (Dial & Avery, 1991; Richter, 1938; Woodside, 2007). For example, rats typically show intense feeding immediately after lights out and just prior to lights on with intermittent bouts in between (Ter Harr, 1972). This typical feeding rhythm breaks down during lactation so that ingestion continues after lights on (Strubbe & Gorissen, 1980). These behavioural changes suggest that the mechanisms controlling energy balance are modified in lactation.

Our understanding of how brain mechanisms drive food intake is an ongoing endeavour and a brief review of what is currently understood is important as it will provide background and context needed for the dissertation studies that follow. The hypothalamus has long been acknowledged as one of the most important brain areas involved in the control of ingestive behaviour. Within this region, there are several nuclei that have been implicated in food consumption, including the ARC, ventromedial hypothalamus (VMH), PVN, lateral hypothalamus (LaH), and dorsomedial hypothalamus (DMH) (see Grill & Kaplan, 2002). Direct connections between the hypothalamus and other nuclei (such as the NTS) have also been noted (Zheng, Patterson, Pfifer, & Berthoud, 2005).

The central control of feeding begins with the transmission of information from the digestive system to the CNS by way of the bloodstream via hormonal/peptide signals such as cholecystokinin (CCK), peptide YY, leptin, ghrelin, insulin, oxytomodulin, glucagon-like peptide-1, and pancreatic polypeptide (for review see Murphy & Bloom, 2006). These signals either act peripherally upon the nodose ganglion of the vagus nerve which then terminates centrally within the nucleus tractus solitaries (NTS), or more directly by affecting areas such as the area postrema which is outside the blood brain barrier (BBB) (Buyse et al., 2001), or finally by crossing the BBB in areas where it is weak, such as the ARC (Ciofi, 2011). There is also evidence that some peripheral signals of energy balance gain access to the brain via active transport mechanisms although the process underlying this are still not clear (Banks, 2012).

Peripheral Signals of Energy Balance

Among the peripheral signals of energy balance leptin, insulin and ghrelin have been the most studied in the context of lactation and will be the focus here. Leptin is a product of both white and brown fat tissues, being released into the blood stream at levels proportionate to adiposity (Friedman & Halaas, 1998). There are six leptin receptor subtypes that are found within several hypothalamic sites that include the PVN, VMH, DMH, LaH and the ARC nuclei (Mercer et al., 1996). Of these six isoforms, the one longform subtype ObRb, being coupled to the JAK/STAT pathway has been identified as being most critical in leptin's effects on energy balance. The function of the remaining shorter isoforms remain unknown or have been observed to act as a soluble circulating receptor (ObRe) or possibly assist leptin transport across the BBB (ObRa) (Friedman & Halaas, 1998; Golden, Maccagnan, & Partridge, 1997). It has been proposed that leptin is able to cross into the brain from the periphery via an active transport mechanism allowing entry through the BBB (Banks, DiPalma, & Farrell, 1999). In normal weight animals and humans, exogenous leptin administration has been found to reduce food intake and body weight (Campfield, Smith, Guisez, Devos, & Burn, 1995; Friedman & Halaas, 1998; Halaas et al., 1995; Pelleymounter et al., 1995).

Insulin is another peripheral signal that plays an important role in the regulation of food intake. This hormone is generated by pancreatic Beta-cells and much like leptin, its levels within

an animals circulation correlate with the amount of fat to signal the amount of glucose available in the body (Schwartz, Woods, Porte, Seeley, & Baskin, 2000). In the periphery, insulin inhibits glucose production and release by acting on the liver while encouraging uptake. It is also similar to leptin in that central administration serves to reduce feeding and weight by acting on the insulin receptor phosphoinositide 3-kinase (PI3K) pathway (Zhao et al., 2000).

Ghrelin's discovery was aided by the identification of the receptor known as the growth hormone secretagogue receptor (GHS-R) which is abundant in the pituitary, hippocampus, ARC, VMH, and infundibular hypothalamus. From this work it was reasoned that there was an endogenous hormone awaiting discovery (Howard et al., 1996). As predicted, the 28 amino acid peptide which attached to this receptor was extracted from the stomach of the rat and humans (Kojima et al., 1999). These authors also identified expression of its receptor in the intestine as well as adipose tissue and from this, correctly assumed that it has a significant role in the regulation of metabolism. The discovery of this hormone aroused much interest from the scientific community regarding its function. Researchers found that both daily peripheral and ICV administration of ghrelin to various rodents stimulated hyperphagia and resulted in an increase in adiposity (Tschop, Smiley, & Heiman, 2000). Evidence suggests that ghrelin, like leptin, is transported across the BBB into the CNS by way of both saturable and non-saturable mechanisms (Banks, Tschop, Robinson, & Heiman, 2002).

Neuropeptides Implicated in the Control of Energy Balance

Although numerous neuropeptides have been found to be involved in feeding, there are several that have emerged as major players in the central control of food intake. The appetite inducing peptides include melanin-concentrating (MC) hormone (MCH), orexins, neuropeptide Y (NPY), ghrelin, and agouti-related peptide (AgRP). There are also appetite suppressing neuropeptides that include cocaine and amphetamine regulated transcript (CART), pro-opiomelanocortin (POMC), alpha-melanocyte-stimulating hormone (α-MSH), and leptin (see Williams & Elmquist, 2012).

Among the orexigenic neuropeptides, NPY stands as one of the most crucial and potent stimulators of food intake (Tatemoto, Carlquist, & Mutt, 1982). NPY is found to have wide

distribution throughout the rat brain, with a particularly large number of cells located in the ARC, peri and paraventricular nuclei, as well as the NTS (Adrian et al., 1983). Levine and Morley (1984) were among the first groups of researchers to report that endogenous NPY administered into the ventricle of male rats induced substantial food intake in these subjects. Subsequent research led to the discovery of the receptors to which NPY attaches (with Y1 and Y5 receptors being important for food intake in the rat), leading to a significant increase in feeding, and more specifically a preference for carbohydrates (Fetissov, Kopp, & Hokfelt, 2004; Polidori, Ciccocioppo, Regoli, & Massi, 2000; Welch, Grace, Billington, & Levine, 1994). Much of the research above has been conducted in male rats, but work from our own lab suggests that chronic stimulation of a Y5 receptor agonist also increases feeding in female rats. In contrast, administration of a Y2 receptor agonist specifically decreases carbohydrate and protein consumption in male rats (Leibowitz & Alexander, 1991) but fails to reduce feeding in similarly treated cycling female rats (Toufexis, Kyriazis, & Woodside, 2002).

AgRP colocalizes with NPY in the ARC and functions as an endogenous antagonist at the MC1r, MC2r, MC3r, MC4r, MC5r receptors. Its primary actions on the regulation of food intake and body weight are mediated through the Mc3r and Mc4r, however. Messenger ribonucleic acid (mRNA) for AgRP has been found in the hypothalamus, mainly within the median eminence and ARC where it colocalizes with NPY (Cone, 2005; Kucera, Bortner, & Rosenberg, 1996; Ollmann et al., 1997). Central administration of this neuropeptide increases feeding and reduces energy expenditure (Goto et al., 2003).

The orexins (A and B), as their name implies, are two neuropeptides that have been found to promote feeding in a dose dependent fashion when infused into the ventricle of male rats (Sakurai et al., 1998). Orexin A contains 33 amino acids while orexin B has only 28, with both sharing 46% of their sequence and being structurally distinct (Sakurai, 1999). This neuropeptide is derived from prepro-orexin which is a 130-residue polypeptide (Sakurai, 1999). Both these peptides bind to G protein-coupled cell surface receptors (OX1R and OX2R; with orexin B binding primarily to OX2R). The mRNA for these peptides are localized mainly in the VMH for OX1R and in the PVN for OX2R (Trivedi, Yu, MacNeil, Van der Ploeg, & Guan, 1998).

Neurons found to express the orexins have been located in the LaH and the posterior hypothalamic area, with projections being received by the PVN and ARC (Sakurai, 1999).

α-MSH is produced by cleavage of POMC, which is present within neuronal populations of the ARC (Gantz et al., 1993). Similar to AgRP, α-MSH is an endogenous neuropeptide that acts upon the MC receptors (MC1r, MC3r, MC4r, and MC5r). However unlike AgRP, it acts as an agonist at these sites and serves to reduce feeding activity in rats following ICV administration (Ludwig et al., 1998).

Interaction between Peripheral Signals of Energy Balance and Neuropeptides

Studies have revealed the ARC to be the gateway into the hypothalamus as this nucleus is the primary target of peripheral signals within the neural circuit that mediates energy balance and food intake. Located in the ARC are two clusters of neurons that project to other nuclei within the hypothalamus. One of these groups releases both NPY and AgRP which act to stimulate food intake, while the other neuronal population expresses CART and POMC and release α-MSH to suppress feeding. These two cell populations exert mutually inhibitory influences such that activation of NPY/AgrP resulting in increased gamma-aminobutyric acid (GABA) input to POMC neurons, with their activation resulting in opioid-mediated inhibition of NPY/AgrP neurons (Abizaid, Gao, Horvath, 2006). When leptin or insulin bind to the receptors located on the NPY/AgRP cluster of neurons, they inhibit the release of these appetite stimulating neuropeptides. Conversely, when leptin binds to POMC neurons, it increases phosphorylation of signal transducer and activator of transcript 3 (PSTAT3) and the subsequent release of α-MSH, which suppresses feeding by acting on the MC4 receptor within the PVN, MPOA, and DMH (Kim et al., 2000). Insulin works through the same pathway but binds to an insulin receptor and then an insulin receptor substrate to produce similar effects (Niswender & Schwartz, 2003). In the absence of leptin or insulin, the orexigenic peptides are released and promote feeding while the POMC neurons are inhibited and α-MSH expression and actions are reduced and prevented from acting on the MC system (Schwartz, Woods, Porte, Seeley, & Baskin, 2000). The neuronal clusters that release NPY/AgRP also express growth hormone secretagogue receptor 1 a (GHSR-1a), to which ghrelin binds. The postsynaptic attachment of ghrelin to these receptors promotes

the release of orexigenic peptides to encourage food intake. In a similar fashion, ghrelin inhibits the activity of the CART/POMC neurons within the ARC via GABA released from NPY and AgRP neurons to increase feeding (Cowley et al., 2003; Fry & Ferguson, 2010).

The neurons in the ARC have projections that extend within the hypothalamus to the VMH, PVN, DMH and the LaH (Bouret, Draper, & Simerly, 2004). The VMH has connections to the DMH and the DMH neurons project to the PVN. The communications that occur between these nuclei to control food intake are mediated by neuropeptides such as NPY, AgRP, and α-MSH (Sahu, 2011). For example, all three of these neuronal populations innervate orexin neurons located within the LaH and thus these are likely regulated by activity within the ARC (Elias et al., 1998) The hypothalamus also projects to many other brain regions (e.g. the brainstem) to communicate information related to energy balance and feeding to other brain nuclei (Blevins & Baskin, 2010).

Orexigenic and Anorexigenic Neuropeptides during the Peripartum Period

As might be expected there are changes in both peripheral signals of energy balance and the neural circuitry modulating it as a function of reproductive state (see Figure 1.1). What remains elusive is how these various hormones and neuropeptides interact both centrally and peripherally beginning in pregnancy to produce a degree of food consumption that is unparalleled to any other time in the life of a rat and that ceases soon after the termination of lactation.

The increase in food intake seen during gestation is accompanied by changes in both orexigenic and anorectic peptides. Serum levels of AgRP were found to be increased in pregnant rats compared to cycling from days 13 to after parturition and that this was accompanied by an increase in AgRP in whole hypothalamus (Szczepeankiewicz et al, 2009). Similarly, Roach et al reported a 51% increase in expression of AgRP gene protein in the hypothalamus on day 19 of gestation (Rocha et al, 2003). However Ladyman and her colleagues found no difference between pregnant and cycling rats in AgRP mRNA, levels specifically within the ARC (Ladyman, Tups, Augustine, Swahn-Azavedo, Kokay, & Grattan, 2009). During mid to late pregnancy serum levels of orexin-A have been found to be double or more than those of virgin

rats during mid to late gestation (Sun, Tian, Yao, & Higuchi, 2006). Prepro-orexin mRNA in the entire hypothalamus was found to be higher on days 10 and 20 of pregnancy than in cycling rats, although as with AgRP this effect does not seem to be apparent in all hypothalamic nuclei because while less prepro-orexin mRNA expression is seen in the lateral hypothalamus on day 18 of gestation than in cycling controls (Garcia et al, 2003) The increase in prepro-orexin mRNA expression observed by some authors does not necessarily correspond with an increase in protein however. Lower levels of orexin-A immunoreactivity were observed on days 16 and 21 of pregnancy (Kanenishi et al, 2004; Sun et al, 2006). Lateral hypothalamic levels of MCH mRNA expression on day 18 of pregnancy were found to be significantly than in cycling rats (Garcia et al, 2003). As with other orexigenics, ghrelin is also affected by reproductive state. During pregnancy, serum levels of ghrelin have been found to increase in concentration on day 13 and 18, followed by a drop to conception levels at parturition. The GHSR 1a mRNA levels follow a similar pattern in the hypothalamus (Szczepankiewicz, Skrzypski, Pruszynska-Oszmalek, Zimmerman, Andralojc, Kaczmarek, Wojciechowicz, Sassek, Nowak, 2010). Others have found there to be no difference between plasma levels of ghrelin during across gestation and proestrus controls (Taylor, Patterson, Ghatei, Bloom, & Wilson, 2009). The effect of ghrelin plasma levels was compared in food restricted and ad libitum fed during days 8, 12, 16, 19 and 21 of gestation and in cycling rats. Plasma levels were not significantly different between the groups with the exception of days 16 and 21, when they were notably higher compared to the ad lib pregnant rats. This corresponded with an increase in gastric ghrelin mRNA expression (Gualillo et al, 2002).

Studies of the anorectic peptide CART across reproductive states have revealed that its expression is increased in the anteroventral periventricular nucleus on gestation day 19 with the hypothesis that it could be related encouraging the emergence of maternal care (Valera, Cavalcante, Elias, & Felicio, 2006). Alpha-MSH levels also fluctuate according to reproductive state. Immunoreactivity was found to be lower in the mediobasal hypothalamus during late pregnancy, and further decreased during lactation (Khorram, DePalatis, & McCann, 1984). Within the arcuate nucleus, POMC mRNA content was found to decrease on the final day of

gestation (Mann, Rubin, & Bridges, 1997). Trujillo and colleagues also found a reduction in POMC mRNA when the hypothalamus was analyzed as a whole (Trujillo, Spuch, Carro, & Senaris, 2011). In contrast, POMC mRNA was found not to differ between pregnant and virgin rats on days 10, 16, and 21 of gestation (Douglas, Bicknell, Leng, Russell, & Meddle, 2002). A later study by Ladyman replicated these results and further investigated how administering alpha-MSH would influence food intake during pregnancy. The authors discovered that while this hormone was able to reduce food intake in dioestrus females when centrally administered but did not do the same in rats that were in their 14th day of gestation (Ladyman, Tups, Augustine, Swahn-Azavedo, Kokay, & Grattan, 2009). Leptin is also affected by reproductive state. Over the course of gestation, serum concentration levels of this neuropeptide steadily rise from approximately mid until day 20 of pregnancy, when they drop to levels found in virgins on the day of parturition (Amico, Thomas, Crowley, & Burmeister, 1998; Terada, Yamakawa, Sugaya, & Toyoda, 1998; Kawai et al, 1997; Chien et al., 1997; Herrera, Lasuncion, Huerta, Martin-Hidalgo, 2000). Suspecting the possibility of leptin resistance occurring during pregnancy, Garcia (2000) examined hypothalamic ObRb receptor mRNA on gestation day 18 and discovered concentrations were lower than that of non-lactating rats while other short forms of the Ob receptor were unchanged (Borgan et al 2000). Given its potent ability to stimulate feeding, increased levels of NPY mRNA expression compared to those of virgin rats are observed in the hypothalamus during gestation and the pp period. This increase is first seen on gestation day 13, is still apparent on gestation day 18 (Trujillo, Spuch, Carro, & Senaris, 2011) but appear to dissipate by gestation day 19; however this might be related to varying methodologies (In situ hybridization vs. reverse transcription polymerase chain reaction (RT-PCR) (Rocha, Bing, Williams, & Puerta, 2003).

Following parturition, peptides and their associated receptors that are implicated in the hyperphagia of pregnancy also altered. During NPY mRNA increases have been found in the median eminence as well as in different sub-divisions of the ARC on day 10 pp (Smith, 1993) and day 14 -15 pp NPY mRNA in the ARC as well as the DMH (Garcia et al., 2003; Suzuki et al., 2014) but were not observed on day 5 pp (Suzuki et al., 2014). At least part of the increased

expression of NPY mRNA depends on suckling stimulation rather than loss of energy to milk. Li and her colleagues used an acute re-suckling paradigm, and showed that after 48 hours of mother- litter separation, three hours of suckling was enough to significantly increase NPY mRNA in hypothalamic nuclei during mid-lactation (Li, Chen, & Smith, 1998), even though no milk was produced. Using this same paradigm, the team was able to establish that blocking milk delivery with bromocriptine did not reduce NPY mRNA expression in the ARC suggesting that the neural stimulation produced by nursing rather than energy drain was responsible for the observed increases in this area (Li, Chen, & Smith, 1999).

Alterations in AgRP mRNA expression have also been observed in the ARC during lactation. When compared to virgin females, AgRP mRNA was found to significantly increase within the ARC following 12 to 13 days of lactation (Ladyman et al., 2009; Chen, Li, Haskell-Luevano, & Smith, 1999). Subsequent research by Chen (1999) demonstrated significant increases in AgRP mRNA in this same hypothalamic region during both early (day 5 pp) and later lactation (day 15 pp; Suzuki et al., 2014).

Given the involvement of the orexins in the control of food intake, these have also been investigated during the nursing period. One group examined prepro-orexin, orexin 1 as well as orexin 2 receptor mRNA throughout lactation and reported that LaH prepro-orexin mRNA content increased on the first day of lactation (Wang, Murata, Narita, Honda, & Higuchi, 2003). However, Garcia et al. (2003) reported a decrease in the mRNA expression for this peptide in the LaH on day 14 pp compared to cycling controls. Brogan, Grove & Smith (2000) found no difference in orexin-B concentrations in rats in mid-lactation (day 10 pp) although they did report increases in concentrations of this peptide in rats food restricted for 48h, suggesting that levels of this peptide are sensitive to energetic challenge (Brogan, Grove, & Smith, 2000; Cai et al., 2001). Together these studies suggest that the orexins may not have as strong of a role in the hyperphagia of lactation. However Sun (2003) and colleagues reported an increased in orexin A immunoreactivity mRNA expression within the hypothalamus during days 11 and 12 pp (Sun, Narita, Murata, Honda, & Higuchi, 2003).

Changes in expression levels of anorexigenic neuropeptides have also been observed in

lactation. POMC mRNA expression in the ARC is down regulated during early (day 5 pp) and late lactation (days 12 and 15 pp) (Mann, Rubin, & Bridges, 1997; Smith, 1993; Suzuki et al., 2014). This effect seems to be limited to lactation because Ladyman (2009) and her team has reported no difference in the expression of POMC mRNA between non-lactating and pregnant rats. Interestingly central administration of α-MSH fails to reduce feeding on gestation day 14 (Ladyman et al., 2009).

Less research has focused on the actions of melanin-concentrating hormone (MCH) during lactation. In an examination of MCH mRNA in pp rats on days 11 to 12 in the zona incerta and LaH was notably upregulated (Sun et al., 2004). Lactating rats have also been found to display increased MCH mRNA content within the MPOA, PVN and the periventricular preoptic nucleus between days 8 and 21 pp (Knollema, Brown, Vale, & Sawchenko, 1992), but this change has been linked to the effects of this hormone on GnRH release rather than on food intake.

Changes in levels of peripheral signals of energy balance as well as the appropriate receptors within the hypothalamus have also been studied across lactation often with mixed results. For instance, one group examining plasma ghrelin concentrations during lactation found no difference in levels between nursing rats sampled on days 1, 5, 10, 15, 20 pp or five days after weaning and those seen in proestrus rats (Taylor, Patterson, Ghatei, Bloom, & Wilson, 2009). In contrast, Shibata and colleagues (2004) found lower circulating concentrations of ghrelin on days 3 and 8 pp compared to cycling female rats. Abizaid, Schiavo, & Diano (2008) also compared plasma concentrations of ghrelin between lactating rats that had their pups removed two days prior to sacrifice and a group that had their pups removed on the first day pp and found no difference. However, acute removal of the litter did result in an increase in circulating ghrelin levels. This group also found significant increases in GHSR-1a mRNA within the hypothalamus, and pituitary of both lactating rats and females whose litter had been removed 48h prior compared to females whose litter was removed on day 1 pp (Abizaid et al., 2008). Preliminary findings from our own lab found that GHS-R mRNA was increased in the ARC during lactation compared to non-lactating rats, together with a trend for the same effect in the PVN (Budin,

Wellman, Abizaid, & Woodside, 2010). Finally the effects of ghrelin on the dam seem to influence their offspring; with one research team observing that treating lactating dams with daily subcutaneous ghrelin from parturition to day 8 pp increased milk production and resulted in heavier pups (Nakahara et al., 2003). Ghrelin also serves to promote increases in plasma levels of growth hormone, which stimulates milk production (Flint, Tonner, Beattie, & Panton, 1992).

Not unexpectedly, circulating levels of leptin also change as a function of reproductive state. Consistent with the increase in adiposity across pregnancy (Vernon & Pond, 1997), serum concentrations of leptin also rise steadily from approximately mid pregnancy until gestation day 20 and then drop to levels found in virgins on the day of parturition (Amico, Thomas, Crowley, & Burmeister, 1998; Chien et al., 1997; Herrera, Lasuncion, Huerta, & Martin-Hidalgo, 2000; Kawai et al., 1997; Terada, Yamakawa, Sugaya, & Toyoda, 1998). Suspecting the possibility of leptin resistance occurring during gestation, Garcia (2000) examined hypothalamic ObRb receptor mRNA on gestation day 18 and discovered concentrations were lower than those of non-lactating rats while short forms of the Ob receptor were unchanged.

There is a marked drop in adipose tissue from gestation to lactation, with fat pad weights being lower than those of cycling females, thereby reflecting the use of body fat by the dam as an energy source during nursing (Denis, Williams, & Vernon, 2003; Johnstone & Higuchi, 2001; Woodside, Abizaid, & Walker, 2000). Thus it is not surprising that leptin levels tend to decrease from late gestation to lactation (Amico, Thomas, Crowley, & Burmeister, 1998). Woodside and her colleagues (2000) found that from days 5 to 25 pp, serum concentrations of leptin remain lower than those of virgin controls, with similar results obtained by Johnstone & Higuchi (2001).

Interestingly, despite the difference in leptin blood serum concentrations between lactating and non-lactating rats, cerebral spinal fluid (CSF) levels are the similar between cycling rats and rats at mid-lactation, consistent with the idea of a saturable transport mechanism conveying leptin from the periphery to the brain (Suzuki et al., 2010). As in pregnancy, the expression of ObRb mRNA has been found to be reduced in the VMH during lactation whereas changes were not apparent in other regions of the hypothalamus (Brogan, Grove, & Smith, 2000).

Changes in Response to Exogenous Peripheral and Central Administration of Modulators of Energy Balance during Lactation

In addition to the studies measuring circulating signals of energy balance and expression of their receptors together with levels of expression of orexigenic and anorexigenic neuropeptides as a function of reproductive state described above, there have also been investigations of how the response to these factors changes across pregnancy and lactation. For example, Ladyman and her colleagues found that central administration of leptin failed to reduce food intake in food restricted gestation day 14 rats but did so in rats tested on gestation day 7 and in non-pregnant rats. The lack of this behavioural effect was associated with reduced PSTAT3 in the VMH but not the ARC, consistent with a decrease in ObRb in that area (Ladyman & Grattan, 2004, Ladyman & Grattan, 2005). Interestingly in subsequent studies, this group showed that the leptin resistance shown by pregnant rats in the third trimester of pregnancy was a function of the chronically high prolactin receptor activation at this time (Augustine & Grattan, 2008) consistent with the ability of tonically high prolactin to induce a state of leptin resistance in cycling rats (Naef & Woodside, 2007).

Most of the research examining the influence of exogenous leptin during lactation has used peripheral administration. For example, Lins, Passos, Lisboa, Bonomo, & de Moura (2005) gave daily injections of leptin (80 μg/Kg of body weight) or saline on days 18-20 pp and found no effects on maternal body weight or food intake. Similarly intravenous leptin administration at concentrations of 0.2 and 0.4 mg/kg of body weight did not decrease food intake in rats in in mid-lactation although they were effective in non-lactating animals (Suzuki et al., 2010). However much higher concentrations of leptin were (2.5 mg/kg) able to reduce food intake in both non-lactating rats and lactating rats on days 13 and 14 pp (Woodside, Abizaid, & Jarrerali, 1998). Other investigators have adopted a strategy of using chronic infusion of leptin at doses aimed to produce circulating levels of leptin in lactating rats similar to those seen in cycling animals to investigate changes in leptin responsivity during lactation. Thus Xu, Kirigiti, Grove, & Smith (2009) administered leptin via osmotic minipumps at a concentration of 500 ng/μl/hr

from days 8-11 pp. They found that neither leptin treatment alone nor in combination with insulin affected food intake or body weight, although insulin alone and in combination with leptin decreased NPY and AGRP expression and increased POMC expression in the ARC. In a similar study Crowley and colleagues (2004) examined the effects of peripheral leptin administration (400 ng/μl/hr) and found that 24 hours of leptin administration produced a reduction in feeding compared to saline treatment that although small, was notable statistically. This treatment did reduce NPY release into the PVN but had no effect on expression of the mRNA of NPY, AgRP or POMC in the ARC (Crowley, Ramoz, Torto, & Kalra, 2004). Woodside (2000) and her team examined the effects of chronic pheripheral administration of 48 μg of leptin daily between days 8 to 22 pp and found that although food intake and litter weight were unaffected, the body weight of dams was reduced (Woodside et al., 2000).

While not as extensive, the effect of central administration of leptin has also been examined during different reproductive states. One team sought to examine the effects of ICV injections of leptin on food intake and body weight during pregnancy, beginning on gestation day 18 and continuing until the first day of lactation. Pregnant rats were unaffected by leptin in terms of body weight and food intake when compared to vehicle treated animals. This group also found leptin treatment during lactation and found that leptin treatment during lactation (days 6 and 13 pp) failed to reduce feeding or body weight in these animals until 6 consecutive days of administration into the ventricle (Johnstone & Higuchi, 2001). Suzuki also examined the effects of ICV leptin administration (10 μg) as well as a leptin antagonist (15 μg) during mid-lactation (day 10 pp), with the authors reporting that food intake was lower in leptin treated dams than in saline treated dams 24 hours after leptin administration (Suzuki et al., 2010). These findings represent a replication of work by Woodside et al. (2000), who demonstrated that continuous ICV leptin treatment using the same concentration of leptin (10 μg daily) later utilized by Suzuki along with a lower concentration (1 μg daily) from postpartum days 8 until 22 resulted in reductions in feeding as well as dam body and litter weights (Woodside et al., 2000). Evidence that leptin signaling pathways may be altered in lactation comes from a study by Roy et al. Using an in vitro approach they applied leptin (1 μg/ml for 10 min) to micropunched ARC of lactating

rats which failed to phosphorylize STAT3 as seen via Western Blot analysis in contrast to non-lactating females who exhibited such activation in response to similar treatment . From these results the authors suggested that this may be evidence of leptin resistance occurring during lactation, although the specific period during nursing was not noted (Roy et al., 2007).

Overall, the review of the literature presented above suggests that much remains to be clarified about the mechanisms driving the hyperphagia of lactation. What is also apparent from the preceding review is that the pp period is dynamic hormonally, physiologically and behaviourally suggesting that mechanisms of energy balance and the factors controlling them may also change across lactation. Given the significant role of both leptin and ghrelin in the control of food intake, it is likely that their contribution also changes across lactation; but precisely how and when their contributions might be modified remains to be elucidated. In the case of leptin, evidence from studies of peripheral administration (some acute and others chronic) have yielded mixed results such that in some cases leptin treatment can influence hyperphagia, body and litter weight, while in others it does not, even when applied during the same pp period. When it comes to central leptin administration, behavioural evidence from Johnstone & Higuchi (2001) along with the signaling results provided by Roy (2007) suggests that lactation may represent a time during which the brain's response to leptin is altered. Specifically, the results from these studies suggest that much like gestation, there may be reduced sensitivity or resistance in response to this anorectic. In contrast, Suzuki and Woodside's findings that nursing rats remain responsive to central leptin administration contradicts such a proposal.

Contradictory findings about the role of leptin in the hyperphagia of lactation most likely reflects differences in methodology including differing concentrations, routes and modes of administration (e.g. chronic vs. acute) as well as differences in the time at which leptin was administered (early, mid, and late lactation). The lack of comparison between the different stages of lactation when attempting to understand the influence of neuropeptides on energetic balance is problematic given that the energetic needs of the young and hence the mother along with the hormonal state of the latter is evolving from birth to weaning. In particular, given that

high prolactin receptor activation has been linked to central leptin resistance, changes in prolactin concentrations across lactation might be expected to influence the contribution of leptin to food intake regulation. It seems clear, then, that studies examining these changes across the pp period are required to shed further light on the mechanisms underlying the control of food intake at this time. Furthermore, many investigations have focused on just one point within the signaling cascade involving leptin (e.g. the JAK/STAT pathway), and effects at later stages in the pathway, for instance using neuropeptides implicated as signals within second order neuronal populations (e.g. α-MSH) remain sparse, if not entirely absent. Thus the aim of the current dissertation was to further elucidate the effects of various neuropeptides on feeding, body weight, and pup growth across lactation. More specifically, the series of studies presented here sought to examine how the appetite stimulant ghrelin and anorexigenics leptin as well as MTII might affect these variables when administered centrally, and in the case of leptin, peripherially. Furthermore, how ICV administration of leptin during early and late lactation effected the phosphorylation of the STAT3 molecules within the hypothalamus, a marker indicative of ObRb binding was examined. In light of findings that the activation of PSTAT3 is suppressed during mid-gestation and represents a state of leptin resistance, we wanted to study whether the hyperphagia of lactation is a behavioural product of similar central mechanisms.

Given previous findings, some predictions can be made with regard to the behavioural responsiveness to leptin during lactation. Based on Johnstone and Higuchi's (2001) investigation, acute central administration of leptin during early (days 0 to 5 pp) lactation should not affect food intake or be significantly attenuated compared to controls and is likely supported by the higher than virgin levels of prolactin present at this time. The effects of leptin administration during mid to late lactation is less clear given the contradictory findings between the Johnstone study and findings reported by Suzuki. Peripheral treatment was also investigated across lactation allowing us to determine if resistance (if apparent) is central or not. Predictions regarding peripheral leptin administration during mid to late lactation are difficult given the contradictory findings in the literature, while this has not been investigated in the early nursing period. In line with the above predictions, peripheral leptin treatment during this time might be

expected to have a reduced effect compared to that seen in late lactation or in cycling rats. In addition, the possibility of central resistance was examined by looking at the JAK/STAT pathway through PSTAT3 activation along with the responsiveness of second order neurons by way of MTII administration. Given results by Roy with regard to STAT3 signaling, it is hypothesized that a lack of phosphorylation of STAT3 will be observed during early lactation, thereby corresponding with the time of predicted resistance or reduced sensitivity to leptin. Finally, based on the preliminary findings of increases of GHS-R mRNA and the equal or lower plasma concentrations of ghrelin in lactating rats, it is predicted that lactating rats will exhibit increased behavioural sensitivity to central injections of ghrelin compared to non-lactating rats.

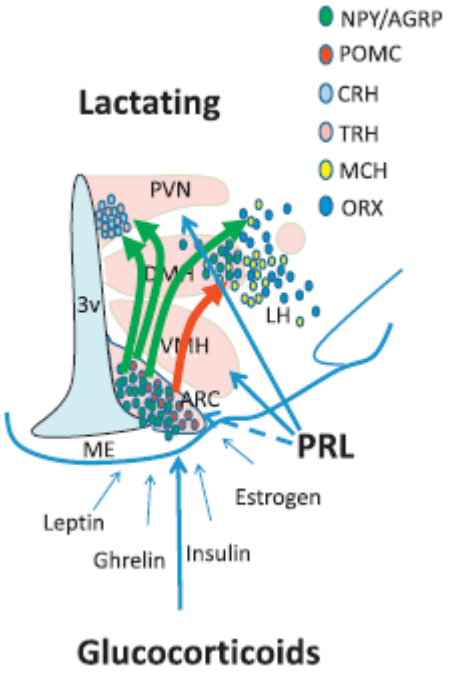

Figure 1.1. The contributions of various peripheral hormones on the ARC of the hypothalamus, their subsequent effects on neuropeptides, neurons, and receptors within this region along with their projections to other hypothalamic nuclei (Adapted from Woodside et al., 2012).

CHAPTER 1

The Effect of Stage of Lactation on the Response of Postpartum Rats to the Administration of Anorectic Peptides

Radek Budin
Barbara Woodside
Center for Studies in Behavioral Neurobiology/Groupe de recherché on neurobiology comportementale
Concordia University, Montreal, Quebec,
Canada

Abstract

The aim of the following experiments was to investigate whether responses to peripheral and intracerebroventricular (ICV) administration of anorexigenic hormones are influenced by stage of lactation. Animals that were either cycling, in early or late stages of lactation were administered either leptin or a melanocortin agonist (MTII) at varying doses and their effects on food intake, body weight and litter size were examined. In addition the ability of central leptin administration to induce the phosphorylation of STAT3 in hypothalamic nuclei was compared across reproductive states. Central leptin administration reduced food intake and body weight, with the higher of the two doses administered having a more pronounce effect on the latter measure. There was a trend toward litter weights change based on leptin treatment. In contrast, chronic peripheral leptin administration at levels previously shown to reduce food intake in virgin rats had no effect on any measures in lactating rats. Central administration of 1μg leptin produced increased PSTAT3-IR in ARC and the VMH across all groups tested, with a trend in a similar direction for the PVN. MTII resulted in a dose dependent suppression of food intake and body weight across all reproductive states, with the higher dose resulting in a more pronounced effect. MTII administration of both doses was found to reduce litter growth. These data suggest that in contrast to the complete lack of response to central leptin injections shown in late pregnancy, postpartum dams remain responsive to central leptin administration throughout lactation, but an attenuated effect to this treatment emerges during early lactation.

Introduction

The ability to provide nutrients to young by producing milk from internal glands is a distinguishing characteristic of mammals (Dall & Boyd, 2004). Providing sufficient energy to support milk production and hence the development of young requires the coordination of a multitude of factors that include behavioural, physiological and neurochemical adaptations that begin during pregnancy and continue until weaning (Woodside et al, 2008). Not surprisingly, studies in rats have shown that many of these adaptations involve the mechanisms controlling energy balance and include increases in expression of the orexigenic neuropeptides NPY and AgRP that begin around mid-lactation as well as decreases in POMC expression within the ARC that are seen both early and late pp (Chen et al., 1999; Crowley et al., 2004; Ladyman et al., 2009; Mann et al., 1997; Smith, 1993). The most obvious behavioural correlate of these physiological adaptations in rat dams is a litter size dependent increase in food intake that peaks at the end of the second week of lactation when it reaches levels as much as 350 % above of that observed in cycling rats, (Malabu, Kilpatrick, Ware, Vernon, & Williams, 1994; Morrison, 1956; Strubbe & Gorissen, 1980; Wilding, Ajala, Lambert, & Bloom, 1997; Woodside et al., 2000; Woodside, 2007).

Peripheral metabolic signals such as leptin are important modulators of central energy balance pathways. Leptin is the protein product of the Ob gene that is produced primarily in white adipose tissue and circulates in the blood stream at levels proportional to stored triglycerides (Maffei et al., 1995). This neuropeptide binds to ObRb receptors distributed throughout several hypothalamic nuclei to stimulate increased energy expenditure and reduce appetite (Tartaglia et al., 1995). There is evidence to suggest that both circulating levels of this hormone and responsivity to its effects changes across pregnancy and lactation in rats (Ladyman & Grattan, 2005; Seeber et al., 2002). For example, there is a 1.8 fold increase in serum concentrations of leptin during the second half of pregnancy in rats; however despite the surge of this typically anorexigenic peptide, these animals still exhibit food consumption that is approximately 20-27% higher than that observed in cycling rats. Moreover, ICV administration of leptin to rats on Day 14 of pregnancy does not elicit the expected reduction in food intake

observed in male or cycling female rats. This lack of behavioral response corresponds with reduced STAT3 phosphorylation, typically used as a marker of leptin signaling, in the VMH but not in the ARC. Together, these findings are strongly indicative of leptin resistance at this point in pregnancy (Ladyman & Grattan, 2005). Subsequent studies from the same group (Augustine & Grattan, 2008) demonstrated that chronic prolactin receptor activation associated with the second half of pregnancy induced the leptin resistant state since pseudopregnant rats chronically administered prolactin failed to show a decrease in food intake and body weight following an acute ICV leptin infusion. These data are consistent with previous results showing that prolactin acts in the brain to increase food intake in both birds and rats (Gerardo-Gettens, Moore, Stern, & Horwitz, 1989; Li, Kelly, & Buntin, 1995; Noel & Woodside, 1993; Sauve & Woodside, 1996; Sauve & Woodside, 2000). In addition, Naef and Woodside (2007) reported that compared to saline infused rats, cycling Wistar rats receiving chronic infusion of prolactin into the lateral ventricles produced a reduction in both the behavioural response to central leptin administration and in the ability of this hormone to induce phosphorylation of STAT3 in the paraventricular nucleus and in the ventral medial nucleus of the hypothalamus.

Studies of leptin responsiveness during lactation have produced mixed results. Xu and colleagues found peripheral leptin treatment of lactating, suckled, ovariectomized rats via subcuteous minipumps failed to produce a change in body mass or food consumption (Xu et al., 2009). In contrast, another group found that similar treatments after 24 hours served to reduce food intake in pp animals and affected central NPY (Crowley et al., 2004). Administering a higher dose of leptin to dams between days 8 to 10 pp, on the other hand, produced a transient reduction in food intake (Woodside et al., 2000). Studies of the effects of central leptin treatment have also obtained mixed results. For instance, Suzuki et al 2010 found that 10 µg leptin ICV reduced food intake in postpartum females, while Woodside et al., 2000 reported that ICV administration of 10 µg reduced both food intake and weight gain in both mothers and their offspring. In contrast, Johnstone and Higuchi (2001) reported that leptin only reduced food intake and body weight after six days of ICV administration. Changes in leptin signaling have also been reported pp. Using Western blot analysis, Roy and his colleagues discovered that leptin

treatment of brain slices resulted in significant increases of PSTAT3 in the ARC of virgin rats but failed to do so in a lactating group (Roy et al., 2007).

Contributing to the variation in the results of these studies are not only differences in routes of administration and dose used but also the time postpartum when rats were tested. Both the hormonal and metabolic state of the lactating female change across lactation. During lactation prolactin is released from the pituitary of the rat dam in response to suckling stimulation from the pups (Grosvenor & Mena, 1969) and there is evidence to suggest that prolactin enters the brain from the periphery to influence food intake via a transport mechanism within the choroid plexus (Walsh, Slaby, & Posner, 1987). Circulating levels of prolactin are influenced by litter size and are higher in early than in late lactation (Grosvenor et al., 1986). Circulating leptin levels also vary with stage of lactation. Despite the significant increase in circulating leptin during late gestation, lactation brings with it a subsequent reduction in the levels of this hormone resulting from the utilization of fat stores in response to the energy drain of nursing. As a result, dams exhibit plasma levels of leptin that are either equal to (during early lactation) or lower (late lactation) than that of non-lactating animals. Given changes in the circulating levels of prolactin and leptin across lactation one might predict a change in the response to leptin across pp. Thus, In the current studies we compared the behavioural response of early and late lactating rats as well as cycling females to both peripheral and central administration of leptin. The ability of central leptin administration to induce phosphorylation of STAT3 in hypothalamic nuclei was also compared across reproductive state. In addition, because the major route through which leptin is thought to decrease food intake is by stimulating increased release of α-MSH from POMC neurons in the ARC and the subsequent activation of MC3/MC4 receptors (Hsiung et al., 2005) we evaluated whether any changes observed in the response to leptin administration were a function of alterations in this second leg of the anorectic pathway. Thus, responses of cycling rats and that of rats in early or late lactation to the central administration of a synthetic form of α-MSH, a melanocortin agonist known as melanotan II (MT II), were also compared. In all experiments the potential effects of administration of these peptides on milk production as reflected in litter weight gain was evaluated.

Methods

Subjects

All rats used in these studies were obtained from Charles River Breeding farms, St. Constant, Quebec. Virgin female Wistar rats weighing between 220 - 240 g upon arrival were randomly assigned to either the cycling or lactating condition and housed in groups (four to five) in large cages. For all experiments, rats were maintained on a 12-hour light dark/cycle. For experiments 2 and 4 lights went on at 8 am and switched off at 8 pm. In the remaining experiments lights went off at 12 pm and on at 12 am. The room was maintained at a temperature of 20 - 22 C. Rat chow (Agway Ltd. Syracuse, NY) in either pellet or powdered form and water were provided ad libitum throughout the experiments with the exception of the pair feeding study. Rats assigned to the lactating groups were mated by introducing a sexually experienced Wistar male into the group cage. Approximately 10 to 14 days later, all subjects, including virgins, were separated into clear individual plastic cages (20 X 45 X 50 cm) with beta chip bedding. The Concordia University Animal Research Ethics Committee approved all procedures under the guidelines of the Canadian Council on Animal Care.

ICV Cannula Implantation

Ten to 14 days following the introduction of the stud male to the lactating group, all subjects, including cycling controls, underwent surgery to implant stainless steel cannulae into the right lateral ventricle. Subjects were anesthetized using ketamine-xylazine (5.7 mg ketamine and .86 mg xylazine/ 100 g body weight). The head was shaved covering an area slightly posterior to the eyes to the base of the skull. The subjects were then placed in a stereotaxic apparatus. A sagittal incision approximately 2.5 cm in length was made to expose the skull, which was subsequently cleaned. Four holes were drilled and three jeweller screws were placed into the skull. 22 Gauge cannulae (Plastics One Inc. Roanoke, VA) were implanted into the lateral ventricle using the following co-ordinates AP: 0.00 mm; ML: 0.16 mm; V: 0.50 (Paxinos & Watson 1996), and were then fixed to the skull using dental cement anchored with three stainless steel screws. The correct placement of cannulae was assessed in Experiments 1 and 3 by ICV injection of angiotensin (50ng in 2 μl) at the end of the experiment. If the injection of

angiotensin stimulated immediate drinking of water (within one minute) that continued for at least 30 seconds following administration, then the cannula placement into the lateral ventricle was considered accurate. In Experiment 5 correct cannulae placement was assessed histologically. Only data from rats with accurate placement of the cannulae were included in the analysis.

Osmotic Minipump Implantation

Osmotic minipumps (Alzet Model # 1003D) were implanted under isoflurane anaesthesia. Incisions were made in the skin at the back of the neck and the pumps, after washing in ethanol and sterile saline, inserted between the scapulae. The incision was closed with surgical staples

Drugs

Leptin (Peprotech, New Jersey) was dissolved in dH2O, buffered with a 1 M tris solution administered at a dose of 0, 1µg or 4µg/4µl. MTII (Sigma) was dissolved in distilled water and administered at doses of 0, 50 pg or 100 pg/µl.

Experiment 1: Behavioral Responses to Central Leptin Administration

The day of parturition was designated as day 0 pp. Within a day of birth, the litters of the dams were culled to 8 pups and females assigned to one of 6 conditions (according to the phase in lactation in which they were tested): Early (days 4-6 pp) or Late (days 14 – 16 pp); and dose 0, 1µg or 4µg leptin. Cycling females were randomly assigned to one of the three dose conditions. To facilitate measurement of food intake, all rats were presented with powdered rat chow in glass food jars anchored to metal supports thereby preventing tipping. Food intake, body weight and, where appropriate, litter weight were monitored daily throughout the experiment. On the test day, subjects were injected with either the appropriate drug amount or vehicle. Infusions were made at the rate of 1µl/min and the injector was left in place for 1 minute after to ensure full dispersal of drug. Daily data collection and infusions were carried out within one hour of lights off.

Experiment 2: Behavioural Responses to Peripheral Leptin Administration

Mating, housing conditions and litter size adjustment proceeded as described above. Rats were assigned to either an early or late lactation group, and within each condition rats were

assigned to receive either vehicle or leptin (1000ng/µl/hr) administered via an Alzet mini-pump (Model # 1003D). This concentration was chosen because in pilot work it was found to produce a decrease in food intake of approximately 3g/day in cycling rats. For rats in the early lactation group, mini-pumps were implanted on day 4 pp and those in the late lactation group were implanted on day 12 pp. As in experiment 1, food intake, body weight and, where appropriate, litter weight were monitored daily from day 1 pp until the end of the experiment.

Experiment 3: Behavioural Responses to Central MTII Administration

Female rats were assigned to one of 6 groups according to reproductive state: cycling, early lactation (days 4 and 5 pp), and late lactation (days 14 and 15 pp) and either saline or a dose of MTII was administered at 50 pg or 100 pg. Since pilot data revealed no carry over effects of MTII treatment on food intake in the days following administration, the effects of MTII were assessed by comparing food intake on the drug day with food intake on the saline day. The order of administration of saline and MTII was counterbalanced within groups. Injections were delivered within the hour before lights out. Food intake, dam body weight, and, where appropriate, litter weight were monitored daily. Following the two days of ICV injections, cannula placement was verified using the angiotensin test as described above.

Experiment 4: Pair-feeding

To assess whether the decrease in food intake induced by MTII administration was sufficient to produce the decrease in litter growth observed (see below), rats were assigned to one of three groups: ad lib fed, pair-fed to the food intake of MTII- treated rats in Experiment 3 on day 4 pp or pair-fed on day 14 pp. Body weight, litter weight and food intake were measured daily throughout the experiment. Rats in the day 4 pp pair-fed groups were given access to 51% of the amount that they had eaten on day 3 pp and those in the day 14 pp pair fed group were given 66% of the amount eaten on day 13 pp.

Experiment 5: Effect of Stage of Lactation on Central Leptin Signaling

The ability of leptin to induce pSTAT3 was compared amongst six groups of rats: cycling rats treated with either 1 ug leptin or saline; lactating rats on day 4 pp treated with either 1 ug leptin or saline and lactating rats on day 14 pp treated with either 1 ug leptin or saline. Leptin

(Peprotech, NJ) was dissolved in dH2O and buffered with a 1 M tris solution to a ph of 7.4 and administered at a dose of 1 ug in a volume of 2 ul. Rats in saline groups were injected with a similar volume. Thirty minutes following the infusion, subjects were given an overdose of approximately 0.7 ml of Euthanyl (sodium pentobarbital) and transcardially perfused with saline followed by 2% paraformaldehyde. After brains were extracted, they were post-fixed overnight at room temperature in 2% paraformaldehyde. Brains were then placed in a 30% sucrose solution until they sank or for a maximum of 72 hours and then stored at –80 C until sectioned on a cryostat. Three sets of 40 µm sections were obtained throughout the hypothalamus and stored in Watson's cryoprotectant at -20C until processed for immunohistochemistry.

PSTAT3 immunohistochemistry

Sections through the hypothalamus were washed (3 X 5 min in PBS), incubated in 1% H2O2 and 1% NaOH in PBS for 20 min, and then 0.03% sodium dodecyl sulphate for 10 min. Following 3 X 5-min washes in PBS, sections were placed in blocking solution (4% NGS in 0.25% Triton X-100 and 0.02% sodium azide in PBS) and incubated at room temperature for 1 h. Overnight, sections were placed in primary antibody directed against pSTAT3 (1:1000 dilution; Cell Signaling Technology, Inc. Beverly, MA) in blocking solution at 4 C. The next day, sections were washed incubated in 3% NGS and secondary antibody (biotinylated goat-antirabbit; Vector) in blocking solution without sodium azide for 1 h, and washed once more. Afterwards, sections were incubated in ABC reagent (Vector) for 2 h and developed with a DAB Vector kit.

Image Analysis

A Sony XC77 camera (Sony Corp., Kanagawaken, Japan) mounted upon a light microscope (LaboluxLeitz GMBH, Wetzlar, Germany) was used to visualize the brain sections obtained in Experiment 5. National Institutes of Health Image Analysis Software (1.60 b) (Bethesda, MD) installed on a Power Macintosh computer (G4; Apple Computer, Inc., Cupertino, CA) was used to capture images of the sections and these were saved as Tagged Image File Format picture files.

For estimates of pSTAT3 expression in PVN, only sections in the medial area of the nucleus, (-1.80 mm from bregma) were used (Paxinos and Watson, 1986) and an average over all

the sections for each rat was obtained. To obtain an estimate of pSTAT3 in the ARC and VMH, sections from -2.56 mm to -2.80 mm from bregma, were counted and the mean number of stained cells/section for each rat was calculated. To identify stained cells; sections from each experimental group across each assay that appeared to be stained were selected and the relative density of each cell was recorded. An average of the density of the cells was then calculated to establish the criterion for each brain region across all experimental groups. The observer counting cells was blind to group membership.

Statistical Analysis

2- way ANOVAs with reproductive state and treatment as the independent variables were used to compare the effects of central and peripheral leptin treatment as well as MTII administration on food intake, body weight and litter weight gain. In experiments 1 and 3, three levels of reproductive state (cycling, early lactation, late lactation) were compared and in Experiment 1 three levels of treatment (saline, leptin 1 µg, leptin 4 µg); and Experiment 3 two levels (MTII 50 pg, MTII 100 pg). In Experiment 2 comparisons were made between two reproductive states (early lactation vs late lactation) and two treatments (saline vs leptin 1 µg/h). In Experiment 4, t-tests were used to compare litter weight gain in groups pair-fed to MTII treated rats in Experiment 3 with that of litters of ad lib fed females. Experiment 4 examined food intake, body and litter weights following treatment analyzed using a univariate ANOVA. 2-way ANOVA with reproductive state (cycling, early lactation, late lactation) and treatment (saline or 1 µg leptin) as the independent variable was used in Experiment 5 to compare the ability of central leptin administration to induce phosphorylation of STAT3 immunoreactivity in the VMH, PVN and ARC. Where appropriate, Fisher's least significant difference and pairwise comparisons were carried out. In addition, given our a priori hypothesis that there would be changes in response to leptin across lactation a planned comparison was used to compare early and late lactating groups in Experiment 1.

Results

Experiment 1: Behavioral Responses to Central Leptin Administration

As Figure 1 shows, central leptin administration reduced food intake in all groups

resulting in a significant main effect of treatment $F(2, 89) = 18.063$; $p < .001$. Pairwise comparisons for each of these effects showed that, overall, treatment with both the 1 µg and 4 µg doses of leptin resulted in a significant reduction of food intake compared to saline treatment ($p < 0.01$) but there was no significant difference in the effects produced by the 1 µg and 4 µg doses. There was also a significant main effect of reproductive state, $F(2, 89) = 3.418$; $p < .037$. Further analysis of this results showed that overall rats in the early lactation group showed a smaller reduction in food intake than those in the cycling group ($p = .01$), but there were no significant differences in food intake between the cycling and late lactation group or between the lactating groups. The fact that saline injections alone produced a decrease in food intake in the cycling groups, perhaps because of their greater stress responsivity obviously makes the interpretation of any difference between the response of the cycling group and that of lactating rats problematic. Although the dose x reproductive state interaction term did not reach statistical significance, inspection of Figure 1 suggests that early lactating rats were less responsive to the low dose of leptin than were late lactating rats. Given our a priori hypothesis that there would be a change in leptin responsivity across lactation a planned comparison was performed to compare the response of the early and late lactation groups to the 1µg dose of leptin, this revealed a significant difference between these groups ($t(12) = 2.257$, $p<0.05$).

As expected, there was a clear difference in baseline intake amongst the groups (cycling group: M = 26.41 (SE = .97) grams; early lactation: mean =40.28 (SE = 1.18) grams; late lactation group: mean = 72.56 (SE = 1.77) grams). To assess the relative contribution of the effects of leptin treatment to overall food intake, we examined the relative decrease in food intake by calculating food intake as a percentage of saline controls for all groups. These data are shown in Figure 2. The analysis of these data revealed a significant main effect for dose, $F(2, 89) = 18.063$; $p < .001$, and reproductive state, $F(2, 89) = 3.418$; $p < .04$, but no significant interaction, $F(4, 89) = 1.258$, $p = .292$. Pairwise analysis of the reproductive state effect revealed that leptin administration induced a greater relative decrease in food intake in cycling rats than rats late lactation ($p < .006$), a similar pattern was seen when rats in early lactation were compared to those in late lactation ($p = .061$).

Leptin treatment reduced body weight in all groups (significant main effect of treatment $F(2, 89) = 13.845$; $p < .001$). Pairwise comparisons showed that both doses of leptin produced a significantly greater effect than saline ($p = .0072$; $p < .001$) and that the higher dose of leptin had a greater effect than the lower dose ($p < .01$) (see Figure 3). There was no significant effect of either reproductive state or the treatment x reproductive state interaction.

As for litter weight gain there was a significant main effect of reproductive state, $F(1, 59) = 9.866$; $p = .003$ (See Figure 4). The litters of late lactating rats ($M = 19.96$, $SE = 1.02$) gained more weight compared to litters of mothers in early lactation ($M = 14.86$, $SE = 1.01$). As well there was a main effect of leptin dose, $F(2, 59) = 2.815$; $p = .068$. Pairwise comparisons indicated that change in litter weight was similar between saline ($M=18.33$, $SE = 1.37$) and leptin 1 µg ($M=18.77$, $SE = .83$) treatments, $t(43) = -.20$, $p = .84$. In contrast, litters of those mothers receiving the highest dose of leptin ($M= 14.66$, $SE = 1.09$) weighed less than litters of mothers who received saline, but this was at the level of a statistical trend, $t(49) = 1.980$, $p = .062$. Likewise litters of mothers that received the the highest does of leptin weighed less compared to the lowest dose of leptin, $t(32) = 2.764$, $p = .009$.

Experiment 2: Behavioural Responses to Peripheral Leptin Administration

In contrast to the effects of central leptin administration, peripheral leptin treatment at the dose used here did not effect food intake ($F(1, 20) = .766$, $p = .39$) but overall there was a greater increase in food intake across testing in the early lactating than late lactating group ($M = 7.53$, $SE = 1.54$:$M = .55$, $SE = 1.57$), respectively resulting in a significant main effect of reproductive state, $F(1, 20) = 7.624$, $p = .021$.

Neither peripheral leptin treatment, reproductive state nor their interaction had significant effects on change in body weight; treatment: leptin $M = 0.00$, $SE = 1.20$; saline $M = 2.00$, $SE = 2.53$; $F(1, 20) = .701$, $p = .412$; reproductive state: early $M = 2.00$ $SE = 1.78$; late $M= -0.66$, $SE = 1.40$; $F(1, 20) = 2.463$, $p = .132$. Reproductive state x treatment interaction: $F(1, 20) = 1.85$, $p = .189$. Similarly, change in litter weight was similar in early and lactating groups ($M = 2.78$, $SE = 1.06$. $M = 2.45$, $SE = 1.48$ respectively $F(1, 20) = .022$, $p = .884$) and was not affected by leptin treatment (saline: $M = 3.21$, $SE = 2.04$, leptin $M = 2.31$, $SE = .92$; $F(1, 20) = .902$, $p = .354$).

Lastly the reproductive state x treatment interaction was not statistically significant for this parameter.

Experiment 3: Behavioural Responses to Central MTII Administration

Both doses of MTII reduced food intake in rats in all reproductive states, and there was a significant effect of treatment; $F(1, 43) = 13.126$, $p = .001$ (Figure 5). Animals receiving MTII 50 pg ($M = -9.47$, $SE = 1.98$) and MTII 100 pg ($M = -18.85$, $SE = 1.70$) exhibited decreases in food intake, but the decrease was larger for animals receiving MTII 100 pg relative to animals receiving MTII 50 pg. However neither the main effect of reproductive state ($F(2, 43) = 1.859$, $p = .168$) nor the reproductive state x treatment interaction effect ($F(2, 43) = .939$, $p = .399$) reached statistical significance. A similar pattern of effects was seen with respect to body weight, overall MTII administration reduced body weight and this effect was greater at the MTII 100 pg dose; $F(1, 42) = 7.571$, $p = .009$ (Figure 6). There were no significant effects of either reproductive state; $F(2, 42) = .396$, $p = .676$ or of the reproductive state x treatment interaction on body weight; $F(2, 42) = .720$, $p = .492$, although inspection of Figure 6 suggests little effect of the lower dose of MTII on body weight in late lactation.

As in Experiment 1, the relative effectiveness of MTII in decreasing food intake was assessed by comparing percent of saline intake across groups and doses. These data are shown in Figure 7. As with absolute change, the higher dose had a greater effect, main effect of dose: $F(1, 45) = 20.683$; $p < .001$. There was also a significant main effect of reproductive state, $F(2, 45) = 6.458$; $p < .01$; and further investigation of this effect showed that as with the leptin administration there was a greater effect of MTII treatment on relative intake in cycling than in late lactating rats ($p < .001$).

MTII administration reduced litter weight gain at both doses and this effect was greater at the 100 pg than the 50 pg dose was also statistically significant; $F(1, 29) = 6.172$, $p = .019$ (Figure 8). In addition, litters of mothers in late lactation lost more weight in response to MTII than those of mothers in early lactation; ($F(1, 29) = 6.176$, $p = .01$. However, the reproductive state x treatment interaction was not statistically significant; $F(1, 29) = 1.878$, $p = .181$.

Experiment 4: Pair-feeding

To establish whether the marked effects of MTII administration on litter weight were solely attributable to the decrease in maternal food intake we investigated the effect of pair-feeding lactating rats to levels induced by the higher dose of MTII administration on litter growth rate. Figure 9 shows that compared to that of ad lib fed subjects, pair feeding to the level of intake in MTII treated rats did not have an effect on growth rate on day 4 pp ($t(12) = 1.22$; $p > .05$), but a similar manipulation on day 14 pp did reduce growth rate ($t(12) = 5.226$; $p < .001$) (Figure 10).

Experiment 5: Effect of Stage of Lactation on Central Leptin Signaling

As expected ICV leptin administration produced a significant increase in PSTAT3-IR within the ARC and VMH compared to the saline treatment, (ARC: $F(1, 19) = 21.59$, $p < .001$; (Figure 11) VMH $F(1, 19) = 21.68$, $p < .001$; (Figure 12)). Only a trend towards this effect was observed in the PVN, $F(1, 17) = 3.17$, $p = .09$; (Figure 13). The ability of leptin to induce PSTAT3-IR was not significantly affected by reproductive state or by an interaction between reproductive state and treatment in any of the brain areas examined.

Discussion

The studies described here were designed to compare the response of lactating rats at various times pp and cycling rats to anorectic signals. To accomplish this, we monitored the food intake and body weight of early lactating, late lactating and cycling virgin female rats following central and peripheral administration of leptin as well as central administration of MTII. Because administration of MTII decreased milk delivery, as reflected in pup growth, a pair feeding study was also conducted to investigate whether this effect was simply a by-product of the reduction in food intake or reflected a more direct role for MC4 r activation in milk production and delivery. In further experiments, we examined whether differences in leptin signaling during lactation could be detected by comparing the effect of central administration of 1 µg leptin on PSTAT3 induction within the ARC, VMH, and PVN in cycling and lactating rats.

Central leptin administration reduced both food intake and body weight in all three groups tested. Consistent with our a priori hypothesis that higher prolactin levels would be

associated with a reduction in the ability of central leptin to reduce food intake, rats in early lactation showed a smaller response than those in late lactation. Previous studies examining the effects of leptin administration on food intake in lactating rodents have also reported a reduction in food intake but have examined differing time points in lactation, doses, routes of administration or species, (i.e. Mistry and Romsos, 2002: pp day 7, 1 ug leptin via ICV, mice). Thus previous work by Suzuki (2014) and Woodside reported reductions in feeding (in the former study), as well as body and litter weights in the latter study by utilizing doses that were anywhere from 2.5 to almost 4 times that of the highest treatment administered in the context of the current research. Furthermore, although Woodside (2000) administered a dose that was equal to our lowest dose in the current study, this was done chronically rather than acutely in the present experiment. Finally, although Johnstone & Higuchi's (2001) leptin treated animals did not differ from controls the significance of their findings is not entirely clear as the dose that was used was not readily apparent. The data presented here suggests that in contrast to the central leptin resistance shown in late pregnancy, rats retain leptin sensitivity throughout lactation. Consistent with the behavioural effects observed, administering 1µg leptin icv induced increased PSTAT3-IR in the ARC and VMH, while trending towards statistical significance in the PVN for all reproductive states tested.

As expected, baseline levels of food intake differed markedly among the groups. Overall, lactating rats ate more than rats in the cycling group and rats in late lactation ate more than those tested in early lactation. As a result, central leptin administration produced a greater relative decrease in food intake in the cycling rats than in rats in late lactation, and in early vs late lactating groups. Overall this suggests that leptin has less of an effect on the overall energy budget in lactating animals compared to cycling conspecifics. However, although the relative decrease in food intake induced in lactating rats by the higher dose of leptin administered was relatively small (approximately 30% in early lactation and 15% in late lactation) it was sufficient to induce a decrease in litter weight.

Our finding that dams in early lactation do not respond to an acutely centrally administered low dose of leptin with the same degree of hypophagia as reproductive states

(cycling and late lactation) may be indicative of reduced leptin sensitivity during this time. Circulating prolactin levels are higher in early than late lactation (Walker et al., 1995; Matheij, 1984) and thus these data are consistent with the notion that prolactin can induce a state of leptin insensitivity. Data in support of such an idea include studies in which chronic ICV prolactin administration resulted in loss of the behavioral response to leptin. In addition, the central leptin resistance of late pregnancy (Ladyman & Grattan, 2005), which has been shown to depend on the high levels of circulating lactogen associated with the latter half of pregnancy (Augustine & Grattan, 2008). In terms of prolactin mRNA expression, the long form of the receptor does not seem to differ from cycling rats (during diestrus) across most of pregnancy with the exception of an upregulation the day of birth and in post-weaning as observed within the ARC (Augustine, Kokay, Andrews, Ladyman, & Grattan, 2003). The results of these studies differed from those described here, however, in that a virtual total lack of response to leptin administration was reported and that this was associated a decrease in leptin signaling, as reflected in reduced pSTAT3 induction within the VMH and/or PVN. Indeed, it is somewhat surprising that in the current study we saw a reduction in the behavioral effect of leptin but could not detect any reduction in leptin signaling as reflected in pSTAT3 induction. These results also contrast to findings reported by Roy whereby a failure of pSTAT3 activation was noted in the ARC during lactation. However it is important to note that they employed Western Blot analysis while immunohistochemistry was used in the current study, with methodology and differences in detection sensitivity (with the former method being more sensitive) explaining the varying results (Roy et al, 2007).

In contrast to the response of lactating rats to acute central leptin administration, the results of Experiment 2 suggest that chronic peripheral leptin administration at levels previously shown to reduce food intake in virgin rats had no effect on food intake, body weight, or pup litter weight in either early or late lactating rats. These results are, in general, consistent with other literature. Xu et al. (2009) found that restoring blood serum levels of leptin in dams on days 8-9 pp to those similar to cycling animals using osmotic minipumps failed to alter their food intake or body weight (Xu et al., 2009). Rat mothers between days 18 to 20 pp given daily injections of

leptin (8 ug/100 g) also failed to exhibit a significant reduction in food intake, body weight or lactational performance (Lins, Gaspar de Moura, Lisboa, Bonomo, & Passos, 2005). By contrast, the results of one study showed that restoration of serum leptin levels in dams (days 8 to 13 pp) to those seen prior to pregnancy resulted in a significant reduction in food consumption, however, this effect was variable and minimal (Crowley et al., 2004). An exception to the general finding that lactating rodents are less sensitive to peripherally administered leptin than cycling females was obtained a study of Brandt's voles, in which peripheral infusion of leptin to dams between days 10-17 pp was observed to produce a dose dependent decrease in body mass and eating (Cui, Tang, Wang, & Speakman, 2011) perhaps reflecting species specific variability in this effect.

Overall the results of the current studies suggest that lactating rats retain their ability to respond to central leptin administration but are resistant to the effects of peripheral leptin administration. These findings raise the issue of the mechanisms through which such differences arise. One possibility is that lactation is associated with a reduction in the ability of leptin to cross the BBB. Such a mechanism has been proposed to underlie other forms of leptin resistance and would result in an inability of fat stores to signal their levels to the hypothalamus; consequently an inhibitory signal on food intake pathways would be lacking. Banks and his colleagues (2004) have reported that IP injections of bovine whole milk into mice resulted in a significant reduction in the transport of leptin into the brain via the blood barrier. The authors suggested that the high triglyceride content of the milk resulted in the inhibition of transport of leptin into the brain by either acting competitively at or controlling the leptin transporter across the BBB. Alternatively these triglycerides could attach directly to leptin within the bloodstream thus preventing its ability to be carried into the brain via transporters (Banks et al., 2004). Rats typically lose fat across lactation and display serum triglyceride levels that are lower than those of controls, thus it is unlikely that this would provide an explanation for our results (Smith et al., 1998).

Although Xu et al. (2009) found no effect of peripheral leptin administration on food intake in mid-pp, they did show that peripheral leptin administration reversed the increase in

hypothalamic POMC mRNA expression typical of lactating rats, suggesting that some central effects of leptin remain unchanged. The fact that they did not observe an effect of this treatment on hypothalamic NPY gene expression, however, suggests that there was incomplete penetration of leptin or that some other factor that is altered during lactation contributes to the elevated NPY expression seen at this time. Notably Xu and colleagues showed that normalizing peripheral insulin levels was sufficient to decrease NPY gene expression to cycling levels, although this manipulation also failed to reduce food intake in lactating rats. Our current results are also similar to those found by peripheral leptin administration results reported by Suzuki (2010) and Lins (2005) during mid lactation and close to weaning, respectively. However they do stand in contrast to findings suggesting peripheral leptin treatment reduced food intake during mid (Crowley et al., 2004) and late lactation (Woodside et al., 1998), as well as decreasing dam body weight from mid lactation to weaning (Woodside et al., 2000); suggesting that an effect may emerge with higher peripheral doses. Although it is difficult to outline the reasons for why our findings failed to replicate the latter set of studies even when administration occurred in similar doses and time of lactation (notably in contrast to Suzuki et al., 2010), the current study stands unique in being the first to report that peripheral leptin administration shortly following birth fails to produce effects on behaviour (food intake and weight) in both the dam and litter. Given the lack of similar investigations during this lactational period using similar methodology, future researchers will need to confirm our findings. Future research might bring clarity to the inconsistency of findings outlined above by examining differing doses of peripheral leptin administration across all of lactation.

As expected, MTII, which is a synthetic analog of α-MSH, produced a dose dependent suppression of food intake and body weight across all reproductive states, with the higher dose resulting in a more pronounced effect. MTII effects on body weight and the consumption of food has been found in a variety of rodents, including mice, rats and hamsters (Chen et al., 2000; Cettour-Rose & Rohner-Jeanrenaud, 2002; Schuhler et al., 2003). Findings by Chen and colleagues (2004) that involved administration of MTII bilaterally and directly into the DMH on day 11 pp also reported significant reductions in food intake. Together these findings imply that

it is unlikely that the mechanism of action responsible for the reduced behavioural response to leptin observed in our early lactation group occurred at the level of the MC system and its related nuclei such as the DMH.

MTII administration reduced milk production and delivery as reflected by litter growth. Because milk production in part depends on adequate food intake, this might be expected to be a result of their effects on food intake. Typically, however, the effects of food restriction are mitigated, at least in early lactation, by the dam's ability to utilize stored body fat (Naismith, Richardson, & Pritchard, 1982). There was a dose dependent reduction of litter weight gain following MTII administration. Interestingly the results of the pair-feeding study found that feeding rats in early lactation to the amount consumed by their highest dose MTII treated counter parts did not produce a significant effect on litter weight gain. Although some of these differences might reflect variation across cohorts, this difference between the litter weights of the same groups in the two studies point to the possibility that MC3/MC4 activity can influence milk delivery, possibly though effects on OT release (Fan et al., 2004; Wu et al., 2012).

In sum, the results of this series of experiments suggest that unlike pregnancy, lactation does not represent a leptin resistant state. This is further reflected in the ability of leptin to induce PSTAT3 activation during lactation within the nuclei critical for the regulation of food intake. Sensitivity to leptin is maintained despite higher levels of serum leptin and prolactin during early lactation. Furthermore, applying a synthetic form of α-MSH similarly influenced the behaviour of all treated animals by reducing food intake and indicates that second order neurons remain responsive throughout the postpartum period. Follow up studies should further examine the differential effects of leptin and MTII treatment on litter weights to understand the mechanisms driving this and if pups are behaviourally more sensitive to the central effects of these various peptides than the dam herself.

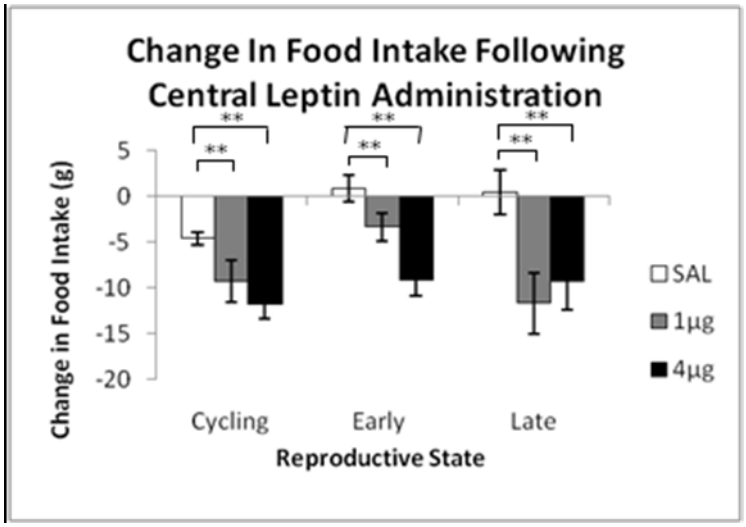

Figure 1. Mean (+/- SEM) change in food intake of animals in grams following central administration of leptin. Leptin reduced food intake in all groups. Both the 1 µg and 4 µg doses of leptin produced a significant reduction in food intake compared to saline treatment $p < .01$. ** $p < .01$.

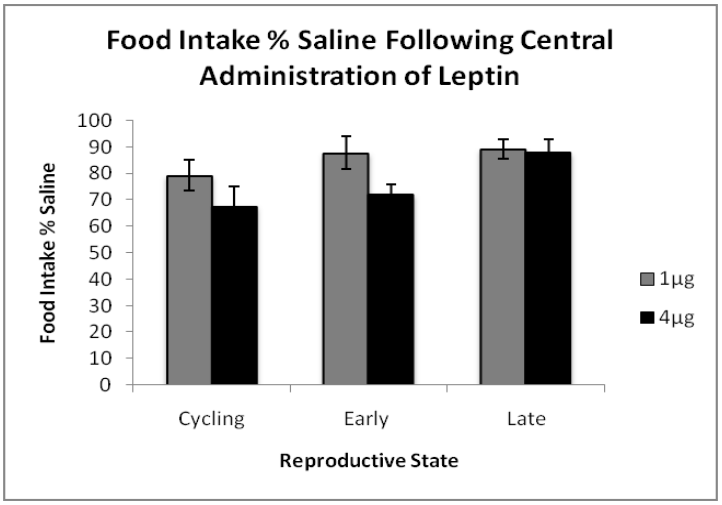

Figure 2. Percent of saline change in food intake following central administration of leptin. Leptin administration induced a greater relative decrease in food intake in cycling than in late lactation $p < .01$. There was also marginally significant difference between early and late groups $p = .061$.

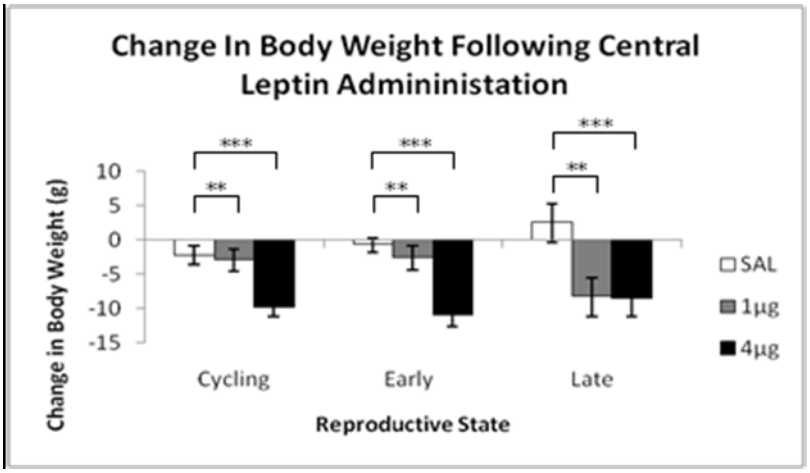

Figure 3. Mean (+/- SEM) change in body weight of animals in grams following central administration of leptin. The 4 µg dose of leptin resulted in a decrease body weight when compared to saline treated animals $p < .01$. ** $p < .01$, *** $p < .001$.

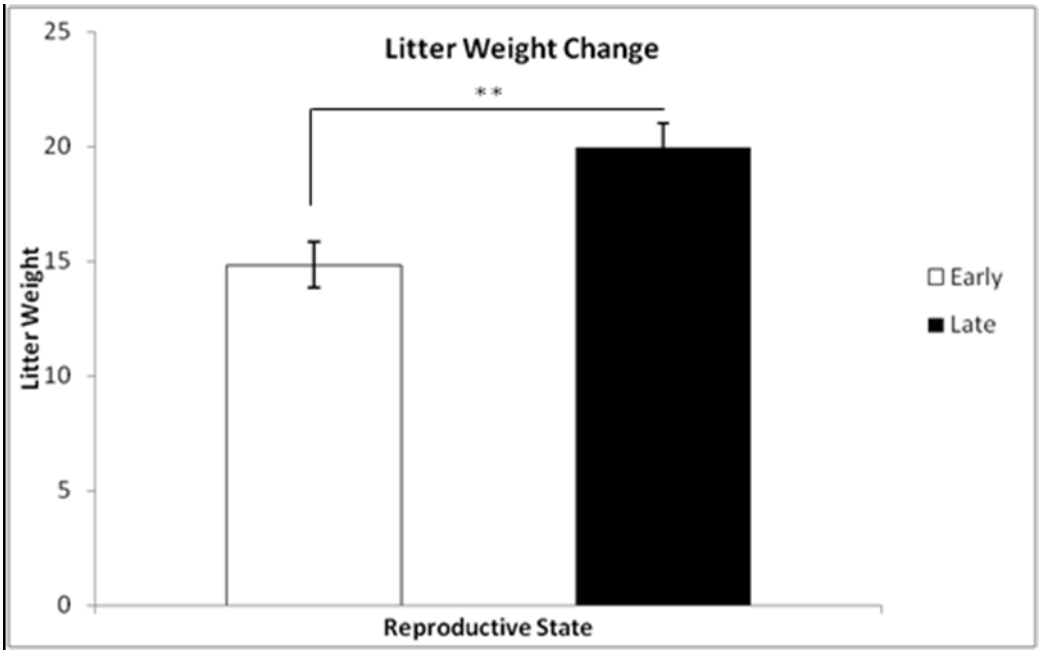

Figure 4. Mean (+/- SEM) change of litter weight change in grams as a function of reproductive state. There was more litter weight gain during late lactation compared to litters in early lactation $p = .003$. ** $p < .01$.

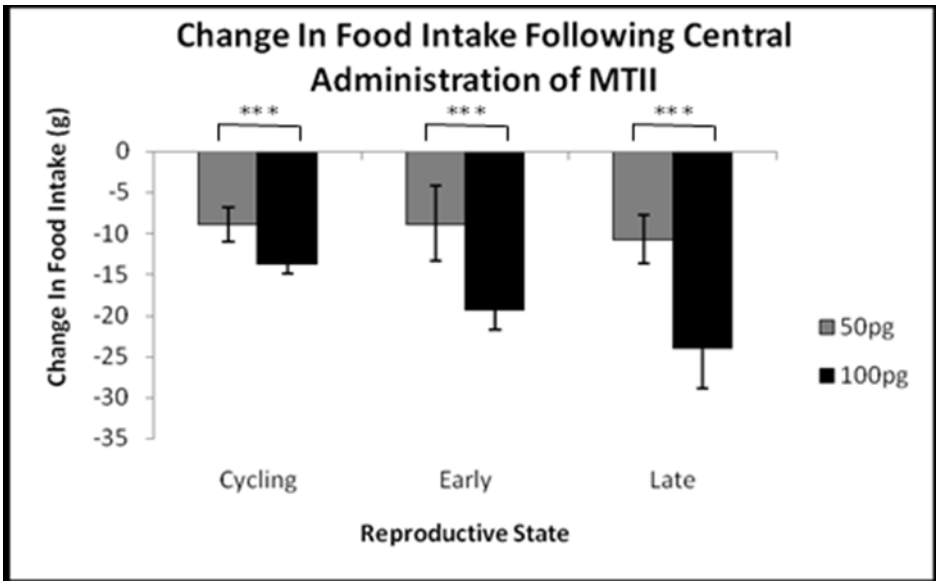

Figure 5. Mean (+/- SEM) change in food intake of animals in grams following central administration of MTII. Both 50 pg and 100 pg doses of MT II reduced food intake in all reproductive states. The effect on food intake was larger at the MT II 100 pg dose $p < .001$. *** $p < .001$.

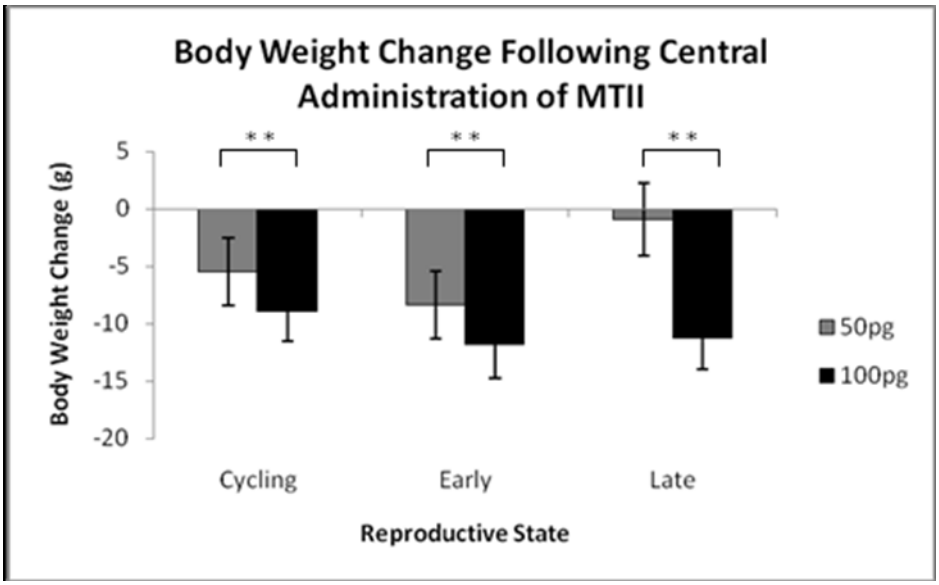

Figure 6. Mean (+/- SEM) change in body weight of animals in grams following central administration of MTII. MT II reduced body weight in all groups, $p = .009$. ** $p < .01$.

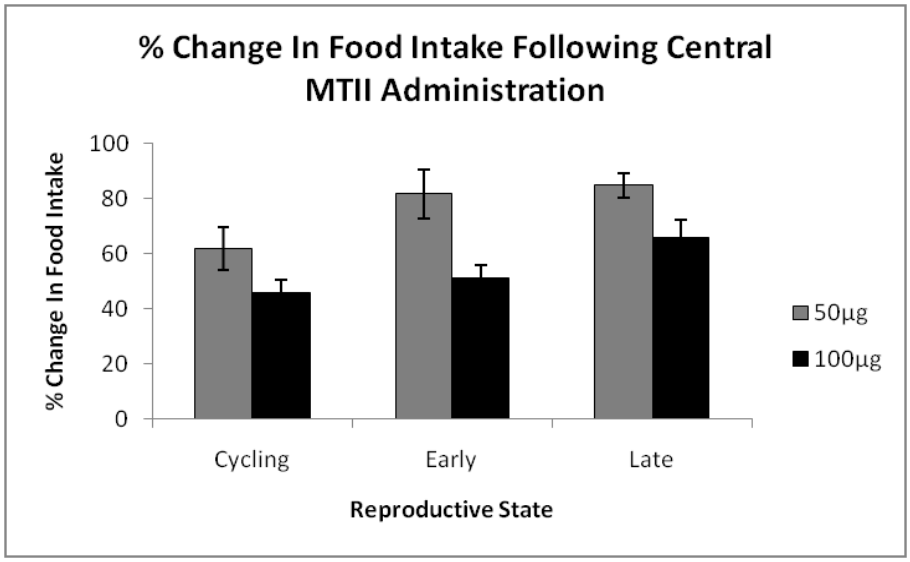

Figure 7. Percent of saline change in food intake following central administration of MTII. There was a greater effect of MTII treatment on relative intake in cycling rats than in late lactating rats $p < .001$.

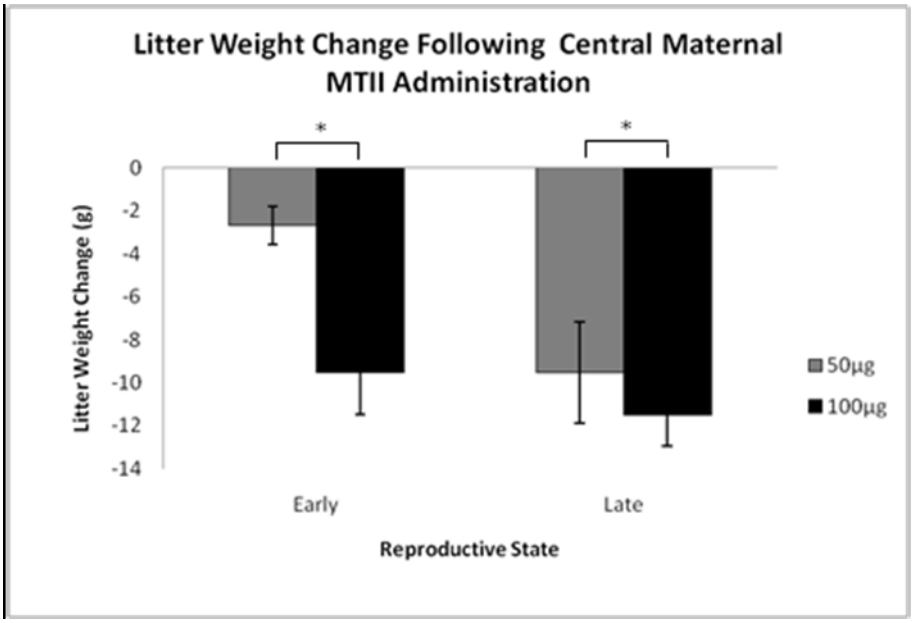

Figure 8. Mean (+/- SEM) change of litter weight in grams following central maternal MTII administration. Litter growth rate decreased more in response to MTII in late lactation than in early lactation $p < .05$. Litters of mothers receiving the higher dose of drug also exhibited growth rate decrease $p < .019$. * $p < .05$.

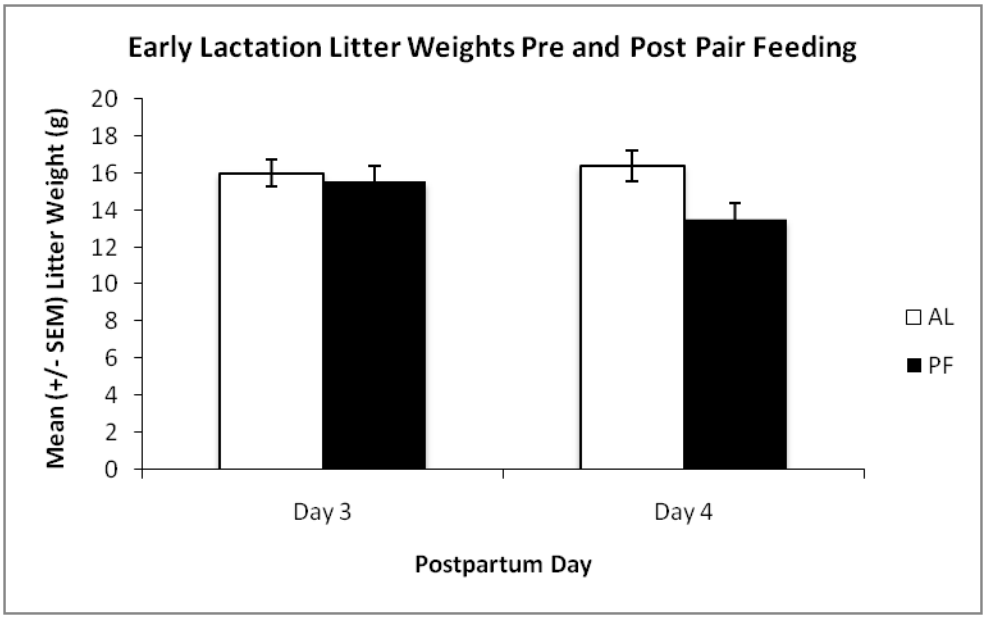

Figure 9. Litter weight of dams provided with ad lib or MTII pair-feeding. Early lactation dams paired with the amount of food that MT II 100 pg treated animals had consumed. Litters of the pair-fed mothers did not differ in weight compared to dams provided with ad lib food $p > .05$.

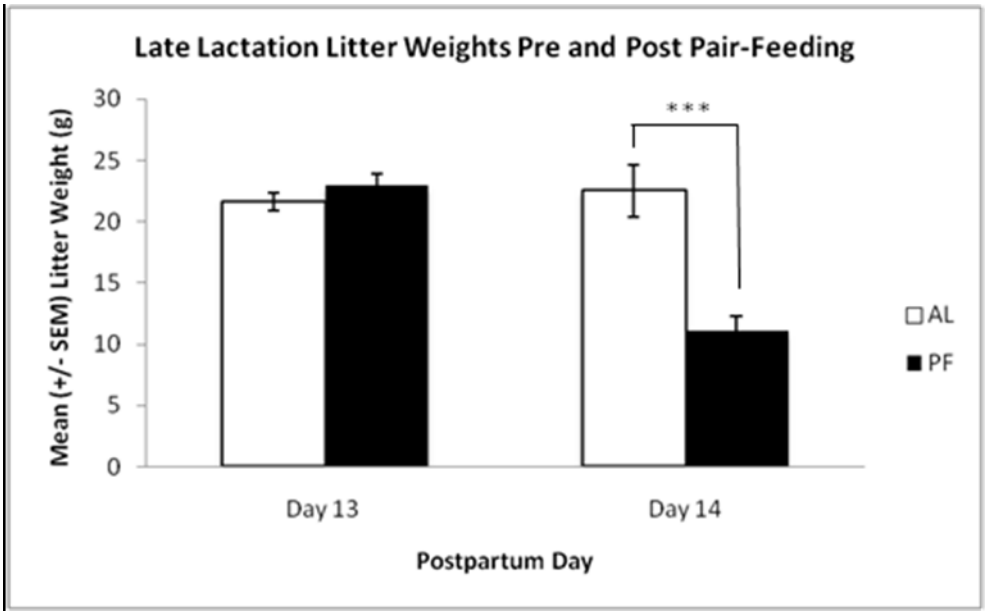

Figure 10. Litter weight of dams provided with ad lib or MTII pair-feeding. Late lactation dams paired with the amount of food that MT II 100 pg treated animals had consumed. Litters of the pair-fed mothers weighed less compared to dams provided with ad lib food in late lactation $p < .001$. *** $p < .001$.

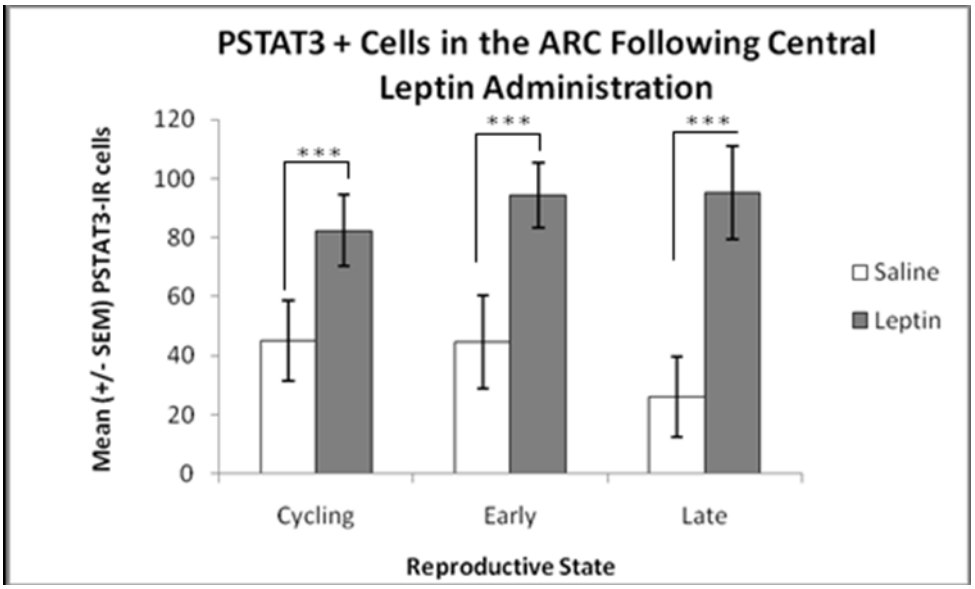

Figure 11. Mean (+/- SEM) number of PSTAT3 + cells in the ARC. Rats that received leptin had an increased number of PSTAT3 + cells $p < .001$ compared to saline treated rats. *** $p < .001$.

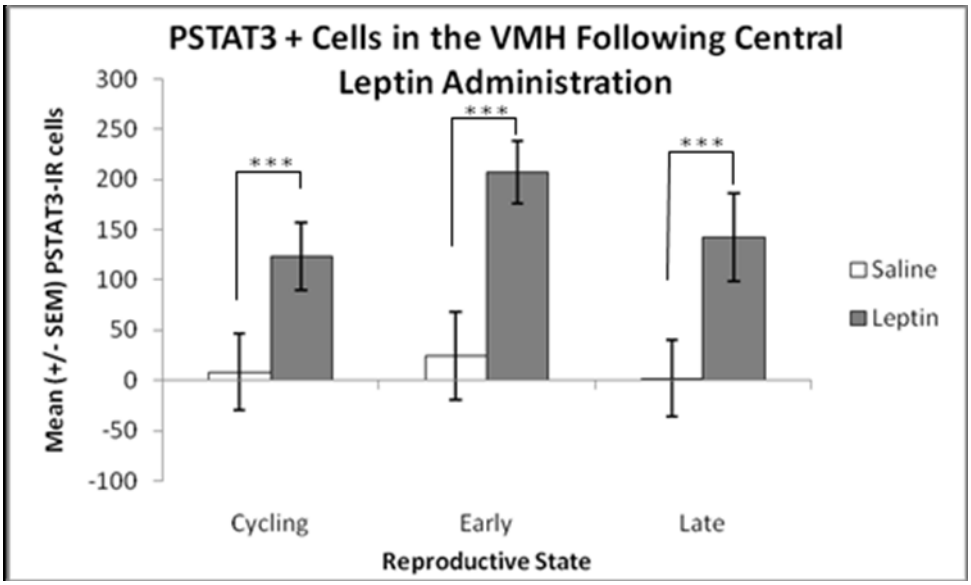

Figure 12. Mean (+/- SEM) number of PSTAT3 + cells in the VMH. Compared to saline treated rats, those that received leptin had an increased number of PSTAT3 + cells $p < .001$.

*** $p < .001$.

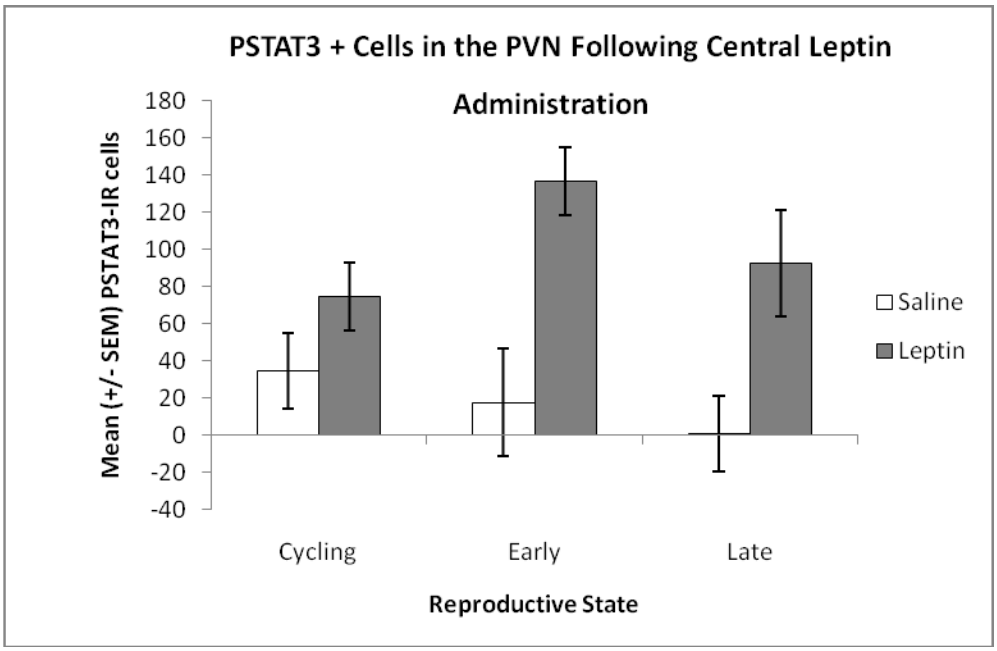

Figure 13. Mean (+/- SEM) number of PSTAT3 + cells in the PVN. There was a trend towards statistical significance such that rats that receiving leptin had an increased number of PSTAT3 + cells $p = .09$ relative to saline treated animals.

CHAPTER 2

The Effect of Central Administration of Ghrelin and a Ghrelin Antagonist on the Behavioural Response of Postpartum Rats Across Lactation

Radek Budin
Barbara Woodside
Center for Studies in Behavioral Neurobiology/Groupe de recherché on neurobiology comportementale
Concordia University, Montreal, Quebec,
Canada

Abstract

The purpose of these experiments was to investigate the contribution of the orexigenic hormone ghrelin to the hyperphagia of lactation and whether this contribution changes as a function of time postpartum. In the first experiment, the effects of ICV administration of varying doses of ghrelin (0, 0.25 µg, 0.5 µg, 1 µg) on food intake in cycling and rats in mid-lactation were compared. In the second and third experiments the food intake of cycling rats following the ICV administration of ghrelin (0.25 µg or 1 µg) or a ghrelin antagonist (JMV 3002, 0.4 µg) was compared to the effects of similar treatments in rats in early or late lactation. Central ghrelin administration resulted in a dose dependent increase in food intake in cycling rats. This pattern of response differed from that seen in rats in mid-lactation, which showed a peak effect at the lowest dose of ghrelin administered. In contrast, rats tested in early and late lactation responded to ghrelin similarly to cycling rats. Finally, ghrelin antagonist administration produced a greater reduction in food intake in dams in late lactation than in either cycling rats or those tested in early lactation. The importance of these findings are discussed in relation to possible mediating factors including endogenous neuropeptides and energy balance during lactation.

Introduction

The nourishment that a mother provides to her offspring during lactation creates in her a state of unparalleled energy drain. For instance, in the developed world, the energetic cost of nursing for a typical mother with a single child is 2.62 MJ per day (Woodside, Augustine, Ladyman, Naef, & Grattan, 2008). In the case of rats, which give birth to multiple young, mothers lose up to 60% of the fat they accumulated during pregnancy by day 14 pp (Naismith, Richardson, & Pritchard, 1982). To meet the energetic costs of milk production and to compensate for this loss of energy stores, there is a dramatic increase in feeding, with lactating rats ingesting four times more than non-nursing females (Malabu, Kilpatrick, Ware, Vernon, & Williams, 1994; Woodside et al., 2000; Woodside, 2007). These behavioral changes are accompanied by alterations in both peripheral and central energy balance signals, but precisely how each of these changes contributes to the dramatic hyperphagia of lactation remains to be elucidated (For review see Woodside et al., 2012)

Recently, considerable attention has been paid to the role of ghrelin in the stimulation of food intake. This 28-amino acid peptide is produced primarily in the fundus of the stomach and is released into the periphery at heightened levels prior to meal onset or during food deprivation; conversely, levels are decreased following the termination of feeding (Tschop et al., 2000). Central administration of ghrelin has been found to promote food intake in male rats that were both sated and fasted, resulting in increases in body weight in both groups (Date et al., 2001). In the brain, ghrelin receptors (GHSR1a) are distributed widely, with those primarily implicated in the regulation of food intake being located within the hypothalamus, more specifically the ARC, VMH, DMH and PVN nuclei (Nakazato et al., 2001; Zigman et al., 2006). Ghrelin acts at the ARC to promote NPY and AgRP expression and stimulate food intake (Nakazato et al., 2001; Goto et al., 2006). These effects are believed to be important mediations of ghrelin's orexigenic effects. Ghrelin receptors have also been located in the VTA, and administration of ghrelin into this area also increases food intake (Abizaid et al., 2006; Guan et al., 1997).

Given that lactation is characterized by increased food intake, one might suspect that it would be accompanied by changes in circulating levels of ghrelin and /or its receptors, and

several studies have examined this possibility. A recent investigation by Taylor and her colleagues (2009) compared plasma ghrelin levels between proestrus female rats and lactating rats on days 1, 5, 10, 15 and 20 pp and reported no significant differences in plasma concentrations among the groups (Taylor et al., 2009). Similarly, a study comparing lactating (between weeks 4 and 5 pp) and non-lactating women found no differences in plasma ghrelin during fasting conditions or immediately following the ingestion of meals (Larson-Meyer et al., 2010). Abizaid et al. (2008) also found that in rats, plasma levels of ghrelin in late lactation rats were similar to those of dams whose had been litters removed immediately after birth, but noted that separation of mother and young in late lactation led to a significant increase in circulating ghrelin levels (Abizaid et al., 2008).

Lactation has, however, been associated with a suckling dependent increase in GHSR mRNA expression within the pituitary as well as the hypothalamus (Abizaid et al., 2008). In addition, preliminary evidence examining GHSR mRNA within specific nuclei of the hypothalamus during lactation found higher levels in the ARC of lactating rats than in those of non-lactating rats and a trend in the same direction was found in the PVN (Budin et al., 2010).

In sum, the data suggest that increases in circulating ghrelin levels do not contribute to the hyperphagia of lactation. It is possible, however that the observed changes in ghrelin receptor levels do facilitate increased food intake at this time by increasing sensitivity to the orexigenic effects of ghrelin. Thus a series of experiments were carried out to examine possible changes in sensitivity to ghrelin in nursing rats by comparing the effects of ICV administration of various doses of ghrelin across lactation with that seen in cycling rats. The contribution of ghrelin to the hyperphagia of lactation was also investigated by comparing the ability of a ghrelin antagonist to suppress food intake between cycling and lactating rats.

Methods

Subjects

All rats used in these studies were obtained from Charles River Breeding farms, St. Constant, Quebec. Virgin female Wistar rats weighing between 220 - 240 g upon arrival were randomly assigned to either the cycling or the lactating conditions and housed in groups of 4-5.

After mating, rats were placed into individual cages where they remained for the duration of the study. All rats were maintained on a 12-hour light/dark cycle at a room temperature between 20 - 22 C. In Experiment 1 and 2 lights went on at 12:00 and went off at 24:00 h, and in Experiment 3 lights went on at 24:00 h and went off at 12:00. Pellets, powdered food (Agway Ltd. Syracuse, NY) and water were provided ad libitum throughout the experiments. Rats assigned to the lactating groups were mated by introducing a sexually experienced Wistar male into the group cage. Approximately 10-14 days later, all subjects, including virgins, were separated into clear individual plastic cages (20 X 45 X 50 cm) and provided with beta chip bedding. The Concordia University Animal Research Ethics Committee approved all procedures under the guidelines provided by the Canadian Council on Animal Care.

Drugs

Ghrelin (Piproteomics) was dissolved in saline and administered at doses of 0, 0.25 µg, 0.5 µg, and 1 µg in a volume of 2 µl. A ghrelin antagonist, JMV 3002 (Cayman Chemical) was dissolved in methyl acetate (0.25 mg/ml, 1:3 ethanol solution: PBS pH 7.2) and administered at doses of 0.4 µg in a volume of 2 µl. Pilot work indicated that the dissolving solution had no effect on any of the dependent variables that were measured.

ICV Cannula Implantation

Ten to 14 days following the introduction of the stud male to the lactating group, all subjects, including cycling controls underwent surgery to implant stainless steel cannulae into the right lateral ventricle. Subjects were anesthetized using ketamine-xylazine (5.7 mg ketamine and .86 mg xylazine/ 100 g body weight). The head was shaved covering an area slightly posterior to the eyes to the base of the skull. The subjects were then placed in a sterotaxic apparatus. A sagittal incision approximately 2.5 cm in length was made to expose the skull, which was subsequently cleaned. Four holes were drilled and three jeweller screws were placed into the skull. 22 Gauge cannulae (Plastics One Inc. Roanoke, VA) were implanted into the lateral ventricle using the following co-ordinates AP: 0.02 mm; ML: 0.16 mm; V: 0.50 mm (Paxinos & Watson, 1996), and were then fixed to the skull using dental cement.

Assessment of Cannula Placement

The correct placement of cannulae was assessed by ICV injection of angiotensin (50ng in 2 µl) at the end of the experiment. If the injection of angiotensin stimulated drinking almost immediately after administration (within one minute) and continued for at least a 30 second duration then the cannula placement into the lateral ventricle was considered accurate. Only data from rats with accurate placement of the cannula were included in the analysis.

Experiment 1: Behavioral Responses to Ghrelin Administration during Mid-lactation

The day of parturition was designated day 0 pp. Within a day of birth, subjects in the lactating group had their litters culled to 8 pups. Both lactating and cycling subjects were assigned to one of four doses (0, 0.25 µg, 0.5 µg, 1 µg ghrelin). To facilitate measurement of food intake, all rats were presented with powdered rat chow in glass food jars anchored to metal supports to prevent tipping. Food intake, body weight and, where appropriate, litter weight were monitored for 5 days following surgeries. On the appropriate day pp (or 10 days following surgery in the case of non-lactating rats), infusions were made at approximately 14:00, at a rate of 1µl/min. The injector was left in place for 1 minute after infusion to ensure full dispersal of the drug. Food intake was monitored at 1 hr and 2 hr following ghrelin or saline administration.

Experiment 2: Behavioral Responses to Ghrelin Administration during Early and Late Lactation

The general methods for this experiment were as described for experiment 1 except that on day 1 pp lactating females were assigned to either be tested in early (days 4-6 pp) or late (days 14 – 16 pp) lactation at doses of 0.25 µg or 1 µg of ghrelin. Cycling females were assigned to one of the two dose conditions. All rats received ICV injections of saline and one dose of ghrelin at approximately14:00 in counterbalanced order so that dose and reproductive state were independent factors. The effect of ghrelin on food intake was assessed by calculating the difference in intake in the 2 hrs following the saline injection and that measured 2 hrs following the ghrelin injection.

Experiment 3: Behavioural Responses to a Ghrelin Antagonist during Early and Late Lactation

The general methods used and the design were as described for Experiment 2, however, all rats were administered one dose, 0.4 µg, of the ghrelin antagonist JMV 3002 (Cayman

Chemical Company). This dose was chosen based on the results of Salome et al. (2009). Both saline and JMV 3002 were administered to all rats in counterbalanced order 1 hr prior to lights out and the effectiveness of the drug was assessed by calculating a difference score between food intake in the 2 hrs following injection of saline and that measured 2 hrs after ghrelin antagonist injection.

Statistical Analysis

In each experiment, 2-way ANOVAs with reproductive state and drug treatment being independent variables were used to compare means across groups. When appropriate pairwise analyses such as Fisher's least significant difference or t-tests were utilized to investigate significant effects. Separate ANOVAs were conducted for measures of food intake, body weight and litter weight. In experiment 1, a repeated measures ANOVA was conducted, with time (1 hr, 2 hr) as the within subject effect, while dose (0, 0.25 µg, 0.5 µg, 1 µg ghrelin) and reproductive state (cycling vs lactating) were included as between subject effects. In experiments 2 and 3, the response of either cycling, in early lactation, or late lactation to either 0, 0.25 µg, 1 µg ghrelin (experiment 2) or the ghrelin antagonist JMV 3002 at doses 0, 0.4 µg were compared (experiment 3).

Results

Experiment 1: Behavioral Responses to Ghrelin Administration during Mid-lactation

Overall, ICV ghrelin administration increased food intake such that there was a significant main effect of dose, $F(3,58) = 13.557$, $p < .001$. There was also a main effect of reproductive state, $F(1,58)$, $F = 16.238$, $p < .001$. Importantly these effects were qualified by a significant dose x reproductive state interaction, $F(3, 58) = 3.435$; $p < 0.03$. As can be seen in Figure 14, this reflected a different pattern of dose responsiveness in lactating and cycling rats. Cycling animals treated with saline (M= 1.20, SE .60) had lower food intake compared to all doses of ghrelin (0.25 µg ghrelin: M=2.96, SE = .56; 0.5 µg ghrelin: M = 3.72, SE = .60; 1.0 µg ghrelin: M =6.15, SE = .60; ps < .05. Animals treated with 0.25 µg ghrelin consumed the same amount of food as those treated with 0.5 µg ghrelin, $p = .36$. However, animals treated with 1 µg ghrelin consumed more food than animals treated with the two lower doses of ghrelin, ps < .01.

For lactating rats a different pattern emerged. Specifically rats treated with saline (M = 2.73, SE = .72) consumed less food than all doses of ghrelin (0.25 µg ghrelin: M = 6.61, SE = .72; 0.5 µg ghrelin: M = 6.36, SE = .68; 1.0 µg ghrelin: M = 5.79, SE = .72), all ps < .01, while there were no differences in food intake between rats treated with different doses of ghrelin, all ps > .41. Thus lactating rats exhibited the same degree of behavioural sensitivity to ghrelin in regard to food intake at the lowest dose as that of the highest.

In addition, as the data in Figure 14 show, rats in all groups increased their food intake from hour 1 to hour 2 with a significant main effect of time $F(1,58) = 62.905$, $p < .001$. This effect was greater in the lactating group with a significant time X group interaction ($F(1,58) = 9.363$, $p = 0.03$, reflecting the overall greater intake in the lactating group compared to the cycling animals (significant main effect of group ($F(1,58) = 16.238$, $p<0.001$).

Experiment 2: Behavioral Responses to Ghrelin Administration during Early and Late Lactation

Rats in all groups ate more following ghrelin administration than after saline injection ($p<0.05$). However, no significant effects of either reproductive state or dose of ghrelin were observed (Figure 15).

Experiment 3: Central Administration of the Ghrelin antagonist

As can be seen in Figure 16, the analysis revealed a trend for reproductive state, $F (2,23) = 2.753$, $p = .085$. Pairwise comparisons for these effects showed that the ghrelin antagonist produced a greater reduction in food intake in rats on days 14-15 pp than in cycling rats ($p < .04$), while cycling and rats in early lactation did not differ on this measure. There was also a trend toward the day 14 pp group exhibiting a greater reduction of food intake than rats tested on day 4 pp ($p = .075$).

Discussion

The aim of this study was to investigate whether changes in central responsiveness to the orexigenic actions of the gastric peptide ghrelin might contribute to the increased food intake seen in lactating rats and whether these responses would change as a function of stage of lactation. This possibility was examined in three experiments in the first of which the food intake response of cycling rats and lactating rats on day 10 pp was compared across 4 doses of

ghrelin (0, 0.25 µg, 0.5 µg and 1µg) administered ICV, in the second the increase in food intake of rats in early and late lactation to that of cycling rats to the ICV administration of 0.25 µg and 1 µg ghrelin was compared. In the final experiment the effect of administering a ghrelin antagonist (0.4 µg JMV 3002) in early and late lactation was compared with its effect in cycling rats.

As expected, ICV ghrelin administration increased food intake in both lactating and cycling rats. Interestingly however, the dose response curve of this relationship differed markedly between cycling and lactating rats. Rats on day 10 of lactation showed a similar food intake response to the 0.25 µg ghrelin dose as they did to the 0.5 µg and 1.0 µg ghrelin doses whereas food intake in virgin rats increased with higher ghrelin doses. These data suggest an increased sensitivity to ghrelin at this point in lactation and are consistent, at least in part, with the observations of Crowley et al. (2007) who demonstrated that chronic (2 day) administration of a ghrelin antagonist reduced NPY and AGRP mRNA expression in the arcuate nucleus of lactating but not cycling females, although, in their study, this manipulation reduced food intake in both groups.

An increase in behavioural sensitivity to ghrelin would be consistent with preliminary data suggesting that suckled dams showed increased expression of GHS-R1a mRNA in both hypothalamic and extra hypothalamic nuclei on day 15 pp (Budin et al., 2010). An increase in ghrelin receptor sensitivity might also explain the lack of relationship between the hyperphagia that is typical of lactation and circulating ghrelin levels. For example, Shibata and colleagues (2004) reported that on day 8 pp lactating dams exhibited plasma ghrelin levels that were significantly lower than those of a control group that had been weaned of pups at parturition. Abizaid et al., (2008) reported that at the time of peak food intake, day 15 pp, that plasma ghrelin concentrations were similar to those of pup weaned controls.

Ghrelin originates in the peripheral systems with this hormone being produced and released primarily by the gastrointestinal system into general circulation, with this process being initiated by excitation from the vagus nerve (see Yin et al., 2009). Given that circulating ghrelin levels are typically increased in response to fasting or in anticipation of the onset of food intake, it is perhaps not surprising that circulating ghrelin levels should be decreased or equal to cycling

rats in a reproductive state associated with hyperphagia such as lactation. In addition, one of the behavioural adaptations exhibited by rats to the foraging demands of lactation is an increase in meal number. This includes an increased tendency to eat during the light phase of the cycle (Munday & Williamson, 1983). As a result increased presence of food in the stomach might contribute to the maintenance of low levels of ghrelin during lactation.

Surprisingly, in Experiment 2, when the effect of ghrelin on food intake on day 4-5 pp and days 14-15 pp was compared to that produced in cycling rats, no differences were observed. It is possible that the increased response to ghrelin that we observed in lactating rats on day 10 pp in Experiment 1 is a function of the particular energetic and hormonal state of postpartum females at this time that might result in greater availability of receptors for activation by exogenous ghrelin. One might suppose, for example, that the increase in hypothalamic GHS-R1a observed at the end of the second week postpartum is not present in early lactation. Moreover, the failure to detect an effect in late lactation, a time at which food intake is approximately 350% of that seen in cycling rats might reflect the fact that the orexigenic system is already at a high level of stimulation (Suzuki et al., 2010; Higuchi et al., 1985).

If the failure to find an increased effect of exogenous ghrelin administration in late lactation were indeed due to a high level of endogenous ghrelin receptor activation at that time, then one might expect a greater effect of antagonist administration during this nursing state. Thus in experiment 3, the contribution of ghrelin to the increased food intake of lactating rats was investigated by administering the ghrelin antagonist JMV 3002 to cycling rats and rats in early or late lactation. Salome et al. (2009) demonstrated that administering this compound centrally prevents the hyperphagia that is typically observed following fasting or ghrelin treatment. The results of this study revealed that central administration of a ghrelin antagonist resulted in a marginally significant greater reduction in food intake in late lactation dams compared to cycling animals; whereas no significant difference was observed between the cycling and early lactation groups. These data are consistent with the idea that ghrelin makes a greater contribution to food intake at the time of peak milk production than it does earlier in lactation when the energetic demands of milk delivery are smaller.

Together, the results of this experiment, although preliminary, suggest that ghrelin does contribute to the hyperphagia of lactation, but that its effects vary across this highly dynamic stage of the life cycle such that responsiveness to exogenous administration of ghrelin or to administration of a ghrelin antagonist is similar in early lactating and cycling females. By the middle of the second week pp, however, sensitivity to exogenous ghrelin administration is increased. Finally in late lactation endogenous ghrelin is making a more significant contribution to food intake than it does to in rats in the early postpartum period, thus exogenous ghrelin administration can stimulate no further increase in food intake. Whether these changes in responsivity are a result of alterations in hypothalamic receptor levels and/or circulating ghrelin levels across lactation or to changing levels in other modulators of energy balance such as leptin remains to be determined. Clearly more studies are needed both to support the pattern of results obtained here and to clarify when ghrelin receptor levels increase in lactation.

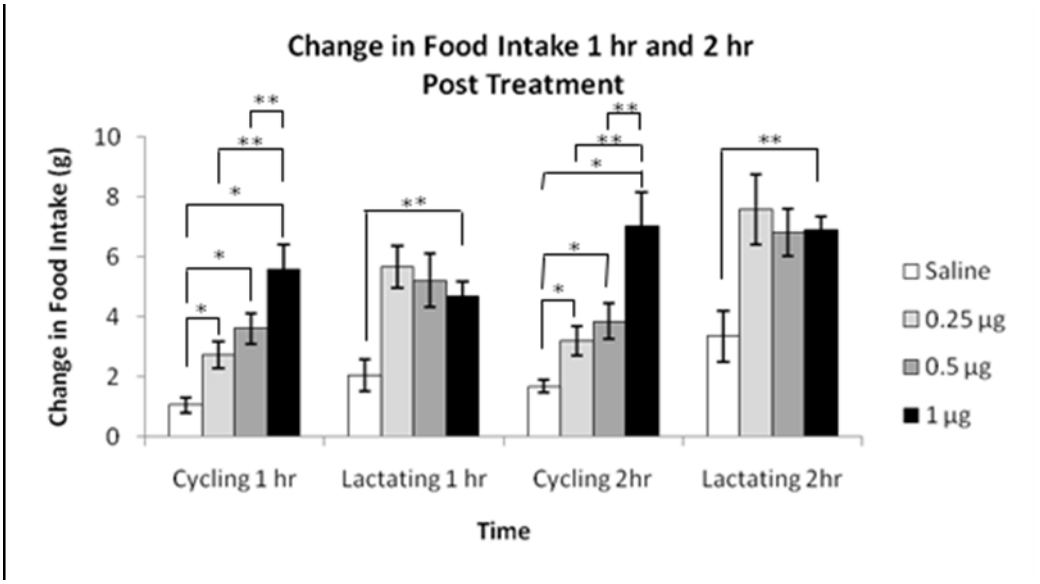

Figure 14. Mean (+/- SEM) change in food intake (g) of animals in grams following central administration of ghrelin. Ghrelin increased food intake in both groups and at all doses in both lactating and cycling rats when compared to saline treated animals. Rats in the lactation group ate more than those in the cycling group at the 0.25 µg and 0.5 µg doses after two hours ($p < .001$). * $p < .05$, ** $p < .01$.

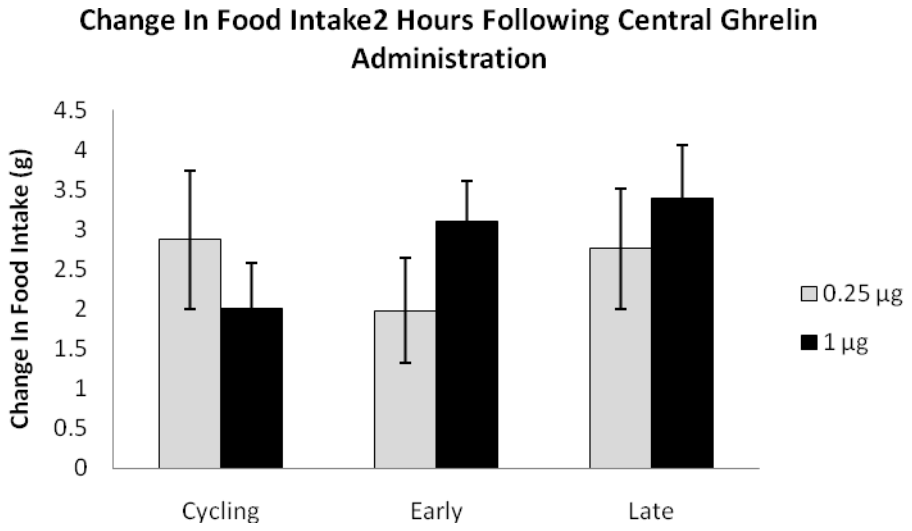

Figure 15. Mean (+/- SEM) change in food intake of animals in grams following central administration of ghrelin. Ghrelin increased food intake in all groups. Both doses of ghrelin produced a significant increase in food intake compared to saline treatment ($p < .05$).

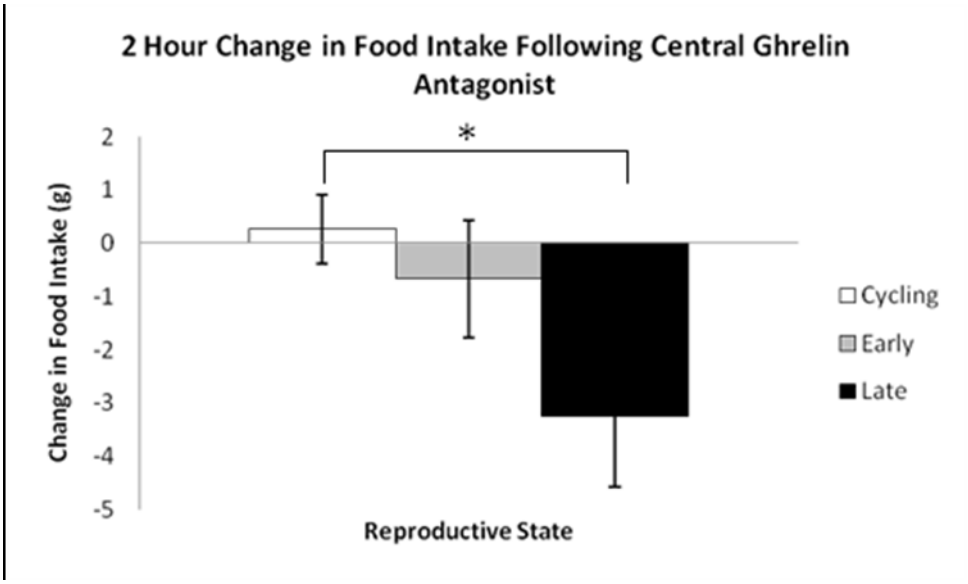

Figure 16. Mean (+/- SEM) change in food intake of animals in grams two hours after central administration of ghrelin antagonist. There was a statistical trend toward a main effect for reproductive state ($p = .085$). Post hoc comparisons indicated a greater reduction in food intake for the late lactation animals when compared to cycling rats ($p < .04$), and there was a trend in this direction when early and late lactation rats were compared ($p = .075$). * $p < .05$.

GENERAL DISCUSSION

The postpartum period in the rat is characterized by a myriad of behavioural and physiological changes, but here the focus will be on the energetic demands and associated adaptations of this time. The behavioural profile of lactation represents a unique period in the animal's life, reflected by an immense consumption of food. However, how this increase in energy demand is associated with neuroplastic/hormonal changes remains elusive (Woodside, 2006). The aim of the preceding studies was to understand behavioural and central signaling changes in response to the administration of various neuropeptides (leptin, MTII, ghrelin, ghrelin antagonist) associated with energy balance across the lactational period and in cycling rats. Several hypotheses were forwarded given previous research findings. This included the prediction that acute central administration and chronic peripheral treatment of leptin during early lactation would not influence food intake or that this would be somewhat reduced compared to cycling rats. In line with this it was reasoned that PSTAT3 activation would be reduced during the early postpartum period. Finally ghrelin was expected to increase behavioral sensitivity given increased GHS-R mRNA during mid-lactation.

Experiments from the first chapter demonstrated that central leptin administration decreased food intake and body weight in all groups, with food intake demonstrating a dose dependent effect. Interestingly, rats in early lactation exhibited a smaller reduction of food intake in response to the lowest dose of leptin than those in the late lactation group, suggesting reduced sensitivity to leptin in the first week after parturition. Consistent with these findings, central leptin administration induced greater PSTAT3 immunoreactivity in the areas of the ARC and VMH, together with a trend towards increased activation in the PVN. In contrast, peripheral leptin treatment did not affect any parameters measured suggesting resistance outside the CNS.

A major neural pathway through which leptin suppresses food intake is believed to occur through the activation of POMC neurons in the ARC resulting in the release of the anorectic peptide α-MSH. To determine whether lactation was associated with any changes in this respect, we compared the behavioural response of cycling and lactating rats to MTII, a synthetic analog of α-MSH. It was revealed that both doses of MTII produced significant reductions in food

intake, body and litter weights compared to control animals during early and late lactation.

Ghrelin increased food intake in both cycling and animals across lactation. However, during mid-lactation, dams exhibited increased behavioural sensitivity to ghrelin administration because the lowest dose of ghrelin used here induced as great an increase in food intake as the highest, while cycling animals showed a dose dependent response. The ability of ghrelin antagonist JMV 3002 to reduce food intake was greater in late lactating than in cycling rats, suggesting that ghrelin makes a more important contribution to food intake in late lactating rats than in either early lactating or cycling females.

Unlike late pregnancy which is characterized by a complete state of central leptin resistance, rats in early and late lactation responded to both doses of leptin used here, although there was some attenuation of the behavioural response to the lower dose during early lactation. The maintenance of leptin responsivity during lactation is consistent with previous work conducted in our lab in which chronic ICV leptin administration from days 8 to 15 pp decreased food intake and body weights of dams and litters (Woodside, Abizaid, & Walker, 2000). It is also in line with the finding in the current studies that the ability of leptin to induce phosphorylation of STAT3 is maintained during lactation.

The dramatic differences in response to leptin between pregnant and lactating rats probably reflects the difference in hormonal and metabolic state in these conditions. Although both reproductive states are associated with hyperphagia, fundamental differences emerge in energetic demands. During gestation the animal builds fat stores, while lactation requires the use of these energy reservoirs to address litter feeding demands (Naismith et al., 1982). Since leptin is produced mainly by white adipose tissue, increases in fat that accompany pregnancy increase plasma leptin compared to the cycling state (Seeber et al., 2002). In contrast, leptin plasma decreases during lactation such that levels are equal or lower than virgins due to use of fat stores (Woodside, Abizaid, & Walker, 2000; Suzuki et al, 2014). This leptin non-responsiveness during gestation allows for fat build up despite high leptin in preparation for motherhood. Our findings suggest lactation does not require central leptin resistance to the same degree as gestation since the energy demands of litter reduces leptin levels via the depletion of fat. This renewed leptin

responsiveness seen in early lactation from the resistance in gestation may be adaptive in cases of the sudden loss of a litter which would allow the dam to quickly return to a cycling state and increase chances of reproductive success since leptin resistance produces reproductive abnormalities (Hausman, Barb, & Lents, 2012). Reduced responsivity to leptin in the early lactation period may represent residual resistance brought over from late pregnancy, although this is less likely given our central signaling findings indicating continued responsiveness in the JAK/STAT pathway as observed through PSTAT3-ir. It more probably reflects decreased leptin sensitivity to lower doses rather than outright resistance. A similar pattern of response was reported in an early study by Widdowson (1997) who showed that diet-induced obese rats showed only an attenuated response to central leptin administration. Our finding that animals during early lactation seem to match those those by Widdowson (1997) who present a diet-induced obesity model whereby animals remain responsive to central leptin, albeit in an attenuated way, in contrast late lactation animals that present as fully responsive to leptin. Decreased sensitivity to leptin during early lactation occurs in parallel with increased release of various orexigenic peptides (such as orexin, AgRP, NPY, and MCH), including prolactin (as a result of pup suckling) (Smith, True, & Grove, 2011). Notably, prolactin has been shown to increase feeding and body weight, while inhibiting leptin signaling within various hypothalamic nuclei (Augustine & Grattan, 2008; Gerardo-Gettens, 1989; Li et al., 1995; Naef & Woodside, 2007; Stern & Horwitz, 1989; Suave & Woodside, 1996; Sauve & Woodside, 2000). The maintenance of leptin responsiveness during early lactation might be considered surprising given that this is a time of high circulating prolactin levels and this has been associated with leptin resistance in both cycling and late pregnant rats. It is possible however, that the induction of leptin resistance by prolactin depends on chronically high activation of the prolactin receptors.

The leptin resistance that begins during mid-pregnancy is likely driven by the chronic increase in placental lactogens that are secreted in the latter half of pregnancy. By contrast prolactin secretion during lactation is pulsatile. The necessity for chronic prolactin receptor activation to induce this state was demonstrated by Augustine and Grattan (2008), who showed that neither the bidaily prolactin surges typical of early pregnancy nor bidaily prolactin

administration were sufficient to induce leptin resistance. The ability of prolactin to induce leptin resistance has been linked to co-expression of prolactin and leptin neurons within the MPOA and ARC (Nagaishi et al., 2014). These authors proposed that coupled neurons could be the way by which prolactin and leptin could influence one another during pregnancy, with high levels of the former affecting the latter hormone by way of increased SOCS expression, leading to impairment in leptin's effectiveness (Nagaishi et al., 2014). If this is the mechanism by which resistance occurs, than examining levels of prolactin receptors during pregnancy and early lactation might explain differences in leptin responsiveness.

Indeed Augustine et al., (2003) found that prolactin mRNA expression in the ARC was higher in late pregnant than cycling rats but that levels did not differ between cycling and rats at the end of the first week of lactation, while day 7 pp (closely approximating our early lactation period) did not differ on this measure (Augustine, Kokay, Ladyman, & Grattan, 2003). It is possible then, that differences in leptin responsiveness between pregnant and lactating rats depends in part on prolactin receptor density in the hypothalamus.

In contrast to intact central responsiveness, our findings demonstrate that in the postpartum period, as in late pregnancy, peripheral leptin administration does not reduce food intake. Chronic subcutaneous administration of leptin at a dose shown in pilot work was found to reduce food intake in cycling females and twice previously found to induce reductions in both feeding and body weight in cycling rats (Crowley et al., 2004), failed to reduce food intake in lactating rats in both early and late lactation. The current results are similar to those of early reports in which administration of leptin (500 and 400 ng/ul/hr subcutaneously) in mid-lactation (days 8-9 pp and days 8-10 pp respectively) was shown to induce plasma leptin to levels higher than those of cycling rats but did not alter food intake, or body weight (Xu et al., 2009; Crowley et al., 2004). The current results extend these earlier findings by showing that a similar state of peripheral leptin resistance is also present in both early and late lactation. This lack of response to peripheral leptin administration is probably not absolute because earlier work from our lab found that chronic subcutaneous leptin at twice the concentration used in the current study from days 8 to 22 pp reduced the body weights of dams while not affecting feeding or pup growth

(Woodside, Abizaid, & Walker, 2000).

Peripheral leptin resistance during gestation and the pp period at the concentration presented here may suggest a common underlying mechanism. There some evidence that lactogens may prevent leptin from exerting its effect by altering the permeability of the BBB, with Banks (1999) proposing that leptin resistance may involve dysfunction of leptin transporter mechanisms (Banks, DiPalma, & Farrell 1999). There are several lines of evidence to support the hypothesis that prolactin may alter the permissiveness of the BBB to leptin and thereby contribute to peripheral resistance. For example, individuals presenting with pituitary tumors which cause hyper-secretion of prolactin have increased incidents of obesity (Greenman, Tordjman, & Stern, 1998). Furthermore, one study reported a group of individuals with such tumors having unchanged levels of leptin within the CSF despite high leptin plasma levels (Poiana et al., 2005).

Possible mechanisms are more apparent if the structure of the BBB is examined. The capillaries of the cerebrum are lined by endothelial cells with membranes that are conjoined with many types of proteins (Hawkins & Davis, 2005). Together these form close connections which act as a natural barrier to various substances gaining entrance into the central nervous system (CNS) from the blood (Guerra, et al., 2011). A study by Rosas-Hernandez (2013) and colleagues found that the presence of prolactin effected tight junctions of the BBB by increasing the expression proteins and reducing permeability (Rosas-Hernandez et al, 2013). Although central leptin resistance develops during gestation, evidence suggests that the BBB may aid this process further by preventing the high levels of plasma leptin from crossing into the brain as pregnant rats were found to have lower levels of CSF leptin during gestation day 13 and gestation day 18 compared to cycling rats. The authors suggested that prolactin may be responsible for this effect since high levels of this hormone were shown to decrease leptin translocation in an in vitro BBB model (Trujillo et al., 2011).

Although our results support the theory that lactation is a state of peripheral leptin resistance, further examination is required to determine if there is a prolactin induced change in BBB permeability to leptin. Such an investigation could utilize radioactively labeled leptin and

administer it peripherally to determine levels crossing the BBB into the CNS (Banks et al., 1999; Price et al., 2010). By examining differences in transportation of leptin this way, prolactin induced changes in BBB permeability could then be compared during different reproductive states as well as in cycling animals chronically treated with prolactin (Noel & Woodside, 1993).

Progesterone may also influence BBB permeability. In an model of cerebral ischema, Wang and colleagues were able to alter BBB permeability to inflammatory agents with peripheral administration of progesterone. Specifically progesterone increased the expression of claudin 5 and altered the integrity of the BBB (Wang, Jaing, Li, Liu, Cheng, & Hao, 2009). As with prolactin, serum progesterone is notably elevated during pregnancy, drops after birth and begins to rise until peaking on day 12 of lactation (Valdez, Penissi, Deis, & Jahn, 2007). Progesterone may alter BBB permeability by preventing passage of leptin into the brain from the periphery. Progesterone might also influence the release of leptin such that adipose tissue maintained in a medium treated with progesterone for 72 hours exhibited reduced leptin release compared to controls (Abelenda & Puerta; 2004).

Progesterone may also reduce the central effectiveness of leptin. Chronic administration of progesterone to female rats resulting in levels that resemble late pregnancy are associated with increases in food intake and body weight despite similar CSF leptin levels in both groups. Interestingly a study carried out by Augustine and Grattan found that nine days of pseudopregnancy in addition to progesterone implants failed to induce resistance to ICV leptin, but that the later addition of prolactin produced this effect (Augustine & Grattan, 2008). The difference in findings between these two studies likely reflects differing methodologies, however there is some suggestion that progesterone alone or in combination with prolactin can change central sensitivity to leptin and encourage hyperphagia.

A role for progesterone in inhibiting the actions of leptin is also consistent with the metabolic and hormonal profile of Koletsky rats, which have a single gene mutation strain that results in obesity, hyperleptinemia and increased serum progesterone levels. These rats exhibit peripheral and central leptin resistance as well as the upregulation of ObRb, NPY, and AgRP mRNA. In contrast, anorexigenic neuropeptide mRNA such as CART, POMC and α-MSH are

noted to be reduced in these animals (Keen, Rhinehart, Kalra, & Kalra 2004). A lack of leptin response in these mutant animals is apparent by the failure of ObRb receptors to respond to leptin and this likely contributes to changes in hypothalamic signaling described above; but the implications of increased circulating levels of progesterone, to either hyperphagia or changes in hypothalamic neuropeptides have not been investigated in this animal model.

Given that progesterone has been found to cross the BBB without issue, it may be able to exert effects within the hypothalamus (Pardridge, Lawrence, & Mietus; 1979). Although limited, there is some evidence showing that chronic peripheral progesterone treatment in mice increases concentrations of the hormone centrally while altering central expression of NPY receptors within the medial amygdala by way of its metabolite allopregnanolone (Ferrara et al. 2001). Furthermore, progesterone has been found to increase NPY mRNA within the hypothalamus (O'Conner, Wade, Brann, & Mahesh, 1995) and receptors for this hormone have been localized within this nucleus (Genazzani et al 2000). One study investigated the possibility of progesterone increasing the expression of NPY, Agrp, and leptin mRNA by treating mouse hypothalamic explants with progesterone for 24 hours. Although no changes emerged, if slices were treated with progesterone for the same length of time observed in pregnancy or lactation perhaps differing results may have been produced (Olofsson, Pierce, & Xu, 2009). Finally progesterone and NPY immunoreactive neurons have been noted to overlap within the ARC of sheep, suggesting such colocalization may encourage the hyperphagia that is observed in both gestation and lactation. However at this time it is not known if similar neuronal coupling exists in rats (Dufourny, Caraty, Clarke, Robinson, & Skinner, 2005). Bringing these ideas back to the current study, given that progesterone begins to rise after birth and there is some evidence to show that it may suppress the effectiveness of leptin, this may contribute to the attenuated effect to central leptin in early lactation that was noted in our findings thus allowing for hyperphagia. It may also interfere with leptin being released by adipose tissue, and finally might reduce its ability to cross the BBB. In line with this, one would expect that once progesterone begins to decline on day 12 pp, that there would be a restoration of leptin responsiveness, which was demonstrated in late lactation within the context of our findings.

Our results also revealed that MTII is a potent suppressor of food intake and body weight in cycling animals and lactation. This represents a replication of findings by Chen et al (2004) who reported direct infusion into the DMH resulted in decreased food intake in both cycling females and in rats during mid-lactation (pp day 9) (Chen, Williams, Grove & Smith, 2004). We extend their findings to include both early and late lactation. This represents an important finding since it highlights the continued responsiveness of second order neurons during lactation and indicates that the attenuation of reduced food intake in response to leptin observed during early lactation relative to other states is not driven by this part of the central energetic pathway. Unsurprisingly, MTII also affected the litter weight of pups presumably through its ability to suppress feeding and body weight gain in their mothers.

Findings from the second chapter revealed that ghrelin acted as a significant stimulator of food intake across all reproductive states. This partially contradicts our prediction that lactating rats would respond with increased sensitivity to ghrelin compared to cycling females. Instead, early and late lactating animals exhibited similar behavioural responses to the various doses of ghrelin when compared to cycling rats since all animals increased their food intake in response to doses administered. In regard to cycling females, this represents a replication of previous research which has found that ICV ghrelin administration increased food consumption (Clegg et al., 2007; Sakurazawa, Mano-Otagiri, Nemoto, & Shibasaki, 2013). Furthermore, although difficult to compare, our results are similar to findings reported by Nakahara and colleagues (2003) who also treated dams with ghrelin (peripherally) and discovered feeding increased compared to controls during the first eight days pp (overlapping with our early lactation period; Nakahara et al., 2003).

Although it is clear that ghrelin acts to increase food intake across lactation, in line with our hypothesis, the results of the first experiment suggests that mid-lactation represents a unique time in relation to ghrelin's impact on energy balance and feeding regulation. Thus mid-lactation may represent a time of increased ghrelin sensitivity since lactating animals receiving a 0.25 µg dose consumed as much as those being given the two higher doses, as opposed to cycling animals who exhibited a dose-response relationship. Why mid-lactation represents a period of

increased ghrelin sensitivity for dams is not entirely clear. However this does correspond with a unique up-regulation of NPY mRNA within the ARC and may provide indirect evidence of increased sensitivity that would directly impact NPY since the majority of ghrelin neurons contain NPY responsive ones (Kohno, Gao, Muroya, Kikuyama, & Yada, 2003; Woodside, Budin, Wellman, & Abizaid, 2012). Previous work revealed that despite plasma ghrelin levels being similar to non-lactating females, those dams sacrificed on day 15 pp have higher pituitary and hypothalamic concentrations of GHS-R 1a transcript relative to cycling rats and litter removed dams (day 13 pp) sacrificed on day 15 pp (Abizaid, Schiavo, & Diano, 2008). While removal of the litter results in a significant reduction in GHS-R la transcript within a 48 hour period, it is unclear when upregulation of these receptors begins during lactation. With the findings of receptor upregulation by Abizaid et al., (2008), an increased sensitivity to ghrelin at the lower dose during late lactation might be expected; however this was not the case. Despite not finding an increase in behavioural sensitivity to ghrelin during early or late lactation when compared to cycling rats, the results from the ghrelin antagonist study point to late lactation being unique in that dams during this time responded with an increased reduction in feeding compared to cycling rats and there was a trend toward this when compared to early pp animals. This supports the contention that blocking the effects of ghrelin by way of an antagonist had a greater effect in late lactation than at other times and is likely driven by the increases of hypothalamic concentrations of GHS-R 1a transcript. Our findings are also supported by differences in GHSR mRNA fold change in the ARC during lactation such that this was significantly increased on day 15 pp compared to virgins, while there was a trend in this direction within the PVN of lactating rats, which in the presence of ghrelin would encourage increases in feeding, and when being blocked via an antagonist, would decrease such behaviour (Budin, Wellman, Abizaid, & Woodside, 2010).

Another consideration is that estrogen might interfere with ghrelin sensitivity in late lactation. For example, Clegg (2007) and her colleagues compared intact females (fluctuating estrogen levels) to ovariectomized (absence of estrogen) rats in their responses following various centrally administered doses of ghrelin (0.01 nmol, 0.1 nmol, and 1 nmol). The authors reported

that intact females responded to the two highest doses with similar amounts of food intake, while feeding levels were the same as controls at the lowest. In contrast, ovariectomized rats increased food intake to all doses relative to saline controls. From this it was suggested that estrogen within intact cycling females reduced ghrelin's potency (Clegg et al., 2007). In lactating animals, estrogen is significantly decreased on day 2 pp and slowly increases until day 10 pp when it reaches what has been observed to be the lowest levels in cycling females. Beyond this, estrogen continues to increase until concentrations compare to females in diestrus-2 (Smith & Neill, 1977; Woodside, 2007). In our experiment the slow increase in estrogen after day 10 pp may have contributed to the absence of increased sensitivity to ghrelin in late lactation as was noted in mid-lactation. The reason for the lack of differential sensitivity to ghrelin during early lactation in comparison to cycling animals in the face of our mid-lactation findings is less apparent. One hypothesis would be that increased sensitivity during mid-lactation may take some time to develop, requiring a reduction of fat stores. A clear answer will require concentrations of GSH-R 1a to be examined throughout lactation to test this idea.

Our findings represent the first study of its kind to centrally administer a ghrelin antagonist to female rats during various reproductive states. The only other study to administer a ghrelin antagonist to cycling female rats (who had undergone a sham operation) did so by administering it in the periphery via daily subcutaneous injections for a week. Even though within group differences were not examined specifically, their data seem to suggest that the sham operated cycling females increased their feeding following this treatment (Abdel-Hakim, Ibrahim, Ibrahim, & Ibrahim, 2014). These results bear closer resemblance to what was reported by Salmone (2009), whose team noted food intake in male rats following an acute central treatment with a ghrelin and followed subsequently by a ghrelin antagonist stopped increases in food intake that are typical of ghrelin administration. What is of interest is our discovery that late lactation resulted in a reduction of food intake when compared to cycling animals. In light of Abizaid's (2008) report of increased GSH-R 1a receptors in late stage nursing dams relative to non-lactating females, perhaps our findings of reduced food intake in the former case is a reflection of these same changes. The absence of such an effect in early lactation is still not

clear, but can be better understood once the concentrations of GSH-R 1a receptors are characterized throughout the nursing period. Future research examining ghrelin antagonists should administer a wider range of doses to across lactation as our treatment may have too low to produce an effect in early pp. Finally, given our findings from the effects of ghrelin during mid-lactation, prospective studies should target this time given this period's unique sensitivity to ghrelin.

When one examines the data presented in this dissertation as a whole, it appears as though separate mechanisms within the postpartum rat are acting in parallel to ensure that the energetic demands of lactation are met. A multiple system adaptation to support hyperphagia appears to be similar to what occurs during pregnancy. For example during gestation, the effects of leptin, administered at pharmacological levels, fail to reduce food intake or result in relevant signaling and similar lack of a behavioural or signaling response following central α-MSH administration, the second leg of the anorectic pathway suggests that multiple steps on this cascade are affected (Ladyman et al., 2016). A similar change in the mechanisms underlying energy balance occurs during the nursing period. Previous work has demonstrated a number of alterations that include increases in hypothalamic NPY mRNA (from mid to late pp) and AgRP mRNA (days 5, 12, 13, 15 pp), as well as reductions of POMC mRNA (early and late pp) to encourage feeding (Garcia et al., 2003; Ladyman et al., 2008; Chen et al., 1999; Mann et al., 1997; Smith, 1993; Suzuki et al., 2014). These changes may reflect peripheral resistance to leptin that is apparent during gestation and in the pp period. This form of resistance likely aided by changes to the blood brain barrier resulting in a reduction in leptin transport. The results of the current studies suggest that other facets of the central orexigenic and anorexigenic systems appear to be working sequentially to encourage the increase in food intake observed after parturition. Thus initial hyperphagia is encouraged by the attenuated behavioral effects of central leptin in early pp (as seen in Chapter 1), which corresponds to a time when endogenous leptin levels are similar to those of cycling rats and are at their relative peak during lactation; therefore this represents a systems response to this internal climate. Although our PSTAT3 study failed to identify the mechanisms underlying this attenuated behavioural response to leptin, it is

possible that other signaling pathways might exhibit evidence of attenuated signaling and might represent the mechanism behind the changes observed in our results (Woodside et al., 2012). Our findings of central leptin responsiveness in late lactation (pp day 15) correspond to a time when fat stores are at their lowest and represents the energetic systems adjustment to low endogenous plasma levels of leptin such that the rat becomes once again reactive to this hormone as resistance is no longer necessary (Woodside et al., 2000). This is also a time when the hyperphagia of lactation is at its peak (Abizaid et al, 2008).

In parallel to these changes in the anorectic pathways, the orexigenic system as represented by ghrelin adjusts such that hypothalamic GHS-R1a receptors upregulate during early lactation, possibly in response to low levels of this hormone. This upregulation allows for increased behavioural sensitivity to ghrelin beginning at around mid-lactation as observed from the results described in Chapter 2. During late lactation, when hyperphagia is at its peak, although plasma ghrelin levels are low, the increased concentrations of GHS-R1a mRNA levels allow for low endogenous levels of ghrelin to take maximal effect. The absence of an enhanced behavioural response to exogenous ghrelin in late lactation as seen in mid pp likely reflects GHS-R1a being at maximal endogenous occupancy since this is a time of heightened feeding. The pronounced reduction of food intake in response to a ghrelin antagonist during late lactation as shown by our study offers evidence of such an idea. Suckling induced prolactin release appears to play an important role in the regulation ghrelin since removal of pups during late lactation significantly increases ghrelin plasma levels while reducing GHS-R1a mRNA (Abizaid et al, 2008). The involvement of multiple systems and mechanisms to encourage feeding during lactation ensures the survival of the offspring throughout lactation. Although insulin was not formally examined in this thesis, it represents another possible mechanism by which the the hyperphagia of lactation might be encouraged (Woodside et al., 2012).

The aim of this dissertation was to examine the role of orexigenic and anorexigenic neuropeptides in the context of the energetic challenges brought on by motherhood and to investigate the effects these compounds have on maternal behavioural responses throughout lactation. Answers to these questions were sought given the contradictory results in the literature

driven in part by differing methodologies (doses, reproductive states, routes of administration) as well as the rapidly changing hormonal and neural states of rats throughout lactation. In an attempt to resolve the differences in previous findings, we administered leptin, MTII, ghrelin at different doses (with the exception the ghrelin antagonist) across the lactational period (mainly during early and late pp and including mid-lactation for ghrelin). Findings suggest that although animals respond similarly and consistently to certain neuropeptides across lactation, that there are notable differences in responses to others. Future studies examining other anorectic and orexigenic peptides during lactation would benefit from taking such an approach as it allows for better understanding of the effects of such compounds on behaviour and physiology throughout this time and might better point to underlying mechanisms.

REFERENCES

Abdel-Hakim, S.M., Ibrahim, M.Y., Ibrahim, H.M., & Ibrahim, M.M. (2014). The effect of ghrelin antagonist (D-Lys3) GHRP-6 on ovariectomy-induced obesity in adult female albino rats. *Endocrine Regulations, 48(3)*, 126-134.

Abelenda, M., & Puerta, M. (2004). Leptin release is decreased in white adipocytes isolated from progesterone-treated rats. *Endocrine Research, 30(3)*, 335-342.

Abizaid, A., Gao, Q., & Horvath, T.L. (2006). Thoughts for food: brain mechanisms and peripheral energy balance. *Neuron*, *51(6)*, 691-702.

Abizaid, A., Liu, Z.W., Andrews, Z.B., Shanabrough, M., Borok, E., Elsworth, J.D., Roth, R.H., Sleeman, M.W., Picciotto, M.R., Tschop, M.H., Gao, X.B., & Horvath, T.L. (2006). *Journal of Clinical Investigation, 116(12)*, 3229-3239.

Abizaid, A., Schiavo, L., & Diano, S. (2008). Hypothalamic and pituitary expression of ghrelin receptor message is increased during lactation. *Neuroscience Letters*, *440(3)*, 206-210.

Adrian, T.E., Allen, J.M., Bloom, S.R., Ghatei, M.A., Rossor, M.N., Roberts, G.W., Crow, T.J., Tatemoto, K., & Polak, M. (1983). *Nature*, *306(5943)*, 584-586.

Amenomori, Y., Chen, C.L., & Meites (1970). Serum prolactin levels in rats during different reproductive states. *Endocrinology*, *86*, 506-510.

Amico, J.A., Thomas, A., Crowley, R.S., & Burmeister, L.A. (1998). Concentrations of leptin in the serum of pregnant, lactating, and cycling rats and of leptin messenger ribonucleic acid in rat placental tissue. *Life Sciences*, *63(16)*, 1387-1395.

Anderson, S.T., Barclay, J.L., Fanning, K.J., Kusters, D.H., Waters, M.J., & Curlewis, J.D. (2006). Mechanisms underlying the diminished sensitivity to prolactin negative feedback during lactation: reduced STAT5 signaling and up-regulation of cytokine-inducible SH2 domain-containing protein (CIS) expression in tuberoinfundibular dopaminergic neurons. *Endocrinology, 14(3),* 1195-1202.

Anderson, G.M., Beijer, P., Bang, A.S., Fenwick, M.A., Bunn, S.J., & Grattan, D.R. (2006). Suppression of prolactin-induced signal transducer and activator of transcription 5b signalling

and induction of suppressors of cytokine signalling messenger ribonucleic acid in the hypothalamic arcuate nucleus of the rat during late pregnancy and lactation. *Endocrinology, 147(10)*, 4996-5005.

Arumugam, R., Fleenor, D., & Freemark, M. (2007). Lactogenic and somatogenic hormones regulate the expression of neuropeptide Y and cocaine- and amphetamine-regulated transcript in rat insulinoma (INS-1) cells: interactions with glucose and glucocorticoids. *Endocrinology, 148(1)*, 258-267.

Augustine, R.A., & Grattan, D.R. (2008). Induction of central leptin resistance in hyperphagic pseudopregnant rats by chronic prolactin infusion. *Endocrinology*, 149(3), 1049-1055.

Augustine, R.A., Kokay, I.C., Andrews, Z.B., Ladyman, S.R., & Grattan, D.R. (2003). Quantitation of prolactin receptor mRNA in the maternal rat brain during pregnancy and lactation. *Journal of Molecular Endocrinology, 31(1)*, 221-232.

Banks, W.A. (2012). Brain meets body: the blood-brain barrier as an endocrine interface. *Endocrinology*, *153(9)*, 4111-4119.

Banks, W.A., Coon, A.B., Robinson, S.M., Moinuddin, A., Shultz, J.M., Nakaoke, R., & Morley, J.E. (2004). Triglycerides induce leptin resistance at the blood-brain barrier. *Diabetes, 53(5)*, 1253-1260.

Banks, W.A., DiPalma, C.R., & Farrell, C.L. (1999). Impaired transport of leptin across the blood-brain barrier in obesity. *Peptides*, *20(11)*, 1341-1345.

Banks, W. A., Tschop, M., Robinson, S.M., & Heiman, M.L. (2002). Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *Journal of Pharmacology and Experimental Therapeutics*, 302(2), 822-827.

Barinaga M. (1995). Obese protein slims mice. *Science*, *269(5223)*, 475-476.

Bealer, S.L., Armstrong, W.E., & Crowley, W.R. (2010). Oxytocin release in magnocellular nuclei: neurochemical mediators and functional significance during gestation. *American Journal of Physiology*, *299(2)*, R452-R458.

Benedetto, L., Pereira, M., Ferreira, A., & Torterolo, P. (2014). Melanin-concentrating hormone in the medial preoptic area reduces active components of maternal behavior in rats. *Peptides*, *58*, 20-

25.

Blanco, C.A. (2014). The principle sources of William James' idea of habit. *Frontiers in Human Neuroscience*, *8*, 274.

Blevins, J.E., & Baskin, D.G. (2010). Hypothalamic-brainstem circuits controlling eating. *Forum of Nutrition*, *63*, 133-140.

Bolles, R., & Woods, P. (1964). The ontogeny of behaviour in the albino rat. *Animal Behaviour, 12(4)*, 427-441.

Bosch, O.J., Pfrotsch, J., Beiderbeck, D.I., Landgraf, R., & Neumann, I.D. (2010). Maternal behaviour is associated with vasopressin release in the medial preoptic area and bed nucleus of the stria terminalis in the rat. *Journal of Neuroendocrinology*, *22(5)*, 420-429.

Bouret, S.G., Draper, S.J. & Simerly, R.B. (2004). Formation of projection pathways from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the neural control of feeding behavior in mice. *Journal of Neuroscience*, *24(11)*, 2797-2805.

Bridges, R.S. (1984). A quantitative analysis of the roles of dosage, sequence, and duration of estradiol and progesterone exposure in the regulation of maternal behavior in the rat. *Endocrinology*, *114(3)*, 930-940.

Bridges, R.S. (2015). Neuroendocrine regulation of maternal behavior. *Frontiers in Neuroendocrinology*, *36*, 178-196.

Bridges, R.S. (2016). Long-term alternations in neural and endocrine processes induced by motherhood in mammals. *Hormones & Behavior*. 77, 193-203.

Bridges, R.S., DiBiase, R., Loundes, D.D., & Doherty, P.C. (1985). Prolactin stimulation of maternal behavior in female rats. *Science*, *227(4688)*, 782-784.

Bridges & Ronsheim (1990). Prolactin (PRL) regulation of maternal behaviour in rats: bromocriptine treatment delays and PRL promotes the rapid onset of behaviour. *Endocrinology*, 126, 837-848.

Brogan, R.S., Grove, K.L., & Smith, M.S. (2000). Differential regulation of leptin receptor but not orexin in the hypothalamus of the lactating rat. *Journal of Neuroendocrinology*, *12(11)*, 1077-1086.

Brunton, P.J., & Russell, J.A. (2008). Keeping oxytocin neurons under control during stress in

pregnancy. *Progress in Brain Research*, *170*, 365-377.

Budin, R., Wellman, M., Abizaid, A., & Woodside, B. Functional role of ghrelin during lactation. Presented at the 4th International Conference on the Parental Brain: Neurobiology, Behaviour and the Next Generation, Edinburgh, Scotland.

Buyse, M., Ovesjo, M.L., Goiot, H., Guilmeau, S., Peranzi, G., Moizo, L., Walker, F., Lewin, M.J., Meister, B., & Bado, A. (2001). Expression and regulation of leptin receptor proteins in afferent and efferent neurons of the vagus nerve. *European Journal of Neuroscience*, *14(1)*, 64-72.

Cai, X. J., Denis, R., Vernon, R. G., Clapham, J. C., Wilson, S., Arch, J. R. S., & Williams, G. (2001). Food restriction selectively increases hypothalamic orexin-B levels in lactating rats. *Regulatory Peptides*, *97*, 163-168.

Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R., & Burn, P. (1995). Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science*, *269(5223)*, 546-549.

Caughey, S.D., Klampfl, S.M., Bishop, V.R., Pfoertsch, J., Neumann, I.D., Bosch, O.J., & Meddle, S.L. (2011). Changes in the intensity of maternal aggression and central oxytocin and vasopressin V1a receptors across the peripartum period in the rat. *Journal of Neuroendocrinology*, *23(11)*, 1113-1124.

Cettour-Rose, P., &Rohner-Jeanrenaud, F. (2002). The leptin-like effects of 3-d peripheral administration of a melanocortin agonist are more marked in genetically obese Zucker (fa/fa) than in lean rats. *Endocrinology, 143(6)*, 2277-2283.

Champagne, F., Diorio, J., Sharma, S., & Meaney, M.J. (2001). Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. *PNAS*, *98(22)*, 12736-12741.

Chan, J.S., Robertson, H.A., & Friesen, H.G. (1978). Maternal and fetal concentrations of ovine placental lactogen measured by radioimmunoassay. *Endocrinology*, 102(5), 1606-1613.

Chen, A.S., Marsh, D.J., Trumbauer, M.E., Frazier, E.G., Guan, X.M., Yu, H., Rosenblum, C.I., Vongs, A., Feng, Y., Cao, L., Metzger, J.M., Strack, A.M., Camacho, R.E., Mellin, T.N., Nunes, C.N.,

Min, W., Fisher, J., Gopal-Truter, S., MacIntyre, D.E., Chen, H.Y., & Vander Ploeg, L.H. (2000). Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nature Genetics,26(1)*, 97-102.

Chen, P., Li, C., Haskell-Luevano, C., Cone, R.D., & Smith, M.S. (1999). Altered expression of agouti-related protein and its colocalization with neuropeptide Y in the arcuate nucleus of the hypothalamus during lactation. *Endocrinology*, *140(6)*, 2645-2650.

Chen, P., & Smith, M.S. (2003). Suckling-induced activation of neuronal input to the dorsomedial nucleus of the hypothalamus: possible candidates for mediating the activation of DMH neuropeptide Y neurons during lactation. *Brain Research*, *984(1- 2)*, 11-20.

Chen, P., Williams, S.M., Grove, K.L., & Smith, M.S. (2004). Melanocortin 4 receptor- mediated hyperphagia and activation of neuropeptide Y expression in the dorsomedial hypothalamus during lactation. *Journal of Neuroscience, 24(22)*, 5091-5100.

Chien, E.K., Hara, M., Rouard, M., Yano, H., Phillippe, M., Polonsky, K.S., & Bell, G.I. (1997). Increase in serum leptin and uterine leptin receptor messenger RNA levels during pregnancy in rats. *Biochemical and Biophysical Research Communications*, *237(2)*, 476- 480.

Ciofi, P. (2011). The arcuate nucleus as a circumventricular organ in the mouse. *Neuroscience Letters*, *487(2)*, 187-190.

Clegg, D.J., Brown, L.M., Zigman, J.M., Kemp, C.J., Strader, A.D., Benoit, S.C., Woods, S.C., Mangiaracina, M., & Geary, N. (2007). Estradiol-dependent decrease in the orexigenic potency of ghrelin in female rats. *Diabetes, 56(4)*, 1051-1058.

Cole, H. H., & Hart, G. H. (1938). The effect of pregnancy and lactation on growth in the rat. *American Journal of Physiology*, *123*, 589-597.

Connor, J. R., & Davis, H. N. (1980). Postpartum estrus in norway rats. I. Behavior. *Biology of Reproduction*, *23*, 994-999.

Cowley, M.A., Smith, R.G., Diano, S., Tschop, M., Pronchuk, N., Grove, K.L., Strasburger, C.J., Bidlingmaier, M., Esterman, M., Heiman, M.L., Garcia-Segura, L.M., Nillni, E.A., Mendez, P., Low, M.J., Sotonyi, P., Friedman, J.M., Liu, H., Pinto, S., Clomers, W.F., Cone, R.D., & Horvath, T.L. (2003). The distribution and mechanism of action of ghrelin in the CNS

demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron*, *37(4)*, 649-661.

Cramer, C.P., Pfister, J.F., & Blass, F.M. (1984). Transitions in the dehydration-induced inhibition of milk intake in suckling rats. *Physiology & Behavior*, 32(4), 691-694.

Cramer, C.P., Thiels, E., & Alberts, J.R. (1990). Weaning in rats: I. Maternal behavior. *Developmental Psychobiology*, *23(6)*, 479-493.

Cripps, A.W., & Williams, V.J. (1975). The effect of pregnancy and lactation on food intake, gastrointestinal anatomy and the absorptive capacity of the small intestine in the albino rat. *British Journal of Nutrition*, *33(1)*, 17-32.

Crowley, W.R., Ramoz, G., Torto, R., & Kalra, S.P. (2004). Role of leptin in orexigenic neuropeptide expression during lactation in rats. *Journal of Neuroendocrinology*, *16(7)*, 637-644.

Crowley, W.R., Ramoz, G., Torto, R., Keefe, K.A., Wang, J.J., &Kalra, S.P. (2007). Neuroendocrine actions and regulation of hypothalamic neuropeptide Y during lactation. *Peptides, 28(2)*, 447-452.

Cui, J.G., Tang, G.B., Wang, D.H., &Speakman, J.R. (2011). Effects of leptin infusion during peak lactation on food intake, body compostion, litter growth, and maternal neuroendocrine status in female Brandt's voles (Lasiopodomysbrandtii). *American Journal of Physiology. Regulatory, Integrative and Comparitive Physiology, 300(2)*, R447-R459.

Dall, S.R., & Boyd, I.L. (2004). Evolution of mammals: lactation helps mothers to cope with unreliable food supplies. *Proceedings of the Royal Society: Biological Sciences, 271(1552)*, 2049-2057.

Date, Y., Nakazato, M., Murakami, N., Kojima, M., Kangawa, K., &Matsukura, S. (2001). Ghrelin acts in the central nervous system to stimulate gastric acid secretion. *Biochemical and Biophysical Research Communications, 280(3)*, 904-907.

D' Cunha, T.M., King, S.J., Fleming, A.S., & Levy, F. (2011). Oxytocin receptors in the nucleus accumbens shell are involved in the consolidation of maternal memory in postpartum rats. *Hormones & Behavior*, *59(1)*, 14-21.

Denis, R.G., Williams, G., & Vernon, R.G. (2003). Regulation of serum leptin and its role in the hyperphagia of lactation in the rat. *Journal of Endocrinology*, *176(2)*, 193-203.

Deschamps, S., Woodside, B., & Walker, C.D. (2003). Pups presence eliminates the stress hyporesponsiveness of early lactating females to a psychological stress representing a threat to the pups. *Journal of Neuroendocrinology*, *15(5)*, 486-497.

Devito, W.J., Avakian, C., Stone, S., & Ace, C.I., (1992). Estradiol increases prolactin synthesis and prolactin messenger ribonucleic acid in selected brain regions in the hypophysectomized female rat. *Endocrinology*, *131*, 2154-2160.

Dial, J., & Avery, D.D. (1991). The effects of pregnancy and lactation on dietary self-selection in the rat. *Physiology & Behavior*, *49(4)*, 811-813.

Doidge, N. (2007). *The brain that changes itself*. New York, New York: Penguin Books.

Douglas, A.J., Bicknell, R.J., Leng, G., Russell, J.A., & Meddle, S.L. (2002). B-endorphin cells in the arcuate nucleus: projections to the supraoptic nucleus and changes in expression during pregnancy and parturition. *Journal of Neuroendocrinology*, *14*, 768-777.

Dufourny, L., Caraty, A., Clarke, I.J., Robinson, J.E., & Skinner, D.C. (2005). Progesterone- receptive dopaminergic and neuropeptide Y neurons project from the arcuate nucleus to gonadotropin-releasing hormone-rich regions of the ovine preoptic area. *Neuroendocrinology, 82(1)*, 21-31.

Elias, C.F., Saper, C.B., Maratos-Flier, E., Tritos, N.A., Lee, C., Kelly, J., Tatro, J.B., Hoffmann, G.E., Ollmann, M.M., Barsh, G.S., Sakurai, T., Yanagisawa, M., & Elmquist, J.K. (1998). Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. *Journal of Comparative Neurology*, *402*, 442-459.

Erskine, M.S., Barfield, R.J., & Goldman, B.D. (1978). Intraspecific fighting during late pregnancy and lactation in rats and effects of litter removal. *Behavioral Biology*, *23(2)*, 206-218.

Fan, W., Ellacott, K.L., Halatchev, I.G., Takahashi, K., Yu, P., & Cone, R.D. (2004). Cholecystokinin-medicated suppression of feeding involves the brainstem melanocortin system. *Nature Neuroscience, 7(4)*, 335-336.

Farrell, W.J., & Alberts, J.R. (2007). Rat behavioral thermoregulation integrates with nonshivering thermogenesis during postnatal development. *Behavioral Neuroscience*, *121(6)*, 1333-1341.

Ferrara, G., Serra, M., Zammaretti, F., Pisu, M.G., Panzica, G.C., Biggio, G., & Eva, C. (2001). Increased expression of the neuropeptide Y receptor Y(1) gene in the medial amygdala of

transgenic mice induced by long-term treatment with progesterone or allopregnanolone. *Journal of Neurochemistry, 79(2)*, 417-425.

Ferris, C.F., Kulkarni, P., Sullivan, J.M. Jr., Harder, J.A., Messenger, T.L., & Febo, M. (2005). Pup suckling is more rewarding than cocaine: evidence from functional magnetic resonance imaging and three-dimensional computational analysis. *Journal of Neuroscience*, *25(1)*, 149-156.

Fetissov, S.O., Kopp, J., Hokfelt, T. (2004). Distribution of NPY receptors in the hypothalamus. *Neuropeptides*, *38*, 175-188.

Fleming, A.S. (1986). Psychobiology of rat maternal behaviour: how and where hormones act to promote maternal behaviour at parturition. *Annals of the New York Academy of Sciences*, *474*, 234-251.

Fleming, A.S., & Luebke, C. (1981). Timidity prevents the virgin female rat from being a good mother: emotionality differences between nulliparous and parturient females. *Physiology & Behavior*, *27(5)*, 863-868.

Fleming, A.S., Miceli, M., & Moretto, D. (1983). Lesions of the medial preoptic area prevent the facilitation of maternal behavior produced by amygdala lesions. *Physiology & Behavior*, *31(4)*, 503-510.

Fleming, A.S., & Rosenblatt, J.S. (1974). Maternal behavior in the virgin and lactating rat. *Journal of Comparative and Physiological Psychology*, *86(5)*, 957-972.

Flint, D.J., Tonner, E., Beattie, J., & Panton, D. (1992). Investigation of the mechanism of action of growth hormone in stimulating lactation in the rat. *Journal of Endocrinology*, *134(3)*, 377-383.

Fischer, D., Patchev, V.K., Hellbach, S., Hassan, A.H., & Almeida, O.F. (1995). Lactation as a model for naturally reversible hypercorticalism plasticity in the mechanisms governing hypothalamo-pituitary-adrenocortical activity in rats. *Journal of Clinical Investigation*, *96(3)*, 1208-1215.

Frederich, R.C., Hamann, A., Anderson, S., Lollmann, B., Lowell, B.B., & Flier, J.S. (1995). Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nature Medicine, 1(12),* 1311-1314.

Freund-Mercier, M.J., & Richard, P. (1984). Electrophysiological evidence for facilitatory control of oxytocin neurons by oxytocin during suckling in the rat. *Journal of Physiology*, *352*, 447-466.

Friedman, J.M., & Halaas, J.L. (1998). Leptin and the regulation of body weight in mammals. *Nature*, *395(6704)*, 763-770.

Fry, M., & Ferguson, A.V. (2010). Ghrelin: central nervous system sites of action in regulation of energy balance. *International Journal of Peptides*, doi: 10.1155/2010/616757.

Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S.J., DelValle, J., & Yamada, T. (1993). Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *Journal of Biological Chemistry*, *268(20)*, 15174-15179.

Gammie, S.C. (2005). Current models and future directions for understanding the neural circuitries of maternal behaviors in rodents. *Behavioral and Cognitive Neuroscience Reviews*, *4(2)*, 119-135.

Gammie, S.C., & Stevenson, S.A. (2006). Effects of daily and acute restraint stress during lactation on maternal aggression and behavior in mice. *Stress*, *9(3)*, 171-180.

Garcia, M.D., Casanueva, F.F., Diequez, C., & Senaris, R.M. (2000). Gestational profile of leptin messenger ribonucleic acid (mRNA) content in the placenta and adipose tissue in the rat, and regulation of the mRNA levels of the leptin receptor subtypes in the hypothalamus during pregnancy and lactation. *Biology of Reproduction*, *62(3)*, 698-703.

Garcia, M.C., Lopez, M., Gualillo, O., Seoane, M., Dieguez, C., & Senaris, R.M. (2003). Hypothalamic levels of NPY, MCH, and prepro-orexin mRNA during pregnancy and lactation in the rat:role of prolactin. *The Journal of the Federation of American Societies for Experimental Biology*, *17(11)*, 1392-1400.

Genazzani, A.R., Stomati, M., Morittu, A., Bernardi, F., Moteleone, P., Casarosa, E., Gallo, R., Salvestroni, C., & Luisi, M. (2000). Progesterone, progestagens, and the central nervous system. *Human Reproduction, 15,(1)*, 14-27.

Gerardo-Gettens, T., Moore, B.J., Stern, J.S., & Horwitz, B.A. (1989). Prolactin stimulates food intake in a dose-dependent manner. *The American Journal of Physiology, 256(1 pt 2)*, R276-R280.

Ghamari-Langroudi, M., Colmers, W.F., & Cone, R.D. (2005). PYY3-36 inhibits the action potential firing activity of POMC neurons of arcuate nucleus through postsynaptic Y2 receptors. *Cell Metabolism, 2(3)*, 191-199.

Golden, P.L., Maccagnan, T.J., & Patridge, W.M. (1997). Human blood-brain barrier leptin receptor.

Binding and endocytosis in isolated human brain microvessels. *Journal of Clinical Investigation, 99(1)*, 14-18.

Goto, K., Inui, A., Takimoto, Y., Yuzuriha, H., Asakawa, A., Kawamura, Y., Tsuji, H., Takahara, Y., Takeyama, C., Katsuura, G., & Kasuga, M. (2003). Acute intracerebroventricular administration of either carboxyl-terminal or amino-terminal fragments of agouti-related peptide produces a long-term decrease in energy expenditure in rats. *International Journal of Molecular Medicine, 12(3)*, 379-383.

Goto, M., Arima, H., Watanabe, M., Hayashi, M., Banno, R., Sato, I., Nagasaki, H, & Oiso, Y. (2006). Ghrelin increases neuropeptide Y and agouti-related peptide gene expression in the arcuate nucleus in rat hypothalamic organotypic cultures. *Endocrinology, 147(11)*, 5102-5109.

Greenman, Y., Tordjman, K., & Stern, N. (1998). Increased body weight associated with prolactin secreting pituitary adenomas: weight loss with normalization of prolactin levels. *Clinical Endocrinology, 48(5)*, 547- 553.

Grill, H.J., & Kaplan, J.M. (2002). The neuroanatomical axis for control of energy balance. *Frontiers in Neuroendocrinology, 23(1)*, 2-40.

Grota, L.J., & Eik-Nes, K.B. (1967). Plasma progesterone concentrations during pregnancy and lactation in the rat. *Journal of Reproduction and Fertility, 13(1)*, 83-91.

Grota, & Ader (1969). Effects of litter size on emotionality, adrenocortical reactivity, and susceptibility to gastric erosions in the rat. *Psychological Reports, 24(2)*, 547-549.

Grosvenor, C.E., & Mena, F. (1969). Effect of auditory, olfactory and optic stimuli upon milk ejection and suckling-induced release of prolactin in lactating rats. *Endocrinology, 80(5)*, 840- 846.

Grosvenor, C.E., Mena, F., & Whitworth, N.S. (1977). Sensory stimuli from pups involved in inhibition of milk secretion in rats during late lactation. *Hormones & Behavior, 8(3)*, 287-296.

Grosvenor, C.E., Mena, F., & Whitworth, N.S. (1979). Ether releases large amounts of prolactin from rat pituitaries previously "depleted" by short term suckling. *Endocrinology, 105(4)*, 884-887.

Grosvenor, C.E., Shyr, S.W., Goodman, G.T., & Mena, F. (1986). Comparison of plasma profiles of oxytocin and prolactin following suckling in the rat. *Neuroendocrinology, 43(6)*, 679- 685.

Grueso, E., Rocha, M., & Puerta, M. (2001). Plasma and cerebrospinal fluid leptin levels are

maintained despite enhanced food intake in progesterone-treated rats. *European Journal of Endocrinology, 144(6)*, 659-665.

Gualillo, O., Caminos, J.E., Nogueiras, R., Seoane, L.M., Arvat, E., Ghigo, E., Casanueva, F.F., & Dieguez, C. (2002). Effect of food restriction on ghrelin in normal-cycling female rats and in pregnancy. *Obesity Research, 10(7)*, 682-687.

Guan, X.M., Yu, H., Palyha, O.C., McKee, K.K., Feighner, S.D., Sirinathsinghji, D.J., Smith, R.G., Van der Ploeg, L.H., & Howard A.D. (1997). Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripherial tissues. *Brain Research. Molecular Brain Research, 48(1)*, 23-29.

Gubernick, D.J., & Alberts, J.R (1983). Maternal licking of young: resource exchange and proximate controls. *Physiology & Behavior, 31(5)*, 593-601.

Gubernick, D.J., & Alberts, J.R. (1985). Maternal licking by virgin and lactating rats: water transfer from pups. *Physiology & Behavior, 34(4)*, 501-506.

Guerra, A., King, J.M., Alajajian, B., Isgor, E., & Digicaylioglu, M. (2011). Occludin and claudin-5 are abundant and co-localized in the rat's blood brian barrier from late gestation to adulthood. *Neonatology Research, 1(1)*, 31-43.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., & Friedman, J.M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. *Science, 269(5223)*, 543-546.

Hall, W.G., & Bryan, T.E. (1980). The ontogeny of feeding in rats: II. Independent ingestive behavior. *Journal of Comparative and Physiological Psychology, 94(4)*, 746-756.

Hallack, M.D., & Hotra, J.W. (2003). Maternal serum hormone concentrations in long-evans rats. *Journal of Trace and Microprobe Techniques, 21(1)*, 103-110.

Hansen, S., Sodersten, P., & Eneroth, P. (1983). Mechanisms regulating hormone release and the duration of dioestrus in the lacatating rat. *Journal of Endocrinology, 99(2)*, 173-180.

Hapon, M.B., Simoncini, M., Via, G., & Jahn, G.A. (2003). Effect of hypothyroidism on hormone profiles in virgin, pregnant and lactating rats, and on lactation. *Reproduction, 126*, 371-382.

Hausman, G.J., Barb, C.R., & Lents, C.A. (2012). Leptin and reproductive function. B*iochimie, 94(10)*,

2075-2081.

Hawkins, B.T., & Davis, T.P. (2005). The blood-brain barrier/neurovascular unit in health and disease. *Pharmacological Reviews, 57(2)*, 173-185.

Herrera, E., Lasuncion, M.A., Huerta, L., Martin-Hidalgo, A. (2000). Plasma leptin levels in rat mother and offspring during pregnancy and lactation. *Biology of the Neonate, 78(4)*, 315-320.

Higuchi, T., Honda, K., Fukuoka, T., Negoro, H., Hosono, Y., & Nishida, E. (1983). Pulsatile secretion of prolactin and oxytocin during nursing in the lactating rat. *Endocrinologia Japonica, 30(3)*, 353-359.

Higuchi, T., Honda, K., Fukuoka, T., Negoro, H., & Wakabayashi, K. (1985). Release of oxytocin during suckling and parturition in the rat. *Journal of Endocrinology, 105(3)*, 339-346.

Higuchi, T., Uchide, K., Honda, K., & Negoro, H. (1985). Functional development of the oxytocin release mechanism and its role in the initiation of parturition in the rat. J*ournal of Endocrinology, 106(3)*, 311-316.

Hosoda, H., Kojima, M., & Kangawa, K. (2006). Biological, physiological, and pharmacological aspects of ghrelin. *Journal of Pharmacological Sciences, 100 (5)*, 398- 410.

Howard, A.D., Feighner, S.D., Cully, D.F., Arena, J.P., Liberator, P.A., Rosenblum, C.I., Hamelin, M., Hreniuk, D.L., Palyha, O.C., Anderson, J., Paress, P.S., Diaz, C., Chou, M., Liu, K.K., McKee, K.K., Pong, S.S., Chaung, L.Y., Elbrecht, A., Dashkevicz, M., Heavens, R., Rigby, M., Sirinathsinghij, D.J., Dean, D.C., Melillo, D.G., Patchett, A.A., Nargund, R., Griffin, P.R., DeMartino, J.A., Gupta, S.K., Schaeffer, J.M., Smith, R.G., Van der Ploeg, L.H. (1996). A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science, 273(5277)*, 974-977.

Hsiung, H.M., Hertel, J., Zhang X.Y., Smith, D.P., Smiley, D.L., Heiman, M.L. Yang, D.D. Husain, S., Mayer, J.P., Zhang, L., Mo, H., Yan, L.Z. (2005). A novel and selective beta-melanocyte stimulating hormone-derived peptide agonist for melanocortin 4 receptor potently decreased food intake and body weight gain in diet- induced obese rats. *Endocrinology, 146(12),* 5257-5266.

Imagawa, W., Yang, J., Guzman, R.C., Nandi, S. (1994). *Control of mammary gland growth and*

development in The Physiology of Reproduction, eds Knobil, E., & Neill, J.D. (Raven, New York), 1033-1063.

Insel, T.R., & Harbaugh, C.R. (1989). Lesions of the hypothalamic paraventricular nucleus disrupt the initiation of maternal behavior. *Physiology & Behavior, 45(5)*, 1033-1041.

Jans, J.E., & Woodside, B. (1987). Effects of litter age, litter size, and ambient temperature on the milk ejection reflex in lactating rats. *Developmental Psychobiology, 20(3)*, 333-344.

Jeffers, K.R. (1935). Cytology of the mammary gland of the albino rat. *American Journal of Anatomy, 56(2)*, 257-277.

Johnstone, L.E., & Higuchi, T. (2001). Food intake and leptin during pregnancy and lactation. *Progress In Brain Research, 133*, 215-227.

Kanenishi, K., Ueno, M., Momose, S., Kuwabara, H., Tanaka, H., Sato, C., Kobayashi, T., Hino, O., Sakamoto, H., & Haha, T. (2004). Prepro-orexin mRNA expression in the rat brain is increased during pregnancy. *Neuroscience Letters, 368*, 73-77.

Kawai, M., Yamaguchi, M., Murakami, T., Shima, K., Murata, Y., & Kishi, K. (1997). The placenta is not the main source of leptin production in pregnant rat: gestational profile of leptin plasma and adipose tissues. B*iochemical and Biophysical Research Communications, 240(3)*, 798-802.

Keen Rhinehart, E., Kalra, S.P., & Kalra, P.S. (2004). Neuropeptidergic characterization of the leptin receptor mutated obese Koletsky rat. *Regulatory Peptides, 119(1-2)*, 3-10.

Keer, S.E., & Stern, J.M. (1999). Dopamine receptor blockade in the nucleus accumbens inhibits maternal retrieval and licking, but enhances nursing behavior in lactating rats. *Physiology & Behavior, 67(5)*, 659-669.

Khorram, O., DePalatis, L.R., & McCann, S.M. (1984). Changes in hypothalamic and pituitary content of immunreactive alpha-melanocyte-stimulating hormone during the gestational and postpartum periods in the rat. *Proceedings of the Society for Experimental Biology and Medicine, 177(2)*, 318-326.

Kim, M.S., Rossi, M., Abusnana, S., Sunter, D., Morgan, D.G., Small, C.J., Edwards, C.M., Heath, M.M., Stanley, S.A., Seal, L.J., Bhatti, J.R., Smith, D.M., Ghatei, M.A., & Bloom, S.R. (2000). *Diabetes, 49(2)*, 177-182.

Klampfl, S.M., Neumann, I.D., & Bosch, O.J. (2013). Reduced brain corticotrophin-releasing factor receptor activation is required for adequate maternal care and maternal aggression in lactating rats. *European Journal of Neuroscience, 38*, 2742-2750.

Knollema, S., Brown, E.R., Vale, W., & Sawchenko, P.E. (1992). Novel hypothalamic and preoptic sites of prepro-melanin-concentrating hormone messenger ribonucleic acid and peptide expression in lactating rats. J*ournal of Neuroendocrinology, 4(6)*, 709-717.

Kohno, D., Gao, H.Z., Muroya, S., Kikuyama, S., & Yada, T. (2003). Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca2+ signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes, 52(4)*, 948-956.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature, 402(6762)*, 656-660.

Kucera, G.T., Bortner, D.M., & Rosenberg, M.P. (1996). Overexpression of an Agouti cDNA in the skin of transgenic mice recapitulates dominant coat color phenotypes of spontaneous mutants. *Developmental Biology, 173(1)*, 162-173.

Ladyman, S.R., Augustine, R.A., Scherf, E., Phillips, H.R., Brown, C.H., & Grattan, D.R. (2016). Attenuated hypothalamic responses to α-melanocyte stimulating hormone during pregnancy in the rat. *Journal of Physiology, 594(4)*, 1087-1101.

Ladyman, S.R., & Grattan, D.R. (2004). Region-specific reduction in leptin-induced phosphorylation of signal transducer and activator of transcription-3 (STAT3) in the rat hypothalamus is associated with leptin resistance during pregnancy. *Endocrinology, 145 (8)*, 3704-3711.

Ladyman, S.R., & Grattan, D.R. (2005). Suppression of leptin receptor messenger ribonucleic acid and leptin responsiveness in the ventromedial nucleus of the hypothalamus during pregnancy in the rat. *Endocrinology, 146(9)*, 3868-3874.

Ladyman, S.R., Tups, A., Augustine, R.A., Swahn-Azavedo, A., Kokay, I.C., & Grattan, D.R. (2009). Loss of hypothalamic response to leptin during pregnancy associated with development of melanocortin resistance. *Journal of Neuroendocrinology, 21*, 449-456.

Ladyman, S.R., & Woodside, B. (2014). Food restriction during lactation suppresses Kiss1 mRNA

expression and kisspeptin-stimulated LH release in rats. *Reproduction, 147(5)*, 743-751.

Larson-Meyer, D.E., Ravussin, E., Heilbronn, L., &DeJonge, L. (2010). Ghrelin and peptide YY in postpartum lactating and nonlactating women. *The American Journal of Clinical Nutrition, 91(2)*, 366- 372.

Lee, A., Clancy, S., & Fleming, A.S. (2000). Mother rats bar-press for pups: effects of lesions of the mpoa and limbic sites on maternal behavior and operant responding for pup-reinforcement. *Behavioural Brain Research, 108(2)*, 215-231.

Leibowitz, S.F., & Alexander, J.T. (1991). Analysis of neuropeptide Y-induced feeding: dissociation of Y1 and Y2 receptor effects on natural meal patterns. *Peptides, 12(6)*, 1251-1260.

Lenard, N.R., & Berthoud, H. (2008). Central and peripheral regulation of food intake and physical activity: pathways and genes. *Obesity, 16(S3)*, S11-S22.

Levine, A.S., & Morley, J.E. (1984). Neuropeptide Y: a potent inducer of consummatory behavior in rats. *Peptides, 5(6),* 1025-1029.

Li, C., Chen, P., & Smith, M.S. (1998). The acute suckling stimulus induces expression of neuropeptide Y (NPY) in cells in the dorsomedial hypothalamus and increases NPY expression in the arcuate nucleus. *Endocrinology, 139(4)*, 1645-1652.

Li, C., Chen, P., & Smith, M.S. (1999). Neuropeptide Y and tuberoinfundibular dopamine activities are altered during lactation: role of prolactin. *Endocrinology, 140(1)*, 118-123.

Li, C., Kelly, P.A., & Buntin, J.D. (1995). Inhibitory effects of anti-prolactin receptor antibodies on prolactin binding in brain and prolactin-induced feeding behavior in ring doves. *Neuroendocrinology, 61(2)*, 125-135.

Lightman, S.L. (1992). Alterations in hypothalamic-pituitary responsiveness during lactation. *Annals of the New York Academy of Sciences, 12(652)*, 340-346.

Lincoln, D.W., & Paisley, A.C. (1982). Neuroendocrine control of milk ejection. *Journal of Reproduction and Fertility, 65(2)*, 571-586.

Lincoln, D.W., Hill, A., & Wakerly, J.B. (1973). The milk-injection reflex of the rat; An intermittent function not abolished by surgical levels of anesthesia. *Journal of Endocrinology, 57*, 459-476.

Lincoln, D.W. & Wakerly, J.B. (1975). Neurosecretory activation in the rat: correlation of the

suckling stimulus with the pulsatile release of oxytocin. *Journal of Physiology, 245(2)*, 42P-43P.

Lins, M.C., de Moura, E.G., Lisboa, P.C., Bonomo, I.T., Passos, M.C. (2005). Effects of maternal leptin treatment during lactation on the body weight and leptin resistance of adult offspring. *Regulatory Peptides, 127(1-3)*, 197-202.

Lins, M.C., Passos, M.C., Lisboa, P.C., Bonomo, I.T., de Moura, E.G. (2005). Leptin treatment during lactation increases transfer of iodine through the milk. *Journal of Nutritional Biochemistry, 16(3)*, 138-143.

Liu, X., Brown, R.S., Herbison, A.E., & Grattan, D.R. (2014). Lactational anovulation in mice results from a selective loss of kisspeptin input to GnRH neurons. *Endocrinology, 55(1)*, 193-203.

Ludwig, D.S., Mountjoy, K.G., Tatro, J.B., Gillette, J.A., Frederich, R.C., Flier, J.S., & Maratos- Flier, E. (1998). Melan-concentrating hormone: a functional melanocortin antagonist in the hypothalamus. *American Journal of Physiology, 274(4 Pt 1)*, E627-633.

Lyons, D.J., Hellysaz, A., & Broberger, C. (2012). Prolactin regulates tuberoinfundibular dopamine neuron discharge pattern: novel feedback control mechanisms in the lactotrophic axis. *Journal of Neuroscience, 32(2)*, 8074-8083.

Maffei, M., Halaas, J., Ravussin, F., Pratley, R.E., Lee G.H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S., et al. (1995). Leptin levels in human and rodent: Measurement of plasma leptin and ob RNA in obese and weight reduced subjects. *Nature Medicine, 1(11)*, 1155- 1161.

Malabu, U.H., Kilpatrick, A., Ware, M., Vernon, R.G., & Williams, G. (1994). Increased neuropeptide Y concentrations in specific hypothalamic regions of lactating rats: possible relationship to hyperphagia and adaptive changes in energy balance. *Peptides, 15(1)*, 83-87.

Maneckjee, R., & Moudgal, N.R. (1975). The onset of oestrus and ovulation in lactating rats. *Journal of the Society for Reproduction and Fertility, 44*, 313-315.

Mann, P.E., Rubin, B.S., & Bridges, R.S. (1997). Differential proopiomelanocortin gene expression in the medial basal hypothalamus of rats during pregnancy and lactation. *Molecular Brain Research, 46*, 9-16.

Mann, P.E., & Bridges, R.S. (2001). Lactogenic hormone regulation of maternal behavior. *Progress In*

Brain Research, 133, 251-262.

Mattheij, J.A., Gruisen, E.F., & Swarts, J.J. (1979). The suckling-induced rise of plasma prolactin in lactating rats: its dependence on stage of lactation and litter size. *Hormone Research, 11(6)*, 325-336.

Mattheij, J.A., Swarts, H.J., &Verstijnen, C.P. (1984). The response of plasma prolactin to suckling during normal and prolonged lactation in the rat. *Hormone Research, 20(4)*, 261-268.

Mattson, B.J., Williams, S., Rosenblatt, J.S., & Morrell, J.I. (2001). Comparison of two positive reinforcing stimuli: pups and cocaine throughout the postpartum period. Behavioral *Neuroscience, 115(3)*, 683-694.

Mayer, A.D., Reisbick, S., Siegel, H.I., Rosenblatt, J.S. (1987). Maternal aggression in rats: Changes over pregnancy and lactation in Sprague-Dawley strain. *Aggressive Behavior,13*, 29-43.

Mayer, A.D., Monroy, M.A., & Rosenblatt, J.S. (1990). Prolonged estrogen-progesterone treatment of nonpregnant ovariectomized rats: factors stimulating home-cage and maternal aggression and short-latency maternal behavior. *Hormones & Behavior, 24(3)*, 342-364.

McNeilly, A.S. (1980). Prolactin and the control of gonadotrophin secretion in the female. *Journal of Reproduction and Fertility, 58(2)*, 537-549.

Mercer, J.G., Hoggard, N., Williams, L.M., Lawerence, C.B., Hannah, L.T., Morgan, P.J., & Trayhurn, P. (1996). Coexpression of leptin receptor and preproneuropeptide Y mRNA in arcuate nucleus of mouse hypothalamus. *Journal of Neuroendocrinology, 8(10)*, 733-735.

Meyer, C., Freund-Mercier, M.J., Guerne, Y., & Richard, P. (1987). Relationship between oxytocin release and amplitude of oxytocin cell neurosecretory bursts during suckling in the rat. *Journal of Endocrinology, 114(2)*, 263-270.

Mistry, A.M., & Romsos, D.R. (2002). Intracerebroventricularleptin administration reduces food intake in pregnant and lactating mice. *Experimental Biology and Medicine, 227(8)*, 616-619.

Moltz, H., Levin, R., & Leon, M. (1969). Prolactin in the postpartum rat: synthesis and release in the absence of suckling stimulation. *Science, 163(3871)*, 1083-1084.

Morrison, S.D. (1956). The total energy and water metabolism during pregnancy in the rat. *Journal of Physiology, 134*, 650-664.

Moss, F.A. (1924). Study of animal drives. *Journal of Experimental Psychology, 7(3)*, 165-185.

Munday, M.R., & Williamson, D.H. (1983). Diurnal variations in food intake and in lipogenesis in mammary gland and liver of lactating rats. *Biochemical Journal, 214(1)*, 183-187.

Murphy, K.G., Dhillo, W.S., & Bloom, S.R. (2006). Gut peptides in the regulation of food intake and energy homeostasis. *Endocrine Reviews, 27(7)*, 719-727.

Naef, L., & Woodside, B. (2007). Prolactin/Leptin interactions in the control of food intake in rats. *Endocrinology, 148(12),* 5977-5983.

Nagaishi, V.S., Cardinali, L.I., Zampieri, T.T., Furigo, I.C., Metzger, M., & Donato, J. Jr. (2014). Possible crosstalk between leptin and prolactin during pregnancy. *Neuroscience, 259*, 71-83.

Nagasawa, H., & Yanai, R. (1972). Changes in serum prolactin levels shortly before and after parturition in rats. *Endocrinologia Japonica,19(2)*, 139-143.

Nakahara, K., Hayashida, T., Nakazato, M., Kojima, M., Hosoda, H., Kangawa, K., & Murakami, N. (2003). Effect of chronic treatments with ghrelin on milk secretion in lactating rats. *Biochemical and Biophysical Research Communications, 303(3)*, 751-755.

Naismith, D.J., Richardson, D.P., & Pritchard, A.E. (1982). The utilization of protein and energy during lactation in the rate, with particular regard to the use of fat accumulated in pregnancy. *The British Journal of Nutrition, 48(2)*, 433-441.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., &Matsukura, S. (2001). A role for ghrelin in the central regulation of feeding. *Nature, 409 (6817)*, 194-198.

Nelson, R.J., Demas, G.E., Huang, P.L., Fishman, M.C., Dawson, V.L., Dawson, T.M., & Snyder, S.H. (1995). Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. *Nature, 378(6555)*, 383-386.

Niswender, K.D. & Schwartz, M.W. (2003). Insulin and leptin revisted: adiposity signals with overlapping physiological and intracellular signaling capabilities. F*rontiers in Neuroendocrinology, 24(1)*, 1-10.

Noel, M.B., & Woodside, B. (1993). Effects of systemic and central prolactin injections on food intake, weight gain, and estrous cyclicity in female rats. *Physiology & Behavior, 54(1)*, 151- 154.

Numan, M. (2006). Hypothalamic neural circuits regulating maternal responsiveness toward infants.

Behavioral and Cognitive Neuroscience Reviews, 5(4), 163-190.

Numan, M., Stolzenberg, D.S. (2009). Medial preoptic area interactions with dopamine neural systems in the control of the onset and maintenance of maternal behavior in rats. *Frontiers in Neuroendocrinology, 30(1)*, 46-64.

Numan, M., & Smith, H.G. (1984). Maternal behavior in rats: evidence for the involvement of preoptic projections to the ventral tegmental area. *Behavioral Neuroscience, 98(4)*, 712-727.

Paxinos, G., & Watson, C. *The Rat Brain in Stereotaxic Coordinate, Compact Third Edition*. New York: Academic Press, 1996.

Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., & Collins, F (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. *Science, 269*, 540-543.

Pi, X.J., & Grattan, D.R. (1999). Increased expression of both short and long forms of prolactin receptor mRNA in hypothalamic nuclei of lactating rats. *Journal of Molecular Endocrinology, 23*, 13-22.

O' Conner, J.L., Wade, M.F., Brann, D.W., & Mahesh, V.B. (1995). Evidence that progesterone modulates anterior pituitary neuropeptide Y levels during the progesterone-induced gonadotropin surge in the estrogen-primed intact immature female rat. *Journal of Steroid Biochemistry and Molecular Biology, 52(5)*, 497-504.

Oliet, S.H., & Bonfardin, V.D. (2010). Morphological plasticity of the rat supraoptic nucleus cellular consequences. *European Journal of Neuroscience, 32(12)*, 1989-1994.

Ollmann, M.N., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I., & Barsh, G.S. (1997). Antagonism of central melancortin receptors in vitro and in vivo by agouti-related protein. *Science, 278(5335)*, 135-138.

Olofsson, L.E., Pierce, A.A., & Xu, A.W. (2009). Functional requirement of AgRP and NPY neurons in ovarian cycle-dependent regulation of food intake. *Proceedings of the National Academy of Sciences of the United States of America, 106(37)*, 15932-15937.

Ota, K., & Yokoyama, A., (1967). Body weight and food consumption of lactating rats nursing various sizes of litters. *Journal of Endocrinology, 38*, 263-268.

Pedersen, C.A., Ascher, J.A., Monroe, Y.L., & Prange, A.J. Jr. (1982). Oxytocin induces maternal behavior in virgin female rats. *Science, 216(4546)*, 648-650.

Pepe, G., & Rothchild, I. (1974). A comparative study of serum progesterone leves in pregnancy and various types of pseudopregnancy in the rat. *Endocrinology, 95*, 275-279.

Pickavance, L., Tadayyon, M., Williams, G., & Vermon, R.G. (1998). Lactation suppresses diurnal rhythm of serum leptin. *Biochemical and Biophysical Research Communications, 248*, 196-199.

Poiana, C., Stefanescu, A.M., Caragheorgheopol, A., Badiu, C., Galoiu, S., & Coculescu, M. (2005). Blood-brain barrier by-pass produced by pituitary adenomas for pituitary peptides does not involve leptin. *Acta Endocrinologica (Buc), 1(2)*, 157-166.

Polidori, C., Ciccocioppo, R., Regoli, D., & Massi, M. (2000). Neuropeptide Y receptor(s) mediating feeding in the rat: characterization with antagonists. *Peptides, 21(1)*, 29-35.

Price, T.O., Farr, S.A., Yi, X., Vinogradov, S., Batrakova, E., Banks, W.A., & Kabanov, A.V. (2010). Transport across the blood-brain barrier of pluronic leptin. *Journal of Pharmacology & Experimental Therapeutics, 333(1)*, 253-263.

Quinones-Jenab, V., Batel, P., Schlussman, S.D., Ho, A., & Kreek, M.J. (1997). Cocaine impairs maternal nest building in pregnant rats. *Behavioural Brain Research, 101(1)*, 15-20.

Ramakrishnan, S., Strader, A.D., Wimpee, B., Chen, P., Smith, M.S., & Buntin, J.D. (2007). Evidence for increased neuropeptide Y synthesis in mediobasal hypothalamus in relation to parental hyperphagia and gonadal activation in breeding ring doves. *Journal of Neuroendocrinology, 19(3)*, 163-171.

Rees, T. (2010). Being neurologically human today: Life and science and adult cerebral plasticity (an ethical analysis). *American Ethnologist, 37,* 150-166.

Richter, C.P., Holt, L., & Barelare, B. (1938). Nutritional requirements for normal growth and reproduction in rats studied by the self-selection method. *American Journal of Physiology, 122*, 734.

Riddle, O., Bates, R.W., Dykshorn, S.W. (1933). The preparation, idenitification and assay of prolactin-a hormone of the anterior pituitary. *American Journal of Physiology, 105*, 191-216.

Riediger, T., Traebert, M., Schmind, H.A., Scheel, C., Lutz, T.A., & Scharrer, E. (2003). Site-specific effects of ghrelin on the neuronal activity in the hypothalamic arcuate nucleus. *Neuroscience Letters, 341(2)*, 151-155.

Rivier, C., & Rivest, S. (1991). Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. *Biology of Reproduction, 45(4)*, 523-532.

Rocha, M., Bing, C., Williams, G., & Puerta, M. (2003). Pregnancy-induced hyperphagia is associated with increased gene expression of hypothalamic agouti-related peptide in rats. *Regulatory Peptides, 114*, 159-165.

Rosas-Hernandez, H., Cuevas, E., Lantz, S.M., Hamilton, W.R., Ramirez-Lee, M.A., Ali, S.F., Gonzalez, C. (2013) Prolactin and blood-brain barrier permeability. *Current Neurovascular Research, 10(4)*, 278-286.

Rosenblatt, J.S. (1967). Nonhormonal basis of maternal behaviour in the rat. *Science, 156*, 1512-1514.

Roy, A.F., Benomar, Y., Bailleux, V., Vacher, C.M., Aubourg, A., Gertler, A., Dijiane, J., & Taouis, M. (2007). Lack of cross-desensitization between leptin and prolactin signaling pathways despite the induction of suppressor of cytokine signaling 3 and PTP-1B. *Journal of Endocrinology, 195(2)*, 341-350.

Russell, J.A. (1980). Milk yield, suckling behaviour and milk ejection in the lactating rat nursing litters of different sizes. *Journal of Physiology, 303*, 403-415.

Sahu, A. (2011). Intracellular leptin-signaling pathways in hypothalamic neurons: The emerging role of phosphatidylinositol-3-kinase-phosphodiesterase-3B-cAMP pathway. *Neuroendocrinology, 93*, 201-210.

Sakurai, T. (1999). Orexins and orexin receptors: implication in feeding behaviour. *Regulatory Peptides, 85*, 25-30.

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, C.S., Richardson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R.S., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R. S., McNulty, D.E., Liu, W., Terrett, J.A., Elshourbagy, N.A., Bergsma, D.J., & Yanagisawa, M. (1998). Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behaviour. *Cell, 92*, 573-

585.

Sakurazawa, N., Mano-Otagiri, A., Nemoto, T., & Shibasaki, T. (2013). Effects of intracerebroventricular ghrelin on food intake and Fos expression in the arcuate nucleus of the hypothalamus in female rats with estrous cycle phase. *Neuroscience Letters, 541*, 204-208.

Salome, N., Haage, D., Perrissoud, D., Moulin, A., Demange, L., Egecioglu, E., Fehrentz, J.A., Martinez, J., & Dickson, S.L. (2009). Anorexigenic and electrophysiological actions of novel ghrelin receptor (GHS-R1A) antagonists in rats. *European Journal of Pharmacology, 612(1-3)*, 167-173.

Schuhler, S., Horan, T.L., Hastings, M.H., Mercer, J.G., Morgan, P.J., &Ebling, F.J. (2003). Decrease of food intake by MC4-R agonist MTII in Siberian hamsters in long and short photoperiods. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology, 284(1)*, R227-R232.

Schwartz, M.W., Woods, S.C., Porte Jr, D., Seeley, R.J., & Baskin, D. G. (2000). Central nervous system control of food intake. *Nature, 404(6)*, 661-671.

Seeber, R.M., Smith, J.T., & Waddell, B.J. (2002). Plasma leptin-binding activity and hypothalamic leptin receptor expression during pregnancy and lactation in the rat. *Biology of Reproduction, 66(6)*, 1762-1767.

Shibata, K., Hosoda, H., Kojima, M., Kangawa, K., Makino, Y., Makino, I., Kawarabayashi, T., Futagami, K., & Gomita, Y. (2004). Regulation of ghrelin secretion during pregnancy and lactation in the rat: possible involvement of hypothalamus. *Peptides, 25(2)*, 279- 287.

Shirley, B. (1984). The food intake of rats during pregnancy and lactation. *Laboratory Animal Science, 34(2)*, 169-172.

Simmons, R. (1924). The relative effectiveness of certain incentives in animal learning. *Comparative Psychology Monographs, 2(7)*, 1-79.

Smith, M.S. (1993). Lactation alters neuropeptide-Y and proopiomelanocortin gene expression in the arcuate nucleus of the rat. *Endocrinology, 133(3)*, 1258-1265.

Smith, J.L., Lear, S.R., Forte, T.M., Ko, W., Massimi, M., & Erickson, S.K. (1998).Effect of pregnancy and lactation on lipoprotein and cholesterol metabolism in the rat. *Journal of Lipid Research,*

39(11), 2237-2249.

Smith, M.S., & Neill, D. (1977). Inhibition of gonadotropin secretion during lactation in the rat: Relative contribution of suckling and ovarian steroids. *Biology of Reproduction, 17*, 255-261.

Stern, J.M., Goldman, L., & Levine, S. (1973). Pituitary-adrenal responsiveness during lactation in rats. *Neuroendocrinology, 12(3)*, 179-191.

Stern, J.M., & Voogt, J.L. (1974). Comparison of plasma corticosterone and prolactin levels in cycling and lactating rats. *Neuroendocrinology, 13(3)*, 173-181.

Strader, A.D., & Buntin, J.D. (2001). Neuropeptide-Y: a possible mediator of prolactin-induced feeding and regulator of energy balance in the ring dove (Streptopelia risoria). *Journal of Neuroendocrinology, 13(4)*, 386-392.

Strubbe, J.H., & Gorissen, J. (1980). Meal patterning in the lactating rat. *Physiology & Behavior; 25*, 775-777.

Sun, G., Narita, K., Murata, T., Honda, K., & Higuchi, T. (2003). Orexin-A immunoreactivity and prepro-orexin mRNA expression in hyperphagic rats induced by hypothalamic lesions and lactation. *Journal of Neuroendocrinology, 15(1)*, 51-60.

Sun, G., Tian, Z., Yao, Y., Li, H., & Higuchi, T. (2004). Central and/or peripheral immunoreactivity of orexin-A in pregnant rats and women. *Journal of Molecular Endocrinology, 36*, 131-138.

Suzuki, Y., Kurose, Y., Kakahashi, H., Asakuma, S., Azuma, Y., & Kobyashi, S. (2010). The differences in feeding-inhibitory responses to peripheral and central leptin between non- lactating and lactating rats. *Journal of Endocrinology, 207(1)*, 105-111.

Suzuki, Y., Nakahara, K., Maruyama, K., Okame, R., Ensho, T., Inoue, Y., & Murakami, N. (2014). Changes in mRNA expression of the arcuate nucleus appetite-regulating peptides during lactation in rats. *Journal of Molecular Endocrinology, 52(2)*, 97-109.

Szczepankiewicz, D., Pruszynska-Oszmalek, E., Kaczmarek, P., Skrzypski, M., Andralojc, K., Wojciechowicz, T., Sassek, M., & Nowak, K.W. (2009). Changes of agouti-related protein in hypothalamus, placenta, and serum during pregnancy in the rat. *Journal of Endocrinology, 202*, 35-41.

Szczepankiewicz, D., Skrzypski, M., Pruszynska-Oszmalek, E., Zimmerman, D., Andralojc, K.,

Kaczmarek, P., Wojciechowicz, T., Sassek, M., & Nowak, K.W. (2010). Importance of ghrelin in hypothalamus-pituitary axis on growth hormone release during normal pregnancy in the rat. *Journal of Physiology and Pharmacology, 61(4)*, 443-449.

Tachi, N., Tomogane, H., & Yokoyama, A. (1981). Diurnal patterns of food intake and plasma corticosterone levels in lactating rats. *Physiology and Behavior, 27(3)*, 481-486.

Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards,G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriarty, A.,Moore K.J., Smutko, J.S., Mays, G.G., Wool, E.A., Monroe, C.A., Tepper, R.I.(1995). Identification and expression cloning of a leptin receptor, OB-R. *Cell, 83(7),* 1263-1271.

Tatemoto, K., Carlquist, M., & Mutt, V. (1982). Neuropeptide Y- a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature, 296(5858)*, 659-660.

Taya, K., & Greenwald, G.S. (1982). Mechanisms of suppression of ovarian follicular development during lactation in the rat. *Biology of Reproduction, 27(5)*, 1090-1101.

Taylor, V.J., Patterson, M., Ghatei, M.A., Bloom, S.R., & Wilson, C.A. (2009). Ghrelin and peptide YY (PYY) profiles in gastrointestinal tissues and the circulation of the rat during pregnancy and lactation. *Peptides, 30*, 2213-2220.

Terada, Y., Yamakawa, K., Sugaya, A., & Toyoda, N. (1998). Serum leptin levels do not rise during pregnancy in age-matched rats. *Biochemical and Biophysical Research Communications, 253(3)*, 841-844.

Ter Harr, M.B. (1972). Circadian and estrual rhythms in food intake in the rat. *Hormones and Behavior, 3(3)*, 213-219.

Theodosis, D.T., Chapman, D.B., Montagnese, C., Poulain, D.A., & Morris, J.F. (1986). Structural plasticity in the hypothalamic supraoptic nucleus at lactation affects oxytocin, but not vasopressin-secreting neurones. *Neuroscience, 17(3)*, 661-678.

Thiels, E., Alberts, J.R., & Cramer, C.P. (1990). Weaning in rats: II. Pup behavior patterns. *Developmental Psychobiology, 23(6)*, 495-510.

Tonkowicz, P., Robertson, M., & Voogt, J. (1983). Secretion of rat placental by the fetal placenta and its inhibitory effect on prolactin surges. *Biology of Reproduction, 28(3)*, 707-716.

Toufexis, D.J., Kyriazis, D., & Woodside, B. (2002). Chronic neuropeptide Y Y5 receptor stimulation suppresses reproduction in virgin female and lactating rats. *Journal of Neuroendocrinology, 14(6)*, 492-497.

Trayhurn, P. (1989). Thermogenesis and the energetics of pregnancy and lactation. *Canadian Journal of Physiology and Pharmacology, 67(4)*, 370-375.

Trivedi, P., Yu, H., MacNeil, D.J., Van der Ploeg, L.H.T., & Guan, X-M. (1998). Distribution of orexin receptor mRNA in the rat brain. *Federation of European Biochemical Societies Letters, 438*, 71-75.

Trujillo, M.L., Spuch, C., Carro, E., & Senaris, R. (2011). Hyperphagia and central mechanisms for leptin resistance during pregnancy. *Endocrinology, 152(4)*, 1355-1365.

Tschop, M., Smiley, D.L., & Heiman, M.L. (2000). Ghrelin induces adiposity in rodents. *Nature, 407(6806)*, 908-913.

Uvnas-Moberg, K., Eriksson, M. (1996). Breastfeeding: physiological, endocrine and behavioural adaptations caused by oxytocin and local neurogenic activity in the nipple and mammary gland. *Acta Paediatrica, 85(5)*, 525-530.

Vaisse, C., Halaas, J.L., Horvath, C.M., Darnell, J.E. Jr., Stoffel, M., & Friedman, J.M. (1996). Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nature Genetics, 14(1)*, 95-97.

Valdez, S.R., Penissi, A.B., Deis, R.P., & Jahn, G.A. (2007). Hormonal profile and reproductive performance in lactation deficient (OFA hr/hr) and normal (Sprague-Dawley) female rats. *Reproduction, 133(4)*, 827-840.

Valera, A.G., Cavalcante, J.C., Elias, C.F., & Felcio, L.F. (2006). Cocaine- and amphetamine-regulated transcript is overexpressed in the anteroventral periventricular nucleus of pregnant rats. *Journal of Neuroendocrinology, 18(9)*, 711-714.

Van Heek, M., Compton, D.S., France, C.F., Tedesco, R.P., Fawzi, A.B., Graziano, M.P., Sybertz, E.J., Strader, C.D., & Davis, H.R. Jr. (1997). Diet-induced obese mice develop peripheral, but not central, resistance to leptin. *Journal of Clinical Investigations, 99(3)*, 385-390.

Vernon, R.G., & Pond, C.M. (1997). Adaptations of maternal adipose tissue to lactation. *Journal of*

Mammary Gland Biology and Neoplasia, 2(3), 231-241.

Wang, J., Jiang, C., Li, X., Liu, C., Cheng, N., & Hao, Y. (2009). The protective mechanism of progesterone on blood-brain barrier in cerebral ischemia in rats. *Brain Research Bulletin, 79(6)*, 426-430.

Wang, J., Murata, T., Narita, K., Honda, K., & Higuchi, T. (2003). Variation in the expression of orexin and orexin receptors in the rat hypothalamus during the estrous cycle, pregnancy, parturition, and lactation. *Endocrine, 22(2)*, 127-134.

Walker, C.D., Mitchell, J.B., & Woodside, B.C. (1995). Suppression of LH secretion in food- restricted lactating females: effects of ovariectomy and bromocryptine treatment. J*ournal of Endocrinology, 146(1)*, 95-104.

Walker, C.D., Salzmann, C., Long, H., Otis, M., Roberge, C., & Gallo-Payet, N. (2004). Direct inhibitory effects of leptin on the neonatal adrenal and potential consequences for brain glucocorticoid feedback. *Endocrine Research, 30(4)*, 837-844.

Walsh, R.J., Slaby, F.J., & Posner, B.I. (1987). A receptor-mediated mechanism for the transport of prolactin from blood to cerebrospinal fluid. *Endocrinology, 120(5)*, 1846-1850.

Welch, C.C., Grace, M.K., Billington, C.J., & Levine, A.S. (1994). Preference and diet type affect macronutrient selection after morphine, NPY, norepinephrine, and deprivation. *American Journal of Physiology, 266(2 Pt 2),* R426-R433.

Widdowson, P.S., Upton, R., Buckingham, R., Arch, J., & Williams, G. (1997). Inhibition of food response to intracerebroventricular injection of leptin is attenuated in rats with diet-induced obesity. *Diabetes, 46(11)*, 1782-1785.

Wilding, J.P., Ajala, M.O., Lambert, P.D., & Bloom, S.R. (1997).Additive effects of lactation and food restriction to increase hypothalamic neuropeptide Y mRNA in rats. T*he Journal of Endocrinology, 52(3)*, 365-369.

Williams, K.W., & Elmquist, J.K. (2012). From neuroanatomy to behavior: central integration of peripheral signals regulating feeding behavior. *Nature Neuroscience, 15(10)*, 1350-1355.

Wilsoncroft, W.E. (1968). Babies by bar-press: maternal behavior in the rat. *Behavior Research Methods, 1(6)*, 229-230.

Windle, R.J., Shanks, N., Lightman, S.L., & Ingram, C.D. (1997). Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats. *Endocrinology, 138(7)*, 2829-2834.

Woodside, B. (1991). Effects of food restriction on the length of lactational diestrus in rats. *Hormones & Behavior, 25(1)*, 70-83.

Woodside, B. (2006). Morphological plasticity in the maternal brain: comment on Kinsley et al.; motherhood and the hormones of pregnancy modify concentrations of hippocampal neuronal dendritic spines. *Hormones and Behavior, 49(2)*, 129-130.

Woodside, B. (2007). Prolactin and the hyperphagia of lactation. *Physiology and Behavior, 91*, 375-382.

Woodside, B., Abizaid, A., & Jafferali, S. (1998). Effect of acute food deprivation on lactational infertility in rats is reduced by leptin administration. *American Journal of Physiology, 274(6)*, R1653-R1658.

Woodside, B., Abizaid, A., & Walker, C. (2000). Changes in leptin levels during lactation: implications for lactational hyperphagia and anovulation. *Hormones & Behavior, 37(4)*, 353-365.

Woodside, B., Augustine, R.A., Ladyman, S.R., Naef, L., Grattan, D.R. (2008). Role of prolactin in the metabolic adaptations to pregnancy and lactation. In: *Neurobiology of the Parental Brain*, Academic Press, p. 249-268.

Woodside, B., Budin, R., Wellman, M.K., & Abizaid, A. (2012). Many mouths to feed: the control of food intake during lactation. *Frontiers in Neuroendocrinology, 33(3)*, 301-314.

Woodside, B., & Popeski, N. (1999). The contribution of changes in milk delivery to the prolongation of lactational infertility induced by food restriction or increased litter size. *Physiology and Behavior, 65(4-5)*, 711-715.

Wu, Z., Xu, Y., Zhu, Y., Sutton, A., Zhao, R, Lowell, B.B., Olson, D.P., & Tong, Q. (2012). An obligate role of oxytocin neurons in diet induced energy expenditure. *PLoS One, 7(9)*, e45167.

Xu, J., Kirigiti, M.A., Grove, K.L., & Smith, S.M. (2009). Regulation of food intake and gonadotropin-releasing hormone/luteininzing hormone during lactation: role of insulin and leptin. *Endocrinology, 150(9)*, 4231-4240.

Yin, X., Li, Y., Xu, G., An, W., & Zhang, W. (2009). Ghrelin fluctuation, what determines its production? *Acta Biochim Biophys Sin, 41(3)*, 188-197.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J.M. Positional cloning of the mouse obese gene and its human homologue. *Nature, 372*, 425-431.

Zhao, A.Z., Shinohara, M.M., Huang, D., Shimizu, M., Eldar-Finkelman, H., Krebs, E.G., Beavo, J.A., & Bornfeldt, K.E. (2000). Leptin induces insulin-like signaling that antagonizes cAMP elevation by glucagon in hepatocytes. *Journal of Biological Chemistry, 275(15)*, 11348-11354.

Zheng, H., Patterson, L.M., Pfifer, C.B., Berthoud, H.R. (2005). Brainstem melanocortinergic modulation of meal size and identification of hypothalamic POMC projections. *American Journal of Physiology. Regulatory, Integrative and Comparitive Physiology, 289(1)*, R247-R258.

Zigman, J.M., Jones, J.E., Lee, C.E., Saper, C.B., & Elmquist, J.K. (2006). Expression of ghrelin receptor mRNA in the rat and mouse brain. *Journal of Comparative Neurology, 20(3)*, 528-548.