# The role of satiety hormones in developmental programming of obesity, stress, and neuroimmune function

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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## DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work is not submitted previously, in whole or in part, to qualify for any academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and ethics procedures and guidelines have been followed.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Signed: Ilvana Ziko

Date: 26/10/2018

## THESIS CONTRIBUTIONS

I would like to thank all the colleagues that contributed to my completion of this thesis.

This thesis was conceived and designed by Ilvana Ziko, Associate. Prof. Sarah J. Spencer and Dr Luba Sominsky. Ilvana Ziko and Dr Luba Sominsky conducted the animal studies and collected samples for Chapters 2, 3, 4, 5 and 6. Ilvana Ziko and Dr Luba Sominsky performed the animal work and conducted the *in vitro* experiments. Dr Luba Sominsky performed the growth hormone, adrenocorticotropic hormone and ghrelin assays, as well as qRT-PCR analysis on adult female samples that are reported in Chapter 5, Ilvana Ziko performed all of the neonatal analysis in Chapter 5. Ilvana Ziko with assistance from Dr Simone N. De Luca and Mr Alita Soch collected tissue samples for Chapter 6 and Dr Simone De Luca performed the triglyceride assay in Chapter 4. Ilvana Ziko and Ms Thai Xinh Nguyen together conducted the signal transducer and activator of transcription 3 immunohistochemical studies for Chapters 2 and 3. All other laboratory work, data analysis, and writing was conducted by Ilvana Ziko under the guidance of Associate Prof. Sarah J. Spencer and Dr Luba Sominsky.

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## PUBLICATIONS

**The work in this thesis has lead into the following publications:**

**1.** Ziko I, Sominsky L, De Luca S, Lelngei F, Spencer SJ. Acylated but not des-acylated ghrelin suppresses the cytokine response to lipopolysaccharide and does so independently of the HPA axis. Brain Behaviour and Immunity. 2018; pii: S0889-1591(18)30332-5.

This work forms the basis of Chapter 6.

**2.** Ziko I, Sominsky L, Nguyen TX, Yam KY, De Luca SN, Korosi A, Spencer SJ. Hyperleptinemia in neonatally overfed female rats does not dysregulate feeding circuitry, Frontiers in Endocrinology. 2017; 8: 287.

This work forms the basis of Chapter 4.

**3.** Sominsky L, Ziko I. Spencer SJ. Hypothalamic effects of neonatal diet: reversible and only partially leptin-dependent. Journal of Endocrinology. 2017; 234 41-56.

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**4.** Sominsky L, Ziko I, Sarah J. Spencer. Neonatal overfeeding disrupts pituitary ghrelin signalling in female rats long-term; implications for the stress response. PLOS One. 2017; 12(3):e0173498.

This work forms the basis of Chapter 5.

**5.** Sominsky L* & Ziko I*, Andrew ZB, Sarah J. Spencer. Early life disruption to the ghrelin system with over-eating is resolved in adulthood in male rats. Journal of Neuropharmacology. 2017; 113: 21e30. *Equal contribution.

This work forms the basis of Chapter 3.

**First- and co-authored publications that are not explicitly part of this thesis:**

**1.** Sominsky L, Ong LK, Ziko I, Dickson PW, Spencer SJ. Neonatal overfeeding increases capacity for catecholamine biosynthesis from the adrenal gland acutely and in the long-term in the male rat. Molecular and Cellular Endocrinology. 2017; 470: 295-303. My contribution to this publication consists of assistance with Western blotting technique and draft editing.

**2.** Yam KY, Ruigrok SR, Ziko I, De Luca SN, Lucassen PJ, Spencer SJ, Korosi A. Ghrelin and hypothalamic NPY/AgRP expression in mice are affected by chronic early-life stress exposure in a sex-specific manner. Psychoneuroendocrinology. 2017; 86:73-77. My contribution to this paper consists of assistance with immunohistochemical techniques, image analysis, generation of results and draft editing.

**3.** Sominsky L, Ziko I, Soch A, Smith J, Spencer SJ. Neonatal overfeeding induces early decline of the ovarian reserve: Implication for the role of Leptin. Molecular and Cellular Endocrinology. 2016; 15;431:24-35. In this publication I assisted with generation of results on the protein expressions and also with draft editing.

**4**. De Luca SN, Ziko I, Dhuna K, Sominsky L, Tolcos M, Stokes L, Spencer SJ. Neonatal overfeeding by small-liter rearing sensitizes hippocampal microglial responses to immune challenge: reversal with neonatal repeated injections of saline as well as minocycline. Journal of Neuroendocrinology. 2017; 29 (11):e12540. My contribution to this paper consists of assistance with generation of results from corticosterone assay and draft editing.

**5.** De Luca S, Ziko I**,** Sominsky L, Nguyen JCD, Dinan T, Miller AA, Jenkins TA, Spencer SJ. Early life overfeeding impairs spatial memory performance by reducing microglial sensitivity to learning. Journal of Neuroinflammation. 2016; 18;13(1):112. My contribution to this publication consists of assistance with animal sample collection and draft editing.

**6.** Cai G, Ziko I**,** Barwood JM, Soch A, Sominsky L, Molero JM, Spencer SJ. Overfeeding during a critical postnatal period exacerbates hypothalamic-pituitary-adrenal axis responses to immune challenge: a role for adrenal melanocortin 2 receptors. Scientific Reports. 2016; 12;6:21097. My contribution to this publication consists of assistance with animal sample collection and draft editing.

**7.** Cai G, Dinan T, Barwood JM, De Luca S, Soch A, Ziko I, Chan SMH, Zeng XY, Li S, Ye J, Molero JM, Spencer SJ. Neonatal overfeeding attenuates acute central pro-inflammatory but not metabolic effects of short-term high fat diet. Frontiers in Neuroscience. 2015; 13;8:446. My contribution to this publication consists of assistance with animal sample collection and draft editing.

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Ziko I, Sominsky L, Spencer SJ. Neonatal overfeeding effects on ghrelin signalling. Students of Brain Research Symposium, Melbourne AUS, November 2016. (Poster)

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## ABBREVIATIONS

**A**

| | |
|---|---|
| AB | Antibody |
| ABC | Avidin-Biotin Horseradish Peroxidase Complex |
| *Actb* | The gene for *actin beta* |
| ACTH | Adrenocorticotropic hormone |
| AD | Alzheimer's disease |
| AG | Acyl ghrelin |
| AgRP | Agouti related protein |
| AMP | Adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| ANOVA | Analysis of variance |
| ARC | Arcuate nucleus of the hypothalamus |
| AVP | Vasopressin |

**B**

| | |
|---|---|
| BBB | Blood brain barrier |
| BAT | Brown adipose tissue |
| BMI | Body mass index |
| BSA | Bovine serum albumin |

**C**

| | |
|---|---|
| CART | Cocaine-amphetamine regulated transcript |
| cDNA | Complementary deoxyribonucleic acid |
| CNS | Central nervous system |
| CL | Control litter |
| CORT | Corticosterone |

| | |
|---|---|
| CRH | Corticotropin releasing hormone |
| *Crhr1* | The gene for *corticotropin-releasing hormone receptor 1* |
| CRF | Corticotropin releasing factor |
| C(t) | Threshold cycle |
| CV | Coefficient of variation |
| *Cx3cr1* | The gene for *C-X3-C motif chemokine receptor 1* |
| Cy5 | Cyanine5 |
| **D** | |
| DAB | Diaminobenzidine |
| DAG | Des-acyl ghrelin |
| DAPI | 4',6-diamidino-2-phenylindole |
| DIO | Diet induced obesity |
| DMEM/F-12 | Dulbecco's modified Eagle's medium/Nutrient mixture F-12 |
| DMH | Dorsomedial hypothalamus |
| **E** | |
| E | Embryonic day |
| E. coli | Escherichia coli |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK | Extracellular signal-regulated kinase |
| **F** | |
| FITC | Fluorescein isothiocyanine |
| FSH | Follicle-stimulating hormone |
| **G** | |
| GD | Gestational diabetes |

| | |
|---|---|
| GH | Growth hormone |
| *GHSR1a* | The gene for *growth hormone secretagogue receptor* |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GnRH | Gonadotropin-releasing hormone |
| GOAT | Ghrelin O-acyltransferase |
| **H** | |
| HFD | High fat diet |
| $H_2O_2$ | Hydrogen peroxide |
| HMGB1 | High-mobility group box 1 |
| HPA | Hypothalamic-pituitary-adrenal |
| HPG | Hypothalamic-pituitary-gonadal |
| **I** | |
| ICV | Intracerebroventricular |
| IF | Immunofluorescence |
| IHC | Immunohistochemistry |
| IGF-2 | Insulin-like growth factor-2 |
| IFN-γ | Interferon-γ |
| IL | Interleukin |
| i.p. | Intraperitoneal |
| **J** | |
| JAK | Janus kinase |
| **L** | |
| LH | Lateral hypothalamus |
| *LEPR* | The gene for *leptin receptor* |
| LPS | Lipopolysaccharide |

**M**

| | |
|---|---|
| Mal | MyD88 adaptor-like |
| MAPK | Mitogen activated protein kinase |
| MBH | Mediobasal hypothalamus |
| MBOAT | Membrane-bound O-acyltransferase |
| MC4R | Melanocortin-4 receptor |
| MDD | Major depressive disorders |
| MG | Magnocellular |
| MP | parvocellular |
| mRNA | Messenger ribonucleic acid |
| α-MSH | α-melanocyte-stimulating hormone |
| β-MSH | β-melanocyte-stimulating hormone |
| mTOR | Mammalian target of rapamycin |
| MyD88 | Myeloid differentiation primary response gene 88 |

**N**

| | |
|---|---|
| NE | Norepinephrine |
| NPY | Neuropeptide Y |
| NHS | Normal horse serum |
| NFκB | Nuclear factor κB |
| NTC | No template control |
| NTS | Nucleus tractus solitarius |
| *Nr3c1* | The gene for *nuclear receptor subfamily 3 group C member 1* |
| *Nr3c2* | The gene for *nuclear receptor subfamily 3 group C member 2* |

**O**

| | |
|---|---|
| OBR | Obese gene/leptin receptor |

**P**

| | |
|---|---|
| P | Postnatal day |
| PBS | Phosphate buffered saline |
| PBMC | Peripheral blood mononuclear cells |
| PCR | Polymerase chain reaction |
| PFA | Paraformaldehyde |
| PGE2 | Prostaglandin-2 |
| POMC | Proopiomelanocortin |
| PTSD | Post-traumatic stress disorder |
| PVN | Paraventricular nucleus of the hypothalamus |

**Q**

| | |
|---|---|
| qRT-PCR | Quantitative real-time PCR |

**R**

| | |
|---|---|
| RT | Room temperature |

**S**

| | |
|---|---|
| Sal | Saline |
| s.c. | Subcutaneous |
| SEM | Standard error of the mean |
| SH2B1 | Src Homology 2 adaptor protein 1 |
| SL | Small litter |
| SMLA | Super mice leptin antagonist |
| SRLA | Super rat leptin antagonist |
| STAT3 | Signal transducer and activator of transcription 3 |

**T**

| | |
|---|---|
| TLRs | Toll-like receptors |

| | |
|---|---|
| TNFα | Tumor necrosis factor-α |
| TRAM | TRIF-related adaptor molecule |
| **V** | |
| VMH | Ventromedial hypothalamus |
| VTA | Ventral tegmental area |
| **W** | |
| WHO | World Health Organisation |
| WHR | Waist-to-hip ratio |

## ABSTRACT

Ghrelin and leptin, two important metabolic hormones involved in regulation of energy balance in the adult, have recently been shown to have a neurotrophic role during early life development in that they regulate neuronal connectivity within regions of the hypothalamus responsible for control of energy balance. Disturbances to the early life nutritional environment including overnutrition have been highly correlated with long-life metabolic problems, such as childhood obesity and its complications. (Throughout this thesis the term overnutrition refers to the neonatally overfed animal model due to litter manipulation and not as a form of malnutrition, which may elicit similar offspring phenotypes.) In addition to their metabolic roles, leptin and ghrelin appear to be important for early brain development, with leptin stimulating and ghrelin inhibiting the establishment of appetite regulatory circuitry. Recent research suggests that the neurodevelopmental actions of leptin and ghrelin are restricted to a crucial developmental period that occurs during the first two weeks after birth in rodents, which in humans this critical period encompasses the third trimester of pregnancy and approximately 1000 days after birth. However, the effects of overnutrition on the developing brain during this period and the mechanisms that underlie neurodevelopmental actions of leptin and ghrelin are currently unknown. It is also unclear whether overnutrition affects brain development in males and females to the same extent. The early developmental period is associated with enhanced neuronal plasticity, and disturbances during this critical time have the potential to program later life health outcomes. It is therefore important to shed further insight into the roles of leptin and ghrelin, to develop interventions that alleviate the incidence of childhood obesity and associated comorbidities.

An animal model of childhood obesity in rats was used to investigate the short- and long-term effects of neonatal overfeeding on the leptin and ghrelin system. The animal model was achieved by manipulating litter sizes into control litters of 12 pups and small litters of 4 pups, the later representing the neonatal overfeeding environment. The reduction of litter size immediately after birth, led to a significant increase in dietary intake. Consequently, neonatally overfed pups experienced accelerated weight gain and disrupted circulating leptin and ghrelin levels in comparison to control counterparts. Using this model we examined the short- and long-term effects of neonatal overnutrition on the leptin and ghrelin systems in males and females. We investigated here the effects of neonatal overnutrition on hypothalamic orexigenic and anorexigenic neuronal fibre immunoreactivity and if these changes were mediated by leptin in male rats. We also investigated whether these effects could be normalised by a leptin antagonist. We found that neonatal overnutrition in males was associated with short-term central leptin resistance, altered orexigenic neuronal fibre immunoreactivity and no changes in anorexigenic immunoreactivity. We also found that short-term neonatal leptin antagonism did not reverse excess body weight or hyperleptinemia. Our findings suggest that factors other than leptin contribute to the obese phenotype. Most importantly, we found that in males the early life effects of neonatal overfeeding are partly resolved in adulthood, emphasising the importance of brain plasticity. Interestingly, neonatally overfed females, despite an obese phenotype characterised by hyperleptinemia and increased body weight compared to controls, did not show the same changes in the central feeding circuitry as observed in males. These findings are suggestive of sex differences in the effects of neonatal overfeeding and of differences in the ability of

the male and female central systems to respond to challenges in the early life nutritional environment.

Similarly to leptin, ghrelin along with its appetite-stimulating role in adults is involved in the establishment of hypothalamic feeding pathways during neonatal development in rodents. Here was investigated long-term effect of neonatal overfeeding on the ghrelin system including the ability of both forms of ghrelin (acyl ghrelin and des-acyl ghrelin) to access the hypothalamus in male rats. We demonstrated that neonatal overfeeding affects the ghrelin system during early life by suppressing circulating ghrelin levels and increasing hypothalamic responsiveness to exogenous acyl but not des-acyl ghrelin. Neonatal overnutrition also affects the ability of acyl ghrelin to reach the hypothalamus. Importantly, the influences of neonatal overnutrition observed during early life were resolved in adulthood. In contrast to males, where neonatal overnutrition resulted in compromised hypothalamic ghrelin signalling, in females, the central ghrelin system and circulating ghrelin remained intact. However, neonatal overnutrition in females was associated with long-term alterations in the capacity for the pituitary gland to respond to ghrelin.

Ghrelin has recently been shown to have additional properties to those involved in satiety signalling, including regulation of hypothalamic-pituitary-adrenal (HPA) axis responses to psychological stress, as well as being a potent anti-inflammatory agent. Here were investigated both forms of ghrelin (acyl and des-acyl ghrelin) and their anti-inflammatory activity hypothesising that this activity is mediated via the HPA axis. In experiments where male rats were concomitantly injected with acyl or des-acyl ghrelin and lipopolysaccharide (LPS) acyl, but not des-acyl ghrelin, suppressed

the inflammatory cytokine response to LPS. Des-acyl ghrelin also had no effects on components of the HPA axis. Acyl ghrelin, despite stimulating neuronal activation in the paraventricular nucleus of the hypothalamus *in vivo* and stimulating adrenocorticotropic hormone release from the pituitary *in vitro*, did not affect the HPA axis response to LPS. These findings suggest acyl ghrelin's anti-inflammatory effects are independent of its actions on the HPA axis and have implications for the potential use of this peptide for treatment of inflammatory conditions without compromising HPA axis activity.

In conclusion, findings from this thesis indicate that metabolic hormones leptin and ghrelin have immense importance in the development of central centres of energy control and metabolism. Altered nutritional environment during early development affects both leptin and ghrelin systems in the rat, however it resolves to a certain extent in adulthood. Importantly, these central developmental changes mediated by leptin and ghrelin have a strong sex-specific factor. In this thesis we also added to the understanding of the mechanisms by which ghrelin exerts its anti-inflammatory properties, which is of great importance in development of therapeutic strategies for treatment of inflammatory conditions.

# Chapter 1

# Introduction and Background

## Childhood obesity

Childhood obesity is one of the world's greatest health challenges. The incidence of childhood obesity has been increasing during the last decades and there are over 42 million children under the age of five who are overweight or obese (WHO, 2016). Childhood obesity is associated with a series of complications such as type 1 and 2 diabetes, cardiovascular diseases, some types of cancers, hypertension, early puberty, steatohepatitis, sleep apnoea, asthma, musculoskeletal disorders as well as psychological problems, amongst other conditions (Figure 1.1. (Barton 2012; Kim, Despres, and Koh 2016)), making childhood obesity one of the leading causes of death in the United States of America and Europe (Mokdad et al. 2005; Park et al. 2012). Although, in some countries such as United States of America, Western European countries, Japan and Australia included, levels of childhood obesity may have reached a plateau in the last 10 years, the prevalence of childhood obesity remains very high especially in the low and middle income countries. This represents a significant health issue particularly in the more vulnerable groups in the population (Ogden et al. 2014; de Onis, Blossner, and Borghi 2010). There is now strong evidence that childhood obesity leads to adult obesity and its related comorbidities (Kelsey et al. 2014).

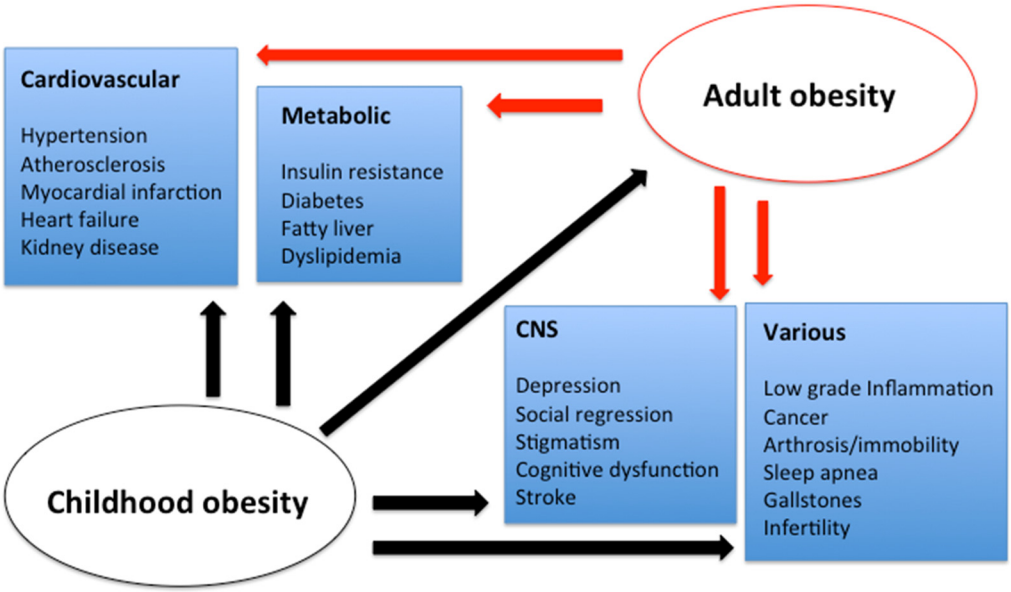


**Figure 1.1 Childhood obesity-related diseases.**
Childhood obesity poses a lifetime risk for other disease conditions including cardiovascular, metabolic, and problems with the central nervous system (CNS) during both childhood and adulthood. Modified from (Barton 2012).

Similarly to other countries, in Australia percentages of obese children have increased dramatically in the last decades with around 20% of children under the age of five being overweight and around 5% of them classified as obese (Pearce et al. 2016). According to the Centers for Diseases Control and Prevention, a child is considered to be overweight between the 85-95$^{th}$ percentile and obese above the 95$^{th}$ percentile (de Onis et al. 2013). Based on survey analyses, the World Health Organisation (WHO) estimated that the prevalence of children under the age of five years old with a body mass index (BMI) of over the 98$^{th}$ percentile, increased from 4.2% in 1990 to 6.7% in 2010, and is expected to increase to 9.1% by 2020 (Lakshman, Elks, and Ong 2012). These increased numbers of obese children result in increased health costs compared to those of normal healthy children of the same age (Brown et al. 2017). The prevalence of childhood obesity in many countries including Australia continues to rise, despite concerted efforts and recognition of the problem, making it a major focus

of research.

The interaction of several factors including behavioural, environmental, physiological and hereditary has contributed to the increasing prevalence of obesity (Hruby and Hu 2015). Twin studies, for instance, have estimated a heritability impact on BMI in children of between 40-70% (Farooqi and O'Rahilly 2000; Wardle et al. 2008). A number of mutations in the leptin receptor gene (*LEPR),* also known as *OBR* for obese gene, melanocortin 4 receptor (*MC4R*), as well as reduced methylation of the insulin-like growth factor 2 (*IGF2)* gene are associated with obesity (reviewed in (Willyard 2014)). However, the genetic contribution to disease risk has been shown to be smaller than the crucial role lifestyle plays in the development of obesity. Studies investigating the gene-lifestyle interactions in obesity have suggested that a healthy lifestyle may partially or totally eliminate the effects of genetic predisposition (Temelkova-Kurktschiev and Stefanov 2012).

## Perinatal programming of obesity – animal models

As is predicted by the strong risk of obesity in children progressing to obesity in later life, early life events including the perinatal and postnatal environment can significantly contribute to the likelihood of an individual becoming obese later in life (Robillard and Segar 2006). A number of animal models of maternal overnutrition or under-nutrition during pregnancy or post-pregnancy have been used to study the early impact of the nutritional environment. One animal model that allows the study of effects of postnatal nutritional status in rodents is by manipulation of litter sizes. Larger than normal litter sizes with less access to maternal milk mimics an under-nutritional environment, whereas a smaller than normal litter size with exposure to

excess maternal milk mimics an over-nutritional environment (Fiorotto et al. 1991). In rodents one of the main periods of developmental changes to brain pathways governing feeding and metabolism is the first two weeks after birth and any perturbations to the nutritional status during this time permanently affects their metabolic programming (Plagemann 2006). Essential metabolic hormones that regulate energy balance via their action in the hypothalamus are leptin, ghrelin, insulin and glucose, and during important developmental periods all of these hormones have their specific roles in modulating axonal innervation of important sites involved in metabolic control (Srinivasan et al. 2008).

Insulin, for instance, inactivates orexigenic neuropeptide Y (NPY) and agouti related peptide (AgRP) neurons in the ARC (Benoit et al. 2002) and upregulates the anorexigenic proopiomelanocortin (POMC) in order to control energy intake (Qiu et al. 2014; Loh et al. 2017). Furthermore, insulin has a neurotrophic role during important periods of neurodevelopment (the first three weeks after birth). Plagemann and colleagues have shown that hypothalamic injection of insulin to rodents during the second or the eighth day of life is associated with long-term changes in weight regulation (Plagemann et al. 1992). Vogt and colleagues used an animal model of maternal high fat diet during lactation to establish the crucial role of insulin in formation of hypothalamic neuronal projections (Vogt et al. 2014). Human studies have shown that children from diabetic mothers experience impaired glucose tolerance and hyperinsulinemia, which further relates to adult obesity (Plagemann et al. 1997; Simerly 2008).

The focus of this thesis was on the roles of leptin and ghrelin during development. These hormones play an important role in regulation of hypothalamic development in the formation of neural circuits (Bouret, Draper, and Simerly 2004b). Leptin, in addition to its role in metabolic regulation, has shown to initiate the growth of neuronal growth and connectivity within regions of the hypothalamus related to feeding and metabolism (Bouret, Draper, and Simerly 2004b). Ghrelin in adulthood plays an important role in central control of energy expenditure by controlling feeding, adiposity and glucose metabolism (Tschop, Smiley, and Heiman 2000; Wren et al. 2001; Druce et al. 2005). In addition to this, ghrelin has an important role in programming feeding-related neurocircuitry during development, that of suppressing leptin-initiated neuronal outgrowth (Steculorum et al. 2015)

Leptin levels in circulation positively correlate with fat deposition in adults. In neonates, naturally occurring increases in levels of leptin (known as the leptin surge) are crucial in initiating and supporting hypothalamic developmental changes during the first weeks of life in rodents (Ahima, Prabakaran, and Flier 1998). Once differentiated, neurons send axonal projections to target cells. Arcuate nucleus of the hypothalamus (ARC) neurons have relatively short projections limiting their connections within the hypothalamus. Comprehensive research has defined the timing of neuronal projections from the ARC towards other regions of the hypothalamus responsible for regulation of feeding. The paraventricular nucleus of the hypothalamus (PVN) is not innervated from ARC neurons until after postnatal day (P)10 (Bouret, Draper, and Simerly 2004a). At this age the leptin receptor is absent from PVN neurons and the timing of the appearance of its expression coincides with ARC axonal innervation of the PVN (reviewed in (Bouret 2017)).

Leptin and ghrelin are both important during these neuronal developmental periods, but how they are influenced by early life nutritional environment, the specific effects of this on females and if developmental perturbations to this circuitry are permanent have not been thoroughly studied. We defined here the role of these hormones in the early life overfeeding context. In these studies we consider the possibility that males and females respond differently to early life environmental insults. Additionally, the roles of different forms of ghrelin, acyl ghrelin (AG) and des-acyl ghrelin (DAG), have not been characterized during neonatal life, especially DAG due to the current lack of information on its receptor. The studies described in this thesis give insights into the role of DAG during development.

## The role of leptin in metabolism

Leptin, discovered in 1994 (Zhang et al. 1994), is secreted mainly by adipocytes in white adipose tissue and is found in circulation in proportional amounts to fat stores in the organism (Considine et al. 1996). Leptin exerts its effect by binding to its receptors, which exist in several isoforms as a result of alternative splicing (*LEPR a-f*). *LEPR a*, the short isoform of the leptin receptor is important in leptin transport across the blood-brain barrier (BBB) (Bjorbaek et al. 1998), whereas the long isoform, *LEPR b,* that is mainly expressed in the hypothalamus (the only isoform with the full length intracellular domain required for cell signaling) is important in mediating signal transduction (Elmquist et al. 1998; Friedman and Halaas 1998). Binding of leptin to its receptor activates a series of signal transduction pathways including the Janus kinase-signal transducers and activators of transcription 3 (JAK-STAT3) pathway, important in regulation of energy homeostasis, mammalian target

of rapamycin (mTOR), adenosine monophosphate (AMP) activated protein kinase (AMPK) and mitogen activated protein kinase (MAPK) as reviewed in (Robertson, Leinninger, and Myers 2008). Leptin activates JAK, which leads to the phosphorylation of proteins of the JAK family (particularly STATs 1 and 3). In hypothalamic neurons, the STATs dimerize and translocate into the nucleus where they induce transcription of genes encoding proopiomelanocortin (POMC) and decrease transcription of genes encoding neuropeptide Y (NPY) and agouti related protein (AgRP) (reviewed in (Gurzov et al. 2016)), collectively resulting in the suppression of feeding behavior.

Leptin has an important role in maintaining energy homeostasis via interactions with specific regions of the hypothalamus, particularly the ARC, but also the lateral hypothalamus (LH), ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH), which are all involved in central control of feeding and energy expenditure (Myers et al. 2009). In the hypothalamus leptin activates two major antagonizing groups of neurons: anorexigenic POMC / cocaine and amphetamine regulated transcript (CART) and orexigenic NPY / AgRP neurons (Cowley et al. 2001; Morrison et al. 2005). These neurons then project to secondary neurons in the PVN, LH and VMH (reviewed in (Timper and Bruning 2017; Myers and Olson 2012; Waterson and Horvath 2015)).

Leptin-deficient humans and animals develop obesity as a result of a combination of reduced energy expenditure and increased calorie intake as a result of aberrant signalling of these pathways and this often progresses to diabetes (St-Pierre and Tremblay 2012; D'Souza A et al. 2014). Leptin deficiency, furthermore, is associated

with infertility due to abnormal synthesis of gonadotropins and hormones related to gonadal development (Elias and Purohit 2013). Additionally, leptin deficient animals experience increased glucocorticoids levels, contributing to disrupted linear growth (reviewed in (Park and Ahima 2015)), and also differences in regulating body temperature compared to wild type animals (Fischer et al. 2016). These conditions are reversed with administration of exogenous leptin if given during the neonatal period. However, obesity in individuals that are not leptin deficient is associated with high circulating leptin levels indicating a malfunction at central levels of leptin signaling, known as leptin resistance.

Two possible sites at which leptin resistance occurs are the BBB and the leptin intracellular signaling pathway. In order to reach the *Lepr b* receptor in the hypothalamus, peripheral leptin needs to pass through the BBB via a saturable transporter (Lopez 2016). In obesity, this transporter becomes saturated due to continuously high circulating leptin levels, resulting in a reduced capacity to respond to further increases in leptin, and thus leptin resistance (Crujeiras et al. 2015). In the early stages of obesity, leptin resistance is observed at the level of the BBB as well as in the ARC (Munzberg, Flier, and Bjorbaek 2004), whereas in advanced obesity leptin resistance occurs in the BBB, ARC as well as in other hypothalamic and extra-hypothalamic regions such as ventral tegmental area (VTA) (Matheny et al. 2011). Obesity-induced leptin resistance at the BBB level can also be mediated by high levels of triglycerides. Triglycerides cross the BBB and induce central leptin resistance by blocking the ability of leptin to stimulate its receptors in the hypothalamus (Banks et al. 2017). Tanycytes, specialized glial cells at the median eminence, have an important role in regulating leptin transport through the BBB by

activating the extracellular signal-regulated kinase (ERK) pathway. In healthy subjects these cells successfully transport leptin towards the mediobasal hypothalamus (MBH), where it applies its anorexigenic effects. In diet-induced obesity these cells retain leptin, disrupting its entry to and action in the MBH (Balland et al. 2014; Gao, Tschop, and Luquet 2014). Megalin, a low-density lipoprotein receptor-related protein-2, is also thought to play an important role in leptin transport through the BBB. Animals that lack megalin in brain endothelial cells, experience hyperleptinemia, increased adiposity and decreased hypothalamic leptin signaling (Bartolome et al. 2017). The second contributor to leptin resistance may be a failure of leptin signaling pathways in specific neurons. The disruption of JAK-STAT3 signaling has been shown to be a contributor to leptin resistance. In obesity, leptin has an impaired ability to induce STAT3 phosphorylation (reviewed in (Ladyman and Grattan 2013)). Leptin signaling is negatively regulated by suppressor of cytokine signaling 3 (SOCS3) and protein tyrosine phosphatase 1B (PTP1B), which are both increased during obesity. Leptin signaling is positively regulated by Src Homology 2 adaptor protein 1 (SH2B1) and genetic deletion of this protein induces severe leptin resistance (Ren et al. 2005).

## Leptin in neurodevelopment

Recent literature has brought to light an additional role of this hormone, one that occurs during neonatal neuronal development. At this time leptin regulates the growth of neuronal connectivity within specific regions of the hypothalamus related to feeding and metabolism (Bouret, Draper, and Simerly 2004b). At birth, a rodent's hypothalamic connectivity is functionally immature. As mentioned above, the ARC signals feeding information via the DMH, LH, and PVN, and the pathways by which

these brain regions communicate do not become fully developed until after the second week of life; more specifically between day 12 and 14 in mice (Bouret, Draper, and Simerly 2004a) and between day 12 and 17 in rats (Bouret and Simerly 2006; Bouret 2010); Figure 1.2). During the early neonatal period animals maximize caloric intake to ensure growth and survival. At this time, there is a marked increase in circulating leptin, originally described as a leptin surge, which occurs from P7 to P14 peaking at P10 in mice and P4 to P14 with again a peak at P10, in rats (Ahima, Prabakaran, and Flier 1998; Delahaye et al. 2008). During this period, the ARC does not transmit leptin signals to the other parts of the hypothalamus. Consistent with this finding, administration of leptin during neonatal life does not decrease food intake or body weight until after weaning (separation from the dam on postnatal day 21) (Bouret 2013). Instead, this leptin surge acts on the brain to trigger the maturation of satiety communication pathways.

Leptin's importance in the development of neuronal projections has been studied in the leptin-deficient mice (ob/ ob), showing that leptin deficiency causes a significant delay in formation of projections from the ARC, especially towards the PVN (Bouret and Simerly 2004), with both orexigenic NPY/AgRP and anorexigenic POMC/CART projections being affected by leptin deficiency ((Proulx, Richard, and Walker 2002; Mistry, Swick, and Romsos 1999); Figure 1.2)). Moreover, leptin treatment in adult leptin-deficient mice is ineffective in reversing innervation deficiency, whereas supplementation during the neonatal period fully restores the density of ARC projections (reviewed in (Bouret et al. 2008)). These results suggest that leptin is a very important neurotrophic factor in controlling initiation and maturation of neuronal circuitry.

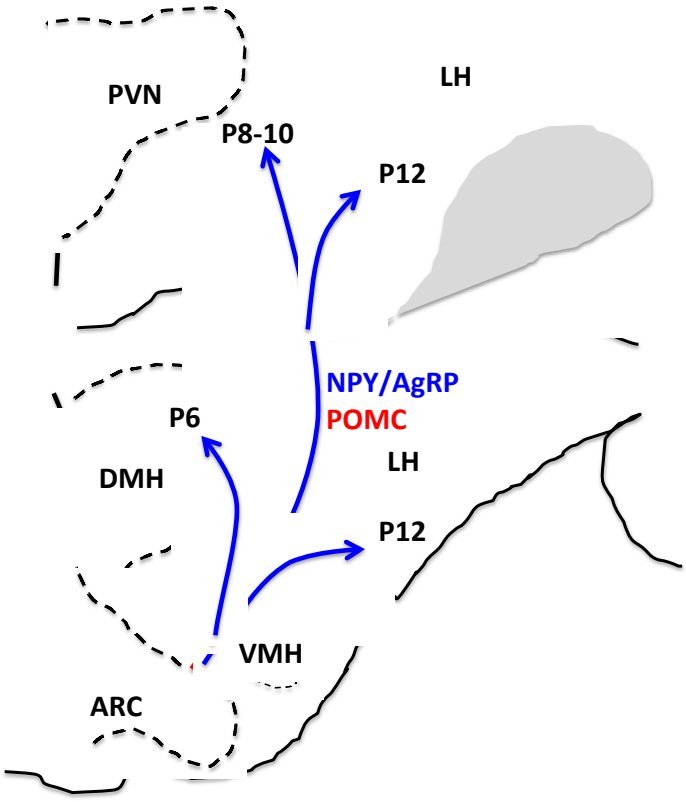


**Figure 1.2 The main hypothalamic nuclei involved in feeding regulation and programming of hypothalamic feeding circuits.**
Neuropeptide Y (NPY), agouti related peptide (AgRP) and proopiomelanocortin (POMC) neurofibres projecting from arcuate nucleus of the hypothalamus (ARC) towards other hypothalamic nuclei including dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH) paraventricular nucleus of the hypothalamus (PVN) and lateral hypothalamus (LH) and timing in postnatal days (P) of detection of these projections (Modified from (Bouret and Simerly 2006))

In the absence of the leptin surge at the crucial time, these signaling pathways do not properly develop and the animal is permanently impaired in its ability to regulate body weight and adiposity (Bouret and Simerly 2007). Alterations to maternal diet during pregnancy or lactation can affect the magnitude and onset of this leptin surge, leading to long-term alterations in body weight regulation in the animal (Proulx, Richard, and Walker 2002). The mother's milk is an important source of leptin for postnatal rodents. Pups suckled in small litters have greater access to mother's milk and greater than normal amounts of leptin (reviewed in (Spencer 2013)). This early

access to excessive amounts of leptin may trigger the leptin surge prematurely, leading to permanent differences in how these pathways communicate (Bouret and Simerly 2004). In this thesis it is tested whether neonatal overfeeding affects the establishment and long-term maintenance of this connectivity by assessing hypothalamic NPY, AgRP and POMC fibre density and its functional outputs during development.

## The role of ghrelin in metabolism

Ghrelin is a peptide hormone consisting of 28 amino acids and in adults is principally synthesised in P/D1-type cells in humans (Rindi et al. 2002) or X/A-like cells in rodents in the fundus of the stomach, which account for approximately 20-30% of the endocrine cell population of the oxyntic glands (Kojima et al. 1999; Date et al. 2000). In adults, ghrelin is also expressed in small amounts in duodenum, jejunum, ileum, colon, lung, heart, pancreas, kidney, testis, ovary and pituitary (Ghelardoni et al. 2006). There are also reports of ghrelin being expressed in the hypothalamus but such findings are inconsistent and whether it is produced there in meaningful amounts is still not clear (Furness et al. 2011; Abizaid and Horvath 2012)). Ghrelin mediates growth hormone (GH) secretion from the pituitary but regulation of GH secretion is not dependent on ghrelin's orexigenic role in the hypothalamus.

The ghrelin gene is located on the short arm of chromosome 3 at position 25-26 and is a 7.2kb region consisting of six exons, four (exons 1-4) of which code for the 117 amino acid peptide preproghrelin, which produces two mature peptides. Within this precursor peptide, part of exon 1 encodes for the preproghrelin signaling peptide; the remainder of exon-1, along with part of exon-2, codes for the 28 amino-acid-peptide

ghrelin; and the reminder of exon 2, with 3 and 4, codes for the C-terminal peptide C-ghrelin, which contains the sequence for the 23 amino-acid peptide obestatin (Seim et al. 2007). In the stomach, ghrelin, derived from preproghrelin, is modified by acylation with octanoate (an eight-carbon fatty acid) (Kojima et al. 1999). This esterification process is mediated by an acyl-transferase named ghrelin O-acyltransferase (GOAT), which is part of the membrane-bound O-acyltransferase (MBOAT) family (Gutierrez et al. 2008; Yang et al. 2008). The acylated form of ghrelin (acyl-ghrelin (AG)), acting via its receptor growth hormone secretagogue receptor (*GHSR1a*), was traditionally thought of as the active form with its effects on GH release and energy balance.

Other data suggest that the non-octanoylated form (des-acyl ghrelin (DAG)) has its own roles, despite the fact that the receptor(s) through which DAG produces biological effects remain unknown. DAG induces short-term food intake when centrally administered, but, contrary to AG, it does not activate GHSR1a or NPY, so such effects may be orexin mediated (Toshinai et al. 2006). Other studies, however, have shown an ability of DAG to reduce food intake and stomach motility under fasting conditions (Chen et al. 2005). Human studies have pointed to a role for DAG in improving insulin sensitivity. Individuals undergoing an intensive long-term exercise intervention have increased levels of DAG and decreased fasting insulin (Cederberg et al. 2012). Also, insulin levels are negatively associated with plasma DAG levels in individuals with metabolic syndrome (Barazzoni et al. 2007). Furthermore, DAG exhibits cardio-protective effects (Li et al. 2006; Baldanzi et al. 2002), it has vasodilator properties (Ku et al. 2015), and it has recently been shown to be involved in activation of the hypothalamic-pituitary-adrenal (HPA) axis responses

to stress (Stark et al. 2016b). Therefore, ghrelin exists in circulation in two active forms, AG and DAG.

The pathways through which circulating gastric ghrelin signals the hypothalamus, are not clearly understood. It is suggested that ghrelin transmits orexigenic signals from the stomach to the brain through a vagal pathway. The majority of afferent nerve fibres comprising the vagus nerve are connections to the nucleus tractus solitarius (NTS), which is one of the main brain regions where feeding related signals from gastro-enteric system are integrated (Emond, Schwartz, and Moran 2001). Peripheral ghrelin signaling that travels to the NTS via the vagus nerve activates the noradrenergic ARC receptors and consequently induces feeding (Date et al. 2006). Blockage of vagal afferent pathways or vagotomy (severance of the vagus nerve) leads to an insensitivity to the orexigenic effects of ghrelin and suppression of ghrelin-induced feeding, including in humans (le Roux et al. 2005). Pharmacokinetic studies in mice have shown that ghrelin crosses the BBB by saturable transport. Human acyl-ghrelin can cross the BBB, although mouse acyl-ghrelin has a decreased ability to do so. This ability is altered by factors such as obesity, serum triglyceride levels and fasting (Banks et al. 2002).

Reaching the brain from the periphery, ghrelin activates hypothalamic NPY/AgRP neurons, which highly express GHSR1a, to stimulate release of NPY and AgRP, leading to an increase in food intake (Kamegai et al. 2001). One of the mechanism by which ghrelin activates NPY and AgRP neurons is by increasing intracellular calcium via pathways depending on phospholipase C and adenylate cyclase-protein kinase-A (Kohno et al. 2007; Coiro et al. 2006). Both routes of ghrelin administration,

intracerebroventricular (ICV) or peripheral, induce feeding via activation of NPY and AgRP neurons. Administration of ghrelin ICV in mice activates the main hypothalamic appetite centres including ARC, PVN, DMH and LH (Lawrence et al. 2002) and ghrelin-induced feeding is completely abolished in mice lacking both NPY and AgRP (Jensen-Seaman et al. 2004). Peripherally injected ghrelin would still exert its orexigenic effects by activating hypothalamic NPY and AgRP neurons, however to do this it requires intact ARC (Cabral et al. 2014). NPY and AgRP fibres arising from the ARC further innervate other hypothalamic appetite centres to convey food related signals (Cowley et al. 2003).

Ghrelin is one of the only peripherally produced feeding signals that stimulates food intake. Ghrelin levels are high in times of fasting and this is associated with increased hunger, with levels decreasing following absorption of nutrients. An increase in plasma ghrelin indicates negative energy balance and consequently stimulates feeding and low energy expenditure (Cummings et al. 2001). Ghrelin levels are inversely correlated with body mass index BMI. As such, human and animal studies have shown that ghrelin levels are decreased in the obese compared to normal body weight controls (Haqq et al. 2003; Wadden et al. 2012; Uchida et al. 2014).

Obesity is usually associated with ghrelin resistance, which is explained by a reduced NPY/AgRP responsiveness to circulating ghrelin in order to reduce further food intake (Briggs et al. 2010), however why this occurs is unclear. It is hypothesised that ghrelin resistance is the body's mechanism of ensuring sufficient energy reserves during times of food scarcity (Zigman, Bouret, and Andrews 2016). In support of this hypothesis, ghrelin knockout mice have reduced body weight regain after a calorie-

restricted diet (Briggs et al. 2013). Contrary to its orexigenic effects, circulating ghrelin levels after a meal do not decrease in obese humans (English et al. 2002; Tschop et al. 2001). Furthermore, obesity in mice is associated with impairments in ghrelin transport through the BBB and reduced hypothalamic sensitivity to ghrelin, consequently leading to central ghrelin resistance (Banks, Burney, and Robinson 2008; Briggs et al. 2010). In addition, weight loss is associated with an increase in plasma ghrelin levels, with consequent further stimulation of hunger, making it difficult to maintain a calorie-restricted diet (Cummings et al. 2002).

## Ghrelin in early life neurodevelopment in rodents

In the rat embryo, ghrelin is expressed as early as the morula stage and continues to be expressed in the fetus (Nakahara et al. 2006; Kawamura et al. 2003). In human and rodent fetuses, pancreas is the main source of ghrelin, with low levels of ghrelin detected in the fetal stomach (Wierup et al. 2002; Chanoine and Wong 2004). However, stomach ghrelin increases to reach adult levels by 3-5 weeks after birth (Hayashida et al. 2002; Torsello et al. 2003) and pancreatic ghrelin levels decrease from birth to weaning becoming almost undetectable in adulthood (Wierup et al. 2002).

Ghrelin may play a role in development as early as the embryonic stage. It stimulates blastocyst development and its receptor, GHSR1a, is also detected in mouse embryos as early as the morula stage. In addition, ghrelin is expressed in the uterus and is secreted in uterine fluid at levels that are affected by maternal nutrition. As such, increased fasting ghrelin levels in the mother are reflected in increased ghrelin levels in the fetus (Kawamura et al. 2003). Postnatally, exogenous ghrelin does not

significantly promote milk intake nor body weight in the first 2-3 weeks in rats and mice (Piao et al. 2008). A possible explanation for this lack of response is that the neonatal brain is relatively insensitive to ghrelin and this lack of effect may represent ghrelin resistance. However, *GHSR1a* is found in the nuclei of neurons known to regulate feeding, including the ARC, and acute peripheral ghrelin treatment activates ARC neurons during early postnatal life (Steculorum et al. 2015). Further supporting a functional role of ghrelin in the postnatal rodent ARC, the mRNA levels of POMC and NPY mRNA in the ARC are decreased and increased, respectively, following acute ghrelin injection to mouse pups at P10 (Steculorum and Bouret 2011). Given that ghrelin receptors are present and functional in the developing hypothalamus, these results support the hypothesis that ghrelin in early life has different function from that in adulthood. A number of *in vitro* studies have shown that the exposure of cultured hypothalamic cells to ghrelin markedly increases cell proliferation and these effects are greater when the cells are taken from E17 embryos instead of P2 pups (Inoue et al. 2010). Both the AG and DAG forms of ghrelin promote cell proliferation and neurogenesis, with AG exerting greater proliferative effects than DAG (Sato et al. 2006).

As suggested by Steculorum and colleagues, ghrelin may also act as a brake to leptin-induced axonal growth in the postnatal period, in order to stop the overgrowth and maintain optimal functioning of hypothalamic circuitry in appetite regulation (Steculorum et al. 2015). Thus, leptin initiates axonal growth during the leptin surge, and ghrelin terminates the development of ARC projections (Steculorum et al. 2015). Based on the above results, it is suggested that an exacerbated leptin surge and disruption of ghrelin's normal elevation during postnatal life can lead to long-term

metabolic problems. Despite a large number of studies in this area, there are considerable gaps in our knowledge in how the nutritional environment during development affects long-term metabolism and what are the specific roles of leptin and ghrelin during development. Further studies are also needed to investigate the developmental effects of neonatal ghrelin and leptin in extra-hypothalamic brain regions such as the pituitary gland. Since ghrelin and leptin receptors are expressed in these regions it remains possible that these hormones are involved in cognitive or HPA axis functions and behavioral regulation (Llorente et al. 2009). Such suggestions need to be verified and the site(s) of action for the developmental effects of ghrelin and leptin need to be further investigated.

**The role of ghrelin in reproduction, regulation of HPA axis and inflammation**

Ghrelin has recently been shown to have an important role in reproduction (reviewed in (Sominsky, Hodgson, et al. 2017)). As a metabolic hormone, ghrelin directly or indirectly affects the hypothalamic-pituitary-gonadal (HPG) axis by acting as an inhibitory agent (Lebrethon et al. 2007). It informs the hypothalamus when there is insufficient energy and consequently disrupts or delays fertility (Martini et al. 2006). The hypothalamus is responsible for the release of gonadotropin releasing hormone (GnRH) from GnRH neurons located at the anterior hypothalamic area, from where they project to the median eminence and stimulate the pituitary gland to release luteinizing hormone and follicle stimulating hormone (FSH). The pituitary hormones then further regulate gonadal steroidogenesis. Absence or inappropriate release of these hormones at any level of the HPG axis is associated with fertility issues (reviewed in (Acevedo-Rodriguez et al. 2018)). Both male and female rats, have shown a disruption of HPG axis functioning in the presence of high levels of ghrelin

(Dupont et al. 2010). Excess ghrelin suppresses luteinizing hormone pulse frequency and testosterone secretion (Wang et al. 2011), in both men and women (Lanfranco et al. 2008; Kluge et al. 2012) preventing reproduction until sufficient energy is available. This suppressive effect of ghrelin on luteinizing hormone pulsatility is associated with increased circulating cortisol levels (Vulliemoz et al. 2004), indicative of ghrelin's interactive role between both HPG and HPA axes (Sominsky, Hodgson, et al. 2017).

Recent research, including from our group, has indeed shown that ghrelin along with its role in regulating metabolism and reproduction has an important role in the HPA axis regulation in response to stress. During acute physical or psychological stress, cells in the PVN secrete corticotropin-releasing hormone (CRH), which acts on the anterior pituitary to stimulate adrenocorticotropic hormone (ACTH) release. Increased circulating ACTH levels stimulate the adrenal cortex to synthesise and secrete glucocorticoids, which then initiate a series of events to aid the organism to cope with the stress (Nicolaides et al. 2015). Ghrelin activates the HPA axis at all levels. At the hypothalamus ghrelin activates CRH neurons in the PVN and increases circulating glucocorticoid levels without affecting GHSR1a on these neurons, suggestive of ghrelin's indirect effect on the apex of the HPA axis (Cabral et al. 2012; Spencer et al. 2012). Our group has shown that ghrelin stimulates the HPA axis at the level of the pituitary by targeting the *GHSR1a* to facilitate ACTH release (Spencer et al. 2012). Ghrelin can also directly activate pituitary ACTH cells (Stevanovic et al. 2007). In humans, ghrelin administration increases ACTH and cortisol release from the adrenal gland (Arvat et al. 2001; Takaya et al. 2000; Locatelli et al. 2010). Similarly, ghrelin also stimulates increases in circulating ACTH and corticosterone in rodents (Spencer

et al. 2012). Under normal conditions the absence of ghrelin (i.e. in ghrelin knockout animals) has no effect on adrenal responses to exogenous ACTH and normal PVN responses to dexamethasone (agent that mimics glucocorticoid release). However, in response to acute stress these animals have exacerbated PVN neuronal activation and reduced glucocorticoid secretion from adrenals compared to wild types (Spencer et al. 2012). Despite some limited knowledge of the role ghrelin plays in the regulation of stress responsivity, it remains unknown how nutritional environment during neonatal period affects ghrelin's ability to regulate the HPA axis and whether this is different between males and females.

It is important to note that the role of ghrelin as an anti-inflammatory agent has recently received great interest. A number of studies have pointed out a potential role for ghrelin in pro-inflammatory disease states in humans and animal models including sepsis (Maruna et al. 2005), pancreatitis (Kerem et al. 2007), Crohn's disease (a form of inflammatory bowel disease) (Ghomraoui et al. 2017), colitis (Tiaka et al. 2011) and rheumatoid arthritis (Koca et al. 2008). The presence of the ghrelin receptor on peripheral blood mononuclear cells (PBMC) such as in human lymphocytes (T- and B-cells) (Gnanapavan et al. 2002; Dixit et al. 2004) and human and rodent immune organs such as thymus and bone marrow (Hattori 2009; Taub, Murphy, and Longo 2010) suggests that ghrelin may at least partly activate these cells and organs. Indeed, Dixit and colleagues have shown that ghrelin suppresses expression of proinflammatory cytokines such as interleukin 1 beta (IL-1β), IL-6 and tumor necrosis factor alpha (TNFα) by human T-lymphocytes and monocytes (Dixit et al. 2004). The anti-inflammatory role of ghrelin has also been observed in a mouse model of colitis where ghrelin administration significantly suppressed a number of

inflammatory cytokines including: TNFα and several pro-inflammatory interleukins. Also, increased anti-inflammatory IL-10 levels in colonic mucosa were associated with significant improvement in survival rate (Gonzalez-Rey, Chorny, and Delgado 2006). One of the suggested mechanisms by which ghrelin exerts its anti-inflammatory effects is via the vagus nerve, as is seen in animal models of traumatic brain injury, where ghrelin administration suppresses circulating TNFα and IL-6 (Bansal et al. 2012). Other suggested mechanisms are via inhibition of high-mobility group box 1 (HMGB1) secretion from macrophages. HMGB1 is an important protein involved in a number of inflammatory conditions including sepsis. Ghrelin significantly inhibits secretion of this protein from macrophages by blocking its cytoplasmic translocation (Chorny et al. 2008). The mechanisms via which ghrelin achieves these anti-inflammatory effects however, remain to be further elucidated.

**Sex differences in developmental programming of metabolism and stress**

Males and females respond differently to metabolic and stressful situations, making sex differences an important, and often under-investigated area of study. For instance, fat distribution in women is mainly located in the gluteal and femoral region, whereas in men the highest proportion of body fat is accumulated in the abdominal area (Karastergiou et al. 2012; Fuente-Martin et al. 2013). A number of studies have shown that there is a high correlation between obesity and mood disorders (Doyle et al. 2007; Scott et al. 2008) and obese women are more likely to develop emotional disorders than obese men (Barry and Petry 2008). We have previously seen that neonatal overfeeding in females, alongside an obesigenic phenotype, is associated with unique changes in female HPA axis. Females, but not males, have exacerbated PVN responses to psychological stress accompanied by increased open arm

exploration in the elevated plus maze (Spencer and Tilbrook 2009), potentially related to an impaired ability of the female pituitary gland to respond to ghrelin. These disorders are not only affected by overfeeding, but also by underfeeding in rodents, which also differentially affects males and females (Fuente-Martin et al. 2012; Mela et al. 2012; Granado et al. 2014).

Important contributors to stress responses and related disorders include the metabolic hormones leptin and ghrelin. As previously mentioned, the leptin surge that initiates at approximately P4 and P7 in mice and rats is crucial not only for optimal neurodevelopmental function, but also for optimal development of the endocrine system (Ahima, Prabakaran, and Flier 1998). The absence or disruption of the leptin surge is related to long-term altered HPA axis activity. For instance, rats overfed during early life develop hyperleptinemia and show an accelerated maturation of the HPA axis compared to normally fed counterparts (Boullu-Ciocca et al. 2005). When challenged with physical or psychological stress, these neonatally overfed animals display exacerbated HPA axis responses to the challenge (reviewed in (Spencer 2013)). Other studies have demonstrated that chronic leptin treatment in early life is followed by suppressed CRH and increased glucocorticoid receptor expression from the PVN (Oates, Woodside, and Walker 2000; Proulx et al. 2001). Very little is known about the relationship between stress and metabolic hormones leptin and ghrelin during development with regard to sex and nutritional status. However, both stress and obesity induce sex-dependent susceptibilities to metabolic disorders (Murphy and Loria 2017). In girls and women circulating ghrelin levels differ from those in boys and men, such that females have higher total and acyl-ghrelin in comparison to males (Hagobian and Braun 2010; Espelund et al. 2005; Ostergard et

al. 2003; Soriano-Guillen et al. 2016). Initially, Lutter and colleagues (Lutter et al. 2008) and later Meyer and Harmatz (Meyer et al. 2014; Harmatz et al. 2017) showed that exposure to chronic stress increases circulating ghrelin levels. On the other hand, alterations in ghrelin levels can affect behaviour and mood, with recent studies showing an anxiolytic and anxiogenic role of ghrelin in regulating anxiety-related behaviours in response to acute stress (Spencer et al. 2015; Spencer et al. 2012). Ghrelin thus promotes anxiety at low concentrations and suppresses anxiety and stress at higher concentrations (Spencer et al. 2012). It is proposed that increased levels of ghrelin during stress may be required to prevent excess anxiety and therefore to facilitate food-seeking. However, the mechanism by which ghrelin modulates anxiety-related behaviours in males and females is not fully studied. Despite the clear sex differences in many parameters, the majority of current research uses males, mainly to avoid the possible influences of the female hormonal cycle. However, it is critical to consider both sexes in order to better understand the effects of sex-dependent metabolic and stress-related disorders.

In the current work I will address four major aims to assess the roles of leptin and ghrelin in developmental programming of obesity and in adult stress and inflammation.

**Aim 1:** To determine the impact of early life overfeeding on the hypothalamic circuitry and the long-term role of leptin in hypothalamic programing in male Wistar rats.

To test this aim I will examine the metabolic effects of neonatal overfeeding on body weight and circulating leptin. I will also examine central effects of neonatal

overfeeding on hypothalamic responsiveness to exogenous leptin and leptin-mediated NPY/AgRP and POMC neurons and whether these effects could be normalised by neonatal leptin antagonism. It has been previously shown that neonatal overfeeding amongst other effects is associated with altered levels of circulating leptin. This, and leptin's neurotrophic effects during neurodevelopment, led us to hypothesise that altered leptin levels would be associated with long-term disrupted connectivity within hypothalamic regions responsible in controlling metabolism.

This chapter has been published in the Journal of Endocrinology in 2017, entitled "*Hypothalamic effects of neonatal diet: reversible and only partially leptin dependent"*. (Sominsky, Ziko, et al. 2017)

**Aim 2:** To examine the effects of neonatal overfeeding on the ghrelin system, including acyl and des-acyl ghrelin accessibility to the hypothalamus in male Wistar rats.

To test this aim I will examine whether low circulating ghrelin levels in the neonatally overfed rats affected the hypothalamic responsiveness to exogenous ghrelin and hypothalamic responsiveness to fluorescently labeled acyl and des-acyl ghrelin. It has been previously shown that neonatal overfeeding is associated with altered ghrelin levels, however it is not clear whether this alterations are associated with long-term disruptions on the central or peripheral ghrelin system.

This chapter has been published in Neuropharmacology in 2016, entitled "*Early life disruption to the ghrelin system with over-eating is resolved in adulthood in male rats"*(Sominsky, Ziko, Nguyen, et al. 2016). (Co-authors Sominsky L. and Ziko I. have equally contributed to this publication).

**Aim 3:** To define the role of early life overfeeding on the peripheral and central leptin systems in female Wistar rats.

As the previous studies had shown that neonatal overfeeding in males, along with an obesigenic phenotype, were associated with a disruption of hypothalamic orexigenic NPY/AgRP circuitry. We hypothesised in this aim that the same outcomes would occur in females.

This chapter has been published in Frontiers in Endocrinology 2017, entitled "*Hyperleptinemia in neonatally overfed female rats does not dysregulate feeding circuitry*" (Ziko et al. 2017).

**Aim 4:** To examine the effects of neonatal overfeeding on the ghrelin system in female Wistar rats long-term.

Previous studies have demonstrated that ghrelin affects the activity of the HPA axis at the pituitary level via activation of its receptor. I hypothesised that neonatal overfeeding in females would compromise the pituitary ghrelin system, further affecting HPA axis responses to stress.

This chapter has been published in PLOS One 2017, entitled "*Neonatal overfeeding disrupts pituitary ghrelin signaling in female rats long-term; implications for the stress response*" (Sominsky, Ziko, and Spencer 2017).

**Aim 5:** To examine the contribution of different forms of ghrelin on the anti-inflammatory responses to an immune challenge. I hypothesised that ghrelin's anti-inflammatory activity is mediated via the HPA axis, and acylated and des-acylated ghrelin would differently contribute to cytokine suppression in response to an immune challenge.

This chapter has been published in Brain, Behaviour, and Immunity 2018 entitled "*Acylated ghrelin suppresses the cytokine response to lipopolysaccharide and does so independently of the hypothalamic-pituitary-adrenal axis*" (Ziko et al. 2018).

# Chapter 2

# Hypothalamic effects of neonatal diet: reversible and only partially leptin-dependent

Luba Sominsky**, Ilvana Ziko,** Thai-Xinh Nguyen, Julie Quach and Sarah J. Spencer,

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## Introduction

The early life nutritional environment affects long-term regulation of body weight with both under- and over-nutrition leading to metabolic problems. An unintended experiment in humans arose from the Dutch famine, which occurred in the winter of 1944-45. This tragedy has shown us that the fetus in the mother's uterus can adapt and survive after a minimal and poor nutritional diet, however the challenge imposed by the poor nutrition perinatally exposes the offspring to metabolic problems, including the inability to control their eating, a preference for high calorie foods, as well as a reduction in physical activity later in life (Roseboom, de Rooij, and Painter 2006; Ravelli, Stein, and Susser 1976). Reflecting these data, the "thrifty gene hypothesis" was proposed as early as 1962 by James V Neel, according to which, genes that predispose to obesity have a selective advantage in populations that have continuously experienced starvation (Neel 1962).

Overnutrition during important developmental periods has also been associated with metabolic mal-programming and offspring predisposition to chronic conditions in adulthood, such as type two diabetes and increased susceptibility to obesity (reviewed in (Carolan-Olah, Duarte-Gardea, and Lechuga 2015; Tarry-Adkins and Ozanne 2017)). Prenatal under-nutrition followed by postnatal overnutrition in animal models is highly associated with obesity and leptin resistance as a result of catch-up growth (Dellschaft et al. 2015; Vickers and Sloboda 2012; Sebert et al. 2009). The combination can result in significant alterations in neuropeptide Y (NPY), agouti related peptide (AgRP) and proopiomelanocortin (POMC) within the hypothalamus (Ikenasio-Thorpe et al. 2007).

Environmental factors can thus influence the way our metabolism responds to nutritional challenges especially when insults happen during important developmental periods. As early as 1979 Dobbing and Sands found that major brain growth spurts happen at around birth in humans and around postnatal day (P)7 in rats, based on the increasing weight of the brain (Dobbing and Sands 1979). This important developmental period has been extensively studied ever since. In 1998, Ahima and colleagues found that circulating leptin levels are highly increased around P7-P10 in mice irrespective of food intake or fat mass, reinforcing the idea that leptin may have a developmental role (Ahima, Prabakaran, and Flier 1998). Bouret and Simerly thereafter found that this leptin surge coincided with the maturation of neuronal projections innervating the hypothalamus and established the particular importance of leptin as a trophic factor in formation of these projections (Bouret, Draper, and Simerly 2004b).

As previously mentioned in the main introduction of this thesis (Section 1.3: *The role of leptin in metabolism*), leptin in adult males centrally regulates metabolism by activation of anorexigenic neurons and inactivation of orexigenic neurons in the arcuate nucleus of the hypothalamus (ARC). The leptin surge in neonates, however, initiates neuronal maturation, and disruption or exacerbation of this leptin surge (as shown with the neonatal overfeeding animal model (Stefanidis and Spencer 2012)) may lead to permanent disruption of connections and long-term obesity (Bouret, Draper, and Simerly 2004a, 2004b). We observed in our animal model of childhood obesity that the hyperleptinemia and body weight increase with neonatal overfeeding are maintained throughout the animal's life (Stefanidis and Spencer 2012). Davidowa and colleagues have shown that this hyperleptinemia is associated with reduced ARC

responsiveness to leptin, indicative of leptin resistance (Davidowa and Plagemann 2000). Results from the same group have also shown that neonatal overfeeding induces POMC hypermethylation within the hypothalamus (Plagemann et al. 2009). A limited number of studies have observed that neonatal overfeeding induces an increase in *NPY* and *AgRP* gene expression (Lopez et al. 2005) and also suppresses NPY responsiveness to ghrelin (Collden et al. 2015). However, it still remains unknown how the neonatal over-nutritional environment affects the NPY/AgRP wiring and POMC expression in the hypothalamus in the short or long-term, and these factors are examined here. We also inspected the early life overnutrition effects on hypothalamic responsiveness to leptin and the ability of a leptin antagonist to re-establish hypothalamic circuitry under neonatal overfeeding conditions.

## Materials and methods

### *2.1.1 Animals*

All our timed pregnant Wistar rats were obtained from the Animal Resources Centre, WA, Australia. The rats were delivered to our facility between E14 and E16. On arrival at the RMIT University Animal Facility, they were housed at 22°C on a 12 hr light/darkness cycle (07:00–19:00 hr) and provided with standard pelleted rat chow and water *ad libitum*. All procedures were conducted in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals and RMIT University Animal Ethics Committee approval.

### *2.1.2 Litter size manipulation*

On the day of birth (P0) pups were removed from their dams and numbers were recorded. After sex identification, pups were reallocated to litters of 4 (2 males and 2

females) called small litter (SL), representing the neonatally overfed model. Sex was determined by a larger genital papilla and longer anogenital distance in male pups than in female pups. The control litter (CL) consisted of 12 pups (6 males and 6 females). Care was taken that none of the dams obtained their own pups and the procedure was conducted quickly (within 5-10 min) so that pups maintained their body temperature. Excess pups were culled or utilized for other research projects. Our group has previously shown that this litter size manipulation results in SL pups being significantly heavier as early as P7 and they remain heavier throughout their lives (Spencer & Tilbrook, 2009; Ziko et al., 2014). Following pup reallocation, the litters were weighed as whole litters on P0 (all litters) and P7 (litters culled on P14) and individually on the day of the experiment. The pups were otherwise left undisturbed with mothers, except for the usual animal husbandry, until experimentation. Only male animals were examined in this chapter. All experimental groups were derived from three or more litters, using a maximum of two pups from the same litter for an experimental treatment to control for maternal effects (Spencer and Meyer 2017).

### *2.1.3 Brain collection*

Neonatally overfed and control animals on P7, P14 and ~P70 were deeply anaesthetised with Lethabarb (150 mg/kg sodium pentobarbitone, i.p.) then decapitated. Another cohort of animals at P12 and ~P70 were deeply anaesthetised with Lethabarb then perfused transcardially with phosphate buffered saline (PBS; 4°C, pH 7.4) followed by 4% PFA in PBS (4°C, pH 7.4). All brains were removed and placed in 4% paraformaldehyde (PFA) in (PBS) for 24 hr then into 20% sucrose in PBS at 4°C. Brains were sectioned at 30 μm (adults) or 40 μm (neonates) and

stored at 4°C in PBS containing 0.02% sodium azide until further processed for immunohistochemistry.

### *2.1.4 Immunohistochemistry*

Hypothalamic sections were immunolabelled for a variety of markers as summarised in Table 2.1. Samples from different treatment groups were randomly selected and processed in batches. To block endogenous peroxidases phospho signal transducer and activator of transcription 3 (pSTAT3) sections were incubated with hydrogen peroxide ($H_2O_2$) in PBS for 15 min. After the primary and secondary antibody incubation as detailed in Table 2.1., pSTAT3 sections were then incubated with avidin-biotin horseradish peroxidase (HRP) complex (ABC; 45 min; Vector Elite Kit; Vector), followed by incubation with diaminobenzidine (DAB), intensified with 1% cobalt chloride and 1% nickel sulphate for 10 min. Then 0.05% $H_2O_2$ was added to the DAB solution to visualise HRP activity. The reaction was stopped when the contrast between specific labelling and background was optimal. The sections were then mounted on gel-coated slides, dried, dehydrated in a series of increasing concentrations of alcohols then cleared in histolene and coverslipped. After blocking non-specific protein binding with 3% bovine serum albumin (BSA) in 0.1 M PBS with Triton X-100, sections were incubated with NPY, AgRP and POMC primary and secondary antibodies as detailed in Table 2.1. Next, sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 15 min then mounted on slides with DAKO anti-fading solution. Slides were kept in the dark at 4°C until visualised.

**Table 2.1 Primary and secondary antibodies used in immunohistochemistry**

| Antigen | Primary Antibody | Secondary Antibody | Incubation Time/Temperature |
|---|---|---|---|
| Signal transducer and activator of transcription 3 (pSTAT3) | Anti-rabbit pSTAT3 (Abcam)<br>[1: 5000] | Biotinylated anti-rabbit (Vector Laboratories, Burlingame, CA, USA<br>[1:500] | 1° AB: overnight, 4°C<br>2° AB: 1.5 h, Room Temperature |
| Neuropeptide Y (NPY) | Anti-rabbit NPY (Sigma-Aldrich)<br>[1:1000] | Alexa-fluor 488 goat anti-rabbit (Thermo Scientific)<br>[1:500] | 1° AB: overnight, 4°C<br>2° AB: 2 h, Room Temperature |
| Agouti related protein (AgRP) | Anti-goat AgRP (Neuromics Inc., MN, USA)<br>[1:500] | Alexa-fluor 594 rabbit anti-goat (Thermo Scientific)<br>[1:200] | 1° AB: 42 h, Room Temperature<br>2° AB: 12 h, Room Temperature |
| Proopiomelanocortin (POMC) | Anti-rabbit POMC (Phoenix Pharmaceuticals, Burlingame, CA, USA)<br>[1:5000] | Alexa-fluor 488 goat anti-rabbit (Thermo Scientific)<br>[1:500] neonate<br>[1:200] adult | 1° AB: overnight, 4°C<br>2° AB: 1h, Room Temperature |

An experimenter blinded to treatment groups assessed hypothalamic sections for numbers of pSTAT3 positive cells in the ARC and ventromedial hypothalamus (VMH). The summed counts of positive cells throughout three sections 120 μm apart between 2.76 and 3.48 caudal to bregma were taken according to the Paxinos and Watson Rat Brain Atlas (Paxinos and Watson 2009). pSTAT3 immunohistochemistry and cell counts were performed by Ms Thai Xinh Nguyen as part of her Honours project from sections generated by me and under my supervision. NPY, AgRP and

POMC photomicrographs were taken on an upright Nikon Eclipse 90i confocal laser microscope using 561 nm lasers (605/75 filter set) for AgRP labeling, 488 nm (513/30 filter set) for NPY and AgRP and 408 nm laser for DAPI. Images were viewed under the 20x magnification lens under Galvano scanner using a 512 x 512 pixels scan size. A thresholding method was used with NIS Elements Advanced Research Software in order to analyse NPY and AgRP fibre density. After background subtraction, the upper and lower threshold limits were defined based on the signal intensity values from the control group of animals. NPY and AgRP labelling of the cell bodies was only observed in the neonatal ARC and not in the neonatal PVN or adult ARC or PVN. The perikaryon labelling of NPY and AgRP was excluded from the neonatal ARC analysis as follows. The thresholds for each of the channels were separately defined and the program subtracted the DAPI layer from the NPY or AgRP threshold layer generating a new layer with the cell bodies excluded. A region of interest constant for all the samples was then analysed for fibre density from this layer. POMC-positive cells were manually counted using Image J (National Institutes of Health) in four brain sections with the summed counts from all brain sections taken as our graphed result.

The specificities of the antibodies used in our study have been previously validated by manufacturers and other researchers in pre-absorption and Western blotting experiments and further supported in our own validation experiments by incubation of experimental tissue without a primary or without a secondary antibody. Negligible positive labelling was seen in these negative controls. Positive labelling was confirmed in the brain regions of interest. More specifically, the NPY antibody (N9528) has been validated by the manufacturer and used in (Garcia et al. 2011;

Kobelt et al. 2008; Konieczna et al. 2013; Pekala et al. 2011). The AgRP antibody (GT15023) has been validated by the manufacturer and used by (Betley et al. 2015; Cao et al. 2011; Garfield et al. 2015; Kim et al. 2016; Kobelt et al. 2008; Li et al. 2012; Shibata et al. 2016). The POMC antibody (H-029-30) specificity has been confirmed by (Wittmann, Hrabovszky, and Lechan 2013) and used by (Cheng, Chu, and Chow 2011; Evans et al. 2007; Gotoh et al. 2006; Pandit et al. 2016; Reyes et al. 2006; Singru et al. 2012; Tavares, Maldonado, and Minano 2013; Zhan et al. 2013). The pSTAT3 antibody (ab76315) has been previously validated by the manufacturer by Western blotting in HeLa cell lysate and used in the following publications (Desai et al. 2007; Mao et al. 2016; McGuckin et al. 2013).

### *2.1.5 Effects of neonatal overfeeding on hypothalamic gene expression*

Quantitative real-time PCR (qRT-PCR) was used to assess neonatal overfeeding effects on hypothalamic gene expression of the leptin receptor (*Lepr*) in the ARC and hypothalamus from the animals described above (see Table 2.2 for primer details). Brains were immediately removed and the hypothalamus was isolated with razor blade from the fresh brain. Initially a coronal cut was made anterior to the optic chiasm and another coronal cut was made 4mm caudal to the first cut. Then the slice was placed with its posterior surface upside and two lateral cuts were made above the optic tracts. Finally, a horizontal cut above the third ventricle finalised the hypothalamic dissection. This isolation technique was adapted by (Reyes et al. 2003) and it can also be found at (Udvari et al. 2017). The arcuate nucleus was dissected at the base of the hypothalamus using a small dissecting knife. The brain was dissected into the ARC and hypothalamus that does not contain the ARC. These samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C until use. RNA was

purified using QIAzol reagents and RNeasy Mini Kits (QIAGEN, Valencia, CA, USA). The RNA concentration was determined by spectrophotometer measurements at 260nm and 280nm (NanoDrop 2000/2000c, Thermo Scientific, Waltham, MA, USA). 1 μg RNA was transcribed to cDNA using iScript cDNA synthesis kits (QIAGEN) according to the manufacturer's instructions. A qRT-PCR Taqman Gene Expression Assay (Applied Biosystems, Mulgrave, VIC, Australia) was performed on a Rotor-Gene Q instrument (Qiagen GmbH) and the relative quantitative measure of the target gene expression was compared with an endogenous control, *18s*. RNA expression was determined using the double delta (C(t)) equation 2-ΔΔC(t), where threshold cycle (C(t)) values were the values at which fluorescence was first detected significantly above background, as previously described. Minus reverse-transcriptase (-RT), with omitted reverse transcriptase reactions, and no template controls (NTC), with omitted primer reactions, were run simultaneously with the samples in order to verify that no genomic DNA contamination was present. The mean C(t) value of our -RT test samples for *18s* was more than ten cycles different from the mean C(t) value of our test samples, indicative of a 2-fold difference in the initial template amount, therefore allowing us to assume 100% efficiency and the presence of negligible genomic DNA. NTC were undetermined (>40 C(t)), suggesting there was no genomic DNA contamination. Data are presented as fold increase relative to P7 CLs (neonates) or adult CLs (adults). We initially tested both of the housekeeping genes 18S and β-actin, and based on the analysis of variability, β-actin was chosen as an endogenous control due to its stability in the neonatal hypothalamus. We can confidently confirm the reliability of our reference gene in this study.

**Table 2.2 Primer details for qRT-PCR**

| Primer name | NCBI Reference Sequence | TaqMan Assay ID | Product size |
|---|---|---|---|
| *18s* | X03205.1 | 4319413E | 187 |
| *Lepr* | NM_012596 | Rn01433205_ml | 94 |

### *2.1.6 Effects of neonatal overfeeding on food intake and hypothalamic responsiveness to exogenous leptin*

To assess whether neonatal overfeeding affected the ability of the hypothalamus to control feeding and body weight after an exogenous stimulus with leptin (3 mg/kg of leptin i.p. (PeproTech, Rocky Hill, NJ, USA)) nocturnal, diurnal and 24 hr food intake were measured. In order to assess neonatal overfeeding acute (neonatal) and long-term (adulthood) effects on hypothalamic responsiveness to exogenous leptin P12 and ~P70 CL and SL animals were injected with 1 mg/kg i.p. leptin (PeproTech, Rocky Hill, NJ, USA) or 0.9% sterile saline and pSTAT3 immunoreactive cells in the ARC and VMH were assessed. ~P70 animals were given 7 days of normalisation before a second injection of 1 mg/kg leptin. Body weight measurements were also conducted before and 45 min post injection. All of the experiments were conducted between 09:00 and 13:00 hr to avoid potential effects of circadian rhythms on any of the parameters measured.

### *2.1.7 Leptin ELISA*

Leptin concentrations were determined using a standard commercially available rat-specific leptin ELISA, following the manufacturer's instructions (Millipore, Ballerica, MA, USA). Leptin ELISAs were conducted by Dr Luba Sominsky from samples generated by me. Intra-assay variability was 1.9-2.5% CV, inter-assay variability 3.0-

3.9% CV, and lower limit of detection 0.04 ng/mL. All compared samples were assayed in duplicate and processed in the same assay.

### *2.1.8 Leptin antagonist*

Super rat leptin antagonist (SRLA) (SLAN-4; Protein Laboratories Rehovot Lt. Rehovot, Israel) used in this study was prepared similarly to super mice leptin antagonist (SMLA) described in (Shpilman et al. 2011). SRLA is a polypeptide chain containing 146 amino acids, with mutations D23L, L39A/D40A/F41A, resulting in a powerful antagonist with high affinity for the leptin receptor. To assess whether antagonising the exacerbated leptin surge at its onset (~P4) would ameliorate the disruption in NPY/AgRP neuronal fibre density, body weight, and circulating leptin levels long-term in our neonatally overfed animals compared to controls, 5 mg/kg leptin antagonist (i.p.) or saline was injected daily to a cohort of CL and SL animals from P4 to P7. These animals were allowed to grow into adulthood (~ P70) and body weight and circulating leptin were assessed. We also assessed POMC positive cells as well as hypothalamic NPY/AgRP fibre density as described above.

### *2.1.9 Statistical analysis*

Statistical analyses were performed separately based on age. For neonates, pre-weaning body weights, leptin receptor expression and hypothalamic responses to leptin were compared using multifactorial analyses of variance (ANOVA)s (using SPSS software) with age (P7/P14) and litter size manipulation (CL/SL) as between factors. For neonatal NPY, AgRP, POMC and pSTAT3 immunoreactivity, and for the adults Student's unpaired t-tests (one variable) or two-way ANOVAs (more than one variable) were used, also including treatment factors where appropriate (saline/leptin

or saline/SRLA). Where significant interactions were found, Tukey *post hoc* tests were performed. Data are presented as the mean ± SEM. Statistical significance was assumed when $p \leq 0.05$. All data were tested for homogeneity of variance and normality, using the Levene's test for Equality of Variance and the Shapiro-Wilks test, respectively, complemented by the assessment of skewness and kurtosis. These assessments and all other statistical analyses were conducted using SPSS. Outliers were determined using the Grubbs' test ($\alpha = 0.05$) in GraphPad Prism.

## Results

### *2.1.10 Neonatal overfeeding effects on the peripheral and central leptin systems*

Our group has previously shown that overfeeding during early life is associated with increased body weight (Smith and Spencer 2012; Spencer and Tilbrook 2009; Stefanidis and Spencer 2012). Here it is shown again that neonatally overfed male pups have significantly increased body weight at P14 compared to normally fed pups of the same age ($F_{(2,28)} = 20.75$, $p < 0.001$; n = 8; Figure 2.1 A). This increase in body weight is associated with a significant increase in circulating leptin levels at P14 as previously described (Stefanidis and Spencer 2012). While there were no differences in the expression of the receptor in the hypothalamus excluding the ARC, in the ARC, there was a significant increase in the leptin receptor mRNA expression at P14 compared to P7 (significant effect of age: $F_{(1,23)} = 51.37$, $p < 0.001$; n = 6-8; Figure 2.1 C).

To test the effects of neonatal overfeeding on the hypothalamic responsiveness to exogenous leptin, P12 pups were given a leptin injection and measured leptin's ability to activate pSTAT3 cells in the ARC and VMH. Leptin significantly increased numbers of pSTAT3 positive cells in both ARC (significant effect of leptin: $F_{(1,15)} = 8.54$, $p = 0.011$; n = 3-6; Figure 2.1 D, F) and VMH (significant effect of leptin: $F_{(1,15)} = 18.82$, $p = 0.001$; n = 3-6; Figure 2.1 E) of normally fed pups, but failed to do so in the overfed ones, indicative of central leptin resistance.

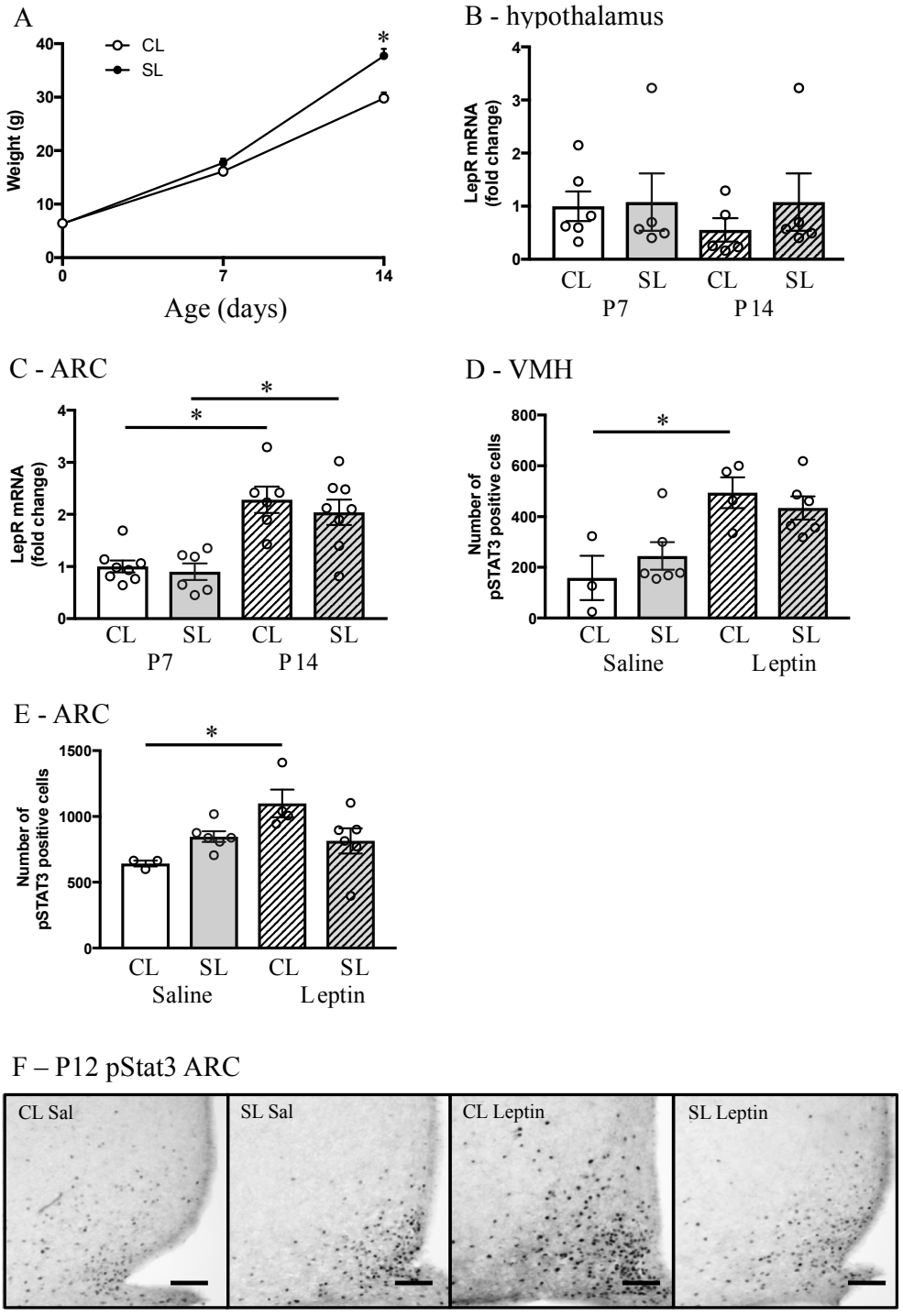


**Figure 2.1 Neonatal overfeeding effects on the peripheral and central leptin systems.**

(A) Pre-weaning body weights of rats raised in control (CL) and small (SL) litters (n = 8). (B) Hypothalamic and (C) arcuate nucleus of the hypothalamus (ARC) leptin receptor expression (n = 6 - 8). (D) Neuronal activation in response to leptin injection at P12; numbers of phosphorylated signal transducer and activator of transcription (pSTAT3) positive cells in the ventromedial hypothalamus (VMH) (n = 3 - 6) and (E) in the ARC (n = 3 - 6). (F) Representative photomicrographs showing leptin-induced pSTAT3 positive cells in the ARC of CL and SL rats (n = 3 - 6). Data are mean ± SEM. * $p < 0.05$. Scale bars = 200 μm.

### *2.1.11 Neonatal overfeeding affects hypothalamic orexigenic NPY and AgRP, but not the anorexigenic POMC*

To determine whether the effects of neonatal overfeeding on circulating leptin levels and body weight were associated with modification of key hypothalamic neuropeptides such as anorexigenic POMC and orexigenic NPY and AgRP, numbers of POMC-positive cells and NPY/AgRP fibre density in the ARC and PVN of overfed and normally fed pups at P12 were measured. Neonatal overfeeding had no effects on ARC POMC positive cells (Figure 2.2 B, E), however it significantly increased both NPY ($t_{(10)} = 2.33$, $p = 0.042$; n = 6 per group Figure 2.2 D, G) and AgRP ($t_{(9)} = 5.18$, $p = 0.001$; n = 5-6 per group Figure 2.2 C, F) fibre density when compared to controls. In the PVN neonatal overfeeding did not affect AgRP fibre density (Figure 2.2 I, K) and in contrast to the ARC it suppressed NPY fibre density in comparison to controls ($t_{(10)} = 2.3$, $p = 0.044$; n = 6 per group Figure 2.2 J, L).

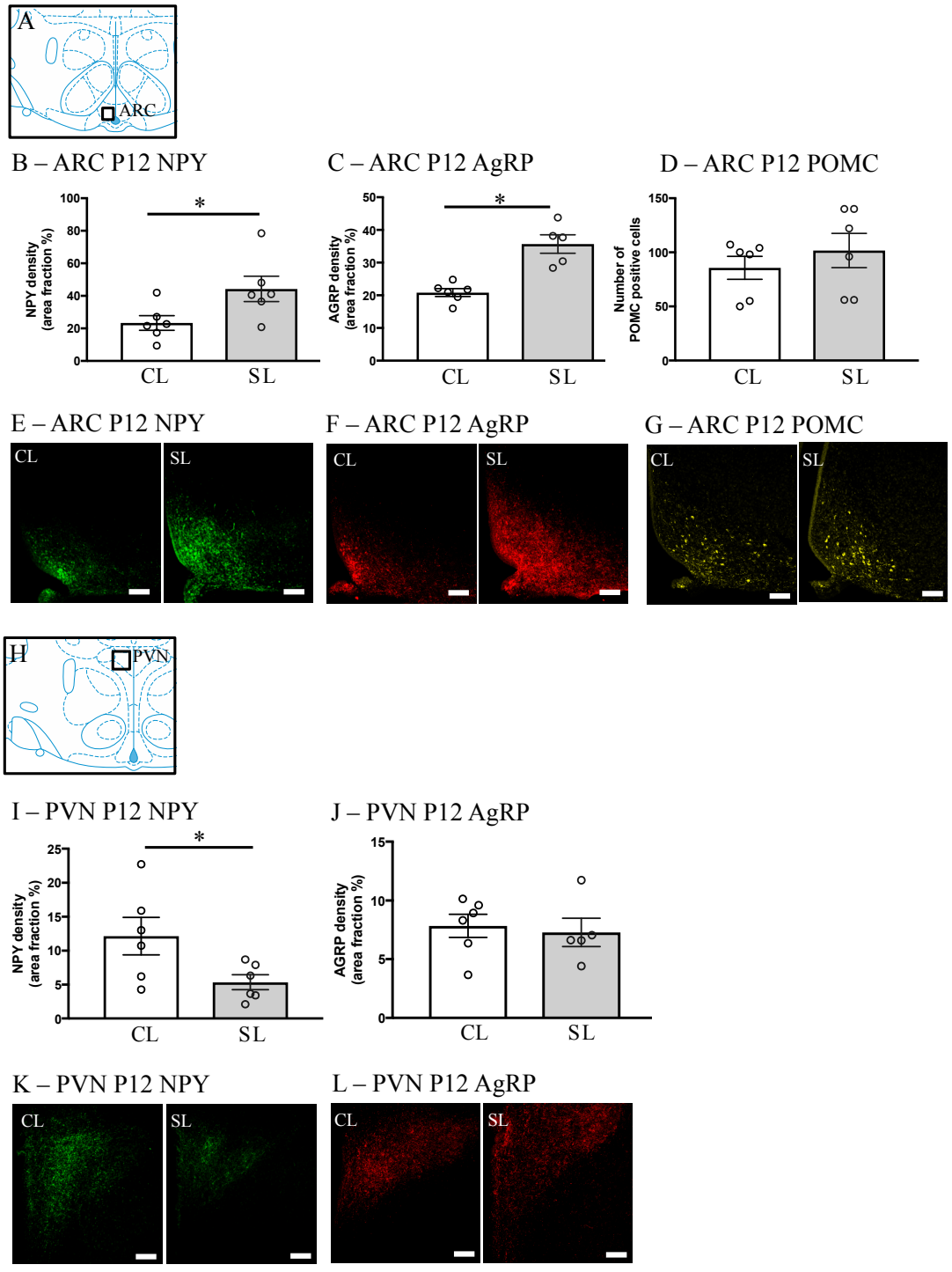


**Figure 2.2 Neonatal overfeeding affects the hypothalamic neuropeptide Y (NPY) and agouti related peptide (AgRP) fibre density, but not proopiomelanocortin (POMC) immunoreactivity.**
(A, H) schematic diagram illustrating the region of interest analysed for NPY, AgRP and POMC. (B) NPY (n = 6), (C) AgRP (n = 6) and (D) POMC immunolabeling in the arcuate nucleus (ARC) (n = 6). Representative photomicrographs of (E) NPY (n = 6), (F) AgRP (n = 6) and (G) POMC labeling in the ARC of control (CL) and small litter (SL) animals on postnatal day (P)12 (n = 5 - 6). (I) NPY and (J) AgRP labelling in the paraventricular nucleus of the hypothalamus (PVN). Representative

photomicrographs showing NPY (K) and AgRP (L) labelling the PVN. Data are mean ± SEM. * $p < 0.05$. Scale bars = 100 μm.

### *2.1.12 Neonatal overfeeding effects on the leptin system long-term*

To determine the long-term peripheral and central effects of neonatal overfeeding on the leptin system leptin parameters were measured in adult animals that were overfed during the first weeks of their life and compared them to control animals. Neonatally overfed animals remained overweight despite weaning them onto a normal diet ($t_{(21)}$ = 2.24, $p$ = 0.036; n = 11-12; Figure 2.3 A). Previous data from our group (Stefanidis and Spencer 2012) have shown that, similar to body weight, neonatal overfeeding affects circulating leptin levels long-term. Therefore, hypothalamic gene expression of the leptin receptor was measured. We observed that adult animals that were overfed during neonatal life had similar levels of *Lepr* expression in the hypothalamus compared to controls (Figure 2.3 B). Since we observed that neonatal overfeeding was associated with a disruption of neonatal NPY and AgRP neurons in the ARC and PVN, we assessed whether these changes were maintained until adulthood. Neonatal overfeeding did not affect ARC (Figure 2.3 D) or PVN (Figure 2.3 G) AgRP fibre density in adult rats. Neonatal overfeeding not only did not induce an increase in adult ARC NPY fibre density, but it significantly suppressed it ($t_{(10)}$ = 2.32, $p$ = 0.043; Figure 2.3 C). No effects of neonatal overfeeding on the adult PVN NPY fibre density (Figure 2.3 F) or ARC POMC positive cells (Figure 2.3 E) were seen. Overall, the above results indicate that neonatal overfeeding effects on NPY/AgRP fibre density observed during neonatal life are resolved by adulthood.

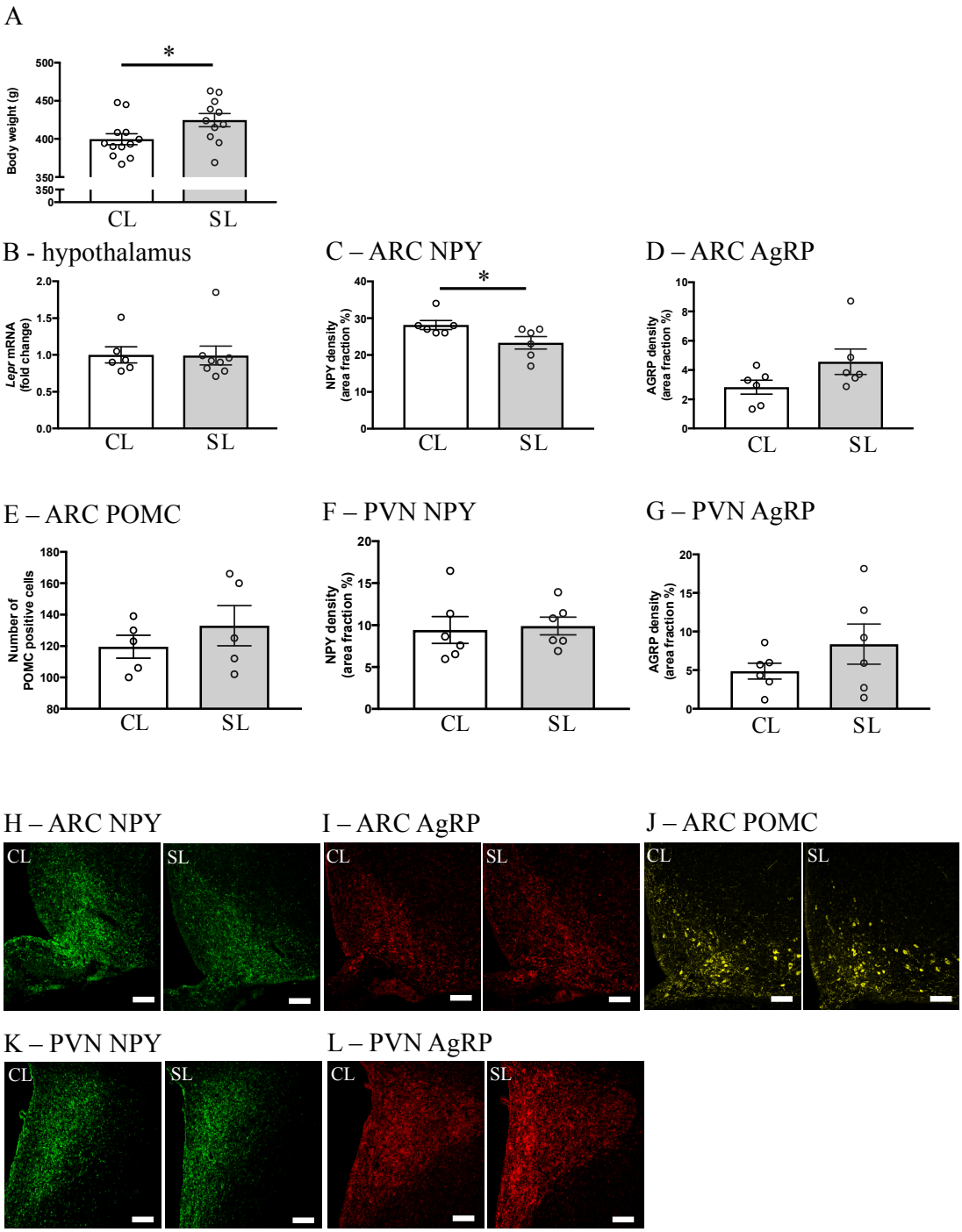


**Figure 2.3 Long-term effects of neonatal overfeeding on the leptin system.**
(A) Body weight of adult control (n = 11 – 12) (CL) and small litter (SL) animals. (B) Leptin receptor expression in the hypothalamus (n = 6 - 10). (C) Neuropeptide Y (NPY) (n = 6), (D) agouti related peptide (AgRP) (n = 5 - 6) and (E) proopiomelanocortin (POMC) immunolabeling in the arcuate nucleus (ARC) (n = 5). Representative photomicrographs of (H) NPY, (I) AgRP and (J) POMC labeling in the ARC. Representative photomicrographs of (K) NPY and (L) AgRP labelling in the PVN. Data are mean ± SEM. * $p < 0.05$. Scale bars = 100 µm.

Given the neonatal leptin resistance in the ARC with neonatal overfeeding, it would be expected a long-term hypothalamic inability to respond to exogenous leptin. There was an overall suppression of nocturnal (significant effect of leptin: $F_{(1,46)} = 3.97$, $p = 0.052$; Figure 2.4 A) and 24 hr (main effects of leptin: $F_{(1,46)} = 4.42$, $p = 0.041$; Figure 2.4 C) food intake with exogenous leptin. However, no effects of exogenous leptin on diurnal food intake (Figure 2.4 B), or any effects of litter size manipulation were observed. Similarly, neonatal overfeeding had no effect on hypothalamic responsiveness to leptin in adulthood, as seen in similar numbers of pSTAT3 positive cells in the ARC of both CL and SL animals (significant effect of leptin: $F_{(1,18)} = 4.92$, $p = 0.040$; Figure 2.4 D and E). These results overall indicate that leptin resistance observed during neonatal life with neonatal overfeeding does not persists into adulthood.

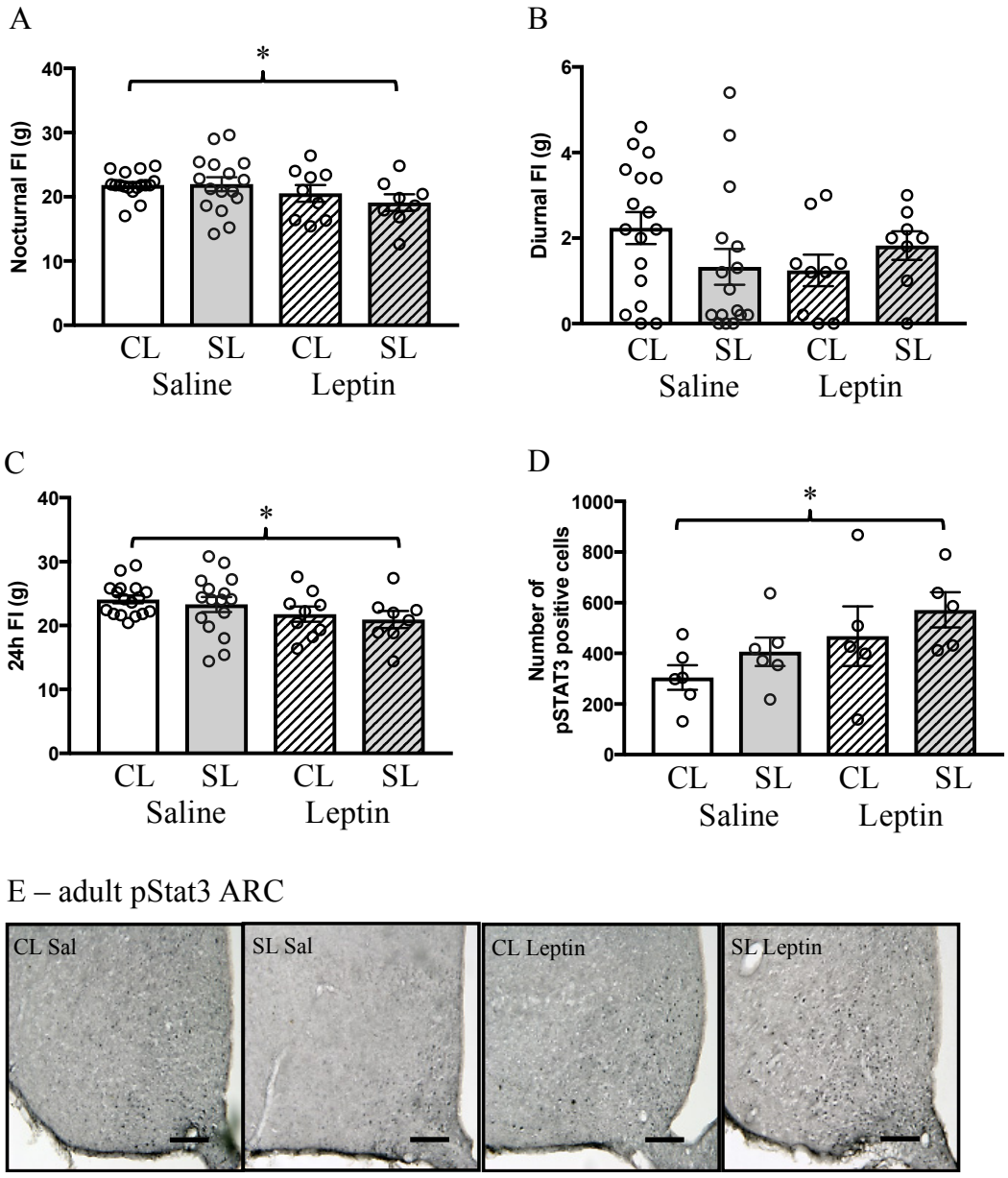


**Figure 2.4 Neonatal overfeeding effects on long-term leptin responsiveness.** Nocturnal (A) (n = 8 - 17), diurnal (B) and 24 hr (n = 8 - 17) (C) food intake after exogenous leptin in control (CL) and small litter (SL) animals (n = 8 - 17). (D) Numbers of phosphorylated signal transducer and activator of transcription (pSTAT3) positive cells in the arcuate nucleus of the hypothalamus (ARC) (n = 5 - 6). (E) Representative photomicrographs showing leptin-induced pSTAT3 positive cells in the ARC of CL and SL rats. Data are mean ± SEM. * $p < 0.05$. Scale bars = 100 μm.

### *2.1.13 Short- and long-term effects of early-life leptin antagonist*

The neonatal leptin antagonist was not able to normalise neonatal body weight at P21 (main effect of litter size: $F_{(1,18)}$= 34.50, $p < 0.001$; n = 5-6, Figure 2.5 A) or

adulthood (main effect of litter size: $F_{(1,18)}$= 9.54, $p$ = 0.006; n = 5-6, Figure 2.5 B) in animals that were overfed during their neonatal life. The leptin antagonist also failed to normalise circulating leptin (main effect of litter size: $F_{(1,15)}$= 11.13, $p$ = 0.005; n = 5-6, Figure 2.5 C). Neonatal leptin antagonist was also not able to affect POMC positive cells in the ARC (Figure 2.5 D). It significantly suppressed AgRP fibre density in the ARC irrespective of litter size (main effect of leptin antagonist: $F_{(1,19)}$= 6.82, $p$ = 0.017; n = 5-6, Figure 2.5 F), however it did not affect ARC NPY fibre density (main effect of litter size: $F_{(1,19)}$= 5.31, $p$ = 0.033; n = 5-6, Figure 2.5 E) or PVN NPY and AgRP fibre density (Figure 2.5 G and H). These leptin antagonist data suggest that there are possibly metabolic factors other than hyperleptinemia affecting long-term body weight in the neonatally overfed rat.

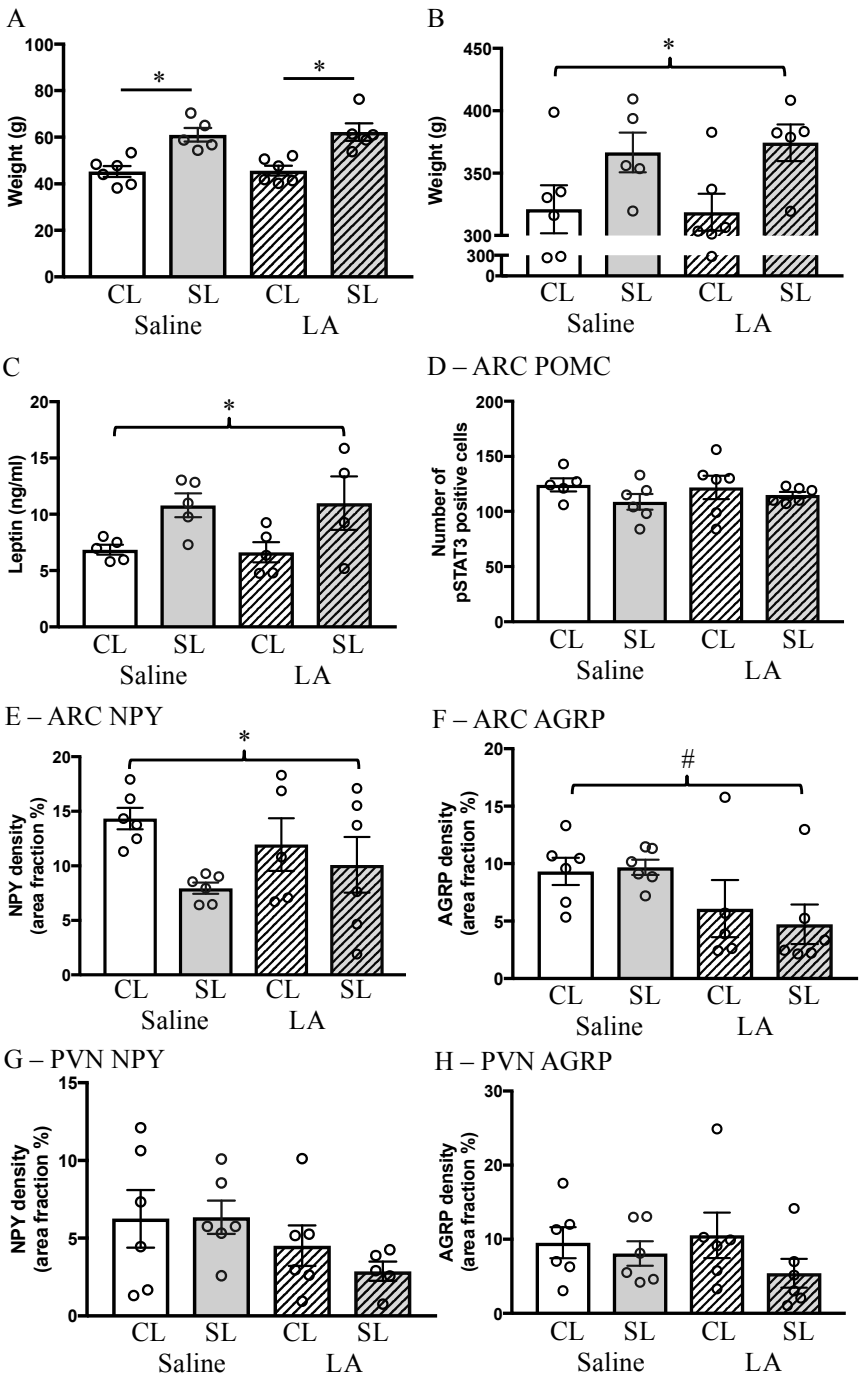


**Figure 2.5 Long-term effects of a leptin antagonist on neonatal overfeeding-induced changes in the leptin system.**
Body weight on (A) postnatal day 21 (n = 5 - 6) and (B) adulthood in control (CL) and small litter (SL) animals given leptin antagonist as neonates (n = 5 - 6). (C) Circulating leptin levels (n = 5 - 6). (D) Numbers of proopiomelanocortin (POMC) positive cells in the arcuate nucleus of the hypothalamus (ARC) (n = 5 - 6). Neuropeptide Y (NPY) immunolabeling in the ARC (n = 5 - 6) (E) and paraventricular nucleus of the hypothalamus (PVN) (n = 6) (G). Agouti related peptide (AgRP) immunolabeling in the ARC (n = 5 - 6) (F) and PVN (n = 6) (H). Data are mean ± SEM. * # $p < 0.05$. * indicates *post hoc* effects (* in B, C, and E

indicates main effect of litter size and no *post hoc* group differences. # in F indicates main effect of leptin antagonist and no *post hoc* group differences).

## Discussion

Here, for the first time, it is observed that neonatal overfeeding along with an increase in body weight and an exacerbated leptin surge was associated with an acute (neonatal) disruption of the NPY and AgRP fibre density in the ARC and PVN as well as acute hypothalamic leptin resistance. These changes being specific to the early life period and being resolved by adulthood emphasise the brain's plasticity and ability to adapt to environmental changes. Encouraging evidence from this study shows that in adulthood, the neonatally overfed animals not only have recovered into optimal NPY/AgRP fibre density, but they show significant reduction in ARC NPY fibre density when compared to their control counterparts. Alongside these changes, neonatally overfed adults show recovered hypothalamic leptin resistance despite maintaining elevated leptin levels (Stefanidis and Spencer 2012). Antagonising leptin activity during neurodevelopment in neonatally overfed pups, despite the inability to ameliorate body weight or hyperleptinemia, led to suppressed ARC NPY fibre density in CL animals to levels similar to those seen in SL.

POMC in the ARC is critical in maintaining energy homeostasis and the inability of leptin to optimally activate POMC neurons leads to obesity. It is demonstrated here that overfed pups with hyperleptinemia do not show the expected increase in hypothalamic POMC immunoreactivity or gene expression. Similarly, a study by Plagemann and colleagues' (Plagemann et al. 2009) showed that neonatal overfeeding was associated with no differences in hypothalamic POMC gene and protein expression, despite hyperleptinemia which is expected to lead to increased POMC

expression (Elias et al. 1999). The results from Plagemann are inversely correlated to POMC promoter methylation (epigenetic modification resulting in transcriptional repression) leading to lack of upregulation in POMC gene expression in a setting of hyperleptinemia. These and our results may reflect an altered capacity of the system to respond to different energy balance conditions.

Work from Ahima and colleagues has highlighted the importance of the leptin surge during the first weeks of development in rodents (Ahima, Prabakaran, and Flier 1998). Others (Schmidt et al. 2001; Lopez et al. 2007) and our group (Stefanidis and Spencer 2012) have shown that neonatal overfeeding is associated with an exacerbation of this leptin surge during the neonatal period and persistent hyperleptinemia throughout life. The leptin surge, as explained by Bouret and colleagues (Bouret, Draper, and Simerly 2004b; Bouret and Simerly 2007) is not involved in feeding during this P7-P10 neurodevelopmental window, but it is crucial in maturation of hypothalamic wiring that controls feeding in the long-term. Therefore it is expected that an exacerbated leptin surge would be associated with an increase in NPY/AgRP fibre density. Indeed, here we observed that both ARC NPY and AgRP fibre density are increased with neonatal overfeeding. We also observed a decrease in PVN NPY, but not AgRP in neonatally overfed pups. While NPY and AgRP are highly co-localised (99%) in the ARC (Hahn et al. 1998), they do not co-localise to the same degree in the PVN. The PVN, apart from ARC-derived neurons is innervated by (~50%) NPY fibres derived elsewhere (Sawchenko et al. 1985). Furthermore, NPY and AgRP neurons of the PVN differently respond to certain stimuli (Kas et al. 2005).

Overweight and obesity are associated with leptin resistance, here we examined whether neonatal overfeeding affected hypothalamic responsiveness to exogenous leptin at P12. We observed that despite no changes in the gene expression of the hypothalamic leptin receptor, neonatal overfeeding suppressed the ARC and VMH neurons' ability to respond to exogenous leptin determined as the number of neurons positive for pSTAT3 in response to this stimulus. This finding is consistent with other studies showing that neonatal overfeeding leads to acute leptin resistance (Glavas et al. 2010; Bouret et al. 2008).

It has been shown in a number of studies that neonatally overfed adults maintain increased body weight and hyperleptinemia despite being fed a normal diet after weaning (Lopez et al. 2007; Schmidt et al. 2001; Stefanidis and Spencer 2012). This hyperleptinemia is usually associated with long-term leptin resistance (Glavas et al. 2010). However, here we saw that despite this increased body weight and hyperleptinemia, neonatal leptin resistance observed in ARC and VMH in response to overfeeding is resolved by adulthood. A study from Lopez and colleagues further supports our finding (Lopez et al. 2007). It is important to mention our observation here of a reduction in NPY fibre density in adult rats that were overfed during early life. This is likely indicative of an adaptation to the neonatal overfeeding with a compensatory response in this orexigenic circuitry. In this regard, previous studies have shown that NPY and AgRP are particularly able to adapt to the effects of overfeeding in genetic models of impaired leptin signalling (Bouret *et al*. 2012), neonatal overfeeding (Lopez *et al*. 2007) and diet-induced obesity (Ziotopoulou *et al*. 2000), with a compensatory response to conditions of positive energy balance.

Despite a resolution of the central effects of neonatal overfeeding adulthood, these adult neonatally overfed rats remain overweight and maintain hyperleptinemia throughout life. We therefore hypothesised that a leptin antagonist introduced at the initiation of the leptin surge would ameliorate the long-term effects related to the neonatal overfeeding. The naturally occurring leptin surge initiates at around P4 and peaks at around P7 – P10 (Delahaye et al. 2008). At P14 there is a greater than ten-fold increase in leptin levels in SL animals compared to controls, as previously shown (Stefanidis and Spencer 2012), indicating that neonatal overfeeding exacerbates or disrupts this naturally occurring leptin surge. It is observed that while the leptin antagonist did not normalise body weight or hyperleptinemia in adult animals that were overfed in early life, it reduced AgRP density in both overfed and control animals. This outcome may depend on the dosage and timing of administration of the leptin antagonist. Previous studies have administered the same leptin antagonist for a slightly prolonged period of time compared to the study reported here (P2 - P13) (Attig et al. 2008; Benoit et al. 2013), but this regimen was associated with leptin resistance and negative effects on development and maturation of many organs (Attig et al. 2011). A study from our group using the same leptin antagonist dose and injection regime showed that the antagonist was able to reverse hyperleptinemia and rescue the decline of primordial follicles caused by neonatal overfeeding in female rats (Sominsky, 2016).

The low animal numbers in some of the groups are explained by tissue loss and exclusion of statistical outliers (Grubb's test; no more than one per group). The criteria of outlier exclusions are described below. The exclusion of outliers, however, has not compromised the effect sizes. For instance, for data depicted on Fig. 2.1 D, F we have seen that although the control group consisted of n = 3, these data show a

strong and significant interaction between the effects of litter size and drug ($F_{(1,15)}$ = 8.54, p = 0.011), with a partial ETA squared of 0.363 and observed power of 0.78, indicating that these data explain over 36% of the overall variance (effects and error), with a power of 78% to detect these effects. Similarly, pStat3 expression in the VMH was not affected by any outlier exclusion, so that a strong and significant effect of leptin ($F_{(1,15)}$ = 18.82, p = 0.001) was seen, with a partial ETA squared of 0.56 and observed power of 0.982, indicating that these data explain over 56% of the overall variance, with a power of 98% to detect these effects. Original studies from the Spencer group where the neonatal overfeeding model was first described (Stefanidis and Spencer 2012; Clarke, Stefanidis, and Spencer 2012), provided extensive observations of changes in body composition. I have referred to this study where this was necessary.

Overall our results suggest that neonatal overfeeding along with a permanent increase in body weight and hyperleptinemia acutely alters central leptin systems related to metabolic control, with most of this disrupted functionality being resolved by adulthood. Attenuating the effects of hyperleptinemia at this time with a leptin antagonist failed to reverse the acute or long-term weight gain and hypothalamic effects suggestive of contributions of other factors in the effects of neonatal overfeeding on satiety pathways.

# Chapter 3

# Early life disruption of the ghrelin system with over-eating is resolved in adulthood in male rats

Luba Sominsky*, **Ilvana Ziko***, Thai-Xinh Nguyen, Zane B. Andrews and Sarah J. Spencer, Journal of Neuropharmacology. 2017; 113: 21e30, (* Equal contribution).

## Introduction

Developmental programming of homeostatic feeding behaviour occurs during the first weeks of development in rodents and is integrated within the hypothalamus. As previously discussed in this thesis (Chapter 1, Introduction: "*Leptin in development*"), the main nuclei that regulate energy intake and expenditure within the hypothalamus are the arcuate nucleus of the hypothalamus (ARC), the paraventricular nucleus of the hypothalamus (PVN), the ventromedial nucleus of hypothalamus (VMH), dorsomedial nucleus of the hypothalamus (DMH) and the lateral hypothalamus (LH) (Gao and Horvath 2008). These nuclei are important integration sites for a number of peripheral hormones such as insulin, leptin and ghrelin, as well as nutrients such as glucose and free fatty acids (reviewed in (Bouret 2017)). In the previous chapter we discussed the role of leptin in developmental programming. The role of insulin is also very well established. Unlike the roles of insulin (Plagemann et al. 1997) (previously explained under Chapter 1, "*Perinatal programming of obesity – animal models*") and leptin (Chapter 2), little is known of the role of ghrelin in developmental programming of the hypothalamus. The main focus here is on the role of ghrelin in particular, as one of the most important metabolic hormones that, not only regulates homeostatic food intake, but may also act as a neurotrophic factor in neurodevelopment.

Ghrelin is mainly secreted from the fundus of the stomach where ghrelin *O*-acyl transferase (GOAT) acylates ghrelin on the third serine residue (Goitein et al. 2012). This acylated form of ghrelin is crucial for ghrelin's biological function via its growth hormone secretagogue receptor (GHSR). The two different forms of circulating

ghrelin: acyl ghrelin and des-acyl ghrelin, have separate contributions to physiology. Des-acyl ghrelin does not have any effect on GHSR function, however it is known to antagonise acyl ghrelin's effects on feeding (as reviewed on (Delhanty, Neggers, and van der Lely 2012)). Des-acyl ghrelin is also able to stimulate proliferation of rat spinal cord and hypothalamic fetal cells, suggesting its involvement in neurogenesis and neurodevelopment (Inoue et al. 2010; Sato et al. 2006).

In adulthood acyl ghrelin plays an important role in central control of energy expenditure by controlling feeding, adiposity and glucose metabolism. Adult humans and rodents injected acutely with ghrelin, increase their food intake and body weight (Tschop, Smiley, and Heiman 2000; Wren et al. 2001; Druce et al. 2005). Aside from ghrelin's role in regulating feeding and metabolism in the adult, evidence from Bouret's group has demonstrated an important role of ghrelin in programming feeding-related neurocircuitry during development, that of suppressing leptin-initiated neuronal outgrowth (Steculorum et al. 2015). Ghrelin significantly suppresses neurite projections from neonatal ARC explants *in vitro*. *In vivo*, anti-ghrelin compounds stimulate an increased density of neuronal projections initiating from the ARC and migrating towards the PVN (Steculorum et al. 2015) further supporting ghrelin's function in suppressing excessive axon development. This exclusive maturation of hypothalamic pathways occurs during very precise developmental periods of time - during the first postnatal weeks after birth in rodents. During this time, the leptin surge that initiates at P4 and peaks at P7-P10 is followed by a gradual increase of circulating ghrelin levels that curtails leptin's neurotrophic effects on axonal growth and prevents over-growth of these projections (Steculorum et al. 2015).

Overnutrition during postnatal life may contribute to the onset of obesity and metabolic-related diseases later in life (Cruz et al. 2005; Taveras et al. 2011). For instance, in an animal model of childhood obesity where rat pups are over-nourished during the first weeks of development, there is an increase in body weight, which is maintained throughout life (Clarke et al. 2013; Ziko et al. 2014; Collden et al. 2015). Limited research has shown that neonatal overfeeding suppresses circulating total and acyl ghrelin levels, which leads to a lack of central responsiveness to exogenous ghrelin (Collden et al. 2015). There is also some limited evidence for a role of des-acyl ghrelin during development, at least during the very early phases. Des-acyl ghrelin is able to stimulate proliferation of rat spinal cord and hypothalamic fetal cells, suggesting its involvement in neurogenesis and neurodevelopment (Inoue et al. 2010; Sato et al. 2006). However, the role of desacyl in later development has not yet been investigated, and acyl-ghrelin's contribution to hypothalamic programming in the rat is similarly unknown.

Here we hypothesised that neonatal overfeeding would affect the ghrelin system acutely (during postnatal development) and long-term (until adulthood) and that acyl and des-acyl ghrelin might be differentially affected. To test this hypothesis we examined early life overfeeding's effects on the peripheral and central ghrelin systems including acyl and des-acyl ghrelin's ability to access the hypothalamus. Our results showed that neonatal overfeeding acutely affected the ghrelin system by reducing circulating ghrelin, increasing hypothalamic *Ghsr* expression and hypothalamic responsiveness to exogenous acyl but not des-acyl ghrelin. Interestingly, while acyl ghrelin's ability to access the ARC was not affected by neonatal overfeeding, acyl ghrelin's ability to reach the PVN was attenuated. Importantly, however, these acute

effects of neonatal overfeeding on ghrelin's ability to signal the brain were resolved by adulthood.

## Materials and methods

### *3.1.1 Animals*

As described in Chapter 2, we obtained timed pregnant Wistar rats from the Animal Resources Centre, WA, Australia. On arrival at the RMIT University Animal Facility, we housed them at 22°C on a 12 hr light/dark cycle (07:00–19:00 hr) and provided them with standard pelleted rat chow and water *ad libitum*. We conducted all procedures in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals and RMIT University Animal Ethics Committee approval.

### *3.1.2 Litter size manipulation*

Litter size manipulation was performed as previously described in Chapter 2 (Materials and Methods: "*Litter size manipulation*") to create the neonatally overfed animal model. Only male animals were used in this study, which were culled at postnatal day (P) 7, 12, 14 and at ~P70. Females were kept for use in other experiments (as described in Chapter 4). The adult animals, after separation into same sex littermate pairs at weaning (P21), were left undisturbed until experimentation.

### *3.1.3 Neonatal overfeeding effects on circulating ghrelin*

On the day of experimentation ~P70, a cohort of rats were deeply anesthetized with Lethabarb (150 mg/kg sodium pentobarbitone, i.p.). P70 is a period of young adulthood in rats; ~4 weeks past the attainment of reproductive maturity. Animals at

P7 or P14 were decapitated and trunk blood was collected for later assessment of serum ghrelin. To accurately measure plasma acyl and des-acyl ghrelin, blood collection tubes that contained no anticoagulant were treated with Pefabloc (Roche Applied Science) to achieve a final concentration of 1 mg/mL. Blood was left to clot at room temperature for 30 min then centrifuged at 2500 g for 15 min at 4 °C ± 2 °C. The collected serum was transferred into a fresh tube then acidified with HCl to a final concentration of 0.05 N. The samples were mixed, aliquoted, and stored at -20 °C avoiding freeze-thaw cycles. Serum acyl and des-acyl ghrelin concentrations were determined using a standard ghrelin enzyme-linked immunoassay (ELISA) for total and acyl ghrelin (Millipore, Billerica, MA, USA) following the manufacturer's instructions. The intra-assay variability was 0.3-7% and 0.7-1.3% CV, inter-assay variability 1-10% and 1.8-4.5% CV, and lower limit of detection 0.8 pg/mL and 0.04 ng/mL for acyl and total ghrelin respectively. Samples were assayed in duplicate and were randomized across two plates. Acyl ghrelin concentrations were subtracted from total ghrelin concentrations to derive a value for serum desacyl ghrelin (Hosoda et al. 2004).

### *3.1.4 Neonatal overfeeding effects on hypothalamic gene expression*

To assess changes in expression of genes involved in ghrelin central pathways brains were collected and were then dissected into ARC and hypothalamus not containing the ARC. Samples were immediately snap-frozen into liquid nitrogen and stored at -80 °C until use. RNA was purified and qRT-PCR was performed. The specific primer details are shown in Table 3.1. A relative quantitative measure of the target gene expression, compared with endogenous control *18s* mRNA expression, was analysed

as fully described in Chapter 2 (Materials and Methods: "*Effects of neonatal overfeeding on hypothalamic gene expression*").

**Table 3.1 Primer details for qRT-PCR**

| Primer name | NCBI Reference Sequence | TaqMan Assay ID | Product size |
|---|---|---|---|
| *18s* | X03205.1 | 4319413E | 187 |
| *Agrp* | NM_033650.1 | Rn01431703_g1 | 67 |
| *Ghsr* | NM_032075.3 | Rn00821417_m1 | 61 |
| *Mboat4* | NM_001107317.2 | Rn02079102_s1 | 93 |
| *Npy* | NM_012614.2 | Rn00561681_m1 | 63 |
| *Pomc* | NM_139326.2 | Rn00595020_m1 | 92 |

### *3.1.5 Weight assessment in response to exogenous ghrelin*

Weight changes in response to 1 mg/kg i.p. acyl ghrelin, des-acyl ghrelin (or saline) in CL and SL neonatal and adult animals were assessed. Body weight was taken immediately before and 2 hr after injection for all animals, food intake was assessed at this time in adults only. 2 hr post-injection all animals were deeply anaesthetised with 150 mg/kg sodium pentobarbitone (i.p.) then transcardially perfused as described in Chapter 2 (Materials and methods: "*Brain collection"*).

### *3.1.6 Neonatal overfeeding effects on neuronal activation in response to ghrelin c-Fos immunohistochemistry*

Sections through the hypothalamus were immunolabelled for c-Fos. Randomly selected sections from each treatment group (a single one in five series of 30 μm sections from each animal) were processed at the same time in batches. Briefly, sections were pre-treated with $H_2O_2$, treated with blocking solution and then

incubated in primary antibody (overnight; 4 °C; 1:10 000; rabbit; Santa Cruz Biotechnology, Santa Cruz, CA, USA). This was followed by secondary antibody (1.5 hr; 1:500, biotinylated anti-rabbit; Vector Laboratories, Burlingame, CA, USA) and an avidin-biotin horseradish peroxidase (HRP) complex (ABC; 45 min; Vector Elite kit; Vector). The tissue was then incubated in diaminobenzidine (DAB) intensified with nickel to visualize the HRP activity. The reaction was stopped when the contrast between specific cellular and non-specific background labelling was optimal. Air-dried brain sections were dehydrated in a series of alcohols, cleared in histolene and coverslipped. Hypothalamic sections were assessed by an experimenter blinded to treatment groups for the numbers of c-Fos-positive cells in the ARC and PVN. Four sections 120 μm apart between 2.76 and 3.48 mm caudal to the bregma per animal were analysed and the summed counts of the four sections was taken as our sampled result.

### *3.1.7 Fluorescently labelled ghrelin*

To determine if the ability of ghrelin to reach the brain was affected by neonatal overfeeding P12 pups were injected subcutaneously with 1 mg/kg (x 2, 90 min apart) of Cyanine5 (Cy5)-labelled acyl or desacyl ghrelin dissolved in saline (0.9% NaCl). The Cy5 fluorescently labelled ghrelins were synthesized as previously detailed (Douglas et al. 2014; McGirr et al. 2011) and provided by Dr. Leonard Luyt from the Department of Chemistry, The University of Western Ontario, Canada. Brains were collected 2 hr after the first injection via decapitation, were immersion-fixed in 4% paraformaldehyde for 24 hr and incubated in 20% sucrose in PBS until processed for fluorescence microscopy. 40 μm brain sections were mounted on slides with DAKO mounting medium and constantly protected from light. Fluorescence images were

acquired on an A1R+ confocal laser scanning microscope (Nikon, Tokyo, Japan) using 640 nm excitation and detection using a 700 ± 37.5 band-pass filter. Through-focus series (z-stacks) of ARC and PVN images were taken under 40 x magnification lenses (photoactivation, perfect focus system), under a Galvano scanner, spectral image of 512 x 512 pixels, 2 μm z-stacks. Negative controls that did not have injections of fluorescently labelled ghrelin had no signal under the same conditions. To estimate the total number of Cy5 fluorescently labelled acyl and desacyl ghrelin-positive cells in the ARC and PVN, cell numbers and intensity signal were quantified. Fluorescently labelled acyl and desacyl ghrelin-positive cell numbers were manually counted using Image J (National Institutes of Health, Bethesda, MD, USA) in four brain sections per animal covering the bregma levels -1.44 mm to -3.60 mm throughout all the z-stacks and total cell numbers were summed. NIS Elements Advanced Research Software (Nikon, Tokyo, Japan) was used to estimate fluorescence signal intensity in the ARC and PVN. The fluorescence signal from blood vessels was manually excluded from the intensity analysis based on shape. Finally, the mean fluorescence intensity was quantified per cell throughout the whole ARC and PVN.

### *3.1.8 Data Analysis*

Neonatal and adult changes were analysed separately. For neonates weights, serum ghrelin, gene expression, fluorescently labelled cells and hypothalamic responses to ghrelin were compared using multi-factorial analyses of variance (ANOVA)s with neonatal nutritional environment (CL/SL) and age (P7/12/14) as between factors. Treatment was also included where appropriate (saline/acyl/desacyl ghrelin). Where significant interactions were found, Tukey *post hoc* tests were performed. For the

adults Student's unpaired t-tests or two-way ANOVAs were used. Data are presented as the mean ± SEM. Statistical significance was assumed when $p \leq 0.05$. All data were tested for homogeneity of variance and normality, using the Levene's test for Equality of Variance and the Shapiro-Wilks test, respectively, complemented by the assessment of skewness and kurtosis. These assessments and all other statistical analyses were conducted using SPSS. Outliers were determined using the Grubbs' test ($\alpha = 0.05$) in GraphPad Prism.

## Results

### *3.1.9 Neonatal overfeeding effects on circulating ghrelin*

Neonatal overfeeding significantly reduced serum ghrelin at P7 (significant effect of litter size: $F_{(1,28)} = 8.2$, $p = 0.008$ and age: $F_{(1,28)} = 14.56$, $p = 0.001$; Figure 3.1 A), specifically due to a decrease in des-acyl ghrelin levels (significant effect of litter size: $F_{(1,28)} = 9.17$, $p = 0.005$ and age: $F_{(1,28)} = 17.72$, $p < 0.001$; Figure 3.1 B, C). Significant increase in the AG /DAG ratio at P7 (significant litter size by age interaction: $F_{(1,28)} = 4.90$, $p = 0.035$; Figure 3.1 D) was also observed. By P14 however, ghrelin levels and AG/DAG ratio were no longer different between groups.

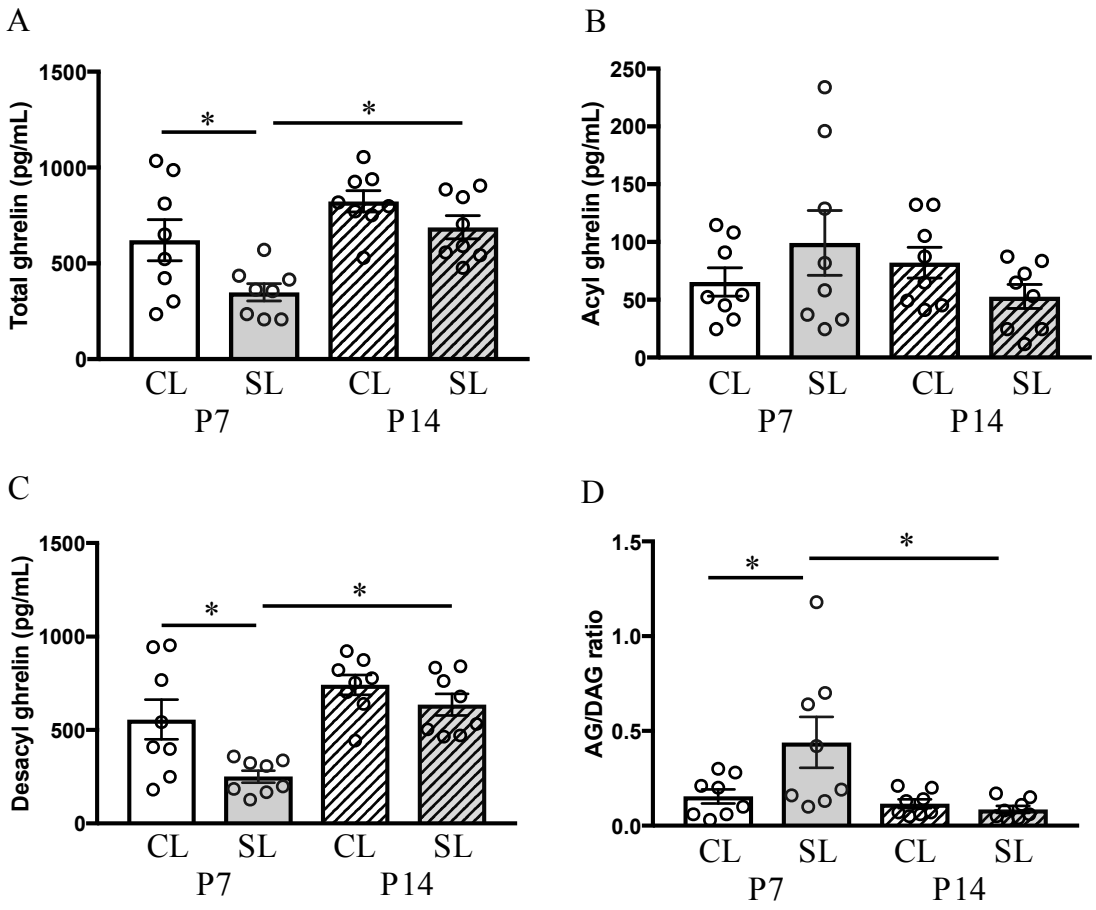


**Figure 3.1 Neonatal overfeeding effects on circulating ghrelin.**
(A) Total ghrelin serum concentration (n = 8), (B) acyl ghrelin (n = 8) (AG) concentration and (C) des-acyl ghrelin (DAG) concentration in the control (CL) and small litter (SL) rats on postnatal day (P) 7 and 14 (n = 8). (D) Ratio of ghrelin at P7 and P14 in CL and SL animals (n = 8). Data are mean ± SEM. * $p < 0.05$.

### *3.1.10 Effects of neonatal overfeeding on the hypothalamus*

To test neonatal overfeeding effects on the hypothalamus mRNA expression of a number of genes (as described in Table 3.1) was measured. In the ARC, neonatal overfeeding significantly increased *Ghsr* expression at P7 (significant effect of litter size: $F_{(1,25)} = 9.003$, p = 0.006 and age: $F_{(1,25)} = 11.52$, p = 0.002; Figure 3.2 A) and also significantly increased *Mboat4 (Goat)* expression at P7 (significant litter size by age interaction: $F_{(1,25)} = 5.127$, $p = 0.032$; Figure 3.2 B), indicating a potential for neonatal overfeeding to enhance hypothalamic responsiveness to endogenous ghrelin.

Similarly to circulating ghrelin levels, ARC gene expression of *Ghsr* and *Mboat4* was normalised by P14. No effects of neonatal overfeeding were observed on *Ghsr* (Figure 3.2 C) or *Mboat4* (Figure 3.2 D) expression in the hypothalamus not containing the ARC. Apart from an increase on *Agrp* (main effect of age: $F_{(1,25)} = 26.15$, $p < 0.001$, Figure 3.2 F) and an increase on *Pomc* (main effect of age: $F_{(1,25)} = 15.16$, $p = 0.001$ Figure 3.2 G) expression in the ARC, neonatal overfeeding was not associated with any changes in *Npy*, *Agrp* or *Pomc* gene expression in this region or in the hypothalamus not containing the ARC.

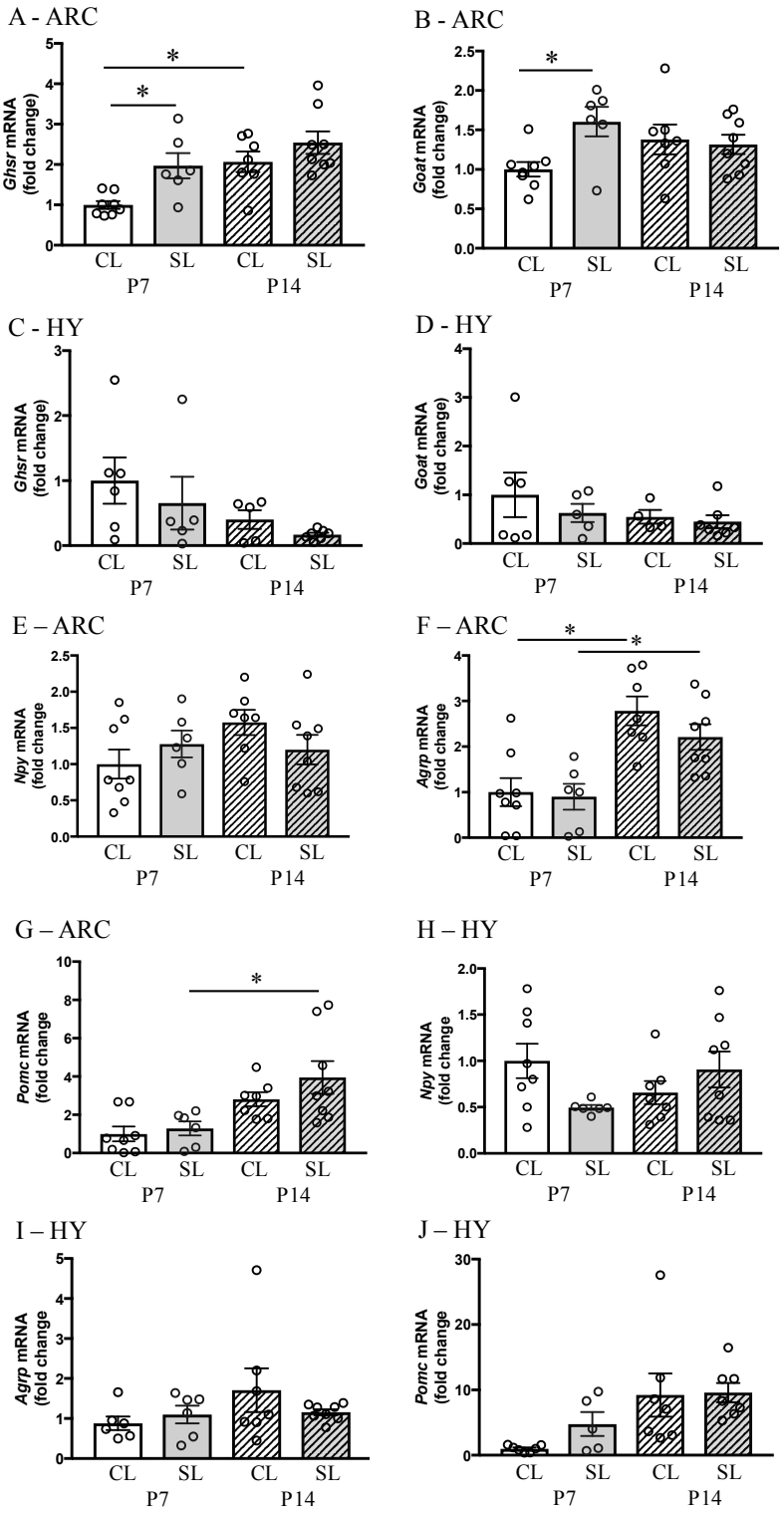


**Figure 3.2 Hypothalamic gene expression in response to neonatal overfeeding.** Growth hormone secretagogue receptor (*Ghsr*) gene expression in the arcuate nucleus of the hypothalamus (ARC) (n = 6 - 8) (A) and the hypothalamus not contain the ARC (HY) (n = 5 - 7) (C) of control (CL) and small litter (SL) animals at postnatal day (P) 7 and 14. Ghrelin *O*-acyl transferase (*Goat*) expression in the ARC (n = 6 - 8) (B) and HY (n = 4 - 7) (D). Gene expression of neuropeptide Y (*Npy*) (n = 6 - 8) (E), agouti related protein (*Agrp*) (F), proopiomelanocortin (*Pomc*) (G) in the ARC and

HY *Npy* (n = 6 - 8) (H), *Agrp* (n = 6 - 8) (I) and *Pomc* (n = 5 - 7) (J). Data are mean ± SEM. * $p < 0.05$.

### *3.1.11 Effects of neonatal overfeeding on hypothalamic responsiveness to exogenous ghrelin*

Overfeeding-induced changes in the availability of ghrelin associated with increased *Ghsr* expression in the ARC, and hence a potential for increased responsiveness to ghrelin, prompted us to test whether neonatal overfeeding also affected hypothalamic ability to respond to an exogenous stimulus. Exogenous acyl ghrelin significantly increased neuronal activation in the ARC of P12 pups compared to controls (significant effect of litter size: $F_{(1,28)} = 5.88$, $p = 0.02$; and drug $F_{(2,28)} = 7.51$, $p = 0.002$; Figure 3.3 A). Similarly, acyl ghrelin stimulated an increase in neuronal activation in the PVN of SL pups compared to CLs (significant litter size by drug interaction: $F_{(2,30)} = 3.55$, $p = 0.041$; Figure 3.3 B). However, exogenous ghrelin did not affect body weight (as a neonatal proxy for food intake) (Figure 3.3 C). No effect of neonatal overfeeding after exogenous des-acyl ghrelin was observed on neuronal activation or body weight.

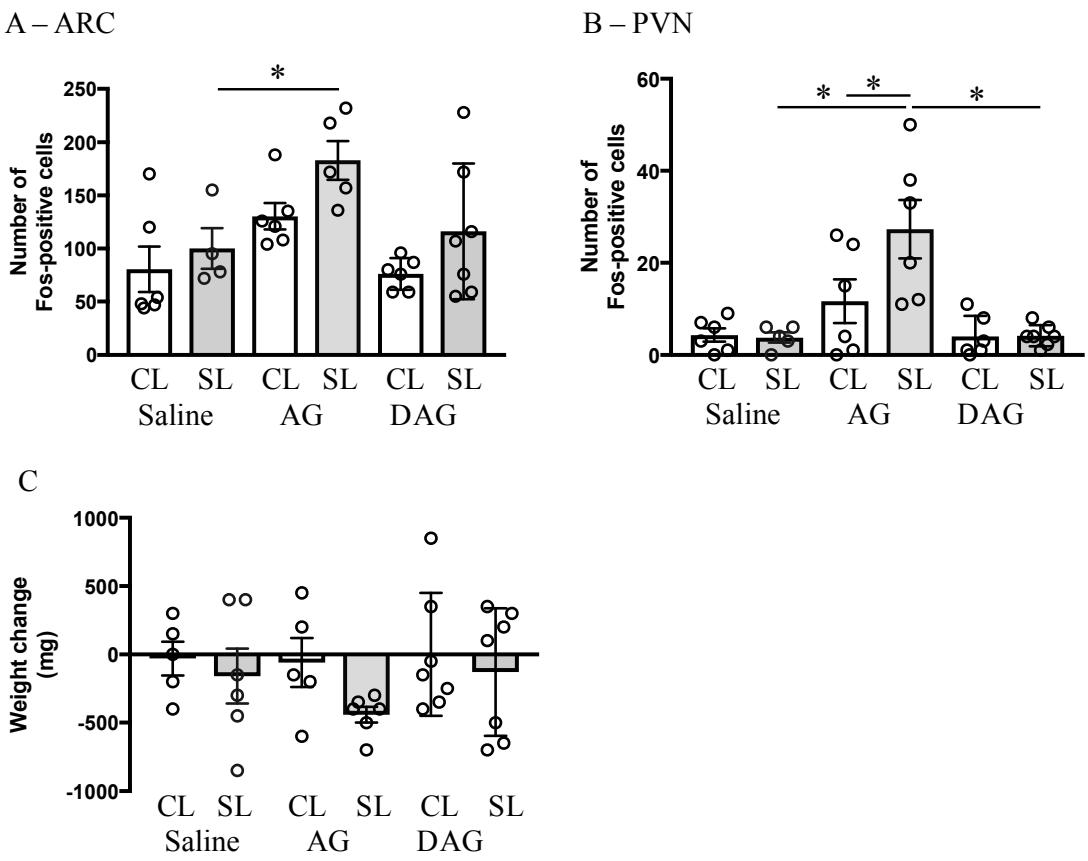


**Figure 3.3 Effects of neonatal overfeeding on the weight change and hypothalamic responsiveness after exogenous ghrelin.**
c-Fos positive cells in the arcuate nucleus of the hypothalamus (ARC) (n = 4 - 7) (A) and paraventricular nucleus of the hypothalamus (PVN) (n = 5 - 7) (B) of control (CL) and small litter (SL) rats on postnatal day (P) 12. (C) Body weight change 2h after 1 mg/kg acyl ghrelin (AG), des-acyl ghrelin (DAG) or saline injection (n = 5 - 7). Data are mean ± SEM. * $p < 0.05$.

### *3.1.12 Neonatal overfeeding effects on ghrelin's accessibility to the hypothalamus*

We observed here that neonatal overfeeding exacerbates hypothalamic responsiveness to exogenous ghrelin. The possibility that neonatal overfeeding affects the blood brain barrier (BBB) permeability as a potential mechanism for this effect by assessing numbers and intensity of cells labeled by fluorescently-labeled acyl and des-acyl ghrelin in the ARC and PVN was tested. Neonatal overfeeding had no effects on the ability of acyl or des-acyl ghrelin to reach the ARC, since there were similar numbers

of positive cells (Figure 3.4 A-D, I, J) and intensity of labelling per cell (Figure 3.4 E-H) in the CL and SL animals at P12. In the PVN, however, there were fewer fluorescently labeled acyl ghrelin cells in SL animals compared to CLs ($t_{(11)} = 2.22$, $p = 0.049$, Figure 3.4 K, O) and lower fluorescence intensity per cell ($t_{(11)} = 4.59$, $p = 0.0008$, Figure 3.4 L). No effects of neonatal overfeeding were observed on the fluorescently labeled des-acyl ghrelin positive cells or intensity per cell in PVN (Figure 3.4 M, N, P).

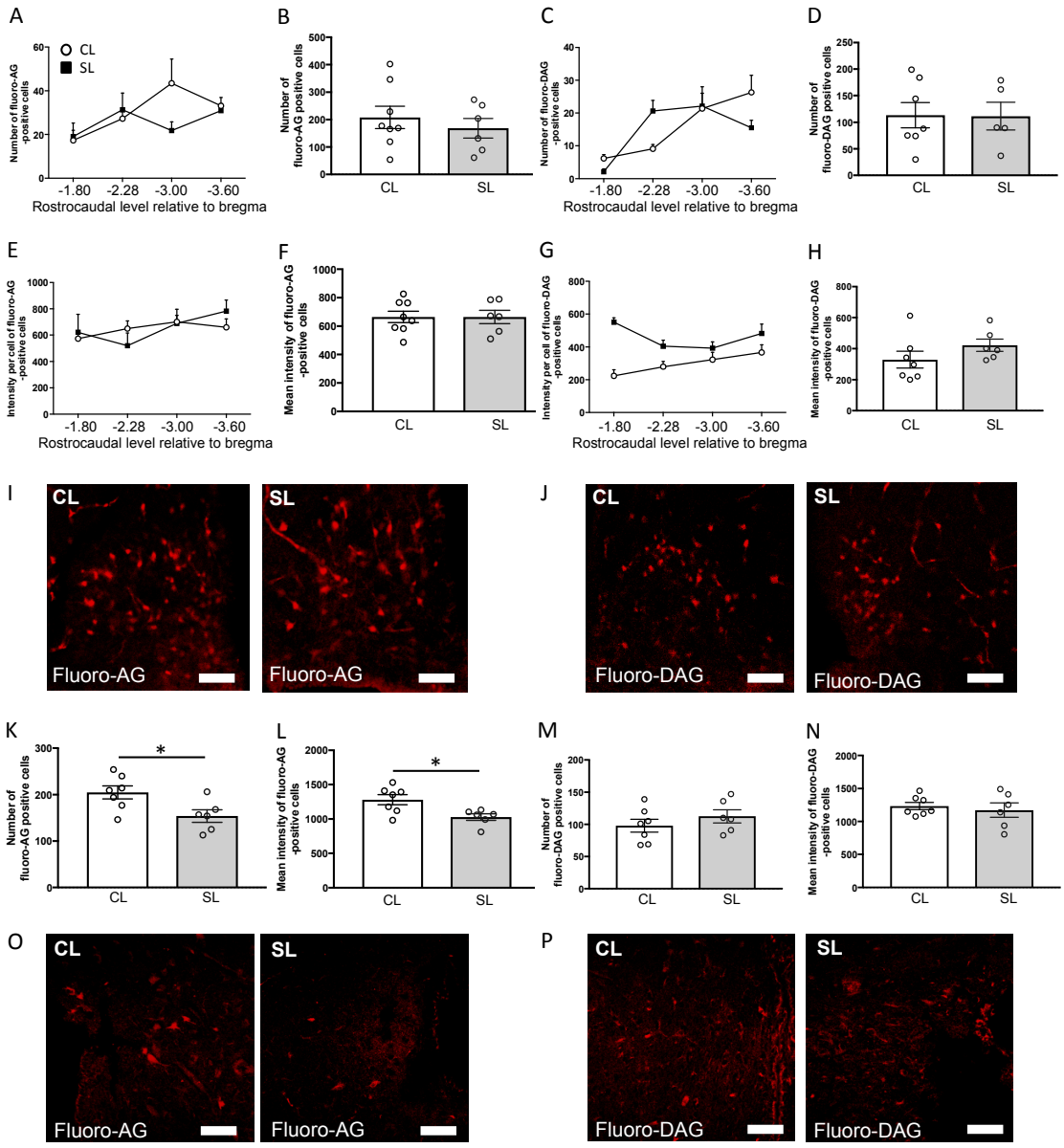


**Figure 3.4 Neonatal overfeeding effects on peripheral ghrelin's ability to access the hypothalamus.**

(A, B) Number of fluorescently labeled acyl ghrelin (AG) positive cells in the arcuate nucleus of the hypothalamus (ARC) of control (CL) and small litter (SL) animals on postnatal day (P) 12 (n = 6 - 8). (C, D) Number of fluorescently labeled des-acyl ghrelin (DAG) positive cells in the ARC of CL and SL animals on P12 (n = 5 - 7). (E, F) Intensity per cell of AG positive cells in the ARC (n = 6 - 8). (G, H) intensity per cell of DAG positive cells in the ARC (n = 6 - 7). (I) Photomicrographs of AG positive cells and (J) DAG positive cells in the ARC. (K) Number, (L) intensity (n = 6 - 7) and (O) photomicrographs of AG positive cells in the paraventricular nucleus of the hypothalamus (PVN). (M) Number, (N) intensity (n = 6 - 7) and (P) photomicrographs of AG positive cells in the PVN. Scale bars = 50 µm. Data are mean ± SEM. * $p < 0.05$.

### *3.1.13 Long-term effects of neonatal overfeeding on the ghrelin system*

Optimal functioning of the ghrelin system during the early life developmental period is important for long-term metabolism (Steculorum et al. 2015). Therefore, we tested here whether changes in the ghrelin system with neonatal overfeeding were reflected in adulthood. Our group has previously shown that neonatally overfed rats remain significantly heavier than controls as adults (Smith and Spencer 2012; Spencer and Tilbrook 2009). Despite a permanent weight change with neonatal overfeeding and suppressed des-acyl ghrelin levels on P7, circulating ghrelin levels in adulthood were no longer different between groups (Figure 3.5 A). Similarly, hypothalamic *Ghsr* expression in adulthood was not different between groups (Figure 3.5 B). However, *Mboat* mRNA expression was significantly increased in the neonatally overfed adult animals relative to controls ($t_{(12)} = 2.70$, $p = 0.019$, Figure 3.5 C). Interestingly, hypothalamic *Npy* mRNA expression was suppressed in the neonatally overfed adults ($t_{(11)} = 2.21$, $p = 0.049$, Figure 3.5 D), without any effects on the hypothalamic *Agrp* and *Pomc* gene expression (Figure 3.5 E, F).

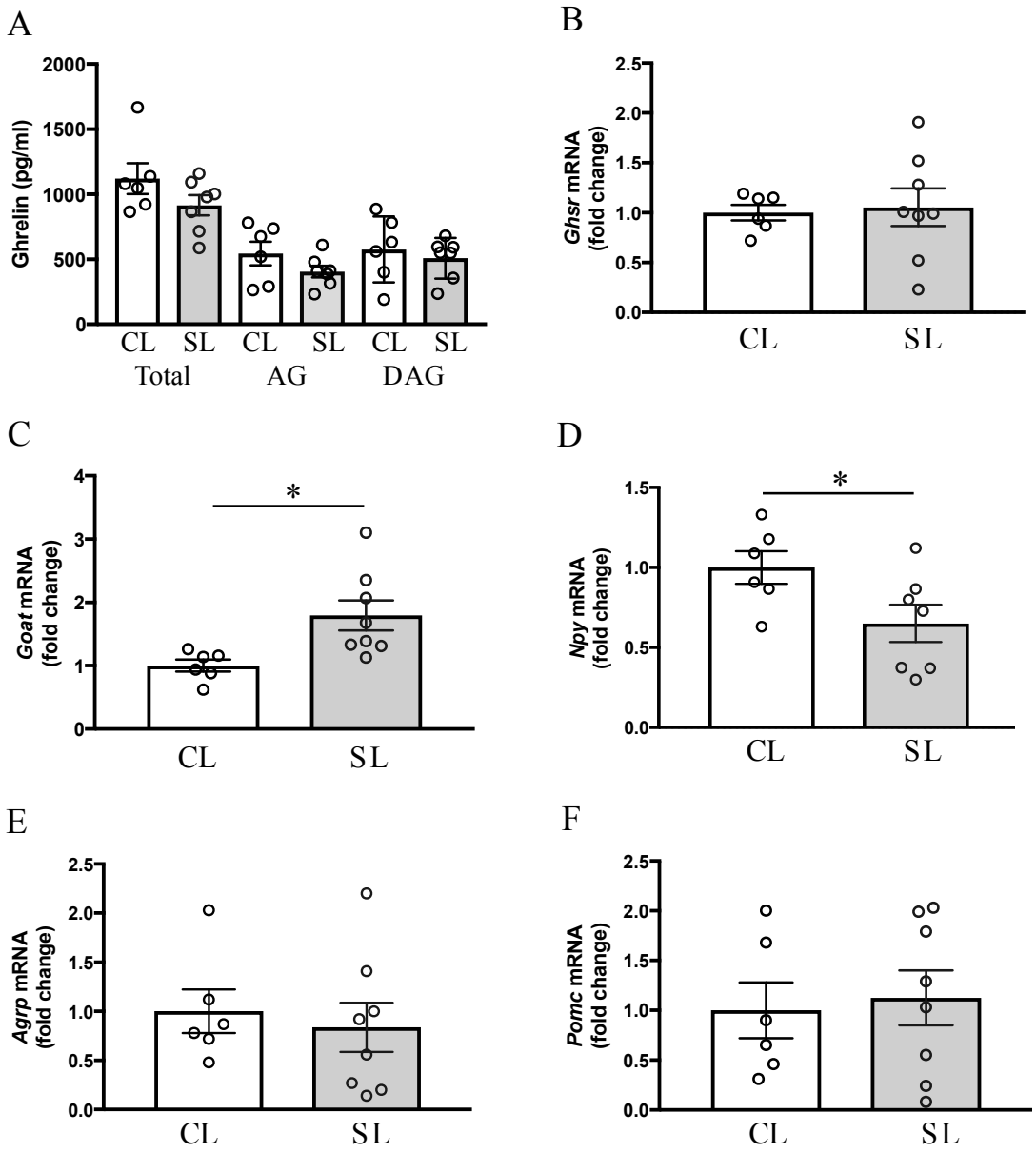


**Figure 3.5 Neonatal overfeeding effects on adult ghrelin system.**
(A) Circulating total, acyl ghrelin (AG) and des-acyl ghrelin (DAG) in control (CL) and adult animals overfed (SL) during neonatal life (n = 6 - 7). Hypothalamic growth hormone secretagogue receptor (*Ghsr*), (B) ghrelin *O*-acyl transferase (*Goat*) *Mboat* (n = 6 - 8), (D) neuropeptide Y (*Npy*) (n = 6 - 7), (E) agouti related peptide (*Agrp*) (n = 6 - 8) and proopiomelanocortin (*Pomc*) gene expression (n = 6 - 8). Data are mean ± SEM. * $p < 0.05$.

Given the changes observed with neonatal overfeeding on hypothalamic *Mboat* expression, there would be anticipated changes to feeding and weight after exogenous acyl or des-acyl ghrelin injection. However, no effects of either ghrelin were observed

on weight (Figure 3.6 A) or food intake (Figure 3.6 B). Acyl but not des-acyl ghrelin mediated an increase in c-Fos positive cells in the ARC, but without any effect of neonatal overfeeding (significant effect of ghrelin: $F_{(2,27)} = 26.31$, $p < 0.001$, Figure 3.6 C). In the PVN, there was no effect of neonatal overfeeding on Fos expression, but only an effect of drug ($F_{(2,29)} = 25.44$, $p < 0.001$, Figure 3.6 D) and, interestingly an effect of des-acyl along with acyl ghrelin in increasing c-Fos positive cells compared to controls (Figure 3.6 D).

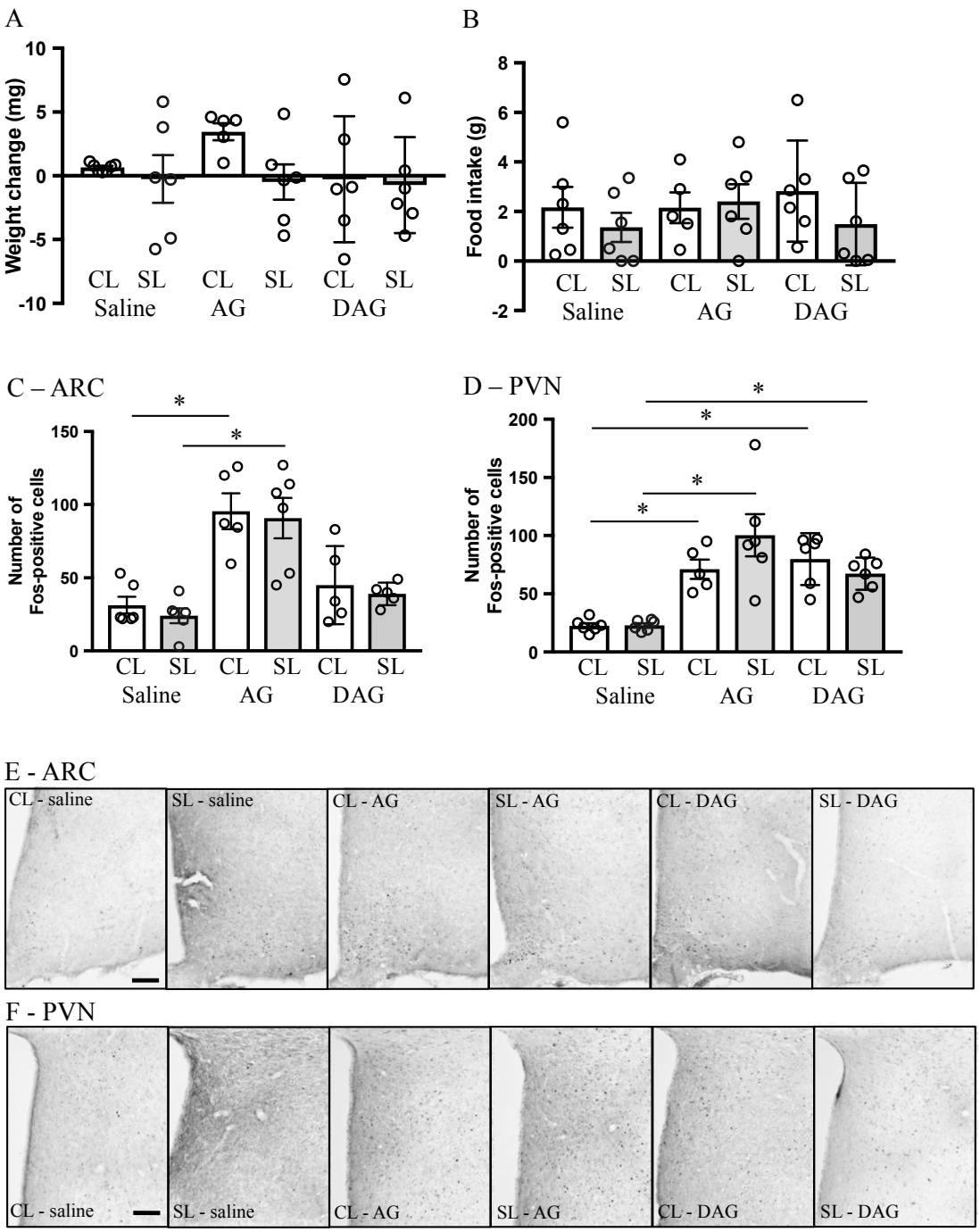


**Figure 3.6 Neonatal overfeeding effects on body weight change and hypothalamic responsiveness to exogenous (1mg/kg) acyl ghrelin (AG), des-acyl ghrelin (DAG) or saline in adulthood.**

(A) Body weight change (n = 5 - 7) and (B) food intake 2 hr after injection in control (CL) and small litter (SL) adult animals (n = 5 - 6). (C) Arcuate nucleus of the hypothalamus (ARC) (n = 5 - 6) and (D) paraventricular nucleus of the hypothalamus (PVN) c-Fos positive cells (n = 5 - 6). Photomicrographs of (E) ARC and (F) and PVN. Scale bars = 100 μm. Data are mean ± SEM. * $p < 0.05$.

## Discussion

Ghrelin, traditionally known for its role in appetite stimulation and energy balance (Tschop, Smiley, and Heiman 2000), has recently been implicated in other roles, including neurodevelopment (Collden et al. 2015). It has been thought likely that acyl ghrelin acts as a brake to the leptin surge-initiated growth of neuronal connectivity within regions of the hypothalamus that regulate feeding and metabolism (Steculorum et al. 2015). Des-acyl ghrelin, on the other hand, has not been studied extensively due to lack of knowledge about the receptor through which it acts. In the present study we show that postnatal overnutrition (during the first three weeks after birth) in the rat alters endogenous ghrelin's ability to act on the hypothalamus during an important developmental period. Neonatal overfeeding suppresses des-acyl ghrelin levels during the first week after birth, increases the ratio between acyl ghrelin and des-acyl ghrelin, increases hypothalamic expression of ghrelin (*Ghsr*) and the enzyme that breaks down ghrelin into its active form (*Goat*) and, more importantly, decreases accumulation of acyl ghrelin in the PVN, which may be due to decreased ability to reach the PVN.

We, and others have previously shown that early life overnutrition is associated with long-term weight gain (Cai et al. 2016; Clarke et al. 2013; Lopez et al. 2007; Stefanidis and Spencer 2012; Ziko et al. 2014; Glavas et al. 2010). Here we aimed to understand whether the permanent body weight change associated with neonatal overnutrition was a consequence of an acute or long-term disruption to the ghrelin system. Our results indicate that neonatal overfeeding has pronounced effects on suppressing circulating ghrelin levels during the first week of development. A similar reduction of acyl ghrelin levels has been reported in neonatally overfed mice (Collden et al. 2015; Soares et al. 2012). It is important to note that these studies have not considered whether des-acyl ghrelin measurements have been affected by early life

overnutrition. It is also important to consider the differences between species in regards to ghrelin des-acylation by the responsible enzymes, such as butyrylcholinesterase in humans and carboxylesterase in rodents (De Vriese et al. 2004). Furthermore, carboxylesterase blood levels, differ greatly between mice and rats (Rudakova, Boltneva, and Makhaeva 2011).

Similarly to what observed here, decreased des-acyl ghrelin levels, unchanged levels of acyl ghrelin and a higher acyl ghrelin to des-acyl ghrelin ratio are reported in obese individuals (Delhanty et al. 2013; St-Pierre et al. 2007; Longo et al. 2008; Pacifico et al. 2009). An increase in the acyl ghrelin to des-acyl ghrelin ratio may explain the increased sensitivity to acyl ghrelin's effects, such as increased hypothalamic gene expression of *Ghsr* and *Goat,* that was observed in our study. Additionally our neonatally overfed pups exhibited exacerbated hypothalamic responses to exogenous acyl ghrelin, but not des-acyl ghrelin. Given the suppressive effect of des-acyl ghrelin on acyl ghrelin's functions (Delhanty et al. 2013; Ozcan et al. 2014) the reduced des-acyl ghrelin levels seen in neonatally overfed animals could attenuate acyl ghrelin's usual "brake" on hypothalamic neurite outgrowth and lead to acyl ghrelin-mediated hyper-activation of hypothalamic neuronal growth.

We showed here that there was no effect of neonatal overfeeding on the ability of ghrelin to access the hypothalamus based on the similar numbers of fluorescently labeled acyl or des-acyl ghrelin-positive cells seen in the ARC. However, neonatal overfeeding affected PVN accessibility of circulating, fluorescently-labeled acyl ghrelin as shown by the reduced positive cell numbers and intensity per cell in this region of the hypothalamus. These results contrasted with the observation of an

exacerbated neuronal activation in the PVN by the same acyl ghrelin dose in neonatally overfed pups compared to normally fed counterparts. A potential explanation for this discrepancy is that the PVN can be remotely activated, for example by ARC and DMH glutamatergic neuronal projections (Ziegler and Herman 2000; Csaki et al. 2000). Furthermore, the PVN is innervated by different populations of neurons and despite the fact that the ghrelin receptor is expressed in PVN neurons, these neurons are a different population from those activated by stress (Spencer et al. 2012). In support of these findings, Cabral and colleagues have shown that corticotropin releasing factor (CRF) neurons not containing *Ghsr* show increased neuronal activation in colchicine treated animals, although indirectly activated by ghrelin (Cabral et al. 2012).

Taken together our results suggest that neonatal overfeeding exacerbates hypothalamic sensitivity to endogenous acyl ghrelin, possibly due to the lack of des-acyl ghrelin's antagonising effects on acyl ghrelin. Importantly, these neuroendocrine defects are restricted to the early life developmental period without life-long effects. The only long-term effect with neonatal overfeeding observed in our study was an increased expression of hypothalamic *Mboat4*, however this was not sufficient to affect circulating ghrelin levels or hypothalamic responsiveness to exogenous ghrelin. *Mboat4* can be locally produced and regulated depending on relevant metabolic states (Gahete, Cordoba-Chacon, et al. 2010) and so it is possible that this difference in *Mboat4* expression could lead to differences in coping with a metabolic challenge, but this remains to be determined. Ghrelin has also been shown to affect feeding not only via the ARC, but also via other extra-hypothalamic sites such as the paraventricular thalamus, amygdala and brainstem (Faulconbridge et al. 2003; Kanoski et al. 2013).

Further investigations are needed to define the role of neonatal overfeeding on extra-hypothalamic influence of ghrelin long-term. Nonetheless, our results here further support those seen with the impact of neonatal overfeeding on leptin-mediated regulation of the hypothalamus in that many of the major effects resolve by adulthood. These findings are potentially encouraging for people having had poor diets in early life.

# Chapter 4

# Hyperleptinemia in neonatally overfed female rats does not dysregulate feeding circuitry

**Ilvana Ziko**, Luba Sominsky, Thai-Xinh Nguyen, Kit-Yi Yam, Simone N. De Luca, Aniko Korosi and Sarah J. Spencer, Frontiers in Endocrinology. 2017; 8: 287.

## Introduction

The early life nutritional environment can permanently affect homeostatic pathways, therefore programming an individual's life-long health. Over- or under-nutrition *in utero* and during the neonatal period in male humans and rodents is associated with increased risks of developing metabolic disorders later in life (as reviewed in (Spencer 2012)). The nutritional environment in early life also alters sensitivity to the metabolic hormones leptin and ghrelin in males (Bouret 2010; Collden et al. 2015; Davidowa, Li, and Plagemann 2003; Plagemann 2006). We have seen that the nutritional environment during postnatal development in males leads to a permanent disruption of metabolic parameters such as body weight, circulating leptin and ghrelin, with central changes to the leptin and ghrelin systems (Sominsky, Ziko, et al. 2017; Sominsky, Ziko, Nguyen, et al. 2016). However, whether similar effects are present in females is much less well known.

Obesity affects women as much, if not more than men. Despite a great variability between and within countries, globally there are more obese women than men (Kanter and Caballero 2012). In boys the relation between body mass index (BMI) and puberty is non-linear. Being overweight in boys is associated with early puberty, whereas severe obesity is associated with late puberty. In girls, obesity is always associated with early puberty (Wang 2002; Rasmussen et al. 2015). Aside from the myriad diseases and disorders common to both sexes that are caused or precipitated by obesity, there are several obesity-related complications that are particular to females. Thus, it is established that being overweight and obese during childhood in girls leads to fertility problems including polycystic ovarian syndrome (Lee et al. 2007; Maisonet et al. 2010; Teede, Deeks, and Moran 2010). Being overweight and

obese increases the risk of pregnancy complications such as gestational diabetes mellitus (Chu et al. 2007). Obesity also differentially affects circulating leptin levels in males and females with the latter having significantly increased leptin compared to males after controlling for BMI, age and percentage of body adiposity indicating that more leptin is released from the same adipose mass in women compared to men (Azar et al. 2002). In the context of mood, anxiety and depression there is a positive relationship between the above disorders and obesity (defined by BMI) in women but not in men (de Wit et al. 2010; Barry, Pietrzak, and Petry 2008).

Early life events including nutrition or exposure to stress also affect females and males differently. Both male and female offspring born from mothers exposed to the Dutch Hunger Winter during pregnancy had increased BMI and adiposity, however females were affected to a greater extend compared to males (Stein et al. 2007). A number of animal studies have shown that maternal obesity is associated with hypothalamic gene dysregulation in both males and females, however this dysregulation in males is greater than in females (Dearden and Balthasar 2014). Likewise, male offspring overfed during the neonatal period develop higher hypothalamic inflammation compared to females (Argente-Arizon et al. 2018). Other early life perturbations, including stress, are associated with significantly increased risk of developing post-traumatic stress disorders, depression and anxiety in females compared to males (Pratchett, Pelcovitz, and Yehuda 2010; Olino et al. 2010; Breslau 2009). As such it seems likely that females are equally if not more vulnerable than males to the early programming effects of diet on later metabolic function.

Unfortunately, most of the key animal studies elucidating the programming effects of early diet and the roles of hormones such as leptin have been performed in males without consideration of females. Bouret and colleagues have shown that large litter rearing in female rats bred to develop diet-induced obesity ameliorates central leptin sensitivity and protects from adverse metabolic effects of obesity in comparison to normal litter rearing females (Patterson et al. 2010). Furthermore, our group and others have shown that males that are neonatally overfed also have disruptions in both circulating ghrelin and the ability of ghrelin to act at the hypothalamus to regulate feeding circuitry (Sominsky, Ziko, Nguyen, et al. 2016; Collden et al. 2015); an effect that is present during the neonatal period but partly normalised by adulthood. Like males, neonatally overfed females are hyperleptinemic in juvenile and adult life (Stefanidis and Spencer 2012; Sominsky, Ziko, Soch, et al. 2016). Our group also shown that increased prepubertal weight and hyperleptinemia are associated with earlier puberty onset in female (Sominsky, Ziko, Soch, et al. 2016), but not male rats (Smith and Spencer 2012). However, it is currently unknown whether hyperleptinemia in females leads to disruptions in hypothalamic feeding circuitry as it does in males (Sominsky, Ziko, et al. 2017), and the precedent for major sex differences in hypothalamic outcomes after neonatal overfeeding (Spencer and Tilbrook 2009) suggests a similarity to male responses cannot be assumed for females. We therefore aimed to determine if neonatal overfeeding-induced hyperleptinemia would compromise hypothalamic connectivity and hypothalamic responsiveness to leptin in females as it does in males.

## Materials and Methods

### *4.1.1 Animals*

In these experiments time-mated pregnant Wistar rats were used, obtained from the Animal Resources Centre, WA, Australia. On arrival at the RMIT University Animal Facility at day 14-16 of gestation, they were housed at 22 °C, 12 hr light/dark cycle (0700 – 1900 hr). *Ad libitum* pelleted standard rat chow and water was provided to them. All procedures described here were conducted in accordance with National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals and the RMIT University Animal Ethics Committee approval.

### *4.1.2 Litter size manipulation*

As previously described in Chapter 2: Materials and Methods "*Litter size Manipulation*" and (Smith and Spencer 2012; Spencer and Tilbrook 2009; Stefanidis and Spencer 2012) manipulation of litter sizes was performed. Experimental animals were culled at P7, P12 or P14. Only females were used in the experiments described here. Data from the males of the same litters were used in other publications (Sominsky, Ziko, et al. 2017; Sominsky, Ziko, Nguyen, et al. 2016). All experimental groups were derived from three or more litters, using a maximum of two pups from the same litter for an experimental treatment to control for maternal effects (Spencer and Meyer 2017).

### *4.1.3 Effects of neonatal overfeeding on neonatal circulating leptin and triglycerides*

On P7 or P14 the animals were rapidly decapitated and trunk blood was collected for later assessment of plasma leptin and triglycerides. Whole blood was collected in

EDTA-coated tubes, kept on ice and quickly centrifuged to separate the plasma. The plasma samples were aliquoted and stored at -20 °C avoiding freeze-thaw cycles until use.

To determine leptin concentrations in our samples a standard commercial leptin ELISA was performed, following the manufacturer's instructions (Millipore, Ballerica, MA, USA). Intra-assay variability was 1.9-2.5% CV, inter-assay variability 3.0-3.9% CV, and lower limit of detection 0.04 ng/mL. All compared samples were assayed in duplicate and processed in the same assay.

To determine triglyceride concentration in our samples a triglyceride assay (Cayman, Ann Arbor, MI, USA) was performed according to the manufacturer's instructions. Intra-assay coefficient of variation was 1.34%, inter-assay coefficient of variation was 3.17% and the lower limit of detection for this assay was 0.5 mg/dL. All samples were assessed in duplicate and under the same conditions. (This ELISA was conducted by Dr Simone De Luca from samples generated by me).

### *4.1.4 Effects of neonatal overfeeding on hypothalamic gene expression*

Quantitative real-time PCR (qRT-PCR) was used in order to assess whether neonatal overfeeding alters hypothalamic gene expression of the *neuropeptide Y (Npy), agouti related peptide (Agrp), proopiomelanocortin (Pomc)* or leptin receptor (*Lepr*) in the arcuate nucleus of the hypothalamus (ARC) and hypothalamus from the animals described above (See Table 4.1 for primer details). These methods are extensively described in Chapter 2 (Materials and Methods: "*Effects of neonatal overfeeding on hypothalamic gene expression*"). Since POMC cell bodies are found exclusively in

two central nervous system (CNS) nuclei, the ARC and the nucleus tractus solitarius (Joseph, Pilcher, and Bennett-Clarke 1983; Cone 2005), POMC gene expression was not examined in the hypothalamic tissue that did not contain the ARC.

**Table 4.1 Primer details for quantitative real-time PCR.**

| Primer name | NCBI Reference Sequence | TaqMan Assay ID | Product size |
|---|---|---|---|
| *Actb* | NM_031144.2 | 4352340E | 91 |
| *Lepr* | NM_012596 | Rn01433205_ml | 94 |
| *Npy* | NM_012614.2 | Rn00561681_m1 | 63 |
| *Agrp* | NM_033650.1 | Rn01431703_g1 | 67 |
| *Pomc* | NM_139326.2 | Rn00595020_m1 | 92 |

### *4.1.5 Neuronal activation in response to exogenous leptin*

To assess if neonatal overfeeding influences the ability of the hypothalamus to respond to circulating leptin, neuronal activation in response to 3 mg/kg i.p. leptin or an equivalent volume of 0.9% sterile saline at P12 was assessed. Pups were weighed immediately before and 45 min after injection. For immunohistochemistry and analysis of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) as a marker of leptin-induced neuronal activation, NPY, AgRP and POMC please see Chapter 2: Materials and Methods: "*Brain collection*" and "*Immunohistochemistry*". pSTAT3 immunohistochemistry and cell counts were performed by Ms Thai Xinh Nguyen as part of her Honours project from sections generated by me and under my supervision and training. In order to exclude POMC cytoplasmic labelling from the fibre density analysis, area restriction was applied.

This allows for the NIS program to exclude labeling of any objects that were larger than the fibres.

### *4.1.6 Data analysis*

All data were analysed using multi-factorial analyses of variance (ANOVA)s with neonatal nutritional environment (CL/SL) and age (P7/14) or leptin treatment (saline/leptin) as between factors. Where significant interactions were found, we then performed Tukey *post hoc* tests. Immunoreactivity results were analysed with Student's unpaired t-tests. Data are presented as the mean ± SEM. Statistical significance was assumed when $p \leq 0.05$. All data were tested for homogeneity of variance and normality, using the Levene's test for Equality of Variance and the Shapiro-Wilks test, respectively, complemented by the assessment of skewness and kurtosis. These assessments and all other statistical analyses were conducted using SPSS. Outliers were determined using the Grubbs' test ($\alpha = 0.05$) in GraphPad Prism.

## Results

### *4.1.7 Neonatal overfeeding leads to accelerated weight gain*

In this cohort of rats, neonatal overfeeding led to early weight gain. Neonatally overfed (SL) female rats were thus significantly heavier than controls (CL; significant age by litter size interaction: $F_{(1, 32)} = 30.37$, $p < 0.001$; n = 8-10; Figure 4.1 A). *Post hoc* analyses indicated that by P14 the SL were significantly heavier than CL. These findings are similar to those in males (as in Chapter 2: Figure 2.1) and to our previously published data in females (Smith and Spencer 2012; Spencer and Tilbrook 2009; Stefanidis and Spencer 2012).

### *4.1.8 Neonatal overfeeding effects on circulating leptin*

We have previously seen that circulating leptin levels are higher in neonatally overfed than in control female (and male) rats during the neonatal period, and into adulthood (Stefanidis and Spencer 2012; Sominsky, Ziko, et al. 2017; Sominsky, Ziko, Soch, et al. 2016) and we replicated the neonatal findings here (significant effect of litter size: $F_{(1, 20)} = 25.35$, $p < 0.001$; significant effect of age: $F_{(1, 20)} = 6.01$, $p = 0.024$, n = 6 per group, Figure 4.1 B).

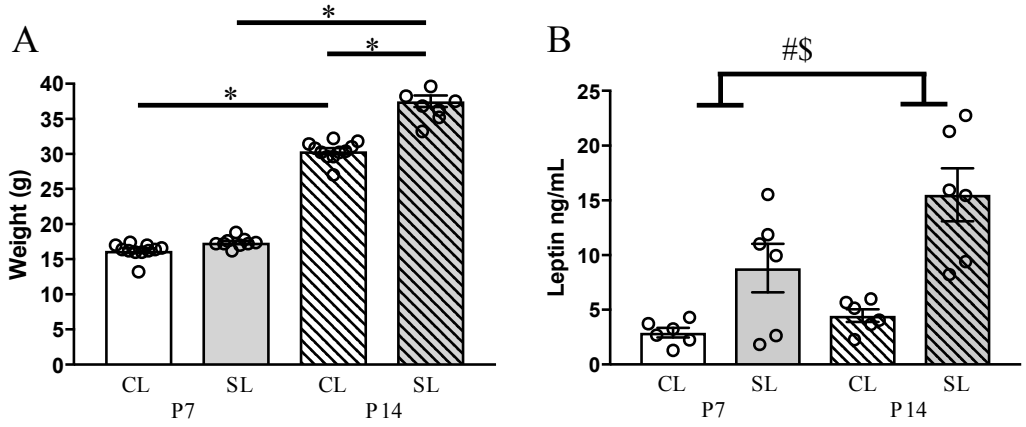


**Figure 4.1 Effects of neonatal overfeeding on the neonatal leptin system.**
(A) Body weights of control (CL) and small litter (SL) rats at postnatal day (P) 7 and 14. (B) Plasma leptin concentrations at P7 and P14. Data are mean ±S.E.M. $n = 6 – 11$ per group. * Tukey's *post hoc*. # significant main effect of litter size. $ significant main effect of age. $p < 0.05$.

### *4.1.9 Neonatal overfeeding effects on neonatal hypothalamic feeding-related gene expression*

Neonatal overfeeding did not affect the hypothalamic satiety-related genes measured here. However, significant age-related changes in these genes were observed. Expression of *Lepr* mRNA was significantly increased at P14 compared to P7 in the ARC (significant effect of age: $F_{(1, 21)} = 23.07$, $p < 0.001$; n = 5-6; Figure 4.2 A) and the hypothalamus (significant effect of age: $F_{(1, 21)} = 13.83$, $p < 0.001$; n =5-7; Figure 4.2 B) in both groups. *Npy* mRNA expression in the ARC was not affected by

neonatal overfeeding, but it was significantly increased at P14 compared to P7 (significant effect of age: $F_{(1, 19)} = 5.84$, $p = 0.026$; n = 5-6; Figure 4.2 C). In the hypothalamus, neonatal overfeeding had no effect on *Npy* expression (Figure 4.2 D). *Agrp* mRNA expression in the ARC was not affected by neonatal overfeeding, however, like *Npy,* it was significantly increased at P14 compared to P7 (significant effect of age: $F_{(1, 19)} = 12.86$, $p = 0.002$; n =5-6; Figure 4.2 E). Neonatal overfeeding also did not affect *Pomc* mRNA expression in the ARC. However, again, there was a significant increase of *Pomc* mRNA in P14 ARC compared to P7 (significant effect of age: $F_{(1, 17)} = 6.16$, $p = 0.024$; n = 5-6; Figure 4.2 F).

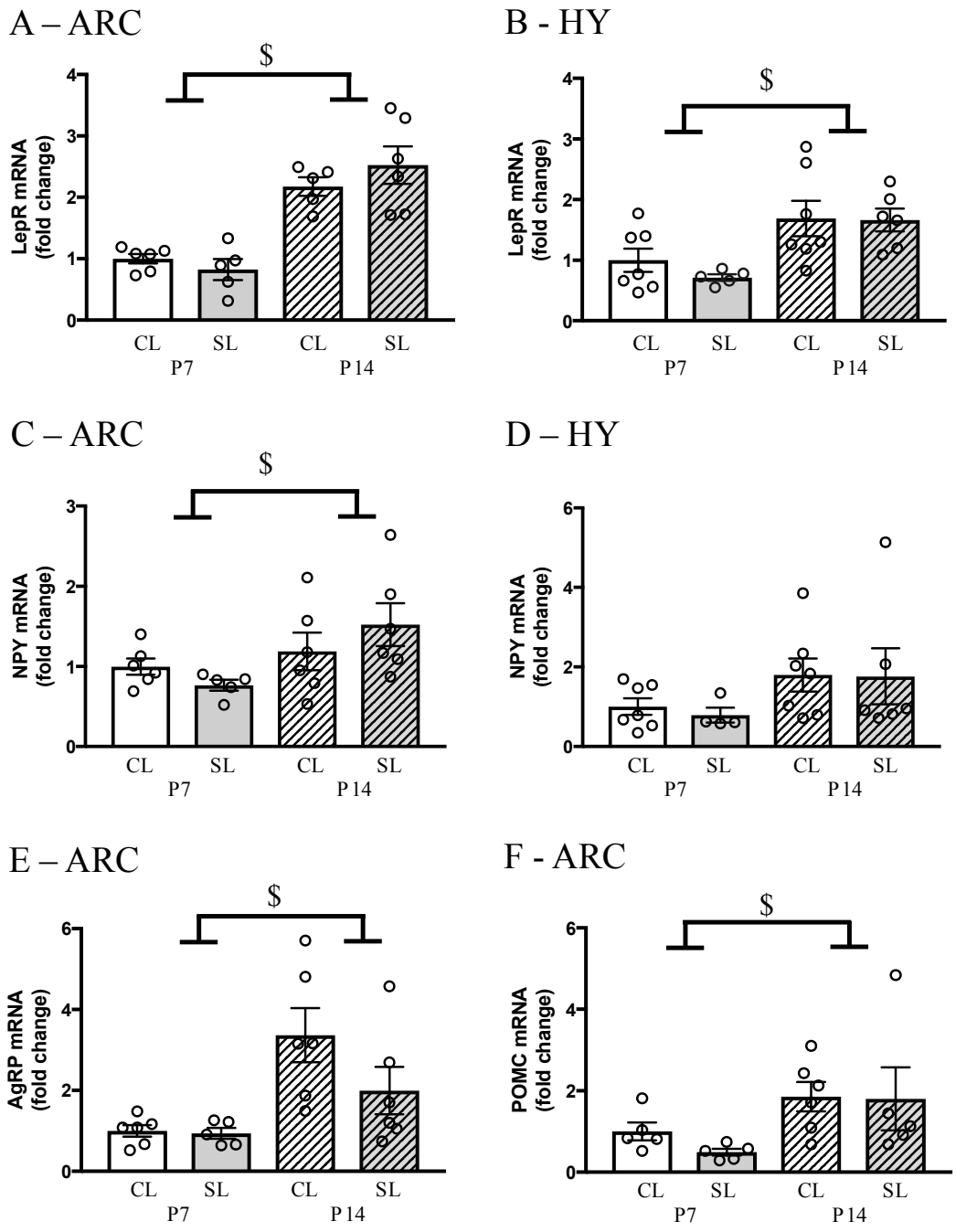


**Figure 4.2 Neonatal overfeeding effects on neonatal hypothalamic feeding-related gene expression.**
(A) Leptin receptor expression in the arcuate nucleus of the hypothalamus (ARC) and (B) hypothalamus (HY) of control (CL) and small litter (SL) rats at postnatal day (P) 7 and P14. (C) Neuropeptide Y (NPY) gene expression in the ARC and (D) in the HY. (E) Agouti-related peptide (AgRP) gene expression in the ARC. (F) Pro-opiomelanocortin (POMC) gene expression in the ARC. Data are mean ± S.E.M. $n$ = 5 – 6 per group. $ significant main effect of age. $p < 0.05$.

### *4.1.10 Neonatal overfeeding effects on neonatal hypothalamic NPY, AgRP, POMC*

To determine if the elevated leptin in neonatally overfed females was associated with a disruption of hypothalamic NPY, AgRP and POMC as previously described in males (Sominsky, Ziko, et al. 2017), we examined NPY fibres (ARC: Figure 4.3 A, B, PVN: Figure 4.3 C, D), AgRP fibres (ARC: Figure 4.3 E, F, PVN: Figure 4.3 G, H), POMC positive cells in the ARC (Figure 4.3 I, J) and POMC fibres in the PVN (Figure 4.3 K, L) at P12. Neonatal overfeeding did not affect NPY, AgRP and POMC immunoreactive fibres or POMC immunoreactive cells in these regions (n = 5-6 per group).

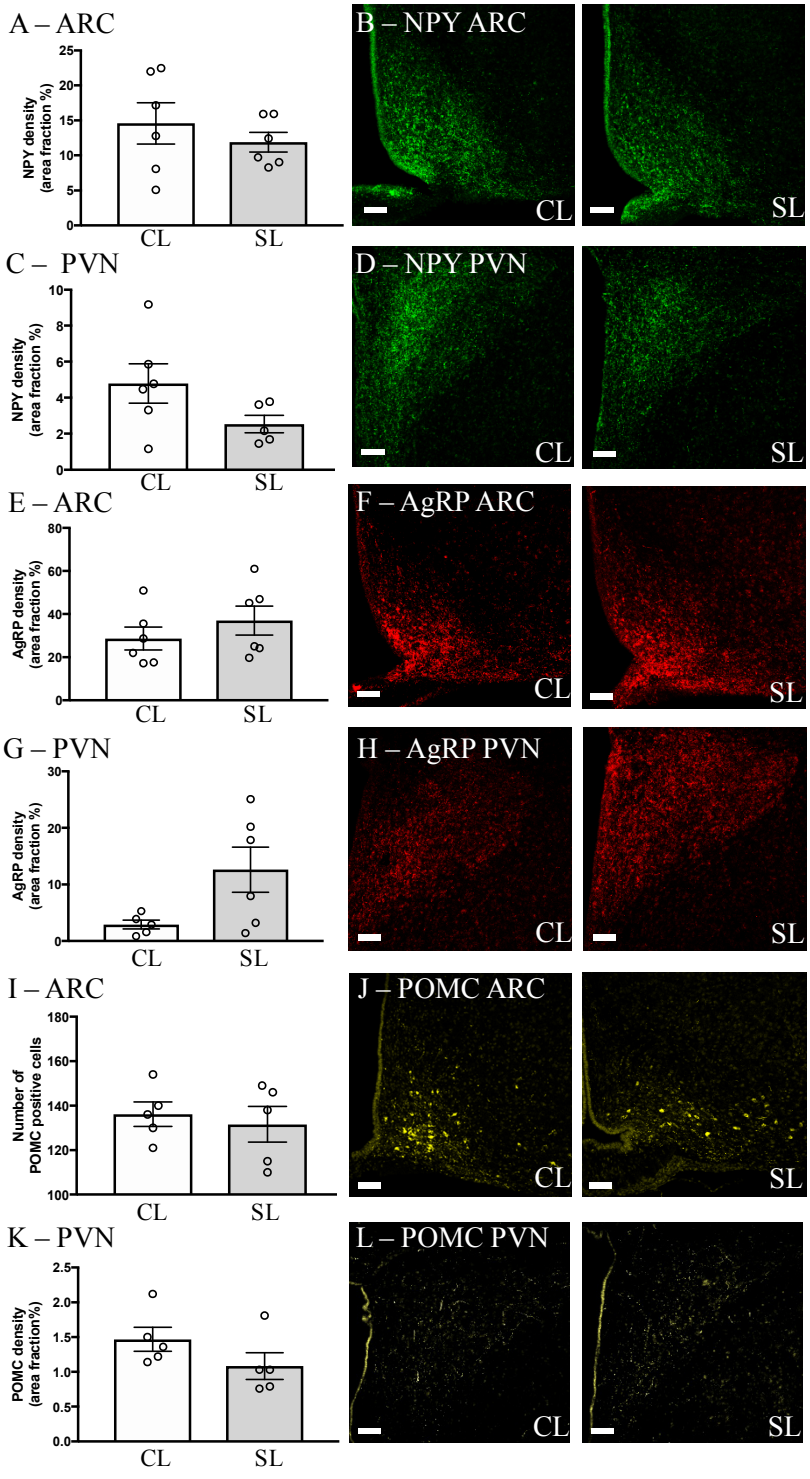


**Figure 4.3 Neonatal overfeeding effects on neonatal hypothalamic circuitry.**
(A, B) Neuropeptide Y (NPY) labelling in the arcuate nucleus of the hypothalamus (ARC) and (C, D) paraventricular nucleus of the hypothalamus (PVN) of control (CL) and small litter (SL) rats at postnatal day (P) 12. (E, F) Agouti-related peptide (AgRP) labelling in the ARC and (G, H) PVN. (I, J) Proopiomelanocortin (POMC) positive cells in the ARC. (K, L) POMC labelling in the PVN. Representative photomicrographs showing NPY labelling in the (B) ARC and (D) PVN, AgRP labelling in the (F) ARC and (H) PVN, POMC labelling in the (J) ARC and (L) PVN. Scale bars = 100 µm. Data are mean ± S.E.M. $n$ = 5 – 6 per group.

### *4.1.11 Neonatal overfeeding effects on neonatal hypothalamic responses to leptin*

We next tested if neonatal overfeeding alters the ability of the neonatal hypothalamus to respond to a leptin signal by giving the pups a single injection of leptin or saline on P12 and measuring weight changes as well as hypothalamic pSTAT3 expression. Leptin stimulated an increase in the number of pStat3 positive cells in the ARC, as expected ($F_{(1, 17)} = 101.30$, $p < 0.001$; n = 5-6). There was also a significant increase in the number of pStat3 positive cells in the ARC by litter size (main effect of litter size ($F_{(1, 17)} = 7.36$, $p = 0.015$; Figure 4.4 A, D) but no interaction. In the ventromedial hypothalamus (VMH) however, leptin injection also induced a significant increase in pSTAT3 positive cells (main effect of leptin: $F_{(1, 17)} = 42.84$, $p < 0.001$; n = 5-6; Figure 4.4 B, E).

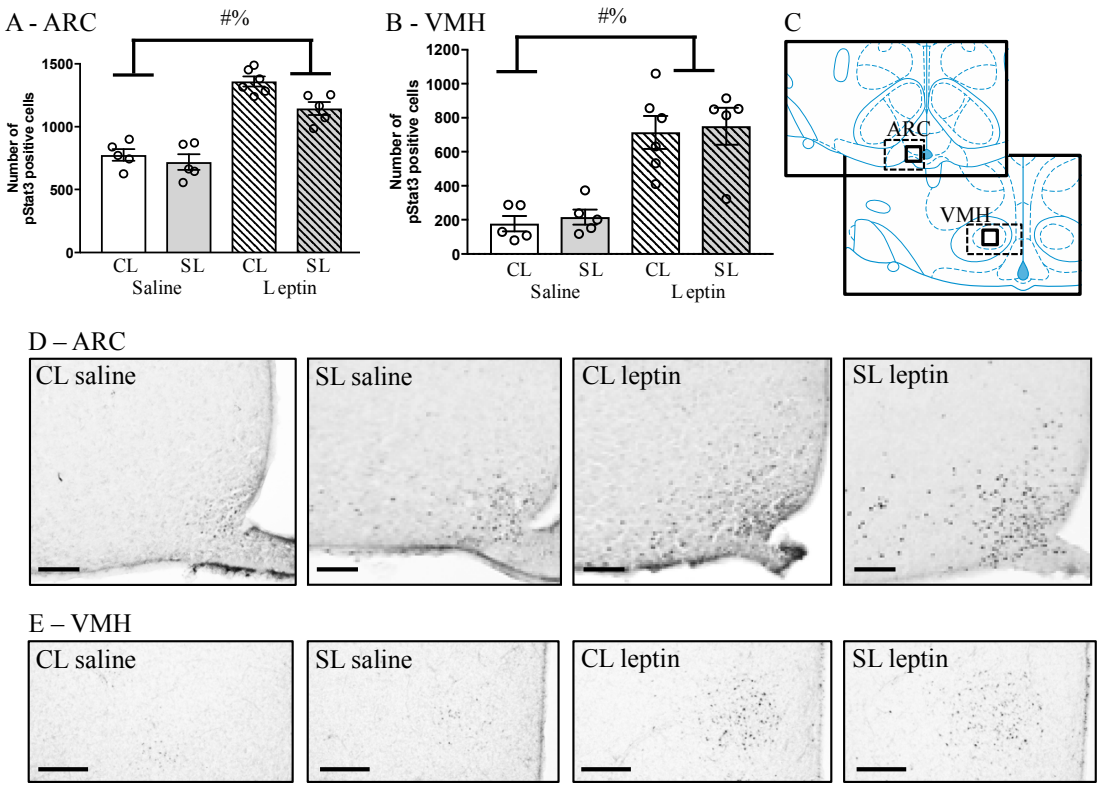


**Figure 4.4 Neonatal overfeeding effects on neonatal hypothalamic responses to leptin.**
(A) Arcuate nucleus of the hypothalamus (ARC) and (B) ventromedial hypothalamus (VMH) neuronal activation in response to leptin injection at postnatal day (P) 12 in control (CL) and small litter (SL) rats as assessed by numbers of signal transducer and activator of transcription (pSTAT3) positive cells. The sum of cell counts in four sections was plotted. (C) Schematic diagram adapted from Paxinos and Watson illustrating the regions of interest (Paxinos and Watson 2009). Thick-line boxes are representative of analysed regions, dotted-line boxes are representative of the photomicrographs. Representative photomicrographs of pSTAT3 after a saline or leptin injection in the (D) ARC (scale bars = 100 µm) and (E) VMH (scale bars = 200 µm). Data are mean ± S.E.M. $n$ = 5 – 6 per group. # significant main effect of litter size. % significant main effect of leptin. $p < 0.05$.

### *4.1.12 Neonatal overfeeding effects on neonatal circulating triglycerides*

Since neonatal overfeeding has minimal effects on hypothalamic feeding circuitry in female rats despite inducing pronounced neonatal hyperleptinemia we next examined circulating triglyceride levels. Triglycerides have been shown to mediate leptin transport to the brain (Banks et al. 2004). We therefore hypothesized neonatal overfeeding might lead to elevated circulating triglycerides that could reduce leptin

transport to the brain and thus reduce the effects of hyperleptinemia. In this regard, neonatal overfeeding led to an increase in circulating triglyceride levels (significant effect of litter size: $F_{(1, 20)} = 5.43$, $p = 0.030$; n = 6 per group, Figure 4.5), without a main effect of age.

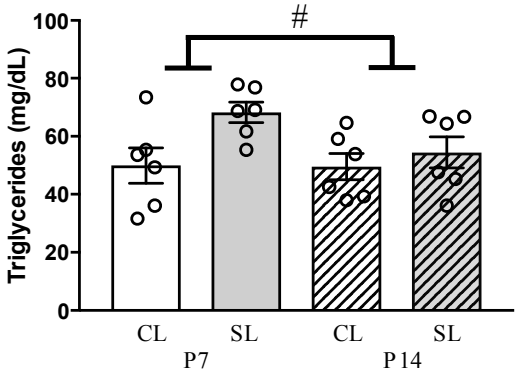


**Figure 4.5 Neonatal overfeeding effects on neonatal circulating triglycerides.** Plasma triglyceride concentration at P7 and P14. Data are mean ± S.E.M. n = 6 – 10 per group. # significant main effect of litter size. $p < 0.05$.

## Discussion

The early life nutritional environment plays a crucial role in metabolism and neurodevelopment. Here, for the first time, we show that neonatal overfeeding in females, despite hyperleptinemia and a corresponding increased body weight, does not affect NPY, AgRP and POMC mRNA or protein in the hypothalamic circuitry responsible for feeding and metabolic control. These observations are different from findings previously shown in neonatally overfed males, where overfeeding leading to hyperleptinemia and increased body weight are associated with early life disruption of hypothalamic neuronal wiring responsible for metabolic regulation (Sominsky, Ziko, et al. 2017; Collden et al. 2015).

Naturally occurring high circulating levels of leptin are seen at approximately P4 to P16 in mice (Ahima and Hileman 2000) and P4 to P14 in rats (Cottrell et al. 2009)

with a peak at P10 (Delahaye et al. 2008). They then decrease towards adult levels after weaning (Ahima, Prabakaran, and Flier 1998). Such an increase in leptin levels is not associated with acute changes in food intake, but is reflective of leptin's neurodevelopmental role in stimulating the growth of hypothalamic connections between the ARC and other hypothalamic regions that are ultimately responsible for controlling energy balance (Bouret, Draper, and Simerly 2004b). Disruptions to this leptin surge can permanently impact upon the development of these hypothalamic connections and lead to aberrant feeding behaviour and metabolism throughout life (Bouret, Draper, and Simerly 2004a, 2004b). Being suckled in a small litter during the first three weeks of life in mice and rats can similarly disrupt hypothalamic circuitry. Thus, early life overfeeding increases body weight, fat mass, and circulating leptin in comparison to the normally-fed. In males, this hyperleptinemia is associated with a disruption in NPY and AgRP fibres and leads to an obese phenotype that is maintained throughout life (Stefanidis and Spencer 2012; Plagemann, Harder, Rake, et al. 1999; Plagemann et al. 2009; Lopez et al. 2007).

It is important to note that most of the current knowledge in regards to the effects of postnatal overfeeding on hypothalamic neuronal development derives from observations in male rodents. However, changes in the neonatal leptin availability as a result of overfeeding appear to induce sex-dependent effects on the development of hypothalamic neuronal connectivity. We observe here that in females, similarly to males, neonatal overfeeding is associated with exacerbated hyperleptinemia during the neonatal leptin surge period, and this is followed by an increase in body weight. However, neonatally overfed females are still responsive to leptin and, importantly, there is no effect of female neonatal overfeeding on the mRNA levels of the leptin

receptor, or in gene or protein expression of NPY, AgRP, or POMC in the hypothalamus. The ARC of small litter animals showed decreased sensitivity to leptin, although there was no interaction between litter size and leptin. However, the magnitude of this difference is small, of the order of 15% of the response in the case of the leptin-treated. We also do not see this difference in the VMH. Is observed here that neonatally overfed females have increased circulating triglyceride levels relative to controls. It is thus possible that hyperleptinemia in females is compensated for by elevated blood triglycerides restricting leptin's access to the brain in the neonatally overfed (Banks et al. 2004), which may also account for the minor reduction in the number of pSTAT3 positive cells in the ARC of these animals. However, this observation does not explain why males are not also resistant to the effects of excess leptin, since we would expect males to also have increased circulating triglyceride levels when neonatally overfed. There is some evidence of sex differences in triglyceride levels in adults after an early food restriction. For instance, there are elevated triglycerides in perinatally food-restricted females relative to males (Lee et al. 2013), while other perinatal insults, such as maternal deprivation, have been shown to increase circulating triglycerides in adult males, but decrease them in females (Mela et al. 2016). However, no direct sex comparison has yet been made during the neonatal phase.

Previous results from our group have demonstrated sex differences in the way that neonatally overfed rats control energy expenditure. Female rats that are overfed as neonates remain obese into adulthood and they do this not by overeating, but by reducing energy expenditure, probably due to reduced activation of brown adipose tissue (BAT) during the first half of the dark phase (Stefanidis and Spencer 2012).

BAT is responsible for the conversion of energy from food into heat primarily via uncoupling protein in mitochondria (Commins et al. 1999), and neonatally overfed females are unable to optimally convert BAT into energy and show reduced energy consumption at the juvenile stage, with normalisation of function in adulthood. BAT uncoupling protein is capable of short-circuiting the electron transport chain and allowing for conversion of mitochondrial membrane potential to heat. This disruption of BAT function at the juvenile stage is not evident in the neonatally overfed males (Stefanidis and Spencer 2012). BAT thermogenesis is reduced in neonatally overfed females until P30 but by adulthood BAT thermogenesis normalises to controls levels (Stefanidis and Spencer 2012); a possible explanation for how these females retain an elevated body weight without any changes in their hypothalamic feeding networks.

Another potential reason for these sex-dependent effects of neonatal overfeeding may lie in the way the male and female brain is wired. Sex steroid hormones such as estrogen and testosterone, in combination with neurotrophins, regulate formation of sexually dimorphic circuits by affecting axonal guidance and synaptogenesis (reviewed in (McCarthy and Arnold 2011)). Significant hormonal changes in the central nervous system in rodents happen partly due to the gonadal steroid hormones during sexual differentiation (Ikeda et al. 2003). For instance, sexually dimorphic nucleus, a cluster of cells in the preoptic area of the hypothalamus responsible for controlling sexual behaviour by affecting sex hormones such as testosterone and estrogens in rats, develops from as early as P1. By P8 this region is almost twice the size in males as that of females (reviewed in (He et al. 2013)). Conversely, the anteroventral periventricular nucleus, a region of the hypothalamus responsible for the pulsatile release of gonadotropin releasing hormone, covers a larger area and consists

of a larger number of neurons in females than in males (reviewed in (McCarthy and Arnold 2011)). Testosterone and estrogens influence energy homeostasis at least partially via hormone receptors, which are co-localised with hunger or satiety neuropeptides located in the hypothalamus (Frank, Brown, and Clegg 2014). This suggests that sexual dimorphism affects the development of hypothalamic regions that control energy balance, and thus may also be reflected in the differential effects of neonatal overfeeding on hypothalamic appetite-regulatory circuitry.

Overall our results suggest females overfed during early life, despite being hyperleptinemic and experiencing an obesogenic phenotype, are not acutely affected in their central neuronal connectivity responsible for metabolic control. These results contrast with our and others' previous findings in males. These findings are potentially reflective of differences in how females and males adapt to early life environmental dietary challenges. Our work further highlights that it is important not to assume female physiology from male data and that different physiological mechanisms may lead to a similar phenotypic outcome, in this case excess body weight.

# Chapter 5

# Neonatal overfeeding disrupts the pituitary ghrelin system in female rats long term; implications for the stress response

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PLOS One. 2017; 12(3):e0173498.

## Introduction

In the previous chapters (Chapters 2-4) we showed that in males, an early life overnutritional environment was associated with peripheral and central alterations to the leptin and ghrelin systems. However, neonatally overfed females despite their obese phenotype, were not affected in terms of the central neuronal connectivity responsible for metabolic control. Despite these findings, the female hypothalamus is still likely to be vulnerable to early life challenge since we have previously shown that females exposed to an over-nutritional environment during the first weeks of life have exacerbated responses to psychological stress later in life. Notably, such responses are unaffected in males (Spencer and Tilbrook 2009).

Both physical and psychological stressors rapidly activate the hypothalamic-pituitary-adrenal (HPA) axis by stimulating release of corticotropin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus (PVN; the apex of the HPA axis). CRH stimulates the anterior pituitary into synthesizing adrenocorticotropic hormone (ACTH), which in return acts via melanocortin 2 receptors in the adrenal cortex to stimulate and secrete glucocorticoids such as corticosterone and cortisol in rodents and humans, respectively. Glucocorticoids negatively feed back at the hypothalamus, hippocampus and pituitary to inhibit secretion of CRH and ACTH and therefore stop further activation of the HPA axis (Papadimitriou and Priftis 2009). Human studies have shown that dysregulation of the HPA axis, especially during development, has been strongly linked to long-term disease risk (Reynolds 2013; Edwards et al. 1993). For instance, low birth weight due to an adverse prenatal environment is associated with increased HPA axis reactivity including increased morning cortisol levels, increased cortisol responses to ACTH challenge,

cardiovascular, metabolic and cognitive disorders long-term (Reynolds 2013; Reynolds et al. 2001). Moreover, girls and women are more likely to display greater stress reactivity than boys and men. As such, preterm females have higher morning cortisol levels compared to preterm and full-term males (Quesada et al. 2014). Likewise, in a study from Yong Ping *et al*, prenatal maternal stress programs greater stress responsivity, as evidenced by increased salivary cortisol after a brief maternal separation in female toddlers compared to male toddlers (Yong Ping et al. 2015). These findings indicate that it is particularly important to consider sex differences in HPA axis responses to stress.

Ghrelin, along with its roles in regulating energy balance and neurodevelopment, has recently been shown to be involved in HPA axis regulation (as reviewed in (Spencer et al. 2015)). Ghrelin is modulated by exposure to stressful situations (Lutter et al. 2008; Zheng et al. 2009). Under stress, circulating ghrelin levels are highly increased, possibly via sympathetic activation of gut ghrelin cells (Mundinger, Cummings, and Taborsky 2006). High ghrelin levels due to ghrelin subcutaneous injection or calorie restriction are associated with anxiolytic-like behaviours, which in turn may help the animals to cope with stress (Lutter et al. 2008; Spencer et al. 2012). Furthermore, ghrelin affects the HPA axis at all its levels. In the hypothalamus, ghrelin indirectly activates the PVN (Cabral et al. 2012); in the anterior pituitary we have shown that ghrelin stimulates ACTH release by recruiting the growth hormone secretagogue receptor (GHSR) (Spencer et al. 2012); and in the adrenal gland ghrelin enhances adrenal cortical cell proliferation (Andreis et al. 2003).

It remains largely unknown how the postnatal nutritional environment affects ghrelin's ability to regulate the HPA axis during development and long-term and whether these effects are sex-dependent. We ((Sominsky, Ziko, Nguyen, et al. 2016) and Chapter 2) and others (Collden et al. 2015) have previously shown that neonatal overfeeding disrupts the ghrelin system in males. However, the effects of early life overnutrition on the ghrelin system and ghrelin's ability to control the HPA axis in females are currently unknown. Here we hypothesised that neonatal overfeeding affects ghrelin's ability to regulate the HPA axis in females and consequently their responses to stress. To test this hypothesis we assessed ghrelin's ability to signal the hypothalamus and the pituitary gland in neonatally overfed female rats. We observed that neonatal overfeeding had no effect on ghrelin signaling in the hypothalamus, however it significantly suppressed the ability of ghrelin to stimulate the pituitary gland, which may be a contributing factor to the exacerbated HPA axis responses to stress we see in these females.

## Materials and methods

### *5.1.1 Animals*

As described in Chapter 2, we obtained timed pregnant Wistar rats from the Animal Resources Centre, WA, Australia. On arrival at the RMIT University Animal Facility, we housed them at 22°C on a 12 hr light/dark cycle (07:00–19:00 h) and provided them with standard pelleted rat chow and water *ad libitum*. We conducted all procedures in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals and RMIT University Animal Ethics Committee approval.

### *5.1.2 Litter size manipulation*

To derive our neonatally overfed animal model we performed manipulation of litter sizes as previously described in Chapter 2 (Materials and methods: "*Litter size manipulation*"). In this study, the animals were culled at postnatal day (P) 7, 12, 14 and at ~P70. The animals that were allowed to grow to adulthood were separated into same sex littermate pairs at weaning (P21) and were fed a standard chow diet until experimentation. The experimental groups were derived from 3 or more litters. Females only were used in these experiments, results collected from males were published in (Sominsky, Ziko, Nguyen, et al. 2016) and reported in Chapters 2 and 3.

### *5.1.3 Ghrelin assays*

Female animals were used in these experiments on P7, P14 and ~P70. Animals were euthanised and blood was collected and processed as previously described in Chapter 3 (Materials and Methods: "*Neonatal overfeeding effects on circulating ghrelin"*).

### *5.1.4 Real-time qPCR analysis*

To assess neonatal overfeeding effects on genes involved in ghrelin-sensitive central pathways we collected pituitaries and brains from which hypothalami were dissected. Samples were processed as previously described in Chapter 2 (Materials and Methods: "*Effects of neonatal overfeeding on hypothalamic gene expression*") using the primers described in Table 5.1.

**Table 5.1 Primer details for qRT-PCR**

| Primer name | NCBI Reference Sequence | TaqMan Assay ID | Product size |
|---|---|---|---|
| *18s* | X03205.1 | 4319413E | 187 |
| *Ghsr* | NM_032075.3 | Rn00821417_m1 | 61 |
| *Mboat4* | NM_001107317.2 | Rn02079102_s1 | 93 |

### *5.1.5 Hypothalamic responsiveness to exogenous ghrelin*

To assess if neonatal overfeeding affects hypothalamic responsiveness to exogenous ghrelin, we injected 1 mg/kg of acyl ghrelin (AG), des-acyl ghrelin (DAG) or saline to control (CL) and small litter (SL) animals on P12. 2 hr post-injection the animals were deeply anaesthetized then transcardially perfused as previously described in Chapter 2 (Materials and methods: "*Brain collection"*) and the tissue was processed for c-Fos immunohistochemistry as described in Chapter 3 (Materials and Methods: "*Neonatal overfeeding effects on neuronal activation in response to ghrelin c-Fos immunohistochemistry"*).

### *5.1.6 Neonatal overfeeding affects pituitary responses to AG and CRH in vitro*

To assess the *in vitro* effects of AG and CRH on the anterior pituitary release of GH and ACTH, we excised the anterior pituitaries from CL and SL adult female rats and stored them in ice-cold Dulbecco's modified Eagle's medium/Nutrient mixture F-12 (DMEM/F-12; Thermo Fisher Scientific, Scoresby, Victoria, Australia) containing 0.1% bovine serum albumin (BSA) until all tissues were collected. Each anterior pituitary was bisected, weighed and pre-incubated for two x 1 hr incubations in 1 mL of DMEM/F-12 at 37 °C in a 95% $O_2$/5% $CO_2$ atmosphere. After the pre-incubation period, we refreshed the medium and collected samples every 15 min for 1 hr to

obtain basal release profiles of growth hormone (GH) and ACTH from the anterior pituitary. To assess the pituitary responsiveness to secretagogue stimuli, AG ($10^{-6}$ M) and CRH (100 ng/mL)-containing media were added in the second fraction. After each 15 min interval, the medium was collected and stored at -20 °C until assayed. GH and ACTH levels were assessed by ELISAs following the manufacturers' instructions. Intra assay variability for the GH ELISA (Millipore, Ballerica, MA, USA) was 1.7 - 4.3% coefficient of variation (CV), inter assay variability was 3.2 – 4.9% CV and the lowest level of detection was 0.07 ng/mL. For the ACTH ELISA (MD Biosciences, St. Paul, MN, USA), intra-assay variability was 3.1 - 4.2% CV, inter-assay variability, 5.8 - 6.2% CV, and lowest limit of detection, 0.46 pg/mL. Samples from all treatment groups were assayed together in duplicate. Data are expressed as percentage of the basal ACTH or GH secretion as measured at the end of the first 15 min period, and set to 100% as previously described in (Cai et al. 2016) and Figure 5.1 (Dr Luba Sominsky assisted me with running the *in vitro* studies and she conducted the ELISAs, which were analysed by me).

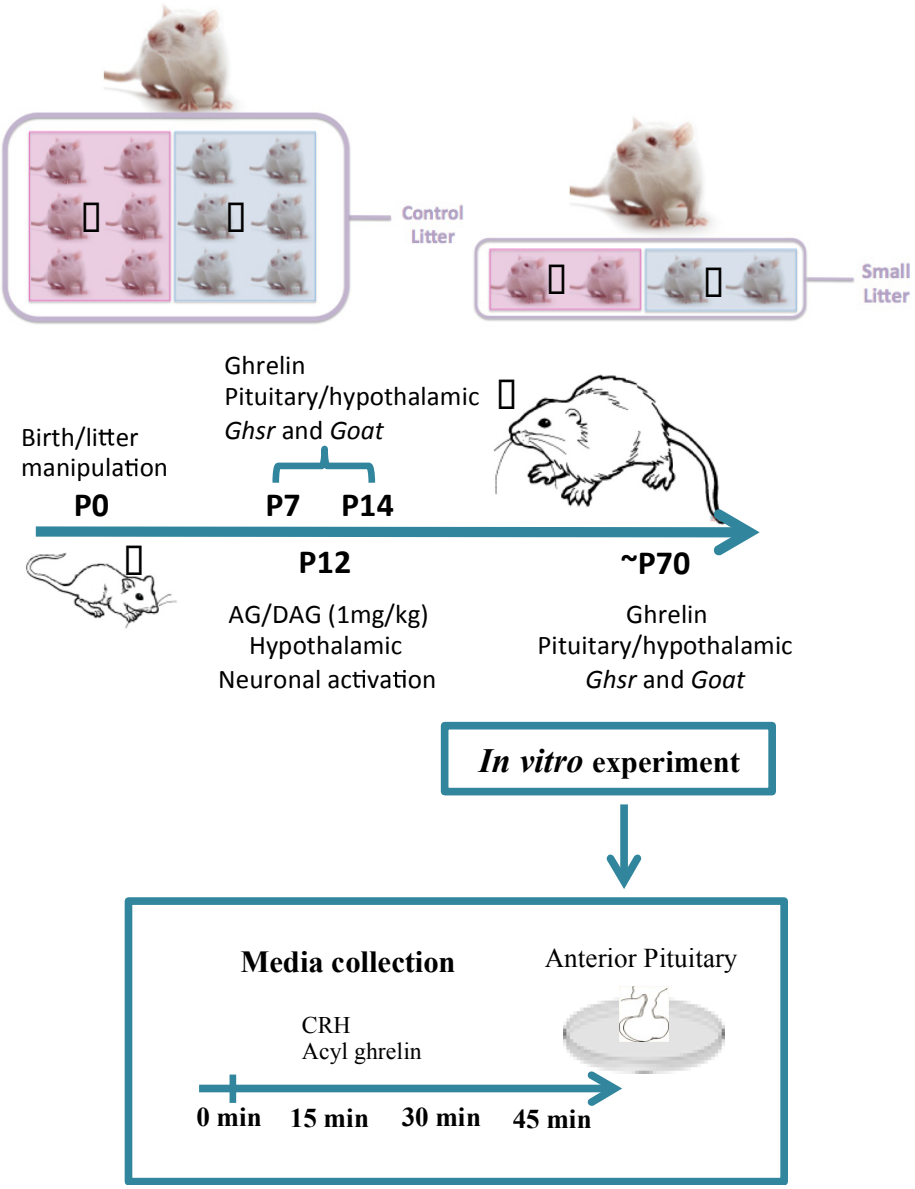



**Figure 5.1 Study design.**
At postnatal day (P) 0 litter size manipulation was conducted and 12 pups (6 males and 6 females) were allocated to dams to form a control litter and 4 pups (2 males and 2 females) to serve as a small litter. Only female offspring were used in this study. At P7, P14 and ~P70 circulating ghrelin concentration, hypothalamic and pituitary mRNA expression of growth hormone secretagogue receptor (*Ghsr*) and ghrelin *O*-acyl transferase (*Goat*) were measured in CL and SL animals. At P12, hypothalamic responsiveness to exogenous acyl ghrelin (AG), des acyl ghrelin (DAG) or saline was assessed. Another cohort of CL and SL adult animals was used for the *in vitro* study. After collection of fraction 1 (0 min), pituitaries were either stimulated with corticotropin releasing hormone (CRH: $10^{-10}$ M) or AG ($10^{-6}$ M); or left unstimulated with media only being refreshed and collected every 15 min. Pituitary growth hormone (GH) and adrenocorticotropic hormone (ACTH) secretion were measured in the media collected every 15 min (for 45 min).

### *5.1.7 Data analysis*

Neonatal and adult data were analysed separately in this study. Neonatal overfeeding effects on ghrelin, gene expression and hypothalamic responses to ghrelin in early life were analysed using multifactorial analysis of variance (ANOVA)s with litter size (CL/SL), age (P7/P14) or treatment (saline/ghrelin) as between factors where appropriate. At P12 data analysis was conducted using Student's unpaired t-tests. For adults we used Student's unpaired t-tests or two-way ANOVAs. When significant interactions were found, Tukey *post hoc* tests were performed. $p$ values of less than 0.05 were considered to be statistically significant. We used repeated measures ANOVAs to analyse *in vitro* pituitary GH and ACTH with AG/CRH and litter size as between factors, and time as the repeated measure. When the assumption of sphericity was violated, we used the Greenhouse-Geisser correction. A Bonferroni correction was applied to adjust for multiple comparisons. We followed this analysis by two-way ANOVA and Tukey *post hoc* tests, where significant interactions were found ($p < 0.05$). Data are presented as the mean ± SEM. All data were tested for homogeneity of variance and normality, using the Levene's test for Equality of Variance and the Shapiro-Wilks test, respectively, complemented by the assessment of skewness and kurtosis. These assessments and all other statistical analyses were conducted using SPSS. Outliers were determined using the Grubbs' test ($\alpha = 0.05$) in GraphPad Prism.

## Results

### *5.1.8 Neonatal overfeeding effects on the female ghrelin system in early life*

It has been previously demonstrated in females and males ((Spencer and Tilbrook 2009; Stefanidis and Spencer 2012; Sominsky, Ziko, Soch, et al. 2016) and Chapter 2, 3 and 4), and here that neonatal overfeeding is associated with significantly increased

body weight at P12 ($t_{(9)}$ = 3.89, $p$ = 0.004, Figure 5.2 A). However, no effects of neonatal overfeeding on circulating total, AG or DAG levels were observed in P7 or P14 female pups. There was a significant litter size by age interaction so that P14 control, but not neonatally overfed animals had increased DAG levels compared to P7 (significant litter size by age interaction, $F_{(1,26)}$ = 4.48, $p$ = 0.044, Figure 5.2 D). Neonatal overfeeding did not affect the hypothalamic gene expression of growth hormone secretagogue receptor (*Ghsr*) or ghrelin *O*-acyl transferase (*Goat*; Figure 5.2 F). However, there was an increase in *Ghsr* expression on P14 compared to P7, irrespective of litter size (significant effect of age; $F_{(1,27)}$ = 42.82, $p$ < 0.001, Figure 5.2 E).

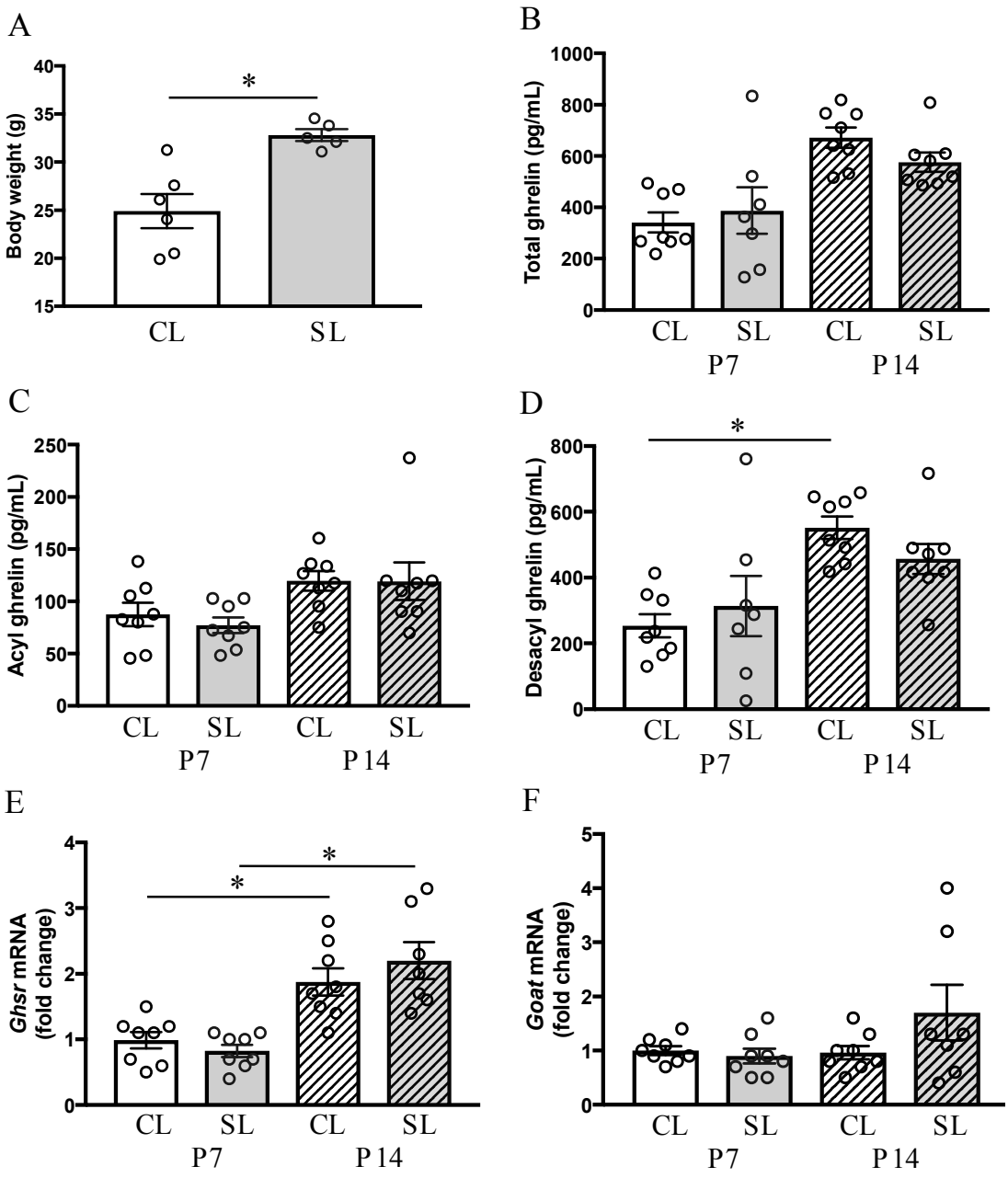



**Figure 5.2 Neonatal overfeeding effects on ghrelin system short-term.**
(A) Body weights in control (CL) and small litter (SL) female animals at P12. (B) Circulating total ghrelin, (C) acyl ghrelin and (D) des-acyl ghrelin at postnatal day (P) 7 and P14 in CL and SL animals. Hypothalamic gene expression of (E) growth hormone secretagogue receptor (*Ghsr*) and (F) ghrelin *O*-acyl transferase (*Goat*) in CL and SL animals at P7 and P14. Data are mean ± SEM. * $p < 0.05$. $n = 5 – 8$ per group.

We also assessed neonatal overfeeding effects on hypothalamic responsiveness to exogenous AG, DAG or saline in P12 females. Neonatal overfeeding had no effect on

ARC neuronal activation as shown by similar numbers of c-Fos positive cells activated in the ARC of CL and SL animals in response to exogenous ghrelin (Figure 5.3 A). In the PVN, neonatal overfeeding also failed to affect hypothalamic responsiveness to exogenous ghrelin, however AG (significant effect of ghrelin; $F_{(2,31)} = 16.45$, $p < 0.001$), but not DAG, induced a significant increase in neuronal activation compared to saline and DAG injected animals, irrespective of litter size (Figure 5.3 B, C). It is important to note that here was used the same AG dosage that induced a significant increase in PVN neuronal activation in SL males compared to CL males (Chapter 3, Figure 3.3 B).

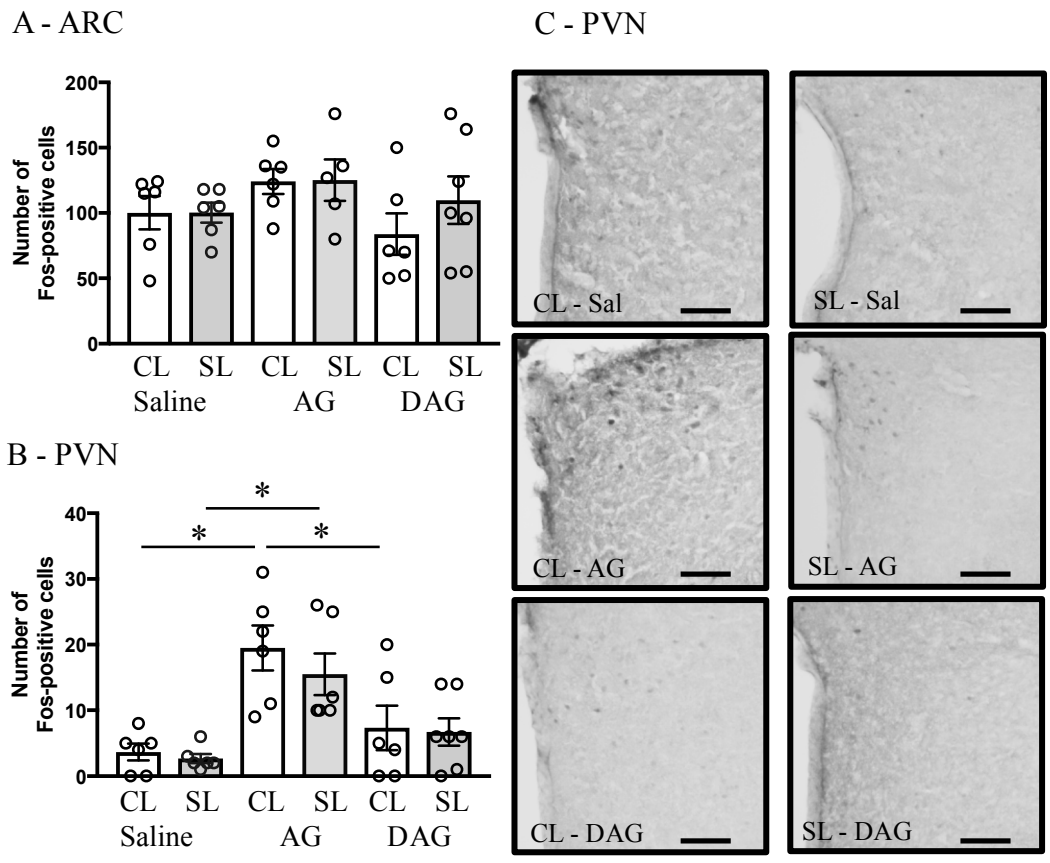



**Figure 5.3 Early life overfeeding effects on hypothalamic responsiveness to exogenous acyl ghrelin (AG), des-acyl ghrelin (DAG) or saline.**
Number of c-Fos positive cells activated by AG, DAG or saline in control (CL) and small litter (SL) females at postnatal day (P) 12 in the (A) arcuate nucleus of the hypothalamus (ARC) and (B) paraventricular nucleus of the hypothalamus (PVN).

(C) Photomicrographs of the PVN. Scale bar = 100 μm. Data are mean ± SEM. * $p <$ 0.05.

### *5.1.9 Long-term effects of neonatal overfeeding on the ghrelin system in females*

To assess peripheral and central effects of neonatal overfeeding on the ghrelin system long-term we measured circulating ghrelin, hypothalamic gene expression of *Ghsr* and *Goat,* as well as body weights. Neonatal overfeeding did not affect circulating ghrelin levels in adult females (Figure 5.4 A) or hypothalamic *Ghsr* and *Goat* gene expression (Figure 5.4 B, C). However, the body weight of neonatally overfed animals remained significantly increased in adulthood relative to controls ($t_{(10)} = 4.36$, $p = 0.001$; Figure 5.4 D). These results indicate that long-term increased body weight with neonatal overfeeding is not likely due to the central ghrelin effects.

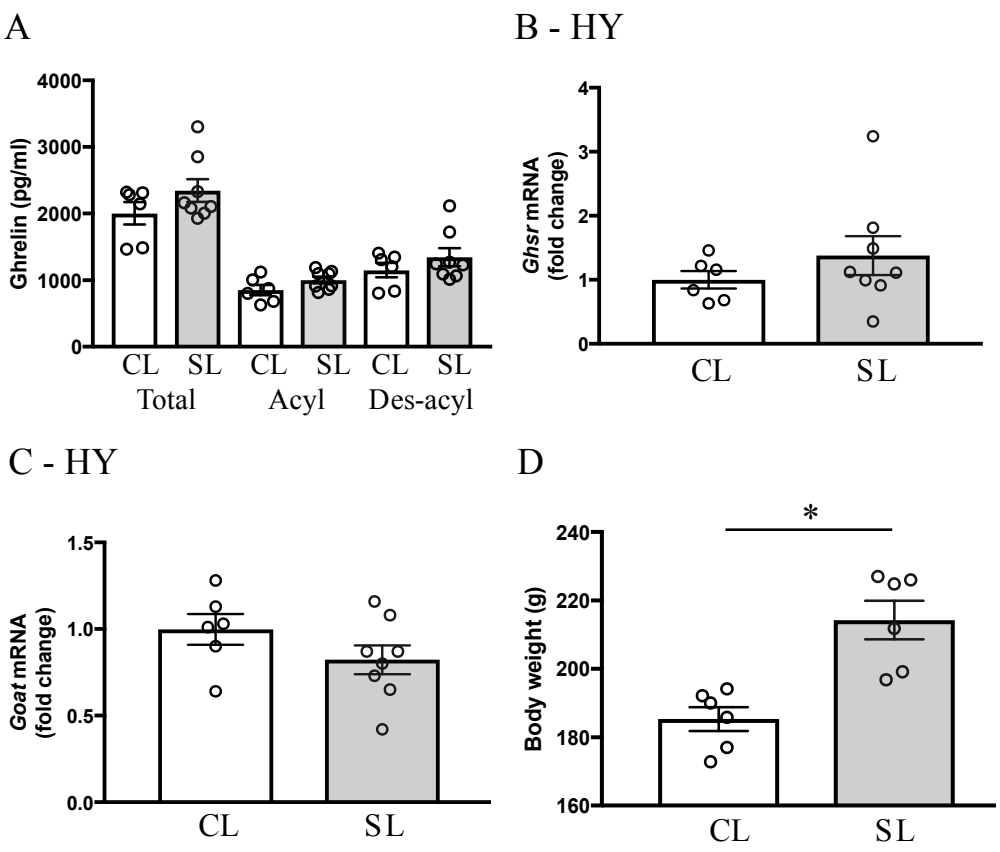



**Figure 5.4 Long-term effects of neonatal overfeeding on the ghrelin system.**
(A) Circulating total, acyl and des-acyl ghrelin in control (CL) and small litter (SL) female adult rats. Gene expression of hypothalamic (B) growth hormone secretagogue receptor (*Ghsr*) and (C) ghrelin *O*-acyl transferase (*Goat)* of CL and SL animals. (D) Body weights of CL and SL adult females. Data are mean ± SEM. * $p < 0.05$. $n = 5 – 7$ per group.

### *5.1.10 Acute and long-term effects of neonatal overfeeding on pituitary ghrelin signaling*

To assess overfeeding effects on ghrelin signaling at the pituitary level we measured gene expression of pituitary *Ghsr* and *Goat*. In the juveniles, was observed that neonatal overfeeding significantly increased pituitary *Ghsr* at P14 relative to normal feeding (significant effect of litter; $F_{(1,25)} = 6.89$, $p = 0.015$, Figure 5.5 A). There was no effect of overfeeding on pituitary *Goat* gene expression in juveniles (Figure 5.5 B). In the adults, neonatal overfeeding significantly suppressed pituitary *Ghsr* ($t_{(12)} = 2.29$, $p = 0.041$, Figure 5.5 C) and *Goat* ($t_{(12)} = 2.61$, $p = 0.023$, Figure 5.5 D) gene

expression. These results indicate that females that are overfed during the neonatal period may have a suppressed ability to respond to ghrelin at the pituitary level in adulthood.

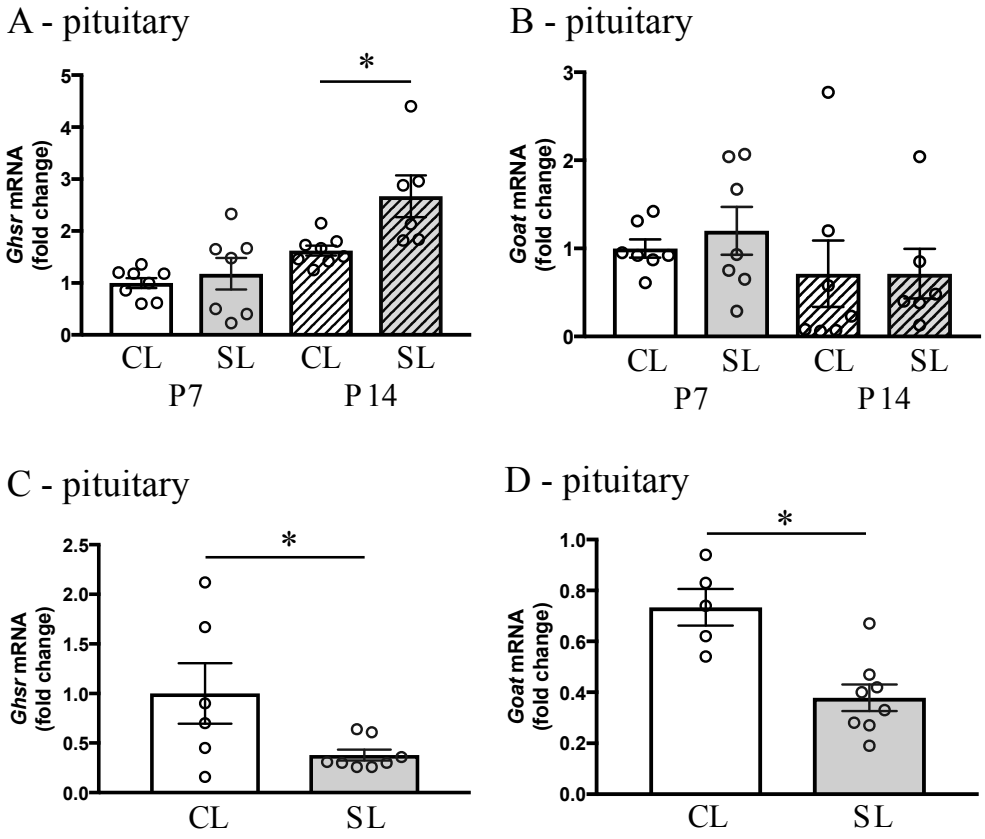



**Figure 5.5 Acute and long-term effects of neonatal overfeeding on potential for pituitary ghrelin signaling.**
Pituitary (A) growth hormone secretagogue receptor (*Ghsr)* and (B) ghrelin *O*-acyl transferase (*Goat*) gene expression at postnatal day (P) 7 and P14 of control (CL) and small litter (SL) female rats. Adult *Ghsr* (C) and *Goat* (D) gene expression at the pituitary of CL and SL rats. Data are mean ± SEM. * $p < 0.05$. $n = 5 – 8$ per group.

### *5.1.11 Pituitary responses to AG and CRH stimulation in vitro*

To test whether neonatal overfeeding affects the ability of the pituitary to release GH and ACTH, we first measured GH and ACTH release from CL and SL non-stimulated adult anterior pituitaries. Under basal conditions both control and neonatally overfed pituitaries released similar levels of GH (Figure 5.6 A) and ACTH (Figure 5.6 B).

When stimulated with AG, CL and SL pituitaries again released similar levels of GH (Figure 5.6 C). However, pituitaries from neonatally overfed rats that were stimulated with AG showed significantly suppressed levels of ACTH secretion at 15 and 30 min time-points relative to controls given AG (Figure 5.6 D), a suppression that was not observed after stimulation with CRH (Figure 5.6 E). The above results indicate that neonatal overfeeding affects the pituitary's ability to respond to AG without affecting its responses to CRH.

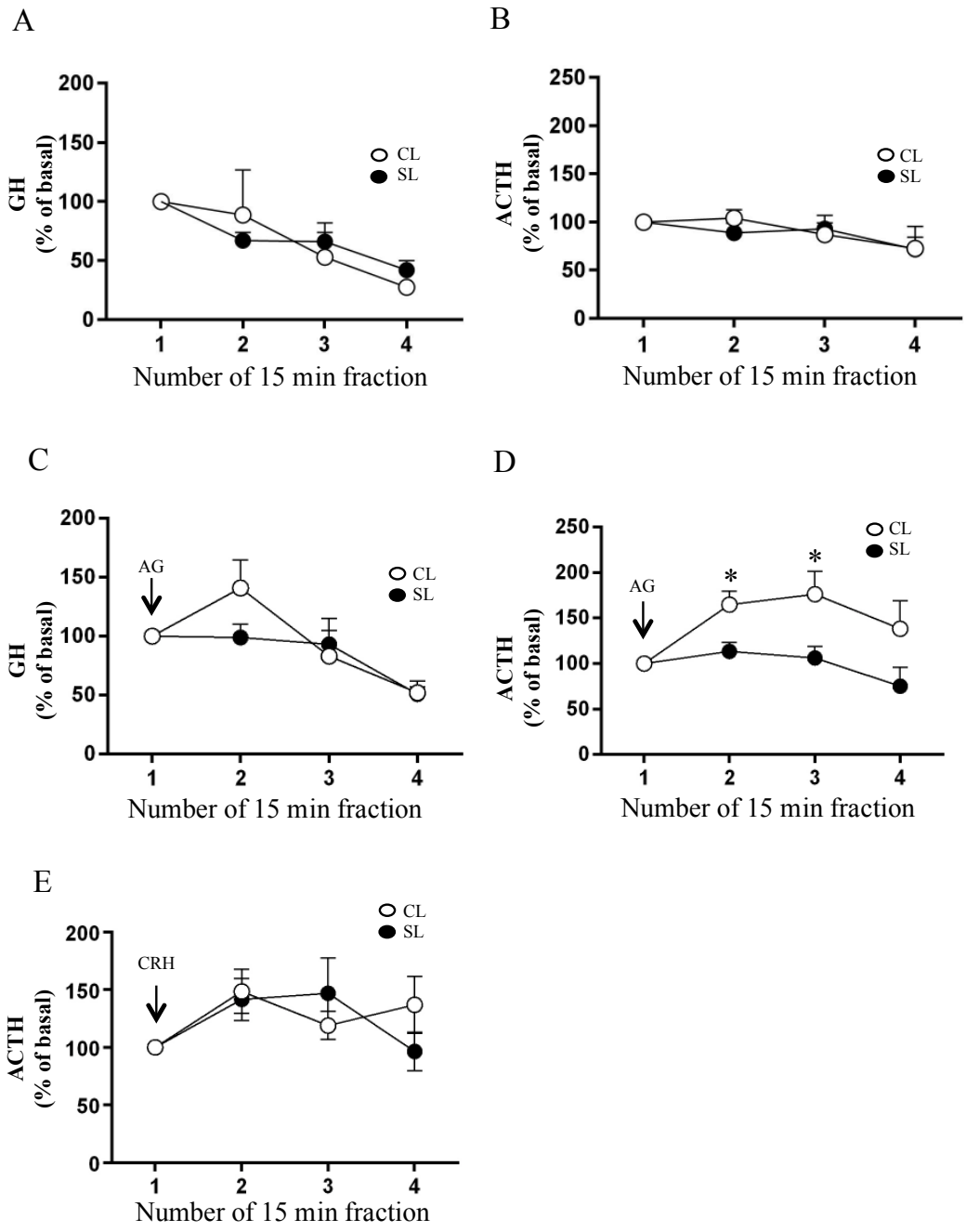



**Figure 5.6 Neonatal overfeeding effects on adult female pituitary responsiveness to acyl ghrelin (AG) and corticotropic-releasing hormone (CRH).**
(A) Pituitary growth hormone (GH) release under basal conditions from control litter (CL) and small litter (SL) animals. (B) Basal adrenocorticotropic hormone (ACTH) release from pituitaries of CL and SL animals. (C) AG-induced GH release from CL and SL pituitaries. (D) AG-induced ACTH release from CL and SL pituitaries. (E) CRH-induced ACTH release from CL and SL pituitaries. Data are mean ± SEM. * $p < 0.05$. $n = 4 – 11$ per group.

## Discussion

We have shown in this study that neonatal overfeeding in females, in contrast to findings previously shown in males, did not affect circulating ghrelin or the hypothalamic responsiveness to exogenous ghrelin in the neonatal or adult periods. However it alters long-term pituitary *Ghsr* and *Goat* expression. In an *in vitro* setting, neonatal overfeeding did not affect basal or AG-stimulated GH secretion from the pituitary. It also did not affect basal or CRH-induced ACTH secretion. However, neonatal overfeeding significantly suppressed pituitary ACTH's responsiveness to AG indicating that early life nutritional environment permanently affects the female pituitary ghrelin system and consequently ghrelin-induced HPA axis function.

Ghrelin, traditionally known for its role in centrally controlling energy metabolism, is the only peripherally produced peptide that induces feeding (Nakazato et al. 2001; Wren et al. 2001). It does this, in part, by centrally activating ARC NPY/AgRP neurons via GHSR expressed by these neurons, and inactivating POMC/CART by initiating gamma-aminobutyric acid (GABA) release from NPY/AgRP/GABA neurons. GABA directly inhibits nearby POMC/CART neurons (reviewed in (Edwards and Abizaid 2017)). Ghrelin also induces feeding by acting at the PVN, where it initiates AgRP secretion, which antagonizes α-melanocyte-stimulating hormone (α-MSH) and β-MSH at melanocortin receptors to abolish anorectic effects from this region (Aponte, Atasoy, and Sternson 2011). Cabral *et al*. have demonstrated that ghrelin indirectly activates PVN CRH neurons, which lack GHSR, consequently activating the HPA axis (Cabral et al. 2012). The mechanism by which ghrelin activates PVN CRH neurons does not necessarily require an intact ARC (Cabral et al. 2016). Studies from our group and others have shown that neonatal

overfeeding at least in males is associated with a disruption of hypothalamic *Ghsr* expression (Soares et al. 2012; Collden et al. 2015; Sominsky, Ziko, Nguyen, et al. 2016). In the present study we show that neonatally overfed females however, in contrast to males, display similar hypothalamic *Ghsr* and *Goat* expression compared to normally fed animals, further emphasizing that there are sex-dependent effects of neonatal overfeeding on the ghrelin system and that females are resistant to the ghrelin-perturbing effects of this early life diet, at least in terms of the hypothalamic appetite regulatory circuitry.

The early life nutritional environment is crucial not only for regulating metabolic programming but, also anxiety and stress responses later in life (Delpierre et al. 2016). We have previously observed that neonatal overfeeding affects anxiety and stress responses with overfed females exhibiting reduced anxiety and increased responses to restraint stress compared to normally fed counterparts (Spencer and Tilbrook 2009). We have also shown that under stress conditions ghrelin specifically stimulates pituitary ACTH release in order to activate HPA axis to consequently suppress stress and anxiety (Spencer et al. 2012). Ghrelin's role in the HPA axis regulation, including after exposure to stress, implies that any abnormality in the ghrelin system may be a contributor to the abnormal functioning of the HPA axis (Spencer et al. 2012; Patterson et al. 2013). Ghrelin stimulates secretion of hormones involved in the stress response, including ACTH and glucocorticoids. In humans and rodents, ghrelin stimulates ACTH and glucocorticoid release, indicating it has effects on both the pituitary (Spencer et al. 2012; Shimon, Yan, and Melmed 1998) and adrenal glands (Barreiro et al. 2002; Rucinski et al. 2009; Andreis et al. 2003). The mechanisms by which ghrelin affects stress, anxiety and mood disorders remain to be

fully elucidated. In this regard, recent work from Goosens' laboratory demonstrates that ghrelin and its stress-induced increased circulating levels are involved in stress-associated fear learning. Interestingly, stress-induced increase in ghrelin, specifically AG, persists long after the cessation of the stress exposure, in both rodents and humans. These elevated AG levels in turn mediate prolonged susceptibility to exhibiting stress-enhanced fear behaviours (Yousufzai et al. 2018). These fear-enhancing actions of AG are likely to occur in the amygdala and are independent of the traditional HPA axis activation (Meyer et al. 2014). Another possible mechanism through which ghrelin is likely to modulate stress-related behaviours is through its potential dual role in promoting neophobia under conditions of positive energy balance (i.e. under low ghrelin levels), and anxiolytic effects at high concentrations (Spencer et al. 2012; Lutter et al. 2008). This adaptation might occur in order to promote food-seeking behaviour when food is scarce and minimize exploratory behaviour when not necessary. In the current study we observe that neonatal overfeeding significantly alters pituitary *Ghsr* and *Goat* expression. It exacerbates *Ghsr* during the second week after birth, and then significantly suppresses both pituitary *Ghsr* and *Goat* expression in adulthood. While we have not assessed stress-induced changes in ghrelin or stress responsivity in the current study, these findings suggest that our previously reported exacerbated PVN responses to restraint stress in neonatally overfed females (Spencer and Tilbrook 2009) could at least partly be due to the suppressed ability of the pituitary to respond to stress-induced ghrelin and hence diminished HPA axis negative feedback.

A number of studies have demonstrated sexual dimorphism in circulating ghrelin levels, with females having higher ghrelin than males and ghrelin levels being

inversely related to body mass index (BMI) in girls/women, but not in boys/men (Soriano-Guillen et al. 2016; Makovey et al. 2007). In animal studies, fasting induces higher ghrelin levels in female adult rats compared to males (Gayle et al. 2006). In terms of the impact of the early life environment on the ghrelin system, little is known about the influences of sex. In male rats, studies have revealed that early life overnutrition greatly affects circulating ghrelin (Soares et al. 2012; Collden et al. 2015; Sominsky, Ziko, Nguyen, et al. 2016), but such studies have not been reported in females. Interestingly, we observe here that neonatal overfeeding in females has no effect on the levels of any of the forms of circulating ghrelin (AG or DAG).

Exposure to nutritional challenges during early life development is associated with long-term complications including metabolic diseases in both sexes (reviewed in (Dearden, Bouret, and Ozanne 2018)). However, females in particular are more likely to experience increased adiposity as well as anxiety behaviours compared to males (Sullivan et al. 2010; Samuelsson et al. 2013; Oostvogels et al. 2017). As reviewed in (Carpenter, Grecian, and Reynolds 2017), not only nutrition but also stress during prenatal life in humans is associated with altered HPA axis responses to stress in a sex-specific manner, with females being more vulnerable than males to stressors later in life. As such, there are sex-differences in depression and major depressive disorders (MDD) with women being more vulnerable to depressive disorders compared to men (Goldstein et al. 2016; Kessler et al. 1993), corresponding with our previous findings indicating increased stress responsivity in neonatally overfed female, but not male rats.

In conclusion, our current results suggest that neonatal overfeeding in females, unlike in males, does not affect hypothalamic ghrelin system, however it suppresses the pituitary's ability to respond to ghrelin long-term. These results are important in further understanding the role of ghrelin in the regulation of HPA axis responses to stress in a sex-specific manner.

# Chapter 6

# Acylated ghrelin suppresses the cytokine response to lipopolysaccharide and does so independently of the hypothalamic-pituitary-adrenal axis

**Ilvana Ziko**, Luba Sominsky, Simone N. De Luca, Francis Lelngei and Sarah J. Spencer, Brain Behaviour and Immunity. 2018; pii: S0889-1591(18)30332-5.

## Introduction

In Chapter 5 we looked at the effects of ghrelin in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis in females. Very recently, additional functions for the peptide have come to light, including its anti-inflammatory properties (reviewed in (Baatar, Patel, and Taub 2011)). Thus, ghrelin decreases mortality in septic shock (Chang, Zhao, et al. 2003; Chang, Du, et al. 2003) and morbidity in models of colitis (Gonzalez-Rey, Chorny, and Delgado 2006; Konturek et al. 2009) and other inflammatory challenges (Collden, Tschop, and Muller 2017). Ghrelin likely does this, at least in part, by acting directly on immune cells, including microglia, macrophages and T-cells, to reduce the pro-inflammatory cytokine response to immune challenge (Waseern et al. 2008; Dixit et al. 2004; Moon et al. 2009).

Ghrelin also regulates HPA axis responses to stress and may thus modify cytokine responses to immune challenge via this mechanism. During acute physical or psychological stress, cells in the paraventricular nucleus of the hypothalamus (PVN) secrete corticotropin-releasing hormone (CRH), which acts on the anterior pituitary to stimulate adrenocorticotropic hormone (ACTH) release. Increased circulating ACTH levels stimulate the adrenal cortex to synthesise and secrete glucocorticoids, which then initiate a series of events to aid the organism to cope with the stress (Nicolaides et al. 2015). In addition to its other roles, glucocorticoids also act to suppress nuclear factor (NF)κB-mediated cytokine transcription in immune cells (Auphan et al. 1995; Scheinman et al. 1995). Ghrelin's role in regulating the HPA axis is still imprecisely described. However, exogenous ghrelin strongly activates CRH neurons in the PVN and increases circulating glucocorticoid levels without affecting growth hormone secretagogue receptors (GHSR) expression on these neurons, suggestive of ghrelin's

indirect effect on the apex of the HPA axis (Cabral et al. 2012). Thus, stress induces high levels of glucocorticoid release in proportion with increased circulating ghrelin (Lutter et al. 2008; Azzam et al. 2017). We have shown that ghrelin stimulates the HPA axis at the level of the pituitary by targeting the GHSR to facilitate ACTH release (Spencer et al. 2012). Ghrelin can also directly activate pituitary ACTH expressing cells (Stevanovic et al. 2007). Therefore, we hypothesized here that ghrelin's anti-inflammatory actions on the cytokine response to an immune challenge (with lipopolysaccharide (LPS)) may be mediated by activation of the HPA axis.

As discussed in previous chapters, ghrelin exists in at least two biologically active isoforms: acylated ghrelin (AG), which is the result of ghrelin-o-acyl-transferase (GOAT)-mediated acylation of pro-ghrelin at serine-3; and des-acylated ghrelin (DAG), which is the unacylated and most abundant form. AG confers its activity via GHSR, and is now known to have important roles in feeding, reward, memory, immune responses, cardiovascular activity, reproduction, and a host of other physiological functions and behaviours, including in regulating the stress response (Spencer et al. 2012; Spencer et al. 2015; Sominsky, Hodgson, et al. 2017). AG appears to have a dual role in stress and anxiety (Spencer et al. 2015; Spencer et al. 2012). It likely promotes neophobia and timely responses to stress at low concentrations, such as occur under conditions of positive energy balance, but suppresses anxiety and stress at high concentrations, as occur with negative energy balance (Lutter et al. 2008; Spencer et al. 2012). This fine interplay would ensure that food-seeking behaviour is promoted when necessary, without encouraging unnecessary exploration and risk under high stress conditions if food is not scarce. DAG has been less well investigated, and its receptor(s) has not yet been identified.

However, our own work has recently suggested DAG also contributes to stress responses and can promote anxiety (Stark et al. 2016a). With these studies, we have shown AG and DAG may have opposing roles in regulating hypothalamic circuitry in response to stress. Since DAG is the most abundant form of ghrelin and AG is quickly metabolized to DAG *in vivo* (Bayliss et al. 2016; Chen et al. 2015), elucidating DAG and AG's relative roles in HPA axis function is crucial. Here we hypothesized that AG and DAG would differentially modulate the HPA axis response to an immune challenge with LPS.

To test this, we gave adult male Wistar rats a concomitant injection of LPS and either AG or DAG and assessed circulating cytokine and HPA axis responses. Circulating pro- and anti-inflammatory cytokines stimulated by LPS were significantly suppressed in the presence of AG, but not DAG. DAG also had no effect on any HPA axis component assessed. *In vitro*, high AG stimulated the release of ACTH from the anterior pituitary and, *in vivo,* AG stimulated HPA axis activation, but the *in vivo* ACTH, glucocorticoid, and PVN responses to LPS were not influenced by AG. These data suggest that AG markedly suppresses the circulating cytokine response to LPS but probably does not do this by modulating the HPA axis response.

## Materials and methods

### *6.1.1 Animals*

In these experiments, we used adult male Wistar rats obtained from the Animal Resources Centre, WA, Australia. After arrival at the RMIT University Animal Facility, at approximately postnatal day (P) 63, they were housed at 22 °C on a 12 hr light/dark cycle (0700 – 1900 hr) and acclimatised for approximately one week prior

to the experiment. We provided them with *ad libitum* pelleted rat chow and water. All procedures were conducted in accordance with the National Health and Medical Research Council Australia Code of Practice for the care of experimental animals and RMIT University Animal Ethics Committee approval.

### *6.1.2 Responses to exogenous AG and DAG after an immune challenge with LPS*

To assess how exogenous AG and DAG alter the hypothalamic and extra-hypothalamic components of the HPA axis after an immune challenge with LPS *in vivo,* we gave the rats a single injection of AG or DAG (1 mg/kg s.c.; PolyPeptide Group, Strasbourg, France), combined with a concomitant injection of LPS (*E. coli*, serotype 0127:B8; Sigma, St Louis, MO, USA; 100 μg/kg i.p.) or pyrogen-free saline (0.9% NaCl Figure 6.1). Immediately after lights on (0700), rats were singly housed and fasted for 2.5 hr prior to ghrelin injection in order to allow for endogenous ghrelin levels to normalise without inducing negative energy balance. A pre-weighed amount of rat pelleted chow was given to the animals immediately after AG, DAG or saline injection. At 2 hr post-injection the weight of the remaining food was recorded and the difference between remaining and received taken as the amount consumed. Body weights were also recorded immediately before and 2 hr after injections. All the experiments were conducted between 0900 and 1300 to limit the potential effects of circadian rhythms on any parameters measured.

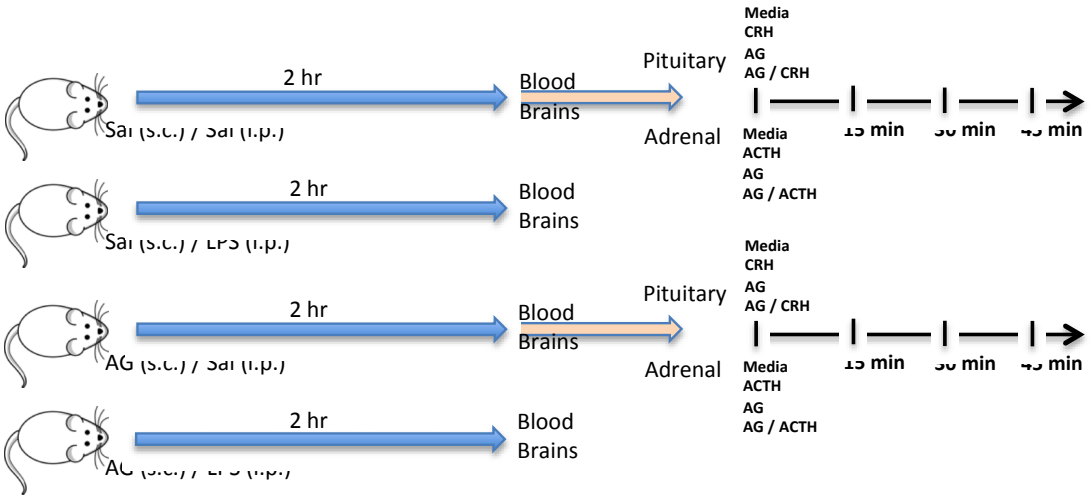



**Figure 6.1 Study design**
Male adult rats were concomitantly injected with LPS and acyl ghrelin (AG), des-acyl ghrelin (DAG) and/or saline and after 2 hr blood and brains were collected. Pituitaries and adrenals from saline and AG injected animals were collected to be stimulated *in vitro* with corticotropin-releasing hormone (CRH; $10^{-10}$ M) and adrenocorticotropic hormone (ACTH; $10^{-7}$ M) accordingly. After collection of fraction 1, AG/DAG ($10^{-6}$ M) alone, the stimulus alone (CRH/ACTH) or the combination of the two were added to the media and samples were collected every 15 min up to 1 hr to test ACTH and corticosterone (CORT) responses of pituitaries and adrenals respectively.

### *6.1.3 Effects of AG and DAG on circulating cytokine responses to LPS*

To assess the effects of ghrelin on peripheral markers of inflammation in response to LPS, we collected cardiac blood into ethylenediaminetetraacetic acid (EDTA) coated tubes, which were kept on ice until the end of the experiment, then centrifuged at 1000 g for 15 min at 4 °C. Plasma was collected, aliquoted and stored at -20 °C until processed for cytokine analysis. Relative concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), interleukin-1α (IL1-α), IL1-β, IL-2, IL-4, IL5, IL-6, IL-10, IL-12, IL-13 and tumor necrosis factor-α (TNFα) in plasma samples were quantified using a 12-plex rat cytokine Bio-Plex Th1/Th2 assay (#171K1002M; Bio-Rad California, USA) and a Bio-Plex MAGPIX™ instrument (Bio-Rad) according to the manufacturer's instructions. Results from samples (in duplicate), blank and standards (in triplicate) were extracted using Bio-

Plex Manager software and analysed by asymmetric sigmoidal, 5PL, X log concentration of mean fluorescence intensity values minus background in Prism (GraphPad Software, Inc.). GM-CSF, IFN-γ, IL-4, IL-12 and IL-13 values are not reported due to their levels being undetectable in this assay.

### *6.1.4 Effects of AG and DAG on pituitary and adrenal gland responses to stimulation in vitro*

To assess the *in vitro* effects of AG and DAG on the anterior pituitary release of ACTH and adrenal gland release of corticosterone (CORT), we excised the anterior pituitaries and the adrenal glands from the AG- and DAG-treated rats that had been given saline only and treated as previously described in Chapter 5 (Materials and methods: *Neonatal overfeeding affects pituitary responses to AG and CRH*). To assess the pituitary responsiveness to secretagogue stimuli, AG or DAG ($10^{-6}$ M) and CRH ($10^{-10}$ M)-containing media were added in the second fraction. To assess the adrenal gland responsiveness to secretagogue stimuli AG or DAG ($10^{-6}$ M) and ACTH ($10^{-7}$ M)-containing media were added in the second fraction. CORT levels were measured with a standard CORT assay (Abnova Corp., Taipei, Taiwan) with intra-assay variability 5.5% CV and inter-assay variability of 10.4% CV. The lower limit of detection for this assay was 0.35 ng/mL. Samples from all treatment groups were assayed together in duplicate. Data are expressed as percentage of the basal ACTH or CORT secretion as measured at the end of the first 15 min period, and set to 100% as previously described in (Cai et al. 2016; Sominsky, Ziko, and Spencer 2017).

### *6.1.5 Effects of exogenous AG and DAG on circulating ghrelin after LPS*

To assess if exogenous AG or DAG had an effect on circulating ghrelin after an immune challenge with LPS we collected cardiac blood for later assessment of serum ghrelin, as well as for plasma CORT and ACTH (assayed as for the media, above). For ghrelin, blood was treated with Pefabloc as previously described in Chapter 3 (Materials and methods: *Neonatal overfeeding effects on circulating ghrelin*).

### *6.1.6 c-Fos immunohistochemistry*

To assess the effects of AG and DAG on hypothalamic neuronal activation after an immune challenge with LPS we examined PVN c-Fos expression. Brains were hemisected into left and right-hand sides. From the left-hand sides, the hypothalami were dissected and immediately snap-frozen in liquid nitrogen to be further processed for mRNA expression analysis. The right-hand side of each brain was immersion-fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS; 4 °C, pH 7.4). These brains were then processed as previously described in Chapter 2, Materials and methods: "*Brain collection"*. Sections throughout the PVN were immunolabelled for c-Fos. Immunofluorescently labeled c-Fos sections were incubated in (1:5 000; ABE 457, MERK Millipore) in a solution of 3% BSA, 0.3% Triton X-100 in PBS with 0.1% Tween-20 for 2 days at 4 °C, after an antigen retrieval (10 mM sodium citrate solution, pH 6.0, 70°C for 10 min) and blocking step (3% BSA, 0.3% Triton X-100, PBS Tween-20, 3 hr at room temperature). Sections were further incubated with secondary antibody (1:500, Alexa-fluor 488 goat anti-rabbit; Thermo Scientific, Rockford, IL, USA) in a solution of 3% BSA, 0.3% Triton X-100, PBS Tween-20 for 2 hr at room temperature. After five washes at 5 min intervals, sections were mounted on slides and coverslipped with DAPI anti-fading

mounting medium (Sigma-Aldrich, St Louis, MS, USA) to be later examined with a confocal microscope. Brain sections were visualised under a 488 nm laser and a 515/30 filter set for c-Fos labeling and under a 408 nm laser for DAPI on an A1 upright confocal laser microscope (Nikon, Tokyo, Japan).

#### *6.1.6.1 Cell counts*

An experimenter blinded to treatment groups assessed PVN sections for the numbers of cells positive for c-Fos. Through-focus series (z-stacks) of PVN images were taken under 20x magnification lenses and one cumulative image of all the z-planes was analysed for each section. c-Fos positive cells were manually counted using ImageJ (National Institutes of Health, Bethesda, MD, USA), which places a number over each counted cell to prevent over- or under- counting. Positive cells were determined by eye, based on the size and round shape, allowing for exclusion of blood vessel staining, which is distinctive (continuous lines of positive staining). Magnocellular (MG) and parvocellular (MP) regions were delineated by overlaying one of two dotted line templates adapted from (Buller, Dayas, and Day 2003) to the images to be analysed, according to the rostrocaudal level. We analysed four sections 120 μm apart between 1.56 and 1.92 mm caudal to the bregma (according to the Paxinos and Watson Rat Brain Atlas (Paxinos and Watson 2009)) for each animal and recorded a mean of c-Fos positive cells from these four sections.

### *6.1.7 Real-time quantitative PCR array*

We used a custom RT2 profiler PCR array (Qiagen, Carlsbad, CA, USA) to examine the mRNA expression from the dissected hypothalami of four genes as in Table 6.1, according to manufacturer's instructions. The RNA (400 ng) was transcribed to

complimentary DNA using an RT2 First Strand Kit (Qiagen). Samples were diluted in RT2 SYBR Green Mastermix, loaded onto 384-well PCR array plates and amplified on an Applied Biosystems™ QuantStudio™ 7 Flex qPCR System instrument (Life Technologies, Carlsbad, Ca, USA). The relative quantitative measure of the target gene expression was compared with an endogenous control, β-actin. RNA expression was determined using the double delta (*C(t)*) equation 2-ΔΔ*C(t)*, where threshold cycle (*C(t)*) values were the values at which fluorescence was first detected significantly above background, as previously described (Sominsky, Ziko, et al. 2017).

**Table 6.1 Gene details for RT-PCR array.**

| Gene symbol | Gene name NCBI | Assay catalog # |
|---|---|---|
| *Actb* | Actin beta | PPR06570 |
| *Nr3c1* | Nuclear receptor subfamily 3 group C member 1 | PPR52805 |
| *Nr3c2* | Nuclear receptor subfamily 3 group C member 2 | PPR44413 |
| *Crhr1* | Corticotropin-releasing hormone receptor 1 | PPR44886 |

### *6.1.8 Statistical Analyses*

Data were analysed using multi factorial analyses of variance (ANOVA)s with immune challenge (saline, LPS) and ghrelin (saline, AG or DAG) treatment as between factors. We used repeated measures ANOVAs to analyse *in vitro* pituitary and adrenal responses to stimulation, with stress hormones (CRH or ACTH, respectively) and AG or DAG treatment as between factors, and time as the repeated measure. When the assumption of sphericity was violated, we used the Greenhouse-Geisser correction. A Bonferroni correction was applied to adjust for multiple

comparisons. We followed the analysis by two-way ANOVAs and Tukey *post hoc* tests, where significant interactions were found. Data are presented as the mean ± SEM. Statistical significance was assumed when $p < 0.05$. All data were tested for homogeneity of variance and normality, using the Levene's test for Equality of Variance and the Shapiro-Wilks test, respectively, complemented by the assessment of skewness and kurtosis. These assessments and all other statistical analyses were conducted using SPSS. Outliers were determined using the Grubbs' test ($\alpha = 0.05$) in GraphPad Prism.

## Results

### *6.1.9 Effects of AG on pituitary and adrenal responses to stimulation*

As previously reported from our group AG stimulates pituitary ACTH release in female rats (Sominsky, Ziko, and Spencer 2017) and in psychologically stressed male mice (Spencer et al. 2012) so we tested if the pituitary ACTH and adrenal CORT response to AG is sensitized in the presence of stress signals CRH and ACTH, respectively. Consistent with our mouse and female rat data, AG treatment robustly affected pituitary ACTH production. CRH at the dose used in these experiments was insufficient to stimulate an increase in pituitary ACTH in the media-alone group. Similarly, there was no effect of CRH and AG combined on ACTH release. However, AG alone stimulated significant ACTH secretion within 15 min. As such, repeated measures ANOVA indicated there were significant fraction x CRH treatment and fraction x AG treatment interactions ($F_{(3,72)} = 5.46$, $p = 0.002$; $F_{(3,72)} = 4.19$, $p = 0.009$), as well as a significant main effect of CRH ($F_{(1,24)} = 6.56$, $p = 0.017$; Figure 6.2 A; n = 6-8). *Post hoc* tests revealed ACTH was increased in the AG-alone group in the 15 min (second) fraction compared with the other groups at this time-point.

ACTH at this dose stimulated an increase in CORT released from the adrenal gland in AG-treated tissue. Repeated measures ANOVA indicated there was a significant fraction x AG treatment interaction ($F_{(3,66)} = 8.08$, $p < 0.001$) and a significant main effect of AG ($F_{(1,22)} = 12.14$, $p = 0.002$; Figure 6.2 B; n = 6-7). *Post hoc* tests revealed CORT was specifically increased in the AG-ACTH group in the 30 and 45 min (third and fourth) fractions compared with the AG-alone group. These data suggest that AG has the capacity to stimulate pituitary ACTH release and to potentiate the effect of ACTH on CORT in male rats but has minimal direct effect on adrenal CORT release.

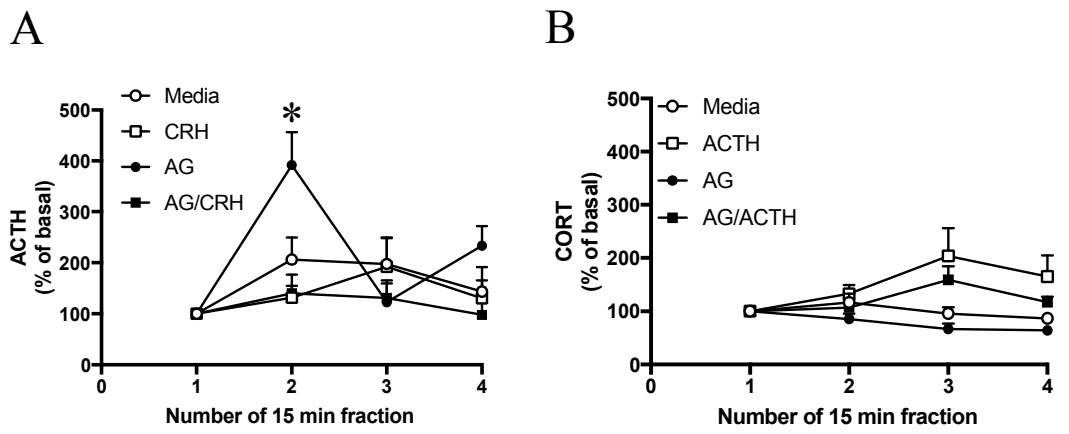



**Figure 6.2 Acyl ghrelin (AG) effects on pituitary and adrenal responses to stimulation *in vitro.***
(A) AG's effect on corticotropin-releasing hormone (CRH)-induced adrenocorticotropic hormone (ACTH) release from anterior pituitaries of adult male rats. AG ($10^{-6}$ M) and CRH ($10^{-10}$ M) were added to the media after collection of fraction 1. B) AG's effect on ACTH-induced corticosterone release from adrenals. Data are mean ± SEM. A) * AG compared with the other groups; $p < 0.05$ (n = 6-8 per group). (B) * ACTH group compared with AG-alone group. $p < 0.05$ (n = 6-7).

### *6.1.10 Effects of exogenous AG on LPS-induced anorexia and circulating cytokines*

To test how AG affects HPA axis responses to immune challenge *in vivo*, we next tested if the pro- and anti-inflammatory cytokine responses to LPS were affected by

elevated AG. Exogenous AG did not significantly affect 2 hr food intake or the anorexic response to LPS (Figure 6.3 A; n = 6-8 per group). However, AG did decrease the levels of several circulating cytokines induced by LPS (n = 5-8 per group). Thus, LPS increased the concentrations of TNFα, IL-1α, IL-1β, and IL-10 under otherwise-untreated conditions, but not when the rats were concomitantly treated with AG. There was a significant interaction between immune challenge and AG on TNFα ($F_{(1,24)}$ = 11.15, $p$ = 0.003; Figure 6.3 B), IL-1α ($F_{(1,23)}$ = 4.31, $p$ = 0.049; Figure 6.3 C) and IL-10 ($F_{(1,27)}$ = 8.55, $p$ = 0.007; Figure 6.3 H) and a significant effect of immune challenge on IL-1β ($F_{(1,22)}$ = 6.34, $p$ = 0.02; Figure 6.3 D). There was no effect of either immune challenge or AG on IL-2 (Figure 6.3 E), IL-5 (Figure 6.3 F) or IL-6 (Figure 6.3 G). The latter were compared using a t-test between the Sal-LPS and AG-LPS groups since no IL-6 was detectable in the saline-treated rats. IL-4, IL-12, IL-13, GM-CSF and IFNγ were not detectable in any groups with this assay.

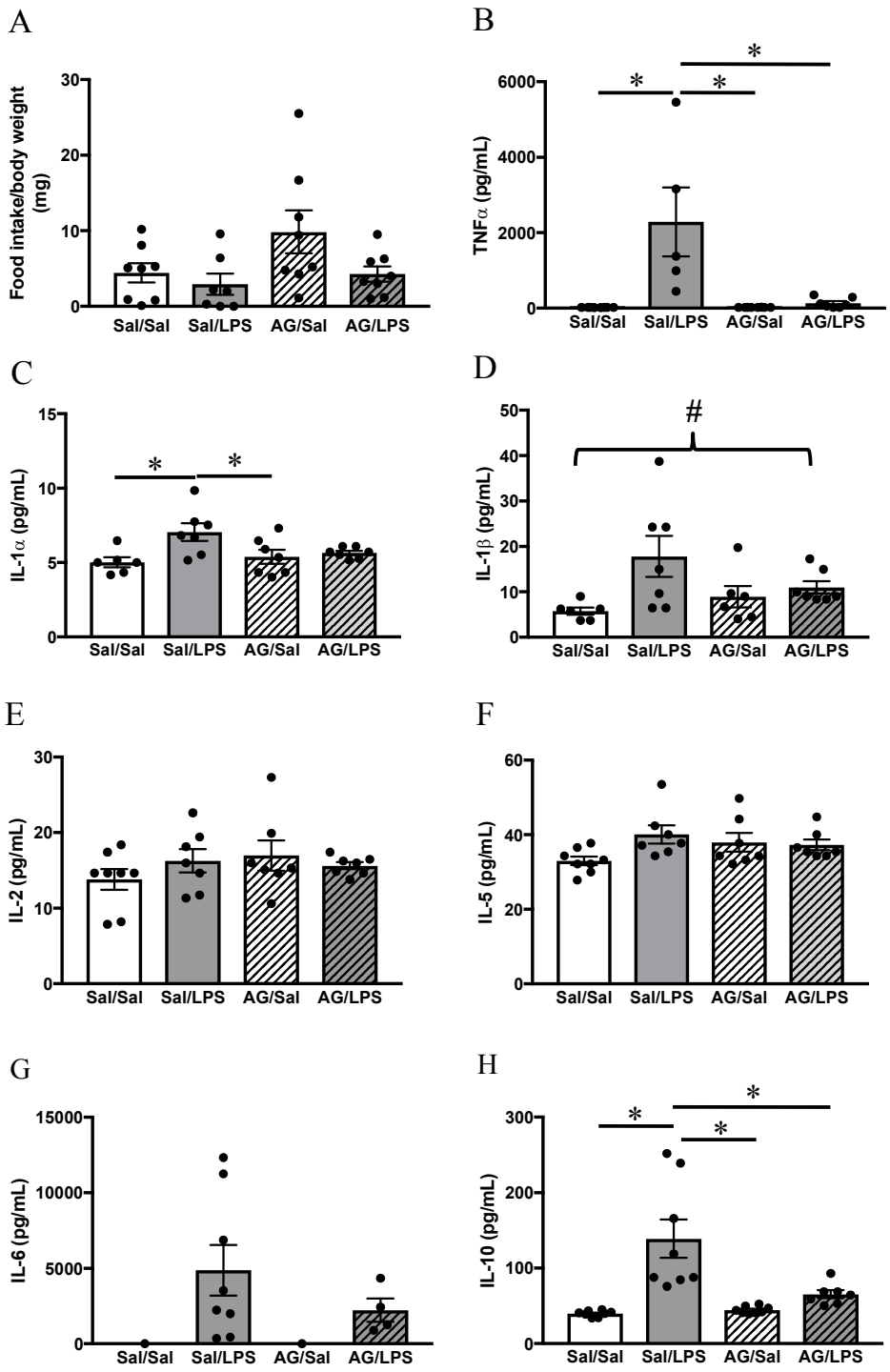



**Figure 6.3 Effects of exogenous acyl ghrelin (AG) on lipopolysaccharide (LPS)-induced anorexia and circulating cytokines**

(A) AG did not affect 2 hr food intake or the anorexigenic effect of LPS. LPS induced a significant increase in circulating cytokines: (B) tumor necrosis factor α (TNFα), (C) interleukin (IL)-1α, (D) IL-1β and (H) IL-10. AG significantly suppressed the response of TNFα (B) and (H) IL-10 to LPS. There was no effect of immune challenge or AG on (E) IL-2, (F) IL-5 or (G) IL-6. # main effect of immune challenge. * significant differences with *post hoc* tests. Data are mean ± SEM, $p < 0.05$ (n = 5-8 per group).

### *6.1.11 Effects of exogenous AG on PVN responses to LPS in vivo*

AG is able to indirectly modulate PVN activation in response to psychological stress (Spencer et al. 2012), in mice, so we next sought to identify if it could influence PVN responses to immune challenge with LPS in rats (Figure 6.4 A-D). As expected, LPS increased neuronal activation in the MP region (significant effect of LPS: $F_{(1, 20)}$ = 7.14, $p$ = 0.015; Figure 6.4 B; n = 5-7 per group), but there was no significant effect of AG. Treatment with AG significantly activated the MG region of the PVN (significant effect of AG: $F_{(1, 22)}$ = 10.97, $p$ = 0.003; Figure 6.4 C; n = 5-8 per group) and did not affect the MG response to LPS. Total PVN neuronal activation was driven by the effect of AG (significant effect of AG: $F_{(1, 21)}$ = 5.83, $p$ = 0.025; Figure 6.4 D; n = 5-8 per group). Exogenous AG, or LPS, also did not affect *Crhr1* mRNA in the hypothalamus (Figure 6.4 E; n = 4-8) together indicating AG does not affect the central HPA axis response to LPS.

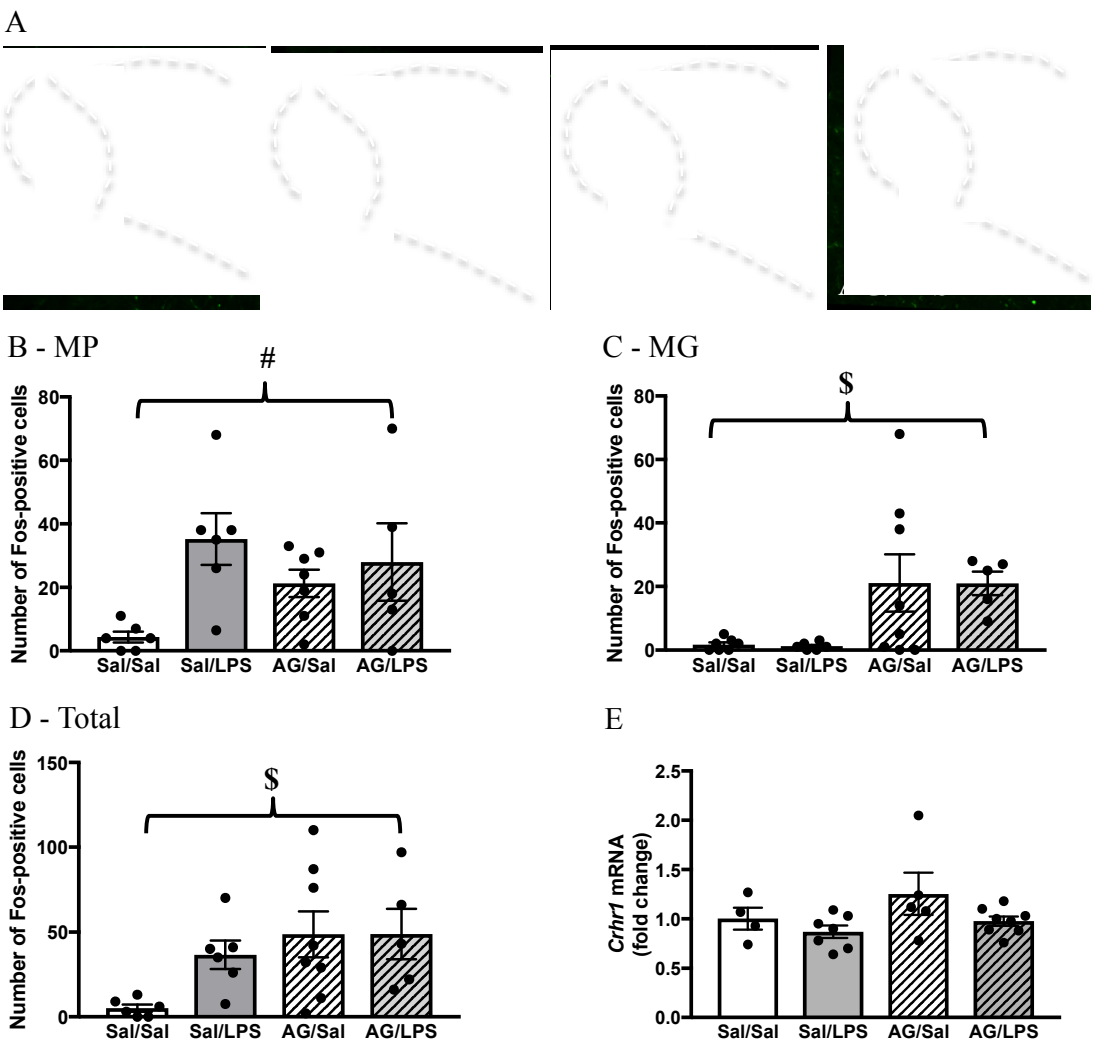



**Figure 6.4 Exogenous acyl ghrelin (AG) does not affect central hypothalamic pituitary adrenal (HPA) axis responses to immune challenge *in vivo.***
(A) Representative photomicrographs of Fos-positive cells in the paraventricular nucleus of the hypothalamus (PVN). (B) Lipopolysaccharide (LPS) increased numbers of Fos-positive cells in the medial parvocellular (MP) region of the PVN without any effect of AG. C) AG significantly activated the magnocellular (MG) region of the PVN without any effect of LPS. D) Total PVN neuronal activation was driven by the effect of AG. E) Hypothalamic mRNA expression of corticotropin-releasing hormone *Crhr1* receptor. # main effect of immune challenge, $ main effect of AG. Data are mean ± SEM, $p < 0.05$. n = 4-8 per group.

### *6.1.12 Effects of exogenous AG on ACTH responses to LPS in vivo*

Since the ACTH response to AG was elevated in vitro, indicating AG has the capacity to regulate at least part of the HPA axis in male rats, we next tested if AG could influence HPA axis responses to LPS in vivo at the level of the pituitary. There was a significant interaction between time and both LPS ($F_{(2, 65)} = 8.03$, $p = 0.001$) and AG

($F_{(2, 65)}$ = 5.34, $p$ = 0.007; Figure 6.5 A; n = 5 - 7 per group), but the only relevant effect revealed with the post hoc tests was a reduction in ACTH at 2 hr compared with 15 min in the saline-saline group. There was no indication at any time point that AG was suppressing the ACTH response to LPS.

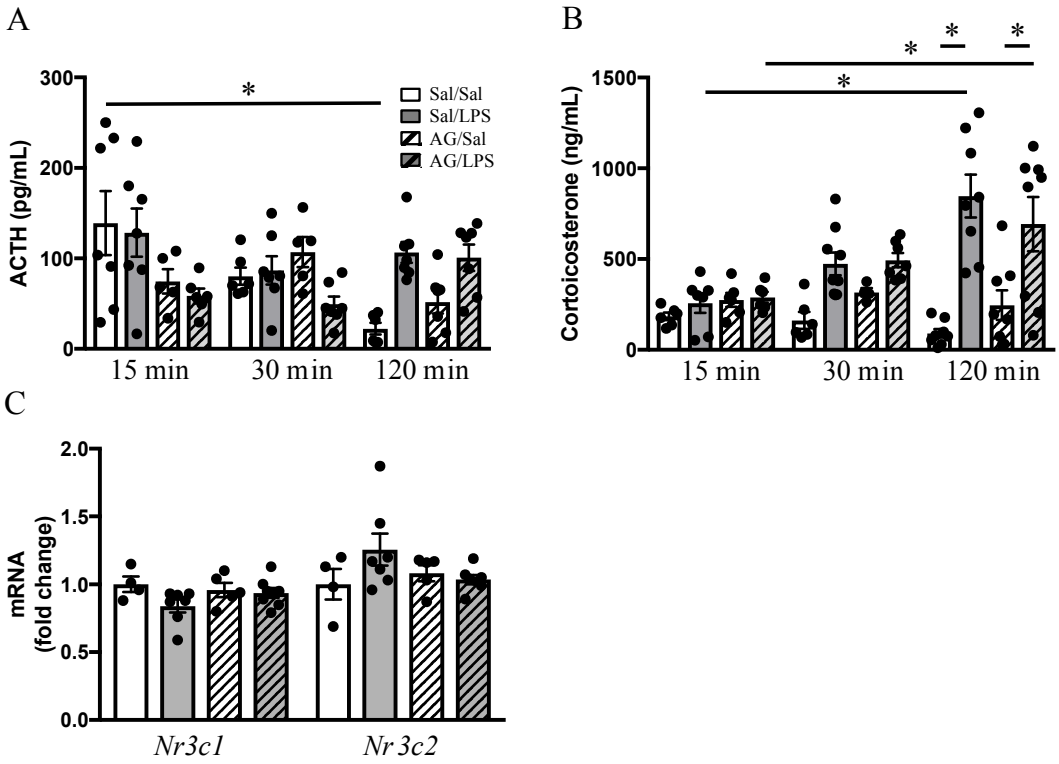



**Figure 6.5 Effects of exogenous acyl ghrelin (AG) on adrenocorticotropic hormone (ACTH), corticosterone (CORT), glucocorticoid receptor (*Nr3c1*) and mineralocorticoid receptor (*Nr3c2*) expression *in vivo.***
(A) AG's effects on circulating ACTH responses to lipopolysaccharide (LPS) at 15 min, 30 min and 120 min. (B) AG's effects on CORT responses to LPS at 15 min, 30 min and 120 min. (C) Hypothalamic *Nr3c1* and *Nr3c2* gene expression. * significant differences with *post hoc* tests. Data are mean ± SEM, $p < 0.05$. n = 4-8 per group.

### *6.1.13 Effects of exogenous AG on CORT responses to LPS in vivo*

Any effect of AG on the HPA axis that mitigates the inflammatory effects of immune challenge would necessarily involve CORT-mediated suppression of NFκB-induced cytokine transcription (Goujon et al. 1997; Auphan et al. 1995). We therefore next assessed if AG could influence the CORT response to LPS, potentially by-passing

both the PVN and pituitary to do so directly. There was a significant interaction between time and LPS on circulating CORT ($F_{(2, 71)} = 13.37$, $p < 0.001$; Figure 6.5 B; n = 5 - 9 per group), but no indication that AG could suppress the response to LPS. *Post hoc* tests revealed CORT was significantly elevated by LPS at 2 hr compared with the LPS-treated groups at 15 min and compared with the saline-treated groups at 2 hr, irrespective of AG treatment. AG and LPS also did not affect expression of *Nr3c1* (glucocorticoid receptor) or *Nr3cr2* (mineralocorticoid receptor) mRNA in the hypothalamus at 2 hr (Figure 6.5 C; n = 4-8). Together these data suggest that the AG-induced cytokine suppression we see after LPS stimulation is not likely due to AG's effects on the HPA axis.

### *6.1.14 Effects of exogenous AG and LPS on circulating ghrelin*

AG has a relatively short half-life of 30 min (De Vriese et al. 2004). Our analyses of the *in vitro* and acute ACTH and CORT responses to AG and LPS were therefore likely to be in the context of high circulating AG whereas the AG concentrations were likely to have dissipated at 2 hr, the time at which we measured cytokine levels. We therefore next tested if there was significant circulating AG still present at 2 hr after AG and LPS administration, to account for the cytokine suppression at this time. Treatment with AG led to a significant increase in circulating total ghrelin levels even at 2 hr after injection in both the saline and LPS-injected groups (significant effect of AG: $F_{(1,27)} = 463.3$, $p < 0.001$; Figure 6.6 A; n = 7-8), with no notable effect of LPS on ghrelin either under basal or AG-injected conditions. Unexpectedly, this increase in total ghrelin was accounted for by DAG, with elevated DAG after AG irrespective of LPS treatment (significant effect of AG: $F_{(1,24)} = 586.0$, $p < 0.001$; Figure 6.6 C; n = 6-8). Circulating AG was even suppressed in those rats that had been given AG

(significant effect of AG: $F_{(1,25)} = 8.50$, $p = 0.007$; Figure 6.6 B; n = 6-8), indicating that exogenous AG is metabolized to DAG and DAG can inhibit AG release (Inhoff et al. 2008).

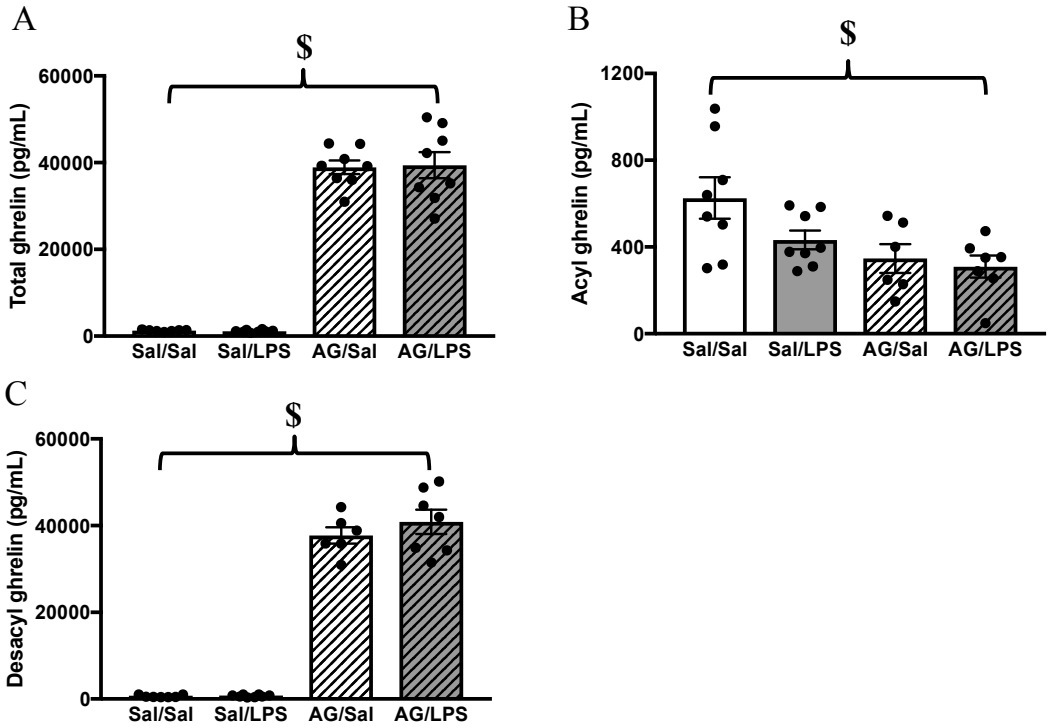



**Figure 6.6 Effects of acyl ghrelin (AG) and lipopolysaccharide (LPS) on circulating ghrelin.**
Exogenous AG is readily metabolized to des-acyl ghrelin (DAG) at 2 hr post-injection. (A) Exogenous AG significantly increased circulating total ghrelin, due to increased circulating DAG levels (C). (B) Exogenous AG significantly suppressed circulating AG without any effect of LPS. # main effect of AG. Data are mean ± SEM, $p < 0.05$. n = 6-8 per group.

### *6.1.15 Effects of exogenous DAG and LPS on circulating ghrelin*

As our results showed exogenous AG is fully metabolized to DAG by 2 hr after injection, it seemed possible that the suppressive effect of AG on the circulating cytokine response to LPS is actually due to an acute effect of DAG at or before the 2 hr time point. As with AG, treatment with DAG also led to a significant increase in circulating total ghrelin levels in both the saline and LPS-injected groups (significant

effect of DAG: $F_{(1,28)} = 3283.4$, $p < 0.001$; Figure 6.7 A; n = 8 per group), with, again, no notable effect of LPS on ghrelin either under basal or DAG-injected conditions. This increase in total ghrelin was again exclusively due to DAG, with elevated circulating DAG after exogenous DAG irrespective of LPS treatment (significant effect of DAG: $F_{(1,27)} = 3135.22$, $p < 0.001$; Figure 6.7 C; n = 7-8). Circulating AG was suppressed to undetectable levels after exogenous DAG in both saline and LPS-treated rats. Since these levels were undetectable, the comparison of the effects of LPS on AG levels under otherwise untreated conditions (Sal-Sal versus Sal-LPS) was assessed for significance using Student t-test and there was a resultant suppression of AG with LPS alone ($t_{(13)} = 3.37$, $p = 0.005$; Figure 6.7 B; n = 7-8), consistent with the trend seen in the exogenous AG experiment.

### *6.1.16 Effects of exogenous DAG on LPS-induced anorexia*

There was no effect of DAG on food intake, but there was a significant main effect of LPS in this experiment such that those exposed to LPS consumed less than the saline-treated rats, consistent with the expected anorexigenic effect of LPS ($F_{(1,22)} = 7.30$, $p = 0.013$; Figure 6.7 D; n = 6-8).

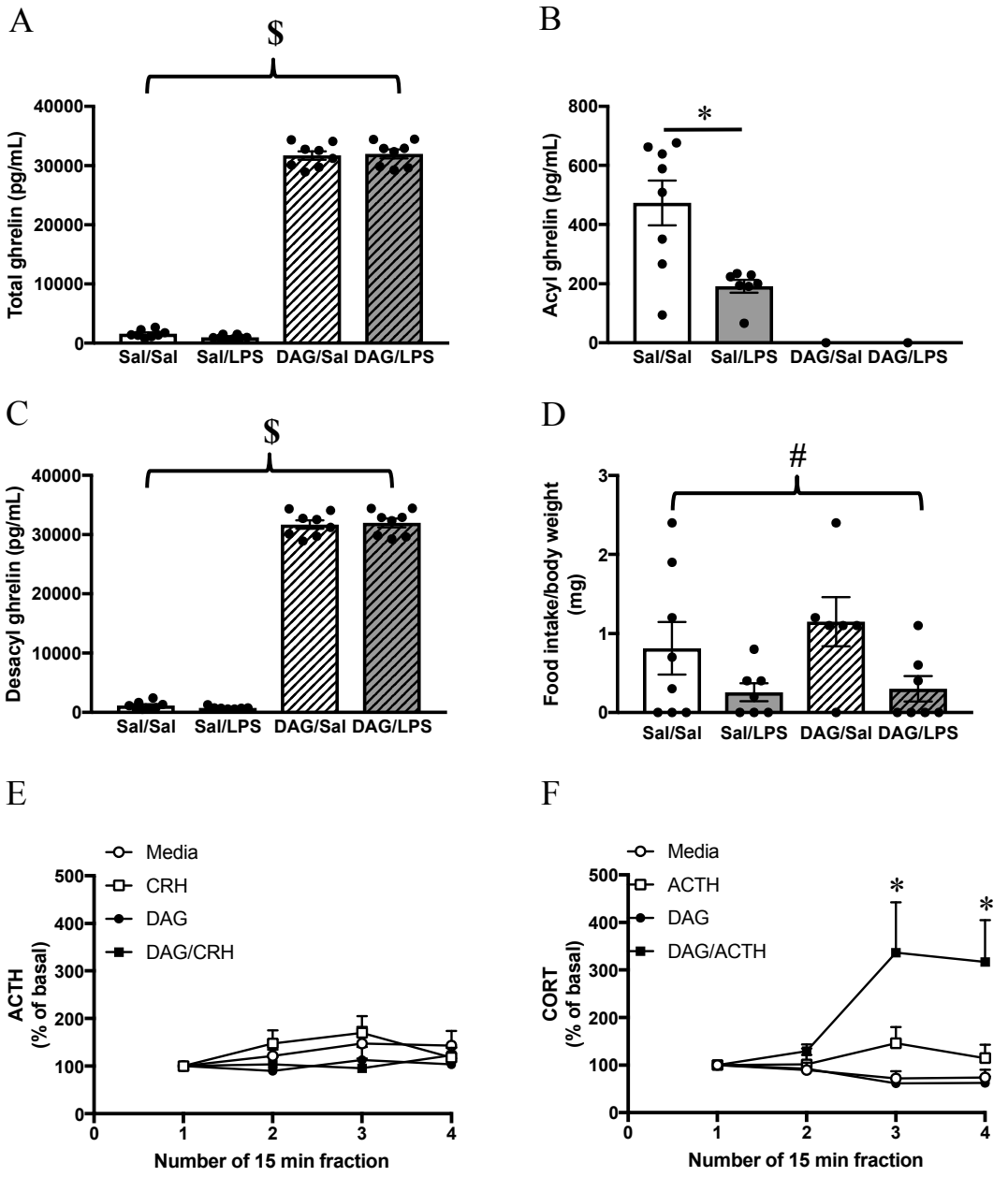
### *6.1.17 Effects of DAG on pituitary and adrenal responses to stimulation*

Neither CRH nor DAG had a significant effect on ACTH release from the pituitary gland (Figure 6.7 E; n = 7-8). In the adrenal gland, there was a significant interaction between fraction, DAG, and ACTH treatments ($F_{(3,69)} = 2.816$, $p = 0.045$; Figure 6.7 F; n = 5-8) on CORT levels. The effect of ACTH on CORT release was not statistically significant with *post hoc* tests, but the magnitude of the difference between the ACTH-alone and media-alone groups was similar to that seen in the AG

experiment. In the presence of DAG, however, ACTH markedly and significantly increased CORT in the 30 and 45 min (second and third) fraction with no effect of DAG alone. Together these results indicate AG has some minimal effect on CORT release from the adrenal gland, slightly potentiating it in the presence of ACTH, while DAG is able to strongly potentiate the effects of ACTH.

### *6.1.18 Effects of exogenous DAG on HPA axis responses to LPS*

Despite this strong *in vitro* effect of DAG on ACTH-induced CORT release, there was no effect of DAG on the central HPA axis response to LPS *in vivo*. LPS significantly increased neuronal activation in the MP (significant effect of LPS: $F_{(1,24)} = 24.42$, $p < 0.001$; Figure 6.7 G; n = 5-8) and MG regions (significant effect of LPS: $F_{(1,24)} = 10.24$, $p = 0.004$; Figure 6.7 H; n = 6-8) of the PVN, as well as in both regions combined (significant effect of LPS: $F_{(1,24)} = 23.20$, $p < 0.001$; Figure 6.7 I; n = 6-8), both under otherwise-untreated conditions and after concomitant treatment with DAG, with no further effect of DAG. Exogenous DAG, or LPS, did not change expression of *Crhr1* (Figure 6.7 J). CORT was elevated at 2 hr in response to LPS, but this was not affected by DAG (significant effect of LPS: $F_{(1,28)} = 46.92$, $p < 0.001$; Figure 6.7 K; n = 8) and neither DAG nor LPS altered *Nr3c1* or *Nr3cr2* mRNA in the hypothalamus (Figure 6.7 L; n = 5-6), except that there was a significant main effect of immune challenge on *Nr3c1* ($F_{(1,20)} = 4.90$, $p = 0.039$) with LPS suppressing expression of this gene.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Total ghrelin (pg/mL)
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800
600
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ACTH (% of basal)
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Number of 15 min fraction
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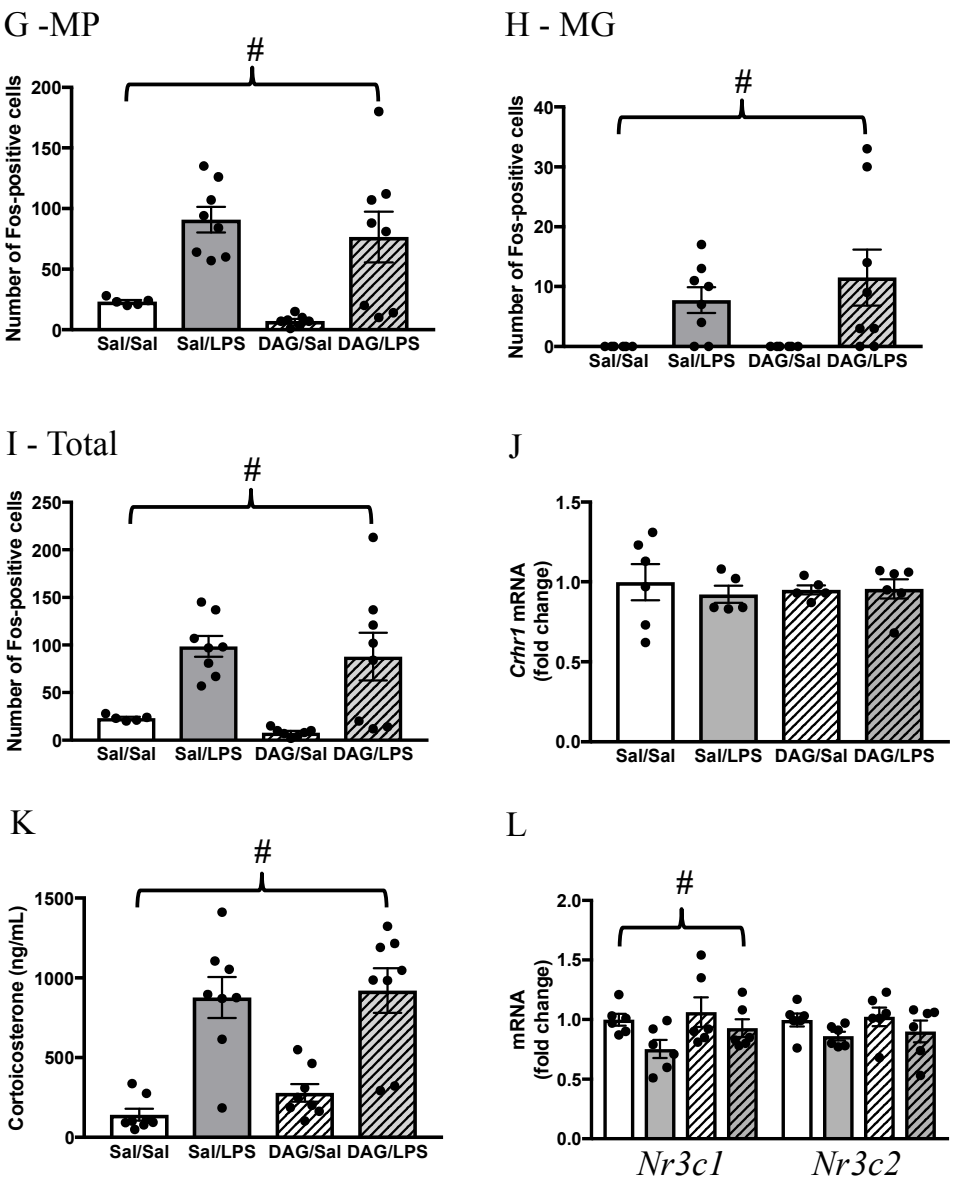


**Figure 6.7 Effect of exogenous des-acyl ghrelin (DAG) and lipopolysaccharide (LPS) *in vivo* and *in vitro*.**

(A) Circulating total ghrelin. (B) Circulating acyl ghrelin (AG). (C) Circulating DAG. (n = 7-8 per group). (D) Food intake. (E) Neither corticotropin-releasing hormone (CRH) nor DAG had an effect on the *in vitro* adrenocorticotropic hormone (ACTH) release from the anterior pituitary. (F) In the presence of DAG, ACTH significantly increased CORT in the 30 min and 45 min fractions without any effects of DAG or ACTH alone. (G) Centrally, LPS induced a significant increase in numbers of Fos-positive cells in the G) medial parvocellular (MP) and (H) magnocellular (MG) regions of the paraventricular nucleus of the hypothalamus (PVN) as well as in both regions combined (I). Neither DAG nor LPS had any effect on hypothalamic (J) corticotropin-releasing hormone receptor *(Crhr1)*, (L) glucocorticoid receptor (*Nr3c1)* or mineralocorticoid receptor (*Nr3cr2)* mRNA expression. (K) LPS significantly increased circulating CORT *in vivo* without any further effects of DAG. # main effect of immune challenge, $ main effect of DAG, Data are mean ± SEM, $p < 0.05$. (n = 5-8 per group.

### *6.1.19 Effects of exogenous DAG on circulating cytokine responses to LPS*

Finally, we tested if the ability of DAG to potentiate the CORT response to ACTH, observed *in vitro*, would lead to a suppression of the cytokine response to LPS such as we saw with AG. As expected, LPS lead to elevated circulating cytokines at 2 hr (n = 5-7), but there were no effects of DAG on this response. There was a significant main effect of immune challenge on TNFα ($F_{(1,24)} = 6.33$, $p = 0.019$; Figure 6.8 A) and IL-1β ($F_{(1,24)} = 16.81$, $p < 0.001$; Figure 6.8 C). There was also a significant effect of DAG on IL-5 ($F_{(1,25)} = 7.08$, $p = 0.013$; Figure 6.8 E), but no effects on IL-1α, (Figure 6.8 B), IL-2 (Figure 6.8 D), or IL-6 (t-test; Figure 6.8 F). The *p* value for IL-10 was $p = 0.058$ (Figure 6.8 G). There were no detectable levels of IL-4, IL-12, IL-13, GM-CSF or IFNγ. Although we saw a significant effect of LPS on IL-1α in the AG experiment (C) and not here (Figure 6.8 B), this effect was small in the former and the absolute IL-1α levels were very similar between experiments. Together these data suggest that AG, but not DAG, can suppress cytokine secretion and can influence HPA axis function, and that the AG-induced cytokine suppression we see after LPS is not due to AG's HPA axis effects but is also not due to a predominant role for DAG.

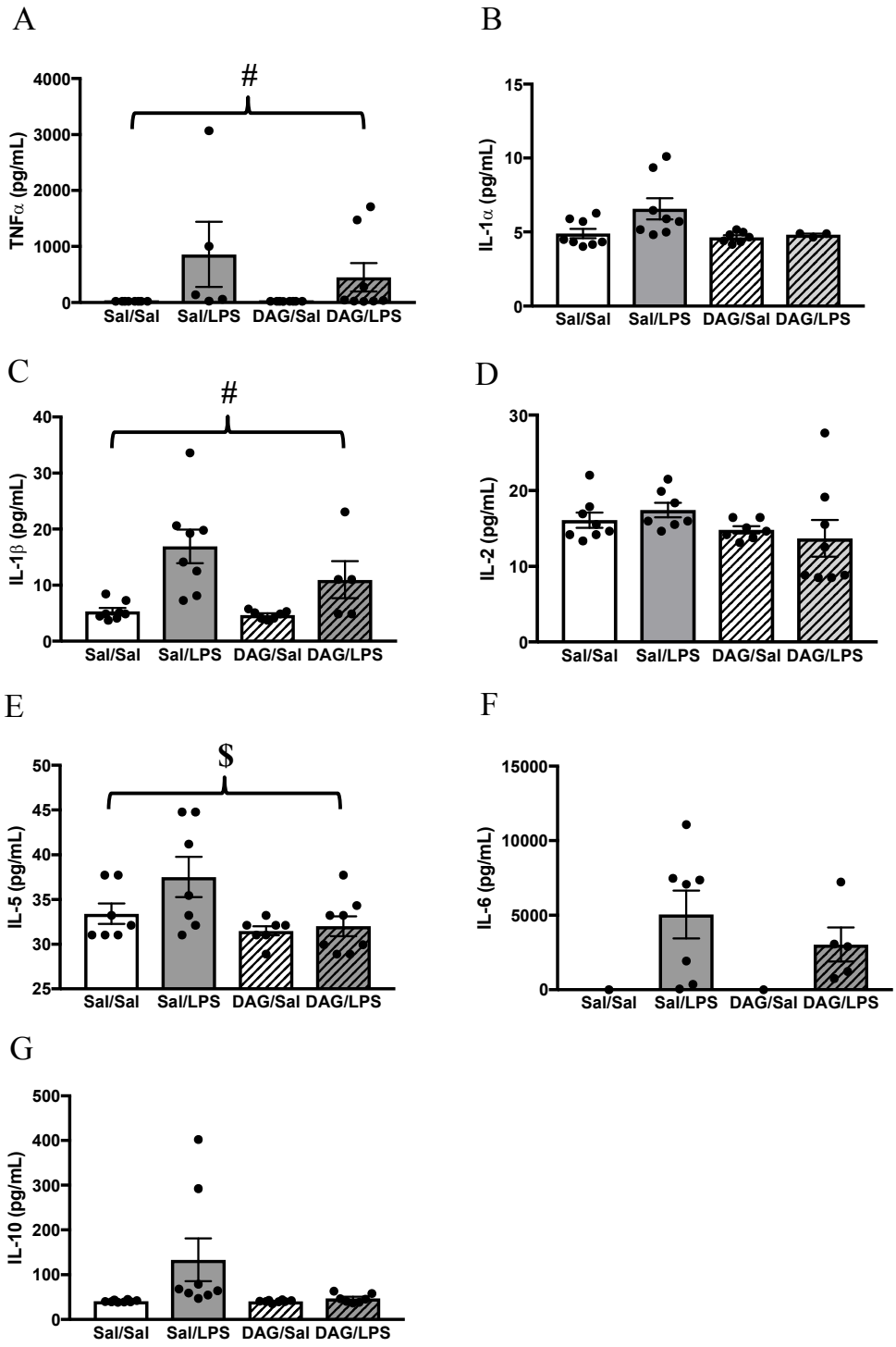


**Figure 6.8 Effects of des-acyl ghrelin (DAG) on circulating cytokine responses to lipopolysaccharide (LPS) *in vivo*.**

LPS significantly increased circulating (A) tumor necrosis factor α (TNFα) and (C) interleukin (IL)-1β, without any further effects of DAG. (E) DAG significantly suppressed IL-5 without any effects of LPS. No effect of LPS or DAG was observed on (B) IL-1α, (D) IL-2, (F) IL-6, or (G) IL-10. # main effect of DAG, $ main effect of immune challenge. Data are mean ± SEM, $p < 0.05$. n = 5-7 per group.

## Discussion

Ghrelin's role in the HPA axis response to psychological stress is well-reported (Lutter et al. 2008; Spencer et al. 2015; Spencer et al. 2012). However, its impact on the HPA axis responses to an immune challenge is less well-characterized. Similarly, research has been lacking into DAG's function in the neuroimmune response. Here we show that AG, but not DAG suppresses LPS-induced pro- and anti-inflammatory circulating cytokines at 2 hr after injection, despite AG being fully metabolized to DAG by this time. However, although AG can clearly stimulate HPA axis activation, this suppressive effect of AG on cytokine release is not likely to be due to its HPA axis effects.

LPS induces a pro-inflammatory response by interacting with cell surface receptors of immune cells such as monocytes/macrophages, neutrophils and lymphocytes (reviewed in (Newton and Dixit 2012)). LPS is transported to pattern recognition receptors toll-like receptor (TLR) 4 and 2 (Kawai and Akira 2011). TLR4 activation initiates a cascade of cell processes including activation of myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like (Mal) and toll-like receptor (TRIF) -related adaptor molecule (TRAM)), which further activate pro-inflammatory transcription factors such as NFκB and extracellular signal-regulated kinase (ERK), initiating transcription of pro- and anti-inflammatory genes, resulting in production of pro- and anti-inflammatory cytokines (Newton and Dixit 2012). This cytokine response then leads to the cyclo-oxygenase 2-mediated conversion of arachidonic acid into prostaglandins, including PGE2 that then act at E-prostanoid receptors throughout the brain to activate the HPA axis (reviewed in (Ricciotti and FitzGerald 2011)). CRH and arginine vasopressin (AVP) cells in the parvocellular region of the

PVN are stimulated to secrete CRH, which reaches the anterior pituitary through the hypothalamo-hypophysial portal blood vessel system from the median eminence and stimulates the release of ACTH. ACTH acts at melanocortin 2 receptors in the adrenal cortex to stimulate release of glucocorticoids that negatively feed back onto glucocorticoid and mineralocorticoid receptors to suppress further activation of the HPA axis (Jacobson and Sapolsky 1991). Glucocorticoids also act to inhibit NFκB-mediated cytokine transcription (Auphan et al. 1995).

At least in psychological stress, AG is known to interact with the HPA axis in order to regulate stress-related behaviours, making this a candidate mechanism for cytokine suppression (Spencer et al. 2015). It has been documented that ghrelin activates the HPA axis at the hypothalamic level, activating hypothalamic explants *in vitro* (Wren et al. 2002; Mozid et al. 2003). Ghrelin also indirectly activates the HPA axis via brainstem inputs to the PVN (reviewed in (Spencer and Tilbrook 2011)) and via indirect activation of CRH neurons of the PVN (Cabral et al. 2012). In humans, ghrelin administration increases ACTH and cortisol release (Arvat et al. 2001; Takaya et al. 2000; Locatelli et al. 2010). Similarly, in rodents ghrelin also stimulates increases in circulating ACTH and CORT (Spencer et al. 2012). As was seen previously (Sominsky, Ziko, and Spencer 2017), we here again show that AG alone is able to stimulate robust ACTH production from the pituitary in an *in vitro* setting. However, in the case of an immune challenge with LPS it seems AG's effects on the HPA axis are insufficient to affect cytokine release.

In support of the idea that AG influences the cytokine response independently of the HPA axis, we saw no significant effects of AG on LPS-induced ACTH levels *in vivo*.

There was even an apparent reduction in ACTH over time. It is likely this is due to a mild effect of the stress associated with handling and injection (Viau et al. 1993) but it is notable that there was no significant effect of LPS or AG on ACTH release. AG also lacked suppressive effects on LPS-induced CORT. Interestingly, an earlier study showed that a much lower dose of ghrelin (0.1 mg/kg) could potentiate the CORT response to LPS (Soriano et al. 2011). In our study, LPS gradually increased CORT release over time, as expected, inducing the highest CORT levels at 120 min, but no AG effects were observed at any time. Centrally, AG alone stimulated a significant increase in neuronal activation in the PVN, including in the magnocellular region of the PVN. Magnocellular neurons are mainly responsible for secretion of oxytocin and AVP into the peripheral circulation via the posterior pituitary. AVP and CRH are both produced in the parvocellular neurons projecting to the anterior pituitary and other brain regions (Ludwig and Leng 2006). Levels of both AVP and CRH are inversely related to glucocorticoid levels, with a role in feedback regulation (Ludwig et al. 2002). AVP, as an ACTH secretagogue, potentiates CRH's effects on ACTH release from the anterior pituitary. However, AG neither significantly attenuated the LPS-induced activation of the PVN, nor significantly stimulated *in vivo* stress hormone secretion despite this PVN activation.

It thus seems clear that AG's suppressive effects on the cytokine response to LPS are independent of its influence on the HPA axis. However, AG clearly suppressed this component of the immune response. Limited literature suggests AG is able to suppress immune activation via the vagus nerve in animal models of traumatic brain injury, sepsis or stroke where ghrelin administration suppressed serum TNFα and IL-6 (Wu, Dong, et al. 2007; Cheyuo et al. 2011; Bansal et al. 2012). However, these

studies did not account for the fluctuation of ghrelin levels based on feeding status or any potential anti-inflammatory role of DAG. Also, the exact neuronal connectivity and involved neurotransmitters that mediate communication between ghrelin and vagus nerve remain to be determined (Veedfald et al. 2018). Another possible mechanism by which ghrelin suppresses inflammatory cytokines is by inhibiting high-mobility group box 1 (HMGB1) release. HMGB1 is an important protein in initiating inflammation in a number of inflammatory diseases including sepsis. It is expressed in almost all cell types, however ghrelin greatly inhibits HMGB1 secretion from macrophages by blocking its cytoplasmic translocation (Chorny et al. 2008). This study, however, tested the delayed effect of ghrelin after endotoxemia was established for a period of more than 2 hr. Whether ghrelin plays an acute role in this regard remains to be tested. Ghrelin can also directly suppress the inflammatory response of a number of immune cells including peripheral macrophages, T-cells and activated microglia (as reviewed in (Hattori 2009)). It is proposed that it does this via a dual effect in reducing pro-inflammatory mediators but also enhancing activation of the anti-inflammatory p38 MAPK pathway through GHSR in order to mediate Th1 and Th2 responses simultaneously (Waseem et al. 2008). The exact cell mechanisms via which ghrelin suppresses inflammation however, need further elucidation.

In the present study, we have shown that unlike AG, DAG does not suppress LPS-induced cytokine activation. It also has limited effect on the HPA axis. These findings are particularly notable given AG is completely metabolized to DAG within 2 hr after injection and suggest that the anti-inflammatory effects of AG on the LPS response are indeed due to an acute AG action and not to excessive accumulation of DAG over the 2 hr. Studies have shown that in the arcuate nucleus DAG exerts its biological

effects via a non-GHSR receptor by impairing AG's orexigenic effect (Fernandez et al. 2016). Here we show that the involvement of DAG in the HPA axis is limited to a potentiating effect on ACTH *in vitro*, resulting in high CORT levels at 30 and 45 min. Otherwise DAG was unable to suppress any LPS-induced effects *in vivo* at any level of the HPA axis. Mozid et al (Mozid et al. 2003) have also shown that DAG has no effect on the HPA axis. We did see a suppressive effect of DAG on IL-5, but this was independent of LPS and not seen with any other tested cytokine. DAG, but not AG, can exert anti-apoptotic effects in cultured neuronal cells (Hwang et al. 2009) and can suppress IL-6 secretion from amyloid-β stimulated microglia (Bulgarelli et al. 2009). Taken together these results indicate that DAG has limited anti-inflammatory effects and, again, does not influence circulating cytokines via the HPA axis. Our findings suggest AG's anti-inflammatory effects are independent of its actions on the HPA axis and are not due to excessive accumulation of DAG. While the exact mechanisms by which ghrelin influences the immune response remain to be determined, the apparent separation between ghrelin's effects on HPA axis responses to psychological versus immune challenges raise the possibility of targeting this peptide for treatments of inflammatory conditions without compromising HPA axis activity.

Chapter 7

# General discussion

In this thesis I investigated the effects of neonatal overfeeding on the metabolic hormones leptin and ghrelin and their role in brain development and long-term function. In terms of developmental programming, it is well established that the early life nutritional environment is crucial in shaping long-term health in humans (Barker 2003). However, the importance of nutrition in programming the developing brain with regards to the metabolic hormones leptin and ghrelin has only recently been addressed and has been largely ignored for females. In this thesis I examined the importance of the early life nutritional environment in establishing optimal neuronal connections in the developing brain, particularly in those regions that are responsible for controlling metabolism.

An over- or under-nutritional environment during important developmental periods leads to metabolic and emotional disorders such as obesity and stress in a sex specific manner, with females having a tendency to experience more stress-related disorders than males (Dearden, Bouret, and Ozanne 2018). However, the effects of an altered nutritional environment during early life development and its impact on the hypothalamic pituitary adrenal (HPA) axis and stress responses remain to be further studied. Along with its role in metabolism, ghrelin is involved, in the adult animal, in modulating stress and anxiety as well as immunity and inflammation (Spencer et al. 2015; Spencer et al. 2012; Pereira, da Silva, and de Moraes-Vieira 2017). However, ghrelin's effects in modulating responses to early life adverse nutrition, its effects in programming the HPA axis, and whether these effects vary in males and females remain largely unstudied. Furthermore, the mechanisms involved in ghrelin's anti-inflammatory roles are not well defined. Leptin, similarly to ghrelin, has been

implicated in the early regulation of neuronal connectivity in hypothalamic regions responsible for feeding and metabolism (Bouret, Draper, and Simerly 2004b). However, there are significant gaps in our knowledge with regards to an altered early life nutritional environment and leptin's role in programming the brain long-term.

To study the effects of nutritional environmental adversity in early life, we used an animal model of litter size manipulation where pups on the day of birth were redistributed into control litters of 12 pups and small litters of 4 pups, with small litter pups having greater access to the dam's milk and consequently having an increased body weight compared to controls. The animal model used in this thesis leads to a long-lasting moderately overweight phenotype, which significantly differs from other animal models of genetic or adult diet-induced obesity. In our model, neonatally overfed animals, when adults, have increased body weight compared to controls despite consuming the same standard diet for most of their lives.

In **Chapter 2,** is demonstrated for the first time that neonatal overfeeding, along with the obese phenotype characterised by elevated body weight, increased the magnitude of the leptin surge and elevated leptin levels long-term. These effects were associated with early life disruptions to hypothalamic neuropeptide Y (NPY) and agouti related peptide (AgRP) fibre density as well as acute hypothalamic insensitivity to leptin. However, in adulthood, central control of appetite regulation was normalised even though the body weight and circulating leptin levels remained elevated in neonatally overfed rats (Sominsky, Ziko, et al. 2017). This persistently elevated leptin and increased body weight in neonatally overfed adult animals could be due to other permanently altered metabolic factors that we have not measured here. For instance,

disturbances to the nutritional environment during development, is associated with disruptions in circulating insulin levels in parallel this with a prolonged leptin surge (Kirk et al. 2009). Furthermore, it has been shown that maternal high fat diet impairs hypothalamic neurocircuitry formation due to affected neonatal insulin levels (Vogt et al. 2014). When in elevated levels, during perinatal life, insulin may program the development of obesity. Similarly to neonatal leptin, malprogramming of neonatal insulin system especially ARC resistance to the insulin satiety signals may explain acquired long-term metabolic problems, including obesity (Plagemann 2008).

Obesity causes a form of low-grade inflammation involving elevated adipocytokines such as leptin, interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNFα) secretion from adipocytes (Pereira and Alvarez-Leite 2014). Our group has previously shown that neonatal overfeeding permanently affects the hypothalamic neuroinflammatory profile manifested in increased numbers of microglia, one of the primary immune cells of the central nervous system (CNS), indicating increased central inflammation long-term (Ziko et al. 2014). Douglass *et al* have shown that increased hypothalamic inflammation is associated with weight gain (Douglass et al. 2017). It is also important to note that metabolic inflammation in the peripheral tissues can be independent of the CNS, as was demonstrated by Valderacos and colleagues in a model of diet induced obesity and microglia ablation, potentially explaining the long-lasting peripheral metabolic disturbances despite the fact that there were no differences in gene expression in central leptin sensitivity in adulthood (Valdearcos et al. 2018).

It has been previously shown that there is a high interrelation between leptin and ghrelin during early life neurodevelopment with ghrelin suppressing the overgrowth of hypothalamic neuronal connections stimulated by leptin in order to fine-tune metabolic central control (Steculorum et al. 2015). In this chapter we examined whether an early life overnutritional environment, in addition to alterations to the leptin system (**Chapter 2**), would affect the ghrelin system in male rats. Indeed, neonatal overfeeding altered the peripheral and central ghrelin system short-term and this effect was partially ameliorated by adulthood (**Chapter 3**) (Sominsky, Ziko, Nguyen, et al. 2016). We have largely focused here on the metabolic hormones leptin and ghrelin however it should be noted that some of the effects could be mediated by insulin and glucose. Plagemann's studies, for instance, in newborn rats injected with insulin from P8-P11, demonstrate life-long increased body weight, impaired glucose tolerance and increased vulnerability to a sub-diabetogenic dose of streptozotocin (Harder et al. 1999). In another study from the same group, gestational diabetes (GD) was induced in mothers on the day of conception with the hypothesis that prenatal hyperleptinemia would affect hypothalamic regulators of body weight and metabolism. It was demonstrated that on P21 offspring of GD mothers were overweight, hyperinsulinemic and they had disrupted differentiation of nuclei within the paraventricular nucleus of the hypothalamus (PVN) and ventromedial hypothalamus (VMH) (Plagemann, Harder, Janert, et al. 1999). These results emphasise the importance of optimal insulin levels during critical periods of postnatal life in hypothalamic regulation of body weight and metabolism and susceptibility to diabetes in later life due to temporary early life hyperinsulinemia.

As observed in **Chapter 2**, neonatal overfeeding in males was associated with acute disruption to hypothalamic metabolic control, changes that had not been studied in females in relation to the early life nutritional environment. Therefore, in **Chapter 4** we aimed to examine whether the same metabolic changes accompanying the early life nutritional status in males would be observed in females; given the importance of sex differences in many aspects. We observed in this chapter that neonatally overfed females, experiencing long-term increased body weight and circulating leptin, had no changes in the central control of feeding circuitry compared to normally fed counterparts (Ziko et al. 2017). Sex-differences in brain structures between males and females have been observed from before birth, with males having larger brain mass than females (Dean et al. 2018). The hypothalamus, being the main energy-regulating brain region, is highly sexually dimorphic. It has been suggested that female sex hormones are responsible for regulating neurogenesis and cell death during the development of the hypothalamus and the absence or overexpression of these hormones during key developmental periods is associated with permanent neuronal changes (Budefeld et al. 2008). Sex hormones are also responsible for regulation of feeding. For instance, male mice have decreased numbers of proopiomelanocortin (POMC) neurons within the arcuate nucleus of the hypothalamus (ARC), which may explain their higher calorie intake and increased body weight when compared to females (Nohara et al. 2011). These changes are at least partially mediated by testosterone during hypothalamic development and, as shown by Nohara and colleagues, female mice injected with testosterone have decreased number and density of POMC neurons within the ARC relative to control females (Nohara et al. 2011). We have previously mentioned that males and females also differ in the location of fat deposition, with males being characterised by abdominal fat deposition whereas

females preferentially deposit subcutaneous fat (Karastergiou et al. 2012). This phenomenon could be explained by the sexually dimorphic way in which the sympathetic nervous system innervates peripheral adipose tissues, with males having more afferent projections to abdominal fat and females having more projections to subcutaneous fat (Frank, Palmer, and Clegg 2018). The above findings suggest that males and females are differently wired not only centrally, but also peripherally, explaining the changes in responses to nutritional challenges in early life. Furthermore, these findings suggest that peripheral factors, including innervation of adipose tissue, may aid the fact that males and females behave differently in the way they centrally control appetite.

In **Chapter 4** we observed that neonatal overfeeding in females, in contrast to males, was not associated with any changes in the central control of energy intake. However, we have previously reported that neonatally overfed females, but not males, display exacerbated HPA axis responses to restraint stress (Spencer and Tilbrook 2009). We have also demonstrated that ghrelin directly affects the anterior pituitary in secreting adrenocorticotropic hormone (ACTH) in order to aid the stress response and help the organism cope with stress (Spencer et al. 2012). In **Chapter 5** we observed that neonatally overfed females have altered ghrelin receptor and *Goat* expression in the pituitary as well as a suppressed ability of the anterior pituitary to respond to ghrelin when stimulated in an *in vitro* setting, indicating that neonatal overnutrition affects at least partly the HPA axis responses to stress in females (Sominsky, Ziko, and Spencer 2017). It has long been shown that the HPA and hypothalamic-pituitary-gonadal (HPG) axis are closely connected (as reviewed in (Goel et al. 2014; Sominsky, Hodgson, et al. 2017)). It has been suggested that sex-specific changes to HPA axis

activity are affected by sex hormones (estrogen, progesterone and testosterone), the end products of the HPG axis, that are produced in significant quantities after the onset of puberty (Panagiotakopoulos and Neigh 2014). However, differences between boys and girls in HPA axis activity are already observed in early childhood, with boys under the age of eight having high salivary cortisol compared to girls of the same age. This pattern is reversed after the age of eight years (van der Voorn et al. 2017). The early life environment including maternal exposure to medications used to treat postpartum stress is an important factor in the neurodevelopmental outcomes of children. For instance, postpartum maternal exposure to fluoxetine (a pharmacological antidepressant used to treat postpartum depression) differently affects male and female offspring in adulthood. Maternal postpartum exposure to fluoxetine induces HPA axis negative feedback in adult male but not female rats, whereas maternal exposure to exercising is associated with increased hippocampal neurogenesis in both sexes, however maternal exposure to exercising impairs HPA axis activity in female offspring (Gobinath et al. 2018). In humans, early life stress, such as with child abuse and neglect, is also associated with impairments in neuronal development and may be one of the leading causes of psychiatric disorders in adulthood (Lajud et al. 2012). There are clear patterns for sex-specific prevalence rates of mental disorders, with conditions such as post-traumatic stress disorder (PTSD) and major depressive disorder (MDD) being more common among women, whereas aggressive behaviour and drug abuse are more common among men (Lundberg 2005; Keane, Marshall, and Taft 2006; Nestler et al. 2002).

The human brain undergoes immense functional and structural transformations between week 24 and 44 after conception (Pomeroy 2004) and also during neonatal

life, early childhood and adolescence (as reviewed in (Karatsoreos and McEwen 2013)). Any alteration to the nutrient state during these times not only affects brain neuroanatomical features, but also its chemistry and physiology, and this can be associated with long-term detrimental effects on physical and mental health (Anda et al. 2010). Having said this, the young brain is also extremely plastic and therefore it may optimise some repair after nutrient alterations. Nutritional insults result in brain dysfunction only when they outbalance brain plasticity. It has been shown that not only the young brain, but also the adult brain has considerable capacity for resilience (Karatsoreos and McEwen 2011). The adult brain has the ability for long-term change and in humans it is altered by behavioural interventions such as cognitive behavioural therapy, which reduces amygdala volume in anxiety disorders and increases grey matter in the prefrontal cortex in chronic fatigue patients (Seminowicz et al. 2013; Mansson et al. 2016). Furthermore, physical activity increases hippocampal volume in elderly people (Erickson et al. 2011). Similarly, in this thesis we observed that adult brain, in our rat model that had been challenged with early life poor nutrition, demonstrates resilience.

Ghrelin, along with its neurotrophic role in regulating neuronal connectivity within energy-controlling hypothalamic centres at early times after birth, has shown a major involvement in regulation of the immune system in adulthood. Acute or chronic inflammatory complications influence ghrelin levels. Increased circulating ghrelin levels have been correlated not only with disease severity, but also with expression of pro-inflammatory cytokines (as reviewed in (Baatar, Patel, and Taub 2011)). In **Chapter 6** we looked at ghrelin's involvement in the endocrine regulation of the immune system as an anti-inflammatory agent. Ghrelin has shown its anti-

inflammatory properties in a number of conditions such as sepsis, inflammatory bowel disease, rheumatoid arthritis and, experimentally, with high dose lipopolysaccharide (LPS) (Baatar, Patel, and Taub 2011; Pereira, da Silva, and de Moraes-Vieira 2017)). Ghrelin's presence in all of the above conditions leads to reduction of serum levels of TNFα and a number of interleukins including IL-6 and IL-8. However, the mechanisms by which ghrelin exerts its anti-inflammatory properties remain to be further elucidated. In **Chapter 6** we aimed to define the mechanism by which ghrelin is suppresses LPS-induced circulating pro- and anti-inflammatory cytokines, hypothesising that ghrelin exerts these effects via the HPA axis (Ziko et al. 2018). Examining the effects of both forms of ghrelin, acylated (AG) and desacylated (DAG) at all of the levels of the HPA axis we observed that des-acyl ghrelin (DAG) was unable to suppress any LPS-induced effects *in vivo* at any level of the HPA axis. Despite AG's ability to stimulate HPA axis activation, our study showed that its suppressive effect on cytokine release was clearly not due to its HPA axis effects.

Some of the mechanisms by which ghrelin suppresses inflammation have been discussed below, however, further studies are crucial in understanding beneficial effects of ghrelin as an anti-inflammatory agent. Initial work suggested that ghrelin may exert its anti-inflammatory effects via the vagus nerve. It is well recognized that ghrelin stimulates the vagus nerve (Okada et al. 2018; Date 2012) and such stimulation has shown to be beneficial in anti-inflammatory complications including sepsis and traumatic brain injury (Jacob, Wu, et al. 2010; Bansal et al. 2012). In support of this, Shah and colleagues have shown that in a rat model of radiation-combined injury and sepsis ghrelin administration significantly reduced plasma TNFα

and IL-6 levels only in non-vagotomized, but not in vagotomized animals (Shah et al. 2009). These studies, however, did not account for fluctuation of ghrelin levels based on feeding status or any effects of desacyl ghrelin on the anti-inflammatory role. Ghrelin has also shown anti-inflammatory effects by suppressing the sympathetic nervous system. Increased cytokine levels, at least partly, are mediated by norepinephrine (NE) release from the gut during sepsis and ghrelin suppresses gut-derived NE release (Jacob, Rajan, et al. 2010). Furthermore, ghrelin has been shown to inhibit activation of nuclear factor-κB (NF-κB), a well-known transcription factor involved in production of pro-inflammatory cytokines during inflammatory insults (Newton and Dixit 2012).

Another potential mechanism by which ghrelin may suppress inflammation is via recruitment of P38 mitogen-activated protein kinase (MAPK). LPS is one of the most well-known stimulators of toll-like receptor (TLR) 4 and TLR4 activation initiates a series of intracellular processes including stimulation of myeloid differentiation primary response (MYD) 88, which further triggers activation of the P38 MAPK cascade (Lamon et al. 2010). MKP-1, the first identified MAPK phosphatase, deactivates P38 MAPK after LPS stimulation (Chen et al. 2002). Increased activation of P38 MAPK leads to overproduction of inflammatory cytokines from the liver and ghrelin upregulates MKP-1 to suppress inflammation (Jacob, Rajan, et al. 2010). In addition, in a study from Slomiany and colleagues, LPS induced increased phosphorylation of P38 in gastric mucosal cells, whereas ghrelin significantly suppressed this LPS effect, indicating that ghrelin counteracts the pro-inflammatory consequences of *Helicobacter pylori* derived LPS (Slomiany and Slomiany 2013).

Ghrelin receptors are largely expressed by macrophages and ghrelin's presence suppresses inflammatory cytokine expression (Chowen and Argente 2017). In contradiction with what previous studies have shown, work from Wu *et al*, demonstrated that LPS treatment alone of Kupffer cells (the resident macrophages of the liver) and peritoneal macrophages, dramatically increases TNFα and IL-6 levels. Ghrelin co-incubation with LPS, however, was unable to further affect the elevated LPS-induced cytokine levels, suggesting that the anti-inflammatory effects of ghrelin may not be due to its direct effect on ghrelin receptors expressed on macrophages (Wu, Dong, et al. 2007). Despite the above-proposed anti-inflammatory mechanisms of ghrelin, its role in the HPA axis responses to an immune challenge has been less well characterised, indicating that further research is necessary to define ghrelin's mechanisms in inflammatory complications as a promising anti-inflammatory agent.

## Future directions

In this thesis we have contributed to the existing knowledge of the importance of the metabolic hormones ghrelin and leptin in the development of hypothalamic feeding circuits (Steculorum and Bouret 2011; Bouret and Simerly 2006; Bouret, Draper, and Simerly 2004a; Steculorum et al. 2015). We further expanded on the effects of an early life over-nutritional environment on such development considering the observations that males and females respond differently to early life nutritional challenges. It is important to note that a direct comparison between sexes of the parameters measured in this thesis needs to be considered for future studies. Despite the work described here, there are considerable gaps in our knowledge with regards to the effects of early life nutritional disturbances centrally; in long-life stress, cognition and whether other extra-hypothalamic regions are involved in such systems. Postnatal

overfeeding and consequently altered leptin and ghrelin levels, are involved in regulation of many systems including endocrine system, reproductive function, HPA axis, adipose tissue, glucose and insulin homeostasis, modification of plasma lipids and proteins, adipocytokines, oxidative stress, kidney and cardiovascular function (as reviewed in (Habbout et al. 2013; Lean and Malkova 2016)).

Of particular interest would be to determine the role of neonatal nutritional insults on the structure and function of adipose tissue. In **Chapters 2, 3, 4 and 5** we saw that early life overnutrition was associated with long-term weight gain consisting of excess adipose tissue. Excessive caloric intake and positive energy imbalance related to the obese phenotype are associated with not only over-accumulation of adipose tissue either as subcutaneous or intra-abdominal, but also with adipose tissue dysfunction including increased cell density, lipolysis and inflammatory cytokine production (from adipocytes and immune cells) and decreased adipose sensitivity to insulin as well as decreased lipogenesis (Ortega and Fernandez-Real 2013)). Inflammation and oxidative stress on the other hand further influences fat accumulation, leading to a continuous positive feedback (Fresno, Alvarez, and Cuesta 2011). Many studies have shown a close relationship between immune and metabolic systems (Schaeffler et al. 2009; Wolowczuk et al. 2008) and leptin, one of the most studied adipokines, exerts both metabolic and anti-inflammatory properties (Fresno, Alvarez, and Cuesta 2011; DiAngelo et al. 2009). However, it is not known whether early life development in combination with nutritional challenges influences this low-grade inflammation by affecting leptin levels. In this thesis we observed that neonatal overfeeding was associated with elevated leptin levels throughout life. Therefore, it will be important in future studies to measure molecular markers of inflammation

including IL-6, IL-1β, c-reactive protein, serum amyloid A and fibrinogen in the metabolically active organs such as adipose tissue, liver and muscle in the neonatally overfed and control animals during development and in adulthood. The presence of these markers has been shown in association with obesity and insulin resistance in the following rodents and human studies (Chou et al. 2014; Shoelson, Lee, and Goldfine 2006; Kwon and Pessin 2013). Furthermore, since recent studies have been suggesting that peripheral inflammation is a result of central inflammation in obesity (Weisberg et al. 2003; Thaler et al. 2012; Mori et al. 2010), it will be important to measure inflammatory markers centrally in the hypothalamus.

Ghrelin and leptin receptors are expressed in extra-hypothalamic regions, including in the hippocampus, and adult rodent studies have demonstrated a direct effect of both these hormones on synaptic plasticity and cognitive function (Beck and Pourie 2013; Harvey 2013). Previous studies have shown that early life exposure to imbalanced nutritional environment or stress can lead to long-life cognitive dysfunctions (as reviewed in (Spencer 2017). Alterations in leptin levels for instance, are associated with emotional and cognitive disorders as observed in pre-clinical and clinical studies (Guo et al. 2013; Milaneschi et al. 2016). Similarly, a reduced ability for local brain ghrelin production, especially in the temporal lobe, has been observed in Alzheimer's Disease (AD) patients (Gahete, Rubio, et al. 2010). In contrast, in animal studies, ghrelin administration in a mouse model of AD is associated with enhanced hippocampal synaptic plasticity and consequently improved learning and memory (Diano et al. 2006). However, the effects of an imbalanced nutrition during early life with regards to ghrelin and leptin's influence on cognitive development long-term remain to be further elucidated. We observed in **Chapter 3** that neonatal overfeeding

was associated with suppressed circulating ghrelin levels, and increased ghrelin receptor expression in the ARC during the first week after birth in male pups. If perturbations to the ghrelin system extend to higher order brain regions involved in cognitive processing this may suggest insufficient ghrelin levels to be associated with increased chances of the offspring developing AD later in life. We indeed have previously seen that neonatally overfed adult rats perform poorly in cognitive behavioural tests and demonstrate impaired sensitivity to learning (De Luca et al. 2016). This is important even if ghrelin levels resolve since the insults have occurred during an important developmental window and may potentially be associated with long-term detrimental effects.

As mentioned in **Chapter 6** and here in **Chapter 7**, ghrelin suppresses inflammation via a number of mechanisms including inhibiting NF-κB pathway (Wu, Zhou, et al. 2007; Tsubouchi et al. 2014), directly suppressing pro-inflammatory cytokines in monocytes and T-cells (Dixit et al. 2004) and through other suggested pathways mentioned in this thesis. Recent research has shown DAG's ability to reverse the pro-inflammatory effects of chronic high fat diet (Gortan Cappellari et al. 2016). Also, DAG is involved in adipose lipid accumulation and suppression of adipose tissue inflammation (Pereira, da Silva, and de Moraes-Vieira 2017). A mechanism via which DAG exerts these effects has been suggested by Au *et al*.. They examined the effects of DAG on breast adipose tissue inflammation in obesity, where the capacity of human adipose tissue macrophages in stimulating aromatase (the enzyme involved in the biosynthesis of estrogens) expression in primary human breast cells (ASCs) was measured. Breast adipose tissue inflammation in obesity is associated with elevated expression of aromatase. DAG treatment significantly suppressed macrophage-

dependent induction of aromatase in ASCs, identifying a new mechanism for DAG in modulating inflammation. However, these studies have not taken into account the expression of the enzymes that hydrolase AG to DAG, such as butyrylcholinesterase, in order to rule out which form of ghrelin is exerting the effects. Altered levels of butyrylcholinesterase are observed in obesity, insulin resistance, hypertension (Chen et al. 2017) and inflammatory diseases (Xu et al. 2018). Understanding the mechanisms via which AG and DAG exert their anti-inflammatory properties would be very important extending to the effects of both forms of ghrelin as promising therapeutic strategies for treatment of inflammatory conditions.

Based on our results we conclude that leptin and ghrelin are two metabolic hormones with great importance in the developmental origins of adult diseases. Nutritional disturbances during early life development alter the levels of these hormones and the systems they control long-term and are associated with long-life metabolic disturbances such as a permanent increase in body weight, despite at least partial central resilience in orexigenic/anorexigenic circuitry as measured here.

Here we also emphasise the importance of studying sex differences. Cellular and molecular changes triggered by steroid hormones and sex chromosomes throughout development define specific physiological functions and behaviours in males and females. Therefore, is very important to consider both sexes to avoid assumption of equal behaviours between sexes without specific assessment.

Important developmental windows that can alter metabolism long-term in humans are the prenatal period, early infancy, 5-7 years of age (known as adiposity rebound

period) and puberty (Power, Kuh, and Morton 2013). Our findings provide further insight into how leptin and ghrelin can affect central and peripheral feeding circuitry during development, which in humans corresponds approximately to the third trimester (Semple et al. 2013), under nutritional challenges. This information could be used to inform the public of the importance of the nutritional environment during important developmental windows in humans in order to minimize the risk of childhood obesity and associated metabolic and cognitive disorders. Therefore, the early life developmental period studied here is not only a period of high vulnerability, but also a very important window for therapeutic interventions.

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# APPENDIX

# Hypothalamic effects of neonatal diet: reversible and only partially leptin dependent

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## Abstract

Early life diet influences metabolic programming, increasing the risk for long-lasting metabolic ill health. Neonatally overfed rats have an early increase in leptin that is maintained long term and is associated with a corresponding elevation in body weight. However, the immediate and long-term effects of neonatal overfeeding on hypothalamic anorexigenic pro-opiomelanocortin (POMC) and orexigenic agouti-related peptide (AgRP)/neuropeptide Y (NPY) circuitry, and if these are directly mediated by leptin, have not yet been examined. Here, we examined the effects of neonatal overfeeding on leptin-mediated development of hypothalamic POMC and AgRP/NPY neurons and whether these effects can be normalised by neonatal leptin antagonism in male Wistar rats. Neonatal overfeeding led to an acute (neonatal) resistance of hypothalamic neurons to exogenous leptin, but this leptin resistance was resolved by adulthood. While there were no effects of neonatal overfeeding on POMC immunoreactivity in neonates or adults, the neonatal overfeeding-induced early increase in arcuate nucleus (ARC) AgRP/NPY fibres was reversed by adulthood so that neonatally overfed adults had reduced NPY immunoreactivity in the ARC compared with controls, with no further differences in AgRP immunoreactivity. Short-term neonatal leptin antagonism did not reverse the excess body weight or hyperleptinaemia in the neonatally overfed, suggesting factors other than leptin may also contribute to the phenotype. Our findings show that changes in the availability of leptin during early life period influence the development of hypothalamic connectivity short term, but this is partly resolved by adulthood indicating an adaptation to the metabolic mal-programming effects of neonatal overfeeding.

**Key Words**
- agouti-related peptide
- developmental programming
- neuropeptide Y
- pro-opiomelanocortin
- obesity

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## Introduction

Overfeeding during development has important effects on long-term body weight regulation, potentially leading to sustained obesity in adulthood. Children born to obese mothers or children who rapidly gain weight after birth are at higher risk of lifelong obesity and have an increased susceptibility to obesity-related comorbidities than those born to healthy-weight mothers and those who gain weight more slowly (Whitaker 2004, Boney *et al.* 2005). Similarly, rats raised in a postnatal environment of overnutrition – in small litters where they are able to consume more milk – become overweight early in life, and this overweight phenotype lasts into adulthood despite a normal diet after weaning (McCance 1962, Spencer & Tilbrook 2009, Clarke *et al.* 2012, Stefanidis & Spencer

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# Early life disruption to the ghrelin system with over-eating is resolved in adulthood in male rats

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ABSTRACT

Early life overweight is a significant risk factor for developmental programming of adult obesity due to changes in the availability of metabolic factors crucial for the maturation of brain appetite-regulatory circuitry. The appetite-stimulating hormone, ghrelin, has been recently identified as a major regulator of the establishment of hypothalamic feeding pathways. Ghrelin exists in circulation in two major forms, as acylated and des-acylated ghrelin. While most research has focused on acyl ghrelin, the role of neonatal des-acyl ghrelin in metabolic programming is currently unknown. Here we assessed the influences of early life overfeeding on the ghrelin system, including acyl and des-acyl ghrelin's ability to access the hypothalamus in male rats. Our data show that early life overfeeding influences the ghrelin system short-term, leading to an acute reduction in circulating des-acyl ghrelin and increased expression of the growth hormone secretagogue receptor (GHSR) in the arcuate nucleus of the hypothalamus (ARC). These changes are associated with increased neuronal activation in response to exogenous acyl, but not des-acyl, ghrelin in the ARC and the paraventricular nucleus of the hypothalamus (PVN). Interestingly, while we observed no differences in the accessibility of the ARC to acyl or des-acyl ghrelin, less exogenous acyl ghrelin reaches the PVN in the neonatally overfed. Importantly, the influences of neonatal overfeeding on the ghrelin system were not maintained into adulthood. Therefore, while early life overfeeding results in excess body weight and stimulates acute changes in the brain's sensitivity to metabolic signals, this developmental mal-programming is at least partially alleviated in adulthood.


## 1. Introduction

Global instances of obesity have reached epidemic proportions in the last few decades, including among children (Ogden et al., 2014). Epidemiological data suggest excess weight gain in the first weeks and months of life is a risk factor for obesity as the child ages (Taveras et al., 2011). Accelerated postnatal weight gain is also predictive of obesity and metabolic dysregulation in animal models (Desai et al., 2014; Habbout et al., 2013; Portella et al., 2015). We have shown that rat pups overfed during the first weeks after birth have increased weight gain compared to normally fed animals as early as postnatal day (P)7 and they maintain increased body weight until adulthood (Clarke et al., 2013; Spencer and Tilbrook, 2009; Ziko et al., 2014), suggesting that a neonatal environment of enhanced nutritional supply may be detrimental to the development and function of hypothalamic body weight-regulatory circuitry long-term.

In the late 1990s and early 2000s Ahima, Bouret and Simerley's groups established a crucial role for leptin in the development of hypothalamic pathways that regulate appetite and control body weight (Ahima et al., 1998; Bouret et al., 2004b). While in adults leptin acts as an anorexigenic signal (Cowley et al., 2001), Bouret and Simerly's work clearly demonstrates a neurotrophic effect of the leptin surge that occurs in rodents around the second week of life (Bouret et al., 2004b). In the absence of leptin, or in the presence of a premature leptin surge, the neuronal connections between the arcuate nucleus of the hypothalamus (ARC), paraventricular nucleus of the hypothalamus (PVN), lateral hypothalamus (LH), and dorsomedial hypothalamic nucleus (DMH) are suppressed, leading to metabolic complications and obesity long-term (Bouret et al.,

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RESEARCH ARTICLE

# Neonatal overfeeding disrupts pituitary ghrelin signalling in female rats long-term; Implications for the stress response

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## Abstract

The hypothalamic-pituitary-adrenal (HPA) axis responses to psychological stress are exacerbated in adult female but not male rats made obese due to overfeeding in early life. Ghrelin, traditionally known for its role in energy homeostasis, has been recently recognised for its role in coordinating the HPA responses to stress, particularly by acting directly at the anterior pituitary where the growth hormone secretagogue receptor (GHSR), the receptor for acyl ghrelin, is abundantly expressed. We therefore hypothesised that neonatal overfeeding in female rats would compromise pituitary responsiveness to ghrelin, contributing to a hyperactive central stress responsiveness. Unlike in males where hypothalamic ghrelin signalling is compromised by neonatal overfeeding, there was no effect of early life diet on circulating ghrelin or hypothalamic ghrelin signalling in females, indicating hypothalamic feeding and metabolic ghrelin circuitry remains intact. However, neonatal overfeeding did lead to long-term alterations in the pituitary ghrelin system. The neonatally overfed females had increased neonatal and reduced adult expression of GHSR and ghrelin-O-acyl transferase (GOAT) in the pituitary as well as reduced pituitary responsiveness to exogenous acyl ghrelin-induced adrenocorticotropic hormone (ACTH) release *in vitro*. These data suggest that neonatal overfeeding dysregulates pituitary ghrelin signalling long-term in females, potentially accounting for the hyper-responsive HPA axis in these animals. These findings have implications for how females may respond to stress throughout life, suggesting the way ghrelin modifies the stress response at the level of the pituitary may be less efficient in the neonatally overfed.

## Introduction

Childhood obesity affects more than 40 million people worldwide and is a significant risk factor for adult obesity and myriad co-morbid diseases and disorders, including hypothalamic-pituitary-adrenal (HPA) axis dysfunction [1]. We have previously shown that neonatal overfeeding in a rodent model induces an overweight phenotype that is maintained into adulthood in both males and females [2–4], with similar increases in fat mass and circulating leptin between the sexes [2, 4, 5]. Both male and female rats overfed *in utero* or postnatally show

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# Hyperleptinemia in Neonatally Overfed Female Rats Does Not Dysregulate Feeding Circuitry

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Neonatal overfeeding during the first weeks of life in male rats is associated with a disruption in the peripheral and central leptin systems. Neonatally overfed male rats have increased circulating leptin in the first 2 weeks of life, which corresponds to an increase in body weight compared to normally fed counterparts. These effects are associated with a short-term disruption in the connectivity of neuropeptide Y (NPY), agouti-related peptide (AgRP), and pro-opiomelanocortin (POMC) neurons within the regions of the hypothalamus responsible for control of energy balance and food intake. Female rats that are overfed during the first weeks of their life experience similar changes in circulating leptin levels as well as in their body weight. However, it has not yet been studied whether these metabolic changes are associated with the same central effects as observed in males. Here, we hypothesized that hyperleptinemia associated with neonatal overfeeding would lead to changes in central feeding circuitry in females as it does in males. We assessed hypothalamic NPY, AgRP, and POMC gene expression and immunoreactivity at 7, 12, or 14 days of age, as well as neuronal activation in response to exogenous leptin in neonatally overfed and control female rats. Neonatally overfed female rats were hyperleptinemic and were heavier than controls. However, these metabolic changes were not mirrored centrally by changes in hypothalamic NPY, AGRP, and POMC fiber density. These findings are suggestive of sex differences in the effects of neonatal overfeeding and of differences in the ability of the female and male central systems to respond to changes in the early life nutritional environment.

**Keywords: hypothalamus, nutrition, leptin, sex, satiety, obesity, neonatal**

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## INTRODUCTION

Early life obesity is associated with increased risk of developing diabetes, cardiovascular complications and, consequently, increased rates of premature death (1, 2). Clinical studies have shown an association between the nutritional environment during early life and obesity, and obesity-related comorbidities, in adulthood (3). Similarly, in a neonatally overfed animal model representative of childhood obesity, both male and female rat pups raised in small litters, with greater access to their mothers' milk, experience significantly increased body weight, accompanied by obesity-related comorbidities in adulthood compared to pups raised in normal litters. These comorbidities include hypothalamic–pituitary–adrenal (HPA) axis dysfunction, impaired reproductive function, memory

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Full-length Article

# Acylated ghrelin suppresses the cytokine response to lipopolysaccharide and does so independently of the hypothalamic-pituitary-adrenal axis

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ABSTRACT

Ghrelin, one of the major metabolic hormones involved in controlling energy balance, has recently been shown to have other properties including regulating the hypothalamic-pituitary-adrenal (HPA) axis response to psychological stress and being a potent anti-inflammatory agent. Ghrelin's HPA axis and anti-inflammatory actions have previously been identified as principally due to the acylated form (AG). However, our recent work has also suggested a role for des-acylated ghrelin (DAG) in these functions. Here we hypothesized ghrelin's anti-inflammatory activity is mediated by the HPA axis and this effect is differentially executed by AG and DAG. We gave adult male Wistar rats a concomitant injection of AG or DAG and lipopolysaccharide (LPS) and measured their effects on circulating cytokines, stress hormones and neuronal activation of the paraventricular nucleus of the hypothalamus (PVN). AG, but not DAG significantly suppressed the pro- and anti-inflammatory cytokine response induced by LPS *in vivo*. DAG also had no effects on any components of the HPA axis. AG, despite stimulating neuronal activation in the PVN *in vivo* and stimulating ACTH release from the pituitary *in vitro*, did not affect the HPA axis response to LPS. These findings suggest AG's anti-inflammatory effects are independent of its actions on the HPA axis and have implications for the potential use of this peptide for treatment of inflammatory conditions without compromising HPA axis activity.

## 1. Introduction

Ghrelin is a satiety hormone, best known for its role in stimulating feeding behavior via excitation of neuropeptide Y neurons at the arcuate nucleus of the hypothalamus. Very recently, additional functions for the peptide have come to light, including its anti-inflammatory properties (reviewed in (Baatar et al., 2011)). Thus, ghrelin dramatically improves mortality in septic shock (Chang et al., 2003a; Chang et al., 2003b) and morbidity in models of colitis (Gonzalez-Rey et al., 2006; Konturek et al., 2009) and other inflammatory challenges (Collden et al., 2017). Ghrelin likely does this, at least in part, by acting directly on immune cells, including microglia, macrophages and T-cells, to reduce the pro-inflammatory cytokine response to immune challenge (Dixit et al., 2004; Moon et al., 2009; Waseern et al., 2008).

Ghrelin is also known to regulate hypothalamic-pituitary-adrenal (HPA) axis responses to stress and may thus modify cytokine responses to immune challenge via this axis. During acute physical or psychological stress, cells in the paraventricular nucleus of the hypothalamus (PVN) secrete corticotropin-releasing hormone (CRH), which acts on the anterior pituitary to stimulate adrenocorticotropic hormone (ACTH) release. Increased circulating ACTH levels stimulate the adrenal cortex to synthesise and secrete glucocorticoids, which then initiate a series of events to aid the organism to cope with the stress (Nicolaides et al., 2015). In addition to its other roles, glucocorticoids also act to suppress nuclear factor (NF)κB-mediated cytokine transcription in immune cells (Auphan et al., 1995; Scheinman et al., 1995). Ghrelin's role in regulating the HPA axis is still imprecisely described. However, exogenous ghrelin strongly activates CRH neurons in the PVN and increases circulating glucocorticoid levels without affecting growth hormone secretagogue receptors (GHSR) on these neurons, suggestive of ghrelin's indirect effect on the apex of the HPA axis (Cabral et al., 2012). Thus, stress induces high levels of glucocorticoid release in proportion with increased circulating ghrelin (Azzam et al., 2017; Lutter et al., 2008). We have shown that ghrelin stimulates the HPA axis at the level of the pituitary by targeting the GHSR to facilitate ACTH release (Spencer et al., 2012). Ghrelin can also directly activate pituitary ACTH cells (Stevanovic et al., 2007). Therefore, we hypothesized here that ghrelin's anti-inflammatory actions on the cytokine response to an immune challenge (with lipopolysaccharide (LPS)) may be mediated by activation of the HPA axis.

Ghrelin exists in at least two biologically active isoforms: acylated ghrelin (AG), which is the result of ghrelin-o-acyl-transferase (GOAT)-

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24 June 2014

Dr Sarah Spencer
School of Health Sciences
RMIT University

Dear Luba,

**AEC 1409: Effects of neonatal overfeeding on reproductive development and function in Wistar rat.**

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from **24 June 2014** until **23 June 2017**. An approved version of the application is attached.

**Animals**
Your application has been approved to use up to **n=1400 Wistar rats (dams + litters, as outlined in your application)** over the duration of the project.

The use of animals in scientific procedures is strictly regulated by the *Australian code of practice for the care and use of animals for scientific purposes*. The above project is conducted under a Scientific Procedures and Premises License issued by the Bureau of Animal Welfare.

**Responsibilities of investigators**

1. **Dr Luba Sominsky**
2. **Dr Sarah Spencer**
3. **Ms Simone De Luca**
4. **Mr William Guohui Cai**
5. **Ms Joanne Barwood**
6. **Ms Ilvana Ziko**
7. **Dr Trisha Jenkins**

Responsibilities of investigators are described in the *Australian code of practice for the care and use of animals for scientific purposes* (section 3). Investigators have a 'personal responsibility for all matters related to the welfare of animals they use and must act in accordance with all requirements of the code. This responsibility begins when an animal is allocated to a project and ends with its fate at the completion of the project' (s.3.1.1).

**Amendments and extensions**
If you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the AEC may only deal with 'minor' amendment requests. Major amendments to projects normally require a new project application.

**Adverse events or unexpected outcomes**
As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the AEC immediately of any

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16 February 2016

Dr Sarah Spencer
School of Health Sciences
RMIT University

Dear Sarah,

**AEC 1605: *Exploring the role of different forms of ghrelin in the stress response in the brain of the Wistar rat.***

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from **1 March 2016** until **1 March 2019**. An approved version of the application is attached.

**Animals**
Your application has been approved to use up to **n=80 rats (Wistar, adult male)** over the duration of the project.

The use of animals in scientific procedures is strictly regulated by the *Australian code for the care and use of animals for scientific purposes*. The above project is conducted under a Scientific Procedures and Premises License.

**Responsibilities of investigators**

1. **Dr Sarah Spencer**
2. **Dr Luba Sominsky**
3. **Mr William Guohui Cai**
4. **Ms Simone De Luca**
5. **Ms Ilvana Ziko**

Responsibilities of investigators are described in the *Australian code for the care and use of animals for scientific purposes* (section 2.4). Investigators have a 'personal responsibility for all matters that relate to the wellbeing of animals that they use, including their housing, husbandry and care. This responsibility extends throughout the period of use approved by the AEC until provisions are made for the animal at the conclusion of their use' (s.2.4.1).

**Amendments and extensions**
If you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the AEC may only deal with 'minor' amendment requests. Major amendments to projects normally require a new project application.

**Adverse events or unexpected outcomes**
As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the AEC immediately of any

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22 January 2018

Associate Professor Sarah Spencer
School of Health and Biomedical Sciences
RMIT University

Dear Sarah,

**AEC 1725: *Exploring the role of ghrelin in the stress response in a rat model.***

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from **22 January 2018** until **22 January 2021**. An approved version of the application is attached.

**Animals**
Your application has been approved to use up to **n=160 rats (Wistar, male)** over the duration of the project.

The use of animals in scientific procedures is strictly regulated by the *Australian code for the care and use of animals for scientific purposes*. The above project is conducted under a Scientific Procedures and Premises License.

**Responsibilities of investigators**

1. **Associate Professor Sarah Spencer**
2. **Dr Luba Sominsky**
3. **Ms Ilvana Ziko**
4. **Ms Simone De Luca**
5. **Mr Alita Soch**

Responsibilities of investigators are described in the *Australian code for the care and use of animals for scientific purposes* (section 2.4). Investigators have a 'personal responsibility for all matters that relate to the wellbeing of animals that they use, including their housing, husbandry and care. This responsibility extends throughout the period of use approved by the AEC until provisions are made for the animal at the conclusion of their use' (s.2.4.1).

**Amendments and extensions**
If you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the AEC may only deal with 'minor' amendment requests. Major amendments to projects normally require a new project application.

**Adverse events or unexpected outcomes**
As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the AEC immediately of any serious or unexpected adverse effects on animals, or unforeseen events, which may affect the ethical acceptability of your project.