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Appetite Regulatory Neural Circuitry in the Prenatal Testosterone Treated and Obese Ewe

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Appetite Regulatory Neural Circuitry in the Prenatal Testosterone Treated and Obese Ewe

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Abstract and Keywords:

Body weight and energy expenditure are regulated by the nervous system. The first aim of this thesis was to characterize appetite regulatory neurons in the sheep brain which express both agouti-related protein (AgRP) and neuropeptide Y (NPY), or proopiomelanocortin (POMC) and cocaine- and amphetamine- regulated transcript (CART). We then asked whether this circuitry is altered in ewes at increased risk of obesity following prenatal testosterone (T) treatment, or in diet-induced obese ewes. Using immunocytochemistry, we found that prenatal T-treated ewes showed increased expression of the appetite stimulatory peptide, AgRP, but not the inhibitory peptide, POMC, consistent with the increased risk of obesity in these animals. In contrast, chronic diet-induced obesity led to decreased AgRP and increased POMC, suggesting compensatory mechanisms to reverse the obese state. Overall, our results suggest that distinct changes in metabolic control neurons are associated with increased predisposition, as well as expression, of obesity in adult sheep.

Keywords:

Prenatal programming, AgRP, POMC, Androgen Receptor, Insulin Receptor, Polycystic Ovarian Syndrome, Obesity, Sheep

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List of Abbreviations, Symbols, Nomenclature

3V	Third Ventricle
α	Alpha
β	Beta
γ	Gamma
ABC	Avidin-biotin-HRP conjugate
ACTH	Adrenocorticotropic hormone
AgRP	Agouti related- peptide
AR	Androgen receptor
ARC	Arcuate nucleus
C	Control
CART	Cocaine- and amphetamine- regulated transcript
DAB	Diaminobenzidine tetrahydrochloride
DHT	Dihydrotestosterone
DMH	Dorsomedial hypothalamus
E	Estrogen
ER	Estrogen receptor
F	Flutamide
f	Fornix
GnRH	Gonadotrophin releasing hormone
IgG	Immunoglobulin G
IR	Insulin receptor

LH	**Lateral hypothalamus**
MBH	**Mediobasal hypothalamus**
MCH	**Melanin concentrating hormone**
MCR	**Melanocortin receptor**
ME	**Median eminence**
MSH	**Melanocyte stimulating hormone**
mr	**Mammilary recess**
mt	**Mammillothalamic tract**
NGS	**Normal goat serum**
NPY	**Neuropeptide Y**
ObR	**Leptin receptor**
oc	**Optic Chiasm**
ot	**Optic Tract**
P	**Progesterone**
PBS	**Phosphate buffered saline**
PCOS	**Polycystic ovary syndrome**
POA	**Preoptic area**
POMC	**Proopiomelanocortin**
scn	**Suprachiasmatic nucleus**
T	**Testosterone**
vmh	**Ventromedial nucleus of hypothalamus**

Chapter 1: Literature Review and Introduction

Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder and a leading cause of infertility in women (1, 2), affecting approximately 6.5 % - 10% of reproductively aged females. The classical symptoms of PCOS include hyperandrogenism, polycystic ovary phenotype and insulin insensitivity (3). Clinically, the appearance of hirsutism or male patterned facial hair, irregularities of the menstrual cycle and increased incidence of acne emerge as distinct characteristics of this disorder (4). Challenges surrounding the understanding of PCOS have stemmed from controversy over defining the illness, due to the heterogeneity of its symptoms, in combination with its complex etiology. In 2003, the diagnosis criteria for PCOS were revised to require at least two of the following: 1. oligo- or anovulation, 2. clinical and/or biochemical signs of hyperandrogenism, and 3. polycystic ovaries with exclusion of other etiologies (such as congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome) (5).

Androgen excess, or hyperandrogenism, resulting from increased production of androgens in the theca cells of the ovary, may be partly responsible for the aforementioned symptoms (6). This hyperandrogenism is driven in part by both luteinizing hormone (LH) hypersecretion and hyperinsulinemia, additional characteristics of PCOS (7, 8). Both LH and insulin can act directly on the theca cells to increase androgen production (9, 10). In PCOS, signalling of gonadotrophin releasing

hormone (GnRH), which acts on LH and follicular stimulating hormone expressing cells of the anterior pituitary, is increased and the responsiveness to this hormone is also enhanced (3, 11). Reduced sensitivity to negative feedback mechanisms involving both estrogen (E) and progesterone (P) to GnRH neurons further contributes to the hypersecretion of GnRH and LH (12). Together these interruptions in signalling lead to profound neuroendocrine dysfunction in women with PCOS.

Insulin resistance is found in 50-70% of PCOS cases, and the incidence of obesity is between 35- 60% in PCOS women (13-15). These correlations demonstrate the significant relationship between PCOS and metabolic dysfunction. Various studies have shown that obesity exacerbates the symptoms and pathology in both PCOS women and animal models of the disorder (16, 17). As such, one of the primary treatment recommendations for these women is exercise, diet and weight loss. Reduction in weight improves menstrual cycle regularity and hyperandrogenism (18, 19). Diminishing insulin levels through weight loss or drug treatment can lead to additional decreases in androgen levels (20, 21). Although the prevalence of obesity, insulin resistance, type II diabetes and the metabolic syndrome have been well documented in PCOS, the mechanisms underlying coordinate development of these associated defects remain unclear.

Current drug treatments for PCOS include the use of metformin, flutamide and oral contraceptives (22). Metformin, an insulin sensitising drug, has been FDA approved for use in type II diabetes patients, and has been shown to improve symptoms of PCOS in women. Metformin decreases circulating androgen levels, improves ovulation and menstruation, and decreases the risk of type II diabetes in women with PCOS (23). Flutamide is an antagonist of androgen receptors (AR), and exerts its effect by blocking the agonistic action of testosterone (T) and its metabolite, dihydrotestosterone (DHT), at AR. In the literature, flutamide has been commonly used as an anti-androgen treatment of prostate cancer (24). Flutamide has been assessed as a treatment option for women with PCOS (25), and has been shown to improve features of obesity in this disorder (26, 27); however, this drug also has many established toxic side effects (28). It is clear that treatment options for women with PCOS need to be improved upon, a goal that will require better understanding of the underlying cause(s) of this disease.

The etiology of PCOS is not fully understood. There are some indications that genetics are implicated (29), and recent investigations have provided significant evidence supporting developmental origins in the manifestation of PCOS. Women with endogenous androgen excess disorders, such as congenital adrenal hyperplasia, stemming from 21 hydroxylase deficiency, or congenital adrenal virilizing tumours, even when these disorders are corrected postnatally, have increased risk of developing PCOS in their lifetime (30, 31). Moreover, when treated prenatally with excess T, sheep,

monkeys and rodents, develop symptoms of PCOS that encompass both its reproductive and metabolic deficits (3). Combined, these human and animal studies involving *in utero* exposure to T, have led to the question: is PCOS a disease of developmental origins?

Prenatal Programming of Adult Disease

The concept of prenatal programming was first proposed by Barker in 1990 (32). His theory suggests that many chronic adult conditions, such as cardiovascular disease, have their origins in early development. By definition, prenatal programming is the response of a system during a critical window of development to an exogenous or endogenous perturbation, thereby leading to an altered persistent change in adult phenotype. As research in this field has developed, certain principles of prenatal programming have been established. As reviewed by Nijland, et al. (33) these suggest that prenatal programming: 1) can permanently affect function later in life, 2) may be passed from generation to generation, and 3) can affect males and females differently. Programming may alter organ size or function, blood flow, gene/protein expression and receptor populations (33).

It is commonly acknowledged that smoking or consuming drugs and alcohol can cause permanent damage to the fetus (34, 35); however, all insults leading to developmental deficits may not be as obvious. Nutritional intake, body weight, and disease state of the

mother can have significant impact on the developing fetus. Recently, bisphenol-A, an endocrine-disrupting industrial chemical that can be found in polycarbonate plastics and that mimics the cellular effects of endogenous estradiol, has received attention for its potential role in prenatal programming (36, 37). The developing fetus can be exposed to environmental steroids in many ways: environmental chemicals, such as bisphenol-A, continued use of contraceptive steroids during pregnancy, use of anabolic steroids or the presence of a concurrently developing male fetus. Recently it was proposed that opposite sex female twins display a lowered risk of eating disorders; this role for androgen exposure in females has been debated (38, 39). The effect of exogenous exposure to prenatal sex steroids is not fully understood at this time, and further research is necessary to fully understand their influence on all physiological systems.

A hypothesis has been developed proposing prenatal programming by testosterone as a key factor in the development of PCOS. This theory derives from studies of animal models showing permanent alterations that mimic the symptoms of PCOS, as well as the clinical epidemiological studies cited above in which early exposure to excess androgens leads to increased risk of PCOS. The prenatally androgenised sheep and rhesus monkey have become well established models of PCOS, encompassing both the reproductive and metabolic dysfunctions associated with this disorder as reviewed by Dumesic, et al. (3). Primates and sheep treated with excess prenatal androgens develop the characteristic polycystic ovaries, hyperinsulinemia, hyperandrogenism, and infertility

associated with PCOS (3, 40) (Table 1.1). Interestingly, the level of T used to induce these programming effects in both female sheep and monkeys (Padmanabhan et al., unpublished) is comparable to that found in male fetuses. As the focus of this thesis is towards the metabolic dysfunction associated with PCOS, the following describes the comparable metabolic alterations between prenatally androgenised animal models and PCOS patients. Rhesus monkeys treated with prenatal T develop greater abdominal and visceral fat than their controls (41), and also exhibit altered insulin sensitivity and function (42). Prenatal T-treated sheep, similarly, demonstrate insulin sensitivity (43) and also reduced birth weight with corresponding catch-up growth (44). The effects of prenatal T on insulin function and adiposity are well described, however, until now the influences of early androgens on the central regulation of appetite and body weight in the sheep at the level of the brain have not been explored.

PCOS models using *in utero* androgenised rodents also produce reproductive and metabolic defects associated with PCOS. Female rats treated with prenatal T shortly after birth, or *in utero* show increased body weight, altered distribution of fat and insulin resistance (45, 46). Rats treated with DHT *in utero* also develop these metabolic symptoms (47). However, one challenge in studying the rodent as a model for prenatal programming of PCOS is that rodents have a shorter gestational period and a significant portion of development continues postnatally, unlike in humans, sheep and primates.

Attributes	Prenatal T-Treated Ewes	Women with PCOS
Anovulation	✔	✔
Hyperandrogenism	✔	✔
Reduced sensitivity to P4 negative feedback	✔	✔
Reduced sensitivity to E2 negative feedback	✔	✔
Reduced sensitivity to E2 positive feedback	✔	✔
Polycystic ovaries	✔	✔
Impaired fertility	✔	✔
Fetal growth retardation	✔	✔
Hypertension	✔	✔
Increased risk of obesity	✔	✔
Insulin resistance	✔	✔
Hyperlipidemia	✔	✔

Table 1.1 Comparisons between prenatal T-treated ewes and women with PCOS in terms of metabolic and reproductive symptoms. These symptoms are also produced in prenatally androgenised female rhesus monkeys (*modified from: Dumesic, et al. 2007*)

Many studies suggest that the development of obesity in adulthood may involve prenatal programming of appetite regulatory systems within the hypothalamus (examples in (48, 49), and reviewed in (50)). More specifically, it is hypothesized that the *in utero* environment of the developing fetus influences the development of appetite regulatory neuronal circuitry and in doing so, impacts its ability to properly coordinate energy balance later in life. In sheep, agouti-related peptide (AgRP), neuropeptide Y (NPY), proopiomelanocortin (POMC), cocaine- and amphetamine- regulated transcript (CART) and leptin receptor (ObR) mRNA are expressed in subsets of hypothalamic neurons prior to birth (51). Importantly, research suggests that, these neurons are

actively regulating energy balance *in utero*, co-ordinately with maternal regulation (52). Maternal nutritional or diabetic status can lead to alterations in offspring and their ability to regulate appetite and energy expenditure. A diabetic environment *in utero* alters NPY expression in rodents (49) and this effect persists into adulthood (53). Pregnant sheep that are overfed give birth to overweight offspring with increased levels of adiposity, reduced expression of leptin receptors, and dysfunctional CART expression in response to increased body mass (48). Rodents that were born to food restricted mothers, showed altered expression of NPY and POMC in response to fasting in adulthood (54). There are many examples in the literature of such perturbations, suggesting that conditions of the *in utero* environment are critical in influencing appetite regulatory circuitry of the developing fetus.

In summary, appetite regulatory neuronal circuitry is present in the hypothalamus before birth and can be programmed by environmental factors, leading to obesity later in life. Prenatal exposure to T can lead to permanent, long term metabolic deficits that mimic the symptoms of PCOS. From these two observations we have hypothesized that the metabolic dysfunction seen in animal models of PCOS may be programmed prenatally by excess androgens acting at the level of the hypothalamus.

Neural Control of Energy Homeostasis

Appetite Regulatory Peptides

The regulation of body weight is complex and involves coordination and communication of both central and peripheral signals. Within the brain, the hypothalamus plays a significant role in homeostasis, particularly in regulating reproduction and body weight. Several areas within the hypothalamus have been linked with energy balance. These include, but are not limited to, the preoptic area (POA), paraventricular nucleus (PVN), dorsomedial nucleus (DMH), lateral hypothalamus (LH), and arcuate nucleus (ARC) (55). The ARC lies adjacent to the third ventricle and neurons within this area, as well as those within the median eminence, receive direct information from blood-borne signalling molecules, as well as from other neurons both locally and at a distance.

Early experiments involving lesion studies provided evidence for the critical role of the hypothalamic nuclei in energy balance (56). Preliminary studies led to the hypothesis of the LH as the 'feeding centre' of the brain, and the ventromedial hypothalamus as the 'satiety centre' (57). As further investigation revealed the complex nature of the appetite regulatory circuitry and pathways, it became apparent that this explanation is much too simplistic; however, the hypothalamus remains the central site of energy control. It is thought that peripheral signals interact with first order neurons that exist in the ARC nucleus to inform the brain of nutrient status and alterations in body weight. Within the ARC exists two populations of neurons, expressing distinct neuropeptides:

orexigenic ARC neurons express the appetite regulatory peptides AgRP and NPY, both of which stimulate appetite, while anorexigenic ARC neurons express POMC and CART, two appetite suppressing peptides (58) (Figure 1.1). These neuropeptides are released from axon terminals arising from these cells, and act as neurotransmitters at target sites such as the PVN, where neuronal and peripheral signals are integrated to form an adaptive response to maintain energy homeostasis. Changes in the expression of AgRP/NPY and POMC/CART occur in response to both long and short term perturbations in food intake (59, 60).

POMC is a polypeptide precursor, which is cleaved to produce both adrenocorticotropic hormone (ACTH) and β-lipotrophic hormone. Further breakdown of these molecules produces the melanocyte-stimulating hormones (MSH) α, β, and γ, as well as β-endorphin and γ-lipotrophic hormone. Collectively, ACTH and α-, β-, and γ-MSH are termed the melanocortins (61). Specific enzymes within the ARC where POMC is expressed, lead to the production of α-MSH, an active appetite regulatory peptide (62). Expression of POMC in the ARC is imperative in the regulation of weight (63) and transgenic mice lacking POMC and its derivatives are significantly overweight compared with wild-type littermates (64). Mutations of the POMC gene have also been noted in obese humans (65); and although α-MSH is the primary derivative of POMC responsible for regulating weight gain in rodents, in humans β-MSH may also play a role in appetite regulation (66). MSH acts on G-protein coupled melanocortin receptors, of which there

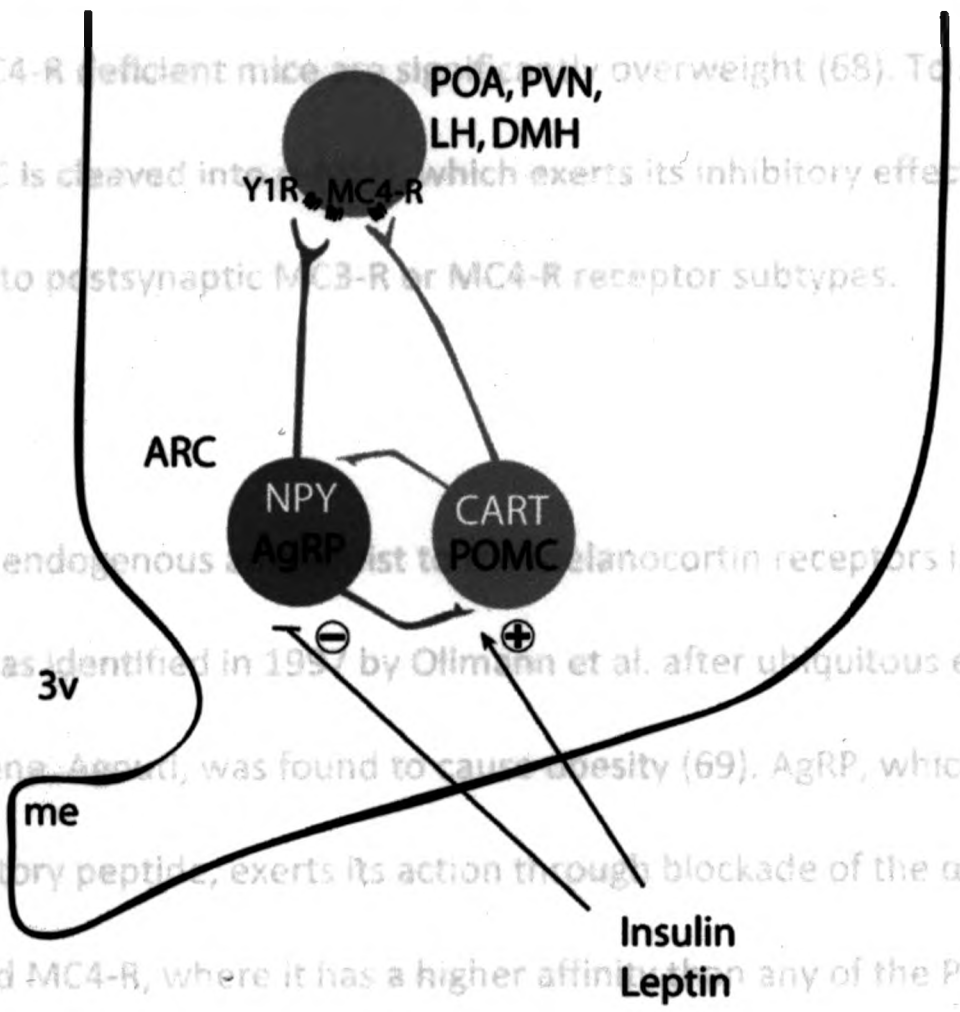

Figure 1.1 The appetite regulatory circuitry of the ARC nucleus. AgRP/NPY and POMC/CART neurons of the ARC are inhibited and activated, respectively, by both insulin and leptin and their signals project to target areas within the hypothalamus. *Abbreviations*: median eminence (me), arcuate nucleus (ARC), third ventricle (3V), neuropeptide Y (NPY), agouti-related peptide (AgRP), cocaine- and amphetamine- regulated transcript (CART), proopiomlanocortine (POMC), melanocortin receptor 4 (MC4-R), preoptic area (POA), paraventricular nucleus (PVN), lateral hypothalamus (LH), dorsomedial hypothalamus (DMH), neuropeptide Y receptor (Y1R).

are 5 known receptor subtypes: MC1-R through MC5-R. MC3-R and MC4-R are targets of α, β, and γ-MSH, and are widely expressed throughout the nervous system in the cortex, brainstem, spinal cord, thalamus and hypothalamus (67). Emphasizing its role in energy homeostasis, MC4-R deficient mice are significantly overweight (68). To summarize, POMC in the ARC is cleaved into α-MSH, which exerts its inhibitory effect on appetite through binding to postsynaptic MC3-R or MC4-R receptor subtypes.

Interestingly, an endogenous antagonist to the melanocortin receptors in the hypothalamus was identified in 1997 by Ollmann et al. after ubiquitous expression of the coat color gene, Agouti, was found to cause obesity (69). AgRP, which acts as an appetite stimulatory peptide, exerts its action through blockade of the α-MSH peptide, at the MC3-R and MC4-R, where it has a higher affinity than any of the POMC-derived peptides (70). AgRP is primarily expressed in the ARC and is upregulated during fasting (71). Increased expression of AgRP leads to obesity and hyperinsulinemia in the mouse (72). Together AgRP and POMC neurons function as a balance to maintain appropriate food intake and body weight.

NPY is another well established potent stimulator of appetite. This peptide has been localized throughout the brain, in the cortex, hippocampus, hindbrain, and hypothalamus (73). Within the ARC of the hypothalamus NPY is co-expressed with AgRP and similar to AgRP, increases appetite and food intake (74). Injection of NPY directly

into the brain produces a robust feeding response, while blocking the action of NPY with antibodies suppresses food intake (75). Various studies have explored the precise mechanism through which NPY leads to increased food intake; possibilities include reducing the latency to eat (76), enhancing the motivation to eat (77), or modulation of dietary preferences (78). NPY acts on receptors distinct from those of AgRP, the Y1, Y2 and Y5 receptors. Both Y1 and Y5 are thought to have orexigenic action, while Y2 may yield anorexigenic action (79).

CART is a neuropeptide that is known largely for its role in the rewarding properties of psychostimulants (80) and for regulating appetite and energy expenditure (81). It is colocalized with POMC neurons in the ARC (82), and has been localized to a wide variety of additional brain areas (83). Interestingly, CART is also found in the gut (84) and there is strong evidence supporting a role for CART in suppressing appetite. Intracerebroventricular injection of CART inhibits feeding in rodents, while blocking the action of CART peptide with antibodies leads to enhanced feeding (85). Fasting decreases levels of CART mRNA in the ARC of rats (86), while chronic infusions of CART reduce body weight and food intake (87). To date, the exact mechanisms underlying the action of CART are unclear, and attempts to identify a specific receptor for CART have been inconclusive.

AgRP/NPY and POMC/CART expressing neurons project to second order neurons located in other hypothalamic areas, including the POA, PVN, LH, or DMH (88), as well as outside of the hypothalamus (89). Within the LH are neurons that express either orexin/hypocretin or melanin concentrating hormone (MCH). MCH expression is increased upon fasting and injection of MCH into the brain increases feeding (90). Orexin is also known for its role in stimulating food intake (91). Anorexigenic peptides, thyrotropin-releasing hormone and corticotrophin-releasing hormone are found in the PVN (reviewed in (92)). Projections from the ARC reach both of these areas suggesting these peptides in the LH and PVN may be regulated by input from AgRP/NPY or POMC/CART ARC neurons.

Insulin and Leptin:

Both AgRP/NPY and POMC/CART neurons have been shown in rodent models to receive input from peripheral signals, such as leptin and insulin (93). Leptin, a product of the *ob* gene, is produced in adipose cells (94), circulates in levels paralleling increases in body weight and adiposity, and signals intracellularly primarily through Stat-3 (95). Leptin is a key regulator of metabolic changes in response to nutritional status, ultimately acting to diminish food intake and body weight (96). Selective knock-out of ObR in neurons leads to increased body weight and percent body fat (97). ObR are expressed in both AgRP/NPY and POMC/CART expressing neurons in the ARC (98, 99), and as reviewed by

Myers, et al. (100), leptin stimulates production of both POMC and α-MSH and, in contrast, inhibits expression of AgRP and NPY.

Although leptin is increased in obesity, excessive weight gain is also correlated with a resistance to leptin at the level of the hypothalamus (100). As such, treatments with leptin have not been overtly successful in counteracting weight gain in humans. Hypotheses of leptin resistance propose inability of leptin to reach its targets or dysfunction at the level of leptin signalling (101, 102). Leptin resistance as a result of increased weight gain and corresponding leptin levels exists at the level of the ARC; it has been shown that the secretion of AgRP, NPY, and α-MSH are insensitive to the effects of leptin in diet-induced obese mice (103).

A reduction in insulin sensitivity is also prevalent in obese and overweight humans and animals. Insulin, a hormone produced in the islets of Langerhans within the pancreas, is responsible for the regulation of glucose energy stores. Insulin resistance is described as a decreased responsiveness of target tissues to insulin and can be measured through glucose tolerance tests (OGTT) and/or fasting baseline insulin levels (I_0). OGTT involves measuring insulin levels in response to a bolus injection of glucose. Like leptin, insulin receptors are localized on AgRP/NPY and POMC/CART neurons of the ARC (104, 105). Selective knock out of insulin receptors (IR) in the brain results in increased adiposity of mice (106).

Other signalling molecules related to control of appetite/body weight:

Peripheral signals exist throughout the body, which function to signal hunger and satiety to the hypothalamus. These include cholecystokinin, peptide YY, ghrelin and the aforementioned insulin and leptin. Both cholecystokinin and peptide YY are produced in the intestine and play a role in suppressing hunger. These satiety signals are sent to the nucleus tractus solitaries through the vagal nerve (92). Ghrelin is produced in the stomach before meals and is known for its role in inducing hunger (107). The ghrelin receptor has been co-localized with NPY in the arcuate nucleus (108), and as such the function of ghrelin is likely intertwined with the previously described appetite regulatory circuitry.

In summary, the regulation of appetite is complex and involves an intricate neural circuitry that predominantly is centered in the hypothalamus but also includes other brain areas such as the limbic system and brainstem. The state of body weight is communicated to the brain through insulin and leptin, as well as other peripheral signals. These hormones signal directly to neurons in the ARC that express appetite regulatory peptides, AgRP/NPY and POMC/CART; these neurons in turn, then project to second-order targets where they can regulate additional orexigenic or anorexigenic peptides. The main function of this circuitry is to maintain body weight within a set range by influencing appetite and energy expenditure. Despite a detailed knowledge of the neuronal regulatory mechanisms underlying the control of body weight, obesity is

still a worldwide health concern that lacks effective cures and preventive treatments, and this is currently the focus of much scientific research.

Obesity

According to the World Health Organization (WHO), obesity and overweight are commonly defined as 'abnormal or excessive fat accumulation that may impair health.' Obesity results from a discrepancy between the amount of energy intake and the amount of energy expenditure. When this balance tips towards increased intake without adequate expenditure the storage of fat occurs. Generally, obesity is characterized by excess adipose tissue and elevated levels of circulating leptin and insulin. However, the abundance of both of these hormones often correlates with resistance to these regulatory signals, as described above. Although there are indications that genetics play a role in the development of obesity (109), modern lifestyle also contributes largely towards this disproportionate weight gain. A lack of exercise and increase in calorie and fat rich diets are significant factors contributing to the increased prevalence of obesity in Western society (110). Globally, greater than 400 million adults suffer from obesity, making this disorder a leading cause of morbidity and mortality worldwide (World Health Organization, 2005).

The etiology of obesity remains complex, with both genetic and environmental contributions. However, the severity of disease and the tremendous impact on health

care costs have provoked a multitude of scientific research in the field of weight regulation. In addition to its direct impact on health, obesity and weight gain are interwoven with many other chronic disorders. These include, but are not limited to, cardiovascular disease (106), stroke (111), hypertension (112), cancer (113), respiratory illness (114), psychiatric disorders (115) and type II diabetes mellitus (116). Furthermore, obesity affects individuals from all races, socioeconomic backgrounds, and geographic locations. Alarmingly, a current rising trend exists in obesity among children and adolescents (117).

Rationale and Hypothesis

The health risks and economic burden associated with the increasing prevalence of obesity is undisputable. As such, in order to most effectively address the development of a treatment strategy for overweight individuals, two important topics must be investigated: the underlying mechanisms leading to the development of obesity and the characterization of chronic state obesity.

The first aim of this thesis was to establish the ewe as a model for investigating neuronal alterations in obesity. Ewes provide a unique animal model for the study of prenatal programming, as they have a long gestational period and are more developed at the time of birth, more comparable with humans than rodent models. Furthermore, sheep

allow for easy and repeated measurements of circulating hormones, as they can repeatedly have blood drawn without stress. Sheep also have a reproductive or 'menstrual' cycle that closely mimics that of human females with respect to hormonal changes and feedback controls – a characteristic that is essential in investigating a disease such as PCOS with metabolic perturbations closely interwoven with reproductive regulation. Finally, similar to humans, sheep become obese naturally, given access to *ad libitum* food, increased long term food consumption and lack of exercise.

We started our work by characterizing the neuronal metabolic control circuitry in the sheep brain; although previous studies had examined this system in the developing sheep brain (51), studies in the adult had not been conducted. Specifically, we used immunocytochemistry to examine AgRP, NPY, POMC, and CART expressing neurons of the ARC and hypothesized that similar to the rodent, AgRP would co-express NPY, while POMC would co-express CART, each in a separate population of neurons in the ARC (Chapter 2). We further asked whether these neurons were direct targets of androgen and insulin action, two key players in the maintenance of PCOS symptoms by examining them for colocalization of androgen (Chapter 2) and insulin (Chapter 3) receptors.

The second objective of this study was aimed towards elucidating the mechanism underlying increased risk of obesity in prenatal androgen treated animals. As described,

prenatal androgens are implicated in the development of PCOS, a disorder that is highly correlated with increased risk of obesity, type II diabetes and metabolic syndrome. We hypothesized that prenatal testosterone exposure may program the appetite regulatory circuitry of the ewe, thereby predisposing these animals to metabolic dysfunction in adulthood. To test this, we examined and compared AgRP and POMC neurons in control ewes and ewes that were prenatally treated with T, DHT, or both T and flutamide (Chapter 2).

The third aim of this thesis involved characterizing AgRP/NPY and POMC/CART expressing neurons in a model of chronic obesity in the ewe. The current literature describes a wide variety of nutritional manipulations designed to further elucidate the function of AgRP/NPY and POMC/CART neurons. Our model is comparable to the development of obesity in humans, as it involves long term *ad libitum* consumption of food and sedentary lifestyle. We hypothesized that chronic obesity would alter the balance of these appetite regulatory peptides such that a drive towards regaining normal body weight would be achieved (Chapter 3).

In summary, the main goals of this thesis were to characterize the AgRP and POMC expressing neuronal populations in the arcuate nucleus of the female sheep, and to determine alterations in this population after either prenatal exposure to excess androgens, or under conditions of chronic diet-induced obesity. Our results

demonstrated a novel and direct role for androgens on the AgRP/NPY and POMC/CART populations of the ARC. Furthermore, both prenatal androgen treatment and chronic diet-induced obesity treatment revealed specific and significant alterations at the level of the appetite regulatory circuitry.

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Chapter 2: The Effects of Excess Prenatal Androgens on AgRP and POMC Expressing Neurons in the Ewe Hypothalamus

Introduction

Polycystic ovarian syndrome (PCOS) is a leading cause of infertility and currently the most prevalent reproductive endocrine disorder seen in reproductively aged women (1-3). PCOS is characterized by a combination of reproductive and metabolic deficits, which include hyperandrogenemia, disrupted menstrual cycles and fertility complications, polycystic ovaries, luteinizing hormone hypersecretion, altered neuroendocrine steroid feedback, insulin resistance and hyperinsulinemia (Reviewed in (4)). PCOS is often associated with additional metabolic morbidities such as obesity, type II diabetes, and the metabolic syndrome (5-7). Such correlations suggest that the etiology of PCOS may be intimately linked with the function of metabolic regulatory systems.

There is increasing evidence that one of the important factors in the etiology of PCOS is prenatal exposure to excess androgens (4, 8, 9). Part of this evidence arises from epidemiological studies showing that women with congenital disorders involving prenatal exposure to androgens, such as adrenal hyperplasia or adrenal virilizing tumors, are at increased risk of developing PCOS (10, 11). In addition, experimental research has shown that both female sheep and monkeys exposed *in utero* to excess levels of androgens develop symptoms in adulthood paralleling those seen in women

with PCOS (4, 12). In sheep, prenatal T exposure leads to impaired insulin sensitivity (13), postnatal catch up growth (14), and hypertension (15), in addition to reproductive symptoms that are exacerbated by excess weight (16). Recent work has focused on neuroendocrine basis for reproductive deficits seen in female sheep exposed to prenatal T, specifically hypothalamic circuitry mediating gonadal feedback control of GnRH secretion (Lehman, et al., submitted). By contrast, underlying mechanisms responsible for metabolic deficits in the prenatal T sheep model have not been previously explored.

Research in the neural control of food intake and metabolism has focused on two key subsets of neurons in the hypothalamus, neurons expressing either the agouti related peptide (AgRP) or proopiomelanocortin stimulating hormone (POMC). Both cell groups are located within the arcuate nucleus (ARC) of the hypothalamus and are largely responsible for stimulating and suppressing appetite, respectively (reviewed in (17)). POMC, a prohormone, is cleaved into a variety of peptides including melanocyte stimulating hormone, which acts directly to regulate appetite (18). Studies in rodents have shown that the majority of POMC expressing neurons co-express cocaine and amphetamine-regulated transcript (CART) (19), an additional appetite suppressing peptide, while the majority of AgRP expressing neurons co-express neuropeptide Y (NPY), which stimulates appetite (20). Leptin and insulin both function to reduce body weight (21, 22), and act on POMC and AgRP neurons, by activating and inhibiting them, respectively (19, 23-25). Together, these neurons function to maintain energy

homeostasis; an imbalance within this neural circuitry can lead to excessive weight loss or gain.

In sheep, appetite regulating peptides, AgRP, NPY, POMC and CART, are present and have been shown to be altered by exogenous factors during *in utero* development (26). In one study, pregnant ewes that were fed in excess produced offspring with altered levels of appetite regulatory peptides, POMC and CART, in the hypothalamus (27). As such, the potential exists for appetite regulatory circuitry to be altered by prenatal factors, such as excess T exposure. Furthermore, although POMC expressing neurons in sheep have been shown to express the gonadal hormone receptor for estrogen (ER) (28), it is currently unknown whether they, nor AgRP neurons, express androgen receptors (AR). Currently, the characterization of these neurons in the ewe, and whether androgens can act directly upon them, remains to be explored.

We hypothesized that prenatal androgen exposure affects the normal development of metabolic control neurons in the ARC in the ewe. This study had two main objectives: first, to characterize the distribution of AgRP and POMC expressing neurons in the female sheep hypothalamus and to determine whether these neurons are direct targets of androgen action; second, to determine whether prenatal T affects the number of immunoreactive AgRP or POMC neurons, and the density of their fiber projections from

these neurons. In addition, since prenatal T can permanently alter brain function by either its aromatization to estrogen, or its reduction to dihydrotestosterone (DHT), we used co-treatment with flutamide, an anti-androgen, to determine whether any effects of prenatal T on metabolic circuitry were mediated by its androgenic action alone. Similarly, a separate group of animals were exposed to DHT to determine whether exposure to androgens alone would be sufficient to produce such changes. The results showed permanent and region-specific effects of prenatal androgens on the number of AgRP neurons and their fiber density, but not on POMC neurons, suggesting a potential basis for the long-lasting metabolic deficits produced by prenatal T exposure.

Materials and Methods

Animals Care and Treatment:

Care of ewes, including nutrition, breeding and lambing have been previously described (14, 29, 30). Animals were raised at the Sheep Research Facility at the University of Michigan (Ann Arbor, MI, 42º 18′N). Hormone treatments of ewes occurred *in utero*, given via the pregnant mother. During a 147 day gestational term pregnant ewes received treatment injections, each week between days 30-90 of the term. Treatments included: T only (T, n=5), T and flutamide co-treatment (T + F, n=4), and dihydrotestosterone only (DHT, n=4). Those assigned to the T group received two weekly injections of 100 mg T propionate (~1.2mg/kg; Sigma-Aldrich Corp., St. Louis, MO, USA), those assigned to T + F received T propionate as described, as well as 15 mg/kg flutamide (Sigma-Aldrich) daily and finally, those assigned to DHT received two weekly doses of 100 mg dihydrotestosterone (DHT) propionate (Steraloids, Inc., Newport, RI, USA). Both T and DHT treatments were given intramuscularly in 2 mL of cottonseed oil, while flutamide was injected subcutaneously dissolved in dimethylsulfoxide (400mg/ml). These doses were chosen based on previous studies describing prenatal androgen treatment in ewes (31). Control ewes (C, n=5) did not require vehicle, as previous experiments have demonstrated no difference between offspring of vehicle versus non-vehicle treated ewes (30). Within maternal circulation, the final concentration of T was comparable to the range found in both adult and fetal males (Padmanabhan V, and Abbott DA, unpublished data). At 3-4 weeks of age, all

ewes were ovariectomized and received estrogen implants to normalize the hormonal milieu, as previously described (31). Female offspring were raised to two years of age, were of comparable body weights at one year of age (Appendix 1, Table A.1), and then sacrificed at two years of age during the breeding season. All experimental procedures have been approved by the University of Michigan Committee for the Use and Care of Animals and are consistent with National Research Council's Guide for the Care and Use of Laboratory Animals (Appendix 3).

Tissue Collection:

Prior to sacrifice each animal received two intravenous injections of 25,000 U heparin at 10 minute intervals. Ewes were then anaesthetized with ~4000 mg of intravenous sodium pentobarbital and sacrificed with rapid decapitation. Each head was perfused with 6L of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.3) mixed with 0.1% sodium nitrite and 10U/mL heparin, through the internal carotid artery. Brains were removed and stored in fixative solution at 4°C. A block of tissue containing the preoptic area and hypothalamus was removed, kept in 30% sucrose at 4°C to complete infiltration, and sectioned coronally using a freezing microtome into six parallel series (45µm slices) for each animal. Tissue was stored in cryoprotective solution (30% ethylene glycol, 1% polyvinylpyrrolidone, 30% sucrose in sodium phosphate buffer) at -20°C until processed for immunocytochemistry.

Experiment 1: Characterization of the AgRP and POMC Neurons

Dual-label immunofluorescence for AgRP/POMC, AgRP/NPY and POMC/CART:

Unless otherwise indicated, tissue sections from the mediobasal hypothalamus of control ewes were used. All incubations were at room temperature; sections were free floating under gentle agitation, and thoroughly rinsed with 0.1M phosphate buffered saline (PBS) between steps. In the first experiment tissue was immunoprocessed for both POMC and AgRP. The tissue was first blocked in incubation solution consisting of PBS with 0.4% Triton X-100 and 20% normal goat serum (NGS) (Jackson ImmunoResearch, West Grove, PA, USA) for one hour. Tissue was then incubated for 17 hours with primary antibodies diluted in incubation solution with 4% NGS: guinea pig anti-AgRP (1:1,000, Antibodies Australia, Melbourne, Australia) and rabbit anti-POMC (1:4,000, Phoenix Pharmaceuticals, Burlingame, CA, USA). Next, tissue was incubated with goat anti-guinea pig Alexa 488 and goat anti-rabbit Alexa 555 (each at 1:200, Molecular Probes, Inc., Eugene, OR, USA) consecutively for 30 minutes each. Finally, tissue was mounted onto positively charged slides and coverslipped with gelvatol (32). Additional tissue was immunoprocessed for both AgRP and NPY using the same protocol, with incubation of the primary antibodies: guinea pig anti-AgRP (1:1,000, Antibodies Australia) and rabbit anti-NPY (1:1,000, Diasorin, Stillwater, MN, USA). Respective secondary antibodies: goat anti-guinea pig Alexa 488 and goat anti-rabbit Alexa 555 (each at 1:200, Molecular Probes) were used.

Finally, additional tissue was immunoprocessed for both POMC and CART. As both the POMC and CART primary antibodies were raised in rabbit, CART was visualized using biotin tyramide amplification as described previously (33, 34). Tissue was incubated with 1% H_2O_2 for 10 minutes and with incubation solution containing 20% NGS for one hour, before incubation with rabbit anti-CART (17 hours; 1:80,000 in incubation solution with 4% NGS, Phoenix Pharmaceuticals). Tissue was then incubated with biotinylated goat anti-rabbit IgG (1 hour; 1:500 in incubation solution with 4% NGS, Vector, Burlingame, CA, USA), ABC-elite (1 hour; 1:500 in PBS, Vector), biotinylated tyramine (10 minutes; 1:250 diluted in PBS with 1µl of 3% H_2O_2 per ml, Elmer Life Sciences, Boston, MA, USA), and Alexa 555 conjugated streptavidin (30 minutes; 1:100, Invitrogen, Carlsbad, CA, USA). Subsequently, tissue was incubated with rabbit anti-POMC (17 hours; 1:4,000 in incubation solution, Phoenix Pharmaceuticals), followed by goat anti-rabbit 488 (30 minutes; 1:500 in PBS, Molecular Probes). Controls included preabsorption with immunizing peptides (see below), or omission of primary antibodies, all of which prevented staining in the appropriate wavelength (Appendix 2).

Dual-label immunoperoxidase staining for AR/AgRP and AR/POMC:

Two series of tissue sections from the mediobasal hypothalamus of control ewes (n=3) were immunoprocessed for AgRP, POMC and AR. As described above, all incubations were at room temperature; sections were free floating under gentle agitation, and thoroughly rinsed with 0.1M PBS between steps. The tissue was first blocked in

incubation solution consisting of PBS with 0.4% Triton X-100 and 20% normal goat serum (NGS) (Jackson ImmunoResearch) for one hour. Tissue was then incubated with rabbit anti-AR antibody (40 hours; 1:200 in incubation solution, Santa Cruz, CA, USA), biotinylated goat anti-rabbit IgG (1 hour; 1:250, Vector) and ABC-elite (1 hour; 1:250, Vector). Nuclear AR staining was visualized with 0.02% 3, 3'-diaminobenzidine tetrahydrochoride (DAB, Sigma), 0.08% nickel sulfate and 0.012% H_2O_2. Next, tissue was incubated with either guinea pig anti-AgRP antibody (17 hours; 1:5,000 in incubation solution, Antibodies Australia), or rabbit anti-POMC antibody (17 hours; 1:120,000 in incubation solution, Phoenix Pharmaceuticals), with secondary goat anti- guinea pig or goat anti-rabbit IgG (1 hour; 1:500, Vector) and ABC-elite (1 hour; 1:500, Vector). Reaction product for AgRP or POMC was visualized with DAB containing 0.012% H_2O_2 (10 minutes, diluted in PB). Sections were mounted onto Superfrost/Plus Microscope Slides (Fisher), dehydrated and cover-slipped with Depex Mountant. Controls included preabsorption with immunizing peptides (see below), which eliminated all staining (Appendix 2).

Data Analysis:

Fluorescent images of sections dual-labeled for AgRP/POMC, AgRP/NPY, and POMC/CART were captured with Leica DM5000B microscope and Leica DFC340fx camera. Each AgRP- or POMC-immunoreactive neuron through the entire mediobasal hypothalamus was examined for co-expression of nuclear AR, and percentage of AGRP

and POMC cells expressing AR was calculated for each animal. Brightfield images were captured using Leica DM5000B microscope and Leica DFC420 camera.

Experiment 2: The Effect of Prenatal Androgens on AgRP and POMC Neurons

AgRP and POMC Cell Counts:

Parallel series of tissue sections containing the preoptic area and hypothalamus from control, T, T + F, and DHT-treated groups were immunoprocessed simultaneously for single-label detection of AGRP or POMC. Again, all incubations were at room temperature; sections were free floating under gentle agitation, and thoroughly rinsed with 0.1M PBS between steps. Tissue was first blocked in incubation solution consisting of PBS with 0.4% Triton X-100 and 20% normal goat serum (NGS) (Jackson ImmunoResearch) for one hour, and then with guinea pig anti-AgRP (1:5,000, Antibodies Australia) or rabbit anti-POMC (1:40,000, Phoenix Pharmaceuticals) and DAB to visualize reaction products.

Analysis:

The number of immunoreactive neurons was counted in sections through the level of the rostral, middle, and caudal ARC (n=3 sections/level) in each animal using Neurolucida© software (Microbrightfield Inc., city, USA). T-tests were used for pair-wise comparisons between each of the experimental groups and the control group. In

addition, T and T+F were compared. A *p* value of less than 0.05 was considered significant.

To analyse the fiber density of AgRP immunolabelled axons, images (n=3-4 images per animal/area of interest) were taken from the preoptic area (POA, 900x900 pixels, 150 threshold, exposure 13.05 ms), paraventricular nucleus (PVN, 800x800 pixels, 140 threshold, exposure 18.46 ms), dorsomedial hypothalamus (DMH, 1280x1024 pixels, 170 threshold, exposure 13.05 ms), and lateral hypothalamus (LH, 800x800 pixels, 185 threshold, exposure 13.05 ms) under brightfield illumination using an Optronics© camara and Neurolucida© software. All images were taken using the same magnification and camera settings. Fiber density was measured using ImageJ© software (NIH, Bethesda, USA) with a fixed threshold. Mean densities were calculated for each area in each animal, and used to calculate the group means for each region. T-tests were used for pair-wise comparisons between each of the experimental groups and the control group. In addition, T and T+F were compared. A *p* value of less than 0.05 was considered significant.

Control for Antibodies

Pre-absorption of the diluted primary antibodies for POMC and CART with their respective peptide antigens (Phoenix Pharmaceuticals) at concentrations of 1μg/mL and 10μg/mL for 24 h at 4° C eliminated all immunostaining corresponding to the appropriate antibody (Fig. A.1). Similarly, pre-absorption of AR antibody with its peptide antigen (Santa Cruz) at concentration of 1 μg/ml yielded no immunoreactive staining (Fig. A.2). AgRP antibody specificity has been previously confirmed by preabsorption of the antisera with 0.5mg/ml of the peptide, which also abolished staining in the ovine ARC (35). The NPY antibody (Diasorin) used in this study has been previously characterized and produces bands on Western blots at the appropriate size for this protein (36).

Results

Distribution of AgRP and POMC neurons, and their colocalization with androgen receptor

AgRP and POMC are expressed in two separate and distinct populations of neurons within the ARC of the ovine hypothalamus (Fig. 2.1). While the distribution of AgRP and POMC neurons overlapped within the ARC, POMC neurons were scattered more laterally and dorsally compared with the AgRP expressing neurons, which were located in more medial and periventricular locations (Fig. 2.2). A small number of both AgRP and POMC neurons extended ventromedially into the internal zone of the median eminence (Fig. 2.2). The majority of all AgRP expressing neurons in the ARC also expressed NPY (Fig. 2.1). Similarly, nearly all neurons expressing POMC also expressed CART (Fig. 2.1).

Sections through the ARC double-labelled for both AR and either AgRP or POMC revealed extensive co-localization of AR with both cell types. In control ewes (n=3), the mean percentage (±SEM) of AgRP expressing neurons throughout the entire ARC which also expressed AR was 78.2±2.3 (Fig. 2.3A), while a slightly smaller percentage of POMC expressing neurons, 64.6±8.3 , were found to co-express AR (Fig. 2.3B). We saw no differences in the percentage of colocalization between AgRP or POMC cells located at different rostral-caudal levels of the ARC (data not shown). However, at each level, AgRP and POMC neurons which colocalized AR were predominantly located medially

within the ARC, while those expressing solely AgRP or POMC were located more laterally.

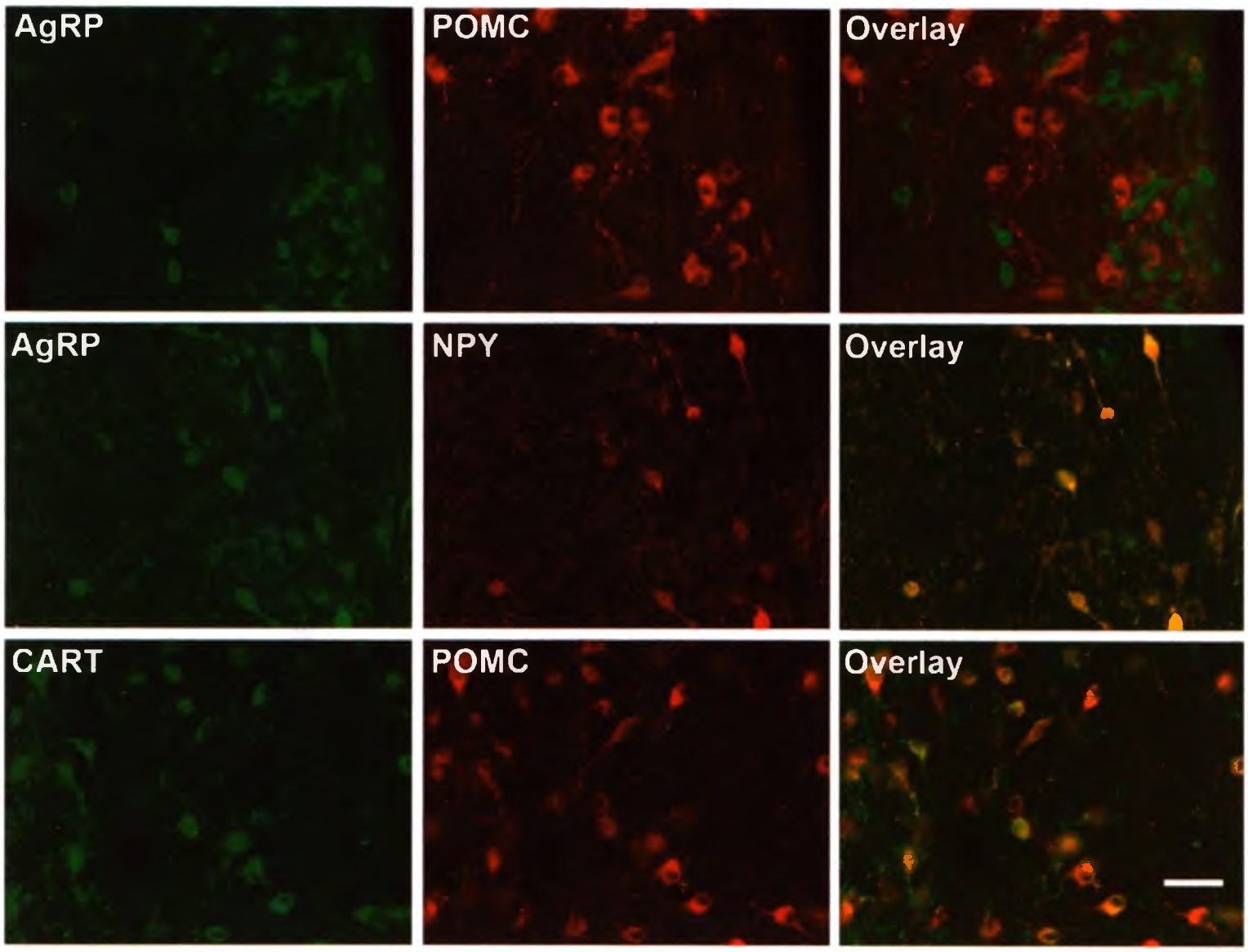

Figure 2.1 Immunofluorescent detection of agouti-related peptide (AgRP), proopiomelanocortin (POMC), neuropeptide Y (NPY) and cocaine- and amphetamine- regulated transcript (CART) in the arcuate nucleus of control ewes. AgRP and POMC expressing neurons formed separate and distinct subpopulations in the ARC (*top row*), while AgRP and NPY (*middle row*) and POMC and CART (*bottom row*) were colocalized in the same neurons. Scale bar = 20um.

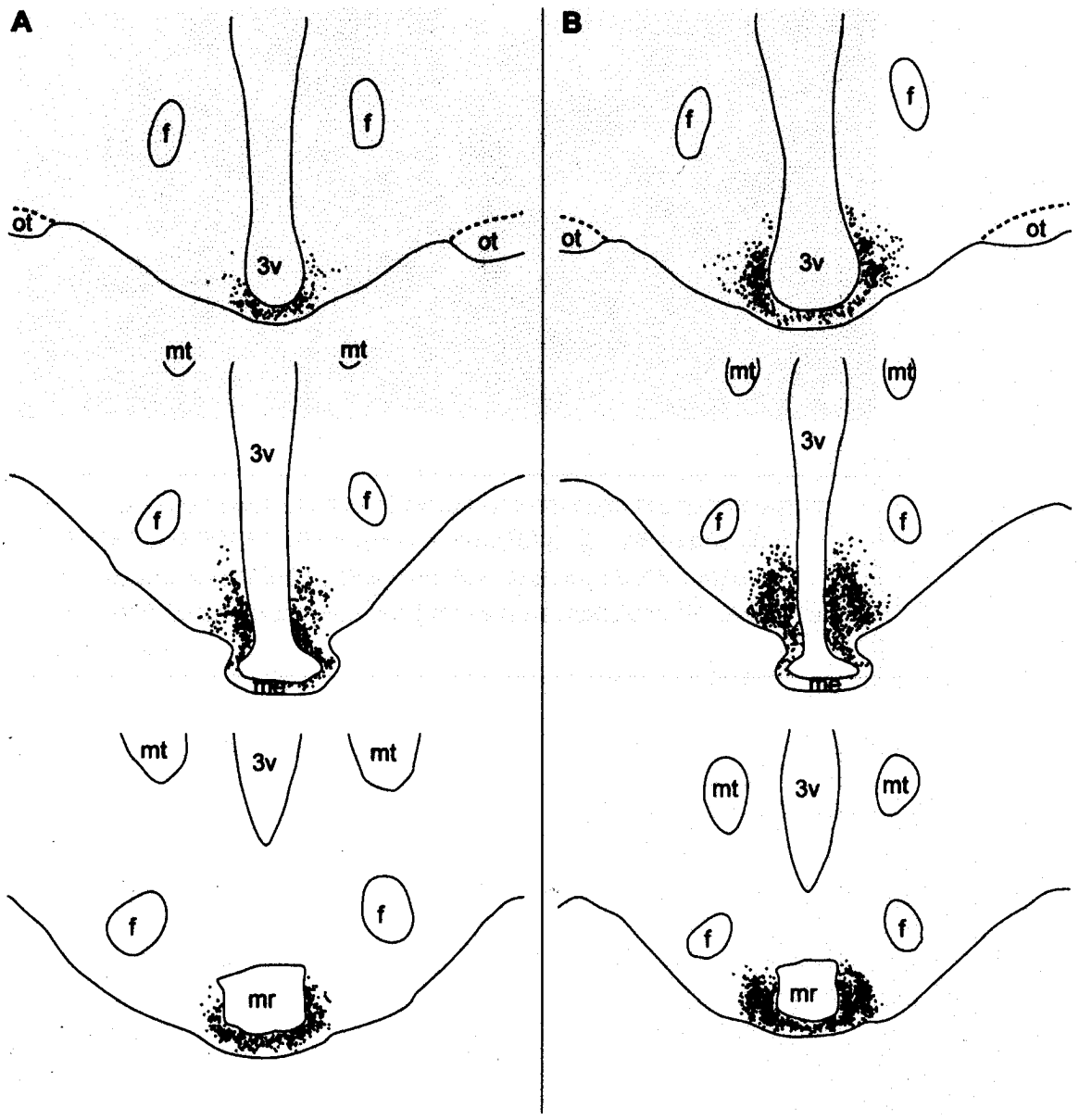

Figure 2.2 Illustrations created using Neurolucida© depicting the rostral (*top*) to caudal (*bottom*) distribution of AgRP (A) and POMC neurons (B) within rostral, middle and caudal levels (top to bottom) of the arcuate nucleus in control ewes. Individual, immunolabelled neurons are shown as filled circles. Abbreviations: fornix (f), mamillary recess (mr), mammillothalamic tract (mt), median eminence (me), optic tract (ot), third ventricle (3v).

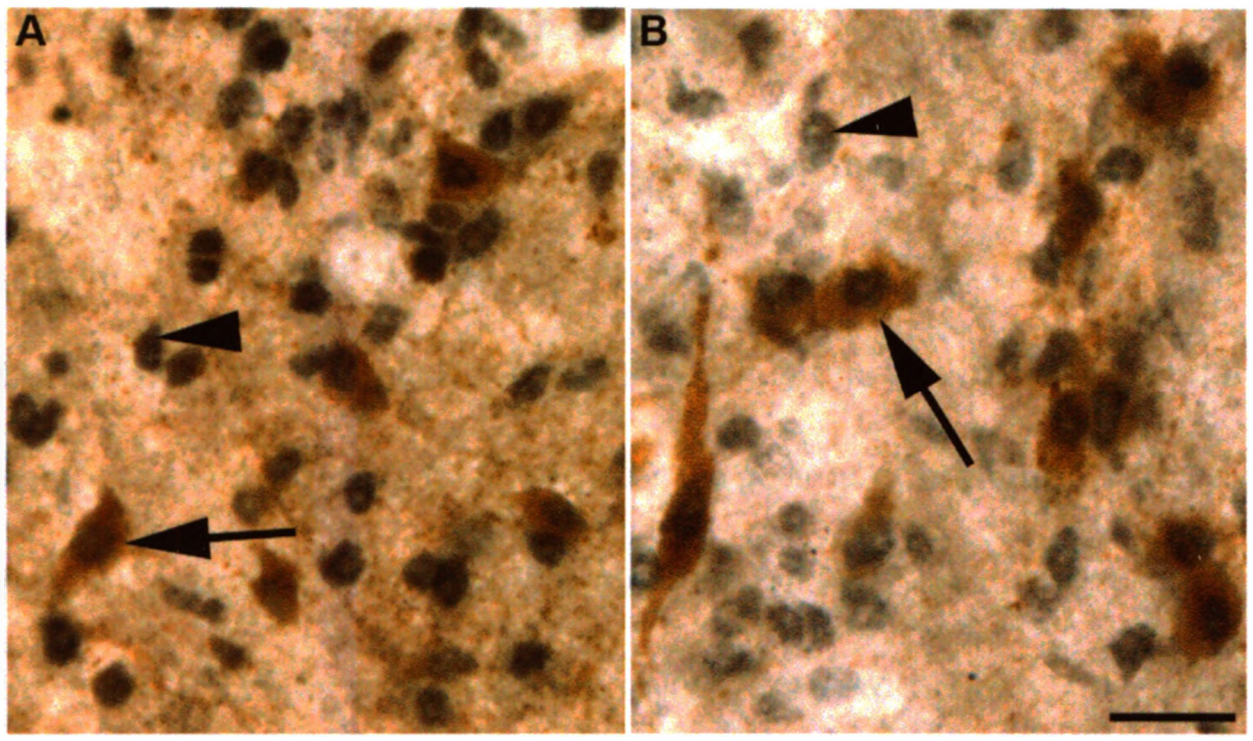

Figure 2.3 Examples of colocalization of androgen receptors within AgRP (A) and POMC (B) neurons in control ewes. AR expressing neurons are identified by black nuclear staining; both single- (*arrowheads*) and dual-labelled (*arrows*) neurons can be seen. Scale bar = 20µm.

Effects of prenatal T, T+flutamide, and DHT

Prenatal T treatment significantly increased the mean number of AgRP neurons in the middle ARC, (control: 107.57 ± 15.10 SEM; prenatal T: 251.00 ± 17.04 SEM; p=0.0002), but did not alter the number of AgRP cells in either the rostral or caudal ARC, when compared with controls (Fig. 2.4A). Co-treatment of prenatal T with an anti-androgen, flutamide, significantly reduced the number of AgRP-labelled neurons (168.75 ± 26.55 SEM; p=0.03) when compared with ewes treated with T only; however, this number was not significantly different from control ewes (Fig. 2.4A). Finally, ewes treated with prenatal DHT also showed a significantly greater number of AgRP-labelled neurons in the middle (215.75 ± 17.63 SEM; p=0.0022), but not the rostral or caudal, ARC, compared with controls (Fig. 2.4A). Thus, at the level of the middle ARC, both T and DHT treatments increased the number of AgRP immunolabelled neurons compared to controls, while co-treatment with flutamide partially blocked the effect of T alone (Fig. 2.4B). In contrast to the effects of prenatal T and DHT on AgRP neurons, we found no significant differences in the number of POMC expressing neurons between any of the treatment groups at any level of the arcuate nucleus (Fig. 2.4C, D).

Because of the changes we observed in AgRP cell number in prenatal T-treated ewes, we analysed the density of AgRP fibers in four projection areas known to be implicated in energy balance and appetite regulation: the POA, PVN, LH, and DMH (Fig. 2.5). In the POA (p=0.0090), PVN (p=0.0027), LH (p=0.0422), and DMH (p=0.0376) prenatal T treated

ewes showed significantly higher AgRP fiber density compared with control animals (Fig. 2.6A-E). In both the DMH (p=0.0222) and the PVN (p=0.0392), animals receiving the T+F treatment showed a significant decrease in AgRP fiber density compared with those receiving T only. A trend was found in both the POA (p=0.0524) and LH (p=0.0623) towards decreased AgRP fiber density in comparison with T treated ewes but these differences did not reach significance.

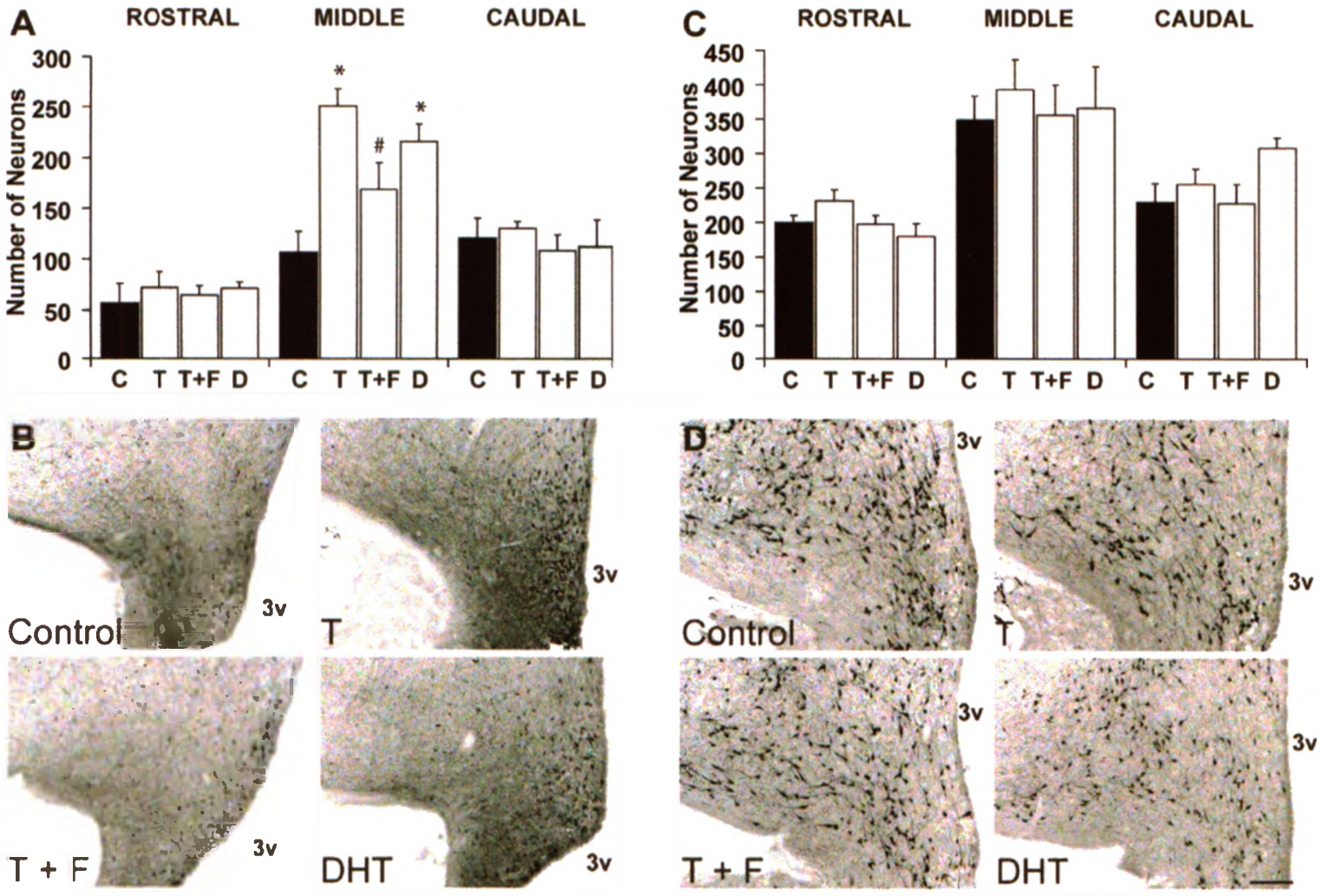

Figure 2.4 Numbers of AgRP and POMC-positive neurons at the level of the rostral, middle and caudal arcuate nucleus. Treatment groups include untreated control (C), testosterone (T), testosterone and flutamide (T+F), and dihydrotestosterone (DHT). Graphs illustrate the mean (±SEM) number of AgRP (A) or POMC (C) expressing neurons. * designates significant difference from control ($p<0.05$), # designates significant difference from T ($p<0.05$). B and D show representative low power (5X magnification) images of AgRP and POMC neurons from the middle arcuate of control and experimental animals. Scale bar = 200µm.

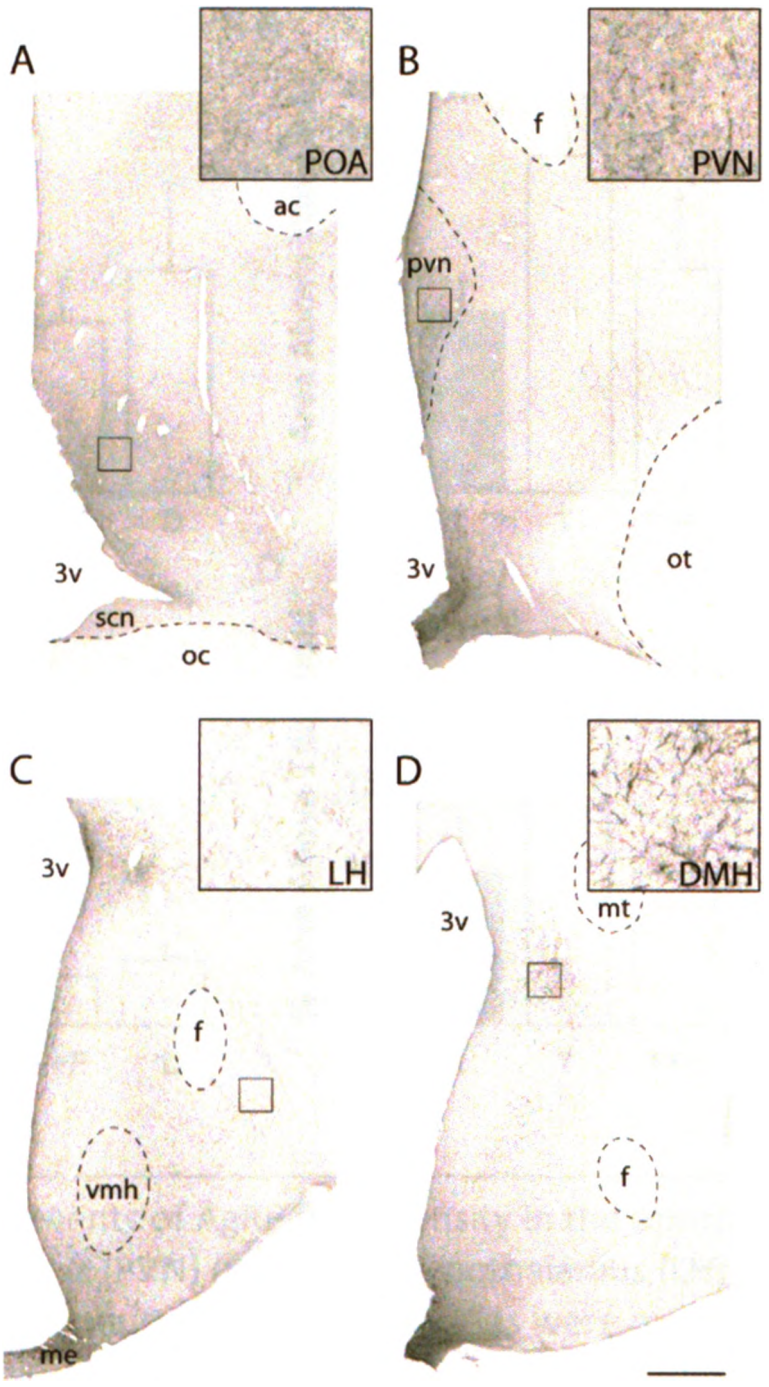

Figure 2.5 Low and high power views of AgRP fibers in the ovine preoptic area (POA) (A), paraventricular nucleus (PVN) (B), dorsomedial hypothalamus (DMH) (C), and lateral hypothalamus (LH) (D). *Black square* indicates the area from which images were captured for analysis. *Inset* - higher magnification of each area of interest. Abbreviations: anterior commissure (ac), fornix (f), mammillothalamic tract (mt), median eminence (me) optic chiasm (oc), optic tract (ot), suprachiasmatic nucleus (SCN), ventromedial hypothalamus (vmh), third ventricle (3v). Scale bar = 1mm.

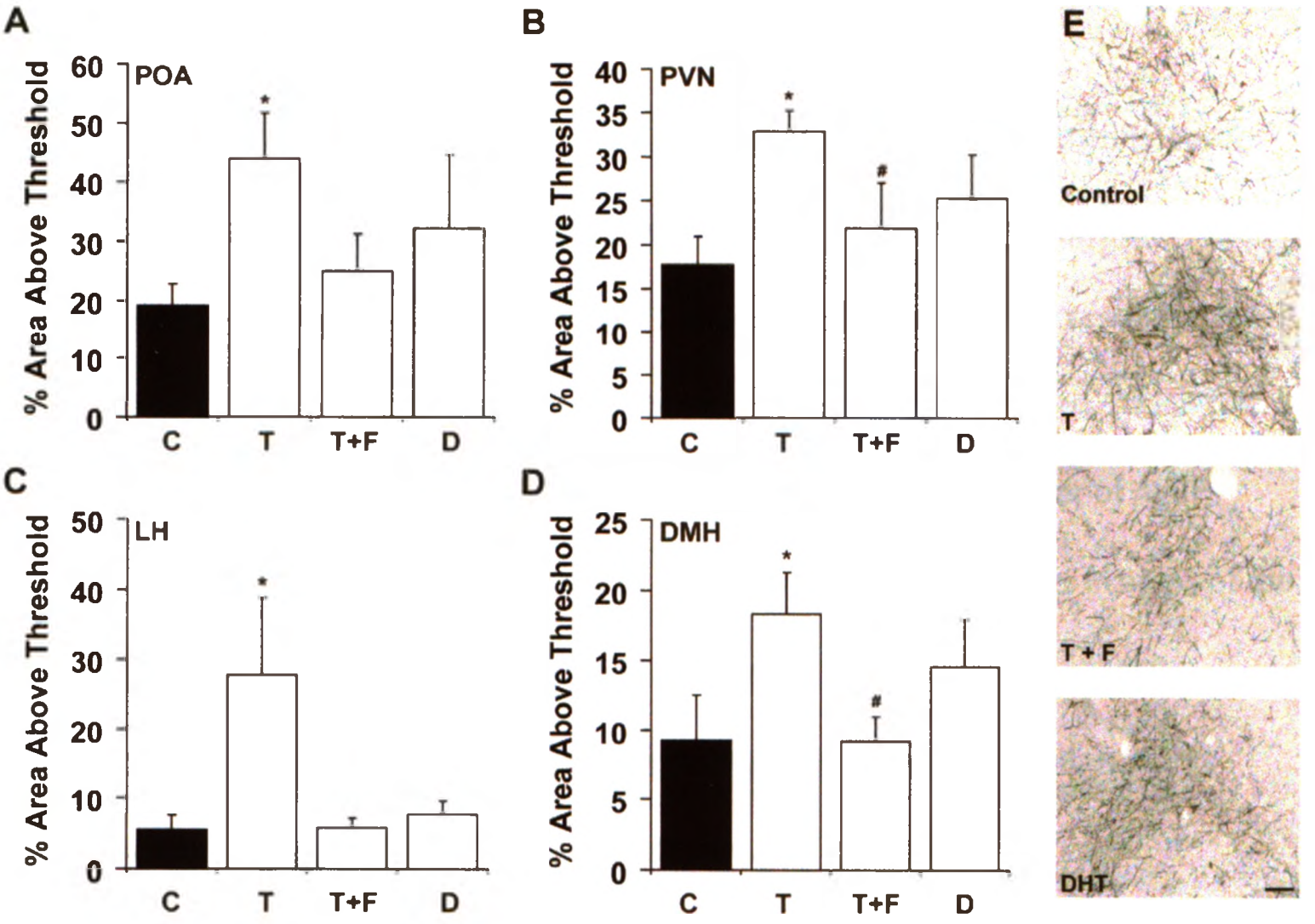

Figure 2.6. Measurements of AgRP fiber density in the preoptic area (POA) (A), paraventricular nucleus (PVN) (B), lateral hypothalamus (LH) (C), and the dorsomedial hypothalamus (DMH) (D). Densities were measured as percentage of area above threshold level (**see Methods**). Treatment groups include control (C), testosterone (T), testosterone and flutamide (T+F), and dihydrotestosterone (DHT) animals. * designates significant difference from control ($p<0.05$), # designates significant difference from T ($p<0.05$). E shows representative images (10X magnification) from the DMH of each treatment group. Scale bar = 100µm.

Discussion

The results from this study present novel evidence that the metabolic control neurons of the ARC are direct targets of androgen action in the ewe. Our results further demonstrated that prenatal treatment with T altered AgRP neurons in a restricted level of the nucleus, while POMC neurons throughout the ARC were unaffected. The effect of prenatal T on AgRP neurons was reflected by changes in both the number of AgRP-immunoreactive neurons and the density of AgRP-immunoreactive fibers in areas of the hypothalamus implicated in energy balance and regulation. Interestingly, these differences were found in the adult ewe, suggesting a permanent alteration in this metabolic circuitry as a result of prenatal exposure.

Characterization of AgRP/NPY and POMC/CART Neurons:

The first objective of this study was to characterize the distribution of AgRP and POMC expressing neurons in the adult ewe. In rodents, AgRP and POMC are expressed in two separate populations, with AgRP neurons co-expressing another orexigenic peptide, NPY (20), whereas POMC neurons co-express another anorexigenic peptide, CART (37). Thus, two distinct cell types exist: AgRP/NPY neurons, which act to stimulate appetite, and POMC/CART neurons, which act to suppress appetite. AgRP/NPY and POMC/CART neurons have also been shown to express receptors for both insulin and leptin (19, 23, 24, 38). In sheep, this system has not been as widely explored. In rams, POMC mRNA has been found to be co-localized with CART mRNA, however, little or no evidence exists

to describe co-localization of AgRP and NPY in this species, although NPY is co-localized with the leptin receptor consistent with its role as a metabolic control neuron (39, 40). Our present observations extend this work, and confirm that co-expression of AgRP/NPY and POMC/CART is conserved in the sheep brain. Further, as in the rodent brain, AgRP/NPY and POMC/CART expressing neurons are present as completely distinct and separate subpopulations of the ARC even though they largely overlap in location within the nucleus.

The role of steroid hormones in the development and function of this metabolic circuitry remains to be fully characterized. Gender differences exist in the neural control of energy balance; for example, females and males differ in their sensitivity to insulin and leptin (41, 42). POMC expressing neurons, specifically, have been shown to respond in a sexually dimorphic manner to leptin (43). Previous studies have established a direct action of steroid hormone, estrogen, on POMC/CART neurons, as well as AgRP/NPY neurons, where each of these cell types express ER (28, 44). However, the role of androgenic steroid hormones on AgRP/NPY and POMC/CART neurons has not been documented.

Our results demonstrate that the majority of both AgRP/NPY and POMC/CART neurons in the arcuate express AR, suggesting a substrate through which testosterone may directly influence energy balance in the ewe at the level of the hypothalamus. Although

colocalization of AR within AgRP neurons has not previously been described, our findings with respect to AR/POMC colocalization are in contrast to a previous study in the rat where only 3% of POMC/CART neurons were found to co-express AR (45). Several factors may have contributed to this controversy, such as: male rats were analysed, colchicine injections were used to enhance peptide detection, and POMC neurons were identified by immunolabelled β-endorphin (βEND), a derivative of POMC. Hence differences in gender, species, protocol pre-treatment, and peptide marker may have each contributed to the differences between these results.

Prenatal Programming of Metabolic Circuitry by Testosterone:

The idea of prenatal programming, first hypothesized by Barker in 1990 (46), suggests that factors present *in utero* can have long lasting effects which increase the risk of disease later in life. Appetite regulatory peptides, as well as leptin receptors, are expressed in the ARC of the sheep hypothalamus before birth (26), and have been shown to be altered by exogenous factors, such as maternal nutritional intake, during development (26, 27). Furthermore, AR is expressed in the POA and hypothalamus of the fetal sheep at day 64 of gestation (47), although the precise cell types have not yet been identified. The presence of AgRP/NPY, POMC/CART, and AR during this critical developmental stage provides the opportunity for prenatal programming by T.

Our results suggest that prenatal T indeed programs this metabolic circuitry, as we found that adult ewes that were treated prenatally with T showed alterations in the number of AgRP neurons, and the density of their axonal projections, when compared to untreated animals. This study does not address potential alterations in NPY, CART, or leptin receptors, all of which, as mentioned above, also hold the potential for prenatal programming. Although we have focused on AgRP, it is possible that alterations involving multiple peptides/receptors may be implicated in the obese phenotype commonly found in PCOS women.

We do not know whether the prenatal T-induced increase in AgRP cell number we observed is due to an increase in AgRP peptide levels resulting in greater numbers of immunodetectable cells, or reflects changes in the absolute number of AgRP cells due to alterations in either cell death and/or proliferation during development. Recent observations of KNDY neurons, a neighboring subset of ARC neurons which co-express three neuropeptides, kisspeptin (KISS), neurokinin B (NKB) and dynorphin (DYN), may be relevant to this issue: prenatal T, in the same paradigm as used here, decreases NKB and DYN in this subpopulation while expression of KISS in the same cells remains unchanged (Lehman, et al. submitted manuscript). This suggests that prenatal T does not affect the number of neurons, but, rather the level of peptide expression, which may also be true of AgRP/NPY subpopulation. In rats however, a greater number of NPY *mRNA expressing neurons are found in the ARC of male brains in comparison to*

females, suggesting a sex differences in the absolute number of these neurons (48). Additional studies in sheep to determine whether prenatal T affects NPY mRNA and peptide expression may help to resolve this question.

T can be either aromatized to estrogen or reduced to DHT, and thus may exert its effect on AgRP neurons through an estrogenic or androgenic action. T and DHT both act on the androgen receptor, with DHT having a more potent effect than T. In this study, to differentiate between the estrogenic and androgenic effects of prenatal T, one group of pregnant ewes was co-treated with both prenatal T and the anti-androgen, flutamide. Flutamide is a drug commonly used in the treatment of prostate cancer as well as androgen excess syndromes in women, where it acts to effectively bind to the androgen receptor, blocking the action of both T and DHT (49). As our results indicate, this co-treatment with flutamide blocked the increase in the number of AgRP immunoreactive neurons in prenatal T treated ewes, suggesting that prenatal T exerts its action on AgRP neurons predominantly through androgenic rather than estrogenic pathways. DHT treatment mimicked that of T treatment with respect to immunolabelled cell numbers, providing further support for the androgenic action of prenatal T. However, analysis of AgRP fiber density showed that flutamide co-treatment only partially reversed the effects of prenatal T and not all areas analysed for fiber density were affected by DHT treatment. Thus, we cannot rule out the possibility that the effects of prenatal T on the

AgRP population are conveyed by a combination of both androgenic and estrogenic actions of T, and further work will be needed to resolve this issue.

The effects of prenatal T on the number of AgRP expressing neurons were restricted to cells at the level of the middle ARC, and not seen in either rostral or caudal levels of the nucleus. It is currently unknown whether there are functional differences between the AgRP/NPY neurons at different levels of the ARC. A rostral-caudal difference has been found in the colocalization of ER in NPY neurons in ewes, with 3% of NPY neurons co-expressing ER in the rostral ARC and 10% in the caudal ARC (44). Our results, however, did not reveal any significant regional differences in the percentage of AR/AgRP or AR/POMC colocalization within the ARC. It may be that AgRP neurons located at different rostral-caudal levels differ in their pattern of efferent projections; although we found significant prenatal T-induced differences in fiber density in each of the four areas we analyzed (POA, PVN, LH, DMH), we did not examine all areas containing AgRP fiber, and it may be that AgRP neurons of the middle ARC share a pattern of efferents that distinguishes them functionally from either rostral or caudal neurons.

In each of the four target areas of AgRP neurons that we analyzed (POA, PVN, LH and DMH) ewes that received prenatal T treatments had a higher density of AgRP fibers compared to controls. It can be inferred from these results that the effect of prenatal T

on AgRP expressing neurons in the ARC involves an increased transport of AgRP peptide towards terminals; however, it is unknown whether this increase in peptide transport is reflected by increased peptide release. In addition, it would be worthwhile in future studies to determine whether prenatal T has any effect on the postsynaptic receptors for AgRP, namely MC4R, which is found in the ARC, POA, PVN, DMH, and LH regions (50, 51). While prenatal T increased fiber density in each of the four AgRP target regions examined, DHT treatment did not completely mimic this effect nor did flutamide co-treatment reverse it, suggesting that in addition to androgenic effects, part of the influence of prenatal T may be conveyed by estrogenic effects (see above).

AgRP, Insulin & PCOS:

As AgRP plays an important role in the control of energy balance and body weight, the increased number of AgRP expressing neurons in the prenatal T-treated ewes may be a factor contributing to the metabolic dysfunction and high risk of obesity seen in these animals. AgRP neurons respond to increased levels of circulating leptin and insulin, by decreasing AgRP expression, contributing to the normal homeostatic control of body weight and metabolism (reviewed in (52)). Thus, one possible explanation for the insulin resistance exhibited by prenatal T animals may be the inability of AgRP neurons to respond to circulating insulin by decreasing AgRP gene expression. Preliminary evidence in our lab has revealed AgRP neurons in the sheep ARC express insulin receptors, and it is interesting to speculate that the increased AgRP cell number seen in the middle ARC is

due to a decrease in insulin receptor expression within that subpopulation. Future studies are needed to determine whether the expression of insulin and/or leptin receptors, or their subsequent cell signalling pathways, is altered in the hypothalamus following prenatal T exposure.

Insulin sensitivity is highly prevalent in women suffering from PCOS and contributes to the high risk of type II diabetes seen in this population. Adolescent girls with PCOS are significantly more likely to exhibit symptoms of the metabolic syndrome than their peers, and this risk is increased with the presence of hyperandrogenemia (7). Weight gain in both prenatal T treated ewes (16) and PCOS women (53) appears to correlate with increased adiposity and ovulatory dysfunction. Elucidating the role of prenatal androgens in programming metabolic circuitry is therefore critical in understanding the complex associations between PCOS and metabolic syndromes, and the mechanistic basis for that association. The present results reveal an important new role for prenatal androgen in programming metabolic neuronal circuitry in the ewe, specifically by altering the number of AgRP neurons and their fibers, and suggests that androgens, T and DHT, may act directly on these and perhaps other metabolic control neurons to permanently alter their structure and function.

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Chapter 3: The Effects of Obesity on AgRP and POMC Expressing Neurons in the Ewe Hypothalamus

Introduction

An increase in the consumption and availability of high fat foods and an increasingly sedentary lifestyle, have together contributed greatly to the increased weight gain throughout Western society (1-3). According to the World Health Organization, globally, approximately 1.6 billion adults are overweight and another 400 million adults are obese. Obesity is currently an extremely prevalent health concern and a leading cause of morbidity and mortality (4). Among its direct impact on health, weight gain is closely linked to development of type II diabetes and cardiovascular disease (5, 6).

Weight regulation and energy homeostasis are known to be centrally controlled by the melanocortin system in the hypothalamus. Within the arcuate nucleus (ARC), proopiomelanocortin (POMC), a prohormone, is cleaved into alpha-melanocyte stimulating hormone (α-MSH), an appetite suppressing peptide. α-MSH acts on melanocortin receptor 3 and 4 (MC3-R and MC4-R) within the hypothalamus, to diminish appetite and increase energy expenditure. Agouti-related peptide (AgRP) is an endogenous antagonist of MC3-R and MC4-R which is expressed by a separate subpopulation of neurons in the ARC. AgRP neurons, compared to POMC neurons, have the opposite effect on body weight, effectively stimulating appetite and decreasing

energy expenditure. POMC neurons co-express another appetite suppressing peptide, cocaine and amphetamine regulated transcript (CART), while AgRP neurons co-express the potent appetite stimulator, neuropeptide Y (NPY). Both POMC/CART and AgRP/NPY expressing neurons are targets of insulin and leptin action. In rodents, the insulin receptor (IR) or its substrate, a downstream molecule of the insulin receptor signalling pathway, have been colocalized with POMC and AgRP expressing neurons in the ARC (7, 8). Leptin receptors have also been colocalized with AgRP/NPY and POMC/CART expressing neurons (9, 10). Both insulin and leptin decrease AgRP mRNA and peptide expression and increase POMC mRNA and peptide expression, thereby leading to an overall decrease in appetite and increase in energy expenditure (reviewed in (11)). Obesity is correlated with an increase in insulin and leptin levels (12, 13) and many overweight patients develop resistance to the high levels of these regulatory signals (12, 14) leading to a dysregulation of energy homeostasis.

The current literature includes studies using many different animal models of obesity, from genetic to dietary manipulations, each of which provides unique and pertinent information in understanding the complex system controlling energy homeostasis. However, genetic anomalies are rare in the general population (15), and perhaps more relevant models of obesity are those that involve high fat, *ad libitum* or restricted diets. The results from these studies remain variable in describing alterations in AgRP/NPY and

POMC/CART populations under diet manipulation, possibly due to variability in the experimental protocols themselves.

Sheep have been a valuable model for studies of the effects of dietary manipulation on appetite regulatory circuitry. Though variability exists in the precise nutritional treatment of animals in these studies, results have been fairly comparable. In one study, ewes fed a restricted diet showed an increase in AgRP mRNA expression, while those fed *ad libitum* expressed no detectable AgRP (16). In a complementary manner, decreases in NPY and AgRP expression have been documented in overfed ewes (17, 18). Notably, none of these studies has documented a change in POMC expression with respect to diet restriction. However, there is one study in lambs in which severe food restriction produced a decrease in the POMC and an increase in AgRP mRNA (19).

In the present study we chose to use a model that replicates two environmental factors, chronic overconsumption of food and lack of exercise, which have been shown to play significant roles in the development of human obesity (1-3, 20). Sheep provide a natural model of obesity as they will continue to consume food until they naturally reach an obese state and they also have a low level of activity (21). The animals in this study were adult ewes fed *ad libitum* over the majority of their life span: from 14 weeks of age to adulthood. Previously published data describes the weight gain over time of the animals

used in this study (22). The purpose of this study was to establish an understanding of the expression of appetite regulatory peptides in chronically obese and normal weight ewes.

The first objective of our study was to co-localize IR within AgRP and POMC neurons in the ARC of the ewe, as this has not yet been documented in sheep. We hypothesized that similar to the previous studies of mRNA expression, the number of AgRP immunolabelled neurons would be fewer, and the number of POMC immunolabelled neurons greater or unchanged, in obese ewes. This hypothesis was derived from the assumption that AgRP expression, as an appetite stimulatory peptide, would be decreased in overweight animals, and POMC, as an appetite suppressor peptide, would be increased in these same animals. Our immunocytochemical analysis characterized AgRP and POMC expression in the rostral, middle and caudal levels of the ARC. The results supported our original hypothesis; obese animals showed decreased AgRP and increased POMC expression, however, these differences were region specific within the rostral-caudal extent of the ARC. Overall, the results suggest that chronic obesity leads to a compensatory mechanism within the hypothalamus which attempts to drive a return to normal weight, and which is associated with changes in peptide expression within different levels of the ARC.

Materials and Methods:

Animal Care:

Care of ewes, including nutrition, breeding and lambing have been previously described (22). Animals were raised at the Sheep Research Facility at the University of Michigan (Ann Arbor, MI, 42º 18´N). Prior to weaning, female lambs were assigned to control (n=5) and overfed (n=6) treatment groups based on date of birth. In the case of twin females, each was assigned to the opposite treatment regime. Procedures were approved by the University Animal Care and Use Committee at the University of Michigan (Appendix 3).

Diet:

Diet has been previously described by Steckler et al. (22). Briefly, from birth until 14 weeks of age, lambs from both treatment groups received *ad libitum* access to both alfalfa hay and commercial feed pellets (Shur-Gain, Elma, NY; contains 18% crude protein). Starting at 14 weeks of age until time of sacrifice, those ewes designated as the overfed group were provided daily 1.7 lb corn, 0.03 lb supplement (36% crude protein), and 1.6 lb hay (equivalent to *ad libitum*). This diet was intended to increase body weight by 25% in comparison to normal weight control ewes. Control ewes were fed a diet that was designed for optimal growth without excess fat deposition. This diet consisted of 1.4 lb corn, 1.4 lb hay, and 0.03 lb supplement (36% crude protein) per day, per ewe.

Tissue Collection:

Ovary-intact ewes were raised to adulthood (2-3 yrs) and then sacrificed during the breeding season. Animals were weighed and insulin levels measured preceding sacrifice. Prior to sacrifice each animal received two intravenous injections of 25,000 U heparin at 10 minute intervals. Ewes were then anaesthetized with ~4000 mg of intravenous sodium pentobarbital and killed by rapid decapitation. Each head was perfused with 6L of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.3) mixed with 0.1% sodium nitrite and 10U/mL heparin, bilaterally through the internal carotid arteries. Brains were removed and stored in fixative solution at 4°C. A block of tissue containing the preoptic area (POA) and hypothalamus was removed, kept in 30% sucrose at 4°C to complete infiltration, and sectioned coronally (45μm) using a freezing microtome into six parallel series for each animal. Tissue was stored in cryoprotective solution (30% ethylene glycol, 1% polyvinylpyrrolidone, 30% sucrose in sodium phosphate buffer) at -20°C until use for immunocytochemistry.

Experiment 1: Insulin Receptor Colocalization with AgRP and POMC

Triple-label Immunofluorescence:

Tissue sections from the mediobasal hypothalamus (MBH) from control ewes were stained for AgRP, POMC, and IR-β. Tissue sections were rinsed with 0.1M phosphate buffered saline (PBS) and then incubated with 1% H_2O_2 for 10 minutes diluted in 0.1M

PBS. Tissue was then blocked for one hour in an incubation solution consisting of 0.1M PBS + 0.4% Triton X-100 and 20% normal goat serum (NGS) (Jackson ImmunoResearch, West Grove, PA, USA), followed by incubation for 17 hours with primary antibody, guinea pig anti-AGRP (1:1000 in incubation solution with 4% NGS, Antibodies Australia, Melbourne, Australia, catalogue # GPAAGRP.1, Lot # AS506, diluted 1:10 and stored at 4°C). With PBS washes between steps, tissue sections were then incubated with goat anti-guinea pig Alexa 488 (1:100, 30 minutes, in PBS, Molecular Probes, Inc., Eugene, OR, USA), followed by 17 hour incubation with rabbit anti- IR-β (1:300, in incubation solution with 4% NGS, Santa Cruz, C-19, SC-711, stored at 4°C). Tissue sections were then incubated with biotinylated goat anti-rabbit (1:500, 1 hour, in incubation solution with 4% NGS, Vector, Burlingame, CA, USA), ABC-elite (1:500, in PBS, Vector, 1 hour, biotinylated tyramine (BT) (1:250, 10 minutes, in PBS with 1 µl of 3% H2O2/mL, Perkin Elmer Life Sciences), alexa 555 conjugated streptavidin (1:100, 30 minutes, in PBS, Invitrogen, Carlsbad, CA, USA) and finally rabbit anti-POMC (1:4000, 17 hours, in incubation solution with 4% normal donkey serum (NDS), Phoenix Pharmaceuticals, Burlingame, CA, USA, catalogue # H-029-30, diluted 1:10 and stored at 4°C). Lastly, sections were incubated with donkey anti-rabbit Cy5 (1:4000, 30 minutes, in PBS, Molecular Probes), rinsed with PB, mounted and coverslipped with gelvatol (23).

Experiment 2: AgRP and POMC Expression in the Obese Ewe

Single-label Immunocytochemistry:

Adjacent series of every sixth section through the MBH from each of the normal weight and obese ewes were immunostained for either AgRP or POMC. Tissue was prepared as described above and incubated for 17 hours with the appropriate primary antibodies; guinea pig anti-AGRP (1:5,000, Antibodies Australia, Melbourne, Australia, catalogue # GPAAGRP.1, Lot # AS506, diluted 1:10 and stored at 4°C) or rabbit anti-POMC (1:40,000, Phoenix Pharmaceuticals, Burlingame, CA, USA, catalogue # H-029-30, diluted 1:10 and stored at 4°C). Tissue sections were then rinsed and incubated with biotinylated goat anti-guinea pig or anti-rabbit IgG (1:500, 1 hour, in blocking solution, Vector), respectively. Finally tissue sections were incubated with avidin-biotin-HRP conjugate (ABC elite,1:500, Vector) for one hour, and reaction product was visualized with 0.02% 3,3'-diaminobenzadine tetrahydrochoride (DAB) (Sigma) and 0.012% H_2O_2. Immunocytochemical controls included preabsorption of diluted antibody with appropriate purified peptides or omission of the primary antibody as described in Chapter 2 (Appendix 2).

Statistical Analysis:

The number of AgRP and POMC immunoreactive neurons were counted in sections through the level of the rostral, middle, and caudal ARC nucleus (n=2-4

sections/anatomical level) in each animal using Neurolucida© software (Microbrightfield Inc., city, USA). Data was analyzed using unpaired t-tests. For all tests the alpha level was set at 0.05.

Results:

Insulin Expression in the ARC Nucleus:

IR-β was found colocalized within both AgRP and POMC neurons in the ARC of control ewes (Figure 3.1). Single labelled AgRP, POMC, and IR-β neurons were also seen in the ARC (Figure 3.1).

Body Weights and Insulin Levels:

Ewes provided the *ad libitum* diet were significantly heavier than those fed the restricted diet, reaching approximately 25% greater body weight (Fig. 3.2A). Sheep fed an *ad libitum* diet also showed a trend (p=0.066) towards increased basal levels of plasma insulin although this difference did not reach significance (Fig. 3.2B).

Appetite Regulatory Peptides:

Immunocytochemical analysis of AgRP and POMC neurons revealed that obese ewes had a significantly fewer immunoreactive AgRP expressing neurons compared with normal weight ewes (p=0.0062) (Fig. 3.3A). This decrease was restricted to the caudal division of the Arc and no differences in AGRP neurons were found at the rostral and middle levels of the ARC. In contrast, obese ewes had a significantly greater number of immunoreactive POMC expressing neurons compared with normal weight ewes. This

effect was observed only in the rostral ARC (p=0.0036) (Fig. 3.3B), and no significant differences in POMC neurons between normal and obese ewes were seen in the middle or caudal ARC.

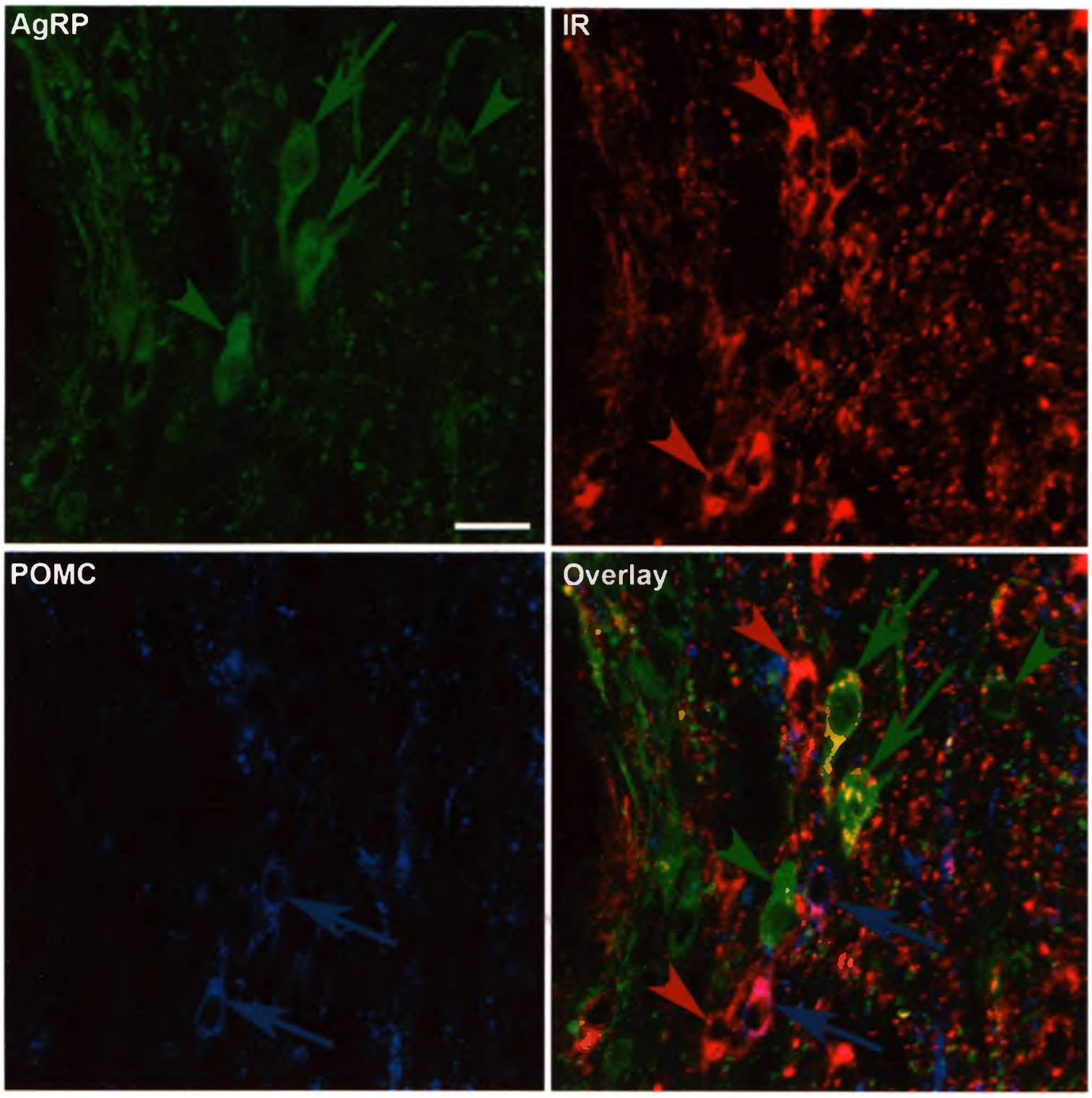

Figure 3.1 Triple labelled immunostaining of AgRP (green), IR-β (red) and POMC (blue). *Green arrows* – double labelled AgRP/IR- β neurons, *Blue arrows* – double labelled POMC/IR- β neurons, *Green arrowheads* – single labelled AgRP neurons, *Red arrowheads* – single labelled IR- β neurons. Scale bar = 20 μm

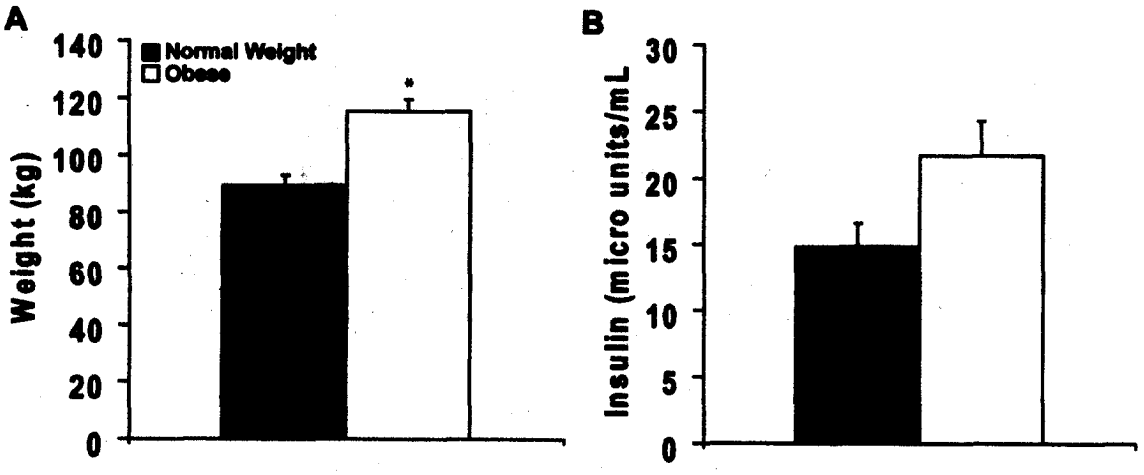

Figure 3.2 (A) Mean body weights and (B) resting levels of plasma insulin in chronically obese and normal weight ewes. Significant difference, $p < 0.05$ (*). Normal Weight (restricted diet), obese (*ad libitum* diet).

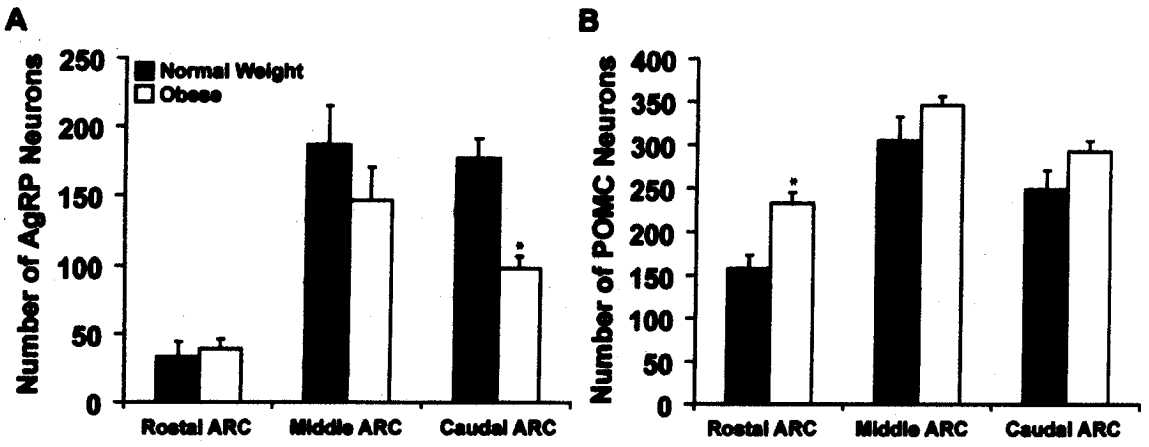

Figure 3.3 Effects of *ad libitum* and restricted access to food on AgRP and POMC expression in the arcuate nucleus (ARC) of the hypothalamus in ewes. A) Number of immunolabelled AgRP expressing neurons in the rostral, middle and caudal ARC of normal weight and obese ewes. B) Number of immunolabelled POMC expressing neurons in the rostral, middle and caudal ARC of normal weight and obese ewes. Significant differences, $p < 0.05$ (*). Normal weight (restricted diet), obese (*ad libitum* diet).

Discussion:

The results from this study suggest that the chronically obese phenotype is accompanied by changes in metabolic circuitry involving the appetite regulatory peptides, AgRP and POMC. Our analysis revealed region specific differences in the number of immunolabelled AgRP and POMC neurons within the ARC of *ad libitum* fed obese ewes compared to lean controls, with AgRP being decreased in the caudal level and POMC being increased in the rostral level of the ARC. The decrease in appetite stimulatory peptide and increase in appetite suppressing peptide in overweight ewes suggests that this system responds to chronic obesity by attempting to reverse weight gain.

The model used in this study provides insight into the effect of *chronic* obesity on appetite regulatory circuitry in the hypothalamus. The obese state of the animals in this study was developed throughout their lifetime, as they were fed *ad libitum* from a few months of age into adulthood. In humans, obesity is frequently measured through BMI, or body mass index, which is defined as the weight in kilograms divided by the square of the height in meters. The World Health Organization has defined 'overweight' and 'obese' using the measure of BMI, where a BMI falling in the range of 25-29 is classified as overweight and a BMI of 30+ is considered obese. Although BMI is a common measure of obesity it does not take into consideration other characteristics of the individual, such as the muscle mass, or adipose tissue composition, and thus is

considered a rough guide. The ewes in this study were not measured using BMI; to our knowledge the range of BMI differentiating 'overweight' and 'obese' in the ewe has not been defined. These ewes were significantly (25%) heavier in terms of body weight compared with control animals.

Thus far, studies investigating dietary induced obesity are highly variable; the time period during which the diet is manipulated, the type of restriction or overfeeding implemented, and the desired final body weights or effects, all contribute to the variability of reported data. Specifically, studies in rodents suggest that the expression of appetite regulatory peptides may be altered differentially during acute and chronic modification of food intake. In rodents that are *acutely* food deprived, NPY and AgRP expression is increased, while POMC and CART expression is decreased (24-26). In rodents that are overfed, POMC expression is increased (27). Longer term diet-induced rodent studies are more ambiguous; rats fed a highly palatable and energy rich diet over several months show increased AgRP, while high fat diet induced obese mice showed a decrease in AgRP mRNA expression. Neither of these studies found a change in α-MSH nor POMC (28, 29). Furthermore, an increase in MC4-R mRNA has been found in diet-induced obese rats, and in the same study both POMC and AgRP mRNA were decreased (30). This is in contrast to what has been found in sheep, where no difference has been found between *ad libitum* and food restricted ewes in the number of MC3-R and MC4-R

labelled cells (30, 31). The variance in these results may be partially accounted for by differences in definition of 'diet-induced obesity', and the severity of food restriction.

As described in the introduction, studies in sheep have shown an increase in AgRP mRNA expression in food restricted ewes *(16)*, and a decrease in AgRP and NPY mRNA expression in overfed animals *(17, 18)*. Furthermore, an increase in the number of NPY expressing cells, as well as a greater number of NPY/ObR expressing neurons have been documented in food restricted sheep (32). Our results describe changes in protein expression that parallel these previous findings in mRNA levels. To our knowledge, this study is the first to show a distinct alteration in POMC expression due to long term over-feeding. Given that few studies have analyzed different levels of the arcuate nucleus, it may be that, in earlier studies, changes in one level were masked by analyses of the entire nucleus.

Our results illustrate colocalization of IR within AgRP and POMC neurons in the sheep model, which complements its previously described colocalization in the rodent (7). Thus, insulin can directly regulate expression of AgRP and POMC in sheep. In rodents, insulin has been shown to inhibit the expression of AgRP and enhance expression of POMC, thereby promoting a reduction in body weight (reviewed in (11)). The obese ewes in this study exhibited a trend towards increased mean levels of plasma insulin,

suggesting that decreased AgRP and increased POMC expression may be a result of increased insulin action. However, unpublished data suggest that the obese ewes used in this study were insulin resistant, as glucose infusion produced a greater increase in plasma insulin levels compared with the normal weight controls, indicating a reduction in insulin sensitivity (Padmanabhan et al, unpublished). Therefore, it is unclear if the alterations in AgRP and POMC levels are directly influenced by elevated insulin levels or reflect a loss of responsiveness to insulin.

Similar to insulin, leptin has a direct affect on AgRP and POMC neurons, and acts to lower body weight (32). Leptin is secreted from adipose cells, and increased adiposity is correlated with increased leptin levels (33). In the obese ewes described in this study, it could be hypothesized that leptin levels were increased in conjunction with the increased weight gain, and in effect contributed to the diminished expression of AgRP and enhanced expression of POMC. Current data suggest that there is a regional, rostro-caudal heterogeneity in the AgRP and POMC populations. A recent paper, described differences between the rostral and caudal AgRP expressing neurons in the mouse hypothalamus in response to leptin, where AgRP expression in the caudal, but not rostral, region was diminished following leptin injections (34). It has also been shown that fasting in mice reduces POMC mRNA expression selectively in the rostral arcuate, and that leptin increases POMC expression in this area (35). As such, it would be of interest to examine leptin receptor colocalization in AgRP and POMC populations in our

chronically obese model. Together these data suggest regional differences in responsiveness to leptin and/or insulin within the population of AgRP and POMC neurons. We do not know whether these regional differences in responsiveness correspond to topographical differences in the axonal projections of rostral vs. caudal AgRp or POMC neurons, nor what the functional implication of such differences might be.

This study provides a description of AgRP and POMC expression in the chronically obese brain; future studies may involve analysing NPY and CART expression, as well as expression of melanocortin receptors, in a similar manner. Moreover, since there is evidence for inheritance of the effects of obesity (36), future studies may determine if dietary-induced changes in the AgRP and POMC populations are transgenerationally expressed in second generation offspring. The ewe provides an exceptional model for studies involving prenatal development, as their gestational period and stage of development at birth is more comparable to that of humans than rodents, and prenatal programming of appetite regulatory circuitry in offspring has been demonstrated by increasing food intake of pregnant ewes (37). Furthermore, the AgRP/NPY and POMC/CART expressing neurons are developed *in utero* and thus have the potential to be programmed by maternal and environmental factors in the ewe (38).

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Chapter 4: Summary and Discussion

A key aspect of this investigation was the use of the female sheep as an animal model. In this study we analysed two distinct models; we first investigated normal weight ewes that were predisposed to obesity, as a consequence of prenatal androgen exposure (a model for PCOS) and secondly we investigated non-hormone treated overweight ewes that were chronically overfed to reach an obese state (a model for chronic obesity). Prenatal programming can be effectively and efficiently studied in sheep because they have a long gestational period paralleling that of humans. An additional benefit is that sheep, although they are a large mammal, have a relatively shorter overall developmental timeline than primates and can therefore be studied from birth into adulthood in a shorter time frame. Finally, sheep provide a unique and clinically relevant model of chronic obesity that can easily be created by allowing *ad libitum* food intake and sedentary lifestyle. The results of the studies described in the preceding chapters provide important baseline data characterizing AgRP and POMC neurons in an obese state, and in a state of predisposition to obesity. However, prior to investigating these models, the first aim of this thesis was to describe the appetite regulatory neuronal circuitry in the ARC of the ewe, creating a basis for future studies of appetite regulation in the ewe.

In characterizing the appetite regulatory neurons, we established similarities that exist between the ewe and previously studied species. Similar to rodents, AgRP/NPY and POMC/CART expressing neurons are distinct and defined populations in the ovine ARC, where each type of neuron also expresses receptors for insulin (Chapter 2 & 3). Furthermore, we have established both AgRP/NPY and POMC/CART neurons as targets for the action of androgens, by demonstrating for the first time the co-expression of AR protein with the majority of both AgRP and POMC expressing neurons in the ARC (Chapter 2). This discovery invokes questions about the direct action of androgens at the level of the ARC that may underlie gender differences in control of food intake and energy balance. Establishing a comprehensive characterization of these neurons was essential before any manipulations of this circuitry could be properly analysed.

In chapter two we demonstrated that prenatal exposure to androgens can permanently program appetite regulatory circuitry in the ewe. More specifically that AgRP, appetite stimulatory peptide, expression in the ARC is increased in the adult ewe following prenatal treatment with excess androgens. Furthermore, we determined that this programming of appetite regulatory peptide expression is likely mediated via androgenic action. Finally we hypothesized that insulin resistance may be partly responsible for the increased expression of AgRP (summarized in Table 4.1).

Insulin resistance is commonly seen in prenatal T treated animals (1) and may contribute to the changes seen in appetite regulatory peptide expression, as insulin has been well established to negatively regulate AgRP expression, leading to an overall decrease in appetite and increase in energy expenditure (reviewed in (2)). Based on these findings, we hypothesized that insulin resistance following prenatal androgen exposure results as a consequence of long-term, permanent changes at the level of the brain, specifically in the decreased responsiveness of AgRP neurons to negative regulation by circulating insulin. One possibility is that prenatal T treatment leads to a decrease in insulin receptor expression within AgRP neurons, specifically in the middle ARC, and that in the absence of the receptor, AgRP peptide levels are chronically increased. Further studies are required to test this and other hypotheses; regardless, it is clear that expression of appetite regulatory peptides are likely implicated in the metabolic dysfunction associated with PCOS, and more specifically that these alterations could be the result of prenatal androgen exposure.

There are additional unanswered questions that arise from the results of our prenatal androgen study. For example, both NPY and CART are implicated in appetite regulation and may also be modulated by androgens during prenatal programming; future studies are necessary to elucidate the possible effects of prenatal androgens on these neuropeptides. Our finding that AgRP expression is upregulated in prenatal T treated ewes suggest that its release at postsynaptic targets should also be enhanced. For this

reason, future studies investigating the activation and expression of MC4-R in the POA, PVN, DMH, and LH would be of particular interest. Finally, as previously described, hyperinsulinemia and hyperandrogenism are common characteristics of PCOS. Although we have described evidence for co-localization of insulin and androgen receptors within AgRP neurons, we do not know whether their co-localization is altered in prenatal T treated ewes, and whether it changes in a regionally-specific manner. As a baseline, we have described altered AgRP expression in the hypothalamus of prenatal T treated ewes, setting the stage for future studies directed towards investigating modulatory inputs to these neurons, as well as possible effects at their postsynaptic sites of action.

Our studies provide support for the current hypothesis for the development of PCOS, specifically the role of prenatal exposure to androgens *in utero*. Prenatal T treatment of fetal ewes produces a metabolic phenotype comparable to that seen in women with PCOS, which includes hyperinsulinemia and an increased risk of type II diabetes and obesity (Table 1.1). It is important to note that in our study of prenatal androgen treatment ewes were fed a restricted diet to maintain a 'normal' body weight in control, prenatal T, DHT and T+F treated ewes. At the time of sacrifice, these ewes were of comparable body weight; however, it is known that prenatal androgen treated ewes are at increased risk of obesity provided *ad libitum* diet (3). As such, we established this as a model of predisposition towards obesity (Figure 4.1). The next appropriate step in this

study would be to investigate the appetite regulatory peptides of chronically obese ewes that also received prenatal androgen treatment.

In Chapter 3, our investigation of AgRP and POMC expression in chronically obese ewes revealed changes in the expression of these peptides, where AgRP expression was decreased and POMC expression increased, differentially at distinct levels of the ARC. This change was hypothesized to be a compensatory mechanism, which may be interpreted as tilting the energy homeostasis balance towards a decrease in appetite and an increase in energy expenditure. It appears this shift of balance is established over time due to excessive weight gain and elevated levels of insulin (summarized in Table 4.1 and Figure 4.2). Although not specifically analysed in this study, it is likely that an increase in leptin also contributes to this imbalance. Curiously, our observations are contrary to the resistance to insulin and leptin that is often reported in both human and animal studies of diet-induced obesity (reviewed (4, 5)). To clarify the roles of insulin and leptin, it would be of value to determine sensitivity to these hormones at the level of the hypothalamus in similar models of chronic obesity.

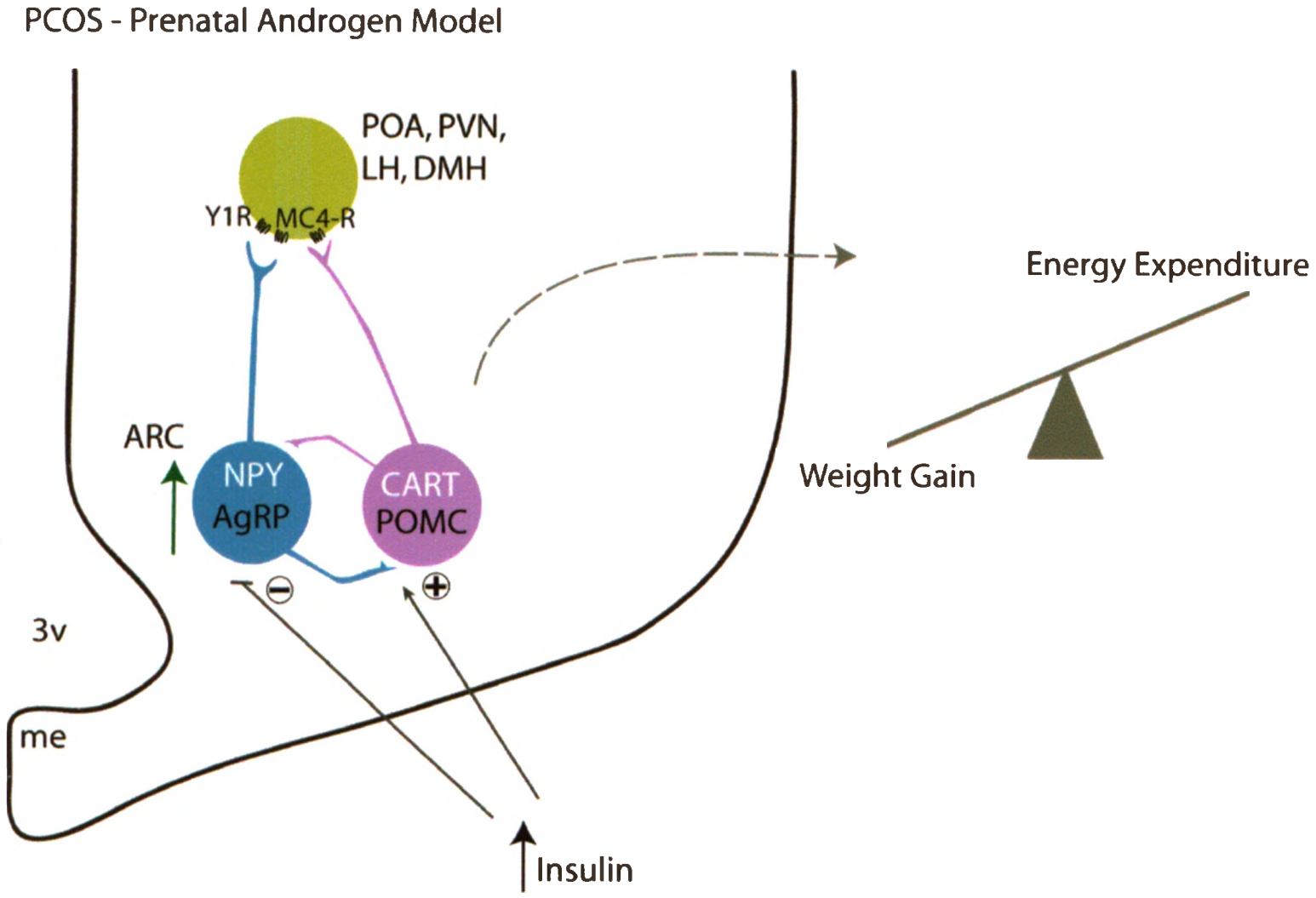

Figure 4.1 The appetite regulatory circuitry of the ARC nucleus of the ewe is altered by exposure to excess testosterone *in utero*. AgRP expression in the ARC is increased. This may be a result of insulin resistance and may contribute to weight gain in these animals. *Abbreviations*: median eminence (me), arcuate nucleus (ARC), third ventricle (3V), neuropeptide Y (NPY), agouti-related peptide (AgRP), cocaine- and amphetamine- regulated transcript (CART), proopiomlanocortine (POMC), melanocortin receptor 4 (MC4-R), preoptic area (POA), paraventricular nucleus (PVN), lateral hypothalamus (LH), dorsomedial hypothalamus (DMH), neuropeptide Y receptor (Y1R).

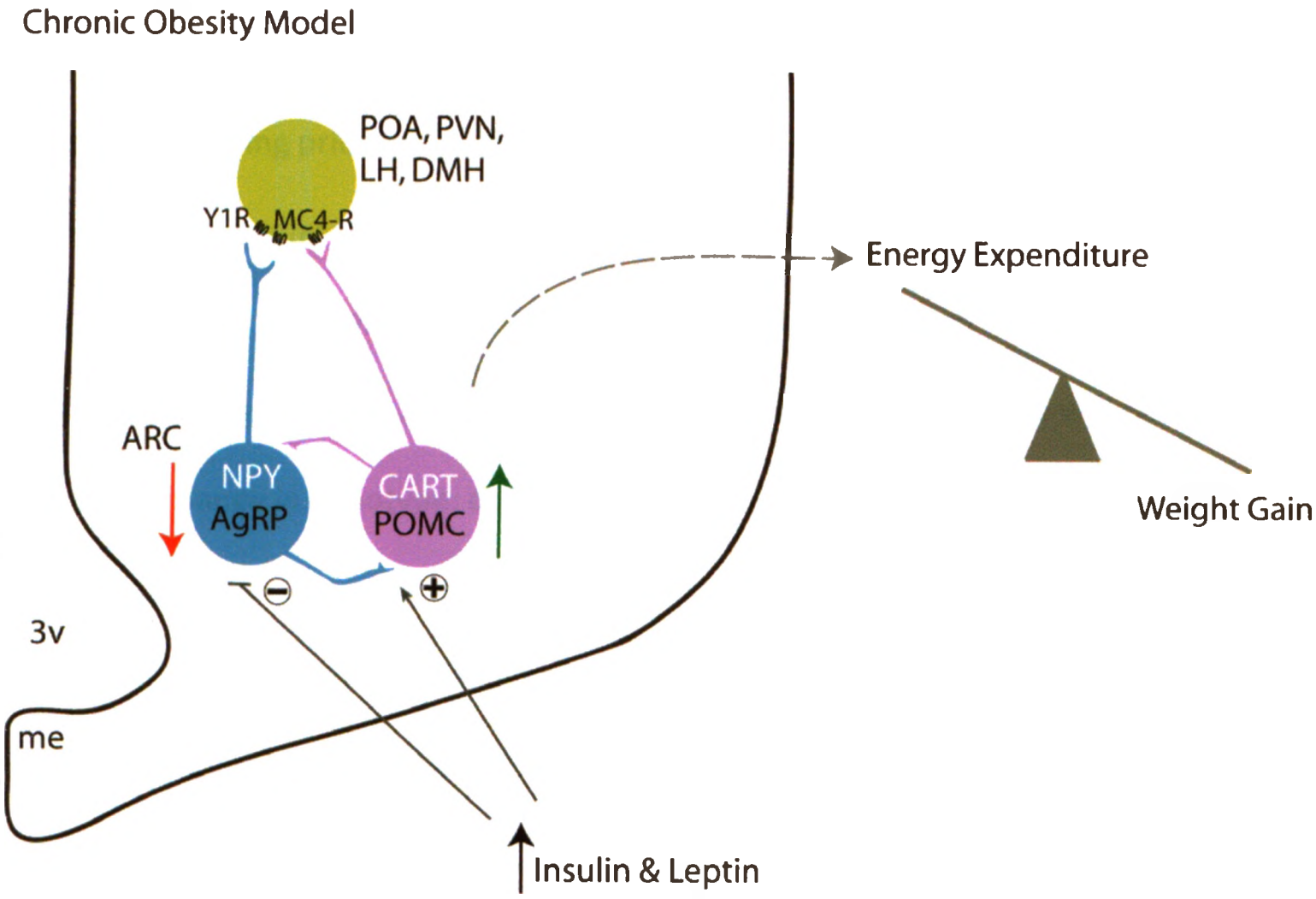

Figure 4.2 The appetite regulatory circuitry of the ARC nucleus is altered in the chronically obese ewe. AgRP and POMC expression in the ARC is decreased and increased, respectively. This may be a result of increased insulin and leptin levels working to drive energy balance towards regaining a normal body weight. *Abbreviations*: median eminence (me), arcuate nucleus (ARC), third ventricle (3V), neuropeptide Y (NPY), agouti-related peptide (AgRP), cocaine- and amphetamine- regulated transcript (CART), proopiomlanocortine (POMC), melanocortin receptor 4 (MC4-R), preoptic area (POA), paraventricular nucleus (PVN), lateral hypothalamus (LH), dorsomedial hypothalamus (DMH), neuropeptide Y receptor (Y1R).

The study of AgRP and POMC expression in chronic obesity, described in Chapter 3, is the first step of a more comprehensive investigation of the potential maternal inheritance of obesity and related defects. The current study provides baseline analysis describing the appetite regulatory circuitry of overweight ewes. These overweight ewes produced offspring prior to sacrifice that will be analysed in the future to determine if the effects of chronic obesity on AgRP and POMC expression are observed in second generation adult females. The rationale for this study is based on the concept of epigenetics. Epigenetics is the study of heritable changes in phenotype without a necessary change in genotype; common mechanisms for epigenetic transmission include chromatin hypermethylation and post-translational modifications of histones (reviewed in (6)). Currently, evidence suggests that programming during prenatal life can lead to epigenetic modifications of many key genes regulating behavior and physiology, and, as a consequence, the effects of prenatal T or long term obesity may be passed between generations resulting in increased risk of disease not only in the first generation but in subsequent generations as well (7, 8). For example, a recent paper suggests that offspring of obese mothers demonstrate both greater percent body fat and insulin resistance at birth (9), and it has also been documented that women with gestational diabetes have daughters who likewise show increased risk of this disease (10). Overfeeding or nutritional deprivation of pregnant ewes affects body weight and appetite regulatory neurons of offspring, as previously reported (11). However, to our knowledge, previous studies have not addressed whether the altered expression of AgRP/NPY or POMC/CART neuropeptides in obese ewes may be subject to

transgenerational transfer. Likewise, prenatal androgen treated ewes produce offspring that also demonstrate a PCOS phenotype (Padmanabhan, et al., unpublished), thus it would also be interesting to determine whether the effect of prenatal androgen on AgRP neurons would be passed on to future generations of females.

Prenatal T (PCOS) Model	Obesity Model
AgRP, appetite stimulatory peptide, expression is increased in adult ewes with prenatal androgen treatment	A chronic state of obesity leads to changes in the expression of AgRP (decreased) and POMC (increased)
Prenatal programming of appetite regulatory peptide expression is mediated via androgenic action	These changes may be a compensatory mechanism to regain a lower body weight in these animals
Insulin resistance in prenatal T treated animals may contribute to alterations in appetite regulatory peptide expression	AgRP and POMC neurons at distinct levels of the ARC are differentially affected by weight gain
Increased AgRP expression may be implicated in the increased risk of obesity in PCOS	This is a relevant model of obesity as it involves long term overeating and lack of exercise

Table 4.1 A summary of the key findings of Chapters 2 and 3.

This thesis is of clinical relevance to the understanding of PCOS, the most common endocrine disorder in women (12), and reaches further to a broader understanding of the development of obesity – currently a leading cause of morbidity and mortality worldwide (13). Excess weight gain severely affects quality of life for many individuals and is frequently associated with social stigma. Furthermore, obesity and its co-

morbidities are a great economic burden to the healthcare system (14). Understanding the mechanisms underlying the development of obesity is imperative in deriving effective prevention and treatment strategies for these individuals. Due to the complex nature of the regulatory system involved in energy homeostasis, and the variety of regulatory inputs from peripheral signals, the study of obesity is both complicated and diverse. Nonetheless, the present work provides a piece of the puzzle using a clinically-relevant animal model, and the data form the basis for many further questions to be addressed in the future.

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Appendices:

Appendix 1. Additional Descriptive Data from Animals

The following tables describe body weights from the animals analysed in Chapters 2 (Table A.1) and 3 (Table A.2) of this thesis.

Treatment	Mean Body Weight (kg)
C	52.5±2.5 SEM
T	62.0±5.3 SEM
T+F	59.0±1.2 SEM
DHT	57.4±1.6 SEM

Table A.1 Mean body weights from treatment groups: control (C), testosterone (T), testosterone and flutamide (T+F) and dihydrotestosterone (DHT) ± standard error of the mean (SEM), measured at one year of age.

Treatment	Mean Body Weight (kg)
Normal Weight	89.6±3.2 SEM
Obese	115.3±4.3 SEM

Table A.2 Mean body weights from treatment groups: normal weight and obese ewes ± standard error of the mean (SEM), measured at two years of age prior to sacrifice.

Appendix 2. Antibody Selection and Controls

The following describes control studies of the antibodies used in both Chapter 2 and 3.

A.1 The specificity of antibodies for POMC and CART were verified by preabsorbing the diluted primary antibodies with their respective peptide antigens (Phoenix Pharmaceuticals), at concentrations of 1µg/mL and 10µg/mL, following the protocols described in Chapters 2 and 3. All immunostaining corresponding to the appropriate antibody was eliminated (Fig. A.1).

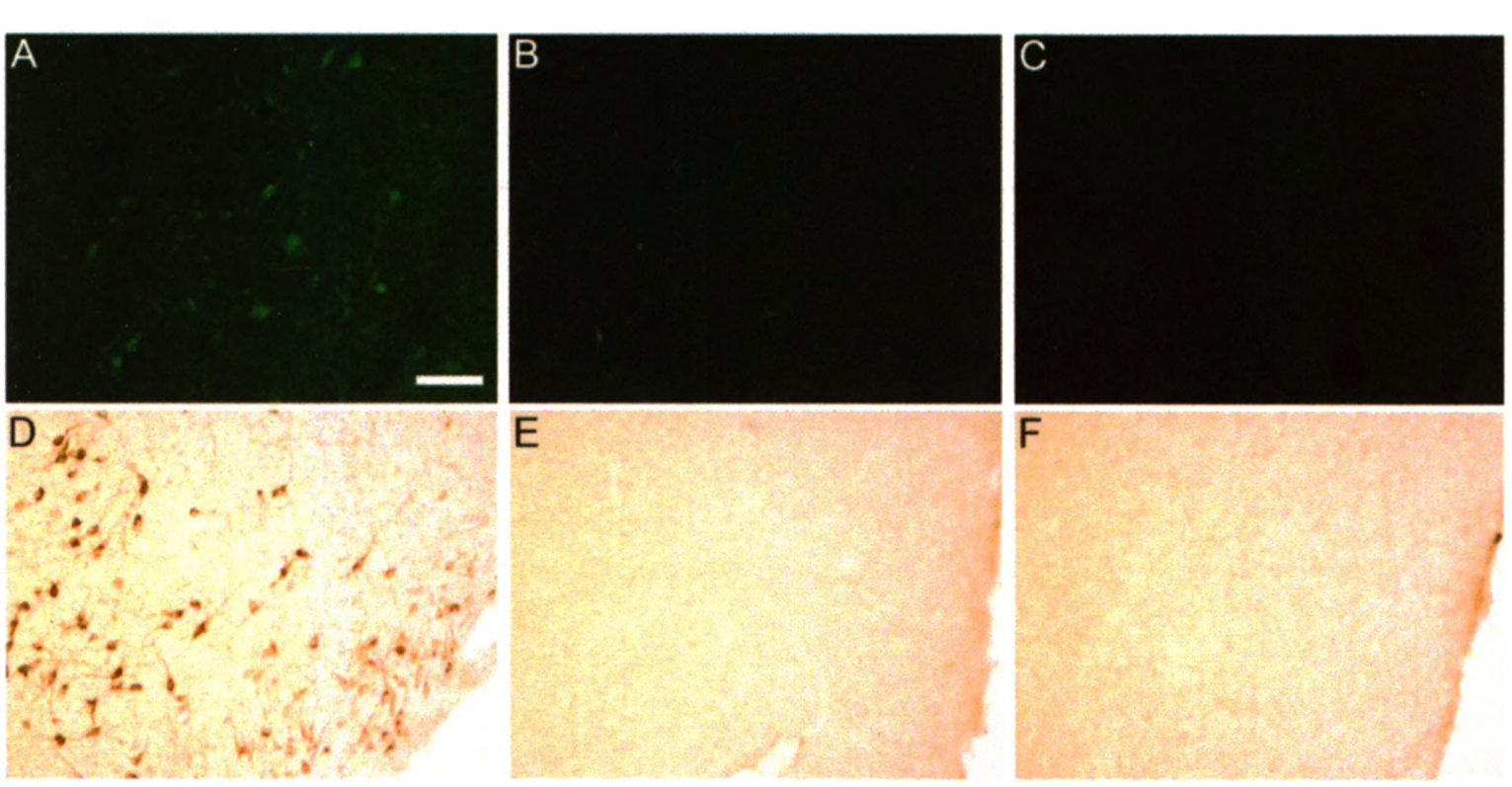

Figure A.1 Controls for CART and POMC antibodies. Preabsorption of CART antibody (1:80,000) with respective peptide antigen at concentrations of 0 µg/mL (A), 1µg/mL (B) and 10µg/mL (C) for 24 h at 4° C, visualized with Alexa 488. Preabsorption of POMC (1:40,000) antibody with respective peptide antigen at concentrations of 0 µg/mL (D), 1µg/mL (E) and 10µg/mL (F) for 24 h at 4° C, visualized with DAB. Scale bar = 100 µm.

A.2 Pre-absorption of AR antibody with its peptide antigen (Santa Cruz) at concentration of 1 µg/ml yielded no immunoreactive staining (Fig. A.2).

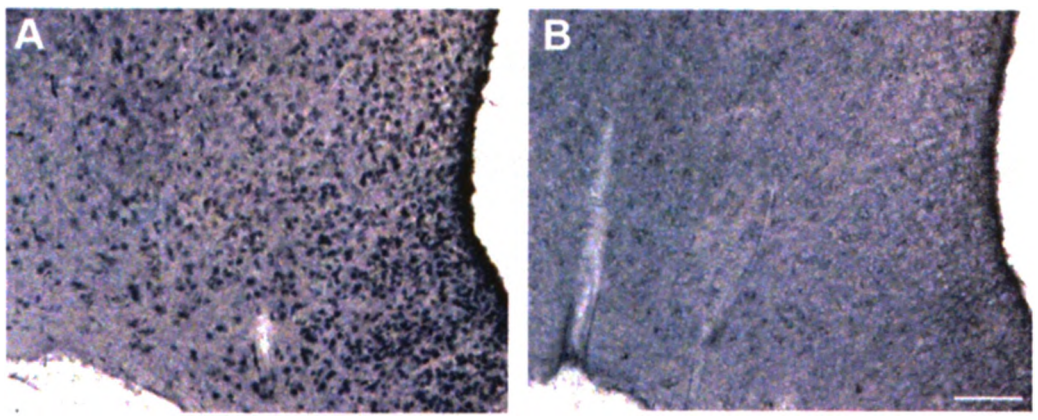

Figure A.2 Control for AR antibody. Preabsorption of AR antibody (1:200) with respective peptide antigen at concentrations of 0 µg/mL (A), and 1µg/mL (B) for 24 h at 4° C, visualized with DAB-nickel as described in Chapter 2. Scale bar = 200µm.

A.3 Both the CART and POMC primary antibodies were raised in rabbit,. Thus, for fluorescent dual labeling of these neuropeptides, CART was visualized using biotin tyramide amplification using a well established protocol in our laboratory, as described in Chapter 3. To verify lack of cross-immunolabelling, the protocol was carried out with omission of POMC antibody, but addition of the Alexa 555-conjugated secondary antibody used to visualize POMC which yielded no staining for POMC (Fig. A.3)

Figure A.3 Omission of POMC antibody in dual labelled staining for CART and POMC. (A) CART neurons immunolabelled with Alexa 488, (B) lack of Alexa 555 immunolabelled POMC neurons, (C) overlay of POMC and CART staining. Scale bar = 100 µm.