The role of hypothalamic kisspeptin regulating genes in polycystic ovary syndrome: Interactions with androgen signalling and diet in a mouse model

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ABSTRACT

Polycystic ovary syndrome (PCOS) is a common endocrine disorder that causes infertility in women of reproductive age. The underlying mechanism of PCOS remains unclear due to its heterogeneous aetiology, and there is no established cure for PCOS. Kisspeptin is a neuropeptide that is vital for fertility in mammals. Kisspeptin is critical for the generation of GnRH pulses and subsequent LH pulses, therefore its role in PCOS aetiology requires attention.

Clearly, androgen signalling plays a key role in PCOS, therefore the first aim and chapter of this thesis explored the role of androgen signalling on hypothalamic gene expression in a mouse PCOS-like model. As androgen signalling is mediated via the androgen receptor (AR), we evaluated whether the absence of AR regulates the gene expression of kisspeptin and related hypothalamic neuropeptides in a PCOS-like state. Using a mouse model of dihydrotestosterone (DHT)-induced PCOS, with global or neuron-specific AR knockout (ARKO) models, we investigated the mRNA expression of the key hypothalamic neuropeptides involved in GnRH pulse regulation. The qRT-PCR results revealed that *Tac2* gene expression (encoding Neurokinin B) is enhanced in the absence of neuron-specific AR signalling compared to the wild type (WT) and global ARKO group. However, the rise in *Tac2* mRNA expression in the neuron-specific ARKO females does not occur following DHT administration. These findings suggest that AR dependant pathways play a role in the regulation of hypothalamic gene expression in the PCOS-like state.

Dietary macronutrient balance plays a role in the regulation of female reproductive function, such as ovarian morphology and estrous cycle regularity, yet the link between diet and PCOS is unknown. Kisspeptin neurons located in the arcuate nucleus (ARC) are known to regulate GnRH pulses, respond to nutritional cues and may play a role in PCOS. To test the hypothesis that diet has an impact on kisspeptin related neuron activity in PCOS, we compared body weight and the expression of key hypothalamic genes among 10 groups of diet manipulation in mice (diets with differing compositions of protein, carbohydrate and fat). These diets were overlaid on control and postnatal DHT treated female mice. Diets with extreme macronutrient balance (towards either protein, carbohydrate or fat) prevented the DHT-induced body weight increase. Analysis showed that the dietary macronutrient balance had a regulatory effect on the androgen response of hypothalamic gene expression in the ARC. Specifically, *Tac2* gene expression was enhanced by DHT administration with a relatively high-fat diet, where fat accounted for 57% of the total caloric input. DHT administration also increased the expression of *Pdyn* and *Npy* in high-protein fed mice, where protein accounted for 42% of the total caloric input. These results indicate that diet and macronutrient

balance interact with androgen signalling to alter hypothalamic gene expression to regulate reproduction and metabolism in females – potentially affecting the PCOS-like state.

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ABBREVIATION

AR: Androgen receptor

ARC: Arcuate nucleus

ARKO: Androgen receptor knockout

AMH: Anti-Müllerian hormone

AVPV: Anteroventral periventricular nucleus

CL: Corpora lutea

CON: Control

DHEA: Dehydroepiandrosterone

DHT: 5α-dihydrotestosterone

Dyn: Dynorphin α

E2: estradiol

ER: Estrogen receptor

FSH: Follicle stimulating hormone

GABA: Gamma aminobutyric acid

GnRH: Gonadotrophin releasing hormone

GPR: G protein-coupled receptor

HPG: Hypothalamic-pituitary-gonadal

IR: Insulin resistance

Kiss1: The gene encoding kisspeptin

Kiss1r: The gene encoding kisspeptin receptor

KNDy: Kisspeptin-Neurokinin B-Dynorphin α

KO: Knockout

KOR: k-opioid receptor

LET: Letrozole

LH: Luteinising hormone

mRNA: Messenger ribonucleic acid

NKB: Neurokinin B

Npy: Neuropeptide Y

OVX: Ovariectomised

qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction

P4: Progesterone

P450: Cytochrome P450

PCOS: Polycystic ovary syndrome

Pdyn: Prodynorphin

PNA: Prenatal androgen treatment

Pomc: Proopiomelanocortin

PR: Progesterone receptor

PPIA: The gene encoding peptidylprolyl isomerase

SDHA: The gene encoding succinate dehydrogenase complex, subunit A, flavoprotein variant

SEM: Standard error of the mean

T: Testosterone

Tac2: Tachykinin 2

Tac3: Tachykinin 3

TBP: The gene encoding TATA-binding protein

TBP: Tris-buffered saline

WT: Wild-type

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Chapter 1. Literature Review

1.1 The Hypothalamic-Pituitary-Gonadal (HPG) axis

1.1.1 The Hypothalamic-Pituitary-Gonadal (HPG) axis in mammals

The reproductive system of mammals is tightly regulated through the coordination of the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotrophin releasing hormone (GnRH) secreted from the hypothalamus stimulates the release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the pituitary (Clarke and Cummins, 1985), which subsequently promotes estradiol (E2)/progesterone synthesis and follicular development/corpora luteal (CL) function in females or testosterone synthesis and spermatogenesis in males (Howles, 2000, Simoni et al., 1999). It is also known that these gonadal steroid hormones have feedback actions on the brain to regulate and maintain the HPG axis. Although this is critical for normal HPG axis function, the steroid feedback does not occur directly through GnRH neurons. Previous studies had failed to identify the co-localisation of requisite steroid receptors on GnRH neurons, including estrogen receptor alpha (ERα) (Lehman and Karsch, 1993), progesterone receptor (PR) (Skinner et al., 2001) and androgen receptor (AR) (Herbison et al., 1996), indicating that there must be another pathway that mediates steroid feedback action on GnRH neurons.

GnRH secretion includes both ‘pulse’ and ‘surge’ phases in females. The pulsatile release of GnRH constantly occurs throughout most of the menstrual cycle and orchestrates with LH pulsatile secretion from the pituitary (Carmel et al., 1976, Clarke and Cummins, 1982). Meanwhile, the GnRH surge occurs when ovarian follicles have matured, and it induces ovulation (Karsch et al., 1997). GnRH and LH pulses are observed in both males and females, whereas the surge typically occurs only in females (Claypool and Foster, 1990, Karsch and Foster, 1975), which suggests that the pulse and surge of GnRH/LH are stimulated and regulated independently.

1.1.1 Kisspeptin as a primary regulator of HPG axis

Since the discovery of GnRH, the mechanism responsible for GnRH neuronal activity and subsequent HPG axis regulation has remained incomplete. This all changed when two breakthrough studies were published almost simultaneously in 2003. These papers revealed that the inactivating mutation of a once orphan G protein-coupled receptor (GPR) 54 causes hypogonadotropic hypogonadism in humans (de Roux et al., 2003) and mice (Seminara et al., 2003). The natural

ligands of GPR54 are a family of neuropeptides collectively called kisspeptin, which was originally found and characterised in human melanoma cell lines (Ohtaki et al., 2001). Kisspeptin is encoded by the *Kiss1* gene, with the major peptide product kisspeptin composed of 54 amino acid residues in humans (Ohtaki et al., 2001), 52 amino acids in rats and mice (Takase et al., 2009, Desroziers et al., 2010) and secreted from kisspeptin neurons, found primarily in the hypothalamus of the brain. Several pieces of evidence suggest that kisspeptin neurons directly stimulate GnRH secretion. First, GPR54 is co-expressed in GnRH neurons in the hypothalamus of mice (Messager et al., 2005, Han et al., 2005), rats (Irwig et al., 2004) and sheep (Li et al., 2012). Second, the administration of kisspeptin increases GnRH neuron output, which is reflected by measurements of enhanced neuron electrophysiological firing in mice (Han et al., 2005) and the elevated GnRH secretion into the hypophysial portal system in sheep (Messager et al., 2005). Finally, kisspeptin-induced increases in plasma LH levels are abolished by the administration of a GnRH neuron antagonist (Irwig et al., 2004, Gottsch et al., 2004). These discoveries strongly implied that kisspeptin is the primary regulator of GnRH neurons in the hierarchical control of the HPG axis in mammals.

Kisspeptin and *Kiss1* gene expression was subsequently observed in the brain (particularly the hypothalamus), placenta, and ovarian granulosa and lutein cells in humans (Ohtaki et al., 2001, Gaytan et al., 2009, Clarkson and Herbison, 2006). Later, the *Kiss1* mRNA was monitored in discrete regions of the hypothalamus of mice, predominantly in anteroventral periventricular (AVPV) and arcuate nucleus (ARC) (Gottsch et al., 2004). The presence of *Kiss1* mRNA in these homologous areas of the human hypothalamus (the preoptic area (POA) and the infundibular nucleus, respectively) was also observed (Rometo et al., 2007). Importantly, AVPV kisspeptin expression in the brain was discovered to be sexually dimorphic, with the AVPV kisspeptin population in females of both rodents (Adachi et al., 2007) and humans (Hrabovszky et al., 2010) being much greater compared with those of males. This sex difference is due to the prenatal androgen exposure in males, which reduces the number of AVPV kisspeptin neurons during this critical developmental period (Kauffman et al., 2007b).

1.1.2 Steroid feedback in the HPG axis is achieved by kisspeptin neurons

As stated previously, GnRH neurons lack the sex steroid receptors that are critical for sex steroid feedback. After the discovery of kisspeptin neurons and their role to directly stimulate GnRH neuron activity, studies were done to determine whether kisspeptin neurons are the central facilitator of the

steroid feedback mechanism – whereby completing the 'missing link' for sex steroid feedback. Firstly, a study using rats revealed that the administration of E2 largely decreased ARC *Kiss1* gene expression in ovariectomised females (Smith et al., 2006). On the other hand, *Kiss1* expression in AVPV was significantly increased by E2 treatment in females (Smith et al., 2006). Then a similar result was reported by Dubois et al. (2015), whereby E2 administration to female mice inhibits ARC *Kiss1* mRNA expression. Importantly, for the stimulatory effect of kisspeptin on GnRH/LH secretion (detailed above), the involvement of ERα in feedback control is essential in both sexes. Indeed, most of the kisspeptin neurons in ARC and AVPV co-express ERα (Smith et al., 2006), and it is clear that ERα plays an important role in the estrogen feedback mechanism – both positive and negative – in females. It is also observed that the regulation of the *Kiss1* expression is completely lost in the ERα gene knockout female mice (Smith et al., 2005a). However, these traits are not observed in ERβ knockouts (Smith et al., 2005a, Smith et al., 2006), suggesting ERα is the main estrogen receptor controlling estrogen feedback mechanism in HPG axis. Additionally, studies indicate the possibility that progesterone is also involved in the steroid feedback regulation. It has been shown that the kisspeptin neurons and PR are co-localised in both the AVPV (Clarkson et al., 2008) and ARC (Smith et al., 2007). The administration of progesterone reduces ARC *Kiss1* expression comparable to E2 administration in ovariectomised female sheep (Smith et al., 2007), which indicates the involvement of progesterone in steroid negative feedback. Furthermore, a recent study using kisspeptin neuron-specific PR knockouts (KO) "KissPRKO" mice revealed that a lack of PR on kisspeptin neurons leads to deficit reproductive function in females (Stephens et al., 2015). In this study, they observed a significantly lower number of CL and the absence of a normal LH surge in KissPRKO females, which was reflected by the reduced AVPV neuronal *Kiss1* expression (Stephens et al., 2015). These results suggest that progesterone directly activates kisspeptin neurons in the hypothalamus and is important for the regulation of both positive and negative feedback mechanism of the HPG axis.

While gonadal steroids are known to have abilities to both stimulate and suppress the activity of kisspeptin neurons, the specific mechanism for this differential regulation is still uncertain. Previous studies have elucidated that kisspeptin neurons in the AVPV (or the homologous preoptic area in sheep) are involved in estrogen positive feedback (Smith et al., 2005a), and they may facilitate the positive feedback-dependant preovulatory GnRH/LH surge. Indeed, the administration of a kisspeptin antibody or kisspeptin receptor antagonist abolishes the LH surge in female rodents and sheep (Adachi et al., 2007, Smith et al., 2011). On the other hand, ARC kisspeptin neurons have been associated with estrogen negative feedback, which is known to be critical for the baseline or 'tonic'

pulsatile release of GnRH/LH (Wakabayashi et al., 2010). Supporting these hypotheses, Dubois et al. (2015) demonstrated that estrogen treatment to ovariectomized female mice reduces *Kiss1* mRNA levels in the ARC. Other studies have also shown that ARC *Kiss1* mRNA expression is decreased during the LH surge, although AVPV *Kiss1* mRNA expression is increased, supporting the idea that ARC kisspeptin neurons mediate estrogen negative feedback, whereas AVPV kisspeptin neurons mediate estrogen positive feedback (Smith et al., 2006). Importantly, while kisspeptin neurons are critical for steroid hormone feedback, other independent neuroendocrine mechanisms may still be at play and feedback directly at the pituitary is also apparent (Clarke, 2015).

1.1.3 Steroid feedback regulation by androgen

In males, testosterone is the major regulator of *Kiss1* expression in the brain. A previous study has found that the ARC *Kiss1* mRNA expression increases in castrated male mice, whereas it decreases following testosterone treatment (Smith et al., 2005b). Conversely, the AVPV *Kiss1* expression is reduced in castrated mice, and it is increased by testosterone administration, similar to the estrogen effects in the female (Smith et al., 2005b). Interestingly, it is again ERα that is the main receptor controlling feedback mechanism in the HPG axis, with testosterone regulation occurring after the aromatisation of testosterone to E2. However, direct testosterone regulation of *Kiss1* expression in the ARC is only partially lost in ERα gene knockout mice, suggesting the added involvement of AR along with ERα in steroid negative feedback mechanism in males (Smith et al., 2005b). Consistent with this, it has been observed that the AR and ERα co-express with kisspeptin neurons in ARC and AVPV in males, and the *Kiss1* expression in AR 'knockdown' male mice varies compared with that of wild type (WT) controls (Smith et al., 2005b).

The androgen pathway also plays an important role in the female reproductive system. It was observed that more than 63% of kisspeptin neurons in ARC co-localize with AR in female rat (Iwata et al., 2017) and we have seen similar data in mice (*Smith JT, unpublished observation*). Interestingly, the kisspeptin-dependent LH surge and pulses were suppressed in 5α-dihydrotestosterone (DHT) administrated female rats (Iwata et al., 2017), which indicates that androgen works independently of sex and it may inhibit kisspeptin neuron activity in the hypothalamus of females.

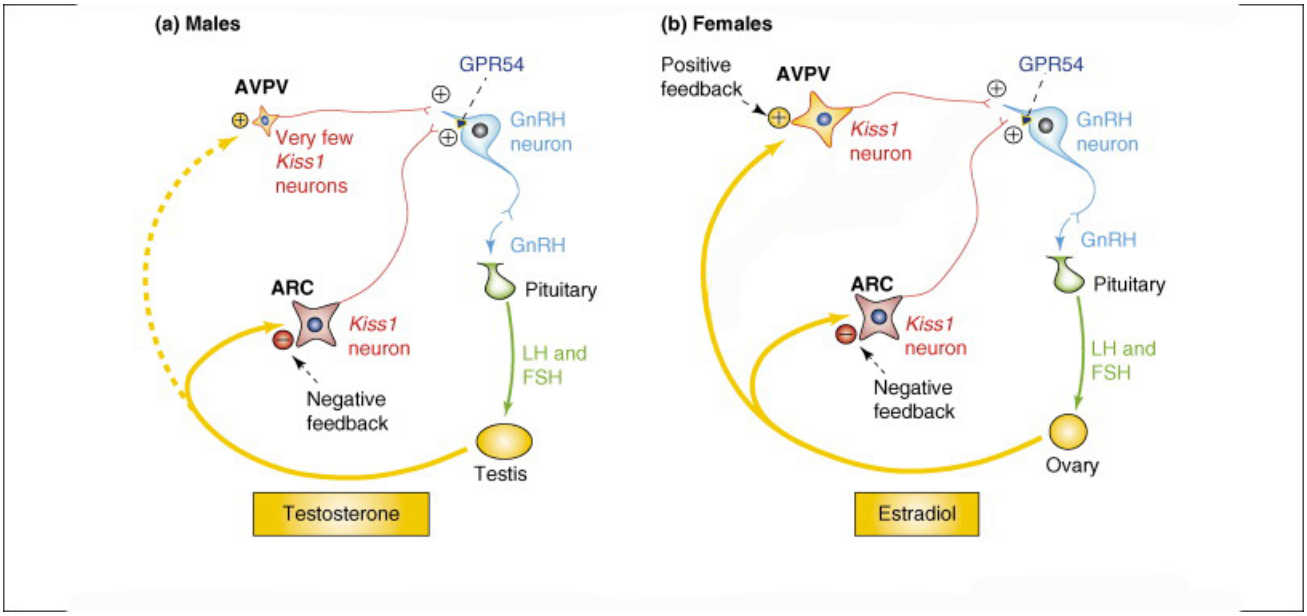

Figure 1. Summary of steroid feedback mechanisms within the HPG axis and the sexual dimorphism between males and females.
Kisspeptin neurons located in the AVPV and ARC activate GnRH neurons, which expresses GPR54 and stimulate GnRH release in the median eminence. GnRH then promotes LH and FSH secretion from the pituitary. These gonadotropins stimulate spermatogenesis in the testis of males and folliculogenesis in the ovary of females respectively, and the production of steroid hormones. Steroid hormones have a positive feedback effect on AVPV kisspeptin neurons, whereas a negative feedback effect on ARC kisspeptin neuron. Steroid positive feedback induces GnRH/LH surge and subsequent ovulation in females, reflected by the very few AVPV kisspeptin neurons in males and the absent GnRH/LH surge and ovulation. (Adapted from (Kauffman et al., 2007a))

1.1.4 'KNDy' neuron

The neuroanatomical characteristic of kisspeptin neurons located in the ARC is unique. An initial study in ewes found that ARC kisspeptin neurons co-express two other neuropeptides, namely neurokinin B (NKB) and dynorphin A (Dyn), which are encoded by the *Tac2* gene in rodents (*Tac3* in humans) and the *Pdyn* gene respectively (Goodman et al., 2007). These ARC kisspeptin neurons are commonly termed as 'KNDy' neuron taking their acronym from the shared neuropeptides. It has also been shown that ARC kisspeptin neurons co-express the receptors of NKB: NKB receptor (NK3R) and those of Dyn: k-opioid receptor (KOR) (Navarro et al., 2009). This colocalization is specific to ARC kisspeptin neurons and is not observed in AVPV kisspeptin neurons (Goodman et al., 2007), indicating that these neurons have the potential for auto-synaptic regulation. Later, it has been suggested that NKB and Dyn pathways are involved in controlling LH pulsatile secretion and necessary for normal reproductive function (Wakabayashi et al., 2010, Goodman et al., 2013, Young et al., 2010). Specifically, evidence implied that Dyn suppresses kisspeptin neuron activity (Weems et al., 2018, Mostari et al., 2013) restricting GnRH/LH pulses. In contrast, NKB stimulates kisspeptin

neuron activity (Billings et al., 2010, de Croft et al., 2013, Ramaswamy et al., 2010), suggesting that the combination of the inhibiting effect of Dyn and stimulating effect of NKB enables the generation of a rhythmic discharge in kisspeptin neurons. This theory is called 'KNDy hypothesis', and it is now one of the most widely accepted hypotheses for the GnRH pulse generator.

Recently, more studies have been conducted to confirm that ARC kisspeptin neurons work as the primary GnRH pulse generator. Experiments using the combination of optogenetic techniques to activate specific neurons and GCamp (green fluorescent protein, calcium and M13 peptide) to measure the targeted neuron activity found that the activity of ARC kisspeptin neurons is almost perfectly synchronized with pulsatile LH secretion in both males and females (Kim et al., 2020, Han et al., 2019, McQuillan et al., 2019, Clarkson et al., 2017), reinforcing the KNDy hypothesis. Furthermore, peripheral administration of KOR antagonist increased GnRH pulse activity in goats, which reaffirmed that Dyn/KOR pathway has an inhibiting effect on the generation of GnRH pulse (Sasaki et al., 2019). Thus, these findings indicate that KNDy neurons play a critical role in regulating GnRH/LH pulsatile secretion and maintaining normal HPG axis function.

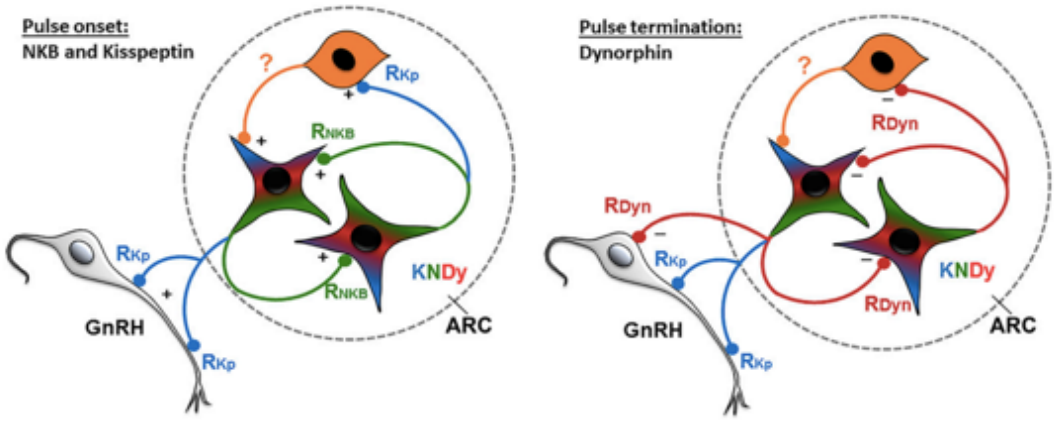

Figure 2. Proposed model of KNDy hypothesis.
Episodic GnRH/LH pulse is regulated by the stimulating effect of NKB (green) and the inhibiting effect of Dyn (red) on kisspeptin (blue) neurons. The GnRH pulse is induced by the activation of KNB neuron, which has projections to KNDy neurons with NKB receptor (R_{NKB}) and stimulates kisspeptin secretion. Kisspeptin acts on GnRH neurons expressing the kisspeptin receptor (R_{Kp}) and promotes GnRH release. Conversely, GnRH neuron activity is attenuated by the Dyn, which is released from KNDy neuron and acts directly on it through Dyn receptor (R_{Dyn}). The inhibition effect of Dyn on KNDy neuron terminate the GnRH neuron activity via R_{Kp}. (Adapted from (Clarkson et al., 2008))

1.2 Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders causing infertility in women of reproductive age (Azziz et al., 2004). There are several broad criteria in the PCOS diagnosis due to its heterogeneous symptoms. However, the Rotterdam definition is the most widely used criteria currently and is based on international evidence and guidelines for the assessment and management for PCOS (Teede et al., 2018). The definition proposes that PCOS can be diagnosed in women who exhibit two of the three following symptoms, in addition to the exclusion of related disorders; 1) oligo- or anovulation, 2) clinical and/or biochemical signs of hyperandrogenism, 3) polycystic ovaries (Azziz, 2006). Under these criteria, it is estimated that almost 20% of women in reproductive age are diagnosed with PCOS (Yildiz et al., 2012). PCOS is known as one of the most common causes of infertility, and many women with PCOS show anovulatory infertility (Teede et al., 2018). They also show a higher risk of developing serious complications during pregnancy, such as gestational diabetes, pregnancy-induced hypertension, pre-eclampsia or preterm birth (Boomsma et al., 2006). Moreover, PCOS is often associated with a number of clinical symptoms such as obesity, insulin resistance, type 2 diabetes mellitus and cardiovascular diseases (El Hayek et al., 2016). Despite the high prevalence of PCOS and its serious impacts on the health, the mechanism responsible remains unclear, and there are no established cures. Therefore, elucidating the aetiology of PCOS is an urgent issue to establish mechanism-based treatments.

1.2.1 Characteristics in PCOS

The characteristics of PCOS are very diverse and complicated, and the different symptoms appear depending on the individual. This chapter addresses the major characteristics of PCOS by categorizing them into reproductive, endocrine and metabolic features.

1.2.1.1 Reproductive features in PCOS

The dysfunction of the reproductive system is a cardinal feature of PCOS as its main outcome is anovulatory infertility. According to the cumulative data of clinical research, around 80% of PCOS women exhibit oligo-amenorrhea (Azziz et al., 2009). Furthermore, menstrual cycle disturbance and irregular ovulation are often observed in PCOS females (Fauser et al., 2012), which is demonstrated

in many studies using PCOS animal models, as will be discussed later (Moore et al., 2015, Osuka et al., 2017, Kauffman et al., 2015, Caldwell et al., 2014).

The cystic ovary is also a key feature of PCOS. In the diagnosis of PCOS, transvaginal ultrasound technique is the most commonly used method to detect cystic ovaries (Figure 3). Using the ultrasound technique, a clinical study revealed that the number of cystic follicles was two-times more in women treated with androgen over a long period than untreated controls (Pache et al., 1991). Likewise, several studies identified the presence of cystic/cyst-like follicles in the ovary of PCOS animal models with an excess level of androgen (Osuka et al., 2017, Kauffman et al., 2015). Moreover, the ovarian volume also increases in PCOS patients (Dewailly et al., 2011). This evidence implies that the hyperandrogenism may play an important role in causing impaired reproductive functions in females, represented in cystic ovaries or alternation in the ovarian morphology.

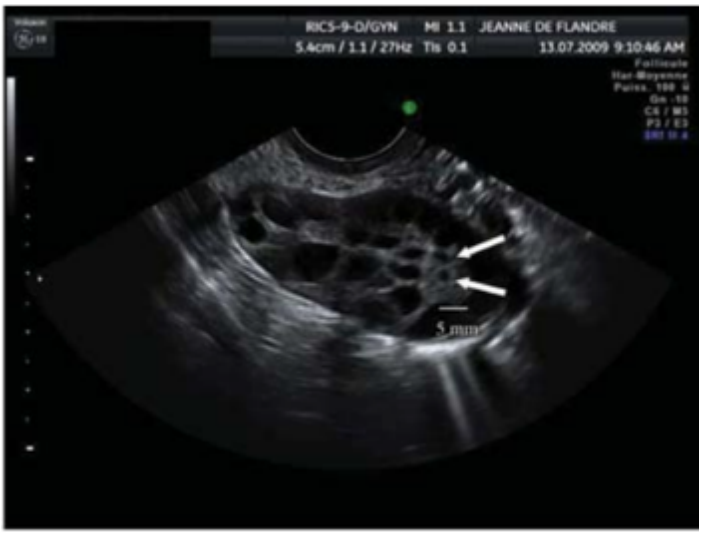

Figure 3. The ultrasound photo of polycystic ovaries.
There are many cystic follicles, which are identified with a dark ellipse. Arrows point the small follicles detected by the ultrasound technique. Adapted from (Dewailly et al., 2011).

1.2.1.2 Neuroendocrine features in PCOS

As PCOS is known as an endocrinopathy, women with PCOS present multiple endocrine dysfunctions. Elevated serum androgen levels, one of the diagnostic criteria of PCOS, is the most consistent feature among patients and thought to be the primary cause of PCOS. In clinical research, the level of circulating testosterone in PCOS girls was significantly higher than that of normal girls (Yoo et al., 2006, Taylor et al., 1997). Paired with this elevation in androgen, an impairment in the secretion of the other gonadal sex steroids (estrogen and progesterone) is often observed in a pre-clinical model of PCOS (Moore et al., 2013, Osuka et al., 2017). It was also reported that the

enhanced serum LH levels had been detected in 75% of PCOS females and 94% exhibited increased LH/FSH ratio (Taylor et al., 1997). This elevated serum LH level is reflected in the increased LH pulse frequency in PCOS females (Yoo et al., 2006), which implies the altered function of upstream hypothalamic neurons that governs gonadotropin secretion. Changes in gonadotropin release patterns affect the downstream folliculogenesis in ovary and steroid output and subsequent feedback mechanism. LH and FSH are responsible for the activity of theca cells and granulosa cells of follicles, respectively. LH acts on theca cells through LH receptor and stimulates biosynthesis of androgen in these cells (Fortune and Armstrong, 1977). Alternatively, FSH acts on granulosa cells via FSH receptor and stimulates the production of the P450 aromatase enzyme, which facilitates the conversion of androgen (specifically androstenedione and testosterone, which has diffused from the theca cells) to estrogen (Fitzpatrick and Richards, 1991, Silva and Price, 2000, Dorrington et al., 1975). In PCOS, it is clear that modified gonadotropin concentration alters the synthesis of steroid hormones in the follicles. Notably, the area of the theca cell, where androgen synthesis occurs increased in DHT-treated female mice (Caldwell et al., 2017). In contrast, the cell wall thickness of granulosa cells, where the conversion of androgen to E2 is facilitated significantly decreases in PCOS female mice compared with untreated controls (Caldwell et al., 2017), indicating that these changes in theca and granulosa cells possibly contribute to the hormonal imbalance in PCOS females. Similar changes in the follicle wall composition and the increased LH pulse frequency are also observed in PNA model (Silva et al., 2018, Moore et al., 2015). The modification of hormonal balance may affect the steroid feedback mechanism that is primarily controlled by hypothalamic neurons – namely kisspeptin neurons. Therefore, it becomes vital to examine hypothalamic neuron activity to determine whether and what hypothalamic neurons (KNDy neurons particularly) are involved in the neuroendocrine dysfunction in PCOS.

1.2.1.3 Metabolic features in PCOS

PCOS is strongly associated with metabolic impairments. Studies indicated that PCOS women have higher risks of multiple metabolic abnormalities, including obesity, type 2 diabetes, dyslipidaemia, hypertension and glucose intolerance (Anagnostis et al., 2018). Specifically, obesity is one of the most pervasive features, and almost 40% of PCOS patients are overweight with significantly higher body mass index (BMI) compared with non-PCOS females (Balen et al., 1995). Additionally, the insulin levels are substantially higher in PCOS women, and 50-70% of PCOS women exhibit insulin resistance (IR) (DeUgarte et al., 2005, Legro et al., 2004). Studies discovered that IR treatment,

including lifestyle changes to lose weight and pharmacological treatment such as Metformin, improved PCOS symptoms such as hyperandrogenemia, menstrual cycles or fertility, indicating that metabolic function may play a vital role in the development of PCOS phenotypes (Teede et al., 2007). Although its distinct correlation between PCOS and metabolic abnormalities, not many studies have examined the mechanism involved, and it remains uncertain until now.

1.2.2 The aetiology of PCOS

The aetiology of PCOS remains unknown due to its complicated mechanism and the variety of symptoms depending on the individual. Various studies have been conducted to elucidate the origin of PCOS, and they suggest several possible factors that may contribute to PCOS development.

One of the most probable factors involved in PCOS aetiology is the androgen exposure of the fetus. An experiment in rhesus monkeys revealed that prenatally androgenised females fulfilled PCOS diagnosis criteria (Abbott et al., 2005). Specifically, females exposed to androgen in early gestational days (between gestational days 40-80, when gonadal differentiation and GnRH neuron distribution occur), during the development of the HPG axis, showed elevated serum LH level in addition to three diagnostic criteria of PCOS (Abbott et al., 2005). On the other hand, females exposed to androgen in later gestational days (later than gestational days 100), did not show endocrine dysfunction (Abbott et al., 2005). Furthermore, evidence has demonstrated that elevated maternal testosterone levels are strongly associated with PCOS symptom onset in female offspring (Sir-Petermann et al., 2002, Caanen et al., 2016). Thus, prenatal exposure to androgen appears to be a critical factor in developing PCOS.

Studies also suggest that genetic factors can be the cause of PCOS. Clinical research data reported that the diagnostic rate of PCOS in sisters of PCOS women was 22%, which is considerably higher than that in the general population (Legro et al., 1998). Another clinical evaluation demonstrated that the prevalence rate of PCOS was significantly higher in the first-degree female relatives of PCOS patients (Kahsar-Miller et al., 2001), indicating that a heritable genetic component is involved in the cause of PCOS. Moreover, epigenetic studies have been conducted to specify the genes that are possibly associated with PCOS aetiology. They suggested a range of candidate genes including *CYP11A*, a gene involved in the androgen biosynthesis, insulin-related genes such as variable number tandem repeat (*VNTR*), insulin receptor gene (INSR) and Calpain-10 (*CAPN10*), which is associated with type 2 diabetes (Urbanek, 2007, Kosova and Urbanek, 2013). However, studies have

failed to determine a critical gene involved due to study limitations such as small sample sizes, the genetic heterogeneity of PCOS and unknown genetic interaction with the environment (Kosova and Urbanek, 2013). Moreover, some studies propose that there is a strong correlation between PCOS aetiology and the environment. For example, Norman et al. (2002) suggested that PCOS symptoms can be improved by modifying the lifestyle with moderate exercise, weight loss or healthy diet. To conclude, it is likely that PCOS is induced by multiple factors and their complex interactions.

1.2.3 Animal model of PCOS

Due to the difficulty to address clinical tissue and organ samples – especially the brain – for PCOS research, various animal models have been used to investigate PCOS aetiology mainly in rodents, ewes or non-human primates. Rodents are the most widely used animal for PCOS research because of the relatively lower cost and simplicity to handle compared with larger animals. Although there are multiple PCOS animal models, the one that perfectly represents all characteristics that are observed in PCOS patients is arguably yet to be established. Again, this is due to the heterogeneous phenotypes belonging to PCOS. Currently, the most widely used method to create PCOS animals is the administration of androgen or aromatase inhibitors to induce an excess level of androgen in circulation. The following chapters discuss the characteristics of major PCOS animal models.

1.2.3.1 Prenatal androgen model

The androgen-induced model is the most popular in PCOS investigations as hyperandrogenism is the most consistent feature among PCOS females and is suggested to be the leading cause of the syndrome (Sullivan and Moenter, 2004). Thus, administration of external androgen to mimic an excessive androgen condition is a standard method to create a PCOS-like animal model. Because of the strong association between prenatal exposure to androgen and PCOS, prenatally androgen (PNA) treated animals have been used as one of the most common models. For general prenatal androgen models in rodents, several androgens are used including testosterone, DHT, or dehydroepiandrosterone (DHEA) are administrated in the third week of pregnancy or 1 to 4 days before delivery, depending on the species used. Then female offspring are analysed as PNA treated animals (Osuka et al., 2019, Walters et al., 2012).

Results of studies using PNA models showed that irregular or prolonged estrous cycles are commonly observed in mice (Moore et al., 2015), rats (Osuka et al., 2017) and sheep (Unsworth et al., 2005). Moreover, PNA treatment affects ovarian morphology in the number of preantral, antral or atretic follicles and CL. Studies demonstrated that the number of preantral, antral and atretic follicles increase, whereas the number of CL decreases in PNA animals (Wu et al., 2010, Osuka et al., 2017, Caldwell et al., 2014). Cystic follicles were also observed in the ovary of the PNA rat (Tehrani et al., 2014), reflecting human PCOS traits. Similarly, PNA female mice show enhanced LH pulse frequency as well as higher serum LH levels, indicating prenatal androgen exposure may contribute to developing PCOS-like HPG endocrine dysfunction (Moore et al., 2015, Osuka et al., 2017). However, there was no change in body weight in PNA mice (Moore et al., 2013, Roland et al., 2010), and rats (Osuka et al., 2017, Tyndall et al., 2012), even though increased body weight is one of the most common features observed in PCOS women. These results imply that PNA treatment reliably induces PCOS-like reproductive and endocrine functions, whereas they fail to cause the associated metabolic dysfunctions.

1.2.3.2 Postnatal androgen model

Postnatal androgen treatment in animals is generally created by subcutaneous implantation of androgens such as DHEA or DHT (Osuka et al., 2019). Estrous cycles of postnatally androgen-treated mice and rats show anovulation or chronic diestrous stage (Osuka et al., 2017, Caldwell et al., 2014). This animal model shows a significantly decreased numbers of CL and increased cystic follicles in the ovary (Osuka et al., 2017, Caldwell et al., 2014). On the other hand, no change in serum LH levels was observed in postnatal DHT treated rats (Osuka et al., 2017) and reduced LH concentrations were detected in postnatal DHEA administrated rats (Iwasa et al., 2016), questioning its PCOS-like HPG endocrine dysfunction. Unlike PNA models, postnatal androgen treated models show PCOS-like metabolic traits including increased body weight, increased body fat, dyslipidaemia and insulin resistance (Osuka et al., 2017, Manneras et al., 2007b, Johansson et al., 2010). There are some inconsistent reports about this model (such as its effects on hypothalamic *Kiss1* expression, detailed below), but the postnatal DHT treated model appears to be attractive to investigate paired reproductive and metabolic traits of PCOS.

1.2.3.3 Letrozole model

Letrozole (LET) is a non-steroidal inhibitor of P450 aromatase, and it is used to mimic the hyper-androgenised condition in PCOS endogenously, by blocking the conversion of testosterone to E2 (Maliqueo et al., 2013). LET models in rodents are created by subcutaneously implanting LET pellets at the age of 3 to 4 weeks in mice (Kauffman et al., 2015) and rats (Maliqueo et al., 2013, Matsuzaki et al., 2017). Exposure to LET causes acyclic estrous cycles withheld in diestrous and abnormal ovarian morphology, with a significantly increased number of cystic and antral follicles and reduced or absent CL (Kauffman et al., 2015, Caldwell et al., 2014, Maliqueo et al., 2013). Serum steroid levels also corresponds to PCOS features with elevated testosterone and LH and decreased FSH and progesterone (Maliqueo et al., 2013, Kauffman et al., 2015). Furthermore, animals treated with LET recapitulate metabolic characteristics of PCOS, including increased body weight, fat mass, blood glucose and insulin (Kauffman et al., 2015, Torres et al., 2019). LET administration model has recently been revealed to cover many of human PCOS traits and to gain more attention and popularity among PCOS researchers.

1.3 Possible neuroendocrine origins of PCOS

1.3.1 Gamma aminobutyric acid (GABA) neurons

In recent studies, it was found that increased activity of gamma-aminobutyric acid (GABA) neurons is likely to play important roles in developing PCOS phenotypes (Sullivan and Moenter, 2004). Using the PNA mouse model, it has been revealed that the projection of GABA neurons originating from the ARC to GnRH neurons in POA is more robust in PNA-PCOS group than the untreated control group (Moore et al., 2015, Silva et al., 2018). Additionally, a follow-up study examined the ARC GABAergic effect on the GnRH neuronal network and discovered that the activation of ARC GABA neurons is strongly associated with increased LH secretion as well as other major reproductive phenotypes of PCOS (Silva et al., 2019). These findings suggest that GABA neurons alter GnRH/LH pulse activity in excessive androgen conditions similar to those in PCOS. Indeed, GnRH neurons are directly influenced by GABA neurons as both GABAA (Sim et al., 2000), and GABAB (Zhang et al., 2009) receptors are expressed on GnRH neurons. Furthermore, GABA neurons receive estrogen signals via estrogen receptors (Leranth et al., 1985), which implies that the GABAergic pathway also regulates steroid feedback mechanisms. Thus, there is increasing evidence to suggest that GABA neurons in the ARC are involved in the development and aetiology of PCOS.

Recently, a novel study focusing on the role of GABAB receptors reinforced the importance of the GABAergic pathway in the reproductive system. This study examined GABAB receptor knockout (GABAB1KO) mice and revealed that the lack of GABAB receptor induced impaired reproductive function (Catalano et al., 2005). This mouse model also exhibited significantly decreased ovulation rates (Di Giorgio et al., 2019). Moreover, the mRNA expression of *Kiss1r* and estrogen receptor 1 (*Esr1*) in the hypothalamus and GnRH receptor 1 (*gnrh1r*) in the pituitary was enhanced in GABAB1KO females (Di Giorgio et al., 2019), which implies the involvement of GABAergic pathway in the regulation of the HPG axis. Therefore, these findings suggest that GABAergic signalling may affect the reproductive and endocrine dysfunctions of PCOS.

1.3.2 Anti-Müllerian hormone (AMH)

Anti-Müllerian hormone (AMH) is a member of transforming growth factor ß-family, which is involved in the regression of Müllerian duct development in the male embryo (Allard et al., 2000). In the ovary, it is produced by granulosa cells in early antral stage follicles and attenuates the FSH induced follicular growth (Broekmans et al., 2008, La Marca and Volpe, 2006). The expression of AMH is influenced by GnRH, and it has a feedback effect on GnRH neuron activities. It has been unveiled that the administration of GnRH agonist stimulates the *AMH* mRNA expression in human granulosa cells (Winkler et al., 2010). Moreover, a study in mice demonstrated that Anti-Müllerian hormone type 2 receptor (AMHR2) is expressed on GnRH neurons and the administration of AMH promotes GnRH neuronal activity and subsequent LH secretion (Cimino et al., 2016). This evidence suggests that AMH plays important roles in follicle growth and the regulation of the HPG axis, including its steroid feedback mechanism.

Other studies proposed that serum AMH level can be an indicator of PCOS because of the correlation between AMH and PCOS symptoms. First, it was reported that patients with high serum AMH concentration have significantly increased serum testosterone and LH levels, LH/FSH ratio, polycystic ovaries and menstrual cycle irregularity (Tal et al., 2014), which are the diagnostic criteria and main characteristics of PCOS. Likewise, women with PCOS show enhanced serum levels of AMH during pregnancy (Piltonen et al., 2019, Tata et al., 2018). Additionally, prenatal AMH treatment induces deficient reproductive function with an acyclic menstrual cycle and a higher number of unhealthy follicles, as well as endocrine abnormalities with higher serum testosterone and LH level and enhanced LH pulse frequency in female offspring (Tata et al., 2018). Thus, elevated

AMH levels appear to be correlated with the development of PCOS and can potentially be a reliable indicator of PCOS.

1.4 The role kisspeptin in PCOS

Endocrine dysfunction is commonly observed among PCOS patients and the hyperactive GnRH/LH pulse secretion is clearly a key characteristic. As GnRH pulsatile secretion is primarily regulated by kisspeptin neurons in the ARC, it is important to investigate kisspeptin expression in the hypothalamus of PCOS females to potentially elucidate the mechanism involved in the impaired endocrine function in PCOS.

1.4.1 KNDy expression in PCOS animal models

KNDy neurons are likely responsible for hyperactive LH pulses because ARC kisspeptin neurons are vital for GnRH – and in turn, gonadotropin release, as discussed above. Also, kisspeptin neuron activity is regulated by steroid hormone feedback through estrogen and androgen receptors (Smith et al., 2005b, Smith et al., 2005a). Therefore, it is highly likely that kisspeptin signalling – or a disturbance in kisspeptin normal functioning – plays a role in the hypersecretion of LH in PCOS patients with excessive levels of androgen. So far, studies have investigated kisspeptin neuron expression using PCOS preclinical animal models, but results are somewhat mixed.

In PNA female rats, a significant increase in kisspeptin and NKB immunoreactive cell number was observed (Osuka et al., 2017). This increase in kisspeptin and NKB neuron expression (perhaps linked with activity) is consistent with the elevated serum LH level, which was demonstrated in the same animal model in this study. As stated above, studies in the PNA animal model strongly implied the involvement of GABA neurons in the development of PCOS. The question can be posed then, in the PNA model, do these GABA neurons expressed in the ARC contain the population of KNDy neurons, and these are responsible for the PCOS-like condition? To answer this question, neuropeptides co-localised with ARC GABA neuron was determined in PNA female mice (Marshall et al., 2017). The result showed that only 2% of ARC kisspeptin neurons co-express GABA (Marshall et al., 2017), which suggests that ARC kisspeptin neurons are not likely to be a critical component for the increased GABA neuron activity observed in the mouse PNA model.

On the other hand, there was no significant change in the kisspeptin positive cell number in a postnatal DHT treatment group compared to control (Osuka et al., 2017). However, other studies in postnatal DHT treated animals showed a significant decrease in the number or complete absence of identifiable kisspeptin neurons and the *Kiss1* mRNA expression in the hypothalamus (Brown et al., 2012, Iwata et al., 2017). Although the data about LH concentration is missing in the study conducted by Brown et al. (2012), the decrease in kisspeptin neuron number was congruent with a reduced LH pulse frequency and plasma LH level detected in this model (Iwata et al., 2017). Thus, some models of postnatal DHT indicate that there is a complete 'shut-down' of the kisspeptin-GnRH regulatory system potentially by negative feedback effects – but this does not always seem to be the case.

In the LET PCOS model, there was a significant increase in the *Kiss1* mRNA expression in ARC, as well as an enhanced serum LH concentration compared to control (Matsuzaki et al., 2017). Additionally, the gene expression of kisspeptin receptor, *Kiss1r* in the POA, where the majority of GnRH neurons are located was increased in letrozole PCOS mice (Kauffman et al., 2015). These results imply that the sensitivity to kisspeptin is enhanced in the PCOS model induced by letrozole, which subsequently increases GnRH and LH release. Clearly, the kisspeptin neuron activity appears to positively correlate with serum LH level in PNA and LET animal model.

In summary, the role of kisspeptin in PCOS, indicated by animal models, is not clearly defined. Results vary greatly depending on the animal model use, and further study is required to conclude any possible involvement of KNDy neuron in PCOS.

1.4.2 Kisspeptin regulation of metabolism

Recent studies have shown that kisspeptin plays a role not only in the reproductive axis but also in energy balance. A study conducted by Tolson et al. (2014) revealed that *Kiss1* KO mice exhibit multiple abnormal metabolic features, including increased body weight, fat mass and plasma leptin levels, which is a hormone secreted from adipocytes and reduces food intake (Schwartz et al., 2000). This publication then shed further light on this novel characteristic of kisspeptin, which is now understood to regulate energy balance in mammals. Interestingly, obesity and elevated fat mass are also distinct characteristics in PCOS patients. Furthermore, because kisspeptin neuron activity is regulated by androgen through AR expressed on KNDy neurons, kisspeptin may be involved in the metabolic impairments in PCOS via an AR dependant pathway.

The precise mechanism for kisspeptin regulation of metabolic functions is not yet completely understood. Previous studies have reported that kisspeptin neurons interacts with other neurons involved in energy balance. Firstly, neuropeptide Y (NPY) is a major metabolic neuropeptide, which engages in positive energy balance by stimulating food intake (Pu et al., 1999). The gene expression of NPY neuron is enhanced in the starved state (Korner et al., 2001), whereas it is reduced by leptin administration (Schwartz et al., 1996). It is widely known that many NPY neurons are found in the hypothalamus, specifically in ARC, a critical hypothalamic area in the control of food intake (Smith, 1993). Recently, numerous studies have been performed to investigate the relationship between kisspeptin and NPY neurons. In ewes, kisspeptin neurons were observed to have neuroanatomical input from NPY neurons (Backholer et al., 2010). Another study that investigated NPY KO mice demonstrated that *Kiss1* mRNA level significantly decreased in the absence of NPY pathway (Luque et al., 2007). Moreover, there seem to be reciprocal influences between kisspeptin and NPY, such as a stimulating effect of kisspeptin on *Npy* mRNA expressing cells (Backholer et al., 2010) and enhanced *Kiss1* expression by NPY treatment (Luque et al., 2007). However, further investigation is required to determine the role of kisspeptin on NPY neuron activity.

Second, pro-opiomelanocortin (POMC) represents an anorexigenic neuropeptide – in contrast to NPY (Zhan et al., 2013). A study in rats demonstrated that *Pomc* mRNA expression was significantly decreased in a fasted group compared with a food-restricted group (Korner et al., 2001). Furthermore, POMC neuron activity is enhanced by leptin administration via leptin receptors, and lack of leptin receptor expression on POMC neurons leads to obesity in mice (Balthasar et al., 2004). POMC neurons are extensively expressed in ARC, separate to but alongside the population of NPY neurons (Smith, 1993). In terms of the relationship between POMC and kisspeptin, POMC neurons also send fibre projections to kisspeptin neurons in sheep (Backholer et al., 2010) and mice (Cravo et al., 2011). Of note, ARC POMC neurons express the kisspeptin receptor, and kisspeptin directly stimulates POMC neuron excitation in mice (Fu and van den Pol, 2010). Another study demonstrated that the administration of kisspeptin significantly reduces food intake in mice (Stengel et al., 2011). These findings indicate that kisspeptin may suppress food intake by stimulating POMC neurons. Thus, it is suggested that kisspeptin plays an active role in energy balance regulation interacting with other ARC neurons such as NPY and POMC, which are more solely associated with metabolism.

1.5 Macronutrient supply and reproductive function

The reproductive system is intimately associated with multiple other pathways that regulate body function. A recent study using mice has revealed that the macronutrient balance has marked effects on the ovarian morphology, including ovarian mass, follicle/CL number or estrous cyclicity (Solon-Biet et al., 2015). Notably, the number of CL, which indicates recent ovulation, is influenced by macronutrient balance, maximised with low protein, low fat and high carbohydrate (Solon-Biet et al., 2015). A similar trend has been observed in the number of estrous cycles (Solon-Biet et al., 2015). This result strongly indicates that female reproductive function is affected by not only the amount of food eaten but also the macronutrient balance of the food.

Interestingly, alteration in the number of CL and estrous cyclicity is a distinct characteristic in PCOS patients, potentially implying that macronutrient balance and PCOS phenotype are likely to be correlated. However, no research has investigated the relationship between macronutrient balance and PCOS phenotype. Therefore, it is worthy of examining whether macronutrient balance has effects on PCOS and its related factors.

1.6 Conclusion

In summary, the reproductive system of mammals is regulated by the intricate interactions among hypothalamic neurons, steroid hormones and their receptors to maintain normal function. Kisspeptin, a neuropeptide predominantly expressed by neurons in AVPV and ARC of the hypothalamus is the primary regulator of the hierarchical control of the HPG axis. Additionally, it has been shown that kisspeptin plays a vital role in energy balance regulation.

Recent studies have implied that kisspeptin is associated with PCOS, which is a prevalent endocrine disorder causing infertility in women of reproductive age. PCOS is characterised by multiple reproductive, endocrine and metabolic dysfunctions and it is an urgent issue to establish a mechanism-based cure. Based on the fact that kisspeptin regulates reproductive and metabolic function in mammals – and is affected by androgen signalling – it is worth investigating the potential role of kisspeptin in PCOS aetiology. Several animal models have been used to elucidate the mechanism of PCOS, yet none of them perfectly reflect the features observed in human PCOS patients. Especially, the correlation between kisspeptin and PCOS remains unclear due to the inconsistent results in each animal model. Therefore, some critical questions remain unanswered;

does androgen signalling have an impact on kisspeptin expression in the hypothalamus to develop PCOS; also, is dietary nutrition involved in the modulation of kisspeptin expression and a contributor to PCOS in females? Currently, it has been revealed that androgen signalling as well as the macronutrient balance in diet play important roles in the development of PCOS-like features. However, whether kisspeptin mediates the signalling of androgen and diet has not been determined yet. The present study has been conducted in an effort to answer these questions and develop better understanding of the neuroendocrine pathology of PCOS, focusing on the relationship with the hypothalamic neuropeptides that have interactive effects with ARC kisspeptin neurons.

Chapter 2. The role of AR signalling on hypothalamic neuropeptide expression in a PCOS mouse model

2.1 Introduction

PCOS is an endocrine disorder that causes infertility in women of reproductive age (Azziz et al., 2004). The 2004 Rotterdam criteria, the most commonly used clinical diagnostic criteria for PCOS requires at least two of the following three symptoms: 1) oligo- or anovulation, 2) clinical and/or biochemical signs of hyperandrogenism, 3) polycystic ovaries (Azziz, 2006). Under these criteria, almost 20% of women of reproductive age are diagnosed as PCOS (Yildiz et al., 2012). PCOS is characterised by multiple reproductive, neuroendocrine and metabolic dysfunctions. Clinical studies report that 95% of adult PCOS patients show oligo-/amenorrhea (Fauser et al., 2012). Neuroendocrine hallmarks of PCOS include the elevated serum level and pulse frequency of LH, reduced FSH and a decline in the level of certain sex steroid production, such as estrogen and progesterone (Taylor et al., 1997, Moore et al., 2013). Furthermore, PCOS women often show metabolic abnormalities, including obesity, insulin resistance and type 2 diabetes (El Hayek et al., 2016). Despite its high prevalence and significant detrimental impacts on the well-being of women, notably in their reproductive health, there is no mechanism-based cure for PCOS due to its heterogeneous aetiology.

Hyperandrogenism is the most consistent feature among PCOS patients, and the exposure to excessive androgen in the early stage of life is suggested to be the main cause of PCOS clinically (Nisenblat and Norman, 2009). As androgen signalling is mediated via the AR, it is strongly implied that AR signalling is involved in the development of PCOS. Previous studies that investigated the characteristics of AR knockout (ARKO) mice implied a clear link between AR pathways and PCOS phenotypes. First, they revealed that neuron specific loss of AR (NeuroARKO) prevented anovulation in PCOS mouse females (Caldwell et al., 2017). Second, NeuroARKO altered the serum LH concentration and the gene expression of neuropeptides associated with LH secretion (Walters et al., 2018). Lastly, the absence of global and neuron specific AR rescued metabolic dysfunction, including increased body weight and fat mass in PCOS females (Caldwell et al., 2017). Thus, AR is highly likely to be a vital factor for the multiple reproductive, neuroendocrine and metabolic impairments in PCOS and it is worth investigating the relationship between AR pathway and PCOS.

AR pathway has also been proven to play an important role in female reproductive function. It is well known that the reproductive system of mammals is tightly regulated by the HPG axis and the

neuropeptide kisspeptin is its central facilitator. Kisspeptin is encoded by the *Kiss1* gene and distributed in the hypothalamus, predominantly in the AVPV and ARC (Gottsch et al., 2004). Interestingly, kisspeptin neurons in the ARC co-expresses two other neuropeptides, NKB and Dyn and these three neuropeptides are shared in neurons termed KNDy neurons (Goodman et al., 2007). NKB and Dyn are encoded by the *Tac2* and the *Pdyn* gene respectively and are responsible for the rhythmic activity or "pulses" of kisspeptin, which in-turn stimulate pulses of GnRH and subsequently causes pulsatile secretion of LH from the pituitary (Clarke and Cummins, 1985). Moreover, ARC kisspeptin neurons in females are colocalised with steroid receptors, such as ER (Smith et al., 2006), PR (Smith et al., 2007) and AR (Iwata et al., 2017), indicating that kisspeptin directly receives the steroid signalling and facilitates the steroid feedback mechanism.

In females, the administration of androgen reduces the mRNA expression of *Kiss1* gene in the ARC (Iwata et al., 2017). Additionally, several studies reported that kisspeptin expression in the ARC was modified in PCOS-like animal models (Osuka et al., 2017, Kauffman et al., 2015, Esparza et al., 2020). Combining the fact that ARC kisspeptin neurons co-expresses AR, we therefore hypothesised that androgen signalling affects the activity of kisspeptin regulating neurons in ARC and contributes to PCOS-like phenotypes. To examine this hypothesis, we sought to be evaluated the mRNA expression of *Kiss1* and related hypothalamic neuropeptides in the ARC using PCOS mouse model with global and neuron specific ARKO lines.

2.1 Materials and Methods

2.1.1 Mice

Mice were maintained under standard housing conditions (ad libitum access to the food and water in a temperature- and humidity-controlled, 12-h light/dark environment) at the ANZAC Research Institute. All surgical procedures were performed under ketamine/xylazine anaesthesia. All procedures were approved by the Sydney Local Health District Animal Welfare Committee within National Health and Medical Research Council guidelines for animal experimentation. We acknowledge the animal work, sample collection and sample shipment by Dr Kirsty Walters from The University of New South Wales.

2.1.2 Experimental design

The purpose of the study was to elucidate the relationship between kisspeptin and its related hypothalamic neuropeptides and the aetiology of PCOS. Two mouse lines were examined: (i) androgen receptor knockout mice with global loss of AR signalling (ARKO) and (ii) neuron-specific androgen receptor knockout mice with brain-specific loss of AR signalling (NeuroARKO) (Caldwell et al., 2017). Each line was compared with the relevant wild-type controls (ARKO OSO and NeuroARKO AOO). All groups were treated without (control) or with long-term exposure to DHT (Figure 4), which induces PCOS-like symptoms as previously reported (Caldwell et al., 2014). At the age of three weeks, animals were weighed and implanted with either a 1cm Silastic implant (i.d., 1,47 mm: o.d., 1.95 mm: Dow Corning: 508-006) containing about 10 mg DHT or empty, blank control. All DHT-treated groups were confirmed to exhibit a significant increase in serum DHT levels (Caldwell et al., 2017). Brain tissues were collected for gene expression analysis by qRT-PCR after 13 weeks of drug administration (WT: control n=7, DHT n=8; ARKO: control n=7, DHT n=7; NeuroARKO: control n=5, DHT n=5), when the mice are ~ 16 weeks of age. Brain tissues were immediately frozen on crushed dry ice while held in aluminium foil. They were shipped to The University of Western Australia by using a Dry Shipper and stored at -80 ˚C.

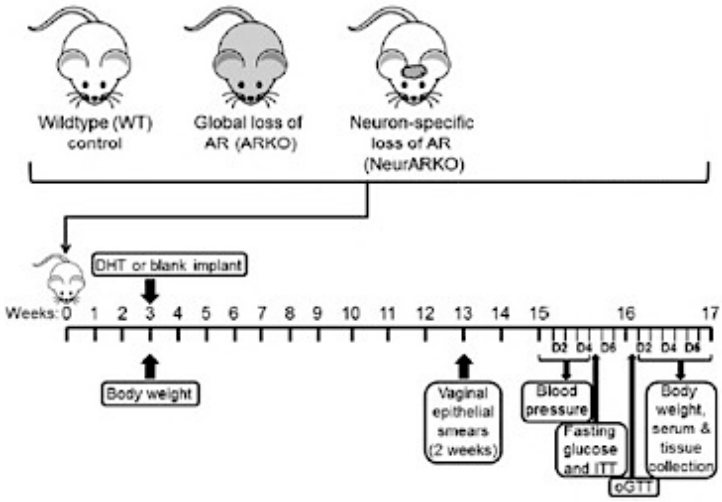

Figure 4. Experimental design.
For this study, PCOS was induced in WT, global and neuron-specific androgen receptor knockout mice by implanting DHT containing tube at the age of 3 weeks. Control mice were implanted blank tubes. The brain tissues were collected after 13 weeks of tube implantation, at the age of 16 weeks. (Caldwell et al., 2017)

2.1.3 Generation of ARKO and NeuroARKO mice

To generate homozygous female mice globally lacking AR, ARflox mice (Notini et al., 2005) were crossed with Sox2-Cre mice (Hayashi et al., 2002) to delete targeted AR sequence (Cheng et al., 2013). Female NeuroARKO mice were generated by crossing ARflox mice (Notini et al., 2005) and CamKllα-Cre mice (Casanova et al., 2001). Mice containing Sox2-Cre or CamKllα-Cre were determined using PCR conditions described in the original article (Caldwell et al., 2017). The confirmation of knockout models was conducted by RT-PCR for tissue-specific AR inactivation for the brain as previously described (Caldwell et al., 2017).

2.1.4 mRNA analysis by qRT-PCR

To examine the gene expression in the PCOS mouse model with the loss of AR signalling, qRT-PCR analysis was performed. Two housekeeping genes, *Tbp* and *PPIA* were used to normalise the data. All brain tissue sectioning, RNA extraction, reverse transcription and qRT-PCR analysis were conducted at the School of Human Sciences at The University of Western Australia.

2.1.4.1 Isolation of posterior ARC tissue

Tissue sectioning was performed in a dedicated RNA room of the molecular biology laboratory. All experimental equipment was wiped with RNAse AWAY™ (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and rinsed with RNase-free water before use.

The hypothalamic was excised with an American safety razor (Personna, Verona, Virginia, USA) by lateral incision 3 mm on each side of the third ventricle, which is indicated in the solid black line in Figure 5. It was then dissected into anterior and posterior portions (contains the medial basal hypothalamus and the ARC) along the dotted line in Figure 5 that intersects with the visible ends of optic chiasm. All dissected brain tissues were stored in 2.0 ml microtubes (Axygen, California, San Francisco, USA) and stored in freezers at -80 ˚C.

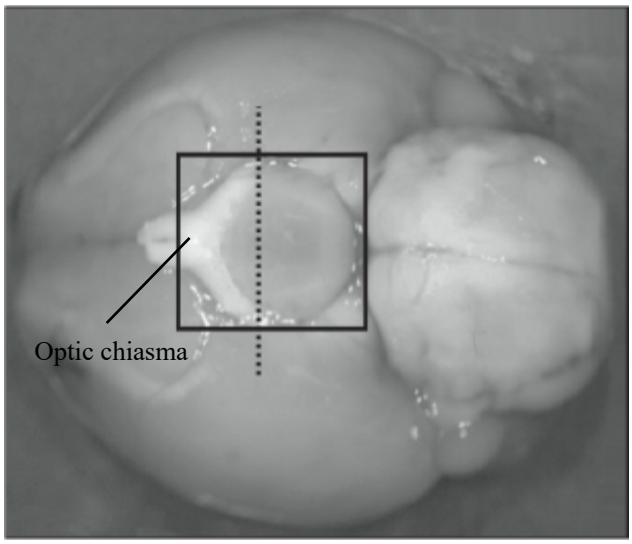

Figure 5. Ventral view of mouse brain. The hypothalamic was excised according to the boundaries of black square. Posterior hypothalamic portion (contains ARC) was dissected out along the dotted line, which intersects with the end of optic chiasma.

2.1.4.2 RNA extraction

500 µl of QIAzol Lysis Reagent (Qiagen, Venlo, Limburg, Netherland) was added to the 2.0 ml tube with the tissue samples and immediately homogenised by the homogeniser for 20-40 seconds. The homogenised samples were incubated at room temperature for 5 minutes. Then 200 µl chloroform was added, vortexed for 15 seconds and incubated at room temperature for 2-3 minutes. The homogenate was centrifuged (12,000 rcf, 4 ˚C, 25 min), and the upper aqueous phase was transferred to the new 1.5 ml tube (Axygen, California, San Francisco, USA). 500 µl isopropanol was added in the tube and incubated at room temperature for 10 minutes after vortexed. It was followed by the centrifuging (12,000 rcf, 4 ˚C, 10 min) again, and the supernatant was carefully discarded. 200 µl of

75% ethanol was then added and centrifuged (7,600 rcf, 4 ˚C, 5 min) to wash the precipitation. The resulting RNA pellet was isolated, air-dried maximum for 5 minutes at room temperature and resuspended in 20 µl of RNase-free water. The resuspended sample was immediately placed on dry ice after vortexed for a few seconds and stored at -80 ˚C.

2.1.4.3 RNA quality control

Quality Control (QC) check using gel-electrophoresis was performed for all samples after RNA extraction and prior reverse transcription to confirm that the extracted RNA sample is at a high purity level without any contamination. The RNA sample was handled on wet ice throughout the procedure. The gel was made by mixing 2% agarose gel (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and SYBR Green fluorescence dye (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) (60 µl for every 50 ml) and left for 20-30 minutes to set at the room temperature. 1 µl of extracted RNA sample and 2 µl ddH_2O was mixed in the 0.2 ml tube (Axygen, California, San Francisco, USA) and incubated in the conventional PCR machine for 5 minutes at 65 ˚C. 6x loading dye (Promega, Madison, Wisconsin, USA) was added in the sample and mixed by gentle repeat pipetting. Then, the sample was loaded into the slot of the gel in the electrophoresis equipment filled with 1x TAE buffer. The electrophoresis was run at 120 V and 200 current for 30 minutes. 100 bp DNA ladder (Promega, Madison, Wisconsin, USA) was used as the indicator of the product size.The outcome was visualised by ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, California, USA). Samples that did not show the clear band were excluded from the qRT-PCR analysis.

2.1.4.4 Removal of contaminating genomic DNA

To avoid interference of PCR due to contaminated genomic DNA, Promega DNase treatment-M610A was performed. The equivalent volume to 2 µg of RNA of each sample was calculated in advance. The calculation was based on the RNA concentration of each sample measured by NanoDrop® (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). 2 µg RNA of each sample was pipetted into 0.2 ml 12-Tube PCR Strips (Qiagen, Venlo, Limburg, Netherland). It was then added to 1 µl of RQ1 RNase-Free DNase 10x Buffer (Promega, Madison, Wisconsin, USA) and 2 µl of RQ1 RNase-Free DNase (Promega, Madison, Wisconsin, USA) and topped up with RNase

free water to the final volume of 10 µl. This solution was incubated for 30 minutes at 37 ˚C and then added 1 µl of RQ1 DNase Stop Solution (Promega, Madison, Wisconsin, USA) to terminate the reaction of previously added DNase. It was then incubated for another 10 minutes at 65 ˚C to deactivate DNase Stop Solution. The solution was carefully mixed by pipetting, and the half of the volume (5.5 µl), which is equivalent to 1 µg RNA was pipetted to the new PCR Strips for the following reverse transcription.

2.1.4.5 Reverse transcription

The reverse transcription protocol for Promega MMLV #M3862 (Promega, Madison, Wisconsin, USA) was used. 1 µg of RNA aliquoted from the Promega DNase treatment was mixed with 8 µl of RNase free water and 0.5 µl of random primers (Promega, Madison, Wisconsin, USA). The solution was heated to 70 ˚C for 5 minutes then immediately chilled on ice for 5 minutes. The premixed solution composed of 5 µl 5x reaction buffer (Promega, Madison, Wisconsin, USA), 1.3 µl 10 mM dNTPs (Promega, Madison, Wisconsin, USA), 1 µl M-MLV (Promega, Madison, Wisconsin, USA)) and 3.7 µl RNase free water was added to the solution. Then it was incubated through 25 ˚C (10 minutes) – 55 ˚C (50 minutes) – 70 ˚C (15 minutes) – 4 ˚C (∞) after mixed gently by pipetting.

To purify the cDNA sample, the product was ‘cleaned’ by using QIAquick® PCR Purification Kit (Qiagen, Venlo, Limburg, Netherland). The sample was mixed with 125 µl (5 volume of the sample) of Buffer PB and moved to the 2ml collection tube equipped with filter-column. Then the solution was centrifuged at 13,000 rpm for 30-60 seconds, and the flow-through was discarded. 750 µl Buffer PE was added to the filter-column and centrifuged (13,000 rpm, 30-60 seconds) to wash the product. The tube was centrifuged again at 13,000 rpm for 1 minute after the flow-through was discarded. The filter-column was then placed in a 1.5 ml tube (Axygen, California, San Francisco, USA), and 50 µl ddH_2O was added to elute DNA. The sample was mixed throughout by vortex and stored at -20 ˚C freezer.

2.1.4.6 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to examine the quantification of genes of interest (GOI). QuantiTect® Primer Assay (QIAGEN, Venlo,

Netherland), which is for use in real-time RT-PCR with SYBR® Green detection was utilised for the analysis.

Posterior hypothalamic mRNA expression for dynorphin (*Pdyn*), neurokinin B (*Tac2*), Proopiomelanocortin (*Pomc*) and neuropeptide Y (*Npy*) was quantified by qRT-PCR on CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). All samples were run in duplicates. Primer pairs for each GOI (Table 3) were purchased as QuantiTect® primers (Qiagen, Venlo, Limburg, Netherland). Gene expression was normalised against reference genes TATA box binding protein (*Tbp*) and peptidylprolyl isomerase A (*Ppia*) that were designed using Primer-BLAST. Their gene sequence and details were shown in Table 3. The optimal annealing temperature for QuantiTect® primers was examined and determined at 62 ˚C. Cycling number is also optimised at 50 times. For the analysis of the result, samples that showed multiple PCR products in melting curve analysis, different melting temperature or no amplification were excluded from the analysis. The qRT-PCR protocols used for GOI and reference genes are shown in Table 1 and Table 2, respectively.

Unfortunately, the mRNA expression of *Kiss1* was unable to be reliably determined (See Appendix) and was removed from the analysis in Chapter 2. This is deemed due to the sample degradation caused by the sample shipment from The University of New South Wales to The University of Western Australia and the poor care while being stored.

Table 1. qRT-PCR protocol for genes of interest (GOI).
This protocol was run by CFX384 Touch™ Real-Time PCR Detection System.

Step	Temperature (˚C)	Duration	
Hold	95 ˚C	10 minutes	
Cycling	95 ˚C	10 seconds	x 50
	62 ˚C	30 seconds	
Melt	70-99 ˚C		

Table 2. qRT-PCR protocol for reference genes.
This protocol was run by CFX384 Touch™ Real-Time PCR Detection System.

Step	Temperature (˚C)	Duration	
Hold	95 ˚C	10 minutes	
Cycling	95 ˚C	1 seconds	x 50
	60 ˚C	15 seconds	
	72 ˚C	5 seconds	
Melt	70-99 ˚C		

Table 3. Primer details of GOI and reference genes used in qRT-PCR.
QuantiTech name, primer sequences (F, forward; R, reverse), product size (base pairs, bp) and accession code (AC) for each primer used for qRT-PCR analysis.

Gene	**QuantiTech name or Primer sequence**	**Size bp**	**AC**
Pdyn	Mm_Pdyn_1_SG	143	NM_018863
Tac2	Mm_Tac2_2_SG	74	NM_009312
Pomc	Mm_Pomc_1_SG	99	NM_008895
Npy	Mm_Npy_1_SG	150	NM_023456
Tbp	F, 5'-GGG AGA ATC ATG GAC CAG AA-3'	113	NM_013684.3
	R, 5'-CCG TAA GGC ATC ATT GGA CT-3'		
Ppia	F, 5'-AGC ATA CAG GTC CTG GCA TC-3'	127	NM_017101
	R, 5'-TTC ACC TTC CCA AAG ACC AC-3'		

2.1.5 Statistical analysis

All data are expressed as mean ± SEM for each group. Group difference of gene expression within each treatment and genotype were analysed by two-way ANOVA followed by Fisher's least significant difference multi-comparison test. Statistical significance was set at $P<0.05$. All analysis was performed with Prism version 8.3.1 for Macintosh.

2.2 Results

Our result suggested that both DHT treatment and AR signalling has significant effects on the *Tac2* gene expression in the hypothalamus (Table 4). No difference was seen for *Pdyn* mRNA expression either between genotypes or treatment (Figure 6). In neuron-specific AR knockout (NeuroARKO) females, the DHT treatment resulted in a significant decrease of the gene expression of the *Tac2* compared to control in the ARC ($P<0.05$; Figure 6). *Tac2* gene expression of DHT-administrated groups tended to decline compared with control groups in all genotypes, yet no significance was observed in WT and ARKO (Figure 6). In control groups, *Tac2* mRNA expression in NeuroARKO females was significantly increased compared with WT and ARKO groups (Figure 6). However, the increase of *Tac2* gene expression in NeuroARKO in control groups disappeared in DHT-treated groups, and there was no difference in *Tac2* gene expression between WT, ARKO and NeuroARKO by DHT administration.

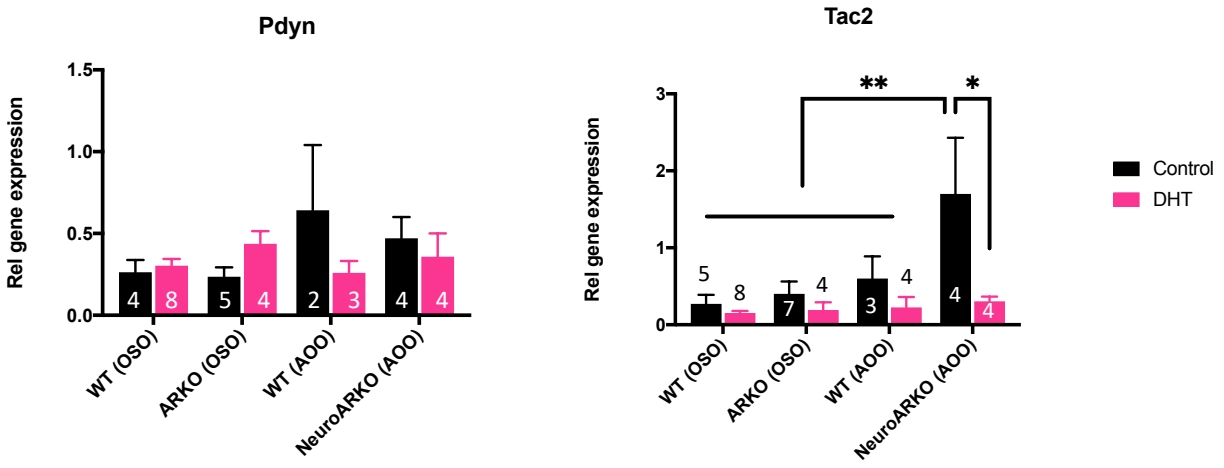

Figure 6.mRNA expression of mPdyn, mTac2 in the global and neuron-specific ARKO female mice.
The results of qRT-PCR in the posterior hypothalamus of control and DHT treated female mice. The black bar represents control groups, and the pink bar represents DHT treated groups. Data are the mean with SEM; n=2 to 8 per genotype/treatment group. (*/**P<0.05, two-way ANOVA; Fisher's LSD test.) Note: OSO and AOO indicate the separate mouse lines for each KO model.

The absence of AR signalling and the treatment with DHT did not affect the mRNA expression of the two hypothalamic genes, *Pomc* and *Npy*, which are involved in the energy balance (Figure 7, Table 4). Additionally, there was a tendency that DHT treatment decreased *Pomc* gene expression in WT group, whereas it tended to increase that of both ARKO and NeuroARKO females.

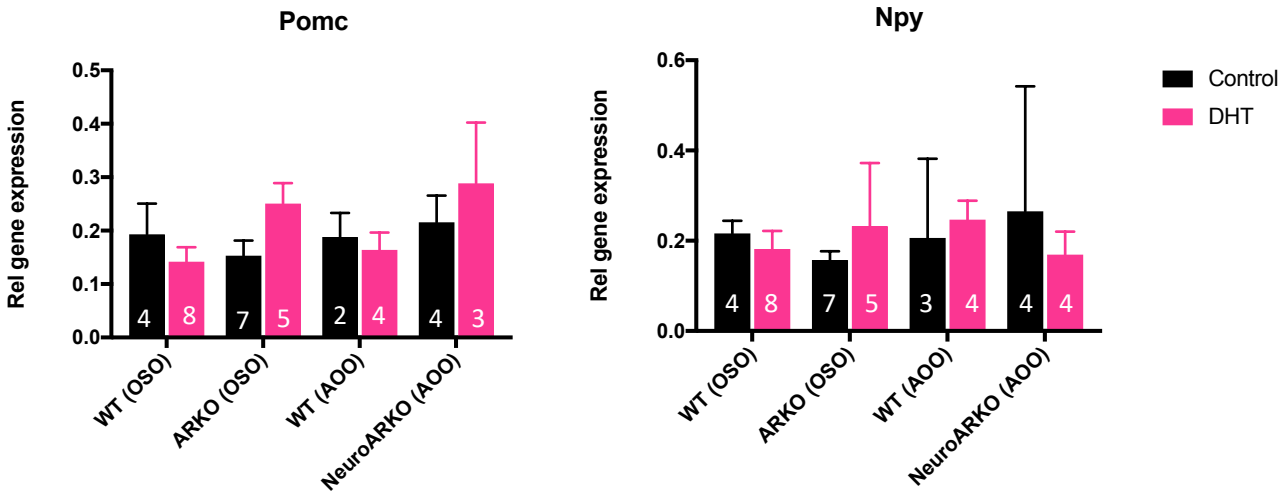

Figure 7. mRNA expression of mPomc and mNpy in the global and neuron-specific ARKO female mice.
The results of qRT-PCR in the posterior hypothalamus of control and DHT treated female mice. The black bar represents control groups, and the pink bar represents DHT treated groups. Data are the mean with SEM; n=2 to 8 per genotype/treatment group. (*P<0.05, two-way ANOVA; Fisher's LSD test.)

Table 4. Significance summary of two-way ANOVA analysis in the global and neuron-specific ARKO female mice.

	Pdyn	*Tac2*	*Pomc*	*Npy*
DHT treatment	ns	* (P=0.0155)	ns	ns
Genotype	ns	** (P=0.0061)	ns	ns
Interaction	ns	ns	ns	ns

2.3 Discussion

This study has demonstrated that androgen signalling through the AR pathway has regulatory effects on the mRNA expression of hypothalamic neuropeptides involved in reproductive function. Based on a qRT-PCR analysis of key hypothalamic genes in the posterior hypothalamus, we found that the neuron-specific loss of AR enhances the expression of *Tac2* mRNA, most likely in the ARC, in the female mouse. Importantly, this supports the observation by Walters et al. (2018) that the serum LH concentrations in NeuroARKO female mice are higher than WT mice in diestrus. Moreover, DHT treatment in NeuroARKO mice resulted in a loss of estrous cyclicity and we pair that with a significant reduction in *Tac2* mRNA. In addition, the administration of DHT significantly decreases *Tac2* gene expression in NeuroARKO females, implying that DHT potentially has direct feedback effects on the pituitary gonadotropes to alter LH release and these in-turn may regulate higher centres governing reproduction.

The *Tac2* gene is expressed widely across the brain, including hypothalamic nuclei such as the ARC, medial preoptic area and periventricular nucleus (Marksteiner et al., 1992). The overall effect of DHT across the experimental groups seems to be the reduction in *Tac2* expression in the posterior hypothalamus. This is consistent with a negative feedback effect and the amount of negative feedback did not seem to change in the WT and global ARKO perhaps indicating a lack of specific response to androgen in the *Tac2* gene. Interestingly, the most robust change was an increase in expression in the untreated NeuroARKO mouse. As stated above, this is consistent with the known serum LH concentrations of NeuroARKO female mice compared to WT in diestrous stage. These results in the NeuroARKO are intriguing and may be related to the possibility of local feedback directly on the pituitary gonadotropes and its flow-on effects (Figure 8). Indeed, the distribution of AR is confirmed in the anterior pituitary of female rats (Handa et al., 1986), which suggests that endogenous gonadal sex steroids in the untreated NeuroARKO may potentially signal in an effort to downregulate LH output. Furthermore, other steroid receptors such as ER and PR are also observed in the pituitary of rodents (Keefer and Holderegger, 1985, Notides, 1970, Scott et al., 2002), implying that other steroid hormones also may have a direct feedback effect on LH release from pituitary. The altered ratio of progesterone/estradiol in PCOS (Azziz et al., 2005) (or potentially DHT decreasing the sensitivity to the steroid receptors ER and PR) possibly attenuates the negative feedback effect on the LH secretion in pituitary and leads to the increased serum LH concentration in PCOS-like females. Additionally, LH receptor co-expresses with GnRH neurons (Toth et al., 1996), and potentially other hypothalamic neurons, implying the reduced LH concentration in

NeuroARKOs without DHT treatment may participate in a 'short-loop' feedback mechanism and increase the neuroendocrine 'tone' of GnRH stimulating systems – including *Tac2* gene expression in the hypothalamus (Figure 8). The question still remains then as to how DHT treatment reduces *Tac2* mRNA expression in the posterior hypothalamus in the NeuroARKO. It may be possible in the presence of high exogenous DHT, estradiol and progesterone concentrations are limited, and these may offer stronger feedback responses to the anterior pituitary gland and potentially limit LH negative and in-turn increase LH 'short loop' feedback effects (Figure 8). Contrary to this hypothesis, in a previous study that examined ARKO and NeuroARKO mice, there was no significant difference in serum LH levels between control and DHT-treated groups (Caldwell et al., 2017). However, this may be because the LH concentration was measured by single blood sample collection not by multiple and frequent blood collection to detect LH pulses, and the LH level may represent a peak or a nadir of a single LH pulse.

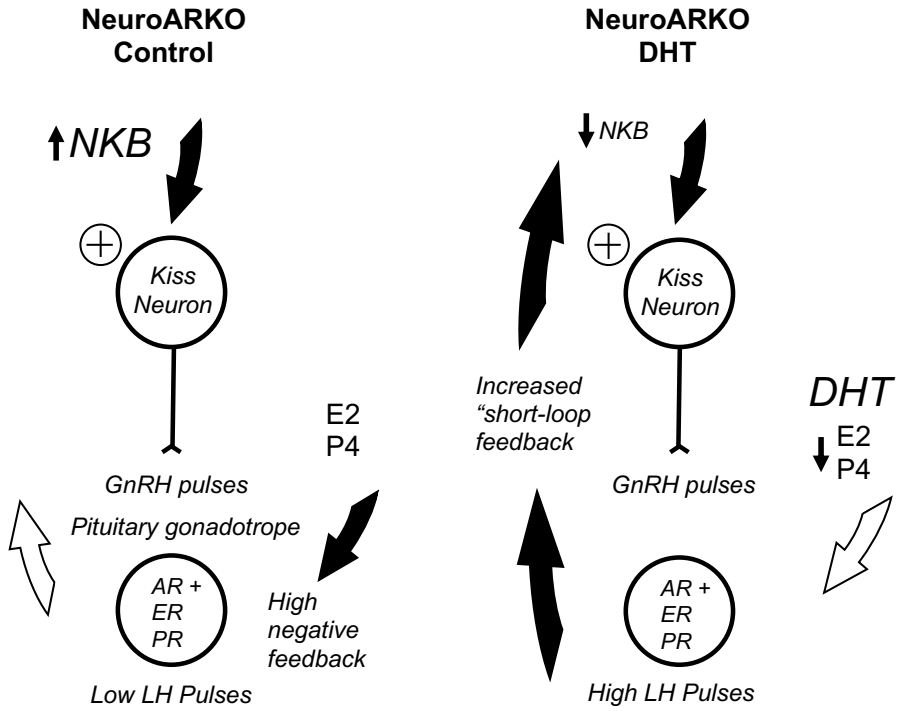

Figure 8. Proposed model indicating the difference between control neural specific (Neuro) androgen receptor (AR) knockout (KO) mice and DHT treated neuroARKO mice.
In the control, endogenous levels of estradiol (E2) and progesterone (P4) inhibit pituitary gonadotrope LH pulses causing a relative increase in neurokin B (NKB) expression in kisspeptin (Kiss) neurons. In DHT treated neuroARKO mice, high androgen levels reduce the E2 and P4 feedback effect on the pituitary and the high pituitary gonadotrope LH pulses cause a relative decline in neurokin B (NKB) expression in kisspeptin (Kiss) neurons. Note: The decrease in E2 and P4 may reflect reduced endogenous concentrations and/or reduced receptor signalling.

Our results also indicated that *Pdyn* mRNA expression in posterior hypothalamus is not influenced by altered AR pathways and DHT treatment. The distribution of dynorphin is observed in several hypothalamic areas, including the supraoptic and paraventricular nucleus (Lightman and Young, 1987), and dynorphin neurons are present beyond those representing kisspeptin neurons (KNDy neurons) in the ARC and this may have led to the lack of significant change in ARKO model and by DHT treatment. In addition, a critical limitation of this study was that we did not succeed in obtaining *Kiss1* mRNA expression data because of the technical issue of qRT-PCR (shown in Appendix 1 below). Due to the absence of *Kiss1* gene expression data, it remains uncertain how kisspeptin neurons respond to the lack of AR signalling or the high exogenous DHT in PCOS-like animal model.

Furthermore, *Pomc* and *Npy* gene expression was unchanged in our ARKO models with and without DHT treatment indicating that both POMC and NPY neurons may not be directly involved in the development of metabolic dysfunction in this PCOS-like regardless of AR status. Thus, another neuronal pathway that mediates AR signalling appears to exist to cause impaired metabolism under the excess androgen condition in PCOS females. Specifically, the postnatally DHT treated mouse model typically shows significant obesity (Caldwell et al., 2014). To clarify the involvement of POMC and NPY neurons in metabolic impairment in PCOS, food intake measurement would also be necessary because POMC and NPY are anorexigenic and orexigenic neurons respectively, and responsible for the balance of food intake behaviour (Pu et al., 1999, Zhan et al., 2013). Additionally, due to the lack of *Kiss1* gene expression data in this study, it is difficult to discuss the potential modulation of kisspeptin in relation to POMC and NPY neuron activity in this PCOS animal model. As POMC neurons are also regulated by leptin to control body weight (Balthasar et al., 2004), it is worthy of investigating the effect of leptin signalling on POMC neuron and energy balance in PCOS models. Moreover, a small number of samples in some groups was also a potential limitation of the statistics for the current study. Further investigation is required to determine the role of these key hypothalamic neurons in the development of PCOS.

Generally speaking, for the study of endocrine regulation on PCOS phenotypes, the animal model needs to be carefully chosen as the method to create a PCOS-like state seems to have a considerable impact on the development of reproductive, endocrine and metabolic abnormalities in PCOS animal models. Evidence shows that features detected in PCOS animal models vary depending on the animal model use (Caldwell et al., 2014, Manneras et al., 2007a). Importantly, a previous study in the postnatal DHT model, which is used in the current study, has failed to observe a major endocrine

traits of PCOS, represented in the elevated serum LH concentration and increased LH pulses (Osuka et al., 2017, Caldwell et al., 2017). However, a recent study using the letrozole implanted model strongly implied the correlation between increased LH pulse/release and the enhanced KNDy neuron activity in PCOS (Esparza et al., 2020). These findings lead to a hypothesis that KNDy neurons are affected by aromatisable androgens including testosterone, but not unaromatisable androgen such as DHT (Osuka et al., 2017). Alternatively, imbalance of multiple hormones caused by the inhibition of androgen aromatic conversion may alter KNDy expression in the hypothalamus in the LET model as a result of modified feedback effects on the hypothalamic neurons. Therefore, the diversity of PCOS-like states induced among conventional PCOS animal models is likely to be due to the difference of neuronal pathways involved to mediate hormonal signalling. This may simply reflect limitations in the models and their application, but it may equally reflect the heterogeneity of the PCOS state and offer translatability, but only to specific criteria of the disease.

One possible candidate neuron that may significantly contribute to PCOS development is the GABA neuron. Recent studies that examined the role of GABA neurons in PCOS animal models suggest that GABA neurons in the hypothalamus interact with androgen and are involved in the development of PCOS. First, in prenatal androgen treated PCOS females, the anatomical input from ARC GABA neurons to GnRH neurons significantly increases (Moore et al., 2015, Silva et al., 2018), supporting the hypothesis that the androgen stimulates GABA neuron activity and subsequently induce increased GnRH and LH pulse/release in PCOS females. Second, the activation of GABA neurons in ARC induces multiple PCOS-like symptoms in female mice, including elevated serum testosterone level, increased LH secretion, irregular estrous cycles and cystic follicles (Silva et al., 2019). Third, the administration of androgen receptor antagonist ameliorates irregular estrous cycles and increased synaptic drive of GABA neuron to GnRH neuron induced by prenatal androgen treatment (Sullivan and Moenter, 2004). Finally, GABAB receptor KO female mice exhibit impaired reproductive function and reduced ovulation rate, which are common features observed in PCOS (Catalano et al., 2005, Di Giorgio et al., 2019), suggesting that the GABAergic pathway is a significant player in the regulation of the female reproductive system. Therefore, GABA neurons in ARC have reciprocal influences with androgen and play a vital role in inducing major reproductive and endocrine abnormalities in PCOS. In addition, GABA colocalises with GnRH neurons in the nasal area during fetal development and plays a vital role in the developmental GnRH neuron migration from the olfactory area to the forebrain (Tobet et al., 1996). Another study also suggested GABAergic regulation of GnRH neurons over the process of sexual maturation (Han et al., 2002). Combining these findings, the evidence indicates that the GABAergic effect is critical for the development of

reproductive system controlled by HPG axis, and the prenatal exposure to the excess level of androgen may alter GABA neuron activity, which then causes impaired reproductive function in adulthood such as PCOS.

Despite the clear correlation between the GABA neuron and the development of reproductive and endocrine PCOS-like traits, there is no distinct evidence that shows the role of GABA neuron in metabolic traits of PCOS. The previous study found that PNA mice do not exhibit major PCOS-like metabolic traits such as increased body weight and fat mass though they show increased GABA activity and impaired reproductive and endocrine functions (Caldwell et al., 2014, Moore et al., 2015, Roland et al., 2010). Interestingly, the administration of GABA or antagonist of GABAA receptor to lateral hypothalamus suppresses food intake and reduces body weight in mice (Stanley et al., 2011), suggesting that GABAergic pathway is involved in the negative energy balance regulation. Thus, GABA neurons are not likely to play a direct role in the metabolic dysfunction in PCOS, and this could be related to its stimulating effect specifically on GnRH neuron (Watanabe et al., 2009), which is a central regulator of reproductive and endocrine function through HPG axis. Furthermore, another study revealed that the food intake increases levels of GABA in the lateral hypothalamus in mice (Yonemochi et al., 2019), implying that increased food intake may enhance GABA neuron activity, then lead to the development of PCOS phenotypes. In the same study, they also showed that the administration of GABAA receptor agonist significantly decreased the gene expression of the orexin precursor, whereas no change was observed in *Npy* and *Pomc* gene expression by the GABAergic pathway activation (Yonemochi et al., 2019). Orexin is a neuropeptide that is predominantly expressed in the lateral hypothalamus including ARC and the paraventricular nucleus in the hypothalamus and stimulates food intake (Edwards et al., 1999, Dube et al., 1999). Taken together, these findings imply that orexin may play a role in the metabolic impairment in PCOS interacting with GABAergic pathway. However, both GABA and orexin neuron activities in PCOS and their relationship with PCOS-related obesity remain unknown. Further research is expected to conduct to examine the role of GABAergic and orexigenic pathway in the metabolic dysfunction in PCOS.

Another possible factor involved in PCOS aetiology is progesterone. The expression of progesterone-regulated genes significantly decreases in the endometrium of PCOS women, providing evidence of progesterone resistance in PCOS patients (Savaris et al., 2011). In animal studies, reduced PR immunoreactivity was observed in the hypothalamus of PNA female mice (Moore et al., 2015), which indicates that progesterone sensitivity decreases in PCOS mice. However, no change in

PR expression was detected in PNA rats, and postnatally DHT treated rats (Osuka et al., 2017). The possibility that progesterone signalling is involved in PCOS is also suggested by the study conducted by Kondo et al. (2016), which revealed that the administration of RU486, an antagonist of PR, induces reproductive dysfunction such as disrupted estrous cycles and cystic follicles. Thus, progesterone resistance caused by decreased progesterone-regulated genes or reduced PR sensitivity potentially has an impact on PCOS. In addition, altered progesterone feedback is associated with PCOS. For instance, a decrease in serum progesterone level is detected in postnatal DHT treated rats (Osuka et al., 2017). Moreover, the administration of DHT interferes the negative feedback effect of progesterone on GnRH neurons in female mice (Pielecka et al., 2006), indicating hyperandrogenism in PCOS may have a significant impact on progesterone feedback as well as the endocrine system. If that is the case, there should be changes in progesterone production under the effect of androgen signalling. Indeed, DHT treatment increases the thickness of theca cells (Caldwell et al., 2017), where the conversion of progesterone to androgen takes place (Walters et al., 2019). Furthermore, the change in theca cell-layer thickness appears to be rescued by the absence of AR signalling (Caldwell et al., 2017). Taken together, the endocrine mechanism that progesterone involves is likely to be affected by androgen signalling and potentially contributes to developing PCOS like abnormalities.

In summary, we have shown that *Tac2* gene expression is regulated by androgen signalling in female mice exposed to a PCOS-like state. *Tac2* mRNA expression increases in the female NeuroARKO, most likely due to the loss of androgen negative feedback to the KNDy neuron, and the elevated *Tac2* gene expression in NeuroARKO is reduced by DHT administration. We suggest that a short-loop negative feedback mechanism involving altered LH dynamics may be involved in the regulation of ARC *Tac2* gene expression through AR expressed in pituitary. No change was noted in *Dyn* mRNA or *Npy* and *Pomc* mRNA. Furthermore, other endocrine pathways, including GABA neuron or progesterone are expected to play a role and could also be influencing in the development of the PCOS-like state via androgen signalling.

Chapter 3. The role of macronutrient balance on hypothalamic neuropeptide expression in a PCOS mouse model

3.1 Introduction

PCOS is an endocrine disorder (Azziz et al., 2004) that affects almost 20% of women of reproductive age (Yildiz et al., 2012). PCOS is characterised by multiple reproductive, endocrine and metabolic dysfunctions. Reproductive dysfunction includes the increased number of cystic follicles and anovulation, the latter represented by the absence of CL or irregular menstrual cycles (Caldwell et al., 2014). Furthermore, endocrine abnormalities such as hyperandrogenism and hyper LH concentration/pulses are commonly observed in PCOS women (Fauser et al., 2004). It is also important that PCOS patients often exhibit metabolic symptoms, including overweight/obesity, insulin resistance (IR) and type 2 diabetes (El Hayek et al., 2016). Despite the high prevalence and serious impacts on the health of women, there is no established cure for PCOS because its underlying causal mechanisms remain unknown.

For the treatment of PCOS, health management of symptoms has been considered to be an effective method. Clinical studies discovered that IR treatment, including lifestyle changes to lose weight and pharmacological treatment to improve glucose resistance, mitigated PCOS symptoms, such as hyperandrogenemia, menstrual cycles or fertility (Teede et al., 2007), which indicates that the metabolic system may play an important role in the development of PCOS. Furthermore, a study that examined the effect of macronutrients (protein, carbohydrates and fat) on reproductive function revealed that the dietary macronutrient balance has significant impact on female reproductive outcomes such as uterine mass, ovarian follicle morphology and estrous cycles (Solon-Biet et al., 2015). Specifically, they showed that the number of CL is increased with a high relative carbohydrate diet (Solon-Biet et al., 2015). Moreover, the ratio of protein and carbohydrate in the diet has a marked influence on estrous cycling (Solon-Biet et al., 2015). Notably, altered number of CL and change in the estrous cycle are also common features of PCOS (Caldwell et al., 2014). Therefore, these results imply that the balance of macronutrients in the diet contributes to the reproductive dysfunctions in PCOS.

The reproductive system of mammals is tightly regulated by the HPG axis and a neuropeptide called kisspeptin is the central facilitator. Kisspeptin is encoded by the *Kiss1* gene and a ligand of GPR54 (Ohtaki et al., 2001). Kisspeptin neurons that release kisspeptin are distributed in the hypothalamus, predominantly in the AVPV and ARC of the hypothalamus (Gottsch et al., 2004). Interestingly,

kisspeptin neurons in the ARC co-expresses two other neuropeptides, NKB and Dyn and these three neuropeptides have resulted in these neurons being termed as 'KNDy' neurons (Goodman et al., 2007). NKB and Dyn are encoded by the *Tac2* and the *Pdyn* gene respectively and are thought to be responsible for the rhythmic activity of kisspeptin and in-turn of gonadotropin releasing hormone (GnRH), which subsequently stimulates pulsatile secretion of LH from pituitary (Clarke and Cummins, 1985). Moreover, ARC kisspeptin neurons in females are colocalised with steroid receptors, such as ER (Smith et al., 2006), PR (Smith et al., 2007) and AR (Iwata et al., 2017), implying that kisspeptin directly receives the steroid signalling and facilitates the steroid feedback mechanism. Combining the fact that both kisspeptin and the macronutrient balance regulate reproductive function in females, it is postulated that kisspeptin may mediate the nutrient signalling to maintain the normal reproductive system but also PCOS pathology.

In a recent study, it has been unveiled that kisspeptin is also involved in metabolic regulation. A study conducted by Tolson et al. (2014) showed that the lack of kisspeptin signalling leads to metabolic dysfunctions, including increased body weight, fat mass and leptin concentrations, which is a hormone secreted from adipocytes and downregulates food intake (Schwartz et al., 2000). Of note, obesity and high fat mass are also the common characteristics of PCOS, as mentioned above. To regulate metabolism, kisspeptin interacts with other hypothalamic neurons. For example, NPY is a neuropeptide stimulating food intake and positive energy balance (Pu et al., 1999). Studies have shown that NPY neurons in the ARC have reciprocal connectivity with kisspeptin neurons (Backholer et al., 2010, Luque et al., 2007, Padilla et al., 2017). On the other hand, POMC is the major neuropeptide that inhibits food intake promoting negative energy balance (Zhan et al., 2013). POMC neurons located in ARC express GPR54, and they directly receive kisspeptin signalling (Fu and van den Pol, 2010). Furthermore, ARC kisspeptin neurons receive fibre projections from POMC neurons (Cravo et al., 2011). These findings show that kisspeptin plays an important role in controlling energy balance interacting with other hypothalamic neurons to stimulate energy expenditure (Tolson et al., 2014). However, their roles in the metabolic dysfunctions of PCOS have not been well examined. Thus, it is worth investigating if kisspeptin, as well as other energy balance related neurons that closely interact with kisspeptin, are involved in the metabolic dysfunctions in PCOS.

This Chapter has three experimental aims: To determine whether 1) macronutrient balance has an effect on kisspeptin and related neuropeptide gene expression in the hypothalamus, 2) diet composition affects the hypothalamic gene expression for neurons responsible for energy balance

and 3) kisspeptin and other hypothalamic neurons interact with nutrient signalling to influence the PCOS phenotype. To achieve these aims, we have examined the mRNA expression of key hypothalamic neurons under different macronutrient balanced diets in mice with or without DHT treatment.

3.2 Materials and Methods

3.2.1 Mice

A diet manipulation protocol that was used in a previous study (Solon-Biet et al., 2015) was applied paired with our DHT-treated PCOS-like mouse model to create the experimental design. For the animal model, CC57BL7/J female mice were purchased from the Animal Resources Centre at the age of three weeks and housed in a specific pathogen-free facility at the ANZAC Research Institute. Mice were maintained under *ad libitum* access to water and one of 10 diets varying in protein (P), carbohydrate (C) and fat (F) (Solon-Biet et al., 2015) (Table 5, Figure 8). Animals were anesthetized with a 1:1 ketamine and xylazine and euthanized at the age of 15 weeks to collect tissues for the analysis. All experiments were done following the protocol of Sydney Local Health District Animal Welfare Committee. The diet manipulation, body weight measurement and tissue collection were conducted by our collaborators from The University of New South Wales, and we acknowledge the animal work and the sample shipment done by Dr Kirsty Walters from University of New South Wales.

Table 5. The macronutrient ratio of the 10 diets. The % of protein (P), carbohydrate (C) and fat (F) of each diet (as a % of total energy).
The diets are isocaloric to 4kcal/g. The content of the diet varied in P (casein and methionine), C (sucrose, wheatstarch and dextrinized corn starch) and F (soya bean oil). Other ingredients included cellulose, a mineral mix (Ca, P, Mg, Na, C, K, S, Fe, Cu, I, Mn, Co, Zn, Mo, Se, Cd, Cr, Li, B, Ni and V) and Vitamin mix (vitamin A, D3, E, K, C, B1, B2, Niacin, B6, pantothenic acid, biotin, folic acid, inositol, B12 and choline).). Note: the inconsistencies in total percentage for diets 4 (101%) 5 (101%) and 10 (99%) are due to rounding to a whole percentage number.

	Diet component		
Diet	%P	%C	%F
1	60	20	20
2	5	75	20
3	5	20	75
4	33	48	20
5	33	20	48
6	5	48	48
7	14	29	57
8	14	57	29
9	43	29	29
10	23	38	38

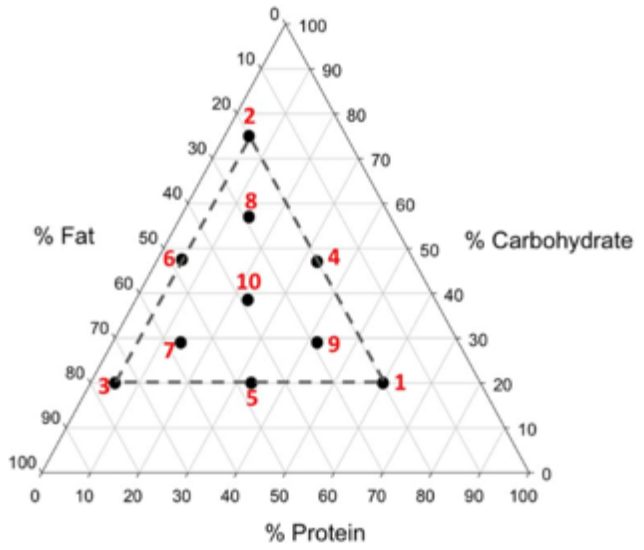

Figure 9. Geometric framework of the macronutrient composition of the diets.
The composition of each diet shown in the geometric framework, which exhibits the ratio of protein, carbohydrate and fat in each line. The numerical number shown in red represents the number of each diet.

3.2.1 mRNA analysis by qRT-PCR

To examine the gene expression in the PCOS mouse model with manipulations of macronutrient composition, qRT-PCR analysis was performed. Two housekeeping genes, *Tbp* and *PPIA* were used to normalise the data. All brain tissue sectioning, RNA extraction, reverse transcription and qRT-PCR analysis were conducted at the School of Human Sciences at The University of Western Australia.

3.2.1.1 Isolation of posterior ARC tissue

Tissue sectioning was performed in the RNA Room of the molecular biology laboratory. All experimental equipment was wiped with RNAse AWAY™ (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and rinsed with RNase-free water before use.

The hypothalamic was excised as previously described in Chapter 2 by lateral incision 3 mm on each side of the third ventricle and then separated into anterior and posterior portions (containing the

ARC) (see Chapter 2 Figure 5). All dissected brain tissues were stored in 2.0 ml microtubes (Axygen, California, San Francisco, USA) and stored in freezers at -80 ˚C.

3.2.1.2 RNA extraction

500 µl of QIAzol Lysis Reagent (Qiagen, Venlo, Limburg, Netherland) was added to the 2.0 ml tube with the tissue samples and immediately homogenised for 20-40 seconds. The homogenised samples were incubated at room temperature for 5 minutes then 200 µl chloroform was added, vortexed for 15 seconds and incubated at room temperature for 2-3 minutes. The homogenate was centrifuged (12,000 rcf, 4 ˚C, 25 min), and the upper aqueous phase was transferred to a new 1.5 ml tube (Axygen, California, San Francisco, USA). 500 µl isopropanol was added, vortexed and incubated at room temperature for 10 minutes. The solution was then centrifuged (12,000 rcf, 4 ˚C, 10 min) again and the supernatant was carefully discarded. 200 µl of 75% ethanol was then added and centrifuged (7,600 rcf, 4 ˚C, 5 min) to wash the precipitation. The resulting RNA pellet was isolated, air-dried maximum for 5 minutes at room temperature and resuspended in 20 µl of RNase-free water. The resuspended sample was immediately placed on dry ice after vortexed for a few seconds and stored at -80 ˚C.

3.2.1.3 RNA quality control

A quality control (QC) check using gel-electrophoresis was performed for all samples after RNA extraction and prior reverse transcription to confirm that the extracted RNA sample is at a high purity level without any contamination. The RNA sample was handled on the ice throughout the procedure. The gel was made by mixing 2% agarose gel (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and SYBR Green fluorescence dye (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) (60 µl drops by 50 ml) and left for 20-30 minutes to set at the room temperature. 1 µl of extracted RNA sample and 2 µl ddH_2O was mixed in the 0.2 ml tube (Axygen, California, San Francisco, USA) and incubated in the conventional PCR machine for 5 minutes at 65 ˚C. 6x loading dye (Promega, Madison, Wisconsin, USA) was added in the sample and mixed by pipetting. Then, the sample was loaded into the slot of the gel in the electrophoresis equipment filled with 1x TAE buffer. The electrophoresis was run at 120 V and 200 current for 30 minutes. 100 bp DNA ladder (Promega, Madison, Wisconsin, USA) was used as the indicator of the product size.The

outcome was visualised by ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, California, USA). Samples that did not show the clear band were excluded from the qRT-PCR analysis.

3.2.1.4 Removal of contaminating genomic DNA

To avoid interference of PCR due to contaminated genomic DNA, Promega DNase treatment-M610A was performed. The equivalent volume to 2 µg of RNA of each sample was calculated in advance. The calculation was based on the RNA concentration of each sample measured by NanoDrop® (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). 2 µg RNA of each sample was pipetted into 0.2 ml 12-Tube PCR Strips (Qiagen, Venlo, Limburg, Netherland). It was then added to 1 µl of RQ1 RNase-Free DNase 10x Buffer (Promega, Madison, Wisconsin, USA) and 2 µl of RQ1 RNase-Free DNase (Promega, Madison, Wisconsin, USA) and topped up with RNase free water to the final volume of 10 µl. This solution was incubated for 30 minutes at 37 ˚C and then added 1 µl of RQ1 DNase Stop Solution (Promega, Madison, Wisconsin, USA) to terminate the reaction of previously added DNase. It was then incubated for another 10 minutes at 65 ˚C to deactivate DNase Stop Solution. The solution was carefully mixed by pipetting, and the half of the volume (5.5 µl), which is equivalent to 1 µg RNA was pipetted to the new PCR Strips for the following reverse transcription.

3.2.1.5 Reverse transcription

The reverse transcription protocol for Promega MMLV #M3862 (Promega, Madison, Wisconsin, USA) was used. 1 µg of RNA aliquoted from the Promega DNase treatment was mixed with 8 µl of RNase free water and 0.5 µl of random primers (Promega, Madison, Wisconsin, USA). The solution was heated to 70 ˚C for 5 minutes then immediately chilled on ice for 5 minutes. The premixed solution composed of 5 µl 5x reaction buffer (Promega, Madison, Wisconsin, USA), 1.3 µl 10 mM dNTPs (Promega, Madison, Wisconsin, USA), 1 µl M-MLV (Promega, Madison, Wisconsin, USA)) and 3.7 µl RNase free water was added to the solution. Then it was incubated through 25 ˚C (10 minutes) – 55 ˚C (50 minutes) – 70 ˚C (15 minutes) – 4 ˚C (∞) after mixed gently by pipetting.

To purify the cDNA sample, the product was ‘cleaned’ by using QIAquick® PCR Purification Kit (Qiagen, Venlo, Limburg, Netherland). The sample was mixed with 125 µl (5 volume of the sample)

of Buffer PB and moved to the 2 ml collection tube equipped with filter-column. Then the solution was centrifuged at 13,000 rpm for 30-60 seconds, and the flow-through was discarded. 750 µl Buffer PE was added to the filter-column and centrifuged (13,000 rpm, 30-60 seconds) to wash the product. The tube was centrifuged again at 13,000 rpm for 1 minute after the flow-through was discarded. The filter-column was then placed in a 1.5 ml tube (Axygen, California, San Francisco, USA), and 50 µl ddH_2O was added to elute DNA. The sample was mixed throughout by vortex and stored at -20 ˚C freezer.

3.2.1.6 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to examine the quantification of genes of interest (GOI). QuantiTect® Primer Assay (QIAGEN, Venlo, Netherland), which is for use in real-time RT-PCR with SYBR® Green detection was utilised for the analysis.

Posterior hypothalamic mRNA expression for dynorphin (*Pdyn*), neurokinin B (*Tac2*), Proopiomelanocortin (*Pomc*) and neuropeptide Y (*Npy*) was quantified by qRT-PCR on CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). All samples were run in duplicates. Primer pairs for each GOI (Table 8) were purchased as QuantiTect® primers (Qiagen, Venlo, Limburg, Netherland). Gene expression was normalised against reference genes TATA box binding protein (*Tbp*) and peptidylprolyl isomerase A (*Ppia*) that were designed using Primer-BLAST. Their gene sequence and details were shown in Table 8. The optimal annealing temperature for QuantiTect® primers was examined and determined at 62 ˚C. Cycling number is also optimised at 50 times. For the analysis of the result, samples that showed multiple PCR products in melting curve analysis, different melting temperature or no amplification were excluded from the analysis. The qRT-PCR protocols used for GOI and reference genes are shown in Table 6 and Table 7, respectively.

Unfortunately, the mRNA expression of *Kiss1* was unable to be reliably determined (See Appendix) and was removed from the analysis in Chapter 2. This is deemed due to the sample degradation caused by the sample shipment from The University of New South Wales to The University of Western Australia and the poor care while being stored.

Table 6. qRT-PCR protocol for genes of interest (GOI).
This protocol was run by CFX384 Touch™ Real-Time PCR Detection System.

Step	Temperature (˚C)	Duration	
Hold	95 ˚C	10 minutes	
Cycling	95 ˚C	10 seconds	x 50
	62 ˚C	30 seconds	
Melt	70-99 ˚C		

Table 7. qRT-PCR protocol for reference genes.
This protocol was run by CFX384 Touch™ Real-Time PCR Detection System.

Step	Temperature (˚C)	Duration	
Hold	95 ˚C	10 minutes	
Cycling	95 ˚C	1 seconds	x 50
	60 ˚C	15 seconds	
	72 ˚C	5 seconds	
Melt	70-99 ˚C		

Table 8. Primer details of GOI and reference genes used in qRT-PCR.
QuantiTech name, primer sequences (F, forward; R, reverse), product size (base pairs, bp) and accession code (AC) for each primer used for qRT-PCR analysis.

Gene	**QuantiTech name or Primer sequence**	**Size bp**	**AC**
Pdyn	Mm_Pdyn_1_SG	143	NM_018863
Tac2	Mm_Tac2_2_SG	74	NM_009312
Pomc	Mm_Pomc_1_SG	99	NM_008895
Npy	Mm_Npy_1_SG	150	NM_023456
Tbp	F, 5'-GGG AGA ATC ATG GAC CAG AA-3'	113	NM_013684.3
	R, 5'-CCG TAA GGC ATC ATT GGA CT-3'		
Ppia	F, 5'-AGC ATA CAG GTC CTG GCA TC-3'	127	NM_017101
	R, 5'-TTC ACC TTC CCA AAG ACC AC-3'		

3.2.2 Statistical analysis

All data are expressed as mean ± SEM for each group. Group difference of gene expression within each treatment and genotype were analysed by two-way ANOVA followed by Fisher's least

significant difference multi-comparison test. Statistical significance was set at $P<0.05$. All analysis was performed with Prism version 8.3.1 for Macintosh.

3.3 Results

3.3.1 Body weight

DHT treatment significantly increased body weight of animals fed with relatively balanced macronutrient diets, which were diets 5, 7, 8, 9 and 10 ($P<0.05$; Figure 9). However, the extreme diets (diets heavily favouring one macronutrient – diets 1, 2 and 3) prevented the body weight increase caused by the DHT administration ($P>0.3$; Figure 9). Similarly, unbalanced diets such as diet 4 with relatively low fat and diet 6 with low protein also inhibited the DHT-induced body weight increase. Of note, the high-fat diet/low protein (diet 3; P:C:F=5:20:75) prevented DHT-induced body weight increase and showed significantly smaller body weights compared with all other diet groups that received DHT administration (Table 9). A similar trend was also observed in the high carbohydrate diet/low protein (diet 2; P:C:F=5:75:20) fed animals with DHT administration, whose body weight was significantly smaller than all other DHT-treated animals, except diet 1 and 3 (Table 9). On the other hand, diet 8 (with relatively high carbohydrate P:C:F=14:57:29) promoted DHT-induced body weight increase the most among all 10 diets. In the DHT treated groups, animals fed with diet 8 gained significantly more weight than those in other diet groups, with the exception of diet 7 (Table 9).

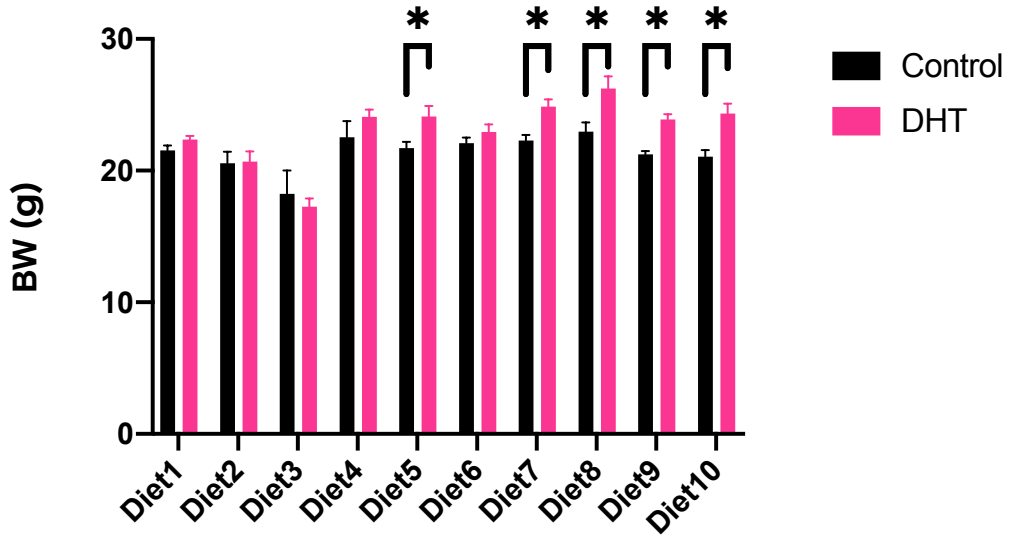

Figure 10. Body weights in DHT or control treatment mice fed varying macronutrient-based diets.
Body weight of animals was measured at the age of 16 weeks, after 13 weeks of either blank or DHT subcutaneous implants. The black bar represents control groups, and the pink bar represents DHT treated groups. Data are the mean with SEM; n=10 (Diet 3 Control and DHT: n=3) per diet/treatment group. (*P<0.05, two-way ANOVA; Fisher's LSD test, multiple comparison between diet groups and control or DHT treatment groups.)

Table 9. Significance summary of two-way ANOVA analysis of body weight in the macronutrient diet model.
The results shown are from the multiple comparison by Fisher's LSD test for diet induced changes, within either Control or DHT treated groups. The number of the diet is shown in the X-axis and the Y-axis, and the asterisk represents the significant difference (*P<0.05) between the diet. Control groups are shown in black asterisk, and DHT-treated groups are in red.

Diet	1	2	3	4	5	6	7	8	9	10
1			*				*	*		*
2			*	*	*	*	*	*	*	*
3	*			*	*	*	*	*	*	*
4		*	*					*		
5			*					*		
6			*				*	*		
7			*							
8		*	*						*	*
9			*							
10			*					*		

3.3.2 Gene expression of hypothalamic neuropeptides

Our result of two-way ANOVA analysis on the hypothalamic gene expression implied that the macronutrient balance in diet has significant effects on the *Tac2* and the *Npy* expression in the lateral hypothalamus (Table 11). However, only *Tac2* gene expression was influenced by the DHT treatment (Table 11).

DHT administration significantly elevated *Pdyn* mRNA expression in diet 9 (relatively high protein diet P:C:F = 42:29:29), compared with the respective control group. (Figure 10). There was no significant change in the *Pdyn* expression associated with DHT treatment in other diet groups. In consideration of DHT treated mice, *Pdyn* gene expression was significantly enhanced in diet 9 (relatively high protein P:C:F = 42:29:29) compared with diet 1 (P:C:F = 60:20:20) and 4 (P:C:F = 33:48:20) (Table 11). In control mice, *Pdyn* mRNA expression was highest in diet 6 (P:C:F = 5:48:48), and it was lowest in diet 5 (P:C:F = 33:20:48). A significant difference was reported in the control diet 6 group compared to control diet 5 in *Pdyn* expression ($P<0.05$, Table 11).

Following qRT-PCR analysis of NKB, DHT treatment significantly increases *Tac2* gene expression compared with the control group in diet 7 (P:C:F=14:29:57), which is relatively low protein diet, yet no other diet groups were affected by the DHT treatment (Figure 11). In control groups, diet 6 (P:C:F = 5:48:48), which has the lowest protein composition, had the highest *Tac2* gene expression, and it was significantly higher than all other diet groups ($P<0.05$, Table 12). In DHT-treated groups, *Tac2* gene expression was highest with diet 10 (P:C:F = 23:38:38), which is the most balanced diet in this study, and it was significantly greater compared with diet 1 (P:C:F = 60:20:20), 2 (P:C:F = 5:75:20), 4 (P:C:F = 33:48:20) and 5 (P:C:F = 33:20:48) (all $P<0.05$, Table 12). Moreover, *Tac2* gene expression appeared to be more heavily influenced by the macronutrient balance than the DHT administration (two-way ANOVA: Diet; $p < 0.0001$, Treatment; $p< 0.025$) (Table 10).

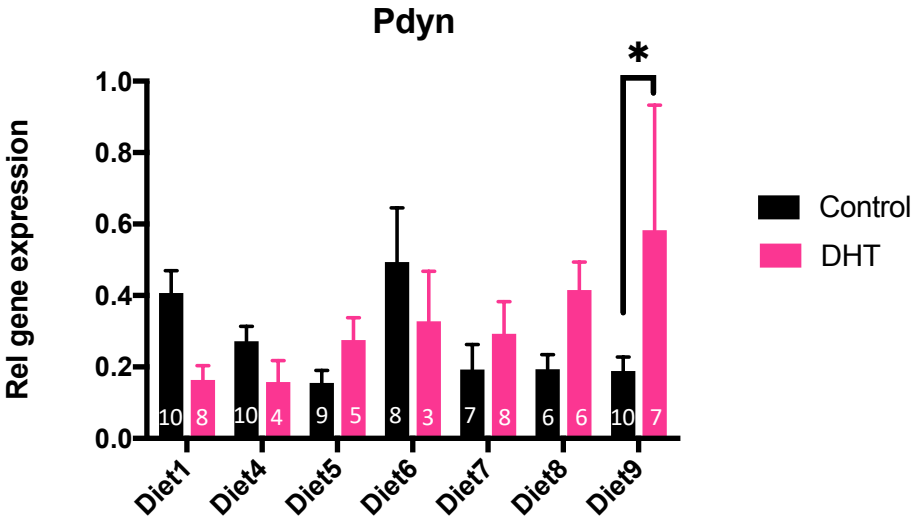

Figure 11. mRNA expression of mPdyn in the macronutrient diet model.
*The result of qRT-PCR in the posterior hypothalamus of control and DHT treated female mice. The black bars represent control groups, and the pink bars represent DHT treated groups. Data are the mean with SEM; n=2 to 8 per genotype/treatment group. (*P<0.05, two-way ANOVA; Fisher's LSD test, multiple comparison between diet groups and control or DHT treatment groups.)*

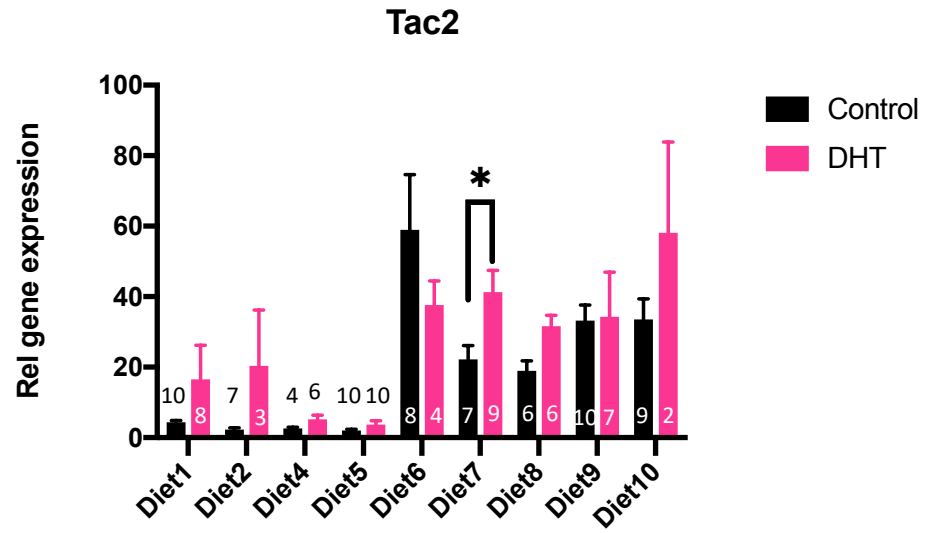

Figure 12. mRNA expression of mTac2 in the macronutrient diet model.
*The result of qRT-PCR in the posterior hypothalamus of control and DHT treated female mice. The black bars represent control groups, and the pink bars represent DHT treated groups. Data are the mean with SEM; n=2 to 8 per genotype/treatment group. (*P<0.05, two-way ANOVA; Fisher's LSD test, multiple comparison between diet groups and control or DHT treatment groups.)*

Table 10. Significance summary of two-way ANOVA analysis in the macronutrient diet model. The results shown are from the multiple comparisons by Fisher's LSD test. The asterisk represents the significant difference (*P<0.05) between the diet and the number of asterisks indicate the degree of the significance.

	Pdyn	*Tac2*	*Pomc*	*Npy*
Diet	ns	**** (P<0.0001)	ns	* (P=0.0181)
DHT treatment	ns	* (P=0.0248)	ns	ns
Interaction	ns	ns	ns	ns

Table 11. Significance summary of two-way ANOVA analysis of the Pdyn mRNA expression in the macronutrient diet model.
The results shown are from the multiple comparisons by Fisher's LSD test for diet induced changes, within either Control or DHT treated groups. The number of the diet is shown in the X-axis and the Y-axis, and the asterisk represents the significant difference (*P<0.05) between the diet. Control groups are shown in black asterisk, and DHT-treated groups are in red.

Diet	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1									*	
2										
3										
4									*	
5										
6					*					
7										
8										
9										
10										

Table 12. Significance summary of two-way ANOVA analysis of the Tac2 mRNA expression in the macronutrient diet model.
The results shown are from the multiple comparison by Fisher's LSD test for diet induced changes, within either Control or DHT treated groups. The number of the diet is shown in the X-axis and the Y-axis, and the asterisk represents the significant difference (*P<0.05) between the diet. Control groups are shown in black asterisk, and DHT-treated groups are in red.

Diet	1	2	3	4	5	6	7	8	9	10
1							*			*
2										*
3										
4						*	*	*	*	*
5						*	*	*	*	*
6	*	*		*	*					
7	*	*		*	*	*				
8						*				
9	*	*		*	*	*				
10	*	*		*	*	*				

Pomc mRNA expression in the ARC was not influenced by the macronutrient balance of diet and DHT treatment (Figure 12).

DHT treatment significantly increased *Npy* gene expression in diet 9 (P:C:F = 42:29:29) compared with the respective control group (P<0.05, Figure 13). In consideration of control mice, diet groups with the lowest fat, which were diet 1 (P:C:F = 60:20:20), 2 (P:C:F = 5:75:20) and 4 (P:C:F = 33:48:20) tended to have the highest *Npy* mRNA expression (Figure 13, Table 13). Specifically, *Npy* gene expression of diet 1 was significantly enhanced compared with relatively balanced diet groups, diet 7 (P:C:F = 14:29:57), 8 (P:C:F = 14:57:29) and 9 (P:C:F = 42:29:29) (Table 13). In DHT-treated mice, *Npy* mRNA expression was lowest in relatively low but not extremely low protein diets, which are diet 7 (P:C:F = 14:29:57) and 8 (P:C:F = 14:57:29) (Table 13).

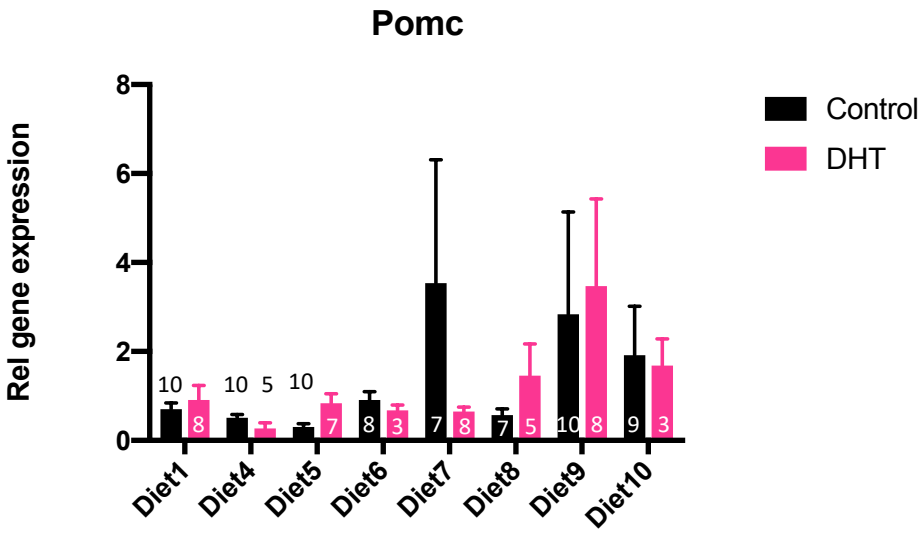

Figure 13. mRNA expression of mPomc in the macronutrient diet model.
*The result of qRT-PCR in the posterior hypothalamus of control and DHT treated female mice. The black bar represents control groups, and the pink bar represents DHT treated groups. Data are the mean with SEM; n=2 to 8 per genotype/treatment group. (*P<0.05, two-way ANOVA; Fisher's LSD test, multiple comparison between diet groups and control or DHT treatment groups.)*

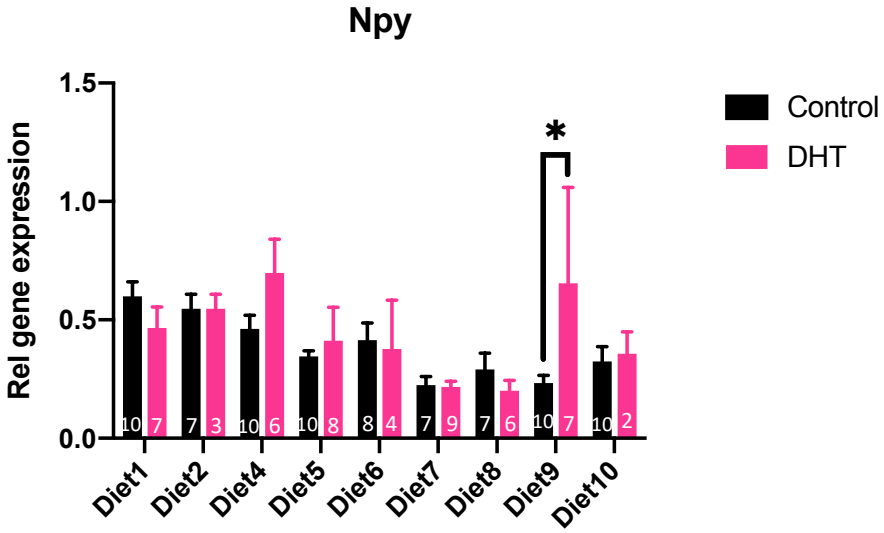

Figure 14. mRNA expression of mNpy in the macronutrient diet model.
*The result of qRT-PCR in the posterior hypothalamus of control and DHT treated female mice. The black bar represents control groups, and the pink bar represents DHT treated groups. Data are the mean with SEM; n=2 to 8 per genotype/treatment group. (*P<0.05, two-way ANOVA; Fisher's LSD test, multiple comparison between diet groups and control or DHT treatment groups.)*

Table 13. Significance summary of two-way ANOVA analysis of the Npy mRNA expression in the macronutrient diet model.
The results shown are from the multiple comparison by Fisher's LSD test for diet induced changes, within either Control or DHT treated groups. The number of the diet is shown in X axis and Y axis, and asterisk represents the significant difference (*P<0.05) between the diet. Control groups are shown in black asterisk and DHT-treated groups are in red.

Diet	1	2	3	4	5	6	7	8	9	10
1										
2							*	*		
3										
4							*	*		
5										
6										
7	*								*	
8	*								*	
9	*	*								
10										

3.4 Discussion

Although health management measures, such as weight loss strategies or pharmacological treatment for glucose resistance are used as a major treatment for PCOS (Teede et al., 2007), the specific factors and mechanisms mediating metabolic signalling and causing PCOS symptoms remain unclear. Recently, a study that examined the effect of dietary macronutrient balance on reproductive function in mice revealed that dietary nutrition composition has an impact on the reproductive outcomes in females, including ovarian morphology and estrous cycles (Solon-Biet et al., 2015). To clarify the role of dietary macronutrient balance in the cause of PCOS, the current study investigated the relationship between dietary macronutrient balance and the key hypothalamic gene expression involved in reproductive function and energy balance regulation, in a PCOS-like animal model. Using DHT-induced PCOS in female mice, our data demonstrated that the ratio of protein, carbohydrate and fat in the diet affects metabolic outcomes in females with an interacting role of the circulating levels of androgen. Furthermore, we showed that the macronutrient balance in diet likely influences the hypothalamic neuron activity, and androgen is also likely to modify these effects caused by diet on hypothalamic gene expression. Firstly, regarding body weight analysis, we found that the 'extremely' unbalanced diets (heavily based on a single macronutrient) prevent DHT-induced body weight increase in female mice. This result could be attributed to the reduction of appetite because of the possible unpalatableness of these extreme diets. However, it is uncertain whether this is the case due to the lack of specific food and caloric intake data currently available.

In our study, the composition of fat in the diet, especially the relatively high fat diets (Diet 3) had an interactive effect with androgen and caused a reduction in the body weight of females. Thus, dietary macronutrient balance appears to interact with androgen signalling and is likely involved in the metabolic regulation in females. Importantly, a limitation of this study was the lack of body composition measurement and locomotive activity information in addition to a lack of food and caloric intake data. Additionally, a small sample size of some groups due to the inability to extract high quality RNA or detect gene expression by qRT-PCR and the fairly large variation in two-way ANOVA analysis could be another limitation in the present study. Because body weight is affected by both energy intake and energy expenditure, which is in-turn influenced by multiple factors such as the fat mass, lean tissue mass, metabolic rate and locomotion (Schutz, 1995), it is difficult to pin-point the exact metabolic response to diet. For example, in a study that investigated metabolic phenotypes of *Kiss1r*KO mouse it was revealed that *Kiss1r*KO females gained more weight than WT even though the food intake was significantly decreased in *Kiss1r*KO (Tolson et al., 2014). The study

also found that locomotor activity and lean tissue mass in *Kiss1r*KO were decreased compared with WT, thus the weight gain of *Kiss1r*KO appears to be attributed predominantly to the reduced energy expenditure (Tolson et al., 2014). Therefore, there may be other factors besides macronutrient balance that contributed to the smaller body weight in extreme diet groups, including food intake amount, decreased locomotive activity or change in body composition that subsequently alter energy expenditure. To get a clearer idea about the relationship between dietary macronutrient balance and metabolism, it is necessary to investigate these features in future research.

Macronutrient balance appears to interact with circulating androgens to influence key hypothalamic neuron activity, which is likely or regulate reproductive and metabolic function. Here, we provide evidence that relatively elevated or preferential protein intake is likely to play a role in the regulation of *Pdyn*, *Tac2* and *Npy* gene expression in the posterior hypothalamus (most likely in the ARC). Specifically, it is indicated that a relatively high protein diet increases *Pdyn* and *Npy* mRNA expression compared to other diets, whereas *Tac2* mRNA expression is increased in a relatively low protein diet compared to other diets. Furthermore, as *Pdyn* gene expression in control females is maximised in the low protein diet group (diet 6; P:C:F ratio = 5:48:48) and minimised in the low carbohydrate diet group (diet 5; P:C:F = 33:20:48), the ratio of protein and carbohydrate potentially has an impact on *Pdyn* expression in females. Regarding the dietary and androgen effect on *Tac2* mRNA expression, 2-way ANOVA analysis indicated that *Tac2* gene expression is significantly influenced by both DHT treatment and diet, and the diet has a statistically 'stronger' effect than the DHT administration. Indeed, *Tac2* mRNA expression is maximised with low protein diet (diet 6; P:C:F = 5:48:48) in the control group, which again implies that protein is an important factor to regulate *Tac2* gene expression. However, with DHT treatment, *Tac2* gene expression was maximised in the group fed with the most balanced macronutrient diet (diet 10; P:C:F = 23:38:38), indicating that the NKB neuron sensitively responds to both dietary and androgen signalling and modulates its activity in ARC.

Although the POMC neuron is a major anorexigenic neuron and a metabolic regulator (Koch et al., 2015), our results however suggested that POMC neuron is not robustly involved in the metabolic modulation caused by diet composition or hyperandrogenism – at least in regard to the expression of *Pomc* mRNA. Therefore, it is likely that other neuronal pathways exist that regulate metabolic endpoints interacting with macronutrient balance and androgen signalling. Alternatively, there may be different neurons that play a role in energy balance responding to the endocrine or metabolic changes caused by hyperandrogenism. On the other hand, the present study indicated that *Npy* gene

expression in NPY neurons, which is known as an orexigenic neuropeptide (Korner et al., 2001) is affected by diet composition. In the control condition, the low fat diet appeared to increase *Npy* gene expression compared to other diets, which is consistent to the observation by Lin et al. (2000) that *Npy* mRNA expression was significantly increased in a mice group fed with low fat diet than high fat diet fed group. Moreover, it appears that unbalanced diets, which are located at the 'outline' of the geometric framework elevate *Npy* gene expression compared with more balanced diets, suggesting that the balance of the nutrition has an impact on *Npy* mRNA expression and potentially orexigenic behaviour. As both POMC and NPY neurons are regulated directly by leptin (Elias et al., 1999), it is important to investigate the leptin receptor expression in response to macronutrient balance in future studies to enable more precise investigation of the role of macronutrient balance in the regulation of hypothalamic gene expression.

Regarding the potential relationship between leptin and PCOS, a clinical study demonstrated that the administration of metformin, which is a drug to enhance insulin sensitivity and used for the treatment of insulin resistance, significantly reduced serum leptin concentration in PCOS patients (Pasquali et al., 2000), which may subsequently affect POMC and NPY neuronal activity in PCOS females. However, there is no clear evidence about the role of leptin and its relationship with insulin resistance in PCOS – so far there have been no clear studies using animal models. In the previous study using PCOS-like mouse models, postnatal DHT administration (which was also used in the present study) did not promote insulin resistance (Caldwell et al., 2014). Similarly, the prevalence rate of insulin resistance in PCOS women is about 64% (DeUgarte et al., 2005), and not all insulin resistant females present with PCOS. Furthermore, Torres et al. (2019) revealed that in the PCOS-like mouse models that are treated with letrozole in the pre-pubertal stage exhibit insulin resistance, whereas adult female mice treated with letrozole do not show insulin resistance, suggesting that the timing of exposure to excess androgen is highly associated with the symptom onset of insulin resistance in PCOS.

In clinical PCOS studies, it has also been suggested that diet plays an important role in PCOS symptoms. A study found that PCOS women who consumed low carbohydrate diet had decreased body weight and fat mass after eight weeks experimental period (Goss et al., 2014). However, in the present study, we did not find consistent body weight decrease in low carbohydrate fed groups. It should be noted that we cannot simply compare clinical and animal study data for many reasons including the different conditions of the study, the nutrient composition of the diet and the experimental period. Moreover, the outcomes of the study that examined low carbohydrate diet

effect on metabolic features in PCOS patients varied depending on the method to produce the low carbohydrate diet. For example, other clinical studies that examined the dietary effect on PCOS traits showed that PCOS patients who consumed a low carbohydrate diet showed significantly reduced fasting insulin levels and increased insulin sensitivity compared to prior the study (Douglas et al., 2006, Gower et al., 2013), indicating that a low carbohydrate diet may improve metabolic abnormalities in PCOS. Importantly, insulin is highly associated with PCOS because insulin resistance is one of the major metabolic features observed in PCOS patients (DeUgarte et al., 2005) and in some animal models (Torres et al., 2019). These findings indicate that decreased insulin sensitivity plays a role in the development of PCOS. Indeed, PCOS patients treated with metformin showed reduced serum insulin level as well as the improvement in the endocrine abnormalities of PCOS, such as decreased serum testosterone and LH concentration (Nestler and Jakubowicz, 1996).

Moreover, they also found that the activity of P450c17α, the enzyme that is responsible for the biosynthesis of androgen in ovarian theca cells (Walters et al., 2019) was significantly reduced in metformin-treated PCOS patients (Nestler and Jakubowicz, 1996). Thus, insulin sensitivity is important to maintain healthy androgen synthesis in females as well as normal endocrine function. As insulin levels appear to be influenced by diet, the dietary nutrient balance may play a role in causing metabolic and endocrine dysfunction in PCOS interacting with insulin signalling. Because the current study did not examine either insulin concentration or the gene expression of the P450 enzyme, further research can be done to clarify the effect of dietary macronutrient balance on insulin level and androgen synthesis.

In summary, the present study showed that dietary macronutrient balance affects metabolism and hypothalamic neuron gene expression interacting with androgen signalling. We found that the extremely unbalanced macronutrient diets interfere with the DHT-induced body weight increase in female mice. Furthermore, our result suggested that nutrition, especially relative protein and fat content in the diet, may play a role in the regulation of hypothalamic gene expression. Due to the lack of data about *Kiss1* mRNA expression, food intake, body composition and locomotive activity of mice in each diet group and treatment, it is challenging to argue deeply about the role of diet in metabolism and modulation of hypothalamic gene expression in PCOS. Additionally, the sample size of some of the groups were very small or several groups were missing in statistics due to the degradation of samples and inability to extract or detect RNA, presumably caused by the low shipping quality and not well-managed sample storage. This was another limitation for our study to discuss the relationship between diet and the hypothalamic gene expressions in PCOS mouse model.

However, these data form a strong basis for ongoing study. Further investigation is required to define the effect of dietary nutrition on PCOS traits and neuron activity that controls endocrine and metabolic function in PCOS females.

Chapter 4. General Discussion and Summary

To summarise broadly the two studies in this thesis, we have shown using a postnatal DHT-treated PCOS-like mouse model that AR pathways and dietary macronutrient balance affect gene expression of key hypothalamic neuropeptides. Thus, it is likely that androgen signalling and diet play key roles in modulating the reproductive and metabolic neuroendocrine characteristics of PCOS.

In the ARKO study (Chapter 2), we demonstrated that *Tac2* mRNA expression in the posterior hypothalamus was increased in NeuroARKO mice compared with WT and ARKO. Moreover, the DHT administration significantly reduced *Tac2* gene expression in NeuroARKO females compared with control group. This unexpected result may indicate the possible involvement of short-loop feedback of androgen on pituitary to regulate LH release and in-turn lead to feedback of higher hypothalamic centres. Furthermore, it was implied that POMC and NPY neurons are not primarily involved in the metabolic dysfunction observed in PCOS animal model thorough an AR dependant pathway. Thus, our results indicated the existence of other neuronal or hormonal pathways that are regulated via AR and responsible for the development of PCOS, including GABAergic and PR pathway.

In the macronutrient diet study (Chapter 3), we showed that the extremely unbalanced macronutrient diets – favouring carbohydrate, protein or fat – prevented the DHT-induced body weight increase in the PCOS-like mouse model. The gene expression analysis conducted by qRT-PCR suggested that the key hypothalamic gene expression, specifically *Pdyn*, *Tac2* and *Npy* but not *Pomc* are influenced by dietary macronutrient balance interacting with androgen treatment. Importantly, the relative composition of protein and fat potentially plays an important role in the regulation of these hypothalamic genes in the hyper androgenised condition. These may offer insights into diet manipulation for managing PCOS-associated symptoms.

The present study had several limitations, including the small sample size, lack of LH pulse data by rapid blood collection in ARKO mouse models and detailed metabolic information of each diet/treatment group in the macronutrient diet study such as food intake, body composition and locomotive activity. In future studies, it is essential to examine these factors to elucidate the precise mechanism involved in the development of PCOS and its correlation with AR signalling or macronutrient balance. Moreover, we were unable to examine *Kiss1* mRNA in these studies – for reasons detailed in Appendix 1. Furthermore, the specific activation of ARC kisspeptin neurons by the combination of GCamp and optogenetic techniques, which was previously used in the

measurement of GABA neuron activity on PCOS phenotypes (Silva et al., 2019), may be an effective method to determine the role of kisspeptin in PCOS aetiology.

There are inherent limitations of the mouse DHT-induced PCOS model. Generally, this model recapitulates the high androgens that occur with PCOS, but these are given exogenously and therefore are unlikely to truly recapitulate the neuroendocrine drivers for PCOS. The model, however, would be an accurate reflection of physiological responses to high androgens – both in the brain and in peripheral tissues. Therefore, we can see from these studies that the neuroendocrine response to androgens is dependant on androgen signalling (not surprisingly) but also, the macronutrient composition of the diet. How diet influences PCOS symptoms is still not completely clear, but from our results in the mouse, we would hypothesise that a diet relatively high in protein would be beneficial to PCOS women and a diet relatively high in carbohydrates would be detrimental. These would valuable and exciting studies to conduct in the future.

Lastly, there is much remaining to be done to clarify the mechanism of PCOS. One of the biggest challenges in PCOS research is to establish an animal model that perfectly reflects human PCOS symptoms and shows the consistent features driving these phenotypes. Particularly, the data available regarding kisspeptin expression in PCOS animal models are somewhat mixed depending on the animal model used. The endocrine system that kisspeptin neurons are involved in is very complex and sensitive to both endogenous and exogenous hormonal changes or the ambient environment (nutrition, circadian rhythms etc.). Therefore, it is important to choose the appropriate animal model that suits the aim of the study and examine the wide range of features that can potentially affect hypothalamic neuron activity. For example, the correlation between kisspeptin and other endocrine factors that are suggested to be involved in PCOS phenotypes such as insulin, leptin, progesterone and P450 have not been determined yet, and it should be investigated in the future studies.

Overall, the aim of this thesis was to utilise the postnatal DHT-treated PCOS-like mouse model to determine the relationship between the key hypothalamic gene expression in the ARC, AR signalling and dietary macronutrient balance. We showed that the AR pathway and macronutrient balance have modulatory effects on the gene expression of hypothalamic neurons, which are involved in reproductive and metabolic regulation associated with a PCOS-like state.

Chapter 5. References

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Chapter 6. Appendix

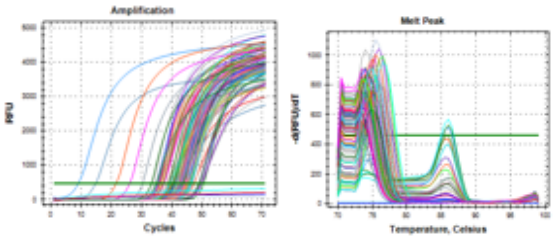
6.1 Extra experiments done to optimise qRT-PCR condition

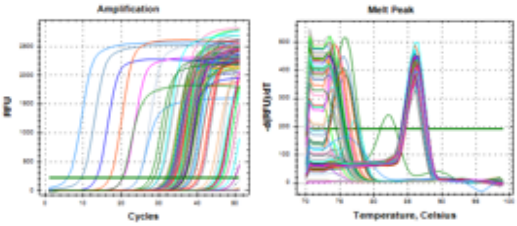
One of the major goals of the present study was to examine the mRNA expression of *Kiss1* in the anterior and posterior hypothalamus to investigate the potential role of kisspeptin in PCOS with the interaction of AR pathway or diet. However, the detection of *Kiss1* gene expression by qRT-PCR technique was extremely difficult presumably due to its low expression and the condition of the brain samples. To gain better outcomes in the PCR analysis, we attempted to optimise qRT-PCR protocol by testing few conditions. Here, we show the results of experiments we have done to optimise the protocol.

6.1.1 Water influence on qRT-PCR outcome in ARKO models

ddH$_2$O | **Qiagen H$_2$O/DMDC**

A.





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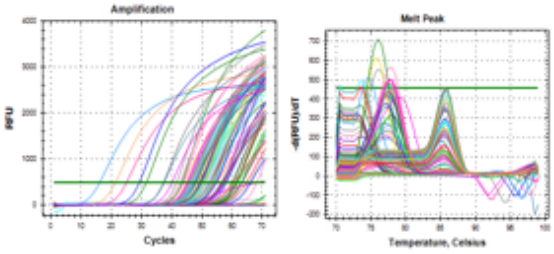
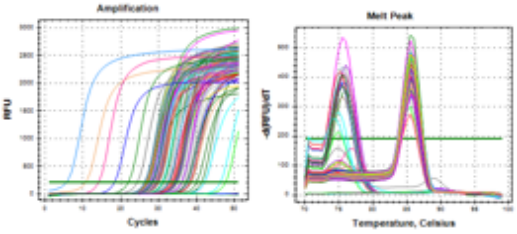
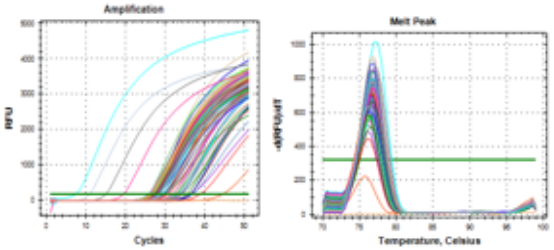
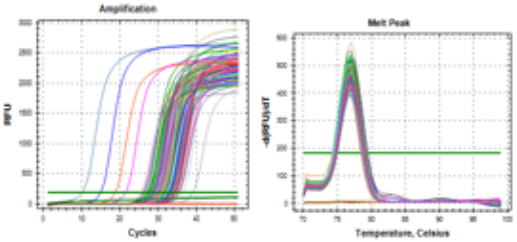




C.





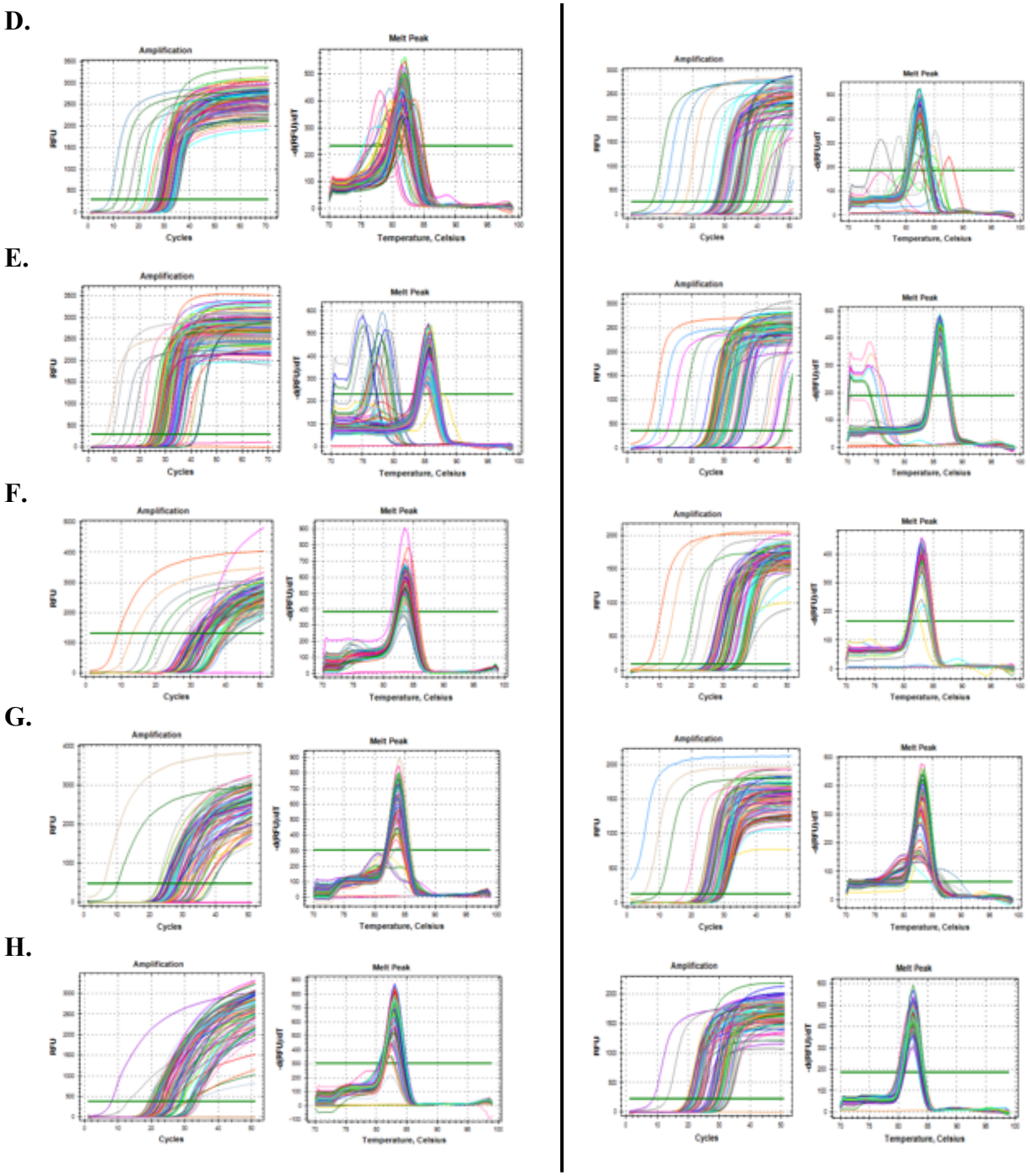


Figure 15. The influence of water for PCR master-mix on the outcome of qRT-PCR in Kiss1, Pdyn, Tac2, Pomc, Npy, TBP, SDHA and PPIA in the mouse hypothalamus of ARKO models. The PCR outcome was compared using ddH2O and Qiagen H_2O or Dimethyldicarbonate (DMDC) for PCR master-mix. (A) Kiss1, (B) Pdyn, (C) Tac2, (D) Pomc, (E) Npy, (F) TBP, (G) SDHA, (H) PPIA. The cycle number to reach plateau and the melt curve analysis outcome have largely improved in all genes when Qiagen H_2O or DMDC was used.

6.1.2 The effect of cycle number and water on the outcome of negative control

The detection of negative control was one of the challenges that prevented us to get the analysable data. Thus, we have tested the effect of cycle number and the water on the outcome of negative control detection in qRT-PCR because the bigger number of the cycle was considered to be the cause of the negative control detection.

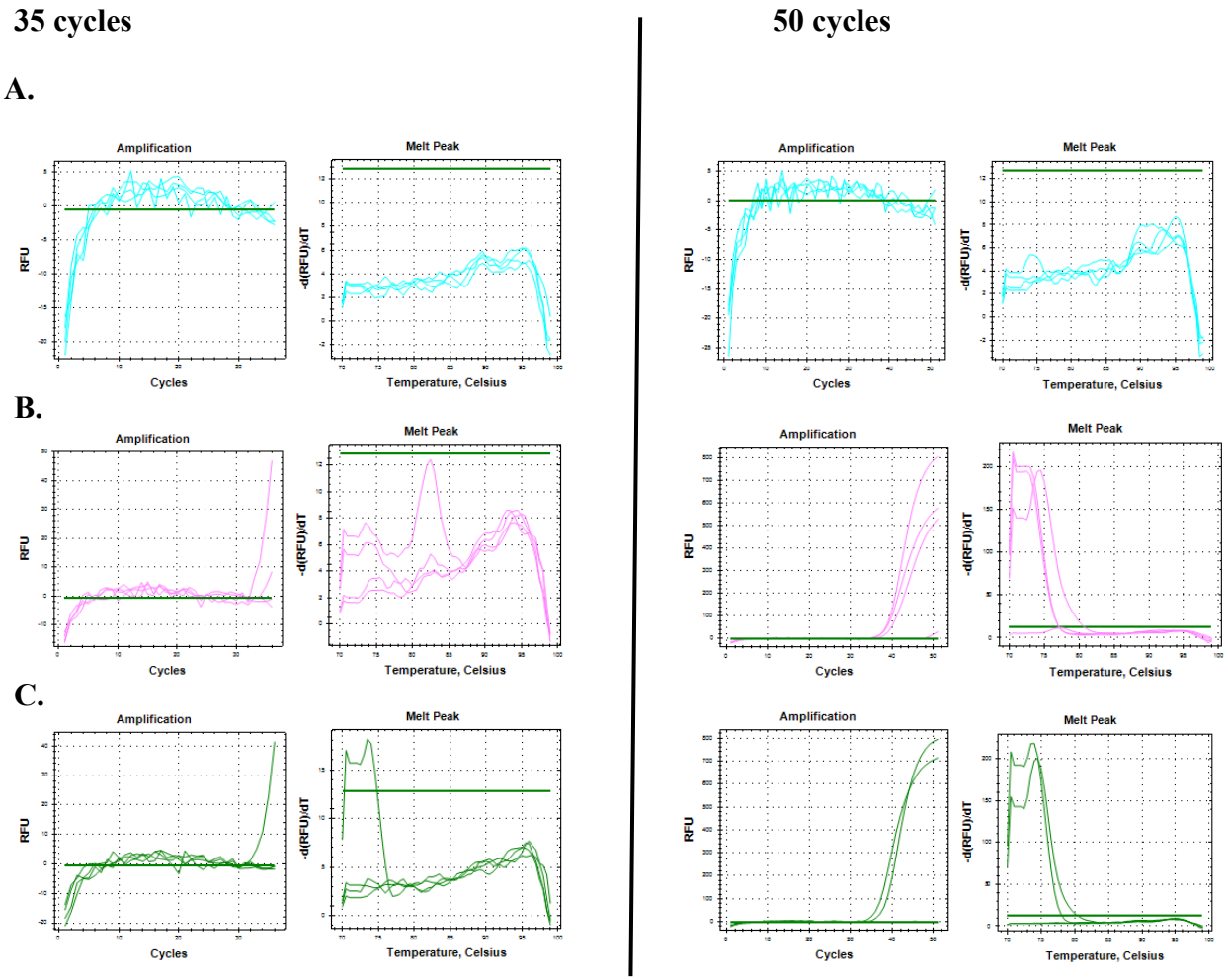


Figure 16. The impact of cycle number and water for negative control on the outcome of qRT-PCR in TBP.
The master-mix was consisted of 5µl SYBR Green Supermix, 1µl forward mTBP primer, 1µl reverese mTBP primer and 1µl Qiagen H_2O. 1µl Qiagen H_2O, 1µl ddH_2O or nothing was added in the master mix and amplified 35 or 50 cycles. (A) Qiagen H_2O, (B) ddH_2O, (C) Master-mix only. The negative control outcome was most stable with Qiagen H_2O. The negative control was more likely to be detected and appear in melt curve analysis as the cycle number increases.

6.1.3 Double amplification effect on *Kiss1* and *Pdyn* qRT-PCR outcome

The low product concentration of the samples was considered to be a cause of failure in melt curve analysis. Therefore, the PCR outcome was examined by amplifying twice using seven random samples. The first amplification was 20 cycles to increase the product concentration, then 1µl of firstly amplified product was amplified 50 cycles in the second amplification with fresh master-mix.

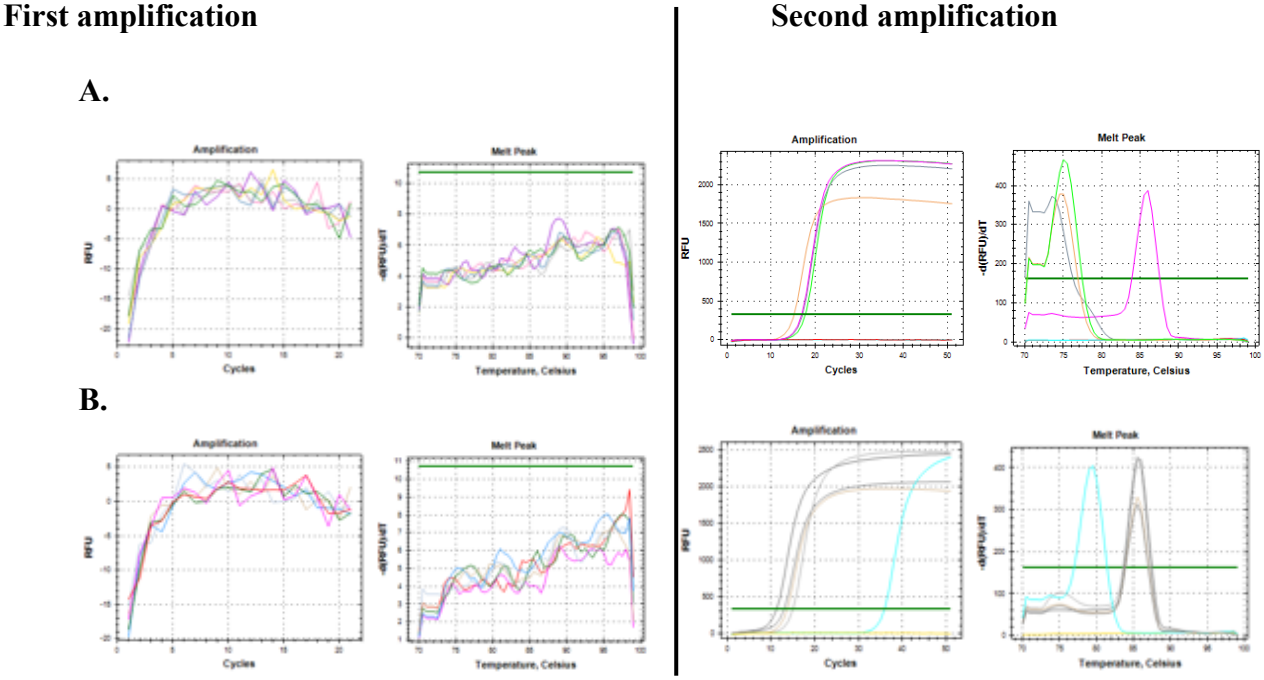
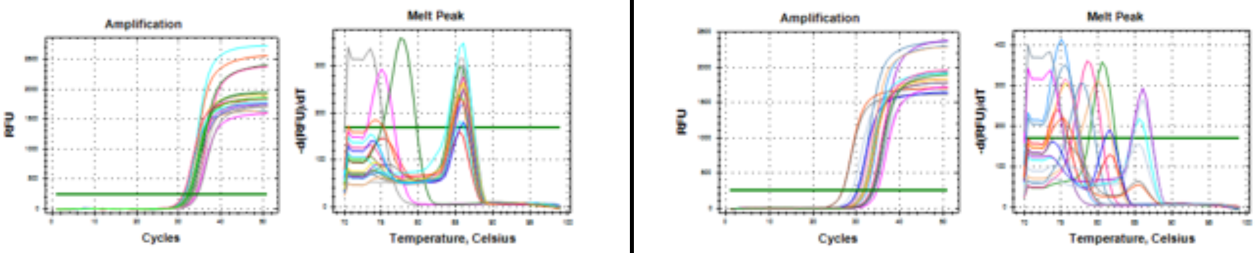


Figure 17. The effect of double amplification on the qRT-PCR outcome of Kiss1 and Pdyn. The result of the amplification curve and melt curve analysis of (A) Kiss1 and (B) Pdyn. The outcome did not improve even though the concentration of the samples was increased by the first amplification.

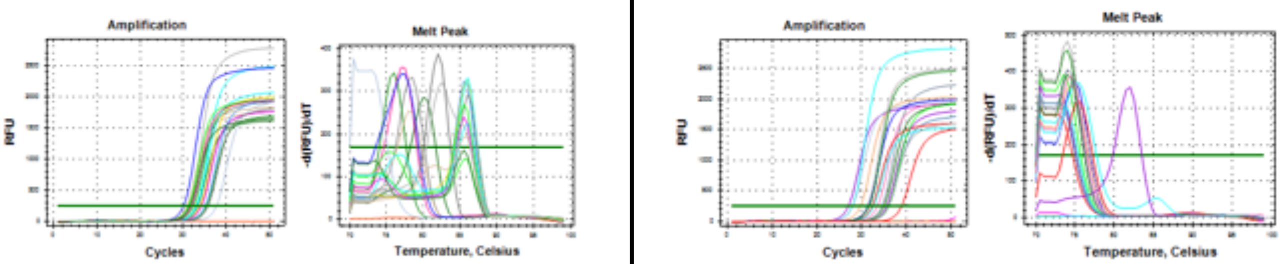
6.1.4 *Kiss1* mRNA expression analysis in 10 diet study with Qiagen *Kiss1* primer

Control | **DHT**

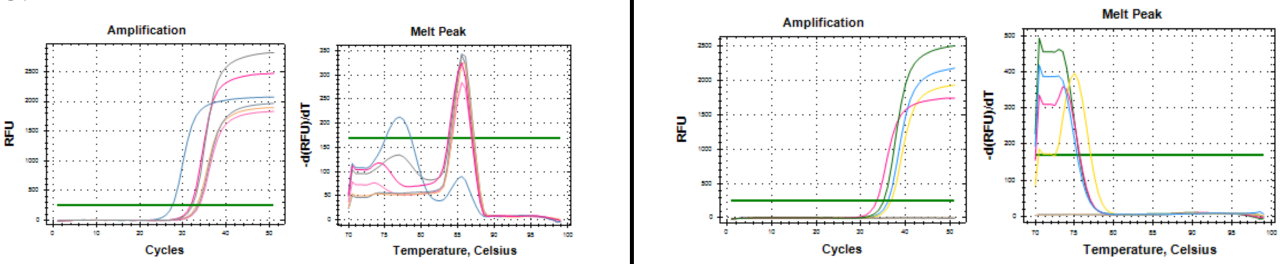
A.



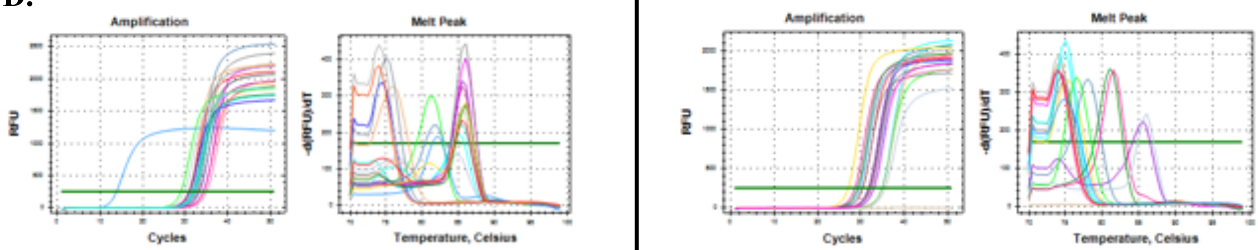
B.



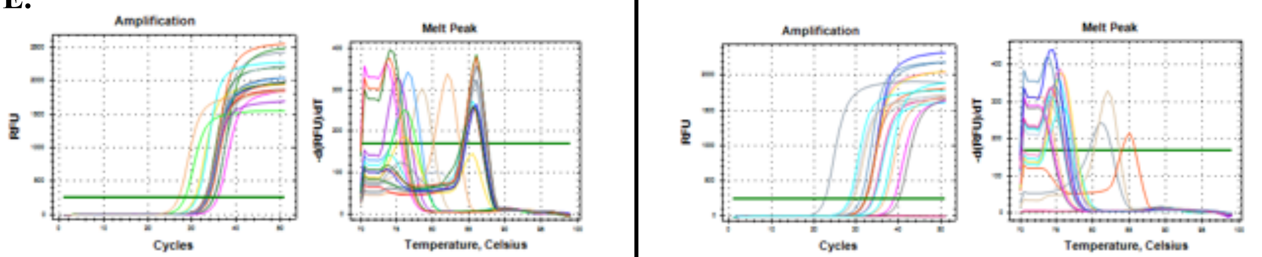
C.



D.



E.



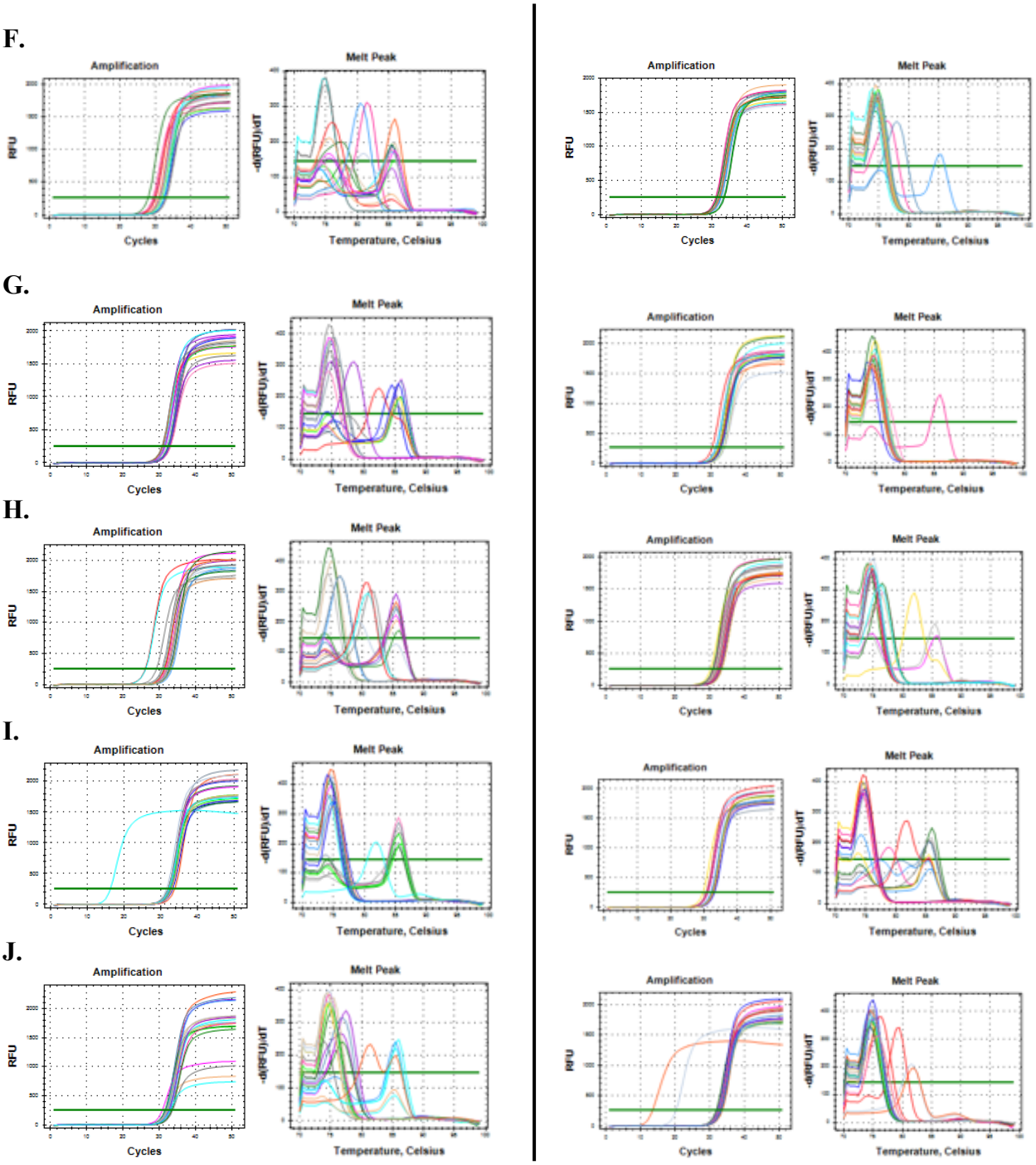


Figure 18. The result of qRT-PCR for Kiss1 in 10 diet study.
The PCR was performed with the annealing temperature 62 ˚C and 50 cycles amplification. (A) Diet1, (B) Diet2, (C) Diet3, (D) Diet4, (E) Diet5, (F) Diet6, (G) Diet7, (H) Diet8, (I) Diet9, (J) Diet10. The amplification generally started at around 30th cycles or later, which is considered to be because the product concentration is very low. The melt curve analysis was mostly unsuccessful as the melt peak temperature was inconsistent across the samples.

6.1.5 Sigma Aldrich *Kiss1* primer test

As the Qiagen *Kiss1* primer was indicated to have issues to detect *Kiss1* mRNA, the *Kiss1* primer by Sigma Aldrich was tested to see whether the PCR outcomes improves.

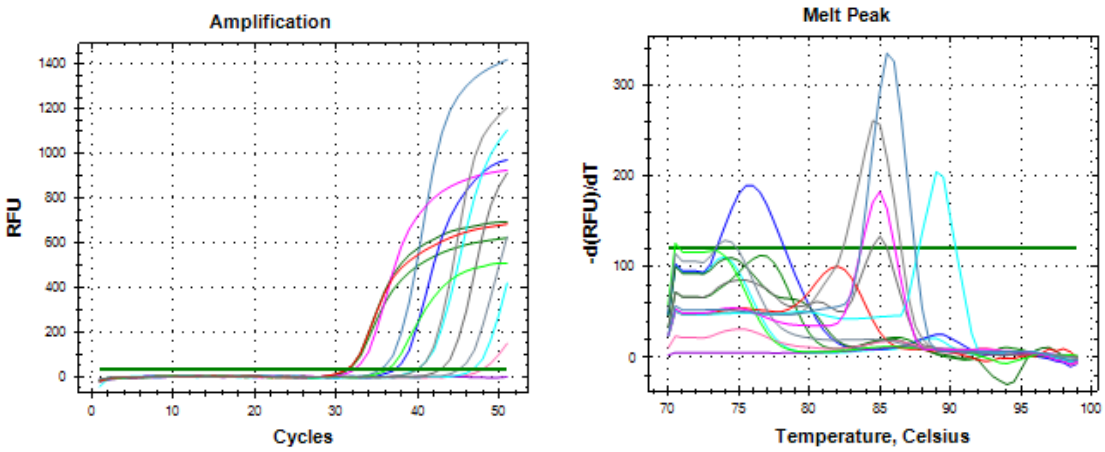


Figure 19. Primer test result of Sigma Aldrich Kiss1 primer.
The mRNA expression of 10 random samples were examined using Sigma Aldrich Kiss1 primer. The annealing temperature was 62˚C and the cycling number was 50. The outcome did not improve compared with Qiagen Kiss1 primer, which is assumed to be due to the low concentration of the product. The amplification of the samples started very late, later than 30th cycles and this seemed to be the cause of unsuccessful melt curve analysis.