Feeding, metabolic rate, and peptide YY regulation in obese-prone and obese-resistant mice

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Certification

I, Gita L. Rahardjo, declare that this thesis, submitted in fulfilment of the requirements for the award Master of Science (Research), in the Faculty of Health & Behavioural Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged.

The document has not been submitted for qualifications at any other academic institute.

Gita L. Rahardjo

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Publications/Presentations

Publications

Rahardjo GL, Huang X-F, Tan YY, Deng C. 2007. Decreased plasma PYY accompanied by elevated PYY and Y2 receptor binding densities in the medulla oblongata of diet-induced obese mice. Endocrinology 148:4704-4710.

Huang XF, Liu Y, Rahardjo GL, McLennan PL, Tapsell LC, Buttemer WA. 2008. Effects of diets high in whey, soy, red meat and milk protein on body weight maintenance in diet-induced obesity in mice. Nutr Dietetics 65 (Suppl. 3):S53-S59.

Presentations

Rahardjo GL, Huang XF, Tan YY, Deng C. The role of PYY and H1 histamine receptor expression in chronic diet-induced obese and diet-resistant mice. Poster presented at: 7th IBRO World Congress of Neuroscience, 2007; Sydney.

Rahardjo G, Huang X, McLennan PL, Buttemer WA. Does obese-prone or resistant mouse differ in their feeding, metabolic rate, and peptide YY regulation? Poster presented at: 10th International Congress on Obesity, 2007; Sydney.

Rahardjo GL, Tan YY, Huang XF. NPY Y2 receptor binding density is reduced in the nucleus accumbens of mice resistant to diet-induced obesity. Oral presentation at: The 26th Annual Meeting of the Australian Neuroscience Society Incorporated, 2006; Sydney.

Rahardjo G, Buttemer W, Huang XF. Progressive change on oxygen consumption and energy intake during development of diet-induced obesity in mice. Oral presentation at: 14th Annual Scientific Meeting of Australasian Society for the Study of Obesity, 2005; Glenelg, South Australia.

Abbreviations

AGRP	Agouti-related peptide
ANOVA	Analysis of variant
Arc	Arcuate nucleus
ATP	Adenosine triphosphate
CART	Cocaine and amphetamine-regulated transcript
DIO	Diet-induced obese
DMH	Dorsomedial hypothalamus
DR	Diet-resistant
g	Grams
h	Hour
kJ	Kilojoule
L	Litre
LF	Low-fat
LH	Lateral hypothalamus
mL	Millilitre
mm	Millimetre
ng	Nanogram
nM	Nanomolar
NPY	Neuropeptide Y
pmol	Picomolar
POMC	Proopiomelanocortin
PYY	Peptide YY

RIA	Radioimmunoassay
RQ	Respiratory quotient
SD	Standard deviation
SEM	Standard error means
µM	Micromolar
VMH	Ventrolateral hypothalamus

Abstract

Some individuals become obese while others remain lean on a high energy diet. The cause of this susceptibility to the development of diet-induced obesity is still unknown. Variations in energy intake, expenditure and the type of substrate being oxidised, as well as Peptide YY (PYY) system regulation are believed to contribute to differential susceptibility to the development of diet-induced obesity.

This project aimed to compare energy balance regulation including energy intake and expenditure, and the PYY system in diet-induced obese (DIO) and diet-resistant (DR) mice.

To investigate energy balance this project measured food intake, body mass gain, spontaneous activity, 24h-metabolic rates and body composition in DIO, DR and low-fat-fed (LF) mice. Plasma PYY was measured by radioimmunoassay and its central binding sites (Neuropeptide Y-Y1, 2 & 5 receptors) were measured by quantitative autoradiography.

This study has shown that body weight gain was significantly (50%) higher in DIO mice compared to DR and LF mice ($F_{2,32}$= 101.5, p<0.001). The higher net energy gain in DIO mice was due to their significantly higher food intake compared to DR mice ($F_{2,33}$=7.79, p=0.002). There were no differences in the metabolic rate, spontaneous activity or type of substrate being oxidized between the DIO and DR mice. The levels of plasma PYY were 32% lower in the DIO mice than in LF mice (p=0.007). PYY and NPY-Y2 receptor binding densities in the DIO mice were significantly higher than DR mice (52% and 24%, respectively) and LF mice (44% and 37%, respectively) at the caudal medulla.

Overall, the major contributing factor to diet-induced obesity in this animal model was increased

energy intake, not a difference in energy expenditure, assimilation efficiency or the substrate types that were oxidized. Reduced plasma PYY in DIO mice may have resulted in the compensatory upregulation of PYY and NPY-Y2 receptor binding density in the caudal medulla. This may contribute at least partially towards the development of diet-induced obesity as this pathway suppresses food intake.

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Literature Review

1.1 Prevalence of obesity

Obesity is a major worldwide health problem. More than 1 billion adults are overweight, and at least 350 million of these people are obese (1). The prevalence of obesity has increased dramatically in the past two decades, as the obesity rates have risen three-fold or more since 1980 in some areas of North America, United Kingdom, Eastern Europe, Middle East, Pacific Islands, Australasia and China (1). In the latest survey by the Australian Bureau of Statistics, the proportion of overweight people in Australia is 49% and alarmingly, a third of them are classified as obese (2). Comparing results of the 2005 Australian National Health Survey with those from previous years shows the proportions of adults classified as overweight or obese has increased dramatically (21%). These increases were recorded in males and females and across all age groups.

1.2 Possible cause of obesity

Obesity is caused by a net positive energy balance, where the amount of energy intake exceeds the amount of energy expenditure. It is believed that human evolution has favoured obtaining a positive energy balance whenever possible to enable the body to store excess energy in the form of fat as a means to support energy-demanding activities

when food was scarcer. However, it is unclear why now, when food is plentiful and the amount of average daily energy expenditure has decreased, some people become obese whilst others do not. There is substantial debate about which part of the energy balance equation is mainly responsible for the excessive positive energy balance in the obese population; whether it is mainly caused by an increase in energy intake, a decrease in energy expenditure, or both (3, 4). This is not an easy issue to resolve because obesity usually results from a slight positive energy imbalance which occurs over a long period of time, which makes the underlying cause hard to distinguish. Furthermore, differences in energy balance in the obese population versus the normal weight population may also be influenced by underlying differences in their neuronal regulation of appetite and physical activity (5). It may be that the obese population has a modified neuronal regulation that can cause them to have an excessive appetite, reduced energy expenditure, or both. In this review, the present knowledge of energy balance and the neuroendocrine factors that can affect it will be reviewed.

1.3 Energy Balance

To understand energy balance, one must examine both energy output and energy input as energy balance represents the difference between these two categories (Figure 1). When energy input is lower than energy output, the net energy balance is negative, which results in an individual in this state tending to lose body weight. On the contrary, when the energy input is higher than the energy output, this individual's energy balance is positive, promoting an increase in body weight as seen in overweight individuals,

pregnant women, and growing individuals. The components comprising energy input and energy output are discussed below in more detail.

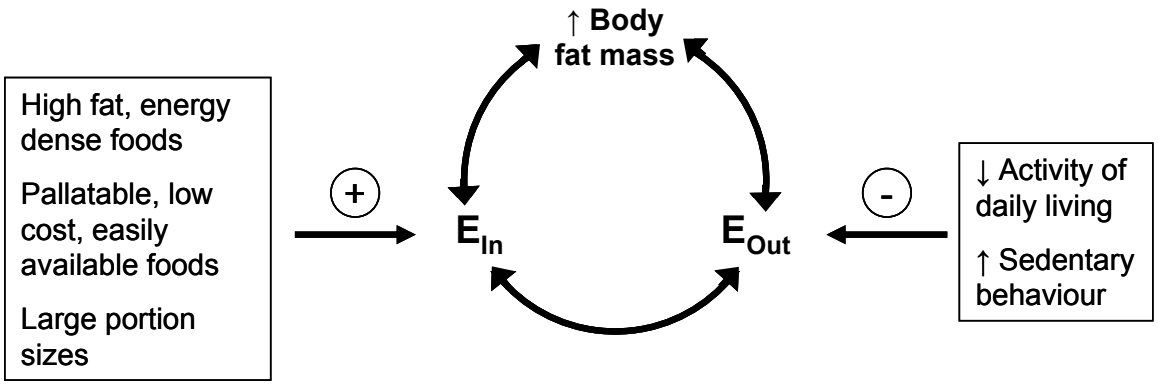

FIGURE 1. The effect of environmental factors on energy balance
When energy intake (E_{in}) equals energy expenditure (E_{out}), the system is in energy balance and body fat mass stable. In the scenario shown above, factors stimulating overfeeding (in circles on the left) are driving E_{in} up, whereas factors on the right are driving E_{out} down, creating a state of positive energy balance, which leads to an increase in the body fat mass (Adapted from Hill et al (6)).

1.3.1 Energy Output (Metabolic Rate)

Differences in individuals' metabolic rate, the rate at which their bodies produce and utilise ATP, may result in differences in energy balance. In measuring metabolic rate, the rate of physical activity has to be taken into account, since during active periods; subjects will have significantly higher metabolic rates. Nevertheless, approximately 60% of the total daily energy expenditure is represented by resting metabolic rate (7).

Interestingly, most studies have found that the total daily energy expenditure of obese humans were higher regardless of their age and gender (8-13) or similar (14) to their lean counterparts when the data are represented as total energy expended daily. When the

energy expenditure is expressed per unit of fat-free body mass, however, obese/obese prone humans population have similar (10, 11, 15) or lower (7) daily energy expenditure compared to their lean counterparts. The discrepancy in these results thus implied that low daily expenditure might not be the main factor that causes obesity as previously thought.

Similar to many total daily energy expenditure studies, resting metabolic rates have been found to be higher in the obese population compared to the lean population (9, 16). Moreover, when considered on a fat-free basis, the resting metabolic rate is similar (8, 10) or even higher (11, 15) in the obese population compared to the lean population. These findings suggest that reduced levels of resting metabolic rate are unlikely to be the cause of obesity as inferred by some obesity studies (17, 18).

1.3.2 Spontaneous Activity

In this study, spontaneous activity is defined as the amount of activity performed by an individual that is not forced upon them. The amount of energy used for spontaneous activity is approximately a third of the total daily energy expenditure, and thus has an important influence on energy balance (7, 8). The amount of energy used for spontaneous activity is determined by both the frequency and the type of movement involved in the activity. Thus, activity-related rises in metabolic rate are higher in situations where the subject does more frequent movement, or when the subject does harder, more difficult movements, such as running and jumping.

It has been hypothesised that the high prevalence of obesity is associated with a reduction of spontaneous activity. However, this hypothesis remains controversial as studies have found conflicting evidence on difference in spontaneous activity between obese population and lean population. For example, obese adolescents were found to perform less physical activity that last for longer than 2 minutes compared to their lean counterparts (8). By contrast, other studies of adults have found spontaneous activity rates to be higher (10) or similar (8, 9, 11) between obese and lean individuals. Based on these results, it is difficult to resolve the influence of spontaneous activity rates on obesity.

1.3.3 Energy Intake

Energy intake is mainly related to the amount of food an individual consumes. At present, many people not only eat bigger food portions, but the food they eat also has a higher energy density, mainly due to higher levels of fat and sugar contents in comparison to the more traditional diets (19, 20). However, not all investigators agree that higher levels of dietary fat are responsible for the prevalence of obesity (21). While dietary surveys indicate that fat as a percentage of energy intake has declined from 37 to 34% over the past decade, fat intake in grams per day has remained essentially unchanged at 80 g/day over the same period (22). On the other hand, the dietary fat estimates from these recent dietary surveys may be lower than the actual consumption since in the wake of public education to reduce fat intake, dietary fat intake may be underreported (19). Not only that, dietary information is hard to measure at a population-wide scale since both normal-weight and obese people commonly underreport their dietary intakes (23-25). This

perhaps explains why in some studies where the food intake is being self-reported by subjects, obese individuals claim less total food intake than lean individuals (13). Due to uncertainty in the amount of dietary intake, the hypothesis that obesity is caused mainly by the excessive energy intake remains unconfirmed.

Apart from the amount of food intake, the type of fuel being oxidised is also believed to affect the state of energy balance. For example, it has been hypothesised that substrate oxidation differs between obese and lean individuals. Accordingly, obese humans show reduced rates of oxidation of lipids ingested, resulting in more dietary fat being shunted into storage depots instead of being used as a metabolic fuel (26). Furthermore, because this bypasses the high-energy process of lipogenesis to produce fat storage from non-fat substrates, they accumulate even more fat storage from a given amount of energy intake (27). This hypothesis gains support from studies showing that a low ratio of fat to carbohydrate oxidation is a good predictor of body weight gain (28, 29). It was also estimated that during recurrence of obesity in weight-loss obese rats, feed efficiency (g of body weight gain/kJ food intake) increased due to a shift toward a preferential use of carbohydrate that shunted fat to storage depots (30). However, not all studies done on obese populations agree with the hypothesis, as some found that the rates of lipid oxidation are higher in obese subjects (31, 32).

Given that energy intake has a significant influence on energy balance, it is very important to investigate the factors that may influence the amount of energy intake. The following paragraphs discuss some the peripheral and central neuroendocrine regulatory processes that are thought to influence energy balance, especially energy intake.

1.4 Peripheral and central neuroendocrine regulatory processes

1.4.1 Leptin

Leptin is known to be one of the primary factors that can influence food intake. It is the product of the *ob* gene and is mainly secreted from white adipose tissue. It acts on receptor sites in the ventromedial nucleus of the hypothalamus (33). Its function was identified from the observation that its absence resulted in state of severe obesity in (*ob/ob*) mice, which led to the conclusion that obesity was a result of leptin deficiency. However, in most human cases of obesity, plasma leptin levels are often significantly higher, which suggests that obesity can also be caused by the state of leptin resistance in obese people (34, 35). Findings from *db/db* (leptin resistant) strains mice strongly support this interpretation as these mice had significantly higher body mass than those of the control group (36, 37). Furthermore, other studies reveal that leptin sensitivity can be affected by the type of diet (38), state of obesity, method of leptin dosing, housing condition, gender and age of mice (39, 40), and strain of mice (41).

Leptin targets some key structures in the hypothalamus that regulate energy intake (42). Within the arcuate nucleus, leptin inhibits the production of Neuropeptide Y (NPY), agouti-related peptide (AGRP) and galanin; these being the substances that are mainly responsible for increasing the food intake. Leptin also stimulates the production of proopiomelanocortin (POMC), cocaine and amphetamine-regulated transcript (CART) and serotonin (42), which, in turn, stimulate appetite as shown in the figure below (Figure 2).

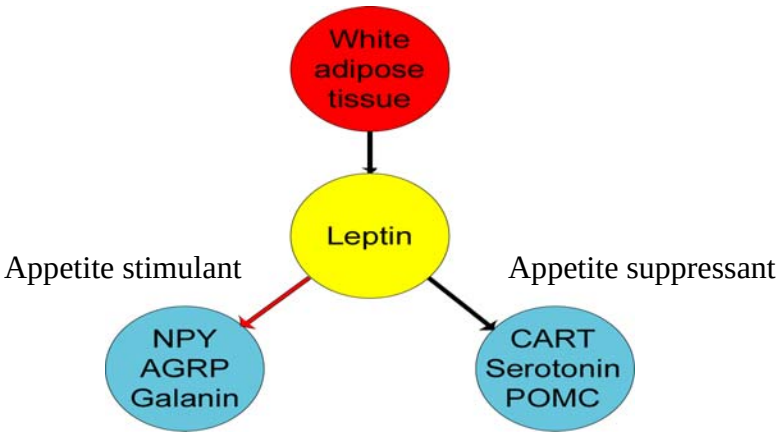

FIGURE 2. The effects of leptin in central regulatory neuropeptides

Red arrow represents an inhibitory effect; black arrow shows that there is an excitatory effect. Abbreviations - NPY: Neuropeptide Y, AGRP: agouti-related peptide, POMC: proopiomelanocortin, CART: cocaine and amphetamine-regulated transcript.

There is, however, no information describing how leptin influences energy expenditure. If spontaneous physical activity acts as a regulator of body weight, it may be that leptin levels, being directly proportioned to the amount of fat in the body, are involved in the regulation of spontaneous physical activity. Despite the fact that a large peripheral dose of leptin has been shown to increase physical activity in *ob/ob* mice, leptin does not appear to influence physical activity levels in humans. For example, when leptin-deficient patients are given leptin, they lost their body fat mainly by altering their eating behaviour, rather than altering their physical activity (43).

1.4.2 Insulin

Insulin is a hormone secreted by the beta cells of the islets of Langerhans of the pancreas. Its secretion is stimulated by a high blood glucose level, which occurs after meals. Insulin

is essential for the use of glucose by cells to produce energy; it lowers the blood glucose level (44). Furthermore, it also functions to reduce food intake (45). However, obese population are mainly insulin resistant where their body cells resist or do not respond to even high levels of insulin (46-48). Currently, it is still unclear whether insulin resistance causes development of obesity. Rather, it is hypothesised that obesity can affect the severity of insulin resistance (49).

1.4.3 Neuropeptide Y Y2 receptor and Peptide YY

Neuropeptide Y (NPY) was isolated for the first time from porcine brain in 1982 by Tatemoto and his colleagues (50). It is a 36-amino acid residue polypeptide which shares high sequence homology with peptide YY (PYY) and the pancreatic polypeptides (50). NPY is present in all major vertebrate groups and is one of the most conserved peptides during evolution (51), suggesting it plays a central role in ensuring basic physiological functions. In mammals, NPY is one of the most abundant peptides found in the central nervous system and it is concentrated in limbic structures and in the hypothalamus (52).

Since its discovery, neuropeptide Y has been studied widely and has been shown to have an important role in both endocrine and neuronal signalling. Functional studies support the involvement of NPY in many physiological functions, such as stimulation of food intake (53), enhancement of memory (54, 55), reduction of locomotor activity (56), shifting of circadian rhythms (57, 58), inhibition of neuronal excitability (59, 60), and modulation of neuroendocrine and cardiovascular functions (61, 62).

The role of NPY in regulating energy intake is clearly revealed by the increased feeding behaviour and food consumption within 10 minutes of NPY injection into the lateral cerebroventricles (63). Similarly, after NPY injection into the hypothalamus, there is an increase in the feeding related activity which has also affected the overall metabolic rate, accompanied by a post-absorptive orientation toward more lipid utilization (64).

The various biological effects of NPY are mediated by the activation of at least five different NPY receptor subtypes, designated as Y1, Y2, Y4, Y5, and Y6 (65, 66). All these receptors have been cloned (53, 65, 66) and are expressed as functional receptors in various rat and human tissues, except for the Y6 subtype which is restricted to mouse and rabbit (*for reviews see* (66)). Some of the NPY receptor types' effects on food intake have been identified from the infusion of different NPY receptor agonists in rats (67-69) as well as in knock-out mice (70-72). From these studies, the Y2 receptor was shown to be a promising component of the pathway regulating energy homeostasis.

Neuropeptide Y2 receptors are expressed in the central and peripheral nervous system with their processes located in many brain regions, including the olfactory bulb, some cortical areas, septum, basal forebrain, nucleus accumbens, amygdala, hippocampus, hypothalamus, substantia nigra compacta, locus coeruleus, and solitary tract nucleus (73, 74). Of all these areas, the functions of Y2 receptor have been most intensively investigated in the areas known to affect energy homeostasis: the hypothalamus and medullary solitary tract nucleus. Microinjection of NPY in hypothalamus and NPY agonist in the fourth ventricle causes an increase in food intake, thus demonstrating the role of NPY in these areas in regulating food intake (64, 75).

The hypothalamus is the key centre in the brain regulating energy balance. It relays the information of energy availability and metabolic need via hormones to the neuroreceptors that are responsible for regulating food intake and energy expenditure. These neuroreceptors are densely located in the arcuate nucleus and are also known to express the leptin receptor (76). They are located in an area accessible to peripheral hormones (77), enabling modulation of hypothalamic circuits involved with the maintenance of energy homeostasis. The receptors for these hormonal signals are primarily expressed on two neurochemically distinct sets of neurons; one neuron group expresses NPY, whilst the other expresses the neuropeptide precursor proopiomelanocortin (POMC), which is processed to melanocortin peptides. Whilst increasing NPY release or activation affects body energy balance by increasing food intake and decreasing energy expenditure, POMC release or activation does the opposite. Working synergistically, these neuron groups will allow an adjustment in the energy intake and energy expenditure to create an energy balance based on the availability or the need of metabolic fuel (78). Long-term signals communicating information from these neurons regarding the energy availability, endocrine status, and general health are predominantly humoral; whilst short-term signals, including gut hormones and neuronal signals from higher brain centres and the gut, regulate meal initiation and termination (79). The signals from these neurons can also affect energy expenditure via sympathetic nervous outflow to brown adipose tissue as well as by affecting the secretion of various pituitary glands (80). In the hypothalamic arcuate nucleus, Y2 receptors are thought to act as an inhibitory autoreceptor that can regulate the expression and secretion of NPY and other neurotransmitter (*for review see*

(78)). This might explain why hypothalamic-specific Y2 receptor knockout mice show a significant increase in food intake (81).

Classical accounts of brain regulation of feeding described two systems balancing each other: the hypothalamus, monitoring the periphery for signals alerting central circuits to diminishing energy stores, and the brainstem, receiving oral and gastrointestinal information as an online signal of the amounts and qualities of the food that are being ingested. This arrangement would allow the hypothalamus to function as a long-term control orchestrating meal initiation and the brainstem serving as a short-term control for meal termination (78).

The brainstem is classically viewed as a channel for visceroceptive information via cranial nerves, in particular the vagus nerve, which carries information from the alimentary organs (82). Vagal afferents synapse onto (83) and excite (84) neurons in the brainstem solitary tract nucleus. From both the hypothalamus and the brainstem, projections fan further into the brain to engage other brain regions in the initiation and organization of food intake (78).

In the solitary tract nucleus, it is known that the Y2 receptor agonist, PYY, suppresses vagally mediated digestive function as a consequence of direct action of the Y2 receptor on the neurons in dorsal medulla (85). By encompassing vago-vagal reflex circuitry, the Y2 receptor can activate inhibition of digestion in the dorsal medullary region, controlling gastric motility and gastric acid secretion (86, 87). However, there is considerable debate concerning the mechanisms and direction of the effects mediated by these receptors (88).

One of the NPY Y2 receptor agonists widely used to study the role of Y2 receptor in regulating food intake is peptide YY (PYY). PYY, a member of the pancreatic polypeptide family, is structurally and functionally related to the neuropeptide Y (NPY) family (89). Discovered before NPY, PYY was first isolated from porcine intestine in 1980 by Tatemoto and Mutt (90). Due to their similarities in structures and functionality, PYY also shares the affinity for NPY receptor binding (91). PYY is secreted from the endocrine L cells of the small and large intestine, and released into the circulation in response to ingestion of food, especially in the presence of a fatty meal (92) (Figure 3A). PYY has also been detected in gastric mucosa, pancreatic islets, myenteric and serosal ganglia, sympathetic neurons, adrenal gland, spinal cord, and brain, including the hypothalamus, pituitary, pons, medulla oblongata, and the brainstem (93, 94). Furthermore, it was been shown that PYY 3-36, one of the two molecular forms of PYY abundant in the blood (107), affects food intake via activation of the auto-inhibitory presynaptic NPY Y2 receptor present on the hypothalamic arcuate nucleus (98).

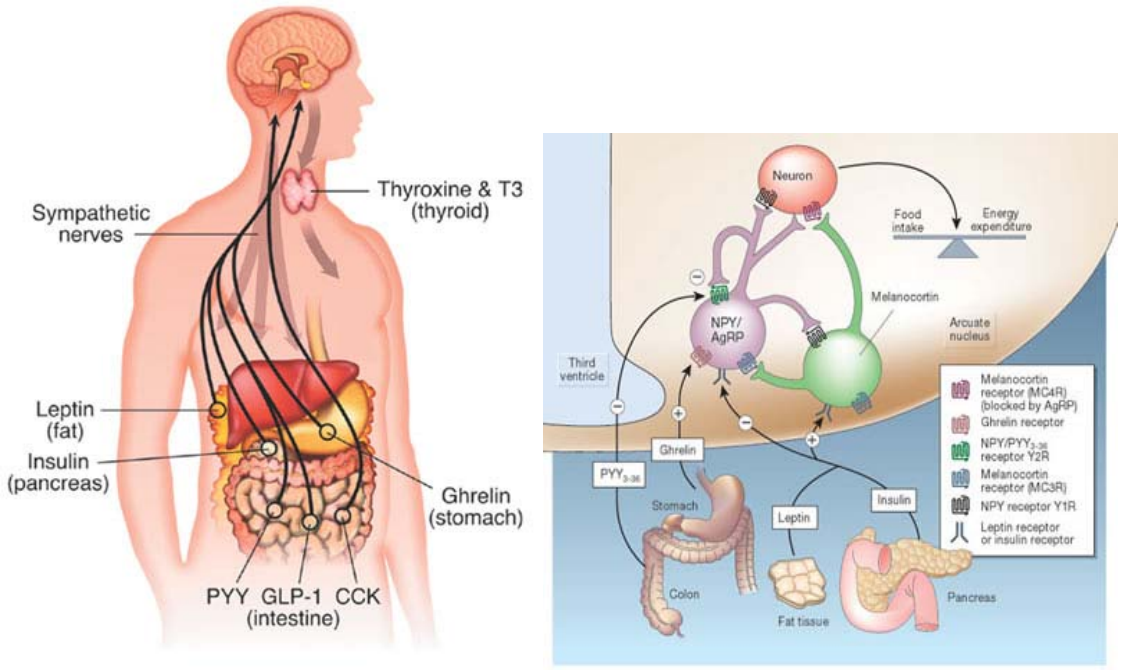

FIGURE 3. The Interactions between leptin, insulin, ghrelin and PYY

(A) The role of peptide YY, leptin, insulin, and ghrelin and (B) the receptors responsible for peptide YY binding. Figure is adapted from (A) publication by Evans et al (108) and (B) publication by Schwartz and Morton (109).

In contrast with the negligible effect on appetite caused by normal daily fluctuations in circulating leptin, the proven effects of PYY on appetite offers an intervention that reduces food intake in healthy as well as obese humans without a subsequent rebound in caloric intake (110).

1.5 The use of animal models to study obesity

As mentioned previously, the results from studies on obesity and energy balance involving human subjects remain inconsistent. This has made it difficult to identify which

factors cause energy imbalance in the development of obesity in humans, that is, whether it is due mainly to high food intake, low metabolic rate, low activity rate or higher fat absorption from the food. These uncertainties in human obesity studies are compounded by flaws in the experimental design that many studies have, such as: 1) inadequate sample size to detect the metabolic imbalance in question; 2) inadequate time points in the experiments resulting in studies rarely examining the onset of weight gain; and 3) inadequate controls to account for environmental influences which may have affected energy balance. Furthermore, studies that incorporate self-reporting of the food intake may be prone to under-reporting (4). Adding to that, it is often that in human obesity studies, researchers have only focused on one part of the energy balance, e.g. the food intake or the energy expenditure, rather than examining both the energy intake and energy expenditure together.

To overcome these shortcomings, statistical power was gained by using an adequate number of subjects. Adding to that, by using animals as the subjects, their metabolic rate and food intake could be monitored more accurately (thus eliminating the under-reporting energy intake) and the effect of external environment could also be controlled carefully.

To eliminate problems in genetic variation, this project examined obesity development using one strain of a single species. This strain should best represent the development of obesity in the human population. Such strains are well established in rodents, e.g. Sprague-Dawley rats (111) and C57Bl/6 mice (112, 113). When these strains were fed a high energy and high fat diet, a certain population became obese (known as diet induced obese (DIO) phenotype) while other individuals remained lean as if they were given a

low fat diet (known as diet resistant (DR) phenotype). In comparison to other strains, C57Bl/6 has been shown to be prone to obesity when given high-fat diet (114, 115). It has been used to study the regulation of leptin (38, 116), insulin, and neuropeptide Y (117) in obese subjects. Apart from this model, many different animal models have been used to investigate development of obesity, such as genetically modified or knock-out model (*ob/ob, db/db, fa/fa*) (34-37). Although those studies have provided a better knowledge of how certain genes can affect obesity, the diet-induced obesity model in DIO C57Bl/6 mouse remains an excellent candidate to study the development of obesity in different phenotypic variations.

Based on the study by Huang et al (112, 113), the development of the obesity in the C57Bl/6 mice can be divided into 3 stages: early stages (0-3 weeks of high-fat diet), middle stage (4-14 weeks of high-fat diet), and late stage (after 15 weeks of high-fat diet) based on the changes in energy conversion efficiency (the amount of body weight gained (g) per amount of energy intake (kJ)) and the difference in the body weight between the DIO and the control mice in comparison to the DR mice and the control mice. However, little is known about what mechanism underlies these different patterns of body weight gains between these two phenotypes since most studies have concentrated solely on the late stage of the development of obesity.

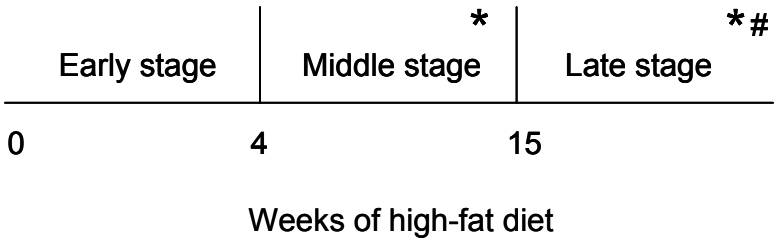

FIGURE 4. The stages of the development of obesity in C57Bl/6 mice
Tested using post-hoc Tukey Kramer, the difference is considered significant when the *p* value is less than 0.05. * Significant difference in the energy conversion efficiency between DIO and DR phenotypes, # significant difference in the energy intake between DIO and DR phenotypes.

To understand the factors underlying the development of obesity, the factors affecting energy balance should be examined during all three stages in this mouse model.

2

Study Aims and Hypotheses

2.1 Experimental aims

The overall aim of this project was to study the influence of variation in energy intake, motor and metabolic activity, and appetite regulation in the development of diet-induced obesity in C57Bl/6 mice. This study focused on PYY/Leptin-NPY and its relationship with motor and metabolic activities.

Specific aims:

- To examine energy metabolism, energy intake, body mass gain and body composition during the development of diet-induced obese and diet-resistant phenotypes
- To examine variation in the PYY system and its related peripheral hormone during the development of diet-induced obese and diet-resistant phenotypes

2.2 Experimental Hypothesis

It was hypothesised that there would be a difference in the spontaneous activity between the DIO and DR mice as early as the middle stage, which would be correlated with differences in their fat-free body masses. It was also hypothesised that PYY, leptin and insulin plasma level would differ between the DIO and DR mice, and would be associated with differences in body weight between the two phenotypes. Furthermore, it

was predicted that there would be a difference in PYY and Y2 receptor binding densities between the DIO and DR phenotypes.

2.3 Expected Outcomes

It was expected that if there was any difference in the metabolic rate between the DIO and DR phenotypes, it might have been correlated to the difference in the rate of spontaneous physical activity and/or by the difference in body composition. These differences in metabolic rate might also be able to be explained by differences in receptor binding densities of NPY and Y2 receptors between these two phenotypes.

By understanding the mechanism of the development of obesity in C57Bl/6 mice, then hopefully we can extrapolate the findings of this study to better understand the mechanism underlying the development of obesity in human.

3

Experiment 1. Changes in energy intake and expenditure during the development of diet-induced obesity in mice

3.1 Introduction

Weight gain results from a positive energy balance and, if excessive may lead to obesity and eventually to obesity-related health problems. This positive energy balance can result from excessive energy intake, decreased energy expenditure, or a combination of both. There are many factors that can influence the amount of energy intake and expenditure (6). The amount of energy intake depends on the amount of food ingested, the amount of the ingested food energy that is absorbed following its digestion, and the types of nutrients absorbed. Rates of energy expenditure, on the other hand, are highly affected by body size and composition, rate and level of physical activity, and the type of fuel source being metabolized.

Given the many variables affecting obesity, it is not surprising that attempts to identify single factors that account for obesity usually fail, both within and, especially, between particular species. For example, the suggestion that obese-prone individuals experience relatively lower metabolic rates than lean phenotypes is supported in some studies (7, 118), but refuted in others (8-10, 15, 32). The hypothesis that obesity is mainly caused by excessive energy intake also remains unresolved. Dietary intake is hard to measure at a population scale, since human subjects, whether obese or lean, commonly underreport

their dietary intakes (23-25). In animals, however, where food intake can be measured accurately, obese subjects tend to have a higher rate of food intake than their lean counterparts (113). Activity effects on obesity also vary among studies, as some claimed that the obese population had higher activity rates (10) while others showed them to be similar to non-obese subjects (8, 9, 11). Because very few obesity studies investigate all variables known to affect obesity in the subjects they studied, they usually provide limited insight into the factors responsible for excessive weight gain. Even with carefully designed studies, discriminating the variables most affecting positive energy balance in obese subjects is not an easy issue to resolve. This is because obesity usually results from a slight positive energy imbalance which occurs over a long period of time, thus making the underlying causes hard to distinguish. The fact that most obesity studies focus mainly on populations who are already obese makes it unclear whether the energy imbalance found in these populations was due to obesity itself, or served as the underlying cause of obesity.

Because of these considerations, an animal model of obesity was used to investigate the factors responsible for differential weight gain. The mouse strain, C57B1/6, was selected as this strain exhibits substantial and distinct phenotypic variation in its tendency to become obese on energy-dense diets. Previous studies on this strain reveal it to have obese and lean phenotypes when provided free access to a high-fat diet (38, 112, 119). These mice are therefore good models for understanding human obesity as members of both populations differ conspicuously in their response to common diets.

The aims of this study were to identify the variables that affect energy balance during the development of obesity in this diet-induced obesity model. By understanding the factors responsible for higher rates of net energy gain among animals developing obesity, insight into the mechanisms of obesity onset in other species, including humans, will be gained.

3.2 Materials and Methods

3.2.1 Animal model and diets

Forty eight nine-week old C57Bl/6 mice were acquired from the Animal Resource Centre (Perth, Western Australia) and kept in a temperature-controlled room at 22^{o}C with a light:dark cycle of 12h:12h. For the first week, all mice were given lab chow *ad libitum*. The mice were then placed singly into standard shoebox mouse cages. Thirty six mice were randomly selected and fed an *ad libitum* high-fat diet (Table 1), whilst the other 12 mice were placed on an *ad libitum* diet of low-fat food (Table 1) and served as control animals (LF). (Table 1). The mice fed with high-fat diet were then assigned into groups based on body mass gain after 22 weeks of dietary intervention; the 12 heaviest mice were assigned to a diet-induced obese (DIO) group and the 12 lightest were designated as a diet-resistant (DR) group (the 12 mice intermediate in mass to these animals were excluded from the experiment).

TABLE 1. Macronutrient composition of the low-fat and high-fat diets given to the experimental mice. The kJ% represents the percent of total kJ provided in the diet by a particular component.

	Low-fat Diet	High-fat Diet
Cornstarch, kJ %	67.73	38.83
Sucrose, kJ %	6.51	5.39
Copha, kJ %	-	15.75
Beef tallow, kJ %	-	15.75
Sunflower oil, kJ%	9.75	8.07
Gelatine, kJ %	5.56	4.60
Casein, kJ %	10.45	11.60
Fibre, g/kg	51	51
Minerals, g/kg	67	67
Vitamins, g/kg	13	13

During the experiment, food intake was determined every 2 days and body mass of all individuals was measured weekly. Food intake rates were based on the 24-h average of the mass difference between the food placed in the cage on a given day and the amount remaining 48-h later. Metabolic rate was also measured over a 24-h period at 2, 8, and 20 weeks after mice began the high-fat diet and was followed by 24-h activity measurements. These sampling dates were chosen as they represent the early, middle and late stages of the development of obesity in the diet-induced obese C57Bl/6 mice (*see* Figure 4)

All procedures were approved by the Animal Ethics Committee of University of Wollongong and accorded with the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.2.2 Indirect calorimetry

Oxygen consumption and carbon dioxide production were monitored for 24 h using an open-flow respirometry system. For these measurements, the holding cages were fitted with airtight, clear plastic lids, which had air inlet and outlet ports. Airflow into each cage was maintained at 500 ml/min by mass flow controllers (Tylan Model FC-280S, Tylan General, USA). At all times, animals had free access to water and food. The cages were housed in an environmental cabinet maintaining a temperature of 22^{o}C and lights were set to the same photoperiod as those in their normal quarters.

Oxygen consumption rate was evaluated by comparing oxygen content of inlet and outlet air of each chamber using a Sable Systems Oxzilla oxygen analyser in combination with an electronic stream selector (Sable Systems Respirometer Multiplexer V 2.0). Voltage output from the oxygen analyser was recorded at 5-s intervals and each mouse was sampled continuously for 20 minutes per hour throughout the 24-h period. The first 2 hours of data were excluded from subsequent analyses to ensure animals were fully settled during metabolic measurement. Before the beginning of each sampling period, the oxygen analyser was calibrated using a known percentage of oxygen as a standard. The experiments were also timed so that they started in the early period of daytime light-cycle (0900-1100).

To measure resting metabolic rate, the mean of the three lowest 5-min running averages of oxygen consumption measured during the 24-h experiment was used. The difference between the amount of oxygen used for resting metabolic rate and the total oxygen used will be determined as non-resting metabolic rate. All reported values of oxygen

consumption are corrected to standard temperature and pressure (STP) conditions. CO_2-production was also measured during this period using a Sable Systems CA-1 carbon dioxide analyser. These measurements permitted calculation of the average respiratory quotient (ratio of volume of oxygen consumed over the volume of carbon dioxide produced) over the entire 24-h period. Before the beginning of each 24-h measurements, the carbon dioxide analyser was calibrated using a precision-mixed gas containing a known concentration of CO_2.

3.2.3 Spontaneous activity measurement

Immediately after the 24-h measurement of metabolic rate, mice were kept in their holding cages and placed under an infrared motion detector in their normal animal quarters. Each cage had partitions placed around them to restrict the angle of each detector's view to the cage beneath it and to minimise social interactions between mice. Voltage outputs from these detectors were recorded every 0.5 sec using an A-to-D converter attached to a computer.

3.2.4 Body composition

After 22 weeks of being on high or low-fat diets, mice were given a lethal injection of sodium pentobarbitone (120mg/kg, intraperitoneal) and, once anaesthetised, blood was collected from the heart. White adipose tissue depots (inguinal, epididymal, retroperitoneal, and mesenteric), brown adipose tissue depot (subscapular), pancreas, stomach, liver, kidneys, and heart were removed and weighed. The organs and the fat pads were then combined with the rest of the body parts to determine the lean dry body mass.

To measure water content and lean dry body mass, each mouse's carcass was weighed before and after being freeze-dried (FD3 Freeze drier, Dynavac Engineering). The dried carcass was then cut into small portions and placed into cellulose thimbles. The total lipid content of each carcass was determined gravimetrically by weighing each thimble before and after placement in a Soxhlett apparatus containing petroleum ether, a solvent known to dissolve neutral lipids (120).

3.2.5 Level of leptin and insulin the plasma

Leptin and insulin levels were measured using commercially ELISA method (Lincoplex Mouse Endocrine Panel (Milipore, Billerica, MA)). Plasma from the mice was prepared following the technical guidelines attached with the kit.

3.2.6 Statistical analysis

Unless stated otherwise, data are presented as means ± SD. An SPSS statistical package (SPSS Inc., Chicago, Illinois) was used for all statistical analyses. Body weight, body weight gain, energy intake, 24-h metabolic rate, resting metabolic rate, and spontaneous activity were assessed by analysis of variance (ANOVA) for repeated measures. For each single time point, an analysis of variance followed by post-hoc Tukey-Kramer Honestly Significant Difference (HSD) test was performed for multiple comparisons among the groups.

Variables on body composition were analysed using ANOVA. When significant differences were found, post-hoc Tukey-Kramer HSD procedures were performed.

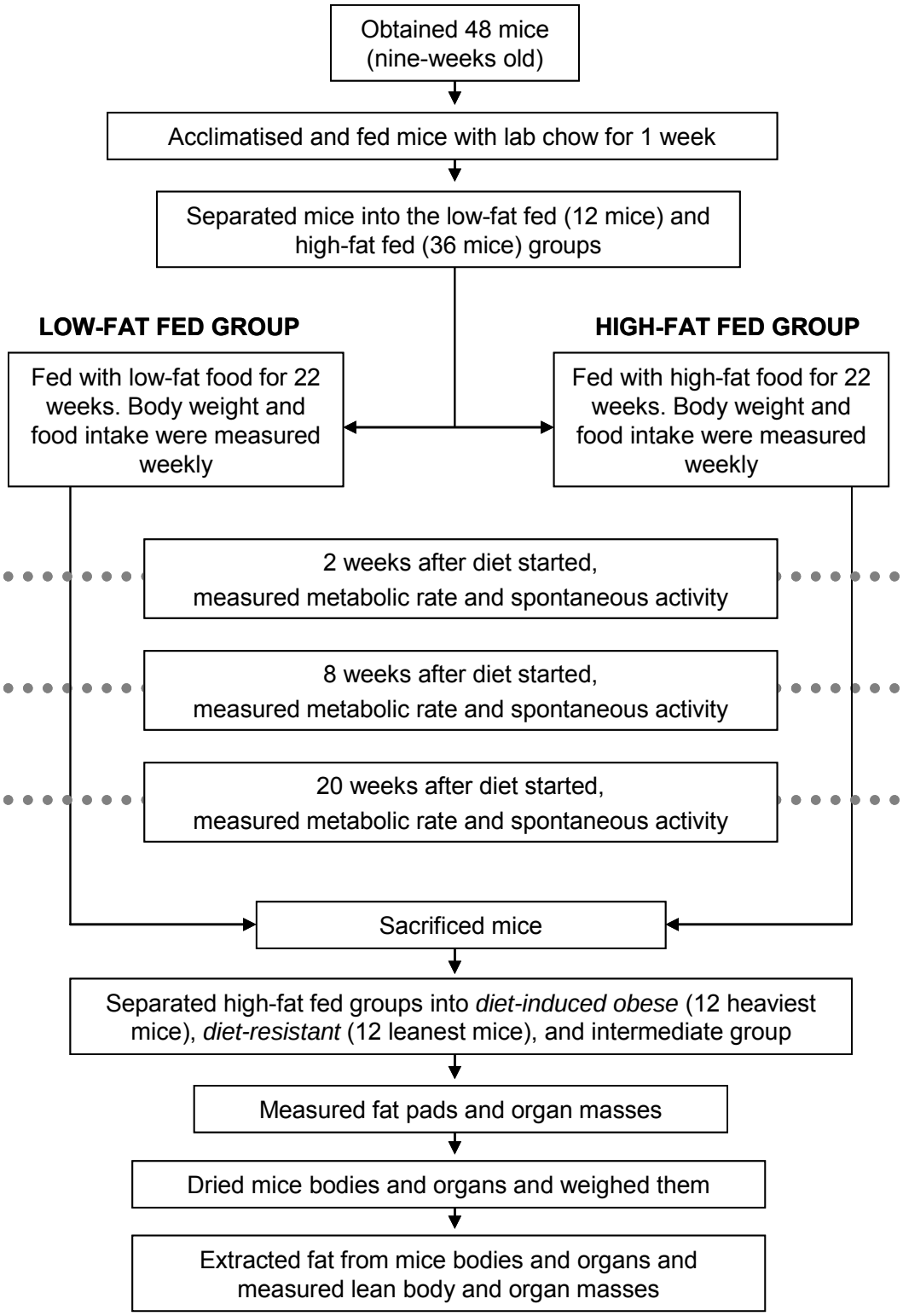

FIGURE 5. Overview of study design and schedule of measurements.

3.3 Results

3.3.1 Body weight gain

Diet and phenotype had significant influences on body weight gain together with the duration of the dietary intervention. A two-way repeated ANOVA of data acquired from weeks 2, 8, and 20 of dietary intervention, revealed significant main effects of 'GROUPS' (three different groups based on diet and phenotypes: diet-induced obese, diet-resistant, and control; $F_{2,33}$=23.84, p<0.001) and repeated measurement with 'TIME' (duration of diet intervention; $F_{2,66}$=473.95, p<0.001) on weight gains. There was also a significant interaction between these two factors ($F_{4,66}$=56.77, p<0.001). Although there was a consistent body weight gain in all groups, the DIO mice had significant difference in body weight gain compared to the DR (p<0.001) and LF (p<0.001) mice through the treatment period, whilst there was no difference in body weight gain between the DR and LF mice (Figure 6).

During week 2 of dietary intervention, there was no significant difference in the amount of weight gain between the groups ($F_{2,33}$=2.35, p=0.111). However, after 8 weeks of dietary intervention, the DIO group gained a significantly more body weight than the DR mice ($F_{2,33}$=4.65, p=0.017; DIO *vs*. DR, p=0.013). Furthermore, by week 20, this difference became greater as the DIO mice had a significantly higher body weight ($F_{2,33}$=56.51, p<0.001) compared to both the DR (p<0.001) and LF (p<0.001) groups.

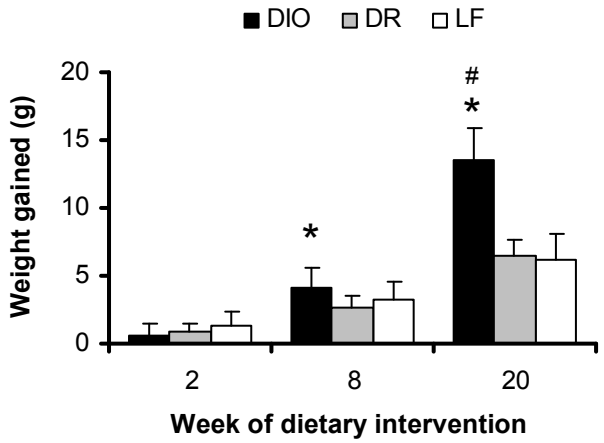

FIGURE 6. Body mass gained in diet-induced mice model

Body mass gained by diet-induced obese (DIO), diet-resistant (DR) and control mice (LF) in relation to weeks of eating either a high-fat (DIO, DR) or low-fat diet (LF; n=12 for each group). Data are presented as means ± SD; * $p<0.05$ vs. DR; # $p<0.05$ vs. LF.

After 22 weeks of dietary intervention, the DIO mice had more body mass gain compared to the DR (42.7±0.8g *vs*. 31.6±0.5g; DIO *vs*. DR; $F_{2,33}=86.54$, $p<0.001$) and LF mice (30.0±0.8g; DIO *vs*. LF, $p<0.001$). By contrast, the skeletal measurements of DIO and DR mice were statistically indistinguishable; implying that differences in body mass between these two groups are not explained by differences in their overall body size, *per se* (Table 2). Consistent with this interpretation, the fat content of the DIO group was substantially higher than either DR or LF mice, and accounted for the major difference in their total body masses. The lean dry mass was also higher in the DIO than the other two groups, suggesting a relative increase in muscular mass in these overweight animals.

TABLE 2. Body weight gain of diet-induced obese (DIO), diet-resistant (DR), and control groups (LF) and their body composition measured 22 weeks after the start of dietary intervention.

	Mean ± SEM			One-way ANOVA	
				F	*P*
	DIO (n = 12)	DR (n = 12)	LF (n = 12)	[2,33]	value
Body weight (g)					
Prior to the diet (*age, 10 weeks*)	25.16 ± 0.55	23.77 ± 0.26	23.49 ± 0.39	4.585	0.017[c]
After 22 weeks diets (*age, 32 weeks*)	42.70 ± 0.79	31.60 ± 0.45	29.95 ± 0.77	77.029	<0.001[a,b]
Lean dry body mass	6.38 ± 0.16	5.52 ± 0.08	5.60 ± 0.10	16.161	<0.001[a,b]
Body fat mass	13.28 ± 0.59	6.53 ± 0.28	5.43 ± 0.38	94.382	<0.001[a,b]
Skeletal measurement (mm)					
Tibia length	22.31 ± 0.13	21.75 ± 0.23	22.04 ± 0.15	2.568	0.092
Radius length	14.23 ± 0.06	13.91 ± 0.08	13.90 ± 0.13	3.946	0.029[d]
Foot length	17.51 ± 0.09	17.73 ± 0.16	17.78 ± 0.11	1.308	0.284
Fat-pad masses (g)					
Epididymal	2.621 ± 0.106	1.247 ± 0.076	0.890 ± 0.072	61.036	<0.001[a,b]
Perirenal	1.018 ± 0.037	0.470 ± 0.035	0.411 ± 0.048	68.455	<0.001[a,b]
Omental	1.276 ± 0.079	0.715 ± 0.052	0.673 ± 0.046	25.640	<0.001[a,b]
Inguinal	1.760 ± 0.106	0.683 ± 0.041	0.566 ± 0.045	57.097	<0.001[a,b]
Interscapular	0.358 ± 0.027	0.166 ± 0.010	0.178 ± 0.014	24.275	<0.001[a,b]

[a]$p<0.001$ DIO vs. DR, [b]$p<0.001$ DIO vs. LF, [c]$p<0.03$ DIO vs. LF, [d]$p=0.049$ DIO vs. DR.

3.3.2 Energy intake

Mouse phenotype and diet affected food intake rates together with the length of dietary intervention. This was revealed in a two-way repeated ANOVA with a significant main effect of 'GROUPS' ($F_{2,33}=7.79$, $p=0.002$) and repeated measurement with 'TIME' ($F_{2,66}=30.66$, $p<0.001$) on energy intake (Figure 7); a significant interaction was also

found between these two factors ($F_{4,66}$=10.62, p<0.001). DIO mice maintained a similar daily energy intake throughout the experiment, whilst daily intake rates decreased with time in the other two groups as the experiment progressed.

On closer inspection, during the 2nd week, the LF group had a significantly higher energy intake compared to the DR and DIO mice ($F_{2,33}$=14.6, p<0.001; LF *vs*. DR, p<0.001; LF *vs*. DIO, p<0.001). However, in the 8th week of dietary intervention, the energy intake rate was significantly higher in DIO mice compared to the DR and LF mice ($F_{2,33}$=5.45, p=0.009; DIO *vs*. DR, p=0.012; DIO *vs*. LF, p=0.035). The same pattern was seen in week 20 of the dietary intervention, with DIO mice showing a significantly higher rate of energy intake than the DR or the LF mice ($F_{2,33}$=8.75, p=0.001; DIO *vs*. DR, p=0.003; DIO *vs*. LF, p=0.003; Figure 7).

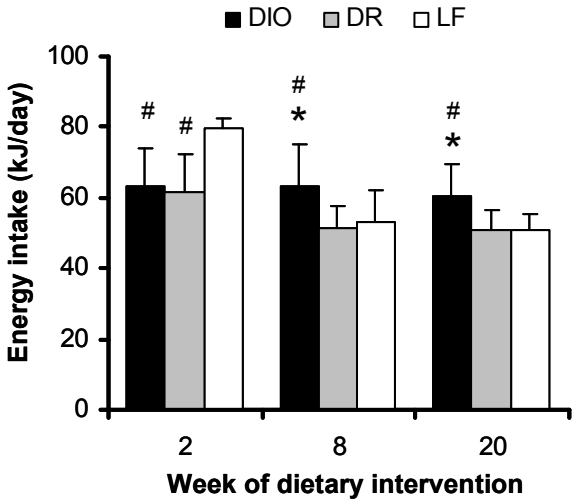

FIGURE 7. The daily energy intake in diet-induced mice model

The daily energy intake of diet-induced obese (DIO), diet-resistant (DR) and the control (LF) mice in relation to weeks of eating either a high-fat (DIO, DR) or low-fat diet (n=12 for each group). Data are presented as means ± SD; * p<0.05 vs. DR; # p<0.05 vs. LF.

3.3.3 Energy expenditure

Energy expenditure was also affected by mouse diet and phenotype as well as the duration of the dietary intervention. Two-way repeated ANOVA revealed significant effects of 'GROUPS' ($F_{2,33}$=4.87, p=0.014) and repeated measurement with 'TIME' ($F_{2,66}$=15.17, p<0.001) on daily energy expenditure. A significant interaction between those two factors was also found ($F_{4,66}$=3.19, p=0.019). Although there was variation in the daily energy expenditure in all groups, the DIO mice had significantly higher daily energy expenditure than the DR (p=0.030) and LF (p=0.026) mice through most of the treatment period (Figure 8a). In week 2 of the dietary intervention, there were no significant differences in the daily energy expenditure between the groups measured ($F_{2,33}$=0.01, p=0.989). In week 8 however, the DIO had higher daily energy expenditure than the DR mice and LF mice ($F_{2,33}$=6.86, p=0.003; DIO *vs*. DR, p=0.002; DIO *vs*. LF, p=0.164; Figure 8a). This trend became more visible in week 20 of the intervention where DIO mice had significantly higher daily energy expenditure than the DR and LF mice ($F_{2,33}$=9.78, p<0.001; DIO *vs*. DR, p=0.019; DIO *vs*. LF, p<0.001; Figure 8a). When metabolic rate is expressed in relation to lean body mass however, the DIO mice had statistically indistinguishable lean mass-specific daily energy expenditure in week 20 of dietary intervention (5.71±0.21 kJ/day.g) compared to DR (5.95±0.18 kJ/day.g) and LF (5.51±0.18 kJ/day.g) mice ($F_{2,33}$=1.35, p=0.272).

The same trend was found in the resting metabolic rate of the mice where the duration of dietary intervention as well as the mouse's diet and phenotype significantly affected the resting metabolic rate ('GROUPS' ($F_{2,33}$=6.20, p=0.005), 'TIME' ($F_{2,66}$=30.843, p<0.001), interaction between these two factors ($F_{2,66}$=3.06, p=0.022)). Similar to

measurements of daily energy expenditure, DIO mice tended to have a higher resting metabolic rate compared to the DR (p=0.058) and LF (p=0.004) mice throughout most of the experimental period (Figure 8b). Also corresponding to the pattern of daily energy expenditure among groups, there was no difference in the resting metabolic rate between the groups at week 2 of the dietary intervention ($F_{2,33}$=1.33, p=0.279). By contrast, in week 8, the DIO mice had a higher resting metabolic rate compared to the DR and LF mice ($F_{2,33}$=6.52, p=0.004; DIO *vs*. DR, p=0.003; DIO *vs*. LF, p=0.058): a trend that was also visible in week 20 of the dietary intervention ($F_{2,33}$=6.46, p=0.004; DIO *vs*. DR, p=0.055; DIO *vs*. LF, p=0.004; Figure 8b). However, these differences disappear when the resting metabolic rate is expressed as per gram of lean body mass, with the DIO mice displaying similar resting metabolic rates in week 20 of dietary intervention (3.82±0.19 kJ/day.g) compared to DR (3.86±0.19 kJ/day.g) and LF (3.52±0.14 kJ/day.g) mice ($F_{2,33}$=1.12, p=0.340).

Interestingly, mouse phenotype and diet did not affect non-resting metabolic rate as two way repeated ANOVA revealed that only 'TIME' ($F_{2,66}$=45.10, p<0.001), but not 'GROUPS' ($F_{2,33}$=1.37, p=0.254) had a significant effect on the non-resting metabolic rate (Figure 8c). No significant differences in the non-resting metabolic rate were found between the DIO, DR or LF mice throughout the experiment.

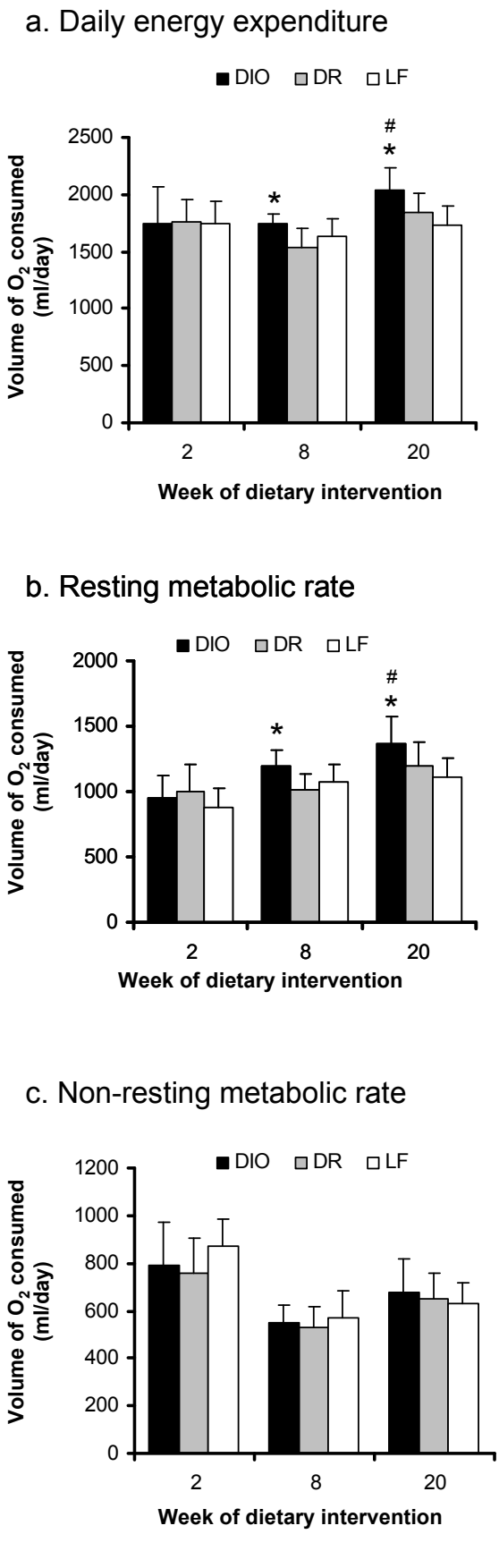

FIGURE 8. Energy expenditure in diet-induced mice model

(A) The total, (B) resting, and (C) activity-related metabolic rate of diet-induced obese (DIO), diet-resistant (DR) and control (LF) mice throughout the experiment (n=12 for each group). Data are presented as means ± SD; * $p<0.05$ vs. DR; # $p<0.05$ vs. LF.

3.3.4 Spontaneous activity rate

Similar to non-resting metabolic rates, spontaneous activity rate during the course of the experiment was not affected by diet or phenotype, with a two-way repeated ANOVA showing 'TIME' ($F_{2,66}$=101.97, p<0.001), but not 'GROUPS' ($F_{2,33}$=0.65, p=0.628) to have a significant effect on the spontaneous activity rate (Figure 9). There was no significant difference in the rate of spontaneous activity between the DIO, DR or LF mice in the weeks activity was measured.

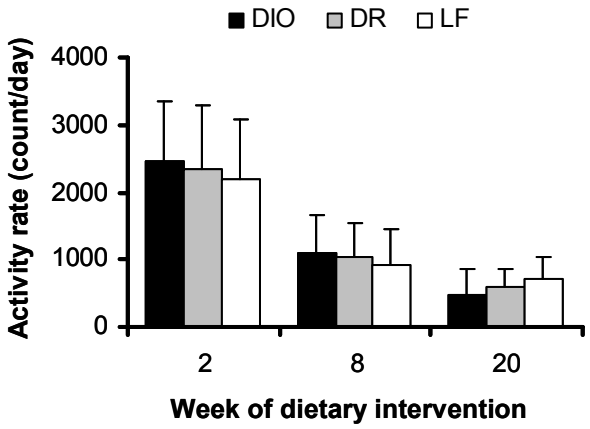

FIGURE 9. Activity rate in diet-induced mice model

Activity rate in diet-induced obese (DIO), diet-resistant (DR) in relation to weeks of eating either a high-fat (DIO, DR) or low-fat diet (n=12 for each group). Data are presented as means ± SD.

3.3.5 Respiratory quotient

Neither duration of dietary intervention nor phenotype affected respiratory quotient, as shown by the two-way repeated ANOVA, 'TIME' ($F_{2,22}$=2.80, p=0.82) and 'GROUPS' ($F_{1,11}$=3.19, p=0.101; Figure 10). Furthermore, no significant difference in the respiratory quotient was found between the DIO and DR mice in any of the weeks it was measured.

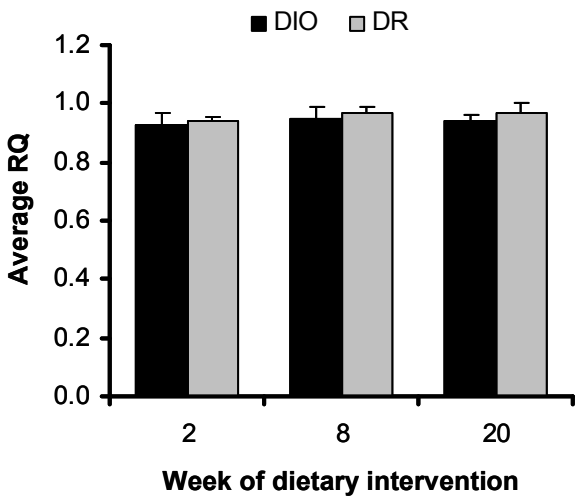

FIGURE 10. The respiratory quotient of diet-induced obese vs. diet resistant mice

Daily average of respiratory quotient in diet-induced obese (DIO), diet-resistant (DR) in relation to weeks of eating either a high-fat (n=12 for each group). Data are presented as means ± SD.

3.3.6 Leptin and insulin

Leptin levels differed significantly among groups ($F_{2,33}$=17.90, p<0.001), with DIO mice having significantly greater leptin levels than both DR (p=0.005) and control mice (p<0.001; Figure 11). Plasma leptin levels were statistically indistinguishable between DR and LF groups (p=0.10).

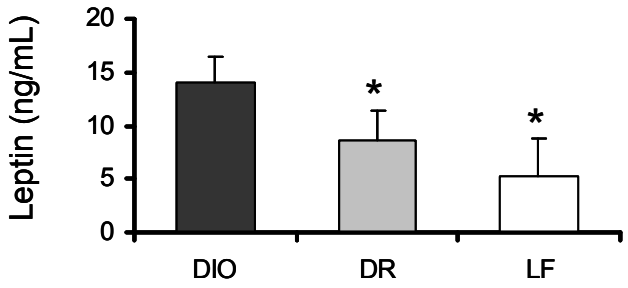

FIGURE 11. The plasma leptin level in diet-induced mice model

The level of leptin in the plasma from diet-induced obese (DIO), diet-resistant (DR) and control (LF) mice. Data are presented as means ± SD; * $p<0.005$ vs. DIO.

The insulin level was significantly higher in DIO mice in comparison to the LF mice ($F_{2,33}=17.90$, $p<0.001$; DIO vs. LF, $p=0.008$; Figure 12). Plasma insulin levels did not differ between DIO and DR mice ($p=0.167$), or between DR and LF mice ($p=0.323$).

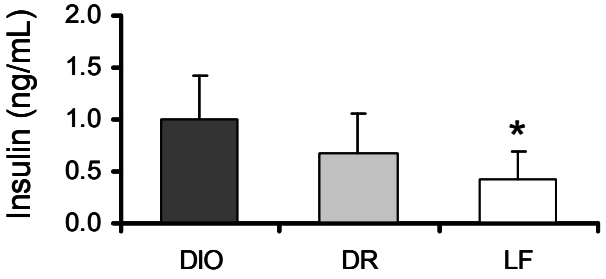

FIGURE 12. The plasma insulin level in diet-induced mice model

The level of insulin in the plasma from diet-induced obese (DIO), diet-resistant (DR) and control (LF) mice. Data are presented as means ± SD; * $p=0.008$ vs. DIO.

3.4 Discussion

Overall, the experiment showed clearly that feeding rates were the only factor that differed significantly between the DIO and the two lean groups, the DR and LF mice. The higher feeding rate in DIO mice therefore appears to be responsible for their significantly higher body weight gain on the high-fat diet compared to the DR group. These differences in feeding rates and associated weight gain were mainly found during the middle stage of the development of obesity (week 8 of dietary intervention), but became more obvious during the later stages obesity development (week 20 of dietary intervention). This experiment also showed that these mice did not differ in respiratory quotient, daily energy expenditure, resting metabolic rate, non-resting metabolic rate or spontaneous activity rate at all stages measured.

The chronology of body weight gain by the DIO, DR and the LF mice were similar to those found in previous studies of diet-induced obesity using this mouse model (112, 113). During the early stage of obesity development (week 2 of high-fat dietary intervention), there was no difference between the DIO and the two lean groups (DR and LF mice). However, as obesity onset progressed towards the middle and late stages (week 8 and 20 of the dietary intervention, respectively), the DIO mice had gained significantly more body weight compared to the lean groups. This further validates the use of this mouse strain to investigate the development of diet-induced obesity. Furthermore, this experiment has given a better insight into the development of obesity and its causes as most of obesity studies mentioned previously focused mainly on a single time point where differences in the amount of body weight gained between the obese and the lean phenotype had already become significant (121).

Furthermore, the type of diet itself may play an important role as a trigger for the development of obesity in this mouse strain given that the difference in the energy intake between the DIO and DR only became significant during the middle and late stages of obesity development where the mice were given more than 5 weeks of high-fat diet. This hypothesis is supported by the finding that obesity can only be identified in C57Bl/6 mice when they are given a high-fat diet (122). Regardless, as obesity developed into more advanced stages, the large amount of energy DIO mice consumed may have caused the immense increase in the body weight gain seen in these mice.

This energy intake rates of the present study were different to those shown in a previous study this strain (38). Although both studies show similarities in early and late stages of the development of diet-induced obesity, this experiment differed in showing that DIO mice consumed significantly more food than lean groups during the middle stage of obesity onset. By contrast, the previous study found that the energy intake of the DIO mice was lower than the lean groups at this stage. The discrepancy between both studies might be due to differences in the age of mice used, as Lin and colleagues (38) used 3-week old mice for their experiment; while in this study, 9-week old mice were used. This might be due to age-related differences in sensitivity of feedback mechanisms to body fat gains. This interpretation is supported by the finding that leptin-based inhibitory systems become desensitized with age in mice (38). Thus the older DIO mice used in this study would have less negative feedback from accumulated fat than those studied by Lin et al. (38) and feed at higher rates throughout their exposure to high-fat diets.

As predicted by findings of previous studies (38), The DIO mice consumed large amounts of food despite having significantly higher leptin levels compared to the DR and LF mice. This is probably a result of development of leptin resistance in these mice as it was found that DIO mice can develop leptin resistance after 7 weeks of consuming a high-fat diet (38, 116). Similar increases were also found in the plasma insulin levels of the DIO mice, As with leptin, this phenomenon may be due to the development of insulin resistance as previously observed in studies of obese subjects (46-48, 123).

It can be stated unequivocally, that decreased rates of energy expenditure does not account for development of obesity in this type of dietary fat-fed DIO mice. Rather, the daily energy expenditure was higher in the DIO mice compared to the DR mice as over the 20 week period of consuming a high-fat diet. Examination of the metabolic results shows that the difference in the daily metabolic rate between the obese and lean phenotypes was due to differences in resting metabolic rates. This does not, however, represent an inherent difference in metabolic intensity between these phenotypes. Firstly, because the overall body size predicted from skeletal measurements of DIO and DR mice were statistically identical, the significantly higher body mass in DIO versus DR mice is not due to differences in size *per se*, but is due mainly to much higher body fat content in the DIO animals. This extra mass has resulted in relatively greater muscle loading which, in turn, has stimulated muscular hypertrophy in the DIO mice. The complete lack of difference between phenotypes in metabolic rate per gram of lean tissue reinforces the interpretation that differences in metabolic rate are due solely to differences in total mass of lean tissue and not because of any difference in metabolic intensity. These results are consistent with those for humans which show that metabolic rate differences between

lean and fat people disappear when corrected for metabolic rat per gram of lean tissue (10, 11, 15). This clearly shows that differential weight gains in DIO versus DR mice are not explained by inherent differences in metabolic energy expenditures.

Differences in spontaneous activity levels were also shown not to play a significant role in the development of obesity in this diet-induced obesity model. Although in this study the measurements of 24-h spontaneous activity suggest that there may be a tendency for DIO mice with advanced onset of obesity to be less active than DR mice, the activity levels of both phenotypes were statistically indistinguishable. Thus, similar to the previous study performed on rats (121), spontaneous activity rate is more likely to be affected by aging rather than diet or body weight. Furthermore, the activity-related energy expenditure determined from the 24-h measurements of oxygen consumption in this experiment also revealed no difference in activity level between the lean and obese phenotypes in any of the weeks measured.

Energy balance can also be affected by potential difference in food conversion efficiencies, which can occur after meals have been fully absorbed. For example, some obese-prone animals are known to oxidise a higher proportion of dietary carbohydrates compared to fats than their lean counterparts, resulting in these animals storing proportionately more fat when consuming high-fat diets (26, 27, 32, 124). Moreover, it has been shown in the diet-induced obesity rat model that more radio-labelled dietary fats were incorporated into adipose tissue in the obese-prone compared to obese-resistant rats when given high-fat diets (125). However, such differences in substrate oxidation would result in divergent respiratory quotients between these groups, a result not seen at any

stages of the development of obesity in the C57B1/6 mice used in this study. Therefore, although higher storage uptake of fat can cause an increase in the amount of adipose tissue seen in obese-prone animals, it is likely that in this diet-induced obesity model, difference in substrate oxidation did not affect the development of obesity as proposed previously.

In conclusion, the development of obesity in DIO C57B1/6 mice is due to an increased in energy intake in the form of food intake, but not to a decreased metabolic rate, lower activity rate, or carbohydrate-biased substrate oxidation compared to their lean counterparts. Furthermore, this difference in energy intake may have been caused by a dysfunction in appetite regulation by peripheral neuroendocrine such as leptin and insulin. Thus, it is important to explore the central appetite regulatory system experimentally in this animal model to better understand the basis of appetite differences among DIO and DR mice.

4

Experiment 2. Decreased plasma PYY accompanied by elevated PYY and Y2 receptor binding densities in the medulla oblongata of diet-induced obese mice

Adapted from the author's publication in Endocrinology (126)

4.1 Introduction

Peptide YY (PYY) is a member of the pancreatic polypeptide family which is structurally and functionally related to the neuropeptide Y (NPY) family (89, 127). PYY is mainly secreted from the endocrine L cells of the small and large intestine and is released into the circulation in response to ingestion of food, especially in the presence of a fatty meal (128, 129). At present, it is known that the peripheral administration of PYY acutely inhibits food intake (67, 98). PYY has a high affinity for NPY Y2 receptors followed by NPY Y1 and Y5 receptors (91). Furthermore, it has been suggested that PYY works via the NPY Y2 receptor to suppress the amount of food intake (87).

In the hypothalamus, many studies have found that PYY acts on NPY Y2 receptors in the arcuate nucleus to decrease food intake (130-133). Additionally, it is known that the medulla has a high level of binding to PYY (68, 94). However, currently, no information is available in regard to hypothalamic and medullary PYY and Y2 receptor regulation in diet-induced obesity. Using a chronic high energy diet-induced obese (DIO) and diet-resistant (DR) mouse model, this study aimed to examine the levels of plasma PYY together with PYY and Y2 receptor binding density in the hypothalamus and medulla oblongata. It is hypothesised that differential regulation exists in the peripheral PYY and

its hypothalamic and medullar binding densities between the mice prone or resistant to diet-induced obesity.

4.2 Materials and Methods

4.2.1 Animal model and diets

The tissues used for this experiment were taken from the same animal model which were utilised for the experiment described previously (*Experiment 1, 3.2 Materials and Method section*).

4.2.2 Tissue preparation and body composition analysis

After 22 weeks of feeding on the high or low-fat diets, mice were given an overdose injection of sodium pentobarbitone (120mg/kg, intraperitoneally). Blood samples were collected from the right ventricle of the heart. Brains were immediately removed after death and frozen in liquid nitrogen. Coronal brain sections (14µm) were cut at -17 °C using a cryostat, and thaw-mounted onto Polysine^{TM} Microscope Slides (Menzel GmbH & Co. KG, Braunschweig).

4.2.3 ^{125}I-PYY binding autoradiography

PYY binding densities were visualized using ^{125}I-PYY as previously described (134). Sections were pre-incubated for 30 minutes in Krebs Henseleit Tris (KHT) buffer (118 mM NaCl, 4.8 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM $CaCl_2$, 50 mM Glucose, 15 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 10 mM Tris, pH 7.3). Slides were then incubated for 120 minutes in KHT buffer containing 0.1% bovine serum albumin, 0.05% Bacitracin, and 25 pM ^{125}I-PYY (Sigma Aldrich, St. Louis, MO). Non-specific binding was determined by

incubating sections in the same incubation buffer plus 1 µM Porcine NPY (Sigma Aldrich, St. Louis, MO). Slides were then washed (3 x 5 min) in ice-cold buffer, dipped in ice-cold distilled water, and dried under a gentle stream of cool air. Slides were stored overnight in desiccators, and then apposed to Kodak X-OMAT AR film in the presence of standard microscales for 12 days. Autoradiographs were developed using Kodak D-19 developer and fixed with Ilford Hypham Rapid Fixer.

4.2.4 Y2 receptor binding autoradiography

To measure Y2 receptor binding density, [Leu^{31}, Pro^{34}]-NPY (Porcine; Sigma Aldrich, St. Louis, MO) was included in the incubating solution to mask the NPY Y1 and Y5 receptors (135). Briefly, sections were pre-incubated for 30 min in KHT buffer. Slides were then incubated for 120 min in KHT buffer containing 0.1% bovine serum albumin, 0.05% Bacitracin, 100 nM [Leu^{31}, Pro^{34}]-NPY, and 25 pM ^{125}I-PYY (Sigma Aldrich, St. Louis, MO). Porcine NPY (1 µM) was used to determine non-specific binding as mentioned above. Autoradiographs were developed as above.

4.2.5 Quantification

Autoradiography images were captured and analysed using a computer assisted image analysis system, Multi-Analysis, connected to a GS-690 Imaging Densitometer (Bio-Rad, USA), as described previously (113). Binding density was calculated with the aid of the standard curve generated from the microscales, which then converted to nCi/mg of tissue equivalent. Individual medullary nuclei were identified with reference to a standard mouse brain atlas (136).

4.2.6 Plasma PYY

A commercially available Peptide YY (Rat, Mouse, Porcine) RIA kit (Phoenix Pharmaceuticals, Belmont, CA) was used to measure the plasma level of PYY. The kit had 100% cross reactivity with both circulating forms of PYY, PYY_{1-36} and PYY_{3-36} (107, 137).

4.2.7 Statistical analysis

Data are presented as means ± SD. Data were analysed using the SPSS statistical package 13.0 (SPSS Inc., Chicago, Illinois). A two way repeated ANOVA (treatment x weeks as repeated measures) was used to analyse data of the weekly body weight and energy intake. Data of PYY binding density, Y2 receptor binding density, and plasma PYY measurements were assessed by one way ANOVA, followed by a post-hoc Tukey-Kramer Honestly Significant Difference (HSD) test for multiple comparisons among the groups. To analyse correlations between variables measured, a Pearson test was performed.

4.3 Results

4.3.1 Plasma PYY

The level of plasma PYY was significantly lower in the DIO group (32%) compared to the LF group ($F_{2,21}$=6.26, p<0.01; DIO *vs.* LF, p=0.007; Figure 13). No significant difference was found in the levels of plasma PYY between the DR and LF groups.

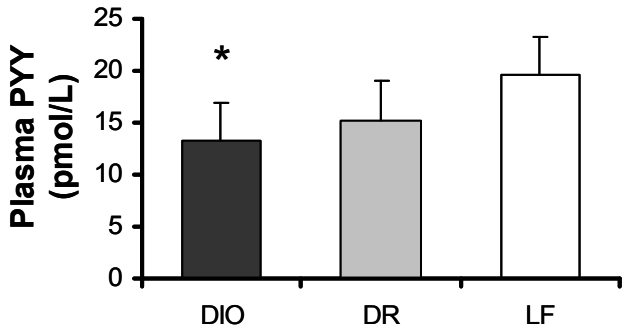

FIGURE 13. The plasma level of Peptide YY in diet-induced mice model

The plasma level of Peptide YY (PYY) in chronic diet-induced obese (DIO), chronic diet-resistant mice (DR) and the control group (LF). Data are represented as means ± SD; *p<0.01 *vs*. LF.

4.3.2 PYY binding density and Y2 receptor binding density in the hypothalamus of DIO, DR and LF mice

Although there was a trend that the obese mice had a higher PYY binding density compared to the lean mice in the dorsomedial and ventromedial hypothalamus, these differences were not significant (Table 3). There was no significant difference in PYY binding density in the arcuate nucleus, and lateral hypothalamus. Furthermore, there were no significant differences of Y2 receptor binding density in any hypothalamic nuclei (Table 3) between the groups.

TABLE 3. ^{125}I-PYY binding sites and Y2 receptor binding density in diet-induced obese mouse model

The ^{125}I-PYY binding sites and Y2 receptor binding density in various areas of the hypothalamus of chronic diet-induced obese (DIO), diet-resistant (DR) and the control (LF) mice

Brain area	Mean ± SD			F	*p* value
	DIO	DR	LF	[2,10]	
^{125}I-PYY binding sites					
Arc	284.8 ± 0.7	198.6 ± 66.5	195.7 ± 38.5	3.00	0.160
DMH	213.1 ± 27.7	123.7 ± 44.7	172.0 ± 20.9	4.39	0.079
LH	224.6 ± 30.1	131.2 ± 46.1	177.9 ± 42.7	3.02	0.138
VMH	248.6 ± 49.3	138.6 ± 41.4	168.3 ± 27.9	5.02	0.064
Y2 receptor binding					
Arc	228.5 ± 17.2	154.3 ± 70.2	170.9 ± 35.5	1.36	0.337
DMH	206.2 ± 16.2	125.1 ± 58.9	154.3 ± 37.4	1.98	0.232
LH	222.1 ± 5.3	131.2 ± 51.0	162.6 ± 42.4	2.83	0.151
VMH	189.5 ± 30.9	139.9 ± 38.2	143.4 ± 23.7	1.72	0.269

Abbreviations - Arc: arcuate nucleus, DMH: dorsomedial hypothalamus, LH: lateral hypothalamus, VMH: ventromedial hypothalamus. Binding densities were quantified at the level of Bregma -1.22 mm, -1.70 mm, and -2.18 mm.

4.3.3 PYY binding density in the medulla of DIO, DR and LF mice

A one-way ANOVA revealed that there were significant differences between the groups for PYY binding density in the dorsal vagal complex (DVC) containing the nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve ($F_{2,10}$=6.76, p=0.019), intermediate reticular zone (IRt; $F_{2,10}$=17.34, p=0.001), and ventrolateral medulla (VLM; $F_{2,10}$=7.60, p=0.014) area (Figure 14 & 15). In the DVC, the DIO mice had higher PYY binding density than that of the DR (68% higher, p=0.015) and LF mice (37%, p=0.079). Similar differences were also observed in the intermediate reticular zone, where the DIO had a significantly higher binding density compared to the DR (171% higher, p=0.001) and the LF group (96%; p=0.004). In the ventrolateral medulla, The DIO mice also had a

significantly higher binding density than the DR (122% higher, p=0.022) and the LF group (134%, p=0.015).

4.3.4 Y2 receptor density in the medulla oblongata of DIO, DR and LF mice

The DIO mice had significantly higher Y2 receptor binding density (Figure 14 & 15) in the DVC compared to the LF group (63% higher; $F_{2,10}$ =4.75, p=0.044; DIO *vs.* LF, p=0.041). In the IRt, the DIO group had 47% higher binding density compared to the DR group ($F_{2,10}$=6.92, p=0.02; DIO vs. DR, p=0.023). In the VLM, there were no differences in the binding density between the DIO, DR and LF groups.

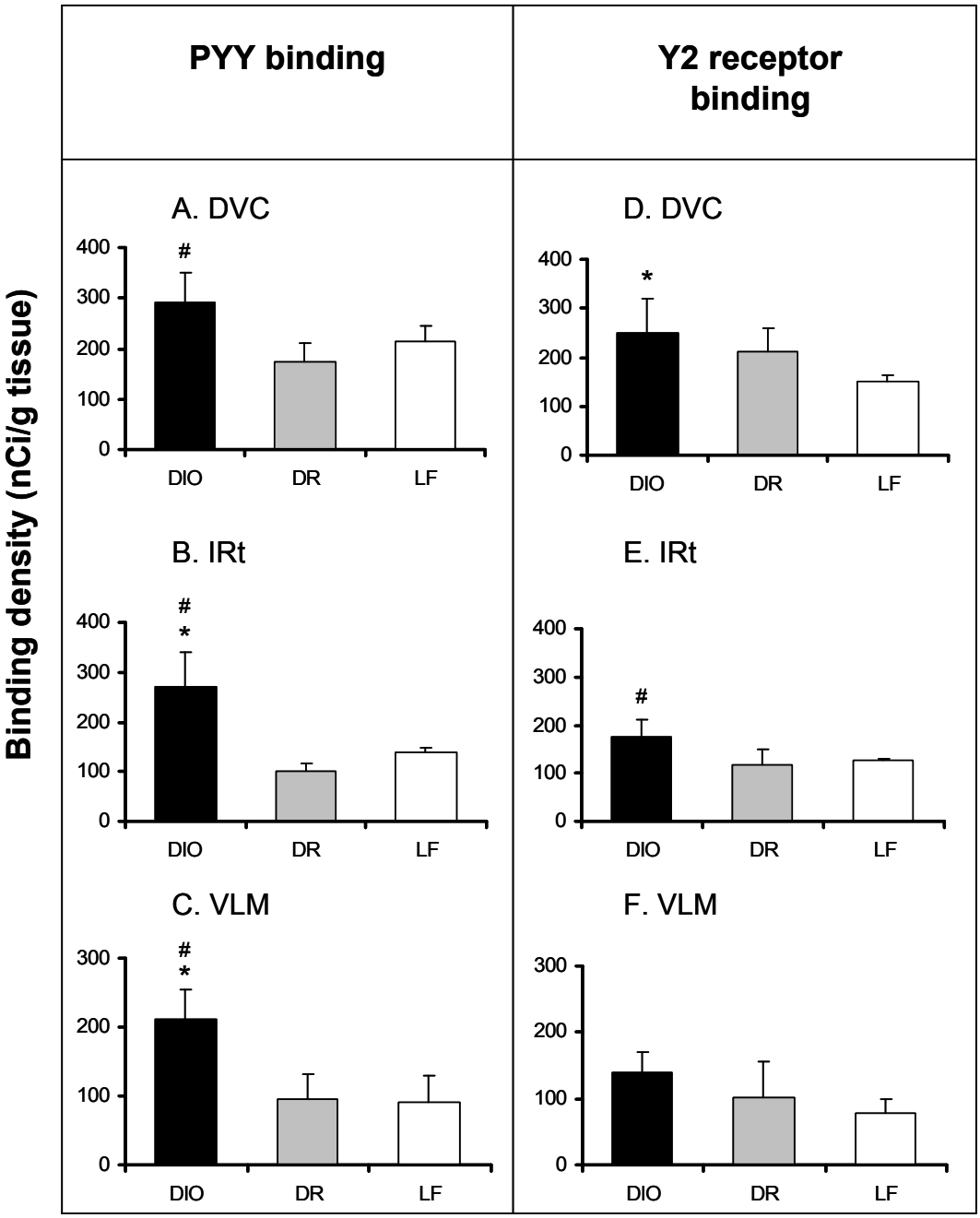

FIGURE 14. ^{125}I-PYY binding sites and Y2 receptor binding density in diet-induced obese mouse model

The binding densities to [^{125}I]-Peptide YY (A to C) and Y2 receptor (D-F) in the dorsal vagal complex (DVC), intermediate reticular zone (IRt), and ventrolateral medulla (VLM) of chronic diet-induced obese (DIO), diet-resistant (DR) and the control (LF) mice. Binding densities were quantified at the level of Bregma -7.32. Data are represented as means ± SD; $^{*}p<0.05$ DIO *vs*. LF, $^{\#}p<0.05$ DIO *vs*. DR.

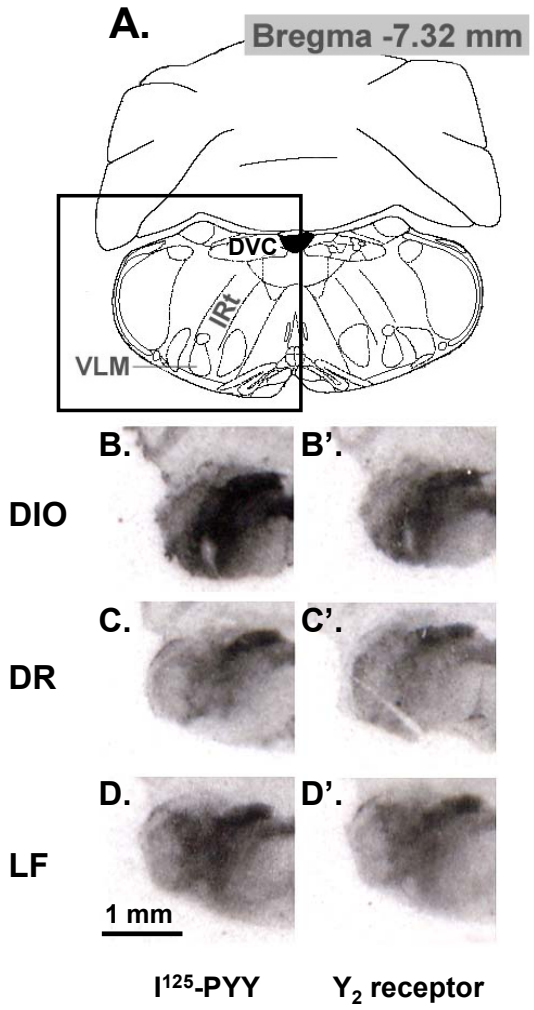

FIGURE 15. ^{125}I-Peptide YY binding and Y2 receptor binding densities in the medulla in diet-induced obese mouse model

Photographs depicting the ^{125}I-Peptide YY ([^{125}I]-PYY) bindings (B to D) and Y2 receptor bindings (B' to D') in the medulla of chronic diet-induced obese (DIO; B and B'), chronic diet-resistant mice (DR; C and C') and the LF (control) group (LF; D and D'). The line box (A) indicates where the section was taken. Abbreviations - DVC: dorsal vagal complex; IRt: the intermediate reticular zone; VLM: ventrolateral medulla.

4.3.5 Relationships between body weight, food intake, plasma PYY and the binding density of ^{125}I-PYY and Y2 receptors in the medullary nuclei

A correlation analysis was carried out between the final body weight, body fat mass, energy intake of the last week of the dietary intervention, the plasma PYY, as well as the medullary PYY binding density and Y2 receptor binding density of the DIO, DR and LF mice (Table 4). Final body weight was highly correlated to the plasma PYY, the PYY binding densities in all measured brain areas, as well as the Y2 receptor binding densities in the IRt and VLM. Total body fat was also highly correlated to the plasma PYY, the PYY binding densities in all measured areas as well as the Y2 receptor binding density in the IRt and the VLM. The energy intake of the last week of the experiment was significantly correlated to plasma PYY concentration and the PYY binding densities and Y2 receptor binding densities in the IRt and the VLM.

TABLE 4. The correlation between the body weight, food intake, and the plasma level of PYY and the binding density of ^{125}I-PYY and Y2 receptor in the medulla nuclei

	After 22 weeks of high-fat diet					
	Final body weight		Total body fat		Energy intake in last week	
	R value	*p* value	R value	*p* value	R value	*p* value
Plasma PYY	-0.468	0.021	-0.475	0.019	-0.462	0.023
Total binding with ^{125}I-PYY						
DVC	0.738	0.010	0.738	0.010	0.516	0.104
IRt	0.842	0.001	0.838	0.001	0.671	0.024
VLM	0.803	0.003	0.817	0.002	0.777	0.005
Y2 receptor binding density						
DVC	0.495	0.122	0.468	0.146	0.431	0.186
IRt	0.727	0.011	0.747	0.008	0.767	0.006
VLM	0.618	0.043	0.634	0.036	0.841	0.001

Abbreviations - DVC: dorsal vagal complex, IRt: the intermediate reticular zone, VLM: ventrolateral medulla

4.4 Discussion

The results of this study revealed a significant increase of medullary PYY and Y2 receptor binding densities in the DIO mice compared to the DR and LF mice. This increased binding density was accompanied by a decrease in plasma PYY levels.

It is known that PYY acts on NPY Y2 receptors in the hypothalamic arcuate nucleus to decrease food intake (91, 130-133). Furthermore, Y2 receptor conditional knock out mice have been shown to have a significant increase of food intake (81). Although there is an abundant amount of NPY receptor in the medulla (138, 139), to the present knowledge, no information is available in respect to whether or not the medullary NPY receptors are involved in the regulation of body weight in high-fat, diet-induced obesity.

This study found that the PYY and Y2 receptor binding densities in the obese mice were significantly higher than the lean DR and LF mice in most areas of the medulla regulating autonomic function (DVC, IRt, and VLM). This study also found that there was a positive correlation between PYY binding density in these areas and final body weight, energy intake, and body fat mass. Furthermore, although a similar trend was found in some of the hypothalamic nuclei (the dorsomedial and ventromedial hypothalamus), the differences between the obese and lean mice were not as significant as the findings in the medullary areas. These findings have demonstrated that PYY can also regulate energy balance via the medulla to control food intake, rather than working exclusively in the arcuate hypothalamic nucleus.

The differences in the binding density in the VLM implied that there might be a difference between the obese and lean mice in their baroreceptor regulation by PYY (140). This is supported by the finding that the VLM is a site that plays a crucial role in baroreceptor regulation in hypertensive obese rats (141). In general, there is a tendency for obese subjects to have higher blood pressure (142-144). However, to the present knowledge, there is no literature available that evaluates baroreceptor regulation in a diet-induced obese mouse model.

Studies have shown that an injection of PYY into the DVC causes similar neuronal inhibition as an injection of a Y2 agonist (68). This implies that PYY binds to Y2 receptors in the medulla to cause these effects. In the present study, the obese mice were found to have a significantly higher Y2 receptor binding density in the DVC and IRt areas of the medulla compared to the lean mice. Since the magnitude of the significance

in the correlation between Y2 receptor binding density and body weight, food intake and body fat mass was less than that of PYY binding density, it is possible that the mechanism in which the PYY regulates the food intake in the medulla, might not only be acting solely on the NPY Y2 receptor binding density as previously thought, but may also by acting through the NPY Y1 and Y5 receptors.

Plasma PYY in the obese mice in this study was found to be lower than the high-fat fed lean and the low-fat fed lean mice. Other studies in humans and rodents have also described a reduced amount of plasma PYY in obese populations (145, 146). This attenuated response of PYY release in obese populations (147) has been shown to cause an insufficient inhibitory effect on feeding (96, 97). Findings from the analysis of PYY *null* mice also confirm that a depleted amount of PYY can cause an increase in food intake (148), whilst transgenic over-expressed PYY mice were protected against diet-induced obesity (149). This study is the first to analyse the peripheral plasma PYY as well as its receptor binding density in a dietary-induced obese animal model. Based on these results, it is suggested that the up-regulation of the medullary PYY binding density in the DIO mice maybe a response to their low level of plasma PYY. However, it is obvious that in the diet-induced obese mice, this compensatory regulation of PYY was not effective enough to reduce the food intake in this group. This is possibly due to the low amount of PYY bound as a result of the low plasma PYY concentration in the obese mice. It is important to note that PYY-immunoreactive neurons have been found in the medulla (150, 151). Therefore, a local effect of PYY neurons on PYY binding and Y2 receptor binding cannot be excluded. Further studies are needed to confirm this issue by measuring the levels of PYY mRNA and protein expression in the medulla of DIO mice.

Furthermore, the results of this study suggested that the elevation of the level of plasma PYY might be effective at decreasing food intake. However, previous studies have shown that there were different effects of the peripheral and central administration of PYY on food intake (79, 152). Peripheral injections of PYY caused significantly lower food intake in humans and rodents (including in a DIO mouse model) (98, 127, 132). Nevertheless, an intracerebroventricular injection of PYY induced higher food intake (153, 154). On the contrary, when PYY was injected directly into the hypothalamic arcuate nucleus, food intake was significantly decreased (98), an effect that was similar to that observed following a peripheral injection of PYY.

As for the difference in the effects of peripheral versus central injections of PYY, one possible explanation could be that when PYY was injected peripherally, it was transported in the blood directly into areas in the brain with a high binding affinity to PYY, such as the hypothalamic arcuate nucleus (and possibly also the DVC), via the highly permeable blood-brain barrier. In these areas, PYY may have bound to the anorexigenic NPY Y2 receptor causing a decrease in food intake, which has been evidenced in Y2 knock-out mice where the anorectic effect of peripheral PYY injection was diminished (98). However, when injected centrally into the ventricle, it was likely that PYY bound to the more orexigenic NPY receptors, such as Y1 and Y5, causing an increase in food intake. This was also supported by the finding that the orexigenic effect of intracerebroventricular PYY was reduced in Y1 and Y5 receptor knock-out mice (154).

In conclusion, it is clear that in diet-induced obese mice, there is a dysfunctional regulation of the PYY system. Even though in DIO mice there was an up-regulation in PYY and Y2 receptor binding in the medulla, the reduced amount of plasma PYY in these mice may not have been sufficient to decrease their food intake thus contributing to their high energy intake and subsequent weight gain.

5

Overall conclusion

Using the diet-induced obese mouse model, this study demonstrated convincingly that the development of obesity following provision of a high-fat diet to DIO mice is due to their greater rate of food intake compared to DR mice. Despite the significant difference in the body weight gained by DIO mice compared to DR mice, both groups have statistically indistinguishable rates of lean-mass-corrected daily energy expenditure, resting energy expenditure, and activity-related energy expenditure, as well as the same levels of spontaneous activity and respiratory quotients. Thus, differences in energy intake are mainly responsible for the greater amount of body weight gained by DIO mice, which may be due to reduced leptin sensitivity. This interpretation is consistent with the higher plasma levels of leptin and insulin in DIO compared with DR mice in this study.

The peptide YY regulation in this model was also observed and it was demonstrated that DIO mice had significantly lower levels of plasma PYY compared to the DR mice. This study also revealed a significant increase of medullary PYY and Y2 receptor binding densities in the DIO mice compared to the DR and control mice. This has demonstrated that medullary PYY receptors may play an important role in determining the phenotypes of diet-induced obesity.

Therefore, based on both experiments, it can be concluded that the amount of food intake is a major factor that can cause a significant body weight gain seen in these DIO mice. An underlying mechanism of the increased food intake is the low plasma level of PYY found in DIO mice. In response to the low level of plasma PYY, the DIO mice have up-regulated PYY and Y2 receptor densities in the medulla oblongata. However, this compensation does not prevent a high food intake.

Although this study has answered some of the questions raised at the development of obesity, it raises a number of issues that require further clarification. For example, it is still not known how PYY regulation and variation in leptin and insulin plasma levels differ among obese-prone and obese-resistant animals during all stages of obesity development. This could be addressed by experimentally altering their levels in both groups and evaluating the consequences. Not only will such studies lead to further understanding of the basis of obesity development in this mouse strain, it may have important implications for targeting research on other obese-prone animals including humans.

6

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