2.9 Pathology and abnormal aspects: appetite

Overnutrition and appetite regulators in mothers and offspring

The World Health Organization (WHO) formally recognized the obesity epidemic in 1997; however, as obesity continues to rise exponentially, it has now reached pandemic proportions. Increasingly around the world, obesity is no longer restricted to adults. Childhood obesity is currently rising at an alarming rate, with 20 million children under five estimated as overweight in 2005 (WHO, 2006). In the USA, the number of overweight children has doubled and the number of overweight adolescents has tripled since 1980 (WHO, 2003). It is increasingly accepted that obesity results from a combination of genetic and environmental factors.

Impact of maternal nutrition on the risk of energy metabolic disorders in offspring

Overconsumption of energy-rich food and a sedentary lifestyle make significant contributions to the rising rate of childhood obesity. However, apart from the social and environmental factors that influence children's behaviour (Nielson *et al.*, 2006), the prenatal maternal condition is also critical to offspring body composition, and can predispose the fetus to the development of obesity after birth (Forsum *et al.*, 2006). Increasing evidence suggests that both maternal phenotype and nutritional state during gestation and postnatal phase are important in promoting obesity in offspring. Human studies suggest that intrauterine factors may be more important than genetic factors in changing gene expression in response to high-fat diet, which may have a longstanding influence postnatally.

Early research revealed the inverse relationship between low birth weight due to gestational malnutrition and the increased risks of obesity and cardiovascular disease (Fall *et al.*, 1995; Law *et al.*, 1992; Ozanne *et al.*, 2004; Painter *et al.*, 2005). Barker first proposed the "fetal origins" hypothesis in 1990, which posits that adverse environmental factors early in life cause disruption of normal growth and development, leading to a more susceptible adult phenotype prone to metabolic disorders, such as cardiovascular disease (Barker, 1990).

However, under global obese pandemic, maternal overnutrition during gestation becomes more prominent to interrupt normal fetal development. The observation of "U-shaped" relationship between birth weight and later obesity (Curhan *et al.*, 1996) makes the interest shift to the potential detrimental impact of a maternal overnutrition/obesity and increased birth weight on the risk of disease in childhood and later on in adulthood. Experimental approaches varies by exploding overnutrition during different period of times, from maternal overnutrition prior to or/and during gestation, or/and lactation, to during lactation.

Maternal obesity and hyperglycemia during pregnancy can lead to large birth weight, increased circulating insulin, glucose, free fatty acid and triglycerides, and glucose intolerance, as well as obesity in offspring (Armitage *et al.*, 2005; Boney *et al.*, 2005; Elahi *et al.*, 2009; Franke *et al.*, 2005; Khan *et al.*, 2005; Merzouk *et al.*, 2000; Paul *et al.*, 2005; Srinivasan *et al.*, 2006; Wu *et al.*, 2006). Offspring of obese mothers are at higher risk of becoming obese and

develop insulin resistance and cardiovascular disease later in life, suggested by both human and animals studies (Clausen *et al.*, 2009; Dorner *et al.*, 1994; Elahi *et al.*, 2009; Mamun *et al.*, 2009; Plagemann *et al.*, 1992; Samuelsson *et al.*, 2007).

A simple increases in body weight gain during pregnancy and lactation due to hyperphagia induced by pharmacological inhibition of melanocortin 3/4 receptor leads to the development of obesity in offspring over time (Heinsbroek *et al.*, 2009). Animal study suggests that overnutrition either starting prior to gestation or from gestation and lactation exert similar effect on the development of obesity and hyperinsulinemia in offspring (Howie *et al.*, 2008), suggesting the critical windows of fetal development that determines the phenotype. Offspring from genetically obesity-prone rats remained obese even when they were suckled by obesity-resistant rats dams (Gorski *et al.*, 2006). Offspring from rat dams who have established obesity induced by high-fat diet consumption prior to gestation developed adiposity and impaired glucose and lipid metabolism as early as postnatal day 20 (Bayol *et al.*, 2007; Bayol *et al.*, 2008; Chen *et al.*, 2008), which were maintained until adulthood (White *et al.*, 2009). Overnutrition during the suckling period can also cause an obese phenotype by consuming fatty milk, even in obesity-resistant rats (Gorski *et al.*, 2006). When these rats were suckled by obese-prone dams with rich milk, they developed obesity by consuming high-fat diet after weaning (Gorski *et al.*, 2006). Increased milk availability through litter size reduction also leads to obese phenotype (Chen *et al.*, 2008; Velkoska *et al.*, 2005); while overfeeding during the suckling period showed additive effects with maternal obesity to promote more severe adiposity and glucose intolerance at weaning (Chen *et al.*, 2008). Thus, both maternal obesity and early postnatal overfeeding can promote obese phenotype in offspring.

Maternal consumption of a typical western 'junk food' diet, such as biscuits, chocolate, muffins, potato crisps, and sweets, in pregnancy and lactation promoted an exacerbated preference for 'junk food' in offspring in adulthood (Bayol *et al.*, 2007). Maternal overnutrition is also necessary to promote, hyperphagia and feeding efficiency when offspring were challenged by post-weaning high-fat diet into adulthood, and thereafter more sever weight gain and adiposity, as well as insulin resistance (Nivoit *et al.*, 2009; White *et al.*, 2009). Rats over-fed during the suckling period also displayed persistence of overweight and hyperphagia throughout the lifespan (Bassett *et al.*, 1988; Plagemann *et al.*, 1992; Voits *et al.*, 1996).

Development of energy homeostasis circuitry in the hypothalamus

Appetite is regulated by a complex and redundant but highly reliable network. The central neural pathways involved in appetite regulation and energy metabolism are well conserved across species. The hypothalamus plays a key role in energy homeostasis. The most commonly studied are two groups of neurons concentrated in the arcuate nucleus (ARH) located at the ventral part of the hypothalamus, one expressing the appetite stimulators, neuropeptide Y (NPY) and agouti-related protein (AgRP), and the other expressing appetite suppressors proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which interact with each other to match caloric intake to energy expenditure. NPY/AgRP and

POMC/CART expressing neurons project to paraventricular nucleus (PVN) to exert their effects (Schwartz *et al.*, 2000).

NPY is the most robust appetite stimulator produced predominantly in the ARH. Physiologically, hypothalamic NPY concentrations are elevated before a meal to stimulate appetite, and continuous or repeated central administration of NPY leads readily to obesity. Activation of the Y1 receptor (major NPY orexigenic receptor (Pralong *et al.*, 2002)) alone can increase food intake even when NPY expression is inhibited (Shintani *et al.*, 2001). POMC derived α-melanocyte-stimulating hormone (MSH) counteracts NPY to inhibit feeding and promote negative energy balance (Ahima *et al.*, 2000). Leptin, an adipose-derived hormone, can directly access the hypothalamic ARH, to suppress NPY/AgRP and activate POMC/CART expression to inhibit feeding and increase energy expenditure via the long form of the leptin receptor (Ob-Rb). Central leptin is commonly observed in dietary obesity (Levin *et al.*, 2004). The hypothalamic NPY/AgRP and POMC/CART expressing neurons are plastic, being modified by chronic overconsumption of high-fat diet, associated with the development of metabolic disorders, such as adiposity and hyperinsulinaemia (Hansen *et al.*, 2004; Huang *et al.*, 2003; Levin *et al.*, 1997; Morris *et al.*, 2008).

The mammalian target of rapamycin (mTOR) is a highly conserved member of the phosphoinositide 3-kinase related kinase family and mTOR functions as a cellular sensor for changes in energy status, such as glucose and amino acid levels to determine cell growth. Hypothalamic mTOR has been recently identified to be involved in brain fuel sensing and feeding regulation (Cota *et al.*, 2006). There are two pathways of mTOR, mTOR complex (mTORC) 1 and mTORC2, whereas mTORC1 signaling has been implicated as a target of leptin in the regulation of energy balance (Cota *et al.*, 2008). Fasting downregulates the phosphorylation of mTOR and its downstream target S6K1 to increase feeding. Activation of the mTOR pathway by leucine (an essential amino acid and activator of mTOR) or leptin has been shown to inhibit food intake and reduce body weight (Cota *et al.*, 2006). Inhibition of mTOR by antagonist rapamycin has been shown to increase energy intake and promote weight gain (Cota *et al.*, 2006). These effects of hypothalamic mTOR have been shown to be due to the selectively inhibition on NPY, as well as facilitation of the central anorexigenic effect of the adipose-derived hormone leptin (Cota *et al.*, 2006).

In the rat, differentiation of the neuronal systems regulating energy homeostasis begins during gestation, and continues until weaning (Grove *et al.*, 2005). In adults, the adipose hormone leptin signals fat stores; however, in the mouse between postnatal days 4 to 16, there is a marked increase in circulating leptin levels, which is called the "***leptin surge***". Leptin surge reaches peak levels at postnatal days 9 to 10. Increased leptin is necessary to support the development and maturation of these brain neurons involved in the regulation of appetite and energy metabolism (Ahima *et al.*, 1998). The role of leptin in neural development in neonatal rodents is shown to be independent of its role in energy homeostasis, given that exogenous leptin does not reduce body weight, milk intake, or metabolic rate during the first two postnatal weeks (Ahima *et al.*, 1998; Mistry *et al.*, 1999; Pinto *et al.*, 2004)

Both leptin deficiency and an abnormally high leptin surge can alter neural development, thus change feeding and energy metabolism, contributing to obesity {Bouret, 2006 #62;Pinto, 2004 #101;Yura, 2005 #42}. The lack of *ob* gene results in leptin deficiency in *ob/ob* mice, whereas higher levels are present in mice with restricted intrauterine nutrition. Leptin deficiency in *ob/ob* mice results in a lower hypothalamic neuron density and innervations, which is thought to contribute to the obese phenotype (Bouret *et al.*, 2006; Pinto *et al.*, 2004). The high leptin surge due to restricted intrauterine nutrition has been shown to be linked to an increased density of hypothalamic neurons (eg. NPY) involved in feeding and energy metabolism, leading to increased weight gain and adiposity when mice were fed a high-fat diet (HFD) after weaning (Yura *et al.*, 2005). Similar correlations have been observed in humans. Thus, any abnormality in the leptin surge will disturb energy homeostasis.

The ontogeny of the NPY and POMC system is most extensively studied in rodents and non-human primates by Grove and colleagues {Grove, 2003 #447}{Grove, 2003 #446}. Because ARH-NPY neurons are the sole source of AgRP, which has become a useful marker of ARH-NPY neurons. There are significant differences between rodent and the nonhuman primate. In the rat, differentiation of the neuronal systems that regulate appetite and energy expenditure begins during the last week of gestation, with development continuing until weaning (Grove *et al.*, 2005). Therefore, projections of ARHNPY and POMC expressing neurons throughout the hypothalamus that do not fully mature until the second and third postnatal weeks {Grove, 2003 #446}{Grove, 2003 #447}. Initial studies demonstrated that NPY was not only abundantly expressed in the ARH, but transient expression of NPY was also observed in the other hypothalamic regions, including the dosalmedial hypothalamic nucleus, PVN, lateral hypothalamus, and the periformical region, which is not evident in adulthood (Singer *et al.*, 2000). NPY is detectable in rat diencephalic neurons from day 15 of intrauterine life (Allen *et al.*, 1984). NPY levels in all areas were low at postnatal day 2, increased rapidly to peak at P15-16 and returned to levels observed in adulthood in the ARC, while in the other areas NPY was no longer apparent after postnatal day 30 {Grove, 2003 #446}{Grove, 2003 #447}{Singer, 2000 #2370}. This process is more completed by birth in humans and primates. NPY mRNA was expressed in the ARH, PVH, and dorsomedial nucleus of the hypothalamus as early as Gestational day 100 (G100) {Grayson, 2006 #311}. ARH NPY projections to the PVH does not initiate until G100, but were limited and variable. However, by G130 there was a modest increase in density and number of NPY-expressing neurons {Grayson, 2006 #311}. By G170, ARH NPY/AgRP fiber projections to efferent target sites were completely developed, but the density continued to increase in the postnatal period, a feature similar as rodent {Grayson, 2006 #311}{Grove, 2003 #447}. Different from NPY/AgRP projections, α-MSH fibers were minimal at G100 and G130 but were moderate at G170. A substantial proportion of the catecholamine fibers did not co-express NPY {Grayson, 2006 #311}.

Impact of maternal nutrition on neural development

The brain is clearly affected by the nutrition state during gestation, which may have a role in producing the long-term behavioral and physiological changes observed in offspring after weaning, including an increase in food intake, preference for fat, hyperlipidemia, and higher body weight. The use of animal models provided fundamental understanding of the developmental aspect of these neural circuits, while modelling maternal obesity in rodents and other species helped to reveal the programming mechanisms during *in utero* and continuing development during the suckling period. The ARH of the hypothalamus is a key component of hypothalamic pathways regulating energy balance, and projection from the ARH to the PVN of the hypothalamus is a well established neural control of energy balance (Elmquist *et al.*, 2005). Increasing evidence suggest that the development of these projections play an essential role in developmental programming associated with maternal nutritional alteration

Animal studies showed that exposure of pregnant dams to a high-fat diet results in changes in gene expression of hypothalamic neuropeptides regulating energy balance in the offspring although the birth weight was not different in offspring between obese and lean mothers. Study using genetically obese prone rats suggested that the hypothalamic neurons involved in energy homeostasis are permanently disrupted by maternal obesity (Bouret *et al.*, 2008). These findings suggest that modifications to the hypothalamic homeostasis circuitry may be a fundamental mechanism underlying the increased risk of obesity by overnutrition during critical windows of early development. In this process, abnormal response to leptin is a critical cause of abnormal development of ARH projections and expression of appetite regulators (Bouret *et al.*, 2004).

i. Impact of maternal obesity on intra uterine neural development

Maternal HFD-feeding from gestational day 8 increased hypothalamic proliferation of different orexigenic peptide-expressing neurons (Chang *et al.*, 2008). The programming of fetal neural development due to maternal overnutrition looks to be initiated in mid-embryo age in the rats, as more active neurogenesis is evident from embryo day 11 to 15 in fetus from high-fat fed dams compared with those from dams fed a balanced diet (Chang *et al.*, 2008). The high-fat diet fed the dams stimulated the proliferation of neuroepithelial and neuronal precursor cells of the embryonic hypothalamic third ventricle. It also stimulated the proliferation and differentiation of neurons and their migration toward hypothalamic areas where ultimately a greater proportion of the new neurons expressed the orexigenic peptides {Chang, 2008 #358}. However, study by Bouret and colleagues suggests that genotype of dietary induced obesity may specifically affect axonal extension of ARH neurons, as opposed to other neurodevelopmental processes specifying neuronal cell number such as neurogenesis, apoptosis, or neuronal migration (Bouret *et al.*, 2008). This increase in neurogenesis and neurite growth, closely associated with a marked increase in lipids in the blood, and was believed to be the first step to make the individual more susceptible to develop obesity and its related metabolic disorders {Bouret, 2008 #300;Chang, 2008 #358}.

As a result, the offspring of rat dams fed a high-fat diet versus balanced diet, from embryonic day 6 to postnatal day 15, showed increased expression of orexigenic peptides, galanin,

enkephalin, and dynorphin, in the paraventricular nucleus and orexin and melanin-concentrating hormone in the perifornical lateral hypothalamus. However, the ARC expression of these genes, including AgRP, showed opposite changes, which were all downregulated in offspring from high-fat diet fed dams (Chang *et al.*, 2008).

In term fetus of high-fat diet fed rat mothers, plasma leptin levels much higher than that in those of lean dams, due to maternal hormones crossing the placenta (Gupta *et al.*, 2009). However, at postnatal day 1, without the support of fetal-placental circulation, blood leptin level dropped dramatically in offspring from obese mother, which ended up to be at a much lower level than that in those from lean dams (Morris *et al.*, 2009). Interestingly, the hypothalamic mRNA expression of most of the appetite regulators, NPY, AgRP, POMC, MC4R, and Ob-Rb were all increased in the fetus of obese dams whereas STAT3 protein level was reduced (Gupta *et al.*, 2009). However, at postnatal day 1, hypothalamic NPY, MC4R, POMC, mTOR, were lower in offspring from obese rat mothers (Morris *et al.*, 2009). Furthermore, Ob-Rb, STAT3, and SOCS3 mRNA expression in the hypothalamus was also downregulated by maternal obesity (Morris *et al.*, 2009). The difference in mRNA expression between term fetus and newborn could be directly due to the circulating leptin level difference. In another words, during early development, either intra uterine or at early postnatal age, leptin directly supports the production of hypothalamic appetite regulators.

ii. Impact of maternal obesity on early postnatal development

At postnatal day 9, leptin levels were observed to be 6 times higher in offspring from obese rat dams with greater fat mass compared with those from lean mothers (Chen & Morris, unpublished data). Maternal milk offers an important source of leptin and milk leptin level was shown to be higher in high-fat diet fed rat dams (White *et al.*, 2009). However, the higher leptin levels in offspring from obese mothers were correlated with their fat mass, suggesting that plasma leptin levels may be independent of milk leptin levels. Nevertheless, milk leptin has been suggested to protect the infant against several chronic diseases later on in life and particularly against obesity and related medical complications (Palou *et al.*, 2009; Pico *et al.*, 2007). Oral leptin supplement in rats during lactation was shown to improve leptin and insulin sensitivity at adulthood by increasing hypothalamic leptin receptor Ob-Rb and reducing the inhibitory signal SOCS-3 (Mistry *et al.*, 1999; Palou *et al.*, 2009; Pico *et al.*, 2007; Sanchez *et al.*, 2008; Vickers *et al.*, 2005). Although leptin injection during prenatal period has been shown to reverse the risk of obesity and its related metabolic disorders in rats subjected to intrauterine undernutrition (Vickers *et al.*, 2008), such approach has not been carried out in animals undergoing intrauterine overnutrition.

Nevertheless, leptin resistance may already exist in neonatal offspring from obese mothers reflecting by a 24% reduction of phosphor-STAT3 immunoreactive cells numbers upon leptin injection at P10 (Bouret *et al.*, 2008), suggesting leptin signalling in Arc neuron is impaired during poatnatal development in offspring of DIO rats. The trophic action of leptin on Arc

neurons is also reduced in DIO neonates, reflected by reduced neurite growth after 36h in vitro incubation with leptin. Interestingly,

By P12, projection pathways from the ARC to the PVN appear fully developed in the offspring of lean dams. In rats from obese dams, labelled fibres extended rostrally through the paraventricular pathway to provide dense innervations to the parvicellular parts of the PVN by P12. There were two to four times fewer labelled fibres compared with lean offspring at P12. The average fibre densities in the PVN remained significantly lower in rats at P16 from obese dams induced by high-fat diet compared to DR animals at the same age. This directly affected the AgRP/NPY neuron density and innervations in adulthood. Because neurons that express AgRP are restricted to NPY-containing neurons of the Arc in adult animals, AgRP-immunoreactive fibres reflect the projections of NPY neurons from Arc to PVN (Bouret *et al.*, 2008). However, projections of α-MSH expressing neuron were not affected.

Juvenile offspring from obese dams had normal or increased Arc Ob-Rb expression (Chen *et al.*, 2008; Gorski *et al.*, 2007), hypothalamic STAT3mRNA expression was also observed to be upregulated by maternal obesity (Chen *et al.*, 2008). However, both increased leptin sensitivity (Gorski *et al.*, 2007) and leptin resistance have been reported in young offspring from obese mothers (Ferezou-Viala *et al.*, 2007), reflecting by the alteration of hypothalamic phosphorylated-STAT3 in response to leptin injection. Also, offspring of obese DIO dams had elevated VMN MC4R, NPY Y1 receptor and DMN NPY Y5 receptor mRNA expression (Gorski *et al.*, 2007).

At postnatal day 20 (normal weaning age) under free feeding state, offspring from obese mothers had reduced NPY and increased POMC mRNA expression in the hypothalamus, in the face of reciprocally altered hypothalamic NPY Y1 receptor and MC4R mRNA expression (Chen *et al.*, 2008). However, hunger is major drive for ingestive behaviour. In rat offspring from obese mothers, reduced hypothalamic NPY and AgRP mRNA expression was upregulated to a similar level as those in rats from lean mothers by overnight (14 hours) fasting (Chen *et al.*, 2009a). The already higher NPY Y1 receptor at free feeding state was further increased after overnight fasting (Chen *et al.*, 2009a). This may also be the fundamental mechanism for an exaggerated feeding response to NPY injection into the lateral brain ventricle in offspring from high-fat diet-fed dams (Kozak *et al.*, 2000). Weakened inhibitory effect of hypothalamic mTOR may contribute the hyperactivity of hypothalamic NPY system (Chen *et al.*, 2008). Although POMC mRNA expression was not altered by fasting, the downregulation of MC4R and its downstream Sim1 was absent in rats from obese mothers. This could directly lead to increased milk consumption during the sucking period, as well as a higher daily energy intake immediately after weaning (Chen *et al.*, 2009a). Furthermore, this could also be the contributing factor of an exacerbated preference for 'junk food' in offspring by maternal 'junk food' diet in pregnancy and lactation promoted (Bayol *et al.*, 2007).

Maternal obesity has been shown to cause reduction in hypothalamic mTOR and GLUT4 expression in offspring at weaning (day 20). As a result, after overnight fasting hypothalamic

NPY signaling in pups from obese dams was more active than that of those from lean dams (Chen *et al.*, 2009a). This was accompanied by increased milk intake during the suckling period and energy intake immediately after weaning (Chen *et al.*, 2009a), which could directly contribute to greater body weight, circulating triglycerides, glucose, and insulin levels in these animals both at weaning (Chen *et al.*, 2008) and later on in adulthood (Chen *et al.*, 2009b). Therefore, we hypothesize that activation of hypothalamic mTOR with leucine could reduce the adiposity and glucose tolerance in offspring from obese mother, especially when they were fed a HFD.

iii. Early postnatal overnutrition

Early postnatal period is also a "critical period" of development. A number of studies have demonstrated that environmental influences during the early postnatal period in humans (Ravelli *et al.*, 1976; Roseboom *et al.*, 2000) and rodents (Chen *et al.*, 2008; Lucas, 1998; Velkoska *et al.*, 2005) can also influence body weight and energy homeostasis in adulthood.

Rodents overfed during the postnatal period induced by reducing litter size, show increased early weight gain and fat deposition, hyperleptinaemia, hyperinsulinaemia (Plagemann *et al.*, 1999b), and central leptin resistance at hypothalamic ARH (Schmidt *et al.*, 2001). Although NPY concentration was only slightly increased in the PVN in over-fed rats during the suckling period, NPY neuron density was significantly increased in the ARC (Plagemann *et al.*, 1999b). This may suggests a lack of inhibition on NPY and an acquired resistance of the hypothalamic NPY system to increased levels of leptin in early postnatally overfed rats. Hypothalamic galanin (GAL) is a stimulator of food intake and body weight gain. On postnatal day 21, in over-fed rats, the number of GAL-neurons was increased in the ARC, positively correlated to their body weight, which persisted even until adulthood (Plagemann *et al.*, 1999a). Furthermore, male rats overfed during the suckling period exhibited a decrease in hypothalamic mRNA levels of OB-Rb in the face of hyperleptinaemia, contributing to their leptin resistance (Lopez *et al.*, 2005). Moreover, this obese model showed an increase in the mRNA expression of CART, NPY and AgRP in the ARC (Lopez *et al.*, 2005). These changes lead to the lifelong persistence of overweight and hyperphagia in those rats over-fed during the suckling period (Bassett *et al.*, 1988; Plagemann *et al.*, 1992; Voits *et al.*, 1996), which may suggest a permanent disturbance of weight regulation.

The impact of maternal obesity appears to be amplified by early postnatal overfeeding, as pups from obese dams showed exaggerated adiposity and glucose intolerance at weaning if they were raised in small litters (Chen *et al.*, 2008; Lucas, 1998). This was closely linked to an alteration in hypothalamic NPY and POMC expression, as well as their functional receptors (Chen *et al.*, 2008).

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