Long-term consequences of early exposure to high-fat on mesolimbic dopamine, hypothalamic-pituitary-adrenal activity and behavior.

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Abstract

Large-scale Canadian epidemiological studies have demonstrated that maternal obesity and excessive weight gain during pregnancy independently increase the child's risk of obesity, although the mechanisms involved in this 'metabolic imprinting' are still unclear. While programming by perinatal overnutrition has been shown to occur in hypothalamic circuits involved in the homeostatic control of energy balance, we have used an animal model of early overnutrition to examine the long-term consequences of early exposure to high-fat on mesolimbic dopamine (DA) function, hypothalamic-pituitary-adrenal (HPA) activity and DA-dependent feeding behavior. In our model, dams are placed on a 30% fat (high-fat) or 5% fat (control) diet from gestation day 13 to postnatal (PND) 22, when offspring are weaned from their mothers and maintained on the 5% control diet until testing in adulthood. The experiments presented in this thesis demonstrate that exposure to high-fat during the perinatal period alters the presynaptic regulation of mesolimbic DA and consequently, modifies the magnitude and pattern of the nucleus accumbens (NAc) DA response to amphetamine administration, the anticipation of high-fat food rewards and in response to stress. Early high-fat exposure also impairs adaptations in NAc DA and adrenocorticotropic hormone (ACTH) responses usually observed with repeated stress. One of the important behavioral consequences of early exposure to high-fat is increased operant responding for fat-enriched, but not sugar-enriched food rewards. We also show that the onset of functional ventral tegmental area (VTA) responsiveness to the anorectic hormone leptin occurs during the time of exposure to the high-fat diet and suggests that one of the possible mechanisms triggering long-term change in DA function with perinatal high-fat involves leptin-induced changes in VTA DA neuronal activity. These findings suggest that the life-long changes in eating patterns observed in offspring exposed to high-fat during early development are mediated, in part, by modifications in mesolimbic DAergic circuits.

Résumé

Des études épidémiologiques canadiennes démontrent que l'obésité maternelle et le gain de poids excessif durant la grossesse qui entraînent un poids élevé à la naissance, augmente le risque d'obésité de l'enfant. Ces études suggèrent que l'environnement nutritionnel et hormonal durant le développement programme les circuits neuronaux impliqués dans l'équilibre énergétique. Bien que la programmation ait été observée dans les circuits hypothalamiques impliqués dans le contrôle homéostatique de l'équilibre énergétique, nous avons utilisé un modèle animal de surnutrition périnatale pour examiner les conséquences à long-terme d'une exposition précoce à une alimentation à haute teneur en gras, sur la fonction dopaminergique mésolimbique (DA), l'activité hypothalamo-hypophyso-surrénalienne (HHS) et le comportement alimentaire. Dans notre modèle, les mères sont soumises à un régime contrôle (5% de graisses) ou un régime alimentaire à haute teneure en graisses (30%), débutant à la 13^{e} journée de gestation et se terminant à la journée postnatale 22. Les ratons sont ensuite sevrés et maintenus sur le régime contrôle de 5% jusqu'à l'âge adulte. Les expériences présentées dans cette thèse montrent que l'exposition à un régime à haute teneur en graisses pendant la période périnatale modifie la régulation présynaptique de DA mésolimbique et, par conséquent, modifie l'ampleur et la structure de la réponse dopaminergique du noyau accumbens (NAc) lors de l'administration d'amphétamine, de l'anticipation de récompenses alimentaires et de la réponse au stress. L'exposition précoce à une alimentation à haute teneur en graisses élimine également les adaptations des réponses du NAc DA et de l'hormone adrénocorticotropique (ACTH) qui sont généralement observées lors d'un stress répété. L'une des conséquences comportementales de l'exposition à ce régime alimentaire est une réponse opérante accrue à des récompenses alimentaires riches en gras. Nous montrons également que l'apparition de la réponse fonctionnelle de la région tegmentale ventrale (VTA) à l'hormone leptine anorexigène se produit pendant la période d'exposition à la diète riche en graisses

et suggère que l'un des mécanismes probables du déclenchement de changements à long terme de la fonction DA pour les régimes périnataux à haute teneur en graisses implique des changements induits par la leptine dans l'activité neuronale du VTA DA. Ces résultats suggèrent que les changements en cours de vie des les habitudes alimentaires observées chez la progéniture exposée à un régime à haute teneur en graisses pendant le développement précoce sont attribuables, en partie, à des modifications dans les circuits DAergiques mésolimbiques.

Contributions to original knowledge

Chapter II: In the published manuscript (Naef et al., 2010) presented in this chapter we show, for the first time, that exposure to high-fat during early development significantly alters the nucleus accumbens dopamine response to the psychostimulant drug amphetamine. We are also the first group to report augmented operant responding for high-fat pellets with early exposure to high-fat.

Chapter III: In the published manuscript (Naef et al., 2012) presented in this chapter, we are the first to demonstrate that early exposure to high-fat significantly reduces anticipatory NAc DA responses in a Pavlovian conditioning paradigm.

Chapter IV: The manuscript presented in this chapter has recently been accepted for publication. This is the first manuscript to examine how early high-fat exposure can modify nucleus accumbens DA and hypothalamic-pituitary-adrenal axis to repeated tail-pinch stress.

Chapter V: The manuscript presented in this chapter is not published. However, we anticipate that it will be published soon. This manuscript will be the first to examine leptin signalling in ventral tegmental area dopamine neurons during development.

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Introduction

Obesity presents a significant health challenge to Canadians. The latest report by the Public Health Agency of Canada (2007-2009) indicates that 24% of Canadian adults are obese (body mass index (BMI): ≥ 30) and 37% are overweight (BMI: 25-29.9). Even more alarming is the high prevalence of overweight and obese Canadian children, with 10% of 6- to 17-year olds estimated to be obese and 18% estimated to be overweight. According to the Canadian Obesity Network, obesity is a risk factor for several chronic diseases including type II diabetes, high blood pressure, heart disease, stroke, arthritis and cancer. Large-scale Canadian epidemiological studies demonstrate that being overweight or obese prior to pregnancy and excessive weight gain during pregnancy independently increases the odds of having a large for gestational age baby (Ferraro et al., 2012), which in turn, increases the odds of being overweight at 4.5 years (Dubois & Girard, 2006). These epidemiological studies provide strong evidence that the metabolic and nutritional environment of developing young plays a crucial role in the establishment of energy homeostasis, thus modulating susceptibility to obesity and obesity-related disorders.

Energy homeostasis is controlled by a complex network of neural circuits which receives inputs from the periphery to modulate feeding. These inputs include vagal and spinal afferents, hormones such as leptin, ghrelin, and insulin, glucose and fatty acids, as well as external stimuli such as the sight, smell, and taste of food (reviewed in Berthoud et al., 2012a). These various inputs target hypothalamic and brainstem areas typically referred to as 'homeostatic' systems as well as 'cognitive/emotional' systems. Of particular importance to my work is the mesolimbic dopamine (DA) system, which mediates the hedonic aspect of feeding. Indeed, what, when, and how much we eat is not simply determined by metabolic need. Food and food cues elicit strong hedonic responses and cravings, which often override homeostatic satiety signals leading to overeating and weight gain. The cell bodies of the mesolimbic DA system originate in the ventral

tegmental area (VTA) and innervate limbic regions including the nucleus accumbens (NAc) and amygdala. The hypothalamic-pituitary-adrenal (HPA) axis, which forms the basis of the neuroendocrine response to stress as well as brain circuits involved in the regulation of stress responsiveness, including the mesocorticolimbic DA system and are also a part of the intricate pathways involved in the modulation of energy homeostasis.

Both animal and human studies suggest that overnutrition during critical periods of development alters life-long feeding patterns, thus increasing susceptibility to obesity. In our laboratory, early overnutrition is achieved by increasing the fat content of the maternal diet of the dam during the last week of gestation and throughout lactation. Feeding the dam a diet rich in saturated fats during this period alters the metabolic, nutritional, and hormonal environment of developing young during the pre-weaning period. High-fat exposed offspring are hyperphagic throughout life, suggesting that early high-fat exposure is altering the development of neural circuits involved in the control of energy homeostasis. While most studies aimed at examining the long-term consequences of perinatal overnutrition on the neural circuits subserving energy balance have focused on the hypothalamus, the experiments presented in this PhD dissertation focus on the programming effects of a perinatal high-fat diet on mesolimbic DA function, HPA axis responsiveness to stress and DA-dependent feeding behavior. The final manuscript presented in this dissertation demonstrates that the onset of leptin responsiveness of VTA DA neurons occurs during the neonatal period, suggesting that one of the possible mechanisms triggering long-term change in DA function with perinatal high-fat involves leptin-induced changes in VTA DA neuronal activity.

The following introduction will highlight pertinent information required for a thorough understanding of the data presented in this thesis. In the first section, I will describe the hypothalamic control of energy balance and the modulation of this circuitry by leptin in the adult and neonate. The second section will examine mesolimbic DA function, the modulation of mesolimbic DA by

leptin, and changes in DA function observed in diet-induced obesity. The third section of the introduction will focus of the HPA stress response, the neural circuits involved in mediating stress responsiveness, and adaptations that occur with repeated stimulation. The fourth section will examine epidemiological studies linking early overnutrition to increased susceptibility to obesity, highlight animal models used to examine the programming of obesity by early dietary manipulations and the ability of these diets to permanently modify neural circuitry. Finally, the experimental model of metabolic programming used in our laboratory will be described and the hypotheses and aims of this PhD dissertation will be stated.

Chapter I: Comprehensive review of the literature

I.1. The neural control of feeding: focus on the hypothalamus and leptin

1.1. Historical perspective: the hypothalamus as the main homeostatic feeding center of the brain

As early as the 1940s, the hypothalamus was identified as the primary feeding center of the brain. In their classic experiments, Hetherington and Ranson (1940) showed that large bilateral electrolytic lesions of the medial hypothalamus, especially the VMH lead to a doubling of body weight and a large increase in adiposity. Conversely, lesions of LH lead to a drastic decrease in feeding and even, death by starvation (Hetherington & Ranson, 1940; Anand & Brobeck, 1951). The results of these lesion studies served as the basis for the Dual Center Hypothesis of feeding, with the LH as a feeding center and the VMH as a satiety center. Thus, the hypothalamus was considered the prime candidate in appetite control. How the hypothalamus receives information regarding satiety and hunger was still unknown.

1.2. The discovery of leptin

The discovery of the anorexigenic hormone leptin (Zhang et al., 1994), the protein product of the *ob* gene represents the most important step in our understanding of how the brain integrates information from the periphery to influence energy homeostasis. In the *ob/ob* mouse, whose mutation of the *ob* gene results in profound obesity and type II diabetes (Zhang et al., 1994), the exogenous administration of leptin rescues the obese phenotype by reducing food intake and increasing energy expenditure (Campfield et al., 1995; Hallas et al., 1995, Pelleymounter et al., 1995). Leptin is primarily synthesized in adipose tissue (Frederich et al., 1995a; Maffei et al., 1995) and circulates in relation to body fat stores in both rodents (Frederich et al., 1995b; Maffei et al., 1995) and humans (Maffei et al, 1995). The brain was quickly identified as an important target of leptin as intracerebroventricular injections of the hormone led to significant reductions in body weight (Campfield et al., 1995; Halaas et al., 1997). The weight loss promoting effects of leptin were quickly identified as a potential

cure for obesity (reviewed in Myers, 2004). However, as described above, leptin concentrations positively correlate with fat mass and are thus high in diet-induced obesity, suggesting a state of leptin insensitivity in obesity. Indeed, obesity is characterized by a state of central leptin resistance (reviewed in Zhou & Rui, 2013).

The receptor for leptin, the Ob-R, was identified one year following its cloning (Tartaglia et al., 1995) and mRNA for the Ob-R was found in the periphery, the choroid plexus and the hypothalamus. Subsequent experiments demonstrated that the Ob-Rb, the long-form of the Ob-R considered the functional receptor, was expressed in several hypothalamic nuclei including the ARC, the VMH, DMH, and LH (Elmquist et al, 1998; Fei et al., 1997; Mercer et al., 1996).

1.3. The hypothalamus – the primary target of leptin

Amid the identification of ARC neuropeptides involved in the regulation of feeding including Neuropeptide Y (NPY), Agouti Related Peptide (AgRP), Proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART), a comprehensive model describing the role of leptin in the hypothalamic control of food intake was provided by Schwartz and colleagues in a Nature review article in 2000. In this model, leptin acts on first order neurons in the ARC, the hub of the hypothalamus, which contains two sets of opposing neurons: 1) the NPY/AgRP neurons which stimulate feeding (orexigenic) and 2) the POMC/CART neurons which inhibit feeding (anorexigenic). ARC neurons project to second order neurons in the PVN, LH, VMH, and DMH. When leptin levels are high, the orexigenic NPY/AgRP neurons are inhibited and the anorexigenic POMC/CART neurons are activated leading to the cessation of feeding. Conversely,when leptin is low, as during starvation, appetite is stimulated via the suppression of anorectic neuropeptides and by increased expression of orexigenic peptides. According to this model, melanocortins, the cleaved product of POMC are critical to the control of food intake by leptin. Activation of POMC neurons by leptin leads to an increase in α-melanocyte-stimulating hormone (α-MSH), which signals through melanocortin 4 receptors (MC4R) to reduce

feeding. Conversely, AgRP is a MC4R antagonist. Thus, when the NPY/AgRP neurons are activated, AgRP inhibits the melanocortin pathway, leading to food intake.

1.4. Leptin receptors and intracellular signaling

The Ob-R is produced in several alternatively spliced forms, including Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf (reviewed in Frühbeck, 2006). All forms share common extracellular and transmembrane domains but differ in their intracellular domains. Based on the length of their intracellular domain, the isoforms have been classified into three classes: short (Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf), long (Ob-Rb) and secreted (Ob-Re) (Baumann et al., 1996). The functional Ob-Rb contains a box 1 and box 2 motif and four important tyrosine residues (Tyr974, Tyr985, Tyr1077 and Tyr1138), which upon phosphorylation, activate different signalling pathways. The effects of leptin in the brain have been shown to be mediated by three signalling pathways. The Janus kinases / signal transducers and activators of transcription (JAK/STAT) signaling pathway was quickly identified as an important mediator of leptin action in the brain via the Ob-Rb (Baumann et al., 1996). The binding of leptin to the Ob-Rb leads to the recruitment, auto-phosphorylation and conformational change of JAK2 (reviewed in Frühbeck, 2006). Activation of JAK 2 also phosphorylates other JAKs and tyrosine residues on the receptor. Phosphorylated Tyr1138 provides a binding site for STAT3 proteins (and other STAT proteins) which are phosphorylated by JAK2, dissociated from the receptor and dimerized, and translocated to the nucleus where they stimulate transcription (reviewed in Frühbeck, 2006). Most regions expressing leptin receptor mRNA also show pSTAT3 activation with leptin administration (Caron et al, 2010), suggesting that this signaling pathway is important in mediating leptin function. Indeed, disruption of pSTAT3 leptin signaling induces hyperphagia and obesity similar to that observed in the *ob/ob* mouse (Bates et al., 2003), showing that leptin signaling via pSTAT3 is required for leptin regulation of energy balance. However, disruption of pSTAT3 leptin signaling did not disrupt reproductive function (Bates et al., 2003), suggesting that

leptin modulation of reproduction is mediating by an alternate signaling pathway. Feedback inhibition of pSTAT3 occurs via suppressor of cytokine signaling 3 (SOCS3, reviewed in Myers, 2004).

Binding of leptin to Ob-R also leads to the activation of the mitogen-activated protein kinase (MAPK) signaling pathway and the PI3K / PDE3B / cAMP (phosphoinositide 3-kinase / phosphodiesterase 3B / cyclic adenosine monophosphate) pathway (reviewed in Fruhbeck, 2006). Contrary to pSTAT3, pERK1/2 a member of MAPK, was not found to be activated in all hypothalamic nuclei with leptin, but rather, restricted to the ARC (Rahmouni et al., 2009). Blockade of hypothalamic ERK1/2 abolishes the anorectic effects of leptin (Rahmouni et al., 2009), suggesting that signaling through pERK1/2 plays a critical role in the control of food intake. The anorectic effects of leptin have also been shown to be mediated by PI3K as blockade of PI3K abolishes hyperpolarization and inhibition of hypothalamic neurons by leptin (Spanswick et al., 1997; Harvey et al., 2000) and blockade of PI3K in the hypothalamus blocks the anorectic effect of leptin (Niswender et al., 2001).

1.5 Hypothalamic development in the rodent

The formation of hypothalamic projections involves three main developmental processes: neurogenesis (the birth of new neurons), the migration of neurons and the establishment of functional circuits (Ishii & Bouret, 2012). Hypothalamic cells are primarily derived from precursors that originate in the proliferative zone surrounding the lower portion of the third ventricle (Altman & Bayer, 1986). In the mouse, the vast majority of ARC, VMH, DMH, LH, and PVN neurons are generated between embryonic days 12 and 16, with a sharp peak of neurogenesis observed on embryonic day 12 (Ishii & Bouret, 2013). The functional establishment of hypothalamic projections appears to be mostly a postnatal event. Axonal tract tracing studies demonstrate that ARC projections innervate hypothalamic nuclei at different postnatal periods, with innervation of the DMH occuring on postnatal day (PND) 6 followed by innervation of the PVN on PND8–10, and the LH on PND12 (Bouret et al., 2004a). NPY/AgRP fibers

from the ARC do not innervate the DMH until PND5-6 and the PVN until PND10-11 (Grove et al., 2003). Together, these studies suggest that the functional integration of hypothalamic circuits involved in the homeostatic control of feeding occurs during the postnatal period in the rodent.

1.6 Leptin in development

In the mouse, leptin concentrations sharply increase during the second postnatal week (Ahima et al., 1998; Devaskar et al., 1997; Rayner et al., 1997), reach their peak on PND10 and return to normal adult levels by PND16 (Ahima et al., 1998). Peak levels on PND10 represent a 10-fold increase from that observed in adults. In the rat, the leptin surge is not as pronounced as in mice (Cottrell et al, 2009; Proulx et al., 2001), although leptin concentrations are 3-5 fold higher than in the adult (Cottrell et al, 2009). Leptin concentrations rise significantly during the second week, peak at PND 7, but remain relatively high throughout the remainder of the pre-weaning period (Cottrell et al., 2009). During this peak period, leptin concentrations do not reflect the amount of fat stores (Ahima et al., 1998; Rayner et al, 1997) and are typically attributed to a rise in *ob* gene expression in the white (Devaskar et al. 1997; Rayner et al., 1997) and brown adipose tissue of the pups (Devaskar et al., 1997) and to transfer from the maternal milk (Casabiell et al., 1997), which is absorbed by the immature stomach (Sánchez et al., 2005).

The temporal pattern of the leptin secretion during the neonatal period, as well as the observation of abnormalities in the hypothalamic circuits of the leptin-deficient *ob/ob* mouse (Bereiter & Jeanrenaud, 1979;) led Ahima and colleagues (1998) to speculate that leptin is involved in the development of the neuroendocrine system. Indeed, in a paper published in Science in 2004, Bouret and colleagues demonstrate that leptin is involved in the establishment of hypothalamic projections in the mouse. While the density of projections from the ARC to the PVN is significantly reduced in the leptin-deficient *ob/ob* mouse, leptin treatment during the neonatal period, but not in adulthood, restores these projections (Bouret et al., 2004b). In this same publication, it is demonstrated that

leptin stimulates neurite outgrowth from organotypic cultures of ARC neurons (Bouret et al, 2004b). More recently, the ability of leptin to stimulate ARC neurite outgrowth was shown to require the Ob-Rb and intact pSTAT3 and pERK signaling (Bouret et al., 2012).

Several lines of evidence suggest that leptin signalling is different during neonatal development in the rodent when circulating leptin is high. Most important to this thesis is the observation that neonatal pups are insensitive to the anorectic effects of leptin (Mistry et al., 1999; Proulx et al., 2002). The mechanisms involved in this insensitivity are still unclear. Leptin transport across the bloob-brain barrier (BBB) is reduced during the neonatal period (Pan et al., 2008) and there is a developmental change in the expression of leptin receptor expression during the pre-weaning period in the rat. In the ARC and VMH, Ob-Rb expression is low during the first two postnatal weeks, increases on PND14 and reaches peak levels on PND15 (Cottrell et al., 2009), suggesting that the ability of leptin to activate hypothalamic circuits might be 'impaired' during the time of hypothalamic development. Interestingly, the ability of leptin to induce the immediate early gene cFOS in the hypothalamic target regions of ARC projections (DMH, PVN and LH) is contingent on the innervation of the nucleus by the ARC (Bouret et al., 2004b). Thus, peak activation of c-FOS is observed when fiber density in the nuclei has also reached peak levels (Bouret et al., 2004b). In addition, there is an overall reduction in pSTAT3-activated neurons during the neonatal period (Caron et al., 2010). These studies suggest that the insensitivity to leptin during early development is mediated, in part, by altered signalling in the hypothalamus.

1.7 Summary

This section focused on the homeostatic control of feeding and body weight regulation by the hypothalamus. In the adult, hypothalamic sensing of peripheral energy reserves is achieved mostly through the adipose-derived hormone leptin, which circulates in relation to body fat stores. Leptin induces its anorectic effect by inhibiting anabolic (NPY/AgRP) and activating catabolic

(POMC/CART) neurons of the ARC via the long form of the Ob-Rb receptor, which signals through three main signaling pathways. During development, high leptin levels are maintained to promote the normal development of hypothalamic projections and developing pups are insensitive to the anorectic affects of leptin. Although this section focused on leptin, the hypothalamus responds to many peripheral signals, including hormones such as insulin, ghrelin, and cholecystokinin, vagal afferents from the gastrointestinal tract and liver (directly and through the hindbrain), as well as nutrients such as glucose and fatty acids. (reviewed in Berthoud, 2002).

I.2. Mesolimbic dopamine in feeding and obesity

The previous section described the homeostatic control of feeding by the hypothalamus. However, feeding can occur without homeostatic need. Examples of this phenomenon can easily be found in daily life when we indulge in palatable foods without feeling hungry or even, when fully satiated following a meal. For this reason, in dissecting the neural circuits subserving the regulation of feeding behavior, a distinction between 'homeostatic' systems, the 'metabolic brain' and 'non-homeostatic' systems, the 'cognitive brain' (Berthoud, 2004; Berthoud, 2007) has been made. While the homeostatic control of feeding is mostly attributed to hypothalamic and brainstem circuits, the hedonic value of food and the ability of food and food cues to induce motivational salience are mediated through mesolimbic DA circuits. DAergic function is modulated by many factors including the 'liking' of food, which is thought to be mediated by opioids in the NAc (Smith et al., 2011), endocannabinoids (Melis et al., 2012), and the hypothalamus (Leinninger et al., 2011). The current obesity epidemic is thought to reflect an overriding of homeostatic systems by the hedonic value of highly palatable, calorically-dense food characteristic of today's food environment (Berthoud, 2007). Thus, individual differences in mesolimbic DA and behavioral responses to food and food cues might mediate susceptibility to obesity. The following section will focus on mesolimbic DA circuitry and its role in mediating the hedonic value of food, psychostimulant drugs and predictive cues. Next, it will focus on the regulation of mesolimbic DA by metabolic hormones and diet-induced obesity.

2.1. Dopamine: from synthesis to feedback

2.1.1. Mesolimbic dopamine projections

Mesolimbic DA neurons originate in the ventral tegmental area (VTA) and send dense projections to the nucleus accumbens (NAc) and olfactory tubercle. The VTA also sends projections to other regions including the septum,

hippocampus, amygdala and prefrontal cortex (PFC) (reviewed in Ikemeto, 2007). The cytoarchitecture of VTA DA neurons provides a basis for its division into distinct nuclei. In the rat, four zones have been identified (Ikemeto, 2007): paranigral nucleus (PN), parabrachial pigmented area (PBP), parafasciculus retroflexus area and ventral tegmental tail (VTT), with the PN and PBP displaying the highest density of DA neurons and the PBP covering the most space. DA neurons are also located along the midline in the interfascicular, rostral linear and central linear nuclei (Ikemeto, 2007). While PN DA neurons selectively project to the ventromedial striatum (medial olfactory tubercle and medial shell of the NAc), PBP DA neurons project to the ventromedial and ventrolateral striatum (lateral olfactory tubercle, NAc core, lateral shell of the NAc) (Ikemeto, 2007).

2.1.2. Dopamine synthesis and release

DA is synthesized from the amino acid precursor tyrosine, and the first step in its synthesis is the hydroxylation of tyrosine to L-dihydroxyphenylaline (DOPA) by tyrosine hydroxylase (TH) (Nagatsu et al, 1964a; Nagatsu et al, 1964b), the rate limiting step in DA synthesis. DOPA is then transformed into DA by DOPA decarboxylase. DA release occurs via two main mechanisms, exocytosis and reverse transport. The exocytotic release of DA is dependent on calcium influx and the fusion of vesicles with the plasma membrane while reverse transport of DA occurs independently of calcium (Heikkila et al, 1975), is mediated by the DA transporter (DAT) (Heikkila et al, 1975), and occurs in response to amphetamine (AMP) administration (Heikkila et al, 1975).

2.1.3. Dopamine receptors

Five DA receptors (D_1 to D_5) have been identified, all of which are G-protein-coupled, and these receptors can been divided into two groups (Garau et al, 1978; Titeler et al, 1978), D_1-like receptors (D_1 and D_5, Seeman & Van Tol, 1994) and D2-like receptors (D_2, D_3, D_4, Seeman & Van Tol, 1994) based on their coupling with adenylyl cyclase, with D_1-like receptors positively coupled to

adenylyl cyclase (all post-synaptic) and D_2-like receptors negatively coupled to adenylyl cyclase (Stoof & Kekabian, 1981). Furthermore, two isoforms of the D_2 receptors have been identified (Dal Toso et al, 1989). While the long-form of the D_2 receptor is expressed post-synaptically (khan et al, 1998), the short-form of the receptor is a pre-synaptic autoreceptor, involved in the regulation of DA synthesis and release, which will be described more thoroughly in the following section on DA regulation. The function of DA receptors can be probed by a multitude of DA receptor agonists and antagonsists, although they are not receptor-specific. For example, the agonist quinpirole acts at the D_2, D_3, and D_4 receptor. Important to this project is that no compounds can discriminate between the long- and short-form of the D_2 receptor, although they can differ in affinity. The D_1 receptor is the most widespread DA receptor and is expressed at high levels in the striatum, the NAc and the olfactory tubercle (Missale et al., 1998). The D_5 receptor is poorly expressed relative to the D_1 receptor, with little or no mRNA detected in the striatum, NAc and olfactory tubercle (Missale et al., 1998). D_2 receptor is mainly found in the striatum, olfactory tubercle, in the core of the nucleus accumbens and in the septal pole of the NAc shell (Missale et al., 1998), but is also present in a variety of other regions, including VTA DA neurons where it functions as an autoreceptor. D_3 receptors are poorly expressed in the striatum and display a specific distribution in limbic areas (Missale et al., 1998).

2.1.4. Local regulation of extracellular dopamine

The uptake of extracellular DA by the DAT is the main process by which DA neurotransmission is inactivated. In mice with genetic deletions of DAT, the clearance of evoked DA from the extracellular space is 100 times slower than in wild-type controls (Giros et al., 1996). The regulation of the DAT occurs via trafficking of DAT from the cell surface where it regulates extracellular DA concentrations, to internalization of the DAT via clathrin-coated vesicles (Zahniser et al, 2004). DAT is an important site of action for psychostimulant drugs such as cocaine and AMP. While cocaine inhibits DAT resulting in

increased extracellular DA, AMP increases DA by inducing the reverse transport of DA through DAT and altering vesicular monoamine transporter-2 (VMAT) uptake of DA (Zahniser et al, 2004). DA neurotransmission is also terminated by the activation of DA D_2 autoreceptors. While somatodendritic D_2 autoreceptors modulate firing rate (Paladini et al., 2003; Ford et al., 2010), D_2 autoreceptors located on nerve terminals in the dorsal striatum and nucleus accumbens regulate the synthesis and release of DA (Cubeddu & Hoffmann, 1982; Wolf & Roth, 1990). Mice lacking D_2 autoreceptors (autoDrd2KO) display elevated synthesis and release of DA, hyperlocomotion under baseline and stimulated (cocaine) conditions and increased operant responses for food rewards (Bello et al., 2011). DA is also regulated by VMAT, a protein integrated in the membrane of intracellular vesicles, which acts to load DA (and other monoamines) into vesicles (Wimalasena, 2011) and monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT), which metabolize DA.

2.2. Dopamine in reward

DA's role in reward was first identified by Roy Wise in a seminal series of articles published in the late 1970s demonstrating that DA receptor blockade results in an attenuation of food reward (Wise et al, 1978), cocaine reinforcement (De Wit et al, 1977) and lateral hypothalamic brain stimulation (Fouriezos, 1978). These findings led Wise to elaborate the DA hypothesis of reward which postulates that the rewarding properties of natural rewards and drugs of abuse are mediated by the same brain circuit, the mesolimbic DA system, with cell bodies arising in the VTA and projecting to the NAc. An intense investigation into DA's role in food reward in the late 80s and early 90s led to the conclusion that although feeding increases NAc DA levels (Hernandez & Hoebel, 1988; Radhakishun et al., 1988; Yoshida et al., 1992), increased NAc DA transmission does not occur as a direct consequence of food consumption (Chance et al., 1987; Blackburn et al., 1989; Weatherford et al., 1991; McCullough & Salamone, 1992; McCullough et al., 1993; Salamone et al., 1994). Rather, NAc DA increases primarily in anticipation of receiving a food reward and terminates as animals

consume the food (Day et al., 2007; Richardson & Gratton, 1996; Richardson & Gratton, 2008). This pattern of NAc DA transmission suggests that, with training (Pavlovian conditioning, for example), NAc DA is activated by the conditioned incentive cues of food and that this activation ceases with presentation and consumption of food rewards.

Thus, the evidence suggests that NAc DA transmission is primarily an anticipatory signal (rather than a consumatory signal) and therefore relies on learning the association between a cue associated with a food reward (conditioned cue) and the arrival of the reward. What is still debated is the psychological, and behavioral significance of the anticipatory DA signal and this debate has given rise to three main hypotheses advocated primarily by the groups of Wolfram Schultz, John Salamone and Berridge & Robinson. Experiments by Schultz and colleagues (Hollerman & Schultz, 1998; Schultz, 2002), which measure the electrophysiological activity of midbrain VTA DA neurons during learning tasks has led them to hypothesize that the timing and magnitude of phasic DA neuron activation to conditioned stimuli reflects the discrepancy between the expectation of reward and the actual reward. This hypothesis is known as the prediction-error hypothesis. The group led by John Salamone (reviewed in Salamone & Correa, 2012) examine the role of DA in instrumental responding (i.e. food seeking behavior). They propose that DA mediates the ability of a predictive stimulus to elicit approach to the stimulus and the amount of work required in obtaining the reward. Finally, the 'liking' vs. 'wanting' hypothesis of Berridge & Robinson (reviewed in Berridge et al., 2009) proposes that while the hedonic impact or subjective pleasure ('liking') of rewards is mediated by opioids in the NAc shell and ventral pallidum, DA mediates 'wanting' of rewards, a type of incentive motivation which promotes the approach and consumption of rewards. More recently, they have demonstrated that cue-induced DA mediates the incentive salience attributed to the conditioned cues themselves and not the predictive value of the conditioned cues (Flagel et al., 2011).

2.3. The regulation of mesolimbic DA by metabolic signals

Several lines of evidence indicate that weight loss amplifies DA-dependent behavior. In rats, food restriction increases lever pressing for lateral hypothalamic stimulation, also known as brain stimulation reward (BSR, Carr, 2007, Fulton et al., 2000), alters the ability of amphetamine to modulate BSR (Carr, 2007) and enhances the locomotor-activating and rewarding properties of psychostimulant drugs, opioids, and DA agonists (reviewed in Carr, 2007). Electrical stimulation enhanced by weight loss is reversed by the administration of leptin (Fulton et al., 2000), suggesting that leptin itself can modulate DA function and behavior. Leptin has been shown to reduce basal and feeding-evoked DA release in the NAc (Krugel et al, 2003), reverse the effect of food deprivation-induced relapse to heroin (Shalev et al, 2001), enhance the reward effect of AMP (Hao et al, 2006), reverse conditioned place preference for high-fat food (Figlewicz et al, 2004), and decrease sucrose self-administration (Figlewicz et al, 2006). Together, the studies suggest that leptin might directly impact mesolimbic DA circuitry.

Indeed, direct modulation of VTA DA neurons by leptin has recently been shown. The long form of the leptin receptor (Ob-Rb) has been detected on VTA DA neurons (Figlewicz et al., 2003; Fulton et al., 2006; Hommel et al, 2006) and leptin has been shown to activate pSTAT3 (Fulton et al, 2006; Hommel et al, 2006; Trinko et al., 2011) and pERK1/2 (Trinko et al., 2011) signaling in the VTA. Leptin reduces the firing rate of VTA DA neurons (Hommel et al., 2006) and this effect is abolished by MEK1/2 kinase which is needed for phosphorylation and activation of ERK1/2 (Trinko et al., 2011). The behavioral relevance of leptin receptor signaling in the VTA is beginning to be unravelled. VTA leptin receptor knockdown increases food intake, locomotion, and sensitivity to high-fat feeding (Hommel et al., 2006). Leptin administered directly into the VTA decreases feeding, an effect that is abolished with the application of MEK1/2 kinase (Trinko et al, 2011), suggesting that the ERK1/2 pathway is critical for leptin-mediated feeding in the VTA.

Research has also investigated the projection areas involved in leptin modulation of DA. Original work using retrograde tracing showed that leptin responsive VTA neurons, as assessed by pSTAT3 activation, project to the NAc (Fulton et al., 2006). More recently, Leshan and colleagues (2010), using LepRb-EGFP mice, have demonstrated that leptin-expressing VTA DA neurons mostly project to the extended central amygdala (extCeA) and not the NAc. Recent work in this field has also demonstrated that leptin can indirectly modulate mesolimbic DA. For example, a recent electrophysiology paper demonstrates that leptin suppresses excitatory synaptic transmission onto VTA DA neurons (Thompson & Borgland, 2013). Leptin modulation of mesolimbic DA function also occurs via neurotensin LH neurons (Leinninger et al., 2011).

Together, these findings suggest that, in addition to its actions on hypothalamic circuits, leptin modulates feeding by acting directly and indirectly on the mesolimbic DA system. Hormonal modulation of DA is not unique to leptin. Studies also demonstrate that insulin (Labouèbe et al., 2013) and ghrelin (Abizaid et al., 2006) directly modulate mesolimbic VTA DA neurons and hedonic feeding.

2.4. Dopamine in obesity

Human and animal studies demonstrate that mesolimbic DA is altered in diet-induced obesity, although the direction of this effect is not clear. Human obesity is associated with blunted striatal responses to the receipt of palatable foods (Green et al., 2011; Stice et al., 2008; Stice et al., 2008b) and a cue predicting sucrose (Frank et al., 2012), but increased striatal responses to visual palatable food stimuli (Martin et al., 2010; Rothemund et al., 2007; Stoecket et al., 2009). Based on these findings, a 'dynamic' (Carnell et al., 2012) model of 'hypersensitivity' to visual stimuli and 'hyposensitivity' to the consumption of food rewards has been proposed. Human obesity has also been associated with decreased (deWeiger et al., 2011; Haltia et al., 2007; Wang et al, 2001; Wang et al, 2004) and increased (Dunn et al., 2012) D_2 receptor availability. In rodents, studies examining the effects of diet-induced obesity on DA function have yielded

mixed results. High-fat feeding and cafeteria feeding have been shown to reduce (Huang et al, 2006; Van de Giessen et al., 2012) and increase (Sharma & Fulton, 2013) striatal D_2 receptor expression. Furthermore, while high-fat and cafeteria feeding have been shown to reduce NAc DA concentrations (Davis et al, 2008; Geiger et al, 2009), diet-induced obesity was also shown to increase NAc DA (Narayanaswami et al., 2012). These discrepant findings might be explained by differences in diet composition, the length of exposure to the diet and whether feeding on the diets lead to diet-induced obesity. The behavioral implications of these changes in DA function in diet-induced obesity include attenuation in AMP reward using a conditioned-place preference paradigm and decreased operant responding for food rewards (Davis et al, 2008; Narayanaswami et al., 2012; Sharma et al., 2012; Shin et al., 2011) in rodents. Deficits in DA function observed in humans and animals has led to the 'reward deficiency' hypothesis of obesity (Berthoud et al., 2012) which proposes that reduced DA tone leads to overeating as an attempt to restore DA levels. Whether differences in DA tone emerge as a consequence of high-fat feeding and/or diet-induced obesity or exist prior to the development of obesity, thus conveying vulnerability to obesity is still unclear.

2.5. The vulnerability of the DA system during development

One of the features that is of critical importance to this research project is the time course of maturation of the mesocorticolimbic DA system in the rodent. Although the total number of DA synapses in the NAc and caudate putamen does not vary during early development (birth to postnatal day 21) the nature of these synapses and the morphology of these neurons undergo significant changes over the three first postnatal weeks (Antonopoulos et al, 2002). Likewise, DA projections to the PFC are still immature during early life and continue to increase past weaning until early adulthood (Benes et al, 2000). The postnatal maturation of DA projections in the rodent makes it vulnerable to environmental stressors occurring during the early perinatal and postnatal periods. For instance, long-term modifications within the mesocorticolimbic DA system have been demonstrated

after a number of perinatal manipulations including, perinatal anoxia (Brake et al, 1997), early tactile stimulation (Lovic et al, 2006), brief perinatal glucocorticoid exposure (McArthur et al, 2005), and prolonged maternal separation (Brake et al, 2004).

2.6. Summary

This section focused on the mesolimbic DA system in reward and feeding, the ability of the metabolic hormone leptin to directly and indirectly modulate NAc DA and changes in DA function observed in diet-induced obesity. Finally, it describes the vulnerability of mesolimbic DA to perinatal manipulations.

I.3. The HPA axis

A discussion regarding feeding habits invariably leads to a discussion about how stress influences these habits. The most salient example of this phenomenon is the relationship between PhD dissertation writing, stress, and weight gain reported by my colleagues and myself. Indeed, the HPA axis, which forms the basis of the neuroendocrine response to stress and the brain circuits involved in regulating the HPA axis, including the mesocorticolimbic DA system are a part of the integrated circuits controlling food intake and energy balance. Interestingly, studies have demonstrated that diet-induced obesity increases anxiety behavior (Sharma & Fulton, 2013) and the neuroendocrine response to stress (Legendre & Harris, 2006; Sharma & Fulton, 2013; Tannenbaum et al., 1997). Thus, one way that changes in the nutritional and hormonal environment of developing young could influence energy balance is by altering responsiveness to stress. This chapter will describe the neuroendocrine response to stress, the neural circuits involved in the stress response and adaptations in these circuits that occur with repeated stress.

3.1. The stress response

It is in Montreal that the physiological response to stress was discovered, although the term 'stress' was not initially used. In a short letter written to Nature in 1936 entitled "A syndrome produced by diverse nocious agents", Hans Selye described a general alarm reaction produced following the administration of extracts of various organs (Selye, 1936). Later, Selye used the word 'stressor' to describe factors and agents that triggers the physiological 'stress' response. According to Selye, stressors can be physical, chemical or psychological in nature (reviewed in Szabo et al., 2012). A 'stressor' is conceptualized as any physically present or perceived threat that disturbs homeostasis.

Stress activates the neuroendocrine and sympathetic nervous system in order to mount adaptive metabolic and behavioral responses to challenges. Physiological responses to stressors occur via activation of the HPA axis. First,

corticotrophin-releasing hormone (CRH) is released from neurosecretory cells of the PVN into the median eminence where it travels through the portal blood system to reach the corticotropes of the anterior pituitary gland. Stored adrenocorticotropic hormone (ACTH) is subsequently released into the general circulation and induces the synthesis and release of glucorticoids (corticosterone in rodents, cortisol in humans) from the adrenal gland. Glucocorticoids released from the adrenal gland act at multiple sites including the pituitary and central nervous system (CNS) via glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) to inhibit the release of CRH and ACTH. Because of the high affinity of glucocorticoids with MRs, MRs are extensively occupied when glucocorticoids levels are low. Conversely, low-affinity GRs are bound when glucocorticoid levels are high and thus, mediate most negative feedback (reviewed in Herman et al., 2012). Negative feedback inhibition by GRs has been shown to occur via two main mechanisms, fast non-genomic feedback in the PVN and neural inhibition by limbic structures (mPFC and hippocampus), although alternate mechanisms have also been identified (Herman et al., 2012).

3.2. The neural control of the neuroendocrine stress response

The brain initiates the response in the HPA axis, but the nature of this initiation depends on the type of stressor. To facilitate categorization, Herman and colleagues (2003) distinguish between threats presenting a direct homeostatic challenge to the organism (infection, pain, etc.) and trigger 'reactive' HPA responses and threats generated in the absence of a disruption in homeostasis arising through anticipated disruptions in homeostasis. 'Anticipatory' HPA responses are generated by species-specific threats such as the presence of a predator or by memory of a previous stimulus. The neural circuits involved in 'reactive' and 'anticipatory' HPA responses were the topic of a recent review by Ulrich-Lai and Herman (2009). Circuits were divided into three main categories: the limbic forebrain (mPFC, ventral subiculum, amygdala) for its role in 'top-down processing', the bed nucleus of the stria terminalis (BST) and hypothalamus considered 'middle management' structures and the brainstem, hypothalamus and

circumventricular organs (CVO) as 'stress response triggers'. Reactive HPA responses (real homeostatic threats) are generated through the 'stress response triggers', which innervate the PVN directly and indirectly through the 'middle management' nuclei. Conversely, anticipatory HPA responses require the limbic forebrain and its projections to the 'middle management' to modulate HPA function. A limbic structure of interest to this project is the mPFC and its role in modulating HPA function will be considered in future sections.

3.3. The mesolimbic DA stress response

There is convincing evidence that mesolimbic DA is involved in the appraisal, integration, and behavioral responses to psychogenic stressors (Cabib et al., 2012), suggesting that individual variations in the pattern of NAc DA responses to stress could translate into important differences in behavioral adaptations to stress and thus confer differential vulnerability to disease. Indeed, individual differences in mesolimbic DA activity and reactivity have been associated with a variety of behavioral traits including novelty-seeking, positive affectivity, impulsivity, drug dependence, and food intake (reviewed in Beauchaine et al., 2011). Tail-shock (Abercrombie et al., 1989; Gresch et al., 1994), tail-pinch (Doherty et Gratton, 1992; Budygin et al., 2012; Rouge-Pont et al., 1998; Laplante et al., 2013), and restraint (Doherty & Gratton, 1992; Imperato et al., 1991) stress significantly modulate NAc DA release, although both increases and decreases have been observed. With the application of a prolonged stressor (120 minutes of restraint in this case), a bi-phasic NAc DA response has been observed, with an initial rise in DA concentrations followed by a decrease below baseline (Imperato et al., 1993). The controllability of the stressor has been reported as a significant factor in determining the direction of the NAc DA response, with controllability associated with a rise in Nac DA and uncontrollability associated with a decline (Cabib et al., 1994). The NAc DA response to stress is regulated in a large part by the mPFC. While mPFC DA exerts an inhibitory influence on efferent inputs to the NAc, thus reducing the NAc DA response to stress (Doherty & Gratton, 1996), mPFC NE, through

activation of alpha-1 adrenergic receptors, exerts the opposite effect leading to an enhancement of the NAc DA response to stress (Nicniocaill & Gratton, 2007).

3.5. Repeated stress: consequences on HPA axis and mesolimbic DA

Attenuation of the neuroendocrine stress response upon removal of the threat and with repeated exposure to the same threat is viewed as an adaptive process protecting the individual from excessive glucocorticoid exposure (Grissom & Bhatnagar, 2009, Nesse et al., 2007). In animals, neuroendocrine habituation with repeated exposure to the same stressor has been frequently demonstrated with a variety of stressors including restraint, exposure to cold, novel environment, immobilization, water immersion, and handling. (reviewed in Grissom & Bhatnagar, 2009). In humans, neuroendocrine habituation has been demonstrated with repeated psychosocial stress (Gerra et al., 2001; Kirschbaum et al., 1995; Wust et al., 2005) and repeated parachute jumps (Deinzer et al., 1997). While habituation to the homotypic (stressor that is repeated) is consistently observed and leads to reduced ACTH and corticosterone secretion, neuroendocrine responses to a novel (heterotypic) stressor after repeated stress are often exaggerated or facilitated. For example, repeated exposure to a cold environment, social stress, and forced swim all lead to facilitated HPA responses to restraint (Akana et al., 1996; Bhatnagar & Dallman, 1998; Bhatnagar & Meaney, 1995; Bhatnagar & Vining, 2003).

Several brain regions have been implicated in the process of neuroendocrine habituation to repeated stress. Significant decreases in early immediate gene expression have been observed in the PVN, ventrolateral septum and medial and central amygdala (Stamp et al., 1999; Umemoto et al., 1994). Habituation to repeated restraint is prevented by lesions of the posterior (Bhatnagar et al., 2002), but not the anterior (Fernandes et al. 2002) paraventricular nucleus of the thalamus, which projects to several portions of the amygdala. The mPFC and other limbic structures, involved in top-down processing also seem important (Grissom & Bhatnagar, 2009). Endocannabinoid signaling in the mPFC, amygdala and hypothalamus is increased with repeated

restraint stress (Patel & Hillard, 2008) and inactivation of the mPFC (Weinberg et al., 2010) or right mPFC lesions (Sullivan & Gratton, 1999) eliminates habituation.

The application of repeated homotypic stress has also been demonstrated to alter NAc DA responses, but the direction of this effect is unclear. While some groups report sensitization with repeated stress (Doherty & Gratton, 1992; Brake et al., 1997), others report a reduction with repeated stress (Imperato et al., 1992). Most studies aimed at investigating these effects focus on the ability of stress to promote behavioral and DA sensitization to psychostimulants, a phenomenon commonly referred to as cross-sensitization. Stress increases behavioral sensitization to repeated amphetamine (Antelman et al., 1980), the acquisition of psychostimulant intravenous self-administration (Piazza et al., 1990), operant responding and bingeing for cocaine (Quadros et al., 2009) and enhances the NAc DA response to cocaine (Garcia-Keller et al., 2013). Thus, stress appears to modify mesolimbic DA circuitry and renders the system more vulnerable to psychostimulant-induced plasticity.

The mPFC is also involved in regulation of the NAc DA response to stress. The DA projection from the VTA to the mPFC is activated by stress as evidenced by increased DA release in the mPFC (Abercrombie et al., 1989; Doherty & Gratton, 1992). DA D1 receptor blockade enhances the NAc DA response (Doherty & Gratton, 1996) and blocks amygdala modulation of the NAc DA response (Stevenson & Gratton, 2003), suggesting that mPFC DA exerts an inhibitory influence on the NAc DA response to stress (Doherty & Gratton, 1996). Conversely, mPFC NE appears to enhance the NAc DA stress response, as alpha (1) adrenergic receptor blockade inhibits the NAc DA response (Nicniocaill & Gratton, 2007).

3.6. The perinatal environment, an important determinant of adult stress

Environmental influences during early life are important determinants of adult stress responsiveness. In rodents, repeated neonatal maternal separation (Brake et al., 2004; Francis et al., 2002), neonatal handling (Brake et al., 2004)

and naturally-occurring variations in maternal care (Liu et al., 1997; Zhang et al., 2005) are associated with significant alterations in hypothalamic-pituitary-adrenal (HPA) axis and mesocorticolimbic dopamine (DA) responses to stress.

3.6. Summary

This section focused on the neuroendocrine and DA stress response, the adaptations in these systems that occur with repeated stress and how stress responsiveness can be modulated by factors during early development.

I.4. Metabolic imprinting

4.1. Early life undernutrition and overnutrition as risk factors for obesity

In 1992, Hales & Barker proposed the 'thrifty phenotype hypothesis' to explain the relationship between low birth weight and the increased risk for Type 2 diabetes in a cohort of men born in Hertfordshire England in the early 1900s (Hales & Barker, 1992). In this seminal paper, the authors suggest that malnutrition during fetal and infant life induces permanent alterations in the structure and function of the pancreas and thus predisposes individuals to the development of Type 2 diabetes. Hales & Barker propose that early undernutrition causes a state of 'nutritional thrift' which is advantageous if undernutrition is maintained. However, when individuals undernourished during early life move into an environment of overnutrition, this 'thrifty' phenotype predisposes them to the development of Type 2 diabetes because the overly efficient metabolic system has been re-programmed to face nutritional challenges and calorie depletion.

Since the postulation of the 'thrifty phenotype hypothesis', the association between low birth weight and altered glucose and insulin metabolism has been replicated in a variety of populations (reviewed in Hales & Barker, 2001) and expanded to several chronic diseases including obesity, hypertension and psychiatric disorders (Calkins & Devaskar, 2011). In 2013 however, maternal undernutrition/malnutrition and low birth weight are not noteworthy problems in the Canadian population. In fact, two large-scale recent Canadian studies suggest that the prevalence of maternal overnutrition, rather than undernutrition and high fetal and infant growth is high in Canada. In a recent analysis of 4321 mother-infant pairs from the Ottawa and Kingston birth cohort, Ferraro and colleagues (2012) demonstrated that prior to pregnancy, 56.2% of mothers were of normal weight, 39.9% were overweight or obese while only 3.9% of mothers in this cohort were underweight. A whopping 57.7% of women in this study exceeded the gestational weight gain guidelines. Being overweight or obese prior to pregnancy and excessive weight gain during pregnancy independently increased the odds of having a large for gestational age baby. To examine factors that might

influence childhood obesity, including birth weight and early weight gain, another large scale Canadian study was conducted by Dubois & Girard (2006) using data from 2103 children born in 1998 in the province of Quebec as part of the Quebec Longitudinal study of Child development. Maternal overweight and obesity, high birth weight, and being in the highest quintiles of weight gain between birth and 5 months significantly increased the odds of being overweight at 4.5 years. Thus, the relationship between birth weight and adult adiposity fits a U-shaped curve, with both low and high birth weight associated with increased risk of obesity (reviewed in Breton, 2013). The term 'metabolic imprinting' has recently been used (Levin, 2006) to describe how alterations in the nutritional and hormonal environment of developing young can predispose individuals to obesity and its associated pathologies.

4.2. *The use of animal models to study the mechanisms of metabolic imprinting*

The association between high birth weight and increased susceptibility to the development of obesity has led to three main questions. First, are alterations in the nutritional and hormonal environment of developing young changing the development of peripheral and central systems regulating energy homeostasis? Second, are there developmental windows for the effect of early diet? And finally, what is mediating the effect between early nutritional environment and changes in energy homeostasis? To answer these questions, scientists have developed a plethora of animal models. To identify developmental windows or critical periods, manipulations in early diet have been restricted to specific developmental periods (pre-conception, gestation, and lactation) in a variety of species (from rodents to rhesus macaques). In rodents, early overnutrition has been achieved by reducing litter size (Plagemann et al., 1999a), artificial rearing of pups which allows for direct control of neonatal diet (pup in a cup model, reviewed in Patel & Srinivasan, 2010) and by altering the diet composition of the mother. Maternal diet manipulations differ in the source and amount of fat and carbohydrates, the fat to carbohydrate ratio and caloric density. Some groups have even used a 'cafeteria diet', which includes a variety of food items (Wright et al., 2011) and

highly palatable liquid diet supplements (Shalev et al., 2010). The overabundance of models, which have sometimes yielded opposite effects, makes it very difficult to directly compare these studies. For this reason, the following sections will focus on litter size manipulation as it is a very well characterized model of postnatal overnutrition and maternal high-fat feeding because it is the model we use to examine the long-term consequences of early overnutrition.

4.3. Overnutrition during postnatal life –long-term consequences on hypothalamic circuitry

In rats, litter size manipulation, which alters nutritional levels during lactation, is one the best characterized model of postnatal overnutrition. Small litter rearing (3-4 pups/litter vs.10 pups/litter) increases the availability of maternal milk, thus inducing early overnutrition in the suckling pups. Rat pups raised in small litters are heavier at weaning and gain more weight as adults, while the opposite is seen in large litters (reviewed in Patel & Srinivasan, 2011). The laboratory of Andreas Plagemann has been especially involved in examining the long-term consequences of small litter rearing on metabolic function in adulthood. Pups reared in small litters are hyperphagic and overweight throughout life, and display increased plasma concentrations of insulin and leptin, impaired glucose tolerance, elevated triglycerides and increased systolic blood pressure as adults (Plagemann et al., 1999a). Subsequently, they demonstrated extensive alterations in the structure and function of the hypothalamus in these animals, with most effects being observed in the ARC and DMH. Overnutrition induced by decreased litter size was associated with an increased number of orexigenic neurons in the ARC (Plagemann et al., 1999a; Plagemann et al., 1999b) and differential ARC responses to leptin (Davidowa et al., 2000a), melanocyte-stimulating hormone (MCH, Davidowa et al., 2002a), insulin (Davidowa et al, 2007), and amylin (Davidowa et al., 2004). Significant alterations in electrophysiological responses to neuropeptides and metabolic hormones were also observed in the VMH (Heidel et al., 1999; Davidowa et al., 2002b; Li et al., 2002; Davidowa et al, 2002a; Davidowa & Plagemann, 2000b) and PVN (Davidowa et al., 2002b). Together,

the studies conducted by Plagemann and colleagues suggest that postnatal overnutition permanently modifies the functional integration of orexigenic and anorexigenic signals in the hypothalamus, thus altering energy homeostasis and predisposing these animals to obesity. Alterations in hypothalamic function with small litter rearing have also been reported by other groups. For example, Rodrigues and colleagues have reported decreased hypothalamic Ob-Rb (2009) and JAK2 (2011) expression, evidence of leptin resistance in adults reared in small litters.

4.4. Maternal high-fat feeding in rodents – long-term consequences on hypothalamic circuitry

Maternal high-fat feeding of rodents during the perinatal period is associated with significant alterations in energy balance in the offspring. This effect has been observed with varying concentrations of maternal dietary fat content: 16% fat (Samuelsson et al., 2008), 24.08% (Shalev et al., 2010), 25.7% fat (Khan et al., 2003), 31% fat (Levin and Govek, 1998), and 60% fat (Tamashiro et al., 2009). At birth, fetuses exposed to high-fat have elevated plasma concentrations of leptin and insulin and increased hypothalamic expression of Ob-Rb, AGRP, NPY, POMC and STAT3 (Gupta et al., 2009), highlighting the importance of the gestational period in the programming of hypothalamic circuits. Prenatal high-fat diet exposure also increased PVN and LH expression of orexigenic neuropeptides by stimulating the proliferation and differentiation of neurons and their migration toward hypothalamic regions (Chang et al., 2008). In addition to having immediate effects on hypothalamic function, high-fat maternal feeding also induces long-lasting changes in hypothalamic function. High-fat feeding of the mother programs hypothalamic leptin resistance (Ferezou-Viala et al., 2007), and increases NPY and decreases POMC hypothalamic mRNA expression (Chen et al., 2008). Interestingly, while gestation appears to be a critical determinant of hypothalamic function, a cross-fostering experiment examining the relative contribution of the prenatal and postnatal period on leptin sensitivity has shown that it is.maternal high-fat exposure during the suckling

period that is more critical in determining metabolic consequences for offspring such as leptin resistance (Sun et al., 2012).

4.5. Summary

The experiments presented in this section demonstrate that overnutrition during early development increases susceptibility to the development of obesity and metabolic disturbances and suggests that this vulnerability is conferred by changes in hypothalamic function. The following section will describe the model of early overnutrition used in our lab to examine the mechanisms of metabolic imprinting

I.5. Our model

5.1. Maternal Diet

To examine the programming effects of early exposure to high-fat, our laboratory increases the fat content of the maternal diet. Access to either high-fat (30% fat) or control (5% fat) diet is provided to mothers from gestation day 13 to PND22 when offspring from both diet groups are weaned from their mothers and maintained on the control diet until testing in adulthood. The late gestational and postnatal period was targeted because it represents a critical period in hypothalamic projection development in the rodent (Bouret et al., 2004a). The full description of the high-fat and control diet is provided in Appendix 1.

5.2. Effect on postnatal metabolic, nutritional and hormonal profile

Studies from our laboratory have demonstrated that increasing the fat content of the maternal diet alters the metabolic, nutritional and hormonal environment of pups during critical periods of development. More precisely, high-fat feeding of the mother increases the lipid and leptin composition of the maternal milk without altering the protein content (d'Asti et al., 2010). By PND10, pups feeding on this high-fat milk are heavier than the control pups and exhibit increased retroperitoneal white adipose tissue weight and significantly elevated plasma concentrations of leptin and corticosterone (d'Asti et al., 2010).

5.3. The adult phenotype

Exposure to high-fat during early development is associated with hyperphagia when given a choice of macronutrients and weight gain in the adult offspring, demonstrating the programming of feeding behavior in these animals (Walker et al., 2008). High-fat exposed adult offspring do not display any signs of leptin resistance when maintained on a control diet post-weaning (Walker et al., 2008). However, when feeding on the macronutrient selection diet, accelerated leptin resistance is observed in the high-fat offspring, suggesting that early high-fat exposure is increasing vulnerability to metabolic disturbances.

While the literature demonstrates that early overnutrition modifies hypothalamic function, little is known about the programming of mesolimbic by early dietary manipulations. The observations that mesolimbic DA function is sensitive to metabolic hormones (Abizaid et al, 2006; Fulton et al, 2006; Hommel et al, 2006; Leinninger et al, 2009; Trinko et al., 2011) and diet-induced obesity in adulthood (Davis et al, 2008; Frank et al., 2012; Geiger et al, 2009; Green et al., 2011; Huang et al, 2005; Sharma & Fulton, 2013; Stice et al., 2008; Stice et al., 2008b; Van de Giessen et al., 2012; Wang et al, 2001; Wang et al), that the projections from the VTA to the NAc are maturing postnatally (Antonopoulos et al, 2002) and modified by other perinatal manipulations (Brake et al, 1997; Brake et al, 2004; Lovic et al, 2006; McArthur et al, 2005) and that exposure to high-fat via the maternal milk is altering the hormonal profile of the young (D'Asti et al, 2010) led us to hypothesize that mesocorticolimbic DA could be permanently altered by early exposure to high-fat. This question was addressed in our previous publication (Naef, 2008) which demonstrates that the offspring of mothers maintained on the high-fat diet display reduced locomotion in response to acute amphetamine administration and decreased behavioral sensitization to repeated amphetamine administration. These behavioral observations were accompanied by increases in TH in the VTA and NAc and increases in DA concentrations and its metabolite 3,4-dihydroxyphenylacetic acid (dopac) in the NAc. No changes in D_1, D_2 or DAT binding were observed using autoradiography. These experiments were conducted in adulthood, long-after the termination of the diet. Since locomotor activation following amphetamine correlates with the amount of DA released into the striatum (Sharp et al, 1987) and behavioral sensitization to AMP (reviewed in Vezina, 2004) involves changes in mesocorticolimbic DA, these data suggest that early exposure to HF is producing permanent alterations in DA function.

I.6. Hypotheses and aims

6.1. Rationale, hypotheses and aims of the dissertation

The rationale, hypotheses and aims of this dissertation are depicted in Figure I-1. High-fat feeding of the mother during the perinatal period alters the nutritional, hormonal and metabolic profile of the pups during critical periods of development and predisposes the offspring to the development of obesity and metabolic disturbances in adulthood by altering patterns of feeding and increasing adiposity. Most studies aimed at examining the mechanisms through which early nutritional environment, and in particular high fat diet can increase susceptibility to obesity have focused on changes in hypothalamic circuits and on the role of leptin in hypothalamic development. The 'programming' effects of early diet and leptin might extend to other neural circuits involved in the regulation of feeding including the mesolimbic DA system and the HPA axis. Mesolimbic DA and HPA activity are changed in obesity and structures within these systems are targets of metabolic hormones such as leptin and ghrelin. Importantly, the functional integration of these systems occurs during the early postnatal period in the rodent and their developmental trajectories are significantly altered by a variety of perinatal manipulations. The goal of this dissertation is to advance our understanding of the mechanisms involved in the programming of obesity. Long-term consequences of early exposure to high-fat on mesolimbic DA function, HPA activity and behavior will be the focus of the first three data chapters. In the last chapter, we will examine the modulation of VTA DA neurons by leptin during neonatal development.

Aim1: We have previously demonstrated that the offspring of mothers feeding on a high-fat diet display, as adults, reduced locomotor responses to acute amphetamine administration and reduced behavioral sensitization to repeated amphetamine. The locomotor-stimulating properties of psychostimulants and behavioral sensitization are mediated, at least in part, by the amount of DA

released into the NAc, suggesting that the NAc response to amphetamine is reduced in high-fat exposed offspring. We therefore hypothesize that early exposure to high-fat is decreasing the NAc DA response to amphetamine. Differences in NAc DA responses to AMP in high-fat offspring might extend to other stimuli known to modulate NAc DA such as food and food cues, as well as stress. The first aim of this PhD dissertation is to measure the NAc DA response to amphetamine, the anticipation and consumption of high-fat food rewards, and stress in control- and high-fat- exposed adult offspring.

Aim 2: Differences in the pattern of NAc DA release with stimulation by psychostimulants, the anticipation and consumption of high-fat food rewards and stress regulation in high-fat exposed offspring suggests that the regulation of DA is altered in these animals. Thus, the second aim is to examine diet group differences in the regulation of NAc DA.

Aim 3: By inducing permanent alterations in DA function and changing the NAc response to the anticipation and consumption of high-fat food rewards (aim 1), we hypothesize that early exposure to high-fat will alter DA-dependent feeding behavior. DA mediates the 'wanting' of rewards, incentive motivation which promotes the approach and consumption of rewards. The third aim is to examine incentive motivation for palatable food rewards in control and high-fat exposed adult offspring.

Aim 4: The adult stress response is influenced by several perinatal manipulations and increased with high-fat feeding in adulthood. We have previously demonstrated that the offspring of mothers feeding on a high-fat diet have increased basal corticosterone concentrations during the early postnatal period (d'Asti et al., 2010), although long-term changes in HPA activity have yet to be examined. Thus, the fourth aim of this PhD dissertation is to examine the long-term consequences of early exposure to high-fat on HPA function.

Aim 5: Leptin inhibits feeding by targeting hypothalamic and dopaminergic circuits involved in the homeostatic and hedonic regulation of feeding behavior. However, leptin function is different in neonates, suggesting that the ability of leptin to modulate hypothalamic and dopaminergic circuits is different than in adulthood. While the ability of leptin to modulate the hypothalamus has been examined in neonates, nothing is known about the ability of leptin to modulate VTA neurons during the neonatal period. Thus, the fifth and final aim of this thesis is to examine leptin signaling in the VTA during neonatal development.

Figure I-1. Rationale and aims of the PhD dissertation

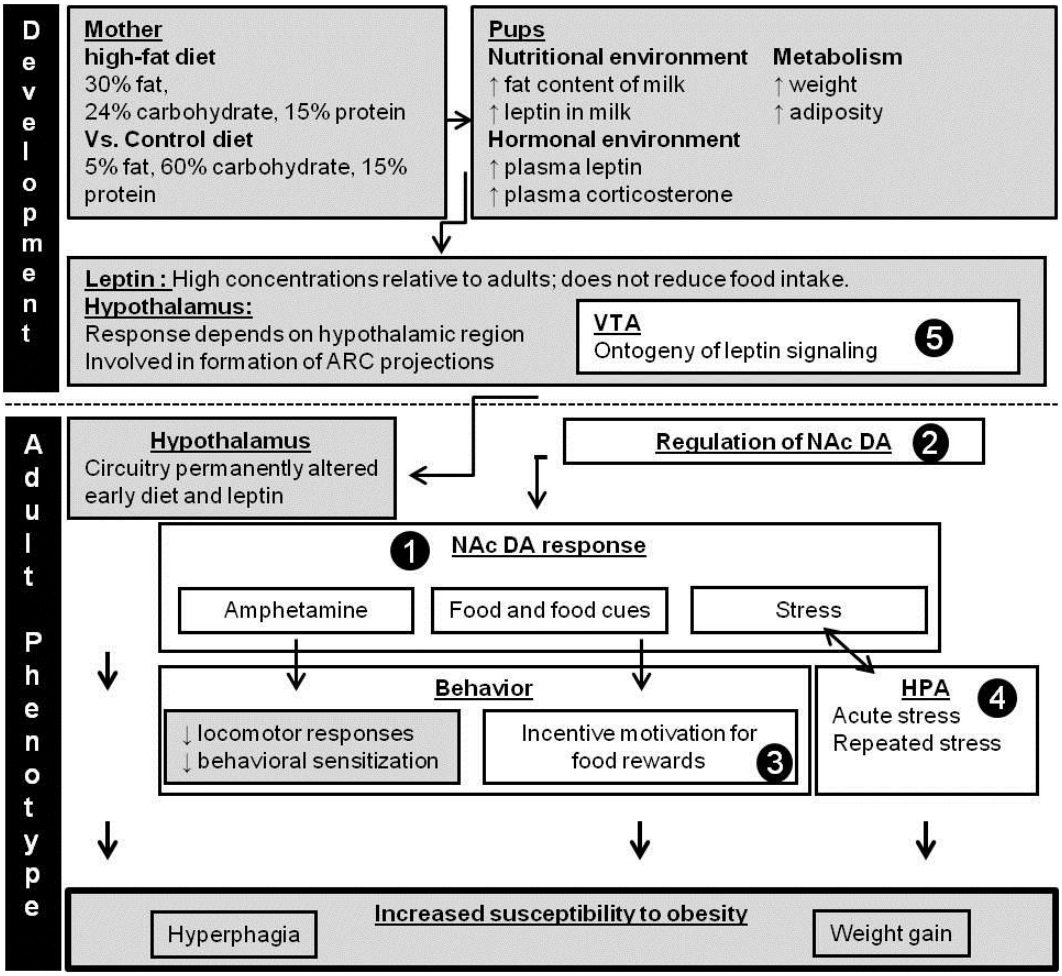

Figure I-1. *Rationale and aims of the PhD dissertation. Aim 1: to measure the NAc DA response to amphetamine, the anticipation and consumption of high-fat food rewards, and stress in control- and high-fat- exposed adult offspring. Aim 2: to examine the regulation of NAc DA in control- and high-fat-exposed offspring. Aim 3: to examine incentive motivation for palatable food rewards in control- and high-fat- exposed offspring. Aim 4: to examine HPA function in control- and high-fat-exposed offspring. Aim 5: to examine the ontogeny of leptin signaling in the VTA during neonatal development. Grey boxes represent known data and concepts.*

Chapter II. Maternal high-fat intake alters presynaptic regulation of dopamine in the nucleus accumbens and increases motivation for fat rewards in the offspring.

II.1 Preface

Experiments conducted during the course of my Master's degree (Naef et al., 2008) demonstrate that the offspring of mothers fed a high-fat diet during the last week of gestation and throughout lactation display, as adults, reduced locomotor responses to amphetamine and reduced behavioral sensitization to repeated amphetamine administration relative to control offspring. The locomotor-stimulating properties of amphetamine are mediated, in part, by NAc DA, suggesting that NAc DA is altered in high-fat exposed offspring. The first manuscript presented in this PhD dissertation examines the long-term consequences of early exposure to high-fat on the NAc DA response to amphetamine (aim 1) and regulation of NAc DA (aim 2) by the PFC, DAT, VMAT, and D_2 presynaptic autoreceptors. Finally, this manuscript will examine incentive motivation for palatable food rewards in control and high-fat exposed offspring (aim 3). The manuscript will be followed by supplemental data examining the contribution of circulating levels of leptin and corticosterone to operant responses for high-fat food rewards in control and high-fat offspring.

II.2 Contribution of authors

L. Naef: Design, execution, analysis of data and writing of the manuscript.

L. Moquin: Dialysate analysis using using high-performance liquid chromatography.

G. Dal Bo and B. Giros: D_2 receptor mRNA using in-situ hybridization.

Dr. Claire-Dominique Walker and Dr. Alain Gratton: design, analysis of data, editing of manuscript.

Acknowledgments: We would like to thank Ms Hong Long for expert technical assistance with the experiments, Dr Benedicte Amilhon for her help with the DAT

and VMAT protocol, and Dr Joseph Rochford for his statistical and behavioral expertise.

II.3 Manuscript

Title: Maternal high-fat intake alters presynaptic regulation of dopamine in the nucleus accumbens and increases motivation for fat rewards in the offspring.

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Keywords: maternal diet, high-fat, development, dopamine, behavior.

Abstract

High caloric intake during early postnatal development can have long term consequences for the offspring. We previously reported that the adult offspring of dams fed a high-fat diet during the last week of gestation and throughout lactation display blunted locomotor responses to amphetamine (AMP) and reduced sensitization to the drug compared to offspring of control diet dams. Here, we report that the subsensitivity of high-fat offspring to AMP's locomotor stimulant action reflects, at least in part, altered regulation of nucleus accumbens (NAc) dopamine (DA) transmission. When compared to controls, the DA response of high-fat animals to AMP, as measured with microdialysis, was attenuated in the NAc, but unaffected in the prefrontal cortex (PFC). A relatively higher activity of NAc synaptosomal DA transporter sites without changes in vesicular monoamine transporter (VMAT) uptake capacity was also observed in high-fat offspring. Moreover, ventral tegmental area (VTA) D_2 receptor mRNA levels were decreased in high-fat offspring, suggesting a reduction in DA release-regulating D_2 autoreceptors in terminal regions such as the NAc. The magnitude of locomotor response to $D_{2/3}$ receptor activation (with quinpirole) was greater in high-fat than in control animals despite having comparable postsynaptic D_2 mRNA levels in the NAc. Finally, while operant responding for a sugar-enriched food reward did not differ between diet groups, high-fat offspring displayed increased operant responding for a fat-enriched reward compared to controls. These findings add to mounting evidence that early life exposure to elevated dietary maternal fat can lead to long lasting changes in DA-mediated behavioral responses to stimulant drugs and fat-enriched foods.

Introduction

"Metabolic imprinting" has recently been used (Levin, 2006) to describe how alterations in the nutritional and hormonal environment of developing young can predispose individuals to obesity and its associated pathologies. In rodents, both gestation and the pre-weaning period represent critical developmental windows with regard to the functional establishment of metabolic-sensitive neural pathways. For instance, the projections from the arcuate nucleus to other hypothalamic nuclei regulating food intake and energy balance are only mature by the 8th to 10th day of postnatal life (Grove & Smith, 2003; Grove et al., 2003; Bouret et al., 2004a). Modifications in these projections have been demonstrated following postnatal overnutrition (Plagemann, 2006) and maternal high-fat feeding (Chang et al., 2008), and in response to variations in leptin (Bouret et al., 2004b) and insulin (Franke et al., 2005) concentrations in rodents and in non-human primates exposed to high-fat *in utero* (Grayson et al., 2010).

Mesocorticolimbic dopamine (DA) consisting of cell bodies arising in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAc) and prefrontal cortex (PFC), has been most intensively studied in the context of reward and addiction. There is increasing evidence that points to a link between metabolic regulation, obesity and DA. Alterations in DA function are associated with human obesity (Wang et al., 2001; Stice et al., 2008) and these findings are supported by evidence of altered D_2 receptor and DA transporter (DAT) (South & Huang, 2008) binding, as well as impaired NAc DA neurotransmission (Davis et al., 2008; Geiger et al., 2009) in adult animals fed a high-fat diet. These findings converge with those derived from studies showing that NAc DA function is modulated by metabolic hormones such as leptin (Fulton et al., 2006; Hommel et al., 2006; Perry et al., 2010) and ghrelin (Abizaid et al., 2006).

While much of the recent research on this topic concerns the interactions between diet, metabolic hormones, mesocorticolimbic DA neurons, and ingestive behavior in adulthood, our interest was to determine the long-term consequences

of early life nutritional manipulations on DA neurotransmision and DA-mediated behaviors. We have previously reported that augmenting the dams' dietary fat content during the last week of gestation and throughout lactation increases maternal milk fat and leptin concentrations, increases the adiposity of the pups (Walker et al., 2008) and skews their hormonal profile towards significantly higher plasma leptin and glucocorticoid concentrations during the early postnatal period (D'Asti et al., 2010) and in adulthood (Walker et al., 2008). The fact that the projections from the VTA to the NAc and PFC are maturing postnatally (Antonopoulos et al., 2002; Kalsbeek et al., 1988), are sensitive to diet-induced obesity in adulthood (Davis et al., 2008; Geiger et al., 2009; South and Huang, 2008) and that exposure to high-fat via the maternal milk is altering the hormonal profile of the young (D'Asti et al., 2010) led us to hypothesize that mesocorticolimbic DA could be permanently altered or "programmed" by early exposure to high-fat. Indeed, we have recently demonstrated that the adult offspring of mothers maintained on a high-fat diet in late gestation and lactation display reduced locomotion in response to acute amphetamine (AMP) administration and decreased behavioral sensitization to repeated AMP administration (Naef et al., 2008). In the present study, we used a variety of molecular and pharmacological manipulations to determine whether the effects of maternal high-fat we observe on the behavioral response of adult offspring to AMP occur as a consequence of altered mesocorticolimbic DA transmission and, if so, to elucidate the mechanism(s) by which it operates. Our second objective was to document whether the effects of maternal high-fat extend to reward-relevant mesolimbic DA function. To test this hypothesis, we compared operant responses of control and high-fat offspring to two highly palatable food rewards (sugar or 35% fat pellets) under continuous, fixed- and progressive-ratio schedules of reinforcement.

Experimental procedures

Animals

Pregnant Sprague–Dawley rats (Charles River, St.-Constant, Canada) were received in our animal facility on gestation day (GD) 12–13 and placed on either a high-fat (30% fat, 24% carbohy- drate, 15% protein, 4.54 kcal/g) or a control diet (5% fat, 60% carbohydrate, 15% protein, 3.45 kcal/g) from arrival until weaning of the pups on postnatal day (PND) 22. Both high-fat and control diets were powdered semi-purified, isocaloric diets from Harlan Teklad (IN, USA). On PND 1, all litters were culled to 10 pups. Upon weaning, male offspring were housed two per cage, respecting maternal diet treatment and were given *ad libitum* access to the control diet until testing in adulthood. Although not assessed in the present cohort of mothers, we have repeatedly found that there are no differences in body weight of the mothers placed on either diet at the time of weaning their pups (unpublished observation). Animals were housed under controlled conditions of light (12:12h light/dark cycle), temperature (24–26 °C) and humidity (70 – 80%). Unless otherwise noted, all tests were conducted during the light phase of the light/dark cycle. All procedures were approved by the Animal Care Committee at McGill University in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

In-vivo microdialysis

Male rats (PND90 –130) were implanted with 20 gauge intracranial cannula aimed at the NAc (AP=0.65 mm, ML=0.14 mm from bregma, DV=–0.65 mm, 10 animals/diet group) or the mPFC (AP=0.27 mm, ML=0.05 mm from bregma, DV=0.27 mm, high- fat n=8, control n=7). Microdialysis probes (active membrane: 2.5 mm) and probe assembly were constructed as previously described (Lupinsky et al., 2010). Flow rate of aCSF was set at 1.5 µl/min and samples were collected every 15 min for 1 h prior to drug administration and 2 h post AMP (1.5 mg/kg, i.p., D-AMP sulfate, Sigma, St-Louis, MO, USA) administration. After the

microdialysis procedure, all rats were decapitated and brains were frozen (−80 °C). Correct probe placement was verified for each animal based on Paxinos and Watson (1998).

Dialysate levels of DA were measured using high-performance liquid chromatography with electrochemical detection. Our system consisted of an ESA pump (model #582), an ESA injector (model #542) and a Luna C18 (2) 75X4.6 mm 3 µm analytical column. The mobile phase (6% methanol, 0.341 mM 1-octanesulfonic acid sodium salt, 168.2 mM sodium acetate, 66.6 mM citric acid monohydrate, 0.025 mM ethylenediamine-tetraacetic acid disodium, 0.71 mM tryethylamine, pH of 4.0-4.1 adjusted with acetic acid) pumped at a rate of 1.5 ml/min and the electrochemical detector (ESA Coularray, model # 5600A) was set at a potential of -250 mV and +300 mV. DA (25 ng/ml, 3-hydroxytyramine hydrochloride, Sigma) standards were loaded alongside samples into a refrigerated ESA autosampler (model #542). Chromatographic peak analysis was conducted using ESA CoulArray software which identified unknown peaks in samples and matched these peaks with the retention time of the known standards.

Activity of the DA transporter (DAT) and vesicular monoamine transporter (VMAT)

For striatal VMAT transport, tritiated serotonin was used as ligand instead of DA because of the greater affinity of the former for VMAT. For each of the four replica experiments, four adult male rats (>PND110) from each diet group were used. Upon rapid decapitation, tissue chunks containing the caudate putamen (CP) and NAc at levels between +2.7 mm to -1.3 mm from bregma were dissected out (Paxinos and Watson, 1998). Vesicles were purified as previously described (Huttner et al., 1983; Amilhon et al., 2010). Protein concentrations were adjusted to 40 µg/10 µl prior to the uptake assay. The transport reaction was started by adding 10 µl of vesicular preparation to 90 µl of transport buffer containing 2.2 mM ATP and 50 nM 5-hydroxy [G-3H]-tryptamine creatinine sulfate (PerkinElmer, SA=28.1 Ci/mmol) with or without

10 µM reserpine (Sigma). After incubation at 37 °C for 10 min, [3H]-serotonin uptake was stopped by dilution with 3 ml of ice-cold KCl 0.15 M, rapid filtration through mixed cellulose esters filters (Millipore, HAWP00010) and four washes with 3 ml ice-cold 0.15M KCl. The radioactivity retained on the filters was measured by scintillation counting in a beta-counter. Each condition was measured in triplicates and four individual uptake experiments were performed on four different vesicular preparations.

DAT uptake was measured as previously described (Martres et al., 1998). 1 mm punches from the NAc and CP were collected from three adult (PND>90) male rats from each diet group. Tissues were processed for synaptosomes as described above for VMAT except that synaptosomes were resuspended in assay buffer (Tris–HEPES 4 mM, NaCl 120 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.2 mM, D-glucose 5.6 mM, ascorbic acid 0.5 mM, pH 7.4) at a concentration of 15 mg wet weight/ml. DA uptake was measured by incubating synaptosomes (100 µl) with assay buffer containing 10 nM of [3H] DA (PerkinElmer, NET673), competing doses of cold DA (20-3000 nM, 3-hydroxytyramine hydrochloride, Sigma, St-Louis) and 10 µM pargyline (Sigma, St-Louis) with or without the addition of nomifensine (1 µM, Sigma) for non specific binding. Incubation was carried out at 37 °C for 5 min and the reaction was quickly stopped by placing the tubes on ice and rapid filtration on GF/B filters (Brandel Inc., Gaithersburg, MD, USA) and three washes with Tris–HEPES buffer (Tris 4 mM–HEPES 6.25 mM, NaCl 120 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.2 mM, pH 7.4) using a cell harvester system. The radioactivity retained on the filters was measured by scintillation counting in a beta-counter. Each condition was measured in triplicates and four individual uptake experiments were performed on four different synaptosomal preparations.

In-situ hybridization for D_1 and D_2 dopamine receptors

In-situ hybridization was carried out as described previously (Herzog et al., 2001). Briefly, six naive adult male rats (PND 90) from each diet condition

were killed by rapid decapitation. Brains were removed, dipped in cold isopentane and frozen at −80°C. Twenty micrometres coronal sections were collected onto Superfrost Plus slides (Fisher), allowed to desiccate overnight under vacuum at 4 °C, and then maintained at −80°C until processed. [^{35}S]-dATP oligonucleotides were synthetized with terminal transferase (Amersham, Biosciences) to obtain a specific activity of $5X10^8$ dpm/µg for each receptor. A mixture of three anti-sense oligonucleotides were selected for D_1 and D_2: for D_1[5=-TGG ACC TCA GGT GTC GAA ACC GGA TGA CGG CCG -3=; 5=-TGT CCT CCA GGG AGG TAA AAT TGC CAT CCA AGG-3=; and 5=-GGT CCT CAG AGG AGC CCA CGG CAT GAG GGA TCA-3=]; and for D_2 [5=-CTG CCT TCC CTT CTG ACC CAT TGA AGG GCC GGC T-3=; 5=-CCA GCT CCT GAG CTC GGC GGG CAG CAT CCA TTC T-3=; and 5=-CCC TGA GCC ATG GGT CCA ACC CCA GAG CTG GTA-3=]. All sections were covered with 70 µl of hybridization mix and $3–5X10^5$ dpm of each labeled oligonucleotide, and incubated overnight at 42 °C. Following washes and dehydratation, slides were air-dried and exposed to a BAS-SR Fujifilm Imaging Plate for 15 days. The plates were scanned with a Fujifilm BioImaging Analyzer BAS-5000 and mRNA levels were analyzed semi-quantitatively by optical density using a ^{14}C-la- belled graded standard (nCi/g). An average of 12 brain sections representing the anterior, middle and posterior striatum and eight brain sections for the VTA was analyzed per animal. Regions identification was based on Paxinos and Watson (1998).

Locomotor responses to $D_{2/3}$ receptor activation by quinpirole

Locomotor activity was monitored in acrylic chambers equipped with an array of photoelectric sensors (AccuScan Instruments, Columbus, OH, USA). Locomotor activity of male rats (PND90 –100, control n=16, high-fat n=13 animals) was analyzed for 90 min and 180 min following s.c. vehicle and quinpirole hydrochloride (0.5 mg/kg subcutaneous, Tocris Bioscience) administration, respectively. Locomotion is expressed as a total distance during

specific time intervals.

Operant responding for fat- and sugar-enriched food rewards

Operant responding for either sugar pellets (Dustless Precision Pellets, 45 mg sugar pellets, Bio-Serv, Product # F0042) or fat pellets (Dustless Precision Pellets, 45 mg 35% fat, Bio-Serv, Product # F05989) was conducted in conditioning chambers (Med-Associates, St. Albans, VT, USA) controlled by a PC running Med Associates software. Ten days prior to testing, weights of all animals were decreased to 85% of initial weight. Operant responding was conducted in the dark phase of the light/dark cycle and their daily meal was provided after the session (18 g). Magazine training and two sessions of a fixed ratio (FR) autoshaping procedure were used to train the animals. Training was followed by two sessions of FR1 reinforcement, two sessions of FR3 and one progressive ratio (PR) session. The start of the operant sessions (FR and PR sessions) was signaled by the house light and the emergence of the active lever. The PR schedule of reinforcement was based on Richardson and Roberts (1996) and followed a modified logarithmic progression. Progressive ratio sessions ended if rats either reached the maximum 2½ h session duration or failed to earn a food reward within a 1 h period. Breaking point was defined as the ratio requirement that each animal failed to achieve. The total number of responses during each session was used to examine group differences.

Statistical analyses

All data were analyzed using one- or two-way analyses of variance (ANOVA), with repeated measures when appropriate. Post-hoc tests (Bonferroni) were performed when significant interactions were found. In some instances two-tailed *t*-tests were performed. The level of significance was set at $P<0.05$. Values are expressed as mean±SEM.

Results

Amphetamine-induced increases in NAc and PFC DA

Only data from animals with histologically confirmed probe placements in the NAc (Figure II-1A) and the PFC (Figure II-1B) were included in the analysis. Basal DA levels were estimated from the pooled means of the four pre-injection dialysate samples. As shown in Figure II-2, the basal NAc (A) and PFC (C) DA levels of high-fat animals did not differ from those of control animals ($P>0.05$). When expressed as a percentage of baseline concentrations, the magnitude of the DA response to AMP was significantly reduced in the NAc of high-fat animals in comparison to control animals (Fig. 2B, $P<0.05$). A two-way ANOVA revealed significant effects of time post-injection ($F(4,72)=11.34$, $P<0.001$) and diet ($F(1,72)=7.17$, $P=0.0153$), but no time x diet interaction. The same analysis was applied to the PFC DA (Fig. 2D) response to AMP, which revealed a significant effect of time post-injection ($F(4,52)=6.96$, $P<0.001$), but no significant effect of diet.

DA uptake via DAT and VMAT

Four independent experiments were performed to investigate diet-induced alterations in DA uptake by the synaptosomal DAT. For the DAT, the kinetic characteristics of DA uptake for each diet condition, Vmax and Km were derived from the Michaelis–Menten regression. While Km describes the affinity of the transporter for the ligand or the concentration at which 50% of the active sites are filled, Vmax represents the maximal amount of DA molecules taken up when the DAT is saturated with DA. Results are expressed as mmol DA transported/mg protein. As shown in Figure II-3, competitive DA uptake via DAT was found to be saturable in both the CP (Figure II-3A) and NAc (Figure II-3B) of high-fat and control animals. There was no effect of diet on the affinity (Km) of DA for either the CP or NAc DAT sites ($P>0.05$). However, NAc Vmax values were noticeably, albeit not significantly ($P=0.0548$) higher in high-fat animals

compared to controls. No diet-related differences in Vmax were observed in the CP. For VMAT (Fig. II-3C), data are represented as nmol 5-HT/mg protein and as for the DAT, four independent experiments were conducted. No differences in VMAT were observed between high-fat and control offspring ($P>0.05$).

In-situ hybridization for D1 and D2 dopamine receptors

Two-way ANOVAs were used to assess diet-induced changes in mRNA levels of D_1 and D_2 DA receptors with region and diet as factors. For D_1 receptors, were observed a significant diet effect ($F(1,30)=5.753$, $P<0.05$) and a significant region effect ($F(2,30)=6.901$, $P<0.001$). However, post hoc analysis using Bonferoni revealed that statistically significant group differences were not reached in any of the regions examined (Figure II-4A). The same analysis was applied for D_2 receptors and revealed a significant diet effect ($F(1,40)=5.83$, $P<0.05$) and a significant region effect ($F(3,40)=90.3$, $P<0.0001$). Expression of D_2 receptor mRNA levels was significantly lower in high-fat offspring compared to control in the VTA ($P<0.05$), but not in the terminal regions of the mesolimbic dopamine system (NAc and CP, Fig. II-4B).

Locomotor responses to $D_{2/3}$ receptor activation with quinpirole

To determine whether changes in VTA D_2 receptors could influence locomotor responses to a $D_{2/3}$ receptor agonist, locomotor activity was examined after s.c. injection of quinpirole. As illustrated in Figure II-5A, locomotor activity following s.c. saline administration did not differ between high-fat and control offspring (two-way ANOVA, $F(1,216)=0.081$, $P>0.05$). In accordance with previous reports (Eilam & Szechtman, 1989; Horvitz et al., 2001; Benaliouad et al., 2009), a biphasic profile of quinpirole-induced locomotion was observed in the current experiment. As illustrated in Figure II-5C, locomotion in the first 10 min following quinpirole administration was reduced to the same extent in control and high fat-animals compared to locomotion observed after saline injection. Using a two-way ANOVA, we find a significant quinpirole effect ($F(1,28)=36.82$, $P<0.001$),

but no diet effect ($F(1,28)=0.2975$, $P>0.05$). In contrast, 90 min following quinpirole administration (Fig. II-5D), we observed a significant increase in locomotor activation with the drug ($F(1,28)=32.19$, $P<0.001$), a significant diet effect ($F(1,28)=4.77$, $P=0.0375$), and a significant drug x diet interaction ($F(1,28)=6.13$, $P=0.0196$). Post hoc analysis reveal that at 90 min post-injection, control and high-fat offspring do not differ in response to saline administration but that high-fat offspring display hyperlocomotion compared to controls following quinpirole ($P<0.01$). Because the stimulatory effect of quinpirole is usually observed starting from 60 min after administration (Benaliouad et al., 2009), we used later timepoints (60 min–180 min following quinpirole administration) to test whether the stimulatory effect of quinpirole on locomotion was diet related. Indeed, in this time interval (60 –180 min), locomotor activity (cm) in high-fat animals was increased compared to their control counterparts ($F(1,286)=4.329$, $P=0.0475$, Figure 5B), and a significant time effect was also revealed ($F(11,286)=9.215$, $P<0.001$, Figure 5B).

Operant responding for sugar- and fat-enriched food rewards

To test whether increased fat during early life alters the reinforcing properties of palatable food rewards, we determined the number of responses during different reinforcement conditions (FR1, FR3, PR) for sugar pellets and 35% fat pellets. Separate groups of animals were used to examine responding for sugar pellets (control: $n=13$; high-fat: $n=14$) and fat pellets (control: $n=14$; high fat: $n=11$). As shown in Figure II-6A, operant responding under the three reinforcement schedules studied (FR1, FR2 and PR) did not differ when the two groups of animals earned sugar- enriched food pellets. Both groups of animals showed the expected increases in response rate with greater operant demands. This was also the case when animals worked for fat-enriched food pellets, except that here, the statistical analysis revealed a significant main effect of maternal diet with high-fat animals showing, relative to control animals, higher response rates across all reinforcement schedules (Figure II-6B). When sugar pellets were

used as reinforcement (Fig. II-6A), a two-way within-subject ANOVA revealed a significant session effect ($F(4,96)=1.04$, $P<0.0001$), but no diet effect ($F(1,96)=0.74$, $P>0.05$) and no session x diet interaction. In contrast, responses to 35% fat pellets (Fig. II-6B) revealed significant session ($F(4,92)=41.02$, $P<0.0001$) and diet effects ($F(1,92)=6.31$, $P=0.0195$), but no session x diet interactions. High-fat offspring displayed increased responding to fat pellets compared to control offspring. Breakpoint did not differ between high-fat and control offspring (data not shown).

We next examined whether the body weight of high-fat and control animals was similarly influenced by the food restriction regimen used in our experiment. As seen in Figure II-6C, high-fat offspring were heavier throughout the experiment (diet effect: $F(1,23)=6.20$, $P=0.0204$) and both control and high-fat offspring lost a comparable amount of weight with the food restriction procedure ($F(1,23)=577.75$, $P<0.0001$). This is confirmed when percent weight loss (II-6C insert) between both groups was examined ($P>0.05$). To determine the contribution of body weight to operant responding for fat pellets, linear regression analysis on the total number of responses during all reinforcement conditions as a function of body weight on the day of PR testing was examined (Figure II-6D). Interestingly, during fat reinforcement, body weight significantly predicted responses in the control animals ($F(1,12)=5.076$, $P=0.0438$, $r^2=0.2973$) but failed to predict responding in the high-fat offspring ($F(1,9)=0.05359$, $P>0.05$, $r^2=0.005919$).

Discussion

In these experiments, we examined whether early life exposure to high-fat could alter adult mesocorticolimbic DA neurotransmission and whether some of these changes could explain the blunted locomotor responses to AMP observed earlier in high-fat offspring (Naef et al., 2008). We report here that adult offspring of mothers fed a high- fat diet from the last week of gestation until weaning exhibit weaker AMP-induced increases in extracellular NAc DA levels, higher NAc DAT activity and reduced D_2 receptor mRNA in the VTA, suggestive of decreased expression of presynaptic D_2 autoreceptors in projection areas such as the NAc. Furthermore, since the mesolimbic DA system is an integral part of the neural circuitry controlling food intake (Shin et al., 2009), we tested whether exposure to high-fat during the perinatal and neonatal periods would alter the reinforcing properties of palatable food rewards. We found indeed, that as adults, high-fat and control offspring differed in their sensitivity to the reinforcing properties of fat-enriched food rewards.

We have previously documented that rats exposed to high-fat during early life show a dampened locomotor response to AMP and reduced behavioral sensitization to the drug with repeated administration compared to their control counterparts (Naef et al., 2008). We also eliminated the possibility that diet-induced changes in AMP pharmacodynamics and brain availability could be an important factor in these behavioral outcomes (Naef et al., 2008). The data presented here suggest that the attenuated locomotor response of high-fat animals to AMP is due, at least in part, to a dampening of the drug's stimulant action on NAc DA neurotransmission as determined by *in-vivo* microdialysis. In adult animals, reductions in NAc DA concentrations have been reported following either acute or chronic exposure to a high-fat diet (Davis et al., 2008; Geiger et al., 2009). The fact that the maternal diet-induced effects reported here were observed more than 2 months after the offspring had been weaned and maintained on the control diet clearly suggests that nutritional factors during early

life can have a significant impact on mesolimbic DA neurons, the functional consequences of which can persist into adulthood. Importantly, the blunted NAc DA response to AMP in the high-fat offspring indicates potential alterations in presynaptic mechanisms caused by early diet, some of which were addressed in our experiments (presynaptic D_2 expression, DAT and PFC DA activity).

NAc DA transmission is indirectly modulated by DA- sensitive PFC glutamate-containing output neurons that project to the NAc and VTA (Del Arco and Mora, 2008). Given that the PFC's DA afferentation reaches maturity relatively late (pre- and peri-pubertally) (Kalsbeek et al., 1988), this cortical structure appears to be especially vulnerable to neurodevelopmental challenges (Brake et al., 2000) and therefore, represent a potential mechanism by which NAc DA could be altered by early diet. To examine the responsiveness of mesocortical projections, DA responses to AMP administration in the PFC were measured with *in vivo* microdialysis in control and high-fat offspring. We observed higher baseline concentrations, but a smaller DA response to AMP administration in the PFC compared to the NAc, in agreement with the lower concentration of the DAT in this region (Moron et al., 2002). Lower concentrations of DAT in the PFC slow the DA uptake process and thus, increase baseline DA levels compared to striatal regions. However, since DAT is a primary target for AMP, lower DAT levels in the PFC also dampen the extracellular DA response to this drug. Contrary to the NAc, no effect of early fat exposure was seen on the PFC DA response to AMP. The finding that maternal diet altered the NAc but not the PFC DA response to AMP is significant because it suggests that the effects of maternal diet on NAc DA transmission are mediated by a mechanism that is unique to meso-NAc DA neurons. This might involve receptors for metabolic hormones such as leptin which are expressed by VTA NAc DA neurons (Fulton et al, 2006) but not by VTA PFC DA neurons. Recent studies have also failed to observe alterations in mesocortical DA after acute or chronic high-fat feeding in adults (Davis et al., 2008; Geiger et al., 2009).

In subcortical structures such as the NAc, reuptake by the DAT is the

primary mechanism by which DA is cleared from the extrasynaptic space (Jones et al., 1998). Thus, DAT is a prime target mechanism through which maternal diet might influence DA neurotransmission in this region. The regulation of the DAT appears to occur via rapid trafficking of the DAT from the cell surface where it is able to uptake DA and therefore regulate extracellular DA concentrations, to internalization of the DAT (reviewed in Zahniser and Sorkin, 2004). Reductions in caudate DAT have been reported in adult rats under multiple and somewhat opposing "metabolic" conditions such as maintenance on a high-fat diet (South and Huang, 2008), food restriction (Zhen et al., 2006) and after insulin administration (Williams et al., 2007). In earlier studies, we failed to detect a significant effect of maternal diet on the density of NAc DAT binding sites as determined by autoradiography, which detects the total amount of DAT binding regardless of location (cell surface vs. internalized, Naef et al., 2008). Here, we used a more functional approach to measure DAT activity by determining DA uptake from synaptosomes isolated from the NAc and the CP. We found a relatively higher uptake capacity (Vmax) of NAc (but not CP) DAT sites in high-fat compared to control animals, suggesting a higher cell surface expression of DAT in the presynaptic terminals of high-fat compared to control offspring. The greater uptake capacity of NAc DAT sites seen in high-fat animals could account for their weaker NAc DA response to AMP, although our ability to speculate on the relationship between NAc DAT activity and locomotor activation after AMP is limited. Positive correlations between these two variables have been demonstrated for cocaine, but not for AMP (Briegleb et al., 2004).

Alternatively, changes in the presynaptic D_2 autoreceptor population in the NAc might also significantly contribute to alterations in AMP-stimulated DA release. Alterations in D_2 receptor binding have been reported in human and animal diet-induced obesity, although the direction of these changes is inconsistent. While human experiments point to a downregulation of striatal D_2 receptors in obese subjects (Wang et al., 2001), high-fat feeding increased D_2 receptor binding in mice (South and Huang, 2008). Two isoforms of the D_2

receptors have been identified (Giros et al., 1989). While the long-form of the striatal D_2 receptor is expressed mostly post-synaptically (Khan et al., 1998), the short-form of the striatal receptor is a pre-synaptic autoreceptor and inhibits DA synthesis and release (Khan et al., 1998). Our data demonstrate no alterations in the expression of postsynaptic D_2 receptors in striatal regions, but a decreased expression of D_2 mRNA in the VTA of high-fat adult offspring although the primers used for the *in-situ* hybridization do not allow to distinguish between expression of the long and short form of the D_2 receptor. However, using immunohistochemistry, Khan and colleagues (1998) have shown that it is primarily the short form of the D_2 receptor that is expressed and is unique to DA neurons in the VTA. Expression of the short form is observed in the cell bodies, distal dendrites and axon terminals and ac- cording to these authors, D_2 receptor mRNA detected in the VTA represents mostly the short form of the DA D_2 receptor and constitutes mainly either VTA somatodendritic D_2 autoreceptors or striatal presynaptic terminal D_2 autoreceptors. In either case, a decrease in D_2 autoreceptors suggests that, in high-fat animals, meso-NAc DA transmission is less stringently regulated. The functional implication of this is that negative feedback regulation of NAc DA transmission is less efficient in high-fat animals. Interestingly, in previous work, we reported increased VTA expression of tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, in the high-fat offspring (Naef et al., 2008), perhaps a consequence of decreased D_2 autoreceptors (Lindgren et al., 2001). How do we reconcile the reduced presynaptic DA autoregulation with reduced DA responses to AMP in high fat offspring? We propose that alterations in NAc DAT represent a compensatory mechanism in response to decreased pre-synaptic inhibition of DA production and release in the high-fat animals. There is also evidence that D_2 autoreceptors directly associate with the DAT and regulate DAT trafficking (Bolan et al., 2007). This blunted regulation may potentially be a significant contributor to the heightened responsivity to fat rewards in high-fat adult offspring.

The present data confirm our previous autoradiography data indicating no effect of early diet on NAc postsynaptic D_2 receptors. This is in contrast to postsynaptic D_1 receptors in this region which are significantly increased in high-fat offspring. It is well recognized that these receptors dose-dependently increase locomotion (Dreher and Jack- son, 1989) and a large body of literature suggests that locomotor activation is mostly dependent on the balance between D_1 and D_2 post-synaptic striatal receptors (Paul et al., 1992). An altered D_1/D_2 ratio might therefore be observed in high-fat offspring, thus modulating the behavioral response to post-synaptic D_2 agonists. Indeed, we found that high-fat offspring are more sensitive than controls, to the locomotor-stimulating effect of quinpirole, a selective $D_{2/3}$ receptor agonist. Our data confirmed earlier reports that quinpirole has an immediate inhibitory effect on locomotion (Figure 5C) followed by a large activation effect (Figure 5D) (Eilam & Szechtman, 1989; Benaliouad et al., 2009). Sixty minutes following s.c. injection of a high dose of quinpirole (0.5 mg/kg), we found that high-fat offspring displayed increased locomotor activation compared to control animals. At this time, we can only speculate as to the implications of these findings but two main explanations seem plausible. Since quinpirole is a $D_{2/3}$ receptor agonist, increases in locomotion in high-fat offspring might reflect a reduction in D_3 receptor expression as this receptor has been reported to have inhibitory effects on locomotion (Waters et al., 1993). Second, although the number of postsynaptic D_2 receptors might not be different between diet groups, intracellular signaling via D_2 receptors might be altered, leading to a more efficient transduction into behavioral effects.

One of the critical consequences of alterations in mesolimbic DA induced by exposure to high-fat during development is that it might "program" behavioral responses to a variety of rewards including palatable food. To investigate the long-term consequences of early exposure to high-fat on DA-dependent feeding, we compared the strength of operant responding of high-fat and control offspring for two highly palatable food rewards (sugar and 35% fat), earned under three

different reinforcement schedules. While no group differences were observed when sugar pellets were used as a reinforcer, high-fat offspring displayed increased responding for 35% fat pellets compared to control offspring suggesting that some components of the fat pellets were more rewarding or "appealing" to offspring of the high-fat group compared to controls. How early exposure to high-fat can selectively increase operant responding for pellets containing 35% fat, but not sugar pellets is unclear at present. Food preferences emerge from the integration of distal cues (sight, smell) along with gustatory (texture, taste) and post-ingestional signals (Gaillard et al., 2008). In the operant paradigm involving a fixed and relatively short session duration, it is likely that the early signals might play a more critical prominent role than delayed ones. It could be argued that familiarity of the high-fat offspring with higher fat content of presented food pellets might increase responding in this group. We believe that this is unlikely for two reasons. First, exposure to the maternal high-fat diet was restricted to the pre-weaning period and all animals were tested after postnatal day 90, long after cessation of the diet and second, both control and high-fat offspring were habituated to the pellets during training sessions before the progressive ratio protocol. Other possible mechanisms dictating a preference for fat in the high fat offspring might be related to changes in the ratio of D_1 and D_2 receptors present in brain regions implicated in reward circuitry. Indeed, it is recognized that antagonism of D_1 vs. D_2 DA receptors differentially impacts the consumption of fat and sucrose in adult rats and these effects are dependent on the feeding conditions of the animals (Corwin and Wojnicki, 2009).

Because differences in body weight might be important predictors of operant responding, we examined changes in weight loss induced by food restriction in both diet groups and the contribution of weight at the time of testing on operant responses. While high-fat animals remained heavier throughout the experimental protocol (before and after our food restriction paradigm), food restriction induced a similar amount of weight loss in both diet groups. Interestingly, weight was a significant predictor of responses towards fat pellets in

control offspring, but operant responding could not be predicted by weight in high-fat offspring. These analyses suggest that the control offspring's responses to fat are sensitive to changes in weight (and perhaps adiposity signals such as leptin and insulin) with heavier animals responding less, while responding in the high-fat offspring is increased compared to controls, regardless of fluctuations in weight between animals. Because some components of the mesolimbic DA system are direct targets for metabolic hormones (i.e. leptin, insulin, ghrelin), it is possible that changes in circulating levels of these hormones at the time of testing in adulthood (Walker et al., 2008) and/or sensitivity of DA neurons to these hormones alter operant responding for fat-rich rewards.

Conclusions

Our data provide evidence for a critical role of early fat exposure on modulation of the adult mesolimbic DA system. We initially demonstrated a reduced NAc DA response to AMP in high-fat offspring, suggestive of a pre-synaptic dysregulation in these animals. We found that DA dysregulation was not a consequence of disrupted PFC DA response, but that a small, but maybe important functional change in the NAc DAT, the main uptake mechanism in this region would contribute to alter DA responses in high-fat animals. Finally, the reduction in D_2 autoreceptors mRNA levels in the VTA suggests that DA regulation at terminal target sites such as the NAc might be modified due to a reduction in D_2 negative feedback inhibition in high-fat offspring. Most interesting in the context of "programming" of metabolic disorders and obesity was our finding that adult offspring of high-fat mothers displayed increased operant responding to fat, but not to sugar pellets and that their operant responses towards fat were not related to their weight at the time of the experiment. These data point out to a complex interaction between early metabolic changes induced at the time of exposure to different maternal diets (D'Asti et al., 2010) and later sensitivity of mesolimbic DA circuitry to the rewarding properties of food. Although the precise mediators and underlying mechanisms are still uncertain, it

is clear from our data that early dietary change during a critical window of brain development leads to behaviors favoring the development of obesity.

Figures

Figure II-1. Microdialysis probe placements in the NAc and PFC.

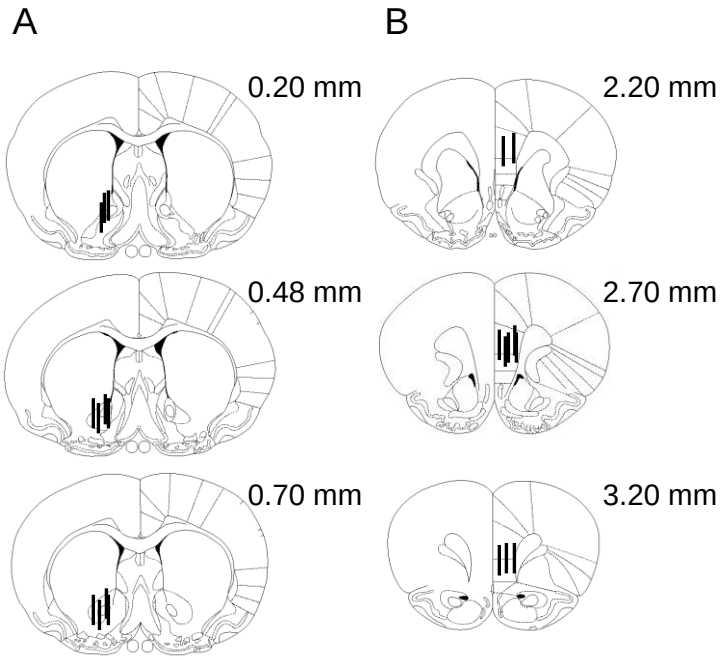

Figure II-1. *Representative drawings of microdialysis probe locations in the NAc (A) and mPFC (B) of six animals in either the high-fat or control diet groups. Drawings are according to the atlas of Paxinos and Watson (1998), in a caudal to rostral distribution using bregma (mm) as a reference point. Paxinos G, Watson C (1998) Adaptation of image published in rat brain in stereotaxic coordinates, Fourth Edition. New York: Academic Press. Copyright was obtained from Academic Press (Elsevier).*

Figure II-2. Baseline and AMP-stimulated NAc and PFC DA.

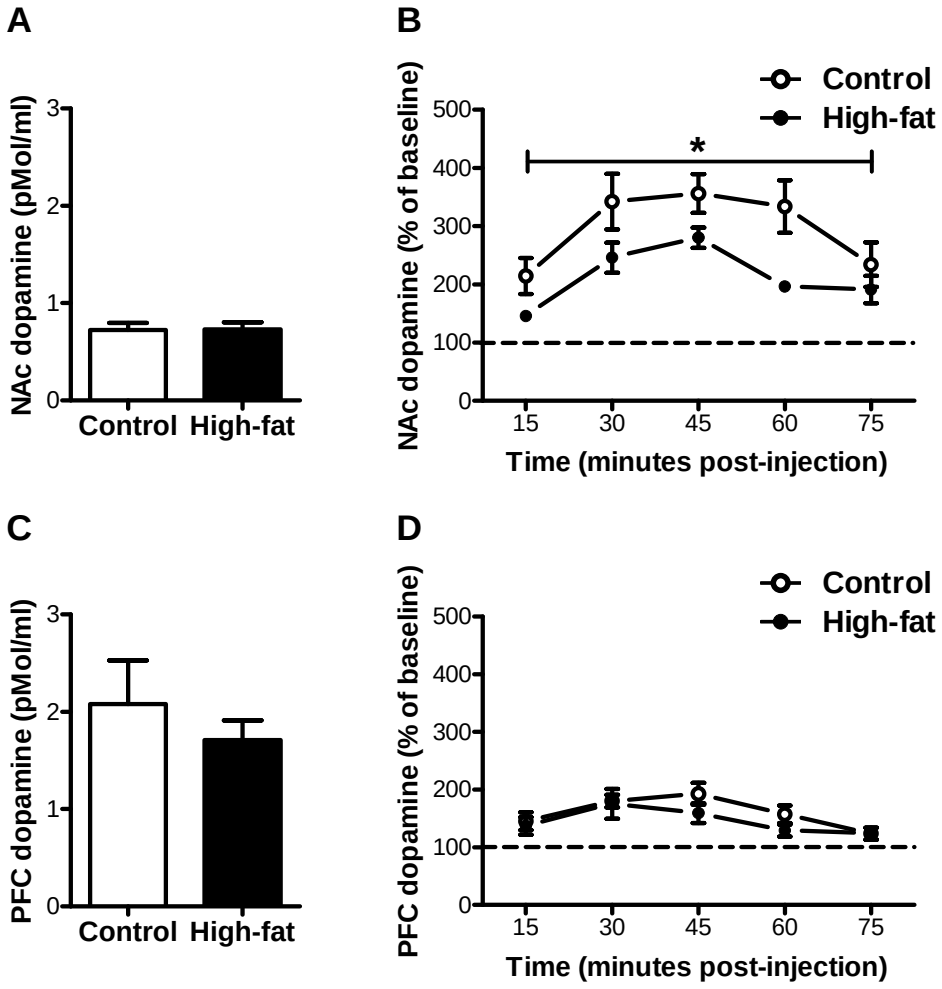

Figure II-2. *Blunted NAc DA response to AMP in high-fat animals. Baseline (A, C) and AMP-stimulated (B, D) DA concentrations in the NAc (A, B) and mPFC (C, D) of adult control and high-fat offspring, as measured by in vivo microdialysis. No differences in baseline DA concentrations were observed in the NAc (A) or mPFC (C) between control and high-fat offspring. DA response to AMP was significantly reduced in high-fat offspring compared to controls ($P<0.05$) in the NAc (B), but not in the mPFC (D). Values represent the average±SEM of 7–10 rats/group.*

Figure II-3. DA uptake through DAT and 5HT-uptake through VMAT.

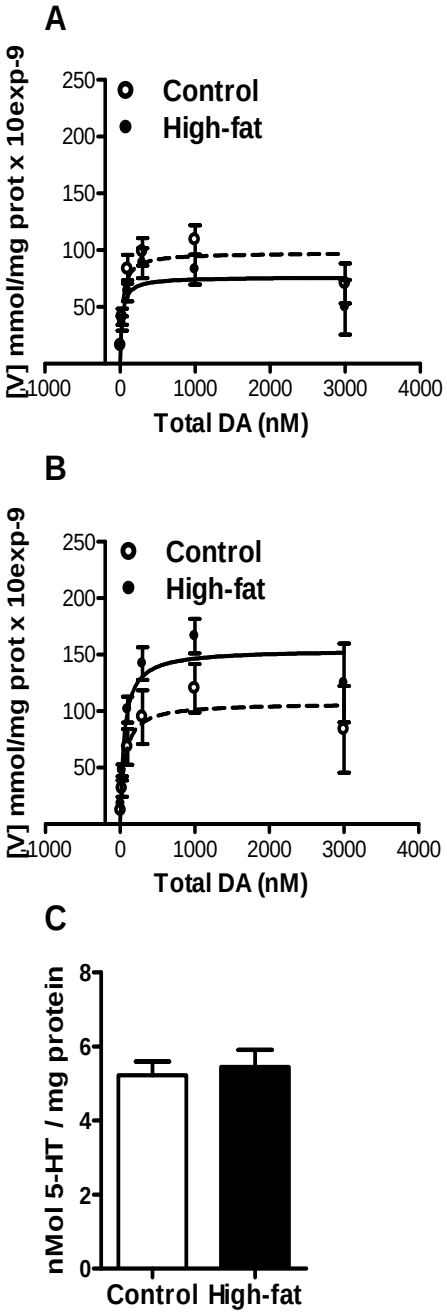

Figure II-3. *Synaptosomal DA uptake through DAT in the CP (A) and NAc (B) and vesicular 5HT-uptake through VMAT in the striatum (C) of high-fat and control offspring. Kinetic characteristics (Vmax and Km) of synaptosomal DAT-mediated DA uptake were determined using the Michaelis–Menten regression in tissue punches. There was no diet- induced difference in the affinity (Km) of DA for DAT in either region, but a close to significant difference in DAT capacity (Vmax) between diet groups in the NAc (B, P=0.0548). There were no diet-induced changes in 5HT uptake through VMAT in tissue chunks containing the CP and NAc. Values represent the mean±SEM of four different uptake experiments.*

Figure II-4. Expression of D_1 and D_2 mRNA levels.

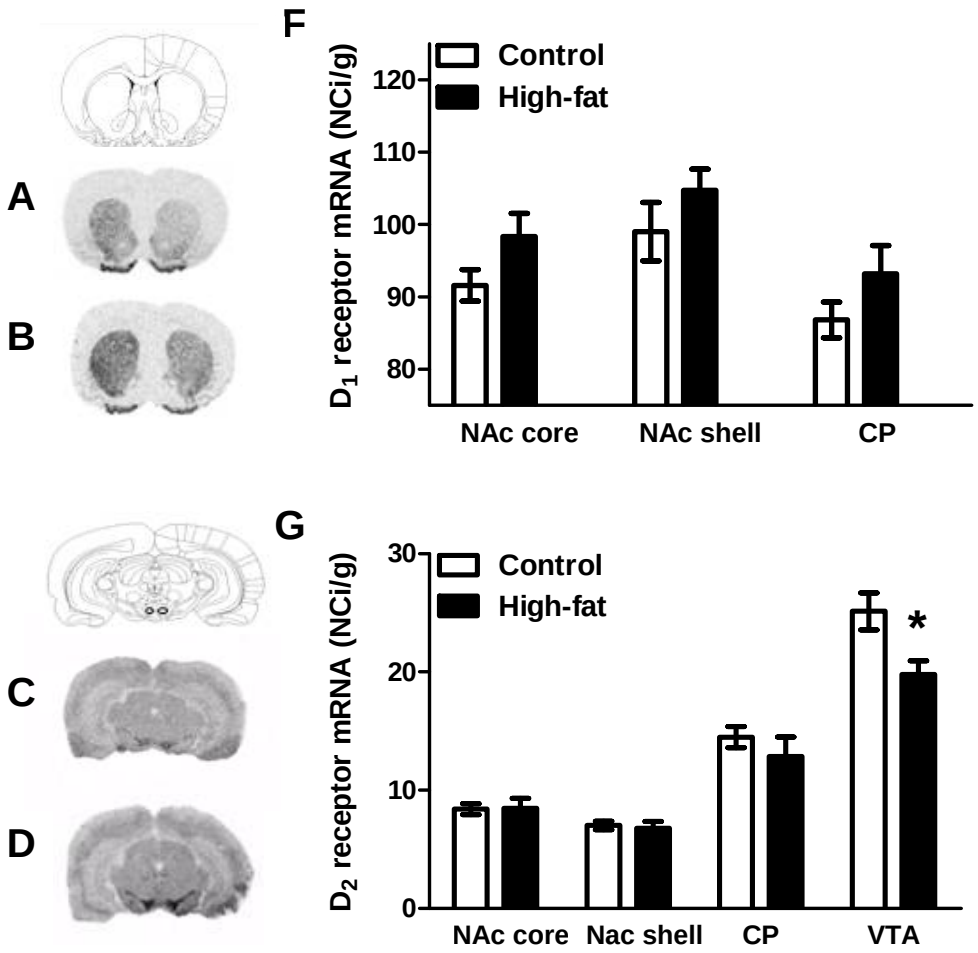

Figure II-4. *Expression of D_1 (E) and D_2 (F) mRNA levels (nCi/g) by in situ hybridization in the NAc (core and shell), CP and VTA of control and high-fat offspring. Representative images of D_1 mRNA detection are given in the CP/NAc region in control (A) and high-fat (B) adult offspring. Similarly, representative images of D_2 mRNA detection are given for the VTA in control (C) and high-fat (D) offspring. Coordinates are according to Paxinos and Watson (1998). D_2 autoreceptors mRNA levels in the VTA of high-fat offspring were significantly reduced ($P<0.01$) compared to control rats. Values represent the mean±SEM of six animals/diet group.*

Figure II-5. Locomotor responses to saline and quinpirole.

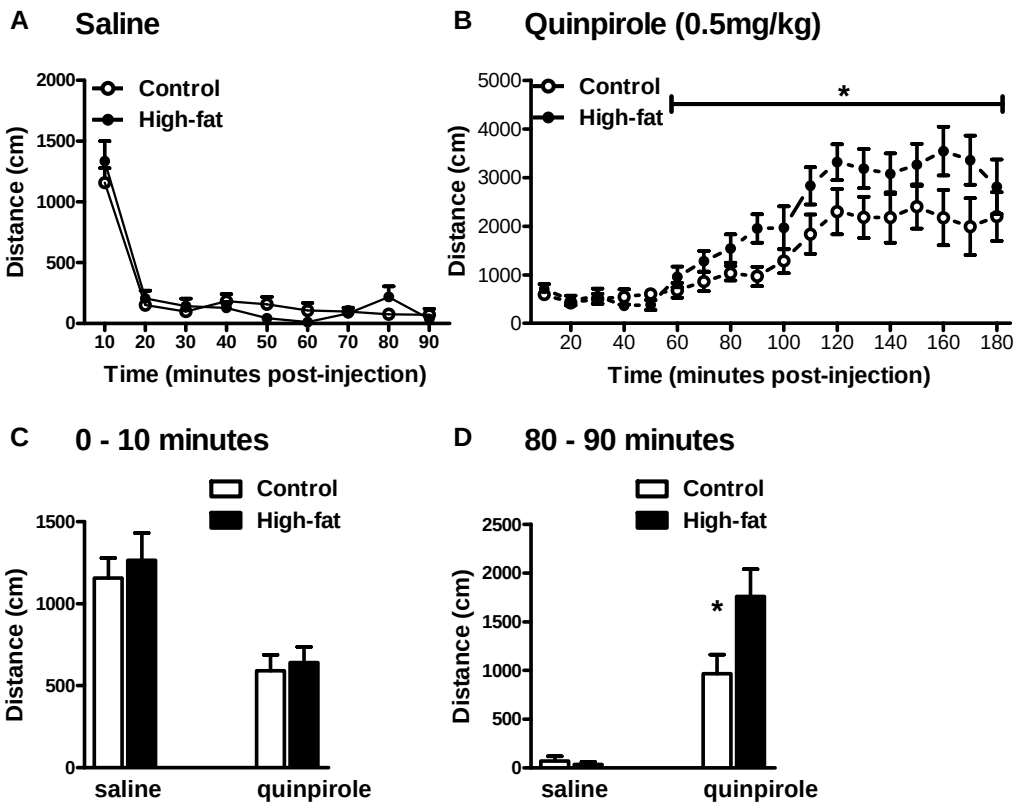

Figure II-5. *Locomotor responses to either saline (A) or quinpirole (B) injection in adult male offspring from control and high-fat diet groups. Locomotor responses (cm) to s.c. saline did not differ between high-fat and control offspring (A) while quinpirole (0.5 mg/kg, s.c.)-induced locomotor responses was higher in high-fat compared to control offspring from 60 min on (shaded area, $P<0.05$). (C) Inhibition of locomotor activity by quinpirole in both diet groups within the first 10 min of treatment (compared to saline). (D) Activation of locomotor responses by quinpirole compared to saline injection at the 80 –90 min time interval. Values represent the mean±SEM of 14 –16 animals for each diet group.*

Figure II-6. Operant responses for sugar and 35% fat pellets.

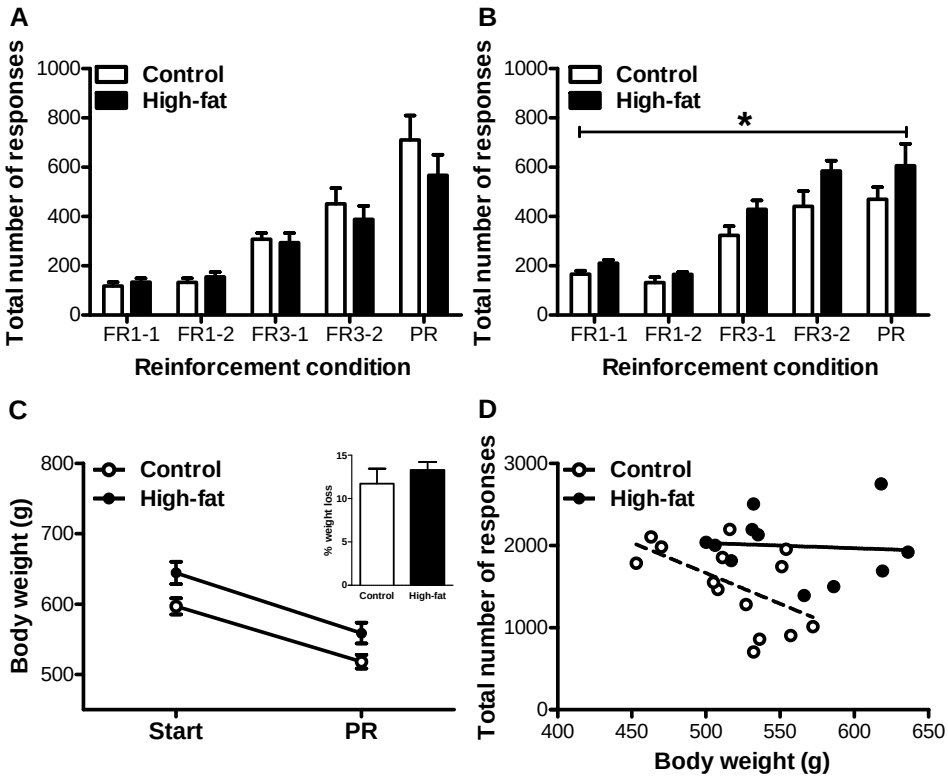

Figure II-6. *Operant responses for either sugar pellets (A) or 35% fat pellets (B) in adult male offspring from the control and high-fat diet groups. The total number of responses during different reinforcement schedules (FR and PR) is depicted. A significant diet effect was observed for fat pellets ($P<0.05$), but not for sugar pellets. (C) Body weight of control and high-fat offspring before food restriction (Start) and on the day of PR testing. High-fat animals were heavier than controls throughout the experiment ($P<0.05$) but lost a similar percentage of their body weight in the course of the experiment (insert). (D) Linear regression analysis of the total number of responses for 35% fat pellets as a function of body weight at the time of PR responding. Body weight was a significant predictor of responses in control offspring ($P=0.0438$, $r^2=0.2973$), but not in high-fat offspring ($P=0.8221$, $r^2=0.005919$). Values represent the mean±SEM of eight control and 11 high-fat animals.*

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II.4 Supplemental data and discussion

Background

In the previous manuscript, we show that high-fat exposed offspring display increased operant responses to 35% fat pellets relative to control offspring (Naef et al., 2010). The acquisition of operant behavior as well as testing under different schedules of reinforcement was conducted following ten days of mild food restriction when both groups of animals were at 85% of their original body weight. Altering the metabolic state of the animal by food restriction amplifies reward-related behavior (Carr, 2007). The enhanced behavioral sensitivity to reward induced by weight loss seems to be mediated, at least in part, by leptin (Fulton et al., 2000) and glucocorticoids (Deroche et al., 1993). Offspring exposed to the high-fat diet display increased plasma concentrations of leptin and corticosterone during the neonatal period (d'Asti et al., 2010) and are heavier in adulthood, suggesting that the increased operant responses of high-fat exposed offspring might not only reflect programming of the DA system by early high-fat, but also differences in the hormonal profile of the high-fat exposed offspring during adulthood and hormonal responses to food restriction and operant training. In this experiment, we hypothesized that alterations in the hormonal profile of adult high-fat offspring might contribute to the increased operant responding to high-fat rewards. The goal of the present experiment was to measure plasma concentrations of leptin and corticosterone prior to and following weight loss, and immediately prior to the progressive ratio test in control and high-fat offspring.

Experimental procedures

Operant responding for high-fat pellets (Dustless Precision Pellets, 45 mg 35% fat, Bio-Serv, product # F05989) was conducted as previously described (Naef et al., 2010). Briefly, following ten days of modest food restriction to decrease body weight to 85% of initial weight, animals were trained to lever press, then tested under three schedules of reinforcement: fixed ratio 1 (FR1), fixed ratio 3 (FR3) and progressive ratio (PR). The PR schedule of reinforcement

was based on Richardson and Roberts (1996) and followed a modified logarithmic progression. The total number of lever presses during each session was used as a measure of incentive motivation. During PR, the number of food rewards earned, which reflects the breakpoint was used as a measure of incentive motivation. To examine the hormonal profile of control and high-fat offspring, a total of 5 blood samples were collected throughout the behavioral experiment. All samples were collected via the tail vein in EDTA-containing tubes. Blood samples were collected at 1 hr before lights off (PM) and at 1 hr after lights on (AM), both prior to and after 10 days of food restriction to determine diurnal variations in hormone levels. In order to determine hormonal concentrations present at the time of PR testing, an additional tail blood sample was collected on the 4th day of operant testing (PR), immediately before placing the rats in the operant boxes. Plasma corticosterone (MP Biomedicals) and leptin (Linco Research Inc, St-Charles MO) concentrations were measured using rat-specific radioimmunoassay kits as previously described (Proulx et al., 2001). The limit of detection of the assays was 0.3125µg/dl (corticosterone) and 0.5 ng/ml (leptin).

Results

Adult male rats were tested under three schedules of reinforcement: FR 1, FR 2, and PR. As illustrated in Figure II-7A, adult high-fat offspring displayed increased operant responses relative to controls across all reinforcement schedules. A two-way repeated measures ANOVA revealed a significant reinforcement condition ($F(4, 68) = 29.86$, $p < 0.0001$) and diet effect ($F(1, 68) = 5.531$, $p = 0.031$). Figure II-7B displays the number of rewards earned during the PR test which were not significantly different between diet groups ($p = 0.0856$) although there was a trend towards high-fat offspring earning more food rewards.

Although the high-fat offspring were heavier throughout the experiment, both diet groups lost a comparable amount of weight following food restriction (Figure II-7C). A time x diet ANOVA conducted on weight (g) showed significant time ($F(11, 132) = 119.3$, $p < 0.0001$) and diet ($F(1, 132) = 13.62$,

p=0.0031) effects. Blood samples were collected at various experimental stages (Figure II-7C) for hormonal determinations. Corticosterone concentrations in control and high-fat offspring displayed diurnal variations that were obliterated after food restriction (Figure II-7D). A two-way repeated measures ANOVA revealed a significant time of day (AM vs PM, $F(1, 17) = 55.77$, $p < 0.0001$) but no diet effect prior to food restriction. While AM corticosterone concentrations increased with food restriction in both control and high-fat offspring ($F(1, 17) = 31.07$, $p < 0.0001$), PM concentrations decreased ($F(1, 17) = 20.87$, $p=0.0003$). A time x diet ANOVA for AM leptin concentrations (Figure 7E) showed a significant time ($F(2, 26) = 54.81$, $p < 0.0001$), but no diet effect. There were no significant group differences in corticosterone or leptin levels immediately prior to the PR session.

Discussion

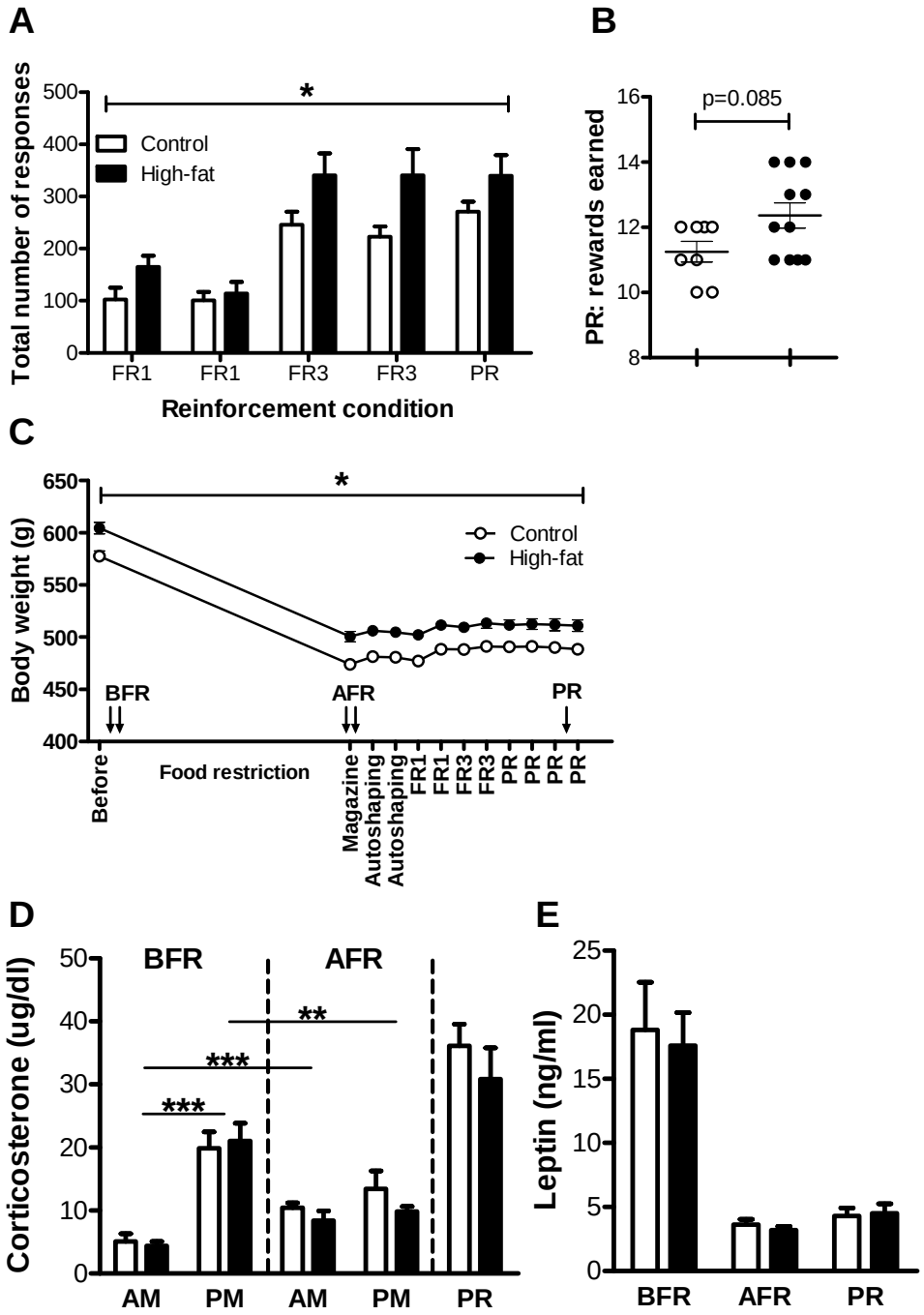
We previously documented that adult offspring exposed to high-fat during early development displayed increased operant responding for fat-enriched (35% fat), but not sugar-enriched rewards compared to control offspring (Naef et al., 2010). Tested under three schedules of reinforcement, we confirmed our previous observation and determined that the increased operant behavior observed in high-fat offspring could not be linked to diet-induced changes in circulating concentrations of either corticosterone or leptin at the time of PR testing. Interestingly, food restriction prior to training elevated plasma levels of corticosterone at the time of the diurnal through (AM) and reduced the diurnal peak secretion (PM), consistent with changes observed under chronic stress conditions. This change in diurnal pattern might be associated with the stress of food restriction and contribute to some aspects of DA-mediated behaviors. Plasma corticosterone levels were high in both diet groups before the PR session, reflecting the anticipation to feeding (PR sessions and daily meal provided following the PR session) and/or the stress related to the context in which testing occurs. However, no diet-induced differences in corticosterone were observed.

Food restriction led to the expected reduction in leptin concentrations in both diet groups and no changes were observed before PR testing. These findings suggest that the increased operant responding observed in the high-fat offspring cannot be attributed to changes in circulating leptin or corticosterone concentrations in these animals during testing.

In conclusion, our results indicate that exposure to high-fat during a critical period of development induces long-lasting perturbations in DA function and behavior and that these changes occur independently of circulating metabolic hormone concentrations at the time of behavioral testing.

Figure II-7. (*A) Total number of responses (lever presses) across different reinforcement conditions (FR1, FR3, PR) in Control (white) and high-fat (Black) adult offspring working for 35% fat 45mg food rewards. High-fat offspring displayed increased responding relative to Controls. (B) The number of rewards earned during the PR test by control and high-fat offspring. A trend (p=0.0856) towards an increase in the number of rewards earned by the high-fat group was observed (C) Evolution of body weight (g) of control and high-fat adult male offspring throughout the experimental protocol. While rats in both diet groups lost similar amounts of weight with the food restriction paradigm, the high-fat animals remained heavier throughout. Arrows represent timing of blood collection before (BFR) and after (AFR) food restriction, and on the last day of PR testing (PM only). (D) Plasma corticosterone concentrations (µg/dl) of control and high-fat offspring at the start of the experiment (BFR, AM and PM), following food restriction (AFR, AM and PM), and on the day of PR testing (PR). (C) Plasma leptin concentrations (ng/ml) before the start of the experiment (BFR, PM), following food restriction (AFR, PM) and immediately prior to PR testing (PM) in control and high-fat offspring. No significant diet group differences in corticosterone or leptin concentrations were observed. Values are means ± SEM of 9-10 rats per group. * p<0.05, ** p<0.01, *** p<0.001.*

Figure II-7. Operant responses for 35% fat pellets and hormones



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Chapter III: Reduced anticipatory Dopamine responses to food in rats exposed to high-fat during early development

III.1 Preface

The previous chapter demonstrates that exposure to high-fat during early development significantly alters the presynaptic regulation of DA and the NAc DA response to amphetamine, with high-fat offspring showing a reduced response relative to controls. In addition to its effect on DA function, exposure to high-fat increased incentive motivation for high-fat food rewards, a behavior that is mediated by NAc DA. The current manuscript examines whether the increased operant responding for high-fat food rewards in high-fat-exposed offspring is the outcome of differences in the pattern and amplitude of NAc DA during the anticipation and consumption of these high-fat food rewards (aim 1).

III.2 Contribution of authors

L. Naef: Design, execution, analysis of data and writing of the manuscript.

L. Moquin: Construction of recording electrodes.

Dr. Claire-Dominique Walker and Dr. Alain Gratton: design, editing of the manuscript.

III.3 Manuscript – Short communication

Title: Reduced anticipatory dopamine responses to food in rats exposed to high-fat during early development

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Journal: Internationl Journal of obesity, e-pub ahead of print

Keywords: high-fat diet, maternal programming, dopamine, food reward, Pavlovian conditioning, nucleus accumbens

Abstract

We have previously demonstrated that exposure to high-fat during early development alters the presynaptic regulation of mesolimbic dopamine (DA) and increases incentive motivation for high-fat food rewards. The goal of the present experiments was to examine the long-term consequences of early exposure to high-fat on anticipatory and consumatory nucleus accumbens (NAc) DA responses to high-fat food rewards. Mothers were maintained on a High-fat (30% fat) or Control diet (5% fat) from gestation day 13 to postnatal day 22 when offspring from both diet groups were weaned and maintained on the Control diet until adulthood. *In vivo* NAc DA responses to food anticipation and consumption were measured in a Pavlovian conditioning paradigm using voltammetry in freely moving rats. High-fat exposed offspring displayed reduced NAc DA responses to a tone previously paired with the delivery of high-fat food rewards. In an unconditioned protocol, consumatory NAc DA responses could be isolated and were similar in High-fat and Control offspring. These data demonstrate that exposure to HF through maternal diet during early development might program behavioral and functional responses associated with mesolimbic DA neurotransmission, thus leading to an increased high-fat feeding and obesity.

Introduction

It is now recognized that pre- and postnatal maternal consumption of calorie dense food increases the offspring's susceptibility to the development of obesity (Levin, 2006) although the underlying mechanisms are still unclear. Mesolimbic dopamine (DA) neurotransmission, which mediates the rewarding properties of food and food cues represents a possible candidate. DA function is altered in diet-induced obesity in both humans (Stice et al., 2008; Frank et al., 2012; Green et al., 2011) and animals (Davis et al., 2008; Geiger et al., 2009; Li et al., 2009; Sharma et al., 2012) and DA projections develops for a large part postnatally (Antonopoulos et al., 2002), making them susceptible to the "organizational effects" of early diet. Along with other groups (Teegarden et al., 2009; Vucetic et al., 2010), we previously demonstrated that exposure of the mother to high-fat during the last week of gestation and throughout lactation blunted locomotor and Nucleus accumbens (NAc) DA responses to amphetamine and reduced presynaptic expression of DA D2 receptors in adult offspring (Naef et al., 2010; Naef et al., 2008). Behaviorally, these functional changes led to an increase in incentive motivation for high-fat food pellets, as measured with three reinforcement conditions in an operant conditioning paradigm (Naef et al., 2010).

A large body of evidence indicates that increased NAc DA transmission is necessary to generate the behavioral responses elicited by food and signals anticipation rather than food consumption (Richardson & Gratton 2008). In the present experiments, we tested the hypothesis that early exposure to high fat through the maternal milk programs NAc DA responses to food and food cues in the adult offspring. We used *in-vivo* voltammetry in freely moving rats to monitor rapid fluctuations in extracellular NAc DA concentrations during the anticipation and consumption of high-fat food rewards in adult offspring exposed to high-fat during early development. We demonstrate that high-fat offspring display a reduction in their anticipatory, but not consumatory DA responses to food, suggesting that the increased operant responding to fat pellets in these rats (Naef et al., 2010) is a consequence of maternally-programmed DA hypofunction.

Experimental procedures

Animals

Female Sprague-Dawley rats (Charles River, Quebec) were placed on powdered diets from Harlan Teklad. High-fat diet (30% fat, 24% carbohydrate, 15% protein, 4. 54 kcal/g, HF) or Control diet (5% fat, 60% carbohydrate, 15% protein, 3. 45 kcal/g) were given from gestation day 13 to postnatal day (PND) 22. Litters we culled to 10 pups, weaned at PND22 and fed the Control diet until testing. Animals were housed under controlled conditions of light (12:12 h light/dark cycle), temperature and humidity. Procedures were approved by the Animal Care Committee at McGill University.

Electrode implantation

Recording electrodes consisted of 30mm Nafion-coated carbon fibers placed in a glass capillary as previously described (14). Prior to implantation, electrodes were calibrated *in vitro*. Specificity was tested with ascorbic acid and measured by increasing concentrations of DA. Only electrodes with a minimum specificity for DA of 1000:1 and a highly linear response to DA (r >.9997) were implanted. Electrodes aimed at the shell of the NAc (lateral: -1.6mm, AP: +1.6, DV: -7.6 mm from bregma) and Ag/AgCL references electrodes aimed at the contralateral parietal cortex were implanted under isoflurane anesthesia. Pin connectors were soldered to both electrodes and inserted into a plastic strip connector anchored to the skull. Electrode placements were confirmed postmortem using the atlas of Paxinos and Watson (1998).

Electrochemical recordings

Electrochemical recordings were performed as previously described (14) using a computer-controlled high-speed chronoamperometric instrument (Quanteon, Lexington KY). An oxidation potential of +0.55 V (with respect to the reference electrode) was applied to the electrode for 100ms at a rate of 5Hz. The amplitude of the oxidation current was digitized and integrated over the last 80ms

of the pulse. Currents were averaged and converted into µM concentrations using the *in vitro* calibrations. The concentration of DA (µM) at the onset of each tone was used to calculate the mean change in signal (µM) for 10 seconds prior and for 60 seconds following this timepoint.

Testing Procedure

Animals were tested in adulthood (>PND 90) in the dark phase of the light/dark cycle and 3-4 days post surgery. Animals underwent four days of conditioning with 15 trials / day. Each trial consisted of a 30 second 90 decibels tone. At 30 seconds, a 'click' arose from the food dispenser and a 45 mg high-fat pellet (Dustless Precision Pellets, 45 mg 35% fat, Bio-Serv) was delivered. DA oxidation currents were measured throughout the 60 trials. In the unpaired condition, trial conditions were similar, but the cues did not predict the delivery of the pellet. A variable inter-trial paradigm was used in both conditions.

Results and discussion

Reduced anticipatory DA responses to food cues in HF vs. Control offspring

Mean change in NAc DA signal (µM) on days 2, 3, and 4 of conditioning is illustrated in Figure III-1A (Controls) and III-1B (HF). In both diet groups, repeated pairing of the compound cue (tone and 'click') with the delivery of a food pellet led to an anticipatory DA response. In both diet groups, modest but significant increases in DA were observed during the 30 second tone. The end of the tone coupled with the 'click' of the food dispenser induced a large rise in DA concentrations with a peak that occurred 2-3 seconds after the 'click'. The dropping of the pellet into the food hopper occurred approximately 2 seconds after the click, which coincides with feeding onset. Thus, we can attribute the large DA increase observed to an anticipatory rather than a consumatory response. Our results agree with previous research demonstrating that NAc DA transmission is activated primarily by conditioned cues that reliably predict a positive outcome (i.e. receiving or earning food) and that this activation ceases once the expected food is presented and consumed (Richardson & Gratton, 2008). More importantly, our results demonstrate that NAc DA responses to the tone (III-1C) and the 'click' (III-1D) were decreased in HF vs. CD offspring on day 4 of conditioning, although no significant differences were observed in earlier days. This effect cannot be attributed to learning deficits since rats in both diet groups immediately consumed the pellet upon delivery on day 4 of testing.

The diminished DA response to the cued presentation and subsequent consumption of high-fat food rewards in adult offspring exposed to HF during early development is remarkable since these offspring were only exposed to high-fat during the preweaning period. This suggests that perinatal and postnatal programming of NAc DA function modified the anticipatory response to food cues, potentially influencing the overall food consumption in the paired condition. Nucleus accumbens DA hyporesponsiveness in HF offspring extends to other modalities than food cues since we previously found reduced locomotor and NAc

DA responses to amphetamine (Naef et al., 2010; Naef et al., 2008).

Our results parallel earlier reports in humans (Frank et al., 2012; Green et al., 2011; Stice et al., 2008;) and rodents (Davis et al., 2008; Geiger et al., 2009; Li et al., 2009; Sharma et al., 2012) showing that diet-induced obesity is associated with reduced DA function. Whether this hypo DA function results from the development of obesity or is a factor predisposing individuals to the development of obesity remains unclear. Our results suggest that maternal diet and the resulting perinatal nutritional environment can program DA function and that NAc DA hypofunction can occur prior to the development of obesity since our HF rats were not obese when tested. Furthermore, electrically evoked DA release was also found to be reduced in mesocorticolimbic terminal regions of obesity prone rats (Geiger et al., 2008). Together, these data support the hypo-sensitivity to reward hypothesis of obesity, which postulates that in individuals with blunted DA function, the excessive consumption of palatable foods serves to reach a threshold of reward contributed by mesolimbic DA activation (Shin et al., 2012). However, the present findings reveal an interesting dissociation between anticipatory DA responses and operant behavior towards the same fat rewards (Naef et al., 2010), which suggests that systems other than mesolimbic DA participate in the incentive motivation in HF offspring.

Similar consumatory DA responses in the unpaired condition between Control and HF offspring

On day 4 of testing in the unpaired condition, when the compound cue did not predict the arrival of the food pellet, DA responses to the cue were close to zero in both Control and HF offspring (Figure III-2A). We found however, that a DA peak could be consistently observed when animals consumed the fat-enriched pellets. This peak was isolated and 15 data points prior to and following this peak were used for analysis (Figure III-2B). No diet group differences were observed in a "pure" consumatory response. Although not directly compared in the present analysis, peak consumatory responses in the unpaired condition were significantly

smaller than anticipatory peak responses in the paired condition (0.4 µM vs. 0.2 µM), especially in Control offspring, again demonstrating that NAc DA transmission is activated primarily by conditioned cues that reliably predict a positive outcome.

Conclusions

Our results indicate that exposure to HF during a critical period of development programs mesolimbic DA function in that adult offspring originating from mothers exposed to HF during the last week of gestation and throughout lactation display reduced anticipatory NAc DA responses to food cues, but no differences in consumatory DA responses. These changes in DA neurotransmission occurred prior to the development of obesity in these animals, suggesting that DA hypofunction programmed in early life might play a causal role in behavioral adaptations geared towards obesity. Our data provide strong evidence for the long term nutritional "programming" of the rewarding properties of fat-enriched rewards and associated mesolimbic DA function. This might lead to alterations in ingestive behavior that favor the development of obesity.

Figures

Figure III-1. *Mean change in DA signal (µM) on days 2 (triangles), 3 (squares) and 4 (circles) of pavlovian conditioning in Control (A) and HF offspring (B). Timepoints located within the grey shaded areas correspond to anticipatory responses. Data were analyzed by repeated measures ANOVA using session time (70 sec) x day of testing. In the Control offspring, mean signal change varied as a function of session time ($F(69, 840 = 2.172, p < 0.0001$) and between days of testing ($F(2, 840 = 150.3, p < 0.0001$). The same was observed in HF offspring (session time effect: $F(69, 980) = 1.416, p < 0.05$; day of testing effect: $F(2, 980) = 33.65, p < 0.0001$). Both Control and HF animals conditioned to the tone, as revealed by a significant day effect (Control: $F(2, 852) = 150.3, p < 0.0001$, HF: $F(2, 994) = 33.65, p < 0.0001$) when analysis was performed on the 30 second tone alone. Mean DA increase during the tone (1C) and following the 'click' (1D) in Control and HF offspring was analyzed using a two-way repeated measures ANOVA. Mean DA increase was increased across days during the tone ($F(2, 26) = 8.061, p < 0.01$) and following the 'click' ($F(2, 26) = 7.15, p<0.01$), but no overall diet effects were observed ($p>0.05$). Bonferroni post hoc analyses reveal that on day 4 of conditioning, HF-exposed offspring show blunted DA responses to the tone and to the 'click' compared to the Control offspring ($p<0.05$) . Values represent mean ± SEM of 7 Control and 8 HF animals. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$*

Figure III-1. The paired condition

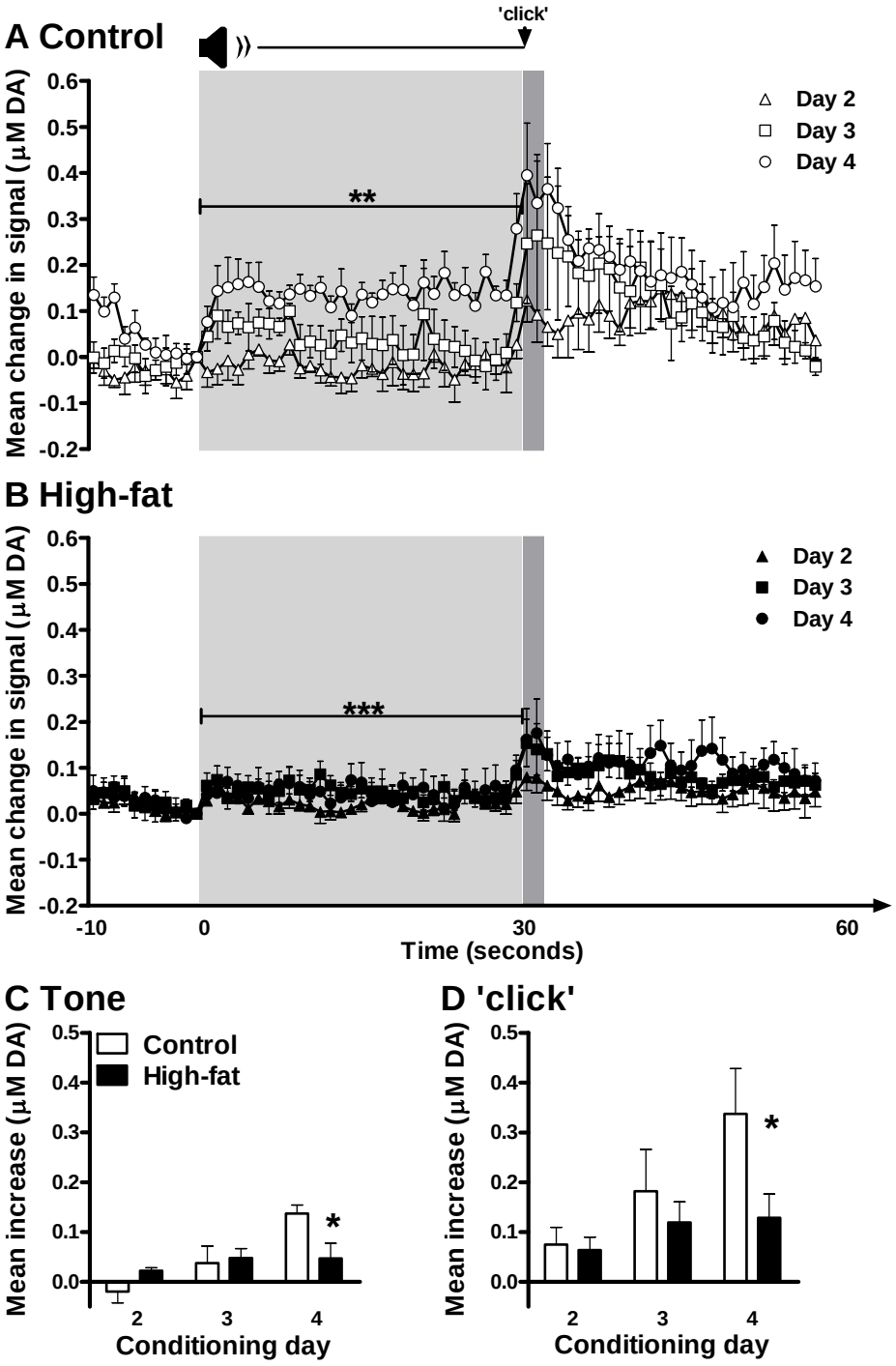

Figure III-2. The unpaired condition

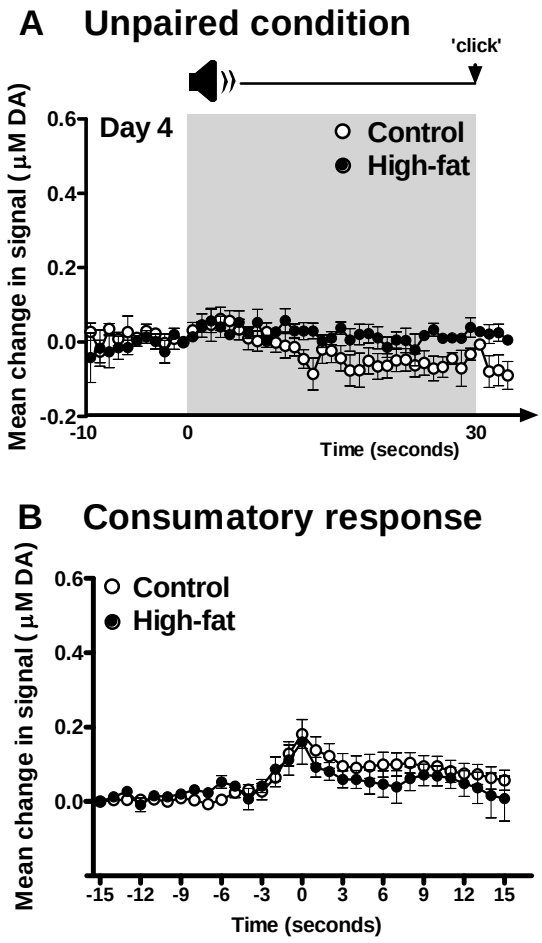

Figure III-2 *(A) Mean change in DA signal (µM) on day 4 of testing in Control (white) and HF (black) offspring in the unpaired condition, in which the tone did not predict the delivery of a food pellet. Timepoints located within the grey shaded area correspond to the 30-second tone. No group differences were observed on day 4 of testing. (B) Similar consumatory DA responses (µM) to the consumption of the high-fat pellets in Control (white) and HF (black) offspring in the unpaired condition. A two-way repeated measures ANOVA showed no diet-related differences in the magnitude of the consumatory DA peak although a significant time effect was observed (F (30,210) = 8.122, p< 0.0001). Values represent mean ± SEM of 6 animals.*

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Chapter IV: Exposure to high-fat during early development impairs adaptations in dopamine and neuroendocrine responses to repeated stress

IV.1 Preface

NAc DA is involved in the appraisal, integration, and behavioral responses to psychogenic stressors. Chapters 2 & 3 demonstrate that early exposure to high-fat significantly reduces the NAc DA response to amphetamine and the anticipation of high-fat food rewards and alters the presynaptic regulation of Nac DA, suggesting that the DA response to stress might also be altered by early exposure to high-fat. Thus, the first aim of this manuscript is to examine the long-term consequences of early exposure to high-fat on the Nac DA response to stress (aim 1). Given that mesocorticolimbic DA is involved in mediating the neuroendocrine stress response, we were also interested in examining stress responsiveness in high-fat vs. control offspring. Thus, the second aim of this manuscript is to examine the long-term consequences of early exposure to high-fat on HPA function (aim 4).

IV.2 Contribution of authors

L. Naef: Design, execution, analysis of data and writing of the manuscript.

Dr. Claire-Dominique Walker and Dr. Alain Gratton: Design, execution, and editing of the manuscript.

Acknowledgments

We would like to thank Ms Hong Long, QuianNi Zhao and Mr Luc Moquin for expert technical help during the course of these experiments.

IV.3 Manuscript

Title: Exposure to high-fat during early development impairs adaptations in dopamine and neuroendocrine responses to repeated stress

Authors: Lindsay Naef, Alain Gratton & Claire-Dominique Walker

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Journal: Stress (accepted for publication April 23, 2013)

Keywords: Maternal high-fat diet, offspring, tail-pinch stress, dopamine, HPA axis

Abstract

Perturbations in the perinatal environment have been shown to significantly alter mesolimbic dopamine (DA) and hypothalamic-pituitary-adrenal (HPA) responses to stressors in adulthood. We have previously demonstrated that adult offspring exposed to high-fat during the last week of gestation and throughout lactation display permanent alterations in mesolimbic DA function and behavior. The goal of the present experiments was to investigate nucleus accumbens (NAc) DA and HPA responses to acute and repeated stress in high-fat (HFD, 30% fat) exposed and control (CD, 5% fat) offspring. Using microdialysis to monitor extracellular DA, we report that adult HFD offspring show an enhanced NAc DA response to acute tail-pinch compared to CD offspring. With repeated tail-pinch, the response of the HFD animals remains unchanged while CD offspring exhibit a sensitized DA response. The pattern of the DA response to both acute and repeated stress is also significantly altered by early diet exposure with an earlier peak and faster return to baseline levels in CD compared to HFD offspring. Similarly, neuroendocrine adaptations to repeated tail-pinch are observed in CD animals, but not in HFD animals. While controls display a habituated adrenocorticotropic hormone (ACTH) response to repeated tail-pinch, and an exacerbated ACTH response to a novel stressor, this effect was not observed in the HFD offspring. Together, our data demonstrate that exposure to high-fat during early development impairs adaptations in NAc DA and HPA responses usually observed with repeated stress.

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Introduction

Environmental influences during early life are important determinants of adult stress responsiveness. For example, repeated neonatal maternal separation (Brake et al., 2004; Francis et al., 2002), neonatal handling (Brake et al., 2004) and naturally-occurring variations in maternal care (Liu et al., 1997; Zhang et al., 2005) are associated with significant alterations in hypothalamic-pituitary-adrenal (HPA) axis and mesocorticolimbic dopamine (DA) responses to stress. The ability of early environmental factors to 'program' adult behavioral, neuroendocrine and autonomic responses to stressors depends, in part, on the postnatal functional integration of the HPA axis (Walker et al., 2001), as well as maturation of mesocorticolimbic DA projections (Benes et al., 2000; Antonopoulos et al., 2002). During this critical period of development, maternal dietary changes can signal available resources to the offspring and alter the nutritional and hormonal environment of developing young. For instance, maternal high-fat feeding increases the lipid content of the maternal milk and subsequently increases plasma concentrations of leptin and corticosterone as early as postnatal day (PND) 10 (D'Asti et al., 2010). We and others have previously demonstrated that increasing the maternal fat content of the diet induces long-lasting alterations in mesolimbic DA function (Naef et al. 2008, 2011, 2012; Vucetic et al., 2010; Teegarden et al., 2009). Adult offspring exposed to 30 % fat (vs. 5%) during early life display blunted locomotor (Naef et al. 2008) and nucleus accumbens (NAc) DA responses to acute amphetamine (Naef et al., 2011), reduced behavioral sensitization to repeated amphetamine administration (Naef et al. 2008) and reduced anticipatory NAc DA responses to high-fat pellets (Naef et al., 2012). The neural substrates subserving the differential responses in high-fat exposed offspring include increased activity of the DA transporter (DAT) and decreased expression of the DA D2 inhibitory pre-synaptic receptor in the VTA (Naef et al., 2011). In addition to its well documented effects on DA function, perinatal high-fat feeding reduces stress responses in neonates (Trottier et al., 1998) although adolescent rats from high-fat feeding mothers display higher ACTH and corticosterone responses

to stress. Exposure to high-fat diet in adulthood consistently increases HPA axis activity (Tannenbaum et al., 1997) and enhances vulnerability to disease, yet the long term effect of perinatal high-fat on adult stress responsiveness is unknown.

In the present experiments, we tested the hypothesis that early exposure to high-fat significantly alters the NAc DA and HPA responses to acute and repeated stress. We used a repeated stress paradigm as it allowed us to test the capacity of the systems to either adapt or sensitize to repeated challenges. We report that, compared to controls, adult offspring exposed to high-fat in early development showed an enhanced NAc DA response to acute stress, but failed to sensitize to repeated stress. Similarly, ACTH responses to tail-pinch stress failed to habituate in high-fat adult offspring and did not show facilitation to a novel stressor as observed in control animals. These data demonstrate that exposure to high-fat during early development impairs adaptations in both NAc DA and HPA responses to repeated stress, highlighting a potential for increased vulnerability to stress-related disorders in these offspring.

Experimental procedures

Animals

Pregnant female Sprague-Dawley rats (Charles River, St-Constant Quebec) were received in our animal facility on gestation day (GD) 13 and immediately placed on either a control diet (CD, 5% fat, 60% carbohydrate, 15% protein, 3.45 kcal/g) or a high-fat diet (HFD, 30% fat, 24% carbohydrate, 15% protein, 4.54 kcal/g) until postpartum day 22. Both diets were powdered semi-purified diets from Harlan Teklad (IN, USA). Litters we culled to 10 pups on PND 1. On PND 22, male offspring were weaned from their mother, caged by two and maintained on the CD until tested in adulthood (Postnatal day 90-120). Animals were housed under controlled conditions of light (12:12 h light/dark cycle, lights on at 08:00hr), temperature (24-26ºC) and humidity (70-80%). All procedures were approved by the Animal Care Committee at McGill University in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

Mesolimbic (NAc) DA responses to acute and repeated tail-pinch stress

In-vivo microdialysis was used to measure Nac DA responses to tail-pinch stress. Adult (> PND 90) male rats (6 animals / diet group) were implanted with 20 gauge cannula aimed at the NAc (AP = 6.5mm, ML = 1.4mm, DV = - 6.5mm from Bregma) according to the atlas of Paxinos & Watson (1998). On the experimental day, rats were placed in opaque circular (30 cm diameter) chambers containing 2 cm of bedding and a microdialysis probe was inserted into the guide cannula. Microdialysis probes (active membrane = 2.5 mm) and probe assembly were constructed as previously described (Lupinsky et al., 2010; Naef et al., 2011). Flow rate of aCSF was set at 1.5 µl/minute (min) and samples were collected every 15 min for 1 hour (hr) prior to stress and for 2 hrs post tail-pinch stress. During the 30 min tail-pinch stress, a plastic clothespin was secured to the animals' tail. Importantly, attachment of the clothespin to the tail of the animals did not elicit a pain response as assessed by lack of audible vocalization and flinching or jumping. Most animals gnawed the clothespin and if they

successfully removed it before the end of the stress session, it was immediately replaced. Rats were exposed to the same tail-pinch stress in the same environment for the next 4 consecutive days, but dialysate collection was only conducted on the first (day 1) and last (day 5) day of repeated stress. Following the experiment, correct probe placement was verified for each animal. Examples of probe placements using these coordinates and these probe assemblies can be found in Naef et al. (2011).

Dialysate levels of DA were measured using high-performance liquid chromatography with electrochemical detection as recently described (Naef et al., 2011). Chromatographic peak analysis was conducted using ESA CoulArray software which identified unknown peaks in samples and matched these peaks with the retention time of the known standards for DA. Baseline measurements of DA were calculated using the mean concentration of four time points prior to stress initiation. For each time point examined, data are represented as a percent of mean baseline concentration.

Neuroendocrine responses to acute and repeated stress

In this experiment, separate groups of animals were tested for either acute or repeated exposure to stress. In the acute stress condition (Day 1: CD n=6, HFD n=7), naive animals were implanted with a jugular cannula, allowed to recover for two days, then subjected to 30 min of tail-pinch stress (as described above) followed 1hr later by 30 min of restraint, which was performed by placing the rat in a plastic restraint bag. For both tail-pinch and restraint, blood samples were collected through the jugular catheter at 5, 15, 30, 60 and 90 min post-stress initiation. The 90 min post tail-pinch sample served as baseline for the restraint stress. In the repeated stress groups (Day 5: CD n=7, HFD n=6), animals were repeatedly stressed with 30 min of tail-pinch stress for 4 days (jugular implantation on day 3), then tested for tail-pinch and restraint, as described above on day 5.

Male rats (600-700g) were equipped with indwelling jugular catheters

under isoflurane anesthesia. A silicone catheter (I.D.: 0.025 inches, O.D.: 0.047 inches) was inserted approximately 3 cm into the jugular vein aimed at the top atrium of the heart, secured to the vein by silk thread and exteriorized from the scapula. Each catheter was filled with heparinized (Hep) saline (50 U/ml) and flushed the day after surgery with Hep-saline. On the day of testing, animals were moved to a separate testing room and the jugular catheter was connected to approximately 30 cm of PE50 tubing that exteriorized to the cage in order to allow for the sampling of plasma without disturbing the animals. Animals were left undisturbed for 90 min to acclimatize to the testing environment before stress onset. At each sampling time, the volume of blood withdrawn (approximately 200ul) was replaced by 150-200ul of heparinized saline.

Hypothalamic tissue collection

Baseline and stress-induced CRH mRNA and hnRNA expression were examined following 30 minutes of tail-pinch (CD n=3, HFD n=4) or 30 minutes of restraint (CD n=7, HFD n=7) in separate cohorts of animals. This time point was chosen to coincide with peak ACTH secretion observed in similar experimental groups. With both stressors, animals were sacrificed by rapid decapitation at 30 minutes post stress and expression levels were compared to unstressed animals (tail pinch: CD n=4 HFD n=5, restraint: CD n= 6, HFD n=8). Upon decapitation, brains were removed, snap frozen with isopentane, and then kept at -80°C until processing for CRH mRNA and hnRNA using *in-situ* hybridization (ISH). ISH was conducted according to previously published methods (mRNA: Mansi et al., 1998; hnRNA: Chen et al., 2001). Twenty µm brain sections were collected onto Superfrost Plus slides (Fisher) and stored at −80 C until processed.

In situ hybridization for hypothalamic PVN CRH mRNA and hnRNA

Synthesis and labelling of the CRH mRNA probes was performed as previously described (Mansi et al., 1998). A plasmid containing a 659 bp fragment of exonic CRH was kindly provided by Dr. G. Drolet (Laval

University). Briefly, radioactive antisense cRNA was synthesized by incubating 8ul (100uCi) ^{35}S-UTP (NEN Life Science) with 1.5 µl specific cDNA (250ng/ul), 1 µl 100mM DTT, 2 µl GTP/ATP/CTP, 1µl Protector RNase inhibitor (Roche), and 1µl SP6 RNA Polymerase for 1 hr at 37°C. Following this initial step, 1µl DNAse (DNA1 RNase-free, Roche) was added and incubation continued for an additional hour. The probe was then purified by column chromatography using a G-25 Sephadex spin column saturated with Tris NaCl EDTA buffer (STE, Roche).

For CRF mRNA ISH, tissue sections were fixed in 4% paraformaldehyde for 20 min, then digested by proteinase K (10 pg/ml in 100 mM Tris-HCl pH 8.0, and 50 mM EDTA) at 37°C for 30 min. Next, the brain sections were rinsed in a solution of 0.1 M triethanolamine (TEA), acetylated in 0.25% acetic anhydride in 0.1 M TEA and dehydrated through graded concentrations of ethanol (50, 70, 95, and 100%). After vacuum drying for 2 h, 100 µl of hybridization mixture containing ^{35}S-CRH cRNA (l0^{7} cpm/ml) was spotted on each slide, sealed under a coverslip, and incubated at 65°C overnight. The following day, coverslips were removed and the slides were rinsed in 4x standard saline citrate (SSC, Roche) at room temperature. Sections were digested by RNase A (10mg/ml, 30 min at 37°C,), rinsed in descending concentrations of SSC (2X, IX, 0.5X SSC + 1M DTT), dipped 10 times in 0.1X SSC+1M DTT, and dehydrated through graded concentrations of ethanol.

For the synthesis and labelling of the CRH hRNA probes, a plasmid containing a 530 bp fragment of intronic CRH was kindly provided by Dr. Tallie Baram (UCI, Chen et al., 2011). Radioactive antisense cRNA was synthesized by incubating T7 RNA polymerase (30U, Promega, Madison, WI) with 1 mg of plasmid linearized with Hind III in 2.5 mM ATP/GTP/UTP, 6 mM ^{35}S-CTP, 10 DTT, 40 mM Tris–HCl (pH 7.5), 6 mM MgCl , mM spermidine, 10 mM NaCl and 40 U RNase inhibitor (RNasin, Promega). After 2 h at 37°C, 3 U of RNase-free DNase (RQ1-DNase, Promega) was added for 15 min at 37°C. The probe was subjected to mild alkaline hydrolysis and purified by column chromatography

using a STE Select-D G-25 spin column from Thomas Scientific.

For CRH hnRNA ISH, the sections were fixed for 20 min in 4% paraformaldehyde, followed by dehydration and rehydration through graded concentrations of ethanol. Sections were then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 8 min, dehydrated through graded ethanol rinses, immersed in chloroform (5 min), and then rehydrated (100% and 95% ethanol). The slides were allowed to dry then 60µl of the pre-hybridization mixture was added and sections were incubated for 1h. Prehybridization and hybridization was performed at 55°C in a solution of 50% formamide, 5x SET, 0.2% SDS, 5XDenhardt's, 0.5 mg/ml salmon DNA, 0.25 mg/ml yeast tRNA, 100 mM DTT, and 10% Dextran sulfate. Following prehybridization, sections were hybridized overnight with 2.5 $x10^7$ cpm/ml 100 µl /slide of ^{35}S labeled ribonucleotide probe. The following day, the slides were rinsed with 2x SSC, RNase digested (30 minutes at 37 °C), rinsed with decreasing concentrations of SSC at 62°C (2x 5 min, 1x 5 min, 0.25x 30 min, 0.1x 1 hr, 0.03x 1 hrs at 62°C), then dehydrated with increasing concentrations of ethanol. Following hybridization for both mRNA and hnRNA, slides were vaccum dried for 2 hrs and exposed to X-ray film (Eastman Kodak, Rochester, NY) for 7 (mRNA) and 17 (hnRNA) days. Hybridization signal was quantified in the PVN using computerized densitometry of X-ray films by means of an MCID image analyzer system (Imaging Research Inc., St Catherine, ON, Canada). Three-four PVN sections per animal were used for analysis.

Hormonal determinations

Plasma concentrations of ACTH and corticosterone were assayed with rat-specific radioimmunoassay kits (ACTH: DiaSorin, Minnesota, corticosterone: MP Biomedicals), as previously described (Buwembo et al., 2012; Proulx et al., 2001). The limit of detection for ACTH and corticosterone were 15 pg/ml and 0.3125 ug/dl, respectively.

Statistical analysis

In repeated sampling experiments, two-way ANOVAs were conducted with time during testing as the repeated measures variable. Bonferroni post-hoc analysis was used when appropriate. Detailed use of statistical tests is described in the results section. Values are reported as means +/- SEM.

Results

NAc DA responses to acute and repeated tail-pinch stress

NAc DA responses to acute and repeated tail-pinch were measured using *in vivo* microdialysis. Application of tail-pinch stress elicited increases in extracellular NAc DA in both CD and HFD offspring (Figure 1), although the pattern and amplitude of these responses were influenced by previous experience with the stressor (day 1 vs. day 5) and early diet (CD vs. HFD). Figure IV-1A (CD n=6, HFD n=6) depicts NAc DA concentrations as a percent of baseline during the 30 min tail-pinch and for 90 min post stress on day 1 and day 5 of tail-pinch. While both diet groups showed a significant time effect when analyzed with a two-way repeated measures ANOVA (across time and days (CD: $F(7, 35) = 7.45$ $p<0.00001$; HFD: $F(7, 35) = 3.65$ $p<0.01$), a significant day effect was only observed in the CD offspring (CD $F(1, 35) = 7.77$, $p<0.05$; HFD ($F(1, 35) = 0.62$).Thus, a sensitized NAc DA response was observed with repeated stress in the CD, but not in the HFD offspring. Peak DA concentrations were observed at 15 min in CD while DA peaked at 30 min in the HFD group and never fully recovered to baseline after 2hrs.

Diet group differences in NAc DA responsiveness to stress (Figure IV-1B) were directly compared as mean increase (mean change from baseline between 15-120min) with a two-way repeated-measures ANOVA. Although NAc mean DA increase did not show a significant diet ($F(1, 10) = 2.506$, $p>0.05$) or day ($F(1, 10) = 2.877$) effect, a significant diet x day interaction was apparent ($F(1, 10) = 6.950$, $p = 0.0249$). Bonferroni post hoc tests revealed that mean NAc DA increases were significantly elevated in HFD offspring compared to CD offspring on Day 1 of stress ($p<0.05$). Baseline DA concentrations (data not shown) did not vary as function of diet ($F(1, 10) = 0.2328$, $p>0.05$) or day of stress ($F(1, 10) = 4.331$, $p>0.05$).

Neuroendocrine responses to acute tail-pinch and restraint stress and repeated tail-pinch stress

Plasma ACTH and corticosterone responses to tail-pinch followed by restraint stress are depicted in Figure IV-2 for Control (2A) and High-fat (2B) adult offspring subjected to stress acutely (Day 1) or after 4 days of repeated tail pinch (day 5). For each diet group, ACTH and corticosterone responses were analyzed using a two-way repeated measures ANOVA with time (0-180 minutes) as the repeated-measure variable. In the CD offspring, ACTH responses to tail-pinch decreased following repeated exposure to the same stressor, but increased in response to a novel stressor (restraint stress). In this diet group, the ANOVA showed a significant time effect ($F(10, 121) = 36.66$, $p<0.0001$) and a time x day interaction ($F(10, 121) = 7.171$ $p <0.0001$). In the repeated stress group (Day 5), t-tests with a Bonferonni correction revealed a significant reduction in ACTH concentrations at 15 and 30 min after the onset of tail-pinch, suggesting habituation to this stressor. However, in response to restraint stress, a significant increase in the ACTH response was observed at 5, 15 and 30 min after restraint onset (95, 105 and 120 minutes in Figure IV-1A) indicative of facilitation of the response to a novel, heterotypic stressor. In contrast, the HFD offspring did not display significant changes in the magnitude of their responses to either stressor when acute and repeated responses were compared (day effect: $F(1, 100) = 0.013$ $p=0.9128$), although as expected, a significant time effect was detected ($F(10, 110) = 30.63$ $p <0.0001$). Corticosterone concentrations showed a significant time effect for both diet groups (CD $F(10, 110) = 11.51$ $p <0.0001$; HFD ($F(10, 120) = 9.693$ $p <0.0001$), but no significant day effect.

To directly compare diet groups, we computed the area under the curve (AUC) for plasma ACTH and corticosterone responses to acute (Day 1) and repeated (Day 5) exposure for each stressor (tail-pinch 0-90 minutes, restraint 90-180 minutes) and a t-test was conducted for each stressor and diet group. We confirmed that the overall stress response in the CD group was lower with the homotypic stressor (tail-pinch) on day 5 ($p=0.017$ t-test) and increased with the novel stressor on day 5 ($p = 0.008$ t-test). No significant differences between days were detected in the HFD offspring. There were no overall differences between

diet groups in either ACTH or corticosterone responses as determined by repeated measures ANOVA. However, while CD offspring displayed habituation to tail-pinch and facilitation to restraint, these neuroendocrine adaptations were not observed in the HFD group.

Hypothalamic responses to acute tail-pinch and restraint stress.

Figure IV-3 depicts CRH mRNA and hnRNA expression measured at 30 minutes post tail-pinch and restraint stress in CD and HFD offspring. Two-way ANOVAs were used for analysis of all measures. The CRH mRNA response to stress showed a small, but non-significant response to tail-pinch (stress vs. non-stressed: $F(1, 12) = 3.466$ $p= 0.0873$) and no response to restraint ($F(1, 24) =0.33$, >0.05). Furthermore, tail-pinch ($F(1, 12) = 0.07$, $p>0.05$) and restraint ($F(1, 22) = 0.04$, $p>0.05$) failed to increase CRH hnRNA at the 30 min time point. No diet group differences were observed in either mRNA or hnRNA CRH measures.

Discussion

The goal of the present experiments was to examine the long-term consequences of early exposure to high-fat on NAc DA and HPA responses to acute and repeated tail-pinch stress. While CD offspring displayed a sensitized NAc DA response with repeated exposure to daily tail-pinch stress (Day 1 vs. Day 5 of tail-pinch stress), this phenomenon was not observed in the HFD offspring, possibly as a result of a higher DA response that was observed on the first day of stress in this group. In the CD offspring, DA sensitization with repeated tail-pinch was associated with habituation of the ACTH response to tail-pinch but facilitation when challenged with a novel stressor, restraint. Together, these findings suggest that early exposure to high-fat through the maternal milk impairs adaptations in HPA activity and DA neurotransmission after repeated stress and suggests that HFD offspring might be more vulnerable to repeated stress-induced pathologies.

NAc dopamine response to acute stress

The mesolimbic DA system has been most extensively studied in the context of drug and food reinforcement. However, there is convincing evidence that mesolimbic DA is also involved in the appraisal, integration, and behavioral responses to psychogenic stressors (Cabib et al., 2012). Tail-shock (Abercrombie et al., 1989; Gresch et al., 1994), tail-pinch (Doherty & Gratton, 1992; Budygin et al., 2012; Rouge-Pont et al., 1998), and restraint (Doherty & Gratton, 1992; Puglisi-Allegro et al., 1991) stress have been previously shown to significantly modulate the release of DA in the NAc. This is confirmed in the present experiment in which we observe significant increases in NAc DA concentrations on day 1 and day 5 of repeated tail-pinch in both CD and HFD adult offspring. However, the NAc DA response of HFD offspring was higher in magnitude on day 1 of testing and a different pattern of responding was observed relative to controls. While the CD DA response peaked at 15 minutes after tail-pinch initiation and quickly returned to baseline, peak DA concentrations were observed

at 30 minutes in HFD animals and DA levels never recovered. An increase in the magnitude of the NAc DA response and a deficit in recovery in the HFD offspring suggest impairments in the regulation of NAc DA in HFD offspring. Several mechanisms might explain this observation, including changes in regulatory inputs to the NAc from the mPFC and local synaptic modulation of extracellular DA concentrations.

The NAc DA response to stress is regulated in a large part by glutamatergic inputs from the mPFC which are themselves the product of a delicate balance between mPFC norepinephrine (NE) and DA activity (Pascucci et al., 2007). While mPFC DA exerts an inhibitory influence on efferent inputs to the NAc, thus reducing the NAc DA response to stress (Doherty & Gratton, 1996), mPFC NE, through activation of alpha-1 adrenergic receptors, exerts the opposite effect leading to an enhancement of the NAc DA response to stress (Nicniocaill & Gratton, 2007). Since HFD adult offspring displayed increased and prolonged DA stress responses, it is tempting to propose that a high mPFC NE tone combined with lower PFC DA might exist in these animals. Although we have not measured mPFC responses to stress in the present study, we previously reported a lack of effect of perinatal diet on baseline and amphetamine-stimulated mPFC DA (Naef et al., 2011) concentrations. This suggests that increased mPFC NE activity might tilt the balance towards a greater input to NAc after stress exposure. Further experiments need to clearly establish this possibility.

Increased NAc DA responses to stress might also be due to local regulation of extracellular DA by the DA transporter (DAT) and presynaptic inhibitory DA D2 receptors. Stimulation of DA with acute (Copeland et al., 2005) and repeated stress (Copeland et al., 2005; El-Khodor & Boksa, 2002) enhances the expression and activity of DAT, which indicates that DAT might help buffer excessive DA release after stress. The higher integrated responses and deficits in the recovery of DA levels with the application of tail-pinch in HFD offspring indicate a potential reduced uptake capacity via DAT. However, under baseline conditions, we observed higher, but not lower DAT activity in HFD offspring

(Naef et al., 2011), suggesting that baseline variations in DAT activity do not contribute to the regulation of DA during and following stress in these animals. Alternatively, the higher DA response and delayed recovery we observe in HFD animals might represent reduced inhibitory feedback via NAc presynaptic D2 receptors. Evidence to support this hypothesis is found in our recent experiment showing that HFD rats had reduced expression of presynaptic D2 receptors mRNA in the ventral tegmental area (VTA) (Naef et al., 2011). This implies that a reduction in the concentration of D2 receptors in target areas such as the NAc and a weaker presynaptic inhibition of DA would allow for increased NAc DA release following stressful stimulation.

DA sensitization to repeated stress

Repeated stress has been reported to both sensitize (Doherty & Gratton, 1992; Brake et al., 1997) and reduce (Imperato et al., 1993) the NAc DA response to stress. Our data indicate that while the CD offspring sensitized to repeated tail-pinch, the DA response to the 1st and 5th episodes of tail-pinch was strikingly similar in HFD animals. It is possible that maximal DA levels were reached on day 1 of tail-pinch in the HFD offspring and thus, precluded further increases on day 5. However, we have observed considerably higher NAc DA concentrations following the administration of amphetamine in these animals (Naef et al., 2011), indicating that HFD animals have the capacity to mount higher responses with pharmacological stimulation than those observed with the application of tail-pinch on day 1. Failure to show DA sensitization with repeated tail-pinch is consistent with our previous study (Naef et al., 2008) showing that, unlike the offspring of CD dams, HFD offspring do not sensitize to amphetamine's locomotor stimulant effect with repeated drug administration. Thus, it would appear that early exposure to high-fat interferes with the cascade of neuroadaptive changes that underlie the development of drug- and/or stress-induced sensitization of meso-Nac DA transmission. We can only speculate as to the nature of these mechanisms, but changes in neurotrophic factors within the mesocorticolimbic

DA circuitry (Pierce & Barri, 2001) might be worth investigating because maternal obesity has been shown to impair hippocampal brain-derived neurotrophic factor (BDNF, Tozuka et al., 2010).

Acute and habituated neuroendocrine and hypothalamic responses to stress

In response to acute stress (day 1), both CD and HFD offspring showed significant and similar ACTH and corticosterone responses to tail-pinch and restraint. However, considerable diet group differences emerged with repeated stress in that HFD offspring failed to display either habituation to the homotypic stressor (tail pinch) or facilitation to the heterotypic stressor (restraint stress). Habituation of neuroendocrine responses to repeated homotypic stress has been well documented for a number of stressors (restraint, cold, novel environment, etc.) and is thought to represent an adaptive protective process to avoid exposure to large amounts of circulating glucocorticoids as well as limit central activation of stress pathways (Nesse et al., 2007). Failure to adapt in response to repeated stressors is linked to psychiatric disorders such as post-traumatic stress disorder (PTSD) and major depression (Grissom & Bhatnagar, 2009). Several brain regions have been implicated in the process of habituation to repeated stress. In particular, lesions of the posterior (Bhatnagar et al.2002), but not the anterior (Fernandes et al. 2002) PVThalamus were found to prevent habituation to repeated restraint. This region projects heavily to several portions of the amygdala (including basolateral and central) which could relay inputs to the paraventricular pPVN neurons and modulate HPA activity (Jankord et al. 2008). It is currently unclear whether early HFD exposure modifies stress-induced activity in these key regions regulating the HPA axis. The participation of the mPFC and other limbic structures for stressor appraisal and learning the familiarity of the stressor appears to be critical in regulating HPA responses (Grissom & Bhatnagar, 2009). Indeed, transient inactivation of the mPFC (Weinberg et al., 2010) or right mPFC lesions (Sullivan & Gratton, 1999) eliminated corticosterone habituation to repeated, but not acute stress, suggesting adaptations in mPFC circuitry with repeated stress.

The observation that HFD rats do not display habituation to repeated stress might thus indicate deficits in the function of the mPFC to regulate neuroendocrine adaptation. Interestingly, the opposite effect of repeated stress that we observed between HPA activity and NAc DA release at least in CD rats might be mediated by region-specific changes in endocannabinoid signaling, specifically via 2-AG production (Patel et al., 2008).

In order to test whether hypothalamic indices of HPA axis activation after acute stress were modified by early diet exposure, we measured CRH hnRNA and mRNA levels in brains from a separate cohort of naïve animals subjected to either acute tail-pinch or restraint. CRH mRNA levels tended to increase 30 min after tail pinch in both diet groups and a modest, non-significant increase in CRH mRNA was observed after restraint only in the CD group, although this time point might not represent maximal CRH mRNA activation (Kovacs et al.1996). There were no stress or diet group effects on CRH hnRNA levels, possibly because of the very transient nature of hnRNA production which might not have been captured by our 30 minute sampling time. Notably in the present experiments, corticosterone secretion did not reflect changes in circulating ACTH levels reported in the repeated stress conditions. In both diet groups and conditions, a significant stress response was observed, but we did not find indices of either habituation or facilitation. Former studies have often reported dissociation between plasma ACTH and corticosterone secretion (Doell et al., 1981) as adrenal corticosterone secretion is non-linearly related to ACTH release and can saturate within the range of ACTH secretion observed in our study. We believe that measurement of plasma ACTH levels represents a more accurate reflection of central stress-induced activation compared to corticosterone and should be used preferentially when experimental procedures allow for a rapid sampling of blood during stimulation.

Facilitation of the ACTH response to a novel stressor

While reduced (habituated) neuroendocrine responses observed with

repeated stress might signal adaptation and coping, under these conditions, the neuroendocrine system displays exacerbated responses to novel stressors, highlighting a state of neuroendocrine sensitization or facilitation. With repeated tail-pinch, we showed a facilitated response to restraint, a heterotypic stressor in the CD, but not in the HFD animals. Facilitation within the HPA axis is also known to involve the recruitment and activation of the basolateral amygdala and the posterior paraventricular thalamus (pPVTh) where there is increased orexigenic neurotransmission originating from the lateral hypothalamic neurons. (Heydendael et al., 2011). Although we have not investigated activation in these structures as a function of perinatal diet, the lack of either habituation or facilitation in HFD offspring suggests that mechanisms underlying adaptability and flexibility in the HPA axis are impaired by early exposure to high-fat.

Early nutritional environment and stress: implications for obesity

The nutritional and hormonal milieu of developing young can have long-lasting consequences on energy homeostasis (Levin 2006). In rodents, exposure of the mother to a high-fat diet promotes the development of obesity in the offspring (Walker et al. 2008; reviewed in Levin 2006). This increased vulnerability to metabolic disturbances is thought to represent long-lasting adaptations within hypothalamic and dopaminergic brain circuits involved in the homeostatic and hedonic control of feeding behavior. In this paper, we demonstrate that exposure to high-fat during the perinatal period leads to reduced functional plasticity in mesolimbic DA circuits and impaired neuroendocrine adaptations to repeated tail-pinch stress. It is possible that such dysregulation in the face of repeated stress represents an inability to successfully adapt to stress and might thus increase vulnerability to develop a number of pathologies, including obesity in animals exposed to high-fat during early development.

Figures

Figure IV-1. NAc DA stress response

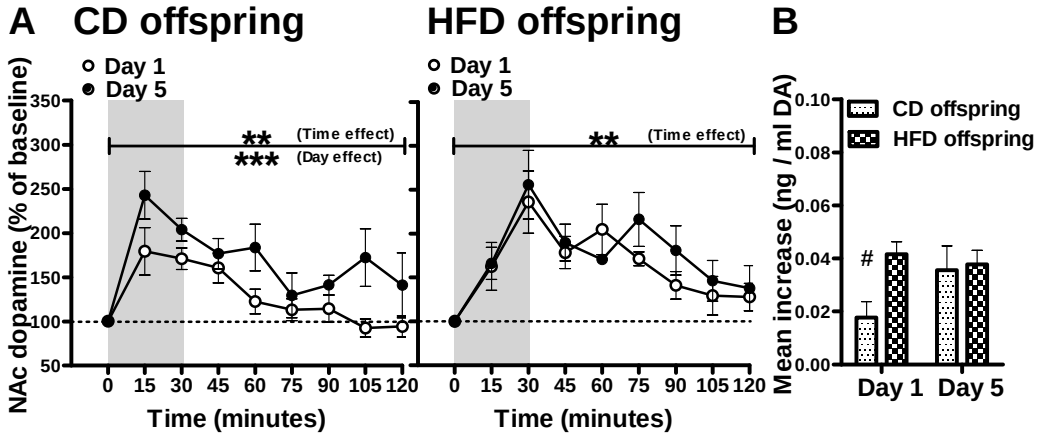

Figure IV-1. *(A) NAc DA responses (% of baseline DA concentrations) of CD and HFD offspring on day 1 (white) and day 5 (black) of 30 min tail-pinch stress (grey shaded area). In the CD offspring, tail-pinch stress led to an increase in NAc DA ($p<0.01$) with peak DA concentrations observed at 15 minutes post-stress and a sensitized DA response was observed on Day 5 vs. Day 1($p<0.0001$). In the HFD offspring, tail-pinch increased NAc DA concentrations ($p<0.01$), but the peak was observed at 30 minutes post-stress and repeated stress did not sensitize NAc DA.* ***(B)*** *Mean increase (mean change from baseline, 15-120 min) in DA concentrations following tail-pinch stress was significantly higher in HFD offspring on Day 1 ($p<0.05$), but not on day 5 of tail-pinch stress. **$p< 0.01$, ***$p< 0.0001$ represent the simple main effects in two-way repeated measures ANOVA, #$p< 0.05$ CD vs. HFD offspring bonferroni posthoc. Values represent the mean +/- SEM of 6 animals / diet group*

Figure IV-2: *Plasma ACTH and corticosterone concentrations of CD (A) and HFD (B) offspring in response to tail-pinch and restraint stress on Day 1 (white) vs. Day 5 (black) of repeated tail-pinch stress. Grey shaded areas represent the timing of stressors' exposure. In the CD offspring, with repeated tail-pinch, we observed significant reductions in ACTH levels at 15 and 30 minutes post tail-pinch, but increased concentrations at 5, 15 and 30 minutes post restraint stress. This effect was not observed in HFD offspring. Repeated tail-pinch stress did not significantly alter corticosterone responses for both CD and HFD offspring. C: Area under the curve in CD and HFD offspring computed for each stressor (tail pinch: 0-90 minutes, restraint: 90-180 min) on day 1 and day 5 of tail-pinch stress. T-tests (day 1 vs. day 5) revealed habituation and facilitation in CD but not in HFD offspring. Values are means +/- SEM of CD (day1: n=6, day 5: n=7) and HFD (day1: n=6, day 5: n=6) adult offspring. *p<0.05 and ***p<0.001 represent significant time points differences (day 1 vs. day 5) using Bonferroni posthoc analysis, #p<0.01 day 1 vs. day 5*

Figure IV-2. Neuroendocrine stress response

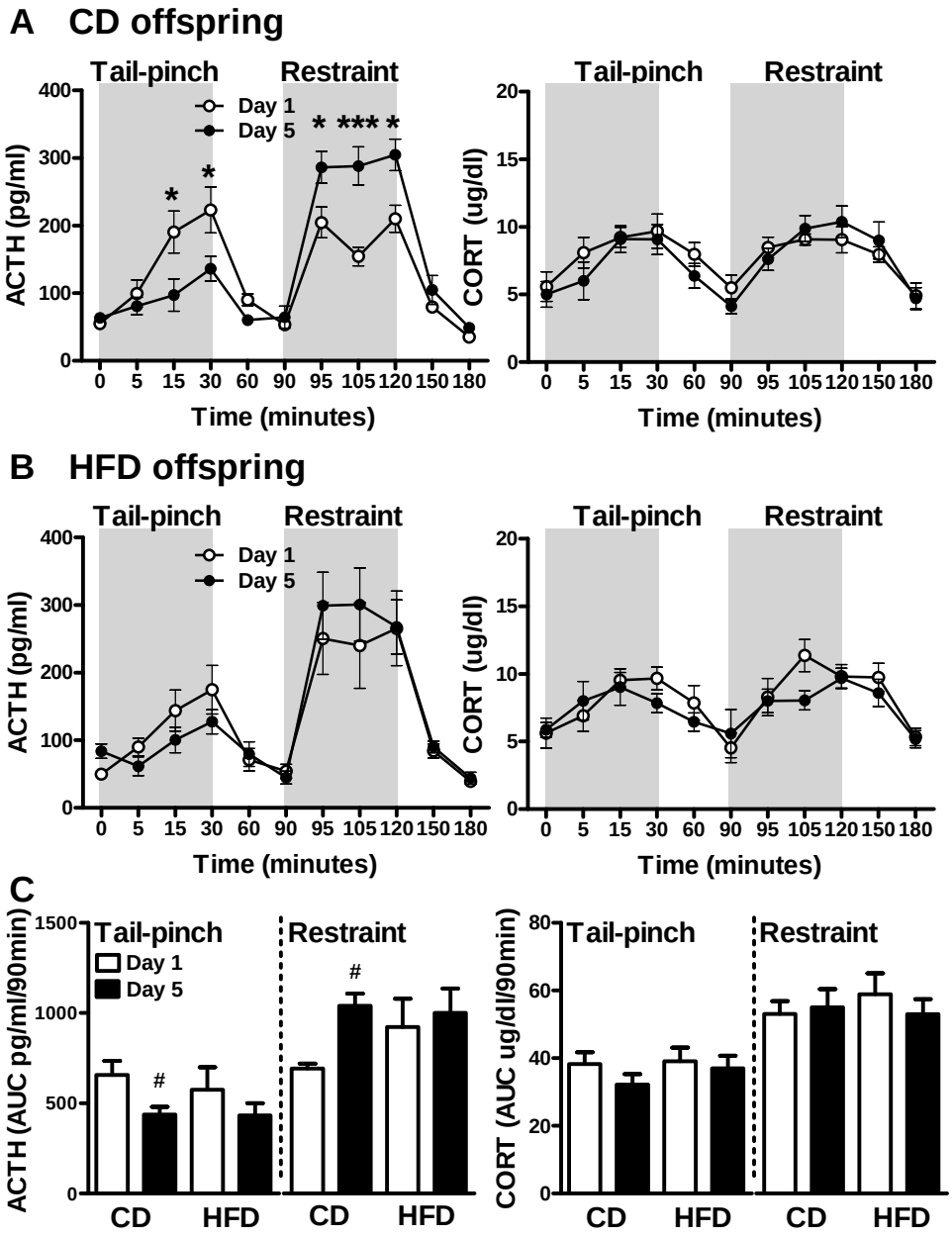

Figure IV-3. CRH stress response

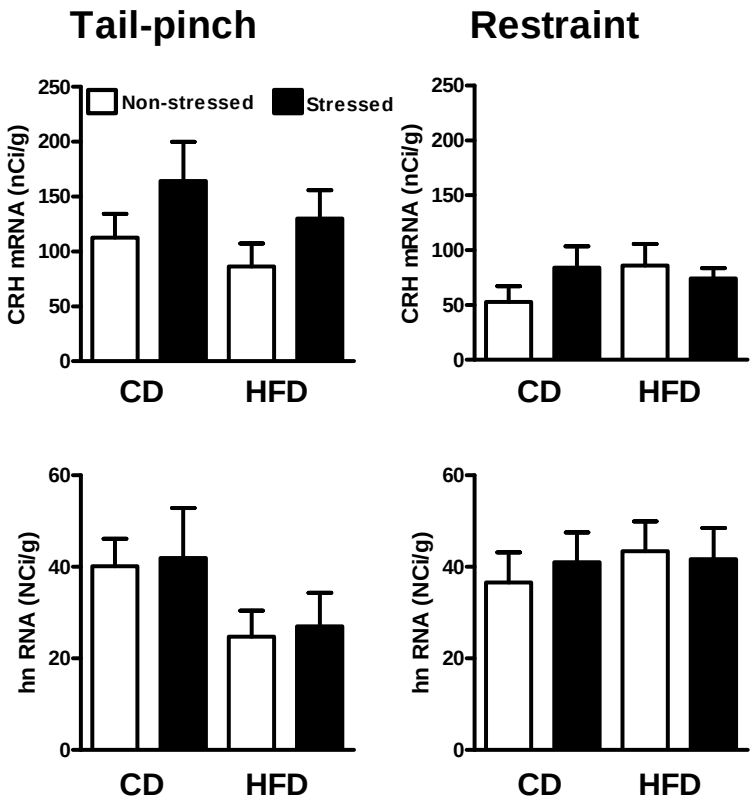

Figure IV-3. *Hypothalamic PVN levels of CRH mRNA and hnRNA in response to either 30 min of tail-pinch (left) or 30 min of restraint (right) in CD and HFD offspring. Brain tissues were collected at the end of the stressor (30min) in both conditions. Tail pinch tended to increase CRH mRNA in both CD and HFD rats although there were no significant differences between diet groups in stress responses of either RNA transcripts. Values represent the means +/- SEM (baseline tail-pinch CD n=4 HFD n=5, tail-pinch CD n=3 HFD n=5; baseline restraint CD n=6 HFD n=7, restraint CD n=7 HFD n=7. 3-4 sections containing the PVN were analyzed per animal*

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Chapter V. Neonatal onset of leptin signaling in dopamine neurons of the ventral tegmental area

V.1 Preface

Previous Chapters demonstrate that early exposure to high-fat induces long-term adaptations in mesolimbic DA function and behavior. The mechanisms through which maternal diet permanently alters DA function in the offspring are unknown. Evidence suggests that the adipocyte-derived hormone leptin could be involved in the organization and 'programming' of DA circuitry during development. High-fat feeding of the mother increases plasma leptin concentrations in the pups during the neonatal period (d'Asti et al., 2010) and is involved in the formation of hypothalamic circuits (Bouret et al., 2004), Thus, it is possible that high leptin concentrations of high-fat feeding pups is altering the development of mesolimbic DA circuitry. For this to occur, mesolimbic DA must be sensitive to leptin during this time. Although leptin modulation of mesolimbic DA neuronal function has been examined in adulthood, we do not know how leptin modulates VTA neurons during early development. Thus, the goal of the present manuscript is to examine leptin signaling in the VTA during neonatal development (aim 5).

V.2 Contribution of authors

L. Naef: Design, execution, analysis of data and writing of the manuscript.

H. Long: Immunocytochemistry protocols

Dr. Richard: leptin receptor *in-situ* hybridization

Dr. Claire-Dominique Walker: Design and execution and editing of the manuscript.

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V.3 Manuscript

Title: Neonatal onset of leptin signaling in dopamine neurons of the ventral tegmental area

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Manuscript in preparation

Keywords: leptin, pSTAT3, pERK1/2, neonatal development, dopamine, VTA

Abstract

Leptin inhibits feeding by acting on hypothalamic and mesolimbic dopamine (DA) pathways involved in the homeostatic and hedonic control of energy balance. In the rodent, the neonatal period is characterized by high circulating leptin concentrations and insensitivity to the anorectic effects of leptin suggesting that the regulation of hypothalamic and DAergic pathways by leptin is altered during this time. Although the ability of leptin to directly regulate intracellular signaling has been examined in adulthood, leptin modulation of neonatal VTA DA neurons is unknown. The goal of the present experiments was to examine the onset of leptin responsiveness of the VTA in neonatal rats and to identify the neuronal phenotype of the activated VTA neurons. We report that leptin administration on postnatal day (PND) 10 fails to increase VTA pSTAT3 immunoreactivity. On PND16, we observed a significant stimulatory effect of leptin on VTA pSTAT3 immunoreactivity, with a subset of these pSTAT3-positive neurons co-localizing with TH, a marker of DA neurons. pERK1/2 was not detected in the VTA of either PND10 or PND16 pups although it was detected in other regions after leptin treatment. These results suggest that the insensitivity of PND10 pups to the anorectic effects of leptin is mediated, at least in part, by a lack of VTA DA response to leptin at this age.

Introduction

Leptin, the protein product of the *ob* gene (Zhang et al, 1994) is synthesized in adipose tissue and circulates in proportion to body fat stores (Frederich et al., 1995; Maffei et al., 1995). Leptin inhibits feeding by targeting hypothalamic circuits involved in the homeostatic control of energy balance including the arcuate nucleus (ARC) and hypothalamic projections areas of the ARC including the dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), paraventricular nucleus (PVN) and lateral hypothalamus (LH). Leptin's ability to reduce feeding is also mediated by hedonic pathways, including the mesolimbic dopamine (DA) system, which originates in the ventral tegmental area (VTA) and projects to the striatum and amygdala complex. Leptin administered directly into the VTA decreases food intake (Bruijnzeel et al., 2011; Hommel et al, 2006; Morton et al., 2009) and RNAi-mediated knockdown of VTA leptin receptors leads to long-term increases in feeding (Hommel et al., 2006). Leptin reduces the firing rate of VTA DA neurons (Hommel et al., 2006; Trinko et al., 2011), and the long form of the leptin receptor (Ob-Rb) has been detected on VTA DA (Figlewicz et al., 2003; Fulton et l., 2006; Hommel et al, 2006; Leshln et al., 2010) and GABA (Fulton et al., 2006) neurons. In the VTA, leptin was found to stimulate the activation, phosphorylation and translocation to the nucleus of signal-transducers-and-activators-of-transcription 3 (STAT3) (Fulton et al., 2006; Hommel et al., 2006), and extracellular-signal-regulated-kinase1/2 (ERK1/2) (Trinko et al., 2006), both of which are downstream mediators of leptin receptor signaling.

While the regulation of mesolimbic DA by leptin has been examined in adulthood, several lines of evidence suggest that leptin modulation of energy balance is different during the neonatal period. Large increases in leptin gene expression and circulating leptin concentrations are observed around postnatal day (PND) 10 in rodents (Ahima et al., 1998; Cotrell et al., 2009; Proulx et al., 2001), and even if leptin regulates neuropeptide Y (NPY) and proopiomelanocortin (POMC) expression in the ARC, at this age, this hormone fails to reduce food

intake, (Mistry et al., 1999; Proulx et al., 2002). This suggests that connections between the ARC and other downstream effector circuits in food intake behavior, such as the DAergic mesolimbic hedonic component, might not be mature by PND10 in the rat. In the hypothalamus, leptin is required for the normal establishment of hypothalamic projections through its stimulatory effect on the outgrowth of ARC neurites (Bouret et al., 2004a). Thus, while the homeostatic arm of the response to food requires early exposure to leptin, little is known about the onset of functional activation of leptin-responsive DA neurons in the VTA, which form the basis of the hedonic response to food. The aim of the present experiments was to examine the onset of leptin responsiveness of the VTA in neonatal rats through the detection of intracellular second messenger molecules and the identification of the neuronal phenotype of the activated VTA neurons. We report that leptin administration to PND10 pups failed to increase pSTAT3 immunoreactivity in the VTA. On PND16, we observed a significant effect of leptin on VTA pSTAT3 immunoreactivity, with a subset of these pSTAT3-positive neurons co-localized with tyrosine hydroxylase (TH), a marker of DA neurons. As a second intracellular signaling molecule for leptin's effects, pERK1/2 was not detected in the VTA of either PND10 or PND16 pups although it was detected in other brain regions. These results suggest that the insensitivity of PND10 pups to the anorectic effects of leptin is mediated, at least in part, by a lack of VTA DA response to leptin at this age.

Experimental procedures

Animals

Pregnant Sprague-Dawley rats (Charles River, St-Constant Quebec) were received in our animal facilities on gestation day 12-13 and immediately placed on a powdered semi-purified control diet purchased from Harlan Teklad (IN, USA, composition by weight: 5% fat, 60% carbohydrate, 15% protein; composition by calories: 13% fat, 69.4% carbohydrate, 17.6% protein; 3.45 kcal/g). Mothers were left undisturbed until parturition. Litters we culled to 10 pups on PND1 and experiments were performed on PND10 and PND16. Animals were housed under controlled conditions of light (12:12 h light/dark cycle), temperature (24-26ºC) and humidity (70-80%). All procedures were approved by the Animal Care Committee at McGill University in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

Neonatal leptin administration and tissue preparation

PND10 or PND16 pups (3-5 pups per group) were given an intraperitoneal (IP) injection of either leptin (3mg/kg recombinant murine leptin, PeproTech) or vehicle (0.9% saline), returned to their mothers, and then perfused transcardially one hour post-injection with 0.9% saline followed by either 2% (pSTAT3 + TH) or 4% (pERK1/2 + TH) paraformaldehyde in 0.1M phosphate buffer (PB). Immediately prior to perfusion, a blood sample was collected by cardiac puncture in EDTA-filled tubes to allow for the determination of plasma leptin concentrations. Following perfusion, brains were carefully extracted, kept in 2% (pSTAT3) or 4% (pERK1/2) paraformaldehyde in PB for 2 hours (4°C), 30% sucrose in 0.1M PB for 48 hours (4°C), and then frozen at -80°C until sectioning. Twenty µm thick frozen coronal sections were collected with a cryostat onto Superfrost Plus slides (Fisher), dried under vacuum overnight, and stored at −80°C until processed for immunohistochemistry. The sections were collected in series of 6 and one series was used for each immunocytochemistry protocol. Plasma leptin levels were measured by specific radioimmunoassay (RIA) using a

kit from Linco Research, Inc. (St. Charles, MO) as previously described (Proulx et al., 2001). The limit of detection was 0.5ng/ml and interassay variability was 9%.

Immunohistochemical detection of leptin-induced pSTAT3 in neonatal VTA DA neurons

Slides containing sections of the VTA and perfused with 2% paraformaldehyde were removed from the freezer and brought to room temperature (RT). Slides were then washed in wash buffer (3x5 minutes, potassium phosphate buffer saline with tween 20 (KPBS-T): 100 ml 10x KPBS (Roche Applied Science catalogue # 11666789001), 900 ml dH20, 1ml Tween 20), treated with 0.5% NaOH + 0.5% H_2O_2 in wash buffer for 20 minutes at RT and then rinsed (4x5 minutes). The slides were then treated with 0.3% glycine (in wash buffer) for 10 minutes (RT), 0.03% sodium dodecyl sulfate (SDS) in dH20 (SDS, 10 minutes at RT), incubated in blocking solution (4% normal goat serum (NGS) + 0.4% Triton x-100 + 1% bovine serum albumin (BSA) in KPBS) for one hour and finally, incubated in primary antibody (Phospho-Stat3 MAPK, Cell Signaling catalogue # 9131, 1:200 dilution in blocking solution) for one hour at RT and then overnight at 4°C. The following day, the slides were rinsed with wash buffer (3x5 minutes), incubated in the secondary antibody (Alexa 568 goat-anti-rabbit, 1:200 in blocking solution) for 2 hours at RT, rinsed (3x5 minutes), then incubated with the primary antibody for TH detection (Mouse-anti-TH, Millipore catalogue # MAB318, 1:1000 dilution in blocking solution) overnight at 4°C. On the third day, the slides were kept in the primary antibody for one hour at RT, rinsed (3x5 minutes), incubated in the TH secondary antibody (Alexa 488 horse-anti-mouse, 1:200 dilution in wash buffer) for 2 hours at RT, rinsed and then coverslipped using H-1500 medium (Vector). The coverslipped slides were preserved in the fridge and imaged quickly to minimize the decay of the fluorescence signal.

Immunohistochemical detection of leptin-induced pERK1/2 in neonatal VTA DA neurons

For pERK1/2, slides perfused with 4% paraformaldehyde and containing sections of the VTA were removed from the freezer and maintained at RT until thawed. The wash buffer (KPBS-T) was the same as used for pSTAT3 with TH detection. Following three rinses (5 minutes each), sections were treated with 1% NaOH (in wash buffer) for 30 minutes, rinsed (3x5 minutes), treated with 0.3% glycine (in wash buffer) for 10 minutes and rinsed again (3x5 minutes). The sections were then treated with 0.03% SDS in dH20, rinsed three times, incubated in 100% methanol at -20°C, rinsed again, then incubated in blocking solution (3% NGS + 0.3% Triton x-100 in wash buffer) for 60 minutes at RT followed by incubation in primary antibody (Phospho-p44/42 MAPK, cell signaling catalogue # 9101, 1:200 dilution in blocking solution) overnight at 4°C. The following day, the slides were removed from the fridge and incubation in the primary antibody continued from one hour at RT. The slides were then rinsed (3x5 minutes), incubated in secondary antibody (Alexa 568 goat-anti-rabbit, 1:200 in blocking solution) for two hours at RT. For the detection of TH in these sections, the protocol and antibodies used were the same as for pSTAT3 + TH. As for pSTAT3 + TH detection, following the immunocytochemistry protocol, the slides were coverslipped with H-1500 medium (Vector), kept in the fridge and imaged rapidly.

In-situ hybridization for Ob-Rb

PND10 pups (n=20, non-injected) were used to examine Ob-Rb expression in the ARC and VTA. Pups were anesthetized with ketamine / xylazine, and then perfused transcardially with 0.9% saline followed by 4% paraformaldehyde made in 0.1M borate buffer. Brains were rapidly removed and post-fixed in 4% paraformaldehyde in borax for 2 hours and 30% sucrose in 4% paraformaldehyde in borax overnight, then frozen at -80°C until processing. Forty-five µm frozen coronal sections were collected into cryoprotectant (30% ethylene glycol, 20%

glycerol, 37.5% diethylpyrocarbonate (DEPC) H_2O in 0.01M phosphate buffer saline (PBS) (8% NaCl, 0.02% KCl, 1.4% Na_2HPO_4, 0.24% KH_2PO_4 in dH_2O water, pH 7.4) and kept at -20°C until processed. Sections were mounted onto Superfrost Plus slides (Fisher) for *in-situ* hybridization, as previously described (Walker et al., 2007). Total cDNA was obtained from rat cerebellum and PCR amplified with specific sense (5-ATGAAGTGGCTTAGAATCCCTTCG-3) and antisense (5TACTTCAAAGAGTGTCCGCTC- 3) primers. Ob-R cDNA (349 bp) was then cloned in pGEM-T vector (Promega, Madison, WI) and sequenced. Sense and antisense RNA probes used *in situ* hybridization experiments were obtained by in vitro transcription of cDNAs using SP6 and T7 polymerase (Promega). The probes were radiolabeled by incorporation of ^{35}S-dUTP (Amersham-Pharmacia Biotech, Piscataway, NJ). Before hybridization, the radiolabeled probes were purified with Qiagen's RNEasy kit (Qiagen Inc., Mississauga, Canada).

For pre-hybridization, sections were fixed for 20 min in 4% paraformaldehyde, digested for 25 min at 37°C with proteinase K (20 mg/ml in 100 mM Tris-HCl containing 50 mM EDTA, pH 8), acetylated 10 min with acetic anhydre (0.1 M triethylamine (TEA), pH 8) and dehydrated through graded concentrations (50, 70, 95, and 100%) of alcohol. After vacuum drying, 90 µl of hybridization solution mixture was applied to each slide. Hybridization buffer contained 500 µl of formamide, 60 µl of 5 M NaCl, 10 µl of 1 M Tris, pH 8.0, 2 µl of 0.5 M EDTA, pH 8.0, 50 µl of 203 Denhart's solution, 200 µl of 50% dextran sulfate, 50 µl of 10 mg/ml tRNA, 10 µl of 1 M DTT, 118 µl DEPC water, and 1 x 10^7 cpm/ml of the ^{35}S labeled RNA Ob-R probe. The slides were sealed with coverslips and incubated at 55°C overnight.

The next day, slides were hydrated with 4x SSC (0.6M NaCl, 60 mM sodium citrate buffer, pH 7), washed with 4x SSC with dithiothreitol (DTT 250µl 0.1M), digested for 30 min at 37°C with RNAse-A (10 mg/ml in 10 mM Tris-500 mM NaCl containing 1 mM EDTA), washed in descending concentrations of SSC with DTT and dehydrated through graded concentrations of alcohol. After 2 hr of

vacuum drying, the slides were exposed to Kodak BioMax for 4 days. Following exposure, the slides were defatted in toluene and dipped in NTB2 nuclear emulsion (Eastman Kodak) for 4 weeks. After developing, slides were rinsed in running tap water for 1 hr, counterstained with thionin (0.25%), and coverslipped with DPX.

Imaging

The imaging system consisted of a Zeiss Imager M1 light microscope with a CCD video camera (DVC-2000C), motorized stage and a computer running StereoInvestigator (MicroBrightField Inc., USA). The midbrain was easily identified with the TH staining in the immunocytochemistry experiments. Pictures of the pSTAT3 and pERK1/2 signals in areas of interest within the VTA were taken to count the pSTAT3 and pERK-positive neurons. Merged pictures (pSTAT3 or pERK1/2 with TH) were taken to examine co-localization which was calculated by counting the number of pSTAT3 or pERK1/2-positive neurons which expressed TH (expressed as a percentage). Counting was conducted by a researcher blind to the experimental groups. Silver grains were visualized in darkfield (low magnification) and brightfield (high magnification). Ob-Rb mRNA-containing cells were identified by clusters of silver grains over single neurons. To visualize the morphological limits of the VTA in PND10 and PND16 pups, we used the neonatal rat brain atlas of Sherwood and Timiras (1970), the adult rat brain atlas of Paxinos and Watson (4^{th} Editon) and a paper by Ikemoto (2007), which separates the VTA into four main divisions (paranigral nucleus (PN), parabrachial pigmented area (PBP), parafasciculus retroflexus area (PFR) and the ventral tegmental tail (VTT)) and illustrates these divisions in TH-stained sections.

Statistical analysis

A two-way ANOVA using age (PND10 vs. 16) and treatment (leptin vs. vehicle) was used to examine group differences in VTA leptin signaling.

Bonferroni post-hoc analysis was used to further dissect our main effects. Detailed use of statistical tests is described in the results section. Values are reported as means +/- SEM.

Results

Leptin administration increases plasma leptin concentrations on PND10 and PND16

Plasma leptin concentrations were determined 60 minutes following the administration of leptin or vehicle in PND10 and PND16 pups perfused with 2% paraformaldehyde (for pSTAT3 detection). Plasma was collected by cardiac puncture at the time of perfusion. As shown earlier (Walker et al. 2007), the administration of 3mg/kg body weight of leptin led to high plasma leptin concentrations 60 minutes after treatment. While plasma leptin concentrations of vehicle treated animals averaged 7.04±0.2 ng/ml in PND10 pups and 5.9 ±0.5 ng/ml in PND16 pups, the mean plasma concentration of leptin-administered pups was 248.58±14.4 ng/ml on PND10 and 223.21±17.4 on PND16.

Leptin receptor (Ob-Rb) mRNA in the VTA on PND10

The presence of leptin receptors in the ARC and VTA was detected with *in-situ* hybridization and silver grains were imaged in darkfield (low magnification) and brightfield (high magnification). Ob-Rb mRNA-containing cells were identified by clusters of silver grains over single neurons in both the ARC and VTA in PND10 pups, although the relative density of leptin receptor mRNA was higher in the ARC compared to the VTA (Figure V-1).

Leptin increases pSTAT3 immunoreactivity in DAergic and non- DAergic VTA neurons on PND16 but not PND10

To examine the onset of leptin signaling through pSTAT3 in VTA DA neurons, we used double immunofluorescent staining for pSTAT3 and TH (Figure V-2 and V-3) in PND10 and PND16 pups treated with either vehicle (PND10 n = 5, PND16 n = 5) or leptin (PND10 n = 5, PND 16 n =5). Based on the work of Ikemoto (2007), we estimate that pSTAT3 staining was restricted to the anterior lateral PBP portion of the VTA, (illustrated in Figure V-2), characterized by dense

and intensely stained TH-positive cell bodies, which are large and medium sized with no unified orientation. Counts represent the mean number of pSTAT3-positive cells (6 sections per animal containing the largest part of the VTA and including the PBP) for each experimental group (Figure V-2). We observed very few pSTAT3-positive neurons in PND10 pups (Figure V-2, less than 7 pSTAT3-positive neurons per section in leptin-treated animals) and a large increase in the number of pSTAT3-positive neurons on PND16 (Figure V-2, over 55 pSTAT3-positive neurons per section in leptin-treated animals). A two-way ANOVA (treatment x age) revealed a significant treatment effect ($F(1, 16) = 75.99$, $p < 0.0001$), age effect ($F(1, 16) = 70.42$, $p < 0.0001$) and a treatment x age interaction ($F(1, 16) = 56.05$, $p < 0.0001$). Bonferroni posthoc analysis further informed us that while a significant effect of leptin on VTA pSTAT3 immunoreactivity was observed in PND 16 pups ($p < 0.0001$), leptin failed to increase the number of pSTAT3-positive cells in this region on PND 10 ($p > 0.05$). To determine whether pSTAT3 activation following leptin occurred in DA neurons (Figure V-3), we counted the number of co-labeled pSTAT3 and TH neurons and used the ratio of pSTAT3/TH positive cells over pSTAT3 cells and expressed it as a percentage. In leptin-treated pups, 19.48 % ± 7.11 and 24.1% ± 4.54 of pSTAT3-positive neurons were TH-positive on PND10 and PND16, respectively. In the vehicle-administered pups, no co-labeling of pSTAT3 and TH was observed on PND10 and only a single pSTAT3 and TH double-labeled neuron was identified on PND16.

Leptin-induced pERK1/2 immunostaining was not detected in the VTA

In contrast to pSTAT3 activation that we observed in the PBP portion of the VTA in PND16 pups and to a lesser extent in PND10 pups, we did not observe any pERK1/2 immunoreactivity in this region of the VTA at any age tested (Figure V-4A). Furthermore, leptin-induced pERK1/2 was not observed in any of the other main subdivisions of the VTA (PN, VTT, PHA, Figure V-4). We did however detect pERK1/2 in PND 10 and PND16 pups in the Edinger

Westphal (EW) nucleus at the level of the VTA (FigureV-4), in the ARC (Figure V-4) and in the caudal linear nucleus of the raphe (CL, FigureV-4), a heart-shaped structure located in the anterior midbrain around the midline and containing medium-sized TH-immunoreactive neurons (Ikemoto, 2007). In the ARC, EW and CL, leptin-induced pERK 1/2-positive neurons did not co-localized with TH.

Discussion

The goal of the present experiments was to examine the onset of the functional VTA response to leptin during the neonatal period (PND10 and PND16) and to characterize the phenotype of leptin-responsive VTA neurons. We focused on two intracellular signaling molecules known to be primarily activated by leptin in the adult VTA, pSTAT3 (JAK/STAT pathway) (Fulton et al., 2006; Hommel et al., 2006) and pERK1/2 (MAPK pathway) (Trinko et al., 2011) and performed double immunofluorescence for these molecules in conjunction with TH, a marker of DA neurons. On PND10, leptin receptors (ObRb) were already present in the VTA, although at a lower density relative to the ARC region. These receptors were modestly functional on PND10 in the VTA since we detected a weak pSTAT3 signal in pups treated with leptin. By PND 16, we observed a large increase in the number of leptin-induced pSTAT3-positive neurons in the anterior lateral PBP of the VTA and a significant effect of leptin administration was revealed. Of the pSTAT3-positive neurons identified, 19.48 % ± 7.11 (PND 10) and 24.1% ± 4.54 (PND 16) colocalized with TH, demonstrating that a subpopulation of DAergic neurons can be activated in response to leptin in the early postnatal period. The response of the DA neurons to leptin was increased on PND16, a time coincident with the onset of independent feeding. In contrast, pERK1/2 was not detected in the VTA of PND10 or PND16 pups in response to leptin treatment, although pERK1/2 immunoreactivity was observed in other areas including hypothalamic (ARC) and midbrain structures (CL and EW) which contain DA neurons. In none of these regions did pERK1/2 co-localize with TH, suggesting that the activation of pERK occurs in non-DA neurons in developing rats.

In adult rodents, leptin has been shown to activate two intracellular signaling pathways in the VTA, the JAK/STAT3 pathway (Fulton et al., 2006; Hommel et al., 2006) and the MAPK/ERK 1/2 pathway (Trinko et al., 2011). The present results demonstrate pERK1/2 signaling in areas other than the VTA on PND10 suggesting that the lack of signal in the VTA does not represent a problem

with immunohistochemical detection, but is representative of the absence of leptin signaling through this molecule in the VTA of young pups. It is possible that leptin signaling via pERK1/2 appears later than PND16, as experiments in adults have shown a significant effect of leptin on VTA pERK1/2 (Trinko et al., 2011). However, Bouret and colleagues (2012) have reported a significant effect of leptin on pERK1/2 in the ARC of PND10 mice. The fact that leptin-induced pERK1/2 activation is absent in the VTA on PND16 might indicate that, contrary to pSTAT3, this intracellular pathway is not implicated in early regulatory mechanisms of food intake in place at the onset of independent feeding. The development of leptin signaling through pERK1/2 in the VTA might serve another function once the pattern of food intake is established in adulthood.

On PND 16, we observed a significant effect of leptin on pSTAT3 immunoreactivity in the VTA, which was mostly observed in the anterior lateral PBP. In this structure, close to 25% of pSTAT3 immunoreactive neurons co-localized with TH. This percentage of DA neurons activated by leptin is low compared to the more widespread activation of pSTAT3 in VTA DA neurons of adult rodents. In the VTA of adult rats, Hommel et al. (2006) estimated that TH and pSTAT3 co-localization ranges between 82% and 95% depending on the level of the VTA examined. More in-line with our findings, another study by Fulton and colleagues (2006) estimated that the co-localization of pSTAT3 and TH in the VTA of adult mice is approximately 42%. The lower pSTAT3 signaling observed in PND16 pups most likely represents reduced leptin receptor expression compared to the adult and the more discrete distribution of pSTAT3-positive cells in neonates might reflect unique features of developmental regulation by leptin on the VTA. In the rat hypothalamus, leptin receptor expression (Ob-Rb) in the ARC and VMH (Cottrell et al., 2009) is developmentally regulated, with a steady increase observed between PND 10 and PND 15. A recent study performed in mice (Caron et al., 2009) shows that the relative expression of VTA Ob-Rb and the pSTAT3 response to leptin is similar between PND10 and adult mice. Although leptin receptor expression was examined and detected in the VTA of

PND10 pups in the present experiments, we did not compare with expression on PND16, neither did we attempt to quantify the expression of leptin receptors. Future studies will be required to establish whether there is a developmental regulation of leptin receptor expression in the VTA of the rat. Species considerations are important when examining neonatal leptin physiology. In the mouse, leptin concentrations sharply increase during the second postnatal week, reach their peak on PND10 (10-fold increase from adulthood) and return to normal adult levels by PND16 (Ahima et al., 1998). The leptin surge of the neonatal rat is not as pronounced as in the mouse (3-5 fold higher than in the adult (Cotrell et al, 2009), with leptin concentrations reaching a peak on PND7 and remaining relatively high compared to adults (approximatively a 3-5 fold increase) throughout the pre-weaning period (Cotrell et al., 2009).

Interestingly, most of the leptin-induced pSTAT3-positive neurons detected on PND 16 were located in the anterior lateral PBP. Detailed mapping of individual VTA DA neurons has revealed that DA neurons originating in this region project mainly to the ventrolateral striatum which includes the NAc core, NAc shell, and lateral tubercle (Ikemoto, 2007). Using retrograde tract tracing to examine leptin-sensitive VTA projections, Fulton and colleagues (2006) demonstrated that pSTAT3 immunoreactivity in the VTA following the administration of leptin co-localizes with tracer retrogradely transported from the NAc core and shell, suggesting that leptin modulation of DA function occurs through the ventrolateral striatum. Taken together, our results suggest that the onset of VTA leptin responsiveness in PND16 occurs primarily in a population of DA neurons projecting to the ventral striatum/NAc. It is possible that as the animal matures further, other DA subpopulations of the VTA that project to other areas become responsive to leptin. Indeed, leptin likely targets diverse populations of DA neurons, projecting to different target areas in adults. For instance, a recent study in mice found that leptin receptor-expressing neurons of the VTA project to the extended central amygdala and not the ventrolateral striatum (Leshan et al., 2010). Further evidence of leptin modulation of the VTA-amygdala projections is

also derived from the studies of Liu and colleagues (2011) using conditional knockout mice with selective deletion of leptin receptors on DA neurons (LepR(DAT-cre). Interestingly, the early responsiveness of VTA neurons to leptin coincides with the onset of independent feeding and suggests that by PND16, some rudimentary component of the hedonic modulation of feeding might already be in place.

Accumulating evidence suggests that even with high circulating levels of leptin (Ahima et al., 1998; Cottrell et al., 2009; Devaskar et al., 1997; Rayner et al., 1997), neonates (PND10) are insensitive to the anorectic effects of leptin. For example, the hyperphagia and obese phenotype of the leptin-deficient *ob/ob* mouse (Zhang et al., 1994) does not emerge until the fourth postnatal week (Mistry et al., 1999) and PND10 pups do not reduce milk intake after leptin injection (Mistry et al., 1999; Proulx et al. 2002). The mechanisms for this leptin insensitivity are currently unclear since at the level of the ARC nucleus, leptin induces functional production of pSTAT3 (Bouret et al., 2012; Caron et al., 2010) and adult-like regulation of NPY and POMC occurs (Proulx et al. 2002). The immaturity of hypothalamic projections has been hypothesized as a potential mechanism. Indeed, the projections from the ARC to other hypothalamic (DMH, PVN, LH) nuclei are developing during the early postnatal period (Bouret et al., 2004b). The ability of leptin to activate these nuclei depends on the unique innervation pattern of each nucleus (Bouret et al., 2004b). While the ARC c-FOS response to leptin is similar on PND10 and PND16 in mice, peak c-Fos labeling in the LH is detected on PND 16 when projections from the ARC to the LH have reached maturity (Bouret et al, 2004b). In the present experiment, we demonstrate that leptin does not activate signaling through the JAK/STAT3 or MAPK/ERK1/2 in the VTA on PND10, suggesting that connections between the ARC-LH and VTA might be required for the anorectic effect of leptin to occur. By PND 16 when independent feeding of the pups starts, these connections might be established and functionally active.

In addition to its direct action on VTA DA neurons, leptin also modulates

DA function indirectly through LH projection to VTA or modulation of glutamatergic tone on VTA DA neurons in adults. For example, leptin acts on neurotensin neurons in the LH, which project to VTA DA neurons and regulate their activity (Leinninger et al., 2011). Recent, electrophysiological experiments demonstrate that leptin treatment causes a presynaptic inhibition of the probability of glutamate release onto VTA DA neurons (Thompson & Borgland, 2013), thus causing an indirect inhibitory effect on DA release. Thus, while we show that leptin does not directly modify leptin signaling in the VTA on PND10, it is still possible that the indirect modulation of DA by leptin is functional at this time. Further experiments are currently performed to address this question.

In conclusion, the data presented in this manuscript suggest that the inability of leptin to reduce feeding in PND10 pups is mediated, at least in part, by the functional insensitivity of the VTA to leptin at this age as evidenced by the lack of either pSTAT3 or pERK1/2 activation following leptin treatment. With the emergence of independent feeding around PND16, VTA DA neurons become responsive to leptin and might actively participate in the emerging anorectic effects of leptin until adulthood.

Figure V-1. Leptin receptor expression

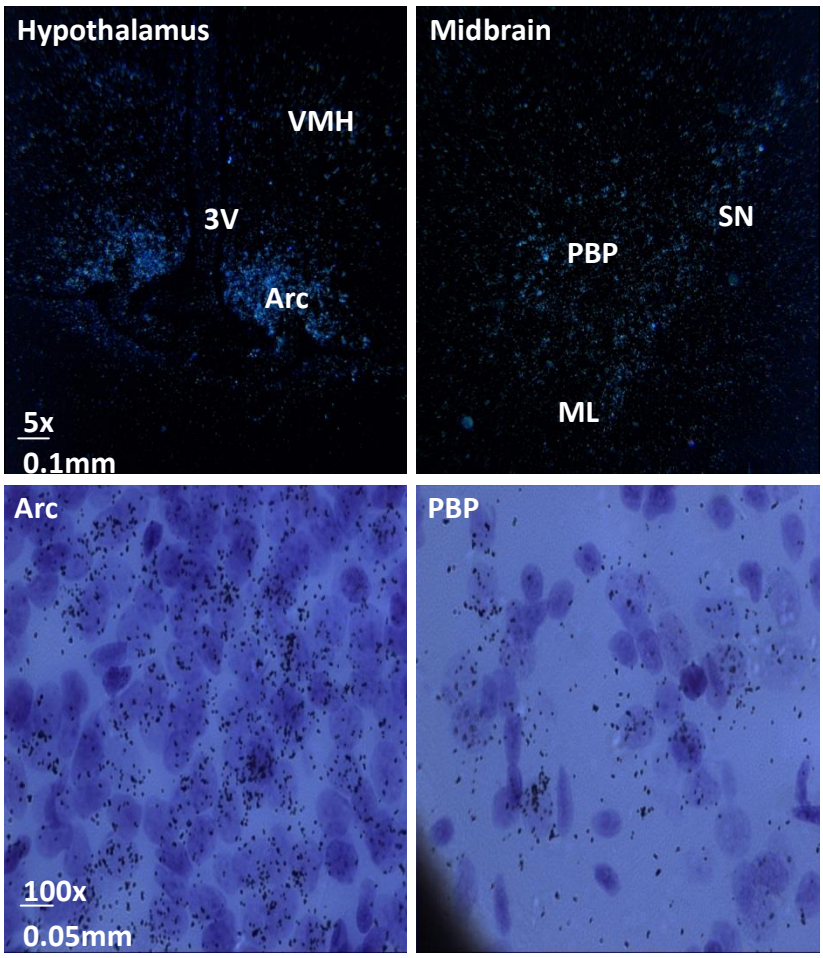

Figure V-1. *Representative images of Ob-Rb expression in the hypothalamus and midbrain of PND10 pups. At high magnification (100x) with brightfield (bottom panels), we observed clusters of silver grains over single neurons in the ARC and the VTA, although the density of these clusters was lower in the VTA compared to the ARC. At low magnification (5x) using darkfield (top panel), we can observe the location of these clusters in hypothalamic nuclei (ARC and VMH) and in the midbrain (PBP and SN).Abbreviations:* ***Arc****, arcuate nucleus,* ***ML****, medial lemniscus,* ***PBP****, parabrachial pigmented area;* ***SN****, substantia nigra;* ***VMH****, ventromedial hypothalamic nucleus.*

Figure V-2. Leptin-induced pSTAT3 in the VTA

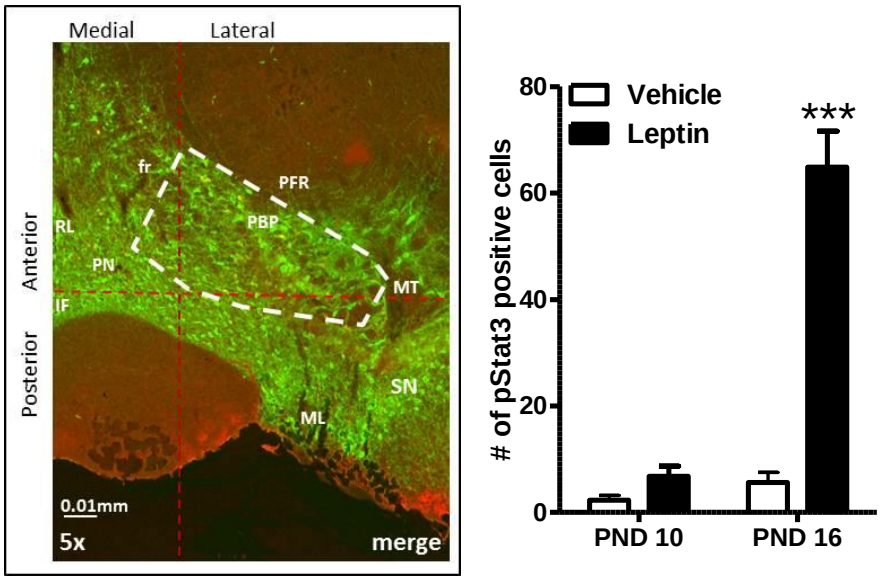

Figure V-2. *Right: Representative image of the localization of pSTAT3 (red) immunoreactive neurons in VTA DA (green) and non-DA neurons of a PND 16 leptin-treated pup. The VTA is divided into anterior/posterior and medial/lateral by red lines according to Ikemoto (2007). Most pSTAT3 immunoreactivity was observed in the anterior lateral PBP of the VTA, highlighted by the dashed white line. Left: The number of pSTAT3-positive neurons in vehicle and leptin-treated PND 10 and PND 16 pups (5 animals per group). While leptin failed to increase pSTAT3 immunoreactivity in the VTA on PND 10, leptin increased the number of pSTAT3-positive neurons on PND 16. Values are means +/- SEM, ***p<0.001 Bonferroni posthoc test. Abbreviations:* ***fr****, fasciculus retroflexus;* ***IF****, interfascicular nucleus;* ***ml****, medial lemniscus;* ***MT****, medial terminal nucleus of the accessory optic tract;* ***PBP****, parabrachial pigmented area;* ***PFR****, parafasciculus retroflexus area;* ***PN****, paranigral nucleus;* ***SN****, substantia nigra.*

Figure V-3. *Representative images of merged PBP DA (green) and pSTAT3 (red) immunoreactivity of PND 10 and PND 16 pups treated with leptin (3mg/kg of body weight.) Notice the increase in the number of pSTAT3-positive neurons from PND 10 to PND 16. In leptin-treated PND 16 pups, 24.1% ± 4.54 of pSTAT3-positive neurons co-localized with TH. 40x images represent zoomed images of the 10x images. Arrows indicate co-localized neurons.*

Figure V-3. pSTAT3 and TH co-localization in the VTA

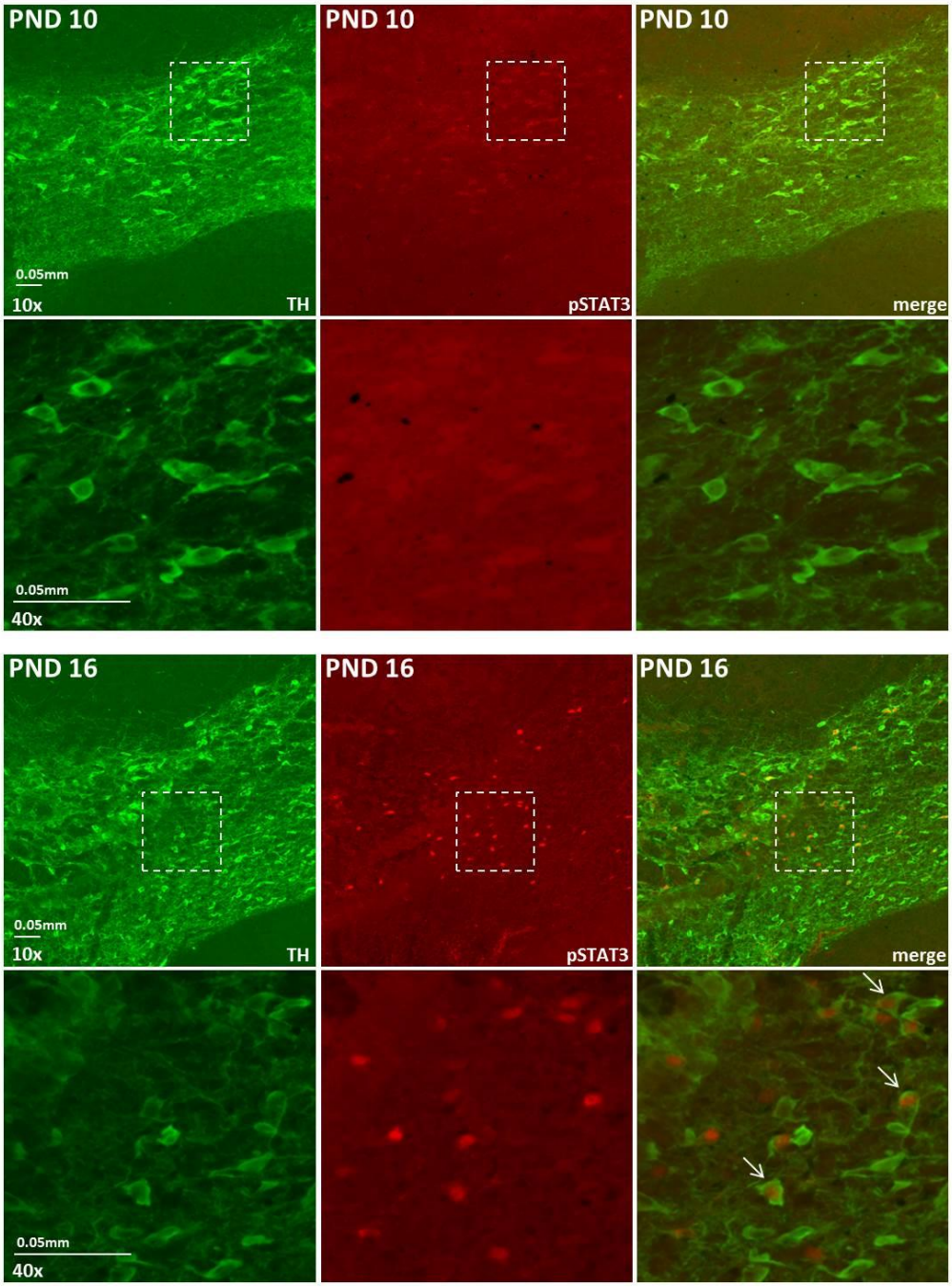

Figure V-4. pERK1/2 and TH co-localization in the VTA

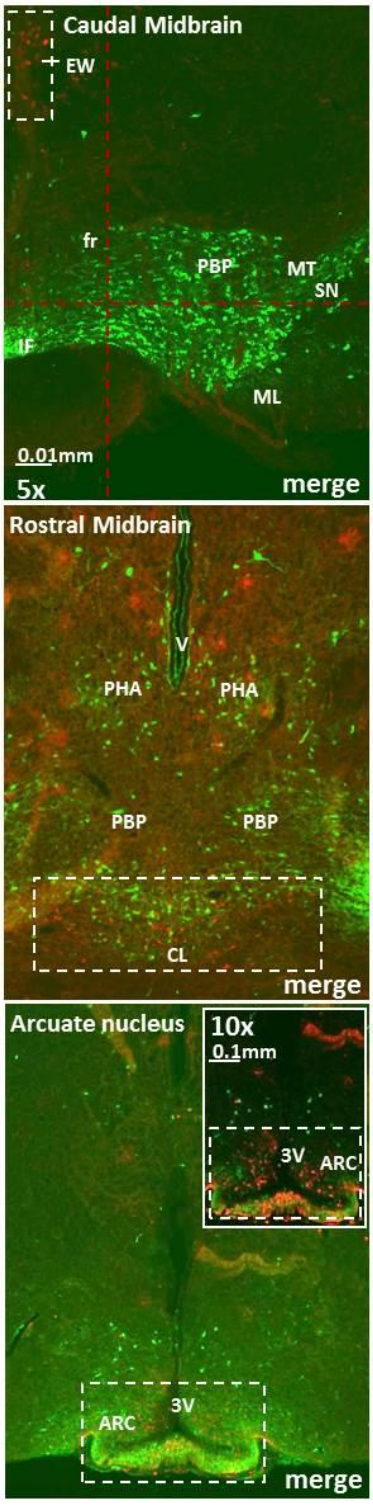

Figure V-4. *Representative images of DA (green) and pERK1/2 (red) immunoreactivity in the caudal, midbrain, rostral midbrain, and ARC of PND 16 pups treated with leptin (3mg/kg of body weight.) Notice the lack of pERK 1/2 in the VTA (caudal midbrain) and the presence of pERK1/2 immunoreactivity in the EW (caudal midbrain), CL (rostral midbrain), and ARC of leptin-treated pups. In neither of these regions did pERK1/2 co-localize with TH. The VTA is divided into anterior/posterior and medial/lateral by red lines according to Ikemoto (2007). 10x images represent zoomed images of the 5x images. Abbreviations:* ***ARC****, arcuate nucleus;* ***EW****, Edinger Westphal;* ***fr****, fasciculus retroflexus;* ***ML****, medial lemniscus;* ***MT****, medial terminal nucleus of the accessory optic tract;* ***PBP****, parabrachial pigmented area;* ***PHA****, posterior hypothalamic area;* ***PFR****, parafasciculus retroflexus area;* ***SN****, substantia nigra;* ***V****, ventricle,* ***3V****, third ventricle.*

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leptin in the ventral tegmental area to decrease food intake is dependent on Jak-2 signaling. Am J Physiol Endocrinol Metab, 297(1): E202-10.

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Chapter VI. General discussion

Large-scale Canadian epidemiological studies have demonstrated that maternal obesity and excessive weight gain during pregnancy resulting in high birth weight increased the child's risk of obesity (Dubois & Girard, 2006; Ferraro et al., 2012). This phenomenon is known as 'metabolic imprinting', which refers to the predisposition to obesity following alterations in the nutritional and hormonal environment of developing young (Levin, 2006). While programming of energy balance has been shown to occur in hypothalamic circuits involved in the homeostatic control of energy balance, we have used an animal model of early overnutrition to examine the long-term consequences of early exposure to high-fat on mesolimbic DA function, HPA activity and DA-dependent feeding behavior. In our model, dams are placed on a 30% high-fat or 5% control diet from gestation day 13 to PND22, when offspring are weaned from their mothers and maintained on the 5% control diet until testing in adulthood. The experiments presented in this thesis demonstrated that exposure to high-fat during the perinatal period altered the presynaptic regulation of mesolimbic DA and consequently, modified the magnitude and pattern of NAc DA release following amphetamine administration, but also during the anticipation of high-fat food rewards and in response to acute stress. Early high-fat exposure also impaired adaptations in NAc DA and ACTH responses usually observed with repeated stress. The behavioral consequences of these changes are that offspring of high-fat fed mothers displayed increased operant responding for fat-enriched food rewards, diminished locomotor responses to acute amphetamine administration (Naef et al., 2008) and reduced behavioral sensitization to repeated amphetamine administration (Naef et al., 2008). While the consequences of maternal high-fat feeding have been mainly evaluated in the adult offspring, the final data chapter of this thesis demonstrated that the onset of functional VTA responsiveness to the anorectic hormone leptin occurs during the time of exposure to the high-fat diet and suggests that one of the possible mechanisms triggering long-term change in DA function with perinatal high-fat involves leptin-induced changes in VTA DA neuronal activity. These findings suggest that the life-long changes in eating patterns observed in offspring

exposed to high-fat during early development are mediated, in part, by modifications in mesolimbic DAergic circuits.

Experiments conducted during the course of my Master's degree demonstrated that adult offspring exposed to high-fat during early development displayed reduced locomotor responses to the psychostimulant drug amphetamine and reduced behavioral sensitization with repeated administration of the drug (Naef et al., 2008). These behavioral studies suggested blunted DA tone in high-fat offspring. However, TH and DA levels were elevated in brain punches in these animals suggesting that DA release with amphetamine administration was attenuated in high-fat offspring. Thus, the first aim of my dissertation was to directly measure DA release in high-fat and control adult offspring using *in-vivo* microdialysis. As predicted, data presented in the first manuscript demonstrated an attenuated NAc DA response with amphetamine administration suggesting that the diminished locomotor response of high-fat animals was due, at least in part, to a dampening of the drug's stimulant action on NAc DA neurotransmission.

Psychostimulant drugs, including amphetamine, 'highjack' the DA system and therefore, provide a good experimental tool to examine DA function. In the context of metabolic imprinting however, what is even more interesting is the programming of DA responses to high-fat food rewards, which was addressed in the second manuscript of this dissertation. To increase the temporal resolution of DA neurotransmission, we used *in-vivo* voltammetry to monitor rapid fluctuations in NAc DA during the anticipation and consumption of high-fat food rewards in control and high-fat exposed offspring. Using a Pavlovian conditioning paradigm, we showed that the repeated pairing of a compound cue (tone + 'click' of the food dispenser) with the delivery of a high-fat food pellet led to a cue-induced anticipatory DA response in control and high-fat offspring. Interestingly, on day 4 of testing, this anticipatory NAc DA response was significantly diminished in the high-fat offspring. Thus, high-fat exposure during early development is reducing the NAc DA response to amphetamine and the anticipation of high-fat food rewards.

Although mostly considered in the context of reward, NAc DA neurotransmission is also modulated by stress. Indeed, in the third manuscript, we showed that tail-pinch stress significantly increased extracellular NAc DA concentrations in both control and high-fat offspring. However, we found that the NAc DA response to tail-pinch was enhanced in the high-fat offspring and failed to sensitize with repeated stress. Thus, DA responses at the level of the NAc are differentially regulated in high-fat offspring depending on the type of stimulation, with blunted responses observed with 'rewarding' stimuli' (amphetamine and high-fat food pellets) and an elevated response observed during and following stress.

The 'reward deficiency' hypothesis of obesity

The majority of human and animal studies demonstrate that diet-induced obesity is associated with deficits in mesolimbic DA function (Davis et al, 2008; Frank et al., 2012; Geiger et al, 2009; Green et al., 2011; Huang et al, 2006; Stice et al., 2008; Stice et al., 2008b; Van de Giessen et al., 2012; Wang et al, 2001; Wang et al, 2004). These findings have led to the 'reward deficiency' hypothesis of obesity (Berthoud et al., 2012) which proposes that reduced DA tone leads to the overeating of palatable foods as an attempt to restore NAc DA levels. Conversely, a few experiments in humans have shown that the presentation of visual palatable food stimuli is associated with increased striatal activation in obese individuals (Martin et al., 2010; Rothemund et al., 2007; Stoecket et al., 2009), suggesting that increased DA responses to food cues is what confers vulnerability to overeating and obesity (Carnell et al., 2011). These findings have led to the speculation of a 'dynamic' model of DA function in obesity, characterized by an initial hyper DA responsiveness to food cues and subsequent hypo DA responsiveness to the consumption of palatable food rewards (Carnell et al., 2011).

Whether differences in DA tone emerge as a consequence of high-fat feeding and/or diet-induced obesity or exist prior to the development of obesity is still unclear. Here, we show for the first time that exposure to high-fat during

early development reduces the NAc DA response to amphetamine and the anticipation and consumption of a high-fat food reward in a Pavlovian conditioning paradigm. Our results suggest that 1) maternal diet and the resulting perinatal nutritional environment can program DA function in the long-term and 2) NAc DA hypofunction can occur prior to the development of obesity. Recent studies examining obesity-prone (vs. obesity resistant) rats have also demonstrated DA hypofunction prior to the development of obesity. Rats classified as 'obesity-prone' based on their weight gain during 5 days of access to a high-fat diet display a reduced NAc DA response to a high-fat meal and lipid emulsion (Rada et al., 2010). Furthemore, in selectively bred obesity-prone rats, there was a 50% reduction in basal extracellular NAc DA concentration and a significant reduction in electrically-evoked DA release (Geiger et al., 2008). Together, these findings suggest that DA hypofunction can occur prior to the development of obesity in animals that are vulnerable to the development of obesity.

However, opposite effects of early diet on DA function have also been reported. Prenatal and postnatal exposure to high-fructose corn syrup (HFCS) or sucrose (Bocorsly et al., 2012) and a maternal diet rich in sugar and fat (Shalev et al., 2010) have been shown to increase locomotor responses to amphetamine administration in the adult offspring. These divergent findings might be explained by differences in the macronutrient composition of the maternal diet, in particular the ratio of fat/sugar. While we used a high-fat/ low carbohydrate diet in order to maintain isocaloric intake between experimental groups, the experiments described above used supplementation with either high carbohydrate (HFCS and sucrose) (Bocorsly et al., 2012) or a combination of a high-fat/high-sugar diet (Shalev et al., 2010). Obviously, the macronutrient ratio is an important factor is determining how the mesolimbic DA system is affected by early diet. Indeed, a recent experiment conducted in adult rodents suggested that it is more the high fat/carbohydrate ratio and not the total energy intake and increased adiposity that is responsible for the reduction in D_2/D_3 receptor expression in diet-induced obesity (de Giessen et al., 2012).

It is also possible that vulnerability to obesity is conferred by both hyper- and hypo- DAergic function or that a simple classification of hyper- or hypo-DA function is misguiding. In our model, offspring exposed to high-fat show increased TH and DA in brain punches, increased NAc DA response to stress, and increased operant responding for high-fat food rewards, suggesting increased DA tone in these animals. On the other hand, these high-fat exposed offspring show reduced locomotor and NAc DA responses to amphetamine and blunted anticipatory responses to food cues, suggesting reduced DA tone. Thus, in these animals, we observe both hyper- and hypo-DA function depending on the stimulation and how this occurs remains unknown. In order to elucidate some of the underlying mechanisms of both hyper- and hypo- DA function, we performed additional experiments to examine the presynaptic regulation of NAc DA in control and high-fat offspring.

Functional alterations in NAc DA in high-fat exposed offspring

The second aim of this PhD dissertation was to examine the long-term consequences of early exposure to high-fat on the regulation of NAc DA. We did not observe any group differences in basal or amphetamine-stimulated PFC DA release, eliminating the PFC DA as a potential mediator of diet group differences in the NAc DA response to amphetamine and we did not observe differences in VMAT, an important mediator of NAc DA regulation. However, we showed a higher uptake capacity of NAc DAT sites in high-fat animals. Increases in DAT have been reported with maternal high-fat (Vucetic et al., 2010) and cafeteria (Ong & Muhlhauser, 2011) feeding. Interestingly, in our experiments, we failed to detect a significant effect of maternal diet on the density of NAc DAT binding sites (Naef et al., 2008). Rather, using a functional approach to measure DAT activity, a higher uptake capacity of NAc DAT was found in high-fat exposed animals. The regulation of the DAT occurs via trafficking of DAT from the cell surface where it regulates extracellular DA concentrations, to internalization of the DAT via clathrin-coated vesicles (Zahniser et al, 2004) and up- or down-regulation of DAT is transient (Copeland et al., 2005). Thus, it is possible that the

differential DA responses of high-fat offspring to amphetamine and the anticipation of high-fat food rewards and stress reflects differences in how these stimuli modulate DAT activity. While AMP increases DA by inducing the reverse transport of DA through DAT (Zahniser et al, 2004), stress increases DAT activity by increasing the production of DAT (Copeland et al., 2005). Thus, the dynamic nature of DAT might partly explain the opposing effects in NAc DA responses with different types of stimulation in high-fat exposed offspring.

The second important finding regarding the regulation of NAc DA is a reduction in presynaptic inhibitory D_2 autoreceptors in high-fat vs. control offspring. Most studies examining D_2 receptor density in obesity and early overnutrition have focused on post synaptic D_2 receptors and have yielded opposing results. For example, human obesity has been associated with both decreased (deWeiger et al., 2011; Haltia et al., 2007; Wang et al, 2001; Wang et al, 2004) and increased (Dunn et al., 2012) D_2 receptor availability. A reduction in the density of D_2 autoreceptors in high-fat offspring suggests diminished regulation of NAc DA and thus, increased extracellular DA. In previous work, we reported increased VTA TH expression, the rate-limiting enzyme in DA synthesis in high-fat offspring (Naef et al., 2008) and here, we report an increased NAc DA response to stress in high-fat exposed offspring, perhaps a consequence of decreased D_2 autoreceptors (Lindgren et al., 2001). How the reduction in D_2 autoreceptor density observed in high-fat offspring modulates DA responses to food and food cues remains to be determined.

Increased operant responses for fat-enriched pellets in high-fat exposed offspring

One of the critical findings of this PhD dissertation is that exposure to high-fat during early development increases incentive motivation for fat-enriched food rewards, an effect that is independent of circulating concentrations of leptin and corticosterone at the time of behavioral testing. This suggests that the hyperphagia and susceptibility to obesity observed in high-fat offspring might be the result of enhanced motivation to seek high-fat food. Interestingly, when sugar pellets were used as a reinforcer, we did not observe any diet group differences in

operant behavior. I think that this finding is very important. Most experiments examining food reward in diet-induced obesity and following manipulations in early dietary environment use sucrose as a reinforcer and make broad generalizations regarding reward function. Here, we show that the macronutrient used in the operant test is an important determinant of diet group differences in behavior. A recent experiment by Shin (2012) also suggests that the concentration of fat and sugar can determine behavioral outcome. While diet-induced obesity was associated with a decreased liking of low concentrations of sucrose and corn oil in the taste reactivity test, higher liking scores were observed when high concentrations of sucrose and corn oil were used. Thus, our experiments demonstrate that food reward in animals exposed to high-fat during early development selectively increases incentive motivation for high-fat food rewards. How this 'selectivity' occurs is still unknown.

Increased operant responding for high-fat pellets indicated that the delivery of high-fat pellet might be more rewarding compared to control offspring or that the anticipatory DA response to food delivery was triggering larger responses in these high-fat offspring. Contrary to our predictions, we found that the anticipatory DA response was reduced in high-fat offspring and therefore one could speculate that because the anticipatory response to conditioned food delivery was lower, rats had to perform more bar pressing in order to achieve a "threshold level" of DA secretion which could be perceived as rewarding. This concept is at the core of the hypothesis of DA hypofunction in obesity.

Exposure to high-fat during early development impairs adaptations in dopamine and neuroendocrine responses to repeated stress

The effect of early maternal diet on pathways regulating the rewarding aspect of food intake is certainly not limited to modulation of the mesolimbic DA system. In fact, we have documented that this early environmental change also affects the activity of the HPA axis, an important system linked to both metabolic and hedonic aspects of food intake. In the third manuscript presented in this PhD dissertation, we found that exposure to high-fat during early development impairs

adaptations in both NAc DA and HPA responses to repeated stress. Compared to controls, fat exposed offspring showed an enhanced NAc DA response to acute stress, but failed to sensitize to repeated stress. Similarly, ACTH responses to tail-pinch stress failed to habituate in high-fat adult offspring and did not show facilitation to a novel stressor as observed in control animals. Increased anxiety has been observed in adult offspring exposed to high-fat during early development in rodents (Bilbo & Tsang, 2010) and non-human primates (Sullivan et al., 2011). However, this manuscript is the first to describe the neuroendocrine and DA stress response of high-fat exposed offspring and show significant alterations with repeated stress. In the context of the programming of behavior, this finding is very interesting. Reductions in HPA activity with repeated exposure to a non-harmful stressor are adaptive for the organism (Grissom & Bhatnagar, 2009) and the impairments observed in the high-fat offspring suggest that they are vulnerable to a number of pathologies, including obesity. Interestingly, Sharma & Fulton (2013) have recently demonstrated that diet-induced obesity in adulthood is associated with an exaggerated corticosterone response to stress as well as increased depressive-like behavior. Although not examined in the experiments presented here, it would be interesting to examine depressive-like behaviors in high-fat exposed offspring, especially following repeated or chronic stress.

Perinatal programming of the mesolimbic DA system

The experiments presented in the first three manuscripts of this dissertation showed that offspring exposed to high-fat during early development display significant alterations in DA function. These experiments were conducted in adulthood, long after the termination of the high-fat diet, suggesting that early exposure to high-fat is permanently altering mesolimbic DA function, although the mechanisms by which this programming occurs are unclear. Increasing the fat content of the maternal diet alters the milk composition of the mother and the hormonal profile of the developing pups, with high-fat exposed pups displaying increased circulating concentrations of leptin and corticosterone relative to pups of mothers feeding on the control diet (d'Asti et al., 2010). Several experiments

implicate leptin in the programming of hypothalamic circuits because leptin is required for the normal development of hypothalamic projections (Bouret et al., 2004). The role of leptin in programming might not be limited to the ARC region, and we propose that this hormone could also be involved in programming DA function. However, this can only be accomplished if leptin receptors are present and functionally coupled to second messengers systems in the VTA during early development. Thus, the final data chapter of this PhD dissertation examined leptin responsiveness of VTA DA neurons during early development. We showed that while no functional intracellular signaling response (pSTAT3 or pERK1/2) to leptin could be detected in the VTA on PND10, leptin induced significant activation of pSTAT3 on PND16, suggesting that leptin could be involved in mediating long-term changes in DA function with high-fat feeding.

How diet and/or leptin could induce long-term changes in DA function is still unclear but epigenetic modifications are an interesting possibility. A recent study reported that exposure to high-fat during early development was associated with decreased TH mRNA expression in adulthood, increased expression of DAT, and decreased global DNA methylation in the VTA and NAc (Vucetic et al., 2010). Epigenetic modifications in offspring exposed to high-fat during early development are currently being investigated in out laboratory.

Conclusions and future directions

The aims and main findings of this thesis are illustrated in Figure VI-1. Our experiments demonstrate that exposure to high-fat through the maternal milk induces long-lasting alterations in DA function and behavior and impairs adaptations in NAc DA and HPA responses to repeated stress, providing novel mechanisms though which early diet can change life-long patterns in feeding behavior and increase susceptibility to the development of obesity.

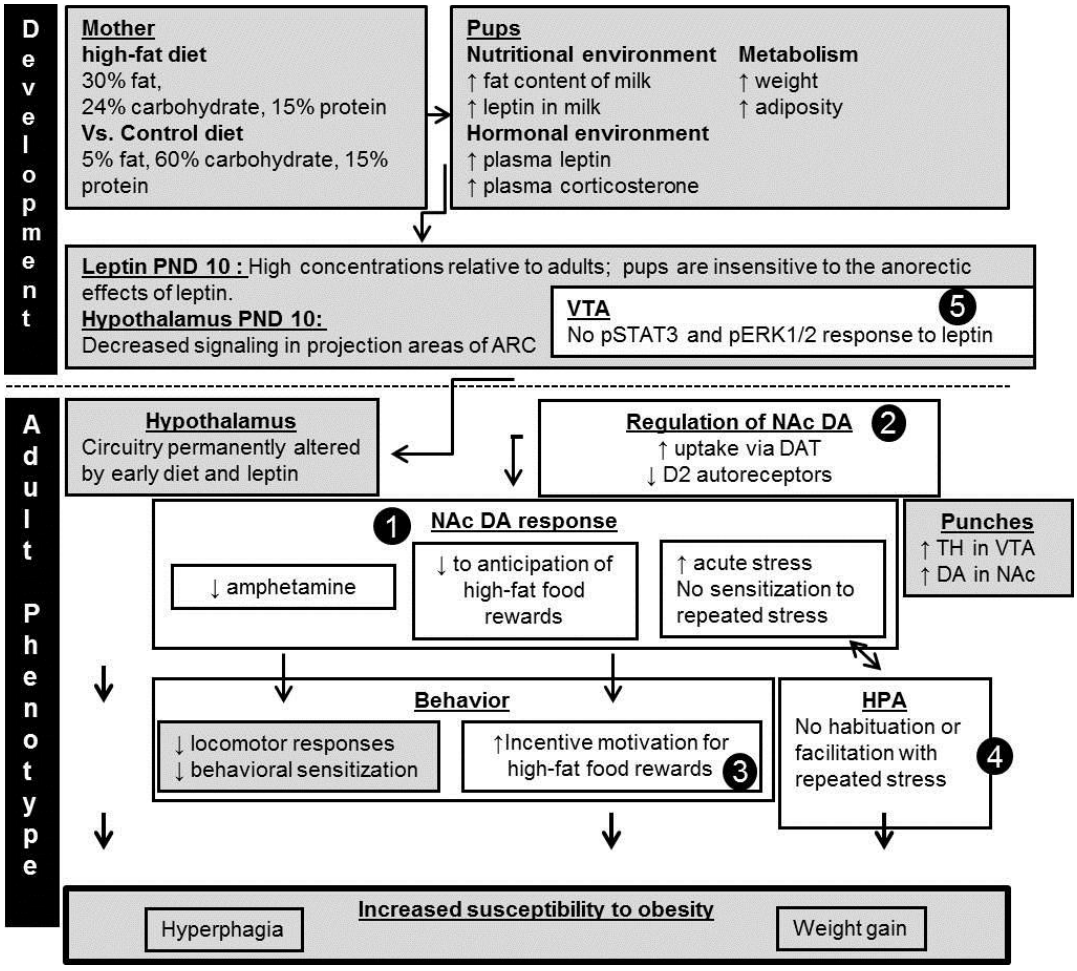

Figure VI: *A summary of the main findings of this PhD dissertation. The white boxes represent data collected during the course of this PhD project with numbers (1-5) corresponding to the aims of the dissertation*

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Appendix A

Table 1: Composition of the diets (g/kg) used in these experiments

Ingredient	Control diet	High-fat diet
Casein	171,5	172,5
L-Cystine	3	3
DL-Methionine		
Corn Starch	328,5	
Maltodextrin	150	80,4
Sucrose	150	150
Soybean oil	24	149
Corn oil		
Lard	24,1	149,3
Cellulose	97,34	234,71
Mineral mix	35	39,9
Calcium phosphate dibasic	6	6,8
Calcium carbonate		
Magnesium oxide	0,3	0,34
Ferric citrate	0,25	0,29
Vitamin mix	10	11,4
Choline bitartrate		2,3
Ethoxyquin (antioxydant)	0,01	0,06
Macronutrient composition (by weight)		
Protein	15%	15%
Carbohydrate	60%	24%
Fat	5%	30%
Macronutrient (%Kcal from)		
Protein	17,6%	14,3%
Carbohydrate	69,4%	22,5%
Fat	13%	63,2%