The long-term effect of juvenile stressor exposure and access to palatable food:
alterations in dopamine and ghrelin signalling

by

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ABSTRACT

Juvenile stressor exposure increases the susceptibility to anxiety-like characteristics in adulthood and it is thought that the consumption of palatable food can dampen the HPA-axis, in part, through the mesolimbic pathway. Ghrelin, an orexigenic peptide, increases food intake and has a role in stress-induced preference for palatable food by modulating dopamine release in the reward pathway. We hypothesize that the combination of juvenile stressor exposure and access to palatable food sensitizes the reward pathway, which will be manifested by altered dopamine receptor mRNA expression and enhanced sensitivity to ghrelin in adulthood. Findings revealed that access to palatable food ameliorated the anxiogenic effects of juvenile stressor exposure in adulthood however at the cost of increased adiposity. Interestingly, our findings displayed a lack of association between the upregulation of dopamine receptor mRNA expression occurring at nucleus accumbens to highly rewarding stimuli (cookie dough and cocaine) in previously stressed rats with access to palatable food. Juvenile stressor exposure enhanced ghrelin signalling for highly hedonic food whereas access to palatable food, regardless of stressor exposure, blunted ghrelin sensitivity.

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LIST OF ABBREVIATIONS

aCSF: Artificial cerebral spinal fluid, **Actβ:** β-Actin, **ACTH:** Adrenocorticotrophic hormone, **AgRP:** Agouti-related peptide, **ANOVA:** Analysis of variance, **ARC:** Arcuate nucleus, **CART:** Cocaine- and amphetamine-regulated transcript, **CPP:** Conditioned place preference, **Cq:** Mean cycle quantification value, **CRH:** Corticotrophin releasing hormone, **CRHR1:** CRH type 1 receptor, **CSD:** Chronic social defeat, **CVS:** Chronic variable stress, **D1:** Dopamine receptor type 1, **D2:** Dopamine receptor type 2, **DA:** Dopamine, **DIO:** Diet-induced obesity, **EPM:** Elevated plus maze, **GABA:** γ-aminobutyrate, **GC:** Glucocorticoid, **GHSR:** growth hormone secretagogue receptor 1a, **GR:** Glucocorticoid receptor, **GRE:** glucocorticoid response element, **HPA:** Hypothalamic-pituitary-adrenal, **icv:** intracerebroventricular, **i.p:** intraperitoneal injection, **LH:** Lateral hypothalamus, **LTP:** Long term potentiation, **α-MSH:** α-melanocyte stimulating hormone, **β-MSH:** β- melanocyte stimulating hormone, **MC3R:** Melanocortin 3 receptor, **MC4R:** Melanocortin 4 receptor, **MCH:** Melanin-concentrating hormone, **MIQE:** Minimum information for publication of quantitative real-time polymerase chain reaction, **mPFC:** Medial prefrontal cortex, **MR:** Mineralocorticoid receptor, **MSN:** Medium spiny neurons, **NA:** Nucleus accumbens, **NPY:** Neuropeptide Y, **PD:** Postnatal day, **PFC:** Prefrontal cortex, **POMC:** Pro-opiomelanocortin, **PVN:** Paraventricular nucleus of the hypothalamus, **RPL-19:** Ribosomal protein L-19, **RT-qPCR:** Real time qualitative polymerase chain reaction, **SEM:** Standard errors of the means, **TRH:** Thyrotropin-releasing hormone, **VTa:** Ventral tegmental area

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INTRODUCTION

Childhood and adolescent obesity has doubled since 1980 and is now a global epidemic as 20 million school aged children are considered overweight or clinically obese (World Obesity, 2014). In Canada, childhood and adolescent obesity has increased from 15% in 1978/79 to 26% in 2004 (Government of Canada, 2010). Notably, overweight and obese adolescents have doubled from 14% to 29% and tripled from 3%-9% respectively (Statistics Canada, 2006). Children and adolescents who are overweight and obese have a higher risk for chronic illnesses such as type II diabetes, cardiovascular disease, atherosclerosis, musculoskeletal conditions and some forms of cancer (Lee et al., 2014; World Health Organization, 2015). In the past two decades overweight and obesity in this demographic have been alarmingly high. The rise in diseases previously only seen in adults have emerged in childhood and in adolescence, such as metabolic syndrome and type II diabetes; this has led to a garnered interest in preventative health research within this demographic (Lobstein, Baur, & Uauy, 2004; Statistics Canada, 2006; Wilkinson & McCargar, 2008). Importantly, childhood and adolescent obesity can lead to a prognosis of adult obesity, as it is a persistent and detrimental phenotype throughout the lifespan (Karnik & Kanekar, 2012).

The current escalation of obesity is attributed to a combination of genetics, environmental factors, socioeconomic status, an increase in sedentary behaviors and a decline in physical activities (Bouret, Levin, & Ozanne, 2015; Karnik & Kanekar, 2012; Zhang, Wang, & Zhao, 2014). Of particular interest is the role that stress plays in the genesis of obesity as daily life stressors, perceived and experienced, are continually occurring (Michels et al., 2013). There is evidence that stress influences a change in

caloric intake where approximately 40% of people have reported to increase their food intake and 40% decrease their food intake; this bidirectional pattern results from various factors like individual differences, genetics and the severity and duration of stressor exposure (Block, He, Zaslavsky, Ding, & Ayanian, 2009; Dallman, 2010; Yau & Potenza, 2013). In particular, negative life events and stress experienced during childhood has been positively correlated to higher body mass index, adiposity and a change in diet preference towards more highly caloric palatable food (Michels et al., 2013, 2015; Vanaelst et al., 2013). A possible reason for a change in diet preference could be due to the proposed stress-buffering effects of palatable food through the reward pathway (Chuang & Zigman, 2010; Kenny, 2011; Lee, Kim, & Jahng, 2014). However, there is a gap in the literature investigating the implications of juvenile stress and access to a palatable diet when one reaches adulthood. In this thesis, the long-term consequences of juvenile stress and the combination of a palatable diet and its effect on the reward pathway will be examined.

The Stress Response

The stress response is associated with a number of physiological changes that include the stimulation of the hypothalamic-pituitary-adrenal (HPA) axis in order to cope with a real or perceived threat. The activation of the HPA axis gives rise to a series of neuroendocrine events that modulate the biological and behavioral reactions to stress such as digestion, immune response, emotion, and homeostasis (Smith & Vale, 2006).

Upon an encounter with a stressor, hypophysiotropic neurons in the parvocellular division of the paraventricular nucleus of the hypothalamus (PVN) synthesizes

corticotrophin releasing hormone (CRH) that is secreted into the portal blood system accessible to the anterior pituitary gland (Kyrou, 2009; Smith & Vale, 2006; Ulrich-Lai & Herman, 2009). CRH type 1 receptor (CRHR1) binding by CRH stimulates the release of adrenocorticotrophic hormone (ACTH) from pituitary corticotropes into the blood. ACTH then binds to melanocortin type 2 receptor in the adrenal cortex stimulating the release of glucocorticoids (GC) such as cortisol in humans and corticosterone in rodents (Chrousos, Kino, & Charmandari, 2009; Dallman, Akana, Strack, Hanson, & Sebastian, 1995; Smith & Vale, 2006). GC are pleiotropic and are a major subclass of steroids that have a role in the regulation of metabolic, cardiovascular, immune and behavioral responses that are mediated through the glucocorticoid receptors, that are widely expressed in the brain and periphery (Sapolsky, Romero, & Munck, 2000).

Elevated levels of GC in response to stressors facilitate the capacity to adapt accordingly as well as providing negative feedback onto the HPA axis to return the body to homeostasis, a crucial phenomenon for survival. GC antagonize the pathways involved in energy storage, growth and reproduction. Circulating levels of cortisol initiate catabolic effects such as hepatic gluconeogenesis and stimulate lipolysis and proteolysis. This is to increase circulating glucose levels to readily supply energy to muscles while inhibiting the storage of glucose by insulin (Kyrou, 2009; Sapolsky et al., 2000). GC act on two receptors, GC receptors (GR) and mineralocorticoid receptors (MR), and GC have a higher affinity for GR during periods of elevated GC secretion. GR binding acts on positive or negative glucocorticoid response elements (GRE) mediating the transcription of numerous genes involved in metabolism, such as upregulating genes encoding for glucose, lipid and amino acid metabolism (Kyrou, 2009). GR are distributed widely in the

brain and most importantly in regions that are involved in the stress response like the PVN. Elevated levels of GC initiate the negative feedback onto the HPA axis by decreasing the expression of CRH, reducing its synthesis and secretion, as well as inhibiting ACTH release (Kyrou, 2009; Smith & Vale, 2006). In addition, when GC bind to GR, excitatory synaptic inputs in CRH neurons are suppressed which is independent of GC transcriptional effects (Tasker & Herman, 2011). The downregulation of CRH diminishes the release of ACTH from the pituitary gland, which in turn decreases the release of GC, culminating with the inhibition of the HPA axis. In addition, there are limbic structures such as the prefrontal cortex (PFC), hippocampus and amygdala that contributes to the regulation of HPA activity. The PFC and hippocampus both highly express GR and when activated by stress have an overall inhibitory effect on the HPA axis. The amygdala is believed to stimulate HPA activity by inhibiting the effects of GC on CRH production in the PVN (Herman & Cullinan, 1997; Smith & Vale, 2006).

The activation of the HPA axis is required to deal with acute stressors. However, the experience of chronic stress can dysregulate the HPA axis thereby leading to detrimental pathologies such as anxiety, depression and metabolic syndrome (Kyrou, 2009). The negative impact incurred psychologically and metabolically is due to HPA over-activity, demanding for the constant use of energetic resources eventually leading to allostatic overload (McEwen, 1998, 2004; Yau & Potenza, 2013).

The Juvenile Period and Stress

In humans, the juvenile and adolescent period is a time of transition where there are many changes, biologically and psychologically, that occur before adulthood. The juvenile

brain is distinctly different from the adult brain as many structures are under continuous development, such as the PFC, hippocampus and amygdala, all of which are important to emotional and learning processes (Spear, 2000; Tsoory, Cohen, & Richter-Levin, 2007). Adverse childhood experiences, such as abuse, have shown to decrease PFC and hippocampal volume and heighten HPA axis activity (Danese & McEwen, 2012). Also there is an increased risk of developing mental illnesses such as depression and anxiety in adulthood (Duhig et al., 2015; Holz et al., 2015; Martins, Von Werne Baes, Tofoli, & Juruena, 2014). There is a need to understand the impact of stress during this critical period and how it can provoke changes in adulthood.

The juvenile period of development

There is a vast array of literature dedicated to early life stressors and their evident effects in adulthood in rodents however there are stark neurobiological differences in the rodent early life brain and the juvenile brain. Much of the focus has been on the pre-weaning period postnatal days (PD) 3-14 however, this period of development has been said to be synonymous to the 23rd week of gestation in humans (Spear, 2000; Tsoory et al., 2007). The juvenile period (PD 27-35) in rodents is very much reflective of children and early adolescents behaviorally, such as reduced maternal dependence, increased independence and playful behaviors (Spear, 2000). Comparing the neurobiology of the early life period and the juvenile period, major differences are evident especially in structures that are involved in and are affected by stress. In pups, the HPA axis is hyporesponsive and premature (Vázquez, Van Oers, Levine, & Akil, 1996) whereas in juvenile (as well as adolescent) rats the HPA axis is presumed to be fully developed (Foilb, Lui, & Romeo, 2011). Though the HPA axis is considered to be fully developed,

it is more reactive and sensitive towards stressors than in adult rats, such as a slower negative feedback response, due to the ongoing synaptic growth in other brain regions (Foilb et al., 2011; Jankord et al., 2011; Spear, 2000). As such the PFC, hippocampus and amygdala are continuously developing and maturing because there is an overproduction and pruning of synaptic connections between these structures (Spear, 2000; Tsoory et al., 2007). There is also evidence of alterations in dopamine signalling during the juvenile and adolescent period, such as an overproduction of DA receptor 1 (D₁) and 2 (D₂), especially in the nucleus accumbens (NA), a phenomena that is not evident in pre-weaning pups and is lost in adults (Spear, 2000). Given the neurobiological differences between rodents in the pre-weaning and juvenile period, there is translational relevance in understanding the effect of stressors experienced during childhood and early adolescence that can arise in adulthood.

Immediate impact of juvenile stress

The immediate impact of juvenile stress is quite distinguishable from the long-term outcomes when looking at behavior and HPA activity. Jacobson-Pick and Richter-Levin (2010) found contrasting behavioral profiles between stressed juvenile rats and adult rats that were stressed during the juvenile period. Six hours after the last stressor, juvenile rats displayed reduced anxiety-like characteristics as there was more time spent in open arms of the elevated plus maze (EPM) and in the center of the open field test. However, the same rats displayed the opposite behavior when evaluated for anxiety-like traits using the same tests in adulthood. There was a significant decrease in time crossing and time spent in open arms on the EPM and less time in the center of the open field test. Administration of corticosterone in naïve juvenile and adult rats showed the same

contrasting behavioral profiles. All in all, the behavioral differences induced by stress concur that there are diverse adaptations to stress between these ages (Jacobson-Pick & Richter-Levin, 2010).

Long-term impact of juvenile stress

Stress during the preadolescent period and learning to cope and manage challenges when encountered is part of the normal experience of transitioning to adulthood. However negative stressors that are experienced intensely and/or chronically such as bullying, childhood abuse, conflicting family and peer dynamics have notably produced long-term effects in adulthood. These negative stressors have shown to increase the susceptibility and diagnosis of psychopathologies like anxiety and depression (Takizawa, Maughan, & Arseneault, 2014).

Animal models of juvenile stressor exposure can induce anxiety-like characteristics in adulthood. Immediate and enduring effects of chronic variable stress (CVS) in pre-pubertal rats (PD 21-32) have produced enhanced acoustic startle response (Maslova, Bulygina, & Popova, 2002). In addition to these findings, re-exposure to stressors during adulthood in rats that experienced juvenile stress had increased anxiety-like characteristics, such as greater time crossing in the periphery of the open field test and a greater startle response than the controls or rats that were stressed twice in adulthood only (Avital and Richter-Levin, 2005).

Along with the long-lasting behavioral outcome of juvenile stress, there are neurobiological changes that occur as well. In adult rats, CVS caused very complex and different changes in dopaminergic, serotonergic and noradrenalinergic pathways and

these systems undergo rapid and significant changes during the juvenile period (Moll et al., 2000; Murrin, Sanders, & Bylund, 2007). For example, juvenile CVS increased levels of DA and decreased levels of 5-hydroxyindoleacetic acid, a metabolite of serotonin, in the medial PFC (mPFC) (Luo et al., 2014). In addition, there was atrophy in the CA3 region of the hippocampus as well as in the PFC in chronic juvenile stressed adult rats (Tsoory et al., 2007). Interestingly, re-exposure to stressors after a month downregulated γ -aminobutyrate (GABA) subunit $\alpha 1$ protein expression in the hippocampus and amygdala (Jacobson-Pick & Richter-Levin, 2011). In addition, juvenile stress has varying effects on hippocampal plasticity, such as impaired long-term potentiation (LTP) in the dorsal hippocampus and increased LTP in the ventral hippocampus with differential sensitivity to β -adrenergic agonists (Grigoryan, Ardi, Albrecht, Richter-Levin, & Segal, 2015). Equally important, chronic social defeat (CSD) during adolescence in rats caused reduced DA release in the mPFC when assessed in early adulthood (Watt, 2014). Given the marked neuronal effects of juvenile stress in adulthood, it provides further reason to understand these long-term implications.

Models of juvenile stress

As evident, many stressor paradigms are implemented to understand the long-term effects of juvenile stress, all of which have varying outcomes behaviorally and biologically. The following will discuss a few of the common paradigms in the literature and the most applicable paradigm employed in this thesis.

Chronic Social Defeat: CSD is an intense psychosocial stressor that involves the introduction of an intruder (the test animal) to the home cage of the resident who is a

sexually experienced and aggressive male rodent. Every day, both animals interact and establish a dominant-submissive relationship such that the resident maintains its dominance and the intruder as the subordinate. After a brief interaction, a sensory barrier is placed between both animals for the rest of the day and this is repeated over a period of 14-21 days. Adolescent rats that were subjected to CSD and re-exposed to the defeat context (without the resident) in adulthood, displayed increased locomotor activity during EPM testing and developed more risk-assessment behaviors like head swaying without ambulation in the open field test (Watt, Burke, Renner, & Forster, 2009). On the contrary, other studies have seen expression of anxiety-like characteristics in the EPM in adult mice that were exposure to CSD during adolescence (Kovalenko et al., 2014; Vidal, Buwalda, & Koolhaas, 2011). Conversely, the same risk-assessment behavior was also seen in male adult rats that were defeated as adults and exposed to the defeat context, meaning that risk-assessment is not singularly unique to adolescent stressor exposure (Buwalda et al., 2005; Razzoli, Carboni, Guidi, Gerrard, & Arban, 2007). The neurobiological effects are also inconsistent with other stressor paradigms, as monoamine and monoamine metabolite levels have been reported to be unchanged in numerous brain regions (Watt et al., 2009) whereas different stressors like, CVS, can induce monoamine changes in similar brain regions (Luo et al., 2014). Besides, the duration of this stress paradigm does not strictly occur during juvenile development and usually extends into adolescence; this can be confounding as different effects of stressor exposure have been seen at various time points in adolescence (Arakawa, 2007; Jankord et al., 2011; Spear, 2000; Varlinskaya, Truxell, & Spear, 2013). As such, CSD may not produce findings that are reflective of the anxiety producing effects of juvenile stress in adulthood.

Chronic Variable Stress: CVS consists of unpredictable and variable daily stressors (both physical and psychosocial) occurring during the juvenile period (Luo et al., 2014; Maslova et al., 2002) or for a prolonged period of time (for example, 28 days or more) (Isgor, Kabbaj, Akil, & Watson, 2004; Restrepo & Armario, 1987; Ricon, Toth, Leshem, Braun, & Richter-Levin, 2011). The unpredictability and varying intensities of CVS in the juvenile period have consistent findings on measures of anxiety evaluated by EPM and the open field test as well as coinciding neurobiological effects as described earlier. The challenge with this paradigm is the time period, as the start and termination of stressor exposure varies between studies. Some studies start the stress on PD 21 (Maslova et al., 2002), others during the juvenile/pre-pubertal period (between PD 27-35) (Luo et al., 2014) and many of the stressors proceed into adolescence (Isgor et al., 2004; Restrepo & Armario, 1987; Ricon et al., 2011). There is overlap between the prepubertal and adolescent developmental periods and this is confounding because the time of which the stressors occur can produce different effects. Even the time window between early adolescence and mid adolescence have shown differences in measures of anxiety, such as enhanced levels of corticosterone induced by stress seen in late but not early adolescence (Jankord et al., 2011). Moreover, PD 33-35 can be considered as early adolescence (Tsoory & Richter-Levin, 2006). Altogether, juvenility is a short time period that is before the onset of puberty, therefore CVS may not be best model used to consider the long-lasting effects exclusive to juvenile stress.

3-day episodic stressors: This stressor schedule established by Tsoory and Richter-Levin (2006), confines the stress exposure to the first three days of the prepubertal juvenile period of development, PD 27-29. Three different consecutive episodic stressors are

implemented during this period; a swim stress on the first day, an elevated platform stress on the second and finally a foot shock or a two hour restraint on the last day. All three stressors have robust effects on activating stress response and significantly elevating corticosterone levels. These stressors on a variable schedule were chosen for their robust and consistent effects in adult rats (Tsoory & Richter-Levin, 2006) and that this prepubertal time frame produce more prominent long-lasting effects in adulthood (Avital & Richter-Levin, 2005; Romeo, Lee, Chhua, McPherson, & McEwen, 2004). The value of this stressor schedule is that it occurs strictly during the juvenile phase and does not extend to early adolescence. A study conducted in our lab employed a modified version of this stressor paradigm (a shortened restraint period) and had consistently produced long-term anxiogenic outcomes evaluated by EPM and social interaction during adulthood (MacKay et al., 2014). Considering that this paradigm has been replicated by others to study the effects of juvenile stress in adulthood (Grigoryan et al., 2015; Jacobson-Pick & Richter-Levin, 2010; MacKay et al., 2014; Tsoory et al., 2007) this stressor paradigm is fitting for the investigation of the long-term effects of juvenile stress and access to palatable food.

Systems regulating food intake and energy balance

Integrated neuronal pathways through homeostatic and hedonic systems regulate maintenance of homeostasis, energy intake and expenditure. Stressors act with these same neuronal pathways which can lead to metabolic dysregulation.

Homeostatic food intake: hypothalamic regulation

The hypothalamus regulates the intake and demand of energy to maintain homeostasis determined by environmental, nutritional and energetic status. This structure allows for the integration of central signals (orexigenic and anorectic) as well as peripheral signals (adiposity and nutrients) conveying nutritional status.

The arcuate nucleus of the hypothalamus (ARC) plays an integral role in energy balance regulation. Given its close proximity to the fenestrated capillaries, the ARC is sensitive to peripheral signals modulating energy balance and contains receptors for these signals (Cerdan et al., 2001; Cone, 2005). The ARC contains two neuronal populations responsible for these opposing signals; neurons that express agouti-related peptide (AgRP) and neuropeptide-Y (NPY) stimulate food intake whereas neurons that express pro-melanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) promote satiety (Abizaid, Gao, & Horvath, 2006; Date et al., 2001; Valassi, Scacchi, & Cavagnini, 2008). The ARC projects to neurons in the PVN, which produces thyrotropin releasing hormone (TRH), and to the lateral hypothalamus (LH), which secretes melanin-concentrating hormone (MCH) and orexin. The second order neurons in the PVN and in the LH can reduce and increase feeding respectively. In sum, these two sets of neurons within the ARC have opposing functions on food intake regulated through their output projections.

During a catabolic state (where energy stores are met), POMC/CART afferents inhibit orexin and MCH neurons in the LH while stimulating TRH release in the PVN and this cascade of signalling can terminate food intake. This inhibition is mediated by

α - and β - melanocyte-stimulating hormones (α - and β -MSH), which are cleaved products of POMC, reducing food intake and increasing energy expenditure through its actions on melanocortin receptor subtypes 3 and 4 (MC3R and MC4R). These receptors are found throughout the brain however are most abundant in the ARC, PVN, LH and dorsomedial hypothalamus and their inhibitory actions on food intake are central to the PVN and the LH. In contrast, when the body is in a state of negative energy balance, the orexigenic response takes precedence to restore energy supply in the body. AgRP/NPY neurons in the ARC, as mentioned briefly above, are mainly responsible for this signalling. AgRP antagonizes MC3R and MC4R receptors in the PVN blocking the anorectic effect of α - and β -MSH released by the POMC/CART neurons. This reduces TRH release in the PVN and stimulates orexin and MCH release in the LH in order to promote feeding. In addition to this, AgRP/NPY neurons inhibit the anorectic response of POMC/CART neurons via GABA release (Abizaid, Gao, et al., 2006; Abizaid & Horvath, 2008; Valassi et al., 2008).

The activation and inhibition of POMC/CART and AgRP/NPY neurons in the ARC are modulated by the varying concentrations of circulating levels of leptin and ghrelin (depending on the energy balance state) which act directly on these neurons through their respective receptors. In a positive energy state, the body has sufficient energy stores and this message is relayed by leptin, a peptide released by adipocytes. Leptin regulates long-term adiposity and metabolic adaptations during nutritional state changes of the body. Leptin also modulates short-term energy intake and meal size to accommodate energy balance fluctuations (Coll, Farooqi, & O'Rahilly, 2007). When leptin crosses the blood brain barrier, it binds to its receptors that are present on

AgRP/NPY and POMC/CART neurons. In an anabolic state, higher levels of leptin binding in the ARC stimulates POMC/CART neurons, thereby decreasing food intake and increasing energy outflow while inhibiting the actions of AgRP/NPY neurons (Cerdan et al., 2001). When energy demands are unsatisfied or low, there are lower levels of leptin and an increase in gastrointestinal ghrelin secretion. When ghrelin binds to its receptor growth hormone secretagogue receptor-1a (GHSR), on AgRP/NPY neurons, food intake is stimulated (Abizaid & Horvath, 2008, 2012; Date et al., 2001).

In addition to the potent actions of leptin and ghrelin on feeding, other peripheral signals during states of negative or positive energy states contribute to the homeostatic regulation by the ARC. When energy requirements have been met, anorexigenic signalling in gastrointestinal tract is triggered. Secretion of cholecystokinin, bombesin, glucagon-like peptide-1 and peptide YY(3-36) help induce satiation by signalling to the nucleus of the solitary tract via vagal afferents which then stimulate the POMC/CART neurons. Insulin is also integral to glucose storage and initiation of catabolic events and can bind to receptors on POMC/CART and AgRP/NPY neurons to terminate a feeding response. Interestingly, ghrelin is the only gastrointestinal peripheral signal to initiate food intake when considering homeostatic energy intake pathways (Abizaid & Horvath, 2008; Date et al., 2001; Valassi et al., 2008).

Hedonic food intake-regulation by the reward pathway

Even when energy demands have been met, food intake (especially food with hedonic value like sugar and palatable food) can be elicited by the activation of the mesolimbic pathway that are associated with a number of processes including motivation,

addiction, reward and stress. This pathway consists of dopaminergic afferents that originate from the ventral tegmental area (VTA), in the mid brain, innervating the NA (in the ventral striatum). The neurons within the NA are primarily GABAergic with projections relaying to the VTA and to the ventral pallidum. Furthermore, the NA receives glutamatergic signalling from the PFC (responsible for cognition and motivation), amygdala and hippocampus and the VTA has dopaminergic projections to these same brain regions as well (Russo & Nestler, 2013; Wise, 2005). DA mediates its actions by binding to D₁-like and D₂-like family of receptors.

The D₁-like family: Consists of D₁ and D₅ receptors coded by *DRD1* and *DRD5* genes respectively and are coupled to G protein G_{sα} to activate adenylyl cyclase. Specific for this discussion, D₁ receptors are highly expressed in the NA and the frontal cortex (Beaulieu & Gainetdinov, 2011).

D₂-like family: This group is comprised of D₁-D₄ receptors encoded by the respective *DRD2*, *DRD3* and *DRD4* genes. This family of DA receptors inhibits adenylyl cyclase via coupled G protein G_{iα} (Beaulieu & Gainetdinov, 2011). The D₂ receptors are also significantly expressed in the VTA and the NA and the D₃ receptor has the highest distribution of receptors in the shell of the NA and lower levels have been detected in the VTA (Beaulieu & Gainetdinov, 2011; Missale, Nash, Robinson, Jaber, & Caron, 1998).

The mesolimbic pathway is also sensitive to peripheral signals. Leptin, insulin and ghrelin play a role in the preference and consumption of palatable food by modulating dopaminergic activity in the mesolimbic pathway (Lockie & Andrews, 2013).

Leptin and insulin: Both leptin and insulin receptors are expressed on dopaminergic cells within the VTA and have been implicated in reducing the incentive salience and preference for palatable food (Figlewicz, Evans, Murphy, Hoen, & Baskin, 2003; Hommel et al., 2006). Insulin administered in the VTA decreases hedonic driven consumption of sweetened high fat diet in satiated mice (Figlewicz & Sipols, 2010; Mebel, Wong, Dong, & Borgland, 2012). Direct administration of leptin in the VTA decreased a preference for palatable food by inhibiting the firing of VTA DA neurons therefore decreasing DA release (Hommel et al., 2006). In contrast, knockdown of the leptin gene increased short-term consumption for HFD and total sucrose intake in the two-bottle choice paradigm. Furthermore, there was a reduced preference for a chamber paired with the high fat diet in conditioned place preference (CPP) with microinjections of insulin or leptin (in the third ventricle) compared to vehicle treatment in both satiated and food restricted rats on test day (Figlewicz et al., 2004).

Ghrelin: A negative state of energy balance can influence the preference for a diet that is palatable and/or highly caloric to meet energy demands. For example, food restricted and fasting animals had a preference for reinforcers associated with caloric value, like sugar and ethanol, while there was no change in preference for non-caloric reinforcers, like saccharin (Fedorchak & Bolles, 1987; Lockie & Andrews, 2013). A possible mediator for this preference is ghrelin, where hunger induces higher circulating levels. Like leptin and insulin, ghrelin receptors are also found on dopaminergic neurons within the VTA and are involved in hedonic eating (Mani et al., 2014; Zigman, Jones, Lee, Saper, & Elmquist, 2006). Central and peripheral administration of ghrelin increased the motivation for mice to work for palatable food in operant bar pressing and increased time

spent in a chamber paired with palatable food in CPP (Chuang et al., 2011; Egecioglu et al., 2010; Skibicka, Hansson, Egecioglu, & Dickson, 2012). In addition, ghrelin binding at the VTA stimulated DA activity and DA turnover at the NA as well as stimulated feeding all of which were GHSR dependent (Abizaid, Liu, et al., 2006). Moreover, it has been suggested that ghrelin promotes the consumption of highly caloric food because it signals for the evaluation of energy content of food rather than its rewarding qualities, to meet energy demands (Bomberg, Grace, Wirth, Levine, & Olszewski, 2007). Taken together, evidence suggests that ghrelin is an important factor in homeostasis and hedonic food intake.

Stress and Feeding

Stimulation of the stress response can promote changes in appetite and altered metabolism. In particular, chronic exposure to stress and increased levels of GC result in increased visceral fat accumulation and in some cases increased weight gain and food intake in addition to glucose tolerance (Kyrou, 2009; Yau & Potenza, 2013). As mentioned before, GC act on GRE to activate the transcription of enzymes and proteins to initiate the metabolism of lipids and amino acids while suppressing other mediators of stress, like CRH, in order to adapt to the stressor successfully (Kyrou, 2009). Unfortunately the long-term activation of the HPA-axis, due to intense or chronic stress, leads to a dysregulated and a reduced negative feedback efficacy, increasing the levels of CRH and ACTH and perpetuating the constant release of GC (Dallman et al., 2006).

Acute and chronic stressors have varying effects on food intake. Acute stress can stimulate the release of CRH in the ARC to inhibit AgRP/NPY neurons to reduce appetite

(Richard, Lin, & Timofeeva, 2002; Sominsky & Spencer, 2014). In contrast, chronic stress mobilizes the body to maintain a constant positive energy state as energy is needed to cope with the stressor. Chronically elevated levels of GC enhance lipoprotein lipase activity in adipose tissues, increasing fat accumulation, especially visceral fat. GC stimulate food intake by upregulating the expression of NPY and AgRP in the ARC (Sominsky & Spencer, 2014; Yau & Potenza, 2013). In addition, long-term elevation of GC can reduce the sensitivity of leptin's satiety signal in the brain (Sominsky & Spencer, 2014). Excessive GC production is seen in Cushing's disease and Prader-Willi Syndrome where adiposity is exacerbated (Weaver, 2008). Concerning stress-induced feeding, exposure to chronic stressors, such as CSD, can increase food intake, increase caloric intake derived from palatable food and induce larger adipocytes (Bartolomucci et al., 2009; Chuang & Zigman, 2010).

Stress and the reward pathway

The mesolimbic DA system is extremely sensitive to stress and stress itself can elicit DA release in this system. Footshock, tail pinch, restraint and exposure to a novel environment has been observed to release DA in the NA and in the frontal cortex mediated by VTA activation and is considered a temporary response (Cabib & Puglisi-Allegra, 1996). Microdialysis studies showed that a mild foot shock increased the DA outflow in the NA (Abercrombie, Keefe, DiFrischia, & Zigmond, 1989; Kalivas & Duffy, 1995; Wu, Yoshida, Emoto, & Tanaka, 1999) however, prolonged exposure reduced DA in the NA, such as 60 or 120 minutes of restraint stress or daily 60 minute restraint sessions (Cabib & Puglisi-Allegra, 1996). Whole-cell patch clamp revealed that CSD reduced the activity of D₁ medium spiny neurons (MSN) and increased D₂ MSN activity,

thereby reflecting an inhibitory effect of chronic stress (Francis et al., 2015). The bidirectional DA response due to the duration of stress can possibly represent the notion that DA release is a coping response to acute aversive stimuli exposure and acts as a defense mechanism. Yet the inhibition of DA release occur if these adaptations fail to work due to a chronic stressor.

Palatable food can dampen HPA axis

Stress has been associated with increased intake of palatable food and is linked to overweight and obesity (Dallman, Pecoraro, & la Fleur, 2005; Dallman et al., 2003; Morris, Beilharz, Maniam, Reichelt, & Westbrook, 2014). As previously discussed in humans, stress can alter feeding patterns in a bidirectional manner and those that experience an increase in food intake may shift their food preferences towards calorically dense palatable foods that are high in sugar and fat (Dallman, 2010; Yau & Potenza, 2013). This emotional eating has been suggested as a method of coping and self-medication, relieving the feelings of stress.

In rodent studies, the majority of stressor paradigms evoke a decrease in food intake. Nevertheless, when given access to a calorically dense palatable diet (high fat and/or high sugar) there is a shift in preference and an increased intake for this type of food (Chuang et al., 2011; Ulrich-Lai, Ostrander, & Herman, 2011; Machado et al., 2013). Access to palatable food after stress has anxiolytic effects and can dampen the HPA axis response to acute and chronic stressful events (Dallman et al., 2003; Pecoraro, Reyes, Gomez, Bhargava, & Dallman, 2004). Adrenalectomized rats, that were not stressed, were given access to a 30% sucrose solution and administered varying

concentrations of corticosterone. Interestingly, increased concentrations of corticosterone led to greater sucrose solution consumption (Dallman et al., 2005). In the context of stress, restraint stress in rats induced elevated levels of GC and increased palatable food intake (Pecoraro et al., 2004). Interestingly in the same study, palatable food was associated with diminished ACTH at one and three minutes of restraint and diminished corticosterone after five minutes (Pecoraro et al., 2004). In concurrence, chronically stressed rats with six weeks access to a cafeteria diet had normalized basal corticosterone levels. Even with access to a palatable drink, such as sucrose water, decreased plasma corticosterone 60 minutes post-acute restraint in addition to reduced CRH mRNA expression in the PVN after chronic stress (Ulrich-Lai et al., 2007, 2011). A sucrose and lard diet also decreased CRH expression in the PVN and in the central amygdala after restraint (Foster et al., 2009). Together, these studies demonstrated how palatability of a diet reduced the HPA response during and after a stressor. A proposed mechanism of how GC regulates the preference for palatable food is through its interaction with catecholamine systems, where GC can increase DA and norepinephrine signalling by inhibiting transporter activity (Figlewicz et al., 1999).

The metabolic consequences of a combination of palatable food and stress promotes weight gain and adiposity. During periods of sustained or repeated chronic stress rodents reduced their chow intake and started losing weight, however a palatable diet increased weight and caloric efficiency (Dallman et al., 2005; Pecoraro et al., 2004). A study observed how chronic exposure to a high fat and high sucrose diet and chronic restraint stress increased adiposity when compared to chow fed stressed rats. This group also had significantly higher levels of total plasma cholesterol and low-density

lipoprotein, indicating a change in metabolic processes induced by the combination of stress and palatable food (Fachin et al., 2008).

There is a small body of literature investigating the effect of a cafeteria diet in early life stressor exposure. Prolonged maternal separation (180 minutes) and access to a high fat cafeteria diet reversed anxiety-like characteristics and increased hedonic behavior, evaluated using sucrose preference test, when tested at 12 weeks. In these rats, GR mRNA expression increased in the hippocampus when compared to the chow fed counterpart, yet CRH mRNA expression was normalized when compared to the controls (chow and no early life stress). This observation linked the upregulation of GR expression as a mechanism to reduce HPA activity and decrease anxiety (Machado et al., 2013). Access to chocolate cookies during juvenility/early-adolescence (starting on PD 28) after neonatal separation, reduced anxiety (evaluated with EPM), normalized basal plasma corticosterone and increased corticosterone levels after a restraint stress. This showed that the palatable diet changed stress sensitivity, considering that neonatal separated rats given chow only had a blunted HPA response to an acute stress (Krolow et al., 2013). Together, palatable food can induce long-term changes ameliorating the behavioral and neurochemical effects of early life stressor exposure. These long-term effects of palatable food consumption need to be investigated for juvenile stressor exposure, as there is paucity in the literature focusing on this developmental window.

Ghrelin

Ghrelin plays a role in hedonic feeding and reward and recent research has revealed that ghrelin contributes to changes in diet preference induced by stress. It is an

interesting factor to consider when investigating the combination of palatable food and juvenile stress because these systems are all integrated and are sensitive during this critical period. Here we discuss in detail the role that ghrelin has in stress, feeding and reward.

Ghrelin is a 28-amino acid peptide secreted by the X/A cells of the intestinal epithelium gut lumen of the rodent stomach and P/D1 enteroendocrine cells of the gastric fundus of humans (Kojima et al., 1999; Kojima, Hosoda, & Kangawa, 2004; Stengel, Wang, & Taché, 2011). The precursor protein, preproghrelin, encoded by the *GHRL* gene, is cleaved twice to produce the final product of ghrelin (Seim et al., 2010). The esterification of a medium chain fatty acid at the serine-3 residue by ghrelin-*O*-transferase leads to an acylated form of ghrelin also known as active ghrelin (Kojima et al., 2004; Seim et al., 2010; Stengel et al., 2011). Through this modification ghrelin can accomplish its physiological functions by binding to its receptor, GHSR, which is distributed widely in the body and the central nervous system (Kojima et al., 1999, 2004; Zigman et al., 2006).

Food Intake

Ghrelin is a gastrointestinal peptide that stimulates feeding (Mani et al., 2014; Stengel et al., 2011). Preprandial secretion of ghrelin is higher and correlates with the onset of feeding, and then decreases after consumption of food as a mechanism to replenish energy stores and maintain homeostasis (Abizaid & Horvath, 2012; Lindqvist, de la Cour, Stegmark, Håkanson, & Erlanson-Albertsson, 2005). Fasting states also lead

to higher circulating levels of ghrelin in addition to increased mRNA expression of GHSR in brain areas responsible for homeostasis (Abizaid & Horvath, 2012).

Ghrelin's role in reward

GHSR mRNA is highly expressed on dopaminergic neurons in the VTA and immunohistochemistry show lower expression in the PFC providing evidence that ghrelin can directly modulate the mesolimbic dopaminergic pathway for rewarding stimuli like drugs of abuse (Mani et al., 2014; Zigman et al., 2006). Brain sections exposed to biotinylated ghrelin followed a similar distribution to that of GHSR expression with particular abundance in the VTA. When administered, ghrelin increased the activity of dopaminergic cells in this region, promoted excitatory synaptic remodelling of DA neurons and increased DA turnover in the ventral striatum, events that are reflective of reward (Abizaid et al., 2006).

Considering the above, the role that ghrelin plays in the sensitization of drugs of abuse, such as cocaine, has been investigated. For example, rats pretreated with ghrelin displayed greater locomotor activity when administered with cocaine. This effect was attenuated with the administration of a GHSR antagonist and in ghrelin-null animals (Abizaid et al., 2011; Jang, Kim, Cho, Lee, & Kim, 2013; Wellman, Clifford, & Rodriguez, 2013; Wellman, Davis, & Nation, 2005). Moreover, systemic administration of ghrelin can enhance CPP at lower doses of cocaine (Davis, Wellman, & Clifford, 2007). There is evidence that ghrelin can modulate DA release induced by drugs in structures within the mesolimbic system that have very low levels GHSR expression, such as the NA. Infusions of ghrelin in the NA core enhanced the systemic cocaine-

induced hyperlocomotor activity (Jang et al., 2013). In addition, an increase in extracellular DA in the NA shell following an acute administration of morphine was significantly and dose-dependently reduced by GHSR antagonist administration, implying that central ghrelin activity is involved in opioid stimulated DA release (Sustkova-Fiserova, Jerabek, Havlickova, Kacer, & Krsiak, 2014).

Ghrelin can induce mesolimbic DA-dependent behavioral responses, such as novelty seeking and CPP. In this vein, a study conducted by Hansson et al. (2012) examined how ghrelin could influence novelty seeking. Microinjection of ghrelin in the VTA increased novel object exploration and locomotor activity which was reduced by a GHSR antagonist. Systemic injection of ghrelin in high responder rats (rats that displayed greater locomotor activity), showed a greater preference for novel environment in novelty place preference paradigm. Moreover, intraperitoneal (i.p.) administration of ghrelin significantly increased locomotor activity, CPP and accumbal DA release (Jerlhag, 2008). Accumbal DA release has been associated with motivated behaviors such as reward and novelty seeking, suggesting that ghrelin may be partially responsible for this outcome (Berridge, 1996; Castro & Berridge, 2014; Jerlhag, 2008).

Stress, reward and palatable food preference

Recently there has been growing evidence that ghrelin influences stress-induced food intake and when accessible, may lead towards a preference for calorically dense palatable food. Stressors such as repeated tail pinch, daily restraint and CSD in mice and rats all induce an increase in plasma ghrelin, ghrelin mRNA and ghrelin cell number in the stomach (Spencer, Emmerzaal, Kozicz, & Andrews, 2014; Chuang & Zigman, 2010;

Asakawa et al., 2001; Lutter et al., 2008). In humans, women that were anticipating a psychosocial stressor (preparing a speech that was to be presented immediately) had increased plasma levels of ghrelin and interestingly in emotional eaters, ghrelin levels did not decline after the consumption of food (Raspopow, Abizaid, Matheson, & Anisman, 2014). In mice, basal ghrelin levels remained elevated 30 days after the last CSD exposure (Chuang et al., 2011). Chuang and colleagues (2011) have investigated this shift towards the consumption of palatable food caused by stress and how ghrelin mediates DA signalling in the reward pathway. Stressed mice spent greater time in the chamber associated with a palatable diet than with the chow when evaluated with CPP, an observation not seen in unstressed mice (as indicated by no difference in preference between diet-paired chambers). However, this was reversed in GHSR-null mice and in fact there was a decrease in palatable food intake and body weight. When looking at HPA response, GHSR-null mice displayed a significant reduction in plasma corticosterone, implying a potential role for ghrelin on HPA responsivity. To observe if this preference was mediated by ghrelin-dependent dopaminergic activity in the mesolimbic pathway, particularly at the VTA, CRE-dependent expression of GHSR on tyrosine hydroxylase producing neurons was used in mice who were then subjected to CSD. The selective expression of GHSR in these stressed mice was enough to fully restore the preference for palatable food, even though there was partial restoration of GHSR expression in regions of the brain that express that receptor. Clearly, the interaction of ghrelin and its receptor in the mesolimbic pathway plays a role in palatable food preference when stress is introduced. In summary, ghrelin's role in hedonic feeding, reward and stressor-induced

changes in diet preference lends itself to be a point of investigation when looking at the long-term effects of juvenile stress and palatable food consumption.

Rationale for Thesis

Recent work from our lab conducted by MacKay et al. (2014) revealed a stress buffering effect on both behavioural and physiological measures of stress and anxiety in adulthood when juvenile stressed rats were given access to a palatable diet. In summary, rats subjected to three days of episodic stressor exposure during the juvenile period with daily-restricted access to palatable food from PD 21 and onwards, displayed reduced anxiety like-behavior in the EPM and social interaction test compared to juvenile stressed rats with access to chow only. Interestingly, palatable diet fed rats displayed lower basal plasma levels of corticosterone, irrespective of whether or not they were exposed to stress. Yet, the palatable diet combined with stress exaggerated glucose challenge response, increased adiposity and weight when compared to unstressed diet matched rats. These findings demonstrated the ability for palatable food to reduce HPA-axis activity and to produce indices of metabolic syndrome and obesity as a long-term consequence of juvenile stressor exposure.

The anxiolytic effects imparted by palatable food consumption may be due, in part, to neurochemical changes within the mesolimbic system which is highly sensitive during juvenile development (Andersen, Napierata, Brenhouse, & Sonntag, 2008). Thus the first objective of this thesis was to determine if juvenile stressor exposure and/or palatable food consumption elicited long-term changes in central DA receptor mRNA expression. Given ghrelin plays an integral role in reward and in mediating palatable food

preference during stress, the second objective was to investigate how ghrelin signalling had been modified long-term after juvenile stressor exposure and access to palatable food.

To achieve these objectives, two experiments both using a modified stress paradigm as outlined in Jacobson-Pick and Levin (2010) and the feeding and testing regime as depicted by MacKay et al. (2014) were conducted. In the first experiment we assessed a) behavioral alterations (expression of anxiety-like behavior) and b) mRNA expression changes of DA receptors at relevant brain regions (NA and PFC) in rats with access to palatable food previously exposed to the episodic stressor regime from PD 27-29. For the second experiment, we assessed the long-term impact of juvenile stressor exposure and/or palatable food consumption on the sensitivity of the reward pathway using a pharmacological treatment of ghrelin to assess a) palatable food versus chow intake with an intracerebroventricular (icv) injection of ghrelin and b) cocaine-induced hyperlocomotor activity with and without a systemic pretreatment of ghrelin. We hypothesized that the combination of juvenile stress and palatable food sensitized the reward pathway which would be manifested by altered DA receptor mRNA expression as well as enhanced sensitivity to ghrelin administration in adulthood.

MATERIALS AND METHODS

Experiment 1

Figure 1: Summary of Experiment 1 design: Animals were assigned to four groups: 1) Stress+Chow, 2) Stress+Palatable Food, 3) Control (No Stress)+Chow, 4) Control+Palatable Food. Groups assigned to the stressor condition were exposed to a different episodic stressor per day from PD 27-29 while control animals received daily handling. All animals were behaviorally tested in social interaction. Each group had an N=10.

Subjects

21-day old male Wistar rats (N=40) were provided by Charles River (Quebec, Canada) and were randomly assigned four conditions each with an N=10: 1) Stress+Chow, 2) Stress+Palatable Food, 3) Control+Chow and 4) Control+Palatable Food. Control was referred to animals not undergoing juvenile stressor exposure. All procedures met the guidelines established by the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa Institute of Mental Health Research. Rats were single-housed in standard plastic cages with bedding at room temperature of $22 \pm 1^\circ\text{C}$ on a 12 hour light-dark cycle with lights on at 7:00. The weight of the rats were taken on PD 21, PD 25, every day of the stressors (PD 27-29) and every five days starting on PD 30.

Diet

All groups received *ad libitum* access to standard chow diet (3.4 kcal/g, 4.5% fat, 18.1% protein, 57.3% carbohydrate, Charles River Rodent Diet 5075, Agribrand Purina Canada, Woodstock, Ontario, Canada). Palatable food diet consisted of 45% kcal fat pellets (4.7 kcal/g, 23.2% fat, 17.3% protein, 47.6% carbohydrates, TD.08811, Harlan Laboratories, Madison, Wisconsin, USA). Rats in palatable food groups were given daily limited access to the palatable diet for two hours from 8:00 to 10:00 starting on PD 21. Rats had access to the palatable diet from PD 21 until they were sacrificed on PD 75 or 76. Total caloric intake was calculated by multiplying the energy content (kcal/g) of the specific diet by the amount (g) consumed. Preference for palatable diet was a ratio of calories of palatable food consumed over total calories consumed.

Initial amount of chow and palatable diet was weighed on PD 21. Chow consumed was measured PD 24-26 to establish a baseline, every day of the stressors (PD 27-29) and every five days and onwards starting PD 30. Chow consumed was measured by subtracting the food weight that was measured 24 hours earlier. Palatable diet was measured PD 21, PD 25 and every day of the stressors (PD 27-29) before and after the two-hour access to the diet. Palatable diet was removed before the onset of stressor exposure. Palatable diet consumed on PD 30 and onwards was measured every five days.

Juvenile Stress

Juvenile stress paradigm was modified from the three day procedure described by Jacobson-Pick & Richter-Levin (2010). Rats were exposed to three consecutive days of episodic stressors during PD 27-29. All stressors have been validated to induce elevated

corticosterone levels and the variable schedule was chosen for its consistent robust effects in adult rats (Tsoory & Richter-Levin, 2006).

PD 27 Forced swimming: Rats were individually placed in circular bins filled with water at a depth of 29cm (bin diameter 48cm and height 42cm; and water temperature $22\pm 2^{\circ}\text{C}$) for 10 minutes. Rats were immediately removed and placed back into their home cages.

PD 28 Elevated platform: Rats were individually placed on a small elevated platform (12cm x 12cm; 70cm elevation from water level) for three 30 minute sessions. Platforms were placed in a pool filled with water. Animals that fell during the testing session were immediately placed back on their assigned platform until the testing session was complete. After each session, rats were placed back in their home cage for 60 minutes.

PD 29 Restraint: Rats were placed in plastic restraining bags for 30 minutes that restricted side to side and forward-backward movement. After restraint, rats were removed from bags and returned to their home cages.

Behavioral testing-Social Interaction

Behavioral testing was conducted under low lighting (30-40 lux) in an arena (60cm x 60cm; 30cm height of walls) in two testing periods, PD 61-63 and PD 63-65. Rats were diet and stress conditioned matched based on their body weight to ensure that there was <10g difference between pairs. This was done to prevent anxiety induced by intimidation as an effect of significant size differences between matched rats (File, 1980; File, Lipka, Beer, & Lipka, 2005). Social interaction was conducted over a period of three days consisting of habituation on the first two days and the last day being testing. On day 1 of habituation, paired rats were placed in the arena for five minutes to familiarize with each

other and to reduce initial social anxiety since rats were single-housed throughout the study. On day 2 of habituation, each rat was individually placed in the arena for three minutes to acclimatize to the environment. On test day, paired rats were placed in the arena and the behaviors of both rats were observed for seven minutes. Total time spent engaged in social interaction (sniffing, following, climbing over and under, allogrooming and play fighting), grooming and squares crossed were recorded. Rats were immediately returned to their home cages and apparatus was thoroughly cleaned with 70% ethanol between each test session.

Figure 2: Summary of social interaction paradigm. Paired rats habituated to one another as well as the arena on day 1 and day 2 respectively. On test day, time spent engaged in social interaction (sniffing, following, climbing over and under, allogrooming and play fighting), grooming and squares crossed were recorded.

Tissue Processing

On PD 75 and 76, animals were sacrificed by rapid decapitation and brains were immediately extracted and placed on a stainless steel brain matrix on an ice block with slots that were 0.5mm apart. Coronal brain sections were taken and the NA and PFC were

micropunched according to the brain atlas of Paxinos & Watson (1998). Plate # 8, located 3.70mm away from bregma, was referenced when the PFC was excised using a 15 gauge needle and Plate #10, located 2.20mm away from bregma, was referenced to punch the NA with a 18 gauge needle. Brain regions were immediately stored at -80°C for mRNA expression analyses. Brain extractions and punching occurred in an RNase free environment to reduce contamination and RNA degradation. Carcasses were stored at -80°C until fat pads weight analyses.

Analysis of basal plasma ghrelin

At sacrifice, trunk blood was collected and centrifuged for 10 minutes at 4000 RPM. 1mL of plasma was transferred and treated with a 25µL of AEBSF protease inhibitor (Sigma-Aldrich, California, USA) and 25µL of 2N HCl. The detection and quantification of immunoreactive ghrelin were performed by high-sensitivity solid-phase radioimmunoassay. Briefly, protein A/G (Calbiochem, La Jolla, California, USA)-coated Immulon-4 wells (Dynatech, Chantilly, Virginia, USA) were incubated with ghrelin antibody (Phoenix Pharmaceuticals, Burlingame, California, USA) for 24 hours at room temperature. After incubation, the wells were washed three times with wash buffer. Samples, standards and blanks (to determine non-specific binding) were pipetted into the designated wells and incubated for 24 hours at 4 °C. Next, 25µl of KRB solution containing 5000–6000 CPM of [125I-His]ghrelin (Phoenix Pharmaceuticals, Burlingame, California, USA) was added to each well and incubated for an additional 24 hour period at 4 °C. Finally, the wells were rinsed and separated, and their residual radioactivity was counted using a gamma counter (Canberra Packard, Cobra II Auto-gamma, model D5002). A four-parameter logistic curve fit model was used for interpolation of the

standard curves with a dynamic range of 1562-.75pg. The intra- and interassay coefficient of variability were 7.7% and 9.9% respectively.

Adiposity

Animal carcasses were thawed at 4°C and the following fat pads of rats were dissected and weighed: visceral, retroperitoneal, subcutaneous and inter-scapular brown fat.

Reverse-Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR)

Brain tissue was submerged and homogenized in TriZol[®] and total RNA was extracted according to the manufacturer's instructions (Invitrogen, Burlington, Ontario, Canada). Briefly, chloroform was used to isolate the RNA and isopropanol precipitated the RNA using linear polyacrylamide as a co-precipitant. Resulting RNA pellet was then dissolved in 10µL of Tris-EDTA buffer. Total RNA concentrations and quality were analyzed using the Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Scientific, Rockford, Illinois, USA) at an absorbance of 260nm. Total RNA was reverse-transcribed using the 5x iScript[™] RT Supermix (Bio-Rad Laboratories, Hercules, California, USA). Nuclease free water was added to adjust to a total volume of 20uL for each sample and samples were run on Bio-Rad T100 Thermal Cycler (Bio Rad Laboratories, Hercules, California, USA) at 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes and then stored at -20°C.

Afterwards, 4uL aliquot of sample cDNA along with 5uL of Sso Advanced Universal[™] SYBR[®] Green Supermix (Bio-Rad Laboratories, Hercules, California, USA), 0.4uL of forward and reverse primer and 0.2uL of nuclease-free water were plated and analyzed in simultaneous quantitative polymerase chain reaction (qPCR). All

samples, as well as non-template controls (all reagents mentioned above replacing cDNA with 4uL of nuclease free water) were run in duplicate. Plates were run on CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) using the following thermal cycler program: initial denaturation at 95°C for 30 seconds, secondary denaturation at 95°C for 10 seconds and annealing at optimal primer specific temperature for 30 seconds. Secondary denaturation and annealing were repeated 39 times. Final elongation starting at 65°C for 5 seconds with a rise in temperature 0.5°C per cycle ramping at a rate of 0.5°C/s for 60 times.

Primers that amplify β -actin (Act β) and ribosomal protein L-19 (RPL19) were used as reference genes and were validated for their stability under the stress and diet conditions for this study. Reference genes were utilized for gene expression normalization such that the mean cycle quantification value (C_Q) of the two reference genes were subtracted from the C_Q of the target gene. Calculation of the mRNA fold changes using the $2^{-\Delta\Delta C_Q}$ method (Livak & Schmittgen, 2001) then compared gene expression with treatment groups (stress or diet) relative to the control chow fed group. Minimum information for publication of quantitative RT-PCR experiments (MIQE) guidelines were followed such that selected primers were optimized for annealing temperatures and a standard curve was conducted. Using a gradient of temperatures, an optimal range of annealing temperatures for the primer was selected for each tissue type. Afterwards, a standard curve was run to find the appropriate dilution factor for the tissue samples using the optimal annealing temperature. Primer efficiency and the cycle threshold were determined from the slope in relation to the absolute copy of RNA

quantity and the C_Q values using Bio-Rad Amplification CFX Manager™ Software version 3.0. All primer pairs had a minimum efficiency of 90%.

Primer sequences selected for RT-qPCR analysis as follows:

Actβ forward: 5'TAT GCC AAC ACA GTG CTG TCT GG 3'

Actβ reverse: 5'TAC TCC TGC TTC CTG ATC CAC AT 3'

RPL19 forward: 5' TGC AGC CAT GAG TAT GCT TAG 3'

RPL19 reverse: 5' GAG AGT TGG CAT TGG CGA TT 3'

DRD1 forward: 5' GGT CCA AGG TGA CCA ACT TCT 3'

DRD1 reverse: 5' CCC AGA TGT TAC AAA AGG GAC C 3'

DRD2 forward: 5' AAG ACA CCA CTC AAG GGC AAC 3'

DRD2 reverse: 5' ATC CAT TCT CCG CCT GTT CAC 3'

DRD3 forward: 5' AGT CTG GAA TTT CAG CCG CA 3'

DRD3 reverse: 5' ACC GCT GTG TAC CTG TCT ATG 3'

Statistical Analyses

Behavioral measures, adiposity and mRNA expression were analysed using two-way analysis of variance (ANOVA). Diet, caloric intake and weight were analysed using repeated measures two-way ANOVA, where diet (chow and palatable food) and stress (three day episodic stress or control) were the between group variables and time as the repeated within group variable. Follow up comparisons between and within the control and treatment groups were conducted using simple effects analyses. Significance was set at $p < .05$.

Experiment 2

Subjects, diet, juvenile stressor paradigm and statistical analysis were conducted with the same methodology as described in Experiment 1 unless otherwise described.

Figure 3: Summary of Experiment 2 design. Animals were assigned to four groups: 1) Stress+Chow, 2) Stress+Palatable Food, 3) Control+Chow and 4) Control+Palatable Food. Groups assigned to the stressor condition were exposed to a different episodic stressor per day from PD 27-29 while control animals received daily handling. All animals underwent surgery on PD 50-51. After recovery, feeding tests were conducted with chow and a novel palatable diet (cookie dough) following a central treatment of saline and ghrelin from PD 62-65. Afterwards, locomotor activity with a pretreatment of ghrelin and an injection of cocaine was measured from PD 70-72. Sacrifice occurred on PD 86.

Surgery

On PD 50 and 51, animals were anesthetized with isoflurane in 100% oxygen and mounted on a stereotaxic frame with a heated pad underneath to maintain body temperature throughout the surgery. Surgical area was shaved and a stainless steel guide cannula was implanted in the third ventricle at the following coordinates:

4.4 mm posterior to bregma, 0 mm lateral, and 4.4 mm below the skull surface. The cannula was anchored to the skull with 3 stainless steel screws and dental cement.

Animals were given 10 days to recovery and removable stylets (Plastics One, Roanoke, Virginia, USA) were inserted into the guide cannula until behavioral testing. During the

recovery period, animals were subjected to mock central injections to habituate them to the injection procedure.

Food Preference Assessment

From PD 62 to 65, an icv injection of 10 μ g ghrelin (Tocris, Bristol, UK), dissolved in artificial cerebral spinal fluid (aCSF), or aCSF (also referred to as saline), was administered and the animals were then placed in their home cage with four hour limited access to pre-weighed chow or a novel palatable food (Pillsbury[®] chocolate chip cookie dough). A small amount of the dough was given for two days prior to testing to eliminate neophobia. On PD 62 and 63 rats had access to chow only and on PD 64 and 65, rats had access to the cookie dough only, with one injection of ghrelin or saline on each day, ensuring that all animals received both treatments. The weight of the chow or cookie dough was recorded before the microinjection, at 30 minutes and every hour for four hours. Pharmacological treatment was delivered in a volume of 3 μ L over 30 seconds via injection cannula extended 0.5mm from the bottom of the guide cannula and connected to an infusion pump through polyethylene tubing (Harvard Apparatus, Massachusetts, USA). After infusion, the injection cannula was left for another 30 seconds to ensure drug diffusion.

Locomotor Activity

From PD 70-72 locomotor activity was measured based on a modified procedure described by (Wellman et al., 2005). In general, rats received a pretreatment (i.p.) of either saline or ghrelin (5nmol per rat) at T=-45 minutes and were returned to their home cage. Afterwards, at T=0 an i.p. injection of cocaine (10mg/kg) was administered and

animals were immediately placed in a dimly lit locomotor activity chamber (28.8cm x 23.6cm x 27.6cm) and beams crossed were recorded every five minutes for 45 minutes. Afterwards, animals were removed and placed in their home cage. All animals were subjected to each pretreatment.

Figure 4: Summary of locomotor activity test. Rats were pretreated with an i.p. injection of saline or ghrelin (5nmol) at T=-45mins and returned back to their home cage. At T=0min, rats were given an i.p. injection of cocaine (10mg/kg) and immediately placed in the locomotor activity chamber for 45 minutes. Beams crossed were recorded every five minutes. All rats received both treatments across PD 70-72.

Statistical Analyses

Analyses of feeding data and locomotor activity for Experiment 2 were conducted with two-way ANOVA (unless indicated) where diet (chow and palatable food) and stress (three day episodic stress or no stress) were the between group variables. Follow up comparisons between and within the control and treatment groups were conducted using simple effects analyses. Significance was set at $p < .05$.

RESULTS

Experiment 1: The long-term effects of juvenile stressor exposure and access to palatable food on physiological indices, behavior and mRNA expression of dopamine receptors

All 40 animals were included for the statistical analysis for weight, caloric intake and plasma ghrelin. Control referred to rats that were not exposed to juvenile stress. The following tests had rats that were excluded from statistical analysis due to data points that were ± 3 standard deviations from calculated means and were considered as outliers and (Taylor, 1997) or because of experimental difficulties (PCR analysis). Social interaction (n=1 from control), fat pad weight measurement (n=2 from Control+Palatable Food) and basal corticosterone analysis (n=1 from Control+Palatable Food, n=1 from Stress+Chow and n=1 from Stress+Palatable Food). For PCR analysis, NA (n=2 from Control+Chow, n=4 from Stress+Chow and n=3 from Stress+Palatable Food), PFC, *DRD1* (n=5 from Control+Chow, n=1 from Stress, n=2 from Control+Palatable Food and n=2 from Stress+Palatable Food) and *DRD2* (n=2 from Control+Chow, n=1 from Control+Palatable Food, n=2 from Stress+Palatable Food)

Weight

All means and standard errors of the means (SEM) for weight gain are depicted in Figure 5. Mauchly's test indicated that the assumption of sphericity was violated for Age $\times$ Diet (65)=766.73. Therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\epsilon=.13$). Repeated measures ANOVA indicated a significant Age $\times$ Diet interaction, $F(5, 53)=4.41$ $p<.05$. As expected, all rats gained weight as they aged

and graphical analysis suggested that previously stressed rats with access to palatable food gained more weight relative to previously stressed chow fed rats however, follow up simple effects failed to show a difference in weight as a result of diet.

Total caloric Intake

Figure 6A depicts all means and SEM for total caloric intake across groups. Mauchly's test indicated that the assumption of sphericity was violated for Age $X^2(27)=68.19$, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\epsilon=.60$). A significant effect for Age x Stress x Diet was present for total caloric intake, $F(4, 25)=2.69$ $p<.05$. Follow up simple effects revealed that on PD 30 ($p<.05$), 35 ($p<.05$) and 55 ($p<.05$), previously stressed rats with access to chow displayed lower caloric consumption relative to the chow fed controls. A similar decrease in caloric consumption was not observed in the palatable food fed rats, in fact on PD 50 follow up simple effects showed that previously stressed rats with access to palatable food consumed more calories than non-stressed rats fed the palatable food ($p<.05$).

Palatable food preference

All means and SEM for palatable food preference across groups are depicted in Figure 6B where all values represent a percentage of palatable food caloric intake over total caloric intake. There was no Stress x Age interaction, on preference for palatable food, $F(7,126)=.71$ $p>.05$, and no main effect of Stress, $F(1,18)=.13$ $p>.05$, indicating that preference for the palatable diet was comparable between stress and control groups across all ages evaluated.

Adiposity

All means and SEM for total fat and fat pad weights are represented in Figure 7. Two-way ANOVA revealed a significant Stress x Diet interaction, $F(1,35)=7.42$ $p<.05$, for total fat pads. Follow up simple effects showed that stressed rats with access to the palatable food had greater accumulation of fat (in total weight of fat pads) relative to their diet matched controls ($p<.05$) and this was not observed in previously stressed chow fed rats.

For visceral fat, two-way ANOVA revealed no main effect of Stress, $F(1,35)=.36$ $p>.55$, or Diet, $F(1,35)=2.51$ $p>.05$, and a Stress x Diet interaction that only approached significance $F(1,35)=3.39$ $p=.057$. Two-way ANOVA for retroperitoneal fat confirmed no main effect of Stress, $F(1,35)=1.43$ $p>.05$, however a significant main effect of Diet, $F(1,35)=5.66$ $p<.05$, was observed. Though the Stress x Diet interaction did not reach significance, $F(1,35)=3.16$ $p=.084$, based on a priori predictions follow up simple effects were examined, comprising the interaction, which indicated that stressed rats with access to palatable food accumulated more retroperitoneal fat than their chow counterparts ($p<.05$) and this effect was not observed in control rats fed a palatable diet. For brown fat, two-way ANOVA revealed no main effect of Stress, $F(1,35)=.62$ $p>.05$, and no Stress x Diet interaction, $F(1,35)=.02$ $p>.05$. There was however a main effect of Diet, $F(1,35)=13.75$ $p<.05$, as access to palatable food, regardless of previous stressor exposure, led to greater accumulation of brown fat compared to rats with access to chow only. Two-way ANOVA for subcutaneous fat pad showed a Stress x Diet interaction, $F(1,35)=6.99$ $p<.05$. Follow up simple effects revealed that stress and access to palatable

food led to increased subcutaneous fat relative to diet matched control rats ($p < .05$) and this was not evident in previously stressed rats fed chow only.

Basal plasma ghrelin

All means and SEM for basal plasma ghrelin are represented in Figure 8. Two-way ANOVA revealed no main effect of Stress, $F(1,36) = .57$ $p > .05$, and no Stress x Diet interaction, $F(1,36) = .01$ $p > .05$, on basal plasma ghrelin. A main effect of Diet, $F(1,36) = 14.92$ $p < .05$, was observed indicating that rats with access to a palatable diet had lower basal levels of ghrelin than rats with access to chow regardless of previous stressor exposure.

Social interaction

All means and SEM are depicted in Figure 9 for total time spent engaged in social interaction across groups, squares crossed and a breakdown of the individual social behaviors. Juvenile stressor exposure induced long-term anxiety-like characteristics measured in the social interaction test. Two-way ANOVA revealed a Stress x Diet interaction, $F(1,35) = 9.53$ $p < .05$. Follow up simple effects showed that previously stressed rats with access to chow had an overall decrease in social interaction relative to the chow fed controls ($p < .05$) and this was not evident in previously stressed rats that had access to palatable food. There were no main effects of Stress, $F(1, 35) = 1.02$, $p > .05$, Diet, $F(1,35) = 2.85$ $p > .05$, or a Stress x Diet interaction, $F(1,35) = .85$ $p > .05$ for the number of squares crossed in the arena, suggesting similar locomotor activity across groups.

For the individual social behaviors, two-way ANOVA indicated a Stress x Diet interaction for sniffing, $F(1,35) = 7.54$ $p < .05$, and over/under behaviors, $F(1,35) = 8.32$

$p < .05$. Follow up simple effects showed that juvenile stress exposed rats with access to chow sniffed less and displayed fewer over/under behaviors relative to their controls ($p < .05$) and this was not evident in previously stressed rats that had access to palatable food. Two-way ANOVA for following indicated there were no main effects of Stress, $F(1,35)=2.60$ $p > .05$ and Diet, $F(1,35)=2.41$ $p > .05$, and no Stress x Diet interaction, $F(1,35)=2.69$ $p > .05$. However, based on a priori hypothesis that stress can decrease social behaviors, follow up simple effects were completed which revealed that previously stressed rats given access to chow displayed less following behavior relative to their controls ($p < .05$) and this was not observed in the palatable diet counterpart. Two-way ANOVA for allogrooming revealed a main effect of Stress, $F(1,35)=5.77$ $p < .05$, no effect of Diet, $F(1,35)=.57$ $p > .05$, and a Stress x Diet interaction that did not approach significance, $F(1,35)=2.98$ $p = .093$. Follow up simple effects was examined which indicated that stressed rats with access to chow displayed less allogrooming relative to non-stressed rats fed chow ($p < .05$). However, there was no difference in allogrooming when compared to palatable diet fed rats exposed to juvenile stress. Furthermore, there were no main effects of Stress, $F(1,35)=2.86$ $p > .05$, and Diet, $F(1,35)=.21$ $p > .05$, or a Stress x Diet interaction for playfighting $F(1,35)=.23$ $p > .05$. Finally, there were no main effects of Stress, $F(1,35)=1.59$ $p > .05$, Diet, $F(1,35)=1.83$ $p > .05$, or a Stress x Diet interaction, $F(1,35)=.88$ $p > .05$, for grooming.

Dopamine receptor expression in the PFC and NA

All means and SEM are depicted in Figure 10 for fold changes in mRNA expression of DA receptors in the PFC. There were no significant differences in *DRD1* mRNA expression in the PFC. Two-way ANOVA revealed no main effects of Stress,

$F(1,26)=.29$ $p>.05$, Diet $F(1,26)=.12$ $p>.05$, and no Stress x Diet interaction, $F(1,26)=2.23$ $p>.05$. For *DRD2* mRNA expression, two-way ANOVA indicated a significant Stress x Diet interaction, $F(1,31)=7.37$ $p<.05$, and follow-up simple effects revealed that control rats fed the palatable diet displayed downregulated *DRD2* mRNA expression relative to their chow fed controls ($p<.05$) and this was not observed in previously stressed rats with access to palatable food.

All means and SEM are depicted in Figure 11 for fold changes in mRNA expression of DA receptors in the NA. Two-way ANOVA indicated a Stress x Diet interaction, $F(1,21)=4.99$ $p<.05$, for the expression of *DRD1* mRNA expression. Follow up simple effects analysis revealed that the combination of stress and palatable food increased *DRD1* mRNA expression relative to its control diet counterpart ($p<.05$) and this effect was not seen in previously stressed chow fed rats. For *DRD2* mRNA expression there was a main effect of Stress, $F(1,21)=4.22$ $p<.05$, and a Stress x Diet interaction, $F(1,21)=3.60$ $p=.072$, that did not reach statistical significance. There was also no main effect of Diet, $F(1,21)=4.22$ $p>.05$. Simple effects comprising the interaction were still examined based on a priori predictions. These follow up tests revealed that stressed rats with access to palatable food had greater *DRD2* mRNA expression relative to control rats that had access to the palatable diet ($p<.05$). Two-way ANOVA for *DRD3* expression revealed no significant main effects of Diet, $F(1,21)=.10$ $p>.05$, Stress, $F(1,21)=2.19$ $p>.05$ and no Stress x Diet interaction, $F(1,21)=.01$ $p>.05$.

Figure 5: Effect of juvenile stress and diet on body weight. Mean ($\pm$ SEM) weight (g) measured every five days. Stressor exposure occurred on PD 27-29 (shaded area).

Figure 6: Effect of juvenile stress and diet on feeding behaviour. A) Juvenile stressor exposure decreased caloric intake on PD 30, 35 and 55. Stressed rats with access to palatable food consumed more calories on PD 50. Data represents mean $\pm$ SEM caloric intake (kcal). B) Juvenile stress did not elicit a preference of palatable food over time. Data represents mean $\pm$ SEM % preference for palatable food. Stressor exposure occurred on PD 27-29 (shaded area). * Significant difference from diet matched counterpart ($p < .05$).

Figure 7: Effect of juvenile stress and diet on adiposity. A) Palatable food increased adiposity in adulthood in rats exposed to juvenile stressor. B) The combination of juvenile stress and access to palatable food resulted in greater retroperitoneal and subcutaneous fat pad weight. Only rats fed with a palatable diet had significantly heavier brown fat. Data represents mean $\pm$ SEM weight (g). * Significant difference from diet matched controls ($p < .05$). τ Significant difference relative to chow fed groups ($p < .05$). Retro = retroperitoneal fat pad. Brown = Inter-scapular brown fat. SubQ = Subcutaneous inguinal white fat pad.

Figure 8: Effect of juvenile stress and diet on basal plasma ghrelin levels. Rats with access to palatable food had lower levels of basal plasma ghrelin. Data represents mean $\pm$ SEM plasma ghrelin (pg/25 μ L). τ Significant difference from chow fed groups ($p < .05$).

Figure 9: Impact of juvenile stress and diet on social interaction in adulthood. A) Juvenile stressor exposure decreased total time in social interaction, an effect not observed in previously stressed rats with access to a palatable diet. B) No differences in squares crossed across groups were observed. Data represents mean $\pm$ SEM squares crossed. C) Juvenile stressor exposure elicited anxiety-like characteristics on different measures of social interaction in chow fed rats: sniffing, over/under behavior, following and allogrooming. There was no effect on playfighting and grooming. Data represents mean $\pm$ SEM time (s) *Significant difference from diet matched controls ($p < .05$).

Figure 10: Effect of juvenile stress and diet on fold changes for DA receptors mRNA expression in the PFC. A) There were no significant changes in D₁ receptors mRNA expression across groups. B) There was a downregulation of D₂ expression in control rats fed palatable food. Data represents mean ± SEM fold changes. * Significantly different from chow fed controls (p<.05 value).

Figure 11: Effect of juvenile stress and diet on fold changes for DA receptors mRNA expression in the NA. A) There was an upregulation in D₁ receptor mRNA expression in juvenile stressed rats with access to palatable food B) D₂ receptor mRNA expression was upregulated in juvenile stressed rats with access to palatable food C) No observed changes in D₃ receptor mRNA expression across groups. Data represents mean $\pm$ SEM fold changes.* Significantly different from diet matched control group ($p < .05$ value).

Experiment 2: The long-term effect of juvenile stressor exposure and access to palatable food on the sensitivity to ghrelin

For the chow feeding tests with icv administration of saline, six rats were excluded from the analysis as they showed signs reaching experimental endpoints (i.e. loss of weight, decreased feeding, lethargy, loss of surgical headcap) (n=1 from Control+Palatable Food, n=2 from Stress+ Control+Palatable Food, n=2 from Stress+Chow and n=1 from Control+Chow). Furthermore, for the chow feeding test with icv ghrelin an additional two rats met experimental endpoints (n=1 from Control+Palatable Food and n=1 from Control+Chow). Of the remaining 32 animals, five rats were excluded from analysis for locomotor activity as they either showed signs of reaching experimental endpoints or their data points were ± 3 standard deviations from calculated means and were considered as outliers (Taylor, 1997) (n=2 from Stress+Palatable Food, n=2 from Control+Chow and n=1 from Stress+Chow).

Feeding test: Chow and cookie dough intake with icv administration of ghrelin

All means and SEM are represented in Figure 12 for total chow intake following central administration of saline or ghrelin. For total chow intake, two-way ANOVA revealed a significant effect of Treatment, $F(1,30)=260.33$ $p<.05$, where administration of ghrelin increased total chow intake in all groups compared to saline injection. There was also a main effect of Diet, $F(1,30)=37.41$ $p<.05$, as total chow intake was significantly greater in chow fed rats compared to rats with access to the palatable diet irrespective of previous stressor exposure. There was however, no main effect of Stress, $F(1,30)=.05$ $p>.05$, or a Stress x Diet interaction, $F(1,30)=3.06$ $p>.05$.

Figure 13 represents all means and SEM for total cookie dough intake in all groups following a central injection of saline and ghrelin. Two-way ANOVA showed a main effect of Diet, $F(1,26)=44.09$ $p<.05$, where rats with access to palatable food consumed less cookie dough than rats with access to chow, regardless of previous stressor exposure for both saline and ghrelin treatments. There was no main effect of Stress, $F(1,26)=2.32$ $p>.05$, and no Stress x Diet interaction, $F(1,26)=.22$ $p>.05$. However, based on a priori hypothesis, simple effects analyses were conducted which indicated that stressed rats with access to chow only ate more cookie dough following ghrelin injection compared to cookie dough intake following saline injection ($p<.05$).

Total cocaine locomotor activity with pretreatment of ghrelin

All means and SEM are represented in Figure 14 for total beams crossed following a pretreatment of saline or ghrelin and then an injection of cocaine. Two-way ANOVA revealed significant main effects of Treatment, $F(1,21)=15.03$ $p<.05$, Diet, $F(1,21)=8.33$ $p<.05$, and a Treatment x Diet interaction, $F(1,21)=10.77$ $p<.05$. Simple effects displayed that ghrelin pretreatment augmented cocaine-induced locomotor activity in rats that were fed a chow diet, regardless of prior stressor exposure ($p<.05$) when compared to the cocaine treatment (with no ghrelin pretreatment). A similar ghrelin-induced increase in locomotor activity was not observed in rats with access to the palatable diet.

Figure 12: Long-term effect of juvenile stressor exposure and diet on chow consumption following central ghrelin administration. Central injection of ghrelin (10ug) increased chow consumption across all groups after four hours. Rats with access to palatable food consumed less chow (regardless of treatment) than rats fed chow only. Central injection administered at T=0. Data represents mean $\pm$ SEM weight (g). τ Significantly different from chow fed rats fed ($p < .05$).

Figure 13: Long-term effect of juvenile stressor exposure and diet on cookie dough consumption following central ghrelin administration. Central injection of ghrelin (10ug) increased cookie dough consumption after four hours in previously stressed rats fed chow. Rats with access to palatable food consumed less cookie dough (regardless of treatment) than chow fed rats. Central injection administered at T=0. Data represents mean $\pm$ SEM weight (g). *Significantly different from saline treatment ($p < .05$). τ Significantly different from rats fed chow only ($p < .05$).

Figure 14: Long-term effect of juvenile stressor exposure and diet on cocaine hyperlocomotor activity with a ghrelin pretreatment. Systemic pretreatment of ghrelin (5nmol) augmented cocaine (10mg/kg) hyperlocomotor activity in rats fed on a chow diet when compared to saline pretreatment. Pretreatment of ghrelin prior to i.p. injection of cocaine did not augment locomotor activity in rats fed a palatable diet. Data represents mean $\pm$ SEM beams crossed. τ Significantly different from rats fed on chow diet only ($p < .05$).

DISCUSSION

The purpose of this study was to investigate the long-term effects of juvenile stressor exposure and access to a palatable diet. We have previously shown that access to a palatable diet mitigated the anxiogenic effects of juvenile stressor exposure when tested in adulthood. We contend that this stress-buffering characteristic is mediated, in part, by neurochemical changes within the mesolimbic pathway. The first experiment explored long-term changes in DA receptor expression in structures within the mesolimbic pathway following juvenile stressor exposure in rats with or without limited access to a palatable diet. For the second experiment, we investigated whether juvenile stressor exposure changed central ghrelin sensitivity in rats that had access to the palatable diet. Stressor exposure elicits a rise in circulating levels of ghrelin which then may shift a preference for palatable food intake via increasing dopaminergic activity in the reward pathway (Chuang et al., 2011; Chuang & Zigman, 2010). Thus, we wanted to evaluate if the sensitivity to central ghrelin had changed in adulthood in rats previously exposed to juvenile stress and if the diet they consumed (chow vs palatable) impacted these changes. We hypothesized that the combination of juvenile stressor exposure and a palatable diet would sensitize the reward pathway which would be manifested by changes in DA receptor mRNA expression within the mesolimbic pathway in adulthood and by enhanced responsiveness to ghrelin administration. Interestingly, our results revealed changes in the DA receptors mRNA expression in the reward pathway that may be consistent with a sensitization type effect in previously stressed rats with access to the palatable diet. However, sensitivity to ghrelin administration in the palatable food fed rats appeared blunted suggesting a lack of association between these measures.

Juvenile stressor exposure and access to a palatable diet did not induce significant differences in weight gain throughout adolescence and into adulthood. This was not expected as previous findings using the same stressor paradigm and feeding regime showed that juvenile stressor exposure decreased weight gain across time and that in adulthood the weight of stressed rats with access to palatable food was comparable to the control chow fed group (MacKay et al., 2014). A difference with this current study is that animals were single-housed and isolation is a stressor in itself which may have contributed to the lack of significant differences in weight gain between groups. It should be noted however that social isolation had been shown to have no effect on body weight after weaning and during adolescence (Hong et al., 2012; Weiss, Pryce, Jongen-Rêlo, Nanz-Bahr, & Feldon, 2004). Although our weight data displayed no significant difference between groups, we did observe an effect of stress in rats with access to the palatable diet when looking at adiposity. In this regard, prior stressor exposure induced an overall increase in adiposity in rats with access to palatable food and specifically an increase in retroperitoneal and subcutaneous fat. In addition, there was an increase in visceral fat pad however this did not reach significance for previously stressed rats fed on a palatable diet. This is in line with the common findings that have shown that access to a densely caloric diet can lead to increased adipose accumulation (Gentile et al., 2015). In addition, access to a high fat and/or high carbohydrate diet led to heavier mesenteric and subcutaneous fat pad when compared to animals given chow only (Foster et al., 2009; Pecoraro et al., 2004). Furthermore, increased GC levels due to chronic stress have been associated with increased visceral fat storage (Dallman et al., 2005; Peters et al., 2004). As well, chronic stress in adolescent mice increased subcutaneous fat when measured in

adulthood (Schmidt et al., 2009). For the current experiment juvenile stressor exposure in combination with a palatable diet led to greater adiposity in adulthood, suggesting that stress exacerbated adipose accumulation.

Juvenile stressor exposure reduced caloric intake in late adolescence and into early adulthood. This coincided with findings that displayed reduced caloric consumption after juvenile stressor exposure (MacKay et al., 2014). In addition, specific stressors applied in adulthood have shown to reduce caloric intake, in particular acute restraint stress and immobilization (Haque, Akbar, Yasmin, Haleem, & Haleem, 2012).

Superimposing the palatable diet with the juvenile stress resulted in an overall increase in total caloric intake in mid to late adolescence which was likely attributable to the increased calories derived from the palatable diet. It is interesting to note that the effect of stress on caloric intake for rats with access to chow only and rats with access to palatable food were quite persistent through adolescence and at the onset of adulthood. This emphasized that juvenile stressor exposure has differential effects on caloric intake based on diet. Similarly, rats subjected to social isolation during the juvenile period displayed increased caloric intake five weeks post-stressor exposure when only allowed to consume a palatable diet and not regular chow (Krolow et al., 2013). Though in this experiment, juvenile stressor exposure did not prompt a preference for the palatable diet, other studies have shown this stress-induced preference shift (Dallman et al., 2005; Dallman, 2010; Pecoraro, Reyes, Gomez, Bhargava, & Dallman, 2004). The lack of preference could be due to the constricted two-hour access to the diet given in the early light phase. The timing of the palatable diet may have also prevented this preference, as it has been observed that rats increased their intake of a sucrose solution substantially over the

course of 3.5 hours after restraint stress (Foster et al., 2009). Since the palatable diet was given before induction of juvenile stress, a possible preference might have been observed if the food was given immediately after the stress.

Access to the palatable diet led to lower levels of total plasma ghrelin which is in agreement with studies using rodent models of diet-induced obesity (DIO), where reduced total and acylated ghrelin levels have consistently been observed in animals consuming a high fat diet (Briggs, Enriori, Lemus, Cowley, & Andrews, 2010). Also, access to a high fat/high sugar diet reduced ghrelin and ghrelin mRNA expression in the stomach of rodents and GHSR mRNA expression in the ARC (Beck & Richy, 2008; Levin, Dunn-Meynell, Ricci, & Cummings, 2003; Lindqvist et al., 2005; Moesgaard et al., 2004). Surprisingly however, while an effect of diet was observed, exposure to juvenile stress did not further impact ghrelin levels. An increase in basal plasma levels of ghrelin in mice that were subjected to CSD was observed and these levels remained elevated for four weeks after the stressor exposure (Chuang et al., 2011; Chuang & Zigman, 2010; Lutter et al., 2008). It is possible that our juvenile stressor paradigm may have not been potent enough to elicit a sustained increase of basal plasma ghrelin levels.

Exposure to stressors during the juvenile period elicited a long-term anxiogenic effect thereby reduced the overall time spent in social interaction in chow fed rats; this effect not present in juvenile stressed rats fed on the palatable diet. This finding corresponded with a previous study that implemented the same juvenile episodic stressors with access to a palatable diet (MacKay et al., 2014). It brings to light that daily limited access to a palatable diet appeared to mitigate anxiogenic results of juvenile stressor exposure in adulthood. This suggests that long-term access to palatable diet can act as

self-medication for stress, however at the cost of increased adiposity, as seen in this study (Dallman et al., 2003; MacKay et al., 2014). As already mentioned, early-life stress increased anxiety-like characteristics and HPA axis activity, however these effects were reduced when animals were provided with a cafeteria diet (Maniam & Morris, 2010). Similar stress-buffering behavioral and endocrine effects of palatable food or drink have been observed when stressors were applied in adulthood as well (Bell et al., 2002; la Fleur, Houshyar, Roy, & Dallman, 2005). Though not measured in this present study, we have also observed that access to a palatable diet reduced basal corticosterone levels (MacKay et al., 2014). Interestingly, intragastric gavage of a sucrose solution did not reduce HPA axis activity after restraint, suggesting that perceived palatability may play a role in blunting the effects of stress through the reward pathway (Ulrich-Lai et al., 2010). The HPA axis can directly influence the mesolimbic pathway as release of CRH has shown to stimulate DA activity in the VTA, increasing motivation to seek out pleasurable foods as a coping method during stress (Morris et al., 2014). As such, it appears that the reward pathway, in part, may have contributed to the stress-buffering effects of palatable food.

To investigate this contention, we examined the long-term impact of juvenile stressor exposure and access to palatable food on mRNA expression of D₁ and D₂ receptors in the PFC and NA. These structures were selected as they play important roles in cognitive processes and reward respectively and are particularly sensitive to stress during juvenile and adolescent development. In terms of D₂ receptor mRNA expression at the PFC, results showed a downregulation in control rats that had access to palatable food. Similarly, a significant decrease in D₂ mRNA expression in the PFC was observed

in DIO mice fed on a 60% fat diet beginning in early life for 12 weeks when compared to DIO mice fed chow. Lower levels of DA were also observed in this study which was thought to be attributed to disrupted DA homeostasis due to decreased DA transporter expression (Carlin, Hill-Smith, Lucki, & Reyes, 2013). Other studies have also reported a reduction in basal extracellular DA levels in the PFC in DIO rats fed a high fat diet (long-term) when compared to DIO rats fed chow (Geiger et al., 2008; Hansen, Jensen, Overgaard, Weikop, & Mikkelsen, 2013). Although we did not measure DA content, future studies may help elucidate whether the reduction of D₂ receptors in the PFC in non-stressed rats fed palatable food could be indicative of a hypodopaminergic state resulting from daily consumption of calorically dense food.

Interestingly, there was an upregulation of both D₁ and D₂ receptor mRNA expression in the NA of palatable food fed rats that were exposed to juvenile stress. Access to a high fat and a high sucrose diet have typically been associated with decreased expression D₁ and/or D₂ receptors in the mesolimbic pathway (Alsiö et al., 2009; Kovalenko et al., 2014; Ong, Wanasuria, Lin, Hiscock, & Muhlhausler, 2013). Surprisingly we observed no changes in D₁ or D₂ mRNA expression at the NA resulting from palatable food consumption in unstressed rats. Although it was not investigated in this current experiment, it is also possible that the combination of previous stressor exposure and access to a palatable diet decreased DA levels that may have led to a compensatory upregulation of DA mRNA expression. In terms of stress, chronic treatment with corticosterone during adolescence in rats that were previously exposed to neonatal stress, increased D₂ receptor expression at the NA (Hill et al., 2014). In addition, prenatal stressor exposure also elicited an increase in D₂ receptor expression at

the NA in adulthood (Lakehayli et al., 2015; Said et al., 2015). Considering the effect of a palatable diet, chronic exposure to a high fat diet has shown to decrease DA in the NA (Carlin et al., 2013) as well as decrease DA turnover (Davis et al., 2008).

It is conceivable that juvenile stressor exposure cross-sensitized with natural food reward in rats fed the palatable diet. Indeed, palatable food activates similar brain reward circuits to that of drugs of abuse (Volkow, Wang, Tomasi, & Baler, 2013; Volkow & Wise, 2005). There is evidence showing that stressor exposure during adolescence produced a sensitization for drugs of abuse (Burke & Miczek, 2014). For example, stressors experienced during adolescence, such as CSD and CVS, potentiated adult locomotor activity to acute amphetamine (Burke, Forster, Novick, Roberts, & Watt, 2013; Burke & Miczek, 2014; Peleg-Raibstein & Feldon, 2011) and cocaine (Lepsch et al., 2005) administration. However, for the current investigation there was no indication from a behavioral perspective that previously stressed palatable food fed rats displayed behavioral sensitization. On the contrary, as will be discussed shortly, the cocaine induced locomotor activity results from Experiment 2 were more in keeping with behavioral desensitization in the palatable food fed rats. It is also not clear if the upregulation of DA receptor mRNA expression was related to the stress-buffering effects of palatable food consumption in terms of reversing the stress-induced decrease in social interaction. Although there is evidence of D₁ receptor involvement at the NA in social behavior (Gunaydin et al., 2014), the fact that DA receptor mRNA expression was unchanged in the NA in the chow fed rats previously exposed to stress (despite reduced levels of social interaction compared to controls) suggests a lack of association between these measures. While more work is needed to determine the functional significance of

the DA receptor mRNA expression changes, these results do show that the combination of juvenile stressor exposure and palatable food diet elicited long-term neurochemical changes within the mesolimbic pathway.

The consumption of the palatable diet provided stress buffering effects in rats previously exposed to juvenile stress and altered DA receptor mRNA expression in the mesolimbic pathway; it was of further interest to investigate if there were alterations in ghrelin signalling for highly rewarding food. Ghrelin is central to homeostatic and hedonic feeding and has a role in stress-induced preference for calorically dense foods. Therefore, for Experiment 2 we monitored the intake of chow and a highly hedonic food (cookie dough), for four hours following a central injection of saline and ghrelin, to evaluate if juvenile stressor exposure and access to palatable food changed ghrelin sensitivity in adulthood. Following a central saline administration, rats that had access to the palatable diet consumed less chow than chow fed rats, irrespective of previous stressor exposure. As expected, central injection of ghrelin increased chow intake in all groups, however similar to what was observed with the saline injection, rats with access to palatable food, regardless of previous stressor exposure, displayed significantly less consumption of chow when compared to chow fed rats. This lower consumption of chow in palatable diet fed rats could be due to their daily access to palatable food, where a portion of their caloric intake was derived from, though in adulthood (PD 60) the total caloric intake were not significantly different between the groups. In addition, a reason for this pattern of reduced caloric intake in palatable food fed rats is that the homeostatic set point is maintained and is reflective of the current energy balance. Therefore, rats with access to the palatable diet may have a much lower threshold due to their positive energy

balance that is based on their diet (which is high in fat and sugar) (Finger, Dinan, & Cryan, 2011; Lindqvist et al., 2005; Perez-Tilve et al., 2011; Perreault et al., 2004). In keeping with our findings, rats fed on a high fat diet displayed reduced hyperphagia when given access to chow with both an i.p. and a central injection of ghrelin (Briggs et al., 2010). In addition, a downregulation of hypothalamic GHSR and ghrelin mRNA expression has been observed with long-term high fat diet consumption, and this was associated with the reduced feeding response to exogenous administration of ghrelin (Briggs et al., 2010; Finger et al., 2011). Further investigation is needed to confirm if there was downregulation of GHSR and ghrelin mRNA expression in hypothalamic regions due to the long-term access to the palatable diet, to correspond to the observed reduced hyperphagic effect of ghrelin on chow feeding.

When rats were microinjected with saline and were presented with cookie dough we again observed a differential rate of consumption. Rats that had access to the palatable diet ate significantly less than chow fed rats over the four hours, regardless of previous stressor exposure. Interestingly, following a central injection of ghrelin, only previously stressed rats on the chow diet increased cookie dough consumption; in all other groups, ghrelin had no effect. These observations suggest that juvenile stressor exposure sensitized central ghrelin signalling for highly rewarding food in adulthood in chow fed rats. Consistent with this contention, stress such as social defeat in adult mice have produced a CPP for a palatable diet. However this effect was blunted in rats that lacked GHSR suggesting that intact ghrelin signalling was required for this preference (Chuang et al., 2011). To our knowledge, these findings were the first to show this sensitivity to central ghrelin in adulthood after juvenile stressor exposure when a hedonic food source

was presented. A future extension of this study would be to apply an acute and/or a chronic stress during adulthood and investigate if there is a correlation between plasma ghrelin and cookie dough feeding immediately after stressor exposure. Stress in rodents is known to elicit increased levels of ghrelin (Chuang et al., 2011; Patterson, Ducharme, Anisman, & Abizaid, 2010; Schellekens, Finger, Dinan, & Cryan, 2012). A proposal would be that juvenile stress enhanced the effect of stress-induced ghrelin in adulthood, prompting a heightened preference for ‘comfort’ food. For example, early-life stressor exposure was also associated with increased preference for densely caloric foods in adulthood (Machado et al., 2013). In contrast, ghrelin did not increase cookie dough feeding in non-stressed chow fed rats and rats with access to palatable food. This result was surprising for the control chow fed rats as it was contrary to a study that showed that central and i.p. injection of ghrelin produced a preference for a palatable diet (Egecioglu et al., 2010). In contrast however, the lack of effect of ghrelin on cookie dough intake in palatable food fed rats may actually be in keeping with the literature. In this regard, DIO rats and obese mice fed a high fat diet displayed lower breakpoints for a palatable treat on progressive ratio with central injections of ghrelin, suggesting a blunted sensitivity to ghrelin (Finger et al., 2011). Chronic access to a high fat diet blunted ghrelin signalling is also independent of the obese phenotype. For example, rats chronically fed a high fat diet had delayed ghrelin secretion upon fasting similar to Zucker rats (genetically bred to be obese). However, after 13 days of access to a high fat diet there was a loss of sensitivity to a peripheral injection of ghrelin (blunted hyperphagia), even though weights were similar to rats fed on a low fat diet (Perreault et al., 2004). A finding similar to our study observed a failed increase in high fat diet consumption following a central injection of

ghrelin (10 μ g) in rats that had acute free access to densely caloric food, consistent with a reduced sensitivity to ghrelin (Perez-Tilve et al., 2011).

To further investigate the impact of juvenile stressor exposure and access to palatable food, we evaluated if juvenile stressor exposure affected how ghrelin enhanced locomotor responses to cocaine. Our findings displayed a diet effect as ghrelin pretreatment augmented cocaine-induced locomotor activity in rats that were fed chow however this effect was not observed in rats that had access to the palatable food. The increased locomotor activity was expected for the chow fed rats which is in agreement with previous studies (Wellman et al., 2005; Wellman, Hollas, & Elliott, 2008). Yet, we predicted greater locomotor activity in previously stressed chow fed rats to parallel the findings in the cookie dough feeding test, following a ghrelin injection when compared to unstressed rats fed chow. Though the cookie dough test and the locomotor activity test both measure the response to rewarding stimuli, it is likely that the mechanism of action for ghrelin to further stimulate palatable food intake and increase locomotor activity is distinct. For the cookie dough feeding test, ghrelin was administered centrally whereas in the locomotor activity test ghrelin was delivered with an i.p injection prior to the cocaine injection; therefore it is very likely that different regions were stimulated. In contrast, ghrelin pretreatment failed to potentiate further locomotor activity in rats that had access to the palatable diet, regardless of prior stressor exposure. Similar to the feeding study, we observed a blunted sensitivity to ghrelin administration in the palatable food fed rats which was consistent with the literature as outlined above (Perez-Tilve et al., 2011; Perreault et al., 2004). It is also worthy to note that locomotor activity induced by cocaine alone was comparable across all conditions. Cocaine administration was used as an

indirect measure of DA activity as cocaine is a DA transporter inhibitor, thereby increasing extracellular DA levels. An increase in sensitivity to cocaine in juvenile stressor exposed rats with access to palatable food was not observed; this was opposite of what was expected considering the upregulation of DA receptors mRNA expression in the NA in the same group. Similarly, findings have shown that long-term high fat feeding reduced locomotor-sensitizing effects of amphetamine (Hryhorczuk et al., 2015) and cocaine (Baladi, Horton, Owens, Daws, & France, 2015). Therefore, in previously stressed palatable food fed rats, there is a lack of association between the upregulation of DA receptors in the NA and cocaine locomotor activity. Further exploration may reveal other mechanisms at play for example changes in monoamine clearance and synthesis, DA transporter activity and DA receptor protein expression.

There are other limitations to be considered for this study, For instance, we limited our investigation of the expression of DA receptors to two brain regions involved in the reward pathway. The VTA is another area of interest as it sends dopaminergic signals to the NA and there is a high expression of the ghrelin receptor. It would be important in future studies to evaluate possible differential changes that are occurring at the VTA (such as GHSR expression and DA activity) that are imposed by the palatable diet and the combination of juvenile stressor exposure and the palatable diet. There is evidence where access to a high fat diet activated c-fos expression in the VTA (Valdivia, Patrone, Reynaldo, & Perello, 2014), decreased tyrosine hydroxylase mRNA expression (Li et al., 2009) and attenuated DA release when cocaine was administered (Cone, Chartoff, Potter, Ebner, & Roitman, 2013), yet none have looked at both palatable food and stress. Ghrelin and its interaction with GHSR in the VTA were responsible for the

intake of a high fat diet (King, Isaacs, O'Farrell, & Abizaid, 2011), stress-induced place preference for highly caloric food (Chuang et al., 2011) and was crucial for augmenting cocaine hyperlocomotor activity (Davis et al., 2007). Considering the increase in cookie dough consumption in previously stressed chow fed rats following ghrelin treatment, it would be of interest to investigate GHSR expression at the VTA. It can be postulated that there was an increase in GHSR expression that may be in part responsible for this enhanced sensitivity to ghrelin. To our knowledge, there is a gap in the literature resolving if stressor exposure and/or a palatable diet can evoke changes in GHSR mRNA expression in the VTA.

In summary, consumption of palatable food mitigated the anxiogenic effects of juvenile stressor exposure in adulthood however at the cost of increased adiposity. Interestingly, our findings displayed a lack of association between the upregulation of DA receptor mRNA expression occurring at NA to highly rewarding stimuli, such as cookie dough and cocaine, in previously stressed rats with access to palatable food. Juvenile stressor exposure enhanced ghrelin signalling for highly hedonic food whereas access to palatable food, regardless of stressor exposure, blunted ghrelin sensitivity. Indeed juvenile stressor exposure and access to palatable food elicited long-term consequences and further studies are needed to understand the interplay of ghrelin signalling, juvenile stress and long-term access to palatable food in the mesolimbic pathway.

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