Modeling depression in the rat: the development and usefulness of a female-centric approach

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For all of your help and hindrance but especially for your love,
I dedicate this work to my little Bubba and his incredible Daddy.

For the opportunity to pursue this work, the gentle encouragement, and the confidence, I will be forever grateful to you Cate, my ever-patient supervisor.

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ABSTRACT

Women are twice as likely to suffer from depression as men, yet stress and depression research has relied primarily on the responses of males. Early life stress is hypothesized to influence the development of vulnerability to depression while adult stress exposure can act as a trigger in those predisposed. This relationship is mediated by other environmental factors. Maternal care and the social environment appear to be particularly important for mammals. The purpose of this thesis was twofold: to develop an animal model of depression for use in female rats based on the chronic mild stress (CMS) model previously validated for use in male rodents, and to apply this model in female offspring of mothers exposed to physical restraint in the second half of gestation representing an early life insult. Results indicate that a modified CMS model was able to alter hedonic and physiological responses not present in the original model. Housing condition interacted with CMS in that effects were evident only in singly housed rats. While gestational stress (GS) altered maternal weight and behavioural profiles related to offspring care and anxiety, little to no behavioural effects were noted in juvenile or adult offspring. Applying the modified CMS model to adult female offspring resulted in an anhedonic-like response that recovered rapidly prior to the third week of CMS. Weight in GS female rats was attenuated throughout life beginning post weaning. When taken together, these results demonstrate that stress-based models, previously established in males, must be altered to accommodate the hormonally intact female rat in two ways: first, to eliminate extraneous variables that may interfere with the estrous cycle and mask possible stress effects, and secondly, to consider the appropriateness of individual stressors to induce a stress response in females. While a general lack of

effect was noted in response to CMS, this was interpreted as a strong influence of housing and supportive early life experiences in protecting the female rat from the establishment of stress effects related to depression and anxiety. The housing practices employed here may be considered a model of stress-resilience and represents an encouraging avenue of future research.

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Foreword

The author preformed all experimental procedures discussed in this thesis unless specified below and is the primary author of all sections and published studies presented here. The contributions of fellow collaborators are presented in detail below; note that daily maintenance of the animals was performed by Sylvie Émond, Animal Care Technician.

Study 1

This study has been published as *Behavioral and physiological effects of chronic mild stress in female rats* and was submitted in collaboration with A.C. Kentner, A.T.M. Konkle, L.S.-M. Barbagallo, and C. Bielajew to the journal, *Physiology & Behavior* 87 (2006) 314-322. Study design was developed by the author and A. Kentner with guidance from A. Konkle and C. Bielajew. Small animal surgery was conducted by the author and A. Kentner under the instruction of A. Konkle. Animal care and testing procedures were carried out by the author and A. Kentner with the help of L. Barbagallo. The manuscript was prepared by the author with the assistance of C Bielajew and input from the other coauthors.

Study 2

This study entitled, *Influence of housing on the consequences of chronic mild stress in female rats*, appears in *Stress* 10 (2007) 283-293 and was coauthored by C.Bielajew who supported study design and manuscript preparation.

Study 3

Effects of gestational stress: 1. Evaluation of maternal and juvenile offspring behaviour was published in *Brain Research* 1213 (2008) 98-110 in collaboration with M. Chebli, S. Rees, N. LeMarec, R. Godbout, and C. Bielajew. Project design was developed in collaboration with M. Chebli, N. LeMarec, and R. Godbout – gestational stress and juvenile monitoring and measures, S. Rees – maternal measures, and overseen by C. Bielajew. Animal care and all procedures were conducted by the author, M.Chebli – gestational stress procedures and all juvenile measures (as fulfillment of the requirement of honour's thesis), and S. Rees- maternal measures. Scoring of behavioural data was conducted by the author, M. Chebli, and S. Rees with help from Elizabeth Quon and Jessica Sparling. The manuscript was prepared by the author under the supervision of C. Bielajew and input from coauthors.

Study 4

This study appears in *Brain Research* 1302 (2009) 194–204 entitled, *Effects of gestational stress: 2. Evaluation of male and female adult offspring* and coauthored by S. Rees, M. Chebli, N. LeMarec, R. Godbout, V. Huta, and C. Bielajew. Animal care and procedures were carried out by the author, S. Rees, M. Chebli with help from Jacinthe Faucher, Elizabeth Quon, and Jessica Sparling. Data analysis was planned by the author and performed with V. Huta; related manuscript sections were written in collaboration. The remaining sections were prepared by the author under the supervision of C. Bielajew with input from S. Rees, N. Le Marec, and R. Godbout.

Study 5

In preparation for submission, this study is entitled, *Effects of gestational stress: 3. Evaluation of interactions between maternal gestational stress and offspring Chronic Mild Stress exposure in adult female rats* and coauthored by V. Huta, and C. Bielajew. Animal care and procedures were carried out by the author with help from Jacinthe Faucher, Elizabeth Quon, and Jessica Sparling. The radio-immune-assay for the detection of corticosterone from blood spots was performed by Marc Milot and Johnathan James. Data analysis was planned by the author and performed with V. Huta and related manuscript sections prepared with her collaboration. The remaining sections were prepared by the author under the supervision of C. Bielajew.

General Introduction

Depressive disorders are characterized by intense feelings of sadness, guilt, hopelessness and worthlessness, as well as a diminished ability to find pleasure in once previously enjoyable activities. Termed anhedonia, this state often results in significant changes in appetite, sleep, activity levels, and cognitive functioning (American Psychiatric Association [*DSM-IV-TR*] 2000). Currently considered one of the most prominent global causes of disease related disability, depression during the middle stage of life has been ranked as producing the greatest burden on individuals, families, and society, both in developed and undeveloped nations. By 2020, it is estimated that depression will be the second leading cause of worldwide disability in those aged five and up, second only to ischemic heart disease (Murray, & Lopez 1996). Those affected suffer an overall diminished quality of life, health, and productivity and have significantly higher rates of mortality, particularly from suicide, and morbidity and mortality from medical conditions (Ramasubbu, & Patten 2003). The economic burden of depression is thought to be under-estimated at 53 billion dollars in the U.S. alone (Greenburg et al. 2001; 1996). The lifetime prevalence rate is just over 12 percent for the Canadian population (Patten et al. 2002) and is similar in other developed countries (Olfson et al. 2002; Kessler et al. 1994).

Generally considered a stress-related illness, most depressive episodes are triggered by the experience of stressful life events. When exposure occurs early in life, even while in the womb, pathological neural pathways may be established creating vulnerability to psychopathology. The link between stress and depression is a well-established finding in clinical research and has received extensive review (Hammen

2005; Liu, & Alloy 2010; Paykel 2003).

Around the world, women and children are at a great risk for experiencing trauma and stress that may precipitate or trigger a pathological neuropsychiatric response such as depression. According to the World Health Organization, women and children represent approximately 80 percent of the estimated 50 million people who are affected by violent conflicts, civil wars, disasters, and displacement globally (WHO 2011). They are also at the greatest risk of sexual and physical victimization. Twenty percent of women report being sexually abused as children compared to 5 to 10 percent of men, and 20 to 50 percent of children report being physically abused (WHO 2010). The lifetime prevalence rate of violence against women ranges from 16 to 50 percent, and rape or attempted rape occurs in one in five women (WHO 2011).

In all developed countries studied, women are at the greatest risk of developing depression and are affected at a rate double that of men (Bhugra, & Mastrogianni 2004; Le et al. 2003; Kendler et al. 2000; Sprock, & Yoder, 1997; Kessler et al. 1993; Earls 1987; Nolen-Hoeksema 1987) - the most robust finding in psychiatric epidemiology. Depressive disorders account for almost 42 percent of disability from neuropsychiatric illness in women compared to 29 percent in men (Piccinelli, & Homen 1997) making it the most prevalent women's mental health problem. Furthermore, depressive disorders may be more persistent in women with female gender being a significant predictor for relapse (Bracke 2000; Kuehner 1999).

Depression can disturb all relationships in an affected woman's life. Most costly is the damage it can have on the mother-child dyad, the most important relationship in early development. When impaired, negative consequences on normal developmental

trajectories of health and well-being are altered in offspring often perpetuating the intergenerational transmission of mental illness and associated problems.

In summary, depression is a devastating illness causing suffering in individuals, families, and society at large; women are more likely to experience mood disorders; and there is a strong link between stress and depression both in the initiation of depressive symptomatology and the creation of depressogenic vulnerabilities. These themes, well-established in the clinical literature, have guided the development of the animal research conducted in this thesis. The main purpose was to develop a model of stress-induced depression with efficacy in female rats that would allow for the exploration of female sensitivity to depression and its interaction with maternal stress during gestation.

Before the presentation of experiments conducted in this thesis, a brief overview of important themes will be presented: applicability of animal research to human psychological disorders, stress, the stress response, gender imbalance in stress and depression research, stress and depression, maternal factors in stress and depression research, the chronic mild stress (CMS) paradigm. An overview of the development of the five studies of the thesis will follow. These studies are presented in manuscript style with the content unchanged, save for formatting, from the published versions, each including the following order of sections: abstract, introduction, methods, results, discussion, and acknowledgments; figures and tables are embedded throughout the text in proximity to the appropriate content. A general discussion integrating findings from all studies, and a global summary and analysis follows.

Applicability of animal research to human psychological disorders

Neuropsychiatric disorders such as depression are a consequence of a myriad of intricately woven, diverse biological and environmental factors occurring throughout an entire lifetime. The examination of the role of individual component factors is impossible in human studies where the needed control of variables is unobtainable and unethical; therefore, animal models are critical and vital in furthering scientific understanding of such disorders.

Behavioural expressions are made through the integration of multiple types of information from both the nervous and endocrine systems; small disturbances at any level can yield significant behavioural modification. Animal behavioural responses to stress exposure are quite characteristic among individual species and are easily observable and quantifiable in a variety of tests. Similarly, the primary players in the stress response, the sympathetic adrenomedullary system and the hypothalamic pituitary adrenal (HPA) axis, are highly conserved across mammalian species and can thus be modeled in laboratory *in vitro* and *in vivo* models. And while there are important differences, for example corticosterone is the primary stress hormone in rodents while cortisol has this function in humans, the underlying mechanisms controlling the steroid hormones involved in the stress response are similar (Norris 1997).

Although depression is a highly subjective experience characterized by individual feelings (e.g. sadness, worthlessness, and guilt) that are not measureable in animals, objective measures related to the behaviour and physiology of other characteristic symptoms of depression such as anhedonia, sleep, cognition, and appetite are readily

observed in response to rodent models of depression. Anxiety, often comorbid with depression (Sartorious et al. 1996), is readily observed and quantified in rodents.

Consistency in the order of neural developmental events permits comparison between species with gross differences in lifespan: prenatal period, infancy, juvenile period, adolescence, puberty, and adulthood with reproductive capacity, followed by senescence allowing for the full range of life stages to be examined. For example, neural structure and connectivity of an infant at birth is comparable to the rodent brain at weaning (Bayer et al 1993). Furthermore, the quality and quantity of maternal care received by offspring in both species affects neuroendocrine and behavioural development (Mousseau, & Fox 1998, Reid 1994). Animal models are therefore informative and essential to understand how normal human processes can be affected by stress in such a way as to cause neuropsychiatric disease.

Stress defined

Despite decades of use in common language and scientific research, stress remains poorly characterized. It is often still defined as any stimulus that alters an organism's homeostasis as originally proposed by Seyle (1959); in preclinical laboratory rodent models involving stress, a recently proposed and highly useful description of stress is that it is a cognitive perception of a stimuli, expressed in both physiological and behavioural responses, of uncontrollability and/or unpredictability (Koolhaas et al. 2011). This view differs from the original definition of a stressor by focusing on the subjective, cognitive evaluative role the organism plays in deeming a stimulus as beyond prediction or control, and thus a true *threat* to homeostasis. The cognitive

appraisal of the situation as aversive and the corresponding behavioural response distinguish stimuli as stressors.

Highlighting the perceptive appraisal component is highly useful given that the primary players that together mount the physical stress response, the HPA axis and sympathetic adrenomedullary system, are activated during both appetitive and aversive stimuli to a similar degree (Koolhaas et al. 2011). For example, corticosterone levels (the primary glucocorticoid in the rodent stress response) of adult male Wistar rats were found to be the highest during mating among a range of stimuli including well-established rodent stressors: social defeat, forced swimming, restraint, and footshock (Koolhaas et al. 1997). This is not surprising given that the HPA axis is involved in the performance of natural behaviours, for example feeding (Krahn et al. 1986), reproduction (Schiml, & Rissman 2000), and maternal care (Rees et al. 2004) and fluctuates naturally in a diurnal rhythm in a species specific manner in addition to reacting to stimuli deemed stressful. It is important to remember that it is not exclusively involved in stress.

The hypothalamus contains sex hormone receptors and both mineralocorticoid receptors and glucocorticoids receptors; the relationship between glucocorticoids and sex steroids is responsible for many hypothalamic functions (Patchev et al. 1999). Neuroendocrine cell groups in this region are involved in regulating and maintaining homeostasis and coordinating the timing of physiological functions within specific environmental conditions through its role in growth, metabolism, stress response, reproduction, lactation, osmoregulation, learning and memory, and circadian rhythms. Such cell groups release neurohormones directly into the circulatory system via the

anterior pituitary that exert actions in target cells throughout the body. Each neuroendocrine system is composed of neurosecretory cells and corresponding pituitary hormone(s) which can be categorized into five general axes linked to distinct, often sexually dimorphic behaviours: pituitary-thyroid axis controlling metabolism; somatropic axis, growth; brain-pituitary-adrenal axis (HPA), stress; brain-pituitary-gonadal axis, reproduction; pituitary-lactotrophic axis, lactation and maternal behaviour. (Gore, & Roberts 2003) Maturation of the hypothalamus and many of its axes is not complete until puberty making these circuits vulnerable to variations in the neuroendocrine environment.

The stress response

The stress response is produced through HPA-stress axis activation in close conjunction with the sympathetic and parasympathetic systems. A series of feed-back and -forward mechanisms allows energy and resources to be mobilized and redistributed via a bi-directional relationship between the central nervous system and peripheral organs. This prepares an organism to mount an appropriate response to internal and external environmental demands via the modulation of metabolic and cardiovascular functions and, following threat, return to a homeostatic, resting state.

While magnitude and duration of neuroendocrine response may vary by sex, both males and females of all mammalian species display a similar basic neuroendocrine profile in response to an acute stressor: a rapid release of hormones from the hypothalamus, namely, corticotropin releasing factor (CRF). This release activates the adrenal medulla triggering the release of the catecholamines

norepinephrine and epinephrine which affects various target organs resulting in a sympathetic response, e.g., pupil dilation, inhibited salivation and digestive system, increased heart rate and sweating, stimulated glucose release. The HPA axis stress response involves the release of CRF and other hormones by the hypothalamus which stimulates anterior pituitary adrenocorticotropin hormone (ACTH) release and, in turn, ACTH causes the release of corticosteroids, particularly cortisol in humans and corticosterone in rats from the adrenal cortex. (Sapolsky, 1992 a,b; Jezova et al. 1995)

In situations of immediate threat and acute stress, activation of the HPA axis is adaptive and often necessary for survival. Normal, effective HPA response to stress involves rapid onset to mobilize the body by shutting down activities that would distract from dealing with the immediate threat; for example, cortisol increases the delivery of fuel to the muscles while CRF decreases appetite for appetitive stimuli such as food and sex while increasing alertness. Once danger has passed, rapid cessation of this response is essential to allow for normal physiological processes to occur. The termination of the neuroendocrine stress cascade occurs through rapid feedback responses initiated by circulating glucocorticoids that act to inhibit the continued release of CRH and ACTH via neural glucocorticoid receptors. In contrast, continued activation of the HPA axis or diminished ability to terminate the stress response is maladaptive and overtime leads to suppression of anabolic processes, decreased energy stores, suppression of the immune system and, it appears depression (Sapolsky 1992). Indeed many elements of depression bring to mind a stress-response gone awry: HPA hyperactivity, HPA insensitivity to corticosteroid feedback, hypersecretion of corticotropin-releasing factor, decreased sleep, appetite, and libido (Gold and Chrousos

1999; Nemeroff, & Vale 2005).

A female focus: addressing parity in stress and depression research

The female stress response has only quite recently become the focus of scientific study. There is a growing body of evidence suggesting that the standard 'fight-and-flight' theory of stress response (Cannon 1932), developed and established in male rodents over the past 80 years, does not adequately encompass the female experience. Despite similarities in basic neuroendocrine HPA and sympathetic responses to acute stress, 'fight-or-flight' responses are less likely to occur in females, particularly those pregnant and lactating - the primary focus of energy and resources of most mature female mammals of reproductive age (modern humans being the exception). Rather than fighting or fleeing, the 'tend-or-befriend' paradigm proposed by Taylor and colleagues (2000) posits that acute stress in females is characterized by behavioural expressions of nurturance, especially for young, and seeking social contact. This model therefore incorporates the critical role of social support well documented for psychological health in human literature and more recently, preclinical studies (e.g., Mastorici et al. 2009; Konkle et al. 2003; Belz et al. 2003; Haller et al. 1999).

The emergence of new models to explain the female stress response is a step towards parity in research involving stress and depression. Until very recently, research at all levels has focused almost exclusively on the male-typical, 'fight-or-flight', response with the assumption that it would translate to the female and avoid the *noise* associated with the reproductive cycle. Greuenewald and colleagues (1999) report that, prior to 1995, only 17 percent of participants in human lab studies of physiological and

neuroendocrine responses to stress were women; this value increased to 30 percent in 1999 with women being overrepresented in niche topics such as affiliative responses to stress and underrepresented in neuroendocrine studies. This limited focus on male responses in models of stress-related illness has resulted in a well-established literature on the characteristics of typical masculine stress response including numerous well-validated paradigms and measures. These often fail, however, to produce expected outcomes in females.

This gender disparity is surprising given the dramatic gender difference that exists in the clinical population; many reports suggest that the ratio of women to men diagnosed with depression is as high as 2 to 1 (Bhugra, & Mastrogianni 2004; Le et al. 2003; Kendler et al. 2000; Sprock, & Yoder, 1997; Kessler et al. 1993; Earls 1987; Nolen-Hoeksema 1987). The exclusion of female animals in depression research may be due to the belief that the gender imbalance is the result of social factors. However, sex differences in incidence rates are quite stable across cultures with higher levels of female depression being observed primarily during the reproductive years (Wade et al. 2002).

While social variables cannot be ignored, there are clearly gender-dependent biological factors involved in female sensitivity to depression. This is may be due largely to elevated/fluctuating levels of sex steroids, particularly estrogen, known to interact with the sympathetic nervous system and the HPA axis. High levels of estrogen in women compared to men (.05 mg/day in men vs .07-.6 mg/day in women) and during cycle phases characterized by high levels of estrogen – (proestrus in the rat and preceding ovulation in humans) results in greater activation of the HPA axis: increased

corticosterone and ACTH secretions both basally and in response to stress (Miller, & Sita 2000; McCormick et al. 1995) and impairs negative feedback (Burgess, & Handa 1992). The symptoms and severity of depression have been observed to vary with the menstrual cycle (Betha et al. 1999) perhaps explaining why the effectiveness of some antidepressants (most notably, SSRIs) is greater in women than in men (Joyce et al. 2003; Martenyi et al. 2001). Support for these effects comes from animals (Leuner et al. 2004). Female rats also show elevated basal and stress-induced corticosterone secretions (Saele et al. 2004). Further, sex differences have been noted in the locus coereleus norepinephrine system, known to be disrupted in depression, with females displaying greater CRF receptor sensitivity, and a diminished capacity to adapt to high levels of this neuropeptide (Bangasser et al. 2010).

Stress and depression

During the past decade it has become increasingly clear that conditions and events occurring in early development resulting in changes to the neuroendocrine system have implications for the long term health of individuals. This was originally known as the fetal origin of adult disease or developmental programming and proposed by Barker (2003) following his observation of a consistent link between low birth weight and increased rates of coronary heart disease in adulthood (Barker, & Osmond, 1986). This finding has since been replicated in a number of other diseases: hypertension, type II diabetes, cancer, and chronic lung disease (Barker 1995ab; Hales et al. 1991), for example. Low weight during year one has also been associated with adult suicide and depression after accounting for social status and early life nutrition (Barker et al. 1995).

Similarly, the neurodevelopmental hypothesis for neuropsychiatric disorders postulates that disturbances during brain maturation can lead to the activation of pathologic neural circuits later in life favouring the development of psychopathological symptoms during adolescence or adulthood; further, the double-hit hypothesis posits that emotional or physical stress in early life can act as a second insult by increasing susceptibility to psychiatric disorders in naturally predisposed individuals (reviewed in Marco, Macrì, & Laviola 2011).

The key feature of these theories is the long latency between exposure and apparent disease or dysfunction which may not occur until puberty or later in life, often decades later. Such theories find support in retrospective studies which have established a strong link between trauma before the onset of puberty and the development of depression in adulthood (e.g., Heim et al. 2004; 2003; Agid et al. 2000), and the seriousness of depression has been correlated with the severity of the early abuse experience (Mullen et al. 1996). Exposure to a major earthquake during gestation has been associated with an increase in the rate of severe depression from 5.5% to 13.3% (Watson et al. 1999).

The perinatal period is marked by increased neural plasticity, particularly in the HPA axis, and is sensitive to stress-induced disturbances of brain and behaviour. The early development of the mammalian nervous system comprises a series of sensitive periods or critical windows for normal anatomical, functional organization and behaviour (Michel, & Tyler 2005). Such processes are sensitive to small perturbations of sensorial, hormonal, and social environments. The effects of steroids on neuroanatomy and function during development are considered irreversible as they are organizational in

nature while effects in adults, activational and reversible (vom Saal 1983). Normal neural development requires a high degree of sensitivity to minute changes in hormone levels making it vulnerable to compounds that can interfere with steroid signalling, even at exceedingly low levels. Such disruption could have dramatic consequences on the organization of neural circuits and related behaviour. For example, the hypothalamus of the adult male rat is ‘masculinized’ neonatally by testicular testosterone which is aromatized to estradiol by the enzyme P450 (Gore, & Roberts 2003). In adulthood, normal male sex-type behaviour (mounting, intromission, and ejaculation) is elicited by circulating levels of testosterone. Neonatal castration eliminates male sexual behaviour even when testosterone is delivered in adulthood, whereas the reduced sexual behaviour observed in rats castrated as adults can be reinstated via testosterone replacement (Gore, & Roberts 2003). Early alterations in neuroendocrine levels can thus have permanent effects on structure, connectivity, and behaviour.

Preclinical research linking stress with depression typically addresses the onset of depression in face of a current challenge, but does not consider prior stress exposure. The importance of early life experience is becoming increasingly recognized. Childhood sexual abuse, unfortunately a common early stress in women, has been associated with long-term changes in the HPA stress axis and has been correlated with the development of anxiety and mood disorders later in life (Weiss, 1999). Recent evidence from human and animal studies suggests that the HPA axis may be affected during the earliest stages of development, in the womb. Increased activity of the maternal HPA axis, in humans, results in decreases foetal blood pressure and oxygenation through hormonal exchange between the mother and foetus (Rohde 1989;

Myers 1975). Persistent, long-term activation of the maternal stress system elevates her basal levels of circulating stress hormones which, in turn, increases release of stress-related hormones, catecholamines, cortisol, and β-endorphins, from the placenta (Petreglia 1996). This, coupled with poorer blood flow and oxygen content, is thought to sensitize the brain to stress, especially if it occurs during the development of the neonatal HPA axis in late gestation (Weinstock 2001). The womb environment may create a brain less tolerant to stress which may increase an individual's vulnerability to mood and anxiety disorders when confronted with stress later in life. This may be further exacerbated by the fluctuating hormonal environment found in adult females.

Certainly, the experience of stress does not generally lead to depression, but for those individuals with a genetic or phenotypic predisposition, the experience of stress puts them at high risk for depression. For example, in one community sample, 80% of depressed cases were preceded by major life events causing stress (Mazure 1998). This is a well-established research finding based on a variety of methodological designs and samples from childhood to old age and has been extensively reviewed (Hammen 2005; Liu, & Alloy 2010; Paykel 2003). This is true both for acute stress, particularly if it is rated as moderate to severe, as well as mild chronic stress (Kendler, Karkowsk, & Prescott 1998) with the latter being a stronger predictor of depression (McGonagle, & Kessler 1990).

Maternal factors require consideration in stress and depression research

As described, experiences in the perinatal environment shape neural development and related behaviour termed perinatal programing (Barker et al. 1999). Prenatal

experiences are almost exclusively mediated by maternal behaviour, nutrition, and neuroendocrine status and prepare offspring for the postnatal environment. Similarly, early postnatal life is marked by maternal mediation of environmental stimuli; this is particularly important for rodents in which significant neural development continues during the lactation period and appears to be highly influenced by maternal care behaviour (Lenz, & Sengelaub 2009). Such epigenetic mechanisms permanently alter gene expression without affecting the genetic code and are thought to mediate stable changes in neural function and behaviour (Darnaudéry, & Maccari 2008). Variability in neuroendocrine systems and resulting behaviour - particularly in responses to stress - expressed in adulthood are highly affected by natural variations in the maternal care that an animal receives in the early postnatal period (i.e. licking and grooming of offspring, nursing positions, and time spent in contact with pups) (Francis et al. 1999; Francis, & Meaney 1999). Increased maternal care has been shown to increase transcription of the glucocorticoid receptor gene involved in the HPA feedback loop as a result of changes in DNA methylation and chromatin structure (Meaney, & Szyf, 2005). Such differences can be found within the expressions of adequate maternal care that promote offspring survival and growth rather than an effect of severely neglectful nurturance (Champagne et al. 2007) and can be mediated by maternal diet (e.g. Trottier et al. 1998) and prenatal stress exposure (e.g. Baker et al. 2008).

Variability in the expression of maternal care behaviours are created by environmental factors (e.g. number of pups, sex ratio, nutritional status, and stress state), and individual factors, namely the heritable tendency to express high versus low levels of maternal care behaviours passed from mother to daughter (Francis et al.

1999). Maternal behaviour occurs in response to hormonal changes that occur during pregnancy and parturition and the early postnatal period and is maintained, postnatally, by interactions between offspring and dam (Fleming et al. 1999). Behaviour in the dam may therefore be modulated by stress via direct neuroendocrine system disruption or alterations in early offspring behaviour and plays a role in the development of stress responsiveness of offspring. Anxiety and coping behaviours under stress in adult rats have been show to depend on variations in the maternal care that animals received as pups (reviewed in Champagne et al. 2008; Levine, 2002; Francis et al. 1999). The effects of early life factors, especially stress, can thus be mediated by maternal behaviour.

Similarly, the critical role of quality maternal care can be observed in humans, and is highlighted by the effects of maternal depression in children. Maternal depression has been found to be the most potent predictor of children's cortisol in the first year of life and at 4.5 years of age (Essex et al. 2002). It is associated with a parenting style characterized by inconsistency, insensitivity, unresponsiveness, and rejection; both maternal depression and attachment style predict the development of insecure attachment in children (Carter et al. 2001; Coyl, Roggman, & Newland, 2002). In turn, insecure attachment in children is associated with the development of personality vulnerabilities to depression: dependency and self-criticism (reviewed in Zuroff et al., 2004).

The Chronic Mild Stress rodent model of depression

There are a variety of animal models of depression, most of which have been developed in rats. Such models do not attempt to recreate the entire human experience of depression in the species of interest. Rather, one behavioural aspect of depression is typically exploited, the goal being to manipulate that behaviour in a way that mirrors the human experience but, at the same time, is ecologically relevant to the species of study. Often, other behavioural and physical changes, beyond the initial target behaviour, occur in the same direction of those observed in the clinical population. These effects can provide further support for the usefulness of the specific model. The most common form of manipulation is through exposure to stress, so most animal models of depression can be considered ethological models based on the theory that depression is caused by exposure to environmental stress.

Since its introduction in the 1980's, the most widely used manipulation to induce behaviours and symptoms modelling depression in animals is the chronic mild stress (CMS) paradigm. As the name implies, rodents are subjected to a variety of mild stressors that are administered over a period of time, usually several weeks. This is an attempt to mimic the daily hassles that are often reported to contribute to the onset of a depressive episode in a vulnerable individual (Hammen 2005; Kessler 1997). This type of stress regime exploits the roles that unpredictability and uncontrollability both have on the impact of stress. The behavioural target of this model has traditionally been a measure indicating the hedonic value of a rewarding stimulus. The preferred consumption of a low concentration sucrose solution is the most common measure of hedonia (Willner et al. 1987). The reduced preference, compared to a control condition

is intended to mimic, in rats, the subjective, qualitative human experience of anhedonia, the core feature of the melancholic subtype of major depressive disorder (APA 1994).

In addition to anhedonia, CMS has been found to induce a variety of other changes that resemble common symptoms of human depression (mainly in male rats) and is considered the model that most closely resembles the human condition (McArthur, & Borsini 2006). CMS induced changes have been reported in sleep architecture (eg. Grønli et al. 2004; Moreau et al. 1995), cardiovascular function (Grippo et al. 2004; 2003; 2002), social behaviour (eg. Ossowska et al. 2004), cognition (eg., Wood et al. 2001), sexual and exploratory behaviour (Brotto et al. 2001; D`Aquila, Brain, & Willner 1994), immune responsiveness (Kubera et al. 2001; 1995; Azpiroz et al. 1999; Anisman, & Zacharko 1992), and corticosterone release (Konkle et al. 2003; Bielajew et al. 2002; Ayensu et al. 1995). These effects, in addition to the administration of CMS as the agent of behavioural change, provide strong face validity for the model, at least in male rats. Furthermore, chronic administration of antidepressant pharmacotherapy has been shown to reverse a variety of these effects (e.g.: Barlow et al. 2004-sucrose preference; Ossowska et al. 2004-social behaviour; Brotto et al. 2001-male sexual behaviour). This lends predictive validity to the model (Willner 2005).

Reliability has been problematic in that some laboratories, including our own (Konkle et al. 2003), have had difficulty generating the expected behavioural change after CMS exposure (Neilsen et al. 2000; Murison, & Hansen 2001; Matthews et al. 1995). Reasons cited for this include strain variability, a lack of consistency across laboratories in the type and length of application of stressors employed, the sensitivity of the behavioural marker, or some combination of all factors. Notwithstanding the

challenges associate with this model, the wealth of positive results (which may be overestimated due to the relative absence of published negative findings), ethological validity, and the ability for CMS to induce other changes in line with a depressive profile suggest its worthiness as a model. Further, it is a highly adaptable and useful base to develop a paradigm for the investigation of female sensitivity to depression.

In summary, understanding the nature of stress susceptibility stemming from experiences in the womb and environmental variables occurring over the course of the female life cycle is critical in understanding the possibility of transmission of stress system dysfunction from mother to female offspring. Furthermore, characterizing the female stress response may help identify the cause of female vulnerability to depression which, in turn, may be important for understanding this devastating disorder.

Project development

The goal of the thesis research is twofold: to establish a CMS-based model of depression in female rats and to investigate the interaction between CMS exposure and maternal gestational stress. A table containing the components of each of the five studies is presented below (Table 1).

Table 1. Summary of experiments.

Study	Rats	Housing	Stress Paradigm	Measures
1	• S-D & LE • Adult • Female	• Individual	Original CMS paradigm	• Sucrose test • Brain stimulation reward (BSR) • Weight • Estrous cycle
2	• LE • Adult • Female	• Individual • Paired • Enrichment Objects	Female-specific CMS paradigm	• Sucrose test *modified* • Social Interaction • Weight • Estrous cycle
3	<u>Dams</u> • LE • Adult • Female <u>Offspring</u> • LE • Juvenile • Male & Female	• Family groups to weaning • Same sex sibling groups post weaning • Nestlets® and Tubes	Gestational stress	<u>Dams</u> • Weight • EPM • Maternal care <u>Offspring</u> • Weight • EPM • Emergence • T-maze
4	• LE • Early adulthood • Male & Female *Study3 Offspring*	• Same sex sibling groups • Nestlets® and Tubes	*None*	• Weight • EPM • Emergence • T-maze • Estrous cycle
5	• LE • Mid adulthood • Female *Study3&4 Offspring*	• Same sex sibling groups • Nestlets® and tubes	Female-specific CMS model	• Sucrose test *modified* • Weight • Estrous cycle • EPM

S-D: Sprague-Dawley ; LE: Long Evans

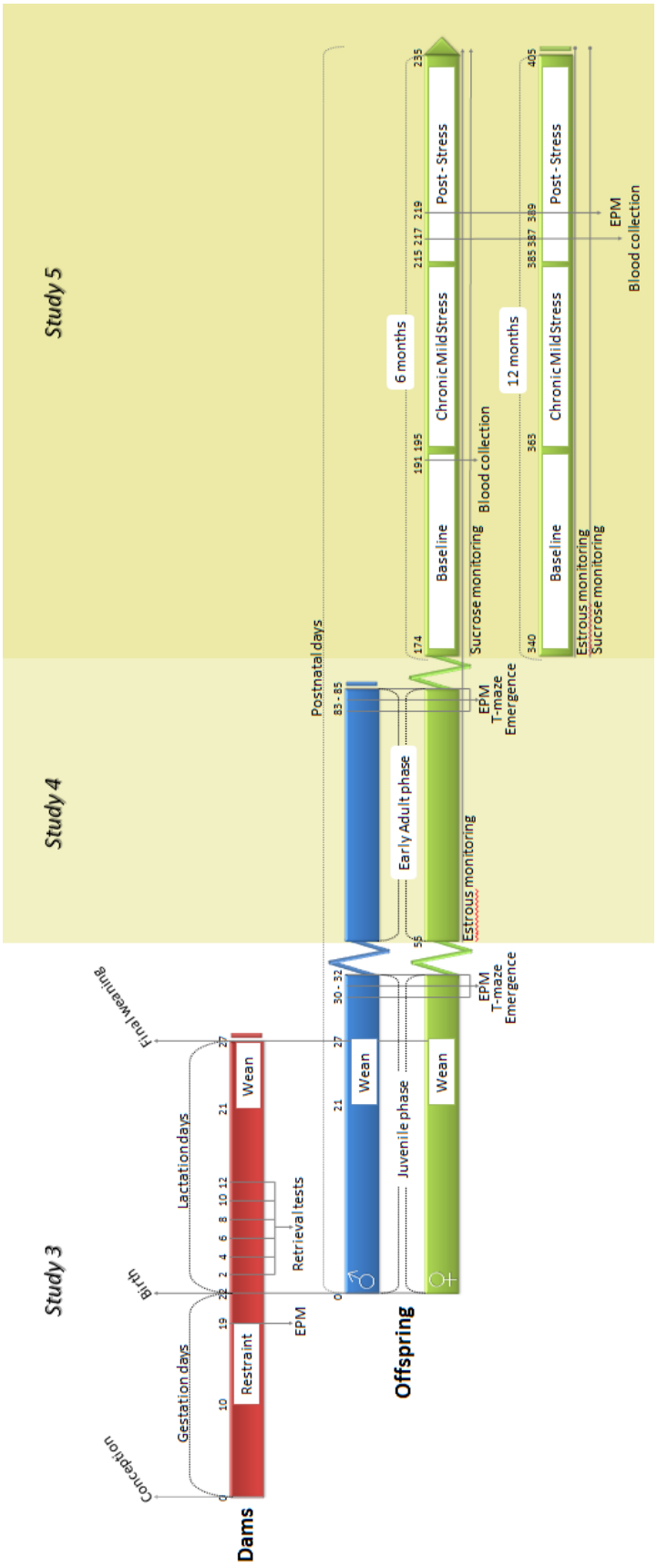
Studies 1 and 2 focused on the development of a female-appropriate CMS model based on a male model that was previously employed with limited success in our laboratory (Konkle et al. 2003). The original CMS procedure was applied in Study 1, and two measures of anhedonia (sucrose preference and intracranial self-stimulation) were examined in two strains of female rats (Long Evans and Sprague-Dawley). Food deprivation and restriction as well as reversing the light cycle were not included in the

revised CMS procedure that was used in Study 2; these were thought to interfere with the regularity of the estrous cycle for physiological reasons beyond the effects of stress. Exposure to stroboscopic light and white noise were added to the CMS regime in Study 2. Long Evans rats were employed exclusively in studies 2-5 based on the general observation that they were more reactive to handling than Sprague-Dawley rats, displayed higher disruption of the estrous cycle in Study 1 (data not shown) and previously by this lab (Konkle et al. 2003), and show high levels of maternal care behaviour (Moore et al. 1997).

Given the mild effect obtained in the sucrose data in Study 1, a modified method of sucrose monitoring was employed in Study 2 with the expectation that it would be more sensitive to the hedonic status of the animals. Social behaviour was added as an additional measure, and intracranial self-stimulation was removed. There was a strong focus on the effects of housing in the second study. Standard housing may be a form of stress, particularly for female rats, and therefore not an appropriate control group. Two housing groups were therefore employed - single and paired. Furthering the attempt to limit stress due to standard husbandry practices, enrichment objects were provided in the cages of all rats which permit the rats to express a more naturalistic repertoire of behaviours than possible under typical laboratory housing practices.

The remaining three experiments – Study 3, Study 4, and Study 5 – are part of a long term project designed to investigate the effect of maternal stress exposure during gestation on measures of offspring parameters and female sensitivity to the modified CMS regime developed in the earlier studies. The timeline of this long-term project is outlined in Figure 1 presented below.

Figure. 1. Timeline for studies 3, 4, & 5



Studies 3 and 4 were based on one group of mothers that were stressed during gestation and a subset of their male and female offspring. The primary focus of these projects was to track the effects of stress during gestation on progeny. Offspring were observed at two distinct time points: juvenile phase (Study 3) and early adult phase (Study 4). In studies 3, 4, and 5, there was a focus on anxiety measures as it is often comorbid with depression (Breslau et al. 1995) and a common precursor to the onset of an episode of depression (Paul 1988). Anxiety was also assessed in the mother and her maternal behaviour was monitored (Study 3). Tests of cognitive function were included in Study 3 and Study 4 to address the impairment often observed in depressed patients, and the link between gestational stress, maternal care, and cognitive outcomes. Additionally, Studies 3 and 4 allowed for the comparison of gender on these measures.

In Study 5, the CMS paradigm developed in Study 1 and Study 2 was applied to the female offspring used in studies 3 and 4. Only female offspring were included in this final experiment. This decision was based on the observation of sex differences in the literature where by exposure to moderate to mild restraint regimes during pregnancy (particularly late in gestation) elicits the expression of anxiogenic and depressive like profile and HPA axis alterations in females to a greater extent than in males (Zagron, & Weinstock 2006; McCormick et al. 1995; Weinstock et al. 1992; Alonso et al. 1991). The goal here was to study a group of female rats in which a complete stress profile, beginning with conception, was known. It was our belief that knowledge of the stress history of each animal would provide superior predictive ability of the model to induce behavioural and physical changes akin to human depression.

Study 1

Behavioural and physiological effects of chronic mild stress in female rats

Baker, Kentner, Konkle, Barbagallo, & Bielajew.
2006. Physiology & Behavior, 87, 314 – 322.

Abstract

Anhedonia, a core symptom of clinical depression, refers to the loss of interest in normally rewarding stimuli; the chronic mild stress paradigm, an animal model of depression, was designed with this as an underlying feature. The procedure consists of the administration of a variety of ecologically relevant stressors over long durations. Its effects have been thoroughly investigated in male but not female rats. This study examines the appropriateness of stressors designed to evaluate the development and progression of depression in two strains of female rats, the effectiveness of two measures of anhedonia, and the relationship between stress reactivity and the estrous cycle. Changes in hedonic status were indexed for three weeks following a three week baseline period using two standard behavioural measures of anhedonia: sucrose intake and preference and thresholds for brain stimulation reward. Decreases in 24 h sucrose intake were observed in both strains during the first week of stress manipulations, and continued to decline thereafter for the remainder of the stress phase; in contrast, sucrose preference was unaffected by the stressors, indicating an overall reduction in fluid intake. No changes in the thresholds for brain stimulation reward were observed. The cyclical pattern of estrous was altered in both strains with a significant reduction in the number of regular cycles as a consequence of both the stressors and brain stimulation reward. Furthermore, cyclicity was not reinstated in many animals even six weeks after stress manipulations and behavioural tests had ceased. While the physiological measures suggest that the mild stressors are disruptive to female rats, the results of the behavioural tests are not consistent with the notion that the stressors induce an anhedonic state.

Keywords: Chronic mild stress; Estrous cycle; Female rats; Brain stimulation reward; Sucrose intake and preference; Long Evans; Sprague–Dawley

Introduction

Depression is a debilitating disorder targeting women twice as often as men (American Psychiatric Association [DSM IV] 1994). In the female population, its incidence increases significantly between menses and menopause, declining rapidly thereafter (Wade, Cairney, Pevalin 2002). Such findings suggest that hormone-related differences may be at the root of some psychiatric problems such as depression. It is known that gonadal hormones communicate bidirectionally with neurotransmitter systems that are implicated in depression. Depleted estrogen levels have been associated with clinical depression, and estrogen has been successfully used as a treatment for depression in women (Flink et al. 1996).

In spite of this literature, animal studies designed to model depression have relied almost exclusively on data from male subjects, in order to avoid the potential confound of hormonal fluctuations on the measure of interest. However, given the pattern and occurrence of this disorder in women, the influence of hormonal activity on depressive symptoms begs examination.

In animal models, the CMS paradigm was developed with anhedonia, defined as the loss of interest in normally rewarding stimuli, as the underlying feature (Willner et al. 1992). While there has been some debate regarding the core features of depression (Auriacombe, Reneric, LeMoal 1997), anhedonia is generally accepted as belonging to the spectrum of depressive symptomatology. The CMS procedure uses the administration of unpredictable, mild stressors that are designed to mimic the daily hassles reported to contribute to the onset of depression in some humans (Willner 1997). Paralleling the symptoms of clinical depression, exposure to CMS induces

several physiological disruptions in animals, some of which include reduced REM sleep latency and increased REM sleep (Morreau et al. 1995; Grønli et al. 2004), cardiovascular changes (Grippo, Moffitt, Johnson 2002; Grippo, Beltz, Johnson 2003; Grippo et al. 2004), diminished sexual and exploratory behavior (D'Aquila, Brain, Willner 1994), overactive immune responsiveness (Azpiroz et al. 1999; Kubera et al. 2001; Silbermanh et al. 2004), and increased activity in corticosterone hypersecretion (Ayensu et al. 1995; Konkle et al. 2003; Bielajew, Konkle, Merali 2002), all of which provide face validity for the model.

It has been demonstrated that stress alters responsiveness to rewarding stimuli in male rodents (Katz 1982; Willner et al. 1991; Willner et al. 1991; Zacharko, Bowers, Prince 1983). The intake or preference of a mildly sweet sucrose solution has been the most extensively used behavioural measure to gauge the anhedonic effects of CMS e.g. (Papp, & Moryl 1996; Pucilowski et al. 1993; Willner et al. 1987; Willner, Muscat, Papp 1992). Generally, these studies demonstrate that sucrose consumption decreases after exposure to CMS, interpreted as an indication of anhedonia (Benelli et al. 1999; Cheeta, Broekkamp, Willner, 1994; Willner 1997a,b).

Brain stimulation reward (BSR) is a well known paradigm that has been employed since the early 1950s as a model for studying the neurobiology of motivation (Olds, & Milner 1954). In the context of the CMS procedure, it has been shown in some laboratories that thresholds for BSR are increased in male rats following administration of stressors, data interpreted as evidence for anhedonia (Moreau et al. 1992; Moreau et al. 1993; Moreau et al. 1994; Moreau et al. 1995; Moreau et al. 1996). Others, including ourselves, however report little or no threshold change following CMS exposure (Lin et

al. 2002; Nielsen, Arnt, Sànchez 2000; Konkle et al. 2003). One group (Nielsen, Arnt, Sànchez 2000) has demonstrated decreases in sucrose intake in male PVG hooded rats but not in Wistar rats, while neither strain showed any overall increase in BSR thresholds. However, individual differences were observed in that two of the 14 rats receiving CMS elicited an increase in BSR thresholds two to four weeks into the stress schedule, suggestive of differences in susceptibility to stressors; this phenomenon, the idea that certain individuals are more sensitive to the effects of stressors, has been addressed in the clinical literature (see Anisman, & Zarcharko 1992 for review).

Notwithstanding the failure to reliably document alterations in the thresholds for rewarding brain stimulation, we do consistently observe the appropriate metabolic, biochemical, and endocrine responses to the stressors (Bielajew, Konkle, Merali 2002; Konkle et al. 2003). It may be that the measure and/or stressors are not selective for anhedonia. All previous work, including our own, has evaluated sucrose and BSR measures in separate groups of animals. In this study, we assessed both sucrose intake and preference as well as BSR thresholds in the same animals in order to determine if changes in sucrose consumption or preference were paralleled by comparable changes in BSR thresholds. The expectation was that individual animals demonstrating stress sensitivity, as shown by a reduction in sucrose intake and preference, should also show corresponding fluctuations in BSR thresholds. To mirror the experimental conditions of our earlier work (Bielajew, Konkle, Merali 2002; Konkle et al. 2003), both Sprague–Dawley (n =10) and Long Evans (n =9) strains were used in this study. In order to explore the relationship between stress reaction and hormonal status, the estrous cycle was tracked before, during, and after the administration of the stressors. Because we

have observed in our laboratory alterations in the estrous cycle due to BSR alone, an additional four groups of Long Evans and Sprague–Dawley rats were added in order to distinguish between the effects of stress and rewarding brain stimulation on the estrous cycle. These included the following — 1: a group that received no manipulation and sucrose tests (n =10), 2: no manipulation and BSR tests (n =14), 3: CMS and sucrose tests (n =9), and 4: CMS and BSR tests (n =14).

Methods

Animals and surgery

Ethical approval for this project was obtained by the Animal Care Committee's Protocol Review Group of the University of Ottawa.

Sixty-six animals (42 Long Evans and 24 Sprague–Dawley) were singly housed and maintained on a 12 h light /12 h dark cycle with food and water available at all times unless otherwise specified in the CMS procedure. All rats were weighed once weekly. Forty-seven female rats (23 Long Evans and 24 Sprague–Dawley), weighing between 242 and 329 g (mean weight of 307 g) at the time of surgery, were implanted with a bilateral pair of electrodes aimed at the ventral tegmental area (VTA) using standard stereotaxic techniques. Each animal received a subcutaneous injection of atropine sulfate (0.05 ml), in order to reduce respiratory distress, followed by the continuous administration of the inhalant anaesthetic, halothane. The VTA coordinates were 4.8 mm posterior to bregma, 0.7 mm lateral to the midsagittal suture, and 8.0 mm below the skull surface (Paxinos, & Watson 1998). The electrodes, (Plastics 1 Inc.), were constructed from stainless steel wire, 250 µm in diameter, and insulated with polyimide

to the rounded tip. The current return consisted of a pliable stainless steel wire wrapped around four stainless steel skull screws. Dental acrylic was applied around the base of the crown in order to secure the electrode assembly to the skull.

Chronic mild stress procedure

This procedure was adapted from Moreau et al. (Moreau et al. 1996) to accommodate both behavioural tests. The stressors used during the day included the following: confinement in a standard mouse cage during which standard kitchen timers would emit short bursts (approximately 2 s) of loud noise approximately every 10 min throughout the hour (8 h/week); exposure to an empty water bottle following a period of 21 h of water deprivation (1 h/week); one half hour restricted access to food in the form of 10 Noyes pellets (.2 g/pellet) following 22 h of food deprivation (30mins/week).

Stressors were also applied overnight; these began at 4 PM and ended at 8 AM. Animals were paired with an unfamiliar rat and placed in one half of a clean rat cage containing warm wet bedding (which cooled to room temperature over time); a Plexiglas barrier with holes separated the pair so that physical contact was prevented (16 h/week). Water was removed for a total of 16 h/week. Both food and water were removed before the day of the sucrose test (22 and 21 h, respectively/week). Home cages were tilted horizontally at 30° and the room was illuminated overnight (16 h/week). During the weekend, the light / dark cycle was reversed so that the room was illuminated Friday, Saturday, and Sunday nights (7 PM–7 AM) and lights were off during the day on Saturday and Sunday (7 AM–7 PM) (64 h total/week). Please refer to Table 2 for the complete schedule of stressors.

Following the collection of baseline data, the CMS procedure was applied for three weeks. During this period, both behavioural measures were collected as shown in the table.

Table 2. Schedule of stressors and behavioural tests.

Time	Monday	Tuesday	Wednesday	Thursday	Friday
8:00 AM	Confine 1 h	BSR test	Empty bottle exposure 1 h	Confine 1 h	BSR test
9:00 AM			Return water		
Noon		Confine 1 h	Confine 1 h	Confine 1 h	Confine 1 h
1:00 PM				2 bottle sucrose test begins	Measure 24 h consumption Return water
2:00 PM	Confine 1 h	Confine 1 h		Measure 1 h consumption Offer limited food	
2:30 PM			Weigh animals	Return food ad lib	
4:00 PM	Overnight pairing in wet bedding	Overnight water deprivation	Remove food and water	Overnight illumination 30- cage tilt	Reversed light / dark cycle 64 h

Sucrose intake and preference

Rats were initially exposed to a one percent sucrose solution for 48 h in order to acclimatize to the test procedure. They were then presented with two bottles, one filled with water and one with the sucrose solution. Intake and preference data were collected five times over two weeks before baseline tests began, as a stabilization measure. Before each test, animals were deprived of food and water for 20 h; tests were administered at about midday. After one hour of exposure to the two bottles, intake was recorded and then food returned. Twenty-three hours later, intake was recorded again,

and test bottles removed and replaced with regular water bottles. Preference for sucrose, determined by the ratio of sucrose solution to total fluid intake, was calculated for both the one and 24 h measures and converted to a percent score.

Brain-stimulation reward

Following a one week recovery period from surgery, screening for BSR was conducted in a wood and Plexiglas box with dimensions 27 cm deep x 37 cm wide x 51 cm high. All animals were trained to press a lever for brain stimulation using conventional shaping procedures. Stimulation consisted of 300 or 500 ms trains of square-wave monophasic cathodal pulses, 0.1 ms in duration. Stimulation was provided by constant-current amplifiers (Mundle 1980) and custom built pulse generators. The 300 ms train was used in some animals in order to reduce stimulation-induced motor artefacts. The lowest current and frequency of pulses to elicit responding at a rate of 30 lever presses/min were determined for each rat. Stimulation parameters were continuously observed on an oscilloscope by reading the voltage drop across a 1 kΩ precision resistor in series with the rat.

Following training, stabilization of the threshold procedure began, employing the method of limits. The current was held constant and stimulation frequencies delivered in a descending sequence, beginning with a value that elicited maximum responding, and reduced in equal logarithmic 10 steps (0.1log10 between adjacent values) each 60 s trial, until a frequency value that yielded less than 10 responses per minute (example of a typical sequence — 50, 40, 32, 25, 20 Hz). The frequency threshold was interpolated from the rate-frequency function and defined as the value that supported half the

maximum response rate. Four rate-frequency thresholds were collected each session; the first was considered a warm-up and discarded. The average threshold per session was based on the three remaining rate-frequency functions. Across rats, the current ranged from 250 to 630 μA. Animals were considered stable when the average frequency threshold did not vary by more than 0.1 log 10 units over three consecutive test days. After stabilization, thresholds were collected twice weekly for six weeks, the first three corresponding to the baseline phase and the last three, the CMS phase.

Estrous cycle

Throughout the study, vaginal swabs were collected each weekday morning in all groups in order to track the estrous cycle during baseline, CMS, and post-CMS phases with one exception. In groups 2 and 4 (BSR without sucrose tests), the estrous cycle was monitored only occasionally in the post-CMS phase to insure that the normal cycle rhythm was not re-established. From our experience, even a few BSR sessions appears to permanently deregulate the cycle pattern.

Histology

After completion of the experiment, all rats were given a lethal dose of sodium pentobarbital. Animals were then perfused intracardially with 0.9% saline followed by a formalin solution containing 10% sucrose. The brains were removed and stored in the formalin/sucrose solution at 6°C until processed. Brains sections were mounted on slides and treated with cresyl violet. The Paxinos and Watson atlas (1998) was used as a guide to locate the electrode tips.

Statistical analysis

Body weight, sucrose intake, and sucrose preference data were analysed via a mixed ANOVA design with two levels of the independent factor, strain, and six levels of the repeated factor time. The Hundt–Felt correction for violations to the assumption of sphericity (Howell 2002) was applied where appropriate. Thus, the degrees of freedom reported in the Results section are adjusted if the correction was used. Significant interactions were further dissected by means of paired t tests with Bonferroni modification of critical alpha level. Data pertaining to BSR–maximum rates, frequency thresholds, and charge values–were analysed with a mixed ANOVA design with one independent factor, strain, and 12 levels of the repeated factor time. All data were analysed using the SPSS (V.12) software package (SPSS 2004). In order to account for the differences in current and train duration across animals, charge was calculated using the formula: $Q = I\,N\,d$ [21] where Q is the charge in microcoulombs, I is the current in microamperes, N is the number of pulses in the train, and d is the pulse duration in seconds.

Results

All electrode tips were located in the vicinity of the intended coordinates, in or around the ventral tegmental area. The data associated with one animal were removed from the following analysis due to unstable sucrose intake scores during the baseline phase. The top of Figure 2 shows the 24 h sucrose intake (left) and preference (right), collected over the six weeks of the study (three weeks baseline and three weeks CMS) in each strain. Note that the functions describing the 1 h data are not shown; neither the 1 h

intake nor 1 h preference results were significant. Statistical analyses of the 24 h data revealed no effects of CMS on sucrose preference; however, the time and interaction terms were significant in the case of intake (time— $F(4, 61) = 9.169$, $p = .0001$; time x strain—$F(4, 61) = 2.88$, $p = .048$). Post hoc tests showed significant pair-wise differences between week 3 (last baseline week) and weeks 5 and 6 (CMS weeks) in both strains (week 3 vs. week 5, $t(8) = 3.219$, $p = .012$ for Sprague–Dawley and $t(8) = 3.091$, $p = .015$ for Long Evans; week 3 vs. week 6, $t(8) = 5.159$, $p = .001$ for Sprague–Dawley and $t(8) = 3.221$, $p = .012$ for Long Evans). Note that statistical analyses were performed on adjusted error terms as required in a repeated measures design; therefore plotted error bars are much larger, not reflecting the removal of between subjects error.

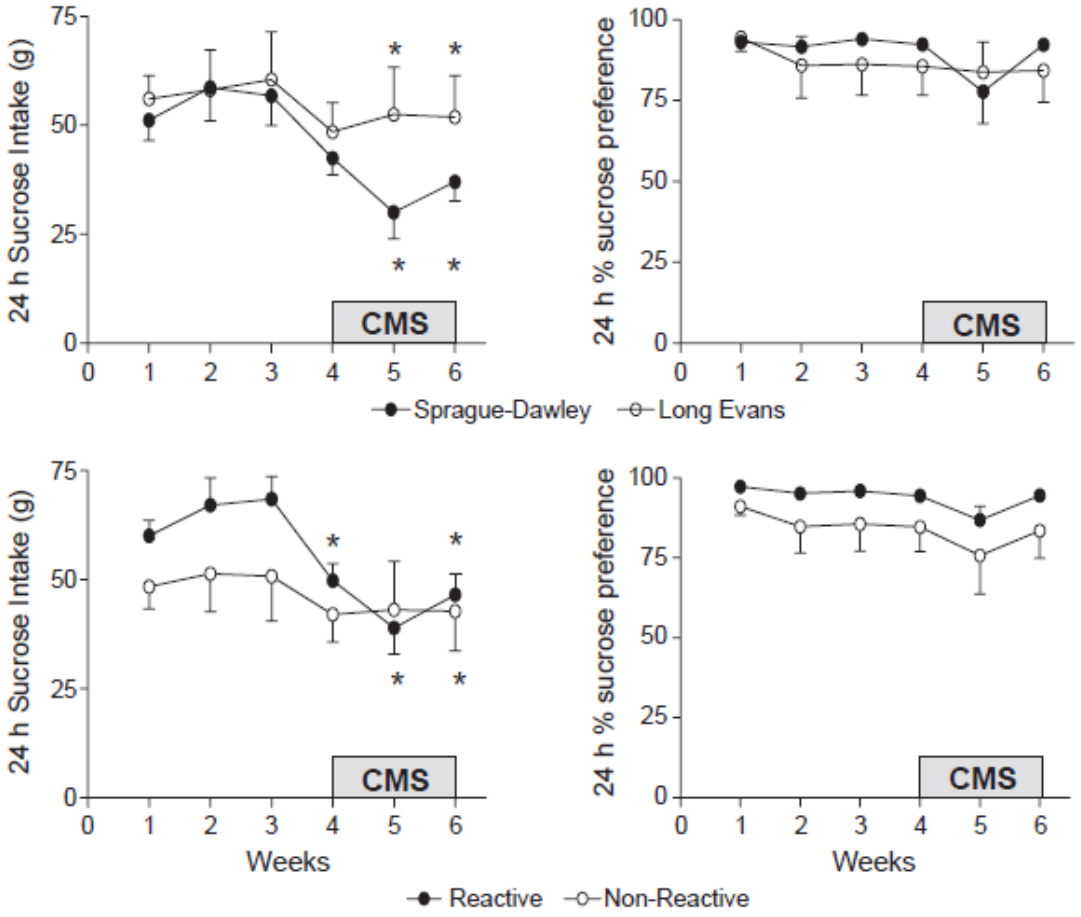


Figure. 2. *Sucrose intake and preference.*
Graphs plot 24 sucrose intake (left panels) and sucrose preference (right panels) for three weeks before and three weeks during CMS exposure. The top half of the figure shows these data for each strain— Sprague–Dawley (filled circles) and Long Evans (unfilled circles). The bottom half shows the same data rearranged as a function of reactivity, ignoring strain. Filled circles represent the reactive group and unfilled circles, the non-reactive group.

Because we were also interested in examining individual responses, we divided the rats into ‘‘reactive’’ and ‘‘nonreactive’’ groups, based on their 24 h intake values. Animals with significant differences between the mean values obtained during the three week baseline and three week CMS phases were categorized as reactive. On this basis, 44% of the animals met the criterion for reactive. Intake and preference for

sucrose in the 24 h test were plotted as a function of reactivity—the data are shown in Figure 2, intake (left) and preference (right). An ANOVA conducted on these groups gave rise to significant time and time x group effects (time — $F(4, 58) = 9.95$, $p = .001$; time x group—$F(4, 58) = 2.57$, $p = .052$. Organizing the preference data in this manner (reactive vs. non-reactive groups) did not yield significant results.

Because the amount of training needed to acquire lever pressing and stabilize BSR thresholds is quite variable across animals, we redid the analysis examining sucrose intake as a function of reactivity, but this time adding ‘‘total number of BSR sessions’’ as a covariate. The result was a slight increase in the probability associated with the interaction effect — from 0.052 to 0.095 (a decrease in power from .662 to .574).

None of the BSR related measures—maximum response rates, frequency thresholds, or charge values were significantly altered as a consequence of the stressors. Data collected during baseline and CMS weeks had similar values with little variability across animals. Unlike the sucrose results, analysis of individual BSR data provided no basis on which to distinguish the groups in terms of animals that showed reactivity to stress (as indexed by BSR) and those that did not as no rat displayed a change in BSR threshold. Furthermore, comparing the BSR responses of rats designated as reactive or non-reactive on the basis of their sucrose data yielded no significant difference. Plots of the maximum response rates, frequency thresholds, and charge values as a function of week are shown in Figure 3.

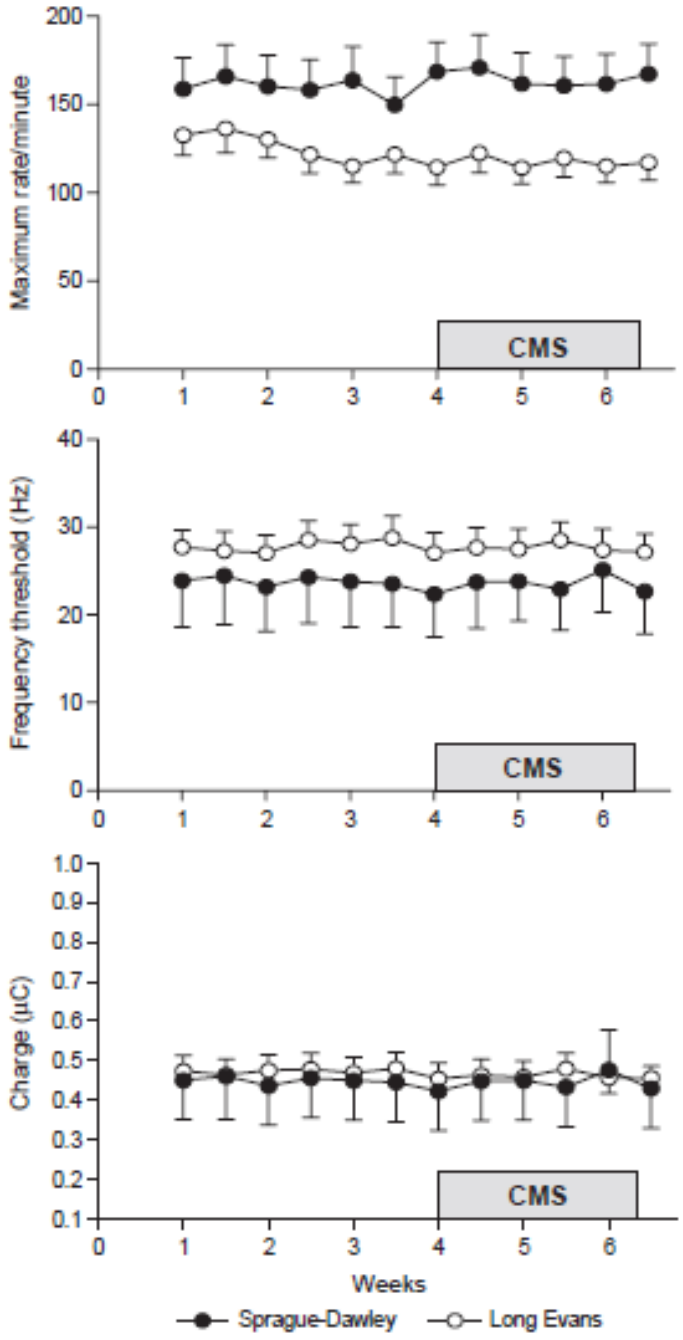


Figure. 3. *BSR.*
Measures collected in BSR trials are shown for each strain. Top graph depicts the mean maximum rate of response per minute, middle, the mean frequency threshold (Hz), and bottom, the mean charge value (µC) before and during the CMS phase in each strain. Data associated with Sprague–Dawley rats are represented by filled circles, and Long Evans rats, unfilled circles.

Examination of body weight revealed no significant difference between strains with respect to initial weight or weight on subsequent weeks. The weight gain over the course of the study accounted for the main effect of time $F(2, 34) = 9.144$; $p = .001$. An additional analysis was conducted on the weight data which included (1) the control group that received no manipulation, (2) the group that underwent CMS exposure and sucrose tests alone, and (3) the primary group in this study — rats that were stressed and evaluated for both sucrose intake and preference and BSR thresholds. Only a significant group difference was obtained due to a consistently higher weight gain in control rats versus the two CMS groups ($F(2, 34)=4.658$, $p = .016$). This amounted to a roughly 5% gain in the control group, measured from the final baseline to the final CMS week, and 2% in the CMS groups over the same period.

Figure 4 shows the percentage of normal cycles observed during the final baseline week, CMS phase, and post-CMS phase, as identified by daily vaginal swabs. Note that although estrous cycle was not formally monitored in the post-CMS period for rats receiving BSR tests alone (bottom graph), the animals in this group were periodically swabbed and found to be acyclic weeks after the cessation of behavioural tests.

In this figure, for purposes of presentation, estrous data pertaining to the different strains were consolidated, as no between strain differences in the number of cycles were observed (chi-square tests). Note that animals receiving BSR trials showed a rapid decline in the number of cycles during the baseline phase. This occurred within the first week of the stabilization period and approached asymptotic values even before baseline tests began. The data associated with the groups shown in the middle and bottom

graphs allow us to distinguish between BSR and stress related changes in estrous cycle frequency. Control animals not exposed to stressors or BSR tests maintain relatively stable frequency rates (see unfilled bars in center graph), while the percentage of cycles is reduced in animals exposed to BSR, whether the group received CMS or not (bottom graph). The frequency of estrous cycles in stressed animals administered sucrose tests alone, progressively declined during the CMS phase, and showed a gradual reinstatement during the post-CMS phase (filled bars —center graph). The majority of animals that displayed irregular cycles in this study tended to become stalled in the estrus and diestrus phases of the cycle in the case of the BSR groups while other stressed animals lingered mostly in diestrus.

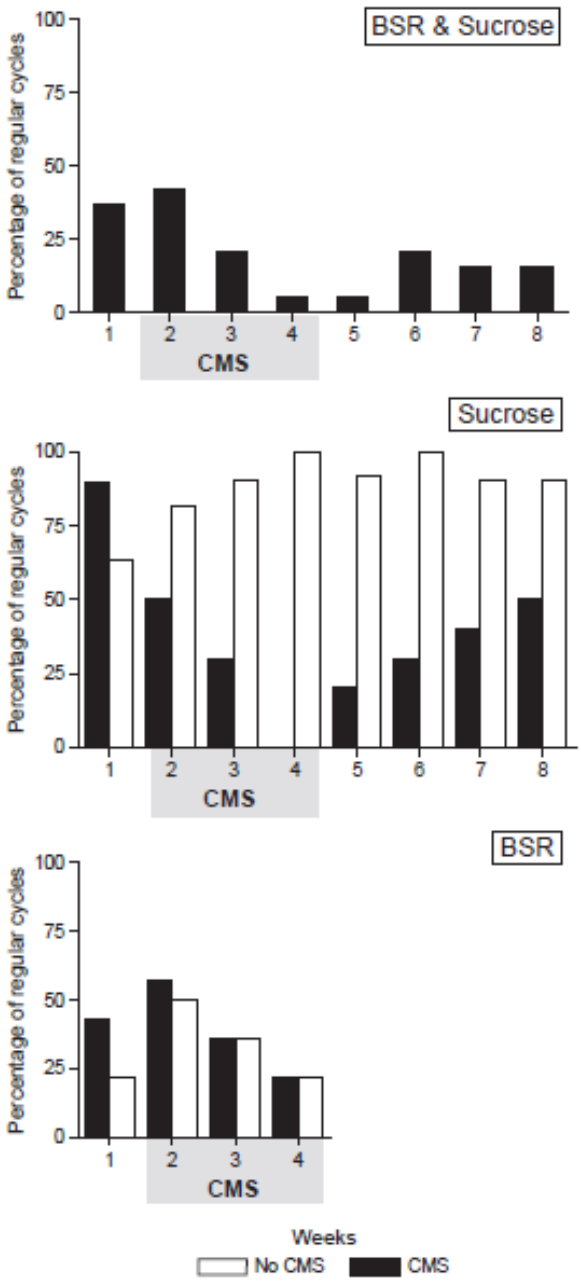


Figure. 4. *Estrous cycle.*
Plots show percent frequency of estrous cycles in all groups (collapsed across strains) over the course of the study. Three separate conditions are displayed. Top graph represents the group that received CMS exposure and both sucrose and BSR tests, the middle graph, sucrose test only, and the bottom graph, BSR tests only. Filled bars refer to animals that were exposed to the stressors, and unfilled bars, control animals that received no stressors.

Discussion

The purpose this work was to evaluate the effects of chronic mild stressors on the development of anhedonia in two female rat strains, as interpreted from behavioural (sucrose and BSR) and physiological indices (estrous cycle, frequency, and weight). Willner et al. (1987) set the precedent by observing in rats a reduction in one hour intake and preference for a mild sucrose solution, following exposure to CMS, arguing that the result reflected a hedonic deficit. Since then, some groups have reported reliable CMS-induced decreases in sucrose intake and preference (Dunčko et al. 2001; Grippo, Moffitt, Johnson 2002; Grippo, Belz, Johnson 2003) while others have observed negligible changes in these measures (D'Aquila, Newton, Willner 1997; Konkle et al. 2003; Matthews, Forbes, Reid 1995). Some of the variability in the response has been attributed to methodological differences between investigators in the choice of stressors and the application of the measurement procedures, making comparisons between laboratories quite arduous.

Dunčko et al. (2001) have found sex differences with respect to stress induced behaviours. While both male and female rats showed a reduction in preference for a 1% sucrose solution, other stress indicators revealed some differences between the two groups. Males demonstrated both a significant reduction in weight gain, independent of baseline weights, and more ‘‘anxious’’ behaviours in the open field test; females were relatively unaffected according to these markers. We have reported a similar pattern, that is, a slower rate of weight gain following six weeks of CMS exposure in male rats (Sprague–Dawley and Long Evans); the difference between control and stressed groups was much less pronounced in the female rats (Konkle et al. 2003). However, in

our hands, CMS did not alter intake or preference for a mild sucrose solution in the one hour test.

In this study, only the 24 h sucrose intake data gave rise to decreased intake, significant in the case of Sprague–Dawley animals, and timed with the introduction of the stressors. A corresponding preference decrease was also observed in this strain in the middle (week 5) of the stressor phase, although the overall pattern was not significant. These results compare well to our previous data (Konkle et al. 2003) using this paradigm in which we observed reductions in 24 h sucrose intake in both strains of female rats during CMS exposure, with slightly lower consumption in Sprague–Dawley animals. Sucrose preference however was minimally affected. Others have also reported similar strain differences as a consequence of CMS with respect to this measure with Sprague–Dawley rats tending to show overall greater intake reductions than that observed in the Long Evans strain (Dunčo et al. 2001; Dunčo et al. 2001; Valverde et al. 1997).

Following the strategy suggested by Nielsen et al. (Nielsen, Arnt, Sànchez 2000) who found individual differences in stress susceptibility, in that only a small sub-set of rats responded to CMS by appropriately increasing thresholds for BSR, we employed the same procedure in this study and examined the 24 h sucrose intake data associated with individual animals. Almost half of the animals met the criterion for ‘‘reactive’’ which was a significant difference between the means representing the baseline and CMS phases in the predicted direction — CMS intake less than baseline intake values. Indeed, the opposite pattern (CMS intake greater than baseline intake) was not observed in any rat. However, the fact that preference was essentially unaffected by this

categorization indicates that reactive animals reduce overall fluid consumption during CMS exposure, and not sucrose intake specifically.

Recently Dunčko et al. (2003) applied the same approach and separated male rats on the basis of their individual sucrose preference scores, calling these groups ‘‘hedonic’’ and ‘‘anhedonic.’’ Rats were considered hedonic if preference values exceeded 60% and anhedonic otherwise which included 30% of the sample. We saw very little variability in preference scores in our sample with none below 80%.

The same strategy of selecting animals on the basis of individual variability was not applied to the BSR data (see Figure 3) as all measures–rate, threshold, and charge–were impressively stable during baseline and unchanged as a result of CMS trials, replicating our earlier findings (Konkle et al. 2003) and that of others (Lin et al. 2002; Nielsen, Arnt, Sànchez 2000). Neilsen has highlighted the importance of individual differences as a contributing factor to such variability (Nielsen, Arnt, Sànchez 2000). However, she observed CMS induced changes in BSR thresholds in only two of 14 Wistar rats.

Recall that our interest was in documenting both sucrose intake/preference and BSR thresholds in animals exposed to CMS, reasoning that if both measures index hedonic status and CMS produces a state of anhedonia, then the results from sucrose and BSR tests should covary. Our data suggests that they do not, at least in the case of sucrose intake in that animals with significant decreases in this measure did not concurrently increase BSR thresholds. Indeed, we have yet to observe stress-induced alterations in thresholds in our laboratory and conclude that this measure is too robust in the face of mild stressors. Severe stressors such as uncontrollable footshock, known

to induce behavioral and neuronal suppression (Simson, & Weiss 1987), has been observed to produce decreased responding for BSR (Zarcharko, Bowers, Anisman 1984; Bowers, Zarcharko, Anisman 1987) but little if any decrement in the threshold for rewarding brain stimulation, the index used to interpret anhedonic effects. In a later study, the authors did report some differentiation between dorsal and ventral aspects of the VTA, in that severe footshock produced current threshold increases in response to severe footshock in the dorsal VTA only (Zarcharko et al. 1990). Regional functional differences in dopaminergic as well as other transmitter systems may account for this dichotomy.

Finally, while there are methodological differences across laboratories in the stressor schedule employed (Morreau 1997), Willner (1997a;b) argues that there is no evidence that differences in the stressor schedule accounts for the poor reliability.

It has been suggested that the decreased rate of body weight gain contributes to the reduction in sucrose intake observed during stressor application (Matthews, Forbes, Ried 1995), while others argue that there is no correlation between these two factors (Nielsen, Arnt, Sànchez 2000). In our hands, the difference in overall weight gain between the stressed and control groups was minimal—about 3%. Not surprisingly, we have observed larger differences in male rats in other studies (Bielajew, Konkle, Merali 2002; Bielajew et al. 2003; Konkle et al. 2003) given that their full adult weight is much higher than that of female rats.

The most potent effect of CMS in this study was the alteration in the estrous cycle, once the influence of BSR on cycle activity was removed. Recall that all animals in groups that included BSR as a measure received stimulation trials for several weeks

before baseline data were collected as part of the screening and stabilization procedures. This alone caused disruption of the estrous cycle that did not recover during the subsequent baseline phase even though stimulation trials at that point were delivered only twice weekly and for a relatively short session—30 min. The introduction of CMS suggests that the frequency of cycles was further reduced; however, the control group data plotted in the bottom graph of Fig. 3 indicates that stimulation alone could have caused the further decline in cycle frequency—see top of Fig. 3. Thus, the CMS contribution can only be gleaned from the data in the middle of Fig. 3; no BSR trials were administered to these groups.

The effects of BSR on estrous cyclicity were still present up to four weeks after stressor manipulations had ceased, evident in the top graph, and a phenomenon that we have documented reliably in our laboratory, even months following the end of BSR trials. Interestingly, many of these animals became stalled in the estrus or diestrus phases of the cycle, which seems an inappropriate and maladaptive response to stress given that most species become infertile when the environment, internal or external, is unfavourable. It would be of interest to know if this phenomenon is site-specific or a general property of rewarding brain-stimulation. In addition, the relationship between hormonal status and cytological profile must be established in this context.

Rewarding stimulation of the medial forebrain bundle has been shown to induce estrous interruption, independent of stressors (Drewett, & Herberg 1975; Hitt, & Gerall 1969; Konkle et al. abstract). Hitt and Gerall 1969 observed that reduced estrous cyclicity recovered to normal levels following cessation of rewarding stimulation delivered to the basal central hypothalamic structures; animals that exhibited estrous

disruption without recovery of regular cycling had electrodes implanted in more rostral and caudal structures adjacent to the hypothalamic areas. In addition, electrodes located in the paraventricular hypothalamic nucleus did not induce suppression of estrous cyclicity at all. One group has reported that medial forebrain bundle stimulation does not induce estrous cycle disruption (Stratmann, & Craft 1997). However, in that study, the estrous cycle was tracked only briefly during BSR stabilization sessions and not beyond.

The influences of stress on reproductive hormones have been well-documented. In the clinical literature, stress has been reported to induce amenorrhea and menstrual cycle disruptions in female patients (Genazzani, Petraglia, DeRamundo 1991). It is well known that female athletes frequently experience cycle disruptions, although reduced fat stores as a consequence of a restricted caloric diet in that example also contributes to this condition (Warren, & Goodman 2003). In animal studies, chronic exposure to physical stressors has been reported to induce a constant diestrus phase (Grippo, Santos, Johnson 2004), not unlike the pattern that we observed in this study in the animals not subjected to BSR tests. One group found that female rats exposed to unpredictable stressors such as tail pinch, electrical shock, and cold stress in adulthood experience incessant diestrus (González et al. 1994). The same group reported similar results arising from unpredictable emotional stressors such as individual housing, white noise, and forced swimming. The pattern observed was that the physical stressors induced a marked disruption in cycling while the emotional stressors induced constant diestrus in a small subset of animals (Rodriguez et al. 1988). Axelson (1987) also noted that forced swimming or swimming and running disrupted the estrous cycles of the rats.

In general, the stressors used in the present study were milder in comparison to those just described. In an earlier study, we found that female rats exposed to the CMS schedule often displayed a disruption in regular cycle activity, typically becoming stalled in one phase of the estrous cycle (Konkle et al. 2003). Once stress manipulation had ceased, a normal pattern of cycling eventually resumed in most animals. Likewise in this study, recovery of estrous began immediately following the cessation of CMS. Very few investigators have tracked CMS-induced estrous activity. The one study that we are aware of reported no effect of mild foot shock delivered for 14 days on cycle regularity in most of their sample of female rats (Anderson et al. 1996).

One explanation for the estrous cycle disruption in response to CMS may be related to the food and water deprivation that is a component of the procedure. Tropp and Markus (Tropp, & Markus 2001) noted that animals that were food deprived only developed irregular cycles. As a result, we include this feature in control animals and have not witnessed the same phenomenon. That is, food and water deprivation, in the absence of the other stressors, does not disrupt the estrous cycle — see middle plot of Fig. 3 (unfilled bars).

There may also be a photoperiodic influence on the estrous cycle (Sharp, & La Regina 1998). For example, constant light exposure has been reported to cause age-related estrous disorders and other disturbances in female mice (Anismov et al. 2004). Our procedure was much milder, incorporating 24 h of continuous exposure to light, once weekly. It remains to be seen if this factor alone, in the absence of stressors, influences cycle activity.

While thresholds for BSR and sucrose preference scores failed to be altered by a regimen of CMS, our observations of fluid reduction, estrous cycle interruption, and reduced weight gain suggest that there are stress-induced metabolic and hormonal alterations that are consistent with a stress response, but not anhedonia, at least as determined by these measures. Behavioural tests need to be developed that more reliably index any anhedonic consequences of CMS, in order to make the model a more useful paradigm for studying the underlying mechanisms of depression.

Study 2

Influence of housing on the consequences of chronic mild stress in female rats

Baker, & Bielajew. 2007. Stress, 10, 283 – 293.

Abstract

The chronic mild stress (CMS) paradigm was developed to induce anhedonia in animals. The repeated administration of a series of unpredictable, mild stressors attempts to mimic the daily stress is associated with the onset of clinical depression in humans. Male animals are predominantly used in these investigations despite significant, well- documented sex differences in human depression. In this study, the CMS procedure was modified to be more ecologically relevant to female animals. The effects of stress on sucrose preference, social interaction, rate of weight gain, and regularity of the estrous cycle in female Sprague–Dawley rats were evaluated in both single- and paired-housed rats, during 3 weeks each of baseline, CMS, and post-CMS phases. The results indicate that only single-housed rats exposed to stressors have a reduced rate of weight gain, significantly attenuated sucrose preference levels, and increased social interaction scores during the CMS phase of the study. Housing condition more than exposure to stress appeared to contribute to the disruption of estrous cycling in some animals. These data suggest that housing affords some protection from the negative consequences of CMS, at least in female rats, and that lack of social interaction in the single-housing condition may render females more vulnerable to stress-related illnesses. The development of paradigms that model human depression should emphasize sex-specific differences.

Keywords: Anxiety, chronic mild stress, depression, sucrose intake and preference, social interaction test, anhedonia

Introduction

In psychiatric disorders with stress and anxiety components, such as depression, there is a notable sex difference in the incidence, with females outnumbering males by two to one (American Psychiatric Association [DSM-IV] 1994). This is primarily evident during the reproductive years, and the occurrence of depression increases dramatically between menses and menopause and declines rapidly thereafter (Wade et al. 2002). Animal studies designed to model depression have relied almost exclusively on the responses of male subjects or ovariectomized female animals in order to avoid the potential confound of hormone fluctuations on the measure of interest. While there are many social and environmental factors likely at play, the finding that sex differences in depression rates are quite stable across cultures suggest that biological differences, such as hormonal fluctuation, contribute significantly to this phenomenon (Gater et al. 1998). Intact female animals are, therefore, a necessary component of depression research.

The CMS model is a validated and widely employed method of inducing a depressive state in rats similar to anhedonia, the core feature of the melancholic subtype of major depressive disorder (American Psychiatric Association [DSM-IV] 1994). Anhedonia is defined as the inability to derive pleasure from events that in a non-depressed state would be enjoyed, and it is interpreted primarily from behavioural measures, such as the place preference test (Benelli et al. 1999a,b), rewarding brain stimulation (Morreau et al. 1992), and the consumption of a mildly sweet sucrose solution (Willner et al. 1987), with the latter being the most frequently employed.

Despite its widespread use, there is no convention for administering the sucrose test or determining hedonic status on the basis of its results. We have adapted a method similar to that described by Dunčko et al. (2003). It consists of a two bottle (0 and 1% sucrose) 3 h test, preceded by overnight water deprivation, from which both sucrose preference and intake are evaluated.

Although the CMS model has been validated in male rodents, reliability has been a problem. While many laboratories have been able to elicit the pattern that is indicative of anhedonia, that is, a significant decrease in consumption (Cheeta et al. 1994; Kim et al. 2003; Grønli et al. 2004) and preference (Willner et al. 1987; Dunčko et al. 2001a,b) of a 1% sucrose solution after CMS exposure, other investigators have been unable to replicate this finding (Matthews et al.1995; Neilsen et al. 2000; Murison and Hansen 2001; Konkle et al. 2003). Dunčko et al. (2003) have suggested that such paradoxical results may reflect individual differences in susceptibility to stress within strain. For example, his group found about 30% of rats to be anhedonic at baseline, using a criterion of 60% sucrose preference to categorize rats as either hedonic (above 60%) or not.

While the results of behavioural tests are not always dependable in this context, metabolic, biochemical, and endocrine responses to chronic mild stressors are generally consistent with the expectations of a paradigm that models human depression (Harris et al. 1998; Dunčko et al. 2001a,b; Bielajew et al. 2002; Konkle et al. 2003). For example, in animals CMS exposure has been reported to cause sleep disturbances (Morreau et al. 1995; Grønli et al. 2004), cardiovascular changes (Grippo et al. 2002, 2003, 2004), decreased sexual behaviour (D'Aquila et al. 1994; Brotto et al. 2001),

decreased locomotor activity (Harro et al. 1999), immune disturbances (Kubera et al. 2001; Siberman et al. 2004), and increased corticosterone secretion (Harris et al. 1998; Bielajew et al. 2002), all of which have been observed in the clinically depressed population.

Our laboratory has been generally unsuccessful in establishing sucrose intake or preference deficits following weeks of stressor challenge in male rats. We have, however, had limited success applying these procedures to female rats, more so with the Sprague–Dawley than Long Evans strain. Recently, using a modification of the scheme of Dunčko et al. (2003) to identify what we termed reactive (significant difference in sucrose intake between baseline and CMS phases) and non-reactive (no significant difference between the two phases) female rats, we obtained the same strain difference pattern (Baker et al. 2006). Thus, Sprague–Dawley was the strain of choice in the current study.

Given the dearth of studies employing female rats as subjects in CMS designs, the adequacy of the paradigm as a model for studying depression in women has not been fully explored. There is ample behavioural evidence to suggest that there are sex differences in stress reactivity. For example, male and female (intact) rats react differently in response to inescapable foot shock (Leuner et al. 2004), tail shock (Wood et al. 2001), and some behaviours in the forced swim test (Drossopoulou et al. 2004), with females overall appearing to be more vulnerable to the effects of stress encounters. One concern with this paradigm is that the stress manipulations may have sex-specific consequences. The original CMS procedure employed pairing as one of the stressors, which is known to cause distress in male, but not female rats (Westenbroek

et al. 2003a). Indeed, regular pairing may even provide a protective effect against stress in females (Westenbroek et al. 2003a,b). To examine this issue, we included two housing conditions in this study, single and paired. Another concern is that stressors such as food and water deprivation (Anderson et al. 1996; Tropp and Markus 2001) and overnight illumination (Sharp and LaRegina 1998; Anisimov et al. 2004) have each been associated with estrous cycle disruptions. Consequently, these were replaced by short exposures to stroboscopic light and white noise, which are stressors that have been included in other CMS reports (Neilsen et al. 2000; Konkle et al. 2003; Grippo et al. 2004, 2005; Baker et al. 2006). We reasoned that these modifications would be more effective in inducing a state of anhedonia in female rats and allow us to identify more clearly stress and estrous cycle interactions. To ensure that these modifications did not simply elicit a general anxiety-like response, exclusive of anhedonia, the anxiogenic profile was assessed using a test of social interaction, a naturalistic animal model of anxiety (File and Hyde 1978; File 1980).

Thus, this study examines the effects of social housing in female rats on the development of anhedonia after exposure to chronic mild stressors. The measures assessed were sucrose preference, social interaction, rate of weight gain, and regularity of the estrous cycle. We hypothesized that the presence of a cage mate would attenuate the effects of stress.

Methods

The use of animals was in accordance with the guidelines of the Canadian Council on Animal Care. All protocols received institutional approval.

Animals

A total of 43 Sprague–Dawley female rats (Charles River Laboratories, St-Constant, QC, Canada), ranging in weight from 258–390 g (average 320 g) upon arrival, were used. They were immediately randomly assigned to one of four groups, based on housing and stress conditions. The groups comprised the following: single-housed control group (n = 11), single-housed stress group (n = 10), paired-housed control group (n = 10), and paired-housed stress group (n = 12). Single-housed rats were individually maintained in standard size plastic cages (24 cm wide, 43 cm long, 20 cm high) and paired rats in larger cages (37 cm wide, 48 cm long, 20 cm high). All rats had free access to food and water, with one exception. Before each sucrose test, water was removed overnight to encourage intake during the morning test (approximately 16 h of deprivation/test). All rats received enrichment objects in the form of a black PVC tube, a Kong® Toy, and Nestlets® in the home cage except during stress exposure. Access to such objects has previously been associated with a reduction in chemical markers of stress that tends to increase as a result of single housing in female rats (Belz et al. 2003). A 12 h light/12 h dark cycle with lights on at 7:00 a.m. was maintained throughout the study. The estrous cycle was monitored daily via vaginal lavage taken between 9 and 11 a.m. using methods described previously (Marcondes et al. 2002). Estrus cycles were considered to be regular if the pattern of stages followed the standard criteria outlined by Long and Evans (1922) and if cycle lengths of individual animals were consistent with pre-baseline monitoring. Body weight was recorded weekly.

Procedures

Chronic mild stress.

Table 3 provides an outline of the study phases and stress schedule delivery and description. Following three weeks of baseline monitoring, the CMS procedure was applied for 3 weeks. During this time, all enrichment objects were removed from animals in the stress groups. The CMS phase was immediately followed by a 3-week period during which baseline conditions were reinstated. Enrichment items were returned to the home cage and rats were handled only for daily vaginal lavages, weekly weight recording, and cage changes.

Table 3. Twenty day schedule of chronic mild stressors.

BASELINE			CMS			POST-CMS		
Week1	Week 2	Week 3 test	Week1	Week 2	Week 3 test	Week1	Week 2	Week 3 test

Time	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
10:00	confine 1h	noise 1h	confine 1h	strobe 2h	noise 1h	confine 1h	noise 1h
12:00	strobe 1h	confine 1h	strobe 1h	noise 1h	confine 1h	strobe 1h	confine 1h
14:00	noise 1h	strobe 2h	noise 1h	confine 1h	strobe 1h	noise 1h	strobe 2h
16:00		Cage tilt	wet bed	cage tilt	wet bed		cage tilt
Time	**DAY 8**	**DAY 9**	**DAY 10**	**DAY 11**	**DAY 12**	**DAY 13**	**DAY 14**
10:00	confine 1h	strobe 2h	noise 1h	confine 1h	noise 1h	confine 1h	strobe 2h
12:00	strobe 1h	noise 1h	confine 1h	strobe 1h	confine 1h	strobe 1h	noise 1h
14:00	noise 1h	confine 1h	strobe 1h	noise 1h	strobe 2h	noise 1h	confine 1h
16:00	wet bed	cage tilt	wet bed		cage tilt	wet bed	cage tilt
Time	**DAY 15**	**DAY 16**	**DAY 17**	**DAY 18**	**DAY 19**	**DAY 20**	**DAY 21**
10:00	noise 1h	confine 1h	noise 1h	confine 1h	strobe 2h	noise 1h	strobe 2h
12:00	confine 1h	strobe 1h	confine 1h	strobe 1h	noise 1h	confine 1h	noise 1h
14:00	strobe 1h	noise 1h	strobe 2h	noise 1h	confine 1h	strobe 1h	confine 1h
16:00	wet bed		cage tilt	wet bed	cage tilt	wet bed	cage tilt

Label	Stressor	Description	Time per week
confine	Confinement with noise	Rats are individually placed in mouse cages timers are set to ring at random intervals.	5hrs
strobe	Stroboscopic light	In home cage, exposed to 300 flashes/min of white light (in dark room).	6 – 7hrs
noise	White noise	An untuned radio set at max volume.	5hrs
wet bed	Wet bedding	Warm water is mixed into bedding to saturation.	2 x 17hrs
cage tilt	Tilted cage	Cage is tilted horizontally 30° overnight.	2 x 17hrs

Sucrose preference test

Following a procedure similar to that previously described by Dunčko et al. (2003), a 3h sucrose test was conducted on each of the last 4 days of every phase (baseline, CMS, and post-CMS) following overnight water deprivation. All tests were administered in the home cage and paired rats were separated from each other by a metal barrier with holes (rats were acclimatized to this separation before the baseline weeks began). The rats were exposed to two test bottles, one containing tap water and the other, a 1% sucrose solution, for 3 h beginning at 8:30 a.m. At 11:30 a.m., the volume in each bottle was measured. Preference for sucrose was calculated by dividing the amount of sucrose solution consumed by the total intake of fluid and converting this value into a percentage. After baseline sucrose preference had been calculated, rats were assigned to experimental groups. Those displaying at least a 60% sucrose preference were placed in the stress groups and those displaying a preference below 60% were placed in the control groups.

Social interaction test

During the last 2 days of each phase, the social behaviour towards a non-familiar stimulus rat during a 5 min test of social interaction was filmed and later scored by an observer blind to treatment. A second individual re-scored half of the data in order to determine inter-rater reliability.

Seven female rats were used as stimulus animals (average weight at each test: Baseline-281 g, CMS-308 g, post-CMS-311 g); they did not belong to any experimental group and did not receive any prior treatment with the exception of daily handling and

vaginal lavage. The test arena was a novel environment (25 cm wide, 44 cm long, 28 cm high) to which all animals were acclimatized for 15 min approximately 30 min before the test. The social interaction tests were conducted during the dark/active phase (between 7 and 10 p.m.) in a room lit by ambient red light. The duration spent engaging in active social behaviour with the stimulus rat and the frequency of amicable, agonistic and nonsocial behaviours were recorded so that each test could be categorized in terms of active social time and the primary behaviour expressed. Figure 5 provides a detailed summary of the behaviours of interest recorded in the social interaction test. These have been described previously (Kiyokawa et al. 2004).

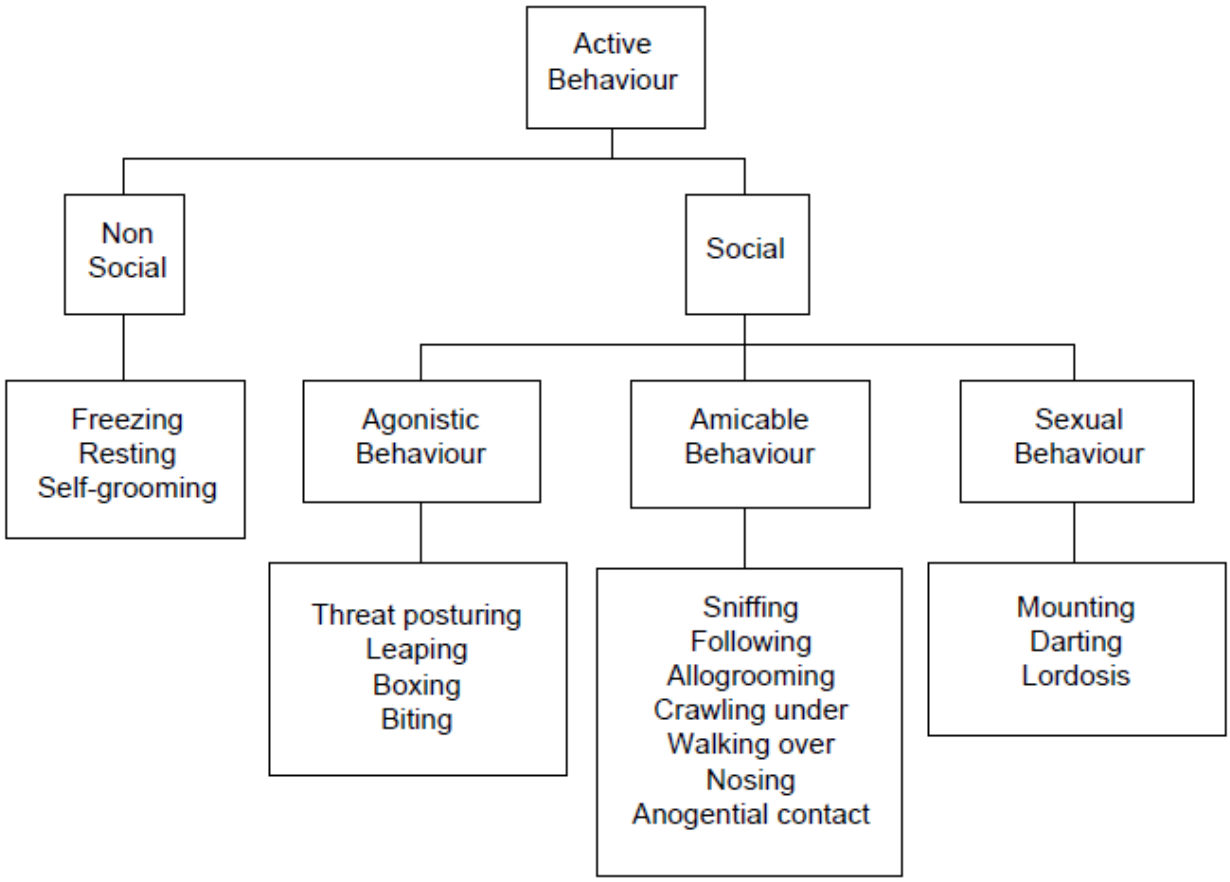


Figure. 5. *Social interaction behaviours.*
This figure shows a breakdown of the social interaction behaviours that were recorded during the last week of each phase; the behaviours were categorized as social and non-social. Their constituents include the following: Freezing: immobile. Threat posturing: arched back, all limbs extended with flanks turned toward opponent. Leaping: Jumping in air and landing on opponent. Boxing: upright posture facing opponent. Allogrooming: grooming stimulus rat. Anogenital contact: sniffing or nosing the anal and or genital area of stimulus rat. Lordosis: coccygeal region raised and the tail to one side (position which permits intromission and is stimulated by pressure of male's forelimbs on the flanks during mounting). Darting: rat in estrus runs a short distance from conspecific and pauses for mounting and then repeats.

Statistical analyses

All analyses were conducted on the raw values and the CMS and post-CMS data expressed as mean differences ± SEM from baseline. The weekly body weight data were analysed (SPSS 2005) via a mixed ANOVA design with group as the independent factor (four levels: single-housed control, single-housed stress, paired-housed control and paired-housed stress) and time as the repeated factor (nine levels of week). The significant interaction was followed-up by pair-wise post-hoc tests in each group in order to assess phase differences. A one-way randomized group ANOVA was used to assess baseline differences and weight gain overall across the course of the study. Pair-wise tests were then conducted to further delineate group differences.

For each phase, the average sucrose preference score, based on three tests, was calculated for each rat and these data were evaluated using a mixed ANOVA design (four groups and three phases). Significant interaction was followed up with pair-wise post-hoc CMS and housing in female rats 285 tests in each group. Note that the average value was calculated on fewer than three tests in some animals, due to spillage. Baseline differences were evaluated as described above for the weight data.

The social interaction data were evaluated via a three by four mixed ANOVA design, with three levels of phase (baseline, CMS and post-CMS) and four levels of group (single- and paired- housed control and stress groups). The significant interaction was followed up by pair-wise post hoc tests in each group.

Huynh-Feldt correction for violations of the assumption of sphericity was applied as required (Howell 2002) to all repeated variables having more than two levels. The adjusted degrees of freedom based on this correction are reported in the results

section.

The estrous cycle data were subjected to a chi square test of independence (3 (phases) x 4 (groups) contingency table). These data were based on the number of rats that generally displayed regular cycles during baseline, CMS and post-CMS weeks.

The alpha level was set at 0.05 for all omnibus analyses and a Bonferroni correction applied to all follow-up tests.

Results

The body weight data are shown in Figure 6. The boxed plot shows the average weight gain in each group over the course of the study (9 weeks) and includes the three weeks each of baseline, CMS, and post- CMS phases. The pattern suggests that control rats demonstrated a greater rate of weight gain over time while in comparison, rats in the stress groups gained less weight (paired-housed) or showed negligible weight gain (single-housed) during the CMS phase. Weight gain over the course of the study was significant across groups ($F(3, 39) = 5.273$, $p = 0.004$) due to pair-wise differences between the single-stressed and each paired group ($p = 0.037$, to paired stressed; $p = 0.005$, to paired control).

The control and stress groups in each housing condition had similar baseline weights; however, the housing manipulation itself altered the baseline weights in that rats in the paired-housing condition had significantly lower body weights ($F(1, 41) = 18.35$, $p = 0.0001$), either due to higher activity levels or lower food intake than that associated with single-housed groups. The average group weight, as a function of housing condition was 280.5 ± 3.12 g in paired-housed rats and 315.5 ± 7.7 g in single-

housed rats.

The mixed ANOVA conducted on the raw body weight values produced significant main effects of group ($F(3,39) = 3.022$, $p = 0.041$) and time ($F(1.9, 75.6) = 40.621$, $p = 0.0001$) and the interaction between the two ($F(5.8, 75.6) = 5.426$, $p = 0.0001$). Pair-wise post-hoc tests to examine differences in weight between phases in each group revealed no significant difference between any pair in the single-housed stressed group indicating negligible weight change in this group over the course of the study. In the remaining groups, CMS and post-CMS weight were significantly different from baseline weight ($p < 0.0001$). Furthermore, in the paired-housed groups, post-CMS weight was significantly greater than CMS weight ($p < 0.0001$).

Within housing conditions, there were significant differences from baseline in weight gain during the CMS phase between the control and stressed groups; this applies to both the single ($p = 0.013$) and paired-housed ($p = 0.001$) groups. At the post-CMS phase, these differences were not significant.

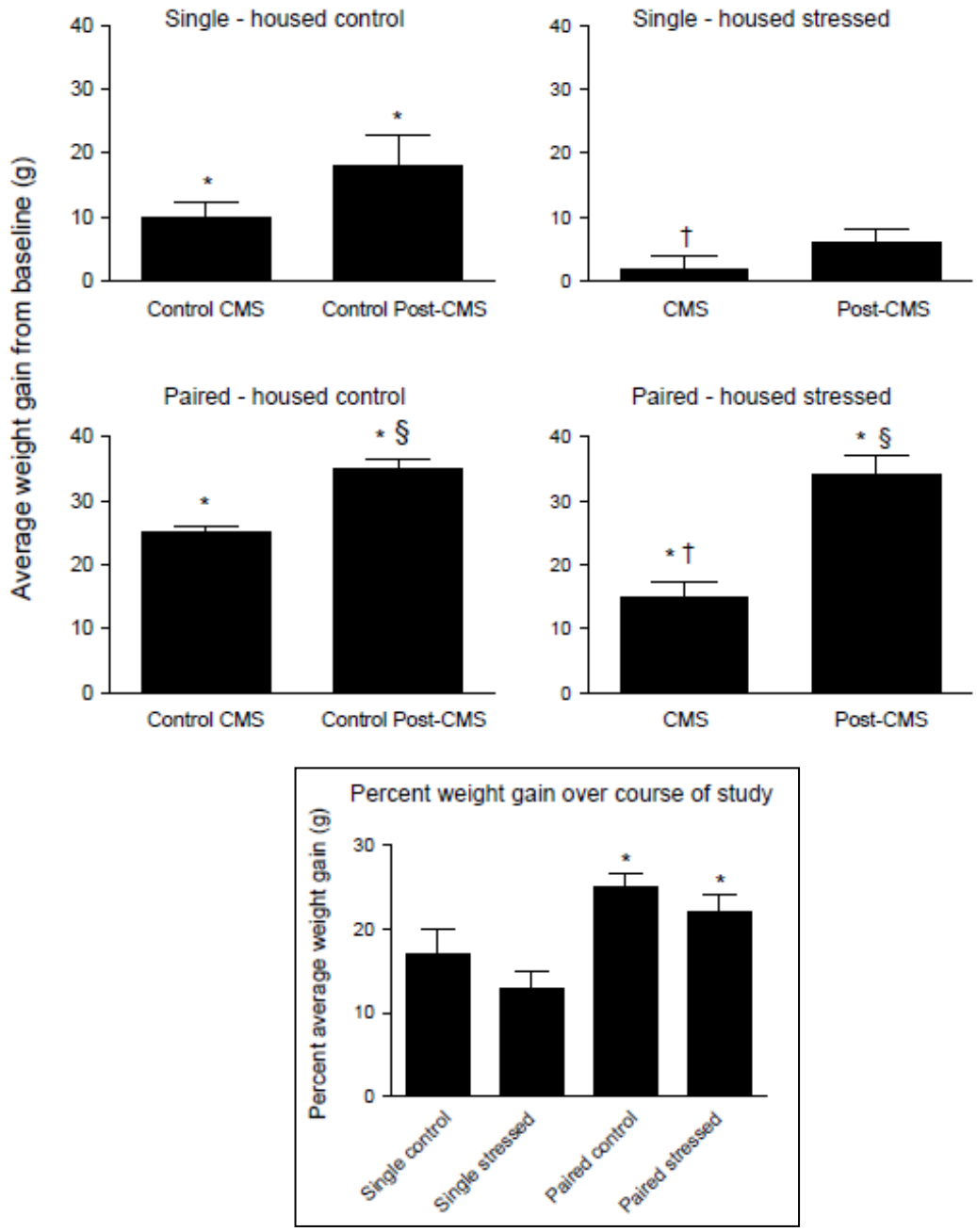


Figure. 6. *Weight.*
*The difference in body weight from baseline at the CMS and post-CMS phases. Data are group means ± SEM. * p < 0.01 vs. baseline; § p < 0.01 vs. CMS. Within housing condition, significant differences between control and stressed groups are denoted by the symbol †. The boxed graph shows the percentage of average weight gain over the course of the study for each group. The * above each paired group indicates that they were both significantly different from the single-stressed group. The sample size was 11, 10, 10, and 12 rats in the single-housed control, single-housed stressed, paired-housed control, and paired-housed stressed groups, respectively.*

Figure 7 shows the average sucrose preference data (plotted as differences from baseline) for each group. The baseline data are shown at the bottom of the figure. Note that rats with low sucrose preference were assigned to the control groups and those with high preference, the stressed groups, thus forcing baseline group differences. An analysis of the baseline data produced an overall significant difference ($F(3,39) = 10.670$, $p = 0.0001$). Pair-wise differences were found between the single-stressed and all other groups ($p = 0.006$, single control; $p = 0.0001$, paired control; $p = 0.044$, paired stressed). In addition, the comparison of the paired groups was significant ($p = 0.028$).

The full analysis produced a significant interaction between group and phase ($F(6, 54) = 2.7$, $p = 0.023$). Follow-up post hoc tests indicated a difference in the preference pattern over phase in the single-housed stress group and paired-housed control groups. That is, the average preference during CMS was significantly reduced relative to its baseline value in the single-housed stressed group ($p = 0.014$). In contrast, the paired-housed control group showed elevated preference scores during both CMS ($p = 0.007$) and post-CMS phases ($p = 0.007$). The single-housed control and paired-housed stress groups maintained a fairly consistent level of preference from phase to phase.

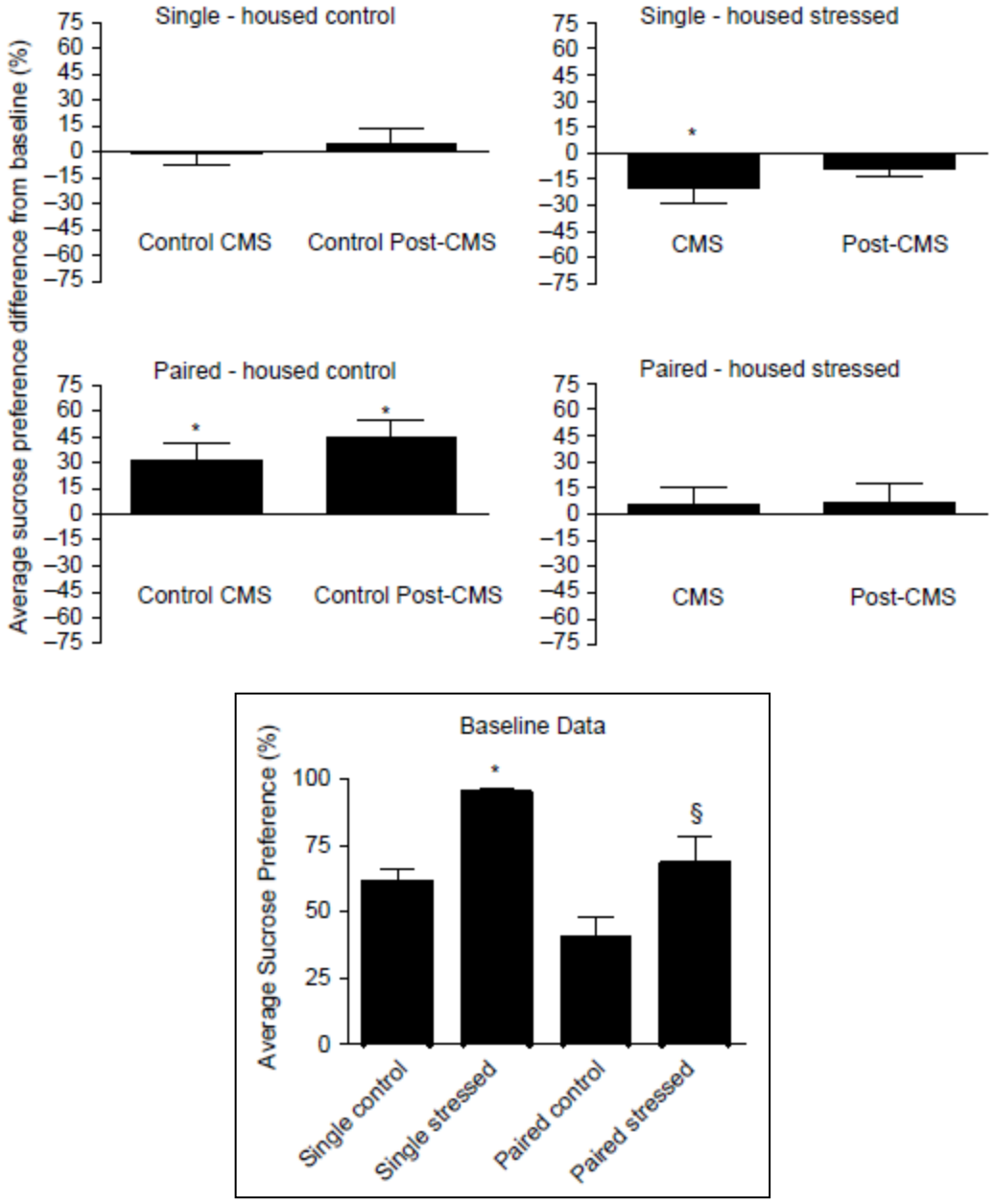


Figure. 7. *Sucrose preference.*
*The percent difference in sucrose preference from baseline at CMS and post-CMS phases. The boxed graph shows the baseline values for each group. Data are group means ± SEM. Significant differences from baseline are indicated by an * above the relevant group. The single stressed group was significantly different from all other groups (*) and the paired stressed group was significantly different from its control counterpart (§) at baseline. The sample size was 11, 10, 10 and 12 rats in the single-housed control, single-housed stressed, paired-housed control, and paired-housed stressed groups, respectively.*

The results of the social interaction test are shown in Figure 8 for each group with baseline data shown at the bottom of the figure. The inter-rater reliability, assessed using Pearson's r correlation, was 0.82 ($p = 0.0001$). The mixed ANOVA conducted on these data gave rise to significant main effects of group ($F(2, 74) = 4.57$, $p = 0.013$), phase ($F(6,74) = 3.80$, $p = 0.002$), and their interaction ($F(3,74) = 5.12$, $p = 0.005$). A significant pair-wise difference was found between baseline and post-CMS scores in the single-housed control group only ($p = 0.0001$); in this group, social interaction scores were also significantly elevated in the post-CMS phase compared to CMS values ($p = 0.037$). The behavioural phenotype did not differ between groups at any phase of the study, and no correlations were found between estrous cycle phase and interaction time, or test and stimulus animal weight.

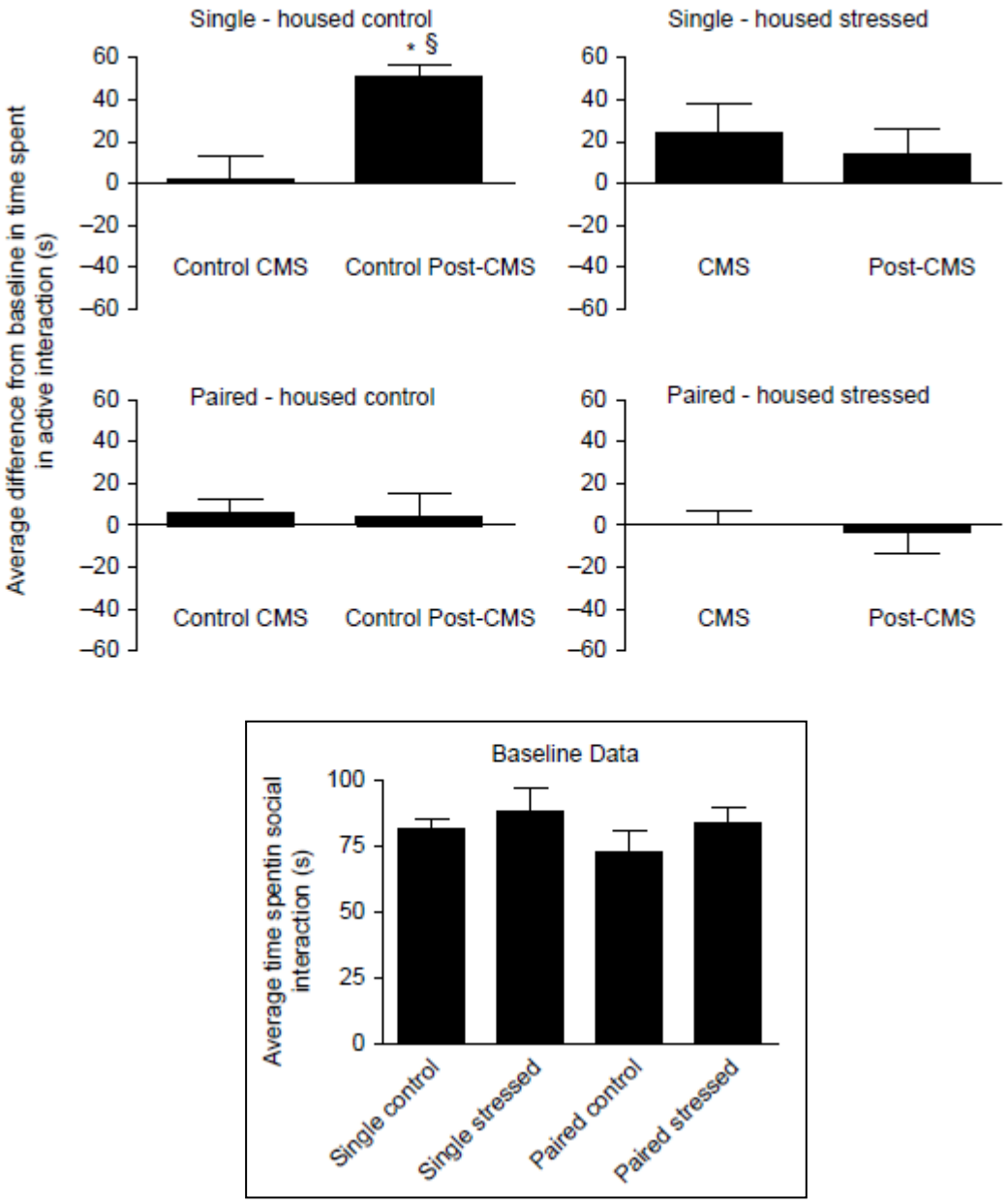


Figure. 8. *Social interaction.*
*The difference from baseline time spent in active social interaction at CMS and post-CMS phases. The boxed graph shows the baseline data for each group. Data are group means ± SEM. *p < 0.01 vs. baseline; §p < 0.01 vs. CMS. The sample size was 11, 10, 10 and 12 rats in the single-housed control, single-housed stressed, paired-housed control, and paired-housed stressed groups, respectively.*

Figure 9 shows the percentage of rats in each group that displayed regular cycles. The units refer to decreases from baseline. The smallest change in this value occurred in the paired-housed control group. Irregular cycles were characterized by extended estrus or diestrus days, or an abnormal pattern of cyclicity. The chi-square test of independence that was performed on these data (all groups versus the three phases) was not significant. Goodness of fit tests to examine the effects of housing alone approached significance ($p = 0.083$), while the same test applied to the stressed versus non-stressed groups did not ($p = 0.879$).

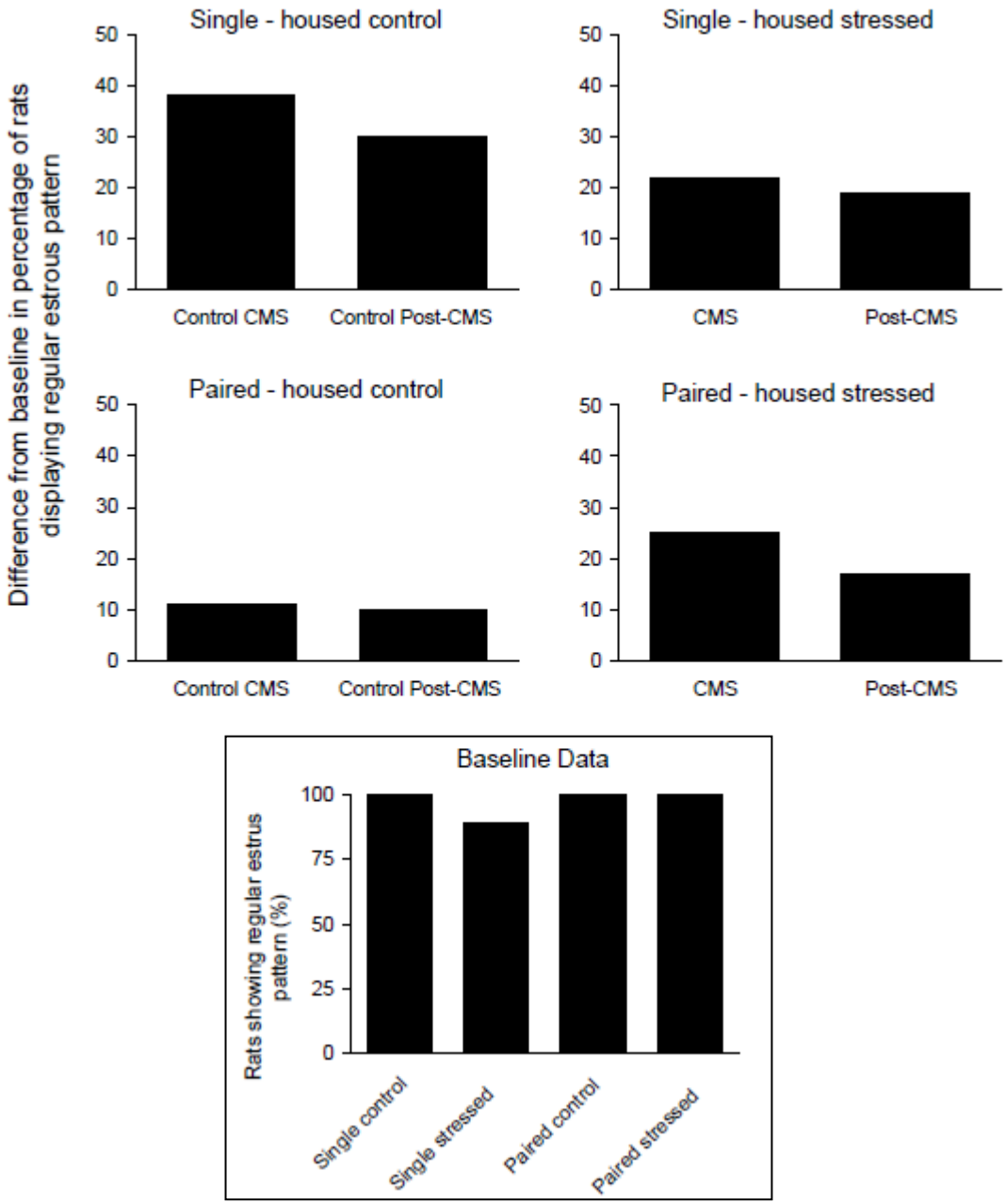


Figure. 9. *Estrous cycle.*
The difference from baseline in the percentage of rats displaying regular estrous cycles at the CMS and post-CMS phases. Increasing values represent decreasing cyclicity. The boxed graph shows the baseline data for each group. The sample size was 11, 10, 10 and 12 rats in the single-housed control, single-housed stressed, paired-housed control, and paired-housed stressed groups, respectively.

Discussion

The present study was designed to evaluate the responses of female rats in different housing conditions to a regime of chronic mild stressors, excluding ones that independently influence the estrous cycle. The consequences of CMS were assessed via behavioural (sucrose preference and social interaction) and physiological (weight and estrous cycle regularity) measures. The rats were assigned to stress and non-stress groups at the baseline phase on the basis of sucrose preference. This was necessary to ensure that baseline preference levels in the stressed groups were sufficiently high to allow observable reductions following experimental manipulations but not so low as to suffer from floor effects.

The CMS procedure induced a mild anhedonic state only in the single-housed stressed rats as interpreted from the sucrose preference data. The paired-housed control group showed a surprising increase in sucrose preference during both the CMS and post-CMS phases. This might have been because some rats require longer periods of acclimatization to sucrose. Nonetheless, given the intentional group differences in baseline preference scores, the appropriate comparisons were within-group. Three weeks of CMS exposure reduced the preference for sucrose by 19.6% in the single-housed stressed group. During the post-CMS phase, when mild environmental enrichment was reinstated, sucrose preference values returned nearer to baseline levels. This overall pattern is typically found in studies of this nature (Willner et al. 1987; Dunčko et al. 2001a,b; Genedani et al. 2001; Grippo et al. 2002, 2004). A difference in sucrose preference between intact and ovariectomized female rats has been reported by one group who found a decrease in preference after CMS exposure in

intact rats only (Dunčko et al. 2001b), suggesting that hormonally unaltered females are more sensitive to the effects of CMS. Few CMS studies employ intact female rats, and of those that have, sucrose intake rather than preference was typically measured and shown to decrease after CMS exposure (Benelli et al. 1999a,b; Dunčko et al. 2001a,b; Konkle et al. 2003; Baker et al. 2006). A study employing a four week CMS regime demonstrated a decrease in both sucrose intake and preference in male and intact female rats (Grippo et al. 2004).

Similar to the sucrose data, the single-housed stressed rats showed the lowest rate of weight gain over the course of the study. In male rats, reduced weight gain following CMS has been frequently observed (Matthews et al. 1995; Dunčko et al. 2001b; Neilsen et al. 2001; Bielajew et al. 2002). Our laboratory had previously reported similar findings in intact females (Konkle et al. 2003); however, others have also observed no change in weight gain in this context (Murison and Hansen 2001; Dunčko et al. 2001a). Data pertaining to the estrous cycle were most surprising. Regardless of group, all rats showed a reduction in regular cycling following the baseline phase, an effect that persisted into the third week of the post-CMS phase. In our previous CMS studies using female rats, we observed a large proportion of acyclic rats after exposure to CMS (Konkle et al. 2003). Grippo et al. 2004 reported similar findings: the cycles of animals exposed to 4 weeks of CMS manipulations experienced a 40% lengthening of the estrous cycle relative to control groups. Although the stressors may influence estrous cycle regularity, the possibility that food and water deprivation or overnight illumination contribute to irregular cycles is equally viable (Sharp and La Regina 1998; Tropp and Markus 2001; Anisimov et al. 2004). In our study the modified CMS regime,

which did not include these particular stressors, resulted in no difference between control and stressed rats in terms of the degree of cycle disruption. The graph (Figure 9) that shows plots of the estrous pattern in individual groups suggests that cycle regularity was more influenced by the housing condition than by the stressors. Almost all control rats in the paired-housed condition displayed regular cycles throughout the study, and the application of stressors was associated with some reduction in cyclicity in rats housed together. On the other hand, both groups of single-housed rats showed the greatest cycle disruptions during and post-CMS. We (Konkle et al. 2003; Baker et al. 2006) have observed abnormal cycle patterns in a high proportion of individually housed rats; in contrast, almost 100% of our group housed rats displayed regular cycles over months of observation (Konkle et al. 2003).

Responses of all rats in the social interaction test suggested that CMS did not induce a general anxiety response as no group displayed decreased social interaction after exposure to the paradigm. This is consistent with responses of male rats (D'Aquila et al. 1994). A housing difference did emerge in our case, however. Regardless of stress experience, the paired rats maintained consistent times across phase (all within a range of 10 s). The pattern in Figure 8 suggests that single-housed rats were generally more variable in the time engaged in social interaction compared to their baseline performance level and that of paired rats during the CMS and post-CMS phases. Unexpectedly, the single-housed control rats displayed a significant spike in interaction during the post-CMS phase. None of these observations can be attributed to weight or cycle phase differences between the housing groups as neither variable correlated with social interaction time. It is possible that the manner in which the test was administered

did not elicit much anxiety because the rats had been acclimatized to the test box prior to baseline tests, and tests were conducted in the dark; both of these factors have been found to increase exploration and social interaction (File and Hyde 1978). Furthermore, the social deprivation experienced by single-housed rats may have made them more motivated to seek out social interaction with the stimulus rat than their paired-housed counterparts, especially during subsequent tests when the presentation of an unfamiliar rat was less novel.

The social nature of rats naturally makes standard, single-housing practices highly stressful, particularly for female rats that display little to no aggression towards conspecifics regardless of familiarity. Housing female rats in pairs or groups tends to diminish stress effects that are normally observed in single-housed rats (Haller et al. 1999; Konkle et al. 2003; Belz et al. 2003; Westenbroek et al. 2003a,b). Presumably this is due to the lack of both social interaction, and the ability to display naturalistic behaviour in the impoverished environment of standard laboratory husbandry. Providing stimulating objects has been found to decrease hormonal indicators of stress in individually housed male rats (Belz et al. 2003). In our case, both single-and paired-housed groups were provided items in their home cage in an attempt to observe the effects of social housing more clearly. While the enrichment objects may have diminished some stress induced by living in typical ‘sterile’ housing conditions, they did not appear to alleviate the behavioural outcomes (notably sucrose preference) of stress associated with isolation or the exposure to CMS in this group. Indeed, the concept of social buffering, whereby anxiety-provoking stimuli elicit a diminished behavioural and hormonal response in animals that are kept in social groups

(Kiyokawaet al. 2004), could be applied to explain the lack of CMS effect in behavioural tests in paired rats. This is consistent with clinical findings: the level of perceived social support is negatively related to the presence and severity of depressive symptoms (George et al. 1989).

Our modified version of the CMS paradigm, which excludes stressors that impact on the reproductive cycle, induces behavioural and body weight changes indicative of an anhedonic state but not anxiety. However, like humans, social support, via paired-housing, appears to provide some protection against the influence of the stressors, suggesting that single-housing, an unnatural living condition in female rats, induces a vulnerability to environmental stressors. These results highlight the importance of tailoring research tools to address gender differences and husbandry practices in animal models of anxiety and stress.

Study 3

Effects of gestational stress:
1. Evaluation of maternal and juvenile offspring behaviour

Baker, Chebli, Rees, LeMarec, Godbout, & Bielajew.
2008. Brain Research, 1213, 98 – 110.

Abstract

In both humans and animals, stress experienced during gestation is associated with physiological changes and disruptions in emotional function and cognitive ability in offspring; however, much less is known about the effects of such stress in mothers. In animal models, physical restraint is commonly employed to induce stress during gestation and results in elevated postpartum maternal anxiety and changes in maternal care. The purpose of the current study was to evaluate the consequences of restraint stress applied on gestation days 10 through 19 in mother rats and their juvenile offspring. Progeny were reared by birth mothers. Preterm anxiety was assessed in the elevated plus maze and maternal behaviour in the retrieval test. Cognitive (T-maze) and anxiety measures (elevated plus maze and emergence) were applied to a subset of male and female offspring at 30 – 31 days of age. Weight and litter characteristics were also recorded. Mother rats exposed to stress during gestation had attenuated weight gain, elevated anxiety-like behaviour, and reduced maternal care. Stressed mothers also had fewer pups and an elevated offspring mortality rate. The consequences of gestational stress in offspring were subtle and gender dependent. Only juvenile females displayed marginal effects of gestational stress in the form of elevated anxiety-like behaviour and attenuated weight gain. In the current study, although gestational stress had robust effects in the mother rat, these did not translate to similar changes in offspring behaviour. The importance of focusing research on maternal responses to gestational stress is highlighted by these findings.

Keywords: Stress; Pregnancy; Maternal behaviour; Juvenile rat; Anxiety; Cognition

Introduction

It has been recognized for some time that women are vulnerable to emotional disturbances in response to stress exposure during pregnancy (Steiner, 1979). For example, the experience of stressful life events during gestation has been linked to an increased risk of developing postpartum depression (Llewellyn et al., 1997). This occurs despite the fact that, in normal pregnancy (Kammerer et al., 2002) and lactation (Heinrichs et al., 2001), ACTH and cortisol levels are decreased in response to stress indicating that the hypothalamic-pituitary-adrenal (HPA) axis is hyporesponsive. Stress experienced during pregnancy, therefore, appears to interfere with this normal adaptive response and may promote the development of abnormal emotional conditions.

Stress also interferes with pregnancy outcome and offspring development. Women exposed to stress during pregnancy are at a greater risk for spontaneous abortion, obstetric complications, preterm delivery, low birth weight and infant mortality, growth abnormalities, and unbalanced sex ratio in the short term (Catalano et al., 2005; Dole et al., 2003; Orr et al., 1996; Paarlberg et al., 1995). Long term emotional (O'Connor et al., 2002; Van den Bergh, & Marcoen, 1994; Huttenen, & Niskanen, 1978; Stot, 1973) and cognitive (Mulder et al., 2002; Brouwers et al., 2001; Van Os, & Selten, 1998; King, & Laplante, 2005) impairments are reported in progeny.

The effects of stress during pregnancy in offspring have been extensively investigated mainly in rat models using physical restraint. Similar short term effects as those reported in humans can be observed using this method (e.g. Barlow et al., 1978). Offspring behavioural impairments have been reported during the juvenile phase: decreased motor ability (Grimm, & Frieder, 1987; Barlow et al., 1978), diminished

spatial learning in the Morris Water maze (Yaka et al., 2007), increased defence-withdrawal behaviour (Dickerson et al., 2005), increased activity, decreased spontaneous alternations in the Y-maze, decreased delayed alternation in the T-maze (Gué et al., 2004), and reduced social play behaviour (Morley-Fletcher et al., 2003). A variety of long term effects of prenatal stress have been observed in the adult rat also mirroring the human condition: cognitive impairments (Lordi et al., 2000), altered sexual behaviour (Herrenkohl, 1986), increased anxiety levels (Vallée et al., 1997), and symptoms akin to depression (Alonso et al., 1991) that coincide with dysfunction of the HPA stress axis (Bosch et al., 2006). Also see Maccari et al., 2003, and Weinstock, 2001 for review.

Conversely, the impact of stress during pregnancy on behaviour of mother rats has received little attention limiting our understanding of the restraint model. The effects of stress during pregnancy in offspring are theorised to be mediated by both prenatal and postnatal environments; maternal care is the predominant contributing factor of the latter (Peters, 1998; Powers, & Moore, 1986). It is well documented that the quality of maternal care is a strong determinant of offspring emotional and cognitive outcome in both humans (De Bellis, 2002; Heim et al., 2000) and animals (Uriate et al., 2007; Champagne, & Meany, 2006; 2001; Champagne et al., 2003; Cirulli et al., 2003; Meany, 2001; Pryce et al., 2001; Liu et al., 2000). Alterations in maternal care following gestational stress may also contribute to or mediate the effects of such stress on offspring development. In many studies, the quality and frequency of maternal care is observed to be diminished by exposure to restraint stress (Champagne, & Meaney, 2006; Patin et al., 2004; Smith et al., 2004; Maccari et al., 1995; Moore and Power,

1986; Power and Moore, 1986), while in others, a change in maternal behaviour is not observed (Polytrev, & Weinstock, 2005; Pardon et al., 2000; Fameli et al., 1995; Herrenkohol, & Whitney, 1976). These inconsistencies are likely caused by a combination of two factors expected to influence the response to stress in this context. First, the ability of stress to alter maternal behaviour could vary between strains of rat and individuals of the same strain. Second, variability in stress regimes would alter the outcome; of particular relevance, the delay between the end of stress exposure and parturition (a greater delay may lead to a diminished impact on maternal behaviour), and the severity of the procedure. The latter point is affected by a number of variables, namely stressor type, exposure duration within stress periods and across gestation, and whether the stress schedule protects against anticipation and habituation.

Understanding the effects of stress during gestation in the mother rat is also important for its own sake. In virgin female rats, the HPA axis is compromised by prolonged exposure to chronic stress resulting in the expression of depressive-like behaviours (e.g., Baker, & Bielajew, 2007). This appears to be the case in the pregnant rat as well; restraint and intruder stress applied between gestation day (GD) 4 and 18 significantly elevated ACTH and corticosterone levels to a mild stressor in a strain of rat bred for high anxiety (Neumann et al., 2005). Behaviourally, mother rats exposed to stress in late gestation have been observed to display enhanced immobility in the forced swim test during the lactation period (Smith et al., 2004). This finding suggests that gestational stress increases depressive-like symptoms during the postpartum period. The effect, however, appears to be transient and specific to the lactation period. Mothers tested after weaning show no such effect as reported by Darnaudéry et al.

(2004). Interestingly, in the latter study, anxiety-like behaviour was also examined and observed to be increased in the Elevated Plus Maze (EPM) test, indicating that gestational stress exposure may have a longer lasting influence on symptoms related to anxiety than to depression (Darnaudéry et al., 2004).

As changes in emotionality before parturition have not previously been examined in pregnant rats, it was our interest to determine if differences in anxiety would be detected between groups before birth and the complex, demanding tasks of motherhood began. This would more effectively link a change in anxiety with the stressor and eliminate the interaction between stress and mothering. To this end, the current study applied a quasi-random protocol of restraint to pregnant rats between GD 10 and 19. Stress was applied during the second half of gestation so as to limit the global impact of the manipulation on offspring and focus it on a period during which higher brain systems, such as the stress axis, are developing in the foetus (Weinstock, 2001). Restraint was applied daily in a random fashion in an attempt to mirror more closely the human condition where stress is often persistent, uncontrollable, and unpredictable. Maternal anxiety was assessed in the EPM before parturition on G 20 and maternal behaviour was monitored during retrieval tests administered on postnatal (PN) days 2, 4, 6, 8, 10, and 12. Litter composition (initial weight and number of offspring and sex ratio) was also recorded. The EPM and emergence tests were employed to assess anxiety, and the T-maze test was used to assess cognitive function in a subset of juvenile offspring between PN 30 and 31. Weight was also monitored throughout the study in both the mother rats and offspring. The experimental procedures are represented in a timeline in Figure 10.

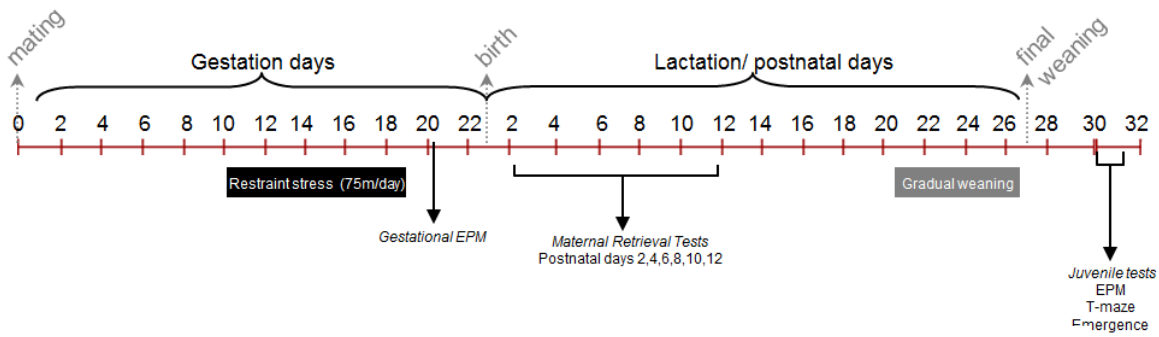


Figure. 10. *Timeline of events.*
Gestation lasted for 23 days. Chronic restraint stress was applied to stressed females from G 10 - 19 after which they were tested in the EPM. After parturition, maternal behaviour was assessed in dams (G 2-12). Culled offspring were kept in the maternal cage until weaning between PN 21 and 27 (lactation period). Anxiety and cognition were assessed in juvenile offspring 4-5 days post weaning.

Methods

Ethical approval for this project was obtained by the institutional protocol review group at the University of Ottawa in accordance to the guidelines from the Canadian Council on Animal Care. The experimental protocol is schematized in Figure 10 and in relation to the long term project, Figure 1 (left).

Prenatal Manipulations and Measures

Animals and Breeding Procedures

Twenty-one virgin female and fourteen, male Long Evans rats were obtained from Charles River Laboratories, St.-Constant, Québec. All rats were housed in standard-sized cages (24 cm wide x 43 cm long x 20 cm high) and maintained on a 12h light/12h dark cycle (lights on at 7:20 A.M.). Food and water were available ad libitum, cages were changed weekly, and clean nesting material (Nestlets®) was provided in the females' cages.

Once females were determined to be in proestrous, based on vaginal lavage examination, breeding began. A detailed description of the vaginal lavage technique has been reported previously (Marcondes et al., 2002). Rats were placed together in a 2:1 female: male ratio in large translucent, plastic cages (37 cm wide x 48 cm long x 20 cm high). Vaginal samples were collected the following morning (9AM). A successful breeding was indicated by the presence of spermatozoids and cells typical of the receptive stage of the cycle, estrous, and was counted as postnatal day one. When pregnancy was determined, the female rats were removed from the breeding cages and housed individually in standard cages containing bedding and nesting material.

Gestational Stress Procedure

Pregnant rats were randomly assigned to either a stressed (n=9) or control group (n=12). In the stressed group, restraint was applied during late gestation (PN 10-19); control rats received no such manipulation. Both groups were otherwise maintained in identical conditions. They were weighed daily between 9 and 11AM (before conception until GD 22), and every second day beginning on day 2 of the postpartum period. All pregnant rats delivered on GD 23. One rat from the stressed group had a sudden weight loss coinciding with the onset of the restraint, suggesting a spontaneous abortion and was thus removed from the study.

Restraint stress was applied in one, two, or three blocks per day for a total daily duration of 75 minutes using a random schedule. Stress was administered in the home cage during the light phase, between 9AM and 6PM. The restraint apparatus was created from a clear plastic water bottle (18 cm tall, 6.5 cm in diameter).

Gestational EPM

On GD 20 (the day after the end of stress period), maternal anxiety was assessed in the EPM. This test creates a conflict between the aversion to an exposed, open, and raised platform and the motivation to explore new environments. Highly anxious rats are expected to spend less time exploring the open arms than less anxious rats (Pellow et al. 1985).

The maze consisted of two open and two enclosed arms, each 38 cm long X 14 cm wide with the enclosed arm wall height of 30 cm. The arms extended from a center platform (14 cm^2) with an elevation of 33 cm from the floor. At the beginning of each 15 minute test, the rat was placed in the center platform facing a closed arm. The entire session was recorded via a digital recorder. All tests were conducted during the light phase. A blind observer scored the tapes and recorded the duration and frequency of the following: entries in the open arms, closed arms, and middle platform, as well as grooming, rearing, and edge behaviour (leaning over or rearing at the edge of the open arm). The rat was considered to have entered an arm when all four paws were within its boundaries. At the end of each test, the interior of the maze was cleaned with a 95% alcohol solution.

Postnatal Measures

Litter Composition

The number of live and non-surviving offspring per litter, sex ratio, and litter weight were recorded on PN 2 prior to the first maternal test. At this time, each litter was culled to five males and five females when possible; only occasionally was this ratio off by one.

Maternal behaviour

Maternal care during the first two weeks postpartum was assessed via maternal retrieval tests that were conducted every second morning (between 9 and 11AM) beginning on PN 2. Prior to the test, the nest site was noted and the dam and litter were removed from the cage and weighed. The mother was then returned to the home cage, and her pups were placed in a location diagonally opposite the nest site. The duration and frequency of the following behaviours were recorded every five seconds for ten consecutive minutes: retrieving (carrying a pup to the nest), non-retrieval pup carrying, pup licking, pup sniffing, hovering (over pups without nursing while engaged in another behaviour such as pup-licking or sniffing), nursing postures (with a distinction between low crouch or high crouch), nest building, sniffing air, and self grooming. Each test was recorded on a digital video camera and later scored by a blind observer.

Two hours after the end of the retrieval test, a spot check was performed in which the mother's behaviour was directly observed and recorded every minute for ten consecutive minutes. The behaviours on the check list included those described above in addition to the notation of the number of pups in the nest.

Offspring Weaning

To minimize the stress induced by separation during weaning, a gradual weaning procedure was used similar to that previously described (see Cook, 1999). Pups were removed in same sex sibling groups from the dam once daily from PN 21 until final weaning on PN 27. The length of separation doubled every second day (PN 21 & 22-3hrs, PN 23 & 24 – 6hrs, PN 25 & 26 – 12hrs). All offspring were removed from the

maternal cage and placed into a similar sized cage that contained new bedding mixed with bedding from the maternal cage; the same cage was used for all separations. Each cage contained two small, plastic opaque tubes and nesting material. After the final weaning, all cages were changed weekly and individual weights were recorded every four days.

Offspring behaviour

Behavioural tests were conducted in offspring from five stressed and six control mothers. To minimize the effects of repeated testing and in order to assess all offspring during PN 30 and 31, each same sex litter group was randomly split into two groups. Anxiety-related behaviour was examined in the first group via the EPM. The second group was tested in a delayed alternation task of spatial memory (T-maze) and a test of anxiety (emergence). Half of the animals were evaluated in the T-maze first followed by the emergence test, and the other half in the reverse order.

Juvenile EPM

A smaller EPM was constructed for the juvenile test. The open and enclosed arms were each 25 cm long X 10 cm wide extending from a middle platform (10 cm^2). The wall height of the enclosed arms was 31 cm. The platform was elevated 32 cm from the floor. The procedure for testing juveniles in the EPM was identical to that described above for mothers with one exception – the inclusion of measures of risk assessment stretch attends (the rat stretches forward and retracts without moving its paws) and head dips (the rat leans over the edge of the open arm) in the offspring groups.

Juvenile emergence test

The emergence test consisted of a dark, opaque plastic box (17 x 14 x 9.5 cm^3) with a small opening (6cm wide x 5 cm high) set in a well-lit open field (37 cm wide x 48 cm long x 20 cm high). At the beginning of the test, a rat was placed inside the box and the latency to fully emerge (all four paws) into the open field was recorded. The frequency of nose pokes and half-body emergences (two front paws) was also recorded. The test ended after the rat fully emerged or 10 minutes had expired.

Juvenile T-maze

The T-maze apparatus was constructed of Plexiglas® with the main branch 55 cm x 15 cm and the two secondary branches each 40 cm x 15 cm. The sides were opaque and a translucent lid covered the entire maze. Three trials were administered separated by a 20-30 minute interval between trials. Each trial consisted of a forced choice test followed within 30 seconds by a free choice test. During the forced choice tests, left and right secondary arms were alternately blocked across trials and sex and family groups. In the free choice trial, both arms were open for exploration and responses were either scored as correct - the unexplored, previously blocked arm was selected or as incorrect - the explored arm was selected. Rats that remained in the main branch for more than five minutes during the forced choice session were removed from the maze and returned 20 to 30 minutes later. Occasionally, some rats failed to respond even after a second exposure; these trials were excluded from the analysis. At the end of each test, the rat was removed and the entire maze cleaned with a 95% alcohol solution to mask olfactory cues.

Statistical analyses

All data were analysed with SPSS (V. 15) software (SPSS Inc., 2007). Weight and duration data were evaluated using parametric procedures (typically mixed ANOVAs) and frequency data via non-parametric statistics (chi-square or Mann-Whitney *U*). The Huynh-Feldt correction for violations of sphericity (Howell, 2007) was applied to parametric analyses when appropriate (repeated factors having more than two levels). The degrees of freedom reported in the Results section are adjusted if the correction was used. The family-wise error rate was maintained at 0.05 in the case of multiple post-hoc comparisons using a Bonferroni correction.

ANOVA designs were used to assess the following data: maternal daily weight during the first 20 days of gestation and maternal weight from PN 2 to weaning; male and female juvenile offspring weights PN 2 to weaning based on the average weight per pup per family group and male and female individual pup weight after weaning on PN 28, 32, 36, and 40; the duration of maternal care; maternal and juvenile EPM duration in open, closed, and center platform and the duration of any other scored behaviours in these compartments (e.g., grooming – see methods for detail); and latency to emerge from the dark box.

Non-parametric procedures were applied to litter size and composition (male:female ratio); frequency of pup retrieval; number of maternal and juvenile open and closed arm entries in the EPM test; counts of juvenile nose pokes and two paw exits from the dark box; juvenile correct choices in the T-maze.

Results

Maternal weight

The maternal weight data from GD 1 to PN 12 are plotted in Figure 11, showing the expected weight gain during gestation ($F(19, 304) = 380.63$, $p = 0.001$). An interaction between group and time was also found, at this time, due to a significantly lower rate of weight gain in the stressed rats relative to their control counterparts ($F(19, 304) = 16.67$, $p = 0.001$). Based on pair-wise comparisons, group differences began on GD10 and continued until GD 20 (all comparisons were significant at the 0.001 level). Note that there was no significant difference between groups before mating. The group difference on GD 20 was not accounted for by between group variations in litter composition, using pup weight as a covariate. In the analysis of the post parturition weight data, dams from both groups gain weight at a similar rate over time ($F(5,47.546)=33.78$, $p = 0.0001$). The overall group difference was persistent, however, with all pairwise between-group comparisons significant at the 0.008 level with the exception of week eight.

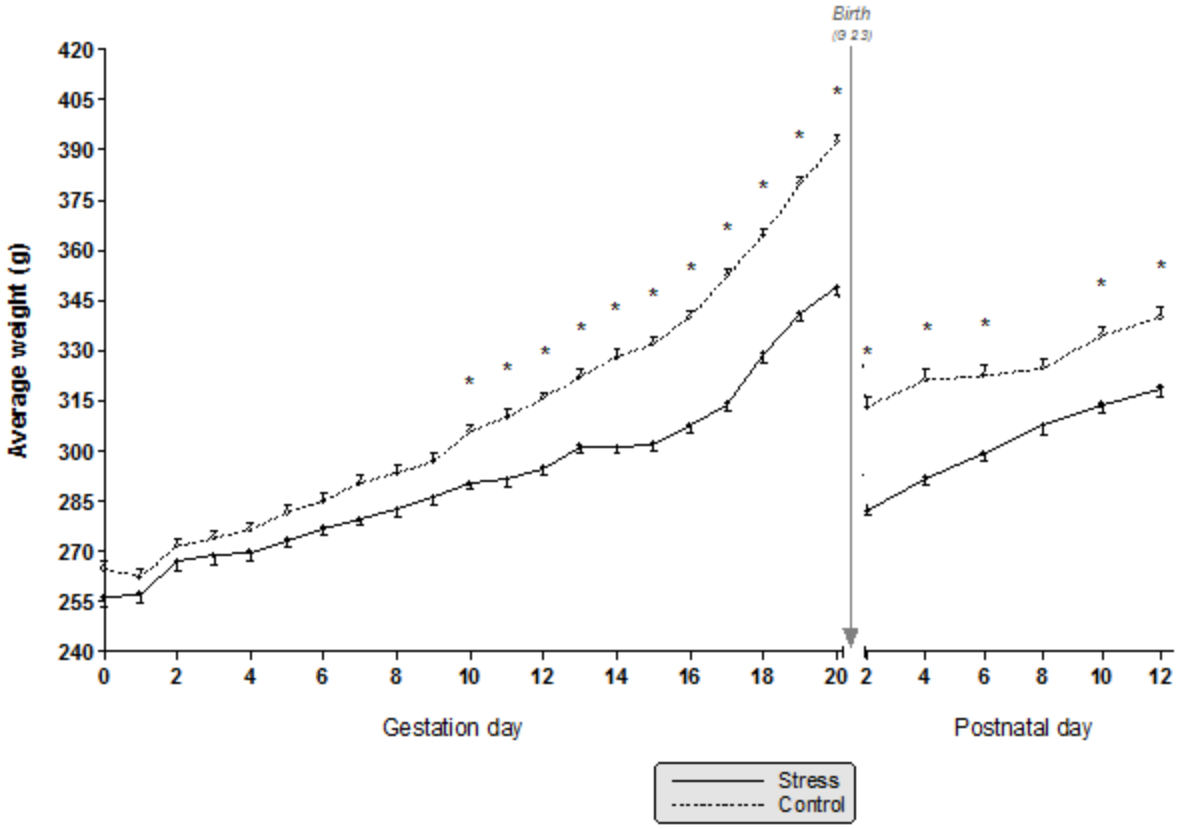


Figure. 11. *Maternal weight.*
*Data represent the average body weight (±SEM) plotted over time, daily from conception to G 22 and every second day from PN 2 to 12. * indicates p < 0.001, stress group versus control group.*

Gestational EPM test

Duration data associated with the gestational EPM tests are located in Figure 12 illustrating time spent in open arm, closed arm, and middle sections (part A). The same data are shown in 5 minute segments (part B). A main effect of time was obtained in all three analyses: closed arm ($F(11.05, 165.78) = 2.58$, $p = 0.005$), open arm ($F(12.08, 181.24) = 2.34$, $p = 0.008$), and middle section ($F(7.79, 116.79) = 3.66$, $p = 0.001$); ignoring group membership, rats increased time in the closed arm across minutes while the opposite pattern was obtained for both the middle and open arms. Significant group

differences were only found in the analysis of the middle section ($F(1, 15) = 4.40$, $p = 0.053$), with stressed mothers spending less time there. Time and group interactions were obtained for both the closed ($F(11.05, 165.78) = 2.07$, $p = 0.025$) and open arms ($F(11.05, 165.78) = 2.07$, $p = 0.025$). Overall, stressed pregnant females spent significantly more time (13%) than control rats in the closed arms. Interestingly, time distribution among maze locations is almost identical between groups when only the first 5 minutes are considered. A striking pattern emerges between groups after five minutes in the maze: stressed rats increased time and control rats decreased time spent in the closed arm.

The frequency of arm entries is shown in Figure 12 C. The control group was generally more active than the stressed group in that they displayed a higher number of arm entries ($\chi^2 = 11.27$, $p = 0.001$). Separate analyses conducted on the open and closed arms revealed a group difference in only closed arm activity ($\chi^2 = 9.41$, $p = 0.002$); this was due to a greater number of entries by control animals.

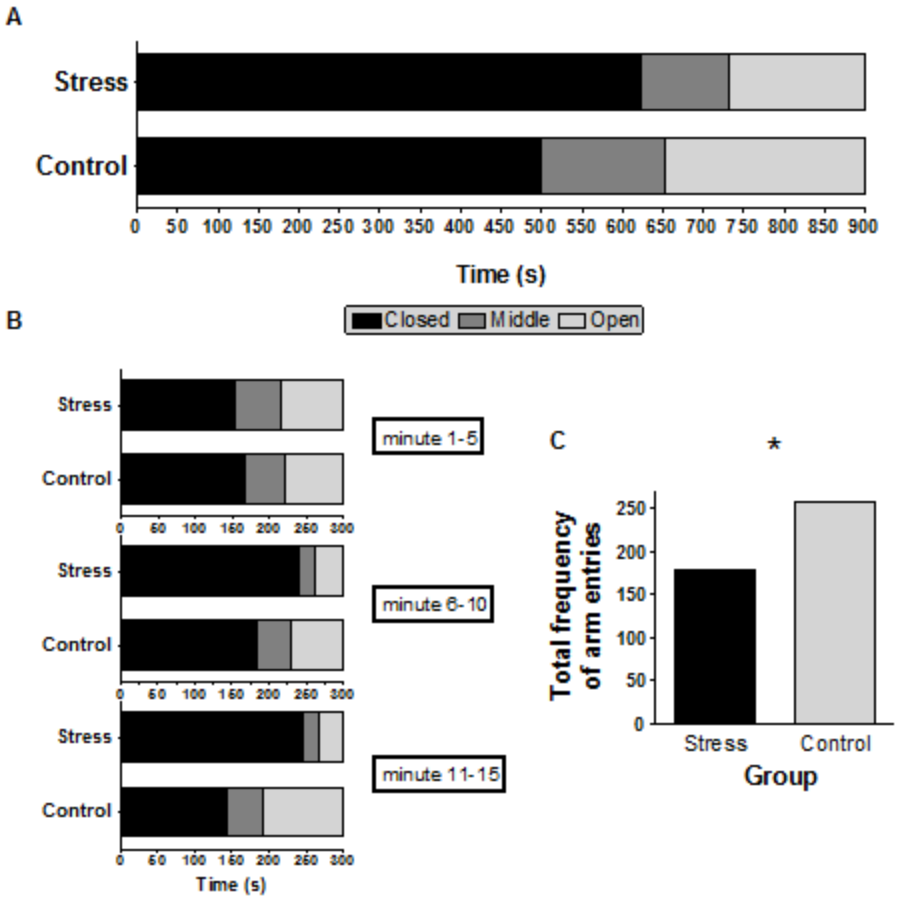


Figure. 12. *Gestational EPM.*
*A represents group means of total duration in seconds recorded in closed, middle, and open sections of the maze. The same data are displayed in five minute segments in B. The total frequency of arm entries for each group is displayed in C. Significant effects are not identified for A or B; * denotes $p < 0.001$ in C.*

Analyses were performed only on behaviours in the closed arms that were observed for more than 20 seconds; negligible values were obtained for behaviours in the open and middle sections. Control animals spent significantly more time rearing ($F(1, 15) = 4.70$, $p = 0.047$), while stressed animals spent more time self-grooming ($F(1, 15) = 32.44$, $p = 0.00004$) (see Figure 13). No other effects were found.

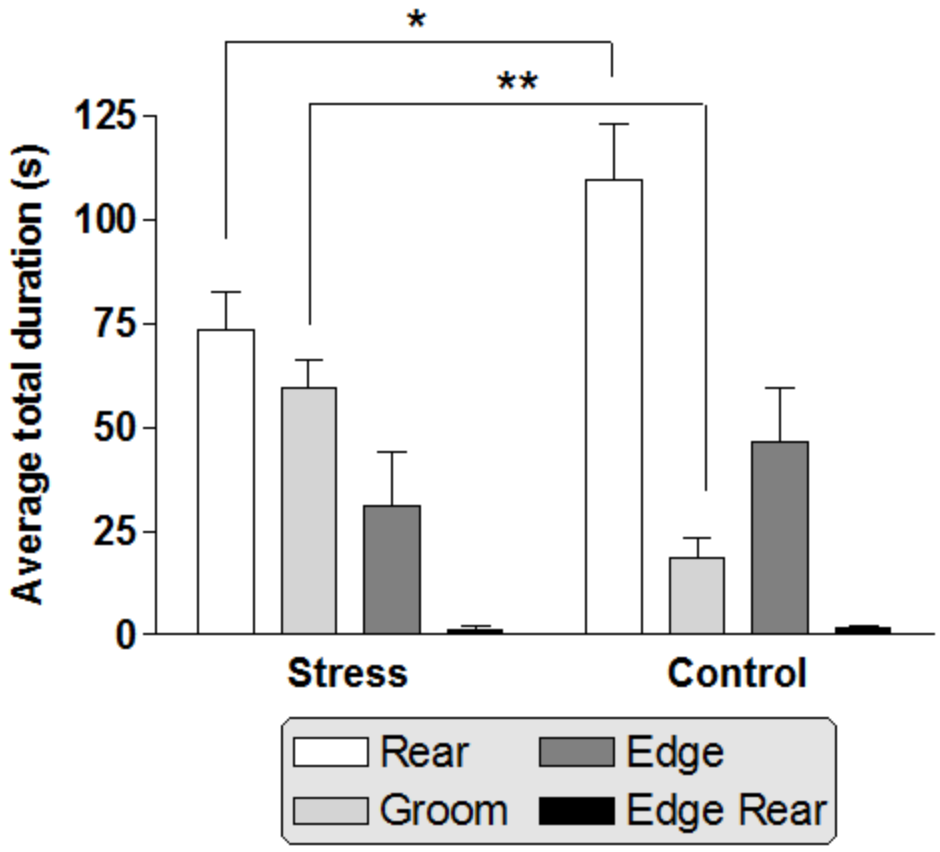


Figure. 13. *Gestational EPM, behaviour.*
*Average (±SEM) total duration engaged in rearing, grooming, and edge behaviour is divided by group. * p < 0.05, ** p < 0.0001 group differences.*

Litter characteristics

The pre-culled litter size was significantly different between groups (U = 5.50, p = 0.026). Stressed mothers delivered fewer offspring, including both live- and still-births. The pre-culled average weight per pup, however, was not different between groups, although the variability in pup weight was higher in the stressed group. The offspring mortality rate in the stressed group was 5 of 67 almost twice that of the control group – 3 of 93 (7.46% versus 3.23%, respectively). See Figure 14 for graphs pertaining to initial litter number (A) and weight per pup (B).

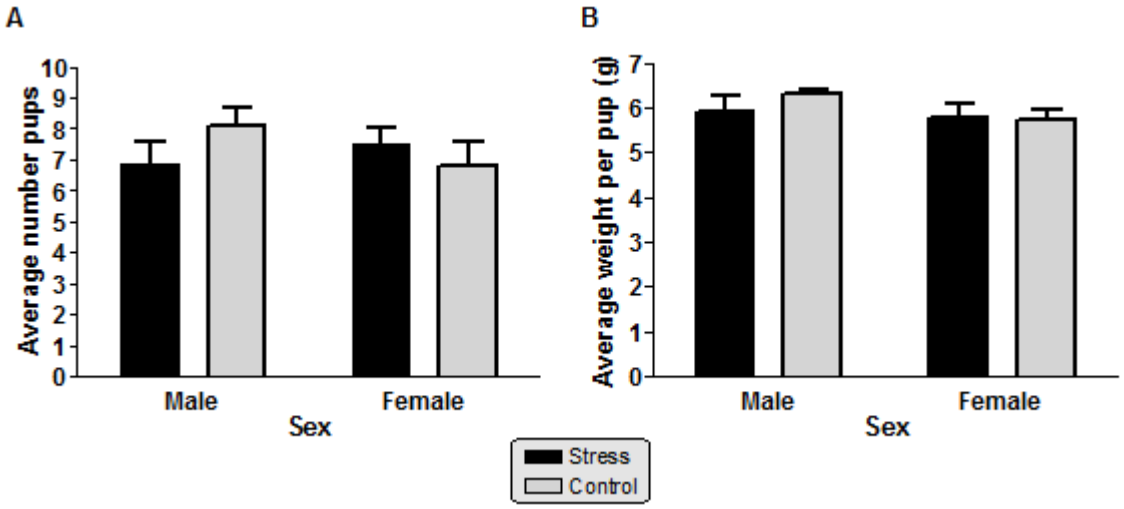


Figure. 14. *Litter composition.*
Mean (±SEM) numbers of preculled live offspring per group are plotted in A. B represents preculled average (±SEM) of weight per pup. In both graphs, the data associated with male groups are on the left and female groups on the right.

Maternal Retrieval test

On almost every test, all dams retrieved at least 50% of their displaced pups during the 10 minute test; there was no difference in the number of pups retrieved between groups. The duration of maternal behaviours scored is presented in Figure 15; the values reflect within-group averages across the six tests. Unstressed mothers spent significantly more time engaged in overall pup care (an average of time spent licking, hovering over, carrying, and sniffing the pups) than stressed mothers ($F(1, 16) = 7.41$, $p = 0.015$). The analyses of individual behaviours revealed that this difference was due primarily to significant group differences in licking ($F(1, 16) = 4.27$, $p = 0.055$) and hovering ($F(1, 16) = 5.37$, $p = 0.034$).

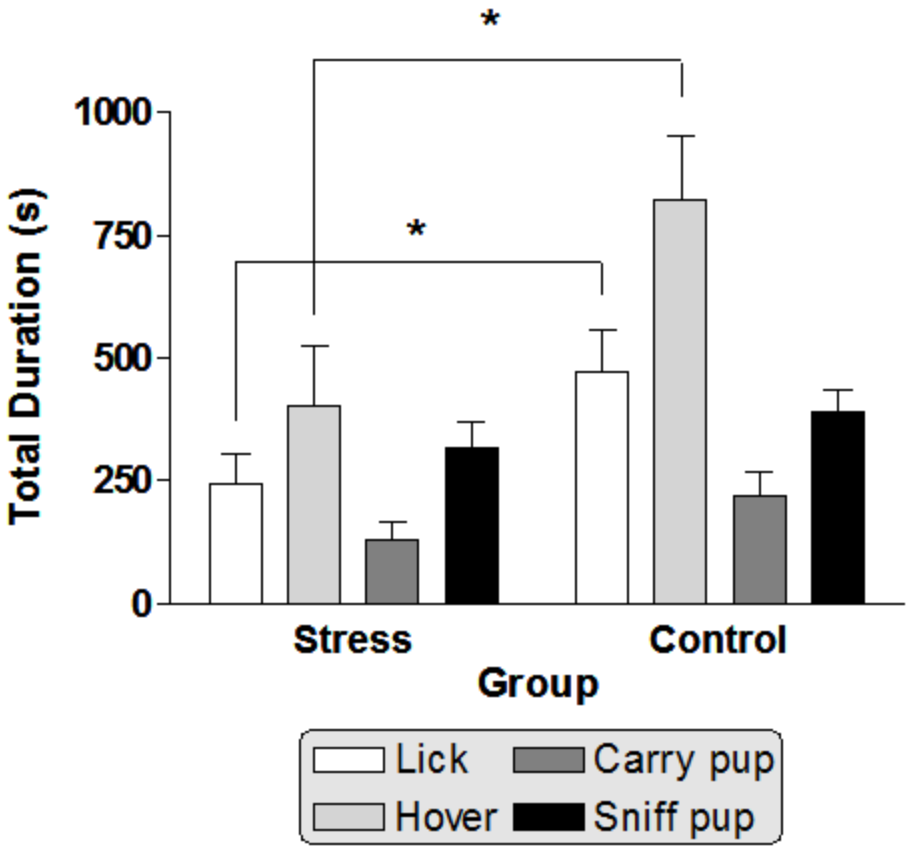


Figure. 15. *Retrieval test.*
*Data represent the mean (±SEM) total duration in seconds in four pup-directed care behaviours averaged across the six tests for stressed (left bars) and non-stressed (right bars) dams. The * indicates group differences p < 0.05.*

Offspring weight

The average weight per pup from PN 2 to 40 is represented in Figure 16. The analysis of weight per pup before weaning (PN 2 – 24) showed the expected weight gain over time in all groups (male analysis: $F(2.02, 18.19) = 566.06$, $p = 0.0001$; female analysis: $F(1.70, 15.27) = 462.14$, $p = 0.000001$). As evident in the figure, no group differences occurred in the analyses of male or female weight in the preweaning period; note, a near significant difference was obtained between the male groups, $p = 0.066$. The analysis of individual weights from PN 28 (weaning) to 40 in both male and female

offspring resulted again in significant effects of time (male analysis: F (1.55, 80.76) = 2023.67, p = 0.0001; female analysis: F (1.40, 71.39) = 1519.22, p = 0.0001). A main effect of group (F (1, 51) = 7.19, p = 0.01) and an interaction between time and group (F (1.4, 71.39) = 6.27, p = 0.008) were also obtained in the analysis of female weight. Follow up tests performed on female post weaning weights revealed that female offspring from stressed mothers showed a reduced rate of weight gain after weaning relative to their control cohort on PN 36 and 40 ($p < 0.0083$).

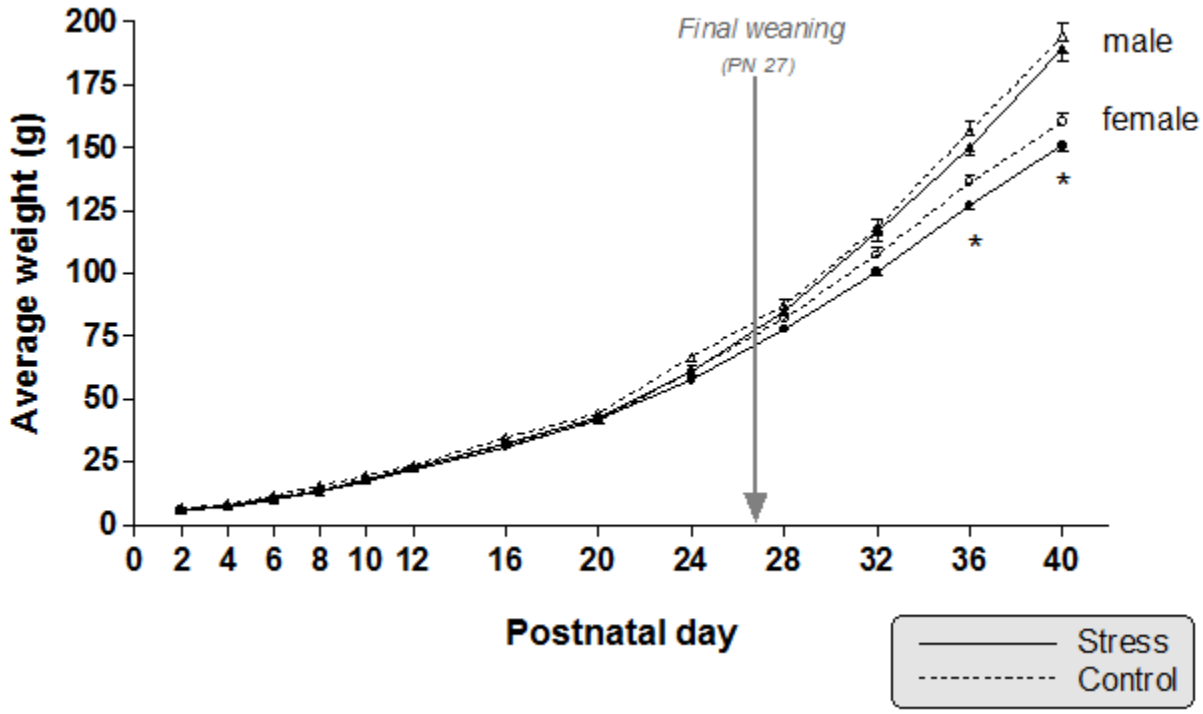


Figure. 16. *Offspring weight.*
*Mean (±SEM) offspring group weight over time. PN 2 – 12 data represent weight measures recorded every second day and PN 12 – 40, every fourth day. Preweaning weights PN 2 – 24 are based on the total male and female litter weight divided by number, and post weaning weights (PN 28 - 40) are based on the average of individual pup weights. * indicates significance between female stressed and control groups, $p < 0.05$.*

Offspring EPM test

Figure 17 shows the time spent in the open, middle, and closed sections of the EPM. Generally, time spent in the open arm tended to decrease throughout the test. The analyses of these data produced a significant three way interaction between group, sex, and time ($F (3.89, 170.97) = 2.40$, $p = 0.054$). Follow up tests did not reveal any consistencies across time between groups as a function of sex or manipulation. A significant group effect was obtained for the analysis of time spent in the middle section ($F (3.04, 133.92) = 20.48$, $p = 0.0001$). There was a difference across minutes in the analysis of the closed arms ($F (4, 176) = 11.35$, $p = 0.0001$), with an increase after the first minute. All groups spent roughly 80% of the test period in the closed arms.

Given the time distribution it was not surprising that the duration of individual behaviours was negligible in almost all groups in the open and middle sections – most were below 1 second. Behaviour in the closed arm was therefore only examined. A main effect of time was obtained for the analyses of rearing $F (3.36, 147.61) = 2.95$, $p = 0.029$ and grooming ($F (1.77, 77.73) = 21.13$, $p = 0.0001$) behaviours. Rats generally increased duration of these behaviours after the first minute. The examination of the data on the basis of group showed that rats from different groups demonstrated distinct patterns of behaviour. Males from stressed mothers increased the amount of time displaying protected head dips across minutes while their control counterparts maintained a consistent level. In the female groups, rats from stressed mothers were stable over time while control animals decreased time performing this behaviour. The results of analyses performed on stretch attend behaviour were not reported as these values were negligible (below 1 second).

Activity level within the EPM in juvenile rats, as measured by total frequency of arm entries, was dependent upon group ($\chi^2 = 18.37$, $p = 0.00002$). Male and female rats displayed opposite patterns of activity. Juvenile males from prenatally stressed mothers had a significantly lower number of arm entries compared to control male rats ($\chi^2 = 4.58$, $p = 0.032$), whereas the opposite pattern was observed in the female groups ($\chi^2 = 79.97$, $p = 0.01X10^{-6}$).

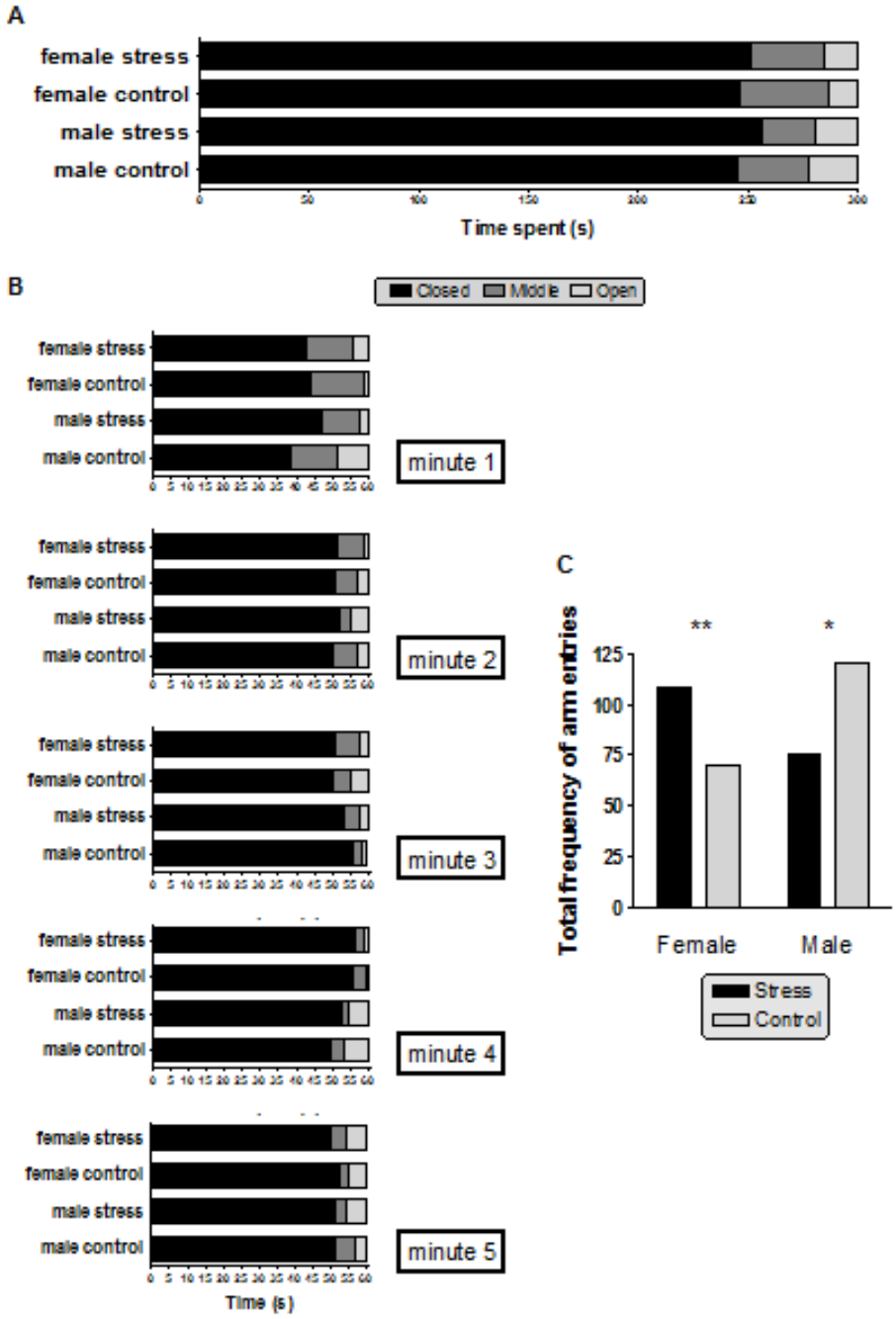


Figure. 17. *Offspring EPM.*
*A represents group means of the total duration in closed, middle, and open sections of the maze. The same data are shown in figure B, divided into one minute segments. The total frequency of arm entries (±SEM) for each group is displayed in C. * $p < 0.05$, ** $p < 0.0001$ group differences in C. Significant effects are not indicated in A or B.*

Offspring Emergence test

Data associated with the emergence test are depicted in Figure 18, showing the average group frequency of nose pokes and two paw exits from the dark box. There were no significant differences in latency to emerge across groups or sex; however there was a trend for females from the stressed group to take longer to emerge when compared to the female control group ($p = 0.082$). The analyses performed on frequency of nose pokes revealed a significant effect of group ($\chi^2 = 7.94$, $p = 0.005$). This was due primarily to the increase in nose pokes observed in the stressed female offspring which was significantly different from the female control group ($\chi^2 = 6.26$, $p = 0.012$). This pattern was not evident in the analysis of the two-paw frequencies.

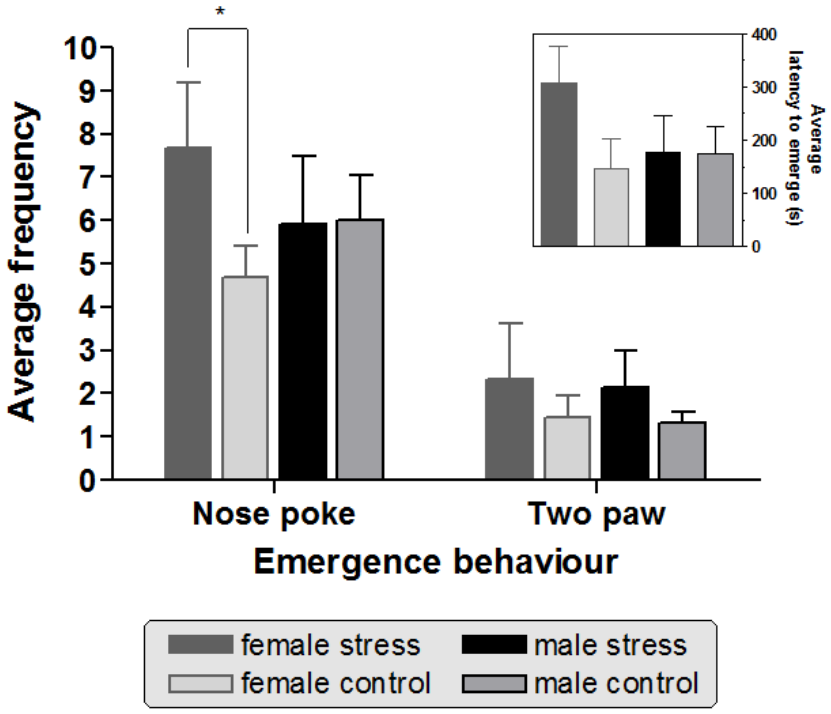


Figure. 18. Offspring emergence test.
Main graph represents average frequency (±SEM) of nose pokes and two paw emergences per group. Insert graph displays the mean (±SEM) latency to emerge as a function of group. The * indicates group difference $p < 0.01$.

Offspring T-maze test

All groups responded at or just above 50%, chance level. Although the female offspring showed the greatest discrepancy between stress groups in number of correct responses, no significant differences were found. There was a trend for the female stressed group to fail to respond during the free choice test.

Discussion

The aim of this study was to assess the physical and behavioural effects of gestational stress in mothers and their juvenile offspring. Restraint stress applied on GD 10 through 19 had physiological and behavioural consequences in the mother rat. It attenuated prenatal weight gain, an effect that persisted after birth, induced one spontaneous abortion, increased anxiety-like behaviour, elevated the mortality rate, diminished total number of offspring, and reduced pup-directed licking and hovering behaviours. The effects in the offspring were subtle and only evident in juvenile females: post weaning weight was reduced and anxiety-like behaviour was marginally elevated.

Maternal Outcomes

The maternal weight data from the current study suggest that chronic restraint during gestation had a pronounced, lasting effect. Stressed mothers had a significantly decreased rate of weight gain; the group difference endured beyond stress exposure into the second postpartum week. This effect has been reported by others (Van den Hove et al., 2006; Darnaudéry et al., 2004; Barlow et al., 1978). Given that stress altered pregnancy success, in the current study, by reducing the number of offspring

produced, it was important to consider if our group difference in maternal weight gain reflected this effect. The covariate analysis of body weight on GD 21 indicates that the effects of gestational stress on maternal weight were independent of its effect on litter size and weight.

As food intake was not monitored, it is unclear if the stress-induced decrease in weight was caused by a reduction in caloric consumption. A possible group difference in food access may be a contributing factor. Food and water were not available during restraint (total of 90 minutes of daylight) in the stressed group, while control animals had constant access. This would not be expected to produce the robust group effects observed, given the relatively brief stress period, but it does present a difference between groups. Another possibility may be the reaction to the restraint procedure itself. Body temperature may have increased during restraint coupled with some physical struggle, which could have reduced weight gain. Others have linked the elevated temperature during restraint stress with weight loss (Alonso et al., 1991). However, we regularly observe similar decreases in weight gain in non-pregnant female rats in response to chronic non-restraint stress (Baker, & Bielajew, 2007; Baker et al., 2006) suggesting that the observed decrease in weight gain was a result of the stress procedure.

Stress during the second half of gestation also had a profound effect on behaviour in the EPM. To our knowledge, this is the first report to administer the EPM late in the gestation period. Findings of Darnaudéry et al. (2004) suggest that anxiety induced by gestational stress may last well into the postnatal period. They observed

elevated anxiety in a five minute EPM test administered after weaning in addition to a reduction of exploration in the open field.

Our analysis of behaviour in a 15 minute EPM test indicated that both groups displayed similar profiles in the first five minutes, after which stressed rats decreased and control rats increased time in the open arm. It appears that control rats overcame anxiety induced by the initial exposure to the EPM while stressed rats did not. Typically, behaviour in the EPM test is observed for five minutes. Our results suggest that longer test periods are needed to determine the effects of stress on anxiety. The initial five minutes might reflect anxiety provoked by a novel stimulus, which in our case was not different between groups. A longer observation period, such as the 15 minute test employed in the current study, may be more telling of the general state. In this view, pregnant rats exposed to stress in the current study had a general state of elevated anxiety.

Maternal care measured in the retrieval test was also affected by gestational stress; specifically, licking and hovering were diminished in stressed mothers. Previous studies employing chronic gestational stress regularly show an effect of stress on pup licking (Champagne, & Meaney, 2006; Patin et al., 2004; Moore, & Power, 1986; Power, & Moore, 1986). Other behaviours that have been reported to be altered in response to gestational stress not observed in the current study include arched-back nursing and nesting/grouping pups (Smith et al., 2004), and retrieval (Patin et al., 2004). A few reports have observed no changes in maternal behaviour in response to gestational stress (Poltyrev, & Weinstock, 2005; Fameli et al.,1995; Herrenkohol, & Whitney, 1976). Effects observed in the mothers from the current study demonstrate that stress during

late parturition has both physiological and behavioural consequences that are present before motherhood begins. The prepartum anxiogenic profile is a possible cause of reduced maternal care behaviour expressed in the postpartum period.

Offspring Outcomes

In contrast to the dramatic findings in mother rats, the results from offspring measures were less clear. There was a minimal range of behaviour exhibited in the EPM making it difficult to interpret these findings. In the present experiment, stress had gender-dependent effects on activity (frequency of arm entries) with stress decreasing activity in male rats and increasing it in females. Stress and sex also interacted in the emergence test – female offspring from stressed mothers exhibited an anxiety-like response as they had a strong tendency for longer emergence latencies than all other groups. Furthermore, weight gain was altered in female offspring after weaning. These data support previous findings in adult rats that demonstrated sex dependent consequences of prenatal stress with females appearing to be more vulnerable (Weinstock et al., 1992; McCormick et al., 1995; Nischio et al., 2001; Bowmann et al., 2004; Gué et al., 2004). Furthermore, adult female rats typically display more anxiety and depressive-like behaviours than males in response to stress (Richardson et al., 2006; Bowman et al., 2004; McCormick et al., 1995; Alonso et al., 1991).

Increased anxiety-like behaviour has been reported in prenatally stressed male rats in the EPM at PN 60 (Patin et al., 2005) and PN 90 (Lordi et al., 2000). Studies employing both male and female rats suggest that sex differences in anxiety behaviour, based on open field and EPM performance, do not emerge until around 60 days of age

(Fernandes et al., 1999; Imhof et al., 1993; Masur et al., 1993; Johnston, & File, 1991). It is possible that, in the present experiment, effects could not be detected because offspring were too young to display robust behaviour in the anxiety measures used. Smith and Morrell report that comparisons between infant (PN 1 - 28) and adult (PN 55 <) behaviour in the EPM were inconclusive because infants slept during the test (2007). Furthermore, differential levels of anxiety-like behaviour have been reported between adolescent (PN 28 - 55) and adult rats in the light-dark box (Slawecki et al., 2005). Certainly, the underdeveloped nervous system may limit behavioural capacity under some test conditions and must be carefully considered. Contrary to our findings, in Wistar rats a few days older than those in our study, female offspring from mothers stressed by restraint displayed elevated anxiety in a five minute EPM test, but no change in activity (Zagron et al., 2006). The restraint procedure was milder that that employed in our study; however, all mothers were adrenalectomized or sham-operated prior to the stress period. Arguably, the stress experienced by these animals was much greater than in our study and may have produced more robust anxiety effects in the offspring. Similar to our findings, offspring in this report also spent the majority of the test period in the closed arm (70-85%); likely this time distribution observed in both studies reflects an age-related phenomenon.

In the current study, no effects of stress on T-maze performance at PN 30 were observed, unlike that of another group (Gué et al., 2004; Meunier et al., 2004) who report a gestational stress-induced impairment in younger rats (PN 25). This discrepancy may be due, in part, to differences in maze size – theirs was significantly smaller than ours. Gestational stress has induced deficits in other tests of spatial

learning and memory in both young (Y-maze (Gué et al., 2004); water maze (Nishio et al., 2001; Lemaire et al., 2000)), and mature (water maze (Zargon et al., 2006; Lemaire et al., 2000)) male offspring. However, others have reported no such impairments (Szuran et al., 2000). Together, this work suggests that gestational stress may have subtle effects on learning and cognition, at least in the juvenile period, such that variations in stress and testing procedures have significant effects on the outcome.

Conclusion

While the experimental design used in our report does not allow the independent effects of stress and maternal care on offspring measures to be analysed separately, the consequences of gestational stress in the offspring likely reflect the quality or quantity of maternal care experienced by these offspring (Neumann et al., 2005; Patin et al., 2002). This idea supports the work employing a cross-fostering program between control and mothers stressed during gestation (Pardon et al., 2000; Maccari et al., 1995; Power & Moore, 1986), and the maternal mediation hypothesis proposed for the effects of early handling on offspring development (Macri, & Wurbel, 2006). In the current study, we are unable to identify the path(s) of action of the stress regime: did it alter the mothers' behaviour toward her offspring, did it alter the behaviour of the offspring in some way as to elicit the type of care received or, most likely, were both affected and interacted.

Additionally, relationships between variations in maternal care received on offspring development also support this theory. Compared to offspring that receive low levels of maternal care, those that receive high levels of maternal care have an attenuated response to stress in terms of both physiology (HPA axis) (Liu et al., 2000;

Caldji et al., 1998) and behaviour (anxiety and maternal behaviour – Champagne, & Meaney, 2006; maternal behaviour - Champagne et al., 2003; fearfulness in response to novelty - Caldji et al. 1998).

Results from the current study indicate that elevated anxiety and diminished maternal care observed in stressed mothers do not necessarily translate into clearly observable changes in juvenile offspring behaviour. However, it is possible that effects may be latent in juvenile rats yet detectable in adulthood, an issue that will be addressed by examining the behavioural profile of these animals in a follow-up report. Finally, these findings also highlight the relevance of focusing on maternal responses to gestational stress for their own sake and will yield a more complete profile of the etiology of postnatal effects in offspring. Elucidating the impact of stress during pregnancy on mothers will further our understanding of this unique period and the potential development of stress-related conditions, like postpartum depression.

Study 4

Effects of Gestational Stress: 2. Evaluation of Male and Female Adult Offspring

Baker, Rees, Chebli, LeMarec, Godbout, Huta, & Bielajew. 2009. Brain Research, 1302, 194 - 204.

Abstract

Physical restraint applied during gestation is a commonly employed animal model of human pregnancy stress. The consequences of such a paradigm have been extensively investigated in adult male rats using a variety of physiological and behavioural measures. The behavioural repertoire of female offspring, however, has been largely ignored. The current study examines adult offspring - male and female Long Evans rats (55 – 90 days of age) and is a follow-up report to the consequences of maternal restraint (gestation days 10 through 19) in mother rats and their juvenile offspring. Physiological measures included weight and estrous cycle regularity. Elevated plus maze and emergence tests were used to measure anxiety, and the t-maze test, cognition. Data were analyzed via hierarchical linear modeling to account for the nesting of offspring within litters. Compared to same sex controls, males from stressed mothers displayed a progressive attenuated weight gain over experimental weeks while females from stressed mothers maintained a stable, lower weight throughout. Twenty-five percent of females in the stressed group and none in the control group displayed irregular cycles in the first week of testing; on subsequent weeks, this group discrepancy ranged from 1 - 11%. Subtle effects were observed in anxiety measures: an interaction between sex and stress group in the analysis of head dip behaviour in the elevated plus maze and decreased emergence latencies in stress groups. Results demonstrate the importance of examining the effects of maternal stress in offspring of both sexes at various developmental stages.

Keywords: Gestational stress; Estrous cycle; Weight; Anxiety; Cognition; Adult rat

Introduction

Epidemiological evidence has demonstrated a link between stress experienced by a mother during pregnancy and a variety of adverse outcomes in her progeny in both the short term - preterm delivery, low birth weight, infant mortality and growth abnormalities (Catalano et al., 2005; Dole et al., 2003; Orr et al., 1996; Paarlberg, et al., 1995), and throughout life - behavioral/emotional disturbances (O'Connor et al., 2005; O'Connor et al., 2002; Van den Bergh and Marcoen, 1994; Huttenen and Niskanen, 1978; Stott, 1973) and cognitive impairments (King and LaPlante, 2005; Mulder et al., 2002; Brouwers et al., 2001; Van Os and Selten, 1998) suggesting that prenatal stress may result in a general susceptibility to psychopathology in adulthood (Huizink et al., 2004). Furthermore, the immediate consequences of stress experienced during gestation may have negative effects in later life; for example, low weight at birth and poor growth within the first year is a strong predictor of the development of a variety of cardiovascular and metabolic disorders later in life, an effect that is independent from other environmental risk factors (for review see Barker, 2004). Taken together, human data suggest that pregnancy stress can be considered to have an important role in the direct or indirect prenatal programming of adult pathophysiology and psychopathology (Barker, 2004; Huzink et al., 2004).

Experimental gestational stress research has been pursued most extensively with a rat model using various types of stressors: conditioned avoidance training, tail suspension, forced swimming, predator exposure, crowding, tail shock, saline injection, immobilization, and, most commonly, physical restraint. Experimental findings generally parallel those in the clinical literature. Reported effects in juvenile offspring include

decreased motor ability (Grimm and Frieer, 1987; Barlow et al., 1978), spatial learning (Yaka et al., 2007), and social play (Morley-Fletcher et al., 2003), and in adult offspring, cognitive impairments (Lordi et al., 2000), altered sexual behaviour (Herrenkohl, 1986), elevated anxiety-like behaviour (Vallée et al., 1997), and symptoms akin to depression (Alonso et al., 1991), coinciding with dysfunction of the hypothalamic pituitary adrenal stress axis (Bosch et al., 2006).

In general, animal research has had a relatively narrow experimental focus with the emphasis on birth parameters such as sex ratio, litter size, weight, and the physiological, endocrine, and behavioural profile of adult, male progeny. Only a small number of reports have examined the responses of females (for example, see Burton, Lovic, & Fleming, 2006; Nishio et al. 2001; Szuran et al. 2000). The lack of inclusion of female responses is not representative of clinical findings: in humans, stress-related psychopathology such as anxiety and depression is more commonly experienced by women than men (Eaton et al., 2008; Kendler et al., 2000; Bebbington et al., 1998; Sprock, & Yoder, 1997). Furthermore, such disturbances are often apparent throughout life. Despite this, there is limited research on various developmental stages. Most studies investigate the consequences of gestational manipulations in offspring during early adulthood or, less commonly, the juvenile period. It cannot be assumed that gestational stress effects are static throughout life; thus, the examination of both male and female offspring at various developmental time points is critical for a more complete understanding of the impact of gestational stress throughout the lifespan.

There is evidence to suggest that the feto-placental unit may be altered during gestational stress, likely by elevated levels of glucocorticoids (Emack et al., 2008;

Mairesse et al., 2007; Hougaard et al., 2005); however, the role of postnatal environmental factors, namely maternal care, cannot be ignored. Like humans at birth, rats are not fully developed and maternal care is critical for normal development (deKloet et al., 2005). For example, the function of the hypothalamic pituitary adrenal stress axis can be altered by maternal care (Liu et al., 1997). Indeed, variations in maternal care alone have been shown to alter offspring anxiety and fearful behaviour (Champagne and Meaney, 2006; Caldji et al., 1998). The inclusion of measures of maternal care is necessary to fully understand the role of prenatal and postnatal factors in the consequences of stress during pregnancy.

The present study adds to the small but growing literature documenting the responses of rat offspring of both sexes following the application of gestational restraint stress. It is the second report in a series of experiments relaying the consequences of gestational restraint stress and attenuated maternal care behaviour in progeny at various developmental stages. Physiological indices included weight and estrous cycle regularity. Elevated plus maze and emergence tests were used to measure anxiety, and the t-maze test, cognition. These measures were chose to replicate those used in the juvenile phase (Baker et al, 2008) and because they are commonly employed in stress research. In addition, the execution of these measures is relatively brief allowing for all animals to be tested in close temporal proximity which yields more consistent results when evaluating rapidly developing juvenile rats and reducing the impact of estrous cycle stage in female rats (females tend to cycle together). The effects of gestational restraint stress applied on days 10 – 19 on maternal and juvenile measures are described in Baker et al. (2008). In this early report, mother rats stressed during

gestation displayed attenuated weight gain, elevated anxiety-like behaviour, and reduced maternal care; they also had fewer pups and an elevated offspring mortality rate. In the offspring, only juvenile females displayed marginal effects of gestational stress in the form of elevated anxiety-like behaviour and attenuated weight gain. The purpose of the current study was to determine if the results obtained in the juvenile test phase would translate into the adult phase, as hypothesized.

Method

This project was approved by the institutional protocol review group at the University of Ottawa in accordance with the guidelines of the Canadian Council on Animal Care.

Animals

Fifty four male and 53 female adult Long Evans rats, the offspring of parents (obtained from Charles Rivers Laboratories, St.-Constant Québec) bred in-house, were used in the current study. At postnatal day two, each litter was culled to five males and five females if possible; all culled offspring were employed in the current study. Roughly half of these rats (31 males and 27 females) were from unstressed mothers (n=6), and the remaining offspring (23 males and 26 females) were from mothers (n=5) exposed to a random schedule of physical restraint during gestation days 10 through 19. Restraint stress was applied to mothers in one, two, or three blocks per day for a total daily duration of 75 minutes using a random schedule – the shortest block was 15 minutes and longest 75 minutes. Stress was administered in the home cage during the light

phase, between 9AM and 6PM. The restraint apparatus was created from a clear plastic water bottle (18 cm tall, 6.5 cm in diameter).

Throughout the study, all adult rats were housed in large, translucent cages (37 cm wide x 48 cm long x 20 cm high) in same sex sibling groups after a gradual, seven day, weaning period on postnatal days 21-27, previously described in Baker et al. (2008). Offspring were culled on postnatal day 2 to a litter size of 10 - 5 males and 5 females, if possible. Most groups, therefore, consisted of 5 rats of the same sex per cage with a few exceptions: females groups of 3, 4, and 6 rats and male groups of 4, 4, and 6 rats. The housing room was maintained on a 12h light/12h dark cycle with lights on at 7:20 AM. Food and water were available ad libitum. Weight was recorded weekly for six weeks beginning at 55 days of age. Cages were cleaned weekly, and each cage was provided with two opaque PVC tubes and nesting material (Nestlets®). The estrous cycle was monitored every other day via vaginal lavage in all female rats beginning on day 55 until the end of the experiment. A detailed description of the vaginal lavage technique employed here has been described previously by others (Marcondes et al., 2002). Briefly, a sample of vaginal fluid was collected with a plastic pipette containing 10mL of saline and placed onto a glass slide to allow the cytology of the sample to be examined under microscope. Cycle stages are categorized by the predominance of cell types: an estrous (sexually receptive) phase sample consists primarily of anucleated cornified cells; metestrus contains roughly equal proportions of leukocytes, cornified, and nucleated epithelial cells; diestrus, leukocytes; and proestrus, nucleated epithelial cells (Long, & Evans, 1922; refer to Marcondes et al., 2002 for images). Individual rats were considered to have regular cycles if they alternated through all four phases within

4-5 days, the normal length of estrous cycles (Long, & Evans, 1922). Irregular cycles were characterized by samples consistently displaying cells of only one cycle stage or failing to follow the typical sequence within the standard 4-5 days.

Behavioural Tests

In the present study, rats were evaluated between 83 and 85 days of age. Each same sex litter group was randomly split into two groups. Anxiety-related behaviours were examined in the first group via the elevated plus maze (EPM) test (11 males and 11 females from stressed mothers and 16 males and 13 females from control mothers). The second group was tested in a delayed alternation task of spatial memory (T-maze) and the Emergence test of anxiety (10 males and 13 females from stressed mothers and 15 males and 12 females from control mothers). Half of these animals were evaluated in the T-maze first followed by the emergence test, and the other half in the reverse order. This regime was also applied during the juvenile test phase (results reported in Baker et al., 2008). To avoid carry over effects that may reduce test validity, as previously documented for the EPM test (File and Zangrossi, 1993), all tests were administered only once, either in the juvenile or adult phase. Tests were conducted during the light phase. Note that the influence of estrous cycle stage was not assessed on any behavioural measure given that most rats were tested during their diestrus phase.

EPM

The maze consisted of two open and two enclosed arms, each 38 cm long X 14 cm wide; the wall height of the enclosed arms was 30 cm. The arms extended from a center platform (14 cm^2) with an elevation of 33 cm from the floor. At the beginning of each 5 minute test, the rat was placed in the center platform facing a closed arm. The entire session was recorded via a digital video recorder (JVC). A blind observer scored the tapes and recorded the duration and frequency of the following behaviours in the offspring groups: entries in the open arms, closed arms, and middle platform, as well as grooming, rearing, edge rearing (rearing at the edge of an open arm), and measures of risk assessment: stretch attends (the rat stretches forward and retracts without moving its paws) and head dips (the rat leans over the edge of the open arm). The measures of risk assessment were categorized as protected – performed while the body of the rat was in the closed or middle area of the maze, or unprotected – while the body was in the open areas of the maze. An arm entry was counted when all four paws were within its boundaries. Before and after each test, the interior of the maze was wiped with a 95% alcohol solution and allowed to dry.

Emergence

The emergence test consisted of a dark, opaque plastic box (37 cm wide x 48 cm long x 20 cm high) with a small opening (9.0 cm high x 10.5 cm wide) set in a well lit open field, a plastic translucent large rat cage (37 cm wide x 48 cm long x 20 cm high) containing standard wood chip bedding on the floor. At the beginning of the test, the rat was placed inside the box and the latency, in seconds, to fully emerge (all four paws)

into the open field recorded. The frequency of nose pokes and half-body emergences (two front paws) was also recorded. The test ended after the rat had fully emerged or 10 minutes had passed.

T-maze

The T-maze apparatus was constructed of Plexiglas® with the main branch 55 cm in length x 15 cm wide and the two secondary branches each 40 cm long x 15 cm wide. The sides were opaque and a translucent lid covered the entire maze. Three trials were administered, separated by a 20-30 minute interval between trials. Each trial consisted of a forced choice test followed within 30 seconds by a free choice test. During the forced choice tests, left and right secondary arms were alternately blocked across trials and sex and family groups. In the free choice trial, both arms were open for exploration and responses were either scored as correct - the unexplored, previously blocked arm was selected or as incorrect - the explored arm was selected. Statistical analyses were based on the percent frequency of successful alternations: the average number of correct choices made during the free choice trials. Rats that remained in the main branch for more than five minutes during the forced choice session were removed from the maze and returned 20 to 30 minutes later for a retest. Two rats failed to respond, even after a second exposure on two trials, and these trials were excluded from the analysis. Before and after each test, the entire maze was cleaned with a 95% alcohol solution to mask olfactory cues.

Statistical analyses

Analyses of weight and behaviour data were performed using HLM V6 software (Raudenbush, Bryk, and Congdon, 2000). The HLM approach was selected as these methods have been developed over the past 20 years to address nested, multilevel/hierarchical data (Raudenbush and Bryk, 2002). The HLM approach to regression was appropriate for the current analyses for several reasons. First, data in the current study had a nested structure such that there were multiple mothers (higher level of the hierarchy) that each had multiple offspring (lower level of the hierarchy). Preliminary HLM analyses were conducted to determine if any of the dependent variables or their relations with gender, time, or the gender-by-time interaction varied from family to family; this was found to be the case in a number of circumstances: weight, weight gain over time, and the interaction between gender and weight gain over time; protected and unprotected head dips and its link with gender; and time spent in the open arm (marginally). Details can be seen in Table 4. Note that the proportion of variance due to the stress manipulation (extreme right column of Table 4) was very high in a number of cases even if the family-to-family variation was significant (for example, weight and weight coupled with gender and time). With a small sample size, even marginally significant variances are indicative of substantial variation between families. If the analyses had been conducted by simply pooling the observations for all offspring and ignoring the fact that offspring within a family are often more similar than offspring from different families, the degrees of freedom would be overestimated and Type I error inflated. Though some measures were fairly consistent from family to family, it made sense to apply HLM to all of the analyses for consistency and comparison.

Table 4. Variance Results of HLM analyses.

Random effect	Between family group variance	df	Chi-square	P-value	Proportion of variance due to maternal stress
Weight	460.48	10	75.57	**0.000**	47%
Its link with gender	200.47	10	14.11	0.168	0%
Its link with time	9.23	10	64.16	**0.000**	55%
Its link with gender-by-time interaction	7.02	10	19.03	**0.040**	79%
Time spend in the open arm	7.13	10	12.95	0.226	0%
Its link to gender	42.26	10	15.08	0.129	0%
Rearing in the closed arm	3.58	10	13.42	0.201	0%
Its link with gender	0.12	10	5.69	>.500	0%
Rearing in the open arm	0.01	10	9.96	>.500	0%
Its link with gender	2.96	10	13.99	0.173	2%
Unprotected head dip	0.63	10	9.85	>.500	21%
Its link with gender	23.47	10	29.16	**0.001**	50%
Protected head dip	1.58	10	24.68	**0.006**	25%
Its link with gender	5.33	10	21.84	**0.016**	0%
Emergency Latency	7.69	10	11.56	0.315	98%
Its link with gender	4.67	10	7.95	>.500	90%
Successful alternations in T-maze	0.12	10	15.08	0.129	0%
Its link with gender	0.54	10	15.74	0.107	0%

In addition, weight was measured repeatedly over time, raising the possibility that there was variation from individual to individual as well. This is precisely what was found – there was significant variance across individuals in weight and in weight gain over time, even after controlling for gender. If this variation proved to be influenced by the stress manipulation, it would be an important finding. If a repeated-measures ANOVA were used to asses weight changes over time, observations would be deleted list wise, so that data from individuals with even a single missing observation would be ignored, or such missing cases would need to be estimated; HLM, on the other hand, retains all of the data. Furthermore, repeated-measures ANOVA assumes sphericity (that measurements close together in time are no more correlated than measurements far apart in time), and various sub-optimal corrections need to be used when this assumption is violated, as is often the case, particularly with a parameter such as weight; HLM does not require the presence of sphericity.

Finally, the HLM approach was also more suitable than reducing each family's data to a single data point, for a number of reasons: the number of observations varied from family to family, so that an optimal solution would be one that weighted each family's data based on its sample size which is precisely what HLM does; further, retaining all of the details provided by multiple observations within each family, as HLM does, would provide greater power than reducing the number of observations to equal the number of families.

Statistical procedures

Initially in each HLM analysis, intercepts (e.g., mean weights for each family, or mean weights for each individual) and slopes (e.g., relation between time and weight for each family) were allowed to vary because this likely reflected reality – outcomes likely do vary somewhat from family to family and from individual to individual. In a few analyses, however, the ratio of meaningful variance to error variance in a coefficient was excessively small (less than the recommended 5%), likely due to the small sample sizes in the study. This does not bias the coefficients, but it does make it more difficult for the program to converge on a solution because it is trying to estimate a variance with too little information (HLM must estimate the variances in order to estimate the coefficients of interest). In such cases, as identified in the results section, the coefficient's residual variance was treated as if it were fixed (i.e., non-randomly varying) allowing the program to more efficiently converge on a solution.

All independent variables at lower levels of the hierarchy were group mean centered (i.e., the group mean was subtracted from each observation) so that intercepts would represent group means and thus be easily interpretable.

Most of the continuous dependent variables were reasonably normally distributed (an assumption underlying HLM) with the exception of emergence latency, rearing in the open arm, and both unprotected and protected head dips which were positively skewed. Detailed results for these variables are reported as untransformed for ease of interpretation; in addition, results for these variables based on transformed data to achieve normal distributions are also noted.

In all HLM analyses, restricted maximum likelihood estimation was used because it has higher power than full maximum likelihood for the sample sizes of this nature, and it is less sensitive to deviations from normality.

Details regarding the model employed for each dependent variable are provided in the results section, and all statistically significant results are indicated in the following format: mean difference ± standard error, t-ratio (degrees of freedom), significance level.

Results

Weight

In studying weight as the dependent variable, a three-level Hierarchical Linear Models (HLM) analysis was performed. Repeated measures of weight over six experimental weeks were the observations at the lowest level, with the time point being the independent variable at this level; multiple offspring within each family were the second

level, with gender being the independent variable at this level; multiple families were the highest or third level, with maternal stress condition being the independent variable at this level. Also included in the model were all potential interactions: the moderating effect of the maternal stress condition on the relationship between time and weight (also called a cross-level interaction as the maternal stress condition was assessed at level three while time was assessed at level one); the moderating effect of gender on the relationship between time and weight; the moderating effect of stress on the relationship between gender and weight; and, finally, the moderating effect of the maternal stress condition on the interaction between gender and time in predicting weight (the three-way interaction between stress, gender, and time). A preliminary plot of weight by time indicated that a linear model could provide a reasonable approximation of weight gain over time; although, as expected, the rate of weight gain decreased somewhat over time. For this reason, and for parsimony, we did not include higher order relationships (i.e., quadratic) with time in the model.

Results indicate that offspring from stressed mothers weighed significantly less than those from non-stressed mothers (by 29.52 ± 10.81g on average, $t(9) = -2.73$, $p = 0.024$), and also gained weight less rapidly (4.50 ± 1.49g less per week, $t(9) = -3.03$, $p = 0.015$). As expected, males had a significantly higher mean weight than females (by 167.37 ± 6.83g $t(9) = 24.51$, $p \leq 0.0001$), and males gained weight more rapidly over time (18.91 ± 0.90g more per week, $t(9) = 20.92$, $p \leq 0.0001$). Maternal stress condition played no role in determining the weight difference between males and females, but it did lead to an interaction between sex, gender, and time ($t(9) = -2.664$, $p = 0.026$) whereby males from stressed dams gained weight less rapidly than males from control

mothers (4.83 ± 1.82g less per week in those from stressed mothers) while all females gained weight at a similar rate. Naturally, offspring tended to gain weight over time (26.95 ± 0.74g per week, t (9) = 36.38, p ≤ 0.0001). These results are graphically depicted in Figure 19.

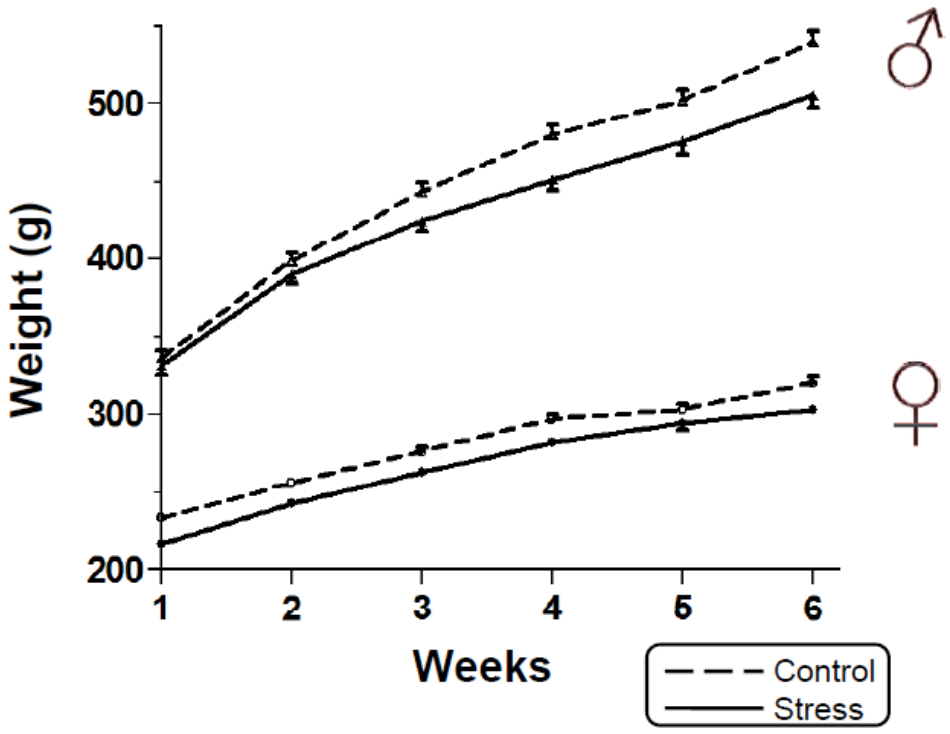


Figure. 19. *Weight.*
Average body weight (± SEM) is plotted over experimental weeks as a function of gender and maternal stress condition. Individual groups include male control (n = 31), male stress (n = 23), female control (n = 27), and female stress (n = 26).

Estrous Cycle

Figure 20 represents the percentage of rats in each experimental group that displayed regular weekly estrous cycles. In the first week of observation, 24% of stressed female rats were acyclic or had abnormal patterns of estrous cycle activity. One family group contributed significantly to this value: only 2 of 6 siblings displayed regular cycle activity.

In week two, 8% of rats in the stressed group had irregular cycles, and regular estrous cycle patterns were observed in 85% or more of the stressed females for the remainder of the monitoring period. All control rats displayed regular patterns in week one. In the second week, this value dropped to 81% but returned to 93% in week 3 and remained at this level.

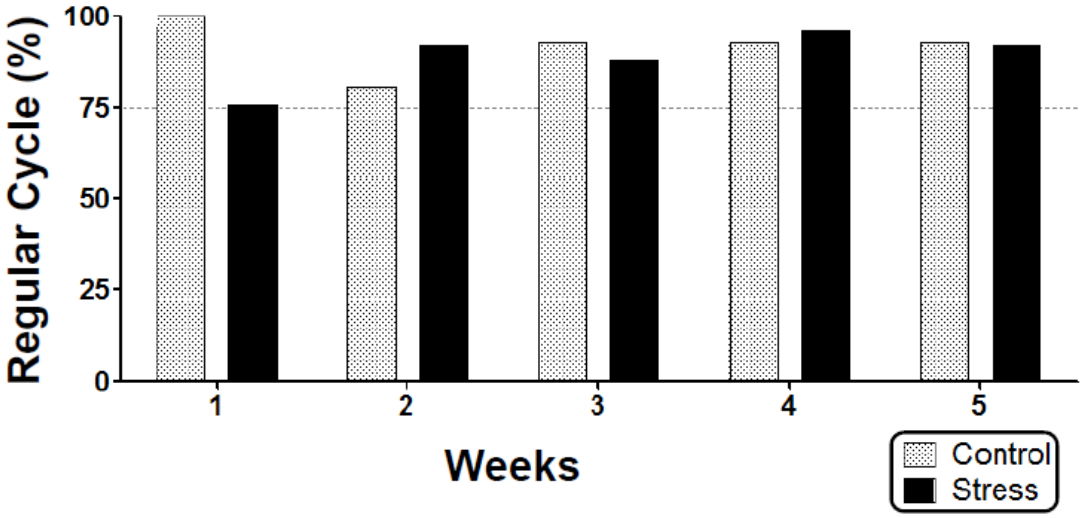


Figure. 20. *Estrous cycle.*
The percent average number of female rats in each group displaying regular estrous cycles is plotted across experimental weeks. Week 1 corresponds to 55 days of age. Filled bars represent the control group (n = 27) and grey bars the stress group (n = 26).

Behaviour

EPM

Due to the small amount of time spent in the middle section of the maze and the redundancy (almost mirror opposite pattern) of the time spent in both open and closed arms, only open arm time was analysed. Using time spent in the open arm as the dependent variable, a two-level HLM was executed. Mean time spent in the open arm

for multiple offspring was the observations at the lower level, with gender as the independent variable; multiple families were the higher level, with the maternal stress manipulation as the independent variable. The potential interaction between stress and gender was also examined.

Neither stress nor gender nor the stress-by-gender interaction influenced the time spent in the open arm. Of the specific behaviours recorded in the EPM test, only rearing in the closed and open arms and protected and unprotected head dips were analysed. Other behaviours yielded negligible values (below 2 s mean total). The analyses were based on standardized scores in the form of percent time in seconds: data were transformed by calculating the quotient of the time spent engaged in a specific behaviour within one area of the maze divided by the total time spent in that maze region and then converted to a percent score. This effectively removed the influence of time distribution among various locations in the EPM so that behaviours performed in these areas could be more appropriately compared. Note that data from two rats (one male and one female, both from stressed mothers) were removed from the analysis of the percent time performing unprotected head dips as they contributed scores that were significantly extreme; thus, this analysis was based on an n of 10 in both the male and female stress groups. For the same reason, in the analysis of protected head dips, the female stress group was reduced by one data point, yielding an n of 10.

Using rearing in the closed arm as the dependent variable, a two-level HLM was performed. Time spent rearing in the closed arm for multiple offspring were the observations at the lower level with gender as the independent variable; multiple

families were the higher level with maternal stress condition as the independent variable. The interaction between stress and gender was also included. Note that gender differences in time spent rearing in the closed arm for each family was treated as non-randomly varying.

Neither stress nor the interaction between stress and gender affected time spent rearing in the closed arm, though males spent a smaller proportion of the time engaged in rearing behaviour than females (6.03 ± 2.04% less time on average, $t(47) = -2.95$, $p = 0.005$).

Time spent rearing in the open arm was examined via a two-level HLM as described above for closed arm rearing. Note that, in the case of data from the open arm, mean open arm rearing duration for each family was treated as non-randomly varying.

Results indicate that neither stress, nor gender, nor their interaction affected rearing duration in the open arm. When this data was log transformed making it normally distributed, mean time spent raring in the open arm for each family no longer needed to be treated as non-randomly varying; however, all of the effects remained non-significant.

A two-level HLM was performed to examine the dependent variable, time spent performing unprotected head dips, as described above in the analyses of time spent rearing. Here, the maternal stress condition did not influence time spent performing unprotected head dips on average, but it did interact with gender ($t(9) = -2.44$, $p = .038$) such that female offspring of stressed mothers spent the greatest proportion of time

engaging in unprotected head dips as evident in Figure 21. Finally, gender had no main effect on this measure.

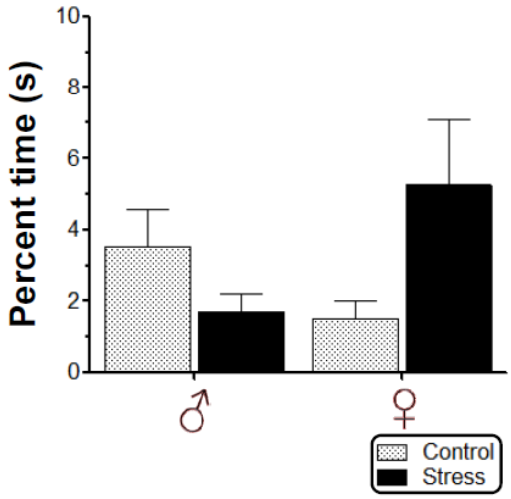


Figure. 21. *EPM.*
Average (± SEM) percent time engaged in unprotected head dips as a function of gender and maternal stress condition. Individual groups include male control (n = 16), male stress (n = 10), female control (n = 13), and female stress (n = 10).

Also similar to the above analyses of rearing time and unprotected head dips, a two-level HLM was applied to analyze the dependent variable, time spent performing protected head dips. Neither the maternal stress condition nor the maternal stress condition-by-gender interaction had an effect on this measure. Males spent a marginally greater percentage of the time engaging in protected head dips (1.97 ± 0.99 % on average, t (9) = 1.98, p = .079) than females. Note that when a log transformation was applied to this variable making it normally distributed, the coefficient was positive at .29, indicating that stress lead to a marginally greater proportion of time spent engaging in protected head dips. Additionally, the effect of gender became significant (p = .014).

Emergence

When studying emergence latency as the dependent variable, a two-level HLM was performed. Emergence latencies for multiple offspring were the observations at the lower level with gender being the independent variable at this level, and multiple families were the higher level, with maternal stress condition being the independent variable at this level. Also included in the model was the potential interaction between stress and gender. Note, mean emergence latencies for each family and gender differences in emergence latencies for each family were treated as non-randomly varying.

Offspring from stressed mothers had lower emergence latencies (emerging faster on average by 7.57 ± 3.25s, $t(9) = 3.25$, $p = .024$). Stress did not, however, interact with gender in predicting emergence latencies. Males had marginally longer emergence latencies than females (by 6.20 ± 3.30s, $t(45) = 1.88$, $p = .066$). Note, when a square root transformation was applied to emergence latencies, the mean values for each family and gender differences in emergence latencies for each family no longer needed to be treated as non-randomly varying; the only change in the results was that the main effect of stress approached significance ($p = .063$); males still had marginally longer latencies ($p = .094$).

T-maze

Examining the T-maze performance as the dependent variable, a two-level HLM was performed where the dependent variable was represented by a binomial distribution as it was assessed as count data (the number of successful alternations out of three trials).

Odds of success for multiple offspring were the observations at the lower level with gender being the independent variable; multiple families were the higher level with maternal stress condition being the independent variable. The interaction between stress and gender was also included in the analysis.

Neither stress nor gender nor their interaction affected the odds of making the correct choice in the free-choice trial; all groups responded at above chance (50% - no memory) levels.

Discussion

The purpose of this study was to perform a follow-up assessment of the long-term consequences of gestational stress exposure and attenuated maternal care in adult offspring. The findings of the current study compliment the initial observations made in mothers and juvenile offspring reported earlier in Baker et al (2008). Mirroring the juvenile data, the analysis of physiological measures yielded a strong effect of gestational stress while behavioural results were subtle. Ignoring gender, offspring from stressed dams weighed less overall and gained weight more slowly than those from control mothers. Males from stressed mothers gained weight less rapidly than males from non stressed mothers; however, females from stressed mothers maintained a consistently lower weight across weeks than their control counterparts and displayed altered estrous regularity in the first week of observation. Anxiety measures revealed a gender difference in the EPM with males displaying less exploratory behaviour, rearing less frequently than females in the closed arms, and performing more head dips form the closed arms (protected head dips) than females. Females from stressed mothers

performed more unprotected head dips than females from control mothers and offspring from stressed dams, regardless of gender, had shorter emergence latencies; both observations are suggestive of an anxiolytic effect in gestational stressed groups. Finally, behaviour in the T-maze was unaffected by gender or maternal treatment during gestation.

Attenuated birth weight is one of the most commonly reported effects of gestational stress in newborn humans (e.g., Orr et al., 1996). Low birth weight is associated with the development of a variety of metabolic-related diseases in adulthood – coronary heart disease, stroke, hypertension, diabetes, and obesity (Barker, 2004). Surprisingly, most studies examining the effects of early life manipulations in adult rat offspring fail to report this measure; of those that have, birth weight has been demonstrated to be largely unaffected by gestational stress (reviewed in Weinstock, 2008). Accordingly, offspring from both sexes, in the current study, did not display an effect of the gestational stress manipulation on weight at postnatal day two (Baker et al., 2008). A difference in weight was observed between the female groups beginning around 36 days of age (female offspring from stressed mothers weighing less than those from control mothers) (Baker et al., 2008); this effect persisted (up to 91 days of age) as reported in the current study. A similar pattern is evident in male offspring but only in the adult data reported in the current study.

Consistent with our findings of a difference in weight among male rats not appearing until the adult phase, Bhatnagar, Lee, and Vining report a weight difference at 79 days of age, but not earlier, in Sprague-Dawley male offspring from mothers subjected to a similar restraint paradigm beginning on GD 15 until 21 (2005). In

contrast, female offspring weight in this study was unaffected by the maternal manipulation (Bhatnagar et al., 2005). An earlier report in which a similar stress protocol was employed in Sprague-Dawley rats demonstrated no effects of stress on the body weight of male or female offspring from 20 to 80 days of age (Bowman et al., 2004).

Two other groups have reported similar results in gestational stress adult male groups (female offspring were not examined) (Bosch et al., 2006; Drago et al., 1999). In the latter report, the weight reduction was counteracted by maternal administration of diazepam prior to gestational stress exposure. This suggests that the stress experience of the mother was the direct or indirect contributing factor to the weight result (Drago et al., 1999). Others have observed no weight differences due to maternal stress in adult male rats (Richardson et al., 2006; Estanislau and Morato, 2005; Van den Hove et al., 2005; Fride and Weinstock, 1989).

This is one of the few reports examining the estrous cycle in female offspring over time in the context of gestational stress research. Beginning estrous monitoring at 55 days of age was clearly too late to mark the beginning of cycling in these rats; this age was chosen with the expectation that puberty and regular cycle patterns would be established (Sharp and LaRegina, 1998) so that animals displaying consistent irregular cycles could be detected. The observation that 25% of rats from stressed mothers and none from the control group had irregular cycles at the beginning of monitoring (55 days of age) may suggest that some rats experienced a delayed onset of puberty or a lengthier period to establish a regular cycle pattern. As no data were collected before this time, this hypothesis cannot be verified. Furthermore, the percent of rats with regular cycle patterns in the stress group ranged between 81 and 96% on subsequent

observation weeks which was at or above the level of the control group. Clearly the observations from the first week must be interpreted with caution. The possibility that gestational stress may delay the acquisition of regular cycling patterns merits further investigation as this could have dramatic consequences for reproduction success in female offspring. This idea finds support in early work in mice demonstrating that a prenatal stress protocol involving heat, light, and restraint stress applied during the same period as the current study increased the length of the estrous cycle, specifically the estrus-metestrus stage (Herrenkohl and Politch, 1978) and delayed the time of vaginal opening, a marker of puberty onset in female rodents (Politch and Herrenkohl, 1984). Indeed it has been demonstrated that elevating fetal glucocorticoid exposure, which is thought to occur during prenatal stress, by inhibiting the placental glucocorticoid barrier can delay the time of vaginal opening, a marker of puberty onset in female rats (Smith and Waddell, 2000). More recently, other reports demonstrate that prenatal stress can alter plasma concentrations of gonadal hormones in offspring during adulthood (Richardson et al., 2006; Ward, 1984).

In contrast to the physiological results, gestational stress influences on behaviour were subtle and, in some tests, in the unexpected direction. Neither independent factor, gestational stress or gender, influenced the traditional measure of anxiety, total or percent total (data not reported) time spent in the open arms of the EPM. Contradictory result were obtained in the secondary anxiety measure, the emergence test, where offspring from stressed dams emerged faster than their control counterparts which suggests an anxiolytic effect of gestational stress, and it is difficult to reconcile these findings. An interesting gender difference was observed in the behaviours performed

within the maze; regardless of group, males spent less time rearing and more time performing head dips from the protected/closed arm than females suggestive of a lower level of exploration and risk.

An increase in anxiety-like behaviour is more commonly reported in the gestational stress literature which has been mainly conducted in males: EPM (Bosch et al., 2006; Patin et al., 2005; Rimondini et al., 2003; Lordi et al., 2000; Vallée et al., 1997; Fride and Weinstock, 1988), open field (Dickerson et al., 2005; Poltyrev et al., 1996); and emergence test (Van den Hove et al., 2005). Contradictory reports of no change in anxiety behaviour as a consequence of gestational stress are also prevalent: EPM (Gotz and Stefanski, 2007; Zagron and Weinstock, 2006; Chapman and Stern 1979) and open field (Tazumi et al., 2005; Polytrev et al., 1996). Few studies have examined both male and female responses in the EPM as a consequence of gestational stress, and none have done so in Long Evans rats. A stress regime similar to that of the current study applied to CD (Richardson et al., 2006) and Wistar (Zagron and Weinstock, 2006; Ordyan and Pivina, 2004) rats has produced anxiety effects in the EPM, but only in female offspring. The consequences of gestational stress in males differed among these reports: no difference was detected between CD male groups (Richardson et al., 2006), but time spent in the open arms was elevated in Wistar males from the stressed group (Ordyan and Pivina, 2004). The latter study also analysed head dip behaviour which was mediated by sex; in groups from stressed mothers, it was elevated in male and attenuated in female rats (Ordyan and Pivina, 2004).

Similar gestational manipulations as those in the present study were administered during late gestation in these reports (Richardson et al., 2006; Zagron and

Weinstock, 2006; Ordyan and Pivna, 2004); therefore, differences in findings may be attributable to major procedural differences in the administration of the EPM between studies. Richardson's group tested during the dark, active phase (2006) while the other groups tested during the light phase but primed the rats with a five minute exposure to an open field immediately prior to the EPM test (Ordyan and Pivina, 2004). A similar priming effect with 60 minutes of restraint prior to EPM administration was responsible for the presence (Rimondini et al., 2003) or exaggeration (Estanislau and Morato, 2005) of an anxiety-like effect in male offspring from stressed mothers. The fact that priming may be required to elicit an anxiety response in some strains following similar maternal treatment may, as suggested by others (Rimondini et al., 2003), be due to a genetically based decreased sensitivity to develop behavioural alterations induced by gestational stress.

Cognitive effects of gestational stress have received much less attention than anxiety/emotionality, and reports involving both genders are sparse. Predominantly, performance in the Morris water maze has been used to assess learning and memory effects. Gestational restraint stress applied throughout the last week of gestation was found to disrupt performance in the Morris water maze test in male but not female Wistar offspring (Zagron and Weinstock, 2006; Szuran et al., 2000, 1994). In contrast, similar to the T-maze results in the current study, restraint applied in the last 4 days preceding parturition did not produce a change in water maze performance of Sprague-Dawley rats of either sex (Meunier et al., 2004). A milder restraint paradigm (confinement in a small-sized cage) for a similar duration yielded an improvement in the radial arm maze performance of Sprague-Dawley males (Fujioka et al., 2001). As in

tests of anxiety/emotion, variations in gestational protocols and behavioural paradigms interact in tests of cognition. In the T-maze test, acute severe stress (foot shock or predator stress) on days 10 or 19 of gestation was found to diminish percent alternation, long-term spatial memory (Lordi et al., 2000 – day 10 only; Lordi et al., 1997 - both). In our hands, spatial memory was not significantly altered by gestational stress group membership. Given that behavioural outcomes tend to be subtle following gestational stress, particularly in the adult phase, perhaps the Morris water maze is a more sensitive behavioural measure in this context. The methodology involves numerous trials over a number of consecutive days and employs an escape task which may be more ecologically relevant to the rat than the tendency to visit unvisited arms of a maze in the spontaneous alternation measure of the T-maze (Morris, 1984).

It is curious that such robust effects were observed in body weight between groups while only subtle effects were obtained in the behavioural measures of emotionality and none in the test of cognition in both juvenile (Baker et al., 2008) and adult offspring. This is particularly interesting as it has been demonstrated previously that a naturally occurring lower level of maternal care alone can increase anxiety and fear behaviour in offspring (Champagne and Meaney, 2006; Caldji et al., 1998, respectively). In the current design, unlike most reports of gestational stress, maternal behaviour was examined and found to be attenuated by the gestational manipulation (reported in Baker et al., 2008) without elevating anxiety or diminishing cognitive behaviours in juvenile offspring. It is important to note that dams in the current study were not previously characterized in terms of their natural level of maternal care (i.e. as low or high in pup-directed licking and grooming) prior to stress as was the case in work

cited above. Given that natural variation observed in a normal rat population would produce groups heterogeneous for maternal care, this characteristic, as measured in this study, may be confounded by the natural level of maternal care prior to stress. Thus, any postulated decrease in maternal care due to gestational stress may not have been consistent or powerful enough in every mother to produce such robust findings in offspring. Further studies may consider working with a more homogeneous subgroup of dams, for example, those expressing low levels of maternal care which may represent an ‘at risk’ population. While it is not possible to differentiate gestational stress effects from those of postnatal maternal care in the current design, it highlights the importance of investigating the interaction between gestational stress and postnatal factors.

In conclusion, the current findings demonstrate that gestational maternal restraint and or decreased maternal care behaviour in Long Evans rats can cause impaired growth throughout development, may delay the establishment of regular estrous cycle activity, and may have sex-mediated influences on anxiety. These data emphasize the importance of investigating the responses of both male and female animals to early life manipulations, and the relevance of considering other factors in interpreting results, namely, age, genetics, stress and behavioural test protocols, as well as maternal care. Moreover, parametric factors such as stress timing and intensity also need to be resolved in gestational stress models. Further investigations focused on delineating the role of such factors will result in a more complete understanding of the implications of stress experienced during pregnancy on progeny throughout life.

Study 5

Effects of gestational stress: 3. Evaluation of chronic mild stress exposure in adult female rat offspring from stressed pregnancies

Baker, Huta, & Bielajew.
2011. For submission.

Abstract

The causes of depression and related disorders remain elusive; however, there is a clear risk associated with being female and exposed to stress with evidence found in both clinical and preclinical literature. Stress exposure early in life may lay the foundation for the development of sensitivities to psychiatric disorders while later stress exposure may trigger their expression. The purpose of this study was to test this hypothesis. Two commonly employed animal models of stress were used, gestational stress (GS) and chronic mild stress (CMS), to evaluate their independent and synergistic effects in Long Evans female rats. Care was taken to reduce stress effects extraneous to the paradigms through gentle husbandry practices including gradual weaning, group housing, and access to objects promoting the expression of naturalistic behaviours. Gestational stress (GS), in the form of maternal physical restraint during late gestation in rodents, has been shown to alter maternal behaviour, the HPA stress axis of both mother and offspring, and related anxiogenic and depressive behaviours. The chronic mild stress (CMS) model of depression is a method of inducing a depressogenic profile and has been well established in male rats. Similar to results obtained during the juvenile and early adult period, GS groups showed lower weight across the entire study but had no effect on EPM anxiety measures. GS rats also had higher corticosterone levels at six and 12 months compared to controls. Exposure to a three week CMS paradigm induced only subtle changes in anhedonic behaviour as indexed by 24h sucrose preference and did not affect estrous cycle regularity or EPM behaviour. It did, however, induce transient decreases in weight gain. GS and CMS were not observed to interact on any measure examined. These results highlight the importance of early life factors in exerting protective effects in models involving stress.

Keywords: CMS, gestational stress, female, adult rat, Long Evans, anxiety, weight, social housing

Introduction

It is becoming increasing clear that environmental factors can alter brain development and are important in the establishment of vulnerabilities to or protection from neuropsychiatric disorders (Marco, MacrÌ, & Laviola 2011). Much attention is being given to the role of environmental influences in the context of stress-related disorders highlighting the importance of the interaction between genes and environment in the expression of pathophysiology. Perinatal life is marked by increased plasticity, particularly in the stress system, and may therefore represent a period of heightened sensitivity to stress mediated by exposure to abnormal maternal hormones and/or maternal care behaviour resulting in permanent epigenetic change (Darnaudéry, & Maccari 2008).

The most robust and well-established findings in psychiatric epidemiological research are that being female and exposed to stress significantly increase one's risk of depression. The stress-depression link is observed across the lifespan (Hammen 2005; Liu, & Alloy 2010; Paykel 2003) with exposure to chronic mild stress being the strongest predictor of the development of depression (McGonagle, & Kessler 1990). The role of stress in the expression of illness is hypothesized to vary depending on timing of exposure; before maturation, normal neural trajectories may be altered resulting in depressogenic sensitivities (a programming effect) while after maturity, the neurochemical milieu may be transiently altered leading to an increased likelihood of its expression (an activational effect). This stress-related vulnerability to depression may be related to epigenetic factors associated with increased activation of the HPA axis (Tsankova et al. 2007).

Gestational stress in the form of physical restraint (GS) and Chronic Mild stress (CMS) are rodent models of stress-induced depression that allow for the examination of this hypothesis. Both GS and CMS models have been extensively evaluated and responses in male rodents have been well established (GS reviewed in Weinstock 2008; CMS reviewed in Willner 2005). Less information is available concerning female responses; however, GS appears to exert sexually dimorphic effects on behaviour whereby males tend to display greater deficits in learning and memory and females show greater effects in emotionality (reviewed in Weinstock 2001). Furthermore, females appear more sensitive to alterations in the regulation of the HPA axis (Richardson et al. 2006; Szuran et al. 2000; McCormick et al. 1995; Weinstock et al. 1992) and show greater depressive-like behaviour (Polytrev et al. 2005; Alonso et al. 1991) Similarly and in line with human data, female rats appear to be more vulnerable to the effects of CMS as indexed by decreased sucrose intake and open field activity, increased corticosterone levels, and decreased activity in the hippocampus and hypothalamus (Dalla et al. 2005).

The current study represents the third instalment in a series of experiments that have documented the consequences of gestational restraint stress on maternal and offspring responses. In an effort to reduce extraneous stress effects associated with standard laboratory practices, all animals were maintained in supportive housing conditions that promoted the expression of naturalistic social and physical behaviours following a gentle weaning procedure. Maternal weight, care and anxiety behaviour, and offspring anxiety and cognitive behaviour and physiological responses of both sexes have been reported previously in the juvenile period (Baker et al. 2008) and in early

adulthood (Baker et al. 2009). Findings of early studies revealed an effect of GS on maternal behaviour and offspring post weaning weight, but little behavioural response. The current study focused on female responses to CMS at six and 12 months of age. The purpose was to observe if the effects on weight would be maintained, if latent effects of GS on anxiety would be expressed in female offspring during mid-adulthood, and if GS would interact with CMS exposure.

Methods

Overview

The present study reports the behavioural and biochemical profiles of female Long Evans rat offspring following exposure to CMS at six and 12 months of age and is part of a longitudinal project examining the effects of maternal gestational stress in offspring throughout the lifespan. The experimental protocol of all phases of the long term project is schematized in Figure 22. Previous reports detail the procedures for statistical analyses of breeding, gestational stress regime, maternal behaviours (maternal care and EPM), litter characteristics, weaning, weight, estrous cycle regularity, and behavioural outcomes (EPM, t-maze, and emergence test), in male and female offspring during the juvenile period (postnatal day 30) (Baker et al., 2008, Figure 22 - left), and during the early adult period (postnatal day 83) (Baker et al., 2009, Figure 22 - middle). The current study (Figure 22 - right) consisted of a set of three phases, each lasting approximately three weeks – baseline, CMS, and post-stress. Rats progressed through each phase at the same time, and in the same order at both six and 12 month testing periods. Weight gain and anhedonic-like behaviour (preference for a 1% sucrose

solution) were monitored throughout the study; offspring stressed groups were exposed to the CMS procedure during the CMS phase. The behavioural measure of anxiety, the EPM, was applied at the beginning of each post-stress phase (PN219 and PN401 for six and 12 month phases, respectively), and blood was collected at the end of the six month baseline phase (PN191) and beginning of the post-stress phase at both six and 12 months (PN217 and PN387 for six and 12 months, respectively). Ethical approval for this project was obtained by the institutional protocol review group at the University of Ottawa in accordance with the guidelines from the Canadian Council on Animal Care.

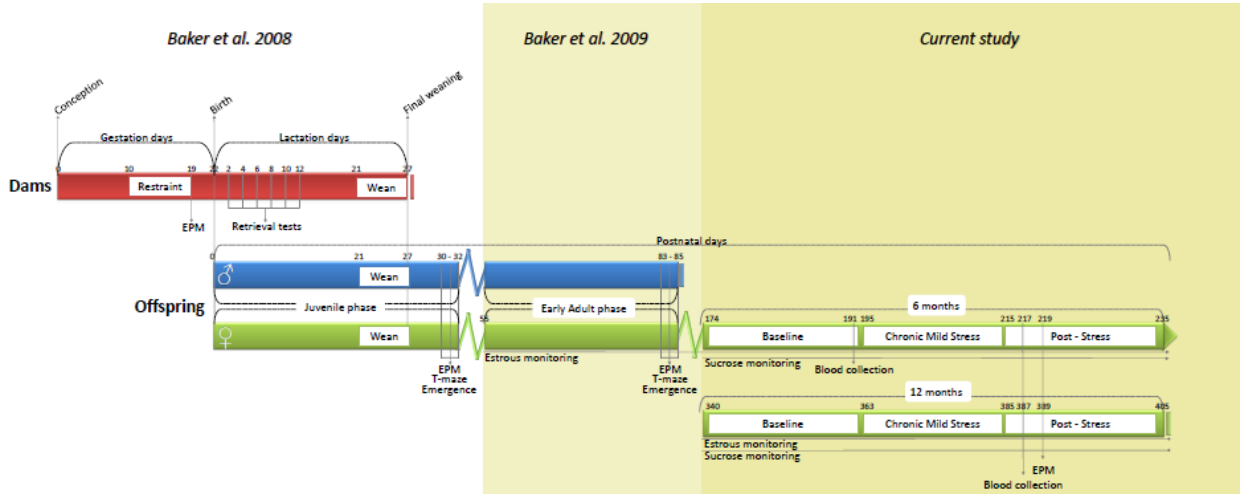


Figure. 22. *Timeline of long-term project.*
Timeline of events for all three phases of the long-term study from conception to offspring endpoints with publication reference indicated at the top of each boxed section; methodology for the current study appears in the far right box. Please note that this image is identical to Figure 1 on page 25 with the exception of the section headings.

Animals

Long Evans female rat offspring from parents obtained from Charles River Laboratories, St.-Constant, Québec and bred in-house were used in the current study. The maternal gestational stress protocol consisted of exposing dams to a random schedule of physical restraint during gestation days 10 through 19 for a total daily duration of 75 minutes; please refer to Baker et al., 2008 for a detailed description of this regime.

A two by two design was used representing maternal treatment (GS and noGS) and female offspring treatment (CMS and noCMS) resulting in the following groups: GS/CMS, GS/noCMS, noGS/CMS, noGS/noCMS. These groups were maintained throughout the duration of the study. Group sizes and attrition are reported in Table 5. Note that due to the long term nature of the study and the limited lifespan of the laboratory rat, a decline in the number of rats due to death by unknown causes or euthanasia following the localization of a tumour and/or decline in health status (generally indicated by rapid weight loss) occurred throughout the period of observation in the current study, from four to 14 months of age. The cause of death and number of dead was random as these factors were found to be equally distributed among both maternal gestational and adult stress groupings. One female rat from the gestational stress group died at five months; 15 rats were euthanized or died between eight and 14 months of age - six from noGS dams and seven from GS dams. Four rats from gestational stress group and five from control mothers expired between eight and 12 months and three from each group between 12 and 14 months. Eight families out of 12 had sibling loss.

Table 5. Group size and attrition.

6 months	CMS	noCMS
GS	13	12
noGS	13	13

12 months	CMS	noCMS
GS	11 *(-1)*	11 *(-1)*
noGS	12 *(-1)*	9 *(-4)*

Husbandry

Housing conditions remained constant across groups from weaning (postnatal day 27). All females were maintained in same sex sibling groups with a maximum of 5 per cage. Large, transparent polycarbonate cages were used (37 cm wide x 48 cm long x 20 cm high), and free access to food and fluids was allowed. Each cage contained two large black PVC tubes and Nestlets® to promote the expression of naturalistic behaviours; such items were removed during the stress phase in the cages of CMS groups.

Regular monitoring

Weights were recorded weekly (with the exception of months nine, 10, and 11 during which three weights were recorded per month), and the estrous cycle was monitored every second day via vaginal lavage. A detailed description of the vaginal lavage technique employed has been described previously by others (Marcondes et al., 2002). Briefly, a sample of vaginal fluid was collected with a plastic pipette containing 10 mL of saline and placed onto a glass slide to allow the cytology of the sample to be examined under a microscope. Cycle stages are categorized by the predominance of cell types: an estrous (sexually receptive) phase sample consists primarily of anucleated cornified cells; metestrus contains roughly equal proportions of leukocytes, cornified, and

nucleated epithelial cells; diestrus, leukocytes; and proestrus, nucleated epithelial cells (Long and Evans, 1922; refer to Marcondes et al., 2002 for images used for stage categorization). Individual rats were considered to have regular cycles if they alternated through all five phases, in order, within four to five days, the normal length of estrous cycles (Long and Evans, 1922). Irregular cycles were characterized by samples consistently displaying cells of only one cycle stage or failing to follow the typical sequence within the standard four to five days.

Chronic mild stress

Please refer to Table 6 for the schedule of stressor administration applied at both six and 12 months employed in the CMS regime and a detailed description of each individual stressor. After three weeks of baseline monitoring of weight, estrous cycle, and sucrose preference, CMS was applied to both stressed groups (GS/CMS and NoGS/CMS) for three weeks. Tubes and Nestlets® were removed from the cages of animals undergoing CMS exposure and returned at the beginning of the three week post-CMS monitoring period.

Table 6. Schedule of chronic mild stressors.

BASELINE *3 weeks*	CMS *3 weeks*	POST-CMS *3 weeks*

Time	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
10:00		strobe 2h	white noise 2h	confine 2 2h			strobe 21h
12:00	white noise 1h			strobe 1h			Confine 1 1h
14:00	confine 1 2h	Confine 2 2h	strobe 2h				
16:00	Crowding	wet bed	wet bed	cage tilt	cage tilt	cage tilt	crowding
Time	**DAY 8**	**DAY 9**	**DAY 10**	**DAY 11**	**DAY 12**	**DAY 13**	**DAY 14**
10:00	strobe 2h	strobe 2h	crowding2 2h	White noise 3h			white noise 1h
12:00		confine 1 1h	strobe 1h				
14:00	confine 2 1h			strobe 2h			confine 1 1h
16:00	wet bed	crowding	wet bed	cage tilt	cage tilt	cage tilt	crowding
Time	**DAY 15**	**DAY 16**	**DAY 17**	**DAY 18**	**DAY 19**	**DAY 20**	**DAY 21**
10:00	white noise 3h	confine 2 1h	white noise 1h	strobe 3h			strobe 2h
12:00		strobe 1h		confine 1 1h			
14:00	strobe 2h		confine 1 2h				confine 2 1h
16:00	crowding	wet bed	crowding	cage tilt	cage tilt	cage tilt	wet bed

Label	Stressor	Description
confine 1	Confinement with noise	Rats are individually placed in mouse cages timers are set to ring at random intervals.
confine 2	Confinement with noise	Rats are individually placed in mouse cages an untuned radio is set at max volume.
strobe	Stroboscopic light	In home cage, exposed to 300 flashes/min of white light (in dark room).
white noise	White noise	An untuned radio set at maximum volume.
wet bed	Wet bedding	Warm water is mixed into bedding.
crowding	Crowding	Sibling group placed in a small cage overnight.
cage tilt	Tilted cage	Cage is tilted horizontally 30° throughout the weekend.

Throughout CMS period, Nestlets® and opaque PVC tubes were removed from cages and home cage position was rotated daily in animal housing room.

Sucrose preference monitoring

Preference for sucrose over water was used as an index of anhedonia. All groups were given free access to two bottles per group cage, one containing tap water and the other a 1% sucrose solution. Bottles were weighed every second day, refilled when necessary, and replaced weekly. During baseline, CMS, and post-stress phases, fluid intake was monitored Monday to Friday, at 9AM. A value representing the daily group preference for sucrose was calculated by dividing the amount of the sucrose solution consumed by the total fluid intake and converting it into a percent score. Constant access to the sucrose solution began at four months to allow for acclimatization; bottle fluid levels were monitored and refilled as necessary. Following the Post-Stress phase at six months, access to the sucrose solution ceased until the 12 month experimental phase. Group rather than individual scores were measured to avoid the possible stress effects of separation that would have been necessary for individual intake assessment, and to allow for continuous 24 hour monitoring for the duration of the study.

Elevated Plus Maze

At both the six and 12 month test phases, the EPM test was administered at the beginning of the post-CMS monitoring period during the light phase. All test sessions lasted five minutes and were recorded (JVC digital video recorder) and target behaviours were scored by a blind observer using the software ODlog®. The maze consisted of two open and two enclosed arms, each 38 cm long x 14 cm wide; the wall height of the enclosed arms was 30 cm. The arms extended from a center platform (14 cm^2) with an elevation of 33 cm from the floor. At the beginning of each test, the rat was

placed in the center platform facing a closed arm. A blind observer scored the tapes and recorded both the duration and frequency of the following behaviours: entries in the open arms, closed arms, and middle platform, as well as grooming, rearing, edge rearing (rearing at the edge of an open arm), and measures of risk assessment: stretch attends (the rat stretches forward and retracts without moving its paws) and head dips (the rat leans over the edge of the open arm). The measures of risk assessment were categorized as protected or unprotected: performed while the body of the rat was in the closed or middle areas of the maze, or while in the open arms, respectively. An arm entry was counted when all four paws were within its boundaries. Before and after each test, the interior of the maze was wiped with a 95% alcohol solution and allowed to dry.

Blood spot collection and corticosterone assay

Tail blood spot collection and blood spot assay were based on methods previously described by Worthman and Stallings 1997). Blood was collected during the first part of the light cycle (9am - noon) on # 903 filter paper (Schleicher & Schuell, Keene, NH) using the tail nick procedure. Samples were taken at the end of the six month baseline phase (PN191), to establish a basal measure, and after the CMS period at both six and 12 month test periods. The procedure involved the brief application of gentle pressure in a downward motion across the rat's tail while transferring the rat from the home cage to a flat workstation covered with a cotton cloth and swabbing the distal portion of the tail with alcohol. A small, longitudinal, superficial nick was made near the tip of the rat's tail using a scalpel blade held approximately at a 45° angle. Initial exudate was wiped away with clean gauze. A large droplet was then allowed to form and placed onto the

preprinted circle of the filter paper without touching the tail to the paper. Two samples were taken from each rat at every sample interval. Occasionally, more than one drop was required to fill the circle due to poor circulation, very shallow tail nick, or a highly active rat. Any blood on the tail was wiped away with water-moistened gauze, and light pressure was then briefly applied to the wound using clean gauze until bleeding ceased. This generally occurred immediately, and the rat was returned to the home cage. This procedure was rapid and caused little to no apparent distress in the rats which were accustomed to being handled. Samples were air-dried in a horizontal position at room temperature for a minimum of 12 hours before being placed in plastic bags containing desiccants and kept at -14°C until processing.

Blood was eluted from the sample paper by placing a 2.5mm circle of sample paper into labeled tubes, adding 200 µL of Dulbecco's phosphate buffer (Sigma Diagnostics, Inc.) with 0.1% gelatine and placed in the fridge overnight. The following day, tubes shook for 1 hour on an orbital shaker at room temperature. Of the resulting eluent, 25 µL was pipetted into duplicate tubes and assayed via radio immune assay using the Corticosterone kit for mice and rats (MP biomedicals, Solon, Ohio, USA) as per kit instructions.

Statistical analyses

Hierarchical Linear Modeling (HLM) was employed for the analyses of weight, sucrose preference, EPM behaviour, and corticosterone levels using the HLM V6 software (Raudenbush, Bryk, and Congdon, 2000). This approach to regression is appropriate for data with a nested structure (Raudenbush and Bryk, 2002), and its specific application

for use in designs involving multiple family groups of genetically similar siblings has been reviewed in detail previously (Baker et al., 2009).

For the dependent variable weight, a three-level HLM analysis was performed with multiple time points nested within each individual, and multiple individuals nested within each family. For the dependent variable, sucrose preference, a two-level HLM was performed with multiple time points nested within each family. For behavior in the EPM and for corticosterone levels, two-level HLMs were performed with multiple individuals nested within each family.

All independent variables at lower levels of a hierarchy were group mean centered, so that the intercepts represented group means. Given the sample sizes in the present study, restricted maximum likelihood estimation was used as it yields higher power in comparison to full maximum likelihood. Initially in each HLM analysis, intercepts (e.g. mean weight for each family or for each individual) and slopes (e.g., mean change in weight over time for each family or change in weight over time for each individual) were allowed to vary (i.e., their error terms were included), since it is reasonable to expect these to vary to some degree from family to family and from individual to individual. The variability of interest in this study was between families as entire family groups were assigned to the different experimental conditions. If the variance across families of an intercept or slope was large enough to be statistically significant, it would have two implications: it would provide statistical support for using HLM rather than a regular/non-nested regression, and it would provide statistical support for adding predictors that might explain some of that variance, i.e., for adding GS, CMS, or GSxCMS to the model. Even if the variance were not significant, however,

HLM could still be applied to the data on theoretical grounds, and predictors of the variance could also be added on theoretical grounds.

The first column of Table 7 shows the results of chi-squared tests of the variances of the intercepts (i.e., mean values for each family) and of the variances of the slopes (i.e., mean change over time for each family). The majority of these variances were at least marginally significant. In cases where the chi-squared test of variance was not significant, HLM analysis was still employed, and the roles of GS and CMS were examined. This was based on theoretical grounds predicting that there would be some difference between families as a function of GS and CMS, and also for consistency and comparability.

Occasionally, the reliability of a parameter was so low (below .05, indicated by an 'X' in the chi-squared column of Table 7), that it was not feasible to include the error term (Raudenbush and Bryk, 2002). That is, a separate parameter for each family could not reliably be estimated; only mean parameters across groups of families could be estimated. In this data set, the most likely reason for these instances was the restricted sample size. In these instances, predictors (GS, CMS, and the interaction) could be added to the model (i.e., may account for some of the variance in the parameter), but any further variance in the parameter would be set to zero.

For all outcome variables, the effect of GS was determined while controlling for the effect of CMS, the effect of CMS was determined while controlling for the effect of GS, and the effect of GSxCMS was determined while controlling for both GS and CMS. As none of the interactions tested proved to be statistically significant, only the main effects of GS and CMS are included in Table 7.

Effect size was computed as percentage of between-family variance in a parameter (intercept or slope) that was explained by a predictor (GS, CMS, or interaction between GS and CMS). Effect sizes were only computed for analyses that had a significant or marginal effect for the predictor as well as a significant or marginal chi-squared test for the variance, since effect sizes cannot be obtained consistently and reliably in the other cases.

Results

Table 7. Results of HLM analyses.

DV	Analysis	Chi-square for between-family variance *df* = 11	Effect of GS		Effect of CMS	
			Coefficient *df* = 9	% of variance explained	Coefficient *df* = 9	% of variance explained
Weight	Over 41 weeks					
	Mean weight	28.42**	-34.78 t	22%	6.65	
	Rate of weight gain	23.57*	-.57		.35	
	At 6 months					
	Change in rate of weight gain from pre-CMS to CMS	48.22**	-2.62		-7.16**	75%
	At 12 months					
	Change in rate of weight gain from pre-CMS to CMS	29.05**	.36		-6.81 t	50%
Sucrose Preference	At 6 months					
	Mean sucrose preference	79.88**	-.01		-.03*	54%
	Change in sucrose preference from pre-CMS to CMS	34.37**	.03		.04	
	At 12 months					
	Mean sucrose preference	84.29**	.00		-.04 t	29%
	Change in sucrose preference from pre-CMS to CMS	14.30	.01		-.01	
Elevated Plus Maze	At 6 months					
	Total activity	25.31**	-.77		-.64	
	% time in open arm (1st 5 min.)	12.57	1.97		1.29	
	Frequency in open arm	34.56**	.62		-.51	
	% protected head dips	12.21	6.22		.36	
	% protected stretch attends	8.82 X	-1.59		4.86	
	At 12 months					
	Total activity	27.40**	-2.46		.14	
	% time in open arm (1st 5 min.)	14.30	4.50		1.16	
	Frequency in open arm	34.30**	1.35		-.20	
	% protected head dips	12.10	-9.03		-3.73	
	% protected stretch attends	6.89 X	-23.58 t		12.42	
Corticosterone	At initial baseline	7.67 X	5.30		-46.55	
	At 6 months					
	Mean corticosterone post-CMS	39.97**	226.05 t	30%	-4.94	
	Change from initial baseline to post-CMS	26.79**	210.59 t	35%	38.23	
	At 12 months					
	Mean corticosterone post-CMS	22.20*	224.94*	55%	-187.99*	36%
	Change from initial baseline to post-CMS	17.16 t	281.48**	88%	-109.40	

*** p < .05; ** p < .01; t p < .10**

X – The error term was removed due to low coefficient's reliability; thus, the percent of variance explained could not be computed and df is altered (df = 26, %protected stretch attends EPM; df = 48, corticosterone at initial baseline).

Weight

Average weight and rate of weight gain, across 41 observation weeks, from four to 14 months of age (PN 105 to PN 420) was examined first. GS had a marginally significant effect on an individual rat's mean weight, reducing it by 34.78 grams, on average. Twenty-two percent of the variance in weight that existed between families was explained by GS, a moderate effect size. Please refer to Figure 23 for a detailed plot of weight gain over time; as evident here, average GS weight for both groups was never higher than either control group at any of the 41 weekly weights.

In the next set of analyses, the change in slope (i.e., rate) of weight gain from the six weeks preceding CMS to the three weeks during CMS was examined for the six month period. CMS exposure had a significant effect, such that rats in the CMS condition showed attenuated weight gain – they gained 7.16 fewer grams per week – during the CMS phase compared to rats unexposed to CMS. The effect size of CMS was very large explaining 75% of all the variance across families in the degree to which weight gain changed.

Similarly, the change in the rate of weight gain from the six weeks preceding the 12 month CMS to the three weeks during the 12 month CMS was evaluated. The CMS exposure had a marginal effect, reducing weight gain by 6.81 grams per week. The effect size was again very large; it accounted for 50% of the variance.

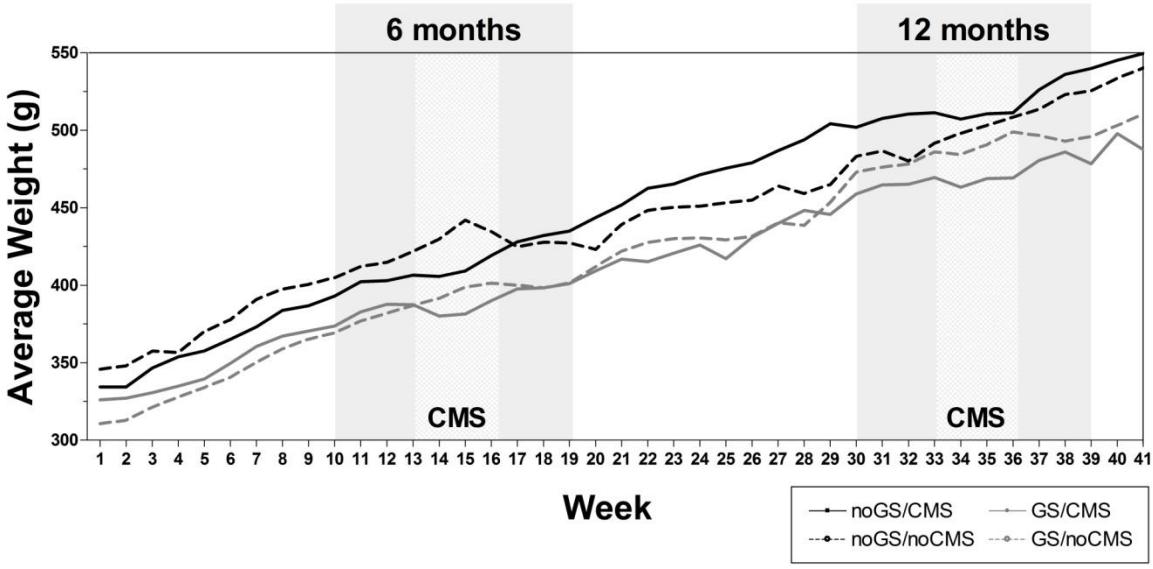


Figure. 23. *Weight gain over time.*
Mean weight, in grams, across all 41 experimental weeks spanning six to 12 month experimental periods. Data associated with six month test period is highlighted in the box on left and 12 months, on right. The CMS phase is indicated by a light bar in the center of each.

Estrous cycle

No group differences were observed at six months with 85% or more of individuals in all groups displaying regular cycles across the six month monitoring period. Between the end of the six month monitoring period and the beginning of the 12 months monitoring period, nearly all animals displayed a cessation of regular cycling patterns.

Preference for Sucrose

Figure 24 shows sucrose preference during baseline (“B”), during each of the three weeks of CMS (1, 2, 3) and during post-CMS (“P”). The degree to which rats preferred sucrose over water was compared for six weeks prior to the CMS phase versus three weeks of the CMS phase and was assessed at both the six and 12 month

periods. This provides an indication of the change in sucrose preference from baseline to CMS, hence the ability for CMS to induce anhedonia. A significant decrease from baseline preference rates is considered an anhedonic response on this measure. Also examined in this model was the overall average preference for sucrose ignoring phase distinction. CMS had a significant effect on mean sucrose preference: CMS exposed animals had 3% lower rate in sucrose preference overall and explained a full 54% of the variance in sucrose preference observed across families. Similarly, at 12 months, CMS exposure had a marginally significant effect on mean sucrose preference producing a 4% decrease in sucrose preference overall and accounted for 29% of the variance in sucrose preference. These effects were due primarily to the tendency for the GS/CMS group to have lower preference scores and the decrease in preference coinciding with CMS; neither effect reached statistical significance. As evident in figure 24, the decrease in preference at six months due to CMS exposure ranged from 6 to 10% and was observed only in the first week in the noGS/CMS group and the first and second week in the GS/CMS group and was fully recovered by the third week. Thus, a strong anhedonic profile was not observed.

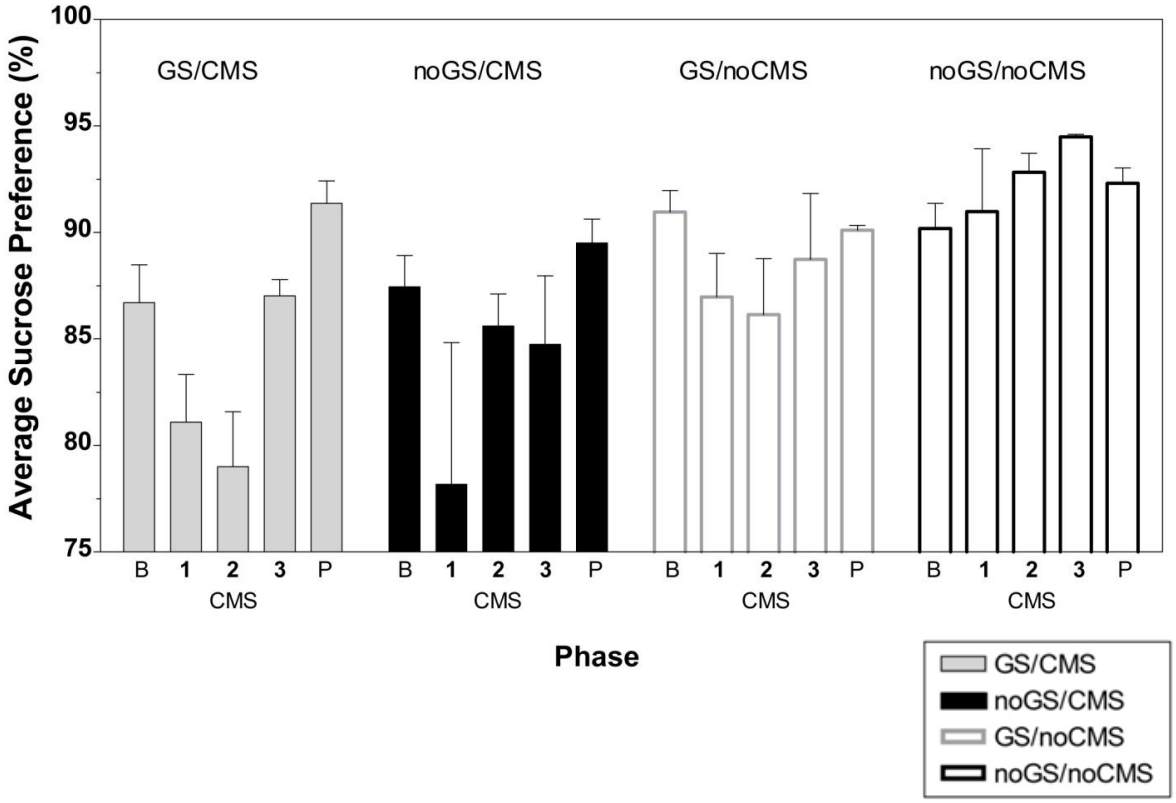


Figure. 24. *Preference for sucrose at six months.*
Group averages (±SEM) for 24h sucrose preference for mean baseline phase (B), each week during the stress phase (CMS 1,2,3), and mean post stress phase (P). GS/CMS group appears on far left, noGS/CMS group appears middle left, GS/noCMS on middle right, and noGS/noCMS on far right.

Elevated Plus Maze

For the EPM data collected following CMS, at six and 12 months, we examined the average total activity in the maze, the number of arm entries ignoring open or closed distinction; the total percent time spent in the open arm of the EPM which was calculated as a percent score ((Time in open arm/Total maze time)*100)); as well as the total frequency of entries into the open arm of the EP ((frequency of open arm entries/frequency of total arm entries)*100), across individuals in each family. Low

levels of these activities served as indices of anxiety. There were no effects of GS, CMS, or GSxCMS on these measures.

The total percent time spent performing either protected head dips ((frequency protected head dips/total frequency head dips) * 100) or protected stretch attends ((frequency protected stretch attends/total frequency stretch attends) *100) at six and 12 months was subsequently analyzed. High levels serve as indices of anxiety. At 12 months, GS had a marginal effect on protected stretch attends, though in the direction contrary to expectations– GS was linked to a decrease in protected stretch attends. In addition, at 12 months, there was a marginal interaction in predicting protected head dips, such that families from GS/CMS group had slightly higher percentages of protected head dips than would be expected from the combined effects of GS and CMS. Thus, although the chi-squared values for these analyses show that the variance across families was too small to reach significance, some of that variance may have been explained by our predictors. These findings should be seen as tentative, however, requiring replication.

Corticosterone

Figure 25 shows the cortisone levels at baseline, six months, and 12 months. Average corticosterone level, across individuals in each family, was assessed at baseline, and the degree of change from baseline to the post-CMS measure was also assessed at both six and 12 months. High levels were indicative of a heightened stress response. While GS had no effect on corticosterone at initial baseline, it resulted in marginally higher corticosterone post-CMS, at six months – by 226.05 units, on average

and accounted for 30% of the variance in corticosterone levels across families. Further, GS resulted in a marginally significant greater degree of change from initial baseline to post-CMS by 210.59 units more in GS rats, on average, and it accounted for 35% of the variance in this change across families. A similar but stronger pattern was observed at 12 months: GS resulted in significantly higher corticosterone post-CMS – by 224.94 units – and accounted for a full 55% of the variance. Corticosterone increased by a significantly greater degree from initial baseline to post-CMS in the GS group: by 281.48 units, and it accounted for nearly all of the variance, 88%.

While CMS had no effect on corticosterone at initial baseline or at the six month post-CMS measure, it resulted in significantly lower corticosterone levels in the post-CMS 12 month measure compared to noCMS – by 187.99 units, on average – and accounted for 36% of the variance in corticosterone levels across families. Note that CMS groups maintained almost perfectly consistent patterns from six months to 12 months, but the effect was driven by the noCMS groups that had increased corticosterone levels in the 12 month measure compared to 6 months and was especially robust in the GS/noCMS group.

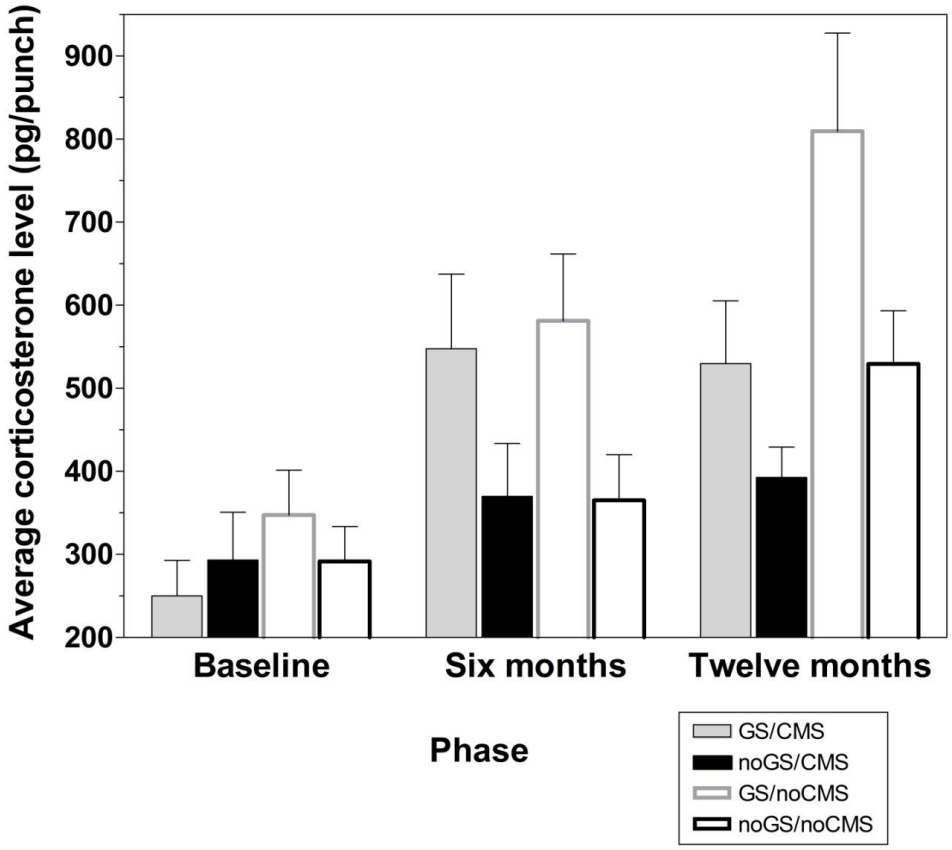


Figure. 25. *Corticosterone levels.*
Average group (±SEM) corticosterone (pg/punch) is displayed at all measurement time points: Baseline phase (left), post-CMS phase at Six months (middle), and Twelve months (right).

Discussion

The current study was conducted to examine if chronic stress exposure during mid and late adulthood would produce latent GS effects not evident in these female offspring in prior testing phases (Baker et al. 2008, 2009). In these earlier studies, a profile, similar to the one observed presently, was obtained; an effect of GS on weight with limited influence on behavioural measures of anxiety or the estrous cycle was found in both the juvenile (Baker et al. 2008) and early adult period (Baker et al. 2009).

Preference for sucrose, weight, and estrous cycle regularity were monitored throughout the current study; anxiety-like behaviour in the EPM and corticosterone levels were monitored following the administration of CMS at six and 12. Results from the present phase of study demonstrated that offspring from mothers stressed during gestation showed a consistent pattern of lower weight over time compared to those from control mothers. GS elevated both average corticosterone levels and the degree of change from baseline to the post-CMS measures. CMS reduced the rate of weight gain during the stress exposure periods and modestly reduced sucrose preference. The latter was suggestive of a very mild anhedonic-like effect that recovered rapidly prior to the cessation of stress. CMS exposure was also associated with consistent corticosterone levels across the six and 12 month post-CMS measures; interestingly, the lack of CMS exposure (noCMS group) was associated with elevated levels of corticosterone at 12 months. Also contrary to expectation, the two grouping variables (GS and CMS) did not interact on any measures examined.

The most robust pattern, consistently lower weight in GS offspring, was present from weaning until 14 months. Statistical significance was observed during juvenile and early adult periods (Baker et al. 2008; 2009) and marginally so in the current observation period, after accounting for the effects of sibling group. Thus GS reduction in weight appears to be a stable effect over the lifespan of these rats and represents a novel finding. While most studies examining the effects of early life manipulations in the adult rat fail to report weight, our findings suggest that monitoring weight may be an important factor for inclusion in GS research.

In previous reports that included GS effects on adult weight, two groups report reduced weight in males (Bosch et al. 2006; Drago et al. 1999). No effects due to GS have also, and more commonly been reported in adult male rats (Richardson et al. 2006; Estanislau and Morato, 2005; Van den Hove et al., 2005; Fride, & Weinstock 1989). Little comparison data exists, however, for females and none that examined weight for the duration reported in the current study. No effect of GS on weight at 79 or 80 days of age was reported for female Sprague-Dawley rats using a similar GS protocol to that employed in the current study (Bhatnagar et al. 2005, Bowman et al. 2004).

We report previously that maternal weight following GS exposure was reduced in the perinatal period (Baker et al. 2008). Maternal weight, like other relevant maternal factors, is not typically included in GS studies. Chronic restraint in late gestation has been shown to reduce maternal food intake and body weight gain (Luz et al. 2005; Ward, & Wainwright, 1988; Kinsley, & Svare 1986); moreover, decreased nutrition alone can produce fetal intrauterine growth retardation resulting in lower birth weights (Lesage et al. 2001). In our case, weights on postnatal day two remained largely unaffected; however, GS was associated with greater variability in individual pup weight within litters, fewer male pups per litter, and elevated pup mortality rate (Baker et al 2008) suggesting that there was likely some degree of nutrient transfer disruption, inconsistency, or inefficiency as a result of the stress manipulation. It remains unclear to what extent the role of maternal nutritional factors may play; however, we see no initial difference in preweaning weight and have a lack of effect on typically altered behavioural measures of anxiety and cognition.

CMS also exerted a temporary attenuation of weight gain over the course of stress. This effect is regularly observed in our laboratory using a similar CMS regime in female Long Evans and Sprague–Dawley rats (Konkle et al. 2003; Baker et al. 2006; Baker, & Bielajew 2007) and by our group (Bielajew et al. 2002) and others in male rats (Matthews et al. 1995; Dunčko et al. 2001b; Nielsen et al. 2001). This weight effect is present despite the lack of food and water deprivation in the CMS model employed in the current study. Food deprivation is often included in CMS models to stimulate ingestion accommodating a one hour sucrose preference measure. As food intake was not monitored here, it remains unclear if the weight change was a result of decreased intake or changes in metabolism induced by CMS exposure. Clearly the interaction between stress, maternal factors, and weight is a complex one and deserving of future investigation.

The regularity of the estrous cycle was not influenced by either stress manipulation. This is in line with previous findings in our laboratory. In the same rats from the current study during early adulthood, no effect of the GS manipulation was observed on estrus regularity (Baker et al. 2009). Sprague–Dawley rats housed in social groups and exposed to a similar CMS regime as that used in the current study did not include alterations to light cycle or bouts of food and water deprivation (Baker, & Bielajew 2007). These factors may be the cause of estrous cycle effects reported previously by our laboratory (Konkle et al. 2003; Baker et al. 2006) and others (Mattioli, Funari, & Perfumi 2009; Dalla et al. 2005; Grippo et al., 2005). The cessation of regular cycling just prior to 12 months may have been caused by the large size these rats reached by this stage - between 450 and 500 g. This could be attributed to the constant

access to a 1% sucrose solution that was consumed in significant quantities by all rats. Obesity in humans is well known to induce alterations in menstrual cycle regularity and induce complete cessation of regular cycling patterns. This is due to the elevated levels of estrogen produced by fat cells that mirrors the physiological state of pregnancy.

The lack of or limited effects seen in the behavioural measures here could be due to a variety of factors - limited effectiveness of GS paradigm, CMS paradigm, or lack of sensitivity in the EPM measure to subtle behavioural variation, for example. Given that the GS clearly induced effects on maternal weight, anxiety and care behaviours, and the CMS paradigm has been previously shown to be modestly effective in Long Evans rats using a similar protocol in our laboratory (Baker et al. 2006), findings in the current study may be due largely to the protective effect of group housing conditions and gentle husbandry practices. This environment could be considered supportive or even enriching compared to standard individual housing practices. It was our aim to eliminate or control, as much as possible, stress effects from alternative sources beyond the GS and CMS manipulations. To this end, animals were maintained in same sex sibling groupings and provided access to objects in an effort to promote positive natural behaviours. Group housing alone has been shown to exert protective effects in the context of both GS (Laviola et al. 2004) and CMS (Baker, & Bielajew, 2007) and is especially important in female animals. Enriched housing may also promote neural plasticity (Brown et al. 2003) and may therefore be a mechanism of neuroprotection.

The gentle weaning procedure carried out over seven days has been shown to reduce corticosterone response to stress (Cook, 1999), and sibling groups were

maintained in the same room in large cages identical to those used during lactation. Rats were accustomed to handling and general laboratory practices from birth never experiencing shipping trauma. Indeed even personnel were relatively consistent. In mice, shipping has been shown to increase corticosterone levels, decrease immune function, weight loss, and dehydration (Landi et al. 1982; Wallace 1976) in the short-term, and when shipping occurs prior to maturity, alterations in HPA axis functioning and sexual behaviour are observed in adulthood (Laroche et al. 2009).

Consistency may have been the critical factor in reducing or counteracting the effects of GS, particularly in the female offspring. As suggested by Weinstock (2001), gestational stress may interfere with the normal adaptive process to novelty. It has been shown previously that GS female offspring have a more marked response to stress than their male counterparts (McCormick et al. 1995; Weinstock et al. 1992). The corticosterone response of GS animals also fails to adapt to novelty even after repeated exposure – up to eight days exposure to the same open field – while control animals show rapid adaptation (Fried 1986). GS offspring may be unable to adapt to changes in facility and housing conditions and general laboratory practices such as handling and cage cleaning which have been shown to activate the stress response in naïve animals (Balcombe, Barnard, & Sandusky 2004). Such procedures, considered routine and not part of a stress regime, may therefore be chronic stressors in GS offspring and a potential root of some GS effects observed on behaviour. Our methods involving consistency throughout the life span may have eliminated this possibility and protected offspring from continued exposure to perceived stress and HPA axis activation.

Although we posit that the supportive nature of the rearing environment most likely exerted a protective effect, ineffectiveness of the paradigms cannot be ruled out. The gestational stress paradigm employed here during late gestation can be considered moderate in nature, involving three random intervals of restraint totalling 75 minutes per day during the light phase. Others using a similar paradigm in Wistar rats do report subtle behavioural effects following a similar procedure in CD (Richardson et al. 2006) and Wistar rats (Zagron, & Weinstock 2006; Ordonia, & Pivina 2004). The discrepancy may be related to differences in strain reactivity.

Given that the effects of a physical restraint stress on plasma corticosterone is progressively attenuated with repeated exposure, a variable stress paradigm may be preferable as described by some (Simpkiss, & Devine 2003) or a similar random presentation in both duration and timing, as conducted here, may satisfy this concern. Corticosterone levels were not monitored in mothers in the current study; however, maternal corticosterone levels were found to be elevated both at GD17 and 20 following the repeated presentation of restraint stress (Zagron, & Weinstock 2006). Despite the findings above and the unpredictable nature of the restraint stress presentation, it is possible that corticosterone levels may have been reduced over subsequent days due to adaptation. In this case, the fetal effects of GS would be limited to the initial exposures rather than late gestation and may have missed the late gestation critical window for GS induced HPA axis alteration.

Other final considerations may be related to methodological issues associated with variability. Fluctuating levels of hormones in normally cycling animals have been observed to influence behaviour in the EPM (Severino et al. 2004; Marcondes et al.

2001). Generally females cycle together, but given the sample size, it was not possible to compare behavioural responses during various estrous cycle stages in the current study. This was a potential problem for the six month measure; however, because nearly all animals had stopped cycling by the 12 month tests, this factor was limited and yielded similar results.

The use of HLM allowed for the removal of variance due to sibling grouping and represents a strong statistical tool for use with such designs. Large variability between individuals exists, however, even within sibling groups. We have shown previously that only a subset of individuals will demonstrate a strong CMS effect and will often be missed when grouped along with others (Baker, & Bielajew 2007). In the current study, to eliminate stress imposed by individual housing required to obtain individual sucrose preference scores, only a group measure was used and therefore did not permit the examination of individual differences.

In conclusion, the current study highlights the importance of considering housing conditions throughout life in stress-related research in both the creation of and protection from stress-related disruptions. It also suggests that reporting weight may be a useful measure in such studies and highlights the usefulness of the HLM statistical technique to deal with nested designs such as those encountered in studies of early life manipulation.

General Discussion

The studies reported in this thesis were conducted with the primary goal of developing a CMS paradigm that would elicit behavioural and physiological alterations in female rats. A secondary purpose was to investigate the possible interactions between CMS and environmental factors of housing conditions and maternal restraint exposure. This thesis focused on the female rat to address the appropriateness of applying models, well-established in male rodents, to females without consideration of known gender differences in response to stress stimuli. This is a common practice in the stress and depression literature. Robust evidence in the clinical population demonstrates a greater incidence and increased sensitivity to develop anxiety and depression in response to stress in women. Therefore, the striking gender difference that exists in preclinical research that, for decades, had focused almost exclusively on male responses is not warranted. This female-centric approach was also taken to modify male-based models to be more appropriate for use with intact female animals and to allow for the examination of CMS effects on the estrous cycle. Stress-related menstrual cycle disruption is a well-known phenomenon reported in the clinical population (Genazzani et al. 1991).

To begin, the CMS model, along with common indices of detecting anhedonia-BSR and sucrose intake and preference - commonly used in males, was applied in female Long Evans and Sprague-Dawley rats. In Study 1, only a change in sucrose intake was noted in the measures of hedonic status, and the estrous cycle was disrupted by both CMS and BSR; severe disruption was caused by the latter. The general lack of an anhedonic profile in this study was hypothesized to be caused, in

part, by the use of overnight pairing as a stressor in the original, male-based, CMS regime. We considered that this condition might have a protective effect against stress in individually housed female rats. Exposure to an unfamiliar conspecific is an appropriate stressor in males who show a defense/attack response, but it is likely not suitable for virgin females who are typically not distressed in the presence of unfamiliar rats (Westenbroek et al. 2003 a, b). While BSR initiated permanent, severe estrous cycle disruption, it was unclear if such disruption in rats unexposed to BSR was a general effect of CMS exposure or induced by changes to the light/dark cycle or food and water availability included in the regime but known to exert estrous cycle alterations, independently.

The model was subsequently revised to address these issues. In Study 2, pairing, overnight illumination, and food and water deprivation were removed from the CMS model and replaced with other stressors well known in the literature. Housing was manipulated to test the hypothesis generated by Study 1 that pairing may exert protective effects in females by including both single- and paired-housed groups. Given the consistent inability to detect CMS effects on BSR measures by our group, the invasiveness of the procedure, and labor intensive nature of the measure, the use of BSR was discontinued. The sucrose preference test was also altered to accommodate the lack of deprivation and a three hour measure was employed. The social interaction test was added to observe possible CMS-induced changes in social behaviour to further our understanding of the interaction between social contact and stress. Single housing facilitated CMS effects; individually housed rats had attenuated weight gain, increased social behaviour, and a change in hedonic status demonstrated by the expected

decrease in sucrose preference following CMS exposure. Rats housed in pairs were unaffected. The estrous cycle was not significantly disrupted once deprivation and changes in light availability were removed as stressors; although, there was a visible trend in the data for a reduction in estrous cycle regularity that was more prominent in the single-housed group. In this and subsequent studies, all animals had access to an opaque tube, nesting material, and a chew toy or similar object to facilitate the expression of naturalistic behaviours; as part of the CMS regime, these objects were removed.

After establishing a model that appeared to be effective in a vulnerable population, the single-housed female rat, we embarked on a long-term project in which the stress history of each animal was known. This entailed creating a housing environment that limited stress – gradual weaning, group housing, access to objects facilitating naturalistic behaviours, and eliminating shipping trauma and acclimatization to handling and a novel facility. In the first installment, Study 3, pregnant rats were exposed to restraint during late gestation and effects on weight, maternal care behaviour, and anxiety in the EPM and juvenile offspring behaviours in the EPM, emergence, and t-maze were observed. Here, GS resulted in reduced maternal weight and care behaviours and an anxiogenic profile in the EPM. These results suggested that GS was effective in inducing stress in the mother. Despite this, only limited evidence of GS effects were observed in male and female juvenile offspring anxiety measures which were often contrary to our hypotheses. Litter size was reduced, mortality was increased, and post weaning weight was attenuated in females, however, suggesting some degree of influence in the offspring.

Following this, Study 4 reported similar measures in these offspring in the early adult phase in which, again, a strong effect was evident on weight, in both sexes, but limited expected behavioural outcomes were observed.

The fifth and final study of this thesis brought GS and CMS together. Here, adult female offspring were exposed to the modified CMS regime at both six and 12 months with the intention to observe possible latent effects of GS at these stages and a possible interaction between early life stress and chronic unpredictable stress experienced in adulthood, a hypothesized recipe for depression in females. In this study, sucrose preference was monitored as a group measure with constant access allowed throughout all phases in an effort to observe, more precisely, a change in hedonic status through daily measurements. Behaviour in the EPM was also included for consistency with Studies 1 and 2 and because anxiety is often found to be comorbid with depression (Linzer et al., 1996). Corticosterone levels were also measured prior to and following CMS exposure at six and 12 months to observe possible differences in HPA activity. Again, weight was consistently lower in GS animals and was transiently diminished by CMS exposure. A change in hedonic status was observed initially but was fully recovered by the third week of CMS. No effects of either measure were evident in responses in the EPM and the estrous cycle was not affected by stress. GS did coincide with elevated corticosterone levels following CMS in both CMS exposed and control rats. Unexpectedly, GS and CMS did not appear to interact on any measure.

Taken together, these results show that stress-based models, previously established in males, must be altered to accommodate the cycling female rat in two ways: 1) to eliminate extraneous variables that may interfere with the estrous cycle and

mask possible stress effects and 2) to consider the appropriateness of individual stressors to induce a stress response in females. While a general lack of effects abound, it is likely that social housing, environmental stability, and supportive early life experiences exerted a protective effect against the establishment of stress effects related to depression and anxiety. The housing practices employed here may be considered a model of stress-resilience and represent a new avenue of pursuit.

Over the course of pregnancy, rats show elevated levels of anxiety that peak during late gestation and are blunted during the postpartum period. This effect is hypothesized to contribute to the expression of postpartum maternal behaviour by reducing the fear response seen in inexperienced female rats towards rat pups (Lonstein 2005; Neumann 2001; Fleming, & Luebke, 1981).There are contradictory reports regarding how GS affects maternal behaviour (Champagne, & Meaney, 2006; Smith et al., 2004; Meek et al., 2001; Pardon et al. 2000; Maestripieri et al. 1991; Fride et al. 1985; Moore, & Power 1986; Power, & Moore, 1986; Herrenkohl, & Whitney, 1976), but generally, it appears to decrease maternal care towards offspring as observed in Study 3. We also reported that anxiety behaviour in the EPM was increased in response to GS in the pregnant rat just prior to birth. This finding suggests that GS effects on maternal behavior may be mediated by a general effect on anxiety and finds support in studies reporting elevated corticosterone levels in response to GS (Zagron, & Weinstock 2006). A similar finding was observed in GS dams during the post-weaning period indicating that this effect may last through the lactation period (Darnaudery et al., 2004).

The most common method of GS delivery is repeated exposure, over multiple days (timing and duration depend on the targeted system in offspring), to physical restraint. This is typically conducted under bright illumination during the light phase for 30 min up to many hours daily. The restraint stress employed in Study 3 can be considered mild in comparison to many paradigms given that it was not conducted under direct exposure to illumination that causes a marked rise in body temperature. Restraint is typically conducted via physical containment in a plastic apparatus; even without illumination, this procedure can elevate body temperature. High temperature can disrupt the feto-placental unit and represents an extraneous variable that could be easily controlled through monitoring and is worthy of future consideration in studies employing this technique. Since maternal behavioural indices were altered in response to GS, the paradigm used here appears to be successful in inducing stress in the mothers. This may have been due, in part, to the unpredictable nature of the stress delivery which was random in both time of day and duration of each restraint session, but always totaled 75 minutes per day. This approach overcomes some of the issues regarding the presentation of repetitive stressors which have been found to cause adaptation in non pregnant rats (Simpkiss, & Devine 2003). Adaptation might be especially problematic when delivered during the light cycle as it is the typical resting period of the rat and some have suggested greater effectiveness of GS when applied during the dark/active phase (Koolhaas et al. 2011).

The CMS model consists of a series of stimuli that are perceived as stressful to the animal when presented in a random, unpredictable manner. It is the combined effect of stimuli in conjunction with the method of presentation that is important rather than any

particular effect of an independent stressor. The critical factor being that the animal perceives the experience as unpredictable and beyond control which creates a sense of threatened homeostasis and consequently, stress. Because of this feature, a variety of stressors have been used in CMS models successful in establishing an anhedonic-like effect (reviewed in Willner 2005). This flexibility allowed for stressor substitution resulting in the development of a model more suitable for use in female rats.

While individual stressors are not necessarily important, for the paradigm to be successful, the stimuli used ought to be relevant not only to the species of study, but also to the sex. A factor that has remained largely ignored in the CMS literature. Furthermore, the model is a more powerful tool if the stimuli do not interfere with normal physiological functioning of the animal independently from the stress effect. Results from Study 2 suggest that the removal of pairing as a stress stimulus, which was possibly not stressful to female rats, as well as light cycle disruption and food and water deprivation, both known to be estrous cycle disruptors, yielded positive results. This was seen both in the induction of an anhedonic response marked by the reduction in sucrose preference not previously observed in our laboratory in females, and a failure to observe robust effects on the estrous cycle previously obtained using the original model. While we do report success in achieving an anhedonic-like profile in the sucrose preference measure, this effect was evident, only in single housed rats. Rats maintained in pairs did not express attenuation of the rewarding value of sucrose. When subsequently applied to rats kept in groups of five per cage, the hedonic status was reduced only marginally in the initial weeks followed by rapid recovery prior to the cessation of stress. Physical isolation, therefore, appears to be an important component

of the CMS regime in females. In future studies, it may prove useful to include individual housing as part of a female-specific CMS model. Indeed this observation is consistent with clinical research - a negative correlation between social support and depression has been documented in women (George et al. 1989).

While single housing is a common laboratory practice, the fact that it was able to induce a depressive-like profile following three weeks of CMS exposure suggests that it may be an inappropriate control group for stress research involving female rats. Indeed others report stress effects observed in individually housed rats (Belz et al. 2003; Westenbrek et al. 2003a,b; Haller et al. 1999). When establishing a 'normal' control condition, group housing appears to be more appropriate in this context while social deprivation appears to be useful in creating vulnerability to developing depressogenic responses.

Housing effects may also have been at the root of the limited GS effects observed on behaviour throughout the long term component of the thesis (Studies 3, 4, and 5). Environmental enrichment including complex objects, environments, and social contact has been shown to reverse cognitive deficits, anxiety-like behaviour, elevated corticosterone levels, and impaired hippocampal synaptic plasticity induced by GS (Yang et al. 2007; Koehl et al. 2002; Morley-Fletcher et al. 2003). While the housing conditions of the offspring from studies 3, 4, and 5 were not 'enriched' in comparison to these studies, they were certainly more so than standard individual housing. Providing social contact may be the important element in preventing behavioural impairments related to emotionality. The lack of strong behavioral responses generally observed throughout the thesis may, therefore, be tentatively interpreted as evidence of a

protective effect rather than a lack of effect of the GS paradigm, a theory requiring further replication with individually housed control groups.

Similar to the CMS model, the primary index of anhedonia, the sucrose measure, was subject to modification throughout the progress of the thesis research. Alterations were made typically to accommodate the housing component of the study, but also to address theoretical concerns. In Study 1, we employed a standard one hour measure preceded by food and water deprivation, a procedure that has been used extensively throughout the literature. In addition, a 24 hour measure was included. The latter has been the practice of our laboratory to address concerns regarding the possible change in motivation for sucrose in the food deprived animal that might consume it for its caloric rather than rewarding value. Because we were interested in studying paired housing in Study 2, obtaining individual scores required separation for the duration of the test. To reduce the amount of time the animals were separated, we opted to use a three hour measure that had been employed successfully by others (Dunčko et al. 2003). Food deprivation prior to the test was eliminated to address the concern raised above. In the final study, a group measure was taken. While it would have been ideal to obtain individual animal sucrose profiles, the logistics involved and the risk of creating stress due to separation made a group measure reasonable. In an effort to improve the sensitivity of the measure and improve statistical power due to low numbers per group based on using a group measure, we opted to allow constant access to sucrose alongside water so that daily values could be recorded. The desired improvements of power and, to a certain degree, sensitivity were attained; however, the consequence of constant access to sucrose was an elevation in body weight due to high consumption.

This effect was not entirely unexpected, but substitution was not made for a non-caloric sweetener as we wanted to maintain consistency with previous studies. This was especially important for comparison between studies because modifications were being made to the methods of delivery. In future, the use of a calorie-free substitute may be advisable in this context. Saccharine, for example, has been shown by others to be a suitable replacement for sucrose (Harris et al. 1998; Pucilowski et al. 1993; Willner et al. 1987).

An additional reason for employing a non-caloric sweetener in place of sucrose is based on evidence that sucrose consumption may regulate the HPA axis (reviewed in Walker 2005). For example, sucrose, but not saccharine (Bhatnagar et al. 2000), has been shown to normalize hypothalamic CRF (Laugero et al. 2001) expression and ACTH release (Bell et al. 2000) following adrenalectomy. In addition, rats that had low sucrose preference displayed elevated basal corticosterone levels compared to those with high preference (Dunčko et al. 2003). Furthermore, it has been suggested that corticosterone increases the willingness to ingest sweet solutions in adrenalectomized rats (Bhatnagar et al. 2000). It is possible that the practice of providing rats with access to sucrose through the sucrose preference test may interfere, to a certain degree, with CMS effects, particularly if it is available ad libitum. The role of rich versus calorie–void rewarding consumables is an interesting avenue of continued investigation.

The other behavioural measures used as indices of stress effects were the social interaction test and EPM. Anxiety-related behaviour in the EPM remained largely unaffected by either stress paradigm (dams being the exception). Because the EPM was only included in studies lacking an individual housing component (studies 3-5), the

usefulness of this measure in this context requires further investigation. Indeed others report anxiogenic profiles in both CMS and GS exposed rats (reviewed in Willner 2005; Weinstock 2008, respectively). The inclusion of this measure is particularly useful in designs involving female responses given that anxiety is commonly comorbid with depression and a significant gender difference exists in the rate of comorbidity (Linzer et al., 1996). Active social behaviour measured in the interaction test was also not affected by CMS as employed in Study 2. While not directly comparable, this measure is an index of anxiety commonly used in male rodents. The lack of results may reflect a lack of anxiety, or alternatively, may suggest that this measure is not an appropriate test of anxiety in female rats. There was a pattern for single-housed stressed rats to increase time spent in social contact. Such an increase may reflect a female-specific response to stress hypothesized by the 'tend-or-befriend' paradigm and accounts for the female-typical response of seeking social contact in the face of stress (Taylor et al. 2000). Thus, the social interaction test may not be useful in detecting anxiety-like behaviour; however, its usefulness as a measure in female rats in the context of seeking social contact may prove beneficial.

In summary, although limited behavioural effects were observed, robust results were generally found in physiological measures, namely weight. As discussed above, it is likely that factors such as housing condition acted to counteract CMS effects and prevented the expression of behavioural disruption; however, it is also possible that group size played a role. Groups sizes used throughout this work were in line with reported effects in the literature, but in detecting subtle behavioural responses, a

greater sample sizes may be necessary. This is particularly true in the case of behaviours that express high individual variability.

Physiological measures of weight and the estrous cycle are often over looked in the context of stress and depression research. We demonstrate compelling evidence that weight should be included. Given that it is commonly recorded as befitting proper animal care practices, it is a measure requiring little additional effort that may reveal useful trends. Using the modified CMS paradigm, weight was consistently reduced in all exposed animals with single housed rats appearing to show the greatest reduction in response to CMS and slowest recovery post stress. Once elements of deprivation and light cycle alterations were removed, the estrous cycle appeared to be generally unaffected by CMS; however, given the well-known interaction between gonadal hormones and the stress response and limited data regarding CMS effects in intact female rats, continued investigation is warranted.

This work represents a promising avenue in which to explore the relationship between stress throughout the lifespan and depression within the context of female sensitivity. It expands upon the CMS literature and supports the usefulness of this approach. Furthermore, supportive postnatal environments appear to limit the expression of negative behavioural consequences in female rats. These studies highlight the significant role of the postnatal social environment in female responsiveness to stress and may represent a model with which to investigate stress resilience.

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