The Effects of Environmental Enrichment and Impoverishment on an Animal Model of Depression and Anxiety: Brain, Behaviour and Immune Function

Guergana Mileva

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of

Ph.D. Degree in Psychology

School of Psychology
University of Ottawa

ABSTRACT

While women are diagnosed with depression at twice the rate of men, pre-clinical research on this topic has relied heavily on the responses of male animals. This thesis examined the behavioural and physiological effects of environmental manipulation in the female Wistar-Kyoto rat, a putative animal model of depression. At postnatal day 52, baseline behavioural measures were collected in 36 Wistar and 36 Wistar-Kyoto female rats using the following tests: the elevated plus maze to assess anxiety, the forced swim test for depression-like behaviour, and sucrose preference test to assess hedonic status. At postnatal day 62, the rats were randomly assigned to one of three environments for 30 days: 1) standard housing - 3 rats in one large cage 2) isolated housing - 1 rat per small cage, or 3) environmental enrichment - 6 animals in a multistory cage filled with novel objects and a running wheel. Following 30 days in their housing condition, the same behavioural measures were again collected. Large differences between strains were found with the Wistar-Kyoto females showing significantly less mobility and activity in both the forced swim test and elevated plus maze. Sucrose preference was significantly higher after enrichment in both strains. Post-environment immune cytokine and corticosterone levels were also assessed in these animals at baseline and after the forced swim test. No difference in corticosterone between strains was found at baseline. However, Wistar-Kyoto females had significantly higher corticosterone levels than their Wistar counterparts after the forced swim test. In contrast, Wistar-Kyoto females showed significantly lower serum levels of the pro-inflammatory cytokine IL-1β than Wistar females. In the hippocampus, astroglial staining intensity was significantly increased in the CA1 of Wistar females after environmental enrichment. Glucocorticoid receptor staining in the CA3 was also increased after environmental enrichment in both the Wistar-Kyoto and Wistar animals. Finally there was a trend towards

higher levels of glucocorticoid receptors in the amygdala in Wistar-Kyoto animals who experienced environmental enrichment. Taken together, this thesis provides evidence for the effect of environment, specifically enrichment, on behaviour and physiological systems. These results suggest that incorporating social and physical enrichment as part of clinical intervention may benefit individuals with depression.

ACKNOWLEDGEMENTS:

First and foremost I would like to thank my supervisor Cate Bielajew who graciously accepted me into her lab and guided me through the PhD process. You have always made yourself available when needed and are there to talk about experiments, future studies, and confusing statistical findings. Not to mention our often hour long tangents on which healthy food we're trying that week! Most of all, thank you for being patient and understanding as I worked to complete this journey.

I would also like to thank my committee members Nafissa Ismail, Hélène Plamondon and Shawn Hayley for providing valuable input to the studies in this thesis during my proposal defense. Thank you Nafissa for helping me with the immune cytokine paper as well as being there for me when life got a little bit tough. Hélène, thank you for letting us borrow the reagents needed to finish up the immunohistochemistry chapter of this thesis and being a source of support. Thank you to my lab mates for being good sounding boards for the many trials and tribulations throughout the PhD. The friends I have made while doing my PhD will stay with me for life, thank you Jacinthe and Tessa!

A huge thank you to my parents, Lili and Roumen, and my sisters Vicky and Viara for being a constant source of support, fun and laughs the last 6 years as I wonder whether I'll ever finish, and what I'll do with myself after. Thank you to my husband, Peter, who finished his PhD two years before me and showed me that there is indeed a light at the end of the tunnel! Thank you for the love, kindness, and support you have in spades. This section wouldn't be complete if I didn't thank Astrid, our rescue pup. Your unconditional love, huge ears, big brown eyes and goofy smile have been helpful through the stressful times!

TABLE OF CONTENTS:

LIST OF TABLES AND FIGURES

1. General Introduction

2. Environmental manipulation affects depressive-like behaviours in female Wistar-Kyoto rats

3. Corticosterone and immune cytokine characterization following environmental manipulation in female WKY rats

LIST OF ABBREVIATIONS

µm	Micrometer
5-HTT	Serotonin transpoter
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
ANOVA	Analysis of variance
AVP	Argenine vasopressin
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
CMS	Chronic mild stress
CRH	Corticotropin releasing hormone
EE	Environmental enrichment
EPM	Elevated plus maze
FSL	Flinders sensitive line
FST	Forced swim test
GAD	Generalized anxiety disorder
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptor
HPA	Hypothalamic pituitary adrenal
Iba1	Ionized calcium-binding adapter molecule 1
IH	Isolation housing
IL-1β	Interleukin one beta
IL-6	Interleukin six
IL-10	Interleukin ten
LPS	Lipopolysaccharide
MANOVA	Multivariate analysis of variance
MDD	Major depressive disorder
ml	Millilitre
mRNA	Messenger ribonucleic acid
ng	nanogram
pg	picogram
PND	Postnatal day
S.E.M	Standard error of the mean
SES	Social economic status
SH	Standard housing
SSRI	Selective serotonin reuptake inhibitor
SPT	Sucrose preference test
TNF-α	Tumor necrosis factor alpha
WKY	Wistar Kyoto

1. General Introduction

1.1 The thesis

This thesis examines the effects of environmental manipulation on behavioural, immune system, and brain biomarkers of depression and anxiety. In the following introductory sections, the societal impact of depression and anxiety will be highlighted, followed by an overview of depression and anxiety as the products of chronic and acute stressful life events. The use of the Wistar Kyoto (WKY) strain as a valuable animal model of depression will also be discussed and why it was chosen over other strains. Finally the last section will outline the impact of depression on the immune system and its function and the ameliorative effects of environmental enrichment.

1.2 Depression and anxiety as a global problem:

Depression is one of the most prevalent psychiatric disorders today; it has an impact on both an individual's quality of life and places an enormous burden on the economy (Cuijpers, 2011; Kessler et al., 1994; Waraich, Goldner, Somers, & Hsu, 2004). The Global Burden of Disease study by the World Health Organization (WHO) shows that major depressive disorder (MDD) has the largest global burden of disease of all the non-fatal neuropsychiatric disorders. Furthermore, MDD is predicted to become one of the top three diseases with the highest burden of disease by the year 2030 (Mathers & Loncar, 2006). The WHO has defined 'global burden of disease' as a measure of both premature mortality and the number of years lost in states of less than optimal health. Major depression is also a leading cause of years lived with disability and coupled with dysthymia accounts for almost 11% of total years lived with disability (Ferrari et al., 2013; Ustun, Ayuso-Mateos, Chatterji, Mathers, & Murray, 2004). According to the WHO study on mental health, the highest lifetime prevalence rates for MDD are found in the United States (17%) with the lowest in the Czech Republic (1%) (Kessler & Bromet, 2013). The cause

of these geographic differences could be due to differential diagnoses or methodological issues. Taken together these studies paint a grim picture indicating that both the prevalence and burden of depression are rising.

Major depression and anxiety disorders often show significant comorbidity (Hirschfeld, 2001). In fact, the presence of anxiety disorders is one of the strongest risk factors for developing depression (Hranov, 2007; Mineka, Watson, & Clark, 1998). It is no surprise that twin studies find strong correlations between genes associated with both depression and generalized anxiety disorder (Kendler, Neale, Kessler, Heath, & Eaves, 1992a, 1992b).

Women are diagnosed with MDD at roughly twice the rate of that as men (Nolen-Hoeksema, 1987; Piccinelli & Wilkinson, 2000). The lifetime prevalence rate for a major depressive episode in women is approximately 21% as compared to 13% for men (Kessler, McGonagle, Swartz, Blazer, & Nelson, 1993). A recent meta-analysis indicates that women report higher rates of depression in all 23 European countries studied (Van, Bracke, & Levecque, 2010). This 2:1 ratio is exacerbated in Eastern and Southern European countries while it is lowest in Nordic countries. This gender difference has been attributed to an increased likelihood of sexual abuse in childhood (Cutler & Nolen-Hoeksema, 1991), hormonal fluctuations associated with menstrual cycle, child bearing and menopause (Noble, 2005), as well as greater genetic vulnerability to stressful life events (Kendler et al., 1995). Lifetime prevalence of generalized anxiety disorder is also roughly 2:1 (female:male), with females having significantly higher rates of comorbid mood disorders and other anxiety disorders (Vesga-Lopez et al., 2008). Generalized anxiety disorder (GAD) is the most frequently diagnosed anxiety disorder in primary care (22%) and in turn strains the health care system due to the typically frequent visits of patients with this disorder (Nutt, Ballenger, Sheehan, & Wittchen, 2002; Wittchen, 2002).

Quality of life is also impacted by depression and generalized anxiety. Patients with MDD report significantly lower scores on perceived quality of life measures as compared to either those who had chronic physical illness or healthy controls (Bonicatto, Dew, Zaratiegui, Lorenzo, & Pecina, 2001; Saarijarvi, Salminen, & Toikka, 2001). Further affecting quality of life is the significant comorbidity in patients with MDD and GAD with alcoholism, suicide, and substance abuse (Bronisch & Wittchen, 1994; Henriksson et al., 1993). Given this complex relationship between anxiety and depression, their effects on perceived quality of life as well as their burden on health care, it is important to examine their comorbid relationship with a specific focus on women as they account for nearly double the diagnoses.

1.3 Depression and anxiety as a product of stressful life experiences:

The hypothalamic pituitary adrenal (HPA) axis is the transduction pathway which mounts the 'stress response'. In other words, the HPA axis is responsible for the colloquially termed 'fight or flight' reaction in response to a stressor. Activity of the HPA axis begins upon perception of a stressor with the secretion of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus. These signal secretion of adrenocorticotrophic hormone (ACTH) from the pituitary, which in turn stimulates the secretion of glucocorticoids from the adrenal cortex (Figure 1.1). These glucocorticoids (cortisol in humans and corticosterone in rodents and birds) bind to receptors on target tissues causing a cascade of physiological responses, including increased heart rate and blood flow to muscles. Glucocorticoids also provide negative feedback to the hippocampus and hypothalamus by binding to glucocorticoid receptors (GRs). Whereas this cascade is typically associated with acute stressors, such as the appearance of a predator, chronic stress affects the same pathway and has also been associated with a dysregulation of the HPA axis.

Many hormones and neuropeptides connected with HPA signal transduction show diurnal fluctuations. Circulating cortisol, for example, is highest approximately 30 minutes after waking, and decreases throughout the day to its lowest levels before sleep. This natural rhythm of cortisol secretion can be negatively impacted by many exogenous events such as acute and chronic stressors. Feedback loops throughout the HPA axis serve as quality control measures and provide bottom up information to the hypothalamus and pituitary (Pariante & Lightman, 2008; Varghese & Brown, 2001).

Genes, the environment, and the interactions between the two can significantly impact the HPA axis by creating increases or decreases in circulating cortisol through changes in glucocorticoid receptor gene expression (for review see Weaver et al., 2001). These changes, if prolonged, can affect the body's response to stressful stimuli and changes in HPA function may predispose a person to develop psychopathology and vice versa. Many patients suffering from MDD and anxiety show hyperactivity in the HPA axis and faulty glucocorticoid signalling (governed by GRs in the brain) has been implicated in this hyperactivity (for review see Holsboer, 2000; Steckler, Holsboer, & Reul, 1999). Peripheral measures of HPA axis function also show marked changes with depression and anxiety. Salivary cortisol is significantly increased after a stressful task in patients with social phobia as compared to non-phobic control participants (Condren, O'Neill, Ryan, Barrett, & Thakore, 2002). Basal and peak cortisol levels also tend to be higher in elderly patients with GAD as opposed to non-anxious individuals (Mantella et al., 2008). A review by Juruena and colleagues (2004) suggests that since impaired glucocorticoid feedback in the HPA axis is implicated in depression, antidepressants may act on this impaired feedback mechanism by resetting the HPA axis to its original pre-stressed state (Juruena, Cleare, & Pariante, 2004).

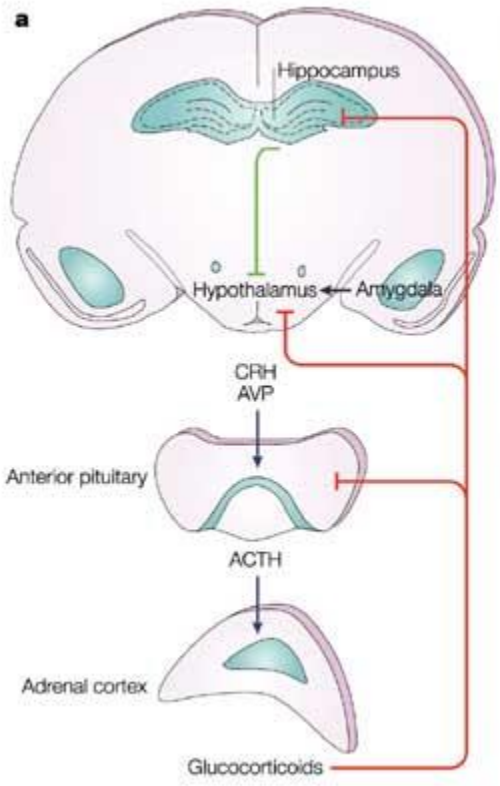

Figure 1.1 The Hypothalamic Pituitary Adrenal (HPA) Axis signal transduction and feedback pathway. Activation results in production of corticotrophic releasing hormone (CRH) and arginine vasopressin (AVP) which in turn stimulate adrenocorticotropic hormone (ACTH) release from the pituitary. Following this, ACTH activates glucocorticoid production and release from the adrenal cortex. These glucocorticoids create changes in the body to respond to the stressor as well as providing negative feedback to the hippocampus and the hypothalamus. Adapted from Sandi (2004).(Sandi, 2004)

The above studies highlight the importance of the HPA axis in regulating glucocorticoids and that a persistent dysfunction of the HPA axis plays a large role in mental health disorders.

Stressful life events such as loss, trauma, and social humiliation are often associated with bouts of depression (for review see Brown, 1998; van Praag, 2004) in some but not all individuals. These differential responses may stem from natural genetic variation associated with the stress response or combined gene by environment interactions. One example of how natural genetic variation can affect susceptibility to depression comes from Caspi and colleagues (2003). The authors found that people carrying a certain allele in the serotonin transporter (5-HT T) gene have an increased overall biological reactivity to stress, putting them at risk for depression, as compared to those who do not carry this allele, but only in the context of a stressful life event (Caspi et al., 2003; Gotlib, Joormann, Minor, & Hallmayer, 2008). This increase in reactivity is often associated with a dysregulation in the HPA axis.

Environmental adversity in early life in the form of neglectful parenting, poverty, and maternal anxiety can also influence the HPA axis and stress response in later life. An extreme example comes from Gunnar and colleagues (2001) who carried out a longitudinal study of children reared in Romanian orphanages. Hundreds of thousands of children during the late 1980s and early 1990s were housed in these orphanages due to the regime imposed by Nicolae Ceausescu which forbade both contraception and abortion. This led to a sharp increase in birth rates and resulted in the abandonment of many children to orphanages in which extreme social isolation, physical, and sexual abuse were the norm. Children who had spent more than eight months in their first year of life at these orphanages had significantly higher daytime cortisol levels six years after being adopted than that of their Canadian-born counterparts (Gunnar, Morison, Chisholm, & Schuder, 2001). However, children who were adopted within their first

four months showed similar cortisol levels to that of Canadian-born children (Gunnar, Morison, Chisholm, & Schuder, 2001). Some children in these institutions were less likely to have the stereotyped diurnal pattern of cortisol and showed a generally more blunted cortisol response throughout the day (Carlson & Earls, 1997; Gunnar & Vazquez, 2001). This phenomenon is known as 'hypocortisolism' and has been correlated with post-traumatic stress as well as some bodily disorders such as fibromyalgia, chronic fatigue syndrome, and asthma (Fries, Hesse, Hellhammer, & Hellhammer, 2005; Heim, Ehlert, & Hellhammer, 2000). It is possible that hypocortisolism observed in institutionalized children may be a risk factor for the development of posttraumatic stress disorder in later life. In contrast, children who show *increased* HPA activity may be more likely to show anxious and depressive phenotypes in later life (Heim, Newport, Mletzko, Miller, & Nemeroff, 2008).

Both hypocortisolism and elevated cortisol are indicators of HPA axis dysregulation. Studies of children reared in impoverished environments have shed light on how early life conditions such as severe maternal and social deprivation can impact the HPA axis and the stress response in later life. To our knowledge, there are no longitudinal studies following institutionalized children into adulthood, so the effect of social deprivation on psychopathology in later life is hard to assess. However, animal studies of maternal and social deprivation in both rodents and primates have found notable effects on behaviour and neurochemistry in adult life (for review see Sanchez, Ladd, & Plotsky, 2001).

Early research by Plotsky and Meaney (1993) found that male rats who experienced maternal separation had increased CRH mRNA levels in the hypothalamus (Plotsky & Meaney, 1993). Increased CRH has been implicated in both depression and anxiety disorders for its role in creating the stereotyped hyperactivity of the HPA axis (for review see Arborelius, Owens,

Plotsky, & Nemeroff, 1999). Finally, a 2.5-5 times increase in corticosterone secretion has been found in response to a mild handling stressor in maternally separated males relative to that of their non-maternally separated counterparts (Kalinichev, Easterling, Plotsky, & Holtzman, 2002). These studies provide substantial support for the notion that early environment can severely impact the HPA axis and its regulatory components.

In humans, one of the most common types of environmental adversity comes from having low social economic status (SES) at or below the poverty level. The prevalence of major depression decreases from 12.4% in low SES communities to 1.9% in high SES communities (Adler et al., 1994). Furthermore, the number of childhood years spent living in poverty correlates positively with hyperactivity of the HPA axis and results in elevated cortisol levels (Evans & Kim, 2007). Children of low SES families not only tend to have higher cortisol levels but also interpret both positive and negative stimuli in a significantly more negative light than that of high SES children (Lupien et al., 2005).

A child's cortisol levels are also tied to maternal depressive symptomatology (Lupien, King, Meaney, & McEwen, 2000), which is not surprising given the link between SES and maternal depression. At the behavioural level, children raised in poverty tend to manifest antisocial behaviours and also show higher levels of depression (Mcleod & Shanahan, 1996). Thus early adversity (e.g. social deprivation or an impoverished environment) may create hyperactivity in the HPA axis and this may influence not only immediate, but also later life psychopathology.

Developing interventions in adolescence or adulthood could be a step towards lowering the prevalence of depression and anxiety. For example, adopting lifestyle changes such as

healthier eating, increased exercise, and quitting smoking, have protective effects on both physical and mental health (Harrington et al., 2010). Yet it is less clear if these lifestyle alterations work in clinically depressed populations, partly due to the high rates of antidepressant prescription (Jureidini & Tonkin, 2006). In other words, it is important to examine whether a change in environment alone, without pharmaceutical intervention, is sufficient to help improve depressive symptoms. There is some evidence that increases in physical exercise (Dunn, Trivedi, Kampert, Clark, & Chambliss, 2005) and social support (Jané-Llopis, Hosman, Jenkins, & Anderson, 2003) can alleviate depressive symptoms in mild to moderate major depression. Unfortunately these studies are few and often do not take comorbidity into account. If simple changes in lifestyle can alleviate depressive symptomatology, the burden on health care systems worldwide would be significantly decreased. Although human research has provided clues to the relationship between environmental adversity, lifestyle changes, and psychopathology, they are limited by inherent ethical and practical concerns. This is one reason mammalian models of anxiety and depression can be particularly useful.

1.4 Contribution of Animal Models to the field of Depression and Psychiatric Disorders:

While depression is one of the most prevalent mood disorders, its pathogenesis is unclear compared with somatic diseases such as Parkinson's and Alzheimer's (Yan, Cao, Das, Zhu, & Gao, 2010). In the search to develop animal models of depression, two lines of research have been predominantly used with success. The first involves the application of chronic stressors to induce depressive behaviours, while the second uses genetic/pharmaceutical manipulations to create animal models of depression.

In the early 1980's, Katz and colleagues used a series of chronic stressors (e.g. electric shocks, tail pinches etc.) for one week and found changes in rats' performance on tests of anxiety, as well as hormonal alterations which could be reversed with antidepressants (Katz, 1981a, 1981b, 1982; Katz & Hersh, 1981; Katz, Roth, & Carroll, 1981). Willner and colleagues (1987) investigated the time course for which a chronic mild stress (CMS) procedure (soiled bedding, 45 degree cage tilt, stroboscopic light) might create these depressive behaviours (Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). Through decades of subsequent studies, CMS has been validated for its ability to create depressive behaviours in rodents. Chronic mild stress also affects hedonic behaviour as interpreted from a decrease in sucrose preference and increased immobility in the forced swim test (both measures of depression) (Baker & Bielajew, 2007; Baker, Kentner, Konkle, Santa-Maria Barbagallo, & Bielajew, 2006; Bielajew et al., 2003; Konkle, 2003; Muscat & Willner, 1992; Willner, Muscat, & Papp, 1992; Willner, 2005). Prenatal stress paradigms have also been used to create depressive and anxious behaviours in rats (for review of both human and rodent work see, Baker et al., 2008, 2009; Glover, O'Connor, & O'Donnell, 2010). Finally, there is evidence that repeated social defeat in mouse and rat models can also create depressive-like behavioural outcomes (Becker et al., 2008; Keeney & Hogg, 1999). Collectively, though the above paradigms are useful for creating an animal model of depression, they are often time-consuming, costly, and frequently accompanied by unwanted confounding factors due to the level of manipulation required.

More recently, depressive behaviours (e.g. high immobility in FST and few open arm entries in EPM) have been observed in four different strains of rats - the Flinders sensitive line (FSL) rat, the fawn-hooded rat, the learned helpless rat, and the Wistar Kyoto (WKY) rat (for review see Overstreet, 2012). These strains were originally bred for purposes other than their

depressive tendencies. Due to the importance of early environment such as maternal care in the development of a healthy HPA axis, it is informative to consider the type of maternal care exhibited by WKY and FSL dams. Two studies by Cierpial and colleagues (1987, 1990) have addressed the issue of maternal care in the WKY rat, with WKY dams being shown to nurse and groom their pups significantly less than observed in control animals (Cierpial, Shasby, & McCarty, 1987). Cross fostering studies show that dams will switch their maternal behaviours to those of the opposite strain (Cierpial, Murphy, & McCarty, 1990). In other words, control dams are more likely to show the maternal behaviours expressed by WKY dams if they are given WKY pups. Both WKY and FSL pups show decreases in ultrasonic vocalizations (USV) and do not seek to be close to their mothers compared to that of the control group (Braw et al., 2008). Due to the nature of the questions addressed in this thesis, as well as the feasibility of obtaining the animals, the studies described here focus specifically on the WKY model and its control, the Wistar strain.

There are differences between the WKY and FSL rat lines in their HPA axis responsivity and anxious behaviours (for review see Malkesman & Weller, 2009; Malkesman et al., 2006). In WKY males the diurnal patterns of corticosterone and ACTH remain higher after they peak as compared to Wistar males (Solberg, Olson, Turek, & Redei, 2001). Furthermore, in male WKY rats, there is an increased corticosterone response after the Y-Maze as compared to the FSL rats (Braw et al., 2006). Differences in hippocampal and hypothalamic monoamine metabolites and transporter levels between WKY and Wistar rats have been implicated in the HPA axis hyper-reactivity (Scholl, Renner, Forster, & Tejani-Butt, 2010).

The WKY rats show greater socially inhibited, depressive-like behaviours when exposed to social isolation than that of control rats or rats from the FSL rat line (Malkesman et al., 2005;

Malkesman, Braw, et al., 2006). The WKY strain also shows higher plasma levels of corticosterone and ACTH and much lower levels of social play as compared to that of FSL and control rats (Malkesman, Maayan, Weizman, & Weller, 2006; Pare & Redei, 1993a, 1993b). Taken together, WKY rats appear to present co-morbid depressive and anxious phenotypes as opposed to their FSL counterparts who exhibit an almost purely depressive phenotype. The WKY strain might thus represent a model of childhood or chronic depression starting from early life while the FSL rats might better constitute a model of depression seen only after exposure to chronic mild stress (Malkesman & Weller, 2009). Because depression in humans is often co-morbid with anxiety disorders, the WKY line is a better fit for the research described here. For this reason, we have chosen to study the effects of environment on both depressive and anxious behaviours using the WKY and Wistar strains.

The WKY rats are also used as an animal model of attention deficit and hyperactivity disorder (ADHD), specifically the inattentive subtype, because they show no accompanying hyperactivity (Adriani, Caprioli, Granstrem, Carli, & Laviola, 2003; Sagvolden et al., 1992; Sagvolden & Johansen, 2012). In humans, the comorbidity between ADHD and anxiety can be as high as 50% (Biederman et al., 1993; Mancini, Van Ameringen, Oakman, & Figueiredo, 1999; Schatz & Rostain, 2006). As many as 33% of patients are diagnosed concurrently with both depression and ADHD (Biederman et al., 1993). For example, in youth diagnosed with internet addiction, concurrent diagnoses of ADHD, depression, and anxiety are common (Yen, Ko, Yen, Wu, & Yang, 2007). Thus depression, anxiety, and ADHD are often inextricably linked making the WKY strain useful as a model of all three of these disorders.

Many parallels exist between the behaviours observed in WKY pups and children of depressed parents. For instance, children of depressed parents show emotional dysregulation in

the form of withdrawal, depressive-like behaviours, and are more likely to develop psychiatric disorders (Field et al., 1988; Goodman & Gotlib, 1999; Maughan, Cicchetti, Toth, & Rogosch, 2007). Consequently, the following series of studies focus primarily on depression and anxiety with WKY rats our model animal of choice.

Thus far, most studies involving WKY rats have been carried out using male rats. Animal work has often shied away from work on females since their estrus cycle can complicate the interpretation of behaviour (For review see Zucker & Beery, 2010). This does not offer a robust model for the study of depression and anxiety, since animal studies indicate a complex relationship between menstrual cycle and hormonal fluctuations in females which can influence the development of psychopathology (Mileva, Bielajew, & Konkle, 2013; Palanza, 2001). In fact, many key structures of the limbic system, including the hippocampus and amygdala show large sexual dimorphism (for review see Mileva et al., 2013). In this thesis depressive and anxious behaviours were investigated in female rats while controlling for their estrus cycle.

1.5 The immune system and depression

In response to infection, pro (i.e. TNF-α, IL-1β, IL-6) and anti-inflammatory (i.e. IL-10) cytokines are released from peripheral macrophages and act to either increase or decrease the inflammatory response, respectively. The functions of these macrophages, also called microglia, are many and they have also been implicated in the pruning and remodelling of synaptic pathways (Schafer et al., 2012; Tremblay et al., 2011). Within the last two decades, the immune system has been hypothesized to effect the pathophysiology of depression and anxiety (for review see Miller, Maletic, & Raison, 2009). This is called the neuroinflammatory theory of depression and it posits that the effects of immune cytokines on neural structures end in the

development of psychopathology. One of the first clues came from observing animals after lipopolysaccharide injection (LPS) (for review see Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). These animals exhibit sickness behaviour which has been likened to the behavioural aspects of depression, including social and physical withdrawal and anhedonia (Dantzer et al., 2008). Though this sort of peripheral infection can create sickness behaviour which mimics depressive behaviour, it has been found that stress can also exacerbate the inflammatory response. More recently it has been shown that if an infection is chronic, the increase in peripheral pro-inflammatory cytokines will travel across the blood-brain barrier and promote further inflammation. This in turn causes a decrease in monoamines and trophic factors as well as an increase in excitotoxicity (Miller et al., 2009).

A recent meta-analysis found that TNF-α and IL-6 were indeed significantly higher in patients with major depression (for review see Dowlati et al., 2010). Similarly, treatment with interferon, a pro-inflammatory cytokine, for Hepatitis C leads to the development of major depression (for review see Udina et al., 2012). In fact, simply having higher levels of inflammation, even if they are subclinical, is a risk factor for de novo depression specifically in female patients (Pasco et al., 2010). From a treatment perspective, selective serotonin reuptake inhibitors (SSRI's) are reported to significantly decrease the levels of pro-inflammatory cytokines (Maes et al., 1999). Taken together these paint a well-rounded picture in which immune system dysregulation can increase the likelihood of developing depression. An increase in pro-inflammatory cytokines may not be limited to depression as patients with schizophrenia (Miller, Buckley, Seabolt, Mellor, & Kirkpatrick, 2011), mania (Maes, Bosmans, Calabrese, Smith, & Meltzer, 1995) and post-traumatic stress disorder (Gola et al., 2013) all show increases in pro-inflammatory cytokines.

Although the above provide evidence for the neuroinflammatory theory of psychopathology the question of where the inflammation come from remains. A recent review by Berk and colleagues (2013) eloquently addresses this question. In their review, they highlight research to support the idea that stress, trauma, diet, smoking, sleep, gut microbiota, and obesity may all serve to increase inflammation in the body which can result in depression (Berk et al., 2013). Specifically, stressors in animal models including acute stress, foot shock, and social isolation increase IL-1 mRNA levels (Maier & Watkins, 1995; Möller et al., 2013; Nguyen et al., 1998) indicating that stressful life experiences, both physical or social, can affect the immune system. In this thesis, we examined whether an animal model of depression would have higher levels of pro-inflammatory cytokines at baseline, and whether environmental manipulation would have any effect on circulating cytokine level.

1.6 Effects of Environmental Enrichment on Stress and Depression:

Environmental enrichment (EE) in animal research can be broadly defined as any physical, cognitive, or social stimulation that is superior to 'standard housing' conditions (for review see Simpson & Kelly, 2011). For instance, many zoological parks implement EE to promote animal welfare by reducing animal stress and boredom (Carlstead & Shepherdson, 2000). Although most research scientists who employ rats as an animal model in their studies agree that EE should be considered the standard setting (as their wild relatives experience a complex external environment), the current 'standard' laboratory conditions in most laboratories are actually impoverished. Currently, 'control' animals are those caged in pairs with very little physical enrichment, while EE is thought to be the gold standard for improving many aspects of rodent cognitive functioning, behaviour, and physiology. Whether animals exposed to EE express the 'natural' or 'improved' phenotype in comparison to their controls remains to be seen.

In rats, EE can be likened to the adoption of a healthy lifestyle in humans (Laviola, Hannan, Macri, Solinas, & Jaber, 2008; Petrosini et al., 2009) due to the cognitive and physical benefits associated with EE. For instance, EE significantly delays the onset of Alzheimer's and Parkinsons' disease in mice (for review see Nithianantharajah & Hannan, 2006) increases neurogenesis in the hippocampus (Kempermann, Gast, & Gage, 2002; van Praag, Kempermann, & Gage, 2000), increases brain volume (Bennett, Rosenzweig, & Diamond, 1969), and can also increase dendritic arborisation in the parietal cortex (Leggio et al., 2005). Environmental enrichment is also associated with increased long-term potentiation in neurons of a brain pathway important for learning and memory (Huang, Huang, Wu, & Boucheron, 2006).

In addition to the dramatic changes in the brain, EE has also been shown to produce positive changes in behaviour, especially after pre- and post-natal adversity. For example, EE is able to reverse the anxiogenic consequences of early maternal separation and prenatal stress (Morley-Fletcher, Rea, Maccari, & Laviola, 2003). Pups exposed to prolonged maternal separation remain hyper responsive to stress even four days after the exposure (Rosenfeld, Wetmore, & Levine, 1992; Wigger & Neumann, 1999), and these deleterious effects persist into adulthood (Wigger & Neumann, 1999). Yet postnatal exercise, a form of EE, reverses the depressive behaviours in maternally separated rat pups, by inhibiting apoptosis and enhancing cell proliferation in the hippocampus (Baek et al., 2012). Not surprisingly, prenatally stressed animals in standard housing have increased levels of corticosterone secretion and decreased levels of social behaviours as compared to EE conditions in prenatally stressed animals (Morley-Fletcher et al., 2003). Enrichment can also reverse the stereotyped increase in hypothalamic CRH and alterations in behavioural responses after maternal separation (Francis, Diorio, Plotsky, & Meaney, 2002). As a whole, EE during development can alleviate the negative behaviours

associated with early adversity as well as increase resilience to challenges (Konkle, Kentner, Baker, Stewart, & Bielajew, 2010).

One aspect of EE in the form of exercise has proved useful in decreasing depressive symptomatology in both humans and animal models (Greer & Trivedi, 2009). In fact, exercise is a key component of EE, as it correlates with increased neurogenesis and neurotrophic factors in the brain (Kobilo et al., 2011). In mice, two months of living in an enriched environment not only increases the number of immature hippocampal neurons, but also decreases the amount of immobility in the forced swim test (Llorens-Martin et al., 2007). These studies provide evidence for the beneficial effects of EE on both behaviour and brain morphology.

Little is known about the effects of EE on immune cytokines. One study in male mice has offered a glimpse into the changes which occur with 'super enriched' housing in which the pro-inflammatory to anti-inflammatory ratio is much higher than that of male mice in standard housing (Marashi, Barnekow, Ossendorf, & Sachser, 2003). However, this finding was confounded by the inherent aggressive tendencies of male mice and the increase in circulating stress hormones this may have created. More recent studies have shown that physical exercise, an important component of EE, has an anti-inflammatory effect and that the novel objects in EE can decrease both IL-1β and TNF-α (Singhal, Jaehne, Corrigan, & Baune, 2014). In sum, EE has many positive effects on behaviour, brain chemistry, and the immune response. In the following section, the question of whether environment manipulation has an effect on an animal model of depression will be tested through a series of studies which make up this thesis.

1.7 Thesis Studies

The following studies examined the effects of environmental manipulation on an animal model of depression and anxiety using female WKY rats and as a control arm, female Wistar rats. Based on previous literature, it was hypothesized that an enriching environment will alleviate depressive- and anxiety-like behaviours, as compared to animals not housed in an enriched environment.

Table 1 describes the three papers that comprise this thesis. The same animals were used in all three papers addressing a separate but complimentary aspect of the effects of environment on depression. Paper 1 "Environmental manipulation affects depressive-like behaviours in female Wistar-Kyoto rats" (Mileva & Bielajew, 2015) examined the behavioural outcomes of 3 housing types 1) Enrichment, 2) Standard, 3) Isolation. Paper 2 "Corticosterone and immune cytokine characterization following environmental manipulation in female WKY rats" (conditionally accepted in Behavioural Brain Research) looked at circulating serum pro and anti-inflammatory cytokines and corticosterone while paper 3 "Environmental enrichment affects astroglia, glucocorticoid receptors and microglia immunofluorescence in female Wistar and WKY animals" (submitted to Brain Research) used immunohistochemistry methods to report the staining intensity and area of microglia, astroglia and glucocorticoid receptors in the hippocampus and amygdala. Each paper has its own detailed introduction, specific detailed methodology, and discussion points.

TABLE 1.1 Summary of papers in thesis

Paper title	***Animal Model***	***Housing***	***Measures***
1) Environmental manipulation affects depressive-like behaviours in female Wistar-Kyoto rats (BBR, 2015)	- Female Wistar Kyoto and Wistar	- Environmental enrichment - Standard (3 in each cage) - Isolation	- Sucrose Preference Task (Anhedonia) - Elevated Plus Maze (Anxiety) - Forced Swim Test (Depression)
2) Corticosterone and immune cytokine characterization following environmental manipulation in female WKY rats (BBR, 2017)	- Female Wistar Kyoto and Wistar	- Environmental enrichment - Standard (3 in each cage) -Isolation	- TNF-α - IL-1β - IL-10 - Corticosterone
3) Environmental enrichment affects astroglia, glucocorticoid receptors and microglia immunofluorescence in female Wistar and WKY animals	- Female Wistar Kyoto and Wistar	- Environmental enrichment - Standard (3 in each cage) -Isolation	- Astroglia - Microglia - Glucocorticoid Receptors

Published in Behavioural Brain Research Volume 293, October 2015

2. Environmental manipulation affects depressive-like behaviours in female Wistar-Kyoto rats

Guergana R. Mileva and Catherine Bielajew

Abstract:

While the efficacy of pharmacological interventions to treat depression has been well-studied in animal models, much less work has been done to shed light on how changes in the immediate environment can impact behaviour. Furthermore, most studies have focused on male rodents despite the prevalence of mood disorders in women. In this study, 36 Wistar Kyoto (validated animal model of depression) and 36 Wistar (control) female rats were used to examine the effects of environmental manipulation on depressive- and anxiety-like behaviours. Animals were assigned to one of three groups: standard (3 rats/cage), enriched (6 rats/cage plus physical enrichment), and isolation (1 rat/cage) housing. The elevated plus maze (EPM) and forced swim test (FST) were conducted prior to, and four weeks after environmental assignment to measure anxiety-like and depressive-like behaviours, respectively. Sucrose preference assessed anhedonia both before and after environmental assignment. Weight was measured every week to monitor weight-gain over time. Post-environment sucrose preference was significantly increased in animals in enriched housing as compared to those in isolated housing in both strains. While there were significant differences between strains in measures of open arm duration in the EPM and immobility in the FST, there appeared to be no differences between environmental groups. The results of this study highlight the importance of environmental factors in the expression of anhedonia. Enrichment appears to reduce anhedonia while isolation increases anhedonia. These effects should be studied further to assess whether longer periods of social and physical enrichment alleviate other symptoms of depression.

Keywords: Environmental enrichment, Wistar-Kyoto, Depression, Anxiety, Sucrose preference, Isolation

2.1 Introduction:

Depression is one of the most prevalent mental health disorders in the world today and places an enormous burden on the economy and healthcare systems worldwide (Cuijpers, 2011; Kessler et al., 1994; Waraich et al., 2004). According to The Global Burden of Disease study started by the World Health Organization in 1990, major depressive disorder (MDD) has the largest global burden of disease of all the non-fatal neuropsychiatric disorders, and is predicted to become one of the top three burdens of disease along with HIV/AIDS and ischaemic heart disease by the year 2030 (Mathers & Loncar, 2006). Major depression and anxiety disorders often show significant comorbidity (Hirschfeld, 2001). While there is debate as to whether these are distinct or overlapping disorders, it is clear that the presence of anxiety disorders is one of the strongest risk factors for developing depression (Hranov, 2007; Mineka et al., 1998).

Women are diagnosed with MDD at roughly twice the rate of that observed in men (Nolen-Hoeksema, 1987; Piccinelli & Wilkinson, 2000). In fact women have a 21% lifetime prevalence for a major depressive episode as compared to 13% of men (Kessler et al., 1993). The apparent gender differences in MDD worldwide have been attributed to an increased likelihood of sexual abuse in childhood (Cutler & Nolen-Hoeksema, 1991), hormonal fluctuations associated with menstrual cycle, child bearing, and menopause (Noble, 2005), as well as greater genetic vulnerability to stressful life events (Kendler et al., 1995). However, in studies that employ animal models of depression and anxiety, there exists a large male bias, again due to the hormonal fluctuations at play during the estrous cycle which cause significant changes in both physiology and behaviour (for review see Mileva, Bielajew, & Konkle, 2013). Zucker and Beery (2010, 2011) found that even in studies of diseases that predominantly affect women such as anxiety and depression, the research is primarily carried out with male animals.

The environment can also play a large role in the development of depression. There is an association between developing depression and the quality of the environment (Galea, Ahern, Rudenstine, Wallace, & Vlahov, 2005). Socioeconomic status, stressful life events, social support, and physical exercise are many facets of the immediate environment that can affect well-being and in turn lead to depression and anxiety. There is much evidence to support the benefits of physical exercise (Carek, Laibstain, & Carek, 2011; Dunn et al., 2005; Salmon, 2001) and social support (Brown, Andrews, Harris, Adler, & Bridge, 1986; Jané-Llopis et al., 2003) on mild to moderate depression in humans.

Environmental enrichment (EE) has been used as a way to incorporate both physical and social stimuli in animal models. In this paradigm, multiple animals are housed together in a large cage with novel stimuli changed every 2 to 6 days (Simpson & Kelly, 2011). Studies utilizing EE have shown it to have an anti-depressant-like effect in rats by creating changes in both behaviour and neurophysiology. For instance, EE increases serotonin concentrations in the prefrontal cortex (Brenes, Rodriguez, & Fornaguera, 2008) and can ameliorate the effects of chronic stress by promoting cell survival and differentiation and increasing glucocorticoid receptor expression in the hippocampus (Sampedro-Piquero, Begega, & Arias, 2014; Veena, Srikumar, Mahati, et al., 2009; Veena, Srikumar, Raju, & Shankaranarayana Rao, 2009). Taken together, it appears that EE can exert not only a protective effect against multiple types of stressors (for review see Fox, Merali, & Harrison, 2006) but can in some cases reverse the effects of these stressors altogether (Escorihuela, Tobena, & Fernandez-Teruel, 1994; Francis et al., 2002; Morley-Fletcher et al., 2003). While pharmaceutical agents are often used to treat depression and anxiety, the current study aimed to explore whether environmental changes, without pharmaceutical manipulation, can have an impact on an established animal model of depression.

The Wistar Kyoto rat (WKY) was originally bred to be the control animal for the spontaneously hypertensive rat (Okamato & Aoki, 1963) but has more recently been used as a genetic model of depression (Lahmame & Armario, 1996, for review see Overstreet, 2012). The WKY rats show a combination of depressive- and anxiety-like behaviours as well as a marked decrease in locomotion and social withdrawal (Nam, Clinton, Jackson, & Kerman, 2014). Because even pre-pubertal WKY animals exhibit both depressive and anxious symptoms, they are considered a model of childhood depression (Malkesman, Braw, et al., 2006; Malkesman & Weller, 2009). Furthermore, the depressive-like behaviour in WKY rats appears to be a model of treatment-resistant depression as these animals tend not to respond to selective serotonin reuptake inhibitors (Lahmame, del Arco, Pazos, Yritia, & Armario, 1997; Lopez-Rubalcava & Lucki, 2000; Tejani-Butt, Kluczynski, & Pare, 2003). However, their depressive-like behaviours in the forced swim test (FST) do appear to be alleviated in response to low doses of ketamine (Tizabi, Bhatti, Manaye, Das, & Akinfiresoye, 2012), resveratrol (Hurley, Akinfiresoye, Kalejaiye, & Tizabi, 2014) and some tricylclic antidepressants and monoamine oxidase inhibitors (Will, Aird, & Redei, 2003). Given what is known about the response of WKY rats to pharmaceuticals, it is surprising that there are no studies on the effects of environment on behaviour in the WKY rat.

This study examined whether changes to the environment can impact depressive- and anxiety-like behaviours in WKY and Wistar female rats. To our knowledge, this is the first study to examine the impact of environmental manipulation on behavioural measures observed in WKY female animals. Of particular interest was the question whether something as relatively 'mild' as an increase in social and physical stimuli through EE could have an effect on measures of anxiety and depression. Animals were randomly assigned to one of three environments -

standard, enriched, or isolated. Depressive and anxiety-like behaviours were assessed using the FST and elevated plus maze (EPM), respectively (for reviews of FST see Lucki, 1997 and EPM see Hogg, 1996). Anhedonia was assessed through the use of the sucrose preference test. Weight and estrus cycle were monitored both before and after environmental assignment and analysed to explore whether the type of environment had an effect on both behavioural and physiological markers.

2.2 Materials and methods:

2.2.1 Animals

A total of 36 Wistar and 36 Wistar Kyoto (WKY) female rats were obtained from a local supplier (Charles River Laboratories, Québec, Canada). All procedures were approved by the University of Ottawa Animal Care Committee. Due to space and time constraints, animals were acquired in two cohorts: a group of 18 Wistar and 18 WKY female rats were first tested and sacrificed, followed by a second identical cohort. Animals arrived to the facility at three to four weeks of age or post natal days (PND) 21-28 and were housed three per cage in standard housing conditions with a day/night cycle of 12h:12h and temperature of 21.5±1^{o}C (humidity ~40%). Throughout the experiment, animals were provided with food and water ad libitum. They were allowed to acclimate to the home cage for 5-7 days and then were handled for five consecutive days. At PND 40, animals were acclimated to the taste of a 1% sucrose solution for five days. Sucrose was then withdrawn for three days and sucrose preference was assessed twice over the next four days. Behavioural testing commenced four days later to ensure that sucrose withdrawal would not affect their behaviour (for review on sucrose addiction see Avena, 2010). At PND 55, animals completed the EPM, and two days later the FST. The FST was carried out after the EPM as not to cause the animals undue stress. Animals were then randomly assigned to their housing

condition at PND 62 and were kept there until sacrifice at PND 96. Combining both cohorts, there were six groups (three Wistar and three WKY) with 12 animals in each group. Animals in each strain were housed separately; in other words, Wistar-Kyoto animals were always housed only with other Wistar-Kyoto animals. Housing in the assigned conditions lasted for a period of four weeks before behavioural testing commenced. All animals were kept in their respective environment during the second set of tests to avoid the potential stress from switching environments. This is referred to as the post-environment test throughout the study. There was minimal interaction between investigators and animals during the four week period, apart from weekly cage and daily food/water changes. Animals were tested again following the environmental manipulation using the same behavioural measures as described above. Sucrose preference was assessed at PND 79 and 81 with EPM and FST at PND 85 and 87 respectively. See Figure 2.1 for a diagram of the study timeline. Therefore, all rats were tested twice, once before assignment to their housing condition (pre-environment) and again four weeks following housing assignment (post-environment). On the day of testing, animals were acclimated to a room outside the test room in their respective cages for one hour. Pre-environment measures were taken during adolescence while post-environment measures were taken during adulthood. Pre-environment EPM and FST were carried out to make the case that there are large behavioural differences between these strains. Post-environment comparisons were done to examine the effect of environment on the behavioural measures with the standard housing condition serving as a baseline.

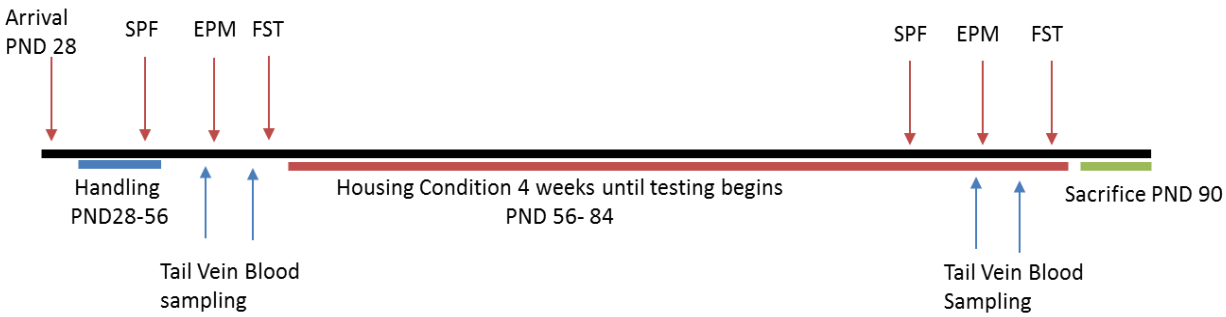

Figure. 2.1:Experimental timeline. PND: post natal day, FST: forced swim test, EPM: elevated plus maze and SPT: sucrose preference test.

2.2.2 Housing

Environmental Enrichment (EE):

A total of 12 WKY and 12 Wistar female rats were randomly assigned to the environmental enrichment (EE) condition. The EE condition combined both physical and social stimulation. Six animals were placed together in a large multistory cage [137.5cm X 61cm X 69cm] with toys and chain link bridges for climbing (approximately 20cm in length). Nestlets®, wooden chewing blocks, multicoloured Plexiglas® houses, and cardboard tubes were permanently available. Toys were varied and new ones added every third day to provide access to novel stimuli. A running wheel was also freely available for use throughout the duration of their stay in the EE cage.

Standard and Isolation conditions:

Animals in standard housing were placed in groups of three in guinea pig cages [50cm x 38cm x 20cm] with one black Plexiglas® tube and no other source of physical stimulation. Those in the isolated housing condition were housed singly in standard shoe box rat cages [45cm x 22cm x 20cm] with one black Plexiglas® tube.

2.2.3 Sucrose preference test:

In this study, animals were acclimated to the taste of a 1% sucrose solution in their home cage for five days prior to the sucrose preference test (SPT). Water was also freely available on those days. On the test day, animals were presented with two bottles, one containing the sucrose solution and one containing water. The weights of both bottles were recorded prior to the start and 24 hours later. This test was performed twice before animals were assigned to their respective environment and twice more three and a half weeks after assignment for a total of four

tests. The overall sucrose consumed by animals in each environment was converted to an average sucrose/water consumption value per animal. Preference for sucrose was determined by the quotient of sucrose consumption at 24hr to total liquid consumption of the combined water and sucrose solutions.

Since the EE cage differed significantly from the standard and isolated cages, the bottles differed between the environments. However, the nozzle size, shape, angle and height from the ground were similar between both types of bottles and environments.

2.2.4 Elevated plus maze (EPM):

Animals were placed on the middle platform of the EPM facing an open arm. Behaviour was recorded from above for five minutes after which animals were returned to their home cage. Behaviours were analyzed using ODlog2®. Scoring was done by one individual with no knowledge of which group the animal was in and intra rater reliability was higher than 90%. The apparatus was cleaned thoroughly with 70% ethanol between tests to avoid individual olfactory and gustatory cues. The duration and frequency of open arm and closed arm entries as well as ethological measures including stretch attend (moving body forward without lifting paws off the ground), head dip postures, and rearing were examined in this study. The latter are considered 'risk assessment' behaviours and are believed to reflect the animal's tendency to assess risk in its environment (Carobrez & Bertoglio, 2005).

2.2.5 Forced Swim Test (FST):

Animals were gently placed into a clear Plexiglas® cylinder (20cm diameter and 45cm in height) filled with room temperature water (24±1^{o}C) up to a level of 30cm. They were removed

15 minutes later, gently dried, and placed in an incubator set at 28^{o}C for ten minutes and then returned to their home cage. After 24 hours, the same animals were again monitored in the apparatus, this time for five minutes, and the frequency and duration of immobility, struggling, and escape attempts videotaped and later scored. The post-environment FST procedure was identical to the pre-environment FST. Only the behaviours recorded during the second 5 minute tests were scored and analysed using ODLog2®. All videos were scored by two individuals with an intra- and inter-rater reliability higher than 90%.

2.2.6 Weight and estrous cycle:

Animals were weighed once a week for a total of eight weeks to track weight gain or loss as a function of housing condition. Estrous cycle stage was confirmed by collecting vaginal fluid as described by (Marcondes, Bianchi, & Tanno, 2002). Briefly, on the day of behavioural testing (i.e. EPM or FST) 50µl of warm water was inserted into and then withdrawn from the vaginal canal using a sterile pipette. Microscopic examination of cell type was used to categorize cycle stage (Marcondes et al., 2002). When behavioural testing commenced, all animals were cycling, indicating that they were in various stages of the estrous cycle and had reached sexual maturity.

2.2.7 Statistical Analysis:

IBM SPSS statistics V. 20 software was used for all statistical analyses. The 2 x 3 mixed ANOVAs were performed to evaluate the effects of strain (2 levels) and environment (3 levels) on sucrose preferences and weight. MANOVAS were conducted to assess strain differences for the measures associated with the FST and EPM tests. Paired samples t-tests were carried out to examine the change in behaviours in the FST and EPM from baseline between environments. Although estrous cycle should be considered a time varying covariate, it was excluded from the

analyses because it did not correlate significantly with any of the DVs in question (i.e. immobility in the FST, open arm entries in the EPM), and therefore did not meet the criterion for inclusion as a covariate. For the sucrose preference analyses, sucrose and water consumption were averaged across the number of animals in each group. In other words, the total sucrose consumption was divided by six for animals in EE and that number was used as an index of sucrose consumption for each animal. For all follow-up statistical analyses, a Bonferroni correction was used to adjust the alpha level depending on the number of tests performed. Finally, a mixed ANOVA was used to examine weight change over time. Huynh-Feldt corrections were used to adjust the degrees of freedom for violation to sphericity.

2.3 Results:

2.3.1 Sucrose preference:

Strain differences:

Figure 2.2 shows the difference in sucrose preference between strains both before and after environmental manipulation. There was no significant strain difference in sucrose preference pre-environment $F(1,22)=1.88$, $p=0.184$, $\eta_p^2=0.079$ and after-environment $F(1,22)=0.021$, $p=0.886$, $\eta_p^2=0.001$.

Group differences:

There were no group differences in sucrose preference in either strain before environmental manipulation (WKY: $F(2,9)=0.261$, $p=0.776$, $\eta_p^2 = 0.055$; Wistar: $F(2,9)=4.160$, $p=0.053$, $\eta_p^2=0.480$. A 2 x 3 x 2 mixed ANOVA examining strain (2 levels; WKY and Wistar), environment (3 levels; enrichment, standard and isolation) and sucrose preference (2 levels; pre

and post- environment) showed significant main effects of environment in the WKY $F(2,9)=4.766$, $p=0.039$, $\eta_p^2=0.514$ and Wistar $F(2,9)=11.207$, $p=0.004$, $\eta_p^2= 0.714$ strains. Interactions between environment and sucrose preference were also found in both the WKY strain $F(2,9)=7.56$, $p=0.012$, $\eta_p^2=0.627$ and control animals $F(2,9)=11.714$, $p=0.003$, $\eta_p^2=0.722$.

Pair-wise comparisons were conducted to examine the effect of environment on sucrose preference. There was a trend for sucrose preference to increase from baseline after EE in both the WKY ($p=0.066$) and Wistar ($p=0.077$) animals. This change matches the hypothesis that EE can increase sucrose preference, and therefore reduce anhedonia in these animals. In contrast, there was a significant decrease in sucrose preference from baseline after isolation in both WKY ($p=0.011$) and Wistar ($p=0.001$) animals. In examination of sucrose preference after environment, animals in EE had significantly higher values than those in isolation both in WKY ($p=0.015$) and Wistar ($p=0.001$) strains. Furthermore, Wistar animals in the EE group had significantly higher sucrose preference as compared to those in standard housing ($p=0.021$). Overall, sucrose preference was positively affected by enrichment in both WKY and Wistar strains while isolation had a negative impact.

2.3.2 Elevated plus maze:

Strain differences:

A MANOVA was used to assess the differences between strains in time spent in the open and closed arms as well as the number of entries into each arm (Figure 2.3). These measures were assessed both before and after housing assignment. Before assignment, there were significant main effects of strain on the number of entries into the open arm $F(1,69)=30.198$, $p<0.001$, $\eta_p^2=0.304$, duration $F(1,69)=16.42$, $p<0.001$, $\eta_p^2=0.192$ and the number of entries

$F(1,69)=188.85$, $p<0.001$, $\eta_p^2=0.732$ into the closed arm. Figure 2.3, panels B and D, highlight the differences between strains in the frequency of open and closed arm entries respectively. Similar trends were seen post-environment with WKY animals showing significantly fewer entries into the open $F(1,69)=23.49$, $p<0.001$, $\eta_p^2=0.254$ and closed arms $F(1,69)=120.52$, $p<0.001$, $\eta_p^2=0.636$ and significantly less time spent in the closed arm $F(1,69)=11.26$, $p<0.001$, $\eta_p^2=0.140$.

These results may be due to the overall decreased activity of WKY animals and may be less related to anxiety-like behaviour then general motor activity. For instance, there was a significant difference between strains in considering total duration of movement during the EPM (these data are presented in Figure 2.4, top panel). This stain difference was observed both before ($F(1,70)=41.833$,$p<0.001$, $\eta_p^2=0.374$) and after ($F(1,70)=107.381$,$p<0.001$, $\eta_p^2=0.605$) environmental assignment. Furthermore, when total arm crosses completed by each strain were examined, it is clear that animals in the Wistar control strain completed significantly more arm crosses both pre- (=30.6) and post-environment assignment (=22.8) as compared to the WKY animals (before =13.2 and after =4.4) (Pre-environment $F(1,69)=147.2$, $p<0.01$, $\eta_p^2=0.681$ and post-environment $F(1,69)=84.62$, $p<0.01$, $\eta_p^2=0.551$) (Figure 2.4, lower panel).

Group Differences:

Paired samples t-tests were used to examine pre- and post-environment differences within each strain. Most of the changes observed were potentially due to practice effects as almost all open arm duration and entry measures decreased between the first and second test time. Table 2.1 shows the direction of change in the mean durations from pre to post environment with the corresponding p-values. The critical p-value based on the number of tests was p=0.0009 based on a Bonferroni correction.

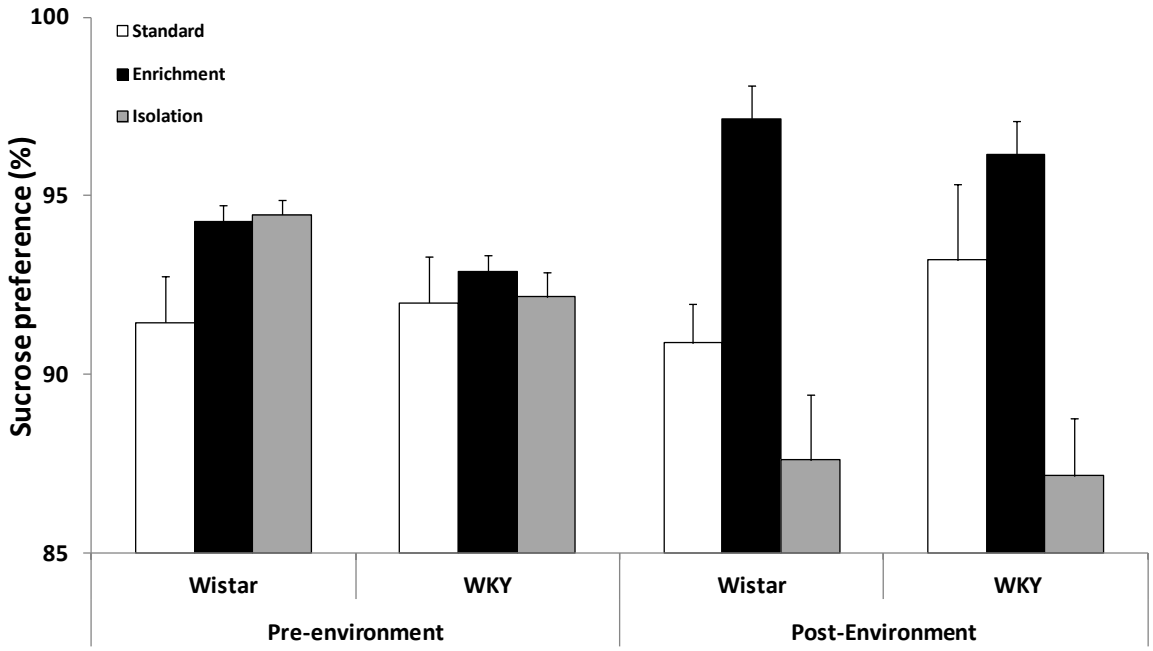

Figure. 2.2. Sucrose preference in the Wistar and WKY strains both before and after environmental manipulation. Error bars represent SEM. Asterisk denotes significance in the pairwise comparisons between groups post-environment ($p < 0.05$).

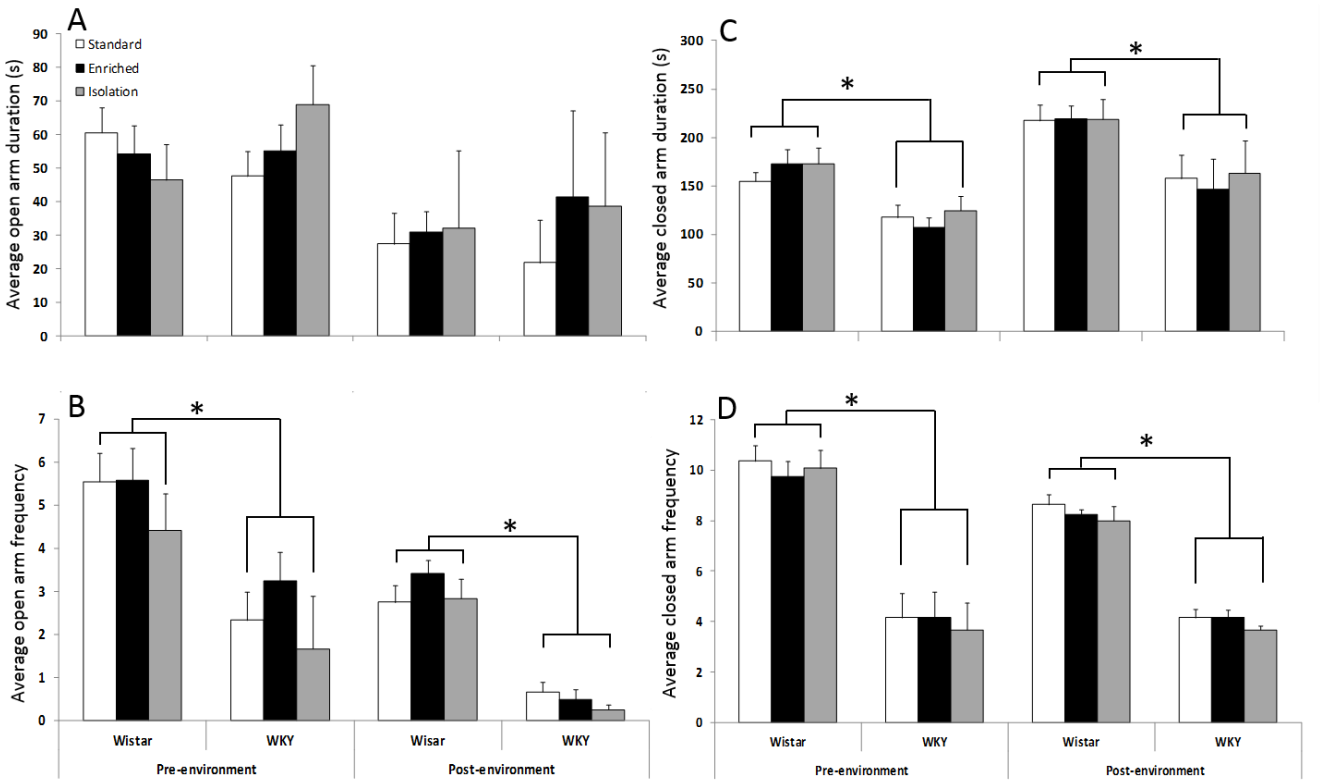

Figure. 2.3. Open arm duration (A) and frequency (B) and closed arm duration (C) and frequency (D) in the elevated plus maze separated by both strain and pre-post environmental manipulation. Error bars represent standard error.

As can be seen from Table 2.1, the only measure which increased significantly across all groups was the time spent in the closed arm, potentially indicative of the animals' familiarity with the maze. The decrease in open arm entries and duration as well as the increase in closed arm duration has been shown in previous studies after repeated testing (Almeida, Garcia, & de Oliveira, 1993).

2.3.3 Forced Swim Test:

Strain difference:

A MANOVA was conducted to examine the differences between strain and the duration of immobility, swim frequency and duration, and total struggle frequency and duration in the FST before and after housing assignment. Results of strain differences are listed in Table 2.2 for both pre- and post- environmental assignment. Pairwise comparisons indicated that all comparisons between WKY and Wistar groups were significant at $p<0.001$.

Some of the most striking results were related to total struggle and immobility durations. In this analysis, WKY had means of 2.7 and 292.7 s respectively for total struggle and immobility duration as compared to means of 89.7 and 207.1 s for the Wistar animals. Given that the test itself is 300 s, the fact that the WKY animals were immobile an average of 293 s indicates that they differ significantly from the Wistar animals which struggle to escape for a much larger proportion of time.

Group differences:

Group differences between pre and post environment were analysed via paired t-tests. With or without the Bonferroni adjusted alpha level due to the multiple comparisons ($p= 0.0009$),

there were no differences in behaviours before and after environment in the WKY strain; however that was not the case in the Wistar strain. Across all environments, these animals showed a decrease in the duration of struggling and an increase in immobility; this is common with repeated testing in the FST.

2.3.4 Weight:

Strain differences:

Weight gain over time in each strain is illustrated in Figure 2.5. A 2 x 8 ANOVA was performed to assess the effects of strain (two levels) on weight gain over time (eight time points). There was a significant main effect of strain $F(1,70)=422.69$, $p<0.001$, $\eta_p^2= 0.858$ and significant interaction between strain and weight gain over time $F(1.99,139.8)=97.95$, $p<0.001$, $\eta_p^2= 0.583$ due to WKY animals gaining weight more slowly than that of control animals. This was expected as WKY animals tend to have a much lower overall body weight throughout their lifespan.

Group differences:

A 3 x 8 mixed ANOVA examining the effects of environment (three levels) on weight over eight time points within each strain revealed no significant main effects of environment in either the WKY $F(2,33)=0.760$,$p=0.467$, $\eta_p^2=0.044$ or Wistar strains - $F(2,33)=0.313$, $p=0.313$, $\eta_p^2= 0.068$. There was a significant interaction between rate of weight gain and environment in the WKY strain $F(5.73,94.51)=5.03$, $p<0.001$ but not in Wistar animals $F(3.99, 65.92)= 1.988$,$p=0.115$. This finding is likely the result of differences during week 13 between the EE and SH groups ($p=0.047$). Examining week 13 alone, it is clear that there is a significant effect of environment in the WKY animals $F(2,33)=3.48$,$p=0.042$ while no significant effect of

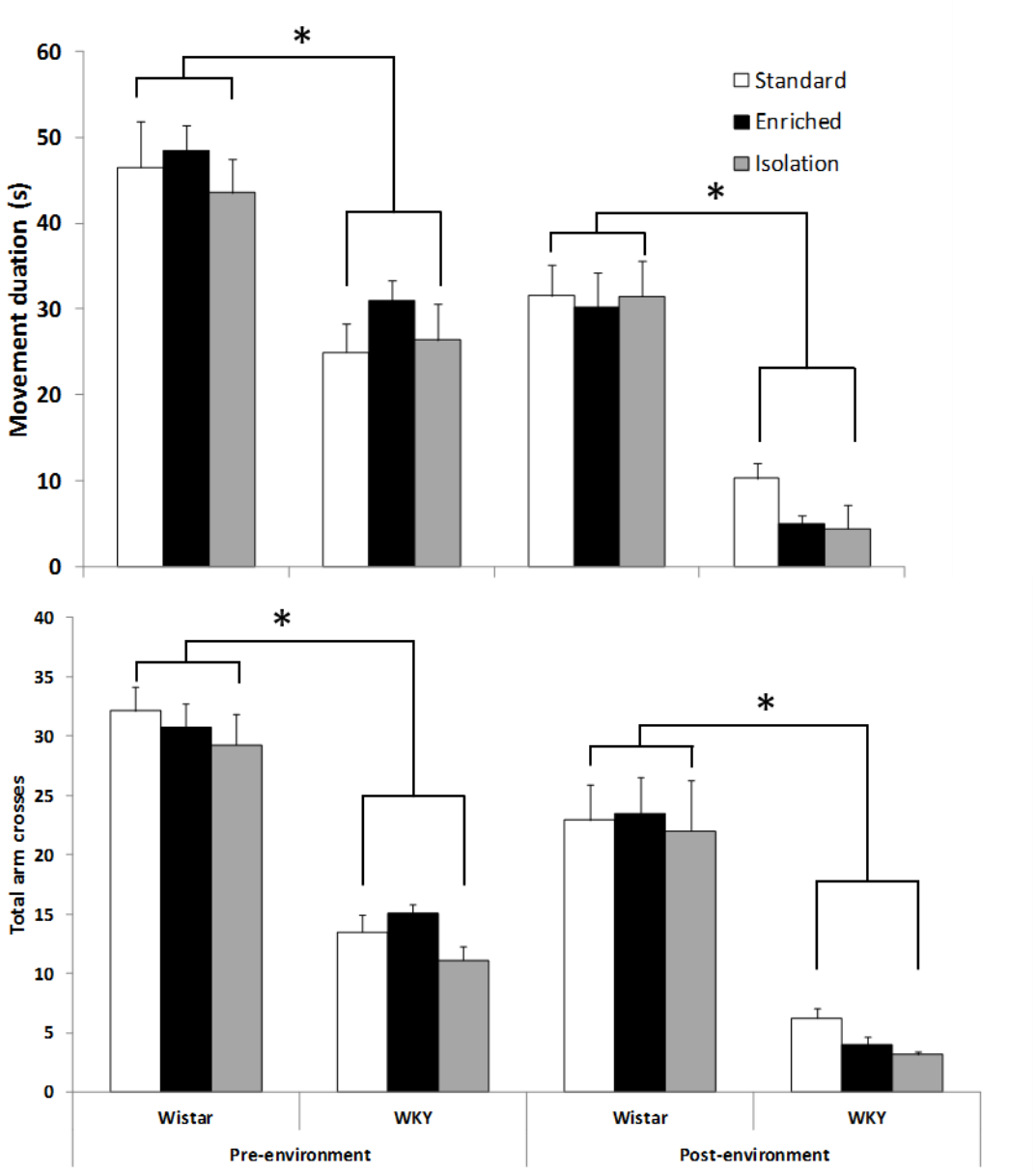

Figure. 2.4. Movement duration in seconds and total arm crosses performed by WKY and Wistar animals both before and after environmental assignment. Error bars represent SEM and asterisk indicates significance at the$p < 0.05$ level.

TABLE 2.1. Change in anxiety-like and ethological measures in the EPM following environmental assignment.

	Wistar			Wistar Kyoto		
Measure	EE	SH	IH	EE	SH	IH
EPM						
Open arm						
Duration	↓ (0.029)	↓ (0.008)	↓ (0.019)	↓ (0.58)	↓ (0.14)	↓ (0.29)
Entries	↓ (0.05)	↓ (0.006)	↓ (0.043)	↓ ($9x10^{-6}$)*	↓ (0.003)	↓ (0.003)
Closed arm						
Duration	↑ (0.005)	↑ (0.002)	↑ (0.001)	↑ (0.28)	↑ (0.092)	↑ (0.246)
Entries	↓ (0.18)	↓ (0.121)	↓ (0.062)	↓ (<$1x10^{-6}$)*	↓ (0.002)	↓ (0.001)
Total arm crosses	↓ (0.076)	↓ (0.012)	↓ (0.02)	↓ (<$1x10^{-6}$)*	↓ ($4x10^{-4}$)*	↓ ($3x10^{-5}$)*
Head Dip						
Duration	↓ (0.045)	↓ (0.091)	↓ (0.002)	↓ ($1x10^{-4}$)*	↓ ($9x10^{-6}$)*	↓ ($7x10^{-6}$)*
Frequency	↓ (0.063)	↓ (0.003)	↓ ($5.2x10^{-5}$)*	↓ (0.001)	↓ ($2.7x10^{-4}$)	↓ ($2x10^{-6}$)*
Stretch Attend						
Duration	↑ (0.72)	↑ (0.266)	↓ (0.841)	↓ (0.059)	↑ (0.042)	↓ (0.271)
Frequency	= (1)	↑ (0.348)	↓ (0.207)	↓ (0.006)	↑ (0.442)	↓ (0.095)

* $p<0.0009$
↓ Decrease in mean behaviour from pre to post environment
↑ increase in mean behaviour from pre to post environment
= no change in mean behaviour from pre to post environment
Critical Bonferroni adjusted p value = 0.0009 for EPM measures

TABLE 2.2. Strain differences in measures of immobility and escape behaviour in the FST.

Measure	**Means**		**F-value**
Pre-Environment	Wistar	WKY	
Swim			
Duration	10.9	0.94	F(1,69)=25.584**
Frequency	5.22	0.47	F(1,69)=35.015**
Climb			
Duration	77.64	1.59	F(1,69)=111.197**
Frequency	9.25	0.64	F(1,69)=130.473**
Immobility			
Duration	207.1	292.7	F(1,69)=101.66**
Frequency	6.81	2.19	F(1,69)=34.126**
Total Struggle			
Duration	89.71	2.76	F(1,69)=118.59**
Frequency	15.13	1.31	F(1,69)=81.405**
Post-Environment			
Swim			
Duration	13.38	1.09	F(1,69)=25.584**
Frequency	4.83	0.639	F(1,69)=35.015**
Climb			
Duration	34.4	1.54	F(1,69)=111.197**
Frequency	8.25	0.81	F(1,69)=130.473**
Immobility			
Duration	251.1	295.4	F(1,69)=101.66**
Frequency	6.81	1.8	F(1,69)=34.126**
Total Struggle			
Duration	48.14	2.63	F(1,69)=118.59**
Frequency	13.34	1.44	F(1,69)=81.405**

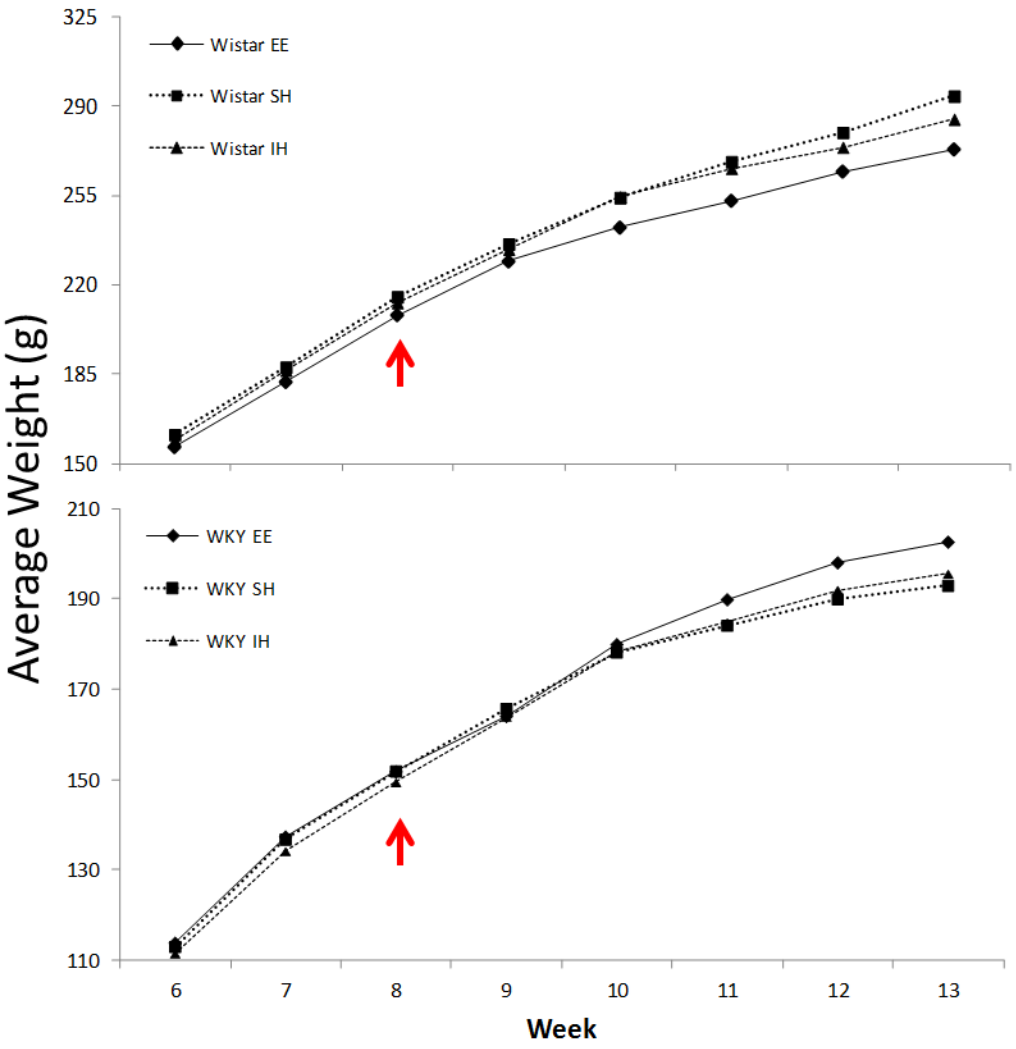

Figure. 2.5. Rate of weight gain (gr) over time in Wistar (top panel) and WKY (bottom panel) animals. Red arrows indicate start of environmental assignment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

environment was observed in the Wistar animals $F(2,33)=1.95, p=0.159$. Post hoc comparisons revealed that in the WKY strain, weight at week 13 differed significantly between EE and SH ($p=0.042$) groups with EE animals being heavier on average ($p=0.042$). Although not significant, the opposite trend is true in the Wistar strain with animals in SH being on average heavier than those in EE ($p=0.173$).

2.4. Discussion:

Large differences in behaviour were found between the two strains in almost all measures of anxiety- and depressive-like behaviours in the EPM and FST respectively. This could be due to the fact that WKY animals were naturally less active and more timid than their Wistar counterparts. As compared to the Wistar strain, WKY animals had significantly fewer entries into the open and closed arms as well as fewer total arm crosses and less movement in the EPM. In addition, WKY animals struggled far less than the Wistar animals and had significantly greater immobility times in the FST. Taken together, most findings in this study show that the behaviour of WKY rats is very different from that of the Wistar control rats and further validates the WKY as a putative model of depression.

The most notable finding in this study was the change in sucrose preference in response to environmental manipulation. A decrease in sucrose preference has been considered as the animal manifestation of anhedonia, a characteristic symptom of depression. It is well documented that animals exposed to stress experience a decrease in sucrose preference (Baker & Bielajew, 2007; Baker et al., 2006; Willner et al., 1992, 1987). At baseline, prior to environmental assignment, all animals had a similar sucrose preference. After assignment, rats in isolated housing had significantly decreased sucrose preference (interpreted as an increase in

anhedonia) as compared to those housed in EE conditions. Furthermore, as the pre- and post-environment sucrose preference of animals in standard housing did not differ significantly, this indicates that it was indeed environmental and not time effects which caused a change in preference. This is in line with previous studies which suggest that social isolation can create an animal model of depression (Brenes Sáenz, Villagra, & Fornaguera Trías, 2006) specifically in female animals who may be more sensitive to isolation (Grippo, Gerena, et al., 2007; Grippo, Cushing, & Carter, 2007). The difference in sucrose preference observed in both strains between EE and isolated housing conditions could be due to the broad anxiolytic effect of EE (for reviews see Fox, Merali, & Harrison, 2006 and Laviola et al., 2008). In our Wistar group, sucrose preference was significantly increased by EE as compared to the group in standard housing. A similar but not significant trend was seen in the WKY equivalent group. This could be due to the effects of physical exercise on behaviour. Exercise has been found to decrease depressive symptomatology in both humans and animal models (Greer & Trivedi, 2009). In fact, exercise is a key component of EE, as it correlates with increased neurogenesis and neurotrophic factors in the brain (Kobilo et al., 2011). These could all contribute to the difference seen in sucrose preference between the standard and EE housing conditions.

Contrary to expectation, there was no significant difference in sucrose preference at baseline between the WKY and Wistar rats. A difference in sucrose preference was expected since the WKY is an animal model of depression and therefore anhedonia would be a likely symptom from the outset of the experiment. A previous study examining pre-pubertal male rats reported a significant strain difference in saccharin preference between WKY and Wistar rats (Malkesman et al., 2005). In their study, animals were not in their home cage for the saccharin preference test and had been exposed to a physical stressor prior to the test. These conditions

could modify the animals' behaviour in a way that creates a significant difference between strains. For instance, not only could the negative effects of the physical stress affect WKY rats for a longer period of time than Sprague-Dawley or Wistar rats but also the novelty of moving to a different room from their home cage could have decreased their saccharin preference.

In the EPM, there was a significant difference between strains on multiple behavioural and ethological measures. This could be due to the lack of movement observed in the WKY strain which is in line with previous studies comparing the WKY strain to Wistar and Sprague-Dawley rats (Ferguson & Gray, 2005). In the current study, it was difficult to assess the WKY rats in the EPM as they tended to freeze in a portion of the maze and remain immobile for long periods of time. Therefore, within strain differences could not be easily observed. This lack of movement and tendency to freeze in the WKY strain also made analyses of environmental groups difficult within the WKY strain and any differences very difficult to interpret.

No protective effect of EE was found in the Wistar rats in the duration or frequency of open arm visits in the EPM. Previous studies using Sprague-Dawley rats have also found no protective effect of EE in the EPM (Brenes, Padilla, & Fornaguera, 2009). This result was attributed to the length of enrichment or isolation which could be a factor in the current study. However, four weeks duration for EE is quite common, with a large proportion of studies that employ EE choosing this time frame (Simpson and Kelly, 2011). While not significant, there was a reduction in both open arm duration and frequency, and an increase in closed arm duration in both the WKY and Wistar animals across all environments. As a whole, this may be indicative of a practice effect.

An alternative explanation is that the EPM may not be the best measure of anxiety-like behaviour in the female rat. In fact, the EPM may be measuring different behavioural dimensions between the sexes. For instance, Frenandes and colleagues (1999) performed a factor analysis on male behaviour in the EPM and showed that it was related to anxiety, while in females, EPM behaviour was more indicative of their activity level (Fernandes, González, Wilson, & File, 1999). Due to limited studies on female rats, there is little evidence that the EPM reliably measures anxiety-like behaviour in females, especially if the stage of the estrous cycle is taken into account. Altogether, it is important in future research to question whether the EPM is an applicable test of female anxiety-like behaviour and to carefully consider the age and estrus cycle stage of the animals.

The results from the FST showed significant differences between the two strains but not between the environments within strain. All escape and struggle behaviours were significantly lower in the WKY animals, both before and after environmental manipulation. This is consistent with previous studies showing that WKY rats are immobile for a significantly larger portion of time as compared to control animals (Paré, 1989; Rittenhouse, López-Rubalcava, Stanwood, & Lucki, 2002). In our study, there appeared to be no effect of environment on FST behaviours in either strain. In the WKY rats, this could be because they tend to be very inactive and even in some places have been bred to show immobility in the FST so that the environmental manipulation is ineffective. However, it was expected that EE and isolation would influence the Wistar rats. Upon further examination it appeared that there were significant differences across FST behaviours between cohorts. As much as possible, both cohorts were kept in the same conditions and tests were carried out at the same time in both cohorts. However, Wistar rats in cohort 1 who were assigned to EE housing showed a significant increase in escape behaviour and

decrease in immobility after environmental manipulation as compared to those in isolation. This is expected based on previous studies (Brenes & Fornaguera, 2008; Brenes et al., 2009). However, no significant difference was observed in cohort 2 animals. When combining the two cohorts, the effects of environment disappeared. While the FST is a validated model of antidepressant activity, it may not be effective in examining the effects of a non-pharmaceutical intervention such as the environmental manipulation used in this study.

Weight also differed significantly between strains. This was not surprising given the difference in growth trends of each strain. The pattern observed in the Wistar rats is more congruent with past research as animals in EE tend to be more physically active and are thus lighter than their standard housing or isolated housing counterparts (Zaias, Queeney, Kelley, Zakharova, & Izenwasser, 2008). However, in the current study, the opposite trend was found in WKY females. The significant increase in weight at week 13 as compared to animals in standard housing is noteworthy as similar effects have been found in previous studies. For instance, the comparison of enriched to impoverished WKY animals indicates enrichment was associated with higher weights in these animals (Hunziker, Saldana, & Neuringer, 1996). These results indicate a further need to study the impact of physical and social environment on body weight.

There were limitations in this study as only female animals and two separate cohorts could be used due to space and time constraints. The use of female animals, as well as the potential variability between cohorts could have impacted the results of this study. Furthermore, the effects of repeated testing in the EPM and FST may have influenced the post-environment behavioural measures. To avoid this, future studies should increase the inter-test interval and physical location of the test between trials. Finally, variability inherent in the WKY strain could

make interpreting small behavioural differences in response to environmental assignment difficult (Overstreet, 2012).

2.5 Conclusions

The environment can have both positive and negative effects on mental illness. This study highlights the importance of the combination of social and physical enrichment in an animal model of anhedonia. Future studies should examine the effects of EE and isolation on both male and female WKY animals to explore the relationship between environment, sex, and depressive- and anxiety-like behaviours. If the social and physical components of EE can improve negative behaviours in an animal model of depression, then perhaps enrichment in the form of a healthy lifestyle, for example, can be used in human work as an intervention for clinical depression either by itself or in conjunction with pharmaceuticals.

Acknowledgements:

We thank the Natural Sciences and Engineering Research Council of Canada for its support of this project.

Published in Behavioural Brain Research Volume 316, Jan 2017

3. **Corticosterone and immune cytokine characterization following environmental manipulation in female WKY rats**

Guergana R Mileva, Jasmine Rooke, Nafissa Ismail, and Catherine Bielajew.

Abstract:

This study investigated the effects of environmental manipulation on female Wistar Kyoto (WKY), an animal model of depression, and female Wistar rats. It explored the function of the hypothalamic-pituitary-adrenal axis (HPA) and immune system, as they have both been implicated in the pathophysiology of depression. A further goal was to characterize the immune cytokine concentrations of female WKY rats as this has, to our knowledge, never been documented. Animals were assigned to enriched, standard, or impoverished housing for four consecutive weeks. Following this, serum was collected at baseline and post-stress periods to measure the concentration of corticosterone, TNF-α, IL-1β, and IL-10. WKY animals had significantly higher corticosterone levels at the post-stress time-point than their Wistar counterparts. WKY rats in isolation tended to have the lowest corticosterone levels which may indicate that they prefer a solitary environment, a symptom of depression. We observed a significant decrease in TNF-α after enrichment in the Wistar strain. A similar decrease in TNF-α was found in the WKY strain, but there was no difference between environmental conditions. There was a significant increase in pre- to post-stress IL-10 level in both Wistar and WKY animals. WKY rats had a significantly lower level of IL-1β as compared to the Wistar animals at both pre- and post-stress time points. Given this strain difference, it is likely that the WKY rats had a dysregulated HPA axis which further influenced their circulating cytokine levels. Further studies are needed to examine how this pattern of findings plays a role in the pathophysiology of depression.

Keywords: Stress, immune response, cytokine, corticosterone, depression, hypothalamic-pituitary-adrenal axis,

3.1. Introduction:

Depression is a serious disorder with a large impact on both an individual's quality of life and society as a whole. The behavioural symptoms include anhedonia, fatigue, changes in sleep, and withdrawal from social situations (Andrews et al., 2007). However, the pathophysiological changes in the brain which occur during depression are still not well understood. For instance, some of the issues that have yet to be resolved are related to the slow onset of the therapeutic response of antidepressants and their clinical efficacy in only a subset of patients (Connolly & Thase, 2011). Moreover, the scientific evidence linking a chemical imbalance in the brain and depression has recently been called into question (Lacasse & Leo, 2005). The view that depression is a chemical imbalance in the brain is likely one piece of a much more complex puzzle and other mechanisms and their interaction in the pathophysiology of depression should be explored.

Chronic stress has long been linked to depression, typically through the dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (Checkley, 1996). One frequently studied marker of stress is the amount of circulating cortisol. Atypical diurnal and reactive stress patterns can reveal an impairment of the HPA axis. For example, an increase in serum cortisol, known as hypercortisolemia, is reported in patients with depression (Carroll et al., 2007; Gillespie & Nemeroff, 2005). However, the mechanism behind this HPA dysregulation leading to elevated circulating cortisol is still unknown (Carroll et al., 2012). A decrease in serum cortisol, known as hypocortisolism, is also common in patients with major depressive disorder (Maripuu, Wikgren, Karling, Adolfsson, & Norrback, 2014). The findings that both too much and too little circulating cortisol are reported in depression illustrates the complicated mechanisms that underlie mood disorders and the need for more studies.

More recently, a role for the immune system in the pathophysiology of major depression has been uncovered (Miller et al., 2009). In response to infection, peripherally produced pro- and anti-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 10 (IL-10) respectively are released. These pass though the blood-brain barrier and directly affect neural structures (Vitkovic et al., 2000). For instance, pro-inflammatory cytokines can increase excitotoxicity and decrease monoamines and trophic factors in the brain (Miller et al., 2009). Not only are serum pro-inflammatory cytokine levels higher in people with depression, but there appears to be an imbalance between the pro- and anti-inflammatory cytokine system (Song, Halbreich, Han, Leonard, & Luo, 2009). The above suggest that a dysregulation in the immune system and the resulting elevation in pro-inflammatory cytokines may predispose an individual to developing depression.

One way to characterize depression-related immune mechanisms is via animal models. For example, rats treated with lipopolysaccharide (LPS), the major component of gram-negative bacteria (Layé, Parnet, Goujon, & Dantzer, 1994) exhibit sickness behaviour which has similarities to the symptoms of depression including fatigue, anhedonia, pain, and both social and physical withdrawal (Dantzer et al., 2008; Kentner et al., 2008). It has been hypothesized that 'depression' or 'sickness behaviour' has evolved as a motivational state in response to infection as it promotes immune system activation and compensatory energy saving mechanisms (Dantzer, 2001; Dantzer, 2009; Stieglitz et al., 2015). The connection between depression and sickness behaviour is strengthened through studies showing that chronic treatment with anti-depressants can alleviate sickness behaviour in rodents (Charlton, 2000; Yirmiya, 1996; Yirmiya et al., 2001). In fact, if an increase in cytokines is chemically blocked with anti-inflammatories such as dexamethasone, no sickness behaviour is exhibited since there is no triggering signal

(Dantzer, 2001; de Paiva et al., 2010). While sickness behaviour can be adaptive in response to infection, it can be the cause of psychopathology when it, or its antecedents, are not deactivated (Dantzer et al., 2008).

While infection can potently activate the immune system, research has shown that stressful stimuli can also trigger the immune response. Chronic stress early in life can affect inflammatory responses and again contribute to a dysregulation of the HPA axis even in medically healthy patients (Danese et al., 2008) without exposure to infection. It is known, via a feedback mechanism, that stress-induced inflammatory responses increase circulating cortisol through HPA axis stimulation (Miller et al., 2009). Taken together, there is powerful evidence that stress-induced immune activation and its effects on the HPA axis play a large role in the pathophysiology of anxiety and depression.

Physical exercise and social support have been found to help alleviate mild to moderate depression in humans and have been successfully used as interventions (Carek et al., 2011; Paykel, 1994). However, it is not clear whether social and physical interventions are helpful in cases of severe depression and how this would affect circulating stress hormone and immune cytokine levels. One way to examine this relationship in animal models is by incorporating environmental enrichment (EE) as a form of physical and social enrichment. While EE currently has no standardized format, it typically involves exposing laboratory animals to physical and social stimuli that they would not normally experience in a standard housing condition (Rosenzweig & Bennett, 1996). EE has been shown to significantly increase neurogenesis as well as decrease anxiety- and depressive-like behaviour (Simpson & Kelly, 2011). EE can affect neurotransmission (Solinas, Thiriet, Chauvet, & Jaber, 2010) as well as increase neurotrophic factors in the hypothalamus of female rats (Bakos et al., 2009). Furthermore, environmental

enrichment, when coupled with brain stimulation reward, has been shown to buffer the effects of LPS injection and the consequent sickness behaviour (Kentner et al., 2008).

Animal models of depression have been used as research tools to study the factors and mechanisms underlying depression. For example, the Wistar Kyoto strain specifically has been utilized as an animal model of depression for the past two decades (Overstreet, 2012). This strain was originally bred to be the normotensive control of the spontaneously hypertensive rat (Okamato & Aoki, 1963). They exhibit depressive-like behaviour in the forced swim test (Mileva & Bielajew, 2015), have abnormal circadian rhythm activity (Solberg et al., 2001), are more prone to stress-induced ulcers (Paré, 1989), and are considered the most likely candidates as an animal model of antidepressant-resistant depression (Lahmame et al., 1997; Willner & Belzung, 2015). While many diseases are more prevalent in women, most animal studies have relied on studies of male animals (for review of sex bias in animal studies see (Beery & Zucker, 2011; Zucker and Beery, 2010). This is also the case in depression and anxiety research. While women have almost twice the likelihood of developing depression in their lifetime, pre-clinical studies of depression have largely employed male animals (Piccinelli & Wilkinson, 2000; Zucker & Beery, 2010). This bias is due to the hormonal fluctuations inherent in female animals during the estrous cycle which can affect both physiology and behaviour (Mileva et al., 2013). In recent years, this issue has been gaining more traction and concrete steps are being taken to balance the use of female and male animals although there is still much work to be done (Clayton & Collins, 2014).

The study described here addressed whether environmental manipulation in the form of enrichment or impoverishment affects the peripheral concentration of corticosterone and the immune cytokines TNF-α, IL-1β, and IL-10 in the WKY strain as compared to that of the Wistar

strain. Due to the dearth of studies investigating female depressive responses, particularly in the WKY strain, it is the wider objective of this study to produce data that can be used as a reference for future studies of these strains.

3. 2. Methods:

3.2.1 Animals and environmental conditions

A total of 36 WKY and 36 Wistar female rats at age post-natal day (PND) 28 were obtained from a local supplier (Charles River Laboratories, Québec, Canada). Figure 3.1 shows the timeline of the study. At PND 28 rats arrived at the facility and were placed in groups of 3 and left to acclimate for 4 days. Following this, animals were handled between PND 32 and PND 62 and behavioural testing as described in Mileva et al 2015 was carried out. A day/night cycle of 12h:12h and temperature of 21.5±1^{o}C with humidity ~40% kept conditions standardized. Animals were cycling at PND 55 at which age they are considered sexually mature (Sengupta, 2013). At PND 62, they were randomly assigned to one of three environments for 30 days: 1) Standard housing (SH) - 3 rats in one guinea pig cage, with dimensions 50cm x 38cm x 20cm, 2) Isolated housing (IH) - 1 rat per small cage, dimensions 45cm x 22cm x 20cm, or 3) Environmental enrichment (EE) - 6 animals in a multistory cage with toys, chain bridges, Nestlets®, wooden chewing blocks, multicoloured Plexiglas® houses, and cardboard tubes. Animals in EE also had unlimited access to a running wheel. In each environment, food and water was provided ad libitum and cages were cleaned once a week to provide fresh bedding. Each environmental condition had twelve animals. Therefore, 12 WKY animals and 12 Wistar animals were in the EE condition, another 12 WKY and 12 Wistar animals in the SH condition, and a final 12 WKY and 12 Wistar animals in the IH condition.

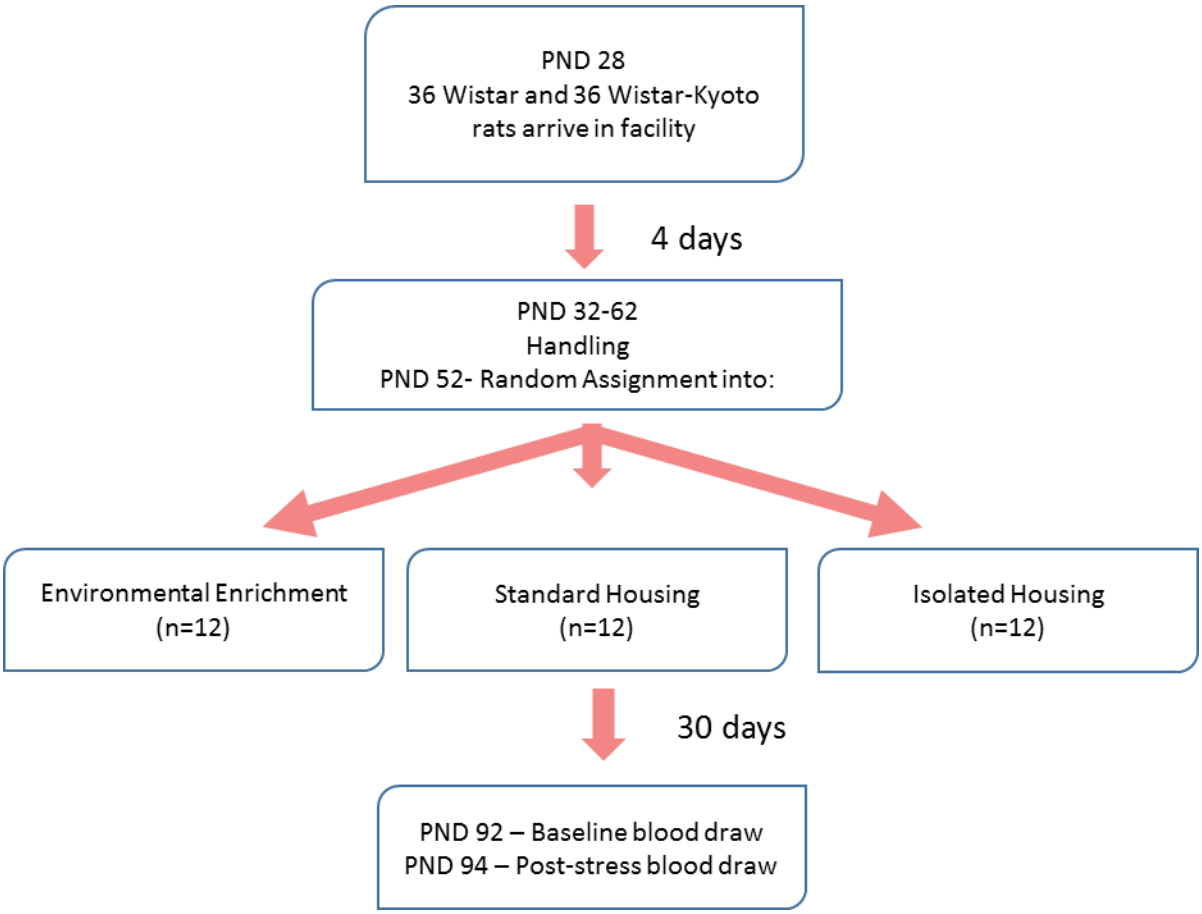

Figure 3.1. Timeline of experiment including arrival at the facility at post natal day (PND) 28, handling, environmental assignment at PND 62 for 30 days and finally baseline and post-stress blood draws at PND 92 and 94 respectively.

3.2.2 Estrous cycle monitoring

Details of estrous cycle stage monitoring are detailed in Mileva et al 2015. Briefly, vaginal fluid was collected by inserting and withdrawing 50µl of warm water into the vaginal canal using a sterile pipette and analysing the cell type (Marcondes et al., 2002).

3.2.3. Immune Cytokine analysis

At PND 92, animals were restrained and baseline blood was withdrawn from the tail vein. This procedure took a maximum of three minutes per animal, minimizing the effects of restraint stress on circulating corticosterone and immune cytokines (Vahl et al., 2005). At PND 94, after a 15-minute forced-swim, animals were placed in an incubator at 28^{o}C and allowed to warm up for 5 minutes so blood collection would be easier. Therefore, post-stress blood was withdrawn approximately 20-30 minutes after exposure to the stressor. Blood was centrifuged and serum was aliquoted and stored in a -80^{o}C freezer. After thawing, the serum was analysed for immune cytokines using an EMD Millipore analyte 96 well plate sensitive to IL-1β, TNFα, and IL-10. Serum was also analysed for corticosterone levels using an Enzo Corticosterone ELISA 96 well plate. All procedures were approved by the Animal Care Committee at the University of Ottawa, Canada.

3.2.4 Statistical Analyses

Statistical analyses were performed using IBM SPSS V 20 software. 2x3x2 ANOVAs were performed accounting for strain (2 levels- Wistar and WKY), environment (3 levels- EE, SH, IH) and time-point (2 levels- baseline and peak). Bonferroni correction was used for all pairwise comparisons as needed. Estrus cycle did not qualify for use as a covariate because it did not significantly correlate ($r < 0.4$) with mean corticosterone, IL-1β, TNF-α, and IL-10 (Ferguson & Takane, 1989).

3.3 Results:

3.3.1 Corticosterone:

3.3.1.1 Comparing Wistar and WKY strains

A 2x3x2 ANOVA comparing strain, environment and time-point found significant main effects in corticosterone levels between strains $F(1,111)=23.501$, $p<0.001$, $\eta^2=0.175$, time-points $F(1,111)=683.954$, $p<0.001$, $\eta^2=0.860$, and there was an interaction between strain and time $F(1,111)=13.488$, $p<0.001$, $\eta^2=0.108$ (Figure 3.2). Pairwise comparisons revealed no significant differences in serum corticosterone at baseline between the Wistar (58 ng/ml) and WKY (76 ng/ml) animals ($p=0.406$). Conversely, there was a significant difference at the post-stress time-point with the Wistar animals having a much lower level of corticosterone (410ng/ml) than the WKY (544ng/ml) rats ($p<0.001$).

Figure 3.3 shows the baseline (panel A) and post-stress levels (panel B) of corticosterone in the Wistar and WKY animals across the three environments. There was no main effect of environment $F(2,103)=2.058$, $p=0.133$, $\eta^2= 0.038$ or a strain by environment interaction $F(2,103)=2.269$, $p=0.109$, $\eta^2= 0.042$. However, at baseline, a marginally significant interaction between strain and environment was found $F(2,52)=3.027$, $p=0.057$ (panel A). Planned comparisons showed that Wistar animals had a significantly lower level of corticosterone (45ng/ml) than that of their WKY counterparts (106ng/ml) in the SH condition $F(1,18)=5.140$, $p=0.037$.

At the post-stress time-point, corticosterone levels were significantly different between strains $F(1, 51)=23.988$, $p<0.01$ (panel B). Planned comparisons at post-stress levels found significant differences between strains in the EE and IH groups. In the EE groups, Wistar animals had a significantly lower levels of corticosterone (426ng/ml) than the WKY (585ng/ml)

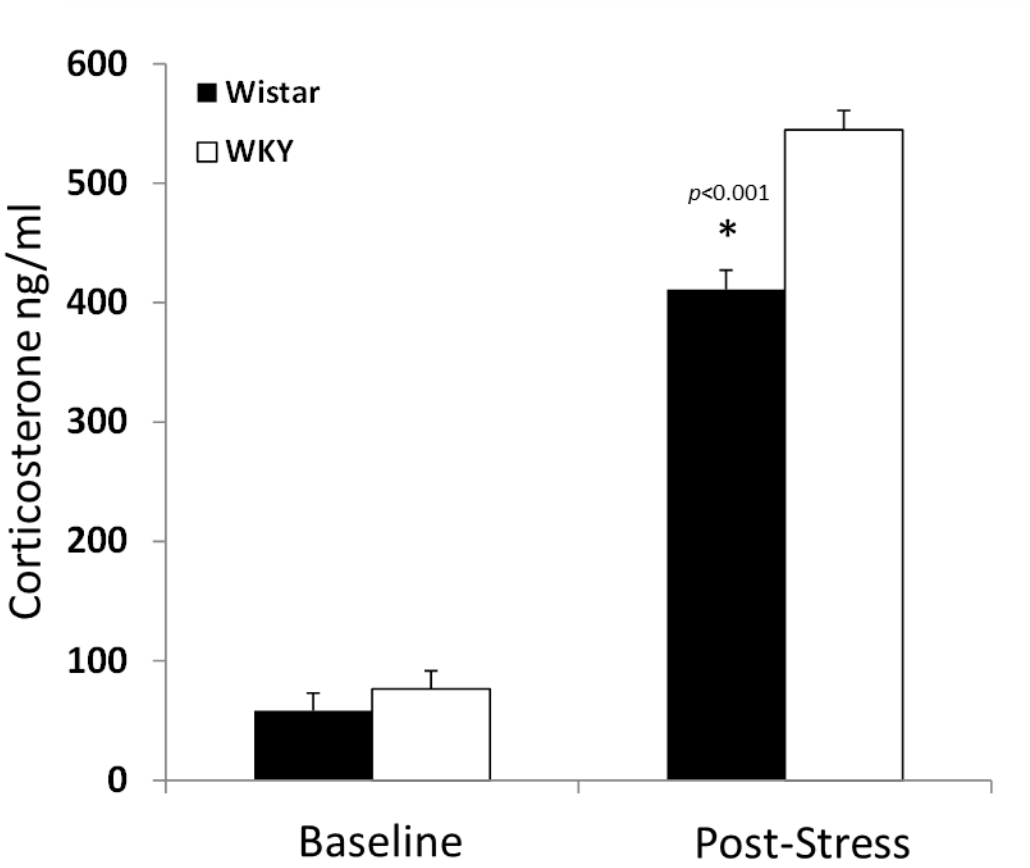

Figure 3.2. Serum corticosterone (ng/ml) in Wistar and Wistar Kyoto female rats at baseline and peak stress levels. Animal numbers were as follows; at baseline Wistar (n=32), WKY (n=26); at the post-stress time-point Wistar (n=31), WKY (n= 26). Error bars represent SEM. Asterisk denotes significance in the pairwise comparisons at (p<0.05).

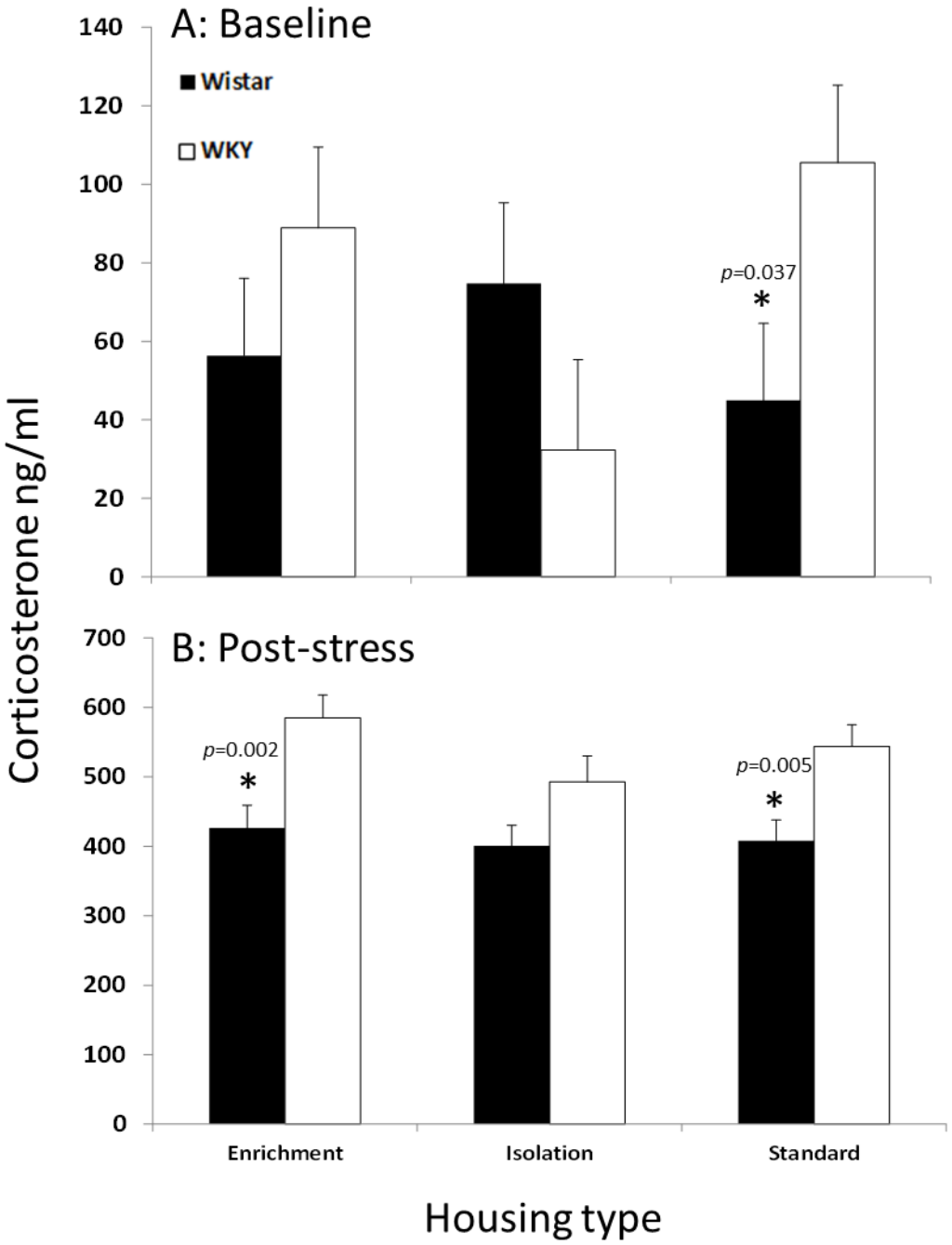

Figure 3.3. Serum corticosterone (ng/ml) in Wistar and Wistar Kyoto female rats at baseline (panel A) and peak (panel B) stress levels across three environments. Sample sizes were as follows; baseline Wistar EE (n=11), IH (n=10), and SH=(11); WKY EE(n=10), IH (n=8) and SH (n=8). At the post-stress time-point Wistar EE (n=9), IH (n=11) and SH (n=11); WKY EE (n=9), IH (n=7) and SH (n=10). Error bars represent SEM. Asterisk denotes significance in the pairwise comparisons at($p<0.05$).

animals (F(1,17)=12.986, *p*=0.002). Similarly, in the standard housing condition Wistar (408ng/ml) animals had lower levels of corticosterone than WKY (543ng/ml) rats (F(1,20)=10.289, *p*=0.005).

3.3.1.2 Within strain comparisons

Using a one-way ANOVA, within strain differences were examined. As shown in Figure 3.2, there was a significant increase in corticosterone from pre- to post- stress time-points in both the Wistar (F(1,62)=422.25, *p*<0.001) and WKY strains (F(1,51)=287.35, *p*<0.001). There was no significant difference between corticosterone levels and environmental placement in either the Wistar (F(2,57)=0.264, *p*=0.77, η^2= 0.009) or WKY strains (F(2,46)=2.686, *p*=0.079, η^2= 0.105). Planned comparisons gave rise to a trend in which the lowest corticosterone levels for WKY animals were found in the IH condition at both baseline (F(29,31)=2.184, *p*=0.135), and at post-stress (F(23,25)=1.235, *p*=0.310).

3.3.2: TNF-α

The top panel (A) in Figure 3.4 illustrates the relationship between TNF-α, strain, and environmental condition. A 2x3x2 ANOVA examining the effects of strain, environment and time on TNF-α levels revealed a significant main effect of time (F(1,127)=5.95, *p*=0.016, η^2= 0.045). Pairwise comparisons displayed a significant decrease in TNF-α between baseline (4.9pg/ml) and post-stress (3.9pg/ml) time-points (p=0.016). There was a significant environment by time interaction F(2, 127)=3.36, *p*=0.038, η^2=0.05 as well as a trend in the environment by strain interaction F(2,127)=2.807, *p*=0.064, η^2=0.042. Pairwise comparisons revealed that TNF-α decreased from baseline (5.45pg/ml) to the post-stress (3.0pg/ml) time-point only in the EE condition (p=0.001). Furthermore, there was a marginal main effect of environment at the post-stress time-point in the Wistar animals (F(2,33)=2.974, *p*=0.065).

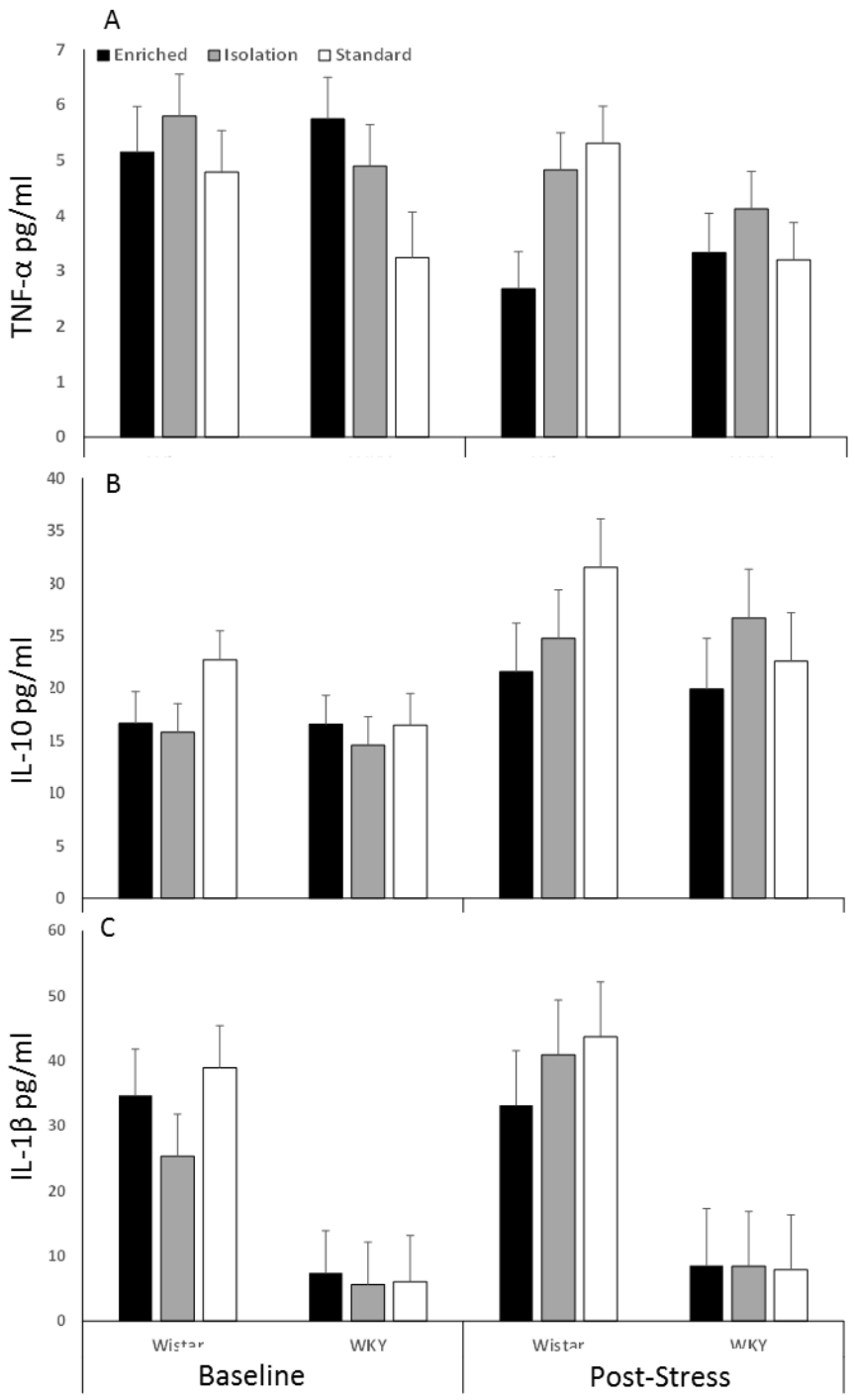

Figure 3.4. Serum cytokine levels (pg/ml) in Wistar and Wistar Kyoto female rats at baseline and peak stress levels across three environments. The sample size for all groups was n=12, expect at baseline for Wistar animals in EE who had n=10, and WKY animals in SH who also had n=10. Error bars represent SEM.

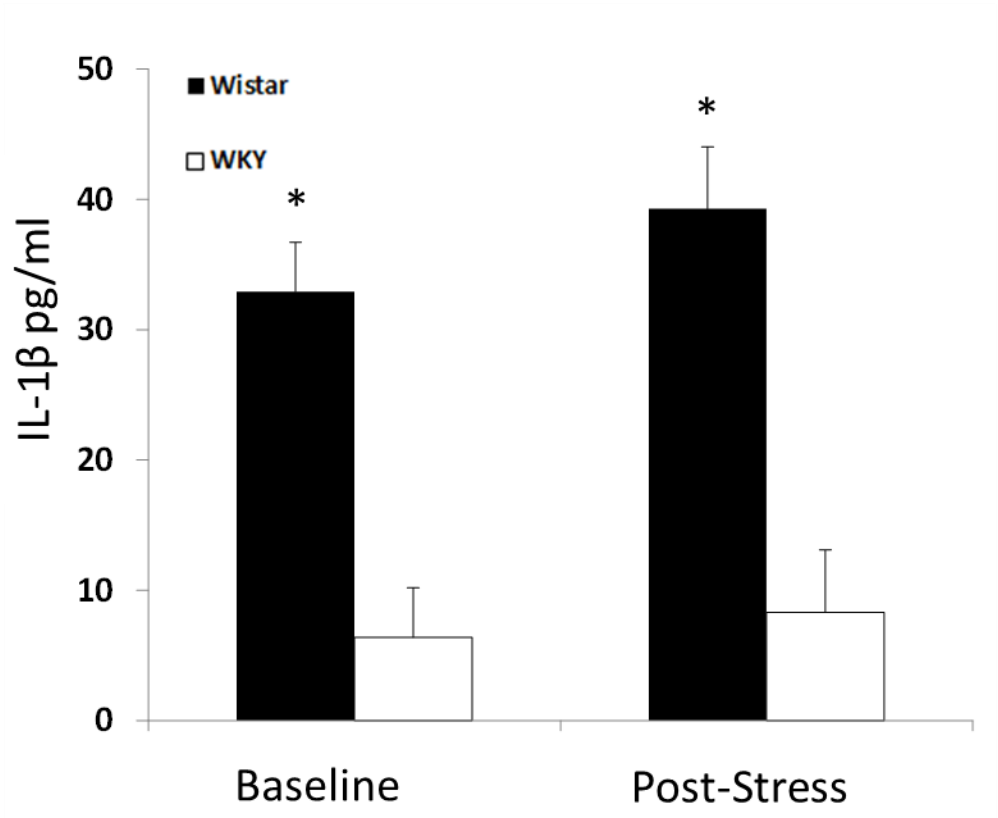

Figure 3.5. Serum IL-1β concentration (pg/ml) in Wistar and Wistar Kyoto female rats baseline and peak stress level. Error bars represent SEM. Animal numbers were as follows; at baseline Wistar (n=34), WKY (n=34); at the post-stress time-point Wistar (n=36), WKY (n= 35). Asterisk denotes significance in the pairwise comparisons at(p<0.05).

Pairwise comparisons reported a trend in which animals in EE (2.6pg/ml) had the lowest level of TNFα as compared to those in SH (5.3pg/ml); p=0.086.

3.3.3 IL-10

The relationship between environment, strain and time and IL-10 serum concentration is shown in the middle panel (B) of figure 3.4. There was a main effect of time on IL-10 serum levels ($F(1,127)=10.68$, p=0.001, η^2=0.078). Pairwise comparisons showed a significantly higher IL-10 serum level at the post-stress time-point (24.5pg/ml) as compared to that of baseline (17.14pg/ml);p=0.001.

3.3.4 IL-1β

The lower panel (C) of Figure 3.4 shows the association between IL-1β, environmental placement, time, and strain. A 2x3x2 ANOVA examining the effects of strain, environment and time on serum concentration of IL-1β showed a significant main effect of strain $F(1,127)=42.12$, $p<0.001$, $\eta^2=0.25$. As illustrated in Figure 3.5, Wistar animals had a significantly higher serum IL-1β concentration at both baseline (32.9pg/ml) and post-stress (39.2pg/ml) time-points than WKY animals at baseline (6.3pg/ml) and post-stress (8.2) (Baseline: $F(1,67)=24.2$, $p<0.001$ and Post-stress: $F(1,70)=21.06$, $p<0.001$).

3.4. Discussion:

The purpose of this study was two-pronged: to examine the effects of environmental condition as well as characterize immune cytokine levels in an animal model of depression. We found significant increases in serum corticosterone and IL-10 concentration at the post-stress time-point for both Wistar and WKY strains. WKY animals had significantly higher post-stress levels of serum corticosterone as compared to that of Wistar animals indicating a potential

dysregulation of the HPA axis. The pro-inflammatory cytokine TNF-α decreased between pre- and post- stress time-points, specifically in the EE condition in Wistar animals. WKY animals also showed significantly lower than normal levels of the pro-inflammatory cytokine IL-1β.

The HPA axis and its metabolites can profoundly affect the immune response. Cortisol has an inhibitory effect on the immune system, such that as circulating cortisol increases the immune response is dampened in order to allow for stress-induced physiological changes to occur and to stop all non-essential processes (for review see Miller, 2009). The inhibitory effect of cortisol on the immune system is decreased after exposure to chronic stress. This allows for the increased release of pro-inflammatory cytokines which access the brain and promote excitotoxicity, a decrease in monoamines and trophic factors. These have strong implications for psychopathology and many studies have shown increases in circulating pro-inflammatory cytokines in depressed patients (Liu, Ho, & Mak, 2012; Song et al., 2009; Sutcigil et al., 2007; Tuglu, Kara, Caliyurt, Vardar, & Abay, 2003). Similarly, chronic stress and an increase in circulating cortisol have been found in patients with depression (Carroll et al., 2012; Checkley, 1996; Gillespie & Nemeroff, 2005). In fact, it may be that the increase in circulating cortisol has no effect on depression but rather that through chronic stress the HPA axis is dysregulated and can no longer act to inhibit and in turn suppress the downstream effects of pro-inflammatory cytokines on the brain. Research on the administration of IL-1β has shown it is able to potently activate the HPA axis by stimulating ACTH and CRH release (for review see Dunn, 2000; Uehara, Gottschall, Dahl, & Arimura, 1987). Taken together, it may be that an increase in cytokines activates the HPA axis and in turn a dysregulation of the HPA axis potentiates the immune response. The evidence points to an innate relationship between the HPA axis and

immune system and the dysregulation of either can play a pivotal role in the development of psychopathology, and in particular depression.

Results from previous studies have reported increased corticosterone levels in WKY male and female rats as compared to Sprague-Dawley rats and may be indicative of an HPA axis dysregulation (Armario, Gavaldà, & Martí, 1995; Rittenhouse, López-Rubalcava, Stanwood, & Lucki, 2002). Studies examining corticosterone response following an acute stressor have also found that WKY males have a sustained higher corticosterone level than their Wistar counterparts (De La Garza & Mahoney, 2004). The largest differences between Wistar and WKY animals in this study were found between the EE and SH housing types. Serum corticosterone levels in EE were the highest for the WKY animals suggesting that the frequent changes in their environment, by way of adding novel objects to their cage, as well as the forced social contact with five other animals could have been more stressful than was the case for animals in the IH environment.

There was a trend for WKY animals in IH towards low corticosterone levels at both the baseline and post-stress time-points. Conversely, corticosterone levels in the Wistar strain were highest in IH at baseline. This could be because female animals are typically more stressed after social isolation than their community housed counterparts (Hatch et al., 1965). In fact, some laboratories use social isolation as a stressor and have shown increases in corticosterone in rodents following these manipulations (Heinrichs & Koob, 2006). The finding that WKY animals have lower corticosterone levels in isolation suggests that the withdrawal inherent in depression may keep their physiological stress response low and that forced social and physical enrichment may in itself be a form of chronic stress for this strain.

While TNF-α has been implicated in the pathophysiology of depression, there is debate as to the relationship of TNF-α and the HPA axis, with some researchers finding that elevated HPA axis activity supresses the production of TNF-α (Berthold-Losleben & Himmerich, 2008; Himmerich et al., 2006). In addition, some laboratories have reported both decreased (Song et al., 2009) and increased (Liu et al., 2012; Tuglu et al., 2003) levels of TNF-α in patients with major depression. In this study, there was a significant decrease in TNF-α between baseline and post-stress time-points, perhaps due to a corticosterone-induced suppression of TNF-α. In our study, Wistar animals in EE tended to have the lowest levels of TNF-α suggesting the physical and social enrichment they experienced in their environment can have an immunomodulatory effect and acts as a buffer to stress-induced inflammation. This is supported by studies of male mice which found that TNF-α and IL-1β were significantly lower in animals in the EE condition after LPS injection (Williamson, Chao, & Bilbo, 2012).

The balance between pro-inflammatory and anti-inflammatory cytokines has been linked to depression (Elenkov & Chrousos, 1999). Studies often show an increase in proinflammatory (IL-1) and decrease in anti-inflammatory (IL-10) cytokines in untreated patients with depression (Song et al., 2009; Sutcigil et al., 2007). In this study, there was no impact of environmental condition on serum concentration of IL-10 in either Wistar or WKY female rats. In both strains IL-10 increased between pre and post-stress time-points. Studies have shown that IL-10 is an inhibitor of TNF-α production (Armstrong, Jordan, & Millar, 1996). The IL-10 mediated suppression of TNF-α may be why we see a decrease in TNF-α as described above. In addition, an increase in IL-10 in response to psychosocial stress has been reported in mouse studies and it has been suggested that stress-induced IL-10 secretion acts as an immunosuppressant (Curtin,

Mills, & Connor, 2009). Therefore, both the Wistar and WKY strains show some degree of stress induced IL-10-mediated immunosuppression.

Typically, an increase in IL-1β serum concentration has been found in patients with depression (Song et al., 2009). In animals, it has been shown that injection with IL-1β can trigger immobility in the FST, a measure of depressive-like behaviour (Simmons & Broderick, 2005). In a previous behavioural study in the same subset of animals, we showed that the WKY female animals were immobile significantly longer than their control counterparts in the FST (Mileva & Bielajew, 2015). Consequently, the goal here was to examine whether this immobility was associated with circulating IL-1β serum concentration. As shown in Figure 4, there was a significant difference in serum IL-1β between strains opposite to our expectations. Other studies using male and female Wistar rats have found similar serum levels of IL-1β to those found in our control Wistar animals at baseline (Gullaiya et al., 2013; Silva et al., 2000). Therefore, it appears that the levels we observed in the WKY female rats were significantly lower than what would be considered 'normal' and may indicate a dysregulation in the IL-1β pathway specifically. To our knowledge, this is the first report of IL-1β serum concentration in female WKY animals. More studies will need to replicate this finding in order to parse out how significantly lower serum levels of IL-1β could affect the HPA axis and stress response. Future studies will need to examine the mechanism responsible for this unusually low level of IL-1β and what this means for the pro- and anti-inflammatory cytokine balance important in the pathophysiology of depression.

The complicated findings of this study may be due to the interaction of estrogen and immune cytokines. This interaction is intricate and depending on which cell or tissue type is examined, estrogen can have either inhibitory or excitatory effects (for review see Straub, 2007).

As an example, TNF- α levels can be either stimulated or suppressed by estrogen (Gilmore, Weiner, & Correale, 1997). Furthermore, an interaction between gonadal hormones and estrous cycle has been found in which progesterone can increase IL-1β induced fever in contrast to animals which were not administered progesterone (Mouihate, Chen, & Pittman, 1998). This indicates a relationship not only between estrogen but also progesterone and immune cytokine response. Others have suggested that estrogen can increase the levels of anti-inflammatory cytokines such as IL-10 during the course of pregnancy, to supress the maternal immune response (Thaxton & Sharma, 2010). Taken together, these studies highlight the complex role of gonadal hormones on immune cytokine secretion. As female rats have large fluctuations in gonadal hormones throughout their estrous cycle, it is possible that an interaction of these hormones and immune cytokines produced the variability we see in our sample and that they negated any potential effects of environment that may have occurred. However, as there was no correlation between estrous cycle phase and measures of immune cytokines or corticosterone, it is unclear whether gonadal hormones did have an effect in this study. Future studies examining estrogen and progesterone will be necessary to define the role of each in the immune system, especially in response to changes in the environment.

3.5. Conclusions

This study characterized corticosterone and immune cytokine serum levels in response to environmental manipulation in female WKY and Wistar rats. Female WKY rats appear to have an altered stress response as they tend to produce more corticosterone following exposure to a stressor than their control counterparts. In addition, housing in isolation seems to be the least stressful environment for the WKY rats seeing as they are prone to withdrawal and avoidance behaviours (Servatius, Jiao, Beck, Pang, & Minor, 2008).

This study provides evidence for a disrupted IL-1β pathway, in the WKY female rat. Future studies could examine the mechanism for the disruption in the IL-1β pathway and if there is an additive effect of pharmacotherapy and enrichment in serum corticosterone and cytokine levels. Pharmacotherapy may be effective in pushing the animals into a more receptive state towards the positive effects of physical and social enrichment. Finally it is important to examine the effects of circulating gonadal hormones on pro- and anti- inflammatory cytokines in female WKY rats during each stage of the estrous cycle. Taken together, the novel characterization of corticosterone and immune cytokine serum levels in the female WKY rat strongly suggest a dysregulation of the IL-1β pathway and the HPA axis.

Acknowledgements:

We thank the Natural Sciences and Engineering Research Council of Canada for its support of this project.

(Submitted to Neuropsychobiology)

4. Environmental enrichment increases GFAP in Wistar females and glucocorticoid receptor immunoreactivity in the amygdala of female WKY rats

Guergana R. Mileva, Carinna Moyes, Shaezeen Syed & Catherine Bielajew

Abstract:

Depression and anxiety are often associated with an increase in HPA axis reactivity and immune response. To investigate this relationship, we examined the consequences of environmental manipulation on the neural correlates of the HPA axis and immune response in an animal model of depression, the Wistar-Kyoto (WKY) rat. Furthermore, given the underrepresentation of females in animal models, we collected data on female Wistar and WKY rats. Female animals are often overlooked in pre-clinical research due to the changes in physiology and behaviour that go hand in hand with the hormone fluctuations inherent in the estrous cycle. The rats were randomly assigned to one of three environments for 30 days: 1) Environmental enrichment (EE), 2) Standard housing (SH), and 3) Isolated housing (IH). Immunoreactivity of astrocytes (GFAP), glucocorticoid receptors (GR), and microglia (Iba1) in the hippocampus and amygdala were measured using immunohistochemistry. In enriched Wistar rats, GFAP staining intensity and area were greater in the CA1. A trend towards a greater percent of area stained with GR was found in WKY animals as compared to that of the Wistar animals. This was due to WKY females in EE having significantly higher GR staining intensity and area in the amygdala as compared to that of animals in SH. The findings of this study suggest that EE can influence the number of GR receptors in an animal model of depression as well as increase astroglia and GRs in control animals. More social interaction and physical activity may have positive neural consequences in animals that express depressive-like behaviour. Such data provide evidence that enrichment may be a viable behavioural and physical intervention in the treatment of depression.

Keywords: Depression, anxiety, glucocorticoid receptors, astroglia, microglia, enriched environment, Wistar-Kyoto, Wistar

4.1. Introduction:

Depression and anxiety disorders are often co-morbid and negatively impact both the individuals' quality of life and society as a whole. In most western countries, females are twice as likely to suffer from depression and anxiety as their male counterparts (Piccinelli & Wilkinson, 2000). However, animal studies use male animals to study depression significantly more often than female animals (Zucker and Beery, 2010). For example, Beery and Zucker (2011) reported that the male to female ratio in neuroscience research is close to 5:1 with male animals used to study disorders with a higher prevalence in females. One reason for this discrepancy is that females have much larger fluctuations in their hormone levels throughout their lifespan, which can cause behavioural and physiological changes that make the interpretation of data difficult (Mileva et al., 2013). More recently, there has been a push to incorporate female animals in pre-clinical research, especially in studying disorders whose prevalence in much higher in females. For these reasons we wanted to examine the effects of environmental manipulation on biomarkers associated with depression and anxiety in female animals. The link between the endocrine, specifically the hypothalamic-pituitary-adrenal (HPA) axis, and immune systems of the body has been investigated in the past 30 years with evidence that both systems act on each other and can be implicated in various disease models (Chryssikopoulos, 1997; Kiess & Belohradsky, 1986; Procaccini, Pucino, De Rosa, Marone, & Matarese, 2014).

Depression and anxiety are known to cause hyperactivity in the HPA axis (Varghese & Brown, 2001; Vreeburg et al., 2009). This hyperactivity in turn leads to an increase in circulating glucocorticoid hormones which bind to glucocorticoid receptors (GRs). When glucocorticoids bind to GRs in the hippocampus, they serve to inhibit the continued activation of the HPA axis

(for review see Hyman, 2009). Decreases in glucocorticoid receptor expression in the hippocampus following stressful life events such as child abuse (McGowan et al., 2009) as well as in the serum of patients with major depression (Carvalho et al., 2014) suggest that chronic stress as well as depression affect GRs and may contribute to the pathophysiology of depression. Similar findings are evident in animal models, in which the adult offspring of mothers with low maternal care have significant decreases in GR expression (Hellstrom, Dhir, Diorio, & Meaney, 2012; Liu et al., 1997). Furthermore, the dexamethasone suppression test, in which a synthetic glucocorticoid that preferentially binds to GRs is administered, does not inhibit cortisol secretion in depressed patients while it does so in non-depressed individuals (Juruena et al., 2006). The above suggests that depressed patients have an impaired glucocorticoid negative feedback pathway and this is most likely mediated by changes in GRs.

Pharmacotherapy with antidepressants appears to decease the hyperactivity of the HPA axis, possibly through the modulation of feedback through the GRs (Anacker et al., 2011) as well as a GR-dependent increase in hippocampal neurogenesis (Anacker et al., 2011). GRs have also been innately tied to the immune system. In a recent study by Cohen and colleagues (2012) chronic stress was shown to be linked to glucocorticoid receptor resistance, defined as a decrease in the sensitivity of immune cells to circulating glucocorticoids, which in turn increases the inflammatory response (Cohen et al., 2012). This may be due to the stress-mediated glucocorticoid resistance of microglia (Stark et al., 2001). Thus, it is important to explore the potential effects of depression and anxiety on microglia.

While much research has been done on the behavioural, molecular, and cellular links to the pathophysiology of depression, comparatively little is known about how non-neuronal cells such as microglia and astrocytes can affect the behavioural or physiological outcomes of

depression. The neuroinflammatory theory of depression posits that cerebral inflammation is central in the development of depression and other neuropsychiatric diseases (for review see Miller and Raison, 2015). Fundamental to this theory is that macrophages, also called microglia in the brain, release pro-inflammatory cytokines which in turn contribute to a decrease in trophic factors and increase in excitotoxicity (for review see Miller et al., 2009). Markers specific for macrophages, such as ionized calcium binding adaptor molecule 1 (Iba1) have shown that the number of primed microglia are increased in the dorsal anterior cingulate white matter of depressed patients who commit suicide (Torres-Platas, Cruceanu, Chen, Turecki, & Mechawar, 2014). Additionally, microglial process density decreases significantly after electroconvulsive shock in the hippocampus (Jinno & Kosaka, 2008) indicating that the therapeutic action of electroconvulsive shock may be due to its effects on microglia.

Astroglia serve a vital role in their interactions with neurons and are responsible for energy metabolism and neuroprotection (Benarroch, 2005). In fact, hippocampal astroglia are instrumental in regulating neurogenesis and synaptic transmission (Song, Stevens, & Gage, 2002) indicating that they are indeed critical in normative brain function. Research into how psychopathology can affect astroglia is just emerging. It has been shown that glial fibrillary acidic protein (GFAP), an astrocyte marker, is significantly decreased in the brains of patients with depression (for review see Rajkowska and Stockmeier, 2013) suggesting that astrocytes are indeed affected in neuropsychiatric disorders. For example, astrocyte reductions have been observed in the amygdala of patients with major depressive disorder (Altshuler et al., 2010). In addition, recent research on the downstream effects of antidepressants has found that astroglial biochemistry changes in response to antidepressants (for review see Peng et al., 2015).

Environmental enrichment (EE) is a paradigm that has been employed since the early 1960's as an effective model to study changes in behavior and physiology as a result of social and physical intervention. Currently, EE does not have a standardized paradigm between laboratories with both the time-frame and level of physical and social enrichment differing significantly (for review see Simpson and Kelly, 2011). Regardless of this lack of consistency, many laboratories use EE to examine changes in biochemistry, physiology and behaviour. Some of the most well-known studies of EE have found that it is neuroprotective (During, Young, Lawlor, Leone, & Dragunow, 1999), increases neurotrophin levels in the brain (Ickes et al., 2000), increases neurogenesis in the hippocampus (Olson, Eadie, Ernst, & Christie, 2006; Segovia, Yagüe, García-Verdugo, & Mora, 2006), and can even reverse the negative effects of early maternal separation (Francis et al., 2002) in rodents. Collectively, because of the positive effects of EE, it may be used as a potential intervention for animal models of depression.

In this study, female rats were used in order to examine the effects of environment on astrocytes, microglia, and glucocorticoid receptors (GR) in the hippocampus and amygdala. The same animals were used in a previous study describing their behavioural outcomes in response to environmental manipulation (Mileva & Bielajew, 2015). Because of the volume loss in the hippocampus associated with major depressive disorder, this study focused on the hippocampus as well as the amygdala, a key component of the limbic system responsible for much of the emotional reactions to stimuli. Using immunohistochemistry, we hypothesized that female WKY rats, a putative animal model of depression (Overstreet, 2012), would show greater baseline levels of fluorescence for the microglial markers while lower fluorescence of both GR and astrocyte markers. Furthermore, based on the expansive literature detailing the positive effects of enrichment, we expected to see more astrocyte and GR fluorescence in animals who had

experienced an enriched environment vs. those who were in isolation as enrichment may have an antidepressant-like effect on these animals (Pariante & Miller, 2001; Simpson & Kelly, 2011).

4.2. Results

4.2.1 Hippocampus - CA1 and CA3:

Figure 4.1 shows representative photomicrographs of GFAP, GR and Iba1 in the CA1 (blue shaded area) and CA3 (yellow shaded area) across EE, SH and IH in Wistar animals. Photomicrographs of Wistar animals were used as the differences between environmental conditions was clearer in this strain. The results of a 2x3 independent ANOVA examining two levels of strain and three levels of environment were a main effect of environment $F(2,28)=3.832$, $p=0.034$, $\eta^2= 0.215$ and a strain x environment interaction $F(2,28)=4.629$, $p=0.018$, $\eta^2= 0.248$ in GFAP CTCF in the CA1 (Figure 4.2, A, left panel). Pairwise comparisons revealed that Wistar animals in EE had significantly higher GFAP CTCF than animals in both SH ($p=0.006$) and IH ($p=0.006$). Conversely, there was no significant difference in the WKY strain between environments ($p>0.05$). No main effects or interactions on the GFAP data were found in the CA3.

Figure 4.2 (A, right panel) shows the percent area covered by GFAP immunofluorescence. There were no main effects of environment or strain in either the CA1 or CA3; however an environment by strain interaction was found in the CA1 $F(2,28)=4.578$, $p=0.019$, $\eta^2= 0.246$. This interaction was likely due to the fact that Wistar animals in EE had a significantly higher percent area of GFAP immunofluorescence than that of animals in SH ($p=0.046$) and marginally higher percent area than that of animals in IH ($p=0.056$), whereas

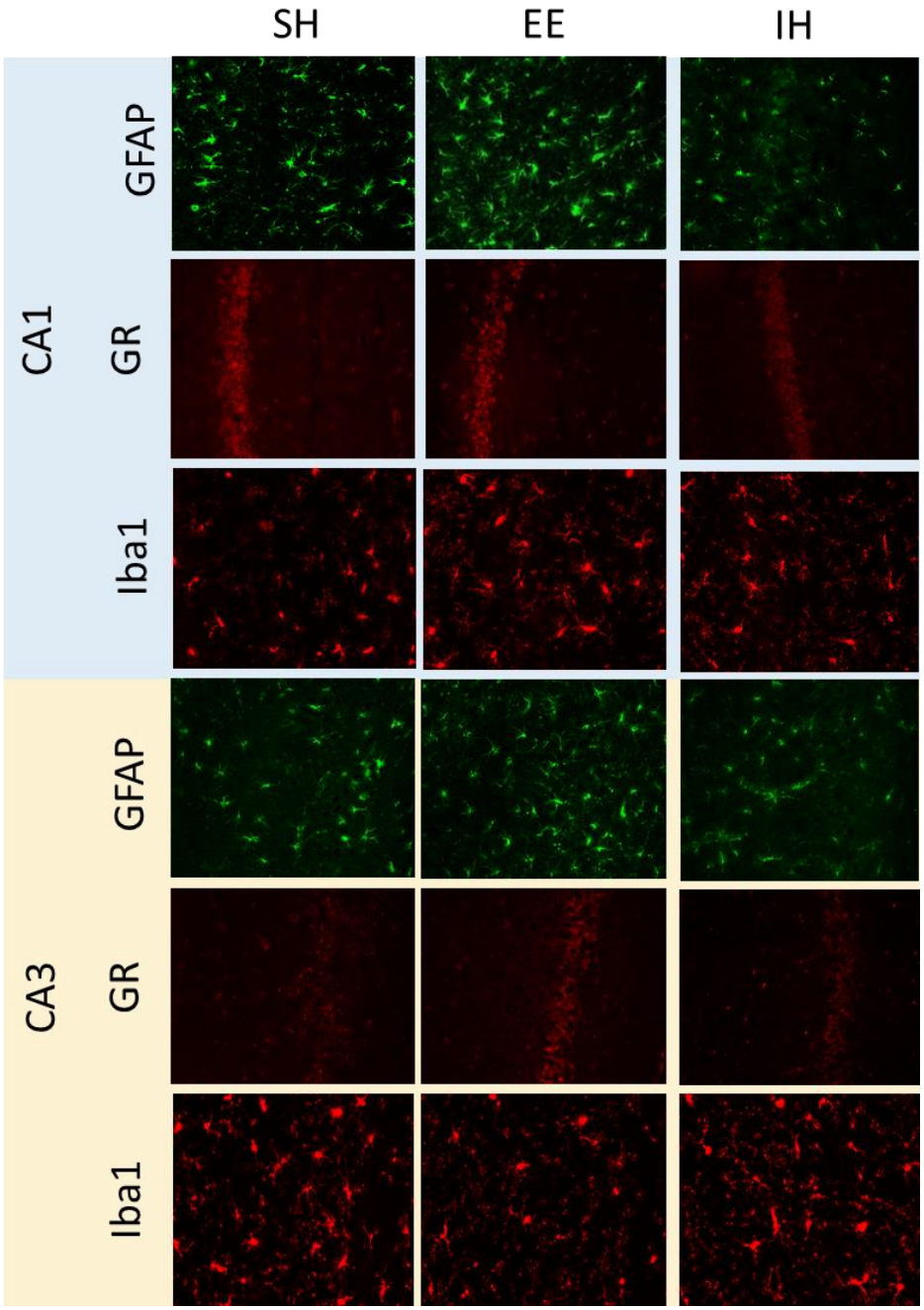

Figure 4.1. Immunoreactivity of astroglia (GFAP), glucocorticoid receptors (GR), and microglia (Iba1) in the CA1 and CA3 of the hippocampus across three different environmental conditions. Representative photomicrographs of GFAP (green, top), GR (red, middle) and Iba1 (red, bottom) are shown in the CA1 (blue shaded area) and CA3 (yellow shaded area) of Wistar animals across enriched (EE), standard (SH) and isolated housing (IH). Each photomicrograph represents 200x magnification of the distinct region of interest.

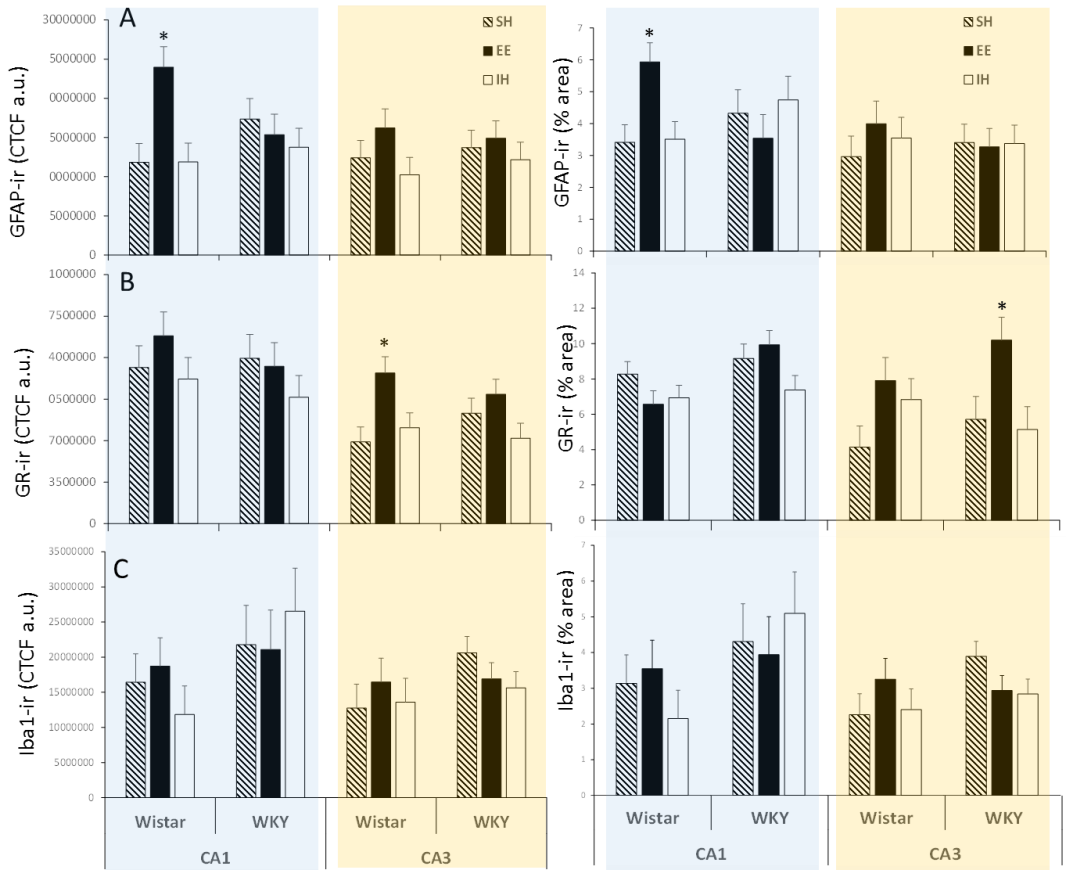

Figure 4.2. Corrected total cell fluorescence (CTCF) and mean percent staining area in the CA1 (blue shaded area) and CA3 (yellow shaded area) of the hippocampus across the three environments and both the Wistar and WKY strain. The graphs represent GFAP CTCF (A, left) and mean percent area (A, right); GR CTCT (B, left) and GR mean percent area (B, right); Iba1 CTCF (C, left) and mean percent area (C, right). Animal numbers in each group were: Wistar SH (n=6), IH (n=6), EE(n=5), WKY SH(n=6), IH(n=5), EE(n=6).CTCF is expressed in arbitrary units. Data are expressed as mean ± SEM. *$p < 0.05$

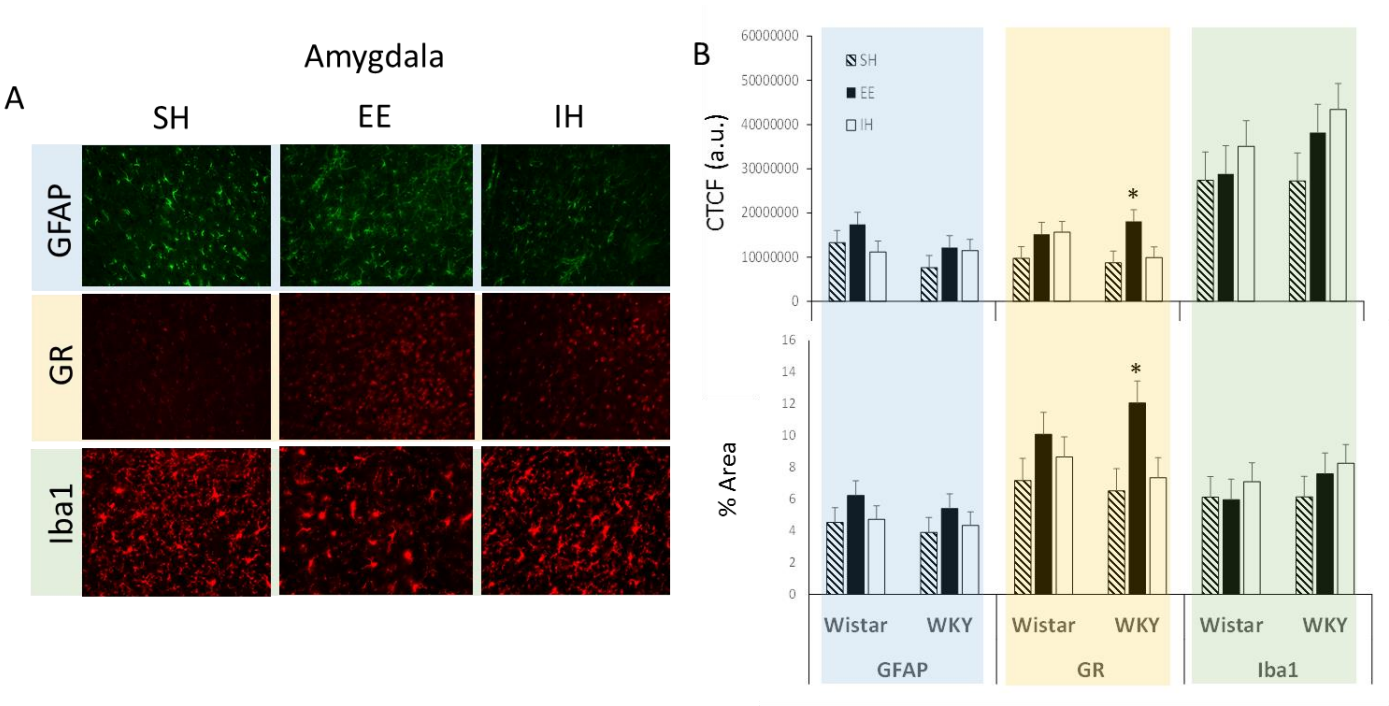

Figure 4.3. Photomicrographs and histograms showing staining in the amygdala across three environmental conditions. A) Photomicrographs of GFAP (green, top), GR (red, middle) and Iba1 (red, bottom) at 200x magnification are shown in the Wistar rats. B) Graphs represent the CTCF (top panel) and mean percent area (bottom panel) of GFAP (blue shaded area), GR (yellow shaded area), and Iba1 (green shaded area). Animal numbers in each group were: Wistar SH (n=5), IH (n=6), EE(n=5), WKY SH(n=5), IH(n=6), EE(n=5).CTCF is expressed in arbitrary units. CTCF is expressed in arbitrary units. Data are expressed as mean ± SEM. *$p < 0.05$.

WKY animals had no such differences and in fact animals in EE had the lowest percent area of GFAP immunofluorescence on average.

Figure 4.2 (B) shows the CTCF (left panel) and mean percent area (right panel) of GR immunoreactivity. A main effect of environment on GR was found in the CA3 only $F(2,29)=6.152$, $p=0.006$, $\eta^2= 0.298$. Pairwise comparisons showed that in the CA3, animals in EE had significantly more GR CTCF than SH ($p=0.023$) or IH ($p=0.009$) animals. For the CA3, planned comparisons within the Wistar strain revealed that animals in EE had significantly higher levels of GR staining intensity as compared to those in SH ($p=0.013$). Similarly, planned comparisons in the CA3 of WKY animals showed that GR area of staining was significantly higher in the EE animals than those in SH($p=0.05$) and IH($p=0.023$).

Figure 4.2 (C) characterizes the immunoreactivity of Iba1 in the CA1 and CA3 by examining CTCF (left panel) and mean percent area (right panel). No statistical differences were found in either brain region.

4.2.2 Amygdala

Figure 4.3 (A) shows representative photomicrographs of GFAP, GR and Iba1 staining in the amygdala across EE, SH, and IH. As shown in Figure 3, B (blue shaded area) a strain difference approached significance $F(1,28)=3.403$, $p=0.076$, $\eta^2=0.108$, with the Wistar animals having more GFAP CTCF than observed in WKY animals ($p=0.076$).

A main effect of environment on GR was found in the amygdala $F(2,28)=5.006$, $p=0.014$, $\eta^2= 0.263$ (Figure 4.3 B, yellow shaded area). Animals in EE had significantly higher levels of GR CTCF than those in SH ($p=0.011$). Within the WKY strain, GR staining intensity was higher in the EE animals in comparison to those in SH ($p=0.014$) for the amygdala. GR

staining area was also significantly higher in WKY animals in EE as compared to animals in SH ($p=0.012$).

Figure 4.3 B (green shaded area) displays the CTCF and mean percent area of Iba1 staining between the Wistar and WKY animals across EE, SH and IH. No significant differences were found when comparing these parameters.

4.3. Discussion:

This study examined both the intensity of staining and total area of immunofluorescence of biomarkers connected to depression and anxiety in an animal model of depression. As mentioned above, female strains were used given the considerable lack of research focused on female data in this context. The hippocampus and amygdala were examined specifically because of their importance in the processing of emotional stimuli as well as the critical role of the hippocampus in the GR-mediated inhibitory feedback to the hypothalamus. In this study, we discovered significantly higher levels of GFAP in the CA1 of the hippocampus following EE in Wistar females, while the area of GR staining was significantly higher in WKY females following EE in the amygdala.

The hippocampus is a vital brain structure responsible for learning, memory, anxiety and stress regulation and shows great plasticity throughout the lifespan (for review see Leuner and Gould, 2010). We examined the CA1 and CA3 separately based on recent findings that the CA1 and CA3 areas of the hippocampus have differential functions. These suggest that the CA1 is required for contextual memory retrieval while both CA1 and CA3 are important for context-dependent extinction (Ji & Maren, 2008). The CA1 and CA3 are also involved in the formation of memories for non-spatial events; however the time-scales differ between these regions

(Farovik, Dupont, & Eichenbaum, 2010). The electrophysiological properties of pyramidal cells of the CA1 and CA3 are also distinct (Collingridge, Kehl, & McLennan, 1983; Harris & Cotman, 1986). Thus, we felt it essential to examine both areas independently. It is important to realize that most of the above studies on the variety of functions of the CA1 and CA3 are done on male animals. Morphological sex-differences in the limbic system, including the hippocampus and amygdala, have been described in previous studies (for review see Madeira and Lieberman, 1995) and may influence their function.

While it is well-known that EE increases neuronal neurogenesis in the hippocampus as a whole (Olson et al., 2006; Segovia et al., 2006), less is known about its effects on astroglia, especially between different hippocampal regions. In this study, female Wistar rats in the EE condition showed significantly higher staining intensity and percent area of staining for astroglia in the CA1, but not CA3. While another study of male mice (Viola et al., 2009) found no change in immunoreactivity or density of astroglia following EE, it did report structural changes in the hippocampal CA1 following EE. In the motor cortex of adult female mice, EE has been shown to increase the number of astroglia (Ehninger & Kempermann, 2003). In addition, EE increases the number of GFAP positive stem cells in juvenile male and female mice following hypoxia (Salmaso et al., 2012). These studies suggest that EE does indeed affect astroglial proliferation, morphology, and the neuron-astroglial relationship. The data presented in this paper are consistent with the hypothesis that physical and social enrichment in the form of EE can have profound positive effects on astroglia, specifically in the Wistar strain. Future studies could examine the glial:neuron ratio as it is important to determine whether both are increasing together. This could indicate that learning and memory are indeed affected by enrichment. Interestingly, there were no significant differences in GFAP staining intensity or area of staining

in the WKY strain. While this could be due to the small sample size in this study, it may be because the constant changes and forced social and physical contact associated with EE are stressful to WKY animals who are prone to withdrawal and isolation (Servatius et al., 2008).

Similar to our findings with GFAP staining, GR staining intensity was the highest in EE in the CA3 for the Wistar female rats. Comparable results for GR staining area were found in the WKY CA3. Based on the importance of GRs for inhibitory feedback of the endocrine system, this indicates that EE may have been responsible for increasing GRs and in turn influence the regulation of circulating glucocorticoids. Furthermore, GRs in the hippocampus play a role in altering synaptic dynamics. For example, while activation of GRs of the CA1 is thought to prolong afterhyperpolarization in the pyramidal cells, less is known about the effects of GRs in the CA3 (Pavlides, Ogawa, Kimura, & McEwen, 1996). Corticosterone activated GRs in the CA3 may also play a part in enhancing the excitatory communications from the dentate gyrus (Yoshiya et al., 2013). The above suggest a distinct role for GRs in CA1 and CA3, specifically in their ability to influence synaptic dynamics. There is some evidence for an increase in GR in the hippocampus but not in other brain areas such as hypothalamus, amygdala, and pituitary following social housing as compared to isolated housing (Meaney et al., 1985). It is possible that the combined effects of social and physical enrichment in this study had similar effects on hippocampal GRs and act to help regulate inhibitory feedback.

In this study, there was significantly more GR staining in the amygdala in the WKY strain. GRs of the amygdala may be implicated in the hyperactivity of the HPA axis by signalling the paraventricular nucleus of the hypothalamus and in turn increase CRH secretion (Hyman, 2009). In the same subset of animals, circulating corticosteroids were lowest in the WKY animals housed in IH while highest in those in EE (Mileva, Rooke, Ismail, & Bielajew, 2017).

Moreover, the WKY females had significantly more circulating corticosterone after a 15 minute forced swim stressor than their Wistar counterparts. In human research, a recent study characterizing the distribution of GRs in the human amygdala found that patients with major depression had significantly more GR-containing astrocytes and GR protein level in the amygdala than either bipolar patients or control subjects (Wang et al., 2014). This study may suggest that it is not the number of GRs in the amygdala but rather their altered function that influences the development of depression. Conversely, other studies observe decreases in GR immunoreactivity or mRNA in the amygdala after stress (Arnett et al., 2015; Han, Ding, & Shi, 2014) or in suicide victims (Pérez-Ortiz, García-Gutiérrez, Navarrete, Giner, & Manzanares, 2013). It is unclear why some studies show an increase in GR in the amygdala while others show the opposite. Within the context of these discrepant findings, it is difficult to interpret the amygdala data in this study with certainty. In any case, changes in the amygdala following environmental manipulation are indicative of an effect of environment on the WKY females. Future studies will be necessary to replicate these findings and parse out their direction.

The neuroinflammatory theory of depression has been gaining traction throughout the past two decades which prompted us to examine the changes caused by environmental manipulation on microglia. The data in this study were inconclusive regarding the changes in staining intensity or area of the microglial biomarker Iba1. One of the only studies on microglia in the WKY rat found that microglial expression is attenuated after stress (Sherwin, Gormley, McGuinness, & Harkin, 2014). Notably, the authors did not specify whether female or male animals were used. Another very recent study examining the number, distribution, and morphology of microglia in brainstem nuclei has reported that microglial morphology and percent area covered do not differ significantly among the three strains they examined -

Sprague-Dawley, Wistar-Kyoto, and spontaneously hypertensive rats (Kapoor, Bhandare, Mohammed, Farnham, & Pilowsky, 2016). While the insignificant findings in our study might suggest little effect of environmental manipulation, we have shown, in the same animals, a significant decrease in TNF- α serum concentration following enriched housing (in press). This indicates that it may not be the microglial number but rather that their function in the brain that is altered after environmental manipulation.

Some of the limitations of this study included the small sample size. Future studies could expand on the promising data presented in this paper by employing more animals and potentially both female and male subjects. Coupling pharmaceutical or nutraceutical intervention with EE would also be an interesting course of study.

In conclusion, the data in this paper show that there is an increase in astroglia after enrichment as compared to either standard or isolated housing. We also report an increase in GR immunoreactivity in enriched WKY animals in the amygdala. Taken together, the immediate environment does have an effect on astroglia and glucocorticoid immunoreactivity, hinting that behavioural and physical intervention may be important in the treatment of depression and anxiety.

4.4. Experimental procedure

4.4.1 Animals and environmental placement

A total of 36 Wistar Kyoto (WKY) and Wistar female rats were obtained from Charles River Laboratories (Quebec, Canada). Figure 4.4 shows the timeline of this experiment. The animals arrived at post-natal day (PND) 28 and were left to acclimate in the facility for four days. Following this, they were handled for five minutes a day for 20 days. Baseline behavioural

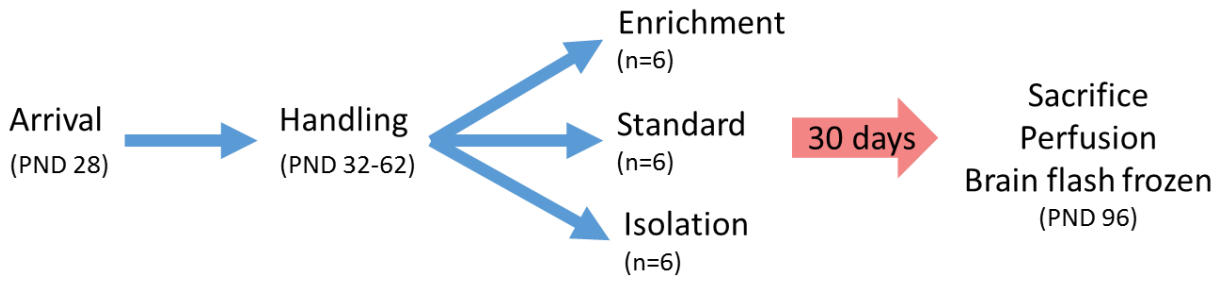

Figure 4.4. Timeline of experiment. Animals arrived at the lab on PND 28, and were handled for 20 days before being randomly assigned to one of three environments for 30 days. They were then sacrificed and immunological analyses conducted.

tests of anxiety and depression (elevated plus maze, forced swim test, and sucrose preference) described in Mileva et al (2015) were carried out prior to environmental manipulation. Six animals from each strain were randomly assigned to one of three environmental conditions for 30 days : 1) Environmental enrichment (EE) - six animals in a multistory cage with toys, chain bridges, Nestlets®, wooden chewing blocks, multicoloured Plexiglas® houses, cardboard tubes, and unlimited access to a running wheel; 2) Standard housing (SH), - 3 rats in one guinea pig cage, with dimensions 50cm x 38cm x 20cm, 3) Isolated housing (IH) - 1 rat per small cage, dimensions 45cm x 22cm x 20cm During PND (85) and PND (87), animals were again subjected to the elevated plus test and forced swim test as described in Mileva et al (2015). Animals were sacrificed at PND 96 by myocardial perfusion with 4% paraformaldehyde. Food and water was provided ad libitum, and the room temperature was kept at 21 ±1 ^{o}C with a humidity of ~41%. Light and dark cycle was regulated at 12hrs of light and 12hrs of dark. Brains were flash frozen and stored at -80^{o}C. A Leica Cryostat was used to obtain 16 µm brain sections which were then mounted on polarized Superfrost slides (Fisher Scientific, Canada).

4.4.2 Immunohistochemistry

The slides with the regions of interest were located and blocked for 30 minutes using a phosphate buffered saline (PBS), Triton (0.002%) and bovine serum albumin (BSA) solution. The sections were incubated at 4^{o}C with monoclonal mouse GFAP (1:400, ab10062, Abcam Inc.) and polyclonal rabbit GR (1:200, SC-8992 Santa Cruz Biotechnology) for 24 hours. The sections were then rinsed in PBS, and incubated with the secondary antibodies Alexa 488-conjugated donkey anti-mouse IgG (1:500, Invitrogen Canada Inc.) and Alexa 594-conjugated donkey anti-rabbit IgG (1:500, Invitrogen Canada Inc.) at 37.5^{o}C for 35 minutes. Following secondary antibody incubation, sections were stained with Hoechst (1:20000 Hoechst 33342,

Invitrogen Canada Inc.) for 10 minutes at room temperature and subsequently rinsed and mounted. In addition, the slides containing the areas of interest were later stained for 24 hours at 4^{o}C for microglia using rabbit anti Iba1 (1:1000, Wako, 019-19741). The rinsed sections were then incubated for 35 minutes at 37.5^{o}C with Alexa 594-conjugated donkey anti-rabbit IgG (1:500, Invitrogen Canada Inc.) secondary antibody. Finally, the sections were stained with Hoechst (1:20000 Hoechst 33342, Invitrogen Canada Inc.) for 10 minutes, rinsed, and mounted.

Labelling was examined using an Olympus DX51 microscope (Center Valley, PA, USA) and Progress Pro 2.7.6 software under 200 x magnifications. Images were analyzed using Image J software (Image J, National Institutes of Health, USA). Optical densities in the form of mean grey values were obtained using the Otsu thresholding algorithm. From there, corrected total cell fluorescence (CTCF) was calculated using the equation: CTCF= Integrated density – (Area of selected cells x Mean fluorescence of background readings). Percent area was also calculated using area fraction x 100. Both staining intensity measured by CTCF and area of staining measured by percent area are important to consider and that is why both are examined in this paper.

4.4.3 Statistical analysis:

Statistical analyses were performed using IBM SPSS V 20 software. The 2x3 independent ANOVAs were performed on the factors strain (2 levels- Wistar and WKY) and environment (3 levels- EE, SH, IH). Bonferroni correction was applied to all pairwise and planned comparisons.

Acknowledgements: We would like to thank the Natural Sciences and Engineering Research Council of Canada grant to CB for its support of this project.

5. General Discussion

The three studies that comprise this thesis were designed to examine the behavioural and physiological effects of environmental manipulation on an animal model of depression. The manipulations included both enrichment and impoverishment to investigate their consequences on behaviour, brain chemistry, and immune function. Data from these studies suggest that the environment does affect behaviour by decreasing anhedonia. In addition, the proinflammatory cytokine TNF-α is significantly lower following EE as compared to IH or SH. Finally, a positive effect of enrichment on astroglia and glucocorticoid receptors was found, hinting that it may help regulate the HPA axis.

Though many studies have examined the effects of various antidepressants on the WKY strain (Lahmame & Armario, 1996; Lahmame et al., 1997; Tizabi et al., 2012; Will et al., 2003), few have addressed interventions such as changes to the social and physical components of the environment. In human work, social and physical changes in the form of social support and physical exercise are often enough to alleviate mild to moderate depression (Carek et al., 2011; Jané-Llopis et al., 2003); however little is known whether these interventions can also help clinically depressed individuals. The literature on EE is very promising, including its ability to decrease anxiety- and depressive-like behaviour as interpreted from EPM and FST results respectively (Simpson & Kelly, 2011), increase neurogenesis (Olson et al., 2006; Veena, Srikumar, Mahati, et al., 2009), and immunity (Benaroya-Milshtein et al., 2004; Marashi et al., 2003). The question of whether EE alone can be used as an intervention in clinical models of depression has not been studied and was the central topic for this thesis.

5.1 Summary of findings

The first paper in this thesis analysed the effects of four weeks of EE, SH, and IH on behavioural outcomes (Mileva & Bielajew, 2015). Many behavioural differences between strains

became apparent throughout this study. Activity in the FST and EPM was significantly lower in female WKY than in control animals, with WKY animals remaining immobile for almost the entire duration of the FST. Although not significant, the decrease in post-environment open arm duration and frequency and increase in closed arm duration in both strains suggests a practice effect. However, as discussed in paper one, other studies have shown that an interval of 28 days is sufficient to extinguish previous memory of the test (Schneider, Ho, Spanagel, & Pawlak, 2011). Another explanation may be that the EPM is not an appropriate test of anxiety for female animals. The possibility that currently employed 'gold standard' behavioural tests may be catering to male animals will be discussed in more detail below. One of the major findings in this article was that female WKY and Wistar rats had significantly higher sucrose preference after exposure to EE than those in IH. A key physiological difference reported in this paper is that WKY animals gain weight at a significantly slower rate than Wistar animals and have opposite weight trends post-environment. This could be due to the naturally slower rate of weight gain and weight at adulthood in WKY rats.

The second paper in this thesis addressed the immune response in the same animals as in the first study following four weeks in each environmental condition (Mileva et al, 2016). To our knowledge, this is the first time the immune cytokine concentration of female WKY rats has been quantified with or without environmental intervention. Regardless of environmental condition, female WKY animals had significantly higher corticosterone levels than their Wistar counterparts only at the post-stress time-point. The discovery that WKY females had significantly lower levels of the proinflammatory cytokine IL-1β as compared to Wistar animals suggests a latent dysregulation either in the production of IL-1β or of the relationship between the HPA axis and the immune system. Though effects of environment were scarce in the above

analysis, the variability between strains highlighted the difference between the animal model of depression exploited in our study and animals in the control group. These results reinforce previous literature on the use of the WKY strain as a putative model of depression.

The third paper in this series once again examined the same subset of animals and the effects of environment on key brain structures implicated in depression and anxiety (Mileva et al 2016). These included the CA1 and CA3 areas of the hippocampus and the amygdala. Female WKY and Wistar rats both showed significantly more GFAP staining after EE as compared to either of the other environments. A significant increase in GR after EE was found in the Wistar and WKY CA3. Finally, WKY animals in EE had the highest levels of GR receptors in the amygdala only. Whereas GRs in the hippocampus are necessary for inhibitory feedback of the HPA axis, the function of GRs in the amygdala is less well known. In sum, the studies above describe the three pronged approach of this thesis in investigating the effects of environmental manipulation on behaviour, the HPA axis, and the immune response.

5.2 The relationship between the HPA axis and immune response can affect psychopathology

Circulating glucocorticoid hormones released by the HPA axis and its corollaries can profoundly affect the immune response. Typically, cortisol, the end product of the HPA axis in response to stress, has an inhibitory effect on the immune system, such that as circulating cortisol increases, the immune response is dampened in order to allow for stress-induced physiological changes to occur and to stop all non-essential processes (for review see Miller et al., 2009). However, chronic stress decreases the inhibitory effect of cortisol on the immune system allowing for an increase in macrophage-mediated release of proinflammatory cytokines which in turn access the brain and promote excitotoxicity, a decrease in monoamines and trophic factors. These have strong implications for psychopathology. For example, many studies have shown that

increases in circulating pro-inflammatory cytokines are correlated with depression (Liu et al., 2012; Song et al., 2009; Sutcigil et al., 2007; Tuglu et al., 2003). Similarly, chronic stress and an increase in circulating cortisol have been found in patients with depression (Carroll et al., 2012; Checkley, 1996; Gillespie & Nemeroff, 2005). In fact, it may be that the increase in circulating cortisol has no effect on depression but rather that through chronic stress the HPA axis is dysregulated and can no longer act to inhibit and in turn suppress the downstream effects of proinflammatory cytokines on the brain. As a whole, the evidence points to an innate relationship between the HPA axis and immune system and the dysregulation of either can play a pivotal role in the development of psychopathology, and in particular depression.

The studies in this thesis suggest that environmental manipulation can affect components of both the HPA axis and the immune response. The changes observed in study three with regard to GR concentration in the brain and circulating corticosterone are indicative of regulatory differences in the HPA axis whereas changes in circulating immune cytokine and microglial staining in the brain are indicative of functional changes in the immune system. The increase of GRs in the CA3 after EE suggests that forced physical and social intervention in the form of enrichment can increase the inhibitory effects of the hippocampus on the HPA axis. How to interpret the increase of GRs in the amygdala is less clear with findings in both directions common in depression research (Arnett et al., 2015; Wang et al., 2014). More research on the function of GRs in the amygdala will be necessary to understand how they fit into the neuroinflammatory theory of depression.

5.3 Male-female differences in physiology and response to environment

When discussing psychopathology, it is important to consider the sex differences inherent in the prevalence of depression as well as the response to environment and stress. It is well

known that the prevalence of depression is roughly twice as high in women as it is in men (Whiteford et al., 2013). Whereas fluctuating hormones have been implicated as a possible answer to the differences in the pervasiveness of depression between men and women (for review see Albert, 2015), little is known about how the immediate environment influences this difference. As enrichment and impoverishment can affect many aspects of functioning, it is important to determine whether males and females respond differently to these circumstances and whether this could precipitate the development of depression.

A review of housing situation and self-reported heath status indicates that women are more vulnerable to socioeconomic dimensions of housing than men who only report negative health status through over-crowding (Dunn, Walker, Graham, & Weiss, 2004). Another example of sex differences in response to the environment can be found in the obesity literature. These studies have found that fewer years of education and lower SES increase risk of obesity in both men and women, but that only female obesity is correlated with lower occupational status (Wardle, Waller, & Jarvis, 2002). Moreover low SES is tied to higher morbidity of myocardial infarction for women than for men (Vogels, Lagro-Janssen, & van Weel, 1999). It is clear that low SES can affect individuals in a sex-dependent fashion with females tending to be at higher risk of obesity, to report lower health status, and to have increased risk of mortality. As a whole, this literature provides evidence that males and females respond to their immediate environment in potentially different ways. In the above examples, low SES neighbourhoods are comparable to impoverished housing in animal studies as the infrastructure for optimal development is often lacking (Hood, 2005).

Studies of enrichment in male and female rats have reported that enrichment, specifically the social aspect, improves performance in the open field test in both sexes (Elliott & Grunberg,

2005), a little more in males than in females generally. In contrast, in male mice, enrichment increases aggression and can be anxiogenic (Haemisch, Voss, & Gärtner, 1994; Marashi et al., 2003). Thus, there is great variability within the Muridae family with respect to outcomes of enrichment and appears highly sex-dependent.

Variability between males and females has also been shown with respect to stress and immunity. For example, in a sample of 45 healthy subjects exposed to the Trier Social Stress test, researchers found that despite similar salivary cortisol concentrations for both men and women, women had a significantly lower level of glucocorticoid sensitivity as compared to men 1 hour after stress (Rohleder, Schommer, Hellhammer, Engel, & Kirschbaum, 2001). In the same study, women also showed a significant increase in cytokine production. In other words, after a psychosocial stressor, HPA axis feedback was decreased and immune activity was increased in women but not in men. Notably, all women were in the luteal phase of their menstrual cycle in the study providing strength to the notion that cycle phase needs to be accounted for in measures of HPA axis and immune response.

Modest changes to circulating cytokines are found in the plasma throughout the murine estrous cycle (Orsi, Gopichandran, Ekbote, & Walker, 2006; Orsi, Ekbote, Walker, & Gopichandran, 2007) and may affect immunity differentially between male and female animals. For example, one study reported an increase in immune responsiveness in female mice as compared to male mice following haemorrhagic shock and resuscitation (Wichmann, Zellweger, DeMaso, Ayala, & Chaudry, 1996). Moreover, females in proestrus showed a decrease in plasma corticosterone following shock as compared to their male counterparts who showed the opposite pattern. Another report from the same laboratory once again found that female mice in proestrus were better able to tolerate septic shock than that observed in male animals (Zellweger et al.,

1997). Taken together, it is unlikely that males and females respond in the same fashion to stress or immune challenges and this needs to be accounted for with appropriate methodological consideration.

5.3.1 Are our current behavioural tests appropriate for female animals?

Is it possible that the currently held 'gold-standard' behavioural tests such as the FST and EPM are not measuring the same thing in males and females? For instance, it has been suggested that the FST tests learning and coping behaviours and not despair and depression (West, 1990). The rationale behind this theory is that after the first test, animals have realized that they cannot escape and then incorporate energy conserving behaviours, or what is typically interpreted as immobility. This immobility may not reflect a 'depressed' state but rather an adaptive measure employed by the animals to survive the immediate environment. Previous studies from our laboratory also suggest sex-dependent effects of stress on behavioural outcomes in the FST (Bielajew et al., 2003). For this reason, a multidimensional approach is necessary when studying depression which incorporates not only physiological measures, but also multiple behavioural tests. Therefore, this thesis employed the FST and sucrose preference tests as behavioural measures of depression and the EPM as a measure of anxiety. In addition to the typically quantified measures in the EPM including the frequency and duration of open and closed arm visits, paper one also analysed the overall activity of each animal. This added dimension was important, specifically as a way to analyse the withdrawal and isolation common in the WKY animals.

To our knowledge, the question of whether the currently available behavioural tests are appropriate for female animals has been addressed in very few studies. For instance, factor

analysis of the EPM reported that only male behaviour in the EPM is anxiety-related while female behaviour in the EPM is more indicative of overall activity levels (Fernandes et al., 1999; Wall & Messier, 2000, 2001). In light of this finding, it puts into question whether other currently employed behavioural tests are equally useful for studies of male and female animals. This reinforces the idea that, when possible, the incorporation of as many different behavioural tests as possible to measure different dimensions of a behaviour is advisable.

5.4 Is enrichment really enriching?

As extrapolation from pre-clinical to clinical research is common, it is important to question whether EE in preclinical research can translate to the same in humans. For instance, it is rare that human individuals are devoid of all physical and social interaction as is common in some 'standard' animal housing. Therefore, is the level of enrichment equivalent to that of the natural environment of the animal? In other words, animals in the typical 'standard' housing condition may actually be housed in a form of physical or social impoverishment due to the lack of novelty in their environment. In fact, studies have found precisely that animals in standard housing often display symptoms similar to those of animals in impoverished environments (Baker & Bielajew, 2007; Würbel, 2001). Following these findings, there has been a general push towards stating animal-welfare in the methods section of publications which should include some form of enrichment for all animals (Würbel, 2007). Often, animal housing is not discussed in the methodology or is stated briefly with little consideration for its effects on behaviour, physiology, disease or health related measures (Laviola et al., 2008). Thus, most studies of animal models of neurodegenerative disease and psychopathology should consider utilizing EE as their standard housing condition unless specifically studying the effects of impoverishment.

In this thesis, EE was meant to represent a form of forced social and physical intervention as animals were housed in a large multistory cage with five other conspecifics and were therefore unable to isolate themselves. In addition, they were subjected to changes in the objects in their environment every two to three days again adding an element of forced novelty. As depression tends to manifest itself with withdrawal and isolation, it was hypothesized that the EE utilized in these studies could ameliorate these tendencies, especially given the promising results of EE in previous literature (for review see Simpson and Kelly, 2011). As reported in Mileva et al 2015, sucrose preference is significantly higher after enrichment as compared to isolated housing in both Wistar and WKY strains. This suggests that enrichment does have the potential to decrease anhedonia in both strains. Weight was also affected by EE in this study with Wistar animal in EE showing a lower weight than those in IH. Due to the exercise wheel available in EE, a lower weight was expected; however it is interesting that the WKY animals had the highest weight gain in EE. This could be partially due to the increased sucrose intake in EE; however this trend would also be expected in Wistar animals and was not found. Future studies may find clues to shed light on these puzzling results.

Effects on the physiology of animals across housing condition was also evident in papers two and three of this thesis. Housing condition can affect corticosterone and TNF-α concentration. The concentration of astrocytes increased greatly in the CA1 of Wistar animals in EE, while GR receptors were the elevated in both WKY and Wistar animals. The possible reasons for these increases were discussed in more detail above, but it is important to reiterate that changes in the environment can affect both behaviour and biomarkers of the HPA axis and the immune response in the studies presented in this thesis.

5.5: Limitations

There were some limitations in this thesis. In this series of studies, for practical reasons, two cohorts of female animals were used. While all attempts were made to keep both cohorts equal, it is possible that conditions beyond experimenter control were experienced by one of the cohorts and resulted in potential behavioural or physiological differences. This was evident in paper one as Wistar animals exposed to EE in cohort one had significantly different behaviours in the FST than the same group in cohort two. In the same vein, a larger sample size, specifically in paper three would have been beneficial.

Utilizing both male and female animals in the study of environmental intervention could elucidate any potential hormone-mediated effects of environment on depression. Male animals could experience enrichment in a different manner to female animals and this could affect behavioural and physiological outcomes. Furthermore, hormonal fluctuations would be negligible in male animals and therefore any hormone-environment interactions that female animals experience during the estrus cycle would be highlighted. Another way to examine this relationship would be to use ovariectomized female rats and examine their responses to the environment in contrast to control animals.

5.6 Future directions

A detailed comparison of male and female behavioural, physiological, and immune responses to enrichment would be ideal for future studies. Examining the effects of environmental enrichment or impoverishment on a large sample of both male and female animals would verify the findings of this study and contrast them to that of male animals. A thorough examination of depressive- and anxiety-like behaviour using multiple behavioural tests such as the open field test and social interaction test in addition to the EPM and FST would provide clues

to the change in social interactions after enrichment or impoverishment. In the brain, it would be interesting to examine the differences in neurogenesis and trophic factors in the hippocampus, amygdala, and GRs in the anterior cingulate cortex. An alternate direction may involve keeping the animals in an enriched environment for a longer period of time which may be more likely to produce behavioural outcomes. Finally, studies of the combined effects of environmental manipulation and pharmaceutical or nutraceutical intervention could reveal an additive effect and would be a valuable addition to the current literature.

5.7 Conclusions

The work highlighted in this thesis represents a promising avenue of research in which to explore the relationship of depression and the environment, specifically targeting women who show the highest rates of prevalence. The results of the studies in this thesis provide evidence for the usefulness of EE as an intervention, particularly in animal models of depression. Moreover, this thesis expands on the EE literature and supports its use to ameliorate the effects of impoverishment. Extrapolating to clinical intervention, it may be beneficial for clinicians to address the immediate environment of their patients in conjunction with pharmaceutical treatment. Encouraging patients to seek social support and increase their level of physical exercise may help to regulate the HPA axis and immune system and improve overall function and alleviate depressive symptoms.

6. References:

Adler, N. E., Boyce, T., Chesney, M. A., Cohen, S., Folkman, S., Kahn, R. L., & Syme, S. L. (1994). Socioeconomic status and health. The challenge of the gradient. *The American Psychologist*, *49*(1), 15–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8122813

Adriani, W., Caprioli, A., Granstrem, O., Carli, M., & Laviola, G. (2003). The spontaneously hypertensive-rat as an animal model of ADHD: evidence for impulsive and non-impulsive subpopulations. *Neuroscience and Biobehavioral Reviews*, *27*(7), 639–651.

Albert, P. R. (2015). Why is depression more prevalent in women? *Journal of Psychiatry & Neuroscience : JPN*, *40*(4), 219–21. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/26107348

Almeida, S. S., Garcia, R. A., & de Oliveira, L. M. (1993). Effects of early protein malnutrition and repeated testing upon locomotor and exploratory behaviors in the elevated plus-maze. *Physiology & Behavior*, *54*(4), 749–752.

Altshuler, L. L., Abulseoud, O. A., Foland-Ross, L., Bartzokis, G., Chang, S., Mintz, J., … Vinters, H. V. (2010). Amygdala astrocyte reduction in subjects with major depressive disorder but not bipolar disorder. *Bipolar Disorders*, *12*(5), 541–549. http://doi.org/10.1111/j.1399-5618.2010.00838.x

Anacker, C., Zunszain, P. A., Carvalho, L. A., & Pariante, C. M. (2011). The glucocorticoid receptor: pivot of depression and of antidepressant treatment? *Psychoneuroendocrinology*, *36*(3), 415–25. http://doi.org/10.1016/j.psyneuen.2010.03.007

Anacker, C., Zunszain, P. A., Cattaneo, A., Carvalho, L. A., Garabedian, M. J., Thuret, S., … Pariante, C. M. (2011). Antidepressants increase human hippocampal neurogenesis by activating the glucocorticoid receptor. *Molecular Psychiatry*, *16*(7), 738–50. http://doi.org/10.1038/mp.2011.26

Andrews, G., Brugha, T., Thase, M. E., Duffy, F. F., Rucci, P., & Slade, T. (2007). Dimensionality and the category of major depressive episode. *International Journal of Methods in Psychiatric Research Int. J. Methods Psychiatr. Res*, *16*(S1), 41–51. http://doi.org/10.1002/mpr.216

Arborelius, L., Owens, M. J., Plotsky, P. M., & Nemeroff, C. B. (1999). The role of corticotropin-releasing factor in depression and anxiety disorders. *The Journal of Endocrinology*, *160*(1), 1–12. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9854171

Armario, A., Gavaldà, A., & Martí, J. (1995). Comparison of the behavioural and endocrine response to forced swimming stress in five inbred strains of rats. *Psychoneuroendocrinology*, *20*(8), 879–890. http://doi.org/10.1016/0306-4530(95)00018-6

Armstrong, L., Jordan, N., & Millar, A. (1996). Interleukin 10 (IL-10) regulation of tumour necrosis factor alpha (TNF-alpha) from human alveolar macrophages and peripheral blood monocytes. *Thorax*, *51*(2), 143–9. Retrieved from

http://www.ncbi.nlm.nih.gov/pubmed/8711645

Arnett, M. G., Pan, M. S., Doak, W., Cyr, P. E. P., Muglia, L. M., & Muglia, L. J. (2015). The role of glucocorticoid receptor-dependent activity in the amygdala central nucleus and reversibility of early-life stress programmed behavior. *Translational Psychiatry*, *5*(4), e542. http://doi.org/10.1038/tp.2015.35

Baek, S. S., Jun, T. W., Kim, K. J., Shin, M. S., Kang, S. Y., & Kim, C. J. (2012). Effects of postnatal treadmill exercise on apoptotic neuronal cell death and cell proliferation of maternal-separated rat pups. *Brain & Development*, *34*(1), 45–56. http://doi.org/10.1016/j.braindev.2011.01.011

Baker, S., & Bielajew, C. (2007). Influence of housing on the consequences of chronic mild stress in female rats. *Stress (Amsterdam, Netherlands)*, *10*(August), 283–293. http://doi.org/10.1080/10253890701265362

Baker, S., Chebli, M., Rees, S., LeMarec, N., Godbout, R., & Bielajew, C. (2008). Effects of gestational stress: 1. Evaluation of maternal and juvenile offspring behavior. *Brain Research*, *1213*, 98–110. http://doi.org/10.1016/j.brainres.2008.03.035

Baker, S. L., Kentner, A. C., Konkle, A. T. M., Santa-Maria Barbagallo, L., & Bielajew, C. (2006). Behavioral and physiological effects of chronic mild stress in female rats. *Physiology & Behavior*, *87*(2), 314–22. http://doi.org/10.1016/j.physbeh.2005.10.019

Baker, S., Rees, S., Chebli, M., LeMarec, N., Godbout, R., Huta, V., & Bielajew, C. (2009). Effects of gestational stress: 2. Evaluation of male and female adult offspring. *Brain Research*, *1302*, 194–204. http://doi.org/10.1016/j.brainres.2009.09.023

Bakos, J., Hlavacova, N., Rajman, M., Ondicova, K., Koros, C., Kitraki, E., … Jezova, D. (2009). Enriched environment influences hormonal status and hippocampal brain derived neurotrophic factor in a sex dependent manner. *Neuroscience*, *164*(2), 788–97. http://doi.org/10.1016/j.neuroscience.2009.08.054

Becker, C., Zeau, B., Rivat, C., Blugeot, A., Hamon, M., & Benoliel, J.-J. (2008). Repeated social defeat-induced depression-like behavioral and biological alterations in rats: involvement of cholecystokinin. *Molecular Psychiatry*, *13*(12), 1079–1092. http://doi.org/10.1038/sj.mp.4002097

Beery, A. K., & Zucker, I. (2011). Sex bias in neuroscience and biomedical research. *Neuroscience and Biobehavioral Reviews*, *35*(3), 565–72. http://doi.org/10.1016/j.neubiorev.2010.07.002

Benaroya-Milshtein, N., Hollander, N., Apter, A., Kukulansky, T., Raz, N., Wilf, A., … Pick, C. G. (2004). Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity. *The European Journal of Neuroscience*, *20*(5), 1341–7. http://doi.org/10.1111/j.1460-9568.2004.03587.x

Benarroch, E. E. (2005). Neuron-Astrocyte Interactions: Partnership for Normal Function and Disease in the Central Nervous System. *Mayo Clinic Proceedings*, *80*(10), 1326–1338.

http://doi.org/10.4065/80.10.1326

Bennett, E. L., Rosenzweig, M. R., & Diamond, M. C. (1969). Rat brain: effects of environmental enrichment on wet and dry weights. *Science (New York, N.Y.)*, *163*(3869), 825–826.

Berk, M., Williams, L. J., Jacka, F. N., O'Neil, A., Pasco, J. A., Moylan, S., … Berk, M. (2013). So depression is an inflammatory disease, but where does the inflammation come from? *BMC Medicine*, *11*(1), 200. http://doi.org/10.1186/1741-7015-11-200

Berthold-Losleben, M., & Himmerich, H. (2008). The TNF-alpha system: functional aspects in depression, narcolepsy and psychopharmacology. *Current Neuropharmacology*, *6*(3), 193–202. http://doi.org/10.2174/157015908785777238

Biederman, J., Faraone, S. V, Spencer, T., Wilens, T., Norman, D., Lapey, K. A., … Doyle, A. (1993). Patterns of psychiatric comorbidity, cognition, and psychosocial functioning in adults with attention deficit hyperactivity disorder. *The American Journal of Psychiatry*, *150*(12), 1792–1798.

Bielajew, C., Konkle, A. T. M., Kentner, A. C., Baker, S. L., Stewart, A., Hutchins, A. A., … Fouriezos, G. (2003). Strain and gender specific effects in the forced swim test: effects of previous stress exposure. *Stress (Amsterdam, Netherlands)*, *6*(4), 269–80. http://doi.org/10.1080/10253890310001602829

Bonicatto, S. C., Dew, M. A., Zaratiegui, R., Lorenzo, L., & Pecina, P. (2001). Adult outpatients with depression: worse quality of life than in other chronic medical diseases in Argentina. *Social Science & Medicine (1982)*, *52*(6), 911–919.

Braw, Y., Malkesman, O., Merenlender, A., Bercovich, A., Dagan, M., Overstreet, D. H., & Weller, A. (2008). Withdrawal emotional-regulation in infant rats from genetic animal models of depression. *Behavioural Brain Research*, *193*(1), 94–100. http://doi.org/10.1016/j.bbr.2008.04.026

Braw, Y., Malkesman, O., Merlender, A., Bercovich, A., Dagan, M., Maayan, R., … Weller, A. (2006). Stress hormones and emotion-regulation in two genetic animal models of depression. *Psychoneuroendocrinology*, *31*(9), 1105–1116. http://doi.org/10.1016/j.psyneuen.2006.07.003

Brenes, J. C., & Fornaguera, J. (2008). Effects of environmental enrichment and social isolation on sucrose consumption and preference: associations with depressive-like behavior and ventral striatum dopamine. *Neuroscience Letters*, *436*(2), 278–282. http://doi.org/10.1016/j.neulet.2008.03.045

Brenes, J. C., Padilla, M., & Fornaguera, J. (2009). A detailed analysis of open-field habituation and behavioral and neurochemical antidepressant-like effects in postweaning enriched rats. *Behavioural Brain Research*, *197*(1), 125–137. http://doi.org/10.1016/j.bbr.2008.08.014

Brenes, J. C., Rodriguez, O., & Fornaguera, J. (2008). Differential effect of environment enrichment and social isolation on depressive-like behavior, spontaneous activity and

serotonin and norepinephrine concentration in prefrontal cortex and ventral striatum. *Pharmacology, Biochemistry, and Behavior*, *89*(1), 85–93. http://doi.org/10.1016/j.pbb.2007.11.004

Brenes Sáenz, J. C., Villagra, O. R., & Fornaguera Trías, J. (2006). Factor analysis of Forced Swimming test, Sucrose Preference test and Open Field test on enriched, social and isolated reared rats. *Behavioural Brain Research*, *169*(1), 57–65. http://doi.org/10.1016/j.bbr.2005.12.001

Bronisch, T., & Wittchen, H. U. (1994). Suicidal ideation and suicide attempts: comorbidity with depression, anxiety disorders, and substance abuse disorder. *European Archives of Psychiatry and Clinical Neuroscience*, *244*(2), 93–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7948060

Brown, G. W. (1998). Genetic and population perspectives on life events and depression. *Social Psychiatry and Psychiatric Epidemiology*, *33*(8), 363–372.

Brown, G. W., Andrews, B., Harris, T., Adler, Z., & Bridge, L. (1986). Social support, self-esteem and depression. *Psychological Medicine*, *16*(4), 813–831.

Carek, P. J., Laibstain, S. E., & Carek, S. M. (2011). Exercise for the treatment of depression and anxiety. *International Journal of Psychiatry in Medicine*, *41*(1), 15–28.

Carlson, M., & Earls, F. (1997). Psychological and neuroendocrinological sequelae of early social deprivation in institutionalized children in Romania. *Annals of the New York Academy of Sciences*, *807*, 419–28. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9071367

Carlstead, K., & Shepherdson, D. (2000). Alleviating stress in zoo animals with environmental enrichment. In *The biology of animal stress: Basic Principles and Implications for Animal Welfare* (pp. 337–354). Wallingford: CABI. http://doi.org/10.1079/9780851993591.0337

Carobrez, A. P., & Bertoglio, L. J. (2005). Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. *Neuroscience and Biobehavioral Reviews*, *29*(8), 1193–1205. http://doi.org/10.1016/j.neubiorev.2005.04.017

Carroll, B. J., Cassidy, F., Naftolowitz, D., Tatham, N. E., Wilson, W. H., Iranmanesh, A., … Veldhuis, J. D. (2007). Pathophysiology of hypercortisolism in depression. *Acta Psychiatrica Scandinavica*, *115*(SUPPL. 433), 90–103. http://doi.org/10.1111/j.1600-0447.2007.00967.x

Carroll, B. J., Iranmanesh, A., Keenan, D. M., Cassidy, F., Wilson, W. H., & Veldhuis, J. D. (2012). Pathophysiology of hypercortisolism in depression: pituitary and adrenal responses to low glucocorticoid feedback. *Acta Psychiatrica Scandinavica*, *125*(6), 478–91. http://doi.org/10.1111/j.1600-0447.2011.01821.x

Carvalho, L. A., Bergink, V., Sumaski, L., Wijkhuijs, J., Hoogendijk, W. J., Birkenhager, T. K., & Drexhage, H. A. (2014). Inflammatory activation is associated with a reduced glucocorticoid receptor alpha/beta expression ratio in monocytes of inpatients with

melancholic major depressive disorder. *Translational Psychiatry*, *4*(1), e344. http://doi.org/10.1038/tp.2013.118

Caspi, A., Sugden, K., Moffitt, T. E., Taylor, A., Craig, I. W., Harrington, H., … Poulton, R. (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science (New York, N.Y.)*, *301*(5631), 386–389. http://doi.org/10.1126/science.1083968

Charlton, B. G. (2000). The malaise theory of depression: major depressive disorder is sickness behavior and antidepressants are analgesic. *Medical Hypotheses*, *54*(1), 126–30. http://doi.org/10.1054/mehy.1999.0986

Checkley, S. (1996). The neuroendocrinology of depression and chronic stress. *Br Med Bull*, *52*(3), 597–617. http://doi.org/10.3109/09540269609051552

Chryssikopoulos, A. (1997). The relationship between the immune and endocrine systems. *Annals of the New York Academy of Sciences*, *816*, 83–93. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9238258

Cierpial, M. A., Murphy, C. A., & McCarty, R. (1990). Maternal behavior of spontaneously hypertensive and Wistar-Kyoto normotensive rats: effects of reciprocal cross-fostering of litters. *Behavioral and Neural Biology*, *54*(1), 90–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2378609

Cierpial, M. A., Shasby, D. E., & McCarty, R. (1987). Patterns of maternal behavior in the spontaneously hypertensive rat. *Physiology & Behavior*, *39*(5), 633–637.

Clayton, J., & Collins, F. (2014). NIH to balance sex in cell and animal studies. *Nature*, *509*, 282–3.

Cohen, S., Janicki-Deverts, D., Doyle, W. J., Miller, G. E., Frank, E., Rabin, B. S., & Turner, R. B. (2012). Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(16), 5995–9. http://doi.org/10.1073/pnas.1118355109

Collingridge, G. L., Kehl, S. J., & McLennan, H. (1983). Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *The Journal of Physiology*, *334*, 33–46. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6306230

Condren, R. M., O'Neill, A., Ryan, M. C., Barrett, P., & Thakore, J. H. (2002). HPA axis response to a psychological stressor in generalised social phobia. *Psychoneuroendocrinology*, *27*(6), 693–703.

Connolly, K. R., & Thase, M. E. (2011). If at First You Don't Succeed. *Drugs*, *71*(1), 43–64. http://doi.org/10.2165/11587620-000000000-00000

Cuijpers, P. (2011). Prevention of depressive disorders: towards a further reduction of the disease burden of mental disorders. *Early Intervention in Psychiatry*, *5*(3), 179–180. http://doi.org/10.1111/j.1751-7893.2011.00282.x; 10.1111/j.1751-7893.2011.00282.x

Curtin, N. M., Mills, K. H. G., & Connor, T. J. (2009). Psychological stress increases expression of IL-10 and its homolog IL-19 via beta-adrenoceptor activation: reversal by the anxiolytic chlordiazepoxide. *Brain, Behavior, and Immunity*, *23*(3), 371–9. http://doi.org/10.1016/j.bbi.2008.12.010

Cutler, S., & Nolen-Hoeksema, S. (1991). Accounting for sex differences in depression through female victimization: Childhood sexual abuse. *Sex Roles*, *24*(7–8), 425–438. http://doi.org/10.1007/BF00289332

Danese, A., Moffitt, T. E., Pariante, C. M., Ambler, A., Poulton, R., Caspi, A., … M, V. (2008). Elevated Inflammation Levels in Depressed Adults With a History of Childhood Maltreatment. *Archives of General Psychiatry*, *65*(4), 409. http://doi.org/10.1001/archpsyc.65.4.409

Dantzer, R. (2001). Cytokine-induced sickness behavior: mechanisms and implications. *Annals of the New York Academy of Sciences*, *933*, 222–234.

Dantzer, R. (2009). Cytokine, sickness behavior, and depression. *Immunology and Allergy Clinics of North America*, *29*(2), 247–64. http://doi.org/10.1016/j.iac.2009.02.002

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W., & Kelley, K. W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci*, *9*(1), 46–56. Retrieved from http://dx.doi.org/10.1038/nrn2297

De La Garza, R., & Mahoney, J. J. (2004). A distinct neurochemical profile in WKY rats at baseline and in response to acute stress: implications for animal models of anxiety and depression. *Brain Research*, *1021*(2), 209–18. http://doi.org/10.1016/j.brainres.2004.06.052

de Paiva, V. N., Lima, S. N. P., Fernandes, M. M., Soncini, R., Andrade, C. A. F., & Giusti-Paiva, A. (2010). Prostaglandins mediate depressive-like behaviour induced by endotoxin in mice. *Behavioural Brain Research*, *215*(1), 146–151. http://doi.org/10.1016/j.bbr.2010.07.015

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E. K., & Lanctôt, K. L. (2010). A Meta-Analysis of Cytokines in Major Depression. *Biological Psychiatry*, *67*(5), 446–457. http://doi.org/10.1016/j.biopsych.2009.09.033

Dunn, A. J. (2000). Cytokine Activation of the HPA Axis. *Annals of the New York Academy of Sciences*, *917*(1), 608–617. http://doi.org/10.1111/j.1749-6632.2000.tb05426.x

Dunn, A. L., Trivedi, M. H., Kampert, J. B., Clark, C. G., & Chambliss, H. O. (2005). Exercise treatment for depression: Efficacy and dose response. *American Journal of Preventive Medicine*, *28*(1), 1–8. http://doi.org/10.1016/j.amepre.2004.09.003

Dunn, J. R., Walker, J. D., Graham, J., & Weiss, C. B. (2004). Gender differences in the relationship between housing, socioeconomic status, and self-reported health status. *Reviews on Environmental Health*, *19*(3–4), 177–195. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-13144253153&partnerID=tZOtx3y1%5Cnhttp://www.scopus.com/inward/record.url?eid=2-

s2.0-13144253153&partnerID=40&md5=9196db203039ec12dc865b1e5ab2cdd3

During, M. J., Young, D., Lawlor, P. A., Leone, P., & Dragunow, M. (1999). Environmental enrichment inhibits spontaneous apoptosis, prevents seizuresand is neuroprotective. *Nature Medicine*, *5*(4), 448–453. http://doi.org/10.1038/7449

Ehninger, D., & Kempermann, G. (2003). Regional Effects of Wheel Running and Environmental Enrichment on Cell Genesis and Microglia Proliferation in the Adult Murine Neocortex. *Cerebral Cortex*, *13*(8), 845–851. http://doi.org/10.1093/cercor/13.8.845

Elenkov, I. J., & Chrousos, G. P. (1999). Stress Hormones, Th1/Th2 patterns, Pro/Anti-inflammatory Cytokines and Susceptibility to Disease. *Trends in Endocrinology & Metabolism*, *10*(9), 359–368. http://doi.org/10.1016/S1043-2760(99)00188-5

Elliott, B. M., & Grunberg, N. E. (2005). Effects of social and physical enrichment on open field activity differ in male and female Sprague–Dawley rats. *Behavioural Brain Research*, *165*(2), 187–196. http://doi.org/10.1016/j.bbr.2005.06.025

Escorihuela, R. M., Tobena, A., & Fernandez-Teruel, A. (1994). Environmental enrichment reverses the detrimental action of early inconsistent stimulation and increases the beneficial effects of postnatal handling on shuttlebox learning in adult rats. *Behavioural Brain Research*, *61*(2), 169–173.

Evans, G. W., & Kim, P. (2007). Childhood poverty and health: cumulative risk exposure and stress dysregulation. *Psychological Science*, *18*(11), 953–7. http://doi.org/10.1111/j.1467-9280.2007.02008.x

Farovik, A., Dupont, L. M., & Eichenbaum, H. (2010). Distinct roles for dorsal CA3 and CA1 in memory for sequential nonspatial events. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *17*(1), 12–17. http://doi.org/10.1101/lm.1616209

Ferguson, G., & Takane, Y. (1989). *Statistical analysis in psychology and education* (6th ed.). New York: McGraw-Hill.

Ferguson, S. A., & Gray, E. P. (2005). Aging effects on elevated plus maze behavior in spontaneously hypertensive, Wistar-Kyoto and Sprague-Dawley male and female rats. *Physiology & Behavior*, *85*(5), 621–8. http://doi.org/10.1016/j.physbeh.2005.06.009

Fernandes, C., González, M. ., Wilson, C. ., & File, S. . (1999). Factor Analysis Shows That Female Rat Behaviour Is Characterized Primarily by Activity, Male Rats Are Driven by Sex and Anxiety. *Pharmacology Biochemistry and Behavior*, *64*(4), 731–736. http://doi.org/10.1016/S0091-3057(99)00139-2

Ferrari, A. J., Charlson, F. J., Norman, R. E., Patten, S. B., Freedman, G., Murray, C. J. L., … Koretz, D. (2013). Burden of Depressive Disorders by Country, Sex, Age, and Year: Findings from the Global Burden of Disease Study 2010. *PLoS Medicine*, *10*(11), e1001547. http://doi.org/10.1371/journal.pmed.1001547

Field, T., Healy, B., Goldstein, S., Perry, S., Bendell, D., Schanberg, S., … Kuhn, C. (1988). Infants of depressed mothers show "depressed" behavior even with

nondepressed adults. *Child Development*, *59*(6), 1569–79. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3208568

Fox, C., Merali, Z., & Harrison, C. (2006). Therapeutic and protective effect of environmental enrichment against psychogenic and neurogenic stress. *Behavioural Brain Research*, *175*(1), 1–8. http://doi.org/10.1016/j.bbr.2006.08.016

Francis, D. D., Diorio, J., Plotsky, P. M., & Meaney, M. J. (2002). Environmental enrichment reverses the effects of maternal separation on stress reactivity. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(18), 7840–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12223535

Fries, E., Hesse, J., Hellhammer, J., & Hellhammer, D. H. (2005). A new view on hypocortisolism. *Psychoneuroendocrinology*, *30*(10), 1010–1016. http://doi.org/10.1016/j.psyneuen.2005.04.006

Galea, S., Ahern, J., Rudenstine, S., Wallace, Z., & Vlahov, D. (2005). Urban built environment and depression: a multilevel analysis. *Journal of Epidemiology and Community Health*, *59*(10), 822–827. http://doi.org/10.1136/jech.2005.033084

Gillespie, C. F., & Nemeroff, C. B. (2005). Hypercortisolemia and depression. *Psychosomatic Medicine*, *67 Suppl 1*(17), S26–S28. http://doi.org/10.1097/01.psy.0000163456.22154.d2

Gilmore, W., Weiner, L. P., & Correale, J. (1997). Effect of estradiol on cytokine secretion by proteolipid protein-specific T cell clones isolated from multiple sclerosis patients and normal control subjects. *Journal of Immunology (Baltimore, Md. : 1950)*, *158*(1), 446–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8977221

Glover, V., O'Connor, T. G., & O'Donnell, K. (2010). Prenatal stress and the programming of the HPA axis. *Neuroscience and Biobehavioral Reviews*, *35*(1), 17–22. http://doi.org/10.1016/j.neubiorev.2009.11.008

Gola, H., Engler, H., Sommershof, A., Adenauer, H., Kolassa, S., Schedlowski, M., … Rey, A. del. (2013). Posttraumatic stress disorder is associated with an enhanced spontaneous production of pro-inflammatory cytokines by peripheral blood mononuclear cells. *BMC Psychiatry*, *13*(1), 40. http://doi.org/10.1186/1471-244X-13-40

Goodman, S. H., & Gotlib, I. H. (1999). Risk for psychopathology in the children of depressed mothers: a developmental model for understanding mechanisms of transmission. *Psychological Review*, *106*(3), 458–490.

Gotlib, I. H., Joormann, J., Minor, K. L., & Hallmayer, J. (2008). HPA axis reactivity: a mechanism underlying the associations among 5-HTTLPR, stress, and depression. *Biological Psychiatry*, *63*(9), 847–851. http://doi.org/10.1016/j.biopsych.2007.10.008

Greer, T. L., & Trivedi, M. H. (2009). Exercise in the treatment of depression. *Current Psychiatry Reports*, *11*(6), 466–472.

Grippo, A. J., Cushing, B. S., & Carter, C. S. (2007). Depression-like behavior and stressor-induced neuroendocrine activation in female prairie voles exposed to chronic social

isolation. *Psychosomatic Medicine*, *69*(2), 149–157. http://doi.org/10.1097/PSY.0b013e31802f054b

Grippo, A. J., Gerena, D., Huang, J., Kumar, N., Shah, M., Ughreja, R., & Carter, C. S. (2007). Social isolation induces behavioral and neuroendocrine disturbances relevant to depression in female and male prairie voles. *Psychoneuroendocrinology*, *32*(8–10), 966–980. http://doi.org/10.1016/j.psyneuen.2007.07.004

Gullaiya, S., Nagar, A., Dubey, V., Singh, V., Kumar, A., Tiwari, P., & Agrawal, S. S. (2013). Modulation of disease related immune events by demethoxycurcumin against autoimmune arthritis in rats. *Biomedicine & Aging Pathology*, *3*(1), 7–13. http://doi.org/10.1016/j.biomag.2013.01.005

Gunnar, M. R., Morison, S. J., Chisholm, K., & Schuder, M. (2001). Salivary cortisol levels in children adopted from romanian orphanages. *Development and Psychopathology*, *13*(3), 611–628.

Gunnar, M. R., & Vazquez, D. M. (2001). Low cortisol and a flattening of expected daytime rhythm: potential indices of risk in human development. *Development and Psychopathology*, *13*(3), 515–538.

Haemisch, A., Voss, T., & Gärtner, K. (1994). Effects of environmental enrichment on aggressive behavior, dominance hierarchies, and endocrine states in male DBA/2J mice. *Physiology & Behavior*, *56*(5), 1041–1048. http://doi.org/10.1016/0031-9384(94)90341-7

Han, F., Ding, J., & Shi, Y. (2014). Expression of amygdala mineralocorticoid receptor and glucocorticoid receptor in the single-prolonged stress rats. *BMC Neuroscience*, *15*, 77. http://doi.org/10.1186/1471-2202-15-77

Harrington, J., Perry, I. J., Lutomski, J., Fitzgerald, A. P., Shiely, F., McGee, H., … Shelley, E. (2010). Living longer and feeling better: healthy lifestyle, self-rated health, obesity and depression in Ireland. *The European Journal of Public Health*, *20*(1), 91–95. http://doi.org/10.1093/eurpub/ckp102

Harris, E. W., & Cotman, C. W. (1986). Long-term potentiation of guinea pig mossy fiber responses is not blocked by N-methyl D-aspartate antagonists. *Neuroscience Letters*, *70*(1), 132–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3022192

Hatch, A. M., Wiberg, G. S., Zawidzka, Z., Cann, M., Airth, J. M., & Grice, H. C. (1965). Isolation Syndrome in the Rat. *TOXICOLOGY AND APPLIED PHARMACOLOGY*, *7*, 737–745.

Heim, C., Ehlert, U., & Hellhammer, D. H. (2000). The potential role of hypocortisolism in the pathophysiology of stress-related bodily disorders. *Psychoneuroendocrinology*, *25*(1), 1–35.

Heim, C., Newport, D. J., Mletzko, T., Miller, A. H., & Nemeroff, C. B. (2008). The link between childhood trauma and depression: insights from HPA axis studies in humans. *Psychoneuroendocrinology*, *33*(6), 693–710. http://doi.org/10.1016/j.psyneuen.2008.03.008

Heinrichs, S. C., & Koob, G. F. (2006). Application of experimental stressors in laboratory

rodents. *Current Protocols in Neuroscience / Editorial Board, Jacqueline N. Crawley ... [et Al.]*, *Chapter 8*, Unit8.4. http://doi.org/10.1002/0471142301.ns0804s34

Hellstrom, I. C., Dhir, S. K., Diorio, J. C., & Meaney, M. J. (2012). Maternal licking regulates hippocampal glucocorticoid receptor transcription through a thyroid hormone-serotonin-NGFI-A signalling cascade. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *367*(1601), 2495–510. http://doi.org/10.1098/rstb.2012.0223

Henriksson, M. M., Aro, H. M., Marttunen, M. J., Heikkinen, M. E., Isometsa, E. T., Kuoppasalmi, K. I., & Lonnqvist, J. K. (1993). Mental disorders and comorbidity in suicide. *The American Journal of Psychiatry*, *150*(6), 935–940.

Himmerich, H., Binder, E. B., Künzel, H. E., Schuld, A., Lucae, S., Uhr, M., … Ising, M. (2006). Successful antidepressant therapy restores the disturbed interplay between TNF-alpha system and HPA axis. *Biological Psychiatry*, *60*(8), 882–8. http://doi.org/10.1016/j.biopsych.2006.03.075

Hirschfeld, R. M. (2001). The Comorbidity of Major Depression and Anxiety Disorders: Recognition and Management in Primary Care. *Primary Care Companion to the Journal of Clinical Psychiatry*, *3*(6), 244–254.

Hogg, S. (1996). A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacology, Biochemistry, and Behavior*, *54*(1), 21–30.

Holsboer, F. (2000). The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *23*(5), 477–501. http://doi.org/10.1016/S0893-133X(00)00159-7

Hood, E. (2005). Dwelling disparities: how poor housing leads to poor health. *Environmental Health Perspectives*, *113*(5), A310-7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15866753

Hranov, L. (2007). Comorbid anxiety and depression: illumination of a controversy. *International Journal of Psychiatry in Clinical Practice*, *11*(3), 171–189. http://doi.org/http://dx.doi.org/10.1080/13651500601127180

Huang, F. L., Huang, K.-P., Wu, J., & Boucheron, C. (2006). Environmental enrichment enhances neurogranin expression and hippocampal learning and memory but fails to rescue the impairments of neurogranin null mutant mice. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *26*(23), 6230–7. http://doi.org/10.1523/JNEUROSCI.1182-06.2006

Hunziker, M. H., Saldana, R. L., & Neuringer, A. (1996). Behavioral variability in SHR and WKY rats as a function of rearing environment and reinforcement contingency. *Journal of the Experimental Analysis of Behavior*, *65*(1), 129–144. http://doi.org/10.1901/jeab.1996.65-129

Hurley, L. L., Akinfiresoye, L., Kalejaiye, O., & Tizabi, Y. (2014). Antidepressant effects of

resveratrol in an animal model of depression. *Behavioural Brain Research*, *268*, 1–7. http://doi.org/10.1016/j.bbr.2014.03.052; 10.1016/j.bbr.2014.03.052

Hyman, S. E. (2009). How adversity gets under the skin. *Nature Neuroscience*, *12*(3), 241–243. http://doi.org/10.1038/nn0309-241

Ickes, B. R., Pham, T. M., Sanders, L. A., Albeck, D. S., Mohammed, A. H., & Granholm, A.-C. (2000). Long-Term Environmental Enrichment Leads to Regional Increases in Neurotrophin Levels in Rat Brain. *Experimental Neurology*, *164*(1), 45–52. http://doi.org/10.1006/exnr.2000.7415

Jané-Llopis, E., Hosman, C., Jenkins, R., & Anderson, P. (2003). Predictors of efficacy in depression prevention programmes: Meta-analysis. *British Journal of Psychiatry*, *183*(NOV.), 384–397. http://doi.org/10.1192/bjp.183.5.384

Ji, J., & Maren, S. (2008). Differential roles for hippocampal areas CA1 and CA3 in the contextual encoding and retrieval of extinguished fear. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *15*(4), 244–51. http://doi.org/10.1101/lm.794808

Jinno, S., & Kosaka, T. (2008). Reduction of Iba1-expressing microglial process density in the hippocampus following electroconvulsive shock. *Experimental Neurology*, *212*(2), 440–447. http://doi.org/10.1016/j.expneurol.2008.04.028

Jureidini, J., & Tonkin, A. (2006). Overuse of antidepressant drugs for the treatment of depression. *CNS Drugs*, *20*(8), 623–632.

Juruena, M. F., Cleare, A. J., Papadopoulos, A. S., Poon, L., Lightman, S., & Pariante, C. M. (2006). Different responses to dexamethasone and prednisolone in the same depressed patients. *Psychopharmacology*, *189*(2), 225–35. http://doi.org/10.1007/s00213-006-0555-4

Juruena, M. F., Cleare, A. J., & Pariante, C. M. (2004). The hypothalamic pituitary adrenal axis, glucocorticoid receptor function and relevance to depression. *Revista Brasileira de Psiquiatria (Sao Paulo, Brazil : 1999)*, *26*(3), 189–201. http://doi.org//S1516-44462004000300009

Kalinichev, M., Easterling, K. W., Plotsky, P. M., & Holtzman, S. G. (2002). Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. *Pharmacology, Biochemistry, and Behavior*, *73*(1), 131–140.

Kapoor, K., Bhandare, A. M., Mohammed, S., Farnham, M. M. J., & Pilowsky, P. M. (2016). Microglial number is related to the number of tyrosine hydroxylase neurons in SHR and normotensive rats. *Autonomic Neuroscience*. http://doi.org/10.1016/j.autneu.2016.05.005

Katz, R. J. (1981a). Animal model of depression: effects of electroconvulsive shock therapy. *Neuroscience and Biobehavioral Reviews*, *5*(2), 273–277.

Katz, R. J. (1981b). Animal models and human depressive disorders. *Neuroscience and Biobehavioral Reviews*, *5*(2), 231–246.

Katz, R. J. (1982). Animal model of depression: pharmacological sensitivity of a hedonic deficit. *Pharmacology, Biochemistry, and Behavior*, *16*(6), 965–968.

Katz, R. J., & Hersh, S. (1981). Amitriptyline and scopolamine in an animal model of depression. *Neuroscience and Biobehavioral Reviews*, *5*(2), 265–271.

Katz, R. J., Roth, K. A., & Carroll, B. J. (1981). Acute and chronic stress effects on open field activity in the rat: implications for a model of depression. *Neuroscience and Biobehavioral Reviews*, *5*(2), 247–251.

Keeney, A. J., & Hogg, S. (1999). Behavioural consequences of repeated social defeat in the mouse: preliminary evaluation of a potential animal model of depression. *Behavioural Pharmacology*, *10*(8), 753–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10780291

Kempermann, G., Gast, D., & Gage, F. H. (2002). Neuroplasticity in old age: sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Annals of Neurology*, *52*(2), 135–143. http://doi.org/10.1002/ana.10262

Kendler, K. S., Kessler, R. C., Waiters, E. E., MacLean, C., Neale, M. C., Heath, A. C., & Eaves, L. J. (1995). Stressful life events, genetic liability, and onset of an episode of major depression in women. *American Journal of Psychiatry*, *152*(6), 833–842. http://doi.org/10.1176/ajp.152.6.833

Kendler, K. S., Neale, M. C., Kessler, R. C., Heath, A. C., & Eaves, L. J. (1992a). Generalized anxiety disorder in women. A population-based twin study. *Archives of General Psychiatry*, *49*(4), 267–272.

Kendler, K. S., Neale, M. C., Kessler, R. C., Heath, A. C., & Eaves, L. J. (1992b). Major depression and generalized anxiety disorder. Same genes, (partly) different environments? *Archives of General Psychiatry*, *49*(9), 716–722.

Kentner, A. C., Takeuchi, A., James, J. S., Miki, T., Seino, S., Hayley, S., & Bielajew, C. (2008). The effects of rewarding ventral tegmental area stimulation and environmental enrichment on lipopolysaccharide-induced sickness behavior and cytokine expression in female rats. *Brain Research*, *1217*, 50–61. http://doi.org/10.1016/j.brainres.2008.04.041

Kessler, R. C., & Bromet, E. J. (2013). The epidemiology of depression across cultures. *Annual Review of Public Health*, *34*, 119–138. http://doi.org/10.1146/annurev-publhealth-031912-114409; 10.1146/annurev-publhealth-031912-114409

Kessler, R. C., McGonagle, K. A., Swartz, M., Blazer, D. G., & Nelson, C. B. (1993). Sex and depression in the National Comorbidity Survey. I: Lifetime prevalence, chronicity and recurrence. *Journal of Affective Disorders*, *29*(2–3), 85–96.

Kessler, R. C., McGonagle, K. A., Zhao, S., Nelson, C. B., Hughes, M., Eshleman, S., … Kendler, K. S. (1994). Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. *Archives of General Psychiatry*, *51*(1), 8–19.

Kiess, W., & Belohradsky, B. H. (1986). Endocrine regulation of the immune system. *Klinische Wochenschrift*, *64*(1), 1–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3512901

Kobilo, T., Liu, Q. R., Gandhi, K., Mughal, M., Shaham, Y., & van Praag, H. (2011). Running is the neurogenic and neurotrophic stimulus in environmental enrichment. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *18*(9), 605–609. http://doi.org/10.1101/lm.2283011; 10.1101/lm.2283011

Konkle, A. T. M., Kentner, A. C., Baker, S. L., Stewart, A., & Bielajew, C. (2010). Environmental-Enrichment-Related Variations in Behavioral, Biochemical, and Physiologic Responses of Sprague-Dawley and Long Evans Rats. *Journal of the American Association for Laboratory Animal Science*, *49*(4), 427–436.

Konkle, a. (2003). Evaluation of the effects of chronic mild stressors on hedonic and physiological responses: sex and strain compared. *Brain Research*, *992*(2), 227–238. http://doi.org/10.1016/j.brainres.2003.08.047

Lacasse, J. R., & Leo, J. (2005). Serotonin and Depression: A Disconnect between the Advertisements and the Scientific Literature. *PLoS Medicine*, *2*(12), e392. http://doi.org/10.1371/journal.pmed.0020392

Lahmame, A., & Armario, A. (1996). Differential responsiveness of inbred strains of rats to antidepressants in the forced swimming test: are Wistar Kyoto rats an animal model of subsensitivity to antidepressants? *Psychopharmacology*, *123*(2), 191–198.

Lahmame, A., del Arco, C., Pazos, A., Yritia, M., & Armario, A. (1997). Are Wistar-Kyoto rats a genetic animal model of depression resistant to antidepressants? *European Journal of Pharmacology*, *337*(2–3), 115–23. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9430405

Laviola, G., Hannan, A. J., Macri, S., Solinas, M., & Jaber, M. (2008). Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. *Neurobiology of Disease*, *31*(2), 159–168. http://doi.org/10.1016/j.nbd.2008.05.001

Layé, S., Parnet, P., Goujon, E., & Dantzer, R. (1994). *Peripheral administration of lipopolysaccharide induces the expression of cytokine transcripts in the brain and pituitary of mice*. *Molecular Brain Research* (Vol. 27). http://doi.org/10.1016/0169-328X(94)90197-X

Leggio, M. G., Mandolesi, L., Federico, F., Spirito, F., Ricci, B., Gelfo, F., & Petrosini, L. (2005). Environmental enrichment promotes improved spatial abilities and enhanced dendritic growth in the rat. *Behavioural Brain Research*, *163*(1), 78–90. http://doi.org/10.1016/j.bbr.2005.04.009

Leuner, B., & Gould, E. (2010). Structural plasticity and hippocampal function. *Annual Review of Psychology*, *61*, 111–40, C1-3. http://doi.org/10.1146/annurev.psych.093008.100359

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, a, … Meaney, M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-

adrenal responses to stress. *Science (New York, N.Y.)*, *277*(5332), 1659–1662. http://doi.org/10.1126/science.277.5332.1659

Liu, Y., Ho, R. C.-M., & Mak, A. (2012). Interleukin (IL)-6, tumour necrosis factor alpha (TNF-α) and soluble interleukin-2 receptors (sIL-2R) are elevated in patients with major depressive disorder: A meta-analysis and meta-regression. *Journal of Affective Disorders*, *139*(3), 230–239. http://doi.org/10.1016/j.jad.2011.08.003

Llorens-Martin, M. V, Rueda, N., Martinez-Cue, C., Torres-Aleman, I., Florez, J., & Trejo, J. L. (2007). Both increases in immature dentate neuron number and decreases of immobility time in the forced swim test occurred in parallel after environmental enrichment of mice. *Neuroscience*, *147*(3), 631–638. http://doi.org/10.1016/j.neuroscience.2007.04.054

Lopez-Rubalcava, C., & Lucki, I. (2000). Strain differences in the behavioral effects of antidepressant drugs in the rat forced swimming test. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *22*(2), 191–199. http://doi.org/10.1016/S0893-133X(99)00100-1

Lucki, I. (1997). The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. *Behavioural Pharmacology*, *8*(6–7), 523–532.

Lupien, S. J., Fiocco, A., Wan, N., Maheu, F., Lord, C., Schramek, T., & Tu, M. T. (2005). Stress hormones and human memory function across the lifespan. *Psychoneuroendocrinology*, *30*(3), 225–242. http://doi.org/10.1016/j.psyneuen.2004.08.003

Lupien, S. J., King, S., Meaney, M. J., & McEwen, B. S. (2000). Child's stress hormone levels correlate with mother's socioeconomic status and depressive state. *Biological Psychiatry*, *48*(10), 976–980.

Madeira, M. D., & Lieberman, A. R. (1995). Sexual dimorphism in the mammalian limbic system. *Progress in Neurobiology*, *45*(4), 275–333. http://doi.org/10.1016/0301-0082(94)00052-J

Maes, M., Bosmans, E., Calabrese, J., Smith, R., & Meltzer, H. Y. (1995). Interleukin-2 and interleukin-6 in schizophrenia and mania: Effects of neuroleptics and mood stabilizers. *Journal of Psychiatric Research*, *29*(2), 141–152. http://doi.org/10.1016/0022-3956(94)00049-W

Maes, M., Song, C., Lin, A.-H., Bonaccorso, S., Kenis, G., Jongh, R. De, … Scharpé, S. (1999). Negative Immunoregulatory Effects of Antidepressants Inhibition of Interferon-γ and Stimulation of Interleukin-10 Secretion. *Neuropsychopharmacology*, *20*(4), 370–379. http://doi.org/10.1016/S0893-133X(98)00088-8

Maier, S. F., & Watkins, L. R. (1995). Intracerebroventricular interleukin-1 receptor antagonist blocks the enhancement of fear conditioning and interference with escape produced by inescapable shock. *Brain Research*, *695*(2), 279–82. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8556346

Malkesman, O., Braw, Y., Maayan, R., Weizman, A., Overstreet, D. H., Shabat-Simon, M., …

Weller, A. (2006). Two different putative genetic animal models of childhood depression. *Biological Psychiatry*, *59*(1), 17–23. http://doi.org/10.1016/j.biopsych.2005.05.039

Malkesman, O., Braw, Y., Zagoory-Sharon, O., Golan, O., Lavi-Avnon, Y., Schroeder, M., … Weller, A. (2005). Reward and anxiety in genetic animal models of childhood depression. *Behavioural Brain Research*, *164*(1), 1–10. http://doi.org/10.1016/j.bbr.2005.04.023

Malkesman, O., Maayan, R., Weizman, A., & Weller, A. (2006). Aggressive behavior and HPA axis hormones after social isolation in adult rats of two different genetic animal models for depression. *Behavioural Brain Research*, *175*(2), 408–414. http://doi.org/10.1016/j.bbr.2006.09.017

Malkesman, O., & Weller, A. (2009). Two different putative genetic animal models of childhood depression--a review. *Progress in Neurobiology*, *88*(3), 153–169. http://doi.org/10.1016/j.pneurobio.2009.03.003

Mancini, C., Van Ameringen, M., Oakman, J. M., & Figueiredo, D. (1999). Childhood attention deficit/hyperactivity disorder in adults with anxiety disorders. *Psychological Medicine*, *29*(3), 515–525.

Mantella, R. C., Butters, M. A., Amico, J. A., Mazumdar, S., Rollman, B. L., Begley, A. E., … Lenze, E. J. (2008). Salivary cortisol is associated with diagnosis and severity of late-life generalized anxiety disorder. *Psychoneuroendocrinology*, *33*(6), 773–781. http://doi.org/10.1016/j.psyneuen.2008.03.002; 10.1016/j.psyneuen.2008.03.002

Marashi, V., Barnekow, A., Ossendorf, E., & Sachser, N. (2003). Effects of different forms of environmental enrichment on behavioral, endocrinological, and immunological parameters in male mice. *Hormones and Behavior*, *43*(2), 281–292. http://doi.org/10.1016/S0018-506X(03)00002-3

Marcondes, F. K., Bianchi, F. J., & Tanno, A. P. (2002). Determination of the estrous cycle phases of rats: some helpful considerations. *Brazilian Journal of Biology = Revista Brasleira de Biologia*, *62*(4A), 609–14. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12659010

Maripuu, M., Wikgren, M., Karling, P., Adolfsson, R., & Norrback, K. F. (2014). Relative hypo- and hypercortisolism are both associated with depression and lower quality of life in bipolar disorder: A cross-sectional study. *PLoS ONE*, *9*(6). http://doi.org/10.1371/journal.pone.0098682

Mathers, C. D., & Loncar, D. (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Medicine*, *3*(11), e442. http://doi.org/10.1371/journal.pmed.0030442

Maughan, A., Cicchetti, D., Toth, S. L., & Rogosch, F. A. (2007). Early-occurring maternal depression and maternal negativity in predicting young children's emotion regulation and socioemotional difficulties. *Journal of Abnormal Child Psychology*, *35*(5), 685–703. http://doi.org/10.1007/s10802-007-9129-0

McGowan, P. O., Sasaki, A., D'Alessio, A. C., Dymov, S., Labonté, B., Szyf, M., … Meaney,

M. J. (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nature Neuroscience*, *12*(3), 342–348. http://doi.org/10.1038/nn.2270

Mcleod, J. D., & Shanahan, M. J. (1996). Trajectories of poverty and children's mental health. *Journal of Health and Social Behavior*, *37*(3), 207–220.

Meaney, M. J., Aitken, D. H., Bodnoff, S. R., Iny, L. J., Tatarewicz, J. E., & Sapolsky, R. M. (1985). Early postnatal handling alters glucocorticoid receptor concentrations in selected brain regions. *Behavioral Neuroscience*, *99*(4), 765–770. http://doi.org/10.1037/0735-7044.99.4.765

Mileva, G., Bielajew, C., & Konkle, A. T. M. (2013). Sex Differences in Physiology and Behaviour: The Importance of Hormones and Rearing Environment. In *Estrous Cycle: Physiology, Endocrinology and Role in Breeding and Reproductive Management* (pp. 1–44).

Mileva, G. R., & Bielajew, C. (2015). Environmental manipulation affects depressive-like behaviours in female Wistar-Kyoto rats. *Behavioural Brain Research*, *293*, 208–216. http://doi.org/10.1016/j.bbr.2015.07.035

Mileva, G. R., Rooke, J., Ismail, N., & Bielajew, C. (2017). Corticosterone and immune cytokine characterization following environmental manipulation in female WKY rats. *Behavioural Brain Research*, *316*, 197–204. http://doi.org/10.1016/j.bbr.2016.09.004

Miller, A. H., Maletic, V., & Raison, C. L. (2009). Inflammation and Its Discontents: The role of Cytokines in the Pathophysiology of Major Depression. *Biological Psychiatry*, *65*(9), 732–741. http://doi.org/10.1016/j.biopsych.2008.11.029.Inflammation

Miller, A. H., & Raison, C. L. (2015). The role of inflammation in depression: from evolutionary imperative to modern treatment target. *Nature Reviews Immunology*, *16*(1), 22–34. http://doi.org/10.1038/nri.2015.5

Miller, B. J., Buckley, P., Seabolt, W., Mellor, A., & Kirkpatrick, B. (2011). Meta-analysis of cytokine alterations in schizophrenia: Clinical status and antipsychotic effects. *Biological Psychiatry*, *70*(7), 663–671. http://doi.org/10.1016/j.biopsych.2011.04.013

Mineka, S., Watson, D., & Clark, L. A. (1998). Comorbidity of anxiety and unipolar mood disorders. *Annual Review of Psychology*, *49*, 377–412. http://doi.org/10.1146/annurev.psych.49.1.377

Möller, M., Du Preez, J. L., Viljoen, F. P., Berk, M., Emsley, R., & Harvey, B. H. (2013). Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. *Brain, Behavior, and Immunity*, *30*, 156–67. http://doi.org/10.1016/j.bbi.2012.12.011

Morley-Fletcher, S., Rea, M., Maccari, S., & Laviola, G. (2003). Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. *The European Journal of Neuroscience*, *18*(12), 3367–74. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14686910

Mouihate, A., Chen, X., & Pittman, Q. J. (1998). Interleukin-1beta fever in rats: gender difference and estrous cycle influence. *The American Journal of Physiology*, *275*(5 Pt 2), R1450-4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9791060

Muscat, R., & Willner, P. (1992). Suppression of sucrose drinking by chronic mild unpredictable stress: a methodological analysis. *Neuroscience and Biobehavioral Reviews*, *16*(4), 507–517.

Nam, H., Clinton, S. M., Jackson, N. L., & Kerman, I. A. (2014). Learned helplessness and social avoidance in the Wistar-Kyoto rat. *Frontiers in Behavioral Neuroscience*, *8*, 109. http://doi.org/10.3389/fnbeh.2014.00109; 10.3389/fnbeh.2014.00109

Nguyen, K. T., Deak, T., Owens, S. M., Kohno, T., Fleshner, M., Watkins, L. R., & Maier, S. F. (1998). Exposure to acute stress induces brain interleukin-1beta protein in the rat. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *18*(6), 2239–46. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9482808

Nithianantharajah, J., & Hannan, A. J. (2006). Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nature Reviews. Neuroscience*, *7*(9), 697–709. http://doi.org/10.1038/nrn1970

Noble, R. E. (2005). Depression in women. *Depression: Genetics, Pathophysiology, and Clinical Manifestations*, *54*(5, Supplement), 49–52. http://doi.org/10.1016/j.metabol.2005.01.014

Nolen-Hoeksema, S. (1987). Sex differences in unipolar depression: Evidence and theory. *Psychological Bulletin*, *101*(2), 259–282. http://doi.org/10.1037/0033-2909.101.2.259

Nutt, D. J., Ballenger, J. C., Sheehan, D., & Wittchen, H. U. (2002). Generalized anxiety disorder: comorbidity, comparative biology and treatment. *The International Journal of Neuropsychopharmacology / Official Scientific Journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)*, *5*(4), 315–325. http://doi.org/10.1017/S1461145702003048

Okamato, K., & Aoki, K. (1963). Development of a strain of spontaneously hypertensive rats. *Japanese Circulation Journal*, *27*, 282–93. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/13939773

Olson, A. K., Eadie, B. D., Ernst, C., & Christie, B. R. (2006). Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. *Hippocampus*, *16*(3), 250–60. http://doi.org/10.1002/hipo.20157

Orsi, N. M., Ekbote, U. V., Walker, J. J., & Gopichandran, N. (2007). Uterine and serum cytokine arrays in the mouse during estrus. *Animal Reproduction Science*, *100*(3–4), 301–310. http://doi.org/10.1016/j.anireprosci.2006.08.016

Orsi, N. M., Gopichandran, N., Ekbote, U. V., & Walker, J. J. (2006). Murine serum cytokines throughout the estrous cycle, pregnancy and post partum period. *Animal Reproduction Science*, *96*(1–2), 54–65. http://doi.org/10.1016/j.anireprosci.2005.11.010

Overstreet, D. H. (2012). Modeling depression in animal models. *Methods in Molecular Biology*

(Clifton, N.J.), *829*, 125–44. http://doi.org/10.1007/978-1-61779-458-2_7

Palanza, P. (2001). Animal models of anxiety and depression: how are females different? *Neuroscience and Biobehavioral Reviews*, *25*(3), 219–233.

Paré, W. P. (1989). Stress ulcer susceptibility and depression in Wistar Kyoto (WKY) rats. *Physiology & Behavior*, *46*(6), 993–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2634265

Pare, W. P., & Redei, E. (1993a). Depressive behavior and stress ulcer in Wistar Kyoto rats. *Journal of Physiology, Paris*, *87*(4), 229–238.

Pare, W. P., & Redei, E. (1993b). Sex differences and stress response of WKY rats. *Physiology & Behavior*, *54*(6), 1179–1185.

Pariante, C. M., & Lightman, S. L. (2008). The HPA axis in major depression: classical theories and new developments. *Trends in Neurosciences*, *31*(9), 464–468. http://doi.org/10.1016/j.tins.2008.06.006

Pariante, C. M., & Miller, A. H. (2001). Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. *Biological Psychiatry*, *49*(5), 391–404. http://doi.org/10.1016/S0006-3223(00)01088-X

Pasco, J. A., Nicholson, G. C., Williams, L. J., Jacka, F. N., Henry, M. J., Kotowicz, M. A., … Berk, M. (2010). Association of high-sensitivity C-reactive protein with de novo major depression. *The British Journal of Psychiatry : The Journal of Mental Science*, *197*(5), 372–7. http://doi.org/10.1192/bjp.bp.109.076430

Pavlides, C., Ogawa, S., Kimura, A., & McEwen, B. S. (1996). Role of adrenal steroid mineralocorticoid and glucocorticoid receptors in long-term potentiation in the CA1 field of hippocampal slices. *Brain Research*, *738*(2), 229–235. http://doi.org/10.1016/S0006-8993(96)00776-7

Paykel, E. S. (1994). Life events, Social support and depression. *Acta Psychiatrica Scandinavica*, *89*(s377), 50–58. http://doi.org/10.1111/j.1600-0447.1994.tb05803.x

Peng, L., Verkhratsky, A., Gu, L., & Li, B. (2015). Expert Review of Neurotherapeutics Targeting astrocytes in major depression Targeting astrocytes in major depression. *Expert Review of Neurotherapeutics*, *15*(11), 1299–1306. http://doi.org/10.1586/14737175.2015.1095094doi.org/10.1586/14737175.2015.1095094

Pérez-Ortiz, J. M., García-Gutiérrez, M. S., Navarrete, F., Giner, S., & Manzanares, J. (2013). Gene and protein alterations of FKBP5 and glucocorticoid receptor in the amygdala of suicide victims. *Psychoneuroendocrinology*, *38*(8), 1251–1258. http://doi.org/10.1016/j.psyneuen.2012.11.008

Petrosini, L., De Bartolo, P., Foti, F., Gelfo, F., Cutuli, D., Leggio, M. G., & Mandolesi, L. (2009). On whether the environmental enrichment may provide cognitive and brain reserves. *Brain Research Reviews*, *61*(2), 221–239. http://doi.org/10.1016/j.brainresrev.2009.07.002

Piccinelli, M., & Wilkinson, G. (2000). Gender differences in depression. Critical review. *The British Journal of Psychiatry : The Journal of Mental Science*, *177*, 486–492.

Plotsky, P. M., & Meaney, M. J. (1993). Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Brain research.Molecular Brain Research*, *18*(3), 195–200.

Procaccini, C., Pucino, V., De Rosa, V., Marone, G., & Matarese, G. (2014). Neuro-Endocrine Networks Controlling Immune System in Health and Disease. *Frontiers in Immunology*, *5*, 143. http://doi.org/10.3389/fimmu.2014.00143

Rajkowska, G., & Stockmeier, C. A. (2013). Astrocyte pathology in major depressive disorder: insights from human postmortem brain tissue. *Current Drug Targets*, *14*(11), 1225–36. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23469922

Rittenhouse, P. A., López-Rubalcava, C., Stanwood, G. D., & Lucki, I. (2002). Amplified behavioral and endocrine responses to forced swim stress in the Wistar–Kyoto rat. *Psychoneuroendocrinology*, *27*(3), 303–318. http://doi.org/10.1016/S0306-4530(01)00052-X

Rohleder, N., Schommer, N. C., Hellhammer, D. H., Engel, R., & Kirschbaum, C. (2001). Sex differences in glucocorticoid sensitivity of proinflammatory cytokine production after psychosocial stress. *Psychosomatic Medicine*, *63*(6), 966–972. http://doi.org/10.1097/00006842-200111000-00016

Rosenfeld, P., Wetmore, J. B., & Levine, S. (1992). Effects of repeated maternal separations on the adrenocortical response to stress of preweanling rats. *Physiology & Behavior*, *52*(4), 787–791.

Rosenzweig, M. R., & Bennett, E. L. (1996). Psychobiology of plasticity: effects of training and experience on brain and behavior. *Behavioural Brain Research*, *78*(1), 57–65. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8793038

Saarijarvi, S., Salminen, J. K., & Toikka, T. B. (2001). Alexithymia and depression: a 1-year follow-up study in outpatients with major depression. *Journal of Psychosomatic Research*, *51*(6), 729–733.

Sagvolden, T., & Johansen, E. B. (2012). Rat models of ADHD. *Current Topics in Behavioral Neurosciences*, *9*, 301–315. http://doi.org/10.1007/7854_2011_126; 10.1007/7854_2011_126

Sagvolden, T., Metzger, M. A., Schiorbeck, H. K., Rugland, A. L., Spinnangr, I., & Sagvolden, G. (1992). The spontaneously hypertensive rat (SHR) as an animal model of childhood hyperactivity (ADHD): changed reactivity to reinforcers and to psychomotor stimulants. *Behavioral and Neural Biology*, *58*(2), 103–112.

Salmaso, N., Silbereis, J., Komitova, M., Mitchell, P., Chapman, K., Ment, L. R., … Vaccarino, F. M. (2012). Environmental enrichment increases the GFAP+ stem cell pool and reverses hypoxia-induced cognitive deficits in juvenile mice. *The Journal of Neuroscience : The*

Official Journal of the Society for Neuroscience, *32*(26), 8930–9. http://doi.org/10.1523/JNEUROSCI.1398-12.2012

Salmon, P. (2001). Effects of physical exercise on anxiety, depression, and sensitivity to stress: a unifying theory. *Clinical Psychology Review*, *21*(1), 33–61.

Sampedro-Piquero, P., Begega, A., & Arias, J. L. (2014). Increase of glucocorticoid receptor expression after environmental enrichment: relations to spatial memory, exploration and anxiety-related behaviors. *Physiology & Behavior*, *129*, 118–129. http://doi.org/10.1016/j.physbeh.2014.02.048; 10.1016/j.physbeh.2014.02.048

Sanchez, M. M., Ladd, C. O., & Plotsky, P. M. (2001). Early adverse experience as a developmental risk factor for later psychopathology: evidence from rodent and primate models. *Development and Psychopathology*, *13*(3), 419–449.

Sandi, C. (2004). Stress, cognitive impairment and cell adhesion molecules. *Nature Reviews Neuroscience*, *5*(12), 917. http://doi.org/10.1038/nrn1555

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., … Stevens, B. (2012). Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron*, *74*(4), 691–705. http://doi.org/10.1016/j.neuron.2012.03.026

Schatz, D. B., & Rostain, A. L. (2006). ADHD with comorbid anxiety: a review of the current literature. *Journal of Attention Disorders*, *10*(2), 141–149. http://doi.org/10.1177/1087054706286698

Schneider, P., Ho, Y. J., Spanagel, R., & Pawlak, C. R. (2011). A novel elevated plus-maze procedure to avoid the one-trial tolerance problem. *Frontiers in Behavioral Neuroscience*, *5*, 43. http://doi.org/10.3389/fnbeh.2011.00043; 10.3389/fnbeh.2011.00043

Scholl, J. L., Renner, K. J., Forster, G. L., & Tejani-Butt, S. (2010). Central monoamine levels differ between rat strains used in studies of depressive behavior. *Brain Research*, *1355*, 41–51. http://doi.org/10.1016/j.brainres.2010.08.003

Segovia, G., Yagüe, A. G., García-Verdugo, J. M., & Mora, F. (2006). Environmental enrichment promotes neurogenesis and changes the extracellular concentrations of glutamate and GABA in the hippocampus of aged rats. *Brain Research Bulletin*, *70*(1), 8–14. http://doi.org/10.1016/j.brainresbull.2005.11.005

Sengupta, P. (2013). The Laboratory Rat: Relating Its Age With Human's. *International Journal of Preventive Medicine*, *4*(6), 624–30. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23930179

Servatius, R. J., Jiao, X., Beck, K. D., Pang, K. C. H., & Minor, T. R. (2008). Rapid avoidance acquisition in Wistar–Kyoto rats. *Behavioural Brain Research*, *192*(2), 191–197. http://doi.org/10.1016/j.bbr.2008.04.006

Sherwin, E., Gormley, S., McGuinness, B., & Harkin, A. (2014). 105. Enhanced glucocorticoid sensitivity coupled with reduced microglial activation in a genetically predisposed animal

model of depression. *Brain, Behavior, and Immunity*, *40*. http://doi.org/10.1016/j.bbi.2014.06.125

Silva, J. C. R., Rocha, M. F. G., Lima, A. A. M., Brito, G. A. C., de Menezes, D. B., & Rao, V. S. N. (2000). Effects of pentoxifylline and nabumetone on the serum levels of IL-1β and TNFα in rats with adjuvant arthritis. *Inflammation Research*, *49*(1), 14–19. http://doi.org/10.1007/PL00000198

Simmons, D. A., & Broderick, P. A. (2005). Cytokines, stressors, and clinical depression: Augmented adaptation responses underlie depression pathogenesis. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *29*(5), 793–807. http://doi.org/10.1016/j.pnpbp.2005.03.009

Simpson, J., & Kelly, J. P. (2011). The impact of environmental enrichment in laboratory rats-Behavioural and neurochemical aspects. *Behavioural Brain Research*. http://doi.org/10.1016/j.bbr.2011.04.002

Singhal, G., Jaehne, E. J., Corrigan, F., & Baune, B. T. (2014). Cellular and molecular mechanisms of immunomodulation in the brain through environmental enrichment. *Frontiers in Cellular Neuroscience*, *8*, 97. http://doi.org/10.3389/fncel.2014.00097

Solberg, L. C., Olson, S. L., Turek, F. W., & Redei, E. (2001). Altered hormone levels and circadian rhythm of activity in the WKY rat, a putative animal model of depression. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *281*(3), R786-94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11506993

Solinas, M., Thiriet, N., Chauvet, C., & Jaber, M. (2010). Prevention and treatment of drug addiction by environmental enrichment. *Progress in Neurobiology*, *92*(4), 572–92. http://doi.org/10.1016/j.pneurobio.2010.08.002

Song, C., Halbreich, U., Han, C., Leonard, B. E., & Luo, H. (2009). Imbalance between pro- and anti-inflammatory cytokines, and between Th1 and Th2 cytokines in depressed patients: the effect of electroacupuncture or fluoxetine treatment. *Pharmacopsychiatry*, *42*(5), 182–8. http://doi.org/10.1055/s-0029-1202263

Song, H., Stevens, C. F., & Gage, F. H. (2002). Astroglia induce neurogenesis from adult neural stem cells. *Nature*, *417*(6884), 39–44. http://doi.org/10.1038/417039a

Stark, J. L., Avitsur, R., Padgett, D. A., Campbell, K. A., Beck, F. M., & Sheridan, J. F. (2001). Social stress induces glucocorticoid resistance in macrophages. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *280*(6), R1799-805. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11353685

Steckler, T., Holsboer, F., & Reul, J. M. (1999). Glucocorticoids and depression. *Bailliere's Best Practice & research.Clinical Endocrinology & Metabolism*, *13*(4), 597–614.

Stieglitz, J., Trumble, B. C., Thompson, M. E., Blackwell, A. D., Kaplan, H., & Gurven, M. (2015). Depression as sickness behavior? A test of the host defense hypothesis in a high pathogen population. *Brain, Behavior, and Immunity*, *49*, 130–9.

http://doi.org/10.1016/j.bbi.2015.05.008

Straub, R. H. (2007). The complex role of estrogens in inflammation. *Endocrine Reviews*, *28*(5), 521–74. http://doi.org/10.1210/er.2007-0001

Sutcigil, L., Oktenli, C., Musabak, U., Bozkurt, A., Cansever, A., Uzun, O., … Sengul, A. (2007). Pro- and anti-inflammatory cytokine balance in major depression: effect of sertraline therapy. *Clinical & Developmental Immunology*, *2007*, 76396. http://doi.org/10.1155/2007/76396

Tejani-Butt, S., Kluczynski, J., & Pare, W. P. (2003). Strain-dependent modification of behavior following antidepressant treatment. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *27*(1), 7–14.

Thaxton, J. E., & Sharma, S. (2010). Interleukin-10: A Multi-Faceted Agent of Pregnancy. *American Journal of Reproductive Immunology*, *63*(6), 482–491. http://doi.org/10.1111/j.1600-0897.2010.00810.x

Tizabi, Y., Bhatti, B. H., Manaye, K. F., Das, J. R., & Akinfiresoye, L. (2012). Antidepressant-like effects of low ketamine dose is associated with increased hippocampal AMPA/NMDA receptor density ratio in female Wistar-Kyoto rats. *Neuroscience*, *213*, 72–80. http://doi.org/10.1016/j.neuroscience.2012.03.052; 10.1016/j.neuroscience.2012.03.052

Torres-Platas, S. G., Cruceanu, C., Chen, G. G., Turecki, G., & Mechawar, N. (2014). Evidence for increased microglial priming and macrophage recruitment in the dorsal anterior cingulate white matter of depressed suicides. *Brain, Behavior, and Immunity*, *42*, 50–59. http://doi.org/10.1016/j.bbi.2014.05.007

Tremblay, M.-E., Stevens, B., Sierra, A., Wake, H., Bessis, A., & Nimmerjahn, A. (2011). The Role of Microglia in the Healthy Brain. *Journal of Neuroscience*, *31*(45), 16064–16069. http://doi.org/10.1523/JNEUROSCI.4158-11.2011

Tuglu, C., Kara, S. H., Caliyurt, O., Vardar, E., & Abay, E. (2003). Increased serum tumor necrosis factor-alpha levels and treatment response in major depressive disorder. *Psychopharmacology*, *170*(4), 429–433. http://doi.org/10.1007/s00213-003-1566-z

Udina, M., Castellví, P., Moreno-España, J., Navinés, R., Valdés, M., Forns, X., … Martín-Santos, R. (2012). Interferon-induced depression in chronic hepatitis C: a systematic review and meta-analysis. *The Journal of Clinical Psychiatry*, *73*(8), 1128–38. http://doi.org/10.4088/JCP.12r07694

Uehara, A., Gottschall, P. E., Dahl, R. R., & Arimura, A. (1987). Interleukin-1 stimulates ACTH release by an indirect action which requires endogenous corticotropin releasing factor. *Endocrinology*, *121*(4), 1580–1582. http://doi.org/10.1210/endo-121-4-1580

Ustun, T. B., Ayuso-Mateos, J. L., Chatterji, S., Mathers, C., & Murray, C. J. (2004). Global burden of depressive disorders in the year 2000. *The British Journal of Psychiatry : The Journal of Mental Science*, *184*, 386–392.

Vahl, T. P., Ulrich-Lai, Y. M., Ostrander, M. M., Dolgas, C. M., Elfers, E. E., Seeley, R. J., …

Herman, J. P. (2005). Comparative analysis of ACTH and corticosterone sampling methods in rats. *American Journal of Physiology. Endocrinology and Metabolism*, *289*(5), E823-8. http://doi.org/10.1152/ajpendo.00122.2005

Van, de V., Bracke, P., & Levecque, K. (2010). Gender differences in depression in 23 European countries. Cross-national variation in the gender gap in depression. *Soc Sci Med*, *71*(2), 305–313. Retrieved from http://europepmc.org/abstract/MED/20483518

van Praag, H., Kempermann, G., & Gage, F. H. (2000). Neural consequences of environmental enrichment. *Nature Reviews. Neuroscience*, *1*(December), 191–198. http://doi.org/10.1038/35044558

van Praag, H. M. (2004). Can stress cause depression? *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *28*(5), 891–907. http://doi.org/10.1016/j.pnpbp.2004.05.031

Varghese, F. P., & Brown, E. S. (2001). The Hypothalamic-Pituitary-Adrenal Axis in Major Depressive Disorder: A Brief Primer for Primary Care Physicians. *Primary Care Companion to the Journal of Clinical Psychiatry*, *3*(4), 151–155. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15014598

Veena, J., Srikumar, B. N., Mahati, K., Bhagya, V., Raju, T. R., & Shankaranarayana Rao, B. S. (2009). Enriched environment restores hippocampal cell proliferation and ameliorates cognitive deficits in chronically stressed rats. *Journal of Neuroscience Research*, *87*(4), 831–843. http://doi.org/10.1002/jnr.21907; 10.1002/jnr.21907

Veena, J., Srikumar, B. N., Raju, T. R., & Shankaranarayana Rao, B. S. (2009). Exposure to enriched environment restores the survival and differentiation of new born cells in the hippocampus and ameliorates depressive symptoms in chronically stressed rats. *Neuroscience Letters*, *455*(3), 178–182. http://doi.org/10.1016/j.neulet.2009.03.059; 10.1016/j.neulet.2009.03.059

Vesga-Lopez, O., Schneier, F. R., Wang, S., Heimberg, R. G., Liu, S. M., Hasin, D. S., & Blanco, C. (2008). Gender differences in generalized anxiety disorder: results from the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC). *The Journal of Clinical Psychiatry*, *69*(10), 1606–1616.

Viola, G. G., Rodrigues, L., Américo, J. C., Hansel, G., Vargas, R. S., Biasibetti, R., … Amaral, O. B. (2009). Morphological changes in hippocampal astrocytes induced by environmental enrichment in mice. *Brain Research*, *1274*, 47–54. http://doi.org/10.1016/j.brainres.2009.04.007

Vitkovic, L., Konsman, J., Bockaert, J., Dantzer, R., Homburger, V., & Jacque, C. (2000). Cytokine signals propagate through the brain. *Molecular Psychiatry*, *5*, 604–615. Retrieved from www.nature.com/mp

Vogels, E. A., Lagro-Janssen, A. L., & van Weel, C. (1999). Sex differences in cardiovascular disease: are women with low socioeconomic status at high risk? *The British Journal of General Practice : The Journal of the Royal College of General Practitioners*, *49*(449), 963–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10824339

Vreeburg, S. A., Hoogendijk, W. J. G., van Pelt, J., Derijk, R. H., Verhagen, J. C. M., van Dyck, R., … Penninx, B. W. J. H. (2009). Major depressive disorder and hypothalamic-pituitary-adrenal axis activity: results from a large cohort study. *Archives of General Psychiatry*, *66*(6), 617–26. http://doi.org/10.1001/archgenpsychiatry.2009.50

Wall, P. M., & Messier, C. (2000). Ethological confirmatory factor analysis of anxiety-like behaviour in the murine elevated plus-maze. *Behavioural Brain Research*, *114*(1–2), 199–212. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10996061

Wall, P. M., & Messier, C. (2001). Methodological and conceptual issues in the use of the elevated plus-maze as a psychological measurement instrument of animal anxiety-like behavior. *Neuroscience and Biobehavioral Reviews*, *25*(3), 275–86. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11378181

Wang, Q., Verweij, E. W. E., Krugers, H. J., Joels, M., Swaab, D. F., & Lucassen, P. J. (2014). Distribution of the glucocorticoid receptor in the human amygdala; changes in mood disorder patients. *Brain Structure and Function*, *219*(5), 1615–1626. http://doi.org/10.1007/s00429-013-0589-4

Waraich, P., Goldner, E. M., Somers, J. M., & Hsu, L. (2004). Prevalence and incidence studies of mood disorders: a systematic review of the literature. *Canadian Journal of psychiatry.Revue Canadienne de Psychiatrie*, *49*(2), 124–138.

Wardle, J., Waller, J., & Jarvis, M. J. (2002). Sex differences in the association of socioeconomic status with obesity. *American Journal of Public Health*, *92*(8), 1299–304. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12144988

Weaver, I. C., La Plante, P., Weaver, S., Parent, A., Sharma, S., Diorio, J., … Meaney, M. J. (2001). Early environmental regulation of hippocampal glucocorticoid receptor gene expression: characterization of intracellular mediators and potential genomic target sites. *Molecular and Cellular Endocrinology*, *185*(1–2), 205–218.

West, A. P. (1990). Neurobehavioral studies of forced swimming: The role of learning and memory in the forced swim test. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *14*(6), 863–IN4. http://doi.org/10.1016/0278-5846(90)90073-P

Whiteford, H. A., Degenhardt, L., Rehm, J., Baxter, A. J., Ferrari, A. J., Erskine, H. E., … al., et. (2013). Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. *The Lancet*, *382*(9904), 1575–1586. http://doi.org/10.1016/S0140-6736(13)61611-6

Wichmann, M. W., Zellweger, R., DeMaso, C. M., Ayala, A., & Chaudry, I. H. (1996). Enhanced Immune Responses in Females, As Opposed To Decreased Responses in Males Following Haemorrhagic Shock and Resuscitation. *Cytokine*, *8*(11), 853–863. http://doi.org/10.1006/cyto.1996.0114

Wigger, A., & Neumann, I. D. (1999). Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. *Physiology & Behavior*, *66*(2), 293–302.

Will, C. C., Aird, F., & Redei, E. E. (2003). Selectively bred Wistar-Kyoto rats: an animal model of depression and hyper-responsiveness to antidepressants. *Molecular Psychiatry*, *8*(11), 925–932. http://doi.org/10.1038/sj.mp.4001345

Williamson, L. L., Chao, A., & Bilbo, S. D. (2012). Environmental enrichment alters glial antigen expression and neuroimmune function in the adult rat hippocampus. *Brain, Behavior, and Immunity*, *26*(3), 500–10. http://doi.org/10.1016/j.bbi.2012.01.003

Willner, P. (2005). Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. *Neuropsychobiology*, *52*(2), 90–110. http://doi.org/10.1159/000087097

Willner, P., & Belzung, C. (2015). Treatment-resistant depression: are animal models of depression fit for purpose? *Psychopharmacology*, *232*(19), 3473–3495. http://doi.org/10.1007/s00213-015-4034-7

Willner, P., Muscat, R., & Papp, M. (1992). Chronic mild stress-induced anhedonia: a realistic animal model of depression. *Neuroscience and Biobehavioral Reviews*, *16*(4), 525–534.

Willner, P., Towell, A., Sampson, D., Sophokleous, S., & Muscat, R. (1987). Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology*, *93*(3), 358–364.

Wittchen, H. U. (2002). Generalized anxiety disorder: prevalence, burden, and cost to society. *Depression and Anxiety*, *16*(4), 162–171. http://doi.org/10.1002/da.10065

Würbel, H. (2001). Ideal homes? Housing effects on rodent brain and behaviour. *Trends in Neurosciences*, *24*(4), 207–211. http://doi.org/10.1016/S0166-2236(00)01718-5

Würbel, H. (2007). Publications should include an animal-welfare section. *Nature*, *446*(7133), 257. http://doi.org/446257a [pii]\n10.1038/446257a

Yan, H.-C., Cao, X., Das, M., Zhu, X.-H., & Gao, T.-M. (2010). Behavioral animal models of depression. *Neuroscience Bulletin*, *26*(4), 327–337. http://doi.org/10.1007/s12264-010-0323-7

Yen, J. Y., Ko, C. H., Yen, C. F., Wu, H. Y., & Yang, M. J. (2007). The comorbid psychiatric symptoms of Internet addiction: attention deficit and hyperactivity disorder (ADHD), depression, social phobia, and hostility. *The Journal of Adolescent Health : Official Publication of the Society for Adolescent Medicine*, *41*(1), 93–98. http://doi.org/10.1016/j.jadohealth.2007.02.002

Yirmiya, R. (1996). Endotoxin produces a depressive-like episode in rats. *Brain Research*, *711*(1–2), 163–74. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8680860

Yirmiya, R., Pollak, Y., Barak, O., Avitsur, R., Ovadia, H., Bette, M., … Weidenfeld, J. (2001). Effects of Antidepressant Drugs on the Behavioral and Physiological Responses to Lipopolysaccharide (LPS) in Rodents. *Neuropsychopharmacology*, *24*(5), 531–544. http://doi.org/10.1016/S0893-133X(00)00226-8

Yoshiya, M., Komatsuzaki, Y., Hojo, Y., Ikeda, M., Mukai, H., Hatanaka, Y., … Kawato, S. (2013). Corticosterone rapidly increases thorns of CA3 neurons via synaptic/extranuclear glucocorticoid receptor in rat hippocampus. *Frontiers in Neural Circuits, 7*, 191. http://doi.org/10.3389/fncir.2013.00191

Zaias, J., Queeney, T. J., Kelley, J. B., Zakharova, E. S., & Izenwasser, S. (2008). Social and physical environmental enrichment differentially affect growth and activity of preadolescent and adolescent male rats. *Journal of the American Association for Laboratory Animal Science : JAALAS*, *47*(2), 30–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18351719

Zellweger, R., Wichmann, M. W., Ayala, A., Stein, S., DeMaso, C. M., & Chaudry, I. H. (1997). Females in proestrus state maintain splenic immune functions and tolerate sepsis better than males. *Critical Care Medicine*, *25*(1), 106–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8989185

Zucker, I., & Beery, A. K. (2010). Males still dominate animal studies. *Nature*, *465*(7299), 690–690. http://doi.org/10.1038/465690a