Developmental Programming and Experience-Dependent Plasticity of Brain and Behavior in the Rodent

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Abstract

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Experiences, both in early life and adulthood, can profoundly modify brain and behavior in mammals. In the Long-Evans rat, two salient components of early life experiences are i) the quality of maternal care received and ii) environmental housing parameters/conditions, both of which are capable of modifying numerous components of the developing CNS, including stress or hypothalamic pituitary adrenal axis (HPA) functioning. The following studies address modifications to both brain and behavior (i.e. stress physiology and behavior, executive function, and reproduction) in Long-Evans rat offspring due to variation in the aforementioned parameters. Understanding how basic environmental variables are capable of influencing brain and behavior is important not just in the study of basic neurobiology but also for the exploration of potential sources of psychopathology. Section 2 of this dissertation provides a review of HPA axis regulation, while Sections 3 and 4 discuss relevant primate and rodent models of early life stress axis calibration. Section 6 addresses how early life maternal care is capable of differentially programing sexual behavior and the underlying hypothalamic pituitary gonadal axis (HPG). Section 7 focuses on early life maternal care and developmental programming of stress physiology and executive function. Finally, Section 8 investigates the role of housing parameters, specifically the role of animal bedding, in regulating stress sensitive behaviors. All the studies demonstrate the powerful effect of early life experience and developmental programming of brain and behavior in the rodent.

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List of Abbreviations:

ABN	arched-backed nursing
AC	anterior cingulate cortex
ACE	adverse child experiences
ACTH	adrenocorticotropic hormone
ASST	attentional set shifting task
AVP	arginine vasopressin
AVPV	anteroventral paraventricular nucleus
BDNF	brain-derived neurotrophic factor
BLA	basolateral nucleus of the amygdala
BnST	bed nucleus of the stria terminalis
CB1	endocannabinoid receptor 1
CD	complex discrimination
CeA	central nucleus of the amygdala
CNS	central nervous system
CRH	corticotropin-releasing hormone
CRH-1	corticotropin-releasing hormone receptor 1
CRH-2	corticotropin-releasing hormone receptor 2
CSF	cerebral spinal fluid
DEX	dexamethasone
ELS	early life stress
EnD	endocrine disruption
ER	estrogen receptor
GABA	gamma-aminobutyric acid
GC	glucocorticoid
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptor
H	handled
HFD	high foraging demand
HPA	hypothalamic- pituitary- adrenal axis
HPG	hypothalamic-pituitary-gonadal axis
ID	intra-dimensional discrimination
IL	infra-limbic cortex
IPT	interpersonal trauma
LFD	low foraging demand
LG	licking and grooming
LTP	long-term potentiation
MeA	corticomedial nucleus of the amygdala
MPOA	medial preoptic area
MR	mineralocorticoid receptor
MS	maternal separation
NE	norepinephrine
NH	non-handled
NPC	neural precursor cell

PFC/mPFC	prefrontal cortex / medial prefrontal cortex
PL	pre-limbic cortex
PND	postnatal day
PR-ir	progesterone receptor immunoreactivity
PVN	paraventricular nucleus of the hypothalamus
R1, R2, R3	reversals 1-3
SAM	sympathetic-adrenomedullary axis
SD	simple discrimination
VCS	vaginal cervical stimulation
VFD	variable foraging demand
VMH	ventromedial nucleus of the hypothalamus

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Section 1: General Overview

Research from the Francis and Kaufer laboratories has focused on the relationship between early life events and later health and behavioral outcomes, particularly as it pertains to stress. Two potent driving forces in the development of neural systems that underlie stress reactivity are early life maternal care and environmental housing parameters (including bedding materials). In this dissertation, I investigate the contributions of each of these factors to offspring phenotypes reared under varying environmental conditions (see Section 5 – Aims of dissertation). A major theme of this dissertation pertains to how behavioral and physiological developmental trajectories can be altered in response to differential environmental input early in life, and how these changes may affect later life health **(Figure 1)**.

The HPA axis is very well conserved across mammalian species. Here, I employ rodent models of developmental programming to inform the human condition. Similar to rodents, the human early life period is particularly sensitive to environmental input from variations in parental care or early life stress, which can influence the organization of the brain at multiple (e.g. molecular, neural, system) levels.

Various components of parenting have been associated with children's emotional and psychological development later in life. Studies suggest that low levels of parental care can be a significant stressor in childhood, and can have long-lasting effects on brain development and mental health (Anda et al., 2006; Hill et al., 2001; Repetti et al., 2002). Similar to findings in animal models (see Section 3 & 4 – Primate and Rodent Models of Early Life Stress), low levels of maternal care are correlated with increased risk of depression among offspring in adulthood (Parker, 1993). This effect is particularly salient among female offspring, as low levels of maternal care increase the risk of major depression by four-fold compared to male siblings, adjusting for other childhood factors (Oakley-Browne et al., 1995). Furthermore, families with neglectful parenting, lack of support and nurturance, or high levels of conflict are reported to put children on an altered, often deleterious, developmental trajectory (Anda et al., 2006; Enlow et al., 2012; Hildyard and Wolfe, 2002; Repetti et al., 2002). Children who come from such family contexts, and are thus exposed to repeated stress, exhibit altered hypothalamic-pituitary-adrenal (HPA) axis, and sympathetic-adrenomedullary (SAM) axis functioning (Gunnar et al., 2009; Hildyard and Wolfe, 2002; Repetti et al., 2002). Adverse family environments are associated with blunted diurnal cortisol patterns and elevated cortisol responses to stress, which put children at risk for poor mental and physical health outcomes later in life, including anxiety and depression (Chapman et al., 2004; Repetti et al., 2002).

Many studies have demonstrated an association between harsh, chronic early life stress (ELS) and mental health in adulthood (Felitti et al., 1998; Kessler and Magee, 1993; Stang et al., 1988). ELS includes exposures such as child abuse, maltreatment, parental loss, and other forms of trauma. Roughly 49% of individuals exposed to ELS develop symptoms of depression and/or anxiety (Afifi, 2012; Anda et al., 2006; Tonmyr et al., 2011; Widom et al., 2007). In aggregate, studies demonstrate increased risk following childhood adversity, particularly in regards to anxiety and mood disorders (Anda et al., 2006; Comijs et al., 2007; Kessler, 1997; Widom et al., 2007). Importantly, there appears to be a dose-response relationship between ELS and mental illness, with increasing risk associated with higher number of stressful experiences (Anda et al., 2006; Kessler, 1997; Slopen et al.,

2010; Widom et al., 2007). This outcome is captured in the concept of allostatic load, or the build-up of stressful experiences, which affects health outcomes. One such study that captures this effect elegantly is the Adverse Childhood Experiences (ACE) study (Felitti et al., 1998).

The ACE study assesses the effects of adverse experiences during childhood on a range of health behaviors and outcomes. ACE categories include physical, emotional, or sexual abuse, maternal abuse, household substance abuse, parental separation or divorce, criminal household member, mental illness in the household, and household dysfunction. Tallying the total number of adverse experiences an individual was exposed to before age 18, an ACE score is generated. This provides both a conceptualization of early life stress (generated by retrospective self-report) and also allows for analysis of the cumulative, dose-response effects of ELS. The ACE score has a dose-response relationship with a variety of mental health outcomes, including depression, anxiety, panic reactions, suicide attempts, and substance abuse (Anda et al., 2006), as well as overall mental health (Edwards et al., 2003). Findings from the ACE study suggest that 54% of current depression and 58% of suicide attempts in women could be attributed to childhood adversity (Brown et al., 2009; Dube et al., 2001; Felitti, 2009). The dose-response relationship of the ACE score and the variety of mental health outcomes suggests that the neurobiological effects of childhood adversity are non-specific, influencing a variety of physiological parameters (including HPA reactivity), brain structures, and their functions (Anda et al., 2006). In adulthood, individuals who experienced ELS as children show alterations in HPA axis regulation, including altered diurnal cortisol patterns (Hart et al., 1996; Tarullo and Gunnar, 2006) as well as increased HPA reactivity to laboratory stressors (both psychological and pharmacological) (Danese and McEwen, 2012). Childhood maltreatment is also associated with decreased volume of the prefrontal cortex and hippocampus in adulthood.

From converging lines of evidence *across* species, it is becoming increasingly clear that early life stress and exposure to high levels of stress hormones can have deleterious outcomes on adult physical and mental health outcomes. Understanding how early-life experiences become 'biologically embedded' and represented in an organism becomes increasingly important. The use of the laboratory rat to study developmental programming of the stress-axis has been an invaluable tool. The ultimate goal, however, is to understand how developmental programming occurs so we can then begin to devise interventions to ameliorate the deleterious consequences of adverse early-life experiences, in rats and humans.

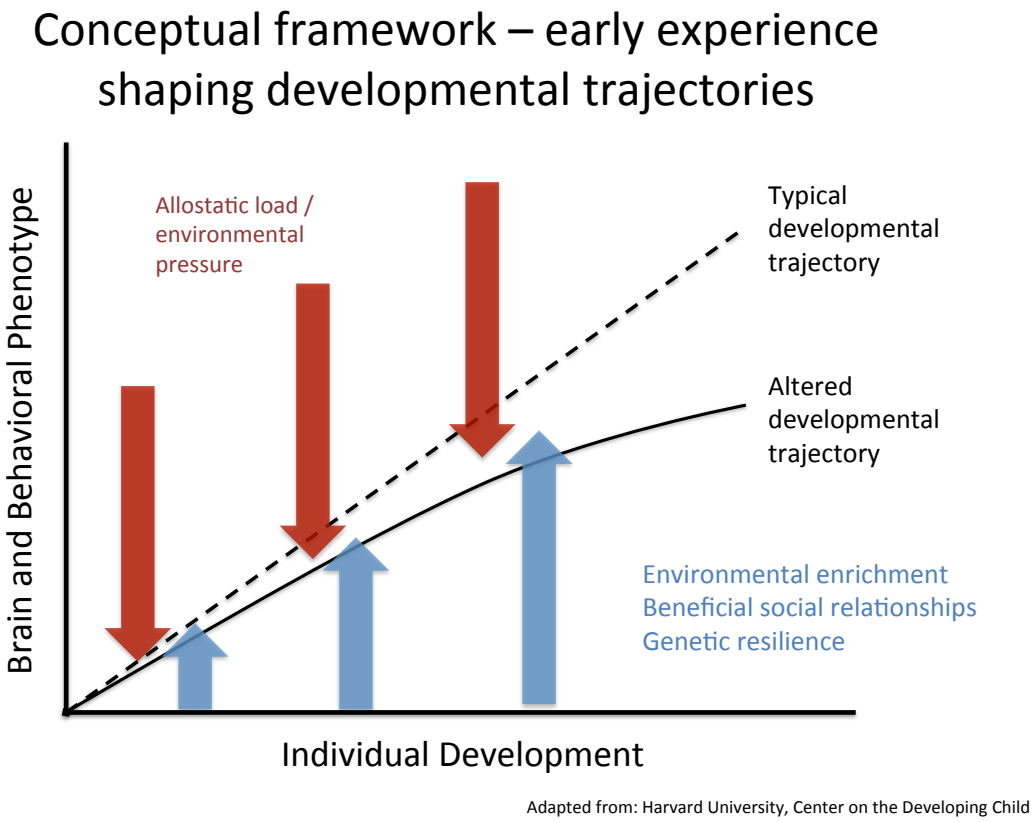


Figure 1: Conceptual figure illustrating how stressful experiences and environmental pressure early in life can skew developmental trajectories to alter individual brain and behavioral phenotypes.

Section 2: Introduction to the Stress Response

Introduction

Organisms face a wide variety of environmental conditions that can perturb homeostasis. To effectively respond to these "stressors," the organism must initiate a coordinated response across a variety of physiological systems. For example, the organism must perceive the stressor and select appropriate behavioral strategies (brain) and optimize energy resources towards a "fight, flight or freeze" response (cardiac, respiratory, skeletal) in part by shutting down systems that are not immediately essential (digestive, immune, growth)[1]. In the vertebrate stress response, the activation of these various systems is initiated by the release of glucocorticoid (GC) stress hormones from the adrenal glands, and also by catecholamine signaling. Importantly, the stressful situations that an organism encounters are not stereotypical. Stressors may be acute and severe (e.g. predation threat), chronic and severe (e.g. drought), or relatively mild (e.g. social interactions) with each type of stressor requiring a unique adaptive response. On the other hand, some types of challenges are predictable, and in these cases GC secretion can allow the organism to prime its physiological response in anticipation of the pending challenge. For example, in diurnal animals GCs are secreted in a daily circadian cycle, with high GC secretion inducing arousal during the day and a GC trough promoting rest during the evening. To respond to these wide variety of environmental challenges, ranging from mild to severe and predictable to unpredictable, vertebrates have evolved a complex regulatory system, the hypothalamic-pituitary-adrenal (HPA) axis, to perceive the severity of environmental challenge and release an appropriate amount of GCs for a measured, homeostatic behavioral response.

In this dissertation chapter, I describe the central role of the brain in the GC-mediated stress response. I describe the mechanisms by which the brain is involved in the initiation, maintenance and termination of an appropriate systemic response – in other words, regulation via the HPA axis. Secondly, I describe how various sub-systems of the brain respond to GC signaling to regulate stress behavior. In particular, I focus on the hippocampus, pre-frontal cortex, and amygdala, where GCs can induce a series of changes **(Figure 1)**. These include alterations that underpin behavioral responses such as alertness and cognitive function, appetitive versus aversive thresholds to various threatening stimuli and rewards (i.e. motivation vs. avoidance), fear, and memory formation. On a cellular and molecular level, this entails modulations of neurotransmitter levels, alterations in dendritic morphology, receptor density, and changes in signal transduction. Thirdly, I briefly discuss an apparent paradox in GC signaling: while exposure to glucocorticoids promotes the survival of an organism during stress, these same hormones can also cause damage and promote illness. Chronic stress is a risk factor for multiple diseases, including diseases of central and peripheral nervous systems such as stroke, mental illness, and multiple

[1] Hans Selye, the father of modern stress research defined stress as the "nonspecific response of the body to any demand made upon it" (Selye, 1976).

sclerosis (Bebbington et al., 1993; Brindley and Rolland, 1989; Cohen and Herbert, 1996; Johnson et al., 1990; Lupien et al., 2009; Sternberg et al., 1992). Within the CNS, chronic glucocorticoid exposure can suppress neurogenesis, bias cell fates of neural precursor cells, contribute to dendritic atrophy, and alter neuronal excitability in key regions of the brain involved in anxiety and depression. Therefore, an organism's best option is to mount as efficient a stress response as possible, limiting its exposure to high levels of catabolic and metabolically demanding glucocorticoids. Fine-tuning of stress response can have a dramatic influence on health. Importantly, the calibration and reactivity of the stress response is partly dependent upon early life environmental contexts and developmental programming, which help prepare organisms for future and current environmental challenges.

2.1 The Hypothalamic-Pituitary Adrenal Axis

As a first step in activating the HPA axis, the brain integrates external and internal sensory information pertaining to the immediate challenge, and this information is transduced into endocrine responses within the paraventricular nucleus of the hypothalamus (PVN) (Herman and Cullinan, 1997). The hypophysiotropic neurons within the PVN secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal system, a system of blood vessels that link the hypothalamus with the pituitary gland. Upon reaching the anterior pituitary, CRH stimulates the release of adrenocorticotropic hormone (ACTH) into circulation. Elevated ACTH levels, in turn, stimulate the synthesis and release of glucocorticoids via binding to melancortin-2 receptors within the cortex of the adrenal glands (Abdel-Malek, 2001). HPA activation results in a maximal rise in circulating GCs after 15-30 minutes, and returns to baseline levels at roughly one hour after the termination of a stressor (Sapolsky et al., 1984). The crucial ability to terminate the stress response, or inhibit the secretion of CRH and ACTH, is via glucocorticoid negative feedback on key neural regions, such as the PVN, anterior pituitary, mPFC, and hippocampus.

The stress response, as a whole, does not solely depend on GCs to alter physiology and behavior—it also requires the concerted actions of several other neuropeptides. These include: urocortins, which interact with CRH systems; vasopressin, which is implicated in stress-related social memory and emotionality; and orexins, which are involved with stress-related energy and circadian homeostasis. Furthermore, CRH interacts with many other brain regions outside of the PVN of the hypothalamus. For example, CRH is released in the bed nucleus of the stria terminalis (BnST) where it plays a role in stress-related anxiety. In the nucleus accumbens, CRH acts to suppress dopamine release in response to rewards, and shift appetitive and aversive behaviors (Lemos et al., 2012; Wanat et al., 2013) while in the amygdala and in the hippocampus it is involved in stress-related emotional memories, anxiety, and learning processes (Roozendaal et al., 2009; Sapolsky, 2003). A review of the actions of GCs on the brain is incomplete without considering the coordinated influence of the aforementioned peptide mediators, however, its discussion exists outside the scope of this chapter. For an excellent review, see (Joels and Baram, 2009).

2.2 HPA Negative Feedback

The ability of the HPA axis to respond dynamically to stress or to tonic secretion of glucocorticoids via circadian rhythm is determined, in part, by the ability of glucocorticoids to adjust ACTH secretion. This negative feedback occurs when GCs penetrate the blood-brain barrier and exert rapid (non-genomic) and slower (genomic) effects on the various neural regions that regulate ACTH release (Di et al., 2003; Giguère et al., 1986; Stahn and Buttgereit, 2008). Two classes of brain steroid receptors mediate negative feedback: the mineralocorticoid receptor (MR), and glucocorticoid receptor (GR). Both MR and GR belong to the nuclear receptor superfamily and function as transcription factors regulating gene expression (Reul and de Kloet, 1985).

MRs have a relatively limited distribution, exhibiting the highest expression within the subiculum/CA1 field and dentate gyrus of the hippocampus (Reul and de Kloet, 1985) **(Figure 2A)**. GRs are expressed nearly ubiquitously **(Figure 2B)**. There are, however, areas of greater GR density within the hippocampus, amygdala, cerebellum, hypothalamus (most notably the PVN), neurons of the ascending aminergic pathways of the brainstem, and to a lesser extent, the caudate nucleus and putamen (Fuxe et al., 1985; Patel et al., 2000). While both receptor subtypes bind corticosterone, MRs have a roughly tenfold greater affinity for GCs relative to GRs (Kd of ~0.5 nM for MR vs. Kd ~2.0-5.0nM for GR) (Reul and de Kloet, 1985). Consequently, GCs preferentially bind MRs over GRs and reach near-saturation levels during troughs of the circadian cycle (i.e. low basal levels), and are fully saturated during circadian peaks and stress. GRs are activated only when glucocorticoid levels reach a high concentration beyond the level that saturates MRs, such as during an acute stressor or during the zenith of the circadian rhythm. It is hypothesized that MRs are a critical component of the circadian regulation of baseline HPA tone (via fast-feedback, non-genomic actions), while GRs, occupied at higher corticosteroid concentrations, mediate feedback actions following stress (Atkinson et al., 2008). Thus, the balance of MR and GR receptor types, their occupancy, and their associated mechanism of action are intricately involved in HPA regulation.

2.3 Major Brain Structures involved in HPA Regulation

Four brain regions are strongly implicated as sites for HPA regulation and CRH and AVP synthesis. These include the PVN of the hypothalamus, frontal cortex, amygdala and hippocampus.

<u>Paraventricular Nucleus of the Hypothalamus</u>

The PVN is the main gateway for initiating the hormonal stress response, and thus a primary target for regulating HPA negative feedback. It contains one of the densest populations of CRH neurons, which express GRs (Chen et al., 2014; Uth et al., 1988). Exogenous application of GCs in the PVN results in a rapid decrease in CRH mRNA expression (Keller-Wood and Dallman, 1984) leading to a corollary decrease in HPA activation. Conversely, lesioning PVN afferents serves to increase expression of CRH and AVP mRNA demonstrating that neuronal inhibitory pathways are also necessary for the maintenance of HPA tone (Herman, 1995; Herman and Cullinan, 1997).

Non-genomic, fast feedback inhibition of the HPA axis within the PVN is dependent on both endocannabinoid and GABAergic mechanisms (Tasker et al., 2006). GCs stimulate the synthesis and release of endocannabinoids within the PVN by binding to membrane-bound MRs. These endocannabinoids then bind to presynaptic CB1 receptors to suppress glutamatergic transmission, thus inhibiting the activation of PVN neurons and reducing secretion of CRH (Di et al., 2003; Evanson et al., 2010; Hill and McEwen, 2010). GCs also bind to receptors on inhibitory magnocellular neurons of the PVN to stimulate fast, G-protein-dependent release of GABA to inhibit downstream CRH secretion (Tasker et al., 2006). In this fashion, MRs act in a rapid, non-genomic pathway for negative feedback within the PVN of the hypothalamus.

Medial Prefrontal Cortex

In rodents, the medial prefrontal cortex (mPFC) is comprised of infra-limbic (IL), pre-limbic (PL), and anterior cingulate cortices (AC) (based on structural connectivity and function, these areas are thought to be homologous to human Brodman areas 25, 32 and 24b respectively) (Uylings et al., 2003; Wallis, 2012). The mPFC is a region of the brain that is involved in cognitive and executive functioning, including working memory, the ability to shift attention across perceptual dimensions, and rule-guided action to plan and guide behavioral sequences. Receiving diverse afferent inputs from the amygdala and ventral hippocampus, as well as providing direct efferent connections to hypothalamic and monoamine brain nuclei, the mPFC is well situated to regulate cognitive, emotional and physiological responses to stress (Cerqueira et al., 2008).

The PFC is highly involved in autonomic control and HPA inhibition (Ahima and Harlan, 1990; Arnsten, 2009; Mizoguchi et al., 2004). Evidence for HPA suppression arises from lesion studies in which mPFC lesions can significantly increases plasma levels of ACTH and corticosterone following restraint stress and increase c-Fos activation in the PVN and medial amygdala (Brake et al., 2000; Diorio et al., 1993; Figueiredo et al., 2003; Sullivan and Gratton, 1999). Furthermore, local injections of corticosterone into the mPFC are capable of dampening plasma levels of these same hormones (Akana et al., 2001). Negative feedback effects may be attributable to GR and MR as chronic stressors, such as 4 weeks of daily restraint, leads to a down regulation of GR mRNA expression and protein levels in the PFC, resulting in attenuated PFC-mediated HPA negative feedback (Mizoguchi et al., 2003). Of note, the primate brain expresses significantly altered GR and MR distributions compared to rodents. In primates, GR levels are in greater abundance in the mPFC than the hippocampus, where there is a relative paucity in expression (Sanchez et al., 2000). This suggests that the primate PFC may play a larger role in GR mediated feedback than the hippocampus.

The mPFC influence over the HPA axis is both intra-region specific and exhibits hemispheric functional lateralization (Radley et al., 2006; Sullivan and Gratton, 1999). Pre-limbic and infra-limbic cortices exert opposing control over HPA tone. The pre-limbic cortex can be thought to be the 'brakes' whereas the infra-limbic cortex can be considered as the 'gas pedal' of HPA regulation. Electrical stimulation of pre-limbic cortex activates parasympathetic systems, whereas infra-limbic stimulation results in robust HPA activation. More recent work has confirmed that this dual control of HPA regulation that is GR dependent. GR knockdown in pre-limbic or infra-limbic cortices via short-hairpin RNA

leads to differential regulation of HPA secretion, such that infra-limbic disruption leads to HPA hyper-reactivity, while pre-limbic GR knockdown contributes to stress hypo-reactivity in response to an acute psychogenic stressor (McKlveen et al., 2013).

HPA control also exhibits hemispheric lateralization. HPA activation is markedly lower after right mPFC lesions, but not left (Sullivan and Gratton, 1999). HPA axis down-regulation after right mPFC lesion was found to be greater in response to chronic stress than to acute stress, suggesting that the mPFC is associated with regulating HPA activity during highly stressful conditions (Cerqueira et al., 2008).

Intra-region specificity of the mPFC (brake vs. gas) is stressor specific. It modulates its responses based on the nature of the environmental challenges presented, such as psychological stress vs. physical stress. The pre-limbic cortex ('brake') is of particular note in its role in inhibiting the HPA axis, especially with respect to psychogenic stressors. This is evidenced by inhibition of CRH and AVP expression after GC infusion into mPFC during restraint stress, a psychological stressor, but not the anesthetic ether, a physical stressor. Conversely, the infra-limbic cortex can initiate HPA activity. It responds robustly to both physical and psychogenic stress (McKlveen et al., 2013; Radley et al., 2006; Sullivan and Gratton, 2002). For instance, repeated social stress, but not noise stress, significantly increases ΔFosB expression, an immediate early gene used as a marker for neuronal activation, within the infra-limbic mPFC (Hinwood et al., 2011). This suggests that the mPFC plays a role in the ability to discriminate between psychogenic and physical stressors, thereby increasing the efficiency and specificity of HPA axis regulation (Diorio et al., 1993; Figueiredo et al., 2003; Radley et al., 2006).

Similarly to the PVN, mPFC-mediated HPA inhibition is dependent in part on GR-mediated endocannabinoid signaling. CB1 receptor antagonism within the mPFC up-regulates HPA activity and results in prolonged GC secretions. Furthermore, GC exposure results in endocannabinoid release, indicating homeostatic negative feedback. Mechanistically, increases in endocannabinoids lead to a decrease in GABA release. This results in a net gain in excitation on pre-limbic ('brake') neurons (Hill et al., 2011). The pre-limbic cortex has direct afferents onto GABAergic neurons within the BnST, which in turn send projections to the neurosecretory cells of the PVN. In this fashion, GC-induced CB1 activation of the pre-limbic mPFC results in an activation of inhibitory BnST-PVN circuitry. The net result is the suppression of HPA activity.

Responding to psychogenic and physiological stressors, the mPFC is a key component in the top-down regulation of the HPA axis. Part of this regulation is mediated by serotonergic mPFC-amygdala connectivity (Fisher et al., 2009). For example, decoupling serotonergic mPFC-amygdala circuitry leads to alterations in stress related behaviors (Andolina et al., 2013; Wellman et al., 2007). However, the amygdala also regulates the mPFC, thus is another critical component in the emotional guidance of behavior (Dilgen et al., 2013).

Amygdala

Receiving direct and indirect connections from limbic structures, including mPFC and hippocampus, the amygdala is thought to be a major integrating center for emotional and arousing stimuli. The amygdala is highly involved in the systemic stress response and is sensitive to both glucocorticoids and catecholamines. Direct stereotactic infusions of GC

to the amygdala greatly increase CRH mRNA expression within the PVN during psychogenic stress, illustrating the amygdala's capacity to alter HPA activity during elevated GC exposure (Shepard et al., 2003) .

Like the mPFC, the amygdala's influence over HPA systems is region specific. The amygdala is a complex of many sub-nuclei, often segregated into three regions: corticomedial (MeA), central (CeA), and basolateral (BLA) nuclei groups (Solano-Castiella et al., 2010). The CeA is further divided into a lateral component (CeL), and a medial (CeM) component. The balance of excitation and inhibition in each of these sub-regions modulates HPA reactivity (Carlsen, 1988; McDonald, 1982; Smith and Pare, 1994; Solano-Castiella et al., 2010). The amygdala has both "anxiogenic" and "anxiolytic" pathways. Stimulation of the BLA itself has been shown to increase HPA activity (anxiogenic), while direct stimulation of the CeM, results in anxiolytic effects. Since both the CeA and the BLA sends projections to the PVN via the BNST, the net effect of amygdala activation on the HPA axis is contingent upon the circuitry that is invoked (Pitts and Takahashi, 2011; Ulrich-Lai and Herman, 2009).

Hippocampus

Involved in cognition and memory formation, the hippocampus is a critical locus in HPA regulation. It exhibits tremendous plasticity to stress and glucocorticoids. Within the rodent brain, the hippocampus expresses the highest level of GR and MR, hence, it is of little surprise that it serves as an important negative feedback center and regulator of the stress response (Herman et al., 1992). Evidence for HPA negative feedback arises from early studies in which lesioning or blocking hippocampal GC receptors results in an up-regulation of CRH and AVP mRNA within the PVN. The consequence of this is hypersecretion of glucocorticoids (Jacobson and Sapolsky, 1991; Sapolsky et al., 1984). Conversely, activation of hippocampal GC receptors results in HPA axis inhibition (Sapolsky et al., 1984).

However, the relationship between hippocampus and HPA regulation are specific to particular hippocampal sub-regions. Structurally and functionally heterogeneous, the hippocampus can be segregated across a septotemporal axis (Bannerman et al., 2004; Fanselow and Dong, 2010). In rodents, the dorsal hippocampus appears to be more involved in learning and memory, while the ventral hippocampus is implicated in the modulation of anxiety-like behavior and HPA regulation (Bannerman et al., 2004). Lesions to dorsal hippocampus result in spatial memory deficits, whereas ventral hippocampal lesions result in alterations anxiety-like behaviors (Bannerman et al., 2004; Fanselow and Dong, 2010; Pentkowski et al., 2006). These differences in function can be explained in part by the connectivity of each region. For instance, regions of the ventral hippocampus project to areas involved in emotional regulation, most notably, the mPFC, amygdala, BnST, and the PVN (Fanselow and Dong, 2010).

The hippocampus is also one of two brain regions in which resident populations of neural stem cells (NSCs) produce new neurons in adult animals (Friedman and Kaufer, 2013; Gage, 2000). These new neurons are thought to contribute to the plasticity of hippocampal networks and have roles in the classical hippocampal functions of learning and memory. However, several lines of recent evidence also suggest that NSCs play a role in HPA regulation. First, chronic, but not acute, activation of hippocampal GRs is associated

with a general *increase* in HPA reactivity (Ridder et al., 2005). This failure in HPA axis negative feedback may be due, in part, to a reduction of the neurogenic pool resulting from chronic GC exposure. More direct evidence comes from ablation of hippocampal neurogenesis, through the use of techniques such as irradiation or by transgenic animal models, which results in impaired HPA negative feedback and elevated GC levels following recovery from restraint stress (Snyder et al., 2011). Animals with impaired neurogenesis also exhibit a depressed phenotype at baseline that can be reversed by antidepressant treatment (Snyder et al., 2011). Lastly, hippocampal neurogenesis appears to be required for antidepressants to restore HPA axis inhibition following chronic stress (Surget et al., 2011). Taken as a whole, these and other findings have implicated hippocampal neurogenesis as a component of HPA axis regulation.

2.4 Effects of Glucocorticoids on Brain and Behavior

As described above, many brain regions that integrate sensory information, such as the mPFC, amygdala, and hippocampus, can exert control of the PVN to fine tune the stress response according to the immediate experiences of the animal. In turn, once the stress response is initiated, the animal also has to enact appropriate behavioral strategies to cope with the stressor. Thus, beyond acting as a negative feedback signal, GCs also modulate brain function in these same regions to coordinate appropriate stress-response behaviors.

Medial Prefrontal Cortex

Stress, both mild and severe, can lead to functional and structural changes in the prefrontal cortex (Arnsten, 2009; Goldwater et al., 2009; Holmes and Wellman, 2009). This includes alterations in dendritic arborization and spine density in all regions of the mPFC (IL, PL, and AC) and neighboring orbitofrontal cortex, which is driven in part, by GR signaling (Butts et al., 2011; Cerqueira et al., 2005; Liston and Gan, 2011; Liston et al., 2006; Popoli et al., 2011; Yuen et al., 2009). For instance, 3 weeks of corticosterone administration (Wellman, 2001) or daily restraint stress (Cook and Wellman, 2004), is capable of reducing dendritic arborization and spine density in the mPFC and dorsomedial striatum (Czeh et al., 2007; Dias-Ferreira et al., 2009). Functionally, both glucocorticoid administration and stress leads to deficits in working memory (Butts et al., 2011; Cerqueira et al., 2005; Mizoguchi et al., 2000; Roozendaal et al., 2004), mPFC dependent set-shifting (Liston et al., 2006), as well as reversal learning (Cerqueira et al., 2007; Cerqueira et al., 2005; Lapiz-Bluhm et al., 2009).

Paradoxically, under some conditions, chronic stress can facilitate reversal learning (Graybeal et al., 2011; Graybeal et al., 2012). One hypothesis by Dias-Ferreira and colleagues as well as Schwab and Wolf, posits that stress leads to a disinhibition of PFC functions and towards striatal mediated learning (Dias-Ferreira et al., 2009). The effect is bias in an organism's behavior towards habit formation (Dias-Ferreira et al., 2009; Graybeal et al., 2012; Schwabe and Wolf, 2009, 2011). Indeed, severe, repeated stressors result in an increase in apical dendrite arborization in both the dorsolateral striatum and orbitofrontal cortex, regions involved in habitual strategies, reward valuation, and reversal learning(Dias-Ferreira et al., 2009; Liston et al., 2006). These studies suggest that the

effects of stress and glucocorticoids may be beneficial to shift behaviors toward optimal behavioral adaption to environmental stress.

Amygdala

Its activation by GCs can lead to alterations in learning and memory (Roozendaal, 2000). However, it is important to note that amygdala contributions to autonomic functioning are with respect to emotional arousal, not circadian or homeostatic HPA regulation.

Both acute and chronic stress results in the remodeling of synapses and dendritic branching within the amygdala (Mitra et al., 2005; Mitra and Sapolsky, 2008). Stress induced synaptic plasticity is modulated by GABAergic inputs (Davis et al., 1994). These changes are correlated with an increase in anxiety-like behaviors and enhanced fear conditioning (Conrad et al., 1999; Mitra and Sapolsky, 2008; Wood et al., 2008). High levels of corticosterone reduce GABA transmission, which results in an increase in the firing rate of excitatory neurons in the basolateral amygdala (Duvarci and Pare, 2007). This suggests that high levels of glucocorticoids can change the balance between excitation and inhibition, resulting in modifications in synaptic connectivity. These changes can influence neuronal plasticity even in distal brain regions. Recent findings demonstrate that the BLA can alter synaptic plasticity and long-term potentiation in the striatum and hippocampus. Therefore, it is becoming increasingly clear that glucocorticoids within the amygdala can be far-reaching and impactful (Akirav and Richter-Levin, 2002; Popescu et al., 2007).

Finally, the amygdala also plays a central role in enhancing memory consolidation following emotionally arousing events. High levels of circulating GCs can improve the recall of a stressful event (Bohannon, 1988; Neisser et al., 1996; Roozendaal et al., 2009). However, GCs effects may be mediated by ß-adrenergic activation; blockade of ß-ardrenergic receptors within the BLA prevents memory enhancements following GR activation. Furthermore, activation of BLA via emotional arousal is critical in GC-mediated memory enhancements (Quirarte et al., 1997). Enhanced memory performance following a stressful event can be advantageous, as future encounters to similarly arousing stimuli would result in a feed-forward HPA activation to prime physiological systems in anticipation of a stressor. However, more investigations are needed to fully determine how stress, NE, and GCs influence different phases of fear learning and its expression in memory.

Hippocampus

The hippocampus responds dynamically to changes in GCs levels by modulating neuronal structure and function. GCs directly influence hippocampal function by acting as neuromodulators to influence neural excitability and signaling (Chameau et al., 2007; Karst and Joëls, 2005; Karst et al., 2000; Krugers et al., 2005; Maggio and Segal, 2009). More broadly, GCs also affect the structural connectivity of the hippocampus by affecting dendritic arborization and formation of synapses (Watanabe et al., 1992; Woolley et al., 1990; Yoshiya et al., 2013). Together, a model has emerged from these studies in which mild or acute stress increases hippocampal dendritic branching and long-term potentiation to boost hippocampal learning and memory, while chronic or high GC concentrations have

opposite effects (Conrad et al., 1999; Herman and Spencer, 1998; Kirby et al., 2013; Sapolsky, 2003). However, the distribution of MR and GR receptors differs in dorsal versus ventral hippocampus, with ventral hippocampus having a much higher relative concentration of MR (Robertson et al., 2005). This suggests that the effect of stress on hippocampal function may be more nuanced and region-specific, such that high levels of GCs do not simply suppress hippocampal memory function in general, but rather specifically suppress the contextual memory functions of dorsal hippocampus while promoting the emotional cognitive functions of the ventral hippocampus (Maggio and Segal, 2010, 2012). This model fits well with the overall paradigm of the stress response as an adaptive mechanism that manifests stress-specific behavioral strategies suited to overcoming stressful challenges (Maggio and Segal, 2010, 2012).

Stress effects on hippocampal-mediated behaviors may also regulated through the contributions of hippocampal NSCs. NSCs express functional GRs (but not MRs) (Chetty et al., 2014; Garcia et al., 2004), and their rate of proliferation and differentiation, as well as the survival of the new neurons that they produce, are altered by GCs (Gould et al 92; Kirby et al 2013; Dranovsky and Hen 2006; Wong and Herbert 2004; Wong and Herbert 2006). The effects can be via direct activation of GR in the NSC (Garcia et al., 2004) or indirectly, through activation of GR-dependent mechanisms in other cells in the hippocampal niche. For instance, acute corticosterone exposure elicits release of fibroblast growth factor-2 from astrocytes in the dorsal hippocampus, leading to increased proliferation of neural stem cells in the area (Kirby et al., 2013).

The effects of stress on adult neurogenesis can be divided into the effects of acute stress and repeated, chronic stress. Chronic, repeated stressors inhibit NSC survival, proliferation, and neuronal differentiation within the dentate gyrus (Kirby and Kaufer, 2009; Mirescu and Gould, 2006; Wong and Herbert, 2004). However, the effects of acute stress display a more mixed picture, ranging from a decrease, increase, or no change in NSC proliferation (Dagytė et al., 2009; Hanson et al., 2011; Thomas et al., 2007; Thomas et al., 2006). One explanation for discrepancies in the literature may be that, like cognitive performance in response to stress, adult hippocampal neurogenesis follows an inverted U function—increasing in response to acute stressors and decreasing in response to high, chronic GC exposure. For example, high levels of transient GCs can inhibit NSC proliferation in the SGZ, and this effect can be blocked through adrenalectomy (Tanapat et al., 2001). Additionally, a recent study demonstrates that mild, acute stress can *increase* dorsal hippocampal cell proliferation via astrocytic mediated fibroblast growth factor 2 secretion (Kirby et al., 2013). Beyond proliferation, high levels of GCs may also reduce the total number of new neurons by decreasing the survival of immature neurons as they begin to incorporate into the network (Wong and Herbert 2004). Furthermore, GCs cause a shift in the cell fate of differentiation NSCs, causing them to more frequently differentiate into oligodendrocytes at the expense of neurogenesis (Chetty et al 2014). Given that new neurons ultimately confer additional plasticity onto hippocampal networks, by forming new synaptic connections and showing enhanced capacity for LTP (Ge et al., 2007; Gu et al., 2012; Marín-Burgin et al., 2012), the reductions in neurogenesis in response to elevated GCs may be one of the mechanisms underlying reduced memory capacity in stressed animals.

2.5 Conclusion

Glucocorticoids regulate the brain and behavior in multiple domains. They help adjust basal and peak HPA axis reactivity (Sapolsky et al., 1984), as well as alter limbic structures (PVN, mPFC, amygdala, hippocampus), both structurally and functionally. This includes alterations in synaptic plasticity, long-term potentiation, and neurogenesis, which result in changes in appetitive and avoidant behaviors, and modifications in learning and memory. Additionally, the limbic system is responsible for the top-down and bottom-up regulation of the HPA axis through its complex micro-circuitry. In this sense, HPA regulation can be a recursive process since glucocorticoids modulate both initiators and terminators of the stress response.

Ultimately, stress is necessary for the optimization of behavior to environmental pressures. With respect to humans, it is HPA axis dis-regulation that is implicated in the pathogenesis of many disease phenotypes such as anxiety and depression (McEwen, 1998). It is of paramount importance to efficiently initiate a stress response, as it promotes survival, while equally important to terminate the stress response, as glucocorticoids are metabolically demanding and can lead to disease.

Figure 1: Selected limbic structures involved with HPA axis regulation

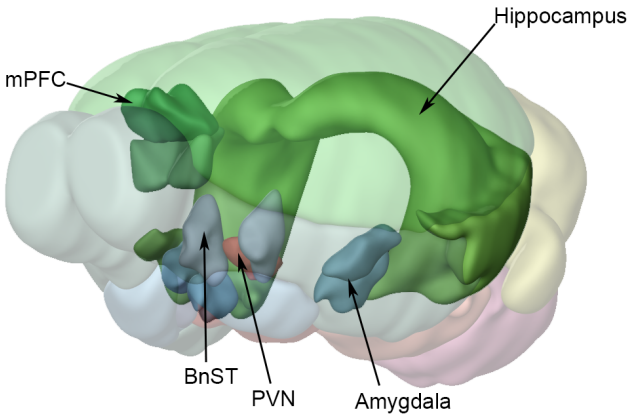


Figure 2A: MR distribution in the mouse brain

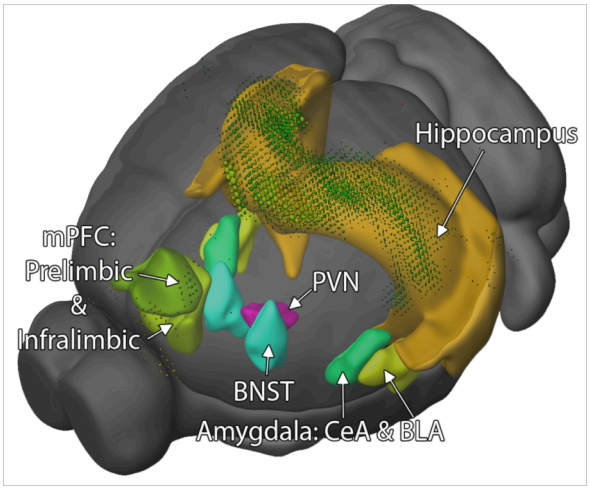


Figure 2B: GR distribution in the mouse brain.

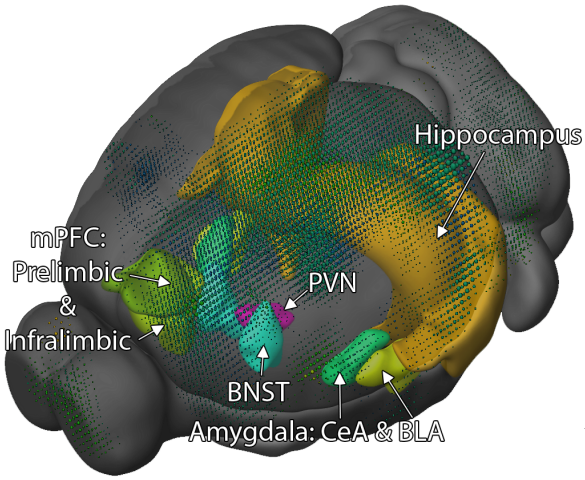


Figure Credits: Shawn Shirazi
Images rendered from the Allen Brain Atlas

Section 3: Models of Early Life Stress in Primates

Introduction

In Section 2 of this dissertation, the role of glucocorticoids in modifying neural and behavioral systems, as well as the principles of HPA regulation, were reviewed. In this section, we examine models of early life environments and their contributions to physiology, stress reactivity, and behavior in non-human primates. The impetus for this section is to emphasize different models of adverse early environments in non-human primates (hitherto referred to as primates) and subsequent developmental outcomes. Much like the rodent work (to be reviewed in Section 4), data from early life models in primates highlight the sensitivity of infancy and childhood to the long lasting effects of developmental programming on behavior and biology. In animal models, changes in mammalian biology due to adverse or altered early life experiences, whether social or psychological, have been shown to influence the regulation of the HPA axis, neural development, gene expression, methylation profiles, patterns of neural connectivity, structural changes in the central nervous system, and ultimately, the behavior of the organism (Floeter and Greenough, 1979; Greenough and Black, 1992; Kandel, 1999; Post, 1992). More recent work from the last three decades has focused on how experiences during "sensitive" developmental periods can become instantiated at the level of the brain to influence future behavior. Early life models in primates have shown a clear relationship between disturbances early in rearing, particularly as they pertain to attachment and later behavioral and physiological changes (Fahlke et al., 2000; Fairbanks, 1989; Schneider et al., 1998). Part of these changes involves alterations in HPA axis and related neuroendocrine systems, shifting basal and recovery cortisol profiles (Andrews and Rosenblum, 1991; Clarke et al., 1998; Higley and Linnoila, 1997; Lyons et al., 2010; Maestripieri, 2001; Parker et al., 2012). Physiological alterations documented in primates are not restricted to the HPA axis, as structural changes in CNS function have been documented by altered early life experience as well. For instance, in squirrel monkeys, early life psychosocial stress reduces GR expression profiles (but not MR) in layer I and II of dorsolateral PFC, while adult psychosocial stress can alter GR hippocampal CA1 distributions (Patel et al., 2008). Given the profound developmental outcomes of early life, it should not be surprising that adverse environments can serve as a major risk factor towards adult psychopathology, including mood and anxiety disorders, substance abuse, and post-traumatic stress disorder (Sameroff, 2000) (**Figure 1**). In the primate, the majority of early life research has centered on the mother-infant dyad, attachment, and the development of stress physiology due to pragmatic and historical causes.

3.1 Benefits of Primate Models (or the constraints of rodent models)

Primate models of early life stress and pathology provide advantages over rodent studies while escaping constraints of human observational work. Shorter lifespans allow researchers to observe the entire life course within an experimental setting. Despite greater maturity at birth, infant primates share similar gestational characteristics and early development to humans (Schneider et al., 2002). Primates also live in complex social

groups with mothers generally giving birth to one infant, which spends virtually all of its first month of life in tight physical contact with its mother (Higley and Linnoila, 1997; Levine, 2005; Suomi, 2006). These small mother-infant dyads ease observation of parental care, which has shown strong reciprocal attachment between mothers and infants during a critical period of development. For example, infants will increase vocalizations in protest to separation and exhibit immediate HPA reactivity that is not prone to adaptation even after more than 80 separations (Hennessy, 1986; Simons et al., 1968). These factors provide a strong model in which to study environmental and biosocial factors of early life stress (Harlow, 1969; Higley and Danner, 1988; Maestripieri, 2001; Simons et al., 1968).

The close evolutionary heritage between humans and primates also provides a firm foundation on which to draw inference towards humans in experimental paradigms. Humans, apes, and old world monkeys share roughly 90-95% of our genome (Goodman, 1999). A large portion of our physiology is homologous, including HPA physiology. Despite a large degree of conservation between mammalian species, rodent models are limited by differences in stress neuroendocrinology from humans and primates. Differences include incongruence in primary stress hormone, in which rodents possess the main glucocorticoid corticosterone, while humans and primates possess cortisol (Gunnar and Quevedo, 2007). Glucocorticoid receptor (GR) distributions between rodent, human, and primate brain also vary, with rodents expressing the majority of GR in the hippocampus, while humans and primates have a relative paucity in GR density in the hippocampus, and more of abundance in the frontal cortex (Ahima and Harlan, 1990; Galeeva et al., 2006; Sanchez et al., 2000; Seckl et al., 1991). Structural and functional differences in prefrontal anatomy also constrain interpretation of results from rodent studies, particularly as explanations of stress reactivity are often investigated from a perspective of top-down models of cognitive control (Amat et al., 2005). Together, these differences may have implications for HPA programming between species as well as the influence of stress on cognition and behavior.

Compared to rodents, humans and primates also exhibit developmental differences in stress reactivity. Rodent early life is hallmarked by a period of altered stress responsivity in which they have reduced or absent physiological responses to mild, specific stressors coupled with low circulating corticosterone levels. Humans and primates early in life do not exhibit marked differences in stress reactivity between infancy and adulthood (Gunnar, 1992; Molet et al., 2014). This is of particular importance when discussing animal models of early life stress, as maternal separation paradigms in rodents generally take place during this period of altered, HPA hypo-reactivity. Paradoxically, lack of maternal stimulation during longer separations of three hours or more results in increased corticosterone reactivity despite being in a stress hypo-responsive developmental period (Huot et al., 2002; Schmidt et al., 2003). These animals are also more stress hyper-responsive later in life (Schmidt et al., 2003). One interpretation of maternal care in rodents posits that maternal stimulation during this period may be actively inhibiting HPA activation, effectively shielding offspring from the 'slings and arrows' of early life and the damaging effects of glucocorticoids (Korosi and Baram, 2010). The mechanisms of action in HPA physiology are better understood for rodents than for primates, and there is little consensus regarding the long-term influence of early life experiences, and the nature of those experiences, on primate HPA physiology. Although a general pattern of altered HPA functioning has emerged in primates raised in adverse environments, enduring and consistent results regarding the influence of early life on primate HPA systems has been

more controversial. Conflicting literature due to methodological differences in timing, intensity, and duration of challenging experiences have only highlighted how malleable and complex the psychobiology of an organism can be.

3.2 Foundations of Early Life Primate Models

The idea that early experiences serve as the foundation of adult behavior has been a key feature of many developmental theories for the last century. Freud's concepts of early life and personality development, the work of Konrad Lorenz on early imprinting in geese, and Seymour Levine's work in rodent stress reactivity to handling were all predecessors of early life primate models. Primate models of early life gained particular notoriety in the 1960's by the work of Harry Harlow, who at the time was investigating learning capabilities in rhesus macaque. After creating a lab-breeding program in order to assess developmental differences in learning, Harlow sequestered newborn infants from their mothers shortly after birth to prevent the spread of disease, to limit confounding variables of rearing, and in consequence bottle-fed infants. Researchers soon observed that the infants who were given cheesecloth blankets fiercely clung to them, and were visually upset when the blankets were removed for cage cleaning. These observational eventually led to a test of "derived-drive theory", in which infant attachment to mothers was theorized to be a learned trait and derived from instant satisfaction of higher motivations such as food, and thirst. To assess this, separated infants were exposed to a cloth "mother" mannequin and a wire "mother", the former providing comfort, while the other provided milk. Infant monkeys shunned the food-providing wire mother, and attached, quite literally, to the cloth mother. Further work explored infant preferences, with infants attaching to rocking mothers over stationary ones, and warm mothers over cold moms (quite literally. In one experiment, Harlow and colleagues pumped ice water through the metallic hollow tubes of the wire mothers to assess preferences) encased in terry cloth. The results dismantled derived drive theory, and brought the idea of contact and comfort as a primary motivation in infants. Infant behavior after separation was also markedly changed, including high stress reactivity and stereotyped movements. These early works served as a spring board for both "attachment theory" in humans, in which infant relations and initial attachment with their mother is capable of fundamentally influencing future social relationships, as well as a model of early life stress and deprivation in non-human primates (Bowlby and Ainsworth, 1988). With the exception of studies investigating genetic polymorphisms and inheritance on stress phenotypes (Suomi, 1997, 2006), primate stress neurobiology research has mainly focused on altering maternal attachment as a model. This includes paradigms of rearing in isolation, peer rearing, and maternal separation, as well as manipulations directed at influencing maternal care via environmental change, with the aim of influencing offspring through maternal mediation.

3.3 Maternal Deprivation/Isolation Paradigms

Used primarily as a primate model of depression, maternal deprivation/isolation conditions often resulted in extreme and often permanent behavioral deficits. Behavioral abnormalities developed by isolated infant monkeys resembled a nondescript profile of extreme psychopathology. Behavioral abnormalities included gross deficits in social and

emotional functioning, that included, but was not limited to, changes in aggressive, reproductive, and parenting behaviors (Harlow et al., 1965). In such investigations, infants were generally separated from mothers at one week of life until later time points (3 – 12 months) of age and kept in social isolation from natal groups. Monkeys subjected to isolation after three months exhibited an exaggerated and prolonged response to stress, appetitive changes, sexual dysfunction, and select learning deficits (Harlow et al., 1971). Idiosyncratic behavior of isolates included self-grasping, huddling, self-rocking, and self-mouthing, in a stereotyped fashion. Some in isolation would freeze in their cage clenching their hand and raising it into the air only to leave it immobile for a lengthened period of time. Fear and aggressive behavior was also increased. Total isolation for 12 months resulted in almost complete removal of play behavior and exploration. Animals displayed little to no aggression, huddling in corners or against walls of the room. Upon reintroduction with control animals, one experiment was stopped as controls were literally "tearing up the 12 month old isolates". Interventions designed to cultivate normal social behavior in these animals were not successful (Harlow et al., 1971).

Changes in in offspring neuroendocrine function were significant in their scope as well, including changes in biosynthesis pathways for dopamine and norepinephrine in the substantia nigra, striatum, and ventral tegmental area, diminished dendritic branching in primary motor and somatosensory cortex, reduced cerebellar purkinje soma cell size, and alternations in neurofilament protein expression, which provide integrity of cell structure support, in the hippocampal formation (Floeter and Greenough, 1979; Martin et al., 1991; Siegel et al., 1993; Struble and Reisen, 1978).

Fortunately, no human cases of social isolation meet the scope, length, and developmental period of isolation paradigms used in monkeys. The lack of specificity and applicability of isolation models to human psychopathology lead to significant criticism of this paradigm. For this reason, along with significant ethical considerations of animal care, primate maternal deprivation is used less often in today's research.

3.4 Peer-Rearing, Maternal Separation Paradigms and Stress Inoculation

Other studies have attempted to develop more nuanced procedures that provide a greater degree of social stimulation during infancy than early isolation paradigms. Monkeys reared in environments of acute maternal separation and peer-rearing exhibit aspects of species normative behavior and develop little of the bizarre and stereotyped behavior of isolated animals (Parker et al., 2012). Still, short periods of maternal separation are capable of altering behavioral and physiological phenotypes over the short and long term.

The majority of rearing paradigms have involved separating infants from their mothers and natal groups for short periods of time (a few hours) up to several weeks (maternal separation). In the case of peer-rearing paradigms, infants were separated at birth, hand reared in a nursery for the first month, and then reared with peers of the same age until puberty (Arabadzisz et al., 2010; Harlow, 1969; Higley and Danner, 1988; Lyons et al., 2002; Spencer-Booth and Hinde, 1971). These peer groups can contain mother-reared infants, as well as older adults (Suomi, 1997).

The behavioral and neuroendocrine effects of separation and peer rearing have generally produced opposite results in anxiety behavior, HPA reactivity, and behavior. Peer rearing paradigms have generally resulted in stress hyper-reactivity, while 'milder'

maternal separation paradigms result in 'stress resilience' or decreased HPA reactivity later in life. Separations from mothers across both paradigms yield strong and consistent results in triggering an increase in ACTH and cortisol upon initial and future separations from mothers (Suomi, 1997).

Peer Rearing

After separation from mothers, peer-reared rhesus monkeys develop strong attachment bonds to one another (Suomi, 1997). These attachment relationships tend to be 'anxious' in nature as peers prove to be ineffective replacements for typical monkey mother. Lacking a "secure base" provided by motherhood, peer-reared infants are unable to balance free exploration while reducing fear responses to novelty, social pressure, and stress. In consequence, peer-reared monkeys show normal motor and physical development; however, as adults, they are highly anxious, less socially competent, and show a reduction in play behavior that may be a consequence of increased anxiety (Chamove et al., 1973; Higley and Linnoila, 1997; Higley et al., 1996). Biochemically, young adult peer-reared animals exhibit chronic elevation of cortisol and ACTH compared to mother-reared animals and display greater HPA reactivity when socially separated from peers (Champoux et al., 1989; Clarke et al., 1998; Fahlke et al., 2000; Fairbanks, 1989; Higley et al., 1996). Socially, peer-reared animals are less likely to engage in normative social huddling behaviors as adults, instead exhibiting infant-like clinging behavior. They are also more likely to engage in disruptive social behaviors and typically fall to the bottom of the social hierarchy when grouped with mother-reared monkeys of their age (Raleigh and McGuire, 1991, 1994).

Peer rearing also has long-term developmental consequences on behavioral profiles of impulse control, particularly as it pertains to aggression and reward. Peer-reared animals are more aggressive than maternally reared counterparts. Aggression, which initially begins in juvenile play behavior, exceeds levels of mother-reared animals as puberty approaches. These aggressive behaviors are also more likely to be directed in socially inappropriate contexts, suggesting deficits in impulse control (Higley et al., 1994). Furthermore, peer-reared offspring consume more alcohol per kilogram of body weight (Higley et al., 1996). Physiologically, peer-reared animals also show abnormal CNS monoamine activity, exhibiting increased cerebral spinal fluid (CSF) serotonin metabolite 5-HIAA and norepinephrine metabolite MHPG. Peer-reared animals also have enlarged medial prefrontal volume than mother-reared monkeys (Higley et al., 1991; Spinelli S, 2009). Taken together, these results suggest that peer rearing is capable of fundamentally altering reward processing and impulse control, possibly at the level of the orbito-frontal striatal circuits.

Maternal Separation and Stress Inoculation

A commonality between peer rearing, attachment, and deprivation studies is the idea that maternal care serves as a link between environments and offspring. Thus, the mother is thought to be the main developmental arbiter of infant HPA responsivity. The maternal mediation hypothesis posits that lack of proper attachment or maternal care leads to stress hyper-reactivity later in life. An alternative to the maternal mediation

hypothesis is the stress inoculation hypothesis, which contends that mild stress early in life is necessary for the development of stress resilience later on in development (Parker et al., 2006). The term "inoculation" was given as an analogy to immunity, in which natural or therapeutic exposure to a mild pathogen can strengthen immune resistance. In this vein, early exposure to mild stress may "inoculate" organisms to future stressors, lead to stress resilience, and reduce risk of stress-related psychopathology (Parker et al., 2006). In primates for instance, infants exposed to the stress of weekly one-hour maternal separations develop stress resistance in young adulthood characterized by reduced basal cortisol levels, lowered anxiety, and dampened sympathetic responses to a variety of stressors, including restraint stress and social separations compared to un-manipulated monkeys (Arabadzisz et al., 2010; Feng et al., 2011; Levine, 2005; Parker et al., 2012). Separations from natal groups also increased cortisol induced suppression of CRH stimulated ACTH secretion, suggesting that animals from postnatal environments of 'mild' separation exhibit more efficient stress negative feedback capacity at eight years of age (Lyons et al., 2000). Importantly, findings of reduced HPA activity before and after an acute stressor was not predicted by maternal care, but to prior stress exposure, lending evidence to the phenomena of stress inoculation (Parker et al., 2006).

More evidence for the benefits of mild early life stress independent of the mother has been shown in marmosets. Short term maternal separations have been shown to increase levels of maternal contact (Parker et al., 2012; Parker et al., 2006) upon reunion; however, mothers *do not show an increase in overall care* despite increased solicitations, contact, and ventral clinging by more anxious infants (Arabadzisz et al., 2010; Spencer-Booth and Hinde, 1971). As adults, these animals exhibit decreased basal cortisol levels, a finding in support of stress inoculation. Lowered anxiety-like behavior in stress-inoculated monkeys may also be due to changes in noradrenergic functioning, as these animals show decreased levels of norepinephrine metabolite MHPG (3-methoxy-4-hydroxyphenylethylene glycol) in cerebrospinal fluid compared to non-inoculated monkeys. However, whether dampened HPA response is due to alternations in stressor perception (i.e. less challenging or fearful), or due to a neurobiological deficit in mounting an appropriate physiological response to a challenge is still an unresolved question.

In addition to changes in HPA activity, stress-inoculated monkeys show greater aspects of cognitive control and behavioral inhibition on tasks that assess prefrontal cortex function. They also exhibit an 8-14% enlargement in portions of the medial prefrontal cortex (McEwen, 2008; Parker et al., 2012). This suggests, that like peer rearing, mild stress early in life can alter reward processing and impulse control.

3.5 Variable Foraging Demand, Environmental Unpredictability, and Maternal Care

Environmental context is crucial to understanding variation in offspring behavior. In rodent studies, levels of maternal care seem to increase with greater environmental demands; however, when environmental demands become severe, maternal care is reduced influencing HPA reactivity. Still, independent of maternal effects, environmental pressures can influence offspring directly (Cameron et al., 2005; Coutellier et al., 2009). These effects are consistent with ecological theories of behavior, which hypothesize conflict between maternal motivations to rear offspring, reproduce, and forage, all of which

can be impinged upon by harsh or competitive environments (Trivers, 1974). In consequence, levels of parental investment are hypothesized to be an adaptive signal to offspring; preparing them for future environments they may encounter[2]. Environmental influences on maternal behavior are evident in baboon studies in which maternal stress has been shown to diminish levels of maternal care (Brent et al., 2002; Maestripieri and Carroll, 1998). Furthermore, in pigtail macaques, higher rates of infant abuse were reported in mothers after acute stressors, despite mothers possessing adequate mothering skills and social behavior compared with control animals (Maestripieri and Carroll, 1998).

Laboratory studies have shown that short-term changes in environmental demands can have long-term lasting consequences on offspring behavior and physiology. Congruent with the maternal mediation hypothesis, which states that maternal care serves as the link between environments and offspring, changes in infant behavior seem to be driven by disruptions to the mother-infant relationship. Influential studies by Rosenblum and colleagues tested the hypothesis of environmental influence on parental behaviors by exposing mothers and infant bonnet macaques to conditions that altered the effort to obtain food. These foraging demand conditions varied between relative unpredictability and periods of easier access. Mothers were required to forage in conditions with relatively stable, plentiful food availability (low foraging demand condition; LFD), in environments with low yet predictable food availability (high foraging demand conditions; HFD), or in settings that were designed to emulate unpredictability (variable foraging demand condition; VFD) (Rosenblum and Paully, 1984). Importantly, manipulations of foraging demand did not result in food deprivation or altered growth across groups, enabling interpretation of results to focus on environmental regulation of parental care.

Mothers in the VFD conditions displayed changes in both peer-oriented social behavior and more disorganized maternal investment compared with HFD and LFD animals. Socially, VFD moms displayed altered patterns of dominance, increasing hierarchical behavior, and lowering rates of affiliation towards peers; however, levels of aggression towards each other or infants did not differ between groups (Rosenblum and Paully, 1984). Changes in group hierarchy and group affiliation may only exacerbate maternal stress, further altering maternal behavior and offspring interactions. Much like short-term separation paradigms, maternal interactions with infants (e.g. contact and adjacency to infants) were significantly *increased* in VFD mothers; however, patterns of maternal care were more erratic /disorganized compared to HFD or LFD groups. VFD mothers displayed reduced responsiveness to infant solicitations for contact and attention (Andrews and Rosenblum, 1991), while establishing and breaking contact more often than LFD and HFD groups despite infant protest (Rosenblum and Paully, 1984).

Disorganized patterns of maternal care in turn influence offspring behavior. Bonnet macaque infants reared by VFD mothers, but not LFD or HFD, exhibited unstable anxiety

[2] Interestingly, perturbations in maternal investment in offspring are not only reflective of environmental influences, but also past experiences in motherhood. Mother vervet monkeys, for example, who failed in their last pregnancy, invested more in their next offspring than those who had success. Increased investment included higher levels of ventral contact, attention, and hyper-vigilance towards their infants' movements, so much so that they were more restrictive mothers (Fairbanks, 1993).

related phenotypes (Andrews and Rosenblum, 1991; Coplan et al., 1992; Rosenblum and Paully, 1984). For instance, during time out of contact with mothers in a novel room, VFD infants show high levels of disturbed behavior, including self-clasping, clinging, huddling with peers, and maintaining diminutive hunched postures, compared with HFD and LFD animals who did not differ from each other. Furthermore, VFD animals showed the least amount of play behavior, object exploration, and autonomy when placed in a novel stressful situation, indicating changes in stress responsivity, as well altered attachment to mothers as a secure base for exploration (Andrews and Rosenblum, 1991; Rosenblum and Paully, 1984; Rosenblum et al., 1994). Long-term consequences of VFD environments in offspring later emerged in HPA and neuroendocrine responses. Bonnet macaques raised by mothers in LFD and HFD conditions exhibited decreased HPA stress reactivity compared with VFD animals (Coplan et al., 2001). Concomitant with physiological measures, animals raised in VFD conditions were also more behaviorally anxious and hyper-responsive to stress as adults (Coplan et al., 1992; Rosenblum and Paully, 1984). Differences in adult phenotypes may have been due to alterations in monoamine responsivity, as VFD offspring at three years of age exhibited hyper-reactive behavior compared with LFD animals when injected with the noradrenergic probes yohimbine, and exhibited hypo-reactive behavior when injected with the serotonergic compound m-cholorphenylpiperazine (Rosenblum et al., 1994). Moreover, at four years of age, VDF offspring exhibited chronically elevated basal levels of CRH in cerebrospinal fluid compared with LDF and HDF animals. Rearing in VDF conditions accounted for two-thirds of the variance in predicting cerebrospinal fluid CRH measures, emphasizing the power of seemingly minor environmental perturbations in influencing physiological and behavioral outcomes in bonnet macaque (Rosenblum et al., 1994).

One assumption of variable foraging models is that differences in offspring behavioral and neuroendocrine phenotypes are being driven, in part, by environmental alterations in maternal care and not by differential foraging of the infants themselves. Also of note, are the similarities in stereotyped behavior and physiology between VFD animals and peer-reared infants. In both models, deficits seem to be driven by alterations in early attachment between mothers and infants.

3.6 Conclusion

Early life mother-infant interactions can influence neurobiological and behavioral outcomes later in life, whether as a result of maternal deprivation, separation, or disruptions in the mother-infant relationship. The nature of these dyadic relationships can be altered by contextual environmental circumstance, which can influence infants directly, or by altering levels of parental care. In the next section of this dissertation, we will focus on rodent models of early life stress.

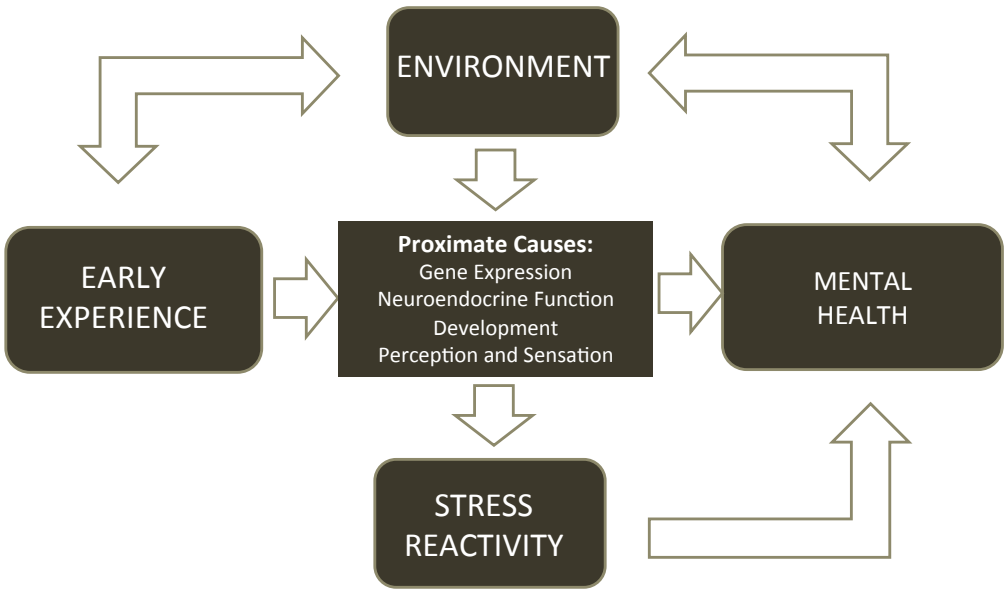


Figure 1: Early environments, either mild or severe, can influence developmental outcomes. This schema illustrates the relationship between early experience, environments, stress reactivity, and later life behavior and mental health.

Section 4: Models of Early Life Stress in Rodents

Introduction

Principles of HPA regulation, the role of glucocorticoids in modifying neural and behavioral systems, and the plasticity of the HPA axis to early-life environmental conditions in non-human primates was reviewed in Sections 2 and 3 of this dissertation. Similar to the work conducted in primates, data from early life models utilizing rodents emphasizes the sensitivity of postnatal biology to the long-lasting effects of environmental input. The current section will examine rodent models of early life stress and subsequent influences on neural systems, particularly HPA function. The rationale for using rodent models of early life stress, including neonatal handling, maternal separation, natural variations in maternal care, and maternal mediation/moderation paradigms of early life stress, are briefly reviewed.

4.1 Why Employ Early Life Rodent Models?

In humans, epidemiological data suggest that harsh or adverse early experiences are often associated with later life psychopathology (e.g. depression, anxiety, PTSD) (Lupien et al., 2009). Adverse experiences include parental neglect and abuse, poverty, parental substance abuse, and maternal depression (Halligan et al., 2007; Lupien et al., 2009; Repetti et al., 2002; Schore, 2000). Akin to these experiences, rodent models commonly involve disturbances in parental care, either via daily maternal separation or by environmental disruption. To accurately model human conditions, diverse models of early-life stress are used to measure biological embedding of environmental factors.

Compared with human and primate models, rodent studies allow for more extensive experimental manipulation to study causality between early-life experiences and developmental outcomes (Korosi and Baram, 2010). Both prenatal and postnatal experiences can be controlled throughout the life course of an animal. Given the large variability that exists in stress-related illnesses (that is, not all individuals get sick under stressful conditions), one powerful application of rodent models is the ability to manipulate and investigate environmental contributions to individual differences in stress reactivity. Rodent models also allow researchers to probe underlying biological mechanisms through the use of neuroanatomical, biochemical, and genetic approaches. This makes it possible to decipher the specific brain regions, circuits, signaling cascades, and mediating responses that might contribute to the effects of developmental programming. In addition, parameters of interest such as genetic backgrounds can be manipulated.

Incontestably, ethics prevent direct manipulation of early environments in children, and concern regarding invasive or ethically ambiguous work in non-human primates is growing (Beauchamp et al., 2014). Combined with the relative cost-effective nature of rodent studies, rodent models provide numerous advantages to primate and human work.

As briefly discussed in Section 3, one common critique of using rodent models to investigate the impact of early-life stress on CNS function is the phenomenon of the neonatal HPA hypo-responsive period. Early work on stress neurobiology suggested that the first two weeks of rodent life are characterized by a period in which the HPA response

is less robust or non-existent (Rosenfeld et al., 1992). The concept of the stress hypo-responsive period was based on work in which neonatal rats and mice displayed reduced sensitivity to CRH, low basal corticosterone levels, and the absence of a stress response to a variety of stressors which would normally lead to HPA activation in an adult rodent (Levine et al., 1967; Rosenfeld et al., 1992; Schapiro et al., 1962; Schoenfeld et al., 1980; Walker et al., 1986). Periods of stress hypo-reactivity are not present in the primate; for example, human infants will respond to pain throughout the neonatal period (Stang et al., 1988). However, rather than neonatal rodents being unresponsive to stress, it may be that their stress system is regulated specifically by stressors that are relevant to the early-life period. Alternatively, the neonatal stress response may not respond to stressors that require higher perceptual facets. For instance, rodent neonates mount a 300-400% increase in plasma corticosterone levels in response to maternal separation or cold exposure, but not restraint stress, a more psychological and less ecologically valid stressor (Pihoker et al., 1993; Plotsky and Meaney, 1993; Schoenfeld et al., 1980; Walker et al., 1986; Yi and Baram, 1994). In contrast, adult rodents display a robust corticosterone response to restraint stress, as opposed to cold exposure (Harbuz et al., 1991; Lightman and Young, 1988). Importantly, the neonatal stress response is mediated by activation of hypothalamic CRH neurons and subsequent CRH release, indicating an intact HPA axis (Dent et al., 2000; Hatalski et al., 1998). For these reasons, the idea that rodent models are not satisfactory due to a stress hypo-responsive period is not fully founded.

4.2 Neonatal Handling Paradigms

In the late 1960's, Levine, Denenberg, Ader, and colleagues first demonstrated that adult behavioral and endocrine responses to stress are modifiable by experiences during the early postnatal period (Denenberg, 1964; Grata and Ader, 1969; Levine, 2005; Levine et al., 1967). This effect was demonstrated using a neonatal handling paradigm, which consists of separating the mother and pups for a period of 15 minutes per day, once per day, for the first three weeks of life. Parameters of this model have since varied tremendously across laboratories. Pups may spend 1 to 15 minutes out of the home cage (Denenberg and Karas, 1959; Padoin et al., 2001; Plotsky and Meaney, 1993), the procedure could be repeated across the second and third weeks of life (Meaney et al., 1985; Padoin et al., 2001), and/or the pups can be handled individually or as a litter (Ladd et al., 2005; Padoin et al., 2001). Regardless of variations in procedure, handled animals (H), as adults, exhibit an attenuated increase in corticosterone in response to varied stressors as well as reduced anxiety-like behaviors in comparison to non-handled controls (NH). Thus, mild handling stimulation in infancy results in reduced HPA activity in response to various stressors.

The most studied consequence of neonatal handling has been in regards to alterations in the adult stress response. Notably, NH and H animals do not vary in basal hormone levels; however, they do differ in HPA reactivity in response to acute challenge (Hess et al., 1969; Liu et al., 2000a). Compared with NH controls, H animals show reduced CRH, ACTH and plasma corticosterone to a wide range of stressors. These stressors include restraint, time in an open field, ether exposure, or re-exposure to fear conditioning chambers (Hess et al., 1969; Levine et al., 1967; Liu et al., 2000a; Meerlo et al., 1999; Plotsky and Meaney, 1993; Weinberg and Levine, 1977). H animals also exhibit increased GR density and receptor binding in the hippocampus and mPFC, which may explain their

more finely tuned HPA regulation to stress (Avishai-Eliner et al., 2001; Meaney et al., 1985). As reviewed in Section 2, both hippocampus and mPFC play a pronounced role in HPA negative feedback and inhibition (Diorio et al., 1993; Sapolsky et al., 1984). Given these findings, it is therefore not surprising that H animals exhibit reduced anxiety-like behaviors, particularly when considering that neonatal handling also increases amygdala and PFC expression of $GABA_A$ receptors, as well as decreased neurotransmitter levels of serotonin, dopamine, and noradrenaline (Arborelius and Eklund, 2007; Caldji et al., 2000b).

Neonatal handling can also have profound consequences for learning and memory. Compared with NH controls, H animals exhibit increases in spatial learning (as tested on Morris water maze, radial arm maze, and T-maze) as adults (Fenoglio et al., 2005; Vallée et al., 1999; Wong and Judd, 1973). Remarkably, these effects have been reported to last into old age, as H animals display a less steep decline in memory performance compared with NH animals. Intriguingly, despite an increase in performance in spatial memory, H animals do not show an increase in adult hippocampal neurogenesis (Meaney et al., 1988; Mirescu et al., 2004). This suggests that improvements in spatial memory are not driven by the production of new neurons, but possibly by the integration of these neurons into existing hippocampal circuits, their excitability within the hippocampus, or even by modifications elsewhere in the brain.

While spatial memory is improved, neonatal handling has been shown to impair aversive learning. When tested on both cue and context learning to conditioned fear responses, H animals display impaired aversive learning (Kosten et al., 2006; Madruga et al., 2006; Wilber et al., 2009). H animals are also behaviorally less avoidant of behaviorally conditioned aversive stimuli, as measured by inhibitory avoidance, eye blink conditioning, and conditioned taste aversion (Kosten et al., 2007; Weinberg et al., 1978; Wilber et al., 2007). Taken as a whole, these results have been interpreted as beneficial to the animal, as enhanced spatial learning and memory is seen as a positive effect, while impaired aversive learning and memory is seen as a negative one (Kosten et al., 2012) . However, these findings can also be considered to be maladaptive, as more exploratory behavior and a decreased learning to aversive stimuli could fatally swing the balance of 'approach-avoid' behaviors in more ecological settings (Beery and Francis, 2011; Raineki et al., 2014). Thus, alterations in phenotypic plasticity due to early life conditions must be considered within a given environmental context.

The neonatal handling model has been instrumental in increasing understanding of the effects of early-life experiences on development. It should be noted that neonatal handling effects have been shown to extend outside of the arena of HPA axis and modifications in learning and memory, but also influence reproductive (Gomes et al., 2006; Gomes et al., 2005; Lucion et al., 1996; Moore, 1995; Padoin et al., 2001), social (Todeschin et al., 2009; Veenema, 2012), and feeding behaviors (Portella et al., 2010; Silveira et al., 2010; Silveira et al., 2008). Neonatal handling as also been used as an intervention to early adverse experiences such as prenatal alcohol exposure, prenatal stress, and perinatal malnutrition, with varying outcomes (Raineki et al., 2014). From these works, it is becoming clearer that handling during the early postnatal period has both "advantageous and disadvantageous" outcomes driven by the infant's experience – it is the environmental context that matters. For an excellent review on these topics, please see Raineki, Lucion and Weinberg, 2014.

4.3 Maternal Separation Paradigms

The effects of short periods of neonatal handling on HPA function (described above) have been well characterized in the literature. In contrast to brief periods of maternal separation, are longer periods of separation that are thought to model maternal neglect (Plotsky and Meaney, 1993). Maternal separation protocols remove the pup from the mothers once per day for several hours. Similar to neonatal handling paradigms, multiple protocols of varying periods of maternal separation have been used across laboratories, ranging from 1-24 hours and numbering 1 – 14 days in length during the first 2 weeks of life (Dalmaz et al., 2015; Kosten et al., 2012; Todeschin et al., 2009). The result of longer bouts of maternal absence, and subsequent loss of care, are increases in HPA axis tone and hyperactivity throughout the life course. As adults, maternally separated animals (MS; i.e. those separated for 180-360 minutes) display elevated plasma ACTH and corticosterone responses to restraint or novelty stress compared with non-handled and handled control animals (Dalmaz et al., 2015).

HPA hyper-responsivity in animals subjected to maternal separation can be attributed, at least in part, to a decrease in hippocampal GR and subsequent GC mediated negative feedback sensitivity (Ladd et al., 2004; Plotsky and Meaney, 1993). However, maternal separation has also been associated with decreased GR levels in the hypothalamus and frontal cortex (Ladd et al., 2004; Liu et al., 2000a; Meaney et al., 1996). Administration of dexamethasone (DEX), a synthetic glucocorticoid, suppresses basal HPA activity in H rats to a greater extent than in MS animals. This suggests that GC negative feedback mechanisms are made less efficient by repeated or extended periods of maternal separation during the neonatal period (Meaney et al., 1996). Hypothalamic CRH mRNA levels are also elevated in MS rats compared with H and NH groups (Plotsky and Meaney, 1993). Elevated CRH has been shown under both basal conditions and after acute stress, in which MS animals (6 hrs. of daily separation from PND 2-20) demonstrate a 125% increase in hypothalamic CRH immunoreactivity (Plotsky and Meaney, 1993). These results are consistent with the idea that CRH neurons and GR expression are particularly sensitive to regulation by neonatal experience.

Not surprisingly, prolonged periods of maternal separation during the neonatal period result in altered behavioral responses to stress in adulthood. MS rats are considerably more stress reactive than NH and H animals, and exhibit less time in an open-field and an exaggerated startle response to high decibel tones (Kosten et al., 2012; Meerlo et al., 1999; Vallée et al., 1999). However, the effects of maternal separation on HPA axis regulation of adult rats are not consistent among studies (Dalmaz et al., 2015). A recent analysis of the literature by (Jahng, 2011) provides a potential explanation, arguing that the effects of maternal separation on adult HPA axis reactivity may depend on the type of stressor used. Another potential point of variation in results is the MS protocol used (Kosten et al 2012). Regardless, these studies support the idea that early-life adverse experience can fundamentally alter stress reactivity in adulthood.

The maternal separation model has shed much light on the effects of early-life experiences on development. Similar to handling paradigms, maternal separation effects extend outside of the arena of HPA axis, and influence learning and memory (Kosten et al., 2012; Kosten et al., 2006). MS animals show improved performance on emotional recollection while exhibiting deficits in spatial memory and cognition. For instance, MS

animals exhibit enhanced cued-fear conditioning (Oomen et al., 2010). However, they present with impaired spatial memory as measured by Morris Water Maze learning (Aisa et al., 2007) and object recognition (Rice et al., 2008). MS animals also show deficits in cognitive function and flexibility (Lejeune et al., 2013).

Deficits in spatial memory and cognition are coupled with alterations in neuronal structure and function in both the hippocampus and PFC. In adulthood, MS animals possess reduced dendritic complexity, spine density, and synaptic function in these regions (Bock et al., 2005; Brunson L et al., 2005; Huot et al., 2002; Monroy et al., 2010). Given its role in HPA regulation, spatial memory, and emotional processing, researchers have also investigated changes in adult neurogenesis as a function of early life maternal separation. These studies have been mixed, reporting decreases (Aisa et al., 2009; Mirescu et al., 2004; Oomen et al., 2010), increases (Oomen et al., 2009) or no effect (Greisen et al., 2005). However, discrepancies in these studies may have been due to the length of maternal separation. As the MS paradigm becomes more severe, a decrease in neurogenesis is observed. Functionally, MS animals also show altered sensitivity to synaptic plasticity as adults (Oomen et al., 2010). These results show that maternal separation can influence behavior by altering structural and functional plasticity at the level of the synapse.

In conclusion, prolonged periods of maternal separation can fundamentally alter stress reactivity, learning and memory, and underlying neural function. Of note, maternal separation also affects reproductive (Akbari et al., 2008; Rhees et al., 2001), social (Levy et al., 2003; Todeschin et al., 2009), and feeding behaviors (Ryu et al., 2009). Please see (Dalmaz et al., 2015) for a review of these topics.

4.4 Natural Variations in Maternal Care in the Laboratory Rat

Both the neonatal handling and separation paradigms have been important models for investigating how early environment can program the development of multiple physiological systems, including an organism's response to stress. Both of these manipulations also fundamentally alter levels of parental care. Indeed, one hypothesis suggests that the long-term effects of postnatal manipulations may be mediated in maternal behavior in response to cues emitted from the pups (Hofer et al., 1989; Smotherman et al., 1977) .

The dam may change levels of care after the removal of her pups, or in response to changes in the pups themselves while they are separated. For example, pups will increase ultrasonic vocalizations while away from the mother and this will increase levels of maternal care (Bell et al., 1974). Furthermore, changing the body temperature of pups, malnourishment, or ear punching pups has been shown to influence maternal behaviors (Barnett and Burn, 1967; Wiener et al., 1977; Young, 1965). Thus, it has been proposed that alterations in the mother-infant dyad, at least in part, mediate the effect of handling on neuroendocrine alterations in emotionality and HPA activation (Levine, 1975).

Increases in maternal behavior (i.e. licking and grooming of offspring; LG) are observed after handing (Lee and Williams, 1974; Liu et al., 1997; Oomen et al., 2009; Reis et al., 2014). Over the first week of life, mothers of H pups have twice the frequency of pup LG compared to NH pups (Liu et al., 1997). Furthermore, pretreatment of a rat mother with an anxiolytic before handling offspring eliminates the handling effects on pups (D'Amato, 1997). Similarly, when a dam is provided with a foster litter during separation from

offspring, the effects of maternal separation are eliminated (Huot et al., 2004). This suggests that allowing a mother to be maternal prevents alterations in care following reunion with her natural pups. These findings implicate the dam as a mediator of neonatal handling effects[3].

In the past two decades, work from the laboratory of Michael Meaney, at McGill University, has investigated the effects of natural variations in maternal care on stress-responsiveness. This work has helped elucidate a pathway in which variations in rodent maternal care can alter behavioral and endocrine responses to stress in offspring. By behavioral observation, the Meaney group quantified the suite of maternal behavior exhibited by a dam. Rodent mothers were classified as High licking and grooming (High LG) or Low licking and grooming (Low LG) based on levels of anogenital licking directed at pups. As adults, offspring of High LG mothers are behaviorally less fearful and exhibit more tightly regulated HPA axis. Compared with Low LG animals, High LG animals have significantly reduced levels of ACTH and corticosterone in response to stress. Upstream of ACTH, High LG animals have less hypothalamic CRH mRNA levels, while expression of both hippocampal GR mRNA and protein levels is higher compared with Low LG offspring (Bredy et al., 2003; Liu et al., 2000b; Liu et al., 1997). In consequence, adult offspring of High LG maternal environments demonstrate enhanced GC negative feedback sensitivity **(Figure 1).** Changes in hippocampal GR expression seem to be driven, at least in part, by the epigenetic methylation of the GR (Nr3c1) promoter during the first two weeks post birth (Szyf et al., 2005; Weaver et al., 2004a; Weaver et al., 2004b). This finding of epigenetic regulation of GR expression based on early life maternal care has provided critical insight into the potential pathways by which experiences can become biologically embedded.

The effects of maternal care described above can be reversed via cross fostering of pups at birth. Rat pups born to Low LG mothers but reared by High LG animals display stress reactivity that is similar to High LG offspring and vice versa (Francis et al., 1999b). Furthermore, individual levels of maternal care *within* a litter correlate with the expression of GRs (van Hasselt et al., 2012). These findings cement the idea that the quality and quantity of maternal care provided to offspring early in life is directly involved in the developmental programming and calibration of the HPA axis.

In addition to changes in stress reactivity, early life maternal care has been shown to alter a host of functions, including learning and memory. For instance, contextual fear conditioning is enhanced in Low compared to High LG animals indicating improved emotional memory formation (Champagne et al., 2008). However, Low animals display worse spatial memory performance on hippocampal dependent tasks, such as Morris Water Maze (Liu et al., 2000a), and object recognition (Bredy et al., 2003). Paralleling findings of worse spatial memory and impaired negative feedback function, Low LG animals have been reported to have reduced rates of neuronal survival compared to High LG (Bredy et al., 2003). Structurally, maternal care can also alter hippocampal dendritic arborization and complexity, such that Low LG animals show reduced dendritic complexity,

[3] Akaysha Tang and colleagues put a competing hypothesis forth. They provide evidence that the experience of a novel environment during separation as a driving factor in predicting effects on offspring (Tang et al., 2006). For an informative review, see (Tang et al., 2014).

spine density, and synaptic function compared with High animals (Bredy et al., 2003; Champagne et al., 2008). These structural changes also lead to changes in synaptic function and plasticity. Low LG animals exhibit deficits in hippocampal long-term potentiation in the CA1 area and dentate gyrus of the hippocampus (assessed via slice culture). However, application of high levels of corticosterone reverses this effect, with Low LG animals showing stronger synaptic plasticity (Champagne et al., 2008). Low LG animals also show enhanced NMDA currents in comparison to High LG animals (Bagot et al., 2012). These results suggest that not only can maternal care influence behavior by altering neuronal function and structure, but low levels of maternal care can potentially render synapses more efficient under stressful conditions. These results are of significance when considering the adaptive significance of maternal care.

In summary, early life maternal care, both in quantity and quality, can profoundly modify adult responses to stress, cognitive and emotional systems. Maternal care has also been shown to effect aspects of reproductive (Cameron et al., 2008a; Champagne et al., 2006), sociality (Starr-Phillips and Beery, 2014), and feeding behaviors (Portella et al., 2007). As a whole, these studies have shown that early life parental care, particularly tactile stimulation from the mother, is capable of programming future offspring behavior via changes in gene expression and epigenetic processes. Differences in response to stress may alter ones susceptibility to pathology later in life as well as help prepare them for varying environmental demands.

4.5 Maternal Mediation / Moderation of Offspring Phenotypes

Environments can influence offspring directly, or indirectly via changes in parental care. Deciphering the relative contributions of each of these factors in programming offspring phenotypes has spurred much debate. The research discussed above (i.e. handling and maternal care) suggests to maternal influences as a *mediator* of the association between environmental manipulations and neural systems involved in stress, emotion, and cognition, among other faculties. Based on these findings, a significant amount of work in rodent early life research has manipulated various aspects of the maternal environment to assess the "maternal mediation hypothesis". That is, the mother's environment at the time of conception, gestation, and parturition is capable of altering levels of parental investment towards offspring. Offspring, in turn, 'use' parental cues as signal of future environmental adversity, programming behavioral and neural phenotypes (e.g. neuroendocrine systems) for optimal future adaptation (Meaney, 2001; Smotherman et al., 1977).

Studies investigating "maternal mediation" range tremendously in their scope. In general, researchers manipulate pre-natal or post-natal environments by altering rodent housing parameters to create moderate or severe stress conditions (Macrì et al., 2011; Würbel, 2001). Moderate stress conditions include handling (Fenoglio et al., 2005; Plotsky and Meaney, 1993), moderately challenging foraging demand (i.e. food availability)(Macrì and Würbel, 2007), or exposure of mothers to low doses of corticosterone, for example (Catalani et al., 1993). Alternatively, severe environmental conditions include maternal separation (Avishai-Eliner et al., 1999; Plotsky and Meaney, 1993), strenuous foraging demands (Macrì and Würbel, 2007), removal of nesting materials (Ivy et al., 2008), simulating predation threat (Mashoodh et al., 2009), or exposure of mother to high doses of corticosterone (Yorty et al., 2004). In some studies, a role for the mother in mediating the

association between environments and adult offspring phenotypes is observed. In other experiments, environmental variation has a more direct impact on the offspring, as the mother serves as a *moderator* of offspring phenotypic plasticity (Tang et al., 2014), or the relationship is ambiguous (Sakhai et al., 2013). Because the literature is too vast, each of these models cannot be reviewed in detail; however, the remainder of this section strives to provide evidence for maternal meditation of environmental signals on offspring phenotypes.

Evidence for maternal mediation comes from studies in which maternal care is altered by environmental stress with subsequent changes in offspring physiology and behavior. For instance, dams exposed to cat odor during parturition display altered levels of maternal care. In turn, their offspring (who were cross-fostered and therefore not exposed to the odor), go on to display altered levels of anxiety-like behaviors (Mashoodh et al., 2009). Furthermore, prenatal stress is capable of turning High LG mothers into Low LG mothers, with later influence on offspring HPA axis physiology and behavior (Champagne and Meaney, 2006). Similarly, maternal care can be decreased when dams experience long periods of social isolation or stress during pregnancy (Champagne and Meaney, 2006).

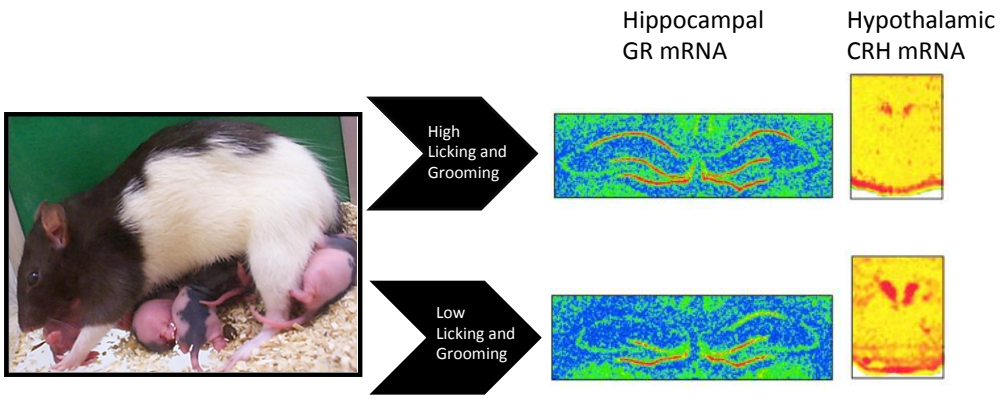
Enrichment also can positively regulate levels of parental care. Increases in maternal care have been observed after housing post-weaning females in environmental enrichment before mating (Champagne and Meaney, 2007). Indeed, environmental conditions designed to "enhance" levels of maternal care, both in quality and quantity, report improved stress reactivity, cognition, and resilience of offspring (Fenoglio et al., 2005; Fenoglio et al., 2006; Kosten et al., 2007), while those that reduce it lead to adverse outcomes (Ivy et al., 2008; Korosi and Baram, 2010; Rice et al., 2008)

One interesting paradigm devised by the lab of Tallie Baram at UC Irvine, provides further support for maternal mediation of environmental signals (Molet et al., 2014) . This model of "chronic early life stress" involves the removal of bedding/nesting material during the neonatal period. The lack of material resources prevents the dam from constructing an adequate nest and leads to significant maternal stress. This stress is associated with alterations in the pattern of maternal care (not specific aspects of maternal behavior such as LG or nursing), which is grossly fragmented and disorganized (Ivy et al., 2008). This disrupted pattern of maternal care is a source of chronic stress in offspring. Offspring chronic stress is manifested by an elevation in plasma corticosterone and adrenal hypertrophy, which disappears once dams and pups are returned to normal bedding/nesting cages (Ivy et al., 2008). As adults, offspring show alterations in cognitive and emotional functioning (Dalle Molle et al., 2012; Raineki et al., 2012) with accompanying brain changes, including augmented CRH hippocampal expression, hippocampal synaptic plasticity (Ivy et al., 2010), dendritic atrophy (Brunson L et al., 2005), and enhanced amygdala activity (Raineki et al., 2012). Results from this model provide more evidence of maternal mediation. Further evidence for maternal mediation in primates is provided in Section 3 of this dissertation.

4.6 Conclusion

While clearly not capable of mimicking the complex nature of human development, rodent models provide many advantages compared to primate and human investigations of early life stress. Rodent work provides widespread tractability and adaptability of methods to

answer varied questions regarding the nature of early life mother-infant interactions. Early life mother-infant interactions can influence neurobiological and behavioral outcomes later in life, whether as a result of brief or prolonged maternal separation, or via natural variations in care. A great deal of evidence suggests that maternal behaviors are plastic and can be modulated by manipulating pre- and post-natal environments. Changes in maternal behaviors are thought to be used by pups as an adaptive signal to program offspring behavior. However, in many cases maternal care cannot be considered the sole mediator between neonatal experiences and adult phenotypes. Furthermore, phenotypic plasticity in behavioral outcomes should not be viewed as "good" or "bad", but viewed within an adaptive context of the organism's environment.



Receptor Autoradiography and In-situ hybridization data courtesy of Dr. Darlene Francis

Figure 1: Natural variations in maternal care can have profound influences on offspring HPA axis development. Offspring of High LG animals exhibit increased hippocampal GR mRNA and enhanced corticosterone negative feedback compared to Low LG animals. In response to acute stressors, High LG animals exhibit decreased hypothalamic CRH mRNA expression and are less behavioral anxious than Low LG counterparts. These findings highlight the importance of maternal care during the neonatal period in the development of individual differences in HPA axis reactivity.

5. Aims of Dissertation

Given the varied effects of maternal care on adult behavioral and biological phenotypes (reviewed in Section 3 and 4), the next three empirical sections of this dissertation aim to answer the following questions regarding the effects of natural variations in maternal care:

1. In what ways can maternal care alter sexual behavior in Long-Evans rat offspring? What are, if any, a neural correlate mediating an effect?

2. Can natural variations in maternal care influence adult cognitive behavior in rodents as measured by attentional set shifting? Furthermore, are developmental differences in offspring stress reactivity modifiable by training/handling?

3. Can environmental housing variables, such as bedding, influence levels of maternal care? What about behavioral phenotypes of developing offspring?

Section 6: Maternal Programming of Sexual Attractivity in Female Long Evans Rats

Sakhai, S. A., Kriegsfeld, L. J., & Francis, D. D. (2011). Maternal programming of sexual attractivity in female Long Evans rats. *Psychoneuroendocrinology, 36*(8), 1217-1225. doi: 10.1016/j.psyneuen.2011.02.016

Abstract: In mammals, maternal care influences the developing offspring across multiple domains. In Long Evans rats, for example, the quality of maternal care received as a pup influences later cognitive function, neuroendocrine responses to stress and behavioral measures of emotionality. Data from humans, non-human primates, and rodents also suggest that early life events may similarly perturb measures of sexual reproduction, with possible consequences for reproductive fitness. The current study examined whether or not male conspecifics differentially prefer females, as adult mating partners, that were reared under varying maternal conditions (assessed via the quantity of licking and grooming received; LG). Additionally, the impact of maternal care on adult female sexual motivation and behavior were quantified to determine if these behavioral characteristics are associated with any preference observed. In a mate preference task, male rats chose, almost exclusively, to mount, copulate and ejaculate with female rats reared under Low LG conditions. Under non-paced mating conditions, female Low LG rats display significantly more paracopulatory and copulatory behaviors compared to High LG rats. Due to its critical role in female paracopulatory behavior, progesterone receptor immunoreactivity (PR-ir) in the ventromedial nucleus of the hypothalamus (VMH) was also assessed in both groups of female rats. Estradiol induced PR-ir in the VMH was significantly higher in Low LG relative to High LG rats. Together, these data suggests that early life parental care may developmentally program aspects of behavior and physiology that subsequently influence sexual attractivity and behavior in adult females.

6.1 Introduction

Early-life environments and experiences, both pre and post natal, shape the development of numerous biological processes in humans and other mammals (Boyce et al., 2006; Evans and Schamberg, 2009; Fish et al., 2004; Lyons et al., 2010). Diverse disciplines such as social epidemiology and behavioral neuroscience have documented that challenging and/or adverse experiences that occur early in life can influence developmental processes to impact an individual throughout its lifetime. These effects include changes in reproductive behavior and corresponding biology, both of which have been documented in insects, reptiles, amphibians, fish, birds, rodents, and primates (Bass, 1992; Danforth, 1991; Denver, 1997; Rhen and Crews, 2002; Tollrian, 1995). In humans, childhood sexual abuse is strongly associated with early pubertal maturation (Turner et al., 1999; Wierson et al., 1993; Zabin et al., 2005). In rats, lower levels of parental investment markedly change reproductive phenotypes, including precocial puberty (Cameron et al., 2008b; Moore, 1984; Uriarte et al., 2007). Despite these well-established effects of early experience on adult physiology and behavior, the means by which these early-experiences are biologically embedded and persist long term is not well understood. The present experiments

investigate the impact of variations in early maternal care on physiological and behavioral measures of sexual attractivity and behavior in female laboratory rats.

Life History Theory postulates that humans have evolved to be responsive to early childhood environments that subsequently bias children to adopt different behavioral and reproductive strategies (Belsky, 1991; Boyce, 2005; Coall and Chisholm, 2003; Crews, 2003). Environments may *directly* influence child development or, alternatively, changes may occur through perturbations in parental care and investment that arise in response to varying ecological demands. For example, girls raised with increased exposure to paternal absence are at significantly elevated risk for early sexual activity and adolescent pregnancy (Ellis et al., 2003; Surbey, 1990). Early sexual activity has been shown to have several deleterious effects that include: i) having more and older sexual partners throughout the teen years ii) increased risk of sexually transmitted disease and iii) increased risk of adolescent pregnancy (Coker et al., 1994; Sandfort et al., 2008). Thus, the acceleration and maturation of the hypothalamo-pituitary-gonadal (HPG) sex axis in young girls has profound consequences that persist throughout the life course.

Research employing animal models has focused on the role of early maternal care on the regulation and programming of sexual development in offspring, allowing cause-effect relationships between early care quality and adult phenotypes to be established. In rats, for example, developing offspring are very sensitive to the quality of parental care received during the early postnatal period. Maternal licking and grooming (LG) of offspring during the first week of life has been shown to developmentally program the hypothalamo-pituitary-adrenal (HPA) axis of Long Evans rats, later impacting adult stress-reactivity profiles (Francis et al., 1999b). Specifically, offspring that experience Low maternal LG as pups are more stress reactive, and less exploratory than their High LG counterparts as adults (Fish et al., 2004; Francis et al., 1999b; Weaver et al., 2004a; Weaver et al., 2004b). Recent findings (Cameron et al., 2005; Moore, 1995; Uriarte et al., 2007) suggest that a similar process occurs for developmental programming of the HPG axis in rats, subsequently influencing adult sexual behaviors.

Disruptions to early postpartum maternal care in rats can perturb neuroendocrine and behavioral processes in the offspring that influence sexual activity and reproduction. For example, female rats subjected to handling as pups exhibit less copulatory behavior and increased anovulatory estrous cycles compared to non-handled controls when assessed later in life (Gomes et al., 2006; Gomes et al., 2005; Raineki et al., 2008). Similar to the influence of maternal care on the developing HPA axis, Cameron (2005, 2008, 2010) reports that variations in maternal care received during the early postnatal period can also significantly influence aspects of female sexual behaviors later in life. Offspring of Low LG females are reported to reach puberty at an earlier age relative to High LG females. Low LG females exhibit a higher lordosis rating, a lower (but not significant) inter-intromission interval and have a higher incidence of pregnancy compared to High LG animals. With the exception of pregnancy rate and pubertal onset, these are measures of copulatory behaviors, behaviors that result in the successful transfer of male gametes to the female (Blaustein, 2008). Whereas paracopulatory behaviors, behaviors performed by female rats to elicit mounting behavior from males, are subject to early-life regulation have been explored using a paced mating paradigm (Cameron et al., 2008b). In the present series of studies, we sought to determine if female sexual behaviors, specifically, paracopulatory behaviors potentially lead to differences in male preference based on maternal history.

The aim of the following study was to investigate the role of maternal care in the developmental regulation of physiological and behavioral measures implicated in female paracopulatory behavior. Specifically, we hypothesized that sexually-experienced male rats, when allowed to choose between High or Low LG females in estrus will prefer to copulate preferentially with Low females. We hypothesized that Low female rats will exhibit increased paracopulatory and copulatory behaviors during estrus relative to High LG female rats, behaviorally rendering them more attractive to males when compared to High LG females. Attractivity, a term coined by Frank Beach (Beach, 1976), is defined by the stimulus value of the female in evoking male sexual behavior. As progesterone treatment potentiates the effects of estradiol on female sexual behaviors (specifically paracopulatory behaviors) we also assessed whether or not estradiol-induced progesterone receptor immunoreactivity (PR-ir) in the ventromedial nucleus of the hypothalamus (VMH) paralleled behavioral differences observed.

6.2 Methods and materials

6.2.1 Animals and Housing

Male Long Evans rats used in the study were purchased from Charles River Breeding Laboratories (Wilmington, MA) and pair housed in polypropylene cages (27.8 × 17.5 × 13.0 cm). Female rats (n=48) used in the study were born in our colony that was generated using Long Evans rats originally purchased from Charles River. For all animals, temperature was kept constant at 20 ± 2 °C and relative humidity was maintained 50 ± 5%. Rats were maintained on a 12-h light–dark cycle (lights on 0700 h to 1900 h) and allowed access to food (Purina Rat Chow, Purina Mills, St. Louis, Missouri) and tap water *ad libitum*. Female offspring underwent behavioral testing between PND 85-90. Housing and care of the rats were carried out in accordance with the standards and practices of the UC Berkeley Animal Care and Use Committee.

6.2.2 Observations of Maternal Behavior

Female rats were bred and permitted to give birth. Day of birth was marked as postnatal day (PND) 0. Maternal observations were performed beginning on PND 1 and continued until PND 8 (Champagne et al., 2003a; Francis et al., 1999a; Liu et al., 1997). Each litter was observed for five hours a day at the following times; 0600 h – 0800 h, 1200 h – 1300 h and 1800 h – 2000 h. During each observation session, litters were observed and behaviors recorded every two minutes (in sum, each litter was observed 150 times per day for eight days). Behaviors recorded included: mother on/off the nest and maternal licking behaviors directed at self or at pups. A maternal care distribution curve was generated by calculating the frequency with which pup-directed maternal licking was observed. Maternal licking was expressed as a percentage of the total number of observations performed for each litter. The mean ($\pm$SD) frequency of maternal licking was calculated for the cohort and High or Low maternal designations were made relative to this mean. High and Low licking litters were assessed as those falling one SD above or below the mean, respectively. Animals were weaned on PND 22, and pair housed with same sex littermates.

6.2.3 Male Mate Preference Task

All testing was performed during the beginning of the dark phase of the light-dark cycle (between 1900 h – 0200 h) and digitally recorded. All animals were examined in a counterbalanced manner with an equal number of animals from both maternal conditions being tested at each time point. The testing apparatus consisted of three transparent plastic Plexiglas chambers (21 x 15 x 10 inches) connected by two cylinders (8" x 4" dia.) in a linear manner. The outer chambers housed High and Low LG tethered female rats in estrus (n=11 pairs of High/Low females) that were behaviorally screened prior to the task. Females were screened by pairing with an established stud male. Females who did not exhibit lordosis upon male stimulation were excluded from testing. The middle chamber was neutral. A vasectomized male rat was placed in the neutral chamber. Female rats were given 10 minutes to acclimate, while males were given 5 minutes prior to testing with access to females prohibited. They were otherwise left undisturbed. After the short acclimation period, males were given 15 minutes to explore, inspect, investigate and "select" a female. Male behaviors scored included the following: i) time spent in proximity to High or Low female rat ii) frequency of mounts iii) intromission frequency iv) inter-mount interval v) inter-intromission interval and vi) ejaculations.

6.2.4 Female Sexual Behaviors

Similar to the Male Mate Preference Task, High and Low LG adult female rats in estrus (behaviorally confirmed) were tethered to the outer-chambers of the testing apparatus and all behaviors were digitally recorded. Females were acclimated to the tethers for at least ten minutes before the onset of the task, and were tethered behind the forelimbs using an 8" nylon harness. The location of the female rats was counterbalanced, with High and Low females being equally represented in the left and right chambers among trials. During the 15 minute test, the following proceptive and receptive behaviors were scored: i) frequency of ear-wiggling ii) frequency of hopping/darting and iii) lordosis quotient (lordosis responses/mounts and intromissions).

6.2.5 Male Olfactory Preference Task

To assess if male mate selection was due to olfactory cues emitted rather than female behaviors, a simple olfactory preference task was performed. Using the three-chambered apparatus described above, soiled bedding from High and Low LG estrus female rats was placed in the outer test chambers. Males were placed in the neutral chamber, allowed to acclimate for five minutes and then were given equal access to the two outer chambers. The placement of the bedding was counterbalanced in the outer chambers for each trial to minimize lateralization effects. Total time spent in the High LG, Low LG and neutral chambers was recorded.

6.2.6 Hormone Treatment and Perfusion

Upon completion of behavioral testing, 20 female rats (n = 10 High and 10 Low) were bilaterally ovariectomized and allowed to recover for seven days. After recovery, females

were injected (s.c.) with 10 ug of estradiol benzoate (EB) in 0.1 ml sesame oil vehicle followed 48 hours later by a 500 ug (s.c.) injection of progesterone in 0.1 ml vehicle. This hormone treatment regimen is sufficient to facilitate the expression of sexual behavior in female rats as well as induce progesterone receptor immunoreactivity in brain regions critical to sexual behavior (Blaustein, 1989; Boling and Blandau, 1939). Four hours after the final hormone treatment, rats were screened for behavioral estrus and subsequently administered a lethal dose of sodium pentobarbital (50 mg/ml). Rats were perfused using 0.9% saline followed by 4% fresh paraformaldehyde in 0.1M PBS. Brains were removed, post-fixed in 4% paraformaldehyde in 0.1M PBS for three hours and subsequently placed in 30% sucrose/0.1 M PBS solution overnight at 4°C until saturated. Forty μm sections were then cut on a cryostat and saturated in anti-freeze cryoprotectant at -20°C until immunohistochemistry (IHC) was performed to label progesterone receptors (PR).

6.2.7 Progesterone Receptor Immunocytochemistry and Image Analysis

For the PR IHC, sections were first washed (6X) in .01M PBS for ten min each wash. Sections were then incubated in 0.5% hydrogen peroxide (10 min) in order to reduce endogenous peroxidase activity. The hydrogen peroxide rinse was followed by three (10 min) PBS washes and incubated in 20% normal goat serum with 0.3% PBT in order to reduce non-specific staining. Sections were then incubated overnight in a rabbit polyclonal primary antiserum generated against the DNA binding domain of human progestin receptor (1:500, A0098, DAKO, Carpinteria, CA) in 0.3% PBT for 72 hours. Residual primary antibody was removed by three (10 min) washes in 0.3% PBT. Sections were then incubated in a biotinylated goat anti-rabbit secondary antibody (1:200, T0411, Vector, Burlingame, CA) for one hour, followed by three (10 min) PBT washes. Next, sections were incubated in avidin- biotin complex (Vectastain Elite, Vector Laboratories, Burlingame, CA) for one hour followed by three (10 min.) PBS washes. Sections were exposed to diaminobenzine (DAB, SK-4100, Vector Laboratories, Burlingame, CA) for five minutes. Sections were immediately washed in three (10 min.) washes in PBS to stop the reaction. Sections were mounted onto gelatin-coated slides and cover slips were applied using Permount mounting medium (ProSciTech, Australia).

PR-immunoreactive cells in the VMH were assessed using three matched sections of the VMH (Plate 56, 59, 60, Paxinos and Watson, 6th ed.) for each animal. Sections were analyzed by capturing images under 20X magnification with a Zeiss Axio Imager M1 with an Axio Cam Mrm TV2/cc 0.63x Camera. Cells were counted using NIH Image J to label cells with a pixel density darker than 130 (where 0 =black and 255 white). Where cells were clustered and difficult to individually discriminate, images of the masked region were captured at 40x magnification and analyzed in the same manner using Image J. The numbers of immunoreactive cells were quantified by observers blind to the experimental conditions. A total of six hemi sections were assessed per animal (three matched sections/animal), averaged for a total count and expressed as the mean ± SEM. Sections were counted by two independent investigators to ensure reliable and valid quantification of immunoreactive cells.

6.2.8 Data Analysis

All behavioral measures were analyzed by a one-way analysis of variance (ANOVA) or a Student t-test. Prior to analysis, a D'Agostino-Pearson omnibus test for normality was conducted. If behaviors failed normality ($p < .05$), a Mann-Whitney t-test was used in order to account for non-Gaussian distributions. The following data was determined to be non-parametric: intromission frequency, inter-intromission interval, and male olfactory preference. All other data was parametric. Densitometric measurements of PR-ir were determined by Student's t-test. Results were considered statistically significant when $p < 0.05$.

6.3 Results

6.3.1 Maternal Observations

Dams naturally differed in frequency of licking/grooming and arched-back nursing over the first six–eight days postpartum. A frequency distribution of maternal licking across all litters was created as previously described (Francis et al., 1999b). A maternal care score was generated by calculating the frequency of maternal licking observed relative to the total number of observations performed over the entire observation period. Percent licking in this cohort ranged from 4.00 – 12.33 % with a mean licking score of 6.84% across all litters. Female rats reared in litters that fell ± 1 SD away from the mean were used in the remainder of the study. In sum, five High and seven Low LG litters were generated out of a total of 30 litters.

6.3.2 Male Mate Preference Task

Male rat sexual preference for female rats reared under a High or Low LG maternal conditions was tested. Males spent significantly more time in proximity to Low LG females compared to High LG females ($p < 0.01$). Indeed, males spent nearly twice the amount of time with Low LG females relative to High LG females (6 min 52 s vs. 3 min 28 s, respectively) (**Figure 1A**). Males also exhibited greater mounting ($p < 0.05$) and intromission frequencies ($p < 0.05$) with Low LG females compared to High LG females (**Figures 1B, and C**) as well as exhibiting a shorter inter-mount interval ($p < 0.01$; **Figure 1D**). Whereas male behavior did not differ in inter-intromission intervals ($p > 0.05$; **Figure 1E**), a general trend of shorter latency between intromissions exists towards Low LG females. Furthermore, across all trials (n=11/group) ejaculations occurred exclusively with Low females (n= 3 total ejaculations) ($p < 0.05$; **Figure 1F**).

6.3.3 Female Sexual Behaviors

Low LG females exhibited a significantly higher lordosis quotient (number of lordosis responses/number of mounts; $p < 0.05$) compared to High females (**Figure 2A**). High and Low LG females also differed significantly in paracopulatory behaviors. Low females engaged in more hopping and darting ($p < 0.01$) compared to High LG females, whereas ear wiggling was not significantly different ($p > 0.05$) (**Figures 2B and 2C**). Collectively, Low

females appear to exhibit more paracopulatory and copulatory behaviors compared to High females.

6.3.4 Male Olfactory Preference Task

When placed in the neutral arena of a three-chambered testing apparatus and allowed to explore olfactory cues (soiled bedding) from High and Low LG females, males did not demonstrate a preference for odors from females from either maternal condition ($p > 0.05$). Males did, however, spend significantly more time in the chambers containing female olfactory cues relative to the neutral center chamber ($p < 0.0001$; **Figure 3**).

6.3.4 Progesterone Receptor Immunoreactivity

Estradiol-induced PR was quantified in the VMH for both Low and High LG females. Consistent with the behavioral data, the number of cells expressing PR-ir in the VMH was significantly higher in estrogen-primed Low LG females compared to High LG females ($p < 0.05$; **Figure 4**).

6.4 Discussion

The findings presented suggest that the quality of maternal care received early in life may differentially program physiological and behavioral measures implicated in rat female sexual function in adulthood. Specifically, female rats raised in a litter that received low levels of maternal care, when tested later as adults, demonstrated higher levels of paracopulatory and copulatory behaviors when compared to females raised in a litter that received high levels of maternal care. Interestingly, females reared under conditions of varying maternal care were also differentially 'attractive' to novel male rats. Males, when allowed to select between High or Low LG female rats in estrus, chose primarily to mount, intromit, and ejaculate with Low LG females. This was not due to differences in pheromonal/odor cues emitted by the Low LG females as males spent the same amount of time exploring olfactory cues from High and Low LG animals. Differences between High and Low LG female rats were not limited to behavior, but also extended to neurophysiology. In female rats, the ventromedial nucleus of the hypothalamus (VMH) has been studied extensively for its role in sexual behavior. Interestingly, Low LG females, as adults, had greater estrogen-induced PR-ir in the VMH compared to High LG females. In accordance with the literature, these results confirm that early life maternal care may influence female sexual behavior; these findings further demonstrate that differences in female sexual behavior can drive male mate preference through behavioral and not pheromonal signaling.

6.4.1 Neuroendocrine Underpinnings of Female Sexual Behavior

Our results suggest that male preferences for Low LG females is guided, in part, by the pattern of female sexual behavior (**Figures 1a-f and 2a-c**) rather than changes in pheromonal cues emitted by females (**Figure 3**). During proestrus, female copulatory and

paracopulatory behaviors are dependent on estrogen and progesterone stimulation of the neural circuits driving sexual behavior (Blaustein, 2002; Boling and Blandau, 1939). The present findings suggest that differences in maternal care manifest in adulthood as changes in the activity of this hormone-dependent circuitry (**Figure 4a**) and consequent changes in paracopulatory and copulatory behaviors that drive male partner preference.

Activation of VMH progestin receptors by estrogens influences many neuroendocrine processes including pre-ovulatory gonadotropin secretion and female sexual behavior (Olster and Blaustein, 1988; Rubin and Barfield, 1983). One mechanism by which maternal care may be influencing the immunoreactivity of PR in the VMH is by influencing the pathway leading to PR induction by estradiol. Several co-regulators of PR induction involved in female sexual behavior may be regulated by differential maternal care in the VMH. Potential targets include steroid receptor co-activator 1 (SRC-1), SRC-2, and cAMP binding protein, all of which have been shown to play a modulatory role in PR and estrogen receptor (ER) induction involved in female sexual behavior (Molenda et al., 2002; Molenda-Figueira et al., 2006). Similarly, estrogen receptor beta (ERβ), like estrogen receptor alpha (ERα), can influence the expression of female sexual behavior by conferring estrogen sensitivity and PR induction (Helena et al., 2009). Whereas, ERβ has not been shown to influence PR-ir in the VMH, this receptor has been shown to act in the locus coeruleus to mediate sexual behavior.

The previously mentioned transcription factors may provide proximate mechanisms for the role of maternal care on PR-ir and sexual behavior. However, maternal care has also been explicitly implicated in altering the neuroendocrine circuits involved in regulating gonadotropin-releasing hormone (GnRH), a key peptide involved in regulating the HPG axis. This is believed to be mediated by epigenetic regulation of ERα in the medial preoptic area (MPOA) and increasing ERα immunoreactivity in the anteroventral paraventricular nucleus (AVPV) of the female LE rat (Cameron et al., 2008c; Champagne et al., 2003b; Champagne et al., 2006). Previous studies have shown that Low LG females exhibit higher ERα expression in both the MPOA and AVPV and can increase the likelihood of female paracopulatory behaviors (Cameron et al., 2008b; Champagne et al., 2003b; Champagne et al., 2006). The MPOA is rich in GnRH cell bodies and input to this nucleus from estrogen-responsive cells in the AVPV can influence GnRH and downstream luteinizing hormone (LH) and estradiol. As estradiol enables PR-ir in the VMH, feedback signaling may increase PR-ir in the VMH and related paracopulatory behaviors shown in the present work. In accordance with our findings, Cameron et al. 2008 report that Low LG females exhibit greater GnRH immunoreactivity, higher estradiol and progesterone profiles during proestrus, and a significantly higher amplitude LH surge after ovariectomy and estrogen replacement. Thus, Low LG animals have greater LH, estradiol and progesterone release, likely due, at least in part, to greater ERα expression in the AVPV. In such a fashion, natural differences in copulatory and paracopulatory behaviors may arise that can influence male preference.

High and Low LG animals expressed similar levels of copulatory and paracopulatory behaviors following ovariectomy and hormonal replacement (data not shown, also reported in Cameron 2008). This finding suggests that early life maternal care may have an organizing effect on the developing neuroendocrine systems responsible for female sexual behavior whereas the downstream targets of these sex steroids remains intact. It should be noted, however, that the arcuate nucleus, a neural locus largely implicated in regulating

anterior pituitary hormonal release and GnRH, exhibits no differences in PR-ir between groups, indicating that the impact of differential maternal care is selective for progesterone sensitive targets that are specifically involved in female sexual behavior. The means by which early life experience leads to these neuroendocrine changes represents an important area for further inquiry.

6.4.2 Strengths and Limitations

The results from this study are in agreement with those investigating the effects of neonatal handling on offspring sexual behavior as well as reports using cross-fostering paradigms to study similar phenomena (Cameron et al., 2008b; Gomes et al., 2006; Gomes et al., 2005; Moore, 1984; Padoin et al., 2001; Uriarte et al., 2007). However, several caveats should be considered in interpreting the present findings: i. ecological validity in the method of testing female sexual behavior in a constrained environment, ii. vaginal cervical stimulation (VCS) received by females during the male preference task may potentiate any effects observed, and iii. female auditory cues, which may influence male preference behaviors (in conjunction with paracopulatory behaviors), were not recorded.

i. The current findings, unlike previous studies, document differences in High/Low LG female paracopulatory behaviors in addition to the previously noted differences in copulatory behavior. Female sexual behaviors are most commonly studied through the use of a paced mating procedure. This is traditionally assessed using a pacing chamber, in which the female regulates the timing and number of interactions with male conspecifics by approaching and withdrawing. Females pace interactions with males in order facilitate fertility and fecundity (Frye and Erskine, 1990). The current experiments did not utilize a female paced mating paradigm, but rather used a protocol in which female rats were tethered. This limited the control exerted by the female over the 'sexual repertoire' but allowed for the assessment of male mate selection between two female rats. Whereas this task has allowed us to gain some insight into female sexual behavior and male mate selection that could not be assessed using conventional methods, it does constrain the ecological validity of our findings.

ii. Another caveat lies in the manner in which male rats approached, mounted, intromitted, and ejaculated with High or Low LG females. Female copulatory behaviors are regulated, in part, by VCS in conjunction with steroid hormones. VCS, including male intromissions and ejaculations can, over time, facilitate the expression of lordosis in the absence of estradiol or progesterone (Blaustein et al., 2009). Furthermore, male copulatory stimulation and non-intromissive copulatory behavior can facilitate female copulatory and paracopulatory behaviors (Blaustein et al., 2009). Consequently, the order in which male conspecifics "chose" females in our task may have affected female behavior. This point did not likely impact the present findings, as our data indicate that males initially "chose" High and Low LG females with an equal probability.

iii. Finally, whereas olfactory and visual cues were assessed to determine if these cues influence female attractivity, auditory cues emitted by the female were not investigated. It is possible that Low LG rats attract male conspecifics through this mechanism (White and Barfield, 1989).

6.4.3 Conclusion

The present findings reveal that early life experiences in female rats influence multiple aspects of later sexual functioning. The quality of maternal care received early in life influences later paracopulatory behaviors, copulatory behaviors and progesterone receptor immunoreactivity in relevant neural loci. By extension, these differences in sexual function result in male rats choosing, almost exclusively, to partner with female rats reared under Low LG maternal conditions. While it remains to be determined if research on sexual behavior in rodents has predictive validity for aspects of human sexual function it is compelling to consider the possibility that similar processes of developmental programming are at play in humans. The findings from the current study suggest that social interventions targeted at ameliorating conflict and strife in the home and improving parental support could have a powerful impact on the sexual maturity and function of young girls at the level of behavior *and* physiology.

Section 6 Figures

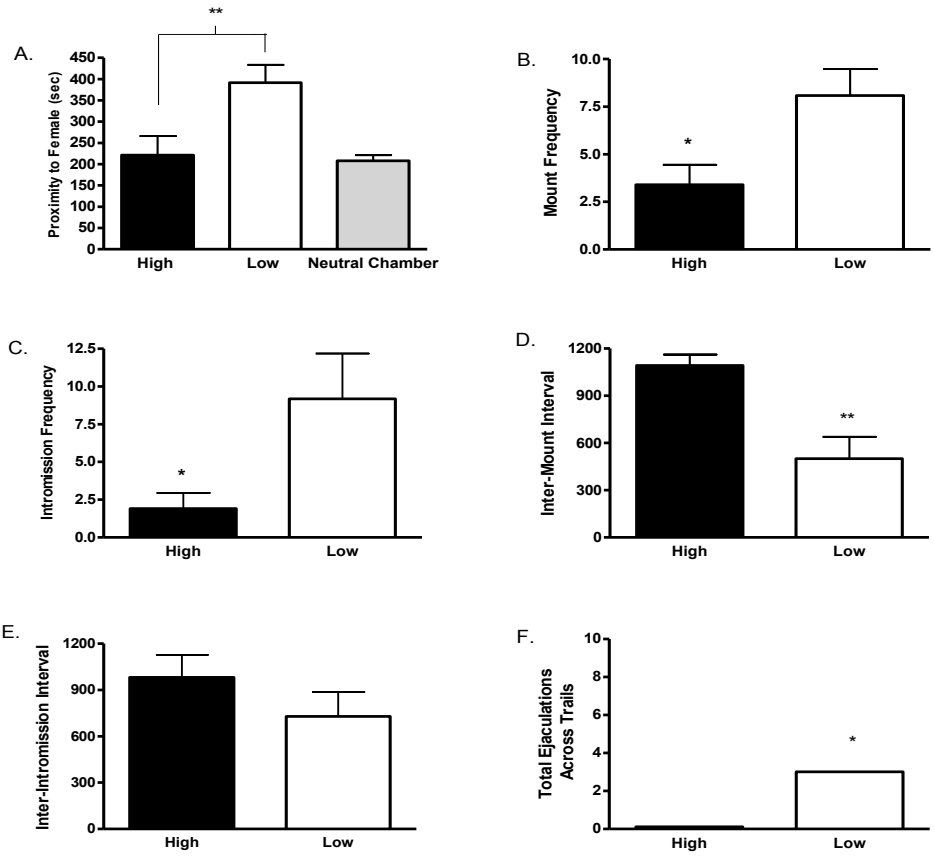


Figure 1 – Male Mate Preference. (A) Mean (±SEM) time spent by male rats in proximity to High or Low LG females. The filled bars represent females reared in High maternal litters and the open bars represents females reared in Low maternal litters. The grey bar represents the neutral chamber where no females were present. Males spent significantly more time investigating Low females relative to Highs (n=11). (B) Mean (±SEM) mount frequency with High and Low female rats. (C) Mean (±SEM) intromission frequency with High and Low female rats. (D) Mean (±SEM) inter-mount interval with High and Low female rats. (E) Mean (±SEM) inter-intromission interval with High and Low female rats. (F) Total number of ejaculations across all trials (n=11) in both High and Low female rats. All observed ejaculations (n = 3) occurred with Low LG females. *p<0.05; **p <0 .01

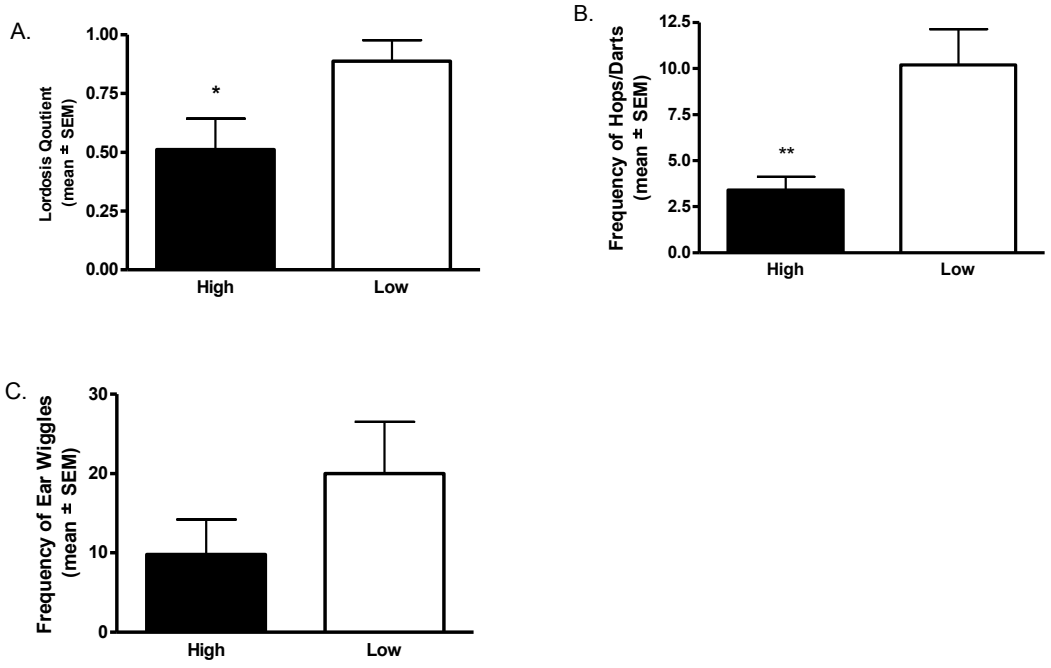


Figure 2: Female Behavior. (A) Mean (±SEM) lordosis quotient for High and Low LG females exposed to a novel male rat. (B) Mean (±SEM) frequency of hopping and darting in High and Low LG females exposed to a novel male. (C) Mean (±SEM) ear wiggle frequency of High and Low females exposed to a novel male. *p<0.05; **p <0 .01.

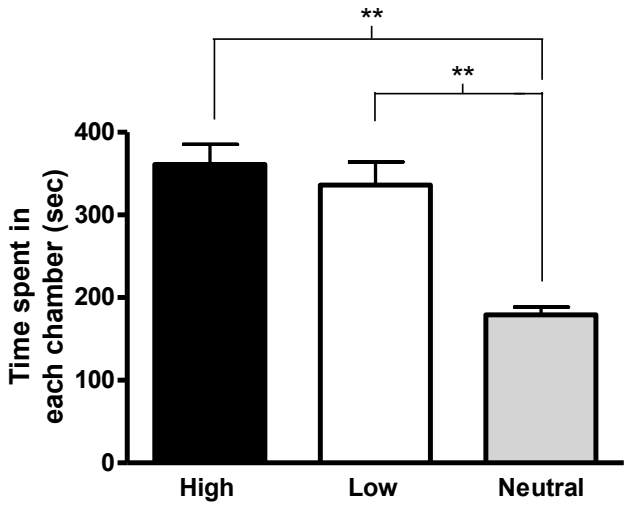


Figure 3: Male olfactory preference. Mean (±SEM) time spent in one of three chambers that contained different olfactory cues. Males spent roughly equal time investigating olfactory cues from High and Low females and significantly less time in the neutral chamber. $^{**}p < 0.01$.

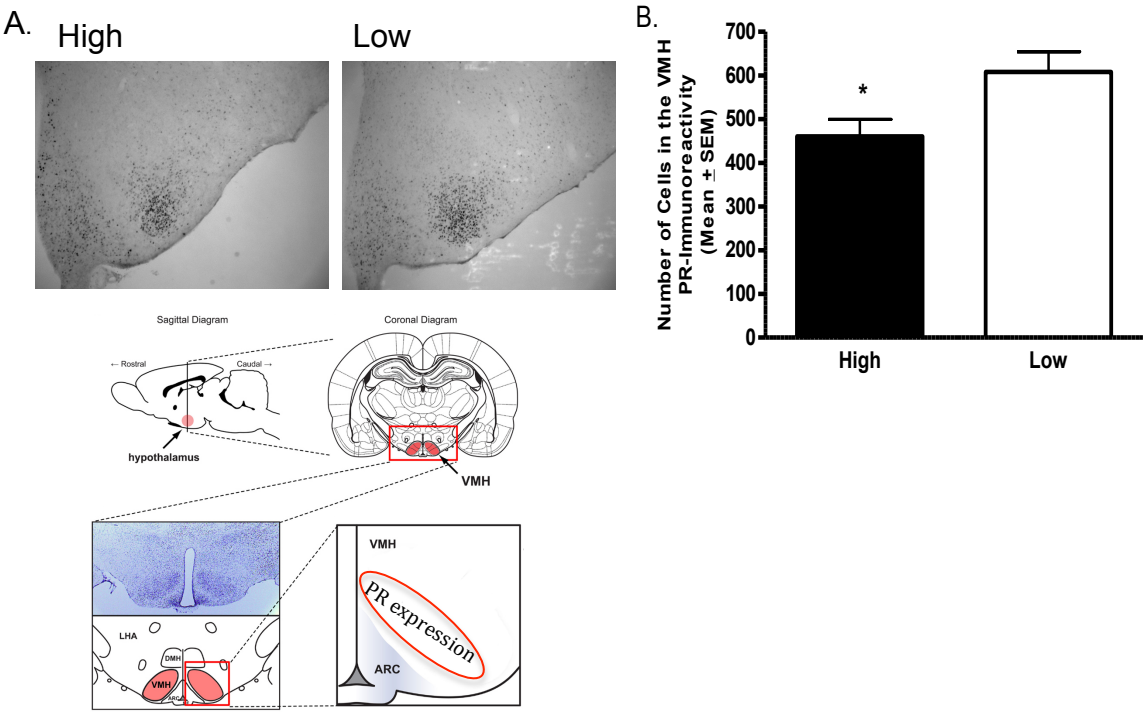


Figure 4: Effects of early maternal rearing on estrogen-induced progesterone receptor expression in the ventromedial hypothalamus (VMH) of female rats. (A) Low-power photomicrograph depicting representative immunostaining for PR in High and Low females (left, upper panel) and plate 56 of Paxinos and Watson (2007), depicting location of the VMH (left, lower panel). (B) The mean (±SEM) number of cells expressing PR immunoreactivity in High and Low LG female rats (n=7/group). *p<0.05.

Section 7: Early-Life Programming of Stress Response is Modifiable by Behavioral Training

Sakhai, S.A., Margerum, L.A., Francis, D.D. (Unpublished). Early-life programming of stress response is modifiable by behavioral training.

Abstract:

Stress influences a wide variety of outcomes including cognitive processing. Individual variability in stress measures may contribute significantly to performance on cognitive tasks. In the rat, early life maternal care can program developing offspring to influence stress reactivity and cognitive processes in adulthood. The current study assessed if variations in early life maternal care can influence cognitive performance on a task dependent on the medial prefrontal cortex (mPFC), the ability to switch cognitive sets. Early in life, offspring were reared under High or Low maternal Licking and Grooming (LG) conditions. As adults, they were trained daily (~25 min/day; 20 days) and then tested on an Attentional Set-Shifting Task (ASST) which measures mPFC function and cognitive flexibility in rodents. Stress-sensitive behavioral and neural markers were assayed before and after the ASST. High and Low LG offspring performed equally well on the ASST (assessed by the number of trials needed to learn the task and the number of errors). Low animals, however, took ~35% longer to complete the task. Interestingly, general training on the ASST task decreased overall stress phenotypes in Low LG offspring. Differences in hormonal and behavioral stress measures between High and Low LG adult offspring existent at the beginning of ASST training were attenuated when assessed at the end of ASST training/testing. These results highlight the potential for amelioration of stress effects, instantiated during the early developmental period, through the application of cognitive training.

7.1 Introduction

Stressful experiences influence a wide variety of health outcomes, both mental and physical. Chronic or repeated activation of the stress axis, or the hypothalamic-pituitary-adrenal (HPA) axis, increases levels of circulating glucocorticoids (Gunnar and Quevedo, 2007; Gunnar, 1992). Acting directly and indirectly on neuroanatomical regions, elevated glucocorticoids can influence the amygdala, hippocampus, orbitofrontal cortex, nucleus accumbens and medial prefrontal cortex function (Brown et al., 2005; Conrad, 2009; Goldwater et al., 2009; Holmes and Wellman, 2009; Kirby et al., 2013; Lemos et al., 2012; Liston et al., 2009; Liston et al., 2006; Mitra and Sapolsky, 2008). Both in humans and animals, the net effect is an alternation in emotional and cognitive function, which may contribute to the onset of numerous psychopathologies including depression, anxiety, and addictive disorders (McEwen, 2005, 2008; McEwen and Morrison, 2013). Neurobiology of

these disorders involves the dysregulation or disruption of frontostriatal-limbic processes; the same processes implicated in the regulation of stressful experiences (Amat et al., 2005; Bouret and Sara, 2004).

One potent regulator of the stress axis, or hypothalamic-pituitary adrenal (HPA) axis, is exposure to compromised or challenging early-life experiences, which can set the stage for individual differences in adult phenotypes. Abuse or neglect early in childhood has been demonstrated to influence a wide-variety of outcomes later in adulthood including stress-reactivity, psychiatric morbidity and mortality (e.g. increased incidence of depressive symptoms and anxiety), and memory impairments (Anda et al., 2006; Bernet and Stein, 1999; Brown et al., 1999; Hildyard and Wolfe, 2002; Valentino et al., 2009). Consequently, patients with anxiety and depressive disorders often show deficits in cognitive domains, including prefrontal dependent cognitive tasks such as the Wisconsin Card Sort Test (Austin et al., 2001; Merriam et al., 1999). Developing mammals are also influenced by more subtle changes in the early-life period. In the laboratory rat, for example, differences in the quality of care pups receive by dams have a very large effect on later adult phenotypes. Offspring reared in Low maternal care (i.e. licking and grooming; LG) litters exhibit increased anxiety-like phenotypes, diminished HPA negative feedback capacity in response to an acute stressor, and decreased expression of glucocorticoid receptors (GR) in the hippocampus relative to rats reared under High maternal care conditions later as adults (Champagne et al., 2003a; Francis et al., 1999b; Liu et al., 1997; Weaver et al., 2004a; Weaver et al., 2004b). All rat dams provide adequate care to their offspring, however, the quality of care is important to later neuroendocrine and behavioral outcomes (Akers et al., 2008).

Evidence from a variety of disciplines demonstrates the powerful role early life experiences play in the lives of young mammals in the realm of cognitive and physical health outcomes. The prefrontal cortex, a region of the brain central to cognitive flexibility and executive functioning (working memory, the ability to shift attention across perceptual dimensions, and rule-guided action to plan and guide behavioral sequences) (Arnsten, 2009; Birrell and Brown, 2000; Dalley et al., 2008; DeSteno and Schmauss, 2008; Goldman-Rakic, 1995; Hauber and Sommer, 2009; McEwen and Morrison, 2013; Wallis et al., 2001), is highly susceptible to environmental experience and glucocorticoids, particularly during early childhood and adolescence (Cook and Wellman, 2004; Dias-Ferreira et al., 2009; Evans and Schamberg, 2009; Hackman and Farah, 2009; Mizoguchi et al., 2000; Radley et al., 2008; Watson et al., 1996). For instance, prenatal and postnatal maternal stress in rodents results in alterations in prefrontal cortex development and dendritic arborization, alterations that can influence behavior upon stress exposure in adulthood (Green et al., 2011; McEwen and Morrison, 2013; Muhammad et al., 2012; Quirk et al., 2006). Early life maternal care is also capable of shifting α-1 $GABA_A$ receptor mRNA expression in the rodent mPFC as well as stress induced dopamine release; potentially contributing to observed alterations in adult anxiety behavior and sensorimotor gating in High and Low offspring (Caldji et al., 2000a; Caldji et al., 2000b; Zhang et al., 2005). In non-human primates, early experiences, both mild and severe are also capable of altering stress responsivity, pre-frontal glucocorticoid expression, pre-frontal cortex volume, monoamine metabolism, and cognitive behaviors dependent on the pre-frontal cortex (Feng et al., 2011; Lyons et al., 2002; Lyons et al., 2000; Parker et al., 2012; Patel et al., 2008; Pryce et al., 2004; Schneider et al., 1998; Spinelli S, 2009). It has become increasingly clear that early

life stressors can augment PFC development in humans as well. For example, children from impoverished backgrounds exhibit diminished activity in PFC activity as measured by EEG (Kishiyama et al., 2009). Damaged or compromised cognitive function is associated with low academic achievement, lower IQ, and deficits in memory and attention (Pechtel and Pizzagalli, 2011).

Across species, converging evidence demonstrates the influence early-life experiences can have on executive function. In the current study we predicted that rats reared under poor maternal conditions will have impaired executive function as measured by a perceptual attentional set-shifting task. We also predicted that Low LG offspring will differ in stress reactivity profiles, and potentially, that these stress profiles may influence executive functioning compared to High LG animals.

7.2 Methods and materials

7.2.1 Animals and Housing

Male rats used in the study were born in our home colony. They were generated from Long Evans rats purchased from Charles River Breeding Laboratories (Wilmington, MA). Female Long-Evans rats were bred in-house at UC Berkeley, allowed to give birth and maternal behaviors recorded as described below. Animals were weaned on post-natal day (PND) 22, and pair housed in polypropylene cages (27.8 x 17.5 x 13.0 cm) and left undisturbed until adulthood (PND 80) upon which they were assessed on several stress sensitive tasks and for mental flexibility (ASST task described below). Temperature was kept constant at 20 ± 2 °C and relative humidity was maintained 50 ± 5%. Rats were maintained on a 12-h light–dark cycle (lights on 0700 h to 1900 h), housed on wood pulp bedding, and allowed access to food (Purina Rat Chow, Purina Mills, St. Louis, Missouri) and tap water *ad libitum*, except during ASST testing. Two weeks prior to ASST, animals were maintained on a restricted diet of 20 grams of food per day and maintained to 85% of their original starting weight to increase motivation to complete the task. Housing and care of the rats were carried out in accordance with the standards and practices of the UC Berkeley Animal Care and Use Committee.

7.2.2 Observations of Maternal Behavior

Observations of maternal behavior were performed with slight modification as previously described in Sakhai, Kriegsfeld, and Francis, 2011 (Sakhai et al., 2011). Female rats were bred and permitted to give birth (n = 54). Maternal observations were performed the day following birth, beginning on PND 1 and continued until PND 5. Each litter was observed for five hours a day at the following times; 0600 h – 0800 h, 1200 h – 1300 h and 1800 h – 2000 h. During each observation session, litters were observed and behaviors recorded every two minutes. Calculating the frequency of pup-directed maternal licking and grooming (LG), a maternal care distribution curve was generated. Maternal LG was expressed as a percentage of the total number of observations performed for each litter. High and Low LG litters were assessed as those falling one SD above or below the mean frequency of maternal licking, respectively. Offspring were weaned, pair housed with same sex littermates, and left undisturbed until stress phenotyping and mPFC dependent set-shifting behavior in adulthood.

7.2.3 mPFC Dependent Set-Shifting Behavior

In adulthood, animals were trained on an mPFC dependent Attentional Set-Shifting Task (ASST) (n = 7 High and n = 15 Low); the overall maternal LG distribution skewed low LG, leading to unequal group numbers. The ASST is a rodent version the Wisconsin Card Sorting Test, a neuropsychological task used to assess PFC function in humans (Robinson et al., 1980). It necessitates an intact mPFC, requiring animals to shift between varying response rules, and is thought to be a measure of cognitive flexibility and executive functioning (Birrell and Brown, 2000; Ng et al., 2007). Because animals must learn one set of rules and behavioral actions before testing, the ASST requires extensive training and handling to test mPFC set shifting integrity (Birrell and Brown, 2000; McAlonan and Brown, 2003). Testing protocol and equipment was modified from Birrell and Brown, 2000 (Birrell and Brown, 2000).

Animals were trained to retrieve a food reward by digging in small terra-cotta pots (6 cm diameter x 5 cm height) filled with medium (e.g. type of bedding). Pots may carry carries two types of information that is salient to the animal to receive reward - either medium type or odor. Animals were trained to dig for reward using information from a single dimension (i.e. odor). The number of errors, perseveration effects, and time to complete the task was assessed before and after the dimension shift occurred. Animals then had to attend to the new relevant dimension of the stimulus (i.e. medium). See Table 1 for the order of discriminations for the ASST.

Initial Training and Simple Discrimination: Starting on PND 115, animals were handled daily for ~5 min per day for 10 days in preparation for ASST testing. After initial handling, animals were trained to dig to retrieve a food reward over 7 days. A minimum of 20 consecutive digs was used as a threshold of learning. For two consecutive days, animals were then trained on a simple discrimination (SD) in which they learned to respond to a specific odor cue to receive a reward. Testing continued until a criterion of six consecutive correct trials were made. Rats were allowed to approach and sniff the pot or climb over it; however, if medium was displaced by digging or by sniffing, the response was recorded. All rats were trained on the same discriminations, in similar order. The exemplars, medium and odor, were not used again during testing. See Table 2 for a list of exemplars.

Training Experience and ASST Testing: During the SD, rodents learned that one odor is relevant and rewarded. In the next stage of the task, the compound discrimination (CD), stimuli were made more complex. Odor continued to remain the rewarded dimension while the animal had to discount extraneous information regarding the medium. The first of three reversals (R1) followed the CD, in which the animal learned that the previously correct exemplar was now incorrect within the same dimension (i.e. odor remained the relevant dimension; however, the previously rewarded odor exemplar was now irrelevant). Animals had to learn to switch learned odor associations to receive reward. For both the intra-dimensional (ID) and extra-dimensional shift (ED), new exemplars were used for the relevant and irrelevant dimensions. For the ID, odor was reinforced as the relevant dimension using new stimuli, solidifying odor-reward associations. For the ED, animals were required to shift rule contingencies with the previously relevant dimension (odor) no

longer being rewarded, that is, the medium is now rewarded). Testing was counterbalanced to minimize order effects. In total, each animal received 20 days of consecutive training ranging from 5 minutes for handling sensitization to 3 hours of cognitive stimulation on testing day.

7.2.4 Anxiety-Like Behavior

Between 01000 – 01300 h beginning on PND 80 and again after mPFC training (n = 7 High and n= 15 Low), animals were tested in two stress-sensitive behavioral tasks: the Open-Field and the Light-Dark Box Test (Archer, 1973; Hall, 1934). Animals were tested on non-consecutive days.

Open Field: To assess anxiety-like behavior, animals were exposed to an open field. The open field consisted of a large circular polypropylene arena 140 cm in diameter, 61 cm in height. Each animal was placed in the open-field for five minutes and subsequent behaviors recorded. The arena was cleaned between animals with 1% NPD and 70% ethanol. Frequency of crosses between the outer arena (14cm width) and the interior inner arena (112 cm diameter) and amount of time spent in the inner-arena of the open field was quantified. The behavior of each rat was recorded and analyzed by an experimenter blinded to group conditions.

Light-Dark Box: Similar to the open field, the light-dark box is used to assess anxious behavior in rodents. The light-dark box consists of two contiguous rectangular arenas (76 x 40 cm) joined by an entrance (10 x 10 cm). One arena, the light box, is open and exposed, while the second arena is dark and sheltered providing a less aversive space for the animal. Animals were initially placed within the dark chamber and given five minutes of exploration time. Latency to emerge from the dark box and time spent in the light box was recorded by an experimenter blind to group conditions.

7.2.5 HPA Measures

Plasma Corticosterone: Tail blood was collected from two subsets of High and Low offspring, before and after training experience (n = 18 High and n = 26 Low). Under basal and stress conditions, blood samples were collected between 0700 h – 01000 h (to control for the diurnal rhythm in corticosterone secretion).To assess basal corticosterone values, animals were removed from home cage and tail bled within two minutes. For stress values, animals underwent 15 minutes of restraint stress, and blood rapidly collected. Upon completion of acute restraint stress, animals were released to home cage and blood collected every 30 minutes for 4 recovery time points. Samples were spun at 14,000 RPM in a micro-centrifuge for 20 minutes at 4 °C, plasma aliquoted, and frozen at -20 °C until assayed using corticosterone enzyme immunoassay (Enzo Life Sciences, Ann Arbor, MI). Samples were diluted 1:20 with assay buffer and aliquoted into a 96 well plate and run in duplicate. Prior to analysis of data, one animal was removed due to insufficient volume of blood sample.

Glucocorticoid Receptor Western Blot: To assess alterations in HPA physiology, before and after training experience, two subsets of High and Low offspring were assayed for

glucocorticoid receptor (GR) protein expression in the frontal cortex and hippocampus via western blot (n = 15 High and n = 14 Low). Whole hippocampus was removed and frontal cortex dissection was restricted to infralimbic, prelimbic, and anterior cingulate cortices. Tissue was dissected immediately after euthanasia and snap frozen in liquid nitrogen. Tissue was then homogenized in RIPA buffer solution with 1% protease inhibitor (Calbiochem protease inhibitor cocktail set iii, EDTA free). RIPA buffer contained 50 mM tris HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate at a pH of 8.0 at room temperature. The homogenate was then centrifuged at 14,000 g for 30 min. Cytosol supernatant was extracted and total protein concentrations determined via Pierce BCA protein assay kit (Thermo Scientific, product # 23227). Twenty ug of protein was then loaded and separated with a 7.5% Tris-SDS polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA). Proteins were transferred to PVDF membrane (Amersham Hybond-P; GE Healthcare) and blocked with 5% non-fat milk in 1× TBS-t (Tris-Buffered Saline, 0.1% Tween-20, pH 7.6) for 1 h. Membrane was incubated with polyclonal rabbit anti-GR at 1:2000 (Santa Cruz Biotechnology, #sc-1004) and mouse anti-actin at 1:10,000 (Sigma Aldrich, #A1978) overnight at 4 C. The signal was detected using horseradish peroxidase conjugated anti-rabbit and anti-mouse antibodies at a concentration of 1:5000 (JacksonImmuno Research, catalog # 711-035-152 and #715-035-151). The GR signal was subsequently enhanced via Western Lightning ECL Kit (PerkinElmer; Waltham, MA) and exposed to autoradiography film for visualization. Western band optical density was determined by gel imaging system (MCID Basic, Version 7.0; Imaging Research Inc.) and normalized with the band optical density value of actin as an internal control.

7.2.6 Data Analysis

Prior to analysis of behavioral data, a D'Agostino—Pearson omnibus test for normality was conducted. If data did not pass normality, a Mann-Whitney U-test was used to account for a non-Gaussian distribution. Otherwise, data was analyzed by a Student's t-test. Plasma corticosterone and ASST performance were analyzed by a two-way analysis of variance (ANOVA). A Šidàk-Bonferroni multiple comparisons post-test was used to assess groups across conditions. Measurements of glucocorticoid receptor expression were determined by Student's t-test. Results were considered statistically significant when $p < 0.05$.

7.3 Results

7.3.1 Maternal Observations

Dams naturally differed in frequency of licking/grooming and arched-back nursing over the first week post-partum. A maternal care score was generated by calculating the frequency of maternal licking observed relative to the total number of observations performed over the entire observation period. Percent licking ranged from 3.33 –11.94% and 4.93% – 14.4% with a mean licking score of 6.8% (SD of 1.7%) and 7.3% (SD 1.8%) across all litters respectively. Litters that fell ± 1 SD away from the mean in each cohort were used in the remainder of the study (n = 6 High and 8 Low LG litters).

7.3.2 Attentional Set Shifting Task

High and Low LG animals did not differ across groups in set-shifting performance on the ASST. The interaction between Maternal care and Stage of the ASST (e.g. SD, CD, R1, ID etc.) for both trials to criterion and errors was not significant, $F(6, 140) = 0.8947$, $p = 0.5007$ and $F(6, 140) = 0.5285$, $p = 0.7859$ respectively (**Figure 1A and 1B**). The main effect of Maternal care was also not significant for trials needed to reach criterion on the ASST, $F(1, 140) = 0.8213$, $p = 0.3664$ or errors on each stage of the ASST, $F(1, 140) = 0.03920$, $p = 0.8433$.

Remarkably, animals reared by Low LG mothers take ~35% more time to complete the ASST task than High LG offspring, $U(20) = 7.5$, $p = 0.0006$ (**Figure 1C**). While there is no interaction between the time to complete each Stage of the ASST and Maternal care, $F(6, 140) = 0.7925$, $p = 0.5773$, main effects of Maternal care, $F(1, 140) = 16.36$, $p < .0001$ and Stage of ASST, $F(6, 140) = 2.187$, $p = 0.0481$ are significant (**Figure 1D**). Šidàk-Bonferonni post-hoc tests show Low LG animals spend significantly more time than High animals to complete the second reversal of the ASST ($p < 0.05$). This trend was also observed, although not significantly, with the CD, R1, ID, ED, and R3.

7.3.3 Pre-Training Stress Phenotype

Behavior

Open Field: As adults, High animals spent significantly more time exploring the inner arena of the open field than Low animals before training on the ASST, $U(20) = 19.50$, $p = 0.0179$ (**Figure 2A)**. More time exploring the inner area of this arena is interpreted as lower levels of anxiety in these animals.

Light-Dark Box: As adult, High animals spent significantly more time exploring the light arena of the Light-Dark box before training, $U(20) = 16.50$, $p = 0.0083$ (**Figure 2B**). Similar to the open field, greater time exploring the exposed arena of a light-dark box apparatus suggests lower levels of anxiety.

HPA Measures

Plasma Corticosterone: Prior to training experience, High and Low animals significantly differed in HPA reactivity to an acute stressor. The main effect of Maternal care was significant, $F(1, 118) = 10.68$, $p = 0.0014$, as was the main effect of Time, $F(5, 118) = 16.99$, $p < .0001$. The interaction of these two factors was not significant, $F(5, 118) = 1.159$, $p = 0.3337$. Post hoc testing shows that Low animals displayed a significantly higher corticosterone peak 30 minutes after restraint stress than High animals ($p < .05$) (**Figure 2C**). Integrated corticosterone values across the two hour period was significantly higher in Low LG offspring, suggesting less robust negative feedback compared to High animals, $t(20) = 2.124$, $p = 0.0463$ (**Figure 2D**). Intra-assay and inter-assay variability for corticosterone ELISA was 3% and 7.8%, respectively.

Glucocorticoid Receptor Expression: Hippocampal glucocorticoid receptor expression was significantly different between High and Low animals before ASST testing ($U(11) = 3$, $p = 0.0082$) (**Figure 2E**). However, glucocorticoid receptor expression in the frontal cortex was insignificant ($U(13) = 28$, $p > 0.9999$) (**Figure 2F**).

7.3.4 Post-Training Stress Phenotype

Behavior
Open Field: As adults, groups are no longer significantly different on behavioral indices of anxiety as measured by the open field. U (20) = 42, p = 0.4786 (**Figure 3A**).
Light-Dark Box: As adult, High and Low animals spend equivalent time exploring the light arena of the Light-Dark box, U (20) = 40, p = 0.5346 (**Figure 3B**).

HPA Measures
Plasma Corticosterone: Plasma corticosterone values did not significantly differ between High and Low LG animals, main of effect of maternal care, F (1, 114) = 1.354, p = 0.2471) (**Figure 3C**). Time, as a main effect, remained significant, F (5, 114) = 21.67, p = < 0.0001. There was no interaction between Time and Maternal care, F (5, 114) = 0.9904, p = 0.4269. Furthermore, integrated corticosterone values over the two hour period were not significant, U (19) = 37, p = 0.5399 (**Figure 3D**). Intra-assay variation for corticosterone ELISA was 3% and 7.8% inter-assay.
Glucocorticoid Receptor Expression: After ASST testing, glucocorticoid receptor expression in the hippocampus remained significantly different, U (14) = 6, p = 0.0052, (**Figure 3E**). Glucocorticoid receptor expression in the frontal cortex was not significant, U (14) = 25, p = 0.5117, (**Figure 3F**).

7.4 Discussion

In the current study we predicted that rats reared under reduced maternal conditions early in life, when tested later in adulthood, would exhibit impaired executive function in a rodent version of the Wisconsin Card Sorting Task, an attentional set- shifting task. However, we report that both groups were capable of successfully performing the ASST task, regardless of maternal rearing condition. Performance on the ASST was assessed as i) the number of trials it took each animal to reach criterion and ii) the number of incorrect choices made by individual rats. This finding was unexpected given the known effects of early-life programming (including variations in maternal care) on cognitive function (Green et al., 2011; Liu et al., 2000b; Muhammad et al., 2012). We did find that adult rats reared under Low maternal licking and grooming conditions took significantly longer to complete the overall task as compared to the High LG offspring. We also predicted that High and Low LG offspring would differ in stress reactivity profiles when tested as adults. This hypothesis was confirmed as animals differed in stress reactivity profiles as adults, however, these group differences disappeared after cognitive training /handling and testing. These results are noteworthy as they suggest that stress effects, instantiated during the early developmental period, can be ameliorated through the application of cognitive training/handling.

We predicted that High LG adult offspring would perform better than Low LG offspring on the ASST, a rat 'version' of the human Wisconsin Card Sorting Task based on a variety of data from humans and rodents exhibiting a putative link between early life experiences and cognitive function (Meaney, 2010). These links include variability between High and Low rat offspring in multiple physiological and behavioral domains,

including behavioral differences in hippocampal-dependent tasks, neuronal survival, HPA functioning and stress reactivity, sociality, reproductive behavior, and sensorimotor functioning (Bredy et al., 2003; Champagne et al., 2008; Engert et al., 2009; Fish et al., 2004; Francis et al., 2000; Liu et al., 2000b; Sakhai et al., 2011; Starr-Phillips and Beery, 2014; Zhang et al., 2005). In humans, a correlation between early-life experiences and cognitive function also suggests a putative role for early-experiences in PFC development. In children, exposure to early-life trauma is associated with deficits in cognitive function. For example, children exposed to interpersonal trauma (IPT), particularly during the zero-2 years, had significantly lower child intelligent quotient (IQ) scores at 24, 64 and 96 months of age compared to children not exposed to IPT in the first 2 years of life (Enlow et al., 2012). Children exposed to child neglect, the most common form of maltreatment, also have deleterious effects on children's development including cognitive function. Neglect that occurs early in life is particularly damaging to developmental trajectories and can be uniquely dissociated from the effects due to physical abuse. Compared to other maltreatment groups, emotionally neglected children have the largest decrease in scores on the Bayley Scales of Infant Development between 9 and 24 months old (Egeland and Sroufe, 1981). By kindergarten age neglected children have the lowest scores of all maltreatment groups on tests of intellectual functioning and academic achievement (Hildyard and Wolfe, 2002).

In laboratory rats, naturally occurring variations in maternal behavior are related to the development of individual differences in cognitive performance of offspring when tested later in adulthood. Specifically, spatial learning and memory is enhanced in adult offspring reared in a High LG relative to Low LG maternal condition. This is a persistent effect that lasts into old age for the rats. As spatial learning and memory are hippocampal dependent processes, it is not surprising that markers of hippocampal synaptic plasticity (synaptophysin and N-CAM) are significantly higher in High LG rats when measured at PND 18. In this same model, hippocampal acetylcholine (Ach) release is greater in High LG offspring compared to Low under both basal and stimulated conditions (Liu et al., 2000b). To date, there is no evidence that PFC-dependent cognitive tasks differ across rats reared under different maternal conditions. Our current findings are consistent with this statement. What has been reported recently, however, is the ability of rat infant-caregiver interactions to epigenetically mark genes demonstrated to play a prominent role in cognition and psychiatric disorders within the mPFC (Blaze and Roth, 2013; Blaze et al., 2013). These epigenetic changes within the PFC, however, have yet to be linked to changes in cognitive function.

The significant difference in time it took for High and Low LG animals to complete each stage of the ASST, with equivalent levels of accuracy across groups, merits further discussion. There are many hypotheses to interpret time differences across groups. Variance could reflect information-processing deficits, stress effects, possible differences in appetitive behaviors (reward and motivation), or differences in food required to achieve satiety. Interestingly, with the exception of the latter, variations in maternal care has been shown to impact all of these processes. For example, animals from Low maternal care backgrounds exhibit increased myelin distribution in the hippocampus and corpus callosum, suggesting alterations in information processing (Taravosh-Lahn et al., 2013). Variations in early-life maternal care have also been shown, in our current study as well as in existing literature, to alter stress reactivity profiles and dopaminergic tone (Brake et al.,

2004; Zhang et al., 2005) (Francis et al., 1999b). During an acute challenge, glucocorticoids work in synergy with catecholamines to regulate many physiological processes, which include increased attention and reduced impulsivity (Arnsten, 2009; Arnsten and Li, 2005). The arousal involved in training and testing the animals could lead to groups that are differentially motivated to seek food reward. In essence, stress effects may confound performance on cognitive tasks. As all animals were food restricted to 80-85% of their body weight before the start of testing we believe they were all motivated to complete the task. Ultimately, further research is required to investigate the relative contribution of each variable to our observed time difference.

One unexpected, yet exciting finding is the convergence of stress phenotypes across High and Low LG animals by the end of the testing period. Baseline measures of stress-sensitive behaviors (open-field and light-dark box performance) and hormones (corticosterone; basal and stress-induced) were measured prior to the training and administering of the ASST task and again after the task (a period of several weeks). High LG offspring had greater exploration in the behavioral tasks and lower corticosterone levels following an acute stressor relative to Low LG offspring. Following the ASST training and trialing period High and Low LG offspring stress phenotypes were no longer significantly different. One potential explanation for the observed amelioration of stress-sensitive behavioral and HPA differences across groups is the robust training, handling, and cognitive enrichment provided to all animals to implement the ASST task. Animals received, on average, ~25 minutes of handling per day for 20 consecutive days, ranging from 5 minutes to 3 hours of stimulation on testing day. In congruence with this hypothesis, previous animal studies have shown an ostensible reversal of the effects of early life programming by handling, training, and enrichment. In mice, for example, handling in adulthood has also been shown to reverse anxiety-like behavior incurred by the stress of solitary housing (Heredia et al., 2012). In rats, postnatal handling is capable of reversing the effects of prenatal stress both behaviorally and via development of the HPA axis (DeNelsky and Denenberg, 1967; Maccari et al., 1995; Vallée et al., 1999; Weinstock, 1997). Environmental enrichment post-weaning is also capable of reversing the adult anxiogenic HPA and behavioral responses to stress generated by maternal separation models (Francis et al., 2002; Whimbey and Denenberg, 1967). Finally, and most relevantly, environmental enrichment during adolescence and early adulthood has been shown to eliminate deficits in cognitive function (assessed by Morris water maze learning and object recognition tasks; hippocampal dependent tasks) between High and Low LG offspring. However, like our current study, mechanisms of this reversibility/compensation were not elucidated (Bredy et al., 2003). Collectively, these studies demonstrate that, at the level of behavior, the long-term effects of early-life maternal programming are, indeed, reversible. These results are of particular relevance to those conducting research in the behavioral neurosciences. We provide evidence that careful attention should be paid to enrichment effects that occur following training/testing, particularly for more cognitively intensive behavioral tasks. Although our data cannot provide more specificity related to which component of training, handling, or enrichment is leading to a convergence of HPA responses, our results do demonstrate that the process of training enrichment is capable of attenuating HPA responses across groups. Potential sources of variation between studies may be inherent in the training and handling that is used to acclimate animals to more cognitive stimulating or demanding tasks.

In the current study, it appears that handling/training enrichment is leading to a reversal of stress-reactive phenotypes at a behavioral level, however what remains undetermined is what cellular and molecular mechanisms are driving this reversal at the level of the brain? Would we expect to see a concomitant reversal of neuronal processes to account for changes in the stress-sensitive behaviors observed? Or, perhaps compensatory effects are at play, influencing the effects of early life experiences? Compensation and reversibility are not mutually exclusive hypothesis. Our current data suggests that some measure of compensation is occurring in animals as a result of the training and handling regimen. Frontal cortex and hippocampal glucocorticoid receptor gene expression, which mediates the magnitude and efficacy of the stress response, remained the same after handling/training enrichment relative to pre-training levels. Indeed, these findings suggest that alterations to hippocampal and frontal cortex GR expression may be resistant to subsequent environmental influences, maintaining the residues of early life programming.

The scope of the proposed compensatory effect remains a matter of speculation, but the hippocampus and prefrontal cortex are interesting sites for consideration due to their role in mediating and moderating the stress response.

We initially hypothesized that adult rats reared under low maternal conditions would be impaired on a PFC dependent task relative to rats raised in high maternal care conditions. This turned out not to be the case, however, we provide evidence that stress-sensitive developmental programming effects in rats are influenced by later handling/training effects. The most highly reported differences between High and Low LG reared rats are related to stress-sensitive measures and behaviors. Low LG rats, as adults, are more anxious, fearful and stress-reactive relative to High LG animals. Here we report that a handling/training regimen on a cognitive task is sufficient to ameliorate stress-sensitive differences across groups. This may be of particular importance for those interested in the capacity for behavioral change after early life adversity or trauma. Our work also provides insight into potential sources of variability in the behavioral sciences.

Section 7 Figures and Tables

Table 1. Order of Discriminations for Attentional Set Shift Task

Training Discriminations	Dimensions Relevant	Dimensions Irrelevant	Exemplar Combinations +	Exemplar Combinations -
Simple (SD)	Odor	Medium	**O1**	O2
Testing Discriminations	**Dimensions Relevant**	**Dimensions Irrelevant**	**Exemplar Combinations +**	**Exemplar Combinations -**
Simple (SD)	Odor	Medium	**O3**	O4
Compound (CD)	Odor	Medium	**O3**/M1 **O3**/M2	O4/M2 O4/M1
Reversal (Rev 1)	Odor	Medium	**O4**/M1 **O4**/M2	O3/M2 O3/M1
Intra-Dimensional Shift (ID)	Odor	Medium	**O5**/M3 **O5**/M4	O6/M4 O6/M3
Reversal (Rev 2)	Odor	Medium	**O6**/M3 **O6**/M4	O5/M4 O5/M3
Extra-Dimensional Shift (ED)	Medium	Odor	**M5**/O7 **M5**/O8	M6/O8 M6/O7
Reversal (Rev 3)	Medium	Odor	**M6**/O7 **M6**/O8	M5/O8 M5/O7

Example of stimulus combination pairs for attentional set shift task. The correct exemplar, which the rat must choose, is shown in bold, with an irrelevant exemplar paired. For each discrimination a new set of exemplars was used. Odor remained the relevant dimension until the extra dimensional shift, upon which the medium became relevant and the odor irrelevant. (Modified from Birrell and Brown, 2000.)

Table 2

Odor Pairs	Medium Pairs
Vanilla vs. Cranberry	
Pine vs. Strawberry	Moss vs. Felt
Honey Dew vs. Cinnamon	Styrofoam vs. Shredded Sand Paper
Lavender vs. Pomegranate	Shredded Paper vs. Plastic beads

Examples of exemplar stimulus pairs used.

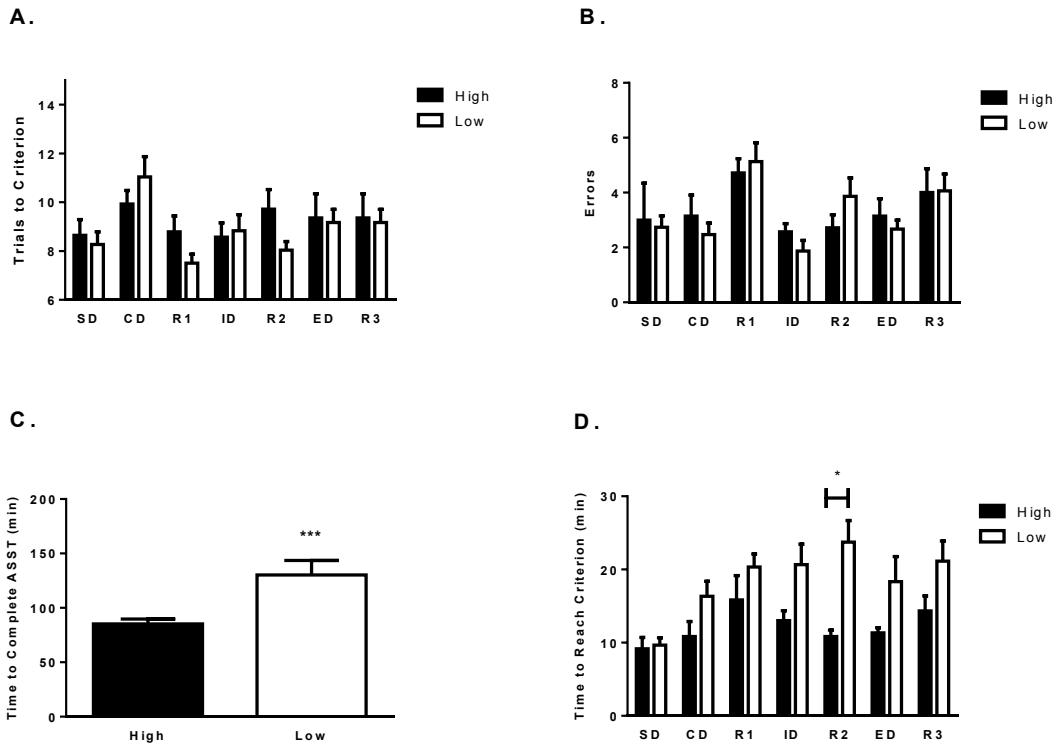


Figure 1: Offspring attention set-shifting behavior. The number of A) Trials to criterion and B) Errors on each component of the attentional set shifting task across maternal care conditions ($p > .05$). C) Animals reared by a Low LG mother spend significantly more time to reach criterion on the second reversal of the ASST ($p < 0.05$) D) Low LG animals spend ~35% more time to complete the task in its entirety ($p < 0.05$) than High LG animals. (SD – simple discrimination, CD – compound discrimination, R – reversal, ID – intra-dimensional shift, ED – extra-dimensional shift).

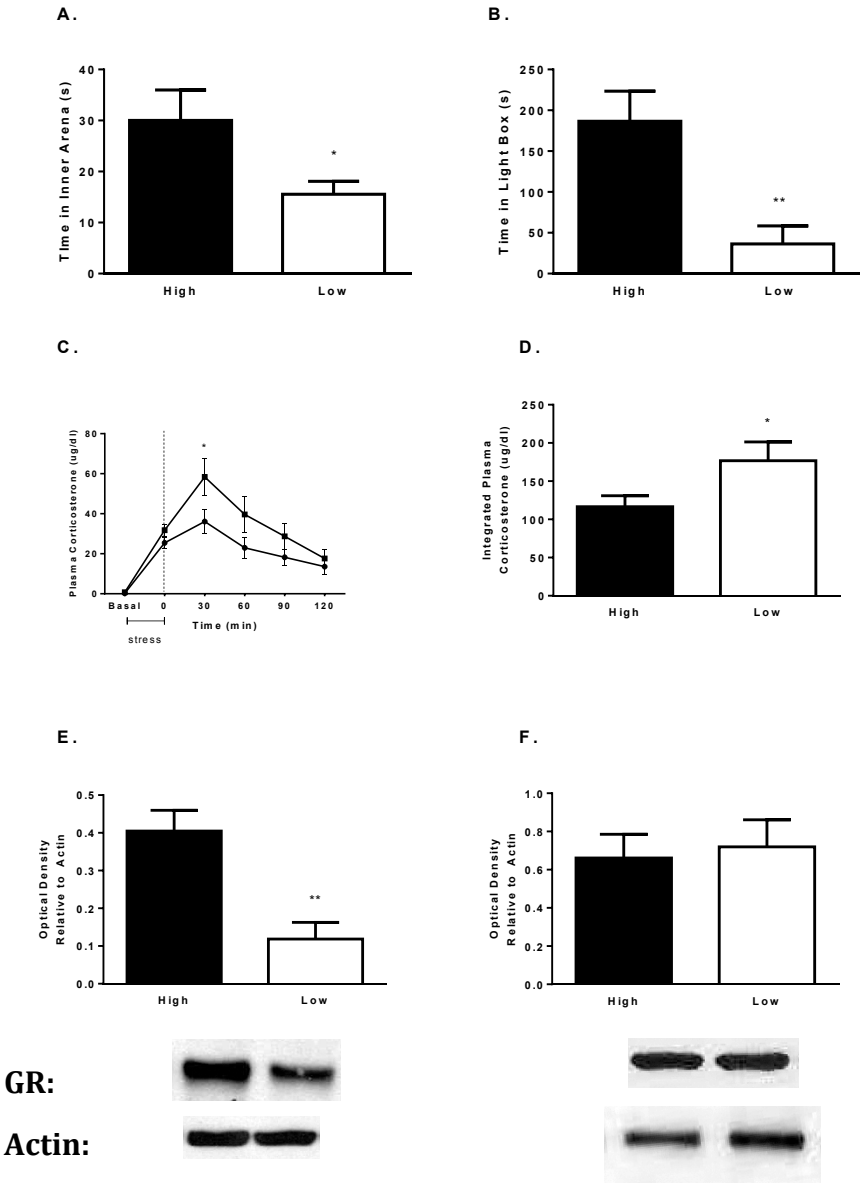


Figure 2: Offspring behavior in adulthood prior to training-experience. A) Open Field and B) Light Dark Box. Animals reared by a Low LG mothers spend significantly more time on behavioral indices of anxiety as indicated by the open field and light-dark box task ($p < .05$). C) When exposed to acute restraint stress, Low LG animals exhibit significantly greater peak corticosterone profiles after 30 minutes recovery as well as D) across the 120 minute recovery period ($p < 0.05$). Basal corticosterone was not significant between groups. E) Hippocampal glucocorticoid receptor protein levels were decreased in the Low LG animals compared to High LG ($p < 0.05$), however, F) glucocorticoid receptor protein levels did not differ in the mPFC between groups.

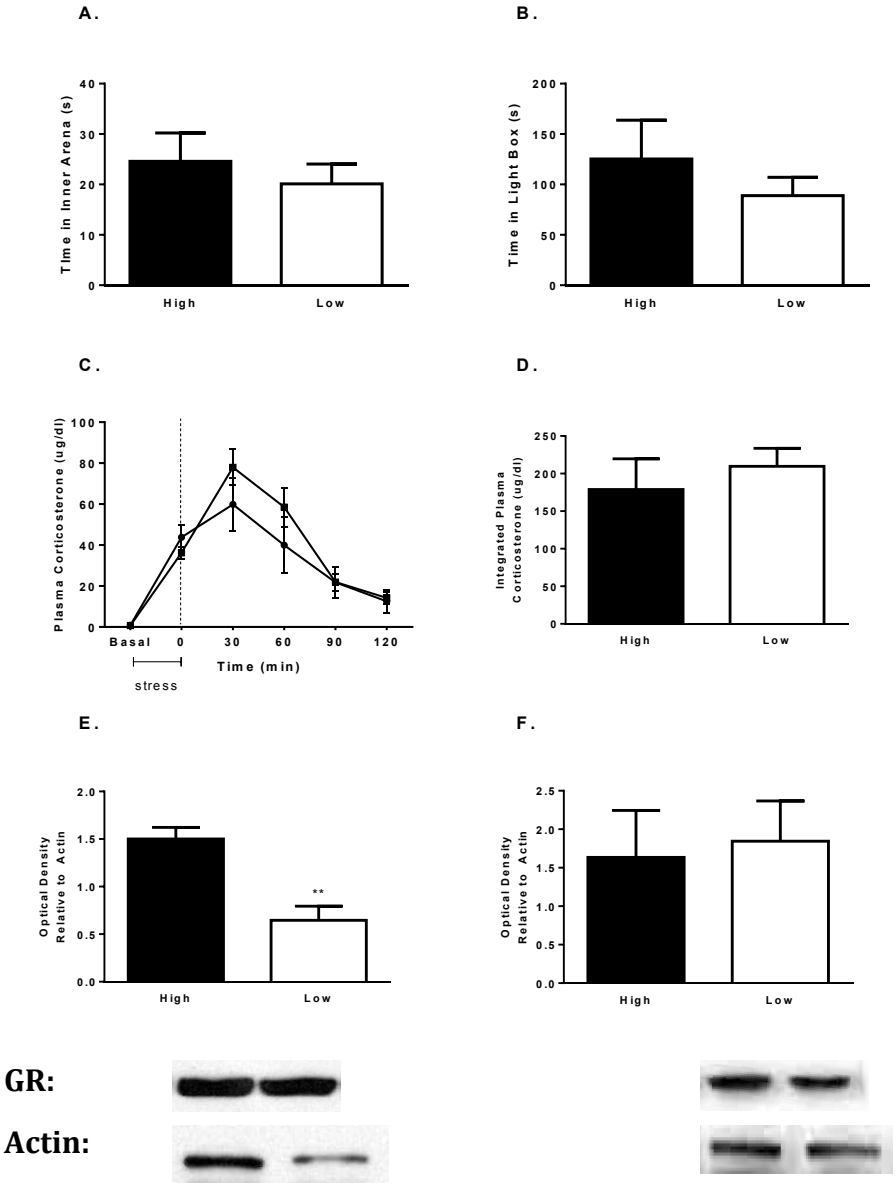


Figure 3: Offspring behavior in adulthood after ASST training-experience. A) Open Field and B) Light Dark Box. Animals reared by Low or High LG mothers did not differ in behavioral indices of anxiety as measured by the open field and light-dark box ($p > .05$). C) When exposed to acute restraint challenge, Low and High groups were no longer significantly different at either peak stress ($p > 0.05$) or D) across the 120 minute recovery period ($p > 0.05$). E) Hippocampal glucocorticoid receptor protein levels remained reduced in the Low LG group ($p < 0.05$) F) glucocorticoid receptor protein levels also remained unchanged in the mPFC. Compared to High LG animals.

Section 8: Influence of housing variables on the development of stress-sensitive behaviors in the rat

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Abstract

Diverse environments early in mammalian life can have profound influences on the physiology and behavior of developing offspring. Environmental factors can influence offspring development directly, or, through perturbations in parental care. In the current study, we wished to determine if the influence of a single environmental variable, type of bedding material used in laboratory cages, is capable of altering physiological and behavioral outcomes in offspring. Female rats were housed in cages containing wood pulp or corncob bedding and allowed to mature. These rats, while housed on assigned bedding material, were bred and allowed to give birth. At weaning, male offspring were housed on one of the two bedding conditions and tested later in adulthood on stress-sensitive behavioral measures. Postmortem analysis of glucocorticoid receptor expression and CRH mRNA levels were also measured. Maternal care directed at the pups reared in the two different bedding conditions was also recorded. Rats reared from birth on corncob bedding exhibited decreased anxiety-like behavior, as adults, in both open field and light-dark box tasks compared to wood pulp reared animals. Animals that received similar overall levels of maternal care, regardless of bedding condition, also differed in anxiety-like behaviors as adults, indicating that the bedding condition is capable of altering phenotype independent of maternal care. Despite observed behavioral differences in adult offspring reared in different bedding conditions, no changes in glucocorticoid receptor expression at the level of the hippocampus, frontal cortex, or corticotrophin releasing hormone (CRH) mRNA expression in the hypothalamus were observed between groups. These results highlight the importance of early life housing variables in programming stress-sensitive behaviors in adult offspring.

8.1 Introduction

Across mammalian species, early life is a time of heightened susceptibility to environmental input, capable of altering the development of offspring behavior and physiology (Francis, 2009; Meaney, 2007; Sakhai et al., 2011; Trivers, 1974). Environmental input, particularly during periods of heightened neuronal plasticity, can increase neuron numbers, synapses, dendritic branching, as well influence neuroendocrine systems such as the hypothalamic-pituitary-adrenal (HPA) or stress axis to further alter animal behavior(Champagne et al., 2003a; Cui et al., 2006; Diamond Mc Fau - Ingham et al.; Francis et al., 1999b; Renner Mj Fau - Rosenzweig and Rosenzweig; Rosenzweig and Bennett, 1996; van Praag H Fau - Kempermann et al.). In the laboratory, different animal housing conditions at various points in the lifespan of the organism (such as providing

more complex/enriched cages, or conversely, deprivation) can also influence stress-axis function and behavior. Many studies, including a seminal paper by Crabbe et al (1999) illustrate how minor changes in standard laboratory environments can abolish or reverse genetic effects of behavior in laboratory mice (Crabbe et al., 1999; Würbel, 2001). Providing enriched housing conditions to mice typically housed under standard laboratory housing conditions is sufficient enough to attenuate non-spatial memory impairments in NMDA knockout mice, possibly by increasing synaptogenesis (Rampon et al., 2000; Würbel, 2001). Little is known about which specific features of laboratory housing contribute to changes in rodent phenotypes. One fundamental environmental variable; the type of bedding used in cages, may be a source variation in laboratory tests of animal behavior. Rodent bedding materials have been demonstrated to influence stress and immune reactivity profiles,(Freed et al., 2008; Sanford et al., 2002) thermoregulation processes,(Gaskill et al.; Gordon, 2004) vocalizations,(Natusch and Schwarting, 2010), body mass (Burn et al., 2006), as well as liver enzyme levels in laboratory rats and mice (Armstrong et al., 1998; Buddaraju and Van Dyke, 2003). Research focusing on corncob bedding, which contains measurable levels of phytoestrogens, report alterations in slow-wave sleep, suppression of male and female reproductive behavior, acyclicity in female estrus cycles, as well as changes in estrogen receptor alpha expression in regions of the brain implicated in aggression and sexual behavior (Landeros et al., 2012; Leys et al., 2012; Markaverich, 2002). These studies suggest that housing conditions can fundamentally alter animal behavior and CNS function, results that emphasize the sensitivity of CNS developmental plasticity as well as fundamentally alter conclusions drawn from animal studies.

Environmental manipulations of standard laboratory housing parameters can influence offspring directly as described above, or indirectly, through perturbations in parental care. For example, alterations in early post-natal maternal care in the laboratory rat can program the developing HPA-neuroendocrine pathways and behavioral fearfulness when rodents reach adulthood (Champagne et al., 2008; Fish et al., 2004; Weaver et al., 2004a). These effects persist throughout the life of the animal and alter risk for stress-related disease (Francis, 2009; McEwen and Sapolsky, 1995; Meaney, 2007). Manipulation of the physical environment, including access to nesting sites and bedding, perturb parental care which subsequently influence neuroendocrine and behavioral phenotypes of developing offspring. For example, rat mothers with restricted access to bedding material during the postpartum period displayed more disorganized/fragmented levels of maternal care than controls (Ivy et al., 2008). Rat dams themselves, with restricted access to bedding material during the postpartum period, also display an increase in HPA reactivity, more stressful behavioral phenotypes and altered hypothalamic CRH expression suggesting that environmental alterations increase maternal stress. While offspring behavior was not reported (Ivy et al., 2008), other studies in which pups whose mothers were given restricted access to nesting and bedding material had deficits in spatial memory, reduced body mass, and an increase in depressive-like behavior that were accompanied by changes in hippocampal CA1 long term potentiation (Cui et al., 2006).

Using a simple manipulation of environmental parameters, we wished to investigate if the use of different housing materials during the early life period of the laboratory rat, was capable of altering offspring behavior as adults, and, if observed changes in offspring behavior can be accounted for by alterations in maternal care. We reared Long Evans rats

on wood pulp or corncob bedding, assessed maternal care during the early postpartum period and subsequently assessed stress-sensitive measures later in adulthood. We hypothesized that animals raised on wood pulp bedding conditions would differ significantly in anxiety-like behavior as adults than animals raised on the corncob bedding. We predicted that rat dams provided with wood-pulp materials would provide greater levels of maternal care to offspring which, in turn, would result in lower stress-reactivity phenotypes as adults.

8.2 Methods and Materials

8.2.1 Animals and Housing

Female Long Evans rats used in this study were purchased from Charles River Breeding Laboratories (Wilmington, MA). Adolescent female rats were pair housed in standard polypropylene cages (27.8 × 17.5 × 13.0 cm) containing either wood pulp or corncob bedding material (1/8" Purelite Sanitized Corncob Bedding and Tek-Fresh Laboratory Animal Bedding, Harlan, Hayward, CA). Animals were allowed to mature for three months on the assigned bedding material. Females were then mated with male stud animals also purchased from Charles River. Male studs were housed on wood pulp bedding prior to mating. For all animals, temperature was kept constant at 20 ± 2 °C and relative humidity was maintained at 50 ± 5%. Rats were kept on a 12-h light–dark cycle (lights on 0700 h to 1900 h) and allowed access to food (Tekland Global Diet #2918) and tap water *ad libitum*. Females were allowed to give birth and maternal behavior recorded as described below. A single 9.5 in. x 5.5 in. paper towel was provided for nesting material to all groups. At PND 21, male offspring from across litters (n = min. 14 / group) were weaned and pair housed in either corncob or wood pulp bedding conditions. Housing conditions at weaning were the same as that of the postpartum period. After 12 weeks of housing, animals were assessed on several stress-sensitive behavioral tasks described below. A subset of naïve animals (n = 10) housed on wood pulp bedding were switched to the opposite bedding and behaviorally tested after two weeks. Animals were euthanized and post-mortem markers assessed within 48 hours of completing behavioral tasks. Breeding, weaning, and rearing of animals was performed simultaneously rather than sequentially. Housing and care of the rats were carried out in accordance with the standards and practices of the UC Berkeley Animal Care and Use Committee.

8.2.2 Observations of maternal behavior

Female rats were bred and permitted to give birth (n = 12). Day of birth was marked as postnatal day (PND) 0. Maternal observations were performed beginning on PND 1 and continued until PND 5 (Champagne et al., 2003a; Francis et al., 1999b; Liu et al., 1997). Each litter was observed for 3 h a day at the following times: 0700—0800h, 1200—1300h and 1900— 2000 h. During each observation session, litters were observed and behaviors recorded every 1 min (i.e. each litter was observed 180 times per day for five days). Behaviors recorded included: mother on/off the nest and maternal licking behaviors directed at self or at pups. A distribution curve was generated by calculating the frequency with which pup-directed maternal licking was observed. Maternal licking was expressed as a percentage of the total number of observations performed for each litter. The mean

frequency of maternal licking was calculated for the cohort. Animals were weaned on PND22, and pair housed with same sex littermates as described above.

8.2.3 Behavior

All animals were tested in two stress-sensitive behavioral tasks as adults: the Open-Field Test and the Light-Dark Box Test. Animals were tested on non-consecutive days.

8.2.4 Open-Field Test

To assess anxiety-like behavior, animals were exposed to an open field (a large circular polypropylene arena 140 cm in diameter, 61 cm in height). Each animal was placed in the open-field for five minutes and subsequent behaviors recorded. The arena was cleaned between animals. Frequency of crosses between the outer arena (14cm width) and the interior inner arena (112 cm diameter) and amount of time spent in the inner-arena of the open field was quantified. The behavior of each rat was recorded and analyzed by an experimenter blinded to group conditions. The greater amount of time spent in the inner arena was interpreted as a less anxious phenotype (Dallas, 1985; Hall, 1934; John, 1973).

8.2.5 Light-Dark Box Test

Similar to the open field, the light-dark box is used to assess anxious behavior in rodents (Dallas, 1985; John, 1973). The light-dark box consists of two contiguous acrylic rectangular arenas (76 x 40 cm) connected by a 10 x 10 cm entrance. One arena, the dark box, is black acrylic and sheltered with a black acrylic cover while the second arena, the light box, is constructed of transparent acrylic and is open and uncovered. Animals were initially placed within the dark chamber and allowed five minutes of open exploration. The behavior of each rat was recorded and analyzed by an experimenter blind to the conditions. Time spent in the light box was quantified and interpreted as a behavioral marker of less anxious behavior (Dallas, 1985; John, 1973).

8.2.6 Postmortem Neuronal Markers
8.2.7 Western Blots

Glucocorticoid receptor (GR) protein expression in the frontal cortex and hippocampus was assayed via western blot in all animals (n = 8 per group). Tissue was dissected immediately after euthanasia and snap frozen in liquid nitrogen. Whole hippocampus was removed and frontal cortex dissection was restricted to infralimbic, prelimbic, and anterior cingulate cortices. Upon assay, tissue was homogenized with motor driven pestle in RIPA buffer solution with 1% protease inhibitor (Calbiochem protease inhibitor cocktail set iii, EDTA free). RIPA buffer contained 50 mM tris HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate at a pH of 8.0 at room temperature. The homogenate was then centrifuged at 14,000 g for 30 min. The supernatant was extracted and total protein concentrations determined via Pierce BCA protein assay kit (Thermo Scientific). Twenty micrograms of protein was then loaded and separated with a 7.5% Tris-SDS polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA). Proteins were

transferred to PVDF membrane (Amersham Hybond-P; GE Healthcare) and blocked with 5% non-fat milk in 1× TBS-t (Tris-Buffered Saline, 0.1% Tween-20, pH 7.6) for 1 h. Membrane was then incubated with polyclonal rabbit anti-GR at 1:2000 (Santa Cruz Biotechnology, #sc-1004) and mouse anti-actin at 1:10,000 (Sigma Aldrich, #A1978) overnight at 4 C. The signal was detected using horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies at a concentration of 1:5000 (JacksonImmuno Research). The signal was subsequently enhanced via Western Lightning ECL Kit (PerkinElmer; Waltham, MA) and exposed to autoradiography film for visualization. Western band optical density was determined by gel imaging system (MCID Basic, Version 7.0; Imaging Research Inc.) and normalized with the band optical density value of actin as an internal control.

8.2.8 RT-qPCR

Hypothalamic tissue (n = 8 per group) was analyzed via RT-qPCR. Specific rat primers for several different mRNAs were designed by blasting the primer sequence against NCBI genomic databases and then checking for specificity. Primers were created by Integrated DNA Technologies. Primers are detailed in the table below:

Gene	Direction	Sequence
CRH	+	5'-GGA GCC GCC CAT CTC TCT-3'
	-	5'-TCC TGT TGC TGT GAG CTT GCT-3'
CRH-1R	+	5'-TCC ACC TCC CTT CAG GAT CA-3'
	-	5'-TGC AGG CCA GAA ACA TTG C-3'
CRH-2R	+	5'-CTG GAA CCT CAT CAC CAC CT-3'
	-	5'-AGG TAG CAG CCT TCC ACA AA-3'
BDNF exon IX	+	5'-GAG AAG AGT GAT GAC CAT CCT-3'
	-	5'-TCA CGT GCT CAA AAG TGT CAG-3'
RPLP	+	5'-ATC TAC TCC GCC CTC ATC CT-3'
	-	5'-GCA GAT GAG GCT TCC AAT GT-3'

CRH, corticotrophin releasing hormone peptide; CRH-1R; corticotrophin releasing hormone receptor 1; CRH-2R; CRH receptor 2; BDNF, brain derived neurotrophic factor; RPLP, 60s ribosomal protein 1

Briefly, hypothalamic regions were dissected and rapidly snap-frozen in liquid nitrogen. Tissue was homogenized with motor pestle using Trizol reagent (Invitrogen) and removed of decontamination via DNase kit protocol (Applied Biosystems). RNA quality was assessed via gel electrophoresis, and 1 ug RNA reverse-transcribed into complimentary DNA using the iScript cDNA synthesis kit (Bio-Rad). cDNA product was analyzed by BioRad CFX96 Real Time PCR machine using a two-step PCR and SsoAdvanced SYBR Green Supermix (BioRad) per manufacturer's instructions. Sso7d Fusion DNA polymerase was activated at 95 C for 30 seconds. cDNA was then denatured at 95 C for an additional 30 seconds following annealing and extension at 55 C for 40 cycles. After the PCR was complete, specificity of each primer pair was confirmed using melt curve analysis in which each amplicon yielded a single peak. A cycle threshold (ΔΔCt) analysis was BioRad CFX96 Data analysis software and normalized to the reference ribosomal RNA, RPLP.

8.2.9 Statistical Analysis

Prior to analysis of behavioral data, a D'Agostino—Pearson omnibus test for normality was conducted. If data from behavioral tasks were not normally distributed, a Mann-Whitney U-test was used to account for non-Gaussian distributions. Otherwise, data was analyzed using a student's t-test between conditions. Results were considered statistically significant when $p < 0.05$. Post-mortem GR optical density values and RT-qPCR mRNA values were normalized to respective housekeeping controls (actin and RPLP gene).

8.3 Results

8.3.1 Offspring Anxiety-Related Behaviors

Open Field: As adults, animals reared and subsequently housed on corncob bedding spent significantly more time exploring the inner arena of the open field relative to wood pulp reared animals ($U (42) = 66.50$, $p < .0001$) **(Figure 1A)**. More time exploring the inner area of this arena suggests lower levels of anxiety in these animals. Latency to enter the inner arena of the open field as well as number of crosses between quadrants was not significant. Light-Dark Box: As adults, animals reared and housed on corncob bedding spent significantly more time exploring the illuminated portion of the light-dark box compared with wood pulp raised animals ($U (32) = 38.00$, $p = .0003$) **(Figure 1B)**. Greater time exploring the open end of a light-dark box apparatus suggests lower levels of anxiety. Open field and light-dark box data was not significant for animals placed on respective bedding as adults. Animals which were reared on wood pulp and placed on corn cob in adulthood (Open Field: $U (26) = 83.00$, $p = 0.7544$ and Light-Dark Box: $U (22) = 45.50$, $p = 0.1440$) as well as animals reared on corn cob and placed on wood pulp in adulthood (Open Field: $t (34) = 1.0$, $p = 0.3170$ and Light-Dark Box: $t (28) = 1.3$, $p = 0.216$) did not differ in behavior **(Figure 2A – 2D)**. This suggests that differences in anxiety related behaviors are developmental in nature.

8.3.2 Glucocorticoid Receptor Expression and CRH Hypothalamic mRNA

As adults, hypothalamic and frontal cortex glucocorticoid receptor expression did not differ between rats housed on corncob or wood pulp bedding conditions ($t (14) = 0.5$, $p = 0.659$

and t (14) = 0.7, p = .4929 respectively) **(Figure 3A and 3B).** Similarly, CRH peptide, CRH-1R, CRH-2R, and BDNF hypothalamic mRNA was not significantly different between groups (t (14) = 0.27, p = 0.7935; t (14) = 0.23, p = .8250; t (14) = 0.19, p = 0.8358; and t (14) = 0.24, p = 0.8164 respectively) **(Figure 3C)**.

8.3.3 Maternal Behaviors

The mean licking and grooming percentages for dams rearing pups on corncob or wood pulp bedding was significantly different. Corncob-housed rat mothers spent significantly more time licking and grooming offspring compared to wood pulp housed dams (U (10) = 3.5, p = 0.0412) **(Figure 4A)**. Rat dams across the two groups did not differ in additional measures including i) percent of time arch-back nursing (U (10) = 14, p = 0.8081) or ii) percent time being on/off nest (U (10) = 11, p = 0.4606) **(Figure 4B and 4C)**.

To control for the putative effects of varying levels of maternal care on adult anxiety measures, animals from both housing conditions were statistically matched for overall levels of maternal care (mean LG score of 7.4%) and performance on anxiety measures assessed. Animals matched for overall maternal care received during the first five postnatal days performed significantly different on both the open field and light-dark box tasks. Rats reared on corncob bedding that had received equivalent amounts of maternal LG as those reared on wood pulp bedding spent significantly more time exploring the inner area of the open-field (U (17) = 12, p = 0.0079) **(Figure 5A)** and more time in the light portion of the light-dark box (t (18) = 3.40, p = 0.0032) **(Figure 5B)**.

8.4 Discussion

A robust literature highlights the importance of early life environmental variables that influence stress responsivity in adult offspring, primarily using the laboratory rat as a model (Fish et al., 2004; Francis et al., 2000; Korosi et al., 2010; Lyons et al., 2010; Sakhai et al., 2011; Szyf et al., 2005). One environmental variable demonstrated to influence offspring development is the material on which rodents are housed (Rice et al., 2008). In the current study, we employed two different bedding materials commonly used in standard laboratory housing conditions to assess how they may influence anxiety related behaviors in the Long Evans rat. We hypothesized that rearing offspring on qualitatively different bedding materials would influence later measures of adult anxiety-like behaviors and, potentially, underlying neurobiological correlates. Our results demonstrate that varying the bedding on which a laboratory rat is reared contributes to significant differences in anxiety-like behaviors later in adulthood. Rats reared (and subsequently housed) on corncob bedding exhibited significantly less-anxious phenotypes compared to those reared (then housed) on wood pulp bedding material. As glucocorticoid receptor, BDNF, CRH, CRH-R1, and CRH-R2 expression (in various neuronal regions) have all been implicated in the expression of fear and anxiety and are sensitive to early life environmental factors, we wished to assess if bedding conditions during early life influenced the expression of these genes (Francis et al., 1999b; Ivy et al., 2008; Korosi and Baram, 2010; Roth et al., 2009; Roth and Sweatt, 2011). Hypothalamic CRH, CRH-R1, and CRH-R2 mRNA levels were not significantly different across conditions. Similarly, glucocorticoid receptor expression in the hippocampus and frontal cortex was not

significantly different across bedding conditions when measured using western blot. However, this does not preclude the possibility that differences in GR mRNA levels may exist in these regions.

The study of early developmental programming of stress-sensitive phenotypes has most recently focused on the relationship between the quality of early life environments and parental care. The extent to which environments directly influence offspring brain and behavior or are mediated by alterations in parental behavior, which subsequently influences offspring development, is subject to much debate **(Figure 6)**. Evidence supporting direct maternal programing of offspring stress physiology is supported by studies in which an increase in early maternal care has been demonstrated to decrease stress reactivity and anxiety profiles of Long Evans rats later in adulthood (Caldji et al., 2000a; Fish et al., 2004; Liu et al., 1997). Ostensibly, our results are consistent with this literature and a 'maternal mediation' model, in which maternal investment serves as a link between environments and offspring. We report that offspring reared on corncob bedding, overall, received higher levels of maternal care as infants, and exhibited lower levels of anxiety-like behaviors as adults relative to offspring reared on wood pulp bedding (mean LG scores of 8.8% and 5.5 %, respectively). Interestingly, differences in anxiety related behaviors related to bedding materials are only evident in rats *reared* under these different conditions. Long Evans rats placed on wood pulp or corncob bedding, as adults, do not differ in anxious behavior phenotypes **(Figure 2A – 2D)**. This suggests that both maternal care and housing conditions during the early postnatal period are involved in regulating the development of stress-sensitive phenotypes in young offspring. While maternal care provided by rat dams differed across developmental bedding conditions, this factor alone did not fully account for observed differences between groups. To assess direct environmental regulation of stress phenotypes, we matched offspring from both bedding conditions for the quantity of maternal licking and grooming offspring received developmentally. Rats reared/housed on corncob bedding exhibited significantly less-anxious phenotypes compared to those reared/housed on wood pulp bedding material despite receiving similar levels of maternal care early in life. The bedding material itself, regardless of the maternal care received by the offspring, was sufficient to influence adult stress-sensitive behaviors consistent with direct environmental regulation of adult stress phenotypes **(Figure 5 and Figure 6)**.

In rodents, previous studies have shown similarly complex associations between early environments, the quantity of maternal care received, and offspring behavior. For instance, in C57BL/6 mice subject to high and variable foraging demand conditions (i.e. an unpredictable stressor) maternal care was more active and intense when compared with control mothers in low foraging demand conditions, consistent with a maternal mediation model. However, offspring anxiety-like behavior as adults was varying across gender and condition, with male and female mice responding to environmental cues differentially regardless of overall amounts of maternal care (Coutellier et al., 2009). Similar effects were observed in predation threat paradigms, in which rodent mothers are exposed to predator cues. Exposure to predator odor during the first day of life increases both nursing and licking and grooming provided by rat dams to the offspring during the postpartum period compared with controls. Yet, female offspring of predator odor-exposed mothers display a more anxious behavioral phenotype compared with males, emphasizing the mixed role of environmental regulation of maternal care on offspring behavior (Coutellier

et al., 2008). Our results are similar to these findings, showing a nuanced relationship between direct environmental programming and maternal mediation.

Our results clearly emphasize the importance of housing variables in influencing commonly assessed stress-sensitive rodent behaviors, while highlighting the importance of sensitive periods in rodent development. The findings of this paper have important implications for animal husbandry and housing standardization. Behavioral testing across laboratories does not always yield similar results despite rigorous attempts at standardization (Lewejohann et al., 2006; Wahlsten et al., 2003). One unknown variable that may contribute to be disparate behaviors across laboratories may be bedding. Standardization of bedding materials may help reduce inter-experimental variability within and across laboratories by reducing behaviorally anxious phenotypes. Importantly, bedding standardization can also enhance animal welfare by diminishing adult anxiety-like behavior in animals, which is assumed to be deleterious and maladaptive in the laboratory setting (Würbel, 2001) (Beery and Francis, 2011). Equally, the conditions in which environments are involved in programming animal behavior may serve as important criteria to refine future research (Würbel, 2001). For instance, when strong anxious phenotypes are required for research purposes, bedding type may also be considered as one avenue to potentiate behavioral effects. For these reasons, we suggest that animal housing parameters, including the type/variety of bedding used in cages, be reported by researchers studying animal physiology and behavior.

The results of this study are in agreement with the few reports examining the effect of bedding materials on adult stress-sensitive measures. Our results are also in line with findings, which demonstrate that early-life maternal care received influences offspring behavior later in adulthood. However, some limitations should be considered when interpreting the current results. First, the independent contributions of maternal behavior and bedding type on future offspring behavior require further investigation. We cannot conclude, from the current study, if maternal care and bedding materials are working synergistically or independently to influence the developmental programming of the stress axis. Our data suggest that bedding material is a key component of the developmental programming effect, as animals matched for overall levels of maternal care received early in life still differed in anxiety-like behaviors as adults. We cannot comment more extensively on the role of maternal care in this paradigm, as we did not systematically vary the amount of maternal care provided the offspring; we simply 'controlled' for the amount of maternal care received. As mentioned above, while maternal effects on offspring behavior and physiology have been shown extensively in the literature, environmental effects on offspring have also been demonstrated to occur independent of the mother (Coutellier et al., 2009).

In the current study we did not investigate the powerful estrogenic properties of corncob bedding. We do not know if early phytoestrogen exposure in rats reared on corncob bedding may be contributing to altered development of the stress-axis and the observed anxiolytic phenotype; however, this is a strong possibility. Phytoestrogens (and the active component, tetrahydroflourane) can alter estrogen signaling and estrogen receptor (ER) alpha neuronal expression profiles and may be directly ingested by animals or potentially indirectly absorbed trans-dermally through contact with bedding (Landeros et al., 2012; Markaverich, 2002; Markaverich et al., 2002). While tetrahydroflourane does not bind ER alpha, it has been shown to influence ER levels in the brain and alter behavior

by an unknown mechanism of action (Landeros et al., 2012; Markaverich et al., 2002). Notably, developmental exposure to estrogens (and progestins) has been shown to influence anxiety-like behavior in male and female rodents, acting as an anxiolytic (Díaz-Véliz et al., 1997; Llaneza and Frye, 2009; Lucion et al., 1996; Zimmerberg and Farley, 1993). Likewise, through development, intake of phytoestrogens via diet has been shown to decrease anxiety-like behavior in Long-Evans rats in a similar direction as shown in this manuscript (Lephart et al., 2004). The anxiolytic effect of corncob bedding on rats may be due to perturbations in developmental estrogenic signaling.

In an elegant series of studies using the California mouse, animals housed on corncob bedding have altered estrogenic profiles and perturbed estrogen dependent behaviors (Landeros et al., 2012). These effects have also been reported in rats (Markaverich, 2002). The most recent paper using the California mouse as a model provides evidence, in line with our own, demonstrating that the effects of corncob bedding on adult stress-relevant behaviors appears to be generated during the postnatal, developmental window and not in adulthood (Trainor et al., 2013). An increasing body of research demonstrates that exposure to endocrine-disrupting compounds, particularly during critical developmental windows, may influence sexually-dimorphic neuroendocrine pathways controlling reproductive behaviors. While the bulk of the research using animal models has focused on the role of endocrine disruptors (EnD) on various aspects of reproductive physiology, considerably less is known about the role of EnD and the stress-axis (Frye et al., 2012).

As a final point of consideration, given the strong estrogenic properties of both corncob bedding and laboratory rodent diet, an interactive effect of both food and corncob bedding may result in adult rats with anxiolytic phenotypes. In the current study, all rats were fed a diet containing 150-250mg/kg of isoflavones (Tekland Global Diet #2918). Patisaul et al (2012) recently reported that developmental exposure to the EnD Bisphenol A (BPA) results in an anxiogenic phenotype in adulthood in rats. However, this anxiogenic phenotype was mitigated if rats were provided with a soy-based diet, demonstrating a strong interaction between EnDs in the environment and soy in the diet (Patisual, 2012).

In summary, these results suggest that seemingly innocuous environmental variables, such as the choice of bedding material to use in a cage, can drastically alter stress phenotypes of offspring reared on particular types of bedding. It remains to be determined if variables such as phytoestrogen levels or maternal care mediate this effect. We conclude that adult rodent behavior is modifiable by early exposure to differential housing conditions. Lack of attention paid to the potent role this variable plays in the developmental programming of laboratory animals will have deleterious consequences for experimenters and researchers.

Section 8 Figures

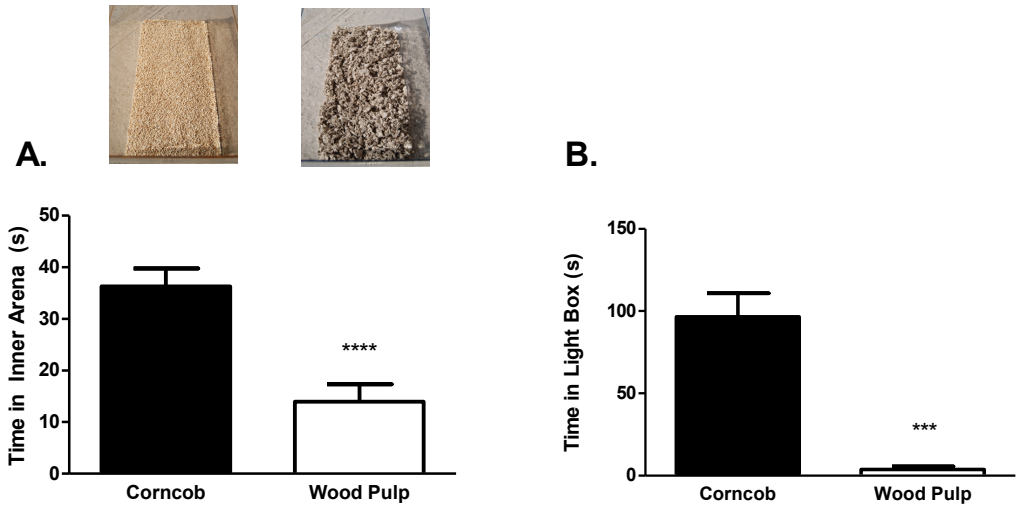

Figure 1: Offspring behavior in adulthood after rearing on either Corncob or Wood Pulp bedding. A) Open Field ($p < .0001$) and B) Light Dark Box ($p < .001$). Results are reported as mean time exploring inner arena of open field and exposed area of light box ± SEM. Animals raised on wood pulp spend significantly less time on behavioral indices of anxiety as indicated by the open field and light-dark box task.

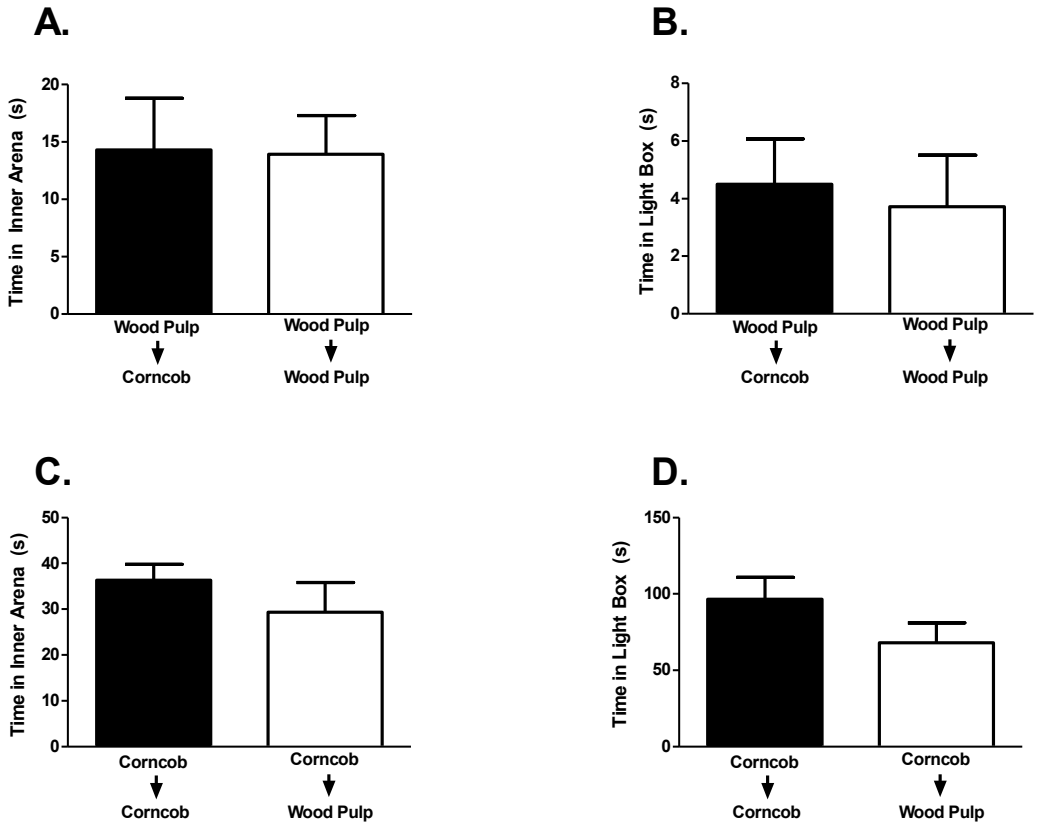

Figure 2: Offspring behavior reared on Corncob or Wood Pulp bedding and placement on opposite bedding in adulthood. A) Open Field ($p > .05$) and B) Light Dark Box ($p > .05$). Animals were reared on Wood Pulp and moved to Corncob bedding in adulthood. C) Open Field ($p > .05$) and D) Light Dark Box ($p > .05$). Animals were reared on Corncob and moved to Wood Pulp bedding in adulthood. Results are reported as mean time exploring inner arena of open field and exposed area of light box ± SEM.

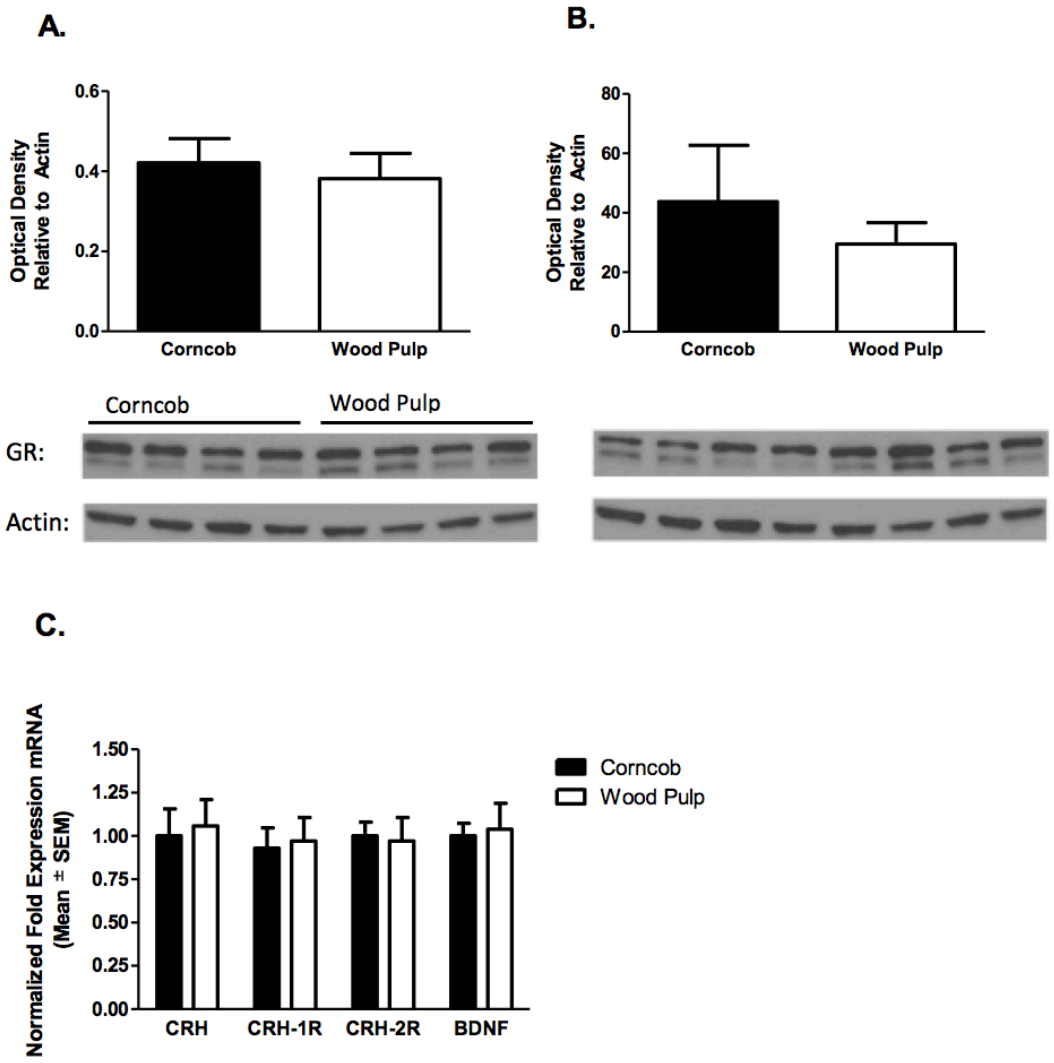

Figure 3: Post-Mortem Neural Markers. Western Blot: A) hippocampal and B) frontal cortex glucocorticoid receptor optical density. Real Time-PCR: C) hypothalamic mRNA expression relative to house keeping control gene RPLP. CRH peptide, CRH-1 receptor, CRH-2 receptor, and BDNF mRNA ($p > .05$).

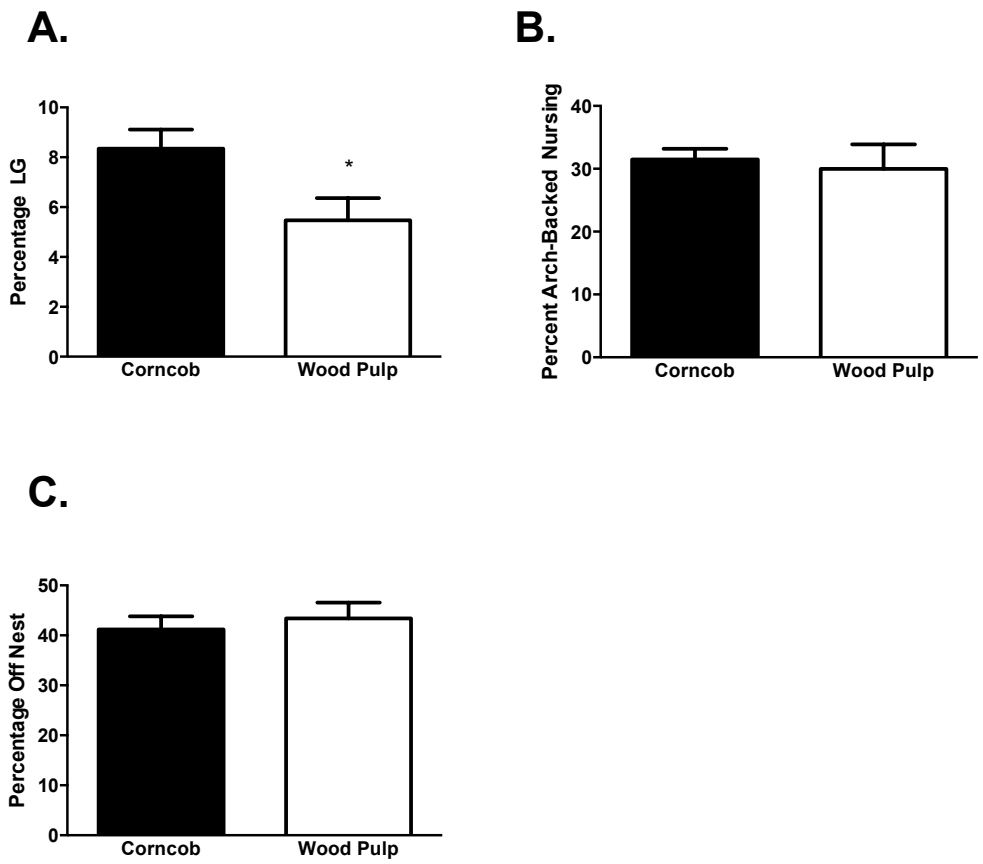

Figure 4: Maternal Behavior observed postpartum. Maternal behavior was observed across the first 5 days post-partum. Behaviors included A) maternal licking ($p < .05$) B) arched-backed nursing and C) time off nest expressed as a percentage of the total number of observations.

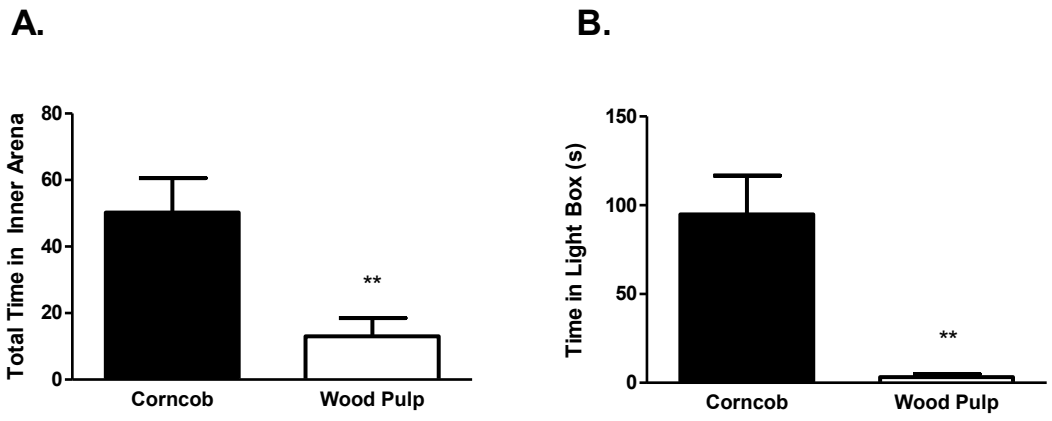

Figure 5: Behavior of offspring matched for overall levels of maternal care. A) Open field and B) light dark box. Results are reported as mean time exploring inner arena of open field and exposed area of light box ± SEM.

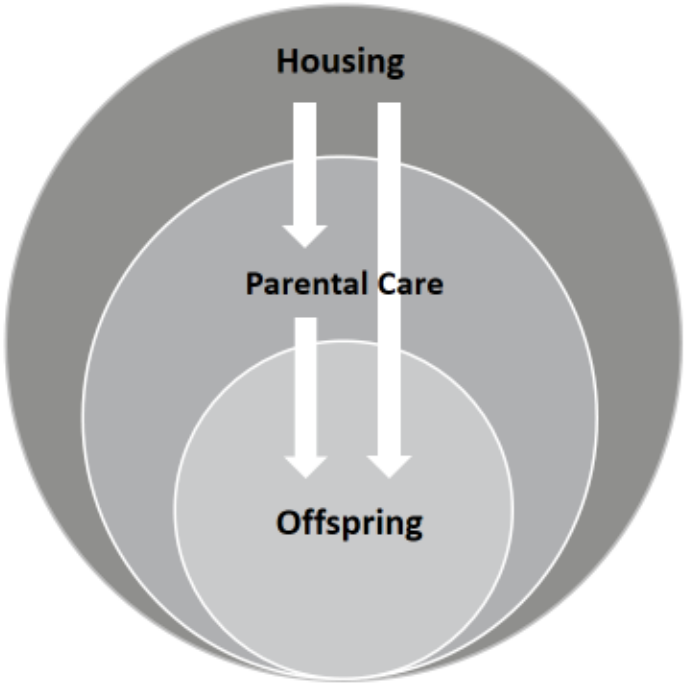

Figure 6: Schematic representation of housing and parental effects on offspring behavior and physiology.

Section 9: Conclusions

Early life experiences during the neonatal period affects the development of endocrine and behavioral responses to stress in offspring. Findings from this dissertation demonstrate that i) early life maternal care is capable of altering offspring HPG axis and can influence adult female sexual behavior, ii) the quality of early life maternal care does not alter set-shifting behaviors in rodents, but may influence aspects of motivated behaviors. Furthermore, data suggest that offspring adult HPA reactivity may be modifiable by behavioral training/handling, and iii) housing variables in early life, such as bedding type, can alter levels of parental care as well as influence offspring stress reactivity in adulthood. These results are significant in that they demonstrate that early life environmental conditions (i.e. maternal care and housing environments) are capable of profound modifications in brain and behavior in multiple domains. These findings are of particular relevance when considering environments, both rich and impoverished, in the health and development in people.

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