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Maternal Sleep Loss During Fetal Development Alters Offspring Endocrine Responses to Stress Throughout Life

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MATERNAL SLEEP LOSS DURING FETAL DEVELOPMENT ALTERS OFFSPRING ENDOCRINE RESPONSES TO STRESS THROUGHOUT LIFE

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By

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ABSTRACT

The hypothalamic-pituitary-adrenal (HPA) axis releases glucocorticoids, including corticosterone (CORT), in response to stress. CORT then negatively feeds back to inhibit its own production by binding to glucocorticoid receptors (GRs) in the hypothalamus and pituitary gland. The HPA axis is subject to "programming" by abnormal stimuli during early development, which may permanently alter how the HPA axis responds to stress. These altered responses have been linked to an increased risk for human psychiatric and metabolic disorders in later life, but the mechanism by which this happens is not fully understood. This study tests the hypothesis that changes to GR expression patterns persisting into adulthood may be playing a role using mice as a model. Pregnant mice were exposed to one of two treatments: 24h of sleep deprivation (SD) or no sleep deprivation. This produced maternally sleep deprived (MSD) pups and non-maternally sleep deprived pups (No-MSD), which were reared to adulthood (>8 weeks of age). Offspring from each group were then exposed to either 24h SD (OSD) or no SD (no-OSD). Hypothalami, hippocampi, liver, and adrenal tissue were taken from these resulting four groups of mice (1. No-MSD:OSD, 2. No-MSD:No-OSD, 3. MSD:OSD, 4. MSD:No-OSD) and RT-PCR was conducted to examine relative GR expression. Mice in the MSD:OSD group showed significant GR reduction in the

hypothalamus compared to other groups. Both No-MSD:OSD and MSD:OSD groups showed significant GR reduction in the hippocampus. No significant changes were found in the liver. No-MSD:OSD, MSD:OSD, and MSD:No-OSD groups all showed significant GR reduction in the adrenal glands compared to No-MSD:No-OSD animals. These data provide evidence that acute stress during pregnancy can alter the offspring's baseline HPA axis function and HPA axis functioning in response to stress. These alterations to how an organism restores homeostasis by activation of the HPA axis when facing a stressor could contribute to the development of disease.

Keywords: Stress, Sleep, Corticosterone, HPA

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PUBLICATIONS

1. Ashley, N.T., *et al.,* Novel environment influences the effect of paradoxical sleep deprivation upon brain and peripheral cytokine gene expression. *Neurosci Lett*. 2016. 615: 55-59.

2. Dumaine, J.E. and Ashley, N.T. Acute sleep fragmentation induces tissue-specific changes in cytokine gene expression and increases serum corticosterone concentration. *Am J Physiol-Reg I.* 2015. (308)12: 1062-1069.

FIELDS OF STUDY

Major Field: Biology

Major Field: Chemistry

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CHAPTER 1

INTRODUCTION

The neuroendocrine system controls many important functions of the body in order to maintain homeostasis, including regulating reproduction, metabolism, and the stress response. During early development, such as during the prenatal period in humans, this system is subject to organizational effects in response to stimuli, meaning that hormone actions during critical stages of development have permanent effects on neuroendocrine responses (Davis and Sandman 2010). Mammals typically live their adult lives in environments similar to those experienced by a mother during fetal development, making the ability to program long-lasting effects on the neuroendocrine system likely an adaptive feature (Harris and Seckl 2010). For example, if a pregnant mother faced stressors due to a highly predatory environment, exaggerated HPA axis responses in the offspring may trigger greater activation of the stress response allowing for more successful escape from predators.

The timing of stimulus exposure during embryonic development is crucial in determining what effects will be produced. For example, elevated glucocorticoids (GCs) late in gestation accelerate development in preparation for neonatal life. Earlier exposure to stressors, however, has been linked to an increased risk for psychological and metabolic

disorders later in life (Buchholz 2015). The programmability of this earlier exposure period was the focus of this study.

The HPA axis controls neuroendocrine responses to stress through negative feedback by GC stress hormones (Herman 2012). In response to stress, the hypothalamus releases corticotropic-releasing hormone (CRH), which acts on the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH then stimulates the adrenal cortex to release GCs. The major GC is cortisol in humans, and corticosterone in mice. These GCs bind to the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily. When GCs bind to GR on the hypothalamus and anterior pituitary they inhibit the production of CRF and ACTH, respectively (Meany 1996). Through this negative feedback mechanism GCs regulate their own production (Figure 1).

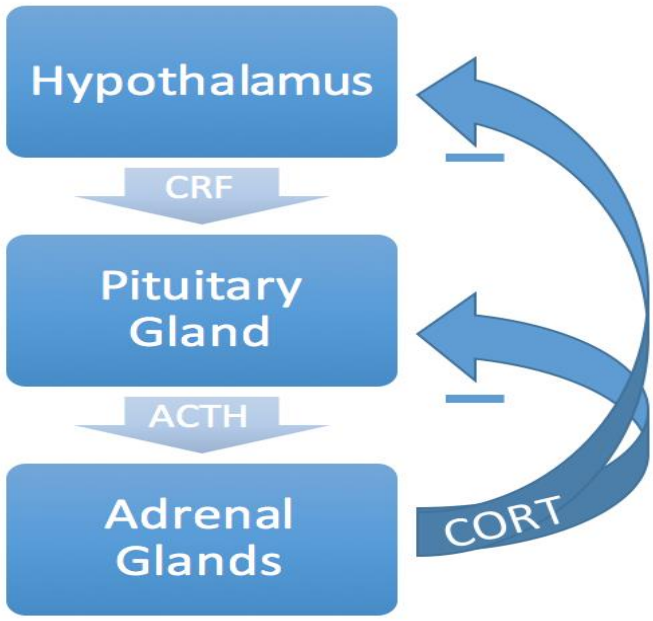

Figure 1. An overview of the glucocorticoid negative feedback loop

While excess exposure to GC during development increases the risk of pre-term birth and low birth weight in humans, this exposure during fetal development can also make alterations to HPA axis responses to stress that persist throughout an organism's life (Noorlander 2006). Dysfunction of the HPA axis due to this 'programming' is linked to later life consequences including anxiety, depression, diabetes, and cardiovascular disease (Buchholz 2015, Davis 2011). It is poorly understood how early life stress leads to these disorders, but a proposed mechanism is through epigenetic changes in gene expression.

Studies in adult rodents have shown that after exposure to acute stress, GR expression levels drop significantly as CORT levels rise. Additionally, early developmental stress can result in decreased HPA responsiveness to stress later in life (Meany 1996). This study aims to test the hypothesis that mice exposed to inappropriately high levels of GCs embryonically will have an altered HPA axis response to stress during adulthood, characterized by less down-regulation of GR expression levels in the hypothalamus, hippocampus, adrenal glands, and liver in maternally SD offspring compared to non-maternally SD offspring. If mice exposed to higher GC levels intrauterinely have a reduced HPA response to stress and so are unable to decrease GR expression to the levels of non-maternally SD mice, then this could be harmful as it may inadequately transduce the effects of CORT, thereby inhibiting the hormone's important roles in controlling glucose release, lipid storage, and immune response activation (Bellevance 2014, Boyea 2012).

Stress is ubiquitous in modern society and can be induced by a diverse array of stimuli. Increasing work demand and prevalence of shift-work have resulted in a 20% decrease in the average amount of time Americans sleep over the past century (Colton

2006). Nearly one-quarter of the United States population is now considered chronically sleep deprived (Kripke 2002). Disrupted and restricted sleep activates the HPA axis' stress response (Meerlo 2008). The prevalence of sleep loss makes it a relevant method of induced stress response worthy of scientific study. This study on the effect of prenatal stress due to maternal sleep deprivation (SD) on the offspring's HPA axis functioning will have pertinent implications for understanding the early development of HPA axis responses and from the perspective of sleep loss during pregnancy, a common phenomenon that often occurs during the third trimester in human pregnancy (Pien and Schwab 2004).

CHAPTER 2

METHODS

Breeding and Sleep Deprivation:

C57BL/6j mice were used from a breeding colony at Western Kentucky University maintained at 12L:12D light cycle at 20±1°C. Food (Rodent RM4 1800 pellets) and tap water were provided *ad libitum*. General methods of sleep deprivation were as follows: mice were taken from their home cages and sleep deprived for 24h using an automated sleep fragmentation cage (Lafayette Instrument) (Figure 2). This cage continuously wakes subjects by sweeping across the bottom of the cage every 20s, requiring the mouse to move over the bar. SD was always started at 11am. This time was chosen because mice are nocturnal, so starting SD when mice would normally be entering sleep produced the greatest amount of deprivation from sleep. Food and water were provided *ad libitum*. Using this cage set at this frequency of awakening for this time period has been shown to cause significant increases is serum CORT levels (Dumaine 2015). While 11β hydroxysteriod dehydrogenase partially protects a developing fetus from increased GC levels by metabolizing maternal CORT in the placenta and fetal tissues, approximately one-third of fetal CORT is derived from placental transfer (Kota 2013).

To obtain mice exposed to inappropriately high levels of corticosterone during development, adult mice were bred in trios of two females and one male. All female mice

were identified with number-stamped ear tags to permit individual identification. Female mice were checked each day for the presence of a mating plug, and first identification of a plug. Day 0 of gestation was recorded upon discovery of a mating plug. At Day 15 of gestation the pregnant female was placed in the sleep deprivation cage described above. After 24 hours, dams were returned to their home cages and allowed to give birth and rear their litter normally.

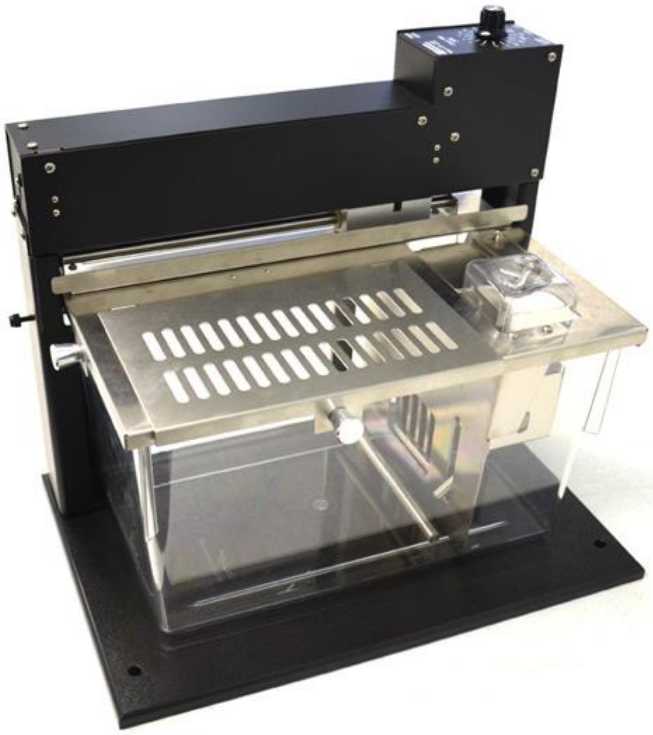

Figure 2. An automated sleep fragmentation chamber from Lafayette Instruments.

Due to the difficulty of consistently identifying mating plugs after each breeding, No-MSD maternal mice were not placed in the sleep deprivation cage without bar movement during their pregnancy to serve as a true control for the effect of the cage's novel environment. A recent study suggests that a novel environment alone can induce marked increases in CORT levels (Ashley 2016). Thus the methods used in this experiment allowed an examination of the activation of the stress response due to sleep deprivation and placement in a novel environment, of the SD cage.

The resulting pups from both maternally sleep deprived (MSD) and non-maternally sleep deprived (No-MSD) mothers were reared to adulthood (≥8 males per treatment). Male offspring of both groups were then assigned one of two treatments: *1.* 24h SD as described above (offspring sleep deprived referred to as OSD) *2.* Take adult males directly from their home cage (no-offspring sleep deprivation referred to as No-OSD). The sole difference in the sleep deprivation method used for this section compared to the maternal mouse SD, was that each group was SD with fellow littermates from their home cage (N=4). This small sample size was also due to mating plug identification difficulties resulting in a limited number of male MSD pups.

Tissue Collection:

Mice were anesthetized with isoflurane gas and decapitated at the completion of the 24h period for SD groups or directly from the home cage. All 24 h periods started and ended at 11am. Additionally all Non-SD mice were taken for tissue collection from their home cage at 11am. The time of day is not relevant, but because CORT levels fluctuate in

a circadian rhythm and it is known CORT levels affect GR expression, it was necessary to take all tissue samples at the same time every day that tissues were taken (Barriga 2001).

Tissue samples were taken from the brain, liver, and adrenals and stored in RNA*later* Solution (Qiagen) at 4°C. The hypothalmus and hippocampus were then dissected from each brain and also placed in RNA*later* and stored at 4°C.

RNA Isolation and Reverse Transcription

Tissues were homogenized and RNA was extracted from the liver, adrenals, hypothalamus, and hippocampus using an RNeasy Mini Kit (Qiagen). The resulting total RNA concentrations were measured using a NanoDrop 2000 (UV/Vis Spectrophotometer, Thermo Scientific). 250 ng of RNA per 20μl reaction was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific) yielding 12.5 ng/μl cDNA. The reactions were run using a thermal cycler according to the manufacturer's specifications.

RT-PCR

Real-Time PCR was run using a GR primer/probe (Nr3cl; Mm00433832_ml) and Taqman Gene Expression RT-PCR Master Mix in an Applied Biosystems 7300 machine. The amplification protocol was the following: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The reactions were carried out according to manufacturer protocol except the reaction volume was halved from 50μl to

25µl. A standard curve of pooled cDNA serial dilutions (1:1, 1:10, 1:100, 1:1000) were run on each plate to determine relative expression. Each sample was run in duplicate and C_t scores were averaged. Mean sample C_t scores were normalized to cDNA input by dividing by the average of the No-MSD:No-OSD group C_t values. This resulted in the No-MSD:No-OSD group serving as a baseline reference where this group's relative expression equaled 1.

Statistical Analysis

Results were analyzed using a two-way ANOVA test. The two independent variables analyzed were fetal treatment (MSD or No-MSD) and adult treatment (OSD or No-OSD) while relative expression values was the continuous variable. Each group contained pups from at least two different dams to limit family effects.

CHAPTER 3

RESULTS

Brain Tissues

In the hypothalamus, relative GR expression was significantly reduced when mice were exposed to both abnormal stressors during development and exposed to acute stress in adulthood (P=0.0001) (Table 1). Compared to the No-MSD:No-OSD baseline reference, hypothalamus GR expression in the MSD:OSD group was reduced ~45%. The average relative expression values of each group were as follows: No-MSD:OSD: 0.957, No-MSD:No-OSD: 1.000, MSD:OSD: 0.560, MSD:No-OSD: 1.136 (Figure 3).

In the hippocampus, GR expression was significantly reduced from SD in adulthood in the No-MSD and MSD groups (P=0.0030) (Table 2). Reduction compared to baseline was ~55% and ~40% for MSD:OSD and No-MSD:OSD groups, respectively. Average relative expression values for each group were as follows: No-MSD:OSD: 0.590, No-MSD:No-OSD: 1.000, MSD:OSD: 0.462, MSD:No-OSD: 0.945 (Figure 4).

ANOVA Table Hypothalamus	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Dam	1	.067	.067	3.245	.0968	3.245	.369
Offspring	1	.383	.383	18.502	.0010	18.502	.984
Dam by Off-spring	1	.283	.283	13.687	.0030	13.687	.937
Residual	12	.248	.021				

Table 1. Statistical analysis of variance for hypothalamus RT-PCR results.

ANOVA Table Hippocampus	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Dam	1	.034	.034	1.314	.2739	1.314	.176
Offspring	1	.797	.797	31.066	.0001	31.066	1.000
Dam by Off-spring	1	.005	.005	.206	.6582	.206	.070
Residual	12	.308	.026				

Table 2. Statistical analysis of variance for hippocampus RT-PCR results.

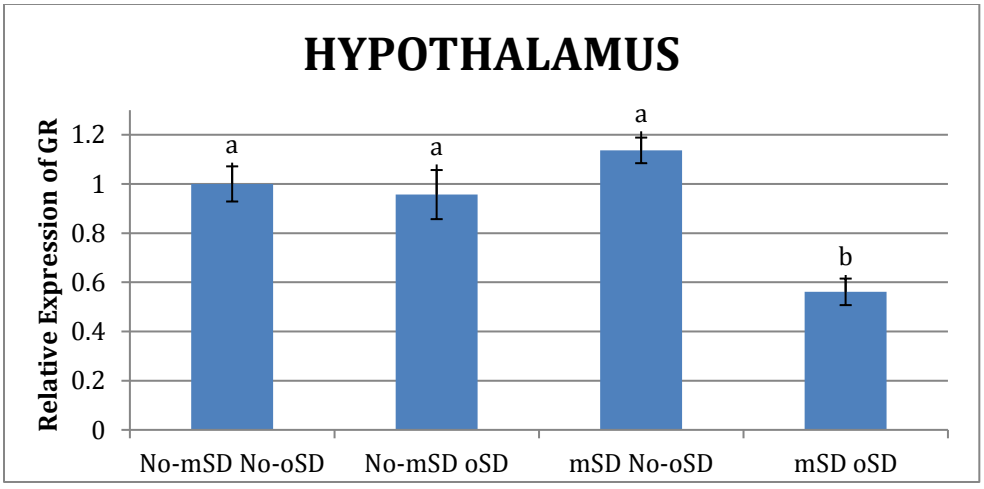

Figure 3. The interaction between abnormal early developmental stress and exposure to acute stress during adulthood significantly reduces GR expression.

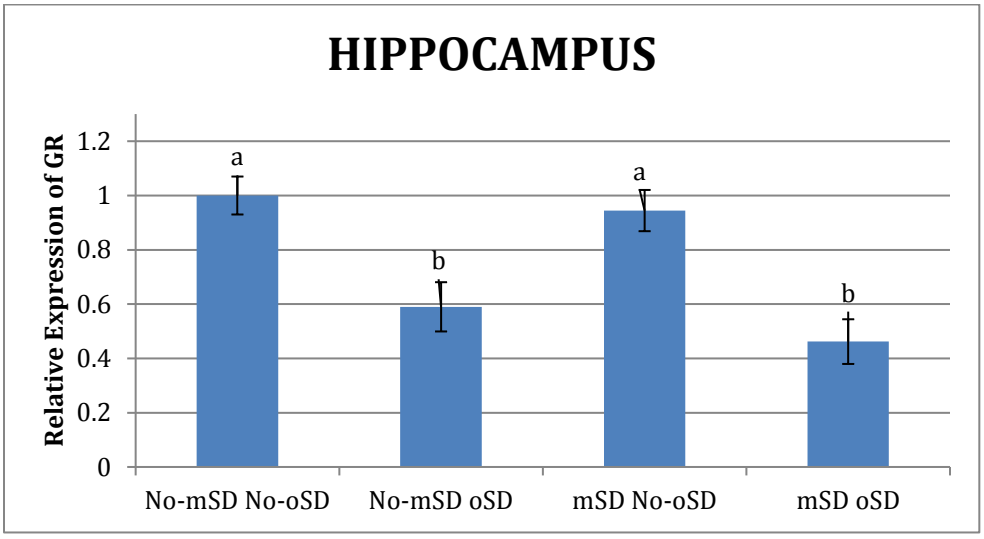

Figure 4. Exposure to acute stress in adulthood significantly reduces hippocampal GR expression regardless of early life experience.

Peripheral Tissues

In the liver, there were no significant differences among groups (Table 3). Average relative expression values were as follows: No-MSD:OSD: 0.461, No-MSD:No-OSD: 1.000, MSD:OSD: 0.510, MSD:No-OSD: 0.492 (Figure 5).

The adrenal glands experienced significant reduction in GR expression as a result of both fetal stress treatment (P=0.0092) and adult stress treatment (0.0261) (Table 4). Reduction from baseline was ~30% for No-MSD:OSD, MSD:OSD groups, and MSD:No-OSD groups. No-MSD:OSD: 0.724, No-MSD:No-OSD: 1.000, MSD:OSD: 0.691, MSD:No-OSD: 0.693 (Figure 6).

ANOVA Table Liver	**DF**	**Sum of Squares**	**Mean Square**	**F-Value**	**P-Value**	**Lambda**	**Power**
Dam	1	.271	.271	1.995	.1832	1.995	.244
Offspring	1	.210	.210	1.545	.2376	1.545	.199
Dam by Offspring	1	.310	.310	2.278	.1571	2.278	.273
Residual	12	1.632	.136				

Table 3. Statistical analysis of variance for liver RT-PCR results.

ANOVA Table Adrenal Glands	**DF**	**Sum of Squares**	**Mean Square**	**F-Value**	**P-Value**	**Lambda**	**Power**
Dam	1	.115	.115	9.598	.0092	9.598	.822
Offspring	1	.077	.077	6.434	.0261	6.434	.644
Dam by Offspring	1	.075	.075	6.277	.0276	6.277	.633
Residual	12	.144	.012				

Table 4. Statistical analysis of variance for adrenal RT-PCR results.

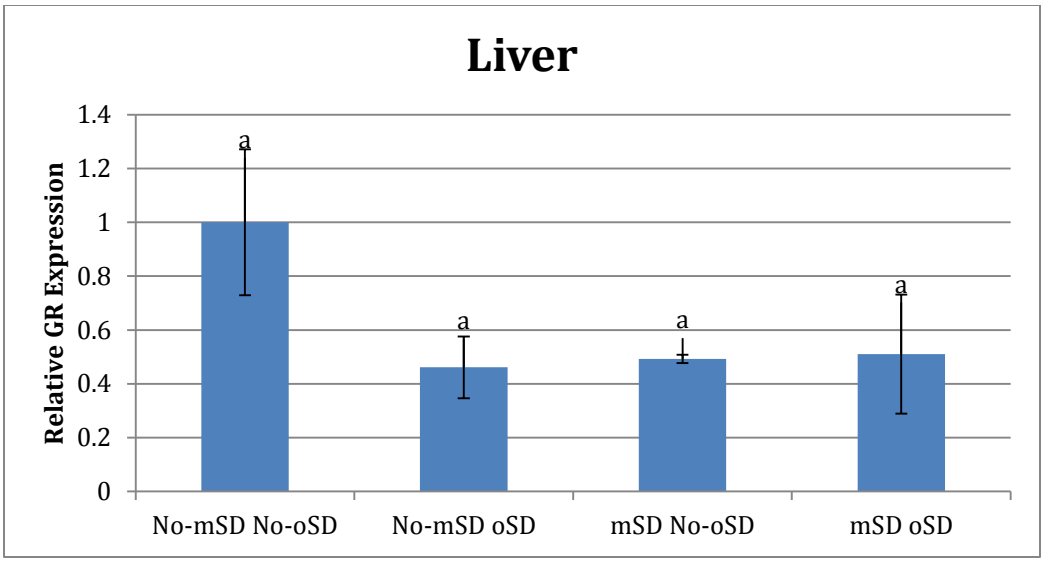

Figure 5. GR expression levels in the liver did not change significantly but tended to decrease in response to acute stress during adulthood or exposure to inappropriate stress during fetal development.

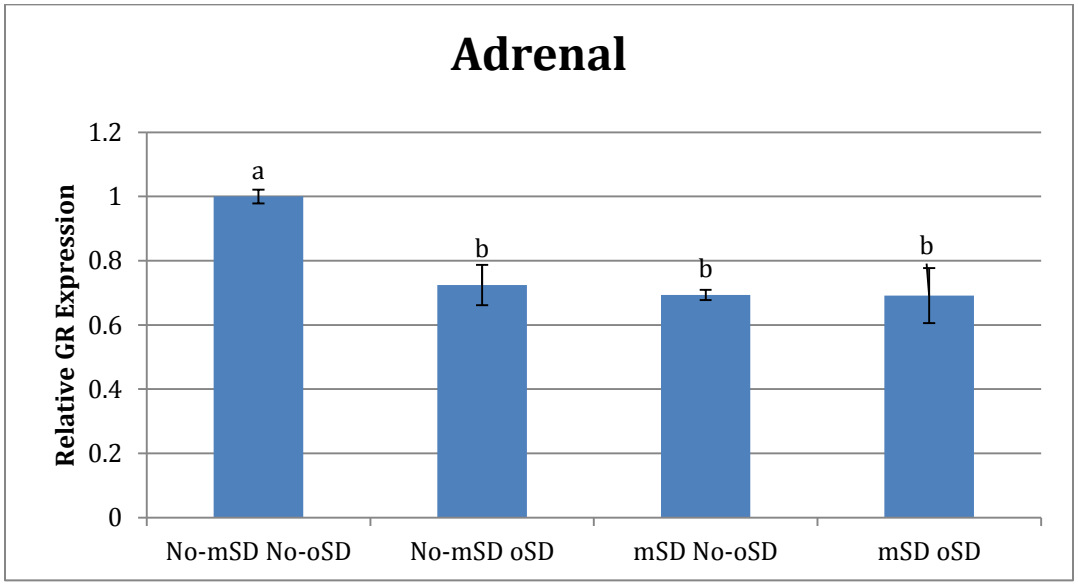

Figure 6. Both acute stress during adulthood or exposure to inappropriate stress during fetal development independently reduced GR adrenal expression significantly.

CHAPTER 4

DISCUSSION

I hypothesized that mice born in the No-MSD group would have significant reduction in GR levels in adulthood upon induction of acute stress based on previous literature (the No-MSD:OSD group) while MSD mice exposed to this same adult stress would lack the ability to similarly decrease GR expression. This is quite opposite of what the data showed.

RT-PCR data indicate that there is significant reduction of GR expression in hypothalamic tissue only when mice were in the MSD:OSD group. This result implies that GR expression in the hypothalamus is affected by early developmental challenge. Given that the hypothalamus regulates the HPA axis, which is sensitive to early life events, these data seem logical. A decrease in GR expression in the MSD:OSD group, instead of the anticipated higher levels, may be explained by CORT levels. If maternally stressed offspring produce higher levels of CORT in response to acute stress, they could see more significant GR lowering because increased CORT decreases GR expression. Measures of CORT plasma concentrations in these groups would be needed to confirm this possibility.

Knowing CORT plasma levels may also give insight into why the No-MSD:OSD group failed to exhibit a reduction in GR expression. The hypothalamus in No-MSD

animals may be less sensitive to CORT upon adult acute stress, therefore the levels brought about by 24 hours of SD were not adequate. A longer period of SD for these animals, such as 48-72 hours, would result in higher concentrations of CORT allowing for further conclusions on this to be drawn.

The hippocampus showed significant reduction in GR expression in both groups exposed to acute stress as adults. The *in utero* treatment did not have an affect on GR reduction. These results follow the pattern of GR reduction in response to stress previous research has shown (Meany 1996). The MSD:OSD group did not show an inability to reduce GR expression to the levels seen in No-MSD:OSD mice. This may be explained because the hippocampus in not an organ in the HPA axis and thus may not be affected by neuroendocrine organizational effects.

In liver tissue, no significant differences were detected between any of the groups, however, there is a trend of GR expression reduction in groups exposed to adult acute SD and in MSD pups normal GR levels. This points toward both *in utero* and adulthood treatment having effects on their own. A larger sample size would likely produce a smaller error leading to clearer answers on liver GR expression patterns. Thus, future studies on this topic are encouraged to use larger sample sizes ($N \geq 8$) as a means of deriving greater insights into this response.

Adrenal gland tissue showed significant reduction in GR attributed to both *in utero* SD and adulthood SD. This indicates that the adrenal glands are affected by the expected reduction of GR in response to CORT increase, and also to organizational effects of early

developmental stress. This programmability is consistent with the hypothalamus results because the adrenal glands are also involved with the HPA axis.

In this study, I predicted that early developmental stress would ameliorate the GR reduction in response to CORT increases, resulting in excessive CORT inhibition. The trend was found, however, that maternally stressed offspring tended to have lower GR expression than their No-MSD counterparts. This may still contribute to explaining how early developmental stress is linked to long-term health consequences because the pattern of GR expression and potentially CORT levels were altered. This implies the normal neuroendocrine function in restoring homeostasis in response to stress is disturbed. Abnormally high GR expression can overly transduce the effect of CORT resulting in above average demand on the systems CORT affects, such as immune and metabolic functions.

The RT-PCR results from these four tissues show that a single acute stress event imparted on a pregnant dam can have effects on the offspring's baseline and stress-induced GR expression that persist into adulthood. An organism's capacity to properly respond to stress and restore homeostasis is vital to good health, and without this ability, unnecessary burden may be required of an animal leading to an increased risk for the development of disease.

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