BEHAVIORAL, COGNITIVE, AND BIOCHEMICAL CONSEQUENCES OF EARLY LIFE STRESS IN LATER LIFE: INSIGHTS FROM AN ANIMAL MODEL

A Dissertation Presented to

The Faculty of the Pharmacological and Pharmaceutical Sciences Department

University of Houston, College of Pharmacy

In Partial Fulfillment

Of the Requirements for the Degree

Doctor of Philosophy

By

Hesong Liu

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ABSTRACT

Adverse experiences during early life contribute to the development of psychiatric conditions in later life. In fact, young children who directly experience or witness traumatic event(s) during early life, a sensitive developmental period, are considered highly vulnerable to psychiatric disorders during adult life. Interestingly, not all children who experience traumatic events are equally at risk of developing later life psychiatric disorders. Some are resilient despite being exposed to the same risk factors, while others are susceptible. The relationship between early life trauma exposure and development of later life psychiatric symptoms is not fully understood, and the mechanistic basis for resilience is also not clear. Clinical and preclinical studies have suggested that defects in stress-adaptive mechanisms potentially contribute to etiology of later life psychiatric conditions. Preclinical data from our laboratory has indicated poor oxidative/antioxidative balance as a critical component of maladaptive stress responsiveness in rodents. Our published work has demonstrated that induction of psychological stress leads to behavioral and cognitive deficits in rats. These impairments correlate with an increase in oxidative stress markers in the periphery as well as in selected regions of the brain including the hippocampus, amygdala, and the prefrontal cortex. Moreover, heightened oxidative stress was associated with decreased levels of key antioxidant enzymes. It seems like that early life stress causes behavioral and cognitive deficits via an oxidative stress-mediated weakening of neuronal connections.

The central hypothesis of this Dissertation is that the ability to acquire susceptibility or resistance to stress-induced behavioral and cognitive deficits

resides in oxidative-antioxidative balance within the CNS. This balance is maintained by transcriptional and epigenetic mechanisms. Therefore, our long-term goal is to investigate a) the role of early life stress on behavior and cognition across different ages in rats, b) reveal resilience and susceptible phenotypes and **c)** to identify the role of oxidative mechanisms in the regulation of behavioral and cognitive function and resilience. We propose to utilize a comprehensive approach to address our goals.

In Aim 1, the effect of induction of early life trauma was examined using a rat model of early-life stress on later life behaviors. Sprague Dawley (SD) rats were exposed to single prolonged stress (SPS) at postnatal day (PND) 25. Behavior tests to assess anxiety-like behavior, depression-like behavior, and learning and memory function were performed at different stages of development during PND 32, 60 and 90. Resilience and susceptibility phenotypes also were examined. In Aim 2 we examined the effect of early life stress on oxidative stress mechanisms as well as transcriptional and epigenetic regulation of specific genes that presumably control antioxidative capacity. We focused explicitly on Keap1-Nrf2 and NF-κB pathway. SD rats were exposed to SPS at PND25. One group of rats were sacrificed at PND32, the other group of rats was sacrificed at PND90. Blood was collected, and plasma was used to examine systemic markers of oxidative stress and physiological stress. Brains were harvested, and specific brain areas were isolated, and homogenates were prepared for conducting biochemical analysis to determine the effect of early life SPS on oxidative-antioxidant balance, and activation of redox-sensitive pathways such as Nrf2 and NF-κB pathways. Studies proposed in aim 1 revealed

that rats exposed to SPS exhibited both anxiety- and depression-like behavior at PND32. Moreover, short-term (STM) but not long-term memory (LTM) was impaired. Rats exposed to SPS at PND60 exhibited anxiety- but not depression-like behavior. STM but not LTM was impaired. Rats exposed to SPS at PND90 exhibited fearful (as indicated by elevated plus maze test) but not an overall anxiety-like behavior (in light and dark test). These rats also displayed significant depression-like behavior with no changes in STM or LTM. Interestingly, when data was further analyzed, two subsets of PND90 rats exposed to SPS were identified, "susceptible": with depression-like behavior and "resilient": without depression-like behavior. Importantly, while *resilient* group expressed early signs of anxiety- (at PND32 and PND60) and depression-like behavior (at PND32), these behavioral deficits were absent at PND90. On the other hand, *susceptible* PND90 rats exposed to SPS expressed later onset of anxiety-like behavior (at PND60), while depression-like phenotype was evident only later on at PND90. At the biochemical level, SPS exposure at PND25 led to an increase in oxidative stress in specific regions of the brain (pre-frontal cortex), as indicated by the increased level of oxidative stress marker at PND32 and PND90. SPS exposure at PND25 also led to an initial increase in antioxidant enzyme expression at PND32 and a decrease in antioxidant enzyme expression at PND90. The increase in oxidative stress and the decrease in antioxidant enzymes at PND90 correlates with the depressive phenotypes in SPS rats at PND90. Further biochemical studies revealed a state of a compromised Nrf2 pathway and activated NF-κB pathway in the pre-frontal cortex (PFC) homogenates. The state of compromised Nrf2 pathway and activated NF-κB pathway was

indicated by a decrease in the levels of Nrf2 and increased levels of NF-κB, as well as NF-κB-mediated increased levels of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) in PFC.

In summary, our findings suggest that early life stress caused co-occurrence of anxiety and depression-like behavior at PND32 (mimics human early-adolescent period). This co-occurrence was lost at PND60 with a demonstration of anxiety- but not depression-like behavior. Later, depression but not the anxiety-like behavior was observed at PND90. It seems that behavioral adaptations occur at the critical PND60 stage (mimics human late-adolescent period) (Sengupta 2013), where behavioral and cognitive switching occurs, thereby, expressing susceptible and resilient phenotypes. Moreover, susceptible phenotype correlates with an increase in oxidative stress and a decrease in antioxidant enzymes in the emotion-regulating brain region of the PFC. The correlation between susceptible phenotype and increased oxidative stress markers suggests that the early life stress causes a buildup of oxidative stress, which negatively affects neuronal circuitry that contributes to depressive phenotypes. The increase in oxidative stress induced by early life stress activates NF-κB pathway, which triggers cellular inflammatory responses.

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ABBREVIATIONS

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

SD: Sprague Dawley

SPS: Single Prolonged Stress

PND: Post natal day

IL-6: Interleukin-6

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

PTSD: Post-traumatic stress disorder

BSO: L-Buthionine-(S, R)-sulfoximine

BDNF: Brain derived neurotrophin factor

SOD: superoxide dismutase

GPx: Glutathione peroxidase

ACE: Adverse childhood experiences study

GXE: Gene-environment interaction

DISCI1: Disrupted-in-Schizophrenia-1

SNS: Sympathetic nervous system

HPG: Hypothalamic-pituitary-gonadal

CpG islands: dinucleotides consisting of cytosine alternating with guanine

5hmC: 5-hydroxymethylcytosine

5fC: 5-formylcytosine

5caC: 5-carboxycytosine

Tet: Ten-eleven translocation

TCAs: Tricyclic antidepressants

MAOIs: Monoamine oxidase inhibitor

SSRIs: Selective serotonin reuptake inhibitors

SNRIs: Serotonin-norepinephrine reuptake inhibitors

NRIs: Norepinephrine reuptake inhibitors

5-HT: Serotonin

CRF: Corticotropin-releasing factor

GR: Glucocorticoid receptors

MR: Mineralocorticoid receptors

H_2O_2: Hydrogen peroxide

CBP: CREB-binding protein

HDAC: Histone deacetylase

DNMT: DNA methyltransferase

DNPH: 2,4-dinitrophenylhydrazine

TNF-α: Tumor necrosis factor-α

HO-1: Heme oxygenase-1

H_2O_2: Hydrogen peroxide

PFC: Prefrontal cortex

Cu-Zn SOD: Copper-zinc superoxide dismutase

Mn-SOD: Manganese superoxide dismutase

Nrf-2: Nuclear factor (erythroid-derived 2)-like 2

ARE: Antioxidant response element

OFT: Open field test

EPM: Elevated plus maze

LD: Light dark

FST: Forced swim test

RAWM: Radial arm water maze

STM: Short-term memory

LTM: Long-term memory

EDTA: Ethylene diamine tetraacetic acid

ELISA: Enzyme-linked immune sorbent assay

ACTH: Adrenocorticotropic hormone

HPA: Hypothalamic-pituitary axis

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1. INTRODUCTION

Adverse experiences during early life can contribute to the development of psychiatric conditions later in life. Adults with a history of experiencing traumatic experiences of either childhood abuse or other traumatic events are considered to be at increased risk of developing depression or post-traumatic stress disorder (PTSD) later in life (McNally, Heeren, and Robinaugh 2017). In the United States alone, 1 in 58 children experience maltreatment (Fourth National Incidence Study of Child Abuse and Neglect). Approximately 15.5 million children witness physical or emotional abuse of a parent every year, becoming vulnerable to developing psychiatric conditions including PTSD and depression (Margolin and Vickerman 2007).

Not all children who experience traumatic events are at equal risk of developing later life psychiatric disorders. Some are resilient despite being exposed to the same risk factors, while others remain susceptible (Masten 2001; Silk et al. 2007; Miller et al. 2011). The relationship between early life trauma exposure and development of psychiatric symptoms in later life is not well understood, and the mechanistic basis for resilience is also not clearly understood While examining the link between early life stress and later life behavioral and cognitive well-being is crucial, conducting such studies in children with abuse or trauma history are difficult to carry out. Therefore, animal models are valuable in studying the

behavioral consequences of early life stress across the developmental course and in distinguishing the occurrence of different developmental trajectories.

Previous studies in rodents and monkeys have revealed that early life stress induced by maternal separation has adverse effects on behavioral and neurobiological phenotype in adulthood (Lyons et al., 2010; Vetulani, 2013). There is also a large body of literature suggesting that prenatal stress in rodents predisposes the animals towards anxiety- and depression-like behavioral phenotype in adulthood (Morley-Fletcher et al., 2003; Lee et al, 2007). However, the role of early life stress remains unclear with lot of variations reported in different studies (Boersma et al., 2014; Tamashiro 2015). Notably, information on the role of early life stress in causing behavioral changes over the developmental course is lacking.

Using a well-established single prolonged stress (SPS) rodent model of PTSD, in the present study we examined the consequences of early life stress across different stages of development in rats. The objective of this study was to determine the impact of early life stress by using SPS exposure at post-natal day (PND) 25 in rats and to examine the behavioral and cognitive consequences at different developmental stages (early adolescent: PND32, late adolescent: PND60, and adult stage: PND90). Male Sprague-Dawley rats were either exposed to SPS or control procedures on PND25, following which specific behavioral and cognitive parameters were examined at different time points of development. Examination of anxiety-like behavior, depression-like behavior, and learning and memory function were performed at PND32, 60 and 90 (Rodent lifespan corresponding to young,

adolescent and adult stage). We hypothesize that early life SPS exposure leads to distinct age-specific behavioral phenotypes in rats.

The next logical question that arises from this work relates to how early life trauma causes psychiatric problems in later life, including anxiety disorders, depression, and cognitive impairment? We have focused our attention on oxidative stress, which is an important biochemical distress mechanism and is primarily considered damaging to the brain. It is well known that psychological stress promotes oxidative damage (Salim. 2014), which is associated with biological aging and affects different organ systems (Cui, Kong and Zhang 2011). Overall, oxidative damage, including lipid peroxidation, protein modification, enzyme inactivation and DNA breaks, are associated with aging and diseases such as inflammation, cancer, diabetes, and cardiovascular diseases (Pham-Huy He, and Pham-Huy 2008). The brain is especially vulnerable to oxidative stress due to its high lipid content (Uttara et al. 2009) which is the primary target for oxidation by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Di Meo et al. 2016). ROS are especially active in the brain. The high oxygen consumption of neuronal tissues due to the metabolism of excitatory amino acids and neurotransmitters leads to a large number of redox reactions, which often results in a high rate of free radical production in the brain (Wang and Michaelis. 2010). Increased ROS attacks glial and neuron cells, leading to neuronal damage or even programmed cell death.

Earlier work has suggested that oxidative stress plays a major role in the development of anxiety-like behavior of rats (Patki et al. 2013). Pharmacologically-

induced oxidative stress with L-Buthionine-(S, R)-sulfoximine (BSO) treatment increased anxiety-like behavior in rats (Sarraj et al. 2010). Treatment with the antioxidant tempol reduced anxiety-like behavior caused by multiple anxiogenic drugs in the rat (Patki et al. 2013). Also, in another study, psychological stress induced by chronic social defeat led to elevated oxidative stress as well as behavioral deficits (Solanki et al. 2017). Other studies have suggested that stress-induced behavioral and cognitive deficits in rats were associated with heightened oxidative stress in the brain and diminished antioxidant response in neuronal tissues (Vollert at al. 2011, Allam et al. 2013, Patki et al. BBR 2013, Solanki et al BR 2013),

Considering a strong correlation between oxidative stress and stress-induced behavioral and cognitive impairment, we think the balance between oxidative stress and antioxidant capacity is essential in regulating behavioral and cognitive function. It is likely that early life exposure to SPS leads to increase in production of ROS in the brain resulting in increased oxidative stress in brain regions that are essential for regulation of behavior and cognition. This postulation seems more compelling considering previous studies including from our lab, which has established a causal relationship between increased oxidative stress in brain and behavioral and cognitive impairments (Patki et al 2013; Patki et al 2014)). Furthermore, increased levels of oxidative stress and inflammation in the hippocampus and cerebral cortex have been associated with the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Multiple Sclerosis (Fischer and Maier 2015)). Oxidative stress has also been reported to be involved in major depression.

These studies reported a significant decrease in glial cells and neurons in cortico-limbic regions of depressed patients, which could be a consequence of increased oxidative stress which in turn leads to cell death. (Michel TM et al. 2012). Increased oxidative stress has also been implicated in learning and memory deficits (Fukui et al. 2001). Numerous animal and clinical studies have described that sleep deprivation or chronic stress exposure can increase hippocampal oxidative stress, which plays a significant role in memory deficits. Therefore, a possible correlation between stress, oxidative stress and behavioral and cognitive deficits exists.

Early life is an important period for neuronal development (Stiles and Jernigan 2010). Thus, early life experiences can have significant effects on the brain development, which can have an impact throughout the lifespan. Animal models of early life stress have shown that early life maternal separation can lead to depression-like behavior in adulthood, mediated by hyper-methylation in the promoter region of key genes such as brain derived neurotrophic factor (BDNF) (Cirulli et al 2009). Exposure to early life stress alters the behavior in adulthood, and large individual differences exist in the effects of early life stress. Some individuals seem vulnerable, others seem to be resistant despite early life exposure to same kind of stress. In this project, behavioral, cognitive, and biochemical consequences of early life stress in later life were examined using a single prolonged stress (SPS) model. Sprague-Dawley (SD) rats were exposed to SPS at PND25. One group of rats were sacrificed at PND32 after conducting a comprehensive behavior analysis; another group of rats was sacrificed at PND90 following behavior analysis. At PND90, susceptible (depressive) and resilient (non-depressive) subgroups were

separated in within a group of SPS rats based on their performance in forced swim stress test (FST). An attempt to investigate the association between oxidative stress and depressive phenotype was also made. This study provides a unique method for testing our hypothesis and suggests that the ability to acquire susceptibility or resilience to early life stress-induced behavioral and cognitive deficits resides in oxidative-antioxidative balance.

We suggest that the imbalance between oxidative stress and antioxidant defense system is the primary force that drives early life SPS induced depressive phenotype in later life. As a next step, the focus of our research is on redox-sensitive transcription factors Nrf2 and NF-κB. These transcription factors can be activated in response to the increase in ROS production as a means to regulate antioxidant defense or inflammatory response respectively (Ganesh Yerra et al. 2013). Nrf2 has a significant role in antioxidant defense system (Gorrini, Harris and Mak 2013). It is a transcription factor that binds to antioxidant response elements (ARE) in the promoter region of genes that regulate the expression of a group of antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, heme oxygenase-1 (OH-1), and non-enzymatic antioxidants (Vomhof-Dekrey and Picklo 2012). Under normal conditions, Keap1 is constitutively active and interacts with Nrf2, promoting its ubiquitination by Cullin 3. During oxidative stress, Nrf2/Keap1/Cullin3 interaction is disrupted, so Nrf2 can translocate into the nucleus to bind to the ARE of the promoter regions of genes that encode a large group of antioxidants (Nguyen, Nioi and Pickett 2009). If the Keap1/Nrf2 pathway is disrupted, the antioxidative capacity of the Keap1/Nrf2 pathway is dampened,

leading to increased oxidative stress (Sykiotis and Bohmann 2008). It is plausible that the ability to acquire susceptibility or resilience to trauma-induced behavioral and cognitive deficits resides in oxidative-antioxidative balance maintained by Nrf2. When there is increased oxidative stres**s,** ROS can lead to the phosphorylation of IκB, which is the negative regulator of P65, a subunit of NF-κB (Morgan and Liu. 2010). The phosphorylation of IκB leads to the release of p65. The released p65 translocates into the nucleus, binds to the promoter region of κb related genes such as Interleukin-6 and TNFα, inducing inflammatory responses. Several studies have suggested that activation of Nrf2 can lead to suppression of NF-κB, and vice versa (Wakabayashi et al. 2010; Bellezza, Mierla and Minelli 2010). Our data suggest that early life exposure to SPS leads to increase in oxidative stress. Therefore, increased levels of ROS might activate redox-sensitive transcription factors. We postulate occurrence of a cross-regulation of NF-κB and Nrf2 pathways, with activation of NF-κB pathway, and inhibition of Nrf2 pathway. The inhibition of Nrf2 pathway and activation of NF-κB pathway cause an increase in the inflammatory response and suppression of antioxidant enzyme expression, ultimately leading to sustained oxidative stress, contributing and leading to behavioral and cognitive impairments in later life.

STATEMENT OF PROBLEM

Early life stress has been linked to adulthood mental and physical problems, including diabetes and cardiovascular disease, neurodegenerative disease and early death (Entringer, Buss, and Heim. 2016)). Children subjected to traumatic events

during childhood are reported to exhibit behavioral and cognitive deficits later in life, often leading to post-traumatic stress disorder (PTSD) and major depression (Wang, Shelton, and Dwivedi. 2017). The association between early life stress and behavioral deficits in adulthood is complex. Interestingly, some children continue to remain normal despite being exposed to the same risk factors. These trauma-related behavioral and cognitive profiles across different stages of life are not well understood. Animal studies can offer useful insights. The first goal of this study was to determine the impact of early life exposure to traumatic events on behavioral and cognitive profile in rats by tracking the behavior of each rat at different ages. The primary hypothesis is that early life exposure to SPS leads to increased anxiety- and depression-like behavior as well as impaired memory in rats at PND32, and a depressive phenotype can be found in a subgroup of SPS rats at PND90. The second hypothesis is the susceptibility or resilience to early life SPS induced depressive phenotype at PND90 resides in oxidative/antioxidative balance.

The significance of this study is that our study reveals the behavioral and cognitive consequences of early life stress across life, as well as individual differences in the effects of early life stress exposure at PND90 and the association between behavioral phenotypes and biochemical changes, including the level of oxidative stress and antioxidant enzymes. In the long term, this study provides an innovative model which can be used to probe underlying neurobiological mechanisms, reveal novel molecular drug targets and explore potential therapeutic interventions for early life stress-induced behavioral deficits across developmental stages.

2. LITERATURE REVIEW

2.1. Early Life Stress

According to the Fourth National Incidence Study of Child Abuse and Neglect. 2005-2006), 1 in 58 children experience maltreatment in the United States. Furthermore, nearly 5.5 million children in the United States are abused every year, and approximately 15.5 million children witness domestic violence every year. The type of childhood trauma includes physical abuse, sexual abuse, emotional abuse, physical neglect, emotional neglect, parental substance abuse, parental separation or divorce, or the loss of a parent or a sibling. According to the Adverse Childhood Experiences Study (ACE Study) conducted by the Center of Disease Control and Prevention, adverse childhood experiences including living in a stressful environment of violence, crime, abuse, poverty, and neglect, are strongly associated with the occurrence of various types of diseases throughout life. In general, stress is believed to be a significant factor that impacts health and disease **(Figure 1)**, and harmful effects of stress are believed to occur when an individual is not able to maintain physiological equilibrium when faced with aversive stressors, usually accompanied by cognitive and behavioral changes. Prolonged exposure to stressors, particularly during early life, can be damaging by interfering with brain developmental processes such as synaptic pruning and remodeling, might affect healthy brain development and also cause other harmful effects (inflammatory responses, oxidative stress, epigenetic methylation, and gene expression). These events might eventually lead to disastrous consequences on health later in life. How

early life stress affects health outcomes in adulthood has long been the focus of many investigations. During the past several decades, the understanding of the effects of early life stress in later life has significantly advanced. These studies can be characterized in several categories.

The first wave of research indicated the link between stress and health by the observations that sudden deaths often are associated with significant stressful events. The second wave of early life stress research provided more evidence of the relationship between stress and health outcomes in later life. Holmes and Rache (Holmes and Rahe 1967) suggested that stressful environment increase the risk of illness. In addition to this, both clinical and preclinical research showed that social support is essential as well. However, the underlying mechanism of the relationship between stress and health outcomes was not clear. The third wave of research provided mechanistic information suggesting the involvement of stress-induced physiological changes occurring in the body. For example, changes in the immune functions have been observed in students facing stress at school. Stressful experiences are associated with changes in the number of total t-lymphocytes and natural killer cell activity. The Forth wave of research mostly focused on examining gene-environment interaction (G×E), and how it affects the effects of early life stress on health outcomes in later life. Recent research indicates that both early life environment and genetic predispositions act as joint determinants to predict the response to stress, ways to cope with stress, and psychological effects of stress. These, in turn, regulate autonomic, neuroendocrine, and immune responses to

stress. These biological alterations subsequently predispose individuals to a higher risk of mental and physical illness (**Figure 2**).

Figure1. Relationship between adverse childhood experiences and disease susceptibility. Modified from *American Jouranl of Preventive Medicine* 1998 14, 245-258DOI: (10.1016/S07 49-3797(98)00017-8)

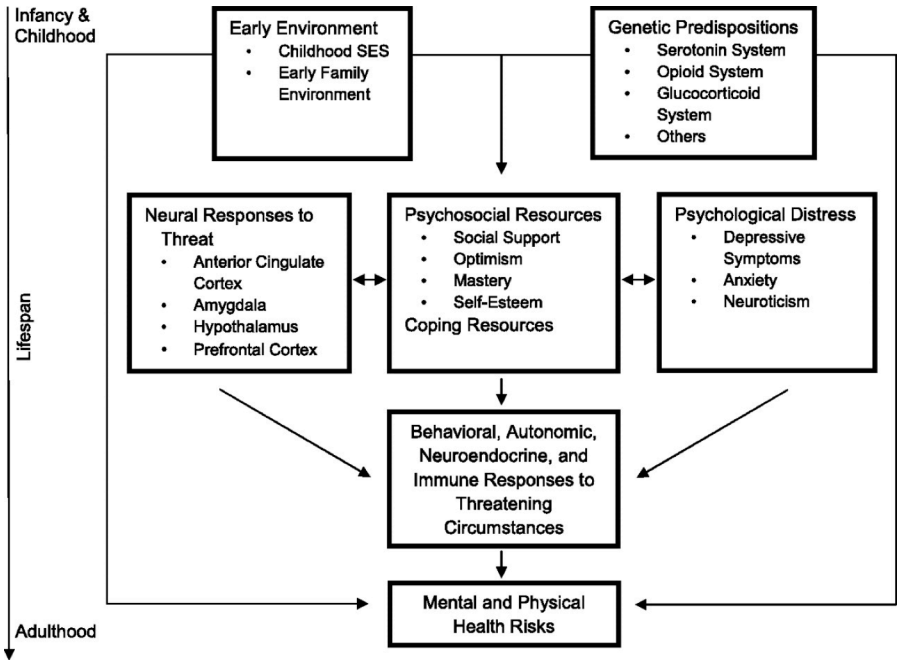


Figure 2. Stress and health outcomes across the lifespan. Modified from Shelley E. Taylor PNAS 2010;107:8507-8512

2.2. Types of Early Life Stress

In 1998, the Adverse Childhood Experience (ACE) study described the long-term effects of childhood experiences on health. (Felitti, V.J.M.D. et al. 1998) The relationship between adverse childhood exposure and the occurrence of adulthood diseases including heart disease, cancer, chronic lung disease, metabolism disorders, and liver disease, were studied. Early life stress was categorized as below in **Table 1**.

Categories	Sub-Categories
Abuse	Emotional abuse
	Physical abuse
	Sexual abuse
Household Challenges	Mother treated violently
	Household substance abuse
	Mental illness in household
	Parental separation or divorce
	Criminal household member
Neglect	Emotional neglect
	Physical neglect

Table1. The Types of Early Life Stress.

Emotional abuse: When a parent, step-parent, or an adult, insults, intimidates, or bullies a child.

Physical abuse: When a parent, step-parent, or an adult living in the house with the child is physically abusive to the child (pushes, grabs, slaps, or hits the child leaving marks of injury on the body of the child).

Sexual abuse: When an adult relative, a family friend, or a stranger at least five years older than the child touches or fondles the body of a child in a sexual manner.

Mother treated violently: When the mother or stepmother is pushed, grabbed, slapped, kicked, hit or ever threatened by the father (or stepfather) or mother's boyfriend.

Household Substance abuse: When a household member abuses alcohol and uses street drugs.

Mental illness in the household: When a parent or a sibling is depressed or suffers from a mental health problem and attempts or commits suicide.

Parental separation or divorce: If parents are separated or divorced.

Criminal household member: Imprisonment of a member of the household.

Emotional neglect: When the child is not made to feel important, special, or loved.

Physical neglect: Absence of an adult in the life of a child to take care of child's physical needs; food, health care and necessities.

2.3 Consequences of Early Life Stress

Both preclinical and clinical studies strongly suggest that early life exposure to stress can lead to long-term negative effects on mental and physical health. Childhood abuse and neglect might put the children on the trajectory to improper brain development, cognitive deficits, lower language development, heart disease, lung disease, liver disease, obesity, cancer, high blood pressure, high cholesterol, anxiety, depression, and substance abuse. If the hypothesis that a high percentage of these diseases have earlier roots in childhood experiences is right, the health care cost relevant to childhood stress has been widely underestimated.

2.3.1 Early Life Stress and Mental Illness

Based on the information presented above, adult vulnerability to emotional disorders seems to mainly derive from exposure to the early environment during development. Brain maturation involves multiple dynamic processes, which can be regulated by environmental stimuli. The early postnatal period is especially sensitive to early life stress, because the early postnatal brain undergoes significant development including axonal and dendritic growth, forming neural connections and pruning. As shown in **Figure 3**, the early postnatal period represents a critical stage for the development of several brain regions that are essential in regulating emotions and cognition. When stress is experienced during this rapid developing

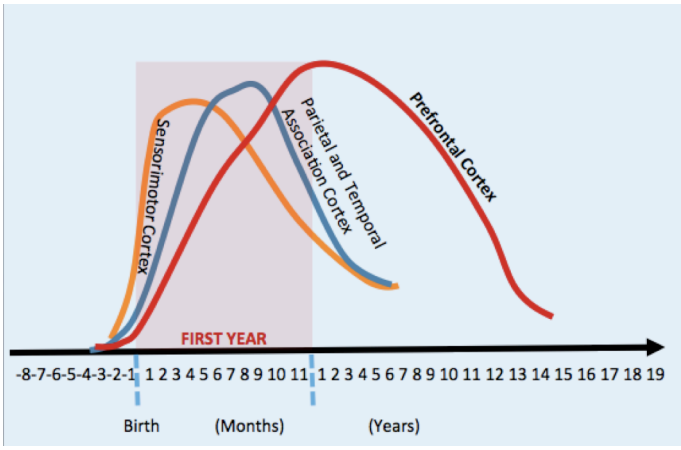


Figure 3. Human brain development: Neurogenesis in brain regions through experience-dependent synapse formation. (Modified from Gerry Leisman et al. 2015)

period, its effects can be long-term, compared with the temporary impact of stress on the adult brain.

2.3.1.1 Early Life is a Critical Period of Brain Development

The early developmental period is critical; therefore, a better understanding of the relationship between environment and brain development is essential not only for identifying the mechanisms of adulthood vulnerability to mental disorders but also for prevention approaches in neuropsychiatry. Early life was first described as a critical period in the "imprinting" phenomenon described in birds, which stated that young precocial bird develops appropriate mate preferences by exposure to conspecifics within hours of hatching (Lorenz 1937). Early life social isolation in monkeys during the first three months after birth had long-lasting developmental effects (Harry Harlow 1965). The early developmental period is associated with neural plasticity, which is less observed in adulthood (Nowakowski and Hayes 1999). This feature of the nervous system makes the brain more responsive to experience throughout gestation and early postnatal period, until adulthood. Maturation processes including neurogenesis, differentiation, and survival occur at different times, determined by the brain regions and neural circuits. Therefore, the critical period for specific brain regions might be slightly different, which determines the specific vulnerability of each brain region to external influences (Andersen and Teicher 2008). The concept that there exists a potentially critical neurodevelopmental period which determines neuroplasticity mechanisms leads us to postulate that early life development has two probable courses. Exposure to early life stress might change the development trajectory and predispose an individual to later psychiatric diseases; or, exposure to favorable conditions or sub-stress might

help the brain to cope with stress, adapting to the environment, developing a more resistant neuronal phenotype.

2.3.1.2. Neurodevelopmental Hypothesis of Mental Disorders

Increasing body of work has focused on the effects of early life stress on later life mental health. It is reported that adverse experiences during brain developmental period may disrupt the maturation of neuron circuit, leading to the formation of pathologic neuron circuit and emergence of psychiatric diseases in later life (Fatemi and Folsom 2009).

This hypothesis is further supported by several epidemiological studies. Patients with symptoms of mental disorder such as schizophrenia and autism often report a history of early life adversities including abuse, neglect, and other types of early life stress (Kendler et al. 2002; Biederman 2005; Cannon et al. 2002; Weinstock 2008). Specifically, Loss of a family member during childhood increases the risk of major depression in adulthood (Kendler et al. 2002). Victims of suicide had higher rates of childhood physical abuse and sexual abuse. A consistent finding across different studies is that there was an association between borderline personality disorder and sexual abuse in early life (Grover et al. 2007; Bandelow et al. 2005; Johnson et al. 2001). Furthermore, many genes that are associated with psychiatric disorders also have essential roles in neurodevelopment, such as Neuroligins, Reelin, Dysbindin, Disrupted-in-Schizophrenia-1 (DISCI1) and SHANK3 (Fatemi and Folsom 2009, Sawamura and Sawa 2006). More recent evidence suggests that the interaction between environmental exposure to early life adversity and individual genetic risk can predispose an individual to psychiatric diseases throughout life

(Gottesman and Hanson 2005). Even though genetic factors play significant roles in determining the risk of mental disease, experiences in early developmental stage also set the vulnerability of an individual to later life mental diseases (Crirulli et al. 2009, Baes et al. 2012). Early life stress exposure may act as "second hit" in addition to genetic factors, making those who were already predisposed to psychiatric diseases more susceptible (Laviola et al. 2009).

Under stressful circumstances, children can have elevated levels of cortisol for a prolonged period. Both clinical and preclinical studies show that long-term elevation in cortisol levels can affect the function and architecture of neural systems that are essential for learning and memory function (Kumari et al. 2013). For example, brain-derived neurotrophic factor (BDNF), which is a candidate target of early life stress, is implicated in early life stress. It is suggested that early life stress via modulation of BDNF levels can regulate neural plasticity and structural integrity (Branchi et al. 2004). Preclinical studies of early life stress with maternal separation have reported that maternal separation decreases the levels of BDNF in the hippocampus region and striatum of rodents exposed to maternal separation, suggesting a role of BDNF in early life stress.

Taken together, we believe that the early environment has a critical role in neurobiological development. Adverse experiences during early developmental period affect behavioral and cognitive functions at adulthood.

2.3.2 Early Life Stress and Physical Health

It is evident that early life stress is harmful to children and adolescents. Physical abuse by an adult, being bullied at school, or witnessing domestic violence, can lead

to the development of behavioral, emotional, or cognitive problems in many, though not all, young people. What is less known is that early life exposure to adversity can also leave hidden alterations that can predispose an individual to physical disorders (Lupien et al. 2009; Shonkoff et al. 2012). Currently, a growing body of multidisciplinary research is focusing on the effects of early life stress on different systems. Such studies have made significant advances in finding biomarkers that can link early life stress with increased susceptibility to heart disease, metabolic diseases, immune diseases, and neurodegenerative diseases in later life (Danese &McEwen, 2012; Miller, Chen & Parker, 2011). These biological changes that induced by stressful experiences in early life are responsible for the onset of physical illness in adulthood. Taking into consideration the prevalence of these chronic diseases and the financial burden they bring to public health care system, such studies are meaningful specifically for early prevention.

2.3.2.1 Early Life Stress and Cardiovascular Disease

Although it is well accepted that early life stress induces alterations in brain development and chemistry, which subsequently can increase the susceptibility to stress-related disorders in later life, less attention is paid in the correlation between these alterations and the risk of cardiovascular diseases. Stress can activate a variety of neurochemical systems that have effects on the body. The most well-known are the hypothalamic- pituitary- adrenocortical (HPA) system and the sympathetic nervous system (SNS). These systems activated by stress also have effects on cardiovascular reactivity (Aduilera and Rabadan-Diehl, 2000; Mangiafico et al. 2002), reflected by the increased response in blood pressure and heart rate.

Importantly, it was reported that higher cardiovascular reactivity after stress exposure is associated with higher risk of the development of cardiovascular diseases such as hypertension and coronary artery disease (Beutel et al. 2014; Carroll et al. 2013). Moreover, clinical studies provide epidemiological evidence linking early life stress exposure to later life onset of cardiovascular disease. A clinical study that first described the association between early life stress and ischemic heart disease reported that there is a positive correlation between the level of adverse childhood experiences and the rate of ischemic heart disease in later life (Dong et al. 2004). A follow-up study with human subjects indicates that individuals that were exposed to multiple adverse childhood events had higher blood pressure levels in later life when compared with the control individuals. Moreover, the level of endothelin-1, which can be released in response to stress, was associated with early life stress and later life elevations in blood pressure (Su et al. 2015). Another clinical study that exposed boys aged 8-17 to a series of laboratory stressors reported that the boys from less supportive families showed higher heart rate responses (Matthews et al. 1990). In addition to clinical studies, there is growing evidence from preclinical studies using animal models of early life stress that early life stress exposure is connected to later life cardiovascular disease susceptibility. The model that was widely used for such studies is maternal separation. It is well established that maternal separation can induce depression and anxiety-like behaviors in adulthood (Lehmann et al. 2000; Lippmann et al. 2007). Similar to the findings from human studies, the behavioral outcomes of early life stress were accompanied by increased risk of developing cardiovascular

symptoms in adulthood (Loria et al. 2013). The mechanism that contributes to the development of cardiovascular dysfunctions after exposure to early life stress is reported to be related to the effects of early life stress on the multiple systems, such as developmental plasticity of central nervous system and the heart itself, inflammation, and epigenetic regulations (Branchi et al. 2004; Trombini et al. 2012; Gershon and High. 2015).

Early life stress seems to be a significant factor that affects the programming of the cardiovascular system and increases the risk of developing chronic cardiovascular disease.

2.3.2.2 Early Life Stress and Immune System

Similar to the central nervous system, the immune system undergoes developmental processes even after birth, continues through-out childhood to adolescent period. The initial evidence that supports the association between early life stress and immune system function emerged from the observation that handling before weaning reduces the development of a transplanted tumor (Ader and Friedman, 1965). After this, emerging research in the developmental psychoneuroimmunology field started to pay attention to the association between early life stress and immune functions in later life. In both non-human primates and rodents, elevated production of pro-inflammatory cytokines in plasma and increased activity of macrophages were observed in animals that were exposed to maternal separation (Coe et al. 1988 and 2012; Hennessy et al. 2010; Wieck et al. 2013). A longitudinal study with children in England and Wales showed that inflammatory biomarkers in blood were elevated in children who were maltreated

and showed symptoms of depression by age 12 years when compared to controls (Danese et al. 2011). Studies with patients with a history of early life childhood maltreatment reported that childhood maltreatment was associated with higher

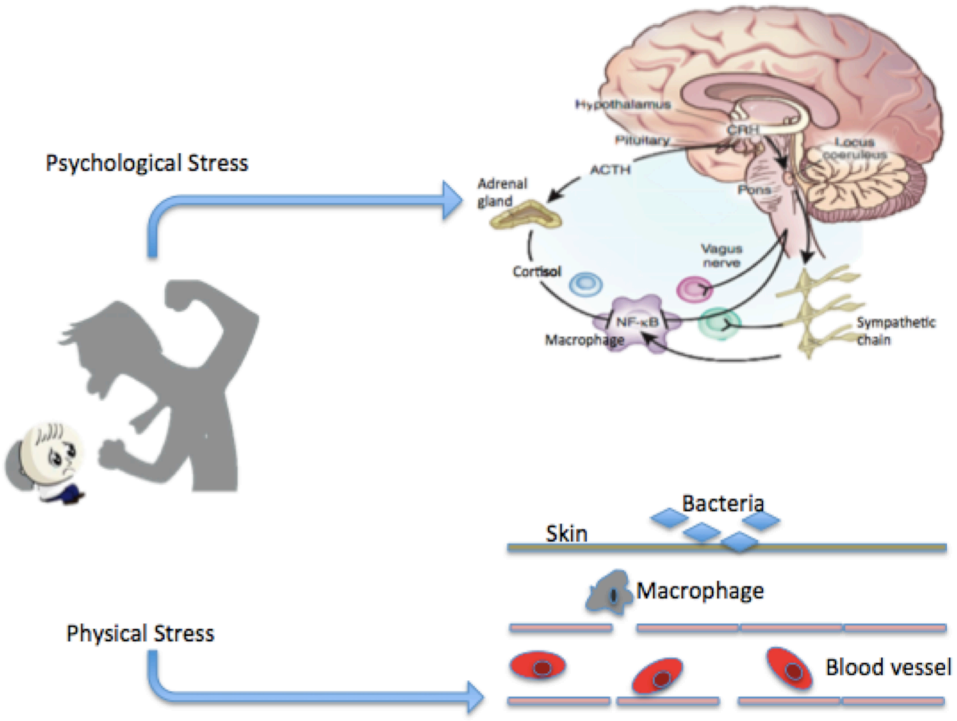


Figure 4. Early life stress-induced acute inflammatory response mechanisms. Psychological stress induced by early life stress can lead to acute inflammatory responses though the activation of sympathetic nervous system; physical stress induces inflammatory response due to injury and pathogen infection (Modified from Danese and Lewis, 2016).

rate of major depression and increased the level of IL-6 and NF-κB DNA binding (Pace et al. 2006; Carpenter et al. 2010). The potential mechanism(s) of how childhood trauma is related to inflammatory response is shown in **Figure 4**.

Interestingly, the co-occurrence of inflammatory responses and long-lasting behavioral consequences after early life exposure to stress indicates that

inflammation induced by early life stress might be the underlying mechanism for the later onset of psychopathology.

2.3.2.3 Early Life Stress and Cancer

Life-course studies on early life stress suggested that later life development of cancer might be rooted in the early life adverse experiences. A study reported that patients who reported adverse childhood experiences had a twofold increased risk of developing cancer before the age of 50 in women (Michelle Kelly-Irving et al. 2013). There is plenty of evidence suggesting that cancer has emotional roots. Stress, especially during early life, plays a crucial role in the development of different types of cancer in later life.

Several lines of research indicate that stress response favors tumor growth (Lutgendorf, Sood, and Antoni, 2010), perhaps due to the suppression of immune system and elevating glucocorticoid and catecholamine levels (Khong and Restifo, 2002). A study in a mouse model of ovarian cancer indicates that stress-induced beta-adrenergic activation enhances tumor angiogenesis (Thaker et al. 2006). In a mouse model of pancreatic cancer, stress-induced activation of the sympathetic nervous system was postulated to increase the growth and invasion of tumor (Kim-Fuchs et al. 2014). Several recent studies have suggested that stress during the critical period of early life induces long-lasting changes in the neuroendocrine function of the hypothalamic-pituitary-adrenal (HPA) and Hypothalamic-pituitary-gonadal (HPG) axes, which is purported to modulate breast tumorigenesis later in life (Boyd et al. 2010; Schuler and Auger. 2010). Thus, epidemiological and preclinical studies have both suggested implication of stress in processes of tumor

growth. However, the biological mechanisms of how early-life stress contributes to the etiology of cancer in later life remains unclear. A better understanding of what happens in biochemical aspect when exposed to adverse early life events and how these changes might predict malignancy is likely to improve cancer prevention strategies.

2.3.3 Early Life Stress and Epigenetics

Epigenetic modifications, especially DNA methylation, is beginning to be appreciated as a plausible biological mechanism that presumably links early life stress with development of later negative health conditions including psychiatric disorders, cardiovascular complications, and cancer (Mitchell, Scheneper, and Notterman, 2015). Epigenetic modifications, including DNA methylation, histone modifications, and noncoding RNA, are the mechanisms that are known to be responsible for cellular diversity and differentiation by regulating mRNA transcription, despite the same set of DNA carried by the cells (Razin A et al. 1998). Epigenetics have been the focus of recent studies in stress research because epigenome can be modified through environmental regulations (Miller GE. 2009).

Regions of genes, including promoters, enhancers, and silencers that are implicated in transcriptional regulation of the genes, usually consist of a greater proportion of dinucleotides consisting of cytosine alternating with guanine (CpG islands). The cytosine is susceptible to addition of a methyl (CH3) group via DNA methyltransferases. The resulting 5-methylcytosine can be subsequently oxidized to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) by the ten-eleven translocation (Tet) family of proteins. The products 5fC

and 5caC can then be replaced with unmethylated cytosine. The methylation of the CpG islands in the promoters of genes inhibits the transcription. Because the addition of methyl group to cytosine is a covalent chemical modification, DNA methylation has been studied extensively as environmental changes occurring during development can leave "hidden" marks on genomic DNA and affect life-long health conditions. During DNA replication, the methylation marks can be passed down from parent cell to daughter cell so that the stable mark will be carried on across generations of cells. Moreover, epigenetic modifications can be heritable (Weaver, Meaney, et al.). This does not mean that the methylation state of CpG islands of the parent's genome can be directly transferred to a new generation. The mechanisms by which epigenetic modifications can be heritable is that the epigenetic state of the parents' genome can affect the mental or physical conditions of the parents, which subsequently affect the developmental environment of the next generation. One clinical research showed that childhood abuse could lead to methylation of CpG islands in many genes that have a significant role in different diseases (Yang BZ et al. 2013). Childhood maltreatment history was associated with hypermethylation in the promoter of glucocorticoid receptor gene in the brain (McGowan et al. 2009). Animal models of maternal separation showed that the long-lasting effect of early life stress is associated with DNA methylation in the brain as well as later life health during adulthood (Pena CJ et al. 2014). Stress-related hypothalamic-pituitary-adrenal (HPA) axis can be the target of epigenetic modifications in response to early life stress. Many genes regulating HPA axis, including glucocorticoid receptor, AVP, CRH, were shown to be hyper methylated

after exposure to early life stress (Weaver et al. 2004; Murgatroyd et al. 2014; Elliot et al. 2010). Neurotrophic factor, BDNF, is also regulated by DNA methylation following early life stress in a rat model of poor maternal care (Roth et al. 2009).

Thus, it is clear that environmental conditions can leave similar DNA methylation patterns in certain genes during the developmental stage, and can remain stable throughout life. The information about how different types of early life stress leads to specific DNA methylation marks on specific genes may be useful in determining a therapeutic target for diseases related to early life stress.

2.4 Statistics of Early Life Stress

Exposure to violence is the most common source of stress for children. According to an epidemiological report, about one-quarter of children experience some early life trauma (Koenen et al. 2010). As discussed before, there are different forms of early life trauma, such as physical abuse, sexual abuse, and witnessing of domestic violence. These different forms of early life stress and their association with later life adverse health outcomes have been comprehensively studied. According to a population-based study conducted in developed countries, 5-35% of children were reported to experience physical abuse, 5-30% experienced sexual abuse, and 10-20% witnessed domestic violence (Gilbert et al. 2009). A United States nationwide study indicated that 60.6% of children and youth in the U.S. experienced physical trauma or witnessed some type of traumatic event. 46.3% of these young victims were physically abused, 10.2% of them experienced child maltreatment, 6.1% were sexually abused, and 25.3% experienced indirect trauma (Finkelhor et al. 2009). Similarly, a study from the United Kingdom reported that

approximately 20% children experienced severe maltreatment. 1 in 3 children experienced sexual abuse. In addition to this, a strong correlation between these early life traumatic experiences and poor emotional wellbeing in later life also was observed (Radford et al. 2011). A retrospective study conducted in 21 countries by The World Health Organization reported that 5-11% respondents reported a history of physical abuse in their childhood, 1-2% respondents recalled childhood experience of sexual abuse, and 4-8% reported violent household environment during childhood (Kessler et al. 2010).

The prevalence of childhood traumatic events worldwide and the strong associations between different forms of trauma and occurrence of later life negative health conditions deserves attention, as it can be a contributing factor for the increasing healthcare burden worldwide.

2.5 Current Interventions for Early Life Stress Related Mental Problems

Both clinical and preclinical studies have suggested that early life stress is associated with poor health outcomes in later life, with depressive disorder as the most prevalent and significant outcome. However, the knowledge of the underlying biology of this connection, the etiology of depression as well as early therapeutic intervention strategies are limited.

Current treatment of depression mainly involves psychotherapy and pharmacotherapy. A vast number of studies were conducted to evaluate the efficacy or effectiveness of both psychotherapeutic and pharmacological interventions (Wolf and Hopko 2008). Problem-Solving Therapy for Primary Care (PST-PC) is designed to assist patients in developing skills to alleviate the depressive memories of

traumatic events and improve psychological functioning. PST-PC strategy has been reported to be effective when used in combination with various pharmacological interventions (Katon, Unutzer, and Simon, 2004). Cognitive-behavioral therapy has been shown in some studies to be equally effective when coupled with antidepressant medications (Schoenbaum et al. 2001; Scott and Freeman, 1992). Interestingly, counseling approaches for depression, including Rogerian psychotherapy, supportive psychotherapy, or psychoeducational techniques, have been proven to be equivalent to antidepressant medications in some studies (Chilvers et al. 2001; Ward et al. 2000). The primary antidepressant treatments fall into six classes, including tricyclic antidepressants (TCAs), monoamine oxidase inhibitor (MAOIs), selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), norepinephrine reuptake inhibitors (NRIs) and atypical antidepressants.

Most of the currently used antidepressants are still based on the monoamine theory of depression that was first proposed in 1965 by Schildkraut et al. The monoamine theory is that depression is caused by the functional deficit of monoamine transmitters, noradrenaline, and serotonin (5-HT). This theory was based on the observation that drugs that have known neurochemical mechanism on monoaminergic transmission are effective to alleviate the symptoms of depression (Manji et al. 2001). However, clinical researches have shown that the direct neurochemical effects of antidepressants appear rapidly after medication, while the therapeutic effects might take weeks to appear. The delayed therapeutic effects of antidepressants suggests that the primary effect of antidepressants may not be

responsible for the antidepressant effect, it might be some secondary responses of the primary effects that directly mediate the antidepressant effects (Manji et al. 2001). Clinically, current antidepressants are reported to be more effective for chronic moderate and severe depression, but not for mild depression. About 20-40% of depressive patients had noticed improvement within six to eight-week treatment of placebo, while about 40-60% of depressive patients showed improvement in their symptoms within six to eight weeks of treatment with antidepressants (Arroll B et al. 2009). The fact that only low percentage of patients respond to these antidepressants indicates that current antidepressants are not effective in all patients.

The side effects of current antidepressants is another concern. More than half of the patients who take antidepressants reported side effects during the first few weeks. The side effects that are commonly seen with SSRIs treatment are diarrhea, sleep problems, headaches, and nausea. TCAs are more likely to cause dizziness, dry mouth, constipation, vision problems, and urination problems (von Wolff et al. 2013). New therapeutic interventions are possible only when new drug targets are revealed. Therefore, mechanistic studies can offer new clues in this regard.

2.6 Preclinical Studies of Early Life Stress

The idea that adversities experienced in early developmental stage of life predict later life phenotype has been widely accepted since decades ago. Several human studies support this idea. Many retrospective studies suggest that a higher risk of developing psychopathology in adulthood is associated with a history of early life abuse or neglect (Heim and Nemeroff 2001; Teicher et al. 2006). The early

preclinical studies are in line with this idea. Neonatal maternal separation in rodents and monkeys demonstrated that stress in early life had negative effects on the developmental process and the effects persisted throughout the lifespan. An association between maternal separation and persistent changes in behavioral and neuroendocrine system were reported (Weininger 1954; Harlow et al. 1964). Recently, using animal models, especially rodent models, scientists have confirmed the critical role of early life experience in neurobiological development and long-term behavioral, emotional, cognitive and physiological outcomes in adulthood.

2.6.1 Animal Models of Early Life Stress

Animal models are valuable experimental tools to study early life stress and its effects on neurodevelopment. They can provide the information regarding the causal link between specific neurodevelopmental abnormalities and specific type of behavioral deficits in later life.

Prenatal period is considered as an important stage of neurodevelopment. Adversities during the perinatal period may have significant effects on the development of health complications throughout an individual's lifespan. There are a lot of early life stress studies focusing on examining the effects of prenatal stress on the health outcomes of the offspring in later life. Human epidemiological studies suggested that maternal infection is strongly associated with the development of schizophrenia in the offspring (Boksa 2008). Preclinical experimental rodent models of immune challenge also suggest that there is a link between prenatal infection and later emergence of behavioral deficits, including abnormalities in working memory, social behavior, and exploration impairment (Meyer et al. 2009).

Specifically, maternal exposure to bacterial agents is reported to be associated with an increased risk of schizophrenia or autism in the offspring (Borrell et al. 2002; Fortier et al. 2004). Neonatal rats administered with proinflammatory cytokines or leukemia inhibitory factors can develop behavioral and cognitive deficits in later life (Tohmi et al. 2004; Watanabe et al. 2004). These studies indicate that immune response during neonatal period may be associated with brain development, and subsequently to abnormalities in neurobiological functions and behavioral deficits in adulthood. In agreement with this, maternal exposure to retrovirus, and influenza can increase the risk of developing behavioral abnormalities in the offspring (Yolken et al. 2000; Shi et al. 2003). In humans, psychological stress during pregnancy is observed to have significant effects on emotional, behavioral, and cognitive outcomes of the offspring (Talge et al. 2007). In the investigation of the neurobiological and neurobehavioral effects of stress during pregnancy, rodent models of stress during gestation have been widely used (Coe et al. 2002; Koening et al. 2005; Weinstock 2008). Restrained stress during the last week of gestation is one of the most commonly used models of prenatal stress. One study with a rat model of prenatal restraint stress showed that the adolescent male offspring of the stressed dam showed increased latency to approach a novel object and impaired basal corticosterone levels (Laviola et al. 2004). Moreover, studies with prenatal restrain stress rodent model also indicate that the effects can last into adulthood. Increased anxiety-like behavior, depression-like behavior, and increased corticosterone response have been observed in adult offspring of the stressed dam (Zuena et al. 2008; Morley-fletcher et al. 2003).

Similarly to perinatal period, neonatal and early childhood period is also significant in neuronal development. Stressful situations during early life can also result in emotional, cognitive, and behavioral abnormalities. A consistent body of preclinical studies addressed the effects of stress administration during early postnatal life. Maternal separation has been used by hundreds of studies to investigate the relationship between mother-offspring separation on the development of behavioral deficits as well as neurobiological changes (Pryce and Feldon 2003; Law et al. 2009). The protocols of maternal separation, mainly the duration and time point of the separation, as well as the time point of behavioral evaluation, varies from study to study. However, there is a consensus that early postnatal adversities have a significant effect on neurobiological development, and subsequently affect adult psychopathology (Levine 2005). During the early postnatal period, the neonatal rat is dependent upon the dam for physical and psychological support, including thermoregulation, nutrition, and protection (Dell and Rose. 1987). The licking and grooming of the dam is especially important in the developmental of brain and stress response system. (Anway MD et al. 2005, Jaenisch and Bird. 2003). Maternal separation in animals has been reported to induce depression-like behavior in adulthood (Pruessner et al. 2004; Lyons-Ruth, Bronfman, and Parsons et al. 1999; Moore and Power. 1986), as well as abnormal HPA axis function. Moreover, antidepressant treatments have been tested to be able to reverse the changes induced by maternal separation (Maestripieri. 2005; Maestripieri, Wallen, and Carroll. 1997; Gonzalez et al. 2001). The quality of maternal care is also an crucial factor that affects the early developmental

environment and may have significant effects on later life psychological and physical health. A vast body of evidence using animal models suggests that low quality of maternal care also increases the risk of behavioral deficits such as anxiety-like behavior, depression-like behavior, and cognitive impairments in adulthood (Vetulani et al. 2013). Offspring of low licking-grooming dams resulted in decreased exploratory behavior as well as neuroendocrine changes (Caldji et al. 1998).

Taken together, the animal models mimicking different aspects of childhood adversities are valuable in the investigations of early environmental disturbances on the development of brain and behavior-associated abnormalities.

2.6.2 Early Life Single Prolonged Stress Model

Single prolonged stress (SPS) animal model is widely used as an animal model for posttraumatic stress disorder (PTSD). It was first developed by the Liberzon lab in 1997. SPS involves several consecutive physical and psychological stressors. The SPS model comprises of three different types of stressors: 2 h restraint stress, 20 min forced swim stress and 2-3 min of ether anesthesia, which are expected to induce psychological, physical, and endocrinological stress respectively (Yamamoto, Morinobu et al. 2009). SPS can be useful in modeling early life trauma because those who experience multiple traumas, especial in early life, are more susceptible to developing PTSD-like behavior in later life (Anda et al. 2006; Maercker et al. 2004). SPS provides an intense one-time stressor, this allows the exposure of rats to early life trauma in rats as early as postnatal day 25, and the intensity of stress is enough to produce long-lasting effects. SPS rat model provides an opportunity to investigate how severe traumatic events at a specific time point during early developmental

stage can predispose individuals to the development of behavioral impairments in adulthood (Souza, Noble, and Mclntyre 2017).

2.7 Mechanistic Insights from Current Studies

Early life stress, is considered as an established predictor of health problems across the lifespan, including cardiovascular disease, metabolic dysfunctions, neurodegenerative disease, and mental disorders (Danese et al. 2009; Heim and Nemeroff 2002; Irish et al. 2009). A large body of preclinical studies using different types of animal models have investigated the pathogenesis of these disorders in response to the exposure of early life stress (Alleva and Francia 2009; Gardner et al. 2009; Kolber et al. 2010). Understanding early life stress has tremendous clinical significance as knowing the neurobiological impact of early life stress on an individual has the potential to inform and predict later life health outcomes, and also provides the opportunities for potential intervention before the onset of full-blown abnormalities.

2.7.1 Early Life Stress and Stress Axis

Most cases of depressive episodes are preceded by previous exposure to stressors, either recent or early life adversities (Hines 2017). Psychological stress during early life can induce adaptive physiological responses. These responses can maintain the internal homeostatic state of an individual. When there is an acute disturbance in this balance, pathological changes occur. The hypothalamic-pituitary-adrenal (HPA) axis is one of the major endocrine systems that respond to stress and maintain homeostasis. Stress-induced activation of HPA axis can stimulate the release of corticotropin-releasing factor (CRF) from the paraventricular nucleus of

the hypothalamus, CRF subsequently stimulates the release of adrenocorticotropic hormone (ACTH) from anterior pituitary cells. The increased ACTH stimulates the release of glucocorticoid from the adrenal cortex. Increase in glucocorticoid can regulate the activity of HPA axis by negative feedback regulation through glucocorticoid receptors (GR) or mineralocorticoid receptors (MR). When cortisol remains at a basal level, the negative feedback is mainly regulated by MR. While, when there is a high level of cortisol in response to stress, the negative feedback is regulated by both MR and GR. In depression patients, hypercortisolemia due to impaired HPA negative feedback is often observed and believed to be a potential explanation for some of the features of depression (Gold, Goodwin, and Chrousos. 1988). There is evidence suggesting that early life stress can lead to modifications in the HPA axis. Since the HPA axis also undergoes early development, the changes in the function of HPA axis persist into adulthood and increase the susceptibility of an individual to depression (AR Tarullo and MR Gunnar. 2006). A study in mice showed that maternal separation during early life induced acute stress and elevated HPA–axis responsiveness, which triggered later behavioral life deficits (Daskalakis et al. 2014).

2.7.2 Early Life Stress and Epigenetic and Genetic Factors

In addition to long-lasting changes in the HPA-axis, early life stress in rodents was reported to induce epigenetic modifications in the promoter regions of stress-related genes (Weaver et al. 2004). A lot of preclinical studies have used maternal separation or maternal stress to induce early life stress. These studies have suggested that early life stress leads to long-lasting behavioral effects, some

observed even in the adulthood. These behavioral effects are associated with a range of epigenetic modifications in genes that are important in regulating stress response and brain development, such as Crh, glucocorticoid receptor, Avp, BDNF, and Gad1 (Weaver et al 2004; Elliott et al. 2010; Murgatroyd and Spengler. 2014; Zhang et al. 2010; Roth et al. 2009). In clinical studies patients with a reported history of childhood trauma also indicate that early life stress is associated with epigenetic modifications in stress-related genes and adult psychiatric disorders (McGowan et al. 2009). Studies with postmortem brain sample from suicide victims with childhood abuse history showed that early life stress is associated with decreased hippocampal volume and hypermethylation in the regulatory regions in the brain samples (McGowan et al. 2009).

Interestingly, recent studies have shown that genetic background also plays an important role in mediating the effect of early life stress. The Gene X Environment theory of early life stress supports the idea that there is an interplay between genetic background and early life stress exposure. Some genetic factors can predispose an individual to be more susceptible to epigenetic modifications when exposed to early life stress (Klengel et al. 2013). Current Gene X Environment studies have involved genetic polymorphisms in genes that regulate brain serotonin system (Lesch e al. 1996; Caspi et al. 2003), HPA axis (Bradley et al. 2008), neurotrophins (Kaufman et al. 2006), and other gene candidates such as dopaminergic genes and GABARA2 (Haeffel et al. 2008; Nelson et al. 2009).

2.7.3 Psychological Stress and Oxidative Stress

Previous publications from our lab have suggested that oxidative stress plays a significant role in the development of anxiety-like behavior in rats. Pharmacologically-induced oxidative stress with L-Buthionine-(S, R)-sulfoximine (BSO) treatment increased anxiety-like behavior in rats (Patki et al. 2013). Treatment with the antioxidant tempol reduced anxiety-like behavior caused by multiple anxiogenic drugs in the rat (Patki et al. 2015). Also, in another study, psychological stress induced by chronic social defeat led to elevated oxidative stress as well as behavioral deficits in rats (Solanki et al. 2017; Patki et al. 2014). The relationship between oxidative stress and early life stress is not known and is the focus of one of the aims of this project.

2.7.3.1 What is Oxidative Stress?

Oxidative stress is caused by an imbalance between the levels of reactive oxygen species (ROS) and the capacity of antioxidant systems to scavenge and detoxify ROS (**Figure 5**). The oxygen we breathe in, plays an important role in generating energy in the form of ATP. Mitochondrial electron transport chain (O_2 + $4e^-$ +$4H^+$ $\rightarrow 2H_2O$) drives the production of ATP, which makes that mitochondrial "the powerhouse" that produces ATP from oxygen (Cadenas and Davies. 2000). However, this process is accompanied with side products; the oxygen can directly generate superoxide anion ($O_2^{\cdot-}$), which can subsequently form hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) (**Figure 6**). To fight against the deleterious effects of these free radicals as well as to keep cellular homeostasis, the cells have developed multiple antioxidant mechanisms. Primary biological reactive oxygen

species are listed in **Table 2**. The endogenous antioxidant system is comprised of antioxidant enzymes and endogenous small molecule antioxidants.

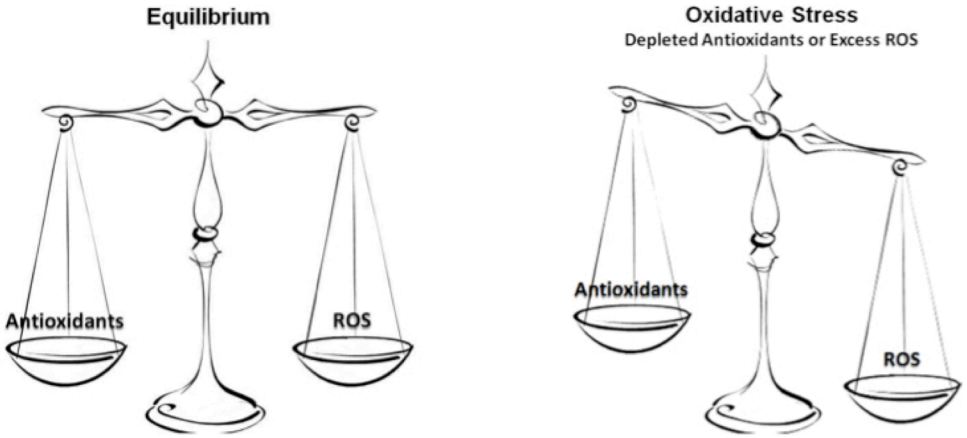


Figure 5. Schematic representation of equilibrium between ROS and antioxidants and oxidative stress conditions (http://www.horsehealthproducts.com/Horsemans_Report/Entry/oxidative_stress)

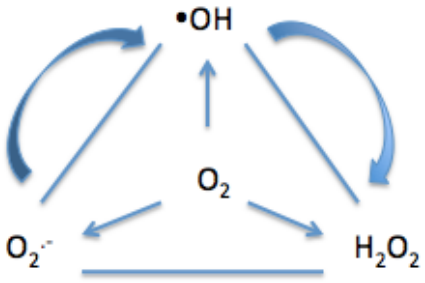


Figure 6. The generation of reactive oxygen species (Modified from Cadenas and Davies. 2000)

Table 2. Primary biological reactive oxygen species

Radicals	Non-radicals
Superoxide	Hydrogen peroxide
Hydroxyl radical	Single oxygen
Hydroperoxyl radical	Lipid peroxide
Lipid peroxyl radical	Hypochlorite acid

2.7.3.2 Cellular Processes in Response to Oxidative Stress

Oxidative stress-mediated injury is one of the primary underlying mechanisms for several diseases involving renal and liver diseases (Small et al. 2012), cardiovascular diseases (Hori and Nishida. 2009), neurodegenerative diseases (Giulia et al. 2017), and respiratory diseases (Cheresh et al. 2013). Once the levels of ROS increase to the extent that the equilibrium between ROS and antioxidants is broken, oxidative stress leads to modifications in cellular macromolecules such as the lipids, protein, and DNA. The results of these processes can be toxic. The lipid and lipoproteins are abundantly present in the cell membranes, making them vulnerable to free radical-mediated lipid peroxidation. This process generates toxic products such as malondialdehyde (Halliwell 2007). Free radical-mediated protein oxidation modifies protein structure and alters their activity, whereas DNA oxidation leads to breakage in DNA strands and increases mutation rates (Pham-Huy et al. 2008). Homologous DNA repair after DNA damage has been reported to change DNA methylation patterns at the repaired sites (Russo et al. 2016). Therefore, Oxidative stress induced by early life stress may induce DNA breakage, the subsequent DNA repair process modifies methylation pattern of the gene. The methylation patterns further govern the expression of certain genes,

which determains the phenotypes of an organism. The modifications mediated by free radicals on these macromolecules are considered as deleterious effects of oxidative stress. However, they can also be considered as biological signals to regulate cellular response and to maintain cellular homeostasis (Adler et al. 1999).

The regulation of cellular responses usually involves activation of transcription factors and their binding to the regulatory regions of target genes. Transcription factors that are in the cytoplasm occurs in an inactive state. Once activated by signals such as phosphorylation, transcription factors translocate into the nucleus and promote transcription of target genes (Adler et al. 1999). Redox-sensitive transcription factors such as nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are gaining interest in oxidative research over the years.

2.7.3.2.1 Oxidative stress mediate the regulation of transcription factor Nrf2

Nrf2 has a significant role in antioxidant defense. It is a transcription factor that binds to antioxidant response elements (ARE) in the promoter region of genes, which regulates the expression of a group of antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, heme oxygenase-1 (OH-1), and non-enzymatic antioxidants. Under normal conditions, Keap1 is constitutively active and interacts with Nrf2, promoting its Ubiquitination by Cullin 3. During oxidative stress, Nrf2/Keap1/Cullin3 interaction is disrupted, so Nrf2 can translocate into the nucleus to bind to the ARE of the promoter regions of genes that encode a large group of antioxidants. If the Keap1/Nrf2 pathway is

disrupted, the antioxidative capacity of the Keap1/Nrf2 pathway will be dampened, leading to increased oxidative stress.

A growing body of animal and in *vitro* publications shows that Nrf2 pathway plays an important role in protecting against the effects of oxidative stress in multiple diseases such as neurodegenerative diseases (Alzheimer's disease and Parkinson's disease) (Yamazaki et al. 2015), airways diseases (McGovern et al. 2016), cardiovascular diseases (Bai Yang et al. 2015), and kidney diseases (Xiao Li et al 2017). Recently, many cancer studies indicate that Nrf2 is epigenetically regulated, in particular, hypermethylated in the promoter region in cancer. The hypermethylation of Nrf2 promoter region leads to a decrease in the production of antioxidant enzymes and an increase in oxidative stress (Kang et al. 2014).

2.7.3.2.2 Oxidative stress mediates the regulation of transcription factor NF-κB

NF-κB is one of the most important transcription factors that respond directly to reactive oxygen species to regulate related inflammatory genes such as tumor necrosis factor (TNF) and interleukins (IL) (Bai Yang et al. 2015; Baldwin 2001). Under oxidative stress conditions, ROS leads to phosphorylation and degradation of IκBα, the negative regulator of NF-κB (P65). The released P65 then can then translocate into the nucleus and regulate pro-inflammatory genes (Haddad 2002). A study in epithelial cells reported that pro-oxidants activate NF-κB pathway (Haddad, Olver, and Land. 2000). Activation of NF-κB pathway contributes to increased expression of proinflammatory cytokines (Schwartz et al. 1996). Furthermore, antioxidant treatments in lipopolysaccharide-endotoxin induced lung

injury were observed to suppress NF-κB pathway and cytokine production (Blackwell et al. 1996). Many Nrf2 induced antioxidants genes also have strong anti-inflammatory functions (Dinkova-Kostova et al. 2005). In addition to these, Nrf2 knockout mice have increased the risk of developing inflammatory lesions (Ma, Battelli, and Hubbs. 2006). All these suggest that there are crosstalks between Nrf2 regulated antioxidative pathway and NF-κB regulated proinflammatory pathway. However, the interaction between Nrf2 and NF-κB remains unclear. Evidence suggests that activation of Nrf2 can lead to suppression of NF-kB, and vice versa (**Figure 7**).

HO-1 is believed to be the key molecule that mediates the interplay between Nrf2 and NF-κB pathways. HO-1 is a target gene of Nrf2. Nrf2 activation leads to expression of HO-1, which can subsequently regulate heme metabolism with following products Fe^{2+}, carbon monoxide and biliverdin, which are then converted into bilirubin. Increase in HO-1 activity inhibits the NF-κB mediated transcription

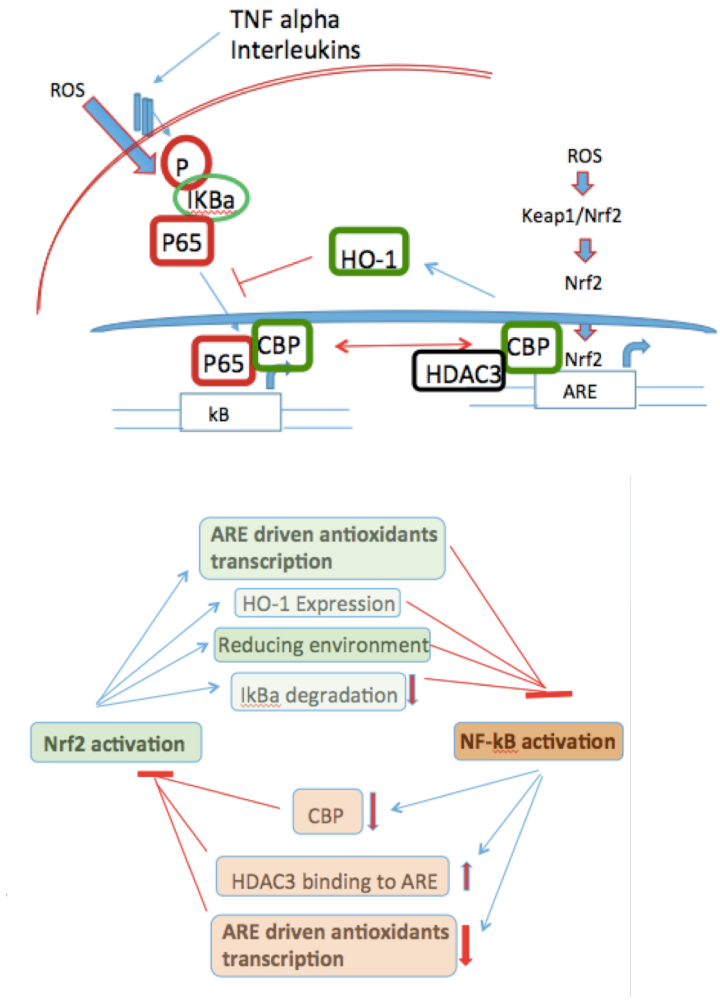


Figure 7. A general schematic showing the interaction between Nrf2 pathway and NF-κB pathway (Modified from Wardyn et al. 2015)

activities, probability through the action of bilirubin (Soares et al. 2004). This may explain the anti-inflammatory role of Nrf-2. However, NF-κB activation also negatively regulates Nrf2 mediated pathway. NF-κB activation increase nucleus level of P65, which competes with Nrf2 for the transcriptional co-activator CBP (CREB-binding protein). CBP has an intrinsic histone acetyltransferase activity that can acetylate both histones as well as non-histone proteins (Liu, QU, and Shen 2008). The lysine residues in both Nrf2 and NF-κB are the targets for CBP mediated acetylation, which enhances gene transcription (Wang et al. 2012). Furthermore, P65 also recruits co-repressor HDAC3 to suppress the transcription activity of Nrf2 and decrease the expression of antioxidant genes (Liu, Qu, and Shen 2008). A summary of molecular cross-talk between Nrf2 and NF-κB is presented in **Figure 7**.

2.7.3.3 Oxidative Stress and Mental Health

The reasons why the central nervous system is especially vulnerable to oxidative stress are listed below:

1. The brain has high-energy demand, so there is high oxygen consumption in the brain, resulting in excessive ROS generation.

2. Neuronal membranes are rich in polyunsaturated fatty acids, which are the preferred target for ROS-induced damage.

3. The neurotransmitter, glutamate, is a primary factor that can cause oxidative stress.

4. The excessive intracellular Ca^{2+} is a contributor to oxidative stress.

5. The antioxidant system in the CNS is not very robust with lower levels of catalase, glutathione peroxidase, and vitamin E.

Oxidative stress has been a known risk factor to numerous disorders. There is clear evidence showing that increased oxidative stress is involved in the development of multiple diseases in different systems. Researches showed that oxidative stress is associated with neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Boskovic et al. 2011; Giulia et al. 2017), renal-cerebrovascular disorders (Matough et al. 2012), metabolic dysfunction related diseases such as diabetes, obesity, and liver diseases (Cichoz-Lach and Michalak 2014; Small et al. 2012), and psychiatric disorders (Sorce S et al. 2010). Since the brain is comparatively more vulnerable to oxidative damage, oxidative stress might be a common underlying mechanism for many mental disorders. Studies to examine the link between oxidative stress and mental disorders by checking oxidative stress markers in patients with psychiatric disorders show a correlation between oxidative stress and psychiatric symptoms. Increased oxidative stress markers such as NO metabolites (Akyol et al. 2002), isoprostanes (Dietrich-muszalska and Olas, 2007), and DNA damage (Nishioka and Arnold, 2004) were observed in patients with schizophrenia. Moreover, decreased antioxidants such as Glutathione (Altuntas et al. 2000), SOD (Ben Othmen et al. 2007), and catalase (Ranjekar et al. 2003) were observed in patients with mental disorders. Clinical trials with antioxidant treatment for schizophrenia showed that antioxidants improve negative symptoms and reverse the decrease in antioxidants (Sivrioglu et al. 2007; Zhang et al. 2001). Preclinical studies also indicate there is a link between oxidative stress and neurobehavioral disorders (Salim, 2014). Several lines of studies in our lab proved this link. Pharmacologically-induced oxidative stress with L-Buthionine-(S, R)-

sulfoximine (BSO) treatment increased anxiety-like behavior in rats (Patki et al. 2013). Treatment with the antioxidant tempol reduced anxiety-like behavior caused by multiple anxiogenic drugs in rat (Patki et al. 2015). Also, in another study, psychological stress induced by chronic social defeat led to elevated oxidative stress as well as behavioral deficits in rats (Solanki et al. 2017; Patki et al. 2014).

3. METHODS AND MATERIALS

3.1 Single Prolonged Stress Model

Single prolonged stress (SPS): The SPS model comprises of three different types of stressors: 2 h restraint stress, 20 min forced swim stress and 2-3 min of ether anesthesia, which are expected to induce psychological, physical, and endocrinological stress respectively (Yamamoto, Morinobu et al. 2009). The rats at PND25 were subjected to a one time combined stress paradigm applied consecutively in one day: two-hour immobilization (compression with plastic Ziploc bag fastened with paper tapes with an opening at the nose of the rat) followed immediately by 20 min of forced swimming stress (in a tall cylindrical tank filled with water 50*20 cm), rest for 15 minutes, and a final 2-3 min exposure to ether anesthesia (with diethyl ether until loss of consciousness). (**Figure. 10**) The animals were then returned to their home cages and left undisturbed for seven days. Control animals were not subjected to any stress except a gentle, brief handling at PND25 and were kept in an undisturbed environment in the same room where SPS protocol and behavior experiments were conducted.

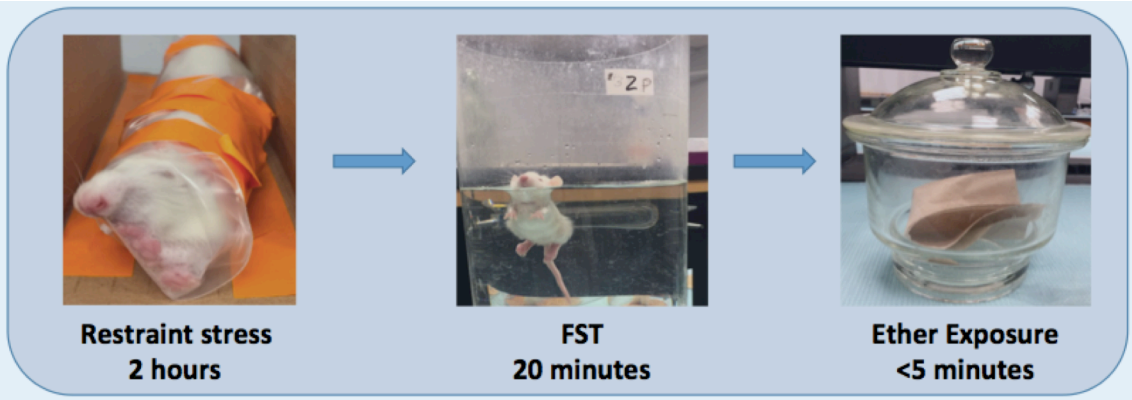


Figure 10. SPS rat model: Restraint-forced swim-anesthesia combined stressor

3.2 Rationale for SPS as a Model of PTSD-like Behaviors

The single prolonged stress (SPS) animal model is considered to be a validated model for PTSD. SPS model contains a battery of physical and psychological stressors. It mimics the pathophysiological abnormalities and behavioral consequences of PTSD as well, such as heightened anxiety-like behavior, depression-like behavior, and negative glucocorticoid feedback (Anda et al. 2006; Maercker et al. 2004). In this project, we have used SPS model to induce early life stress. The rationale for using this model as an early life stressor is the following. First, SPS model comprises of both physical and psychological stress, which mimics childhood traumatic experiences often encountered by children. Second, SPS provides an intense one-time stressor, allowing us to expose the rats to trauma within at an early postnatal day period. Finally, the intensity of stress elicited by SPS is enough to produce long-lasting effects (Solanki et al. 2013).

3.3 Animal and Housing Conditions

Male Sprague-Dawley consolidated litters at postnatal day (PND) 12 were purchased from Charles River Laboratories (200 Charendon Street Boston, MA 02116-5092), USA.

The pups were separated from the female rats at PND21 and split into control and Single Prolonged Stress (SPS) groups. Control group was subjected to control exposures while SPS group was subjected to SPS procedures at PND25 **(Figure. 11)**. Rats were housed with a 12-hour light/dark cycle (lights on at 0600 h) in a climate-controlled room with ad libitum food and water. Experiments were conducted in accordance with the National Institutes of Health (NIH) guidelines, using protocols approved by the University of Houston Animal Care and Use Committee.

3.4 Experimental Design

Two experimental designs were followed. In one, behavior and cognitive tests were conducted at different ages including at PND32, 60 and 90 and rats were sacrificed after the conclusion of the last behavior test at PND90. In the other experimental design, rats were sacrificed after the conclusion of the last behavior test at PND30 and PND90.

1) To study the behavioral and cognitive impact of early life stress across different ages using SPS model.

In this study, the rats were randomly divided into two groups:

Control: Gently handled and put back into their home cages in an undisturbed environment for 2 hours at PND25.

Single Prolonged Stress (SPS): Exposed to SPS at PND25.

Male Sprague-Dawley consolidated litters were acclimatized for one week and separated from mother at PND21. The rats were then subjected to single prolonged stress (SPS: 2 h restraint stress, 20 min forced swim stress and 2-3 min anesthesia) at PND25 as previously published (Patki, Li et al. 2014) with some modifications.

Behavior tests including light and dark (LD), elevated plus maze (EPM) and forced swim test (FST) were conducted using our published protocols (Patki, Salvi et al. 2015). Short-term (STM) and long-term memory (LTM) was examined using radial arm water maze (RAWM) test according to our previously published protocols (Patki, Salvi et al. 2015). The same set of behavior tests were carried out at PND32, 60, and 90. The behavioral performances of each rat at PND32, 60, and 90 were tracked and analyzed. General body parameters such as body weight, food and water intake of the rats were monitored throughout the study. Rats were sacrificed

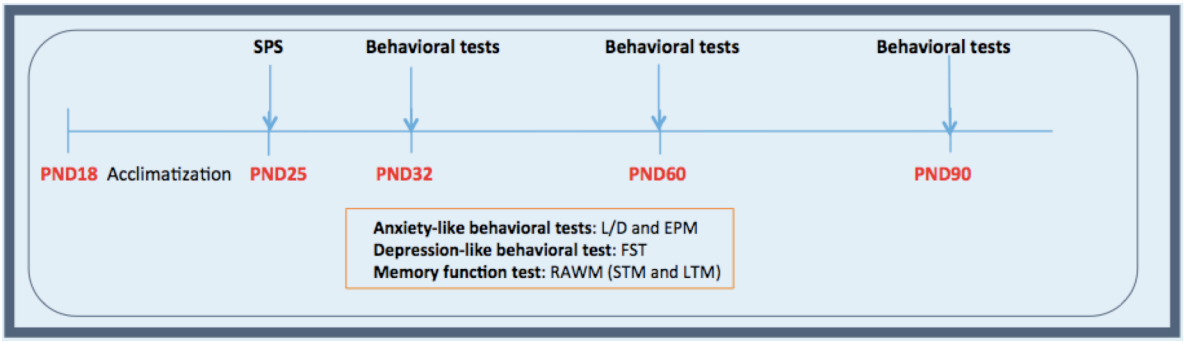


Figure 11. Schematic representation of the experimental regimen for specific aim 1

after the conclusion of all behavior tests at PND90. The schematic experimental design for the study is provided in **Figure 11**.

2) To study the effects of early life stress on oxidative stress mechanisms at different ages.

The same experimental design as depicted above was followed. Rats were randomly divided into three groups:

Control: Gently handled and put back into their home cages in an undisturbed environment for 2 hours at PND25, half of the control group were sacrificed at PND32, the other half of control group were sacrificed at PND90.

SPS32: Exposed to SPS at PND25 and were sacrificed at PND32.

SPS90: Exposed to SPS at PND25 and were sacrificed at PND90.

Male Sprague-Dawley consolidated litters were acclimatized for one week and separated from mother at PND21. The rats were then subjected to single prolonged stress (SPS: 2 h restraint stress, 20 min forced swim stress and 2-3 min anesthesia) at PND25 as previously published (Patki, Li et al. 2014) with some modifications. Behavior tests including light and dark (LD), elevated plus maze (EPM) and forced swim test (FST) were conducted using our published protocols (Patki, Salvi et al. 2015). Short-term (STM) and long-term memory (LTM) was examined using radial arm water maze (RAWM) test according to our previously published protocols (Patki, Salvi et al. 2015). The same set of behavioral tests were conducted before sacrifice for control, SPS32, and SPS90. General body parameters such as body weight, food and water intake of the rats were monitored throughout the study. The schematic experimental design for the study is provide in **Figure 12**.

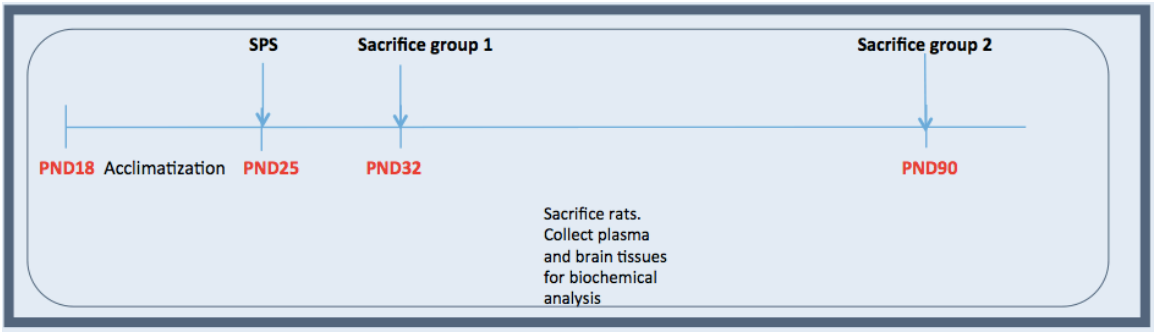


Figure 12. Schematic representation of the experimental regimen for specific aim 2

3.5 Behavior and Cognitive Assessments

All behavior tests were conducted and analyzed by the same person between 9 a.m. and 4 p.m. The experimenter was blinded to treatment. Anxiety-like behavior tests were conducted in the order such that the least stressful test is conducted first, followed by the most stressful one. There was a gap of 24h between each test. In our experience, twenty four hour rest period has proven to be sufficient to remove the memory of previous stress. The order of the tests was the following; (1) Light-dark test —24h rest —(2) elevated-plus maze —24h rest —(3) short-term memory test —24h rest —(4) long-term memory test —24h rest —(5) forced swim test.
We have published that if this order of anxiety behavior tests is maintained allowing a gap of twenty-four hours between each test, the behavioral performance of rats is not affected (Liu et al. 2017, Kochi et al. 2017, Solanki et al. 2017).

3.5.1 Measurement of Anxiety-like Behavior

Two different anxiety-like behavior tests were carried out. First, light-dark (LD) test was conducted followed by elevated-plus maze (EPM) test, as previously published by us (Patki, Salvi et al. 2015).

3.5.1.1 Light-Dark (LD) test: The less time the rat spends in light is considered as an indication of anxiety-like behavior (Patki, Salvi et al. 2015). The LD box consists of two compartments, one light compartment (27 x 27 x 27 cm), and one dark compartment (27 x 18 x 27 cm) (**Figure 13**). The barrier between the two compartments has a single opening (7 x 7 cm) for the rat to explore each compartment freely. Each session of LD test lasted 5 min, started by placing the rat

into the light compartment. Total time spent in each compartment was recorded. The LD test was performed two days after the SPS protocol.

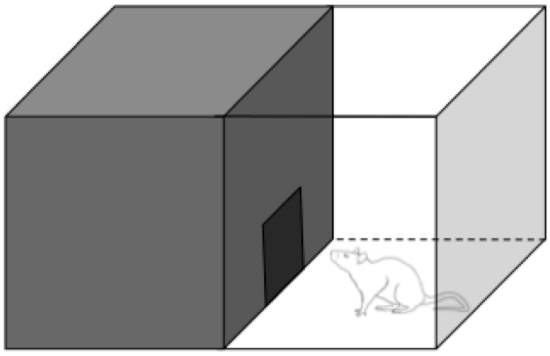


Figure 13. The Light-Dark test apparatus

3.5.1.2 Elevated Plus Maze (EPM) test: The less time the rat spends in the open arms of the EPM apparatus is considered as an indication of anxiety-like behavior (Patki, Salvi et al. 2015). The EPM consisted of four 43 cm long arms extending from a 10 cm^2 central area, with two arms open and two arms closed, placed 90 cm above the floor (Med Associates Inc.) (**Figure14**). The rat was placed in the center area facing the open arms of the maze and allowed to explore each arm freely for 5 min. Total time spent in both open and closed arms was recorded manually by an experimenter blinded to treatment. The EPM test was performed three days after SPS protocol.

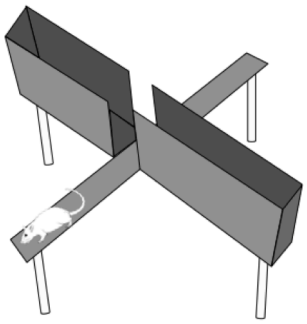


Figure 14. The Elevated Plus Maze Apparatus

3.5.2 Measurement of Depression-like Behavior

3.5.2.1 Forced Swim test (FST): More time spent by the rat in an immobile position is considered as an indication of depression-like behavior (Patki, Salvi et al. 2015). The apparatus of FST comprised of a water tank measuring 24 cm in diameter and 50 cm in height. Rats were individually placed in a water tank for 5 min at 25 ℃. In response to the threat of drowning, the rat keeps swimming. When the rat stops struggling and floats motionlessly, it is a sign of "giving up," which can be interpreted as despair-like behavior (**Figure 15**). The total time of immobility was recorded. The FST was performed four days after the SPS protocol. An important point to be mentioned is that FST was utilized not to induce depression-like behavior in our animals but to evaluate depression-like behavior. Therefore, the pre-test phase was omitted. In our case, the exposure to SPS already introduced stress in the rats. Approach similar to what was used in this project has been used before by others in rats (Detke MJ et al. 1995; Hédou, G., et al., *2001)*

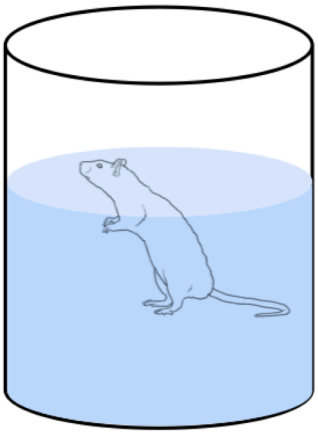


Figure 15. The Forced Swim test apparatus (www. Scielo.br)

3.5.3 Measurement of Short and Long Term Memory Impairments

3.5.3.1 Radial Arm Water Maze (RAWM) test: RAWM test comprised of a circular black water pool that had six swimming arms and an open central area. The test was performed in a dimly lit room. Each rat was randomly assigned a goal arm with a hidden platform at the end of the arm that was submerged 1 cm under water. The test began with 12 practice trials in which the rat was dropped from one of the arms (except the goal arm) and was given 1 min to swim through the pool and find the hidden platform. The room had visual cues on the walls to help the rats in locating the platform. If the rat could not find the platform in 1 min, they were manually guided to the platform. Once on the platform, the rat was given a rest period of 15 sec before the next learning trial began. The purpose of the practice trials was to help the rat learn and memorize the location of the platform. 30 min following practice trials, short-term memory (STM) test was conducted wherein the rat was again dropped from a randomly selected arm and given 1 min to find the hidden platform. Cognitive function of the rat was quantified based on a number of

errors the rat made in locating the platform. Every time the rat entered halfway into any arm, or entered the goal arm but failed to reach the platform, was counted as an error. If the rat failed to find the platform in 1 min, it was given a score of 7. The same procedure was repeated 24 hours after the STM test and was denoted as long-term memory (LTM) test. The timeline for all learning trials, STM and LTM tests is depicted in the schematic representation in **Figure 16**.

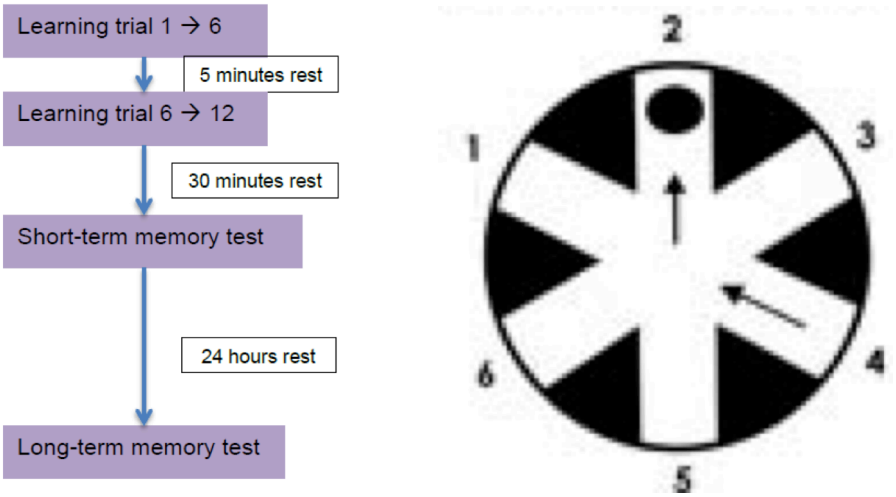


Figure 16. The Radial Arm Water Maze test. Schematic representation of the timeline for practice and test trials and pictorial representation of the RAWM pool (Patki et al. 2013).

3.6 Brain Dissections and Collection of Plasma

24 hours after termination of behavioral and cognitive function tests, rats were anesthetized using isoflurane (cat. # 57319-479-06, Phoenix Pharmaceuticals). Blood was collected from the left ventricle of the heart following which plasma was separated by centrifugation and stored at -80° C. The rats were quickly decapitated, and brains were removed. Three brain regions, namely, pre-frontal cortex (PFC), hippocampus and amygdala were dissected out according to Paxinos and Watson

(Watson 1997). The regions were immediately flash frozen in liquid nitrogen and later stored at -80 C.

3.7 Tissue Homogenization and Protein Estimation

The brain tissues were homogenized using lysis buffer containing 20 mM Tris-HCl, four mM ethylenediaminetetraacetic acid (EDTA), protease inhibitors, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), one µg/ml leupeptin, one µg/ml aprotinin and one µg/ml pepstatin (Salim and Dessauer 2004). The protein concentration of the lysates was estimated using a micro BCA assay kit from Pierce (Pierce, Rockford, IL).

3.8 Statistical Analysis

3.8.1 Statistical Test between Groups

All values are reported as mean ± SEM. Comparison between groups was made either by student's t-test (behavioral and biochemical analysis) or one-way ANOVA (mitochondrial oxygen consumption) with subsequent Tukey's posthoc test where appropriate (GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was used to denote statistically significant groups.

3.8.2 K-Mean Clustering Analysis

The forced swim test (immobility time) data was used to identify two clusters using K-means cluster analysis (SPSS Statistics version 23, IBM, Armonk, North Castle, NY). ANOVA test followed by Tukey's multiple comparison tests were then performed on the control group and the two clusters using GraphPad Prism (GraphPad Software, La, Jolla, CA). The clusters were considered significantly different when $p<0.05$.

3.9 Indices of Systemic and Neuronal Oxidative Stress

3.9.1 Measurement of 8-Isoprostane Levels

The level of 8-isoprostane in plasma was measured using ELISA kit (cat # 516351, Cayman Chemical, MI). Oxidative stress and subsequent increase in free radicals lead to oxidation of tissue phospholipids, generating isoprostanes. Therefore, 8-isoprostane is considered to be a marker of oxidative stress.

3.9.2 Measurement of Protein Carbonylation Levels

The level of protein carbonylation was measured by detecting the addition of carbonyl groups to the proteins by oxidation reactions. Oxygen free radicals under oxidative stress can lead to oxidative modification of proteins. As a consequence of the modification, carbonyl groups are added to the protein side chains. 2,4-dinitrophenylhydrazine (DNPH) can transfer the carbonyl groups into 2,4-dinitrophenylhydrazone (DNP-hydrazone). The DNP-derivatized protein samples can be dot blotted onto a membrane filter. The membranes then were incubated with a horseradish peroxidase-antibody conjugate directed against the primary antibody (secondary antibody: goat anti-rabbit IgG). The intensity of the blotted dot was determined using Alpha Ease FC 4.0 (Alpha Innotech Corp., San Leandro, CA).

3.9.3 Protein Expression of Antioxidant Enzymes-Western Blotting

Western blotting was used to measure protein expression of antioxidant enzymes, including Cu/Zn SOD, Mn SOD, Catalase, and Heme oxygenase1 (HO-1) in the brain region homogenates. Samples for western blotting were prepared by diluting the respective brain region homogenates in 2x Laemmli buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS and 0.1 mg/ml bromophenol blue). Samples

(approximately 30 µg of total protein per sample) were resolved on standard 10-well 8-16% SDS-PAGE gels. The proteins were transferred to PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) and then detected as immunoreactive bands using specific primary antibodies and horseradish peroxidase-conjugated secondary antibody. Beta-actin was used as a loading control. Antibodies dilutions used have been listed in **Table 3**.

Chemiluminescence reagent (Cat# 1705060, Biorad, CA) was used in the development of the protein blot. Chemiluminescence was detected using Alpha Innotech imaging system (Alpha Innotech, San Leandro, CA, USA) and densitometric analysis was performed using Fluorochem FC8800 software.

Table 3. Antibodies, dilutions, and sources. Details of primary and secondary antibodies used for detecting the levels of specific proteins

	Primary Antibody	Secondary Antibody
Cu/Zn SOD	Ab13498 (Abcam, MA) 1:1000	7074S (Cell Signalling, MA) 1:2000, goat anti-rabbit
Mn SOD	Ab13533 (Abcam, MA) 1:1000	7074S (Cell Signalling, MA) 1:2000, goat anti-rabbit
Catalase	Ab16731 (Abcam, MA) 1:2000	7074S (Cell Signalling, MA) 1:2000, goat anti-rabbit
H0-1	Ab13248 (Abcam, MA) 1:1000	7076 (Cell Signaling, MA) 1:2000, anti-mouse

3.10 Measurement of Nrf-2, Keap-1, NF- κB, IL-6, and TNF-α Protein Expression

Protein expression of Nrf-2, Keap-1, NF- κB, IL-6, TNF-α and BDNF was measured in brain region homogenates using western blotting as described in section 3.9.3. using a 1:1000 dilution of primary antibodies.

3.11 Measurement of DNA Methylation Level in Promoter Region of Nrf2

500ng of genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. The samples were eluted in 40ul of M-Elution Buffer, and 2ul (equivalent to 25ng of bisulfite-modified DNA) were used for each PCR reaction. Both bisulfite conversion and subsequent pyrosequencing analysis were done at the DNA Methylation Analysis Core, The University of Texas M.D. Anderson Cancer Center.

PCR primers for the genomic area proximal to the transcription start site (TSS) of Nfe2l2 gene, interrogating four CpG sites between -818-bp -843-bp from TSS (assay 1) and -six CpG sites between +30-bp to +58-bp from TSS (assay 2), were designed using the Pyromark® Assay Design SW 2.0 software (Qiagen, Hilden, Germany). In brief, a sequencing primer is identified within 1 to 5 base pairs near the CpG sites of interest, with an annealing temperature of 40± 50^{o}C. Next, forward and reverse primers are identified upstream and downstream to the sequencing primer, with a target annealing temperature ranging from 50^{o}C to 60^{o}C and amplicon product size ranging from 100bp to 200bp. Optimal annealing temperature was tested using gradient PCR. Controls for high methylation (SssI-

treated DNA), low methylation (WGA-amplified DNA), and no-DNA template were included in the PCR reaction.

Duplicated PCR reactions were performed in a total volume of 20 μl, and the entire volume was used for each pyrosequencing reaction, as previously described (Estecio et al., 2007). Briefly, PCR product purification was done with streptavidin-sepharose high-performance beads (GE Healthcare Life Sciences, Piscataway, NJ), and co-denaturation of the biotinylated PCR products and sequencing primer (3.6 pmol/reaction) was conducted following the PSQ96 sample preparation guide. Sequencing was performed on a PyroMark Q96 ID instrument with the PyroMark Gold Q96 Reagents (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The degree of methylation for each CpG site was calculated using the PyroMark Q96 software (Biotage AB, Uppsala, Sweden). The average methylation of all sites and duplicates was reported for each sample.

4 RESULTS

4.1 General Body Parameters

Stress is well known to affect body weight, food intake, and water intake in preclinical studies (Jeong, Lee, and Kang 2013). Clinical studies also show that chronic stress is associated with multiple metabolism disorders, including obesity, and diabetes(Dnyanraj Choudhary et al. 2016). Therefore, the body weight, food intake, and water intake were measured throughout the developmental period to examine if early life SPS exposure leads to changes in these parameters. It was observed that body weight gain, food intake, and water intake remained unchanged

after SPS exposure (**Figure. 17 A**, **B**, and **C**). These results suggests that early life SPS exposure does not affect general body parameters.

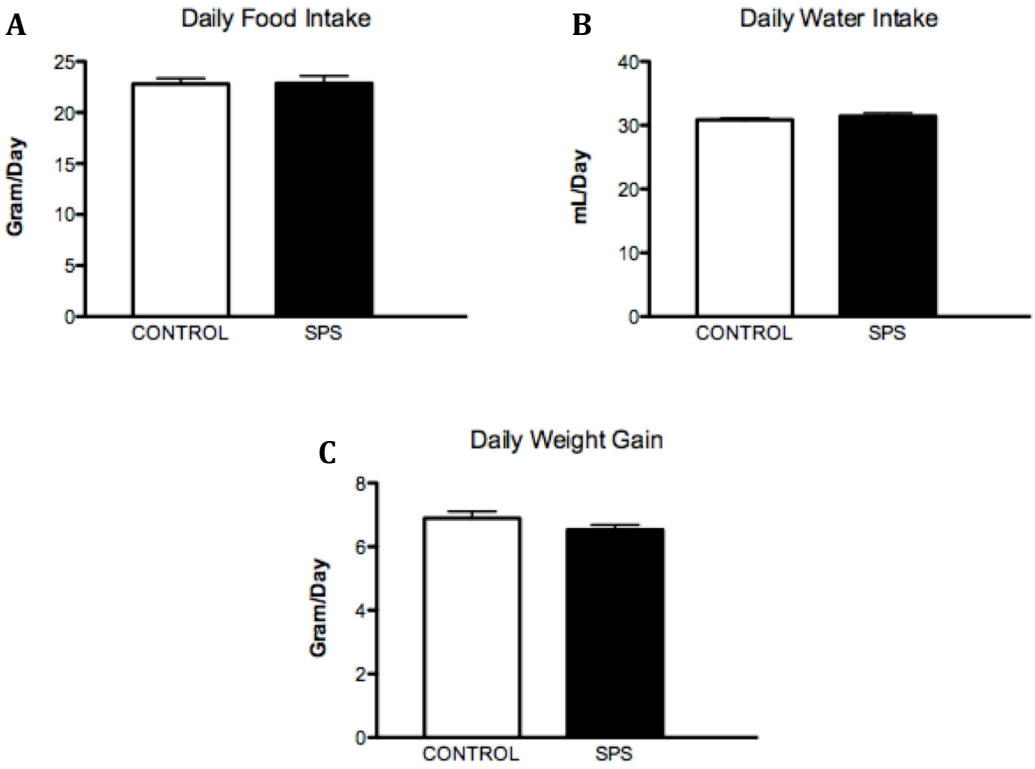


Figure 17. Body weight, food and water intake measurements following early life SPS exposure at PND25, CONTROL: n=12, SPS: n=30.

4.2 Behavioral and Cognitive Effects of Early Life Stress

4.2.1 Examination of the Behavioral and Cognitive Effect of Early Life SPS Exposure at PND32:

First, behavior and cognitive analysis were conducted seven days after the conclusion of SPS procedure at PND32 (rodent age mimicking human adolescent period). In the LD test, the SPS rats spent significantly less time in the light compartment as compared to control rats (CON: 42.60 ± 6.437 seconds, SPS: 24.27 ± 3.859 seconds; $p<0.05$), indicative of increased anxiety-like behavior **(Figure. 18A)**. Early life SPS rats also spent significantly reduced time in the open arms in the EPM test as compared to control rats (CON: 25.00 ± 4.098, SPS: 15.91 ± 2.263; $p<0.05$), indicating increased anxiety-like behavior **(Figure. 18B)**. In the FST, early life SPS caused a significant increase in the time of immobility in SPS rats compared to their matched controls during a 5-min session test (CON: 7.240 ± 2.510, SPS: 39.24 ± 6.528; $p<0.05$), indicative of depression-like behavior **(Figure. 18C)**. The SPS rats made more errors when compared to their matched controls in the STM test (CON: 1.043 ± 0.4145, SPS: 1.527 ± 0.3102; $p<0.05$), **(Figure. 18D)**. In the LTM test (24 h after the STM test), there was no significant difference between SPS rats and their matched controls **(Figure. 18E)**. In summary, after SPS procedure conducted at PND25, SPS rats exhibited heightened anxiety- and depression-like behavior, and short-term memory impairment at PND32 **(Figure. 18A-D)**

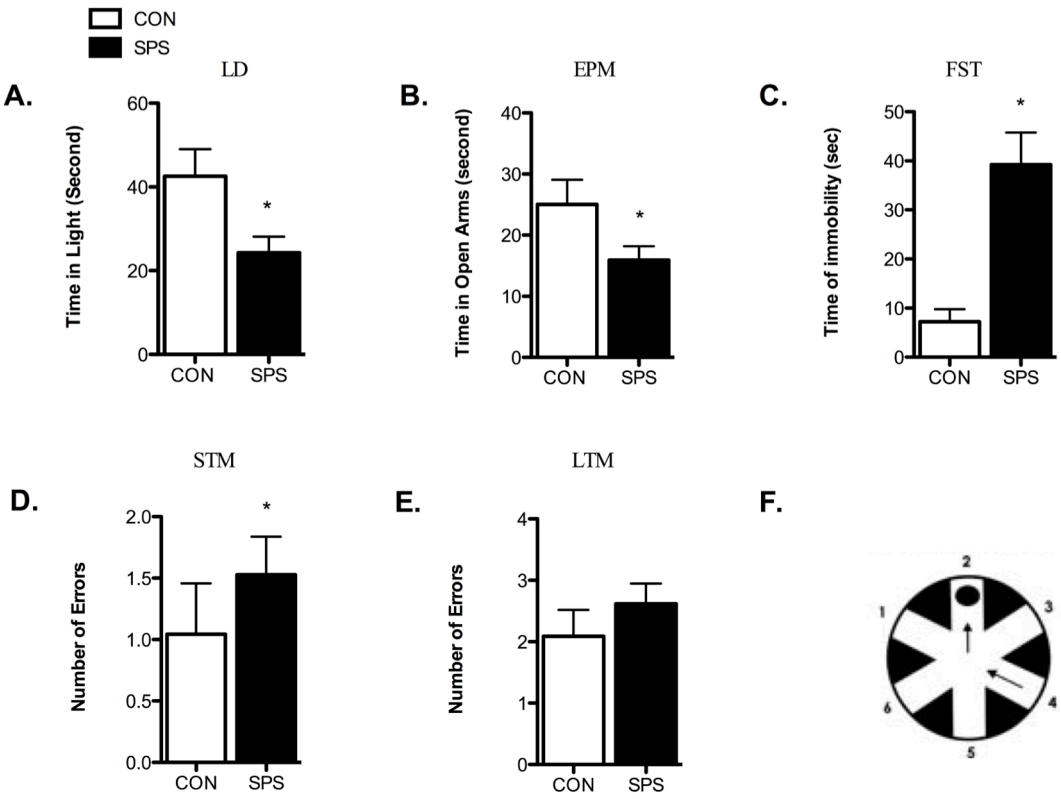


Figure 18. ***Examination of anxiety- and depression-like behavior and short and long term memory function tests conducted at PND32****.* Male Sprague-Dawley rats were subjected to control or SPS exposures at PND25. One week later, behavior and cognitive tests were conducted at PND32. Total time spent in the light compartment in light-dark (LD) test **(A)** and in the open arms in the elevated plus maze (EPM) test **(B)** measured anxiety-like behavior respectively. Total immobility time in the Forced swim test (FST) was used to examine depression-like behavior **(C)**. Number of errors made in the short-term memory (STM) test **(D)** and long-term memory (LTM) test **(E)** examined learning and memory function in radial arm water maze apparatus (RAWM) comprising of six swim paths **(F)**. Group designations: Control exposures (CON: open bars, n=25 rats); early life single prolonged stress (SPS: black bars, n=55 rats). (*) indicates significantly different from control at $p<0.05$. Bars represent means ±SEM.

4.2.2 Examination of the Behavioral and Cognitive Effect of Early Life SPS Exposure at PND60:

After the conclusion of the SPS procedure at PND25, behavioral tests were carried out at PND60 to assess if the effects of SPS lasted until PND60. In the LD test, SPS rats continued to exhibit significant anxiety-like behavior when compared to respective controls (CON: 65.88 ± 12.22, SPS: 26.44 ± 3.068; $p<0.05$) **(Figure. 19A)**. In the EPM test, SPS rats also exhibited anxiety-like behavior compared to their matching controls, indicated by significantly less time spent in the open arms (CON: 64.96 ± 9.415, SPS: 34.29 ± 3.895; $p<0.05$) **(Figure. 19B)**. However, in the FST test, SPS rats spent equal time staying immobile when compared to their matched controls (CON: 20.56 ± 5.161, SPS: 24.76 ± 2.36; $p<0.05$) **(Figure. 19C)**, indicative of no depression-like behavior at PND60. In learning-memory function tests, the SPS rats showed impairments in STM but not LTM, similar to the results obtained at PND32. SPS rats made more errors in STM when compared to their matched controls (CON: 0.1200 ± 0.06633, SPS: 0.6852 ± 0.1710; $p<0.05$) **(Figure. 19D)**, but they made a comparable number of errors compared to controls in LTM test **(Figure. 19E)**. In summary, following SPS procedure at PND25, anxiety-like behavior and short-term memory impairment persisted until PND60 **(Figure. 19A, B, D, and E)**. However, depression-like behavior was not evident at PND60 **(Figure. 19C)**.

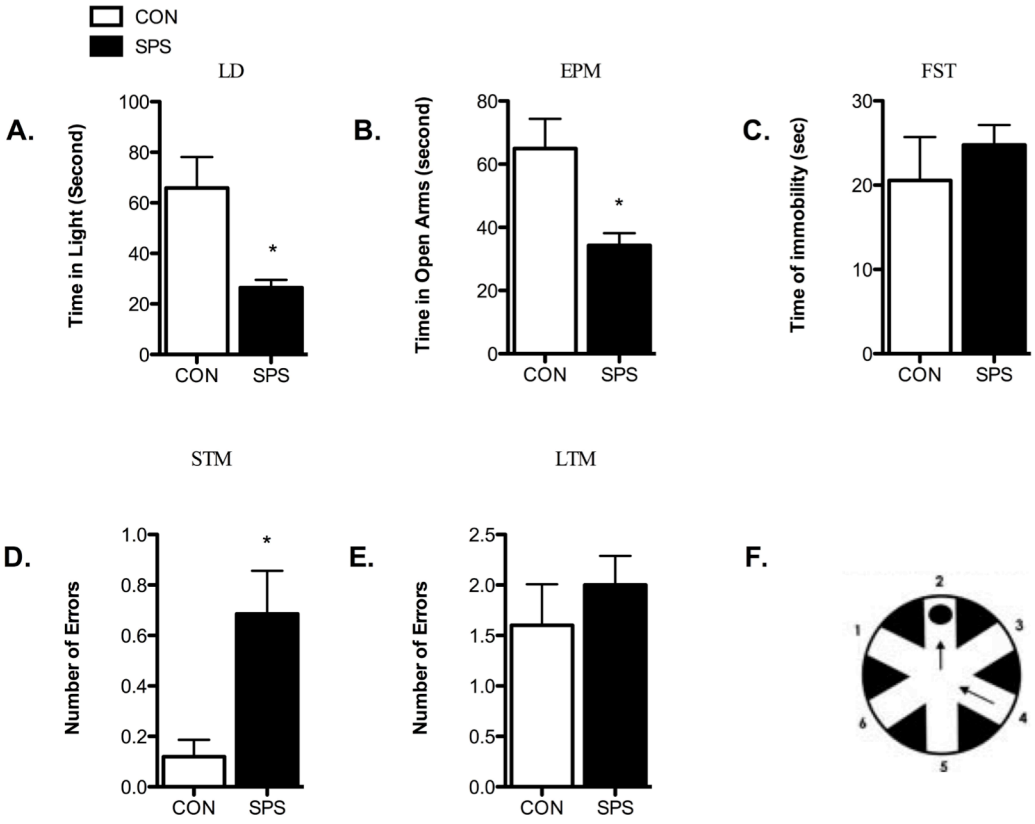


Figure 19. ***Examination of anxiety- and depression-like behavior and short and long term memory function tests conducted at PND60.*** Male Sprague-Dawley rats were subjected to control or SPS exposures at PND25. Five weeks later, behavior and cognitive tests were conducted at PND60. Total time spent in the light compartment in light-dark (LD) test **(A)** and in the open arms in the elevated plus maze (EPM) test **(B)** measured anxiety-like behavior. Total immobility time in the Forced swim test (FST) was used to examine depression-like behavior **(C)**. Number of errors made in the short-term memory (STM) test **(D)** and long-term memory (LTM) test **(E)** examined learning and memory function in the radial arm water maze apparatus (RAWM) comprising of six swim paths **(F)**. Group designations: Control exposures (CON: open bars, n=25 rats); early life single prolonged stress (SPS: black bars, n=55 rats). (*) indicates significantly different from control at $p<0.05$. Bars represent means ±SEM.

4.2.3 Examination of the Behavioral and Cognitive Effect of Early Life SPS Exposure at PND90:

At PND90, i.e., 68 days after first exposure to SPS, the same set of behavior tests were carried out on the rats to assess whether behavioral impairments lasted into this stage. In LD and EPM test, heightened anxiety-like behavior that we observed at PND32 and 60 were not observed at this age. At PND90, SPS rats spent comparable time in the illuminated area in LD test compared to their age-matched controls (CON: 55.44 ± 7.820, SPS: 44.98 ± 4.619; p=0.2297) **(Figure. 20A)**. The time SPS rats spent in the open arms was significantly different from that of the control rats (CON: 42.28 ± 7.524, SPS: 24.95 ± 3.203; $p<0.05$) **(Figure. 20B)**. Interestingly, in the FST, SPS rats exhibited significantly increased immobility time during the 5-min test when compared to control rats (CON: 22.80 ± 3.126, SPS: 43.82 ± 3.762; $p<0.05$) **(Figure. 20C)**. Short-term memory impairment observed at PND32, and 60 did not last at PND90. SPS rats made comparable numbers of errors in both STM (CON: 0.8182 ± 0.2244, SPS: 0.7818 ± 0.1735; p=0.9067) **(Figure. 20D)** and LTM (CON: 1.909 ± 0.4648, SPS: 2.073 ± 0.3112; p=0.7761) **(Figure. 20E)** tests when compared to their matched controls. In summary, following SPS procedure at PND25, while anxiety-like behavior and short-term memory impairment did not persist until PND90 **(Figure. 20A, B, and D)**, depression-like behavior was observed at PND90 **(Figure. 20C)**.

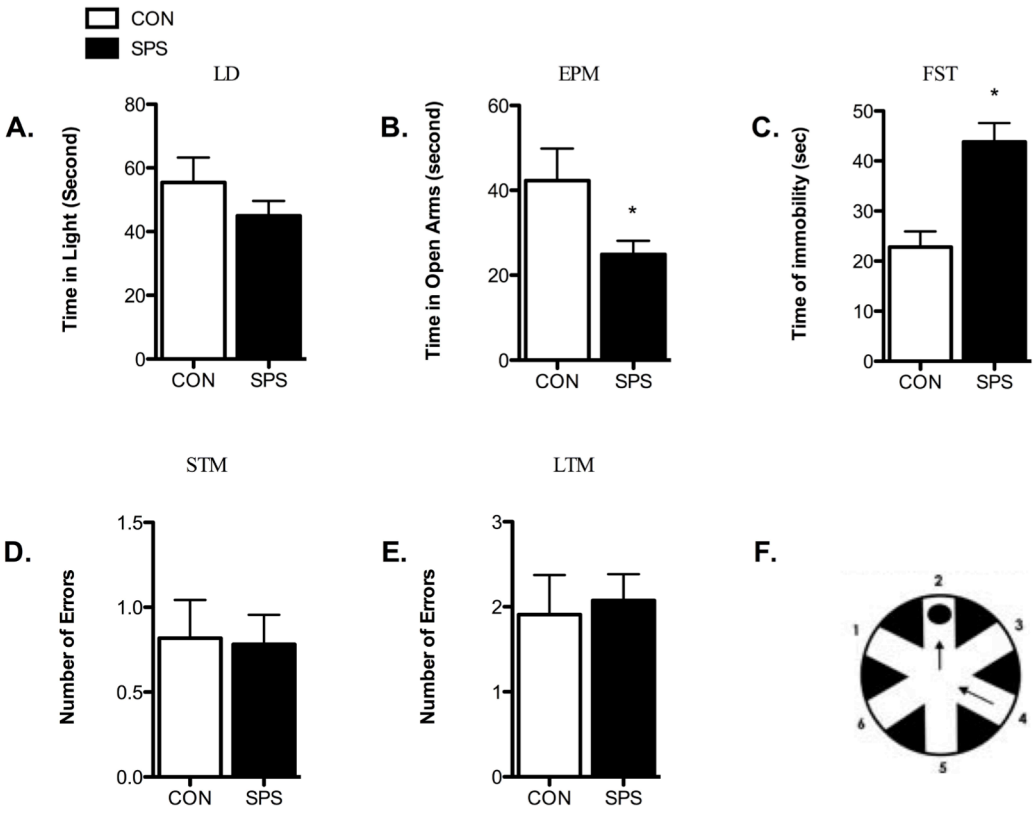


Figure 20. ***Examination of anxiety- and depression-like behavior and short and long term memory function tests conducted at PND90.*** Male Sprague-Dawley rats were subjected to control or SPS exposures at PND25. Nine weeks later, behavior and cognitive tests were conducted at PND90. Total time spent in the light compartment in light-dark (LD) test **(A)** and in the open arms in the elevated plus maze (EPM) test **(B)** measured anxiety-like behavior. Total immobility time in the Forced swim test (FST) was used to examine depression-like behavior **(C)**. Number of errors made in the short-term memory (STM) test **(D)** and long-term memory (LTM) test **(E)** examined learning and memory function in radial arm water maze apparatus (RAWM) comprising of six swim paths **(F)**. Group designations: Control exposures (CON: open bars, n=25 rats); early life single prolonged stress (SPS: black bars, n=55 rats). (*) indicates significantly different from control at $p<0.05$. Bars represent means ±SEM

4.2.4 Clustering Analysis of PND90 FST Data:

We further examined PND90 FST data using K-mean cluster analysis utilizing IBM SPSS (IBM, Armonk, NY) to identify subgroups in SPS rats. Based on the time of immobility in FST, the SPS rats appeared to occur in two clusters. From a total of 55 SPS rats, 28 rats exhibited less immobility time and belonged to the same cluster as controls. We considered these rats to be the "*resilient type*." The other 27 rats belonged to the second cluster and exhibited longer immobility time in the FST. These rats were considered as the "*susceptible type*," **(Figure. 21 panel I)**. We then performed ANOVA test on the control group and the two clusters (resilient and susceptible), followed by Tukey's multiple comparison tests using GraphPad Prism (GraphPad Software, La, Jolla, CA). ANOVA test indicated the two clusters were significantly different ($p<0.05$). Tukey's multiple comparison tests indicated that the 27 rats in the susceptible group had a significantly longer time of immobility when compared to their matched controls, or to the 28 rats in the resilient group (**Figure. 21 panel II**). However, the 28 rats within the resilient group were not significantly different from their matched controls (**Figure. 21**).

I

SPS PND 90 Immobility Time in FST (seconds)		
Control	**Resilient**	**Susceptible ***
13	29	42
13	33	42
20	25	47
13	22	42
12	35	90
25	25	52
23	27	64
12	20	40
15	25	96
3	11	46
9	37	119
59	9	126
4	6	50
20	6	53
36	8	46
18	15	42
18	15	100
52	35	55
30	33	58
27	15	56
60	27	78
89	30	56
27	16	78
49	18	64
29	23	40
	23	48
	20	80
	34	

II

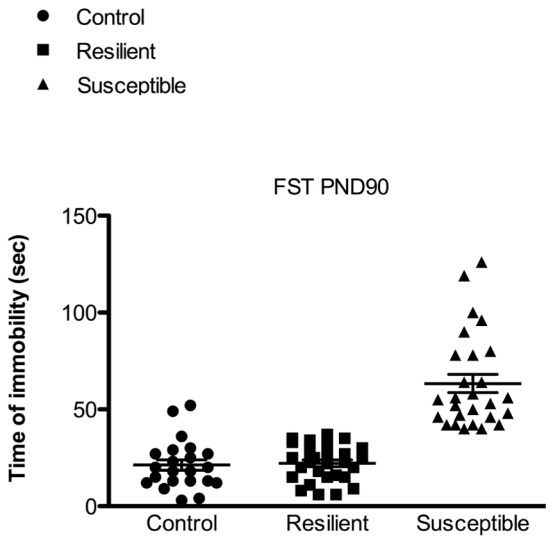


Figure 21. ***Cluster analysis of depression-like behavior at PND90***. PND90 FST data was examined using K-mean cluster analysis with IBM SPSS (IBM, Armonk, NY) **(panel I)**. Two clusters were identified in cluster test at PND90 as a function of immobility time in FST **(panel II)**. Control group n=25; Resilient group n=28; Susceptible group n=27. (*) indicates significantly different from control as well as resilient groups at $p<0.05$.

4.2.5 Correlation Between Emergence of Behavioral Phenotype and Different Stages of Development:

After identification of susceptible (with depression-like behavior) and resilient (without depression-like behavior) phenotypes of PND90 rats in FST test, we back-tracked behavior performance of these rats at PND32 and PND60 period. Both depression- and anxiety-like behaviors were assessed within each group at different stages of development *i.e.* susceptible rats at PND32, 60 and 90 and resilient groups at PND32, 60 and 90.

PND90 susceptible rats (depressive phenotype) with significantly longer FST immobility time than control or resilient rats (Control: 42.60 ± 6.437, Resilient: 21.83 ± 4.682, Susceptible: 30.05 ± 6.991) **(Figure. 22 panel IC)**, did not exhibit greater FST immobility at PND32 (Control: 7.240 ± 2.510, Susceptible: 14.00 ± 1.984; p=0.0820) **(Figure. 22 panel IA)**, or at PND60 (Control: 20.56 ± 5.161, Susceptible: 29.42 ± 2.879; p=0.1762) **(Figure. 22 panel IB)**. On the other hand, PND90 resilient rats (no depressive phenotype) with no demonstration of higher FST immobility time (Control: 22.80 ± 3.126, Susceptible: 27.52 ± 2.084; p=0.1988) **(Figure. 22 panel IC)**, exhibited significant immobility time at PND32 (Control: 7.240 ± 2.510, Resilient: 37.31 ± 6.24 8; p<0.05) **(Figure. 22 panel IA)**, but not at PND60 (Control: 20.56 ± 5.161, Resilient: 22.31 ± 3.233; p=0.7640) **(Figure. 22 panel IB)**.

PND90 susceptible rats (no anxiety phenotype) spent comparable time in light at PND32 when compared to controls (Control: 42.60 ± 6.437, Susceptible: 30.05 ± 6.991; p=0.1972) **(Figure. 22 panel IID)** and significantly less time in the

light compartment at PND60 (Control: 65.88 ± 12.22, Susceptible: 32.42 ± 4.732; $p<0.05$) **(Figure. 22 panel IIE)**. On the other hand, PND90 resilient rats (no anxiety-like behavior) spent less time in light compartment at both PND32 (Control: 42.60 ± 6.437, Resilient: 21.83 ± 4.682; $p<0.05$) and 60 (Control: 65.88 ± 12.22, Resilient: 23.28 ± 3.910; $p<0.05$) **(Figure. 22 panel IID, F)**. Upon mapping the presence/absence of behavioral impairments over time in susceptible and resilient rats, a period of behavioral switching emerged at PND60 (**Figure. 22 panel IIIG**).

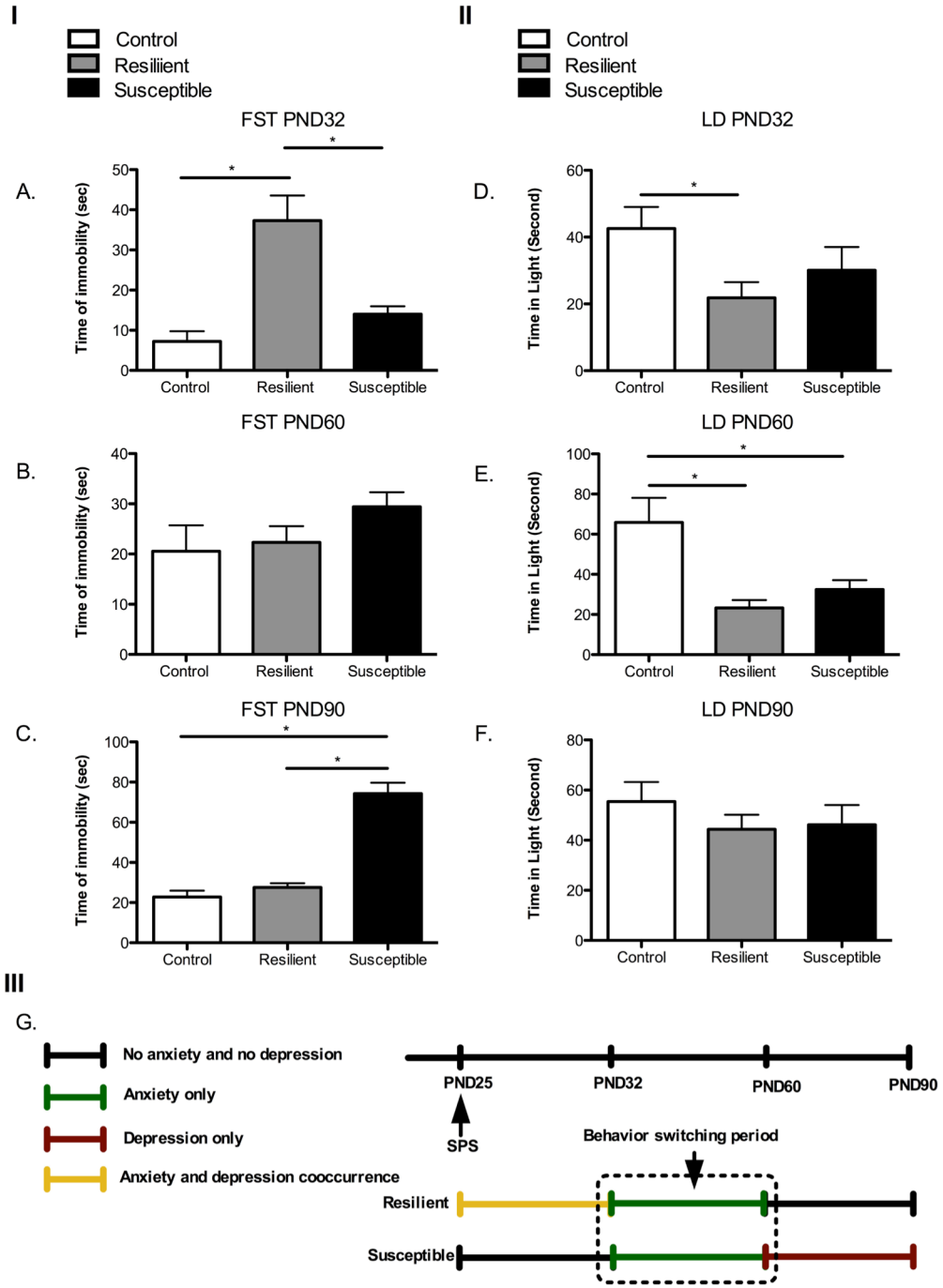


Figure 22. ***Retrospective data analysis of susceptible and resilient rats.*** Performance of susceptible and resilient PND90 rats was traced back in FST **(panel I)** and LD **(panel II)** tests at PND32 (**A** and **D**), 60 (**B** and **E**) and 90 (**C** and **F**). A diagrammatical representation of occurrence of either anxiety- and depression-like behavior or their co-occurrence is provided in different colors **(panel IIIG)**. Presence of anxiety-like behavior is indicated in green and its absence in black. Presence of depression-like behavior is indicated in maroon and its absence in black, while co-occurrence of the two behaviors is indicated in yellow. A black colored dotted box in the center indicates a potential behavior switch period **(panel IIIG)**. Group designations: Control exposures (CON: open bars, n=25 rats); Resilient (Grey bars, n=28 rats); Susceptible (Black bars, n=27 rats). (*) Significantly different at $p<0.05$. Bars represent means ±SEM.

4.3 Biochemical Effects of Early Life Stress

4.3.1 Indices of Oxidative Stress Markers

4.3.1.1 Effect of Early Life SPS Exposure on 8-isoprostane Levels:

Increased levels of free radicals in the body due to increase in oxidative stress lead to oxidation of free fatty acids and production of isoprostanes (Betteridge 2000). Therefore, the level of plasma 8-isoprostane can be used as a systemic oxidative stress marker. ELISA assay for 8-isoprostane levels indicated that 8-isoprostane did not vary between the control group and the SPS group at different ages, suggesting that early life exposure to SPS did not induce systemic oxidative stress (**Figure. 23 A and B**). Also, after identification of susceptible and resilient phenotypes of PND90 rats in FST test, we analyzed the 8-isoprostane levels of susceptible and resilient groups. There was no difference among the control, susceptible, and resilient groups (**Figure. 23 C**). The mean and SEM values are as follows: PND32 (CONTROL: 147.1 ± 26.89, SPS: 139.8 ± 13.73); PND90 (CONTROL: 132.1 ± 25.80, SPS: 148.3 ± 14.41).

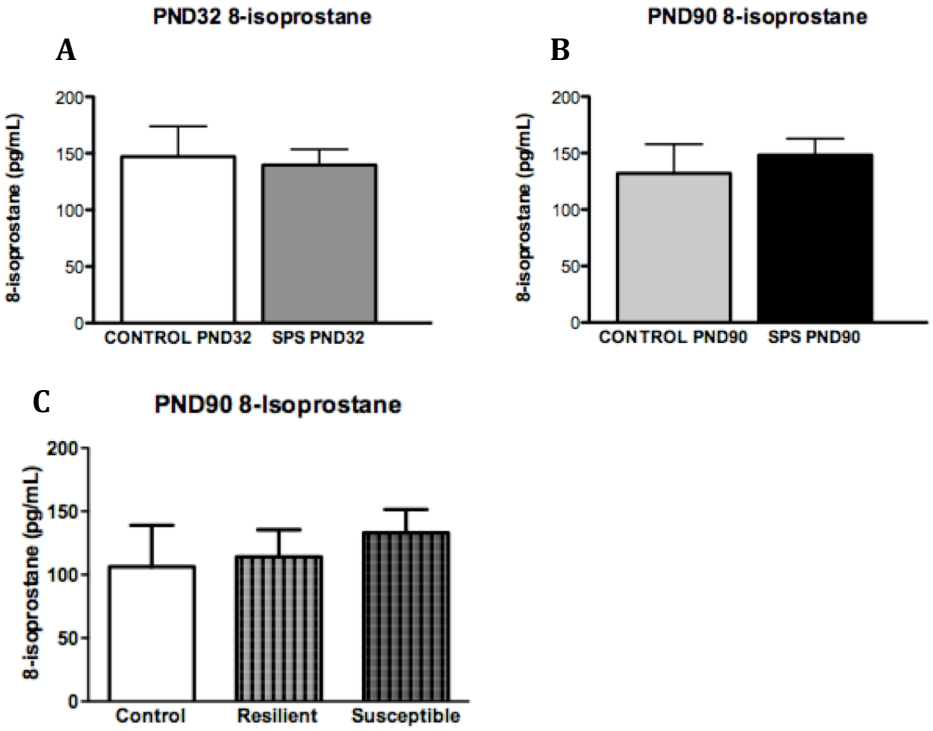


Figure 23. ***Effect of early life SPS exposure on 8-isoprostane levels in control/SPS rats.*** Rats subjected to SPS in ealry life showed no differences in plasma levels of 8-isoprostane at PND32 (A) ot PND90 (B) as compared to control rats. PND90 Susceptible or Resilient rats showed no differences in plasma levels of 8-isoprostane at PND90 (C). Control: n=28, SPS: n=55.

4.3.1.2 Effect of Early Life SPS Exposure on Protein Carbonylation Levels in the Brain:

Under oxidative stress conditions, the build-up of free radicals can promote the formation of reactive ketones or aldehydes that can subsequently react with 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. Antibody against DNPH-derivatized proteins can identify carbonylated proteins (Suzuki, Carini, and Butterfield. 2009). Therefore increase in protein carbonylation levels is an indication of increased neuronal oxidative stress. Early life SPS exposure led to significant increase in the levels of protein carbonylation in prefrontal cortex (PFC) region of SPS rats at both PND32 (**Figure. 24 A**) and 60 (**Figure. 24 B**). These results suggest that early life SPS exposure increased neuronal oxidative stress in the PFC regions at PND32, and persisted into PND90. Early life SPS exposure did not increase the neuronal level of protein carbonylation in the hippocampus and amygdala regions in SPS rats at PND32 (**Figure. 24 C and E**) or PND90 (**Figure. 24 D and F**). The mean and SEM values are as follows: PND32 PFC (CONTROL: 1.086e+006 ± 164384, SPS: 1.645e+006 ± 118157); PND32 hippocampus (CONTROL: 1.246e +006 ± 46315, SPS: 1.136e+006 ± 35841); PND32 amygdala (CONTROL: 883813 ± 169453, SPS: 1.061e+006 ± 95811); PND90 PFC (CONTROL: 1.096e+006 ± 150267, SPS: 1.426e+006 ± 49439); PND90 hippocampus (CONTROL: 1.119e+006 ± 101456, SPS: 1.120e+006 ± 49568); PND90 amygdala (CONTROL: 1.013e+006 ± 72961, SPS: 1.035e+006 ± 101395).

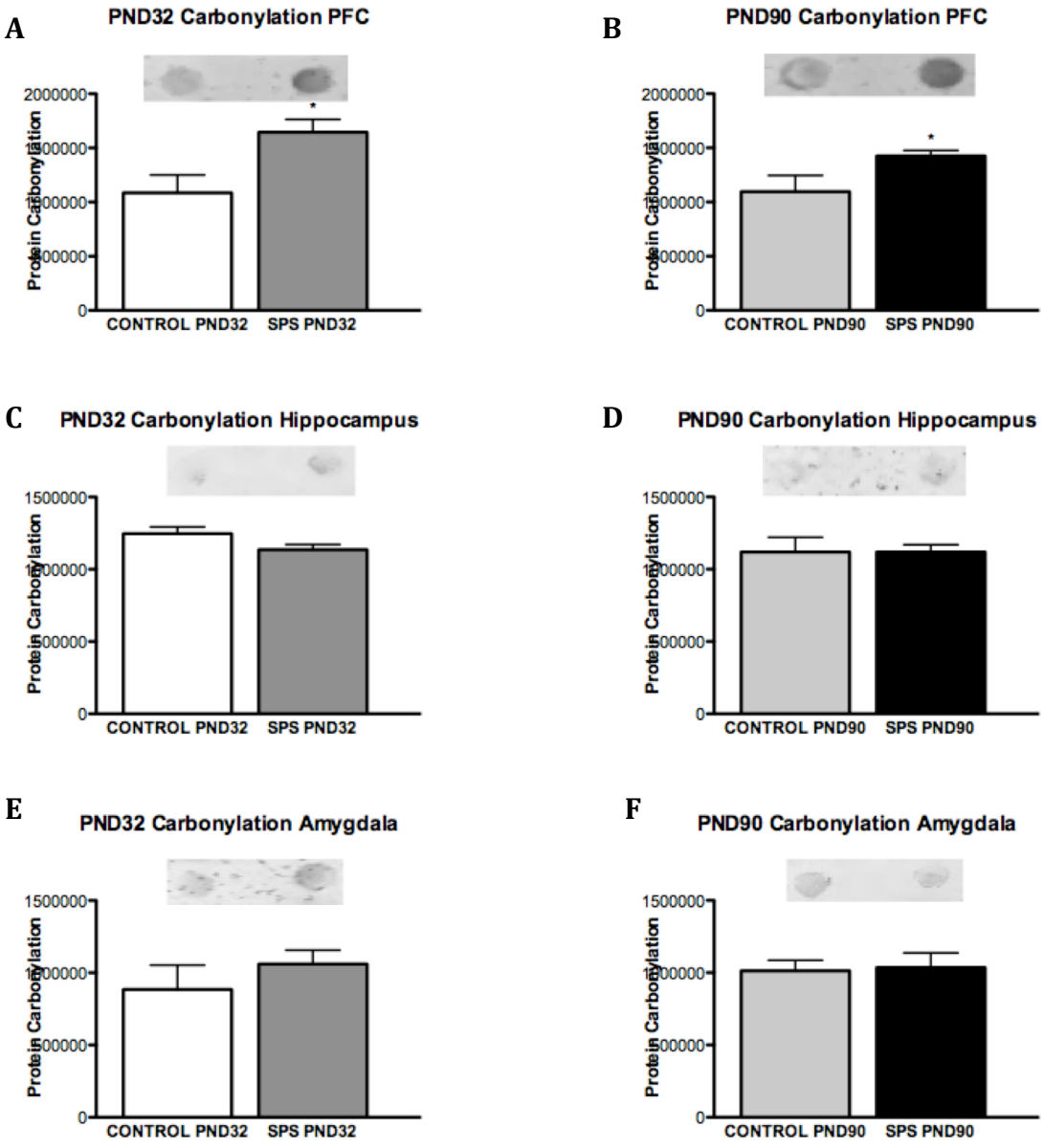


Figure 24. ***Effect of early life SPS on protein carbonylation levels in Control/SPS rats at PND32 and 90.*** Rats subjected to early life SPS exposure showed significant increase in neuronal protein carbonylation levels as compared to control rats in PFC regions at PND32 and 90 (**A** and **B**). However, no difference was observed between control rats and SPS rats in the hippocampus region (**C** and **D**) or amygdala region (**E** and **F**). (*) $P<0.05$, significantly different from Control. Values are mean ± SEM, Control: n=10, SPS: n=30.

We further examined PND90 protein carbonylation data using K-mean cluster analysis utilizing IBM SPSS (IBM, Armonk, NY) to identify subgroups in SPS rats using protein carbonylation levels as variable. Based on the levels of protein carbonylation, the SPS rats appeared to occur in two clusters. From a total of 20 SPS rats, 12 rats exhibited lower levels of protein carbonylation and belonged to the same cluster as controls. The other eight rats belonged to the second cluster and exhibited higher levels of protein carbonylation. We then performed ANOVA test on the control group and the two clusters, followed by Tukey's multiple comparison tests using GraphPad Prism (GraphPad Software, La, Jolla, CA). ANOVA test indicated the two clusters were significantly different ($p<0.05$). Tukey's multiple comparison tests indicated that the eight rats in the high protein carbonylation cluster had a significantly higher level of protein carbonylation when compared to their matched controls, or to the 12 rats in the low protein carbonylation cluster (**Figure. 25 A**). However, the 12 rats within the low protein carbonylation cluster were not significantly different from their matched controls (**Figure. 25 A**). Next, we compared the clusters that were defined using protein carbonylation levels as variable and the clusters that were defined using the time of immobile in FST as variable (**Figure 25 B)**. We found the *resilient* cluster in FST at PND90 had a 100% overlap with the low carbonylation cluster in protein carbonylation assay (**Figure. 25 C)**; the *susceptible* cluster in FST at PND90 had a 66.67% overlap with the high carbonylation cluster in protein carbonylation assay (**Figure. 25 D)**. The overlaps between these clusters indicates a strong association between susceptible phenotype and high neuronal oxidative stress in PFC at PND90.

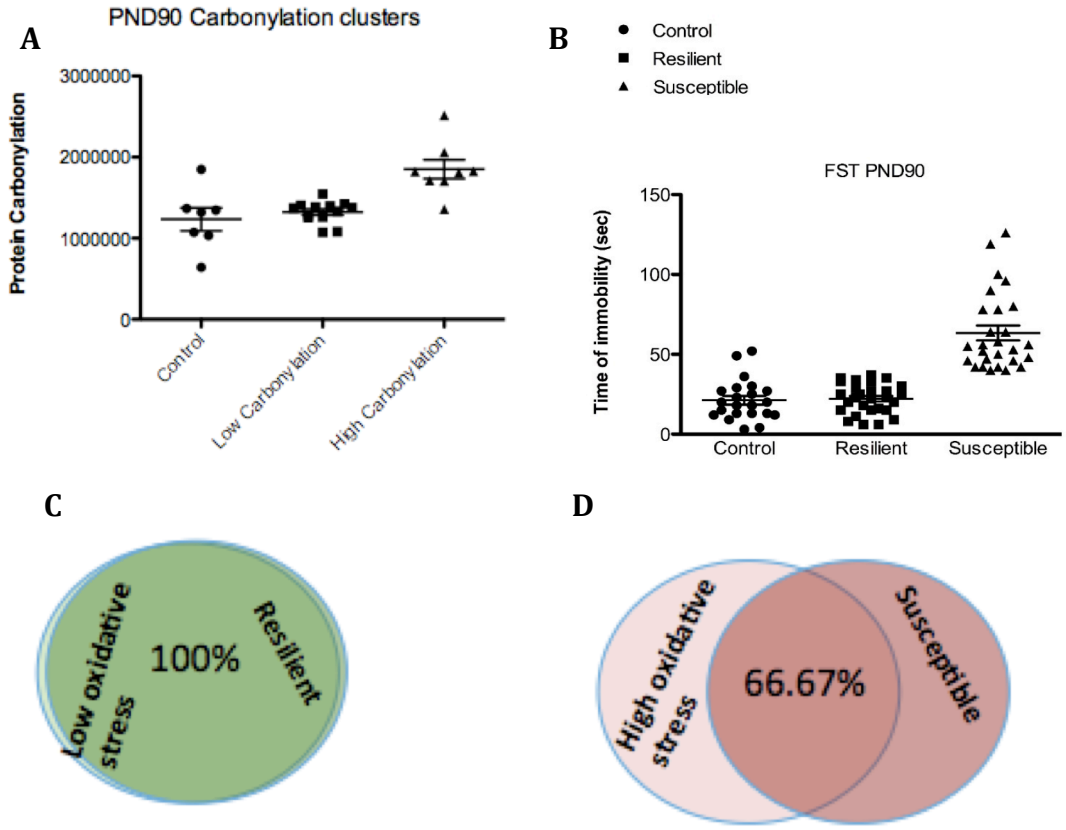


Figure 25. ***The overlaps between the clusters defined by protein carbonylation levels and the clusters defined by behavioral test.*** The protein carconylation levels were used to define clusters within SPS group. Two clusters were found: One with significant high levels of protein carbonylation as compared to control rats; one with similar levels of protein carbonylation as compared to control rats (**A**). The low carbonylation group had 100% overlap (**C**) with the resilient cluster (**B**) in FST. The high carbonylation cluster had a 66.67% overlap with the Susceptible cluster in FST (**D**).

4.3.2 Effect of Early Life SPS Exposure on Antioxidant Defense System

Oxidative stress is caused by an imbalance between the levels of reactive oxygen species (ROS) and the capacity of antioxidant systems to scavenge and detoxify ROS. The antioxidant defense system comprises of both enzymatic and non-enzymatic antioxidants to fight against free radicals (Birben et al. 2012).

4.3.2.1 Effect of Early Life SPS Exposure on the Protein Expression of Antioxidant Cu/Zn SOD:

Cu/Zn SOD is one of the key antioxidant enzymes that are critical for maintaining a balance between free radicals and antioxidant defense system (Stralin and Marklund. 1994). Western blotting was used to detect the protein expression of Cu/Zn SOD in selected brain regions, including PFC, hippocampus, and amygdala. Protein expression of Cu/Zn SOD was higher in PFC at PND32, but lower in PFC and hippocampus at PND90 (**Figure. 26 A, B, and D**). However, Cu/Zn SOD protein expression remained unaltered in hippocampus and amygdala at PND32 and in amygdala at PND90 (**Figure. 26 C, E, and F**). These changes in Cu/Zn SOD levels suggest that early life SPS exposure initially led to an increase in Cu/Zn SOD in PFC at PND32, but eventually a decrease in PFC and hippocampus at PND90. No changes were observed in hippocampus and amygdala at PND32 or amygdala at PND90. The mean and SEM values are as follows: PND32 PFC (CONTROL: 2.453 ± 0.5399, SPS: 3.740 ± 0.2712); PND32 hippocampus (CONTROL: 1.101 ± 0.1063, SPS: 1.032 ± 0.08788); PND32 amygdala (CONTROL: 2.317 ± 0.1035, SPS: 2.102 ± 0.1349); PND90 PFC (CONTROL: 1.861 ± 0.1759, SPS: 1.454 ± 0.07995); PND90 hippocampus

(CONTROL: 1.542 ± 0.1862, SPS: 1.033 ± 0.09347); PND90 amygdala (CONTROL: 7.578 ±2.364, SPS: 6.456 ±1.348).

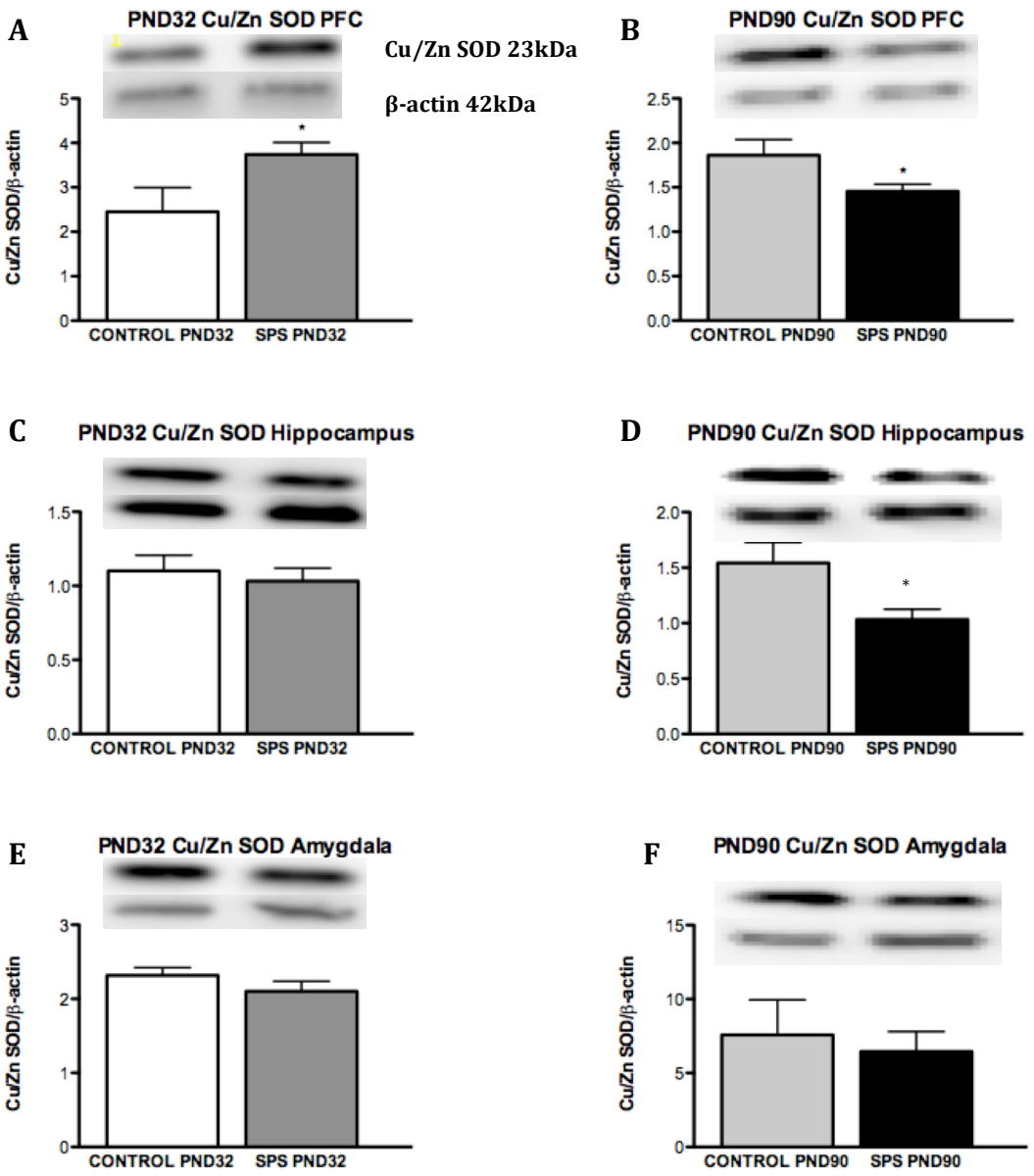


Figure 26. ***Examination of Cu/Zn SOD protein expression in Control/SPS rats at PND32 and 90.*** Rats subjected to early life SPS exposure showed marked increase in expression of Cu/Zn SOD in PFC at PND32 (**A**), but significant decrease in expression of Cu/Zn SOD in PFC and hippocampus at PND90 (**B** and **D**). The expression levels of Cu/Zn SOD in the hippocampus and amygdala were not different from that of the control rats (**C** and **E**) at PND32. Cu/Zn SOD levels of SPS rats were not changed in amygdala at PND 90 (**F**). (*) $P<0.05$, significantly different form Control. Values are mean ± SEM, Control: n=10, SPS: n=30.

We further examined PND90 Cu/Zn SOD protein expression data using K-mean cluster analysis utilizing IBM SPSS (IBM, Armonk, NY) to identify subgroups in

SPS rats using Cu/Zn SOD expression levels as variable. Based on the levels of Cu/Zn SOD, the SPS rats appeared to occur in two clusters. From a total of 20 SPS rats, seven rats exhibited higher levels of Cu/Zn SOD and belonged to the same cluster as controls. The other 13 rats belonged to the second cluster and exhibited lower levels of Cu/Zn SOD. We then performed ANOVA test on the control group and the two clusters, followed by Tukey's multiple comparison tests using GraphPad Prism (GraphPad Software, La, Jolla, CA). ANOVA test indicated the two clusters were significantly different ($p<0.05$). Tukey's multiple comparison tests indicated that the 13 rats in the high Cu/Zn SOD cluster had a significantly lower level of Cu/Zn SOD protein expression when compared to their matched controls, or to the seven rats in the low Cu/Zn SOD cluster (**Figure. 27 A**). However, the seven rats within the high Cu/Zn SOD cluster were not significantly different from their matched controls (**Figure. 27 A**). Next, we compare the clusters that were defined using Cu/Zn SOD protein expression levels as variable with the clusters that were defined using the time of immobile in FST as variable (**Figure. 27 B**). We found that the *resilient* cluster in FST at PND90 had a 70% overlap with the high Cu/Zn SOD cluster in Cu/Zn SOD protein expression western blotting assay (**Figure. 27 C**); the *susceptible* cluster in FST at PND90 had a 90% overlap with the low Cu/Zn SOD cluster in Cu/Zn SOD protein expression western blotting assay (**Figure. 27 D**). The overlaps between these clusters indicates a strong association between susceptible phenotype and low Cu/Zn SOD protein expression in PFC at PND90.

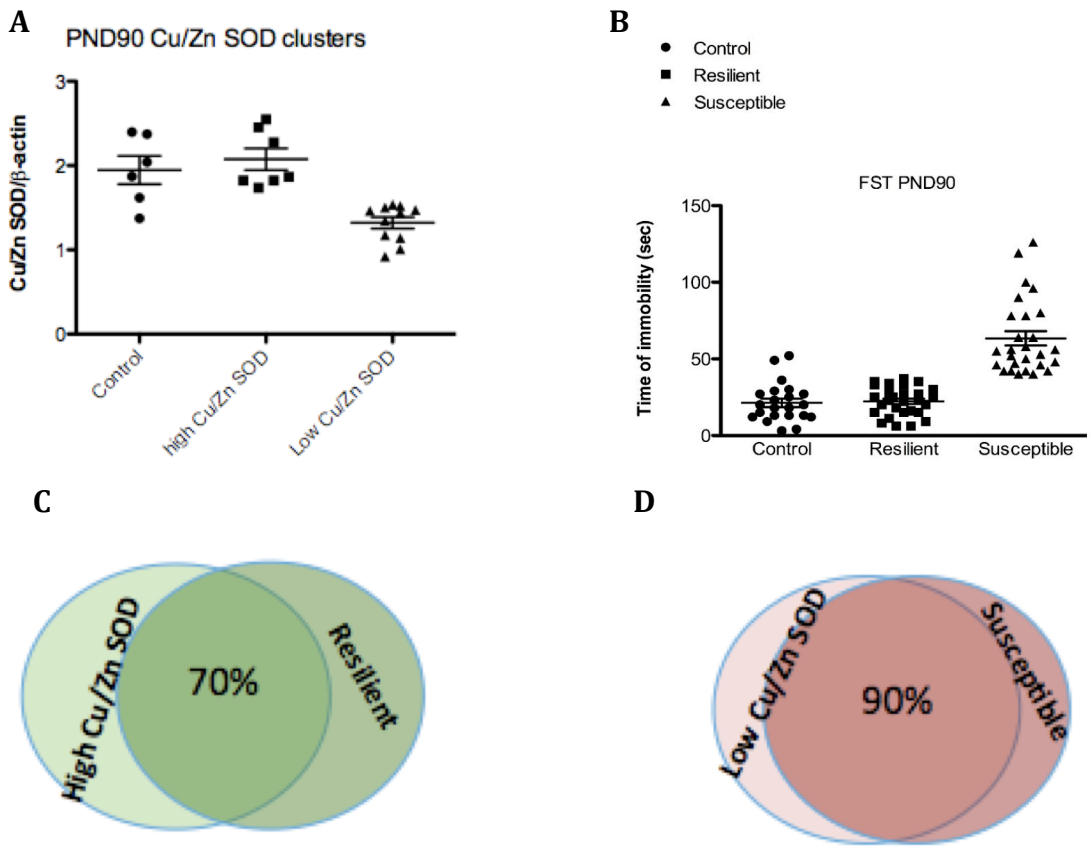


Figure 27. ***The overlaps between the clusters defined by protein expression levels of Cu/Zn SOD and the clusters defined by behavioral test.*** The protein expression levels of Cu/Zn SOD were used to define clusters within SPS group. Two clusters were found: One with significant high levels of Cu/Zn SOD as compared to control rats; one with similar levels of Cu/Zn SOD as compared to control rats (**A**). The high Cu/Zn SOD cluster had 100% overlap (**C**) with the resilient cluster (**B**) in FST. The low Cu/Zn SOD cluster had a 66.67% overlap with the Susceptible cluster in FST (**D**).

4.3.2.2 Effect of Early Life SPS Exposure on the Protein Expression of Antioxidant Mn-SOD

Mn-SOD is a mitochondrial antioxidant that has an important role in protecting the cells against free radicals under elevated oxidative stress conditions (Candas and Li. 2014). Western blotting was used to detect the protein expression of Mn-SOD in selected brain regions, including the PFC, hippocampus, and the amygdala. Protein expression of Mn-SOD was higher in PFC at PND32 (**Figure. 28 A**). However, Cu/Zn SOD protein expression remained unaltered in hippocampus and amygdala at PND32 and in all three regions at PND90 (**Figure. 28 B, C, D, E, and F**). Suggesting that early life SPS exposure initially led to an increase in Mn-SOD in PFC at PND32, but eventually went back to normal at PND90. No changes were observed in hippocampus and amygdala at PND32 or PND90. The mean and SEM values are as follows: PND32 PFC (CONTROL: 2.358 ±0.4484, SPS: 3.168 ± 0.1644); PND32 hippocampus (CONTROL: 1.457 ± 0.1845, SPS: 1.517 ± 0.09028); PND32 amygdala (CONTROL: 2.014 ± 0.2909, SPS: 1.661 ± 0.2695); PND90 PFC (CONTROL: 1.157 ± 0.1532, SPS: 1.193 ±0.07137); PND90 hippocampus (CONTROL: 1.247 ± 0.3073, SPS: 1.107 ±0.1059); PND90 amygdala (CONTROL: 0.4911 ±0.06616, SPS: 0.4517 ±0.03309).

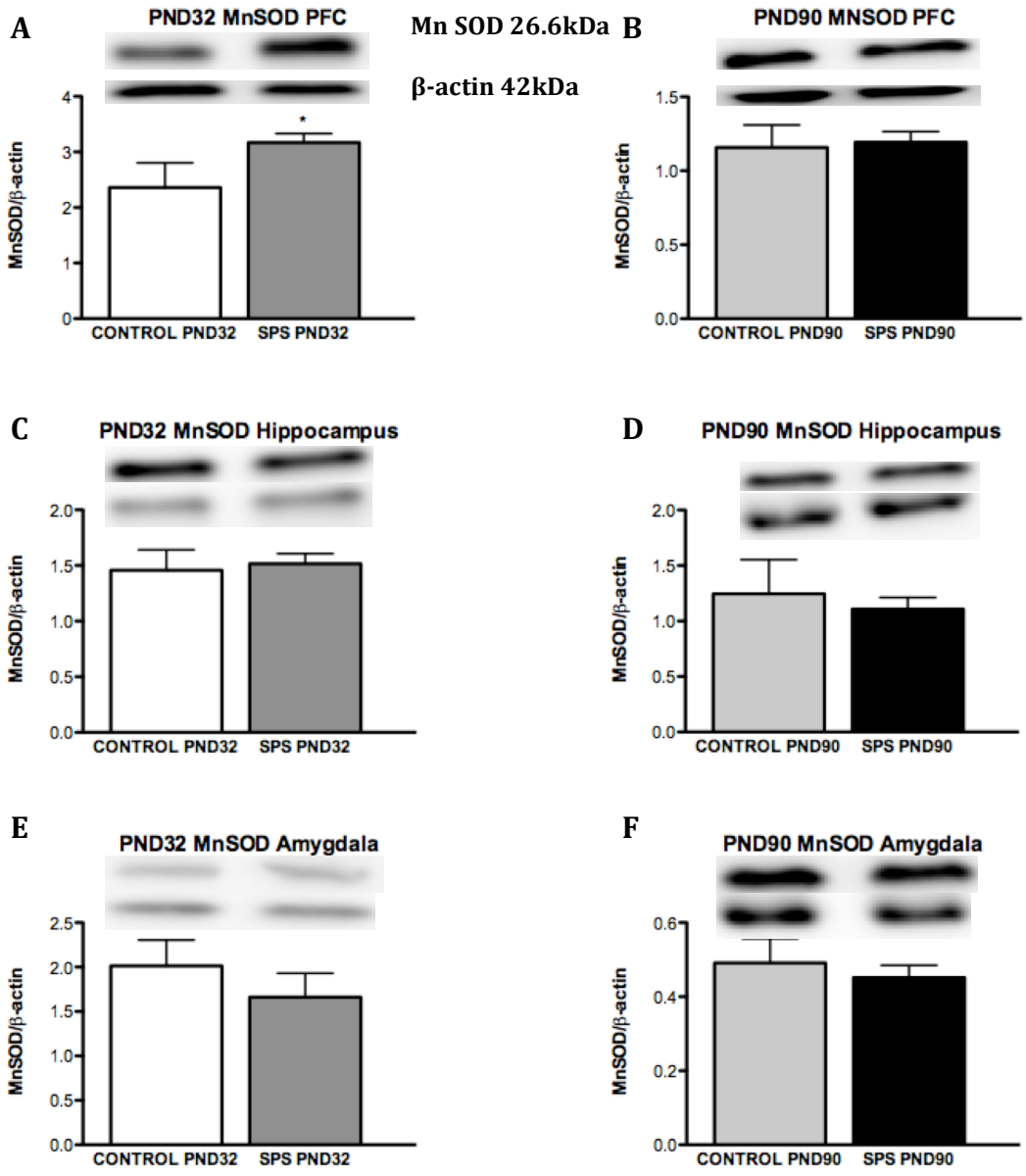


Figure 28. ***Examination of Mn SOD protein expression in Control/SPS rats at PND32 and 90.*** Rats subjected to early life SPS exposure showed marked increase in expression of Mn SOD in PFC at PND32 (**A**), but no change was observed at PND90 (**B**). The expression levels of Mn SOD in the hippocampus and amygdala were not different from that of the control rats (**C** and **E**) at PND32 and PND90 (**D** and **F**). (*) $P<0.05$, significantly different form Control. Values are mean ± SEM, Control: n=10, SPS: n=30.

We further examined PND90 Mn-SOD protein expression data using K-mean cluster analysis utilizing IBM SPSS (IBM, Armonk, NY) to identify subgroups in SPS rats using Mn-SOD expression levels as variable. Based on the levels of Mn-SOD, there were no clusters within the SPS group.

4.3.2.3 Effect of Early Life SPS Exposure on the Protein Expression of Antioxidant Catalase

Catalase is an antioxidant enzyme that presents in most aerobic cells to prevent the cell from oxidative stress damages (Al-Abrash, Al-Quobaili, and Al-Akhras. 2000). Western blotting was used to detect the protein expression of catalase in selected brain regions, including PFC, hippocampus, and amygdala. Protein expression of catalase was higher in PFC at PND32 (**Figure. 29 A**), but lower in PFC and hippocampus at PND90 (**Figure. 29 B and D**). However, catalase protein expression remained unaltered in hippocampus and amygdala at PND32 and in amygdala at PND90 (**Figure. 29 C, E, and F**), suggesting that early life SPS exposure initially led to an increase in catalase in PFC at PND32, but eventually a decrease in PFC and hippocampus at PND90. No changes were observed in hippocampus and amygdala at PND32 or amygdala at PND90. The mean and SEM values are as follows: PND32 PFC (CONTROL: 2.802 ± 0.5823, SPS: 5.147 ± 0.6396); PND32 hippocampus (CONTROL: 1.577 ± 0.1562, SPS: 1.566 ± 0.08641); PND32 amygdala (CONTROL: 2.673 ± 0.2132, SPS: 2.752 ± 0.2659); PND90 PFC (CONTROL: 0.4159± 0.04710, SPS: 0.3100 ± 0.02292); PND90 hippocampus (CONTROL: 0.3407 ± 0.05875, SPS: 0.2376 ± 0.02487); PND90 amygdala (CONTROL: 0.3933 ± 0.08036, SPS: 0.3850 ± 0.05150).

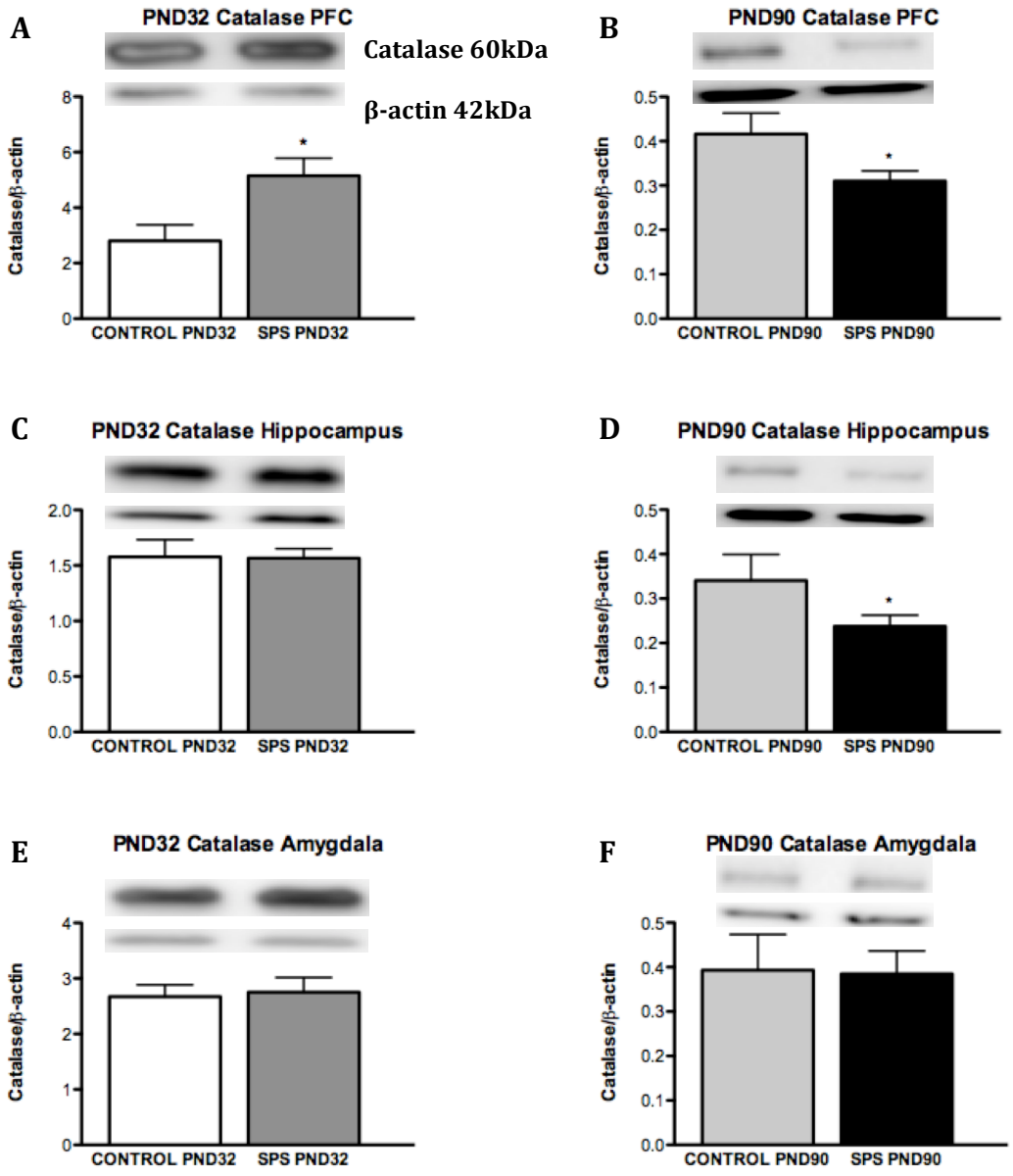


Figure 29. ***Examination of catalase protein expression in Control/SPS rats at PND32 and 90.*** Rats subjected to early life SPS exposure showed marked increase in expression of catalase in PFC at PND32 (A), but significant decrease in expression of catalase in PFC and hippocampus at PND90 (B and D). The expression levels of catalase in the hippocampus and amygdala were not different from that of the control rats (C and E) at PND32. Catalase levels of SPS rats were not changed in amygdala at PND 90 (F). (*) $P<0.05$, significantly different form Control. Values are mean ± SEM, Control: n=10, SPS: n=30.

We examined PND90 catalase protein expression data using K-mean cluster analysis utilizing IBM SPSS (IBM, Armonk, NY) to identify subgroups in SPS rats using catalase expression levels as variable. the SPS rats appeared to occur in two clusters. From a total of 20 SPS rats, seven rats exhibited higher levels of catalase and belonged to the same cluster as controls. The other 13 rats belonged to the second cluster and exhibited lower levels of catalase. We then performed ANOVA test on the control group and the two clusters, followed by Tukey's multiple comparison tests using GraphPad Prism (GraphPad Software, La, Jolla, CA). ANOVA test indicated the two clusters were significantly different ($p<0.05$). Tukey's multiple comparison tests indicated that the 13 rats in the high catalase cluster had a significantly lower level of catalase protein expression when compared to their matched controls, or to the seven rats in the low catalase cluster (**Figure. 30 A**). However, the seven rats within the high catalase cluster were not significantly different from their matched controls (**Figure. 30 A**). Next, we compare the clusters that were defined using catalase protein expression levels as variable with the clusters that were defined using the time of immobile in FST as variable (**Figure. 30 B**). We found that the *resilient* cluster in FST at PND90 had a 50% overlap with the high catalase cluster in catalase protein expression western blotting assay (**Figure. 30 C**); the *susceptible* cluster in FST at PND90 had a 90% overlap with the low catalase cluster in catalase protein expression western blotting assay (**Figure. 30 D**). The overlaps between these clusters indicates a strong association between susceptible phenotype and low catalase protein expression in PFC at PND90.

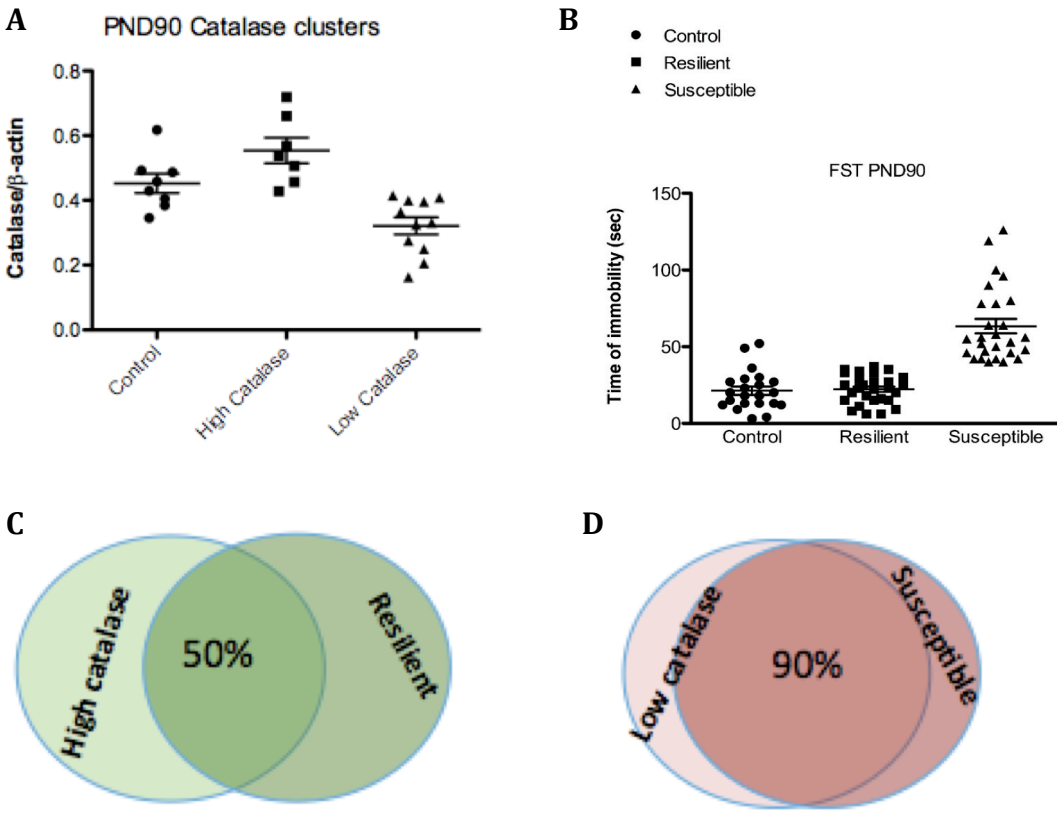


Figure 30. The overlap between the clusters defined by protein expression levels of catalase and the clusters defined by behavioral test. The protein expression levels of catalase were used to define clusters within SPS group. Two clusters were found: One with significant high levels of catalase as compared to control rats; one with similar levels of catalase as compared to control rats (**A**). The high catalase cluster had 50% overlap (**C**) with the resilient cluster (**B**) in FST. The low catalase cluster had a 66.67% overlap with the susceptible cluster in FST (**D**).

4.3.2.4 Effect of Early Life SPS Exposure on the Protein Expression of Antioxidant Heme Oxygenase-1 (HO-1)

HO-1 is a stress response protein that can be induced by oxidative stress to protect the cell against the damage from free radicals (Choi and Alam. 1996). Western blotting was used to detect the protein expression of HO-1 in selected brain regions, including PFC, hippocampus, and amygdala. Protein expression of HO-1 was higher in PFC at PND32, but lower in PFC at PND90 (**Figure. 31 A and B**). However, catalase protein expression remained unaltered in hippocampus and amygdala at PND32 and PND90 (**Figure. 31 C, D, E, and F**). The changes in catalase levels suggest that early life SPS exposure initially led to an increase in catalase in PFC at PND32, but eventually a decrease in PFC at PND90. No changes were observed in hippocampus and amygdala at PND32 or PND90. The mean and SEM values are as follows: PND32 PFC (CONTROL: 0.5305 ± 0.1278, SPS: 1.096 ± 0.09476); PND32 hippocampus (CONTROL: 3.609 ± 0.8980, SPS: 4.137 ± 0.7745); PND32 amygdala (CONTROL: 8.986 ± 3.174, SPS: 8.958 ± 1.827); PND90 PFC (CONTROL: 0.8226 ± 0.01665, SPS: 0.5547 ± 0.06883); PND90 hippocampus (CONTROL: 3.507 ± 1.157, SPS: 6.637 ± 1.500); PND90 amygdala (CONTROL: 0.8687 ± 0.06312, SPS: 0.9522 ± 0.05144).

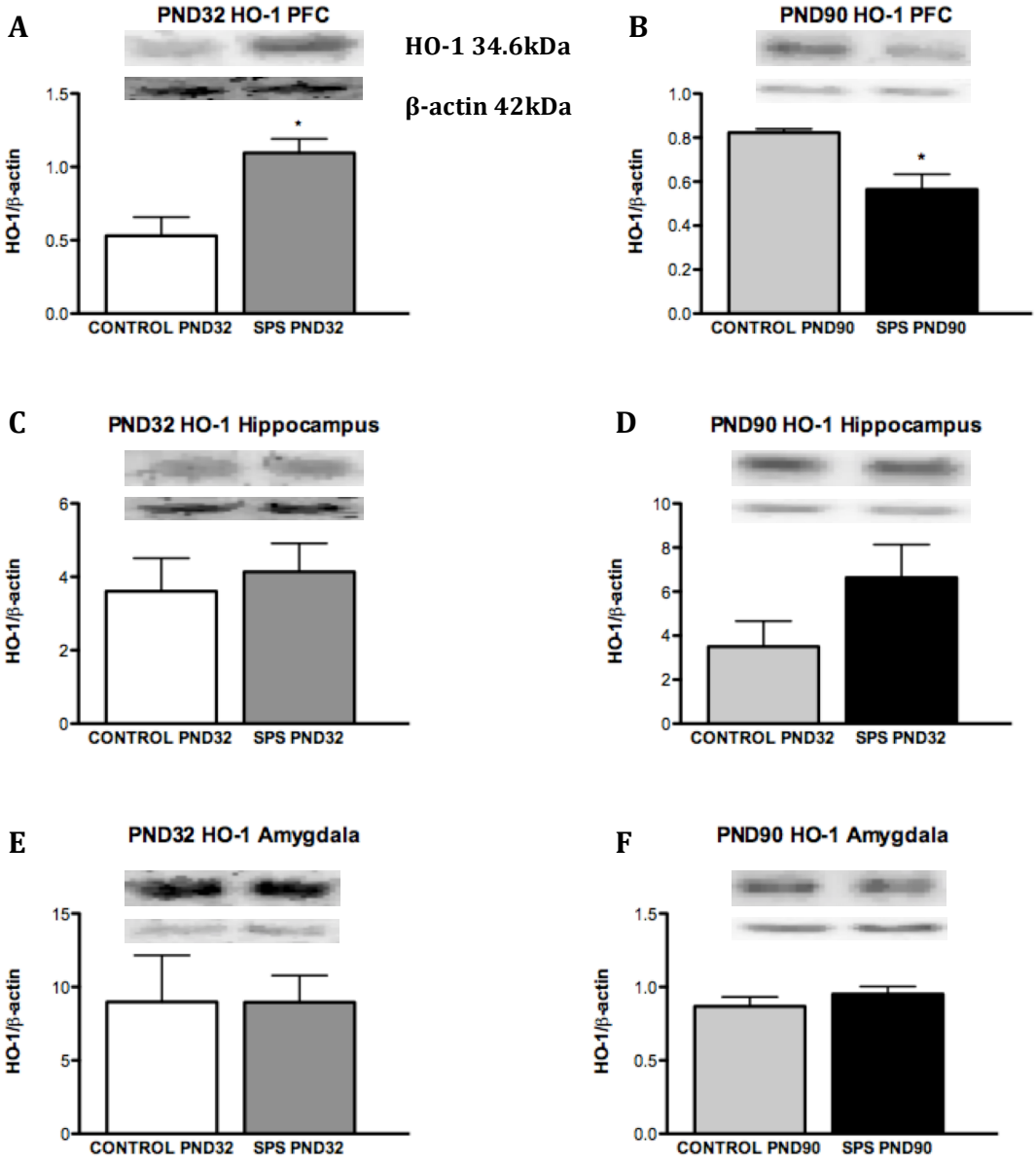


Figure 31. ***Examination of HO-1 protein expression in Control/SPS rats at PND32 and 90.*** Rats subjected to early life SPS exposure showed marked increase in expression of HO-1 in PFC at PND32 (A), but a significant decrease in HO-1 was observed in PFC at PND90 (B). The expression levels of HO-1 in the hippocampus and amygdala were not different from that of the control rats (C and E) at PND32 and PND90 (D and F). (*) $P<0.05$, significantly different form Control. Values are mean ± SEM, Control: n=10, SPS: n=30.

We further examined PND90 HO-1 protein expression data using K-mean cluster analysis utilizing IBM SPSS (IBM, Armonk, NY) to identify subgroups in SPS rats using HO-1 expression levels as variable. Based on the levels of HO-1, the SPS rats appeared to occur in two clusters. From a total of 18 SPS rats, nine rats exhibited higher levels of HO-1 and belonged to the same cluster as controls. The other nine rats belonged to the second cluster and exhibited lower levels of HO-1. We then performed ANOVA test on the control group and the two clusters, followed by Tukey's multiple comparison tests using GraphPad Prism (GraphPad Software, La, Jolla, CA). ANOVA test indicated the two clusters were significantly different ($p<0.05$). Tukey's multiple comparison tests indicated that the nine rats in the low HO-1 cluster had a significantly lower level of HO-1 protein expression when compared to their matched controls, or to the nine rats in the high HO-1 cluster (**Figure. 32 A**). However, the nine rats within the high HO-1 cluster were not significantly different from their matched controls (**Figure. 32 A**). Next, we compare the clusters that were defined using HO-1 protein expression levels as variable with the clusters that were defined using the time of immobile in FST as variable (**Figure. 32 B**). We found that the *resilient* cluster in FST at PND90 had a 100% overlap with the high HO-1 cluster in HO-1 protein expression western blotting assay (**Figure. 32 C**), the *susceptible* cluster in FST at PND90 had a 90% overlap with the low HO-1 cluster in HO-1 protein expression western blotting assay (**Figure. 32 D**). The overlaps between these clusters indicate a strong association between susceptible phenotype and low HO-1 protein expression in PFC at PND90.

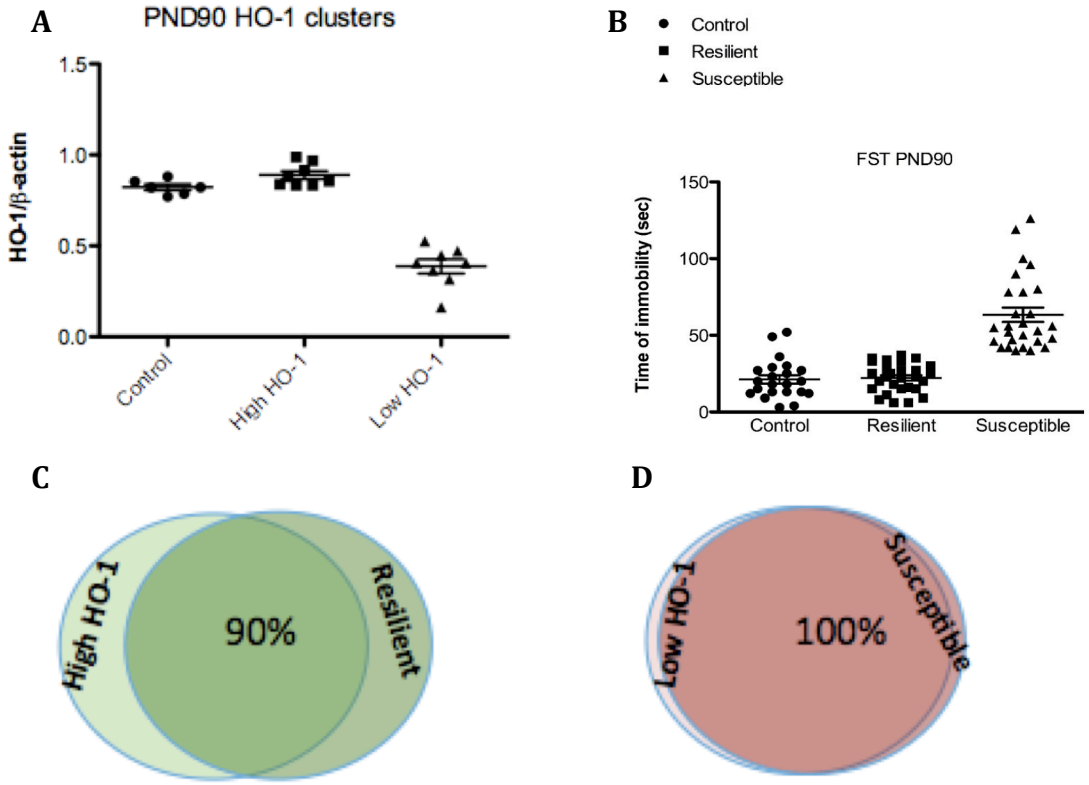


Figure 32. ***The overlaps between the clusters defined by protein expression levels of HO-1 and the clusters defined by behavioral test.*** The protein expression levels of HO-1 were used to define clusters within SPS group. Two clusters were found: One with significant high levels of HO-1 as compared to control rats; one with similar levels of HO-1 as compared to control rats (**A**). The high HO-1 cluster had 90% overlap (**C**) with the resilient cluster (**B**) in FST. The low HO-1 cluster had a 100% overlap with the susceptible cluster in FST (**D**).

4.3.3 Effect of Early Life SPS Exposure on Nrf2 and NF- kB Regulated Pathways

Under oxidative stress conditions, increased levels of reactive oxygen species can lead to activation or suppression of redox-sensitive transcription factors (Haddad. 2002; Surh et al. 2005). Oxidative stress induced Nrf2 pathway can lead to the expression of antioxidant enzymes and cytoprotective genes, while oxidative stress induced NF-kB pathway can regulate pro-inflammatory genes (Korashy and

El-Kadi. 2008). Evidence suggests that activation of Nrf2 can lead to suppression of NF-kB, and vice versa (Wakabayashi et al. 2010).

4.3.3.1 Effect of Early Life SPS Exposure on Nrf2 levels

Based on the evidence we obtained regarding the differential of expression of phase II antioxidant enzymes, we focused our attention on Nrf-2, a key transcription factor known to regulate phase II antioxidant enzymes transcription in response to overproduction of reactive oxygen species (Qiang Ma. 2013). We found that nuclear and cytosol levels of Nrf-2 were not changed in SPS rats at PND32 (**Figure. 33 A and B**), but Nrf-2 protein expression level was decreased at PND90 in SPS rats at PND90 (**Figure. 33 E**). The nuclear and the cytosolic levels of Keap1, the regulator of Nrf-2, were unaltered at neither PND32 nor 90 (**Figure. 33 C, D, and F**). The mean and SEM values are as follows: PND32 Nrf2 cytosol (CONTROL: 2.155 ± 0.1128, SPS: 2.144 ± 0.330); PND32 Nrf2 nuclear (CONTROL: 8.653 ± 0.3058, SPS: 10.10 ± 0.4103); PND32 keap1 cytosol (CONTROL: 3.089 ± 0.3256, SPS: 3.516 ± 0.3123); PND32 keap1 nuclear (CONTROL: 7.630 ± 0.1994, SPS: 7.079 ± 0.5905); PND90 Nrf2 (CONTROL: 43.39 ± 4.023, SPS: 34.46 ± 2.985); PND90 keap1 (CONTROL: 11.35 ± 1.388, SPS: 10.51 ± 1.058).

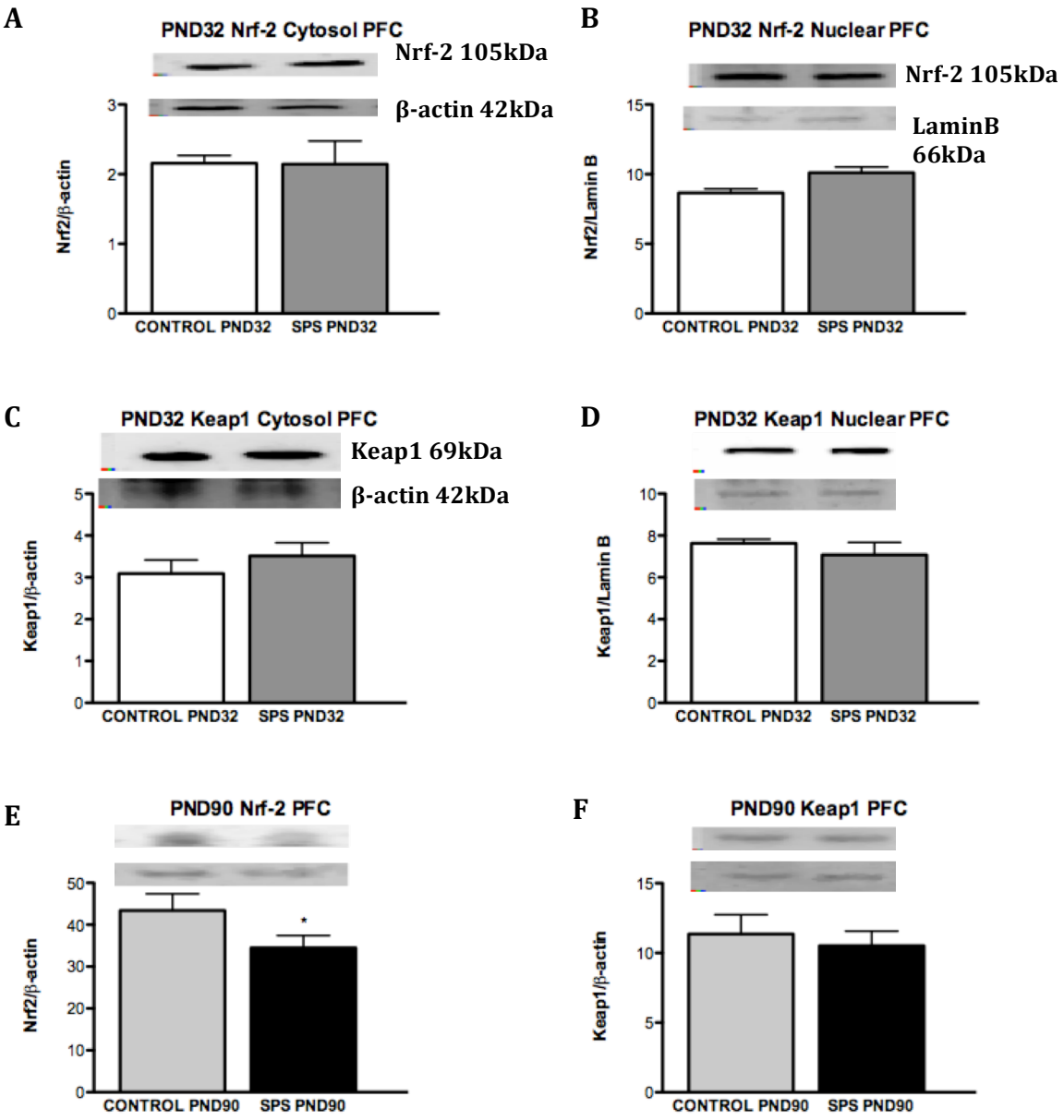


Figure 33. ***Examination of Nrf-2 levels in Control/SPS rats at PND32 and 90.*** Rats subjected to early life SPS exposure showed marked decrease in expression of Nrf-2 in PFC at PND90 (**E**). The cytosol (**A**) and nuclear (**B**) levels of Nrf-2 were not changed in the SPS rats compared to control rats. The expression levels of Keap1 were not different from that of the control rats at PND32 (**C** and **D**) and PND90 (**F**). (*) $P<0.05$, significantly different form Control. Values are mean ± SEM, Control: n=10, SPS: n=30.

4.3.3.2 Effect of Early Life SPS Exposure on HDAC and DNMT

Epigenetic modifications are beginning to be appreciated as plausible biological mechanisms that presumably link early life stress with the development of later negative health conditions including psychiatric disorders, cardiovascular complications, and cancer (Mitchell, Scheneper, and Notterman, 2015). The mechanisms that mediate epigenetic modifications involve DNA methylation and histone modifications (Murgatroyd and Spengler. 2014). DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) play an important role to mediate epigenetic modifications that repress gene transcription (Murgatroyd and Spengler. 2014). The measurement of these enzyme levels thus indicates the involvement of ELS-responsive epigenetic modifications in regulating biochemical changes following SPS exposure. A significant increase in the levels of HDAC2 and DNMT1 was observed in PFC of the SPS rats at PND90 as compared to control rats (**Figure. 34 B and D**), while the levels of HDAC2 and DNMT1 were unaltered in PFC of the SPS rats at PND32 (**Figure. 34 A and C**). The mean and SEM values are as follows: PND32 HDAC2 (CONTROL: 0.8336 ± 0.1108, SPS: 0.9402 ± 0.1019); PND32 DNMT1 (CONTROL: 3.894 ± 0.5497, SPS: 4.541 ± 0.3345); PND90 HDAC2 (CONTROL: 0.3902 ± 0.02421, SPS: 0.6609 ± 0.1358); PND90 DNMT1 (CONTROL: 2.898 ± 0.6271, SPS: 3.580 ± 0.5216).

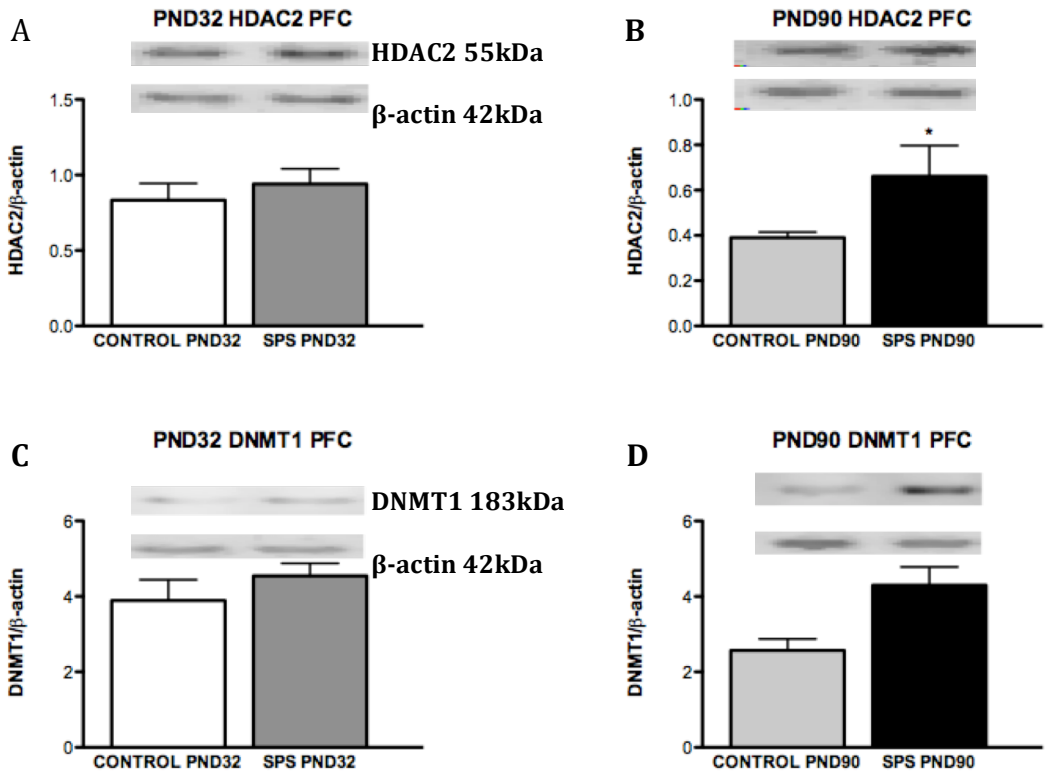


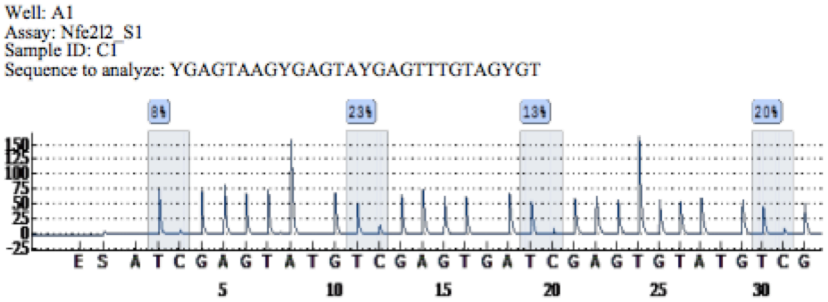
Figure 34. ***Examination of HDAC2 and DNMT1 levels in Control/SPS rats at PND32 and 90.*** Rats subjected to early life SPS exposure showed marked increase in expression of HDAC2 and DNMT1 in PFC at PND90 (**B** and **D**). The levels of HDAC2 and DNMT1 were not changed in SPS rats as compared to control rats at PND32 (**A** and **C**). (*) $P<0.05$, significantly different form Control. Values are mean ± SEM, Control: n=6, SPS: n=10.

4.3.3.3 Effect of Early Life SPS Exposure on DNA methylation in the promoter region of Nrf-2

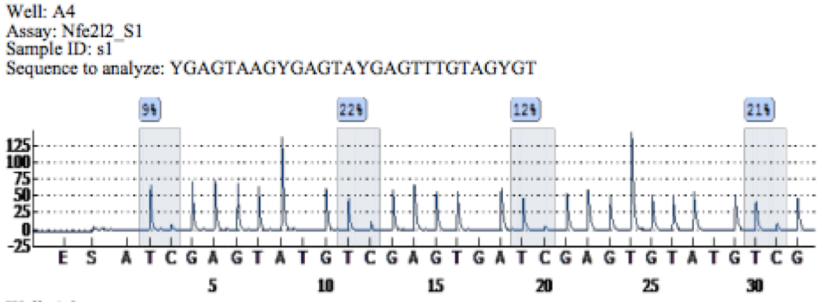
Current literature shows that there is an association between early life stress exposure and gene methylation in the promoter region of specific genes (Mitchell, Scheneper, and Notterman. 2015). A cancer study with mice indicates that decreased Nrf-2 expression level is associated with hypermethylation in the promoter region of Nrf-2 (Khor et al. 2014). DNA methylation analysis was performed to evaluate the methylation levels in the promoter region of Nrf-2. It was observed that early life SPS exposure did not lead to any changes in the level of methylation in the promoter region of Nrf-2 in SPS rats as compared to control rats at PND32 (**Figure. 35 A and B**)

A

Control



SPS



B

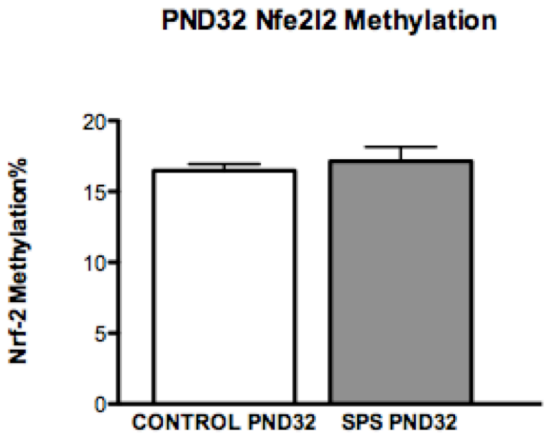


Figure 35. ***Examination of DNA methylation levels in the promoter region of Nrf-2 in Control/SPS rats at PND32 and 90.*** Rats subjected to early life SPS exposure did not show difference in DNA methylation in the promoter region of Nrf-2 as compared to control rats at PND32 (**A** and **B**). Control: n=3, SPS: n=6.

4.3.3.4 Effect of Early Life SPS Exposure on NF-kB Pathway

A build-up of reactive oxygen species due to disturbed oxidative-reduction homeostasis has been acknowledged to be a key element in the development of diseases (Adler et al. 1999). Increased free radicals can serve as a signal to activate redox-sensitive transcription factors. NF-kB is one of the most important transcription factors that directly responds to oxidative stress, and subsequently regulates immune and inflammatory responses (Haddad. 2002). Western blotting analysis was used to evaluate the levels of P65 (a subunit of NF-kB), Interleukin -6 (IL-6), and tumor necrosis factor (TNF)- α in PFC of both control and SPS rats. The levels of cytosol and nuclear P65 protein expression were markedly increased in PFC of SPS rats as compared to control rats (**Figure. 36 A and B**). Protein expression levels of IL-6 were significantly increased in PFC of SPS rats at PND32 and 60 (**Figure. 36 C and D**) as compared to control rats. Protein expression levels of TNF-α were increased in PFC of SPS rats at PND32, (**Figure. 36 E**), but not at PND90 (**Figure. 36 F**). The mean and SEM values are as follows: PND32 P65 cytosol (CONTROL: 2.572 ± 0.08353, SPS: 5.808 ± 0.7401); PND32 P65 nuclear (CONTROL: 1.850 ± 0.3422, SPS: 4.132 ± 0.8931); PND 32 IL-6 (CONTROL: 0.3203 ± 0.04925, SPS: 0.6744 ± 0.1180); PND32 TNF-α (CONTROL: 1.408 ± 0.3514, SPS: 2.273 ± 0.1967); PND90 IL-6 (CONTROL: 1.366 ± 0.2508, SPS: 2.849 ± 0.3477); PND90 TNF-α (CONTROL: 0.1954 ± 0.01480, SPS: 0.2462 ± 0.03471).

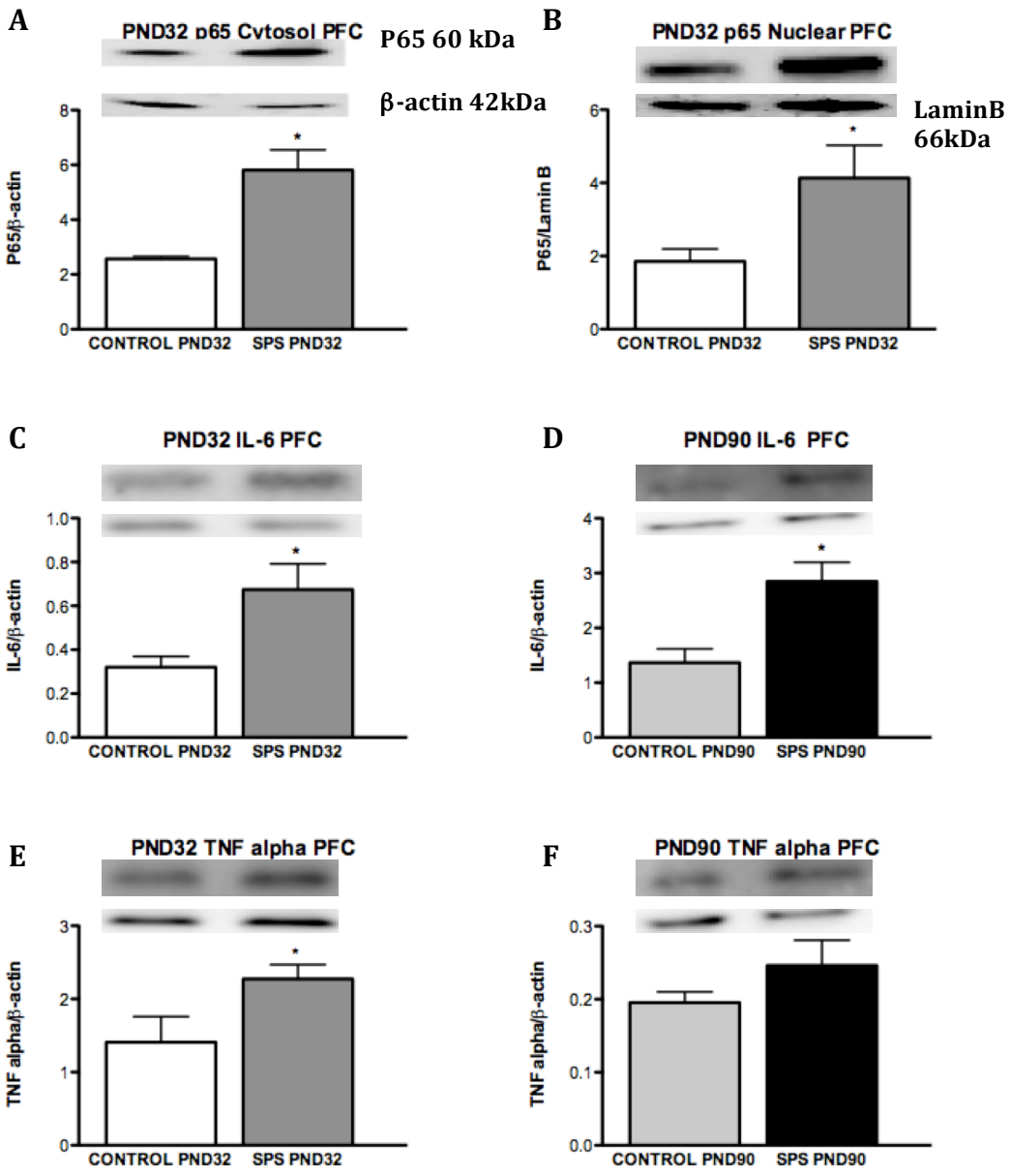


Figure 36. ***Examination of P65, IL-6, and TNF-α in Control/SPS rats at PND32 and 90.*** The levels of P65 were increased in SPS rats as compared to control rats (**A** and **B**). IL-6 protein expression levels were higher in PFC of SPS rats at PND32 and 90 as compared to control rats (**C** and **D**). TNF-α levels were increased in PFC of SPS rats at PND32 (**E**), but not at PND90 (**F**). (*) $P<0.05$, significantly different form Control. Values are mean ± SEM, Control: n=6, SPS: n=10.

4.3.3.5 Effect of Early Life SPS Exposure on BDNF

Early life stress is associated with structural and biological changes in specific brain regions, which underlies later life behavioral and cognitive alterations. Preclinical and clinical studies show that early life stress leads to a decreased volume of the hippocampus region of the brain as well as decreased level of brain-derived neurotrophin factor (BDNF) (Spinelli, Chefer, and Suomi. 2009; Sarabdjitsingh et al. 2017). Neurotrophins have important roles in the central nervous system such as mediating neurogenesis, facilitating synaptic connection, and increasing neuronal plasticity (Lee et al. 2002). Western blotting analysis was used to evaluate the levels of BDNF protein expression in PFC of both control and SPS rats. The levels of BDNF were markedly decreased in PFC (**Figure. 37 A**), and hippocampus (**Figure. 37 B**) in SPS rats at PND32 as compared to age-matched control rats but were not changed in Amygdala (**Figure. 37 C**). No changes were observed in all the three regions at PND90 as compared to control rats (**Figure. 37 D, E, and F**). The mean and SEM values are as follows: PND32 PFC (CONTROL: 79.74 ± 13.95, SPS: 57.31 ± 3.125); PND32 hippocampus (CONTROL: 44.17 ± 10.42, SPS: 35.46 ± 4.379); PND32 amygdala (CONTROL: 5.520 ± 0.9064, SPS: 5.374 ± 0.8959); PND90 PFC (CONTROL: 1.445 ± 0.07775, SPS: 1.577 ± 0.05888); PND90 hippocampus (CONTROL: 1.419 ± 0.2396, SPS: 1.340 ± 0.1405); PND90 amygdala (CONTROL: 0.8741 ± 0.06596, SPS: 1.003 ± 0.08500).

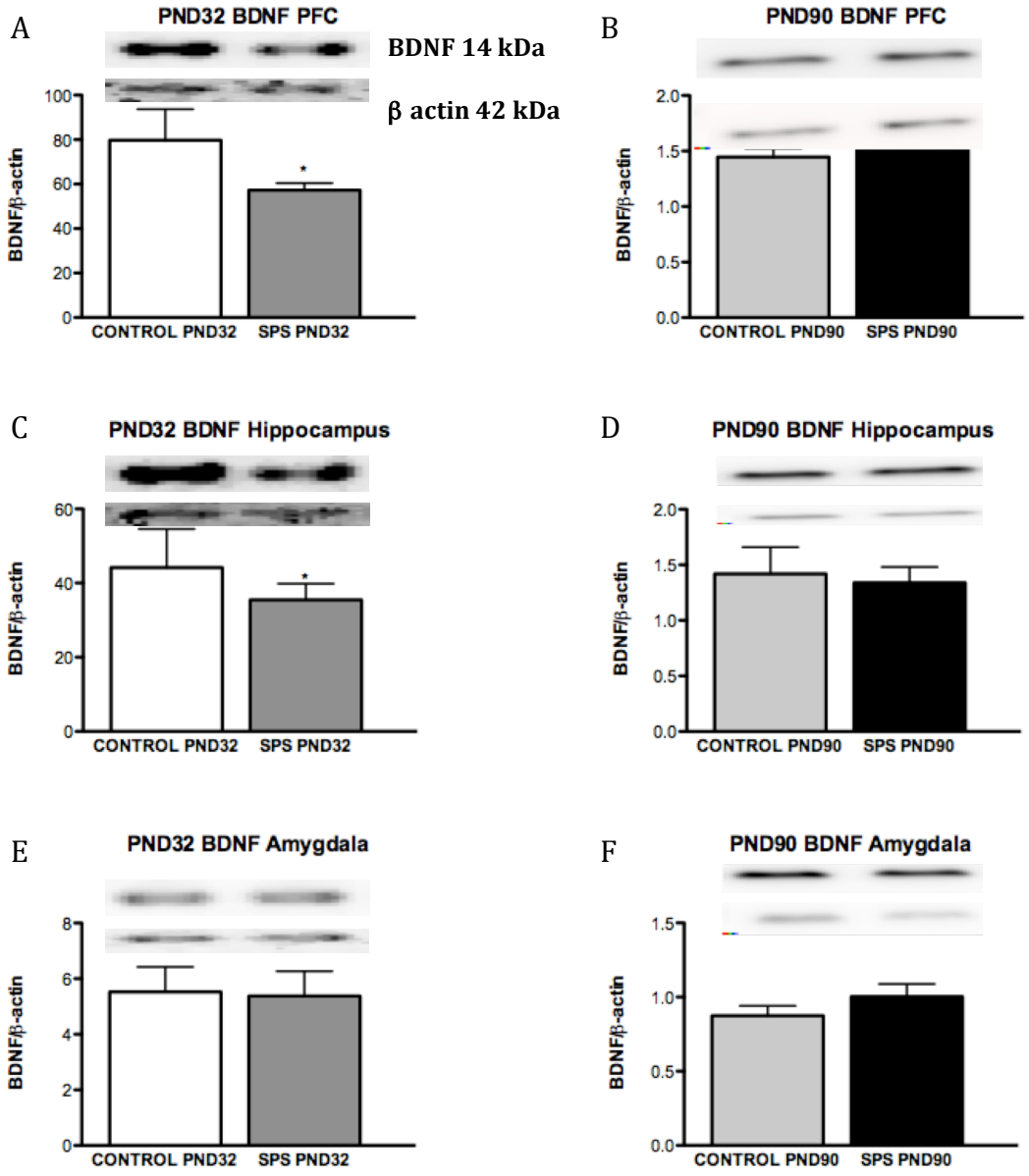


Figure 37. ***Examination of BDNF protein expression in Control/SPS rats at PND32 and 90.*** Rats subjected to early life SPS exposure showed marked decrease in expression of BDNF in PFC and hippocampus at PND32 (A and C), but no change was seen in the expression levels of BDNF in amygdala at PND32 and in PFC, hippocampus, and amygdala of the SPS rats at PND90 (E, B, D, and F) (*) $P<0.05$, significantly different form Control. Values are mean ± SEM, Control: n=10, SPS: n=30.

5. DISCUSSION

5.1 Behavioral and Cognitive Changes in SPS Rats Following Early Life SPS Exposure at PND25.

A growing body of research focusing on childhood trauma has established the correlation between early life experiences and later life health outcomes. Early life traumatic experiences, including physical, psychological, or combined, alter susceptibility to mood disorders in later life, especially major depression (Scheller-Gilkey, Moynes et al. 2004, Brunson, Kramar et al. 2005, Pietrek, Elbert et al. 2013). Although negative consequences of early life trauma are well recognized (Kessler, Davis et al. 1997), yet, many aspects remain unknown. For example, what is the most vulnerable age when early life trauma exerts most severe and long-lasting effects? Whether early life trauma causes one psychiatric condition such as anxiety or depression and whether one or both persist over time or does one disorder transform into another? Whether early life trauma causes learning and memory impairments? Are these deficits permanent? Answers to these questions cannot be simply sought, as conducting these studies in humans are quite challenging. Although animal models cannot accurately reveal the impact of early life traumatic events on psychiatric symptoms occurring in later life, rodent models are excellent tools that can provide useful insights. In this study, using a modified version of a previously published model of PTSD (SPS)(Patki, Li et al. 2014), we have examined the role of early life stress in the regulation of behavioral and cognitive function as well as examined the phenomenon of resilience. After exposing the rats to SPS at PND25 (equivalent to human childhood), behavioral tests were conducted at PND32

(equivalent to early human adolescent or late childhood), PND60 (equivalent to early human adulthood or late adolescent), and PND90 (equivalent to human adulthood) (Sengupta 2013). Learning and memory function also was analyzed at each stage.

We observed that early life SPS induced anxiety-like behavior and short-term memory impairment at initial stages of development (PND32 and 60). When the SPS rats reached the adult stage, anxiety-like behavior evident at PND32 and 60 transformed into depression-like behavior at PND90. The short-term memory impairment observed at PND32, and 60 was not evident at PND90. Early life stress is well known to affect memory (Raine, Park et al. 2001, Morris, Le et al. 2016). Memory function deficit observed in our study during the PND32-60 period may be due to a stress-induced elevation of glucocorticoids, which can accelerate hippocampal cell loss because of stress-induced acceleration of neurotoxicity mechanisms (Finsterwald and Alberini 2014). Restorative processes (Hoffman, Krigbaum et al. 2011) occurring later in the developmental course most likely dominate over toxicity mechanisms and hence restore learning and memory mechanisms at PND90. Therefore, STM and LTM were no longer evident at PND90. Interestingly, there appears to be a critical period between PND32-PND60 where anxiety-like behavior switches to depression-like behavior. PND60 also seems to be a critical window when cognitive features become permanent, beyond which learning cannot be compromised, and memories are solidified. This data has clinical relevance as it informs us of a behaviorally sensitive period during which potentially

critical biological changes occur, which are responsible for triggering the onset of depression in the later course of life.

Furthermore, at PND90, one subgroup of SPS rats were more susceptible to depression-like behavior. Using our FST data collected at PND90, we tracked the behavioral performance of each rat in each behavioral test. The idea was to gain information about the developmental stage at which behavioral impairments become evident before the occurrence of susceptible and resilient phenotypes. We observed that resilient rats showed heightened anxiety-like behavior at both PND32 and 60, and depression-like behavior at PND60. However, the susceptible rats did not show anxiety and depression-like behavior at PND32 but exhibited anxiety-like behavior at PND60. Our data indicate that after early life exposure to SPS, rats at earlier stages (PND32 and 60) showed an onset of anxiety-like behavior but did not continue to have a behavioral impairment at PND90. However, those rats that remained normal at earlier stages (PND32) showed anxiety-like behavior at PND60 and later continued to have depression-like behavior at PND90.

Behavioral and cognitive analysis conducted throughout developmental stages after initial exposure to early life stress has high clinical relevance since our data suggest that exposure to early life SPS leads to behavioral alterations. For some rats, the alterations seem to be temporary; while the alterations for some rats seems to be permanent and has long-last neurobiological mechanisms in specific brain regions underlying the behavioral alterations. Even though the exact underlying mechanisms are unknown, biological disturbances in brain regions such as PFC, hippocampus, and amygdala seem plausible. Previous studies from our lab indicated

that increased levels of oxidative stress in PFC, hippocampus, and amygdala are associated with behavioral and cognitive deficits (Patki et al. 2013b). PFC is responsible for executive functions such as decision making, social and emotional controls (Wen-Jun Gao et al. 2012). PFC is also one of the most vulnerable regions to environmental factors, and dysfunctions in this brain region are associated with many cognitive and behavioral disorders such as schizophrenia, depression, substance addiction, autism, and anxiety (Goldman-Rakic. 1999). Hippocampus modulates memory formation and stress regulation (Leuner and Gould. 2010). The amygdala plays a significant role in the regulation of emotions and behaviors (Shin and Liberzon 2010). Thus it is possible that elevated levels of oxidative stress in these brain regions triggers redox-sensitive signaling pathways that eventually result in behavioral and cognitive impairments. Involvement of epigenetic modifications in the regulation of this behavioral phenotype seems an attractive possibility. Perhaps, epigenetic modifications occur in specific brain areas at the particular developmental period of life, positively regulating anxio-depressive circuits (Stankiewicz, Swiergiel et al. 2013) to cause recovery of anxiety-like behavior. Reports of an interaction between individual genetic vulnerabilities and environmental risk factors are already available (Wu, Feder et al. 2013, Zannas and West 2014, Nestler 2016). Thus, stress in early life can potentially affect neurodevelopmental processes by epigenetically modifying gene expression during specific developmental stages, permanently changing brain structure and function. Our next step was to investigate the effects of early life stress on oxidative stress mechanisms and their correlations with behavioral and cognitive phenotypes.

5.2 Biochemical Alterations in SPS Rats Following Early Life SPS Exposure at PND25

Psychological stress induced by chronic social defeat and single prolonged stress is known to be associated with elevated oxidative stress as well as behavioral deficits in rats (Solanki et al. 2017; Patki et al. 2014). In our study, we found that the behavioral alterations caused by early life SPS exposure led to long-lasting behavioral deficits. Therefore SPS exposure in early life could lead to long-lasting oxidative stress as well. As mentioned previously, PFC, hippocampus, and amygdala are the core regions of the brain that regulate memory, social and emotional functions, and coordinate stress responses (Wen-Jun Gao et al. 2012; Leuner and Gould. 2010; Shin and Liberzon 2010). These regions are sensitive to stressful stimuli, especially during developmental stages (Bremner 2006). Knowing that normal brain undergoes structural and functional changes across the lifespan, we think that trauma at different stages of life may have different effects on the developing brain.

In the measurement of oxidative stress levels after early life stress exposure, different markers of oxidative stress were examined. Increased levels of free radicals in the body due to increase in oxidative stress lead to oxidation of free fatty acids and production of isoprostanes (Betteridge 2000). Therefore, the level of plasma 8-isoprostane is used as biomarkers of oxidative stress. After early life exposure to SPS at PND25, we measured 8-isoprostane levels in plasma samples of the rats at PND32 and PND90. Plasma 8-isoprostane levels remained unaltered at

both PND32 and PND90 following SPS exposure at PND25. On the other hand, under oxidative stress conditions, the build-up of free radicals can promote the formation of carbonyl groups, which is relatively stable and can be permanent. The carbonyl groups can subsequently react by 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. Antibody against DNPH-derivatized proteins can identify carbonylated proteins (Suzuki, Carini, and Butterfield. 2009). So we measured the level of protein carbonylation in brain regions of the rats at PND32 and 90. Neuronal protein carbonylation levels were elevated in PFC of the SPS rats at both PND32 and PND90. However, early life SPS exposure did not change protein carbonylation levels in hippocampus and amygdala at PND32 or 90. The changes in protein carbonylation levels confirms our previous hypothesis that early life stress can induce the build-up of oxidative stress in brain regions that are more vulnerable to free radicals, in this case, in the PFC region only. This oxidative stress is relatively permanent, presented at PND32 and persisted into PND90. Systemic oxidative stress was not observed along with neuronal oxidative stress. The unaltered systemic oxidative stress markers is possibly due to compensatory mechanisms that restored the systemic oxidative stress levels. While neuronal oxidative stress is more permanent, as in this case, PFC continued to show increased oxidative stress levels at PND32 and 90. Interestingly, the two clusters we found in the SPS group based on immobility time in FST, namely depressive and nondepressive, had considerable overlap with the high carbonylation and low carbonylation clusters we found in protein carbonylation assays. The association bwteen depressive phenotype and high level of protein carbonylation levels suggests that SPS in early life leads to long lasting-

behavioral deficits and augments neuronal oxidative stress levels in the PFC in subgroups of SPS rats. The overlap between these two subgroups indicates the behavioral deficits are strongly correlated with increased neuronal oxidative stress in PFC.

It is well established that build up of oxidative stress can mediate injury by introducing modifications to macromolecules such as proteins, lipids, and DNAs (Halliwell 2007; Pham-huy et al. 2008; Adler et al. 1999). These modifications change the structure and function of these macromolecules, which are believed to be the deleterious effects of oxidative stress. However, these modifications are now viewed as biological signals that can initiate signaling pathways in response to oxidative stress to maintain cellular homeostasis (Adler et al. 1999). Oxidative stress can be caused by overproduction of reactive oxygen species or decrease in the production or activity of antioxidants (Salim et al. 2014). Since we observed increased oxidative stress markers in the brain, we hypothesized that the increase in oxidative stress might be caused by the decreased generation of antioxidants. In agreement with this, measurements of protein expression levels of Cu/Zn SOD, catalase, and HO-1 revealed a decrease in these enzyme levels. Moreover, the decreases in these antioxidant enzymes were strongly correlated with increased levels of neuronal oxidative stress in PFC as well as the presence of depression-like behavior at PND90. However, the protein expression levels of Cu/Zn SOD, Mn SOD, catalase, and HO-1 were increased in SPS rats at PND32. The reason for the observed increase in antioxidant enzymes initially at PND32 might be a compensatory response that was activated upon the initial increase in free radicals.

An increase in neuronal oxidative stress was still observed despite the increase in antioxidant enzymes at PND32 suggests that the balance between oxidants and antioxidants was not achieved. The free radicals might continue to build up at this stage due to this imbalance.

The increase in oxidative stress leads to modifications of macromolecules, especially in proteins, that act as signals of stress to initiate biological responses. In this study, there were changes in the expression levels of antioxidant enzymes such as Cu/Zn SOD, Mn SOD, catalase, and HO-1. These antioxidant enzymes are known to be regulated by transcription factor Nrf2. Interestingly, Nrf2 is one of the most crucial redox-sensitive transcription factors. Therefore it seems plausible that early life SPS exposure leads to elevation of reactive oxygen species, which subsequently affects Nrf2 pathway and lead to alterations in the expression levels of Nrf2 and consequent Nrf2-dependent ARE antioxidant enzyme expression levels. Therefore, we measured Nrf2 levels in response to early life SPS exposure. As expected, a decrease in Nrf2 level was observed in PFC in SPS rats at PND90, but not at PND32. The changes in Nrf2 levels suggest that early life SPS exposure induced changes in Nrf2 levels in PFC at PND90, which can explain the observed decrease antioxidants enzymes in PFC at PND90 as well as the long-lasting depression-like behavior.

Epigenetic modifications are currently acknowledged to be plausible biological mechanisms that predispose an individual to later life adverse health conditions in response to early life adversities (Mitchell, Scheneper, and Notterman. 2015). DNMTs and HDACs are the main enzymes that mediate epigenetic modifications to repress gene transcription. Many studies indicate that decreased

Nrf2 expression under the diseased condition is caused by hypermethylation in the promoter region of Nrf2 (Khor et al. 2014). Therefore, it is possible that the decrease in Nrf2 is caused by epigenetic suppression and hypermethylation in the promoter region. In agreement with this, we observed increase levels of DNMT1 and HDAC2 in PFC regions of SPS rats at PND90. However, the methylation levels in the promoter region of Nrf2 gene in PFC regions of SPS rats were not significantly different from control rats. The unaltered methylation levels in the promoter region of Nrf2 suggests that changes in methylation levels in the promoter region of Nrf2 are not the reasons for the decrease in Nrf2 or the decrease in levels of antioxidant enzymes. The decrease in Nrf2 might be via histone modifications or other unknown mechanisms.

In addition to the Nrf2 pathway, NF-κB pathway is another critical transcription factor that responds directly to reactive oxygen species to regulate inflammatory genes such as tumor necrosis factor (TNF) and interleukins (IL) (Bai Yang et al. 2015; Baldwin 2001). Moreover, studies are suggesting that there is crosstalk between Nrf2 and NF-κB pathway. The activation of Nrf2 pathway suppresses the activation of NF-κb pathway, and vice versa (Wakabayashi et al. 2010). Upon increased oxidative stress, which pathway is activated depends on the level of oxidative stress. When there is a low level of oxidative stress, the Nrf2 pathway will be activated to enhance the production of the antioxidant enzymes and to maintain homeostasis. However, in situations of higher levels of oxidative stress, NF-κB is activated, to produce more pro-inflammatory cytokines as well as to suppress Nrf2 mediated pro-survival pathway (Wardyn et al. 2015). It is possible

that early life SPS exposure leads to oxidative stress which activates NF-κB pathway, increase pro-inflammatory cytokines and suppress Nrf2 pathway. Therefore, we measured the levels of NF-κB and pro-inflammatory cytokines including IL-6 and TNF-α in PFC region of the rat brains. In agreement with the hypothesis, the levels of NF-κB and IL-6 were increased in PFC of SPS rats at both PND32 and 90. An increase in the levels of TNF-α was observed in PFC of SPS rats at PND32, but not at PND90.

Early life stress has been linked with structural alterations in stress-sensitive brain regions that are essential for regulation of behavior. Adverse early life experiences decrease synaptogenesis and BDNF (Liu et al. 2000). BDNF is a member of neurotrophic factors, which is crucial for neurogenesis, synaptic structure and neuronal plasticity (Ghosh and Greenberg. 1995). Both BDNF and oxidative stress are reported to be involved in the pathophysiology of mental disorders such as schizophrenia. A negative correlation between BDNF and antioxidant defense system has also been observed in patients with mental disorders (Xiang Yang Zhang et al. 2014), suggesting oxidative stress may negatively affect the BDNF system, contributing to the behavioral and cognitive impairments in mental disorders. So it is possible that the increased oxidative stress observed in SPS rats may be accompanied by decreased BDNF system. Therefore, we measured BDNF levels in rats following early life exposure to SPS at both PND32 and 90. The levels of BDNF were decreased in PFC and hippocampus of SPS rats at PND32, but not in the amygdala samples. No difference was observed in the levels of BDNF in all three brain regions of SPS rats at PND90. The changes in BDNF leves indicates that early

life stress leads to decreased levels of BDNF, which affects the neuronal development in this early developmental stage and predisposes the rats to later life behavioral and cognitive impairments.

In conclusion, our study examined the psychological and biological effects of early life stress using a rat model. We followed up the rats' behavioral performances from PND32-PND90 (mimicking human childhood to adulthood period) after a one-time exposure to SPS at PND25. By identifying the resilient and susceptible subgroups in rats that were exposed to early life stress and tracking their behavioral performances, we were able to map the behavioral phenotypes throughout the lifespan. Levels of oxidative stress markers and antioxidant enzymes were also tracked for both resilient and susceptible rats. Depressive phenotype has a positive correlation with the levels of oxidative stress marker, but a negative correlation with the levels of antioxidant enzymes. Oxidative stress-induced cellular responses, such as Nrf2 and NF-κB, were also found altered in SPS rats. The postulation of this study is that early life SPS exposure regulates cellular responses, including NF-κB and Nrf2. NF-κB was activated, which induced pro-inflammatory responses and dampened Nrf2 mediated anti-oxidative responses. These alterations resulted in build-up of oxidative stress and long-lasting behavioral impairments (**Figure 37**).

Our study provides a premise for future investigations of various underlying cellular pathways that might be involved in the development of disorders in response to early life stress. We investigated the behavioral phenotypes and biochemical phenotypes that are related to early life stress exposure. We also tracked each rat for the behavioral performance and biochemical alterations at

different developmental stages. As mentioned in section 2, in response to oxidative stress, many redox-sensitive transcriptional pathways are activated. In this case, we measured Nrf2 and NF-κB. There are many other transcription factors also reported being the primary targets of oxidative stress, such as activator protein 1 (Surh et al. 2005), protein 53, glucocorticoid receptor, and estrogen receptor (Mohora et al. 2009). It will be interesting to know how these redox-sensitive transcription factors are regulated in response to early life stress-induced oxidative stress.

6. LIMITATIONS AND FUTURE DIRECTIONS

We recognize the limitations of our study. For example, the DNA methylation assays were conducted in the samples collected from rats at PND32, but not at PND60 or 90. Whether age exerts differential effects on DNA methylation lead to changes in gene expression and subsequent behavioral phenotypes was not tested in the present study. Therefore, in future studies, examining methylation profiles between PND32-PND90 would be important. Examining methylation pattern within the promoter region of Nrf2 also would be interesting.

In previous studies from our lab, oxidative stress was reported to be accompanied with mitochondria impairment, which was postulated to be responsible for the alteration in mitochondrial dynamics and cognitive deficits. In the present study, we did not check the involvement of mitochondrial function in response to early life stress and oxidative stress. It is known that the mitochondrial Krebs cycle, which generates energy from oxidative reactions, can produce intermediates such as 2-2 oxoglutarate, citrate, and fumarate. These intermediates are reported to regulate gene expression involving epigenetic modification patterns. Therefore, disruption in mitochondrial function can possibly disturb Krebs cycle

and change epigenetic regulation patterns in certain genes (Manev and Dzitoyeva, 2013). In future studies, mitochondrial function also need to be examined following early life stress exposure.

The samples used in the present study were isolated from the prefrontal cortex, hippocampus, and the amygdala. All the biochemical experiments were performed using the tissue homogenates of these brain tissues. It is well known that there are different cell types within the same brain area forming unique circuits, leading to different functions. For instance, glutamatergic neurons and GABAergic in the same region of the brain exhibit different response to stimuli (Alitto and Dan, 2012). Therefore, it is possible that the changes induced by early life stress might only present in certain population of neurons . We measured protein carbonylation levels and the antioxidant enzymes levels in the whole brain regions, and we found there are associations between increased oxidative stress levels and depression-like behaviors. However, we are not able to predict if the changes in oxidative stress markers and antioxidant enzymes were in the same neurons in the PFC of the depressive rats.

We observed a decrease in the level of Nrf-2 expression levels, which might explain for the decrease in antioxidant enzyme levels and increased oxidative stress. However, we did not find changes in the methylation levels in the promoter regions of Nrf-2 in the PFC of SPS rats. One reason may be that DNA hypermethylation in the promoter region of Nrf-2 is not responsible for the decreased level of Nrf-2. Another possibility is that the promoter region selected for methylation assays is not the only region that was hypermethylated in response to early life stress. It is well established in cancer research that hypermethylation in the enhancer sequences correlates with decreased gene expression better than methylation levels in the promoter regions. Therefore, the fact that there were no changes in the methylation levels in the promoter region of Nrf-2 is not enough to rule out the possibility that

Nrf-2 can be methylated in response to early life exposure to SPS. Future studies need to address the methylation status of different regulatory regions in Nrf2, perhaps to predict unexplored biomarkers for stress susceptibility.

7. SUMMARY AND CONCLUSIONS

1. Our findings suggest that early life stress caused long-lasting behavioral deficits and biochemical changes in rats.
2. Two phenotypes were obtained, resilient and the susceptible. We suggest that in the resilient phenotype the response to early life stress is normal while in the susceptible phenotype the stress response system is defective. Moreover, the biochemical reason behind this defective stress responsiveness is the oxidant-antioxidant-inflammation connection.
3. The oxidative stress level (indicated by protein carbonylation level) and the decrease in antioxidant enzyme expression levels selectively in PFC but not in the hippocampus or the amygdala, positively correlated with the depressive phenotype of SPS rats.
4. The oxidant-antioxidant imbalance seems to play a key role in mediating the depressive phenotype.
5. The depressive phenotype was associated with a decrease in Nrf-2 and an increase in NF-kB pathway. The association between Nrf-2 pathway and NF-κB pathway is an interesting relationship. It seems that early life stress induces an initial rise in oxidative stress, which causes activation of both redox-sensitive transcription factors, Nrf-2 and NF-kB, however, one

dominates over the other and NF-kB with its pro-damage effects supersedes the pro-survival act of Nrf2.

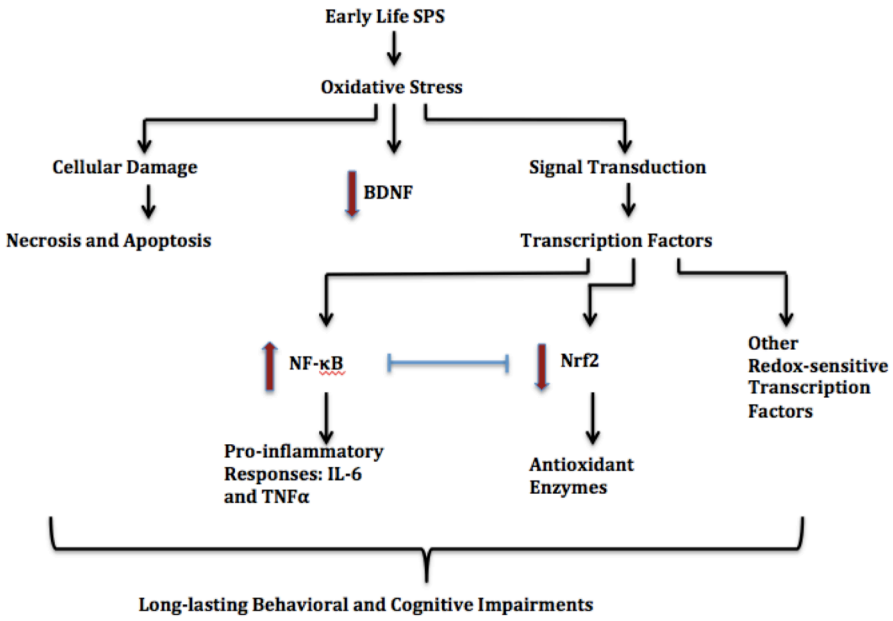


Figure 38. Schematic showing the regulation of cellular responses mediated by oxidative stress induced oxidative stress.

8. REFERENCES

Ader, R., Effects of early experience and differential housing on behavior and susceptibility to gastric erosions in the rat. Journal of Comparative and Physiological Psychology, 1965. ***60****(2): p. 233-238.*

Adler, A., et al., Gonadectomy in adult life increases tyrosine hydroxylase immunoreactivity in the prefrontal cortex and decreases open field activity in male rats. Neuroscience, 1999. ***89****(3): p. 939-54.*

Aguilera, G. and C. Rabadan-Diehl, Vasopressinergic regulation of the hypothalamic-pituitary-adrenal axis: implications for stress adaptation. Regul Pept, 2000. ***96****(1-2): p. 23-9.*

Akyol, M., et al., The effects of vitamin E on the skin lipid peroxidation and the clinical improvement in vitiligo patients treated with PUVA. Eur J Dermatol, 2002. ***12****(1): p. 24-6.*

Al-Abrash, A.S., F.A. Al-Quobaili, and G.N. Al-Akhras, Catalase evaluation in different human diseases associated with oxidative stress. Saudi Med J, 2000. ***21****(9): p. 826-30.*

Allam, F., et al., Grape powder supplementation prevents oxidative stress-induced anxiety-like behavior, memory impairment, and high blood pressure in rats. J Nutr, 2013. ***143****(6): p. 835-42.*

Alleva, E. and N. Francia, Psychiatric vulnerability: suggestions from animal models and role of neurotrophins. Neurosci Biobehav Rev, 2009. ***33****(4): p. 525-36.*

Altuntas, I., et al., Erythrocyte superoxide dismutase and glutathione peroxidase activities, and malondialdehyde and reduced glutathione levels in

schizophrenic patients. Clin Chem Lab Med, 2000. ***38****(12): p. 1277-81.*

Anda, R.F., et al., The enduring effects of abuse and related adverse experiences in childhood: A convergence of evidence from neurobiology and epidemiology. European archives of psychiatry and clinical neuroscience, 2006. ***256****(3): p. 174-186.*

Andersen, S.L. and M.H. Teicher, Stress, sensitive periods and maturational events in adolescent depression. Trends Neurosci, 2008. ***31****(4): p. 183-91.*

Anway, M.D., et al., Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science, 2005. ***308****(5727): p. 1466-9.*

Arroll, B., et al., Antidepressants versus placebo for depression in primary care. Cochrane Database Syst Rev, 2009(3): p. Cd007954.

Bai, Y., et al., Sulforaphane Protects against Cardiovascular Disease via Nrf2 Activation. Oxidative Medicine and Cellular Longevity, 2015. ***2015****: p. 13.*

Baldwin, A.S., Jr., Series introduction: the transcription factor NF-kappaB and human disease. J Clin Invest, 2001. ***107****(1): p. 3-6.*

Bandelow, B., et al., Early traumatic life events, parental attitudes, family history, and birth risk factors in patients with borderline personality disorder and healthy controls. Psychiatry Res, 2005. ***134****(2): p. 169-79.*

Bellezza, I., A.L. Mierla, and A. Minelli, Nrf2 and NF-kappaB and Their Concerted Modulation in Cancer Pathogenesis and Progression. Cancers (Basel), 2010. ***2****(2): p. 483-97.*

Ben Othmen, L., et al., Altered antioxidant defense system in clinically stable patients with schizophrenia and their unaffected siblings. Prog Neuropsychopharmacol

Biol Psychiatry, 2008. ***32****(1): p. 155-9.*

Beutel, A., et al., F<sc>lexi</sc>F<sc>a</sc>CT: Scalable Flexible Factorization of Coupled Tensors on Hadoop, in Proceedings of the 2014 SIAM International Conference on Data Mining. p. 109-117.

Biederman, J., Attention-deficit/hyperactivity disorder: a selective overview. Biol Psychiatry, 2005. ***57****(11): p. 1215-20.*

Birben, E., et al., Oxidative Stress and Antioxidant Defense. The World Allergy Organization journal, 2012. ***5****(1): p. 9-19.*

Blackwell, T.S., et al., In vivo antioxidant treatment suppresses nuclear factor-kappa B activation and neutrophilic lung inflammation. J Immunol, 1996. ***157****(4): p. 1630-7.*

Boersma, G.J. and K.L. Tamashiro, Individual differences in the effects of prenatal stress exposure in rodents. Neurobiology of Stress, 2015. ***1****: p. 100-108.*

Boksa, P., Maternal infection during pregnancy and schizophrenia. J Psychiatry Neurosci, 2008. ***33****(3): p. 183-5.*

Borrell, J., et al., Prenatal immune challenge disrupts sensorimotor gating in adult rats. Implications for the etiopathogenesis of schizophrenia. Neuropsychopharmacology, 2002. ***26****(2): p. 204-15.*

Boskovic, M., et al., Oxidative stress in schizophrenia. Curr Neuropharmacol, 2011. ***9****(2): p. 301-12.*

Boyd, R., H. Gintis, and S. Bowles, Coordinated Punishment of Defectors Sustains Cooperation and Can Proliferate When Rare. Science, 2010. ***328****(5978): p. 617-620.*

Bradley, R.G., et al., Influence of child abuse on adult depression: moderation by the corticotropin-releasing hormone receptor gene. Arch Gen Psychiatry, 2008. ***65****(2): p. 190-200.*

Branchi, I., et al., Early interactions with mother and peers independently build adult social skills and shape BDNF and oxytocin receptor brain levels. Psychoneuroendocrinology, 2013. ***38****(4): p. 522-532.*

Branchi, I., et al., Early social enrichment shapes social behavior and nerve growth factor and brain-derived neurotrophic factor levels in the adult mouse brain. Biol Psychiatry, 2006. ***60****(7): p. 690-6.*

Brunson, K.L., et al., Mechanisms of Late-Onset Cognitive Decline after Early-Life Stress. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2005. ***25****(41): p. 9328-9338.*

Cadenas, E. and K.J. Davies, Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med, 2000. ***29****(3-4): p. 222-30.*

Caldji, C., et al., Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proceedings of the National Academy of Sciences, 1998. ***95****(9): p. 5335-5340.*

Candas, D. and J.J. Li, MnSOD in oxidative stress response-potential regulation via mitochondrial protein influx. Antioxid Redox Signal, 2014. ***20****(10): p. 1599-617.*

Cannon, C.P., et al., Safety of Anacetrapib in Patients with or at High Risk for Coronary Heart Disease. New England Journal of Medicine, 2010. ***363****(25): p. 2406-2415.*

Carpenter, L.L., et al., Association between plasma IL-6 response to acute stress and early-life adversity in healthy adults. Neuropsychopharmacology, 2010. ***35****(13):*

p. 2617-23.

Carroll, A., M.J. Nash, and J. Thachil, Haemophilia stress fractures – an increasing complication with better orthopaedic care. Haemophilia, 2013. ***19****(3): p. e178-e180.*

Caspi, A., et al., Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science, 2003. ***301****(5631): p. 386-9.*

Cheresh, P., et al., Oxidative Stress and Pulmonary Fibrosis. Biochimica et biophysica acta, 2013. ***1832****(7): p. 1028-1040.*

Chilvers, C., et al., Antidepressant drugs and generic counselling for treatment of major depression in primary care: randomised trial with patient preference arms. Bmj, 2001. ***322****(7289): p. 772-5.*

Choi, A.M. and J. Alam, Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol, 1996. ***15****(1): p. 9-19.*

Cichoz-Lach, H. and A. Michalak, Oxidative stress as a crucial factor in liver diseases. World J Gastroenterol, 2014. ***20****(25): p. 8082-91.*

Cirulli, F., et al., Early life stress as a risk factor for mental health: role of neurotrophins from rodents to non-human primates. Neurosci Biobehav Rev, 2009. ***33****(4): p. 573-85.*

Cirulli, F., et al., Early life stress as a risk factor for mental health: role of neurotrophins from rodents to non-human primates. Neurosci Biobehav Rev, 2009. ***33****(4): p. 573-85.*

Coe, C.L., L.T. Rosenberg, and S. Levine, Effect of maternal separation on the

complement system and antibody responses in infant primates. Int J Neurosci, 1988. ***40****(3-4): p. 289-302.*

Cui, H., Y. Kong, and H. Zhang, Oxidative Stress, Mitochondrial Dysfunction, and Aging. Journal of Signal Transduction, 2012. ***2012****: p. 13.*

Danese, A. and B.S. McEwen, Adverse childhood experiences, allostasis, allostatic load, and age-related disease. Physiol Behav, 2012. ***106****(1): p. 29-39.*

Danese, A. and S. J Lewis, Psychoneuroimmunology of Early-Life Stress: The Hidden Wounds of Childhood Trauma? Neuropsychopharmacology, 2016. ***42****: p. 99.*

Danese, A., et al., Biological embedding of stress through inflammation processes in childhood. Molecular Psychiatry, 2010. ***16****: p. 244.*

Daskalakis, N.P., et al., Early Life Stress Effects on Glucocorticoid—BDNF Interplay in the Hippocampus. Frontiers in Molecular Neuroscience, 2015. ***8****: p. 68.*

Dell, P.A. and F.D. Rose, Transfer of effects from environmentally enriched and impoverished female rats to future offspring. Physiology & Behavior, 1987. ***39****(2): p. 187-190.*

Di Meo, S., et al., Role of ROS and RNS Sources in Physiological and Pathological Conditions. Oxid Med Cell Longev, 2016. ***2016****: p. 1245049.*

Dietrich-Muszalska, A., et al., The Oxidative Stress May be Induced by the Elevated Homocysteine in Schizophrenic Patients. Neurochemical Research, 2012. ***37****(5): p. 1057-1062.*

Dinkova-Kostova, A.T., et al., Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. Proc Natl Acad Sci U S A, 2005. ***102****(12): p. 4584-9.*

Dong, M., et al., The interrelatedness of multiple forms of childhood abuse, neglect, and household dysfunction. Child Abuse Negl, 2004. ***28****(7): p. 771-84.*

Elliott, E., et al., Resilience to social stress coincides with functional DNA methylation of the Crf gene in adult mice. Nat Neurosci, 2010. ***13****(11): p. 1351-3.*

Elliott, E., et al., Resilience to social stress coincides with functional DNA methylation of the Crf gene in adult mice. Nat Neurosci, 2010. ***13****(11): p. 1351-3.*

Entringer, S., C. Buss, and C. Heim, [Early-life stress and vulnerability for disease in later life]. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz, 2016. ***59****(10): p. 1255-61.*

Fatemi, S.H. and T.D. Folsom, The neurodevelopmental hypothesis of schizophrenia, revisited. Schizophr Bull, 2009. ***35****(3): p. 528-48.*

Finkelhor, D., The prevention of childhood sexual abuse. Future Child, 2009. ***19****(2): p. 169-94.*

Finsterwald, C. and C.M. Alberini, Stress and glucocorticoid receptor-dependent mechanisms in long-term memory: from adaptive responses to psychopathologies. Neurobiol Learn Mem, 2014. ***112****: p. 17-29.*

Fischer, R. and O. Maier, Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. Oxid Med Cell Longev, 2015. ***2015****: p. 610813.*

Fortier, M.E., et al., Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring. J Psychiatr Res, 2004. ***38****(3): p. 335-45.*

Fukui, K., et al., Impairment of learning and memory in rats caused by oxidative stress

and aging, and changes in antioxidative defense systems. Ann N Y Acad Sci, 2001. ***928****: p. 168-75.*

Ganesh Yerra, V., et al., Potential therapeutic effects of the simultaneous targeting of the Nrf2 and NF-κB pathways in diabetic neuropathy(). Redox Biology, 2013. ***1****(1): p. 394-397.*

Gardner, K.L., et al., Adverse early life experience and social stress during adulthood interact to increase serotonin transporter mRNA expression. Brain research, 2009. ***1305****: p. 47-63.*

Gershon, N.B. and P.C. High, Epigenetics and child abuse: Modern-day Darwinism--The miraculous ability of the human genome to adapt, and then adapt again. Am J Med Genet C Semin Med Genet, 2015. ***169****(4): p. 353-60.*

Ghosh, A. and M.E. Greenberg, Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science, 1995. ***268****(5208): p. 239-47.*

Gilbert, R., et al., Burden and consequences of child maltreatment in high-income countries. Lancet, 2009. ***373****(9657): p. 68-81.*

Gold, P.W., F.K. Goodwin, and G.P. Chrousos, Clinical and biochemical manifestations of depression. Relation to the neurobiology of stress (2). N Engl J Med, 1988. ***319****(7): p. 413-20.*

Goldman-Rakic, P.S., The physiological approach: functional architecture of working memory and disordered cognition in schizophrenia. Biol Psychiatry, 1999. ***46****(5): p. 650-61.*

Gonzalez, A. and A.S. Fleming, Artificial rearing causes changes in maternal behavior and c-fos expression in juvenile female rats. Behavioral Neuroscience, 2002.

116*(6): p. 999-1013.*

Gorrini, C., I.S. Harris, and T.W. Mak, Modulation of oxidative stress as an anticancer strategy. Nat Rev Drug Discov, 2013. ***12****(12): p. 931-47.*

Gottesman, II and D.R. Hanson, Human development: biological and genetic processes. Annu Rev Psychol, 2005. ***56****: p. 263-86.*

Grover, S.A., et al., Patient knowledge of coronary risk profile improves the effectiveness of dyslipidemia therapy: the CHECK-UP study: a randomized controlled trial. Arch Intern Med, 2007. ***167****(21): p. 2296-303.*

Guidotti, G., L. Brambilla, and D. Rossi, Cell-Penetrating Peptides: From Basic Research to Clinics. Trends in Pharmacological Sciences. ***38****(4): p. 406-424.*

Haddad, J.J., R.E. Olver, and S.C. Land, Antioxidant/pro-oxidant equilibrium regulates HIF-1alpha and NF-kappa B redox sensitivity. Evidence for inhibition by glutathione oxidation in alveolar epithelial cells. J Biol Chem, 2000. ***275****(28): p. 21130-9.*

Haddad, J.J., Redox regulation of pro-inflammatory cytokines and IkappaB-alpha/NF-kappaB nuclear translocation and activation. Biochem Biophys Res Commun, 2002. ***296****(4): p. 847-56.*

Haeffel, G.J., et al., Measuring cognitive vulnerability to depression: Development and validation of the cognitive style questionnaire. Clinical psychology review, 2008. ***28****(5): p. 824-836.*

Halliwell, B., Biochemistry of oxidative stress. Biochem Soc Trans, 2007. ***35****(Pt 5): p. 1147-50.*

Harlow, H.F., R.O. Dodsworth, and M.K. Harlow, Total social isolation in monkeys.

Proceedings of the National Academy of Sciences of the United States of America, 1965. ***54****(1): p. 90-97.*

Heim, C. and C.B. Nemeroff, Neurobiology of early life stress: clinical studies. Semin Clin Neuropsychiatry, 2002. ***7****(2): p. 147-59.*

Heim, C. and C.B. Nemeroff, The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol Psychiatry, 2001. ***49****(12): p. 1023-39.*

Hennessy, M.B., et al., Maternal separation produces, and a second separation enhances, core temperature and passive behavioral responses in guinea pig pups. Physiol Behav, 2010. ***100****(4): p. 305-10.*

Hines, P.J., Early life stress in depression susceptibility. Science, 2017. ***356****(6343): p. 1134-1135.*

Hoffman, A.N., et al., Chronic stress disrupts fear extinction and enhances amygdala and hippocampal Fos expression in an animal model of post-traumatic stress disorder. Neurobiology of learning and memory, 2014. ***112****: p. 139-147.*

Hori, M. and K. Nishida, Oxidative stress and left ventricular remodelling after myocardial infarction. Cardiovasc Res, 2009. ***81****(3): p. 457-64.*

Irish, M. and O. Piguet, The Pivotal Role of Semantic Memory in Remembering the Past and Imagining the Future. Frontiers in Behavioral Neuroscience, 2013. ***7****: p. 27.*

Jaenisch, R. and A. Bird, Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet, 2003. ***33 Suppl****: p. 245-54.*

Johnson, J.G., et al., Childhood verbal abuse and risk for personality disorders during

adolescence and early adulthood. Compr Psychiatry, 2001. ***42****(1): p. 16-23.*

Kang, K.A., et al., Epigenetic modification of Nrf2 in 5-fluorouracil-resistant colon cancer cells: involvement of TET-dependent DNA demethylation. Cell Death Dis, 2014. ***5****: p. e1183.*

Katon, W., et al., Cost-Effectiveness and Net Benefit of Enhanced Treatment of Depression for Older Adults With Diabetes and Depression. Diabetes Care, 2006. ***29****(2): p. 265-270.*

Kaufman, J., et al., Brain-derived neurotrophic factor-5-HTTLPR gene interactions and environmental modifiers of depression in children. Biol Psychiatry, 2006. ***59****(8): p. 673-80.*

Kelly-Irving, M., et al., Childhood adversity as a risk for cancer: findings from the 1958 British birth cohort study. BMC Public Health, 2013. ***13****: p. 767.*

Kendler, K.S., C.O. Gardner, and C.A. Prescott, Toward a comprehensive developmental model for major depression in women. Am J Psychiatry, 2002. ***159****(7): p. 1133-45.*

Kessler, R.C., C.G. Davis, and K.S. Kendler, Childhood adversity and adult psychiatric disorder in the US National Comorbidity Survey. Psychol Med, 1997. ***27****(5): p. 1101-19.*

Kessler, R.C., et al., Childhood adversities and adult psychopathology in the WHO World Mental Health Surveys. The British Journal of Psychiatry, 2010. ***197****(5): p. 378-385.*

Khong, H.T. and N.P. Restifo, Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol, 2002. ***3****(11): p. 999-1005.*

Kim-Fuchs, C., et al., Chronic stress accelerates pancreatic cancer growth and invasion: a critical role for beta-adrenergic signaling in the pancreatic microenvironment. Brain Behav Immun, 2014. ***40****: p. 40-7.*

Klengel, T., et al., Allele-specific FKBP5 DNA demethylation mediates gene–childhood trauma interactions. Nature Neuroscience, 2012. ***16****: p. 33.*

Kochi, C., et al., Prior treadmill exercise promotes resilience to vicarious trauma in rats. Prog Neuropsychopharmacol Biol Psychiatry, 2017. ***77****: p. 216-221.*

Koenen, K.C. and M. Uddin, FKBP5 Polymorphisms Modify the Effects of Childhood Trauma. Neuropsychopharmacology, 2010. ***35****(8): p. 1623-1624.*

Koenig, L.B., M. McGue, and W.G. Iacono, Rearing Environmental Influences on Religiousness: An Investigation of Adolescent Adoptees. Personality and individual differences, 2009. ***47****(6): p. 652-656.*

Kolber, B.J., et al., Transient early-life forebrain corticotropin-releasing hormone elevation causes long-lasting anxiogenic and despair-like changes in mice. J Neurosci, 2010. ***30****(7): p. 2571-81.*

Korashy, H.M. and A.O. El-Kadi, NF-kappaB and AP-1 are key signaling pathways in the modulation of NAD(P)H:quinone oxidoreductase 1 gene by mercury, lead, and copper. J Biochem Mol Toxicol, 2008. ***22****(4): p. 274-83.*

Kumari, P., et al., An essential role for maternal control of Nodal signaling. eLife, 2013. ***2****: p. e00683.*

Laviola, G., et al., Beneficial effects of enriched environment on adolescent rats from stressed pregnancies. Eur J Neurosci, 2004. ***20****(6): p. 1655-64.*

Laviola, G., et al., Gene-environment interaction during early development in the

heterozygous reeler mouse: clues for modelling of major neurobehavioral syndromes. Neurosci Biobehav Rev, 2009. ***33****(4): p. 560-72.*

Law, C.T. and J.I. Gold, Reinforcement learning can account for associative and perceptual learning on a visual-decision task. Nat Neurosci, 2009. ***12****(5): p. 655-63.*

Lee, J., W. Duan, and M.P. Mattson, Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. J Neurochem, 2002. ***82****(6): p. 1367-75.*

Lee, P.R., et al., Prenatal stress generates deficits in rat social behavior: Reversal by oxytocin. Brain Res, 2007. ***1156****: p. 152-67.*

Lehmann, J. and J. Feldon, Long-term biobehavioral effects of maternal separation in the rat: consistent or confusing? Rev Neurosci, 2000. ***11****(4): p. 383-408.*

Lesch, K.P., et al., Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science, 1996. ***274****(5292): p. 1527-31.*

Leuner, B. and E. Gould, Structural plasticity and hippocampal function. Annu Rev Psychol, 2010. ***61****: p. 111-40, c1-3.*

Levine, S., Developmental determinants of sensitivity and resistance to stress. Psychoneuroendocrinology, 2005. ***30****(10): p. 939-46.*

Lippmann, M., et al., Long-term behavioural and molecular alterations associated with maternal separation in rats. Eur J Neurosci, 2007. ***25****(10): p. 3091-8.*

Liu, G.H., J. Qu, and X. Shen, NF-kappaB/p65 antagonizes Nrf2-ARE pathway by

depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. Biochim Biophys Acta, 2008. ***1783****(5): p. 713-27.*

Liu, H., et al., Behavioral and cognitive impact of early life stress: Insights from an animal model. Prog Neuropsychopharmacol Biol Psychiatry, 2017. ***78****: p. 88-95.*

Lorenz, K.Z., The Companion in the Bird's World. The Auk, 1937. ***54****(3): p. 245-273.*

Loria, A.S., D.H. Ho, and J.S. Pollock, A mechanistic look at the effects of adversity early in life on cardiovascular disease risk during adulthood. Acta physiologica (Oxford, England), 2014. ***210****(2): p. 277-287.*

Lupien, S.J., et al., Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci, 2009. ***10****(6): p. 434-45.*

Lutgendorf, S.K., A.K. Sood, and M.H. Antoni, Host factors and cancer progression: biobehavioral signaling pathways and interventions. J Clin Oncol, 2010. ***28****(26): p. 4094-9.*

Lyons-Ruth, K., E. Bronfman, and E. Parsons, Atypical attachment in infancy and early childhood among children at developmental risk. IV. Maternal frightened, frightening, or atypical behavior and disorganized infant attachment patterns. Monogr Soc Res Child Dev, 1999. ***64****(3): p. 67-96; discussion 213-20.*

Lyons, D.M., K.J. Parker, and A.F. Schatzberg, Animal Models of Early Life Stress: Implications for Understanding Resilience. Developmental psychobiology, 2010. ***52****(5): p. 402-410.*

Ma, Q., L. Battelli, and A.F. Hubbs, Multiorgan autoimmune inflammation, enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen species in mice lacking the antioxidant-activated transcription factor Nrf2. Am J Pathol,

2006. ***168****(6): p. 1960-74.*

Ma, Q., Role of Nrf2 in Oxidative Stress and Toxicity. Annual Review of Pharmacology and Toxicology, 2013. ***53****(1): p. 401-426.*

Maestripieri, D., K. Wallen, and K.A. Carroll, Genealogical and demographic influences on infant abuse and neglect in group-living sooty mangabeys (Cercocebus atys). Dev Psychobiol, 1997. ***31****(3): p. 175-80.*

Maestripieri, D., On the Importance of Comparative Research for the Understanding of Human Behavior and Development: A Reply to Gottlieb & Lickliter (2004). Social Development, 2005. ***14****(1): p. 181-186.*

Mangiafico, S., et al., Neutral endopeptidase inhibition and the natriuretic peptide system: an evolving strategy in cardiovascular therapeutics. Eur Heart J, 2013. ***34****(12): p. 886-893c.*

Manji, H.K., W.C. Drevets, and D.S. Charney, The cellular neurobiology of depression. Nat Med, 2001. ***7****(5): p. 541-7.*

Margolin, G. and K.A. Vickerman, Post-traumatic Stress in Children and Adolescents Exposed to Family Violence: I. Overview and Issues. Professional psychology, research and practice, 2007. ***38****(6): p. 613-619.*

Masten, A.S., Ordinary magic: Resilience processes in development. American Psychologist, 2001. ***56****(3): p. 227-238.*

Matough, F.A., et al., The Role of Oxidative Stress and Antioxidants in Diabetic Complications. Sultan Qaboos University Medical Journal, 2012. ***12****(1): p. 5-18.*

Matthews, G., K. Coyle, and A. Craig, Multiple factors of cognitive failure and their relationships with stress vulnerability. Journal of Psychopathology and

Behavioral Assessment, 1990. ***12****(1): p. 49-65.*

McGovern, T.K., et al., Neutrophilic oxidative stress mediates organic dust-induced pulmonary inflammation and airway hyperresponsiveness. Am J Physiol Lung Cell Mol Physiol, 2016. ***310****(2): p. L155-65.*

McGowan, P.O., et al., Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat Neurosci, 2009. ***12****(3): p. 342-8.*

McIlwrick, S., et al., Late-Onset Cognitive Impairments after Early-Life Stress Are Shaped by Inherited Differences in Stress Reactivity. Frontiers in Cellular Neuroscience, 2017. ***11****(9).*

McNally, R.J., A. Heeren, and D.J. Robinaugh, A Bayesian network analysis of posttraumatic stress disorder symptoms in adults reporting childhood sexual abuse. Eur J Psychotraumatol, 2017. ***8****(sup3): p. 1341276.*

Meyer, J.S., et al., Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proceedings of the National Academy of Sciences, 2009. ***106****(39): p. 16698-16703.*

Michel, T.M., D. Pulschen, and J. Thome, The role of oxidative stress in depressive disorders. Curr Pharm Des, 2012. ***18****(36): p. 5890-9.*

Miller, G.E., E. Chen, and K.J. Parker, Psychological Stress in Childhood and Susceptibility to the Chronic Diseases of Aging: Moving Towards a Model of Behavioral and Biological Mechanisms. Psychological bulletin, 2011. ***137****(6): p. 959-997.*

Miller, G.E., E. Chen, and K.J. Parker, Psychological stress in childhood and susceptibility to the chronic diseases of aging: moving toward a model of behavioral and

biological mechanisms. Psychol Bull, 2011. ***137****(6): p. 959-97.*

Miller, G.E., N. Rohleder, and S.W. Cole, Chronic interpersonal stress predicts activation of pro- and anti-inflammatory signaling pathways 6 months later. Psychosom Med, 2009. ***71****(1): p. 57-62.*

Mitchell, C., L.M. Schneper, and D.A. Notterman, DNA methylation, early life environment, and health outcomes. Pediatr Res, 2016. ***79****(1-2): p. 212-9.*

Mitchell, C., L.M. Schneper, and D.A. Notterman, DNA methylation, early life environment, and health outcomes. Pediatr Res, 2016. ***79****(1-2): p. 212-9.*

Mohora M, Greabu M, Totan A, Mitrea N, Battino M (2009)

Moore, C.L. and K.L. Power, Prenatal stress affects mother–infant interaction in norway rats. Developmental Psychobiology, 1986. ***19****(3): p. 235-245.*

Morgan, M.J. and Z.G. Liu, Reactive oxygen species in TNFalpha-induced signaling and cell death. Mol Cells, 2010. ***30****(1): p. 1-12.*

Morley-Fletcher, S., et al., Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. Eur J

Morley-Fletcher, S., et al., Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. Eur J Neurosci, 2003. ***18****(12): p. 3367-74.*

Morris, M.J., V. Le, and J. Maniam, The impact of poor diet and early life stress on memory status. Current Opinion in Behavioral Sciences, 2016. ***9****(Supplement C): p. 144-151.*

Murgatroyd, C., Epigenetic programming of neuroendocrine systems during early life. Experimental Physiology, 2014. ***99****(1): p. 62-65.*

Murgatroyd, C.A., et al., Transgenerational Social Stress, Immune Factors, Hormones, and Social Behavior. Frontiers in Ecology and Evolution, 2016. ***3****(149).*

Nelson, J.A., et al., Family stress and parental responses to children's negative emotions: tests of the spillover, crossover, and compensatory hypotheses. J Fam Psychol, 2009. ***23****(5): p. 671-9.*

Nestler, E.J., Transgenerational Epigenetic Contributions to Stress Responses: Fact or Fiction? PLoS Biol, 2016. ***14****(3): p. e1002426.*

Neurosci, 2003. ***18****(12): p. 3367-74.*

Nguyen, T., P. Nioi, and C.B. Pickett, The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem, 2009. ***284****(20): p. 13291-5.*

Nishioka, N. and S.E. Arnold, Evidence for oxidative DNA damage in the hippocampus of elderly patients with chronic schizophrenia. Am J Geriatr Psychiatry, 2004. ***12****(2): p. 167-75.*

Nowakowski, R.S. and N.L. Hayes, CNS development: an overview. Dev Psychopathol, 1999. ***11****(3): p. 395-417.*

Pace, T.W., et al., Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress. Am J Psychiatry, 2006. ***163****(9): p. 1630-3.*

Patki, G., et al., Depression, anxiety-like behavior and memory impairment are associated with increased oxidative stress and inflammation in a rat model of social stress. Brain Res, 2013. ***1539****: p. 73-86.*

Patki, G., et al., Moderate treadmill exercise rescues anxiety and depression-like

behavior as well as memory impairment in a rat model of posttraumatic stress disorder. Physiology & behavior, 2014. ***130****: p. 47-53.*

Patki, G., et al., Tempol Treatment Reduces Anxiety-Like Behaviors Induced by Multiple Anxiogenic Drugs in Rats. PLoS ONE, 2015. ***10****(3): p. e0117498.*

Pena, C.J., et al., Epigenetic signaling in psychiatric disorders. J Mol Biol, 2014. ***426****(20): p. 3389-412.*

Pham-Huy, L.A., H. He, and C. Pham-Huy, Free Radicals, Antioxidants in Disease and Health. International Journal of Biomedical Science : IJBS, 2008. ***4****(2): p. 89-96.*

Pham-Huy, L.A., H. He, and C. Pham-Huy, Free Radicals, Antioxidants in Disease and Health. International Journal of Biomedical Science : IJBS, 2008. ***4****(2): p. 89-96.*

Pietrek, C., et al., Childhood adversities in relation to psychiatric disorders. Psychiatry Res, 2013. ***206****(1): p. 103-10.*

Pruessner, J.C., et al., Dopamine release in response to a psychological stress in humans and its relationship to early life maternal care: a positron emission tomography study using [11C]raclopride. J Neurosci, 2004. ***24****(11): p. 2825-31.*

Pryce, C.R., et al., Comparison of the effects of early handling and early deprivation on conditioned stimulus, context, and spatial learning and memory in adult rats. Behav Neurosci, 2003. ***117****(5): p. 883-93.*

Raine, A., et al., Reduced right hemisphere activation in severely abused violent offenders during a working memory task: An fMRI study. Aggressive Behavior, 2001. ***27****(2): p. 111-129.*

Ranjekar, P.K., et al., Decreased antioxidant enzymes and membrane essential polyunsaturated fatty acids in schizophrenic and bipolar mood disorder

patients. Psychiatry Res, 2003. ***121****(2): p. 109-22.*

Razin, S., D. Yogev, and Y. Naot, Molecular Biology and Pathogenicity of Mycoplasmas. Microbiology and Molecular Biology Reviews, 1998. ***62****(4): p. 1094-1156. Redox sensitive signaling factors and antioxidants. Farmacia 57(4):399–410*

Roth, T.L., et al., Lasting epigenetic influence of early-life adversity on the BDNF gene. Biol Psychiatry, 2009. ***65****(9): p. 760-9.*

Salim, S., et al., Identification of RGS2 and type V adenylyl cyclase interaction sites. J Biol Chem, 2003. ***278****(18): p. 15842-9.*

Salim, S., Oxidative Stress and Psychological Disorders. Current Neuropharmacology, 2014. ***12****(2): p. 140-147.*

Sawamura, N., et al., Nuclear DISC1 regulates CRE-mediated gene transcription and sleep homeostasis in the fruit fly. Molecular psychiatry, 2008. ***13****(12): p. 1138-1069.*

Sawyerr, A. and C. Bagley, Child Sexual Abuse and Adolescent and Adult Adjustment: A Review of British and World Evidence, with Implications for Social Work, and Mental Health and School Counselling. Advances in Applied Sociology, 2017. ***Vol.07No.01****: p. 15.*

Scheller-Gilkey, G., et al., Early life stress and PTSD symptoms in patients with comorbid schizophrenia and substance abuse. Schizophrenia Research, 2004. ***69****(2): p. 167-174.*

Schoenbaum, M., et al., Cost-effectiveness of practice-initiated quality improvement for depression: results of a randomized controlled trial. Jama, 2001. ***286****(11): p. 1325-30.*

Schuler, L.A. and A.P. Auger, Psychosocially influenced cancer: diverse early-life stress experiences and links to breast cancer. Cancer Prev Res (Phila), 2010. ***3****(11): p. 1365-70.*

Schwartz, G.N., et al., Early suppressive effects of chemotherapy and cytokine treatment on committed versus primitive haemopoietic progenitors in patient bone marrow. British Journal of Haematology, 1996. ***92****(3): p. 537-547.*

Scott, A.I. and C.P. Freeman, Edinburgh primary care depression study: treatment outcome, patient satisfaction, and cost after 16 weeks. Bmj, 1992. ***304****(6831): p. 883-7.*

Seay, B., B.K. Alexander, and H.F. Harlow, MATERNAL BEHAVIOR OF SOCIALLY DEPRIVED RHESUS MONKEYS. J Abnorm Psychol, 1964. ***69****: p. 345-54.*

Sengupta, P., The Laboratory Rat: Relating Its Age With Human's. Int J Prev Med, 2013. ***4****(6): p. 624-30.*

Shi, L., et al., Maternal Influenza Infection Causes Marked Behavioral and Pharmacological Changes in the Offspring. The Journal of Neuroscience, 2003. ***23****(1): p. 297-302.*

Shonkoff, J.P., et al., The Lifelong Effects of Early Childhood Adversity and Toxic Stress. Pediatrics, 2012. ***129****(1): p. e232-e246.*

Silk, J.S., et al., Resilience among children and adolescents at risk for depression: Mediation and moderation across social and neurobiological contexts. Dev Psychopathol, 2007. ***19****(3): p. 841-65.*

Sivrioglu, E.Y., et al., The impact of omega-3 fatty acids, vitamins E and C supplementation on treatment outcome and side effects in schizophrenia

patients treated with haloperidol: an open-label pilot study. Prog Neuropsychopharmacol Biol Psychiatry, 2007. ***31****(7): p. 1493-9.*

Small, G.W., et al., Prediction of cognitive decline by positron emission tomography of brain amyloid and tau. Arch Neurol, 2012. ***69****(2): p. 215-22.*

Soares, M.P., et al., Heme Oxygenase-1 Modulates the Expression of Adhesion Molecules Associated with Endothelial Cell Activation. The Journal of Immunology, 2004. ***172****(6): p. 3553-3563.*

Solanki, N., et al., Grape powder prevents cognitive, behavioral and biochemical impairments in a rat model of posttraumatic stress disorder. Nutrition research (New York, N.Y.), 2015. ***35****(1): p. 65-75.*

Solanki, N., et al., Grape powder prevents cognitive, behavioral, and biochemical impairments in a rat model of posttraumatic stress disorder. Nutrition Research, 2015. ***35****(1): p. 65-75.*

Solanki, N., et al., Modulating Oxidative Stress Relieves Stress-Induced Behavioral and Cognitive Impairments in Rats. International Journal of Neuropsychopharmacology, 2017. ***20****(7): p. 550-561.*

Sorce, S., et al., The NADPH oxidase NOX2 controls glutamate release: a novel mechanism involved in psychosis-like ketamine responses. J Neurosci, 2010. ***30****(34): p. 11317-25.*

Souza, R.R., L.J. Noble, and C.K. McIntyre, Using the Single Prolonged Stress Model to Examine the Pathophysiology of PTSD. Frontiers in Pharmacology, 2017. ***8****: p. 615.*

Spinelli, S., et al., Early-life stress induces long-term morphologic changes in primate

brain. Arch Gen Psychiatry, 2009. ***66****(6): p. 658-65.*

Stiles, J. and T.L. Jernigan, The basics of brain development. Neuropsychol Rev, 2010. ***20****(4): p. 327-48.*

Strålin, P. and S.L. Marklund, Effects of oxidative stress on expression of extracellular superoxide dismutase, CuZn-superoxide dismutase and Mn-superoxide dismutase in human dermal fibroblasts. Biochemical Journal, 1994. ***298****(Pt 2): p. 347-352.*

Su, S., et al., The Role of Adverse Childhood Experiences in Cardiovascular Disease Risk: a Review with Emphasis on Plausible Mechanisms. Current cardiology reports, 2015. ***17****(10): p. 88-88.*

Suzuki, Y.J., M. Carini, and D.A. Butterfield, Protein Carbonylation. Antioxidants & Redox Signaling, 2010. ***12****(3): p. 323-325.*

Sykiotis, G.P. and D. Bohmann, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. Dev Cell, 2008. ***14****(1): p. 76-85.*

Talge, N.M., C. Neal, and V. Glover, Antenatal maternal stress and long-term effects on child neurodevelopment: how and why? J Child Psychol Psychiatry, 2007. ***48****(3-4): p. 245-61.*

Tamashiro, K.L.K., Developmental and environmental influences on physiology and behavior — 2014 Alan N. Epstein Research Award. Physiology & Behavior, 2015. ***152****(Part B): p. 508-515.*

Tarullo, A.R. and M.R. Gunnar, Child maltreatment and the developing HPA axis. Horm Behav, 2006. ***50****(4): p. 632-9.*

Taylor, S.E., Mechanisms linking early life stress to adult health outcomes. Proceedings

of the National Academy of Sciences, 2010. ***107****(19): p. 8507-8512.*

Teicher, M.H., A. Tomoda, and S.L. Andersen, Neurobiological consequences of early stress and childhood maltreatment: are results from human and animal studies comparable? Ann N Y Acad Sci, 2006. ***1071****: p. 313-23.*

Thaker, P.H., et al., Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nature Medicine, 2006. ***12****: p. 939.*

Tohmi, M., et al., Perinatal inflammatory cytokine challenge results in distinct neurobehavioral alterations in rats: implication in psychiatric disorders of developmental origin. Neurosci Res, 2004. ***50****(1): p. 67-75.*

Trombini, M., et al., Early maternal separation has mild effects on cardiac autonomic balance and heart structure in adult male rats. Stress, 2012. ***15****(4): p. 457-70.*

Urban, K.R. and W.-J. Gao, Performance enhancement at the cost of potential brain plasticity: neural ramifications of nootropic drugs in the healthy developing brain. Frontiers in Systems Neuroscience, 2014. ***8****: p. 38.*

Uttara, B., et al., Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol, 2009. ***7****(1): p. 65-74.*

Vetulani, J., Early maternal separation: a rodent model of depression and a prevailing human condition. Pharmacol Rep, 2013. ***65****(6): p. 1451-61.*

Vetulani, J., Early maternal separation: a rodent model of depression and a prevailing human condition. Pharmacol Rep, 2013. ***65****(6): p. 1451-61.*

Vollert, C., et al., Exercise prevents sleep deprivation-associated anxiety-like behavior in rats: potential role of oxidative stress mechanisms. Behav Brain Res, 2011.

224*(2): p. 233-40.*

Vomhof-Dekrey, E.E. and M.J. Picklo, Sr., The Nrf2-antioxidant response element pathway: a target for regulating energy metabolism. J Nutr Biochem, 2012. ***23****(10): p. 1201-6.*

Von Werne Baes, C., et al., Assessment of the hypothalamic–pituitary–adrenal axis activity: glucocorticoid receptor and mineralocorticoid receptor function in depression with early life stress – a systematic review. Acta Neuropsychiatrica, 2012. ***24****(1): p. 4-15.*

von Wolff, A., et al., Selective serotonin reuptake inhibitors and tricyclic antidepressants in the acute treatment of chronic depression and dysthymia: a systematic review and meta-analysis. J Affect Disord, 2013. ***144****(1-2): p. 7-15.*

Wagner, B., S. Forstmeier, and A. Maercker, Posttraumatic Growth as a Cognitive Process with Behavioral Components: A Commentary on Hobfoll et al. (2007). Applied Psychology, 2007. ***56****(3): p. 407-416.*

Wakabayashi, Y., et al., Correlation of vascular endothelial growth factor with chemokines in the vitreous in diabetic retinopathy. Retina, 2010. ***30****(2): p. 339-44.*

Wakabayashi, Y., et al., Correlation of vascular endothelial growth factor with chemokines in the vitreous in diabetic retinopathy. Retina, 2010. ***30****(2): p. 339-44.*

Wang, Q., R.C. Shelton, and Y. Dwivedi, Interaction between early-life stress and FKBP5 gene variants in major depressive disorder and post-traumatic stress disorder: A systematic review and meta-analysis. Journal of Affective Disorders, 2018.

225*(Supplement C): p. 422-428.*

Wang, X. and E.K. Michaelis, Selective Neuronal Vulnerability to Oxidative Stress in the Brain. Frontiers in Aging Neuroscience, 2010. ***2****: p. 12.*

Wang, Z., et al., Melatonin activates the Nrf2-ARE pathway when it protects against early brain injury in a subarachnoid hemorrhage model. Journal of Pineal Research, 2012. ***53****(2): p. 129-137.*

Ward, E., et al., Randomised controlled trial of non-directive counselling, cognitive-behaviour therapy, and usual general practitioner care for patients with depression. I: clinical effectiveness. Bmj, 2000. ***321****(7273): p. 1383-8.*

Wardyn, Joanna D., Amy H. Ponsford, and Christopher M. Sanderson, Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. Biochemical Society Transactions, 2015. ***43****(4): p. 621-626.*

Watanabe, D., et al., Suppressor of cytokine signalling-1 gene silencing in acute myeloid leukaemia and human haematopoietic cell lines. Br J Haematol, 2004. ***126****(5): p. 726-35.*

Watson, C., et al., Specificity of volumetric magnetic resonance imaging in detecting hippocampal sclerosis. Arch Neurol, 1997. ***54****(1): p. 67-73.*

Weaver, I.C.G., et al., Epigenetic programming by maternal behavior. Nature Neuroscience, 2004. ***7****: p. 847.*

Weininger, O., Physiological Damage under Emotional Stress as a Function of Early Experience. Science, 1954. ***119****(3087): p. 285-286.*

Weinstock, M., The long-term behavioural consequences of prenatal stress. Neurosci Biobehav Rev, 2008. ***32****(6): p. 1073-86.*

Weinstock, M., The long-term behavioural consequences of prenatal stress. Neurosci Biobehav Rev, 2008. ***32****(6): p. 1073-86.*

Wieck, A., S.L. Andersen, and H.C. Brenhouse, Evidence for a neuroinflammatory mechanism in delayed effects of early life adversity in rats: relationship to cortical NMDA receptor expression. Brain Behav Immun, 2013. ***28****: p. 218-26.*

Wolf, N.J. and D.R. Hopko, Psychosocial and pharmacological interventions for depressed adults in primary care: a critical review. Clin Psychol Rev, 2008. ***28****(1): p. 131-61.*

Wu, G., et al., Understanding resilience. Frontiers in Behavioral Neuroscience, 2013. ***7****: p. 10.*

Xiao, L., et al., The mitochondria-targeted antioxidant MitoQ ameliorated tubular injury mediated by mitophagy in diabetic kidney disease via Nrf2/PINK1. Redox Biology, 2017. ***11****: p. 297-311.*

Yamamoto, S., et al., Single prolonged stress: toward an animal model of posttraumatic stress disorder. Depress Anxiety, 2009. ***26****(12): p. 1110-7.*

Yamazaki, H., et al., Role of the Keap1/Nrf2 pathway in neurodegenerative diseases. Pathol Int, 2015. ***65****(5): p. 210-9.*

Yang, B.Z., et al., Child abuse and epigenetic mechanisms of disease risk. Am J Prev Med, 2013. ***44****(2): p. 101-7.*

Yolken, R.H., H. Karlsson, and E.F. Torrey, Endogenous retroviruses and schizophrenia — A solution to the nature/nurture dilemma? Schizophrenia Research. ***41****(1): p. 86-87.*

Zannas, A.S. and A.E. West, Epigenetics and the regulation of stress vulnerability and

resilience. Neuroscience, 2014. ***264****: p. 157-70.*

Zhang, T.Y. and M.J. Meaney, Epigenetics and the environmental regulation of the genome and its function. Annu Rev Psychol, 2010. ***61****: p. 439-66, c1-3.*

Zuena, A.R., et al., Prenatal Restraint Stress Generates Two Distinct Behavioral and Neurochemical Profiles in Male and Female Rats. PLoS ONE, 2008. ***3****(5): p. e2170.*