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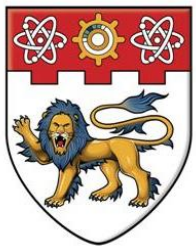

NANYANG TECHNOLOGICAL UNIVERSITY

EARLY LIFE REARING OF STRESSED ANIMALS IN COMPLEX HOUSING ENVIRONMENT DRIVES STRESS RESILIENCE IN PHYSIOLOGY, BEHAVIOR AND BRAIN PLASTICITY DURING ADULTHOOD

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SCHOOL OF BIOLOGICAL SCIENCES

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
ARC	Activity-regulated cytoskeleton-associated protein
AUC	Area under the curve
BDNF	Brain derived neurotrophic factor
BLA	Basolateral Amygdala
CeA	Central Amygdala
CNS	Central Nervous System
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
EE	Environmental Enrichment/ Enriched Environment
EPM	Elevated plus maze
Erk	Extracellular signal-regulated kinases
FST	Forced swim task
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAD	Glutamate Decarboxylase
GC	Glucocorticoids
GR	Glucocorticoid receptor
HPA	Hypothalamic-Pituitary-Adrenal
IL	Infralimbic Region
pMAPK	Phosphorylated Mitogen Activated Protein Kinase
MDD	Major Depressive Disorder
MeA	medial Amygdala
MFT	Mossy Fibre Terminals
mPFC	medial Prefrontal Cortex
MR	Mineralocorticoid Receptor
mRNA	Messenger Ribonucleic Acid
MRI	Magnetic Resonance Imaging

NAc	Nucleus Accumbens
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate Receptor
OD	Optical density
OFT	Open field test
PBS	Phosphate buffered saline
PFC	Prefrontal cortex
PrL	Prelimbic Region
PTSD	Post-Traumatic Stress Disorder
PV	Parvalbumin
PVN	Paraventricular nucleus
ROI	Regions of interest
SC	Standard conditions
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of the mean
SIT	Social interaction test
SNS	Sympathetic Nervous System
tPA	tissue Plasminogen activator
TrkB	Tyrosine kinase receptor B
vPrL	ventral Prelimbic Region
VTA	Ventral Tegmented Area

ABSTRACT

Early-life maternal separation leads to hyper-reactive stress-response in adulthood along with long-lasting anxiogenic and depressive-like behavior. Maternal separation occurring during the vulnerable postnatal period interferes with the normal development of brain and its neuronal morphology. Most studies on maternal separation during early life highlight the dysregulation in emotional behavior of an individual in various stages of life. Emotional learning and memory are processed in amygdala. Thus, exploring the amygdala in maternally separated rats is crucial in understanding the mechanisms that drives emotional dysregulation in early-life stress model. Interestingly, recent studies showed that short-term enriched environment can enhance prominent innate behaviours like avoidance of predator odour and attractiveness of adult male rats. Additionally, short-term enrichment during adulthood can reverse the negative impacts of repeated early life maternal separation on behavior and morphology of basolateral amygdala neurons. In this thesis, rat pups were exposed to concomitant maternal separation stress and enriched environment during early life (P2-P21) and their stress-response and behavior were yested during adulthood. Changes in neuronal morphology using Golgi staining was analyzed. Protein regulation of GR, BDNF and p-MAPK in the basolateral amygdala (BLA) using western blot and immunostaining was also examined. Through the above series of experiments, it was demonstrated that exposure to early-life enriched environment can ameliorate the effects of maternal separation stress by renormalizing the underlying neurobiological substrate (namely neuronal morphology and protein regulation) as well as physiology and behavior.

Chapter 1

1. Background

1.1 Over-riding influence of the early life stress

Individual animals show remarkable variance in their behavior depending on the incipient environment during the early period of their life. Behavioral and physiological phenotypes for the same genetic backgrounds are often changeable (plastic) in response to changes in the environment [1-3]. Pigliucci et al. (2006) defined *phenotypic plasticity* as 'the ability of a single genotype to give rise to various phenotypes when exposed to differential environmental conditions'[4]. This involves the possibility to change the developmental pathway *(developmental plasticity)* of an individual in response to a particular environment cue and also the ability of an individual to alter their phenotypic activity (e.g., metabolism) in response to differential environmental conditions [5]. It is seen that, the earlier an organism's development is disturbed, higher the amplitude of detrimental effects [6, 7]. Given the rate at which the environment is varying globally, studying the consequence of the changing environment and the relationship between the environment and the phenotypic plasticity is of enormous importance. Animals have evolved by adapting themselves to various dynamic environments through temporary or permanent changes in the physiology and behavior of an individual, depending on the environment that they were exposed to during the early stage of life. For example, aging was delayed, and lifespan was increased in rats that were exposed to a particular diet during adulthood [8] and It was hypothesized that animals have an adaptive response to famine wherein the resources are shifted

away from reproduction and focused on increased cellular maintenance to maintain a healthy physiology. This further increased the chances of survival during the famine and permits fertility to be preserved for the post-famine period [9, 10].

During the early life, the time window that is vulnerable to the changing environment is commonly referred to as the '*critical'* or '*sensitive'* period [11]. An organism process various stimuli of visual, olfactory and social cues from the surroundings that are required for its normal development during the sensitive period [12]. Observations show that during the sensitive period, development of an individual depends on the type of cue perceived from the environment [13]. The organism utilizes the best options from the available environment and this helps in development [14]. Some studies suggest that, sensitive period during early life is crucial for development due to the complication involved in reversing developmental mechanisms later in life [15]. If the phenotype obtained in early life is not compatible with the conditions later in life, developmental plasticity might have detrimental effects on the survival and reproductive performance of the individual in future. Prior studies have shown that developmental plasticity is beneficial to numerous species but can have a different impact during a degree of variation in the environment later in life [16-19]. At this juncture, it is very clear that environment during the *sensitive period* plays a crucial role for the normal development of an individual.

Among the numerous factors available in the environment, a fundamental essence during an individual's early life is, 'the mother'. In several bird species, the mother can change multiple components of her egg, like the size of the egg,

hormones, nutrients and many other factors including the sex of the embryo according to the changes in the environment [20-22]. In certain birds and mammals, depending on the availability of food and quality of their mating partners, their maternal effects can vary. This variation has a significant impact on the developmental phenotypic plasticity of their offspring that persist across various generations even if the conditions have changed later on [23, 24]. Mothers in numerous vertebrates have an enormous impact on growth and breeding performance of their offspring, depending on their own early life experience [25]. Mothers in mammals that have undergone food restriction in their early life, give birth to offspring that have low birth-weight [26]. This shows that the interaction between a mother and the offspring plays an important role in its development process.

1.2 Mother-infant interactions

In embryonic life, the prenatal environment within the mother's womb also plays a crucial role in an offspring's survival and reproductive success. In humans, studies have shown that the brain morphology and physiology of the embryo depends on the nutritional circumstances of the mother [27, 28]. For example, a foetus develops in a specific pattern, in response to the environmental cues provided by the mother in the womb. This in turn prepares the baby for an environment that it is likely to be born into. If the 'prenatal conditions' and 'early-life conditions' of a baby are in contrast with each other, the consequences could be harmful [15] It has been observed that babies with low birth weights (due to stressful prenatal period) that grew up in a favourable environments later on, developed multiple health problems including heart disease, diabetes and hypertension [27, 29, 30]. Additionally, if the birth weight

is high and the later life has a favourable environment, the risk of having health problems is very low [31]. Therefore, the cues an individual receives prenatally has a huge influence on the early development and health success [32]. A classic illustrator of this idea is the Dutch hunger during the Second World War. Pregnant women in the Dutch famine in 1944 underwent starvation and this caused adverse foetal environment due to lack of nutrition and energy supplements. These prenatal harsh conditions caused low body weight of the babies at birth and these individuals were susceptible to compromised cognitive functions, cardiovascular disease and obesity despite adequate food intake later in life. This was due to the food deprived environment that persisted during their foetal stage, which programmed their bodies to conserve nutrients throughout the course of their life [33]. The information above shows that early life focuses on preparing an offspring for a future environment based on the cues it receives during the crucial period of development even before the birth.

Mother-infant relationship during postnatal life plays a cardinal role in phenotypic plasticity too. Bowlby stated that, a mother's love during early life is as crucial for 'mental health' as proteins and vitamins are for 'physical health' [34, 35]. Multiple studies have focused on the importance of mother-infant relationship for the behavioural and cognitive development of infants [34, 36, 37]. Studies have shown that mothers with post-partum depression (PPD) have long-term issues that affect the positive interaction with their child. This can have damaging effects on the child's cognitive and emotional development [38-40]. Mothers who have undergone early life stress due to childhood abuse have problems in interacting with their children and this impacts negatively on the child's development [41, 42]. Therefore, mother-infant relationships have a

long-term effect on a child's future. It has also been reported that effective intervention at an earlier stage is attainable, if the problem is detected early on [43]. A positive mother-infant relationship (postnatal environment) is beneficial for development of the affective behaviour of the infant [44, 45]. Therefore, the manner in which an infant establishes the relationship with the mother influences the manner in which it establishes relationships with every other facet in the environment throughout the rest of her/his life [46]. Based on all information available on mother-infant interaction, this relationship has been validated in rodents in lab conditions as well and is described below.

1.3 Mother-infant relationship validated with rodents

An interesting body of work by Meaney on rodents confirms that alteration in maternal care can have a major impact in development of the offspring's physiology. An individual's physiology is regulated by "Hypothalamic-Pituitary-Adrenal (HPA) axis" (Figure 1). The HPA axis *(detailed discussion of HPA axis is available in section 2)* releases glucocorticoids (stress hormones) that mediate the stress response that help the body to return to basic physiological balance after an encounter of stress episode [19]. Meaney's study on rats showed that offsprings that received 'high maternal care' (licking and grooming) from the mother during initial 10 days of development (postnatal day 10), displayed low corticosterone (glucocorticoid) responses to acute stress and expressed more glucocorticoid receptors messenger RNA (mRNA) when compared to their counterparts with 'low maternal care'. The amount of care in rats during that early stage was crucial in programming the Hypothalamus-Pituitary-Adrenal (HPA) axis response to stress in the individual [47].

Additionally, they showed that adverse conditions in early life could cause long-term behavioural deficits and epigenetic changes in glucocorticoid receptor in rat hippocampus [48]. Thus, the interaction between mother and pup is as compelling as merely the presence of the mother. RM Sullivan et al., (2006) determined that, in the absence of the mother, rat pups displayed odour avoidance when exposed to odour-shock conditioning along with their amygdala activation. The same conditioning initiates a preference to the odour in the presence of mother without the amygdala activation. They also determined that this variation in behaviour due to mother's presence is regulated by the modulating the corticosterone which in turn helps in the moderation of the amygdala activity [49, 50]. These studies show that in the presence of mother, the pups do not have a fear/stress response. The proximate mechanism underlying this fear/stress response have been shown to involve the amygdala and the corticosterone [51]. The role of amygdala in modulating stress response is described in the next section.

1.4 Amygdala and emotion

Stress is known to induce dendritic hypertrophy in the basolateral amygdala (BLA) [52, 53] and to enhance anxiety [53, 54]. Stress also causes increase in secretion of glucocorticoids (GC) that has harmful impact on an individual [55, 56]. It was observed that a single acute dose of corticosterone was sufficient to produce dendritic hypertrophy in the BLA and anxiogenic behavioural output. Additionally, this pattern of dendritic hypertrophy after acute treatment was comparable to that caused by chronic treatment of corticosterone [57]. These studies affirm the crucial role of amygdala in modulating stress response making it an interesting region to explore in early life stress models. As

mentioned earlier, in RM Sullivan's study [49], fear response in rat pups due to mother's absence is regulated by the modulation the corticosterone and amygdala activity. The amygdala situated in the temporal lobe is crucial regulator of fear and emotions [58].

Unlike the hippocampus, very little is known about how early life stress affects the amygdala and the nature of its role in modulating stress response. Amygdala constitutes a group of nuclei that helps in modulation of various functions related to initiation, processing and consolidation of fear memory [59]. Each nucleus of amygdala comprises of different neuronal population ranging in structure and function. The function of amygdala has been extensively studied in context of Pavlovian fear conditioning [60, 61]. The fear stimulus reaches amygdala from the thalamus or the cortex. This information is further transmitted from the basolateral amygdala (BLA) to the central amygdala that is crucial for expression of fear induced by the stimuli [62-64]. BLA being the primary nucleus to receive signal from the fear stimulus can be considered as an essential brain region for formation of fear induced behavioural response and this makes the BLA intriguing. BLA activation relays projections to switch on the HPA axis for generating a stress response [65]. The BLA comprises of seven specific types of cell populations [66] out of which the predominating pyramidal neurons, stellate neurons and GABAergic local interneurons are three major kind of cell populations. Pyramidal and stellate neurons are glutamatergic, which are excitatory in nature and the GABAergic local interneurons, are inhibitory in nature [67, 68]. Activation of BLA mediates the activation of the central amygdala (CeA) or the medial amygdala (MeA) via the excitatory neurons mentioned earlier [69]. This further causes

activation of the paraventricular nucleus (PVN) of the hypothalamus via the bed nucleus of stria terminalis (BNST), to switch on the HPA axis for generating a stress response [69, 70]. Additionally, studying the BLA in context of early life and stress is more important than studying other cortical regions because the development of amygdala takes place during the critical early life period, which is much earlier than cortical regions [71].

As mentioned earlier, BLA takes part in the consolidation of fear-induced memories by processing information from the environment. This helps them to use prior experience and related memories to engage in similar situations the may arise in the future [72]. This highlights an interesting concept of "behavioural engagement" that sheds light on crucial processes that regulates interactions between individuals and the environment. The decision of an animal to engage or disengage to the threatening environment is extremely crucial for an animal to decide on how to respond to various situations like foraging, unexpected encounter of predator and other similar interactions with the environment meaningfully [73]. Considering that amygdala's structural plasticity occurs much earlier in life, the engaging and disengaging behaviour might also be formed, processed and organised earlier in life [73]. This makes it important to learn the impacts of varying environment during early life.

1.5 Impact of harsh environment

While the presence of the mother is crucial in early life development, a mother's presence under 'impoverished conditions' has proven to be a counterproductive. It is observed that the dam and litter living in resource-deprived conditions are distressed. When children and infants are exposed to severe poverty, famine, war or drug-abusing mothers, the stress is typically

chronic in nature. In this situation, the mother is typically present, but her behavior is abnormal due to stressful environment [74, 75]. An animal model of chronic early-life stress, "Limited Nesting model" was developed to recreate these important elements of the human condition. Manipulation of the environment and the quality of care from the mother in this model causes chronic 'early life stress' [76]. The experimental paradigm, consisting of limited nesting material recapitulates this pattern in rodents by replacing the bedding and nesting material with wire mesh. Hence, it is established that an impoverished environment in the presence of mother during early life induces detrimental effects on rat pups due to early life stress. Though multiple models have been established to study short-term 'early life stress', no models have been established to study short-term 'early life enrichment'. This study is the first study that attempts to understand the impact of early life enrichment on the animal's behavior during adulthood.

1.6 The 'best' way out of the worst environment

Despite the fact that a stressful developmental stage causes various abnormal phenotypes that is harmful for the offspring, a school of thought expresses that these abnormalities could be beneficial considering the environment is the cause of the abnormality. Certain compromised developmental experiences can heighten memory processes and learning under stressful situations [77]. In certain cases, it is observed that this kind of putative abnormal and irregular morphological, physiological alterations can stimulate defensive and reproductive system for survival [78, 79]. Hence, early life adaptive coping mechanism towards a stressful environment allows the individual to make "the

best use out of the worst situation" to alleviate the inescapable health consequences. Additionally, it is important to note that even if an individual has adapted a mechanism to cope with stress during development, it might not be the "best mechanism" as it may cause harmful effects later in life. Such mechanisms are definitely not the ideal conditions of health and survival [80, 81]. Furthermore, Belsky suggests that natural selection might have designed human development to adapt to adversity in a certain manner that is now considered to be a putatively abnormal, non-ideal developmental phenotype [82]. Although this perspective opens avenues to think about early life stress from another angle (help to cope up with harsh environment), it certainly does not mean that we have to discard our attempts to discover novel strategies to mitigate the costs involved in adaptive response to stress (could cause detrimental impacts on later life health status) [78, 83, 84]. In fact, we need to address all the facets of early life stress and find ways to alleviate the detrimental effects associated with early development. It is suggested that the efficacy of interventions against developmental stress abnormalities can be strengthened by considering the cost and benefits of the altered (naturally selected) phenotype instead of concluding it as a putative physiological, morphological or mental health complication [82, 85, 86]. It is important to note that, though many studies suggested that an adaptive response in adulthood following early-life stress is difficult, studies are also trying to establish otherwise. A recent study shows that a short-term implementation of the enriching environment in adult rats can reverse the detrimental effects of early life stress [87]. The effects of reduced maternal care on cognitive function are reversed with peripubertal environmental enrichment [88]. This ensures that

the detrimental effects caused due to early life stress can be reversed in adulthood by manipulating the environment for a short-term. Stress, resilience and associated topics will be discussed in detail below in the next section.

Chapter 2

2. Introduction

2.1 Homeostasis & Allostasis

Homeostasis originated from the Greek words, “same” and “steady” which means maintaining a steady and stable state of vital physiological processes that are important for an individual’s survival. Walter Cannon coined this term in 1930 and described it as various ways a body maintains steady states of various vital functions namely; pH, temperature, glucose levels, oxygen levels, etc., in response to varying environment [89]. A process that maintains homeostasis is called allostasis. **Allostasis** refers to the active processes that the body undergoes to re-establish homeostasis when exposed to a stressor. This could involve production of hormones or various mediators (e.g., adrenaline, noradrenaline, glucocorticoids, cytokine, etc.,) that help an organism to adapt to different situations [90-92]. If we consider heart rate as an example, homeostasis refers to maintaining the heart rate at a stable “set point” for survival and allostasis is elevating the heart rate with the help of mediators (adrenaline) to adapt to a challenging situation (stress). Allostatic state promotes adaptation for a short-term but when this occurs chronically, it leads to an **allostatic load** that generates disease like hypertension due to chronic levels of elevated heart rate and constricted blood vessels. Therefore, chronic stress leads to detrimental problems in an individual due to allostatic load [92, 93].

2.2 Stress & Resilience: Two sides of a coin

Stress is defined as a condition that disturbs physiological or psychological homeostasis of an individual. Cannon [94] and Selye [95] proposed early conception of stress and its consequence on humans. In their theories, stress was described as a behavioural and physiological response due to interaction with the environment for the first time in 1930s.

Excessive degree of stress leads to negative social, psychological, and biological outcomes. For example, stress-related disorders including, anxiety disorders, post-traumatic stress disorder (PTSD), major depressive disorders (MDD) [96, 97]. Chronic exposure to stressful events causes surge in secretion of stress hormones (Glucocorticoids) (Figure 1) leading to negative impact on brain, cognition and behavior, whether it occurs during early life, adolescence, adulthood or old age [52, 98-101]. The magnitude of effect of stress in an individual varies with the age and extent of the stress exposure. Glucocorticoids (GC) are one of the primary mediators of allostasis and are crucial to initiate stress response to cope up with a stressful situation.

Though adverse stress leads to different disorders, there are individuals who can cope better without facing negative consequences. The variation in this coping strategy (active coping strategies) that helps the organisms deal well with the environment is called “resilience”. In this context, resilience refers to the capacity of an individual to counter successfully, the harmful effects of stress, successfully.

Resilience is the ability of an individual to sustain homeostasis when exposed to an extraordinary level of stress and trauma [102]. In a previously reported study, when mice were exposed to inescapable foot-shocks to induce learned

helplessness, majority of them were stressed but a subset (≈30%) exhibited resilient (*active coping*) feature wherein their escape latencies (successful escape) were comparable to non-stressed counterparts [103]. A study on consequences of premature children in poverty revealed that, around 10% of the affected kids displayed resilience in cognition, growth and overall health [104]. In another study, around 40% of maltreated children displayed resilience in spite of their stressful childhood [105]. Thus, it is intriguing to focus on previously mentioned long-term resilience [102]. Though resilience exists in certain individuals innately, it is present in minute percentage. This makes it interesting to explore various ways and methods to induce resilience in vulnerable individuals, that can help them to cope up with stress, better.

Why study stress & resilience?

Suicide is the second leading cause for premature death worldwide and violence is also one of the leading causes of death that accounts for about 14% of deaths in males and 7% of deaths in females [106, 107]. Since stress is one of the major causes for suicide [108] and violence [109], there is a need to target this problem and understand the basis of resilience as a counter-strategy. Early life is the critical period of life characterized by rapid growth, development and maturation of organs and systems. Variation in the quality of nurture and nature of environment can exert permanent and powerful effects upon later stages of life [110].

In the preceding paragraphs, we understand that both stress-vulnerable and stress-resilient are two contrasting outcomes that can emerge from the same stressful experience of an individual. As mentioned earlier, resilience is obtained in a *small percentage* naturally in response to a stressful situation.

Hence, it is necessary and important to study the underlying neurobiological mechanisms of resilience to counter the devastating effects of stress. In this study, resilient characteristics will be referred to as the active coping features. To determine the methods to counter stress, it is essential to understand how stress affects the internal system of an individual. We will now look into the various parameters that plays a crucial role in response to stress.

2.3 The internal system: HPA axis

Activation of Hypothalamic-Pituitary-Adrenal (HPA) axis in response to stress is initiated by corticotropin-releasing factor (CRF), which is synthesized and secreted by hypophysiotropic neurons localized in paraventricular nucleus of the hypothalamus. CRF, the principle regulator of the axis, activates CRF receptors in anterior pituitary causing the release of Adrenocorticotropic (ACTH) hormone into the systemic circulation. ACTH then activates the receptor on adrenal cortex to stimulate the Glucocorticoid (GC) synthesis and further causes the release of Glucocorticoids from zona fasciculata into the bloodstream, which regulates the physiological and behavioral changes in response to stress and brings it back to baseline, when the stressor is gone. Incomplete or exaggerated activation of the HPA axis leads to emergence of pathological conditions [111, 112]. “Hypothalamic-Pituitary-Adrenal (HPA) axis” (Fig.1) releases glucocorticoids (stress hormones) to induce a flight or fright response and helps the body to return to homeostatic equilibrium after the stress episode is over [113].

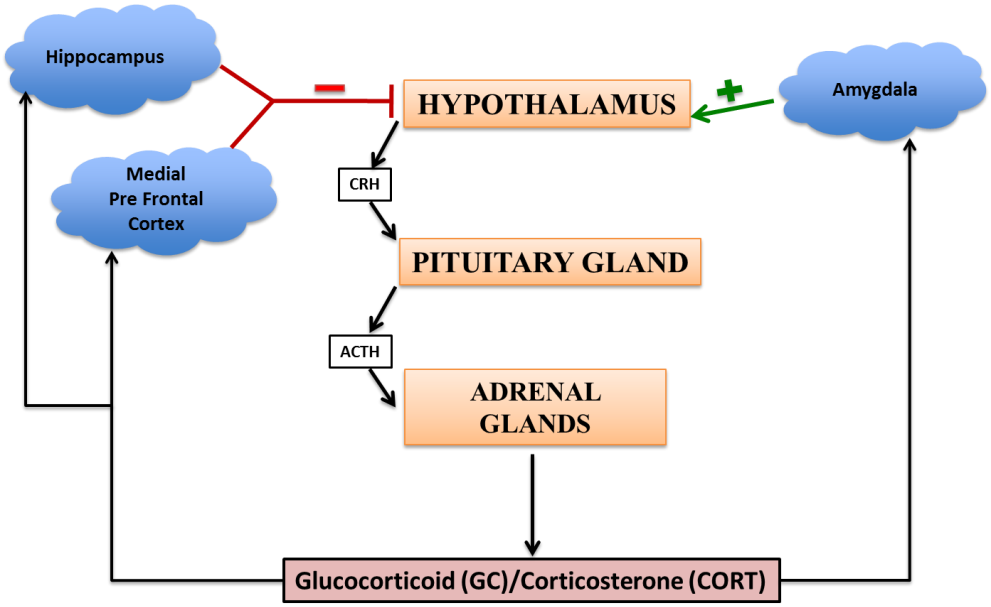


Figure 1: Hypothalamic-Pituitary-Adrenal axis

In addition to the neuroendocrine HPA axis as mentioned above, another captivating "Brain-HPA" is, **H**ippocampus, **P**refrontal cortex and **A**mygdala. These are the brain structures that contribute in the regulation of HPA axis. They have a significant effect on the release of GC and behavioral response to a stressful event [114, 115].

Hippocampus is located in the medial temporal lobe of the brain and is mainly responsible for spatial, episodic and contextual memory formation [116]. As shown in the figure, hippocampus is the primary brain region that provides negative feedback to the HPA axis. Hippocampectomy also results in increase level of CRF mRNA [117, 118]. Hippocampus was one of the most extensively explored brain regions in most of the primitive brain-related studies due to its wide involvement in cognitive processes [119, 120]. Glucocorticoid (GC) cascade hypothesis proposes that increase in glucocorticoids causes hippocampal neuronal loss, which in turn causes decrease in glucocorticoid receptor (GR) loss [121]. The structural plasticity of this region is highly dependent on the number and impact of stressful episodes experienced by an

individual (level of glucocorticoids in the systemic circulation) [122, 123]. Additionally, stress induced impairment of learning and memory is closely associated with alteration in hippocampal neurons [124, 125]. Early life stress induces alterations in CA3 region of hippocampal neurons [126, 127].

Another brain structure shown in the figure is amygdala, which is present deep in the temporal lobes and is most commonly called the "almond-shaped amygdala". Bilateral radiofrequency lesions in amygdala showed low levels of corticosterone following adrenalectomy which was expected result (positive control) and interestingly, these animals also had reduced ACTH secretion [128, 129]. Therefore, disruption of amygdala influenced impaired expression of ACTH. This showed the involvement of amygdala in providing a positive feedback to the HPA axis with respect to the amount of GC present in the circulation. There are other studies that support this idea wherein lesions of the central amygdala inhibited the response of the HPA axis to stressful stimuli. Additionally, the electrical stimulation of the amygdala impaired the glucocorticoid negative feedback following neural stressful stimuli [130]. Amygdala is the emotional center of the brain and it helps in formation, processing and consolidation of fear memory [131]. Amygdala mediates fear and anxiety and this in turn drives the HPA axis to release stress hormones [59, 65, 132, 133] Fear and emotion related memories are known to be consolidated in this region which in turn helps in long-term adaptability [134]. The amygdala is activated by corticosterone released during a stress response and this causes dendritic morphological changes and these changes have been correlated with anxiety and stress in prior studies [52, 53, 57, 135]. Neuronal morphology is associated with anxiety-like behavior and general stress

response.[52, 54, 57]. Acute and/ or chronic stress, as well as exogenous supplementation corticosterone results in neuronal hypertrophy in basolateral nucleus of amygdala (BLA) [136]. Additionally, rats subjected to chronic immobilization stress in both early life and adulthood has altered spine population in BLA [7, 17, 21].

Apart from hippocampus and amygdala, another brain region that plays a critical role in regulation of HPA axis is medial pre-frontal cortex (mPFC). It has an inhibitory role on the axis and the involvement of this region depends upon the magnitude of stressor. The evidence for involvement of mPFC in regulation of HPA axis was shown in studies based on lesions in mPFC that regulates ACTH and corticosterone in response to stress [137]. The mPFC indirectly provides a negative feedback to the HPA axis [138-140] and modulates fear responses [117, 139, 140]. Prelimbic (PrL) and Infralimbic (IL) regions are the two main sub regions that form mPFC. PrL is shown to play a role in fear-conditioning and learning while fear extinction is associated with IL [141]. mPFC region is sensitive to GC, which regulates stress response in a biphasic manner. This GR:MR ratio in mPFC is high which reflects the high traffic of stress hormones in this region [142]. Hence, the primary brain regions regulating HPA axis include the hippocampus, the amygdala and the medial prefrontal cortex (Brain-HPA).

Therefore, the interaction of the brain **H**ippocampus-**P**FC-**A**mygdala axes and **H**ypothalamus-**P**ituitary-**A**drenal axes influences the behavioral read out of an individual. As seen earlier, stress has major impacts on the *structure of brain*, *neuroendocrine system* and *behavioral outcome*, which are the interacting triad in response to stress. In light of this, my study will focus on environment-

induced resilience associated with early life stressful experiences. The upcoming paragraphs will highlight the influences of differential early life environment on the interacting triad discussed above.

2.4 Influences of differential early-life environment

The environment plays a crucial role in molding BLA, hippocampus and mPFC; the regions of the brain that are involved in regulating HPA axis as mentioned above. Social attachment is an integral environmental component that influences an individual's behaviour. The 'social brain hypothesis' explains the large brain size of primates. It states that a primate's social group size has a positive correlation with the volume of its prefrontal cortex [143]. This correlation between PFC volume and social network size was found in humans as well, which is reflected in their social cognitive abilities [144]. This shows that the environment plays a major role in modulating brain regions. Of all the stages in life, early life is the most crucial one because this is the developmental stage wherein the environment plays a crucial role in driving behaviour and health for rest of the life. Exposure to chronic or severely stressful events in early life is a potential threat to physiological and psychological well-being. An individual's early life contributes to shaping the physical and emotional states in adulthood. Adverse experiences during this period result in manifestation of emotional disorders [145, 146]. A growing body of evidence shows that a disturbed relationship between parent and child, childhood negligence, abuse, and any such adverse childhood experience causes physical and behavioural dysfunctionalities that reflects on adulthood [147-149]. Adverse childhood experiences are seen to increase the risk of committing suicide [150-152]. Apart from physical and emotional damage,

neurological damage involving brain structures are also observed. For example, chronic stress causes reduced size of corpus callosum, disruption in development of regions that play an important role in modulating stress response namely neocortex, amygdala, and hippocampus [153, 154].

The influence of environment doesn't just commence from birth; it starts affecting the individual right from the foetal stage. A classic illustrator of this idea is the Dutch hunger during Second World War. In the Dutch famine of 1944, pregnant women underwent starvation, resulting in an adverse foetal environment inside the womb due to lack of nutrition and energy supplements. These harsh prenatal conditions resulted in babies with low body weights at birth, who were susceptible to decline of cognitive functions, cardiovascular diseases, and obesity despite adequate food intake in the later stages of life. This was due to the food-deprived environment that persisted during their foetal stage, which programmed their bodies for forty or more years, to be unable to get over the early period of malnutrition. [155].

As mentioned earlier in the background, a crucial need of an individual during early life is, maternal care as it helps in the sound regulation of the HPA axis, which imparts emotional stability in the long run [156, 157]. Orphans growing up in institutions, deprived of social attachment during their early life display physical, psychological, and sexual aggression [158]. Impacts of mother and infant attachment were studied in the 1950s in a famous experiment on Rhesus Monkeys by a psychologist, Harry Harlow. He showed that the mother-infant relationship was more psychological than biological. The study suggested that mothers were important not just for supplementing food but also crucial for

touch-sensitive attachment with the infant that helped the long-term emotional and mental stability of the child [159].

In contrast, positive influences in early life show a healthy pattern of development in an individual. The impact of strong parental bonding, characterized by high affection towards children was linked to a lower risk of PTSD in adulthood, suggesting that emotional enrichment protects against PTSD in adulthood [160]. Contact behavior is particularly crucial for mother-infant interaction. Besides parental touch, face-to-face interaction is vital for infants to be an active participant in social encounters during their growing years [161]. Another good example of early life environment promoting learning and social interactions is speech. New evidence reveals that starting from the first months of life, listening to speech is a reliable tool to support the attainment of fundamental psychological processes like pattern learning, categorizing the formation of objects, identifying partners for communication, improving social interactions and developing social cognition [162].

2.5 Intervention

We need to address the important point of developing interventions against concerns associated with early life stress. Individuals vary in reacting to an adverse situation wherein not all individuals react in a similar manner towards adversity. When adults or children are exposed to a stressful environment, most of them are maladaptive while few of the individuals respond in a well adaptive manner and are resilient [163]. Therefore, processes and mechanisms driving resilience needs to be studied thoroughly so that these mechanisms can be utilised as an intervention to all the detrimental effects caused by early life stress [164]. The adults who are resilient (well adaptive) towards a stressful

episode might have been exposed to a stimulatory and enriched environment during early life or a mixture of stress and enriched environment during early life. This is a question that has not been addressed so far. There is a gap we need to fill in order to understand the connection between early life experiences and its impact on adulthood behaviours. My efforts would be to gain information on how early life exposure to different environments impact the adulthood of an individual.

To define and understand neurobiology of resilience, we need to focus on the other side of the coin, "stress", from where different coping strategies/resilience starts. Maternal separation is a well-established early life stress model [165] and Enriched environment (EE) is a paradigm that induces pro-resilient behavioural and physiological features [166, 167] as well as adaptive behavioural and neural features in animals [168, 169]. So far, only two studies have determined that enriched environment can rescue the damaging effects of early life stress. One reported that environmental enrichment during the peri-pubertal period rescues the harmful effects of maternal separation on HPA and behavioural response to stressors [167]. Another study reported that, short term enrichment reverses the anxiety and hypertrophy of BLA neurons in adulthood [87]. Since there is such limited information about how enriched environment can be applied in early life to counter stress and promote resilience, it makes it a fascinating area to explore.

The hypothesis of this study: Maternally separated rats housed concurrently in enriched environment in early life (P2 to P21) shows active-coping behavior in adulthood and ameliorate the harmful impacts of maternal

separation on physiological response, neuronal structure, mRNA abundance and protein levels during adulthood.

Animal models are invaluable for an understanding of the mechanisms involved in sequelae of early life environment. An interesting early-life study in rodents showed that natural fear of predator odor develops after postnatal day (PND) 10 and this is correlated with the function of amygdala and regulation of corticosterone (CORT) [170]. As mentioned in the background, the study highlighted that maternal presence acts as an environmental switch during infancy for stabilizing fear responses [171]. At this point we know that harmful effects of MS can be reversed via short-term enrichment in adulthood [87] and my effort is to find out if short-term EE given in early life concomitant to maternal separation (MS) will alleviate the physiological, behavioral, neuronal morphological and molecular changes insults. The following paragraphs will discuss about the background of the two important paradigms that was used in this study, MS and EE.

2.6 Maternal Separation (MS)

Various studies have shown that any form of alteration in maternal care can have a lasting effect on the stress response, behavior, and emotion in rats, as it is one of the most crucial influences during development [172]. Manipulations in early environment cause acute and long-term effects on endocrinology and behavior, which have been studied in various models including rats, mice, primates, etc. Bonnet infant monkeys show a compromised behavioral and physiological response to maternal separation. Similarly, pig-tailed and rhesus infant monkeys exhibit a maladaptive response to maternal separation wherein the mother and the conspecifics are removed [173-176].

589 In rats, one of the most well-studied environmental manipulations that have
590 been reported is a brief daily separation of the pups from the mother (dam)
591 before and after weaning. Levine et al. introduced a protocol where they
592 separated the rat pups from dams for only 15 minutes, which was typically
593 referred to as “early handling” (EH) but still considered a short maternal
594 deprivation [177]. In the mid-1970’s a different but related procedure was
595 introduced, namely that of prolonged maternal separation (MS). Maternal
596 separation is a collective term used to describe various experimental methods
597 that involve separation of a pup from the dam for different time periods. This
598 study will be following separation for 180 min/day from PND 2 -14 as it is one
599 of the most well-studied chronic stress paradigm in early life [165]. Hence, MS
600 (used in this study) is undoubtedly a promising model to study early life stress.

601 *2.7 Effects of MS on Behavior and GC Levels*
602

603 A large body of evidence shows that MS during postnatal weeks causes a
604 permanent increase in anxiety-related disorders [178]. Lifelong hyperactivity of
605 HPA (hypothalamus-pituitary-adrenal) axis is another consequence of this
606 postnatal separation [179]. Maternal care involving licking/grooming (LG) and
607 arched-back nursing (ABN) of their pups (LG-ABN) is a strong influence on
608 early life development, as the maternal care is the critical environment that
609 moulds the pup’s long lasting coping with stress. Such maternal behaviors play
610 a positive role in regulating the HPA axis of the pup [180] and these high LG
611 offsprings exhibit similar maternal behaviour later on [181].
612 It has been found that when compared to control rats, MS rats show depressive-
613 like behavior in the forced swimming test in adulthood [182] and anxiety-like
614 behavior in the elevated plus maze [183]. MS rats also have increased HPA

axis responsiveness to stressors and elevated CRF mRNA in the PVN even in later stages of life [184]. As stated earlier, hippocampal GR causes glucocorticoid negative feedback, and hippocampal GR loss causes elevated GC levels. Therefore, there is a decrease in inhibitory signaling to the PVN-CRF neurons due to downregulation of hippocampal GR [121]. Apart from anxiety, MS affects cognitive and social behaviors too [185, 186]. A study on the behaviour of MS rats exhibited a significant cognitive impairment in two different tests, the Morris water maze task and the novel object recognition test [179, 187]. Therefore, neonatal MS in rats can be considered as a suitable animal model for stress-vulnerable organisms during earlylife that resulted in depression-like syndrome and enhanced stress responsiveness [188].

2.8 Effects of MS on neuronal plasticity and molecules in the brain

Early-life stress leads to reductions in hippocampal neurogenesis as well as retraction of dendrites and spine density of the hippocampus, medial prefrontal cortex (mPFC) and nucleus accumbens [126]. A single episode of MS for 24 h at postnatal day 3 does not affect dendritic complexity in the basolateral amygdala (BLA) [127]. 3 hours of MS from P2-P14 caused hypertrophy of BLA neurons which was observed both in dendritic length and branch points [87]. These are the only two studies that gives us information about the BLA neuronal morphology with respect to MS paraidgm. This limited information available about MS and BLA makes it intriguing to explore further.

Various research using rodent models provides evidence for the role of early life stress on the epigenome. Poor maternal care in rats alters DNA methylation at a particular sequence of the glucocorticoid receptor gene (Nr3c1) in the

hippocampus of the pup, causing epigenetic and behavioral changes in adulthood. MS caused downregulation of hippocampal GR [189]. However, unaffected epigenetic changes have also been reported in MS rats where DNA methylation in the same region of Nr3c1 of the hippocampus does not occur [190]. MS rats are seen to have long-term adverse effects like increased level of plasma ACTH. Effects of maternal deprivation were not only limited to the HPA axis, but elevated CRH mRNA expression was also seen in the amygdala, which most certainly plays a role in the stress response [191]. This shows the influence of molecules in amygdala mediating stress response in MS rats. MS rats exposed to acute restraint stress were reported to have elevated levels of Fos positive cells (an estimate of neuronal activation) in the central nucleus of the amygdala, PVN and bed nucleus of the stria terminalis and each of these brain regions play a critical role in organizing the behavioral response to stress [192-194]. Though very little is known about the CRF receptors, few studies have shown that after MS for 24 hours CRF receptor two is down-regulated in ventromedial hypothalamus and biomedical amygdala [195]. Prior studies show that global effect of early life experience on the hippocampal transcriptome alters more than 900 genes in response to maternal care that in turn causes anxiety mediated behavior. Few of these changes seen in gene expression is reversed using histone deacetylase inhibitor and methyl donor. Such epigenetic changes due to MS have been widely studied in the hippocampus [190, 196, 197].

2.9 Enriched Environment (EE)

Though the positive effects of environmental stimulation and its enrichment have been studied for many years, it was Hebb in the late 1940's who proposed

"enriched environment" as an experimental concept. He reported that his pet rats displayed behavioral improvements as compared to his laboratory rats [198]. So to test this in an experimental setup, an "enriched" environment about a standard cage was designed, where the cages were much larger with more complex social interaction, and it also consisted of the exploratory environment with tunnels, toys, nesting material and running wheels in some cases [199-201]. This study has no voluntary wheel as the focus of the study was on the effects of housing condition and not exercise [166, 202, 203].

2.10 Effects of EE on behavior and GC levels

The environment affects the shape and organization of animals which are more adaptable to it and are selected naturally to be propagated to further generations. These traits might be associated with behaviour of animals as well; particularly those, which are sensitive to environmental change. Mice exposed to the enriched environment had enhanced memory function in various learning tasks. These mice performed better in a spatial memory task (water maze task) than controls in standard housing cage [204]. Rodents exposed to an enriched environment, both before and after a cerebral insult show improved cognitive performance [205]. Recent studies have demonstrated that EE can influence prominent innate behaviors like avoidance of predator odor and attractiveness of males [206]. Clinical evidence showing reduced risk of dementia in individuals who have Parkinson's and Alzheimer's disease indicates that a stimulating environment has positive effects on cerebral health [207]. To see the benefits of EE, the same strain of mice was divided into two groups based on their exploratory behavior in open field task namely, high exploratory (HE) and low exploratory (LE). These animals were exposed to EE in adulthood for

692 two months to test if it modifies the existing behavior. The behaviors were
693 examined on an open field to test exploratory behavior, novel object
694 recognition to quantify the memory and the inhibitory avoidance task to test
695 learning/memory. LE animals without enrichment showed low exploratory
696 behavior and less memory performance in other tests, but enrichment provided
697 to these LE animals enhanced the exploratory behavior in the open field and
698 also increased the memory and learning performance in novel object
699 recognition and inhibitory avoidance task [208]. Enrichment is also seen to
700 reverse the effects of maternal separation on HPA axis where the MS rats were
701 subjected to EE during the peripubertal stage. When the plasma corticosterone
702 levels were compared between the MS and MS-EE rats, the latter showed much
703 lower corticosterone levels than the MS rats [209].
704 In 1942, Robert Tyron raised two groups of “bright” and “dull” mice by
705 repeatedly breeding a “bright” male (few mistakes made in the Hebb-William
706 maze test) with a “bright” female and “dull” male (multiple mistakes made in
707 the Hebb-William maze test) with a “dull” female. He took seven generations
708 to achieve a state where the resultant progenies of both the lines were all
709 “bright” and “dull” respectively [210]. Following the same line of research,
710 Cooper and Zubek (1958) exposed a group of “bright” and “dull” to an
711 enriched environment (spacious and stimulatory living conditions) and another
712 group to the standard housing to compare the effects of housing on the rat’s
713 behavior. This led to an interesting result where EE elevated the learning
714 capacity in “dull” rats while EE did not affect “bright” rats. The standard
715 conditions alleviated the learning ability in “bright” mice and did not influence
716 “dull” rats. The ability of a simple environment to change the phenotype in one

generation that took seven generations of inbreeding previously is an outstanding observation [211]. Research on benefits of EE is being applied in zoos and farms where the animals are seen to thrive better in a more enriched environment [212].

2.11 Effects of EE on neuronal plasticity and molecules in the brain

Prior studies showed that genes were the sole reason responsible for driving individual behaviors and structure of the brain. Eventually, many investigators found that the environment also played a significant role in modulating an individual's behavior and brain's structure. The cerebral cortex is the area that takes part in higher cognitive processing, and this region is more receptive than other areas of the brain to environmental enrichment. A study was carried out to check the differences in the cortex region of a rat in EE and rats in impoverished conditions. Results showed that the cortex from the enriched group had increased in thickness compared with the standard group while the brains from the impoverished group decreased compared to the latter [213]. These changes were thought to be due to alterations in the dendritic branching. More detailed studies revealed that the increase in cortical thickness was due to several factors including increased nerve cell size, number and length of dendrites, dendritic spines, and length of postsynaptic thickening as measured on electron microscopic pictures of synapses [214, 215].

Environmental enrichment and voluntary exercise exhibit similar effects in the brain. Studies showed that this change in environment and playing on wheels affected neurogenesis. Running enhanced the survival of the newborn neurons in the dentate gyrus [216, 217]. In addition to its effects on neurogenesis, studies have shown that enriched environment reduces spontaneous apoptotic

743 cell death in a rodent's hippocampus by 45% [205]. Moreover, these
744 environmental conditions have a protective effect against kainate-induced
745 seizures and excitotoxic injury.
746 The first molecular player to focus concerning HPA axis is glucocorticoid
747 receptor (GR), GC activated transcription factors that maintain the regulation
748 of HPA axis in response to various stressors [142, 218, 219]. The density of
749 GR population across amygdala, hippocampus, and mPFC differ and has a link
750 in providing feedback regulation via HPA axis [219]. The recruitment of
751 activated GR in the nucleus as a transcription factor is a crucial step that
752 initiates a relay of downstream molecular pathways in various brain regions to
753 mediate stress response [112, 220]. Prior studies show that activation of GR
754 through GC causes phosphorylation of $Erk1/2^{MAPK}$. This activated $Erk1/2^{MAPK}$
755 causes relay of downstream molecular pathways that are crucial in mediating
756 behavioral effects of GC [221, 222]. Another important molecule that interacts
757 with GC-GR and $Erk1/2^{MAPK}$ is BDNF that mediates molecular cascade to
758 enhance fear related memories. The enrichment also induces expression of
759 glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic
760 factor (BDNF) [205, 223]. It also increases phosphorylation of the transcription
761 factor cyclic-AMP response element binding protein, indicating that the
762 influence of the enriched environment on spontaneous apoptosis may be
763 mediated through transcription factor activation [207].
764 In a study, stroke-induced a decrease in nerve growth factor-induced gene A
765 (NGFI-A) mRNA expression in cortical areas and the CA1 subregion of the
766 hippocampus two to three days after ischemia. However, when these rats were
767 housed in an enriched environment, NGFI-A expression increased compared to

standard housing and the stroke-induced reduction of the hippocampal glucocorticoid receptor (GR) mRNA was reverted. By altering these molecules after the stroke, an improved functional outcome was attained by exposure to long-term EE [224-226]. A lot of focus in research is directed towards the response of EE in the hippocampus in a broad range of biological variables. The neurotrophic factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are some of the major factors to regulate the dendritic growth and the arborization of the pyramidal neurons in the hippocampus. NGF regulates structural plasticity in neurons and is upregulated under an enriched environment in the hippocampus to give rise to increased dendritic arborization and spine density [227]. NT-3 mRNA is also upregulated in the hippocampus of enriched rats. BDNF has a crucial role in modulating apical dendritic spine density and number of spines. This neurotrophic factor also has a high expression in hippocampus in response to EE [228].

Overall, EE induces the hippocampal changes in gene/protein expression of neurotrophins, glucocorticoid receptors, immediate early gene, serotonin receptors, etc., to cause the various changes in an individual[229]. These studies prove that EE has numerous beneficial effects and it can be a promising treatment as a counter-strategy to stress response.

Though numerous studies have been conducted using EE in adulthood as shown earlier, very few studies have been conducted using EE in early life, wherein long-term EE; lead to a conspicuous acceleration of visual system development appreciable at behavioral, electrophysiological and molecular level [230], slowed the rate of loss of one of the first identifiable neurochemical

deficits of Huntington's disease [231], prevented the prenatal stress-induced cognitive disorders [232] and overcame the effects of early life stress due to limited nesting on young adult rats [233]. None of the studies conducted so far have exposed the pups to short-term EE from P2-P21 to determine the beneficial effects of EE during development. This is an important gap in knowledge because most of the early life stress model have been establised in this critical window (P2-P21) of development. The study focussed in this thesis is the first ever study to examine the effects of EE from P2-P21.

2.12 Rationale behind this study

From the above information, it was apparent that differential environment plays a crucial role in shaping an individual's health. Therefore, the rats were exposed to positive (EE) and stressful (MS) environment in early life and determined if EE can alleviate the detrimental effects of MS. Since, most of the behavioural pathologies in various species like rodents, primates and humans are associated with stressful/traumatic events in early life, we studied about the individuals under stressful condition with concurrent EE as one of the paradigms to promote resilience.

The study focused on the three primary nodes of a single triad that regularly interacts with each other: neuroendocrine output, morphology, and behavior under the influence of environment. Here, each node offered a vantage point to understand its critical role in shaping stress-coping strategies using enriched environment. Another crucial vantage point was the regulation of genes as mentioned earlier.

The study was broadly divided into the following specific aims, elaborating the objectives of this study:

<u>Specific aim 1 (S.A.1):</u> To analyze the effect of early life MS, EE and MSEE on behavior and physiological readouts in adult male rats.

<u>Specific aim 2 (S.A.2):</u> To determine the effect of early life MS, EE and MSEE on morphological changes in Basolateral amygdala, hippocampal and mPFC neurons in adult male rats.

<u>Specific aim 3 (S.A.3):</u> To identify the role of candidate genes in driving behavioral changes in S.A.1 and morphological changes in S.A.2 (e.g., GR, BDNF, CRF, etc.,) in adult male rats.

<u>Specific aim 4:</u> To delineate underlying possible epigenetic changes that drives S.A.1, S.A.2 and S.A.3 in adult male rats.

Chapter 3

3. Materials and Methods

3.1 Animals

Male and female Wistar rats (7 weeks old and housed 2/cage) were procured from Charles River, Kingston, North America. Breeding was initiated between a pair of rats after at least two weeks of habituation in Nanyang Technological University vivarium. Animals were maintained in a 12h light-dark cycle (0700-1900 h light on, 1900-0700 h light off) with *ad libitum* food and water. The institutional animal care and use committee (IACUC) of the NTU approved all experimental procedures.

Breeding protocol

Naïve female breeders were procured at 7-8 weeks of age. Females were allowed to acclimatize for at least five days before breeding was set up. All breeders were housed in the corner of room away from the door and high traffic areas. Bedding was changed once a week, but with gentle handling of the females, in case of pregnancy. A handful of bedding from the old cage was added to the new cage to reduce novelty stress in females. Ten days after the breeding pairs were set up, females were checked once or twice a week for signs of pregnancy (swollen abdomen). Once pregnancy was confirmed (approx. 2 weeks), males were removed. 19 days after breeding pairs were set up (or if females were visually heavily pregnant); cages were checked daily for the presence of litters.

Day of birth was assigned as P0. Following birth, cages were left undisturbed by (no cage cleaning). Feeding and watering were handled by me until weaning.

On P2, pups were sexed. If more than 12 pups were born to a single female, female pups were culled to obtain a litter size less than or equal to 12. Cages were cleaned by me on P2, P9, P14 and P18 (if required). On P21, pups were weaned (seperated from dam/mother). After weaning the animals, the males were housed two animals per cage and habituated for a week in NTU Animal facility. The animals were fed and watered ad-libitum and were maintained in a light-dark cycle of 12 h (0700-1900 h light on, 1900-0700 h light off). The animals not subjected to any treatment were considered as animal facility rearing (AFR) group/control group. For AFR controls, litters were undisturbed, except for cage changing on P2, P9 and P14. After P14, litters were handled the same way as all other group's litters.

For every experiment conducted in this study, multiple litters were used. The litters were taken from different dams for each group and each dams was bred only once. Pups from different dams were chosen for each experiment to avoid bias of one particular maternal grooming in experiment as licking pattern can vary for each dam [181]. Since there were different cohorts used for all the experiments, the numbers of pups vary for each experiment. The number of animals used for each experiment is indicated in each figure legend.

Experimental paradigms

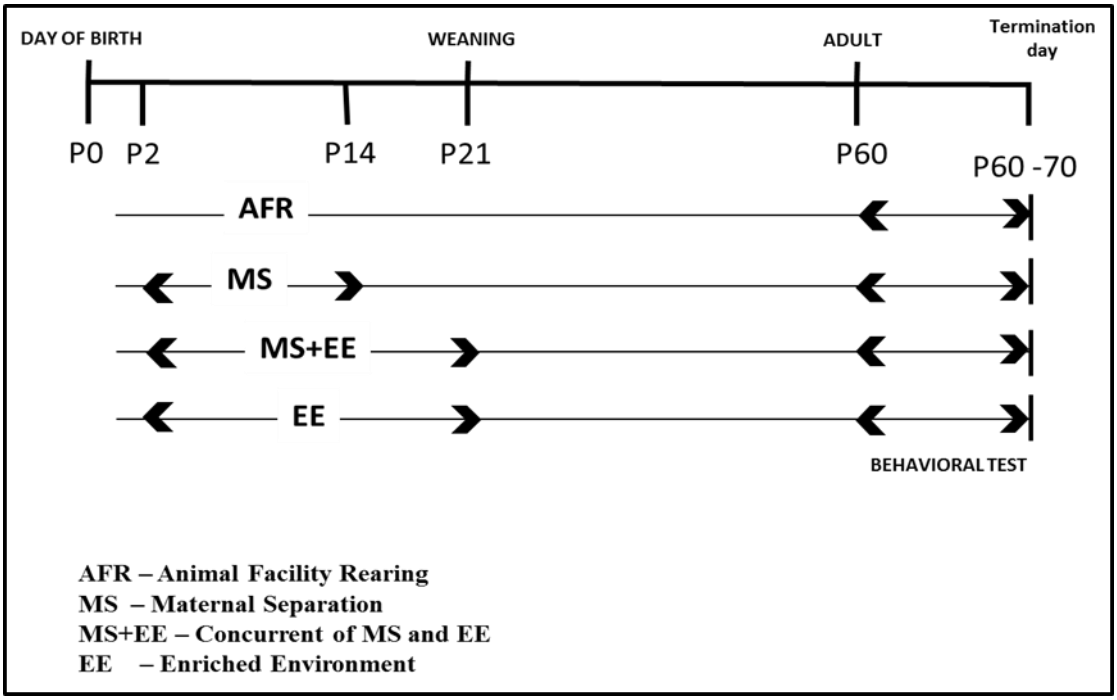


Figure M.1: Timeline of experiments showing the duration of early life treatments as well as the timeline of behavioral tests. Termination day varies depending on the endpoint of the study. Please refer to the respective schedule for each experiment.

3.2 Maternal separation (MS)

On P2-P14 (daily), using a gloved hand, the mother was placed in a clean cage with fresh bedding and returned to the shelf. Pups were then moved one-by-one into a clean cage as a group, brought to another room, kept on a heat pad and monitored for three hours. After separation, pups were returned to the home cage, placed in the nest and sprinkled with dirty bedding. The mother was then returned to the home cage.

On P2, P9, and P14, following separation, the animals were placed into a clean cage and sprinkled with a handful of bedding and nesting material from the old cage. This was repeated on P18 if due to litter size the cage was visibly dirty. On P21, the pups were weaned.

Figure M.2: Maternal separation model. Dam is kept in a clean cage in a room and pups were kept in a clean cage as a group in another room and monitored for three hours.

3.3 Environmental enrichment (EE)

The EE groups were housed in Enrichment (EE) cages on P2-P21 (1 dam and her litter). The EE cages (72 x 51 x 110 cm^3) were more spacious than standard cages. They were equipped with plastic pipes for burrowing, plastic and wooden toys, jingles, platforms, ladder for climbing, baskets, and nesting material. EE cages were also wire meshed for rats to climb. The arrangement of the various stimulatory objects within the EE cage were changed every 4th day, and some fruits loops and sunflower seeds were also dispersed every 4^{th} day.

Figure M.3: Environmental enrichment cage. EE cages (72 x 51 x 110 cm^3) were equipped with plastic pipes for burrowing, plastic toys, wooden toys, jingles, platforms, ladder for climbing, baskets, and nesting material.

3.4 Maternal separation + Enriched Environment (MSEE)

The MSEE group underwent maternal separation just like MS group, but was housed in EE cage instead of the standard cage from P2-P21. The EE cages used for this group were similar to EE group.

3.5 Home cage emergence test

The home cage emergence test was adapted and modified from Prickaerts, J. et al., (1996) [234]. A rat in its home cage was moved from the holding room to the test room and habituated for 5 minutes. The whole area of the test was well lit. The home cage was left open, and the rat was offered the possibility of emerging from its home cage via a grid. This was observed for 5 minutes. The latency to emerge from the home cage (i.e., the time until the rat was on the grid outside its home cage with all four legs) was scored. The acquisition and the test trials were video recorded and scored blinded manually afterward**.**

Apparatus was cleaned with 70% ethanol after each animal's emergence and allowed to dry before the next test.

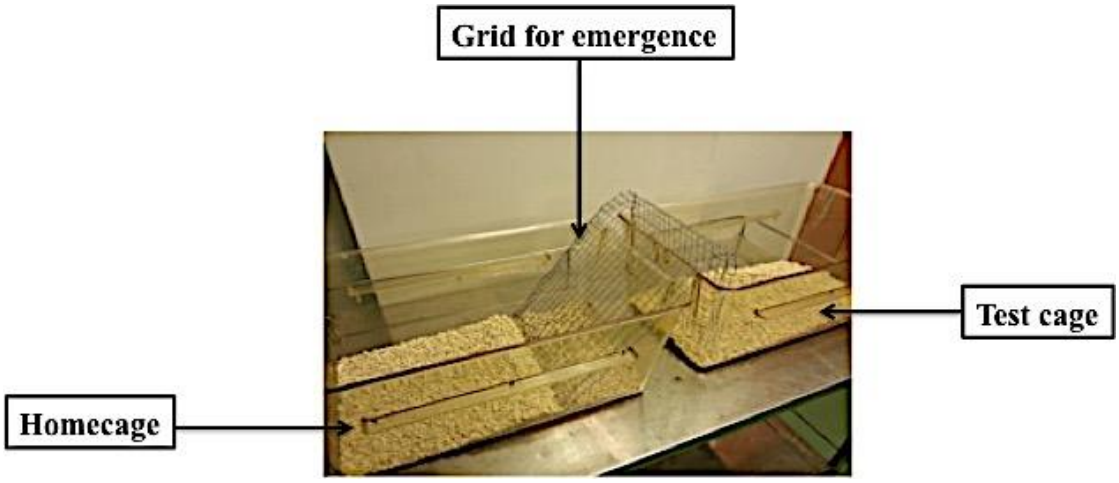


Figure M.4: Home cage emergence setup. The home cage was left open, and the rat was offered the possibility of emerging from its home cage via a grid to move to the test cage.

3.6 Open field test

Open field test was conducted in a circular arena. Animals were placed in an open arena (radius=120 cm, trial duration=300 s, diffused dim light conditions). Time spent in the center of the field was quantified as the reciprocal proxy of the anxiety (center defined as a concentric circle to the arena with 0.33 m radius). All tests were conducted between 09.00AM and 12.00PM (duration = 300s). Apparatus was cleaned with 70% ethanol after each animal and allowed to dry before the next test.

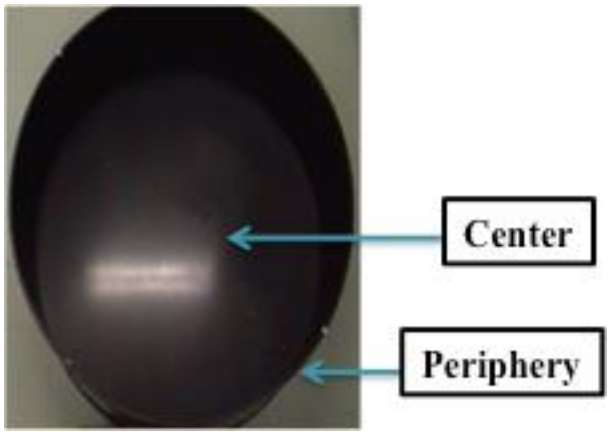


Figure M.5: Open field test setup. Open field test was conducted in a circular arena. Animals were placed in an open arena (radius=120 cm, trial duration=300 s, diffused dim light conditions).

3.7 Light dark box test

The light dark exploration test was conducted as previously described [235]. The apparatus consisted of a polypropylene cage (separated into two compartments by a partition, which had a small opening (10 X 8 cm^2) at floor level. The light compartment (28 cm long) was open-topped, and very brightly illuminated by a desk lamp (500 lx). The dark compartment (24 cm long) was also open-topped and painted black without light. Rats were individually placed in the center of the black compartment, facing away from the partition, and allowed to explore the apparatus for 5 minutes freely. The total time spent in the light and dark compartment were video recorded and scored blinded manually afterward. Apparatus was cleaned with 70% ethanol after each animal and allowed to dry before the next test.

Figure M.6: Light dark box setup. The apparatus consisted of a polypropylene cage, separated into two compartments by a partition, which had a small opening at floor level. The white compartment was open-topped and very brightly illuminated. The dark compartment was also open-topped and painted black without light.

3.8 Elevated plus maze

Anxiety was measured using an elevated plus-maze (EPM, [236]). The EPM consisted of a plus-shaped arena with two open (75 x 11cm, 1cm wall, 3-4 lux illumination) and two enclosed arms (75 x 11 cm, 26 cm wall, 0 lux illumination). The arena was elevated to 60 cm above the ground. The animal was placed at the center at the start of trial. Exploration in the open and enclosed arms was quantified for the duration of 5 minutes. Apparatus was cleaned with 70% ethanol after each animal and allowed to dry before the next test. Open arm exploration (entries and occupancy time) relative to sum of open and enclosed arm exploration was used as an index for anxiety [236]. Entry in an arm was defined as the presence of the whole body including head, four paws and at least base of the tail inside the open arm. Studies using factor analysis have shown that the number of closed-arm entries corresponds to the general locomotion of rats [237]. Therefore, the number of closed-arm entries

was also measured as an internal control. Also, the number of head dips was quantified. Head dip is defined as downward movement of the head toward the floor, extending completely out of the open arm [236]. It has been defined as a measure of exploratory, and goal-directed behavior. Its absence indicates passive behavior [238-240]. Such an exploratory trait representing active coping serves as a potential marker for stress-resilience. Trials were videotaped and coded before offline analysis.

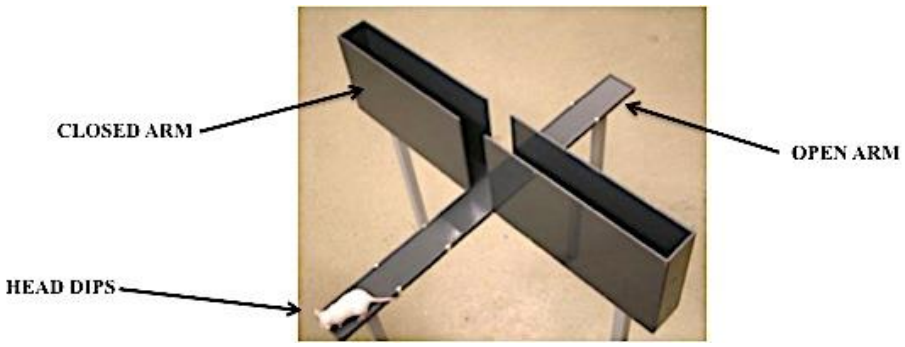


Figure M.7: Elevated plus maze setup. The EPM consisted of a plus-shaped arena with two open and two enclosed arms. The arena was elevated to 60 cm above the ground.

3.9 Social interaction test

The social preference test was adapted from the protocol described by Vandana and collaborators to investigate social affiliation in male rats [241]. The test was carried out in a rectangular, three-chambered box (a center 20 × 35 × 35 cm^3; a left and a right compartment 30 × 35 × 35 cm^3). Dividing walls had retractable doorways allowing access to each chamber. Left and the right compartment were separated from the central compartment with the help of transparent Plexiglas with small holes, where either a social (unfamiliar rat of similar weight) or a non-social stimulus (yellow plastic box) was placed. The Plexiglas permits visual, tactile, auditory and olfactory communication. The

juvenile rats were first habituated to the three-chambered apparatus by placing them individually in the central compartment for 10 min during the three consecutive days preceding the social test. The doorways into the two side chambers were closed during this habituation phase.

On the testing day, the unfamiliar juvenile was placed in one of the side chambers and the object on the other side. The experimental rat was first placed in the middle chamber and allowed to explore for 5 min. The location of the juvenile and the object in the left vs. right side chamber was counter-balanced. The session was video-recorded and the time spent sniffing each cylinder containing the object or unfamiliar juvenile was scored offline to evaluate the level of preference for the unfamiliar juvenile as compared to the object. The rats were considered to have explored the object and the juvenile when their behaviour complied with the following criteria: (1) when they were approaching the respective compartment with their nose at a distance less than approximately 2 cm (2) when their nose was oriented toward the contents of the compartment (i.e., juvenile rat or object). These criteria of proximity and orientation were followed and quantified as sniffing behavior. The entire apparatus was cleaned with 70% ethanol solution and dried properly between each test.

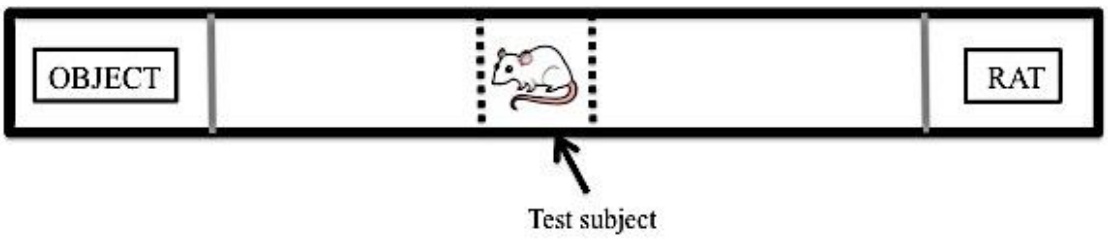


Figure M.8: Schematic of the three-chambered box used to perform social interaction test. The test was carried out in a rectangular, three-chambered box. Left and the right compartment were separated from the central compartment with the help of transparent Plexiglas with small holes, where either a social (unfamiliar rat of similar weight) or a non-social stimulus (yellow plastic box) was placed.

3.10 Porsolt's forced swim test

The animals were tested for depressive-like symptoms (despair) using Porsolt's forced swim task [242] . On the first day (T1) of the test, each animal was put in a water container (diameter = 20 cm, height = 50 cm) for 15 minutes for habituation. The temperature of the water was maintained at 21-23ºC and was filled up to 33 cm of the container. On the second day (T2) of the test, each animal was tested for forced swim and videotaped for 10 minutes (conducted between 09.00AM-12.00PM). After each test, the animal was returned to its home cage, and the container was cleaned thoroughly with water. I was blind to treatments during offline analysis. Parameters such as swimming and immobility (an indication of despair or depressive-like behavior) were timed and compared across groups.

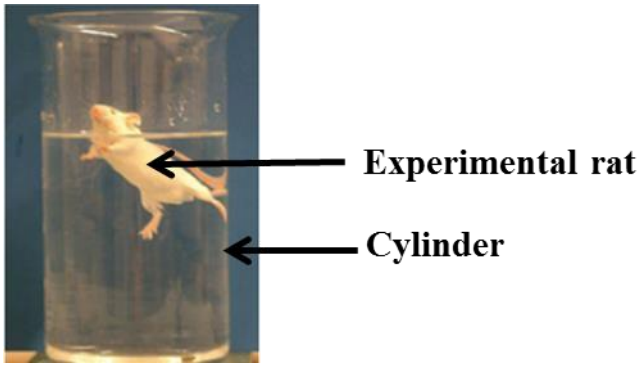
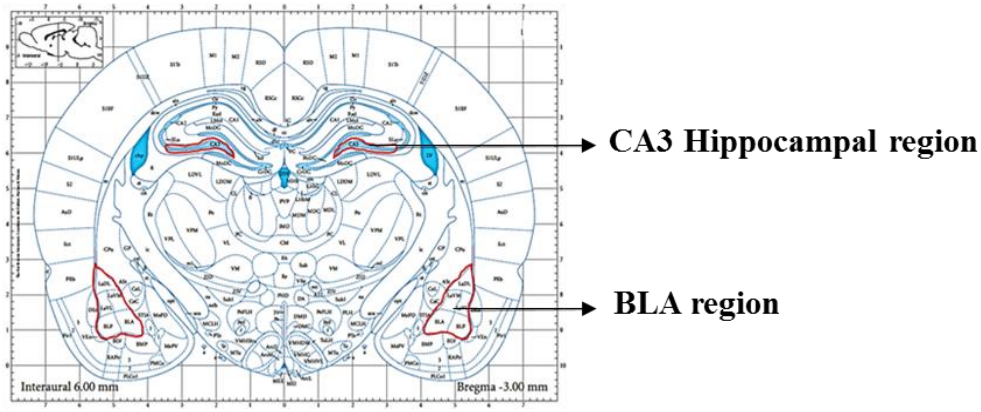


Figure M.9: Forced swim test setup. The animals were tested for depressive-like symptoms (despair) using Porsolt's forced swim task. The temperature of the water was maintained at 21-23ºC and was filled up to 33 cm of the container.

3.11 Measurement of dendritic arborization

Freshly harvested brains were obtained by sacrifice through decapitation. Blocks of brain tissue were processed for Golgi-Cox stain using a commercial kit (FD Neurotechnologies, Columbia, USA). The golgi solution (solution A and B, mixed in equal amount) was prepared 24 hours before tissue extraction. The freshly harvested tissue was immersed in Golgi solution soon after the extraction and these brains were transferred to fresh golgi solution after 24 hours. The brains were kept in the golgi solution for 2 weeks and then frozen using dry ice. The tissue was then cryo-sectioned (100-µm thick) and further processed using cresyl violet staining. The stained 100-µm thick coronal sections were mounted on glass slides. One batch of animals was used to obtain sections containing both BLA and hippocampus (Bregma -2.04 to -3.36, Figure M.10A [243]) and another one for sections containing mPFC (Bregma 3.72 to 3.00, Figure M.10B, The Rat Brain, Paxinos and Watson [243]). For mPFC, neurons from the prelimbic and infralimbic regions were analyzed separately. Therefore all sections in the second batch contained these regions. The slides were counter-stained with cresyl violet, dehydrated using an ascending series of alcohol and xylene, and coverslipped in non-aqueous medium (Permount, Thermofisher Scientific, USA).

A



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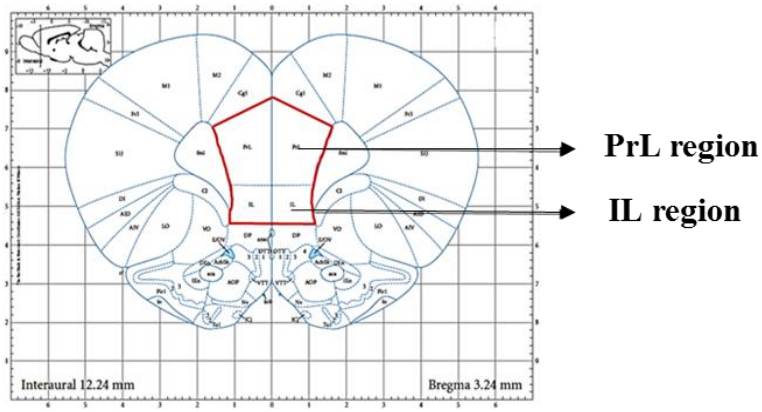
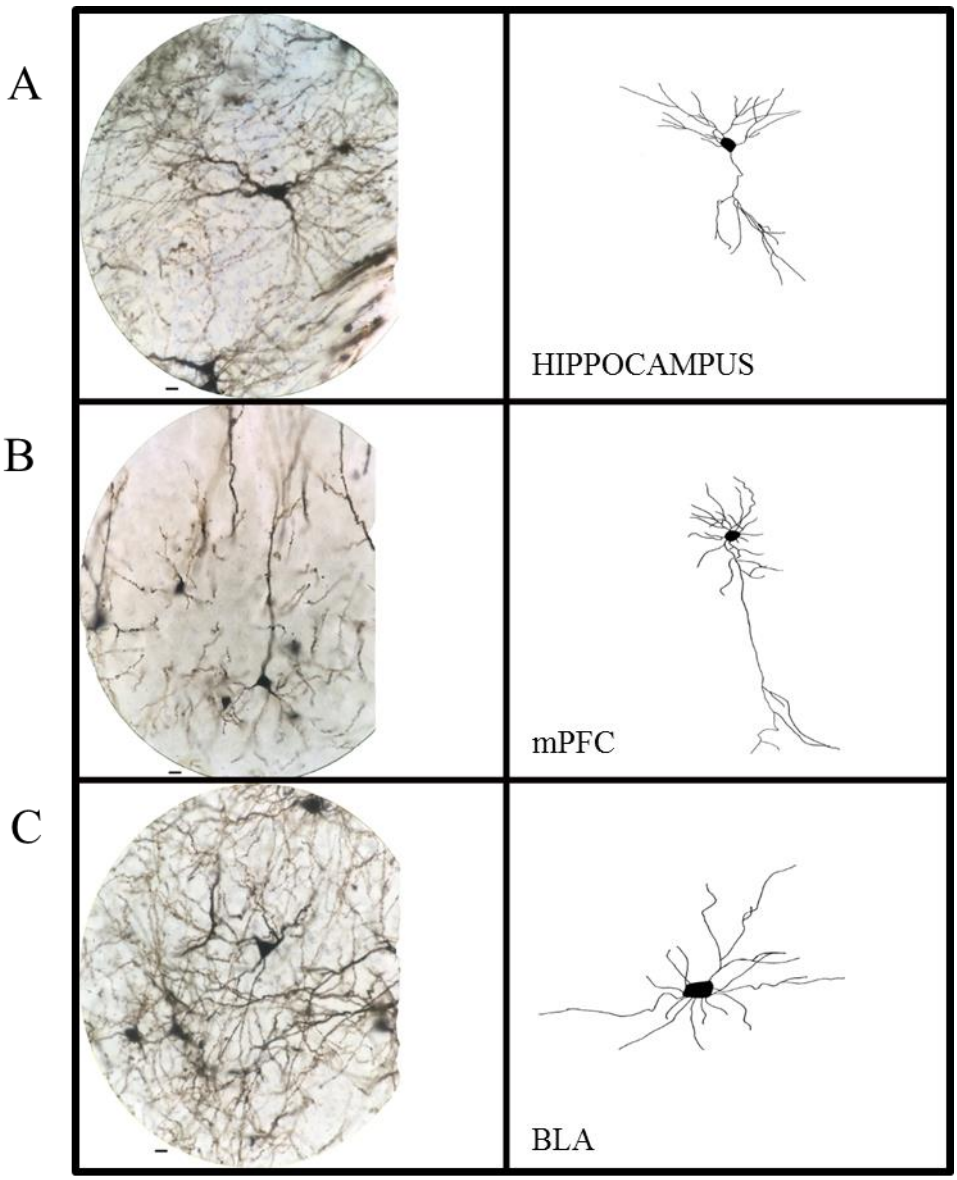


Figure M.10: Coronal brain section showing sampled BLA, mPFC and hippocampal region. A) Representative coronal brain section used for sampling neurons from the basolateral amygdala (BLA) and the hippocampus. B) Representative coronal brain section used for sampling neurons from the Prelimbic (PrL) and the Infralimbic (IL) regions. Image source: The Rat Brain, Paxinos and Watson [243].



1084

1085 **Figure M.11: Representative images of neurons from the hippocampus,**

1086 **mPFC, and BLA.** Scale bar is 20 μm and represents all images.

Two-dimensional traces of neurons (10 neurons per animal) from three regions: BLA, hippocampus, and medial prefrontal cortex were obtained at 400X magnification using a camera lucida attachment on the optical microscope (Olympus BX43, Japan). Randomly chosen neurons from each region were analyzed for neuronal complexity and spine analysis (Table M.11). Traces were scanned (300 dpi, 8-bit greyscale tiff) along with a calibrated scale for subsequent computerized estimation of dendritic arbors using custom-designed routine embedded in ImageJ (http://rsb.info.nih.gov/ij/). Dendritic length and number of branch points were quantified as a function of radial distance from the cell soma (Sholl analysis, Figure M.12) [244, 245]. This is represented as both dendritic length and total branch points in results.

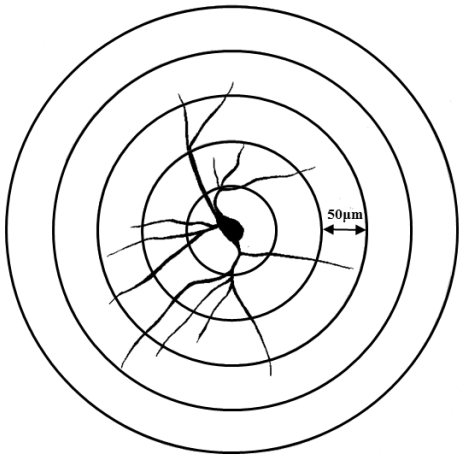


Figure M.12: Sholl analysis of a BLA neuron sample. Sholl analysis includes quantification of dendritic length and number of branch points in each segment of the overlying concentric circles (50µm radius) starting from the soma till the distal ends of the neuron.

Dendritic spines were counted using the 1000X oil-immersion magnification using Olympus BX43 microscope. Dendrites directly originating from cell soma were classified as primary dendrites, and those arising from primary dendrites were classified as secondary dendrites [246]. Starting from the origin of the branch, and continuing away from cell soma, spines were counted along a 60-80µm stretch of dendrite depending upon the type of neurons (Table M.1). One dendrite of each kind (primary/secondary) per cell and 4-10 cells per animals for dendritic spines were analyzed (Figure M.13). Since morphology and spine analysis were done independently, but from the same slides, there is a good chance of an overlap among the cells that underwent analysis for both morphology and spine analysis.

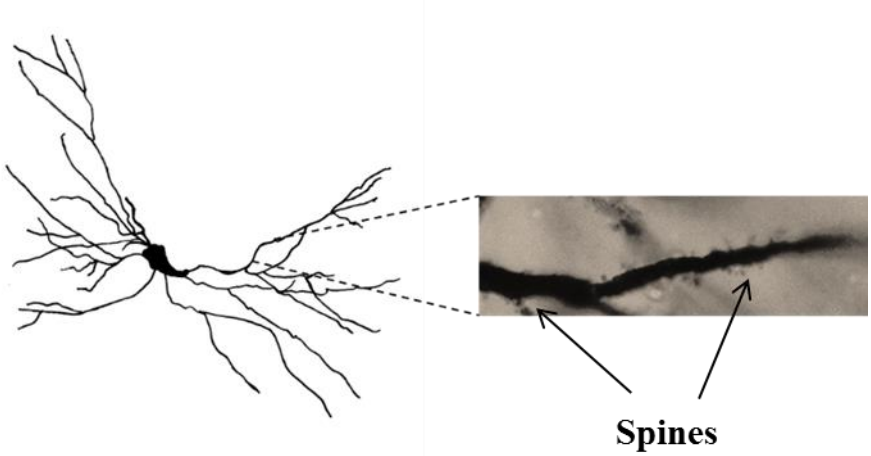


Figure M.13: Illustrative representation of neuronal spine analysis. Highlighted region indicates a stretch of primary dendrite (at 1000X magnification) selected from a sample pyramidal neuron for spine analysis.

Table M.1: Details of neuronal types in each brain region selected for morphological analysis.

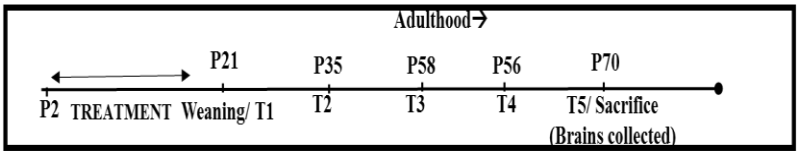
Brain Region	**Type of neurons**	**Bregma**	**Length of dendrite analyzed for spines**
Basolateral Amygdala	Pyramidal and stellate neurons	-2.04 to -3.36	60 µm
Hippocampus	CA3 Pyramidal neurons	-2.04 to -3.36	60 µm
Prelimbic region (mPFC)	Pyramidal neurons	3.72 to 3.00	80 µm
Infralimbic region (mPFC)	Pyramidal neurons	3.72 to 3.00	60 µm

3.12 Measurement of corticosterone, weight of adrenal gland, and body weight.

The various time points for blood collection are summarised in the figure below (Figure M.14B). Certain time points were taken post-stress exposure. The stress exposure was 2 mL of cat urine (predator odor) added to a cotton pad and placed in the home cage for 5 mins. Tail vein blood samples were collected from animals for corticosterone measurement. Animals were lightly restrained in a soft cotton towel, and 50-200 µl blood was drawn from tail nick at lateral vessels. This method is known to result in minimal stress [247]. Blood

was centrifuged (4700 rpm, 15 minutes; Thermo Scientific Sorvall Legend XTR, Germany) and separated serum was stored at -80ºC until further analysis. The concentration of serum corticosterone was quantified using an enzyme-linked immunoassay based commercial kit (Enzo life sciences, NY, USA). Also, adrenal glands were collected bilaterally using forceps (wiped dry and clean) during sacrifice and dry weight was taken. Body weights of all animals were recorded at various time points throughout the experiment to assess weight gain. The different time points for weighing are summarised in the figure below (Figure M.14A). Area under the curve (AUC) for-weight gain was compared across groups.

A



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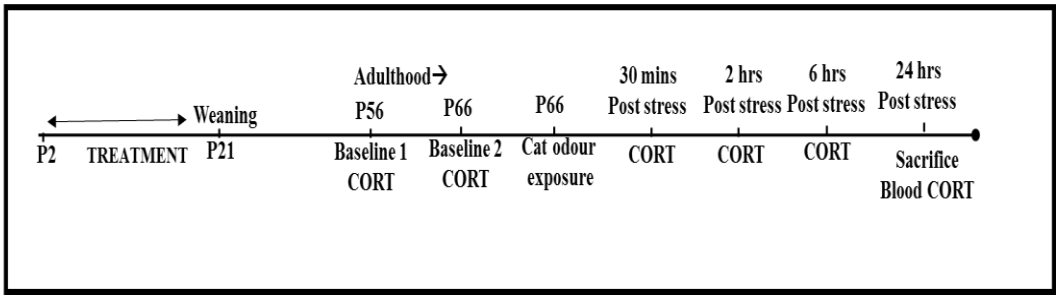

Figure M.14: Experimental timelines for Physiological endpoints. A) Experimental timeline for weight measurements. B) Experimental timeline for blood collection to determine plasma corticosterone levels.

3.13 Quantification of mRNA abundance in the basolateral amygdala

Brain tissue was harvested post-decapitation and flash-frozen in liquid nitrogen. The tissue was stored at -80˚C until further analysis. Subsequently, mRNA abundance was quantified for GR, BDNF, Trk B, Arc, Erk-1 and MR. A standard method based on SYBR green dye was used as described before [248, 249]. PCR cycles needed to reach a predetermined fluorescence threshold at the early linear phase of the amplification were determined (Ct, threshold cycle number). Greater mRNA abundance in this assay results in lower Ct values. Ct values for all molecules were normalized by subtracting corresponding Ct values of 2 housekeeping genes (HPRT and GAPDH). Three technical replicates were used for each determination. Median coefficient of variation between technical replicates was ≤ 15%. 'No-template control' and 'no-RT control' were maintained in every plate used in the experiment. Multiple primers were designed before choosing the final primer. The primer efficiency of the final primer that was considered in this experiment was >90%. The baseline threshold was automatically set using CFX Maestro™ Software, which was reliable and unbiased between the groups and this was confirmed after each run. The dissociation curve was performed for every single primer to avoid primer-dimers. For clarity, an example of acceptable and unacceptable dissociation curve is given in Figure. M.15. Refer to figure M.16 for the schedule of the experiment.

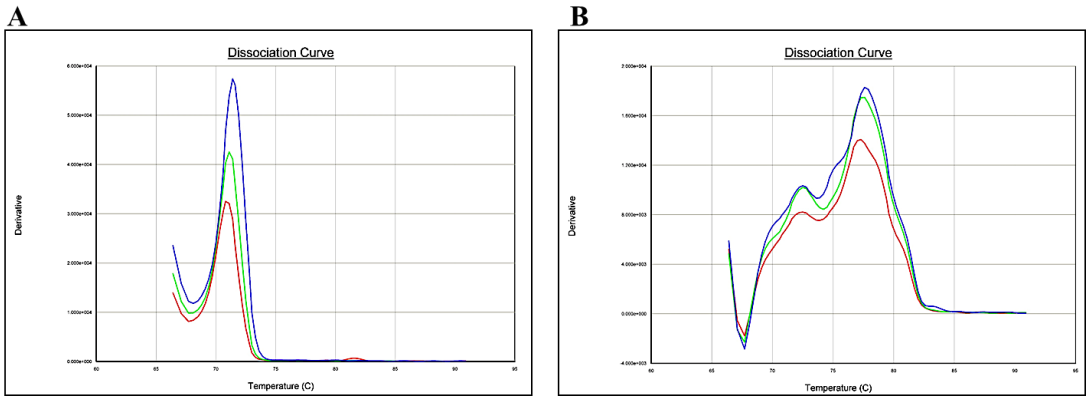

Figure M.15: Dissociation curve for qPCR. The dissociation curves displayed above are examples of acceptable (A) and unacceptable (B) dissociation curves used for selecting a suitable primer for each gene of interest.

Table M.2: List of Primers used for qPCR and their sequences

Primer Name	Sequence 5' to 3'
CRF_F	ATCAAGGTGTCTGAGGGCACCTAC
CRF_R	TGGCCTAAATCTGCTTGGCGAAC
Nr3c2_F	AGGCTTCTGGGTGTCACTATGG
Nr3c2_R	ACACAGATAGTTGTGTTGCCCTTC
BDNF_F	GAGAAGAGTGATGACCATCCT
BDNF_R	TCACGTGCTCAAAAGTGTCAG
Arc_F	ACAGAGGATGAGACTGAGGCAC
Arc_R	TATTCAGGCTGGGTCCTGTCAC
GAPDH_F	TCACCACCATGGAGAAGGC
GAPDH_R	GCTAAGCAGTTGGTGGTGCA
HPRT_F	AGGCCAGACTTTGTTGGATT
HPRT_R	GCTTTTCCACTTTCGCTGAT
ERK_F	TCCCAAACGCTGACTCCAAAGC
ERK_R	GCCAGAGCCTGTTCAACTTCAATC
TrKB_F	AGCTGACGAGTTTGTCCAGGAG
TrKB_R	ACGGATTACCCGTCAGGATCAG
GR_F	AGGCTGGAATGAACCTTGAAGCTC
GR_R	TCCTGCAGTGGCTTGCTGAATC

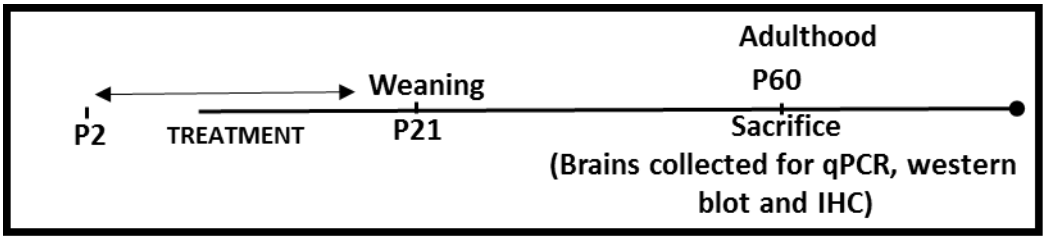

Figure M.16: Schedule of experiment for qPCR, western blot and immunohistochemistry (IHC) endpoints.

3.14 Protein expression studies in the basolateral amygdala

Tissue preparation for immunostaining

Rats were sacrificed through transcardial perfusion of phosphate-buffered saline (PBS) followed by 4% paraformaldehyde dissolved in buffered saline. Harvested brains were post-fixated and equilibrated with 30% sucrose in PBS. These brains were cryosectioned in coronal planes for 40µm thickness and collected in 24-well plate with anti-freeze media at -20ºC. Representative sections from Bregma -2.76 to -2.92 were used for each animal and subjected to free-floating staining (The Rat Brain, Paxinos, and Watson) for the following:

Study of colocalized expression of BDNF and pMAPK: Brain-derived neurotrophic factor with phosphor-mitogen activated phosphate kinase.

Study of activated glucocorticoid receptor (GR) expression: Colocalization of GR with DAPI for nuclear GR expression

Immunofluorescence staining for BDNF and pMAPK

For all staining, sections (with BLA) in series of 6 were selected for each animal and placed in a 12-well plate. The sections were rinsed three times (10 mins/wash) in 2.5mL phosphate buffer saline (PBS). 2.5 mL per well of blocking solution (1XPBS + 5% BSA) was added to the sections and incubated for one hour at room temperature. 2.5 mL of primary antibody with 2 primary antibodies simultaneously—Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (1:200, Cell Signalling Technology, MA, USA) and mouse anti-BDNF antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa) diluted in PBT (PBS + 0.1%Tx100 + 3% BSA was added, and the plate was incubated for 48 hours at 4˚C on a shaker at low speed. Sections were washed twice with 2.5 mL (10 mins/ wash) 1X PBS at room temperature. The secondary antibodies were DyLight®549 goat anti-rabbit IgG (1:1000, Vector laboratories, CA, USA) and DyLight®488 horse anti-mouse IgG (1:1000, Abcam, CA, USA) diluted in (PBS+5% BSA) was added to the wells and incubated for 2 hours in the dark (2.5mL/well). Sections were washed three times with 2.5 mL (10 mins/ wash) 1X PBS at room temperature and high-speed. For co-labeling with 4', 6-diamidino-2-phenylindole (DAPI, a nuclear counterstain for blue-fluorescence, Sigma-Aldrich, USA), the sections were incubated with 2.5 mL/well DAPI solution for 1 minute in the dark. Sections were washed four times with 2.5 mL (10 mins/ wash) 1X PBS at room temperature and high-speed. Sections were mounted on Superfrost slides dried and fixed in a nonaqueous medium (PVA-DABCO, Sigma-Aldrich, USA) on each section and coverslip. Slides were stored at -20˚C until imaging. The images were analyzed using Image J, (NIH)

to quantify average percentage area of BDNF and pMAPK signals. Refer to figure M.16 for the schedule of the experiment.

Immunofluorescence staining for nuclear GR

For all staining, sections (with BLA) in series of 6 were selected for each animal and placed in a 12-well plate. The sections were rinsed three times (10 mins/wash) in 2.5mL phosphate buffer saline (PBS). 2.5 mL per well of blocking solution (1XPBS + 5% BSA) was added to the sections and incubated for one hour at room temperature. 2.5 mL of primary antibody, rabbit anti-GR antibody (1:500, Santa Cruz, CA, USA) diluted in PBT (PBS + 0.1%Tx100 + 3% BSA was added, and the plate was incubated for 48 hours at 4˚C on a shaker at low speed. Sections were washed twice with 2.5 mL (10 mins/ wash) 1X PBS at room temperature. The secondary antibody, DyLight®549 goat anti-rabbit IgG (1:1000, Vector laboratories, CA, USA) diluted in (PBS+5% BSA) was added to the wells and incubated for 2 hours in the dark (2.5mL/well). Sections were washed three times with 2.5 mL (10 mins/ wash) 1X PBS at room temperature. For co-labeling with 4', 6-diamidino-2-phenylindole (DAPI, a nuclear counterstain for blue-fluorescence, Sigma-Aldrich, USA), the sections were incubated with 2.5 mL/well DAPI solution for 1 minute in the dark. Sections were washed four times with 2.5 mL (10 mins/ wash) 1X PBS at room temperature. Sections were mounted on Superfrost slides dried and fixed in a nonaqueous medium (PVA-DABCO, Sigma-Aldrich, USA) on each section and coverslip. Slides were stored at -20˚C until imaging.

Confocal Imaging

Four randomly selected regions of interest (ROI) for each animal was imaged at 400X magnification using the confocal laser scanning microscope LSM 710 (Carl Zeiss, Oberkochen, Germany). Immunofluorescent images were obtained for a 40µm thick cross-section set as the ROI (zoomed to 2X) which was imaged into a z-stack of 35-40 slices (interval: 1µm). Each z-stack was imaged at a fixed area which was identical across all the ROIs in all sections in this experiment using Image J, (NIH) to quantify average percentage area of GR. The open-source Vaa3D (Allen Institute for Brain Sciences, Washington, USA software [250] was used to quantify HD-GR. Refer to figure M.16 for the schedule of the experiment.

The same images were used to quantify "percentage high density GR cells". The cells that were quantified as *high density* manually based on the amount of signal present in the nucleus and these cells are represented in figure M.17. VAA3D was used to visualize the DAPI-stained nucleus with high density GR (HD-GR). These HD-GR cells were counted manually.

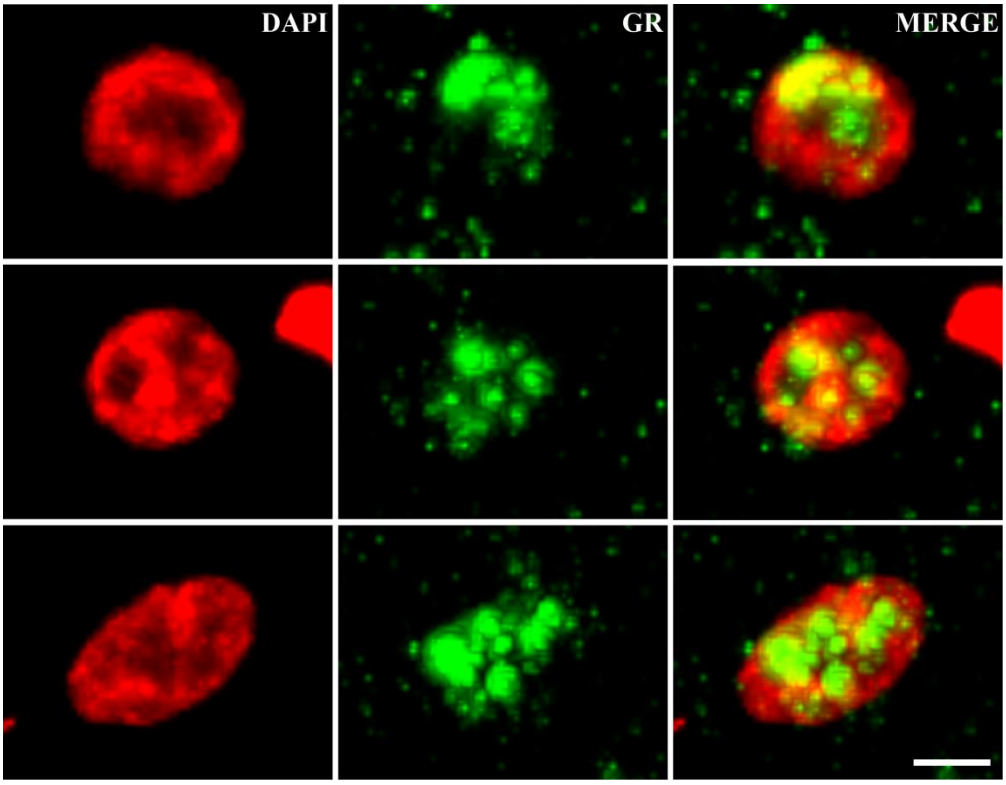

Figure M.17: Panel representing high-density GR stained cells.

Immunofluorescence staining of the nucleus with DAPI (red) and GR (green). These are the representative images of the cells that contained high-density GR and were considered for high-density GR analysis. The image was pseudocolored with red and green for clarity. Scale bar represents 5 µm.

Tissue preparation for Western Blotting

Freshly harvested brains were obtained by sacrifice through decapitation, which was snap-frozen using liquid nitrogen. Harvested brains were cryosectioned in coronal planes at 100 µm thickness, and the BLA was collected by bilateral punches using biopsy micro punch and Pasteur pipette tubes in sections from Bregma -2.04 to -3.36 (The Rat Brain, Paxinos, and Watson). Lysis buffer (1ml TPER + 10µL of 100x EDTA + 10µL of a protease inhibitor; 80µL per sample) was added to each tube at room temperature. After ten minutes, tissue was homogenized and centrifuged at 14,000 RPM for 20

mins at 4˚C. The aliquot supernatant containing the protein was stored as samples at -80˚C.

Western Blotting

Expression of GR, TrkB, BDNF, pro-BDNF and Arc were determined by Western blotting.

Bovine serum albumin (BSA) standards were prepared and used to generate a standard curve for the estimation of sample protein concentration. MilliQ water was used to make a 1:10 dilution of the sample and 10 µL of diluted sample was added to a 96 well plate in duplicates. Protein assay dye (200 µL) was added to each of the above wells and incubated for 15 minutes. Absorbance was measured at 595 nm in the Tecon 200 machine with the help of i-software. The following sequence was followed: choose plate type → select well reading → 15 seconds shaking → Reading 3x3 for each well → 595nm. Samples were then loaded into pre-cast gels and run for 45 mins at 145V. The Transfer turbo cassette (Bio-rad, Singapore) was used to transfer the gel for 7mins. Blot was cut based on required size and blocked in 5% BSA (made in TBST 1X) for 1 hour. Blot was incubated with primary antibody for ~14 hours (diluted with three %BSA made in TBST 1X). Blot was washed three times with 1X TBST (10 min/ well). Blot with incubated with secondary antibody for 1 hour (diluted with 3 %BSA made in TBST 1X). Blot was washed three times with 1X TBST (10 min/ well). The substrate was added to blot (A+B; 1:1) and developed. Blot was saved by placing in 10X TBS. Blots were first probed with specific primary antibody for all the proteins and beta actin (internal control). The bands were visualized using ECL select (G.E. Healthcare, UK). Blots were

developed using LAS4000 image reader version 2.02 (Fujifilm, Japan). The relative intensity of the protein bands was quantified using NIH ImageJ (version 1.50b; NIH, USA). Refer to figure M.17 for the schedule of the experiment.

Table M.3: Details of primary and secondary antibodies used in Western Blotting

Protein of interest	Molecular weight (kDa)	Primary antibody (Dilution, Manufacturer)	Secondary antibody (Dilution, Manufacturer)
Glucocorticoid receptor (GR)	90-95	Rabbit (1:500, Santa cruz)	Goat anti-rabbit (1:2000, Santa cruz)
Tyrosine kinase receptor B (TrkB)	145	Mouse (1:500, BD Biosciences)	Donkey anti-mouse (1:1000, Santa Cruz)
pro-Brain derived neurotrophic factor (pro-BDNF)	32	Goat (1:1000, Santa Cruz)	Donkey anti-goat (1:2000, Santa Cruz)
Brain derived neurotrophic factor (BDNF)	14-15kDa	Mouse (1:300, University of Iowa)	Donkey anti-mouse (1:2000, Santa Cruz)
Beta-actin	37	Mouse (1:1000, Santa Cruz)	Donkey anti-mouse (1:2000, Santa Cruz)

3.15 Determination of methylation status for GR and BDNF

On Day 60, rats were taken from the housing room, weighed and immediately decapitated. The rat brains were extracted from the skull, and the basolateral amygdala was dissected on ice and frozen at −80°C. DNA extraction was done for each sample, and the DNA was shipped to QIAGEN GmbH, Hilden,

Germany where it was assayed by a scientist who was blind to the treatment groups

DNA was extracted from the brain tissue using an AllPrep DNA/RNA kit (Qiagen). Methylation status was assessed via direct bisulfite DNA sequencing PCR (BSP) on bisulfite-modified DNA (Qiagen). Bisulfite-treated samples were amplified by primers that targeted a CpG-rich region within Bdnf exon IV (NW_047673.1: 251864–251926) and GR (Nr3c1; See Figure M.18). PCR products were purified using a gel extraction kit (Qiagen) and sequenced using the reverse primer at the Qiagen, Singapore facility. The percent methylation of each CpG site within the region amplified was determined by the ratio between peaks values of G and A (G/[G+A]), and these levels on the electropherogram were determined using Chromas software. To confirm that direct bisulfite sequencing was adequately sensitive to detect methylation, universally unmethylated and methylated standards (EpigenDx) were run in parallel with samples and analysed. Analysis of data generated from the standards indicated that the ratio of cytosine methylation increased proportionately with expected methylation rates (R2=0.947) and that there was small sample deviation (slope=0.963) [251].

A

251864- GGCAGAGGAGGTATCATATGACAGCTCA**[1]CG**TCA
AGGCAG**[2]CG**TGGAGCCCTCT**[3]CG**TGGACTCCC -251926

B

GTTCTCTG**[1]CG**GCA**[2]CG**CCCACTTCTAGCAGATAAGGC**[3]CG**GG**[4]CG**GGC
GA

Figure M.18: Sequence of A) Bdnf exon IV amplicon and its first 3 CG dinucleotides and B) GR amplicon and its first 3 CG dinucleotides

3.16 Statistical analysis

All experimental data were analyzed using two-way analysis of variance (inter-subject source of variance = stress and EE). More importantly, since the aim of the experiment is to compare the non-stressed individuals to their stressed counterparts in the differential housing environments, planned comparisons were conducted for effects of stress in absence and presence of the EE (two orthogonal comparisons, independent sample t-test). The reason for including two-way ANOVA results is to show the main effects of EE-housing, which is relevant due to the use of two mutually exclusive treatments in this 2x2 design. This helps in understanding how EE-housing is overall affecting the non-stressed and stressed subjects. Non-orthogonal comparisons were not used in order to avoid spurious effects of multiple testing. The level of significance was set at $p < 0.05$ for all the statistical tests performed in this study. Figures represent mean and SEM (standard error of the mean). N is stated within the figure legends.

Methods (For supplementary chapters only)

Novel Object Recognition Test

The test was adapted from previously described protcol by Redrobe et al., Briefly, rats were habituated to the test room for 24 h before testing. The test was performed in a dimly lit open field arena of the size 1.2m in diameter. On day one, the rats were habituated to the arena for 20mins individually. 24 hours

later, on the second day, acquisition and test trials were performed. Rats were placed in the Open field alone for acquisition trial and allowed to investigate two similar objects for 3 min before being returned to their home cage. After 90 min break, (inter-trial interval) the rats were placed in the open field again for the test trial, and one of the familiar object was replaced by a novel object. Rats were allowed to investigate the familiar object and the novel object for another 3 min. The objects were glass/ceramic material placed opposite to each other 20cm away from the wall of the arena. The objects were cleaned with 70% ethanol between each trial. After each trial, the faeces and urine were removed with a wet paper towel and the arena was wiped with 70% ethanol. The acquisition and the test trials were video recorded. The videos were scored manually afterwards. I was blinded throughout the experiment. Exploration was defined as sniffing, licking and touching the object while facing it. Acquisition requirements were a minimum of 15 seconds (s) exploring the objects, with a minimum of 1 s spent on a single object. In the test trial the difference between time spent investigating the novel and the familiar object divided by the total time spent investigating the objects was calculated as a measure of memory performance (Discrimination index, DI). A high score indicated good memory, whereas a low score indicated poor memory formation.

Sucrose Intake Test

On the day 1 of the experiment, the rats were tested for depression-like by measuring their sucrose intake. The experiment was performed in standard laboratory cages (1 animal/cage). One day before the test, the animals were habituated for sucrose intake (1% sucrose) as they were exposed to sucrose

solution for few hours without any access to food or plain water for 1 hour. The animals were fasted overnight (1900 -09.00AM h; 14 hours). On the day 2 of the experiment, the test was performed for 1 hour (09.00AM-1000 hr) in which each animal was provided sucrose ad-libitum without any access to food or water. Weights of the animals and the sucrose bottles was recorded pre- and post-test in order to calculate percentage change in sucrose per body weight. After the test, the animals were returned back to their respective home cages.

Limited nesting (LN)

The experimental dams with their pups were placed in the cage containing a limited amount of nesting material (One dam with the litter/cage). This nesting material was placed on a fine-gauge aluminum mesh platform (dimension: 36.06x20.52x2 cm^3), layered approximately 2.5 cm above the cage floor. The cage floor was covered with a small amount of corn husk bedding (~500 g). This setup permitted rat droppings to fall below the platform without trapping the pups [252]

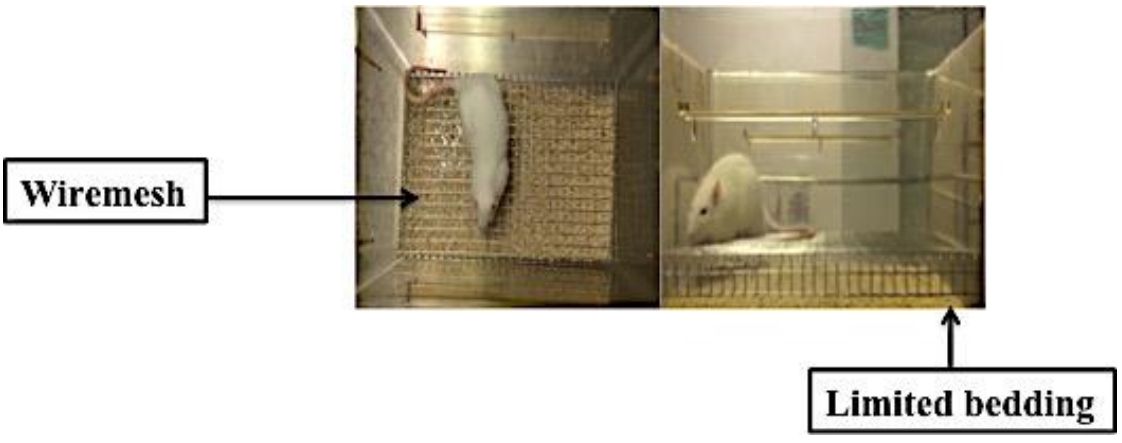

Figure M.19: Limited nesting model. The rats were placed on an aluminium wire mesh with limited nesting material and the cage floor was covered with a small amount of bedding.

Chapter 4

4. Results

The results section is broadly classified into the following sections; Physiological measurements, behavioral assays, qPCR, protein analysis: western blot & immunohistochemistry, epigenetics: methylation in promoter sites and dendritic morphology.

4.1. Physiological readouts

In order to investigate the influence of EE and stress on physiological parameters pertinent to activation of the stress-HPA axis, we measured **plasma corticosterone (CORT) concentration in blood of all animals**; **the weight of adrenal glands** in adulthood (P56) and **body weight** throughout the experiement. The results of these physiological measurements are presented as a comparison between different experimental groups (AFR, MS, EE, and MSEE). The various physiological outputs assessed in this study, will be highlighted in this section.

Please note that, AFR refers to animal facility rearing without maternal separation stress or enriched environment (EE). MS refers to animal facility rearing with maternal separation stress but without enriched environment. EE refers to the enriched environment without maternal separation stress, and MSEE refers to enriched environment along with maternal separation stress.

Body weight: Stress and Enrichment had a significant effect on body weight

Chronic stress is known to lead to an increase in body weight [253]. To investigate the body weight profile in our stress paradigm, body weight was quantified during the course of the experiment. Total body weight (animal weight) was monitored every fortnight from weaning till the day of sacrifice in adulthood (Figure R.1). Repeated measures ANOVA was conducted with body weight as the within subject factor and treatment group as the between subject effect. Both stress ($F_{(1,28)} = 4.69$, $p = 0.039$) and EE ($F_{(1,28)} = 7.74$, $p = 0.010$) had a significant main effect on body weight. The interaction between stress and EE ($F_{(1,28)} = 6.05$, $p = 0.020$) also had a significant effect on body weight.

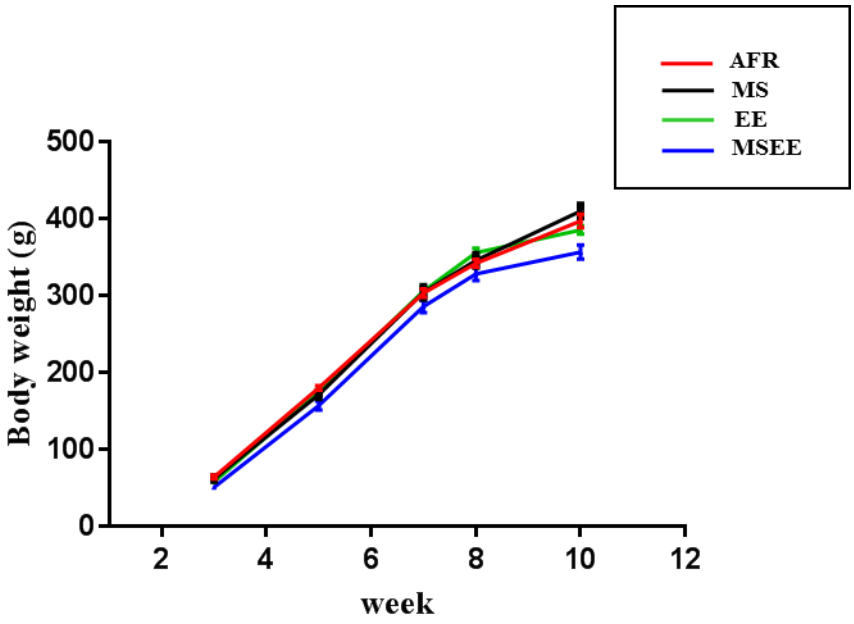

Figure R.1: Mean body weights of male animals. Repeated measures ANOVA revealed a significant effect of stress, EE, and interaction between stress and EE on the weights of animals. Y-axis represents the mean weight in grams (g) of animals. Graph

represents mean values for each group during experiment. N = 8 for AFR, 8 for Stress, 8 for EE and 8 for MSEE.

Moreover, a two-way ANOVA was conducted for body weight 'area under curve' (AUC) with stress and EE as two between subject sources of variance. This was done to determine the impact of 2 different treatments on the body weight throughout the experiment. Both stress ($F_{(1,28)} = 5.355$, $p = 0.03$) and EE ($F_{(1,28)} = 4.786$, $p = 0.04$) had significant main effects on AUC of body weight. However, the interaction between stress and EE had a marginal significance ($F_{(1,28)} = 8.983$, $p = 0.06$).

To analyze the effects of stress in absence or presence of the EE, orthogonal planned comparisons were conducted (independent samples t-tests). In absence of EE, the effect of stress on body weight did not reach statistical significance ($t_{14} = 0.234$, $p = 0.82$; Cohen's $d = 0.117$). In contrast, stressed animals displayed significant lower body weight in presence of EE ($t_{14} = 2.941$, $p = 0.01$; Cohen's $d = 1.47$; Figure R.2).

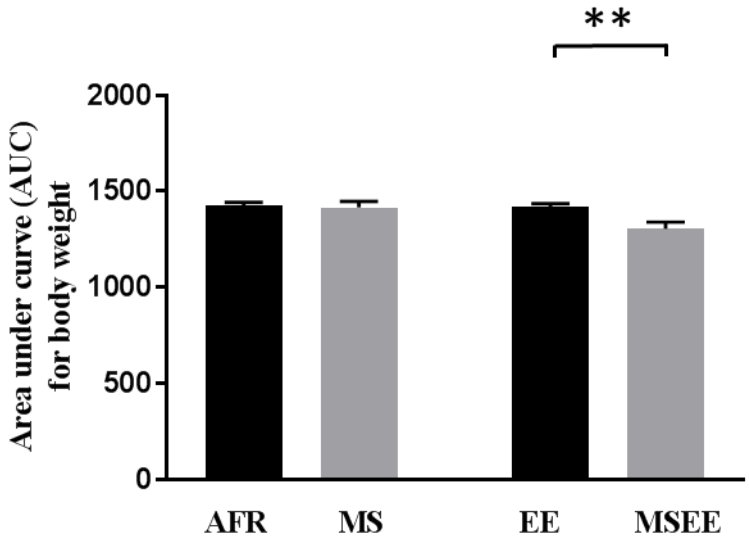

Figure R.2: Effect of early life stress and EE on body weight: MSEE animals displayed lower body weight. Graphs represent Mean ± SEM. N = 8 for AFR, 8 for Stress, 8 for EE and 8 for MSEE; $^{**}p \leq 0.01$.

Additionally, we analyzed the body weight just before sacrifice to assess the impact of the early life paradigm specifically at the end of the experiment. A two-way ANOVA was conducted for body weight at sacrifice with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,28)} = 1.055$, $p = 0.31$) on body weight at sacrifice did not reach statistical significance. Both EE ($F_{(1,28)} = 17.207$, $p < 0.00$) and the interaction between stress and EE ($F_{(1,28)} = 7.114$, $p = 0.01$) had significant main effects on AUC of body weight.

To analyze the effects of stress in absence or presence of the EE, orthogonal planned comparisons were conducted (independent samples t-tests). In absence of EE, the effect of stress on body weight at sacrifice did not reach statistical significance ($t_{14} = -1.155$, $p = 0.27$; Cohen's d =). In contrast, in the presence of

EE, stressed animals displayed significant lower body weight at sacrifice (t_{14} = 2.624, p = 0.02; Cohen's d = 7; Figure R.2b).

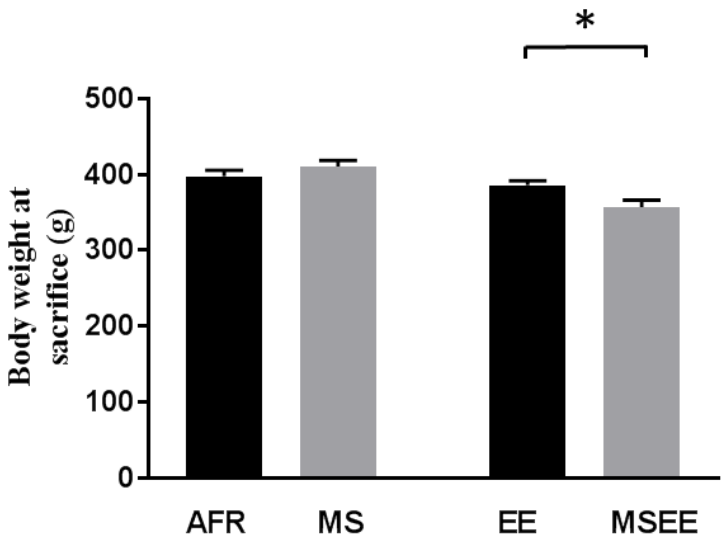

Figure R.2b: Effect of early life stress and EE on body weight at sacrifice: MSEE animals displayed lower body weight. Graphs represent Mean ± SEM. N = 8 for AFR, 8 for Stress, 8 for EE and 8 for MSEE; *$p \leq 0.05$.

In our paradigm, enriched environment housing reduced body weight along the experiment unlike previously reported stress-induced weight gain by stressed rats during the course of the experiment. Rats exposed to prenatal stress and then housed in environmental enrichment conditions showed a similar decrease in body weight [254]. Therefore, stress paradigm in this experiment did not have a significant impact on body weight unlike previous studies. This could be due to the increase in locomotor activity (climbing, exploring, etc) involved in EE cages [255].

Weight of adrenal glands: Stress significantly increased adrenal gland weights

Early life stress affects various physiological parameters, particularly those which are involved in the hypothalamus-pituitary-adrenal (HPA) axis. Adrenal glands are the terminal arm of the HPA axis that initiates stress response [167]. Adrenal glands are vital organs that produce corticosterone in response to stressful stimuli and undergo hypertrophy in the process [256, 257]. So, it was crucial for us to test if MS in our experiment induced any prolonged hypertrophy to the adrenals glands. The weights of the adrenal glands were tested before sacrifice of the animals.

A two-way ANOVA was conducted for adrenal gland weight with stress and EE as two between subject sources of variance. Both stress ($F_{(1,32)} = 17.05$, $p < 0.00$) and EE ($F_{(1,32)} = 21.59$, $p < 0.00$) had significant main effects on the adrenal gland weights. Similarly, the interaction between stress and EE had a significant effect ($F_{(1,32)} = 9.42$, $p < 0.00$) on adrenal gland weights.

Orthogonal planned comparisons (independent samples t-tests) showed that, in absence of EE, stress significantly increased the adrenal gland weights ($t_{19} = -7.104$, $p < 0.00$; Cohen's d = 3.218). However, in the presence of EE, the effect of stress on adrenal gland weights failed to reach statistical significance ($t_{13} = -0.555$, $p = 0.59$; Cohen's d = 0,275; Figure R.3).

MS caused adrenal gland hypertrophy in male rats living in standard animal facility housing when compared to AFR male rats which is in line with previous

studies [256, 258]. Interestingly, early life stress in enriched housing (interaction) ameliorated the increase in the adrenal weights of animals (Figure R.3).

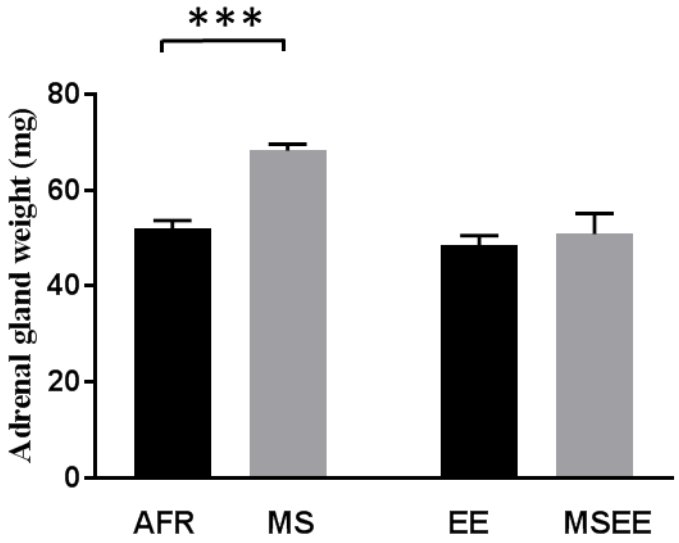

Figure R3: Effect of early life stress and EE on adrenal weight: Stress induced a significant increase in adrenal weights in the absence of EE. Graphs represent Mean ± SEM. N = 12 for AFR, 7 for Stress, 9 for EE and 5 for MSEE; ***$p \leq 0.001$.

Stress significantly increased baseline plasma corticosterone levels

As mentioned earlier, "Hypothalamic-Pituitary-Adrenal (HPA) axis" (Fig.1) that releases glucocorticoids (stress hormones) mediates allostasis that help the body to return to homeostatic equilibrium after an encounter of stress episode [113]. Along with adrenal glands, it is cardinal to check the profile of the stress hormone in a study involving stress paradigm. To investigate the effects of stress and EE on this

crucial parameter, plasma corticosterone was measured at various time points (Figure R.4) during the course of the study, namely: baseline1 = baseline plasma corticosterone (effect of early life treatment) in adulthood; baseline 2 = basal corticosterone levels 24 hours prior to predator odour exposure; post-30 minutes, post-2 hours, post-6 hours after exposure to predator odour and at sacrifice (24 hours post stress exposure). All these different time points were used to plot the 'area under cure' (AUC). This signifies the profile of stress hormone post stress indicating recovery efficiency. The result with statistics is displayed below.

A two-way ANOVA was conducted for corticosterone with stress and EE as two between subject sources of variance. This shows the effects of the treatments throughout the course of study that gives us a holistic picture of the corticosterone profile. The effects of stress ($F_{(1,22)} = 0.614$, $p = 0.44$) and EE ($F_{(1,22)} = 0.723$, $p = 0.40$) on the AUC of corticosterone failed to reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,22)} = 0.725$, $p = 0.40$) on the AUC of corticosterone failed to reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence ($t_9 = -1.591$, $p = 0.146$; Cohen's $d = 0.951$) of EE, the effect of stress on the AUC of corticosterone failed to reach statistical significance. Similarly, in the presence ($t_{13} = 0.044$, $p = 0.97$; Cohen's $d = 0.023$; Figure R.4) of EE, the effect of stress on the AUC of corticosterone failed to reach statistical significance. Additionally, two baseline time-points of the corticosterone concentration are highlighted below.

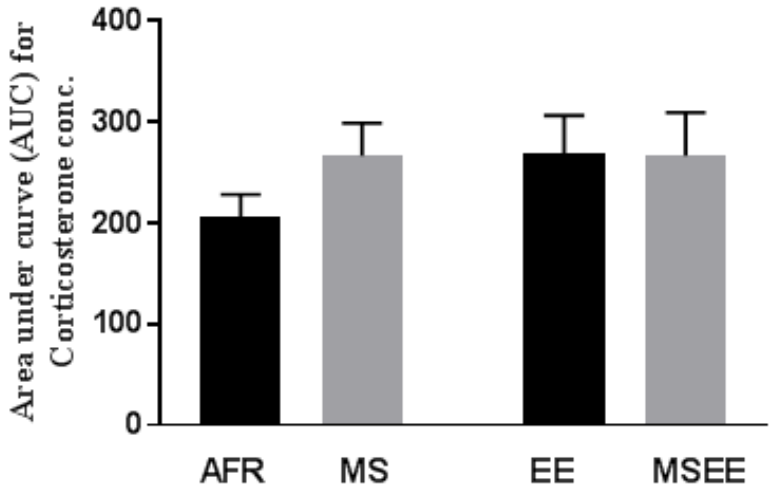

Figure R4: Area under the curve for plasma corticosterone concentration for pre-and post-predator odor exposure in animals exposed to stress and/ or enrichment in early life: EE and stress had no significant effect on AUC of corticosterone. Graphs represent Mean ± SEM. N = 6 for AFR, 5 for Stress, 9 for EE and 6 for MSEE.

The first baseline plasma corticosterone concentration highlights the corticosterone concentration levels in animals during adulthood, which are exposed to the early life treatment. So, this time point captures the prolonged effect of early environment on the levels of corticosterone in individuals

A two-way ANOVA was conducted for corticosterone with stress and EE as two between subject sources of variance. Stress ($F_{(1,22)} = 4.666$, $p = 0.04$) had a significant main effect on the baseline corticosterone levels. However, the effect of EE ($F_{(1,22)} = 0.769$, $p = 0.39$) and the interaction between stress and EE ($F_{(1,22)} =$

0.007, p = 0.94) on baseline corticosterone levels failed to reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that, in absence of EE, stress significantly increased baseline corticosterone levels at adulthood (P56) (t_9 = -2.432, p = 0.04; Cohen's d = 1.443) However, in the presence of EE, the effect of stress on baseline corticosterone levels failed to reach statistical significance (t_{13} = -1.408, p = 0.18; Cohen's d = 0.712; Figure R.5).

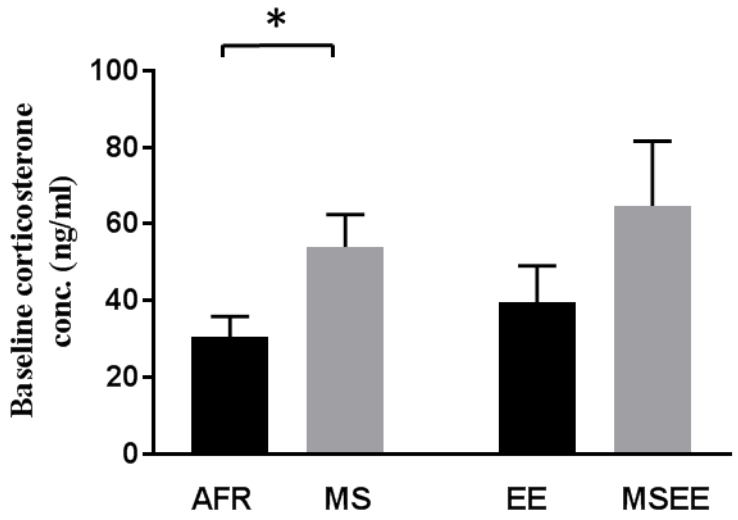

Figure R.5: Baseline plasma corticosterone concentration during adulthood in animals exposed to stress and/ or enrichment in early life: Stress significantly increased baseline corticosterone levels in the absence of EE. Graphs represent Mean ± SEM. N = 6 for AFR, 5 for Stress, 9 for EE and 6 for MSEE; *$p \leq 0.05$.

Additionally, the second baseline plasma corticosterone concentration shows the levels of stress hormone after being exposed to numerous behavioral experiments (at sacrifice). This emphasizes on the baseline corticosterone level of early life

1601 treatments that is obtained after multiple behavioral exposure. A two-way ANOVA
1602 was conducted for corticosterone levels 24 hours after exposure to predator stress,
1603 with stress and EE as two between subject sources of variance. Stress ($F_{(1,19)}$ =
1604 5.876, p = 0.03) and EE ($F_{(1,19)}$ = 4.349, p = 0.05) had a significant main effect on
1605 corticosterone levels at sacrifice showing a strong indication of pro-resilient
1606 feature in animals reared in EE. However, the effect of the interaction between
1607 stress and EE ($F_{(1,19)}$ = 2.808, p = 0.11) on corticosterone levels at sacrifice failed
1608 to reach statistical significance.

1609 Orthogonal planned comparisons (independent samples t-tests) showed that in the
1610 absence of EE (t_8 = -1.836, p = 0.10; Cohen's d = 1.180) the effect of stress on
1611 corticosterone levels 24 hours after predator stress, failed to reach statistical
1612 significance. Similarly, in the presence of EE (t_{11} = -1.641, p = 0.13; Cohen's d =
1613 0.875; Figure R.6), the effect of stress on corticosterone levels at sacrifice, failed to
1614 reach statistical significance

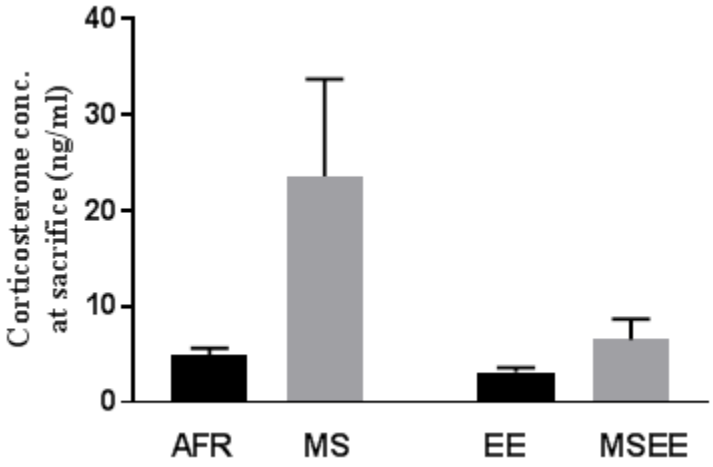

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Figure R.6: Plasma corticosterone concentration at sacrifice in animals exposed to stress and/ or enrichment in early life: Stress had no significant effect on post-sacrifice corticosterone levels. Graphs represent Mean ± SEM. N = 5 for AFR, 5 for Stress, 7 for EE and 6 for MSEE.

Though there was no significant difference between groups for AUC during the course of the study, results for maternal separation stress alone displays enhanced corticosterone concentration in baseline blood (Figure R5.). This is similar to what has previously been observed in rats exposed to chronic stress having elevated baseline plasma corticosterone [257, 259] when compared to control animals. This effect of maternal stress was not observed in rats that were placed in the enriched environment simultaneously (interaction effect, Figure R5). This observation is indicative of the modulatory effect of environmental enrichment on the HPA axis. Furthermore, Roy and colleagues (2001) showed that male mice assigned to the environmental enrichment treatment had significantly less plasma corticosterone compared to animals in standard animal house housing after exposure to stress [260] which is in line with our study.

All in all, our physiological measurements show that MS alone has detrimental effects but concurrent exposure of MS and EE displays reduced damage. This highlights the impact of concurrent exposure of stress and EE.

4.2. Behavioral assays

The effect of early–life stress and enriched environment on behavior was tested in adult animals. Responses to stress results in a spectrum of outcome from detrimental immediate and long-term effects to resilience and active coping behavior in response to future stressors. Stress can be assessed by behavioral tests [261]. In this section, we investigated various behavioral outputs in response to stress and EE. For testing anxiety-like behavior, the home cage emergence test (HCE), the open field test (OFT) and the elevated plus maze (EPM) were employed. For testing social behavior, the social interaction test (SIT) was employed. To test depressive-like behavior, the forced swim test (FST) was used. The results of these behavioral endpoints are presented as a comparison between different experimental groups (control, stress, EE, and MSEE).

Stress significantly reduced the inner zone exploration in open field test

To analyze the effect of early life stress and EE on anxiety-like behavior, animals were subjected to various anxiety tests in adulthood (P56). Open field test will be the first test discussed here wherein the time spent in inner zone of an open arena was assessed. The open field is a well-established behavioral test of anxiety-like behavior. It has been validated with anxiolytic drugs [262]. In this test, lower exploration of inner zone indicated higher anxiety. The following statistics highlights the anxiety-like behavior of animals in our experiment.

A two-way ANOVA was conducted for inner zone exploration in the open field test with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,28)} = 0.157$, $p = 0.69$) on time spent exploring the inner zone in the open field test failed to reach statistical significance. However, the effect of both EE ($F_{(1,28)} = 27.09$, $p < 0.00$) and the interaction between stress and EE ($F_{(1,28)} = 9.018$, $p = 0.01$) had significant effect on the time spent exploring the inner zone in open field test.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE, stress significantly decreased time spent exploring the inner zone of the open field test ($t_{17} = 2.403$, $p = 0.03$; Cohen's $d = 1.305$). In contrast, the effect of stress on time spent exploring the inner zone of the open field test in the presence of EE ($t_{11} = -2.029$ $p = 0.07$; Cohen's $d = 1.088$; Figure R.9) failed to reach statistical significance.

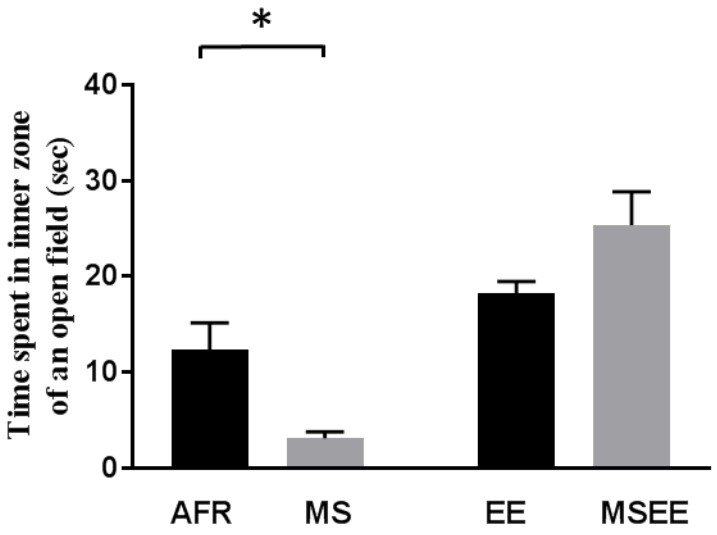


Figure R.8: Effect of early life stress and EE on time spent in the inner zone of OFT: Stress significantly decreased the time spent exploring the inner zone in open field test in the absence of EE. Graphs represent Mean ± SEM. N = 12 for AFR, 7 for Stress, 7 for EE and 6 for MSEE; *$p \leq 0.05$.

My results showed that early life maternal separation induced anxiogenic avoidance of the inner zone of the open field. This represents the anxiogenic effect of animals exposed to MS alone in standard housing in early life. This result is in line with previous reports that showed MS to result in anxiogenic behavior in adulthood [263, 264]. However, this effect was not seen in animals exposed to MS in the enriched environment (Figure R.8). This emphasizes the impact of EE along with MS on alleviating anxiogenic behavior.

Stress or EE had no significant effect on escape latency in home cage emergence test

The second test used to assess anxiety-like behavior was, home cage emergence wherein the latency to escape from home cage was analyzed. Home cage emergence was used to quantify exploratory activity and anxiety in rodents [265, 266]. In this test, higher escape latency denoted higher anxiety. A two-way ANOVA was conducted for escape latency in the home cage emergency test with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,28)} = 0.291$, $p = 0.59$) and EE ($F_{(1,28)} = 1.102$, $p = 0.30$) on escape latency in home cage emergence test failed to reach statistical significance. Similarly, the

interaction between stress and EE ($F_{(1,28)}$ = 0.017, p = 0.90) on escape latency in home cage emergence test failed to reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in absence (t_{17} = -0.264, p = 0.80; Cohen's d = 0.119) of EE, the effect of stress on escape latency in the home cage emergence test, failed to reach statistical significance. In the presence (t_{11} = -0.953, p = 0.36; Cohen's d = 0.557; Figure R.8) of EE, the effect of stress on escape latency in the home cage emergence test, failed to reach statistical significance.

The results revealed that the latency to escape from the home cage emergence test was not significantly affected by MS, EE or their interaction.

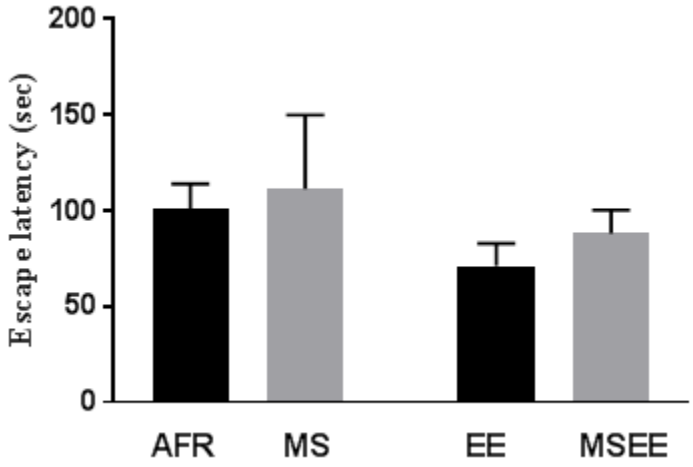


Figure R.9: Effect of early life stress and EE on escape latency in HCE in adulthood: Stress (in presence and absence of EE) had no significant effect on escape latency in home cage emergence test. Graphs represent Mean ± SEM. N = 10 for AFR, 8 for Stress, 9 for EE and 5 for MSEE.

Stress along with EE significantly increased percentage open arm time in the EPM

Another test to study the anxiety like behavior was the elevated plus maze (EPM). Open arm exploration (entries and occupancy time) relative to sum of open and enclosed arm exploration was used as index for the anxiety [267]. In EPM, % open arm entries and % open arm time were assessed, wherein lower percentage of time and entries in open arm indicate greater anxiety.

A two-way ANOVA was conducted for percentage open arm time in the elevated plus maze with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,32)} = 2.123$, $p = 0.15$) and EE ($F_{(1,32)} = 0.149$, $p = 0.70$) on the percentage time spent exploring the open arm of the elevated plus maze failed to reach statistical significance. Similarly, the interaction between stress and EE ($F_{(1,32)} = 3.393$, $p = 0.07$) on the percentage time spent exploring the open arm of the elevated plus maze failed to reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE, the effect of stress on the percentage time spent exploring the open arm of the elevated plus maze ($t_{19} = 0.287$, $p = 0.78$; Cohen's $d = 0.1223$) did not reach statistical significance. In contrast, stress significantly increased the percentage time spent exploring the open arm of the elevated plus maze in the presence of EE ($t_{13} = -2.310$, $p = 0.04$; Cohen's $d = 1.294$; Figure R.10).

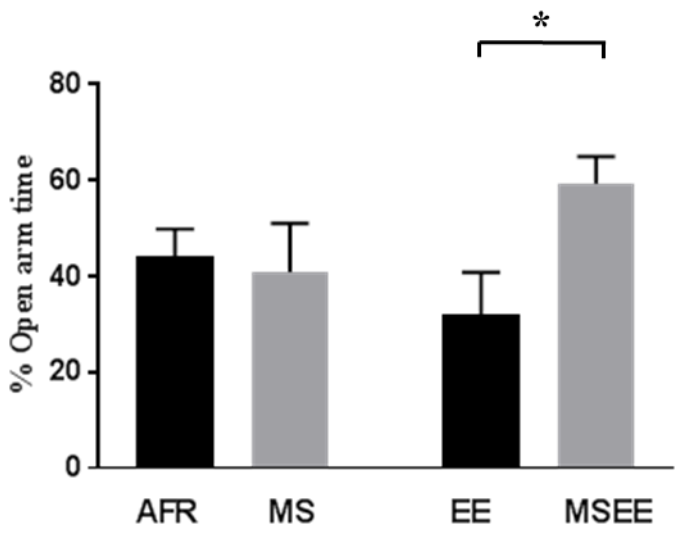


Figure R.10: Effect of early life stress and EE on percentage open arm time in EPM in adulthood: stress along with EE significantly increased the percentage time spent exploring the open arm of the elevated plus maze. Graphs represent Mean ± SEM. N = 12 for AFR, 9 for Stress, 9 for EE and 6 for MSEE.

Stress along with/in the presence of EE significantly increased percentage open arm entries in the EPM

A two-way ANOVA was conducted for percentage open arm entries in the elevated plus maze with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,28)} = 0.491$, $p = 0.49$) and EE ($F_{(1,28)} = 0.407$, $p = 0.53$) on the number of entries into the open arm of the elevated plus maze failed to reach statistical significance. Similarly, the interaction between stress and EE ($F_{(1,28)} = 3.276$, $p = 0.08$) on the number of entries into the open arm of the elevated plus maze failed to reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE, stress had no significant effect on the number of entries into the open arm of the elevated plus maze ($t_{18} = 0.777$, $p = 0.45$; Cohen's $d = 0.349$). In contrast, stress significantly increased the number of entries into the open arm of the elevated plus maze in the presence of EE ($t_{10} = -2.615$, $p = 0.03$; Cohen's $d = 1.510$; Figure R.11).

The results show that MS and EE alone did not affect the anxiety-like behavior in EPM but MSEE group displayed anxiolytic behavior. Rats subjected to both MS and EE spent more time exploring (% time and % entries) the open arm of the EPM compared to rats exposed only to environmental enrichment (figure R.10 and R.11). Thus, it appears that the presence of both MS and EE has a cumulative effect on the increase in anxiolytic behavior. This is corroborated by reports show

that both short-term [87] and long-term [167] enrichment in adulthood after early life maternal separation increases anxiolytic behavior.

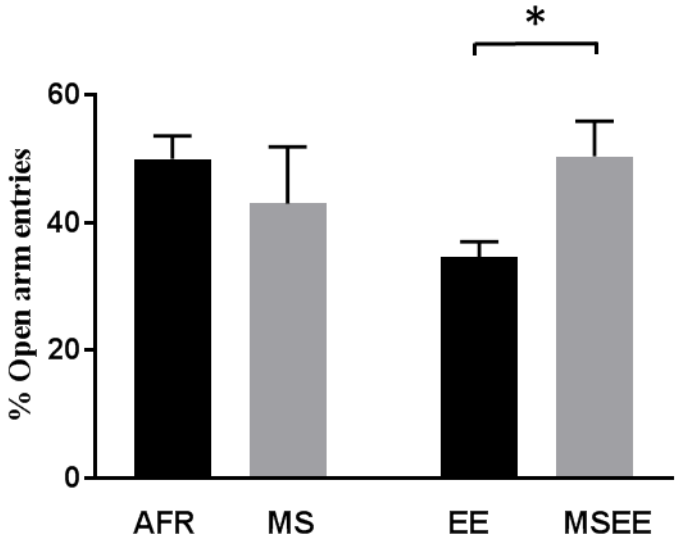


Figure R.11: Effect of early life stress and EE on percentage open-arm entries in EPM: Stress along with EE significantly increased the number of entries into the open arm of the elevated plus maze. Graphs represent Mean ± SEM. N = 11 for AFR, 9 for Stress, 6 for EE and 6 for MSEE; *$p \leq 0.05$

Stress or EE did not alter number of head dips in the elevated plus maze

A resilient individual highlights certain behaviors in the form of active coping strategy that involves assessment of risks and threats in the surrounding [268]. Risk assessment behavior in this study was measured using number of head-dips

performed by each subject during the test. In this test, higher number of head dips indicates elevated risk assessment behavior.

A two-way ANOVA was conducted for the number of head dips in the elevated plus maze with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,32)} = 1.652$, $p = 0.21$) and EE ($F_{(1,32)} = 1.924$, $p = 0.18$) on the number head dips performed in the elevated plus maze failed to reach statistical significance. Similarly, the interaction between stress and EE ($F_{(1,32)} = 0.020$, $p = 0.89$) had no significant effect on the number head dips performed in the elevated plus maze.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE ($t_{19} = -1.081$, $p = 0.29$; Cohen's $d = 0.46$), the effect of stress on the number head dips performed in the elevated plus maze failed to reach significance. Similarly, in the presence of EE ($t_{13} = -0.778$, $p = 0.45$; Cohen's $d = 1.328$; Figure R.12) the effect of stress on the number of head dips performed in the elevated plus maze failed to reach significance.

The results of head dips in EPM test revealed that MS, either in absence or presence of EE did not significantly affect risk assessment behavior.

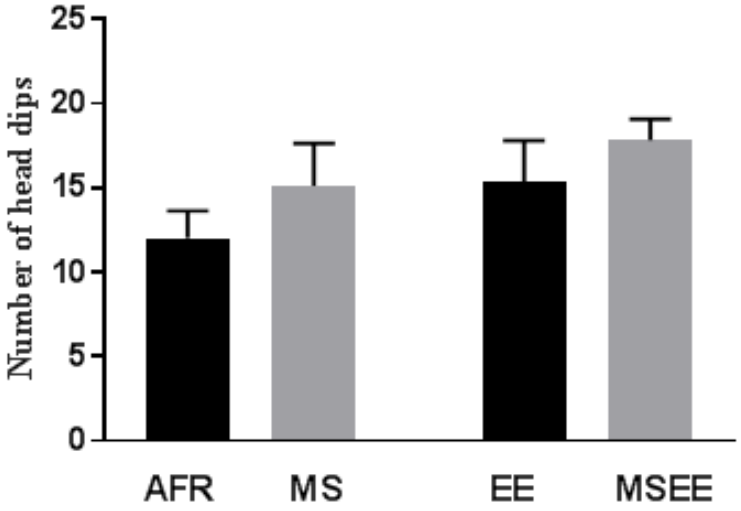


Figure R.12: Effect of stress and EE on a number of head dips in EPM: Stress had no significant effect on the number head dips performed in the elevated plus maze in presence and absence of EE. Graphs represent Mean ± SEM. N = 12 for AFR, 9 for Stress, 9 for EE and 6 for MSEE.

Stress or EE did not alter closed arm entries in the elevated plus maze

Number of closed arm entries indicates the influence of locomotion of rats in the maze as indicated by factor analysis studies on EPM [269]. To assess the influence of locomotion on the results of EPM, number of closed arm entries was analyzed.

A two-way ANOVA was conducted for percentage closed arm time in the elevated plus maze with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,32)} = 1.702$, $p = 0.20$) and EE ($F_{(1,32)} = 1.168$, $p = 0.29$) on the number of entries into the closed arm of the elevated plus maze failed to reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,32)} = 0.035$, $p = 0.85$) on the number of entries into the closed arm of the elevated plus maze failed to reach statistical significance.

Orthogonal planned comparisons (Independent samples t-tests) showed that in the absence of EE ($t_{19} = 0.842$, $p = 0.41$; Cohen's $d = 0.359$) the effect of stress on the number of closed arm entries in the elevated plus maze failed to reach significance. Similarly, in the presence of EE ($t_{13} = 1.026$, $p = 0.32$; Cohen's $d = 0.520$; Figure R.13) the effect of stress on the number of closed arm entries in the elevated plus maze failed to reach significance.

The result indicates the absence of any influence of general locomotion on the results of the EPM test as a result of different early life housing conditions.

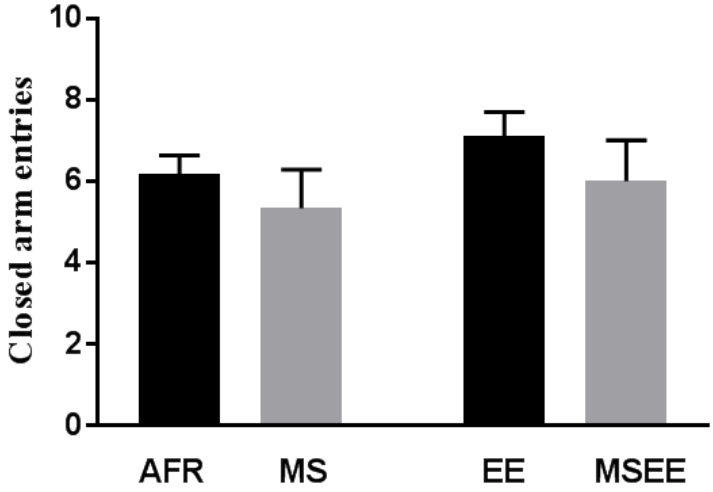


Figure R.13: Effect of early life stress on number of closed arm entries in EPM in presence and absence of EE: Stress had no significant effect on the number of entries into the closed arm of the elevated plus maze in presence and absence of EE. Graphs represent Mean ± SEM. N = 12 for AFR, 9 for Stress, 9 for EE and 6 for MSEE.

Stress along with EE significantly increased total percentage exploration in the SIT

Social interaction test was performed in this study to assess the social motivation in rats of varying early life environment. Previous study has shown that stress causes decline in social exploration in rats where they are motivated to explore objects in comparison to unfamiliar conspecifics [241].

A two-way ANOVA was conducted for total percentage exploration in the social interaction test with stress and EE as two between subject sources of variance. The effect of EE ($F_{(1,30)} = 2.347$, $p = 0.14$) on percentage total exploration in the social

interaction test failed to reach statistical significance. However, both stress ($F_{(1,30)}$ = 11.39, p<0.00) and the interaction between stress and EE ($F_{(1,30)}$ = 5.656, p = 0.02) had significant effect on the time spent interacting with the stimulus animal in the social interaction test.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE (t_{18} = -0.796, p = 0.44; Cohen's d = 0.403), the effect of stress on percentage total exploration in the social interaction test failed to reach statistical significance. However, in the presence of EE (t_{12} = -3.661, p < 0.00; Cohen's d = 2.000; Figure R.14) stress significantly increased the time spent interacting with the stimulus animal in the social interaction test.

Rats subjected to both MS and EE spent significantly more time exploring unfamiliar conspecifics in comparison to wooden objects. This shows that presence of both environments has a combined effect on elevating the social interaction behaviour. Though stress did not show a significant decline in social interaction like previous studies, MS in presence of/along with EE has a pro-social behaviour. This could be because enriched environment provided a complex environment including exploratory, social, emotional and motor stimulation to the animals early in life and also helped induce well adaptive behaviours [169].

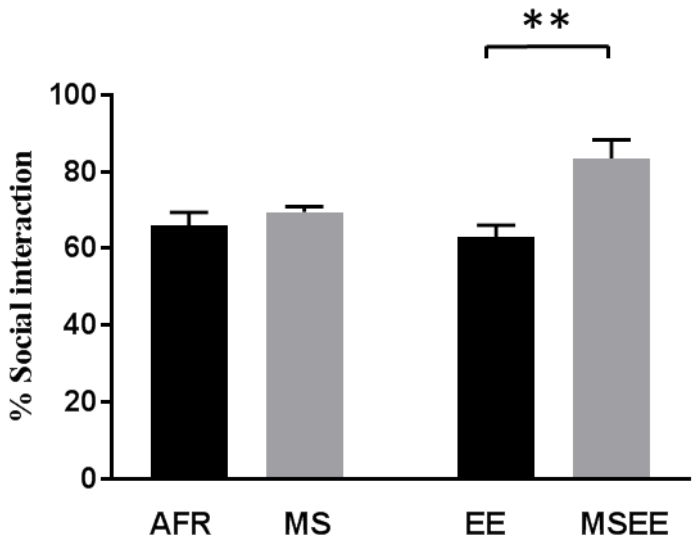


Figure R.14: Effect of early life stress and EE on social exploration in social interaction test: Stress along with EE significantly increased the time spent interacting with the stimulus animal in the social interaction test. Graphs represent Mean ± SEM. N = 12 for AFR, 8 for Stress, 9 for EE and 5 for MSEE; $^{**}p \leq 0.01$.

Stress decreased T1 immobility but not swimming in the forced swim test (FST)

Chronically administered corticosterone reduced immobility time in the FST [270]. As an index for learned helplessness (depressive-like symptoms), immobility (time in seconds) in Porsolt's forced swim task (FST) was measured in our study. MS induced depression-like behaviour was associated with decreased swimming behaviour in the FST [271-273]. This positive stress-coping behavior such as swimming (time in seconds) was also measured in our study shown below. Immobility and swimming were measured at two time points, T1 and T2. T1 was the first day where the animals were habituated to the test and T2 was the time

point for the actual test day. In FST, the initial exposure to the swim tank on day 1 (T1) is known to induce a state of "behavioral despair," such that these rats have *altered emotional reactivity* in response to a new emergency represented by swimming. So, day 2 (T2) is considered as the test day where the depression-like behavior can be well captured, after T1 habituation [274]. In this study, we quantified T1 behavioral response to capture the initial response to novelty stress and T2 behavioral response to capture the stabilized emotional response to forced swim test.

A two-way ANOVA was conducted for T1 (Day-1) immobility and swimming in the forced swim test with stress and EE as two between subject sources of variance. The effect of both EE ($F_{(1,30)} = 2.797$, $p = 0.11$) and stress ($F_{(1,30)} = 2.075$, $p = 0.16$) on immobility in the forced swim test failed to reach statistical significance. However, the interaction between stress and EE ($F_{(1,30)} = 3.966$, $p = 0.06$) had a marginal significant effect on the T1 immobility in the forced swim test.

For swimming, the effect of EE ($F_{(1,32)} = 0.334$, $p = 0.57$) and the interaction between EE and stress ($F_{(1,32)} = 0.420$, $p = 0.52$) did not reach statistical significance. However, stress ($F_{(1,32)} = 3.923$, $p = 0.06$) had a marginal significant effect on the T1 swimming in the forced swim test.

Orthogonal planned comparisons (independent samples t-tests) for T1 immobility showed that, in the absence of EE ($t_{18} = 2.138$, $p = 0.05$; Cohen's $d = 1.061$), stress significantly decreased immobility in the forced swim test. However, in the presence of EE ($t_{12} = -0.849$, $p = 0.42$; Cohen's $d = 0.528$; Figure R.15A) the effect

of stress on T1 immobility in the forced swim test failed to reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) for T1 swimming showed that, in the absence of EE (t_{19} = 1.759, p = 0.10; Cohen's d = 0.736), the effect of stress on T1 swimming in the forced swim test failed to reach statistical significance. Similarly, in the presence of EE (t_{13} = 1.254, p = 0.23; Cohen's d = 0.649; Figure R.15B) the effect of stress on swimming in the forced swim test failed to reach statistical significance.

The results revealed that MS significantly decreased immobility in T1 when compared to controls. The response to the first period of immobility is proposed as an indicator of the length of the first effort to escape from the stressful situation that the forced swim test represents. The *altered emotional reactivity* when confronted with an emergency (swim test) for the first time of could be the reason why the MS rats displayed less immobility on day 1 (T1). The T1 result observed in this study has already been reported before [275, 276]. However, there are studies that show that stress increases immobility time [277, 278] but reduced immobility is not always associated with a compromised behavior due to chronic stress as shown in earlier studies. MS in this study did not alter T1 swimming time in FST.

Stress decreased T2 swimming but not immobility in the forced swim test (FST)

As mentioned earlier, T2 was considered as the test day wherein the rats were habituated already. Immobility on day 2 (T2) is considered as an indicator that the animal lacks motivation for solving a problem [279]. A two-way ANOVA was conducted for T2 (day 2) immobility and swimming in the forced swim test with stress and EE as two between subject sources of variance. The effect of EE ($F_{(1,26)}$ = 2.167, p = 0.15), stress ($F_{(1,26)}$ = 1.050, p = 0.32) and the interaction between stress and EE ($F_{(1,26)}$ = 3.348, p = 0.08) on T2 immobility in the forced swim test failed to reach statistical significance.

For swimming, the effect of EE ($F_{(1,26)}$ = 27.829, p < 0.00) and stress ($F_{(1,26)}$ = 4.929, p = 0.04) had a significant effect on T2 swimming in the forced swim test. Similarly, the interaction between EE and stress ($F_{(1,26)}$ = 13.334, p < 0.00) had a significant effect on the T2 swimming in the forced swim test.

Orthogonal planned comparisons (independent samples t-tests) for T2 immobility showed that, in the absence of EE (t_{13} = 1.583, p = 0.14; Cohen's d = 1.108), the effect of stress on T2 immobility in the forced swim test failed to reach statistical significance. Similarly, in the presence of EE (t_{13} = -0.830, p = 0.42; Cohen's d = 0.435; Figure R.16A) the effect of stress on T2 immobility in the forced swim test failed to reach statistical significance. Orthogonal planned comparisons (independent samples t-tests) for T2 swimming showed that, in the absence of EE (t_{13} = 3.350, p = 0.01; Cohen's d = 2.110), stress significantly decreased T2 swimming in the forced swim test. However, in the presence of EE (t_{13} = -1.372, p = 0.19; Cohen's d = 0.769; Figure R.16B) the effect of stress on swimming in the forced swim test failed to reach statistical significance.

In T2 experiments, MS significantly decreased swimming time in the FST. MS induced depression-like behaviour is associated with decreased swimming behaviour in the FST [271-273]. Thus, MS induced depression-like behaviour in the FST, which is in agreement with existing literature. MS in this experiment did not alter T2 immobility time in FST.

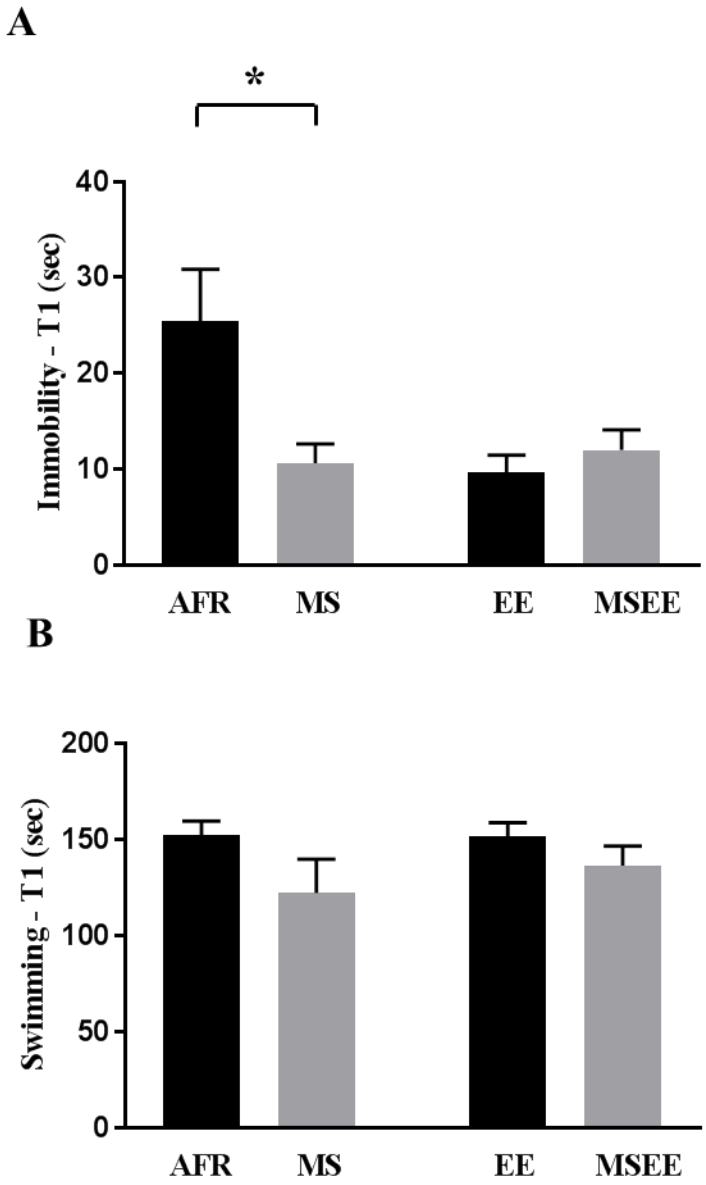
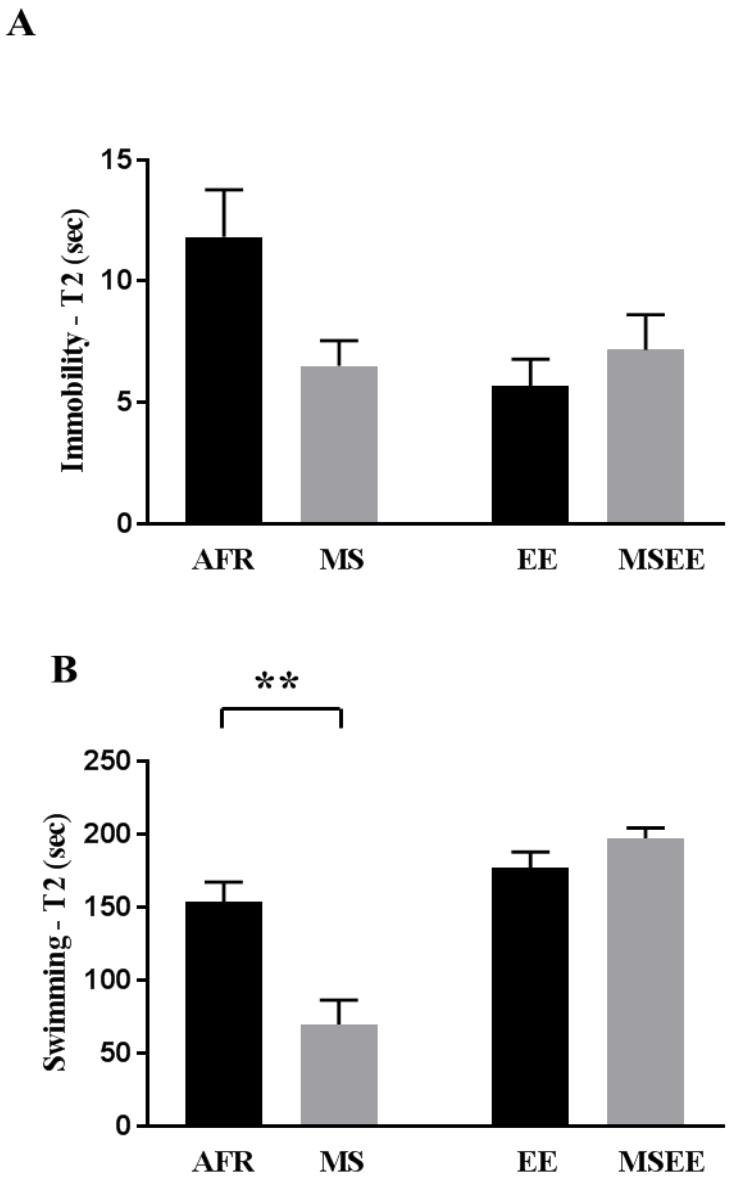


Figure R.15: Stress significantly decreased T1 immobility but not swimming in the forced swim test. A) Stress significantly decreases immobility time in the forced swim test. Graphs represent Mean ± SEM. N = 12 for AFR, 8 for Stress, 8 for EE and 6 for MSEE; $*p \leq 0.05$. **B)** Stress had no significant effect on swimming time in the forced swim test. Graphs represent Mean ± SEM. N = 12 for AFR, 8 for Stress, 8 for EE and 6 for MSEE.



1942

1943 **Figure R.16: Stress significantly decreased T2 swimming but not immobility**
1944 **in the forced swim test. A)** Stress had no significant effect on immobility time in
1945 the forced swim test. Graphs represent Mean ± SEM. N = 12 for AFR, 9 for Stress,
1946 9 for EE and 6 for MSEE. **B)** Stress significantly decreased swimming time in the
1947 forced swim test. Graphs represent Mean ± SEM. N = 12 for AFR, 9 for Stress, 9
1948 for EE and 6 for MSEE; $^{**}p \leq 0.01$.

4.3. quantitative PCR

GC activated glucocorticoid receptor (GR) has a central role in maintaining the regulation of HPA axis in response to various stressors [142, 218, 219]. The density of GR population across amygdala, hippocampus, and mPFC differ and is shown to have a link in providing feedback regulation to HPA axis [219]. The recruitment of activated GR in the nucleus as a transcription factor is a crucial step that initiates a relay of downstream molecular pathways to mediate stress response [112, 220]. Preliminary evidence suggests that in the basolateral amygdala (unlike in the hippocampus), GR activation has lasting excitatory rather than suppressive and normalizing effects on cell firing [280]. This makes it interesting to look at the GR expression in the BLA with respect to stress. Various proteins have been shown to be involved in amygdala-dependent fear and learning. Acquisition of fear conditioning is known to involve the interaction of neural inputs from the conditioned stimulus (CS) and unconditioned stimulus (US) pathways onto Lateral Amygdala (LA) cells during fear conditioning [281-283]. In the LA, ERK/MAPK gets activated by phosphorylation that is initiated within an hour after conditioning [284]. BDNF activation via the tyrosine kinase TrkB receptor is important in the acquisition fear conditioning in the LA [285]. The mRNA abundance levels of glucocorticoid receptor (GR) and brain derived neurotrophic factor (BDNF) were measured using quantitative polymerase chain reaction (qPCR). GC activated glucocorticoid receptor (GR) were focused in this study because of its central role in maintaining the regulation of HPA axis in response to various stressors [142, 218, 219]. Another important molecule that interacts with glucocorticoid and its

receptor (GC-GR) is BDNF, that activates molecular cascade to enhance fear related memories [286].

Thus we looked into the most prominent molecules that might be playing role in regulating emotional response to stress. GR being the primary driver of plasticity within BLA, we studied expression, regulation and nuclear activation of GR that has been shown to be crucial for BLA-dependent response to stress. Additionally we looked into the expression of BDNF and MAPK, which has been previously shown to modulate emotional response to stress. Since these molecules are important mediators of the stress response, a candidate-based approach was used to select these molecules for analysis of my animal models (MS, EE and MSEE).

Our first aim was to determine the effect of early-life stress and enriched environment on the transcription levels of these molecules. This gave us information about how early life environment plays an important role in altering the transcriptional regulation of the animals during adulthood and the translational regulation will be discussed in the next section. The Ct values obtained for GR and BDNF were normalized using the geometric mean of the Ct value of two reference genes. The qPCR results are presented as delta Ct values (Ct value of gene of interest – geometric mean of reference genes Ct values) for each experimental group. The delta Ct values of the target genes with the statistical analysis are given below.

Based on previous literature, the most likely mediator candidates TrkB, Arc, Erk, and MR of stress response were also analyzed were. Please refer to appendix for p values of TrkB, Arc, Erk and MR.

Stress or EE did not alter total GR mRNA levels in BLA

A two-way ANOVA was conducted for total GR mRNA abundance in the BLA with stress and EE as two between subject sources of variance. EE ($F_{(1,19)}$ = 7.521, p = 0.01) had a significant main effect on mRNA transcript level of GR in the basolateral amygdala. However, the effect of both stress ($F_{(1,19)}$ = 0.021, p = 0.89) and the interaction between stress and EE ($F_{(1,19)}$ = 0.111, p = 0.74), on the mRNA transcript level of GR in the basolateral amygdala failed to reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence (t_9 = 0.599, p = 0.56; Cohen's d = 0.368) of EE, the effect of stress on the mRNA transcript level of GR in the basolateral amygdala failed to reach statistical significance. Similarly, in the presence of EE (t_{10} = -0.107, p = 0.92; Cohen's d = 0.064; Figure R.17) the effect of stress on the mRNA transcript level of GR in the basolateral amygdala failed to reach statistical significance.

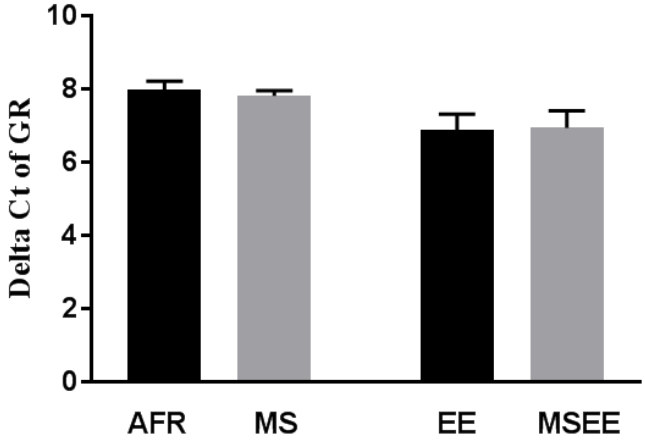


Figure R.17: Effect of early life stress and EE on GR mRNA expression: EE had a significant effect on mRNA transcript level of GR in the basolateral

amygdala in presence and absence of EE. Graphs represent Mean ± SEM. N = 6 for AFR, 5 for Stress, 6 for EE and 6 for MSEE.

Stress or EE did not alter total BDNF mRNA levels in BLA

A two-way ANOVA was conducted for total BDNF mRNA abundance in the BLA with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,18)} = 0.153$, $p = 0.70$) and EE ($F_{(1,18)} = 1.609$, $p = 0.22$) on the mRNA transcript levels of BDNF in the basolateral amygdala did not reach statistical significance. However, the interaction between stress and EE ($F_{(1,18)} = 4.036$, $p = 0.06$) had a marginally significant effect on the mRNA transcript levels of BDNF in the basolateral amygdala.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE ($t_9 = 1.455$, $p = 0.18$; Cohen's $d = 0.842$), the effect of stress on the mRNA transcript levels of BDNF in the basolateral amygdala did not reach statistical significance. Similarly, in the presence of EE ($t_9 = -1.444$, $p = 0.18$; Cohen's $d = 0.912$; Figure R.18), the effect of stress on the mRNA transcript levels of BDNF in the basolateral amygdala did not reach statistical significance.

MS did not alter the mRNA expression of GR and BDNF either in presence or absence of EE. It was interesting to note that EE had a marginal significant effect ($p = 0.06$) on the mRNA levels of BDNF.

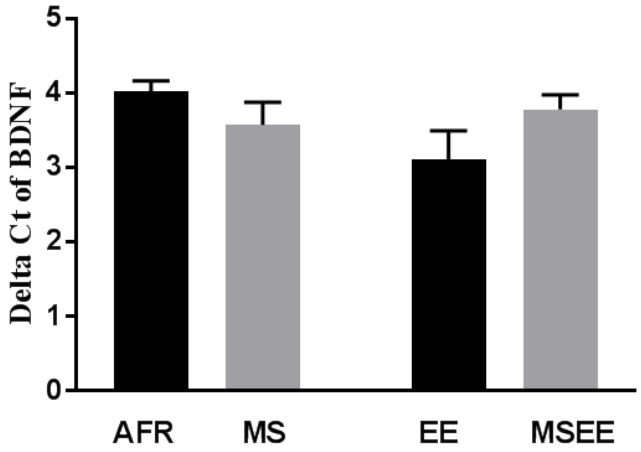


Figure R.18: Effect of early life stress and EE on BDNF mRNA expression: EE had a marginal significant (p = 0.06) effect on mRNA transcript level of BDNF in the basolateral amygdala in presence and absence of EE. Graphs represent Mean ± SEM. N = 6 for AFR, 5 for Stress, 6 for EE and 5 for MSEE.

Geometric mean of reference genes qPCR

Two housekeeping genes were used to alleviate the errors caused due to usage of one reference gene [287]. Therefore, the geometric mean of two reference genes was used for normalization in this experiment. To assess the influence of the geometric mean on the results of qPCR, two-way ANOVA and orthogonal planned comparison was performed for these values.

A two-way ANOVA was conducted for geometric means of reference genes in the BLA with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,19)} = 0.033$, $p = 0.86$) and EE ($F_{(1,19)} = 1.722$, $p = 0.21$) on the geometric mean of Ct values of the reference genes used to normalize qPCR Ct values did not reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,19)} = 0.038$, $p = 0.85$) on the

geometric mean of Ct values of the reference genes used to normalize qPCR Ct values did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE (t_9 = -0.539, p = 0.60; Cohen's d = 0.349), the effect of stress on the geometric mean of Ct values of the reference genes used to normalize qPCR Ct values did not reach statistical significance. Similarly, in the presence of EE (t_{10} = 0.008, p = 0.99; Cohen's d = 0.008; Figure R.19) the effect of stress on the geometric mean of Ct values of the reference genes used to normalize qPCR Ct values did not reach statistical significance.

The results revealed the absence of any influence of the geometric mean itself on the results of qPCR as there was no significant difference between the reference genes between groups. Hence, we were confident of using housekeeping genes as a reference for comparison of candidate genes in our study.

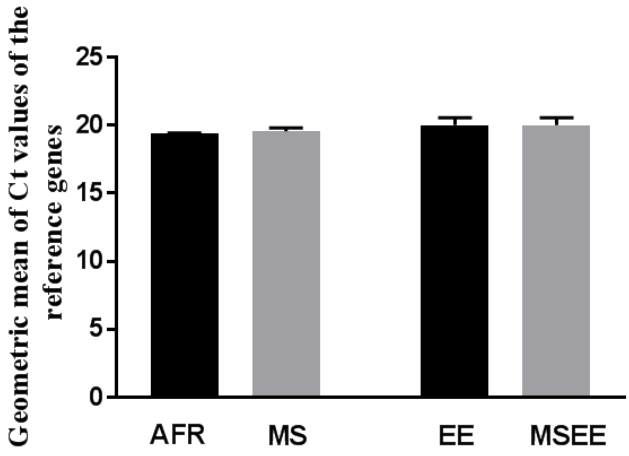


Figure R.19: Effect of early life stress on the geometric mean of Ct values of the reference genes: Stress had no significant effect on the geometric mean

of Ct value of the reference genes used to normalize qPCR Ct values. Graphs represent Mean ± SEM. N = 5 for AFR, 6 for Stress, 6 for EE and 6 for MSEE.

4.4. Protein analysis: Western Blot and Immunohistochemistry

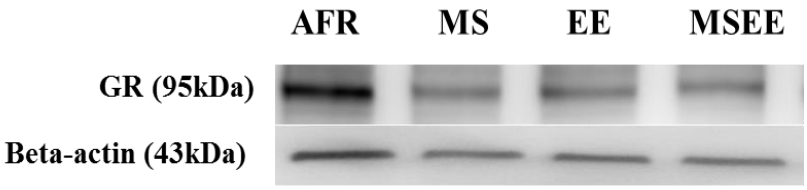
As mentioned in the previous section, the translational regulation of above-mentioned genes was investigated in this section. Candidate-based approach was applied to determine the protein expression levels of glucocorticoid receptor (GR), pro-brain derived neurotrophic factor (pro-BDNF) and brain derived neurotrophic factor (BDNF) in the basolateral amygdala (BLA) using western blot. Immunohistochemistry was used to further visualize the expression pattern of GR, Pro-BDNF, BDNF and phosphorylated mitogen-activated protein kinase (pMAPK) in neurons of the BLA. There is very limited information of the expression of these proteins in BLA when compared to hippocampus. Our study focuses on exploring the effect of early life environment on the expression of candidate- based proteins specifically in BLA, as it remains understudied in spite of BLA's crucial role in mediating stress response and emotional behavior.

Once the effect of early-life stress and enriched environment on mRNA transcription was determined, it was important to understand whether the results of qPCR had an impact on protein levels in the different group of animals. Protein expression levels of these molecules were determined in the BLA of animals assigned to different experimental groups and compared, statistically for difference between them.

Stress or EE did not alter total GR protein levels in BLA

A two-way ANOVA was conducted to measure relative intensity of GR in the BLA with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,19)} = 1.399$, $p = 0.25$) and EE ($F_{(1,19)} = 0.053$, $p = 0.82$) on the relative intensity of GR in the basolateral amygdala did not reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,19)} = 0.400$, $p = 0.53$) on the relative intensity of GR in the basolateral amygdala did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in absence of EE ($t_9 = 1.232$, $p = 0.25$; Cohen's $d = 0.782$) the effect of stress on the relative intensity of GR in the basolateral amygdala did not reach statistical significance. Similarly, in the presence of EE ($t_{10} = 0.405$, $p = 0.69$; Cohen's $d = 0.226$; Figure R.20) stress had no significant effect on protein level of GR in the basolateral amygdala.



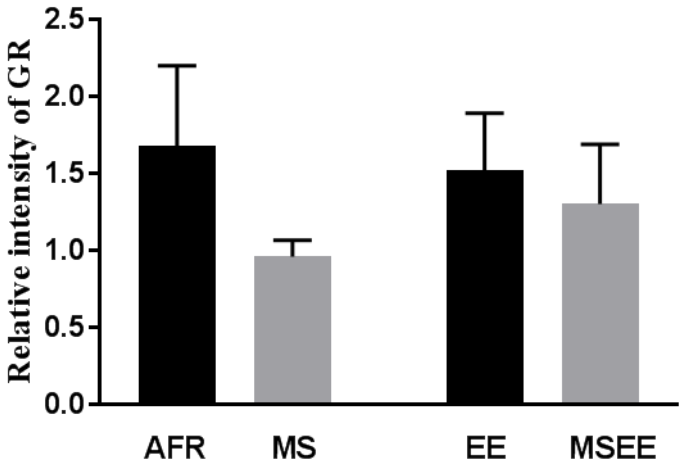
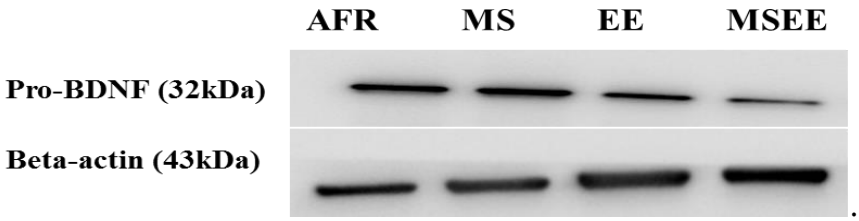


Figure R.20: Effect of stress on the relative intensity of GR in presence and absence of EE: Stress or EE had no significant effect on protein level of GR in the basolateral amygdala in presence and absence of EE. Graphs represent Mean ± SEM. N = 6 for AFR, 5 for Stress, 6 for EE and 6 for MSEE.

Stress or EE did not affect Pro-BDNF protein levels in BLA

A two-way ANOVA was conducted for relative intensity of Pro-BDNF in the BLA with stress and EE as two between subject sources of variance. EE ($F_{(1,19)} = 5.007$, $p = 0.04$) had a significant main effect on the relative intensity of Pro-BDNF in the basolateral amygdala. However, the effect of both stress ($F_{(1,19)} = 0.359$, $p = 0.56$) and the interaction between stress and EE ($F_{(1,19)} = 0.230$, $p = 0.64$) on the relative intensity of Pro-BDNF in the basolateral amygdala did not reach statistical significance.

Orthogonal planned comparisons (independent sample t-tests) showed that in the absence of EE ($t_{10} = 0.067$, $p = 0.95$; Cohen's d = 0.040) the effect of stress on the relative intensity of Pro-BDNF in the basolateral amygdala did not reach statistical significance. Similarly, in the presence of EE ($t_9 = 1.506$, $p = 0.17$ Cohen's d = 0.890; Figure R.21) the effect of stress on the relative intensity of Pro-BDNF in the basolateral amygdala did not reach statistical significance.



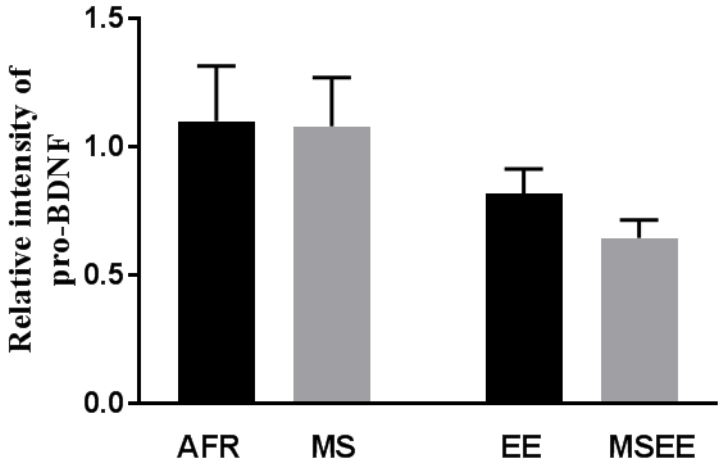


Figure R.21: Effect of early life stress and EE on the relative intensity of pro-BDNF: Stress or EE had no significant effect on protein level of pro-BDNF in the basolateral amygdala in presence and absence of EE. Graphs represent Mean ± SEM. N = 6 for AFR, 6 for Stress, 5 for EE and 6 for MSEE.

Stress alone did not affect BDNF protein levels in BLA, but interaction between stress & EE had significant influence on BDNF.

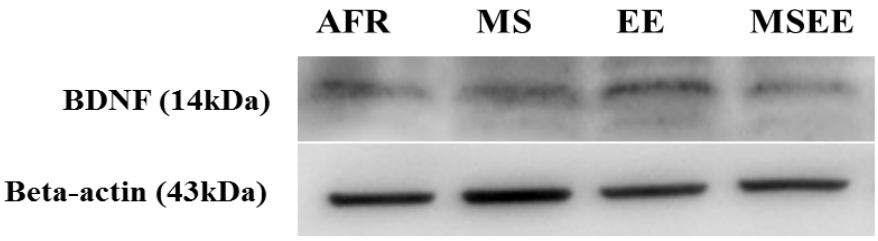
A two-way ANOVA was conducted for BDNF relative intensity in the BLA with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,20)} = 1.676$, $p = 0.21$) and EE ($F_{(1,20)} = 0.060$, $p = 0.81$) on the relative intensity of BDNF in the basolateral amygdala did not reach statistical significance.

However, the interaction between stress and EE ($F_{(1,20)} = 7.187$, $p = 0.01$) had a significant effect on the protein expression level of BDNF in the basolateral amygdala.

Orthogonal planned comparisons (independent samples t-tests) showed that in absence of EE ($t_{10} = -0.871$, $p = 0.40$; Cohen's $d = 0.498$) the effect of stress on the relative intensity of BDNF in the basolateral amygdala failed to reach statistical significance. However, in the presence of EE ($t_{10} = 3.282$, $p = 0.01$ Cohen's $d = 1.880$; Figure R.22) stress significantly decreased the protein level of BDNF in the basolateral amygdala.

The results revealed that MS did not alter the above mentioned protein levels in BLA. Expression of pro-BDNF, the precursor product of mature BDNF, was significantly decreased in rats placed in environmental enrichment. Previous work has associated increased expression of pro-BDNF in the hippocampus to depression in humans [288].The reduced pro-BDNF levels in my study could have anti-depressive like effect driven by BLA because hippocampus and BLA are known to display opposite effects to each other in response to stress [53]. Similarly, the hippocampus and BLA might have displayed contrasting effects in response to EE. Environmental enrichment was not observed to affect the expression of BDNF protein in the BLA. Prior reports have shown that BDNF levels are elevated in response to enriched environment conditions. Only one report demonstrated that short-term enriched environment during stress rescues the stress-induced elevated BDNF mRNA expression in BLA [166]. Finally, concurrent exposure to both short-term environmental enrichment and early life

maternal stress resulted in lower BDNF protein expression in the BLA. This is an important interaction effect that is not observed when rats are subjected to only maternal separation. Chronic immobilization stress (CIS) in adulthood increases mRNA abundance of BDNF in BLA. However, the presence of both CIS and EE rescues the stress induced increase in BDNF mRNA levels [166].



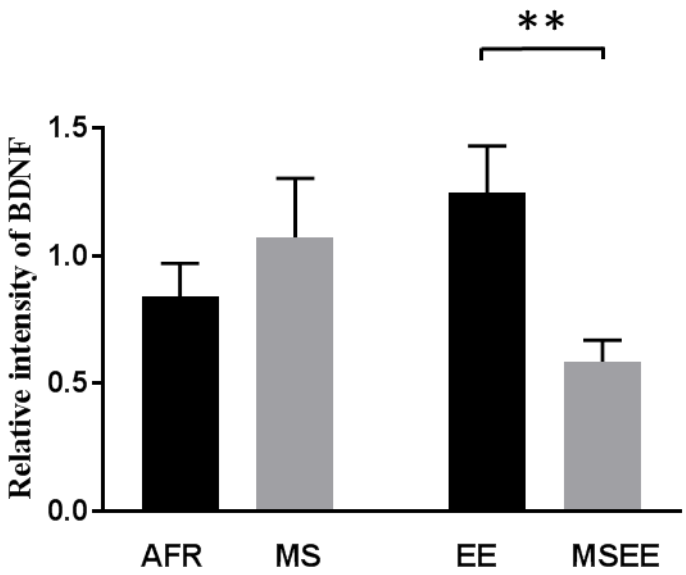


Figure R.22: Effect of stress on the relative intensity of BDNF in presence and absence of EE: Stress + EE significantly decreased the protein level of BDNF in the basolateral amygdala. Graphs represent Mean ± SEM. N = 6 for AFR, 6 for Stress, 6 for EE and 6 for MSEE; $^{**}p \leq 0.01$.

Stress increased the percentage of intra-nuclear GR in BLA neurons (Immunohistochemistry)

As mentioned earlier, immunohistochemistry was used to further visualize the expression pattern of GR, Pro-BDNF, BDNF and phosphorylated mitogen-activated protein kinase (pMAPK) in neurons of the BLA. The results are displayed below.

While analyzing total GR protein, there was an interesting observation wherein the density of GR signals was varying between samples. Hence, we decided to manually count the high-density GR signal cells to infer if the pattern of this peculiar (different density) signal was varying between groups.

Percentage of intra-nuclear GR was quantified by manually counting the high-density GR signals colocalized with DAPI stained cells. A two-way ANOVA was conducted for intra-nuclear GR in BLA neurons with stress and EE as two between subject sources of variance. Stress ($F_{(1,19)} = 7.870$, $p = 0.01$) and EE ($F_{(1,19)} = 9.537$, $p = 0.01$) had a significant main effect on the percentage of intra-nuclear GR in basolateral amygdala neurons. However, the effect of the interaction between stress and EE ($F_{(1,19)} = 0.356$, $p = 0.56$) on the percentage of intra-nuclear GR in basolateral amygdala neurons did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that, in absence of EE ($t_9 = 2.641$, $p = 0.03$; Cohen's d = 1.653) stress significantly decreased the percentage of intra-nuclear GR in basolateral amygdala neurons. However, in the presence of EE ($t_{10} = 1.469$, $p = 0.17$; Cohen's d = 0.848; Figure

R.23) the effect of stress on the percentage of intra-nuclear GR in basolateral amygdala failed to reach statistical significance.

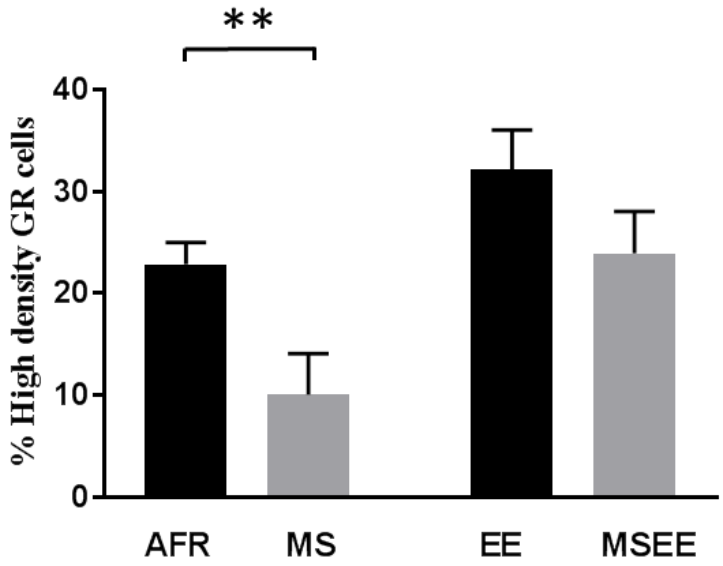


Figure R.23: Effect of early life stress and EE on the percentage of intra-nuclear GR/high-density cells: Stress significantly decreased the percentage of high-density GR cells in the basolateral amygdala in the absence of EE. Graphs represent Mean ± SEM. N = 5 for AFR, 6 for Stress, 6 for EE and 6 for MSEE; $^{**}p \leq 0.01$.

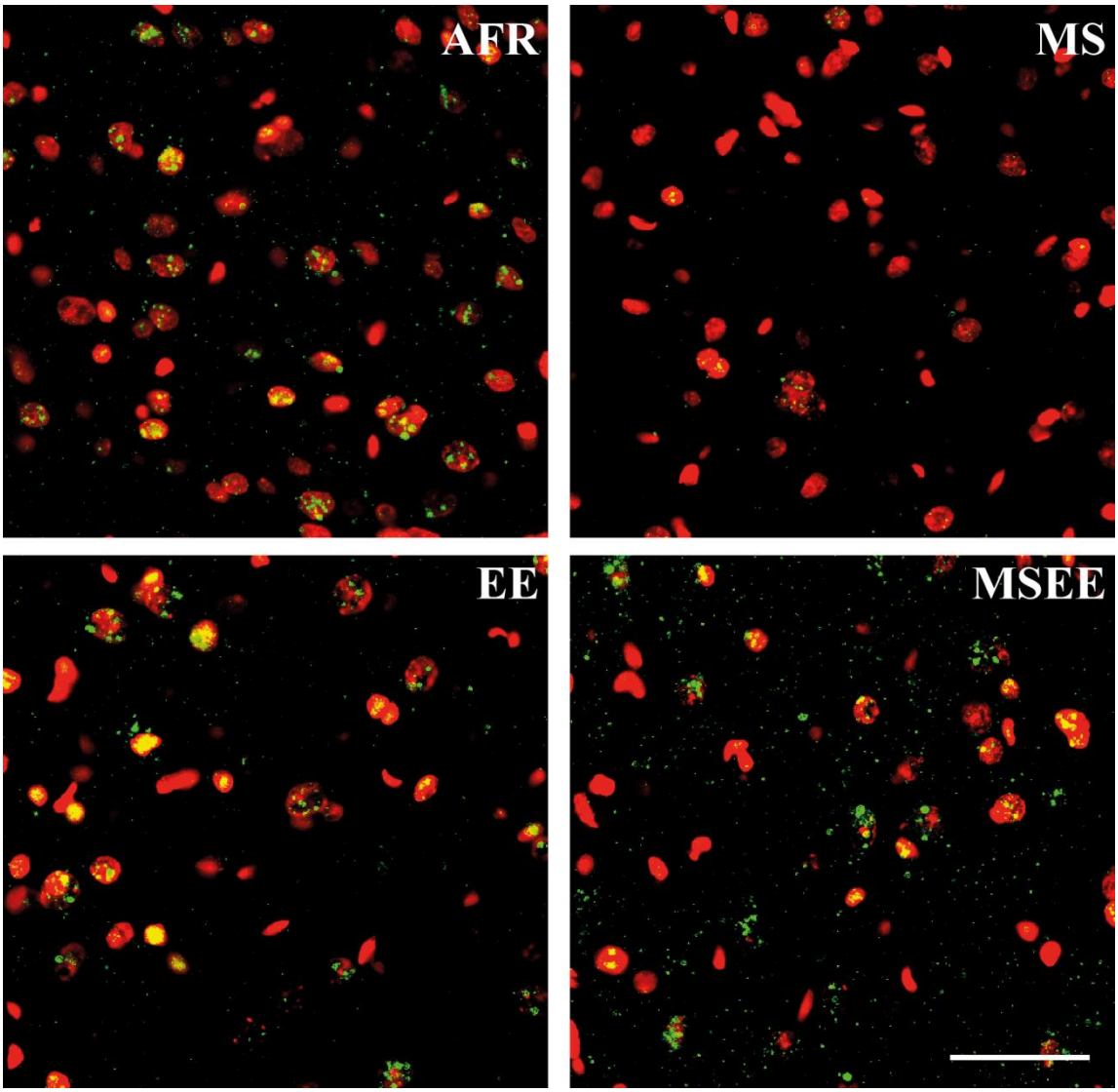


Figure R.24A: Representative images of GR stained cells in the BLA for all the four groups. Immunofluorescence staining of the nucleus with DAPI (red) and GR (green). These are the representative images of the cells that contained GR and were considered for GR analysis. The image was pseudocolored with red and green for clarity. Scale bar represents 50 µm.

Stress or EE did not alter the total GR signal in BLA (Immunohistochemistry)

Total GR signal was quantified by estimating the average percentage area of GR signals using ImageJ software. It was crucial to determine the total GR signal because we compared this with information about high density GR (in the previous section). This helped us infer if MS increased total GR or only activated GR (intra-nuclear) in BLA. A two-way ANOVA was conducted for total GR signal in BLA neurons with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,17)} = 1.600$, $p = 0.22$) and EE ($F_{(1,17)} = 0.010$, $p = 0.92$) on the total GR signal in the basolateral amygdala did not reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,17)} = 3.543$, $p = 0.08$) on the total GR signal in the basolateral amygdala did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE ($t_8 = 1.690$, $p = 0.13$; Cohen's $d = 1.103$) the effect of stress on the total GR signal in the basolateral amygdala did not reach statistical significance. Similarly, in the presence of EE ($t_9 = -0.696$, $p = 0.50$; Cohen's $d = 0.372$; Figure R.24) the effect of stress on the total GR signal in the basolateral amygdala did not reach statistical significance.

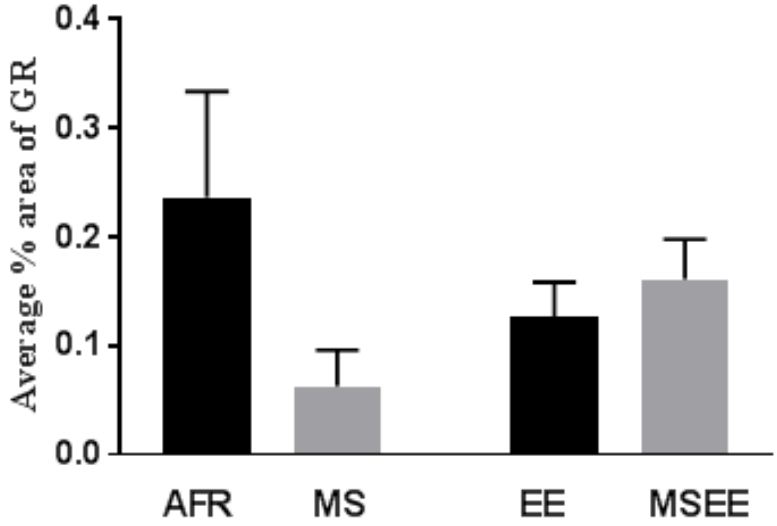


Figure R.24B: Effect of early life stress and EE on the total GR signal in the BLA: Stress or EE had no significant effect on the average percentage area of GR signals in the basolateral amygdala in presence and absence of EE. Graphs represent Mean ± SEM. N = 5 for AFR, 6 for Stress, 6 for EE and 6 for MSEE.

Stress or EE did not alter the total BDNF signal in BLA (Immunohistochemistry)

Total BDNF signal was quantified by estimating the average percentage area of BDNF signals using ImageJ software. This information further helps us visualize the expression of BDNF and validate the results shown in western blot. A two-way ANOVA was conducted for total BDNF signal in BLA neurons with stress and EE as between subject sources of variance. The effect of stress ($F_{(1,19)} = 0.861$, $p = 0.37$) and EE ($F_{(1,19)} = 3.261$, $p = 0.09$) on the total BDNF signal in the basolateral amygdala did not reach statistical significance. Similarly, the effect of the

interaction between stress and EE ($F_{(1,19)}$ = 3.079, p = 0.10) on the total BDNF signal in the basolateral amygdala did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE (t_9 = -0.793, p = 0.45; Cohen's d = 0.464) the effect of stress on the total BDNF signal in the basolateral amygdala did not reach statistical significance. Similarly, in the presence of EE (t_{10} = 1.628, p = 0.14; Cohen's d = 0.921; Figure R.25) the effect of stress on the total BDNF signal in the basolateral amygdala did not reach statistical significance.

Stress or EE had no significant effect on the average percentage area of BDNF signals in the basolateral amygdala.

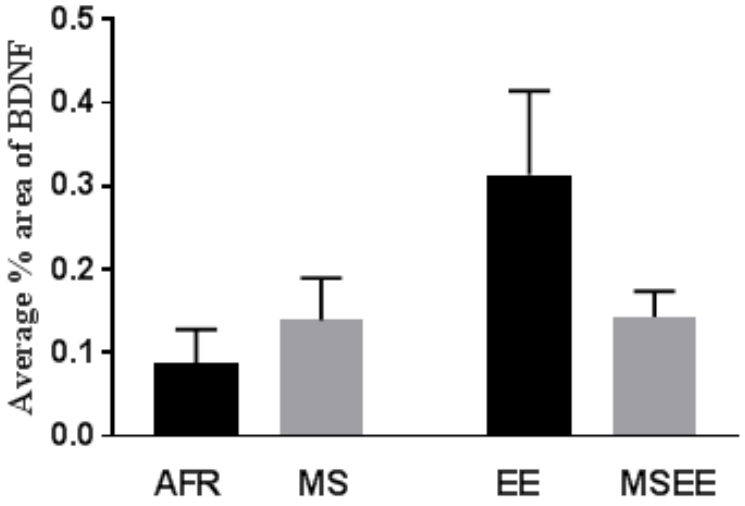


Figure R.25B: Effect of early life stress and EE on the total BDNF signal in the BLA: Stress or EE had no significant effect on the average percentage area of BDNF signals in the basolateral amygdala. Graphs represent Mean ± SEM. N = 5 for AFR, 6 for Stress, 6 for EE and 6 for MSEE

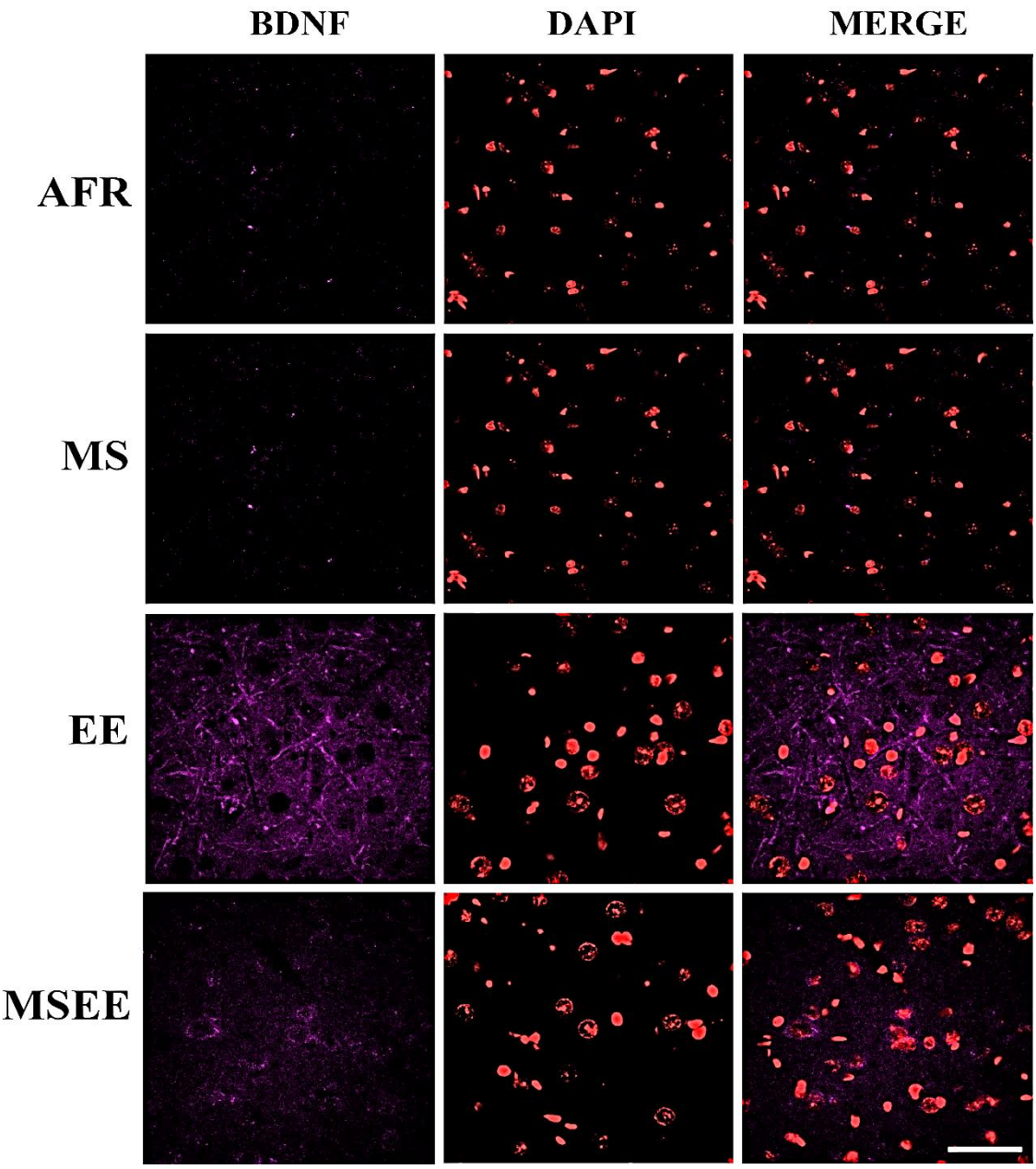


Figure R.25A: Representative images of BDNF stained cells in the BLA for all the four groups. Immunofluorescence staining of the nucleus with DAPI (red) and BDNF (magenta). These are the representative images of the cells that contained BDNF and were considered for BDNF analysis. The image was pseudocolored with red and magenta for clarity. Scale bar represents 50 μm.

Stress or EE did not alter the total pMAPK signal in the BLA (Immunohistochemistry)

The total pMAPK signal was quantified by estimating the average percentage area of pMAPK signals using ImageJ software. This information further helps us visualize the expression of pMAPK to understand the translational regulation of pMAPK in various treatment groups. A two-way ANOVA was conducted for total pMAPK signal in BLA neurons with stress and EE as between subject sources of variance. The effect of stress ($F_{(1,18)} = 2.654$, $p = 0.12$) and EE ($F_{(1,18)} = 0.126$, $p = 0.73$) on the total pMAPK signal in the basolateral amygdala did not reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,18)} = 0.647$, $p = 0.43$) on the total pMAPK signal in the basolateral amygdala did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE ($t_8 = 1.407$, $p = 0.20$; Cohen's $d = 0.890$) the effect of stress on the total pMAPK signal in the basolateral amygdala did not reach statistical significance. Similarly, in the presence of EE ($t_{10} = 0.730$, $p = 0.48$; Cohen's $d = 0.485$; Figure R.26) the effect of stress on the total pMAPK signal in the basolateral amygdala did not reach statistical significance.

Stress or EE had no significant effect on the average percentage area of BDNF signals in the basolateral amygdala.

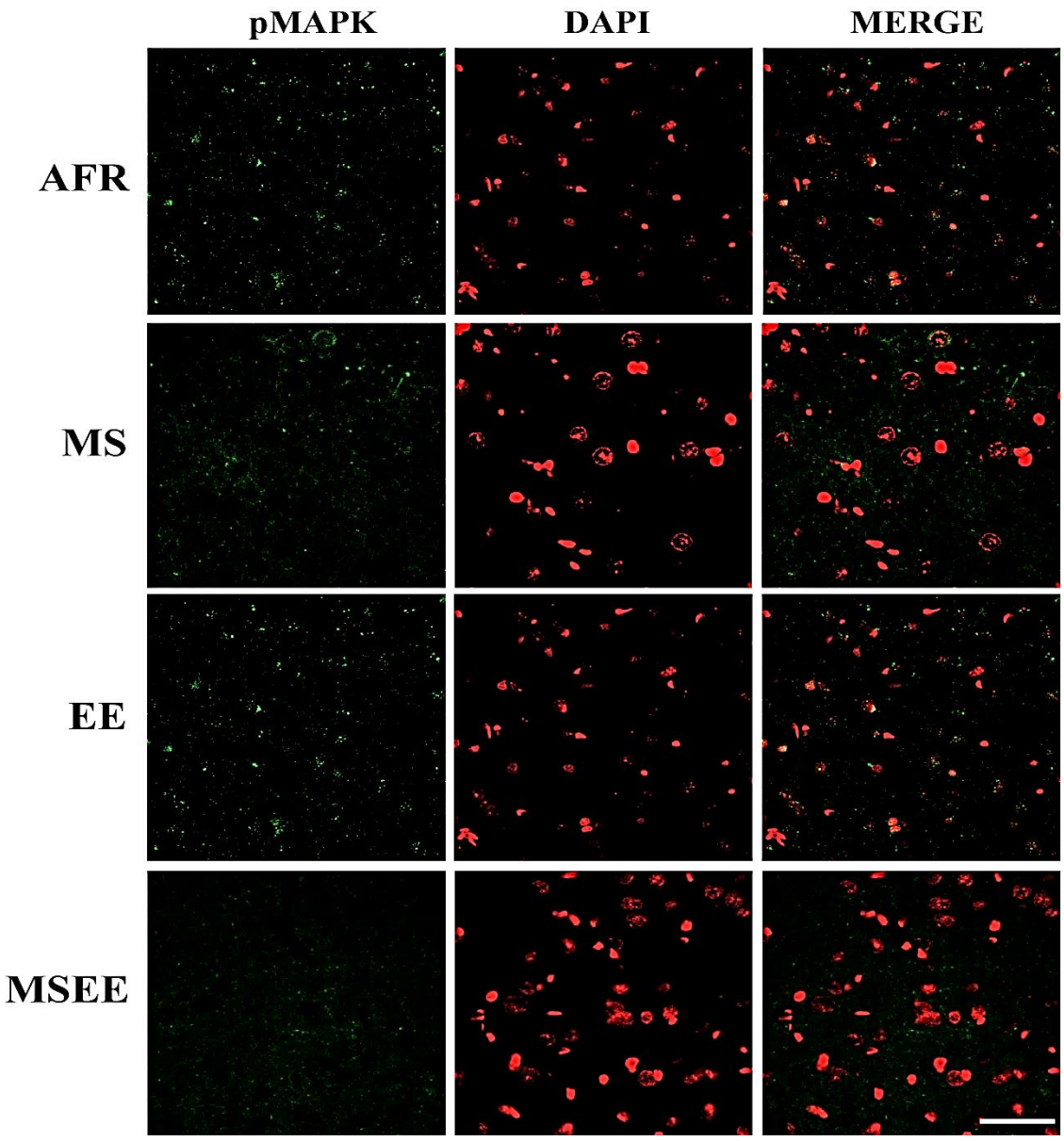


Figure R.26A: Representative images of pMAPK stained cells in the BLA for all the four groups. Immunofluorescence staining of the nucleus with DAPI (red) and pMAPK (green). These are the representative images of the cells that contained pMAPK and were considered for pMAPK analysis. The image was pseudocolored with red and green for clarity. Scale bar represents 50 μm.

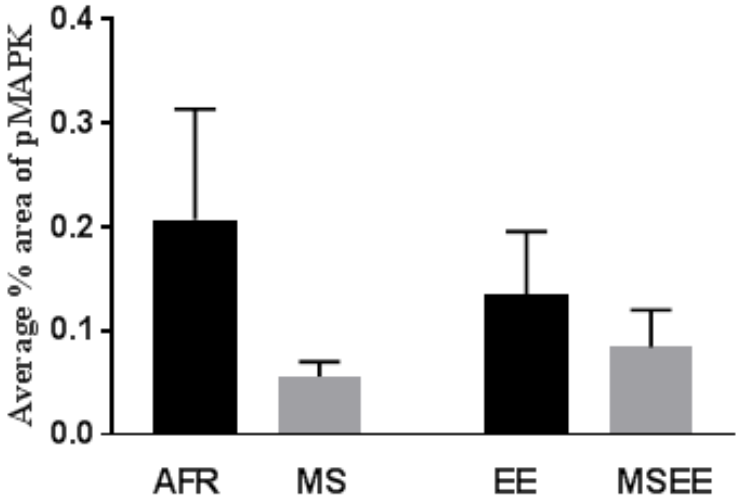


Figure R.26B: Effect of early life stress and EE on the total area of pMAPK signals in the BLA: Stress or EE had no significant effect on the average percentage area of BDNF signals in the basolateral amygdala. Graphs represent Mean ± SEM. N = 5 for AFR, 6 for Stress, 6 for EE and 6 for MSEE

The immunohistochemistry results indicate that the early life stress and EE did not alter the levels of the above-mentioned protein expression in BLA. Though total protein levels for GR, BDNF and pMAPK did not vary, the density of GR signals in the nucleus was significantly different in MS animals housed in standard cage. The recruitment of activated GR in the nucleus as a transcription factor is a crucial step that initiates a relay of downstream molecular pathways to mediate stress response [112, 220]. Prior studies show that activation of GR through GC causes phosphorylation of Erk1/2^{MAPK}. This activated Erk1/2^{MAPK} causes relay of downstream molecular pathways that are crucial in mediating behavioral effects of

GC [221, 222]. The stress response cascade of these molecules have been widely examined in the hippocampus [221] Unlike hippocampus, BLA remains understudied in spite of its crucial role in mediating stress response and emotional behavior. GR being the primary driver of plasticity within BLA, we studied expression, regulation and nuclear activation of GR, which has been shown to be crucial for BLA-dependent response to stress. In AFR housing conditions, the percentage of cells containing high-density (intra nuclear) GR in the basolateral amygdala was significantly greater in MS rats compared to controls. This effect of MS was not observed in rats exposed to the enriched environment, as shown above. High-density GR refers to activated-GR, which translocate to the nucleus to activate downstream transcription. Thus, although MS did not affect total GR, it significantly decreased the amount of activated GR found in the nucleus. These results clearly highlighted the varying regulation of activated-GR in the BLA. The amount of **activated-GR** could be a crucial mediator in driving stress-induced BLA structural and molecular plasticity under the influence of differential early-life housing environments.

4.5. Epigenetics: Methylation in promoter sites

In the previous section of the results, our data showed a treatment-dependent change in protein expression levels of the candidate molecules, specifically GR and BDNF. Thus, we sought to determine if the observed change in protein levels were a result of underlying epigenetic modifications, specifically DNA methylation of the promoter region of the GR and BDNF gene in the basolateral amygdala. The results are represented as average percentage of cytosine methylation of the candidate with well-characterized CpG sites in rats.

Stress or EE did not alter average percentage cytosine methylation of BDNF promoter in BLA

Overall DNA methylation of a gene was calculated as the average % DNA methylation of all CpGs within the gene in this study. A two-way ANOVA was conducted for average percentage cytosine methylation of BDNF promoter in BLA neurons with stress and EE as between subject sources of variance. The effect of stress ($F_{(1,15)} = 2.784$, $p = 0.12$) and EE ($F_{(1,15)} = 0.0004$, $p = 0.99$) on the average percentage cytosine methylation at the promoter region of BDNF in the basolateral amygdala did not reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,15)} = 2.413$, $p = 0.14$) on the average percentage cytosine methylation at the promoter region of BDNF in the basolateral amygdala did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE ($t_8 = 1.775$, $p = 0.11$; Cohen's $d = 1.120$) the effect of stress on the average percentage cytosine methylation at the promoter region of BDNF in the

basolateral amygdala did not reach statistical significance. Similarly, in the presence of EE ($t_7 = 0.210$, $p = 0.84$; Cohen's $d = 0.133$; Figure R.27) the effect of stress on the average percentage cytosine methylation at the promoter region of BDNF in the basolateral amygdala did not reach statistical significance.

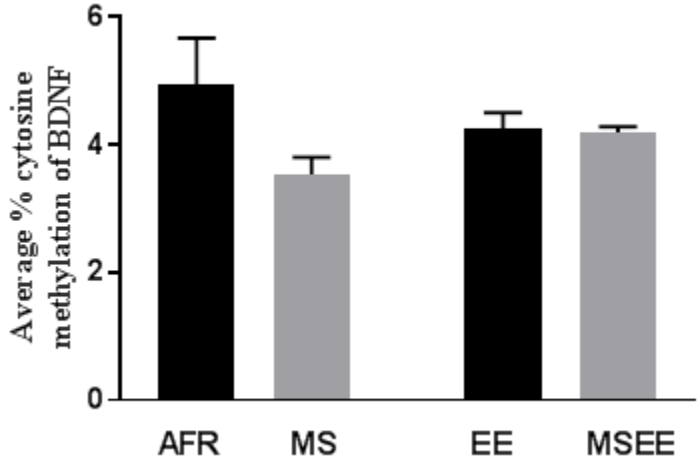


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Figure R.27: Effect of early life stress and EE on average percentage cytosine methylation at the promoter region of BDNF in the BLA: stress or EE had no significant effect on the average percentage cytosine methylation at the promoter region of BDNF in the basolateral amygdala in presence and absence of EE. Graphs represent Mean ± SEM. N = 5 for AFR, 5 for Stress, 4 for EE and 5 for MSEE

Stress or EE did not alter average percentage cytosine methylation of GR promoter in BLA

A two-way ANOVA was conducted for average percentage cytosine methylation of GR promoter in BLA neurons with stress and EE as between subject sources of variance. The effect of stress ($F_{(1,12)} = 2.400$, $p = 0.15$) and EE ($F_{(1,12)} = 0.730$, $p = 0.41$) on the average percentage cytosine methylation at the promoter region of GR in the basolateral amygdala did not reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,12)} = 1.030$, $p = 0.33$) on the average percentage cytosine methylation at the promoter region of GR in the basolateral amygdala did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE ($t_6 = -1.320$, $p = 0.24$; Cohen's $d = 0.133$) the effect of stress on the average percentage cytosine methylation at the promoter region of GR in the basolateral amygdala did not reach statistical significance. Similarly, in the presence of EE ($t_6 = -0.926$, $p = 0.39$; Cohen's $d = 0.659$; Figure R.28) the effect of stress on the average percentage cytosine methylation at the promoter region of BDNF in the basolateral amygdala did not reach statistical significance.

Epigenetic changes in GR promoter gene are well reported in the hippocampus for MS models. In particular, methylation studies are widely explored in these models [251]. The results revealed that the methylation status of the CpG sites used in this study did not alter due to stress in the presence and absence of EE. This observation shows that these specific CpG sites are not the major driving force

behind the long-term effect on variation in protein expression of GR and BDNF in BLA.

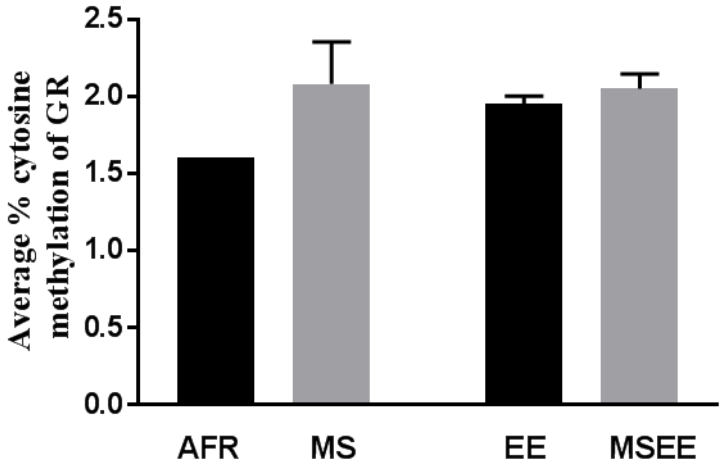


Figure R.28: Effect of early life stress and EE on average percentage cytosine methylation at the promoter region of GR in the BLA: Stress or EE had no significant effect on the average percentage cytosine methylation at the promoter region of GR in the basolateral amygdala in presence and absence of EE. Graphs represent Mean ± SEM. N = 3 for AFR, 5 for Stress, 4 for EE and 4 for MSEE.

4.6. Dendritic Morphology

As mentioned earlier, in addition to the neuroendocrine HPA axis, another captivating *Brain-HPA* is; **H**ippocampus, **P**refrontal cortex and **A**mygdala. These are the structures that contribute in the regulation of HPA axis. They have a significant role on the release of GC and behavioral response to a stressful event [114, 115]. Stress is known to alter the dendritic morphology of neurons in these brain regions. Stress is known to induce dendritic hypertrophy in the basolateral amygdala (BLA) [52, 53] and to enhance anxiety [53, 54]. Amygdala is activated by, corticosterone released during a stress response and this results in dendritic hypertrophy as well as elevated spine density [65]. These morphological changes have been correlated with anxiety and stress in prior studies [52, 53, 57, 135]. These changes in neuronal morphology are known to be associated with anxiety-like behavior and general stress response.[52, 54, 57]. In spite of being a cruicial role player in stress response, amygdala is the least explored region in early-life stress studies. This makes it important to explore amygdala in this study. To assess the effects of early life stress and EE on morphological changes in BLA, we determined the dendritic length and branch points using Golgi staining. We also observed the spine density in primary and secondary dendrites of neurons in BLA. The results with statistics are displayed below.

Stress along with EE increased dendritic length of BLA neurons

A two-way ANOVA was conducted for a dendritic length of BLA neurons with stress and EE as between subject sources of variance. The effect of stress ($F_{(1,275)}$ = 24.383, $p < 0.00$) and the interaction between stress and EE ($F_{(1,275)}$ = 28.404, $p <$

0.00) had a significant effect on the total dendritic length of neurons in the basolateral amygdala. However, the effect of EE ($F_{(1,275)} = 1.992$, $p = 0.16$) on the total dendritic length of neurons in the basolateral amygdala did not reach statistical significance.

Orthogonal planned comparisons (Independent samples t-tests) showed that, in the absence of EE ($t_{159} = 0.296$, $p = 0.77$; Cohen's $d = 0.047$), the effect of stress on the total dendritic length of neurons in the basolateral amygdala did not reach statistical significance. However, in the presence of EE ($t_{116} = -6.926$, $p < 0.00$; Cohen's $d = 1.275$; Figure R.29) stress significantly increased the total dendritic length of neurons in the basolateral amygdala.

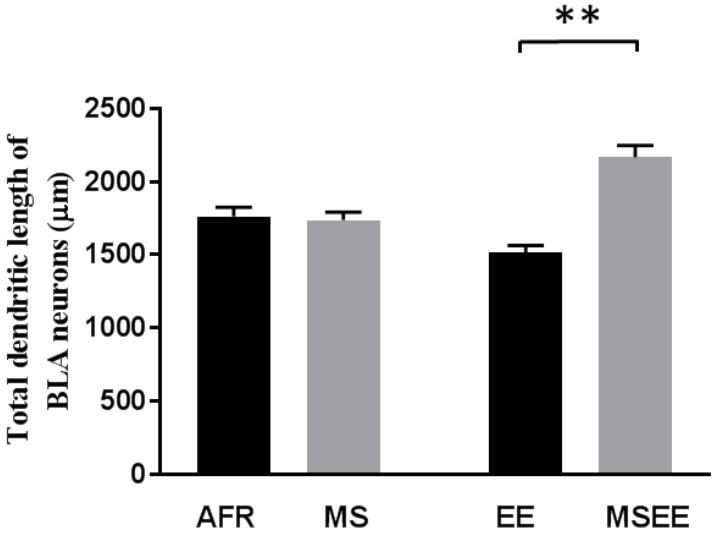


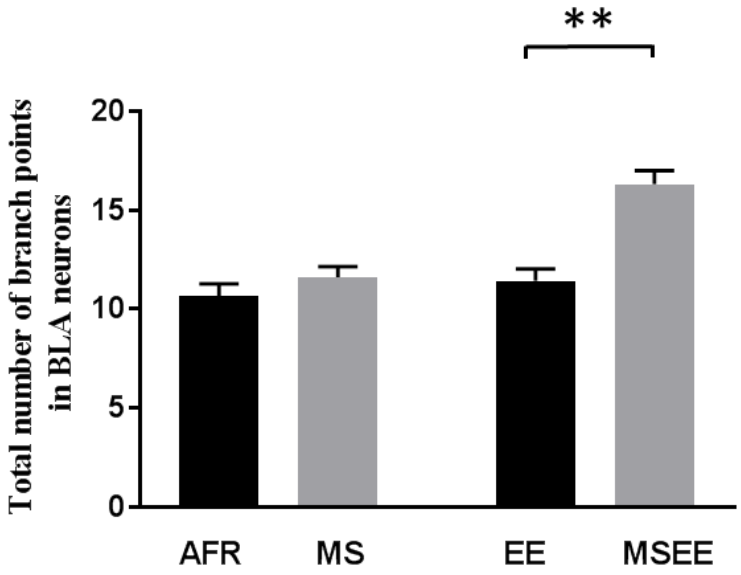
Figure R.29: Effect of early life stress and EE on total dendritic length in the BLA neurons: Stress along with/in the presence of EE significantly increased the total dendritic length of neurons in the basolateral amygdala. Graphs represent

Mean ± SEM. N (number of neurons) = 79 for AFR, 82 for Stress, 59 for EE and 59 for MSEE, 8-10 neurons/animal; $^{**}p \leq 0.01$.

Stress along with EE induced increase in branch points in BLA neurons

A two-way ANOVA was conducted for number of branch points in BLA neurons with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,270)} = 22.494$, $p < 0.00$), EE ($F_{(1,270)} = 19.641$, $p < 0.00$) and the interaction between stress and EE ($F_{(1,275)} = 10.350$, $p < 0.00$) had a significant effect on the total number of branch points of neurons in the basolateral amygdala.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE ($t_{156} = -1.161$, $p = 0.25$; Cohen's $d = 0.185$), the effect of stress on the total dendritic length of neurons in the basolateral amygdala did not reach statistical significance. However, in the presence of EE ($t_{114} = -5.316$, $p < 0.00$; Cohen's $d = 0.985$; Figure R.30) stress significantly increased the total number of branch points of neurons in the basolateral amygdala.



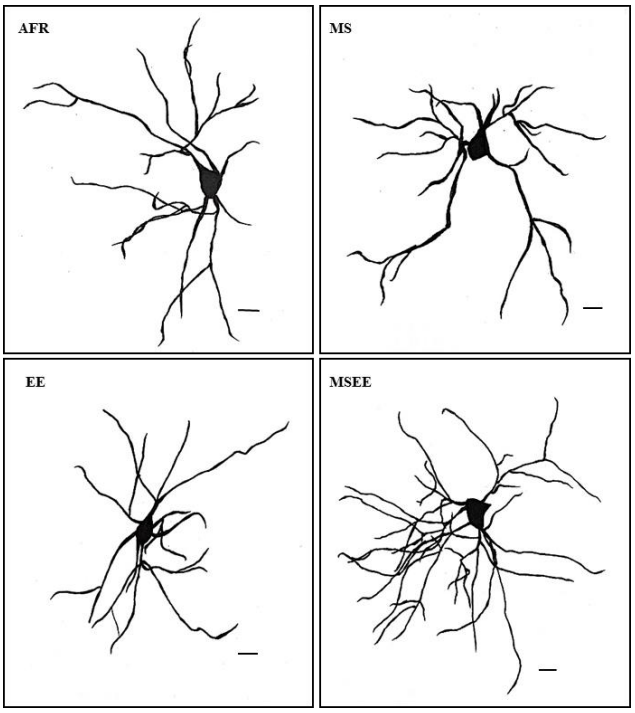


Figure R.30: Effect of early life stress and EE on total number of branch points in BLA neurons: Stress along with EE significantly increased the total number of branch points of neurons in the basolateral amygdala. Graphs represent

Mean ± SEM. N (number of neurons) = 79 for AFR, 79 for Stress, 60 for EE and 56 for MSEE, 8-10 neurons/animal $^{**}p \leq 0.01$. Inset (bottom): Representative images of sample Golgi-stained neurons in the BLA for the 4 groups from optical microscope at 400X magnification. Scale bar represents 20 μm.

Rats exposed to both maternal separation and environmental enrichment show dendritic length hypertrophy, increase in branch points in the BLA compared to rats exposed to only environmental enrichment. Earlier studies have shown that the dendritic complexity of principal neurons in the BLA correlates with high anxiety in rats [135]. In contrast to the previous studies, the neuronal hypertrophy induced by MSEE group in this study does not correspond to anxiety-like behavior in adults. It is possible that early life could have very different on BLA morphology, as there is are no previous early life studies correlating hypertrophy and anxiety. Hence, hypertrophy in this case may denote active-coping behavior.

Stress induced increase in the number of spines on primary dendrites in BLA neurons in presence and absence of EE

Stress exposure (acute or chronic) as well as exogenous corticosterone administration leads to increase in the dendritic arbors and spine density of the principal neurons of BLA and corresponding manifestation of anxiety [52]. To investigate the impact of early life stress and enriched environment on dendritic spines in the BLA, we quantified the number of primary and secondary spines in BLA using Golgi staining.

A two-way ANOVA was conducted for number of spines on primary dendrites in BLA neurons with stress and EE as between subject sources of variance. The

effect of stress ($F_{(1,234)} = 53.186$, $p < 0.00$) and EE ($F_{(1,234)} = 8.610$, $p < 0.00$) had a significant main effect on the number of spines found on primary dendrites in the basolateral amygdala. However, the effect of the interaction between stress and EE ($F_{(1,234)} = 0.536$, $p = 0.47$) on the number of spines found on primary dendrites in the basolateral amygdala did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE ($t_{117} = -5.654$, $p < 0.00$; Cohen's $d = 1.037$) the effect of stress on the number of spines found on primary dendrites in the basolateral amygdala did not reach statistical significance. Similarly, in the presence of EE ($t_{117} = -4.656$, $p < 0.00$; Cohen's $d = 0.855$; Figure R.31) the effect of stress on the number of spines found on primary dendrites in the basolateral amygdala did not reach statistical significance.

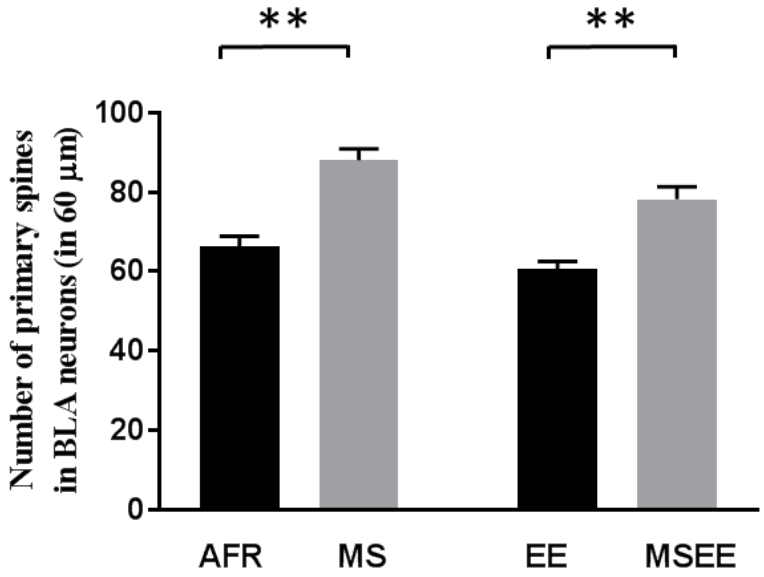


Figure R.31: Effect of early life stress and EE on the number of primary spines in BLA neurons: Stress significantly increased the number of spines found

on primary dendrites in the basolateral amygdala in presence and absence of EE. Graphs represent Mean ± SEM. N (number of neurons) = 79 for AFR, 79 for Stress, 60 for EE and 56 for MSEE, 10 neurons/animal; $^{**}p \leq 0.01$.

Stress induced increase in the number of spines on secondary dendrites in BLA neurons in the absence of EE

A two-way ANOVA was conducted for number of spines on secondary dendrites in BLA neurons with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,234)} = 5.194$, $p = 0.02$) and the interaction between stress and EE ($F_{(1,234)} = 18.240$, $p < 0.00$) had a significant effect on the number of spines found on secondary dendrites in the basolateral amygdala. However, the effect of EE ($F_{(1,234)} = 0.893$, $p = 0.35$) had no significant effect on the number of spines found on secondary dendrites in the basolateral amygdala.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE ($t_{117} = -4.313$, $p < 0.00$; Cohen's d = 0.790), stress significantly increased the number of spines found on secondary dendrites in the basolateral amygdala. However, in the presence of EE ($t_{117} = 1.531$, $p = 0.13$; Cohen's d = 0.294; Figure R.32) the effect of stress on the number of spines found on secondary dendrites in the basolateral amygdala did not reach statistical significance.

Acute and/ or chronic stress, as well as exogenous supplementation of corticosterone results in neuronal hypertrophy and an increase in spine density of BLA principal cells [136]. Rats subjected to chronic immobilization stress in early life and adulthood increases neuronal spine density in the amygdala [7, 17, 21].

Similarly, the results presented above showed significant increase in neuronal spine density in animal's subjected maternal separation compared to control rats in the normal housing. Although the spine density was increased, there was no increase in dendritic length and branch points in normal housing rats that were subjected to maternal separation. Environmental enrichment, irrespective of presence or absence of maternal separation treatment resulted in a general decrease in secondary spine density in the BLA neurons. Rats exposed to both maternal separation and environmental enrichment show dendritic hypertrophy and increase in primary neuronal spine density in the BLA compared to rats exposed to only environmental enrichment. As this treatment is a novel one, such an interesting pattern of neuronal morphology is worth exploring.

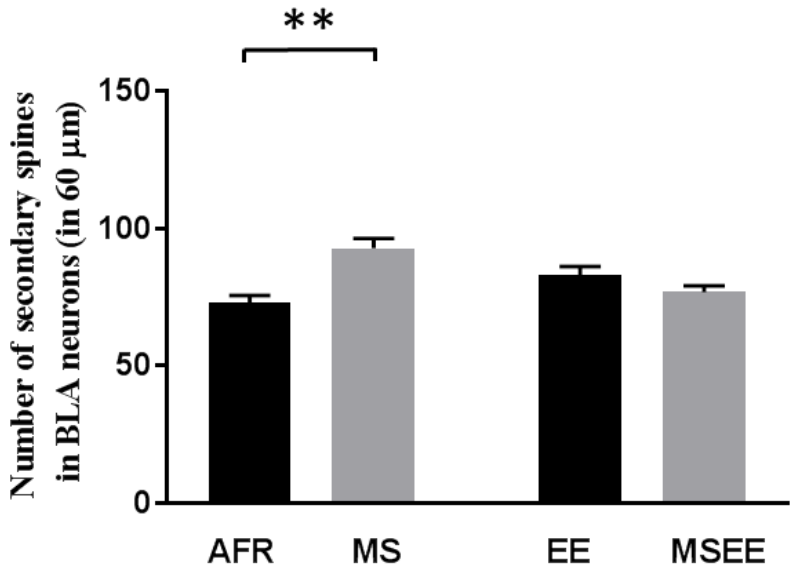


Figure R.32: Effect of early life stress and EE on the number of secondary spines in BLA neurons: Stress significantly increased the number of spines found on secondary dendrites in the basolateral amygdala in the absence of EE. Graphs represent Mean ± SEM. N (number of neurons) = 60 for AFR, 59 for Stress, 60 for EE and 59 for MSEE, 10 neurons/ animal; $^{**}p \leq 0.01$.

Stress did not alter the total dendritic length of hippocampal neurons in presence and absence of EE

Secondly, we look at the neuronal morphology of hippocampus as its involvement is widely reported in stress and cognitive processes [119, 120] It is known to be one of the most plastic regions of the brain and the structural plasticity is highly dependent on the number and impact of stressful episodes experienced by an individual (level of glucocorticoids in the systemic circulation) [122, 123]. To analyze the effects of early life stress and EE on the neuronal morphology of hippocampus, we determine the dendritic length, branch points and spine density of primary and secondary neurons of hippocampus. The results with statistics are given below.

A two-way ANOVA was conducted for a dendritic length of hippocampal neurons with stress and EE as between subject sources of variance. The effect of stress ($F_{(1,255)} = 0.666$, $p = 0.42$) and EE ($F_{(1,255)} = 0.427$, $p = 0.51$) on the total dendritic length of neurons in the hippocampus did not reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,255)} = 2.828$, $p = 0.09$) on the total dendritic length of neurons in the hippocampus did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE ($t_{140} = 0.652$, $p = 0.52$; Cohen's $d = 0.113$) the effect of stress on the total dendritic length of neurons in the hippocampus did not reach statistical significance. Similarly, in the presence of EE ($t_{115} = -1.657$, $p = 0.10$; Cohen's d =

0.306; Figure R.33) the effect of stress on the total dendritic length of neurons in the hippocampus did not reach statistical significance.

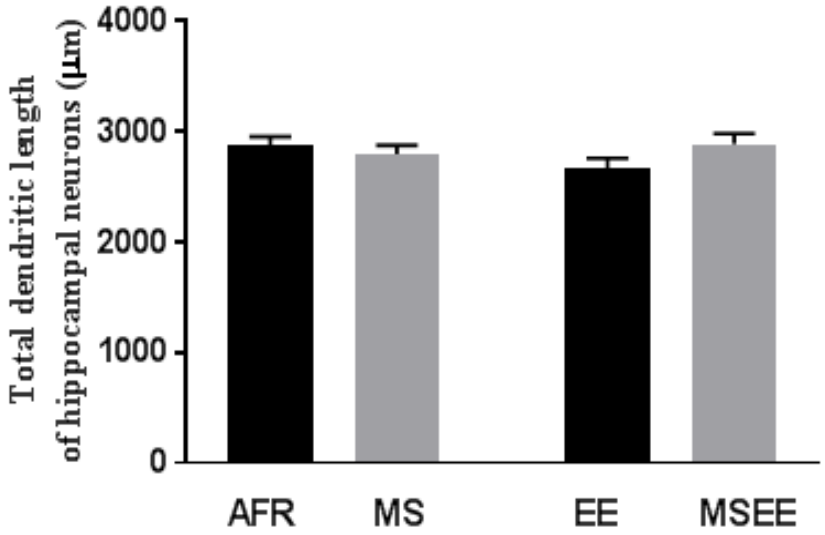


Figure R.33: Effect of early life stress and EE on total dendritic length of hippocampal neurons: Stress had no significant effect on the total dendritic length of neurons in the hippocampus in the presence and absence of EE. Graphs represent Mean ± SEM. N (number of neurons) = 58 for AFR, 84 for Stress, 59 for EE and 58 for MSEE, 8-10 neurons/ animal.

Stress did not alter the number of branch points of hippocampal neurons in presence and absence of EE

A two-way ANOVA was conducted for number of branch points in hippocampal neurons with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,258)} = 2.232$, $p = 0.14$) and EE ($F_{(1,258)} = 0.168$, $p = 0.68$) on the total branch points of neurons in the hippocampus did not reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,258)} = 0.027$, $p = 0.87$) on the total branch points of neurons in the hippocampus did not reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE ($t_{142} = -0.982$, $p = 0.33$; Cohen's $d = 0.168$) the effect of stress on the total branch points of neurons in the hippocampus did not reach statistical significance. Similarly, in the presence of EE ($t_{116} = -1{,}126$, $p = 0.26$; Cohen's $d = 0.207$; Figure R.34) the effect of stress on the total branch points of neurons in the hippocampus did not reach statistical significance.

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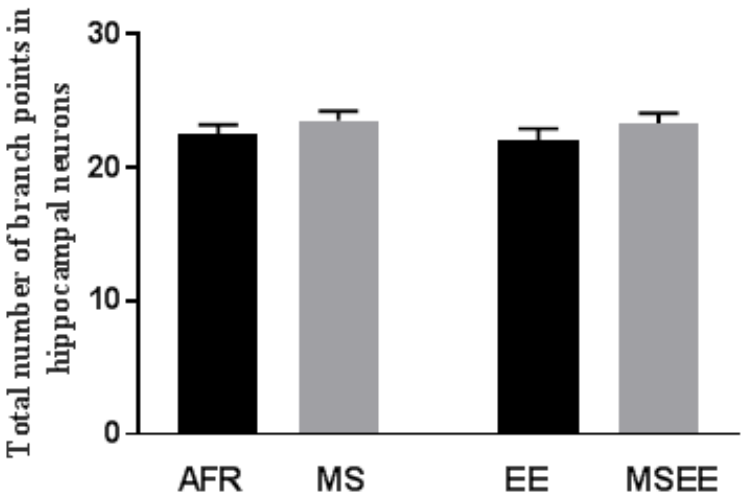

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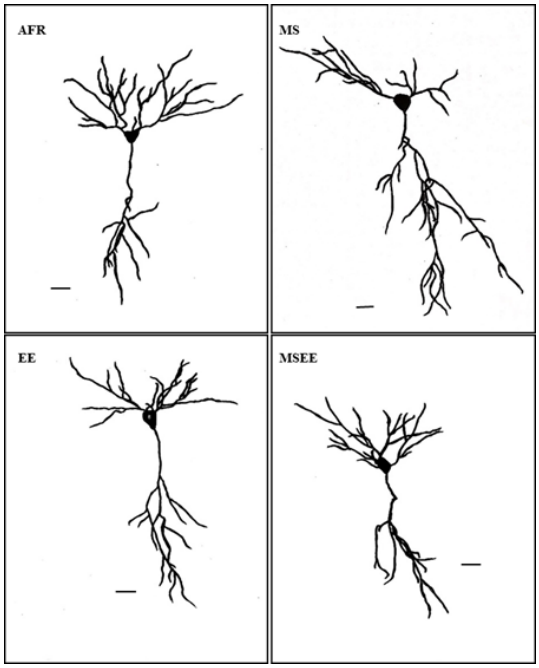

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2608 **Figure R.34: Effect of early life stress and EE on total number of branch**
2609 **points in hippocampal neurons:** Stress did not alter the total number of branch
2610 points of neurons in the hippocampus in presence and absence of EE. Graphs
2611 represent Mean ± SEM. N (number of neurons) = 59 for AFR, 85 for Stress, 59 for
2612 EE and 59 for MSEE, 8-10 neurons/ animal. Inset (bottom): Representative images

of sample Golgi-stained neurons in the hippocampus for the 4 groups from optical microscope at 400X magnification. Scale bar represents 20 μm.

Stress had differential effects on the number of spines on primary dendrites in hippocampal neurons in presence and absence of EE

A two-way ANOVA was conducted for total number of primary spines of hippocampal neurons with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,275)} = 0.405$, $p = 0.53$) on the number of primary spines in the hippocampus did not reach statistical significance. However, EE ($F_{(1,275)} = 7.186$, $p = 0.01$) and the interaction between stress and EE ($F_{(1,275)} = 16.573$, $p < 0.00$) had a significant effect on the number of spines found on primary dendrites in the hippocampus.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE ($t_{147} = -3.853$, $p < 0.00$; Cohen's d = 0.646), stress significantly increased the number of spines found on primary dendrites in the hippocampus. Similarly, in the presence of EE ($t_{128} = 2.123$, $p = 0.04$; Cohen's d = 0.370; Figure R.35) stress significantly decreased the number of spines found on primary dendrites in the hippocampus.

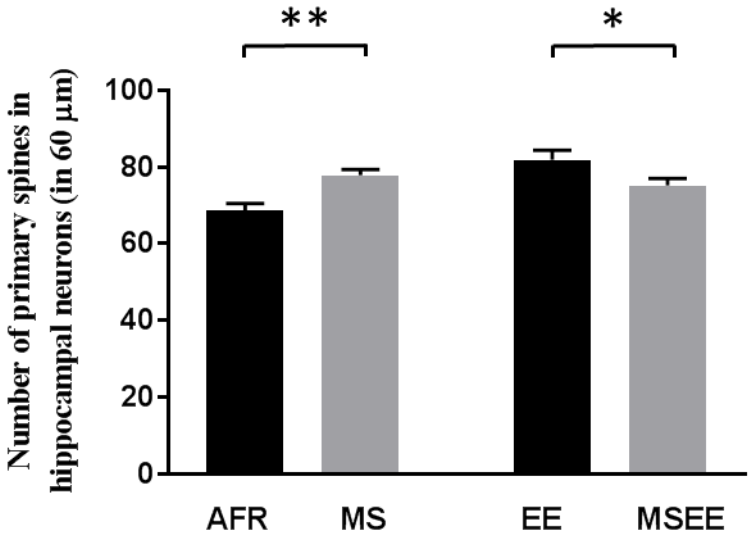


Figure R.35: Effect of early life stress and EE on primary spines in hippocampal neurons: Stress significantly increased the number of spines found on primary dendrites in the hippocampus in the absence of EE and stress significantly decreased the number of spines found on primary dendrites in the hippocampus in the absence of EE. Graphs represent Mean ± SEM. N (number of neurons) = 60 for AFR, 89 for Stress, 60 for EE and 70 for MSEE, 10 neurons/animal; *$p \leq 0.05$, **$p \leq 0.01$.

Stress significantly increased the number of spines on secondary dendrites in hippocampal neurons in the absence of EE

A two-way ANOVA was conducted for a total number of secondary spines of hippocampal neurons with stress and EE as between subject sources of variance. The effect of stress ($F_{(1,275)} = 5.108$, $p = 0.03$), EE ($F_{(1,275)} = 49.604$, $p < 0.00$) and the interaction between stress and EE ($F_{(1,275)} = 25.397$, $p < 0.00$) had a significant effect on the number of spines found on secondary dendrites in the hippocampus.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE (t_{148} = -5.932, $p < 0.00$; Cohen's d = 0.970), stress significantly increased the number of spines found on secondary dendrites in the hippocampus. However, in the presence of EE (t_{127} = 1.724, p = 0.09; Cohen's d = 0.304; Figure R.36) the effect of stress on the number of secondary spines in the hippocampus did not reach statistical significance.

Under normal housing conditions, maternal stress reduces the complexity of hippocampal neurons. Specifically, dendritic length and cell proliferation are known to decrease in MS rats compared to control [126, 127]. While studies report atrophy of CA3 neurons in the hippocampus, Sunanda *et al.,* (1995) observed increased spine density in rats subjected to chronic stress in adult life and has argued that this observed increase in spine density may be a mechanism to counter neuronal atrophy by increasing the postsynaptic membrane area [289]. This observation was corroborated in the above results, where rats subjected to MS showed an increase in spine density of both primary and secondary branches of the neuron. Moreover, MS did not alter neuronal length or number of branch points in normally housed rats. This is congruent with the observation that although atrophy of hippocampal neurons can be observed up until four days post-MS treatment, atrophy is no longer present after ten days post stress treatment because of the plastic recovering nature of hippocampal neurons [290, 291]. Also, MSEE group showed a decrease in both primary and secondary neuronal spine density compared to rats exposed only to the enriched environment. EE paradigm is widely studied on hippocampal neurons and its plasticity, where the neurons are shown to have

elevated neuronal arborization and increased neurogenesis due to the influence of EE [A- 243, 248, 301, 319-324] as seen in our study.

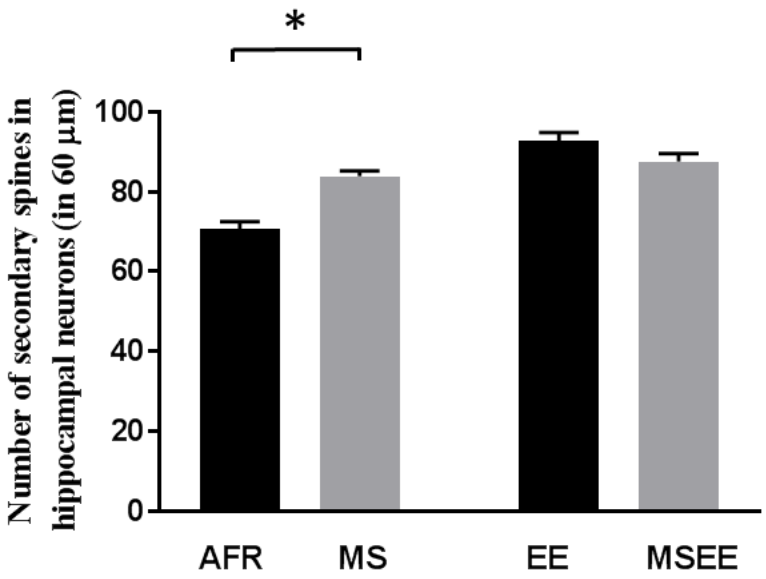


Figure R.36: Effect of early life stress and EE on secondary spines in hippocampal neurons: Stress significantly increased the number of spines found on secondary dendrites in the hippocampus in the absence of EE. Graphs represent Mean ± SEM. N (number of neurons) = 60 for AFR, 90 for Stress, 60 for EE and 69 for MSEE, 10 neurons/animal; *$p \leq 0.05$.

Stress did not alter dendritic length in mPFC neurons in the presence and absence of EE

Together with the BLA and hippocampus, the mPFC modulates fear responses [115, 140, 141]. Prelimbic (PrL) and Infralimbic (IL) regions are the two most important sub-regions that form mPFC [142]. The effect of MS on the neuronal morphology of the prelimbic (PrL) region of medial prefrontal cortex was focused in particular due to their strong projections to the BLA [292]. So, we will focus on the final region of the brain HPA axis, the mPFC to study the effect of early life environment on dendritic length, branch points and spine density of primary and secondary neurons of this region. The results along with the statistics are given below.

A two-way ANOVA was conducted for dendritic length of mPFC neurons with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,259)} = 0.439$, $p = 0.51$) and the interaction between stress and EE ($F_{(1,259)} = 0.715$, $p = 0.40$) on the total dendritic length of neurons in the mPFC did not reach statistical significance. However, EE ($F_{(1,259)} = 5.023$, $p = 0.03$) had a significant main effect on the increase in total dendritic length of neurons in the mPFC.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE $t_{133} = 1.255$, $p = 0.21$; Cohen's $d = 0.213$) the effect of stress on the total dendritic length of neurons in the mPFC did not reach significance. Similarly, in the presence of EE ($t_{126} = -0.113$, $p = 0.91$; Cohen's $d = 0.020$; Figure R.37) the effect of stress on the total dendritic length of neurons in the mPFC did not reach significance.

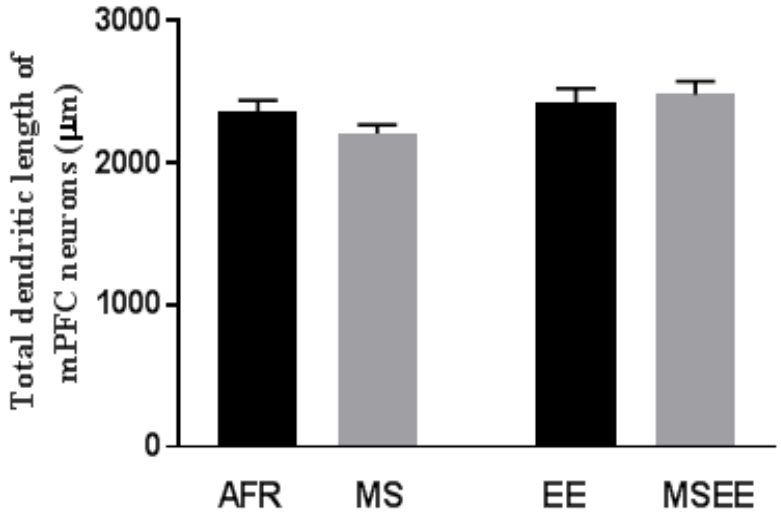


Figure R.37: Effect of early life stress and EE on the total dendritic length of mPFC neurons: Stress had no significant effect on the total dendritic length of neurons in the mPFC in presence and absence of EE. Graphs represent Mean ± SEM. N (number of neurons) = 59 for AFR, 76 for Stress, 64 for EE and 64 for MSEE, 8-10 neurons/animal

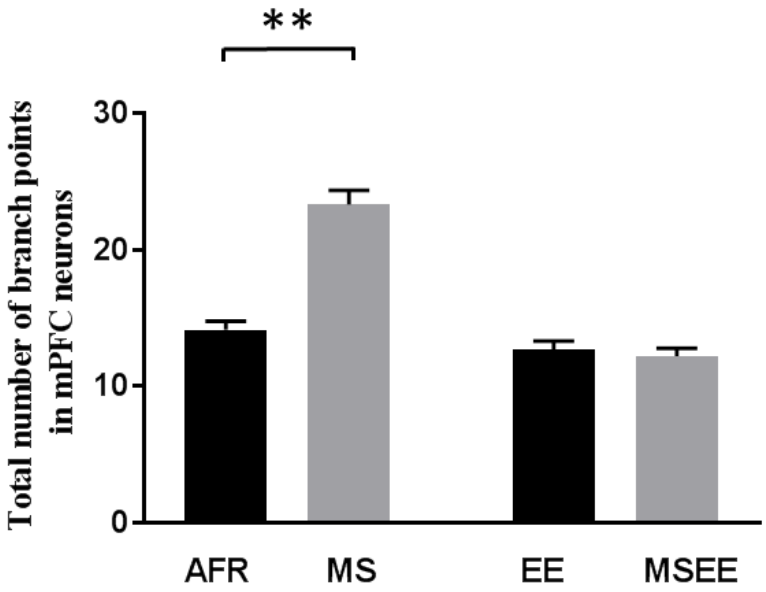
Stress increased the number of branch points of mPFC neurons in the absence of EE

A two-way ANOVA was conducted for number of branch points of mPFC neurons with stress and EE as between subject sources of variance. The effect of stress ($F_{(1,260)} = 31.185$, $p < 0.00$), EE ($F_{(1,260)} = 64.945$, $p < 0.00$) and the interaction

between stress and EE ($F_{(1,260)} = 38.427$, $p < 0.00$) had a significant effect on the total number of branch points of neurons in the mPFC.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE ($t_{135} = -7.215$, $p < 0.00$; Cohen's d = 1.283), stress significantly increased the total number of branch points of neurons in the mPFC. However, in the presence of EE ($t_{125} = -0.555$, $p < 0.58$; Cohen's d = 0.099; Figure R.38) the effect of stress on the total number of branch points of neurons in the mPFC did not reach statistical significance.

The PrL region suffers from dendritic length retraction and loss of spines post-chronic stress or exogenous CORT administration in adult rats. Similar to hippocampal neurons, these cortical neurons belonging to young adult rats also possess the capacity to recover the dendritic length and branching during post-stress recovery phase [293, 294]. In this study, MS rats showed increased number of branch points. This contrasting reversing effect of neuronal morphology has been reported before [293]. Additionally, my observation is in line with a previous study that shows an increase in neuronal spine density after MS [295].



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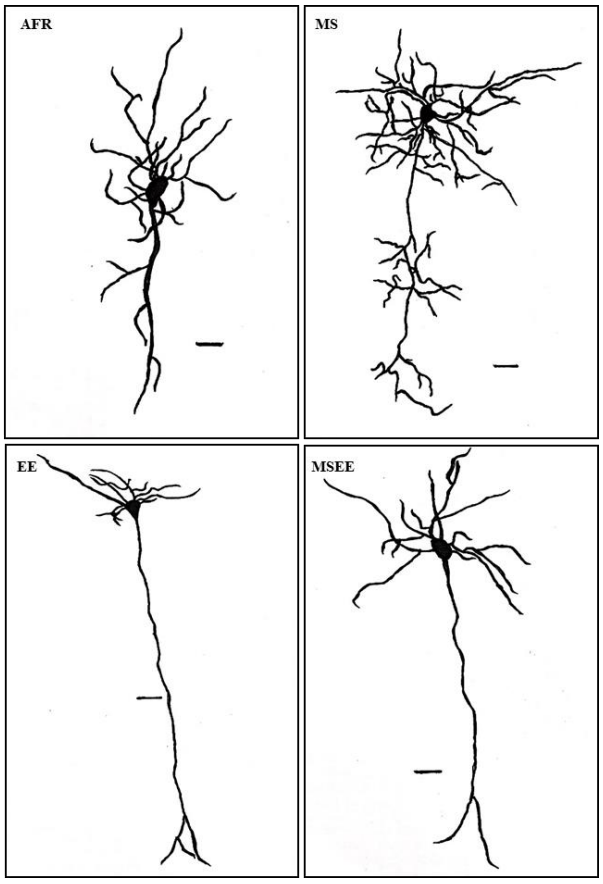

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Figure R.38: Effect of early life stress and EE on total number of branch points in mPFC neurons: Stress significantly increased the total number of branch points of neurons in the mPFC in the absence of EE. Graphs represent Mean ± SEM. N (number of neurons) = 60 for AFR, 77 for Stress, 63 for EE and 64 for MSEE, 8-10 neurons/animal; $^{**}p \leq 0.01$. Inset (bottom): Representative images of sample Golgi-stained neurons in the mPFC for the 4 groups from optical microscope at 400X magnification. Scale bar represents 20 μm.

Stress had differential effects on the number of spines of primary dendrites of mPFC neurons in presence and absence of EE

A two-way ANOVA was conducted for number of primary spines in mPFC neurons with stress and EE as between subject sources of variance. The effect of stress ($F_{(1,260)} = 0.003$, $p = 0.96$) on the number of primary spines in mPFC did not reach statistical significance. However, EE ($F_{(1,260)} = 4.122$, $p = 0.04$) and the interaction between stress and EE ($F_{(1,260)} = 98.948$, $p < 0.00$) had a significant effect on the number of spines found on primary dendrites in the mPFC.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE ($t_{136} = -7.461$, $p < 0.00$; Cohen's $d = 1.298$), stress significantly increased the number of spines found on primary dendrites in the mPFC. However, in the presence of EE ($t_{124} = 6.635$, $p < 0.00$; Cohen's $d = 1.179$; Figure R.39) stress significantly decreased the number of primary spines in mPFC neurons.

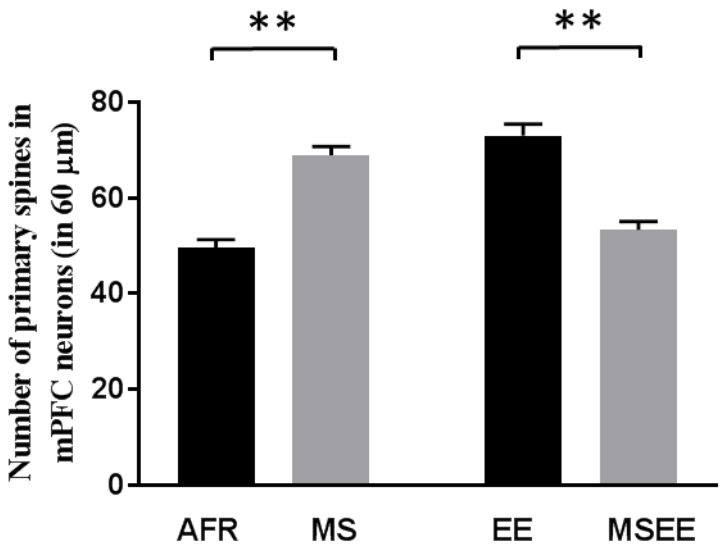


Figure R.39: Effect of early life stress on primary spines in mPFC neurons: Stress significantly increased the number of spines found on primary dendrites in the mPFC in the absence of EE and stress significantly decreased the number of spines found on primary dendrites in the mPFC in the presence of EE. Graphs represent Mean ± SEM. N (number of neurons) = 59 for AFR, 79 for Stress, 62 for EE and 64 for MSEE, 10 neurons/animal; $^{**}p \leq 0.01$.

Stress significantly increased the number of spines on secondary dendrites in mPFC neurons in the absence of EE

A two-way ANOVA was conducted for number of secondary spines in mPFC neurons with stress and EE as between subject sources of variance. The effect of stress ($F_{(1,260)} = 1.268$, $p = 0.26$) and EE ($F_{(1,260)} = 0.520$, $p = 0.47$) on the number of secondary spines in the mPFC did not reach statistical significance. However, the interaction between stress and EE ($F_{(1,260)} = 50.531$, $p < 0.00$) had a significant effect on the number of spines found on secondary dendrites in the mPFC.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE (t_{137} = -5.567, $p < 0.00$; Cohen's d = 0.973), stress significantly increased the number of spines found on secondary dendrites in the mPFC. However, in the presence of EE (t_{123} = 4.519, $p < 0.00$; Cohen's d = 0.813; Figure R.40) stress significantly decreased the number of secondary spines found in the mPFC.

MS rats showed elevated number of branch points and increased neuronal spine density. It is possible that the observed effects of MS are a result of compensation through reorganization of dendrites leading to hypertrophy. On the other hand, it is possible that the observed increase in dendritic arborization is a product of the extended period of recovery and additional compensation (approximately 36 days) given after MS treatment. This reversing effect of neuronal morphology is in consensus with previous reports [293]. Additionally, my observation is in line with a previous study that shows an increase in neuronal spine density after MS [295]. Prior literature shows that the enriched environment enhances the responsiveness of cortical neurons to stress [296, 297]. This is in line with my observation mentioned above where EE increases dendritic length, not branch points. I propose that decrease in branch points may also be a compensatory mechanism to temper the effect of dendritic length hypertrophy. The impact of EE on dendritic morphology is comparable to the pro-resilient features observed in previous work [255]. A decrease in number of branch points as well as primary and secondary neuronal spine density in the mPFC was observed in rats subjected to both MS and EE. This effect was not seen in rats exposed to only environmental enrichment.

Interestingly, there was an unique effect of enriched environment in neuronal morphology in all the three brain regions considered in this study. Further experiments are necessary to understand these interesting results that aid individuals to appropriately respond to their early life environment.

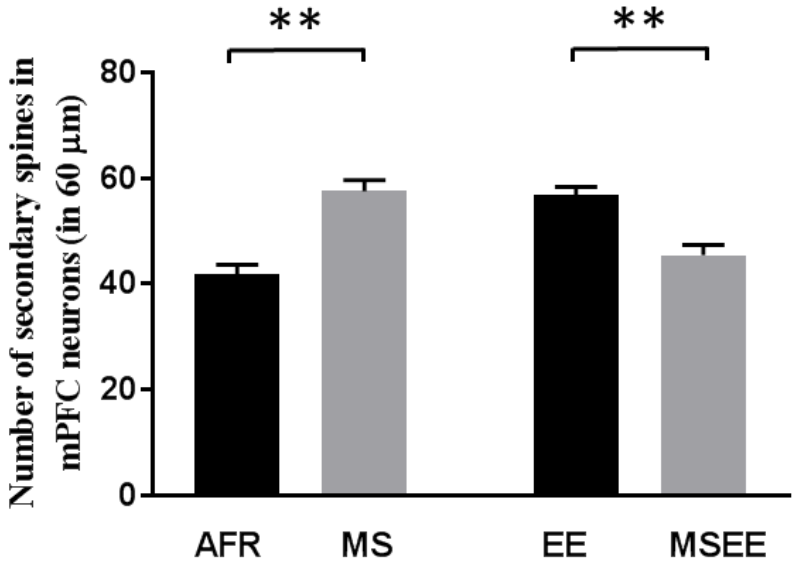


Figure R.40: Effect of early life stress and EE on secondary spines in mPFC neurons: Stress significantly increased the number of spines found on secondary dendrites in the mPFC in the absence of EE. Graphs represent Mean ± SEM. N (number of neurons) = 60 for AFR, 79 for Stress, 60 for EE and 65 for MSEE,10 neurons/animal; $^{**}p \leq 0.01$.

Chapter 5

5. Discussion

Early-life is a critical developmental period for the physical and emotional development of an individual (P0-P21 in rodents) as this is the time-window, which is vulnerable to the varying environment during which certain environment can bias the development of neural systems mediating emotion towards pathology later in life. [13]. Specifically, early life environment programs crucial aspects of neurobiological development including behavioral, emotional, cognitive and physiological development. Thus, exposure to stress during this period has important implications for stress sensitivity and behavioral response throughout the life of an individual [6, 7, 99]. In rats, one of the most well-established models of chronic early life stress is maternal separation [264]. Different interventions were used to counter the detrimental effects of maternal stress. Of all the techniques used to reduce the compromising effects of MS, enriched environment has been the most intriguing one as this is not an invasive method. A mere change in the environment for a short period of time can alleviate the harmful effects of MS significantly. Previous studies on the effect of enriched environment on maternal separation were done using long-term enrichment protocols [167, 298]. However, providing short-term enrichment in adulthood is known to reverse the detrimental effects of chronic immobilization stress [166] and maternal separation[87]. In my study, the focus was to investigate the effect of enriched environment provided in early life, concomitant to the period of maternal separation. The effects of early

environment on physiology, behavior, structural plasticity of neuronal dendrites and molecular changes were evaluated in brain during adulthood.

Three distinct themes emerge from my results. These are,

i) MS is stressful (pages 152 to 160).

ii) EE is beneficial (pages 161 to 166).

iii) MS and EE have interesting combinatorial effect (pages 167 to 173).

5.1 Effects of Maternal Separation

5.1.1 Impact of MS on physiology (S.A.1)

Early life stress affects various physiological parameters, particularly those about the hypothalamus-pituitary-adrenal (HPA) axis [167] and the sympathoadrenomedullary axis [299]. The adrenal glands are common to both these systems and are responsible for the production of corticosterone in response to stressful stimuli. Adverse conditions in early life could cause long-term behavioural deficits and epigenetic changes in glucocorticoid receptor in rat hippocampus [48]. Manipulation of the environment and the quality of care from the mother in this model causes chronic 'early life stress' which has long-term behavioral defects [76]. A large body of evidence shows that MS during postnatal weeks causes a permanent increase in anxiety related disorders [178]. Lifelong hyperactivity of HPA (hypothalamus-pituitary-adrenal) axis is another consequence of this postnatal separation [179]. My results showed that MS caused adrenal gland hypertrophy in male rats living in standard animal facility housing

when compared to AFR rats. Interestingly, early life stress in enriched housing reduced the increase the adrenal weights of animals (Figure R.3). Similarly, baseline plasma corticosterone levels were significantly increased in adult rats subjected to MS in early life under standard animal housing conditions. However, animals exposed to MS along with enriched environment did not show a significant difference in baseline plasma corticosterone levels (Figure R.5). Our results of MS alone causing enhanced corticosterone and adrenal gland hypertrophy is similar to what has previously been observed in rats. It has been reported that rats exposed to chronic stress with both baseline plasma corticosterone [257, 259] and adrenal weights [256, 258] increased compared to control animals.

5.1.2 Impact of MS on behavior (S.A.1)

Increase in CORT and adrenal hypertrophy indicates high anxiety in MS [99]. Therefore, we tested the impact of early life stress and EE on various behavioral readouts during adulthood. To determine the effect of MS in the absence and presence of enriched environment on anxiety-like behavior, animals were subjected to various anxiety tests in adulthood (P56). This included exposure of experimental animals to open field test (OFT), elevated plus maze (EPM) and home cage emergence test (HCE). Along with anxiety like behavior, depressive like behavior is another common behavioral defect observed in MS rats [300, 301]. To determine the impact of early life stress and EE on depressive-like behavior, the animals were subjected to the forced swim test (FST).

My results showed that early life maternal separation induced anxiogenic avoidance of inner zone in open field test. This represented the high anxiety in animals exposed to MS alone in standard housing during early life. However, this effect was not seen in animals exposed to MS in the enriched environment (Figure R.9). This result is in line with previous reports that show that MS leads to anxiogenic behavior in adulthood [263, 264]. MS had no significant effect on latency to escape in the home cage emergence test irrespective of EE (Figure R.8). Similarly, MS did not alter the percentage of open arm time/entries and risk assessment behavior (measured using the number of head dips) as tested on EPM (Figure R.10, R.11, and R.12). The number of closed arm entries was same across all groups showing no influence of locomotor function (an internal control of EPM) (Figure R.13), on anxiety.

Immobility and swimming were measured at two time points, T1 and T2. T1 was the first day where the animals were habituated to the test and T2 was the time point for the actual test day. In FST, the initial exposure to the swim tank on day 1 (T1) is known to induce a state of "behavioral despair," such that these rats have *altered emotional reactivity* in response to a new emergency represented by swimming. So, day 2 (T2) is considered as the test day where the depression-like behavior can be well captured, after T1 habituation [274]. In this study, we quantified T1 behavioral response to capture the initial response to novelty stress and T2 behavioral response to capture the stabilized emotional response to forced swim test. I observed that MS significantly decreased immobility in T1 (FST) when compared to controls. This effect was not observed in T2 (FST). The T1

immobility result observed in my study has already been reported before [275, 276]. Additionally, chronically administered corticosterone reduced immobility time in the FST [270]. However, this is in contrast to previous studies that showed a stress-induced increase in immobility [277, 278]. This shows that reduced immobility is not always associated with a compromised behaviour due to chronic stress. MS in this study did not alter T1 swimming time in FST (Figure R.15). In T2 experiments, MS significantly decreased swimming time in the FST (Figure R.16). MS induced depression-like behaviour was associated with decreased swimming behaviour in the FST [271-273]. Thus, MS induced depression-like behaviour in the FST, is in agreement with existing literature.

5.1.3 Impact of MS on dendritic morphology (S.A.2)

The BLA is a critical brain region for processing fear and anxiety [60, 61]. This in turn drives the HPA axis to release stress hormones [59, 65, 132, 133] Fear and emotion related memories are known to be consolidated in this region which in turn helps in long-term adaptability [134]. The amygdala is activated by corticosterone released during a stress response and this results in dendritic hypertrophy as well as elevated spine density [65]. These morphological changes have been correlated with anxiety and stress in prior studies [52, 53, 57, 135]. These changes in neuronal morphology are known to be associated with anxiety-like behavior and general stress response [52, 54, 57]. Acute and/ or chronic stress, as well as exogenous supplementation of corticosterone results in neuronal hypertrophy and an increase in spine density of BLA principal cells [136]. Rats subjected to chronic immobilization stress in early life and adulthood increases

neuronal spine density in the amygdala [7, 17, 21]. Similarly, in my paradigm, a significant increase in neuronal spine density was observed in rats subjected maternal separation compared to control rats in the normal housing (Figure R.31 and R.32). Although the spine density was increased, an increase in dendritic length and branch points was not observed in normal housing rats that were subjected to maternal separation (Figure R.29 and R.30).

In addition to BLA, hippocampus was examined due to its involvement in widely reported stress and cognitive processes [119, 120] It is known to be one of the most plastic regions of the brain and the structural plasticity is highly dependent on the number and impact of stressful episodes experienced by an individual (level of glucocorticoids in the systemic circulation) [122, 123]. Additionally, stress induced impairment in learning and performance in short-term memory tasks is closely associated with atrophy of CA3 dendrites in the hippocampus [124, 125]. Under normal housing conditions, maternal stress reduces the complexity of hippocampal neurons. Specifically, dendritic length and cell proliferation are known to decrease in MS rats compared to control [126, 127]. While studies report atrophy of CA3 neurons in the hippocampus, Sunanda *et al.,* (1995) observed increased spine density in rats subjected to chronic stress in adult life and has argued that this observed increase in spine density may be a mechanism to counter neuronal atrophy by increasing the postsynaptic membrane area [289]. This observation was corroborated in our paradigm, where rats subjected to MS showed an increase in spine density of both primary and secondary branches of the neuron in BLA (Figure R.35 and R.36). Moreover, in our paradigm, MS did not alter

neuronal length or number of branch points in normally housed rats (Figure R.33 and R.34). This is congruent with the observation that although atrophy of hippocampal neurons can be observed up until four days post-MS treatment, atrophy is no longer present after ten days post stress treatment because of the plastic recovering nature of hippocampal neurons [290, 291].

mPFC is one of the many GC-sensitive brain regions that regulate stress response by the activity of glucocorticoids. Two types of corticosteroid receptors in the brain are, glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). The MR binds GCs with a 10-fold higher affinity than the GR [302]. The MR activation is considered as neuroprotective to memory performance. In contrast, GR activation is known to damage neurons and impair memory performance especially when prolonged and elevated GR occupancy is observed. These observations suggest that GR/MR ratio is an important parameter in to understand CORT effects in different brain regions [303]. mPFC has a high ratio of GR/MR ratio that indicates the high involvement of stress hormones [142]. The mPFC is reported to play a role in processing cognitive and goal-oriented behavior [304]. Importantly, the mPFC indirectly provides a negative feedback to the HPA axis [138-140]. Together with the BLA and hippocampus, the mPFC modulates fear responses [117, 139, 140]. Prelimbic (PrL) and Infralimbic (IL) regions are the two most important sub-regions that form mPFC. PrL is shown to play a role in fear-conditioning and learning while fear extinction is associated with IL [141]. In my study, the effect of MS on the neuronal morphology of the prelimbic (PrL) medial prefrontal cortex was studied due to their strong projections to the BLA [292]. MS

rats showed increased number of branch points and increased neuronal spine density (Figure R.38, R.39, and R.40). It is possible that the observed effects of MS are a result of compensation through reorganization of dendrites leading to hypertrophy. On the other hand, it is possible that the observed increase in dendritic arborization is a product of the extended period of recovery and additional compensation (approximately 36 days) given after MS treatment. This reversing effect of neuronal morphology is in consensus with previous reports [293]. Additionally, my observation is in line with a previous study that shows an increase in neuronal spine density after MS [295].

5.1.4 Impact of MS at the molecular level (S.A.3/4)

As discussed in earlier paragraphs, the maladaptive behavioral output of early life stress was demonstrated in MS. MS led to adrenal hypertrophy, elevated corticosterone levels, anxiety-like behavior and high spine density in BLA. GC activated glucocorticoid receptor (GR) was analyzed because of its central role in maintaining the regulation of HPA axis in response to various stressors [142, 218, 219]. The density of GR population across amygdala, hippocampus, and mPFC differ and is shown to have a link in providing feedback regulation to HPA axis [219]. The recruitment of activated GR in the nucleus as a transcription factor is a crucial step that initiates a relay of downstream molecular pathways to mediate stress response [112, 220]. Prior studies show that activation of GR through GC causes phosphorylation of $Erk1/2^{MAPK}$. This activated $Erk1/2^{MAPK}$ causes relay of downstream molecular pathways that are crucial in mediating behavioral effects of GC [221, 222]. Another important molecule that interacts with GC-GR and

Erk1/2^{MAPK} is BDNF that activates molecular cascade to enhance fear related memories [286]. All these molecular studies have been widely examined in the hippocampus [221] concerning stress. On the other hand, the basolateral amygdala (BLA) remains understudied in spite of BLA's crucial role in mediating stress response and emotional behavior. It is vital to study the regulation of these molecules. The protein expression level of the glucocorticoid receptor (GR), brain-derived neurotrophic factor (BDNF), and phosphorylated mitogen-activated protein kinase (pMAPK) was determined in the BLA in different group of animals to determine the impact of early life MS in presence and absence of EE.

There was no observed effect of maternal separation on total GR protein expression either using western blot or immunohistochemistry (Figure R.20 and R.24). Conversely, in normal animal housing conditions, the percentage of cells containing high-density GR in the basolateral amygdala was significantly greater in MS rats compared to controls. This effect of MS was not observed in rats assigned to the enriched environment (Figure R.23). High-density GR refers to 'activated' GR, which translocate to the nucleus to activate downstream transcription. Thus, although MS did not affect total GR, it significantly decreased the amount of activated GR found in the nucleus. This indicated that GR alone might be the primary driver of BLA function in MS. Interestingly, protein expression of BDNF (Figure R.25) and pMAPK (Figure R.26) in the basolateral amygdala were not significantly altered by exposure to early life maternal stress. Based on these results, it is clear that the effect of GR in the BLA plays a crucial role in driving stress-induced BLA structural and molecular plasticity in different

housing environments. To investigate the proximate mechanism involved in driving changes of protein expressions, the possible role of epigenetic regulation that might be associated with GR and BDNF expression was determined in BLA. Epigenetic changes in GR promoter gene are well examined in the hippocampus for MS models. In particular, methylation studies are widely explored in these models [251]. The average percentage of cytosine methylation of few critical CpG sites of GR and BDNF was assessed. Interestingly, the methylation status of the CpG sites used in this study did not alter due to stress in the presence and absence of EE. This observation shows that these specific CpG sites are not the major driving force behind the long-term effect on variation in protein expression of GR and BDNF in BLA. This opens up more avenues to explore other epigenetic modifications like histone acetylation, histone methylation, and other modifications. An interesting pathway of molecular activation in response to stress is suppression of GABAergic neuronal activity and elevated hyper-excitability of a smaller subset of BLA neurons leading to elevated memory consolidation [305-307]. GABAergic interneurons constitute 20-30% of the neuronal cell types in the BLA and stress affects these interneurons to induce compromising physiological readout [308-310]. Inhibition of the activity of these interneurons caused by hyperexcitability of the BLA neurons leads to anxiety like behavior and MDD [311-313]. Glutamate decarboxylase (GAD), an enzyme that causes synthesis of GABA from L-glutamic acid, is expressed in the GABAergic interneurons in the BLA [314, 315]. It is a pre synaptic protein marker that causes inhibitory function in GABAergic interneurons to relay information within the BLA. Prior work shows that decrease in GAD67

protein expression in rats is linked to enhanced depressive-like behavior and can be initiated by repeated corticosterone administration in the amygdala [316]. Hence, GAD67 in the inhibitory neurons of the BLA can be regulated by stress, which can lead to aberrant emotional behavior.

Until now, the impact of MS on physiology, behavior, neuronal morphology and the molecular players of the stress response were discussed. The mal-adaptive outcomes induced by the stress paradigm were also indicated. In the next section, the main effect of enriched environment on all the experimental endpoints will be discussed.

5.2 Effects of Enriched Environment (EE)

Exposure to short-term environmental enrichment in adulthood is known to enhance defensive behavior and attractiveness in male rats [206]. Additionally, exposure to short-term environmental enrichment in adulthood is also known to alleviate behavioral compromise induced by MS [87]. Mice exposed to the enriched environment had enhanced memory function in various learning tasks [204]. Research on benefits of EE is being applied in zoos and farms where the animals are seen to thrive better in a more enriched environment [212]. Numerous studies on EE similar to those mentioned above, inspired me to apply this treatment for this study, to investate the impact of early life EE (P0-P21). My paradigm is the first study to investigate the impact of short-term exposure to the enriched environment in early life. EE effects were considered as the main effect (i.e. presence & absence of EE on stress) of treatment across all experimental groups.

5.2.1 Impact of EE on physiology level (S.A.1)

The presence of EE significantly decreased the body weight-area under the curve (AUC) (Figure R.2) of experimental animals. The area under curve depicts the overall change in body weight across the course of development (early life till adulthood). Conversely, maternal separation did not affect the body weight AUC of test animals assigned to the standard cages. Chronic stress is known to lead to an increase in body weight [253]. In our paradigm, enriched environment housing reduced body weight gain (throughout the experiment) instead. Interestingly, rats

exposed to prenatal stress and then housed in environmental enrichment conditions showed a similar decrease in body weight [254]. The decrease in body weight gain could be due to the continuous activity exploring various objects and interacting with fellow cage mates.

Adrenal glands are vital organs that produce corticosterone in response to stressful stimuli and undergo hypertrophy in the process [256, 257]. A two-way ANOVA revealed a significant main effect of enriched environment on adrenal gland weight. Specifically, animals assigned to the enriched environment housing condition had significantly lower adrenal weights compared to animals assigned to animal facility rearing conditions, irrespective of presence or absence of maternal separation (Figure R.3). Similarly, a two-way ANOVA showed a main effect for enriched environment on plasma corticosterone levels at the point of sacrifice during adulthood (Figure R.7). This is in good agreement with another study by Roy and colleagues (2001) who showed that male mice assigned to the environmental enrichment treatment had significantly less plasma corticosterone compared to animals in standard animal house housing after exposure to stress [260]. This emphasized on the active coping output (low CORT) induced by EE.

In other words, animals in the enriched environment had significantly lower plasma corticosterone at the end of the experiment, compared to animals in the normal animal housing conditions, irrespective of absence or presence of early-life maternal stress treatment. In rats assigned to standard cages, maternal stress significantly increased adrenal weights. This effect of maternal stress was not observed in rats that were placed in the enriched environment (MSEE). This

observation is indicative of the modulatory effect of environmental enrichment on the adrenal glands and corticosterone, the crucial arms of HPA axis. On the whole, the study highlighted that EE modulated the HPA axis to regulate the baseline corticosterone levels in the animals to actively cope with stress. Another interesting point is the combinatorial effect of MS and EE wherein the interaction between the two treatments alleviates the harmful effects of MS on certain physiological parameters (as mentioned above).

5.2.2 Impact of EE on behavior (S.A.1)

In general, animals assigned to the enriched environment displayed a more anxiolytic behavior compared to animals in the normal animal house treatment. A two-way ANOVA revealed significant main effects of enriched environment on time spent exploring the inner zone in the open field test (Figure R.9). This observation is congruent with previous studies that reported significantly increased anxiolytic behavior in animals placed in enriched environment housing conditions[317]

5.2.3 Impact of EE on dendritic morphology (S.A.2)

Stress-induced growth of dendrites and synapses in BLA neurons can be rescued with environmental enrichment in adult male rats [166]. So far, only two studies have determined that enriched environment can rescue the damaging effects of early life stress. One reported that environmental enrichment during the peri-pubertal period rescues the harmful effects of maternal separation on HPA and behavioural response to stressors [167]. Another study reported that, short term

enrichment reverses the anxiety and hypertrophy of BLA neurons in adulthood [87]. As mentioned earlier, there is limited information about how enriched environment during early life (P0-P21) can influence dendritic morphological changes. Interestingly, short-term environmental enrichment had main effect on secondary neuronal spine density of BLA and hippocampal neurons (Figure R.32). Environmental enrichment, irrespective of presence or absence of maternal separation treatment resulted in a general decrease in secondary spine density in BLA neurons. Conversely, enriched environment significantly increased secondary neuronal spine density in the hippocampus. This observed increase in the neuronal spine density is in direct agreement with reports on increased spine density as a result of environmental enrichment, found in the literature [318-322]. This is result is opposite to the stress-induced hypertrophy seen in BLA [87].

In my paradigm, an increase in dendritic length but a decrease in branch points of mPFC neurons was observed. Previous literature showed that the enriched environment enhanced the responsiveness of cortical neurons to stress [296, 297]. Similarly, this study revealed that environmental enrichment increases dendritic length but not the branch points. I propose that decrease in branch points may be a compensatory mechanism to dampen the effect of dendritic length hypertrophy. Hypertrophy of mPFC neurons was observed in animals exposed to long-term enriched environment, which displayed active coping behavior [255]. This observation is comparable to the impact of EE on dendritic morphology in this study, which reinstates the role of mPFC in regulating HPA in response to varying environment during early life (P2-P21).

5.2.4 Impact of EE at molecular level (S.A.3/4)

The density of GR concentration across brain regions such as hippocampus, amygdala, and mPFC varies and is linked with its ability to provide feedback regulation [142]. GR is an important regulator stress response [167]. The activation of GR (by corticosterone occupancy) and its localization into the nucleus for its recruitment as transcription factor constitutes one of the key molecular pathways mediating stress response in relevant brain regions [112, 220]. GR receptors in the amygdala induced by stereotactic administration of corticosterone lead to hyperactivation of HPA and anxiety [323]. These compromising behavioral features are reversed by experimentally blocking corticosterone binding to GR in the BLA [324]. These studies indicate the association of enhanced GR activation with potentiation of anxiety and BLA hypertrophy. Hence, in the basolateral amygdala, the effect of environmental enrichment on GR expression is critical. In this study, there was a significant main effect of environmental enrichment on GR mRNA abundance in the basolateral amygdala (Figure R.17). This is congruent to my observation that environmental enrichment increased the percentage of number of cells containing activated GR when compared to animals in normal housing conditions (Figure R.23). As mentioned earlier, the basolateral amygdala is one of the brain regions that provide feedback for regulating corticosterone secretion via HPA axis. Activated GR and its subsequent translocation into the nucleus is a major step in mediating stress as this step initiates the feedback loop in response to stress episodes [112, 220]. Previously, the effect of enriched environment housing on GR expression has been studied mainly in the hippocampus wherein the GR

expression is observed to be upregulated in the presence of environmental enrichment [325, 326]. GR expression in rat pups is reported to vary depending on the mother's care they receive in the form of licking and grooming in hippocampus. In pups that experience high licking and grooming, GR expression is upregulated compared to pups that experience low licking and grooming and it highlighted that increase in GR is beneficial for HPA-GC regulation [181]. This study showed that environmental enrichment significantly increased activation of nuclear GR in the BLA, which plays a significant role in downstream protein regulation, which in turn drives neural plasticity as well as BLA feedback to the HPA axis and by extension, corticosterone secretion.

Brain-derived neurotrophic factor (BDNF) is an important growth factor for regulating survival in a variety of cells. It also plays important role in modulating dendritic growth in various regions of the brain. BDNF mediates intracellular pathways that modulate the synaptic plasticity of neurons [327-329]. Expression of pro-BDNF, the precursor product of mature BDNF, was significantly decreased in rats placed in environmental enrichment (Figure R.21). Previous work has associated increased expression of pro-BDNF in the hippocampus to depression in humans. This study used formalin-fixed paraffin-embedded hippocampal sections from subjects with schizophrenia, major depressive disorder (MDD), bipolar disorder (BPD) and non-psychiatric controls from the Stanley Foundation Neuropathology Consortium to analyze the expression of Pro-BDNF [288]. In contrast, the reduced pro-BDNF levels in my study could have anti-depressive like effect driven by BLA. However, environmental enrichment was not observed to

affect the expression of BDNF protein in the BLA (Figure R.22 and R.25). Prior reports have shown that BDNF levels are elevated in response to enriched environment conditions. Only one report demonstrated that short-term enriched environment during stress rescues the stress-induced elevated BDNF mRNA expression in BLA [166]. This highlights the differential role of BDNF regulation in response to EE during early life and adulthood (S.A.3).

My data supports the notion that MS is indeed a stressful paradigm and short-term EE in early life induces several resilient features in physiological, behavioral, dendritic morphological and molecular parameters. In the next section, the interaction effect between MS and EE on all the experimental endpoints will be discussed.

5.3 Interaction between Maternal Separation and Environmental Enrichment

The previous sections have concentrated on understanding the individual effects of stress and environmental enrichment on various endpoints of physiology, behavior and brain plasticity. In this section, the effect of concurrent effect of environmental enrichment and early life maternal stress on different experimental endpoints will be discussed. Previous studies have shown that when stress and EE are combined, they recue the maladaptive behaviors observed in stress rats [87, 166]. This gives crucial information about the combinatorial impact of two different environments that act in a synergistic way to bring about an active-coping behavior in animals. Hence, MSEE paradigm was used in this study to explore the chances of inducing resilient (active-coping) characteristics in animals exposed to concurrent MS and EE.

5.3.1 Impact of both MS and EE on physiology (S.A.1)

A two-way ANOVA revealed that there was a significant effect of the interaction of stress and environmental enrichment on body weight. Rats subjected to both early life MS and enriched environment had significantly decreased body weights in adulthood (Figure R.2). Previously, early-life maternal stress in normal animal house conditions led to a significant increase in body weight compared to animals that did not undergo MS [253]. This suggests that presence of environmental enrichment along with MS in my study resisted the increase in body weight as observed in rats subjected to only MS. This could be due to the increase in locomotor activity (climbing, exploring, etc) involved in EE cages [255]. An

interesting point to note here is that even a short-term exposure to EE in early life can induce a long-term impact on other physiological parameters like adrenal weight and corticosterone secretion of an individual.

MS without EE housing displayed adrenal hypertrophy and a two-way ANOVA revealed a significant interaction effect of stress and environmental enrichment on adrenal weights with no hypertrophy (Figure R.3). Previous reports showed that early life stress and chronic stress resulted in adrenal hypertrophy [256, 258]. However, there are no current studies specifically linking MS to adrenal hypertrophy. The results in this study showed that in an enriched environment, MS resulted in significant hypotrophy of adrenal glands along with MS main effect.

Environmental enrichment significantly decreased plasma corticosterone levels post-exposure to predator odor (Figure R.6). Interestingly, in environmental enrichment conditions, rats subjected to MS had significantly lower plasma corticosterone concentration compared to rats subjected only to environmental enrichment, when exposed to predator odor. This suggests that in addition to the observed general decrease in plasma corticosterone levels in EE animals, exposure to early life maternal stress served to decrease plasma corticosterone in EE animals further. Thus, the concurrent presence of both stress and environmental enrichment appears to have a potent inhibitory effect on the HPA axis [167]. Parallel maternal stress and environmental enrichment decreased plasma corticosterone and lead to adrenal hypotrophy suggesting that this dual reduces the adrenal glands, the two important elements (GC and adrenal galnds) of the HPA axis.

5.3.2 Impact of both MS and EE on behavior (S.A.1)

Environmental enrichment is reported to have a significant anxiolytic effect [166, 167]. This is corroborated by our observation that rats in environmental enrichment spent significantly more time exploring the inner zone of the OFT compared to rats in normal housing conditions. A two-way ANOVA of OFT data further revealed that concurrent exposure to MS and environmental enrichment resulted in increased anxiolytic behavior (figure R.9). In other words, rats subjected to both MS and environment enrichment spent significantly more time exploring the inner zone of the OFT. This increase in anxiolytic behavior was also observed in EPM. Rats subjected to both maternal separation and environmental enrichment spent more time exploring the open arm of the EPM compared to rats exposed only to environmental enrichment (figure R.10 and R.11). Thus, it is observed that the presence of both maternal separation and environmental enrichment has a cumulative effect on the increase in anxiolytic behavior (active coping). This is corroborated by reports that show both short-term [87] and long-term [167] enrichment in adulthood after early life maternal separation increases anxiolytic behavior.

5.3.3 Impact of both MS and EE on morphology (S.A.2)

In previous sections, it was reported that the concurrent exposure to both maternal separation and environmental enrichment resulted in robust benefit both on physiology and behavior. Surprisingly, analysis of dendritic morphology in the

BLA showed results similar (increased dendritic spines) to what has been reported in the prior literature to occur in rats subjected to maternal separation in the absence of environmental enrichment. Rats exposed to both maternal separation and environmental enrichment showed dendritic length hypertrophy, increase in branch points and increase in primary neuronal spine density in the BLA compared to rats exposed to only environmental enrichment (figure R.29, R.30, and R.31). The increase in BLA dendritic arborization was not expected in this study. As mentioned earlier, increase in dendritic length and branch points are associated with anxiety-like behavior but this was not the case in this study. In other words, in response to early life stress, individuals employ adaptation strategies that would allow them to make "the best out of a bad situation" so to speak [78, 79]. This may be responsible for the increase in dendritic arborization observed in this study. This could also be due to overlap of timeline of this study and amygdala development.

However, extant literature argues that certain negative experiences in the critical developmental phase may improve memory processes and learning under stressful situation [77]. Thus, it is important to note that, stressful experiences are not always associated with maladaptive behavior as shown in previous reports. It is possible that early life experiences could have varying impact on BLA morphology wherein hypertrophy does not always mean impaired response to stress. Hence, hypertrophy in this study may not have highlighted maladaptive output.

Interestingly, secondary neuronal spine density decreased in rats exposed to both maternal separation and environmental enrichment compared to those subjected to only environmental separation (figure R.32). This may be due to the differential

influence of synaptic activity at the distal end of neurons as well as overall compensation for dendritic length for synaptic activity. The formation of dendritic arborization and spine density of the BLA neurons in this study could be characterized by the electrical activity of long cables of neurons harboring decreased synaptic contacts. This could be a compensatory mechanism of the distal neurons to lower the excitability of the neurons under the influence of combined effect of MS and EE. This cable theory of neuronal activity for hypo-responsiveness of BLA neuron was observed in a study which had similar combined effect of stress and EE in adulthood [166]. There results emphasized that; the dendritic arborization in BLA can vary according to different environment provided in different age of the individual (S.A.2). These results shed some light on the limited knowledge in the existing literature about BLA activity and early life stress and EE.

As seen earlier, the dendritic arborization in hippocampus was not affected in this study but spine density showed significant changes in response to MSEE. The hippocampus of rats exposed to both maternal separation and environmental enrichment showed a decrease in both primary and secondary neuronal spine density compared to rats exposed only to the enriched environment (figure R.35 and R.36). Beneficial effects of EE are widely studied on hippocampal neurons and its plasticity. The hippocampal neurons are shown to have elevated neuronal arborization and increased neurogenesis due to the influence of positive effects of EE [A- 243, 248, 301, 319-324]. So, in this study EE had an adaptive main effect

similar to earlier studies but this impact of EE on hippocampal neurons disappeared when MS and EE treatments were combined.

A decrease in number of branch points as well as primary and secondary neuronal spine density was observed in the mPFC neurons (figure R.38, R.39, and R.40) of rats subjected to both maternal separation and environmental enrichment. This effect was not seen in rats exposed to only environmental enrichment. Multiple studies have shown the impact of EE in increasing cortical neuronal responsiveness to stress [296, 297, 330, 331] Interestingly, there was a unique effect of enriched environment in neuronal morphology in all three brain regions considered in this study in spite of having a positive influence of enriched environment on physiological and behavioral outputs. As previously mentioned this may simply be a result of the attempt to "make the best of a bad situation" particularly because of the crucial period in which stress was given [80, 83]. This reinstates the point that early life experience influences the dendritic arborization in a different way when compared to adulthood experiences. The maladaptive readouts obtained in response to stress in adulthood might not be the same in early life (P2 –P21).

5.3.4 Impact of both MS and EE at molecular level (S.A.3/4)

Brain-derived neurotrophic factor (BDNF) is an important growth factor for regulating survival, and dendritic growth in a variety of cells and its pathway modulates synaptic plasticity [327-329] as discussed in earlier section. A two-way ANOVA showed that concurrent exposure to both short-term environmental

enrichment and early life maternal stress resulted in marginal (p=0.06) lower BDNF mRNA expression in the BLA (Figure R.18). This result is further corroborated by another result wherein the expression of BDNF protein in the BLA was significantly decreased when both early life maternal stress and environmental enrichment were provided together (Figure R.22). This is an important interaction effect that is not observed when rats are subjected to only maternal separation or EE. A prior study showed that chronic immobilization stress (CIS) that caused anxiety like behavior in adulthood increases mRNA abundance of BDNF in BLA. However, the presence of both CIS and EE rescues the stress induced increase in BDNF mRNA levels [166] which revealed that lower levels of BDNF expression is an adaptive readout associated to anxiolytic behavior. Similarly, MSEE group in this study displayed lower levels of BDNF expression that highlighted the adaptive behavior shown earlier [166]. As shown earlier, MS did not affect total GR but it significantly decreased the amount of 'activated GR' found in the nucleus. This indicated that 'activated GR' alone might be the primary driver of BLA function in MS. Interestingly, when MS was combined with EE, the significant decrease in 'activated GR' disappeared. This result supported the idea that combinatorial effect of MS and EE renormalized the decrease in activated GR in BLA. Therefore, no significant change in total GR is valuable information, which highlighted that the activity of GR is modulated by translocation to nucleus and not by changing the degree of expression. The protein levels of pMAPK did not vary under the influence of all the treatments. This indicated that pMAPK does not play a direct role in modulating the physiology and

behavioral read out in response to stress and EE. Overall data suggested that the combinatorial effect helped in ameliorating various underlying neurobiological substrates of neural plasticity and behavior (S.A.3). The results in this section also contributed to the limited knowledge present in the existing literature, as there are very few studies of combinatorial effects of stress and EE paradigm.

5.4 Conclusion

Mother-infant relationship is one of the crucial (both physical and psychological) processes in brain development during early life [34, 35] when compared to other components of environment. A positive mother-infant relationship (postnatal environment) is beneficial for development of the affective behaviour of the infant [44, 45]. Harry Harlow showed that mother-infant relationship was more psychological than biological. The study suggested that mothers were important not just for supplementing food but also crucial for touch-sensitive attachment with the infant that helped the long-term emotional and mental stability of the child [159]. Disruption in the mother-infant relationship can cause stress in offsprings. Maternal separation is known to be one of the most potent forms of early life stress. Maternal stress causes detrimental effects on the neurobiological, behavioral, emotional and physiological development as mentioned earlier. In our model, maternal stress under AFR conditions showed compromised physiological effects including increase in adrenal and body weight as well as elevated baseline plasma corticosterone levels when tested in adulthood. However, provision of an enriched environment (EE) reduced the above parameters. Similarly, the interaction of maternal separation and enriched environment (MSEE) showed similar and stronger active coping outcomes compared to only EE conditions in certain experiments as discussed in detail in previous section.

Maternal separation induced anxiogenic behavioral outcome. However, EE and MSEE conditions showed anxiolytic behavior. Moreover, MSEE induced pro-social behavior, which was not observed in animals exposed only to EE conditions.

Thus, from physiology and behavior readouts, MSEE induced active coping response that reflects an adaptive outcome.

In general, MS increased spine density in primary and secondary dendrites in three important brain regions responsible for regulating stress response namely; BLA, Hippocampus, and mPFC. EE treatment induced a decrease in secondary spine density in BLA but increased secondary spine density in hippocampus and dendritic length in the mPFC, which was in consensus with what has been reported in the extant literature [255, 296, 319, 322]. Interestingly, the neuronal morphology of rats subjected to MSEE was congruent to what was observed in MS rats. The unique results highlighted in this study emphasized that, in response to early life stress, individuals employ adaptation strategies that would allow them to make "the best out of a bad situation" as discussed in previous section.

In maternally separated rats, the total mRNA abundance and protein expression levels of GR did not vary. Conversely, the percentage of high density activated GR cells decreased upon exposure to maternal stress but increased in enriched housing conditions. This had important implications for HPA regulation [47]. Another important molecular player in stress response was BDNF and it was observed to decrease in MSEE although both EE and MS alone were not observed to affect the expression of BDNF mRNA and protein. These two molecules have been very well studied in the hippocampus concerning stress response, but, to the best of my knowledge, this work was the first study to report the expression of GR and BDNF in the BLA of maternally separated rats. Prior work by Meaney et al., had reported that MS decreased the expression of GR in hippocampus and GR feedback

sensitivity to the HPA axis [332, 333]. Elevated levels of GR in hippocampus through manipulations have shown to eliminate the compromising effects on HPA function [332, 333]. In the hippocampus, the GR-GC complex acted as a transcription factor that regulated basal BDNF production in response to stress episodes. Thus, specific balance of both GC and BDNF expression is necessary during neurodevelopment of hippocampus in maintaining homeostasis [327]. Another molecular player that is influenced by GR-GR interaction is Erk1/2^{MAPK}. Studies have also shown that activation of GR through GC caused phosphorylation of Erk1/2^{MAPK}. This activated Erk1/2^{MAPK} caused relay of downstream molecular pathways that are crucial in mediating behavioral effects of GC [286, 334]. BDNF is another molecular player that also interacted with GC-GR complex and Erk1/2^{MAPK} mediated molecular cascade to enhance fear related memories [221, 222]. These pathways could be playing a significant role in BLA to induce active coping (resilient) behavior in my model. The pattern of significant lower activated GR levels in BLA of the MS animals in my study was similar to reduced GR levels seen in hippocampal neurons of MS rats observed in other studies [88]. The elevated levels of GR in my EE paradigm could be the major role player in regulating the transcription level of BDNF and in turn causing the lowering of BDNF in the EE animals. The interaction of all above-mentioned molecules is summarized in the schematic representation (Figure D.1).

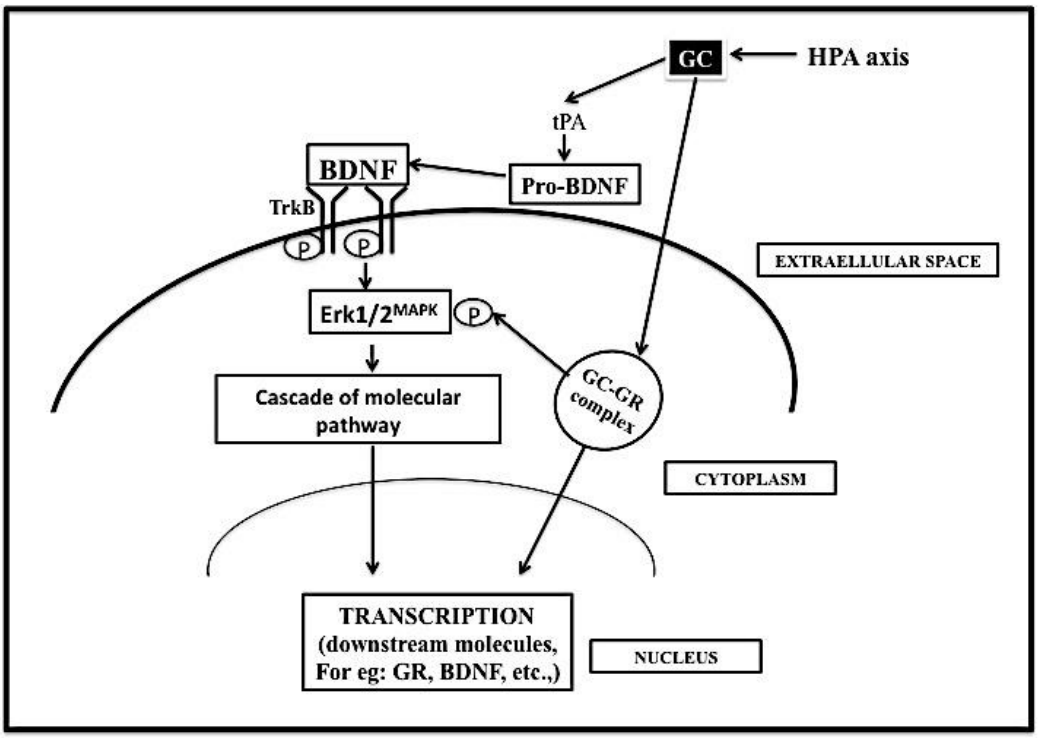


Figure D.1: Putative schematic representation of intracellular molecular interactions: The interaction of numerous molecules discussed above in this section [286, 334] is summarized in this schematic representation. The figure is adapted from the various studies mentioned in the text. GC: Glucocorticoid, GR: Glucocorticoid receptor, BDNF: Brain derived neurotropic factor, tPA: tissue plasminogen activator, Erk: Extracellular signal-regulated kinases, pMAPK: Phosphorylated Mitogen Activated Protein Kinase and TrkB: Tyrosine kinase receptor B.

However, the observed changes in GR and BDNF expression in the BLA was not associated with hypomethylation of specific CpG sites in their respective promoter regions chosen here. Further studies need to be considered to look at other CpG sites in GR and BDNF and other markers of epigenetic modification including

acetylation, histone modification, and chromatin remodeling to conclusively understand the underlying mechanism of the robust changes observed in the expression of these molecules. We have focussed on GR and BDNF due to specific relevance to stress and resilience in our animal models. In addition to GR, BDNF, MAPK, we also studied Arc (activity-regulated cytoskeletal proteins), MR (mineralocorticoid receptor) for their important roles in both stress-regulation and neuronal plasticity [335]. In addition to these, glutamate and GABA-ergic neuronal network of amygdala have dominant influence on effects of stress and associated neuronal plasticity [336, 337]. The signaling pathway of GR activated pathway of BDNF and pMAPK interaction could be causing activation of multiple other signaling pathways that cause protein regulation in our study [338].

Future directions

My study has highlighted the effects of MS and EE on structural physiology (adrenal weight, CORT & body weight), behavior, dendritic morphology and molecular underpinnings within BLA. This opens up far more avenues to explore other parts of the brain including other nuclei of amygdala namely central, medial and BNST, which plays important role in anxiety and stress response. Hypothalamus is the other crucial brain region that needs to be studied due to its role in HPA axis and the limited knowledge available about this brain region. Although hippocampus has been extensively studied in stress field, there are still gaps in the knowledge with regards to how it regulates various behaviors in EE particularly, in my model being the first to be exposed to short-term EE in early life. Many other behavioral studies that are associated with hippocampus and

mPFC can be conducted to determine the effect of short-term stress and EE in early life on these behaviors. Candidate based approach can be used to determine the molecular role players in other regions of the brain in my model. Thus, the EE and MSEE paradigms used in my study could be a potential resilient model of early life. An interesting follow-up from my study would be experiments focusing on the dams that were exposed to EE. Future epigenetics, molecular studies and functional studies of neurons of animals used in this study will be an exciting area to explore. It would be fascinating to investigate the behavior of the mothers in all the different groups used in this study. The licking/grooming pattern of all the four groups would be contributing to the results we have reported in this study. Another exciting question would be, are the "pro-resilient feature/maternal care" patterns seen in all the groups in our study transgenerational?

Summary:

I showed that early life enriched environment, concomitant to maternal separation stress has the potential to ameliorate much of the detrimental effects of MS on adult behavior, physiology, amygdala morphology and underlying molecular players, mainly the glucocorticoid receptor.

Chapter 6

6. Supplementary Experiments (suggested by Thesis Advisory Committee)

Initial Experiments

Introduction

This chapter focuses on the initial approaches taken to establish a potential animal model for resilience. I aimed to take a closer look at the impact of different paradigms during the early life of male rats; particularly on how early life environment affects their behavior during adolescence and adulthood. A comparative study using different models was devised to comprehensively highlight the crucial factors that are characteristic of both vulnerability to stress and resilience against stress. Additionally, I also aimed to investigate if short-term EE exposure during early life has the potential to induce long-lasting resilience to prepare an individual to maintain well-regulated levels of physiological parameters and behavior (homeostasis).

Impacts of different early life environment were investigated on the emotional (affective) behaviors of adolescent and adult rats in this study. The different environments to which the rats were exposed in early life were maternal separation stress (MS), limited nesting (LN), and short-term environmental enrichment (EE). Prior studies have showed that male rats, which experienced maternal separation (MS) in the first two weeks of its life showed higher depressive like behavior in the forced swimming test and had hyper sensitive HPA axis in response to subsequent stressors during adulthood [339, 340]. Studies also showed that MS

produced significant defects in learning tasks, both in the Morris water maze and in the novel object recognition test [341]. Similarly, behavioral changes of increased escape latency in the Morris water maze test and inability to distinguish the novel from a familiar object in the object recognition test was seen in rats subjected to limited nesting, during first two weeks of life [342]. Apart from behavioral abnormalities, physiological variations like elevated plasma corticosterone and increased adrenal weight were observed in limited nesting model [252, 343]. In contrast, EE enhanced memory function in various learning tasks in rodents [344]. Adult mice performed better in a spatial memory task (water maze task) after exposure to EE paradigm than controls in standard housing cage [204]. Previous literature shows that EE had beneficial effects on cognition accompanied by active coping mechanisms in rodents [168]. Mice exposed to long-term EE in early life displayed higher propensity to interact socially [345]. These evidences indicate that stressful early life environment has harmful effects on physiology and behavior of adult rats while EE imparts positive and well-adaptive features to the rats and their physiology and behavior. Though numerous studies had been done using EE in early life, no studies showed the impact of short term EE in rats. This inspired me to study varying environments during early life including early life stress and EE.

Various studies have demonstrated that a single episode (actue) or repeated episodes (chronic) of separation between mother and pups in rodents lead to both acute and long-term effects on behavior and endocrine read out [137, 340, 346-349]. When adult rats that previously underwent MS in early life were tested, they

showed neuroendocrine and behavioral deficits similar to those seen in patients with anxiety related disorders and depression [350-352]. 300-400% increase in plasma corticosterone was observed in rat pups, when they were exposed to maternal separation stress or any maternal care associated stress [339, 347]

The limited nesting environment results in stress in the mother, promoting fragmented care towards the pups due to harsh environment [252]. This aberrant maternal behavior in response to impoverished conditions, induced chronic early-life stress in the pups, evident from elevated plasma glucocorticoids and increased adrenal weight, often associated with modest, transient reduction of weight gain in the pups [252, 343]. So, this was another early life stress model with the presence of mother but impoverished housing. LN was another strss pardigm that was used in this study to investigate the impact of different kind of early life stress on adulthood of male rats.

Along with physiological variations due to exposure to LN, behavioral changes like increased escape latency in the Morris Water Maze test and inability to distinguish the novel object from a familiar object in the Object recognition test was also seen in the pups in this model [342]. Dendritic atrophy of pyramidal cells along with hippocampus mediated cognitive impairments was observed in rats exposed to limited nesting during their early life [342]. Synaptic density decreased in hippocampus and amygdala showed enhanced activity in rats that were exposed to LN [353],[354].

At this point we have discussed both early life stress and EE. In this study, “MS” and ‘LN” were the two early-life stress models in rodents that showed

compromised effects in various studies. and “EE” was another treatment used in early life to determine the beneficial effects of ealy life EE on adulthood in male rats. These three paradigms were used to study physiology, behavior and dendritic morphology during adulthood.

Experimental Plan

It was very intriguing to know how various environment affected the neuronal development of an individual. MS was provided from postnatal day (PD) 2 to PD 14, EE was provided from P2- P16 and LN was provided from P2-P9 and all these time points were adapted from previous studies [206, 339, 343]. Animal facility rearing (AFR) group was considered as the control group without any treatment. The treated rats were housed in AFR (neutral environment) after various treatment till adulthood. This experimental paradigm was well planned to give a variety of environment like positive environment (spacious and elevated sensory activity) in the presence of mother (EE), stress in the absence of mother (MS) and stress (impoverished conditions) in the presence of mother (LN). Various behavioral parameters were quantified for all the groups. Our aim was to analyze the imapct of three differential early life environments on physiology and behavior of an individual during adulthood. Please refer to Figure N for the schematic representation of the experimental flow of this study.

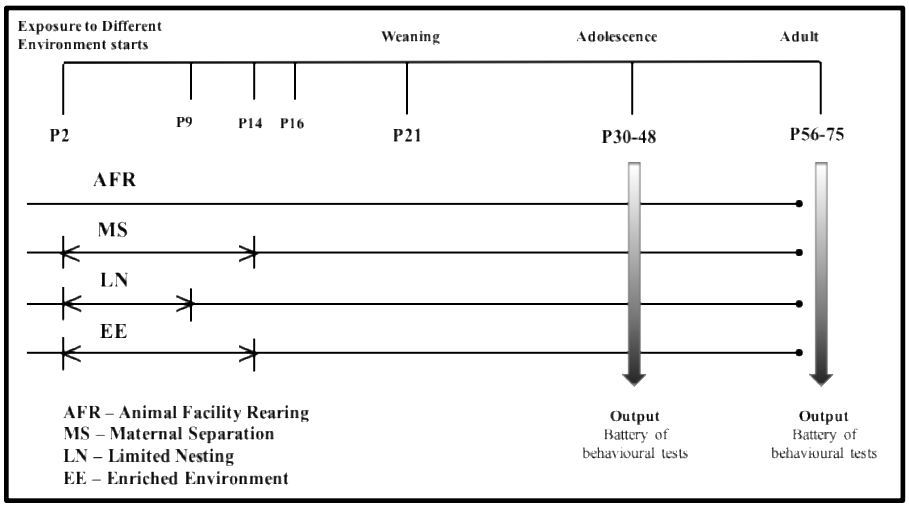


Figure N: Schematic representation of experimental flow in this study.

Results

A. **Anxiety-like behavior**

i) Home cage emergence in adolescent rats

In an attempt to examine the effect of different early life environment on the anxiety-like behavior of the adoloscent rats (P30-48), they were tested for escape latency from home cage during their adolescent stage. Higher latency indicates less exploratory behaviour as it means that the rat spends more time to escape from the home cage. One-way ANOVA, $F_{(3,27)} = 4.6$, $p = 0.01$ revealed a significant increase in time to escape from the home cage in LN when compared to MS and EE groups. Thus, LN was most anxious when compared to MS and EE in adolescent animals (Figure N.1).

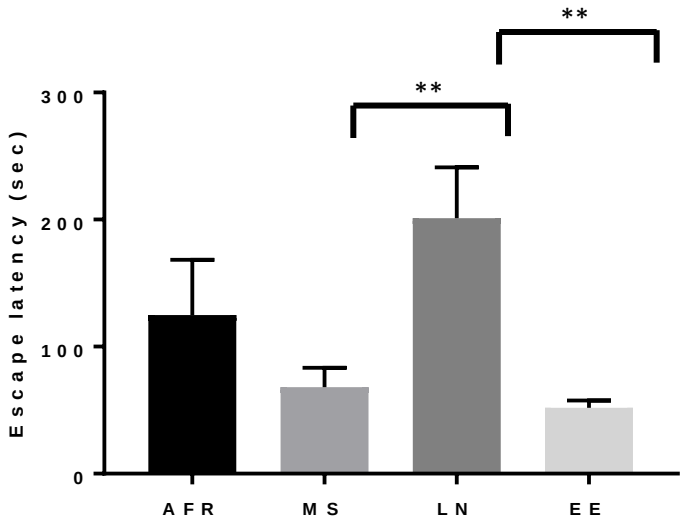


Figure N.1: Effect of different early environment on escape latency from home cage in adolescent rats. The graph represents escape latency for each group (mean ± SEM). LN group showed a significant increase in time to escape from the home cage in comparison to MS and EE groups. One-way ANOVA; $F_{(3,27)} = 4.6$, p=0.01; LSD posthoc test; **, $p < 0.01$. n=7 for MS, n=8 for AFR, LN, and EE. AFR: Animal Facility Rearing, MS: Maternal Separation, LN: Limited Nesting and EE: Environmental Enrichment.

ii) Home cage emergence in adult rats

To examine the effect of different early environment on the anxiety-like behavior of the adult rats, they were tested for escape latency from home cage during their adulthood. One-way ANOVA; $F_{(3,28)} = 3.2$, $p < 0.051$ revealed a significant decrease in escape latency from home cage in MS rats when compared to AFR and LN groups. This data showed that MS was least anxious when compared to AFR and LN groups when tested during adulthood

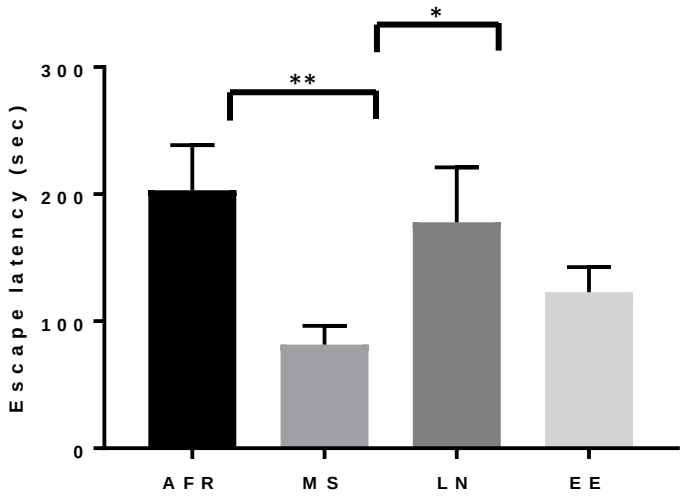


Figure N.2: Effect of different early environment on escape latency from home cage in adult rats. The graph represents escape latency for each group (mean ± SEM). MS group showed a significant reduction in time to escape the home cage in comparison to AFR and LN groups. One-way ANOVA; $F_{(3,28)} = 3.2$, $p<0.05$ for differences between groups; LSD posthoc test; *$p < 0.05$, **$p <0.01$, n= 8/group. AFR: Animal Facility Rearing, MS: Maternal Separation, LN: Limited Nesting and EE: Environmental Enrichment.

iii) Light box exploration in adolescent rats

To examine the effect of different early environment on anxiety-like behavior during adolescence, we subjected the animals to the Light-dark box test. Higher %time spent in the light box indicates anxiolytic behavior as the rat approaches light box when compared to the dark box. No significant differences were observed between the groups in One-way ANOVA; $F_{(3,27)} = 0.58$, $p = 0.6$ (Fig.4). This result does not support the hypothesis that stressful early environment causes an increase in anxiety-like behavior during a later stage of life (Figure N.3).

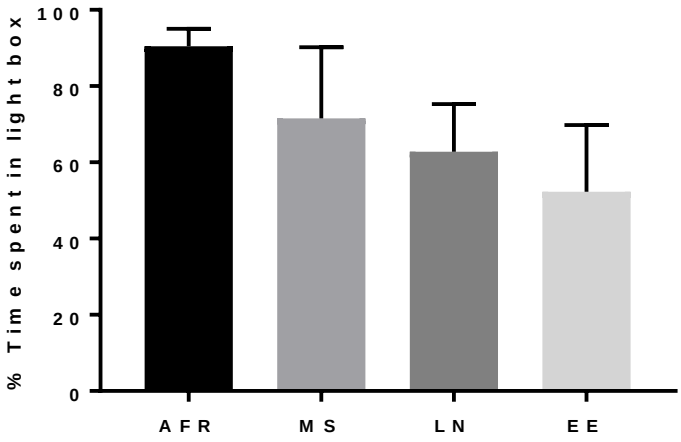


Figure N.3: Effect of different early environment on the anxiety of adolescent rats in the light-dark apparatus. The graph represents percentage time spent in light compartment (mean ± SEM). One-way ANOVA revealed no significant differences between the groups. AFR: Animal Facility Rearing, MS: Maternal Separation, LN: Limited Nesting and EE: Environmental Enrichment.

iv) Anxiety-like behavior in adult rats.

To examine the effect of different early environment on anxiety-like behavior at adulthood, we subjected the animals to elevated plus maze (EPM). One-way ANOVA; $F_{(3,28)} = 3.2$, $p<0.05$ revealed a significant increase in open arm entries in MS group when compared to AFR and LN groups (Figure N.4A). MS group showed a significant increase in open arm time in comparison with all other groups with One-way ANOVA; $F_{(3,28)} = 6.9$, $p = 0.001$ (Figure N.4B). One-way ANOVA; $F_{(3,28)} = 3.6$, $p<0.05$ revealed that MS group showed a significant increase in a number of head-dips when compared to AFR group (Figure N.4C). These results indicate that MS had anxiolytic effect in adulthood when compared to other groups and risk assessment (no. of head dips) was significantly high in MS when compared to AFR.

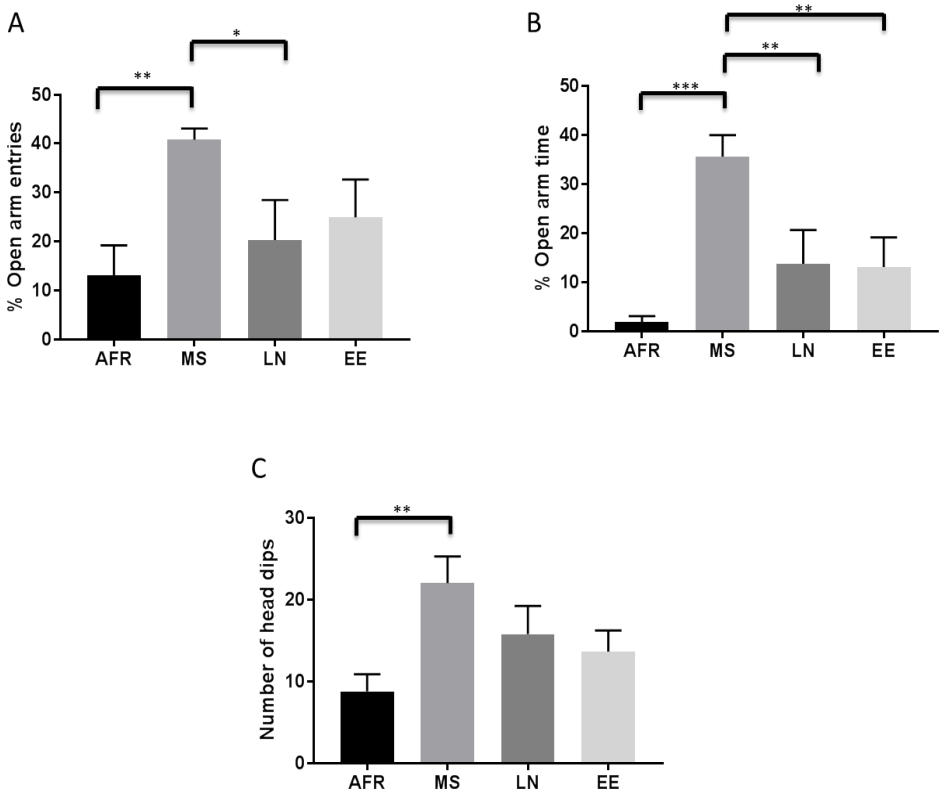


Figure N.4: Effect of different early environment on anxiety in Elevated plus maze in adult rats. A) The graph represents percent time of open arm entry for each group (Mean ± SEM). MS group showed a significant increase in open arm entries in comparison with AFR and LN groups. One-way ANOVA; $F_{(3,28)} = 3.2$, $p < 0.05$ for differences between groups; LSD posthoc test; *$p < 0.05$, **$p \leq 0.01$. n= 8/group. **B)** The graph represents percentage open arm time for each group (Mean ± SEM). MS group showed a significant increase in open arm time in comparison with all other groups. One-way ANOVA; $F_{(3,28)} = 6.9$, $p = 0.001$ for differences between groups; LSD posthoc test; **$P < 0.01$, ***$p \leq 0.001$. n= 8/group. **C)** The graph represents a number of head-dips for each group (Mean ± SEM). MS group showed a significant increase in a number of head-dips when compared to AFR group. One-way ANOVA; $F_{(3,28)} = 3.6$, $p < 0.05$ for differences between groups; LSD posthoc test; **$p \leq 0.01$. n= 8/group. AFR: Animal Facility Rearing, MS: Maternal Separation, LN: Limited Nesting and EE: Environmental Enrichment.

B) Social Interaction

i) Motivation for social exploration in adolescent rats

To assess the effect of different early environment on sociability in adolescent stage, we performed a social preference test. Higher preference towards the stimulus animal in this indicates higher social interaction. One-way ANOVA; $F_{(3,24)} = 5.0$, $p = 0.008$ revealed a significantly high percentage time of social exploration in the EE group as compared to AFR and MS groups (Figure N.5).

This result indicates that EE group had higher levels of social exploration when compared to two other groups.

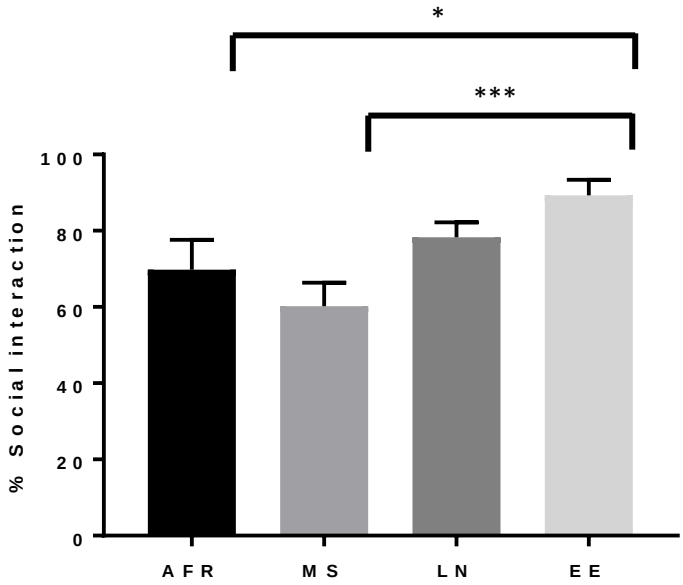


Figure N.5: Effect of different early environment on social preference test in adolescent rats. The graph represents percent time of social interaction for each group. EE group (Mean ± SEM) spent significantly most time in social interaction when compared to AFR and MS group. One-way ANOVA; $F(3,24) = 5.0$, $p = 0.008$ for differences between groups; LSD posthoc test; $^{*}p < 0.05$, $^{***} p \leq 0.001$. n=7 for AFR, n=7 for MS, n=6 for LN and n=8 for EE. Each bar represents mean ± SEM. AFR: Animal Facility Rearing, MS: Maternal Separation, LN: Limited Nesting and EE: Environmental Enrichment.

ii) Motivation for social exploration in adult rats

To assess the effect of different early environment on sociability in adulthood, we performed a social preference test. No significant differences were observed between the groups in One-way ANOVA; $F_{(3,21)} = 2.2$, $p = 0.12$ (Figure N.6). These results do not suggest any conclusive data for social exploratory behavior in adults.

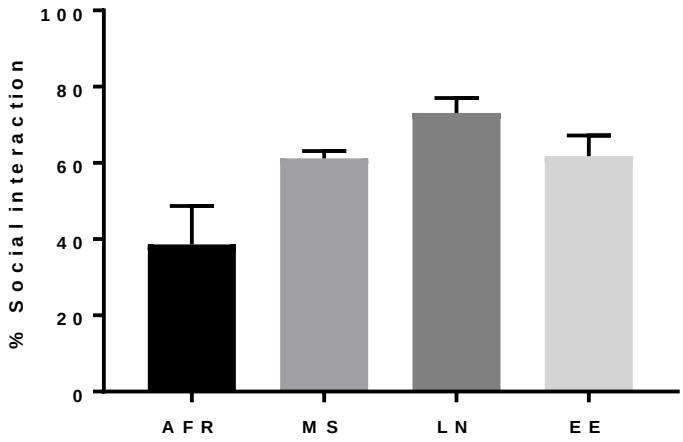


Figure N.6: Effect of different early environment on social preference test in adult rats. The graph represents percent time of social interaction for each group (mean ± SEM). One-way ANOVA revealed no significant differences between the groups. n= 8/group. AFR: Animal Facility Rearing, MS: Maternal Separation, LN: Limited Nesting and EE: Environmental Enrichment.

C) Depression

i) Sucrose intake test in adolescent rats

To evaluate the effect of different early environment on depressive like behavior in adolescent rats, we performed sucrose intake test. One-way ANOVA F (2,14) = 0.4, p=0.71 revealed no significant differences between the groups (Figure N.7). This result indicates that the early environment did not affect the depressive like behavior in adult rats.

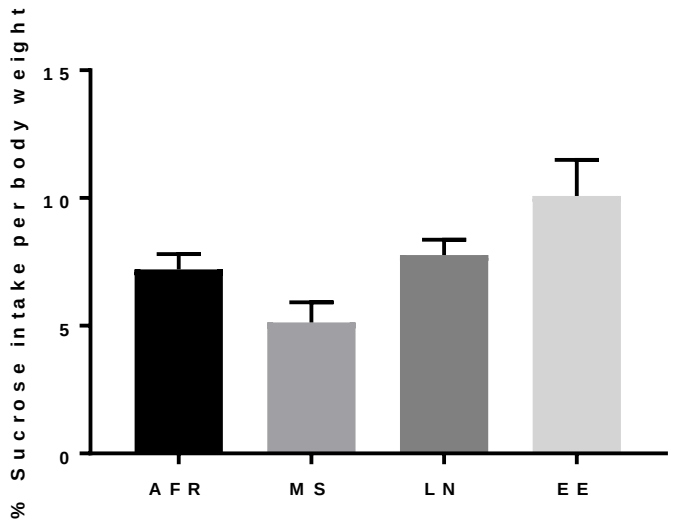


Figure N.7: Effect of different early environment on sucrose intake test in adolescent rats. The graph represents percentage sucrose intake per body weight for all the groups (mean ± SEM). One-way ANOVA revealed no significant differences between the groups. F (2,14) = 0.4, p=0.71. n=6 for AFR, n=7 for LN, n=8 for MS and EE. AFR: Animal Facility Rearing, MS: Maternal Separation, LN: Limited Nesting and EE: Environmental Enrichment.

ii) Forced swim test in adult rats

To evaluate the effect of different early environment on depressive-like behavior in adult rats, we performed forced swim test. MS group spent significantly higher time in swimming when compared to LN and EE with One-way ANOVA; $F_{(3,26)} = 3.1$, $p = 0.04$ (Figure N.8A) suggesting active coping behavior in MS rats. Duration of immobility showed no significant difference between groups, One-way ANOVA; $F_{(3,28)} = 0.82$, $p = 0.49$ (Figure N.8B) showing that the different early life environment did not have any effect on the depressive-like behavior.

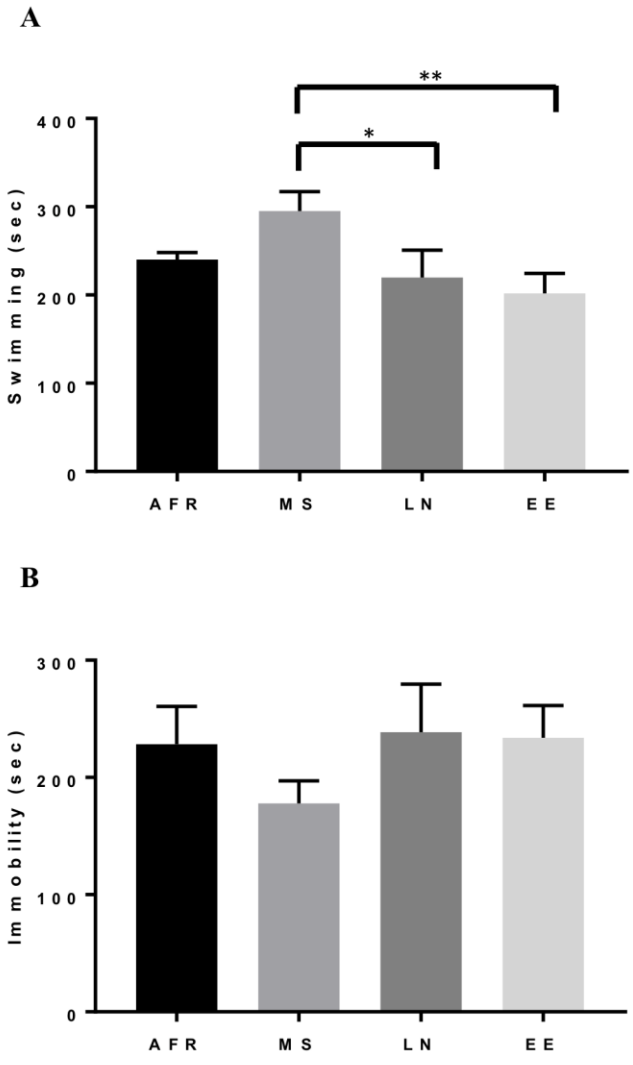


Figure N.8: Effect of different early environment on forced swim test in adult rats. A) The graph represents time spent in swimming for each group (Mean ± SEM). MS group showed a significant increase in time of swimming when compared to LN and EE group. One-way ANOVA; $F_{(3,26)} = 3.1$, $p=0.04$ for differences between groups; LSD posthoc test; **p <0.01, *P ≤ 0.05. n= 8/group. **B)** The graph represents the duration of immobility for each group (Mean ± SEM). One-way ANOVA revealed no significant differences between the groups. n= 8/group. AFR: Animal Facility Rearing, MS: Maternal Separation, LN: Limited Nesting and EE: Environmental Enrichment.

Discussion

In this study, the MS rats displayed affective (emotional) behaviour contradictory to the previous literature as most of MS studies have associated the model with compromised behavioural output. MS rats showed anxiolytic behaviour in HCE and EPM when compared to all other groups. MS rats showed improved risk assessment behaviour when compared to AFR and they also had better coping strategy in forced swim test suggesting less depressive-like behaviour. However, they showed impairment in social exploration. EE rats displayed higher social interaction compared to other groups. Overall behavioural results showed that different early life environment gave rise to distinct behavioural outputs, which is given in detail below.

Anxiety like behaviour was studied using Home cage emergence (HCE), Light dark box (LDB) and Elevated plus maze (EPM). HCE was used to test anxiety in both stages, LDB was used in adolescence and EPM was used in adulthood only. EPM was used only in adulthood to avoid carryover effect [355]. Earlier studies showed that stressed rats tend to take more time to leave their home cage (escape latency) due to anxiety compared to the control rats which take lesser time to escape [234]. In support, this study showed that rats exposed to LN during early life takes significantly longer time to escape the home cage compared to other groups in adolescent stage suggesting anxiogenic behaviour in them. HCE carried out in adulthood showed contradicting results wherein the MS rats had significant low escape latency compared to other groups. We infer that the various behavioural tests done in adolescent stage might have affected the adulthood behavioural analysis. A more likely reason for the

above-mentioned unexpected results was a construction project that took place right next door to the animal facility. All researchers involved in behavioural and endocrine related study reported a similar trend of unexpected results. This was rather unfortunate because when this issue was further examined, there were serious problems with the health status of the rats used in this study. Loss of excess fur, patches of skin without fur in the belly, bleeding nose, damaged skull, etc., were observed with various animals used in this study.

LDB is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stressors, mainly novel environment. This test was used to test anxiety in adolescent stage and anxiety was not affected between different treatments in LDB.

Adult rats were tested on EPM and the results suggested that MS rats were less anxious compared to AFR and LN rats. It was surprising to note that MS rats were displaying anxiolytic behaviour and the AFR rats had abnormally low basal levels of percentage open arm time and entries. Extant literature suggests that in EPM, the baseline value of percentage open arm time ranges from 15 to 30 and percentage open arm entries vary between 25-45 [355, 356]. This information indicated impairement in baseline values of control rats in our study, which might be influencing the unexpected results. Apart from anxiolytic behaviour MS rats showed significantly elevated head-dip score compared to AFR group suggesting higher risk assessing behaviour in MS rats. This unexpected anxiolytic behaviour in adult MS rats could have been also influenced by the battery of behavioural experiments conducted in adolescent

rats but the construction work was a more probable cause for the absurd and unexpected data.

More affective behaviours such as social exploration and depression were also tested in this study. Social interaction test was performed in adolescence and adulthood to study the social motivation in rats. Previous study has shown that stress causes decline in social exploration in rats where they are motivated to explore objects in comparison to unfamiliar conspecifics [241]. EE group had a significantly higher percentage of social exploration in adolescent rats when compared to AFR and MS groups but in adult rats, there was no significant difference in percentage social exploration between any groups. Enriched environment provides a complex exploratory, social, emotional and motor stimulation to the animals and also induces well adaptive behaviours [169]. In support to this, in our study, the enriching environment provided to the dam and pups during postnatal period, induced social motivation in their adolescence. Licking/grooming behaviour of dams provided positive effects that helps to regulate the development of endocrine, emotional and cognitive responses to stress in pups [172]. In our study, the dam was exposed to enriched environment and this could have resulted in her spending adequate amount of time licking and grooming the pups, which in turn could have helped the pups to grow into a socially active adolescense. EE paradigm in this study was administered for 16 days, which is different from the 21 day EE paradigm discussed earlier. This was to assess for EE effects till weaning period only.

In addition to other behaviours discussed above, I investigated depressive like behaviour in adolescent rats by performing sucrose intake test (SIT) and in adults; forced swim test (FST). Stressed rats have previously been found to

have lower consumption of saccharin or sucrose solutions which was referred to as anhedonia [357, 358]. There was no significant difference seen in the percentage of sucrose intake per body weight between the groups. FST is used to quantify immobility, climbing and swimming. Immobility corresponds to depressive like behaviour and there was no significant difference in this trait among the groups. Swimming was assessed in FST and suggested positive coping in response to inescapable shock [359]. Prior studies have shown that maternally separated mice display significantly shorter swim times on the forced swim test [360]. Our study showed that MS rats had higher swimming time when compared to LN and EE group of rats but not to the controls. As FST was the final experiment done in adulthood, all other behavioural tests done prior to it may have affected this behaviour.

Novel object recognition test (NORT) was used to examine the short-term memory of the rat. This test was performed in both adolescence and adulthood. Rodents have a tendency to explore the novel object more than a familiar object and this principle is used to test the memory and learning by evaluating the exploration time of novel and familiar objects [361]. Previous work showed that stress affects learning and memory that are mediated by the hippocampus [362]. Due to the analysis criteria, memory test performed in adolescence did not generate results but adulthood NORT data suggested that MS rats had significantly lesser time exploring novel object compared to all other groups.

The inconsistencies in behavioural results putatively could be due to the nature of this longitudinal study wherein adulthood behaviours were affected by an unfortunate event of construction right next to the animal house from the point

of breeding till adulthood. It had detrimental effects on the development and health status of the rats used in this study.

Due to the unforeseen results mentioned above, efforts were taken to re-plan and deduce a different experimental paradigm. Additionally, we imported the rats from Charles River, to avoid any concomitant health issue of rats during this time. The following chapters will focus on the revised experimental design.

Chapter 7

7. MSEE 14

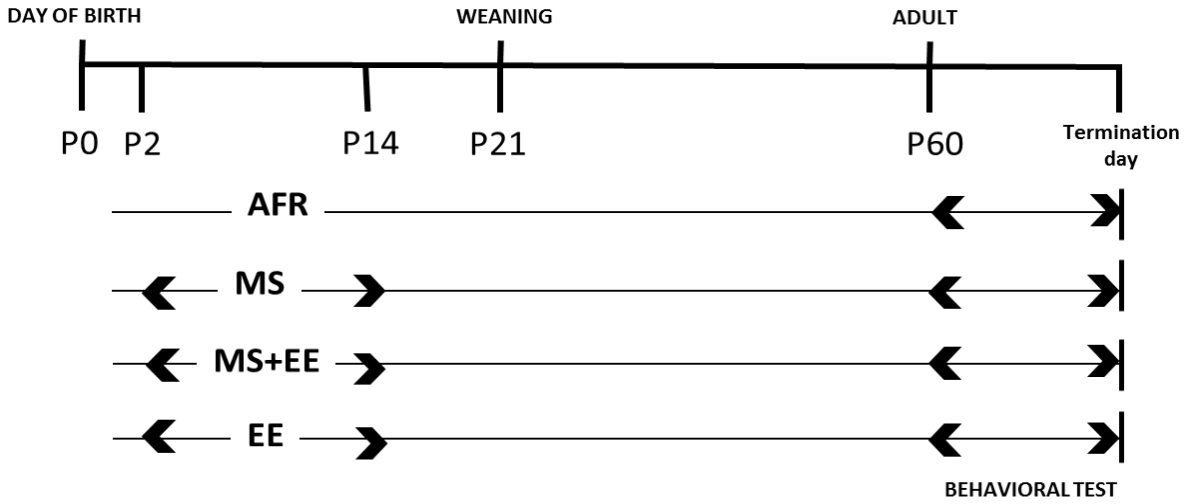
Introduction

As mentioned in earlier chapters, efforts were taken to build a revised early life model to understand stress and resilience in this critical period. Moving on from a longitudinal approach, we shifted the focus from exploring 'the impact of differential treatments in early life' on adolescence and adulthood to studying 'the influence of EE and MS in early life' on adulthood. MS is shown to have detrimental effects on adulthood, and my attempt was to investigate if EE could alleviate the negative repercussion of MS.

This chapter will focus on a stress-EE paradigm designed to establish a potential stress-resilience animal model. I aimed to take a closer look at the impact of concurrent EE and MS in the early life of male rats and particularly on how it affects their physiology, affective behavior, and dendritic arborization during adulthood.

This paradigm was devised to comprehensively highlight the crucial factors that are characteristic to both stress and resilience and also to investigate the role of short-term EE exposure during early life on long-lasting resilience effects that prepare an individual to maintain basal levels of physiology and behavior till adulthood. This experiment displayed interesting results discussed in this chapter but it was hypothesized that this stress-resilience model could be improvised to increase the potential of combinatorial effect of MS and EE. The improvised model was the main experiment of this thesis that was discussed in detail earlier. Nevertheless, I would like to present the results obtained from

this pilot study that helped us to build a better model. I believe the experiment itself was well planned and executed. Please refer to Figure F.1 for the schematic representation of the experimental flow of this study.



AFR – Animal Facility Rearing
MS – Maternal Separation
MS+EE – Co-exposure of MS and EE
EE – Enriched Environment

Figure F.1: Schedule of experiments for MSEE 14 paradigm

The figure above represents the experimental workflow for the MSEE paradigm. The rational for choosing a 14-day enrichment paradigm was because previous reports had shown that exposure to an enriched environment for 14 days was enough to reverse behavioral and physiologic effects of MS [87] and adult chronic stress [166]. Additionally, the maternal separation protocol typically lasts for 14 days (P2 to P16) [263]. Thus, we modeled the length of this EE paradigm based on previous reports and also to coincide with the duration of maternal stress exposure [77, 87]. In subsequent sections, I will present results and very briefly discuss these.

Physiological Measurements

The effect of early life stress and enriched environment on body weight, adrenal weight, and plasma corticosterone levels were analysed in adulthood. The results of these physiological measurements are presented as a comparison between different experimental groups (AFR, MS EE, and MSEE).

Please note that AFR refers to animal facility rearing without maternal separation stress or environmental enrichment. MS refers to animal facility rearing with maternal separation stress but without environmental enrichment. EE refers to the enriched environment without maternal separation stress, and MSEE refers to enriched environment with maternal separation stress.

Enrichment had a significant effect on body weight

Body weight was measured at different time points (Figure F.2), namely: week 3, week 5, week 7, week 8 and at sacrifice.

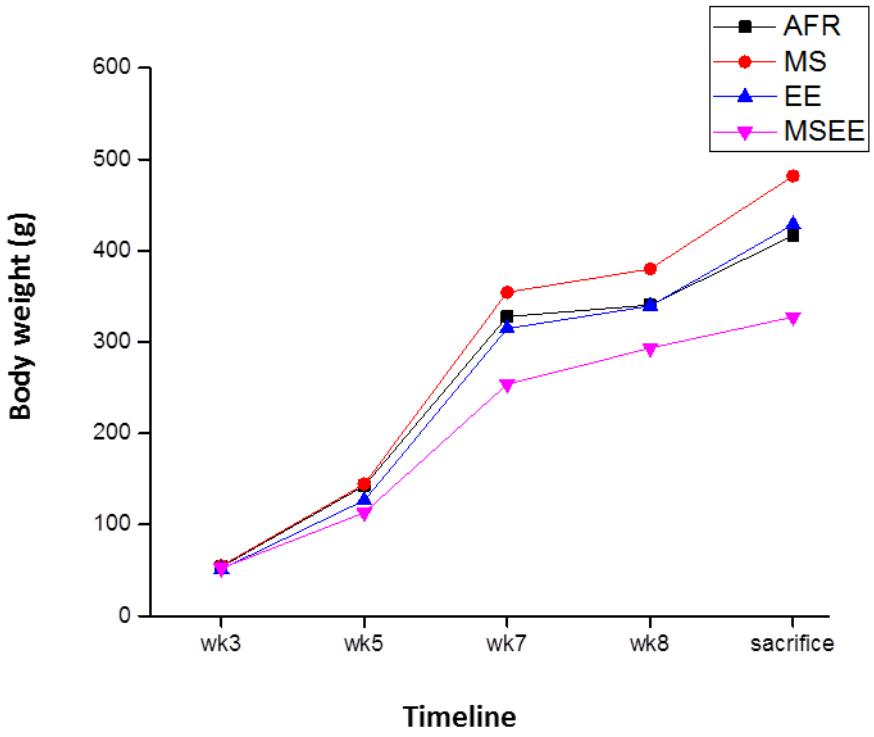


Figure F.2: Mean weights of animals at different time points throughout the course of study for animals exposed to early life stress and enrichment.

Further, a two-way ANOVA was conducted for body weight 'area under curve' (AUC) with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,27)} = 1.785$, $p = 0.193$) on body weight did not reach statistical significance. However, the effect of EE ($F_{(1,27)} = 38.754$, $p = 0.00$) and the interaction between stress and EE ($F_{(1,27)} = 24.818$, $p = 0.00$) had a significant effect on body weight.

To analyze the effects of stress in absence or presence of the EE, orthogonal planned comparisons were conducted (independent samples t-tests). In absence of EE, stress significantly increased body weight ($t_{13} = -3.587$, $p < 0.00$). In the presence of EE, stress significantly decreased the body weight ($t_{14} = 3.756$, $p < 0.00$; Figure F.3).

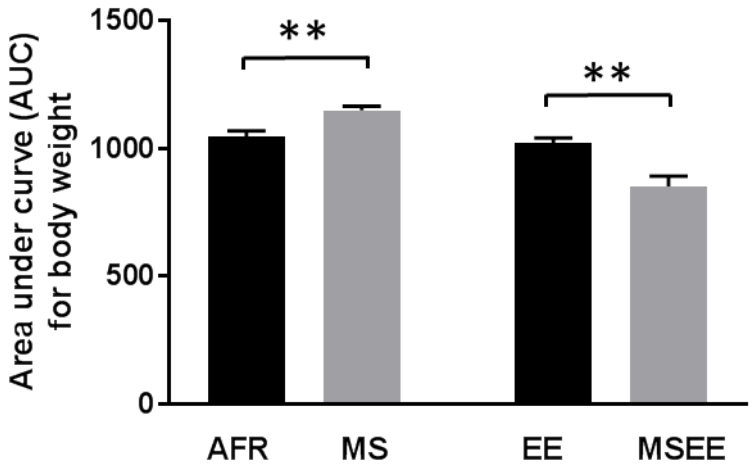


Figure F.3: Effect of early life stress and EE on body weight: stressed animals displayed lower body weight in the presence of EE. Graphs represent Mean ± SEM. N = 7 for AFR, 8 for Stress, 8 for EE and 8 for MSEE; $^{**}p \leq 0.01$.

MSEE significantly decreased adrenal gland weights

A two-way ANOVA was conducted for adrenal gland weight with stress and EE as two between subject sources of variance. Stress ($F_{(1,25)} = 9.930$, $p < 0.00$) had a significant main effect on adrenal gland weight. However, EE ($F_{(1,25)} = 0.830$, $p = 0.37$) and the interaction between stress and EE had a significant effect ($F_{(1,25)} = 0.401$, $p = 0.53$) on adrenal gland weights.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE, the effect of stress ($t_{13} = 1.482$, $p = 0.16$) on adrenal gland weights failed to reach statistical significance. However, in the presence of EE, the effect of stress ($t_{12} = 3.850$, $p < 0.01$; Figure F.4) significantly decreased the adrenal gland weights.

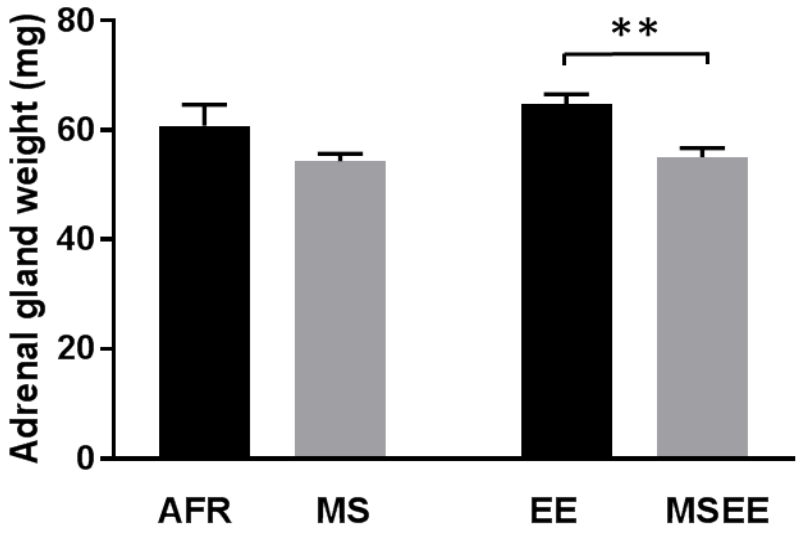


Figure F.4: Effect of early life stress and EE on adrenal weight: stress induced a significant decrease in adrenal weights in the presence of EE. Graphs represent Mean ± SEM. N = 8 for AFR, 7 for Stress, 7 for EE and 7 for MSEE; $^{**}p \leq 0.01$.

Behavioral assays

The effect of early–life stress and enriched environment on behavior was tested in adult animals. For testing anxiety-like behavior, the home cage emergence test (HCE), the open field test (OFT) and the elevated plus maze (EPM) were employed. For testing social behavior, the social interaction test (SIT) was employed. To test depressive-like behavior, the forced swim test (FST) was used. The results of these behavioral endpoints are presented as a comparison between different experimental groups (control, stress, EE, and MSEE).

Stress and EE had no significant effect on escape latency in home cage emergence test

A two-way ANOVA was conducted for escape latency in the home cage emergency test with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,27)} = 0.732$, $p = 0.40$) and EE ($F_{(1,27)} = 0.040$, $p = 0.84$) on escape latency in home cage emergence test failed to reach statistical significance. Similarly, the interaction between stress and EE ($F_{(1,27)} = 1.083$, $p = 0.307$) on escape latency in home cage emergence test failed to reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that in the absence of EE, ($t_{13} = -0.127$, $p = 0.90$) the effect of stress on escape latency in the home cage emergence test, failed to reach statistical significance. In the presence of EE ($t_{14} = 1.379$, $p = 0.19$; Figure F.5), the effect of stress on escape latency failed to reach statistical significance

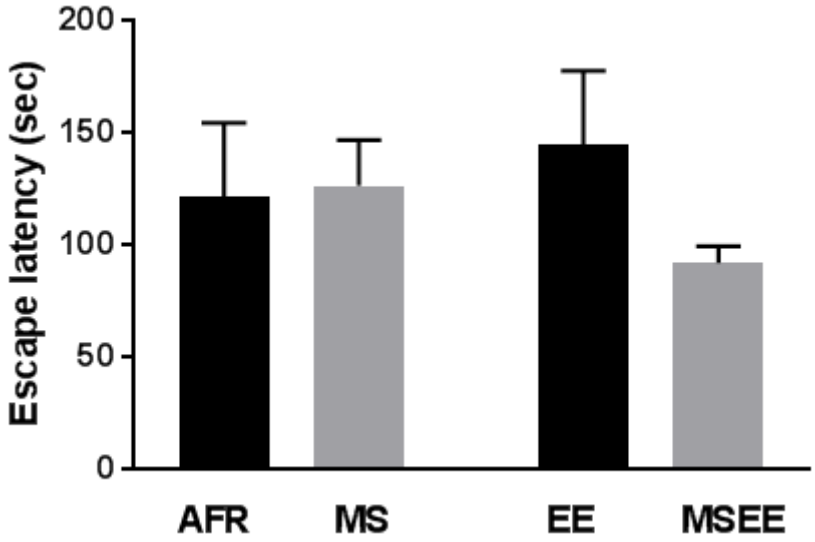


Figure F.5: Effect of early life stress and EE on escape latency in HCE in adulthood: Stress (in presence and absence of EE) had no significant effect on escape latency in home cage emergence test. Graphs represent Mean ± SEM. N = 8 for AFR, 7for Stress, 9 for EE and 7 for MSEE.

Stress did not alter the inner zone exploration in open field test

A two-way ANOVA was conducted for inner zone exploration in the open field test with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,26)} = 0.093$, $p = 0.763$) and EE ($F_{(1,26)} = 1.663$, $p = 0.21$) on time spent exploring the inner zone in the open field test failed to reach statistical significance. Similarly, the effect of the interaction between stress and EE ($F_{(1,26)} = 2.473$, $p = 0.13$) on time spent exploring the inner zone in the open field test failed to reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE, the effect of stress on the time spent exploring the inner

zone in the open field test (t_{13} = 1.100, p = 0.29) failed to reach statistical significance. Similarly, the effect of stress on the time spent exploring the inner zone in the open field test in the presence of EE (t_{13} = -1.221; p = 0.24; Figure F.6) failed to reach statistical significance.

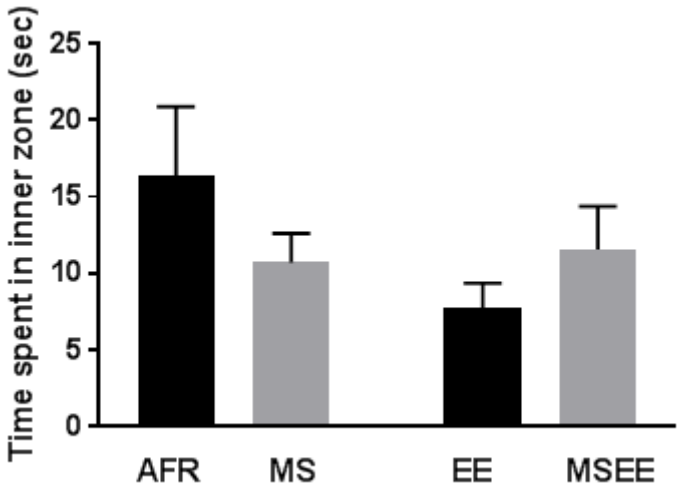


Figure F.6 Effect of early life stress and EE on time spent in the inner zone of OFT: stress significantly decreased the time spent exploring the inner zone in open field test in the absence of EE. Graphs represent Mean ± SEM. N = 8 for AFR, 7 for Stress, 8 for EE and 7 for MSEE.

EE significantly increased and MS significantly reduced percentage open arm time in the EPM

A two-way ANOVA was conducted for percentage open arm time in the elevated plus maze with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,25)}$ = 5.878, p = 0.02) and EE ($F_{(1,25)}$ = 6.017, p = 0.02) had a significant main effect on the percentage time spent exploring the open arm of the elevated plus maze. However, the interaction between

stress and EE ($F_{(1,25)} = 0.387$, $p = 0.54$) on the percentage time spent exploring the open arm of the elevated plus maze failed to reach statistical significance.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE, the effect of stress on the percentage time spent exploring the open arm of the elevated plus maze ($t_{13} = 1.518$, $p = 0.15$) did not reach statistical significance. Similarly, in the presence of EE, the effect of stress on the percentage time spent exploring the open arm of the elevated plus maze ($t_{12} = 1.860$, $p = 0.09$; Figure F.7) did not reach statistical significance.

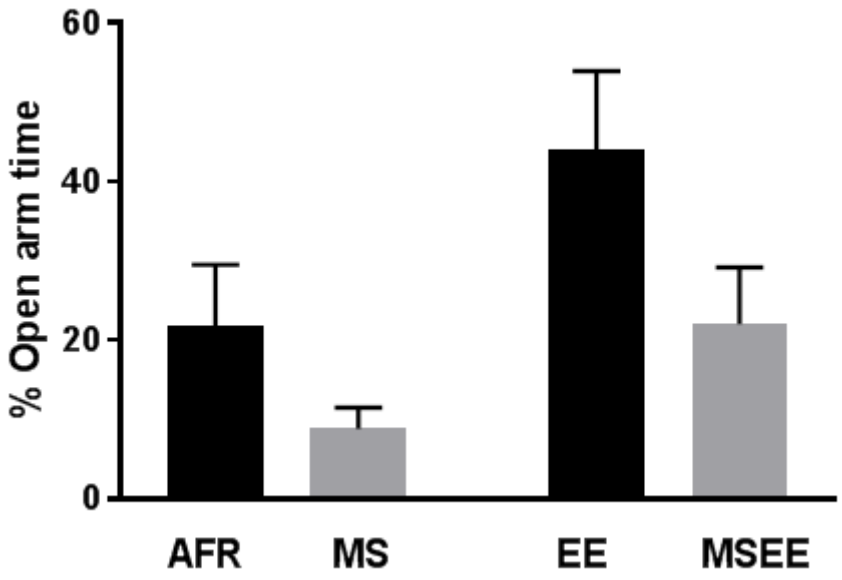


Figure F.7: Effect of early life stress and EE on percentage open arm time in EPM in adulthood: EE significantly increased and MS significantly decreased the percentage time spent exploring the open arm of the elevated plus maze in the presence of EE Graphs represent Mean ± SEM. N = 8 for AFR, 7 for Stress, 6 for EE and 8 for MSEE.

EE significantly increased percentage open arm entries in the EPM

A two-way ANOVA was conducted for percentage open arm entries in the elevated plus maze with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,25)} = 2.748$, $p = 0.11$) and the interaction between stress and EE ($F_{(1,25)} = 0.349$, $p = 0.56$) on the number of entries into the open arm of the elevated plus maze failed to reach statistical significance. However, EE ($F_{(1,25)} = 12.743$, $p < 0.00$) had a significant effect on the number of entries into the open arm of the elevated plus maze.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE, stress had no significant effect on the number of entries into the open arm of the elevated plus maze ($t_{13} = 0.835$, $p = 0.42$). Similarly, in the presence of EE, stress had no significant effect on the number of entries into the open arm of the elevated plus maze ($t_{12} = 1.447$, $p = 0.17$; Figure F.8).

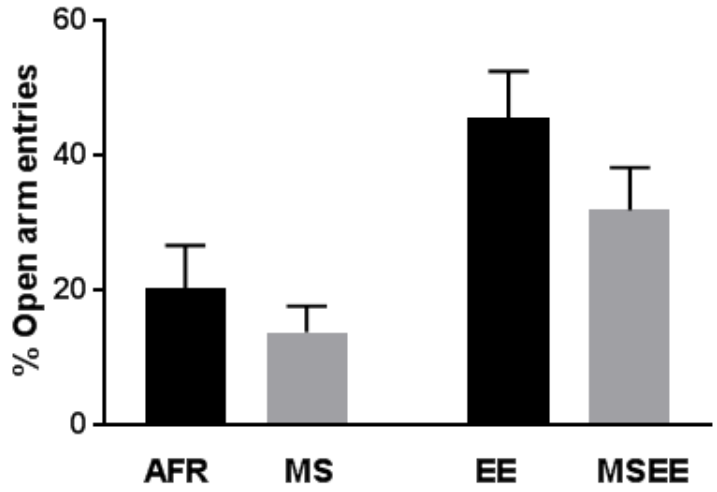


Figure F.8: Effect of early life stress and EE on percentage open-arm entries in EPM: stress significantly increased the number of entries into the

open arm of the elevated plus maze in the presence of EE. Graphs represent Mean ± SEM. N = 8 for AFR, 7 for Stress, 6 for EE and 8 for MSEE.

EE increased closed arm entries in the elevated plus maze

A two-way ANOVA was conducted for percentage closed arm time in the elevated plus maze with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,25)} = 3.426$, $p = 0.08$) and the interaction between stress and EE ($F_{(1,25)} = 0.7.4$, $p = 0.41$) on the number of entries into the closed arm of the elevated plus maze failed to reach statistical significance. However, EE ($F_{(1,25)} = 9.101$, $p < 0.00$) had a significant main effect on the number of entries into the closed arm of the elevated plus maze.

Orthogonal planned comparisons (Independent samples t-tests) showed that in the absence ($t_{13} = -0.728$, $p = 0.48$) the effect of stress on the number of closed arm entries in the elevated plus maze failed to reach significance. Similarly, in the presence of EE ($t_{12} = -1.873$, $p = 0.09$; Figure F.9) the effect of stress on the number of closed arm entries in the elevated plus maze failed to reach significance.

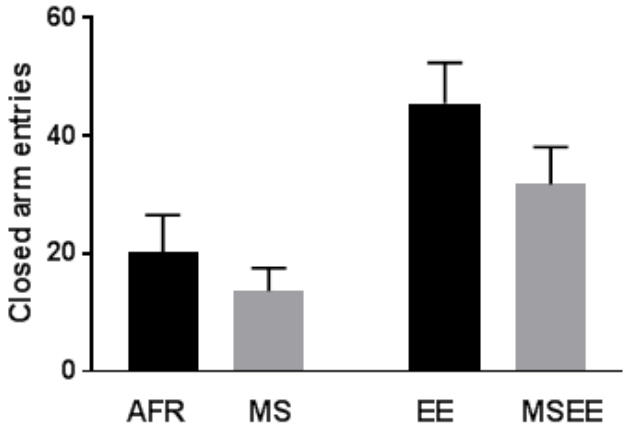


Figure F.9: Effect of early life stress and EE on number of closed arm entries in EPM: EE significantly increased the number of entries into the closed arm of the elevated plus maze in presence and absence of EE. Graphs represent Mean ± SEM. N = 8 for AFR, 7 for Stress, 6 for EE and 8 for MSEE.

Stress in the presence of EE significantly decreased percentage social exploration in the SIT

A two-way ANOVA was conducted for percentage total exploration in the social interaction test with stress and EE as two between subject sources of variance. The effect of EE ($F_{(1,26)} = 0.779$, $p = 0.39$) and the interaction between stress and EE ($F_{(1,26)} = 0.021$, $p = 0.89$) on percentage total exploration in the social interaction test failed to reach statistical significance. However, stress ($F_{(1,26)} = 12.370$, $p < 0.00$) had a significant main effect on the time spent interacting with the stimulus animal in the social interaction test.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE ($t_{138} = 2.087$, $p = 0.06$), stress had a marginal (p=0.06) significant effect on percentage total exploration in the social interaction. However, in the presence of EE ($t_{13} = 3.108$, $p < 0.00$; Figure F.10) stress significantly decreased the time spent interacting with the stimulus animal in the social interaction test.

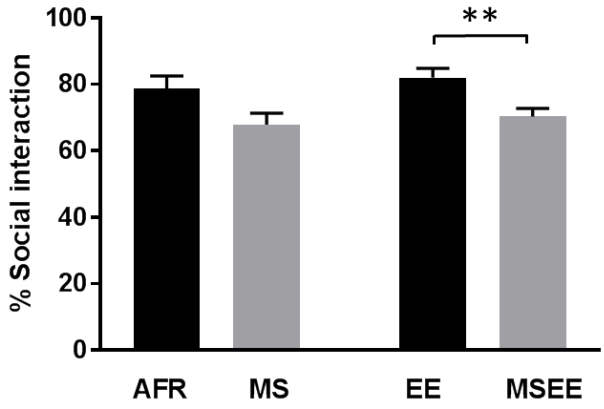


Figure F.10: Effect of early life stress and EE on social exploration in social interaction test: Stress significantly decreased the time spent interacting with the stimulus animal in the social interaction test in the presence of EE. Graphs represent Mean ± SEM. N = 8 for AFR, 7 for Stress, 7 for EE and 8 for MSEE; $^{**}p \leq 0.01$.

Stress alone significantly increased immobility in FST and stress in the presence of EE significantly decreased immobility in the FST

A two-way ANOVA was conducted for immobility in the forced swim test with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,26)} = 9.694$, $p < 0.00$) and EE ($F_{(1,26)} = 14.095$, $p < 0.00$) had a significant main effect on the immobility time in FST. Similarly, the interaction between stress and EE ($F_{(1,26)} = 21.031$, $p < 0.00$) also had a significant main effect on the immobility time in FST.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE ($t_{12} = -4.582$, $p < 0.01$), stress significantly increased immobility in the forced swim test. However, in the presence of EE ($t_{14} = 4.188$, $p < 0.00$; Figure F.11) stress significantly decreased the immobility time in FST.

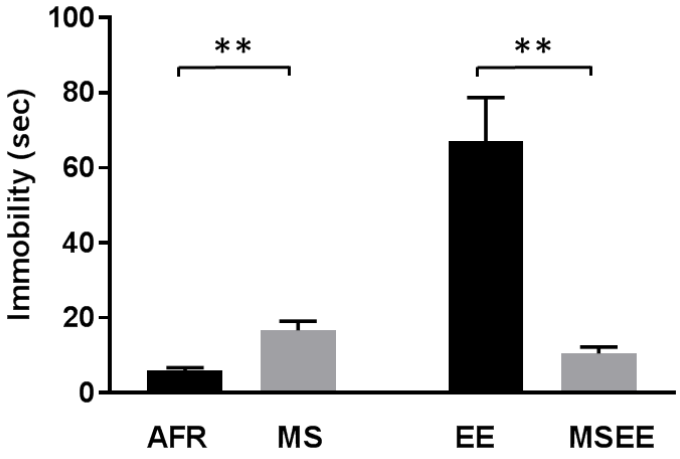


Figure F.11: Effect of early life stress and EE on immobility in the forced swim test: Stress alone significantly increased the immobility time in FST but stress in the presence of EE, decreased immobility time in FST. Graphs represent Mean ± SEM. N = 8 for AFR, 6 for Stress, 9 for EE and 7 for MSEE; $^{**}p \leq 0.01$.

Stress in the presence of EE significantly increased swimming in the FST

A two-way ANOVA was conducted for swimming in the forced swim test with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,26)} = 24.527$, $p < 0.00$) and EE ($F_{(1,26)} = 56.028$, $p < 0.00$) had a significant main effect on swimming time in FST. Similarly, the interaction between stress and EE ($F_{(1,26)} = 9.306$, $p < 0.00$) also had a significant main effect on the swimming time in FST.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE ($t_{12} = -1.029$, $p = 0.32$), the effect of stress on swimming in the forced swim test failed to reach significance. However, in the presence of

EE (t_{14} = -8.396, p < 0.00; Figure F.12) stress significantly increased the swimming time in FST.

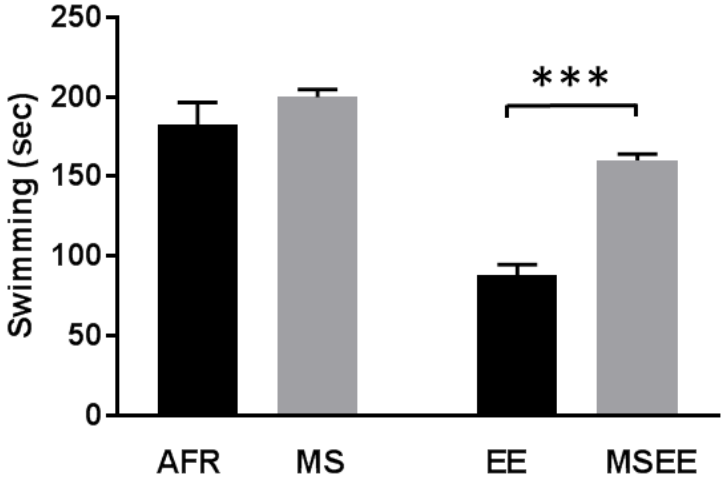


Figure F.12: Effect of early life stress and EE on swimming in the forced swim test: Stress significantly increased the swimming time in FST in the presence of EE. Graphs represent Mean ± SEM. N = 8 for AFR, 6 for Stress, 9 for EE and 7 for MSEE; **p ≤ 0.01.

Dendritic Morphology

Stress is known to alter the dendritic morphology of neurons in the brain. We analyzed the effect of early-life stress and enriched environment on the dendritic morphology of neurons in the basolateral amygdala of animals assigned to each treatment group. The total dendritic length and a total number of branch points were determined for neurons in BLA, hippocampus and mPFC for all animals.

EE induced increase in dendritic length of BLA neurons in the presence of EE

A two-way ANOVA was conducted for dendritic length of BLA neurons with stress and EE as two between subject sources of variance. The effect of stress

($F_{(1,234)} = 1.147$, $p = 0.29$) and the interaction between stress and EE ($F_{(1,234)} = 0.208$, $p < 0.65$) on the total dendritic length of neurons in the basolateral amygdala did not reach statistical significance. However, the effect of EE ($F_{(1,234)} = 11.437$, $p < 0.00$) had a significant main effect on the total dendritic length of neurons in the basolateral amygdala.

Orthogonal planned comparisons (Independent samples t-tests) showed that, in the absence of EE ($t_{120} = -0.471$, $p = 0.64$), the effect of stress on the total dendritic length of neurons in the basolateral amygdala did not reach statistical significance. Similarly, in the presence of EE ($t_{114} = -1.002$, $p = 0.32$; Figure F.13) the effect of stress on the total dendritic length of neurons in the basolateral amygdala did not reach statistical significance.

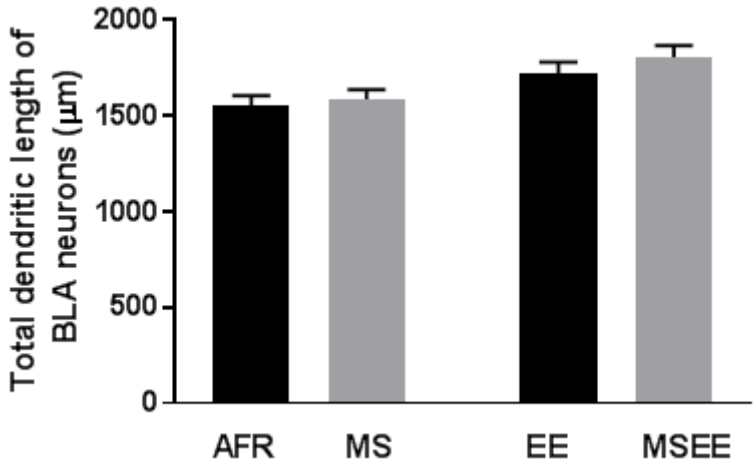


Figure F.13: Effect of early life stress and EE on total dendritic length in the BLA neurons: EE significantly increased the total dendritic length of neurons in the basolateral amygdala. Graphs represent Mean ± SEM. N = 60 for AFR, 62 for Stress, 60 for EE and 56 for MSEE, 8-10 neurons/animal.

Stress induced increase in branch points in BLA neurons

A two-way ANOVA was conducted for number of branch points in BLA neurons with stress and EE as two between subject sources of variance. The effect of stress ($F_{(1,237)} = 64.035$, $p < 0.00$), EE ($F_{(1,237)} = 13.231$, $p < 0.00$) and the interaction between stress and EE ($F_{(1,237)} = 25.280$, $p < 0.00$) had a significant effect on the total number of branch points of neurons in the basolateral amygdala.

Orthogonal planned comparisons (independent samples t-tests) showed that, in the absence of EE ($t_{119} = -2.293$, $p = 0.02$), stress significantly increased the total number of branch points of neurons in the basolateral amygdala. Similarly, in the presence of EE ($t_{118} = -8.550$, $p < 0.00$; Figure F.14) stress significantly increased the total number of branch points of neurons in the basolateral amygdala.

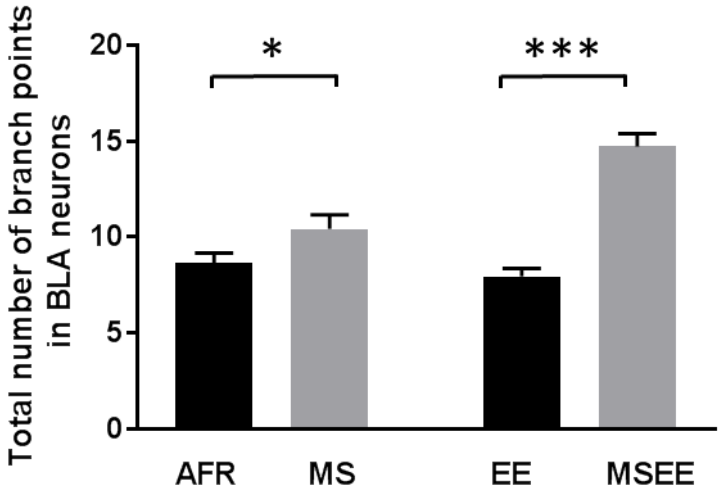


Figure F.14: Effect of early life stress and EE on total number of branch points in BLA neurons: Stress alone and in the presence of EE significantly increased the total number of branch points of neurons in the basolateral

amygdala. Graphs represent Mean ± SEM. N = 79 for AFR, 79 for Stress, 60 for EE and 56 for MSEE, 8-10 neurons/animal.

Discussion

In this chapter, the impact of early life MS, EE and MSEE was studied on physiology, behavior and dendritic morphology during adulthood. This study was designed before the 21-day treatment paradigm discussed as the major results in this thesis was designed. As mentioned earlier, I was curious to study the impact of concurrent exposure of MS and short-term EE during early life. At the onset, we did not know whether exposure to EE for 14 days or 21 days is more effective in inducing pro-resilient features in adulthood. A big difference is the availability of EE one week after treatment (14 days) pre-weaning stage. We detail it further towards the end of this section.

Similar to the result section of the main thesis; physiological, behavioural and morphological output was analysed in this section. To study the impact of early life stress and EE on physiology of adult of male rats, the adrenal glands were investigated, as it is responsible for the production of corticosterone in response to stressful stimuli. MS did not affect the adrenal glands weight, but in the presence of EE, MS decreased the adrenal glands weight. This showed that MSEE group behaved very differently compared to all other groups. Chronic stress is known to lead to an increase in body weight [253]. Additional to the weight adrenal glands, body weight also was studied. In our paradigm, we reported that stress caused a significant increase in body weight, which was already observed earlier [253]. It was also observed that MSEE caused a significant decrease in body weight. MSEE showed varying results when compared to other groups in this study.

Stress did not have any effect on behavioral output but EE induced anxiolytic behavior in EPM, which is comparable to previous literature [166]. The result that was unexpected is that the internal control parameter, closed arm entries has an EE main effect. EE group spent more time in the closed arm indicating the locomotion to be affected. The social exploration of the MSEE group was significantly lower than EE. Interestingly, MS has a significant increase in immobility time in FST which is in line with a previous study [359].

The neuronal morphology analysis showed that EE induced a significant increase in dendritic length in BLA neurons and MS caused a significant increase in branch points in BLA neurons. This is in line with previous experiments where stress induced hypertrophy in BLA neurons [7, 17, 21]. We got results in this paradigm, but the stress effects were not as profound as seen in the previous studies. The problems in this paradigm were,

i) Locomotion (closed arm entries) being affected in EPM, which could be due to too much variation in environment before weaning of rats.

ii) The dendritic length and branch points in the BLA neurons were contradicting each other.

iii) Less pronounced pro-resilient features.

Keeping all these crucial pointers in mind, we took a step back to re-think this model and deduced ways to improve this paradigm. As mentioned earlier in previous chapters, BLA is crucial for understanding resilience and stress response. Hence, we looked at the developmental stage of the BLA and it is shown that until weaning, the amygdala is undergoing dynamic process of development [66]. Hence, the speculation was that, enrichment

might be important till the pups were weaned. This is mainly based on the idea that 14 days EE will not suffice for the whole period until weaning (till 21 days postnatal). Additionally, and most importantly, the change from EE cage to normal cage at 14-day time point most likely would have bought additional novelty stress for the nursing mother, which would have indirectly affected the pups in EE groups (both with and without MS). The pups or the dams might undergo stress soon after 14 days EE treatment when placed under standard housing conditions. Hence, we revised our 14 days model of EE to 21 days of EE to come up with potentially better model of resilience (main thesis, chapter 1-5).

Chapter 8

8. References

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APPENDIX

1. Golgi staining

1.1 Golgi-cox commercial kit: FD Rapid GolgiStain™ Kit.

Block of fresh brain was incubated in a mixture of solution A and B (1:1, kit supply) for 24 hours. The solution was replaced with a fresh batch and incubated for 2 weeks for stain impregnantion. The brains were equilibrated using solution C (kit supply) and cryosectioned at 100µm to obtain brain slices on gelatinised slides. The stain was further fully-developed and counter-stained with cresyl-violet by subjecting the sections to the following sequence of solutions:

1. Deionized water (2 min)
2. Working Solution: mixture of solution D (kit supply), E (kit supply) and deionised water (1:1:2) (10 min)
3. Deionised water (30s)
4. 95% Ethanol (1 min)
5. 100% Ethanol (1 min)
6. Xylene (1 min)
7. 100% Ethanol (1 min)
8. 95% Ethanol (1 min)
9. 70% Ethanol (1 min)
10. Deionized water (1 min)
11. Cresyl violet (6 min)
12. 10% Acetic acid (wash 5 times; 5 sec)
13. Water (dips)
14. 50% Ethanol (30s)
15. 70% Ethanol (30s)
16. 95% Ethanol (30s)
17. 100% Ethanol (2 min)

18. Xylene (30s)
19. Permount and close with coverslip (Fixation)

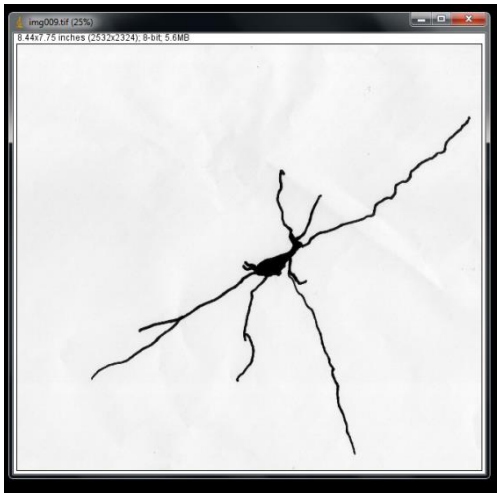
1.2 Cresyl Violet solution

1g of Cresyl Violet (Acros organics, Geel, Belgium) was dissolved in 400ml of deionized water and stirred for 1 hour. It was filtered using a 0.45 µm filter and the pH was adjusted to 3.6 using 10% acetic acid.

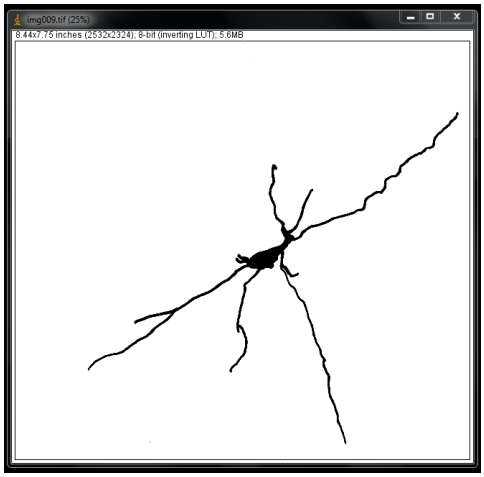
2. Dendritic arborisation

I scripted an ImageJ custom-designed routine for neuronal analysis in JAVA for the dendritic complexity analysis. In order to analyse branch points and total dendritic length via Sholl analysis, JAVA-based ImageJ macro was scripted for following the algorithm (Images represent the resultant execution of corresponding codes on ImageJ:

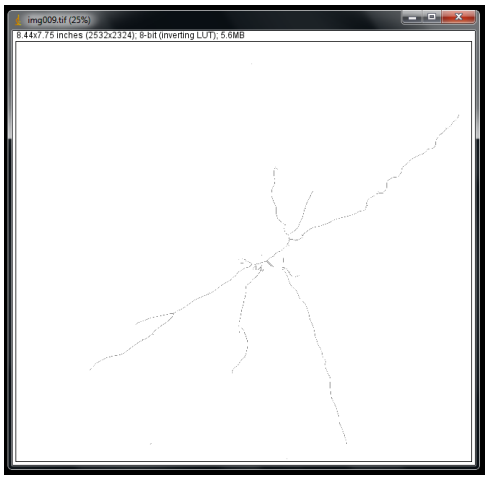
i. Image of a neuronal trace (8-bit TIFF file) is opened on ImageJ and the scale is set to area of 1pixel=1µm2.



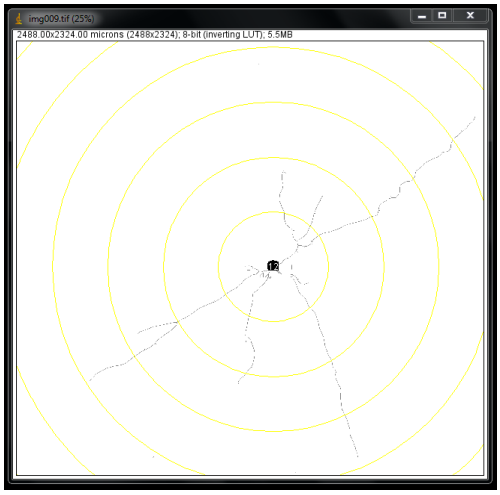
ii. The threshold of image is adjusted automatically and digitized using binary option.



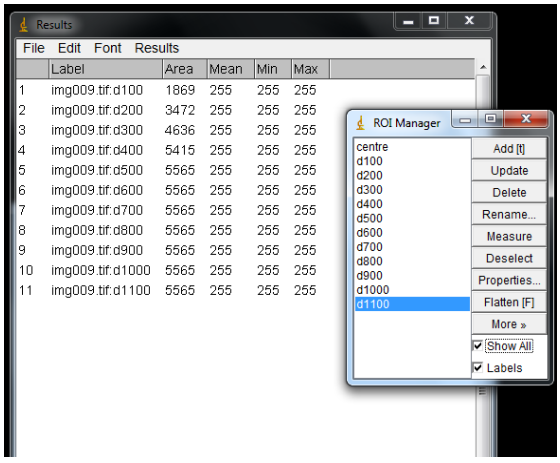
iii. The image is skeletonised to a frame of singular pixels.



iv. An overlay of concentric circular region of interests (ROI) increasing in radius (by 50μm), with soma as the centre, is formed by applying scaling-factor (determined through microscope).



v. The area of pixels in each ROI is calculated by ImageJ. It is then convert into distance (segmental and total) dendritic length by multiplying it with the scaling factor. No. of branch points in each ROI is manually counted.



JAVA code written for the macro:

```
//all commented out lines of code are for user reference of initial processes

//run("Clear Results");
//roiManager("Delete");
run("Set Scale...", "distance=1 known=1 pixel=1 unit=micron");
setAutoThreshold("Default");
//run("Threshold...");
setAutoThreshold("Default");
//setThreshold(0, 135);
//run("Convert to Mask");
run("Close");
run("Make Binary");
//print ("Select centre");
//setTool("point");
//makePoint(1024, 1008);
getCursorLoc(x,y,z,flags);
print ("The centre is"+x+","+y);
makePoint(x, y);
run("Add to Manager");
roiManager("Select", 0);
roiManager("Rename", "centre");
run("Skeletonize");
//incorporate scaling-factor for micron to pixel conversion (where s =
reciprocal of scaling factor);
s=5.885;
for(i=1; i<12; i++)
{
d=100*s*i;
roiManager("Select", 0);
//run("Specify...", "width=100*i height=100*i x=1024 y=1008 oval centered");
makeOval(x-0.5*d,y-0.5*d,d,d);
roiManager("Add");
roiManager("Select", i);
roiManager("Rename", "d"+i+"00");
roiManager("Select", i);
run("Analyze Particles...", "size=20-Infinity circularity=0.00-1.00
show=Nothing display");
//results saved as excel file wherein file location is provided by user in the
paranthesis;
saveAs("Results", "C:\\Program Files\\ImageJ\\results\\neuron1.xls");
}
```

Note: s = pixels/unit micron = 5.885 (in this case)

The scaling factor is the reciprocal of s, which is multiplied with the (total) dendritic length obtained as the result of the macro, which retrieves the final value in microns.

3. Paraformaldehyde (PFA) solution (4%, 1L)

40g of 95% prilled PFA (Sigma-Aldrich, MO, USA) was dissolved in 1X phosphate buffer saline. 5M sodium hydroxide was used for catalysing solubility. The solution was filtered and pH was adjusted to 7.4 using 2M hydrochloric acid.

3.1 Phosphate Buffer Saline (10X, 1L)

80g Sodium Chloride, 2g Potassium Chloride, 14.4g disodium hydrogenphosphate, 2.4g Monopotassium phosphate in 1L (pH adjusted to 7.4)

4. Reagents for Immunofluorescence staining

4.1 Blocking buffer

5% Bovine serum albumin in 1X phosphate buffer saline (PBS).

4.2 Primary antibody buffer

5% Bovine serum albumin, 0.1% Triton-X in 1X phosphate buffer saline (PBS).

4.3 Secondary antibody buffer

5% Bovine serum albumin in 1X phosphate buffer saline (PBS).

4.4 DAPI solution

DAPI solution (5mg/ml) was diluted to 1:1000 in 1X phosphate buffer saline (PBS) containing 5% Bovine serum albumin.

5. Reagents for Western blotting

- Bovine Serum Albumin (BSA) (Biorad, USA, Cat No. 500-0007)
- Mammalian protein extraction reagent (Pierce, USA Cat No. 78503)
- Pico chemiluminescent substrate Kit (Pierce, Thermo Scientific, USA, Cat No.34080)
- Precision Plus Protein Standards Kaleidoscope (Bio-Rad, USA, Cat No. 161-0375)
- Protease inhibitor cocktail kit (Pierce, USA, Cat No. 78410)
- Protein assay kit (Bio-Rad, USA, Cat No. 5000002)
- Stripping buffer (Pierce, Thermo Scientific, USA, Cat No. 0021059)
- Tissue protein extraction reagent (Pierce, USA, Cat No. 78503)

5.1 10X TBS (pH 7.6; 1L)

30g Tris base, 80g Sodium Chloride in deionized water (made upto 1000ml)

5.2 1X TBST

10X TBS diluted to 1X with deionized water and 0.1% Tween-20.

5.3 5% blocking buffer (20ml)

1g of non-fat dry milk powder was thoroughly dissolved in 20ml of 1X TBST

5.4 Antibody buffer (5% non-fat dry milk solution)

250mg of milk powder was thoroughly dissolved in 5ml of 1X TBST and the required amount of stock antibody was replaced with the same amount of milk solution to attain the necessary dilution.

5.5 Stripping buffer (50ml)

- Stock: 3.78g Tris base was dissolved in 500ml deionized H2O (pH 6.7)
- Stripping buffer: 1g of SDS was added to 50 ml of stock and warmed up to dissolve it. On cooling back to room temperature, 350µl of β mercapto-ethanol was added just before use

5.7 Mini-PROTEAN TGX Precast Gel

5.7 Trans-Blot® Turbo™ Midi PVDF Transfer Pack

5.7 Chemiluminiscent substrate

Luminol was used as the chemiluminiscent substrate, commercially available as ECL select, G.E. healthcare, U.K. It consists of light sensitive luminol solution and peroxide solution which were mixed together (1:1) and applied over the protein impregnated membrane.

List of two-way ANOVA p values for qPCR and wester blot

A) qPCR table

Molecule	Stress	Enriched environment	Interaction
TrkB	0.27	0.2	0.84
Arc	0.79	0.17	0.93
Erk	0.29	0.33	0.27
MR	0.09	0.93	0.54

B) Western table

Molecule	Stress	Enriched	Interaction

		environment	
TrkB	0.77	0.27	0.85
Arc	0.48	0.65	0.85

LIST OF PUBLICATIONS

1. **Akshaya Hegde**, Poh Soh Yee, and Rupshi Mitra. "Dendritic Architecture of Principal Basolateral Amygdala Neurons Changes Congruently with Endocrine Response to Stress." *International journal of environmental research and public health* 14.7 (2017): 779.
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