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

**AKSHAYA HEGDE NAGARAJAN
SCHOOL OF BIOLOGICAL SCIENCES**

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AKSHAYA HEGDE NAGARAJAN

School of Biological Sciences

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic 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 Tegmental Area

1 **ABSTRACT**

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

24

25 Chapter 1

26 1. Background

28 1.1 Over-riding influence of the early life stress

29
30 Individual animals show remarkable variance in their behavior depending on
31 the incipient environment during the early period of their life. Behavioral and
32 physiological phenotypes for the same genetic backgrounds are often
33 changeable (plastic) in response to changes in the environment [1-3]. Pigliucci
34 et al. (2006) defined *phenotypic plasticity* as ‘the ability of a single genotype to
35 give rise to various phenotypes when exposed to differential environmental
36 conditions’[4]. This involves the possibility to change the developmental
37 pathway (*developmental plasticity*) of an individual in response to a particular
38 environment cue and also the ability of an individual to alter their phenotypic
39 activity (e.g., metabolism) in response to differential environmental conditions
40 [5]. It is seen that, the earlier an organism’s development is disturbed, higher
41 the amplitude of detrimental effects [6, 7]. Given the rate at which the
42 environment is varying globally, studying the consequence of the changing
43 environment and the relationship between the environment and the phenotypic
44 plasticity is of enormous importance. Animals have evolved by adapting
45 themselves to various dynamic environments through temporary or permanent
46 changes in the physiology and behavior of an individual, depending on the
47 environment that they were exposed to during the early stage of life. For
48 example, aging was delayed, and lifespan was increased in rats that were
49 exposed to a particular diet during adulthood [8] and It was hypothesized that
50 animals have an adaptive response to famine wherein the resources are shifted

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

55 During the early life, the time window that is vulnerable to the changing
56 environment is commonly referred to as the '*critical*' or '*sensitive*' period [11].

57 An organism process various stimuli of visual, olfactory and social cues from
58 the surroundings that are required for its normal development during the
59 sensitive period [12]. Observations show that during the sensitive period,
60 development of an individual depends on the type of cue perceived from the
61 environment [13]. The organism utilizes the best options from the available
62 environment and this helps in development [14]. Some studies suggest that,
63 sensitive period during early life is crucial for development due to the
64 complication involved in reversing developmental mechanisms later in life [15].

65 If the phenotype obtained in early life is not compatible with the conditions
66 later in life, developmental plasticity might have detrimental effects on the
67 survival and reproductive performance of the individual in future. Prior studies
68 have shown that developmental plasticity is beneficial to numerous species but
69 can have a different impact during a degree of variation in the environment
70 later in life [16-19]. At this juncture, it is very clear that environment during the
71 *sensitive period* plays a crucial role for the normal development of an
72 individual.

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

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

88 *1.2 Mother-infant interactions*

89

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

102 is high and the later life has a favourable environment, the risk of having health
103 problems is very low [31]. Therefore, the cues an individual receives prenatally
104 has a huge influence on the early development and health success [32]. A
105 classic illustrator of this idea is the Dutch hunger during the Second World War.
106 Pregnant women in the Dutch famine in 1944 underwent starvation and this
107 caused adverse foetal environment due to lack of nutrition and energy
108 supplements. These prenatal harsh conditions caused low body weight of the
109 babies at birth and these individuals were susceptible to compromised cognitive
110 functions, cardiovascular disease and obesity despite adequate food intake later
111 in life. This was due to the food deprived environment that persisted during
112 their foetal stage, which programmed their bodies to conserve nutrients
113 throughout the course of their life [33]. The information above shows that early
114 life focuses on preparing an offspring for a future environment based on the
115 cues it receives during the crucial period of development even before the birth.
116 Mother-infant relationship during postnatal life plays a cardinal role in
117 phenotypic plasticity too. Bowlby stated that, a mother's love during early life
118 is as crucial for 'mental health' as proteins and vitamins are for 'physical health'
119 [34, 35]. Multiple studies have focused on the importance of mother-infant
120 relationship for the behavioural and cognitive development of infants [34, 36,
121 37]. Studies have shown that mothers with post-partum depression (PPD) have
122 long-term issues that affect the positive interaction with their child. This can
123 have damaging effects on the child's cognitive and emotional development [38-
124 40]. Mothers who have undergone early life stress due to childhood abuse have
125 problems in interacting with their children and this impacts negatively on the
126 child's development [41, 42]. Therefore, mother-infant relationships have a

127 long-term effect on a child's future. It has also been reported that effective
128 intervention at an earlier stage is attainable, if the problem is detected early on
129 [43]. A positive mother-infant relationship (postnatal environment) is
130 beneficial for development of the affective behaviour of the infant [44, 45].
131 Therefore, the manner in which an infant establishes the relationship with the
132 mother influences the manner in which it establishes relationships with every
133 other facet in the environment throughout the rest of her/his life [46]. Based on
134 all information available on mother-infant interaction, this relationship has
135 been validated in rodents in lab conditions as well and is described below.

136 *1.3 Mother-infant relationship validated with rodents*

137

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

152 Additionally, they showed that adverse conditions in early life could cause
153 long-term behavioural deficits and epigenetic changes in glucocorticoid
154 receptor in rat hippocampus [48]. Thus, the interaction between mother and
155 pup is as compelling as merely the presence of the mother. RM Sullivan et al.,
156 (2006) determined that, in the absence of the mother, rat pups displayed odour
157 avoidance when exposed to odour-shock conditioning along with their
158 amygdala activation. The same conditioning initiates a preference to the odour
159 in the presence of mother without the amygdala activation. They also
160 determined that this variation in behaviour due to mother's presence is
161 regulated by the modulating the corticosterone which in turn helps in the
162 moderation of the amygdala activity [49, 50]. These studies show that in the
163 presence of mother, the pups do not have a fear/stress response. The proximate
164 mechanism underlying this fear/stress response have been shown to involve the
165 amygdala and the corticosterone [51]. The role of amygdala in modulating
166 stress response is described in the next section.

167 *1.4 Amygdala and emotion*

168

169 Stress is known to induce dendritic hypertrophy in the basolateral amygdala
170 (BLA) [52, 53] and to enhance anxiety [53, 54]. Stress also causes increase in
171 secretion of glucocorticoids (GC) that has harmful impact on an individual [55,
172 56]. It was observed that a single acute dose of corticosterone was sufficient to
173 produce dendritic hypertrophy in the BLA and anxiogenic behavioural output.
174 Additionally, this pattern of dendritic hypertrophy after acute treatment was
175 comparable to that caused by chronic treatment of corticosterone [57]. These
176 studies affirm the crucial role of amygdala in modulating stress response
177 making it an interesting region to explore in early life stress models. As

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

182 Unlike the hippocampus, very little is known about how early life stress affects
183 the amygdala and the nature of its role in modulating stress response.
184 Amygdala constitutes a group of nuclei that helps in modulation of various
185 functions related to initiation, processing and consolidation of fear memory
186 [59]. Each nucleus of amygdala comprises of different neuronal population
187 ranging in structure and function. The function of amygdala has been
188 extensively studied in context of Pavlovian fear conditioning [60, 61]. The fear
189 stimulus reaches amygdala from the thalamus or the cortex. This information is
190 further transmitted from the basolateral amygdala (BLA) to the central
191 amygdala that is crucial for expression of fear induced by the stimuli [62-64].
192 BLA being the primary nucleus to receive signal from the fear stimulus can be
193 considered as an essential brain region for formation of fear induced
194 behavioural response and this makes the BLA intriguing. BLA activation relays
195 projections to switch on the HPA axis for generating a stress response [65]. The
196 BLA comprises of seven specific types of cell populations [66] out of which
197 the predominating pyramidal neurons, stellate neurons and GABAergic local
198 interneurons are three major kind of cell populations. Pyramidal and stellate
199 neurons are glutamatergic, which are excitatory in nature and the GABAergic
200 local interneurons, are inhibitory in nature [67, 68]. Activation of BLA
201 mediates the activation of the central amygdala (CeA) or the medial amygdala
202 (MeA) via the excitatory neurons mentioned earlier [69]. This further causes

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

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

222 *1.5 Impact of harsh environment*

223

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

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

243

244 *1.6 The ‘best’ way out of the worst environment*

245

246 Despite the fact that a stressful developmental stage causes various abnormal
247 phenotypes that is harmful for the offspring, a school of thought expresses that
248 these abnormalities could be beneficial considering the environment is the
249 cause of the abnormality. Certain compromised developmental experiences can
250 heighten memory processes and learning under stressful situations [77]. In
251 certain cases, it is observed that this kind of putative abnormal and irregular
252 morphological, physiological alterations can stimulate defensive and
253 reproductive system for survival [78, 79]. Hence, early life adaptive coping
254 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

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

283

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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].

320 *2.2 Stress & Resilience: Two sides of a coin*

321

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

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

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

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

346 helplessness, majority of them were stressed but a subset ($\approx 30\%$) exhibited
347 resilient (*active coping*) feature wherein their escape latencies (successful
348 escape) were comparable to non-stressed counterparts [103]. A study on
349 consequences of premature children in poverty revealed that, around 10% of
350 the affected kids displayed resilience in cognition, growth and overall health
351 [104]. In another study, around 40% of maltreated children displayed resilience
352 in spite of their stressful childhood [105]. Thus, it is intriguing to focus on
353 previously mentioned long-term resilience [102]. Though resilience exists in
354 certain individuals innately, it is present in minute percentage. This makes it
355 interesting to explore various ways and methods to induce resilience in
356 vulnerable individuals, that can help them to cope up with stress, better.

357 *Why study stress & resilience?*

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

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

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

377

378 *2.3 The internal system: HPA axis*

379

380 Activation of Hypothalamic-Pituitary-Adrenal (HPA) axis in response to stress
381 is initiated by corticotropin-releasing factor (CRF), which is synthesized and
382 secreted by hypophysiotropic neurons localized in paraventricular nucleus of
383 the hypothalamus. CRF, the principle regulator of the axis, activates CRF
384 receptors in anterior pituitary causing the release of Adrenocorticotrophic
385 (ACTH) hormone into the systemic circulation. ACTH then activates the
386 receptor on adrenal cortex to stimulate the Glucocorticoid (GC) synthesis and
387 further causes the release of Glucocorticoids from zona fasciculata into the
388 bloodstream, which regulates the physiological and behavioral changes in
389 response to stress and brings it back to baseline, when the stressor is gone.
390 Incomplete or exaggerated activation of the HPA axis leads to emergence of
391 pathological conditions [111, 112]. “Hypothalamic-Pituitary-Adrenal (HPA)
392 axis” (Fig.1) releases glucocorticoids (stress hormones) to induce a flight or
393 fright response and helps the body to return to homeostatic equilibrium after
394 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

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

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

458 Therefore, the interaction of the brain **Hippocampus-PFC-Amygdala** axes and
459 **Hypothalamus-Pituitary-Adrenal** axes influences the behavioral read out of an
460 individual. As seen earlier, stress has major impacts on the *structure of brain*,
461 *neuroendocrine system* and *behavioral outcome*, which are the interacting triad
462 in response to stress. In light of this, my study will focus on environment-

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

466 *2.4 Influences of differential early-life environment*

467

468 The environment plays a crucial role in molding BLA, hippocampus and
469 mPFC; the regions of the brain that are involved in regulating HPA axis as
470 mentioned above. Social attachment is an integral environmental component
471 that influences an individual's behaviour. The 'social brain hypothesis'
472 explains the large brain size of primates. It states that a primate's social group
473 size has a positive correlation with the volume of its prefrontal cortex [143].
474 This correlation between PFC volume and social network size was found in
475 humans as well, which is reflected in their social cognitive abilities [144]. This
476 shows that the environment plays a major role in modulating brain regions. Of
477 all the stages in life, early life is the most crucial one because this is the
478 developmental stage wherein the environment plays a crucial role in driving
479 behaviour and health for rest of the life. Exposure to chronic or severely
480 stressful events in early life is a potential threat to physiological and
481 psychological well-being. An individual's early life contributes to shaping the
482 physical and emotional states in adulthood. Adverse experiences during this
483 period result in manifestation of emotional disorders [145, 146]. A growing
484 body of evidence shows that a disturbed relationship between parent and child,
485 childhood negligence, abuse, and any such adverse childhood experience
486 causes physical and behavioural dysfunctionalities that reflects on adulthood
487 [147-149]. Adverse childhood experiences are seen to increase the risk of
488 committing suicide [150-152]. Apart from physical and emotional damage,

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

493 The influence of environment doesn't just commence from birth; it starts
494 affecting the individual right from the foetal stage. A classic illustrator of this
495 idea is the Dutch hunger during Second World War. In the Dutch famine of
496 1944, pregnant women underwent starvation, resulting in an adverse foetal
497 environment inside the womb due to lack of nutrition and energy supplements.
498 These harsh prenatal conditions resulted in babies with low body weights at
499 birth, who were susceptible to decline of cognitive functions, cardiovascular
500 diseases, and obesity despite adequate food intake in the later stages of life.
501 This was due to the food-deprived environment that persisted during their
502 foetal stage, which programmed their bodies for forty or more years, to be
503 unable to get over the early period of malnutrition. [155].

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

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

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

528 *2.5 Intervention*

529

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

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

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

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

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

565 Animal models are invaluable for an understanding of the mechanisms
566 involved in sequelae of early life environment. An interesting early-life study
567 in rodents showed that natural fear of predator odor develops after postnatal
568 day (PND) 10 and this is correlated with the function of amygdala and
569 regulation of corticosterone (CORT) [170]. As mentioned in the background,
570 the study highlighted that maternal presence acts as an environmental switch
571 during infancy for stabilizing fear responses [171]. At this point we know that
572 harmful effects of MS can be reversed via short-term enrichment in adulthood
573 [87] and my effort is to find out if short-term EE given in early life
574 concomitant to maternal separation (MS) will alleviate the physiological,
575 behavioral, neuronal morphological and molecular changes insults. The
576 following paragraphs will discuss about the background of the two important
577 paradigms that was used in this study, MS and EE.

578 *2.6 Maternal Separation (MS)* 579

580 Various studies have shown that any form of alteration in maternal care can
581 have a lasting effect on the stress response, behavior, and emotion in rats, as it
582 is one of the most crucial influences during development [172]. Manipulations
583 in early environment cause acute and long-term effects on endocrinology and
584 behavior, which have been studied in various models including rats, mice,
585 primates, etc. Bonnet infant monkeys show a compromised behavioral and
586 physiological response to maternal separation. Similarly, pig-tailed and rhesus
587 infant monkeys exhibit a maladaptive response to maternal separation wherein
588 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

640 hippocampus of the pup, causing epigenetic and behavioral changes in
641 adulthood. MS caused downregulation of hippocampal GR [189]. However,
642 unaffected epigenetic changes have also been reported in MS rats where DNA
643 methylation in the same region of Nr3c1 of the hippocampus does not occur
644 [190]. MS rats are seen to have long-term adverse effects like increased level of
645 plasma ACTH. Effects of maternal deprivation were not only limited to the
646 HPA axis, but elevated CRH mRNA expression was also seen in the amygdala,
647 which most certainly plays a role in the stress response [191]. This shows the
648 influence of molecules in amygdala mediating stress response in MS rats. MS
649 rats exposed to acute restraint stress were reported to have elevated levels of
650 Fos positive cells (an estimate of neuronal activation) in the central nucleus of
651 the amygdala, PVN and bed nucleus of the stria terminalis and each of these
652 brain regions play a critical role in organizing the behavioral response to stress
653 [192-194]. Though very little is known about the CRF receptors, few studies
654 have shown that after MS for 24 hours CRF receptor two is down-regulated in
655 ventromedial hypothalamus and biomedical amygdala [195]. Prior studies
656 show that global effect of early life experience on the hippocampal
657 transcriptome alters more than 900 genes in response to maternal care that in
658 turn causes anxiety mediated behavior. Few of these changes seen in gene
659 expression is reversed using histone deacetylase inhibitor and methyl donor.
660 Such epigenetic changes due to MS have been widely studied in the
661 hippocampus [190, 196, 197].

662 *2.9 Enriched Environment (EE)*

663

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

666 “enriched environment” as an experimental concept. He reported that his pet
667 rats displayed behavioral improvements as compared to his laboratory rats
668 [198]. So to test this in an experimental setup, an “enriched” environment about
669 a standard cage was designed, where the cages were much larger with more
670 complex social interaction, and it also consisted of the exploratory environment
671 with tunnels, toys, nesting material and running wheels in some cases [199-
672 201]. This study has no voluntary wheel as the focus of the study was on the
673 effects of housing condition and not exercise [166, 202, 203].

674 *2.10 Effects of EE on behavior and GC levels*

675

676 The environment affects the shape and organization of animals which are more
677 adaptable to it and are selected naturally to be propagated to further
678 generations. These traits might be associated with behaviour of animals as well;
679 particularly those, which are sensitive to environmental change. Mice exposed
680 to the enriched environment had enhanced memory function in various learning
681 tasks. These mice performed better in a spatial memory task (water maze task)
682 than controls in standard housing cage [204]. Rodents exposed to an enriched
683 environment, both before and after a cerebral insult show improved cognitive
684 performance [205]. Recent studies have demonstrated that EE can influence
685 prominent innate behaviors like avoidance of predator odor and attractiveness
686 of males [206]. Clinical evidence showing reduced risk of dementia in
687 individuals who have Parkinson's and Alzheimer's disease indicates that a
688 stimulating environment has positive effects on cerebral health [207]. To see
689 the benefits of EE, the same strain of mice was divided into two groups based
690 on their exploratory behavior in open field task namely, high exploratory (HE)
691 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

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

721 *2.11 Effects of EE on neuronal plasticity and molecules in the brain* 722

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

738 Environmental enrichment and voluntary exercise exhibit similar effects in the
739 brain. Studies showed that this change in environment and playing on wheels
740 affected neurogenesis. Running enhanced the survival of the newborn neurons
741 in the dentate gyrus [216, 217]. In addition to its effects on neurogenesis,
742 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

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

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

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

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

801 *2.12 Rationale behind this study*

802

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

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

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

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

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

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

827 **Specific aim 4:** *To delineate underlying possible epigenetic changes that*
828 *drives S.A.1, S.A.2 and S.A.3 in adult male rats.*

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841 Chapter 3

842 3. Materials and Methods

843 3.1 Animals

844 Male and female Wistar rats (7 weeks old and housed 2/cage) were procured
845 from Charles River, Kingston, North America. Breeding was initiated between
846 a pair of rats after at least two weeks of habituation in Nanyang Technological
847 University vivarium. Animals were maintained in a 12h light-dark cycle (0700-
848 1900 h light on, 1900-0700 h light off) with *ad libitum* food and water. The
849 institutional animal care and use committee (IACUC) of the NTU approved all
850 experimental procedures.

851 *Breeding protocol*

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

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

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

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

884

885 Experimental paradigms

886

887

888 **Figure M.1: Timeline of experiments showing the duration of early life**

889 **treatments as well as the timeline of behavioral tests.** Termination day varies

890 depending on the endpoint of the study. Please refer to the respective schedule

891 for each experiment.

892 3.2 Maternal separation (MS)

893

894 On P2-P14 (daily), using a gloved hand, the mother was placed in a clean cage

895 with fresh bedding and returned to the shelf. Pups were then moved one-by-one

896 into a clean cage as a group, brought to another room, kept on a heat pad and

897 monitored for three hours. After separation, pups were returned to the home

898 cage, placed in the nest and sprinkled with dirty bedding. The mother was then

899 returned to the home cage.

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

904
905 **Figure M.2: Maternal separation model.** Dam is kept in a clean cage in a
906 room and pups were kept in a clean cage as a group in another room and
907 monitored for three hours.

908

909 *3.3 Environmental enrichment (EE)*

910

911 The EE groups were housed in Enrichment (EE) cages on P2-P21 (1 dam and
912 her litter). The EE cages (72 x 51 x 110 cm³) were more spacious than standard
913 cages. They were equipped with plastic pipes for burrowing, plastic and
914 wooden toys, jingles, platforms, ladder for climbing, baskets, and nesting
915 material. EE cages were also wire meshed for rats to climb. The arrangement of
916 the various stimulatory objects within the EE cage were changed every 4th day,
917 and some fruits loops and sunflower seeds were also dispersed every 4th day.

918

919 **Figure M.3: Environmental enrichment cage.** EE cages (72 x 51 x 110 cm³)
 920 were equipped with plastic pipes for burrowing, plastic toys, wooden toys,
 921 jingles, platforms, ladder for climbing, baskets, and nesting material.

922 *3.4 Maternal separation + Enriched Environment (MSEE)*

923

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

927 *3.5 Home cage emergence test*

928

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

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

939

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

943 *3.6 Open field test*

944

945 Open field test was conducted in a circular arena. Animals were placed in an
946 open arena (radius=120 cm, trial duration=300 s, diffused dim light conditions).
947 Time spent in the center of the field was quantified as the reciprocal proxy of
948 the anxiety (center defined as a concentric circle to the arena with 0.33 m
949 radius). All tests were conducted between 09.00AM and 12.00PM (duration =
950 300s). Apparatus was cleaned with 70% ethanol after each animal and allowed
951 to dry before the next test.

952

953

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

957 *3.7 Light dark box test*

958

959 The light dark exploration test was conducted as previously described [235].
 960 The apparatus consisted of a polypropylene cage (separated into two
 961 compartments by a partition, which had a small opening (10 X 8 cm²) at floor
 962 level. The light compartment (28 cm long) was open-topped, and very brightly
 963 illuminated by a desk lamp (500 lx). The dark compartment (24 cm long) was
 964 also open-topped and painted black without light. Rats were individually
 965 placed in the center of the black compartment, facing away from the partition,
 966 and allowed to explore the apparatus for 5 minutes freely. The total time spent
 967 in the light and dark compartment were video recorded and scored blinded
 968 manually afterward. Apparatus was cleaned with 70% ethanol after each
 969 animal and allowed to dry before the next test.

970

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

976 *3.8 Elevated plus maze*

977

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

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

998
999 **Figure M.7: Elevated plus maze setup.** The EPM consisted of a plus-shaped
1000 arena with two open and two enclosed arms. The arena was elevated to 60 cm
1001 above the ground.

1002 *3.9 Social interaction test*

1003

1004 The social preference test was adapted from the protocol described by Vandana
1005 and collaborators to investigate social affiliation in male rats [241]. The test
1006 was carried out in a rectangular, three-chambered box (a center $20 \times 35 \times 35$
1007 cm^3 ; a left and a right compartment $30 \times 35 \times 35 \text{ cm}^3$). Dividing walls had
1008 retractable doorways allowing access to each chamber. Left and the right
1009 compartment were separated from the central compartment with the help of
1010 transparent Plexiglas with small holes, where either a social (unfamiliar rat of
1011 similar weight) or a non-social stimulus (yellow plastic box) was placed. The
1012 Plexiglas permits visual, tactile, auditory and olfactory communication. The

1013 juvenile rats were first habituated to the three-chambered apparatus by placing
1014 them individually in the central compartment for 10 min during the three
1015 consecutive days preceding the social test. The doorways into the two side
1016 chambers were closed during this habituation phase.

1017 On the testing day, the unfamiliar juvenile was placed in one of the side
1018 chambers and the object on the other side. The experimental rat was first placed
1019 in the middle chamber and allowed to explore for 5 min. The location of the
1020 juvenile and the object in the left vs. right side chamber was counter-balanced.
1021 The session was video-recorded and the time spent sniffing each cylinder
1022 containing the object or unfamiliar juvenile was scored offline to evaluate the
1023 level of preference for the unfamiliar juvenile as compared to the object. The
1024 rats were considered to have explored the object and the juvenile when their
1025 behaviour complied with the following criteria: (1) when they were
1026 approaching the respective compartment with their nose at a distance less than
1027 approximately 2 cm (2) when their nose was oriented toward the contents of
1028 the compartment (i.e., juvenile rat or object). These criteria of proximity and
1029 orientation were followed and quantified as sniffing behavior. The entire
1030 apparatus was cleaned with 70% ethanol solution and dried properly between
1031 each test.

1032

1033 **Figure M.8: Schematic of the three-chambered box used to perform social**
1034 **interaction test.** The test was carried out in a rectangular, three-chambered
1035 box. Left and the right compartment were separated from the central
1036 compartment with the help of transparent Plexiglas with small holes, where
1037 either a social (unfamiliar rat of similar weight) or a non-social stimulus
1038 (yellow plastic box) was placed.

1039

1040 *3.10 Porsolt's forced swim test*

1041 The animals were tested for depressive-like symptoms (despair) using Porsolt's
1042 forced swim task [242] . On the first day (T1) of the test, each animal was put
1043 in a water container (diameter = 20 cm, height = 50 cm) for 15 minutes for
1044 habituation. The temperature of the water was maintained at 21-23°C and was
1045 filled up to 33 cm of the container. On the second day (T2) of the test, each
1046 animal was tested for forced swim and videotaped for 10 minutes (conducted
1047 between 09.00AM-12.00PM). After each test, the animal was returned to its
1048 home cage, and the container was cleaned thoroughly with water. I was blind to
1049 treatments during offline analysis. Parameters such as swimming and
1050 immobility (an indication of despair or depressive-like behavior) were timed
1051 and compared across groups.

1052

1053 **Figure M.9: Forced swim test setup.** The animals were tested for depressive-
1054 like symptoms (despair) using Porsolt's forced swim task. The temperature of
1055 the water was maintained at 21-23°C and was filled up to 33 cm of the
1056 container.

1057 *3.11 Measurement of dendritic arborization* 1058

1059 Freshly harvested brains were obtained by sacrifice through decapitation.
1060 Blocks of brain tissue were processed for Golgi-Cox stain using a commercial
1061 kit (FD Neurotechnologies, Columbia, USA). The golgi solution (solution A
1062 and B, mixed in equal amount) was prepared 24 hours before tissue extraction.
1063 The freshly harvested tissue was immersed in Golgi solution soon after the
1064 extraction and these brains were transferred to fresh golgi solution after 24
1065 hours. The brains were kept in the golgi solution for 2 weeks and then frozen
1066 using dry ice. The tissue was then cryo-sectioned (100-µm thick) and further
1067 processed using cresyl violet staining. The stained 100-µm thick coronal
1068 sections were mounted on glass slides. One batch of animals was used to obtain
1069 sections containing both BLA and hippocampus (Bregma -2.04 to -3.36, Figure
1070 M.10A [243]) and another one for sections containing mPFC (Bregma 3.72 to
1071 3.00, Figure M.10B, The Rat Brain, Paxinos and Watson [243]). For mPFC,
1072 neurons from the prelimbic and infralimbic regions were analyzed separately.
1073 Therefore all sections in the second batch contained these regions. The slides
1074 were counter-stained with cresyl violet, dehydrated using an ascending series
1075 of alcohol and xylene, and coverslipped in non-aqueous medium (Permount,
1076 Thermofisher Scientific, USA).

1077

1078 **Figure M.10: Coronal brain section showing sampled BLA, mPFC and**
 1079 **hippocampal region.** A) Representative coronal brain section used for
 1080 sampling neurons from the basolateral amygdala (BLA) and the hippocampus.
 1081 B) Representative coronal brain section used for sampling neurons from the
 1082 Prelimbic (PrL) and the Infralimbic (IL) regions. Image source: The Rat Brain,
 1083 Paxinos and Watson [243].

1084

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

1086 **mPFC, and BLA. Scale bar is 20 μ m 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.

1105 Dendritic spines were counted using the 1000X oil-immersion magnification
1106 using Olympus BX43 microscope. Dendrites directly originating from cell
1107 soma were classified as primary dendrites, and those arising from primary
1108 dendrites were classified as secondary dendrites [246]. Starting from the origin
1109 of the branch, and continuing away from cell soma, spines were counted along
1110 a 60-80 μ m stretch of dendrite depending upon the type of neurons (Table M.1).
1111 One dendrite of each kind (primary/secondary) per cell and 4-10 cells per
1112 animals for dendritic spines were analyzed (Figure M.13). Since morphology
1113 and spine analysis were done independently, but from the same slides, there is
1114 a good chance of an overlap among the cells that underwent analysis for both
1115 morphology and spine analysis.

1116
1117 **Figure M.13: Illustrative representation of neuronal spine analysis.**
1118 Highlighted region indicates a stretch of primary dendrite (at 1000X
1119 magnification) selected from a sample pyramidal neuron for spine analysis.

1120

1121

1122 Table M.1: Details of neuronal types in each brain region selected for morphological
1123 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

1124

1125 *3.12 Measurement of corticosterone, weight of adrenal gland, and* 1126 *body weight.*

1127

1128 The various time points for blood collection are summarised in the figure
1129 below (Figure M.14B). Certain time points were taken post-stress exposure.
1130 The stress exposure was 2 mL of cat urine (predator odor) added to a cotton
1131 pad and placed in the home cage for 5 mins. Tail vein blood samples were
1132 collected from animals for corticosterone measurement. Animals were lightly
1133 restrained in a soft cotton towel, and 50-200 μl blood was drawn from tail nick
1134 at lateral vessels. This method is known to result in minimal stress [247]. Blood

1135 was centrifuged (4700 rpm, 15 minutes; Thermo Scientific Sorvall Legend
1136 XTR, Germany) and separated serum was stored at -80°C until further analysis.
1137 The concentration of serum corticosterone was quantified using an enzyme-
1138 linked immunoassay based commercial kit (Enzo life sciences, NY, USA).
1139 Also, adrenal glands were collected bilaterally using forceps (wiped dry and
1140 clean) during sacrifice and dry weight was taken. Body weights of all animals
1141 were recorded at various time points throughout the experiment to assess
1142 weight gain. The different time points for weighing are summarised in the
1143 figure below (Figure M.14A). Area under the curve (AUC) for-weight gain was
1144 compared across groups.

A

B

1145
1146 **Figure M.14: Experimental timelines for Physiological endpoints.** A)
1147 Experimental timeline for weight measurements. B) Experimental timeline for
1148 blood collection to determine plasma corticosterone levels.

1149 *3.13 Quantification of mRNA abundance in the basolateral*
1150 *amygdala*
1151

1152 Brain tissue was harvested post-decapitation and flash-frozen in liquid nitrogen.
1153 The tissue was stored at -80°C until further analysis. Subsequently, mRNA
1154 abundance was quantified for GR, BDNF, Trk B, Arc, Erk-1 and MR. A
1155 standard method based on SYBR green dye was used as described before [248,
1156 249]. PCR cycles needed to reach a predetermined fluorescence threshold at the
1157 early linear phase of the amplification were determined (Ct, threshold cycle
1158 number). Greater mRNA abundance in this assay results in lower Ct values. Ct
1159 values for all molecules were normalized by subtracting corresponding Ct
1160 values of 2 housekeeping genes (HPRT and GAPDH). Three technical
1161 replicates were used for each determination. Median coefficient of variation
1162 between technical replicates was $\leq 15\%$. ‘No-template control’ and ‘no-RT
1163 control’ were maintained in every plate used in the experiment. Multiple
1164 primers were designed before choosing the final primer. The primer efficiency
1165 of the final primer that was considered in this experiment was $>90\%$. The
1166 baseline threshold was automatically set using CFX Maestro™ Software,
1167 which was reliable and unbiased between the groups and this was confirmed
1168 after each run. The dissociation curve was performed for every single primer to
1169 avoid primer-dimers. For clarity, an example of acceptable and unacceptable
1170 dissociation curve is given in Figure. M.15. Refer to figure M.16 for the
1171 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	AGGCTTCTGGGTGTCACATGG
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-fixed and equilibrated with 30% sucrose in PBS. These brains were cryosectioned in coronal planes for 40 μ m thickness and collected in 24-well plate with anti-freeze media at -20°C. 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

1203 *Immunofluorescence staining for BDNF and pMAPK*

1204

1205 For all staining, sections (with BLA) in series of 6 were selected for each
1206 animal and placed in a 12-well plate. The sections were rinsed three times (10
1207 mins/wash) in 2.5mL phosphate buffer saline (PBS). 2.5 mL per well of
1208 blocking solution (1XPBS + 5% BSA) was added to the sections and incubated
1209 for one hour at room temperature. 2.5 mL of primary antibody with 2 primary
1210 antibodies simultaneously—Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)
1211 (D13.14.4E) XP® Rabbit mAb (1:200, Cell Signalling Technology, MA, USA)
1212 and mouse anti-BDNF antibody (Developmental Studies Hybridoma Bank,
1213 University of Iowa, Iowa) diluted in PBT (PBS + 0.1%Tx100 + 3% BSA was
1214 added, and the plate was incubated for 48 hours at 4°C on a shaker at low
1215 speed. Sections were washed twice with 2.5 mL (10 mins/ wash) 1X PBS at
1216 room temperature. The secondary antibodies were DyLight®549 goat anti-
1217 rabbit IgG (1:1000, Vector laboratories, CA, USA) and DyLight®488 horse
1218 anti-mouse IgG (1:1000, Abcam, CA, USA) diluted in (PBS+5% BSA) was
1219 added to the wells and incubated for 2 hours in the dark (2.5mL/well). Sections
1220 were washed three times with 2.5 mL (10 mins/ wash) 1X PBS at room
1221 temperature and high-speed. For co-labeling with 4', 6-diamidino-2-
1222 phenylindole (DAPI, a nuclear counterstain for blue-fluorescence, Sigma-
1223 Aldrich, USA), the sections were incubated with 2.5 mL/well DAPI solution
1224 for 1 minute in the dark. Sections were washed four times with 2.5 mL (10
1225 mins/ wash) 1X PBS at room temperature and high-speed. Sections were
1226 mounted on Superfrost slides dried and fixed in a nonaqueous medium (PVA-
1227 DABCO, Sigma-Aldrich, USA) on each section and coverslip. Slides were
1228 stored at -20°C until imaging. The images were analyzed using Image J, (NIH)

1229 to quantify average percentage area of BDNF and pMAPK signals. Refer to
1230 figure M.16 for the schedule of the experiment.

1231 *Immunofluorescence staining for nuclear GR*

1232

1233 For all staining, sections (with BLA) in series of 6 were selected for each
1234 animal and placed in a 12-well plate. The sections were rinsed three times (10
1235 mins/wash) in 2.5mL phosphate buffer saline (PBS). 2.5 mL per well of
1236 blocking solution (1XPBS + 5% BSA) was added to the sections and incubated
1237 for one hour at room temperature. 2.5 mL of primary antibody, rabbit anti-GR
1238 antibody (1:500, Santa Cruz, CA, USA) diluted in PBT (PBS + 0.1%Tx100 +
1239 3% BSA was added, and the plate was incubated for 48 hours at 4°C on a
1240 shaker at low speed. Sections were washed twice with 2.5 mL (10 mins/ wash)
1241 1X PBS at room temperature. The secondary antibody, DyLight®549 goat anti-
1242 rabbit IgG (1:1000, Vector laboratories, CA, USA) diluted in (PBS+5% BSA)
1243 was added to the wells and incubated for 2 hours in the dark (2.5mL/well).
1244 Sections were washed three times with 2.5 mL (10 mins/ wash) 1X PBS at
1245 room temperature. For co-labeling with 4', 6-diamidino-2-phenylindole (DAPI,
1246 a nuclear counterstain for blue-fluorescence, Sigma-Aldrich, USA), the
1247 sections were incubated with 2.5 mL/well DAPI solution for 1 minute in the
1248 dark. Sections were washed four times with 2.5 mL (10 mins/ wash) 1X PBS at
1249 room temperature. Sections were mounted on Superfrost slides dried and fixed
1250 in a nonaqueous medium (PVA-DABCO, Sigma-Aldrich, USA) on each
1251 section and coverslip. Slides were stored at -20°C until imaging.

1252 *Confocal Imaging*

1253

1254 Four randomly selected regions of interest (ROI) for each animal was imaged
1255 at 400X magnification using the confocal laser scanning microscope LSM 710
1256 (Carl Zeiss, Oberkochen, Germany). Immunofluorescent images were obtained
1257 for a 40µm thick cross-section set as the ROI (zoomed to 2X) which was
1258 imaged into a z-stack of 35-40 slices (interval: 1µm). Each z-stack was imaged
1259 at a fixed area which was identical across all the ROIs in all sections in this
1260 experiment using Image J, (NIH) to quantify average percentage area of GR.
1261 The open-source Vaa3D (Allen Institute for Brain Sciences, Washington, USA
1262 software [250] was used to quantify HD-GR. Refer to figure M.16 for the
1263 schedule of the experiment.

1264
1265 The same images were used to quantify “percentage high density GR cells”.
1266 The cells that were quantified as *high density* manually based on the amount of
1267 signal present in the nucleus and these cells are represented in figure M.17.
1268 VAA3D was used to visualize the DAPI-stained nucleus with high density GR
1269 (HD-GR). These HD-GR cells were counted manually.

1270

1271

1272 **Figure M.17: Panel representing high-density GR stained cells.**

1273 Immunofluorescence staining of the nucleus with DAPI (red) and GR (green).

1274 These are the representative images of the cells that contained high-density GR

1275 and were considered for high-density GR analysis. The image was

1276 pseudocolored with red and green for clarity. Scale bar represents 5 μ m.

1277 *Tissue preparation for Western Blotting*

1278

1279 Freshly harvested brains were obtained by sacrifice through decapitation,

1280 which was snap-frozen using liquid nitrogen. Harvested brains were

1281 cryosectioned in coronal planes at 100 μ m thickness, and the BLA was

1282 collected by bilateral punches using biopsy micro punch and Pasteur pipette

1283 tubes in sections from Bregma -2.04 to -3.36 (The Rat Brain, Paxinos, and

1284 Watson). Lysis buffer (1ml TPER + 10 μ L of 100x EDTA + 10 μ L of a protease

1285 inhibitor; 80 μ L per sample) was added to each tube at room temperature. After

1286 ten minutes, tissue was homogenized and centrifuged at 14,000 RPM for 20

1287 mins at 4°C. The aliquot supernatant containing the protein was stored as
1288 samples at -80°C.

1289 *Western Blotting*

1290

1291 Expression of GR, TrkB, BDNF, pro-BDNF and Arc were determined by
1292 Western blotting.

1293 Bovine serum albumin (BSA) standards were prepared and used to generate a
1294 standard curve for the estimation of sample protein concentration. MilliQ water
1295 was used to make a 1:10 dilution of the sample and 10 µL of diluted sample
1296 was added to a 96 well plate in duplicates. Protein assay dye (200 µL) was
1297 added to each of the above wells and incubated for 15 minutes. Absorbance
1298 was measured at 595 nm in the Tecon 200 machine with the help of i-software.
1299 The following sequence was followed: choose plate type → select well reading
1300 → 15 seconds shaking → Reading 3x3 for each well → 595nm. Samples were
1301 then loaded into pre-cast gels and run for 45 mins at 145V. The Transfer turbo
1302 cassette (Bio-rad, Singapore) was used to transfer the gel for 7mins. Blot was
1303 cut based on required size and blocked in 5% BSA (made in TBST 1X) for 1
1304 hour. Blot was incubated with primary antibody for ~14 hours (diluted with
1305 three %BSA made in TBST 1X). Blot was washed three times with 1X TBST
1306 (10 min/ well). Blot with incubated with secondary antibody for 1 hour (diluted
1307 with 3 %BSA made in TBST 1X). Blot was washed three times with 1X TBST
1308 (10 min/ well). The substrate was added to blot (A+B; 1:1) and developed. Blot
1309 was saved by placing in 10X TBS. Blots were first probed with specific
1310 primary antibody for all the proteins and beta actin (internal control). The
1311 bands were visualized using ECL select (G.E. Healthcare, UK). Blots were

1312 developed using LAS4000 image reader version 2.02 (Fujifilm, Japan). The
 1313 relative intensity of the protein bands was quantified using NIH ImageJ
 1314 (version 1.50b; NIH, USA). Refer to figure M.17 for the schedule of the
 1315 experiment.

1316

1317 **Table M.3: Details of primary and secondary antibodies used in Western**
 1318 **Blotting**

1319

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)

1320

1321

1322 *3.15 Determination of methylation status for GR and BDNF*

1323

1324 On Day 60, rats were taken from the housing room, weighed and immediately
 1325 decapitated. The rat brains were extracted from the skull, and the basolateral
 1326 amygdala was dissected on ice and frozen at -80°C . DNA extraction was done
 1327 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 ($R^2=0.947$) and that there was small sample deviation (slope=0.963) [251].

A

251864- GGCAGAGGAGGTATCATATGACAGCTCA¹CGTCA
AGGCAG²CGTGGAGCCCTCT³CGTGGACTCCC -251926

B

GTTCTCTG¹CGGCA²CGCCCACTTCTAGCAGATAAGGC³CGGG⁴CGGGC
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

1349

1350

1351 *3.16 Statistical analysis*

1352 All experimental data were analyzed using two-way analysis of variance (inter-
1353 subject source of variance = stress and EE). More importantly, since the aim of
1354 the experiment is to compare the non-stressed individuals to their stressed
1355 counterparts in the differential housing environments, planned comparisons
1356 were conducted for effects of stress in absence and presence of the EE (two
1357 orthogonal comparisons, independent sample t-test). The reason for including
1358 two-way ANOVA results is to show the main effects of EE-housing, which is
1359 relevant due to the use of two mutually exclusive treatments in this 2x2 design.
1360 This helps in understanding how EE-housing is overall affecting the non-
1361 stressed and stressed subjects. Non-orthogonal comparisons were not used in
1362 order to avoid spurious effects of multiple testing. The level of significance was
1363 set at $p < 0.05$ for all the statistical tests performed in this study. Figures
1364 represent mean and SEM (standard error of the mean). N is stated within the
1365 figure legends.

1366 **Methods (For supplementary chapters only)**

1367

1368 *Novel Object Recognition Test*

1369

1370 The test was adapted from previously described protocol by Redrobe et al.,
1371 Briefly, rats were habituated to the test room for 24 h before testing. The test
1372 was performed in a dimly lit open field arena of the size 1.2m in diameter. On
1373 day one, the rats were habituated to the arena for 20mins individually. 24 hours

1374 later, on the second day, acquisition and test trials were performed. Rats were
1375 placed in the Open field alone for acquisition trial and allowed to investigate
1376 two similar objects for 3 min before being returned to their home cage. After 90
1377 min break, (inter-trial interval) the rats were placed in the open field again for
1378 the test trial, and one of the familiar object was replaced by a novel object. Rats
1379 were allowed to investigate the familiar object and the novel object for another
1380 3 min. The objects were glass/ceramic material placed opposite to each other
1381 20cm away from the wall of the arena. The objects were cleaned with 70%
1382 ethanol between each trial. After each trial, the faeces and urine were removed
1383 with a wet paper towel and the arena was wiped with 70% ethanol. The
1384 acquisition and the test trials were video recorded. The videos were scored
1385 manually afterwards. I was blinded throughout the experiment. Exploration was
1386 defined as sniffing, licking and touching the object while facing it. Acquisition
1387 requirements were a minimum of 15 seconds (s) exploring the objects, with a
1388 minimum of 1 s spent on a single object. In the test trial the difference between
1389 time spent investigating the novel and the familiar object divided by the total
1390 time spent investigating the objects was calculated as a measure of memory
1391 performance (Discrimination index, DI). A high score indicated good memory,
1392 whereas a low score indicated poor memory formation.

1393

1394 *Sucrose Intake Test*

1395

1396 On the day 1 of the experiment, the rats were tested for depression-like by
1397 measuring their sucrose intake. The experiment was performed in standard
1398 laboratory cages (1 animal/cage). One day before the test, the animals were
1399 habituated for sucrose intake (1% sucrose) as they were exposed to sucrose

1400 solution for few hours without any access to food or plain water for 1 hour. The
1401 animals were fasted overnight (1900 -09.00AM h; 14 hours). On the day 2 of
1402 the experiment, the test was performed for 1 hour (09.00AM-1000 hr) in which
1403 each animal was provided sucrose ad-libitum without any access to food or
1404 water. Weights of the animals and the sucrose bottles was recorded pre- and
1405 post-test in order to calculate percentage change in sucrose per body weight.
1406 After the test, the animals were returned back to their respective home cages.

1407 *Limited nesting (LN)*

1408

1409 The experimental dams with their pups were placed in the cage containing a
1410 limited amount of nesting material (One dam with the litter/cage). This nesting
1411 material was placed on a fine-gauge aluminum mesh platform (dimension:
1412 36.06x20.52x2 cm³), layered approximately 2.5 cm above the cage floor. The
1413 cage floor was covered with a small amount of corn husk bedding (~500 g).
1414 This setup permitted rat droppings to fall below the platform without trapping
1415 the pups [252]

1416

1417 **Figure M.19: Limited nesting model.** The rats were placed on an aluminium
1418 wire mesh with limited nesting material and the cage floor was covered with a
1419 small amount of bedding.

1420

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1426 Chapter 4

1427 4. Results

1428

1429 The results section is broadly classified into the following sections; Physiological
1430 measurements, behavioral assays, qPCR, protein analysis: western blot &
1431 immunohistochemistry, epigenetics: methylation in promoter sites and dendritic
1432 morphology.

1433 4.1. Physiological readouts

1434

1435 In order to investigate the influence of EE and stress on physiological parameters
1436 pertinent to activation of the stress-HPA axis, we measured **plasma corticosterone**
1437 **(CORT) concentration in blood of all animals; the weight of adrenal glands in**
1438 **adulthood (P56) and body weight** throughout the experiment. The results of these
1439 physiological measurements are presented as a comparison between different
1440 experimental groups (AFR, MS, EE, and MSEE). The various physiological
1441 outputs assessed in this study, will be highlighted in this section.

1442 Please note that, AFR refers to animal facility rearing without maternal separation
1443 stress or enriched environment (EE). MS refers to animal facility rearing with
1444 maternal separation stress but without enriched environment. EE refers to the
1445 enriched environment without maternal separation stress, and MSEE refers to
1446 enriched environment along with maternal separation stress.

1447 *Body weight: Stress and Enrichment had a significant effect on body*
1448 *weight*

1449 Chronic stress is known to lead to an increase in body weight [253]. To investigate
1450 the body weight profile in our stress paradigm, body weight was quantified during
1451 the course of the experiment. Total body weight (animal weight) was monitored
1452 every fortnight from weaning till the day of sacrifice in adulthood (Figure R.1).
1453 Repeated measures ANOVA was conducted with body weight as the within
1454 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
1455 on body weight. The interaction between stress and EE ($F_{(1,28)} = 6.05, p = 0.020$)
1456 also had a significant effect on body weight.

1458
1459 **Figure R.1: Mean body weights of male animals.** Repeated measures ANOVA
1460 revealed a significant effect of stress, EE, and interaction between stress and EE on the
1461 weights of animals. Y-axis represents the mean weight in grams (g) of animals. Graph

1462 represents mean values for each group during experiment. N = 8 for AFR, 8 for Stress, 8
1463 for EE and 8 for MSEE.

1464 Moreover, a two-way ANOVA was conducted for body weight ‘area under curve’
1465 (AUC) with stress and EE as two between subject sources of variance. This was
1466 done to determine the impact of 2 different treatments on the body weight
1467 throughout the experiment. Both stress ($F_{(1,28)} = 5.355$, $p = 0.03$) and EE ($F_{(1,28)} =$
1468 4.786 , $p = 0.04$) had significant main effects on AUC of body weight. However,
1469 the interaction between stress and EE had a marginal significance ($F_{(1,28)} = 8.983$,
1470 $p = 0.06$).

1471 To analyze the effects of stress in absence or presence of the EE, orthogonal
1472 planned comparisons were conducted (independent samples t-tests). In absence of
1473 EE, the effect of stress on body weight did not reach statistical significance ($t_{14} =$
1474 0.234 , $p = 0.82$; Cohen’s $d = 0.117$). In contrast, stressed animals displayed
1475 significant lower body weight in presence of EE ($t_{14} = 2.941$, $p = 0.01$; Cohen’s d
1476 $= 1.47$; Figure R.2).

1477

1478

1479

1480

1481

1482 **Figure R.2: Effect of early life stress and EE on body weight:** MSEE animals
 1483 displayed lower body weight. Graphs represent Mean ± SEM. N = 8 for AFR, 8 for
 1484 Stress, 8 for EE and 8 for MSEE; ** $p \leq 0.01$.

1485 Additionally, we analyzed the body weight just before sacrifice to assess the
 1486 impact of the early life paradigm specifically at the end of the experiment. A two-
 1487 way ANOVA was conducted for body weight at sacrifice with stress and EE as
 1488 two between subject sources of variance. The effect of stress ($F_{(1,28)} = 1.055$, $p =$
 1489 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 ($F_{(1,28)} = 7.114$,
 1490 $p = 0.01$) had significant main effects on AUC of body weight.

1492 To analyze the effects of stress in absence or presence of the EE, orthogonal
 1493 planned comparisons were conducted (independent samples t-tests). In absence of
 1494 EE, the effect of stress on body weight at sacrifice did not reach statistical
 1495 significance ($t_{14} = -1.155$, $p = 0.27$; Cohen's $d =$). In contrast, in the presence of

1496 EE, stressed animals displayed significant lower body weight at sacrifice ($t_{14} =$
1497 2.624, $p = 0.02$; Cohen's $d = 7$; Figure R.2b).

1498

1499 **Figure R.2b: Effect of early life stress and EE on body weight at sacrifice:**

1500 MSEE animals displayed lower body weight. Graphs represent Mean $\pm$ SEM. N =
1501 8 for AFR, 8 for Stress, 8 for EE and 8 for MSEE; $*p \leq 0.05$.

1502 In our paradigm, enriched environment housing reduced body weight along the
1503 experiment unlike previously reported stress-induced weight gain by stressed rats
1504 during the course of the experiment. Rats exposed to prenatal stress and then
1505 housed in environmental enrichment conditions showed a similar decrease in body
1506 weight [254]. Therefore, stress paradigm in this experiment did not have a
1507 significant impact on body weight unlike previous studies. This could be due to the
1508 increase in locomotor activity (climbing, exploring, etc) involved in EE cages
1509 [255].

1510 *Weight of adrenal glands: Stress significantly increased adrenal gland*
1511 *weights*

1512 Early life stress affects various physiological parameters, particularly those which
1513 are involved in the hypothalamus-pituitary-adrenal (HPA) axis. Adrenal glands are
1514 the terminal arm of the HPA axis that initiates stress response [167]. Adrenal
1515 glands are vital organs that produce corticosterone in response to stressful stimuli
1516 and undergo hypertrophy in the process [256, 257]. So, it was crucial for us to test
1517 if MS in our experiment induced any prolonged hypertrophy to the adrenals
1518 glands. The weights of the adrenal glands were tested before sacrifice of the
1519 animals.

1520 A two-way ANOVA was conducted for adrenal gland weight with stress and EE as
1521 two between subject sources of variance. Both stress ($F_{(1,32)} = 17.05$, $p < 0.00$) and
1522 EE ($F_{(1,32)} = 21.59$, $p < 0.00$) had significant main effects on the adrenal gland
1523 weights. Similarly, the interaction between stress and EE had a significant effect (F
1524 $_{(1,32)} = 9.42$, $p < 0.00$) on adrenal gland weights.

1525 Orthogonal planned comparisons (independent samples t-tests) showed that, in
1526 absence of EE, stress significantly increased the adrenal gland weights ($t_{19} = -$
1527 7.104 , $p < 0.00$; Cohen's $d = 3.218$). However, in the presence of EE, the effect of
1528 stress on adrenal gland weights failed to reach statistical significance ($t_{13} = -0.555$,
1529 $p = 0.59$; Cohen's $d = 0.275$; Figure R.3).

1530 MS caused adrenal gland hypertrophy in male rats living in standard animal
1531 facility housing when compared to AFR male rats which is in line with previous

1532 studies [256, 258]. Interestingly, early life stress in enriched housing (interaction)
 1533 ameliorated the increase in the adrenal weights of animals (Figure R.3).

1534

1535

1536 **Figure R3: Effect of early life stress and EE on adrenal weight:** Stress induced
 1537 a significant increase in adrenal weights in the absence of EE. Graphs represent
 1538 Mean $\pm$ SEM. N = 12 for AFR, 7 for Stress, 9 for EE and 5 for MSEE; *** $p \leq$
 1539 0.001.

1540

1541 *Stress significantly increased baseline plasma corticosterone levels*
 1542

1543 As mentioned earlier, “Hypothalamic-Pituitary-Adrenal (HPA) axis” (Fig.1) that
 1544 releases glucocorticoids (stress hormones) mediates allostasis that help the body to
 1545 return to homeostatic equilibrium after an encounter of stress episode [113]. Along
 1546 with adrenal glands, it is cardinal to check the profile of the stress hormone in a
 1547 study involving stress paradigm. To investigate the effects of stress and EE on this

1548 crucial parameter, plasma corticosterone was measured at various time points
1549 (Figure R.4) during the course of the study, namely: baseline1 = baseline plasma
1550 corticosterone (effect of early life treatment) in adulthood; baseline 2 = basal
1551 corticosterone levels 24 hours prior to predator odour exposure; post-30 minutes,
1552 post-2 hours, post-6 hours after exposure to predator odour and at sacrifice (24
1553 hours post stress exposure). All these different time points were used to plot the
1554 'area under curve' (AUC). This signifies the profile of stress hormone post stress
1555 indicating recovery efficiency. The result with statistics is displayed below.

1556

1557 A two-way ANOVA was conducted for corticosterone with stress and EE as two
1558 between subject sources of variance. This shows the effects of the treatments
1559 throughout the course of study that gives us a holistic picture of the corticosterone
1560 profile. The effects of stress ($F_{(1,22)} = 0.614$, $p = 0.44$) and EE ($F_{(1,22)} = 0.723$, $p =$
1561 0.40) on the AUC of corticosterone failed to reach statistical significance.
1562 Similarly, the effect of the interaction between stress and EE ($F_{(1,22)} = 0.725$, $p =$
1563 0.40) on the AUC of corticosterone failed to reach statistical significance.

1564 Orthogonal planned comparisons (independent samples t-tests) showed that in the
1565 absence ($t_9 = -1.591$, $p = 0.146$; Cohen's $d = 0.951$) of EE, the effect of stress on
1566 the AUC of corticosterone failed to reach statistical significance. Similarly, in the
1567 presence ($t_{13} = 0.044$, $p = 0.97$; Cohen's $d = 0.023$; Figure R.4) of EE, the effect of
1568 stress on the AUC of corticosterone failed to reach statistical significance.
1569 Additionally, two baseline time-points of the corticosterone concentration are
1570 highlighted below.

1571

1572

1573 **Figure R4: Area under the curve for plasma corticosterone concentration for**
1574 **pre-and post-predator odor exposure in animals exposed to stress and/ or**
1575 **enrichment in early life:** EE and stress had no significant effect on AUC of
1576 corticosterone. Graphs represent Mean $\pm$ SEM. N = 6 for AFR, 5 for Stress, 9 for
1577 EE and 6 for MSEE.

1578 The first baseline plasma corticosterone concentration highlights the corticosterone
1579 concentration levels in animals during adulthood, which are exposed to the early
1580 life treatment. So, this time point captures the prolonged effect of early
1581 environment on the levels of corticosterone in individuals

1582 A two-way ANOVA was conducted for corticosterone with stress and EE as two
1583 between subject sources of variance. Stress ($F_{(1,22)} = 4.666$, $p = 0.04$) had a
1584 significant main effect on the baseline corticosterone levels. However, the effect of
1585 EE ($F_{(1,22)} = 0.769$, $p = 0.39$) and the interaction between stress and EE ($F_{(1,22)} =$

1586 0.007, $p = 0.94$) on baseline corticosterone levels failed to reach statistical
1587 significance.

1588 Orthogonal planned comparisons (independent samples t-tests) showed that, in
1589 absence of EE, stress significantly increased baseline corticosterone levels at
1590 adulthood (P56) ($t_9 = -2.432$, $p = 0.04$; Cohen's $d = 1.443$) However, in the
1591 presence of EE, the effect of stress on baseline corticosterone levels failed to reach
1592 statistical significance ($t_{13} = -1.408$, $p = 0.18$; Cohen's $d = 0.712$; Figure R.5).

1593

1594 **Figure R.5: Baseline plasma corticosterone concentration during adulthood in**
1595 **animals exposed to stress and/ or enrichment in early life:** Stress significantly
1596 increased baseline corticosterone levels in the absence of EE. Graphs represent
1597 Mean $\pm$ SEM. $N = 6$ for AFR, 5 for Stress, 9 for EE and 6 for MSEE; $*p \leq 0.05$.

1598 Additionally, the second baseline plasma corticosterone concentration shows the
1599 levels of stress hormone after being exposed to numerous behavioral experiments
1600 (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

1615

1616 **Figure R.6: Plasma corticosterone concentration at sacrifice in animals**
1617 **exposed to stress and/ or enrichment in early life:** Stress had no significant
1618 effect on post-sacrifice corticosterone levels. Graphs represent Mean $\pm$ SEM. N =
1619 5 for AFR, 5 for Stress, 7 for EE and 6 for MSEE.

1620 Though there was no significant difference between groups for AUC during the
1621 course of the study, results for maternal separation stress alone displays enhanced
1622 corticosterone concentration in baseline blood (Figure R5.). This is similar to what
1623 has previously been observed in rats exposed to chronic stress having elevated
1624 baseline plasma corticosterone [257, 259] when compared to control animals. This
1625 effect of maternal stress was not observed in rats that were placed in the enriched
1626 environment simultaneously (interaction effect, Figure R5). This observation is
1627 indicative of the modulatory effect of environmental enrichment on the HPA axis.

1628 Furthermore, Roy and colleagues (2001) showed that male mice assigned to the
1629 environmental enrichment treatment had significantly less plasma corticosterone
1630 compared to animals in standard animal house housing after exposure to stress
1631 [260] which is in line with our study.

1632 All in all, our physiological measurements show that MS alone has detrimental
1633 effects but concurrent exposure of MS and EE displays reduced damage. This
1634 highlights the impact of concurrent exposure of stress and EE.

1635 4.2. Behavioral assays

1636

1637 The effect of early-life stress and enriched environment on behavior was tested in
1638 adult animals. Responses to stress results in a spectrum of outcome from
1639 detrimental immediate and long-term effects to resilience and active coping
1640 behavior in response to future stressors. Stress can be assessed by behavioral tests
1641 [261]. In this section, we investigated various behavioral outputs in response to
1642 stress and EE. For testing anxiety-like behavior, the home cage emergence test
1643 (HCE), the open field test (OFT) and the elevated plus maze (EPM) were
1644 employed. For testing social behavior, the social interaction test (SIT) was
1645 employed. To test depressive-like behavior, the forced swim test (FST) was used.
1646 The results of these behavioral endpoints are presented as a comparison between
1647 different experimental groups (control, stress, EE, and MSEE).

1648 *Stress significantly reduced the inner zone exploration in open field* 1649 *test*

1650 To analyze the effect of early life stress and EE on anxiety-like behavior, animals
1651 were subjected to various anxiety tests in adulthood (P56). Open field test will be
1652 the first test discussed here wherein the time spent in inner zone of an open arena
1653 was assessed. The open field is a well-established behavioral test of anxiety-like
1654 behavior. It has been validated with anxiolytic drugs [262]. In this test, lower
1655 exploration of inner zone indicated higher anxiety. The following statistics
1656 highlights the anxiety-like behavior of animals in our experiment.

1657 A two-way ANOVA was conducted for inner zone exploration in the open field
1658 test with stress and EE as two between subject sources of variance. The effect of
1659 stress ($F_{(1,28)} = 0.157$, $p = 0.69$) on time spent exploring the inner zone in the open
1660 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
1663 field test.

1664 Orthogonal planned comparisons (independent samples t-tests) showed that, in the
1665 absence of EE, stress significantly decreased time spent exploring the inner zone of
1666 the open field test ($t_{17} = 2.403$, $p = 0.03$; Cohen's $d = 1.305$). In contrast, the effect
1667 of stress on time spent exploring the inner zone of the open field test in the
1668 presence of EE ($t_{11} = -2.029$, $p = 0.07$; Cohen's $d = 1.088$; Figure R.9) failed to
1669 reach statistical significance.

1670

1671 **Figure R.8: Effect of early life stress and EE on time spent in the inner zone of**
1672 **OFT:** Stress significantly decreased the time spent exploring the inner zone in
1673 open field test in the absence of EE. Graphs represent Mean $\pm$ SEM. N = 12 for
1674 AFR, 7 for Stress, 7 for EE and 6 for MSEE; $*p \leq 0.05$.

1675 My results showed that early life maternal separation induced anxiogenic
1676 avoidance of the inner zone of the open field. This represents the anxiogenic effect
1677 of animals exposed to MS alone in standard housing in early life. This result is in
1678 line with previous reports that showed MS to result in anxiogenic behavior in
1679 adulthood [263, 264]. However, this effect was not seen in animals exposed to MS
1680 in the enriched environment (Figure R.8). This emphasizes the impact of EE along
1681 with MS on alleviating anxiogenic behavior.

1682 *Stress or EE had no significant effect on escape latency in home cage*
1683 *emergence test*

1684 The second test used to assess anxiety-like behavior was, home cage emergence
1685 wherein the latency to escape from home cage was analyzed. Home cage
1686 emergence was used to quantify exploratory activity and anxiety in rodents [265,
1687 266]. In this test, higher escape latency denoted higher anxiety. A two-way
1688 ANOVA was conducted for escape latency in the home cage emergency test with
1689 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
1690 home cage emergence test failed to reach statistical significance. Similarly, the
1691

1692 interaction between stress and EE ($F_{(1,28)} = 0.017$, $p = 0.90$) on escape latency in
1693 home cage emergence test failed to reach statistical significance.

1694 Orthogonal planned comparisons (independent samples t-tests) showed that in
1695 absence ($t_{17} = -0.264$, $p = 0.80$; Cohen's $d = 0.119$) of EE, the effect of stress on
1696 escape latency in the home cage emergence test, failed to reach statistical
1697 significance. In the presence ($t_{11} = -0.953$, $p = 0.36$; Cohen's $d = 0.557$; Figure R.8)
1698 of EE, the effect of stress on escape latency in the home cage emergence test,
1699 failed to reach statistical significance.

1700 The results revealed that the latency to escape from the home cage emergence test
1701 was not significantly affected by MS, EE or their interaction.

1702

1703 **Figure R.9: Effect of early life stress and EE on escape latency in HCE in**
1704 **adulthood:** Stress (in presence and absence of EE) had no significant effect on
1705 escape latency in home cage emergence test. Graphs represent Mean $\pm$ SEM. N =
1706 10 for AFR, 8 for Stress, 9 for EE and 5 for MSEE.

1707 *Stress along with EE significantly increased percentage open arm time*
1708 *in the EPM*

1709 Another test to study the anxiety like behavior was the elevated plus maze (EPM).
1710 Open arm exploration (entries and occupancy time) relative to sum of open and
1711 enclosed arm exploration was used as index for the anxiety [267]. In EPM, % open
1712 arm entries and % open arm time were assessed, wherein lower percentage of time
1713 and entries in open arm indicate greater anxiety.

1714 A two-way ANOVA was conducted for percentage open arm time in the elevated
1715 plus maze with stress and EE as two between subject sources of variance. The
1716 effect of stress ($F_{(1,32)} = 2.123$, $p = 0.15$) and EE ($F_{(1,32)} = 0.149$, $p = 0.70$) on the
1717 percentage time spent exploring the open arm of the elevated plus maze failed to
1718 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
1719 elevated plus maze failed to reach statistical significance.

1721 Orthogonal planned comparisons (independent samples t-tests) showed that, in the
1722 absence of EE, the effect of stress on the percentage time spent exploring the open
1723 arm of the elevated plus maze ($t_{19} = 0.287$, $p = 0.78$; Cohen's $d = 0.1223$) did not
1724 reach statistical significance. In contrast, stress significantly increased the
1725 percentage time spent exploring the open arm of the elevated plus maze in the
1726 presence of EE ($t_{13} = -2.310$, $p = 0.04$; Cohen's $d = 1.294$; Figure R.10).

1727

1728

1729 **Figure R.10: Effect of early life stress and EE on percentage open arm time in**
 1730 **EPM in adulthood:** stress along with EE significantly increased the percentage
 1731 time spent exploring the open arm of the elevated plus maze. Graphs represent
 1732 Mean ± SEM. N = 12 for AFR, 9 for Stress, 9 for EE and 6 for MSEE.

1733 *Stress along with/in the presence of EE significantly increased*
1734 *percentage open arm entries in the EPM*

1735 A two-way ANOVA was conducted for percentage open arm entries in the
1736 elevated plus maze with stress and EE as two between subject sources of variance.
1737 The effect of stress ($F_{(1,28)} = 0.491$, $p = 0.49$) and EE ($F_{(1,28)} = 0.407$, $p = 0.53$) on
1738 the number of entries into the open arm of the elevated plus maze failed to reach
1739 statistical significance. Similarly, the interaction between stress and EE ($F_{(1,28)} =$
1740 3.276 , $p = 0.08$) on the number of entries into the open arm of the elevated plus
1741 maze failed to reach statistical significance.

1742 Orthogonal planned comparisons (independent samples t-tests) showed that, in the
1743 absence of EE, stress had no significant effect on the number of entries into the
1744 open arm of the elevated plus maze ($t_{18} = 0.777$, $p = 0.45$; Cohen's $d = 0.349$). In
1745 contrast, stress significantly increased the number of entries into the open arm of
1746 the elevated plus maze in the presence of EE ($t_{10} = -2.615$, $p = 0.03$; Cohen's $d =$
1747 1.510 ; Figure R.11).

1748 The results show that MS and EE alone did not affect the anxiety-like behavior in
1749 EPM but MSEE group displayed anxiolytic behavior. Rats subjected to both MS
1750 and EE spent more time exploring (% time and % entries) the open arm of the
1751 EPM compared to rats exposed only to environmental enrichment (figure R.10 and
1752 R.11). Thus, it appears that the presence of both MS and EE has a cumulative
1753 effect on the increase in anxiolytic behavior. This is corroborated by reports show

1754 that both short-term [87] and long-term [167] enrichment in adulthood after early
1755 life maternal separation increases anxiolytic behavior.

1756

1757

1758 **Figure R.11: Effect of early life stress and EE on percentage open-arm entries**
1759 **in EPM:** Stress along with EE significantly increased the number of entries into
1760 the open arm of the elevated plus maze. Graphs represent Mean ± SEM. N = 11 for
1761 AFR, 9 for Stress, 6 for EE and 6 for MSEE; * $p \leq 0.05$

1762 *Stress or EE did not alter number of head dips in the elevated plus*
1763 *maze*

1764 A resilient individual highlights certain behaviors in the form of active coping
1765 strategy that involves assessment of risks and threats in the surrounding [268].
1766 Risk assessment behavior in this study was measured using number of head-dips

1767 performed by each subject during the test. In this test, higher number of head dips
1768 indicates elevated risk assessment behavior.

1769 A two-way ANOVA was conducted for the number of head dips in the elevated
1770 plus maze with stress and EE as two between subject sources of variance. The
1771 effect of stress ($F_{(1,32)} = 1.652$, $p = 0.21$) and EE ($F_{(1,32)} = 1.924$, $p = 0.18$) on the
1772 number head dips performed in the elevated plus maze failed to reach statistical
1773 significance. Similarly, the interaction between stress and EE ($F_{(1,32)} = 0.020$, $p =$
1774 0.89) had no significant effect on the number head dips performed in the elevated
1775 plus maze.

1776 Orthogonal planned comparisons (independent samples t-tests) showed that, in the
1777 absence of EE ($t_{19} = -1.081$, $p = 0.29$; Cohen's $d = 0.46$), the effect of stress on the
1778 number head dips performed in the elevated plus maze failed to reach significance.
1779 Similarly, in the presence of EE ($t_{13} = -0.778$, $p = 0.45$; Cohen's $d = 1.328$; Figure
1780 R.12) the effect of stress on the number of head dips performed in the elevated
1781 plus maze failed to reach significance.

1782 The results of head dips in EPM test revealed that MS, either in absence or
1783 presence of EE did not significantly affect risk assessment behavior.

1784

1785 **Figure R.12: Effect of stress and EE on a number of head dips in EPM: Stress**
1786 had no significant effect on the number head dips performed in the elevated plus
1787 maze in presence and absence of EE. Graphs represent Mean $\pm$ SEM. N = 12 for
1788 AFR, 9 for Stress, 9 for EE and 6 for MSEE.

1789 *Stress or EE did not alter closed arm entries in the elevated plus maze*

1790 Number of closed arm entries indicates the influence of locomotion of rats in the
1791 maze as indicated by factor analysis studies on EPM [269]. To assess the influence
1792 of locomotion on the results of EPM, number of closed arm entries was analyzed.

1793 A two-way ANOVA was conducted for percentage closed arm time in the elevated
1794 plus maze with stress and EE as two between subject sources of variance. The
1795 effect of stress ($F_{(1,32)} = 1.702$, $p = 0.20$) and EE ($F_{(1,32)} = 1.168$, $p = 0.29$) on the
1796 number of entries into the closed arm of the elevated plus maze failed to reach
1797 statistical significance. Similarly, the effect of the interaction between stress and
1798 EE ($F_{(1,32)} = 0.035$, $p = 0.85$) on the number of entries into the closed arm of the
1799 elevated plus maze failed to reach statistical significance.

1800 Orthogonal planned comparisons (Independent samples t-tests) showed that in the
1801 absence of EE ($t_{19} = 0.842$, $p = 0.41$; Cohen's $d = 0.359$) the effect of stress on the
1802 number of closed arm entries in the elevated plus maze failed to reach significance.
1803 Similarly, in the presence of EE ($t_{13} = 1.026$, $p = 0.32$; Cohen's $d = 0.520$; Figure
1804 R.13) the effect of stress on the number of closed arm entries in the elevated plus
1805 maze failed to reach significance.

1806 The result indicates the absence of any influence of general locomotion on the
1807 results of the EPM test as a result of different early life housing conditions.

1808

1809

1810 **Figure R.13: Effect of early life stress on number of closed arm entries in**
 1811 **EPM in presence and absence of EE:** Stress had no significant effect on the
 1812 number of entries into the closed arm of the elevated plus maze in presence and
 1813 absence of EE. Graphs represent Mean $\pm$ SEM. N = 12 for AFR, 9 for Stress, 9 for
 1814 EE and 6 for MSEE.

1815 *Stress along with EE significantly increased total percentage*
 1816 *exploration in the SIT*

1817 Social interaction test was performed in this study to assess the social motivation
 1818 in rats of varying early life environment. Previous study has shown that stress
 1819 causes decline in social exploration in rats where they are motivated to explore
 1820 objects in comparison to unfamiliar conspecifics [241].

1821 A two-way ANOVA was conducted for total percentage exploration in the social
 1822 interaction test with stress and EE as two between subject sources of variance. The
 1823 effect of EE ($F_{(1,30)} = 2.347$, $p = 0.14$) on percentage total exploration in the social

1824 interaction test failed to reach statistical significance. However, both stress ($F_{(1,30)}$
1825 $= 11.39$, $p < 0.00$) and the interaction between stress and EE ($F_{(1,30)} = 5.656$, $p =$
1826 0.02) had significant effect on the time spent interacting with the stimulus animal
1827 in the social interaction test.

1828 Orthogonal planned comparisons (independent samples t-tests) showed that, in the
1829 absence of EE ($t_{18} = -0.796$, $p = 0.44$; Cohen's $d = 0.403$), the effect of stress on
1830 percentage total exploration in the social interaction test failed to reach statistical
1831 significance. However, in the presence of EE ($t_{12} = -3.661$, $p < 0.00$; Cohen's $d =$
1832 2.000 ; Figure R.14) stress significantly increased the time spent interacting with
1833 the stimulus animal in the social interaction test.

1834 Rats subjected to both MS and EE spent significantly more time exploring
1835 unfamiliar conspecifics in comparison to wooden objects. This shows that presence
1836 of both environments has a combined effect on elevating the social interaction
1837 behaviour. Though stress did not show a significant decline in social interaction
1838 like previous studies, MS in presence of/along with EE has a pro-social behaviour.
1839 This could be because enriched environment provided a complex environment
1840 including exploratory, social, emotional and motor stimulation to the animals early
1841 in life and also helped induce well adaptive behaviours [169].

1842

1843

1844

1845 **Figure R.14: Effect of early life stress and EE on social exploration in social**
 1846 **interaction test:** Stress along with EE significantly increased the time spent
 1847 interacting with the stimulus animal in the social interaction test. Graphs represent
 1848 Mean ± SEM. N = 12 for AFR, 8 for Stress, 9 for EE and 5 for MSEE; $**p \leq 0.01$.

1849 *Stress decreased T1 immobility but not swimming in the forced swim*
 1850 *test (FST)*

1851 Chronically administered corticosterone reduced immobility time in the FST [270].
 1852 As an index for learned helplessness (depressive-like symptoms), immobility (time
 1853 in seconds) in Porsolt's forced swim task (FST) was measured in our study. MS
 1854 induced depression-like behaviour was associated with decreased swimming
 1855 behaviour in the FST [271-273]. This positive stress-coping behavior such as
 1856 swimming (time in seconds) was also measured in our study shown below.
 1857 Immobility and swimming were measured at two time points, T1 and T2. T1 was
 1858 the first day where the animals were habituated to the test and T2 was the time

1859 point for the actual test day. In FST, the initial exposure to the swim tank on day 1
1860 (T1) is known to induce a state of “behavioral despair,” such that these rats have
1861 *altered emotional reactivity* in response to a new emergency represented by
1862 swimming. So, day 2 (T2) is considered as the test day where the depression-like
1863 behavior can be well captured, after T1 habituation [274]. In this study, we
1864 quantified T1 behavioral response to capture the initial response to novelty stress
1865 and T2 behavioral response to capture the stabilized emotional response to forced
1866 swim test.

1867 A two-way ANOVA was conducted for T1 (Day-1) immobility and swimming in
1868 the forced swim test with stress and EE as two between subject sources of
1869 variance. The effect of both EE ($F_{(1,30)} = 2.797$, $p = 0.11$) and stress ($F_{(1,30)} = 2.075$,
1870 $p = 0.16$) on immobility in the forced swim test failed to reach statistical
1871 significance. However, the interaction between stress and EE ($F_{(1,30)} = 3.966$, $p =$
1872 0.06) had a marginal significant effect on the T1 immobility in the forced swim
1873 test.

1874 For swimming, the effect of EE ($F_{(1,32)} = 0.334$, $p = 0.57$) and the interaction
1875 between EE and stress ($F_{(1,32)} = 0.420$, $p = 0.52$) did not reach statistical
1876 significance. However, stress ($F_{(1,32)} = 3.923$, $p = 0.06$) had a marginal significant
1877 effect on the T1 swimming in the forced swim test.

1878 Orthogonal planned comparisons (independent samples t-tests) for T1 immobility
1879 showed that, in the absence of EE ($t_{18} = 2.138$, $p = 0.05$; Cohen’s $d = 1.061$), stress
1880 significantly decreased immobility in the forced swim test. However, in the
1881 presence of EE ($t_{12} = -0.849$, $p = 0.42$; Cohen’s $d = 0.528$; Figure R.15A) the effect

1882 of stress on T1 immobility in the forced swim test failed to reach statistical
1883 significance.

1884 Orthogonal planned comparisons (independent samples t-tests) for T1 swimming
1885 showed that, in the absence of EE ($t_{19} = 1.759$, $p = 0.10$; Cohen's $d = 0.736$), the
1886 effect of stress on T1 swimming in the forced swim test failed to reach statistical
1887 significance. Similarly, in the presence of EE ($t_{13} = 1.254$, $p = 0.23$; Cohen's $d =$
1888 0.649 ; Figure R.15B) the effect of stress on swimming in the forced swim test
1889 failed to reach statistical significance.

1890 The results revealed that MS significantly decreased immobility in T1 when
1891 compared to controls. The response to the first period of immobility is proposed as
1892 an indicator of the length of the first effort to escape from the stressful situation
1893 that the forced swim test represents. The *altered emotional reactivity* when
1894 confronted with an emergency (swim test) for the first time of could be the reason
1895 why the MS rats displayed less immobility on day 1 (T1). The T1 result observed
1896 in this study has already been reported before [275, 276]. However, there are
1897 studies that show that stress increases immobility time [277, 278] but reduced
1898 immobility is not always associated with a compromised behavior due to chronic
1899 stress as shown in earlier studies. MS in this study did not alter T1 swimming time
1900 in FST.

1901 *Stress decreased T2 swimming but not immobility in the forced swim*
1902 *test (FST)*

1903 As mentioned earlier, T2 was considered as the test day wherein the rats were
1904 habituated already. Immobility on day 2 (T2) is considered as an indicator that the
1905 animal lacks motivation for solving a problem [279]. A two-way ANOVA was
1906 conducted for T2 (day 2) immobility and swimming in the forced swim test with
1907 stress and EE as two between subject sources of variance. The effect of EE ($F_{(1,26)}$
1908 $= 2.167$, $p = 0.15$), stress ($F_{(1,26)} = 1.050$, $p = 0.32$) and the interaction between
1909 stress and EE ($F_{(1,26)} = 3.348$, $p = 0.08$) on T2 immobility in the forced swim test
1910 failed to reach statistical significance.

1911 For swimming, the effect of EE ($F_{(1,26)} = 27.829$, $p < 0.00$) and stress ($F_{(1,26)} =$
1912 4.929 , $p = 0.04$) had a significant effect on T2 swimming in the forced swim test.
1913 Similarly, the interaction between EE and stress ($F_{(1,26)} = 13.334$, $p < 0.00$) had a
1914 significant effect on the T2 swimming in the forced swim test.

1915 Orthogonal planned comparisons (independent samples t-tests) for T2 immobility
1916 showed that, in the absence of EE ($t_{13} = 1.583$, $p = 0.14$; Cohen's $d = 1.108$), the
1917 effect of stress on T2 immobility in the forced swim test failed to reach statistical
1918 significance. Similarly, in the presence of EE ($t_{13} = -0.830$, $p = 0.42$; Cohen's $d =$
1919 0.435 ; Figure R.16A) the effect of stress on T2 immobility in the forced swim test
1920 failed to reach statistical significance. Orthogonal planned comparisons
1921 (independent samples t-tests) for T2 swimming showed that, in the absence of EE
1922 ($t_{13} = 3.350$, $p = 0.01$; Cohen's $d = 2.110$), stress significantly decreased T2
1923 swimming in the forced swim test. However, in the presence of EE ($t_{13} = -1.372$, p
1924 $= 0.19$; Cohen's $d = 0.769$; Figure R.16B) the effect of stress on swimming in the
1925 forced swim test failed to reach statistical significance.

1926 In T2 experiments, MS significantly decreased swimming time in the FST. MS
1927 induced depression-like behaviour is associated with decreased swimming
1928 behaviour in the FST [271-273]. Thus, MS induced depression-like behaviour in
1929 the FST, which is in agreement with existing literature. MS in this experiment did
1930 not alter T2 immobility time in FST.

1931

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1935

1936 **Figure R.15: Stress significantly decreased T1 immobility but not swimming**
 1937 **in the forced swim test. A)** Stress significantly decreases immobility time in the
 1938 forced swim test. Graphs represent Mean $\pm$ SEM. N = 12 for AFR, 8 for Stress, 8
 1939 for EE and 6 for MSEE; $*p \leq 0.05$. **B)** Stress had no significant effect on
 1940 swimming time in the forced swim test. Graphs represent Mean $\pm$ SEM. N = 12 for
 1941 AFR, 8 for Stress, 8 for EE and 6 for MSEE.

A

B

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 $\pm$ 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 $\pm$ SEM. N = 12 for AFR, 9 for Stress, 9

1948 for EE and 6 for MSEE; $**p \leq 0.01$.

1949 4.3. *quantitative PCR*

1950

1951 GC activated glucocorticoid receptor (GR) has a central role in maintaining the
1952 regulation of HPA axis in response to various stressors [142, 218, 219]. The
1953 density of GR population across amygdala, hippocampus, and mPFC differ and is
1954 shown to have a link in providing feedback regulation to HPA axis [219]. The
1955 recruitment of activated GR in the nucleus as a transcription factor is a crucial step
1956 that initiates a relay of downstream molecular pathways to mediate stress response
1957 [112, 220]. Preliminary evidence suggests that in the basolateral amygdala (unlike
1958 in the hippocampus), GR activation has lasting excitatory rather than suppressive
1959 and normalizing effects on cell firing [280]. This makes it interesting to look at the
1960 GR expression in the BLA with respect to stress. Various proteins have been
1961 shown to be involved in amygdala-dependent fear and learning. Acquisition of fear
1962 conditioning is known to involve the interaction of neural inputs from the
1963 conditioned stimulus (CS) and unconditioned stimulus (US) pathways onto Lateral
1964 Amygdala (LA) cells during fear conditioning [281-283]. In the LA, ERK/MAPK
1965 gets activated by phosphorylation that is initiated within an hour after conditioning
1966 [284]. BDNF activation via the tyrosine kinase TrkB receptor is important in the
1967 acquisition fear conditioning in the LA [285]. The mRNA abundance levels of
1968 glucocorticoid receptor (GR) and brain derived neurotrophic factor (BDNF) were
1969 measured using quantitative polymerase chain reaction (qPCR). GC activated
1970 glucocorticoid receptor (GR) were focused in this study because of its central role
1971 in maintaining the regulation of HPA axis in response to various stressors [142,
1972 218, 219]. Another important molecule that interacts with glucocorticoid and its

1973 receptor (GC-GR) is BDNF, that activates molecular cascade to enhance fear
1974 related memories [286].

1975 Thus we looked into the most prominent molecules that might be playing role in
1976 regulating emotional response to stress. GR being the primary driver of plasticity
1977 within BLA, we studied expression, regulation and nuclear activation of GR that
1978 has been shown to be crucial for BLA-dependent response to stress. Additionally
1979 we looked into the expression of BDNF and MAPK, which has been previously
1980 shown to modulate emotional response to stress. Since these molecules are
1981 important mediators of the stress response, a candidate-based approach was used to
1982 select these molecules for analysis of my animal models (MS, EE and MSEE).

1983 Our first aim was to determine the effect of early-life stress and enriched
1984 environment on the transcription levels of these molecules. This gave us
1985 information about how early life environment plays an important role in altering
1986 the transcriptional regulation of the animals during adulthood and the translational
1987 regulation will be discussed in the next section. The Ct values obtained for GR and
1988 BDNF were normalized using the geometric mean of the Ct value of two reference
1989 genes. The qPCR results are presented as delta Ct values (Ct value of gene of
1990 interest – geometric mean of reference genes Ct values) for each experimental
1991 group. The delta Ct values of the target genes with the statistical analysis are given
1992 below.

1993 Based on previous literature, the most likely mediator candidates TrkB, Arc, Erk,
1994 and MR of stress response were also analyzed were. Please refer to appendix for p
1995 values of TrkB, Arc, Erk and MR.
1996

1997 *Stress or EE did not alter total GR mRNA levels in BLA*

1998 A two-way ANOVA was conducted for total GR mRNA abundance in the
 1999 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
 2000 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
 2003 reach statistical significance.
 2004

2005 Orthogonal planned comparisons (independent samples t-tests) showed that, in
 2006 the absence ($t_9 = 0.599$, $p = 0.56$; Cohen's $d = 0.368$) of EE, the effect of stress
 2007 on the mRNA transcript level of GR in the basolateral amygdala failed to reach
 2008 statistical significance. Similarly, in the presence of EE ($t_{10} = -0.107$, $p = 0.92$;
 2009 Cohen's $d = 0.064$; Figure R.17) the effect of stress on the mRNA transcript
 2010 level of GR in the basolateral amygdala failed to reach statistical significance.

2011

2012 **Figure R.17: Effect of early life stress and EE on GR mRNA expression:**

2013 EE had a significant effect on mRNA transcript level of GR in the basolateral

2014 amygdala in presence and absence of EE. Graphs represent Mean $\pm$ SEM. N =
2015 6 for AFR, 5 for Stress, 6 for EE and 6 for MSEE.

2016 *Stress or EE did not alter total BDNF mRNA levels in BLA*

2017 A two-way ANOVA was conducted for total BDNF mRNA abundance in the
2018 BLA with stress and EE as two between subject sources of variance. The effect
2019 of stress ($F_{(1,18)} = 0.153$, $p = 0.70$) and EE ($F_{(1,18)} = 1.609$, $p = 0.22$) on the
2020 mRNA transcript levels of BDNF in the basolateral amygdala did not reach
2021 statistical significance. However, the interaction between stress and EE ($F_{(1,18)}$
2022 $= 4.036$, $p = 0.06$) had a marginally significant effect on the mRNA transcript
2023 levels of BDNF in the basolateral amygdala.

2024 Orthogonal planned comparisons (independent samples t-tests) showed that, in
2025 the absence of EE ($t_9 = 1.455$, $p = 0.18$; Cohen's $d = 0.842$), the effect of stress
2026 on the mRNA transcript levels of BDNF in the basolateral amygdala did not
2027 reach statistical significance. Similarly, in the presence of EE ($t_9 = -1.444$, $p =$
2028 0.18 ; Cohen's $d = 0.912$; Figure R.18), the effect of stress on the mRNA
2029 transcript levels of BDNF in the basolateral amygdala did not reach statistical
2030 significance.

2031 MS did not alter the mRNA expression of GR and BDNF either in presence or
2032 absence of EE. It was interesting to note that EE had a marginal significant
2033 effect ($p = 0.06$) on the mRNA levels of BDNF.

2034

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2038 **Figure R.18: Effect of early life stress and EE on BDNF mRNA expression:**

2039 EE had a marginal significant ($p = 0.06$) effect on mRNA transcript level of
 2040 BDNF in the basolateral amygdala in presence and absence of EE. Graphs
 2041 represent Mean $\pm$ SEM. N = 6 for AFR, 5 for Stress, 6 for EE and 5 for MSEE.

2042 *Geometric mean of reference genes qPCR*

2043 Two housekeeping genes were used to alleviate the errors caused due to usage
 2044 of one reference gene [287]. Therefore, the geometric mean of two reference
 2045 genes was used for normalization in this experiment. To assess the influence of
 2046 the geometric mean on the results of qPCR, two-way ANOVA and orthogonal
 2047 planned comparison was performed for these values.

2048 A two-way ANOVA was conducted for geometric means of reference genes in
 2049 the BLA with stress and EE as two between subject sources of variance. The
 2050 effect of stress ($F_{(1,19)} = 0.033$, $p = 0.86$) and EE ($F_{(1,19)} = 1.722$, $p = 0.21$) on
 2051 the geometric mean of Ct values of the reference genes used to normalize
 2052 qPCR Ct values did not reach statistical significance. Similarly, the effect of
 2053 the interaction between stress and EE ($F_{(1,19)} = 0.038$, $p = 0.85$) on the

2054 geometric mean of Ct values of the reference genes used to normalize qPCR Ct
2055 values did not reach statistical significance.

2056 Orthogonal planned comparisons (independent samples t-tests) showed that, in
2057 the absence of EE ($t_9 = -0.539$, $p = 0.60$; Cohen's $d = 0.349$), the effect of stress
2058 on the geometric mean of Ct values of the reference genes used to normalize
2059 qPCR Ct values did not reach statistical significance. Similarly, in the presence
2060 of EE ($t_{10} = 0.008$, $p = 0.99$; Cohen's $d = 0.008$; Figure R.19) the effect of
2061 stress on the geometric mean of Ct values of the reference genes used to
2062 normalize qPCR Ct values did not reach statistical significance.

2063 The results revealed the absence of any influence of the geometric mean itself
2064 on the results of qPCR as there was no significant difference between the
2065 reference genes between groups. Hence, we were confident of using
2066 housekeeping genes as a reference for comparison of candidate genes in our
2067 study.

2068

2069 **Figure R.19: Effect of early life stress on the geometric mean of Ct values**
2070 **of the reference genes:** Stress had no significant effect on the geometric mean

2071 of Ct value of the reference genes used to normalize qPCR Ct values. Graphs
2072 represent Mean $\pm$ SEM. N = 5 for AFR, 6 for Stress, 6 for EE and 6 for MSEE.

2073

2074 *4.4. Protein analysis: Western Blot and Immunohistochemistry*

2075

2076 As mentioned in the previous section, the translational regulation of above-
2077 mentioned genes was investigated in this section. Candidate-based approach
2078 was applied to determine the protein expression levels of glucocorticoid
2079 receptor (GR), pro-brain derived neurotrophic factor (pro-BDNF) and brain
2080 derived neurotrophic factor (BDNF) in the basolateral amygdala (BLA) using
2081 western blot. Immunohistochemistry was used to further visualize the
2082 expression pattern of GR, Pro-BDNF, BDNF and phosphorylated mitogen-
2083 activated protein kinase (pMAPK) in neurons of the BLA. There is very limited
2084 information of the expression of these proteins in BLA when compared to
2085 hippocampus. Our study focuses on exploring the effect of early life
2086 environment on the expression of candidate- based proteins specifically in
2087 BLA, as it remains understudied in spite of BLA's crucial role in mediating
2088 stress response and emotional behavior.

2089 Once the effect of early-life stress and enriched environment on mRNA
2090 transcription was determined, it was important to understand whether the
2091 results of qPCR had an impact on protein levels in the different group of
2092 animals. Protein expression levels of these molecules were determined in the
2093 BLA of animals assigned to different experimental groups and compared,
2094 statistically for difference between them.

2095 *Stress or EE did not alter total GR protein levels in BLA*

2096 A two-way ANOVA was conducted to measure relative intensity of GR in the
2097 BLA with stress and EE as two between subject sources of variance. The effect of
2098 stress ($F_{(1,19)} = 1.399$, $p = 0.25$) and EE ($F_{(1,19)} = 0.053$, $p = 0.82$) on the relative
2099 intensity of GR in the basolateral amygdala did not reach statistical significance.
2100 Similarly, the effect of the interaction between stress and EE ($F_{(1,19)} = 0.400$, $p =$
2101 0.53) on the relative intensity of GR in the basolateral amygdala did not reach
2102 statistical significance.

2103 Orthogonal planned comparisons (independent samples t-tests) showed that in
2104 absence of EE ($t_9 = 1.232$, $p = 0.25$; Cohen's $d = 0.782$) the effect of stress on the
2105 relative intensity of GR in the basolateral amygdala did not reach statistical
2106 significance. Similarly, in the presence of EE ($t_{10} = 0.405$, $p = 0.69$; Cohen's $d =$
2107 0.226 ; Figure R.20) stress had no significant effect on protein level of GR in the
2108 basolateral amygdala.

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2121 **Figure R.20: Effect of stress on the relative intensity of GR in presence and absence**
2122 **of EE:** Stress or EE had no significant effect on protein level of GR in the
2123 basolateral amygdala in presence and absence of EE. Graphs represent Mean $\pm$
2124 SEM. N = 6 for AFR, 5 for Stress, 6 for EE and 6 for MSEE.

2125 *Stress or EE did not affect Pro-BDNF protein levels in BLA*

2126 A two-way ANOVA was conducted for relative intensity of Pro-BDNF in the BLA
2127 with stress and EE as two between subject sources of variance. EE ($F_{(1,19)} = 5.007$,
2128 $p = 0.04$) had a significant main effect on the relative intensity of Pro-BDNF in the
2129 basolateral amygdala. However, the effect of both stress ($F_{(1,19)} = 0.359$, $p = 0.56$)
2130 and the interaction between stress and EE ($F_{(1,19)} = 0.230$, $p = 0.64$) on the relative
2131 intensity of Pro-BDNF in the basolateral amygdala did not reach statistical
2132 significance.

2133 Orthogonal planned comparisons (independent sample t-tests) showed that in the
2134 absence of EE ($t_{10} = 0.067$, $p = 0.95$; Cohen's $d = 0.040$) the effect of stress on the
2135 relative intensity of Pro-BDNF in the basolateral amygdala did not reach statistical
2136 significance. Similarly, in the presence of EE ($t_9 = 1.506$, $p = 0.17$ Cohen's $d =$
2137 0.890 ; Figure R.21) the effect of stress on the relative intensity of Pro-BDNF in the
2138 basolateral amygdala did not reach statistical significance.

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2149 **Figure R.21: Effect of early life stress and EE on the relative intensity of pro-**
 2150 **BDNF:** Stress or EE had no significant effect on protein level of pro-BDNF in the
 2151 basolateral amygdala in presence and absence of EE. Graphs represent Mean $\pm$
 2152 SEM. N = 6 for AFR, 6 for Stress, 5 for EE and 6 for MSEE.

2153 *Stress alone did not affect BDNF protein levels in BLA, but interaction*
 2154 *between stress & EE had significant influence on BDNF.*

2155 A two-way ANOVA was conducted for BDNF relative intensity in the BLA with
 2156 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
 2157 of BDNF in the basolateral amygdala did not reach statistical significance.

2159 However, the interaction between stress and EE ($F_{(1,20)} = 7.187$, $p = 0.01$) had a
2160 significant effect on the protein expression level of BDNF in the basolateral
2161 amygdala.

2162 Orthogonal planned comparisons (independent samples t-tests) showed that in
2163 absence of EE ($t_{10} = -0.871$, $p = 0.40$; Cohen's $d = 0.498$) the effect of stress on the
2164 relative intensity of BDNF in the basolateral amygdala failed to reach statistical
2165 significance. However, in the presence of EE ($t_{10} = 3.282$, $p = 0.01$ Cohen's $d =$
2166 1.880 ; Figure R.22) stress significantly decreased the protein level of BDNF in the
2167 basolateral amygdala.

2168 The results revealed that MS did not alter the above mentioned protein levels in
2169 BLA. Expression of pro-BDNF, the precursor product of mature BDNF, was
2170 significantly decreased in rats placed in environmental enrichment. Previous work
2171 has associated increased expression of pro-BDNF in the hippocampus to
2172 depression in humans [288]. The reduced pro-BDNF levels in my study could have
2173 anti-depressive like effect driven by BLA because hippocampus and BLA are
2174 known to display opposite effects to each other in response to stress [53].
2175 Similarly, the hippocampus and BLA might have displayed contrasting effects in
2176 response to EE. Environmental enrichment was not observed to affect the
2177 expression of BDNF protein in the BLA. Prior reports have shown that BDNF
2178 levels are elevated in response to enriched environment conditions. Only one
2179 report demonstrated that short-term enriched environment during stress rescues the
2180 stress-induced elevated BDNF mRNA expression in BLA [166]. Finally,
2181 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].

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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 $\pm$ SEM. N = 6 for AFR, 6 for Stress, 6 for EE and 6 for MSEE; $**p \leq 0.01$.

2194 *Stress increased the percentage of intra-nuclear GR in BLA neurons*
2195 *(Immunohistochemistry)*

2196 As mentioned earlier, immunohistochemistry was used to further visualize the
2197 expression pattern of GR, Pro-BDNF, BDNF and phosphorylated mitogen-
2198 activated protein kinase (pMAPK) in neurons of the BLA. The results are
2199 displayed below.

2200 While analyzing total GR protein, there was an interesting observation wherein the
2201 density of GR signals was varying between samples. Hence, we decided to
2202 manually count the high-density GR signal cells to infer if the pattern of this
2203 peculiar (different density) signal was varying between groups.

2204 Percentage of intra-nuclear GR was quantified by manually counting the high-
2205 density GR signals colocalized with DAPI stained cells. A two-way ANOVA was
2206 conducted for intra-nuclear GR in BLA neurons with stress and EE as two between
2207 subject sources of variance. Stress ($F_{(1,19)} = 7.870$, $p = 0.01$) and EE ($F_{(1,19)} =$
2208 9.537 , $p = 0.01$) had a significant main effect on the percentage of intra-nuclear
2209 GR in basolateral amygdala neurons. However, the effect of the interaction
2210 between stress and EE ($F_{(1,19)} = 0.356$, $p = 0.56$) on the percentage of intra-nuclear
2211 GR in basolateral amygdala neurons did not reach statistical significance.

2212 Orthogonal planned comparisons (independent samples t-tests) showed that, in
2213 absence of EE ($t_9 = 2.641$, $p = 0.03$; Cohen's $d = 1.653$) stress significantly
2214 decreased the percentage of intra-nuclear GR in basolateral amygdala neurons.
2215 However, in the presence of EE ($t_{10} = 1.469$, $p = 0.17$; Cohen's $d = 0.848$; Figure

2216 R.23) the effect of stress on the percentage of intra-nuclear GR in basolateral
 2217 amygdala failed to reach statistical significance.

2218

2219 **Figure R.23: Effect of early life stress and EE on the percentage of intra-**
 2220 **nuclear GR/high-density cells:** Stress significantly decreased the percentage of
 2221 high-density GR cells in the basolateral amygdala in the absence of EE. Graphs
 2222 represent Mean ± SEM. N = 5 for AFR, 6 for Stress, 6 for EE and 6 for MSEE;
 2223 $**p \leq 0.01$.

2224

2225

2226 **Figure R.24A: Representative images of GR stained cells in the BLA for all the four**
 2227 **groups.** Immunofluorescence staining of the nucleus with DAPI (red) and GR
 2228 (green). These are the representative images of the cells that contained GR and
 2229 were considered for GR analysis. The image was pseudocolored with red and
 2230 green for clarity. Scale bar represents 50 μm.

2231

2232 *Stress or EE did not alter the total GR signal in BLA*
2233 *(Immunohistochemistry)*

2234 Total GR signal was quantified by estimating the average percentage area of GR
2235 signals using ImageJ software. It was crucial to determine the total GR signal
2236 because we compared this with information about high density GR (in the previous
2237 section). This helped us infer if MS increased total GR or only activated GR (intra-
2238 nuclear) in BLA. A two-way ANOVA was conducted for total GR signal in BLA
2239 neurons with stress and EE as two between subject sources of variance. The effect
2240 of stress ($F_{(1,17)} = 1.600$, $p = 0.22$) and EE ($F_{(1,17)} = 0.010$, $p = 0.92$) on the total
2241 GR signal in the basolateral amygdala did not reach statistical significance.
2242 Similarly, the effect of the interaction between stress and EE ($F_{(1,17)} = 3.543$, $p =$
2243 0.08) on the total GR signal in the basolateral amygdala did not reach statistical
2244 significance.

2245 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2246 absence of EE ($t_8 = 1.690$, $p = 0.13$; Cohen's $d = 1.103$) the effect of stress on the
2247 total GR signal in the basolateral amygdala did not reach statistical significance.
2248 Similarly, in the presence of EE ($t_9 = -0.696$, $p = 0.50$; Cohen's $d = 0.372$; Figure
2249 R.24) the effect of stress on the total GR signal in the basolateral amygdala did not
2250 reach statistical significance.

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2254 **Figure R.24B: Effect of early life stress and EE on the total GR signal in the**

2255 **BLA:** Stress or EE had no significant effect on the average percentage area of GR

2256 signals in the basolateral amygdala in presence and absence of EE. Graphs

2257 represent Mean ± SEM. N = 5 for AFR, 6 for Stress, 6 for EE and 6 for MSEE.

2258 *Stress or EE did not alter the total BDNF signal in BLA*

2259 *(Immunohistochemistry)*

2260 Total BDNF signal was quantified by estimating the average percentage area of

2261 BDNF signals using ImageJ software. This information further helps us visualize

2262 the expression of BDNF and validate the results shown in western blot. A two-way

2263 ANOVA was conducted for total BDNF signal in BLA neurons with stress and EE

2264 as between subject sources of variance. The effect of stress ($F_{(1,19)} = 0.861$, $p =$

2265 0.37) and EE ($F_{(1,19)} = 3.261$, $p = 0.09$) on the total BDNF signal in the basolateral

2266 amygdala did not reach statistical significance. Similarly, the effect of the

2267 interaction between stress and EE ($F_{(1,19)} = 3.079$, $p = 0.10$) on the total BDNF
2268 signal in the basolateral amygdala did not reach statistical significance.

2269 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2270 absence of EE ($t_9 = -0.793$, $p = 0.45$; Cohen's $d = 0.464$) the effect of stress on the
2271 total BDNF signal in the basolateral amygdala did not reach statistical significance.
2272 Similarly, in the presence of EE ($t_{10} = 1.628$, $p = 0.14$; Cohen's $d = 0.921$; Figure
2273 R.25) the effect of stress on the total BDNF signal in the basolateral amygdala did
2274 not reach statistical significance.

2275 Stress or EE had no significant effect on the average percentage area of BDNF
2276 signals in the basolateral amygdala.

2277
2278 **Figure R.25B: Effect of early life stress and EE on the total BDNF signal in**
2279 **the BLA:** Stress or EE had no significant effect on the average percentage area of
2280 BDNF signals in the basolateral amygdala. Graphs represent Mean $\pm$ SEM. N = 5
2281 for AFR, 6 for Stress, 6 for EE and 6 for MSEE

2282

2283 **Figure R.25A: Representative images of BDNF stained cells in the BLA for all**
 2284 **the four groups.** Immunofluorescence staining of the nucleus with DAPI (red) and
 2285 BDNF (magenta). These are the representative images of the cells that contained
 2286 BDNF and were considered for BDNF analysis. The image was pseudocolored
 2287 with red and magenta for clarity. Scale bar represents 50 μ m.

2288 *Stress or EE did not alter the total pMAPK signal in the BLA*
2289 *(Immunohistochemistry)*

2290 The total pMAPK signal was quantified by estimating the average percentage area
2291 of pMAPK signals using ImageJ software. This information further helps us
2292 visualize the expression of pMAPK to understand the translational regulation of
2293 pMAPK in various treatment groups. A two-way ANOVA was conducted for total
2294 pMAPK signal in BLA neurons with stress and EE as between subject sources of
2295 variance. The effect of stress ($F_{(1,18)} = 2.654$, $p = 0.12$) and EE ($F_{(1,18)} = 0.126$, p
2296 $= 0.73$) on the total pMAPK signal in the basolateral amygdala did not reach
2297 statistical significance. Similarly, the effect of the interaction between stress and
2298 EE ($F_{(1,18)} = 0.647$, $p = 0.43$) on the total pMAPK signal in the basolateral
2299 amygdala did not reach statistical significance.

2300 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2301 absence of EE ($t_8 = 1.407$, $p = 0.20$; Cohen's $d = 0.890$) the effect of stress on the
2302 total pMAPK signal in the basolateral amygdala did not reach statistical
2303 significance. Similarly, in the presence of EE ($t_{10} = 0.730$, $p = 0.48$; Cohen's $d =$
2304 0.485 ; Figure R.26) the effect of stress on the total pMAPK signal in the
2305 basolateral amygdala did not reach statistical significance.

2306 Stress or EE had no significant effect on the average percentage area of BDNF
2307 signals in the basolateral amygdala.

2308

2309 **Figure R.26A: Representative images of pMAPK stained cells in the BLA for**
 2310 **all the four groups.** Immunofluorescence staining of the nucleus with DAPI (red)
 2311 and pMAPK (green). These are the representative images of the cells that
 2312 contained pMAPK and were considered for pMAPK analysis. The image was
 2313 pseudocolored with red and green for clarity. Scale bar represents 50 μ m.

2314

2315

2316 **Figure R.26B: Effect of early life stress and EE on the total area of pMAPK signals**
2317 **in the BLA:** Stress or EE had no significant effect on the average percentage area
2318 of BDNF signals in the basolateral amygdala. Graphs represent Mean $\pm$ SEM. N =
2319 5 for AFR, 6 for Stress, 6 for EE and 6 for MSEE

2320 The immunohistochemistry results indicate that the early life stress and EE did not
2321 alter the levels of the above-mentioned protein expression in BLA. Though total
2322 protein levels for GR, BDNF and pMAPK did not vary, the density of GR signals
2323 in the nucleus was significantly different in MS animals housed in standard cage.
2324 The recruitment of activated GR in the nucleus as a transcription factor is a crucial
2325 step that initiates a relay of downstream molecular pathways to mediate stress
2326 response [112, 220]. Prior studies show that activation of GR through GC causes
2327 phosphorylation of Erk1/2^{MAPK}. This activated Erk1/2^{MAPK} causes relay of
2328 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.

2351 4.5. Epigenetics: Methylation in promoter sites

2352

2353 In the previous section of the results, our data showed a treatment-dependent
2354 change in protein expression levels of the candidate molecules, specifically GR
2355 and BDNF. Thus, we sought to determine if the observed change in protein levels
2356 were a result of underlying epigenetic modifications, specifically DNA
2357 methylation of the promoter region of the GR and BDNF gene in the basolateral
2358 amygdala. The results are represented as average percentage of cytosine
2359 methylation of the candidate with well-characterized CpG sites in rats.

2360 *Stress or EE did not alter average percentage cytosine methylation of* 2361 *BDNF promoter in BLA*

2362 Overall DNA methylation of a gene was calculated as the average % DNA
2363 methylation of all CpGs within the gene in this study. A two-way ANOVA was
2364 conducted for average percentage cytosine methylation of BDNF promoter in BLA
2365 neurons with stress and EE as between subject sources of variance. The effect of
2366 stress ($F_{(1,15)} = 2.784$, $p = 0.12$) and EE ($F_{(1,15)} = 0.0004$, $p = 0.99$) on the average
2367 percentage cytosine methylation at the promoter region of BDNF in the basolateral
2368 amygdala did not reach statistical significance. Similarly, the effect of the
2369 interaction between stress and EE ($F_{(1,15)} = 2.413$, $p = 0.14$) on the average
2370 percentage cytosine methylation at the promoter region of BDNF in the basolateral
2371 amygdala did not reach statistical significance.

2372 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2373 absence of EE ($t_8 = 1.775$, $p = 0.11$; Cohen's $d = 1.120$) the effect of stress on the
2374 average percentage cytosine methylation at the promoter region of BDNF in the

2375 basolateral amygdala did not reach statistical significance. Similarly, in the
2376 presence of EE ($t_7 = 0.210$, $p = 0.84$; Cohen's $d = 0.133$; Figure R.27) the effect of
2377 stress on the average percentage cytosine methylation at the promoter region of
2378 BDNF in the basolateral amygdala did not reach statistical significance.

2379 .

2380 **Figure R.27: Effect of early life stress and EE on average percentage cytosine**
2381 **methylation at the promoter region of BDNF in the BLA:** stress or EE had no
2382 significant effect on the average percentage cytosine methylation at the promoter
2383 region of BDNF in the basolateral amygdala in presence and absence of EE.
2384 Graphs represent Mean $\pm$ SEM. $N = 5$ for AFR, 5 for Stress, 4 for EE and 5 for
2385 MSEE

2386 *Stress or EE did not alter average percentage cytosine methylation of*
2387 *GR promoter in BLA*

2388 A two-way ANOVA was conducted for average percentage cytosine methylation
2389 of GR promoter in BLA neurons with stress and EE as between subject sources of
2390 variance. The effect of stress ($F_{(1,12)} = 2.400$, $p = 0.15$) and EE ($F_{(1,12)} = 0.730$, $p =$
2391 0.41) on the average percentage cytosine methylation at the promoter region of GR
2392 in the basolateral amygdala did not reach statistical significance. Similarly, the
2393 effect of the interaction between stress and EE ($F_{(1,12)} = 1.030$, $p = 0.33$) on the
2394 average percentage cytosine methylation at the promoter region of GR in the
2395 basolateral amygdala did not reach statistical significance.

2396 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2397 absence of EE ($t_6 = -1.320$, $p = 0.24$; Cohen's $d = 0.133$) the effect of stress on the
2398 average percentage cytosine methylation at the promoter region of GR in the
2399 basolateral amygdala did not reach statistical significance. Similarly, in the
2400 presence of EE ($t_6 = -0.926$, $p = 0.39$; Cohen's $d = 0.659$; Figure R.28) the effect of
2401 stress on the average percentage cytosine methylation at the promoter region of
2402 BDNF in the basolateral amygdala did not reach statistical significance.

2403 Epigenetic changes in GR promoter gene are well reported in the hippocampus for
2404 MS models. In particular, methylation studies are widely explored in these models
2405 [251]. The results revealed that the methylation status of the CpG sites used in this
2406 study did not alter due to stress in the presence and absence of EE. This
2407 observation shows that these specific CpG sites are not the major driving force

2408 behind the long-term effect on variation in protein expression of GR and BDNF in
2409 BLA.

2410

2411 **Figure R.28: Effect of early life stress and EE on average percentage cytosine**
2412 **methylation at the promoter region of GR in the BLA:** Stress or EE had no
2413 significant effect on the average percentage cytosine methylation at the promoter
2414 region of GR in the basolateral amygdala in presence and absence of EE. Graphs
2415 represent Mean $\pm$ SEM. N = 3 for AFR, 5 for Stress, 4 for EE and 4 for MSEE.

2416 4.6. Dendritic Morphology

2417
2418 As mentioned earlier, in addition to the neuroendocrine HPA axis, another
2419 captivating *Brain-HPA* is; **H**ippocampus, **P**refrontal cortex and **A**mygdala. These
2420 are the structures that contribute in the regulation of HPA axis. They have a
2421 significant role on the release of GC and behavioral response to a stressful event
2422 [114, 115]. Stress is known to alter the dendritic morphology of neurons in these
2423 brain regions. Stress is known to induce dendritic hypertrophy in the basolateral
2424 amygdala (BLA) [52, 53] and to enhance anxiety [53, 54]. Amygdala is activated
2425 by, corticosterone released during a stress response and this results in dendritic
2426 hypertrophy as well as elevated spine density [65]. These morphological changes
2427 have been correlated with anxiety and stress in prior studies [52, 53, 57, 135].
2428 These changes in neuronal morphology are known to be associated with anxiety-
2429 like behavior and general stress response.[52, 54, 57]. In spite of being a crucial
2430 role player in stress response, amygdala is the least explored region in early-life
2431 stress studies. This makes it important to explore amygdala in this study. To assess
2432 the effects of early life stress and EE on morphological changes in BLA, we
2433 determined the dendritic length and branch points using Golgi staining. We also
2434 observed the spine density in primary and secondary dendrites of neurons in BLA.
2435 The results with statistics are displayed below.

2436 *Stress along with EE increased dendritic length of BLA neurons*

2437 A two-way ANOVA was conducted for a dendritic length of BLA neurons with
2438 stress and EE as between subject sources of variance. The effect of stress ($F_{(1,275)} =$
2439 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.

2450

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

2454 Mean $\pm$ SEM. N (number of neurons) = 79 for AFR, 82 for Stress, 59 for EE and
2455 59 for MSEE, 8-10 neurons/animal; $**p \leq 0.01$.

2456

2457 *Stress along with EE induced increase in branch points in BLA*
2458 *neurons*

2459 A two-way ANOVA was conducted for number of branch points in BLA neurons
2460 with stress and EE as two between subject sources of variance. The effect of stress
2461 ($F_{(1,270)} = 22.494$, $p < 0.00$), EE ($F_{(1,270)} = 19.641$, $p < 0.00$) and the interaction
2462 between stress and EE ($F_{(1,275)} = 10.350$, $p < 0.00$) had a significant effect on the
2463 total number of branch points of neurons in the basolateral amygdala.

2464 Orthogonal planned comparisons (independent samples t-tests) showed that, in the
2465 absence of EE ($t_{156} = -1.161$, $p = 0.25$; Cohen's $d = 0.185$), the effect of stress on
2466 the total dendritic length of neurons in the basolateral amygdala did not reach
2467 statistical significance. However, in the presence of EE ($t_{114} = -5.316$, $p < 0.00$;
2468 Cohen's $d = 0.985$; Figure R.30) stress significantly increased the total number of
2469 branch points of neurons in the basolateral amygdala.

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2471

2472

2473 **Figure R.30: Effect of early life stress and EE on total number of branch**
 2474 **points in BLA neurons:** Stress along with EE significantly increased the total
 2475 number of branch points of neurons in the basolateral amygdala. Graphs represent

2476 Mean $\pm$ SEM. N (number of neurons) = 79 for AFR, 79 for Stress, 60 for EE and
2477 56 for MSEE, 8-10 neurons/animal $^{***}p \leq 0.01$. Inset (bottom): Representative images
2478 of sample Golgi-stained neurons in the BLA for the 4 groups from optical microscope at
2479 400X magnification. Scale bar represents 20 μ m.

2480 Rats exposed to both maternal separation and environmental enrichment show
2481 dendritic length hypertrophy, increase in branch points in the BLA compared to
2482 rats exposed to only environmental enrichment. Earlier studies have shown that the
2483 dendritic complexity of principal neurons in the BLA correlates with high anxiety in rats
2484 [135]. In contrast to the previous studies, the neuronal hypertrophy induced by MSEE
2485 group in this study does not correspond to anxiety-like behavior in adults. It is
2486 possible that early life could have very different on BLA morphology, as there is
2487 are no previous early life studies correlating hypertrophy and anxiety. Hence,
2488 hypertrophy in this case may denote active-coping behavior.

2489 *Stress induced increase in the number of spines on primary dendrites*
2490 *in BLA neurons in presence and absence of EE*

2491 Stress exposure (acute or chronic) as well as exogenous corticosterone
2492 administration leads to increase in the dendritic arbors and spine density of the
2493 principal neurons of BLA and corresponding manifestation of anxiety [52]. To
2494 investigate the impact of early life stress and enriched environment on dendritic
2495 spines in the BLA, we quantified the number of primary and secondary spines in
2496 BLA using Golgi staining.

2497 A two-way ANOVA was conducted for number of spines on primary dendrites in
2498 BLA neurons with stress and EE as between subject sources of variance. The

2499 effect of stress ($F_{(1,234)} = 53.186$, $p < 0.00$) and EE ($F_{(1,234)} = 8.610$, $p < 0.00$) had a
 2500 significant main effect on the number of spines found on primary dendrites in the
 2501 basolateral amygdala. However, the effect of the interaction between stress and EE
 2502 ($F_{(1,234)} = 0.536$, $p = 0.47$) on the number of spines found on primary dendrites in
 2503 the basolateral amygdala did not reach statistical significance.

2504 Orthogonal planned comparisons (independent samples t-tests) showed that in the
 2505 absence of EE ($t_{117} = -5.654$, $p < 0.00$; Cohen's $d = 1.037$) the effect of stress on
 2506 the number of spines found on primary dendrites in the basolateral amygdala did
 2507 not reach statistical significance. Similarly, in the presence of EE ($t_{117} = -4.656$, p
 2508 < 0.00 ; Cohen's $d = 0.855$; Figure R.31) the effect of stress on the number of
 2509 spines found on primary dendrites in the basolateral amygdala did not reach
 2510 statistical significance.

2511

2512 **Figure R.31: Effect of early life stress and EE on the number of primary**
 2513 **spines in BLA neurons:** Stress significantly increased the number of spines found

2514 on primary dendrites in the basolateral amygdala in presence and absence of EE.
2515 Graphs represent Mean $\pm$ SEM. N (number of neurons) = 79 for AFR, 79 for
2516 Stress, 60 for EE and 56 for MSEE, 10 neurons/animal; $**p \leq 0.01$.

2517 *Stress induced increase in the number of spines on secondary*
2518 *dendrites in BLA neurons in the absence of EE*

2519 A two-way ANOVA was conducted for number of spines on secondary dendrites
2520 in BLA neurons with stress and EE as two between subject sources of variance.
2521 The effect of stress ($F_{(1,234)} = 5.194$, $p = 0.02$) and the interaction between stress
2522 and EE ($F_{(1,234)} = 18.240$, $p < 0.00$) had a significant effect on the number of
2523 spines found on secondary dendrites in the basolateral amygdala. However, the
2524 effect of EE ($F_{(1,234)} = 0.893$, $p = 0.35$) had no significant effect on the number of
2525 spines found on secondary dendrites in the basolateral amygdala.

2526 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2527 absence of EE ($t_{117} = -4.313$, $p < 0.00$; Cohen's $d = 0.790$), stress significantly
2528 increased the number of spines found on secondary dendrites in the basolateral
2529 amygdala. However, in the presence of EE ($t_{117} = 1.531$, $p = 0.13$; Cohen's $d =$
2530 0.294 ; Figure R.32) the effect of stress on the number of spines found on
2531 secondary dendrites in the basolateral amygdala did not reach statistical
2532 significance.

2533 Acute and/ or chronic stress, as well as exogenous supplementation of
2534 corticosterone results in neuronal hypertrophy and an increase in spine density of
2535 BLA principal cells [136]. Rats subjected to chronic immobilization stress in early
2536 life and adulthood increases neuronal spine density in the amygdala [7, 17, 21].

2537 Similarly, the results presented above showed significant increase in neuronal
2538 spine density in animal's subjected maternal separation compared to control rats in
2539 the normal housing. Although the spine density was increased, there was no
2540 increase in dendritic length and branch points in normal housing rats that were
2541 subjected to maternal separation. Environmental enrichment, irrespective of
2542 presence or absence of maternal separation treatment resulted in a general decrease
2543 in secondary spine density in the BLA neurons. Rats exposed to both maternal
2544 separation and environmental enrichment show dendritic hypertrophy and increase
2545 in primary neuronal spine density in the BLA compared to rats exposed to only
2546 environmental enrichment. As this treatment is a novel one, such an interesting
2547 pattern of neuronal morphology is worth exploring.

2548

2549

2550

2551 **Figure R.32: Effect of early life stress and EE on the number of secondary**
2552 **spines in BLA neurons:** Stress significantly increased the number of spines found
2553 on secondary dendrites in the basolateral amygdala in the absence of EE. Graphs
2554 represent Mean $\pm$ SEM. N (number of neurons) = 60 for AFR, 59 for Stress, 60 for
2555 EE and 59 for MSEE, 10 neurons/ animal; $**p \leq 0.01$.

2556 *Stress did not alter the total dendritic length of hippocampal neurons*
2557 *in presence and absence of EE*

2558 Secondly, we look at the neuronal morphology of hippocampus as its involvement
2559 is widely reported in stress and cognitive processes [119, 120] It is known to be
2560 one of the most plastic regions of the brain and the structural plasticity is highly
2561 dependent on the number and impact of stressful episodes experienced by an
2562 individual (level of glucocorticoids in the systemic circulation) [122, 123]. To
2563 analyze the effects of early life stress and EE on the neuronal morphology of
2564 hippocampus, we determine the dendritic length, branch points and spine density
2565 of primary and secondary neurons of hippocampus. The results with statistics are
2566 given below.

2567 A two-way ANOVA was conducted for a dendritic length of hippocampal neurons
2568 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
2569 length of neurons in the hippocampus did not reach statistical significance.
2570 Similarly, the effect of the interaction between stress and EE ($F_{(1,255)} = 2.828$, $p =$
2571 0.09) on the total dendritic length of neurons in the hippocampus did not reach
2572 statistical significance.
2573

2574 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2575 absence of EE ($t_{140} = 0.652$, $p = 0.52$; Cohen's $d = 0.113$) the effect of stress on the
2576 total dendritic length of neurons in the hippocampus did not reach statistical
2577 significance. Similarly, in the presence of EE ($t_{115} = -1.657$, $p = 0.10$; Cohen's $d =$

2578 0.306; Figure R.33) the effect of stress on the total dendritic length of neurons in
2579 the hippocampus did not reach statistical significance.

2580

2581

2582

2583 **Figure R.33: Effect of early life stress and EE on total dendritic length of**
2584 **hippocampal neurons:** Stress had no significant effect on the total dendritic
2585 length of neurons in the hippocampus in the presence and absence of EE. Graphs
2586 represent Mean $\pm$ SEM. N (number of neurons) = 58 for AFR, 84 for Stress, 59 for
2587 EE and 58 for MSEE, 8-10 neurons/ animal.

2588

2589 *Stress did not alter the number of branch points of hippocampal*
2590 *neurons in presence and absence of EE*

2591 A two-way ANOVA was conducted for number of branch points in hippocampal
2592 neurons with stress and EE as two between subject sources of variance. The effect
2593 of stress ($F_{(1,258)} = 2.232$, $p = 0.14$) and EE ($F_{(1,258)} = 0.168$, $p = 0.68$) on the total
2594 branch points of neurons in the hippocampus did not reach statistical significance.
2595 Similarly, the effect of the interaction between stress and EE ($F_{(1,258)} = 0.027$, $p =$
2596 0.87) on the total branch points of neurons in the hippocampus did not reach
2597 statistical significance.

2598 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2599 absence of EE ($t_{142} = -0.982$, $p = 0.33$; Cohen's $d = 0.168$) the effect of stress on
2600 the total branch points of neurons in the hippocampus did not reach statistical
2601 significance. Similarly, in the presence of EE ($t_{116} = -1.126$, $p = 0.26$; Cohen's $d =$
2602 0.207 ; Figure R.34) the effect of stress on the total branch points of neurons in the
2603 hippocampus did not reach statistical significance.

2604

2605 .

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

2613 of sample Golgi-stained neurons in the hippocampus for the 4 groups from optical
2614 microscope at 400X magnification. Scale bar represents 20 μ m.

2615 *Stress had differential effects on the number of spines on primary*
2616 *dendrites in hippocampal neurons in presence and absence of EE*

2617 A two-way ANOVA was conducted for total number of primary spines of
2618 hippocampal neurons with stress and EE as two between subject sources of
2619 variance. The effect of stress ($F_{(1,275)} = 0.405$, $p = 0.53$) on the number of primary
2620 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
2623 primary dendrites in the hippocampus.

2624 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2625 absence of EE ($t_{147} = -3.853$, $p < 0.00$; Cohen's $d = 0.646$), stress significantly
2626 increased the number of spines found on primary dendrites in the hippocampus.
2627 Similarly, in the presence of EE ($t_{128} = 2.123$, $p = 0.04$; Cohen's $d = 0.370$; Figure
2628 R.35) stress significantly decreased the number of spines found on primary
2629 dendrites in the hippocampus.

2630

2631

2632

2633 **Figure R.35: Effect of early life stress and EE on primary spines in**
 2634 **hippocampal neurons:** Stress significantly increased the number of spines found
 2635 on primary dendrites in the hippocampus in the absence of EE and stress
 2636 significantly decreased the number of spines found on primary dendrites in the
 2637 hippocampus in the absence of EE. Graphs represent Mean $\pm$ SEM. N (number of
 2638 neurons) = 60 for AFR, 89 for Stress, 60 for EE and 70 for MSEE, 10
 2639 neurons/animal; * $p \leq 0.05$, ** $p \leq 0.01$.

2640 *Stress significantly increased the number of spines on secondary*
 2641 *dendrites in hippocampal neurons in the absence of EE*

2642 A two-way ANOVA was conducted for a total number of secondary spines of
 2643 hippocampal neurons with stress and EE as between subject sources of variance.
 2644 The effect of stress ($F_{(1,275)} = 5.108$, $p = 0.03$), EE ($F_{(1,275)} = 49.604$, $p < 0.00$) and
 2645 the interaction between stress and EE ($F_{(1,275)} = 25.397$, $p < 0.00$) had a significant
 2646 effect on the number of spines found on secondary dendrites in the hippocampus.

2647 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2648 absence of EE ($t_{148} = -5.932$, $p < 0.00$; Cohen's $d = 0.970$), stress significantly
2649 increased the number of spines found on secondary dendrites in the hippocampus.
2650 However, in the presence of EE ($t_{127} = 1.724$, $p = 0.09$; Cohen's $d = 0.304$; Figure
2651 R.36) the effect of stress on the number of secondary spines in the hippocampus
2652 did not reach statistical significance.

2653 Under normal housing conditions, maternal stress reduces the complexity of
2654 hippocampal neurons. Specifically, dendritic length and cell proliferation are
2655 known to decrease in MS rats compared to control [126, 127]. While studies report
2656 atrophy of CA3 neurons in the hippocampus, Sunanda *et al.*, (1995) observed
2657 increased spine density in rats subjected to chronic stress in adult life and has
2658 argued that this observed increase in spine density may be a mechanism to counter
2659 neuronal atrophy by increasing the postsynaptic membrane area [289]. This
2660 observation was corroborated in the above results, where rats subjected to MS
2661 showed an increase in spine density of both primary and secondary branches of the
2662 neuron. Moreover, MS did not alter neuronal length or number of branch points in
2663 normally housed rats. This is congruent with the observation that although atrophy
2664 of hippocampal neurons can be observed up until four days post-MS treatment,
2665 atrophy is no longer present after ten days post stress treatment because of the
2666 plastic recovering nature of hippocampal neurons [290, 291]. Also, MSEE group
2667 showed a decrease in both primary and secondary neuronal spine density compared
2668 to rats exposed only to the enriched environment. EE paradigm is widely studied
2669 on hippocampal neurons and its plasticity, where the neurons are shown to have

2670 elevated neuronal arborization and increased neurogenesis due to the influence of
 2671 EE [A- 243, 248, 301, 319-324] as seen in our study.

2672

2673 **Figure R.36: Effect of early life stress and EE on secondary spines in**
 2674 **hippocampal neurons:** Stress significantly increased the number of spines found
 2675 on secondary dendrites in the hippocampus in the absence of EE. Graphs represent
 2676 Mean $\pm$ SEM. N (number of neurons) = 60 for AFR, 90 for Stress, 60 for EE and
 2677 69 for MSEE, 10 neurons/animal; * $p \leq 0.05$.

2678 *Stress did not alter dendritic length in mPFC neurons in the presence*
2679 *and absence of EE*

2680 Together with the BLA and hippocampus, the mPFC modulates fear responses
2681 [115, 140, 141]. Prelimbic (PrL) and Infralimbic (IL) regions are the two most
2682 important sub-regions that form mPFC [142]. The effect of MS on the neuronal
2683 morphology of the prelimbic (PrL) region of medial prefrontal cortex was focused
2684 in particular due to their strong projections to the BLA [292]. So, we will focus on
2685 the final region of the brain HPA axis, the mPFC to study the effect of early life
2686 environment on dendritic length, branch points and spine density of primary and
2687 secondary neurons of this region. The results along with the statistics are given
2688 below.

2689 A two-way ANOVA was conducted for dendritic length of mPFC neurons with
2690 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 ($F_{(1,259)} = 0.715$,
2691 $p = 0.40$) on the total dendritic length of neurons in the mPFC did not reach
2692 statistical significance. However, EE ($F_{(1,259)} = 5.023$, $p = 0.03$) had a significant
2693 main effect on the increase in total dendritic length of neurons in the mPFC.
2694

2695 Orthogonal planned comparisons (independent samples t-tests) showed that, in the
2696 absence of EE ($t_{133} = 1.255$, $p = 0.21$; Cohen's $d = 0.213$) the effect of stress on the
2697 total dendritic length of neurons in the mPFC did not reach significance. Similarly,
2698 in the presence of EE ($t_{126} = -0.113$, $p = 0.91$; Cohen's $d = 0.020$; Figure R.37) the
2699 effect of stress on the total dendritic length of neurons in the mPFC did not reach
2700 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 $\pm$ 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

2715 between stress and EE ($F_{(1,260)} = 38.427$, $p < 0.00$) had a significant effect on the
2716 total number of branch points of neurons in the mPFC.

2717 Orthogonal planned comparisons (independent samples t-tests) showed that in the
2718 absence of EE ($t_{135} = -7.215$, $p < 0.00$; Cohen's $d = 1.283$), stress significantly
2719 increased the total number of branch points of neurons in the mPFC. However, in
2720 the presence of EE ($t_{125} = -0.555$, $p < 0.58$; Cohen's $d = 0.099$; Figure R.38) the
2721 effect of stress on the total number of branch points of neurons in the mPFC did
2722 not reach statistical significance.

2723 The PrL region suffers from dendritic length retraction and loss of spines post-
2724 chronic stress or exogenous CORT administration in adult rats. Similar to
2725 hippocampal neurons, these cortical neurons belonging to young adult rats also
2726 possess the capacity to recover the dendritic length and branching during post-
2727 stress recovery phase [293, 294]. In this study, MS rats showed increased number
2728 of branch points. This contrasting reversing effect of neuronal morphology has
2729 been reported before [293]. Additionally, my observation is in line with a previous
2730 study that shows an increase in neuronal spine density after MS [295].

2731

2732

2733

2734 **Figure R.38: Effect of early life stress and EE on total number of branch**
2735 **points in mPFC neurons:** Stress significantly increased the total number of
2736 branch points of neurons in the mPFC in the absence of EE. Graphs represent
2737 Mean $\pm$ SEM. N (number of neurons) = 60 for AFR, 77 for Stress, 63 for EE and
2738 64 for MSEE, 8-10 neurons/animal; $**p \leq 0.01$. Inset (bottom): Representative
2739 images of sample Golgi-stained neurons in the mPFC for the 4 groups from optical
2740 microscope at 400X magnification. Scale bar represents 20 μ m.

2741

2742 *Stress had differential effects on the number of spines of primary*
2743 *dendrites of mPFC neurons in presence and absence of EE*

2744 A two-way ANOVA was conducted for number of primary spines in mPFC
2745 neurons with stress and EE as between subject sources of variance. The effect of
2746 stress ($F_{(1,260)} = 0.003$, $p = 0.96$) on the number of primary spines in mPFC did not
2747 reach statistical significance. However, EE ($F_{(1,260)} = 4.122$, $p = 0.04$) and the
2748 interaction between stress and EE ($F_{(1,260)} = 98.948$, $p < 0.00$) had a significant
2749 effect on the number of spines found on primary dendrites in the mPFC.

2750 Orthogonal planned comparisons (independent samples t-tests) showed that, in the
2751 absence of EE ($t_{136} = -7.461$, $p < 0.00$; Cohen's $d = 1.298$), stress significantly
2752 increased the number of spines found on primary dendrites in the mPFC. However,
2753 in the presence of EE ($t_{124} = 6.635$, $p < 0.00$; Cohen's $d = 1.179$; Figure R.39)
2754 stress significantly decreased the number of primary spines in mPFC neurons.

2755

2756

2757 **Figure R.39: Effect of early life stress on primary spines in mPFC neurons:**

2758 Stress significantly increased the number of spines found on primary dendrites in
 2759 the mPFC in the absence of EE and stress significantly decreased the number of
 2760 spines found on primary dendrites in the mPFC in the presence of EE. Graphs
 2761 represent Mean $\pm$ SEM. N (number of neurons) = 59 for AFR, 79 for Stress, 62 for
 2762 EE and 64 for MSEE, 10 neurons/animal; $**p \leq 0.01$.

2763 *Stress significantly increased the number of spines on secondary*
 2764 *dendrites in mPFC neurons in the absence of EE*

2765 A two-way ANOVA was conducted for number of secondary spines in mPFC
 2766 neurons with stress and EE as between subject sources of variance. The effect of
 2767 stress ($F_{(1,260)} = 1.268$, $p = 0.26$) and EE ($F_{(1,260)} = 0.520$, $p = 0.47$) on the number
 2768 of secondary spines in the mPFC did not reach statistical significance. However,
 2769 the interaction between stress and EE ($F_{(1,260)} = 50.531$, $p < 0.00$) had a significant
 2770 effect on the number of spines found on secondary dendrites in the mPFC.

2771 Orthogonal planned comparisons (independent samples t-tests) showed that, in the
2772 absence of EE ($t_{137} = -5.567$, $p < 0.00$; Cohen's $d = 0.973$), stress significantly
2773 increased the number of spines found on secondary dendrites in the mPFC.
2774 However, in the presence of EE ($t_{123} = 4.519$, $p < 0.00$; Cohen's $d = 0.813$; Figure
2775 R.40) stress significantly decreased the number of secondary spines found in the
2776 mPFC.

2777 MS rats showed elevated number of branch points and increased neuronal spine
2778 density. It is possible that the observed effects of MS are a result of compensation
2779 through reorganization of dendrites leading to hypertrophy. On the other hand, it is
2780 possible that the observed increase in dendritic arborization is a product of the
2781 extended period of recovery and additional compensation (approximately 36 days)
2782 given after MS treatment. This reversing effect of neuronal morphology is in
2783 consensus with previous reports [293]. Additionally, my observation is in line with
2784 a previous study that shows an increase in neuronal spine density after MS [295].

2785 Prior literature shows that the enriched environment enhances the responsiveness
2786 of cortical neurons to stress [296, 297]. This is in line with my observation
2787 mentioned above where EE increases dendritic length, not branch points. I propose
2788 that decrease in branch points may also be a compensatory mechanism to temper
2789 the effect of dendritic length hypertrophy. The impact of EE on dendritic
2790 morphology is comparable to the pro-resilient features observed in previous work
2791 [255]. A decrease in number of branch points as well as primary and secondary
2792 neuronal spine density in the mPFC was observed in rats subjected to both MS and
2793 EE. This effect was not seen in rats exposed to only environmental enrichment.

2794 Interestingly, there was an unique effect of enriched environment in neuronal
 2795 morphology in all the three brain regions considered in this study. Further
 2796 experiments are necessary to understand these interesting results that aid
 2797 individuals to appropriately respond to their early life environment.

2798

2799

2800

2801 **Figure R.40: Effect of early life stress and EE on secondary spines in mPFC**

2802 **neurons:** Stress significantly increased the number of spines found on secondary
 2803 dendrites in the mPFC in the absence of EE. Graphs represent Mean $\pm$ SEM. N

2804 (number of neurons) = 60 for AFR, 79 for Stress, 60 for EE and 65 for MSEE,10

2805 neurons/animal; $**p \leq 0.01$.

2806 **Chapter 5**

2807 **5. Discussion**

2808

2809 Early-life is a critical developmental period for the physical and emotional
2810 development of an individual (P0-P21 in rodents) as this is the time-window,
2811 which is vulnerable to the varying environment during which certain environment
2812 can bias the development of neural systems mediating emotion towards pathology
2813 later in life. [13]. Specifically, early life environment programs crucial aspects of
2814 neurobiological development including behavioral, emotional, cognitive and
2815 physiological development. Thus, exposure to stress during this period has
2816 important implications for stress sensitivity and behavioral response throughout the
2817 life of an individual [6, 7, 99]. In rats, one of the most well-established models of
2818 chronic early life stress is maternal separation [264]. Different interventions were
2819 used to counter the detrimental effects of maternal stress. Of all the techniques
2820 used to reduce the compromising effects of MS, enriched environment has been
2821 the most intriguing one as this is not an invasive method. A mere change in the
2822 environment for a short period of time can alleviate the harmful effects of MS
2823 significantly. Previous studies on the effect of enriched environment on maternal
2824 separation were done using long-term enrichment protocols [167, 298]. However,
2825 providing short-term enrichment in adulthood is known to reverse the detrimental
2826 effects of chronic immobilization stress [166] and maternal separation[87]. In my
2827 study, the focus was to investigate the effect of enriched environment provided in
2828 early life, concomitant to the period of maternal separation. The effects of early

2829 environment on physiology, behavior, structural plasticity of neuronal dendrites
2830 and molecular changes were evaluated in brain during adulthood.

2831 Three distinct themes emerge from my results. These are,

2832 i) MS is stressful (pages 152 to 160).

2833 ii) EE is beneficial (pages 161 to 166).

2834 iii) MS and EE have interesting combinatorial effect (pages 167 to 173).

2835 5.1 Effects of Maternal Separation

2836

2837 *5.1.1 Impact of MS on physiology (S.A.1)*

2838 Early life stress affects various physiological parameters, particularly those about
2839 the hypothalamus-pituitary-adrenal (HPA) axis [167] and the
2840 sympathoadrenomedullary axis [299]. The adrenal glands are common to both
2841 these systems and are responsible for the production of corticosterone in response
2842 to stressful stimuli. Adverse conditions in early life could cause long-term
2843 behavioural deficits and epigenetic changes in glucocorticoid receptor in rat
2844 hippocampus [48]. Manipulation of the environment and the quality of care from
2845 the mother in this model causes chronic ‘early life stress’ which has long-term
2846 behavioral defects [76]. A large body of evidence shows that MS during postnatal
2847 weeks causes a permanent increase in anxiety related disorders [178]. Lifelong
2848 hyperactivity of HPA (hypothalamus-pituitary-adrenal) axis is another
2849 consequence of this postnatal separation [179]. My results showed that MS caused
2850 adrenal gland hypertrophy in male rats living in standard animal facility housing

2851 when compared to AFR rats. Interestingly, early life stress in enriched housing
2852 reduced the increase the adrenal weights of animals (Figure R.3). Similarly,
2853 baseline plasma corticosterone levels were significantly increased in adult rats
2854 subjected to MS in early life under standard animal housing conditions. However,
2855 animals exposed to MS along with enriched environment did not show a
2856 significant difference in baseline plasma corticosterone levels (Figure R.5). Our
2857 results of MS alone causing enhanced corticosterone and adrenal gland
2858 hypertrophy is similar to what has previously been observed in rats. It has been
2859 reported that rats exposed to chronic stress with both baseline plasma
2860 corticosterone [257, 259] and adrenal weights [256, 258] increased compared to
2861 control animals.

2862 *5.1.2 Impact of MS on behavior (S.A.1)*

2863 Increase in CORT and adrenal hypertrophy indicates high anxiety in MS [99].
2864 Therefore, we tested the impact of early life stress and EE on various behavioral
2865 readouts during adulthood. To determine the effect of MS in the absence and
2866 presence of enriched environment on anxiety-like behavior, animals were
2867 subjected to various anxiety tests in adulthood (P56). This included exposure of
2868 experimental animals to open field test (OFT), elevated plus maze (EPM) and
2869 home cage emergence test (HCE). Along with anxiety like behavior, depressive
2870 like behavior is another common behavioral defect observed in MS rats [300, 301].
2871 To determine the impact of early life stress and EE on depressive-like behavior,
2872 the animals were subjected to the forced swim test (FST).

2873 My results showed that early life maternal separation induced anxiogenic
2874 avoidance of inner zone in open field test. This represented the high anxiety in
2875 animals exposed to MS alone in standard housing during early life. However, this
2876 effect was not seen in animals exposed to MS in the enriched environment (Figure
2877 R.9). This result is in line with previous reports that show that MS leads to
2878 anxiogenic behavior in adulthood [263, 264]. MS had no significant effect on
2879 latency to escape in the home cage emergence test irrespective of EE (Figure R.8).
2880 Similarly, MS did not alter the percentage of open arm time/entries and risk
2881 assessment behavior (measured using the number of head dips) as tested on EPM
2882 (Figure R.10, R.11, and R.12). The number of closed arm entries was same across
2883 all groups showing no influence of locomotor function (an internal control of
2884 EPM) (Figure R.13), on anxiety.

2885 Immobility and swimming were measured at two time points, T1 and T2. T1 was
2886 the first day where the animals were habituated to the test and T2 was the time
2887 point for the actual test day. In FST, the initial exposure to the swim tank on day 1
2888 (T1) is known to induce a state of “behavioral despair,” such that these rats have
2889 *altered emotional reactivity* in response to a new emergency represented by
2890 swimming. So, day 2 (T2) is considered as the test day where the depression-like
2891 behavior can be well captured, after T1 habituation [274]. In this study, we
2892 quantified T1 behavioral response to capture the initial response to novelty stress
2893 and T2 behavioral response to capture the stabilized emotional response to forced
2894 swim test. I observed that MS significantly decreased immobility in T1 (FST)
2895 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

2919 neuronal spine density in the amygdala [7, 17, 21]. Similarly, in my paradigm, a
2920 significant increase in neuronal spine density was observed in rats subjected
2921 maternal separation compared to control rats in the normal housing (Figure R.31
2922 and R.32). Although the spine density was increased, an increase in dendritic
2923 length and branch points was not observed in normal housing rats that were
2924 subjected to maternal separation (Figure R.29 and R.30).

2925 In addition to BLA, hippocampus was examined due to its involvement in widely
2926 reported stress and cognitive processes [119, 120] It is known to be one of the
2927 most plastic regions of the brain and the structural plasticity is highly dependent on
2928 the number and impact of stressful episodes experienced by an individual (level of
2929 glucocorticoids in the systemic circulation) [122, 123]. Additionally, stress
2930 induced impairment in learning and performance in short-term memory tasks is
2931 closely associated with atrophy of CA3 dendrites in the hippocampus [124, 125].
2932 Under normal housing conditions, maternal stress reduces the complexity of
2933 hippocampal neurons. Specifically, dendritic length and cell proliferation are
2934 known to decrease in MS rats compared to control [126, 127]. While studies report
2935 atrophy of CA3 neurons in the hippocampus, Sunanda *et al.*, (1995) observed
2936 increased spine density in rats subjected to chronic stress in adult life and has
2937 argued that this observed increase in spine density may be a mechanism to counter
2938 neuronal atrophy by increasing the postsynaptic membrane area [289]. This
2939 observation was corroborated in our paradigm, where rats subjected to MS showed
2940 an increase in spine density of both primary and secondary branches of the neuron
2941 in BLA (Figure R.35 and R.36). Moreover, in our paradigm, MS did not alter

2942 neuronal length or number of branch points in normally housed rats (Figure R.33
2943 and R.34). This is congruent with the observation that although atrophy of
2944 hippocampal neurons can be observed up until four days post-MS treatment,
2945 atrophy is no longer present after ten days post stress treatment because of the
2946 plastic recovering nature of hippocampal neurons [290, 291].

2947 mPFC is one of the many GC-sensitive brain regions that regulate stress response
2948 by the activity of glucocorticoids. Two types of corticosteroid receptors in the
2949 brain are, glucocorticoid receptors (GR) and mineralocorticoid receptors (MR).
2950 The MR binds GCs with a 10-fold higher affinity than the GR [302]. The MR
2951 activation is considered as neuroprotective to memory performance. In contrast,
2952 GR activation is known to damage neurons and impair memory performance
2953 especially when prolonged and elevated GR occupancy is observed. These
2954 observations suggest that GR/MR ratio is an important parameter in to understand
2955 CORT effects in different brain regions [303]. mPFC has a high ratio of GR/MR
2956 ratio that indicates the high involvement of stress hormones [142]. The mPFC is
2957 reported to play a role in processing cognitive and goal-oriented behavior [304].
2958 Importantly, the mPFC indirectly provides a negative feedback to the HPA axis
2959 [138-140]. Together with the BLA and hippocampus, the mPFC modulates fear
2960 responses [117, 139, 140]. Prelimbic (PrL) and Infralimbic (IL) regions are the two
2961 most important sub-regions that form mPFC. PrL is shown to play a role in fear-
2962 conditioning and learning while fear extinction is associated with IL [141]. In my
2963 study, the effect of MS on the neuronal morphology of the prelimbic (PrL) medial
2964 prefrontal cortex was studied due to their strong projections to the BLA [292]. MS

2965 rats showed increased number of branch points and increased neuronal spine
2966 density (Figure R.38, R.39, and R.40). It is possible that the observed effects of
2967 MS are a result of compensation through reorganization of dendrites leading to
2968 hypertrophy. On the other hand, it is possible that the observed increase in
2969 dendritic arborization is a product of the extended period of recovery and
2970 additional compensation (approximately 36 days) given after MS treatment. This
2971 reversing effect of neuronal morphology is in consensus with previous reports
2972 [293]. Additionally, my observation is in line with a previous study that shows an
2973 increase in neuronal spine density after MS [295].

2974 *5.1.4 Impact of MS at the molecular level (S.A.3/4)*

2975 As discussed in earlier paragraphs, the maladaptive behavioral output of early life
2976 stress was demonstrated in MS. MS led to adrenal hypertrophy, elevated
2977 corticosterone levels, anxiety-like behavior and high spine density in BLA. GC
2978 activated glucocorticoid receptor (GR) was analyzed because of its central role in
2979 maintaining the regulation of HPA axis in response to various stressors [142, 218,
2980 219]. The density of GR population across amygdala, hippocampus, and mPFC
2981 differ and is shown to have a link in providing feedback regulation to HPA axis
2982 [219]. The recruitment of activated GR in the nucleus as a transcription factor is a
2983 crucial step that initiates a relay of downstream molecular pathways to mediate
2984 stress response [112, 220]. Prior studies show that activation of GR through GC
2985 causes phosphorylation of $\text{Erk1/2}^{\text{MAPK}}$. This activated $\text{Erk1/2}^{\text{MAPK}}$ causes relay of
2986 downstream molecular pathways that are crucial in mediating behavioral effects of
2987 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

3034 protein expression in rats is linked to enhanced depressive-like behavior and can
3035 be initiated by repeated corticosterone administration in the amygdala [316].
3036 Hence, GAD67 in the inhibitory neurons of the BLA can be regulated by stress,
3037 which can lead to aberrant emotional behavior.

3038 Until now, the impact of MS on physiology, behavior, neuronal morphology and
3039 the molecular players of the stress response were discussed. The mal-adaptive
3040 outcomes induced by the stress paradigm were also indicated. In the next section,
3041 the main effect of enriched environment on all the experimental endpoints will be
3042 discussed.

3043

3044 5.2 Effects of Enriched Environment (EE)

3045

3046 Exposure to short-term environmental enrichment in adulthood is known to
3047 enhance defensive behavior and attractiveness in male rats [206]. Additionally,
3048 exposure to short-term environmental enrichment in adulthood is also known to
3049 alleviate behavioral compromise induced by MS [87]. Mice exposed to the
3050 enriched environment had enhanced memory function in various learning tasks
3051 [204]. Research on benefits of EE is being applied in zoos and farms where the
3052 animals are seen to thrive better in a more enriched environment [212]. Numerous
3053 studies on EE similar to those mentioned above, inspired me to apply this
3054 treatment for this study, to investigate the impact of early life EE (P0-P21). My
3055 paradigm is the first study to investigate the impact of short-term exposure to the
3056 enriched environment in early life. EE effects were considered as the main effect
3057 (i.e. presence & absence of EE on stress) of treatment across all experimental
3058 groups.

3059 *5.2.1 Impact of EE on physiology level (S.A.1)*

3060 The presence of EE significantly decreased the body weight-area under the curve
3061 (AUC) (Figure R.2) of experimental animals. The area under curve depicts the
3062 overall change in body weight across the course of development (early life till
3063 adulthood). Conversely, maternal separation did not affect the body weight AUC
3064 of test animals assigned to the standard cages. Chronic stress is known to lead to
3065 an increase in body weight [253]. In our paradigm, enriched environment housing
3066 reduced body weight gain (throughout the experiment) instead. Interestingly, rats

3067 exposed to prenatal stress and then housed in environmental enrichment conditions
3068 showed a similar decrease in body weight [254]. The decrease in body weight gain
3069 could be due to the continuous activity exploring various objects and interacting
3070 with fellow cage mates.

3071 Adrenal glands are vital organs that produce corticosterone in response to stressful
3072 stimuli and undergo hypertrophy in the process [256, 257]. A two-way ANOVA
3073 revealed a significant main effect of enriched environment on adrenal gland
3074 weight. Specifically, animals assigned to the enriched environment housing
3075 condition had significantly lower adrenal weights compared to animals assigned to
3076 animal facility rearing conditions, irrespective of presence or absence of maternal
3077 separation (Figure R.3). Similarly, a two-way ANOVA showed a main effect for
3078 enriched environment on plasma corticosterone levels at the point of sacrifice
3079 during adulthood (Figure R.7). This is in good agreement with another study by
3080 Roy and colleagues (2001) who showed that male mice assigned to the
3081 environmental enrichment treatment had significantly less plasma corticosterone
3082 compared to animals in standard animal house housing after exposure to stress
3083 [260]. This emphasized on the active coping output (low CORT) induced by EE.

3084 In other words, animals in the enriched environment had significantly lower
3085 plasma corticosterone at the end of the experiment, compared to animals in the
3086 normal animal housing conditions, irrespective of absence or presence of early-life
3087 maternal stress treatment. In rats assigned to standard cages, maternal stress
3088 significantly increased adrenal weights. This effect of maternal stress was not
3089 observed in rats that were placed in the enriched environment (MSEE). This

3090 observation is indicative of the modulatory effect of environmental enrichment on
3091 the adrenal glands and corticosterone, the crucial arms of HPA axis. On the whole,
3092 the study highlighted that EE modulated the HPA axis to regulate the baseline
3093 corticosterone levels in the animals to actively cope with stress. Another
3094 interesting point is the combinatorial effect of MS and EE wherein the interaction
3095 between the two treatments alleviates the harmful effects of MS on certain
3096 physiological parameters (as mentioned above).

3097 *5.2.2 Impact of EE on behavior (S.A.1)*

3098 In general, animals assigned to the enriched environment displayed a more
3099 anxiolytic behavior compared to animals in the normal animal house treatment. A
3100 two-way ANOVA revealed significant main effects of enriched environment on
3101 time spent exploring the inner zone in the open field test (Figure R.9). This
3102 observation is congruent with previous studies that reported significantly increased
3103 anxiolytic behavior in animals placed in enriched environment housing
3104 conditions[317]

3105 *5.2.3 Impact of EE on dendritic morphology (S.A.2)*

3106 Stress-induced growth of dendrites and synapses in BLA neurons can be rescued
3107 with environmental enrichment in adult male rats [166]. So far, only two studies
3108 have determined that enriched environment can rescue the damaging effects of
3109 early life stress. One reported that environmental enrichment during the peri-
3110 pubertal period rescues the harmful effects of maternal separation on HPA and
3111 behavioural response to stressors [167]. Another study reported that, short term

3112 enrichment reverses the anxiety and hypertrophy of BLA neurons in adulthood
3113 [87]. As mentioned earlier, there is limited information about how enriched
3114 environment during early life (P0-P21) can influence dendritic morphological
3115 changes. Interestingly, short-term environmental enrichment had main effect on
3116 secondary neuronal spine density of BLA and hippocampal neurons (Figure R.32).
3117 Environmental enrichment, irrespective of presence or absence of maternal
3118 separation treatment resulted in a general decrease in secondary spine density in
3119 BLA neurons. Conversely, enriched environment significantly increased secondary
3120 neuronal spine density in the hippocampus. This observed increase in the neuronal
3121 spine density is in direct agreement with reports on increased spine density as a
3122 result of environmental enrichment, found in the literature [318-322]. This is result
3123 is opposite to the stress-induced hypertrophy seen in BLA [87].

3124 In my paradigm, an increase in dendritic length but a decrease in branch points of
3125 mPFC neurons was observed. Previous literature showed that the enriched
3126 environment enhanced the responsiveness of cortical neurons to stress [296, 297].
3127 Similarly, this study revealed that environmental enrichment increases dendritic
3128 length but not the branch points. I propose that decrease in branch points may be a
3129 compensatory mechanism to dampen the effect of dendritic length hypertrophy.
3130 Hypertrophy of mPFC neurons was observed in animals exposed to long-term
3131 enriched environment, which displayed active coping behavior [255]. This
3132 observation is comparable to the impact of EE on dendritic morphology in this
3133 study, which reinstates the role of mPFC in regulating HPA in response to varying
3134 environment during early life (P2-P21).

3135 *5.2.4 Impact of EE at molecular level (S.A.3/4)*

3136 The density of GR concentration across brain regions such as hippocampus,
3137 amygdala, and mPFC varies and is linked with its ability to provide feedback
3138 regulation [142]. GR is an important regulator stress response [167]. The activation
3139 of GR (by corticosterone occupancy) and its localization into the nucleus for its
3140 recruitment as transcription factor constitutes one of the key molecular pathways
3141 mediating stress response in relevant brain regions [112, 220]. GR receptors in the
3142 amygdala induced by stereotactic administration of corticosterone lead to
3143 hyperactivation of HPA and anxiety [323]. These compromising behavioral
3144 features are reversed by experimentally blocking corticosterone binding to GR in
3145 the BLA [324]. These studies indicate the association of enhanced GR activation
3146 with potentiation of anxiety and BLA hypertrophy. Hence, in the basolateral
3147 amygdala, the effect of environmental enrichment on GR expression is critical. In
3148 this study, there was a significant main effect of environmental enrichment on GR
3149 mRNA abundance in the basolateral amygdala (Figure R.17). This is congruent to
3150 my observation that environmental enrichment increased the percentage of number
3151 of cells containing activated GR when compared to animals in normal housing
3152 conditions (Figure R.23). As mentioned earlier, the basolateral amygdala is one of
3153 the brain regions that provide feedback for regulating corticosterone secretion via
3154 HPA axis. Activated GR and its subsequent translocation into the nucleus is a
3155 major step in mediating stress as this step initiates the feedback loop in response to
3156 stress episodes [112, 220]. Previously, the effect of enriched environment housing
3157 on GR expression has been studied mainly in the hippocampus wherein the GR

3158 expression is observed to be upregulated in the presence of environmental
3159 enrichment [325, 326]. GR expression in rat pups is reported to vary depending on
3160 the mother's care they receive in the form of licking and grooming in
3161 hippocampus. In pups that experience high licking and grooming, GR expression is
3162 upregulated compared to pups that experience low licking and grooming and it
3163 highlighted that increase in GR is beneficial for HPA-GC regulation [181]. This
3164 study showed that environmental enrichment significantly increased activation of
3165 nuclear GR in the BLA, which plays a significant role in downstream protein
3166 regulation, which in turn drives neural plasticity as well as BLA feedback to the
3167 HPA axis and by extension, corticosterone secretion.

3168 Brain-derived neurotrophic factor (BDNF) is an important growth factor for
3169 regulating survival in a variety of cells. It also plays important role in modulating
3170 dendritic growth in various regions of the brain. BDNF mediates intracellular
3171 pathways that modulate the synaptic plasticity of neurons [327-329]. Expression of
3172 pro-BDNF, the precursor product of mature BDNF, was significantly decreased in
3173 rats placed in environmental enrichment (Figure R.21). Previous work has
3174 associated increased expression of pro-BDNF in the hippocampus to depression in
3175 humans. This study used formalin-fixed paraffin-embedded hippocampal sections
3176 from subjects with schizophrenia, major depressive disorder (MDD), bipolar
3177 disorder (BPD) and non-psychiatric controls from the Stanley Foundation
3178 Neuropathology Consortium to analyze the expression of Pro-BDNF [288]. In
3179 contrast, the reduced pro-BDNF levels in my study could have anti-depressive like
3180 effect driven by BLA. However, environmental enrichment was not observed to

3181 affect the expression of BDNF protein in the BLA (Figure R.22 and R.25). Prior
3182 reports have shown that BDNF levels are elevated in response to enriched
3183 environment conditions. Only one report demonstrated that short-term enriched
3184 environment during stress rescues the stress-induced elevated BDNF mRNA
3185 expression in BLA [166]. This highlights the differential role of BDNF regulation
3186 in response to EE during early life and adulthood (S.A.3).

3187 My data supports the notion that MS is indeed a stressful paradigm and short-term
3188 EE in early life induces several resilient features in physiological, behavioral,
3189 dendritic morphological and molecular parameters. In the next section, the
3190 interaction effect between MS and EE on all the experimental endpoints will be
3191 discussed.

3192 5.3 Interaction between Maternal Separation and Environmental 3193 Enrichment 3194

3195 The previous sections have concentrated on understanding the individual effects of
3196 stress and environmental enrichment on various endpoints of physiology, behavior
3197 and brain plasticity. In this section, the effect of concurrent effect of environmental
3198 enrichment and early life maternal stress on different experimental endpoints will
3199 be discussed. Previous studies have shown that when stress and EE are combined,
3200 they rescue the maladaptive behaviors observed in stress rats [87, 166]. This gives
3201 crucial information about the combinatorial impact of two different environments
3202 that act in a synergistic way to bring about an active-coping behavior in animals.
3203 Hence, MSEE paradigm was used in this study to explore the chances of inducing
3204 resilient (active-coping) characteristics in animals exposed to concurrent MS and
3205 EE.

3206 *5.3.1 Impact of both MS and EE on physiology (S.A.1)*

3207 A two-way ANOVA revealed that there was a significant effect of the interaction
3208 of stress and environmental enrichment on body weight. Rats subjected to both
3209 early life MS and enriched environment had significantly decreased body weights
3210 in adulthood (Figure R.2). Previously, early-life maternal stress in normal animal
3211 house conditions led to a significant increase in body weight compared to animals
3212 that did not undergo MS [253]. This suggests that presence of environmental
3213 enrichment along with MS in my study resisted the increase in body weight as
3214 observed in rats subjected to only MS. This could be due to the increase in
3215 locomotor activity (climbing, exploring, etc) involved in EE cages [255]. An

3216 interesting point to note here is that even a short-term exposure to EE in early life
3217 can induce a long-term impact on other physiological parameters like adrenal
3218 weight and corticosterone secretion of an individual.

3219 MS without EE housing displayed adrenal hypertrophy and a two-way ANOVA
3220 revealed a significant interaction effect of stress and environmental enrichment on
3221 adrenal weights with no hypertrophy (Figure R.3). Previous reports showed that
3222 early life stress and chronic stress resulted in adrenal hypertrophy [256, 258].
3223 However, there are no current studies specifically linking MS to adrenal
3224 hypertrophy. The results in this study showed that in an enriched environment, MS
3225 resulted in significant hypotrophy of adrenal glands along with MS main effect.

3226 Environmental enrichment significantly decreased plasma corticosterone levels
3227 post-exposure to predator odor (Figure R.6). Interestingly, in environmental
3228 enrichment conditions, rats subjected to MS had significantly lower plasma
3229 corticosterone concentration compared to rats subjected only to environmental
3230 enrichment, when exposed to predator odor. This suggests that in addition to the
3231 observed general decrease in plasma corticosterone levels in EE animals, exposure
3232 to early life maternal stress served to decrease plasma corticosterone in EE animals
3233 further. Thus, the concurrent presence of both stress and environmental enrichment
3234 appears to have a potent inhibitory effect on the HPA axis [167]. Parallel maternal
3235 stress and environmental enrichment decreased plasma corticosterone and lead to
3236 adrenal hypotrophy suggesting that this dual reduces the adrenal glands, the two
3237 important elements (GC and adrenal glands) of the HPA axis.

3238

3239 *5.3.2 Impact of both MS and EE on behavior (S.A.1)*

3240 Environmental enrichment is reported to have a significant anxiolytic effect [166,
3241 167]. This is corroborated by our observation that rats in environmental
3242 enrichment spent significantly more time exploring the inner zone of the OFT
3243 compared to rats in normal housing conditions. A two-way ANOVA of OFT data
3244 further revealed that concurrent exposure to MS and environmental enrichment
3245 resulted in increased anxiolytic behavior (figure R.9). In other words, rats
3246 subjected to both MS and environment enrichment spent significantly more time
3247 exploring the inner zone of the OFT. This increase in anxiolytic behavior was also
3248 observed in EPM. Rats subjected to both maternal separation and environmental
3249 enrichment spent more time exploring the open arm of the EPM compared to rats
3250 exposed only to environmental enrichment (figure R.10 and R.11). Thus, it is
3251 observed that the presence of both maternal separation and environmental
3252 enrichment has a cumulative effect on the increase in anxiolytic behavior (active
3253 coping). This is corroborated by reports that show both short-term [87] and long-
3254 term [167] enrichment in adulthood after early life maternal separation increases
3255 anxiolytic behavior.

3256 *5.3.3 Impact of both MS and EE on morphology (S.A.2)*

3257 In previous sections, it was reported that the concurrent exposure to both maternal
3258 separation and environmental enrichment resulted in robust benefit both on
3259 physiology and behavior. Surprisingly, analysis of dendritic morphology in the

3260 BLA showed results similar (increased dendritic spines) to what has been reported
3261 in the prior literature to occur in rats subjected to maternal separation in the
3262 absence of environmental enrichment. Rats exposed to both maternal separation
3263 and environmental enrichment showed dendritic length hypertrophy, increase in
3264 branch points and increase in primary neuronal spine density in the BLA compared
3265 to rats exposed to only environmental enrichment (figure R.29, R.30, and R.31).
3266 The increase in BLA dendritic arborization was not expected in this study. As
3267 mentioned earlier, increase in dendritic length and branch points are associated
3268 with anxiety-like behavior but this was not the case in this study. In other words, in
3269 response to early life stress, individuals employ adaptation strategies that would
3270 allow them to make “the best out of a bad situation” so to speak [78, 79]. This may
3271 be responsible for the increase in dendritic arborization observed in this study. This
3272 could also be due to overlap of timeline of this study and amygdala development.

3273 However, extant literature argues that certain negative experiences in the critical
3274 developmental phase may improve memory processes and learning under stressful
3275 situation [77]. Thus, it is important to note that, stressful experiences are not
3276 always associated with maladaptive behavior as shown in previous reports. It is
3277 possible that early life experiences could have varying impact on BLA morphology
3278 wherein hypertrophy does not always mean impaired response to stress. Hence,
3279 hypertrophy in this study may not have highlighted maladaptive output.

3280 Interestingly, secondary neuronal spine density decreased in rats exposed to both
3281 maternal separation and environmental enrichment compared to those subjected to
3282 only environmental separation (figure R.32). This may be due to the differential

3283 influence of synaptic activity at the distal end of neurons as well as overall
3284 compensation for dendritic length for synaptic activity. The formation of dendritic
3285 arborization and spine density of the BLA neurons in this study could be
3286 characterized by the electrical activity of long cables of neurons harboring
3287 decreased synaptic contacts. This could be a compensatory mechanism of the distal
3288 neurons to lower the excitability of the neurons under the influence of combined
3289 effect of MS and EE. This cable theory of neuronal activity for hypo-
3290 responsiveness of BLA neuron was observed in a study which had similar
3291 combined effect of stress and EE in adulthood [166]. There results emphasized
3292 that; the dendritic arborization in BLA can vary according to different environment
3293 provided in different age of the individual (S.A.2). These results shed some light
3294 on the limited knowledge in the existing literature about BLA activity and early
3295 life stress and EE.

3296 As seen earlier, the dendritic arborization in hippocampus was not affected in this
3297 study but spine density showed significant changes in response to MSEE. The
3298 hippocampus of rats exposed to both maternal separation and environmental
3299 enrichment showed a decrease in both primary and secondary neuronal spine
3300 density compared to rats exposed only to the enriched environment (figure R.35
3301 and R.36). Beneficial effects of EE are widely studied on hippocampal neurons
3302 and its plasticity. The hippocampal neurons are shown to have elevated neuronal
3303 arborization and increased neurogenesis due to the influence of positive effects of
3304 EE [A- 243, 248, 301, 319-324]. So, in this study EE had an adaptive main effect

3305 similar to earlier studies but this impact of EE on hippocampal neurons
3306 disappeared when MS and EE treatments were combined.

3307 A decrease in number of branch points as well as primary and secondary neuronal
3308 spine density was observed in the mPFC neurons (figure R.38, R.39, and R.40) of
3309 rats subjected to both maternal separation and environmental enrichment. This
3310 effect was not seen in rats exposed to only environmental enrichment. Multiple
3311 studies have shown the impact of EE in increasing cortical neuronal
3312 responsiveness to stress [296, 297, 330, 331] Interestingly, there was a unique
3313 effect of enriched environment in neuronal morphology in all three brain regions
3314 considered in this study in spite of having a positive influence of enriched
3315 environment on physiological and behavioral outputs. As previously mentioned
3316 this may simply be a result of the attempt to “make the best of a bad situation”
3317 particularly because of the crucial period in which stress was given [80, 83]. This
3318 reinstates the point that early life experience influences the dendritic arborization
3319 in a different way when compared to adulthood experiences. The maladaptive
3320 readouts obtained in response to stress in adulthood might not be the same in early
3321 life (P2 –P21).

3322 *5.3.4 Impact of both MS and EE at molecular level (S.A.3/4)*

3323 Brain-derived neurotrophic factor (BDNF) is an important growth factor for
3324 regulating survival, and dendritic growth in a variety of cells and its pathway
3325 modulates synaptic plasticity [327-329] as discussed in earlier section. A two-way
3326 ANOVA showed that concurrent exposure to both short-term environmental

3327 enrichment and early life maternal stress resulted in marginal ($p=0.06$) lower
3328 BDNF mRNA expression in the BLA (Figure R.18). This result is further
3329 corroborated by another result wherein the expression of BDNF protein in the
3330 BLA was significantly decreased when both early life maternal stress and
3331 environmental enrichment were provided together (Figure R.22). This is an
3332 important interaction effect that is not observed when rats are subjected to only
3333 maternal separation or EE. A prior study showed that chronic immobilization
3334 stress (CIS) that caused anxiety like behavior in adulthood increases mRNA
3335 abundance of BDNF in BLA. However, the presence of both CIS and EE rescues
3336 the stress induced increase in BDNF mRNA levels [166] which revealed that lower
3337 levels of BDNF expression is an adaptive readout associated to anxiolytic
3338 behavior. Similarly, MSEE group in this study displayed lower levels of BDNF
3339 expression that highlighted the adaptive behavior shown earlier [166]. As shown
3340 earlier, MS did not affect total GR but it significantly decreased the amount of
3341 ‘activated GR’ found in the nucleus. This indicated that ‘activated GR’ alone
3342 might be the primary driver of BLA function in MS. Interestingly, when MS was
3343 combined with EE, the significant decrease in ‘activated GR’ disappeared. This
3344 result supported the idea that combinatorial effect of MS and EE renormalized the
3345 decrease in activated GR in BLA. Therefore, no significant change in total GR is
3346 valuable information, which highlighted that the activity of GR is modulated by
3347 translocation to nucleus and not by changing the degree of expression. The protein
3348 levels of pMAPK did not vary under the influence of all the treatments. This
3349 indicated that pMAPK does not play a direct role in modulating the physiology and

3350 behavioral read out in response to stress and EE. Overall data suggested that the
3351 combinatorial effect helped in ameliorating various underlying neurobiological
3352 substrates of neural plasticity and behavior (S.A.3). The results in this section also
3353 contributed to the limited knowledge present in the existing literature, as there are
3354 very few studies of combinatorial effects of stress and EE paradigm.

3355 **5.4 Conclusion**

3356

3357 Mother-infant relationship is one of the crucial (both physical and psychological)
3358 processes in brain development during early life [34, 35] when compared to other
3359 components of environment. A positive mother-infant relationship (postnatal
3360 environment) is beneficial for development of the affective behaviour of the infant
3361 [44, 45]. Harry Harlow showed that mother-infant relationship was more
3362 psychological than biological. The study suggested that mothers were important
3363 not just for supplementing food but also crucial for touch-sensitive attachment with
3364 the infant that helped the long-term emotional and mental stability of the child
3365 [159]. Disruption in the mother-infant relationship can cause stress in offsprings.
3366 Maternal separation is known to be one of the most potent forms of early life
3367 stress. Maternal stress causes detrimental effects on the neurobiological,
3368 behavioral, emotional and physiological development as mentioned earlier. In our
3369 model, maternal stress under AFR conditions showed compromised physiological
3370 effects including increase in adrenal and body weight as well as elevated baseline
3371 plasma corticosterone levels when tested in adulthood. However, provision of an
3372 enriched environment (EE) reduced the above parameters. Similarly, the
3373 interaction of maternal separation and enriched environment (MSEE) showed
3374 similar and stronger active coping outcomes compared to only EE conditions in
3375 certain experiments as discussed in detail in previous section.

3376 Maternal separation induced anxiogenic behavioral outcome. However, EE and
3377 MSEE conditions showed anxiolytic behavior. Moreover, MSEE induced pro-
3378 social behavior, which was not observed in animals exposed only to EE conditions.

3379 Thus, from physiology and behavior readouts, MSEE induced active coping
3380 response that reflects an adaptive outcome.

3381 In general, MS increased spine density in primary and secondary dendrites in three
3382 important brain regions responsible for regulating stress response namely; BLA,
3383 Hippocampus, and mPFC. EE treatment induced a decrease in secondary spine
3384 density in BLA but increased secondary spine density in hippocampus and
3385 dendritic length in the mPFC, which was in consensus with what has been reported
3386 in the extant literature [255, 296, 319, 322]. Interestingly, the neuronal
3387 morphology of rats subjected to MSEE was congruent to what was observed in MS
3388 rats. The unique results highlighted in this study emphasized that, in response to
3389 early life stress, individuals employ adaptation strategies that would allow them to
3390 make “the best out of a bad situation” as discussed in previous section.

3391 In maternally separated rats, the total mRNA abundance and protein expression
3392 levels of GR did not vary. Conversely, the percentage of high density activated GR
3393 cells decreased upon exposure to maternal stress but increased in enriched housing
3394 conditions. This had important implications for HPA regulation [47]. Another
3395 important molecular player in stress response was BDNF and it was observed to
3396 decrease in MSEE although both EE and MS alone were not observed to affect the
3397 expression of BDNF mRNA and protein. These two molecules have been very
3398 well studied in the hippocampus concerning stress response, but, to the best of my
3399 knowledge, this work was the first study to report the expression of GR and BDNF
3400 in the BLA of maternally separated rats. Prior work by Meaney et al., had reported
3401 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).

3422

3423 **Figure D.1: Putative schematic representation of intracellular molecular**
 3424 **interactions:** The interaction of numerous molecules discussed above in this
 3425 section [286, 334] is summarized in this schematic representation. The figure is
 3426 adapted from the various studies mentioned in the text. GC: Glucocorticoid, GR:
 3427 Glucocorticoid receptor, BDNF: Brain derived neurotropic factor, tPA: tissue
 3428 plasminogen activator, Erk: Extracellular signal-regulated kinases, pMAPK:
 3429 Phosphorylated Mitogen Activated Protein Kinase and TrkB: Tyrosine kinase
 3430 receptor B.

3431 However, the observed changes in GR and BDNF expression in the BLA was not
 3432 associated with hypomethylation of specific CpG sites in their respective promoter
 3433 regions chosen here. Further studies need to be considered to look at other CpG
 3434 sites in GR and BDNF and other markers of epigenetic modification including

3435 acetylation, histone modification, and chromatin remodeling to conclusively
3436 understand the underlying mechanism of the robust changes observed in the
3437 expression of these molecules. We have focussed on GR and BDNF due to specific
3438 relevance to stress and resilience in our animal models. In addition to GR, BDNF,
3439 MAPK, we also studied Arc (activity-regulated cytoskeletal proteins), MR
3440 (mineralocorticoid receptor) for their important roles in both stress-regulation and
3441 neuronal plasticity [335]. In addition to these, glutamate and GABA-ergic neuronal
3442 network of amygdala have dominant influence on effects of stress and associated
3443 neuronal plasticity [336, 337]. The signaling pathway of GR activated pathway of
3444 BDNF and pMAPK interaction could be causing activation of multiple other
3445 signaling pathways that cause protein regulation in our study [338].

3446 **Future directions**

3447

3448 My study has highlighted the effects of MS and EE on structural physiology
3449 (adrenal weight, CORT & body weight), behavior, dendritic morphology and
3450 molecular underpinnings within BLA. This opens up far more avenues to explore
3451 other parts of the brain including other nuclei of amygdala namely central, medial
3452 and BNST, which plays important role in anxiety and stress response.
3453 Hypothalamus is the other crucial brain region that needs to be studied due to its
3454 role in HPA axis and the limited knowledge available about this brain region.
3455 Although hippocampus has been extensively studied in stress field, there are still
3456 gaps in the knowledge with regards to how it regulates various behaviors in EE
3457 particularly, in my model being the first to be exposed to short-term EE in early
3458 life. Many other behavioral studies that are associated with hippocampus and

3459 mPFC can be conducted to determine the effect of short-term stress and EE in
3460 early life on these behaviors. Candidate based approach can be used to determine
3461 the molecular role players in other regions of the brain in my model. Thus, the EE
3462 and MSEE paradigms used in my study could be a potential resilient model of
3463 early life. An interesting follow-up from my study would be experiments focusing
3464 on the dams that were exposed to EE. Future epigenetics, molecular studies and
3465 functional studies of neurons of animals used in this study will be an exciting area
3466 to explore. It would be fascinating to investigate the behavior of the mothers in all
3467 the different groups used in this study. The licking/grooming pattern of all the four
3468 groups would be contributing to the results we have reported in this study. Another
3469 exciting question would be, are the “pro-resilient feature/maternal care” patterns
3470 seen in all the groups in our study transgenerational?

3471 **Summary:**

3472 I showed that early life enriched environment, concomitant to maternal separation
3473 stress has the potential to ameliorate much of the detrimental effects of MS on
3474 adult behavior, physiology, amygdala morphology and underlying molecular
3475 players, mainly the glucocorticoid receptor.

3476 **Chapter 6**

3477 **6. Supplementary Experiments (suggested by Thesis Advisory** 3478 **Committee)**

3479 **Initial Experiments**

3480 **Introduction**

3481 This chapter focuses on the initial approaches taken to establish a potential animal
3482 model for resilience. I aimed to take a closer look at the impact of different
3483 paradigms during the early life of male rats; particularly on how early life
3484 environment affects their behavior during adolescence and adulthood. A
3485 comparative study using different models was devised to comprehensively
3486 highlight the crucial factors that are characteristic of both vulnerability to stress
3487 and resilience against stress. Additionally, I also aimed to investigate if short-term
3488 EE exposure during early life has the potential to induce long-lasting resilience to
3489 prepare an individual to maintain well-regulated levels of physiological parameters
3490 and behavior (homeostasis).

3491 Impacts of different early life environment were investigated on the emotional
3492 (affective) behaviors of adolescent and adult rats in this study. The different
3493 environments to which the rats were exposed in early life were maternal separation
3494 stress (MS), limited nesting (LN), and short-term environmental enrichment (EE).
3495 Prior studies have showed that male rats, which experienced maternal separation
3496 (MS) in the first two weeks of its life showed higher depressive like behavior in
3497 the forced swimming test and had hyper sensitive HPA axis in response to
3498 subsequent stressors during adulthood [339, 340]. Studies also showed that MS

3499 produced significant defects in learning tasks, both in the Morris water maze and
3500 in the novel object recognition test [341]. Similarly, behavioral changes of
3501 increased escape latency in the Morris water maze test and inability to distinguish
3502 the novel from a familiar object in the object recognition test was seen in rats
3503 subjected to limited nesting, during first two weeks of life [342]. Apart from
3504 behavioral abnormalities, physiological variations like elevated plasma
3505 corticosterone and increased adrenal weight were observed in limited nesting
3506 model [252, 343]. In contrast, EE enhanced memory function in various learning
3507 tasks in rodents [344]. Adult mice performed better in a spatial memory task
3508 (water maze task) after exposure to EE paradigm than controls in standard housing
3509 cage [204]. Previous literature shows that EE had beneficial effects on cognition
3510 accompanied by active coping mechanisms in rodents [168]. Mice exposed to
3511 long-term EE in early life displayed higher propensity to interact socially [345].
3512 These evidences indicate that stressful early life environment has harmful effects
3513 on physiology and behavior of adult rats while EE imparts positive and well-
3514 adaptive features to the rats and their physiology and behavior. Though numerous
3515 studies had been done using EE in early life, no studies showed the impact of short
3516 term EE in rats. This inspired me to study varying environments during early life
3517 including early life stress and EE.

3518 Various studies have demonstrated that a single episode (acute) or repeated
3519 episodes (chronic) of separation between mother and pups in rodents lead to both
3520 acute and long-term effects on behavior and endocrine read out [137, 340, 346-
3521 349]. When adult rats that previously underwent MS in early life were tested, they

3522 showed neuroendocrine and behavioral deficits similar to those seen in patients
3523 with anxiety related disorders and depression [350-352]. 300-400% increase in
3524 plasma corticosterone was observed in rat pups, when they were exposed to
3525 maternal separation stress or any maternal care associated stress [339, 347]

3526 The limited nesting environment results in stress in the mother, promoting
3527 fragmented care towards the pups due to harsh environment [252]. This aberrant
3528 maternal behavior in response to impoverished conditions, induced chronic early-
3529 life stress in the pups, evident from elevated plasma glucocorticoids and increased
3530 adrenal weight, often associated with modest, transient reduction of weight gain in
3531 the pups [252, 343]. So, this was another early life stress model with the presence
3532 of mother but impoverished housing. LN was another stress paradigm that was used
3533 in this study to investigate the impact of different kind of early life stress on
3534 adulthood of male rats.

3535 Along with physiological variations due to exposure to LN, behavioral changes
3536 like increased escape latency in the Morris Water Maze test and inability to
3537 distinguish the novel object from a familiar object in the Object recognition test
3538 was also seen in the pups in this model [342]. Dendritic atrophy of pyramidal cells
3539 along with hippocampus mediated cognitive impairments was observed in rats
3540 exposed to limited nesting during their early life [342]. Synaptic density decreased
3541 in hippocampus and amygdala showed enhanced activity in rats that were exposed
3542 to LN [353],[354].

3543 At this point we have discussed both early life stress and EE. In this study, “MS”
3544 and ‘LN’ were the two early-life stress models in rodents that showed

3545 compromised effects in various studies. and “EE” was another treatment used in
3546 early life to determine the beneficial effects of early life EE on adulthood in male
3547 rats. These three paradigms were used to study physiology, behavior and dendritic
3548 morphology during adulthood.

3549 **Experimental Plan**

3550 It was very intriguing to know how various environment affected the neuronal
3551 development of an individual. MS was provided from postnatal day (PD) 2 to PD
3552 14, EE was provided from P2- P16 and LN was provided from P2-P9 and all these
3553 time points were adapted from previous studies [206, 339, 343]. Animal facility
3554 rearing (AFR) group was considered as the control group without any treatment.
3555 The treated rats were housed in AFR (neutral environment) after various treatment
3556 till adulthood. This experimental paradigm was well planned to give a variety of
3557 environment like positive environment (spacious and elevated sensory activity) in
3558 the presence of mother (EE), stress in the absence of mother (MS) and stress
3559 (impoverished conditions) in the presence of mother (LN). Various behavioral
3560 parameters were quantified for all the groups. Our aim was to analyze the impact
3561 of three differential early life environments on physiology and behavior of an
3562 individual during adulthood. Please refer to Figure N for the schematic
3563 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 adolescent 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).

3579

3580 **Figure N.1: Effect of different early environment on escape latency from**

3581 **home cage in adolescent rats.** The graph represents escape latency for each group

3582 (mean $\pm$ SEM). LN group showed a significant increase in time to escape from the

3583 home cage in comparison to MS and EE groups. One-way ANOVA; $F_{(3,27)} = 4.6$,

3584 $p=0.01$; LSD posthoc test; **, $p < 0.01$. $n=7$ for MS, $n=8$ for AFR, LN, and EE.

3585 AFR: Animal Facility Rearing, MS: Maternal Separation, LN: Limited Nesting

3586 and EE: Environmental Enrichment.

3587 ii) Home cage emergence in adult rats

3588 To examine the effect of different early environment on the anxiety-like behavior

3589 of the adult rats, they were tested for escape latency from home cage during their

3590 adulthood. One-way ANOVA; $F_{(3,28)} = 3.2$, $p < 0.051$ revealed a significant

3591 decrease in escape latency from home cage in MS rats when compared to AFR and

3592 LN groups. This data showed that MS was least anxious when compared to AFR

3593 and LN groups when tested during adulthood

3594

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3596

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3598 **Figure N.2: Effect of different early environment on escape latency from**
3599 **home cage in adult rats.** The graph represents escape latency for each group
3600 (mean ± SEM). MS group showed a significant reduction in time to escape the
3601 home cage in comparison to AFR and LN groups. One-way ANOVA; $F_{(3,28)} = 3.2$,
3602 $p < 0.05$ for differences between groups; LSD posthoc test; $*p < 0.05$, $**p < 0.01$,
3603 $n = 8/\text{group}$. AFR: Animal Facility Rearing, MS: Maternal Separation, LN: Limited
3604 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 $\pm$ 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 $\pm$ 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; $*p < 0.05$, $**p \leq 0.01$. $n = 8/\text{group}$. **B)** The graph represents percentage open arm time for each group (Mean $\pm$ 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; $**P < 0.01$, $***p \leq 0.001$. $n = 8/\text{group}$. **C)** The graph represents a number of head-dips for each group (Mean $\pm$ 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; $**p \leq 0.01$. $n = 8/\text{group}$. 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 $\pm$ 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 $\pm$ 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 $\pm$ SEM). One-way ANOVA revealed no significant differences between the groups. $n = 8/\text{group}$. 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 $\pm$ 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 $\pm$ 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; $**p < 0.01$, $*P \leq 0.05$. $n= 8/\text{group}$. **B)** The graph represents the duration of immobility for each group (Mean $\pm$ SEM). One-way ANOVA revealed no significant differences between the groups. $n= 8/\text{group}$. 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 impairment 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 adolescence. 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.

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 $\pm$ 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 $\pm$ 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 $\pm$ SEM. N = 8 for AFR, 7 for 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ SEM. N = 8 for AFR, 6 for Stress, 9 for EE and 7 for MSEE; ** $p \leq 0.01$.

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 $\pm$ 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 $\pm$ 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 brought 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 impregnation. 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=1 μm^2 .

- 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 $50\mu\text{m}$), 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.

	Label	Area	Mean	Min	Max
1	img009.tif.d100	1869	255	255	255
2	img009.tif.d200	3472	255	255	255
3	img009.tif.d300	4636	255	255	255
4	img009.tif.d400	5415	255	255	255
5	img009.tif.d500	5565	255	255	255
6	img009.tif.d600	5565	255	255	255
7	img009.tif.d700	5565	255	255	255
8	img009.tif.d800	5565	255	255	255
9	img009.tif.d900	5565	255	255	255
10	img009.tif.d1000	5565	255	255	255
11	img009.tif.d1100	5565	255	255	255

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: $s = \text{pixels/unit micron} = 5.885$ (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
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		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.
2. **Akshaya Hegde**, Abdulai-Saiku, Samira, Ajai Vyas, and Rupshi Mitra. "Effects of stress or infection on rat behavior show robust reversals due to environmental disturbance." *F1000Research* 6 (2018).
3. Ashokan Archana, **Akshaya Hegde**, and Rupshi Mitra. "Short-term environmental enrichment is sufficient to counter stress-induced anxiety and associated structural and molecular plasticity in basolateral amygdala." *Psychoneuroendocrinology* 69 (2016): 189-196.
4. Archana Ashokan, **Akshaya Hegde**, Anushanthi Balasingham, Rupshi Mitra Housing environment influences stress-related hippocampal substrates and depression-like behavior." *Brain Research* (2018)