**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**SHORT-TERM ENVIRONMENTAL ENRICHMENT
INDUCES ‘RESILIENCE TO CHRONIC STRESS’ IN
ADULTHOOD**

**(A study traversing behavioural, neuronal and molecular
plasticity in brain)**

**ARCHANA ASHOKAN KOLIKIL
SCHOOL OF BIOLOGICAL SCIENCES
2017**

**SHORT-TERM ENVIRONMENTAL ENRICHMENT
INDUCES ‘RESILIENCE TO CHRONIC STRESS’ IN
ADULTHOOD**

**(A study traversing behavioural, neuronal and molecular
plasticity in brain)**

ARCHANA ASHOKAN KOLIKIL

School of Biological Sciences

**A thesis submitted to Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy**

2017

Acknowledgements

Firstly, I would like to express my heartfelt gratitude to my mentor Asst. Prof. Rupshi Mitra. She has taught me innumerable things in this journey of research which has shaped the crux of my research approach and supported me through my tough hours of graduate years. She has perennially been encouraging me about enquiring and pursuing the right questions in the science of animal-behaviour. I will always be thankful to Rupshi for her esteemed guidance and for making me a part of the *Resilio* team.

I would like to express my sincere gratitude to the School of Biological Sciences, Nanyang Technological University and the Ministry of Education, Singapore for providing me this opportunity to pursue a doctoral degree with a scholarship and funding to support this project. I would sincerely like to thank Asst. Prof Albert Chen, Asst. Prof Ayumo Tashiro and Prof. Salil Bose for their prized guidance, suggestions and critical inputs on my research as my Thesis Advisory Committee. Their inputs have always helped me improve my research. I would particularly like to thank Dr. A. Chen for sharing precious antibodies (GAD67) from his lab in order to support a branch of work in this study and providing his precious time and inputs for the imaging studies.

I would like to extend my gratitude to my colleagues (pioneer members of this lab): Akshaya Hegde and Yamini Bhaskar with whom I started as a team guided by Rupshi. Akshaya and Yamini have helped and supported me like a family in this lab. They have always offered timely help especially in setting up behaviour experiments. I would sincerely like to thank Dr. Anthony Lim for his guidance in running behaviour experiments during my training phase, former post-doctoral fellow Dr. Amelia Koe who not only is inspirational and taught me myriad of things in research practices but also is a very close friend, and former post-doctoral fellow Dr. Arnab Datta who also guided me through some research techniques. I would like to thank all my past and current lab colleagues: Linda, Eunice, Meena, Siddharth, Grace, Shruti and Vandana who have always provided me with timely help when needed and supported me in this venture. I would like to express my sincere gratitude to Mr. Ravichandran Annamalai, Animal facility staff, NTU, for the crucial job of helping me in taking care of all the animals used in this project. I would also like to sincerely thank a bunch of NTU

undergraduate students namely Pei Wern, Mei Xuan, Nicholas, Lim Wee Han , Zhou Wei, Melissa and Darshinee who collaborated with us to help out in the morphology experiments in this study. Without their efforts, this study would have not come this far. They were all a pleasure to work with.

I would like to extend special thanks to Assoc. Prof Ajai Vyas for his valued guidance, particularly his support in developing the JAVA-based ImageJ macro necessary for dendritic complexity analysis in this study. His scientific outlook on animal-behaviour has always been inspiring. His critical inputs on statistical approach and various other aspects of the study have always been helpful for my research. I would also like to sincerely thank the former and current members of his lab (Ethoneuro) namely Shantala, Dhiraj, Samira, Wen Han, Vineet, Anand and Donna who have always been kind in providing help and inputs whenever needed.

I would not be here if not for the support and care from my family. I would like to thank them and dedicate this work to my parents who gave me everything they could so that I could pursue my goals, including the strength to steer through hard times. I would also like to thank my brother and sister in law, who have taken care of me like my own parents and supported me through all walks of life.

I would like to mention a special thanks to the two main pillars of my entire journey in graduate school - Vinay and Akshaya. From my undergraduate days till date, Vinay has been a constant source of inspiration, care and support. His scientific, artistic and philosophical outlook has influenced my thinking deeply. He has stood by me through thick and thin, advising me on all fronts, be it work-related or personal. Akshaya has been the real ‘human model of stress-resilience’ as I like to call her. She has been more like a sister than colleague whose cheerfulness and positive outlook in life has toned my HPA response for the better. A special thanks to her mum, who treated me like family as well and ‘enriched’ our lives with home-cooked meals. I cannot begin to put in words the support Vinay and Akshaya have provided me during this phase which is irreplaceable.

This acknowledgment would be incomplete without the three names: Matt Ridley, Francis H. C. Crick and Robert M. Sapolsky. The works of these three personalities kindled my drive and ultimate fascination for the study of life. In my undergraduate days reading ‘*Genome: The autobiography of a species in 23 chapters*’ authored by M.

Ridley as well as '*What Mad Pursuit*' by F. Crick moulded my perspective on life-science which inspired me to pursue research on the same. Thanks to Rupshi, I was introduced to the works of R. Sapolsky, who is such an inspiring figure for any behaviour-biology aspirant. His book, '*Why zebra don't get ulcers*' transcends not just your approach towards stress-research but also your personal life. His research not only inspires me to question what I want to study but why I want to study it and its direct implications in the long run.

I would like to thank my friends Reelina, Siddharth, Nandhini, Margaret, Abhishek, Manish, Ishani, Mrinal, Sharadha, Hari, Laxmi, Rajagopalan, Ravi, and Narendar for supporting me more like a family than just as friends. Being in their company has made me realise that social ties are indeed the actual form of enrichment for human beings.

Lastly but most importantly, I would like to express my heartfelt gratitude to all the rats whose lives have been sacrificed during the course of this study. Learning about stress and environment through this study would have not been possible if not for these animals, the true martyrs of science.

Table of Contents

ABSTRACT.....	i
LIST OF FIGURES	ii
LIST OF TABLES	vi
LIST OF ABBREVIATIONS	vii
LIST OF SYMBOLS	xi
INTRODUCTION	1
1. Overview	2
2. Review of Literature	3
2.1 Allostasis and stress	3
2.2 HPA axis neuroanatomy and role of Glucocorticoids.....	4
2.2.1 Feedback mechanism in HPA: Role of Glucocorticoids.....	5
2.2.2 The stress-regulating triad: Amygdala, Hippocampus and the medial prefrontal cortex (mPFC).....	7
2.3 Stress: Disruption of HPA activity and elevated glucocorticoid levels.	12
2.3.1 Effect of stress on the hippocampus.....	12
2.3.2 Effect of stress on the amygdala	18
2.3.3 Effect of stress on the mPFC.....	23
2.3.4 Basolateral Amygdala: the primary mediator of fear and anxiety; how chronic stress affects it.....	26
2.4 Resilience and its neurobiology	30
2.4.1 Biological co-relates of resilience.....	30
2.4.2 “Are we there?” - Approaches towards a stress-resilient animal model	32
2.5 Environmental Enrichment (EE): A potential resilience inducer.....	34
2.5.1 What is Environmental Enrichment?	35
2.5.2 EE: Effects on brain and behaviour	35
2.5.3 EE: Potential therapeutic effects	41
3. Motivation and proposed hypothesis: Stress-resilient animal model for adult phase using EE	42
MATERIALS AND METHODS	46
1. Animals	47
2. Stress and Enrichment procedures	47
3. Experimental groups	48
4. Elevated plus maze.....	48
5. Laboras™.....	49
6. Sucrose intake test.....	50

7. Porsolt's Forced swim test	50
8. Dendritic arborisation	51
9. Corticosterone measurement, adrenal gland weight and Body weight gain	55
10. Protein expression studies in the Basolateral Amygdala	55
10.1 Tissue preparation for immunostaining	55
10.2 Immunofluorescence staining for inhibitory presynaptic protein markers	56
10.3 Immunofluorescence staining for nuclear GR	57
10.4 Tissue preparation for Western Blotting	58
10.5 Western Blotting	58
11. Statistical analysis	60
RESULTS	61
1. Effect of stress and EE on anxiety and risk assessment.....	62
2. Effect of stress and EE on hedonic responses	67
3. Effect of stress and EE on depressive-like behaviour (learned-helplessness).....	69
4. Effect of stress and EE on basal activities.....	72
5. Effect of stress and EE on dendritic complexity of BLA neurons	75
6. Effect of stress and EE on spine density of BLA neurons	80
7. Effect of stress and EE on dendritic complexity of hippocampal CA3 neurons.....	82
8. Effect of stress and EE on spine density of hippocampal CA3 neurons	84
9. Effect of stress and EE on dendritic complexity of mPFC neurons: PrL and IL	86
10. Effect of stress and EE on spine density of mPFC neurons: PrL and IL	90
11. Effect of stress and EE on corticosterone and adrenal weights.....	94
12. Effect of stress and EE on expression of inhibitory presynaptic-protein markers in BLA (GAD67)	96
13. Effect of stress and EE on expression and activation of Glucocorticoid receptor (GR) protein in the BLA	101
14. Effect of stress and EE on expression of pro - brain-derived neurotrophic factor (pro-BDNF) and Tyrosine Kinase receptor B (TrkB) in the BLA	106
15. Effect of stress and EE on body-weight	108
DISCUSSION	110
1. Specific aim 1: To evaluate the effects of EE on stress-related behavioural outcomes: developing rodent model of stress-resilience.....	111
1.1 EE reduces anxiety and increases active-coping in stressed subjects.	111
1.2 EE reduces depressive-like symptoms irrespective of stress exposure.....	113
1.3 EE reduces hyper-reactivity at resting levels in stressed subjects.....	115
1.4 EE reduces circulating corticosterone in irrespective of stress exposure.....	118

2. Specific aim 2: To investigate characteristic neuronal morphology associated with stress-resilience induced by EE.....	119
2.1 EE induced reorganization of basolateral amygdala neurons in stressed animals.	119
2.2 EE induced reorganization of hippocampal CA3 neurons in stressed animals.....	121
2.3 EE induced reorganization of the medial prefrontal cortex neurons in stressed animals.	124
3. Specific aim 3: To investigate the molecular regulation associated with stress-resilience induced by EE.....	129
3.1 EE reduces activation of glucocorticoid receptor protein in stressed animals.	129
3.2 Stress conditionally reduces expression of inhibitory presynaptic protein (GAD67) of BLA in absence of EE but not in its presence.....	131
3.3 Stress conditionally reduces expression of pro-BDNF protein in the BLA in absence of EE but not in its presence.....	133
CONCLUSION.....	139
SUPPLEMENTARY INFORMATION.....	142
1. Effect of EE and stress on Central amygdala.....	143
2. Effect of EE and stress on BNST.....	146
3. Effect of EE and stress on pro-BDNF and TrkB protein expression in the medial prefrontal cortex.....	150
4. Effect of EE and stress on on exploratory behaviour using Open-field (OFT).....	152
5. Mean and SEM of tested variables for all 4 groups:	156
APPENDIX.....	163
1. Golgi staining.....	164
1.1 Golgi-cox commercial kit: FD Rapid GolgiStain™ Kit.	164
1.2 Cresyl Violet solution	165
2. Dendritic arborisation	165
3. Paraformaldehyde (PFA) solution (4%, 1L).....	169
3.1 Phosphate Buffer Saline (10X, 1L).....	169
4. Reagents for Immunofluorescence staining.....	169
4.1 Blocking buffer	169
4.2 Primary antibody buffer	169
4.3 Secondary antibody buffer.....	169
4.4 DAPI solution	169
5. Reagents and specifications for tissue preparation for western blotting.....	170
5.1 Lysis buffer	170
5.2 Sonication	170
6. Reagents for SDS/PAGE	170

6.1 Resolving gel.....	170
6.2 Stacking gel.....	170
6.3 Polyacrylamide gel (12%).....	170
6.4 10X PAGE buffer (100ml; pH 8.3).....	171
6.5 Sample loading buffer (100µl).....	171
6.6 10% Sodium dodecyl sulphate (SDS) solution (100ml)	171
6.7 10% Ammonium persulphate (APS) solution (10ml).....	171
7. Reagents for Western blotting.....	171
7.1 1X Transfer buffer (1L)	171
7.2 10X TBS (pH 7.6; 1L)	171
7.3 1X TBST.....	171
7.4 5% blocking buffer (20ml).....	172
7.5 Antibody buffer (5% non-fat dry milk solution).....	172
7.6 Stripping buffer (50ml)	172
7.7 Chemiluminiscent substrate	172
REFERENCES	173
LIST OF PUBLICATIONS	193

ABSTRACT

In mammals, expression of fear is an adaptive trait that safeguards individuals from natural threats including predators. However, this expression can be exacerbated by chronic stress into manifestation of negative valence such as anxiety, leading to mental disorders. The potentiation of anxiety is primarily associated with neuronal changes in the basolateral amygdala (BLA). Stress-induced hyperactivation of BLA in the form of dendritic reorganisation corresponds to precipitation of anxiety-states in adult male rats. This study shows that short-term environmental enrichment (EE) in adulthood is sufficient to counter anxiety-related pathologies induced by chronic stress. Stressed subjects housed in EE show lowered anxiety in contrast to those housed in standard-conditions. They differ in dendritic reorganization in the form of lowered spine-density compared to their stressed counterparts without EE. Furthermore, compared to the latter, the EE-exposed stressed animals also differ in protein-expression of activated glucocorticoid-receptors, and pro - brain-derived neurotrophic factor, which play an important role in mediating stress-response. These observations suggest that short-term EE can potentially cultivate resilience in adulthood against stress-induced pathologies at behavioural, neuronal, and molecular levels.

Keyword: Resilience, Basolateral amygdala, Enrichment, Anxiety, Plasticity, Corticosterone, Glucocorticoid receptor

LIST OF FIGURES

FIGURE	TITLE	PAGE
No.		No.
M.1A	Coronal brain section showing sampled BLA and Hippocampal region	51
M.1B	Coronal brain section showing sampled PrL and IL region	52
M.2	Sholl analysis of a BLA neuron sample	53
M.3	Illustrative representation of neuronal spine analysis	54
1A.	Effect of stress on percentage open arm exploration in absence and presence of EE	64
1B.	Effect of stress on percentage closed arm exploration in absence and presence of EE	65
1C.	Effect of stress on percentage open-arm entries and time in absence and presence of EE	65
1D.	Effect of stress on closed arm entries in absence and presence of EE	66
1E.	Effect of stress on number of head-dips in absence and presence of EE	67
2.	Effect of stress on sucrose intake in absence and presence of EE	68
3A.	Effect of stress on immobility in forced-swim test in absence and presence of EE	70
3B.	Effect of stress on latency to first immobility in FST in absence and presence of EE	71
3C.	Effect of stress on swimming in forced-swim test in absence and presence of EE	71

4A.	Effect of stress on immobility in LABORAS™ test in absence and presence of EE	73
4B.	Effect of stress on locomotion in LABORAS™ test in absence and presence of EE	74
4C.	Marginal-mean comparison of immobility in stressful setting vs. resting phase	74
5A.	Effect of stress on number of branch points in the BLA neurons in absence and presence of EE	77
5B.	Effect of stress on total dendritic length in the BLA neurons in absence and presence of EE	78
6.	Effect of stress on total dendritic spines (in 60µm) of the BLA neurons in absence and presence of EE	81
7A.	Effect of stress on total number of branch points in the hippocampal CA3 neurons in absence and presence of EE	83
7B.	Effect of stress on total dendritic length of the hippocampal CA3 neurons in absence and presence of EE	83
8.	Effect of stress on total dendritic spines (in 60µm) of the hippocampal CA3 neurons in absence and presence of EE	85
9A.	Effect of stress on dendritic complexity of the prelimbic neurons in absence and presence of EE	88
9B.	Effect of stress on dendritic complexity of the infralimbic neurons in absence and presence of EE	89
10A.	Effect of stress on total dendritic spines (in 80µm) of the prelimbic neurons in absence and presence of EE	91
10B.	Effect of stress on total dendritic spines (in 60µm) of the infralimbic neurons in absence and presence of EE	93
11A.	Serum corticosterone concentration for day 1, day 3 and day 20 in absence and presence of EE	95

11B.	Effect of stress on adrenal weight in absence and presence of EE	96
12A.	Effect of stress on protein-expression of GAD67 positive and GAD67 negative synapsin within the BLA in absence and presence of EE	99
12B.	Effect of stress on excitation/inhibition in BLA in absence and presence of EE	101
13A.	Effect of stress on nuclear localized GR protein expression within the BLA in absence and presence of EE	103
13B.	Effect of stress on total GR protein expression in BLA in absence and presence of EE	105
14A.	Effect of stress on pro-BDNF protein expression in BLA in absence and presence of EE	107
14B.	Effect of stress on TrkB protein expression in BLA in absence and presence of EE	108
15.	Effect of stress on percentage body-weight gain in absence and presence of EE	109
16	Microscope traces of BLA principal neurons for the 4 groups	121
17	Microscope traces of hippocampal CA3 neurons for the 4 groups	123
18	Microscope traces of prelimbic (PrL) neurons for the 4 groups	127
19	Microscope traces of infralimbic (IL) neurons for the 4 groups	128
S.1A	Effect of stress on dendritic complexity of CeA neurons in absence and presence of EE	144
S.1B	Effect of stress on total dendritic spines (in 60µm) of primary dendrites in CeA neurons in absence and presence of EE	145

S.2A	Effect of stress on dendritic complexity of BNST neurons in absence and presence of EE	148
S.2B	Effect of stress on total dendritic spines (in 60µm) of primary dendrites in BNST neurons in absence and presence of EE	149
S.3	Effect of stress on pro-BDNF and TrkB protein expression within the mPFC in absence and presence of EE	151
S.4A	Effect of stress on time spent in the centre of open field in absence and presence of EE	154
S.4B	Effect of stress on time spent in the periphery of open field in absence and presence of EE	155

LIST OF TABLES

TABLE	TITLE	PAGE
No.		No.
M.1	Details of neuronal types in each brain region selected for morphological analysis	54
M.2	Details of primary and secondary antibodies used in Western Blotting	59
S.1	Mean and SEM of all tested variables	156

LIST OF ABBREVIATIONS

11 β HSD	11 β - Hydroxysteroid Dehydrogenase
ACC	Anterior Cingulate Cortex
ACTH	Adrenocorticotrophic hormone
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ARC	Activity-regulated cytoskeleton-associated protein
AUC	Area under the curve
BDNF	Brain derived neurotrophic factor
BLA	Basolateral Amygdala
BNST	Bed nucleus of Stria Terminalis
BrdU	Bromodeoxyuridine
CaMKII α	Ca ²⁺ /calmodulin-dependent protein kinase II
cAMP	Cyclic Adenosine Monophosphate
CeA	Central Amygdala
ChR2	Channelrhodopsin receptor 2
CIS	Chronic Immobilization Stress
CN	Control
CORT	Corticosterone
CNS	Central Nervous System
CREB	cAMP Response Element Binding
CRF/H	Corticotropin releasing factor/hormone
CRFR1	Corticotropin releasing factor receptor 1
CRS	Chronic Restraint Stress
DAPI	4',6-diamidino-2-phenylindole
dIPFC	Dorsolateral Prefrontal Cortex
dPrL	dorsal Prelimbic Region
DG	Dentate Gyrus

DNA	Deoxyribonucleic Acid
EAA	Excitatory Amino Acids
EC	Enriched cages
EE	Environmental Enrichment/ Enriched Environment
EEST	Environmental Enrichment + Stress
EPM	Elevated plus maze
Erk	Extracellular signal-regulated kinases
fMRI	Functional Magnetic Resonance Imaging
FST	Forced swim task
GABA	γ -aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAD	Glutamate Decarboxylase
GluR1/2/3	Glutamate receptor 1/2/3 subunit
GC	Glucocorticoids
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive elements
HPA	Hypothalamic-Pituitary-Adrenal
IC	Impoverished cages
IE	Impoverished environment
IGF1	Insulin growth factor 1
IgG	Immunoglobulin G
IL	Infralimbic Region
LABORAS	Laboratory Animal Behaviour Observation, Registration and Analysis System
LTP	Long term potentiation
MAPK	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
<i>ns</i>	Non-significant
NAc	Nucleus Accumbens
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate Receptor
NT3	Neurotrophin-3
NTS	Nucleus Tractus Solitarii
OCD	Obsessive Compulsive Disorder
OD	Optical density
OFT	Open field test
Olig2	Oligodendrocyte progenitor marker
PBS	Phosphate buffered saline
PET	Positron Emission Tomography
PFC	Prefrontal cortex
PKM ζ	Protein kinase C, zeta
PrL	Prelimbic Region
PTSD	Post-Traumatic Stress Disorder
PV	Parvalbumin
PVN	Paraventricular nucleus
ROI	Regions of interest
SC	Standard conditions
SDS	Sodium Dodecyl Sulphate
SE	Standard environment
SEM	Standard error of the mean
SIT	Sucrose intake test

SNS	Sympathetic Nervous System
ST	Stress
tPA	tissue Plasminogen activator
TrkB	Tyrosine kinase receptor B
vPrL	ventral Prelimbic Region
VTA	Ventral Tegmental Area

LIST OF SYMBOLS

°C	Degree Celsius
Ca ²⁺	Calcium ion
K ⁺	Potassium ion
β	Beta
Δ	Delta (Difference)
μm	Micron
cm	Centimeter
m	meters
mM	Millimolar
M	Molar
ml	Millilitre
pg	Picogram
ng	Nanogram
μg	Microgram
mg	Milligram
g	Gram
μl	Microlitre
ml	Millilitre
L	Litre
Hz	Hertz
s	Second
lux	SI unit of illuminance
hr	Hour
kDa	Kilo Dalton
rpm	Rotation per minute
$\bar{x}$	Mean

INTRODUCTION

1. Overview

The psychological and physiological discomfort caused by prolonged exposure to stress is enormous. It is the main cause for panic, anxiety disorders, phobias, MDD (major depressive disorder) and depression. Therefore, the need to understand its implications and address ways to alleviate it is of utmost importance. Stress not only induces maladaptive behavioural and physiological changes in individuals, but also gets manifested in altered neuronal structures and their molecular milieu. However, what is more interesting is that not all individuals respond to stress similarly. Some exhibit a certain degree of resistance against its adverse effects, thereby indicating *resilience* in them. Stress-resilience is characterised by the ability of the body to effectively readjust its equilibrium with the dynamic changes (stressors) in the environment. This is achieved by resisting maladaptive stress-induced changes at different biological levels such as: coping behaviour, physiological responses (hormonal levels), and structure and molecular milieu of the neurons in the brain. What could possibly be contributing to this pattern of resilient behaviour? In order to probe into this question, it is important to adopt a suitable paradigm that provides a scope to develop and study *resilience*. ***Enriching the environment*** (EE) of an individual, which has stimulatory and regulatory effects on learning, memory, and emotional behaviour, provides a potential scope in this regard.

Our objective is to employ environmental enrichment (EE) in order to study the biology of resilience against stress. We aim to study and understand the differences in behaviour and structural and molecular pathways of relevant brain regions contributing to the manifestation of resilience. The study can potentially provide a translational edge once the 'resilient markers' are identified and hopefully deployed for developing possible clinically-relevant therapy against stress - disorders.

2. Review of Literature

2.1 Allostasis and stress

Stress is defined as anything that disturbs homeostatic equilibrium of the body. Depending on its magnitude, stress may set an adverse predicament on the body, which may be either physiological or psychological in nature. In response to this, the body tries to stabilize itself to basal level of normal functioning and this process of achieving homeostasis post-exposure to stress is defined as allostasis [1]. Allostasis essentially comprises of a physiological process to cope with the loss incurred due to a stressor. Under normal physiological conditions, when a body meets an acute stressor, a stress-response follows, which helps achieve homeostasis quickly. However, when the stress is chronic and causes too much wear-and-tear in the body, it results in constant and uncontrollable activation of stress-response, which eventually results in dysfunctional management of allostasis. This is referred to as allostatic overload [2].

Stress primarily triggers two systems: the sympathetic nervous system (SNS) releasing catecholamines (adrenaline and noradrenaline) and the hypothalamus-pituitary-adrenocortical (HPA) axis releasing glucocorticoids [3, 4]. The orchestrated activity of these two systems is responsible for mobilizing and managing energy and bodily resources to counter stress and return the body to homeostatic equilibrium. In other words, they help in generating a “fight or flight” response against stress, also broadly termed as a ‘stress - response’ [5]. Stress response can be broadly divided into two parts. The first involves rapid utilization of energy during a threatening situation. At this phase, the sympathetic nervous system is instantaneously triggered, which generates an alert response by the release of adrenaline in response to stress [6]. This leads to an array of physiological outcomes like instantaneous global energy

mobilization by increasing delivery of glucose and oxygen in circulation particularly to the heart and brain, modulating vascular resistance, increasing vasodilation in skeletal muscles, increasing renal, splanchnic, and cutaneous vasoconstriction etc [6]. Under resting state, the same system is responsible for maintaining tonic and reflexive changes in the cardiovascular system via noradrenaline [6]. Upon gaining control over the stressor, the SNS activity requires attenuation which is achieved by another set of nerves called the parasympathetic nervous system. It antagonises the SNS firing and returns the body to normalised tone of energy expenditure [7, 8].

The second step involves focussed restoration of the rapidly utilized energy during a stressor, which will essentially bring the body to homeostasis. The HPA axis is recruited during this phase (stress-response) releasing glucocorticoids that mediate the availability of energy by stepping up the glucose-reserve via a series of physiological processes directed towards gluconeogenesis [9]. Any disruption in the functioning of allostatic modulators can cause stress-vulnerability in individuals [1, 3].

2.2 HPA axis neuroanatomy and role of Glucocorticoids

The mammalian physiology relays a stress response via the Hypothalamus-Pituitary-Adrenocortical axis in association with the Central Nervous System (CNS). Stress-induced activation of HPA system is initiated by the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus that secrete Corticotropin releasing factor (CRF) [10]. CRF is released into hypophysial portal vessels of the anterior pituitary gland wherein they bind to their receptors and in-turn cause the release of Adrenocorticotrophic hormone (ACTH) into the systemic circulation [11]. ACTH binds to its receptors on its primary target - the adrenal cortex resulting in the release of glucocorticoids from the zona fasciculata into the systemic circulation which

regulate physiological changes to counter the stress load and restore homeostatic equilibrium [9].

Glucocorticoids are a class of steroidal hormones mainly constituting of corticosteroids (Corticosterone in rodents and cortisol in humans), which regulate a number of physiological actions that are catabolic in nature. Its primary function is to mobilize energy through gluconeogenesis, proteolysis, and lipolysis in response to the allostatic load [9]. As a compensatory mechanism, it also mediates the suppression of immune system, reproductive functions, and growth processes, thereby leading to focussed restoration and availability of energy post exposure to an immediate challenge caused by a stressful stimulus. The activation of HPA is largely dependent on the GC dose under circulation, which is controlled by a fine-tuned feedback mechanism involving structures from the mid-brain. The feedback mechanism for HPA is regulated by monitoring the GC concentration in circulation which is detected by certain regions in the brain that are sensitive to it [12]. Circadian corticosteroid secretion studied through blood sampling analysis shows that it follows a series of ultradian pulsative pattern of release, which increases drastically on an encounter with stress in order to cope with its impact and is gradually normalized to basal levels by the HPA axis [13].

2.2.1 Feedback mechanism in HPA: Role of Glucocorticoids

The function of GC is mediated by its binding with its cognate receptors, following which a series of downstream molecular pathways are relayed that affect physiological and behavioural parameters. The affinity of GC for its two receptors: Mineralocorticoid receptor (MR) and Glucocorticoid receptor (GR) are different from each other. MR has 10-fold greater binding affinity than GR for GC [14]. The GRs are

bound to GC only after the saturation of MR population in the cell. Hence action of GC on any cell is dependent on the GC concentration in the cellular environment and the ratio of MR to GR population in the cell. MR population regulates the HPA at basal levels of GC [15]. During an encounter with a stressor, the GC concentration generally rises to a dose which saturates all the MRs and a GR-activated stress response is generated consequential to this. GRs are nuclear receptors which upon ligand-binding, undergo a conformational change forming a GR homodimer. This homodimer dissociates itself from its heat-shock protein complex and gets translocated into the nucleus [16, 17]. Within the nucleus, the GR homodimer binds to the promoter region of specific DNA motifs called glucocorticoid responsive elements (GRE). This generates a downstream cascade of transcriptional and translational events leading to physiological changes in metabolism and cardiovascular and behavioural processes [18, 19].

The feedback mechanism in HPA is governed by differences in the MR and GR population [15] occurring in different regions of the brain and their neuronal network associated with the PVN in the hypothalamus [12]. GRs are ubiquitously expressed in many regions of the brain particularly in abundance in certain regions like the hippocampus (CA1 region), dentate gyrus (DG), amygdala, lateral septum, the PVN and nucleus tractus solitarii (NTS) of the hypothalamus. MRs, however, are highly expressed in selective regions of the brain such as the hippocampus and lateral septum and relatively much lesser expressed in regions like the amygdala, PVN and medial prefrontal cortex. [14, 15, 20].

2.2.2 The stress-regulating triad: Amygdala, Hippocampus and the medial prefrontal cortex (mPFC).

Hippocampus is the primary brain structure involved in providing a negative feedback to the HPA axis and therefore tones its activity down via multisynaptic and stressor-specific pathways [21]. Located in the medial temporal lobe underneath the cerebral cortex, this structure is also crucial for spatial, episodic, and contextual memory formation [22]. The hippocampus is present in both the hemispheres and constitutes of different cell layers (CA1, CA2, CA3). The CA1 region (rich in GR) is highly sensitive to GC concentration. Along with ventricle subiculum, it relays signals via afferent projections to GABAergic neurons of bed nucleus of stria terminalis (BNST) and peri-PVN region to inhibit the activity of parvocellular neurons in order to downregulate CRF release which in-turn attenuates the HPA signalling [23]. Lesions in the hippocampal region correspond to feedback that shows exaggerated HPA activation. This leads to elevated levels of downstream GCs in many species with the exception of rats which show corresponding elevated GC levels during lighting conditions only [24]. Studies suggest a 4-fold increase in the CRF mRNA in the PVN corresponding to hippocampectomy or extirpation of the dorsal hippocampus indicating its role in providing negative feedback to the HPA axis [25].

The action of corticosterone in the CA1 region of the hippocampus is biphasic in nature owing to the occurrence of both MR and GR in this area [26]. An acute exposure of corticosterone causes occupancy of MR which mediates a non-genomic response [27]. This accounts for the ascending profile of the biphasic corticosterone-dose dependent functioning of the hippocampus which is essentially mediated by MRs [26]. If the corticosterone concentration is higher such that all MRs are saturated, then the response includes a delay of several hours followed by GR activation via a gene-

mediated pathway after the rapid MR mediated effects have phased out. This accounts for the descending profile of the biphasic corticosterone-dose dependent functioning of the hippocampal neurons which is essentially mediated by GRs [26]. This phenomenon could result in strengthening of the synapses that were active at the time of stress response which signify the encoded information in regard to stress exposure. Studies suggest that this may impart an adaptive value through learning and memory formation mediated by hippocampus; which is an integral process known as long term potentiation (LTP) [26, 27]. LTP is the chief neural mechanism of encoding information associated with spatial and contextual memory by the hippocampus and the concentration of corticosterone is one of the primary feats which modulate the efficiency of this process [28]. In the CA3 neurons of the hippocampus, similar biphasic relationship is observed in response to corticosterone, where MR activation enhances LTP (ascending curve) while GR activation leads to its suppression (descending curve) [29].

Another brain structure crucially involved in the feedback mechanism of HPA, present deep within the temporal lobes of the brain is the almond-shaped **amygdala**. Also known as the emotional centre in the brain, it comprises a group of nuclei responsible for regulation of different functions related to formation, processing, and consolidation of fear memory. Each sub area or nucleus differs in anatomy and function from each other owing to their differences in their neuronal composition. The function of amygdala is best understood in context of Pavlovian fear conditioning [30, 31]. The input for stimulus leading to a fear response enters the lateral amygdala from the thalamus or the cortex [32-34]. This is relayed from the basolateral amygdala (BLA) to the central amygdala, which is important for the expression of fear caused by these stimuli. Since the BLA is the primary nucleus receiving input signal in response to a

fear stimulus from the thalamus or the cortex, it can be regarded as a crucial point for initiating fear response, which makes its study an interesting case. The BLA consists of seven distinct types of cell populations [35] out of which three are most unique: the predominating pyramidal and stellate glutamatergic neurons which are excitatory in nature and the GABAergic local interneurons which are inhibitory in nature [36, 37]. Activation of BLA causes activation of the central amygdala (CeA) or the medial amygdala (MeA) via the excitatory projection neurons in the former [38]. This leads to activation of the PVN via the BNST, to switch on the HPA axis for generating a stress response (fear in most cases) [38, 39].

It provides a positive feedback to the HPA depending upon the amount of GC under circulation and contributes to the enhancement of memory consolidation associated with fear responses [40]. Most of the nuclei in the amygdala also express both MRs and GRs. [15, 41, 42]. Hence it is a major target for the circulating GCs and modulate the HPA activity based on it. In the BLA, GR-mediated pathways stimulated by GCs result in enhanced memory consolidation after inhibitory avoidance training or contextual fear-conditioning via nonadrenergic activation [43, 44]. Moreover, studies suggest that this memory induced by systemic GCs is impaired when BLA is subjected to neurotoxic lesions [45]. The GC-induced GR-mediated pathway also potentiates β -adrenoreceptor-cAMP signalling and thereby contributes to memory consolidation [46, 47].

BLA activation essentially regulates emotional arousal due to elevation of stress hormones under circulation [48, 49]. Memory consolidation pertaining to such emotional arousals renders long-term adaptive value [40, 49]. Rats when exposed to novelty or in case of an object recognition task without habituation experience an emotional arousal that triggers release of noradrenaline in amygdala leading to

memory consolidation which otherwise does not occur for storage of neutral information [50]. This activation of BLA is not observed in case of an object-recognition task that involves habituation which removes the emotional arousal effect, thereby blocking the corticosterone-inducing memory enhancement [51]. Furthermore, BLA establishes connections with other regions of the brain which control different types of memory [52, 53]. The caudate nucleus and the dorsal hippocampus centrally regulate memory associated with visual-cued training and spatial training respectively. Direct and indirect projections of BLA to these structures modulate both of these types of memory consolidation [54, 55].

The third important brain region which participates in the regulation of HPA axis is a cortical region known as the medial prefrontal cortex (mPFC). As the name suggests, it is present in the frontal lobe in the neocortex, posterior to the olfactory bulbs in rodents. However, the functional homolog of this region in humans and non-human primates is the dorsolateral PFC (dlPFC). mPFC plays a role in processing complex cognitive control associated with goal-directed behaviour [56]. It is involved in decision-making, planning and various executive functions [56, 57]. These functions heavily rely on working memory, the processing of which is central to mPFC [58, 59]. It is rather involved in complex valences, such that it participates in both reflexive as well as anticipatory responses [60]. The latter includes reference to past experiences in order to respond in a contextually-specific manner. The rodent mPFC is divided into two main sub-regions: The anterior cingulate cortex (ACC) and the dorsal prelimbic region (dPrL), collectively forming the dorsal mPFC, and the ventral prelimbic region (vPrL) and infralimbic region (IL), together forming the ventral mPFC [61, 62]. These areas in particular mediate functions such as selective attention and attentional set-shifting [63, 64]. The mPFC extends efferent projections to several regions in the

cortex (including cingulate and perirhinal structures), ventral striatum, basal forebrain nuclei, BLA, lateral hippocampus, various thalamic nuclei, periaqueductal grey and the raphe nuclei [61, 62, 65]. Though fewer in number, mPFC neurons also send direct projections to the PVN as well as the extended amygdala and is known to modulate the HPA activity via direct or indirect pathways [60, 66].

The mPFC manages stress-responses not only via HPA activity but also through its influence on the autonomic control, particularly the parasympathetic system [62, 67]. Excitotoxic lesion in the dorsal mPFC (at the cingulate gyrus) in adult male rats shows significant increase in the plasma ACTH and CORT after a stressful experience in comparison to sham subjects, indicating that mPFC provides a negative feedback to the HPA axis [68, 69]. Similarly, lesions restricted to the PrL and IL regions collectively, using ibotenic acid, cause hyperactivity of the HPA axis and an increase in the c-fos expression in the CRH releasing PVN cells [66]. However, the role of dorsal and ventral regions of the mPFC bifurcates with regard to modulation of stress-response. The PVN is a common effector-interface for both the autonomic (sympathoadrenal) and neuroendocrine (HPA) systems [3]. Specific excitotoxic lesions to the dorsal mPFC regions lead to c-fos activation in the PVN cells responsible for HPA activity and subsequent increase in the CRH mRNA [66, 69]. Furthermore it is accompanied by increase in release of both plasma ACTH and CORT. In contrary to this, similar lesions in the ventral mPFC causes a decrease in the c-fos expression in the same PVN neurons mentioned previously as well as CRH mRNA abundance [69]. In fact, it heightens the expression of c-fos in the PVN neurons responsible for autonomic control [69]. Also it does not change the ACTH-rise compared to subjects with non-lesioned mPFC. The CORT rise in dorsal mPFC

lesioned subjects takes longer to normalise than that in ventral mPFC lesioned subjects [69].

2.3 Stress: Disruption of HPA activity and elevated glucocorticoid levels.

The HPA axis is a highly sensitive system with a fine-tuned feedback. However, it is vulnerable to dysfunction on exposure to an acute stressful event or elongated stretches of stressful experience resulting in chronicity characterised by high GC levels of pathological degrees [22]. This invariably manifests a behavioural maladaptation in the form of elevated anxiety or depression [70]. It also influences the neuroplasticity of brain structures closely associated with HPA signalling [22]. An elevated level of endogenous GC is a classic hallmark of stress. The cardinal role of GCs is to mobilize energy from available reserves in the body to counter the stress-induced emergency. Repeated exposure of stress leads to persistent high levels of endogenous GCs which increase the risk of adult onset of diabetes type-2 and hypertension [5]. In extreme cases it can also lead to stunted growth and disruption of reproductive and immune system [5].

2.3.1 Effect of stress on the hippocampus

A. Changes in behaviour associated to hippocampus

The most widely studied brain region with respect to stress is the hippocampus. Due to its dynamic involvement in learning and memory of contextual, episodic and spatial information, it is regarded as one of the most plastic structures in the brain. Its plasticity largely depends on the number and magnitude of stressful events experienced by an individual and the corresponding adrenal steroids in systemic circulation [4, 71]. The amygdala and the prefrontal cortex (PFC) are other brain regions which also undergo structural plasticity in response to stress and

glucocorticoids; however they have not been studied as intensively as the hippocampus. Furthermore, stress-induced atrophy of CA3 dendrites in hippocampus of adult male rats impairs spatial learning and performance in short-term memory tasks (McEwen et al., 1995, Magarinos et al., 1996). Repeated restraint stress continued for 21 days (6hr/day) not only impaired hippocampal – dependent learning but also facilitated fear conditioning (Conrad et al., 1999). This form of chronic stress led to atrophy in the CA3 neurons of the hippocampus which reversed back to basal conditions 10 days after the cessation of stress. In adult male rats, chronic immobilization stress for 10 days resulting in heightened anxiety is also characterised by atrophy in CA3 neurons of the dorsal hippocampus [72].

B. Stress - induced GC effects

Stress and corresponding levels of glucocorticoids manifest its effects not only in physiology and behaviour but also in the anatomy of brain. Structural morphological changes are observed as an adaptive response to stress in brain regions relevant to memory consolidation and homeostasis management. Some of these are reversible while some of them are permanent [71]. GC in systemic circulation is noted to be elevated in case of aged rats, which is often associated with the degeneration of hippocampus and cognitive decline in the same [73]. Patients suffering from Cushing's disease are reported to have a decrease in their hippocampal volume owing to the high level of GC in circulation [74]. Studies report that administration of CORT 12h/day for three months which is equivalent to endogenous GC corresponding to chronic stress resulted in 20 percent loss of CA3 neurons [75]. A range of hippocampal neurons such as CA1 and CA3 are sensitive to GC, however the latter are most affected by stress [76]. A number of factors attribute this stress-specific sensitivity to CA3 neuron which are discussed later in this section (page 14-15) and the next section (page 16-17). Various studies have shown

atrophy in CA3 neurons in response to CORT causing a decrease in the volume and dysfunction of the hippocampus [77-80]. These anatomical and cytological characteristics of neuronal degeneration resembles to that of ageing [73]. Hence, it can be inferred that high levels of endogenous GC accumulated due to stress accelerates hippocampal ageing [81]. Similar neuro-anatomical changes were observed in the hippocampus when rodents were subjected to long period of stress involving six months of aversive learning [82]. Besides neurotoxicity, GC can also lead to neuroendangerment. Deleterious effects of stress leading to elevated GCs exacerbate toxicity due to exposure to kainate by lowering the threshold for kainate-induced epileptiform activity [83]. Furthermore, GCs tend to aggravate striatal damage caused by excitatory amino acids and NMDA receptor [83].

It has been reported that stress or elevated levels of GC up-regulates extracellular EAA in the hippocampus resulting in neurological insults in the form of neuronal atrophy [77]. This GC-induced atrophy was prevented in both rats and primates by administration of an anti seizure drug such as phenytoin which reduces EAA release, thereby confirming the role of GC in modulating glutamatergic cascade of neuronal injury in the hippocampus [84]. Conventional steroidal mechanisms ride the effects caused by GC via GR receptor activation which are necrotic rather than apoptotic in nature [85]. The GRs essentially depend upon the local GC concentration which in-turn is modulated by local conversion of GC catabolites into active GCs by a reductase isoform of 11β – hydroxysteroid dehydrogenase (11β -HSD) in the hippocampus [86]. Stress or excessive GC concentration-induced modulation of extracellular levels of EAA in turn results in modulation of synaptic communication. LTP, an essential mechanism of storing information associated with memory in the hippocampus requires binding of EAA to both NMDA and non-NMDA receptors in order to mobilize postsynaptic cytosolic calcium. The calcium mobilization results in strengthening the synaptic formations by changing

both pre and post synaptic elements [87]. This process in CA3 neurons is therefore adversely affected by stress [75].

A heightened level of GC causes remodelling of CA3 neurons in the hippocampus irrespective of whether corticosterone is administered externally (via injections or passive supplementation through drinking water) or by chronic stress exposure. What is interesting to note is that, the mechanistic aspects related to the mode of increase leading to similar effects are different [79, 88-90]. Both chronic corticosterone treatment by injection or passive administration via drinking water and 21 days of chronic restraint stress leads to retraction of CA3 hippocampal dendrites. However the difference lies in their mechanism of action where, in the former case, there is a reduction in the volume fraction occupied by mitochondria in the CA3 region while in the latter there is an increase in the mitochondrial profile in MFT [88, 91]. The basis of difference in the mechanism of these two approaches leading to the same outcome is still undiscerning. It is important to note that effects of acute/chronic stress essentially depend upon the GC levels and result in a reduction of hippocampal volume. This reduction does not correspond to any neuronal loss, but perhaps a loss of glia cells or atrophy of dendrites and reduction in branching [80].

Social housing with running wheel alters the degree of neurogenesis in the active phase when GC levels are high [92]. It is interesting to note that prolonged GC exposure due to chronic restraint stress suppresses cell proliferation in DG while acute restraint has no quantifiable effect [93]. This implies how an increase in GC level can lead to both positive and negative effects. This is perhaps due to the extreme sensitivity and difference in the mechanistic pathway of two different experimental settings towards the neuro-endocrinal system [4].

C. Structural plasticity and molecular co-relates

There are three basic features of plasticity associated with hippocampus [71]. Firstly, the effect of adrenal steroids at basal diurnal rhythm as well as post-stress is biphasic on excitability, cognitive function, and memory [94]. It can cause a reversible and biphasic structural modulation in hippocampal neurons and the degree of LTP mediated by it [95-97]. Secondly, GCs can affect neurogenesis in granule neurons of DG in association with EAA [98]. Suppression of neurogenesis is observed in case of stressful experience involving fear-related learning and memory owing to the connection between the DG and amygdala [99, 100]. The third feature is the occurrence of reversible stress-induced atrophy of CA3 dendrites in hippocampus of adult male rats. This impairs spatial learning and performance in short-term memory tasks [75, 101]. Interestingly reversible neuronal retraction is observed in case of hibernation and chronic stress suggesting that the structural plasticity in these regions are essential adaptive responses which vary according the degree of stressor in order to protect the brain regions from undergoing permanent damage [71]. Interestingly besides GC, there are other factors that contribute to dendritic remodelling of hippocampus which highlight the importance of glutamate in this process. These contributors include serotonin, GABA, and EAA [71, 102]. EAA released by mossy fibers also contributes in restructuring CA3 dendrites in response to stress [71]. Restrain stress leading to elevated levels of extracellular EAA induces glial glutamate transporters which in turn cause activation of nuclear transcription factors like phospho-CREB [103]. Chronic restrain stress (CRS) lasting upto 21 days causes depletion of clear vesicles from MFT (mossy fiber terminal) and enhanced expression of presynaptic proteins involved in vesicle release. These vesicles populating active

synaptic zones of the MFT, rich in mitochondria suggest the tendency of CRS to enhance glutamate release [88].

NMDA receptors play an important role in the process of neurogenesis and it has been observed that blockade of these receptors prevent the acute actions of GC on neurogenesis in the hippocampus [99, 104]. It could be speculated that the mode of action in these experimental setting varies with regard to NMDA receptor activation, resulting in different downstream trends of neurogenesis. In an experimental setting involving a novel environment rodents experience an emotional arousal in order to enhance object recognition mediated via adrenal steroids [50]. This memory consolidated by hippocampus exhibits an inverted U-shape response and is highly sensitive to the GC dosage, leading to occupancy of both the GR and MR in the hippocampus (as explained in section 2.2.2). This emotional arousal on exposure to novelty is absent post habituation to a novel object/environment due to removal of the very 'novel' feature during the actual test.

It is evident from various studies that stress modulates neuronal plasticity in different brain regions. This implies a role of certain neurotrophic factors facilitating structural changes in neurons of stress-affected brain regions. The brain-derived neurotrophic factor (BDNF) is one such growth factor which is influenced by learning experiences and stress [105-109]. Similar to decrease in spine density, BDNF protein expression also decreases in CA3 neurons of hippocampus in response chronic stress [110]. BDNF insufficiency in transgenic mice leads to retraction of hippocampal neurons [111] while its over-expression results in increase in the dendritic complexity in the dentate gyrus [112]. There is evidence that suggests in presence of EE, BDNF facilitates complexity of the neuronal morphology and functionality of hippocampal neurons in mice [113].

2.3.2 Effect of stress on the amygdala

A. Changes in behaviour associated to amygdala

The BLA is a crucial brain structure for generation of fear and anxiety and for driving HPA to secrete stress hormones [33, 39, 114-116]. It is known to consolidate memories associated with fear and emotional arousal which is favourable for long-term adaptability [117]. However, strong acute stressor or chronic stress which compromises working memory can also induce strong fear - memory consolidation by the BLA [118, 119]. Such a phenomenon is maladaptive leading to heightened anxiety due to hyper-activation of the amygdala [40]. This leads to development of anxiety disorders such as PTSD, MDD etc. The amygdala is affected by glucocorticoid hormones secreted during stress, resulting in prominent increase in dendritic length and branching (hypertrophy) along with formation of new spines [72, 120, 121]. These increases in morphological parameters are correlated with high anxiety in stressed animal [122]. Moreover, increase in aggressive behaviour among stressed animals within the same cage is observed implying hyperactive amygdala [123]. Neuroimaging studies in humans also highlight the role of amygdala in the same direction. fMRI studies indicate hyperactivity of the amygdala in patients with anxiety-states [124-126]. Furthermore, in depressed patients, the amygdalar baseline activity (in the form of glucose metabolism) as well as the responsiveness to an affective stimulus is elevated when tested using fMRI and PET (Positron Emission Tomography) [127, 128].

B. Stress - induced GC effects

Amygdala is sensitive to glucocorticoids due to expression of its cognate receptors (GRs and MRs) [41, 129]. Administration of corticosterone in amygdala shows

increase in HPA activation post stress indicating the role of GC to be crucial in enhancing the stress response [130]. Ten days of daily dose of high physiological corticosterone level leads to BLA neuronal hypertrophy as observed in response to chronic immobilization stress exposure [121]. Increase in the excitability of the principal neurons of BLA is another important effect induced by CORT [131]. These effects are essentially mediated by the binding of CORT with its cognate receptor GRs [132]. Studies have shown that experimental blockade of GR from binding with GC in the BLA leads to reduction in anxiety and hyperactivation of HPA [133, 134]. This makes concentration of GRs in BLA a potential mediator of driving anxiety-like behaviour. However, the activity of BLA also depends on the concentration of GR relative to MR. Studies show that overexpression of MR in the BLA through viral vectors reduces the binding of GR with CORT and thereby lowers anxiety and corresponding stress-induced CORT rise [135]. It also needs to be noted that the function of GR in BLA in mediating stress-pertaining effects is possibly distinct from that of the hippocampus. The stress-induced activation of excitatory neurons in the hippocampus and BLA neurons differ owing to the differential distribution and transcriptional regulation of calcium channel subunits in the neurons of these two regions [136]. The modulation in calcium channel subunits expression is in fact influenced by GR activation in the BLA [137]. As a consequence, it induces an increase in sustained high-voltage calcium currents and differentiates these BLA neurons from those in hippocampus in their pattern of slow after-hyperpolarization [136, 137].

C. Structural plasticity and molecular co-relates

Studies show that excitation of the neurons of the lateral division of amygdala is increased in response to Pavlovian fear conditioning in rodents [138]. These effects are

mediated through activation of β -adrenoreceptors by noradrenaline. This, on one hand enhances potentiation of fear learning and memories from cortical input to lateral amygdala and on the other, inhibits small-conductance Ca^{2+} activated K^{+} channels (SK channels). The final effect of this molecular activation in response to stress is suppression of GABAergic neuronal activity and increased neuronal hyper-excitability of a smaller subset of BLA neurons leading to enhanced memory consolidation [114, 139, 140]. Thus it is not surprising that overexpression of SK channels in the BLA causes a reduction in anxiety, probably through reduction in excitability along with stress-induced GC secretion and dendritic arborisation [141].

Stress also adversely affects the GABAergic interneurons which constitute 20-30% of the neuronal cell types in the BLA [142-144]. Suppression in the net activity of these interneurons due to the hyperexcitability of the principal neuron in the BLA leads to manifestation of anxiety and associated mood disorders [145-147]. Lesions in the GABAergic interneuron subpopulation in the BLA which express neurokinin1 receptors lead to anxiety-like behaviour [148]. Moreover, stress causes disruption in nonadrenergic facilitated GABA release within the BLA which potentially leads to hyperexcitability of this brain region leading to epilepsy and anxiety-like effects [149]. Alongside, an increase in the intrinsic excitability of excitatory neurons, stress-induced corticosterone contributes in the reduction of GABAergic inhibitory synaptic transmission in the BLA [131].

Glutamate decarboxylase (GAD), an enzyme that leads to synthesis of GABA from L-glutamic acid, is also expressed in the local GABAergic interneurons in the BLA [150, 151]. It serves as potential inhibitory presynaptic protein marker in these neurons for GABAergic activity mediated within the BLA. Previous reports have demonstrated that reduction in GAD67 protein expression in rats is linked to increased depressive-

like behaviour and can be induced by repeated corticosterone administration in the amygdala [152]. GAD67 in the inhibitory neurons of the BLA therefore can be modulated by stress, which can lead to emotional dysfunction.

Chronic stress and associated levels of CORT leads to structural remodelling of BLA neurons. These remodelling effects include changes in synapse formation, dendritic length and branching patterns in the BLA (hypertrophy) and MeA (atrophy) along with increase in anxiety [72, 120, 121, 153, 154]. A single episode of acute immobilization stress for 2 hours causes a delayed increase in spine density on principal neurons of the BLA along with manifestation of heightened anxiety in behaviour [120]. This correlation is interestingly observed 10 days post the acute stress episode. Such a delayed pattern of spinogenesis is limited to the dendritic segments lying proximal to the cell body. This indicates that acute stressors trigger delayed responses mediating plasticity at synaptic levels accompanied by delayed anxiogenic behavioural phenotype. However, repeated episodes of 2 hours of immobilization stress for 10 consecutive days increases anxiety within 24 hours after the cessation of stress; indicating the robustness of chronic stress at the cellular and behavioural levels [72, 155]. Structural plasticity corresponding to chronic stress includes increase in spine density in both primary and secondary dendrites of BLA principal neurons, exhibiting a more extensive pattern of effect than acute stress [120]. This is coupled with robust dendritic growth in pyramidal and stellate neurons of BLA which essentially corresponded to manifestation of greater anxiety-like behaviour. Therefore, in response to chronic stress, the cumulative impact (more spines per unit length of dendrite multiplied by more dendrites) is manifested as a significant strengthening of synaptic connectivity in the BLA, which in turn possibly serve as a cellular substrate for enhanced anxiety. Increase in spines enhances LTP in the

amygdala, characterized by potentiation of learning and memories associated to fear [156, 157]. Enhanced LTP can lead to manifestation of anxiety disorders [157]. These findings indicate the differences in acute and chronic episodes of severe stress and their responses towards spinogenesis in the BLA neurons, wherein the former acts in a delayed and restricted pattern while the latter gives rise to robust effects accompanied by hypertrophy in the BLA dendrites with a direct correlation to increased anxiety-like behaviour [40]. Importantly, these effects of chronic stress are remarkable in their persistence, as shown in studies that in spite of 21 days of stress free recovery, chronically stressed animals continue to exhibit high anxiety and dendritic hypertrophy in the BLA [158].

CRF (also known as CRH for corticotrophin releasing hormone) is considered as an important molecule affecting neuro-endocrinal and behavioural responses, and is secreted by PVN in the hypothalamus. Recent findings suggest that extra-hypothalamic CRF secretion by the BLA causes synaptic plasticity in the BLA neurons, which translate into anxiety-like behaviour and emotional disorders [159, 160].

Another critical factor which contributes to the neuro-morphology of different brain regions and poses sensitivity to stress is the brain derived neurotrophic factor (BDNF). In response to different stressors, BLA shows an increase in mRNA abundance of BDNF which strengthens plasticity in BLA neurons via spine formation [110, 161]. This observation is in fact contrary to the lowered BDNF protein expression profile in the hippocampus in response to stress [105]. This further highlight the contrasting effects of stress on hippocampus and amygdala, not only in the neuronal remodeling of excitatory neurons but also in their corresponding BDNF expression profiles. Furthermore, crosstalk between GR activation and BDNF signaling affects stress

response [109, 162]. BDNF is expressed via MAPK/ERK pathway and its activation leads to increase in synaptic proteins [109, 163]. But this process is inhibited by the presence of excessive glucocorticoids [164, 165]. However, GR activity is essential in consolidating long-term inhibitory avoidance memory via activation of BDNF in the hippocampus and is associated with activation of CaMKII α and CREB activation downstream to BDNF signaling [166]. On the other hand in the amygdala, enhancement of contextual fear memory is mediated by GR activation via BDNF signaling but accompanied with the recruitment of tPA, downstream to which Erk1/2 (MAPK) signaling pathway is activated [167]. A dysregulation in this mechanism within the amygdala could potentially lead to psychiatric disorders.

2.3.3 Effect of stress on the mPFC

A. Changes in behaviour associated to mPFC

Medial prefrontal cortex (mPFC) is primarily involved in executive functions including processing working memory, decision making, and fear responses [56, 58, 59]. It is highly responsive to changes in an individual's immediate environment, including acute and chronic stress, given its property of forming strong intrinsic synaptic interconnections [57, 60]. Both acute and chronic stress adversely affect working memory as well as attentional set-shifting performance in rats [56, 168-170]. Stress also negatively affects decision-making abilities in rats when tested via water foraging choice-task and rat-model of Iowa gambling task (which involves reward based decision making) [171, 172]. Chronic stress strongly influences affective behaviour wherein anxiety appears to be comorbid with cognitive impairments particularly extradimensional set-shifting deficits as seen in chronic unpredictable stress rat models [173]. These behavioural pathologies can be reversed upon

administration of antidepressants in these rat models [173]. Similarly, chronic stress increases the immobility of animals in forced swim test, which indicates depressive-like symptoms in these subjects [174]. This behaviour is observable upon GR knockdown in the IL region of the ventral mPFC in rats [175]. Moreover, it has been shown that deep brain stimulation of the ventral mPFC results in attenuation of anhedonia as tested in sucrose preference test in rats [176].

B. Stress - induced GC effects

Similar to most GC-sensitive brain regions, stress or corresponding levels of glucocorticoids act on mPFC in a biphasic manner [15]. The mPFC also has a remarkably high GR:MR ratio [15]. Majority of cell population in the mPFC are glutamatergic in nature (80-90%) and the rest being GABAergic interneuron populations (around 10-20%) [61]. Acute stress tends to increase glutamate transmission and the depolarisation-evoked glutamate release facilitated by GR mechanisms [177]. However under chronic stress, these excitatory effects, particularly the NMDA and AMPA-mediated currents begin to decline through enhanced degradation of postsynaptic glutamate receptors [178]. Besides this, in the ventral mPFC, chronic corticosterone administration reduces the expression of certain NMDAR and AMPA subunits namely NR2B and GluR2/GluR3 respectively [179]. Unlike its effect on the excitatory neurotransmission, acute GC exposure leads to suppression of the inhibitory (GABAergic) transmission in the mPFC through an endocannabinoid –dependent mechanism [180]. On the other hand, upon chronic stress exposure this inhibitory system features enhanced activity and complexity [181]. The cumulative effects of chronic stress and increased GC in circulation leads to a dysfunctional mPFC that fails to provide a negative feedback control over the HPA.

C. Structural plasticity and molecular co-relates

Chronic stress or corticosterone treatment leads to dendritic remodeling in the pyramidal neurons of the mPFC, by increasing complexity of dendrites closer to the soma and decreasing in distal dendrites [182]. These effects are associated with impairments in behavioural flexibility [183]. Chronic restraint stress (CRS) causes significant changes in the apical dendrites of the mPFC neurons but not in the basal dendrites. In the apical dendrites, the number and length of dendritic branches are reduced to 18% and 32% respectively [184]. CRS also causes significant reduction in overall spine density in apical dendrites, an estimated 33% loss of axospinous synapses [185]. Interestingly, mild short-term stress (restrain for 10 minutes daily for 7 days) also decreases complexity in distal dendrites indicating a high level of sensitivity of the mPFC to stress [186]. The apical dendritic atrophy caused by chronic stress is shown to be reversible, indicating that the mPFC neurons exhibit plasticity [187]. In the IL region, chronic immobilization stress in rats leads to atrophy [188]. However after three weeks of recovery from CRS, the proximal apical dendrites (but not the distal dendrites) of these neurons show increased arborisation and spine density [189]. Similar effect is observed in the PrL neurons of young adults which undergo reversible dendritic remodelling post-recovery after CRS that is absent in case of middle-aged and aged rats [190]. Two to three weeks of corticosterone exposure to the PrL neurons through localised implants results in reduction in spine density wherein a higher dose of the same can in fact lead to apical dendritic retraction along with spine loss [191]. In line with these observations, neurotrophic factors such as BDNF, which plays an important role in spine formation and modulation of synaptic plasticity, also decreases in the mPFC in response to chronic stress and elevated GC levels [110, 192]. Chronic stress through footshocks in rats further shows modulation downstream to BDNF

signalling including dendritic phospho-ERK1/2 hyperphosphorylation and reduced expression of phospho-CREB [193]. Acute stress on the other hand, results in increase of both activity-regulated cytoskeleton-associated (arc) mRNA and protein expression which is an early immediate marker [194].

2.3.4 Basolateral Amygdala: the primary mediator of fear and anxiety; how chronic stress affects it

So far three crucial brain regions sensitive to stress have been discussed: the hippocampus, the basolateral amygdala and the medial prefrontal cortex. Out of these three, the BLA - primary emotional centre in the brain, takes the front seat when it comes to fear processing. This is due to its role as the input interface in the brain for any sensory stimulus loaded with the valence of fear/aversion [31]. Quintessential experiments using Pavlovian fear conditioning have shown how memories pertaining to a specific fear stimulus is acquired, consolidated and recalled via the BLA [117, 119, 195]. Such experiments involve coupling of a neutral event (also known as the conditioning stimulus) along with an aversive event (also known as the unconditional stimulus, such as footshocks), which elicits a defensive response from the animal. Fear responses being mediated by the BLA can further be regulated by the direct and indirect projections it sends to and receives from the hippocampus and the mPFC [54, 116, 196]. The latter is particularly involved in extinction of fear by attenuating the BLA activation [138, 197].

Fear is an emotion aroused by specific stimuli, which generates a defensive or escaping behaviour, in other words, a “fight or flight” response [5, 198]. Animals may learn to fear a situation to which they have been exposed previously, that presented an aversive cue/stressor then. Repeated exposure to such a stressor or a strongly single

aversive/stressful stimulus can generate a fear response even in the absence of the stressor itself in the long run [114, 199, 200]. This response is known as anxiety. Therefore anxiety is an exaggerated form of fear response which can be triggered by an unknown threat while fear is focussed to a known external danger [198]. Fear carries an adaptive value in coping with various ethologically relevant dangers. However, anxiety is a mental state where fear is expressed pathologically even in the absence of any perceived threat due to lowered threshold and hyperactivity of the fear circuitry itself [201-204]. Anxiety and hyperactivity of fear circuitry is potentiated by exposure to a severely stressful event or repeated exposure to a stressor (chronic stress) leading to prevalence of psychiatric disorders such as PTSD or generalized anxiety disorder [204, 205]. This anxiety behaviour is mediated by the BLA which further hyperactivates the HPA axis via the central amygdala and the BNST (extended amygdala) [38, 200, 201]. It is important to note that fear and anxiety also share the same endocrine response mediated by stress hormones namely glucocorticoids and catecholamines [5, 45, 130, 167, 206-209]. However, the difference between these two is the threshold of activation and degree of these responses [210]. Severely stressful event such as exposure to predator odour in rats not only cause specific activation in the BLA via c-fos expression but also show hypertrophy and increase in spines in the neurons of this region in animals which co-express heightened anxiety [211, 212]. In male rats, predator-stress induced hypertrophy in the BLA neurons is correlated with their anxiety-like behaviour [122]. This morphological predictor of anxiety in the form of hypertrophy and spinogenesis in BLA neurons is shared by the effects of chronic stress as well [72, 120, 213]. Furthermore in case of chronic stress, these neurons do not normalise over a recovery period, rendering these changes as irreversible [158]. Physiologically as well, chronic stress increases the excitability of the BLA neurons,

making the fear circuitry hyper-responsive and facilitating emergence of anxiety disorders [214-216]. The hyperexcitation of these neurons associated to anxiety is in fact correlated with increase in spines of the same, also suggesting enhanced excitatory synaptic drive in these neurons [215]. Recent study has shown that electrical lesions in the BLA reduce anxiety behaviour induced by sub chronic (stress exposure for 7 consecutive days) and chronic (stress exposure for 21 consecutive days) stress in rodents which confirms that anxiety behaviour induced by chronic stress is in fact mediated by the BLA [217]. Besides this, Section 2.3.2 C also mentions various studies which elaborate on how molecules such as GAD, GR and BDNF in the BLA are essentially involved in mediating fear responses are affected by stress.

The potency of chronic stress in inducing heightened anxiety and related mental pathologies has been demonstrated by various other studies [158, 173, 218, 219]. These studies together, present chronic stress as a potential paradigm for developing animal models for stress-induced anxiety. Such animal models can be studied for (chronic) stress induced BLA morphological hypertrophy that is associated with anxiety. A chronic-stress model could help in investigating a possible association of the above mentioned molecules (GAD, GR and BDNF) with the morphological changes in the BLA (primary brain region responsible for fear processing) that translate into anxiety-like behaviour in rats. Furthermore, exposing these models to stress-relieving agents/conditions could in-turn help in studying resilience.

In summary, the basolateral amygdala is the cardinal brain region responsible for fear-conditioning [33, 117, 119, 195]. The endocrine response to acute fear and anxiety follows a similar course, both being coordinated by the BLA [5, 45, 130, 167, 206-209]. Recent longitudinal human study shows how the activity in the amygdala (measured in the form of resting metabolism via PET/CT scan) is a positive predictor

of stress associated cardiovascular diseases [220]. Stress and associated levels of glucocorticoids (the primary stress hormones) induce anxiety, a symptom of various mental pathologies such as PTSD, generalized anxiety disorders and OCD [72, 121, 124, 173, 199, 208, 209, 221]. This is a resultant of stress induced hyperactivation of BLA [214-216]. At the morphological level, stress and its associated hormones induce hypertrophy in the BLA neurons [72, 120, 213]. This increase in BLA dendritic structures in fact correlates with predator-stress induced anxiety phenotype [122, 212]. Preventing this hypertrophy of neurons within the BLA via molecular means such as, overexpression of SK2 potassium channels helps in attenuating anxiety in rats [141]. Furthermore, lowering the net GR signalling within the BLA by sequestering CORT through ER (estrogen receptor) or MR signalling is sufficient to reduce anxiety in rats [135, 222]. Activity of BLA can further affect fear-processing under stressed conditions in other brain regions to which its neurons innervate directly [116]. As mentioned earlier, hippocampus that is responsible in providing negative feedback to the HPA axis is also sensitive to stress and its associated hormones [24, 71]. Lesion of BLA inputs to hippocampus reduces the impact of stress hormones on hippocampal fear-memory processing [196]. These studies therefore delineate the central role of BLA in mediating fear and anxiety responses. They also highlight the link between fear, anxiety and BLA, and the impact of stress on them.

2.4 Resilience and its neurobiology

Stress resilience is the capacity of an individual to cope by resisting the pathologies caused due to stress [223]. It not only attributes to endurance of the adversities due to stressful experiences but also represents the active process of physical or psychological involvement in minimizing the impact of stress and render well-adaptive behavioural functions. This is termed as *active coping* characterized by exploratory activity, defensive or aggressive behaviour, risk-assessing activity etc. which corresponds to optimism and positive reassessment [224]. This feature is in contrast with passive coping which is characterized by denial, avoidance of conflicts, suppression of emotions and behavioural disengagement which is maladaptive in nature and offers only a short-term resilient response to stress [225]. Stress resilience is potentially mediated by a synchronized discrete interaction between genetic and environmental factors that regulate HPA in an adaptive manner. This in-turn, results in ‘active coping’ behaviour which is a hallmark of a resilient individual. Physiologically, this would correspond to achievement of undergoing homeostasis more effectively such that the body stabilises faster with the changing environment post stress-exposure. Identifying the key neurobiological mechanism and molecular markers that form the basis of resilience would give way to design more effective, natural and therapeutic approaches for countering against stress-induced anxiety and fear disorders.

2.4.1 Biological co-relates of resilience

A number of neuroendocrine mechanisms have been speculated and proven to be pro-resilient in nature. High levels of glucocorticoids are associated with stress-disorders, such that it mediates reduction of dendritic spines density in the hippocampus and

hypertrophy in the BLA, translating to memory-loss, high anxiety, and depression-like symptom [71, 120, 121, 209]. However, a smaller subset of depressed individuals show lower levels of GCs. Hypocortisolemia has been reported widely in PTSD [226, 227], thereby suggesting a need of reassessing the exact role of GC in the context of stress responses. Interestingly, in studies performed to understand role of maternal care, it has been demonstrated that high maternal care correlates to hypomethylation of GR gene in the DNA leading to upregulated expression of the GR in both gene and protein level in the hippocampus; generating stronger feedback inhibition of HPA axis and characterising resilient behavioural features [228]. In contrast, another factor that possibly governs resilient behaviour is the ratio of MRs and GRs in different brain regions. In the BLA, anxiety is probably a function of hyperactivation of GR-associated pathways. A study involving modulation of MR and GR population ratio in the BLA by overexpressing MRs led to reduction in corticosterone secretion and anxiety in rats [135]. Sex hormones also have a role to play in contributing to resilient features which imparts sexual dimorphism in the patterns of stress responses in mammals. Testosterone attributes aggressive behaviour and suppresses social avoidance in males while hormones like oxytocin have positive effects on cognition and regulates protective effects against stress in females [223].

A number of studies in humans have identified anatomical and neuro-cytological markers or mechanisms which mediate resilient features. MRI (or fMRI) studies suggest that in humans, anxiety and depression are controlled by various brain regions. Most studies indicate the manifestation of these disorders is associated with a reduction in volume and lowered activity of the frontal cortex and hippocampus. Consequentially, on one hand this reduces the activity of subcortical regions such as the nucleus accumbens (NAc) while on the other, increases the activity of subgenual

area of the ACC in the PFC [229-232]. fMRI studies also indicate hyperactivity of amygdala in patients suffering from anxiety and depression [124]. The ideal functional/neuroanatomical configuration for these subcortical regions that promotes resilience can be framed from the observations in these studies. However, they typically fail to indicate if the adaptive responses correspond to the activation or inhibition of the circuits in these regions. Therefore, investigators have resorted to study synaptic changes in the glutamate systems in these brain regions. Studies note higher expression of c-Fos, FosB or Δ FosB in glutamatergic neurons of the mPFC in resilient mice after being subjected to chronic predator or social defeat stress [233-235]. Optogenetic stimulation of glutamatergic neurons in the mPFC to NAc (studied via stimulation of channelrhodopsin ((ChR2) tagged glutamatergic neurons) indicate the presence of pro-resilient microcircuitry in the mPFC [233]. In the ventral tegmented area (VTA), dopamine neurons undergo increase in firing rate in response to stress which manifests stress-related deleterious effects. This effect is normalized by increased expression of K^+ channel subunits which buffers the hyper-excitatory currents in these neurons thereby rescuing from the compromise of stress [236, 237]. Therefore, such studies suggest that boosting the activity of various circuits facilitated by dopaminergic neurons lead to alleviation of depression or rather enhancement of resilient-like features characterised by motivational valence. Besides this, one of the recently proposed molecular underpinnings of resilience is changes in epigenetic status of certain genes. For example, gene silencing studies show that hypermethylation of *Crh* gene results in resilience [238].

2.4.2 “Are we there?” - Approaches towards a stress-resilient animal model

In order to study stress resilience, a number of laboratories have attempted to develop resilient animal models. These attempts involve subjecting a population of animals to

a validated stress paradigm, testing for behaviour, selection of well-performing individuals and inbreeding to develop resilient strains. For example, genetically inbred C57BL/6/J male mice when subjected to social defeat stress, give rise to a cohort of heightened stress reactivity responses including increased anxiety, deficits in exploratory behaviour and stress-induced polydipsia. Out of this population, about 35% of the stress mice result as the 'resilient' exhibiting lack of social avoidance or anhedonia [236, 239]. Similarly, exposure to inescapable foot-shocks to induce learned-helplessness in animals result in a subset of animals (~30%), which exhibit resilient features unlike the rest of the stressed population [240]. Deployment of stress paradigms like predator odour exposure and unpredictable chronic mild stress also result in developing a subset of animals, which exhibit resilient features like absence of social avoidance and anhedonia which show high expression of a pro-resilient marker in the brain called neuropeptide-Y [241, 242].

Additionally, more reliable models are also developed upon the demonstration of trials which employ the concept of 'stress inoculation' [243]. The basic principle governing this model is that coping with moderate amount of stress leads to an individual sense of mastery and promotes resilience in the future [223]. Studies on squirrel monkeys employed a moderate stressor in which brief intermittent separation of offspring from mother (1 hour per week spread over 10 sessions) is performed. Testing in adult phase (9 months) shows that the separated monkeys exhibit lower anxiety and more exploratory behaviour compared to the non-separated monkeys [244, 245]. However, in order to induce resilience, it is extremely crucial that the stressor has optimal degree of its impact. A degree of any lower or higher than the optimal would manifest into deleterious effects in response to the stressor [246]. For instance, long hours of maternal separation (3 hours of daily separation) from birth to 2 weeks can result in

induction of depressive-like behaviour upon stressful encounters in future [247, 248]. Hence designing a paradigm for stress inoculation with an objective to tease out resilience features highly depends on the degree of stressor and age of subjects which otherwise would inversely result in increased chances of stress-vulnerability.

2.5 Environmental Enrichment (EE): A potential resilience inducer

All the above mentioned paradigms, involve either follow-up of several generations of individuals to finally attain a cohort featuring resilience or it involves degree of stress-specific treatments as seen in stress-inoculation, which might trigger an individual to be resilient based on only a certain age group or degree of stressor itself. It would be ideal if a single treatment/alteration in an individual irrespective of its age/sex or any specification would give rise to resilient feat(s). It is also important that this alteration is non-invasive, easily accessible and employable under normal conditions.

Such a paradigm is also not too far-fetched. In 1942, Robert Tryon was successful in raising two distinct cohorts of “bright” and “dull” mice by repeatedly testing a large number of animals on a Hebb-William maze. At the beginning of his study, he classified each individual as “bright” (few errors made in the testing phase) and “dull” (many errors made in the testing phase). Along his study, after each series of testing, a bright male was bred with a bright female and a dull male was bred with a dull female. It took him seven generations to reach a point where the resultant progenies of the two lines of breeding were all bright and dull respectively. In 1958, Cooper and Zubek used Tryon’s lines of “bright” and “dull” mice, and they reared them in enriched environment (stimulatory and spacious) separately in comparison to an unstimulatory usual laboratory environment [249]. This resulted in an outstanding observation

wherein, enrichment enhanced the learning ability in dull rats while no effect was seen on the bright rats. An unstimulatory environment on the other hand, decreased the learning capacity in bright rats but had no effect on the dull ones. A distinction that took seven generations to be built was overcome within one generation by a simple intervention of environmental enrichment.

2.5.1 What is Environmental Enrichment?

An important paradigm which potentially induces pro-resilient features is Environmental Enrichment (EE) and this is the model that I used for this study. EE is characterised by a more spacious and exploratory ambience relative to standard laboratory cages. It is enriched with a number of objects like pipes, toys, nesting material, ladders, baskets, food containers and water distributed over a much larger space than a standard cage. The orientation of all these objects is shuffled at regular intervals. The number of cage-mates in an enriched cage is double than in a standard cage. Exposure to EE provides a complex exploratory, social, emotional and locomotory stimulation to the animals. It is a potential paradigm to study and develop a resilient well-adaptive behavioural response to stress. EE has been promising in studies of gene X environment as outlined before. It is also known to induce positive behavioural, physiological and neural features in animals [250, 251].

2.5.2 EE: Effects on brain and behaviour

EE influences plasticity in various areas of the brain such as the cortex (primarily the visual cortex), the hippocampus, the entorhinal cortex, the cerebellum and the amygdala. In order to study the effects of EE on animals, majority of the studies have compared biological variables across three main groups: animals kept in enriched

environment (EE), animals kept in standard environment (SE) with two or three animals per standard laboratory cage and animals kept in impoverished environment (IE) with one animal per cage that is slightly smaller than a standard cage [251]. The earliest trials of EE show increase in the weight of brain of animals in enriched cages (EC) compared to their litter mates in impoverished cages (IC) or standard caging (SC) [252, 253]. However in these studies the body weight of the former is lower than that of latter. The EE group also show an increase in the volume and thickness of the cortex compared to those in standard environment (SE) or impoverished environment (IE) [254-256]. The largest difference between the two groups is observed with respect to the occipital cortex accounting to 9.4%. EE increases dendritic branching and spinogenesis in various cortical layers [251]. The visual cortex shows an increase in dendritic branching and spinogenesis in response to EE in the stellate and pyramidal neurons of occipital cortex [257]. These changes appear to be mediated by neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) which are up-regulated in response to EE in the visual cortex [258, 259]. Increased synapse formations marked by high synaptic density and larger synaptic area in these regions of the brain attribute to enhanced capacity of learning and long-term memory [259].

Recent studies demonstrate that EE can potentially influence prominent innate behaviours like avoidance of predator odor and ability of males to attract mates, characterised by brain plasticity in certain regions of the hypothalamus [260]. Exposure to EE in peripubertal rats also reverses the effects of postnatal maternal separation (exaggerated HPA activity) [261]. Moreover EE decreases social anxiety in juvenile and female rats (dams), which also corresponds with decrease in Glutamate receptor 1 subunit (GluR1) and GABA protein concentration in the prefrontal cortex

[262]. Another interesting study shows the influence of EE in imparting resilience against social defeat stress via pathways associated to the infralimbic cortex. EE facilitates a higher expression of FosB/ Δ FosB in the glutamatergic neuron in this region which is responsible for emotional regulation and reward processing [235]. Furthermore, the entorhinal cortex exhibits more thickness in response to EE compared to exposure to an impoverished environment along with elevation in glutamate levels [263]. Another neurotrophin, insulin growth factor (IGF-1) has its importance in regulating functions like neuronal survival, glial differentiation and facilitating release of acetylcholine [264]. IGF-1 receptors sites increase in occipital cortex layers under a month of EE relative to IE [251].

A. Effects of EE on hippocampus

The most widely studied brain structure in response to the EE is the hippocampus. EE results in increase in spine formation in CA1 neurons [265]. It mediates dendritic growth and arborisation in the pyramidal neurons of the hippocampus which is regulated by various neurotrophic factors. NGF, known to regulate structural plasticity in neurons is enhanced with enriched environment in the hippocampus which also results in increased dendritic arborisation and spine density [266]. NGF exhibits a higher expression at transcriptional level in both the hippocampus and the visual cortex [259]. Similarly NT-3 mRNA is also expressed more in the hippocampus of enriched rats compared to rats in SE in a period of a month's time [258]. The role of BDNF is considered crucial in increasing apical dendritic spine density and number of synapses per neuron [267]. Its expression is enhanced at transcriptional level in the hippocampus in response to EE [268]. Besides this, environmental enrichment potentially reverses the effects of chronic stress on cognitive integrity and dendritic

morphology in the hippocampus; whereby it reinstates the dendritic morphology and cell proliferation to normalised state even in the presence of stress [269, 270]. Furthermore EE protects rats from depressive-like symptoms induced by acute and chronic stress (tested by Porsolt's forced swim task) [271]. This effect of EE is also associated with increased GR levels and protection from increased synaptic GluA2/PKM ζ levels induced by stress in the hippocampus.

To study the contribution of BDNF in plasticity pertaining to EE, BDNF knockout mice were exposed to EE along with wild-type controls for 2 months [251]. The wild-type strains exhibited increased dendritic spines in dentate gyrus neurons of the hippocampus while this plasticity was attenuated in mutant strains. This confirmed the participation of BDNF in modulating plasticity in hippocampal neurons in response to EE. The behaviour correlates of the three types of mice strains were analyzed through open field test wherein, the wild-type enriched strains were most adaptive showing less anxiety, both the EE and SE. BDNF knockout mutants were hyperactive and wild-type SE strains took longer to habituate to the open field. This indicates the combined role of BDNF and other cellular/molecular factors in orchestrating the structural plasticity and corresponding behaviour outcome in response to EE [251]. BDNF abundance is upregulated by EE in the hippocampus and cortical areas, which on the other hand, is downregulated in presence of stress [272, 273].

By the deployment of a proliferation marker known as bromodeoxyuridine (BrdU), studies in both mice and rats demonstrate a remarkable increase in neurogenesis in hippocampal-dependent dentate gyrus in response to 60-90 days of EE [274, 275]. This phenomenon is contradictory to the effect of stress which reduced proliferation of neurons in the dentate gyrus [99]. The cumulative effects of EE on the molecular environment and structural plasticity of the hippocampus therefore, translates to

enhanced learning and memory [276-278]. Rat studies show that stress-induced cognitive deficits and disrupted cell-proliferation in the hippocampus can be restored by exposing them to EE [270]. It also protects mice from chronic mild stress by preserving neurogenic effects in these subjects after stress cessation and preventing decrease of doublecortin-positive cells in the dentate gyrus [279]

B. Effects of EE on amygdala and emotionality

As mentioned in earlier sections, encounter with a novelty induces an emotional arousal in rats and this response is primarily mediated by the amygdala, which regulates emotions and fear responses in individuals. Enriched rats show higher exploratory behaviour compared to rats in standard environment. They habituate quicker to novel conditions, which correlates with a decreased arousal and emotionality relatively. This can be demonstrated by lower locomotor activity by EE rats than SE rats in an open field [268, 277]. Besides this, EE also reduces anxiety towards novel objects and predator-odor in comparison to their non-EE counterparts [280]. Correspondingly these EE subjects show lesser c-fos expression in the BLA following a stressful task performed in the form of water-escape. In response to cat odor, EE animals cope more actively as compared to non-EE counterparts. For instance, EE rats engage more in basal grooming behaviour and active exploration of the cat-odor borne arena and also show lesser freezing behaviour in the same. These behavioural effects correspond to increased expression of PV-positive cells and GAD-67 in the mPFC and basolateral amygdala respectively, which putatively enhances a stronger (inhibitory) control over fear responses and corresponding HPA activation [281].

Moreover, in response to a pheromonal environment, the neurons in the MeA exhibit plasticity changes pertaining to synapses [282]. Studies demonstrate the expression of *c-fos* as one of the immediate early gene markers as well as its translatory product- c-Fos in various brain regions in response to an aversive stimulus [283, 284]. Differential expression of c-Fos has also been observed in the amygdala, thalamus, and the hypothalamus as well in response to stress. Aversive stimuli upregulate c-Fos in these brain regions post 2 hours of their exposure [285]. Interestingly, the c-Fos expression in response to EE was lesser than that expressed in IE or SE indicating that recovery of enriched animals from stressful events was quicker than from of SE. EE also reverses the anxiety-like behaviour in male rats induced by early-life stress (maternal separation) even when the enrichment was provided in the adult phase for a short duration [286]. These effects correspond to reversal of hypertrophy in amygdalar neurons in presence of EE. Recent studies suggest the role of amygdala in inducing anxiolytic characters in mice by downregulating the CRF mRNA expression in BLA and corresponding CRFR1 mediated signalling [287]. Yet another important player is the BDNF which in the BLA, shows an increase in its mRNA abundance and strengthens plasticity in BLA neurons via spine formation [110, 161]. However, studies have shown that EE can reverse the expression of anxiogenic behaviour and changes in emotionality in mice with partial deletion of BDNF when housed under standard cages to basal level of anxiety and emotionality [288, 289].

C. Effects of EE on aged animals

It is interesting to note studies that demonstrate the effect of EE on aged rats. The changes caused by EE during adult phase are long-term and observable even during aging. Aged rats after exposure of EE during adult phase show larger brain weight and longer cortices marked by longer dendrites and increased branching and spine density

compared to standard housing [251]. Positive changes in glial cells are also observed in aged animals exposed to EE [290]. The cumulative effect results in attenuated cognitive dysfunction in animals due to aging compared to those in SE [291, 292]. Better survival of DG neurons is also seen in the former than the latter owing to the feature of enhanced neurogenesis in EE [293]. All three regions: hippocampal DG, entorhinal cortex and the visual cortex have increased dendritic spine density in 22-months old rat exposed to EE compared to those in SE [251]. Recent studies in rats also demonstrate that EE exposure in old age improves hippocampal synaptic plasticity by enhancing BDNF expression through acetylation – dependent mechanisms [294]. Such exposures to EE also aid in slower cessation of neurogenesis in aged subjects and therefore have strong potential in slowing down the eventual cognitive decline in this population [295].

2.5.3 EE: Potential therapeutic effects

EE also has potential therapeutic effects on neuronal insults and brain injuries. EE prevents seizure development and reduces apoptosis in hippocampal neurons [296]. It can potentially improve impairments caused due to hormonally induced distress of brain and behaviour. Neonatal hypothyroid rats are attributed by retarded development of nervous system and deficits in learning ability which is improved by exposure to EE [297]. Moreover, it renders neuroprotection in case of animals suffering from genetic defects. For example, EE causes a reversal of deficits in mutant mice suffering from NMDA receptor lesions in the CA1 hippocampal neurons [298]. Recent studies indicate that EE enhances motor recovery and helps in reducing depression consequent to stroke [299]. Several such studies indicate the potential of EE as a promising treatment for improving physiological as well as behavioural distress suffered by an individual.

3. Motivation and proposed hypothesis: Stress-resilient animal model for adult phase using EE

Earlier sections in this chapter emphasise on the maladaptive effects of chronic stress at different biological levels such as manifestation of anxiety and depressive-like symptoms, increased physiological levels of glucocorticoids, changes in structure and associated protein-expression in neurons of stress-sensitive regions like the BLA, hippocampus, and mPFC. These changes indicate the inability of the body to undergo homeostasis (attain equilibrium with the changes in the environment) effectively post stress-exposure. On the other hand, resilient individuals can be characterised by the ability to resist these stress-induced changes. In other words, stress-resilient individuals would tend to elicit non-anxious/non-depressed behaviour or rather demonstrate active coping, healthy physiological levels of hormones and changes in neuronal patterns which are adaptive in nature. These changes cumulatively would constitute an efficient mechanism for homeostasis in individuals post stress-exposure.

The impact of nurture and stressful experiences is most pronounced in the developing stage owing to the continuous neuronal plasticity underway during this phase [300-304]. However, the extent of plasticity in the brain drops gradually with increase in age [304]. Nevertheless, the impact of negative experiences such as chronic stress on the brain and its plasticity as discussed in this section is evident in adulthood. On the other hand, the use of a positive experience such as enrichment to induce resilience is not far-fetched. In fact, the positive effects of enrichment in different age groups tend to rescue from stress effects in adult rats. In most studies enrichment is used during developmental stages [305]. In animal models of maternal separation stress, enrichment is known to rescue effects of early life stress [261, 286]. One of the prime

reasons of its effectiveness over other drug-based systems to counter psychiatric pathologies is its non-invasive and intuitive nature.

At the same time, in order to maintain ethological relevance, it is also important to note that a naturalistic environment consists of both positive (rewarding) and negative (threatening) elements. With this in consideration, this study aimed at emulating this combination of negative and positive experiences in the immediate environment by housing chronically stressed (negative) animals in environmental enrichment (positive) for a brief duration during adulthood. ***The primary hypothesis of this study is that animals in adulthood would recover from the deleterious effects of chronic stress (exhibit stress-resilience) in a positive environment, such as a briefly long enrichment, which otherwise will persist in standard-cage housing.*** In other words, stressed individuals would exhibit maladaptive features compared to their non-stressed counterparts under standard-cage housing. However under EE housing, stressed individuals would exhibit either comparable or more adaptive features (trait of stress-resilience) relative to their non-stressed counterparts. In order to demonstrate this, chronic immobilization stress (CIS) was provided to subjects in two differential housing conditions: standard laboratory condition versus EE housing. Comparisons were planned orthogonally between non-stressed and stressed individuals in each housing type. The study hereafter employed a top-down approach to investigate the following basic goals: (based on which the entire study is divided):

Specific aim 1: To evaluate the effects of EE on stress-related behavioural outcomes: developing rodent model of stress-resilience: The previous sections mention the potentiation of anxiety through chronic immobilization stress in male rats. The aim of this study was to validate the sufficiency of short-term EE in relieving subjects from CIS-induced anxiety and other behavioural symptoms such as learned

helplessness and hyperactivity at resting state. The study also aimed at investigating the corticosterone profile (primary physiological readout) in response to EE in stressed animals.

Specific aim 2: *To investigate characteristic neuronal morphology associated with stress-resilience induced by EE:* Anxiety is associated with hyperactivity of BLA and neuronal hypertrophy in this region. The aim of this study was to investigate the morphological status of the BLA neurons in response to EE and to see if it protects the neurons from CIS-induced hypertrophy. At the same time, it needs to be noted that hippocampus and mPFC are involved in negative feedback to the HPA. Therefore the study also aimed at investigating the morphological status of stress-affected neurons (CA3 neurons in the hippocampus and PrL and IL neuron in the mPFC).

Specific aim 3: *To investigate the molecular regulation associated with stress-resilience induced by EE:* The literature reviewed in this text highlights the role of Glucocorticoids and their cognate receptors - Glucocorticoid receptors (GR), in regulating stress-responses and mediating stress-induced behavioural deficits such as anxiety and depression. Extensive information is available regarding the status of hippocampal GR in response to stress and EE. However, a comparable status of GR in the amygdala is currently undiscerning. Similarly, the status of BDNF and its cognate receptor - TrkB, were aimed to be investigated in the amygdala in response to short-term EE. These proteins are associated with neuronal plasticity pertaining to stress-sensitivity in brain. Previous literature in this text also highlights the role of inhibitory circuits within the BLA in mediating fear and anxiety. Thus the current study aimed to investigate GAD67: an inhibitory presynaptic marker protein as an index for the responsiveness of the BLA inhibitory neurons under the influence of CIS and short-

term EE. The study also aimed at investigating Synapsin (a generic synaptic marker), which serves as an index for synaptic activity in the BLA neurons.

MATERIALS AND METHODS

1. Animals

Male Wistar rats (7 weeks old, 220-250g, and housed 2/cage) were procured from National University of Singapore and habituated for a week in Nanyang Technological University vivarium. Animals were maintained in light-dark cycle of 12 h (light on 7AM) with *ad libitum* food and water. The institutional animal care and use committee (IACUC) of the NTU approved all experimental procedures.

2. Stress and Enrichment procedures

Stress paradigm consisted of chronic immobilization stress (CIS; 2 hours a day between 09:30-11:30 hours, 10 successive days; [72]). During stress, rats were immobilized in plastic restraint cones (Braintree Scientific, MA, USA). Unstressed animals remained unhandled in their home cage (37 x 22 x 18 cm³) during this period. Both stressed and unstressed animals were housed 2/cage.

The enriched environment consisted of larger cages (72 x 51 x 110 cm³ for 14 successive days) with more individuals per cage (4/cage) and presence of cognitively stimulating objects [306] for 24 hours every day till end of experiment. Enrichment included climbing walls made of wire-net, plastic tunnels, plastic and wooden objects of varied color and texture, ample nesting material, gustatory variety in form of fruit loops and sunflower seeds. There were layered tiers within the cage. Running wheel was not provided in the enriched housing. The arrangement of enrichment objects was changed every fourth day.

3. Experimental groups

After one week of acclimatization to the vivarium, rats were randomly assigned to four groups: CN (control, non-stressed and non-enriched), EE (environmentally enriched non-stressed), ST (non-enriched, stressed) and EEST (enriched and stressed). Number of animals (N) in each group is indicated in figure legends. A number of batches were raised sequentially (each with all the 4 groups of above mentioned treatments). Temporal relation between EE and stress protocols during testing and endpoints (behavioural, morphological and molecular readout) is depicted in inset for each individual figure in experimental results. All the samples were coded before analysis by colleagues. Experimenter was blind to treatment groups.

4. Elevated plus maze

Anxiety was measured using an elevated plus-maze (EPM, [307]). The EPM consisted of a plus-shaped arena with two open (75 x 11cm, 1cm wall, 3-4 lux illumination) and two enclosed arms (75 x 11 cm, 26 cm wall). The arena was elevated to 60 cm above the ground. Animal was placed at the center at start of the trial. Exploration in open and enclosed arms was quantified for duration of five minutes each. Open arm exploration (entries and occupancy time) relative to sum of open and enclosed arm exploration was used as index for the anxiety [307]. Entry in an arm was defined as presence of the whole body including head, four paws and at least base of the tail inside the open arm. Studies using factor analysis have showed that the number of closed-arm entries corresponds to influence of locomotion of rats on the maze [308]. Therefore number of closed-arm entries was also measured. In addition number of head dips was quantified. Head dip is defined as downward movement of head toward the floor, extending completely out of the open arm [307]. It has been a measure of

exploratory and goal-directed behaviour and its absence indicates passive behaviour [309-311]. Such a trait representing active-coping serves as a potential marker for stress-resilience. Trials were videotaped and coded before offline analysis.

5. Laboras™

The Laboratory Animal Behaviour Observation, Registration and Analysis System (LABORAS™, Metris, Netherlands) is a sensor-based system which detects basal readouts for locomotory and posture-related activities of an animal [312]. The animals were fasted overnight (19:00 hr -10:30 hr) prior to the test. The test was spread over 2 days (16 animals/day) in a room with standard lighting conditions (conducted between 10:30-13:30 hours). During the testing, the animals were individually placed into covered polycarbonate/Makrolon type IIIh cages ($37 \times 21 \times 24 \text{ cm}^3$) which were then immediately positioned onto the LABORAS™ sensor-platform. These cages resembled that of the standard housing cages which emulate non-stimulatory environment. Six rats were chosen randomly and tested simultaneously. The duration of test for each rat was 15 minutes. The readout in this system indicated the general activities of these animals at a basal (resting) level, which were quantified as immobility and locomotion within the cage. Such activities during this period indicated the ease of habituation of these subjects in a non-stimulatory (baseline) setting [313]. Food and water was not provided during testing. Data were automatically analyzed by LABORAS™ software for duration of locomotion and immobility (absence of any of the other behaviours) within the entire 15 minutes of testing for each animal.

6. Sucrose intake test

The animals were tested for anhedonia (depression) by measuring their sucrose intake [314]. The experiment was performed in standard laboratory cages (1 animal/cage). A day before the test, the animals were habituated for sucrose intake (1% sucrose) without any access to food or water for 1 hour. The animals were fasted overnight (19:00 hr -09:00 hr; 14 hours). The test was performed the next day for 1 hour (09:00-10:00 hr) in which each animal was provided sucrose *ad-libitum* without any access to food or water. Weights of the animals and the sucrose bottles were recorded pre- and post – test in order to calculate percentage change in sucrose per body weight. After the test, the animals were returned back to their respective home cages.

7. Porsolt's Forced swim test

The animals were tested for depressive-like symptoms (helplessness) using Porsolt forced swim task [315] . On the first day of the test, each animal was put in a water container (diameter = 20 cm, height = 50 cm) for 15 minutes for habituation. The temperature of the water was maintained at 21-23°C and was filled up to 33 cm of the container. On the second day of the test, each animal was tested for forced swim and video-taped for 10 minutes (conducted between 09:00-12:00 hours). After each test, the animal was returned to its home cage and the container was cleaned thoroughly with water. Experimenter was blind to treatments during offline analysis. Parameters such as swimming and immobility (indication of learned-helplessness behaviour) were timed and compared across groups.

8. Dendritic arborisation

Freshly harvested brains were obtained by sacrifice through decapitation. Blocks of brain tissue were processed for Golgi-Cox stain using commercial kit (FD Neurotechologies, Columbia, USA). After processing, 100 μ m thick coronal sections were mounted on glass slides. One batch of animals was used to obtain sections containing both BLA and hippocampus (Bregma -2.04 to -3.36, Figure M.1A [316]) and another one for sections containing mPFC (Bregma 3.72 to 3.00, Figure M.1B, The Rat Brain, Paxinos and Watson [316]). For mPFC, neurons from the prelimbic and infralimbic regions were analysed separately, therefore all sections in the second batch contained these regions. The slides were counter-stained with cresyl violet, dehydrated using an ascending series of alcohol and xylene, and coverslipped in non-aqueous medium (Permount, Thermofisher Scientific, USA).

M.1A)

Figure M.1A. Coronal brain section showing sampled BLA and Hippocampal region. Representative coronal brain section used for sampling neurons from the Basolateral amygdala (BLA) and the hippocampus. Image source: The Rat Brain, Paxinos and Watson [316].

M.1B)

Figure M.1B. Coronal brain section showing sampled PrL and IL region. Representative coronal brain section used for sampling neurons from the Prelimbic (PrL) and the Infralimbic (IL) regions. Image source: The Rat Brain, Paxinos and Watson [316].

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 specific neurons from each region were analysed for neuronal complexity and spine analysis (Table M.1). Traces were scanned (300 dpi, 8-bit grayscale 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 function of radial distance from the cell soma (Sholl analysis, Figure M.2) [72, 317]. This is represented as both total branch points (and segmental branch points for BLA at radial distance) in results.

M.2)

Figure M.2 Sholl analysis of a BLA neuron sample. Sholl analysis includes quantification of no. of branch points in each segment of the overlaying concentric circles (50μm radius) starting from the soma till the distal ends of the neuron.

Dendritic spines were counted using the 1000X magnification. Dendrites directly originating from cell soma were classified as primary dendrites, and those originating from primary dendrites were classified as secondary dendrites [120]. Starting from the origin of branch, and continuing away from cell soma, spines were counted along a 60-80μm stretch of dendrite depending upon the type of neurons (Table M.1). One dendrite of each kind (primary/secondary) per cell and 4-10 cells per animals for dendritic spines was analysed (Fig M.3). Since morphology and spine analysis were done independently but from the same slides, there is a good of an overlap among the cells that underwent analysis for both morphology and spine analysis.

M.3)

Fig M.3. Illustrative representation of neuronal spine analysis. Highlighted region indicates a stretch of primary dendrite (at 1000X magnification) selected from a sample pyramidal neuron for spine analysis.

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

Brain Region	Type of neurons	Bregma	Length of dendrite analysed 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

9. Corticosterone measurement, adrenal gland weight and Body weight gain

Tail vein blood samples were collected at day 1, 3 and 20 from start of the experiment (30 minutes to one hour after end of stress). Animals were lightly restrained in a soft cotton towel and 50-200 µl blood was drawn from tail nick at lateral vessels. This method is known to result in minimal stress [318]. Blood was centrifuged (4700 rpm, 15 minutes; Thermo Scientific Sorvall Legend XTR, Germany) and separated serum was stored at -80°C until quantification. Concentration of serum corticosterone was quantified using an enzyme-linked immunoassay based commercial kit (Enzo life sciences, NY, USA). In addition, adrenal glands were collected bilaterally using forceps (wiped dry and clean) during sacrifice and weighed. Body weights of all animals were recorded at the start and end of experiment. Percentage body-weight gain was compared across groups.

10. Protein expression studies in the Basolateral Amygdala

10.1 Tissue preparation for immunostaining

Rats were sacrificed through trans-cardial perfusion of phosphate buffered saline (PBS) followed by 4% paraformaldehyde dissolved in buffered saline. Harvested brains were post-fixated and equilibrated with 30% sucrose in PBS. These brains were cryosectioned in coronal planes at 20µm thickness and collected on superfrost slides (Thermofisher Scientific, USA) at -20°C. Representative sections from Bregma -2.76 to -2.92 were used from each animal and subjected to slide staining (The Rat Brain, Paxinos and Watson) for the following:

1. Study of colocalized expression of inhibitory presynaptic-marker proteins: Glutamate-decarboxylase 67 (GAD67, GABAergic presynaptic protein) with synapsin (generic synaptic-marker protein).
2. Study of activated glucocorticoid receptor (GR) expression: Colocalization of GR with DAPI for nuclear GR expression.

10.2 Immunofluorescence staining for inhibitory presynaptic protein markers

For all staining, sections mounted on superfrost slides were rinsed 3 times (7 mins/wash) in phosphate buffer saline (PBS) between each incubation step. For GAD67-synapsin double-immunostaining studies, the sections were incubated in the blocking solution, followed by overnight incubation of the sections at 4°C with 2 primary antibodies simultaneously—mouse anti-GAD67 antibody (1:10,000, obtained from Asst./Prof Albert Chen's lab, NTU, Singapore) and goat anti-Synapsin antibody (1:300, Santa Cruz, CA, USA). The secondary antibodies were DyLight®549 horse anti-mouse IgG (1:1000, Vector laboratories, CA, USA) and DyLight®488 donkey anti-goat IgG (1:1000, Abcam, CA, USA) in which the sections were incubated for 2 hours at room temperature. The sections were incubated with 4', 6-diamidino-2-phenylindole (DAPI, a nuclear counterstain for blue-fluorescence, Sigma-Aldrich, USA) for 1 minute. The sections were washed 7 times with PBS (5 mins/wash) to remove excess DAPI, dried and fixed in non-aqueous medium (Permount, Thermofisher Scientific, Belgium). Three randomly selected regions of interest (ROI) in each section were imaged bilaterally at 400X magnification using the confocal laser scanning microscope LSM 710 (Carl Zeiss, Oberkochen, Germany). Immunofluorescent images were obtained for a 4µm thick cross-section set as the ROI (zoomed to 2X) which was imaged into a z-stack of 5 slices (interval: 0.8µm). Each z-stack was imaged at a fixed area which was identical across all the ROIs in all sections

in this experiment using software (ZEN 2010 Light Edition, Carl Zeiss). Images were analysed using puncta analyzer 1.1 (custom-designed macros for Image J, NIH) which quantifies the number of puncta of the necessary signal in a given area (single or co-localized stains) for the corresponding protein of interest [319].

10.3 Immunofluorescence staining for nuclear GR

For nuclear GR-immunostaining studies, the sections were incubated in the blocking solution, followed by overnight incubation of the sections at 4°C with primary antibody- rabbit anti-GR antibody (1:500, Santa cruz, CA, USA). The secondary antibody was DyLight[®]549 goat anti-rabbit IgG (1:1000, Vector laboratories, CA, USA) in which the sections were incubated for 2 hours at room temperature. The sections were incubated with DAPI (Sigma-Aldrich, USA) for 1 minute. The sections were washed 7 times with PBS (5 mins/wash) to remove excess DAPI, dried and fixed in non-aqueous medium (PermOUNT, ThermoFisher Scientific, Belgium). Three randomly selected regions of interest (ROI) in each section were imaged bilaterally at 400X magnification using the confocal laser scanning microscope LSM 710 (Carl Zeiss, Oberkochen, Germany). Immunofluorescent images were obtained for a 20µm thick cross-section set as the ROI (zoomed to 2X) which was imaged into a z-stack of 5 slices (interval: 4µm). Each z-stack was imaged at a fixed area which was identical across all the ROIs in all sections in this experiment using software (ZEN 2010 Light Edition, Carl Zeiss). Images were analysed using puncta analyzer 1.1 (custom-designed macros for Image J, NIH) which quantifies the number of puncta of the necessary signal in a given area (single or co-localized stains) for the corresponding protein of interest [319].

10.4 Tissue preparation for Western Blotting

Freshly harvested brains were obtained by sacrifice through decapitation, which were snap-frozen using liquid nitrogen. Harvested brains were cryosectioned in coronal planes at 100 μ m thickness and the BLA was collected by bilateral punches using biopsy micropunch and Pasteur pipette tubes in sections from Bregma -2.04 to -3.36 (The Rat Brain, Paxinos and Watson). The tissue was homogenized using a glass homogenizer (Kontes, Thermofisher Scientific) in lysis buffer (150mM sodium chloride, 0.1% Triton-X 100, 50mM Tris, pH: 8.0) in order to solubilize total proteins and further sonicated in order to solubilize nuclear proteins. The supernatant was separated, agitated for 2 hours and further centrifuged (12000 rpm) for 20 minutes at 4°C. Protein lysates were stored in aliquots under -20°C until further use. Protein concentrations were estimated using Bradford protein assay (Bio-Rad, CA, USA) [320].

10.5 Western Blotting

Expression of proteins namely Glucocorticoid receptor (GR), Tyrosin-receptor Kinase B (TrkB), and Brain-derived neurotrophic factor (BDNF) was determined by Western blotting. Blots were first probed with specific primary antibody for TrkB and GAPDH (internal control) and they were stripped and re-probed for BDNF specific antibody (Table M.2). GR was run in separate set of blots with GAPDH as the internal control. 15 μ g of protein lysate was resolved in 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane using wet transfer unit (Bio-Rad laboratories, Richmond, CA). Blots were rinsed 3 times (10min/wash) in 1X TBST (20mM Tris Cl, pH 7.6, 150mM sodium chloride, 0.1% tween 20) between each incubation step. Blots were blocked at room temperature for 1 hour in 5% non-fat dry

milk in 1X TBST. They were incubated at 4°C for 14 hours (overnight) with primary antibody (diluted in TBST containing 5% non-fat dry milk) and the next day with secondary antibody conjugated to HRP (diluted in 5% non-fat dry milk in TBST) at room temperature for 2 hours. The bands were visualized using ECL select (G.E. healthcare, UK). Blots were developed using LAS4000 image reader version 2.02 (Fujifilm, Japan). The optical intensity of the protein bands were quantified using NIH ImageJ (version 1.50b; NIH, USA). Details of the reagents used in western blotting have been provided in the appendix.

Table M.2: Details of primary and secondary antibodies used in Western Blotting

Protein of interest	Molecular weight (kDa)	Primary antibody (dilution, manufacturer)	Secondary antibody (dilution, manufacturer)
Glucocorticoid receptor	90-95	Rabbit (1:500, Santa cruz)	Goat anti-rabbit (1:2000, Santa cruz)
TrkB	145	Mouse (1:500, BD biosciences)	Donkey anti-mouse (1:1000, Santa cruz)
pro-BDNF	32	Goat (1:1000, Santa cruz)	Donkey anti-goat (1:2000, Santa cruz)
GAPDH	37	Mouse (1:1000, Santa cruz)	Donkey anti-mouse (1:2000, Santa cruz)

11. Statistical analysis

All experimental data were analyzed using two-way analysis of variance (inter-subject source of variance = stress and EE). More importantly, since the aim of the experiment is to compare the non-stressed individuals to their stressed counterparts in the differential housing environments, planned comparisons were conducted for effects of stress in absence and presence of the EE (two orthogonal comparisons, independent sample t-test). The reason for including two-way ANOVA results is to show the main effects of EE-housing, which is relevant due to the use of two mutually exclusive treatments in this 2x2 design. This helps in understanding how EE-housing is overall affecting the non-stressed and stressed subjects. Non-orthogonal comparisons were not used in order to avoid spurious effects of multiple testing. The level of significance was set at $p < 0.05$ for all the statistical tests performed in this study. Figures represent mean and SEM (standard error of the mean). N is stated within the figure legends. This is also represented in Table S.1. Mean inter-group differences ($\Delta\bar{x} = \bar{x}_1 - \bar{x}_2$, where $\bar{x}_1$ is the mean of unstressed animals in absence or presence of stress and $\bar{x}_2$ is the mean of stressed animals in absence or presence of stress) and their SEM are also reported.

RESULTS

1. Effect of stress and EE on anxiety and risk assessment

As a measure of anxiety, open arm exploration in an elevated plus maze (EPM) was studied, where lower exploration indicates greater anxiety. Open arm exploration was calculated as the mean of % open arm entries and % open arm time (relative to sum of open and enclosed arm entries and time). Single anxiety-related endpoint in the form of open arm exploration was used to avoid the statistical problem of multiple testing and probable lack of sphericity in event of a repeated-measure analysis of variance (ANOVA) for entries and time simultaneously. According to a two-way ANOVA, both stress ($F_{(1,35)} = 4.99, p = 0.032$) and EE ($F_{(1,35)} = 5.814, p = 0.021$) had significant main effects on open arm exploration. However the interaction between stress and EE did not reach statistical significance ($F_{(1,35)} = 2.26, ns$). According to a two-way ANOVA, both stress ($F_{(1,35)} = 4.99, p = 0.032$) and EE ($F_{(1,35)} = 5.814, p = 0.021$) had significant main effects on closed arm exploration. However the interaction between stress and EE did not reach statistical significance ($F_{(1,35)} = 2.26, ns$). Stress increased closed arm exploration while EE decreased the same in EPM. Stress decreased open arm exploration while EE increased the same in EPM.

In order to analyze the effects of stress in absence or presence of the EE, orthogonal planned comparisons were conducted (independent sample t-test). In absence of EE, stress reduced open arm exploration (Figure 1A left; $\Delta\bar{x} = 20.6 \pm 9.2; t_{21} = 2.244, p = 0.036$). In contrast, stress was unable to reduce open arm exploration in presence of EE (Figure 1A right; $\Delta\bar{x} = 4.0 \pm 4.4; t_{14} = 0.9, ns$). Inversely, in absence of EE, stress increased closed arm exploration (Figure 1B left; $\Delta\bar{x} = -20.6 \pm 9.2; t_{21} = -2.244, p = 0.035$). In contrast, stress was unable to change closed arm exploration in presence of EE (Figure 1B right; $\Delta\bar{x} = -4.0 \pm 4.4; t_{14} = 0.9, ns$).

As stated above, % open arm exploration is the mean of % open arm entries and % open arm time. Two-way ANOVA revealed that both EE ($F_{(1,35)} = 12.94, p < 0.001$) and stress ($F_{(1,35)} = 6.09, p = 0.019$) had a main effect on % open arm entries. The interaction between stress and EE did not reach statistical significance ($F_{(1,35)} = 2.32, ns$). According to the same statistical analysis, neither stress ($F_{(1,35)} = 3.66, ns$) nor EE ($F_{(1,35)} = 1.61, ns$) had a main effect on % open arm time. The interaction between stress and EE also did not reach statistical significance ($F_{(1,35)} = 1.97, ns$).

According to orthogonal planned comparisons revealed significant reduction in % open arm entries in absence (Figure 1C left panel, left; $\Delta\bar{x} = 20.84 \pm 8.73; t_{21} = 2.39, p = 0.026$) but not in its presence (Figure 1C left panel, right; $\Delta\bar{x} = 4.93 \pm 4.08; t_{14} = 1.21, ns$) of EE. Orthogonal planned comparisons also revealed significant reduction in % open arm time in absence (Figure 1C right panel, left; $\Delta\bar{x} = 20.31 \pm 10.03; t_{21} = 2.03, p = 0.05$) but not in its presence (Figure 1C, right panel, right; $\Delta\bar{x} = 3.12 \pm 5.45; t_{14} = 0.57, ns$) of EE.

EE had no significant effect ($F_{(1,35)} = 4.0, ns$) on number of closed arm entries in EPM while stress had a significant effect ($F_{(1,35)} = 4.3, p = 0.047$) on it. No interaction ($F_{(1,35)} = 0.21, ns$) was observed between the two treatments on this parameter. Number of closed arm entries indicate the influence of locomotion of rats in the maze as indicated by factor analysis studies on EPM [308]. Number of entries in the closed arms was not significantly affected by stress either in absence (Figure 1D left; $\Delta\bar{x} = -1.43 \pm 1.33, t_{21} = -1.07, ns$) or presence of EE (Figure 1D right; $\Delta\bar{x} = -2.25 \pm 1.11, t_{14} = -2.031, ns$). This indicates the absence of any influence on general locomotion via independent sample t-test between non-stressed and stressed individuals in either housing conditions as tested on the EPM.

1A)

Figure 1A. Effect of stress on percentage open arm exploration in EPM in absence and presence of EE: Stress significantly reduced percentage open-arm exploration in absence of EE (left) but not in presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=17), Stress (N=6), EE (N=8) and EEST (N=8); * $p < 0.05$, independent sample t-test. Inset: Schedule of the experiment; D = day.

1B)

Figure 1B. Effect of stress on percentage closed arm exploration in EPM in absence and presence of EE: Stress significantly increased percentage closed-arm exploration in absence of EE (left) but not in presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=17), Stress (N=6), EE (N=8) and EEST (N=8); * $p < 0.05$, independent sample t-test. Schedule of the experiment same as Fig. 1A.

1C)

Figure 1C. Effect of stress on percentage open-arm entries and time in absence and presence of EE: Stress significantly decreased percentage open-arm entries in absence of EE

(left) but not in presence of EE (right) (**left panel**). Stress significantly decreased percentage open-arm time in absence of EE (left) but not in presence of EE (right) (**right panel**). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=17), Stress (N=6), EE (N=8) and EEST (N=8); * $p \leq 0.05$, independent sample t-test. Schedule of the experiment same as Fig. 1A.

1D)

Figure 1D. Effect of stress on closed arm entries in EPM in absence and presence of EE: Stress did not affect closed-arm entries in the absence (left) or presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=17), Stress (N=6), EE (N=8) and EEST (N=8). Schedule of the experiment same as Fig. 1A.

Risk assessment behaviour was measured using number of head-dips by each subject during the trial. Two-way ANOVA revealed significant main effects of stress ($F_{(1,35)} = 19.52$, $p < 0.001$) and EE ($F_{(1,35)} = 27.48$, $p < 0.001$) on risk assessment. The interaction between stress and EE did not reach statistical significance ($F_{(1,35)} = 0.52$, ns). Stress lowered risk assessment behaviour while EE had an opposite effect. Orthogonal planned comparisons revealed significant reduction in head dips by stress

in both absence (Figure 1E left; $\Delta\bar{x} = 4.2 \pm 1.38$; $t_{21} = 3.06$, $p = 0.006$) and presence (Figure 1E right; $\Delta\bar{x} = 5.9 \pm 1.9$; $t_{14} = 3.06$, $p = 0.009$) of EE.

1E)

Figure 1E. Effect of stress on number of head-dips in EPM in absence and presence of EE: Stress significantly reduced head-dips both in the absence (left) and presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=17), Stress (N=6), EE (N=8) and EEST (N=8); * $p < 0.05$, *** $p < 0.001$, independent sample t-test. Schedule of the experiment same as Fig. 1A.

2. Effect of stress and EE on hedonic responses

As an index for hedonic responses, sucrose intake by animals was measured through the sucrose-intake test (SIT). This was indicated by the difference in the sucrose (in the feed-bottle) per body weight of animal after testing-phase. Two-way ANOVA revealed that only EE ($F_{(1,26)} = 16.99$, $p < 0.001$) had a significant main effect on sucrose intake. However, the effect of stress ($F_{(1,26)} = 2.71$, ns) was found to be statistically insignificant. The interaction between stress and EE also, was significant

($F_{(1,26)} = 11.02$, $p = 0.003$). EE reduced the hedonic responses in animals compared to its non-EE counterparts.

Orthogonal planned comparisons revealed that in absence of EE, stress reduced sucrose intake (Figure 2 left, $\Delta\bar{x} = 1.71 \pm 0.46$; $t_{14} = 3.71$, $p = 0.002$). In contrast, stress was unable to reduce sucrose intake in presence of EE (Figure 2 right, $\Delta\bar{x} = 0.85 \pm 0.43$; $t_{14} = 1.98$, ns). This indicated that in the presence of EE, stress was unable to modulate hedonic responses in these animals.

2)

Figure 2. Effect of stress on sucrose intake in absence and presence of EE: Stress significantly reduced the percentage Δ sucrose intake per body weight in the absence (left) of EE but not in the presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=8), EE (N=8) and EEST (N=6); ** $p < 0.01$, independent sample t-test. Inset: Schedule of the experiment; D = day.

3. Effect of stress and EE on depressive-like behaviour (learned-helplessness)

As an index for learned helplessness (depressive-like symptoms), immobility (time in seconds) in Porsolt's forced swim task (FST) was measured. Two-way ANOVA revealed that only EE ($F_{(1,26)} = 4.60$, $p = 0.042$) had a significant main effect on immobility in FST. The effect of stress ($F_{(1,26)} = 1.50$, *ns*) was found to be statistically insignificant. The interaction ($F_{(1,26)} = 0.091$, *ns*) between stress and EE also failed to reach statistical significance. EE reduced depressive-like symptoms in animals compared to their non-EE counterparts.

Orthogonal planned comparisons revealed that immobility in FST was not significantly affected by stress either in absence (Figure 3A left; $\Delta\bar{x} = -66.25 \pm 55.48$, $t_{14} = -1.19$, *ns*) or presence of EE (Figure 3A right; $\Delta\bar{x} = -40.0 \pm 67.95$, $t_{12} = -0.59$, *ns*).

Another measure to indicate despair behaviour, latency to first immobility in FST was timed. According to a two-way ANOVA, both stress ($F_{(1,26)} = 1.67$, *ns*) and EE ($F_{(1,26)} = 1.17$, *ns*) had no significant main effect on the latency to first immobility in the FST. The interaction between stress and EE also did not reach statistical significance ($F_{(1,26)} = 1.64$, *ns*).

Orthogonal planned comparisons showed that stress did not alter the latency to first immobility in FST both in the absence of EE (Figure 3B left; $\Delta\bar{x} = 44.45 \pm 27.09$; $t_{14} = 1.63$, *ns*) and in its presence (Figure 3B right; $\Delta\bar{x} = 0.17 \pm 19.40$; $t_{12} = 0.009$, *ns*).

Positive stress-coping behaviour such as swimming (time in seconds) was also measured in FST. Two-way ANOVA revealed that only stress ($F_{(1,26)} = 5.033$, $p = 0.034$) had a significant main effect on swimming in FST. The effect of EE ($F_{(1,26)} =$

1.16, *ns*) was found to be statistically insignificant. The interaction between stress and EE also failed to reach statistical significance ($F_{(1,26)} = 0.02$, *ns*). Stress reduced coping in the form of swimming in animals compared to their non-stressed counterparts.

Orthogonal planned comparisons revealed that swimming in FST was not significantly affected by stress either in absence (Figure 3C left; $\Delta\bar{x} = 32 \pm 17.43$, $t_{14} = 1.84$, *ns*) or presence of EE (Figure 3C right; $\Delta\bar{x} = 28.63 \pm 20.93$, $t_{12} = 1.37$, *ns*).

3A)

Figure 3A. Effect of stress on immobility in forced-swim test in absence and presence of EE: EE significantly decreased immobility in animals in FST. Stress did not significantly affect immobility in FST in the absence (left) or presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=8), EE (N=8) and EEST (N=6). Inset: Schedule of the experiment; D = day.

3B)

Figure 3B. Effect of stress on latency to first immobility in FST in absence and presence of EE: Stress did not significantly affect the latency to first immobility in FST in the absence (left) or presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=8), EE (N=8) and EEST (N=6). Schedule of the experiment same as Fig. 3A.

3C)

Figure 3C. Effect of stress on swimming in forced-swim test in absence and presence of EE: Stress did not significantly affect swimming in FST in the absence (left) or presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=8), EE (N=8) and EEST (N=6). Schedule of the experiment same as Fig. 3A.

4. Effect of stress and EE on basal activities

Basal activities such as immobility (time in seconds) and locomotion (time in seconds) at resting levels (in a non-stimulatory environment) were measured using LABORAS™. Two-way ANOVA revealed that only EE ($F_{(1,28)} = 5.37, p = 0.028$) had a significant main effect on immobility in LABORAS. The effect of stress ($F_{(1,28)} = 0.58, ns$) was found to be statistically insignificant. The interaction between stress and EE also failed to reach statistical significance ($F_{(1,28)} = 0.51, ns$). EE increased immobility in animals during basal conditions as compared to their non-EE counterparts.

Orthogonal planned comparisons revealed that immobility at basal conditions was not significantly affected by stress either in absence (Figure 4A left; $\Delta\bar{x} = 1.00 \pm 14.57; t_{14} = 0.07, ns$) or presence of EE (Figure 4A right; $\Delta\bar{x} = 29.38 \pm 37.00; t_{14} = 0.79, ns$).

Along with immobility, locomotion in these animals was also measured. Two-way ANOVA revealed that both stress ($F_{(1,28)} = 4.76, p = 0.038$) and EE ($F_{(1,28)} = 5.69, p = 0.024$) had significant main effects on locomotion at basal levels. The interaction between stress and EE failed to reach statistical significance ($F_{(1,28)} = 1.5, ns$). Stress increased locomotion (indicating hyperactivity) at resting levels while EE reduced this.

Orthogonal planned comparisons revealed that in absence of EE, stress increased locomotion (Figure 4B left, $\Delta\bar{x} = -20.9 \pm 8.7; t_{14} = -2.39, p = 0.031$). In contrast, stress was unable to increase locomotion in presence of EE (Figure 4B right, $\Delta\bar{x} = -5.88 \pm 8.6; t_{14} = -0.68, ns$). This indicates that in the presence of EE, stress does not lead to hyperactivity.

Immobility measures via marginal means of each treatment group (non-stressed, stressed, non-EE and EE) in two different conditions such as non-stressful (tested in LABORAS) and stressful (tested in FST) were compared. Animals devoid of EE (red dot, Fig 4C) exhibited maximum immobility while the EE-housed animals (grey dot, Fig 4C) exhibited minimum immobility compared in a stressful situation (as showed in FST). Conversely, animals devoid of EE (red dot, Fig 4C) exhibited minimum immobility while the EE-housed animals (grey dot, Fig 4C) exhibited maximum immobility in a non-stressful situation (as showed in LABORAS).

4A)

Figure 4A. Effect of stress on immobility in LABORAS™ test in absence and presence of EE: EE significantly increased basal immobility in animals. Stress did not significantly affect immobility in at resting phase in the absence (left) or presence of EE (right). Graphs represent Mean ± SEM. Bars represent Control (N=8), Stress (N=8), EE (N=8) and EEST (N=8). Inset: Schedule of the experiment; D = day.

4B)

Figure 4B. Effect of stress on locomotion in LABORAS™ test in absence and presence of EE: EE significantly basal decreased locomotion in animals. Stress significantly increased locomotion at resting phase in the absence (left) of EE but not in the presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=8), EE (N=8) and EEST (N=8); * $p < 0.05$, independent sample t-test. Schedule of the experiment same as Fig 4A.

4C)

Figure 4C. Marginal-mean comparison of immobility in stressful setting vs. resting phase: Scatter gram comparing marginal means for immobility (LABORAS vs. FST) for all individuals from 4 types of treatment: No EE (red), Stress (blue), No stress (green) and EE (grey). Individuals show maximum immobility in FST (Y axis) and Laboras (X axis) in absence and presence of EE respectively.

5. Effect of stress and EE on dendritic complexity of BLA neurons

Morphological features of relevant brain regions such as BLA, hippocampus and medial prefrontal cortex were investigated. Dendritic complexity of neurons in the form of branching and dendritic length along with spine density was quantified. Number of branch points and dendritic length of BLA neurons (Figure M.1A) were analyzed to study neuronal arbors in the BLA. Two-way ANOVA revealed significant main effects of both stress ($F_{(1,283)} = 6.66, p = 0.01$) and EE ($F_{(1,283)} = 5.64, p = 0.018$) for total number of branch points. A significant interaction between stress and EE ($F_{(1,283)} = 4.48, p = 0.035$) was also observed. Stress caused number of branch points in the BLA neurons to increase while EE decreased the same.

Orthogonal planned comparisons (independent sample t-test) revealed that in the absence of EE, stress increased number of branches of BLA neurons (Figure 5A left; $\Delta\bar{x} = -2.0 \pm 0.67$; $t_{145} = -3.02, p = 0.003$). The stress-induced increase in the branch points was absent when animals were housed in EE (Figure 5A right; $\Delta\bar{x} = -2.0 \pm 0.53$; $t_{138} = -0.37, ns$).

Segmental analysis of number of branch points in the BLA neurons was obtained by Sholl analysis (Figure M.2). Two way ANOVA on segmental-analysis of branch points showed that EE (Figure 5A, Inset: $F_{(1,283)} = 4.67, p = 0.031$) but not stress ($F_{(1,283)} = 0.56, ns$), significantly reduced the number of branch points at a radial distance of 0-100 μ m. On the other hand, stress (Figure 5A, Inset: $F_{(1,283)} = 14.70, p < 0.001$) but not EE ($F_{(1,283)} = 0.75, ns$), increased this at a radial distance between 100 μ m - 200 μ m from the soma. At a radial distance of 0-100 μ m, EE and stress showed significant interaction ($F_{(1,283)} = 7.52, p = 0.006$).

Orthogonal planned comparisons showed that the number of branch points in the radial stretch from 0-100µm was increased by stress in absence of EE (Figure 5A inset, $\Delta\bar{x} = -1.2 \pm 0.51$; $t_{145} = -2.34$, $p = 0.021$) but not in its presence (Figure 5A inset; $\Delta\bar{x} = 0.69 \pm 0.45$, $t_{138} = 1.51$, *ns*). On the other hand, in radial stretch from 100-200µm, there was a stress-induced increase both in absence of EE (Figure 5A inset; $\Delta\bar{x} = -0.52 \pm 0.26$, $t_{145} = -1.97$, $p = 0.05$) and presence of EE (Figure 5A inset; $\Delta\bar{x} = -0.79 \pm 0.21$, $t_{138} = -3.68$, $p < 0.001$).

Both stress ($F_{(1,283)} = 43.87$, $p < 0.001$) and EE ($F_{(1,283)} = 15.41$, $p < 0.001$) treatments resulted in significant main effects of total dendritic length in ANOVA. The interaction between stress and EE did not reach statistical significance ($F_{(1,283)} = 0.798$, *ns*). Stress and EE both resulted in a significant increase in the dendritic length of the BLA neurons. Orthogonal planned comparison showed that stress increased dendritic length of BLA neurons in both absence (Figure 5B left, $\Delta\bar{x} = -298.16 \pm 76.1$; $t_{145} = -3.91$, $p < 0.001$) and presence of the EE (Figure 5B right, $\Delta\bar{x} = -391.15 \pm 70.6$; $t_{138} = -5.54$, $p < 0.001$).

5A)

Figure 5A. Effect of stress on number of branch points in the BLA neurons in absence and presence of EE: Stress significantly increased the number of branch points in the BLA

neurons in the absence (left) but not in the presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=7), Stress (N=7), EE (N=7) and EEST (N=8), 8-10 neurons/animal, $**p < 0.01$, independent sample t-test. Inset (top): Schedule of the experiment (D = day), Inset (bottom): Segmental-analysis for number of branch points in the BLA neurons through Sholl analysis. Stress increases the number of branch points in the radial distance of 0-100 μ m in absence of EE (black square vs. red circle) but not its presence. Stress increases the same in the radial distance of 100-200 μ m both in absence (black square vs. red circle) and presence (blue upright triangle vs. pink inverted triangle) of EE. Graphs represent Mean $\pm$ SEM plotted against no. of branch points (ordinate) and radial distance from the soma (abscissa). Dots represent control (N=7, black squares), stress (N=7, red circles), EE (N=7, blue upright triangles) and EEST (N=8, pink inverted triangles), 8-10 neurons/animal, $*p < 0.05$, $***p < 0.001$, independent sample t-test.

5B)

Figure 5B. Effect of stress on total dendritic length in the BLA neurons in absence and presence of EE: Stress significantly increased the total dendritic length in the BLA neurons both in the absence (left) and presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=7), Stress (N=7), EE (N=7) and EEST (N=8), 8-10 neurons/animal, *** $p < 0.001$, independent sample t-test. Schedule of the experiment same as Fig. 5A. Inset (bottom): Representative images of sample Golgi-stained neurons in the BLA for the 4 groups from optical microscope at 400X magnification. Scale bar represents 20 μ m.

6. Effect of stress and EE on spine density of BLA neurons

Spines were analysed in the BLA neurons for a stretch of 60µm of both primary and secondary dendrites. Two-way ANOVA revealed significant main effect of EE ($F_{(1,165)} = 3.92$, $p = 0.049$) but not stress ($F_{(1,165)} = 2.11$, *ns*) on spine density in primary dendrites of BLA neurons. No significant interaction between stress and EE ($F_{(1,165)} = 3.32$, *ns*) was observed. EE resulted in decrease in spine density of primary dendrites in the BLA neurons. Orthogonal planned comparisons revealed that spine density was not significantly affected by stress in absence (Figure 6 left panel, left; $\Delta\bar{x} = -0.53 \pm 2.03$; $t_{94} = -0.26$, *ns*) of EE, but it led to a significant decrease in the same in presence (Figure 6 left panel, right; $\Delta\bar{x} = 4.75 \pm 1.99$; $t_{71} = 2.38$, $p = 0.02$) of EE.

Two-way ANOVA revealed significant main effect of both EE ($F_{(1,165)} = 3.80$, $p = 0.05$) and stress ($F_{(1,165)} = 6.09$, $p = 0.015$) on spine density in secondary dendrites of BLA neurons. No significant interaction between stress and EE ($F_{(1,165)} = 2.88$, *ns*) was observed. Both EE and stress resulted in decrease in spine density of secondary dendrites in the BLA neurons. Orthogonal planned comparisons revealed that spine density was not significantly affected by stress in absence of EE (Figure 6 right panel, left; $\Delta\bar{x} = 1.21 \pm 1.97$; $t_{94} = 0.61$, *ns*) of EE, however it led to a significant decrease in the same in presence (Figure 6 right panel, right; $\Delta\bar{x} = 6.55 \pm 2.51$; $t_{71} = 2.61$, $p = 0.011$) of EE.

6)

(Inset 1: primary dendritic spines)

(Inset 2: secondary dendritic spines)

Figure 6. Effect of stress on total dendritic spines (in 60μm) of the BLA neurons in absence and presence of EE: Stress significantly reduced the spine density in both primary (**left panel**) and secondary dendrites (**right panel**) of BLA neurons in the presence (right, grey bars) of EE but not in its absence (left, uncoloured bars). Graphs represent Mean ± SEM. Both left and right panel: Bars represent Control (N=6), Stress (N=4), EE (N=4) and EEST (N=4), 4-10 neurons/animal, * $p < 0.05$, independent sample t-test. Schedule of the experiment same as Fig. 5A. Insets: Inset 1: Images of Golgi-stained primary dendritic spines in BLA of the 4 groups from optical microscope at 1000X. Inset 2: Images of Golgi-stained secondary dendritic spines in BLA of the 4 groups from optical microscope at 1000X magnification. Scale bar represents 20 μm.

7. Effect of stress and EE on dendritic complexity of hippocampal CA3 neurons

Number of branch points and dendritic length of hippocampal neurons (Figure M.1A) were analyzed to study neuronal arbors in the hippocampus. Two-way ANOVA revealed significant main effect of stress ($F_{(1,141)} = 13.97, p < 0.001$) but not EE ($F_{(1,141)} = 0.73, ns$) on total number of branch points. Significant interaction between stress and EE ($F_{(1,141)} = 1.18, ns$) was not observed. Stress caused number of branch point in the hippocampal CA3 neurons to increase. Orthogonal planned comparisons revealed that total number of branch points in hippocampal CA3 neurons was not significantly affected by stress in absence (Figure 7A left; $\Delta\bar{x} = -2.74 \pm 1.79$; $t_{65} = -1.52, ns$) but was increased in presence (Figure 7A right; $\Delta\bar{x} = -4.98 \pm 1.12$; $t_{76} = -4.43, p < 0.001$) of EE.

Two-way ANOVA showed that stress ($F_{(1,141)} = 18.44, p < 0.001$) but not EE ($F_{(1,141)} = 2.29, ns$) had a significant main effect on total dendritic length of CA3 hippocampal neurons. The interaction between stress and EE was also statistically significant ($F_{(1,141)} = 7.33, p = 0.008$). Stress increased the total dendritic length of the hippocampal CA3 neurons.

Orthogonal planned comparisons revealed that total dendritic length of hippocampal CA3 neurons was not significantly affected by stress in absence (Figure 7B left; $\Delta\bar{x} = -240.33 \pm 222.76$; $t_{65} = -1.08, ns$) but was increased in presence (Figure 7B right; $\Delta\bar{x} = -1060.65 \pm 206.16$; $t_{76} = -5.15, p < 0.001$) of EE.

7A)

Figure 7A. Effect of stress on total number of branch points in the hippocampal CA3 neurons in absence and presence of EE: Stress significantly increased the number of branch points in the hippocampal CA3 neurons in the presence (right) but not in the absence of EE (left). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=5), Stress (N=4), EE (N=7) and EEST (N=4), 4-10 neurons/animal, *** $p < 0.001$, independent sample t-test. Schedule of the experiment same as Fig 5A.

7B)

Figure 7B. Effect of stress on total dendritic length of the hippocampal CA3 neurons in absence and presence of EE: Stress significantly increased the total dendritic length of the hippocampal CA3 neurons in the presence (right) but not in the absence of EE (left). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=5), Stress (N=4), EE (N=7) and EEST (N=4), 4-10 neurons/animal, *** $p < 0.001$, independent sample t-test. Schedule of the experiment same as Fig 5A. Inset (bottom): Representative images of sample Golgi-stained CA3 neurons in the hippocampus for the 4 groups from optical microscope at 100X magnification. Scale bar represents 20 μ m.

8. Effect of stress and EE on spine density of hippocampal CA3 neurons

Spines were analysed in the hippocampal CA3 neurons for a stretch of 60 μ m in primary dendrites. Two-way ANOVA revealed significant main effects of both stress ($F_{(1,176)} = 7.1, p = 0.008$) and EE ($F_{(1,176)} = 8.2, p = 0.005$) on spine density in primary dendrites of CA3 neurons. A significant interaction between the two treatments ($F_{(1,176)} = 43.3, p < 0.001$) was also observed. Stress resulted in reduction of spine density in the primary dendrites of the CA3 neurons while EE had a reverse effect. Orthogonal planned comparisons revealed that spine density was significantly reduced by stress in

absence (Figure 8 left; $\Delta\bar{x} = 8.8 \pm 1.3$; $t_{97} = 6.83$, $p < 0.001$) of EE, however it led to a significant increase in the same in presence (Figure 8 right; $\Delta\bar{x} = -3.73 \pm 1.4$; $t_{79} = -2.67$, $p = 0.009$) of EE.

8)

(Inset: primary dendritic spines)

Figure 8. Effect of stress on total dendritic spines (in 60μm) of the hippocampal CA3 neurons in absence and presence of EE: Stress significantly reduced the spine density in primary dendrites of the hippocampal CA3 neurons in absence of EE (left) but significantly increased the same in presence of EE (right). Graphs represent Mean ± SEM. Bars represent Control (N=5), Stress (N=5), EE (N=5) and EEST (N=4), 5-15 neurons/animal, ** $p < 0.01$, *** $p < 0.001$, independent sample t-test. Schedule of the experiment same as Fig. 5A. Inset: Images of Golgi-stained primary dendritic spines in CA3 hippocampal neurons of the 4 groups from optical microscope at 1000X magnification. Scale bar represents 20 μm.

9. Effect of stress and EE on dendritic complexity of mPFC neurons: PrL and IL

The mPFC is divided into two functionally distinct sub-regions: the Prelimbic (PrL) and the infralimbic (IL) regions. The neurons from each sub-region (Figure M.1B) were analysed for their neuronal arborization patterns in response to stress and EE. In the PrL neurons (these neurons were traced by Lim Wee Han, NTU undergraduate student, under my mentorship), two-way ANOVA revealed significant main effect of only EE ($F_{(1,174)} = 12.73, p < 0.001$) but not stress ($F_{(1,174)} = 0.93, ns$) on total number of branch points. No significant interaction between stress and EE ($F_{(1,174)} = 0.53, ns$) was observed. EE caused number of branch points to increase in the pyramidal PrL neurons irrespective of stress. Orthogonal planned comparisons revealed that total number of branch points was not significantly affected by stress either in absence (Figure 9A left panel, left; $\Delta\bar{x} = -0.23 \pm 1.28; t_{98} = -0.18, ns$) or presence (Figure 9A left panel, right; $\Delta\bar{x} = -1.71 \pm 1.60; t_{76} = -1.07, ns$) of EE.

Both stress ($F_{(1,169)} = 4.07, p = 0.045$) and EE ($F_{(1,169)} = 51.51, p < 0.001$) treatments resulted in significant main effects of total dendritic length during two-way ANOVA. The interaction between stress and EE did not reach statistical significance ($F_{(1,169)} = 0.01, ns$). Both Stress and EE resulted in increase in total dendritic length of pyramidal PrL neurons. Orthogonal planned comparisons revealed that total dendritic length was not significantly affected by stress either in absence (Figure 9A right panel, left; $\Delta\bar{x} = -223.51 \pm 180.32; t_{73} = -1.24, ns$) or presence (Figure 9A right panel, right; $\Delta\bar{x} = -200.95 \pm 121.68; t_{96} = -1.65, ns$) of EE.

In the IL neurons (these neurons were traced by Nicholas Han, NTU undergraduate student, under my mentorship), two-way ANOVA revealed no significant main effect

of either EE ($F_{(1,138)} = 2.91$, *ns*) or stress ($F_{(1,138)} = 2.67$, *ns*) on total number of branch points. No significant interaction between stress and EE ($F_{(1,138)} = 2.72$, *ns*) was observed as well. Neither EE nor stress affected the number of branch points in the pyramidal IL neurons. Orthogonal planned comparisons revealed that total number of branch points increased significantly following stress in absence (Figure 9B left panel, left; $\Delta\bar{x} = -1.34 \pm 0.59$; $t_{77} = -2.26$, $p = 0.03$) of EE but not in its presence (Figure 9B left panel, right; $\Delta\bar{x} = 0.006 \pm 0.52$; $t_{61} = 0.01$, *ns*).

Neither EE ($F_{(1,138)} = 0.11$, *ns*) nor stress ($F_{(1,138)} = 1.15$, *ns*) had a significant main effect on total dendritic length according to two-way ANOVA. There was no significant interaction between the two treatments ($F_{(1,138)} = 0.12$, *ns*) as well. Orthogonal planned comparisons revealed that total dendritic length was not affected by stress in absence (Figure 9B right panel, left; $\Delta\bar{x} = -60.89 \pm 58.20$; $t_{77} = -1.05$, *ns*) or presence (Figure 9B right panel, right; $\Delta\bar{x} = -30.94 \pm 61.65$; $t_{61} = -0.50$, *ns*) of EE.

9A)

Figure 9A. Effect of stress on dendritic complexity of the prelimbic neurons in absence and presence of EE: EE significantly increased the total number of branch points (**left panel**) and dendritic length (**right panel**) in PrL neurons. Stress had no effect on the number of branch points (**left panel**) or total dendritic length (**right panel**) of the PrL neurons both in the presence (right, grey bars) and absence (left, uncoloured bars) of EE. Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=8), EE (N=7) and EEST (N=6), 4-7 neurons/animal, Inset (top): Schedule of the experiment. Inset (bottom): Representative images of sample Golgi-stained PrL neurons in the hippocampus for the 4 groups from optical microscope at 100X magnification. Scale bar represents 20 μ m.

9B)

Figure 9B. Effect of stress on dendritic complexity of the infralimbic neurons in absence and presence of EE: Stress significantly increased the number of branch points of the IL neurons (**left panel**) in the absence (left, uncoloured bars) but not in the presence (right, grey bars) of EE while stress had no effect on the total dendritic length (**right panel**) of the IL neurons both in absence (left, uncoloured bars) and presence (right, grey bars) of EE. Graphs represent Mean $\pm$ SEM. Bars represent Control (N=6), Stress (N=6), EE (N=6) and EEST (N=6), 4-8 neurons/animal, $*p < 0.05$, independent sample t-test. Schedule of the experiment same as 9A. Inset (bottom): Representative images of sample Golgi-stained IL neurons in the hippocampus for the 4 groups from optical microscope at 100X magnification. Scale bar represents 20 μ m.

10. Effect of stress and EE on spine density of mPFC neurons: PrL and IL

Spines were counted in the PrL neurons of mPFC for a stretch of 80 μ m (since they are comparatively longer) of both primary and secondary dendrites. Two-way ANOVA revealed significant main effect of EE ($F_{(1,232)} = 179.91$, $p < 0.001$) but not stress ($F_{(1,232)} = 0.73$, *ns*) on spine density in primary dendrites of PrL neurons. No significant interaction between stress and EE ($F_{(1,232)} = 2.61$, *ns*) was observed. EE resulted in an increase in spine density of primary dendrites in the PrL neurons irrespective of stress. Orthogonal planned comparisons revealed that spine density was not significantly affected by stress either in absence (Figure 10A left panel, left; $\Delta\bar{x} = -1.35 \pm 1.71$; $t_{158} = -0.79$, *ns*) or presence (Figure 10A left panel, right; $\Delta\bar{x} = 4.39 \pm 3.7$; $t_{74} = 1.19$, *ns*) of EE.

Two-way ANOVA revealed significant main effects of both EE ($F_{(1,167)} = 151.75$, $p < 0.001$) and stress ($F_{(1,167)} = 5.21$, $p = 0.024$) on spine density in secondary dendrites of PrL neurons. A significant interaction between the two treatments ($F_{(1,167)} = 5.8$, $p = 0.017$) was also observed. Stress resulted in a decrease in the spine density of the secondary dendrites in the PrL neurons while EE had a reverse effect. Orthogonal planned comparisons revealed that spine density was not significantly affected by stress in absence (Figure 10A right panel, left; $\Delta\bar{x} = -0.23 \pm 2.03$; $t_{111} = -0.11$, *ns*) of EE, however it led to a significant decrease in the same in presence (Figure 10A right panel, right; $\Delta\bar{x} = 8.33 \pm 2.98$; $t_{56} = 2.8$, $p = 0.007$) of EE. Stress therefore affected the spines located in secondary dendrite of PrL neurons in presence of EE.

10A)

Figure 10A. Effect of stress on total dendritic spines (in 80μm) of the prelimbic neurons in absence and presence of EE: EE significantly increased the total number of spines in both primary (**left panel**) and secondary (**right panel**) dendrites in PrL neurons. Stress had no effect on the spine density in primary dendrites (**left panel**) of the PrL neurons both in absence and presence of EE. But stress decreased the spine density in secondary dendrites (**right panel**) of the PrL neurons in the presence (right, grey bars) but not in the absence of EE (left, uncoloured bars). Graphs represent Mean $\pm$ SEM. Both left and right panel: Bars represent Control (N=8), Stress (N=8), EE (N=4) and EEST (N=5), primary: 5-10 neurons/animal, secondary: 3-9 neurons/animal, $**p < 0.01$, independent sample t-test. Schedule of the experiment same as Fig. 9A. Insets: Inset 1: Images of Golgi-stained primary dendritic spines in PrL neurons of the 4 groups from optical microscope at 1000X magnification. Inset 2:

Images of Golgi-stained secondary dendritic spines in PrL neurons of the 4 groups from optical microscope at 100X. Scale bar represents 20 μm .

Spines were analysed in the IL neurons of mPFC for a stretch of 60 μm of both primary and secondary dendrites. Two-way ANOVA revealed significant main effect of both stress ($F_{(1,167)} = 130.12, p < 0.001$) and EE ($F_{(1,167)} = 268.24, p < 0.001$) on spine density in secondary dendrites of IL neurons. A significant interaction between the two treatments ($F_{(1,167)} = 4.90, p = 0.028$) was also observed. Stress resulted in significant decrease in spine density in secondary dendrites of the IL neurons while EE showed a distinctive opposite effect. Orthogonal planned comparisons revealed that spine density was significantly reduced by stress both in absence (Figure 10B.1 left; $\Delta\bar{x} = 16.56 \pm 1.14; t_{94} = 14.59, p < 0.001$) and presence (Figure 10B.1 right; $\Delta\bar{x} = 11.18 \pm 2.33; t_{73} = 4.79, p < 0.001$) of EE.

Two-way ANOVA revealed significant main effect of both stress ($F_{(1,167)} = 59.73, p < 0.001$) and EE ($F_{(1,167)} = 454.82, p < 0.001$) on spine density in secondary dendrites of IL neurons. A significant interaction between the two treatments ($F_{(1,167)} = 37.23, p < 0.001$) was also observed. Stress resulted in a decrease in the spine density of the secondary dendrites in the IL neurons while EE prevented this effect. Orthogonal planned comparisons revealed that spine density was significantly reduced by stress in absence (Figure 10B.2 left; $\Delta\bar{x} = 15.3 \pm 1.23; t_{94} = 12.42, p < 0.001$) of EE, but was unaffected by stress in presence (Figure 10B.2 right; $\Delta\bar{x} = 1.8 \pm 1.99; t_{73} = 0.93, ns$) of EE.

10B)

Figure 10B. Effect of stress on total dendritic spines (in 60μm) of the infralimbic neurons in absence and presence of EE: EE significantly increased the total number of spines in both primary (**left panel**) and secondary (**right panel**) dendrites of IL neurons. Stress reduced spine density in the primary dendrites (**left panel**) of IL neurons both in the absence (left, uncoloured bars) and presence (right, grey bars) of EE. Stress decreased the same in the secondary dendrites (**right panel**) of the IL neurons in the absence (left, uncoloured bars) but not in the presence (right, grey bars) of EE. Graphs represent Mean ± SEM. Both left and right panel: Bars represent Control (N=6), Stress (N=8), EE (N=6) and EEST (N=5), 6-8 neurons/animal, *** $p < 0.001$, independent sample t-test. Schedule of the experiment same as Fig. 9A. Insets: Inset 1: Images of Golgi-stained primary dendritic spines in IL neurons of the 4 groups from optical microscope at 1000X magnification. Inset 2: Images of Golgi-stained

secondary dendritic spines in IL neurons of the 4 groups from optical microscope at 100X. Scale bar represents 20 μm .

11. Effect of stress and EE on corticosterone and adrenal weights

On Day 1 (start of the experiment), 3 and 20 (end of the experiment), serum corticosterone (CORT) was measured in ng/ml (Figure 11). In order to circumvent statistical limitations involved with lack of sphericity in a repeated measure ANOVA, area under the curve (AUC) for the three time points was calculated and subsequently used for statistical analysis. The CORT readouts for three time points: Day 1, Day 3 and Day 20 are repeated-measure samples across the experimental timeline (from start of the experiment to sacrifice of the rats) Hence, area under the curve (sum of the integral of CORT concentration vs. time curve obtained from day 1 to day 3 and the integral of CORT concentration vs. time curve obtained from day 3 to day 20 for each animal) was calculated. A significant main effect of EE ($F_{(1,23)} = 13.29$, $p = 0.001$) but not stress ($F_{(1,23)} = 0.97$, ns) was observed on serum corticosterone concentration (two-way ANOVA). The interaction between EE and stress was also not significant ($F_{(1,23)} = 2.89$, ns). Orthogonal planned comparisons showed that stress had no significant effect on serum corticosterone either in absence (Figure 11A; $\Delta\bar{x} = -32.41 \pm 17.49$; $t_{13} = -1.85$, ns) or presence of EE (Figure 11A; $\Delta\bar{x} = 8.64 \pm 15.70$; $t_{10} = 0.55$, ns).

11A)

Figure 11A. Serum corticosterone concentration for day 1, day 3 and day 20 in absence and presence of EE: Serum corticosterone concentration for 3 time-points across the experiment: Day 1 (left, start of the experiment), 3 (centre) and 20 (right, end of the experiment) in stressed and unstressed animals in absence (left) and presence (right) of EE. Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=7), EE (N=6) and EEST (N=6). Inset: Schedule of the experiment; D = day.

At the end of the experiment, wet weight of bilateral adrenal glands was measured. Two-way ANOVA showed statistically non-significant main effects of EE ($F_{(1,42)} = 0.01$, *ns*) and stress ($F_{(1,42)} = 1.97$, *ns*). The interaction between stress and EE ($F_{(1,42)} = 1.83$, *ns*) was also non-significant. Orthogonal planned comparisons showed that the adrenal weights were unaffected by stress either in absence (Figure 11B, left; $\Delta\bar{x} = -$

13.75 ± 8.40 ; $t_{22} = -1.64$, *ns*) or presence (Figure 11B, right; $\Delta\bar{x} = -0.25 \pm 5.08$; $t_{20} = -0.05$, *ns*) of EE.

11B)

Figure 11B. Effect of stress on adrenal weight in absence and presence of EE: Stress did not significantly affect wet adrenal gland weight both in absence (left) and presence (right) of EE. Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=16), EE (N=8) and EEST (N=16). Schedule of the experiment same as Fig. 11A.

12. Effect of stress and EE on expression of inhibitory presynaptic-protein markers in BLA (GAD67)

As mentioned before in section 2.2.2., the BLA consists of both excitatory and inhibitory type of neurons in a distinct proportion [36, 37]. As an index for inhibition from the BLA neuronal circuitry, the GABAergic presynaptic protein marker: GAD67 was quantified in representative sections of brain containing BLA. Inhibitory presynaptic vesicles were identified by colabelling GAD67 (red) [321, 322] with a generic presynaptic protein marker such as synapsin (green) [323, 324]. Though currently the literature lacks information about GAD67-synapsin colocalization in the inhibitory synapses of amygdala, studies in hippocampal interneurons show presence

of synapsin isomers in GAD67 positive neurons [325]. The colocalization of GAD67 and synapsin within the BLA neuronal circuitry therefore would likely indicate the presence of inhibitory presynaptic vesicles. The GAD67-unlabelled synapsin signals were rendered to be excitatory in nature, which constituted for about ~78% of the total synapsin signals in the BLA in the absence of any treatment (Figure 12B, left panel, plain white bar for CN). The total number of GAD67-synapsin colabel puncta (yellow) was quantified for a given imaged area. Two-way ANOVA revealed significant main effect of both stress ($F_{(1,164)} = 5.62, p = 0.019$) and EE ($F_{(1,164)} = 5.17, p = 0.024$) on the number of GAD67-synapsin colabelled puncta in a given area. A significant interaction between the two treatments ($F_{(1,164)} = 8.76, p = 0.004$) was also observed. Both stress and EE significantly decreased the GAD67 in the presynaptic vesicles. Orthogonal planned comparisons revealed that the number of GAD67-synapsin colabel puncta was significantly reduced by stress in absence (Figure 12A.2 left; $\Delta\bar{x} = 33.69 \pm 10.25$; $t_{73} = 3.29, p = 0.002$) of EE but it was unaffected by stress in presence (Figure 12A.2 right; $\Delta\bar{x} = -3.72 \pm 7.79$; $t_{91} = -0.48, ns$) of EE. The total number of GAD67 negative synapsin puncta [(Total synapsin – GAD67-positive synapsin); green] was quantified for a given imaged area. Two-way ANOVA revealed significant main effect of EE ($F_{(1,164)} = 9.54, p = 0.002$) but not stress ($F_{(1,164)} = 0.09, ns$) on the number of GAD67 negative synapsin puncta in a given area. No significant interaction between the two treatments ($F_{(1,164)} = 0.72, ns$) was observed. EE significantly decreased the GAD67 negative synapsin within in BLA neuronal circuitry. Orthogonal planned comparisons revealed that the number of GAD67 negative synapsin puncta was unaltered by stress both in absence (Figure 12A.3 left; $\Delta\bar{x} = 21.63 \pm 30.83$; $t_{73} = 0.70, ns$) and presence (Figure 12A.3 right; $\Delta\bar{x} = -10.13 \pm 22.48$; $t_{91} = -0.45, ns$) of EE.

The % excitation and by corollary, % inhibition was quantified as well, wherein, % excitation = [(free synapsin)/(free synapsin + GAD67-synapsin colabels)]. All values were represented in puncta number per given area. Two-way ANOVA revealed significant main effect of only stress ($F_{(1,170)} = 6.15, p = 0.014$) but not EE ($F_{(1,170)} = 1.00, ns$) on % excitation. A significant interaction between the two treatments ($F_{(1,170)} = 4.20, p = 0.042$) was also observed. Stress significantly increased the % excitation in BLA. Orthogonal planned comparisons revealed that the % excitation in the BLA was significantly increased by stress in absence (Figure 12B left; $\Delta\bar{x} = -8.69 \pm 3.07; t_{76} = -2.84, p = 0.006$) of EE but it was unaffected by stress in presence (Figure 12B right; $\Delta\bar{x} = -0.83 \pm 2.40; t_{94} = -0.35, ns$) of EE.

12A)

12A.1)

12A.2)

12A.3)

Figure 12A. Effect of stress on protein-expression of GAD67 positive and GAD67 negative synapsin within the BLA in absence and presence of EE: Confocal images of ROI in BLA showing GAD67-synapsin colocalization (yellow) in all 4 groups: CN (control), ST (Stress), EE and EEST (**12A.1**). Scale bar represents 20 μ m. Stress significantly decreased the number of GAD67-synapsin colabelled puncta per given area in the BLA in absence (left) but not in presence (right) of EE (**12A.2**). Stress did not affect the number of GAD67 negative synapsin puncta per given area in the BLA both in absence (left) and in presence (right) of EE (**12A.3**). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=6), Stress (N=7), EE (N=7) and EEST (N=8), $**p < 0.01$, independent sample t-test. Inset: Schedule of the experiment.

12B)

Figure 12B. Effect of stress on excitation/inhibition in BLA in absence and presence of EE: Stress significantly increased the % excitation (**left panel**) and reduced the % inhibition (**right panel**) in the BLA in absence (left, uncoloured bars) but not in the presence of EE (right, grey bars). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=6), Stress (N=7), EE (N=7) and EEST (N=8), ** $p < 0.01$, independent sample t-test. Inset: Schedule of the experiment.

13. Effect of stress and EE on expression and activation of Glucocorticoid receptor (GR) protein in the BLA

Two types of GR protein expression were tested in the BLA in this study: activated GR protein expression via immunofluorescence (Figure 13A) and total GR protein levels in the BLA tissue via western blotting (Figure 13B). The expression of activated GR protein was determined using immunofluorescence staining by colocalizing GR (red) with a nuclear stain such as DAPI (blue). The colocalized GR-DAPI signal (pink) corresponded to activated GR protein which signifies stress-induced molecular response. In order to normalize data across all quantified ROIs, GR activation was represented as the percentage ratio between the number of GR-DAPI colabelled puncta/given area by number of DAPI puncta/given area within each ROI. Two-way

ANOVA revealed significant main effect of EE ($F_{(1,178)} = 5.38, p = 0.022$) but not stress ($F_{(1,178)} = 2.07, ns$) on activated GR protein expression. There was no significant interaction between the two treatments ($F_{(1,178)} = 1.41, ns$). EE significantly decreased the activation of GR protein. Orthogonal planned comparisons revealed that activated GR remained significantly unaffected by stress in absence (Figure 13A.2 left; $\Delta\bar{x} = 1.11 \pm 7.67$; $t_{86} = 0.15, ns$) of EE but was significantly lowered in presence (Figure 13A.2 right; $\Delta\bar{x} = 11.48 \pm 4.49$; $t_{92} = 2.56, p = 0.012$) of EE.

13A)

13A.1)

13A.2)

Figure 13A. Effect of stress on nuclear localized GR protein expression within the BLA in absence and presence of EE: Confocal images of ROI in BLA showing GR-DAPI colocalization (pink) in all 4 groups: CN (control), ST (Stress), EE and EEST (**13A.1**). Scale bar represents 20 μ m. Stress did not affect the GR nuclear expression in the BLA in absence (left) of EE but significantly lowered it in presence (right) of EE (**13A.2**). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=7), EE (N=8) and EEST (N=8), ** $p \leq 0.01$, independent sample t-test. Inset: Schedule of the experiment; D = day.

The expression of total glucocorticoid receptor (GR) protein in BLA was quantified by means of western blotting. GR protein expression was retrieved in the form of band intensity (OD value) normalized with GAPDH used as the internal control. Two-way ANOVA revealed significant main effect of both stress ($F_{(1,20)} = 4.3$, $p = 0.051$) and EE ($F_{(1,20)} = 5.88$, $p = 0.025$) on GR protein expression. There was no significant interaction between the two treatments ($F_{(1,20)} = 0.59$, ns). Both stress and EE significantly decreased the GR protein expression. However, the effect size of EE was greater than stress as indicated in two-way ANOVA (EE partial $\eta^2 = 0.227$, stress partial $\eta^2 = 0.177$). Orthogonal planned comparisons revealed that GR remained

significantly unaffected by stress both in absence (Figure 13B left; $\Delta\bar{x} = 0.75 \pm 0.42$; $t_{10} = 1.77$, *ns*) and presence (Figure 13B right; $\Delta\bar{x} = 0.34 \pm 0.31$; $t_{10} = 1.10$, *ns*) of EE.

13B)

Figure 13B. Effect of stress on total GR protein expression in BLA in absence and presence of EE: EE significantly lowered the total GR expression in the BLA. Stress did not significantly affect the GR protein expression in the BLA in the absence (left) or presence (right) of EE. Graphs represent Mean $\pm$ SEM. Bars represent Control (CN, N=6), Stress (ST, N=6), EE (EE, N=6) and EEST (EEST, N=6). Inset: Schedule of the experiment; D = day.

14. Effect of stress and EE on expression of pro - brain-derived neurotrophic factor (pro-BDNF) and Tyrosine Kinase receptor B (TrkB) in the BLA

The expression of pro - brain-derived neurotrophic factor (pro-BDNF) and its cognate receptor Tyrosine receptor Kinase B (TrkB) in the BLA was quantified by means of western blotting. Expression of both the proteins was retrieved in the form of band intensity (OD value) normalized with GAPDH used as the internal control. Two-way ANOVA revealed that there was no significant main effect of either stress ($F_{(1,16)} = 2.34$, *ns*) or EE ($F_{(1,16)} = 0.31$, *ns*) on pro-BDNF protein expression. There was no significant interaction between the two treatments ($F_{(1,16)} = 0.15$, *ns*). Orthogonal planned comparisons revealed that pro-BDNF remained significantly unaffected by stress in absence (Figure 14A left; $\Delta\bar{x} = 0.40 \pm 0.67$; $t_8 = 0.60$, *ns*) of EE but was reduced significantly reduced by stress in presence (Figure 14A right; $\Delta\bar{x} = 0.67 \pm 0.21$; $t_8 = 3.22$, $p = 0.012$) of EE.

Two-way ANOVA revealed that there was no significant main effect of either stress ($F_{(1,20)} = 0.03$, *ns*) or EE ($F_{(1,20)} = 2.20$, *ns*) on TrkB protein expression. There was no significant interaction between the two treatments ($F_{(1,20)} = 0.20$, *ns*). Both stress and EE had no effect in altering TrkB protein expression. Orthogonal planned comparisons revealed that TrkB remained significantly unaffected by stress both in absence (Figure 14B left; $\Delta\bar{x} = -0.15 \pm 0.38$; $t_{10} = -0.40$, *ns*) and presence (Figure 14B right; $\Delta\bar{x} = 0.07 \pm 0.31$; $t_{10} = 0.21$, *ns*) of EE.

14A)

Figure 14A. Effect of stress on pro-BDNF protein expression in BLA in absence and presence of EE: Stress did not affect the pro-BDNF protein expression in the BLA in the absence (left) of EE but significantly lowered the same in presence (right) of EE. Graphs represent Mean $\pm$ SEM. Bars represent Control (CN, N=5), Stress (ST, N=5), EE (EE, N=5) and EEST (EEST, N=5), * $p < 0.05$, independent sample t-test. Schedule of the experiment same as Fig. 13B.

14B)

Figure 14B. Effect of stress on TrkB protein expression in BLA in absence and presence of EE: Stress did not affect the TrkB receptor protein expression in the BLA both in the absence (left) or presence (right) of EE. Graphs represent Mean $\pm$ SEM. Bars represent Control (CN, N=6), Stress (ST, N=6), EE (EE, N=6) and EEST (EEST, N=6). Schedule of the experiment same as Fig. 13B.

15. Effect of stress and EE on body-weight

During the course of the experiment, body weight gain was quantified as percentage gain relative to weight at the start of the experiment. Two-way ANOVA showed statistically significant main effect of stress ($F_{(1,26)} = 31.31$, $p < 0.001$) but not of EE ($F_{(1,26)} = 0.04$, ns). The interaction between stress and EE ($F_{(1,26)} = 0.02$, ns) was also non-significant. Stress significantly reduced % body-weight gain irrespective of EE housing. Orthogonal planned comparisons revealed that % body-weight gain was significantly reduced by stress both in absence (Figure 15 left; $\Delta\bar{x} = 11.38 \pm 2.25$; $t_{14} = 5.06$, $p < 0.001$) and presence (Figure 15 right; $\Delta\bar{x} = 11.95 \pm 3.66$; $t_{12} = 3.27$, $p = 0.007$) of EE.

15)

Figure 15. Effect of stress on percentage body-weight gain in absence and presence of EE: Stress significantly reduces the % body-weight gain in animals both in the absence (left) and presence (right) of EE. Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=8), EE (N=8) and EEST (N=6), ** $p < 0.01$, *** $p < 0.001$, independent sample t-test. Inset: Schedule of the experiment, D = day.

DISCUSSION

The Hypothalamic-Pituitary-Adrenal (HPA) axis is highly sensitive to experiences and stressful events such as trauma or chronic stress-exposure. Encounter with such events can result in a long-lasting impact on behaviour of an individual. Chronic stress in particular is known for inducing anxiety and depression in rats and other mammals [4, 71, 72, 205]. Presence of chronic stress in the environment can therefore pose a potential threat to the normal functioning of the HPA axis leading to psychiatric disorders [210]. However, exposure to stress-inducing situations in a lifetime is inevitable. An optimal combination of positive and negative elements in the environment would broaden the scope of successfully coping with life-time stressors. With this as the primary motivation, the study employed a positive environment such as a brief period of enrichment (EE) to house chronically stressed rats and investigate if such a housing effect leads to stress-resilient behaviour associated with an efficiently adaptive HPA response to stressors. For this we compared the stressed and non-stressed populations in each differential housing (standard conditions vs. EE housing) to evaluate if the maladaptive stress effects were relieved or not.

1. Specific aim 1: To evaluate the effects of EE on stress-related behavioural outcomes: developing rodent model of stress-resilience.

1.1 EE reduces anxiety and increases active-coping in stressed subjects.

The basolateral amygdala is one of the main regulators of the HPA axis through its positive neural feedback [3, 114]. Hyperactivation of this brain region is translated into behavioural states of anxiety as well as hyperactivation of HPA [39, 114]. In turn the influence of stress and stress hormones (glucocorticoids) is well established in precipitating anxiety-like responses in mammals by enhancing the responsiveness of

BLA [115, 116, 121]. On the other hand, intervention of positive environments such as EE has adaptive effects on emotional behaviour. Previous studies have shown that brief exposure of animals to EE in adult-life imparts not only enhanced defensive behaviour and attractiveness in male rats than their non EE counterparts but also alleviated behavioural pathologies induced by early-life stress [260, 286]. In this study, chronic stress increased anxiety in animals while EE reduced the same as suggested by the open arm exploration in the EPM. Comparisons within the differential housing conditions (orthogonal planned comparisons) showed that stressed individuals exhibited distinct anxiety-like behaviour when tested on an EPM compared to non-stressed individuals under normal housing condition. This stress-induced anxiety was absent in stressed individuals exposed to short stretch of EE. This finding in particular, is of clinical significance since it has been shown that anxiety imparted by chronic stress through immobilization is irreversible even after a recovery phase of 21 days [158]. Chronic stress also increased locomotion on EPM, while EE had no effect on it. However this increase may not be associated with anxiety-like behaviour since it has an opposite direction to the effect of anxiogenic agents which upon an incremental dosage, tend to decrease this property on EPM [308]. Comparisons within the differential housing conditions (orthogonal planned comparisons) showed that there was no difference in locomotion between non-stressed and stressed individuals in either normal or EE housing.

Goal-directed behaviours encompass an important element of resilience [326]. Such behaviours are reflected in the form of active coping which involve assessment of risks and threats voluntarily by a resilient individual [224]. In this study, head-dips were measured in EPM to indicate risk assessment. Stress led to a decrease in risk assessment behaviour while EE rescued it in these individuals. It is important to note

that, in presence of EE, stressed animals had lower risk assessment behaviour than non-stressed. Risk assessment in the former was in fact comparable to control animals (baseline), which indicated an adaptive level of risk assessment. Further increase in head dips as seen in the EE-exposed non-stressed animals (that exceeds baseline) could override the adaptive value of fear [204]. Consequentially this could increase the vulnerability of these animals particularly in situations with predators in the immediate environment, instilling a negative valence in the form of maladaptive loss of aversion. Therefore, in EE, stressed animals may perform more adaptively than non-stressed ones due to restoration of baseline levels of aversive response. Therefore, short-term EE could successfully give rise to resilient behaviour in stressed rats in the form of lowered anxiety and high risk-assessment.

1.2 EE reduces depressive-like symptoms irrespective of stress exposure.

Anxiety is often co-morbid with depression [327]. A considerable number of studies have reported the positive effects of EE on alleviating the behavioural symptoms of depression [250, 270, 328-331]. But most of these studies involved long-term EE, which last for a month or more. Therefore, the study investigated if depressive-like behaviours were concurrent with the anxiety-state induced in the chronic stress model in this study. The study also investigated the effects of short-term EE on possibly depressive-like symptoms. Depression is characterized by two classic hallmarks - anhedonia and learned helplessness [332]. Sucrose intake is a validated test for hedonic experience in rats and is reduced by chronic stress [314]. According to comparisons within the differential housing conditions (orthogonal planned comparisons) in this study, stress reduced sucrose intake in rats in absence of EE. However, in presence of EE, this effect was nullified. An important point to note here

is that EE had an overall effect in lowering the basal hedonic response to start with. This is in line with other studies that have reported a decline in sucrose intake in EE-housed animals in comparison to standard-housed rats undergoing social isolation stress [333]. Furthermore, studies have reported that adult rats exposed to EE show less anticipatory behaviour and impulsivity during appetitive conditioning and in response to cue-based sucrose seeking [334-336]. These studies perhaps indicate a well-regulated and emotionally-stable appetitive behaviour in enriched rats towards stimuli which otherwise can promote impulsiveness and addictive responses, including sucrose-intake. It is also important to note that yet another effective way to validate the hedonic responses in EE rats is to perform a sucrose-preference test, which would potentially elucidate whether EE rats show a preference for hedonic experiences (intake of sucrose) over a neutral feed (water) or not. This could also be done by saccharine-preference test which could also clarify the confounding effect of EE on sucrose metabolism [337, 338].

Learned helplessness, another characteristic of depressive-like behaviour was measured by FST. Immobility in FST is an indication of learned helplessness, wherein the animal gives up by showing lack of efforts in escaping a threatening situation and stay immobile in the water [315, 339]. EE reduced learned helplessness compared to their non-EE counterparts, in line with other studies that also used short-term EE [271]. Stress, however, did not affect this behaviour. Swimming, another parameter measured in FST, indicates coping against the stressful situation (making efforts to stay up at the surface of the water). Stress led to a decrease in swimming in these animals. Hence, stress lowered discreet active-coping tendencies in challenging situations like forced swimming while EE protected animals from submitting to learned helplessness. Comparisons within the differential housing conditions

(orthogonal planned comparisons) showed that levels of learned-helplessness (immobility) and coping (swimming) in FST were in fact comparable between stressed and non-stressed animals in both types of housings.

1.3 EE reduces hyper-reactivity at resting levels in stressed subjects.

This study so far shows that chronic stress can impair coping behaviour of animals, which can potentially be reversed by EE. Most of the stress-induced psychiatric disorders, particularly anxiety, involve the HPA axis becoming hyperactive even at baseline/resting levels simply at the anticipation of a stress encounter [199, 340]. We measured baseline response using a sensor-based platform called LABORAS™, which monitored immobility and locomotion in a non-stimulatory environment (resembling a standard laboratory cage). In this testing environment, immobility was increased in EE compared to the non-EE animals, while stress had no effect. On the other hand, in the same experimental setup, stress increased locomotion, which suggests hyperactivity in these animals in a non-stimulatory (basal) setting. On the contrary, EE reduced locomotion in these animals, which suggests that perhaps EE imparts a well-regulated homeostatic control on behaviour including an optimal baseline response and accelerated habituation to new yet non-exploratory settings. In other words, it may be imparting better control over the stress axis (HPA axis) in order to stabilise the physiology of the body with respect to the changes in the environment. According to comparisons within the differential housing conditions (orthogonal planned comparisons), stressed individuals exhibited increased locomotion when tested on LABORAS compared to non-stressed individuals under normal housing condition, which was absent in stressed individuals exposed to EE. This observation is in consensus with other studies that have shown lower latency of EE-exposed individuals to habituate to novel settings [329]. This also provides scope for speculating the

adaptive flexibility in energy expenditure in EE-exposed animals. These individuals putatively exhibit a better sensitization and awareness to their immediate environment wherein they invest more physical energy in stressful situations (Fig 4C) such as forced swim test (low immobility) but less physical energy at baseline settings (closed non-stimulatory conditions).

Therefore, modulation in regulation of HPA axis and resilience is much evident in the combinatorial behaviour output observed through various tests of EPM, SIT, FST, and LABORAS trials which is summarized as follows:

1. Stressed individuals had higher anxiety compared to non-stressed individuals in the absence of EE. This difference between stressed and non stressed individuals was not seen in presence of EE (seen in Fig 1A, 1B and 1C).
2. Higher levels of active coping in EE-housed animals under threatening situations (seen in Fig 1E).
3. Lowered levels of sucrose seeking in EE-housed animals (seen in Fig 2).
4. Lowered levels of learned-helplessness in EE-housed animals in response to challenging situation (seen in Fig 3A).
5. Stressed individuals were hyperactive (higher locomotion) in non-stimulatory environment compared to non-stressed individuals in the absence of EE. This difference between stressed and non stressed individuals was not seen in presence of EE (seen in Fig 4B).

Based on these behavioural outputs, the EE-housed stressed individuals successfully coped against anxiety as reflected by their exploration on EPM and lack of hyperactivity (locomotion) at resting conditions in LABORAS. There was no difference between the stressed and non-stressed individuals under EE housing as

opposed to standard-cage housing, which confirms the pattern of stress-resilience in the form of recovery from anxiety.

Hedonic responses (associated with depression) tested in SIT showed difference between non-stressed and stressed animals in standard-cage housing, which was absent in EE housing. However, similar to previous studies [333, 334], the basal level for this behavioural feature was lowered significantly perhaps due to lack of sensitivity of EE-exposed animals to sucrose, making the test unsuitable to test resilience in animals in this study. It is also crucial to note that EE lowered the level for depressive-like symptoms (learned-helplessness in the form of immobility, Fig 3A) as seen in FST for animals (both non-stressed and stressed) via the main effects of EE-housing. However, there was no difference between stressed or non-stressed animals across the differential housing patterns for behavioural features pertaining to depression. This pattern rather seems dissimilar to the anxiety-response in this model. The comparable levels of immobility in FST in both stressed and non-stressed animals were found in both standard-cage and EE housing, with a change in the basal levels of the parameter. Similar pattern of response was observed in the ease of habituation (tested in the form of immobility in LABORAS, Fig 4B). Hence, these behavioural parameters (related to depression) may not be contributing to the specific feature of stress-resilience in the animal model for this study. However, perhaps they are affected in a broader way by contributing in modulation of the HPA axis regulation in EE-housed animals (via significant alteration in the levels of these behavioural features).

1.4 EE reduces circulating corticosterone in irrespective of stress exposure.

In order to investigate the influence of EE and stress on physiological properties pertinent to the HPA axis, we measured serum corticosterone (CORT) concentration for three time points across the experiment (Day 1, 3, and 20). The weight of adrenal glands and percentage body weight gain were also tested at the end of the experiment.

Earlier studies have shown increase in CORT in response to an exposure to a novel object or setting [50]. However, the effect on CORT due to an acute or prolonged exposure to environmental enrichment has still been inconclusive. A number of studies have reported modulation in corticosterone in response to enrichment, wherein some showed increase in the baseline CORT post-EE exposure [341, 342], while some showed decrease or no significant change in CORT [343]. From these studies it appears that such modulations putatively depend on the species-subtype and age of the animals, as well as duration of EE exposure. In response to chronic immobilization stress, previous studies have shown the habituation of the HPA activity, which normalises the CORT back to baseline levels [344, 345]. Similarly in this study, stress had no main effect on modulation of CORT, which might be suggestive of the normalization of CORT back to baseline along the course of experiment. On the other hand, EE animals had lower CORT than non-EE as shown by the AUC (area under the curve) obtained for CORT (including baseline levels). This feature could be putatively supportive of the stronger homeostatic control exhibited by EE-exposed animals compared to the non-EE animals. This is also reflected in the LABORAS trials where EE-exposed animals exhibited lower latencies for habituation.

Furthermore, in this study, stress and EE did not significantly affect the weight of adrenal glands. Stress reduced the body weight gain of all animals irrespective of EE exposure. Previous reports have shown strong association between lack of body weight gain and stress exposure [346]. In our study, EE showed no effect on this physiological parameter.

2. Specific aim 2: To investigate characteristic neuronal morphology associated with stress-resilience induced by EE

2.1 EE induced reorganization of basolateral amygdala neurons in stressed animals.

As mentioned earlier, the basolateral amygdala (BLA) is the primary interface for receiving input and generating a fear-based response [30, 31]. Its hyper-reactivity leads to manifestation of anxiety, which is a maladaptive valence of fear [39, 114].

Earlier studies have shown that the dendritic complexity of principal neurons (primary excitatory neurons) in the BLA correlates with high anxiety in rats [122]. Stress exposure (acute or chronic) as well as exogenous corticosterone administration leads to increase in the dendritic arbors and spine density of the principal neurons of BLA and corresponding manifestation of anxiety. [72, 120, 121]. In this study, EE significantly reduced branching in these neurons but increased the dendritic length. According to comparisons within the differential housing conditions (orthogonal planned comparisons), stress led to hypertrophy in the BLA neurons in absence of EE, in line with previous studies [72]. However stress-induced increase in branching but not dendritic length was lowered in presence of EE. Stressed individuals exhibited increase in number of branch points in BLA neurons compared to non-stressed individuals under normal housing condition, which was absent in BLA neurons of

stressed individuals exposed to EE. BLA neurons of stressed animals devoid of EE showed hypertrophy in the form of increased branching proximal to the soma (0 – 200 μm) and increase in total dendritic length.

In the BLA neurons, interestingly chronic stress affected spines on primary and secondary dendrites non-uniformly. Stress had no affect on spines in primary dendrites while it reduced the same in secondary dendrites. EE on the other hand, decreased spines in the primary dendrites and had a marginal effect on increasing spines in secondary dendrites. Comparisons within the differential housing conditions (orthogonal planned comparisons) showed that, a decrease in the dendritic spines of both primary and secondary dendrites of these neurons was observed in stressed subjects compared to the non-stressed, only in the presence of EE but not in its absence. This suggests that short-term EE in conjunction with chronic stress contributes in reversing BLA neuronal hypertrophy in stressed animals by modulating branching and lowering dendritic spines in these neurons. In presence of EE, BLA neurons in stressed animals therefore presented longer dendritic cables with lowered spines and branches which putatively might be attributing to lowered synaptic activity [215]. These modulations in the morphological substrates of BLA neurons are characterized by the electrotonic effect of longer cables harbouring lowered contacts for synapses. This might lead to lowered net excitability. The summative plasticity therefore prevents the hyper-responsiveness of the BLA neurons which blocks the manifestation of anxiety even after exposure to chronic-stress. In other words, this results in a resilient behaviour. This also perhaps explains the absence of hyper-reactivity of the HPA axis when tested for baseline response in EE animals. The hyperactive baseline response of the stressed animals housed in absence of EE is

associated with increased complexity of the dendritic arbors in the BLA as they are more likely to be excitable relative to the EE-exposed animals.

Figure 16. Microscope traces of sample BLA principal neurons for the 4 groups: Control (A), Stress (B), EE (C) and EEST (D). Scale bar represents 100 μ m. Inset: Magnified region of dendrite to show dendritic spines represented as schematic stars along dendrites.

2.2 EE induced reorganization of hippocampal CA3 neurons in stressed animals.

The model of resilience validated in this study also exhibited alleviation of depressive-like symptoms post stress exposure. This suggests a role of CA3 hippocampal neurons that share an inverse relationship with depressive-like symptoms [71]. A large number of studies have shown the sensitivity of CA3 neurons to chronic restraint stress and high corticosterone in rodents, wherein these neurons undergo atrophy in response, in contrast to hypertrophy seen in BLA neurons [75, 77-80, 89, 347, 348]. However unlike BLA neurons, CA3 hippocampal neurons undergo reversible neuronal plasticity post recovery from chronic stress [77, 101, 158]. Moreover, the influence of

hippocampal neurons on the HPA axis has a strong implication since it is the cardinal brain region responsible for providing negative feedback to HPA axis [24].

Extant literature has shown the positive effects of EE on hippocampal plasticity in the form of increased neuronal arborisation, including enhanced neurogenesis in the dentate gyrus [270, 275, 331, 349-354]. Previous reports have demonstrated the reversibility of stress-induced atrophy upon a recovery phase post stress exposure [347]. Similarly in our study, stress increased both dendritic length and branch points of CA3 hippocampal neurons while EE had no effect. This was expected due to the post-recovery duration after chronic stress (~7 days, Fig 5A inset). The atrophy in CA3 neurons would possibly have been evident if the experiment was terminated shortly after the stress duration (~1-2 days) without providing any recovery time for the neurons to restore their morphology. This stands as an experimental design limitation to observe the singular effect of stress on these neurons (due to the recovery period). However according to comparisons within the differential housing conditions (orthogonal planned comparisons), the study shows that in presence of EE, the stressed subjects showed further increase in dendritic length as well as branch points. This difference in the neuronal arborisation between stressed and non-stressed animals was absent in non-EE housing. Such an increase could be a combination of post-stress recovery and EE housing.

Moreover, spine density in the primary dendrites of these neurons was diminished by stress, which is in consensus with previous reports [111]. It is important to note that this was rescued in presence of EE but could not be recovered even after the post-stress resting phase in the absence of EE. Therefore, according to comparisons within the differential housing conditions (orthogonal planned comparisons), the spine-density increase was pronounced in stressed animals compared to the non-stressed, in

presence of EE. The increase in hippocampal neuronal plasticity is in line with other studies that show increase in branching and spine density in response to EE [353-357].

Figure 17. Microscope traces of hippocampal CA3 neurons for the 4 groups: Control (A), Stress (B), EE (C) and EEST (D). Scale bar represents 20 μ m. Inset: Magnified region of dendrite to show dendritic spines represented as schematic stars along dendrites.

The increased complexity and synaptic sites of these neurons putatively enhanced their responsiveness to various stimuli in the immediate environment. Lowered depressive-like symptoms and optimal HPA reactivity in the behaviours of animals exposed to EE are likely associated with the adaptive synaptic plasticity in modulated spine-density in hippocampal CA3 neurons. The occurrence of this plasticity is capable of providing a stronger negative feedback during stressful encounters, and is probably reflected in the modulation of CORT, which is significantly lowered by EE.

2.3 EE induced reorganization of the medial prefrontal cortex neurons in stressed animals.

Another brain region indirectly responsible for contributing to HPA negative-feedback regulation is the mPFC [66, 68, 69]. The mPFC participates in modulation of emotional responses by extending efferent projections to various subcortical regions such as BLA, lateral hippocampus, various thalamic nuclei, periaqueductal grey and raphe nuclei [61, 62, 65]. Prelimbic (PrL) and Infralimbic (IL) regions: the two main subregions of the mPFC which play a role in regulating fear responses were studied separately due to their functional differences. PrL is known to play a role in fear-conditioning and learning while IL in fear extinction [138]. These two regions in conjunction with their respective neuronal connections with the BLA, control fear responses. It has been shown that BLA has strong projections to both PrL and IL [65]. Several studies have shown that PrL and IL differ in their distributions of projections to the amygdala. The IL projects widely throughout the amygdala whereas the PrL projects relatively stronger to the basolateral nucleus of the amygdala [62]. Although the mechanism of action is still disputed, it has been established that fear extinction is mediated by the connections from the mPFC to the amygdala [358]. This makes the properties of both the PrL and IL projection neurons crucial, such that any maladaptive response at this level could lead to a negative valence such as anxiety/depression.

The influence of chronic stress on PrL and IL projection neurons is well established. Many studies have shown that chronic stress or excessive glucocorticoids leads to atrophy of the mPFC neurons in form of shorter dendrites and loss of spines [182, 184-186, 359]. Specifically, the PrL region suffers from dendritic length retraction and loss of spines post chronic stress or exogenous CORT administration in adult rats [190, 191, 360]. However, similar to hippocampal neurons, these cortical neurons belonging

to young adult rats also possess the capacity to recover the dendritic length and branching during post-stress recovery phase [190]. Such a recovery is not seen in middle-aged and aged rats. The IL neurons also undergo also dendritic remodelling upon chronic stress exposure. [57, 183, 188, 189]. However, post stress, the IL neurons also follow a discreet pattern of reversible dendritic plasticity during post-stress resting phase by expanding the proximal dendritic arbors completely [57, 189]. Extant literature has shown the potency of EE in enhancing cortical neuronal responsiveness in countering stress [235, 281, 361, 362].

In this study, comparisons within the differential housing conditions (orthogonal planned comparisons) showed no difference in the number of branch points or dendritic length of PrL neurons between non-stressed and stressed individuals in either type of housing. However, it is important to note that stress significantly increased the dendritic length of PrL neurons. This effect was expected due to recovery phase post-stress exposures (~10 days, Fig 7A inset) and is in consensus with previous reports [190]. EE resulted in the increase of both dendritic length and branching of PrL. It had a main effect in increasing the general dendritic architecture of the PrL neurons irrespective of stress exposure. This indicates a stronger responsiveness of the PrL neurons of stressed animals, housed in EE. Stress reduced the spine density only in the secondary but not primary dendrites, which means the synaptic activity at the distal ends of the neuron is affected, similar to other reports [360, 363]. However, EE increased the spine density in both primary and secondary dendrites of these neurons compared to all non-EE animals irrespective of stress exposure. This suggests that EE potentially increases the capacity of establishing synaptic contacts by these neurons at a basal level. According to comparisons within the differential housing conditions (orthogonal planned comparisons) there was no difference in the spine density of

primary dendrites in PrL neurons between non-stressed and stressed individuals in either type of housing. But the spine density in secondary dendrites of these neurons was lowered for stressed individuals than its non-stressed counterparts in presence of EE, an effect which was absent in normal housing conditions. This suggests a limited role of PrL neurons in contributing to the trait of resilience in animal in this study.

The dendritic length of IL neurons on the other hand, was unaffected by EE or recovery post-stress exposure. No treatment effect (stress or EE) was observed on the branching of IL neurons. However according to comparisons within the differential housing conditions (orthogonal planned comparisons), in the absence of EE, stressed subjects exhibited increased branching in the IL neurons which did not occur in presence of EE. This effect of stress on IL neurons could be due to dendritic reorganisation and expansion of proximal dendritic arbors (as a compensatory mechanism) as shown by previous studies [57, 189]. EE had a main effect in increasing the spine density in both primary and secondary dendrites of IL neurons at a basal level, irrespective of stress. However, stress reduced spine density in both types of dendrites while EE could reverse this effect in secondary dendrites but not primary. According to comparisons within the differential housing conditions (orthogonal planned comparisons) the stressed individuals had lowered spine density of primary dendrites in IL neurons than their non-stressed counterparts, a pattern that was comparable in both type of housing. EE-housing could not rescue the stress-induced spine loss in the primary dendrites of IL neurons. In case of secondary dendrites in IL neurons the stressed individuals had lowered spines than its non-stressed counterparts in absence of EE, an effect which did not occur in presence of EE where the stressed and non-stressed were comparable. Stressed individuals in EE therefore were rescued from stress induced spine loss at the distal regions (secondary dendrites) of IL

neurons. However it is important to note that at a broader level overall effect of EE in both PrL and IL neurons in increasing the synaptic contacts (in the form of spines), shows a boost in the responsiveness of mPFC neurons in general and increases the chances of better negative feedback control over the HPA axis.

Figure 18. Microscope traces of prelimbic (PrL) neurons for the 4 groups: Control (A), Stress (B), EE (C) and EEST (D). Scale bar represents 20 μ m. Inset: Magnified region of dendrite to show dendritic spines represented as schematic stars along dendrites.

Figure 19. Microscope traces of infralimbic (IL) neurons for the 4 groups: Control (A), Stress (B), EE (C) and EEST (D). Scale bar represents 20 μ m. Inset: Magnified region of dendrite to show dendritic spines represented as schematic stars along dendrites.

EE therefore rescues the stress-induced hypertrophy in principal BLA neurons in the form of lowered spine density and branching. In the hippocampus, CA3 neurons of EE-exposed stressed animals exhibit hypertrophy and increase in spine density. In IL neurons, these individuals are rescued from spine-loss at distal regions. These features associate with stress-resilience at the morphological level. It is also noteworthy that EE broadly affect hypertrophy and increase in spines in the PrL neurons. The combinatorial effect of neuronal plasticity in brain regions such as the BLA and hippocampus, are possibly associated with adaptive behavioural tendencies such as reduced anxiety and learned helplessness respectively. This might be indirectly coordinated by brain structures that are responsible for higher executive function and decision making, ergo emphasizing on the role of medial prefrontal cortex in facilitating these effects and the modulation of its activity by EE [58].

3. Specific aim 3: To investigate the molecular regulation associated with stress-resilience induced by EE

In order to address probable mechanisms underlying the behavioural and morphological changes observed with respect to chronic stress and short-term EE exposure, we studied protein regulation for prospective candidates:

3.1 EE reduces activation of glucocorticoid receptor protein in stressed animals.

The current study demonstrates a poor control over the HPA axis feedback regulation, evident in the form of anxiety and hypertrophied BLA neurons in non-enriched stressed animals. This indicates a potential role of glucocorticoid receptors (GR), the cognate receptor of corticosterone, known to maintain the HPA tone under a range of arousal states [3, 15, 364]. The density of GR concentration across brain regions such as hippocampus, amygdala, and mPFC also varies and is linked with its ability to provide feedback regulation [15, 365]. The activation of GR (by corticosterone occupancy) and its localization into the nucleus for its recruitment as transcription factor constitutes one of the key molecular pathways mediating stress response in relevant brain regions [18, 19].

The GR expression, activation, and its implications on stress responses have been extensively studied with respect to hippocampus [26, 366, 367]. Studies have shown that chronic stress in early life (maternal separation) downregulates the expression of GR in the hippocampus, leading to dysregulation of HPA negative feedback system [368-370]. EE, however, can reverse these effects induced by early-life stress by upregulating GR expression in the hippocampus [228, 261]. The status of GR protein expression and regulation in the basolateral amygdala with respect to EE is still

undiscerning. Previous studies have demonstrated that engaging these receptors in the amygdala directly by stereotactic administration of corticosterone leads to hyperactivation of HPA and anxiety [130, 371]. The same behavioural features are in fact reversed by experimentally blocking corticosterone binding to GR in the BLA [133, 134]. These studies indicate the association of enhanced GR activation with potentiation of anxiety and BLA hypertrophy.

Furthermore, previous studies have established the corticosterone-induced acquisition and consolidation of fear memories facilitated by GR via noradrenergic pathways [43, 372, 373]. This emphasises the necessity of GR in processing fear memories and its potential role in leading to psychiatric conditions like anxiety. Previous studies have shown that chronic immobilization stress fails to modulate GR protein expression in the amygdala and decreases the same in hippocampus and hypothalamus [374]. Similarly, another study showing effects of stress induced by chronic substance-abuse showed no change in the GR status of the amygdala but a reduction in the dorsomedial hypothalamus [375]. In consensus to these studies, this study also showed unaltered GR protein expression in response to chronic stress. On the other hand, EE, which demonstrates efficient HPA activity in our current model, had a significant effect in downregulating both the activation of GR and total GR protein expression in individuals, relative to those unexposed to EE. According to comparisons within the differential housing conditions (orthogonal planned comparisons), stressed individuals had lowered activation of GR (lowered GR nuclear localization) in presence of EE, an effect which was not observed in absence of EE. This is an interesting observation and has not been studied in isolation except a single report which also demonstrated a decrease in GR expression using Indian field male mice in response to EE and is linked to anxiolytic behaviour in these animals [376]. Besides this, subjects with

absence of GR (knockout) in the amygdala show no difference in the anxiety levels relative to wild-type (control subjects) when tested on an EPM [364].

A number of studies have reported the upregulation of GR expression in the hippocampus in response to EE [271, 377-380]. However, the selective information on the GR status for BLA in enriched subjects is lacking currently. In this study we found that GR protein expression and activation in EE-exposed animals was lowered. This is in line with the combined lowering of anxiety-state, depressive-like symptoms, as well as circulating corticosterone. Lowered GR activation in particular might be associated with EE-induced lowered BLA neuronal responsiveness leading to lowered excitability via calcium influx in the principal excitatory neurons of the BLA [137]. Therefore, EE housed stressed animals show lowered nuclear activation in the BLA compared to their non-stressed counterparts which might be contributing to their resilience-trait at a molecular level. It is also important to note that, EE lowers the GR protein expression and activation at a basal level irrespective of stress which may be associated with fine-tuned feedback response of the HPA axis on a broader scale.

3.2 Stress conditionally reduces expression of inhibitory presynaptic protein (GAD67) of BLA in absence of EE but not in its presence.

It is well established that a majority of BLA neurons are excitatory in nature. However, a small population of the local interneurons (20-30%) in the BLA are functionally inhibitory [142, 143]. Though much smaller in population, the local GABAergic neurons have very distinct firing properties compared to principal excitatory neurons in the BLA. The principal neurons in the BLA can fire action potentials up to 20 Hz while the interneurons spontaneously can reach a potential ranging up to 100 Hz.[36, 37, 150, 381]. A balance in the activities of excitatory and

inhibitory neurons in this brain region is essential to maintain BLA-based fear-response regulation. An imbalance on the other hand, particularly hyperactivation of the local excitatory neurons, could override the inhibition provided by the interneurons and lead to manifestation of anxiety [145-147]. Thus it is crucial to evaluate excitatory-inhibitory regulation amidst the dynamic neuronal plasticity seen in stressed animals. It is also crucial to study how this circuit is regulated in presence of EE which successfully rescued many of the stress effects including anxiety.

The local GABAergic interneurons in the BLA express glutamate decarboxylase (GAD), an enzyme that leads to synthesis of GABA from L-glutamic acid [150, 151]. It therefore serves as potential inhibitory presynaptic protein marker to study GABAergic activity mediated within the BLA. Studies have shown that lowered GAD67 protein expression in rats linked to increased depressive-like behaviour was induced by repeated corticosterone administration in the amygdala [152]. Another study in adult mice shows a significant reduction in the expression of GAD67 mRNA in the interneurons of BLA in response to chronic restraint stress [382]. In the current study, GAD67 was quantified in BLA when coexpressed with synapsin (generic presynaptic protein marker) to confirm its presence in presynaptic sites. In support of previously mentioned studies, stress decreased the coexpression of GAD67 with synapsin in the absence of EE but this difference was not seen in presence of EE as per comparisons within the differential housing conditions (orthogonal planned comparisons). The expression of free synapsin (unlabelled with GAD67) complementarily was rendered to be excitatory in nature and the net excitation was therefore retrieved which showed a significant increase in the BLA neurons in the stressed animals in absence of EE. Importantly, this difference was not seen in the presence of EE. Stress conditionally modulated the excitability of the BLA neurons

based on housing. Though GAD67-synapsin coexpression (index of inhibition) in the BLA of EE-exposed animals were comparable to its non-stressed counterparts, an effect which was not seen in absence of EE, its basal level was drastically altered under EE-housing. However the anxiety behaviour does not relate to this molecular pattern. The basal expression of synapsin and GAD67 itself underwent a reduction in presence of EE compared to subjects housed without EE. In fact in EE housed animals, the level of GAD67-negative synapsin itself was lowered. Moreover, stress-exposure in such an enriched housing failed to modulate these proteins. This indicates that differential environments modulate GAD67 and the resultant excitation and inhibition differently in BLA.

It is also important to note, these effects in isolation may not be suggestive of the lowered anxiety behaviour seen in EE-exposed animals. A knockdown of GAD67 in the amygdala, besides disrupting fear extinction, blocks the anxiolytic effects induced by diazepam [383]. EE, similar to diazepam, is also an anxiolytic factor, but leads to downregulation of GAD67 and yet precipitates lowered-anxiety in individuals. This suggests that the EE-induced anxiolytic effects may not be mediated solely by modulation in the local inhibitory circuit of BLA. They could be totally or partially independent of excitatory-inhibitory modulation in BLA that is recruited by stress. The function of GAD67 in this context needs further review and testing of its necessity/sufficiency within the BLA on anxiety in presence of EE.

3.3 Stress conditionally reduces expression of pro-BDNF protein in the BLA in absence of EE but not in its presence.

With respect to the complex dendritic reorganisation (particularly dendritic-spine plasticity) observed in the BLA in response to chronic stress and EE, the next

molecular target of interest was brain derived neurotrophic factor (BDNF) and its cognate receptor tyrosine kinase receptor B (TrkB).

BDNF is a growth factor that promotes survival and dendritic growth in various cell types [384-391]. The BDNF/TrkB molecular pathway controls synaptic plasticity via spine modulation [162, 392]. In humans, under conditions such as MDD and bipolar disorder, hippocampal pro-BDNF levels are significantly lowered [393]. Chronic restraint stress leads to an increase in the BDNF protein expression of BLA neurons reciprocal to its decrease in the hippocampus [105]. Enhancement of fear memories via glucocorticoids also shows lowered BDNF/pro-BDNF levels in the hippocampus [167]. Similar upregulation of BDNF protein expression in the BLA is observed when adult rats are exposed to chronic social-instability stress. In the same model, TrkB increases (recovers) post-stress exposure. EE on the other hand, is known to upregulate the expression of BDNF and cause no change in TrkB protein levels in hippocampus. However, no study has shown a comparable BDNF profile for BLA [272, 273].

In this current study, neither stress nor EE modulated pro-BDNF or TrkB in the BLA. The stress effect on pro-BDNF protein in animals was not observed unlike previous literature perhaps owing to a small sample size (n=5). Another possible reason for the absence of this effect could be the ineffectiveness of CIS in providing stress to these animals or the recovery period that followed it (~7 days, Fig 5A inset) that normalised the protein levels in BLA. However according to comparisons within the differential housing conditions (orthogonal planned comparisons), in presence of EE the pro-BDNF levels of stressed individuals were lower than that of non-stressed ones. This difference in pro-BDNF levels was absent between non-stressed and stressed individuals under normal housing. Such a molecular pattern is perfectly in line with

the spine-pattern in both the primary and secondary dendrites of the BLA neurons, indicating that alterations in the pro-BDNF levels in these neurons might be associated with the spine plasticity in presence of EE. This further could be linked with the lower GR activation that also plays a role in modulating BDNF signalling in facilitating synaptic plasticity via spine formation/regression [109, 162, 164, 394]. Glucocorticoids secreted during stress bind to GR, leading to phosphorylation of TrkB receptors (cognate to BDNF). Higher BDNF signalling results in enhanced synaptic plasticity via spine formation. In the presence of heightened stress, this process would accelerate, giving rise to a feedforward cascade driven by circulating CORT, higher activation of GRs and aggravated BDNF signalling [110]. However, in our model, EE lowers circulating CORT, leading to lowered GR activation. Therefore it attenuates the pro-BDNF signalling, reflected in the expression of the protein itself in stressed animals housed in EE. TrkB also does not show any increase, possibly indicating a heightened stress response by upregulating the receptor concentration. Stress has an effect on phosphorylation of the TrkB protein which indicated the degree of BDNF-signalling [395]. In this model, phosphorylation of TrkB could possibly be modulated by EE which could also be playing a role in modulating the degree of BDNF-signalling.

Short-term EE therefore robustly leads to a cascade of transitions ranging from behaviour to neuronal plasticity to stress-mediating molecular pathways. This study demonstrates that EE lowers circulating CORT and downregulates the activation of its cognate receptor (GR) in stressed individuals which might further influence BDNF signalling. Downregulated BDNF proteins in stressed animals housed in EE perhaps are associated with the dendritic remodelling in the BLA in the form of lowered spines and branching. This type of neuronal rearrangement within the BLA attenuates the

hyper-responsive positive feedback provided to the HPA axis and protects the EE-exposed animals from anxiety-inducing effects of chronic stress. Yet another pattern worth observing is the putative commonalities of EE with inoculation stress [396]. Inoculation stress in the form of constant exposure to novelty, social interaction with conspecifics and exercise (the defining factors of EE) can putatively emulate effects of chronic mild stress in the form of lowered CORT [328, 397-399]. This is still a hypothesis which needs further validation. Currently existing evidences for parallels between chronic mild stress and EE are observed largely in case of rats exposed to EE right after weaning (at P21) for longer durations (≥ 30 days). It is also essential to note that the morphological changes in the BLA neurons and its corresponding protein levels of GR, GAD67 and BDNF have an association with stress-resilient behaviour in EE-housed stressed individual. These constitute as important factors playing a role in modulation of stress-axis in response to differential housing. However, this study does not throw light upon the causal effects for this resilient behaviour observed in these rats. In order to determine the sufficiency or necessity of these factors, causing to lower anxiety/depressive-like symptoms, it is important to test the behaviour via pharmacological, knock-down or overexpression of these factors in rats systematically. For example, providing excitation to BLA neurons pharmacologically or optogenetically in rats under EE-housing should still sustain stressful behaviour in the form of heightened anxiety. Similar effect could be seen upon BLA-specific overexpression of BDNF in EE-housing condition. Such experiments are currently beyond the scope of this study.

In summary, the animal model for resilience obtained in this study using EE showed two main features of stress-coping in the form of:

- a. Recovery from anxiety and enhancement of active coping as established by EPM trials.
- b. Recovery from hyperactivity at resting levels as established by LABORAS.

According to morphological analysis in these animals, branching in the BLA excitatory neurons returned to normalcy (absence of stress-induced increase). They also showed lowered spine-density in excitatory neurons of the BLA compared to their non-stressed counterparts. Similar analysis in these stressed animals also showed reorganization in CA3 hippocampal neurons in the form of hypertrophy and increased spine-density in EE as opposed to standard-cage housing. In EE-housed stressed animals the IL neurons of the mPFC, were rescued from spine-loss at distal regions, an effect which did not occur in standard housing. At a molecular level, stressed animals were showed lowered expression of activated GR and BDNF protein (in line with the spine-density pattern) within the BLA, compared to their non-stressed counterparts under EE housing. Such a difference between non-stressed and stressed individuals was absent in standard housing.

On the other hand, certain findings in this study indicated that not all changes in these animals can be rendered as stress-resilience traits. It is important to note that the specific stress-resilience induced by EE in these animals may not encompass lowered depressive-like symptoms (in the form of learned helplessness in FST) or lower activity at resting levels (in the form of immobility in LABORAS) since these features were comparable to their non-stressed counterparts just as in standard-cage housing. Such patterns of comparison between non-stressed and stressed individuals for these parameters were similar in both standard-cage and EE housing conditions. However, in this study the levels of depressive-like symptoms was lowered and ease of habituation was increased under EE housing, irrespective to stress-exposure (as

indicated by the statistical main effect of EE-housing in Results: 3 and 4; discussed under specific aim 1 – page 117, line 6-20). These animals were also characterized by lowered CORT. At the morphological level, EE-housed animals also showed hypertrophy in PrL neurons. At a molecular level, these animals had lower levels of total GR protein and expression of GAD67-negative synapsin protein (index for excitability of the BLA) within the BLA. Such changes perhaps are associated with optimally lowered activity of BLA under EE housing. These overall effects may be associated with enabling EE-housed animals to fine-tune the HPA axis regulation (irrespective of stress-exposure).

CONCLUSION

The key findings of this study and their implication are summarised as follows:

Specific aim 1: To evaluate the effects of EE on stress-related behavioural outcomes: developing rodent model of stress-resilience.

- Short-term environmental enrichment induced stress-resilience in animals by countermanding the behavioural effects of chronic stress in the form of lowered anxiety (normal exploration on EPM) and lowered hyperactivity at a resting state (normal locomotion in LABORAS)
- Short term EE lowered the levels of depressive-like symptoms (lowered immobility in FST).
- It also lowered the levels of ease of habituation in a non-stimulatory environment (higher immobility in LABORAS).
- The circulating corticosterone was also lowered by EE irrespective of stress-exposure. These effects indicate an overall well-regulated HPA axis with a well-adaptive feedback response induced by EE housing.

Specific aim 2: To investigate characteristic neuronal morphology associated with stress-resilience induced by EE.

- At the morphological level, EE-induced stress-resilience is characterized by complex dendritic reorganization within the BLA, hippocampus and mPFC of stressed subjects. It lowered spine-density and rescued the BLA neurons from undergoing stress-induced increase in dendritic branching. EE-housed stressed animals showed hypertrophy and increase in spines in hippocampal CA3 neurons. In these animals, the IL neurons were rescued from stress-induced loss of spines in the distal regions.

- Animals under EE-housing exhibited neuronal hypertrophy and increase in spines in the PrL neurons of the mPFC. The effect of EE in the mPFC along with the hippocampus might influence their responsiveness in providing negative feedback to the HPA axis.

Specific aim 3: To investigate the molecular regulation associated with stress-resilience induced by EE.

- At the molecular level, EE-induced stress-resilience is characterized by lowered activation of GR and expression of pro-BDNF protein within the BLA, which is in line with lowered number of spines seen in this region.
- Stress aggravated excitation and lowered inhibitory presynaptic proteins within the BLA in absence of EE. However in presence of EE, stress could not substantiate this. This demonstrates the putative influence of EE on modulating the inhibitory circuit within the BLA.
- Activation of GR (nuclear-localization) within the BLA of stressed subjects was reduced in EE housing but not otherwise. The total GR protein expression was also lowered in subjects housed in EE. This could be one of the key factors for optimally regulating the positive feedback to HPA, circumventing its hyperactivation.

Therefore, EE leads to an array of robust effects in the form of modulations in molecules important for stress-reactivity such as GR, pro-BDNF, GAD67 to CORT (HPA axis), and associated neuronal plasticity in BLA in the form of branching and spines. Coupled with its effects on the inhibitory circuit within the BLA, short-term EE renders protection from stress-induced behavioural pathologies such as anxiety in adulthood.

SUPPLEMENTARY INFORMATION

1. Effect of EE and stress on Central amygdala

Background

Central amygdala (CeA) has been found to have direct afferent and efferent connections with hypothalamus and brainstem in the stress pathway [39, 400, 401]. CeA also receives glutamatergic input from the BLA [401]. This indicates that CeA plays a role in mediating fear-responses downstream to BLA. In this study the status of neuronal morphology in CeA with respect to CIS and EE were tested.

Methods:

As explained in section Materials and Methods (Dendritic arborisation, page 45-48) the dendritic arborisation of neurons from CeA (Bregma -1.44 to -2.92, The Rat Brain, Paxinos and Watson) was studied by analysing the total number of branch points, dendritic length and number of spines (in 60µm). I mentored Pei Wern (NTU undergraduate student) in tracing the neurons, counting spines and analysing them for retrieving the relevant end points.

Results:

In the CeA, two-way ANOVA revealed that main effect of stress ($F_{(1,273)} = 18.51, p < 0.001$) but not EE ($F_{(1,273)} = 1.42, ns$) was statistically significant for total number of branch points. A significant interaction between stress and EE ($F_{(1,273)} = 38.25, p < 0.001$) was also observed. Stress resulted in increase in the number of branch points in the CeA neurons.

Orthogonal planned comparisons (independent sample t-test) revealed that stress increased number of branch points in CeA neurons in the absence (Figure S.1A left

panel, left; $\Delta\bar{x} = -2.52 \pm 0.34$; $t_{145} = -7.30$, $p < 0.001$) but not in the presence (Figure S.1A left panel, right; $\Delta\bar{x} = 0.45 \pm 0.33$; $t_{128} = 1.37$, ns) of EE.

Both stress ($F_{(1,271)} = 20.16$, $p < 0.001$) and EE ($F_{(1,271)} = 5.44$, $p = 0.02$) treatments resulted in significant main effects of total dendritic length during ANOVA. The interaction between stress and EE also had a statistical significance ($F_{(1,271)} = 134.21$, $p < 0.001$). Stress resulted in a significant increase in the dendritic length of the CeA neurons and EE prevented this effect. Orthogonal planned comparison showed that stress increased dendritic length of CeA neurons in absence (Figure S.1A right panel, left; $\Delta\bar{x} = -361.64 \pm 31.35$; $t_{144} = -11.57$, $p < 0.001$) of EE but reduced the same in its presence (Figure S.1A right panel, right; $\Delta\bar{x} = 160.07 \pm 32.34$; $t_{127} = 4.95$, $p < 0.001$). Stress led to opposite effects in neuronal morphology depending on EE housing.

S.1A)

Figure S.1A. Effect of stress on dendritic complexity of CeA neurons in absence and presence of EE: Stress significantly increased the total number of branch points in absence (left, uncoloured bars) of EE but not in presence (right, grey bars) of EE (**left panel**). Stress significantly increased the total dendritic length in absence (left, uncoloured bars) of EE but reduced the same in presence (right, grey bars) of EE (**right panel**). Graphs represent Mean $\pm$ SEM. Both left and right panel: Bars represent Control (N=8), Stress (N=7), EE (N=7) and

EEST (N=6), 9-11 neurons/animal, *** $p < 0.001$, independent sample t-test. Schedule of the experiment same as Fig. 5A (Results).

Spines were also analysed in the CeA neurons for a stretch of 60 μ m in primary dendrites. Two-way ANOVA revealed that main effects of stress ($F_{(1,172)} = 4.39$, $p = 0.038$) but not EE ($F_{(1,172)} = 0.23$, ns) was statistically significant for spine density in primary dendrites of CeA neurons. A significant interaction between the two treatments ($F_{(1,172)} = 51.71$, $p < 0.001$) was also observed. Stress resulted in reduction of spine density in the primary dendrites of the CeA neurons while EE had a reverse effect. Orthogonal planned comparisons revealed that spine density was significantly reduced by stress in absence (Figure S.1B left; $\Delta\bar{x} = 5.60 \pm 0.81$; $t_{90} = 6.87$, $p < 0.001$) of EE, however it increased the same in presence (Figure S.1B right; $\Delta\bar{x} = -3.07 \pm 0.89$; $t_{82} = -3.44$, $p = 0.001$) of EE.

S.1B)

Figure S.1B. Effect of stress on total dendritic spines (in 60 μ m) of primary dendrites in CeA neurons in absence and presence of EE: Stress significantly decreased the number of spines in the primary dendrites of CeA neurons in absence (left) of EE but significantly increased the same in presence (right) of EE. Graphs represent Mean $\pm$ SEM. Bars represent Control (N=8), Stress (N=7), EE (N=7) and EEST (N=6), 6-8 neurons/animal, *** $p < 0.001$, independent sample t-test. Schedule of the experiment same as Fig. 5A (Results).

Conclusion:

In this study, we observed that stress induced hypertrophy in neurons of CeA while EE prevented the same. Dendritic spines in these neurons were decreased in stressed animals compared to non-stressed animals without EE. This effect was inversed in EE housing wherein, the dendritic spines were increased in stressed animals compared to non-stressed. The decrease in spines in hypertrophied neurons of CeA in stressed animals (devoid of EE) could be a compensatory mechanism for retaining the net responsiveness and prevent hyper-excitability in these neurons. This effect on CeA might be a reflection of dynamic neuronal-network adjustment associated with BLA neuronal plasticity, which sends projections to CeA for mediating fear responses [400].

2. Effect of EE and stress on BNST

Background

Bed nucleus of Stria terminalis (BNST) has been found to have direct neuronal connections with hypothalamus and BLA in the stress pathway [38, 402]. Hence it is involved in mediating anxiety and related stress-responses downstream to BLA. In this study the status of neuronal morphology in BNST with respect to CIS and EE were tested.

Methods:

As explained in section Materials and Methods (Dendritic arborisation, page 45-48) the dendritic arborisation of neurons from BNST (Bregma -0.20 to -0.80, The Rat Brain, Paxinos and Watson) was studied by analysing the total number of branch points, dendritic length and number of spines (in 60µm). I mentored Zhou Wei (NTU

undergraduate student), in tracing the neurons and analysing them for retrieving the relevant end points. I also mentored Darshinee Raman (NTU undergraduate student), in counting spines and analysing them for retrieving the relevant end points in this project.

Results:

In the BNST, two-way ANOVA revealed significant main effect of stress ($F_{(1,126)} = 13.08, p < 0.001$) but not EE ($F_{(1,126)} = 1.48, ns$) for total number of branch points. No significant interaction between stress and EE ($F_{(1,126)} = 0.88, ns$) was observed. Stress caused number of branch points in the BNST neurons to increase.

Orthogonal planned comparisons revealed that stress increased number of branch points in BNST neurons in the absence (Figure S.2A left panel, left; $\Delta\bar{x} = -1.45 \pm 0.38$; $t_{76} = -3.8, p < 0.001$) but not in the presence (Figure S.2A left panel, right; $\Delta\bar{x} = -0.85 \pm 0.54$; $t_{50} = -1.59, ns$) of EE.

Additionally, two-way ANOVA revealed that main effect of stress ($F_{(1,126)} = 23.99, p < 0.001$) but not EE ($F_{(1,126)} = 0.93, ns$) was statistically significant for total dendritic length of BNST neurons. A significant interaction between stress and EE ($F_{(1,126)} = 15.402, p < 0.001$) was also observed. Stress resulted in a significant increase in the dendritic length of the BNST neurons. Orthogonal planned comparison showed that stress increased total dendritic length of BNST neurons in absence (Figure S.2A right panel, left; $\Delta\bar{x} = -244.53 \pm 35.34$; $t_{76} = -6.92, p < 0.001$) of EE but not in the presence (Figure S.2A right panel, right; $\Delta\bar{x} = -26.97 \pm 43.12$; $t_{50} = -0.63, ns$) of the EE.

S.2A)

Figure S.2A. Effect of stress on dendritic complexity of BNST neurons in absence and presence of EE: Stress significantly increased the total number of branch points in absence (left, uncoloured bars) of EE but not in presence (right, grey bars) of EE (**left panel**). Stress significantly increased the total dendritic length in absence (left, uncoloured bars) of EE but not in presence (right, grey bars) of EE (**right panel**) Graphs represent Mean $\pm$ SEM. Both left and right panel: Bars represent Control (N=8), Stress (N=6), EE (N=7) and EEST (N=5), 4-6 neurons/animal, *** $p < 0.001$, independent sample t-test. Schedule of the experiment same as Fig. 5A (Results).

Spines were analysed in the BNST neurons for a stretch of 60μm in primary dendrites. Two-way ANOVA revealed that main effect of stress ($F_{(1,174)} = 21.78$, $p < 0.001$) but not EE ($F_{(1,174)} = 0.61$, ns) was statistically significant on spine density in primary dendrites of CeA neurons. A significant interaction between the two treatments ($F_{(1,174)} = 80.73$, $p < 0.001$) was also observed. Stress resulted in reduction of spine density in the primary dendrites of the BNST neurons while EE had a reverse effect. Orthogonal planned comparisons revealed that spine density was significantly reduced by stress in absence (Figure S.2B left; $\Delta\bar{x} = 4.51 \pm 1.14$; $t_{98} = 3.95$, $p < 0.001$) of EE, however it led to a significant increase in the same in presence (Figure S.2B right; $\Delta\bar{x} = -14.28 \pm 1.86$; $t_{76} = -7.67$, $p < 0.001$) of EE.

S.2B)

Figure S.2B. Effect of stress on total dendritic spines (in 60μm) of primary dendrites in BNST neurons in absence and presence of EE: Stress significantly decreased the number of spines in the primary dendrites of BNST neurons in absence (left) of EE but significantly increased the same in presence (right) of EE. Graphs represent Mean ± SEM. Bars represent Control (N=7), Stress (N=6), EE (N=7) and EEST (N=5), 4-10 neurons/animal, *** $p < 0.001$, independent sample t-test. Schedule of the experiment same as Fig. 5A (Results).

Conclusion:

In this study, we observed that stress induced hypertrophy in neurons of the BNST while EE had no effect. Dendritic spines in these neurons were decreased in stressed animals compared to non-stressed animals without EE. This effect was inversed in EE housing wherein, the same was increased in stressed animals compared to non-stressed. The decrease in spines in hypertrophied neurons in stressed animals (devoid of EE) could be a compensatory mechanism for retaining the net responsiveness and prevent hyper-excitability in these neurons, similar to the effect seen in BNST. This effect on BNST might be a reflection of dynamic neuronal-network adjustment associated with BLA neuronal plasticity, which sends projections to BNST for mediating fear responses [38].

3. Effect of EE and stress on pro-BDNF and TrkB protein expression in the medial prefrontal cortex

Background

In the current model, EE had a pronounced effect on mPFC neurons in increasing dendritic arborisation in PrL neurons (Fig 9A) and spine density in both PrL (Fig 10A) and IL neurons (Fig 10B). Therefore, neurotrophic factor such as Brain-derived neurotrophic factor (pro-BDNF) and its cognate receptor: Tyrosine receptor Kinase B (TrkB) were quantified for mPFC region in these animals.

Methods:

As explained in section Materials and Methods (Western Blotting, page 52-53), protein expression of pro-BDNF and TrkB in the mPFC region (Bregma 3.72 to 3.00, The Rat Brain, Paxinos and Watson) was quantified. β -actin was used as the internal control for these samples.

Results

Expression of both the proteins was retrieved in the form of band intensity (OD value) normalized with β -actin. Two-way ANOVA revealed that there was no significant main effect of either stress ($F_{(1,16)} = 2.80$, *ns*) or EE ($F_{(1,16)} = 0.44$, *ns*) on pro-BDNF protein expression. There was no significant interaction between the two treatments ($F_{(1,16)} = 0.80$, *ns*). Both stress and EE had no effect in modulating pro-BDNF protein expression. Orthogonal planned comparisons revealed that pro-BDNF remained significantly unaffected by stress in both absence (Figure S.3 left panel, left; $\Delta\bar{x} = 1.25$

± 0.90 ; $t_8 = 1.39$, *ns*) and in presence (Figure S.3 left panel, right; $\Delta\bar{x} = 0.38 \pm 0.38$; $t_8 = 1.0$, *ns*) of EE.

Two-way ANOVA revealed that there was no significant main effect of either stress ($F_{(1,20)} = 0.21$, *ns*) or EE ($F_{(1,20)} = 0.27$, *ns*) on TrkB protein expression. There was no significant interaction between the two treatments ($F_{(1,20)} = 0.27$, *ns*). Neither stress nor EE had any effect in altering TrkB receptor protein expression. Orthogonal planned comparisons revealed that TrkB remained significantly unaffected by stress both in absence (Figure S.3 right panel, left; $\Delta\bar{x} = -0.14 \pm 0.23$; $t_{10} = -0.60$, *ns*) and presence (Figure S.3 right panel, right; $\Delta\bar{x} = 0.01 \pm 0.16$; $t_{10} = 0.05$, *ns*) of EE.

S.3)

Figure S.3. Effect of stress on pro-BDNF and TrkB protein expression within the mPFC in absence and presence of EE: Stress has no effect on the protein expression of pro-BDNF (left panel) or TrkB (right panel) in the mPFC either in the absence (left, uncoloured bars) or presence (right, grey bars) of EE. Both left and right panel: Graphs represent Mean $\pm$ SEM. Both left and right panel: Bars represent Control (CN, N=5), Stress (ST, N=5), EE (EE, N=5) and EEST (EEST, N=5). Schedule of the experiment same as Fig. 13B (Results).

Conclusion

EE or stress did not modulate the pro-BDNF and TrkB protein levels in the mPFC. Though previous studies suggest a downregulation in the BDNF protein levels in mPFC in response to chronic stress [110], the comparable pro-BDNF levels across groups could putatively be due to the recovery phase post-stress cessation (7 days)

4. Effect of EE and stress on on exploratory behaviour using Open-field (OFT)

Background and Method

Open field test is also a validated behavioural test for measuring exploratory behaviour and anxiety in rodents. The animals were tested for anxiety in an open field test (OFT, customized; conducted 09:00-12:00 hours). The behaviour was videotaped remotely and coded before analysis. The OFT is a square arena (100cm × 100cm × 30 cm) made of black Plexiglas base with transparent walls with the surface divided into nine equal squares (classified as inner zone and outer zone). The apparatus was illuminated by 10 lux lighting in the center and 3-4 lux at the corners. The animal was placed at the centre at the start of trial (trial duration = 5 minutes). As an index for exploratory behaviour, time spent in the centre (one-fourth of the entire arena) and periphery of the open-field was measured. Time spent in the centre indicated exploratory behaviour and time spent in the periphery indicated the thigmotactic behaviour of these animals. Trials were video recorded and analysed offline.

Results

Time spent in the centre indicated exploratory behaviour and time spent in the periphery indicated the thigmotactic behaviour of these animals. Two-way ANOVA revealed that neither EE ($F_{(1,45)} = 3.18$, *ns*) nor stress ($F_{(1,45)} = 1.86$, *ns*) had a significant main effect on time spent in the centre. The interaction between stress and EE also failed to reach statistical significance ($F_{(1,45)} = 2.33$, *ns*), showing they had no effect on the exploratory behaviour of these animals.

Orthogonal planned comparisons revealed that time spent in the centre was significantly lowered by stress in absence (Figure S.4A; $t_{31} = 2.43$, $p = 0.021$) of EE but not in its the presence (Figure S.4A; $t_{14} = 0.56$, *ns*).

Two-way ANOVA revealed that neither EE ($F_{(1,45)} = 3.18$, *ns*) nor stress ($F_{(1,45)} = 1.86$, *ns*) had a significant main effect on time spent in the periphery. The interaction between stress and EE also failed to reach statistical significance ($F_{(1,45)} = 2.33$, *ns*). Thus, stress and EE had no effect on the thigmotactic behaviour of these animals in the open-field.

Orthogonal planned comparisons revealed that time spent in the periphery was significantly increased by stress in absence (Figure S.4B; $t_{31} = -2.43$, $p = 0.016$) of EE but not in the presence of EE (Figure S.4B; $t_{14} = -0.56$, *ns*).

S.4A)

Figure S.4A. Effect of stress on time spent in the centre of open field in absence and presence of EE: Stress significantly reduced the time spent in the centre of the open field in the absence (left) of EE but not in the presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=19), Stress (N=15), EE (N=8) and EEST (N=8); * $p < 0.05$, independent sample t-test. Inset: Schedule of the experiment.

S.4B)

Figure S.4B. Effect of stress on time spent in the periphery of open field in absence and presence of EE: Stress significantly increased the time spent in the periphery of the open field in the absence (left) of EE but not in the presence of EE (right). Graphs represent Mean $\pm$ SEM. Bars represent Control (N=19), Stress (N=15), EE (N=8) and EEST (N= 8); * $p < 0.05$, independent sample t-test. Schedule of the experiment same as Fig. 1A.

Conclusion

Stressed individuals devoid of EE housing showed lowered centre exploration and increased thigmotaxis indicating anxiety. This difference in exploration was not observed between stressed and unstressed animals in the presence of EE. Other studies in fact have shown reduced locomotion (faster habituation) of EE-exposed rats in an open field [329, 403]. This might also indicate of OFT to be a non-optimal test for exploratory response in EE rats and might not be a suitable test to measure exploration or anxiety in these animals.

5. Mean and SEM of tested variables for all 4 groups:

Table S.1. Mean and SEM of all tested variables

Variables	Group	Mean	SEM
% Open arm exploration	Control	23.21	5.34
	Stress	2.63	1.74
	EE	28.20	3.47
	EEST	24.18	2.69
% closed arm exploration	Control	76.79	5.34
	Stress	97.37	1.74
	EE	71.18	3.47
	EEST	75.82	2.69
Closed arm entries (n)	Control	6.24	0.65
	Stress	7.67	1.31
	EE	7.63	0.63
	EEST	9.88	0.91
% Open arm entries	Control	24.61	5.03
	Stress	3.77	2.51
	EE	35.43	3.46
	EEST	30.50	2.17
% Open arm time	Control	21.81	5.86
	Stress	1.49	0.97
	EE	20.97	4.27
	EEST	17.85	3.38
Head dips (n)	Control	5.06	0.79
	Stress	0.83	0.48
	EE	11.88	1.73
	EEST	6.00	0.85
Sucrose intake test (%Δsucrose/body weight)	Control	3.17	0.28
	Stress	1.47	0.36
	EE	0.62	0.23
	EEST	1.19	0.51
FST (immobility) (s)	Control	277.50	47.67
	Stress	343.75	28.37

	EE	197.50	35.36
	EEST	237.50	63.24
Latency to immobility in FST (s)	Control	103.63	24.98
	Stress	59.38	10.48
	EE	63	13.58
	EEST	62.83	13.11
FST (swimming) (s)	Control	95.25	14.25
	Stress	63.25	10.04
	EE	108.13	12.79
	EEST	79.50	17.21
Immobility in LABORAS (s)	Control	23.25	10.88
	Stress	22.25	9.69
	EE	83.50	27.03
	EEST	54.13	25.27
LABORAS in Locomotion (s)	Control	35.63	4.35
	Stress	56.50	7.57
	EE	28.50	5.38
	EEST	34.38	6.72
Total no. of branch points (BLA)	Control	8.40	0.40
	Stress	10.42	0.55
	EE	8.29	0.41
	EEST	8.49	0.35
Total dendritic length (BLA) (μm)	Control	1416.21	46.89
	Stress	1714.37	61.11
	EE	1574.00	45.64
	EEST	1965.16	53.95
Branch points at a radial distance of 0-100 μm (BLA)	Control	7.00	0.34
	Stress	8.20	0.39
	EE	7.20	0.36
	EEST	6.51	0.27
Branch points at a radial distance of 100-200 μm (BLA)	Control	1.28	0.15
	Stress	1.80	0.22
	EE	1.00	0.13
	EEST	1.79	0.17

Primary spines (in 60µm) (BLA)	Control	39.88	1.45
	Stress	40.41	1.24
	EE	39.65	1.71
	EEST	34.90	1.12
Secondary spines (in 60µm) (BLA)	Control	41.54	1.40
	Stress	40.33	1.22
	EE	41.15	2.42
	EEST	34.59	1.03
Total no. of branch points (Hippocampus)	Control	17.82	1.35
	Stress	20.55	1.04
	EE	15.82	0.66
	EEST	20.79	0.94
Total dendritic length (µm) (Hippocampus)	Control	2783.11	161.68
	Stress	3023.43	141.67
	EE	2602.42	79.46
	EEST	3663.07	232.77
Primary spines (in 60µm) (Hippocampus)	Control	33.88	1.03
	Stress	25.08	0.76
	EE	30.34	0.89
	EEST	34.07	1.05
Total no. of branch points (Prelimbic region)	Control	19.75	1.03
	Stress	19.98	0.74
	EE	22.62	1.03
	EEST	24.33	1.25
Total dendritic length (µm) (Prelimbic region)	Control	2582.96	76.23
	Stress	2783.91	95.48
	EE	3326.07	123.05
	EEST	3549.58	132.11
Primary spines (in 80µm) (Prelimbic region)	Control	56.57	1.31
	Stress	58.63	1.29
	EE	83.97	2.86
	EEST	79.59	2.39
Secondary spines (in 80µm) (Prelimbic region)	Control	53.64	1.25
	Stress	53.86	1.58

	EE	79.78	2.25
	EEST	71.45	1.97
Total no. of branch points (Infralimbic region)	Control	4.95	0.27
	Stress	6.29	0.50
	EE	4.92	0.33
	EEST	4.92	0.39
Total dendritic length (µm) (Infralimbic region)	Control	1109.26	44.10
	Stress	1170.15	38.41
	EE	1138.38	41.33
	EEST	1169.32	40.68
Primary spines (in 60µm) (Infralimbic region)	Control	55.93	0.83
	Stress	39.36	0.76
	EE	73.15	1.62
	EEST	61.97	1.68
Secondary spines (in 60µm) (Infralimbic region)	Control	53.76	1.15
	Stress	38.45	0.63
	EE	70.60	1.06
	EEST	68.80	1.69
Serum CORT (Day 1, ng/ml)	Control	83.96	14.20
	Stress	159.30	18.99
	EE	42.02	15.93
	EEST	53.78	13.63
Serum CORT (Day 3, ng/ml)	Control	6.33	1.24
	Stress	9.70	1.73
	EE	19.60	4.07
	EEST	10.12	3.41
Serum CORT (Day 20, ng/ml)	Control	83.90	14.62
	Stress	66.62	14.49
	EE	52.47	7.04
	EEST	42.40	4.82
Adrenal weights (mg)	Control	63.50	3.04
	Stress	77.25	5.69
	EE	69.75	3.38
	EEST	70.00	3.30

GAD67-Synapsin colabels (BLA) (puncta/given area)	Control	65.89	8.76
	Stress	32.20	5.76
	EE	32.80	4.73
	EEST	36.53	6.16
GAD67 negative Synapsin (BLA) (puncta/given area)	Control	237.2	19.69
	Stress	215.58	23.12
	EE	163.7	17.04
	EEST	173.83	14.71
% Excitation (BLA)	Control	78.16	2.48
	Stress	86.86	1.88
	EE	84.02	1.33
	EEST	84.85	2.00
% Inhibition (BLA)	Control	21.84	2.48
	Stress	13.14	1.88
	EE	15.98	1.33
	EEST	15.15	2.00
% GR-DAPI colabel/DAPI (BLA)	Control	42.65	5.46
	Stress	41.54	5.32
	EE	37.69	3.52
	EEST	27.78	3.14
Normalised GR Band Intensity (OD) (BLA)	Control	1.99	0.22
	Stress	1.24	0.36
	EE	1.15	0.29
	EEST	0.80	0.12
Normalised pro-BDNF Band Intensity (OD) (BLA)	Control	1.68	0.63
	Stress	1.27	0.23
	EE	1.61	0.16
	EEST	0.94	0.13
Normalised TrkB Band Intensity (OD) (BLA)	Control	1.44	0.27
	Stress	1.60	0.27
	EE	1.19	0.18
	EEST	1.12	0.26
% Body Weight gain	Control	38.09	1.16
	Stress	26.72	1.93

	EE	38.81	1.48
	EEST	26.85	3.78
Total no. of branch points (CeA)	Control	2.91	0.18
	Stress	5.43	0.31
	EE	4.11	0.25
	EEST	3.66	0.21
Total dendritic length (µm) (CeA)	Control	490.10	15.62
	Stress	875.00	36.46
	EE	714.23	29.73
	EEST	538.77	17.42
Primary spines (in 60µm) (CeA)	Control	46.74	0.60
	Stress	41.14	0.53
	EE	42.12	0.65
	EEST	45.19	0.61
Total no. of branch points(BNST)	Control	2.76	0.19
	Stress	4.21	0.38
	EE	2.67	0.34
	EEST	3.52	0.42
Total dendritic length (BNST) (µm)	Control	399.47	12.22
	Stress	644.00	41.19
	EE	534.94	26.53
	EEST	561.92	34.46
Primary spines (in 60µm) (BNST)	Control	38.21	0.79
	Stress	33.63	0.83
	EE	29.56	0.91
	EEST	43.85	1.63
Normalised pro-BDNF Band Intensity (OD) (mPFC)	Control	1.98	0.88
	Stress	0.73	0.19
	EE	1.22	0.28
	EEST	0.84	0.26
Normalised TrkB Band Intensity (OD) (mPFC)	Control	0.74	0.17
	Stress	0.88	0.16
	EE	0.74	0.11
	EEST	0.73	0.12

Time spent in centre in OFT (s)	Control	16.32	5.11
	Stress	2.80	1.22
	EE	4.63	1.81
	EEST	3.50	0.87
Time spent in periphery in OFT (s)	Control	283.68	5.11
	Stress	297.20	1.22
	EE	295.38	1.81
	EEST	296.50	0.87

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 50µm), with soma as the centre, is formed by applying scaling-factor (determined through microscope).

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

JAVA code written for the macro:

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

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

Note: $s = \text{pixels/unit micron} = 5.885$ (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 and specifications for tissue preparation for western blotting

5.1 Lysis buffer

150mM sodium chloride, 0.1% Triton-X 100, 50mM Tris, pH: 8.0 (50µl/mg used for tissue suspension).

5.2 Sonication

Glass-homogenised tissue was sonicated (Diagenode bioruptor, NJ, USA) at 2.5 minutes (pulse on/off every 30s interval) at medium amplitude at 4°C.

6. Reagents for SDS/PAGE

6.1 Resolving gel

M Tris Cl, 9.0825g of Tris base in 50 ml in deionized water (pH 8.8).

6.2 Stacking gel

1M Tris Cl, 6.055g of Tris base in 50 ml in deionized water (pH 6.8).

6.3 Polyacrylamide gel (12%)

Reagents	Resolving gel (15ml)	Stacking gel (10ml)	Source
Deionized water	6.45ml	7.3ml	SBS facility, NTU
40% Acrylamide/Bis Solution	4.5ml	1.25ml	Bio-Rad, CA, USA
Tris (1.5M; pH 8 for resolving buffer) (1.0M; pH 6.8 for stacking buffer)	3.75ml	1.25ml	Home-made
10% Sodium dodecyl sulphate solution	150µl	100µl	Home-made

10% Ammonium persulphate solution	150µl	100µl	Home-made
TEMED	6µl	10µl	Bio-Rad, CA, USA

6.4 10X PAGE buffer (100ml; pH 8.3)

3.03g Tris Base, 14.4g Glycine and 1g Sodium dodecyl sulphate (SDS) dissolved in deionized water (made upto 100ml).

6.5 Sample loading buffer (100µl)

95µl of Laemmli sample buffer (Bio-Rad, CA, USA) mixed with 5µl of β-mercaptoethanol (5µl was added for total volume of 35µl of protein sample load).

6.6 10% Sodium dodecyl sulphate (SDS) solution (100ml)

10g of SDS dissolved in 100ml of deionized water.

6.7 10% Ammonium persulphate (APS) solution (10ml)

1g of APS dissolved in 100ml of deionized water

7. Reagents for Western blotting

7.1 1X Transfer buffer (1L)

3g Tris base, 14.4g Glycine and 200ml Methanol in deionized water (made upto 1L)

7.2 10X TBS (pH 7.6; 1L)

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

7.3 1X TBST

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

7.4 5% blocking buffer (20ml)

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

7.5 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.

7.6 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, 350 μ l of β mercapto-ethanol was added just before use

7.7 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.

REFERENCES

1. McEwen, B.S., *Stress, adaptation, and disease. Allostasis and allostatic load*. Ann N Y Acad Sci, 1998. **840**: p. 33-44.
2. McEwen, B.S. and J.C. Wingfield, *The concept of allostasis in biology and biomedicine*. Hormones and Behavior, 2003. **43**(1): p. 2-15.
3. Herman, J.P. and W.E. Cullinan, *Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis*. Trends Neurosci, 1997. **20**(2): p. 78-84.
4. McEwen, B.S., *Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators*. Eur J Pharmacol, 2008. **583**(2-3): p. 174-85.
5. Sapolsky, R.M., *Why Zebras Dont Get Ulcers*. 2004.
6. Goldstein, D.S., *Adrenaline and Noradrenaline*, in *eLS*. 2001, John Wiley & Sons, Ltd.
7. Ondicova, K. and B. Mravec, *Multilevel interactions between the sympathetic and parasympathetic nervous systems: a minireview*. Endocr Regul, 2010. **44**(2): p. 69-75.
8. Thayer, J.F. and E. Sternberg, *Beyond heart rate variability: vagal regulation of allostatic systems*. Ann N Y Acad Sci, 2006. **1088**: p. 361-72.
9. Munck, A., P.M. Guyre, and N.J. Holbrook, *Physiological functions of glucocorticoids in stress and their relation to pharmacological actions*. Endocr Rev, 1984. **5**(1): p. 25-44.
10. Vale, W., et al., *Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin*. Science, 1981. **213**(4514): p. 1394-7.
11. Rivier, C. and W. Vale, *Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin*. Nature, 1983. **305**(5932): p. 325-7.
12. Smith, S.M. and W.W. Vale, *The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress*. Dialogues Clin Neurosci, 2006. **8**(4): p. 383-95.
13. Walker, J.J., J.R. Terry, and S.L. Lightman, *Origin of ultradian pulsatility in the hypothalamic-pituitary-adrenal axis*. Proc Biol Sci, 2010. **277**(1688): p. 1627-33.
14. Reul, J.M. and E.R. de Kloet, *Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation*. Endocrinology, 1985. **117**(6): p. 2505-11.
15. Joels, M., R.A. Sarabdjitsingh, and H. Karst, *Unraveling the time domains of corticosteroid hormone influences on brain activity: rapid, slow, and chronic modes*. Pharmacol Rev, 2012. **64**(4): p. 901-38.
16. Giguere, V., et al., *Functional domains of the human glucocorticoid receptor*. Cell, 1986. **46**(5): p. 645-52.
17. Cadepond, F., et al., *Heat shock protein 90 as a critical factor in maintaining glucocorticosteroid receptor in a nonfunctional state*. J Biol Chem, 1991. **266**(9): p. 5834-41.
18. Bamberger, C.M., H.M. Schulte, and G.P. Chrousos, *Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids*. Endocr Rev, 1996. **17**(3): p. 245-61.
19. Hollenberg, S.M. and R.M. Evans, *Multiple and cooperative trans-activation domains of the human glucocorticoid receptor*. Cell, 1988. **55**(5): p. 899-906.
20. Joels, M., et al., *Corticosteroid effects on cellular physiology of limbic cells*. Brain Res, 2009. **1293**: p. 91-100.
21. Sapolsky, R.M., L.C. Krey, and B.S. McEwen, *Glucocorticoid-sensitive hippocampal neurons are involved in terminating the adrenocortical stress response*. Proc Natl Acad Sci U S A, 1984. **81**(19): p. 6174-7.
22. McEwen, B.S., *Physiology and neurobiology of stress and adaptation: central role of the brain*. Physiol Rev, 2007. **87**(3): p. 873-904.
23. Herman, J.P., et al., *Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness*. Frontiers in Neuroendocrinology, 2003. **24**(3): p. 151-180.

24. Jacobson, L. and R. Sapolsky, *The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis*. Endocr Rev, 1991. **12**(2): p. 118-34.
25. Herman, J.P., et al., *Evidence for hippocampal regulation of neuroendocrine neurons of the hypothalamo-pituitary-adrenocortical axis*. J Neurosci, 1989. **9**(9): p. 3072-82.
26. Joels, M., *Corticosteroid effects in the brain: U-shape it*. Trends Pharmacol Sci, 2006. **27**(5): p. 244-50.
27. Joels, M., *Impact of glucocorticoids on brain function: relevance for mood disorders*. Psychoneuroendocrinology, 2011. **36**(3): p. 406-14.
28. Lynch, M.A., *Long-term potentiation and memory*. Physiol Rev, 2004. **84**(1): p. 87-136.
29. Pavlides, C. and B.S. McEwen, *Effects of mineralocorticoid and glucocorticoid receptors on long-term potentiation in the CA3 hippocampal field*. Brain Research, 1999. **851**(1-2): p. 204-214.
30. LeDoux, J.E., *Emotion circuits in the brain*. Annu Rev Neurosci, 2000. **23**: p. 155-84.
31. LeDoux, J., *The amygdala*. Curr Biol, 2007. **17**(20): p. R868-74.
32. Romanski, L.M. and J.E. LeDoux, *Information cascade from primary auditory cortex to the amygdala: corticocortical and corticoamygdaloid projections of temporal cortex in the rat*. Cereb Cortex, 1993. **3**(6): p. 515-32.
33. LeDoux, J., *The emotional brain, fear, and the amygdala*. Cell Mol Neurobiol, 2003. **23**(4-5): p. 727-38.
34. Shi, C. and M. Davis, *Visual pathways involved in fear conditioning measured with fear-potentiated startle: behavioral and anatomic studies*. J Neurosci, 2001. **21**(24): p. 9844-55.
35. Faulkner, B. and T.H. Brown, *Morphology and physiology of neurons in the rat perirhinal-lateral amygdala area*. J Comp Neurol, 1999. **411**(4): p. 613-42.
36. Rainnie, D.G., E.K. Asprodini, and P. Shinnick-Gallagher, *Inhibitory transmission in the basolateral amygdala*. J Neurophysiol, 1991. **66**(3): p. 999-1009.
37. Rainnie, D.G., E.K. Asprodini, and P. Shinnick-Gallagher, *Excitatory transmission in the basolateral amygdala*. J Neurophysiol, 1991. **66**(3): p. 986-98.
38. Davis, M., *Are different parts of the extended amygdala involved in fear versus anxiety?* Biol Psychiatry, 1998. **44**(12): p. 1239-47.
39. Davis, M., *The role of the amygdala in fear and anxiety*. Annu Rev Neurosci, 1992. **15**: p. 353-75.
40. Roozendaal, B., B.S. McEwen, and S. Chattarji, *Stress, memory and the amygdala*. Nat Rev Neurosci, 2009. **10**(6): p. 423-33.
41. Pryce, C.R., *Postnatal ontogeny of expression of the corticosteroid receptor genes in mammalian brains: inter-species and intra-species differences*. Brain Res Rev, 2008. **57**(2): p. 596-605.
42. Patel, P.D., et al., *Glucocorticoid and mineralocorticoid receptor mRNA expression in squirrel monkey brain*. J Psychiatr Res, 2000. **34**(6): p. 383-92.
43. Roozendaal, B. and J.L. McGaugh, *Glucocorticoid receptor agonist and antagonist administration into the basolateral but not central amygdala modulates memory storage*. Neurobiol Learn Mem, 1997. **67**(2): p. 176-9.
44. Donley, M.P., J. Schulkin, and J.B. Rosen, *Glucocorticoid receptor antagonism in the basolateral amygdala and ventral hippocampus interferes with long-term memory of contextual fear*. Behav Brain Res, 2005. **164**(2): p. 197-205.
45. Roozendaal, B. and J.L. McGaugh, *Amygdaloid nuclei lesions differentially affect glucocorticoid-induced memory enhancement in an inhibitory avoidance task*. Neurobiol Learn Mem, 1996. **65**(1): p. 1-8.
46. Quirarte, G.L., B. Roozendaal, and J.L. McGaugh, *Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala*. Proc Natl Acad Sci U S A, 1997. **94**(25): p. 14048-53.
47. Roozendaal, B., G.L. Quirarte, and J.L. McGaugh, *Glucocorticoids interact with the basolateral amygdala beta-adrenoceptor--cAMP/cAMP/PKA system in influencing memory consolidation*. Eur J Neurosci, 2002. **15**(3): p. 553-60.

48. Buchanan, T.W. and W.R. Lovallo, *Enhanced memory for emotional material following stress-level cortisol treatment in humans*. Psychoneuroendocrinology, 2001. **26**(3): p. 307-17.
49. Cahill, L., et al., *Amygdala activity at encoding correlated with long-term, free recall of emotional information*. Proc Natl Acad Sci U S A, 1996. **93**(15): p. 8016-21.
50. Okuda, S., B. Roozendaal, and J.L. McGaugh, *Glucocorticoid effects on object recognition memory require training-associated emotional arousal*. Proc Natl Acad Sci U S A, 2004. **101**(3): p. 853-8.
51. Roozendaal, B., et al., *Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala*. Proc Natl Acad Sci U S A, 2006. **103**(17): p. 6741-6.
52. Pikkarainen, M. and A. Pitkanen, *Projections from the lateral, basal and accessory basal nuclei of the amygdala to the perirhinal and postrhinal cortices in rat*. Cereb Cortex, 2001. **11**(11): p. 1064-82.
53. Petrovich, G.D., N.S. Canteras, and L.W. Swanson, *Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems*. Brain Res Brain Res Rev, 2001. **38**(1-2): p. 247-89.
54. McDonald, R.J. and N.M. White, *A triple dissociation of memory systems: Hippocampus, amygdala, and dorsal striatum*. Behav Neurosci, 2013. **127**(6): p. 835-53.
55. Packard, M.G. and J.L. McGaugh, *Double dissociation of fornix and caudate nucleus lesions on acquisition of two water maze tasks: further evidence for multiple memory systems*. Behav Neurosci, 1992. **106**(3): p. 439-46.
56. Arnsten, A.F., M.J. Wang, and C.D. Paspalas, *Neuromodulation of thought: flexibilities and vulnerabilities in prefrontal cortical network synapses*. Neuron, 2012. **76**(1): p. 223-39.
57. McEwen, B.S. and J.H. Morrison, *The brain on stress: vulnerability and plasticity of the prefrontal cortex over the life course*. Neuron, 2013. **79**(1): p. 16-29.
58. Arnsten, A.F., et al., *Dynamic Network Connectivity: A new form of neuroplasticity*. Trends Cogn Sci, 2010. **14**(8): p. 365-75.
59. Goldman-Rakic, P.S., *Cellular basis of working memory*. Neuron, 1995. **14**(3): p. 477-85.
60. McKlveen, J.M., B. Myers, and J.P. Herman, *The medial prefrontal cortex: coordinator of autonomic, neuroendocrine and behavioural responses to stress*. J Neuroendocrinol, 2015. **27**(6): p. 446-56.
61. Gabbott, P.L., et al., *Prefrontal cortex in the rat: projections to subcortical autonomic, motor, and limbic centers*. J Comp Neurol, 2005. **492**(2): p. 145-77.
62. Vertes, R.P., *Differential projections of the infralimbic and prelimbic cortex in the rat*. Synapse, 2004. **51**(1): p. 32-58.
63. Barense, M.D., M.T. Fox, and M.G. Baxter, *Aged rats are impaired on an attentional set-shifting task sensitive to medial frontal cortex damage in young rats*. Learn Mem, 2002. **9**(4): p. 191-201.
64. Birrell, J.M. and V.J. Brown, *Medial frontal cortex mediates perceptual attentional set shifting in the rat*. J Neurosci, 2000. **20**(11): p. 4320-4.
65. Hoover, W.B. and R.P. Vertes, *Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat*. Brain Struct Funct, 2007. **212**(2): p. 149-79.
66. Spencer, S.J., K.M. Buller, and T.A. Day, *Medial prefrontal cortex control of the paraventricular hypothalamic nucleus response to psychological stress: possible role of the bed nucleus of the stria terminalis*. J Comp Neurol, 2005. **481**(4): p. 363-76.
67. Powell, D.A., B. Maxwell, and J. Penney, *Neuronal activity in the medial prefrontal cortex during Pavlovian eyeblink and nictitating membrane conditioning*. J Neurosci, 1996. **16**(19): p. 6296-306.
68. Diorio, D., V. Viau, and M.J. Meaney, *The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress*. J Neurosci, 1993. **13**(9): p. 3839-47.

69. Radley, J.J., C.M. Arias, and P.E. Sawchenko, *Regional differentiation of the medial prefrontal cortex in regulating adaptive responses to acute emotional stress*. J Neurosci, 2006. **26**(50): p. 12967-76.
70. de Kloet, E.R., M. Joels, and F. Holsboer, *Stress and the brain: from adaptation to disease*. Nat Rev Neurosci, 2005. **6**(6): p. 463-75.
71. McEwen, B.S., *Stress and hippocampal plasticity*. Annu Rev Neurosci, 1999. **22**: p. 105-22.
72. Vyas, A., et al., *Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons*. J Neurosci, 2002. **22**(15): p. 6810-8.
73. Sapolsky, R.M., *Do glucocorticoid concentrations rise with age in the rat?* Neurobiol Aging, 1992. **13**(1): p. 171-4.
74. Starkman, M.N., et al., *Hippocampal formation volume, memory dysfunction, and cortisol levels in patients with Cushing's syndrome*. Biol Psychiatry, 1992. **32**(9): p. 756-65.
75. Magarinos, A.M., et al., *Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews*. J Neurosci, 1996. **16**(10): p. 3534-40.
76. McLaughlin, K.J., et al., *The effects of chronic stress on hippocampal morphology and function: an evaluation of chronic restraint paradigms*. Brain Res, 2007. **1161**: p. 56-64.
77. Magarinos, A.M. and B.S. McEwen, *Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: involvement of glucocorticoid secretion and excitatory amino acid receptors*. Neuroscience, 1995. **69**(1): p. 89-98.
78. Watanabe, Y., E. Gould, and B.S. McEwen, *Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons*. Brain Res, 1992. **588**(2): p. 341-5.
79. Sousa, N., et al., *Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement*. Neuroscience, 2000. **97**(2): p. 253-66.
80. Magarinos, A.M., M. Orchinik, and B.S. McEwen, *Morphological changes in the hippocampal CA3 region induced by non-invasive glucocorticoid administration: a paradox*. Brain Res, 1998. **809**(2): p. 314-8.
81. Sapolsky, R.M., *A mechanism for glucocorticoid toxicity in the hippocampus: increased neuronal vulnerability to metabolic insults*. J Neurosci, 1985. **5**(5): p. 1228-32.
82. Kerr, D.S., et al., *Chronic stress-induced acceleration of electrophysiologic and morphometric biomarkers of hippocampal aging*. J Neurosci, 1991. **11**(5): p. 1316-24.
83. Sapolsky, R.M., *Stress, Glucocorticoids, and Damage to the Nervous System: The Current State of Confusion*. Stress, 1996. **1**(1): p. 1-19.
84. Taft, W.C., et al., *Phenytoin protects against ischemia-produced neuronal cell death*. Brain Res, 1989. **483**(1): p. 143-8.
85. Masters, J.N., et al., *Glucocorticoid Endangerment of Hippocampal Neurons Does not Involve Deoxyribonucleic Acid Cleavage*. Endocrinology, 1989. **124**(6): p. 3083-3088.
86. Rajan, V., C.R. Edwards, and J.R. Seckl, *11 beta-Hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity*. J Neurosci, 1996. **16**(1): p. 65-70.
87. Dugan, L.L. and D.W. Choi, *Excitotoxicity, free radicals, and cell membrane changes*. Ann Neurol, 1994. **35 Suppl**: p. S17-21.
88. Magarinos, A.M., J.M. Verdugo, and B.S. McEwen, *Chronic stress alters synaptic terminal structure in hippocampus*. Proc Natl Acad Sci U S A, 1997. **94**(25): p. 14002-8.
89. Magarinos, A.M., A. Deslandes, and B.S. McEwen, *Effects of antidepressants and benzodiazepine treatments on the dendritic structure of CA3 pyramidal neurons after chronic stress*. Eur J Pharmacol, 1999. **371**(2-3): p. 113-22.

90. Woolley, C.S., E. Gould, and B.S. McEwen, *Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons*. Brain Res, 1990. **531**(1-2): p. 225-31.
91. Coburn-Litvak, P.S., et al., *Chronic corticosterone affects brain weight, and mitochondrial, but not glial volume fraction in hippocampal area CA3*. Neuroscience, 2004. **124**(2): p. 429-38.
92. Stranahan, A.M., D. Khalil, and E. Gould, *Social isolation delays the positive effects of running on adult neurogenesis*. Nat Neurosci, 2006. **9**(4): p. 526-33.
93. Mirescu, C. and E. Gould, *Stress and adult neurogenesis*. Hippocampus, 2006. **16**(3): p. 233-8.
94. De Kloet, E.R., et al., *Brain corticosteroid receptor balance in health and disease*. Endocr Rev, 1998. **19**(3): p. 269-301.
95. Kerr, D.S., A. Huggett, and W. Abraham, *Modulation of hippocampal long-term potentiation and long-term depression by corticosteroid receptor activation*. Psychobiology, 1994. **22**(2): p. 123-133.
96. Pavlides, C., et al., *Hippocampal homosynaptic long-term depression/depotential induced by adrenal steroids*. Neuroscience, 1995. **68**(2): p. 379-85.
97. Pavlides, C., et al., *Role of adrenal steroid mineralocorticoid and glucocorticoid receptors in long-term potentiation in the CA1 field of hippocampal slices*. Brain Res, 1996. **738**(2): p. 229-35.
98. Cameron, H.A. and E. Gould, *Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus*. Neuroscience, 1994. **61**(2): p. 203-209.
99. Gould, E., et al., *Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation*. J Neurosci, 1997. **17**(7): p. 2492-8.
100. Ikegaya, Y., H. Saito, and K. Abe, *The basomedial and basolateral amygdaloid nuclei contribute to the induction of long-term potentiation in the dentate gyrus in vivo*. Eur J Neurosci, 1996. **8**(9): p. 1833-9.
101. McEwen, B.S., et al., *Stress and the brain: a paradoxical role for adrenal steroids*. Vitam Horm, 1995. **51**: p. 371-402.
102. McEwen, B.S. and S. Chattarji, *Molecular mechanisms of neuroplasticity and pharmacological implications: the example of tianeptine*. Eur Neuropsychopharmacol, 2004. **14 Suppl 5**: p. S497-502.
103. Wood, G.E., et al., *Stress-induced structural remodeling in hippocampus: prevention by lithium treatment*. Proc Natl Acad Sci U S A, 2004. **101**(11): p. 3973-8.
104. Nacher, J. and B.S. McEwen, *The role of N-methyl-D-aspartate receptors in neurogenesis*. Hippocampus, 2006. **16**(3): p. 267-70.
105. Lakshminarasimhan, H. and S. Chattarji, *Stress leads to contrasting effects on the levels of brain derived neurotrophic factor in the hippocampus and amygdala*. PLoS One, 2012. **7**(1): p. e30481.
106. Smith, M.A., et al., *Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus*. J Neurosci, 1995. **15**(3 Pt 1): p. 1768-77.
107. Tsai, S.F., et al., *Social instability stress differentially affects amygdalar neuron adaptations and memory performance in adolescent and adult rats*. Front Behav Neurosci, 2014. **8**: p. 27.
108. Bai, Y.Y., et al., *ProBDNF Signaling Regulates Depression-Like Behaviors in Rodents under Chronic Stress*. Neuropsychopharmacology, 2016.
109. Numakawa, T., et al., *BDNF function and intracellular signaling in neurons*. Histo Histopathol, 2010. **25**(2): p. 237-58.
110. Bennett, M.R. and J. Lagopoulos, *Stress and trauma: BDNF control of dendritic-spine formation and regression*. Prog Neurobiol, 2014. **112**: p. 80-99.
111. Magarinos, A.M., et al., *Effect of brain-derived neurotrophic factor haploinsufficiency on stress-induced remodeling of hippocampal neurons*. Hippocampus, 2011. **21**(3): p. 253-64.

112. Tolwani, R.J., et al., *BDNF overexpression increases dendrite complexity in hippocampal dentate gyrus*. Neuroscience, 2002. **114**(3): p. 795-805.
113. Novkovic, T., T. Mittmann, and D. Manahan-Vaughan, *BDNF contributes to the facilitation of hippocampal synaptic plasticity and learning enabled by environmental enrichment*. Hippocampus, 2015. **25**(1): p. 1-15.
114. Davis, M., D. Rainnie, and M. Cassell, *Neurotransmission in the rat amygdala related to fear and anxiety*. Trends Neurosci, 1994. **17**(5): p. 208-14.
115. Corodimas, K.P., et al., *Corticosterone potentiation of conditioned fear in rats*. Ann N Y Acad Sci, 1994. **746**: p. 392-3.
116. Rodrigues, S.M., J.E. LeDoux, and R.M. Sapolsky, *The influence of stress hormones on fear circuitry*. Annu Rev Neurosci, 2009. **32**: p. 289-313.
117. Rogan, M.T., U.V. Staubli, and J.E. LeDoux, *Fear conditioning induces associative long-term potentiation in the amygdala*. Nature, 1997. **390**(6660): p. 604-7.
118. Roozendaal, B., J.R. McReynolds, and J.L. McGaugh, *The basolateral amygdala interacts with the medial prefrontal cortex in regulating glucocorticoid effects on working memory impairment*. J Neurosci, 2004. **24**(6): p. 1385-92.
119. Johansen, J.P., et al., *Molecular mechanisms of fear learning and memory*. Cell, 2011. **147**(3): p. 509-24.
120. Mitra, R., et al., *Stress duration modulates the spatiotemporal patterns of spine formation in the basolateral amygdala*. Proc Natl Acad Sci U S A, 2005. **102**(26): p. 9371-6.
121. Mitra, R. and R.M. Sapolsky, *Acute corticosterone treatment is sufficient to induce anxiety and amygdaloid dendritic hypertrophy*. Proc Natl Acad Sci U S A, 2008. **105**(14): p. 5573-8.
122. Mitra, R., R. Adamec, and R. Sapolsky, *Resilience against predator stress and dendritic morphology of amygdala neurons*. Behav Brain Res, 2009. **205**(2): p. 535-43.
123. Wood, G.E., et al., *Acute and chronic restraint stress alter the incidence of social conflict in male rats*. Hormones and Behavior, 2003. **43**(1): p. 205-213.
124. Linden, D.E., *How psychotherapy changes the brain--the contribution of functional neuroimaging*. Mol Psychiatry, 2006. **11**(6): p. 528-38.
125. Mahabir, M., et al., *Emotional face processing in post-traumatic stress disorder after reconsolidation impairment using propranolol: A pilot fMRI study*. J Anxiety Disord, 2015. **36**: p. 127-33.
126. Stark, E.A., et al., *Post-traumatic stress influences the brain even in the absence of symptoms: A systematic, quantitative meta-analysis of neuroimaging studies*. Neurosci Biobehav Rev, 2015. **56**: p. 207-21.
127. Hamilton, J.P. and I.H. Gotlib, *Neural substrates of increased memory sensitivity for negative stimuli in major depression*. Biol Psychiatry, 2008. **63**(12): p. 1155-62.
128. Drevets, W.C., W. Bogers, and M.E. Raichle, *Functional anatomical correlates of antidepressant drug treatment assessed using PET measures of regional glucose metabolism*. Eur Neuropsychopharmacol, 2002. **12**(6): p. 527-44.
129. Han, F., J. Ding, and Y. Shi, *Expression of amygdala mineralocorticoid receptor and glucocorticoid receptor in the single-prolonged stress rats*. BMC Neurosci, 2014. **15**: p. 77.
130. Shepard, J.D., K.W. Barron, and D.A. Myers, *Stereotaxic localization of corticosterone to the amygdala enhances hypothalamo-pituitary-adrenal responses to behavioral stress*. Brain Res, 2003. **963**(1-2): p. 203-13.
131. Duvarci, S. and D. Pare, *Glucocorticoids enhance the excitability of principal basolateral amygdala neurons*. J Neurosci, 2007. **27**(16): p. 4482-91.
132. Hartmann, J., et al., *Forebrain glutamatergic, but not GABAergic, neurons mediate anxiogenic effects of the glucocorticoid receptor*. Mol Psychiatry, 2016.
133. Calvo, N. and M. Volosin, *Glucocorticoid and mineralocorticoid receptors are involved in the facilitation of anxiety-like response induced by restraint*. Neuroendocrinology, 2001. **73**(4): p. 261-71.

134. Yang, Y.L., P.K. Chao, and K.T. Lu, *Systemic and intra-amygdala administration of glucocorticoid agonist and antagonist modulate extinction of conditioned fear*. Neuropsychopharmacology, 2006. **31**(5): p. 912-24.
135. Mitra, R., D. Ferguson, and R.M. Sapolsky, *Mineralocorticoid receptor overexpression in basolateral amygdala reduces corticosterone secretion and anxiety*. Biol Psychiatry, 2009. **66**(7): p. 686-90.
136. Liebmann, L., et al., *Differential Effects of Corticosterone on the Slow Afterhyperpolarization in the Basolateral Amygdala and CA1 Region: Possible Role of Calcium Channel Subunits*. Journal of Neurophysiology, 2008. **99**(2): p. 958-968.
137. Karst, H., et al., *Glucocorticoids alter calcium conductances and calcium channel subunit expression in basolateral amygdala neurons*. Eur J Neurosci, 2002. **16**(6): p. 1083-9.
138. Maren, S. and G.J. Quirk, *Neuronal signalling of fear memory*. Nat Rev Neurosci, 2004. **5**(11): p. 844-52.
139. Faber, E.S., A.J. Delaney, and P. Sah, *SK channels regulate excitatory synaptic transmission and plasticity in the lateral amygdala*. Nat Neurosci, 2005. **8**(5): p. 635-41.
140. Tully, K., et al., *Norepinephrine enables the induction of associative long-term potentiation at thalamo-amygdala synapses*. Proc Natl Acad Sci U S A, 2007. **104**(35): p. 14146-50.
141. Mitra, R., D. Ferguson, and R.M. Sapolsky, *SK2 potassium channel overexpression in basolateral amygdala reduces anxiety, stress-induced corticosterone secretion and dendritic arborization*. Mol Psychiatry, 2009. **14**(9): p. 847-55, 827.
142. McDonald, A.J., *Neurons of the lateral and basolateral amygdaloid nuclei: a Golgi study in the rat*. J Comp Neurol, 1982. **212**(3): p. 293-312.
143. Swanson, L.W. and G.D. Petrovich, *What is the amygdala?* Trends Neurosci, 1998. **21**(8): p. 323-31.
144. Prager, E.M., et al., *The basolateral amygdala gamma-aminobutyric acidergic system in health and disease*. J Neurosci Res, 2016. **94**(6): p. 548-67.
145. Zhang, W. and J.A. Rosenkranz, *Effects of Repeated Stress on Age-Dependent GABAergic Regulation of the Lateral Nucleus of the Amygdala*. Neuropsychopharmacology, 2016. **41**(9): p. 2309-23.
146. Lange, M.D., et al., *Glutamic acid decarboxylase 65: a link between GABAergic synaptic plasticity in the lateral amygdala and conditioned fear generalization*. Neuropsychopharmacology, 2014. **39**(9): p. 2211-20.
147. Tasan, R.O., et al., *Altered GABA transmission in a mouse model of increased trait anxiety*. Neuroscience, 2011. **183**: p. 71-80.
148. Truitt, W.A., et al., *Anxiety-like behavior is modulated by a discrete subpopulation of interneurons in the basolateral amygdala*. Neuroscience, 2009. **160**(2): p. 284-94.
149. Aroniadou-Anderjaska, V., F. Qashu, and M.F. Braga, *Mechanisms regulating GABAergic inhibitory transmission in the basolateral amygdala: implications for epilepsy and anxiety disorders*. Amino Acids, 2007. **32**(3): p. 305-15.
150. Sosulina, L., et al., *Classification of projection neurons and interneurons in the rat lateral amygdala based upon cluster analysis*. Mol Cell Neurosci, 2006. **33**(1): p. 57-67.
151. Soghomonian, J.J. and D.L. Martin, *Two isoforms of glutamate decarboxylase: why?* Trends Pharmacol Sci, 1998. **19**(12): p. 500-5.
152. Lussier, A.L., et al., *Altered GABAergic and glutamatergic activity within the rat hippocampus and amygdala in rats subjected to repeated corticosterone administration but not restraint stress*. Neuroscience, 2013. **231**: p. 38-48.
153. Kim, H., et al., *Regional differences in acute corticosterone-induced dendritic remodeling in the rat brain and their behavioral consequences*. BMC Neurosci, 2014. **15**: p. 65.
154. Lau, T., et al., *Stress-induced structural plasticity of medial amygdala stellate neurons and rapid prevention by a candidate antidepressant*. Mol Psychiatry, 2016.

155. Vyas, A. and S. Chattarji, *Modulation of different states of anxiety-like behavior by chronic stress*. Behav Neurosci, 2004. **118**(6): p. 1450-4.
156. Maroun, M., *Stress reverses plasticity in the pathway projecting from the ventromedial prefrontal cortex to the basolateral amygdala*. European Journal of Neuroscience, 2006. **24**(10): p. 2917-2922.
157. Leuner, B. and T.J. Shors, *Stress, anxiety, and dendritic spines: what are the connections?* Neuroscience, 2013. **251**: p. 108-19.
158. Vyas, A., A.G. Pillai, and S. Chattarji, *Recovery after chronic stress fails to reverse amygdaloid neuronal hypertrophy and enhanced anxiety-like behavior*. Neuroscience, 2004. **128**(4): p. 667-73.
159. Rainnie, D.G., et al., *Corticotrophin releasing factor-induced synaptic plasticity in the amygdala translates stress into emotional disorders*. J Neurosci, 2004. **24**(14): p. 3471-9.
160. Shekhar, A., et al., *Role of stress, corticotrophin releasing factor (CRF) and amygdala plasticity in chronic anxiety*. Stress, 2005. **8**(4): p. 209-19.
161. Boyle, L.M., *A neuroplasticity hypothesis of chronic stress in the basolateral amygdala*. Yale J Biol Med, 2013. **86**(2): p. 117-25.
162. Numakawa, T., et al., *Brain-derived neurotrophic factor and glucocorticoids: reciprocal influence on the central nervous system*. Neuroscience, 2013. **239**: p. 157-72.
163. Rothman, S.M. and M.P. Mattson, *Activity-dependent, stress-responsive BDNF signaling and the quest for optimal brain health and resilience throughout the lifespan*. Neuroscience, 2013. **239**: p. 228-40.
164. Kumamaru, E., et al., *Glucocorticoid prevents brain-derived neurotrophic factor-mediated maturation of synaptic function in developing hippocampal neurons through reduction in the activity of mitogen-activated protein kinase*. Mol Endocrinol, 2008. **22**(3): p. 546-58.
165. Kawashima, H., et al., *Glucocorticoid attenuates brain-derived neurotrophic factor-dependent upregulation of glutamate receptors via the suppression of microRNA-132 expression*. Neuroscience, 2010. **165**(4): p. 1301-11.
166. Chen, D.Y., et al., *Glucocorticoid receptors recruit the CaMKIIalpha-BDNF-CREB pathways to mediate memory consolidation*. Nat Neurosci, 2012. **15**(12): p. 1707-14.
167. Revest, J.M., et al., *BDNF-TrkB signaling through Erk1/2(MAPK) phosphorylation mediates the enhancement of fear memory induced by glucocorticoids*. Molecular Psychiatry, 2014. **19**(9): p. 1001-1009.
168. Liston, C., et al., *Stress-induced alterations in prefrontal cortical dendritic morphology predict selective impairments in perceptual attentional set-shifting*. J Neurosci, 2006. **26**(30): p. 7870-4.
169. Barsegyan, A., et al., *Glucocorticoids in the prefrontal cortex enhance memory consolidation and impair working memory by a common neural mechanism*. Proc Natl Acad Sci U S A, 2010. **107**(38): p. 16655-60.
170. Mizoguchi, K., et al., *Chronic stress induces impairment of spatial working memory because of prefrontal dopaminergic dysfunction*. J Neurosci, 2000. **20**(4): p. 1568-74.
171. Koot, S., et al., *Corticosterone and decision-making in male Wistar rats: the effect of corticosterone application in the infralimbic and orbitofrontal cortex*. Front Behav Neurosci, 2014. **8**: p. 127.
172. Graham, L.K., T. Yoon, and J.J. Kim, *Stress impairs optimal behavior in a water foraging choice task in rats*. Learn Mem, 2010. **17**(1): p. 1-4.
173. Bondi, C.O., et al., *Chronic unpredictable stress induces a cognitive deficit and anxiety-like behavior in rats that is prevented by chronic antidepressant drug treatment*. Neuropsychopharmacology, 2008. **33**(2): p. 320-31.
174. Herman, J.P., *Neural control of chronic stress adaptation*. Front Behav Neurosci, 2013. **7**: p. 61.
175. McKlveen, J.M., et al., *Role of prefrontal cortex glucocorticoid receptors in stress and emotion*. Biol Psychiatry, 2013. **74**(9): p. 672-9.

176. Hamani, C., J.N. Nobrega, and A.M. Lozano, *Deep brain stimulation in clinical practice and in animal models*. Clin Pharmacol Ther, 2010. **88**(4): p. 559-62.
177. Musazzi, L., et al., *Acute stress increases depolarization-evoked glutamate release in the rat prefrontal/frontal cortex: the dampening action of antidepressants*. PLoS One, 2010. **5**(1): p. e8566.
178. Yuen, E.Y., et al., *Repeated stress causes cognitive impairment by suppressing glutamate receptor expression and function in prefrontal cortex*. Neuron, 2012. **73**(5): p. 962-77.
179. Gourley, S.L., et al., *A history of corticosterone exposure regulates fear extinction and cortical NR2B, GluR2/3, and BDNF*. Neuropsychopharmacology, 2009. **34**(3): p. 707-16.
180. Hill, M.N., et al., *Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response*. J Neurosci, 2011. **31**(29): p. 10506-15.
181. Gilabert-Juan, J., et al., *Chronic stress alters inhibitory networks in the medial prefrontal cortex of adult mice*. Brain Struct Funct, 2013. **218**(6): p. 1591-605.
182. Wellman, C.L., *Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration*. J Neurobiol, 2001. **49**(3): p. 245-53.
183. Cerqueira, J.J., et al., *Morphological correlates of corticosteroid-induced changes in prefrontal cortex-dependent behaviors*. J Neurosci, 2005. **25**(34): p. 7792-800.
184. Cook, S.C. and C.L. Wellman, *Chronic stress alters dendritic morphology in rat medial prefrontal cortex*. J Neurobiol, 2004. **60**(2): p. 236-48.
185. Radley, J.J., et al., *Repeated stress induces dendritic spine loss in the rat medial prefrontal cortex*. Cereb Cortex, 2006. **16**(3): p. 313-20.
186. Brown, S.M., S. Henning, and C.L. Wellman, *Mild, short-term stress alters dendritic morphology in rat medial prefrontal cortex*. Cereb Cortex, 2005. **15**(11): p. 1714-22.
187. Radley, J.J., et al., *Reversibility of apical dendritic retraction in the rat medial prefrontal cortex following repeated stress*. Exp Neurol, 2005. **196**(1): p. 199-203.
188. Shansky, R.M., et al., *Stress-induced dendritic remodeling in the prefrontal cortex is circuit specific*. Cereb Cortex, 2009. **19**(10): p. 2479-84.
189. Goldwater, D.S., et al., *Structural and functional alterations to rat medial prefrontal cortex following chronic restraint stress and recovery*. Neuroscience, 2009. **164**(2): p. 798-808.
190. Bloss, E.B., et al., *Interactive effects of stress and aging on structural plasticity in the prefrontal cortex*. J Neurosci, 2010. **30**(19): p. 6726-31.
191. Anderson, R.M., et al., *Prolonged corticosterone exposure induces dendritic spine remodeling and attrition in the rat medial prefrontal cortex*. J Comp Neurol, 2016.
192. Duman, R.S. and L.M. Monteggia, *A neurotrophic model for stress-related mood disorders*. Biol Psychiatry, 2006. **59**(12): p. 1116-27.
193. Kuipers, S.D., et al., *Molecular correlates of impaired prefrontal plasticity in response to chronic stress*. J Neurochem, 2003. **85**(5): p. 1312-23.
194. Mikkelsen, J.D. and M.H. Larsen, *Effects of stress and adrenalectomy on activity-regulated cytoskeleton protein (Arc) gene expression*. Neurosci Lett, 2006. **403**(3): p. 239-43.
195. Fanselow, M.S. and J.E. LeDoux, *Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala*. Neuron, 1999. **23**(2): p. 229-32.
196. Roozendaal, B. and J.L. McGaugh, *Basolateral amygdala lesions block the memory-enhancing effect of glucocorticoid administration in the dorsal hippocampus of rats*. Eur J Neurosci, 1997. **9**(1): p. 76-83.
197. Cho, J.H., K. Deisseroth, and V.Y. Bolshakov, *Synaptic encoding of fear extinction in mPFC-amygdala circuits*. Neuron, 2013. **80**(6): p. 1491-507.
198. Steimer, T., *The biology of fear- and anxiety-related behaviors*. Dialogues in Clinical Neuroscience, 2002. **4**(3): p. 231-249.

199. Shelton, C.I., *Diagnosis and management of anxiety disorders*. J Am Osteopath Assoc, 2004. **104**(3 Suppl 1): p. S2-5.
200. Davis, M. and P.J. Whalen, *The amygdala: vigilance and emotion*. Mol Psychiatry, 2001. **6**(1): p. 13-34.
201. Rosen, J.B. and J. Schulkin, *From normal fear to pathological anxiety*. Psychol Rev, 1998. **105**(2): p. 325-50.
202. Ghosh, S. and S. Chattarji, *Neuronal encoding of the switch from specific to generalized fear*. Nat Neurosci, 2015. **18**(1): p. 112-20.
203. Kindt, M., *A behavioural neuroscience perspective on the aetiology and treatment of anxiety disorders*. Behav Res Ther, 2014. **62**: p. 24-36.
204. Rau, V., et al., *Neurobiological and Neuroethological Perspectives on Fear and Anxiety*

Understanding Trauma. 2007: Cambridge University Press.

205. McEwen, B.S., *Mood disorders and allostatic load*. Biol Psychiatry, 2003. **54**(3): p. 200-7.
206. Galvez, R., M.H. Mesches, and J.L. McGaugh, *Norepinephrine release in the amygdala in response to footshock stimulation*. Neurobiol Learn Mem, 1996. **66**(3): p. 253-7.
207. McGaugh, J.L., *The amygdala modulates the consolidation of memories of emotionally arousing experiences*. Annu Rev Neurosci, 2004. **27**: p. 1-28.
208. Shepard, J.D., K.W. Barron, and D.A. Myers, *Corticosterone delivery to the amygdala increases corticotropin-releasing factor mRNA in the central amygdaloid nucleus and anxiety-like behavior*. Brain Res, 2000. **861**(2): p. 288-95.
209. Kaouane, N., et al., *Glucocorticoids Can Induce PTSD-Like Memory Impairments in Mice*. Science, 2012. **335**(6075): p. 1510-1513.
210. McEwen, B.S., *Protection and damage from acute and chronic stress: allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders*. Ann N Y Acad Sci, 2004. **1032**: p. 1-7.
211. Rajbhandari, A.K., et al., *Graded fear generalization enhances the level of cfos-positive neurons specifically in the basolateral amygdala*. J Neurosci Res, 2016. **94**(12): p. 1393-1399.
212. Adamec, R., et al., *Dendritic morphology of amygdala and hippocampal neurons in more and less predator stress responsive rats and more and less spontaneously anxious handled controls*. Behav Brain Res, 2012. **226**(1): p. 133-46.
213. Vyas, A., S. Jadhav, and S. Chattarji, *Prolonged behavioral stress enhances synaptic connectivity in the basolateral amygdala*. Neuroscience, 2006. **143**(2): p. 387-93.
214. Rosenkranz, J.A., E.R. Venheim, and M. Padival, *Chronic stress causes amygdala hyperexcitability in rodents*. Biol Psychiatry, 2010. **67**(12): p. 1128-36.
215. Padival, M., D. Quinette, and J.A. Rosenkranz, *Effects of repeated stress on excitatory drive of basal amygdala neurons in vivo*. Neuropsychopharmacology, 2013. **38**(9): p. 1748-62.
216. Zhang, W. and J.A. Rosenkranz, *Repeated restraint stress increases basolateral amygdala neuronal activity in an age-dependent manner*. Neuroscience, 2012. **226**: p. 459-74.
217. Ranjbar, H., et al., *Effects of Electrical Lesion of Basolateral Amygdala Nucleus on Rat Anxiety-like Behavior under Acute, Sub-chronic, and Chronic Stresses*. Clin Exp Pharmacol Physiol, 2017.
218. Wood, G.E., et al., *Chronic immobilization stress alters aspects of emotionality and associative learning in the rat*. Behav Neurosci, 2008. **122**(2): p. 282-92.
219. Suvrathan, A., A. Tomar, and S. Chattarji, *Effects of chronic and acute stress on rat behaviour in the forced-swim test*. Stress, 2010. **13**(6): p. 533-40.
220. Tawakol, A., et al., *Relation between resting amygdalar activity and cardiovascular events: a longitudinal and cohort study*. The Lancet. **389**(10071): p. 834-845.

221. Romeo, R.D., et al., *Anxiety and fear behaviors in adult male and female C57BL/6 mice are modulated by maternal separation*. Horm Behav, 2003. **43**(5): p. 561-7.
222. Mitra, R. and R.M. Sapolsky, *Expression of chimeric estrogen-glucocorticoid-receptor in the amygdala reduces anxiety*. Brain Res, 2010. **1342**: p. 33-8.
223. Russo, S.J., et al., *Neurobiology of resilience*. Nat Neurosci, 2012. **15**(11): p. 1475-84.
224. Franklin, T.B., B.J. Saab, and I.M. Mansuy, *Neural mechanisms of stress resilience and vulnerability*. Neuron, 2012. **75**(5): p. 747-61.
225. Sherrer, M.V., *The role of cognitive appraisal in adaptation to traumatic stress in adults with serious mental illness: a critical review*. Trauma Violence Abuse, 2011. **12**(3): p. 151-67.
226. Stetler, C. and G.E. Miller, *Depression and hypothalamic-pituitary-adrenal activation: a quantitative summary of four decades of research*. Psychosom Med, 2011. **73**(2): p. 114-26.
227. Meewisse, M.L., et al., *Cortisol and post-traumatic stress disorder in adults: systematic review and meta-analysis*. Br J Psychiatry, 2007. **191**: p. 387-92.
228. Weaver, I.C., et al., *Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life*. J Neurosci, 2005. **25**(47): p. 11045-54.
229. Price, J.L. and W.C. Drevets, *Neurocircuitry of mood disorders*. Neuropsychopharmacology, 2010. **35**(1): p. 192-216.
230. Murrough, J.W., et al., *Cognitive dysfunction in depression: neurocircuitry and new therapeutic strategies*. Neurobiol Learn Mem, 2011. **96**(4): p. 553-63.
231. Mayberg, H.S., *Targeted electrode-based modulation of neural circuits for depression*. J Clin Invest, 2009. **119**(4): p. 717-25.
232. van Tol, M.J., et al., *Functional magnetic resonance imaging correlates of emotional word encoding and recognition in depression and anxiety disorders*. Biol Psychiatry, 2012. **71**(7): p. 593-602.
233. Covington, H.E., 3rd, et al., *Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex*. J Neurosci, 2010. **30**(48): p. 16082-90.
234. Adamec, R., et al., *A comparison of activation patterns of cells in selected prefrontal cortical and amygdala areas of rats which are more or less anxious in response to predator exposure or submersion stress*. Physiol Behav, 2012. **105**(3): p. 628-38.
235. Lehmann, M.L. and M. Herkenham, *Environmental enrichment confers stress resiliency to social defeat through an infralimbic cortex-dependent neuroanatomical pathway*. J Neurosci, 2011. **31**(16): p. 6159-73.
236. Krishnan, V., et al., *Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions*. Cell, 2007. **131**(2): p. 391-404.
237. Cao, J.L., et al., *Mesolimbic dopamine neurons in the brain reward circuit mediate susceptibility to social defeat and antidepressant action*. J Neurosci, 2010. **30**(49): p. 16453-8.
238. Elliott, E., et al., *Resilience to social stress coincides with functional DNA methylation of the Crf gene in adult mice*. Nat Neurosci, 2010. **13**(11): p. 1351-3.
239. Lutter, M., et al., *The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress*. Nat Neurosci, 2008. **11**(7): p. 752-3.
240. Berton, O., et al., *Induction of deltaFosB in the periaqueductal gray by stress promotes active coping responses*. Neuron, 2007. **55**(2): p. 289-300.
241. Cohen, H., et al., *The neuropeptide Y (NPY)-ergic system is associated with behavioral resilience to stress exposure in an animal model of post-traumatic stress disorder*. Neuropsychopharmacology, 2012. **37**(2): p. 350-63.
242. Delgado y Palacios, R., et al., *Magnetic resonance imaging and spectroscopy reveal differential hippocampal changes in anhedonic and resilient subtypes of the chronic mild stress rat model*. Biol Psychiatry, 2011. **70**(5): p. 449-57.
243. Levine, S., *Plasma-free corticosteroid response to electric shock in rats stimulated in infancy*. Science, 1962. **135**(3506): p. 795-6.

244. Parker, K.J., et al., *Prospective investigation of stress inoculation in young monkeys*. Arch Gen Psychiatry, 2004. **61**(9): p. 933-41.
245. Lyons, D.M., K.J. Parker, and A.F. Schatzberg, *Animal models of early life stress: implications for understanding resilience*. Dev Psychobiol, 2010. **52**(7): p. 616-24.
246. McEwen, B.S. and P.J. Gianaros, *Stress- and allostasis-induced brain plasticity*. Annu Rev Med, 2011. **62**: p. 431-45.
247. Uchida, S., et al., *Early life stress enhances behavioral vulnerability to stress through the activation of REST4-mediated gene transcription in the medial prefrontal cortex of rodents*. J Neurosci, 2010. **30**(45): p. 15007-18.
248. Franklin, T.B., et al., *Influence of early stress on social abilities and serotonergic functions across generations in mice*. PLoS One, 2011. **6**(7): p. e21842.
249. Cooper, R.M. and J.P. Zubek, *Effects of enriched and restricted early environments on the learning ability of bright and dull rats*. Can J Psychol, 1958. **12**(3): p. 159-64.
250. van Praag, H., G. Kempermann, and F.H. Gage, *Neural consequences of environmental enrichment*. Nat Rev Neurosci, 2000. **1**(3): p. 191-8.
251. Mohammed, A.H., et al., *Environmental enrichment and the brain*. Prog Brain Res, 2002. **138**: p. 109-33.
252. Rosenzweig, M.R., et al., *Effects of environmental complexity and training on brain chemistry and anatomy: a replication and extension*. J Comp Physiol Psychol, 1962. **55**: p. 429-37.
253. Bennett, E.L., M.R. Rosenzweig, and M.C. Diamond, *Rat brain: effects of environmental enrichment on wet and dry weights*. Science, 1969. **163**(3869): p. 825-6.
254. Bennett, E.L., et al., *Chemical and anatomical plasticity of brain*. 1964. J Neuropsychiatry Clin Neurosci, 1996. **8**(4): p. 459-70.
255. Diamond, M.C., D. Krech, and M.R. Rosenzweig, *THE EFFECTS OF AN ENRICHED ENVIRONMENT ON THE HISTOLOGY OF THE RAT CEREBRAL CORTEX*. J Comp Neurol, 1964. **123**: p. 111-20.
256. Diamond, M.C., et al., *Increases in cortical depth and glia numbers in rats subjected to enriched environment*. J Comp Neurol, 1966. **128**(1): p. 117-26.
257. Holloway, R.L., Jr., *Dendritic branching: some preliminary results of training and complexity in rat visual cortex*. Brain Res, 1966. **2**(4): p. 393-6.
258. Torasdotter, M., et al., *Expression of neurotrophin-3 mRNA in the rat visual cortex and hippocampus is influenced by environmental conditions*. Neurosci Lett, 1996. **218**(2): p. 107-10.
259. Torasdotter, M., et al., *Environmental enrichment results in higher levels of nerve growth factor mRNA in the rat visual cortex and hippocampus*. Behav Brain Res, 1998. **93**(1-2): p. 83-90.
260. Mitra, R. and R.M. Sapolsky, *Short-term enrichment makes male rats more attractive, more defensive and alters hypothalamic neurons*. PLoS One, 2012. **7**(5): p. e36092.
261. Francis, D.D., et al., *Environmental enrichment reverses the effects of maternal separation on stress reactivity*. J Neurosci, 2002. **22**(18): p. 7840-3.
262. Connors, E.J., et al., *Environmental enrichment models a naturalistic form of maternal separation and shapes the anxiety response patterns of offspring*. Psychoneuroendocrinology, 2015. **52**: p. 153-67.
263. Myhrer, T., et al., *Differential rearing conditions in rats: effects on neurochemistry in neocortical areas and cognitive behaviors*. Brain Res Bull, 1992. **28**(3): p. 427-34.
264. Aberg, M.A., et al., *Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus*. J Neurosci, 2000. **20**(8): p. 2896-903.
265. Moser, M.B., M. Trommald, and P. Andersen, *An increase in dendritic spine density on hippocampal CA1 pyramidal cells following spatial learning in adult rats suggests the formation of new synapses*. Proc Natl Acad Sci U S A, 1994. **91**(26): p. 12673-5.
266. Sofroniew, M.V., C.L. Howe, and W.C. Mobley, *Nerve growth factor signaling, neuroprotection, and neural repair*. Annu Rev Neurosci, 2001. **24**: p. 1217-81.

267. Tyler, W.J. and L.D. Pozzo-Miller, *BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses*. J Neurosci, 2001. **21**(12): p. 4249-58.
268. Falkenberg, T., et al., *Increased expression of brain-derived neurotrophic factor mRNA in rat hippocampus is associated with improved spatial memory and enriched environment*. Neurosci Lett, 1992. **138**(1): p. 153-6.
269. Hutchinson, K.M., et al., *Environmental enrichment protects against the effects of chronic stress on cognitive and morphological measures of hippocampal integrity*. Neurobiol Learn Mem, 2012. **97**(2): p. 250-60.
270. Veena, J., et al., *Enriched environment restores hippocampal cell proliferation and ameliorates cognitive deficits in chronically stressed rats*. J Neurosci Res, 2009. **87**(4): p. 831-43.
271. Zanca, R.M., et al., *Environmental Enrichment Increases Glucocorticoid Receptors and Decreases GluA2 and Protein Kinase M Zeta (PKMzeta) Trafficking During Chronic Stress: A Protective Mechanism?* Front Behav Neurosci, 2015. **9**: p. 303.
272. Ravenelle, R., et al., *Housing environment modulates physiological and behavioral responses to anxiogenic stimuli in trait anxiety male rats*. Neuroscience, 2014. **270**: p. 76-87.
273. Turner, C.A. and M.H. Lewis, *Environmental enrichment: effects on stereotyped behavior and neurotrophin levels*. Physiol Behav, 2003. **80**(2-3): p. 259-66.
274. Kempermann, G., H.G. Kuhn, and F.H. Gage, *More hippocampal neurons in adult mice living in an enriched environment*. Nature, 1997. **386**(6624): p. 493-5.
275. Nilsson, M., et al., *Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory*. J Neurobiol, 1999. **39**(4): p. 569-78.
276. Mohammed, A.K., G. Jonsson, and T. Archer, *Selective lesioning of forebrain noradrenaline neurons at birth abolishes the improved maze learning performance induced by rearing in complex environment*. Brain Res, 1986. **398**(1): p. 6-10.
277. Mohammed, A.K., et al., *Environmental influence on behaviour and nerve growth factor in the brain*. Brain Res, 1990. **528**(1): p. 62-72.
278. Galani, R., et al., *Effects of postoperative housing conditions on functional recovery in rats with lesions of the hippocampus, subiculum, or entorhinal cortex*. Neurobiol Learn Mem, 1997. **67**(1): p. 43-56.
279. Vega-Rivera, N.M., et al., *The neurogenic effects of an enriched environment and its protection against the behavioral consequences of chronic mild stress persistent after enrichment cessation in six-month-old female Balb/C mice*. Behav Brain Res, 2016. **301**: p. 72-83.
280. Lambert, K., et al., *Natural-enriched environments lead to enhanced environmental engagement and altered neurobiological resilience*. Neuroscience, 2016.
281. Sampedro-Piquero, P., et al., *Environmental enrichment as a therapeutic avenue for anxiety in aged Wistar rats: Effect on cat odor exposition and GABAergic interneurons*. Neuroscience, 2016. **330**: p. 17-25.
282. Ichikawa, M., M. Matsuoka, and Y. Mori, *Effect of differential rearing on synapses and soma size in rat medial amygdaloid nucleus*. Synapse, 1993. **13**(1): p. 50-6.
283. Kaczmarek, L., *Molecular biology of vertebrate learning: is c-fos a new beginning?* J Neurosci Res, 1993. **34**(4): p. 377-81.
284. Kaczmarek, L. and A. Chaudhuri, *Sensory regulation of immediate-early gene expression in mammalian visual cortex: implications for functional mapping and neural plasticity*. Brain Res Brain Res Rev, 1997. **23**(3): p. 237-56.
285. Nikolaev, E., et al., *Environmental manipulation differentially alters c-Fos expression in amygdaloid nuclei following aversive conditioning*. Brain Res, 2002. **957**(1): p. 91-8.
286. Koe, A.S., A. Ashokan, and R. Mitra, *Short environmental enrichment in adulthood reverses anxiety and basolateral amygdala hypertrophy induced by maternal separation*. Transl Psychiatry, 2016. **6**: p. e729.

287. Sztainberg, Y., et al., *The anxiolytic effect of environmental enrichment is mediated via amygdalar CRF receptor type 1*. Mol Psychiatry, 2010. **15**(9): p. 905-17.
288. Chourbaji, S., C. Brandwein, and P. Gass, *Altering BDNF expression by genetics and/or environment: impact for emotional and depression-like behaviour in laboratory mice*. Neurosci Biobehav Rev, 2011. **35**(3): p. 599-611.
289. Chourbaji, S., et al., *Nature vs. nurture: can enrichment rescue the behavioural phenotype of BDNF heterozygous mice?* Behav Brain Res, 2008. **192**(2): p. 254-8.
290. Soffie, M., et al., *Behavioural and glial changes in old rats following environmental enrichment*. Behav Brain Res, 1999. **101**(1): p. 37-49.
291. Escorihuela, R.M., A. Tobena, and A. Fernandez-Teruel, *Environmental enrichment and postnatal handling prevent spatial learning deficits in aged hypoemotional (Roman high-avoidance) and hyperemotional (Roman low-avoidance) rats*. Learn Mem, 1995. **2**(1): p. 40-8.
292. Fernandez-Teruel, A., et al., *Neonatal handling and environmental enrichment effects on emotionality, novelty/reward seeking, and age-related cognitive and hippocampal impairments: focus on the Roman rat lines*. Behav Genet, 1997. **27**(6): p. 513-26.
293. Kempermann, G., H.G. Kuhn, and F.H. Gage, *Experience-induced neurogenesis in the senescent dentate gyrus*. J Neurosci, 1998. **18**(9): p. 3206-12.
294. Neidl, R., et al., *Late-Life Environmental Enrichment Induces Acetylation Events and Nuclear Factor kappaB-Dependent Regulations in the Hippocampus of Aged Rats Showing Improved Plasticity and Learning*. 2016. **36**(15): p. 4351-61.
295. Nithianantharajah, J. and A.J. Hannan, *Enriched environments, experience-dependent plasticity and disorders of the nervous system*. Nat Rev Neurosci, 2006. **7**(9): p. 697-709.
296. Young, D., et al., *Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective*. Nat Med, 1999. **5**(4): p. 448-53.
297. Davenport, J.W., et al., *Environmental stimulation reduces learning deficits in experimental cretinism*. Science, 1976. **191**(4227): p. 578-9.
298. Rampon, C., et al., *Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice*. Nat Neurosci, 2000. **3**(3): p. 238-44.
299. Livingston-Thomas, J., et al., *Exercise and Environmental Enrichment as Enablers of Task-Specific Neuroplasticity and Stroke Recovery*. Neurotherapeutics, 2016. **13**(2): p. 395-402.
300. Ryan, S.J., D.E. Ehrlich, and D.G. Rainnie, *Morphology and dendritic maturation of developing principal neurons in the rat basolateral amygdala*. Brain Struct Funct, 2016. **221**(2): p. 839-54.
301. Huttenlocher, P.R., *Synapse elimination and plasticity in developing human cerebral cortex*. Am J Ment Defic, 1984. **88**(5): p. 488-96.
302. Romeo, R.D., *The impact of stress on the structure of the adolescent brain: Implications for adolescent mental health*. Brain Res, 2016.
303. Finnerty, G.T., L.S. Roberts, and B.W. Connors, *Sensory experience modifies the short-term dynamics of neocortical synapses*. Nature, 1999. **400**(6742): p. 367-71.
304. Kolb, B., et al., *Brain plasticity in the developing brain*. Prog Brain Res, 2013. **207**: p. 35-64.
305. Baldini, S., et al., *Enriched early life experiences reduce adult anxiety-like behavior in rats: a role for insulin-like growth factor 1*. J Neurosci, 2013. **33**(28): p. 11715-23.
306. Mitra, R. and R.M. Sapolsky, *Effects of enrichment predominate over those of chronic stress on fear-related behavior in male rats*. Stress, 2009. **12**(4): p. 305-12.
307. Walf, A.A. and C.A. Frye, *The use of the elevated plus maze as an assay of anxiety-related behavior in rodents*. Nat Protoc, 2007. **2**(2): p. 322-8.
308. Cruz, A.P., F. Frei, and F.G. Graeff, *Ethopharmacological analysis of rat behavior on the elevated plus-maze*. Pharmacol Biochem Behav, 1994. **49**(1): p. 171-6.

309. Cole, J.C. and R.J. Rodgers, *An ethological analysis of the effects of chlordiazepoxide and bretazenil (Ro 16-6028) in the murine elevated plus-maze*. Behav Pharmacol, 1993. **4**(6): p. 573-580.
310. Fernandes, C. and S.E. File, *The influence of open arm ledges and maze experience in the elevated plus-maze*. Pharmacol Biochem Behav, 1996. **54**(1): p. 31-40.
311. Fernandez Espejo, E., *Structure of the mouse behaviour on the elevated plus-maze test of anxiety*. Behav Brain Res, 1997. **86**(1): p. 105-12.
312. Quinn, L.P., et al., *LABORAS: Initial pharmacological validation of a system allowing continuous monitoring of laboratory rodent behaviour*. J Neurosci Methods, 2003. **130**(1): p. 83-92.
313. Castagne, V., et al., *Differential behavioral profiling of stimulant substances in the rat using the LABORAS system*. Pharmacol Biochem Behav, 2012. **101**(4): p. 553-63.
314. Papp, M., E. Moryl, and P. Willner, *Pharmacological validation of the chronic mild stress model of depression*. Eur J Pharmacol, 1996. **296**(2): p. 129-36.
315. Porsolt, R.D., M. Le Pichon, and M. Jalfre, *Depression: a new animal model sensitive to antidepressant treatments*. Nature, 1977. **266**(5604): p. 730-2.
316. Paxinos, G. and C. Watson, *The rat brain atlas in stereotaxic coordinates*. San Diego: Academic, 1998.
317. Shankaranarayana Rao, B.S., et al., *Subicular lesions cause dendritic atrophy in CA1 and CA3 pyramidal neurons of the rat hippocampus*. Neuroscience, 2001. **102**(2): p. 319-27.
318. Flutterm, M., S. Dalm, and M.S. Oitzl, *A refined method for sequential blood sampling by tail incision in rats*. Lab Anim, 2000. **34**(4): p. 372-8.
319. Ippolito, D.M. and C. Eroglu, *Quantifying synapses: an immunocytochemistry-based assay to quantify synapse number*. J Vis Exp, 2010(45).
320. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. Anal Biochem, 1976. **72**: p. 248-54.
321. Jin, H., et al., *Demonstration of functional coupling between gamma -aminobutyric acid (GABA) synthesis and vesicular GABA transport into synaptic vesicles*. Proc Natl Acad Sci U S A, 2003. **100**(7): p. 4293-8.
322. Kanaani, J., et al., *Two distinct mechanisms target GAD67 to vesicular pathways and presynaptic clusters*. J Cell Biol, 2010. **190**(5): p. 911-25.
323. Hirokawa, N., et al., *The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin I*. J Cell Biol, 1989. **108**(1): p. 111-26.
324. Landis, D.M., et al., *The organization of cytoplasm at the presynaptic active zone of a central nervous system synapse*. Neuron, 1988. **1**(3): p. 201-9.
325. Song, S.-H. and G.J. Augustine, *Synapsin Isoforms Regulating GABA Release from Hippocampal Interneurons*. The Journal of Neuroscience, 2016. **36**(25): p. 6742-6757.
326. LeDoux, J.E. and J.M. Gorman, *A call to action: overcoming anxiety through active coping*. Am J Psychiatry, 2001. **158**(12): p. 1953-5.
327. Mineka, S., D. Watson, and L.A. Clark, *Comorbidity of anxiety and unipolar mood disorders*. Annu Rev Psychol, 1998. **49**: p. 377-412.
328. Crofton, E.J., Y. Zhang, and T.A. Green, *Inoculation stress hypothesis of environmental enrichment*. Neurosci Biobehav Rev, 2015. **49**: p. 19-31.
329. Brenes, J.C., M. Padilla, and J. Fornaguera, *A detailed analysis of open-field habituation and behavioral and neurochemical antidepressant-like effects in postweaning enriched rats*. Behav Brain Res, 2009. **197**(1): p. 125-37.
330. Mehta-Raghavan, N.S., et al., *Nature and nurture: environmental influences on a genetic rat model of depression*. Transl Psychiatry, 2016. **6**: p. e770.
331. Jha, S., B. Dong, and K. Sakata, *Enriched environment treatment reverses depression-like behavior and restores reduced hippocampal neurogenesis and protein levels of brain-derived neurotrophic factor in mice lacking its expression through promoter IV*. Transl Psychiatry, 2011. **1**: p. e40.

332. Andersen, S.L., *Exposure to early adversity: Points of cross-species translation that can lead to improved understanding of depression*. Dev Psychopathol, 2015. **27**(2): p. 477-91.
333. Brenes, J.C. and J. Fornaguera, *Effects of environmental enrichment and social isolation on sucrose consumption and preference: associations with depressive-like behavior and ventral striatum dopamine*. Neurosci Lett, 2008. **436**(2): p. 278-82.
334. van der Harst, J.E., A.M. Baars, and B.M. Spruijt, *Standard housed rats are more sensitive to rewards than enriched housed rats as reflected by their anticipatory behaviour*. Behav Brain Res, 2003. **142**(1-2): p. 151-6.
335. Wood, D.A., A.K. Siegel, and G.V. Rebec, *Environmental enrichment reduces impulsivity during appetitive conditioning*. Physiol Behav, 2006. **88**(1-2): p. 132-7.
336. Grimm, J.W., et al., *Environmental enrichment attenuates cue-induced reinstatement of sucrose seeking in rats*. Behav Pharmacol, 2008. **19**(8): p. 777-85.
337. Rygula, R., et al., *Pharmacological validation of a chronic social stress model of depression in rats: effects of reboxetine, haloperidol and diazepam*. Behav Pharmacol, 2008. **19**(3): p. 183-96.
338. Brenes Saenz, J.C., O.R. Villagra, and J. Fornaguera Trias, *Factor analysis of Forced Swimming test, Sucrose Preference test and Open Field test on enriched, social and isolated reared rats*. Behav Brain Res, 2006. **169**(1): p. 57-65.
339. Porsolt, R.D., et al., *Rodent models of depression: forced swimming and tail suspension behavioral despair tests in rats and mice*. Curr Protoc Neurosci, 2001. **Chapter 8**: p. Unit 8.10A.
340. McEwen, B.S., *Stressed or stressed out: what is the difference?* J Psychiatry Neurosci, 2005. **30**(5): p. 315-8.
341. Moncek, F., et al., *Effect of environmental enrichment on stress related systems in rats*. J Neuroendocrinol, 2004. **16**(5): p. 423-31.
342. Konkle, A.T., et al., *Environmental-enrichment-related variations in behavioral, biochemical, and physiologic responses of Sprague-Dawley and Long Evans rats*. J Am Assoc Lab Anim Sci, 2010. **49**(4): p. 427-36.
343. Ravenelle, R., et al., *Environmental enrichment effects on the neurobehavioral profile of selective outbred trait anxiety rats*. Behav Brain Res, 2013. **252**: p. 49-57.
344. Lucas, L.R., et al., *Effects of immobilization stress on neurochemical markers in the motivational system of the male rat*. Brain Res, 2007. **1155**: p. 108-15.
345. Galea, L.A., et al., *Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress*. Neuroscience, 1997. **81**(3): p. 689-97.
346. Sweis, B.M., et al., *Individual differences in the effects of chronic stress on memory: behavioral and neurochemical correlates of resiliency*. Neuroscience, 2013. **246**: p. 142-59.
347. Conrad, C.D., et al., *Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy*. Behav Neurosci, 1999. **113**(5): p. 902-13.
348. McEwen, B.S., *Plasticity of the hippocampus: adaptation to chronic stress and allostatic load*. Ann N Y Acad Sci, 2001. **933**: p. 265-77.
349. Paez-Martinez, N., et al., *Environmental enrichment increases doublecortin-associated new neurons and decreases neuronal death without modifying anxiety-like behavior in mice chronically exposed to toluene*. Behav Brain Res, 2013. **256**: p. 432-40.
350. Segovia, G., et al., *Environmental enrichment promotes neurogenesis and changes the extracellular concentrations of glutamate and GABA in the hippocampus of aged rats*. Brain Res Bull, 2006. **70**(1): p. 8-14.
351. Tanti, A., et al., *Region-dependent and stage-specific effects of stress, environmental enrichment, and antidepressant treatment on hippocampal neurogenesis*. Hippocampus, 2013. **23**(9): p. 797-811.

352. Johansson, B.B. and P.V. Belichenko, *Neuronal plasticity and dendritic spines: effect of environmental enrichment on intact and postischemic rat brain*. J Cereb Blood Flow Metab, 2002. **22**(1): p. 89-96.
353. Bindu, B., et al., *Short-term exposure to an enriched environment enhances dendritic branching but not brain-derived neurotrophic factor expression in the hippocampus of rats with ventral subicular lesions*. Neuroscience, 2007. **144**(2): p. 412-23.
354. Beauquis, J., et al., *Short-term environmental enrichment enhances adult neurogenesis, vascular network and dendritic complexity in the hippocampus of type 1 diabetic mice*. PLoS One, 2010. **5**(11): p. e13993.
355. Berman, R.F., et al., *Prenatal alcohol exposure and the effects of environmental enrichment on hippocampal dendritic spine density*. Alcohol, 1996. **13**(2): p. 209-16.
356. Rojas, J.J., et al., *Effects of daily environmental enrichment on behavior and dendritic spine density in hippocampus following neonatal hypoxia-ischemia in the rat*. Exp Neurol, 2013. **241**: p. 25-33.
357. Li, M., et al., *Environmental Enrichment during Gestation Improves Behavior Consequences and Synaptic Plasticity in Hippocampus of Prenatal-Stressed Offspring Rats*. Acta Histochem Cytochem, 2012. **45**(3): p. 157-66.
358. Sotres-Bayon, F., D.E. Bush, and J.E. LeDoux, *Emotional perseveration: an update on prefrontal-amygdala interactions in fear extinction*. Learn Mem, 2004. **11**(5): p. 525-35.
359. Radley, J.J., et al., *Chronic behavioral stress induces apical dendritic reorganization in pyramidal neurons of the medial prefrontal cortex*. Neuroscience, 2004. **125**(1): p. 1-6.
360. Bloss, E.B., et al., *Evidence for reduced experience-dependent dendritic spine plasticity in the aging prefrontal cortex*. J Neurosci, 2011. **31**(21): p. 7831-9.
361. Ronzoni, G., et al., *Infralimbic cortex controls the activity of the hypothalamus-pituitary-adrenal axis and the formation of aversive memory: Effects of environmental enrichment*. Behav Brain Res, 2016. **297**: p. 338-44.
362. Leger, M., et al., *Environmental enrichment enhances episodic-like memory in association with a modified neuronal activation profile in adult mice*. PLoS One, 2012. **7**(10): p. e48043.
363. Radley, J.J., et al., *Repeated stress alters dendritic spine morphology in the rat medial prefrontal cortex*. J Comp Neurol, 2008. **507**(1): p. 1141-50.
364. Furay, A.R., A.E. Bruestle, and J.P. Herman, *The role of the forebrain glucocorticoid receptor in acute and chronic stress*. Endocrinology, 2008. **149**(11): p. 5482-90.
365. Joels, M. and T.Z. Baram, *The neuro-symphony of stress*. Nat Rev Neurosci, 2009. **10**(6): p. 459-66.
366. Joels, M. and E.R. de Kloet, *Effects of glucocorticoids and norepinephrine on the excitability in the hippocampus*. Science, 1989. **245**(4925): p. 1502-5.
367. Djordjevic, A., et al., *Stress type dependence of expression and cytoplasmic-nuclear partitioning of glucocorticoid receptor, hsp90 and hsp70 in Wistar rat brain*. Neuropsychobiology, 2009. **59**(4): p. 213-21.
368. Liu, D., et al., *Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress*. Science, 1997. **277**(5332): p. 1659-62.
369. McCormick, C.M., et al., *Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats*. Brain Res Dev Brain Res, 1995. **84**(1): p. 55-61.
370. Meaney, M.J., et al., *Basal ACTH, corticosterone and corticosterone-binding globulin levels over the diurnal cycle, and age-related changes in hippocampal type I and type II corticosteroid receptor binding capacity in young and aged, handled and nonhandled rats*. Neuroendocrinology, 1992. **55**(2): p. 204-13.
371. Myers, B. and B. Greenwood-Van Meerveld, *Corticosteroid receptor-mediated mechanisms in the amygdala regulate anxiety and colonic sensitivity*. Am J Physiol Gastrointest Liver Physiol, 2007. **292**(6): p. G1622-9.

372. Ferry, B., B. Roozendaal, and J.L. McGaugh, *Basolateral amygdala noradrenergic influences on memory storage are mediated by an interaction between beta- and alpha1-adrenoceptors*. J Neurosci, 1999. **19**(12): p. 5119-23.
373. Roozendaal, B., G. Portillo-Marquez, and J.L. McGaugh, *Basolateral amygdala lesions block glucocorticoid-induced modulation of memory for spatial learning*. Behav Neurosci, 1996. **110**(5): p. 1074-83.
374. Chung, S., et al., *Differential adaptive responses to chronic stress of maternally stressed male mice offspring*. Endocrinology, 2005. **146**(7): p. 3202-10.
375. Mantsch, J.R., et al., *Daily cocaine self-administration under long-access conditions augments restraint-induced increases in plasma corticosterone and impairs glucocorticoid receptor-mediated negative feedback in rats*. Brain Res, 2007. **1167**: p. 101-11.
376. Durairaj, R.V. and E.R. Koilmani, *Environmental enrichment modulates glucocorticoid receptor expression and reduces anxiety in Indian field male mouse Mus booduga through up-regulation of microRNA-124a*. Gen Comp Endocrinol, 2014. **199**: p. 26-32.
377. Sampedro-Piquero, P., A. Begega, and J.L. Arias, *Increase of glucocorticoid receptor expression after environmental enrichment: relations to spatial memory, exploration and anxiety-related behaviors*. Physiol Behav, 2014. **129**: p. 118-29.
378. Sampedro-Piquero, P., J.L. Arias, and A. Begega, *Behavioral testing-related changes in the expression of Synapsin I and glucocorticoid receptors in standard and enriched aged Wistar rats*. Exp Gerontol, 2014. **58**: p. 292-302.
379. Gapp, K., et al., *Potential of Environmental Enrichment to Prevent Transgenerational Effects of Paternal Trauma*. Neuropsychopharmacology, 2016.
380. Lin, E.J., et al., *Environmental enrichment exerts sex-specific effects on emotionality in C57BL/6J mice*. Behav Brain Res, 2011. **216**(1): p. 349-57.
381. Pare, D. and H. Gaudreau, *Projection cells and interneurons of the lateral and basolateral amygdala: distinct firing patterns and differential relation to theta and delta rhythms in conscious cats*. J Neurosci, 1996. **16**(10): p. 3334-50.
382. Gilabert-Juan, J., et al., *Chronic stress induces changes in the structure of interneurons and in the expression of molecules related to neuronal structural plasticity and inhibitory neurotransmission in the amygdala of adult mice*. Exp Neurol, 2011. **232**(1): p. 33-40.
383. Heldt, S.A., L. Mou, and K.J. Ressler, *In vivo knockdown of GAD67 in the amygdala disrupts fear extinction and the anxiolytic-like effect of diazepam in mice*. Transl Psychiatry, 2012. **2**: p. e181.
384. Ahmed, S., B.A. Reynolds, and S. Weiss, *BDNF enhances the differentiation but not the survival of CNS stem cell-derived neuronal precursors*. J Neurosci, 1995. **15**(8): p. 5765-78.
385. Geremia, N.M., et al., *Endogenous BDNF regulates induction of intrinsic neuronal growth programs in injured sensory neurons*. Exp Neurol, 2010. **223**(1): p. 128-42.
386. Geschwind, M.D., et al., *Defective HSV-1 vector expressing BDNF in auditory ganglia elicits neurite outgrowth: model for treatment of neuron loss following cochlear degeneration*. Hum Gene Ther, 1996. **7**(2): p. 173-82.
387. Larimore, J.L., et al., *Bdnf overexpression in hippocampal neurons prevents dendritic atrophy caused by Rett-associated MECP2 mutations*. Neurobiol Dis, 2009. **34**(2): p. 199-211.
388. Lu, J., et al., *SMAD pathway mediation of BDNF and TGF beta 2 regulation of proliferation and differentiation of hippocampal granule neurons*. Development, 2005. **132**(14): p. 3231-42.
389. Mey, J. and N. Rombach, *Retinoic acid increases BDNF-dependent regeneration of chick retinal ganglion cells in vitro*. Neuroreport, 1999. **10**(17): p. 3573-7.
390. Spencer, T.K., W. Mellado, and M.T. Filbin, *BDNF activates CaMKIV and PKA in parallel to block MAG-mediated inhibition of neurite outgrowth*. Mol Cell Neurosci, 2008. **38**(1): p. 110-6.

391. Zhou, Z., et al., *Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation*. Neuron, 2006. **52**(2): p. 255-69.
392. Alonso, M., J.H. Medina, and L. Pozzo-Miller, *ERK1/2 activation is necessary for BDNF to increase dendritic spine density in hippocampal CA1 pyramidal neurons*. Learn Mem, 2004. **11**(2): p. 172-8.
393. Dunham, J.S., et al., *Expression of hippocampal brain-derived neurotrophic factor and its receptors in Stanley consortium brains*. J Psychiatr Res, 2009. **43**(14): p. 1175-84.
394. Numakawa, T., et al., *Functional interactions between steroid hormones and neurotrophin BDNF*. World J Biol Chem, 2010. **1**(5): p. 133-43.
395. Gourley, S.L., et al., *REGIONALLY-SPECIFIC REGULATION OF pERK1/2 MAP KINASE IN A MODEL OF ANTIDEPRESSANT-SENSITIVE CHRONIC DEPRESSION*. Biological psychiatry, 2008. **63**(4): p. 353-359.
396. Ashokan, A., M. Sivasubramanian, and R. Mitra, *Seeding Stress Resilience through Inoculation*. Neural Plast, 2016. **2016**: p. 4928081.
397. Bowling, S.L., J.K. Rowlett, and M.T. Bardo, *The effect of environmental enrichment on amphetamine-stimulated locomotor activity, dopamine synthesis and dopamine release*. Neuropharmacology, 1993. **32**(9): p. 885-93.
398. Bardo, M.T., et al., *Environmental enrichment attenuates locomotor sensitization, but not in vitro dopamine release, induced by amphetamine*. Pharmacol Biochem Behav, 1995. **51**(2-3): p. 397-405.
399. Bowling, S.L. and M.T. Bardo, *Locomotor and rewarding effects of amphetamine in enriched, social, and isolate reared rats*. Pharmacol Biochem Behav, 1994. **48**(2): p. 459-64.
400. Kalin, N.H., S.E. Shelton, and R.J. Davidson, *The role of the central nucleus of the amygdala in mediating fear and anxiety in the primate*. J Neurosci, 2004. **24**(24): p. 5506-15.
401. Sah, P., et al., *The amygdaloid complex: anatomy and physiology*. Physiol Rev, 2003. **83**(3): p. 803-34.
402. Ehrlich, I., et al., *Amygdala inhibitory circuits and the control of fear memory*. Neuron, 2009. **62**(6): p. 757-71.
403. Larsson, F., B. Winblad, and A.H. Mohammed, *Psychological stress and environmental adaptation in enriched vs. impoverished housed rats*. Pharmacol Biochem Behav, 2002. **73**(1): p. 193-207.

LIST OF PUBLICATIONS

- **Ashokan, A.**, A. Hegde, and R. Mitra, *Short-term environmental enrichment is sufficient to counter stress-induced anxiety and associated structural and molecular plasticity in basolateral amygdala*. Psychoneuroendocrinology, 2016. **69**: p. 189-96.
- **Ashokan, A.**, M. Sivasubramanian, and R. Mitra, *Seeding Stress Resilience through Inoculation*. Neural Plast, 2016. **2016**: p. 4928081.
- Koe, A.S., **A. Ashokan**, and R. Mitra, *Short environmental enrichment in adulthood reverses anxiety and basolateral amygdala hypertrophy induced by maternal separation*. Transl Psychiatry, 2016. **6**: p. e729.