CHAPTER 1

MAIN INTRODUCTION
1.1 Introduction

“If you’re stressed like a normal mammal in an acute physical crisis, the stress response is lifesaving. But if instead you chronically activate the stress response for reasons of psychological stress, your health suffers it.”

— Robert M. Sapolsky

Wilder Penfield mentions, “The brain is the organ of destiny. It holds within its humming mechanism secrets that will determine the future of the human race”. The brain has an incredible capacity to support lifetime of learning and memory and reorganize itself by forming new connections between neurons called synapses. It is highly plastic and has the ability to change throughout life. The neuroplasticity of the brain begins when the immature brain continuously reorganizes itself in the course of adulthood through learning and memory.

The brain is the key organ where stress processes take place. It changes both functionally and structurally as a result of stressful experiences that an individual experience. In order to cope with stress response, there is a distributed, dynamic and plastic neural circuitry that coordinates and monitors the behavioral and physiological responses to meet the demands imposed by particular stressors. There exists a bidirectional signaling between the brain and body. Short term changes can become adaptive, but when prolonged can become maladaptive and eventually lead to stress related mental and physical health conditions.

The structural and functional changes in the brain is not uniform across all regions. The magnitude of the changes are region specific leading to complex symptoms in psychiatric disorders. The limbic and cortical circuits are known to play a key role in memory, emotion and cognition (Ledoux, 1989).

Three main regions involved in the etiology of stress and memory circuitry are amygdala (BLA), hippocampus and prefrontal cortex (Lupien and McEwen, 1997). Hence, it becomes important to study the region specific changes caused by stress in these regions. The amygdala, the hippocampus and the prefrontal cortex also critically participate in orchestrating the
hypothalamic–pituitary–adrenal (HPA) axis leading to stress disorders (Mathew et al., 2008). (Fig.1.1).

1.2. The amygdala

The amygdala is an almond-shaped structure located deep within the temporal lobe. It was first identified by Burdach in the early 19th century (Sah et al., 2003). The amygdaloid complex comprises of 13 nuclei, which are further divided into subdivisions that have extensive internuclear and intranuclear connections (Paré et al., 1995). The basolateral complex is the largest subdivision and is composed of the lateral nucleus, basolateral nucleus and accessory basal nucleus. The central amygdala and the medial amygdala are considered as an extended amygdala. While lateral nucleus is the main point of entry for sensory inputs into the amygdala, central amygdala is considered as output nucleus and projects to the brainstem (McDonald, 1992) (Fig.1.2)
Amygdala receives inputs from several of amygdaloid nuclei as well as from medial prefrontal cortex (mPFC), paraventricular nucleus, hippocampus and other regions (Sotres-Bayon and Quirk 2010; Fabrico H.Do-monte 2015). It also projects to several brain regions including the hypothalamus, periaqueductal grey matter (PAG), bed nucleus of stria terminalis (BNST) etc., (Rizvi et al., 1991; Sun et al., 1991).

The amygdala is a locus of fear memories and plays an important role in emotional behavior in the context of aversive experiences (Holland and Gallagher, 1999; LeDoux, 2000). Stress and stress hormones have a great influence on the amygdala and the activation of the same enhances the storage of emotionally arousing experiences (Davis, 1992; Ehrlich et al., 2009; Suvrathan et al., 2014). Amygdala with its huge network modulates different memory functions including retrieval and working memory (Nathan et al., 2004; Roozendaal et al., 2004). On the other hand, stress is known to disturb these interactions and impair memory (McGaugh and Roozendaal, 2002; Nathan et al., 2004; Roozendaal et al., 2004, 2009; Pikkarainen et al., 1999).

1.3. The hippocampus

Hippocampus is a brain structure embedded deep into the temporal lobe. It is a banana shaped structure consisting of two lobes, one lobe in each hemisphere. It has a laminar structure consisting of dentate gyrus, a densely packed cell layer and Cornu Ammonis (CA). The CA is further subdivided into CA1, CA2, CA3, and CA4. Each region has distinct cellular and physiological properties (David and Pierre, 2009) (Fig.1.3).

![Hippocampal anatomy in rodents showing different sub regions](https://bmcneurosci.biomedcentral.com/articles/10.1186/1471-2202-6-36/fig3 and paxinos atlas)
The hippocampus has both afferent and efferent connections and regulated through two pathways i.e., fornix and entorhinal cortex. It has reciprocal connections with amygdala (Jin and Maren, 2015; Pitkanen A, 2000; Saunders and Rosene, 1998).

Hippocampus plays a major role in learning and memory and is studied extensively in rodents and humans. It is known to be important in declarative and episodic memory in humans ((Dolan and Fletcher, 1997; Squire and Zola, 1996; Stella et al., 2012) and contextual and spatial memory in rodents (Morgado-Bernal, 2011; Morris et al., 1982; Stella et al., 2012). Hippocampus is rich in the concentration of receptors for the stress hormones, called glucocorticoids (i.e., cortisol in humans, corticosterone in rodents) and considered as a major regulator of the hypothalamic-pituitary-adrenal (HPA) axis (McEwen et al., 1968; Sapolsky et al., 1984). Hippocampus is important for acquisition of newly formed memories and is one of the unique region in the brain where the neurogenesis continues even through adulthood (Bonfanti L, 2011; Steinworth S, 2005). The spatial memory and the spatial information encoded by hippocampus is reflected in the properties of place cells, that show location specific firing (Tomar A, 2015). Hippocampal neural activity is necessary for both encoding and retrieval of spatial memory both for consolidation and long-term storage (Riedel et al., 1999).

1.4. The medial prefrontal cortex (mPFC)

The prefrontal cortex is involved in cognitive processes that are generally referred to as executive functions. The prefrontal cortex is located in the anterior part of the frontal lobe towards the lateral side occupying the basal part of the brain. The prefrontal cortex (PFC) of the rat has been divided into medial, orbital and lateral parts (Ongür and Price, 2000). The medial PFC (mPFC) consists of the four main divisions i.e., medial precentral, the anterior cingulate (AC), the prelimbic (PL), the infralimbic (IL) cortices (Berendse and Groenewegen, 1991; James P. Ray, 1992; Ongür and Price, 2000). It also plays an important role in social behavior (Myers et al., 1973; Robert D Rogers, 2004) (Fig.1.4).

The mPFC receives projections from hippocampus (Jay et al., 1989, 1996). It has reciprocal anatomical interconnections with amygdala (Krettek; Mcdonald, 1991; McDonald et al., 1996; Porrino et al., 1981). Further retrograde labelling studies show that prelimbic (PL) and infralimbic
(IL) gets and sends projections from the agranular insular cortex, hippocampus, the subiculum, amygdala hippocampal area (Condé and Maire-lepoivre, 1995; Shirley L. Buchanan, 1994).

The medial prefrontal cortex (mPFC) is an important component mediating the neural circuitry of stress and its implications. It is associated with diverse functions including attentional processes, decision making and goal directed behavior. Plays an important role in recognition memory, familiar spatial response, spatial location of sequences, visual object information, executive and cognitive functions, object recognition and object location memory tasks (Chiba et al., 1997; Dalley et al., 2004; Ennaceur et al., 1997; Kesner et al., 1996).

While amygdala is vital for fear memory acquisition, mPFC is important in fear circuitry, further sub regions like infra limbic region is the seat of fear extinction and pre limbic region is important for fear expression (Fabrico H.Do-monte 2015). Along with hippocampus, mPFC plays an important role in anxiety related behavior (Adhikari et al., 2010).

1.2.1. Learning and memory

Dr. James Zull describes, “Learning means the modification, growth and pruning of our neuronal connections or neuronal networks called “synapses” and through experience, we are cultivating our own neuronal networks”. Memory is broadly classified as declarative and non-declarative memory. Declarative memory is classified into factual and episodic memory and involves brain regions like hippocampus and medial temporal lobe. Non-declarative memory is classified into motor learning, associative learning and non-associative learning which involves brain regions like cerebellum, motor cortex and striatum (Squire and Zola, 1996).
1.2.2. Brain regions involved in memory circuitry

Memory circuitry involves mainly communication and connectivity between amygdala, hippocampus and mPFC (Canteras et al., 2010). Both human and animal studies over decades of research reveal that the hippocampus and mPFC play an important role in encoding and retrieval of episodic memories (Alison R.Preston, 2013; ME Hasselmo, 2005; PJ Kennedy, 2004). Amygdala and mPFC are involved in the encoding and expression of learned fear (Nicole M.Lauzon, 2010; Pape HC and Pare D, 2010) and share reciprocal projections which are functionally relevant (Mcdonald, 1991; Pitkanen A, 2000; Rosenkranz and Grace, 2003).

1.2.3. Stress and stress response

The term “stress” was coined by Hans Seyle, an Hungarian endocrinologist in 1936 and defined as “the non-specific response of the body to any demand for change”. Stress can be classified mainly as good stress and bad stress. Good stress is sometimes facing a challenge by taking a risk and feeling rewarded by positive and adaptive changes. Bad stress refers to experiences which are prolonged, physically exhausting, dangerous and inability to cope up and resilience is impaired (McEwen BS, 2000).

The hypothalamic-pituitary-adrenal (HPA) axis mediates the stress response. In response to a stress, hypothalamic neurons release corticotrophin-releasing factors (CRF) and this triggers subsequent secretion and release of ACTH from the pituitary following which corticosteroids are secreted and released from the adrenal glands. The HPA axis response to stress partly determined by the ability of corticosteroids to regulate ACTH and CRF release. Circulating corticosteroids inhibit the secretion of CRF and ACTH leading to homeostasis through feedback mechanisms. Hippocampus and mPFC provides negative feedback (Diorio et al., 1993; Jacobson and Sapolsky, 1991; McEwen and Morrison, 2013) to shut the HPA activity and amygdala provides positive feedback to increase the HPA activity (Feldman S, 1980, 1985) (Fig.1.5).
Prolonged or chronic stress are main causes for mental illness. It leads to various psychiatric and neurodegenerative disorders including major depressive disorder (MDD) and post-traumatic stress disorders (PTSD) (Kessler, 1997). Major symptoms of stress related disorders are hyper vigilance, avoidant behaviors, anger, anxiety, depression (M. Lindau, 2016; Matar et al., 2013).

1.3.1. Animal models of stress

Animal model systems serve as a great platform to study the effects of stress and its implications across various brain areas. There exists different stress paradigms that are tailored to investigate the different aspects of stress and stress induced behaviors. Clinical and neuroimaging studies have shown that stress leads to distinct structural and functional changes in the brain which are region specific (Rauch et al., 2000a; Shin et al., 2005). This poses a great challenge to study the stress related disorders in animal models. Several animal models are proposed to study stress induced plasticity right from behavior to molecular level. Stressors are broadly classified as physical stressors and social stressors. Physical stressors are using rodent bags, restrainer stress, vibration stress on a shaker, tail suspension stress and forced swim stress (Alfaez and Krugers, 2003; Galea et al., 1997; Porsolt et al., 1978; Steru et al., 1985; Vyas et al., 2002). (Fig.1.6). Based on the
duration of stress, there are two categories of physical stressors, acute stress (Mitra et al., 2005a) and chronic stress or restrained stress (McEwen BS, 2000; Vyas et al., 2002). Ethologically relevant stressors of social stimuli which animals are likely to interact in their life time. They are maternal isolation, social defeat, social isolation etc.,(Frisone et al., 2002; Koolhaas et al., 2017).

1.3.2. Implications of stress on learning and memory

Studies in animals and humans have shown that the brain is very sensitive especially during early childhood and old age and undergoes changes during these period and thus modulates the memory process (M. Lindau, 2016). Stressful events are either tangible or mentally evoked, which could be a physical or psychological in nature (Mitra et al., 2005; Vyas et al., 2002). Stress can be a threat to the physiological and psychological integrity of an individual and may result in psychic and behavioral changes (Fuchs et al., 2006). Stress has a great impact on learning and memory and its effects vary across different brain areas. For example stress affects hippocampus and impairs cognition, in amygdala it disturbs the fear memory and in the mPFC, it affects the higher order

Figure 1.6: Different animal models of stress

Image adapted from https://www.nature.com/articles/nn.4115/fig 2

Studies in animals and humans have shown that the brain is very sensitive especially during early childhood and old age and undergoes changes during these period and thus modulates the memory process (M. Lindau, 2016). Stressful events are either tangible or mentally evoked, which could be a physical or psychological in nature (Mitra et al., 2005; Vyas et al., 2002). Stress can be a threat to the physiological and psychological integrity of an individual and may result in psychic and behavioral changes (Fuchs et al., 2006). Stress has a great impact on learning and memory and its effects vary across different brain areas. For example stress affects hippocampus and impairs cognition, in amygdala it disturbs the fear memory and in the mPFC, it affects the higher order

1.3.2. Implications of stress on learning and memory

Studies in animals and humans have shown that the brain is very sensitive especially during early childhood and old age and undergoes changes during these period and thus modulates the memory process (M. Lindau, 2016). Stressful events are either tangible or mentally evoked, which could be a physical or psychological in nature (Mitra et al., 2005; Vyas et al., 2002). Stress can be a threat to the physiological and psychological integrity of an individual and may result in psychic and behavioral changes (Fuchs et al., 2006). Stress has a great impact on learning and memory and its effects vary across different brain areas. For example stress affects hippocampus and impairs cognition, in amygdala it disturbs the fear memory and in the mPFC, it affects the higher order
functions like execution and also impairs fear extinction (Chakraborty and Chattarji, 2018; Lupien and McEwen, 1997).

### 1.4.1. Effects of stress on amygdala

Amygdala is a seat of emotions and shows positive feedback as a result of stress. Stress leads to enhanced anxiety, fear memory at behavioral level (Conrad et al., 1999; Mitra et al., 2005; Vyas et al., 2004, 2006) and increased spinogenesis and dendritic arborization at the structural level (Vyas et al., 2004, Mitra et al., 2005). In vitro electrophysiological studies have revealed that stress enhanced synaptic plasticity in the form of enhanced LTP in the amygdala pyramidal neurons (Suvarthan et al., 2014; Yasmin et al., 2016). Stress also causes an increase in expression of BDNF, a protein that is important for synaptic, morphological, and cognitive plasticity mechanisms (Lakshminarasimhan and Chattarji, 2012).

### 1.4.2. Effects of stress on hippocampus

The hippocampus is vulnerable to stress effects because it contains abundant glucocorticoid receptors (Beylin and Shors, 2003; McEWEN and Wallach, 1973; Rao et al., 2012). Acute or repeated stress leads to morphological, molecular, physiological and behavioral changes in the hippocampus, manifested as cognitive impairments (Diamond et al., 2006; Francis et al., 1995; Lupien et al., 1997). Stress leads to dendritic shrinkage (Watanable Y et al., 1992; Magariños et al., 1995; Sousa N et al., 2000; Vyas et al., 2004) and also reduction in dendritic spines (Chen Y et al., 2010; Silva-Gomez et al., 2003), disrupts synaptic transmission leading to deficits in hippocampal memory (Kim JJ et al., 2002). Stress leads to reduced firing rate of place cells and impaired place cell remapping for different contexts (Tomar et al., 2015).

### 1.4.3. Effects of stress on mPFC

Medial prefrontal cortex (PFC) plays an important role in mediating the effects of stress on both cognition and psychopathology (Goldman-Rakic PS, 1995, Arnsten et al., 2009). Repeated stress causes cognitive impairment by suppressing glutamate receptor expression and function in prefrontal cortex (Yuen EY et al., 2009). Chronic or restrain stress cause impaired temporal order
recognition memory in rats, a cognitive process controlled by the mPFC (Yuen et al., 2012). Chronic restraint stress also impairs working memory and caused spine loss and debranching and shortening of dendrites on mPFC neurons (Cook and Wellman, 2004; Goldwater et al., 2009; Liston et al., 2006; Radley et al., 2004, 2006).

1.5.1. Post traumatic stress disorder (PTSD)

PTSD is a psychiatric condition that occurs as a result of trauma or traumatic incident and is characterized by persistent flashbacks, hyper vigilance, and hyper anxiety (Liberzon and Sripada, 2008; Rao et al., 2012a). Not all individuals who undergo trauma are prone to develop PTSD. The occurrence varies greatly. This is can be attributed partly to the individual’s genetic makeup. In most of the PTSD patients, the observed findings are that they re-experience the trauma very often and this flashbacks in turn lead to nightmares. The patients fail to learn to be safe in the absence of threat (Milad et al., 2006). Much work has focused on defining animal models of PTSD to understand the pathophysiology that reproduce salient features of the human syndrome (Adamec et al., 2006; Cohen et al., 2006a; Siegmund and Wotjak, 2006). The anxiety like behaviors in animals assessed with standardized tests such as the elevated plus maze (EPM), open field, social interaction test. Neuroimaging techniques have been critical in the process of identifying key brain systems in the pathophysiology of PTSD. Decades of neuroimaging research has yielded important information concerning the structure, neurochemistry and function of the amygdala, medial prefrontal cortex and hippocampus in posttraumatic stress disorder (PTSD). The neuroimaging research provides evidence that amygdala activity is heightened, diminished volume in the medial prefrontal cortex and in hippocampus and impaired neuronal integrity in PTSD (Davis, 1992; Gilbertson et al., 2002; Morris et al., 1982; Whalen et al., 1998; Woodward et al., 2006; Yamasue et al., 2003).

1.5.2. Aggression

Aggression is a natural biological form of social behavior and is commonly experienced by both animals and humans. Animals can be used to study aggressive behavior was first mentioned by Darwin. Hess in 1928, by electrically stimulating the hypothalamus of cats proved that an attack behavior can be elicited. Aggressive behaviors in many social organisms play a pivotal role in the
defense of territory, as well as in acquiring social status and resources like food and mates. Furthermore, individual differences in aggressive behaviors have been considered as important indicators of different coping strategies. Coping is the behavioral flexibility or alternative response patterns in reaction, that an animal adapts to combat the challenge and it could be either proactive and reactive coping (Koolhaas et al., 1999) and this is even evident in other species like fish and birds where a different term like shyness and boldness is used (Sloan Wilson et al., 1994). For instance, high-aggressive animals adopt a proactive coping style showing more impulsive, rigid behavioral patterns whereas low-aggressive animals are reactive copers exhibiting more flexible behaviors that depend on environmental cues (de Boer, 2018).

1.5.3. Importance of dendritic spines

Synapses are critical for the functioning of nervous system. Majority of synapses occur on dendritic specializations called ‘spines’ (Grutzendler et al., 2002; Maletic-Savatic et al., 1999). The visualization of neurons and spines with the help of Golgi Cox technique has advanced our understanding of how a complex brain functions and how different environments influence its functioning. The technique was first developed by Golgi and modified by Cox in the 1800s. (Louth et al., 2017). Discovery of this technique was a breakthrough in the field of neuroscience. Its selective staining properties (1-3% of cells) and darkly stained neurons enables identification of complete neuronal morphology. Dendritic spines are neuronal protrusions, which are the major sites of excitatory synaptic transmission in the central nervous system. They contain neurotransmitter receptors, organelles and signaling systems essential for synaptic function and plasticity (Harris and Kater, 1994; Nimchinsky et al., 2002). Spines undergo remodeling in response to perturbations in the form of stress and also through pharmacological interventions.

1.6 Objectives

Earlier work from our laboratory has shown that Acute Immobilisation Stress (AIS) leads to increased anxiety and spinogenesis in BLA, not immediately but after a delay of 10 days (Mitra et al., 2005). The delayed build of anxiety seen in BLA is reminiscent of posttraumatic stress disorder (PTSD) where the effects are both delayed and prolonged (Yehuda and Antelman, 1993; Yehuda et al., 1998) and accompanied by hyperactivity in the amygdala (Yehuda R, 2007). The various stress paradigms employed rely either on acute or chronic stress, but our study is novel in
investigating the temporal progression of stress in a delayed manner. Stress effects studied both in animal models of stress and human studies including PTSD, there are three main regions involved in the stress - amygdala, hippocampus and medial prefrontal cortex (mPFC). Hence my first aim was to study the delayed effects of stress induced plasticity in the hippocampus and the mPFC.

1.6.1 Objective 1

1. Does exposure to acute stress have delayed effects on neuronal morphology in the hippocampus and medial prefrontal cortex? If so, are these effects similar or different compared to those reported earlier in the amygdala?

From my first aim, it is evident that even a single exposure to stress can cause delayed effects in the hippocampus and mPFC. Hence for my second aim, I wanted to explore the stress in a more ethologically relevant model of social stress and investigate the nature of social stress on the structure of BLA and the hippocampus. This study was conducted using murine mice model of PTSD.

1.6.2 Objective 2

To investigate whether psychosocial stress elicits divergent temporal patterns of spine formation in the hippocampus versus the amygdala.

Large number of studies using social stress have focused on the defeated subjects or the intruders and explored the stress induced effects across various levels of neuronal organization. For my third aim, I wanted to investigate the structural remodeling that occurs in the resident rats and explore if successive victory over the intruders leads to a stressful situation in the resident rats. This study was carried out in wild type Groningen rats, which are a highly studied model of aggression.

1.6.3 Objective 3

3. To investigate how social defeat stress affects specific cells and circuits within the amygdala and how they compare with effects of physical stressors using the Wild Type Groningen (WTG) rats.
CHAPTER 2
MATERIALS AND METHODS
2.1 Experimental animals

2.1.1 Morphological Experiments

A total of 12 male Wistar rats weighing around 300-350 grams were used from Institutional animal facility, NCBS, India) were housed (group of 2 per cage) and maintained on a 10/14-hour light/dark cycle with *ad libitum*, access to food and water. All procedures related to animal maintenance and experimentation was approved by the Institutional Animal Ethics Committee (National Centre for Biological Sciences).

2.1.2 Behavioral analysis experiments

A total of 22 male Wistar rats weighing around 300-350 grams used from Institutional animal facility, NCBS, India) were housed (group of 2 per cage) and maintained on a 10/14-hour light/dark cycle with *ad libitum*, access to food and water. All procedures related to animal maintenance and experimentation was approved by the Institutional Animal Ethics Committee (National Centre for Biological Sciences).

2.2 Stress protocol (for chapter 4)

Acute stress was evoked as described previously (Mitra et al., 2005). Briefly, animals were subjected to a single episode of 2 hour complete immobilization (10:00 am–12:00 pm) in plastic bags without access to either food or water. All the animals remained undisturbed for next 10 days. On the 11\(^{th}\) day of experiment, these rats were decapitated and fixed in Golgi Cox solution (Fig.2.1A).

2.3 Golgi Cox fixation protocol modified as part of this thesis and details can be found in Chapter 3

After the termination of the stress or behavior protocol, brains were decapitated and fixed in Golgi Cox fixative. The fixation duration was for 2 weeks followed by 1 week incubation in 6% sucrose solution. The brain tissue was sectioned using Leica vibrotome (Leica 1200S) with 0.1M PB in the bath solution. Sections were directly collected on gelatin coated slides (4 sections on each slide). After sectioning, the sections were washed and subjected to enzymatic reaction and washed again and dehydrated and mounted with DPX.
2.3.1. Microscopy analysis to study the morphology

Once the slides were ready for analysis, the spine density analysis was done using a Neurolucida software (100x, 1.3 numerical aperture) from MicroBrightField Inc., (Williston, Vermont), attached to an Olympus BX61 microscope. All protrusions along the primary dendrite irrespective of their morphological characteristics were analysed as spines along 80µm stretch of the dendrite using NeuroLucida software (Mitra et al., 2005). For dendritic arborization neurons were traced at 40x magnification using the neuron tracing tool from Neurolucida software and later subjected to sholl analysis.

2.4 Protocol for spatial memory experiment

![Diagram of the protocol for spatial memory experiment]

2.4.1 Object displacement task

The hippocampal spatial memory was evaluated using an object displacement task. The task design was modified from previous study (Gobbo and O’Mara, 2004; Rahman et al., 2016). The floor of the apparatus was layered with corncob bedding. The bedding material was the same that was used by the rats in their home cages.

The test was conducted for three days. Two days of context habituation, followed by training session and then finally testing (Fig. 2.1B). To elaborate, the animals were left to habituate in the arena for fifteen minutes on day one. The habituation was repeated on day two as well, for the same duration. The training phase was conducted on day three which consisted of three sessions.
of five minutes each with an inter session interval (ISI) of 2 minutes. During ISI, the animals were kept in a cage in the same room. During training sessions, four objects were placed in square formation approximately 10 cm from the centre of the arena. The objects were of similar dimension and were screwed on their bottom to the apparatus, so that the rats do not move the objects.

![Figure 2.1C: Schematic of Object displacement task](image)

The rats were subjected to a testing phase 6 hours after the end of the training phase. During the testing phase, only one of the objects was displaced by 6 cm towards the centre of the arena. The testing phase consisted of five minutes. After every session the arena and the objects were wiped with alcohol to remove odors. The bedding was changed between controls and stress group. The behavior was recorded with a camera mounted on the top of the apparatus for all the sessions.

### 2.4.2 Temporal order memory task

This task was done to evaluate the short term memory, a cognitive process controlled by the medial prefrontal cortex. The task used was modified from Barker et al., 2007 (Barker et al., 2007) (Fig.2.1B). The apparatus used for this experiment was the same setup that was used for object displacement task. The only differences were the objects used and the duration of the task where only two objects were used for this task. The task consisted of three phases.

![Figure 2B: Schematic of Temporal order memory task](image)
The first phase was the training session, three trials of five minutes each with an ISI of 2 minutes. During ISI, the rats were kept in a cage in the same room. The first phase of training was conducted with a set of 2 similar objects and during the second phase another set 2 other similar objects. There was a gap of one hour between the first phase and the second phase of training. The rats were subjected to a testing phase 3 hours after the end of the second training phase. During testing phase, one object from the first phase and another object from the second phase was kept. The testing session was conducted for 4 minutes. The bedding was changed between controls and stress group. The behavior was recorded with a camera mounted on the top of the apparatus for all the sessions.

2.4.3 Object displacement task behavior analysis

The displaced object explored by the rat was evaluated by the time spent in contact with the object (Gobbo and O’Mara, 2004; Rahman et al., 2016). Total exploration time was normalized to the total session time to obtain the exploration rate. The difference in exploration rate between training and testing was used to evaluate the response showed, exploring the displaced object. The behavior were recorded during all the sessions using Ethovison software from Noldus. The tracks were analysed to calculate the time spent at each point in the arena. The change in exploration rate was calculated by subtracting the exploration rate during the training session from the exploration rate during testing session.

2.4.4 Temporal order memory task analysis

The temporal order memory task used to assess the short term memory, and in this task, the animal’s ability to differentiate between two familiar objects at two different time intervals was evaluated. The discrimination ratio was calculated as the difference in the time spent by each animal exploring the object from phase 1 compared with object from the phase 2 divided by total time spent exploring both objects during the 4 minute test period.
2.5 Protocols for cage-in-cage resident intruder paradigm (for Chapter 5)

2.5.1 Experimental animals

2.5.2 Aggressor mice

SJL mice around 6-week old, weighing 30–35 grams were used. They were housed individually in polycarbonate cages (48 cm × 27 cm × 20 cm) with ad libitum food and water, under a reverse dark-light cycle. The aggressor mice SJL mice were trained to assault intruders.

2.5.3 Subject mice

A total of 65 C57BL/6J male mice around 6-week old, weighing 20–25 grams were used. They were singly housed in a separate room.

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Walter Reed Army Institute of Research and the Medstar Research Institute and were performed in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All mice were purchased from Jackson Laboratory, ME, USA.

![Diagram](image)

**Figure 2.2A Schematic showing Cage-within cage-resident intruder paradigm**
2.6 Stress protocol

2.6.1 ‘Cage-in-cage’ resident-intruder exposure

The subject mice were placed in a wire mesh cage (17.5 cm × 14 cm × 7.5 cm) which was in turn placed within the aggressor’s larger plastic cage (48 cm × 27 cm × 20 cm) for 6-hours per day and repeated for 10 days. The control mice were also subjected to the same daily schedule but without exposure to an aggressor. Both control and socially stressed (SS) mice were deprived of food and liquid during the 6-hour ‘cage-in-cage’ episode. The SS mice were directly exposed to the aggressor mice at three random times during the 6-hour ‘cage-in-cage’ episode. These direct exposures lasted for 1 minute or till the aggressor mice were able to inflict 10 strikes to the SS mice, whichever came first. (Fig.2.2A)

![Immediate effect: Aggressor stress](image)

![Delayed effect: Aggressor stress followed by stress free recovery](image)

Figure 2.2B: Schematic showing Aggressor exposure social stress (Agg-E SS)

2.6.2 Morphology

One-day after the 10-day Agg-E SS, groups of control and Agg-E SS mice were subjected to Golgi Cox analysis to study the immediate effects of stress. Additional groups of control and stress-exposed mice were likewise subjected for Golgi Cox analysis after a 4 week home-cage rest (Fig.2.2B). The brains were carefully removed from the skulls of euthanized mice, impregnated with Golgi-Cox staining solution, and processed according to the protocol established by FD Neurotech, Inc. (Ellicot City, MD). Briefly, the impregnated brains were serially sectioned at a thickness of 100µm. Every third cryostat section (intervals of 300µm) was mounted on gelatin-coated slide, stained, dehydrated in ethanol, cleared in xylene, and cover-slipped in permount.
2.7 Protocol for coping styles in evaluating aggressive behavior (for chapter 6)

2.7.1 Experimental animals

A total of 50 Wild Type Groningen (WTG) rats of 4 month old was used. The animals were kept with 12/12-hour reversed light/dark cycle (lights off at 10:00 h) and food and water were given ad libitum. All behavioral experimental procedures were performed during the dark phase (11:00–15:00 h) of the cycle. All experimental protocols conducted were approved by the Animal Ethics Committee of Groningen University.

The ancestors of Wildtype Groningen were originally wild-trapped and were subsequently bred in laboratory for 49 generations. They exhibit a rich repertoire of social behavior including aggressive behavior. Their natural behavior was studied and measured using the resident intruder paradigm.

2.7.2 The resident WTG rat

The resident WTG rat was housed in a big cage of 80×55×40 cm. One week prior to start of the experimental paradigm, a sterilized female rat was introduced in the same cage. Female rat was introduced to prevent social isolation of the resident rat and also to retain the territorial dominance of the resident rat. The female rat is removed one hour before the experiment.

2.8 Resident Intruder paradigm

An intruder male was introduced into the home cage of the resident. The behavior was recorded using a light sensitive video camera. The test duration was for 10 minutes which is enough to show the offensive behavior. After the test, the intruder was removed and the female rat was reintroduced. The resident rat continues to stay with the female rat. This continues till the completion of the experimental protocol (Fig.2.3A).
2.8.1 Short term aggression or coping style

The above procedure was followed for 4 days and on the day 5, brains were harvested and fixed in Golgi Cox fixative to study the morphology. This study was done to study the baseline differences or the trait characteristics among the WTG rats. They are known to exhibit a high degree of inter-individual variation (Fig.2.3B).

2.8.2 Long term aggression or coping style

This protocol was followed for 22 days, where the intruders were introduced at random days in the resident’s cage and 2 days after the last session, the residents and the introducers were subjected for Golgi Cox fixative to study the morphology. (Fig.2.3B).

2.8.3 Resident intruder paradigm analysis

Offensive behavior, defensive behavior and the violent behavior were evaluated (Koolhaas et al., 2013). Each behavior was evaluated based on the following parameters
2.8.4 Offensive behavior

Attack latency (the time between the introduction of the intruder and the first clinch attack), move towards, social exploration, and ano-genital sniffing, rearing, lateral threat and upright posture, clinch attack, keep down, chase.

2.8.5 Calculation of behavioral score

Total offense score: sum of lateral threat, upright, clinch, keep down and chase. Social exploration score: sum of social explore, ano-genital sniffing and social groom.

2.8.6 Defensive behavior

Submission latency, submissive posture, move away, flight, defensive upright posture, freeze, non-social exploration, rearing.

2.8.7 Calculation of behavioral score

Defense score: Sum of the amount of time spent on flight, defensive upright posture, submission and freeze

2.8.8 Violent behavior

Short latency is a first indicator of violence, increased lateral threat frequency, bite and harm the intruder, attack the anesthetized intruder.

2.8.10 Calculation of behavioral score

Frequencies of the lateral threat and clinch were calculated. A ratio below 1 shows that animals attack without any introductory behavior and forewarning which is a reliable indicator of violence (out of control).
CHAPTER 3

Golgi Cox – a simplified technique to study neuronal morphology
3.1 Abstract

Discovery of Golgi technique in the late 19\textsuperscript{th} century by Camillo Golgi was a breakthrough in the field of neuroscience. Its selective staining properties (1-3\% of cells) and darkly stained neurons enabled identification of complete neuronal morphology. Since then the technique was modified by different laboratories based on their work. Earlier methods used in the laboratory was both time consuming and costly. The modified technique presented here works like a simple histology technique, stains both dendrites and spines with minimal background and has been working across hands in our laboratory.

3.2. Introduction

In the development and plasticity of nervous system, synapses play a crucial role. Vast majority of synapses occur on dendritic specializations called ‘spines’ (Grutzendler et al., 2002; Maletic-Savatic et al., 1999; Van Harreveld and Fifkova, 1975). Dendritic spines are neuronal protrusions, which are the major sites of excitatory synaptic transmission in the central nervous system. They contain neurotransmitter receptors, organelles, and signaling systems essential for synaptic function and plasticity (Harris and Kater, 1994; Nimchinsky et al., 2002). Spines are highly pleomorphic and undergo activity-induced modification of neuronal connections involving alterations in both the functional efficacy of synaptic transmission and the structure of neuronal connections (Matsuzaki et al., 2001). Spine plasticity and maintenance can be modulated by sensory experience and forms the basis for learning and memory (Yuste and Bonhoeffer, 2001).

Spines undergo remodeling in response to perturbations in the form of stress, acute or chronic and also by pharmacological interventions. Studies from our lab and literature show that stress leads to alteration in spine density. Chronic and acute stress leads to increased spine number in the basolateral amygdala which is a seat of emotional memory and dendritic atrophy in hippocampus (Mitra et al., 2005b; Vyas et al., 2004, 2006). Stressful events leading to fear and mental illness are to certain extent regulated by alterations in dendritic and synaptic structure in brain regions involved in memory circuitry like amygdala, hippocampus and mPFC (Leuner and Shors, 2013).

Dendritic spines play an important role in pharmacological studies. Administration of corticosterone before stress prevented the anxiogenic effects there by suppressing the growth of BLA spines demonstrating a strong link between stress-induced spinogenesis in the amygdala and high anxiety (Liston and Gan, 2011; Rao et al., 2012b). Dendritic spines are modified both
structurally and functionally by modulation of different drug exposures (Hill et al., 2013; Robinson and Kolb, 1999) and also in Fragile X pathology (Hayashi et al. 2004; Dölen et al. 2007).

3.3 Golgi Cox fixation protocol

Stock solutions were prepared separately

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dichromate solution (A)</td>
<td>5%</td>
</tr>
<tr>
<td>Mercuric chloride solution (B)</td>
<td>5%</td>
</tr>
<tr>
<td>Potassium chromate solution (C)</td>
<td>4%</td>
</tr>
</tbody>
</table>

Stock solutions were prepared 24hrs before use. Solutions A, B, C were mixed in the ratio of 5:5:4 on the day of sacrificing animals. A pinch of sodium chloride was added to get a clear Golgi Cox solution. Used 50ml of fixative for each brain.

Figure 3.1A: Schematic showing detailed explanation of modified Golgi Cox fixation protocol

1. Preparation of Golgi Cox fixative
2. Fixation duration 2wks +1wk in 6% sucrose solution
3. Sectioning 120um sections (Leica vibrotome)
4. Processing and mounting
Amber colored bottles were used to store the brains as all the chemicals were good oxidizing agents and light sensitive, it’s better to maintain dark.

Brains were changed into fresh solution of Golgi Cox fixative after 24hrs.

Brains were allowed to remain in the same solution for 15 days.

On day 16, brains were transferred into freshly prepared solution of 6% sucrose in 0.1M PB Changed into a fresh solution of sucrose after 24hrs and the brains were allowed to remain in the same solution for 5 days. (Fig.3A)

**0.1M Phosphate buffer - 1000ml double distilled water, pH-7.4**

1. Na2HPO4 - 14.18 g
2. NaH2PO4 - 3.12 g

Above salts were dissolved in 800ml double distilled water, adjusted the pH to 7.4 and then volume made up to1000ml.

**Slide coating solution**

In a glass beaker, 300ml of distilled water was taken and heated on a hot plate to attain a temperature of 60°C and 9 grams of gelatin was added, once the solution was clear 4.5 grams of chrome alum was added and stirred well and slides were coated while the solution was on the hot plate.

**Slides washing and coating**

Glass slides were soaked in 1% Hcl solution for overnight. Next day slides were washed in distilled water, 3 times of 30 minutes each wash and arranged the slides in glass beaker and allowed them to dry in hot air over for 2hrs. Once dried, the slides were placed in a metal cradle and dipped in the hot solution for 5 mins, excess solution was drained off by placing the cradle on a blotting sheet and the slides were arranged in the slide box and kept the slide box in 37°C overnight and used the next day.

**Sectioning**

On day 6 of sucrose solution, brains were sectioned at 120um thickness using a vibrotome (Leica 1200S) with 0.1M PB as the bath solution.
Sections were directly collected on gelatin coated slides. 4 sections were collected on each slide. On completion of the required region of interest or the entire brain, the excess sucrose on the sections was drained off and sections were gently pressed with forefinger with help of a tissue paper. After allowing sections to air dry for 5 mins, they were processed as mentioned below.

_Utmost care was taken to prevent sections from drying off completely._

**Processing and mounting**

Slides were arranged in a metal cradle and processed as follows:

Solutions were arranged in plastic or metal troughs and the metal cradles were dipped sequentially and mounted. The sections were processed in the following manner.

- **Double distilled water** – 5 mins – 2 changes
- **5% Sodium carbonate** – 20 mins.
- **Double distilled water** – 5 mins – 2 changes
- **70% Ethanol** – 10 mins.
- **95% Ethanol** – 3 mins.
- **Xylene** – 2 mins

Mounted with DPX

Studies were allowed to dry for 2 days before proceeding with the analysis.

**3.4 Microscopic analysis**

Once the slides were ready for analysis, the spine density analysis was done using a Neurolucida software (100x, 1.3 numerical aperture) from (MicroBrightField Inc., Williston, Vermont) attached to an Olympus BX61 microscope. All protrusions along the primary dendrite irrespective of their morphological characteristics were analysed as spines along the 80µm stretch using NeuroLucida software. Region of interest was chosen at 4x magnification, neurons were traced at 40x and spine analysis was carried out at 100x magnification. (Fig.3.1B).
Figure 3.1A: Schematic showing detailed explanation of modified Golgi Cox fixation protocol
3.5 Discussion

Golgi’s method of impregnation of nerve cells in late 1800s has led to lot of resurgence in an interest to investigate behavioral and morphological relationships using the technique. Lot of work has been carried out by Golgi technique and this in turn led to modifications of the technique. The visualization of neurons with the help of Golgi Cox technique has advanced our understanding of how a complex brain functions and how different environments influence its functioning. The technique was first developed by Golgi and modified by Cox in the 1800s. (Louth et al., 2017). The Golgi Cox technique presented here is a simple protocol to study the neuronal morphology and dendritic spines. The technique is modified from original study by Shankar Narayan Rao and Gibbs et al., (Gibb and Kolb, 1998). The main motive behind the standardization was to have a staining protocol that is reliable and reproducible. Especially when you do behavior experiments and keen on knowing the underlying mechanisms, it is often difficult to repeat or replace individual animals or groups after the experiment. In case the staining doesn’t work the entire experiment must be repeated, which is both time consuming and expensive. And most importantly, because of less time consumption, it is always convenient to get both behavior and the morphology correlated on a minimal time scale. It is also ideal to have a method that allows for simple embedding of the entire brain so that every section can be saved for later analysis. This has been used by different members of our laboratory and has consistently worked across hands with high reproducibility. Recently published paper from our laboratory used this modified technique (Madan et al., 2018).
CHAPTER 4
DELAYED EFFECTS OF ACUTE STRESS ON
THE HIPPOCAMPUS AND THE MEDIAL PREFRONTAL CORTEX
4.1. Introduction

4.1.1 Hippocampus

Hippocampus is a complex brain structure embedded deep into the temporal lobe. It plays an important role in learning and memory. In humans it is studied with respect to episodic, declarative memories and in rodents, on spatial, contextual memory. It expresses high levels of stress hormones and vulnerable to stress (Bremner et al., 1997; Eichenbaum, 1997; McEwen, 1999; Schmidt and Duman, 2010; Squire and Zola-Morgan, 1991). Clinical studies using functional and structural MRI studies have reported the dysfunction of hippocampus in stress related psychiatric conditions (Bremner et al., 1997; Liberzon and Sripada, 2008). Acute and chronic stress studies have shown that hippocampus undergoes structural and functional plasticity (Bhakta et al., 2017; McLaughlin et al., 2009). Repeated restraint stress leads to remodeling of CA3 neurons (Bremner et al., 1997; Watanabe et al., 1992). Stress leads to shortening of apical dendrites of CA3 neurons (Conrad et al., 1999; Vyas et al., 2002). Stress impairs long term memory and hippocampal dependent memory (Diamond and Rose, 1994) and enhances the long term depression (LTD) in hippocampus (Kim and Diamond, 2002). Sousa et al., showed that there exist an intimate relationship between the behavioral deficits with underlying neural deficits (Sousa et al., 2000).

4.1.2 Medial prefrontal cortex

Prefrontal cortex is a neocortical regions directly involved in high order executive processes, including integration of cognitive information, emotional states and modulation of subcortical systems (Diorio et al., 1993; Groenewegen and Uylings, 2000; Uylings et al., 2003). The medial prefrontal cortex (mPFC) plays an important role in the neural circuitry that mediates stress and implications of stress response. It is known to modulate neuroendocrine function during stress (McEwen et al., 1986; Meaney et al., 1985). Damage to the prefrontal cortex produces poor judgment, planning, and decision-making in humans (Funahashi, 2017). In rodents models, chronic stress results in neuronal morphological alterations in the mFPC as evidenced by retraction of apical dendritic branches in layer II/III pyramidal cells as well as spine loss (Cook and Wellman, 2004; Liston et al., 2006; Radley et al., 2004). However, the effects of stress are studied at the post stress point only, either at the termination of acute stress or chronic or repeated stress.
Therefore to study the delayed effects or the temporal progression of stress, we investigated behavior and morphology on the hippocampus and the mPFC. Rats were subjected for acute Immobilisation stress for 2hrs and after a delay of 10 days, morphological and behavioral analysis were investigated. We did two behavioral observations through object displacement task and temporal order memory task. At morphology, we analysed dendritic spine plasticity and neuronal tracing. Morphological analysis was performed both on CA1 and CA3 neurons of hippocampus and IL and PL neurons of mPFC.

4.2 Results

Rats were subjected to single episode of Immobilisation stress for 2 hours without food and water. At the end of two hours, the rats were kept in their home cage and not disturbed for 10 days. On day 11, the brains were decapitated and fixed in Golgi Cox fixative for morphological analysis. For dendritic spine analysis, spines were counted manually on the apical dendrites and for dendritic branching, neurons were traced at 40x using NeuroLucida software attached to Olympus microscope BX 61. The following criteria was followed for neuron tracing. Each neuron with its process was devoid of other processes was chosen. Neurons traced at 40x magnification and then then subjected for Sholl analysis. Sholl analysis was done using neuro explorer application by considering centre of the cell body as the reference point, at a fixed radial distance, concentric circles were drawn such that the last the circle covers the entire traced neuron and calculates length and branch points. The output was received as an excel sheet and the control and stress groups were decoded and plotted. (Fig.4.1)

![Representative image of traced neuron subjected for Sholl analysis](image1)

![Schematic showing the description of primary dendrite chosen spine for analysis](image2)

**Figure. 4.1: Schematic explaining the neuron tracing and spine analysis**
4.2.1 Delayed effects of acute stress leads to decreased spine number in apical dendrites of CA3 neurons and also dendritic atrophy of both basal and apical dendrites

CA3 pyramidal neurons showed stress induced dendritic shrinkage in both apical (t=2.26, p=0.029) and basal (t=3.69, p=0.007) dendrites and segmental analysis across the length of the dendrite revealed stress effects in both apical $F_{(1,956)}=49.36$, p=0.0007) and basal $F_{(1,34)}=9.99$, p=0.0033) dendrites. The difference was most prominent in the basal dendrites. Spine numbers in apical dendrites also showed a decrease in CA3 neurons (t=2.9, p=0.005) and detailed segmental analysis also showed decrease in spine number along the length of the dendrite $F_{(1,58)}=12.39$, p=0.008) in a two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test. (Fig.4.2)

![Figure 4.2: Acute stress leads to dendritic atrophy and decreased spine number in the CA3 region of hippocampus.](image)

A). Schematic showing anatomical location of CA3 region. B). Representative CA3 neuron at 20x magnification along with dendrite showing spines. C). Bar graph showing decreased spine number in the apical dendrite. D&E). Bar graphs showing dendritic atrophy in the apical and basal dendrites of CA3 neurons. F). Segmental analysis of spines in the apical dendrite showing the distribution along the 100µm length of the dendrite. G&H). Segmental analysis of apical and basal dendritic length showing the distribution of dendritic branching along the length of the neuron.

* p<0.05, **p<0.01 ***p<0.001
4.2.2 Delayed effects of acute stress lead to decreased spine number in apical dendrites of CA1 neurons and also dendritic atrophy of both basal and apical dendrites

In CA1 pyramidal neurons, stress lead to shrinkage in both apical (t=2.026, p=0.05) and basal (t=2.47, p=0.01) dendrites and segmental analysis across the length of the dendrite revealed stress effects in both apical (F_{1,36}=8.58, p=0.005) and basal (F_{1,36}=4.53, p=0.040) dendrites. Spine numbers in apical dendrites also show a decrease in CA1 neurons (t=3.57, p=0.0008) and detailed segmental analysis also show decrease along the length of the dendrite (F_{1,52}=12.74, p=0.08) in a two-way repeated measures ANOVA followed by Bonferroni’s multiple comparisons test. (Fig. 4.3).

Figure 4.3:
Acute stress leads to dendritic atrophy and decreased spine number in the CA1 region of hippocampus.
A). Schematic showing anatomical location of CA3 region. B). Representative CA1 neuron at 20x magnification along with dendrite showing spines. C). Bar graph showing decreased spine number in the apical dendrite. D&E). Bar graphs showing dendritic atrophy in the apical and basal dendrites of CA1 neurons. F). Segmental analysis of spines in the apical dendrite showing the distribution along the 80μm length of the dendrite. G&H). Segmental analysis of apical and basal dendritic length showing

* p<0.05, **p<0.01 ***p<0.001
4.2.3 Delayed effects of acute stress lead to decreased spinogenesis and dendritic atrophy in IL region of mPFC

The IL region of mPFC showed dendritic shrinkage both apical (t=2.264, p=0.029) and basal (t=3.66 p=0.02), followed by segmental analysis in apical $F_{1,46}=11.71$, p= 0.0013) and basal $F_{1,37}=8.87$, p=0.001 showed significant decrease in the stressed group. Spine numbers in apical dendrites show a decrease (t=3.40, p=0.0013) and detailed segmental analysis also show decrease along the length of the dendrite ((F_{1,363}=25.36, p<0.0001) in a two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test. (Fig.4.4)

**Figure.4.4:** Acute stress leads to dendritic atrophy and decreased spine number in the IL region of mPFC.
A). Schematic showing anatomical location of IL region. B). Representative IL neuron at 20x magnification along with dendrite showing spines. C). Bar graph showing decreased spine number in the apical dendrite. D&E). Bar graphs showing dendritic atrophy in the apical and basal dendrites of IL neurons. F). Segmental analysis of spines in the apical dendrite showing the distribution along the 80µm length of the dendrite. G&H). Segmental analysis of apical and basal dendritic length showing the distribution of dendritic branching along the length of the neuron.

* p<0.05, **p<0.01, ***p<0.001
4.2.4 Delayed effects of acute stress did not affect spines and dendrites in the PL region of mPFC

The PL region of mPFC did not show any effects both in apical (t=0.408, p=0.68) and basal (t=0.508, p=0.61) and also spine number (t=1.103, p=0.27) in a two-way repeated measures ANOVA. (Fig.4.5)

Figure.4.5:
Acute stress did not affect both dendrites and spine number in the PL region of mPFC.
A). Schematic showing anatomical location of PL region. B). Representative PL neuron at 20x magnification along with dendrite showing spines. C). Bar graph showing no change in spine number in the apical dendrite. D&E). Bar graphs showing no change in dendritic length in the apical and basal dendrites of PL neurons. F). Segmental analysis of spines in the apical dendrite showing no change in the distribution along the 80µm length of the dendrite. G&H). Segmental analysis of apical and basal dendritic length showing no change in distribution of dendritic branching along the length of the neuron.
4.2.5. Delayed effects of acute stress leads to impairment in locating the displaced object in the object displacement task

The results showed that a single exposure to stress to lead to delayed effects in CA1 and CA3 and IL regions at the structural level. In the light of these results, we investigated if the same stress causes behavioral deficits 10 days later. To this end, we used object displacement task to assess the hippocampal memory and temporal order memory task to investigate short term memory deficits of mPFC. In this task, the male Wistar rats around 55 days old were handled for 3 days and on day 4 subjected for acute Immobilisation stress for 2 hours and after the end of the stress they were kept back in their home cages in separate room apart from the control group. On day 9 and 10, the rats were habituated for the context for 15 minutes and on day 11, object displacement task and on day 12 temporal order memory task were performed respectively. (refer material and methods for detail). (Refer Fig.2.1)

Object displacement task is challenging task where the rat needs to form the memory of the arena, the objects and their locations and should be able to distinguish a small displacement of one of the objects and it is shown to be hippocampal dependent (Mumby et al., 2002). The displaced object explored by the rat was evaluated by the time spent in contact with the object (Gobbo and O’Mara, 2004; Rahman et al., 2016). Gerstein also showed that performance in Morris water maze is equivalent to object displacement task (Gerstein et al., 2013).

We observed that stress did not significantly affect the exploration of different objects during training session (Two-way Repeated Measures ANOVA with RM by rows; Factor Interaction: $F(3, 60) = 0.7438$, $p=0.5302$, Factor Objects: $F(3, 60) = 1.105; p=0.3544$, Factor Stress: $F(1, 20) = 0.5329; p=0.4739$. However, further analysis within the groups revealed that only unstressed or the control group was able to distinguish the displaced object from the other objects. This is demonstrated by change in exploration rate of only displaced object (object B) in the testing session as compared to the training session. This change was noticed only in the control group. The stressed group was unable to distinguish the displaced object from the other objects (Two-way Repeated Measures ANOVA with RM by rows: Factor Objects: $p<0.0001$, Factor Stress $p=0.0929$, Factor interaction $p=0.0483$ Post hoc Tukey’s test (* means $p<0.05$). (Fig.4.6)
Figure 4.6:

**Acute stress impairs the memory to locate the displaced object in the object displacement task**

A) Schematic explaining the behavioral protocol. B) Schematic showing the arrangement of objects in the object displacement task. C) Change in exploration rate of the displaced object (object B) is significantly higher as compared to other objects, only in the control group and not in the stress group.

*N= 11 animals in each group*
4.2.6. Delayed effects of acute stress does not seem to affect the short term memory as revealed by temporal order memory task

The task used is modified from Barker et al., 2007 (Barker et al., 2007). The temporal order memory task scores for the short term memory controlled by mPFC. In this task, the animal’s ability to differentiate between two familiar objects at two different time intervals are evaluated. The discrimination ratio was calculated as the difference in the time spent by each animal exploring the object from phase 1 compared with object from the phase 2 divided by total time spent exploring both objects during the 4 minute test period.

We observed that delayed effects of stress doesn’t seem affect the short term memory, as we did not notice any significant difference between control and stress group in exploring the objects from the 2 phases (2 way repeated measures ANOVA: Factor stress $F_{(1,66)}=1.471$, $p=0.2295$; factor objects $F_{(2,66)}=2.582$, $p=0.0833$; Interaction $F_{(2,66)}=0.2070$, $p=0.8136$. (Fig.4.7)

![Figure 4.7](image)

**Figure 4.7:**

**Acute stress does not seem to impair the short term memory to locate the novel object in the temporal order memory task.** Schematic explaining the behavioral protocol. B). Schematic showing the arrangement of objects in the temporal order memory task. C). No change in discrimination ratio in locating the novel object (A1) between control and stress group. $N=11$ animals in each group.
4.3 Discussion

In our study, we observed alteration of morphology in hippocampus and mPFC which confirms that a single episode of stress causes delayed effect both on the behavior and the morphology. At structural level, we noticed that the stress lead decrease in spine number in CA3 and CA1 regions followed by dendritic atrophy in both. Simultaneously, at the behavioral level the same stress lead to memory impairment in the well-known hippocampal dependent object displacement task. To further corroborate our data, human imaging studies on PTSD patients, have shown smaller hippocampal volumes (Bremner et al., 1997; Gilbertson et al., 2002; Wang et al., 2010) and exhibit impaired performance on hippocampal dependent tasks (Gilbertson et al., 2002; Lindauer et al., 2006; Samuelson, 2011; Shin et al., 2004).

Our results show that delayed effects of acute stress affects spines and dendrites in the IL region in the form of decreased spinogenesis and dendritic shrinkage. But the same stress does not affect the spines and dendrites in the PL region. When tested for short term memory, we did not notice any change in discriminating the novel object between stress and control groups in the temporal order memory task. This could be attributed to the different efferent distribution pattern between the PL/IL and other brain regions. The IL is directly involved in autonomic or visceromotor activity, while, PL sends projections to visceral regions (Brog et al., 1993; Vertes). Also, IL influences anxiety states as shown by the finding that brief uncontrollable stress attenuates the rate of fear extinction and also dendritic retraction of terminal branches in the mouse IL cortex, but not PrL (Izquierdo et al., 2006) suggesting that different sub regions have distinct functions. Further studies are needed to evaluate the distinct functions of IL and PL in memory based tasks.

Exposure to stress has generally been associated with a wide range of deleterious outcomes, including decline in well-being and increased incidence of psychological disorders such as posttraumatic stress disorder and major depression. Chronic stress leads to behavioral deficits and dendritic remodeling leading to shrinkage of dendrites in hippocampus (McEwen, 1999; Vyas et al., 2002; Watanabe et al., 1992). The hippocampal region is prone to damage by insults such as ischemia, hypoglycemia, seizure etc., (Diamond and Rose, 1994; Foy MR, 1987; Jacobson and Sapolsky, 1991). In rodents, while hippocampus has been studied extensively as part of a brain
system responsible for learning and memory, the primary function of prefrontal cortex in animals and humans is known for cognitive functions like working memory, flexibility, decision making, and rewarding learning (Bicks et al., 2015; Goldman-Rakic, 1995; Jay et al., 1996).

Synaptic plasticity in the mPFC is known to be induced by activating hippocampal projections to the mPFC (Burette et al., 1997; Jay and Witter, 1991; Takita et al., 1999). Stress leads to executive dysfunctions in rodents (Holmes and Wellman, 2009) and dendritic reorganization (Liston et al., 2006; Radley et al., 2004; Shansky and Morrison, 2009) in mPFC. Human MRI studies, have shown a reduction in mPFC volume as a result of stress induced psychiatric disorders.

From chronic stress studies, it is known that hippocampus and mPFC show similar effects as a result of stress. And also hippocampus and amygdala show contrasting patterns of structural plasticity.

Findings from the current study on hippocampus and mpfc along the previous finding from the our laboratory investigating the delayed effect of stress in the amygdala (Mitra et al, 200) it becomes evident that there exists contrasting patterns of structural plasticity in the amygdala and the hippocampus and mPFC follows hippocampal pattern, emphasizing the fact that irrespective of the nature of stress, the amygdala, the hippocampus and the mPFC are affected in the similar manner. These findings are further supported by human imaging studies where hippocampus and mPFC show hypoactivity and amygdala show hyper activity in case of psychiatric disorders induced by stress. Dendritic spines being the major sites of excitatory synaptic transmission, the direct effect of stress on the spine plasticity opens up further avenues to probe the downstream protein signaling to understand the disease paradigm better.
CHAPTER 5
PSYCHOSOCIAL STRESS ELICITS DIVERGENT TEMPORAL PATTERNS OF SPINE FORMATION IN THE HIPPOCAMPUS VERSUS AMYGDALA IN A MURINE MODEL SIMULATING POST-TRAUMATIC STRESS DISORDER
5.1. Introduction

Post-traumatic stress disorder (PTSD) causes enduring and cascading adverse consequences in psychiatric and neurobiological outcomes including memory deficits (Bhakta et al., 2017), cognitive impairment (Samuelson et al., 2017), depressive disorder (O’Donnell et al., 2004) and learning disability (Burris et al., 2008). Human studies involving functional and structural neuroimaging in patients implicate the dysfunctions in the limbic brain regions specifically in the amygdala and the hippocampus, brain regions known to be involved in learning and memory (Bremner et al., 1997; Hamilton et al., 2008; Liberzon et al., 1999; Rauch et al., 2000). Rodent studies further emphasize this phenotype and indicate that the hippocampus and the amygdala exhibit contrasting patterns of structural plasticity in response to stress (Akiki et al., 2017; Shin and Liberzon, 2010). For example, chronic stress causes dendritic atrophy in CA3 pyramidal neurons of the hippocampus, whereas dendritic hypertrophy and spinogenesis is observed in the basolateral amygdala (BLA) (Conrad et al., 1999; Mitra et al., 2005b; Roozendaal et al., 2009; Vyas et al., 2002; Wang et al., 2012). The majority of previous rodent studies have employed stress paradigms that involve exposing rodents to a physical stressor and we sought to examine whether these phenotypes might be replicated by employing PTSD-mimicking stress.

The aggressor-exposed social stress (Agg-E SS) model, a modified resident intruder paradigm, is a credible model for the unpredictable stress that typifies some human conflict situations typically precipitate PTSD. Two factors namely the social context and the unpredictable occurrence of potentially life-threatening events distinguish this model from other stress models (Chakraborty et al., 2017). We further hypothesize that these two key factors contribute to the unique spatiotemporal dynamics of spine number in the Agg-E SS mice. We further examined how the neuroanatomic shifts correlated with the cerebral transcriptomic regulations that were done earlier. To this end, dendritic spinogenesis was examined in basolateral amygdala (BLA) and CA1 subregion of hippocampus at two-time points: one day after a 10-day Agg-E SS (immediate effect) and 4-weeks after termination of stress (delayed effect).

Experimental design

One-day after the 10-day Agg-E SS, groups of control and Agg-E SS mice were subjected for Golgi Cox analysis to study the immediate effects of stress. Additional groups of control and stress-
exposed mice were likewise subjected for Golgi Cox analysis after a 4 weeks home-cage rest. (Refer materials and methods section for detailed protocol). The brains were carefully removed from the skulls of euthanized mice, impregnated with Golgi-Cox staining solution, and processed following the protocol established by FD Neurotech, Inc. (Ellicot City, MD). Briefly, the impregnated brains were serially sectioned at a thickness of 100µm. Every third cryostat section (intervals of 300 µm) was mounted on gelatin-coated sides, stained, dehydrated in ethanol, cleared in xylene, and cover-slipped in permount.

5.2. Results
5.2.1. BLA showed increased spinogenesis as an immediate effect of Agg-E SS, which returned back to control levels after 4 week home-cage rest
Spine density was analyzed on the primary dendrites of BLA neurons at two time points, 24 hours after termination of 10-day Agg-E SS to assess the immediate effect as well as after 4 week home-cage rest period, to assess delayed effects. As an immediate effect, BLA showed increased spinogenesis in the SS mice (Controls: 83.22 ± 1.6, n=32; Stress: 97.73 ± 2.3, n=30, Unpaired t test: P< 0.0001), similar to the effects seen in physical models of stress model. After a 4 weeks home-cage rest, however, the dendritic status had reverted to control levels(Controls: 88.34 ± 2.013, n=32; Stress: 84.97 ± 2.196, n=32; Unpaired t test). (Fig.5.1).

5.2.2. CA1 displayed no change in spine number as an immediate effect of Agg-E SS, but spine number significantly increased after 4 weeks of home-cage rest
We examined the effects of Agg-E SS on spine density in CA1 pyramidal neurons. We analyzed the pyramidal cells of the stratum radiatum layer of CA1 sub region of hippocampus. Analysis was done at two time points, 24 hours after termination of 10-day Agg-E SS and 4-week after the withdrawal of Agg-E SS to assess the immediate and delayed effect respectively. As an immediate effect, there was no significant difference in spine number in SS mice (Controls: 86.00 ± 1.9, n=26; Stress: 81.96 ± 2.3, n=26; unpaired t test: P=0.19) when compared to controls. However, four weeks later, the spine density in the stress group was significantly increased (Controls: 86.62 ± 1.8, n=26; Stress: 93.35 ± 2.2, n=26; unpaired t test P<0.5). (Fig.5.2).
Figure 5.1:
BLA showed increased spinogenesis as an immediate effect of Agg-E SS, which returned back to control levels after 30 days of home-cage rest.

A). Schematic of experimental protocol of Immediate Effect. B). Bar graph showing increased spine number in the stress group with respect to control in the BLA on day 11 after a 10 day Agg-E SS C). Representative images of primary dendrites of BLA pyramidal neurons from control and stressed mice. D). Schematic of experimental protocol of Delayed Effect. E). Bar graph showing spine number rescued back to control levels in the stressed group after a 30 day home cage rest F). Representative images of primary dendrites of BLA pyramidal neurons from control and stressed mice. Scale bar: 20µm
Figure 5.2: CA1 showed no change in spine number as an immediate effect of Agg-E SS, but spine number significantly increased after 4-weeks of home-cage rest.

A). Schematic of experimental protocol of Immediate Effect B) Bar graph showing no difference in spine number in the stress and control group on day 11, after a 10 day stress. C). Representative images of primary dendrites of CA1 pyramidal neurons from control and stressed mice. D). Schematic of experimental protocol of Delayed Effect. E). Bar graph showing increased spine number in the stress group after 30 days of home cage rest. F). Representative images of primary dendrites of CA1 pyramidal neurons from control and stressed mice. Scale bar: 20µm
5.3. Discussion
The present study examined the effects of aggressor stress at two different time points as immediate effect and the delayed effect. The spine density was analysed in the BLA and the hippocampus. BLA showed increased spinogenesis as an immediate effect which is in agreement with previous physical models of chronic stress (Vyas et al., 2002). However the same stress did not have any delayed effects where the spine number returned to control group. This is unlike seen in the chronic stress in the BLA where BLA spinogenesis persists even after 21 days of stress recovery. (Vyas et al., 2004). In the hippocampus, CA1 region did not show any change in spine number as an immediate effect unlike the chronic stress (Christian et al., 2011; Luine et al., 1994; Vyas et al., 2002) surprisingly the delayed effect lead to increase in spine number in the stress group.

The aggressor stress model used here to study the spatiotemporal shift of dendritic spinogenesis, a neural correlate of experience dependent plasticity in a murine model, mimics most of the features of PTSD like behaviors. By carefully dissociating the immediate and delayed effects of aggressor stress, the present study describes the structural correlates in the perspective of time and context of conspecific social interaction, eliciting PTSD-like symptoms. After 10-days of aggressor stress, the animals exhibited a range of behavioral alterations, many of which persisted after 30 days (Hammamieh et al., 2012). Increased frequency of freezing implicated a contextual conditioned fear in response to the aggressor stress. Grooming, a marker of a stress and arousal-related behavior, was heightened by the acute effects of aggressor stress, as well. On the other hand, the stressed mice showed no tail rattling (a marker for agonistic behavior) in response the aggressor cues. This pattern of behavior may parallel the avoidance-depression dimension of PTSD (Hammamieh et al., 2012). These acute PTSD-like traits were manifested in concurrence with the increased spine density at the BLA. The current findings highlight that data coming from different stress models potentially converges to suggest that BLA as the key locus to retain fear memory. It further appeared that the BLA neurons are highly sensitive to fear-inducing stress (Davis, 1992).

Aggressor stress failed to cause any change in spine number in CA1 as an immediate effect, unlike what is reported in other chronic stress models mostly eliciting anxiety (Bremner et al., 1997; McEwen, 1999; Pawlak et al., 2005; Sheline et al., 1996). However, delayed effect as aggressor stress elevated CA1 spinogenesis after 30 day home-cage rest. Interestingly, the chronic unpredictable stress model, which has ‘randomness’ as its salient feature like the aggressor stress
model, also failed to perturb hippocampal dendrites (Vyas et al., 2002). Aggressor stress elevated CA1 spinogenesis with 30 days of rest, and most of the negative behavioral attributes of aggressor stress such as freezing, grooming and tail rattling remained significantly different between control and SS mice after same time frame. Our spine density data suggested an opposite longitudinal trend in hippocampal and amygdalar neuroanatomy. In fact, chronic stress also caused an opposite trend of synaptogenesis between these two cerebral loci (Lakshminarasimhan and Chattarji, 2012; Luine et al., 1994; Vyas et al., 2002).

The aggressor stress induced temporal dynamics of both BLA and CA1 dendritic atrophy was supported by transcriptomic analysis as detailed in the Table 1 (Muhie et al., 2015). The table explains the current data with the summary of earlier data (Hammamieh et al., 2012b; Muhie et al., 2015) Table 2.

Table 1. Summary of our findings relevant to the neurohistologic findings

<table>
<thead>
<tr>
<th>Assay</th>
<th>Brain regions</th>
<th>10 day after aggressor stress</th>
<th>40 day after aggressor stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behavior</td>
<td></td>
<td>Increased freezing, grooming and withdrawal duration in cage periphery. Decreasen tail rattling</td>
<td>Persistently increased freezing and grooming and persistently suppressed tail rattling.</td>
</tr>
<tr>
<td>Transcriptomic</td>
<td>Amygdala</td>
<td>Elevated synaptogenesis and synaptic plasticity.</td>
<td>Decreased synaptogenesis, synaptic plasticity and dendritic spine density</td>
</tr>
<tr>
<td>assay</td>
<td>Hippocampus</td>
<td>Increased fear conditioning. Suppressed neuronal loss</td>
<td>Increased synaptogenesis, synaptic plasticity, dendritic spine density</td>
</tr>
<tr>
<td>Morphology</td>
<td>Amygdala</td>
<td>Significantly increased BLA spine numbers</td>
<td>BLA showed no changes in spine numbers</td>
</tr>
<tr>
<td></td>
<td>Hippocampus</td>
<td>CA1 showed no changes in spine numbers</td>
<td>Significantly increased CA1 spine numbers</td>
</tr>
</tbody>
</table>
Amygdala and hippocampus constitute a major cerebral circuitry to process the emotional memory and are believed to be the major locus to process the fear conditioning memory and declarative or explicit memory, respectively (Chavez et al., 2009; Davis and Whalen, 2001; Dolcos et al., 2004). Trauma survivors are typically burdened with negative emotion, explicit memory-dysfunction, and fear memory (Elzinga and Bremner, 2002; Shin et al., 2006a). Typical symptoms of PTSD include re-experiencing or recalling of the traumatic events in the aftermath of the trauma exposure (Samuelson et al., 2017). Voluntary or involuntary exposure to stressful situations is highly likely to affect the neural circuitry leading to morphological alterations. Emerging data, however, has distanced PTSD from the anxiety disorders, in favor of re-classification as “trauma and stressor-related disorder” (Zoellner et al., 2011). This classification of PTSD may explain the dissimilar temporal trends of BLA spinogenesis caused by the CIS and aggressor stress models.

5.4. Conclusion

A large body of preclinical research has utilized observations of neuroanatomic structural correlates to explain complex behavioral changes (Radley et al., 2004, 2006). We suggest that stressors similar to those that elicit PTSD might trigger cerebral loci-specific structural remodeling of neurons in mouse model. Transcriptomic and behavioral analysis support the current findings. It can be postulated that emotional and explicit memory processing unique to PTSD patients might be distinctly different from those of other conditions such as anxiety disorder. Further experiments are warranted to establish this hypothesis.

The above work was done in collaboration with Prof. Nabarun Chakraborty and Prof. James Meyerhoff from Geneva Foundation, Integrative Systems Biology, Geneva Foundation, USACEHR, and 568 Doughten Drive, Fredrick, MD, 21702-5000, USA.
CHAPTER 6

DENDRITIC SPINE DENSITIES IN THE HIPPOCAMPUS AND AMYGDALA REFLECT INDIVIDUAL DIFFERENCES IN AGGRESSIVE AND VIOLENT BEHAVIOR IN WILD-DERIVED MALE RATS
6.1 Introduction

Throughout life all animals, including humans, are continuously confronted with challenges posed by the environment. Whereas some of these challenges are easily dealt with, others can be more difficult and may even cause a serious stress response. Large individual differences exist in the way individuals respond behaviorally and physiologically to these stressors. This also leads to a large variability in the vulnerability to develop stress related pathologies like anxiety or mood disorders (Duman and Monteggia, 2006; Nestler et al., 2002). Less than 25% of all people who are exposed to uncontrollable and potentially traumatic stress actually develop a post-traumatic stress disorder (Kessler, 1997; Liberzon and Sripada, 2008) or a major depression (Shin and Liberzon, 2010). So it is not as much the characteristics of the environmental stressor that defines the susceptibility of the individual to stress pathology but the specific behavioral and physiological trait characteristics (Yehuda et al., 2006). Distinct behavioral and physiological trait characteristics can be observed in many animal species which are considered to reflect different coping strategies or animal personalities (Réale et al., 2010; Sih et al., 2004). Many studies indicate the ecological relevance of these trait characteristics or coping styles as an individual phenotypic adaptations to different environmental challenges (de Boer, 2018; Réale et al., 2010). The difference between vulnerability and resilience may be based upon the perceived environmental demand and the expressed coping style that either matches or mismatches the demand. A match results in successful adaptation and a mismatch in maladaptation which determines the health and disease state of the individual.

Individual differences in trait-aggressiveness, focusing on offensive aggressive behavior, are extensively described in laboratory rodents like rats and mice. Aggressive behavior is a natural, biological and functional form of social behavior aimed at the establishment of a territory, social dominance and defense of resources and is essential for the survival of the individual and thus clearly has evolutionary relevance. Nevertheless, a consistent bimodal distribution in attack latencies was described in rats (De Boer and Koolhaas, 2003) and mice (Van Oortmerssen et al., 1987) that were tested as residents in the resident-intruder paradigm. These differences in offensive aggressive behavior correlate with differences in behavioral responses in a wide variety of environmental challenges (Koolhaas et al. 1999, 2007). In tests measuring proactive behavior, behavioral flexibility and impulse control, general emotional reactivity and reward processing a picture arises that high-aggressive animals are proactively coping with environmental challenges.
aiming to control their environment. Low-aggressive animals are reactively, sometimes also defined as passively, coping with environmental challenges which results more often in avoiding or freezing but also in increased behavioral flexibility (Koolhaas et 1999, 2007, De Boer et al. 2017).

The individual differences in the behavioral and physiological coping styles emerge as a result of underlying neurocircuitry often termed as social decision making network (O’Connell and Hofmann, 2012) which includes prefrontal cortex, amygdala, BNST, hippocampus and their afferent and efferent projections. The functional properties of the circuitry are determined by various neuropeptides that are considered as key signaling molecules such as serotonin, dopamine, noradrenaline and neuropeptides like corticotrophin releasing factor, oxytocin and arginine vasopressin (de Boer et al., 2015; Coppens et al., 2010; De Boer and Koolhaas, 2003). Different lesion studies also show that there exist distinct neuronal circuits that underlie proactive and reactive coping strategies (Bandler et al., 2000; Davidson et al., 2000; Keay and Bandler, 2001) and the neurons become either activated or inhibited during different coping styles (Haller et al., 2006; Kerman et al., 2011).

In the present study we selected rats with either a pro or a reactive coping styles from a wild derived strain, the so-called Wildtype Groningen (WTG) rats, by scoring their offensive aggressive behavior as residential males in the resident-intruder paradigm. This strain has been bred for more than 50 generations in the laboratory of Groningen University and the ancestors were caught in the wild. It was chosen for this study because of the rich social behaviour and the high individual variation in genetics, behaviour, and physiology. As mentioned above this aggressive behavior is a natural social behavior that is highly functional and feral rodents display much higher levels of this behavior than typical laboratory strain animals (De Boer et al. 2003). In the animals that show a proactive coping style having an aggressive phenotype, a small portion of around 10% will show escalated aggressive or even violent behavior when allowed to experience multiple winning episodes in a social conflict (De Boer et al. 2009). These rats developed over the course of 25 winning experiences a type of offensive aggression that disinhibited causing them to neglect appeasement signals, attack vulnerable body parts of the opponent inflicting serious bite wounds and even attack anesthetized animals or females (De Boer et al, 2009, De Boer 2018). Particularly
these rats and mice with escalated aggressive behavior show changes in the underlying neurocircuitry (Miczek et al. 2015).

The studies of the neural circuitry underlying the differences in natural aggressive behavior and coping strategies as well as the individuals that show abnormal and violent forms of aggressive behavior focus on differences in signaling molecules like monoamines (noradrenalin, dopamine, serotonin), neuropeptides (oxytocin, vasopressin and corticotropin releasing factor) and steroid receptors (De Boer et al. Miczek et al.). There is, however, no study available focusing on differences in the structural changes in brain regions involved in the regulation of cognitive and emotional behavior like the amygdala and hippocampal structure with relevance to aggressive behavior. The current study is designed at understanding different coping styles like proactive or aggressive and reactive or docile. This would help us to understand if trait characteristics based upon differences in coping styles and aggressive behavior may underlie individual differences in the vulnerability to develop psychopathologies following exposure to environmental challenges like chronic or intense stress.

6.2. Experimental design

Our study was divided into two parts. In first part, we studied groups of rats that differ in natural offensive aggression as measured in a 4 time exposure to intruder males in the resident-intruder model. These rats represent different coping styles: proactive and reactive. In the second part, we studied a group of rats that were allowed to win from intruder males for more than 20 times and from these animals, we selected 6 rats that were very aggressive and also attacked anesthetized males. For ease of understanding, for the proactive and reacting coping styles, we termed as short term coping style and for rats with violently aggressive trait characteristics, we termed as long term coping style.

6.2.1 Short term coping styles

We used 22 WTG rats to interact with different intruders for 4 consecutive days, 10 minutes every day. Their behavior was recorded and 24 hours after the behavioral assay (Fig.6.1), the brains were decapitated and fixed in Golgi Cox fixative to study the underlying neural morphology.

6.2.2 Long term coping styles

In this group, WTG rats were exposed to intruders for more than 20 times and after 2 days after last interaction, brains were fixed in Golgi Cox fixative (Detailed description in material and
methods section) to study the underlying neural morphology. In this group, we have included violent group and also the intruder group. Violent nature was assessed when the resident rat did not even spare the anesthetized intruder and attacked with a minimum attack latency.

Figure 6.1: Resident and intruder interaction

**Figure 6.1A:**

**WTG rats exhibit different behaviors during their interaction with the intruders**  A) Schematic explaining the resident intruder paradigm.  B,C,D) Bar graphs show different behaviors expressed by the WTG rats during their interaction with the intruders.  N=22 animals.

(This experiment was conducted to investigate the baseline trait characteristics expressed among the resident rats while interacting with the intruders)

*(Behavioral elements were calculated based on the summation of each of the activities mentioned under each behavior during the 10 minute interaction).*
6.3 Results

6.3.1 Short term coping styles

6.3.2 Short term coping styles show different grades of aggression

The following behaviors were assessed to define the grades of aggression for the WTG rats.

**Exploratory behavior:** Explore environment, rear, immobility

**Social behavior:** Investigate opponent, sniff in anogenital region, social groom

**Aggressive behavior:** Clinch, threat, offensive upright, keep down, chase

Summation of time spent in each actions performed would categorize them into that respective behavior.

Based on the above criteria we noticed three grades of aggression. No aggression, mild aggression and high aggression. Out of 22 rats, 8 rats showed non aggressive, exploratory and social behavior, 6 rats showed mild aggressive traits and 8 rats showed high aggression and no exploratory or social behavior.

These rats were then subjected for Golgi Cox analysis to evaluate if there exists any neural correlate for the observed behavioral phenotypes. Dendritic spine analysis was carried out in the hippocampus and the amygdala. We chose only non-aggressive and highly aggressive group for analysis dendritic spine analysis.

6.3.3. Non aggressive and high grades of aggression did not affect the spine number in the BLA in the short term coping styles

Spine density analysis was carried in the pyramidal neurons of BLA. Spines were counted in the primary dendrite along the 80µm length of the dendrite. Results showed that there was no difference between the non-aggressive 106.4±3.05, n=31; highly aggressive 104.0±2.1, n=41, p=0.5139, group in a two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test. (n= number of dendrites on which spines were quantified). (Fig.6.2)

6.3.4. Non aggressive and high grades of aggression did not affect the spine number in the CA1 region of hippocampus, in the short term coping styles

Spine density analysis was carried in the pyramidal neurons in the stratum radiatum layer of CA1 region. Spines were counted in the primary dendrite along the 80µm length of the dendrite. Results
showed that there was no difference between the non-aggressive 147.5±2.26, n=37; highly aggressive 141.4±2.92, n=38, p=0.1064, group. (Fig.6.3)

Figure 6.2:
Non-aggressive and aggressive group do not show any change in the spine number in the BLA. A). Coronal section showing the anatomical location of BLA. B). Schematic showing the experimental protocol. C&D) Bar graphs and Segmental analysis showing no difference in spine number both in total spine number and also across the length of the dendrite between non-aggressive v/s aggressive groups

Figure 6.3:
Non-aggressive and aggressive group do not show any change in the spine number in the CA1 region of hippocampus. E). Coronal section showing the anatomical location of hippocampus. F). Schematic showing the experimental protocol. G&H). Bar graphs and segmental analysis showing no difference in spine number both in total spine number and also across the length of the dendrite between non-aggressive v/s aggressive groups.
6.4 Long term coping styles

Spine density analysis was carried out in BLA and CA1. The groups included were control, defeated and the violent group.

6.4.1 Violent group showed increased spinogenesis in the BLA

Spine density analysis in the violent group showed increases spinogenesis. Control $84.88 \pm 2.69$, $n=39$, defeated $88.71 \pm 1.79$, $n=39$, violent $92.60 \pm 3.03$, $n=39$. P value- control v/s defeated, $p=0.33$; control v/s violent, $p=0.023$ in a two-way repeated measures ANOVA followed by Bonferroni’s multiple comparisons test. ($n$ refers to the number of dendrites). (Fig.6.4)

Figure 6.4:
Violent group exhibit increased spine number in the BLA.
A). Coronal section showing the anatomical location of BLA. B). Schematic showing the experimental protocol. C&D) Bar graphs and segmental analysis showing increased spine number in the violent when compared to the control group. The spine number is noticed more in the proximal dendrites.
6.4.2 Violent group showed decreased spinogenesis in the CA1

Spine density analysis in the CA1 region of hippocampus showed that the violent group showed decreased spinogenesis. Control 86.63 ±1.28, n=37, defeated 77.34± 1.30, n= 38, Violent 78.15±2.03, n=34. P value- control v/s defeated, p=0.00005; control v/s violent, p=0.0055 in a two-way repeated measures ANOVA followed by Bonferroni’s multiple comparisons test. (Fig.6.5)

![Figure 6.5: Violent and defeated group exhibit decreased spine number in the CA1 region of hippocampus.
E). Coronal section showing the anatomical location of hippocampus. F). Schematic showing the experimental protocol. G&H). Bar graphs and Segmental analysis showing decrease in spine number both in both the violent group and the defeated group. The spine number decrease is noticed along 80µm length of the dendrite both in the violent and defeated group.](image-url)
6.4. Discussion

From the current study, we noticed that different kinds of behavioral repertoire were expressed by the WTG rats. In the first of experiments, during short term coping styles we observed that there was various trait characteristics observed by the WTG rats in the form of different grades of aggression. Different grades of aggression observed here, did not show any structural modification as there was no change in spine density in the hippocampus and the amygdala. But very interestingly we observed that during the long term coping, rats exhibited violent behavior which was scored as short latency to attack. At the structural level, violent rats showed decreased spine density in the hippocampus and increase in the amygdala. This data mimics what is seen in stress studies in the rodents and also in human stress induced psychiatric conditions (Akiki et al., 2017; Chattarji et al., 2015; Vyas et al., 2002, 2006). We analysed dendritic spines in the defeated rats as well. While the hippocampus showed similar stress phenotype, decrease in spine number, the amygdala did not show any change in spine number with respect to the control group.

Coping is the behavioral flexibility or alternative response patterns in reaction that an animal adapts to combat the challenge and it could be either proactive and reactive coping (Koolhaas et al., 1999) and this is even evident in other species like fish and birds where a different term like shyness and boldness is used (Sloan Wilson et al., 1994). In proactive coping style animals exhibit offensive behavior towards male conspecific rivals and are impulsive in decision-making and score high in frustration tests (David et al., 2004; Groothuis and Carere, 2005; Steimer and Driscoll, 2005).

One of the main causes for psychopathologies like anxiety and depression are most often due to impairment in social behaviors leading to different levels of anxiety due to disturbed emotional regulation eventually resulting in excessive aggression and violence (Kohn and Asnis, 2003; Swann, 2003). Study from Otto et al., adds light to data where they have showed that individuals with psychiatric disorders often show more aggressive behaviors (Kernberg, 1968). Boer et al., while explaining the neurobiology of coping mentions that there exists a balance between coping and the environmental demands and net outcome could be being resilient or vulnerable depending on the demand (Sietse F. de Boer, Buwalda, and Koolhaas 2017).
Various studies from the literature focus on the signaling molecules or probe with markers for activated gens. Gene mutations could be one of the reasons that predisposes people to aggression, for example, monoamine oxidase mutation is known to one of the cause for impulsive aggression (Kandel, 2013, Brunner HG et al., 1993). An integrated genomic and transcriptomic analysis of human and rodent species provides insights into genes that underlie aggression. Genetic variations in serotonergic system is known to be a main determinant of inter male aggression and epigenetic markers also play a role in aggression in mental illness (Zhang-James et al., 2018; Chen C et al., 2015; Manchia M et al., 2017; Tuvblad et al., 2011; Nelson RJ et al., 2001). Studies also show that c-fos expression goes up as a result of aggression (Haller et al., 2006). There is large body of data available indicating that the coping styles to larger extent are modulated by serotonin or dopamine signaling (de Boer et al., 2015, 2017; Dolan et al., 2001). David Anderson et al., using modern optogenetic methods to study aggression in mice have identified neurons in a region of the hypothalamus whose activity causes males to attack other males, females, and even inanimate objects and this could probably due to the overlapping networks between aggression and mating and the faulty wiring in the network could be the reason some forms of pathological violence in people (Anderson, 2012; Lin et al., 2011). But there are not much studies to study and understand the neural wiring in a region specific manner. Hence in our study we tried to capture the structural correlate to different kinds of grades of aggressive behaviors expressed by the WTG rats in the hippocampus and the amygdala, the two regions involved in cognitive and emotional behavior and part of social circuitry network (O’Connell and Hofmann, 2012).

To conclude, Based on our results, we can hypothesize that short term coping leads to a match with demands leading a reactive behavior by being resilient and hence doesn’t have much impact on the neural circuitry, whereas the long term coping leads to a mismatch with the environmental demands not being able to cope up, leading to a proactive behavior or being violent, eventually disrupting the neural circuitry. Future studies are focused towards investigating the neural circuit that regulates aggressive behavior (de Boer et al., 2015).

**Fig. 6.6. Neuronal network involved in aggressive behavior**
7.1 Discussion

Stress leads to maladaptive changes in the brain and behavior leading to psychiatric disorders. Stress effects are seen at different levels of neuronal organizations, right from molecules to behavior. Most of the stress effects are studied at the termination of the stress paradigm. But there are no studies available that have investigated the delayed effect of stress. Mitral et al., from our laboratory showed that there is increased anxiety at behavioral level associated with increased spine number at structural level in BLA after 10 days of stress (Mitra et al., 2005b). Yasmin et al., showed that the above effects are due to delayed strengthening of synaptic connectivity and it depends on NMDA receptor activation. It was by shown by her that a single episode of stress through whole cell patch clamp recordings in brain slices, enhances frequency, but not amplitude, of miniature excitatory postsynaptic currents (mEPSCs) recorded from principal neurons in the BLA 10 days later (Yasmin et al., 2016). This provided evidence that the acute stress had long lasting effects in the BLA. This made me explore if the same acute stress has any delayed effects in the other two important regions of stress and memory circuitry: Hippocampus and mPFC.

In my thesis, I have shown how a single episode of stress leads to a gradual temporal progression of stress effects and how it affects both morphology and the behavior in the hippocampus and the mPFC. The stress used was single episode of Immobilisation stress for 2 hours. The structural changes was associated with behavioral impairment in hippocampal memory similar to the effects seen in chronic stress models (Francis et al., 1995; Rahman et al., 2016). Findings from my mPFC analysis where IL shows stress induced changes but not PL. This is supported by the earlier finding mentioning that IL is more prone for stress not PL (Izquierdo et al., 2006) needs to be further evaluated as this is the first finding focusing on the delayed effects in the mPFC.

The findings of my first objective has opened up new a avenue to explore the contrasting patterns in BLA and hippocampus as a result of acute stress. To this end, neuronal translation as measured by FUNCAT was evaluated. The results revealed that there was enhancement in neuronal translation in BLA 10 days later, which dropped down to control levels in hippocampus (Madan et al., 2018). Also, our findings are supported by human research involving imaging studies that individuals with psychiatric stress disorders like PTSD, where increased amygdalar volume and have smaller hippocampal mPFC volumes (Bremner et al., 1997; Gilbertson et al., 2002; Shin et
al., 2006b; Wang et al., 2010) were noticed. Future studies are directed towards exploring the role of mPFC in the delayed effects of acute stress.

From my first aim, it is evident that even acute stress has strong delayed effects mimicking PTSD. The second aim was to investigate the stress effects in a more ethological relevant form of stress like social defeat and to investigate how amygdala and hippocampus respond to stress effects. Voluntary or involuntary interaction with stress is highly likely to affect the neuroanatomic connections and morphology. Here, we carefully dissociated the immediate and delayed effects of Agg-E SS, to understand the dynamics of neuroanatomic correlates in the perspective of time and context of conspecific social interaction, eliciting PTSD-like features. After 10-days Agg-E SS, the animals exhibited a range of behavioral alterations, many of which persisted after a 4-week home-cage rest (Hammamieh et al., 2012).

The Immediate effect of these behavioral deficits co-occurred with the increased spine density at BLA similar observations were reported as a consequence of acute effects of chronic stress (Hamilton et al., 2008; Mitra et al., 2005b; Rauch et al., 2000a; Vyas et al., 2002). Thise data potentially suggested a positive correlation between anxiety and BLA hypertrophy. BLA spine number reverted to control levels after 30 days of home-cage rest post Agg-E SS. Agg-E SS failed to cause any spine number in CA1 as an acute effect, unlike what is reported in other chronic stress models mostly eliciting anxiety (Bremner et al., 1997; McEwen, 1999; Pawlak et al., 2005; Sheline et al., 1996). However, there was a delayed effect as Agg-E SS elevated CA1 spine number after 30 days home-cage rest and this was associated with most of the decreased behavioral phenotypes like freezing, grooming and tail rattling between control and SS mice after home cage rest. Our morphology data from the social defeat study again suggested an opposite longitudinal trend in hippocampal and amygdalar neuroanatomy providing insights that this could probably be to maintain homeostasis in some way. This Agg-E SS induced temporal dynamics of BLA and CA1 spine number was supported by our transcriptomic analysis, which was explained in the discussion in the form of a table in chapter 5.

Finally as a third objective, my interest was to focus on coping behavior. As everyone knows that coping is the behavioral flexibility or alternative response patterns in reaction that an animal adapts
to combat the challenge and this could either be proactive or reactive coping (Koolhaas et al., 1999). This is evident even in other species like fish and birds where a different term like shyness and boldness is used (Sloan Wilson et al., 1994). There are two ways of coping either be proactive showing offensive behavior towards male conspecific rivals being impulsive in decision-making or being reactive or docile (de Boer, 2018; David et al., 2004; Groothuis and Carere, 2005; Steimer and Driscoll, 2005).

The results presented here show that short term coping style is the reactive or docile form and may be under controllable form of aggression and does not cause great damage at the neuronal level as we did not notice any changes in spine number in the BLA and hippocampus which are known to undergo contrasting patterns of stress induced structural plasticity. But when the residents were exposed for long duration of interaction, the coping turned out to be proactive or aggressive probably leading to social impairment. Thus violent animals showing increased spine number in BLA and decrease in hippocampus noticed in chronic stress studies (Vyas et al., 2002). This study provides an understanding that violent individuals probably have faulty wiring in the network leading to pathological violence as seen in humans as reported earlier based on the optogenetic studies (Anderson, 2012; Lin et al., 2011). Study from Otto et al., adds light to data where they have showed that individuals with psychiatric disorders often show more aggressive behaviors (Kernberg, 1968).

Going back to our question whether violent behavior or being violent is anxious or stressful, the results of long term coping styles seem to be stressful, showing contrasting patterns of structural plasticity in hippocampus and the amygdala as seen in case of physical stress models, emphasizing the fact that main causes for psychopathologies like anxiety and depression are most often due to impairment in social behaviors leading to different levels of anxiety due to disturbed emotional regulation eventually resulting in excessive aggression and violence (Kohn and Asnis, 2003; Swann, 2003).
References


Nicole M. Lauzon (2010). Dopamine D4-receptor modulation of cortical neuronal network activity and emotional processing: Implications for neuropsychiatric disorders - ScienceDirect.


Publications:


Note: Patel D & Anilkumar S are equal authors

