“Stress” is a word derived from the Latin word ‘Stringere’ meaning to draw tight and was popularly used in the 17th century to mean hardship, strain, adversity or affliction. In modern times, stress is a buzzword used to describe the physical, emotional, cognitive and behavioral response to events that are appraised as threatening and challenging. The term stress was first employed in a biological context by a Canadian endocrinologist Hans Selye in 1930s. Hans Selye, Father of stress, described stress as a non-specific phenomenon representing the intersection of symptoms produced by a wide variety of noxious agents (Selye, 1998). Selye employed various conditions, including fasting, extreme cold, and others as stressors to produce the representative stress response, and defined that the determinants of the stress response are non-specific (Selye, 1998). However, John Wayne Mason modified the original concept of Selye by incorporating the importance of psychosocial and the emotional aspects of stress. He defined stress response as a specific hormonal, behavioral and physiological response rather than the non-specific response as advocated by Selye (Mason, 1968).

Stress is a physical or psychological stimulus that can produce mental tension or physiological reactions to produce illness. The stress response is an orchestrated process which involves various mechanisms for physiological and metabolic adjustments in the body to cope with the demands of a homeostatic challenge. These changes occur at the psychological (emotional and cognitive), behavioral (fight and flight), and biological level (altered autonomic and neuro-endocrine function). Stress mainly acts on the two major pathways i.e. hypothalamic-pituitary-adrenal (HPA) axis and sympathetic adreno-medullary system (SAM) (Figure 1). The ability to cope with a stressor is a crucial determinant of health and the chemical mediators (stress hormones) play an important role in promoting stress adaptation. This blunted response to the stress stimulus during repeated exposure is referred as ‘stress adaptation’ and it has been suggested to be a key protection mechanism against repeated stress exposure (Stone and Platt, 1982; Stone et al., 1985; Rabasa et al., 2011) (Figure 2). Stress is a predecessor and is a causative factor for the development of anxiety and depression. Both anxiety and depression are the result of an inappropriate adaptation to stress and these have been termed as ‘stress-related disorders’, with a causal role of HPA axis. Stress has been postulated to be involved in the pathophysiology of a variety of disease including gastric ulcers, anxiety, depression, memory loss and others (Yadin and Thomas, 1993).
Figure 1: Stress-induced activation of hypothalamic–pituitary–adrenal axis and sympathetic-adrenal-medullary system
3.1 Preclinical models of stress

Animal models are pivotal for understanding the pathophysiological changes occurring in the disease and for the development of new pharmacological agents for the disease management. Accordingly, diverse animal models, acute as well as chronic, have been created to simulate the stress condition in animals akin to humans (Jaggi et al., 2011). The immobilization (Bhatia et al., 2011; Rabasa et al., 2011), restraint (Jackson and Moghaddam, 2006), electric foot shock (Rabasa et al., 2011) and social isolation stress (Serra et al., 2008) models have been used more frequently by different scientists to evaluate the anti-stress activity of pharmacological interventions.

3.1.1 IMMobilIZATION STRESS

Kvetnansky and Mikulai developed a gold standard protocol for inducing stress in laboratory animals by immobilizing rats/mice for a stipulated period of time. Thereafter, it has become one of the most frequently employed stress model for rodents. A typical immobilization procedure involves fixing the four limbs of mouse/rat in the prone position on the plain board with an adhesive tape. The head is also fixed with a metal loop over the neck area to limit the
head motion (Kvetnansky and Mikulai, 1970). This model has been widely used to induce both acute (Kvetnansky and McCarty, 2007; Bhatia et al., 2011; Kumar et al., 2012) as well as chronic stress by varying the duration of immobilization stress (Larco et al., 2012; Xing et al., 2013; Baptista et al., 2014). Immobilization is a complex stressor and has both physical as well as psychological dimensions. The struggling and muscular exertion during immobilization is an intense form of physical exercise. The struggling and muscular exertion in the process to free itself comprise the physical components of stress. On the other hand, limited movement during immobilized position and exposure in an open area comprises the psychological stress. During the first exposure to immobilization stress, rats or mice struggle vigorously to free themselves for 5–10 mins. After that time, they stop struggling and remain motionless. However, on repeated immobilization exposures, the animals tend to develop signs of habituation to stressor and stop struggling much earlier.

Immobilization is well tolerated by laboratory rats and mice, and is used in chronic stress studies extending over weeks or even months. This stressor is sufficiently intense to activate the stress-responsive system in the body, including HPA axis and the sympathetic nervous system. This type of physical and psychological stress is particularly useful for studying stress-induced neurodegeneration, post-traumatic disorders and deregulation of the immune system (Southwick et al., 1994). Immobilization may also be combined with other stressors, such as cold temperature or water immersion (immobilization board may be placed in shallow water such that the animal is partially submerged) (Senay and Levine, 1967; Guth et al., 1979). However, the limitation of the model is that in this model the intensity of the stressor cannot be altered, as with other stresses like foot-shock stress.

### 3.1.1.1 Acute immobilization stress

A single episode of immobilization typically lasts for 120-150 min for acute stress induction (Kvetnansky and Mikulai, 1970; Bhatia et al., 2011; Kumar et al., 2012). Scientists have varied the immobilization time periods for specific investigation. García and coworkers subjected the rats to immobilization stress for 20 min, 1 h and 2 h to study the effects of stressor duration on post-stress recovery (García et al., 2000). Others scientists have employed stressor of 2 h duration in mice (Bhatia et al., 2011), 3 h duration in rats (Takayama et al., 1986), 6 h in mice (Goyal and Anil, 2007) to investigate the anti-stress effects of different pharmacological agents, including components of plant origin. Other research studies involving variable time of
immobilization stress include employment of 1 h stress in mice to study the adaptive response of HPA axis (Rabasa et al., 2011), 2.5 h stress to study the cognition enhancing actions of acute stress in mice (Das et al., 2000), 3 h stress to study the effect of stress on testicular steroidogenesis in adult rats (Orr et al., 1994), 4 h stress to evaluate the significance of pain sensitivity in immobilization stress in rats (Zarubina and Shabanov, 2012) and 4 h stress to determine the effects of odorants on serum levels of adrenocorticotropic hormone (ACTH) and corticosterone in rats (Komori et al., 2003) (Table 1).

3.1.1.2 Chronic immobilization stress

There have been many studies on rats and mice to examine the effects of chronic immobilization stress on various aspects of stress response including disease development pattern. Different scientists have employed variable time periods ranging from 5-22 days to induce chronic stress of varying degree in rats and mice (Bhatia et al., 2011; Larco et al., 2012). The different research-based studies employing immobilization stressor for chronic stress include immobilization for 1h daily for 5-14 days in mice to study the adaptive response of HPA axis in chronic stress model (Rabasa et al., 2011; Haleem et al., 2013), 2.5 h daily for 5 consecutive days to evaluate the cognitive dysfunction of chronic stress in mice (Das et al., 2000), 2 h daily for 10 days to determine the association of stress recovery with the changes in the mesolimbic brain regions (Lucas et al., 2011), 1 h daily for 14 days to study the development of anxiety and depression in mice (Xing et al., 2013), 20 min daily for 21 days to study effects of stress on serum ghrelin levels in rats (Elbassuoni, 2014), 1 h daily for 22 days to evaluate the effects of stress and estradiol on pain tolerance (Cruthirds et al., 2011), 1 h for 30 days to evaluate the cardiovascular risk of stress exposure (Baptista et al., 2014) (Table 1).

3.1.2 RESTRAINT STRESS

Restraint stress is a modified form of immobilization stress in which animals are not allowed to move for a specified period of time. The neural and endocrine responses have described that restraint stress is less intense stressor than immobilization. Nevertheless, restraint stress is one of more commonly employed model for the induction of acute as well as chronic stress in rats (Kumari et al., 2007; Jaggi et al., 2011; Manchanda et al., 2011). Restraint involves placing the test animal in a well-ventilated plastic tube or a wire-mesh container. Although the animal's range of movement is severely limited, yet, the limbs are not secured and the animal remains free within an enclosed area (Southwick et al., 1994). Restraint stress is generally
induced by placing the rats individually in the 5.5 cm diameter and 18 cm long semi-cylindrical, acrylic restrainer with air holes for variable period including 2 h-6 h (Uramoto et al., 1990; Kaur et al., 2010; Manchanda et al., 2011). However, there have been variations among different models of this category with respect to difference in the size of restrainers employed to restrain the animals (Das et al., 2000).

### 3.1.2.1 Acute Restraint stress

Researchers have employed variable acute stress protocols to investigate different aspects of stress and these include 15 min and 2 h restraint stress to investigate the changes in stress responsivity with time of restraint (development of hypersensitivity in 15 min restraint stress and hyposensitivity in 2 h restraint stress) (Zimprich et al., 2014), 15 min stress to investigate the effects of mild calorie restriction on anxiety and HPA axis responses to stress in the male rat (Kenny et al., 2014), 3 h stress to evaluate the effects of physical stress on serum cortisol level in rat (Jameel et al., 2014), 3 and half hour stress to study the beneficial effects of sodium cromoglycate and diethyldithiocarbamic acid in acute stress-induced behavioral alterations in rats (Manchanda et al., 2011) (Table 1).

### 3.1.2.2 Chronic Restraint stress

Chronic stress sessions usually last for 7-30 days with different stress protocols including 60 min/day for 7 days to study the stress adaptive process in mice (Miyagawa et al., 2015), 1 h/day for 21 days to investigate the effect of stress on spatial learning and memory in rats (Abidin et al., 2004), 60 min stress for 20 days to evaluate the nociceptive response in rats (Heidari et al., 2012), 6 h/day for 21 days to study the effects of stress on glial activity in the rostral ventromedial medulla in rat (Imbe et al., 2004). Restraint has also been employed in the development of an animal model of long-term weightlessness. This model involves continuous restriction of the movement of laboratory rats for as long as 60 days to simulate the physiological changes occurring during long-term space flight. However, the restraint apparatus is modified such that the animal can gain access to food and water. This model is similar to studies with humans in which long-term bed rest has been used as a model of weightlessness associated with space flight (Sulzman, 1996) (Table 1).
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Duration</th>
<th>Species</th>
<th>Objective of study</th>
<th>References</th>
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<tbody>
<tr>
<td>1.</td>
<td>Acute Immobilization</td>
<td></td>
<td><strong>Immobilization stress</strong></td>
<td></td>
</tr>
<tr>
<td>a)</td>
<td>20 min, 1 h and 2 h</td>
<td>Rats</td>
<td>To study the effects of stressor duration and intensity on post-stress recovery</td>
<td>García et al., 2000</td>
</tr>
<tr>
<td>b)</td>
<td>2 h, 3.5 h, 6 h</td>
<td>Mice</td>
<td>To investigate the anti-stress effects of different pharmacological agents including components of plant origin</td>
<td>Bhatia et al., 2011; Goyal et al., 2012</td>
</tr>
<tr>
<td>c)</td>
<td>1 h</td>
<td>Mice</td>
<td>To study the HPA axis response during stress</td>
<td>Rabasa et al., 2011</td>
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<tr>
<td>d)</td>
<td>2.5 h</td>
<td>Mice</td>
<td>To study the cognition enhancing actions of acute stress</td>
<td>Das et al., 2000</td>
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<tr>
<td>e)</td>
<td>3 h</td>
<td>Rats</td>
<td>To study the effect of stress on testicular steroidogenesis</td>
<td>Orr et al., 1994</td>
</tr>
<tr>
<td>f)</td>
<td>4 h</td>
<td>Rats</td>
<td>To evaluate the significance of pain sensitivity in immobilization stress</td>
<td>Zarubina and Shabanov, 2012</td>
</tr>
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<td>g)</td>
<td>4 h</td>
<td>Rats</td>
<td>To determine the effects of odorants on serum levels of ACTH and corticosterone</td>
<td>Komori et al., 2003</td>
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## Review of Literature

### 2. Chronic Immobilization

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<tbody>
<tr>
<td>a)</td>
<td>1 h for 5-14 days</td>
<td>Mice</td>
<td>To study the adaptive response of HPA axis response in chronic stress model</td>
<td>Rabasa et al., 2011; Haleem et al., 2013</td>
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<tr>
<td>b)</td>
<td>2.5 h for 5 days</td>
<td>Mice</td>
<td>To evaluate the cognitive dysfunction of chronic stress</td>
<td>Das et al., 2000</td>
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<td>c)</td>
<td>2 h for 10 days</td>
<td>Rats</td>
<td>To determine the association of stress recovery with the changes in the mesolimbic brain regions</td>
<td>Lucas et al., 2011</td>
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<td>d)</td>
<td>1 h for 14 days</td>
<td>Mice</td>
<td>To study the development of anxiety and depression in mice</td>
<td>Xing et al., 2013</td>
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<tr>
<td>e)</td>
<td>20 min for 21 days</td>
<td>Rats</td>
<td>To study effects of stress on serum ghrelin levels</td>
<td>Elbassuoni, 2014</td>
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<td>f)</td>
<td>1 h for 22 days</td>
<td>Rats</td>
<td>To evaluate the effects of stress and estradiol on pain tolerance</td>
<td>Cruthirds et al., 2011</td>
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<tr>
<td>h)</td>
<td>1 h for 30 days</td>
<td>Rats</td>
<td>To evaluate the cardiovascular risk of stress exposure</td>
<td>Baptista et al., 2014</td>
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</table>
## Table 1: Different immobilization and restraint stress protocols employed in preclinical studies to study different aspects of stress

<table>
<thead>
<tr>
<th></th>
<th>Restraint stress</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>3</td>
<td><strong>Acute Restraint stress</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a)</td>
<td>15 min and 2 h Mice</td>
<td>To investigate the changes in stress responsivity with time of restraint</td>
<td>Zimprich et al., 2014</td>
</tr>
<tr>
<td>b)</td>
<td>15 min Rats</td>
<td>To investigate the effects of mild calorie restriction on anxiety and HPA axis responses to stress</td>
<td>Kenny et al., 2014</td>
</tr>
<tr>
<td>d)</td>
<td>3 h Rats</td>
<td>To evaluate the effects of physical stress on serum cortisol level</td>
<td>Jameel et al., 2014</td>
</tr>
<tr>
<td>e)</td>
<td>3.5 h Rats</td>
<td>To study the beneficial effects of sodium cromoglycate and diethylthiocarbamic acid in stress-induced behavioral alterations in rats</td>
<td>Manchanda et al, 2011</td>
</tr>
<tr>
<td>4.</td>
<td><strong>Chronic Restraint stress</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a)</td>
<td>60 min/day for 7 days Mice</td>
<td>To evaluate the stress adaptive process</td>
<td>Miyagawa et al., 2015</td>
</tr>
<tr>
<td>b)</td>
<td>1 h/day for 21 days Rats</td>
<td>To investigate the effect of stress on spatial learning and memory</td>
<td>Abidin et al., 2004</td>
</tr>
<tr>
<td>c)</td>
<td>60 min stress for 20 days Rats</td>
<td>To evaluate the nociceptive response</td>
<td>Heidari et al., 2012</td>
</tr>
<tr>
<td>d)</td>
<td>6 h/day for 21 days Rats</td>
<td>To study the effects of stress on glial activity in the rostral ventromedial medulla</td>
<td>Imbe et al., 2004</td>
</tr>
</tbody>
</table>
3.1.3 SOCIAL ISOLATION STRESS

Early life events have profound consequences on subsequent quality of life (Ladd et al., 2004). It has been shown that the early life stress in the form of neonatal isolation in rats produces immediate and long lasting neural and behavioral effects (Kuhn et al., 1990). Rodents reared in social deprivation exhibit an abnormal behavior that includes hyper-locomotion in response to a novel environment, and disrupted exploratory behaviors (Varty et al., 2009). Although, isolation of pup from its mother does not grossly affect the growth in neonatal stage or in adult stage (Hamm et al., 1983; Kehoe and Bronzino, 1999), yet it induces stress characterized by stimulation of HPA axis and morphological changes in the hippocampus including neurodegeneration (Kosten et al., 2007). Therefore, this stressor has been very useful in evaluating the effect of stress on cognition and memory development. Furthermore, early lifetime stress also increases the vulnerability to addiction and it has been employed for those particular studies (Kosten and Kehoe, 2005). Other significant changes due to maternal deprivation during neonatal period include development of tentative behavior (Kuhn et al., 1990), exacerbation of the severity of trinitrobenzene sulfonic acid (TNBS)-induced colitis (Barreau et al., 2004) and gastric ulcer susceptibility in the adult stage (Ackerman et al., 1978).

In a typical neonatal isolation procedure, the pup is removed from the cage on the second day after its birth and placed individually in an opaque plastic container (9 cm diameter and 8 cm deep) with no bedding for 1 h, which in turn is placed in a temperature and humidity-controlled chamber with white noise to mask the calls of other pups. Thereafter, the litters are placed back in home cage with mother. This isolation procedure is repeated for 8 days to induce chronic stress (Kosten et al., 2000; Kosten et al., 2003; Kosten et al., 2005; Knuth and Etgen, 2007; Kosten et al., 2007).

3.1.4 ELECTRIC FOOT SHOCK STRESS

3.1.4.1 Electric foot shock as physical stressor

A direct application of electric foot shock to animals produces physical stress to induce behavioral and other changes in the body organs (Retana-Márquez et al., 2003; Enkel et al., 2010). The major advantage of this stressor over other commonly used immobilization stress is that its intensity, duration and frequency may be varied to induce stress of variable degree.
Accordingly, different scientists have varied the stress intensity, duration and frequency to study different aspects of stress (Brevet et al., 2010; Rabasa et al., 2011; Sántha et al., 2013).

### 3.1.4.1.1 Electric foot shock-induced acute stress

Four electric shocks of 0.8 mA intensity and 2s duration delivered at inter-stimulus interval of 1 min have been shown to significantly trigger anhedonia-like behavior to produce depression in rats (Enkel et al., 2010). The exposure to acute electric foot shocks with an intensity of 3 mA, duration of 200 ms and a frequency of 1 per second over a 5-min period has been shown to significantly increase the plasma corticosterone levels in rats (Retana-Márquez et al., 2003). Rabasa and co-workers employed electric foot shocks of 0.5 mA intensity for inducing moderate stress or 1.5 mA intensity for inducing severe shock for 1 h with one shock every 60 s in a regular schedule to investigate the stress adaptive response of HPA axis (Rabasa et al., 2011). 120 electric foot shocks of 0.15 mA intensity and 5 s duration over a period of 1-hr session (every 30 s) were delivered to investigate the effects of stress in altering the humoral and cellular immune function (Brevet et al., 2010). Six foot shocks of 1 mA intensity of 750 ms duration within a period of 2 min have been employed to study transcriptional changes in brain (Sántha et al., 2013) (Table 2).

### 3.1.4.1.2 Electric foot shock induced chronic stress

A stress protocol comprising delivery of 60 unpredictable shocks of 0.8 mA intensity and 5s duration in the early life span of rats (14th day and 21st day) has been used to study the deleterious effects of early life stress on spontaneous locomotor activity at maturity (Kim et al., 1999). In our study, application of mild electric foot shock stress of 0.15 mA intensity with duration 0.5s for 4 days was used to investigate effects of mild stress on memory improvement (Bali et al., 2013). A mild extrinsic foot shock stress of 0.1 or 0.3 mA for 1s for 7 days has been used to reactivate the memory of a discriminative avoidance task in mice (Takatsu-Coleman et al., 2013). The delivery of 3 electric foot shocks trials/day for 7 consecutive days with each trial comprising of 0.2 mA intensity, 6 s duration for 10 times with a 30 s interval is shown to induce post-traumatic stress disorder in rats (Kim and Seo, 2013). Ten foot shock shocks of 0.6 mA intensity and each of 2 s duration within a period of 5 min for 7, 14 or 21 consecutive days has been employed to study the effects of stress conditions on the transcription and translation of actin-related cytoskeletal genes in the rat brain (Santha et al., 2013). Scientists have also used this stressor to examine the effects of stress in altering the humoral and cellular immune function.
that include application of shocks of 1 mA of 0.5 s duration every 5 s for 30 min/day for 5 consecutive days (Zhang et al., 2005); 2 mA of 10 s duration with an interval of 50s for 2 hour/day (Yamamoto et al., 2009); 120 electric foot shocks each of 0.15 mA intensity of 5 s duration over a period of 1-hr session (every 30 s) for 5 days (Brevet et al., 2010) (Table 2).

3.1.4.2 Electric foot shock as psychological stressor

Electric foot shock has also been used to produce psychological stress in rats indirectly by visual, olfactory and auditory sensation from the foot shock subjected rats. In other words, the test animal is not subjected to any electric shock. However, this animal is allowed to visualize the stress induction procedure in fellow animal. The visual, olfactory or auditory sensation from foot shock subjected animal indirectly induces stress in test animals. In a typical model, foot shocks of 1 mA intensity once every second for 1 hour/day are provided during acute (1 day) and chronic (15-30 day) stress studies (Greisen et al., 2005; Rostamkhani et al., 2012). Other protocols include delivery of 10 unpredictable shocks of 0.5 mA of 1 s duration at random for 1 day (acute) and 5 days (chronic) (Daniel et al., 2008); 1 mA once every second for 1 hour per day for 5 weeks have also been used to induce psychological stress (Li et al., 2013). However, this type of indirect stressor is relatively mild as compared to direct physical stress.
## Review of Literature

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Duration</th>
<th>Species</th>
<th>Objective of study</th>
<th>References</th>
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<tbody>
<tr>
<td>1.</td>
<td><strong>Acute stress</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a)</td>
<td>Four unsignaled electric shocks (2s, 0.8 mA, pulsed) with interval of 1 min</td>
<td>Rats</td>
<td>To trigger anhedonia-like behavior and depression</td>
<td>Enkels et al., 2010</td>
</tr>
<tr>
<td>b)</td>
<td>Shocks of 3 mA of 200 ms and a frequency of 1 per second over a 5-min period</td>
<td>Rats</td>
<td>To investigate the effect of stress on stress hormones</td>
<td>Retana-Márquez et al., 2003</td>
</tr>
<tr>
<td>c)</td>
<td>0.5 mA foot shocks for 1 h for moderate shock (FS-medium) or 1.5 mA for severe shock (FS-high), one shock every 60 s in a regular schedule</td>
<td>Mice</td>
<td>To investigate adaptive response of the HPA axis</td>
<td>Rabasa et al., 2011</td>
</tr>
<tr>
<td>d)</td>
<td>120 electric foot shocks each of 0.15 mA intensity and 5 s duration over a period of 1-hr session (every 30 s)</td>
<td>Mice</td>
<td>To investigate the effects of stress in altering the humoral and cellular immune function</td>
<td>Brevet et al., 2010</td>
</tr>
<tr>
<td>e)</td>
<td>Six foot shocks of 1 mA intensity and 750 ms duration within a period of 2 min</td>
<td>Rats</td>
<td>To study transcriptional changes in brain</td>
<td>Sa´ntha et al., 2013</td>
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### Table 2: Different acute and chronic electric foot shock stress protocols in preclinical studies to study different aspects of stress

<table>
<thead>
<tr>
<th></th>
<th><strong>Chronic Stress</strong></th>
<th><strong>Animals</strong></th>
<th><strong>Objectives</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td>Mild electric foot shock stress of 0.15 mA intensity with duration 0.5s for 4 days</td>
<td>Mice</td>
<td>To investigate effects of mild stress on memory improvement</td>
<td>Bali et al., 2012</td>
</tr>
<tr>
<td>b)</td>
<td>Mild extrinsic foot shock of 0.1 or 0.3 mA of 1-s duration for 7 days</td>
<td>Mice</td>
<td>To evaluate the effects of stress on memory</td>
<td>Takatsu-Coleman et al., 2013</td>
</tr>
<tr>
<td>c)</td>
<td>60 unpredictable shocks of 0.8 mA intensity and 5s duration in the early life span of rats (14\textsuperscript{th} day &amp; 21\textsuperscript{st} day)</td>
<td>Rats</td>
<td>To study the deleterious effects of early life stress on spontaneous locomotor activity at maturity</td>
<td>Kim et al., 1999</td>
</tr>
<tr>
<td>d)</td>
<td>Exposure to 3 electric foot shocks trials/day for 7 consecutive days with each trial comprising of 0.2 mA intensity, 6 s duration for 10 times with a 30 s interval</td>
<td>Rats</td>
<td>To investigate stress induced post-traumatic stress disorder</td>
<td>Kim and Seo, 2013</td>
</tr>
<tr>
<td>e)</td>
<td>6 shocks, of 1 mA intensity and 750 ms duration in 2 min, for 3 consecutive days and 10 shocks of 0.6 mA intensity of 2 s duration in 5 min for 7, 14 or 21 day</td>
<td>Rats</td>
<td>To study the effects of stress conditions on the transcription and translation of actin-related cytoskeletal genes in the brain</td>
<td>Sa´ntha et al., 2013</td>
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</table>
3.1.5 IMMERSION IN COLD WATER (ICW)

In this method, rats are placed individually in a tank of cold water (depth 15.5 cm; temperature 15–20°C) for 15 min, where they either swim or remain in an upright position, keeping their heads above the water level (Armario et al., 1995; Resstel et al., 2009; Agrawal et al., 2011). A modification has been made in the method by subjecting the animals to cold water immersion stress for 5 min at 4°C (Lee et al., 2002). Stressor may be applied, both acutely (5–15 min) as well chronically (during 4, 12 and 20 days) (Aslan and Meral, 2007). Immersion in cold water elicits an increase in the plasma corticosterone levels (Resstel et al., 2009). For acute stress, rats are killed 30 min after the stress exposure. For chronic stress, animals are exposed to this stressor for 7–10 days and thereafter, the rats are killed 1 h after the last stress session. The major advantage of this type of stressor is that acute stress can be achieved in a relatively short period of time (Kee-Won et al., 2003). However, the major drawback of this model is that the body adapts to change in temperature on chronic exposure to low temperature and hence, stress response gets highly diminished with repeated episodes of stress (Blustein et al., 1998).

3.1.6 COLD ENVIRONMENT ISOLATION

In this method, rats are individually kept in a freezer with a temperature maintained at 4°C for 15 min once for acute stress and for 7–10 days for chronic stress (Kvetnansky et al., 1971; Krizanova et al., 2005). In a modified method, cold exposure in a small refrigerator set at 4 ± 2°C lasting for 30 min has been provided for inducing stress (Varty et al., 2000). In another modification, the rats are exposed to cold stress by placing them in wire mesh cages in a refrigerated compartment at -8°C for 4 h. Rats are exposed to this environment only once and their behavior is observed throughout the stress experiment along with body temperature monitoring (Mednick et al., 2002). This sharp fall in temperature leads to a sharp increase in the levels of adrenocorticoids, culminating in the development of stress response (Park et al., 2011). Unlike the ICW model, rats are prevented from drowning in cold water and hence, it is relatively safe model. However, it also suffers from same drawback of development of resistance/adaptation on chronic exposure.
3.1.7 COLD-WATER RESTRAINT STRESS

It is combination of both restraint and cold stress. The combination of body-restraint and the cold environment drastically increases the occurrence of gastric ulceration in a short period of time (3 h). Two different cold exposures have been employed to enhance gastric ulceration. One of two different cold exposure procedures involve immobilization of the animal, generally in a supine position, and then placing the animal in a cold environment (5 ± 1°C) such as in a refrigerator for 3 h (Pitman et al., 1988; Filaretova et al., 1998; Lyle et al., 2009). The other procedure involves restraining the animal in a cylindrical tube and then immersing vertically in cold water (22°C) for 1 h (Klenerová et al., 2007). It has been shown that combination of these two stressors produce severe form of stress, characterized in terms of activation of the HPA axis, including changes in corticotrophin-releasing factor (CRF), adrenocorticotropic hormone (ACTH) plasma levels and adrenergic receptors in the pituitary (Fernandez, 2004). It has been suggested that combinations of different stressors are better ulcerogenic stimuli, when compared with each one alone.

3.1.8 FORCED SWIMMING-INDUCED STRESS

It is the tendency of the living being to escape or avoid a noxious stimuli/condition. If the animal is not able to escape the stressful stimuli or it feels threatened, the animal will show stress response. This principle is used in developing forced swimming model for inducing stress in laboratory animals. In order to produce swimming-induced stress, rats are made to swim in a cylinder (30 cm in diameter and filled to a height of 20 cm) for a single session of 2-h duration for acute stress, or for 2-h session a day for five consecutive days for chronic stress (Ferry et al., 1991). Some scientists have used forced swimming in warm (20°C) water for 3 min with the total session lasting for 1 h (Kitchen and Pinker, 1990). Other researchers have used forced swimming in cold water (4°C) in a container (15 cm in diameter and 20 cm in height with water filled to a depth of 11 cm) for 3 min (Staratakis and Chrousos, 1995). Although, forced swimming-induced stress is a highly safe model, yet adaptation to chronic swimming-induced stress has been reported and inter-strain differences between rats to forced swimming behavior have also been documented (Armario et al., 1995).
3.1.9 FOOD-DEPRIVED ACTIVITY STRESS

Food-deprived activity stress has been defined as the condition in which rats are forced to run on a wire wheel, while food consumption is restricted. The animals are subjected to forced running on an activity wheel and are also subjected to an additional stress through food deprivation for 22.5 h/day and are permitted to take food and water for 1.5 h/day (Badmaeva et al., 2006).

3.1.10 REPEATED SOCIAL DEFEAT STRESS

Repeated social defeat stress provides a more naturalistic model of stress characterized by aggressive interactions that are intense, unpredictable and inescapable. The social defeat model has been characterized by the physiological and behavior associated with anxiety and depression (Kabbaj et al., 2001). Social defeat is considered an ecologically and ethologically relevant animal model of psychosocial stress that produces enduring behavioral and neurochemical sensitization in defeated individuals (Matsunaga et al., 2011). Social defeat stress consists of a brief aggressive confrontation between experimental intruder rodent and aggressive resident rodent. To induce social defeat stress, a mouse (the ‘intruder’) is transiently placed in the home cage of a resident male mouse (the ‘aggressor’) (Nicholson et al., 1985). Before starting the social stress procedure, the resident male mouse is housed with a normal cycling female to enhance territorial behavior and aggressiveness and is followed by removal of females from the resident’s cage. Thereafter, the intruder mouse is introduced for a 20-min trial and five such trials are given in a day for 3-6 days. Alternatively, rats are exposed to social defeat once every 72 h over the course of 10 days (i.e., four stress exposures) (Fanous et al., 2010). The social defeat behavior is characterized by social defeat posture consisting of immobility; escape; crouching (four paws on ground, not orienting toward resident), and defensive upright stance (standing still and erect with forepaws extended).

In many laboratories, rodents are first choice to model human psychiatric disorders. However, in recent years, evidence has accumulated that chronic psychosocial stress in a non-rodent species, the male tree shrew (Tupaia belangeri), may represent a suitable and naturalistic paradigm to study the causal mechanisms of stress-related disorders (Fernandez, 2004; Wei et al., 2008). From the phylogenetic point of view, the day-active animals are regarded as an intermediate between insectivores and primates.
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(Lai et al., 2008). Tree shrews are widely distributed in Southeast Asian forests and plantation areas, where males vigorously defend their territories against intruding conspecifics (Kaur et al., 2010). This pronounced territoriality of the males can be used to establish a naturally occurring challenging situation under experimental conditions in the laboratory. When living in visual and olfactory contact with a male conspecific by which it has been defeated, the subordinate tree shrew shows dramatic behavioral, physiological and neuroendocrine changes that are comparable to the symptoms observed during episodes of depression in patients. These include persistent hyperactivities of both the HPA system and sympathetic nervous system, disturbances in sleeping patterns and reduced motor activity (Fernandez, 2004). Treatment of subordinate tree shrews with antidepressants resulted in a time-dependent restoration of both endocrine and behavioral parameters (Wei et al., 2008).

3.1.11 NEONATAL ISOLATION STRESS (MATERNAL DEPRIVATION-INDUCED STRESS)

Early life events have profound consequences on subsequent quality of life (Lai et al., 2008). It has been shown that the early life stress of neonatal isolation in rats has immediate and enduring neural and behavioral effects (Kuhn et al., 1990). Rearing rats in isolation (post-weaning) is an animal model of social deprivation that recapitulates features of limbic-based psychopathology in humans. Rodents reared in deprivation of social contact exhibit an abnormal behavioral phenotype that includes hyper-locomotion in response to a novel environment (Hall et al., 1998), altered habituation and disrupted exploratory behaviors (Van de Kar LD and Blair, 1999; Dronjak and Gavrilovic, 2006). Brief isolation of an individual pup from the dam and litter is an effective method to stimulate HPA axis, without altering the growth of neonates (Hamm et al., 1983) and adults (Kehoe and Bronzino, 1999). Such effects may reflect stress-induced morphological changes in the hippocampus and other brain regions (Kosten et al., 2005; Kosten et al., 2007). In fact, the hippocampus provides negative feedback regulation of the HPA axis and hence, neonatal isolation-induced stress can represent the stress response that may lead to neurodegeneration at an early stage of life. This stress procedure is also useful in evaluating the effect of stress on cognition and memory development. In the neonatal isolation procedure, the litter of the inbred strain is removed.
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from the cage on the second day after the birth, weighed and placed individually in an opaque plastic container (9 cm diameter and 8 cm deep) with no bedding for 1 h (between 09.00 and 12.00 hours) in a temperature (30°C) and humidity-controlled chamber with white noise to mask other pups’ calls. The chamber has to be located in a room separate from animal colony facility. Containers are placed 20–30 cm apart (Kosten et al., 2000; Kosten et al., 2007). After 1 h period, the litters are placed back with their dams in home-cage (Kosten et al., 2000). This isolation procedure continues up to 8 days and hence, it is used to induce chronic stress. Neonatal isolation stress model has been used extensively to demonstrate the effect of early lifetime stress on vulnerability to addiction (Kosten and Kehoe, 2005) and response to psycho-stimulants by impairment of hippocampus-dependent context-induced fear in adult male rats (Kosten et al., 2005). Neonatal isolation model has been used extensively to study the persistent changes in the dopamine levels resulting from neonatal stress (Kosten et al., 2003).

3.1.12 PREDATORY STRESS

Direct encounter of an animal with its natural predator is one of the most stressful and anxiogenic event, and it leads to rapid development of ‘flight or fight’ response. Exposure of rodents to natural predators or to their odors may induce stress-like state (Adamec and Shallow, 1993; Adamec et al., 2005). Under such circumstances, there is rapid activation of the sympathetic system leading to rise in the levels of adrenocorticoids in blood. Direct encounter with a predator has been effectively used to evaluate the biochemical and physiological changes produced during such stressful conditions (Marilia et al., 2007). Predatory stress in mice is induced by series of short exposures to natural predator like cat (Blanchard and Blanchard, 1989) or to any substance having the odor of cat like the fecal pellets of cat (Berton et al., 1998). In one of the methods, mice are placed individually in different cages and after four initial 20-min cage habituation sessions, each animal is submitted to two randomly assigned 20-min predator confrontation sessions. The change in the behavioral pattern such as locomotion, shrieking-like voices and endocrinological changes after the stress exposure are observed (Blanchard and Blanchard, 1989).

Marmosets (Callithrix penicillata) have also been employed for induction of predatory stress in a test known as Marmoset Predator Confrontation Test (MPCT) (Cilia
and Piper, 1997). This model compares the behavioral response of experienced versus naive adult black tufted-ear marmosets in a confrontation with a wild-cat predator stimulus. After four initial 20-min cage habituation sessions, each subject is submitted to two randomly assigned 20-min predator confrontation sessions. Confrontation with the predator induces significant behavioral changes; i.e., proximic avoidance and tsik-tsik alarm call. Anti-stress drug administration, concomitant to predator exposure, reverses the behavioral changes observed (Barros et al., 2004). Predator-induced stress is an established model to induce short-term acute stress response, but its major disadvantage is the development of habituation to predator exposure; hence, the use of this model for inducing stress is justified for developing only acute stress.

3.1.13 DAY–NIGHT LIGHT CHANGE-INDUCED STRESS (SLEEP DEPRIVATION-INDUCED STRESS)

The changes in the circadian rhythm have profound effect on physical and psychological well being of an individual (Mednick et al., 2002). Laboratory animals, when subjected to abrupt changes in day-night light pattern, exhibit acute stress response (Nicholson et al., 1985; Galvao et al., 2009). The changes in circadian rhythms are regulated by the pineal gland through the secretion of melatonin (Bernard et al., 1997). Melatonin is released from the pineal gland in response to dark or dim light, whereas its functional antagonist serotonin is secreted in response to bright light. The serotonin–melatonin cycle is responsible for regulation of sleep–awake state of the body (Bermudez et al., 1983; Brzozowski et al., 2000). Sleep deprivation is documented to be responsible for causing several forms of memory deficits (Maquet, 2001). To induce stress, the cages of rat or mice are kept under bright light during night (in the dark phase) and cages are kept in dark room during day (in the light phase) for 180 min for 7–10 days (Marin et al., 2007).

3.1.14 NOISE-INDUCED STRESS

Noise is one of the most widespread sources of environmental stress in living environment (Wallenius, 2004). A large number of people are exposed to potentially hazardous levels of noise levels in daily modern life. Acute noise exposure activates the autonomic and hormonal systems, leading to temporary changes such as increased blood pressure, increased heart rate and vasoconstriction. A prolonged noise exposure in
susceptible individuals may develop permanent effects, such as hypertension and ischaemic heart disease. Experimental studies have demonstrated the ultra structural modifications in rat cardiomyocytes (mainly in mitochondria) due to noise stress which has been related to an imbalance in calcium homeostasis, which is supposed to be sustained by increased catecholamine innervations (Paparelli et al., 1992). When noise of any kind exceeds 90 dB, noise becomes a stressor. Studies have revealed that the exposure to noise stress alters the biogenic amine levels in discrete regions of the brain (Samson et al., 2006). Noise stress in laboratory rats can be produced by loudspeakers (15 W), driven by a white noise generator (0–26 kHz), installed 30 cm above the cage. Thus, a noise level can be set at 100 dB or above uniformly throughout the cage and can be monitored using a sound level meter. Each animal is exposed to noise stress for 4 h/day for 15 days. An acute model has also been developed involving exposure of rats to noise stressor of 10 kHz, 100 dB stress for 30 min (Sembulingam et al., 2003; Sembulingam et al., 2005).
3.2 Clinical models of stress

There have been various types of stress induction methods employed in humans to induce stress (Figure 3; Table 3) and these include the following:

Figure 3: Representation of clinical stress models employed for experimental purposes
3.2.1 TRIER SOCIAL STRESS TEST (TSST)

TSST is the gold standard test amongst the various stress protocols, and most commonly employed acute stress protocol to induce experimental stress in humans (Kirschbaum et al., 1993; Allen et al. 2014). It is one of the main psychological research tool to induce robust and reliable psychological, physiological and neuro-endocrinological changes in both children and adults (Dickerson and Kemeny, 2004; Yim et al., 2010). It elicits a stress response in terms of activation of the HPA and sympathetic adreno-medullary (SAM) axis during the task in a reproducible manner. The TSST test consists of 5 min preparation period, 5 min public speech (generally job interview) and 5 min mental arithmetic task (subtracting a two-digit number, say 25, from a four-digit number, say 4356) in front of a panel of evaluators (2-5 in number who are pre-trained to be completely impartial and provide no encouragement verbally or non-verbally). During test, the participants convince a panel of assessors that they are the perfect candidates for a job (nature of speech varies from laboratory to laboratory). It is demonstrated that tasks associated with social-evaluative threat are very effective in inducing stress and stress response (Dickerson and Kemeny, 2004) (Figure 4).

In addition, scientists have also designed variants of TSST including Repeated measure TSST in which participants are exposed to TSST more than once (Gerra et al., 2001; Von Känel et al., 2006) (Figure 5); Non-stress TSST which do not produce stress and is used to compare and understand the *per se* effects of TSST protocol; Control TSST in which participants are asked to speak on film, novel or other thing (that require less self-presentation and are non-stress inducing), but not in front of an evaluative committee (Het et al., 2009); Friendly TSST in which participants are asked to speak about their education and hobbies in front of a panel (with encouragement by smile, nods and simple questions) (Weimers et al., 2003); TSST-G (group) in which a participant is asked to speak about his job interview (for two min) in a group standing in a line with five other participants (Figure 6).
Figure 4: A typical TSST protocol in which participant is asked to perform in front of a panel of evaluators. The performance includes a speech and mental arithmetic test.

Figure 5: The participants are subjected to TSST stress testing more than once. The development of habituation is effectively controlled by making changes in speech content and mental arithmetic task during each TSST protocol, and having a sufficient gap between two consecutive TSST.
Other variants of TSST

Control TSST Protocol
- Preparation period
- Presentation on light topic
- No evaluative committee

Friendly TSST Protocol
- Preparation period
- Presentation on light topic
- Evaluation committee
  - Friendly
  - Positive feedback
  - Provide encouragement

TSST-G Protocol
- Preparation period
- Speech and MAT as in TSST
- Evaluation committee
  - Video recording
- Participant in a group

Figure 6: Representation of other variants of TSST protocol i.e., control TSST, friendly TSST and TSST-G. Control TSST and friendly TSST are non-stress inducing stress protocols in which participants are asked to speak on a light topic including movies, novel etc. and there is no social evaluative threat by not having an evaluative committee (control TSST) or having a friendly committee (friendly TSST) which provides positive feedback and encouragement. In TSST-G, a group of participants is evaluated by a panel of committee in comparison to individual participant in conventional TSST.

3.2.2 Cold Pressor Test (CPT) and Its Variants

CPT and its modified forms are commonly employed for stress induction in humans. In this test, the participants are instructed to immerse their hands up to the wrist in an ice-cold water (typically 0-2°C) for as long as possible, with a maximum of 3 min. During the test, the experimenter is present in the test room to covertly monitor the participant’s performance. This test reliably activates the SAM axis in terms of increase in blood pressure and skin conductance. However, activation of the HPA axis in terms of
cortisol responses is minimal (Mitchell et al., 2004). The traditional CPT has been modified to SECPT (Socially Evaluated Cold Pressor Test) in which the participants are allowed to perform the hand immersion task in the presence of the experimenter of the opposite sex (social evaluative element). The experimenter closely monitors the test and displays a lack of empathy towards the participants. SECPT produces significantly higher stress responses (more cortisol release) than the standard CPT (Schwabe et al., 2008). Another modification of CPT is the prolonged P-SECPT, in which participants are asked to immerse their hand (never exceeds 90s) in an ice-cold (2°C) water, multiple times alternated with short resting periods (minimum of 45 s) (Figure 7).

**Figure 7:** In CPT, the hand of participant is immersed in cold water for 3 min. In SEPCT, the hand immersion task is monitored by an evaluator of opposite sex. In P-SEPCT, participant is subjected to multiple trials of hand immersion. In this test, uncontrollability is introduced by randomly assigning the time of hand immersion and unpredictability is introduced by randomly varying the test period.
3.2.3 MAASTRICHT ACUTE STRESS TEST (MAST)

Smeets et al combined the stressful features of TSST (psychosocial evaluation threat; unpredictability and uncontrollability) and CPT (physical pain component) to create a physically and psychologically challenging laboratory test termed MAST. In this test, the participants undergo five socially evaluated cold pressor trials of duration (60 s to 90 s), with the water temperature held constant at 2°C. In between the hand immersion trials of variable duration, participants are engaged in a mental arithmetic task analogous to TSST and with every mistake, the participants receive negative feedback and they again have to start from beginning. The duration of the mental arithmetic task is also controlled by computer and comprises of 45 s (Smeets et al., 2012) (Figure 8).

Figure 8: Representation of MAST which combines the physical components of CPT and psychological components of TSST. The procedure is very similar to P-SEPCT, however, in between the hand immersion trials (physical stress), the participant is subjected to mental arithmetic stress (psychological stress).
3.2.4 FUNCTIONAL MAGNETIC RESONANCE IMAGING BASED STRESS

The subjective and neuroendocrine stress parameters suggest that fMRI procedure is uncomfortable to patients and may induce fear and stress. However, fMRI scan *per se* may not be used as stressor because it does not elicit significant activation of the stress response. Scientists add another stress triggering components during MRI scan to potentiate the stress response that include display of emotional pictures, aversive movies, mental arithmetic task and social evaluative threat in different protocols. Before the stress induction protocol of MRI, the participants are subjected to training (20 min) and thereafter, prepared for MRI (preparation of 10 min). Thereafter, the participant is subjected to two experimental sessions of 3T MRI scan, each comprising of 15 min (Muehlhan et al., 2011). In one of these procedures of fMRI-based psychological stress, participants are displayed emotional pictures during fMRI scanning. During scanning of 45 min, participants are randomly presented with 92 pictures varying from neutral scenes of domestic objects to extremely negative scenes of hurt or accidental injury. After each picture, participants are asked to indicate the emotional intensity of picture on a 4 point scale ranging from 1 to 4 (no emotional to extremely emotional) on the screen (Van Stegeren et al., 2006; Yang et al., 2007) (Figure 10).

![fMRI associated stress Protocol](image)

**Figure 9:** fMRI-based stressor in which participant is subjected to multiple stressors by showing aversive movie clips and asking to perform a face task, while being in a MRI scanner.
3.2.5 CO\textsubscript{2} CHALLENGE TEST

Researchers have also employed CO\textsubscript{2} as an acute physiological stressor and utilized as “physiological and psychological tool” to examine the stress response in humans (Griez and Van den Hout, 1983; Kaye et al., 2004; Wetherell et al., 2006). CO\textsubscript{2} readily passes through the blood brain barrier and produces transient effects on stress responsive brain regions. The test involves a rest period of 20 min, followed by inhalation of a single vital capacity breath of a mixture of CO\textsubscript{2} (35\%) and oxygen (65\%). In a typical procedure, participants are subjected to practice normal breath (normal air) followed by test breath (35 \% CO\textsubscript{2} and 65 \% O\textsubscript{2}). In each breath, participants are asked to inhale a full breath, exhale fully and then take a fast full breath. The breath is held for 4 s, after which the participants exhale the air. A care has to be taken that in each breath (practice and test), the inspired vital capacity is maintained to a same level. The cardiovascular parameters including blood pressure and heart rate are measured for 5-10 min before and after the test. The blood and saliva samples are taken before and after performing stress (at different time intervals, including 2, 10, 20 and 30 min post inhalation) for measuring the noradrenaline, cortisol and salivary alpha amylase levels (Wetherell et al., 2006).

3.2.6 COGNITIVE STRESSOR

3.2.6.1 Paced auditory serial addition task (PASAT)

PASAT is a cognitive stressor and has been commonly used as a laboratory psychological stressor (Tanosoto et al., 2012). In this test, participants are asked to listen the single digit numbers and instructed to add the recently listened two numbers. The test is paced by decreasing the time period to solve the task at regular interval. A typical procedure of PASAT includes four sessions (each of 2.5 min) with total time period of 10 min. The first session includes 65 auditory presentations within the stipulated period of time. In subsequent sessions, the pace is increased to 73, 80 and 88 presentations in second, third and fourth sessions, respectively (Tanosoto et al., 2012).

3.2.6.2 Stroop test

The Stroop task is a mental stress test and has been very frequently applied stress paradigm (as a laboratory stress tool) in neuropsychology for investigating the human psychophysiological response to stress (MacLeod, 1991; Renaud and Blondin, 1997).
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Stroop task is the Color-Word interference (a type of subset of interference) and this test assesses the ability of a person to inhibit automatic verbal responses (Stroop, 1935). In the classic form of Stroop test, the participants are initially required to read words representing the names of some basic colors, and then he/she tries to quickly name the colors. After 5 min, the participants are subjected to ‘sub-test of interference’ in which the different word signifies the names of some basic colors represented in a dissimilar manner (e.g. the word ‘red’ is written in ‘green’). The total time period of the test is 8-10 min. The subjects are instructed to identify the color in which the word is displayed as early as possible, rather than the color that the word names. During quick reading, the participant gets into a conflict filled stressful situation because the answer is influenced by the learned reaction (tendency to read words, but not the name the colors). In computerized version of Stroop test, two types of stimuli are presented: Stroop stimuli, which consist of four color names printed in dissimilar colors, and control stimuli, which consist of four color names printed in same color. In the Stroop list, the 12 possible combinations of color names and dissimilar colors are repeated 12 or 13 times and their order are varied randomly with the restriction that no identical stimuli would follow each other (Renaud and Blondin, 1997).

3.2.7 MANNHEIM MULTICOMPONENT STRESS TEST (MMST)

A new stress protocol has been developed in Mannheim (Germany), which consists of several components of stress, and it is named the “Mannheim Multicomponent Stress Test” (MMST) (Kolotylova et al., 2010; Reinhardt et al., 2012). The basic objective of MMST is to develop a high degree of stress, even in the absence of social evaluative threat, by combining different stressors including emotional, acoustic, cognitive and motivational stressors. In this test, the participants are subjected to stress for 5 min in the absence of investigator to avoid the social evaluative threat. During stress period, emotional (total 44 slides for 5 s of negative affective valence - disgust and fear related pictures; total 9 slides for 3 s of positive affective valence- pictures with babies and animals), acoustic (white noise from 78 to 93 dB noise), cognitive (sum up the most recent number flashed on a computer screen with the previous number) and motivational stress (instruction that reimbursement will be reduced after each wrong answer) are used simultaneously. This MMST protocol significantly increases the subjective stress ratings,
changes the cardiovascular parameters, electrodermal activity and HPA axis activity (Kolotylova et al., 2010; Reinhardt et al., 2012).

3.2.8 NOISE STRESS

Noise is widely accepted as a stressful stimulus and it has been employed as laboratory stress to examine the functional processes in the body during stress. Noise stress is an environmental stressor, which produces psychological and physiological effects. The noise levels below 65 dB are generally considered non-stress. However, noise exposure >80 dB has been shown to produce stress in terms of increase in levels of salivary cortisol (Fouladi et al., 2012). Numerous research studies have demonstrated that traffic noise may cause discomfort, sleep disorders, disturb daily-life activities, induce hypertension and other cardiovascular diseases.

In a typical laboratory stress procedure, participants relax for 10 min without any acoustic content and then are exposed to recorded naturalistic road and rail traffic vehicle noise of 48 dB-75 dB via a loudspeaker system for 20 min. The samples are collected immediately before (pre-exposure) and after (post-exposure) noise stress exposure (Wagner et al., 2010). In a modified method, noise stress is combined with vibration (as stressor) in which sinusoidal whole body vibrations of $1.0 \text{ m/s}^2$-$2.5 \text{ m/s}^2$ and recorded noise stimuli containing the sound of helicopter at an intensity level of 77-86 dB are presented. In fact, the combination of these may be used to induce stress of variable intensity and these may include 77 dB noise with $1.0 \text{ m/s}^2$ vibrations, 81 dB with $1.6 \text{ m/s}^2$ vibrations and 86 dB with $1.0 \text{ m/s}^2$ vibrations (Ljungberg et al., 2004).

3.2.9 SING A SONG STRESS TEST

A sing a song stress test is a recently introduced experimental paradigm to induce mental stress in a quick, easy and controlled way (Brouwer and Hogervorst, 2014). This test induces stress by asking participants to sing a song after a designated time interval and during this test, the perceptual inputs and movement are kept constant. In this test of 11 min, participants and confederates (one male and one female participant) are instructed to sit in front of the monitor and silently read the messages that appear on the monitor. The social evaluative threat is imposed on participants by making video of their performance. They are told that one of the messages on the monitor can involve a task that they need to carry out, when the counter reaches 0. The confederates are involved in
the test only to make the participants believe that they really fellow participants, but this may not be necessary. Total nine phrases (without stress) are selected approximately of the same length and structure as the 10th sentence (elicit stress), which may be as following “Task: start singing a song aloud when the counter reaches zero. Keep sitting still until that moment.” The participants are not told earlier that the experiment is about stress or singing. They are instructed to indicate the stress that they experience during a min before singing on a scale of 1 (not stressed at all) to 10 (extremely stressed). The heart rate and skin conductance during the 1 min interval following the sing a song stress message are found to be substantially higher than during intervals following neutral messages (Brouwer and Hogervorst, 2014). This test is advantageous over the TSST as the latter is a relatively complicated procedure to examine neuroendocrine stress response and response may also be strongly affected by posture or body movements such as speech. A sing a song stress test keeps these perceptual inputs and movement constant and only mental stress is varied.
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<th>S. No.</th>
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<tr>
<td>1.</td>
<td>TSST</td>
<td><strong>Advantages</strong>&lt;br&gt;Involvement of social-evaluative stress along with unpredictability and uncontrollability of stress</td>
<td>• Gold standard test and most commonly employed stress protocol&lt;br&gt;• Stress response is higher in children, young adults and men as compared to older adults and women&lt;br&gt;• Number of variants are developed to suit the requirements of a particular stress</td>
<td>Allen et al. 2014; Yim et al., 2010; Kirschbaum et al., 1993</td>
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<td><strong>Disadvantages</strong>&lt;br&gt;Requirement of a panel of judges makes this test more costly and introduces the chances of conflicts among the panel of evaluators</td>
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<td>2.</td>
<td>CPT/ SPECT</td>
<td><strong>Advantages</strong>&lt;br&gt;• CPT and SPECT offer the advantage of being shorter duration (3 min) as compared to TSST tests (15 min)&lt;br&gt;• These tests are most cost effective due to need of single evaluator than a panel of evaluators in TSST</td>
<td>• It mimics stress due to tearing, burning, shooting, aching pain</td>
<td>Dickerson and Kemeny, 2004</td>
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<td><strong>Disadvantages</strong>&lt;br&gt;• CPT involves only physical pain sensation for stress induction without involving</td>
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<td>psychosocial evaluative threat</td>
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<td>• It also lacks the uncontrollability and unpredictability of procedure, which is an essential feature for robust activation of stress response</td>
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<td>• Physical component may be very intense for children and patients with muscle problems</td>
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#### 3. MAST

**Advantages**
- There is involvement of both physical and psychological components of stress in comparison to psychological (TSST) and physical (CPT) components.
- It is relatively economical, as only one experimenter is required

**Disadvantages**
- Physical component may be very intense for children and patients with muscle problems

- Physically and psychologically challenging laboratory test
- MAST elicits the strongest cortisol response in comparison to CPT, SECPT and P-SECPT

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#### 4. CO₂ Challenge test

**Advantages**
- 35% CO₂ challenge is a safe and simple method to provoke panic attacks and a potential laboratory model for the study of
- Safe and specific model for induction of panic state
- CO₂ reliably stimulates the key

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Smeets et al., 2012
Fyer et al., 1987; Wetherell et al.,
### Review of Literature

#### CO₂ Challenge Test
- **Advantages**
  - CO₂ has minimal taste or odor, noninvasive, time efficient and reproducible method to provoke different forms of stress and anxiety
- **Disadvantages**
  - CO₂ challenge test increases the cerebral blood flow and this aspect may interfere with the stress assessment

**Amaral et al., 2013**

<table>
<thead>
<tr>
<th><strong>5.</strong></th>
<th><strong>MMST</strong></th>
<th><strong>Advantages</strong></th>
<th><strong>Disadvantages</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>5.</td>
<td>MMST</td>
<td>• induction of high degree of stress, even in the absence of social evaluative threat, by combining different stressors including cognitive, emotional, acoustic and motivational stressors</td>
<td>This stress paradigm can be used in larger cohorts or multicenter-studies even under field conditions</td>
<td>Reinhardt et al., 2012</td>
</tr>
<tr>
<td>6.</td>
<td>Sing a song stress test</td>
<td>• Sing a song stress test keeps the perceptual inputs and movement constant and only mental stress is varied.</td>
<td>This induces mental stress in a quick, easy and controlled way</td>
<td>Brouwer and Hogervorst, 2014</td>
</tr>
</tbody>
</table>

**Table 3:** Representation of commonly employed models with their advantages and disadvantages
3.3 Role of different brain regions in stress-induced behavioral, neurophysiologic, neuroendocrine changes

The brain has been considered as the key organ of stress reactivity and stress coping. In the brain, interconnected neural circuit determines the stressful situation to the individual (McEwen and Gianaros, 2010). According to the type, intensity and duration of stressor, the two major systems are activated, including the limbic–hypothalamic–pituitary–adrenal (LHPA) system and SAM system. The limbic–hypothalamic–pituitary–adrenal system stimulates the adrenal cortex to release glucocorticoids such as cortisol (in humans) or corticosterone (in rodents) into the blood. The SAM influences the stress response by triggering the release of adrenaline and noradrenaline (Mason, 1968). These systems collectively regulate physiological and behavioral stress processes, which may be adaptive or maladaptive, according to strength and duration of stress exposure. The main systems of this circuitry connect to the hippocampus, prefrontal cortex (PFC) and amygdala regions. These regions are considered as the most sensitive brain regions to stressors such as restraint, fear or exposure to a novel environment. Immobilization as well as electric foot shock induce stress response in terms of activation of the SAM and HPA system.

3.3.1 AMYGDALA

Amygdala has been projected as the central brain region, which links the stressful stimuli to behavioral, neuroendocrine and neurotransmitter changes (Radley et al., 2015). Amygdala comprises almond-shaped groups of nuclei located deep within the medial temporal lobes of the brain and is considered as a part of the limbic system. It includes several nuclei including the lateral, basolateral (BLA) and central nuclei (CeA) (Wilson et al., 2015). It detects and responds to threatened stimuli due to its widespread interconnections with number of important brain regions including hippocampus, median prefrontal cortex, septal area, ventral tegmental area, periaqueductal gray (PAG), thalamus and locus coeruleus (Radley et al., 2015; Wilson et al., 2015). The basolateral amygdaloid nucleus receives sensory information that is processed and relayed to neurons in the central amygdaloid nucleus (Le Doux, 1995).

The amygdala detects threatening stimulus during fear or stress, and the hippocampus and PFC regions of the neural system regulate the emotions through functional connectivity with the amygdala (Radley et al., 2015). The amygdala functioning has been widely implicated in both acute and chronic stress conditions
A number of studies have addressed that anxiety disorders are associated with increased sensitivity to threat and increased amygdala activity (Armony et al., 2005). Electrical stimulation of amygdala is associated with induction of fear, post traumatic stress disorder (PTSD) and startle response, whereas lesion of amygdala completely blocks the fear-potentiated startle response (Hitchcock and Davis, 1991). Foot shock-induced fear-potentiated startle has been linked the activation of amygdala (Rosen et al., 1992). Furthermore, it has been shown that amygdala lesions attenuate foot shock-induced freezing behavior and ultrasonic vocalization (Goldstein et al., 1996). Electric foot shock stress induces neuroendocrinological changes including increase in plasma corticosterone and ACTH, secondary to activation of HPA axis (Swenson and Vogel, 1983). Stress-induced HPA axis activation has also been linked with activation of amygdala as studies have shown that direct electrical activation of amygdala (Mason, 1959) or stress-induced activation of central nucleus of amygdala is involved in adrenocortical activation (Van de kar et al., 1991). Furthermore, lesions in amygdala attenuate foot shock-induced activation of adrenocortical response (Goldstein et al., 1996) suggesting the direct involvement of amygdala in HPA axis activation in response to foot shock stress. Another study demonstrated that a 10 day immobilization stress paradigm elicited hypertrophy of pyramidal neurons in the rat BLA to facilitate anxiety. The chronic immobilization stress induction increased the total dendritic length and number of branch points of BLA pyramidal neurons as compared to normal rats (Vyas et al., 2002). In addition, immobilization stress paradigm of 21 days also produced dendritic hypertrophy of BLA pyramidal neurons as well as increase in spine density that were accompanied by anxious behavior (Vyas et al., 2006).

Animal and human data show abnormal hyper-responsiveness of the amygdala to threatening stimuli and development of abnormal behavior in anxiety is due to inadequate regulation by the medial prefrontal cortex and the hippocampus (Shin et al, 2004). The emotional regulation is achieved when activity of the amygdala is balanced with the activity of the mPFC and hippocampus. The impaired ability of mPFC and hippocampus to regulate the amygdalar emotional responses to any kind of perceived threat lead to anxiety disorder. In addition to this, decoupling of amygdala and mPFC in anxiety related disorders indicates the decreased PFC control over the amygdala activity. Moreover, impaired ability of hippocampus to discriminate
between the safe and threatening stimulus and reduced control of extinction has been reported in anxiety related disorders (Garfinkel et al., 2014; Lissek et al., 2014). Therefore, it may be suggested that stress enhances amygdalar activity, probably due to structural degeneration of PFC and hippocampus. Thus, it may be proposed that the loss of inhibitory control of hippocampus and mPFC over the amygdala leads to defect in the emotional regulation and cognitive impairment.

3.3.2 HIPPOCAMPUS

The hippocampus is the quintessential limbic region, importantly and directly involved in the mediating anxiety reactions in animals (Radley et al., 2015). Numerous studies have shown that the hippocampus normally inhibits the HPA axis, both under basal and stressful conditions, thus considered as prominent site of glucocorticoid receptor-mediated feedback mechanism and essential for restoring glucocorticoids to baseline levels following the termination of stress (Tuvnes et al., 2003). A very high density of glucocorticoid receptors are present in the hippocampus. The hippocampal mineralocorticoid receptors possess very high affinity for glucocorticoids and therefore, these receptors are activated even in small quantity of glucocorticoids. Accordingly, the presence of mineralocorticoid receptors foster cellular activation and maintain basal circadian corticosteroid rhythms. The activation of hippocampal glucocorticoid receptors normally help in controlling the HPA axis via negative feedback mechanism (Herman et al., 2013). During acute stress, glucocorticoid receptors in the hippocampus initiate the negative feedback mechanism and control the HPA axis. However, during chronic stress exposure with recurrent stimulation of LHPA system, there is desensitization of HPA axis (Mamalaki et al., 1992). It results in uncontrolled activation of HPA axis, which in turn leads to excessive release of corticosteroids (Herman et al., 1992). The deficits following chronic stress exposure is evidenced by reduced hippocampal volume, hippocampal atrophy and decreased hippocampal neurogenesis (Rozendaal and McGaugh, 2011). The damage or atrophy of the hippocampus is associated with impairment of organism's ability to inhibit the HPA axis, even when the stressor has been subsided, thus leading to prolonged HPA activation and neuronal damage.

The hippocampus not only plays an important role in memory formation, but, it also regulates emotions through contextual extinction during fear conditioning. The amygdala processes cues and predicts a threatening stimuli during fear or stress,
whereas, the dorsal hippocampus relays contextual information about the specific threat cue through interactions with the amygdala and the mPFC. Thus, dorsal hippocampus allows the organism to discriminate threat from safety cues. Anatomically, the hippocampus makes a direct connection to the amygdala, which mainly regulates the brain system associated with fear and/or anxiety (Marsden, 2013). The evidence from intra-hippocampal infusion studies in animals support a distinctive role of the hippocampus in anxiety and PTSD disorders (Engin and Treit, 2007). Rats with cytotoxic ventral hippocampal lesions, which removed approximately 50% of the hippocampus (including dentate gyrus) starting from the temporal pole, displayed a reduction in freezing behavior following the delivery of an un-signaled foot-shock (Bannerman et al., 2003; Yoon and Otto, 2007). Furthermore, the role of hippocampus in the learned helplessness behavior has also been documented as electric foot shock stress significantly lower c-Fos-like immunoreactivity in the hippocampal dentate gyrus and the lateral septal nucleus in mice and rat model of leaned helplessness (Steciuk et al., 1999; Huang et al., 2004). Other studies have also demonstrated that inescapable shocks impair long-term potentiation in hippocampal slices (Shors et al., 1989). Immobilization stress over 2-3 weeks caused a reduction in rat hippocampal neurogenesis (Xu et al., 2006) and dendritic atrophy of hippocampal CA3 pyramidal neurons (Vyas et al., 2002).

### 3.3.3 PREFRONTAL CORTEX

Based on connectivity and cytoarchitecture, the rodent PFC is classified into three distinct neuroanatomical sub-regions, the anterior cingulate (ACC), prelimbic (pIPFC), and infralimbic (iPFC) cortices (McKlveen et al., 2013). The ACC in rats has mostly been implicated in motor behavior because it sends projections to oculomotor sites and regions involved in spatial navigation. The pIPFC projections to dorsal raphe, dorsal striatum, nucleus accumbens, BLA, anterior bed nucleus of the stria terminalis (aBST) are consistent with a role for the dorsal mPFC in limbic and cognitive functions. The projections of the iIPFC projections to nucleus of the solitary tract (NTS), posterior hypothalamus (PH), central nucleus of the amygdala and parabrachial nucleus suggest a role in visceral/autonomic responses (Vertes, 2004; McKlveen et al., 2013).

Prefrontal cortex has been implicated in the regulation of HPA and autonomic functions (Radley and Sawchenko, 2011). Like the hippocampus, the medial
prefrontal cortex expresses the large numbers of glucocorticoid receptor positive neurons (Ahima and Harlan, 1990). The lesions of the anterior cingulate and prelimbic divisions of the medial prefrontal cortex has been shown to enhance ACTH and corticosterone secretion and c-fos mRNA induction on the paraventricular nucleus (PVN) following restraint stress, suggesting the inhibitory role of mPFC in regulation of HPA axis (Figueiredo et al., 2003). However, during chronic stress exposure, elevated glucocorticoids have been documented to play an important role in prefrontal-dysfunction (Liston et al., 2006; Anderson et al., 2014). During chronic stress exposure, structural and functional degeneration in multiple PFC sub-regions such as anterior cingulate, prelimbic, and infralimbic cortices has been observed. A significant loss of dendrite and spines in the pyramidal cells occurs following repeated stress exposure (Ansell et al., 2012). Evidence suggests that in contrast to loss of dendrites in mPFC, chronic stress results increase in dendritic growth in the amygdala, which in turn initiates the imbalance in the amygdala and PFC functions (Vyas et al., 2002). Moreover, reduced functional connectivity of the PFC and impaired PFC regulation of amygdala has also been observed in subjects with stress related disorders (Mah et al., 2007; Garfinkel et al., 2014). Thus, it may be suggested that stress-induced PFC degeneration may contribute to the development of anxiety related disorders.

3.3.4 OTHER BRAIN AREAS

The other brain regions include locus coeruleus (LC), median eminence, subfornical organ, dorso-medial hypothalamus and nucleus tractus solitarius also respond to stress stimuli. The LC region is the nucleus in the pons (a part of the brain-stem) and is actively involved in controlling the physiological response to stress and panic. It is the site of origin of sympathetic innervation to the cortex and is involved in stress-induced central sympathetic stimulation. The dorso-medial hypothalamus is a nucleus of hypothalamus, and it regulates the initiation of panic-like responses (Carrasco and Van de Kar, 2003; Berridge and Waterhouse, 2003). Paraventricular nucleus of the hypothalamus is another important stress sensitive brain region (located adjacent to the third ventricle in the forebrain) which contains multiple sub-populations of neurons that are activated by a variety of stressful and/or physiological changes. Subfornical organ is a sensory circumventricular organ (situated on the ventral surface of the fornix, at the interventricular foramina outside the blood-brain
barrier in lamina terminalis) and is also involved in regulating the stress response. NTS is a group of cells in the brain-stem (on each side of the upper medulla and dorsal nucleus of the vagus reticular) that receives viscero-sensory information and transmits them to the basal forebrain, which is actively involved in regulating cortical processing of anxiogenic stimuli. Furthermore, additional projections from the nucleus tractus solitarius to the LC, the bed nucleus of the stria terminalis and amygdaloid structures serve to influence the processing of anxiogenic stimuli. The median eminence is an integral part of the hypophyseal portal system, which connects the hypothalamus with the pituitary gland. The projections of neurons from the median preoptic nucleus (a region of the hypothalamus) to the median eminence regulate the stress response by controlling the release of stress hormones (Wang et al., 2004; Bregonzio et al., 2008; Saavedra et al., 2011).

3.4 Renin Angiotensin system

The renin angiotensin system is one of the best characterized systems in the body (Figure 9). The brain generated angiotensin II (Ang II) by the local renin angiotensin system acts as a neuropeptide, neuromodulator, neurotransmitter and neurohormone (Ferguson et al., 2001; McKinley et al., 2003). Central Ang II, being a neuromodulator, facilitates neurotransmission by enhancing the release of norepinephrine (NE) from the catecholaminergic nerve terminals and inhibiting its reuptake (Fabiani et al., 2001). Ang II is also a classical neurohormone and regulates the blood pressure, thirst, water and electrolyte balance. However, it also produces more diverse actions particularly related to behavior by modulating the neuroendocrine system, and it has been regarded as an important “stress hormone” (Saavedra, 1992; Phillips, 1997). Ang II exerts its actions by acting on its two subtypes of receptors, the AT\(_1\) and AT\(_2\). The well-known physiological actions of Ang II are dependent on AT\(_1\) receptor stimulation, while the role of AT\(_2\) receptors in mediating homeostatic functions is controversial (Saavedra, 1999; De Gasparo et al., 2000). The binding of Ang II induces a conformational change in the AT\(_1\) receptors (G protein coupled) to mediate the signal transduction via several plasma membrane localized effector systems. These include enzymes such as phospholipase A\(_2\), C, D; adenylyl cyclase; NADPH oxidase and ion channels such as L- and T-type voltage-sensitive calcium channels (Tsutsumi and Saavedra, 1991; Nguyen Dinh Cat and Touyz, 2011). The AT\(_2\) receptors are clearly distinct from the AT\(_1\) receptors with
respect to molecular weight, diversity in tissue-specific expression and signaling mechanisms. These receptors are also G protein coupled (mostly of Gi type), and the signal transduction pathway involves the activation of protein serine/threonine phosphatase PPA2 and phosphotyrosine phosphatases (PTPases), nitric oxide, and cyclic guanosine monophosphate (Haung et al., 1996; De Gasparo et al., 2000).

**Figure 10:** Representation of Renin Angiotensin System in the body

The AT1 receptors are distributed throughout the brain, including the key areas regulating the stress response such as the HPA axis (Tsutsumi and Saavedra, 1991, Israel et al., 1995). The medial, basomedial, lateral, and basolateral nuclei of amygdala (located deep within the medial temporal lobes and part of the limbic system) control the emotional responses such as fear conditioning and adaptation to danger, and these nuclei are enriched with AT1 receptors (Collister and Hendel, 2003). The other stress responsive brain regions including the different sections of the cortex, hippocampus, LC, median eminence (ME), subformical organ (SFO), dorsomedial hypothalamus (DMH) and nucleus tractus solitarius (NTS) are also enriched with Ang II and its receptors. The different sections of the cortex such as prefrontal
cortex, entorhinal, piriform cortex and neocortex are involved in controlling cognition and emotional behavior (Tsutsumi and Saavedra, 1991, Lenkei et al., 1998). The hippocampus (located deep within the medial temporal lobes of the brain, lateral to amygdala, and a part of the limbic system) is an important structure for storing the cognitive processes during stress and these prior memories may have an important influence on enhancing, suppressing, or independently generating a stress response. The different types of stressors (isolation, immobilization/restraint, cold restraint and immunological) are shown to influence the release of Ang II and expression of its receptors in the brain as well as in the peripheral tissues (Yang et al., 1996; Bregonzio et al., 2008). The stimulation of the PVN of the hypothalamus localized AT₁ receptors increases the secretion of corticotrophin releasing hormone (CRH) and ACTH, which is followed by the release of adrenal glucocorticoids (Aguilera et al., 1995; Castrén and Saavedra, 1988). Accordingly, the studies have documented the critical role of AT₁ receptors in modulating stress-associated anxiety behavior (Braszko et al., 2003a; López et al., 2012). A number of preclinical and clinical studies has shown the usefulness of renin-angiotensin modulating drugs in attenuating stress-associated anxiety and improving mood in depressed patients (Armando et al., 2001; Nasr et al., 2011). The AT₁ receptors have received the greater attention so far, and the scientists have employed the selective AT₁ receptor antagonists to demonstrate their anxiolytic potential (Armando et al., 2001).

### 3.4.1 AT₁ RECEPTORS AND STRESS

The different type of non-immunological (isolation, immobilization/restraint and cold restraint) and immunological stressors are associated with an increased expression of AT₁ receptors in the stress-responsive brain areas both inside and outside the blood-brain barrier (Saavedra et al., 2006; Bregonzio et al., 2008). There has been evidence documenting that stress increases the release of Ang II with in the brain and also increases the expression of AT₁ receptors in both the brain as well as in peripheral tissues suggesting the involvement of the Ang II/AT₁ receptor system in stress-mediated changes (Saavedra, 1992; Yang et al., 1996; Bregonzio et al., 2008). In particular, the significant rise in AT₁ receptor expression in the parvocellular PVN of the hypothalamus (the brain region where cell bodies forming the CRH are present) in response to stress has been demonstrated (Castrén and Saavedra, 1988; Aguilera et
al., 1995). Furthermore, it is also reported that these receptors are transported from the PVN to the ME through the axons co-expressing CRH (Oldfield et al., 2001).

The changes in density of AT1 receptors are related to changes in the hormonal and sympathoadrenal system in response to stress. Stress-induced activation of the HPA axis increases the levels of adrenal glucocorticoids that in turn regulate the expression of AT1 receptors in the PVN region (Castrén and Saavedra, 1988; Aguilera et al., 1995) through stimulation of glucocorticoid response elements (GREs) in the AT1 receptor promoter (Gou et al., 1995). Interestingly, these receptors are also present on the HPA axis (Nguyen Dinh Cat and Touyz, 2011) and the stressors (of different types and variable intensities) are well reported to increase their expression on this axis (Castrén and Saavedra, 1988; Armando et al., 2001; Leong et al., 2002). Therefore, stress-induced increased expression of AT1 receptors leading to an increased response of Ang II may be responsible for the HPA axis over-activation to result in development of vicious cycle and exaggerate the stress response in terms of development of anxiety and depression (Figure 10).

The various studies have shown that the pharmacological blockade of AT1 receptors attenuates the stress-mediated deleterious effects (Table 4). The administration of candesartan (AT1 receptor antagonist) has been shown to prevent isolation stress-induced changes in the hormonal and SAM system by a sustained inhibition of peripheral and brain AT1 receptors (Watanabe et al., 1999). In addition, the pretreatment with candesartan has also been shown to prevent the cold-restraint and isolation-induced gastric ulceration in rats (Saavedra et al., 2005). These gastro-protective effects have been attributed to antagonism of Ang II-mediated inflammation in the gastric mucosa (Saavedra et al., 2005). The peripheral administration of losartan, a selective AT1 receptor antagonist, has also been shown to attenuate the anxiogenic behavior in experimental renal hypertensive as well as in normotensive rats. Furthermore, the administration of enalapril, an angiotensin-converting enzyme inhibitor, has also been shown to selectively attenuate the anxiogenic behavior in the hypertensive rats (Srinivasan et al., 2003).

An intracerebroventricular (icv) administration of losartan has been shown to attenuate Ang II-induced anxiety and motor impairment (Braszko et al., 2003a). Raasch and co-workers demonstrated that the administration of candesartan and ramipril (an angiotensin-converting enzyme inhibitor) decreases the stress sensitivity
of HPA axis in the spontaneously hypertensive rats independent of their anti-hypertensive effects. The chronic treatment with angiotensin antagonists was shown to attenuate CRH-stimulated increase in the plasma levels of ACTH and corticosterone, without affecting their baseline levels (Raasch et al., 2006). Berganzio and co-workers demonstrated that the administration of candesartan abolishes cold-restraint stress-induced increase in tyrosine hydroxylase mRNA in the LC and adrenal medulla of the spontaneously hypertensive rats (Berganzio et al., 2008) suggesting that the decreased sympathetic outflow from the brain and adrenaline release from the periphery may be responsible for the anti-stress effects of AT₁ receptor antagonists. The same study also demonstrated the reduction in AT₁ receptor binding in different brain regions, including the ME and basolateral amygdala in response to cold restraint stress. The reduced AT₁ receptor binding was linked to down-regulation of these AT₁ receptors due to fast receptor internalization following the increased Ang II release during stress (Yang et al., 1996; Berganzio et al., 2008). The reduced AT₁ receptor binding may serve as an endogenous protective mechanism to prevent the deleterious effects of excessively released Ang II in stress subjected animals.

The contention that the increased levels of Ang II induce anxiety has been supported by the studies showing the development of anxiety like behavior in the transgenic rats with an up-regulated RAS system [TGR (mREN2)27] (Krková et al., 2009). In these transgenic rats, the AT₁ receptor binding is significantly increased in most of the regions inside the blood-brain barrier, including the PVN, piriform cortex, lateral olfactory tract, and lateral preoptic area. Furthermore, the functional response of Ang II is also increased in these rats suggesting that these up-regulated AT₁ receptors in the brain are functional. However in the subfornical organ and area postrema regions of circumventricular organs, the AT₁ receptor binding was significantly lower in these rats (Monti et al., 2001). Voigt and co-workers demonstrated that these transgenic rats exhibit more signs of anxiety as compared to normal Sprague-Dawley rats in the elevated plus maze and light/dark box tests (Voigt et al., 2005). The same group of scientists also demonstrated the anxiogenic phenotype of these rats in the canopy test of anxiety-related behavior (Voigt et al., 2010). Müller and co-workers demonstrated an increased stress sensitivity in TGR(ASrAOGEN)680 animals as compared to corresponding normal control rats. In response to CRH or ACTH challenge, the corticosterone release was also more
distinct in these transgenic rats and was independent of concurrent ACTH enhancement. The ACTH independency of enhanced corticosterone release during CRH and ACTH challenge test indicates the possible involvement of adrenal mechanisms in releasing corticosterone (Müller et al., 2010) (Figure 11).

The renin-angiotensin system modulating drugs have also been shown to exert the beneficial effects in patients with behavioral alterations. Zubenko and Nixon reported the improvement in mood and attenuation of depressive symptoms in three hypertensive patients (Zubenko and Nixon, 1984). These results were supported by more studies showing that captopril and enalapril improve the cognition and depressed mood in hypertensive patients (Braszko et al., 2003b). Hertzman and co-workers demonstrated that the administration of lisinopril may augment the anti-depressant response (Hertzman et al., 2005). A more recent study has shown that the use of renin-angiotensin-aldosterone system modifying medication is associated with lower rate of anti-depressant usage (Nasr et al., 2011). A recent clinical study has also suggested the significant association between ACE inhibitor/ARB (angiotensin receptor blocker) medications and decreased PTSD symptoms based on the cross-sectional, observational data from the large number of patients suggesting the key role of RAS in regulating the stress response in patients diagnosed with PTSD (Khoury et al., 2012). Thus, the preclinical as well as clinical reports suggest that the antagonism of peripheral and brain AT₁ receptors could be of therapeutic relevance in controlling stress-associated deleterious effects (Figure 11).

3.4.2 ANXIOLYTIC/ANXIOGENIC EFFECTS OF CENTRALLY ADMINISTERED ANG II

There have been conflicting reports regarding the anxiolytic/anxiogenic actions of centrally administered angiotensin. An icv administration of Ang II has been shown to exert significantly less defensive burying behavior (conditioned fear-related behaviors) in a dose-dependent manner suggesting that Ang II may modulate the central neurotransmitter system to exert the anxiolytic effects (Khoury et al., 2012). The bilateral microinjections of Ang II (0.1, 0.5 and 1.0 µg) into the hippocampal CA1 area has also been shown to exert the anxiolytic effects (Braszko and Wisniewski, 1988; Belcheva et al., 1997). The other studies have also demonstrated the anxiolytic effects of icv administered Ang II (1 nmol) in Wistar rats (Holy and Wiśniewski, 2001). On the contrary, Ciobica and co-workers demonstrated the
anxiogenic phenotype of icv administered Ang II (0.1 mg/kg for seven consecutive days) by employing the elevated plus maze model. The chronic central administration of Ang II-induced anxiety was associated with an increased pro-oxidant status, and these effects were attenuated in the presence of Ang II antagonists (Ciobica et al., 2010). A recent study has also reported that the chronic infusion of Ang II (1900 ng/kg/min) for three weeks in mice produces the anxious behavior along with the learning and spatial memory deficits in the elevated plus maze and open field tests (Duchemin et al., 2013).

Figure 11: Role of Ang II and AT1 receptor in the brain and periphery in stress-induced anxiety and its modulation by AT1 receptor antagonists.
### Table 4: The summarized preclinical and clinical pharmacological effects of ACE inhibitors, AT₁ receptor antagonists in stress-associated anxiety and other behavioral changes

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Intervention</th>
<th>Comments</th>
<th>References</th>
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<tbody>
<tr>
<td>1.</td>
<td>Candesartan</td>
<td>Normalizes hormonal response to isolation stress and cold restraint stress</td>
<td>Armando et al., 2001; Bregonzio et al., 2008</td>
</tr>
<tr>
<td>2.</td>
<td>Candesartan</td>
<td>Attenuates gastric ulceration produced by isolation and cold restraint stress</td>
<td>Saavedra et al., 2005</td>
</tr>
<tr>
<td>3.</td>
<td>Candesartan</td>
<td>Decreases tyrosine hydroxylase mRNA in LC and adrenal medulla, leading to decrease in central sympathetic outflow and adrenaline release, respectively</td>
<td>Seltzer et al., 2004</td>
</tr>
<tr>
<td>4.</td>
<td>Candesartan</td>
<td>Prevents acute inflammatory and restraint stress-induced changes in benzodiazepine receptors</td>
<td>Sánchez-Lemus et al., 2012</td>
</tr>
<tr>
<td>5.</td>
<td>Candesartan</td>
<td>Decreases stress sensitivity of HPA axis</td>
<td>Raasch et al., 2006</td>
</tr>
<tr>
<td>6.</td>
<td>ACE inhibitor</td>
<td>Decreases the incidences of PTSD in patients</td>
<td>Ciobica et al., 2010</td>
</tr>
<tr>
<td>7.</td>
<td>Losartan</td>
<td>Attenuates i.c.v Ang II-induced anxiety</td>
<td>Schinke et al., 1999</td>
</tr>
<tr>
<td>8.</td>
<td>Enalapril</td>
<td>Attenuates anxiogenic behavior in experimental renal hypertension</td>
<td>Srinivasan et al., 2003</td>
</tr>
<tr>
<td>9.</td>
<td>Captopril and enalapril</td>
<td>Improves cognition and depressed mood in hypertensive patients</td>
<td>Braszko et al., 2003</td>
</tr>
<tr>
<td>10.</td>
<td>Lisinopril</td>
<td>Augments the therapeutic response of antidepressants</td>
<td>Hertzman et al., 2005</td>
</tr>
<tr>
<td>11.</td>
<td>ACE inhibitors</td>
<td>Lowers the rate of anti-depressant usage</td>
<td>Nasr et al., 2011</td>
</tr>
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</table>
3.4.3 ROLE OF ANGIOTENSIN II IN ASTROCYTE PROLIFERATION, NEUROINFLAMMATION AND CEREBRAL INJURY

There have been number of studies describing the role of Ang II in astrocyte proliferation, neuroinflammation and cerebral injury (Figure 12). In the CNS, the astrocytes contain all the components necessary for Ang II synthesis and serve as a major source of angiotensinogen, the precursor molecule for Ang II (Stornetta, 1988). Angiotensin mediates astrocyte growth promoting effects mainly via stimulation of extracellular signal regulated kinase 1/2 (ERK) mitogen activated protein kinase (MAPK), which are the terminal serine threonine kinases in MAPK cascade (Figure 13). Ang II has been shown to activate ERK½ in astrocytes cultured from the brain stem and cerebellum in a dose and time-dependent manner. Ang II may produce astrocyte proliferation through intracellular non-receptor-linked protein kinases. Clark and Gonzalez reported that Ang II induces the rapid phosphorylation of Src (in 5 min) and Pyk2 (as early as 1 min) in rat astrocytes cultured from the brainstem and cerebellum. Furthermore, the selective inhibitors of Src (PP2) and Pyk2 (dantrolene) significantly attenuated Ang II-induced phosphorylation of the respective proteins suggesting the direct and selective effect of Ang II on Src and Pyk2. The authors also demonstrated that Src (PP2) and Pyk2 (dantrolene) inhibitors significantly decrease Ang II-induced ERK½ activation suggesting that these two non-receptor tyrosine kinases are upstream signals in Ang II-induced ERK½ activation in cultured astrocytes. Along with it, it was also proposed that these two intracellular tyrosine kinases act in concert with each other in astrocytes and Pyk2 may possibly mediate the actions of Ang II. Together, it may be possible to suggest that Ang II triggers a signaling pathway in the astrocytes in which Src activates ERK½ MAP kinases to stimulate astrocytic growth and this pathway may also involve Pyk2 as one of the intermediate steps (Clark and Gonzalez, 2007a) (Figure 14).

Clark and Gonzalez reported that administration of Ang II significantly stimulates the membrane bound receptor tyrosine kinase for platelet derived growth factor (PDGF) and epidermal growth factor (EGF) on the cultured astrocytes and selective inhibition of these receptors significantly attenuates Ang II-induced activation of membrane bound tyrosine receptors. Furthermore, selective PDGF and EGF inhibitors significantly reduced Ang II-induced ERK½ phosphorylation and
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astrocyte proliferation. However, the combined administration of these inhibitors produced synergistic effect and the overall inhibition of Ang II-induced ERK½ phosphorylation was relatively more significant (91 % vs 49 % and 71 % with PDGF and EGF receptors). Therefore, it may be suggested that the PDGF and EGF receptors synergistically mediate Ang II-induced ERK½ phosphorylation and astrocyte proliferation (Figure 14). Furthermore, there was no effect of PDGF receptor inhibition on Ang II-induced EGF receptor activation suggesting that Ang II directly activates the EGF and PDGF receptors, and there is no transactivation of tyrosine kinase receptors in response to Ang II (Clark and Gonzalez, 2007b).

The brain renin angiotensin system is known to play a major role in the regulation of neuroinflammation and progression of neurological disorders via RhoA/ROCK activation (Villar Cheda et al., 2012; Rodriguez-Perez et al., 2015). Villar-Cheda et al demonstrated an important interaction between the Ang II/AT₁ and the RhoA/ROCK II pathways in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced brain lesion. Injection of MPTP in the substantia nigra was associated with increase in RhoA and ROCK II expressions as well as increase in ROCK activity in mice. However, MPTP failed to induce such changes in the substantia nigra portion in AT₁a null mice suggesting that MPTP modulates Rho kinases through AT₁ receptors. This contention was further supported by the observation that Ang II enhances the MPP⁺-induced dopaminergic neuron death, which in turn is inhibited in the presence of ROCK inhibitor Y-27632 (Figure 15). The inhibition of the Ang II/AT₁ pathway produced similar results to those observed after ROCK inhibitor treatment suggesting that ROCK activation may play role a critical role in Ang II-induced inflammatory response (Villar-Cheda et al., 2012).

Studies have reported that Ang II/AT₁ pathway activation exacerbates the microglial Nicotinamide adenine dinucleotide phosphate-oxidase (NAPDH) oxidase and the glial inflammatory response, which is inhibited by treatment with AT₁ antagonist, candesartan (Rodriguez-Pallares et al., 2008; Joglar et al., 2009). Rodriguez-Perez et al demonstrated that Ang II activates ROCK through NADPH-dependent reactive oxygen species (ROS) generation as inhibition of NADPH-oxidase with apocynin or inhibition of ROS with N-acetyl-cysteine (NAC) abolished Ang II-induced ROCK activation. Moreover, studies have shown the existence of cross-talk signaling between NADPH-oxidase and Rho-kinase via p38 MAPK. Ang II was
shown to activate the ROCK to increase the phosphorylation of p38 MAPK, which in turn increases the NADPH activity as ROCK inhibitor and p38 MAPK inhibitor abolished p38 MAPK and NADPH oxidase activity, respectively (Rodriguez-Perez et al., 2015) (Figure 15).

A major role of Ang II in interleukin 1β (IL-1β)-induced neuronal inflammation in neuronal culture through c-Jun N-terminal kinase (JNK) signaling has also been described (Pang et al., 2012). Telmisartan was shown to reduce the IL-1β-induced increase in NADPH oxidase-4 mRNA expression, NADPH oxidase activity, ROS generation, reduce hydrogen peroxide-induced cyclooxygenase 2 (COX-2) gene expression and JNK activation (Figure 11). It suggests that telmisartan directly ameliorates the neuronal inflammation and injury via regulating JNK/c-Jun and NADPH oxidase pathways (Pang et al., 2012). Moreover, studies have shown that Ang II may be involved in ischemia reperfusion-induced cerebral injury (Zhang et al., 2012) and nociception (Pang et al., 2012). It has been reported that Ang II-induced nociceptive behavior is associated with an increased AT_{1} receptor expression and phosphorylation of p38 MAPK in the lumbar superficial dorsal horn. In addition, the behavioral observation revealed that co-administration of either losartan (AT_{1} inhibitor) or SB203580 (p38 MAPK inhibitor) completely inhibits the Ang II-nociceptive behavior (Pang et al., 2012). Zhang et al demonstrated that administration of losartan attenuates the neural damage following cerebral ischemia reperfusion injury along with inhibition of JNK3, Bcl-2, caspases-3 and cytochrome c (Zhang et al., 2012).
Figure 12: Ang II-triggered signaling cascades in neuronal disorders
Figure 13: Signaling cascades triggered by Ang II during nociception, astrocyte proliferation and cerebral injury: Ang II via Gq protein stimulates ERK dependent pathway via (Rapidly accelerated fibrosarcoma) c-raf and is associated with increased expression of c-myc and c-fos DNA synthesis to induce astrocyte proliferation. On the other hand, Ang II stimulates p-38MAPK to promote nociception via non-transcriptional pathway. Ang II also activates NADPH oxidase, a major source of cellular ROS, and sequentially, NADPH-derived ROS activate the JNK-3, which in turn increases the c-jun expression to induce cerebral injury.
Figure 14: Signaling cascades triggered by Ang II in astrocyte proliferation and neuronal inflammation via receptor and non-receptor tyrosine kinase: Ang II activates the non-receptor tyrosine kinase, Src/Pyk2 followed by ERK½ activation to induce astrocyte proliferation. Ang II also activate non-receptor tyrosine kinase Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway to induce astrocyte proliferation followed by increase in IL-6 secretion to induce neuroinflammation. Ang II-induced AT₁ receptor activation also transactivates non-tyrosine kinase receptors, EGFR and PDGFR to induce cellular effects. The activation of EGFR and PDGFR is followed by stimulation of Ras/Raf proteins to activate ERK½ and p38 MAP kinases to induce astrocyte proliferation.
Figure 15: Signaling cascades triggered by Ang II during cerebral infarction and microglial migration: The Rho protein functions as a molecular switch that cycles between the active GTP-bound form and inactive GDP-bound form. GDP/GTP exchange is facilitated by guanine nucleotide exchange factors (GEFs) that catalyzes the exchange of GDP to GTP and by GTPase activating proteins (GAPs) that accelerate the hydrolysis of the GTP to GDP. Ang II stimulates the ROCK (Rho-associated protein kinase) pathway via acting on AT1/G protein-coupled receptors which in turn induce phosphorylation of its substrate, adducin and MYPT1, to induce cerebral infarction. Ang may also II bind to AT1 receptors to activate NADPH oxidase, which generates ROS and increases NF-κB expression to induce activation of ROCK, which mediates Ang II induced microglial migration/phagocytosis.
3.5 Endogenous opioids as stress modulator

The endogenous opioids are derived from three independent genes that give rise to three precursor proteins known as proopiomelanocortin (POMC), proenkephalin (PENK), and prodynorphin, and their appropriate processing yields β-endorphin, met-enkephalin, leu-enkephalin, dynorphin and nociceptin, respectively. These peptides and their derivatives exhibit different affinity and selectivity for the mu-, delta- and kappa-receptors located on the central and the peripheral neurons, neuroendocrine, immune, mucosal cells and on many other organ systems (Figure 16). Two additional endogenous opioid peptides have been isolated from the bovine brain that include endomorphin-1 and endomorphin-2. Apart from the analgesic actions of opioids, different opioid agonists and antagonists have shown therapeutic actions in diverse diseases of the central nervous system, including depression, stress, anxiety, epilepsy; gastro-intestinal diseases such as ulceration, irritable bowel syndrome, diarrhoea, postoperative ileus; diseases of immune system and related inflammatory disorders such as osteoarthritis and rheumatoid arthritis; and others, including ischemia-reperfusion injury, alcoholism and obesity/binge eating (Sauriyal et al., 2011).

Opioids receptors are widely distributed on different components of the HPA axis such as the hypothalamus, pituitary and adrenal gland. It has been documented that the POMC fibers originating in the arcuate nucleus innervate the stress-responsive hypothalamic nuclei, including the PVN of hypothalamus, median eminence and other limbic structures, including the septum, bed nucleus of stria terminalis (BNST) and amygdala (Palkovits et al., 1987; Sauriyal et al., 2011). Numerous research studies and reviews provide evidence for the role of the endogenous opioid system in regulating and modulating the HPA axis, autonomic nervous system and behavioral responses during stress (Valentino and Bockstaele, 2008). Various clinical and preclinical studies have documented the critical role of endogenous as well as exogenous opioids in modulating stress and stress-associated anxiety, PTSD and depression (Nixon et al., 2010; Szczytkowski-Thomson et al., 2013). Furthermore, stress has also been shown to modulate the response of opioids, including drug craving and reinstatement of drug of abuse (Hays et al., 2012; Haghparast et al., 2014).
3.5.1 EFFECT OF ENDORPHIN/MU OPIOID AGONISTS ON STRESS-RELATED BEHAVIOR

Endorphins (endogenous morphine) are endogenous opioid peptides that function by acting on the mu receptors. The possible involvement of β-endorphin in stress-related psychiatric disorders, including depression, PTSD and fear conditioning has been reported (Merenlender-Wagner et al., 2009). Specifically, the central endorphinergic neurons originate from two nuclei, the arcuate nucleus in the posterior hypothalamus and nucleus tractus solitarius in the brain stem. The endorphinergic neurons have extensive projections to other brain areas, including the hippocampus, midbrain and the amygdala, thus, providing a rich network of POMC fibers throughout the brain, particularly to regions associated with stress (Palkovits, 1987; Narita and Tseng, 1998).

Figure 16: Endogenous opioids with their precursor molecules and binding receptor sites
Research evidence suggests the key role of β-endorphin in stress-coping behavior (Grisel et al., 2008; Barfield et al., 2010). Transgenic mice with low β-endorphin exhibit increased anxious behavior and show deficits in coping ability during an inescapable aversive situation, suggesting the anxiolytic actions of endogenous endorphins (Grisel et al., 2008; Barfield et al., 2010). Mice with low/no β-endorphin have enlarged adrenal glands suggesting that persistent up-regulation of HPA axis may occur with the decreased β-endorphin levels (Rubinstein et al., 1996). The reduction in endogenous opioid tone has been linked with the development of depression (Burnett et al., 1999). Poulin and collaborators demonstrated that the activation of mu-opioid receptors in the intercalated nucleus of amygdala modulates the communication between the basolateral and central amygdala to inhibit stress-induced fear response (Poulin et al., 2006). A very recent study has shown that mice with depleted levels of β-endorphin display a stronger aversion to novelty-feeding in response to stress, suggesting that β-endorphin plays a key role in stress coping behavior (Barfield et al., 2010; 2013). It has been reported that morphine (1 and 5 mg/kg) attenuates acute restraint stress-induced anxiety and potentiates adaptation in response to repeated stress exposure in rats (Anand et al., 2012; Joshi et al., 2014).

Studies have described that the genetic differences influence the stress coping behavior that is associated with variation in endogenous β-endorphin system (Amir, 1982; Yamada and Nabeshima, 1995). The response of β-endorphin system to acute stress is heritable and the genetic variations in mu-opioid receptors mainly contribute to differential stress coping response (Schwandt et al., 2011). A recent study examined the anxious behavior of transgenic mice with varying capacities to synthesize β-endorphin in response to stress. The animals with low/no β-endorphin displayed a stronger aversion to novelty-suppressed feeding test following stress exposure. The above findings suggest that stress induces the release of β-endorphin to promote adaptation at the endocrine and behavioral level, and in the absence of β-endorphin, the over-activation of HPA axis may contribute to maladaptive behavior. Therefore, it is proposed that β-endorphins play an important role in stress-coping behavior and that genotypic variability in the β-endorphin system may contribute to the heritable differences in stress reactivity and vulnerability (Barfield et al., 2013).

Stress-induced β-endorphin release may be attributed to increase in CRH secretion that in turn increases the expression of the POMC gene in the anterior
pituitary to produce ACTH and β-endorphin (Charmandarie et al., 2005). In turn, β-endorphin attenuates the stress response, including stress-induced nociception by inhibiting the secretion of CRH through a negative feedback mechanism (Nakagawasai et al., 1999) (Figure 16). Curtis et al described that both CRH and opioids are involved in fine tuning the LC activity during stressful conditions. The activation of opioid afferents tends to restrain the actions of CRH and facilitates the recovery to pre-stress levels (Curtis et al., 2012).

**Figure 17: Role of brain derived β-endorphin (mu receptor agonists) in stress adaptation:** Stress enhances the release of CRH from hypothalamus to increase the expression of the POMC gene in the anterior pituitary that in turn is converted to ACTH and β-endorphin. The latter binds to mu-opioid receptors in the intercalated nucleus of amygdala to decrease fear response and produce stress adaptation. β-endorphin inhibits the CRH release by a negative feedback mechanism, which may also contribute in attenuating stress response.
3.5.2 POST-TRAUMATIC STRESS DISORDER (PTSD)

A growing body of literature addresses the important relationship between morphine and post-traumatic stress (Holbrook et al., 2010; Nixon et al., 2010; Szczytkowski-Thomson et al., 2013). Administration of morphine following a traumatic event reduces the severity of symptoms and the risk of PTSD development (Bryant et al., 2009; Stoddard et al., 2009). Bryant and collaborators described that acute administration of morphine inhibits the development of fear conditioning following a traumatic injury, suggesting that morphine may be employed as a preventive strategy to reduce the development of PTSD (Bryant et al., 2009). Nixon and coworkers documented that morphine reduces the development of PTSD in children experiencing a traumatic event and the reduction in PTSD symptoms is positively correlated with morphine dosage (Nixon et al., 2010). Stoddard and coworkers described a correlation between the morphine usage and PTSD development in young children (1 to 4 years) admitted to pediatric burn center. Treatment with morphine decreased the development of post-traumatic symptoms, assessed 3 to 6 months after the burn event (Stoddard et al., 2009). A clinical study by Holbrook and collaborators described that the use of morphine (2-20 mg) in the US military personnel after a combat injury reduces the risk of PTSD development (Holbrook et al., 2010). A very recent study has also described a lower prevalence rate of PTSD in patients with traumatic brain injury, who received intravenous morphine within hours of injury (Melcer et al., 2014). A recent study documented that the repeated administration of morphine, but not a single dose, inhibits the enhancement of fear learning in PTSD model. The authors proposed that administration of morphine following a traumatic event may obstruct the memory consolidation and associated fear learning that are necessary for the development of PTSD (Szczytkowski-Thomson et al., 2013) (Table 5).
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Intervention</th>
<th>Response</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Morphine</td>
<td>Limits fear conditioning after traumatic injury</td>
<td>Bryant et al., 2009</td>
</tr>
<tr>
<td>2.</td>
<td>Morphine</td>
<td>Reduces the PTSD development in children experiencing a single traumatic event.</td>
<td>Nixon et al., 2010</td>
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<tr>
<td>3.</td>
<td>Morphine</td>
<td>Decreases post-traumatic symptoms assessed 3 to 6 months after the burn event</td>
<td>Stoddard et al., 2009</td>
</tr>
<tr>
<td>4.</td>
<td>Morphine</td>
<td>Reduces the risk of subsequent PTSD development in US military personnel after combat injury</td>
<td>Holbrook et al., 2010</td>
</tr>
<tr>
<td>5.</td>
<td>Repeated administration of morphine (not with single dose)</td>
<td>Inhibits the enhancement in fear learning in another context suggesting that morphine's mechanism of action is related to consolidation processes occurring around that time</td>
<td>Szczytkowski-Thomson et al., 2013</td>
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**Table 5:** Traumatic stress attenuating effects of morphine in experimental and clinical conditions of PTSD.
3.5.3 EFFECT OF DYNORPHIN SYSTEM ON STRESS-RELATED ANXIETY AND DEPRESSION

The multiple active forms of dynorphin including dynorphin A, dynorphin B, and α/β-neo-endorphin are produced from the precursor prodynorphin by an enzyme proprotein convertase. Dynorphin A and B are primarily present in the hypothalamus, medulla, pons, mid-brain, and spinal cord. Dynorphins exert their effects primarily through G-protein-coupled κ-opioid receptors, κ₁ and κ₂. Although, all dynorphin primarily produce their actions by interacting with κ receptors, yet, these peptides also have some affinity for mu- and δ-opioid receptors (DOR) (Valentino and Bockstaele, 2008).

Studies have shown that stress induces dynorphin release, which subsequently activates κ receptors in the central nervous systems to induce anxiety and depression (McLaughlin et al., 2003; Van't Veer and Carlezon 2013). The literature evidences the association between dynorphin and dysphoria; therefore, the role of dynorphin has also been investigated in the development of depression. The repeated exposure of immobilization stress decreases the motivational behavior in animals and correlate with changes in the dynorphin/κ-opioid receptor system in the different brain regions (Lucas et al., 2011). Shirayama and coworkers documented the importance of both dynorphin A and B in stress-induced depression and reported that during “learned helplessness”, the levels of dynorphin A and B are increased in the hippocampus and nucleus accumbens regions. Furthermore, administration of nor BNI (KOR antagonist) was shown to promote recovery from the learned helplessness suggesting that the release of dynorphin is responsible for stress-induced depression (Shirayama et al., 2004). A single (2 h) or repeated exposures (2 h×10 days) of immobilization stress increases the mRNA levels of κ-opioid receptors in the striatal and nucleus accumbens regions of rats. The κ-opioid receptor related transcriptional changes are diminished only after a longer recovery period (about 9 days) and remain unchanged after a shorter recovery period. Consequently, it is proposed that the prolonged inescapable stress alters the motivational system to produce learned helplessness and dysphoria, which is attributed to an increase in dynorphin in the striatal region (Lucas et al., 2011). Other research studies have also documented that the activation of dynorphin/κ-opioid receptor system may be involved in producing prolonged inescapable stress-induced depression-like behavior in rodents (Land et al., 2008).
Chartoff and coworkers documented that the antidepressant effects of desipramine in the swim stress test are accompanied by a decrease in dynorphin expression and cyclic AMP response element-binding protein (CREB) phosphorylation in the nucleus accumbens, and are independent of the norepinephrine or other monoaminergic inputs (Chartoff et al., 2009). Research evidence suggests that stress activates the dynorphin/κ-opioid receptor system to trigger the intracellular signaling involving the activation of ERK/CREB pathway (Bruchas and Chavkin, 2010) (Figure 18). The activation of the κ-opioid receptor system in the LC region is also important in the development of stress-related problems as an increased gene expression of κ-opioid receptors has been documented in this region of Wistar Kyoto rats (a strain particularly useful for studying stress-related behavior) (Pearson et al., 2006). It is also suggested that CRH activates the dynorphin/κ-opioid receptor system in the mouse basolateral amygdala to produce anxiety-like behavior as pretreatment with κ-opioid receptor antagonist (norbinaltorphimine) is reported to block stress/CRH-induced increase in anxiety (Bruchas and Chavkin, 2010). Administration of kappa opioid receptor antagonist 2-(3,4-dichlorophenyl)-N-methyl-N-[(1S)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl] acetamide hydrochloride (DIPPA) produces anxiolytic effects in two different strains of rats, Wistar Kyoto and Sprague Dawley (Carr and Lucki, 2010). Furthermore, administration of norbinaltorphimine (a selective KOR antagonist) prevents stress-induced decline in learning and memory. Dynorphin gene-disrupted mice do not show the learning and memory deficits in response to stress exposure, suggesting that stress-induced activation of kappa opioids receptors is critical to produce deficits in a novel object recognition test (Carey et al., 2009).
3.5.4 ROLE OF ENKEPHALIN IN STRESS AND ASSOCIATED BEHAVIOR

Enkephalin is distributed throughout the limbic system, including the extended amygdala, cingulate cortex, entorhinal cortex, septum, hippocampus, and the hypothalamus (Drolet et al., 2001). The met-enkephalin peptide sequence is coded by the enkephalin gene; while the sequence of leu-enkephalin peptide is coded by both enkephalin and dynorphin genes. The POMC also contains met-enkephalin sequence.
on the N-terminus of β-endorphin, but the endorphin peptide is not processed into enkephalin. The latter demonstrates its action by acting on the delta opioid receptors (Amir, 1982).

The enkephalin system plays an important role in promoting stress adaptation in an organism (Van Loon and Pierzchala, 1990). The decreased functioning of the enkephalin system produces depression-like symptoms and accordingly, an increase in enkephalin signaling is therapeutically explored in the treatment of depression. Chronic immobilization stress (21 days) has been shown to increase the expression of enkephalin and delta opioid receptors in the hippocampus region of rats (Chen et al., 2004). During acute stress exposure, the reduced activity of soluble enkephalinase in the hippocampus and membrane enkephalinase in the amygdala increases the availability and duration of action of enkephalins to produce anxiolytic actions (Hernández et al., 2009; Kung et al., 2010). Furthermore, preproenkcephalin knockout mice are more sensitive to traumatic stimuli, and develop more severe anxiety and depressive symptoms, suggesting the anti-anxiety influence of enkephalin (Lishmanov et al., 2012). In stress vulnerable individuals, the decreased mRNA expression of enkephalin in the posterior basolateral nucleus of amygdala has been documented. Furthermore, the specific knockout of enkephalin genes in this region increases the anxiety-like behavior. Accordingly, the authors suggested that the enkephalin system in the basolateral nucleus of amygdala is involved in producing specific neuro-adaptation and resilience. In another study, the same group of scientists reported the decrease in mRNA expressions of enkephalin in the basolateral nucleus of amygdala and an increase in mRNA levels of dynorphin in the dorsal and media shell of nucleus accumbens in stress vulnerable rats. It suggests that enkephalin facilitates the behavioral adaptation in chronic social stress model by opposing the actions of dynorphin (Bérubé et al., 2013). A very recent study has documented that the down-regulation of enkephalin in the nucleus accumbens region triggers the development of anhedonia in a chronic stress model. The decrease in preproenkcephalin mRNA expression in the nucleus accumbens region is associated with the development of anxiety in restraint stress-subjected rats suggesting that the down-regulation of enkephalin in the nucleus accumbens might underlie the susceptibility to chronic stress (Poulin et al., 2014) (Figure 19).
3.5.5 ROLE OF NOCICEPTIN ON STRESS-RELATED BEHAVIOR

Nociceptin (orphanin FQ), a relatively newly discovered endogenous heptadecapeptide, is a 17-amino acid peptide, and shows structural homology to opioid peptides, particularly to dynorphin A (Reinscheid and Civelli, 2002). Nociceptin/orphanin FQ (N/OFQ)-expressing neurons are present in the hypothalamus and limbic system to regulate the HPA axis and stress response (Witkin et al., 2014). Nociceptin binds to the opioid receptor like-1 receptors (ORL1 receptors), also termed as nociceptin receptors (NOP)/orphanin FQ receptors, and these receptors lack affinity towards the traditional opioid receptors (Southwick et al., 1994).

Studies have demonstrated the key role of nociceptin in regulating the stress-associated behavioral and psychological alterations (Witkin et al., 2014). N/OFQ is essentially an anxiolytic peptide and plays an important role in stress adaptation.
Transgenic mice lacking the OFQ/N precursor proteins exhibit anxiety-like behaviors and do not habituate to repeated exposure to stress (Reinscheid and Civelli, 2002). A number of nociceptin agonists, including N/OFQ and Ro 64-6198 produce anxio-lytic-like effects in the preclinical models of anxiety (Delaney et al., 2012; Goeldner et al., 2012). Goeldner and coworkers reported that the N/OFQ peptide receptor agonists produce dose-dependent anxiolytic effects similar to benzodiazepine receptor agonists (Goeldner et al., 2012). Both acute and chronic restraint stress produces time-dependent changes in the preproN/OFQ transcript expression in the hippocampus, medio-dorsal forebrain and hypothalamus. Furthermore, acute and chronic stress induces differential changes in the ppNN/OFQ expression, with their decreased expression in the central amygdala in response to acute stressor and increased expression in the bed nucleus and reticular thalamus in response to a repeated restraint stressor (Delaney et al., 2012). Injection of N/OFQ in the central nucleus of amygdala is shown to significantly and selectively reduce anxiety-like behavior (in the elevated plus maze test) in restraint rats, suggesting that acute stress activates the N/OFQ system in this brain region to produce anti-stress effects (Witkin et al., 2014). In response to acute restraint stress, the enhanced expression of N/OFQ in the CA1, CA3, and dentate gyrus of the hippocampus and rise in the plasma corticosterone levels has been documented. However, restraint stress failed to increase the N/OFQ expression in adrenalectomized rats, suggesting that the increased expression of N/OFQ may be secondary to an increase in plasma corticosterone in stress-subjected rats (Nativio et al., 2012).

It has been reported that bilateral microinjections of N/OFQ into the rat perifornical area of the lateral hypothalamus abolish stress-induced analgesia and it has been proposed that N/OFQ attenuates immobilization/restraint stress-induced analgesia via inhibition of hypocretin/orexin (Hcrt) neurons in the lateral hypothalamus (Gerashchenko et al., 2011). In response to a single-prolonged stress (an animal model for PTSD), an increased levels of N/OFQ in the cerebrospinal fluid (CSF) have been correlated with the development of anxiety, hyperalgesia and allodynia (Zhang et al., 2012). It has been shown that N/OFQ antagonizes CRH-induced increase in GABAergic neurotransmission in the central amygdala in alcohol-dependent rats. Therefore, it has been hypothesized that nociceptin may produce anxiolytic effects by inhibiting the actions of CRH (Cruz et al., 2012). In contrast,
Nazzaro and others demonstrated that the potent inhibitory effects of N/OFQ on the dorsal raphe nuclei are independent of CRH and GABA (Nazzaro et al., 2009) (Figure 20).

**Figure 20: Adaptogenic and anxiolytic effects of brain-derived nociceptin:**
Stress increases the nociceptin levels directly in the hypothalamus, hippocampus and medio-dorsal forebrain regions to produce anxiolytic and adaptive effects. The nociceptin levels are also increased indirectly through increased release of CRH and corticosterone, which in turn inhibits the CRH release and prevents HPA axis.

### 3. 6 Glycogen synthase kinase-3β (GSK-3β)

Glycogen synthase kinase-3β (GSK-3β) is a widely expressed serine/threonine kinase, and is the rate-limiting enzyme in glycogen synthesis. GSK-3 phosphorylates and regulates number of critical intracellular signaling pathways (Jope and Johnson, 2004). The GSK-3 family consists of 2 isoforms, α and β, which are 98% identical. Both isoforms have unique regulatory signaling mechanism as absence of GSK-3β prevents activation of NF-κB, but absence of GSK-3α does not show such effect. Unlike other protein kinases, GSK-3 is active in un-stimulated cells and is inhibited in response to a variety of stimuli. Furthermore, GSK-3 is unique as it is reported to be inactivated by phosphorylation (Jope and Roh, 2006). GSK-3 activity is significantly
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reduced by phosphorylation of N-terminal serine at 9\textsuperscript{th} position (SER9) in GSK-3\(\beta\) and Ser21 in GSK-3\(\alpha\). GSK-3\(\beta\) is one of the downstream targets of Akt and is shown to regulate many metabolic and signaling proteins that can influence the survival and the expression of a variety of genes associated with cellular metabolism. Akt phosphorylates the GSK-3\(\beta\) at serine-9 to inactivate the GSK-3\(\beta\) kinase and is considered as one of the survival pathway and this inhibition exerts protective effects and increases neuroplasticity (Li et al., 2002; Gould and Manji, 2005).

3.6.1 GSK-3\(\beta\) IN STRESS

The role of Akt/GSK signaling in acute as well chronic stress-induced behavioral and other systemic effects has been described. GSK-3\(\beta\) is an essential downstream effector of Akt and its activity is inhibited by Akt-mediated phosphorylation of GSK at serine 9 (p-GSK3\(\beta\)-S9) (Stoica et al., 2003). Acute restraint stress exposure of 1h significantly impaired the induction of long term potentiation (LTP) along with inhibition of phosphorylation of Akt at Ser47 and GSK-3\(\beta\) at Ser 9 in the hippocampus of mice (Jin et al., 2015). A significant decline in levels of p-GSK-3\(\beta\)-S9 is indirectly proportional to GSK-3\(\beta\) activity (Gould and Manji, 2005). Thus, the observed decline in p-Akt-S47 and p-GSK3\(\beta\)-S9 levels in response to acute stress reveals an increase in GSK-3 activity. Therefore, it may be suggested that acute restraint stress impairs LTP induction possibly by suppressing Akt activity with consequent decreased GSK-3\(\beta\) phosphorylation (decreased p-GSK-3\(\beta\)-S9 levels) and enhanced GSK-3\(\beta\) activity (Jin et al., 2015).

It has been revealed that chronic exposure to immobilization stress of 6h/day for three weeks significantly activated the GSK-3\(\beta\) signaling, involving the increase in GSK-3\(\beta\) activity and decrease in p-GSK-3\(\beta\)-S9 levels in the hippocampus of rats. Moreover, chronic administration of antipsychotic drugs, olanzapine and aripiprazole, significantly attenuated stress-induced decrease in expression of these proteins in the hippocampus of rats, suggesting that antipsychotic drugs exert beneficial effects by upregualting the p-GSK3-S9 levels and decreasing GSK-3\(\beta\) activity (Park et al., 2011). A recent study by the same group of scientists also demonstrated that immobilization stress of same duration decreases the expression of synapse-associated proteins and p-GSK-3\(\beta\)-S9 in the frontal cortex of rats and treatment with atypical antipsychotics ameliorate these stress induced changes by up-regulation of p-GSK-3\(\beta\)-S9 levels (Seo et al., 2015). A recent study has shown that chronic restraint stress
of 12 h daily for 2 days in BALB/c mice produces apoptosis in the macrophages and increases p38 MAPK phosphorylation along with reduction in Akt/GSK-3β phosphorylation. Furthermore, Toll-like receptor-9 (TLR9) deficiency prevented stress-induced apoptotic effects, also prevented the increment of p38 MAPK phosphorylation and reduction of Akt/GSK-3β phosphorylation. Therefore, it is suggested that chronic stress exposure may induce activation of TLR9, which in turn decrease the Akt/GSK-3β signaling, with consequent increase in the GSK-3β activity to induce apoptosis (Xiang et al., 2015).

3.6.2 GSK-3β IN DEPRESSION

Emerging evidence suggests that GSK-3β is a potential target for the treatment of depression (Rowe et al., 2007). Chen et al demonstrated the important role of GSK-3β signaling in the chronic stress-induced depressive like behavior in rats. Acute swim stress exposure involving 2 forced swim sessions produced the depressive behavior with normal expression of p-GSK-3β levels in the mPFC in rats. In contrast, chronic swim stress exposure of 5-min test for 14 days induced the significant behavioral changes (increase immobility and decrease swimming time) along with decrease in phosphorylated GSK-3β levels in the rat's mPFC. Chronic antidepressant treatment with citalopram improved these behavioral changes (decreased immobility and increased swimming time) in chronically stressed rats and normalized the down regulation of p-GSK-3β levels. It suggests that the up regulation of GSK-3β signaling in stress responsive brain regions may play an important role in the chronic, but not acute stress-induced depression, and down-regulation of GSK-3β activity may be involved in antidepressant effects of citalopram (Chen et al., 2012). Earlier study has also described that administration of AR-A014418, a selective GSK-3β inhibitor, reduces immobility time in rats exposed to the forced swim test, suggesting antidepressant-like effects of GSK inhibitors (Gould et al., 2004). Zhang et al described that pre-injection of lentiviral vector expressing GSK-3β into the hippocampal dentate gyrus in chronic mild stress subjected mice significantly decreased sucrose preferences in the sucrose intake test and increased immobility times in both forced swim and tail suspension tests. The overexpression of GSK-3β produced prodepressant-like effects and increased the sensitivity to chronic mild stress. Furthermore, chronic fluoxetine administration attenuated the prodepressant-like effects and decreased neuronal apoptosis in the GSK-3β over-expressing
hippocampal dentate gyrus cells (Zhang et al., 2013). The study implies that overexpression of GSK may increase GSK-3 activity to produce prodepressant-like effects. GSK-3β knock-in mice (overexpression of GSK-3β) exhibit more helplessness behavior than wild type mice, suggesting that the serine-phosphorylation of GSK3 (p-GSK-3-S9) is an important determinant of the susceptibility of mice to acquire depressive-like behavior during stress exposure. Chronic restraint stress exposure of 3h once a day for consecutive 3 days produced significant increase in the immobility time period and reduction in p-Akt and p-GSK-3β-S9 levels in the hypothalamus of stress subjected mice (Kanno et al., 2014). Furthermore, administration of 1,2-dilinoleoylsn-glycero-3-phosphocholine (DL-PC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PO-PC) reduced the stress-induced increase in immobility time period along with inhibition of decline in p-Akt and p-GSK-3β-S9 levels. It may be suggested that chronic stress-induced significant decrease in p-GSK-3β-S9 levels in hypothalamus and consequent increase in GSK-3β activation may lead to depression (Kanno et al., 2014).

3.6.3 GSK-3β IN PTSD

A recent study by Chen et al demonstrated the association between electric foot shock-induced PTSD and GSK-3β signaling. The authors described that sevoflurane attenuates stress-enhanced fear learning by regulating hippocampal Akt/GSK-3β signaling pathway in a rat model of PTSD. Exposure to 15 electric foot shocks followed by a single foot shock in different environment showed an increase in fear learning response along with significant increase in p-Akt, p-GSK-3β and brain-derived neurotrophic factor (BDNF) expressions. Sevoflurane administration during stress exposure attenuated stress-enhanced fear learning as well as increase in p-Akt, p-GSK-3β and BDNF expressions in the PTSD model. In addition, pretreatment with lithium chloride reversed the inhibitory effects of sevoflurane on stress-enhanced fear learning, p-Akt and p-GSK-3β levels. It suggests that sevoflurane administration during the stress exposure may decrease the Akt/GSK-3β signaling and consequently, increase the GSK-3 activity leading to significant impairment in stress-enhanced fear learning during PTSD (Chen et al., 2015).

3.6.4 GSK-3β, ANGIOTENSIN II AND OPIOIDS

Studies have shown that Ang II may trigger GSK-3β dependent signaling to produce different CNS effects (Tian et al., 2012; Hu et al., 2013; Wang et al., 2014).
Tian et al. reported that central administration of angiotensin II significantly elevates the p-tau levels via GSK-3β. Angiotensin II-induced cognitive impairment and tau phosphorylation was attenuated by losartan and GSK-3β inhibitor (SB216763) suggesting that angiotensin signaling may activate GSK-3β kinase signaling transduction pathway to induce hyper-phosphorylation of tau proteins (Tian et al., 2012). In addition, GSK-3β is involved in telmisartan-mediated neuroprotective effects in glutamate-induced neuronal injury in cerebral granule cells (CGC) in rats (Hu et al., 2013; Wang et al., 2014). Incubation of CGC in the presence of glutamate, reduced the Akt phosphorylation and decreased the GSK-3β (Ser 9) phosphorylation, indicating the activation of GSK-3β. Moreover, telmisartan prevented glutamate-induced decrease in Akt and associated GSK-3β phosphorylation, suggesting the involvement of Ang II/AT₁ receptor signaling through Akt/GSK-3β pathway in glutamate excitotoxicity (Hu et al., 2013; Wang et al., 2014). Pang et al. reported the involvement of Akt-GSK-3β and angiotensin in nutrient deprivation-induced apoptosis in CGC. B27 substitute-induced nutrient deprivation significantly increased the apoptosis of CGC along with increase in expression of AT₁ receptors, inhibition of Akt and activation of caspases and GSK-3β. Administration of telmisartan attenuated the neuronal injury against nutrient deprivation-induced apoptosis in CGCs through activation of the survival Akt/GSK-3β pathway (Pang et al., 2014). Thus it may be suggested that angiotensin induces cell injury by decreasing Akt phosphorylation and associated GSK phosphorylation, which in turn activates GSK-3β to induce cell injury.

Numerous studies have also addressed the significant interaction between the GSK-3β and opioids. Li and colleagues reported the increase GSK-3β activity in the opioid-induced hyperalgesia. The authors observed that µ-receptor agonist, remifentanil, infusion caused mechanical and thermal hyperalgesia along with up-regulation of N-methyl-D-aspartate (NMDA) receptors in dorsal horn of spinal cord. In addition, increased GSK-3β activity in spinal cord dorsal horn was also observed. Administration of GSK-3β inhibitor, TDZD-8, significantly attenuated remifentanil-induced hyperalgesia via modulating the expression and function of NMDA receptors. Therefore, it may be suggested that inhibition of GSK-3β can significantly ameliorate opioids-induced hyperalgesia via regulating NMDA receptors (Li et al., 2013). An earlier study has also observed an increase in GSK-3β activity in terms of increased
expression of GSK-3β mRNA and decreased ratio of pGSK-3β/GSK-3β in remifentanil-induced hyperalgesia in rats (Yuan et al., 2012). Another study revealed the potential role of GSK-3β in morphine tolerance and naloxone-precipitated withdrawal syndrome. The repeated morphine exposure (10 mg/kg twice daily for 6 days) was associated with the development of tolerance, characterized in terms of decrease in tail-flick latencies, suggesting the attenuation of morphine response. Following administration of naloxone (1 mg/kg) on day 7, naloxone-precipitated withdrawal behavior was observed. Furthermore, co-administration with GSK-3β inhibitor, SB216763 or SB415286, significantly prevented morphine tolerance and improved withdrawal behavior including grooming, chewing, and ptosis, suggesting the importance of GSK-3β in reducing chronic morphine-induced tolerance and withdrawal syndrome (Liao et al., 2014).
Figure 21: Role of GSK-3β signaling cascade in Alzheimer’s disease, neuronal inflammation and neuronal injury: Akt is activated in response to phosphoinositide-3 kinase (PI-3 kinase) signaling, and subsequent activation of Akt phosphorylates the GSK-3β at Ser 9, leading to GSK-3β inactivation, thereby regulating PI3-kinase/Akt/GSK-3β, a cytoprotective signaling cascade in normal cells. Injurious stimuli inhibit PI-3K and Akt, and induce dephosphorylation of GSK-3β, ensuing GSK-3β activation. Activation of GSK-3β induces hyper-phosphorylation of tau proteins and deficits in memory formation related to Alzheimer’s disease. On the other hand, GSK-3β activation directly increases the inflammatory cytokines NF-κB, tumor necrosis factor-α (TNF-α) and IL-1, and decreases the anti-inflammatory IL-10 cytokines to induce neuronal inflammation. Activation of GSK-3β also increases the actions of caspases-3 to induce apoptosis and subsequent neuronal injury.
3.7 Nuclear factor-kappa B

NF-kB is a stress-regulated key transcription factor belonging to the Rel family and plays an important role in inflammatory, innate immune responses, cell cycle and cell survival. The five different members compose the NF-κB family, including p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). The activated NF-κB subunits collectively form the homo-or hetero-dimerized transcription factor complexes. The most widely studied form of NF-κB is a heterodimer of the p50 and p65 subunits and is a potent activator of gene transcription (Schmitz and Baeuerle, 1995). Importantly, c-Rel expression in the CNS plays a critical role in anti-apoptosis and reduces the age-related behaviors. NF-kB exists in the cytoplasm in an inactive form by virtue of its association with a class of inhibitory proteins called IκBs. In most of the cells, IκBα is phosphorylated and proteolytically degraded, which leads to activation of NF-κB (Mercurio and Manning, 1999; Piva et al., 2006). NF-κB transcription factors are abundant in the brain and exhibit different functions. Exposure to stress has been documented to trigger the signaling cascade involving the activation and potentiation of NF-kB in different stress sensitive brain regions, including the frontal cortex, hippocampus, amygdala and hypothalamus (Madrigal et al., 2001). Following activation, free NF-kB dimer translocates directly into the nucleus, bind to the promoter regions of target genes, and induce transcription.

3.7.1 NF-kB IN ANXIETY AND DEPRESSION

A number of studies have demonstrated the involvement of NF-kB in the development of anxiety and depression (Manchanda et al., 2011; Shao et al., 2015; Pesarico et al., 2016). Sharma et al described that acute immobilization stress (6 h) increases the NF-κB expression and administration of NF-κB inhibitor was found to produce anti-anxiety like activity in immobilized mice, suggesting the key role of NF-kB in the anxiety (Sharma et al., 2011). A study by our laboratory also demonstrated the anti-stress effects of NF-kB inhibitor in restraint stress-induced behavioral changes (Manchanda et al., 2011). In addition, Shao et al reported that the chronic unpredictable stress-induced depressive like behavior in mice was associated with an increase in NF-κB protein expression in the hippocampus. Furthermore, the acupuncture intervention and fluoxetine administration independently decreased the depressive behavior assessed in terms of decrease in NF-κB protein expression in the
hippocampus. It suggests that stress initiates signaling cascade by involving NF-kB in the hippocampus to produce depression like behavior (Shao et al., 2015).

Another study has reported that antidepressant, icariin, attenuates unpredictable chronic mild stress-induced depression like behavior by inhibiting stress-mediated NF-κB signaling cascade (Liu et al., 2015). A recent study has also documented an increase in NF-kB expression in the prefrontal cortex and corticosterone and ACTH in the serum in the chronic unpredictable mild stress-induced depression in mice. Further, administration of 7-fluoro-1,3-diphenylisoquinoline-1-amine (FDPI), a novel isoquinoline derivative, significantly attenuated depressive behavior along with significant decrease in the levels of NF-kB, corticosterone and ACTH in prefrontal cortex and serum. It further suggests the involvement of NF-kB signaling cascade in stress-induced depressive behavior (Pesarico et al., 2016).

3.7.2 NF-kB IN PTSD

The significant involvement of NF-kB mediated signaling cascade has also been observed in PTSD. The increased hippocampal expression of NF-κB p50 and p65 subunits was observed in stress-induced PTSD-like behavioral patterns in rats. It was observed that extreme behavioral responders have increased hippocampal expression of NF-κB p50 and p65 along with increase in I-κBα, p38, and phospho-p38 levels in hippocampal structures as compared to minimal behavioral responders. Furthermore, immediate post-exposure treatment with NF-kB inhibitor significantly attenuated rate of extreme responders and normalized the expression of altered genes. It suggests that stress-induced upregulation of NF-κB complex in the hippocampus significantly contributes to stress-induced altered behavioral processes (Cohen et al., 2011). Moreover, it was also reported that post-traumatic anxiety-associated inflammation was associated with the failure of innate immune receptor TLR9 (toll-like receptors) to escape the pro-inflammatory NF-κB pathway suggesting the involvement of NF-κB-mediated inflammatory reactions in the post-traumatic phenotype (Zimmerman et al., 2012).

3.7.3 NF-kB, ANG II AND OPIOIDS

Several studies have demonstrated that NF-kB activation plays an important role in Ang II-mediated signaling cascade (Wolf et al., 2002; Agarwal et al., 2013; Rodriguez-Perez et al., 2015). Neuronal Ang II exposure has been shown to increase
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p-NF-κB (Ser-276) levels and reduce phosphorylated CREB (Ser-133), leading to reduced CREB-CBP and increased NFκB-CBP binding. These results were accompanied by increased activation of GSK-3β, suggesting that Ang II-induced dysregulation is mediated by GSK-3β-induced alteration in NFκB and CREB in neuronal cells (Agarwal et al., 2013). Furthermore, the reduction of NF-kB activation in the presence of NADPH inhibitor suggests that NADPH-induced ROS generation activates NF-kB to activate RhoA/ROCK pathway (Rodriguez-Perez et al., 2015). Moreover, a number of studies have demonstrated that Ang II activates NF-kB both in vivo and in vitro after binding to the AT1 receptors (Wolf et al., 2002). It has also been reported that Ang II increases the expression of proinflammatory cytokines through activation of NF-kB in the vascular smooth muscle cells (VSMCs) (Zhang et al., 2005). Agarwal et al demonstrated that Ang II induces the imbalance between proinflammatory cytokines and anti-inflammatory cytokines by modulating downstream transcription factors, NF-κB and CREB in the brain. A study by Rodriguez-Perez et al described that NF-kB activation is also required for Ang II-induced up-regulation of ROCK activity and the latter was abolished in the presence of the NF-kB inhibitor.

Furthermore, a large number of studies have also addressed the significant interaction between the NF-kB and opioids. It was also observed that fentanyl, µ opioid receptor agonist, inhibits the colorectal cancer cell growth along with suppression of NF-κB and increase in expression of Sirt1, suggesting that fentanyl initiates carcinoma cell apoptosis by inhibition of NF-κB activation in a Sirt1-dependent manner (Zhang et al., 2014). Another study reported that activation of µ opioid receptors modulates inflammation in acute experimental colitis. Administration of µ opioid receptors agonist (DAMGO) significantly reduced acute phase of dextran sodium sulfate (DSS)-induced colitis in mice by suppression of NF-kB, cytokines, caspases, and upregulation of Bcl-xL (Anselmi et al., 2015). Furthermore, the potential role of opioid receptors in modulating the TLR induced NF-kB signaling was also reported. Increased expression of myocardial TLR4 and NF-κB was observed in the ischemia reperfusion injury. However, the κ-opioid receptor stimulation with U50, 488H significantly attenuated the expressions of TLR4 and NF-κB along with reduction of myeloperoxidase (MPO) levels, myocardial TNF-α production. In addition, selective κ-opioid receptor antagonist, Nor-BNI, attenuated the protective effects of U50, 488H in ischemia reperfusion injury, suggesting that κ-
opioid receptor stimulation attenuate myocardial ischemia reperfusion injury via down regulating the TLR4/NF-κB signaling in rats (Cai et al., 2014). Another study observed the down regulation of mu and delta opioid receptor mRNAs following chronic constriction injury of sciatic nerve, which was significantly attenuated following administration of NF-κB and MEK1/2 inhibitor. Thus it may be suggested that both inhibitors potentiate morphine analgesia, which was associated with the up-regulation of both mu and delta opioid receptor mRNAs expression in the spinal levels of the model of neuropathy (Popiolek-Barczyk et al., 2014).