BEHAVIORAL AND NEUROCHEMICAL ASSESSMENT OF PARATHYROID HORMONE 2 (PTH2) RECEPTOR AGONIST TUBEROINFUNDIBULAR PEPTIDE OF 39 (TIP39) IN NEUROLOGICAL DISORDERS

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By

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DECLARATION

I hereby certify that I am the sole author of this thesis entitled **"BEHAVIORAL AND NEUROCHEMICAL ASSESSMENT OF PARATHYROID HORMONE 2 (PTH2) RECEPTOR AGONIST TUBEROINFUNDIBULAR PEPTIDE OF 39 (TIP39) IN NEUROLOGICAL DISORDERS "** and that neither any part of this thesis nor the whole of the thesis has been submitted for a degree to any other University or Institution. I certify that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or, are fully acknowledged in accordance with the standard referencing practices. I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis review committee.

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G.Venkatesh

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ABBREVIATIONS

PTSD : Posttraumatic stress disorder

HPA : Hypothalamic pituitary adrenocortical axis

CRF : Corticotrophin releasing factor

PVN : Para ventricular nucleus

ACTH :Adrenocorticotropin hormone

WHO : World Health Organization

CVS : Cardiovascular system

NE : Norepinephrine

5-HT : 5-Hydroxytryptamine

DA : Dopamine

MDD : Depressive disorder;

GABA: Gamma-aminobutyric acid;

Glu : Glutamate;

CSF : Cerebrospinal fluid

GFAP : Glial fibrillary acidic protein

PFC : Prefrontal cortex

GCs : Glucocorticoid

ROS : Reactive oxygen species

AD : Alzheimer's disease

LTP : long term potentiation

PTH2R: Parathyroid hormone 2 receptor

PTH1R: Parathyroid hormone 1 receptor

ABBREVIATIONS

TIP39:Tuberoinfundibular peptide of 39

cAMP: Cyclic adenosine monophosphate

TH : Tyrosine hydroxylase

TCA : Tricarboxylic acid cycle

GAD : Glutamate decarboxylases

GABA-T :GABA transaminase

BDNF: Brain derived neurotrophic factor

CUMS: Chronic unpredictable mild stress

FST : Forced swim test

APA : American Psychiatric Association

BHT :Butylated hydroxyl toluene

BHA : Butylated hydroxyl anisole

SOD : Superoxide dismutase

GPx : Glutathione peroxidise

CAT : Catalase

TBARS: Thiobarbituric acid-reactive substances

HIV : Human immunodeficiency virus

TNF-α : Tumor necrosis factor-alpha

IL-6 : Interleukin-6

IL1β : Interleukin-1β

ATP : Adenosine Triphosphate

mRNA: Messenger RNA

IR- Immunoreactive

ABBREVIATIONS

TIP39-KO: TIP39 knockout

PTH2R-KO: PTH2R knockout

CeA : Central amygdale

MeA : Medial amygdale

PTH : Parathyroid hormone

SIA : Stress induced analgesia

GH : Growth hormone

WT : Wild type

MnPO : Median Preoptic nucleus;

DRG : Dorsal root ganglion

NO : Nitric oxide

INTRODUCTION

Stress is a nonspecific and adaptive response caused by physical, psychological and social environmental factors. Many clinical disorders can either be induced, or aggravated by stress. The extreme levels of stress cause major damage to health. The epidemiological status of stress disorder demonstrated higher degree of prevalence in high income countries and socio-demographic correlates [1, 2]. Recent epidemiological studies from around the world have included low and middle income countries providing novel evidence on the distribution of trauma and posttraumatic stress disorder (PTSD) [3, 4]. Although stress is a necessary mechanism for survival, severe and/or long term stress disrupts normal brain structure and function. It has been reported that stress can cause depression and cognitive impairment possibly by elevating excitatory amino acid, glucocorticoid and oxidative damage, which in turn induce excitotoxicity and hippocampal atrophy [5,6].

Pathophysiology of stress

The neuroendocrine and emotional component of the stress reaction involves activation of limbic and hypothalamic brain structures. The body's principal physiological responses to stressful stimuli are mediated by the sympathoadrenal system and the hypothalamic pituitary adrenocortical (HPA) axis, which are in turn, mediated by the hippocampus [7]. Stress stimulates the release of corticotrophin releasing factor (CRF), from the hypothalamic paraventricular nucleus (PVN), into the hypophysialportal circulation, where it induces the release of adrenocorticotropin hormone (ACTH) from

the anterior pituitary and glucocorticoids (cortisol in humans; corticosterone in rodents) from the adrenal glands [8].

The magnitude of the HPA axis stress response elicited by PVN neurons is limited by neuronal and hormonal mechanisms, which work together to maintain glucocorticoid levels within tolerable limits [9]. Three feedback loops prevent overshooting of the HPA axis: (a) the negative feedback of glucocorticoids to the anterior pituitary, hypothalamus, and hippocampus, (b) the ACTH feedback to the hypothalamus, and (c) the direct feedback of CRF to the hypothalamus. While oxytocin may also have a role in the mediation of the stress response [10].

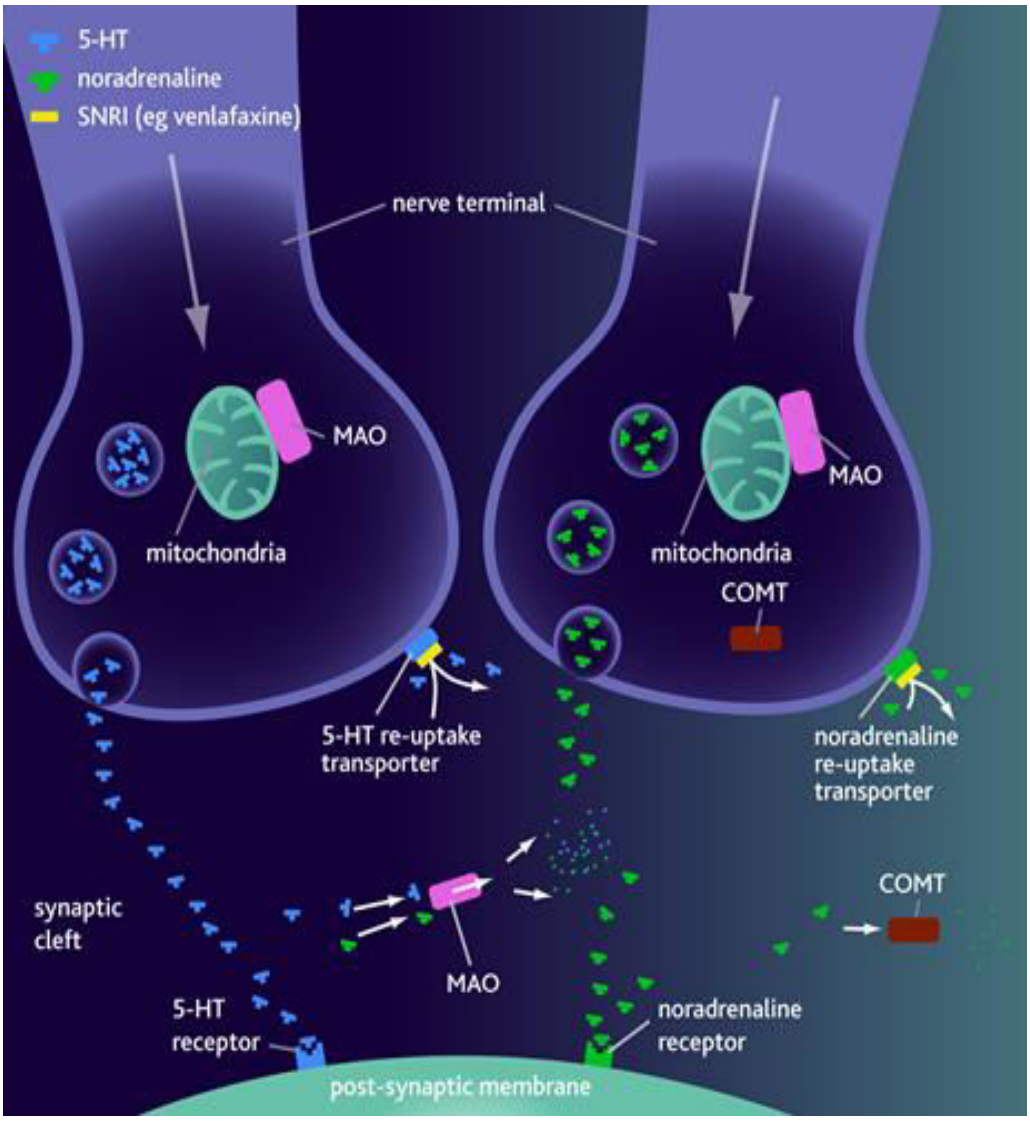
Stress induced Depression

According to a report from World Health Organization (WHO), approximately 300 million people all over the world suffer from depression. This could be predicted to rise by 15% by the year 2020 [11]. The forthcoming scenario shows, depression will be the second most extensive disease around the world, which is aggravated by psychological, physiological, or environmental stress that disturbs the quality of life [2, 3]. Depression is not a homogeneous disorder, but a complex phenomenon, which has many subtypes and probably more than one etiology. It can occur at any age from childhood to late life and is a tremendous cost to society as this disorder causes severe distress and disruption of life and, if left untreated, can be fatal. The psychopathological state involves chain of symptoms with low or depressed mood, anhedonia, and low energy or fatigue. Other symptoms, such as sleep and psychomotor disturbances, feelings

of guilt, low self esteem, suicidal tendencies as well as autonomic and gastrointestinal disturbances are also often present [12].

Several lines of evidence indicated an important relationship between depression and cardiovascular system (CVS) disorders, together with increased mortality rates. Some studies have demonstrated that depression increases the risk of developing cardiac disease, in particular coronary artery disease, and worsens the prognosis after myocardial infarction. Depression also appears to increase the risk for cardiac mortality independently; moreover, the excess mortality risk for major depression was more than twice that for minor depression [13]. Another very important aspect of depression is the high rate of co-morbidity with other psychiatric disturbances. Anxiety, especially panic disorder is often associated with affective disorders, while the magnitude of the association with alcohol or drug abuse is less manifest. Interestingly, the onset of anxiety generally precedes that of depression [14, 15].

Figure 1: Monoaminergic pathway



Major hypothesis of depression was formulated and proposed that the main symptoms of depression are due to a functional deficiency of the brain monoaminergic transmitter's norepinephrine (NE), 5-Hydroxytryptamine (5-HT), and dopamine (DA) at critical synapses in the brain [16]. Most of the serotonergic, noradrenergic and

dopaminergic neurons are located in midbrain and brainstem nuclei and project to large areas of the entire brain. This anatomy suggests that monoaminergic systems are involved in the regulation of a broad range of brain functions, including mood, attention, reward processing, sleep, appetite and cognition [17]. Inhibiting the enzyme monoamine oxidase, which induces an increased availability of monoamines in presynaptic neurons, also has antidepressant effects. These observations led to the pharmacologically most relevant theory of depression, referred to as the monoamine deficiency hypothesis.

Serotonin is the most extensively studied neurotransmitter in depression. The most direct evidence for an abnormally reduced function of central serotonergic system, such reduction leads to the development of depressive symptoms in subjects at increased risk of depression, possibly mediated by increased brain metabolism in the ventromedial prefrontal cortex and subcortical brain regions [18]. There was also evidence for abnormalities of serotonin receptors in depression, with the most solid evidence pointing to the serotonin-1A receptor, which regulates serotonin function.

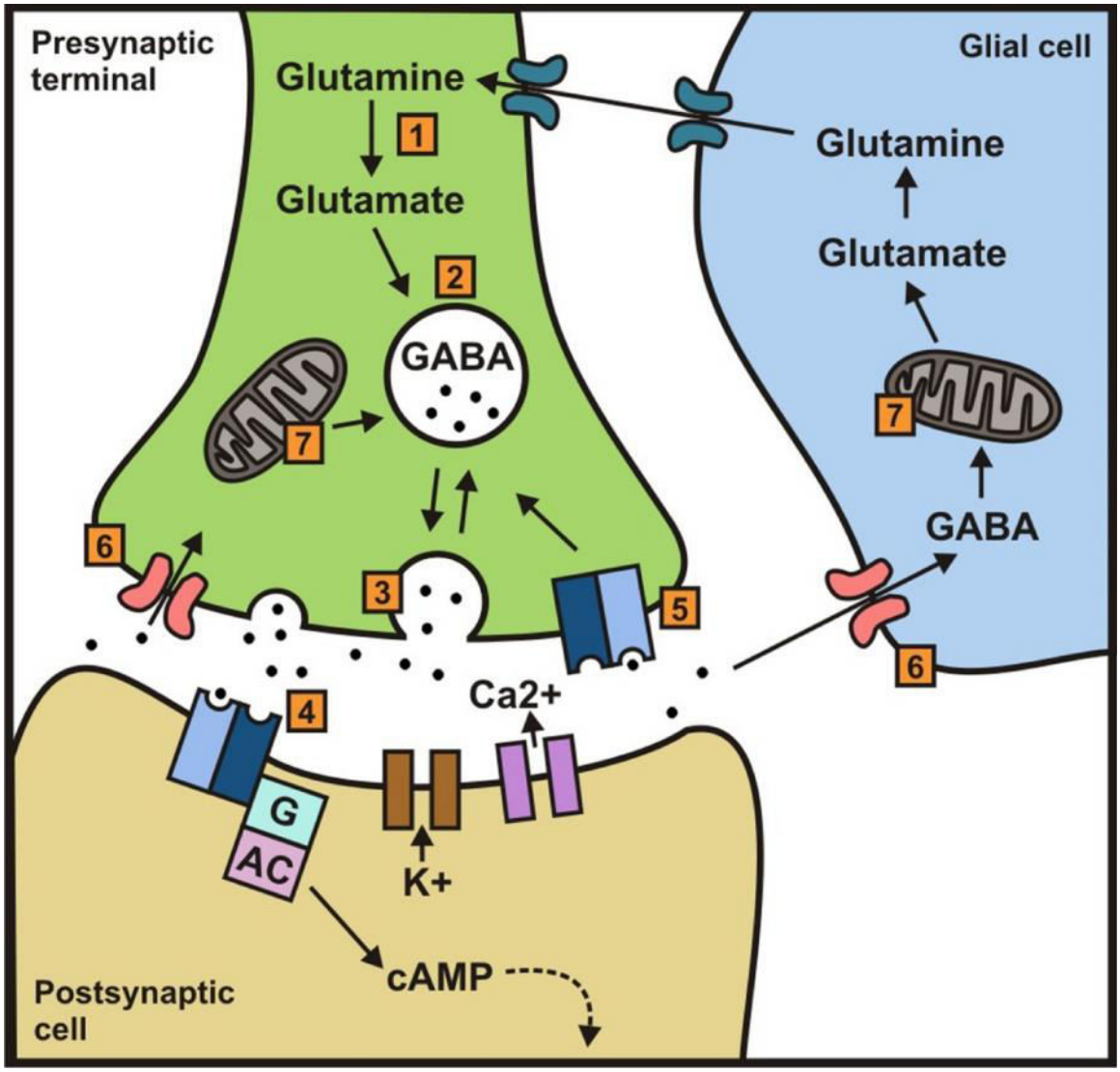
Dysfunction of the central noradrenergic system has been hypothesized to play a role in the pathophysiology of major depressive disorder (MDD), based on decreased norepinephrine metabolism, increased activity of tyrosine hydroxylase and decreased density of norepinephrine transporter in the locus coeruleus in depressed patients. In addition, decreased neuronal counts in the locus coeruleus, increased alpha-2 adrenergic receptor density, and decreased alpha-1 adrenergic receptor density have been found in the brains of depressed patients. [19]

Classical theories of the neurobiology of depression mainly focused on serotonin and norepinephrine, there is increasing interest in the role of dopamine [20]. In the cerebrospinal fluid and plasma levels of dopamine metabolites were consistently reduced in depression, suggesting decreased dopamine turnover [21]. Striatal dopamine transporter binding and dopamine uptake were reduced in MDD, consistent with a reduction in dopamine neurotransmission [22]. Degeneration of dopamine projections to the striatum in Parkinson's disease was associated with a major depressive syndrome [23]. Experimentally reduced dopaminergic transmission into the accumbens has been associated with anhedonic symptoms and performance deficits on a reward processing task in subjects at increased risk of depression [24].

Glutamate, the major excitatory neurotransmitter in the mammalian brain, is in a balance with gamma-aminobutyric acid (GABA), which is the main inhibitory amino acid neurotransmitter in the brain [25]. It can be suggested that dysregulation of Glu/GABA is involved in the pathogenesis of depression and anxiety [26]. An increased level of Glu was found in the brains and cerebrospinal fluid (CSF) of depressed patients as well as in their serum and plasma [27, 28]. Glial cells, especially astrocytes play a crucial role in the maintenance of Glu/GABA balance [29]. These cells are a critical structural and functional part of the tripartite synapses, in which they play a direct and interactive role with neurons in synaptic transmission [30]. A number of evidences have shown that the dysfunction of astrocytes may be involved in the pathogenesis of depression and anxiety. Postmortem studies performed on brains of depressed patients demonstrated that a decrease in the density of glial cells in cortical regions, especially in the prefrontal and

cingular areas [31] and in the hippocampus. These decreases were associated with a reduced level of astrocytic markers, such as Glial Fibrillary Acidic Protein (GFAP) and glutamine synthetase. It is interesting that a reduction in the number of astrocytes in the prefrontal cortex (PFC) was also found in rats exposed to chronic unpredictable stress induced depressive condition [32].

Figure 2: Glutamate and GABA transmission mechanism



There is consistent evidence that the volume loss of the hippocampus and other brain regions is related to the duration of depression [33] suggesting that untreated depression leads to hippocampal volume loss, possibly resulting in increased stress sensitivity [34] and increased risk of recurrence [35]. Glucocorticoid neurotoxicity, glutamatergic toxicity, decreased neurotrophic factors, and decreased neurogenesis have been proposed as possible mechanisms explaining brain volume loss in depression.

Stress induced memory impairment

The impact of chronic stress on cognitive performance is depends on varies factors such as biological, chronobiological factors and others [36]. Chronic social stress has been proposed to be a major cause for cognitive deficits often associated with depression. In many cases, in addition to emotion related symptoms, depressed patients also suffer from cognitive problems including memory loss, attentional deficit, executive dysfunction and poor decision making [37]. Stress induced clinical conditions causes sleeplessness and suicidal tendencies, decreased food intake and body weight loss [38], and more importantly suffer from cognitive functional impairments, such as delayed thinking correlation, reduced computational capability, learning and memory impairment, and reduction in attention, comprehension and judgment [39]. Learning and memory impairment is one of the important residual symptoms, which has a strong impact on function of patient's life. On the other hand, it is becoming increasingly clear that the disturbance of cognitive processes, especially the impairment of learning and memory, plays an important role in the development and complete recurrence of depression [40, 41].

Stress exposure is associated with an activation of HPA axis. Repeated stress exposure is known to lead to cause an excessive HPA axis activation, resulting in an overproduction of glucocorticoids (GCs) [42]. As a consequence, neurochemical and neuroanatomical alterations in several brain regions, including the hippocampus, prefrontal cortex, amygdala, dorsal striatum and brain stem [43]. As result of GCs overproduction, neuronal atrophy as well as decreased neurogenesis have been observed in the dentate gyrus of the hippocampal formation that impairs memory processing [44,45].

There was evidence that the majority of stress induced complications result from overproduction of reactive oxygen species (ROS), including superoxide anion (O_2^-), which induce oxidative stress and reduce antioxidant capacity. Free radicals trigger biologic mechanisms that cause memory impairment in animal models [46]. Also, evidence has shown that inhibition of hippocampal oxidative stress can exert learning and memory impairment in animal models [47]. The reduction of brain oxidative stress has been shown to induce significant improvements in the recognition memory indexes in models [48, 49].

Several studies have demonstrated the detrimental effects of chronic stress on cognitive function, and many studies have found that a higher vulnerability to chronic stress is related to brain aging [50-52]. However, the neurological mechanisms underlying this effect are not well known. The term synaptic plasticity is used to describe several types of activity-dependent alterations in synaptic strength. Synaptic plasticity has long been recognized as the neurobiological basis of cognition. Moreover, the structural and

functional changes of hippocampal synapses lead to cognitive alterations [53]. The hippocampus, which is one of the most important brain regions that are associated with cognition, is also known as the cerebral region that is largely vulnerable to internal and external alterations, including those related to aging, stress, and neurodegenerative diseases [54].

Many experiments have established that chronic stress is a risk factor for the development of cognitive deficits, especially for Alzheimer's disease (AD). Chronic stress not only impaired the early long term potentiation (LTP) in AD model, but it also decreased hippocampal synaptic plasticity and increased amyloid-β plaque deposition [55-57].

Parathyroid hormone 2 receptor (PTH2R) and Tuberoinfundibular peptide 39(TIP39)

The parathyroid hormone 2 receptor (PTH2R) was identified based on its sequence homology to other polypeptide receptors [58]. It is a seven transmembrane domain receptor, which belongs to Type II (or family B) class of G protein-coupled receptors [59, 60]. It has about 50% amino acid sequence similarity with the parathyroid hormone 1 receptor (PTH1R). PTH2R distribution has been identified in the central nervous system and in a number of peripheral organs [60]. PTH2 receptor expressing cells are widely distributed in the subcortical areas and dense PTH2 receptor expression was found in limbic structures, the hypothalamus, certain areas of the thalamus, brainstem and even in the spinal cord [61]. Generally, hypothalamus is particularly rich in neuropeptides and their receptors. Based on its distribution in the brain it may play a role

in regulation of neuroendocrine, emotion, auditory, and pain related processes. Its peripheral distribution suggests involvement in endocrine, cardiovascular and reproductive function [62]. Parathyroid hormone potently activates the human PTH2R in vitro stating that PTH2R is abundantly expressed and widely distributed in rat brain, whereas parathyroid hormone has been reported at very low levels in the brain [63]. In addition, parathyroid hormone binds the rat PTH2R with low affinity act as a partial agonist for PTH2 receptor [64].

Tuberoinfundibular peptide of 39 (TIP39) peptide is a potent neuroendocrine agonist to PTH2R in brain and peripheral region. TIP39 is synthesized by two groups of neurons, one in the subparafascicular area at the caudal end of the thalamus and the other in the medial paralemniscal nucleus within the lateral brainstem. TIP39 neurons are restricted to two brain regions the subparafascicular posterior intralaminar thalamic area, which extends caudolaterally from the periventricular gray of the thalamus to the medial geniculate body, and the medial paralemniscal nucleus at the midbrain-pons junction [65]. In contrast to the restricted distribution of TIP39 cell bodies, amplification immunocytochemistry demonstrated a wide spread distribution of TIP39 fibres in limbic, endocrine, and auditory brain regions [63]. It was purified from bovine hypothalamus on the basis of its stimulation of cAMP formation in a PTH2R expressing cell line. Mouse, rat and human TIP39 were subsequently cloned. TIP39 is a potent agonist and binds to both the rat and the human PTH2Rs with high affinity but it has low affinity and minor agonist at the PTH1R [66-69].

TIP39 containing cell bodies are present only in the posterior intralaminar complex of the thalamus and the medial paralemniscal nucleus of the lateral pons. TIP39 fibers originating in these nuclei reach a number of endocrine, limbic and auditory regions in the brain [70]. TIP39 distribution has been identified, both in the central nervous system and in a number of peripheral organs [71-73]. Based on its distribution in the brain it may play a role in regulation of neuroendocrine, emotion, auditory, and pain related processes. Its peripheral distribution suggests involvement in endocrine, cardiovascular and reproductive function [74]

TIP39 peptide is acting through PTH2R that potentially regulates HPA axis system. TIP39 induced the *fos* gene expression in the infralimbic cortex, preoptic area, lateral hypothalamus, lateral septum, and paraventricular thalamic nucleus; areas believed to be imperative in anxiety and depression [75]. Mice lacking TIP39 or PTH2R signalling significantly displayed increased anxiety, depression, also exhibited fear memory after exposure to an aversive event [76]. Moreover, following TIP39 administration, long term consequence of traumatic event was reduced by controlling amygdale in cerebral region which is known to involve in fear conditioning. In addition to that, TIP39 peptide showed guarding response in the neuropathic and inflammatory pain response by regulating through the inhibition of release of hypoalgesic amount of norepinephrine [77, 78].

Despite the last 60 years of rigorous research in this line, no concrete therapies are available in current practice for the stress induced anxiety, depression and cognitive impairment. Hence, we hypothesize that TIP39 may link the neurological and

endocrinological pathway in stress induced anxiety, depression and cognitive deficit. With the hold of optimistic regulation of TIP39, this study intends to evaluate the neurotransmitter role in the regulation of oxidative changes, proinflammatory cytokines and gene expressions in stressed rats.

CHAPTER 2: AIM & OBJECTIVES

Aim

- To study the behavioural and neurochemical role of PTH2 receptor agonist TIP39 peptide on depression, anxiety and memory impairment model in rats

Objectives

- To study the physiological role of TIP39 peptide in relation with behavioural, neurochemical and morphological changes in rats
- To study the role of TIP39 peptide on acute restraint stress induced depression and anxiety in rats by using PTH2 receptor antagonist
- To study the role TIP39 peptide on chronic unpredictable mild stress induced depression and anxiety in rats
- To study the role TIP39 peptide on chronic unpredictable mild stress induced learning and memory impairments in rat model

Literature Review

Stress and Hypothalamic Pituitary Adrenal (HPA) axis

Stress causes major damage to the neuroendocrine and emotional component that regulates stress reaction involves activation of limbic and hypothalamic structures. Chronic uncontrolled stress increases the allostatic load of an organism and impairs homeostasis that causes abnormal activity of the systems in the body [79]. Chronic stress may suppress the immune system and increases the production of proinflammatory cytokine IL-6. In animals, the chronic psychosocial stress may induce neuroinflammation and apoptosis and reduced neurogenesis. Also, it has an influence on neuroendocrine responses in men [80].

The HPA axis serves as a neuroendocrine stress response system get stimulated by the response to varies stressor; resulted in the release of glucocorticoids from the adrenal cortex that would minimize the long-term activation of HPA axis through a negative feedback pathway [81]. At the central level, HPA axis is regulated by the paraventricular nucleus (PVN) that produce corticotrophin-releasing factor (CRF), arginine vasopressin (AVP) peptides and oxytocin. CRF and AVP intern release adrenocorticotrophic hormone (ACTH) from the anterior pituitary, while oxytocin may have a role in the mediation of the stress response [82]. The HPA axis responds to stress by secreting the corticotrophin-releasing hormone (CRH) from the hypothalamus which then instigates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary into the bloodstream, which in turn causes the synthesis and release of cortisol from the adrenal cortex [83]. Chronic stress can, therefore, lead to elevated levels of cortisol, which also contributes to an elevated risk of stress-related disorders [84]. Cui Y et al., (2017) described several pathways between

elevated cortisol and alteration of prefrontal cortex function, amygdala and hypothalamus hyperactivity and reductions in volume and function of the hippocampus [85].

In addition, CRF plays a neurotransmitter or neuromodulatory role through neurons and receptors distributed in diverse brain regions. CRF neurons, localized in the hypothalamic periventricular nucleus, are a major mediator of stress-induced activation of the HPA axis, whereas pathways innervating limbic and cortical areas are thought to mediate the behavioral effects of CRF [86].

Stress-induced depression

Many clinical reports suggest that prolonged exposure to stressful episodes, as a common risk factor could provoke the development of major depression. Depression is a mental disorder characterized by a pervasive low mood and loss of pleasure or interest in usual activities [87] and is the most common psychiatric illness that involves the disturbance of mood, with 10 to 20% lifetime prevalence [88]. The major depressive disorder affecting more than 120 million people worldwide. The patients also demonstrate sleeplessness and suicidal tendencies, decreased food intake and body weight [89] and more importantly always suffer from obvious cognitive function impairments, such as delayed thinking correlation, reduced computational capability, learning and memory impairment, and reduction in attention, comprehension, and judgment [90]. External stress factors such as stressful life events and internal stress factors such as chronic inflammation may induce inflammatory, oxidative, and nitrosative stress pathways to precipitate depression in susceptible individuals

Monoaminergic theories of Depression

Most of the monoaminergic neurons are located in midbrain and brainstem nuclei and project to large areas of the brain. This anatomy suggests that monoaminergic systems are involved in the regulation of depression. The monoamine deficiency theory hypothesize that the underlying pathophysiological basis of depression is a depletion of the neurotransmitters serotonin, norepinephrine or dopamine in the central nervous system [91]

Serotonin is the most extensively studied neurotransmitter in depression. The most direct evidence for an abnormally reduced function of the central serotonergic system comes from studies using tryptophan depletion, which reduces central serotonin synthesis. Such a reduction leads to the development of depressive symptoms in subjects at increased risk of depression [92], possibly mediated by increased brain metabolism in the ventromedial prefrontal cortex and subcortical brain regions. Evidence for abnormalities of serotonin receptors in depression, with the most solid evidence pointing to the serotonin-1A receptor, which regulates serotonin function. Decreased availability of this receptor has been found in multiple brain areas of patients with MDD [43]. There is preliminary evidence that an increased availability of the brain monoamine oxidase, which metabolizes serotonin may cause serotonin deficiency [93, 94].

Dysfunction of the central noradrenergic system has been hypothesized to play a role in the pathophysiology of MDD, based upon evidence of decreased norepinephrine metabolism, increased activity of tyrosine hydroxylase, and decreased the density of norepinephrine transporter in the locus coeruleus in depressed patients. In addition, decreased neuronal counts in the locus coeruleus, increased alpha-2 adrenergic receptor

density, and decreased alpha-1 adrenergic receptor density have been found in the brains of depressed patients [95].

Mesocortical and mesolimbic dopaminergic systems are known to mediate HPA axis induced glucocorticoid release and other CNS effects. The interplay between corticosterone and the dopaminergic system is linked with various neurological disorders. The number of reports suggested the involvement of glucocorticoids on dopamine-mediated behavioral responsiveness by modulatory effects of corticosterone. Expression of tyrosine hydroxylase (TH); a rate-limiting enzyme of DA biosynthesis is associated with the levels of glucocorticoid [96].

Glutaminergic and GABAergic theory of depression

Glutamate is the major excitatory neurotransmitter is in balance with GABA, which is the main inhibitory neurotransmitter in the brain. It can be suggested that dysregulation of Glu/GABA is involved in the pathogenesis of depression [97]. Astrocytes are a type of g lial cells play a crucial role in the maintenance of Glu/GABA balance. These cells are involved in the direct and interactive role with neurons in synaptic transmission [98]. Studies have shown that the dysfunction of astrocytes may be involved in the pathogenesis of depression and found that there was a decrease in the density of glial cells in cortical regions, especially in the prefrontal and cingular areas and in the hippocampus [99]. These decreases were associated with a reduced level of astrocytic markers, such as glial fibrillary acidic protein and glutamine synthetase. An increased level of Glu was found in the brain and cerebrospinal fluid (CSF) as well as in serum and plasma of depressed patients. [100].

GABA and glutamate are tightly linked intermediary products of energetic metabolism, because they are involved in the same metabolic pathway, the glutamate/GABA-glutamine

cycle [101]. In neurons, glutamate is produced from the tricarboxylic acid (TCA) cycle and from the glutamine released by astrocytes, whereas GABA originates exclusively from glutamate from two glutamate decarboxylases (GAD), GAD-65 and GAD-67 [102]. Cytosolic GABA is catabolized into glutamate by GABA transaminase (GABA-T). Parts of the glutamate and GABA cell pools are released by neurons as neurotransmitters, representing respectively the major excitatory and inhibitory neurotransmitters. Both compounds are taken up by the astrocytes and catabolized through the TCA cycle. The GABA/glutamate tissue ratio may, therefore, reflect the dynamic equilibrium between GABA and glutamate in the context of cell metabolism. This balance is physiologically important since variations in GABA content via enzyme inhibitions are followed by changes in synaptic function [103]. GABA neurotransmission has been suggested to intervene in mood disorders, especially depression. Changes in GABA-glutamate balance may also be involved in these particular disorders. Depressed patients exhibit increased glutamate and decreased GABA cortical content, later that might differ according to depression subtypes [104].

In humans, exposure to a mild stressor such as the threat of shock decreases cortical GABA content. In accordance with this, peritraumatic plasma GABA levels are reduced in subjects with acute stress disorder and post-traumatic stress disorder, when compared to healthy volunteers. Alterations in the cortical GABA-glutamate equilibrium may be also present in post-traumatic stress disorder patients as they present a decrease in GABAergic functioning and an increase in glutamatergic- driven functions [105]. In rats, frontal cortex GABA content remains unchanged after acute stress but decreases after repeated exposure to stress. In mice, chronic mild stress triggers depressive symptoms, while repeated social

defeat leads to anxiety-like symptoms, both reactions being associated to a reduction in prefrontal cortex GABA content and GABA/glutamate ratio, but not in the hippocampus [106]. This suggests that specific brain area stress induced GABA-glutamate equilibrium disturbances. However, rats exposed to a single intense prolonged stress and investigated seven days later exhibit a decrease in glutamate and glutamine content in the medial prefrontal cortex without any change in GABA content. Altogether, intense and/or repeated stress exposures are associated with changes in frontal cortex GABA-glutamate equilibrium [107].

Neurotrophic Hypothesis of Depression

There is a consistent evidence that the volume loss of the hippocampus and other brain regions is related to the duration of depression, suggesting that untreated depression leads to hippocampal volume loss, possibly resulting in increased stress sensitivity and increased risk of recurrence [108]. Glucocorticoid neurotoxicity, glutamatergic toxicity, decreased neurotrophic factors, and decreased neurogenesis have been proposed as possible mechanisms explaining brain volume loss in depression. There is no solid evidence on any of these mechanisms since there are no imaging tools to directly examine neurotoxic and neurotrophic processes, in vivo [109]. Brain-derived neurotrophic factor (BDNF) has attracted considerable interest. Specifically, preclinical studies have shown correlations between stress-induced depressive-like behaviors and decreases in hippocampal BDNF levels, as well as enhanced expression of BDNF following antidepressant treatment [110].

In addition to this, approximately 30% of patients with depression fail to respond to currently available therapies, which mainly influence monoaminergic systems [111]. The difficulties in finding efficient antidepressant treatment due to heterogeneous nature and

associated with different molecular, environmental, and genetic factors. Hence, depression requires long-term treatment [112]. In recent years, studies on depression have shifted from monoamines towards other mechanisms, including glutamatergic neurotransmission.

Stress-induced memory impairment

The mood disorders of depression are often accompanied by cognitive symptoms, such as deficits in learning and memory, difficulty in decisions making, and loss of cognitive flexibility. The increasing evidence indicates that these cognitive deficiencies may be an early episode in depression [113, 114]. Amygdala and CA regions of the hippocampus have been long associated with stress, emotion, learning and memory processes. It is well known that stress induces a rapid rise in corticosterone. Chronically elevated corticosterone impairs memory, reduces neuronal spine density, and decreases hippocampal neurogenesis and volume [115].

The impact of chronic stress on cognitive performance is thought to depend on biological (such as sex) and chronobiological (age) factors. It was reported that the implementation of predictable stressors enhances animals cognitive performance [116].Adding further complexity to this issue, a recent study revealed that the period of the day (diurnal/nocturnal) in which the stress protocol is implemented also modulates cognitive performance [117].

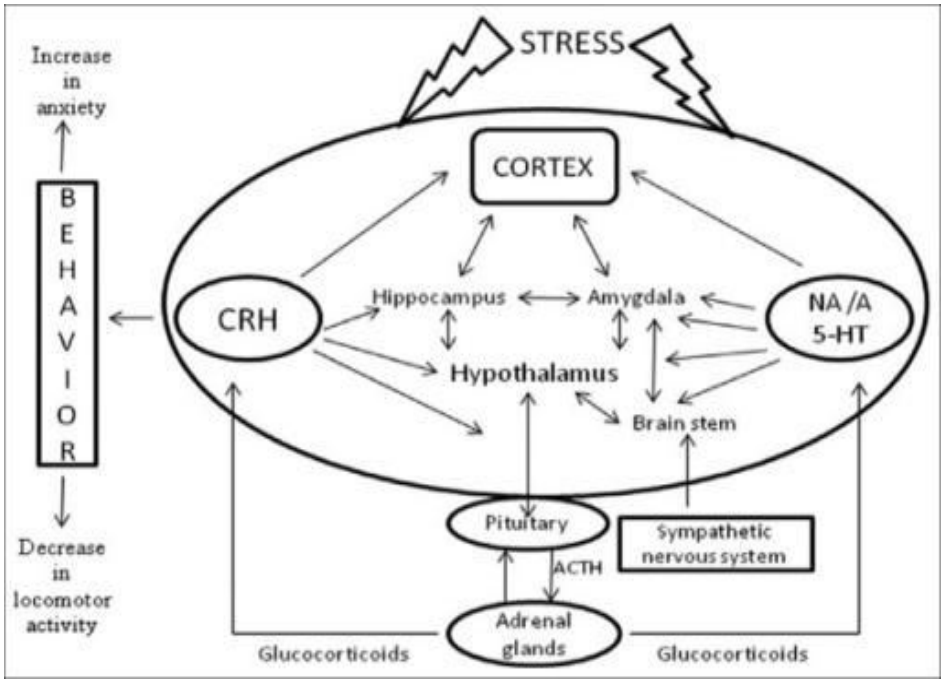
Clinical and preclinical studies have shown that prolonged exposure to stressful conditions impairs structural and functional plasticity of the hippocampal formation related to stress-driven cognitive and mood deficits [118, 119]. A key finding of this animal study is that tau is essential for chronic stress to induce dendritic atrophy and interrupt neuronal connectivity in the hippocampus.

Chronic unpredictable mild stress model

Chronic unpredictable mild stress (CUMS) procedure was performed in animals are consecutively exposed to a series of unpredictable mild stressors such as food deprivation, water deprivation, continuous illumination, tilted cages, and soiled cages in a random unpredictable manner to simulate a series of life stress events [120]. Indeed, a large number of etiological symptoms and neurobiological abnormalities found in CUMS induced animals are similar to those exhibited in human depressed patients. After 2 weeks of CUMS, the animals developed the variety of symptoms similar to depressive symptoms in human beings such as weight loss, altered diurnal rhythms, sleep disturbances, and anhedonia. Thus, CUMS induced depressive animal model has good validity and reliable predictability to screen new antidepressants through a series of behavioral tests [121].

Porsolt et al., (1978) suggested the forced swim test (FST) to evaluate the depressive-like state in animals as a tool to study clinical efficacy of potential antidepressant drugs. Because FST is designed based on a capability of rodents, to exhibit resistance to the repeated action of a strong stressor. In the classical model of FST, a 15 minutes pretest is a stressor suggested to induce a state of behavioral despair [122] which becomes more expressed in the five minutes retest session on the next day. In the initial version of the FST, the time spent in an immobile posture and the latency to the first immobility episode was recorded as an index of depression. The modified version of the FST included swimming, climbing, diving in order to facilitate the differentiation between serotonergic and noradrenergic role in depression [123]

Figure 3: Stress pathway



According to American Psychiatric Association (APA- 2013), Anhedonia is a core feature of major depressive disorder and a key diagnostic criterion. It is another popular approach to assess depressive-like state in laboratory animals [124]. Anhedonia has been considered as a "loss of pleasure", that emphasizes different facets of hedonic function, including desire, effort/motivation, anticipation, and consummatory pleasure. In animal studies, anhedonia was estimated in various models of depression, including chronic unpredictable mild stress (CUMS), social defeat, and others. In animals, anhedonia may be assessed using "primary" reward such as the presentation of palatable food or drink or strong positive reinforcing stimuli such as drug injection or presentation of pups in a specific place. The CUMS model resulting in the development of depressive-like behavior was initially validated as an Anhedonia inducing model [125].

A major limitation of the rodent social defeat due to lack of knowledge of sex differences in responses to this stressor given the vast majority of studies are undertaken in male rats because females exhibit a reduced level of aggression [126]. Yet women have a two-fold lifetime greater risk of developing major depression compared to men. Thus, the omission of social defeat studies in female rats is a scientific gap. [127]

Chronic stress and antioxidant system

Stressful conditions can precipitate cognitive impairment along with anxiety and depression, which can lead to excessive production of free radicals, resulted in oxidative stress, an imbalance in the oxidant/antioxidant system [128]. Antioxidants protect biological systems from deleterious effects of ROS by free radicals scavenging activity. It can mitigate ROS effect by blocking the enzyme generating free radicals and provoking the expression of antioxidant enzymes. Antioxidants include carotenoids, anthocyanidins, catalase, glutathione peroxidase ferritin, superoxide dismutase, ceruloplasmin, catechins, vitamin C, tocopherols vitamin E, glutathione, and flavonoids etc. Synthetic antioxidants like butylated hydroxyl toluene (BHT) and butylated hydroxyl anisole (BHA) cause toxicity to living systems [129]. During the restraint stress process, the production and scavenging of ROS are unbalanced; the excessive free radicals react with proteins, lipids and nucleic acids in the cells. In addition, oxygen free radicals attack the unsaturated fatty acids of the biofilm, resulting in lipid peroxidation and destroying the integrity of the cell membrane structure [20].

Mitochondria are the main sites where ROS (including O_2^-, $OH-$, and H_2O_2) are produced and for cell energy conversion. When dysfunction occurs in the body, hydrogen ions flow out of the respiratory chain, forming a negative transmembrane potential inside relative to outside membrane. During this process, electron leakage will occur, causing the

reduction of O_2 to O_2 free radical and the conversion of partial O_2 free radical to H_2O_2, which further converts to OH−, in turn giving rise to the excessive production of mitochondrial ROS and the occurrence of a series of injuries [31].

An antioxidant study in different tissues of CUMS rats revealed decreased SOD, CAT levels and increased TBARS levels denoting that administration of different stressors triggered free radicals generation. Further, the adrenal ascorbic acid level was elevated in stressed rats which clearly denoted that CUMS rats were under oxidative stress and the biological system exhibits an adaptive mechanism [130]. In stressed condition, similar metabolic alterations were reported resulting in the imbalance of energy utilization and consequently in the generation of superoxide anion, hydrogen peroxide and hydroxyl radical ions as the major reactive oxygen species (ROS) in the bio-system which provoked cell lipid membrane peroxidation. Lipid peroxide products reportedly caused widespread cellular injury [131]. Endogenous antioxidants such as SOD, which dismutases the highly reactive superoxide anion to the less reactive species H_2O_2, CAT, a home containing enzyme, which scavenges hydrogen peroxide to water and molecular oxygen and ascorbic acid, which is a water-soluble antioxidant that protects the biological system from oxidative stress [132].
Generation of reactive oxygen species (ROS) are by-products of stress stimuli (biotic and abiotic) and intrinsic oxygen metabolism that inactivates enzymes and damages vital cellular substances and membranes, as a result causing cancers, aging, chronic inflammation and play a vital role in HIV infection and diabetes etc. [133]. ROS also modulate the principal neurotransmitters involved in the neurobiology of depression, also known to aggravate inflammation and associated pain caused by tissue injury [134].

Proinflammatory cytokines

An increasing body of evidence revealed that activation of the inflammatory response system plays a role in the pathophysiology of depression. Several studies reported increased levels of proinflammatory cytokines, for example, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in depressive disorders [135,136]. Similarly, increased mean plasma levels of proinflammatory cytokines such as the tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL 6) have been reported in patients with clinical depression. In non-human animal models [137], administration of high doses of interferon-α has been reported to induce depressive-like symptoms, which could be attenuated with repeated antidepressant therapy. These pieces of evidence clearly indicate a crosstalk between chronic inflammation and depression. Recent studies have reported a strong association between inflammation and deficits in learning and memory in animal models for neurodegenerative diseases including Parkinson's disease, Alzheimer's disease (AD) and amyotrophic lateral sclerosis. Another study found that anti-inflammatory agent; XPJY has the better effect on improving learning and memory ability in depressive rats than sertraline, which might be related to a reduction of inflammatory factors such as IL-1β, IL-6, and TNF-α, in serum and hippocampus. [138].

ATP and inflammation

Over the past decades, evidence has accumulated indicating that extracellular nucleotides and nucleosides may be important regulators of inflammatory and immune responses in cell lines. Modulation of inflammatory processes and immune responses by extracellular ATP is complex and results from specific effects on a wide variety of both immune and non-immune cells. The immunomodulatory effects of ATP in different immune

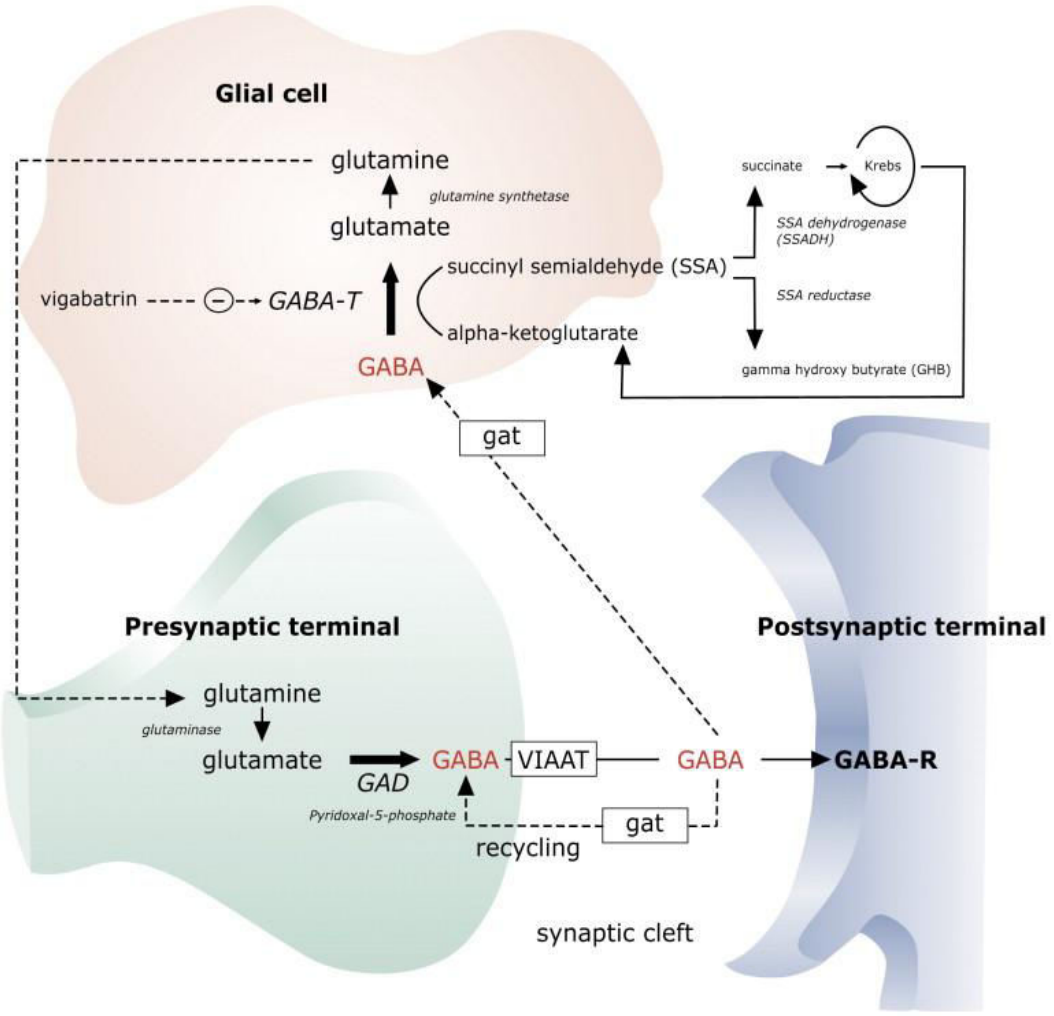
cell types have been described by Di Virgilio et al. [139] and were recently reviewed by the same authors, summarised that ATP and adenosine are important endogenous signaling molecules in immunity and inflammation through activation of purinergic receptors. The immunomodulatory effects of ATP on cytokine release by different immune cells depends on its extracellular concentration, the cellular expression of purinergic receptor subtypes and ectoenzymes can also be affected by various inflammatory mediators [140].

Glutamate alpha decarboxylase (GAD)

Neuronal hydrolysis of glutamine by glutaminase is the main source of the excitatory neurotransmitter glutamate, while glial specific glutamine synthetase is involved in the regulation of glutamate by astrocyte uptake of glutamate and conversion to glutamine. GABA levels are regulated through synthesis from glutamate catalyzed by glutamate alpha decarboxylase (GAD) and metabolism to glutamate by GABA-T, both synthesis, and metabolism occurring in neurons [141].

GAD enzyme activity was significantly decreased in the hippocampus of old rats exposed to CUMS. Specifically, GAD enzyme is expressed in the adult brain in two isoforms, namely GAD-65 and GAD-67. The two isoforms were shown to substantially differ in their response to phosphorylation; GAD-67 is inhibited by phosphorylation and activated by calcineurin mediated dephosphorylation, while the GAD-65 isoform is activated by phosphorylation.[142]. Herman and Larson (2001) reported that exposure to chronic intermittent stress decreased GAD65 mRNA levels in the hippocampal-paraventricular hypothalamic nucleus (PVN) relays of old Fischer rats, which was not seen in young rodents [143].

Figure 4: GAD enzyme mediated Glutamate and GABA neurotransmitters in neuronal cells



Parathyroid hormone 2 receptor and Tuberoinfundibular peptide of 39 residues

The parathyroid hormone 2 receptor was recognized based on its sequence homology with other receptors. It is a seven transmembrane type II G-protein coupled receptors [58]. It has 50% amino acid sequence similarity with the parathyroid hormone 1 receptor (PTH1R). The expression and distribution of PTH2R have also been examined in the rat (Wang et al., 2000). PTH2R-expressing cell bodies were found in a variety of brain areas. However, some discrepancies were reported between PTH2R mRNA-expressing and PTH2R-immunoreactive (ir) cell bodies [59].

Based on pharmacological and distributional data suggesting that parathyroid hormone and parathyroid hormone-related peptide are not endogenous ligands of PTH2R. Tuberoinfundibular peptide of 39 residues (TIP39) is an endogenous ligand that binds to the PTH2R with high affinity. In contrast, it has low affinity and negligible agonism at the PTH1 receptor level [60, 61]. TIP39 was purified from the bovine hypothalamus on the basis of its stimulation of cAMP formation. Mouse and rat TIP39 sequences are identical and share only 4 and 6 amino acid residues with parathyroid hormone-related peptides [62].

Detailed investigation on expression and distribution of TIP39 in the rat has revealed that TIP39 neurons are restricted to two brain regions, the subparafascicular/posterior intralaminar thalamic area and the medial paralemniscal nucleus at the midbrain-pons junction. In contrast to the restricted distribution of TIP39 cell bodies, amplification immunocytochemistry demonstrated a widespread distribution of TIP39 fibers in limbic, endocrine, and auditory brain regions [63, 64]

The central and basolateral nuclei of the amygdala are key components in the neural circuitry of innate and learned fear [65], but little is known about the circuits that underlie fear incubation. The amygdala is likely to be involved, based on its contributions to fear memory. Recent observations suggest that the central amygdala (CeA) plays a critical role in incubation of drug craving, which is a time-dependent increase in cue-induced drug-seeking behaviour after withdrawal [66]. Within the amygdala, PTH2R expression and projections from TIP39 neurons are relatively concentrated in the medial amygdala (MeA) and CeA. Neurons in the MeA are activated by contextual fear conditioning and lesions of the MeA disrupt several fear behaviors, including predator odor-evoked freezing, acute neuroendocrine responses, fear-potentiated startle, and conditioned fear memory [67, 68], suggesting that it may also be involved in fear memory.

Fear incubation was studied in mice and rats. Siegmund and Wotjak (2007) [69] showed that a single foot shock generates hyperarousal, enhanced contextual fear responses and generalized anxiety [70]. Moreover, fear and avoidance behaviors increase from 1 to 28 d of incubation in PTSD condition. Functional studies demonstrated that TIP39 modulates fear incubation stress responses, anxiety, and emotional behaviours [62-65]. Global deletion of TIP39 signaling in TIP39-KO and PTH2R-KO mice enhanced conditioned fear memory 14 d but not 6 d after a single foot shock, indicated potentiation of fear incubation [144].

The role of the PTH2R in the regulation of the catecholamine systems is less pronounced though TIP39 fibers are abundant in the locuscoeruleus and subcoeruleus areas, where noradrenergic neurons exist more. Recent evidence brings up the possibility that TIP39 may interact with noradrenergic pathways.TIP39KO mice and WT mice injected with a PTH2R antagonist demonstrated selective impairment of memory performance during

novelty-induced arousal [145]. Noradrenergic signaling has a biphasic, inverted U shaped, effect in the regulation of cognitive functionalities. The impaired performance of mice without TIP39/PTH2R signaling was restored by propranolol, an antagonist of beta-adrenoceptors, suggesting that PTH2R signalling influences the effect of novelty stress via an interaction with noradrenergic mechanisms [58].

PTH2R signaling in stress-induced analgesia (SIA) using hotplate model, before and after an inescapable foot shock, the response latencies were reduced in the wild-type mice. In contrast, a larger and dramatic increase was found in the response latency in mice without PTH2R signalling [61]. Similarly, TIP39 knockout mice showed elevated pre-shock response latencies in the hot plate test of SIA. These findings suggest that signaling through the TIP39-PTH2R system may normally limit SIA. Stress-induced analgesia induced by high-intensity stressful stimuli, including inescapable foot shock, has been shown to have a predominant for non-opioid component [146]. Indeed the opioid antagonist naloxone did not show any changes, but the cannabinoid receptor antagonist (Rimonabant) significantly decreased the SIA in wild type mice. But, the inhibitory effect of rimonabant on the SIA was much greater in the TIP39-PTH2R KO mice were observed [58].

ICV infusion of TIP39 reduced the plasma AVP levels by acting through PTH2R. Forty-eight hours of water deprivation induced dehydration significantly reduced the plasma AVP level following TIP39 administration. In the same study, it did not alter the plasma total protein content. The effect of TIP39 was not related to change in blood pressure because TIP39 infusion produced fall in mean arterial blood pressure, which would rather stimulate AVP secretion. In turn, the opioid receptor antagonist naloxone significantly reversed the inhibitory effect of TIP39 on dehydration induced AVP release while it had no

significant effect on the plasma AVP level when injected alone. These results suggest that TIP39 inhibits AVP release by central action possibly via an opioid system without influencing hemodynamic and osmotic action [147].

Lateral ventricular administration of TIP39 peptide in male rats blocked the release of growth hormone (GH) in plasma. This finding is consistent with anatomical data showing a high density of TIP39 containing fibers around somatostatin neurons in the periventricular hypothalamic nucleus. Somatostatin neurons that inhibit the release of GH [148]. PTH2R expression was demonstrated on many of these somatostatin neurons in the rat as well as in human providing the anatomical basis for TIP39 stimulating the release of somatostatin, which in turn inhibits GH secretion [149]

TIP39 KO mice demonstrated increased anxiety in the shock-probe defensive burying test as compared to WT controls. In normal or in low-stress conditions, TIP39 KO mice did not differ from WT controls in the arm entries in the elevated plus maze or in the dark-light emergence test of spontaneous anxiety-like behaviours [55]. However, an increase in anxiety-like behavior became evident in TIP39 KO mice that were tested in the elevated plus maze under prior restraint or bright illumination conditions. These results are consistent with a role of endogenous TIP39 in limiting the consequences of stressful perturbations. Furthermore, mice lacking TIP39 or the PTH2R demonstrated increased anxiety and depression-like behaviours after a foot shock in elevated zero mazes, open field, light dark box and forced swim tests [150].

Fear-related anxiety was investigated in relation to the TIP39-PTH2 receptor system using Pavlovian fear conditioning method. TIP39 KO mice showed more freezing than wild-type mice after one tone-shock pairing during conditioning and subsequently, more freezing

during both tone and context recall tests. However, the similar level of freezing was noted during subsequent tone presentation, respectively [151]. Furthermore, foot shock conditioned fear recall was enhanced 14 days but not 6 days after the aversive stimulus in both TIP39 KO and PTH2RKO mice as compared to wild-type controls [55-57]. These results suggest that normal TIP39 signalling lessens the long-term consequences of a traumatic event while the absence of signalling *via* the PTH2R increase the recovery period. Since the amygdala is known to be involved in the fear response [152], the abundant TIP39-PTH2R system in the amygdala, especially in its central and medial nuclei might be involved in these effects.

Infusion of TIP39 into the lateral ventricle increased the core temperature of WT mice while TIP39 injection had no effect in PTH2R KO mice. Furthermore, PTH2R KO mice had impaired heat production upon cold exposure, but no change in basal temperature and no impairment in response to a hot environment suggesting that theTIP39 PTH2R system plays a specific role in temperature conservation in a cold environment [66], Since temperature sensation was normal in PTH2R KO mice, the PTH2R may play a role in the heat production signal. Both seem to be the case because acute intracerebral PTH2R antagonist administration also impaired the heat production response to a cold environment. In addition, the weight of brown adipose tissue (BAT), and its capacity to increase body temperature were reduced. PTH2 receptor in the Median Pre-Optic nucleus (MnPO) seem to be involved in the thermoregulatory action of TIP39 because TIP39 injected into the MnPO produced a larger body temperature increase (2°C) for longer periods of time than injection of the same amount of TIP39 into the lateral ventricle. Furthermore, local injection of TIP39 into the dorsomedial hypothalamic nucleus had no effect on the body temperature. The

MnPO as a site of action is consistent with its high density of TIP39 terminals and PTH2R immunoreactivity as well as with the known role of MnPO neurons in the control body temperature [153] *via* descending mechanism. No data available on the actions of TIP39 on the HPA axis and in central thermoregulation association with fever.

The functional significance of the elevated TIP39 was studied on the release of prolactin because the projection of TIP39 neurons in the PIL toward tyrosine hydroxylase-containing neurons in the mediobasal hypothalamic regions known to regulate prolactin secretion. In rodents, removal of the pups from the dams for 4h results in a decrease in prolactin level, which is in turn dramatically increased upon the return of the litter and the immediate onset of nursing. Injection of a PTH2R antagonist into the lateral ventricle 5 min before the union of mothers with pups potently and dose-dependently inhibited suckling-induced prolactin release in the rat [154]. The physiological significance of this is supported by the observation that in a similar pup removal/return paradigm the weight increase (a measure of milk consumed) of pups suckling PTH2R KO mice was reduced 30 min after the onset of nursing as compared to pups suckling WT mice. Also consistent with less effective suckling by PTH2R KO dams, pups reared by PTH2R KO mice had a lower body weight at the time of weaning than pups reared by WT mice. TIP39 fibers and PTH2Rs are ideally positioned to affect gonadotropin-releasing hormone (GnRH) neurons, whose activity is suppressed during lactation. In addition, the TIP39-PTH2R neuromodulator system might also play a role in conveying the effect of sucking on other systems adapted in the postpartum period. PTH2Rs in the preoptic area, the lateral septum, and the periaqueductal gray could be involved in the control of maternal behaviours [155]. Emotional changes that take place in the postpartum period could also be affected by TIP39 based on the

localization of the TIP39-PTH2R system in the infra limbic cortex, the medial, and central amygdaloid nuclei, the amygdalo-hippocampal transitional zone, the pre-mamillary nuclei, the ventral subiculum, and the periaqueductal gray which are parts of the circuitry of reproductive and emotional regulation [156-157]

Tuberoinfundibular peptide of 39 residues (TIP39) found to have the modulatory effect on an acute nociceptive action. TIP39-PTH2R located most of the central and peripheral region. Many of these regions process pain information [158]. PTH2 receptors are also expressed in dorsal root ganglion (DRG) neurons as well as the spinal dorsal horn suggesting that PTH2 receptors may play a role in pain regulation. Moreover, intraplantar injection of its endogenous agonist TIP39 elicited nociceptive flexor responses in mice. In addition to this, the intrathecal administration of TIP39 potentiated thermal and mechanical responses, as well as a nocifensive response. These findings suggest that TIP39 may have pharmacological and physiological effects on nociceptive fibers. It is also synthesized by a population of DRG cells and by neurons within the dorsal horn of the spinal cord. TIP39 increases cAMP in F-11 cells, which are a DRG neuroblastoma hybrid cell line that possesses some of the properties of peptidergic nociceptors [16,17], whereas the absence of TIP39 signaling, in TIP39 knockout (KO) mice, mice with null mutation of the PTH2R, or following acute PTH2R block, reduces nocifensive responses in animal models of acute thermal and inflammatory pain [11]. These reports suggest that TIP39 signaling modulates sensory thresholds via effects on the glutamatergic transmission to brainstem GABAergic interneurons that innervate noradrenergic neurons. TIP39's normal role may be to inhibit release of hypoalgesic amounts of norepinephrine during chronic pain.

Expression of TIP39 gene in the cardiovascular system was proved that it has strong negative inotropic effect in the presence of L-NA with no vasodilator action through PTH2R, the contrast to PTH1R. This study led to the hypothesis, nitric oxide (NO) contributes to negative inotropic effect and influences the contractility in a positive mode. Similar results were obtained in experiments with isolated heart at the lower concentration. These results suggest that a direct effect of TIP39 on the cellular level and that myocyte derived NO rather than vascular NO is responsible for this effect. Furthermore, study stating that NO mediates its positive inotropic effect via cGMP. These observations revealed that TIP39 activates two pathways. One is NO/cGMP dependent and influences inotropy in a positive manner. Second is the blockade of NO synthesis by L-NA [159]. Therefore, it concludes that NO is responsible for the positive effect of TIP39 on contractility.

PLAN OF WORK

Phase 1: Elucidation of Physiological role of Tuberoinfundibular Peptide 39 (TIP39) in rats

- Assessment of physiological role of TIP39 peptide in rats
- Assessment of behavioural activity of TIP39 treated rats
- Estimation of neurochemical and stress hormone in TIP39 treated rats
- Morphological study on prefrontal cortex and hippocampus in TIP39 treated rats

Phase II: Neurochemical assessment and behavioural role of Tuberoinfundibular Peptide-39 (TIP39) in acute restraint stressed Rats

- Interactive study assessment
- Induction of acute restraint stress (ARS) in rats for two hours
- Behavioural evaluation of TIP39 peptide on acute restraint stressed rats by using PTH2 receptor antagonist
- Estimation of stress hormone in acute restraint stressed rats using PTH2 receptor antagonist
- Estimation of GABA, Glutamate, 5HT and NA release in acute restraint stressed rats using PTH2 receptor antagonist by using HPLC and HPTLC techniques
- Expression of TIP39 gene in normal and stressed rats by using PCR techniques

Phase III: Elucidation of role of TIP39 on chronic unpredictable mild stress induced HPA axis dysregulation, inflammation and oxidative damage in depressive rats

- Behavioural evaluation of TIP39 peptide on CUMS induced anxiety and depression in rats.
- Estimation of sucrose intake in CUMS rats
- Estimation of oxidative markers such as SOD, CAT, GSH and MDA on CUMS rats
- Estimation of energy metabolite in CUMS rats
- Study on pro-inflammatory cytokines (IL-6, IL-1 β and TNF-α) in CUMS rats

Phase IV: Elucidation of role of TIP39 on chronic unpredictable mild stress induced learning and memory impairment in rats.

- Behavioural evaluation of TIP39 peptide on CUMS induced learning and memory impairment in rats.
- Estimation of acetylcholine esterase release in CUMS rats.
- Estimation of glutamic acid decarboxylase enzyme (GAD) in CUMS rats
- Expression of GR and MR genes in CUMS rats
- Histological study on Pre frontal cortex and hippocampus in CUMS rats.

Materials and Methods

Table 1: List of Materials used in this study

Chemicals /Reagents/ Instruments	Manufacturer
Ketamine	Med India, India.
Xylenol	Kruz Pharma, India.
Carboxy methyl cellulose	Thermo Fisher Scientific, USA.
TIP39 peptide	Life Technologies Pvt Ltd, India
5-Hydroxytryptamine (5HT)	Sigma Aldrich, USA
Noradrenaline (NA)	Neon lab, India
Glutamic acid	Sigma Aldrich, USA
GABA	Sigma Aldrich, USA
Acetylecholine esterase (AchE)	Sigma Aldrich, USA
Corticosterone	Sigma Aldrich, USA
Dimethyl sulphide	Thermo Fisher Scientific,USA.
Stereotaxic apparatus	RWD Life Science Co Ltd, China
HYWH	Biomolecules Midwest Inc.USA
ATP	Sigma Aldrich, USA
IL-6, IL-1 β and TNF-α Elisa kit	Hi Media, Bengaluru.
SOD, CAT, GSH and MDA	Hi Media, Bengaluru.
3-(4 5-dimethylthiazol-2)-2 5-diphenyltetrazolium bromide	Sigma Aldrich, USA
Glutamic acid decarboxylase enzyme (GAD)	Sigma Aldrich, USA

Acetyl thiocholine iodide	Sigma Aldrich, USA
GR and MR primers	Thermo Fischer scientific, USA.
Dissection microscope	Micros, Austria.
High Performance Liquid Chromatography (HPLC)	Waters, USA.
High Performance Thin Layer Chromatography (HPTLC)	LAMAG , Switzerland.
GR, MR primers	Hi Media, Bengaluru.
Inverted Trinocular phase contrast fluorescent Microscope	Nikon , Japan
Water Maze	Medi Analytica ,India.
Multimode reader	Thermo scientific, , USA
Reverse transcriptase PCR	Thermo scientific, , USA
Refrigerated ultra centrifuge	Eppendrof, Germany.
Deep freezer	Cryo scientific system, Tamil Nadu
Surgical table with temperature control	RWD life sciences, China
UV Visible spectrophotometer (2)	Shimadzu, Japan

CHAPTER 5: MATERIALS AND METHODS

Methodology

Phase 1: Elucidation of Physiological role of Tuberoinfundibular Peptide 39 (TIP39) in rats

- Behavioural study on open field exploratory behaviour Test (OFT), forced swim test (FST) and elevated plus maze (EPM) in rats
- Estimation of plasma corticosterone level in rats using HPLC method
- Estimation of plasma 5-Hydroxytryptamine (5HT) level and Estimation of plasma noradrenaline (NA) level using HPLC method
- Estimation of brain Glutamate and GABA content using HPTLC method
- Estimation of brain acetylcholine esterase (AchE)
- Histopathological study on prefrontal cortex and hippocampus in rat brain

Phase II: Neurochemical assessment and behavioural role of Tuberoinfundibular Peptide-39 (TIP39) in Acute Restraint Stressed (ARS) Rats

- Induction of acute restraint stress (ARS) in rats for two hours
- Interactive study in forced swim test and elevated plus maze model on HYWH treated rats in acute restraint stressed rats
- Estimation of plasma corticosterone on HYWH treated rats in acute restraint stress model using HPLC method
- Estimation of plasma 5-Hydroxytryptamine (5HT) level and plasma noradrenaline (NA) level on HYWH treated rats in acute restraint stress model using HPLC method
- Estimation of brain Glutamate and GABA content on HYWH treated rats in acute restraint stress model using HPTLC method
- Expression of TIP39 gene in normal and acute restraint stressed rats by using PCR techniques

Phase III: Elucidation of role of TIP39 on chronic unpredictable mild stress (CUMS) induced HPA axis dysregulation, inflammation and oxidative damage in depressive rats

- Chronic unpredictable mild stress induction procedure for 28 days
- Estimation of sucrose consumption on Anhedonia model in CUMS rats
- Behavioural study on open field exploratory behaviour test , forced swim test and tail suspension test (TST) in CUMS rats
- Estimation of SOD, CAT, GSH, and MDA on prefrontal cortex and hippocampus region in CUMS rats
- Estimation of plasma corticosterone in CUMS rats using HPLC method
- Estimation of brain ATP content in CUMS rats using HPLC method
- Estimation of Pro Inflammatory Cytokines like TNFα , IL-1β and IL-6 in CUMS rats using ELISA techniques

Phase IV: Elucidation of role of TIP39 on chronic unpredictable mild stress induced learning and memory impairment in rats.

- Behavioural study on Morris water maze Test(MWM), Y- Maze task, modified elevated plus maze (mEPM) and novel object recognition test (NORT) in CUMS rats
- Estimation of acetyl cholinesterase activity in CUMS rats
- Estimation of GAD enzyme by ELISA method in CUMS rats
- Mineralocorticoid (MR) and Glucocorticoid receptor (GR) gene expression in CUMS rats using PCR techniques

- Morphological study on Pre frontal cortex and Hippocampus in CUMS rats using H&E staining

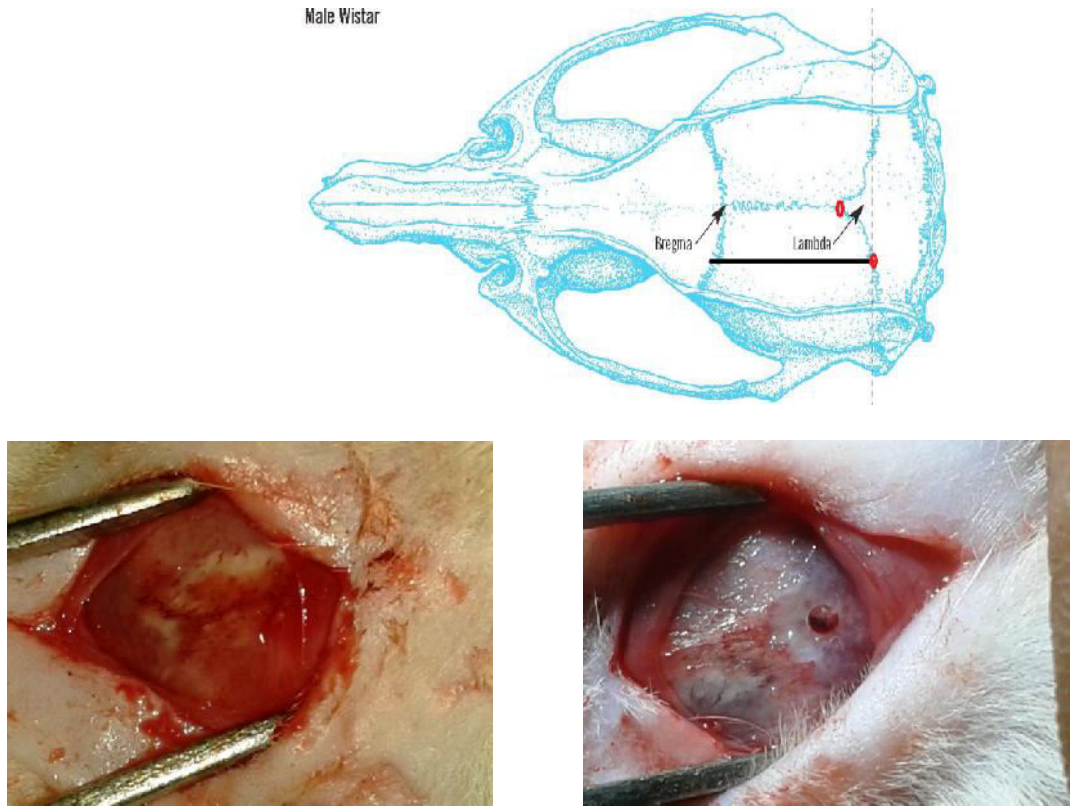
1. Animals

Male Sprague Dawley rats (150-200g) were obtained from PSG IMS&R (205/2013/IAEC, 280/2015/IAEC, 359/2017/IAEC). Experiment was performed according to the experimental protocol approved by the institutional animal ethics committee (IAEC). Rats were maintained in a separate cage (6 animals per cage) with standard diet. Animals were housed under room temperature (25 ± 2º C), 12/12 hrs light-dark cycle, humidity (55±5%) according to CPCSEA guidelines and guide for the care and use of laboratory animals.

2. Surgical procedure and drug treatment

Stereotoxic surgery was made seven days before the drug administration. Rats were anesthetised with ketamine (80 mg/kg, i.m.) xylazine (10 mg/kg,i.m.). Guide cannulae (Stailess steel (OD 0.64 x ID 0.45) RWD Life Science Co Ltd, China) was implanted in the right lateral ventricle at pre-established coordinates, anteroposterior, 0.2 mM from bregma; lateral, 1.5 mM; and vertical, 4.2 mM, and kept under controlled temperature in an individual cages [160]. Two different doses of TIP39 peptide (1 nmol & 10 nmol) was infused through intracerebroventricular (i.c.v) route over 5 min.

Figure 5. Site of Intracerebroventricular (ICV) infusion on bregma region at skull



3. <u>Behavioural study</u>

3.1. Chronic unpredictable mild Stress induction and Anhedonia model

The CUMS model was implemented according to a previous method with a slight modification [172]. During the experiment, the animals were first trained to experience and drink a sweet beverage by presenting them simultaneously with two bottles, during the first 24 h; both bottles contained a 1%sucrose solution. Subsequent 24 h, one bottle contained the standard sucrose solution the other contained water. Following 14 h food and water deprivation, rats received the first baseline sucrose preference test. Each animal was presented simultaneously with two pre weighed bottles, one containing the sucrose

solution and the other containing normal water. Both bottles were removed and weighed at 60 min at the end of the test. Then, animals were given food and water for 2 h. After another period (14 h) of food and water deprivation, animals received a second baseline sucrose preference test. Four days thereafter, following 24 h food and water deprivation, animals received a third baseline sucrose preference tests. After the 1st, 2nd, 3rd and 4th week of CUMS, rats were subjected for sucrose preference test [173].

Followed by third baseline test, animals were divided into various groups (n=6) and were subjected to chronic unpredictable stress. In brief, the CUMS protocol consisted of the chronological application of a variety of mild stressors. These stimulants are shifted every other week in a period of four week experiment. All the control rats were kept under identical conditions in a separate cage away from the stressed rats. (Table 2)

Table 2: Stress induction procedure in CUMS model

Days	**Type/Duration of stress**
Day 1	Food deprivation (24 h) and cold swimming (5 min at 6º C)
Day 2	Water deprivation (24 h), Tail pinch (1min - 2 times interval of 6 h)
Day 3	Soiled bedding (150 ml water per cage) for 12 h , Physically restrained for 2 h
Day 4	Day and night light illumination and restricted food pellets (45 g)
Day 5	Exposure to a novel odour with noise (12 h) and animal isolation (1rat/cage) (12h)
Day 6	Crowded housing (10 rats/cage) for 12 h
Day 7	Cage tilting (45° inclined) for 12 h
Same methodology was followed for 2nd,3rd and 4th week	

3.2. Open Field Exploratory Behaviour Test (OFT)

The open field test (OFT) was executed to check whether the immobility period was in conjugation with any effect of motor activity. Open field [161] apparatus is an arena made of plywood, consisting of a floor (96 × 96 cm) with high walls. The entire apparatus was painted black except for 6 mm thick white lines which divided the floor into 16 squares. Experimental animals were placed in the centre of an open box and allowed to explore the arena. Behavioural parameters were quantified, such as ambulation, rearing, time spent in central compartment, and grooming for 5 min.

3.3. Forced Swim Test (FST)

With slight modifications, forced swim test (FST) was carried out [125]. Rats were forced to swim one by one in a transparent glass vessel (45cm X 12cm X 45cm) with water filled up to 30 cm at room temperature (25 ± 2^{o}C) 24 h ahead test session. A training period of 10 min has given to each animal. The duration of immobility, jumping and dipping responses were observed for five min in a six min test session. On the test day, after the initial 1 min, rats were observed for immobility, jumping and dipping responses for next 5 min. The start of immobility reflects behavioural despair or helplessness.

3.4.Elevated plus maze (EPM)

Elevated plus maze (EPM) was conducted to evaluate the anxiety state. On testing day, the rat was placed in the centre square of the plus maze, facing one of the enclosed arms. The number of entries and the time spent in open and closed arms were noted for next six minutes period. An arm entry was defined when all the four limbs were on the arm [162].

3.5. The tail suspension test (TST)

TST was conducted according to steru et al. [174] where animals were suspended by the bands and hang from a hook mounted 50 cm above the floor for 6 min, approximately 5 cm from the lever. Rats were suspended for a period of 6 min and were considered immobile when rats are completely motionless.

3.6. Morris Water Maze Test (MWM)

Morris water maze employed to evaluate spatial learning and memory [163]. It consisted of large circular pool (150 cm in diameter, 45 cm in height, filled to a depth of 30 cm with water maintained at 28±1^{o}C). During the MWM test, a platform 15 cm in diameter was located 1.5 cm below the water in one of four sections of the pool, approximately 50 cm from the sidewalls. The pool was divided into four quadrants of equal area

3.6.1. Acquisition phase:

The MWM test was initiated after drug administration. The animals received four trials per day. The rats were trained to find the hidden platform. The position of platform was kept unaltered throughout the training session. Each animal was subjected to four consecutive training trials on each day. The rat was gently placed in the water between quadrants, facing the wall of pool and allowed 60 sec to locate submerged platform. Then, it was allowed to stay on the platform for 20 sec. If it failed to find the platform within 60 sec, it was guided gently onto platform and allowed to remain there for 20 sec. Day 6, escape latency was performed (ELT), rats were allowed to locate the hidden platform in water maze was noted as an index of acquisition or learning. Animals were

subjected to training trials for six consecutive days. Between one trail and the next, water was stirred to erase olfactory traces of previous swim patterns.

3.6.2. Retention phase:

On ninth day, the platform was removed and each rat was allowed to explore the pool for 90 sec. Mean time spent in the target quadrants was noted. The mean time spent by the animal in target quadrant searching for the hidden platform was noted as an index of retrieval or memory. The time to reach the target quadrant and the number of crossings the animal made while searching the target quadrant was also noted as index of memory.

3.7. Y- Maze task: Spontaneous alternative performance

Y-maze test was used to assess the immediate working memory performance of the animal by recording their spontaneous alternation behaviours in a single session [180]. The maze was made of 3 identical arms, 40 cm long, 35 cm high and 12 cm wide, positioned at equal angles and labelled A, B, and C. Each rat, unfamiliar with the maze, was placed at the end of one arm and allowed to move freely through the maze during a 5-min session. Spontaneous alternation was examined by visually recording the pattern of entrance into each arm in the maze for each rat. Alternation behaviour was determined from successive entries into three different arms (e.g., ABC, CAB, or BCA). Arm entry was considered to be complete when the hind paws of the rat were completely placed in the arm. The percentage of spontaneous alternations was defined by the following equation:

Spontaneous alternation (%) = [(number of alternations)/(total arm entries−2)]×100.

3.8. Modified Elevated plus Maze (mEPM)

The Elevated plus maze was made of wood and painted black. The apparatus consisted of two opposite open arms (45 cm×10 cm) without sidewalls and two enclosed arms (45 cm×10 cm×30 cm) with sides and end walls, extending from a central square (10 cm×10 cm). The maze was elevated to the height of 60 cm above the floor and placed in a moderately lighted room. On the first day, each rat was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) was defined as the time taken by the animal to move from the open arm into one of the covered arms with all its four legs. TL was recorded on the first day for each animal. If the animal did not enter into one of the covered arm within 90 sec, it was gently pushed into one of the two covered arms and TL was assigned as 90 sec. The rat was allowed to explore the maze for another 2 min and then returned to its home cage. Retention of this learned-task (memory) was examined 24 h after the first day trial [180]

3.9. Novel object recognition test (NORT)

The test consists of three sessions separated by 24 h. In session 1 (habituation), animals allowed to freely explore the open field during 10 min. In session 2 (familiarization), rats allowed to interact with two identical objects placed in the centre of the open field during two 5 min periods. During inter period time (1 hour), rats placed in their home cage. In session 3 (test),after 24 h rats presented 1 familiar object and a novel object that differed in shape, colour, and texture, during 10 min session. The starting position (facing objects) unchanged over sessions and animals were tested in a semi randomized order. Contacts with objects defined as when the animal's nose less than 1

cm from the object and contacts recorded. The results calculated as duration of contact with the novel object and expressed as a percentage of the total contact time with the two objects (TCT). The apparatus cleaned with diluted ethanol (50%) between animals and only 1 rat tested by session [181].

3.10. Blood and brain sample collection

Immediately after the last behavioural test 2 ml of blood was collected through retroorbital puncture for neurochemical estimation. Blood samples were collected in ependroff tubes contains 10% sodium citrate solution. Plasma was separated by centrifuging the blood at 4000 rpm for 10 min and stored at -80ºC until the estimation was done. After blood collection the rats were anesthetized with ether followed by quick cervical dislocation then performed decapitation followed by harvesting of brain. Brains were immediately stored at -80ºC until the estimation was done.

4. Estimation of plasma 5-hydroxytryptamine (5HT) level

5-Hydroxytryptamine was measured in the plasma sample of rat using High Performance Liquid Chromatography (HPLC) techniques [164].

4.1. Preparation of stock solution

The main stock solution was prepared by dissolving the standard drug in water at a concentration of 1 mg/ml. From this stock solution, working standard solutions were prepared in the mobile phase at various concentrations ranging from 10 µg to 50 µg for the calibration curve.

4.2. Preparation of plasma samples

The plasma samples were dissolved in the mobile phase. Internal standard (Paracetamol) was added and vortexed for 5 minutes and centrifuged at 10,000 RPM for

15 min. The supernatant layer was injected into the HPLC for the estimation of 5HT neurotransmitters.

4.3. Preparation of mobile phase

The mobile phase consists of potassium dihydrogen phosphate (0.07mm/lit), dipotassium ethylene diamine tetra acetic acid salt (0.1 mm/lit), heptane sulphonic acid (1.1 mm/lit), tri ethyl amine (3.1 mm/lit) and methanol. The buffer solution was prepared by dissolving all the chemicals in the HPLC grade water, sonicated for 15 min and filtered through 0.22 µm nylon filter. The mobile phase was prepared with the following ratio of acetonitrile: methanol: sodium acetate (pH 3.5) in the range of (10:10:80 % v/v) and the pH was adjusted with saturated citric acid solution, sonicated and filtered prior to the use.

4.4. Chromatographic conditions and procedure

Plasma serotonin level was measured using 10At HPLC systems (shimadzu LC). The mobile phase used for the study was acetonitrile: methanol: sodium acetate (pH 3.5) in the ratio of (10:10:80 % v/v). The stationary phase used was phenomenex C18 (250*4.6mm i.d, 5 µ) at the flow rate of 0.7 ml/min and detected at 275 nm. The samples were prepared with phenomenex strata solid phase extraction cartridge was conditioned with methanol and water (1 ml) sequentially. To this 0.5ml of plasma was added. The cartridge was washed with 2ml of water. The drug was eluted from the cartridge using 0.5 ml of a mobile phase. The standard serotonin was used for the preparation of calibration curve.

5. Estimation of plasma noradrenaline (NA) level

Nor-adrenaline was measured in the plasma sample of rat using HPLC coupled with Electro Chemical Detector [165].

5.1. Preparation of stock solution

The main stock solution was prepared by dissolving the standard drug in water at a concentration of 1 mg/ml. From this stock solution, working standard solutions were prepared in the mobile phase at various concentrations ranging from 10 ug to 50 ug for the calibration curve.

5.2. Preparation of plasma samples

The plasma samples were added in the mobile phase. Internal standard (paracetamol) was added and vortexed for 5 min and centrifuged at 13,000 rpm for 10 min. The supernatant layer was injected into the HPLC-ECD for the estimation of neurotransmitters.

5.3. Preparation of mobile phase

The buffer was prepared based on the procedure mentioned in the section 1.5.4. The mobile phase was prepared in the ratio of buffer: methanol (90:10 v/v) and the pH was adjusted to 3.12 with saturated citric acid solution, sonicated and filtered prior to the use.

5.4. Chromatographic conditions

HPLC was performed using water 515 system coupled with electro chemical detector (waters 2465), which is equipped with binary solvent delivery pump and rheodyne manual injector. The chromatographic separation was performed on C18 column: Luna C18. 110A (50×4.60 mm, 5 µ) (phenomenex) and voltage was measured at

0.7 mV. The system was run in the isocratic mode at a flow rate of 0.5 mL/min. the injection volume was 20 µl while the column was maintained at 35ºC temperature during the run.

6. Preparation of Brain Tissue Samples

Rats were sacrificed under ether anaesthesia and their brains were excised out quickly and stored at -80^{o}C. The brain was homogenized using 0.1 N HCl (for every 10 mg tissue/200 µl) in a manual homogenizer. The homogenates were transferred to polypropylene tubes and centrifuged at 4500 rpm for 20 min at 250^{o}C. The supernatant was then transferred into micro centrifuge tubes and used at the earliest for spot application.

7. Estimation of Glutamate and GABA using HPTLC in rat brain

Neurotransmitters like L-glutamic acid and GABA were measured in rat brain samples using high performance thin layer chromatography (HPTLC) coupled with densitometer [166].

Chromatographic Conditions:

- Stationary phase : HPTLC Silica gel GF254 plates
- Mobile phase : n-butanol: glacial acetic acid: water (60:15:25 v/v)
- Chamber saturation time : 7 hr
- Prewashing : Ethanol
- Instrument : HPTLC (Camag–version 1.3.4)
- Applicator : Linomat V
- Scanner : Camag TLC scanner III
- Developing chamber : Twin trough glass chamber (20 × 10)

- Developing mode : Ascending mode (multiple development)
- Detection reagent : 0.2% Ninhydrin in acetone
- Scanning wavelength : 486 nm
- Experimental condition : Room temperature
- Temp/RH-55 : 65%.

7.1. Preparation of standard solution

0.1N HCl was prepared using 80% ethanol. This was used to dissolve L-glutamic acid and GABA.

7.2. Preparation of stock solution of L-Glutamic acid and GABA

Stock solution of L-Glutamic acid and GABA were prepared by dissolving 1 mg, 2 mg and 4 mg of the respective amino acid in 1 ml of 0.1N HCl (1 mg/ml).

7.3. Preparation of 0.2%Ninhydrin solution

In 100 ml standard flask, 200 mg of ninhydrin was taken and dissolved in 1 ml of acetone. Then added 1 ml of pyridine and make up the volume upto 100 ml with acetone.

8. Estimation of corticosteroids

Corticosteroids are measured in the plasma sample of rat using HPLC coupled with UV detector [167]. Immediately after the last behavioural test, animals were sacrificed by decapitation and blood was collected in sodium citrate containing tubes kept in ice and centrifuged at 1000×gfor 20 min. Plasma was separated and aliquots were stored at −80°C for corticosterone estimation. Corticosterone (HPLC grade) was used as standard for the preparation of calibration curve in the concentration ranging from 400-1400 ng/ml.

Chromatographic conditions:

- Column : 15×4.6 mm C18
- Mobile phase : Distilled water: Acetonitrile: glacial acetic acid (65:35:0.05)
- pH : 4.10 and 4.20.
- Flow rate : 1.2 ml/min
- Sample injection volume : 100µl
- Wavelength :245 nm
- Run time : ± 15 minutes

8.1.Preparation of standards

A 100 µg/ml stock solution of corticosterone was prepared in 20% methanol, in an amber volumetric flask and stored in a refrigerator. Blood of healthy rats were collected in heparin blood tubes and centrifuged. All the plasma was pooled into one glass beaker. The plasma was treated with activated charcoal (0.04 g/ml of plasma) to remove the endogenous corticosterone**.** The suspension was stirred for 90 min at room temperature where after it was pipette into a glass tube and centrifuged at 3000 rpm for 10 min. The top layer of plasma was filtered through a 0.45 µm Millipore filter to remove all the carbon particles. The concentration range of 400-1400 ng/ml was made with the activated charcoal treated plasma for plasma standards.

8.2.**Sample preparation**

500 µl of the test plasma was taken in a glass tube and was extracted with 5 ml of dichloromethane by vortexing it for 2 min where after it was centrifuged at 3000 rpm for 10 minutes. After centrifugation the upper layer which comprised of plasma was removed, and the lower organic layer was transferred to conical tubes and evaporated to

dryness under nitrogen at room temperature. The residue was reconstituted with 150 µl of mobile phase.

9. Acetylcholine esterase assay

Acetylcholine esterase activity was estimated by using artificial substrate, acetylthiocholine (ATC).In the medium, thiocholie released due to the cleavage of ATC by AChE is allowed to react with –SH reagent 5,5' –dithiobis-2-nitrobenzoic acid (DTNB),which is reduced to be a yellow colored anion called thionitrobenzoic acid measureable at a wavelength of 412 nm. Concentration of thionitrobenzoic acid was spectrometrically detected and taken as a direct estimate of AChE activity. Acetylcholine esterase assay was done based on the principle and protocol as described earlier by Ellman et al.[168] with some minor modification. Briefly, rats were sacrificed by cervical dislocation and whole brain was dissected out,20 mg of brain tissue/mL of phosphate buffer(0.1M; pH8) was homogenized and 0.4 mL aliquot of brain homogenate was added to a cuvette containing 2.6 ml of 0.1M phosphate buffer, 100 µL of DTNB reagent (10 mg DTNB in 100 mL of phosphate buffer, pH8.0) The substrate acetylthiocholine iodide 20 µL(75mg of acetylcholine iodide per 50 mL of distilled water) was added and change in optical absorbance was measured every 2 min for 10 min at 412 nm to provide a measure of enzyme activity [169]. The AChE activity was determined by following formula:

$$R=5.74\times10^{-4}\times\Delta A/C$$

Where,

R= Rate of enzymatic activity (in µmoles of acetylthiocholine hydrolysed /min/mg protein)

A=Change in absorbance /min

C = Concentration of tissue homogenate

10. Estimation of SOD, CAT, GSH, and MDA in the brain

Immediately after the last behavioural test, rats were anesthetized with ether followed by quick cervical dislocation then decapitated followed by harvesting of brain. Brain samples were immediately flash freezed using liquid nitrogen and stored at -80^{o}C. The tissue homogenate was prepared by using homogenization buffer (PBS-pH 7.4 with 1 mM EDTA, 5M NaCl, 0.5% triton X100 and protease inhibitor). The homogenised samples were centrifuged at 2000 g for 2 min and the supernatant was used for the enzyme assay. Superoxide dismutase (SOD) activity was estimated spectro photometrically at 550 nm by measuring the generation of superoxide free radicals produced by xanthine and xanthine oxidase, which react with nitro-blue tetrazolium (NTB) system according to the method by Sun et al.,(1988) [175].

The activity of superoxide dismutase was expressed as units/mg protein. According to Aebi et al.,(1984) [17] measurement of catalase (CAT) was carried out based on the ability of catalase to restrict oxidation of the H_2O_2. Initial reaction was carried out by adding 1ml of H_2O_2 (30 mmol/L). The variations in decomposition rate of H_2O_2 were determined spectrophotometry at 240 nm. The activity of catalase was expressed as units/mg protein. Assay of reduced glutathione (GSH) was carried out based on the Elmann procedure. GSH concentrations were determined with the absorbance read at 412 nm. The values were expressed in nmol/mg protein. malondialdehyde (MDA) measurement was carried in compliance with Ohkawa et al.,(1979) [176]. The organic layer was separated and its absorbance was measured at 532 nm by micro plate

spectrophotometry. Protein estimation was conducted according to Lowry et al.,(1951). The data were expressed as nmol/mg protein [177].

11. Estimation of ATP content in brain

ATP level in supernatants was quantified using a reverse-phase HPLC (Shimadzu, Japan). RP-HPLC determination was performed on a reversed-phase Hypersil C18 column (Elite, Dalian, China) attached to two LC-10ATvp pumps, equipped with UV–Vis detector. The detection wavelength 254 nm [178].

12. Measurement of TIP39 expression by Polymerase Chain Reaction

The Cells were lysed using TRI reagent (Sigma-Aldrich) and the total RNA was extracted and quantified by NanoDrop (Thermo Scientific Wilmington, DE, USA) and whose 260/280 ratio found >1.8, were used for cDNA conversion (Applied Biosystems, USA). Primer sequences were synthesized at Sigma-Aldrich, MO, USA. The TIP39 primer sequence is forward: 5'-GCTTCTGGGTGTGATGGTGA-3, Reverse: 'AGCAGCAAAAGCAGCAGCAG-3'.PCR reactions were run in qPCR (Applied Biosystems, USA) system. Reactions were initiated with denaturation at 95 °C for 30s, followed by 40 cycles of two-step reaction, denaturation at 95 °C for 5 s, and annealing and extension for 30 s [171].

13. Measurement of Pro Inflammatory Cytokines by ELISA

After collection, tissues were lysed by using hypotonic lysis buffer composed of 10 mM Tris HCl, 10 mM NaCl and 0.3% NP40 and 10% glycerol along with protease and phosphatase inhibitors. Protein estimation was done by Bradford assay. Plates were coated with capture antibody (diluted in PBS) then washed with 0.05% Tween 20 in PBS (pH7.2- 7.4). After washing plates incubated for blocking with reagent diluents (1% BSA

in PBS pH 7.2-7.4) for minimum one hr. 100 µl of sample and standard were incubated respectively for 2 hr at room temperature. Washing was repeated as above and 100 µl of detection antibody reconstituted in reagent diluents for TNFα (DY-510) along with 2% heat inactivated NGS for IL1β (DY-501) and IL-6(DY-506). Then incubated for 2 h at room temperature. Washing was repeated as above. Substrate solution (Tetra Methyl Benzidine) was added and incubated for 20 minutes. Finally 50 µl of stop solution (2 N H2SO4) was added to each well and plates were gently tapped for thorough mixing. Optical density was determined at 450 nm with 540 nm baseline correction.

14. Estimation of GAD enzyme by ELISA method

Estimation of glutamic acid decarboxylase (GAD) was performed according to the procedure given in ELISA kit purchased from sigma,USA. [182]. GAD enzyme was determined based on enzyme linked immunosorbent assay technique called quantitative sandwich immunoassay. The microtiter plate provided in this kit has been precoated with a monoclonal antibody specific for GAD. Standards or samples are then added to the microtiter plate ells. A standardized preparation of horseradish peroxidase (HRP) conjugated polyclonal antibody was added to each well to "sandwich" the GAD immobilized on the plate. The microtiter plate undergoes incubation, and then the wells are thoroughly washed to remove all unbound components. Next, A and B substrate solution was added to each well. The enzyme (HRP) and substrate were allowed to react over a short incubation period. Only those wells that contain GAD and enzyme conjugated antibody exhibited a change in colour. The enzyme substrate reaction was terminated by the addition of sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450 nm.

15. **Mineralocorticoid (MR) and Glucocorticoid receptor (GR) gene expression**

Cell lysis were done using TRI reagent (Sigma Aldrich, USA) and the total RNA was extracted according to the manufacturer's procedure. The RNA was quantified by NanoDrop. The RNA was converted to cDNA by high capacity cDNA conversion kit (Applied Biosystems). Expressions of Mineralocorticoid receptor (MR) and Glucocorticoid receptor and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were studied.The designed sequences were obtained from Sigma-Aldrich, MO, USA. The primer sequences are given in supplementary (Table 3). PCR reactions were run in qPCR (Applied Biosystems) system [183].

Table 3: Primers of Mineralocorticoid (MR) and Glucocorticoid receptor (GR) genes

Gene	Primer/Sequence
GAPDH	Forward- CAACTTTGGCATCGTGGAAG Reverse - CTGCTTCACCACCTTCTT
MR	Forward- ACCCTCCACACCTGTCAAAG Reverse - ACCTCCTGCCATATTTGCTG
GR	Forward- ATAAAAGCCTGAGGGGAGGA Reverse - GGAGAATCCTCTGCTGCTTG

16. Histopathological Evaluation

The brain was exposed by cutting the skull along the midline. Brains were immediately fixed in 10% phosphate buffered formaldehyde, embedded in paraffin, and 5 µm longitudinal sections were performed. The sections were stained with hematoxylin and eosin (H&E) and examined microscopically [170].

17. Statistical analysis

Data were expressed as Mean ± SD. Statistical analysis was carried out by one way ANOVA followed by post hoc analysis Tukey's multiple comparison test. Weekly sucrose intake were subjected to two way ANOVA followed by Bonferroni post hoc test. $P<0.05$, considered as statistically significant. Data were analysed using Graph Pad Prism, 4.03 (La Jolla, CA. USA).

RESULTS AND ANALYSIS

Phase I: Elucidation of Physiological role of Tuberoinfundibular Peptide 39 (TIP39) in rats

1.1. Effect of TIP39 treatment on behavioural activity in rats

TIP39 (1 &10 nmol) was administered through ICV route in rats; both doses of TIP39 did not altered the behavioural responses significantly such as ambulation ($F(2, 15) = 1.9$, $p > 0.05$), time spent in central compartment ($F(2, 15) = 1.7$, $p > 0.05$), grooming response ($F(2, 15) = 1.2$, $p > 0.05$) and rearing response ($F(2, 15) = 2.1$, $p > 0.05$), when compared to saline treated control group in OFT model (Fig.6). In EPM model (Table 1), TIP39 1nmol & 10 nmol did not show any significant changes on time spent ($F(2, 15) = 2.8$, $p > 0.05$) and arm entries ($F(2, 15) = 3.1$, $p > 0.05$) in both open arm and closed arms as comparison to saline treated control group. In FST model (Fig.7), immobility time ($F(2, 15) = 0.41$, $p > 0.05$), dipping ($F(2, 15) = 0.82$, $p > 0.05$) and jumping response ($F(2, 15) = 1.1$, $p > 0.05$) were not altered significantly in both the doses of TIP39 (1nmol &10 nmol) when compared to saline treated control group. Similarly, in MWM test, animals did not show any significant changes on number of crossings ($F(2, 15) = 2.7$, $p > 0.05$), times spent ($F(2, 15) = 3.5$, $p > 0.05$), and time to reach the target quadrant ($F(2, 15) = 3.2$, $p > 0.05$) as comparison to saline treated control group. Shown in (Fig.8)

Figure 6: Effect of TIP39 treatment on Open field test in rats

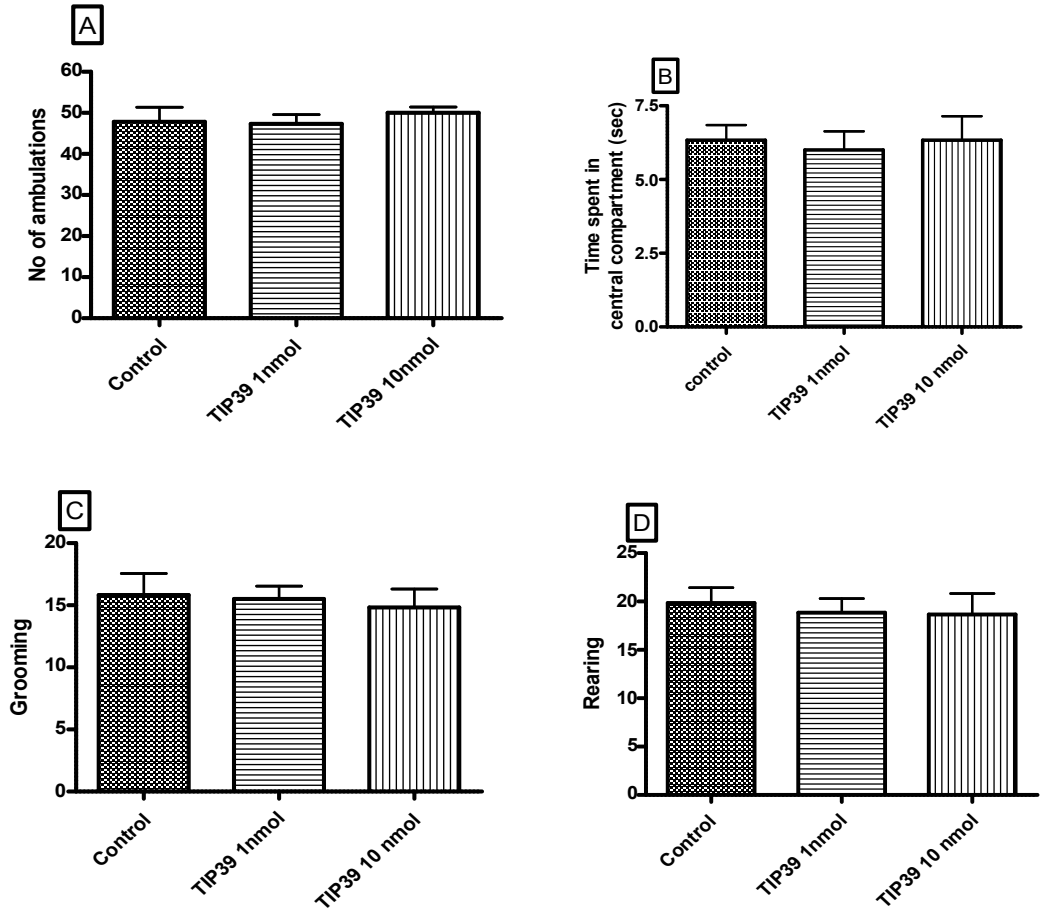


Fig 6. Effect of TIP39 treatment on OFT in rats: A) Ambulations B) time spends in central compartment C). Grooming response D). Rearing response. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests.

Table 4: Effect of TIP39 on EPM in rats

Treatment	Number of entries (counts/6 min)		Time spent (sec/6min)	
	Open Arm	**Closed Arm**	**Open Arm**	**Closed Arm**
Control	11.31±2.01	8.43±1.03	78.41±11.2	183.41191.12
TIP39 1 nmol/rat	12.01±1.24	9.3±0.94	71.23±10.22	190.35±20.32
TIP39 10 nmol/rat	10.91±2.43	8.62±1.1	74.61±10.31	186.23±19.11

Table 4. Effect of TIP39 treatment on EPM in rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests.

Figure 7: Effect of TIP39 treatment on Forced swim test in rats

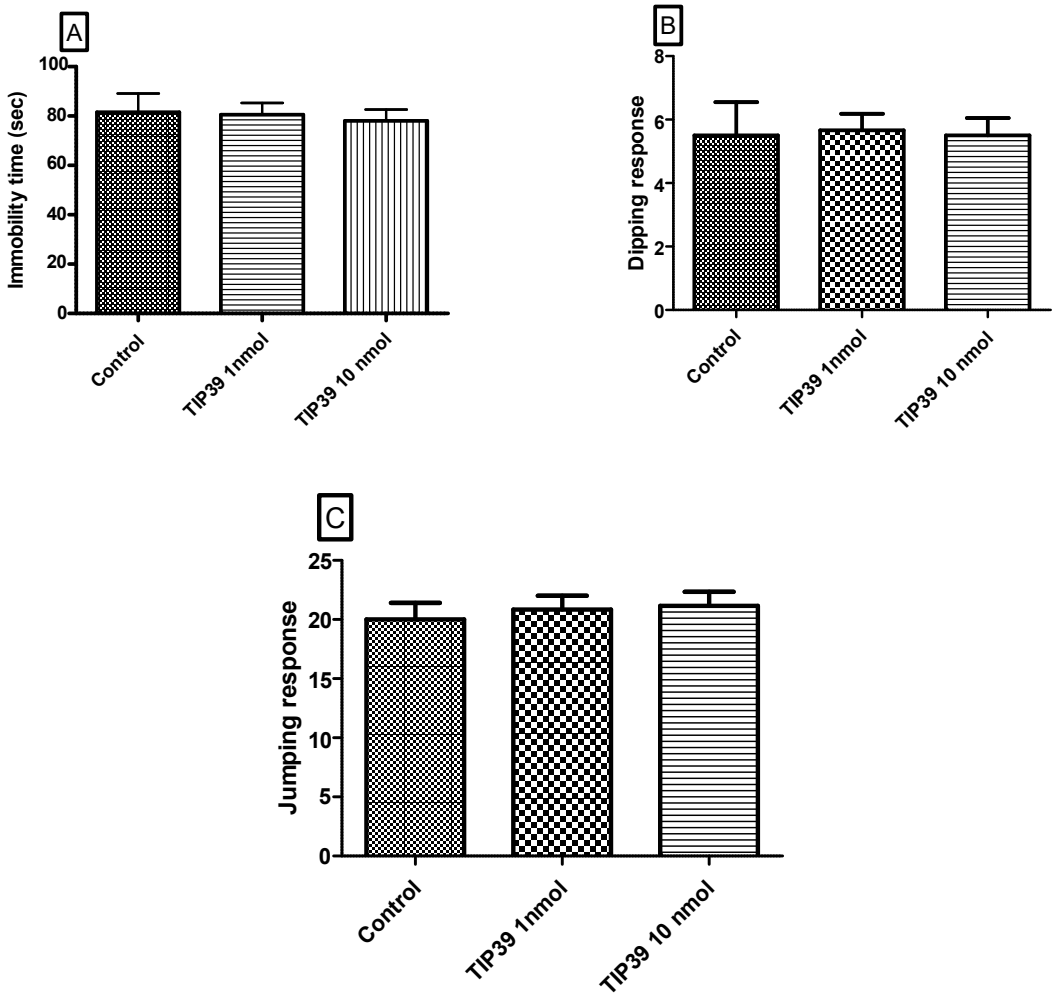


Fig 7. Effect of TIP39 treatment on FST in rats A) Immobility time B) Dipping response C). Jumping response. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests.

Figure 8: Effect of TIP39 treatment on Morris water maze test in rats

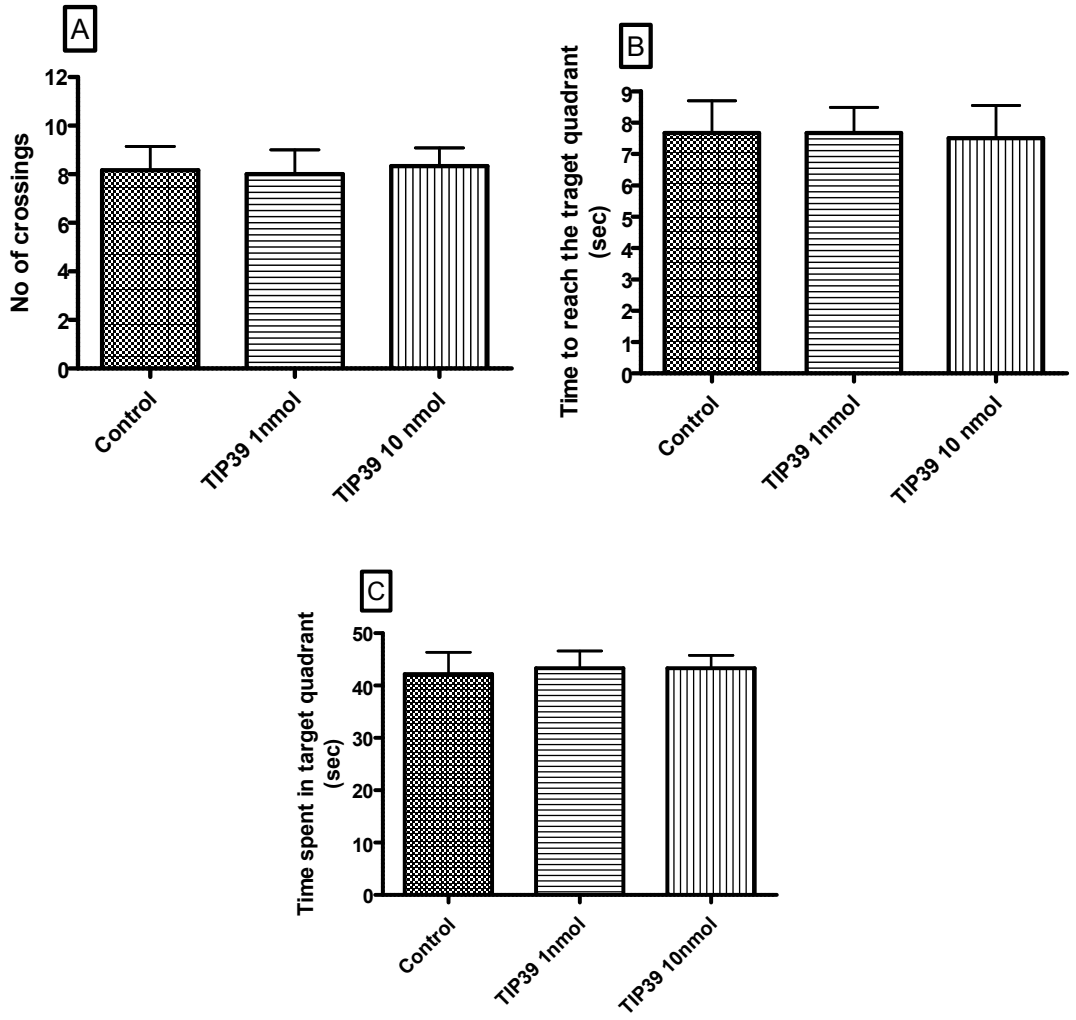


Fig 8. Effect of TIP39 treatment on MWM test in rats. A) No of crossings B) Time to reach the target quadrant C). Time spent in target quadrant. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests.

1.2. Effect of TIP39 treatment on biochemical's and neurotransmitter level in rats

Rats treated with ICV administration of TIP39 (1nmol & 10nmol) did not showed significant changes in plasma 5HT ($F(2, 15) = 1.2$, $p > 0.05$) and NA ($F(2, 15) = 1.4$, $p > 0.05$) level when compared to control group. Similarly, brain Glutamate ($F(2, 15) = 0.82$, $p > 0.05$), and GABA ($F(2, 15) = 0.51$, $p > 0.05$) levels were not altered with TIP39 1 &

10 nmol when compared to control group. Stress hormone like plasma Corticosterone levels also did not changed significantly ($F(2, 15) = 2.9$, $p > 0.05$), as comparison to control group. Similarly, both doses of TIP39 (1nmol & 10nmol) did not alter the acetylcholine esterase level ($F(2, 15) = 3.4$, $p > 0.05$) significantly in comparison to normal saline treated rats (Fig.9).

Figure 9: Effect of TIP39 treatment on plasma and brain neurotransmitter level in rats

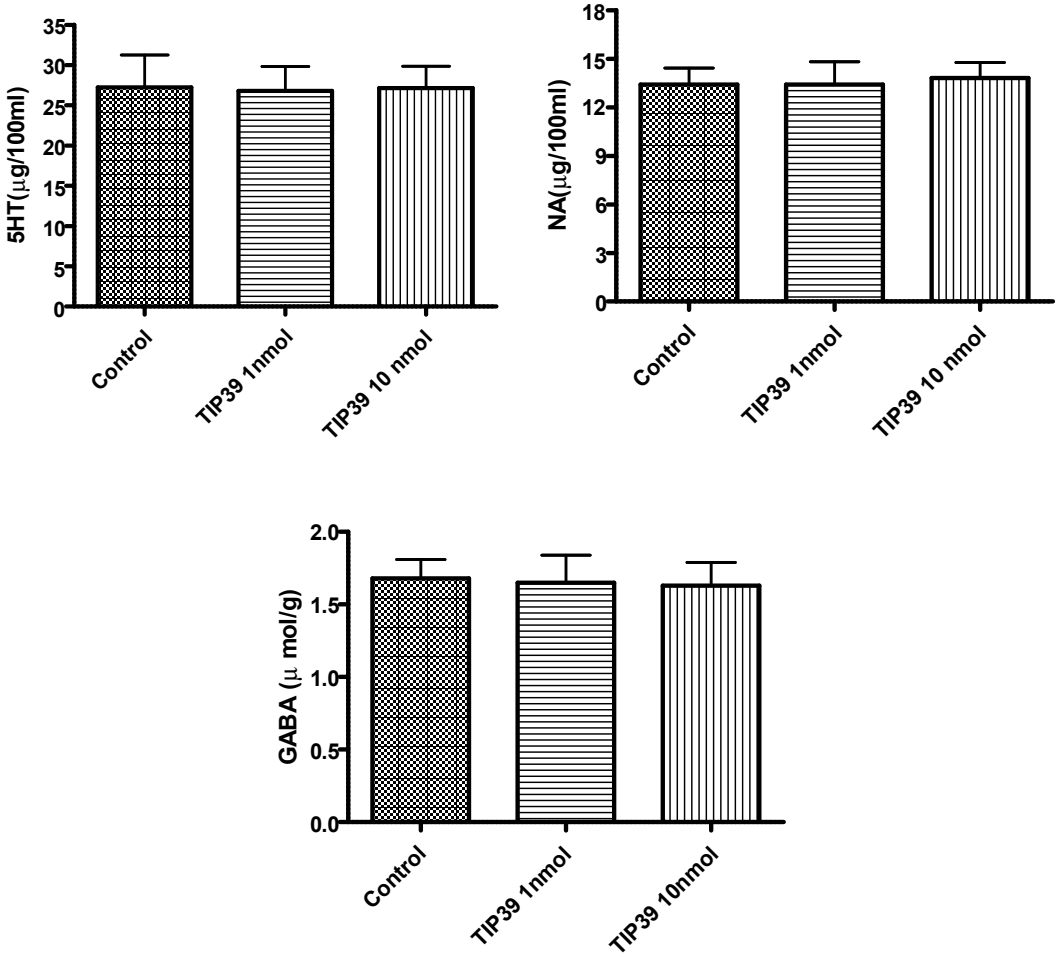


Fig 9. Effect of TIP39 treatment on 5HT, NA, GABA and Glutamate level in rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests.

Table 5: Standard Plasma 5HT estimation

S.No	Standard 5HT concentration (µg/100ml))	Peak area
1	10	30968
2	20	62534
3	30	90628
4	40	114102
5	50	152960

Figure 10: Standard Plasma curve of 5HT

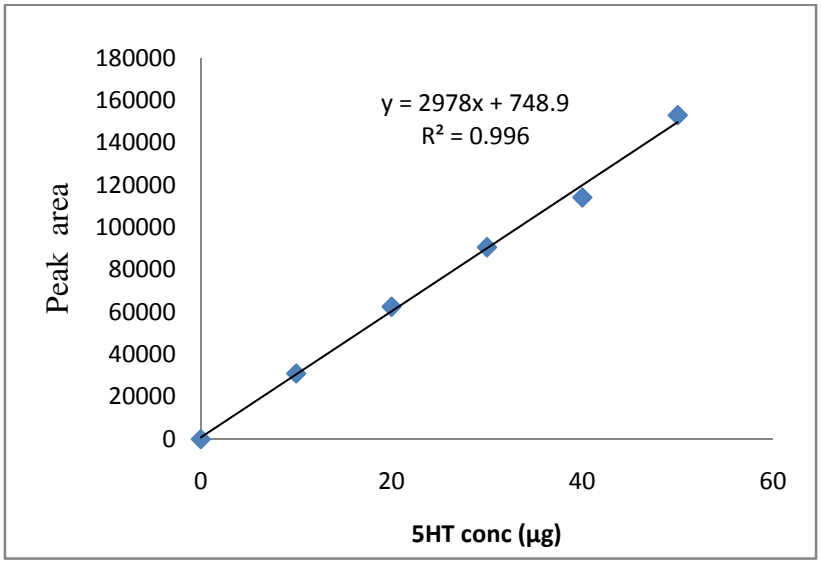


Table 6: Standard plasma Noradrenaline estimation

S.No	Noradrenaline concentration ((µg/100ml)	Peak Area
1	10	18189
2	20	32878
3	30	54926
4	40	73102
5	50	89898

Figure 11: Standard plasma curve of Noradrenaline

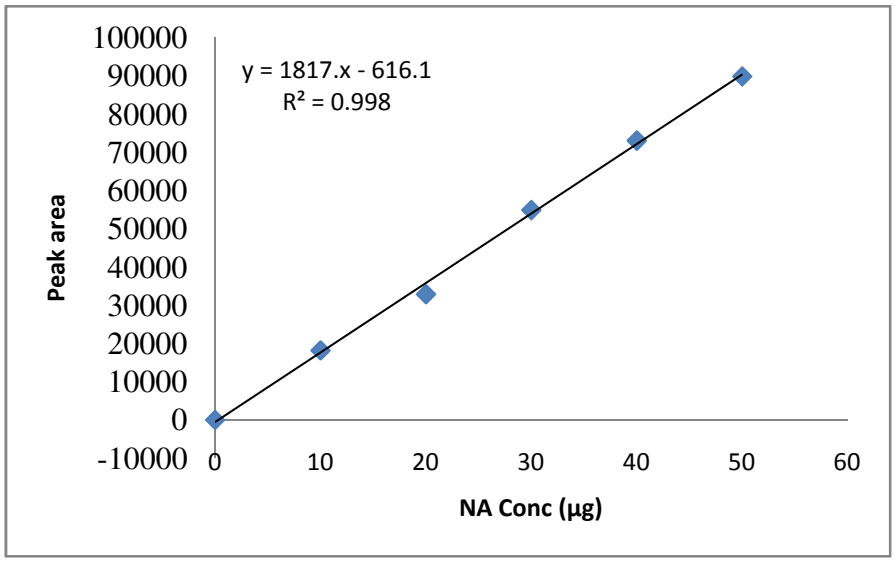


Figure 12: Standard linearity curve of GABA neurotransmitter

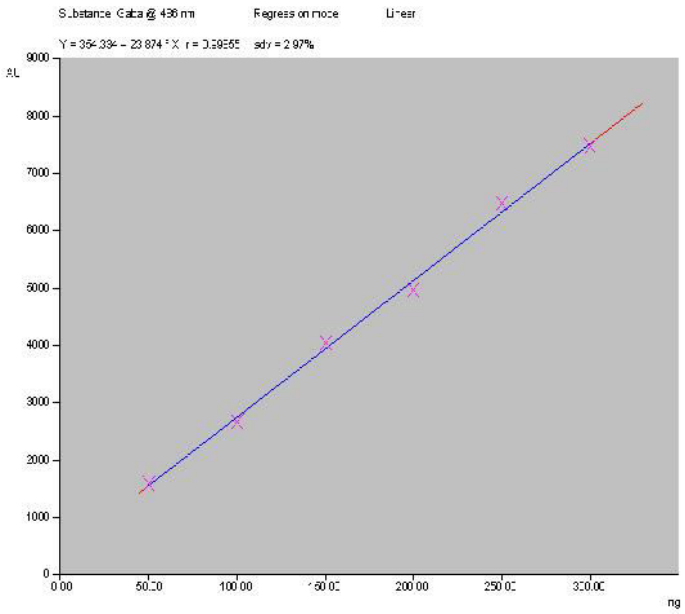


Figure 13: HPTLC- 3D Image of standard GABA

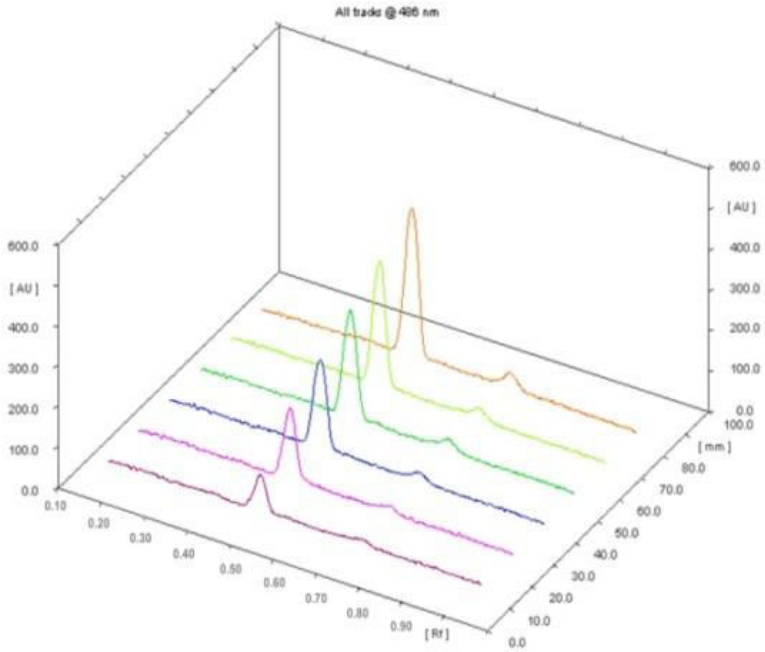
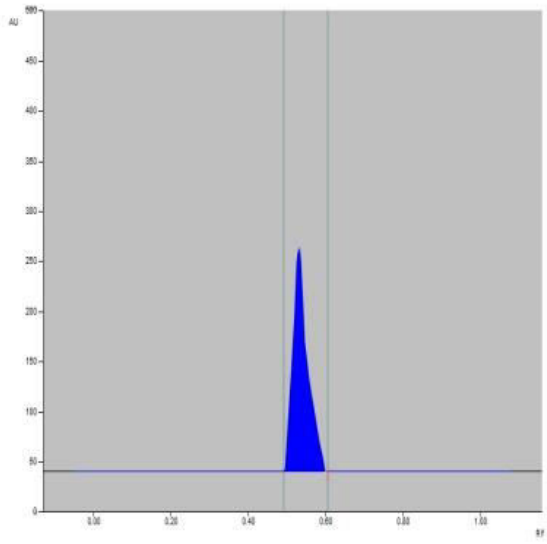
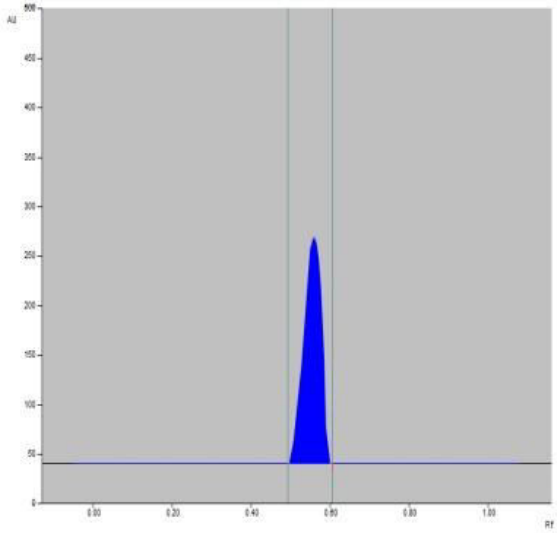


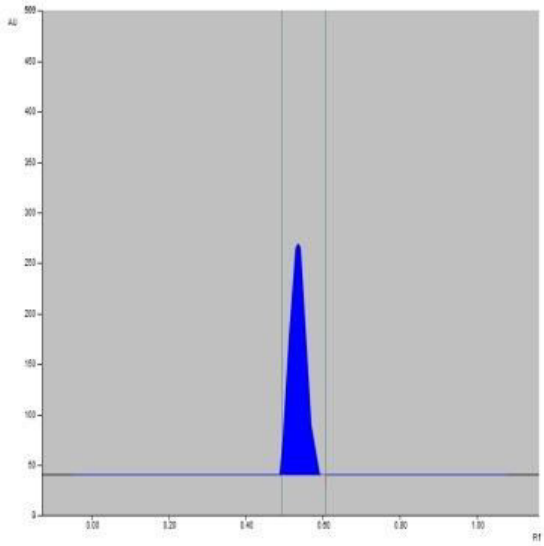
Figure 14: Estimation brain GABA level in rats



Control



TIP39 1 nmol/rat



TIP39 10 nmol/rat

Figure 15: Standard linearity curve of Glutamate neurotransmitter

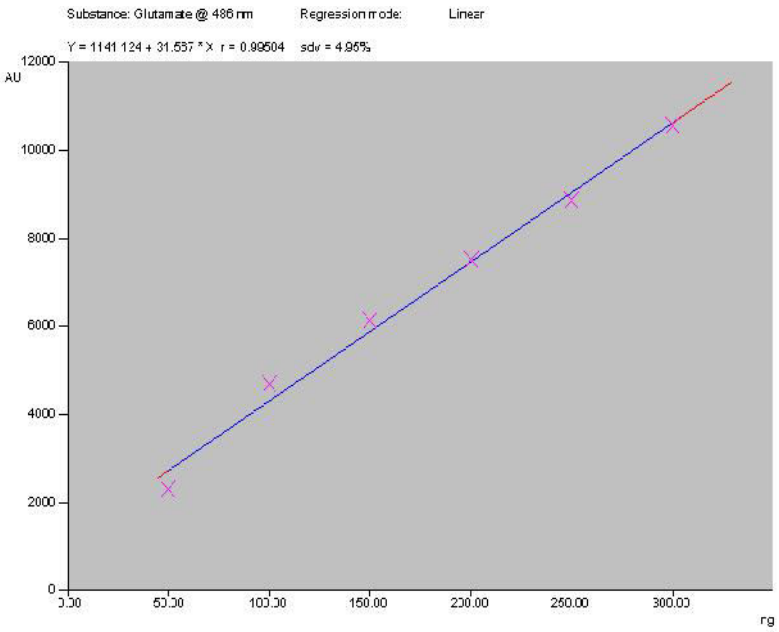


Figure 16: HPTLC- 3D Image of standard Glutamate

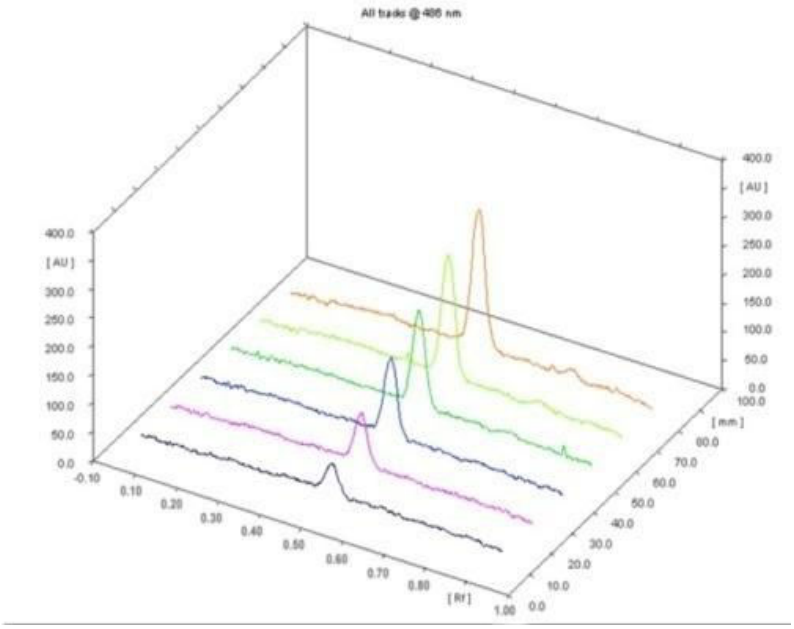
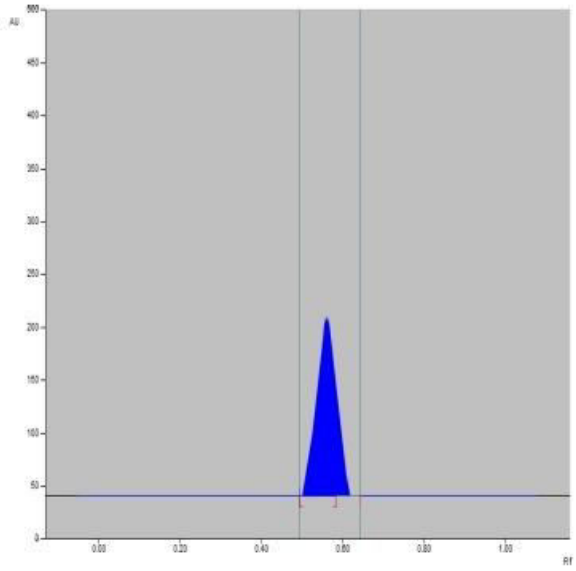
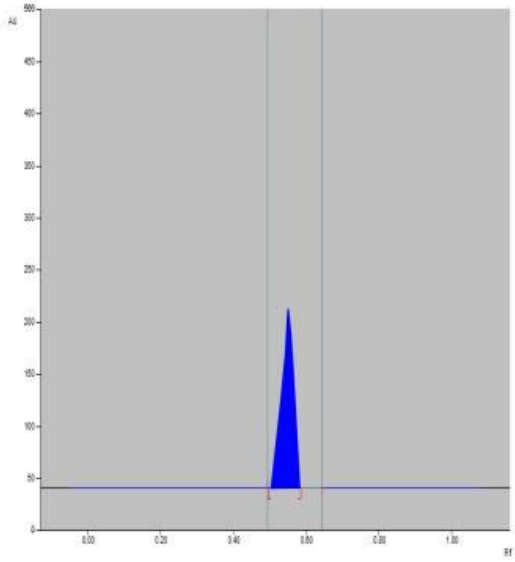


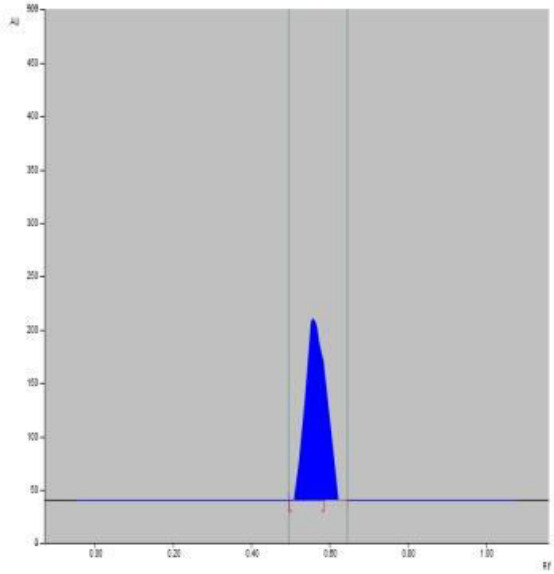
Figure 17: Estimation of brain Glutamate level in rats



Control



TIP39 1 nmol/rat



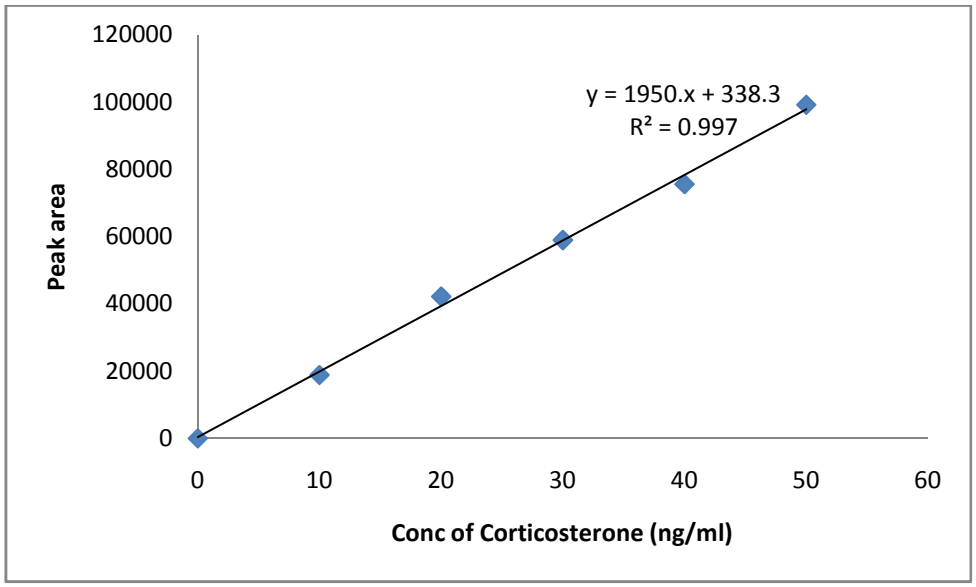
TIP39 10 nmol/rat

1.3.Effect of TIP39 treatment on Plasma corticosterone level in rats

Table 7: Standard plasma Corticosterone estimation

S.No	Corticosterone concentration ((µg/100ml)	Peak Area
1	10	18828
2	20	42157
3	30	58952
4	40	75568
5	50	99160

Figure 18: Standard Plasma curve of Corticosterone



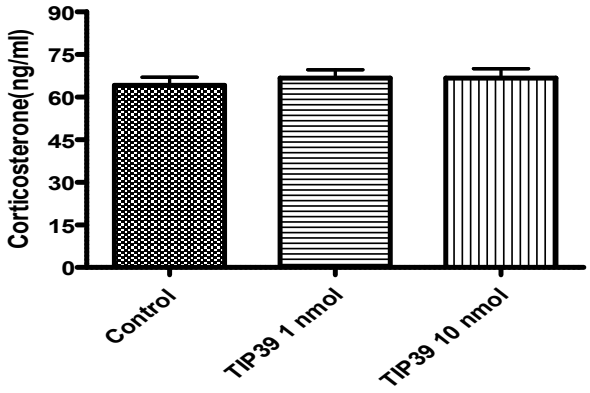


Fig 19. Effect of TIP39 treatment on Plasma corticosterone level in rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests.

Table 8: Effect of TIP39 treatment on brain Acetylcholine esterase level in rats

S.No	Treatment	Change In Absorbance (ΔA/min)	Protein Concentration (mg/ml)	Rate of Enzymatic Activity (µmoles/min/mg protein)
1	Control	0.04745	0.529	0.5146×10^{-4}
2	TIP39 1 nmol/rat	0.04733	0.524	0.5030×10^{-4}
3	TIP39 10 nmol/rat	0.04732	0.521	0.5211×10^{-4}

Figure 20: Effect of TIP39 treatment on brain Acetylcholine esterase level in rats

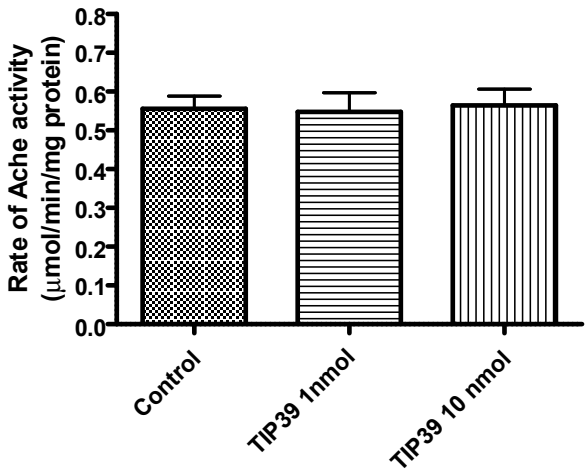
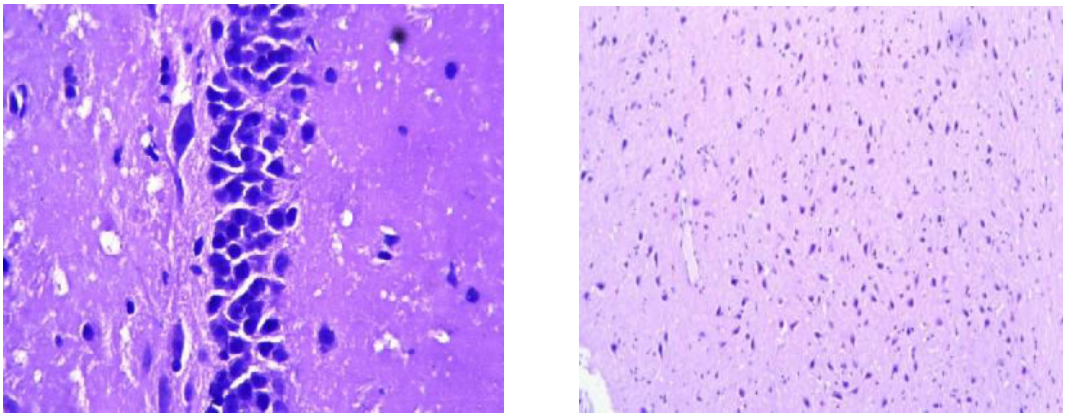


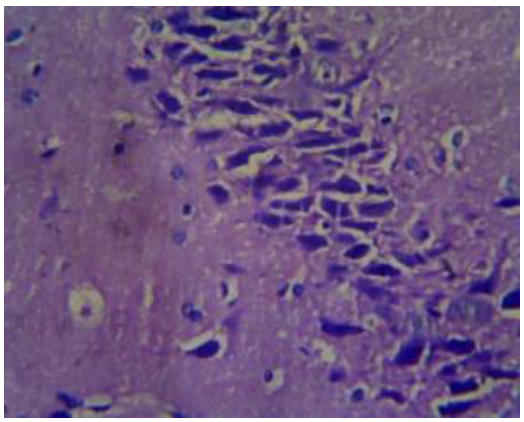
Fig 20. Effect of TIP39 treatment on brain Acetylcholine esterase level in rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests.

Figure 21: Histopathological evaluation TIP39 in rat's brain

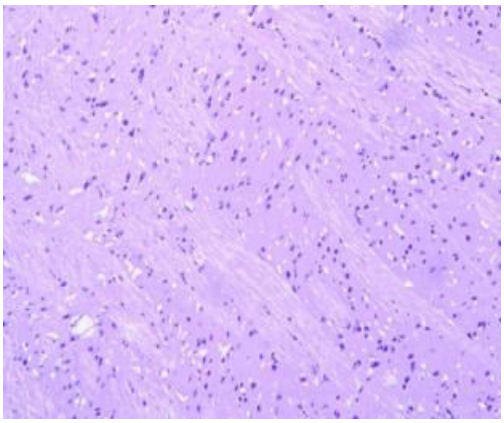
Hippocampus (40X) **Prefrontal cortex (10X)**



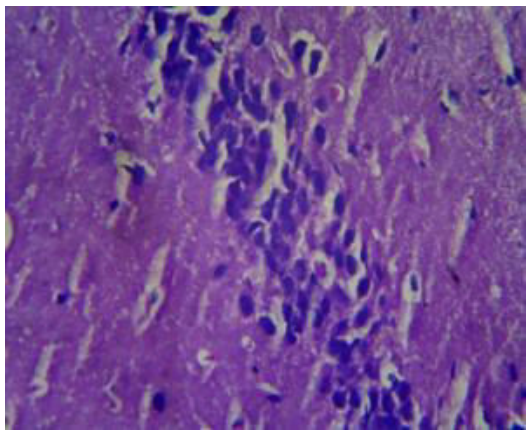
Control **Control**



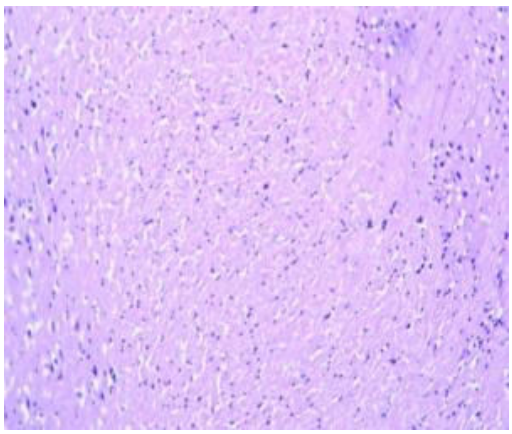
TIP39 1 nmol/rat



TIP39 1 nmol/rat



TIP39 10 nmol/rat



TIP39 10 nmol/rat

Phase II: Neurochemical assessment and behavioural role of Tuberoinfundibular Peptide-39 (TIP39) in acute restraint stressed rats

2.1. Effect of TIP39 on Elevated plus maze PM in ARS rats

There was a significant decrease in open arm entry ($F (6, 35) = 18.1$, $p < 0.001$) and increase in closed arm entry ($F (6, 35) = 21.2$, $p < 0.001$) in ARS rats when compared to control group in EPM model. Diazepam 2mg/kg significantly revered the ARS induced changes in arm entries ($F (6, 35) = 10.4$, $p < 0.001$) when compared to ARS group. Similarly, TIP39 (1nmol &10nmol) significantly reversed ($F (6, 35) = 8.4$, $p < 0.01$) the ARS induced changes in open and closed arm entry. Dose dependent activity was not observed in arm entry level.

In time spent response, ARS rats showed significant decrease in open arm ($F (6, 35) = 9.1$, $p < 0.001$) and increase in closed arm ($F (6, 35) = 12.8$, $p < 0.001$) when compared to control group. Diazepam 2mg/kg significantly revered the ARS induced changes in time spent ($F (6, 35) = 8.9$, $p < 0.001$) when compared to ARS group. Similarly, TIP39 (1nmol &10nmol) significantly reversed the ARS induced changes ($F (6, 35) = 6.2$, $p < 0.01$) in time spent in open and closed arm level. In addition to that, Time spent was significantly altered dose-dependently. Moreover, administration of the PTH2R antagonist (HYWH 1nmol/rat) along with TIP39 did not show significant changes in arm entry and in time spent as the comparison to ARS group. (Table 9)

Table 9: Effect of TIP39 on EPM test in ARS rats

Treatment	Number of entries (counts/6 min)		Time spent (sec/6min)	
	Open Arm	**Closed Arm**	**Open Arm**	**Closed Arm**
Control	9.63±1.21	7.13±0.63	62.54±15.1	193.26±21.32
ARS	3.32±0.73^{c}	13.94±1.14^{c}	21.37±7.83^{c}	271.81±26.51^{c}
Diazepam 2mg/kg	9.54±1.11^{z}	8.74±1.0^{z}	64.64±10.35^{z}	199.69±18.27^{z}
TIP39 1 nmol/rat	7.21±0.94^{a,y,k}	10.21±0.86^{a,y,k}	44.28±9.42^{a,y,k}	234.29±25.36^{a,y,k}
TIP39 1 nmol+ HYWH 1nmol/rat	4.4±1.14$^{c,\ m}$	14.32±1.32$^{\ c\ ,m}$	18.12±8.67$^{\ c,\ m}$	263.61±30.41$^{\ c,m}$
TIP39 10 nmol/rat	7.86±1.32^{a,z}	9.12±0.82^{z}	51.63±9.11$^{a,\ z}$	219.23±19.41$^{a,\ z}$
TIP39 10 nmol+ HYWH 1nmol/rat	4.31±0.94$^{\ c,m}$	14.91±0.91$^{\ c,\ m}$	26.25±7.81$^{\ c,\ m}$	251.41±26.43$^{\ c,m}$

Effect of TIP39 treatment on EPM in ARS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance $^{a,\ c}$ denote p<0.05 and p<0.001 Vs control. ^{y,z} denotes p<0.01 and p<0.001 Vs ARS. $^{k,\ m}$ denotes p<0.05, p<0.01 and Vs diazepam 2mg/kg.

2.2. Effect of TIP39 treatment on FST in ARS rats

Two hours of ARS brought a depressive state in rats, which was quantified by increased immobility time ($F(6, 35) = 13.61$, $p < 0.01$) in FST (Fig.22) in comparison to control rats. Diazepam 2 mg/kg significantly decreased the immobility time when compared to ARS group. Similarly, TIP39 1nmol &10nmol ($F(6, 35) = 5.61$, $p < 0.05$)

significantly decreased the duration of immobility time when compared to ARS rats. Moreover, administration of PTH2R antagonist (HYWH 1nmol/rat) along with TIP39 did not showed significant ($F(6,35) = 1.61$, $p>0.05$) changes on immobility time as comparison to ARS group. There was no dose-dependent activity was noted between the TIP39 1nmol and TIP39 10 nmol treated group.

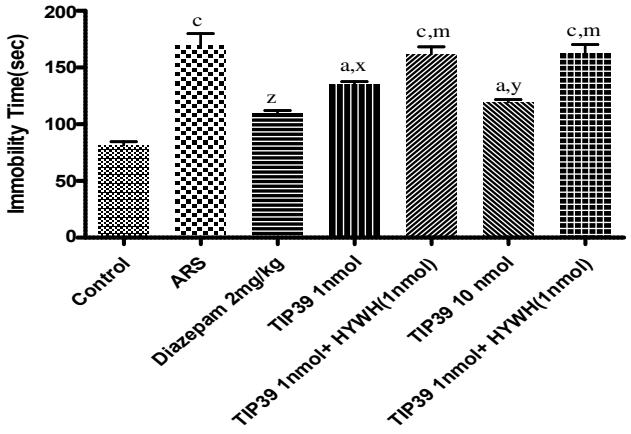


Fig 22. Effect of TIP39 treatment on FST in ARS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. a, c denote $p<0.05$ and $p<0.001$ Vs control. y,z denotes $p<0.01$ and $p<0.001$ Vs ARS. m denotes $p<0.01$ and Vs diazepam 2mg/kg.

2.3. Effect of TIP39 treatment on brain and plasma neurotransmitters in ARS rats.

ARS group showed significant decrease in plasma 5HT ($F(6, 35) = 10.1$, $p < 0.001$) and NA ($F(6,35) = 13.2$, $p < 0.001$) when compared to control group (Fig.23). Treatment with Diazepam 2mg/kg significantly increased the plasma 5HT and NA level when compared to ARS group. Administration of TIP39 (1&10nmol) significantly increased ($F(6, 35) = 4.2$, $p < 0.01$) the NA levels in plasma, where as TIP39 (1&10 nmol/rat) did not showed significant changes in plasma 5HT level ($F(6,35) = 0.46$, $p > 0.72$) when compared to the ARS rats. interestingly, treatment with TIP39 1&10nmol and

TIP39+HYWH (1nmol/rat) did not showed significant changes in plasma 5HT and NA level as compared to the ARS rats($F(6,35) = 1.06$, $p > 0.05$).

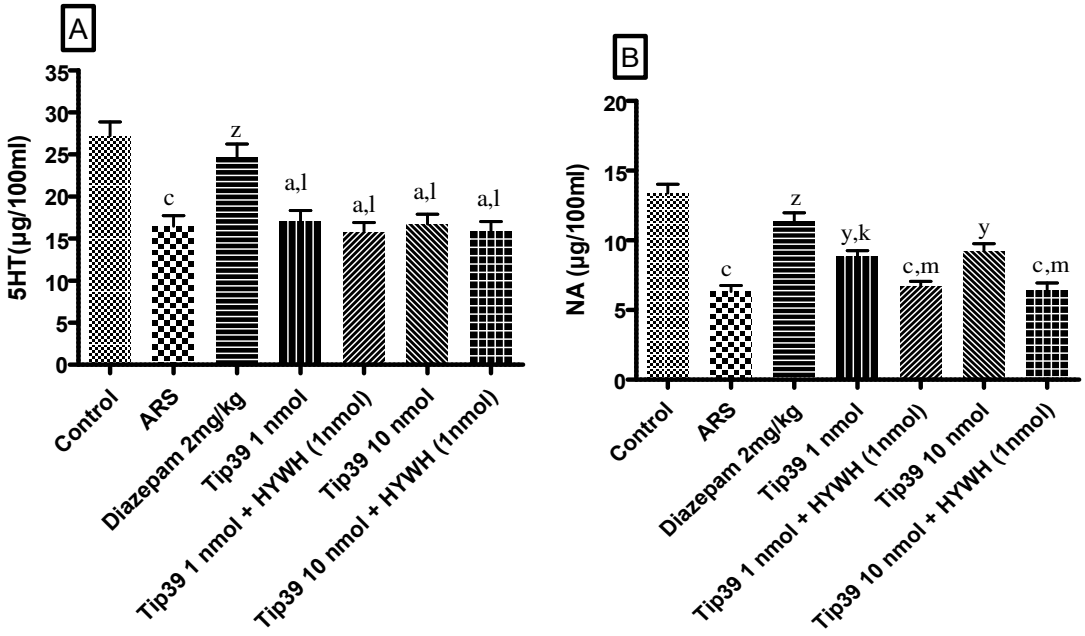


Fig 23. Effect of TIP39 treatment on Plasma 5HT and Noradrenaline in ARS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a,c denote $p<0.05$ and $p<0.001$ Vs control. y, z denotes $p<0.01$ and $p<0.001$ Vs ARS. k, l, m denotes $p<0.05$, $p<0.01$, $p<0.01$ and Vs diazepam 2 mg/kg.

ARS exposure caused a significant increase in glutamate ($F(6, 35) = 15.6$, $p < 0.001$) and decrease in GABA ($F(6, 35) = 11.3$, $p < 0.001$) activity in brain when compared to control group (Fig.24). Treatment with Diazepam 2mg/kg significantly reversed the ARS induced changes in glutamate and GABA level when compared to ARS group. TIP39 doses, i.e., 1&10nmol significantly decreased the glutamate ($F(6,35) = 2.8$, $p<0.05$) and significantly increased the GABA ($F(6,35) = 3.16$, $p < 0.05$) activity in brain;

whereas TIP39+HYWH (1nmol/rat) did not showed significant changes in glutamate and GABA activity when compared to the ARS rats ($F(6,35) = 1.5$, $p > 0.05$). These observations confirmed that TIP39 regulated NA, GABA, and glutamate through PTH2 receptor evident with the application of PTH2 receptor antagonist (HYWH).

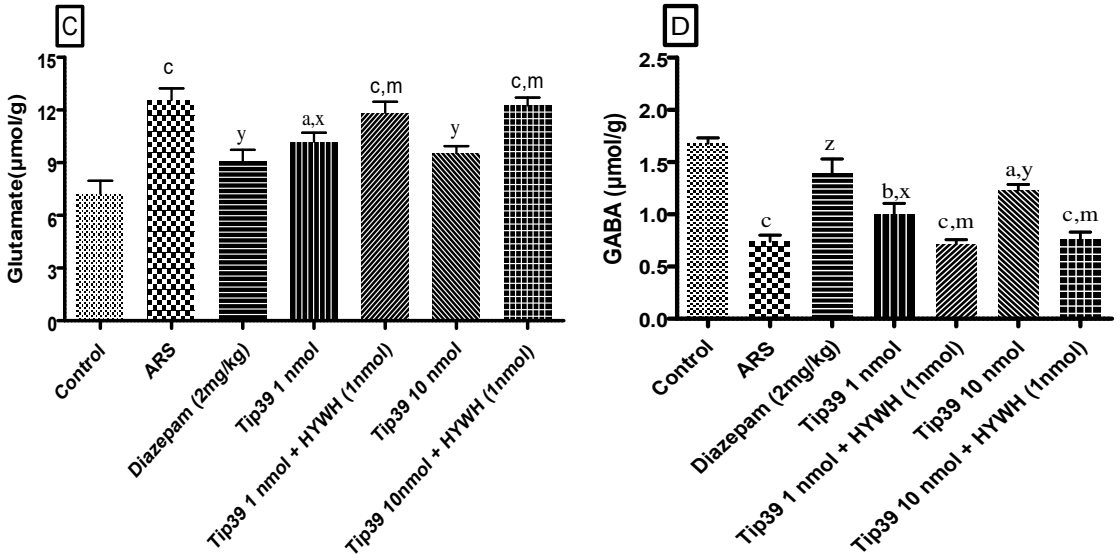


Fig 24. Effect of TIP39 treatment on brain Glutamate and GABA in ARS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a, b, c denote $p<0.05$, $p<0.01$ and $p<0.001$ Vs control. x, y denotes $p<0.05$, $p<0.01$ Vs ARS. m denotes $p<0.001$ and Vs diazepam 2mg/kg.

2.4. Effect of TIP39 treatment on Plasma corticosterone in ARS rats.

Shown in (Fig.25), ARS rats significantly increased ($F(6, 35) = 15.2$, $p < 0.001$) the plasma corticosterone when compared to control group. Diazepam at the dose of 2mg/kg significantly decreased ($F(6, 35) = 17.1$, $p < 0.001$) the corticosterone levels in plasma when compared to ARS group. Similarly, TIP39 both doses, i.e., 1&10 nmol/rat, significantly decreased corticosterone ($F(6, 35) = 8.61$, $p < 0.01$) when compared to the

ARS group. Treatment of HYWH (1nmol/rat) along with TIP39 (1&10 nmol/rat) did not significantly altered (F (6, 35) = 1.81, p>0.05) the plasma corticosterone level as comparable to ARS group.

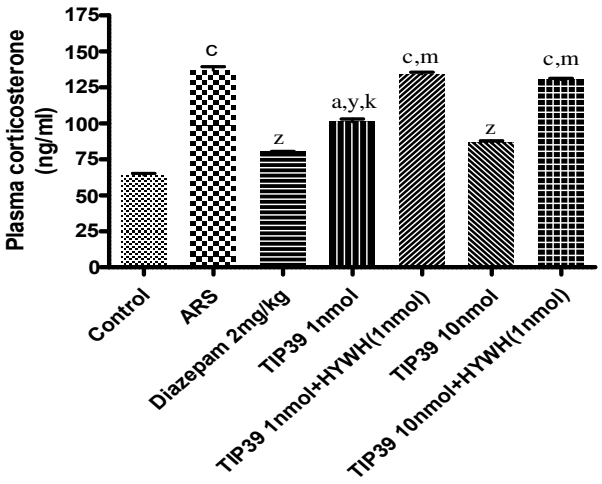


Fig 25. Effect of TIP39 treatment on Plasma corticosterone in ARS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. All data expressed as Mean± SD and statistical significance a,c denote p<0.05 and p<0.001 Vs control. y,z denotes p<0.01 and p<0.001 Vs ARS. k, m denotes p<0.05, p<0.001 and Vs diazepam 2mg/kg.

2.5. Effect of TIP39 treatment on brain TIP39 expression in ARS rats.

Shown in (Fig.26), Upon 2 hrs of acute restraint stress induction, rats have shown a significant decrease of TIP39 gene expression (F (2, 15) = 3.9, p <0.01) in brain tissue when compared to normal rats. In contrast, upon half an hour of acute restraint stress decreased the expression of TIP39 gene is observed when compared to normal rats; but there were no significant changes were exhibited (F (2,15) = 2.16, p >0.05).

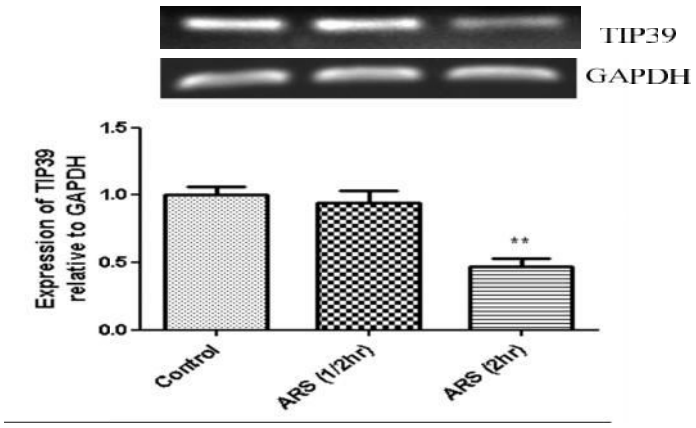


Fig 26. Effect of TIP39 treatment on brain TIP39 expression in ARS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. All data expressed as Mean± SD and statistical significance ** denotes p<0.01 Vs control.

Figure 27: Effect of TIP39 treatment on plasma 5HT in ARS rats by HPLC method.

Standard 5HT curve **Plasma 5HT curve**

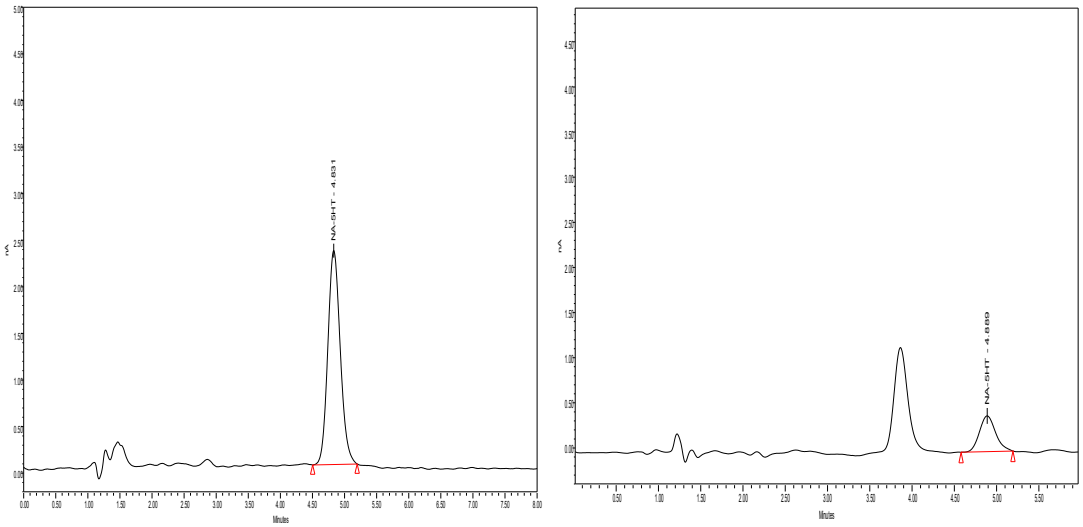


Figure 28: Effect of TIP39 treatment on plasma NA in ARS rats by HPLC method.

Standard NA curve **Plasma NA curve**

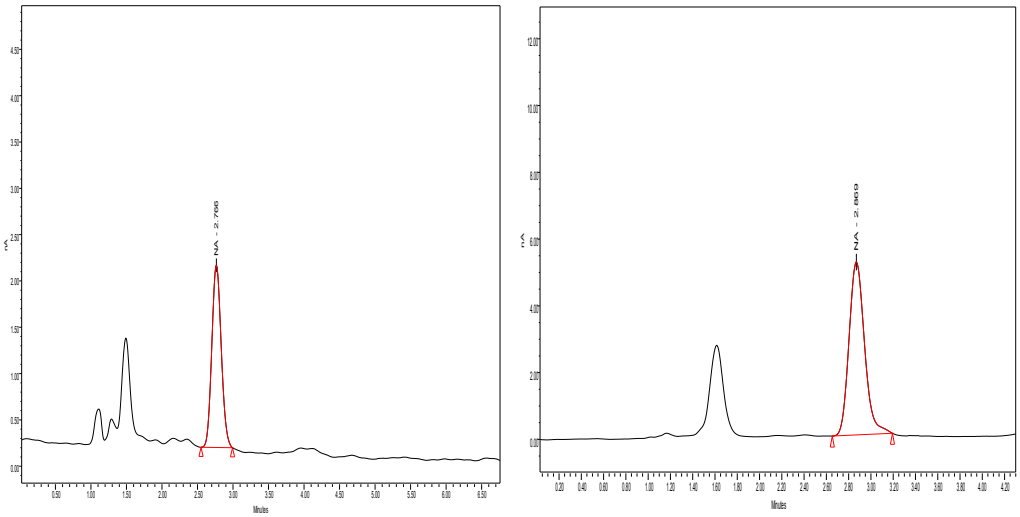
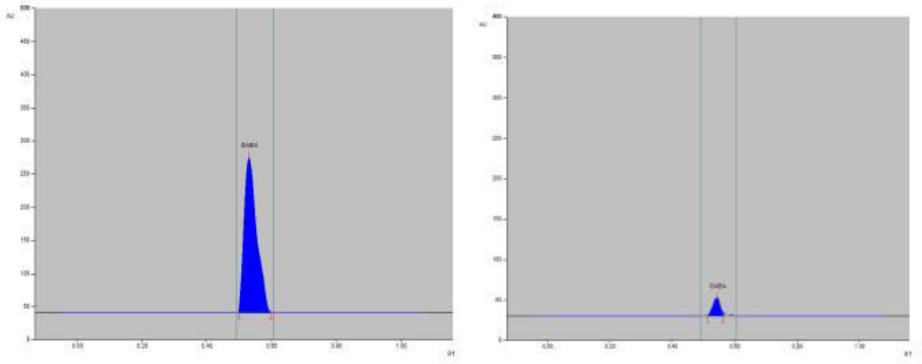
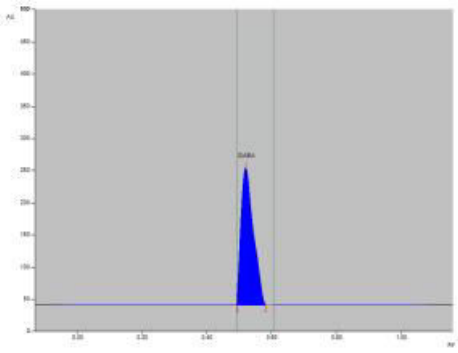


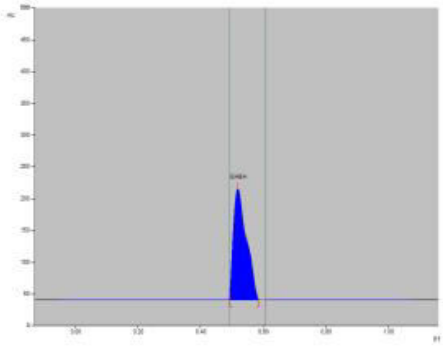
Figure 29: Effect of TIP39 treatment on brain GABA content in ARS rats by HPTLC method



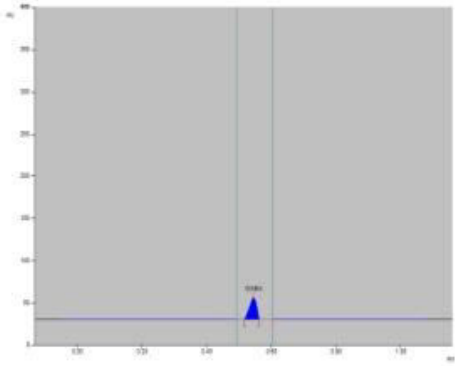
Control CUMS



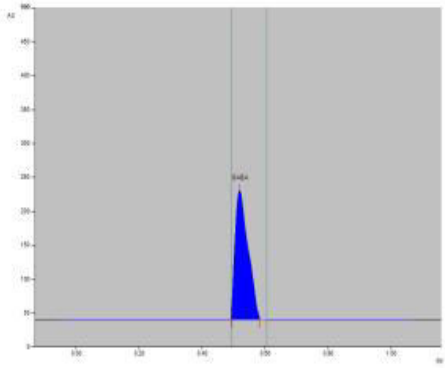
Diazepam 2mg/kg



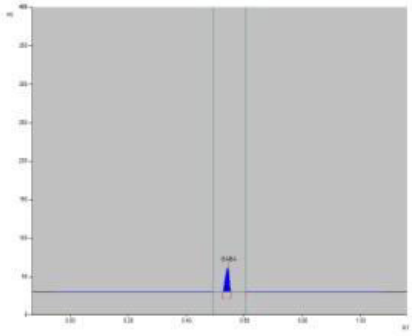
TIP39 1nmol



TIP39 1nmol+ HYWH(1nmol)

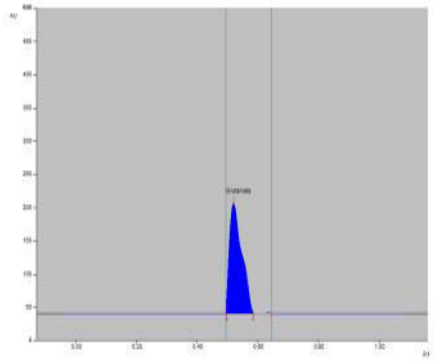


TIP39 10nmol

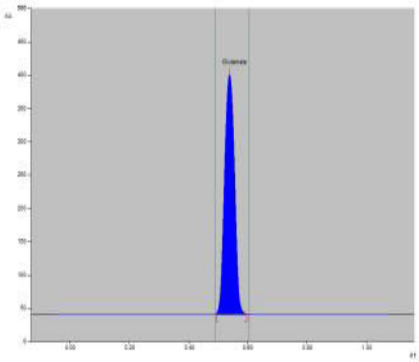


TIP39 10nmol+ HYWH(1nmol)

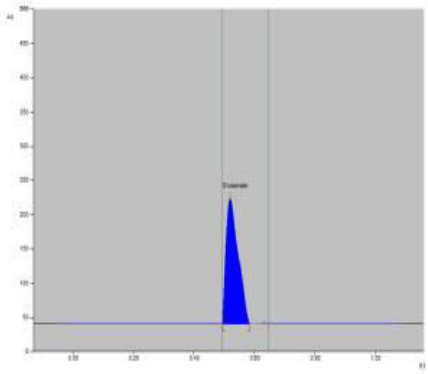
Figure 30: Effect of TIP39 treatment on brain Glutamate content in ARS rats by HPTLC method



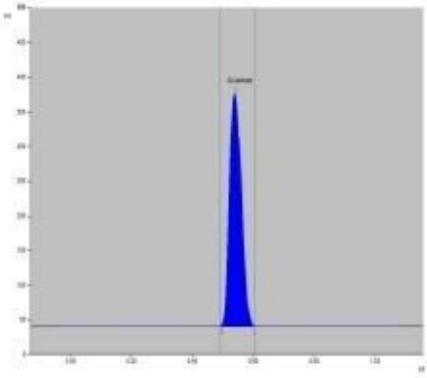
Control



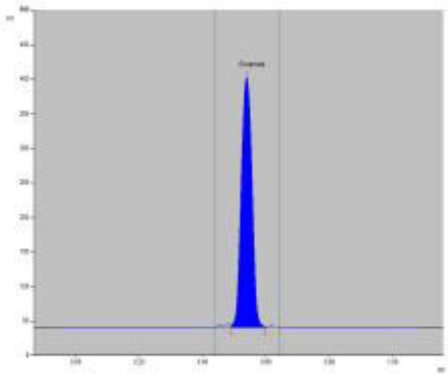
CUMS



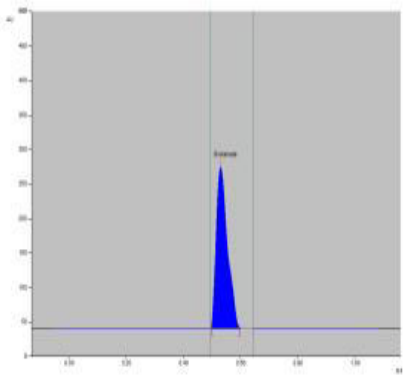
Diazepam 2mg/kg



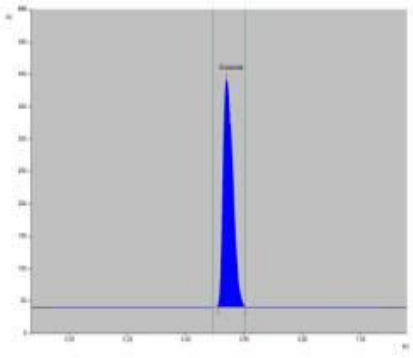
TIP39 1nmol



TIP39 1nmol+ HYWH (1nmol)

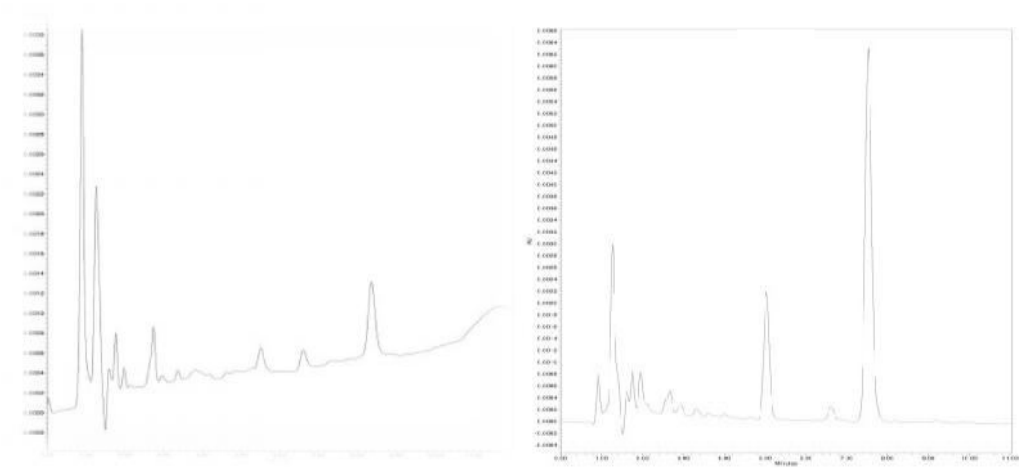


TIP39 10nmol

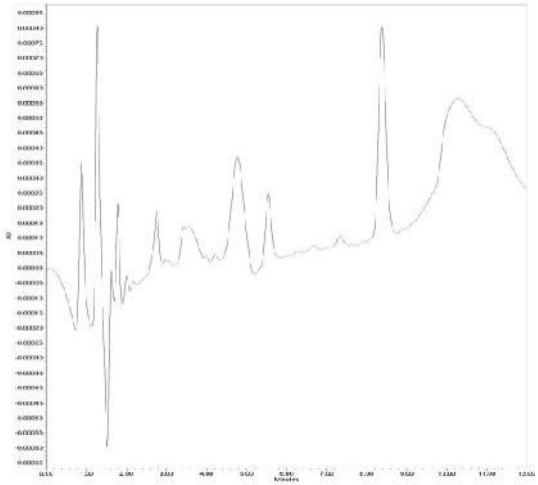


TIP39 10nmol+ HYWH (1nmol)

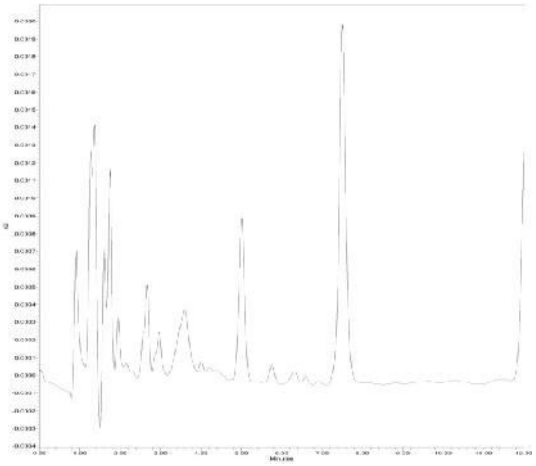
Figure 31: Effect of TIP39 treatment on Plasma corticosterone in ARS rats. By HPLC



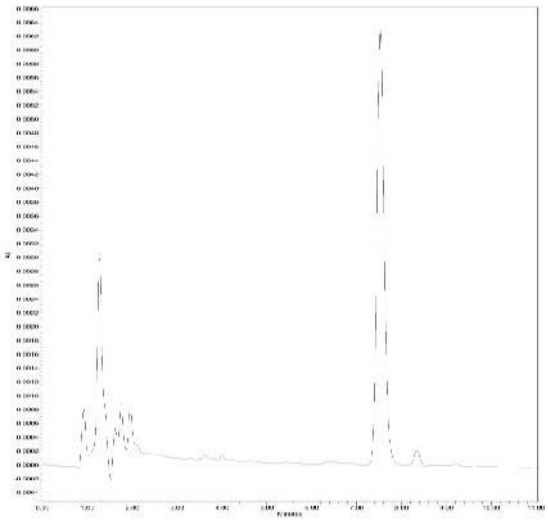
Control CUMS



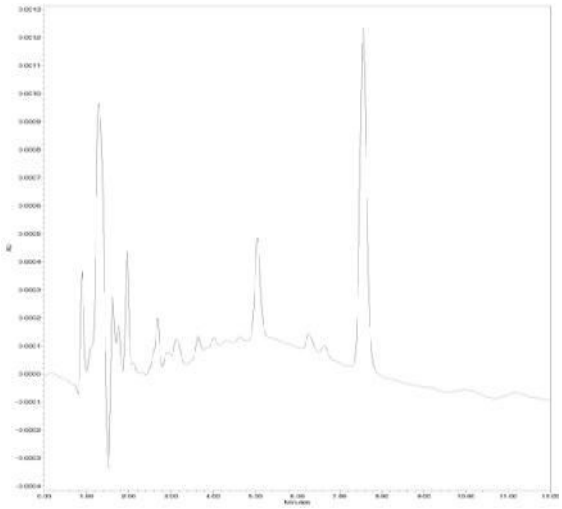
Diazepam 2mg/kg



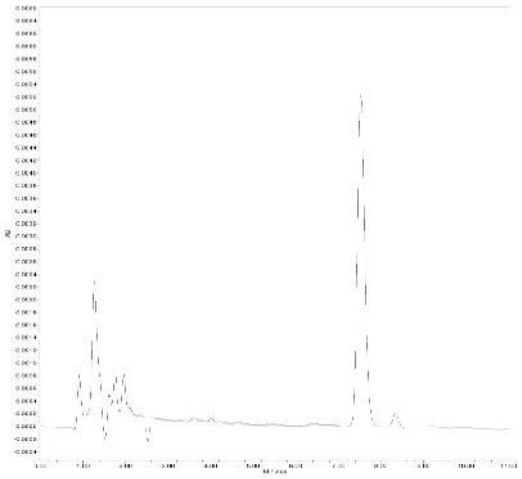
TIP39 1nmol



TIP39 1nmol+ HYWH (1nmol)



TIP39 10nmol



TIP39 10nmol+ HYWH(1nmol)

Phase III: Elucidation of role of TIP39 on chronic unpredictable mild stress-induced HPA axis dysregulation, inflammation and oxidative damage in depressive rats

3.1. Effect of ICV administration of TIP39 on anhedonia model in CUMS rats

Sucrose preference test is a familiar model to distinguish the effect of drugs on anhedonia during depressive condition (Fig.32). There was a gradual decline in the intake of sucrose in subsequent weeks in all the groups. CUMS rats showed a significant reduction in sucrose consumption as a comparison to control group. These changes confirmed the state of depression in stressed rats.

Percentage reduction in sucrose intake was observed in normal (13%), CUMS (54.8%), diazepam 2 mg/kg (19.3%) TIP39 1 nmol (26.5%) and TIP39 10 nmol (20.8%) treated animals from 1st week to 4th week. In week 3 and 4, there was a significant ($F(4, 25) = 11.64$, $P < 0.001$) reduction of sucrose intake in CUMS rats when compared to control group. Diazepam 2mg/kg exhibited significant ($F(4, 25) = 5.86$, $P < 0.01$) increase in sucrose consumption on 3rd and 4th week among rats subjected to stress as

compared to CUMS group. Chronic treatment with TIP39 (1 nmol and 10 nmol) significantly ($F(4, 25) = 3.27$, $P < 0.05$) increased the sucrose consumption when compared to CUMS group. Dose dependent response was observed between the doses of TIP39.

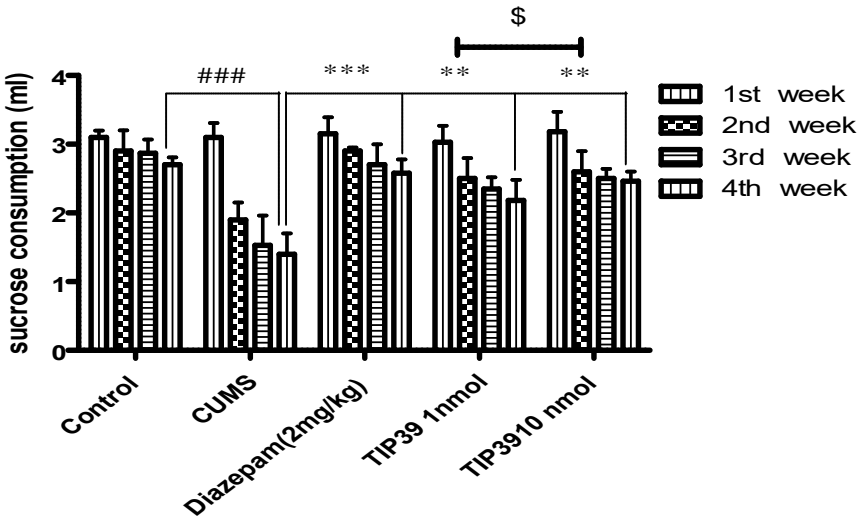


Fig 32. Effect of TIP39 treatment on sucrose consumption in CUMS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance at 4^{th}-week #, ##, ### denotes $p<0.05$, $p<0.01$ and $p<0.001$ Vs control. *, **, *** denotes $p<0.05$, $p<0.01$ and $p<0.001$ Vs CUMS. $ denotes $p<0.05$, TIP39 1nmol Vs TIP39 10 nmol at 4^{th} week.

3.2. Effect of ICV administration TIP39 on Open field test in CUMS rats

CUMS rats exhibited decreased ambulation ($F(4, 25) = 6.26$, $P<0.01$) and rearing ($F(4, 25) = 11.5$, $P<0.001$). Further it increased the grooming ($F(4, 25) =10.02$, $P<0.001$) and time spend in central compartment ($F(4, 25) = 4.8$, $P<0.01$) when compared to unstressed rats (Fig.33). Treatment with TIP39 (1 nmol & 10 nmol) significantly reversed the stress-induced behavioral changes as observed by increased ambulation, rearing and

decreased time spent in the central compartment when compared to the CUMS rats. No significant changes were observed in grooming response after TIP39 administration in comparison to CUMS rats. Results were compared with that of the standard drug Diazepam 2 mg/kg ($F(4, 25) = 5.6$, $P<0.01$).

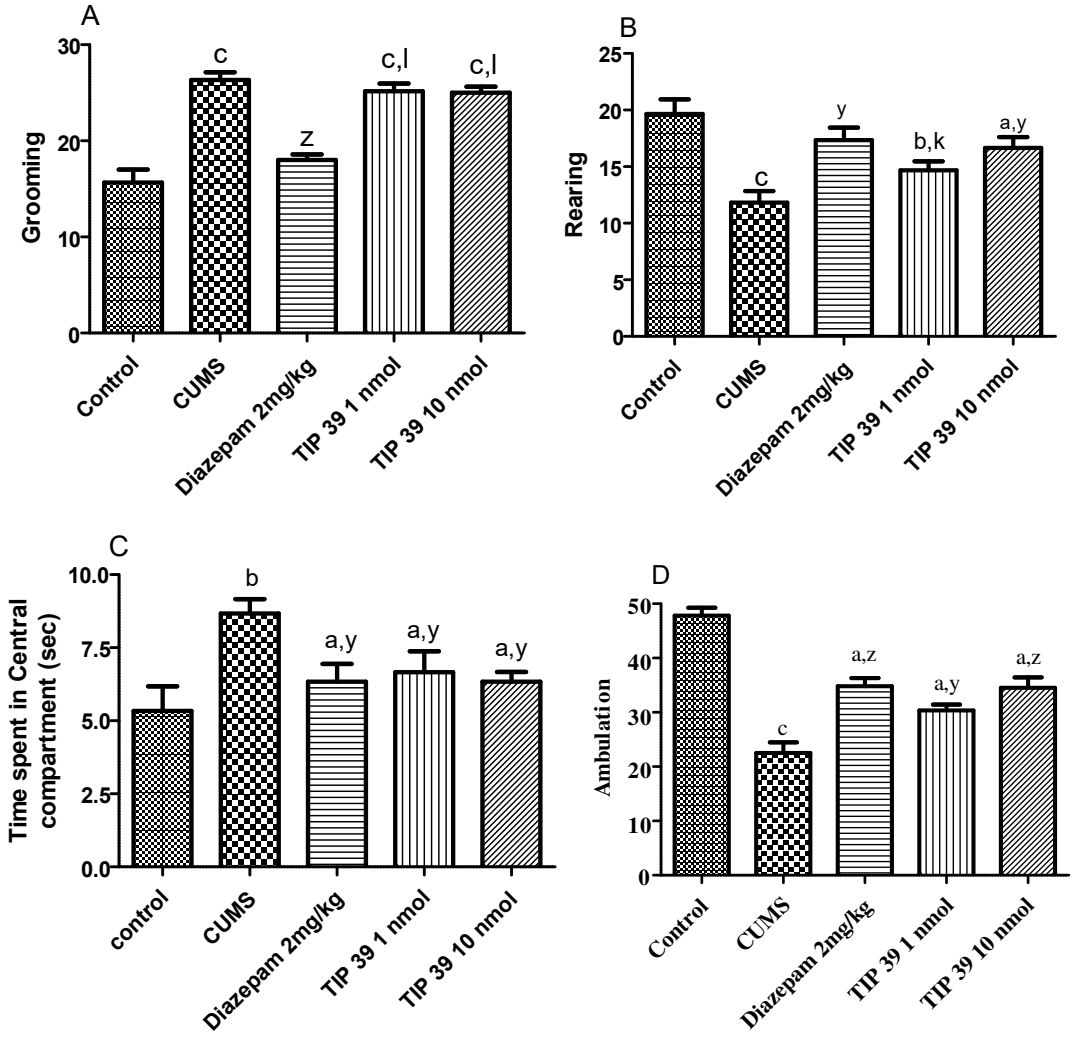
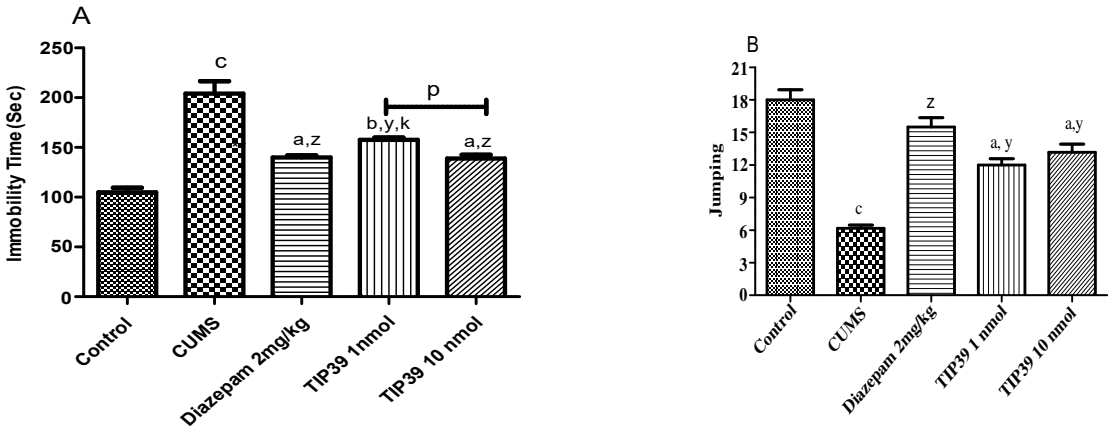


Fig 33. Effect of ICV administration of TIP39 on Open field test. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple

comparison tests. Statistical significance a, b, c denote p<0.05, p<0.01 and p<0.001 Vs control. y, z denotes p<0.01 and p<0.001 Vs ARS. k, l denotes p<0.05, p<0.01 Vs diazepam 2 mg/kg.

3.3. Effect of ICV administration of TIP39 on Forced swim test

Twenty-eight days of CUMS brought a depressive state in rats, which was quantified by parameters such as increased immobility, dipping response and decreased climbing response in FST in comparison to control rats ($F(4,25) = 14$, $P<0.001$). Fig.34 shows that the chronic administration of TIP39 1 nmol ($F(4, 25) = 5.12$, $P<0.01$) & 10 nmol ($F(4, 25) = 9.24$, $P<0.001$) in CUMS rats significantly reduced the duration of immobility time as compared to CUMS rats. In the same manner, the number of jumping responses increased ($F(4, 25) = 6.3$, $P<0.01$) and number of dipping responses decreased ($F(4, 25) = 4.61$, $P<0.01$) significantly as compared to CUMS rats. A similar effect was observed in diazepam ($P< 0.001$) at the dose of 2 mg/kg when compared to CUMS rats. TIP39 (1&10 nmol/rat) dose dependently decreased the immobility time and dipping response in CUMS rats.



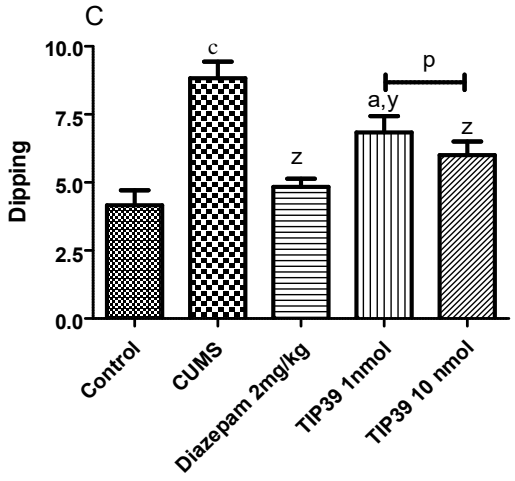


Fig 34. Effect of ICV administration of TIP39 on Forced swim test. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a, b, c denote $p<0.05$, $p<0.01$ and $p<0.001$ Vs control. y, z denotes $p<0.01$ and $p<0.001$ Vs CUMS. k, denotes $p<0.01$ Vs diazepam 2mg/kg. p, denotes $p<0.05$, TIP39 1 nmol Vs TIP39 10 nmol

3.4. Effect of ICV administration of TIP39 on Tail suspension test

Shown in (Fig.28), Dose-dependent effects of TIP39 on the immobility period in the tail suspension test are shown in (Fig. 35). CUMS-induced depressive rats exhibited a significant ($F(4, 25) = 7.65$, $P < 0.001$) increase in immobility period as compare to control group. Administration of Diazepam 2 mg/kg ($F(4, 25) = 6.79$, $P < 0.001$) in CUMS rats significantly reduced the immobility time as compared to CUMS rats. Similar type of results were observed in the TST in which TIP39 at doses of 1 & 10 nmol ($F(4, 25) = 4.91$, $P < 0.01$) significantly reduced the immobility period when compared to CUMS group.

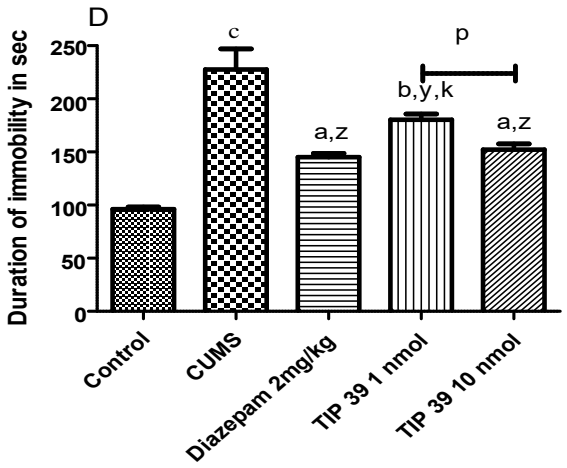


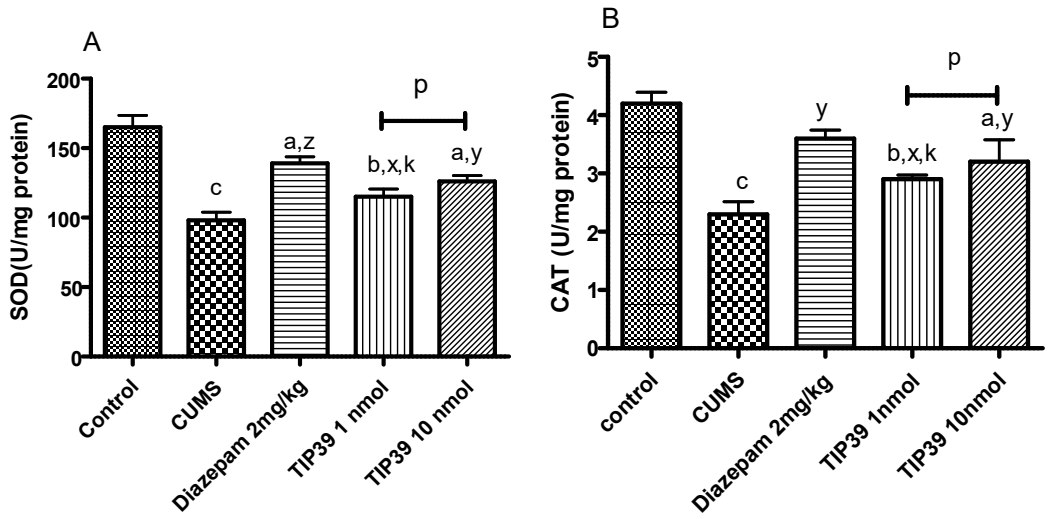
Fig 35. Effect of ICV administration of TIP39 on Tail suspension test. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a, b, c denote $p<0.05$, $p<0.01$ and $p<0.001$ Vs control. y,z denotes $p<0.01$ and $p<0.001$ Vs CUMS. k, denotes $p<0.05$ Vs diazepam 2mg/kg. p denotes $p<0.05$, TIP39 1 nmol Vs TIP39 10 nmol

3.5. Effect of ICV administration of TIP39 on antioxidant biomarkers in prefrontal cortex and Hippocampus

The effect of TIP39 on the hippocampus and prefrontal cortex antioxidant system is shown in (Fig. 36 &37). From the results depicted, diazepam 2 mg/mg significantly alters the oxidative parameters as compared to CUMS group in the hippocampus ($F(4, 25) = 13.03$, $P < 0.001$) and the prefrontal cortex ($F(4, 25) = 4.72$, $P < 0.01$). However, CUMS rats showed a significant elevation in the MDA levels ($F(4, 25) = 12.3$, $P < 0.001$) and a significant turn down in the activities of SOD ($F(4, 25) = 8.14$, $P < 0.001$),

CAT (F (4, 25) = 10.68, P < 0.001), and GSH (F (4, 25) = 9.12, P < 0.001) in the hippocampus and the prefrontal cortex as compared to the control group.

ICV administration of TIP39 at doses of 1 nmol and 10 nmol showed significant increase in the activities of SOD (F (4, 25) = 5.3, P < 0.01) and CAT F (4, 25) = 3.6, P < 0.05), and GSH (F (4, 25) = 3.72, P < 0.05) as compared to CUMS groups in the hippocampus and also treatment with TIP39 showed significant decline in the MDA (F (4, 25) = 4, P < 0.05) levels in the hippocampus. In the prefrontal cortex, TIP39 (1 nmol & 10 nmol) exhibited significant increase in the activities of enzymatic SOD (F (4,25) = 3.9, P < 0.05), CAT (F (4, 25) = 3.63, P < 0.05), GSH (F (4, 25) = 4.6, P < 0.01) and significant decline in the MDA ((F (4, 25) = 6.9, P < 0.01) as compared to CUMS group. Dose dependent activity was observed in all the antioxidant parameters except MDA in prefrontal cortex. Similarly, except in CAT and MDA in hippocampus



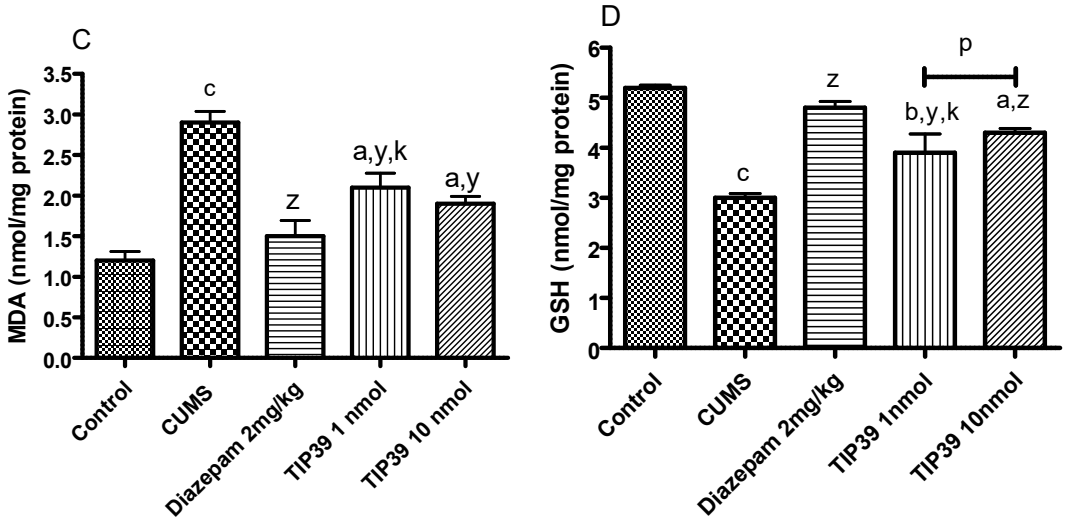
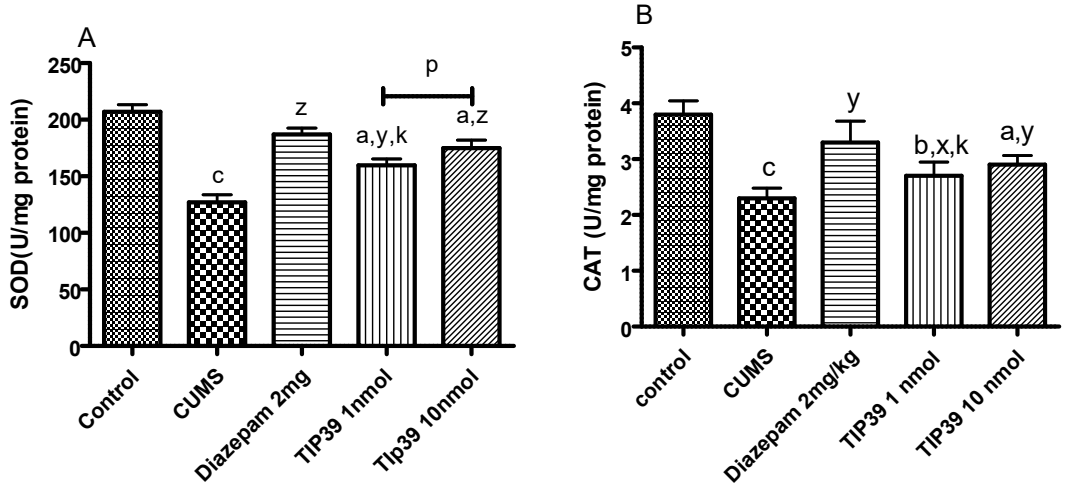


Fig 36. Effect of ICV administration of TIP39 on antioxidant biomarkers in the prefrontal cortex. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a, b, c denote $p<0.05$, $p<0.01$ and $p<0.001$ Vs control. x, y,z denotes $p<0.05$, $p<0.01$ and $p<0.001$ Vs CUMS. k, denotes $p<0.05$, Vs diazepam 2mg/kg. P denotes $p<0.05$, TIP39 1 nmol Vs TIP39 10 nmol.

3.6. Effect of ICV administration of TIP39 on antioxidant biomarkers in Hippocampus



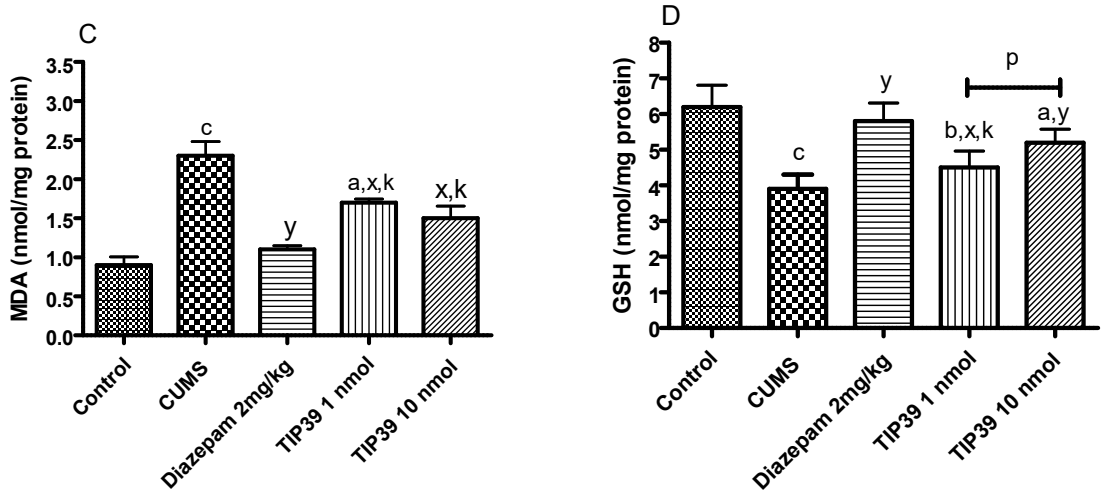


Fig 37. Effect of ICV administration of TIP39 on antioxidant biomarkers in Hippocampus. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a, b, c denote $p<0.05$, $p<0.01$ and $p<0.001$ Vs control. x, y,z denotes $p<0.05$, $p<0.01$ and $p<0.001$ Vs CUMS. k, denotes $p<0.05$, Vs diazepam 2mg/kg. p, denotes $p<0.05$, TIP39 1nmol Vs TIP39 10 nmol

3.7. Effect of ICV administration of TIP39 on proinflammatory markers

Upon CUMS induction, rats have shown a significant increase of IL-6, IL-1β, and TNF-α in brain tissue as compared to normal rats ($F(4, 25) = 11.4$, $P < 0.001$) (Fig.38). No significant difference was observed in the levels of IL-6, IL-1β, and TNF-α between diazepam treated rats and normal rats. However, diazepam 2 mg/kg had significantly ($F(4, 25) = 8.61$, $P < 0.01$) reduced the IL-6, IL-1β, and TNF-α levels in the brain as compared to CUMS rats. Chronic ICV administration of TIP39 1 & 10 nmol significantly reduced the IL-6 ($F(4, 25) = 3.25$, $P < 0.05$), IL-1 β ($F(4, 25) = 8.9$, $P < 0.001$) and TNF-α ($F(4, 25) = 4.42$, $P < 0.05$) levels in brain tissue as compared to CUMS induced rats

(Fig.7). Dose dependent activity was observed between the TIP39 1nmol& 10nmol in IL-6, IL-1β, and TNF-α levels in brain tissue.

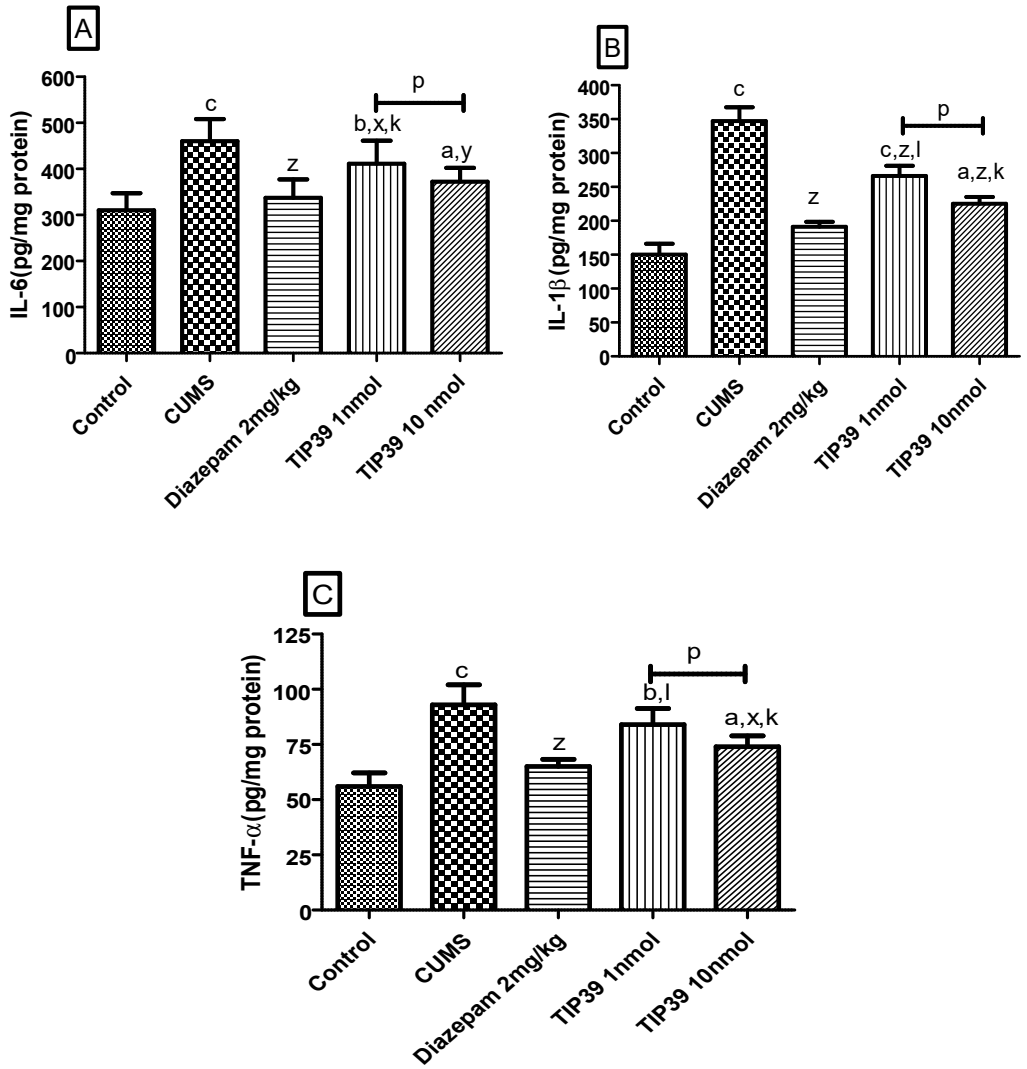


Fig 38. Effect of ICV administration of TIP39 on proinflammatory markers. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a, b, c denote $p<0.05$, $p<0.01$ and $p<0.001$ Vs control. x, y, z denotes $p<0.05$, $p<0.01$ and $p<0.001$ Vs CUMS. k, l denotes $p<0.05$, $p<0.01$, Vs diazepam 2mg/kg. P denotes $p<0.05$,TIP39 1nmol Vs TIP39 10 nmol

3.8. Effect of ICV administration of TIP39 on brain ATP content.

Shown in (Fig.39), Levels of energy molecule were studied after CUMS induction. CUMS group showed significant decrease ($F(4, 25) = 10.9$, $P < 0.001$) in brain ATP content when compared to control group. Administration of diazepam 2mg/kg significantly $F(4, 25) = 8.8$, $P < 0.001$) reversed the CUMS induced brain ATP deprivation as comparison to CUMS group. The chronic administration of TIP39 1 nmol ($F(4, 25) = 6.12$, $P<0.01$) & 10 nmol ($F(4, 25) = 8.24$, $P<0.001$) significantly increased the brain ATP content as compared to CUMS rats. TIP39 (1&10 nmol/rat) dose dependently increased the brain ATP content in CUMS rats.

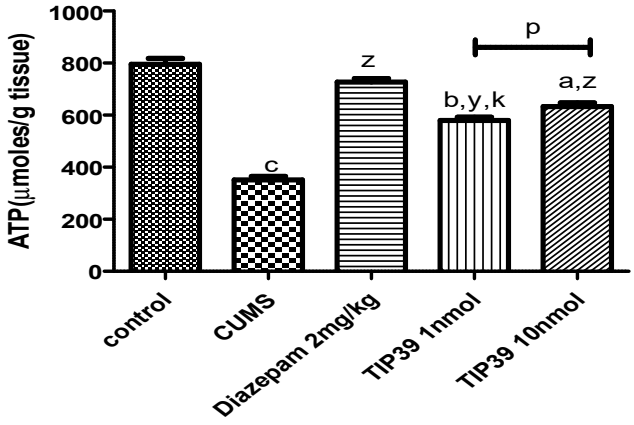


Fig 39. Effect of ICV administration of TIP39 on energy metabolite. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a, b, c denote $p<0.05$, $p<0.01$ and $p<0.001$ Vs control. y,z denotes $p<0.01$ and $p<0.001$ Vs CUMS. K denotes $p<0.01$, Vs diazepam 2mg/kg. p denotes $p<0.05$, TIP39 1nmol Vs TIP39 10 mol.

Phase IV: Elucidation of the role of TIP39 in chronic unpredictable mild stress-induced learning and memory impairment in rats.

4.1. Effect of TIP39 on Morris water maze Performance in CUMS rats

In MWM test, all the groups rapidly learned the location of the submerged hidden platform and reached within 20 sec on day 6 of the trials, reflecting acquisition (learning). All the rats showed a reduction in the escape latency time throughout the training period. The rats significantly spent more time in the target quadrant when compared to other quadrants indicated that memory or retrieval.

CUMS induction significantly reduced the time spent in the target quadrant ($F(4, 25) = 13.4$, $P < 0.001$) in rats during retrieval trial reflecting impairment of memory. Diazepam 2mg/kg treatment showed significant increase ($F(4, 25) = 9.21$, $P < 0.001$) in time spent in target quadrant in comparison with the CUMS rats. Treatment with TIP39 1 &10 nmol exhibited dose dependent increase ($F(4, 25) = 7.32$, $P < 0.01$) in time spent in target quadrant in comparison to CUMS rats. (Fig.40)

The decreased time to reach the target quadrant indicates the memory of the animal in searching missing platform. CUMS rats resulted in significant increase in time taken to reach the target quadrant in comparison to control rats ($F(4, 25) = 9.41$, $P < 0.001$). Diazepam 2mg/kg treatment showed significant decrease ($F(4, 25) = 8.24$, $P < 0.001$) in time taken to reach the target quadrant in comparison to CUMS rats. Similarly, TIP39 both the doses (1&10 nmol) exhibited decrease ($F(4, 25) = 5.9$, $P < 0.01$) in time taken to reach the target quadrant in comparison to CUMS rats.

In addition to this, CUMS rats resulted in significant decrease ($F(4, 25) = 11.24$, $P < 0.001$) in number of crossings of rats in the target quadrant in comparison to control

rats. Treatment with diazepam showed a significant increase ($F(4, 25) = 10.1$, $P < 0.001$) in number of crossings in the target quadrant in comparison to CUMS rats. TIP39 (1&10 nmol) administered rats exhibited increase ($F(4, 25) = 3.4$, $P < 0.05$) in the number of crossings in target quadrant in comparison to CUMS rats.

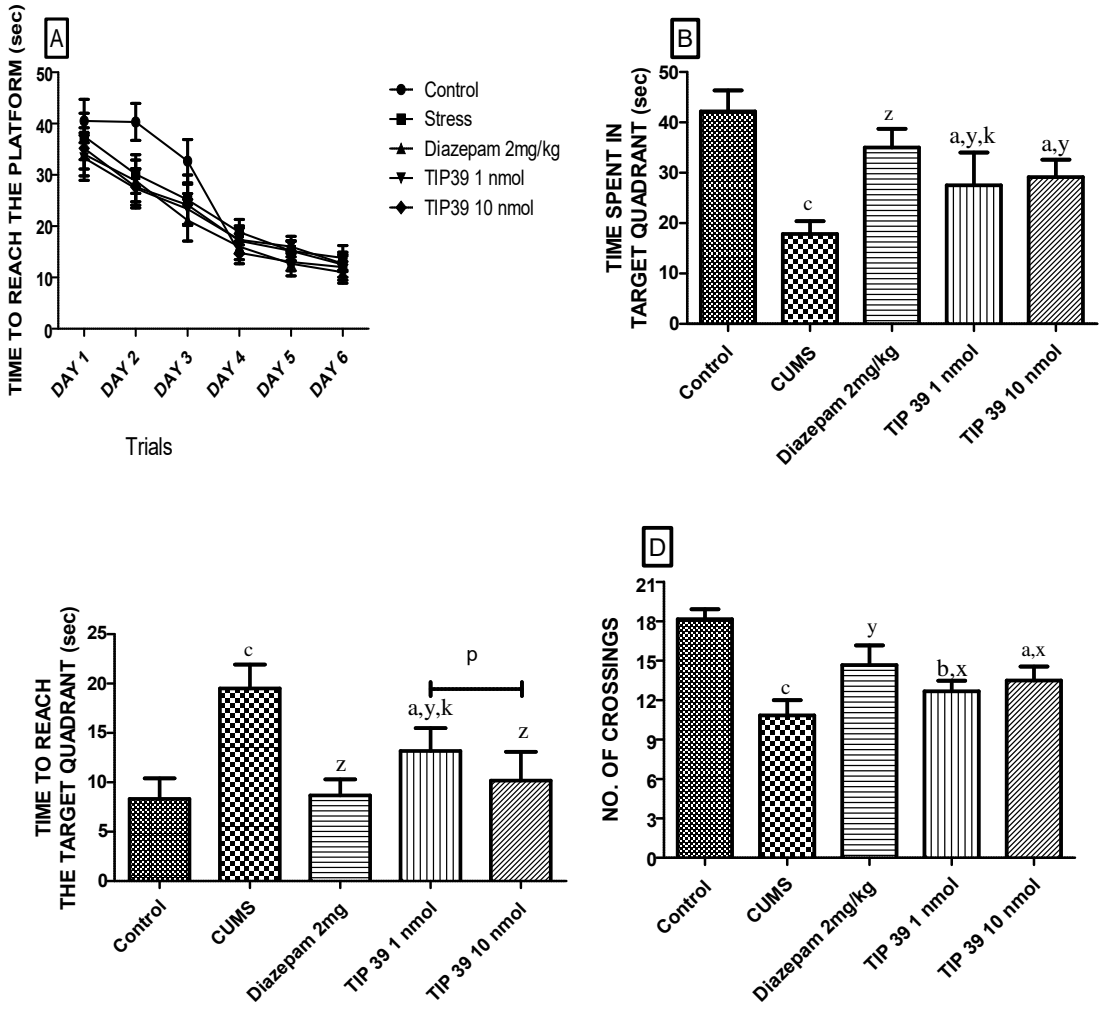
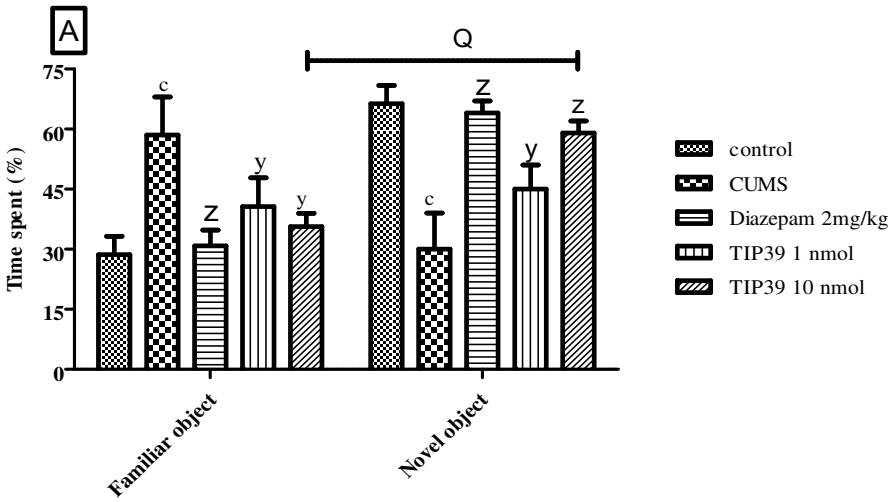


Fig 40. Effect of TIP39 on Morris water maze Performance. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple

comparison tests. Statistical significance a, b, c denote p<0.05, p<0.01 and p<0.001 Vs control. x, y,z denotes p<0.05, p<0.01 and p<0.001 Vs CUMS. k, denotes p<0.05, Vs diazepam 2 mg/kg.

4.2. Effect of TIP39 on Novel object Recognition test in CUMS rats

In NORT test, CUMS rats significantly increased the time to reach and decreased the time spent with novel object ($F(4, 25) = 7.9$, $P < 0.001$); decreased time to reach and increased time spent with familiar object ($F(4, 25) = 6.8$, $P < 0.001$) was observed when compared to normal rats (Fig.41). Diazepam significantly reversed the CUMS induced changes towards the familiar ($F(4, 25) = 12.3$, $P < 0.001$) and novel objects ($F(4, 25) = 10.2$, $P < 0.001$). TIP39 (1 &10 nmol) significantly decreased the time to reach and increased the time to spent with novel object ($F(4, 25) = 6.9$, $P < 0.01$) and significant increase in time to reach and decrease in time spent with familiar object ($F(4, 25) = 5.7$, $P < 0.01$) was observed as comparable to CUMS rats.



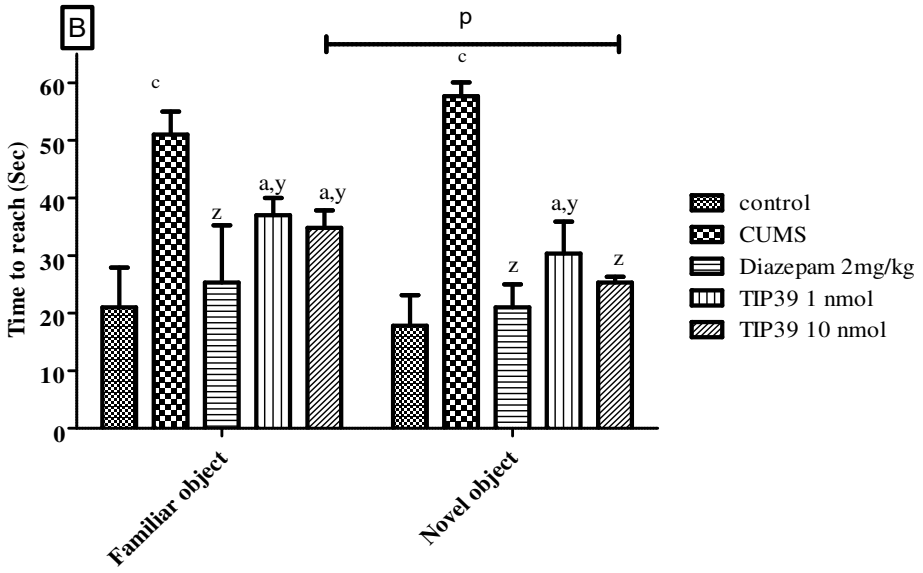


Fig 41: Effect of TIP39 on Novel object Recognition test in CUMS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a,c denote $p<0.05$, and $p<0.001$ Vs control. y,z denotes $p<0.01$ and $p<0.001$ Vs CUMS. p, q denotes $p<0.05$, $p<0.01$,familiar Vs novel at TIP39 10 nmol

4.3. Effect of TIP39 on Y maze performance in CUMS rats.

Y maze test is used to evaluate the spatial working and short term memory performance. Shown in (Fig.42). ICV administration of TIP39 was examined on Spontaneous alternative performance in Y maze. CUMS rats showed ($F(4, 25) = 17.9$, $P < 0.001$) decreased spontaneous alteration performance as compare to control group. Treatment with diazepam 2 mg/kg ($F(4, 25) = 14.2$, $P < 0.001$) exhibited significant increase in alteration performance in comparison with the CUMS group. Similarly, TIP39 1&10 nmol ($P < 0.001$) significantly increased ($F(4, 25) = 5.9$, $P < 0.05$) the Spontaneous alternative performance when compared to CUMS group.

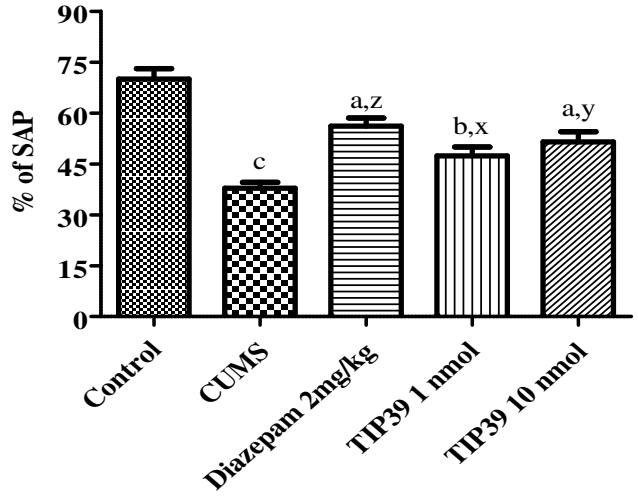


Fig 42: Effect of TIP39 on Y maze performance in CUMS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a, b, c denote $p<0.05$, $p<0.01$ and $p<0.001$ Vs control. x, y,z denotes $p<0.05$, $p<0.01$ and $p<0.001$ Vs CUMS.

4.4. Effect of TIP39 on mEPM in CUMS rats.

In mEPM test (Fig.43), CUMS rats significantly increased the transfer latency time when compared to normal rats ($F(4, 25) = 16.3$, $P < 0.001$). Treatment with diazepam (2 mg/kg) showed significant decrease ($F(4, 25) = 11.6$, $P < 0.001$) in transfer latency as comparable to CUMS rats. Similarly, Administration of TIP39 (1 & 10 nmol) significant decrease ($F(4, 25) = 6.3$, $P < 0.01$) in transfer latency as comparable to CUMS rats. Dose dependent activity was observed between the TIP39 1 & 10 nmol treated rats.

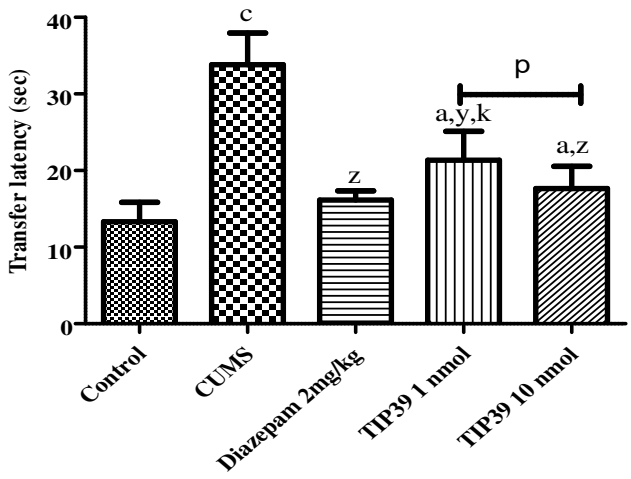


Fig 43: Effect of TIP39 on mEPM in CUMS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a, c denote p<0.05 and p<0.001 Vs control. y,z denotes p<0.01 and p<0.001 Vs CUMS. k, denotes p<0.01,Vs diazepam 2 mg/kg. P denotes p<0.05,TIP39 1nmol Vs TIP39 10 nmol.

4.5. Effect of TIP39 on Acetyl cholinesterase activity in CUMS rats:

Upon CUMS induction, rats have shown significant increase of brain acetyl cholinesterase activity ($F(4, 25) = 5.6$, $P < 0.001$) shown in Table (10). Diazepam at the dose of 2mg/kg significantly ($F(4, 25) = 4.2$, $P < 0.01$) reduced AChE activity in comparison with CUMS group and reaching near values to normal group. Dose dependent activity was noted for TIP39 1&10 nmol ($F(4, 25) = 3.8$, $P < 0.05$) reflected that significant reduction in AChE activity as compared to CUMS group.

Table 10: Effect of TIP39 on Acetyl cholinesterase Activity

S.No	Treatment	Change In Absorbance (ΔA/min)	Protein Concentration (mg/ml)	Rate of Enzymatic Activity (µmoles/min/mg protein)
1	Control	0.05364	0.725	0.5446×10^{-4}
2	CUMS	0.08045	0.558	0.927×10^{-4}
3	CUMS+ Diazepam 2 mg/kg	0.02605	0.521	0.682×10^{-4}
4	CUMS+ TIP39 1 nmol/rat	0.06275	0.810	0.830×10^{-4}
5	CUMS+ TIP39 10nmol/rat	0.04745	0.529	0.724×10^{-4}

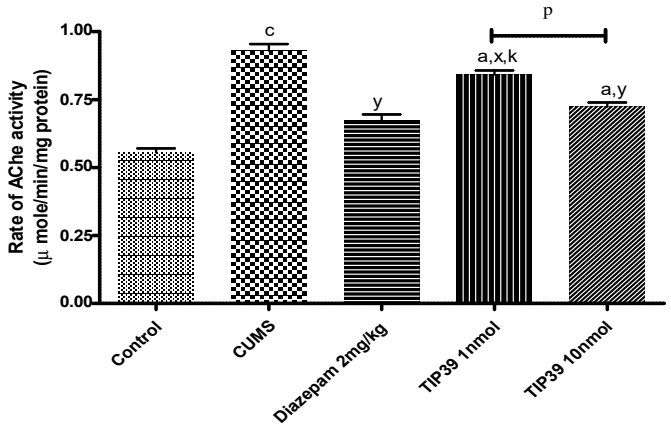


Fig 44: Effect of TIP39 on Acetylcholinesterase activity in CUMS rats. All data expressed as Mean± SD and statistical significance a, b, c denote p<0.05, p<0.01 and p<0.001 Vs control. x, y,z denotes p<0.05, p<0.01 and p<0.001 Vs CUMS. k, l, m denotes p<0.01, p<0.05, p<0.01 and Vs diazepam 2mg/kg. p, q, r denotes p<0.05, p<0.01 and p<0.001,TIP39 1nmol Vs TIP39 10 mol.

4.6. Effect of TIP39 on GR/MR gene expression in CUMS rats.

ICV administration of TIP39 significantly altered the GR and MR expression in brain after chronic stress for 28 days in rats. GR expression was significantly elevated in rats treated with CUMS ($F(4, 25) = 8.4$, $P < 0.001$) when compared to control group. In contrast, diazepam (2 mg/kg) treated rats significantly reversed the CUMS induced GR elevation ($F(4, 25) = 5.6$, $P < 0.001$) in rats. Similarly, significant decrease ($F(4, 25) = 3.6$, $P < 0.05$) was noted on GR expression after TIP39 (1 & 10 nmol) administration in comparison to CUMS group. In MR expression study, CUMS treatment significantly reduced the MR expression ($F(4, 25) = 4.3$, $P < 0.01$) in rat brain when compared to control group. Diazepam (2 mg/kg) significantly increased the MR expression in rat brain ($F(4, 25) = 3.2$, $P < 0.05$) when compared to CUMS treated rats. On treatment with TIP39 (1& 10 nmol), there is no significant changes were observed in comparison to CUMS group ($F(4, 25) = 2.1$, $P > 0.05$).

GR/MR ratio was calculated for all these groups (Fig.45). GR/MR ratio was significantly elevated ($F(4, 25) = 20.3$, $P < 0.001$) in rats treated with CUMS as comparison to control group. Standard drug diazepam (2 mg/kg) treated groups showed significant decrease in GR/MR ratio ($F(4, 25) = 15.4$, $P < 0.001$) when compared to CUMS treated group. Significant decrease of GR/MR ratio was noted after TIP39 (1& 10 nmol) administration ($F(4, 25) = 6.7$, $P < 0.01$) in comparison to CUMS group. Dose dependent activity was observed between the TIP39 1 & 10 nmol treated CUMS rats.

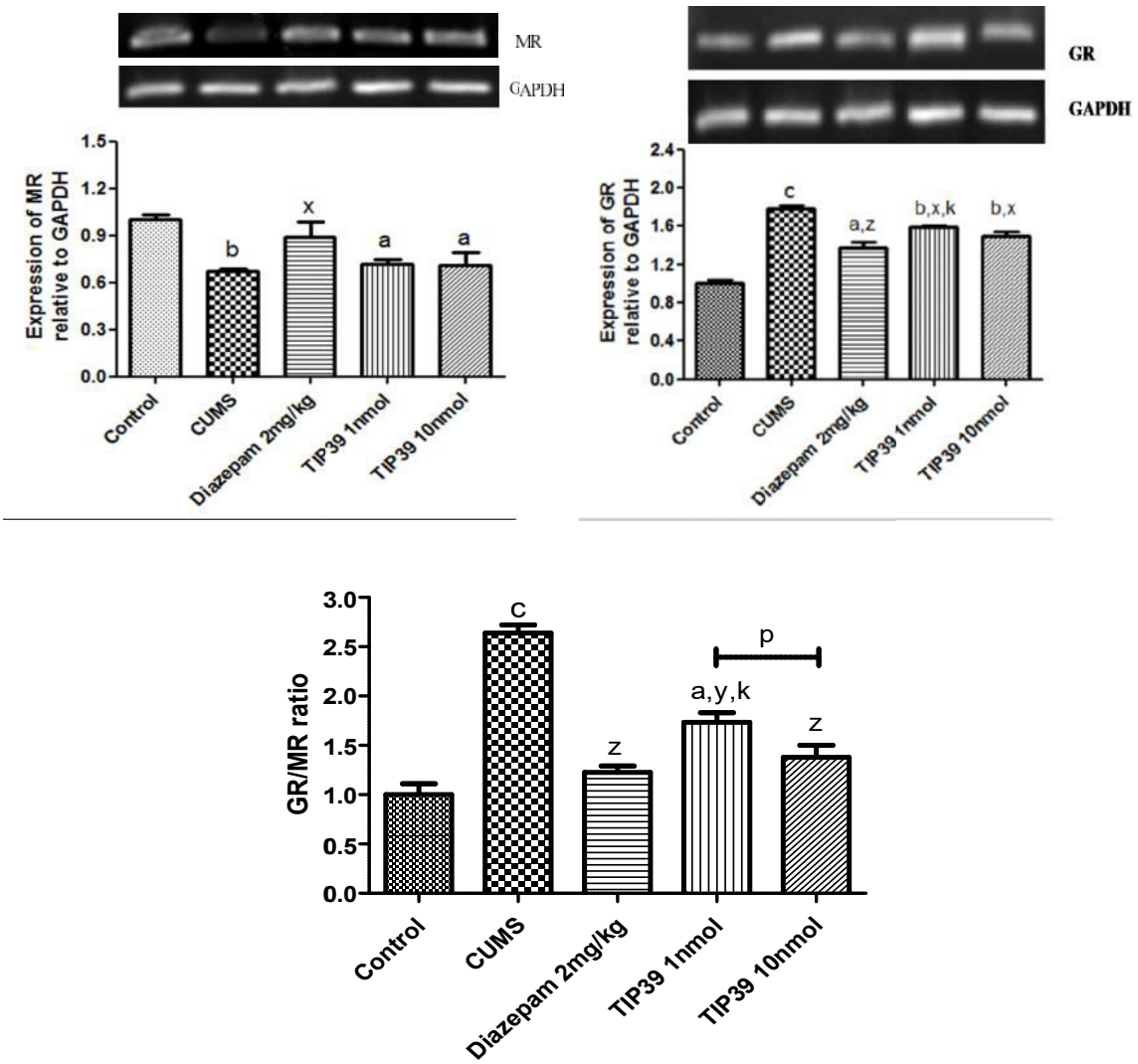


Fig 45: Effect of TIP39 on GR/MR gene expression in CUMS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. Statistical significance a, b, c denote p<0.05, p<0.01 and p<0.001 Vs control. x, y,z denotes p<0.05, p<0.01 and p<0.001 Vs CUMS. K denotes p<0.01, p Vs diazepam 2 mg/kg. p denotes p<0.05TIP39 1nmol Vs TIP39 10 nmol.

4.7. Effect of TIP39 on GAD enzyme activity by ELISA method

CUMS treated rats significantly decreased the brain GAD enzyme expression ($F(4, 25) = 14.7$, $P < 0.001$) as compared to control group (Fig.46). Diazepam (2 mg/kg) significantly increased the GAD enzyme expression ($F(4, 25) = 9.8$, $P < 0.001$) when compared to CUMS group in cerebral regions. Similarly, treatment with TIP39 (1&10nmol) significantly increased ($F(4, 25) = 5.3$, $P < 0.05$) the GAD enzyme expression in brain when compared to CUMS group in cerebral regions. Observations reflected that TIP39 induced GAD enzyme activity indirectly involved in the conversion of glutamate to GABA in the brain, which in turn resulted in stress relieving action in rats. Dose dependently TIP39 1&10 nmol increased the GAD enzyme activity in rats ($p<0.05$) as compared to CUMS group.

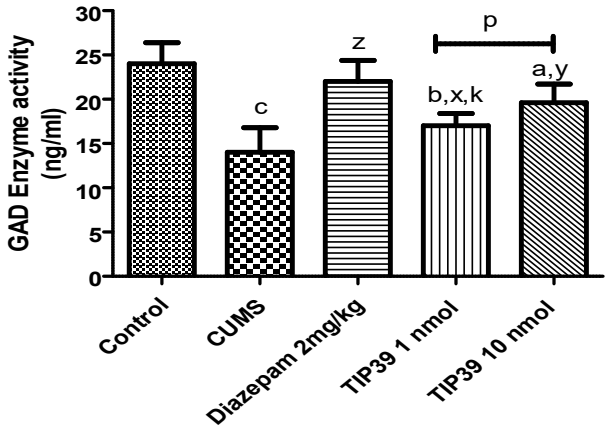
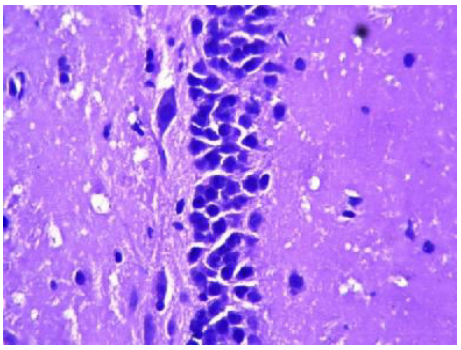
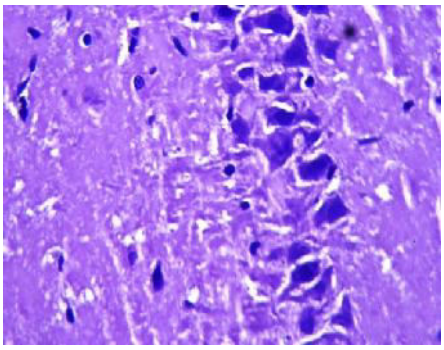


Fig 46: Effect of TIP39 on GAD enzyme activity in CUMS rats. All data expressed as mean± SD, n=6. Statistical analysis was carried out in one way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. a, b, c denotes $p<0.05$, $p<0.01$ and $p<0.001$ Vs control. x, y,z denotes $p<0.05$, $p<0.01$ and $p<0.001$ Vs CUMS. k denotes $p<0.01$ Vs diazepam 2mg/kg, p denotes $p<0.05$, TIP39 1nmol Vs TIP39 10 nmol.

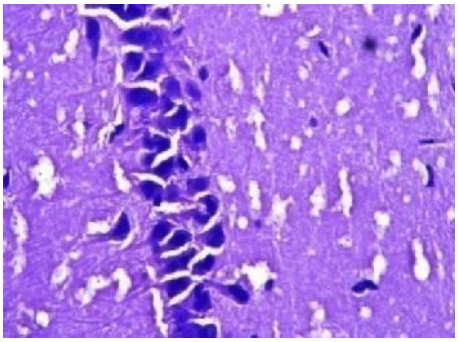
Figure 47: Histopathological evaluation of TIP39 on hippocampus in CUMS rats



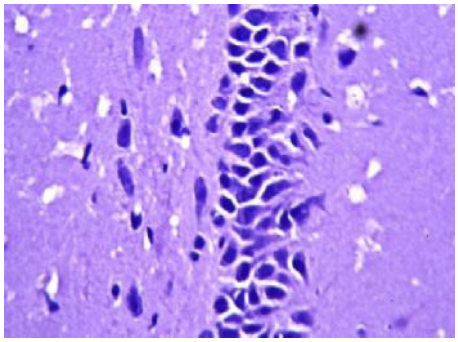
Control



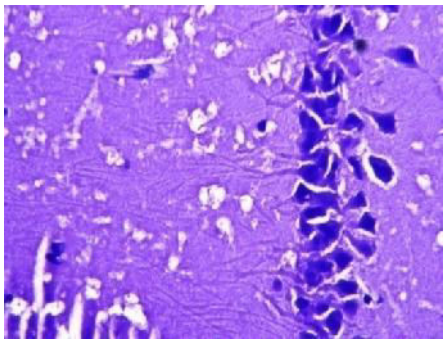
CUMS



Diazepam 2mg/kg

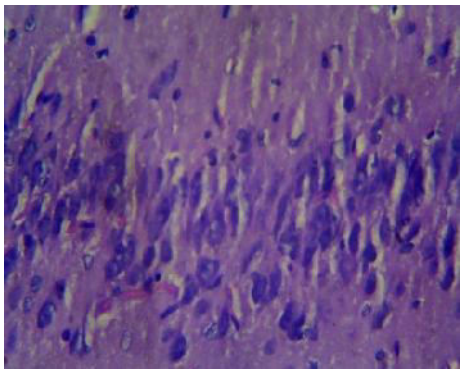


TIP39 1nmol/rat

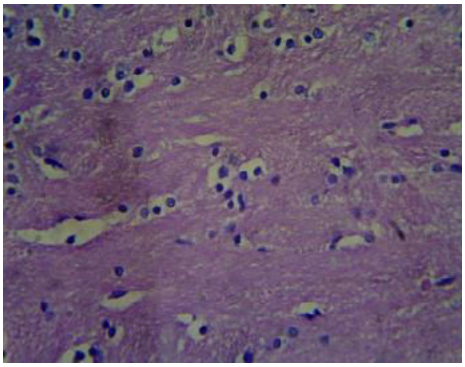


TIP39 10nmol/rat

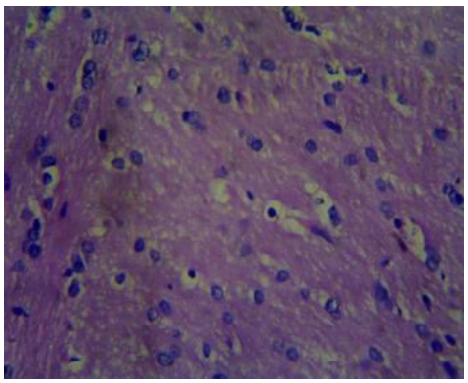
Figure 48: Histopathological evaluation of TIP39 on prefrontal cortex in CUMS rats



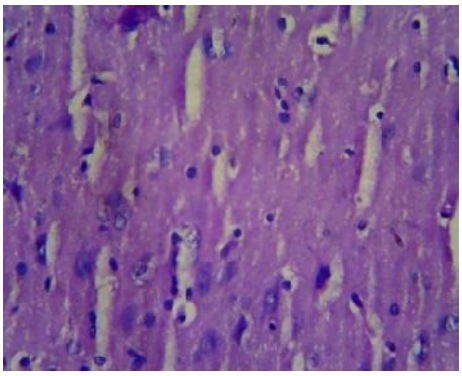
Control



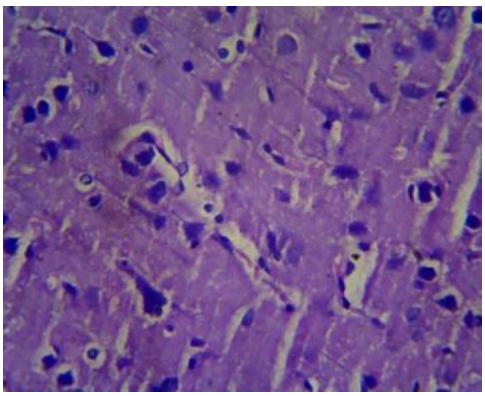
CUMS



Diazepam 2mg/kg



TIP39 1nmol/rat



TIP39 10nmol/rat

DISCUSSION

1. Elucidation of Physiological role of Tuberoinfundibular Peptide 39 (TIP39) in rats

In the present study we planned to elucidate the neuroendocrine role of TIP39 peptide in anxiety, depression and memory markers with respect to the behavioural, neurochemical, gene expression and morphological changes in pre frontal cortex and hippocampus region. OFT and FST models were used to evaluate the exploration, locomotion, ability to escape and state of depression and anxiety in animals which are in connection with monoaminergic circuits [184]. In this line, we demonstrated that administration of TIP39 peptide in normal rats did not alter the ambulation, rearing, grooming and time spent in central compartment in open field test; indicated that TIP39 did not alter the exploration and motor function in normal rats. Similar type of result was obtained in FST model replicating that no change in immobility time by TIP39 peptide as compared to normal rats. It reveals that TIP39 did not influence the serotonergic and noradrenergic system in normal rats [185] which are evident with the same study stating that the level of NA and 5HT in plasma was not changed after TIP39 (1nmol& 10 nmol) administration. Elevated plus maze (EPM) was conducted particularly to evaluate the exploration and awareness during anxiety [186]. TIP39 both the doses did not alter the number of entries and time spent in open and closed arm in EPM model. These observations were in connection with the GABA and glutamate release in brain which was not altered by TIP39 administration in this study. MWM test was conducted to assess the spatial and working memory performance in rats [187]. Both doses of TIP39 did not alter the time to reach the target quadrant; time spent in target quadrant and number of crossings during the test session as

compared to normal rats indicated that peptide did not involved in the spatial learning and memory course in normal state which was evident with brain acetylcholine esterase activity was not altered by TIP39 1& 10 nmol administration. From the above report we conclude that TIP39 peptide administration did not produce any changes on behavioural, neurochemical and brain morphology during the normal state of the animals.

II. Neurochemical assessment and behavioural role of Tuberoinfundibular Peptide 39 (TIP39) in acute restraint stressed Rats.

Bounteous evidence demonstrated that stress is the key factor in the pathogenesis of neuropsychiatric disorders including anxiety, depression, cognitive damage, insomnia and anorexia [188]. In the present study, we demonstrated ARS exposure selectively decreased TIP39 expression in rat brain, and that could mimic depression-like behaviour. TIP39 could activate monoamine like noradrenaline, but not serotonin level in plasma and we showed evidence for a GABA role in terms of controlling the glutaminergic action.

In a pilot study, TIP39 expression was examined in normal and stressed rats. The animal received 30 min of ARS did not establish the significant decrease in TIP39 expression as compared to control group. In contrast, animal received 2h of ARS significantly decreased the TIP39 expression, meant that severe stress has vital relation with the PTH2-TIP39 system. Hence we had chosen 2h of ARS model, to assess the role of TIP39 in depressive rats. In the same work, we further confirmed the relationship between a PTH2R-TIP39 system and neurotransmitter release during stress by administering PTH2 agonist (TIP39) and with the PTH2 antagonist (HYWH). Moreover, our determinations

are consistent with Usdin et al., indicated that TIP39/PTH2R knockout mice showed depression-like behaviour [76]

An effect of TIP39 at the immobility time was studied in FST model which is been really accurate and takes in the greater pharmacological sensitivity **[75].** TIP39 at doses of 1&10 nmol/rat significantly decreased the duration of immobility. However, HYWH treated animal did not demonstrate any real growth as compared to control group, suggesting that TIP39 might potentially have antidepressant-like activity. This is the first interactive study that verifies the antidepressant effect of TIP39 in ARS rats. The present data were consistent with the report of LaBuda et al. indicates that TIP39 can decrease the duration of immobility in the FST model [189].

The elevated plus maze was designed based on exploration and natural aversion of rodents to open spaces. EPM provided measures of two independent factors, one reflecting anxiety by measuring open arm entries and time spent on the open arms and another one reflecting motor activity by measuring number of open and closed arm entries provided a better measure of motor activity [186]. In this study CUMS rats exhibited significant decrease in open arm and increase in closed arm entry vice versa, significant decrease in open arm and increase in closed arm in EPM model that was reversed after administration of TIP39.This result was in accordance with the previously reported study [190], indicating TIP39 improved the anxiety condition and motor function.

Increased CORT level could cause depression-like behaviours that decrease hippocampal neurogenesis [72]. Our results showed that decreased TIP39 expression could increase the serum CORT, which suggests CORT might be involved in TIP39 related depression-like

behaviour. A higher level of CORT and hyperactivity of HPA axis has been implicated in the development of depression. In the present study, the ARS group showed a substantial lift in the CORT level as compared to the control group, which was reversed after TIP39 administration, dose dependently. In contrast, our study report is inconsistent with LaBuda et al., indicated that increased plasma CORT level after TIP39 infusion in the PVN region [189]. This might be ascribable to the deviation in the volume of stress applied. Nevertheless, the detailed mechanisms still need to be vigilant in future research. Many written reports have publicized that Ventral hypothalamus (VH) had glutamatergic input to the paraventricular hypothalamic nucleus (PVN), while PVN is liable for coordinating the regulation of the HPA axis [191]. Consequently adaptations of CORT and ACTH levels may be interrelated with the PVN, which is regulated by the VH directly. Interestingly, neuroanatomical studies implicated that high-density TIP39 fibres project widely in many limbic areas, including the PVN and several hypothalamic nuclei. Earlier studies also implicated that TIP39 peptide potentially modulate the natural process of the HPA axis during stressful condition [192].

Depression invariably accompanied by the reduction in 5-HT, NE, and DA level in the blood and brain tissues which are in close relation with symptoms of depression [193]. In the present work, we found that NE and 5HT were significantly decreased in the ARS group compared to control group. TIP39 administration significantly increased the NE content when compared to ARS group which was not seen in HYWH treated groups. In contrast, 5-HT levels were not significantly different among the treatment and ARS groups, indicating that 5-HT levels in the whole brain were not significantly impressed. Thus, this study linking that elevation of NE in the treatment group might be a cause for

increasing ambulatory behaviour in OFT. An earlier study also has revealed that TIP39/PTH2-R system potentially acting through central noradrenergic signalling pathway [63]. With the aforementioned resolution, we could infer that the antidepressant-like effect of TIP39 may be associated with modulation of the central noradrenergic pathway.

Finding efficient therapy in challenging depression is very difficult may be due to diversity in the origin of mood and mental disorders associated with different genetic and environmental factors. Recent years, studies on depression have focused to a greater extant on the glutaminergic role rather than the monoamines. Studies revealed that high level of brain and plasma glutamate were found in the patient with depression. Also established that inhibition of glutamatergic neurotransmission was strongly correlated with the therapeutic action of a majority of antidepressant drugs [194]. Interestingly, in whole brain tissue, TIP39 significantly reduced the glutamate level as compared to ARS rats. In contrast, GABA content was significantly increased as compared to ARS rats, which were not observed in rats treated with HYWH. Mathew SJ et al stating that imbalance between the glutamate and GABA content in the brain can cause depression-like behaviour which is coherent with the present study. These reports point towards the importance of TIP39 in regulating the glutaminergic and GABAergic system. However, our study report support the previous study implicated that TIP39 peptide can regulate hypothalamic glutaminergic and inhibitory GABAergic neuron in the cerebral region[195]. Recent studies have reported that, besides the limbic area, TIP39 are abundantly expressed in other brain regions also [196]. Hence, this study suggests that the curative role of TIP39 needs to be explored in other brain areas, equally well. This study

provided the first evidence indicating that stimulating TIP39 expression can bring about an antidepressant-like effect by modulating the monoaminergic,GABAergic and glutaminergic release with the support of HPA axis.

III. Tuberoinfundibular Peptide of 39 Attenuates Chronic Unpredictable Mild Stress Induced HPA axis Dysregulation, Inflammation and Oxidative Damage in Depressive Rats

Research studies are focusing wide on stress induced neurological disorders on the basis of nature and origin of the stress generation [197]. Stress is induced due to age, sex, individual differences, types of stimulus, duration, and intensity of stressors in the cerebral region [124,125]. Chronic stress can increase the vulnerability in hippocampus and prefrontal cortex by altering the antioxidant defence system interactions closely with the HPA axis [194]. After 28 days of CUMS induction, animals were examined through the open field model. CUMS induced rats have shown decreased ambulation, rearing and increased time spent in the central compartment reveals less exploratory behaviour and increased grooming activity, that revealed the higher anxiety level, which is sensitive towards serotonergic and noradrenergic activity [198]. The aforesaid activities were reversed after the ICV administration of TIP39. In contrast, no significant changes were observed in grooming activity. TIP39 was studied in the FST and TST models, which are widely used preclinical models to examine antidepressant activity [199,200]. In the modified version of FST model, together with the immobility time, jumping and dipping response were recorded. The dipping and jumping response has connection with the drugs that regulates serotonergic and noradrenergic transmission [201,202]. Decreased Immobility time in TST represents the level of confidence in the animal to overcome the

depressive state. In this study, both the doses of TIP39 significantly decreased the immobility time ,dipping response and increased the jumping response. Similar types of effects were also observed in TST in terms of decreased immobility time. Interpretation of the above results shows TIP39 may regulate catecholamines in general to control the symptoms like depression and anxiety. This study supports the earlier study reported that, the TIP39 is potentially acting on central noradrenergic signalling pathway during stressful conditions [203], implicating that TIP39 significantly increased the plasma noradrenaline level. In contrast, serotonin level did not get altered in acute restraint stressed rats after TIP39 administration. CUMS rats exhibited increased corticosterone level in plasma, which indicates the depressive state of the rats, evident with Wang C et al [203]. Elevation of plasma costicosterone level might be due to dysregulation of HPA axis, which alters the neurochemical and biochemical activities [204]. The purpose of elevation during stress is to maintain the energy levels by utilizing glucocorticoids for demands and compensatory mechanism [205]. Interestingly, TIP39 reversed the elevation of plasma corticosterone level in CUMS rats. The normalizing effect of TIP39 on plasma corticosterone level indicated that, it may possess adaptogenic response in stressed rats. This is due to the activation of hypothalamic glutaminergic neurons that facilitates TIP39 on HPA axis [206]. One of the core symptoms of depression is Anhedonia (decreased responsiveness to rewards), which reduces the sucrose preference in depressive animals [207]. This could be due to the alterations in HPA axis activity and it could be a root cause for hypophagia (decreased food intake) [208,209]. In the four weeks study, gradual decline in sucrose consumption was observed in all the groups and this is might be due to the taste familiarity. Administration of anti stress drug (diazepam 2mg/kg) markedly

increases the sucrose intake in CUMS rats. This could be due to the reduction of stress [75, 210]. In same manner, TIP39 dose dependently increased the sucrose intake in CUMS rats, which was not observed in CUMS alone treated rats.

It was observed that induction of chronic stress causes major cell injury provoked by the lipid peroxidation in cell membrane due to release of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radical [211]. As the ROS content increases, mitochondria complex get damaged led to deprivation of energy molecule such as ATP. The decreased activities of mitochondrial complexes and the impaired function of energy regularization further increased the ROS production in the affected system [212]. Administration TIP39 increased the brain ATP content in the stressed rats by reducing the hydrolysis of ATP to ADP in extracellular membrane. This statement supports the earlier study stating that CUMS potentially disturbed the ATP generation in varies preclinical models (101).

In the present study, the daily exposure of rats to different stressors for 4 consecutive weeks significantly increased the marker of oxidative stress (LPO) and reduced the endogenous antioxidant level (SOD, CAT, and GSH) confirming that CUMS triggers free radical generation in hippocampus and prefrontal cortex [213,214]. Simultaneous treatment with TIP39 rescued CUMS induced disproportion by normalizing SOD, CAT, GSH, and lipid peroxidation marker (MDA). TIP39 administration for four weeks increased the SOD, CAT, GSH levels and reduced the production of MDA, which indicates the inhibition of lipid peroxidation. Many researchers demonstrated that, chronic stress increased the production of IL-6, IL-1β, and TNF-α level that induced pain perception due to glucocorticoid resistance action. This similarly reduced the competence

of glucocorticoids to suppress cytokine production [215]. Subsequent administration of TIP39 in CUMS rats significantly reduced the proinflammatory cytokine levels. This is due to the guarding role of TIP39 by maintaining the central sensitization mechanism [216]. We also believe that suppression of proinflammatory cytokine by TIP39 peptide possibly due to the diminution of oxidative damage.

IV. Elucidation of role of TIP39 in chronic unpredictable mild stress-induced learning and memory impairment in rats.

The impact of chronic stress on cognitive performance is thought to be depend on biological and chronobiological factors [217]. In this line, stress predictability also modulates these effects. For instance, it was reported that the implementation of predictable stressors enhances cognitive performance. In contrast, implementations of unpredictable stressors have shown impairment of cognitive performance [180, 218].

In the present study we proposed to assess the potential effect of TIP39 peptide treatment on spatial working memory, short term memory and recognition memory in CUMS rats. We demonstrated that chronically induced unpredictable mild stress for 28 days exhibited impaired spatial working memory in Morris water maze task. Furthermore, CUMS rats resulted in short term memory impairment in Y maze task and deficit in recognition memory performance in the NORT task. The obtained data were consistent with the previous reports suggesting that deterioration of learning and memory performance in CUMS models [181, 219].

In order to check the spatial working memory on MWM, during the training session, invariably all the rats in the group were allowed to swim and reach the platform from day one to six. The time interval to reach the platform was noted. It reveals that repetitive

investigation of same event could improve the learning and memory performance in naive animals [220]. During the test session, in CUMS induced rats decreased the time spend and number of crossing of target quadrant and increased the time to reach target quadrant [221]. Chronic administration of TIP39 peptide potentially reversed the CUMS induced spatial learning and working memory impairment in MWM task as revealed by increasing time spend and crossing the target quadrant and decreased the time to reach target quadrant.

Percentage spontaneous alternation performance was significantly reduced in CUMS rats. It point towards the deterioration of short term memory processing in rats. In this study TIP39 peptide dose dependently ameliorated the CUMS induced changes in rats by increasing the spontaneous alternation performance indicated that rodents showed more willingness to explore new environments with acquired memory in Y-maze task [62]. This study strongly support the previously reported study, demonstrated that mice lacking TIP39 signalling or TIP39 knockout resulted in decreased spontaneous alternation performance during the novelty induced arousal condition [229].

Fear related memory performance was evaluated in modified elevated plus maze task, decrease in transfer latency indicated improvement of memory and vice versa. In this study, CUMS rats significantly increased transfer latency time as reported before [196]. In contrary, both doses of TIP39 significantly reversed the CUMS induced changes on transfer latency time in rats. Improvement of memory performance possibly due to fear reliving role of TIP39 which was demonstrated in our pervious study report [222].

In order to study the discriminating ability of rodents in normal and abnormal neurological condition, animals were explored to NORT task [223]. Studies also

demonstrated that stressful condition could produce anxiety like behaviour even in familiar environment [224]. In our study, stressed rats exhibited greater time spent with familiar object than novel object vice-versa more time taken to reach the novel object than familiar objects; replicating the previously reported cognitive deficit model [225]. TIP39 treated rats showed spontaneous tendency to spend more time with a novel object than a familiar object. From these report, we can conclude that development of cognitive functionality was improved in rodents after TIP39 treatment. This might be due to anxiolytic property of TIP39 peptide led to improvement in working and short term memory performance [226]. Furthermore, this study supports Usdin et al, demonstrated that TIP39-PTH2R knockout mouse less explored to novel object than in wild type mouse [227].

Acetylcholine is an important neurotransmitter that governs vital aspects of memory and other cognitive functions. The hippocampus, amygdala and cortical regions of the brain are mainly involved in cholinergic transmission to monitor learning and memory processing [228]. Dysregulation of the cholinergic neuronal pathway and memory circuits in the central nervous system; might result in serious impairment in acquisition, immediate retention, working memory [229]. In this study, CUMS was found to significantly elevated the acetylcholine esterase activity (AChE), an enzyme responsible for degradation of acetylcholine. This increased AChE activity was significantly reversed after TIP39 administration. These observations suggest that TIP39 peptide has role on modulation of cholinergic neurotransmission and/or prevention of cholinergic neuronal loss. Studies also demonstrated that persistent induction of stress in response to degradation of acetylcholine and increase the level of AChE enzyme, further responsible

for the production of oxidative stress and pro-inflammatory mediator's viz., cytokines and further activation of these cells [230]. This statement strongly support our previous report stating that TIP39 dose dependently reduced the oxidative stress markers and proinflammatory cytokine levels in brain tissue.

Prolonged stress significantly affects HPA axis function that could increase the plasma corticosterone produces memory impairment and reduced the dendrite spine density which has been considered as important clinical marker in learning and memory circuit [231]. Studies also have suggested that elevated corticosterone reduces the hippocampal neurogenesis and its volume [232,233]. In the present study TIP39 treatment markedly reduced the CUMS induced CORT elevation, this might be due to facilitatory role of TIP39 on HPA axis regulating through hypothalamic glutaminergic neurons [234]. This study result consistent with our previous report revealed that, even lower dose of TIP39 significantly decreased the CORT level on acute restraint stress model.

Prolonged elevation of glucocorticoids by chronic stress is very well known to cause depression that could alter the cognitive process [235]. Studies demonstrated that glucocorticoid and its receptors exerts differential role on cognition, particularly on memory performance. In fact, it has been proposed that glucocorticoids have a dissociative impact on memory consolidation and retrieval [236]. Chronic stress causes imbalance in glucocorticoid and mineralocorticoid receptor expression in brain tissues; this may be due to either over activation of HPA axis or continuous stimulation of NMDA receptor [138]. This statement was accordance with this study reports stated that CUMS rats showed remarkable increase in corticosterone release that increased the expression of glucocorticoid receptor which was reversed after TIP39 administration. In

contrast, TIP39 did not alter the mineralocorticoid receptor expression in brain. However, the ratio of GR/MR expression was maintained dose dependently after TIP39 administration in CUMS rats.

Chronic stress can modulate the release of glutamate and GABA, the principal excitatory and inhibitory neurotransmitters in the brain respectively, are highly complex in which alters the neuronal and glial metabolism that could affect again glutamate and GABA release by GAD (Glutamate alpha decarboxylase) enzyme; responsible for the conversion of glutamate to GABA in neurons[141]. In this study CUMS rats exhibited significant increase in brain glutamate and decrease in GABA. This report was in line with the previously reported studies showed that ratio of glutamate/GABA was found abnormal in clinical and preclinical models [142]. Increased glutamate level was associated with reduced GAD activity, while enhanced GAD activity in the cerebral region may account for the increase in GABA levels [143]. While TIP39 was associated with a considerable increase in GAD enzyme activity which is in relation with marked decrease in the glutamate level in the brain of CUMS exposed rats.

Studies are stating that, histopathological features of CUMS inducted rats showed cellular infiltration, neuronal loss, cytoplasmic vacoulation, chromatin condensation, ghost cells in cerebral cortex, odema, hemorrhage, and gliosis [59]. Our results, on microscopical examination of prefrontal cortex and hippocampus on CUMS rats, showed adverse effects at subcellular level which includes changes in structure of cerebral cortex caused gliosis and lympocytic infiltration; changes in hippocampus causing neuronal cell loss ,vacoulation and disruption of cell membrane. Treatment with TIP39 showed

reduction in the lymphocytic infiltration and no gliosis in cerebral cortex, reduced neuronal cell loss and no vacoulation was seen in hippocampus region.

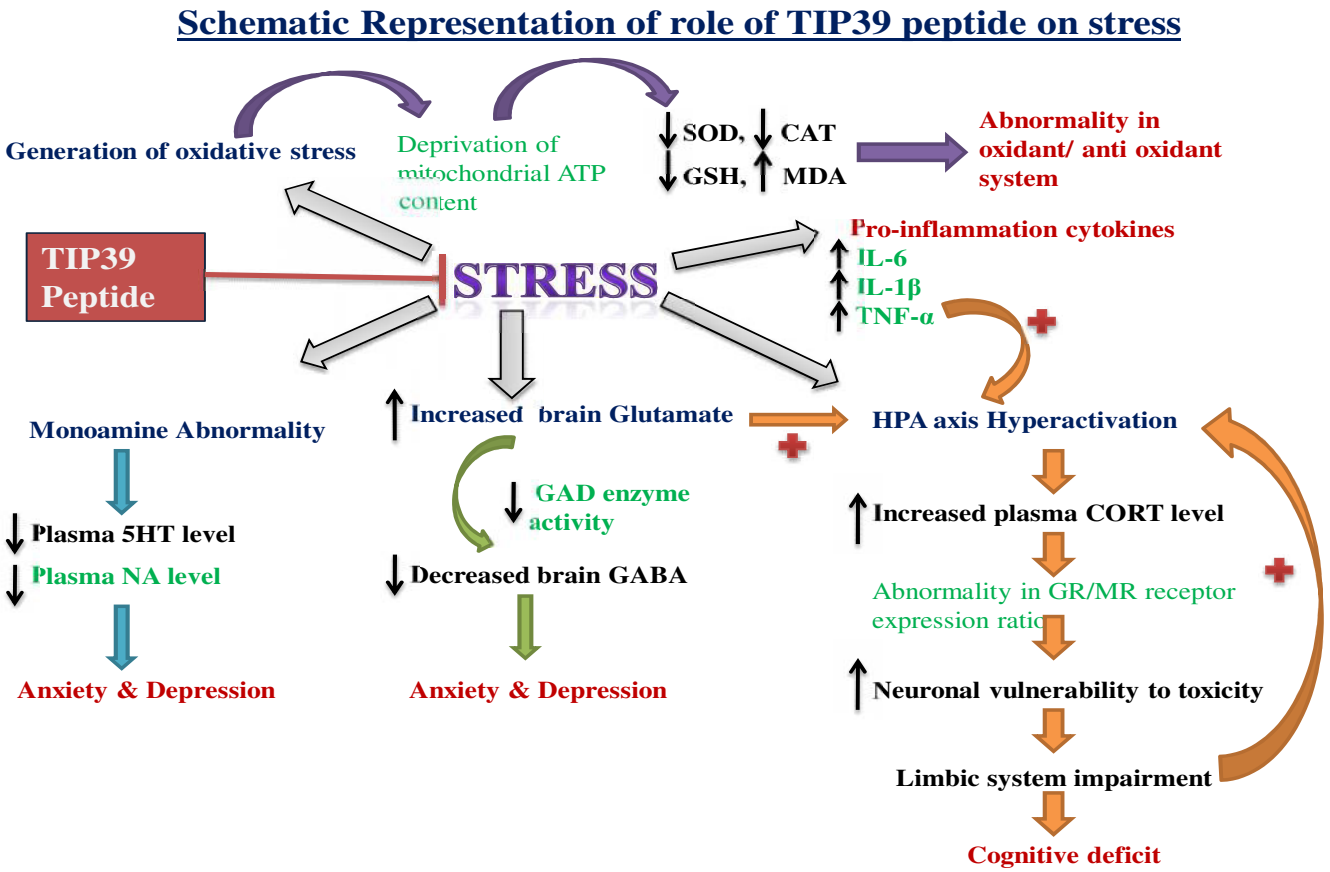
From this study it was clearly evident that TIP39 peptide normalized the stress mediated temporary deregulation of behavioural, acetylcholine esterase activity, Glu/GABA release in relation with GAD activity and in GR/MR expression ratio in brain; along with the structural improvement on prefrontal cortex and hippocampus in cerebral region. Further this study provided an insight into the central mechanisms responsible for learning and memory improving and neuroprotective role of TIP39 peptide.

CHAPTER 7: DISCUSSION

SUMMARY AND CONCLUSION

The objective of the present study is to elucidate the neuroendocrine role of TIP39 peptide in anxiety, depression and memory model with respect to the behavioural, neurochemical, gene expression and morphological changes in rats. In normal rats, ICV administration of TIP39 did not alter the behaviour, physiology and morphology demonstrating that administration TIP39 peptide did not influence the anxiety, depression and memory markers during the normal state. From the interactive study, PTH2R antagonist was used to study the role of TIP39 in ARS model; TIP39 significantly increased the NA and GABA release and significantly decreased the glutamate and CORT level in brain and plasma of ARS rats. This was reversed after PTH2R antagonist administration, indicating that TIP39 has direct role on noradrenergic, glutaminergic/GABAergic and HPA axis system through PTH2 receptor. CUMS rats exhibited significant reduction in sucrose intake, is a potential predictor of depressive state, led to cause energy deprivation, HPA axis abnormality, oxidative damage and inflammation. In this line, TIP39 both the doses (1 nmol & 10 nmol) effectively reversed the CUMS induced abnormalities. In addition to that, TIP39 effectively increased the GAD enzyme activity and decreased the acetyl cholinesterase activity in CUMS rats. CUMS induced abnormality in mineralocorticoid (MR) and glucocorticoid receptor (GR) gene expressions were significantly reversed after TIP39 administration. Moreover, TIP39 both the doses effectively improved the CUMS induced neurodegeneration in hippocampus and prefrontal cortex region.

Figure 49: Mechanism of TIP39 Peptide



IMPACT OF THE STUDY

- In this study, we found that TIP39 was acting through neurological and endocrinological pathway (bi-directionally) during the stressful condition; which is novel and unique to this study. Because of many existing therapies in the treatment of stress exhibited unidirectional approach which was not satisfactory still, since because stress is generated by both brain and endocrine system.
- Moreover, TIP39 did not produce any effect in normal state and it is involved only in pathological condition, hence clinically it can be used as a diagnostic marker in the treatment of stress and stress associated disorders.

CHAPTER 10: BIBLIOGRAPHY

1. Breslau N, Kessler RC, Chilcoat HD, Schultz LR, Davis GC, Andreski P. Trauma and posttraumatic stress disorder in the community: the 1996 Detroit Area Survey of Trauma. Arch Gen Psychiatry. 1998 Jul;55(7):626-32.
2. Rosenman S. Trauma and posttraumatic stress disorder in Australia: findings in the population sample of the Australian National Survey of Mental Health and Wellbeing. Aust N Z J Psychiatry. 2002 Aug;36(4):515-20.
3. Darves-Bornoz JM, Alonso J, De Girolamo G, De Graaf R, Haro JM, Kovess-Masfety V, Lepine JP, Nachbaur G, Negre-Pages L, Vilagut G, Gasquet I; ESEMeD/MHEDEA 2000 Investigators. Main traumatic events in Europe: PTSD in the European study of the epidemiology of mental disorders survey. J Trauma Stress. 2008 Oct;21(5):455-62.
4. Norris FH, Murphy AD, Baker CK, Perilla JL, Rodriguez FG, Rodriguez Jde J. Epidemiology of trauma and posttraumatic stress disorder in Mexico. J Abnorm Psychol. 2003 Nov;112(4):646-56.
5. McEwen BS. Stress and hippocampal plasticity. Annu Rev Neurosci. 1999;22:105-22.
6. McEwen BS. Protective and damaging effects of stress mediators: the good and bad sides of the response to stress. Metabolism. 2002 Jun;51(6 Suppl 1):2-4.
7. Henriquez A, House J, Miller DB, Snow SJ, Fisher A, Ren H, Schladweiler MC, Ledbetter AD, Wright F, Kodavanti UP. Adrenal-derived stress hormones modulate ozone-induced lung injury and inflammation. Toxicol Appl Pharmacol. 2017 Aug 15;329(12):249-258.

8. Hesketh S, Jessop DS, Hogg S, Harbuz MS. Differential actions of acute and chronic citalopram on the rodent hypothalamic-pituitary-adrenal axis response to acute restraint stress. J Endocrinol. 2005 Jun;185(3):373-82.

9. Swanson LW, Sawchenko PE. Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. Annu Rev Neurosci. 1983;6(5):269-324.

10. Neumann ID. Involvement of the brain oxytocin system in stress coping: interactions with the hypothalamo-pituitary-adrenal axis. Prog Brain Res. 2002;139(32):147-62.

11. Santosh P, Venugopl R, Nilakash AS, Kunjbihari S, Mangala L. Antidepressant activity of methanolic extract of Passiflora foetida leaves in mice. Int J Pharm Pharm Sci. 2011Aus;3(1):6–9.

12. Musselman DL, Evans DL, Nemeroff CB. The relationship of depression to cardiovascular disease: epidemiology, biology, and treatment. Arch Gen Psychiatry. 1998 Jul;55(7):580-92.

13. Penninx BW, Beekman AT, Honig A, Deeg DJ, Schoevers RA, Van Eijk JT, Van Tilburg W. Depression and cardiac mortality: results from a community-based longitudinal study. Arch Gen Psychiatry. 2001 Mar;58(3):221-7.

14. Angst J. Depression and anxiety: implications for nosology, course, and treatment. J Clin Psychiatry. 1997;58 Suppl 8:3-5.

15. Merikangas KR, Angst J, Eaton W, Canino G, Rubio-Stipec M, Wacker H, Wittchen HU, Andrade L, Essau C, Whitaker A, Kraemer H, Robins LN, Kupfer DJ. Comorbidity and boundaries of affective disorders with anxiety disorders and substance misuse: results of an international task force. Br J Psychiatry Suppl. 1996 Jun;(30):58-67.

16. Schildkraut JJ. The catecholamine hypothesis of affective disorders: a review of supporting evidence. J Neuropsychiatry Clin Neurosci. 1995;7(4):524-33.

17. Belmaker RH, Agam G. Major depressive disorder. N Engl J Med. 2008 Jan 3;358(1):55-68.

18. Neumeister A, Nugent AC, Waldeck T, Geraci M, Schwarz M, Bonne O, Bain EE, Luckenbaugh DA, Herscovitch P, Charney DS, Drevets WC. Neural and behavioural responses to tryptophan depletion in unmedicated patients with remitted major depressive disorder and controls. Arch Gen Psychiatry. 2004 Aug;61(8):765-73.

19. Saijo T, Takano A, Suhara T, Arakawa R, Okumura M, Ichimiya T, Ito H, Okubo Y. Effect of electroconvulsive therapy on 5-HT1A receptor binding in patients with depression: a PET study with [11C]WAY 100635. Int J Neuropsychopharmacol. 2010 Jul;13(6):785-91.

20. Lambert G, Johansson M, Agren H, Friberg P. Reduced brain norepinephrine and dopamine release in treatment-refractory depressive illness: evidence in support of the catecholamine hypothesis of mood disorders. Arch Gen Psychiatry. 2000 Aug;57(8):787-93.

21. Meyer JH, Krüger S, Wilson AA, Christensen BK, Goulding VS, Schaffer A, Minifie C, Houle S, Hussey D, Kennedy SH. Lower dopamine transporter binding potential in striatum during depression. Neuroreport. 2001 Dec 21;12(18):4121-5.

22. Santamaría J, Tolosa E, Valles A. Parkinson's disease with depression: a possible subgroup of idiopathic parkinsonism. Neurology. 1986 Aug;36(8):1130-3.

23. Hasler G, Fromm S, Carlson PJ, Luckenbaugh DA, Waldeck T, Geraci M, Roiser JP, Neumeister A, Meyers N, Charney DS, Drevets WC. Neural response to

catecholamine depletion in unmedicated subjects with major depressive disorder in remission and healthy subjects. Arch Gen Psychiatry. 2008 May;65(5):521-31.

24. Hasler G, Luckenbaugh DA, Snow J, Meyers N, Waldeck T, Geraci M, Roiser J, Knutson B, Charney DS, Drevets WC. Reward processing after catecholamine depletion in unmedicated, remitted subjects with major depressive disorder. Biol Psychiatry. 2009 Aug 1;66(3):201-5.
25. Wierońska JM, Pilc A. Metabotropic glutamate receptors in the tripartite synapse as a target for new psychotropic drugs. Neurochem Int. 2009 Jul-Aug;55(1-3):85-97.
26. Hashimoto K. Emerging role of glutamate in the pathophysiology of major depressive disorder. Brain Res Rev. 2009 Oct;61(2):105-23
27. Altamura CA, Mauri MC, Ferrara A, Moro AR, D'Andrea G, Zamberlan F. Plasma and platelet excitatory amino acids in psychiatric disorders. Am J Psychiatry. 1993 Nov;150(11):1731-3.
28. Mitani H, Shirayama Y, Yamada T, Maeda K, Ashby CR Jr, Kawahara R. Correlation between plasma levels of glutamate, alanine and serine with severity of depression. Prog Neuropsychopharmacol Biol Psychiatry. 2006 Aug 30;30(6):1155-8.
29. Anderson CM, Swanson RA. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia. 2000 Oct;32(1):1-14.
30. Araque A, Parpura V, Sanzgiri RP, Haydon PG. Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci. 1999 May;22(5):208-15.
31. Cotter D, Mackay D, Landau S, Kerwin R, Everall I. Reduced glial cell density and neuronal size in the anterior cingulate cortex in major depressive disorder. Arch Gen Psychiatry. 2001 Jun;58(6):545-53.

32. Banasr M, Duman RS. Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors. Biol Psychiatry. 2008 Nov 15;64(10):863-70.

33. Sheline YI, Gado MH, Kraemer HC. Untreated depression and hippocampal volume loss. Am J Psychiatry. 2003 Aug;160(8):1516-8.

34. Hasler G, Fromm S, Alvarez RP, Luckenbaugh DA, Drevets WC, Grillon C. Cerebral blood flow in immediate and sustained anxiety. J Neurosci. 2007 Jun 6;27(23):6313-9.

35. Frodl TS, Koutsouleris N, Bottlender R, Born C, Jäger M, Scupin I, Reiser M, Möller HJ, Meisenzahl EM. Depression-related variation in brain morphology over years: effects of stress?. Arch Gen Psychiatry. 2008 Oct;65(10):1156-65.

36. Ricon T, Toth E, Leshem M, Braun K, Richter-Levin G. Unpredictable chronic stress in juvenile or adult rats has opposite effects, respectively, promoting and impairing resilience. Stress. 2012 Jan;15(1):11-20.

37. Pehrson AL, Leiser SC, Gulinello M, Dale E, Li Y, Waller JA, Sanchez C. Treatment of cognitive dysfunction in major depressive disorder-a review of the preclinical evidence for efficacy of selective serotonin reuptake inhibitors, serotonin-norepinephrine reuptake inhibitors and the multimodal-acting antidepressant vortioxetine. Eur J Pharmacol. 2015 Apr 15;753(56):19-31

38. Nemeroff CB. The burden of severe depression: a review of diagnostic challenges and treatment alternatives. J Psychiatr Res. 2007 Apr-Jun;41(3-4):189-206.

39. Patten SB. Major depression prevalence is very high, but the syndrome is a poor proxy for community populations' clinical treatment needs. Can J Psychiatry. 2008 Jul;53(7):411-9.

40. Van Kleef GA, Heerdink MW, Homan AC. Emotional influence in groups: the dynamic nexus of affect, cognition, and behavior. Curr Opin Psychol. 2017 Oct;17:156-161.

41. Sun MK, Alkon DL. Induced depressive behavior impairs learning and memory in rats. Neuroscience. 2004;129(1):129-39.

42. Dias-Ferreira E, Sousa JC, Melo I, Morgado P, Mesquita AR, Cerqueira JJ, Costa RM, Sousa N. Chronic stress causes frontostriatal reorganization and affects decision-making. Science. 2009 Jul 31;325(5940):621-5.

43. Kalivas PW, Duffy P. Selective activation of dopamine transmission in the shell of the nucleus accumbens by stress. Brain Res. 1995 Mar 27;675(1-2):325-8.

44. Sousa N, Almeida OF. Disconnection and reconnection: the morphological basis of (mal)adaptation to stress. Trends Neurosci. 2012 Dec;35(12):742-51.

45. Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S. Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci. 2002 Aug 1;22(15):6810-8.

46. Ceretta LB, Réus GZ, Abelaira HM, Ribeiro KF, Zappellini G, Felisbino FF, Steckert AV, Dal-Pizzol F, Quevedo J. Increased oxidative stress and imbalance in antioxidant enzymes in the brains of alloxan-induced diabetic rats. Exp Diabetes Res. 2012;2012:302682.

47. Vargas HO, Nunes SO, de Castro MR, Vargas MM, Barbosa DS, Bortolasci CC, Venugopal K, Dodd S, Berk M. Oxidative stress and inflammatory markers are associated with depression and nicotine dependence. Neurosci Lett. 2013 Jun 7;544:136-40.

48. De Senna PN, Xavier LL, Bagatini PB, Saur L, Galland F, Zanotto C, Bernardi C, Nardin P, Gonçalves CA, Achaval M. Physical training improves non-spatial memory, locomotor skills and the blood brain barrier in diabetic rats. Brain Res. 2015 Aug 27;1618:75-82.

49. Mesripour A, Moghimi F, Rafieian-Kopaie M. The effect of Cinnamomum zeylanicum bark water extract on memory performance in alloxan-induced diabetic mice. Res Pharm Sci. 2016 Jul;11(4):318-23.

50. Wang XD, Rammes G, Kraev I, Wolf M, Liebl C, Scharf SH, Rice CJ, Wurst W, Holsboer F, Deussing JM, Baram TZ, Stewart MG, Müller MB, Schmidt MV. Forebrain CRF_1 modulates early-life stress-programmed cognitive deficits. J Neurosci. 2011 Sep 21;31(38):13625-34.

51. Kelly JP, Filley CM. Proneness to psychological distress is associated with risk of Alzheimer's disease. Neurology. 2004 Sep 14;63(5):941-45.

52. Nagata K, Nakashima-Kamimura N, Mikami T, Ohsawa I, Ohta S. Consumption of molecular hydrogen prevents the stress-induced impairments in hippocampus-dependent learning tasks during chronic physical restraint in mice. Neuropsychopharmacology. 2009 Jan;34(2):501-8.

53. Wilson RS, Arnold SE, Schneider JA, Kelly JF, Tang Y, Bennett DA. Chronic psychological distress and risk of Alzheimer's disease in old age. Neuroepidemiology. 2006;27(3):143-53.

54. Hasan A, Nitsche MA, Rein B, Schneider-Axmann T, Guse B, Gruber O, Falkai P, Wobrock T. Dysfunctional long-term potentiation-like plasticity in schizophrenia

revealed by transcranial direct current stimulation. Behav Brain Res. 2011 Oct 10;224(1):15-22

55. Gerges NZ, Aleisa AM, Schwarz LA, Alkadhi KA. Chronic psychosocial stress decreases calcineurin in the dentate gyrus: a possible mechanism for preservation of early ltp. Neuroscience. 2003;117(4):869-74.
56. Wang J, Yuan J, Pang J, Ma J, Han B, Geng Y, Shen L, Wang H, Ma Q, Wang Y, Wang M. Effects of Chronic Stress on Cognition in Male SAMP8 Mice. Cell Physiol Biochem. 2016;39(3):1078-86.
57. Joshi YB, Chu J, Praticò D. Stress hormone leads to memory deficits and altered tau phosphorylation in a model of Alzheimer's disease. J Alzheimers Dis. 2012;31(1):167-76.
58. Han B, Yu L, Geng Y, Shen L, Wang H, Wang Y, Wang J, Wang M. Chronic Stress Aggravates Cognitive Impairment and Suppresses Insulin Associated Signaling Pathway in APP/PS1 Mice. J Alzheimers Dis. 2016 Jul 2;53(4):1539-52.
59. Usdin TB, Gruber C, Bonner TI. Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTH2 receptor. J Biol Chem. 1995 Jun 30;270(26):15455-8.
60. Liapakis G, Matsoukas MT, Karageorgos V, Venihaki M, Mavromoustakos T. Family B G Protein-coupled Receptors and their Ligands: From Structure to Function. Curr Med Chem. 2017;24(31):3323-3355.
61. Usdin TB, Bonner TI, Hoare SR. The parathyroid hormone 2 (PTH2) receptor. Receptors Channels. 2002;8(3-4):211-8.

62. Dobolyi A, Palkovits M, Bodnár I, Usdin TB. Neurons containing tuberoinfundibular peptide of 39 residues project to limbic, endocrine, auditory and spinal areas in rat. Neuroscience. 2003;122(4):1093-105.

63. Wang T, Palkovits M, Rusnak M, Mezey E, Usdin TB. Distribution of parathyroid hormone-2 receptor-like immunoreactivity and messenger RNA in the rat nervous system. Neuroscience. 2000;100(3):629-49.

64. Pang PK, Kaneko T, Harvey S. Immunocytochemical distribution of PTH immunoreactivity in vertebrate brains. Am J Physiol. 1988 Oct;255(4 Pt 2):R643-7.

65. Dobolyi A, Ueda H, Uchida H, Palkovits M, Usdin TB. Anatomical and physiological evidence for involvement of tuberoinfundibular peptide of 39 residues in nociception. Proc Natl Acad Sci U S A. 2002 Feb 5;99(3):1651-6.

66. Usdin TB, Hoare SR, Wang T, Mezey E, Kowalak JA. TIP39: a new neuropeptide and PTH2-receptor agonist from hypothalamus. Nat Neurosci. 1999 Nov;2(11):941-3.

67. Hansen IA, Jakob O, Wortmann S, Arzberger T, Allolio B, Blind E. Characterization of the human and mouse genes encoding the tuberoinfundibular peptide of 39 residues, a ligand of the parathyroid hormone receptor family. J Endocrinol. 2002 Jul;174(1):95-102.

68. John MR, Arai M, Rubin DA, Jonsson KB, Jüppner H. Identification and characterization of the murine and human gene encoding the tuberoinfundibular peptide of 39 residues. Endocrinology. 2002 Mar;143(3):1047-57.

69. Usdin TB. The PTH2 receptor and TIP39: a new peptide-receptor system. Trends Pharmacol Sci. 2000 Apr;21(4):128-30.

70. Dobolyi A, Palkovits M, Usdin TB. Expression and distribution of tuberoinfundibular peptide of 39 residues in the rat central nervous system. J Comp Neurol. 2003 Jan 20;455(4):547-66.

71. Fraser RA, Kronenberg HM, Pang PK, Harvey S. Parathyroid hormone messenger ribonucleic acid in the rat hypothalamus. Endocrinology. 1990 Nov;127(5):2517-22.

72. Usdin TB. Evidence for a parathyroid hormone-2 receptor selective ligand in the hypothalamus. Endocrinology. 1997 Feb;138(2):831-4.

73. Hoare SR, Bonner TI, Usdin TB. Comparison of rat and human parathyroid hormone 2 (PTH2) receptor activation: PTH is a low potency partial agonist at the rat PTH2 receptor. Endocrinology. 1999 Oct;140(10):4419-25.

74. Usdin TB, Hoare SR, Wang T, Mezey E, Kowalak JA. TIP39: a new neuropeptide and PTH2-receptor agonist from hypothalamus. Nat Neurosci. 1999 Nov;2(11):941-3.

75. LaBuda CJ, Dobolyi A, Usdin TB. Tuberoinfundibular peptide of 39 residues produces anxiolytic and antidepressant actions. Neuroreport. 2004 Apr 9;15(5):881-5.

76. Coutellier L, Usdin TB. Enhanced long-term fear memory and increased anxiety and depression-like behavior after exposure to an aversive event in mice lacking TIP39 signaling. Behav Brain Res. 2011 Sep 12;222(1):265-9.

77. Sugimura Y, Murase T, Ishizaki S, Tachikawa K, Arima H, Miura Y, Usdin TB, Oiso Y. Centrally administered tuberoinfundibular peptide of 39 residues inhibits arginine vasopressin release in conscious rats. Endocrinology. 2003 Jul;144(7):2791-6.

78. Sah P. Fear, Anxiety, and the Amygdala. Neuron. 2017 Sep 27;96(1):1-2

79. Perveen T, Emad S, Haider S, Sadaf S, Qadeer S, Batool Z, Sarfaraz Y, Sheikh S. Role of Cyclooxygenase Inhibitors in Diminution of Dissimilar Stress-induced Depressive

Behavior and Memory Impairment in Rats. Neuroscience. 2017 Nov 12. pii: S0306-4522(17)30801-1.

80. Hesketh S, Jessop DS, Hogg S, Harbuz MS. Differential actions of acute and chronic citalopram on the rodent hypothalamic-pituitary-adrenal axis response to acute restraint stress. J Endocrinol. 2005 Jun;185(3):373-82.

81. Swanson LW, Sawchenko PE. Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. Annu Rev Neurosci. 1983;6:269-324.

82. Neumann ID. Involvement of the brain oxytocin system in stress coping: interactions with the hypothalamo-pituitary-adrenal axis. Prog Brain Res. 2002;139:147-62.

83. Kiecolt-Glaser JK, Derry HM, Fagundes CP. Inflammation: depression fans the flames and feasts on the heat. Am J Psychiatry. 2015 Nov 1;172(11):1075-91.

84. Kalia M. Neurobiological basis of depression: an update. Metabolism. 2005 May;54(5 Suppl 1):24-7.

85. Cui Y, Ren X, Li J, Zhai Q, Feng Y, Xu Y, Ma L. Effects of ammonia-N stress on metabolic and immune function via the neuroendocrine system in Litopenaeus vannamei. Fish Shellfish Immunol. 2017 May;64:270-275.

86. Carrasco GA, Van de Kar LD. Neuroendocrine pharmacology of stress. Eur J Pharmacol. 2003 Feb 28;463(1-3):235-72.

87. Pechnick RN, Kariagina A, Hartvig E, Bresee CJ, Poland RE, Chesnokova VM. Developmental exposure to corticosterone: behavioral changes and differential effects on leukemia inhibitory factor (LIF) and corticotropin-releasing hormone (CRH) gene expression in the mouse. Psychopharmacology (Berl). 2006 Mar;185(1):76-83.

88. Zoumakis E, Chrousos GP. Corticotropin-releasing hormone receptor antagonists: an update. Endocr Dev. 2010;17:36-43.

89. Alsina FC, Irala D, Fontanet PA, Hita FJ, Ledda F, Paratcha G. Sprouty4 is an endogenous negative modulator of TrkA signaling and neuronal differentiation induced by NGF. PLoS One. 2012;7(2):e32087

90. Green JG, Avenevoli S, Gruber MJ, Kessler RC, Lakoma MD, Merikangas KR, Sampson NA, Zaslavsky AM. Validation of diagnoses of distress disorders in the US National Comorbidity Survey Replication Adolescent Supplement (NCS-A). Int J Methods Psychiatr Res. 2012 Mar;21(1):41-51.

91. Neumeister A, Nugent AC, Waldeck T, Geraci M, Schwarz M, Bonne O, Bain EE, Luckenbaugh DA, Herscovitch P, Charney DS, Drevets WC. Neural and behavioural responses to tryptophan depletion in unmedicated patients with remitted major depressive disorder and controls. Arch Gen Psychiatry. 2004 Aug;61(8):765-73.

92. Iscan Z, Rakesh G, Rossano S, Yang J, Zhang M, Miller J, Sullivan GM, Sharma P, McClure M, Oquendo MA, Mann JJ, Parsey RV, DeLorenzo C. A positron emission tomography study of the serotonergic system in relation to anxiety in depressionEur Neuropsychopharmacol. 2017 Oct;27(10):1011-1021.

93. Neumeister A, Bain E, Nugent AC, Carson RE, Bonne O, Luckenbaugh DA, Eckelman W, Herscovitch P, Charney DS, Drevets WC. Reduced serotonin type 1A receptor binding in panic disorder. J Neurosci. 2004 Jan 21;24(3):589-91

94. Hasler G, Bonwetsch R, Giovacchini G, Toczek MT, Bagic A, Luckenbaugh DA, Drevets WC, Theodore WH. 5-HT1A receptor binding in temporal lobe epilepsy

patients with and without major depression. Biol Psychiatry. 2007 Dec 1;62(11):1258-64.

95. Meyer JH, Ginovart N, Boovariwala A, Sagrati S, Hussey D, Garcia A, Young T, Praschak-Rieder N, Wilson AA, Houle S. Elevated monoamine oxidase a levels in the brain: an explanation for the monoamine imbalance of major depression. Arch Gen Psychiatry. 2006 Nov;63(11):1209-16.
96. Coplan JD, Gupta NK, Karim A, Rozenboym A, Smith ELP, Kral JG, Rosenblum LA. Maternal hypothalamic-pituitary-adrenal axis response to foraging uncertainty: A model of individual vs. social allostasis and the "Superorganism Hypothesis". PLoS One. 2017 Sep 7;12(9):e0184340.
97. Wierońska JM, Pilc A. Metabotropic glutamate receptors in the tripartite synapse as a target for new psychotropic drugs. Neurochem Int. 2009 Jul-Aug;55(1-3):85-97.
98. Pałucha-Poniewiera A, Pilc A. Glutamate-Based Drug Discovery for Novel Antidepressants. Expert Opin Drug Discov. 2016 Sep;11(9):873-83.
99. Gittins RA, Harrison PJ. A morphometric study of glia and neurons in the anterior cingulate cortex in mood disorder. J Affect Disord. 2011 Sep;133(1-2):328-32.
100. Banasr M, Chowdhury GM, Terwilliger R, Newton SS, Duman RS, Behar KL, Sanacora G. Glial pathology in an animal model of depression: reversal of stress-induced cellular, metabolic and behavioral deficits by the glutamate-modulating drug riluzole. Mol Psychiatry. 2010 May;15(5):501-11.
101. Bradford HF, Ward HK, Thomas AJ. Glutamine--a major substrate for nerve endings. J Neurochem. 1978 Jun;30(6):1453-9.

102. Herman JP, Larson BR. Differential regulation of forebrain glutamic acid decarboxylase mRNA expression by aging and stress. Brain Res. 2001 Aug 31;912(1):60-6.

103. Soghomonian JJ, Martin DL. Two isoforms of glutamate decarboxylase: why? Trends Pharmacol Sci. 1998 Dec;19(12):500-5.

104. Torres-Platas SG, Hercher C, Davoli MA, Maussion G, Labonté B, Turecki G, Mechawar N. Astrocytic hypertrophy in anterior cingulate white matter of depressed suicides. Neuropsychopharmacology. 2011 Dec;36(13):2650-8.

105. Benson C, Mifflin K, Kerr B, Jesudasan SJ, Dursun S, Baker G. Biogenic Amines and the Amino Acids GABA and Glutamate: Relationships with Pain and Depression. Mod Trends Pharmacopsychiatry. 2015;30:67-79

106. Bruno V, Caraci F, Copani A, Matrisciano F, Nicoletti F, Battaglia G. The impact of metabotropic glutamate receptors into active neurodegenerative processes: A "dark side" in the development of new symptomatic treatments for neurologic and psychiatric disorders. Neuropharmacology. 2017 Mar 15;115:180-192.

107. Dhir A. Investigational drugs for treating major depressive disorder. Expert Opin Investig Drugs. 2017 Jan;26(1):9-24.

108. Hasler G, Fromm S, Alvarez RP, Luckenbaugh DA, Drevets WC, Grillon C. Cerebral blood flow in immediate and sustained anxiety. J Neurosci. 2007 Jun 6;27(23):6313-9.

109. Frodl TS, Koutsouleris N, Bottlender R, Born C, Jager M, Scupin I, Reiser M, Maller HJ, Meisenzahl EM. Depression-related variation in brain morphology over 3 years: effects of stress?. Arch Gen Psychiatry. 2008 Oct;65(10):1156-65.

110. Martinowich K, Manji H, Lu B. New insights into BDNF function in depression and anxiety. Nat Neurosci. 2007 Sep;10(9):1089-93.

111. Prins J, Olivier B, Korte SM. Triple reuptake inhibitors for treating subtypes of major depressive disorder: the monoamine hypothesis revisited. Expert Opin Investig Drugs. 2011 Aug;20(8):1107-30.

112. Catena-Dell'Osso M, Fagiolini A, Rotella F, Baroni S, Marazziti D. Glutamate system as target for development of novel antidepressants. CNS Spectr. 2013 Aug;18(4):188-98.

113. Rose EJ, Ebmeier KP. Pattern of impaired working memory during major depression. J Affect Disord. 2006 Feb;90(2-3):149-61.

114. Zhao J, Yin D, Rowe J, Badawy S, Nikfar F, Pandey P. Understanding the Factors that Control the Quality of Mini-tablet Compression: Flow, Particle Size and Tooling Dimension. J Pharm Sci. 2017 Dec;S0022-3549(17)30871-7.

115. Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S. Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci. 2002 Aug 1;22(15):6810-8.

116. Egeland M, Zunszain PA, Pariante CM. Molecular mechanisms in the regulation of adult neurogenesis during stress. Nat Rev Neurosci. 2015 Apr;16(4):189-200.

117. Ricon T, Toth E, Leshem M, Braun K, Richter-Levin G. Unpredictable chronic stress in juvenile or adult rats has opposite effects, respectively, promoting and impairing resilience. Stress. 2012 Jan;15(1):11-20.

118. Ter Horst JP, de Kloet ER, Schächinger H, Oitzl MS. Relevance of stress and female sex hormones for emotion and cognition. Cell Mol Neurobiol. 2012 Jul;32(5):725-35.

119. Sousa N, Almeida OF. Disconnection and reconnection: the morphological basis of (mal)adaptation to stress. Trends Neurosci. 2012 Dec;35(12):742-51.

120. Lupien SJ, McEwen BS, Gunnar MR, Heim C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci. 2009 Jun;10(6):434-45.

121. Katz RJ, Roth KA, Carroll BJ. Acute and chronic stress effects on open field activity in the rat: implications for a model of depression. Neurosci Biobehav Rev. 1981 Summer;5(2):247-51.

122. Hill MN, Hellemans KG, Verma P, Gorzalka BB, Weinberg J. Neurobiology of chronic mild stress: parallels to major depression. Neurosci Biobehav Rev. 2012 Oct;36(9):2085-117.

123. Zhang ZJ. Therapeutic effects of herbal extracts and constituents in animal models of psychiatric disorders. Life Sci. 2004 Aug 20;75(14):1659-99.

124. Cryan JF, Markou A, Lucki I. Assessing antidepressant activity in rodents recent developments and future needs. Trends Pharmacol Sci. 2002 May;23(5):238-45.

125. Porsolt RD, Bertin A, Jalfre M. "Behavioural despair" in rats and mice: strain differences and the effects of imipramine. Eur J Pharmacol. 1978 Oct 1;51(3):291-4.

126. Grigoryan GA, Gulyaeva NV. [Animal Models of Depression: Behavior as the Basis for Methodology, Assessment Criteria and Classifications]. Zh Vyssh Nerv Deiat Im I P Pavlova. 2015 Nov-Dec;65(6):643-60.

127. Willner P. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiology. 2005;52(2):90-110.

128. Chaouloff F. Social stress models in depression research: what do they tell us? Cell Tissue Res. 2013 Oct;354(1):179-90.

129. Aroz EE, Bolton P, Gross A, Chan KS, Michalopoulos L, Bass J. Depression symptoms across cultures: an IRT analysis of standard depression symptoms using data from eight countries. Soc Psychiatry Psychiatr Epidemiol. 2016 Jul;51(7):981-91.

130. Benedetti M, Giuliani ME, Regoli F. Oxidative metabolism of chemical pollutants in marine organisms: molecular and biochemical biomarkers in environmental toxicology. Ann N Y Acad Sci. 2015 Mar;1340:8-19.

131. Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K, Taniguchi K, Tsuda S. The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutat Res. 2002 Aug 26;519(1-2):103-19.

132. Arslan-Acaroz D, Zemheri F, Demirel HH, Kucukkurt I, Ince S, Eryavuz A. In vivo assessment of polydatin, a natural polyphenol compound, on arsenic-induced free radical overproduction, gene expression, and genotoxicity. Environ Sci Pollut Res Int. 2017 Nov 12.43(51):231-40.

133. Lee SM, Koh HJ, Park DC, Song BJ, Huh TL, Park JW. Cytosolic NADP(+)-dependent isocitrate dehydrogenase status modulates oxidative damage to cells. Free Radic Biol Med. 2002 Jun 1;32(11):1185-96.

134. Matés JM, Sánchez-Jiménez F. Antioxidant enzymes and their implications in pathophysiologic processes. Front Biosci. 1999 Mar 15;4:D339-45.

135. Arora D, Jain P, Singh N, Kaur H, Bhatla SC. Mechanisms of nitric oxide crosstalk with reactive oxygen species scavenging enzymes during abiotic stress tolerance in plants. Free Radic Res. 2016;50(3):291-303.

136. Winrow VR, Winyard PG, Morris CJ, Blake DR. Free radicals in inflammation: second messengers and mediators of tissue destruction. Br Med Bull. 1993 Jul;49(3):506-22.

137. Maes M. Depression is an inflammatory disease, but cell-mediated immune activation is the key component of depression. Prog Neuropsychopharmacol Biol Psychiatry. 2011 Apr 29;35(3):664-75.

138. Leonard B, Maes M. Mechanistic explanations how cell-mediated immune activation, inflammation and oxidative and nitrosative stress pathways and their sequels and concomitants play a role in the pathophysiology of unipolar depression. Neurosci Biobehav Rev. 2012 Feb;36(2):764-85.

139. Hayley S, Scharf J, Anisman H. Central administration of murine interferon-α induces depressive-like behavioral, brain cytokine and neurochemical alterations in mice: a mini-review and original experiments. Brain Behav Immun. 2013 Jul;31:115-27.

140. Huang HJ, Chen WL, Hsieh RH, Hsieh-Li HM. Multifunctional Effects of Mangosteen Pericarp on Cognition in C57BL/6J and Triple Transgenic Alzheimer's Mice. Evid Based Complement Alternat Med. 2014;2014:813672.

141. Cauwels A, Rogge E, Vandendriessche B, Shiva S, Brouckaert P. Extracellular ATP drives systemic inflammation, tissue damage and mortality. Cell Death Dis. 2014 Mar 6;5:e1102.

142. Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC. Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther. 2006 Nov;112(2):358-404.

143. Marek R, Strobel C, Bredy TW, Sah P. The amygdala and medial prefrontal cortex: partners in the fear circuit. J Physiol. 2013 May 15;591(10):2381-91.

144. Pickens CL, Airavaara M, Theberge F, Fanous S, Hope BT, Shaham Y. Neurobiology of the incubation of drug craving. Trends Neurosci. 2011 Aug;34(8):411-20.

145. Trogrlic L, Wilson YM, Newman AG, Murphy M. Context fear learning specifically activates distinct populations of neurons in amygdala and hypothalamus. Learn Mem. 2011 Oct 3;18(10):678-87.

146. Walker DL, Paschall GY, Davis M. Glutamate receptor antagonist infusions into the basolateral and medial amygdala reveal differential contributions to olfactory vs. context fear conditioning and expression. Learn Mem. 2005 Mar-Apr;12(2):120-9.

147. Siegmund A, Wotjak CT. A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitised fear. J Psychiatr Res. 2007 Nov;41(10):848-60.

148. Pamplona FA, Henes K, Micale V, Mauch CP, Takahashi RN, Wotjak CT. Prolonged fear incubation leads to generalized avoidance behavior in mice. J Psychiatr Res. 2011 Mar;45(3):354-60.

149. Hough LB, Nalwalk JW, Yang W, Ding X. Significance of neuronal cytochrome P450 activity in opioid-mediated stress-induced analgesia. Brain Res. 2014 Aug 26;1578:30-7.

150. Sugimura Y, Murase T, Ishizaki S, Tachikawa K, Arima H, Miura Y, Usdin TB, Oiso Y. Centrally administered tuberoinfundibular peptide of 39 residues inhibits arginine vasopressin release in conscious rats. Endocrinology. 2003 Jul;144(7):2791-6.

151. Luque RM, Park S, Kineman RD. Role of endogenous somatostatin in regulating GH output under basal conditions and in response to metabolic extremes. Mol Cell Endocrinol. 2008 May 14;286(1-2):155-68.

152. Bagó AG, Dimitrov E, Saunders R, Seress L, Palkovits M, Usdin TB, Dobolyi A. Parathyroid hormone 2 receptor and its endogenous ligand tuberoinfundibular peptide of 39 residues are concentrated in endocrine, viscerosensory and auditory brain regions in macaque and human. Neuroscience. 2009 Aug 4;162(1):128-47.

153. Bratincsák A, Palkovits M. Activation of brain areas in rat following warm and cold ambient exposure. Neuroscience. 2004;127(2):385-97.

154. Morrison SF. Central neural control of thermoregulation and brown adipose tissue. Auton Neurosci. 2016 Apr;196:14-24.

155. Cservenák M, Bodnár I, Usdin TB, Palkovits M, Nagy GM, Dobolyi A. Tuberoinfundibular peptide of 39 residues is activated during lactation and participates in the suckling-induced prolactin release in rat. Endocrinology. 2010 Dec;151(12):5830-40.

156. Lonstein JS, Stern JM. Role of the midbrain periaqueductal gray in maternal nurturance and aggression: c-fos and electrolytic lesion studies in lactating rats. J Neurosci. 1997 May 1;17(9):3364-78.

157. Hasen NS, Gammie SC. Differential fos activation in virgin and lactating mice in response to an intruder. Physiol Behav. 2005 Apr 13;84(5):681-95.

158. Eichinger A, Fiaschi-Taesch N, Massfelder T, Fritsch S, Barthelmebs M, Helwig JJ. Transcript expression of the tuberoinfundibular peptide (TIP)39/PTH2 receptor system

and non-PTH1 receptor-mediated tonic effects of TIP39 and other PTH2 receptor ligands in renal vessels. Endocrinology. 2002 Aug;143(8):3036-43.

159. Degenhardt H, Jansen J, Schulz R, Sedding D, Braun-Dullaeus R, Schlüter KD. Mechanosensitive release of parathyroid hormone-related peptide from coronary endothelial cells. Am J Physiol Heart Circ Physiol. 2002 Oct;283(4):H1489-96.
160. Santos GA, Pereira VD, Roman EA, Ignacio-Souza L, Vitorino DC, De Moura RF, Razolli DS, Torsoni AS, Velloso LA, Torsoni MA. Hypothalamic inhibition of acetyl-CoA carboxylase stimulates hepatic counter-regulatory response independent of AMPK activation in rats. PLoS One. 2013 Apr 23;8(4):e62669.
161. Gao S, Cui YL, Yu CQ, Wang QS, Zhang Y. Tetrandrine exerts antidepressant-like effects in animal models: role of brain-derived neurotrophic factor. Behav Brain Res. 2013 Feb 1;238:79-85
162. Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods. 1985 Aug;14(3):149-67.
163. Yazir Y, Utkan T, Gacar N, Aricioglu F. Resveratrol exerts anti-inflammatory and neuroprotective effects to prevent memory deficits in rats exposed to chronic unpredictable mild stress. Physiol Behav. 2015 Jan;138:297-304.
164. Castrogiovanni P, Blardi P, De Lalla A, Dell'Erba A, Auteri A. Can serotonin and fluoxetine levels in plasma and platelets predict clinical response in depression? Psychopharmacol Bull. 2003 Spring;37(2):102-8.
165. Wang Y, Fice DS, Yeung PK. A simple high-performance liquid chromatography assay for simultaneous determination of plasma norepinephrine, epinephrine,

dopamine and 3,4-dihydroxyphenyl acetic acid. J Pharm Biomed Anal. 1999 Nov;21(3):519-25.

166. Babu CS, Ramanathan M. Pre-ischemic treatment with memantine reversed the neurochemical and behavioural parameters but not energy metabolites in middle cerebral artery occluded rats. Pharmacol Biochem Behav. 2009 May;92(3):424-32.
167. Badenhorst NJ, Brand L, Harvey BH, Ellis SM, Brink CB. Long-term effects of pre-pubertal fluoxetine on behaviour and monoaminergic stress response in stress-sensitive rats. Acta Neuropsychiatr. 2017 Aug;29(4):222-235.
168. Ehmann GL, Leung JY, Giangrande PH, Nevins JR. A role for Myc in facilitating transcription activation by E2F1. Oncogene. 2008 Jul 10;27(30):4172-9.
169. Naskar S, Islam A, Mazumder UK, Saha P, Haldar PK, Gupta M. *In Vitro* and *In Vivo* antioxidant potential of hydro methanolic extract of Phoenix dactylifera fruits. J Sci Res. 2010;2(1):144–57.
170. Darshit BS, Ramanathan M. Activation of AKT1/GSK-3β/β-Catenin-TRIM11/Surviving Pathway by Novel GSK-3β Inhibitor Promotes Neuron Cell Survival: Study in Differentiated SH-SY5Y Cells in OGD Model. Mol Neurobiol. 2016 Dec;53(10):6716-6729.
171. Ducottet C, Griebel G, Belzung C. Effects of the selective nonpeptide corticotrophin releasing factor receptor 1 antagonist antalarmin in the chronic mild stress model of depression in mice. Prog. Neuropsychopharmacol. Biol. Psychiatry. 2003March: 27(4), 625-631.

172. Willner P, Towell A, Sampson D, Sophokleous S, Muscat R. Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacology (Berl). 1987;93(3):358-64.

173. Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology (Berl). 1985;85(3):367-70.

174. Sun Y, Larry WO, Ying L. A simple method for clinical assay of superoxide dismutase. Clin. Chem. 1988June; 34(102):497–500.

175. Aebi, H. Catalase in vitro. Methods. Enzymol. 1984; 105:121–126.

176. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979 Jun;95(2):351-8.

177. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein estimation with folin phenol reagent. J. Biol. Chem. 1951Sep;193(47):265–275.

178. Zhan C, Yang J. Protective effects of isoliquiritigenin in transient middle cerebral artery occlusion-induced focal cerebral ischemia in rats. Pharmacol Res. 2006 Mar;53(3):303-9.

179. Vasudevan M, Milind P. Memory-enhancing activity of Coriandrumsativum in rats. Pharmacol online. 2009 Aug;2(4):827-39.

180. Dhingra D, Kumar V. Memory-enhancing activity of palmatine in mice using elevated plus maze and morris water maze. Adv Pharmacol Sci. 2012;2012:357368.

181. Talib F. Abbas. Optimizing Object Recognition Task: A Practical Study in Neurobiology by using C57BL/6 normal mice. Journal of Basrah Researches Sciences.2013;39(2):203-24

182. Dirkx R, Thomas A, Li L, Lernmark A, Sherwin RS, De Camilli P, Solimena M. Targeting of the 67-kDa isoform of glutamic acid decarboxylase to intracellular organelles is mediated by its interaction with the NH2-terminal region of the 65-kDa isoform of glutamic acid decarboxylase. J Biol Chem. 1995 Feb 3;270(5):2241-6.

183. Hala F. Zaki , May A. Abd-El-Fattah, Amina S. Attia. Naringenin protects against scopolamine-induced dementia in rats. Bulletin of Faculty of Pharmacy, Cairo University. 2014;52,15–25.

184. Abdallah CG, Hannestad J, Mason GF, Holmes SE, DellaGioia N, Sanacora G, Jiang L, Matuskey D, Satodiya R, Gasparini F, Lin X, Javitch J, Planeta B, Nabulsi N, Carson RE, Esterlis I. Metabotropic Glutamate Receptor 5 and Glutamate Involvement in Major Depressive Disorder: A Multimodal Imaging Study. Biol Psychiatry Cogn Neurosci Neuroimaging. 2017 Jul;2(5):449-456.

185. Joca SR, Padovan CM, Guimarães FS. Activation of post-synaptic 5-HT(1A) receptors in the dorsal hippocampus prevents learned helplessness development. Brain Res. 2003 Jul 18;978(1-2):177-84.

186. Rodgers RJ, Johnson NJ. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacol Biochem Behav. 1995 Oct;52(2):297-303.

187. Lupien SJ, McEwen BS, Gunnar MR, Heim C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci. 2009 Jun;10(6):434-45.

188. Subakanmani S, Umadevi P. Evaluation of Anxiolytic Potential of Ethanolic Extract Hypericum Hookerianum in Stress Induced Swiss Albino Mice. Mol Neurobiol. 2012 Feb24;3(4):219–25.

189. Dammann F, Kirschstein T, Guli X, Müller S, Porath K, Rohde M, Tokay T, Köhling R. Bidirectional shift of group III metabotropic glutamate receptor-mediated synaptic depression in the epileptic hippocampus. Epilepsy Res.2017 Dec 7. pii: S0920-1211(17)30405-9.

190. Pathan AR, Kothawade KA, Logade MN. Anxiolytic and analgesic effect of seeds of Coriandrum sativum Linn. Int J Res Pharm Chem. 2011Sep 20;1:1087–99.

191. Freitas AE, Egea J, Buendia I, Gómez-Rangel V, Parada E, Navarro E, Casas AI, Wojnicz A, Ortiz JA, Cuadrado A, Ruiz-Nuño A, Rodrigues ALS, Lopez MG. Agmatine, by Improving Neuroplasticity Markers and Inducing Nrf2, Prevents Corticosterone-Induced Depressive-Like Behavior in Mice. Mol Neurobiol. 2016 Jul;53(5):3030-3045.

192. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci. 2009 Jun;10(6):397-409.

193. Lam RW, Iverson GL, Evans VC, Yatham LN, Stewart K, Tam EM, Axler A, Woo C. The effects of desvenlafaxine on neurocognitive and work functioning in employed outpatients with major depressive disorder. J Affect Disord. 2016 Oct;203:55-61.

194. Coutellier L, Logemann A, Kuo J, Rusnak M, Usdin TB. TIP39 modulates effects of novelty-induced arousal on memory. Genes Brain Behav. 2011;10(1):90-9.

195. Mathews DC, Henter ID, Zarate CA. Targeting the glutamatergic system to treat major depressive disorder: rationale and progress to date. Drugs. 2012 ;72(10):1313-33.

196. Dimitrov EL, Kim YY, Usdin TB. Regulation of hypothalamic signaling by tuberoinfundibular peptide of 39 residues is critical for the response to cold: a novel peptidergic mechanism of thermoregulation. J Neurosci. 2011;31(49):18166-79.

197. Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. Nat Rev Immunol. 2005 Mar;5(3):243-51.

198. Kim A, Sung JH, Bang JH, Cho SW, Lee J, Sim CS. Effects of self-reported sensitivity and road-traffic noise levels on the immune system. PLoS One. 2017 Oct 30;12(10):e0187084.

199. Andersen BL, Goyal NG, Westbrook TD, Bishop B, Carson WE 3rd. Trajectories of Stress, Depressive Symptoms, and Immunity in Cancer Survivors: Diagnosis to 5 Years. Clin Cancer Res. 2017 Jan 1;23(1):52-61.

200. Biala G, Pekala K, Boguszewska-Czubara A, Michalak A, Kruk-Slomka M, Budzynska B. Behavioral and Biochemical Interaction Between Nicotine and Chronic Unpredictable Mild Stress in Mice. Mol Neurobiol. 2017 Mar;54(2):904-921.

201. Joca SR, Padovan CM, Guimarães FS. Activation of post-synaptic 5-HT(1A) receptors in the dorsal hippocampus prevents learned helplessness development. Brain Res. 2003 Jul 18;978(1-2):177-84.

202. Detke MJ, Rickels M, Lucki I. Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. Psychopharmacology (Berl). 1995;121(1):66-72.

203. Wang C, Guo R. The Effect of Xingpijieyu Decoction on Depressive Behavior and Serum 5-HT as well as Corticosterone of Depression Rats from Chronic Stress. J. Tradit. Chin. Med. 2014May; 12:1633–35.

204. Prins J, Olivier B, Korte SM. Triple reuptake inhibitors for treating subtypes of major depressive disorder: the monoamine hypothesis revisited. Expert Opin Investig Drugs. 2011 Aug;20(8):1107-30.

205. Rai D, Bhatia G, Sen T, Palit G. Comparative study of perturbations of peripheral markers in different stressors in rats. Can J Physiol Pharmacol. 2003 Dec;81(12):1139-46.

206. Wu LM, Han H, Wang QN, Hou HL, Tong H, Yan XB, Zhou JN. Mifepristone repairs region-dependent alteration of synapsin I in hippocampus in rat model of depression. Neuropsychopharmacology. 2007 Dec;32(12):2500-10.

207. Luo DD, An SC, Zhang X. Involvement of hippocampal serotonin and neuropeptide Y in depression induced by chronic unpredicted mild stress. Brain Res Bull. 2008 Sep 5;77(1):8-12.

208. Becker C, Zeau B, Rivat C, Blugeot A, Hamon M, Benoliel JJ. Repeated social defeat-induced depression-like behavioral and biological alterations in rats: involvement of cholecystokinin. Mol Psychiatry. 2008 Dec;13(12):1079-92.

209. Richardson DK, Reynolds SM, Cooper SJ, Berridge KC. Endogenous opioids are necessary for benzodiazepine palatability enhancement: naltrexone blocks diazepam-induced increase of sucrose-'liking'. Pharmacol Biochem Behav. 2005 Jul;81(3):657-63.

210. Farhan M, Ikram H, Kanwal S, Haleem DJ. Unpredictable chronic mild stress induced behavioral deficits: a comparative study in male and female rats. Pak J Pharm Sci. 2014 Jul;27(4):879-84.

211. Dimitrov E, Usdin TB. Tuberoinfundibular peptide of 39 residues modulates the mouse hypothalamic-pituitary-adrenal axis via paraventricular glutamatergic neurons. J. Comp. Neurol., 2010 April; 518(21):4375-94.

212. Frodl TS, Koutsouleris N, Bottlender R, Born C, Jager M, Scupin I, Reiser M, Moller HJ, Meisenzahl EM. Depression-related variation in brain morphology over 3 years: effects of stress? Arch Gen Psychiatry. 2008 Oct;65(10):1156-65.

213. Chaudiere J, Ferrari-Iliou R. Intracellular antioxidants: from chemical to biochemical mechanisms. Food Chem Toxicol. 1999 Sep-Oct;37(9-10):949-62. Review. PubMed PMID: 10541450.

214. Rajan RK, M SS, Balaji B. Soy isoflavones exert beneficial effects on letrozole-induced rat polycystic ovary syndrome (PCOS) model through anti-androgenic mechanism. Pharm Biol. 2017 Dec;55(1):242-251.

215. Zafir A, Banu N. Antioxidant potential of fluoxetine in comparison to Curcuma longa in restraint-stressed rats. Eur J Pharmacol. 2007 Oct 15;572(1):23-31.

216. Beg AA, Finco TS, Nantermet PV, Baldwin AS Jr. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. Mol Cell Biol. 1993 Jun;13(6):3301-10.

217. Ricon T, Toth E, Leshem M, Braun K, Richter-Levin G. Unpredictable chronic stress in juvenile or adult rats has opposite effects, respectively, promoting and impairing resilience. Stress. 2012 Jan;15(1):11-20.

218. Parihar VK, Hattiangady B, Kuruba R, Shuai B, Shetty AK. Predictable chronic mild stress improves mood, hippocampal neurogenesis and memory. Mol Psychiatry. 2011 Feb;16(2):171-83.

219. McLaughlin KJ, Gomez JL, Baran SE, Conrad CD. The effects of chronic stress on hippocampal morphology and function: an evaluation of chronic restraint paradigms. Brain Res. 2007 Aug 3;1161:56-64.

220. Ramkumar K, Srikumar BN, Shankaranarayana Rao BS, Raju TR. Self-stimulation rewarding experience restores stress-induced CA3 dendritic atrophy, spatial memory deficits and alterations in the levels of neurotransmitters in the hippocampus. Neurochem Res. 2008 Sep;33(9):1651-62.

221. Kim HJ, Shin KY, Chang KA, Ahn S, Choi HS, Kim HS, Suh YH. Dehydroevodiamine·HCl Improves Stress-Induced Memory Impairments and Depression Like Behavior in Rats. Korean J Physiol Pharmacol. 2014 Feb;18(1):55-9.

222. Shishikura Y, Koarai A, Aizawa H, Yamaya M, Sugiura H, Watanabe M, Hashimoto Y, Numakura T, Makiguti T, Abe K, Yamada M, Kikuchi T, Hoshikawa Y, Okada Y, Ichinose M. Extracellular ATP is involved in dsRNA-induced MUC5AC production via P2Y2R in human airway epithelium. Respir Res. 2016 Sep 27;17(1):121.

223. Farhan M, Ikram H, Kanwal S, Haleem DJ. Unpredictable Unpredictable chronic mild stress induced behavioural deficits: A comparative study in male and female rats. Pak. J. Pharm. Sci. 2014 Dec; 27: 879-884.

224. Koizumi M, Kondo Y, Isaka A, Ishigami A, Suzuki E. Vitamin C impacts anxiety-like behavior and stress-induced anorexia relative to social environment in SMP30/GNL knockout mice. Nutr Res. 2016 Dec;36(12):1379-1391.

225. Coutellier L, Logemann A, Kuo J, Rusnak M, Usdin TB. TIP39 modulates effects of novelty-induced arousal on memory. Genes, Brain Behav.2011;10(1):90–9.

226. Fan Y, Hu J, Li J, Yang Z, Xin X, Wang J, Ding J, Geng M. Effect of acidic oligosaccharide sugar chain on scopolamine-induced memory impairment in rats and its related mechanisms. Neurosci Lett. 2005 Feb 21;374(3):222-6.

227. Jeong EJ, Lee KY, Kim SH, Sung SH, Kim YC. Cognitive-enhancing and antioxidant activities of iridoid glycosides from Scrophularia buergeriana in scopolamine-treated mice. Eur J Pharmacol. 2008 Jun 24;588(1):78-84.

228. Hancianu M, Cioanca O, Mihasan M, Hritcu L. Neuroprotective effects of inhaled lavender oil on scopolamine-induced dementia via anti-oxidative activities in rats. Phytomedicine. 2013 Mar 15;20(5):446-52.

229. Woolley CS, Gould E, McEwen BS. Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons. Brain Res. 1990 Oct 29;531(1-2):225-31.

230. Egeland M, Zunszain PA, Pariante CM. Molecular mechanisms in the regulation of adult neurogenesis during stress. Nat Rev Neurosci. 2015 Apr;16(4):189-200.

231. Dinse HR, Kattenstroth JC, Lenz M, Tegenthoff M, Wolf OT. The stress hormone cortisol blocks perceptual learning in humans. Psychoneuroendocrinology. 2017 Mar;77:63-67.

232. Roozendaal B. Stress and memory: opposing effects of glucocorticoids on memory consolidation and memory retrieval. Neurobiol Learn Mem. 2002 Nov;78(3):578-95.

233. De Kloet ER, Van Acker SA, Sibug RM, Oitzl MS, Meijer OC, Rahmouni K, De Jong W. Brain mineralocorticoid receptors and centrally regulated functions. Kidney Int. 2000 Apr;57(4):1329-36.

234. Lakshmi BV, Sudhakar M, Anisha M. Neuroprotective role of hydroalcoholic extract of Vitis vinifera against aluminium-induced oxidative stress in rat brain. Neurotoxicology. 2014 Mar;41:73-9.

235. Amjad S, Umesalma S. Protective Effect of Centella asiatica against Aluminium-Induced Neurotoxicity in Cerebral Cortex, Striatum, Hypothalamus and Hippocampus of Rat Brain-Histopathological, and Biochemical Approac. J Mol Biomark Diagn.2015 Jan3; 6(2): 1212-14 .

236. Sumathi T, Shobana C, Thangarajeswari M, Usha R. Protective effect of L-Theanine against aluminium induced neurotoxicity in cerebral cortex, hippocampus and cerebellum of rat brain - histopathological, and biochemical approach. Drug Chem Toxicol. 2015 Jan;38(1):22-31.

PLAGIARISM CERTIFICATE

This is to certify that this dissertation work titled **BEHAVIORAL AND NEUROCHEMICAL ASSESSMENT OF PARATHYROID HORMONE 2 (PTH2) RECEPTOR AGONIST TUBEROINFUNDIBULAR PEPTIDE OF 39 (TIP39) IN NEUROLOGICAL DISORDERS** of the candidate **G.Venkatesh** with registration Number **141440026** for the award of **Doctor of Philosophy** in the branch of **Pharmacy and Pharmacology.** I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from the title to conclusion pages and result shows **1** percentage of plagiarism in the dissertation.

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Institutional Animal Ethics Committee

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DATE: 19.03.2015

Title of the Project: Effect of TIP39 peptide on chronic unpredictable mild stress induced learning and Memory Impairment in rats

Proposal Number: 280/2015/ IAEC

Name of the Applicant: Mr.G.Venkatesh

Approval date: 19.03.2015

Expiry date (Termination of the Project): 18.03.2016

No. of animals sanctioned with name of species: Thirty Sprague Dawley rats and methodology approved. Forced Swim test to be performed before open field test.

Signature of Chairperson

Date:

Name of the chairperson

Dr.S.Ramalingam

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Signature of the CPCSEA nominee

Date: 19/3/2015

Name of IAEC/CPCSEA nominee

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DATE: 20.05.2017

Title of the Project: Evaluation of role of TIP39 as antidepressant, anxiolytic and Memory enhancement in rats.

Proposal Number:	359 /2017/ IAEC
Name of the Applicant:	G.Venkatesh.
Approval date:	20.05.2017
Expiry date (Termination of the Project):	19.05.2018
Methodology:	Approved.

Name of species: **Swiss albino mice/ Wistar rats/ Sprague Dawley rats/ Guinea pigs/ Newzealand White rabbits.**

Male/Female/Both sex----48----animals approved.

Signature of Chairperson

Date: 20.5.17

Dr.M.Ramanathan

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2 messages

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Dear Prof. M Ahmed
i have submitted my research paper in your journal entitled Neurochemical Assessment and Behavioural Role of Tuberoinfundibular Peptide 39 in Acute Restraint Stress Induced Depression in Rats". Kindly let me know the status regarding this.
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Regards
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NEUROCHEMICAL ASSESSMENT AND BEHAVIOURAL ROLE OF TUBEROINFUNDIBULAR PEPTIDE-39 IN ACUTE RESTRAINT STRESS INDUCED DEPRESSION IN RATS

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Abstract

Objective: Tuberoinfundibular Peptide of 39 (TIP39) is a potent agonist to the parathyroid hormone receptor 2 receptor (PTH2R) abundantly expressed in brain. The current study focused to evaluate the role of TIP39 in acute restraint stress (ARS) induced depression model. **Methods:** Rats were exposed to acute restraint stress for 2 hr to establish the depression and then subjected to open field and forced swim test. TIP39 (1&10 nmol/rat) and HYWH (1nmol/rat) is a PTH2R antagonist were infused through Intracerebroventricular (i.c.v) route. Diazepam (2 mg/kg, i.p) was utilized as reference standard. **Results:**The results depict, ARS significantly diminished the TIP39 expression in cerebral regions, causes depression like behaviour. TIP39 significantly decreased the immobility period in forced swim test (FST). In the open field test (OFT), TIP39 significantly increased the ambulatory activity and did not alter the rearing and grooming activity in comparison to ARS group. After TIP39 treatment, plasma noradrenaline levels were significantly increased, whereas the serotonin levels were unaltered. The corticosterone (CORT) levels also decreased significantly. In rat brain tissues, TIP39 significantly reversed the abnormalities in glutamate and GABA level by ARS induction. In contrast, HYWH treated rats did not show any significant variations in the neurochemical and behavioural parameters in comparison to ARS rats. **Conclusion:** Our reports submitted the primary evidence depicting the stimulation of TIP39 expression could modulate the monoaminergic, GABAergic and glutaminergic release with the support of HPA axis that can be produced an antidepressant-like effect evident with the interactive study.

Key words: TIP39; acute restraint stress; HPA axis; Glutamate; GABA; Noradrenaline

INTRODUCTION

The stress involved in the genesis of depression and is considered a paramount factor. It is estimated that depression will be the second most prevalent disease throughout the world[1].Agreeing to a story from WHO, approximately 300 million people all over the world suffer from depression and is forecast to grow by 15% by the year 2020[2]. TIP39 is a neuroendocrine hormone, acting through PTH2 receptor that potentially regulates HPA axis system. Studies proposed that TIP39 induces fos gene expression in the infralimbic cortex, preoptic area, lateral hypothalamus, lateral septum, and paraventricular thalamic nucleus; areas believed to be imperative in anxiety and depression[3]. Mice lacking TIP39 or PTH2R signalling significantly displayed increased anxiety, depression, also exhibited fear memory after exposure to an aversive event[4]**.** Many hypotheses postulated regarding the pathophysiological base of depression results from lack of monoamine neurotransmitter, 2)

imbalance in Glutamate and GABA level in brain 3) over activation of the HPA axis. In depression, monoamine pathway is seen as the primary pathway embattled by most of the currently available depression therapies [5]. HPA axis hypothesis, which acts as a central part in mediating the responses to various stressful stimuli [6]. Exposure of acute restraint stress can activate the HPA axis, resulting in higher blood levels of corticosterone (CORT) and dysregulates circadian rhythm of CORT secretion. This cascade of event finally stimulates the over expression of glucocorticoids receptors in the hippocampus, which in turn cut the size and functionality of hippocampus. The revival of HPA axis activity has been seen as an indicator of improvement in depression. Studies also proposed that therapy of depression based on the serotonergic and noradrenergic mechanism effectively controls the channels of the HPA axis function [7]. In this setting, extensive research suggests that, studies on depression have shifted from monoamines toward other mechanisms, including glutaminergic neurotransmission[8]. About 80% of neurons in brain found to have glutamate, which is considerably more than monoamines. Glutamate is the major excitatory neurotransmitter which is in a balance with inhibitory neurotransmitter gamma-amino butyric acid (GABA)[9]. Emerging evidence hold up the deficits of GABAergic transmission is also a major cause for stress-induced depression. Also found that downstream modification in GABAergic activity with the currently available monoaminergic antidepressants, suggesting that deregulation of Glutamate and GABA system is involved in the pathogenesis of depression[10]. Though TIP39 have possible relation to the pathogenesis of depression, no direct evidence to authenticate the functional correlation of TIP39 and depression caused by stress. With the hold of optimistic regulation of TIP39, for the first time, this study point towards a specific neurotransmitter involvement in ARS rats, with the support of PTH2 receptor agonist and antagonist.

MATERIALS AND METHODS

Chemicals and reagents: TIP39 peptide purchased from Life Technologies Pvt Ltd, India. HYWH, PTH2R antagonist synthesised from Biomolecules Midwest Inc.USA. Serotonin (5HT), L-Glutamic acid and GABA were purchased from sigma, USA. Noradrenalin (NA) was received as gift sample from Neon lab, India. All other chemical, reagents and solvents were of analytical grade.

Animals: Male Sprague Dawley rats (150-200g) were obtained from PSG IMS&R (205/2013/IAEC). Experiment was performed according to the experimental protocol approved by the institutional animal ethics committee (IAEC). Rats were maintained in a separate cage (6 animals per cages) with standard diet. Animals were housed under room

temperature (25 ± 2º C), 12/12 hr light-dark cycle, humidity (55±5%) according to CPCSEA guidelines and guide for the care and use of laboratory animals.

Surgical procedure and drug treatment: TIP39 peptide was infused through intra-cerebroventricular route over 5 min. stereotoxic surgery was made seven days before the test and standard drug administration. Rats were anesthetised with ketamine (80 mg/kg, i.m.) xylazine (10 mg/kg,i.m.). Guide cannulae (Stailess steel (OD 0.64 x ID 0.45) RWD Life Science Co Ltd, China) was implanted in the right lateral ventricle at pre-established coordinates, anteroposterior, 0.2 mM from bregma; lateral, 1.5 mM; and vertical, 4.2 mM, and kept under controlled temperature in an individual cages [11].

Behaviour study

The animal behaviour was assessed in an open-field apparatus made of plywood, consisted of a floor (96 × 96 cm) with high walls. The entire apparatus was painted black except for 6 mm thick white lines which divided the floor into 16 squares. Experimental animals were placed in the centre of an open box and allowed for free exploration. Behavioural parameters were quantified, such as ambulation, rearing and grooming for 5 min [12]. With the slight modifications of FST was conducted. Rats were allowed to swim individually in a transparent glass vessel (45cmX12cmX45cm) with water filled to 30 cm [13]. 24 h before start of the test session, Animals were allowed to swim for 10 min as training. The duration of immobility was observed for five min in a six min test session. Rats were considered as immobile when they made no attempts to escape.

5HT and Noradrenalin estimation in plasma

Blood samples were collected and mixed with 10% sodium citrate solution and then centrifuged at 3000 rpm for 10 min. supernatant liquid was collected and stored at −10 °C for estimation of 5HT and noradrenaline by HPLC systems using Electro chemical detector (shimadzu LC) [14] .

Glutamate and GABA estimation in brain tissue

Brain samples were isolated and homogenised in 0.1 N HCl in 80% ethanol (10 mg tissue/200 µl) and centrifuged at 4500 rpm for 20 min at 25 °C. The supernatant was collected and estimated by HPTLC (CAMAG version 1.3.4, USA). at 486 nm. Standard solutions of L-glutamic acid (20–200 ng/spot) and GABA (5–80 ng/spot) were prepared for plotting the calibration curve.[15]

Estimation of CORT

Immediately after the stress protocol, animals were sacrificed by decapitation and blood was transferred to sodium citrate and centrifuged at 2000 rpm for 20 min. Plasma was separated

and aliquots were stored at −80°C for CORT estimation by HPLC/UV system according to Ahmad A et al. [16]

Measurement of TIP39 expression by Polymerase Chain Reaction

The Cells were lysed using TRI reagent (Sigma-Aldrich) and the total RNA was extracted and quantified by NanoDrop (Thermo Scientific Wilmington, DE, USA) and whose 260/280 ratio found >1.8, were used for cDNA conversion (Applied Biosystems, USA). Primer sequences were synthesized at Sigma-Aldrich, MO, USA. The primer sequence is Forward: 5'-GCTTCTGGGTGTGATGGTGA-3, Reverse: 5'-AGCAGCAAAAGCAGCAGCAG-3'. PCR reactions were run in qPCR (Applied Biosystems, USA) system. Reactions were initiated with denaturation at 95 °C for 30s, followed by 40 cycles of two-step reaction, denaturation at 95 °C for 5s, and annealing and extension for 30s. [17]

Statistical analysis

Data were expressed as Mean ± SEM and one way analysis of variance (ANOVA) followed by post hoc analysis Tukey's multiple comparison tests was used to analyse the data. $P<0.05$, considered as statistically significant. Data were analysed using Graph Pad Prism, 4.03 (La Jolla, CA. USA).

RESULTS

Effect of PTH2 R agonist and antagonist in Open field exploratory behaviour test

As shown in (Table 1), no significant changes in open field exploratory behaviour were observed following either TIP39 or HYWH administration in ARS rats. ARS inducted rats exhibited significantly decreased ambulation ($F(6,35)=4.2$, $p<0.01$), rearing ($F(6,35)=7.08$, $p<0.001$) and increased grooming ($F(6,35)=2.81$, $p<0.05$) response compared to control and Diazepam 2 mg/kg treated rats. Treatment with TIP39 (1&10nmol/rat) and TIP39+HYWH (1nmol/rat) did not show significant changes in rearing ($F(6,35) = 1.52$, $p>0.05$) and grooming ($F(6,35) =0.09$, $p>0.05$)as compared to the ARS rats but not in ambulation ($F(6,35) =1.8$, $p<0.05$) on TIP39 (1&10nmol/rat) treatment.

Table 1: Effect of TIP39 treatment on OFT & FST in ARS rats

Groups	**Open field exploratory Behaviour**			**Behavioural despair activity**
	Ambulation	**Grooming**	**Rearing**	**Immobility time**

Control	39±2.2	13.5±2.8	22.8±3.1	81.5±13.1
ARS	16.1±4.5$^{\#\#}$	22±3.6$^{\#}$	14.3±1.5$^{\#\#\#}$	169.8±24.8$^{\#\#}$
Diazepam 2mg/kg	27.3±2***	15.8±1.6***	18.6±2.5***	109.8±15.8**
TIP39 1 nmol/rat	23.8±1.4*	22.3±1.8	15±2	135.1±13.7*
TIP39 1 nmol+ HYWH 1nmol/rat	15±1.4	22.6±1.8	13.6±1.6	162±15.2
TIP39 10 nmol/rat	25.6±2**	22.3±1.6	14.8±2.1	119.5±10.6**
TIP39 10 nmol+ HYWH 1nmol/rat	17.8±1.8	21.1±1.7	13.3±1.6	162.6±18.6

Values are expressed in mean ± S.D, n=6. One way ANOVA followed by post hoc analysis Tukey's multiple comparison tests. #,##,### denotes ARS Vs control rats $p<0.05$, $p<0.01$ & $p<0.001$ respectively. *, **,*** treatment Vs ARS, $p<0.05$, $p<0.01$ & $p<0.001$ respectively.

Effect of PTH2 R agonist and antagonist in Forced swim test

The obtained results showed that ARS group significantly ($F(6, 35) = 13.61$, $p < 0.01$) increased the immobility time as compared to Control and Diazepam 2 mg/kg treated rats. TIP39 both doses, i.e., 1&10 nmol/rat, significantly decreased immobility time as compared to the ARS group ($F(6,35) = 5.61$, $p < 0.05$, $F(6,35) = 9.02$, ($p < 0.01$). Treatment of HYWM (1nmol/rat) along with TIP39 (1&10 nmol/rat) did not significantly alter the total immobility time in the forced swim test as comparable to ARS group ($F(6,35) = 1.61$, $p>0.05$, $F(6,35) = 2.1$, ($p >0.05$) (Table 1)

Effect of PTH2 R agonist and antagonist in plasma 5HT and NA level

Shown in (Fig.1A & 1B), ARS group showed significant decreased level of plasma 5HT ($F(6,35) = 10.1$, $p < 0.001$) and NA ($F(6,35) = 13.2$, $p < 0.001$) as compared to control group. Treatment with TIP39 (1&10nmol) significantly increased the NA level in plasma($F(6,35) = 4.2$, $p < 0.01$, $F(6,35) = 6.1$, $p < 0.01$), where as TIP39 (1&10 nmol/rat)+HYWH (1nmol/rat) did not show significant changes in NA level as compared to the ARS rats. In contrast, Treatment with TIP39 1&10nmol ($F(6,35) = 0.46$, $p > 0.72$) and TIP39+HYWH (1nmol/rat) did not show significant changes in plasma 5HT level as compared to the ARS rats ($F(6,35) = 1.06$, $p >0.05$).

Effect of PTH2 R agonist and antagonist in brain Glutamate and GABA activity

As shown in (Fig.1C&1D), ARS exposure caused a significant increase of glutamate ($F(6,35) = 15.6$, $p <0.001$) and decrease of GABA ($F(6,35) = 11.3$, $p <0.001$) activity in brain tissue when compared with control group. Diazepam 2 mg/kg treated rats. with TIP39 doses, i.e.,

1&10nmol significantly decreased the glutamate (F(6,35) =2.8, p<0.05, F(6,35) = 3.6, p <0.01) and significantly increased the GABA ((F(6,35) = 3.16, p <0.05, F(6,35) = 5.3, p <0.01) activity in brain, where as TIP39+HYWH (1nmol/rat) did not show significant changes in glutamate and GABA activity compared to the ARS rats (F(6,35) = 1.5, p >0.05).

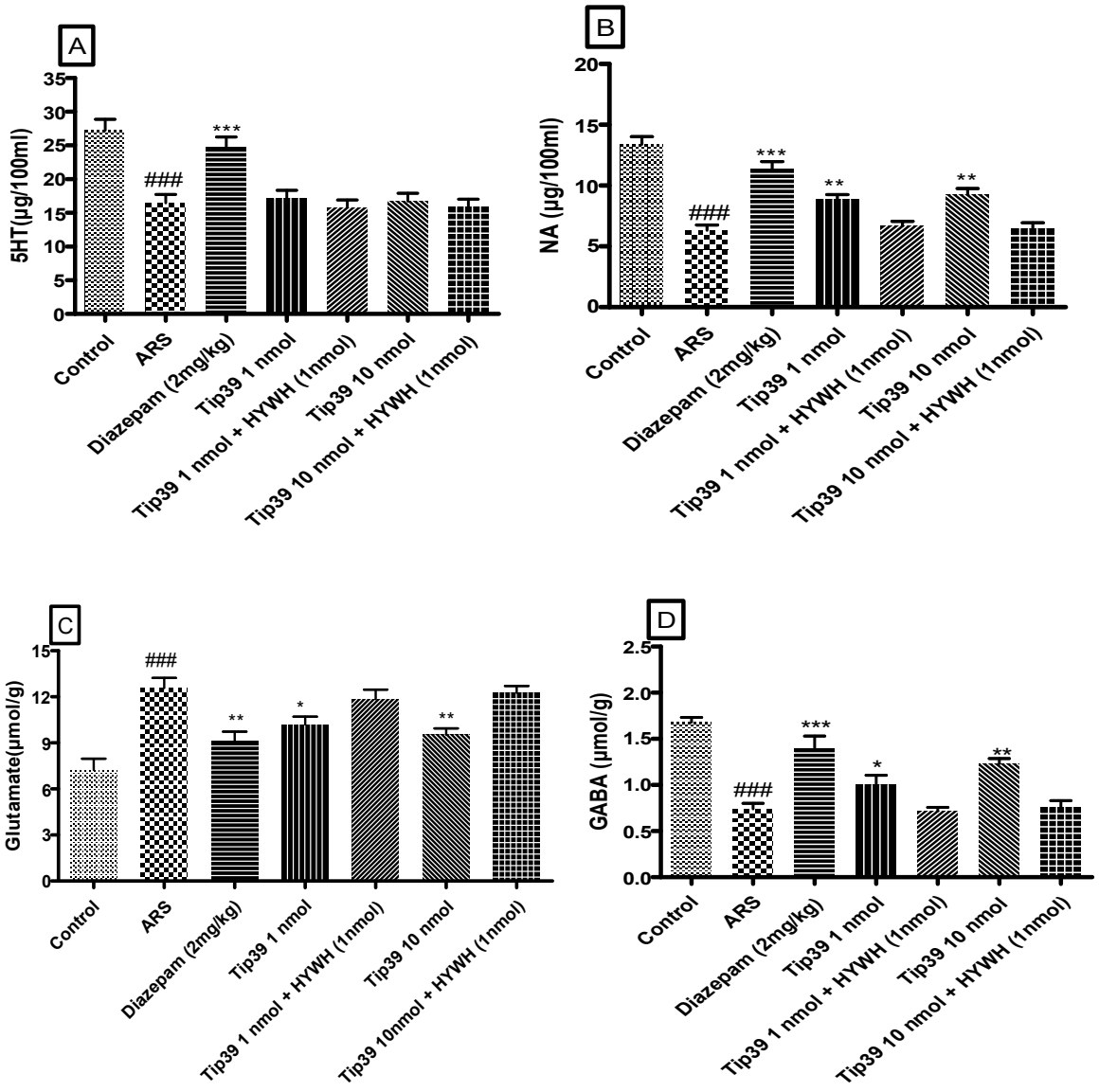

Fig 1: Effect of TIP39 treatment on neurotransmitter level in ARS rats

values are expressed in Mean ± SD, n=6. One way ANOVA followed by post hoc analysis Tukey's multiple comparison test. ### denotes ARS Vs control, p<0.001. *,**,*** denotes treatment Vs ARS, p<0.05, p<0.01 & p<0.001 respectively.

Effect of PTH2 R agonist and antagonist in CORT level

ARS group significantly (F (6, 35) = 15.2, p < 0.001) increased the CORT as compared to Control group. Diazepam at the dose of 2mg/kg significantly decreased the CORT when compared to ARS group (F(6,35) = 17.1, p < 0.001). Similarly, TIP39 both doses, i.e., 1&10

nmol/rat, significantly decreased CORT as compared to the ARS group ($F(6,35) = 8.61$, $p < 0.01$, $F(6,35) = 13.02$, ($p < 0.001$). Treatment of HYWM (1nmol/rat) along with TIP39 (1&10 nmol/rat) did not significantly alter the CORT in the plasma ($F(6,35) = 1.81$, $p>0.05$, $F(6,35) = 2.6$, ($p >0.05$) as comparable to ARS group. (Fig. 2)

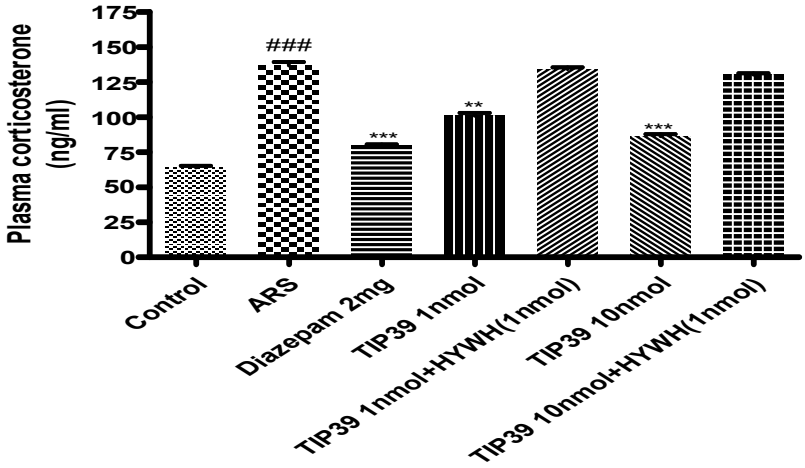

Fig 2: Effect of TIP39 on plasma CORT in CUMS rats

Values are expressed in Mean ± SD, n=6. One way ANOVA followed by post hoc analysis Tukey's multiple comparison test. ### ARS Vs control rats, $p<0.001$ respectively. **. *** Treatment Vs ARS rats, $p<0.01$ & $p<0.001$ respectively.

Estimation of TIP39 expression in rat brain by polymerase chain reaction

Upon 2 hr of acute restraint stress induction, rats have shown significant decrease of TIP39 gene expression in brain tissue as compared to normal rats ($F(2,15) = 3.9$, $p <0.01$). In contrast, upon half an hour of acute restraint stress decreased expression of TIP39 gene was observed as compared to normal rats but there were no significant ($F(2,15) = 2.16$, $p >0.05$) differents were exhibited. (Fig. 3)

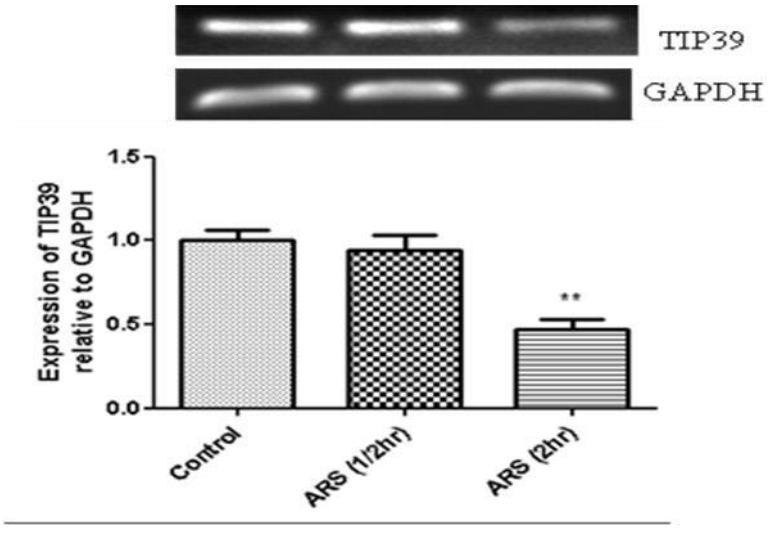

Fig 3: Expression of TIP39 in brain by polymerase chain reaction

Values are expressed in Mean ± SD, n=6. One way ANOVA followed by post hoc analysis Tukey's multiple comparison test. ** denotes control Vs ARS, $p<0.001$.

DISCUSSION

Bounteous evidence demonstrated that stress is the key factor in the pathogenesis of neuropsychiatric disorders including anxiety, depression, cognitive damage, insomnia and anorexia [18]. In the present study, we demonstrated ARS exposure selectively decreased TIP39 expression in rat brain, and that could mimic depression-like behaviour. TIP39 could activate monoamine like nor adrenaline, but not serotonin level in plasma and we showed evidence for a GABA role in terms of controlling the glutaminergic action.

In a pilot study, TIP39 expression was examined in normal and stressed rats. The animal received 30 min of ARS did not establish the significant decrease in TIP39 expression as compared to control group. In contrast, animal received 2h of ARS significantly decreased the TIP39 expression, meant that severe stress has vital relation with the PTH2-TIP39 system. Hence we had chosen 2h of ARS model, to assess the role of TIP39 in depressive rats. In the same work, we further confirmed the relationship between a PTH2R-TIP39 system and neurotransmitter release during stress by administering PTH2 agonist (TIP39) and with the PTH2 antagonist (HYWH). Moreover, our determinations are consistent with Usdin et al., indicated that TIP39/PTH2R knockout mice showed depression-like behaviour [4]

An effect of TIP39 at the immobility time was studied in FST model which is been really accurate and takes in the greater pharmacological sensitivity [19]. TIP39 at doses of 1&10 nmol/rat significantly decreased the duration of immobility. However, HYWH treated animal did not demonstrates any real growth as compared to control group, suggesting that TIP39 might potentially have antidepressant-like activity. This is the first interactive study that verifies the antidepressant effect of TIP39 in ARS rats. The present data were consistent with the report of LaBuda et al. indicates that TIP39 can decrease the duration of immobility in the FST model [3].

The FST combined with the OFT can separate locomotor stimulant drugs from antidepressant drugs [20]. In our work, ARS significantly decreased ambulation, rearing and increased grooming response, lets on less exploratory, lack of interest and anxiety like deportment, which are raw to the monoaminergic system, is accordance with the previous report [21]. Acute ICV administration of TIP39 did not alter the open field exploratory activity significantly except ambulatory behaviour compared to ARS rats, indicating that TIP39 improved the decreased locomotor activity, simply had no psychostimulant effects. The

abovementioned two different behavioral investigations provided convincing evidence to the antidepressant activity of TIP39.

Increased CORT level could cause depression-like behaviours that decrease hippocampal neurogenesis [22]. Our results showed that decreased TIP39 expression could increase the serum CORT, which suggests CORT might be involved in TIP39 related depression-like behavior. A Higher level of CORT and hyperactivity of HPA axis has been implicated in the development of depression. In the present study, the ARS group showed a substantial lift in the CORT level as compared to the control group, which was reversed after TIP39 administration, dose dependently. In contrast, our study report is inconsistent with LaBuda et al., indicated that increased plasma CORT level after TIP39 infusion in the PVN region (3). This might be ascribable to the deviation in the volume of stress applied. Nevertheless, the detailed mechanisms still need to be careful in future research. Many written reports have publicized that Ventral hypothalamus (VH) had glutamatergic input to the paraventricular hypothalamic nucleus (PVN), while PVN is liable for coordinating the regulation of the HPA axis [23]. Consequently adaptations of CORT and ACTH levels may be interrelated with the PVN, which is regulated by the VH directly. Interestingly, neuroanatomical studies implicated that high-density TIP39 fibres project widely in many limbic areas, including the PVN and several hypothalamic nuclei. Earlier studies also implicated that TIP39 peptide potentially modulate the natural process of the HPA axis during stressful condition [24].

Depression invariably accompanied by the reduction in 5-HT, NE, and DA level in the blood and brain tissues which are in close relation with symptoms of depression [25]. In the present work, we found that NE and 5HT were significantly decreased in the ARS group compared to control group. TIP39 administration significantly increased the NE content when compared to ARS group which was not seen in HYWH treated groups. In contrast, 5-HT levels were not significantly different among the treatment and ARS groups, indicating that 5-HT levels in the whole brain were not significantly impressed. Thus, our study demonstrating that elevation of NE in the treatment group might be a cause for increasing ambulatory behaviour in OFT. An earlier study also has revealed that TIP39/PTH2-R system potentially acting through central noradrenergic signalling pathway [26]. With the aforementioned resolution, we could infer that the antidepressant-like effect of TIP39 may be associated with modulation of the central noradrenergic pathway.

Finding efficient therapy in challenging depression is very difficult may be due to diversity in the origin of mood and mental disorders associated with different genetic and environmental factors. Recent years, studies on depression have focused to a greater extant on the

glutaminergic role rather than the monoamines. Studies revealed that high level of brain and plasma glutamate were found in the patient with depression. Also established that inhibition of glutamatergic neurotransmission was strongly correlated with the therapeutic action of a majority of antidepressant drugs [27]. Interestingly, in whole brain tissue, TIP39 significantly reduced the glutamate level as compared to ARS rats. In contrast, GABA content was significantly increased as compared to ARS rats, which were not observed in rats treated with HYWH. Mathew SJ et al stating that imbalance between the glutamate and GABA content in the brain can cause depression-like behaviour which is coherent with the present study. These reports point towards the importance of TIP39 in regulating the glutaminergic and GABAergic system. However, Our study report support the previous study implicated that TIP39 peptide can regulate hypothalamic glutaminergic and inhibitory GABAergic neuron in the cerebral region [28]. Recent studies have reported that, besides the limbic area, TIP39 are abundantly expressed in other brain regions also [29]. Hence, our study suggests that the curative role of TIP39 needs to be explored in other brain areas, equally well. This study provided the first evidence indicating that stimulating TIP39 expression can bring about an antidepressant-like effect by modulating the monoaminergic,GABAergic and glutaminergic release with the support of HPA axis.

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References

1. Budni J, Zomkowski AD, Engel D, Santos DB, dos Santos AA, Moretti M, et al. Folic acid prevents depressive-like behavior and hippocampal antioxidant imbalance induced by restraint stress in mice. Exp Neurol. Elsevier Inc 2013;240(1):112–21.
2. Santosh P, Venugopl R, Nilakash a. S, Kunjbihari S, Mangala L. Antidepressant activity of methanolic extract of *Passiflora foetida* leaves in mice. Int J Pharm Pharm Sci 2011;3(1):6–9.
3. LaBuda CJ, Dobolyi A, Usdin TB. Tuberoinfundibular peptide of 39 residues produces anxiolytic and antidepressant actions. Neuroreport 2004;15(5):881–5.
4. Coutellier L, Usdin TB. Enhanced long-term fear memory and increased anxiety and depression-like behavior after exposure to an aversive event in mice lacking TIP39 signaling. Behav Brain Res 2011;222(1):265–9.
5. Aihara M, Ida I, Yuuki N, Oshima A, Kumano H, Takahashi K, et al. HPA axis dysfunction in unmedicated major depressive disorder and its normalization by pharmacotherapy

correlates with alteration of neural activity in prefrontal cortex and limbic/paralimbic regions. Psychiatry Res - Neuroimaging 2007;155(3):245–56.

6. Barden N. Implication of the hypothalamic-pituitary-adrenal axis in the physiopathology of depression. In: Journal of Psychiatry and Neuroscience 2004. p. 185–93.
7. Nikisch G. Involvement and role of antidepressant drugs of the hypothalamic-pituitary-adrenal axis and glucocorticoid receptor function Neuroendocrinology Letters. 2009;30;11–6.
8. Catena-Dell'Osso M, Fagiolini A, Rotella F, Baroni S, Marazziti D. Glutamate system as target for development of novel antidepressants. CNS Spectr 2013;18(4):188–98.
9. Mathew SJ, Keegan K, Smith L. Glutamate modulators as novel interventions for mood disorders. Vol. 27, Revista Brasileira de Psiquiatria 2005. p. 243–8.
10. Hashimoto K. Emerging role of glutamate in the pathophysiology of major depressive disorder. Brain Res Rev 2009;61(2):105–23.
11. Santos GA, Pereira VD, Roman EAFR, Ignacio-Souza L, Vitorino DC, de Moura RF, et al. Hypothalamic Inhibition of Acetyl-CoA Carboxylase Stimulates Hepatic Counter-Regulatory Response Independent of AMPK Activation in Rats. PLoS One 2013;8(4).
12. Pawar DB, Marathe PA, Rege NN. Antidepressant activity of aqueous extracts of fruits of Terminalia chebula and Phyllanthus emblica in behavioral models of depression: involvement of monoaminergic system. Int J Pharm Pharm Sci 2014;6:615-20.
13. Tarali Devi1, Swarnamoni Das. Evaluation of the protective effect of ethanolic extract of leaves of punica granatum linn. On forced swimming induced chronic fatigue syndrome in mice. Int J Pharm Pharm Sci 2017; 9(1): 207-12.
14. Parrot S, Neuzeret P-C, Denoroy L. A rapid and sensitive method for the analysis of brain monoamine neurotransmitters using ultra-fast liquid chromatography coupled to electrochemical detection. J Chromatogr B 2011;879:3871–8.
15. Tshibangu DS, Divakar S, Ramanathan M, Syamala GG, Ngbolua K, Mudogo V, et al. In vitro Screening of the Leaf Extracts from Gardenia ternifolia (Forest Gardenia) for their Anticancer Activity 2016;1(December 2014):1–7.
16. Badenhorst NJ, Brand L, Harvey BH, Ellis SM, Brink CB. Long-term effects of pre-pubertal fluoxetine on behaviour and monoaminergic stress response in stress-sensitive rats. Acta Neuropsychiatr 2016;1–14.
17. Darshit BS, Ramanathan M. Activation of AKT1/GSK-3??/??-Catenin???TRIM11/Survivin Pathway by Novel GSK-3?? Inhibitor Promotes Neuron Cell Survival: Study in Differentiated SH-SY5Y Cells in OGD Model. Mol Neurobiol 2016;53(10):6716–29.
18. Subakanmani S, Umadevi P. Evaluation of Anxiolytic Potential of Ethanolic Extract

Hypericum Hookerianum in Stress Induced Swiss Albino Mice. 2012;3(4):219–25.

19. Paul IA, Skolnick P. Glutamate and Depression: Clinical and Preclinical Studies. In: Annals of the New York Academy of Sciences 2003. p. 250–72.
20. Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: A new method for screening antidepressants in mice. Psychopharmacology (Berl) 1985;85(3):367–70.
21. Joca SRL, Padovan CM, Guimarães FS. Activation of post-synaptic 5-HT1A receptors in the dorsal hippocampus prevents learned helplessness development. Brain Res 2003;978(1–2):177–84.
22. Freitas AE, Egea J, Buendia I, G??mez-Rangel V, Parada E, Navarro E, et al. Agmatine, by Improving Neuroplasticity Markers and Inducing Nrf2, Prevents Corticosterone-Induced Depressive-Like Behavior in Mice. Mol Neurobiol 2016;53(5):3030–45.
23. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci 2009;10(6):397–409.
24. Dobolyi A, Palkovits M, Usdin TB. Expression and distribution of tuberoinfundibular peptide of 39 residues in the rat central nervous system. J Comp Neurol. 2003;455(4):547–66.
25. Nutt DJ. Relationship of neurotransmitters to the symptoms of major depressive disorder. J Clin Psychiatry 2008;69 Suppl E:4–7.
26. Coutellier L, Logemann A, Kuo J, Rusnak M, Usdin TB. TIP39 modulates effects of novelty-induced arousal on memory. Genes, Brain Behav 2011;10(1):90–9.
27. Mathews DC, Henter ID, Zarate CA. Targeting the glutamatergic system to treat major depressive disorder: Rationale and progress to date. Drugs. 2012
28. Dimitrov EL, Kim YY, Usdin TB. Regulation of Hypothalamic Signaling by Tuberoinfundibular Peptide of 39 Residues Is Critical for the Response to Cold: A Novel Peptidergic Mechanism of Thermoregulation. J Neurosci 2011;31(49):18166–79.
29. Wang T, Palkovits M, Rusnak M, Mezey É, Usdin TB. Distribution of parathyroid hormone-2 receptor-like immunoreactivity and messenger RNA in the rat nervous system. Neuroscience 2000;100(3):629–49.

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RESEARCH ARTICLE

Tuberoinfundibular Peptide of 39 Attenuates Chronic Unpredictable Mild Stress Induced HPA Axis Dysregulation, Inflammation and Oxidative Damage in Depressive Rats

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Abstract: ***Background:*** Tuberoinfundibular Peptide of 39 (TIP39) is a neuroendocrine hormone, potentially acting through parathyroid hormone receptor 2 receptor (PTH2R) abundantly expressed in brain.

Objective: This study aimed to evaluate the neuroendocrine role of TIP39 in chronic unpredictable mild stress (CUMS) induced depression and to elucidate its underlying mechanism.

Method: The depression was induced in rats by CUMS for a period of four weeks. TIP39 was administered through intracerebroventricular (ICV) route at doses (1 & 10 nmol/rat) for four weeks on alternate days, parallel with the daily exposure of stress. At the end of the treatment period, animals were evaluated for sucrose preference, behavioral, biochemical and oxidative changes. Further the molecular mechanism of anti-stress activity of TIP39 confirmed through gene expression study.

Results: TIP39 administration significantly reversed the CUMS induced increased immobility time in depressive rats and increased plasma corticosterone as well as decreased open-field activity and sucrose consumption. CUMS lowers the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) and elevated the production of malondialdehyde (MDA) in hippocampus and prefrontal cortex, which was reversed by the administration of TIP39. Moreover, TIP39 could effectively reverse alteration in interleukin 6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in brain tissue.

Conclusion: Chronic ICV administration of TIP39 alleviated the behavioral deficits of chronic unpredictable mild stress, consanguinity to its concurrent modulatory repercussion on hypothalamic pituitary adrenal axis, inflammation, and oxidative courses.

Keywords: TIP39, CUMS, HPA axis, depression, oxidative stress, inflammation.

1. INTRODUCTION

According to a report from WHO, approximately 300 million people all over the world suffer from depression. This could be predicted to rise by 15% by the year 2020 [1]. The forthcoming scenario shows, depression will be the second most extensive disease around the world, which is aggravated by psychological, physiological, or environmental stress that disturbs the quality of life [2, 3]. Out of various theories postulated for depression, hypothalamic pituitary adrenal (HPA) axis is the most important theory, which links the central and peripheral action during stressful conditions [4]. Dysregulation of HPA axis causes behavioural changes and disruption in cascade of hormonal release that leads to diseases like depression, anxiety, obsessive compulsive disorder, anorexia, insomnia, hyperglycaemia, and reduced immune response [5, 6].

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Cell bodies containing Tuberoinfundibular peptide consisting of 39 (TIP39) amino acid residues are principally located in subpar fascicular area of thalamus and medial paralemniscal nucleus of lateral brainstem. These neurons project towards TIP39's receptor sites, which show closely matching distribution with parathyroid hormone 2 receptor (PTH2R) [7]. Studies showed that TIP39 instigate *fos* gene expression in the infralimbic cortex, preoptic area, lateral hypothalamus, lateral septum, and paraventricular thalamic nucleus and these areas are believed to be imperative in depression and anxiety. Mice lacking TIP39/PTH2R signalling displayed anxiety and depression like behaviour. In addition to that knockout mouse showed increased fear memory after exposure to an aversive event [8]. Sugimura *et al* demonstrated that ICV administration of TIP39 reduced the plasma AVP (Arginin Vasopressin) level via opioid system. Following TIP39 administration, long term consequence of traumatic event was reduced by the regulation of amygdale in cerebral region which is known to involve in fear conditioning. TIP39 peptide showed guarding response during the neuropathic and inflammatory pain by

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regulating through inhibition of release of hypoalgesic amount of norepinephrine. In addition to that activation of TIP39 signalling facilitates the HPA axis function during the stressful condition [9-11].

CUMS has more negative impact than predictable stressor in humans, maybe due to temporal ambiguity and incompetence to anticipate the event. Moreover, CUMS is the most important pathogenic factor that dysregulates HPA axis, which reflects the symptoms of depression akin to those seen in patient with depression [12]. Acute and chronic stress amends sympathetic nervous system and the HPA axis, which in turn precipitate anxiety and depression. These changes results in disproportionate generation of free radicals and further cause disturbances in the oxidant/antioxidant system and contribute to the upliftment of inflammatory mediators and vice versa [13, 14]. Despite the last 60 years of rigorous research in this line, no concrete antidepressant therapies are available in current practice. Depression requires long-term treatment [15] because of the hurdle to identify the exact pathway in depression pathology. In this study, we hypothesize that TIP39 could link HPA axis with inflammatory and oxidative pathway in CUMS model. Hence, the present study is planned to evaluate the neuroendocrine role of TIP39 on rat model of chronic unpredictable mild stress.

2. MATERIALS AND METHODS

2.1. Animals and Surgical Procedure

Male Sprague Dawley rats weighing 150-200 g were collected from the PSGIMS&R (205/2013/IAEC). Rats were kept in separate cages (6 animals per cage) with standard diet unless there was a limitation due to stress protocol. Animals were housed with husk as a bedding material under normal room temperature (25 ± 2º C), 12/12 hr light-dark cycle as well as constant relative humidity (55 ± 5%) throughout the experimental period according to CPCSEA guidelines.

Seven days before the TIP39 administration, stereotoxic surgery was made and Guide cannulae (Stainless steel (OD 0.64 x ID 0.45) RWD Life Science Co Ltd, China) was implanted under anaesthetic condition ketamine (80 mg/kg, i.m.) xylazine (7.5 mg/kg, i.m.) in the right lateral ventricle at pre-established coordinates, anteroposterior, 0.2 mM from bregma; lateral, 1.5 mM; and vertical, 4.2 mM, and kept under controlled temperature in separate cages [16]. TIP39 peptide (SP-101335-1, ATZ lab, Life Technologies Pvt Ltd, India, in 10 ml saline) was injected through ICV route over 5 min.

2.2. Behavioral Experiments

2.2.1. Chronic Unpredictable Mild Stress Induction and Anhedonia model

The CUMS model was implemented according to a previous method with a slight modification [17]. During the experiment, the animals were first trained to experience and drink a sweet beverage by presenting them simultaneously with two bottles, during the first 24 h, both bottles contained a 1%sucrose solution. Subsequent 24 h, one bottle contained the sucrose solution the other contained water. Following 14 h food and water deprivation, rats received the first baseline sucrose preference test. Each animal was presented simultaneously with two pre weighed bottles, one containing the sucrose solution and the other containing normal water. Both bottles were removed and weighed at 60 min at the end of the test. Then, animals were given food and water for 2 h. After another period (14 h) of food and water deprivation, animals received a second baseline sucrose preference test. Four days thereafter, following 24 h food and water deprivation, animals received a third baseline sucrose preference tests. After the 1st, 2nd, 3rd and 4th weeks of CUMS, rats were subjected for sucrose preference test [18].

Followed by third baseline test, animals were divided into various groups (n=6) and were subjected to chronic unpredictable stress. In brief, the CUMS protocol consisted of the chronological application of a variety of mild stressors. These stimulants are shifted every other week in a period of four week experiment. All the control rats were kept under identical conditions in a separate cage away from the stressed rats (Table **1**).

2.2.2. Open Field Exploratory Behavior Test

The open field test (OFT) was executed to check whether the immobility period was in conjugation with any effect of motor activity. Open field [19] apparatus is an arena made of plywood, consisting of a floor (96 × 96 cm) with high walls. The entire apparatus was painted black except for 6 mm thick white lines which divided the floor into 16 squares.

Table 1. Stress induction procedure in CUMS model.

Days	Type/Duration Of Stress
Day 1	Food deprivation (24 h) and cold swimming (5 min at 6º C)
Day 2	Water deprivation (24 h), Tail pinch (1min - 2 times interval of 6 h)
Day 3	Soiled bedding (150 ml water per cage) for 12 h, Physically restrained for 2 h
Day 4	Day and night light illumination and Restricted food pellets (45g)
Day 5	Exposure to a novel odour with noise (12 h) and animal isolation (1rat/cage) (12h)
Day 6	Crowded housing (10 rats/cage) for 12 h
Day 7	Cage tilting (45° inclined) for 12 h

Same methodology was followed for 2nd, 3rd and 4th week.

Experimental animals were placed in the centre of an open box and allowed to explore the arena. Behavioral parameters were quantified, such as ambulation, rearing, time spent in central compartment, and grooming for 5 min.

2.2.3. Forced Swimming Test

With slight modifications, forced swimming test (FST) was carried out [20]. Rats were forced to swim one by one in a transparent glass vessel (45cm X 12cm X 45cm) with water filled up to 30 cm at room temperature (25 ± 2°C) 24 h ahead test session. A training period of 10 min has given to each animal. The duration of immobility, jumping and dipping responses were observed for five min in a six min test session. On the test day, after the initial 1 min, rats were observed for immobility, jumping and dipping responses for next 5 min. The start of immobility reflects behavioral despair or helplessness [21].

2.2.4. Tail Suspension Test

The tail suspension test (TST) was conducted according to steru *et al.* where animals were suspended by the bands and hang from a hook mounted 50 cm above the floor for 6 min, approximately 5 cm from the lever. Rats were suspended for a period of 6 min and were considered immobile when rats are completely motionless [22]

2.2.5. Estimation of SOD, CAT, GSH, and MDA in the Brain

Immediately after the last behavioural test, rats were anesthetized with ether followed by quick cervical dislocation then decapitated followed by harvesting of brain. Brain samples were immediately flash freezed using liquid nitrogen and stored at -80°C. The tissue homogenate was prepared by using homogenization buffer (PBS-pH 7.4 with 1mM EDTA, 5M NaCl, 0.5% triton X100 and protease inhibitor). The homogenised samples were centrifuged at 2000g for 2min and supernatant was used for the enzyme assay. Superoxide dismutase (SOD) activity was estimated spectro photometrically at 550 nm by measuring the generation of superoxide free radicals produced by xanthine and xanthine oxidase, which react with nitro-blue tetrazolium (NTB) system according to the method by Sun *et al.* [23]. The activity of superoxide dismutase was expressed as units/mg protein. According to Aebi *et al.* [24], measurement of catalase (CAT) was carried out based on the ability of catalase to restrict oxidation of the H_2O_2. Initial reaction was carried out by adding 1ml of H_2O_2 (30 mmol/L). The variations in decomposition rate of H_2O_2 were determined spectrophotometry at 240 nm. The activity of catalase was expressed as units/mg protein. Assay of reduced glutathione (GSH) was carried out based on the Elmann procedure [25]. GSH concentrations were determined with the absorbance read at 412 nm. The values were expressed in nmol/mg protein. malondialdehyde (MDA) measurement was carried in compliance with Ohkawa *et al.* [26] the organic layer was separated and its absorbance was measured at 532 nm by micro plate spectrophotometry. Protein estimation was conducted according to Lowry *et al*. [27]. The data were expressed as nmol/mg protein.

2.2.6. Estimation of Corticosterone

After completion of stress protocol, animals were anesthetized with ether and blood was collected through retro orbital route and transferred to sodium citrate containing tubes kept in ice and centrifuged at 1000×g for 20 min. Plasma was separated and aliquots were stored at −70°C. Estimation of were done plasma using HPLC/UV system in compliance to Ahmad A *et al.* [28].

2.2.7. Measurement of Pro Inflammatory Cytokines by Polymerase Chain Reaction (PCR)

Cell lysis were done using TRI reagent (Sigma Aldrich, USA) and the total RNA was extracted according to the manufacturer's procedure. The RNA was quantified by NanoDrop (Thermo Scientific Wilmington, DE, USA) and whose 260/280 ratio found >1.8, were used for cDNA conversion. The RNA was converted to cDNA by high capacity cDNA conversion kit (Applied Biosystems). Expressions of interleukin 6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were studied. Primer designing were done using Primer3 software. The designed sequences were obtained from Sigma-Aldrich, MO, USA. The primer sequences are given in supplementary (Table **2**). PCR reactions were run in qPCR (Applied Biosystems) system. Reactions were initiated with denaturation at 95 °C for 30s, followed by 40 cycles of two-step reaction, denaturation at 95°C for 5s, and annealing and extension for 30s. Gene expression was normalized by reference gene GAPDH. The experiments were conducted in duplicates [29].

2.3. Statistical Analysis

Data were expressed as Mean ± SEM and ANOVA followed by post hoc analysis Turkey's multiple comparison tests was used to analyse the data. Weekly sucrose intake was subjected to two way ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant when $P<0.05$. The obtained data was analysed using Graph Pad Prism, 4.03 (La Jolla, CA. USA).

3. RESULTS

3.1. Effect of ICV Administration of TIP39 on Anhedonia Model

Sucrose preference test is a familiar model to distinguish the effect of drugs on anhedonia during depressive condition (Fig. **1**). There was a gradual decline in the intake of sucrose in subsequent weeks in all the groups. CUMS rats showed significant reduction in sucrose consumption in comparison to control group. These changes confirmed the state of depression in stressed rats. Percentage reduction in sucrose intake was observed in normal (13%), CUMS (54.8%), diazepam 2 mg/kg (19.3%) TIP39 1 nmol (26.5%) and TIP39 10 nmol (20.8%) treated animals from 1st week to 4th week. In week 3 and 4, there was a significant ($F(4, 25) = 11.64$, $P < 0.001$) reduction of sucrose intake in CUMS rats when compared to control group. Diazepam 2mg/kg exhibited significant ($F(4, 25) = 5.86$, $P < 0.01$) increase in sucrose consumption on 3rd and 4th weeks among rats subjected to stress as compared to CUMS group. Chronic treatment with TIP39 (1 nmol and 10 nmol) significantly ($F(4, 25) = 3.27$, $P < 0.05$ & $F(4, 25) = 4.21$, $P < 0.01$) increased the sucrose consumption when compared to CUMS group.

Table 2. Details of Primers: TNF-α, IL-1β and IL-6 gene.

Gene	Primer/Sequence
GAPDH	Forward- CAACTTTGGCATCGTGGAAG Reverse - CTGCTTCACCACCTTCTT
TNF-α	Forward -TCCCAACAAGGAGGAGAAGTTCC Reverse - GGCAGCCTTGTCCCTTGAAGAGA
IL-1β	Forward - AGCAGCTTTCGACAGTGAGGAGAA Reverse -TCTCCACAGCCACAATGAGTGACA
IL-6	Forward -AGGATACCACTCCCAACAGACCT Reverse -CAAGTGCATCATCGTTGTTCATAC

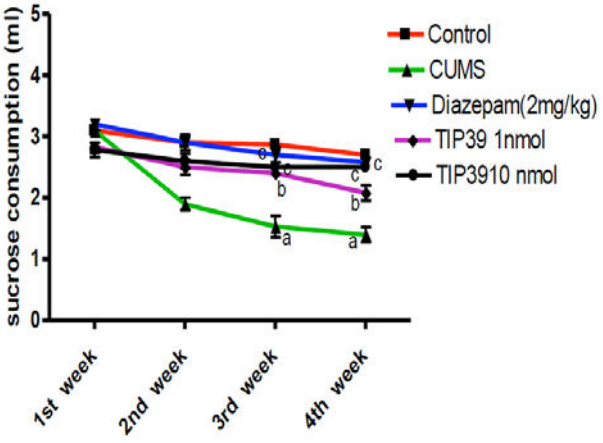

Fig. (1). Effect of TIP39 on Sucrose consumption in CUMS rats. All the values are expressed in Mean ± SD, n=6. Statistical analysis was carried out by Statistical analysis was carried out by Two way ANOVA followed by Bonferroni post test. [a]denotes statistical significance in comparison to control rats at P <0.001. [b,c]denotes statistical significance in comparison to CUMS group at P <0.05 and P <0.01 respectively.

3.2. Effect of ICV Administration of TIP39 on Open Field Test

CUMS rats exhibited decreased ambulation (F (4, 25) = 6.26, P<0.01) and rearing (F (4, 25) = 11.5, P<0.001). Further it increased the grooming (F (4, 25) =10.02, P<0.001) and time spend in central compartment (F (4, 25) = 4.8, P<0.01) when compared to unstressed rats (Fig. **2**). Treatment with TIP39 (1 nmol & 10 nmol) significantly reversed the stress induced behavioural changes in a dose dependent manner as observed by increased ambulation, rearing and decreased time spend in central compartment when compared to the CUMS rats. No significant changes were observed in grooming response after TIP39 administration in comparison to CUMS rats. Results were compared with that of the standard drug Diazepam 2 mg/kg.

3.3. Effect of ICV Administration of TIP39 on Forced Swim Test

Twenty eight days of CUMS brought a depressive state in rats, which was quantified by parameters such as increased immobility, dipping response and decreased climbing response in FST in comparison to control rats (F (4,25) = 14, P<0.001). Fig. (**3**) shows that the chronic administration of TIP39 1 nmol (F (4, 25) = 5.12, P<0.01) & 10 nmol (F (4, 25) = 9.24, P<0.001) in CUMS rats significantly reduced the duration of immobility time as compared to CUMS rats. In the same manner, the number of jumping responses increased (F (4, 25) = 6.3, P<0.01 & F (4, 25) = 5.93, P<0.01) and number of dipping responses decreased (F (4, 25) = 4.61, P<0.01 & F (4,25) = 10.1, P<0.001) significantly as compared to CUMS rats. Similar effect was observed in diazepam (P< 0.001) at the dose of 2 mg/kg when compared to CUMS rats.

3.4. Effect of ICV Administration of TIP39 on Tail Suspension Test

Dose dependent effects of TIP39 on the immobility period in the tail suspension test are shown in (Fig. **3D**). CUMS-induced depressive rats exhibited a significant (F (4, 25) = 7.65, P < 0.001) increase in immobility period as compare to control group. Administration of Diazepam 2 mg/mg (F (4, 25) = 6.79, P < 0.001) in CUMS rats significantly reduced the immobility time as compared to CUMS rats. Similar type of results were observed in the TST in which TIP39 at doses of 1 nmol (F (4, 25) = 4.91, P < 0.01) and 10 nmol (F (4, 25) = 15.82, P < 0.001) significantly reduced the immobility period when compared to CUMS group.

3.5. Effect of ICV Administration of TIP39 on Plasma Corticosterone Levels

Exposure to CUMS causes significant increase of plasma corticosterone level (F (4, 25) = 7.48 & P < 0.001) as comparison to normal rats. Treatment with TIP39 (1 nmol and 10 nmol) significantly (F (4, 25) = 5.53, P < 0.01, & (F (4, 25) = 6.93, P< 0.01) decreased the CUMS induced elevated corticosterone levels, dose dependently. Similar results were obtained for diazepam at the dose of 2 mg/kg in CUMS induced rats (F(4, 25) = 12.16, P < 0.001) (Fig. **4**).

3.6. Effect of ICV Administration of TIP39 on the Brain Antioxidant Biomarkers

The effect of TIP39 on the hippocampus and prefrontal cortex antioxidant system is shown in (Figs. **5** & **6**). From the results depicted, diazepam 2 mg/mg significantly alters the oxidative parameters as compared to CUMS group in the hippocampus (F (4, 25) = 13.03, P < 0.001) and the prefrontal cortex (F (4, 25) = 4.72, P < 0.01). However, CUMS rats showed a significant elevation in the MDA levels (F (4, 25) = 12.3, P < 0.001) and a significant turn down in the activities of SOD (F (4, 25) = 8.14, P < 0.001), CAT (F (4, 25) = 10.68, P < 0.001), and GSH (F (4, 25) = 9.12, P < 0.001) in the hippocampus and the prefrontal cortex as compared to the control group. ICV administration of TIP39 at doses of 1 nmol and 10 nmol showed significant increase in the activities of SOD (F (4, 25) = 5.3, P < 0.01& F (4, 25) = 6.9, P < 0.001) and CAT F (4, 25) = 3.6, P < 0.05& F (4, 25) = 5.81, P < 0.01), and GSH (F (4, 25) = 3.72, P < 0.05 & F (4, 25) = 6.7, P < 0.01) as compared to CUMS groups in the hippocampus and also treatment with TIP39 showed significant decline in the MDA (F (4, 25) = 4, P < 0.05 & F (4, 25) = 5.1, P < 0.01) levels in the hippocampus. In the prefrontal

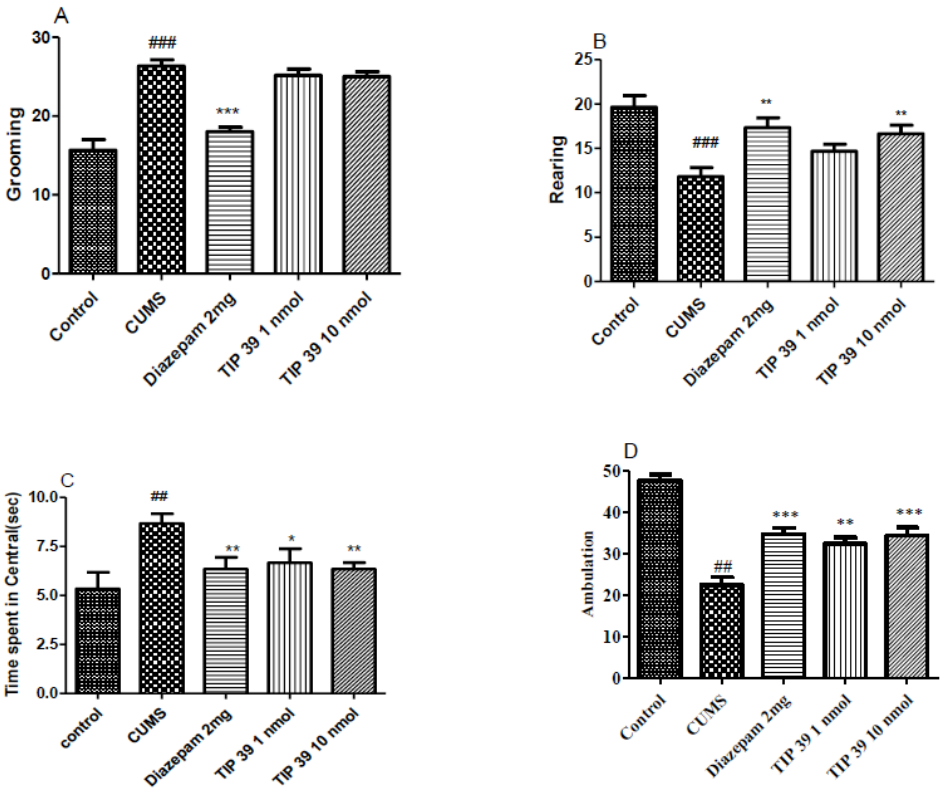

Fig. (2). Effect of TIP39 treatment on OFT in CUMS rats. All the values are expressed in Mean ± SD, n=6.Statistical analysis was carried out by one way ANOVA followed by post hoc analysis Tukey's multiple comparison test. ##, ### denotes statistical significance in comparison to control rats at P<0.01 and P<0.001 respectively. *, **, *** denotes statistical significance in comparison to vehicle treated CUMS group at P<0.05, P<0.01 and P<0.001 respectively.

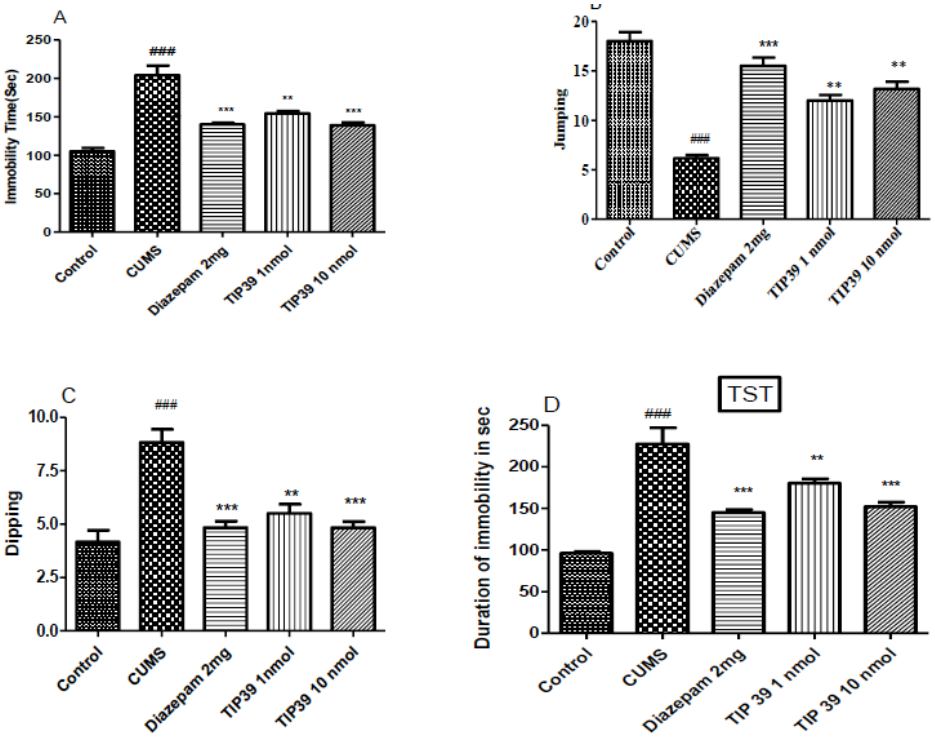

Fig. (3). Effect of TIP39 treatment on FST (A, B, C) &TST (D) in CUMS rats. All the values are expressed in Mean ± SD, n=6.Statistical analysis was carried out by one way ANOVA followed by post hoc analysis Tukey's multiple comparison test. ### denotes statistical significance in comparison to control rats at P <0.001. **, *** denotes statistical significance in comparison to vehicle treated CUMS group at P <0.01 and P <0.001 respectively.

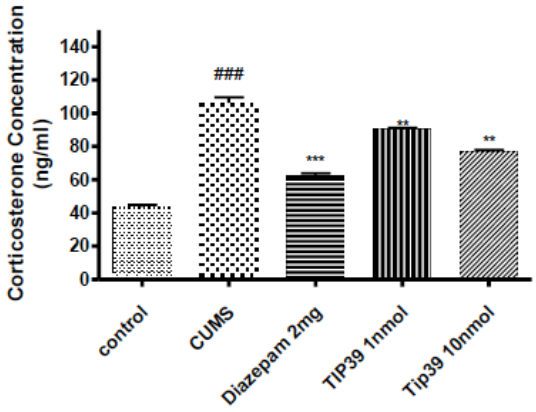

Fig. (4). Effect of TIP39 on plasma corticosterone in CUMS rats. All the values are expressed in Mean ± SD, n=6. Statistical analysis was carried out by one way ANOVA followed by post hoc analysis Tukey's multiple comparison test. ### denotes statistical significance in comparison to control rats at P <0.001. **, *** denotes statistical significance in comparison to vehicle treated CUMS group at P <0.01 and P <0.001 respectively.

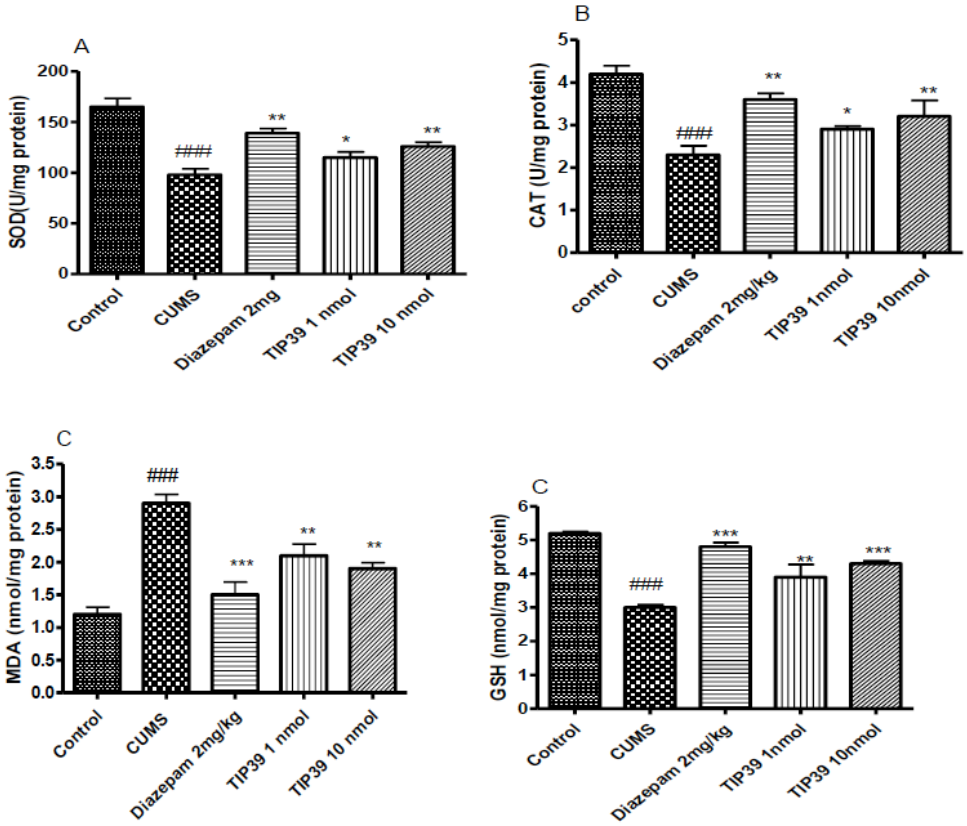

Fig. (5). Effect of TIP39 on SOD, CAT, MDA and GSH on Prefrontal cortex in CUMS rats. All the values are expressed in Mean ± SD, n=6. Statistical analysis was carried out by one way ANOVA followed by post hoc analysis Tukey's multiple comparison test. ### denotes statistical significance in comparison to control rats at P <0.001. *, **,*** denotes statistical significance in comparison to vehicle treated CUMS group at P <0.05, P <0.01 and P <0.001 respectively.

cortex, TIP39 (1 nmol & 10 nmol) exhibited significant increase in the activities of enzymatic SOD ($F(4,25) = 3.9$, $P < 0.05$ & $F(4, 25) = 5.42$, $P < 0.01$), CAT ($F(4, 25) = 3.63$, $P < 0.05$& $F(4, 25) = 67$, $P < 0.01$), GSH ($F(4, 25) = 4.6$, $P < 0.01$& $F(4, 25) = 7.8$, $P < 0.001$) and significant decline in the MDA (($F(4, 25) = 6.9$, $P < 0.01$&$F(4, 25) = 4.74$, $P < 0.01$) as compared to CUMS group.

3.7. Effect of ICV Administration of TIP39 on Inflammatory Markers

Upon CUMS induction, rats have shown significant increase of IL-6, IL-1β, and TNF-α in brain tissue as compared to normal rats ($P < 0.001$). No significant difference was observed in the levels of IL-6, IL-1β, and TNF-α between diazepam treated rats and normal rats. However, diazepam 2

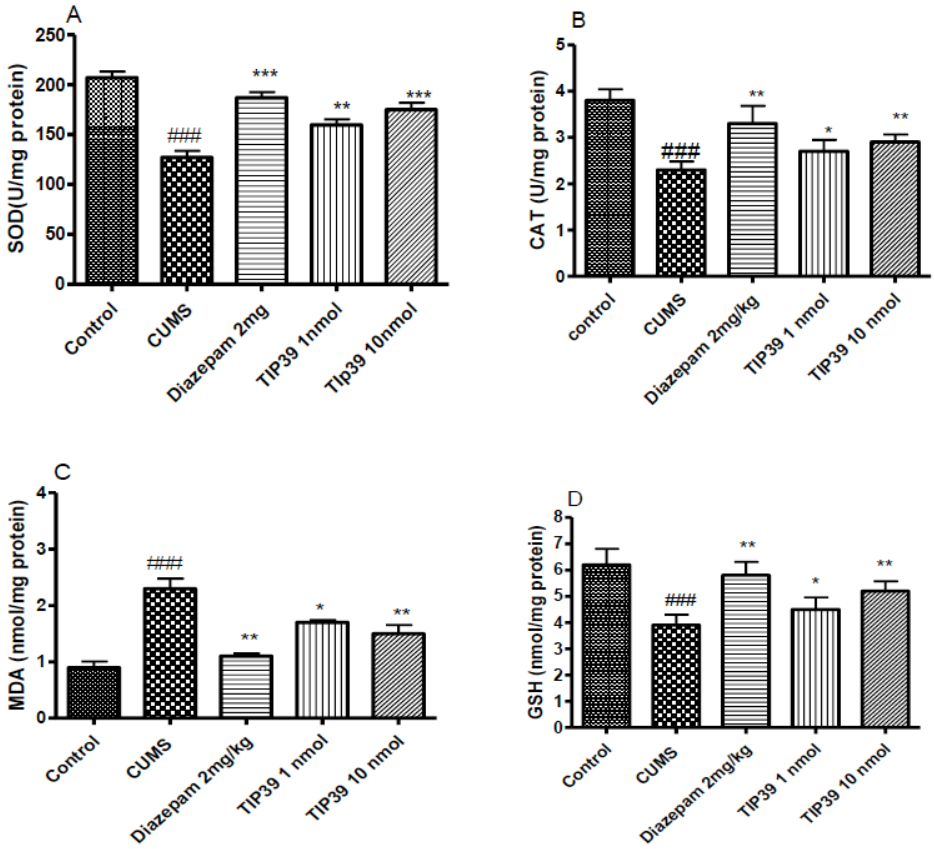

Fig. (6). Effect of TIP39 on SOD, CAT, MDA and GSH on Hippocampus in CUMS rats. All the values are expressed in Mean ± SD, n=6. Statistical analysis was carried out by one way ANOVA followed by post hoc analysis Tukey's multiple comparison test. ### denotes statistical significance in comparison to control rats at P <0.001. *, **, *** denotes statistical significance in comparison to vehicle treated CUMS group at P <0.05, P <0.01 and P <0.001 respectively.

mg/kg had significantly (F (4, 25) = 8.61, P < 0.01) reduced the IL-6, IL-1β, and TNF-α levels in brain as compared to CUMS rats. Chronic ICV administration of TIP39 1 nmol (F (4, 25) = 3.25, P < 0.05) and 10nmol (F (4, 25) = 6.78, P < 0.01) significantly reduced the IL-6, IL-1 β, and TNF-α levels in brain tissue as compared to CUMS induced rats (Fig. **7**).

4. DISCUSSION

Research studies are focusing wide on stress induced neurological disorders on the basis of nature and origin of the stress generation [30]. Stress is induced due to age, sex, individual differences, types of stimulus, duration, and intensity of stressors in the cerebral region [31, 32]. Chronic stress can increase the vulnerability in hippocampus and prefrontal cortex by altering the antioxidant defence system interactions closely with the HPA axis [33]. After 28 days of CUMS induction, animals were examined through the open field model. CUMS induced rats have shown decreased ambulation, rearing and increased time spent in the central compartment reveals less exploratory behaviour and increased grooming activity, that revealed the higher anxiety level, which is sensitive towards serotonergic and noradrenergic activity [34]. The aforesaid activities were reversed after the ICV administration of TIP39. In contrast, no significant changes were observed in grooming activity. TIP39 was studied in the FST and TST models, which are widely used preclinical models to examine antidepressant activity [35, 36]. In the modified version of FST model, together with the immobility time, jumping and dipping response were recorded. The dipping and jumping response has connection with the drugs that regulates serotonergic and noradrenergic transmission [37, 38]. Decreased Immobility time in TST represents the level of confidence in the animal to overcome the depressive state. In this study, both the doses of TIP39 significantly decreased the immobility time and dipping response but increased the jumping response. Similar types of effects were also observed in TST in terms of decreased immobility time. Interpretation of the above results shows TIP39 may regulate catecholamines in general to control the symptoms like depression and anxiety. This study supports the previous study reported that, the TIP39 is potentially acting on central noradrenergic signalling pathway during stressful conditions [39]. These statements also in accordance with our pervious study results implicating that TIP39 significantly increased the plasma noradrenaline level. In contrast, serotonin level did not g*et al*tered in acute restraint stressed rats after TIP39 administration (Data not shown). CUMS rats exhibited increased corticosterone level in plasma, which indicates the depressive state of the rats, evident with Wang C *et al.* [40]. Elevation of plasma costicosterone level might be due to dysregulation of HPA axis, which alters the neurochemical and biochemical activities [12]. The purpose of elevation during stress is to maintain the energy levels by util-

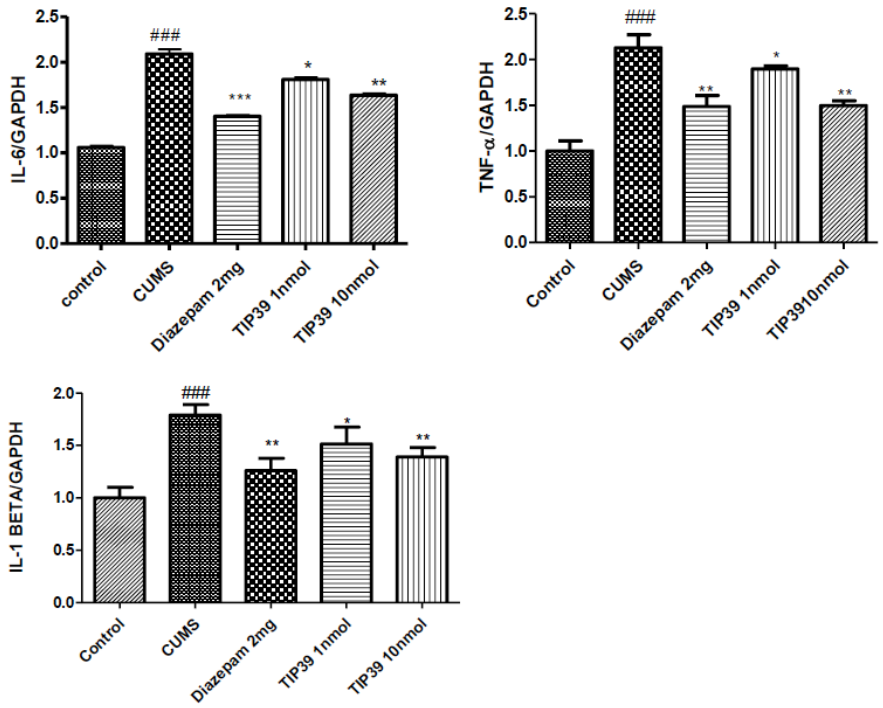

Fig. (7). Effect of TIP39 on IL-6, IL-1 β and TNF-α level in brain tissues of CUMS rats. All the values are expressed in Mean ± SD, n=6. Statistical analysis was carried out by One way ANOVA followed by post hoc analysis Tukey's multiple comparison test. ### denotes statistical significance in comparison to control rats at P <0.001. *, **, *** denotes statistical significance in comparison to vehicle treated CUMS group at P <0.05, P <0.01 and P <0.001 respectively.

izing glucocorticoids for demands and compensatory mechanism [41]. Interestingly, TIP39 reversed the elevation of plasma corticosterone level in stressed rats. The normalizing effect of TIP39 on plasma corticosterone level indicated that, it may possess adaptogenic response in stressed rats. This is due to the activation of hypothalamic glutaminergic neurons that facilitates TIP39 on HPA axis [42]. One of the core symptoms of depression is Anhedonia (decreased responsiveness to rewards), which reduces the sucrose preference in depressive animals [43]. This could be due to the alterations in HPA axis activity and it could be a root cause for hypophagia (decreased food intake) [44, 45]. In the four weeks study, gradual decline in sucrose consumption was observed in all the groups and this is due to the taste familiarity. Administration of anti stress drug (diazepam 2mg/kg) markedly increase the sucrose intake in CUMS rats. This could be due to the reduction of stress [46,47]. In the same manner, TIP39 dose dependently increased the sucrose intake in CUMS rats, which was not observed in CUMS alone treated rats.

It was observed that induction of chronic stress causes major cell injury provoked by the lipid peroxidation in cell membrane due to release of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radical [48]. In the present study, the daily exposure of rats to different stressors for 4 consecutive weeks significantly increased the marker of oxidative stress (LPO) and reduced the endogenous antioxidant level (SOD, CAT, and GSH) confirming that CUMS triggers free radical generation in hippocampus and prefrontal cortex [49, 50]. Simultaneous treatment with TIP39 rescued CUMS induced disproportion by normalizing SOD, CAT, SH, and lipid peroxidation marker (MDA). TIP39 administration for four weeks increased the SOD, CAT, GSH levels and reduced the production of MDA, which indicates the inhibition of lipid peroxidation. Many researchers demonstrated that, chronic stress increased the production of IL-6, IL-1β, and TNF-α level that induced pain perception due to glucocorticoid resistance action. This similarly reduced the competence of glucocorticoids to suppress cytokine production [51]. Subsequent administration of TIP39 in CUMS rats significantly reduced the proinflammatory cytokine levels. This is due to the guarding role of TIP39 by maintaining the central sensitization mechanism [52]. We also believe that suppression of proinflammatory cytokine by TIP39 peptide possibly due to the diminution of oxidative damage.

CONCLUSION

In CUMS rats, ICV administration of TIP39 could effectively improve the changes in FST, TST, sucrose consumption and open-field activity. Moreover, TIP39 could effectively reverse the alterations of plasma corticosterone concentrations, elevated SOD and CAT as well as the level of GSH and reduced MDA levels in the hippocampus and prefrontal cortex**.** Based on our research findings, this study identified the anti stress activity of TIP39 in CUMS model in relation to their simultaneous modulatory effects on HPA axis, inflammatory process, and oxidative courses. It points toward the possible relation between these systems during CUMS.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Reynolds, E.H. Brain and mind: a challenge for WHO. *Lancet,* **2003**, *361*(9373), 1924-1925.

[2] Chrousos, G.P.; Gold, P.W. The concepts of stress and stress system disorders. Overview of physical and behavioural homeostasis. *JAM,* **1992**, *267*(9), 1244-1252.

[3] Vieyra-Reyes, P.; Mineur, Y.S.; Túnez, I.; Vidaltamayo, R.; Picciotto, M.R.; Drucker Colín, R. Antidepressant-like effects of nicotine and transcranial magnetic stimulation in the olfactory bulbectomy rat model of depression. *Brain. Res. Bull.*, **2008**, *77*(1), 13-18.

[4] Mello, A.F.; Mello, M.F.; Carpenter, L.L.; Price, L.H. Update on stress and depression: the role of the hypothalamic pituitary adrenal (HPA) axis. *Rev. Bras. Psiquiatr,* **2003**, *25*(4), 231-238.

[5] Jayanthi, L.D.; Ramamoorthy, S. Regulation of monoamine transporters: influence of psychostimulants and therapeutic antidepressants. *AAPS J.,* **2005**, *7*(3), 728-738.

[6] Filip, M.; Frankowska, M.; Zaniewska, M.; Golda, A.; Przegalinski, E. The serotonergic system and its role in cocaine addiction. *Pharmacol. Rep,* **2005**, *57*(6), 685-700.

[7] Dobolyi, A.; Palkovits, M.; Usdin, T.B. Expression and distribution of tuberoinfundibular peptide of 39 residues in the rat central nervous system. *J. Comp. Neurol.,* **2003**, *455*(4), 547-566.

[8] Satherine, A.; Faber.; Arpád Dobolyi.; Mark sleeman.; Usdin, T.B. Distribution of tuberoinfundibular peptide of 39 residues and its receptor, parathyroid hormone 2 receptor, in the mouse brain. *J. Comp. Neurol*., **2007**, *502*(4), 563-583.

[9] LaBuda, C.J.; Dobolyi, A.; Usdin, T.B. Tuberoinfundibular peptide of 39 residues produces anxiolytic and antidepressant actions. *Neuroreport,* **2004**, *15*(5), 881-5.

[10] Sugimura, Y.; Murase, T.; Ishizaki, S.; Tachikawa, K.; Arima, H.; Miura, Y.; Usdin, T.B.; Oiso, Y. Centrally administered tuberoinfundibular peptide of 39 residues inhibits arginine vasopressin release in conscious rats. *Endocrinology,* **2003**, *144*(7), 2791-2796.

[11] LeDoux, J. The emotional brain, fear, and the amygdala. *Cell. Mol. Neurobiol.,* **2003**, *23*(4-5), 727-738.

[12] Prins, J.; Olivier, B.; Korte, S.M. Triple reuptake inhibitors for treating subtypes of major depressive disorder: the monoamine hypothesis revisited. *Expert. Opin. Investig. Drugs,* **2011**, *20*(8), 1107-1130.

[13] Catena-Dell'Osso, M.; Fagiolini, A.; Rotella, F.; Baroni, S.; Marazziti, D. Glutamate system as target for development of novel antidepressants. *CNS Spectr.,* **2013**, *18*(4), 188-198.

[14] Wang, P.; Xie, K.;Wang, C.; Bi, J. Oxidative stress induced by lipid peroxidation is related with inflammation of demyelination and neurodegeneration in multiple sclerosis. *Eur. Neurol*., **2014**, *72*(3-4), 249-254.

[15] Thompson, S.M.; Kallarackal, A.J.; Kvarta, M.D.; Van Dyke, A.M,; LeGates, T.A.; Cai, X. An excitatory synapse hypothesis of depression. *Trends. Neurosc*. **2015**, *38*(5), 279-294.

[16] Santos, G.A.; Pereira, V.D.; Bacurau, R.F.; Roman, E.A.F.R. Hypothalamic Inhibition of Acetyl-CoA Carboxylase Stimulates Hepatic Counter-Regulatory Response Independent of AMPK Activation in Rat. *PLoS One.,* **2013**, *8*(4), e62669.

[17] Ducottet, C.; Griebel, G.; Belzung, C. Effects of the selective nonpeptide corticotrophin releasing factor receptor 1 antagonist antalarmin in the chronic mild stress model of depression in mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry.,* **2003**, *27*(4), 625-631.

[18] Willner, P.; Towell, A.; Sampson, D.; Sophokleous, S.; Muscat, R. Reduction of sucrose preference by chronic mild stress and its restoration by a tricyclic antidepressant. *Psychopharmacology (Berl).,* **1987***, 93*(3), 358-364.

[19] Gao, S.; Cui, Y.L.; Yu, C.Q.; Wang, Q.S.; Zhang, Y. Tetrandrine exerts antidepressant-like effects in animal models: role of brain-derived neurotrophic factor. *Behav. Brain. Res.,* **2013***, 238,* 79-85.

[20] Porsolt, R.D.; Bertin, A.; Jalfre, M. Behavioural despair in mice: a primary screening test for antidepressants. *Arch. Int. Pharmacodyn. Ther.* **1977**, *229*(2), 327-336.

[21] Santiago, R.M.; Barbieiro, J.; Lima, M.; Dombrowski, P.A.; Andreatini, R.; Vital, M.A. Depressive-like behaviors alterations induced by intranigral MPTP, 6- OHDA, LPS and rotenone models of Parkinson's disease are predominantly associated with serotonin and dopamine. *Prog. Neuropsychopharmacol. Biol. Psychiatry.,* **2010**, *34*(6), 1104-1114.

[22] Steru, L.; Chermat, R.; Thierry, B.; Simon, P. The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl).,* **1985**, *85*(3), 367-370.

[23] Sun, Y.; Larry, W.O.; Ying, L. A simple method for clinical assay of superoxide dismutase. *Clin. Chem.,* **1988**, *34*, 497-500.

[24] Aebi, H. Catalase *in vitro*. *Methods. Enzymol*. **1984**, *105*,121-126.

[25] Elmann, G.L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.,* **1959**, *82*,70-77.

[26] Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.,* **1979**, *95*, 351-358.

[27] Lowry, O.H.; Rosenbrough, N.J.; Farr, A.L.; Randall, R.J. Protein estimation with folin phenol reagent*. J. Biol. Chem.,* **1951**, *193*, 265-275.

[28] Ahmad, A.; Rasheed, N.; Banu, N.; Palit, G. Alterations in monoamine levels and oxidative systems in frontal cortex, striatum, and hippocampus of the rat brain during chronic unpredictable stress. *Stress,* **2010**, *13*(4), 355-364.

[29] Darshit, B.S.; Ramanathan, M. Activation of AKT1/GSK-3β/β-Catenin-TRIM11/Survivin Pathway by Novel GSK-3β Inhibitor Promotes Neuron Cell Survival: Study in Differentiated SH-SY5Y Cells in OGD Model. *Mol. Neurobiol.,* **2016**, *53*(10), 6716-6729.

[30] Glaser. R.; Kiecolt-Glaser, J.K. Stress-induced immune dysfunction: implications for health. *Nat. Rev. Immunol.,* **2005**, *5*(3), 243-251. ·

[31] Webster Marketon, J. I.; Glaser, R. Stress hormones and immune function. *Cellular Immunology*, **2008**, *252*(2), 16-26.

[32] Reiche, E.M.; Nunes, S.O.; Morimoto, H.K. Stress, depression, the immune system, and cancer. *Lancet Oncol*., **2004**, *5*(10), 617-625.

[33] Biala, G.; Pekala, K.; Boguszewska-Czubara, A.; Michalak, A.; Kruk-Slomka, M.; Budzynska, B. Behavioral and biochemical interaction between nicotine and chronic unpredictable mild stress in mice. *Mol. Neurobiol.*, **2017**, *54*(2), 904-921.

[34] Joca, S.R.; Padovan, C.M.; Guimaraes, F.S. Activation of post synaotic 5- HT-1A receptors in the dorsal hippocampus prevents learned helpnessness development. *Brain Res.,* **2003**, *978*, 177-184.

[35] Porsolt, R.D.; Anton, G.; Blavet, N.; Jalfre, M. Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur. J. Pharmacol.,* **1978**, *47*, 379-391.

[36] Cryan, J.F.; Markou, A.; Lucki, I. Assessing antidepressant activity in rodents: recent developments and future needs. *Trends. Pharmaco. Sci*., **2002**, *23*, 238-245.

[37] Cryan,J.F.; Page, M.E.; Lucki, I. Differential behavioral effects of the antidepressants reboxetine, fluoxetine, and moclobemide in a modified forced swim test following chronic treatment*. Psychopharmacology*, **2005**, *182*, 335-344.

[38] Detke, M.J.; Rickels, M.; Lucki, I. Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology,* **1995**, *121*, 66-72.

[39] Coutellier, L.; Logemann, A.; Kuo, J.; Rusnak, M.; Usdin, T.B. TIP39 modulates effects of novelty-induced arousal on memory. *Genes Brain Behav*., **2011**, *10*(1), 90-99.

[40] Wang, C.; Guo, R. The Effect of Xingpijieyu Decoction on Depressive Behavior and Serum 5-HT as well as Corticosterone of Depression Rats from Chronic Stress. *J. Tradit. Chin. Med*., **2014**, *12*, 1633-1635.

[41] Rai, D.; Bhatia, G.; Sen, T.; Palit, G. Comparative study of perturbations of peripheral markers in different stressors in rats. *Can. J. Physiol. Pharmacol*., **2003**, *81*(12), 1139-1146.

[42] Dimitrov, E.L.; Kim, Y.Y.; Usdin, T.B. Regulation of hypothalamic signaling by tuberoinfundibular peptide of 39 residues is critical for the response to cold: a novel peptidergic mechanism of thermoregulation. *J Neurosc.,* **2011**, 31(49), 18166-18179.

[43] Wu, L.M.; Han, H.;Wang, Q.N. Mifepristone repairs regiondependent alteration of synapsin I in hippocampus in rat model of depression. *Neuropsychopharmacology*, **2007**, *32*, 2500-10.

[44] Luo, D.D.; An, S.C.; Zhang, X. Involvement of hippocampal serotonin and neuropeptide Y in depression induced by chronic unpredicted mild stress. *Brain. Res. Bull.,* **2008**, *77*, 8-12.

[45] Becker, C.; Zeau, B.; Rivat, C.; Blugeot, A.; Hamon, M.; Benoliel, J.J. Repeated social defeat-induced depression-like behavioral and biological alterations in rats: involvement of cholecystokinin. *Mol. Psychiatry.*, **2008**, *13*, 1079-1092.

[46] Richardson, D.K.; Reynolds, S.M.; Cooper, S.J.; Berridge, K.C. Endogenous opioids are necessary for benzodiazepine palatability enhancement: naltrexone blocks diazepam-induced increase of sucrose-'liking'. *Pharmacol. Biochem. Behav*., **2005**, *81*(3), 657-663.

[47] Farhan, M.; Ikram, H.; Kanwal, S.; Haleem, D.J. Unpredictable Unpredictable chronic mild stress induced behavioural deficits: A comparative study in male and female rats. *Pak. J. Pharm. Sci.,* **2014**, *27*, 879-884.

[48] Dimitrov, E.; Usdin, T.B. Tuberoinfundibular peptide of 39 residues modulates the mouse hypothalamic-pituitary-adrenal axis via paraventricular glutamatergic neurons. *J. Comp. Neurol.,* **2010**, *518*(21), 4375-94

[49] Rajan, R.K.; Kumar, M.S.S.; Balaji, B. Soy isoflavones exert beneficial effects on letrozole-induced rat polycystic ovary syndrome (PCOS) model through anti-androgenic mechanism. *Pharm. Biol.,* **2017**, *55*(1), 242-251.

[50] Zafir, A.; Banu, N. Antioxidant potential of fluoxetine in comparison to Curcuma longa in restraint-stressed rats. *Eur.J. Pharmacol.,* **2007**, *572*, 23-31.

[51] Beg, A.A.; Finco,T.S.; Nantermet, P.V.; Baldwin, A.S. Jr. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Mol.Cell. Biol*., **1993**, *13*(6), 3301-3310.

[52] Dimitrov, E.L.; Kuo, J.; Kohno, K.; Usdin, T.B. Neuropathic and inflammatory pains are modulated by tuberoinfundibular peptide of 39 residues. *Proc. Natl. Acad. Sci.,* **2013**, *110*(32), 13156-13161.

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Email id:psganimalethics@gmail.com, Registration number: 158/1999/CPCSEA

DATE: 09.08.2013

Title of the Project:

Elucidation of Tuberoinfentebular Peptide 39 (TIP39) in neurological disorders.

Proposal Number: 205 /2013/ IAEC

Name of the Applicant: Mr.G.Venkatesh. PhD scholar

Approval date: **09.08.2013**

Date of Initiation: Immediately after the approval

No. of animals sanctioned with name of species: Twenty eight male Sprague Dawley rats.

Sex of the animal: Male/Female

Expiry date (Termination of the Project): **08.08.2014**

Dr.S.Ramalingam	**Dr.C.Gunasekaran** 9/8/13
Name of the chairperson	Name of IAEC/CPCSEA nominee
Signature of Chairperson	Signature of the CPCSEA nominee
Date: 9/8/2013	Date:

INTERNATIONAL SYMPOSIUM ON NEURODEGENERATIVE DISORDERS

SOCIETY *for* NEUROSCIENCE
BANGALORE CHAPTER

ISND 2017

29TH & 30TH MARCH 2017
NATIONAL INSTITUTE OF MENTAL HEALTH AND NEURO SCIENCES
(NIMHANS) BENGALURU

Certificate

This is to certify that *Mr. Venkatesh Gunasekaran* participated in the symposium as a *Delegate* and presented a paper entitled:

Evaluation of Neuroprotective role of Tuberoinfundibular Peptide of 39 (TIP39) on chronic unpredictable mild stress (CUMS) induced cognitive deficit in animal models

Dr. Laxmi T. Rao	Dr. Phalguni A. Alladi	Dr. T. N. Satyaprabha	Dr. Bindu M. Kutty
Scientific Chair	*Joint-Organizing Secretary*	*Organizing Secretary*	*Chairperson*

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67th INDIAN PHARMACEUTICAL CONGRESS 2015 Mysuru

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This is to certify that

Prof./Dr./Mr./Ms. Venkatesh, P.S.G. College Of Pharmacy, Coimbatore, Tamil Nadu

has presented a paper entitled Tuberoinfundibular Peptide of 39 (TIP 39) Ameliorates Chronic Unpredictable Mild Stress (CUMS) Induced Dysregulation Of Hypothalamic Pituitary Adrenocortical Axis (HPA) On Depressive Model In Rats

in the Scientific ~~Oral~~ / Poster Session of 67th IPC held at

JSS Univeristy, Mysuru, Karnataka, from December 19th to 21st, 2015.

Dr. G. N. Singh	Dr. B. Suresh	Dr. T. K. Ravi	Dr. S. Balasubramanian
President, IPCA	Chairman, LOC	Convener, SSC-IPCA	Chairman, SSC-LOC

Organised by:
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Gmail **Venkatesh Gunasekaren <gvenkatpharma@gmail.com>**

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