# Protective Effects of Ascorbic Acid in Mouse Model of Depression

**Master of Science in Healthcare Biotechnology**

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**Protective Effects of Ascorbic Acid in Mouse Model of Depression**

A thesis submitted in partial fulfillment of the requirement for the degree

of Masters of Science in Healthcare Biotechnology

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*Dedicated to my parents for their endless love, support and belief in me*

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***Sidra Ishtiaq***

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| | |
|---|---|
| **AA** | Ascorbic Acid |
| **ACTH** | Adrenocorticotropic hormone |
| **ANOVA** | Analysis Of Variance |
| **ATF6** | Activating Transcription Factor 6 |
| **BDNF** | Brain Derived Neurotrophic Factor |
| **BiP** | Binding Protein |
| **BrdU** | BromodeoxyUridine |
| **CA3** | Cornu Ammonis 3 |
| **CELF4** | CUGBP Elav-Like Family Member 4 |
| **CHOP** | CCAAT/enhancer-binding protein-homologous protein |
| **CRH** | Corticoliberin Releasing Hormone |
| **CRP** | C Reactive Protein |
| **CSDS** | Chronic Social Defeat Stress |
| **DG** | Dentate Gyrus |
| **DRD2** | Dopamine D2 Receptor |
| **DSM** | Diagnostic and Statistical Manual of Mental Disorders |
| **ER** | Endoplasmic Reticulum |
| **FST** | Forced Swim Test |
| **FXT** | Fluoxetine |
| **GBD** | Global Burden of Disease |
| **GRP** | Glucose-Regulated Proteins |
| **GRP** | Glucose-Regulated Protein |
| **H&E** | Hematoxylin and Eosin |

| | |
|---|---|
| **HPA** | Hypothalamus Pituitary Axis |
| **IL** | Interleukins |
| **IRB** | Institutional Review Board |
| **IRE1α** | Inositol-requiring ER-to-nucleus signaling protein |
| **MAO** | Monoamine Oxidase |
| **MDD** | Major Depressive Disorder |
| **MRI** | Magnetic Resonance Imaging |
| **mTOR** | Mammalian Target of Rapamycin |
| **NeuN** | Neuronal Nuclear Protein |
| **NSFT** | Novelty Suppressed Feeding Test |
| **PCR** | Polymerase Chain Reaction |
| **PERK** | Protein kinase-like Endoplasmic Reticulum Kinase |
| **PET** | Positron Emission Tomography |
| **ROS** | Reactive Oxygen Species |
| **SEM** | Standard Error of Mean |
| **SGZ** | Sub Granular Zone |
| **SNP** | Single Nucleotide Polymorphism |
| **SNRIs** | Selective Norepinephrine Reuptake Inhibitors |
| **SSRIs** | Selective Serotonin Reuptake Inhibitors |
| **SST** | Sucrose Splash Test |
| **TCA** | Tricyclic Antidepressant |
| **TNF** | Tumor Necrosis Factor |
| **TST** | Tail Suspension Test |
| **UCMS** | Unpredictable Chronic Mild Stress |

| | |
|---|---|
| **UPR** | Unfolded Protein Response |
| **WHO** | World Health organization |
| **XBP1** | X-box Binding Protein 1 |
| **YLDs** | Years Lived with Disability |

# ABSTRACT

Major Depressive Disorder (MDD), an intricate neuropsychiatric disorder is marked by diminished mood, low energy, no motivation, anhedonia and self-harm or suicidal thoughts in severe cases. Endoplasmic reticulum (ER) dysfunction, impaired neurogenesis, mitochondrial dysfunction and oxidative stress are implicated in the pathophysiology of MDD. Adverse side effects of the available treatments *i.e.*, Selective Serotonin Reuptake Inhibitors (SSRIs) have been reported including serotonin syndrome, nausea, confusion and dizziness. Ascorbic acid (AA), a potent antioxidant and anti-inflammatory compound has shown antidepressant activity as reported in many studies. The present research sought to explore the neuroprotective advantages of ascorbic acid when compared to the conventional drug fluoxetine (FXT) in a mouse model of clinical depression induced by Unpredictable Chronic Mild Stress (UCMS). The mice were distributed into 8 groups (n=6 each). The 9-week long procedure for the UCMS led to a deteriorated coat of mice ($p<0.001$ for week 4 and $p<0.0001$ from week 5 to 9). A three-week treatment of AA and FXT displayed a significantly lower coat score exhibiting substantial improvement in the coat state. Behavioral assessment was conducted through Sucrose Splash Test (SST), Novelty Suppressed Feeding test (NSFT), Tail Suspension Test (TST), Y maze test and Forced Swim Test (FST). A significant reduction ($p<0.001$) in anhedonia was indicated by increased grooming time (189.5±19.67s) in SST task by UCMS+AA group. While the combination group (FXT+ AA) also exhibited the similar increase in grooming time (195.5±15.31) ($p < 0.001$). The results also indicated the potential ameliorative effect of AA on behavioral despair or depression like behavior and helplessness, assessed through TST. Antidepressant effects of AA were also eminent in FST indicated by significantly delayed latency to immobility. However, a non-significant reduction in

latency to immobility was observed in UCMS+FXT+AA (14.83±5.30s) group ($p<0.05$).

Similarly, anxiety-like behavior was assessed through NSFT where increased latency to eat food was evident in UCMS group. A mild trend ($p>0.05$) showing anxiety like behavior was observed in the UCMS group (185±65.8) In addition, UCMS group exhibited a decrease in percentage of spontaneous alterations, total time period spent in the novel arm and percentage of entries in novel arm in Y-maze test ($p>0.05$ for each), while treatment with AA and FXT increased the percentage spontaneous alterations and time spent in the novel arm indicating that AA and FXT can improve spatial learning and memory. Moreover, the treatment with AA significantly restored the neuronal cell count in the hippocampal dentate gyrus (27.5±1.04) in the UCMS exposed group which was substantially deteriorated. The combinational treatment of FXT and AA also displayed significantly increased neuronal density (24±1.08) as compared to UCMS group (8.75±1.01). The results of qRT-PCR showed the decreased hippocampal expression of NeuN in UCMS group ($p<0.05$) in contrast to the control group, however it was substantially normalized by FXT, and AA. In contrast to the control group, the mRNA expression of activating transcription factor 6 (ATF6), was significantly increased ($p<0.0001$) in UCMS group that is evocative of the higher cellular ER stress. A slight normalization of ATF6 levels in UCMS+AA group ($p>0.05$) was observed whereas FXT ($p<0.0001$) substantially recovered the aberrant expression. Protein-protein interaction was also assessed for ATF6, NeuN with FXT and AA using molecular docking. The analysis revealed that ATF6 and NeuN can interact with both FXT and AA with comparable binding energies. In conclusion, these findings support that the potent neuroprotective, antidepressant and antianxiety effects of ascorbic acid make it an effective alternative and adjuvant treatment approach for MDD.

# INTRODUCTION

Major Depressive Disorder (MDD), commonly known as depression encompasses symptoms including anhedonia, persistent sadness, low energy, diminished mood, insomnia, loss of appetite, reduced concentration, low self-esteem, poor concentration and even suicide ideation, defined by the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM5) (Guha, 2014). In 2015, it was estimated that 320 million people globally experienced depression constituting 4.7% of the world population (GBD and Injury Incidence and Prevalence Collaborators, 2017; World Health Organization (WHO), 2017).

Due to the intricate and heterogenous etiology of MDD, no precise single underlying mechanism can be pinpointed. However, multiple hypotheses have been suggested to elucidate the pathophysiology of Major Depressive Disorder (MDD). Disruption of Hypothalamus Pituitary Axis (HPA) axis in depression caused by the significantly higher cortisol levels produced as a response to stressful stimulus can lead to the decreased tryptophan metabolism which ultimately leads to decrease in levels of monoamines *i.e.,* norepinephrine, serotonin, and dopamine (Sorgdrager *et al.,* 2017). This decrease in the levels of monoamines leads to diminished motivation, concentration and increased aggression (Ashe *et al.,* 2019; Kaltenboeck & Harmer, 2018). Moreover, increased levels of cortisol that also decrease the BDNF levels lead to reduced brain volume and hippocampal neuronal degeneration and loss of neurons as implicated through the postmortem studies on MDD patients (Duman *et al.,* 2021; Tartt *et al.,* 2022). Similarly, impaired synaptic plasticity patterns have also been implicated as the underlying cause of MDD (Duman & Aghajanian, 2012). In MDD, significant reduction in astrocytes, particularly in the frontal areas and limbic circuitry, results in a compromised integrity of the Blood-Brain Barrier. Consequently, this

breakdown triggers the recruitment of monocytes to the brain, fostering an escalation of inflammation mediated by interleukins such as IL-6, and TNFα. (Medina *et al.,* 2018; Petralia *et al.,* 2020; Wu *et al.*,2021).

The mitochondrial dysfunction hypothesis of depression suggests that increased oxidative and nitrosative stress caused by the disbalance in the cellular antioxidant enzymes and Reactive Oxygen Species (ROS) eventually results in inflammation and neuronal apoptosis (Bhatt *et al.,* 2020; Somani *et al.,* 2022). Several physiological conditions including hypoxia, stress, calcium depletion, hypoglycaemia, high fat diet and oxidative stress can contribute to an increased number of unfolded and misfolded proteins resulting in an increase in endoplasmic reticulum stress (Hetz & Papa, 2018). The disrupted cellular homeostasis is then restored by Unfolded Protein Response (UPR) activation. However, if endoplasmic reticulum (ER) stress persists, UPR initiates inflammatory cascades or cellular apoptotic signalling (Hetz *et al.,* 2015). The expression of PERK, CHOP, XBP1 that are the main ER signalling proteins was significantly higher in Chronic Social Defeat Stress (CSDS) depression model suggesting that ER stress plays a role in MDD pathogenesis (Tang *et al.,* 2018).

As reported by Thase *et al.,* 2010, half of the patients treated with the conventional antidepressants do not attain full remission. The main treatment approach for MDD involves the use of Selective Serotonin Reuptake Inhibitors (SSRIs) that mostly alleviate the symptoms only. Fluoxetine (FXT) is one of the most widely prescribed SSRI due to its better tolerability as compared to other antidepressants. It inhibits the Serotonin Transporter (SERT) and reduces the serotonin reuptake. FXT elevates the dopamine and norepinephrine levels in synapse at higher dosages (Edinoff *et al.,* 2021; Bymaster *et al.,* 2002). Several side effects of SSRIs including diarrhoea, excessive

bleeding, nausea, swelling, sleep disturbances, sexual dysfunction, serotonin syndrome, dry mouth syndrome and in extreme cases, suicide ideation have been reported (Hillhouse & Porter, 2015; Wang *et al*., 2018; Ramachandraih *et al*., 2011; Ramic *et al.*, 2020).

Ascorbic acid (AA) or Vitamin C (vit C) is a water-soluble vitamin with potent antioxidant activity (Du *et al.*, 2012). Studies have documented that AA can reverse the neurodegenerative alterations and memory deficits in aluminum-treated rats (Olajide *et al.*, 2017; Sil *et al.*, 2016). Reduction in immobility time in Tail Suspension Test (TST) and Forced Swim Test (FST) with AA treatment is also reported in restraint stress mice (Binfaré *et al.*,2009; Moretti *et al.*, 2013). Moretti *et al.*, 2015 reported that the pretreatment with AA also abolishes the depression induced by TNFα. Considering the anti-inflammatory and antioxidant properties of AA, it can be used as an antidepressant by targeting different pathologies associated with the disease.

UCMS model of depression stands out as the premier method for replicating the human condition in animal models. Despite its susceptibility to minor design alterations, it represents a paramount effort to faithfully mirror the complexities of the human condition (Markov & Novosadova, 2022).

### 1.1 Aim and Objectives

The present study aims to explore the anti-depressive and neuroprotective effects of Ascorbic acid in comparison to Fluoxetine in UCMS model of depression and to evaluate the effect of supplementation of Ascorbic acid with Fluoxetine. The objectives of the study are:

- To investigate the effects of AA on depressive behavior and anxiety levels in mice in comparison to standard drug Fluoxetine.

- Identification of molecular targets of Fluoxetine and AA via *in-silico* analysis.

- To elucidate the effects of AA on neuronal architecture and gene expression associated with depression.

# LITERATURE REVIEW

## 2.1 Major Depressive Disorder

Depression or Major Depressive Disorder (MDD) ranks as the third-leading contributor to the burden of disease described by WHO and is anticipated to ascend to the primary cause by 2030 (Dadi *et al.,* 2020). GBD Injuries and Risk Factors Study revealed that depression accounted for 34.1 million of the overall Years Lived with Disability (YLDs), securing its position as the fifth most substantial cause of YLDs (Carapetis & Dadi, 2017). Approximately, 280 million people are affected from depression worldwide, comprising 5% of adults while it is 50% more prevalent among women than men (WHO 2023).

## 2.2 Pathophysiology of MDD

MDD is an intricate, complex and highly heterogenous condition with unclear etiology but various contributing factors such as genetics, epigenetics, and environmental influences collectively lead to its onset. Depression encompasses various endophenotypes, each characterized by unique pathophysiological mechanisms. Viewing depression as a cohesive syndrome allows for understanding how these mechanisms interact as interconnected nodes within a matrix (Dean & Keshavan, 2017). As diagnosing depression relies on its symptoms rather than the underlying pathology, only 30% of patients respond to the treatment (Caldiroli *et al.,* 2021). Several studies have made attempts to understand the neuropsychiatry and neurophysiology of MDD, yet no single precise mechanism responsible for the MDD has been discovered. This indicates that MDD involves multiple etiologies which need to be understood for the development of therapeutics (Kamran *et al.,* 2022). Numerous

hypotheses and theories have been suggested to elucidate the pathophysiology of Major Depressive Disorder (MDD).

### 2.2.1 Monoamine Theory

The reduced monoamine levels inside brain, *i.e*., norepinephrine (NE), Serotonin 5-HT, and DA (Dopamine) is one of the earliest proposed mechanisms underlying MDD (Hamon & Blier, 2013). The functioning of the prefrontal cortex, responsible for working memory, regulation of behavior, and mindfulness, is closely associated with norepinephrine. Dopamine is involved in modulating reward, working memory, motivation, and mindfulness. On the other hand, serotonin, constituting the largest cohesive system of neurotransmitters, innervates various brain regions and peripheral systems (Girotti *et al.*, 2018). Within the central nervous system, serotonin regulates essential brain functions such as sleep, stress response, autonomic neural activity, mood, body temperature, and appetite (Wu *et al.*, 2019). In the gastrointestinal tract, serotonin performs its action by regulating motor and secretory functions. Additionally, serotonin has significant effects on heart rate, hemostasis, cell growth, respiratory drive and immunity. In response to inflammation, serotonin engages in regulating immune cells in response to inflammation, particularly upon the platelet's activation (Herr *et al.*, 2017). Significantly reduced levels of serotonin in brain samples from depressed patients have been documented in studies. Aberrations in brain dopamine levels can lead to impaired motivation, concentration and aggregation. Deficiency of norepinephrine mediates a wide array of depressive symptoms, including concentration, aggression, mindfulness, appetite and motivation (Ashe *et al.*, 2019). Several studies have offered evidence in support of the monoamine theory of depression, but there are limitations to consider. One notable inconsistency is the

delayed onset of clinical effects associated with antidepressants, which typically take weeks to manifest, in contrast to the nearly instantaneous impact of antidepressants on increasing monoamine levels (Kaltenboeck & Harmer, 2018). This implies that monoamine deficiency is not a factor applicable to all depressed patients, underscoring the significance of considering alternative pathways and neurotransmitters in MDD pathology (Fries *et al.*, 2023).

### 2.2.2 HPA axis and stress response

The Hypothalamus Pituitary Adrenal (HPA) axis regulates the response of the body to stress for maintaining homeostasis. In response to stress, Corticotropin-Releasing Hormone (CRH) is released from the hypothalamus, triggering the anterior pituitary to release Adrenocorticotropic Hormone (ACTH). Acting on the adrenal cortex, ACTH enables cortisol release in the body (Jacobson, 2005). Through a negative feedback loop, elevated cortisol levels halt the release of CRH and ACTH (Joseph & Whirledge, 2017). Released under stressful situations, cortisol serves as a defense response as it stimulates gluconeogenesis and prevents excessive activation of immune response (Heaney, 2020). Most severe subtypes of MDD are associated with cortisol hypersecretion (Nandam, 2020). The excessive adrenal activity results in increased vulnerability to depression by having destructive effects on hippocampus (Qin *et al.*, 2015). Glucocorticoid resistance can result from the elevated levels of cortisol (Slavich & Irwin, 2014) and has been reported in patients suffering from MDD (Bertollo, 2020) as well as in CMS (Chronic Mild Stress) depression model (Andrade *et al.*, 2013). Cerebral blood flow irregularities within the prefrontal cortex as well as limbic cortex have been observed in PET studies (Fuster, 2001). Moreover, the size of amygdala; the

part of brain involved in regulation of glutaminergic signaling, cortical arousal and neuroendocrine signaling is reduced in depression as shown by MRI studies (Yue *et al.*, 2013). Hippocampus, which is the brain region involved in memory, learning and neurogenesis, is closely linked with hypothalamus (Liu *et al.*, 2017). This region is rich in corticosteroid receptors and hence changes in the degree of its plasticity results from stress. These changes include increased neuronal apoptosis and reduced hippocampal volume (Zhang *et al.*, 2012). A meta-analysis showed reduced tryptophan levels in depressed patients in contrast to healthy individuals. In CNS, tryptophan is the substrate for serotonin synthesis (Fernstrom, 2013). HPA axis dysregulation may be responsible for this as it leads to the formation of balance between metabolism of tryptophan and cortisol levels (Sorgdrager *et al.*, 2017).

### 2.2.3 Neurotrophic factor hypothesis

Neurotrophins are actually the growth factors whose key functions include growth, survival and plasticity of neurons. BDNF is an important neurotrophin that facilitates adult hippocampal neurogenesis (Tartt *et al.*, 2022) and activates cell signaling pathways using Trk receptors, axonal growth, dendritic pruning and normal neuronal functions (Su *et al.*, 2014). BDNF is also responsible for mTOR pathway activation that activates protein synthesis in neuronal dendrites (Leal *et al.*, 2014). When there is an increase in cortisol levels as a stress response to such extent that the brain cannot further suppress its release, BDNF levels are decreased (Duman *et al.*, 2021) and eventually the neurogenesis decreases (McLaughlin *et al.*, 2022). In animal models of depression and blood of MDD patients, reduced BDNF levels have been documented (Kishi *et al.*, 2018 ; Tayyab *et al.*, 2018). Deficits in hippocampal neurogenesis, hippocampal size and volume, reduction in cell size, neuronal and glial cell lost have been indicated in

MDD postmortem brains (Tartt *et al.,* 2022). The neurotrophin hypothesis further elucidates the role of other neuroendocrine factors such as endogenous steroids like allopregnanolone. Chronic stress and depression have been linked to reduced levels of allopregnanolone (Walkery *et al*., 2021). Following labor and delivery, there is rapid decrease of this hormone leading to the postpartum depression (Chen *et al.,* 2021). Additionally, hypothyroidism is also a contributive to secondary depression onset (Bauer & Whybrow, 2021).

### 2.2.4 Reduced neurogenesis and neuroplasticity

The process of neurogenesis in adults refers to the development of nascent neurons and ultimately increased synapse formation in specific areas such as lateral ventricles and dentate gyrus of hippocampus (Urbán *et al.,* 2014). In adult brains, the neurotrophic factor BDNF is prominently expressed in limbic regions and ensures the proper survival, proliferation and differentiation of neurons. Cattaneo *et al.,* 2016 reported that MDD patients have shown dysregulated expression of BDNF in their mononuclear blood cells due to hypermethylation in gene promoters of BDNF. Additionally, alterations in morphology of brain as well as synaptic plasticity patterns are also reported (Mariani *et al.,* 2021). A decrease in hippocampal volume has been noted in MDD patients (Chan *et al*., 2016) as well as in animal models of depression (Banasr *et al.*, 2004). Research shows that increased glucocorticoid levels in depression cause this significant hippocampal loss of neurons (Sheline *et al.,* 2011).

Duman *et al.,*1999 explained that the neuronal capacity to adapt individually to their environment is known as neural plasticity, involving the generation of new cells and the genetically programmed, healthy death of cells inside the brain .Activation of each distinct neural circuit occurs in response to learning, memory, stress, or environmental

stimuli. An intracellular signal transduction cascade is triggered by this activation, which constitutes a fundamental process in neural plasticity (Jeon & Kim, 2016). The dendritic complexity of hippocampus and prefrontal cortex neurons is lowered by repeated stress exposure (Radley *et al.,* 2006). Rodents exposed to stress display abnormal synaptic plasticity patterns (Krishnan & Nestler, 2008). Altered levels of synaptic plasticity markers have also been observed in MDD patients (Duric *et al.,* 2013). Together these findings back the abnormal synaptic plasticity can be another cause of depression (Duman & Aghajanian, 2012).

### 2.2.5 The cytokine theory and inflammation

The cytokine theory of depression suggests that MDD and increased inflammation are linked bidirectionally. Under normal circumstances, immune cells detect tissue damage and initiate a cascade of inflammatory responses but if the response sustains for a longer period as in case of infection, autoimmune diseases or malignancy, it may lead to depression (Beurel & Nemeroff, 2020). Proinflammatory cytokines associated with MDD include C-reactive protein (CRP), interleukins such as IL-6, IL-2, IL-1β, IL-2 receptor, IL-10, Transforming Growth Factor-β and TNFα (Petralia *et al*., 2020). IL-18 and IL-1β activation via inflammasome pathway is caused by the endogenous molecules; Damage Associated Molecular Patterns (Alcocer *et al*., 2014; Miller & Raison, 2016). Additionally, nitrosative and oxidative stress, obesity, smoking, altered gut permeability, psychosocial stress, poor diet, sedentary lifestyle can also induce inflammation (Berk *et al.,* 2013). There are reports suggesting that the association of MDD may be linked to a dysfunctional Blood-Brain Barrier (BBB) and neuroinflammation, potentially resulting from the depletion of astrocytes in the frontal and specific limbic brain regions (Medina *et al.,* 2018 ; Wu *et al.,*2021). Increased BBB

permeability allows activated microglia to initiate monocytes recruitment to brain (Leighton *et al.,* 2018) resulting in irreversible oxidation of cofactors needed for monoamine biosynthesis (Kalkman & Feuerbach, 2016). Inflammatory cytokines activate indoleamine 2,3- dioxygenase (IDO) enzyme eventually decreasing the 5-HT synthesis (Catena *et al.,* 2013). Moreover, proinflammatory cytokines have the ability to reduce the expression of neurotrophins and impede the BDNF/TrkB signaling pathway (Jin *et al.,* 2019). HPA axis activation by circulating cytokines ultimately results in increased adrenocorticotropic hormone and glucocorticoid levels (Liu *et al.,* 2017; Bertollo *et al.,* 2020).

### 2.2.6 Mitochondrial dysfunction and Oxidative stress Hypothesis

"Mitochondrial theory of depression" is supported by the studies linking MDD with rare mitochondrial disorders (Klinedinst & Regenold, 2015). This encompasses observed changes in mitochondrial functions and its structure, including a reduction in ATP production (Kuffner *et al.,* 2020) along with disturbances in mitochondrial dynamics, including fusion, fission, and mitophagy (Scaini *et al.,* 2022). Mitochondria play a crucial role in supporting neurotransmission through various mechanisms, including ATP production, $Ca^{2+}$ signaling , neurotransmitters synthesis (Rossi & Pekkurnaz, 2019), establishment and maintenance of excitation of membrane and organization of synaptic vesicles for the release of neurotransmitters. Moreover, mitochondria play a crucial role in generating free nitrogen and oxygen species, which are essential for maintaining synaptic plasticity. In long-term depression, mitochondria contribute to the caspase enzymes inside the neuronal dendrites to get activated thereby resulting in spine elimination of post synaptic neuronal cells (Jeanneteau & Arango, 2016).Triebelhorn *et al.,* 2022 documented the unusual mitochondrial functioning in

reprogramming of the neural progenitor cells isolated from MDD patients' fibroblasts. In contrast to the healthy controls, induced pluripotent stem cells of MDD patients revealed substantial alterations in the membrane capacitance as well as the neuronal electrophysiological properties. Furthermore, dysfunctional mitochondria contribute to increased proinflammatory cytokines production(Hoffmann *et al.,* 2019). Mitochondrial damage triggers cell death cascades in brain samples of MDD patients (Miguel *et al.,* 2014). These events ultimately contribute to activation of chronic inflammation observed in MDD (Osimo *et al.,* 2019).

In MDD, oxidative stress as well as nitrosative stress markers are elevated, whereas the antioxidant activity is diminished due to mitochondrial disruption (Somani *et al.,* 2022). The "oxidative stress hypothesis of depression" suggests that the altered brain structure in MDD is likely due to oxidative stress. Importantly, at normal levels reactive oxygen species (ROS) serve as signaling messengers with significant neuronal cell function role. When present at elevated levels with low antioxidant concentrations, these molecules have the potential to damage neuronal cells. Increased oxidative stress leads to further mitochondrial disruption, escalating apoptosis, and eventually to inflammation (Bhatt *et al.,* 2020).

### 2.2.7 Endoplasmic Reticulum stress and the UPR (Unfolded Protein Response)

Endoplasmic reticulum is a single-membrane bound organelle inside the cell which performs important roles in protein and lipid synthesis. Directing various secretory as well as membrane proteins to appropriate location inside the cell, proper folding of proteins and post translational modifications are some of the important roles of ER (Rasheva & Domingos, 2009; Ron & Walter, 2007). Regulation of cellular levels of

$Ca^{2+}$ ions, maintenance of redox homeostasis and influencing several cellular signaling cascades are important functions of endoplasmic reticulum.ER chaperones, mainly Glucose-Regulated Proteins GRP78 and GRP94 control the proper folding of proteins. However, if ER is unable to fold the proteins as per the requirement of cell, it leads to ER stress (Ron & Walter, 2007). Inflammation, Hypoxia, metabolic disorders, infections and several neurodegenerative disorders can disrupt the proper ER functioning (Hotamisligil, 2010). To remove the defective unfolded proteins by enhancing the capacity of ER for protein folding and restoring the ER homeostasis, UPR; Unfolded Protein Response is initiated (Hetz, 2013). If ER equilibrium is not established, UPR can initiate cell death (Ron & Walter, 2007). The main ER stress receptors in mammals include: IRE1α (inositol-requiring ER-to-nucleus signaling protein), PERK (protein kinase-like endoplasmic reticulum kinase) and ATF6 (Carrara & Ali, 2013). GRP78 also refered to as BiP (Binding Protein) is bound to these proteins under homeostatic conditions. Under ER stress, the misfolded proteins bind to BiP which leads to the release of IRE1,ATF6 and PERK. Homodimerization and autophosphorylation results in activation of IRE1 after being released (Harding,2000). Activation of PERK subsequently induces the eukaryotic translation initiation factor 2α (eIF2α) phosphorylation, initiating the activation of various transcription factors, including ATF4. The activity of phosphorylated eIF2α is restrained in the initial stress phase, causing a decelerated protein synthesis and translation across the cell, thereby diminishing the protein folding task in the endoplasmic reticulum (ER) (Carrara *et al.*,2015). Similarly, IRE1 after dissociating from GRP78 gets activated by autophosphorylation process and splices the messenger RNA of X-box binding protein 1(XBP1) XPB1 restores ER homeostasis by activating ERAD (Endoplasmic Reticulum Associated Degradation) program by promoting CHOP (CCAAT/enhancer-binding

protein-homologous protein) transcription which is a pro-apoptotic gene (Lee *et al.*,2003). ATF6 after being dissociated from BiP, gets translocated into Golgi bodies. Golgi site-1 and -2 proteases (Hillary & Gerald, 2018),cleave the ATF6α transcription factor and liberate it. Collaborating with XBP1, ATF6α stimulates the transcriptional activation of target genes *i.e.,* GRP78, thus counteracting ER stress and promoting cell survival (Shoulders *et al.*, 2013). Through negative feedback these signaling pathways collectively work to alleviate ER stress, diminishing the presence of unfolded proteins and supporting cell survival (Oakes & Papa, 2015). However, if this regulatory mechanism falters, a substantial release of calcium into the cytoplasm triggers apoptosis (Shore *et al.,* 2011). Persistent UPR signaling can lead to chronic ER stress. The UPR triggers cell death via apoptosis. This apoptosis can be carried out by activating CHOP, caspases, and the Bcl-2-like protein 11 (Bim); a proapoptotic protein.

UPR response activation results in initiation of inflammatory pathways and transcriptional activation of cytokines (Ron,2002). There is well-documented evidence of a robust association of depression with UPR (Zhang *et al.*, 2014). As UPR activates inflammation, higher levels of interleukins and TNFα in depressed patients are reported (Lotrich,2012). Higher expression of GRP94 and GRP78 has been documented in temporal cortex of patients suffering from MDD who attempted suicide and died of it in contrast to the patients after non-suicidal deaths (Behnke *et al.*, 2016). Some studies suggest that neuronal regeneration and survival depends on chronically activated ER stress (Wu *et al.,* 2016) as long-term stress can damage the amygdala sensitivity to negative stimuli, normal hippocampal function and nerve regeneration (Dillon & Pizzagalli, 2018).

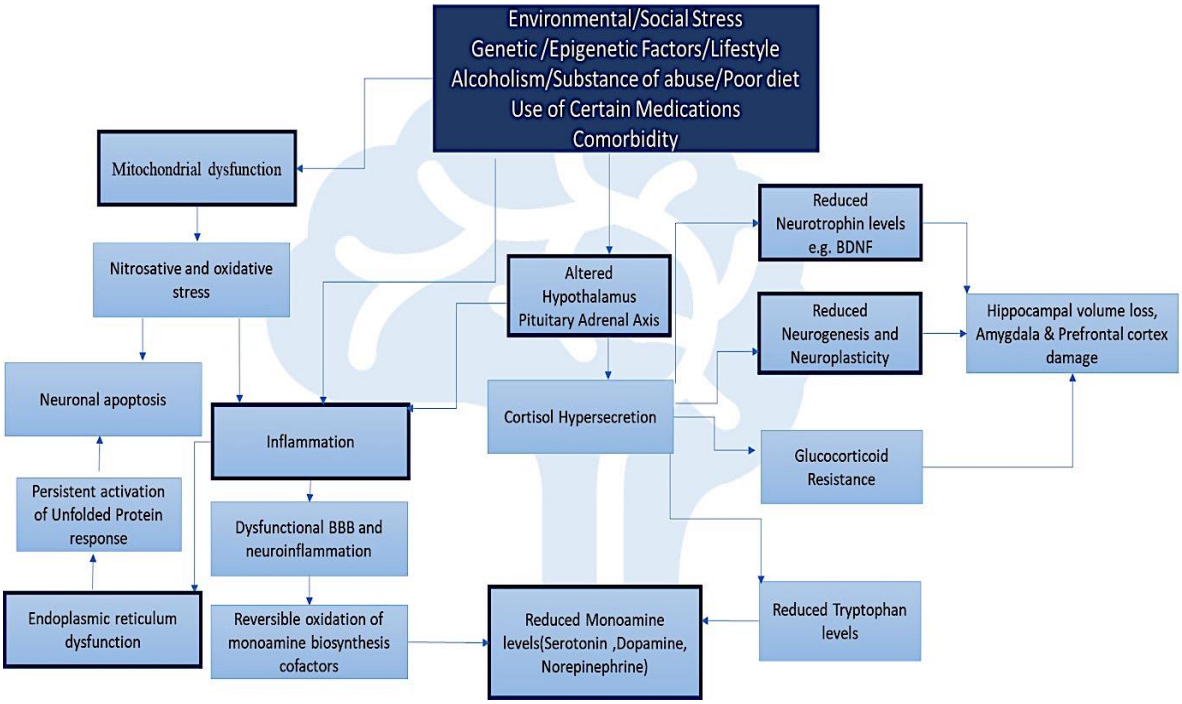


**Figure 2.1 Pathophysiology of Major Depressive disorder**

## 2.3 Risk Factors for MDD

### 2.3.1 Environmental Factors

Numerous factors contribute to the development of MDD, with life experiences being the most extensively studied among them. Risk factors associated with life events and changes encompass various circumstances, including harassment, social seclusion, financial constraints , bullying , a serious medical disorder, social stress, childbirth and loss of closed ones (He *et al.*, 2021). Alcoholism and substance abuse are the additional factors that contribute to the onset of MDD (Evans *et al.*, 2021). Moreover, the utilization of various medications such as α-interferon, rimonabant, and isotretinoin leads to elevated risk of developing MDD (Pa, 2013).

### 2.3.2 Genetic and Epigenetic Basis

MDD exhibits a predominantly polygenic mode of inheritance, where numerous loci with minor effects interact both among themselves and with environmental triggers as depicted by family and twin studies (Shadrina *et al.*, 2018). Studies involving twins and similar research indicate a moderately heritable component (37%) for MDD, ranging from 26% to 49%. (Calker & Serchov, 2021). The most extensive Genome-Wide Association Study (GWAS) conducted on depression until now, involving more than 1.2 million Africans and Europeans, found out that 178 loci were associated with genetic risk . Moreover, 223 independent SNPs were linked to MDD (Levey *et al.*, 2020). Based on single nucleotide polymorphisms (SNPs), MDD heritability was calculated as approximately 11.3% which encompass the biological functions such as the assembly and functioning of synapses and nervous system development. The key genes associated with MDD encompass LAMB2 (Laminin Subunit Beta 2), which maintains cellular adhesion and embryonic development; neuronal RNA-binding proteins such as CELF4 that is involved in maintaining synaptic plasticity and neuronal transmission (Wagnon *et al.*, 2012); FADS1 (Fatty acid desaturase 1), participating in fatty acid regulation; NEGR1 (Neuronal Growth Regulator 1), governing dendritic maturation and synapse number (Noh *et al.*, 2020); DRD2 (Dopamine D2 Receptor), regulates pruning of the synapses and activation of mTOR (Zhang *et al.*, 2021); CCDC71 (Coiled-Coil Domain Containing 71), involved in metabolism of lipids; proteins involved in glycosylation processes like SPPL3 (Signal peptide peptidase-like 3) (Levey *et al.*, 2021).

Environmental stressors and these genetic variants together may promote subtle modifications at the organelle, cellular and even physiological levels. This interplay has the potential to heighten an individual's susceptibility to stressful events in the future.

Several epigenetic modifications in MDD have been documented, including the DNA methylation of target genes associated with the projection, formation, and plasticity of neuronal circuits (Starnawska *et al.*, 2019). For example, histone deacetylase 4 hypermethylation aligns with its role in dendritic branching, neuronal morphology (Litke *et al.,* 2018) and hippocampal-dependent learning and memory (Kim *et al.,* 2012). Additional epigenetic modification mechanisms comprise non-coding RNAs and modifications in histone protein (Deussing & Jakovcevski, 2017). Notably, miR-132 which is among the prominently upregulated microRNAs in MDD across various studies (Homorogan *et al.,* 2021), acts as a synaptic plasticity regulator (Xu *et al.,* 2019).

### 2.3.3 Diagnosis of MDD

The diagnostic criterion DSM5 was developed by American Psychiatric Association . It is used by mental health practitioners for MDD diagnosis (Guha, 2014). An overall physical examination is conducted, along with an inquiry about the patient's health by the mental health professional and underlying medical conditions which could contribute to MDD are identified. This is followed by a psychiatric assessment in which the patient must provide detailed responses regarding symptoms, thoughts, emotions, and behavioral patterns. According to DSM5 criteria, MDD is characterized by an individual enduring a persistently diminished mood or is unable to take pleasure from enjoyable routine tasks for a minimum period of two weeks. Additionally, the diagnosis is also made under the condition that the specified symptoms, including disturbances in sleep, energy levels, appetite, focus, and self-esteem are predominantly present (Dubovsky *et al.*, 2021).

## 2.4 Treatment of MDD

Several pharmacological and non-pharmacological interventions can help in alleviating the symptoms of MDD. Psychotherapy which includes Interpersonal Therapy and Cognitive Behavioral Therapy (CBT) is an effective intervention along with pharmacological treatment (Park *et al*., 2019). Transcranial management stimulation and Electroconvulsive Therapy (ECT) can be used for managing severe cases of MDD (Li *et al*., 2021). A 30% of those individuals undergoing appropriate pharmacological treatment achieve complete symptomatic recovery. Whereas 70% of patients with MDD often exhibit a partial or poor response to medication without achieving full remission or do not show response at all, leading to the onset of Treatment-Resistant Depression (TRD) (Caldiroli *et al.*, 2021).

The pharmacological treatments employed for managing MDD exhibit variability and can be categorized using various criteria. This includes classification based on the chemical structure of antidepressants or more importantly, based on their pharmacological mechanism of action. Presently, antidepressants in use operate through thirteen distinct mechanisms (Fasipe, 2018). Notably in 2020, a novel class of antidepressants was identified, introducing an additional mechanism of action, thereby expanding the total number of antidepressant drug classes to fourteen (Fasipe *et al*., 2020). Out of these fourteen classes, Monoamine Oxidase (MAO) inhibitors, tricyclic antidepressants (TCAs), Selective Norepinephrine Reuptake Inhibitors (SNRIs) and SSRIs are usually prescribed due to their better tolerability and efficacy.

### 2.4.1 Selective Serotonin Reuptake Inhibitors

SSRIs inhibit the serotonin reuptake into serotonergic neurons, consequently boosting the concentration of serotonin in presynaptic terminals. In comparison to TCAs, SSRIs

offer superior tolerability, minimal anticholinergic effects, lower incidences of seizures, and enhanced safety for both the cardiovascular system and instances of overdosage (; Wang *et al*., 2018; Ramachandraih *et al*., 2011). Furthermore, the selectivity of SSRIs for inhibiting serotonin reuptake over norepinephrine reuptake is notably higher, ranging from 20 to 1500 times, as reported by Hillhouse & Porter, 2015. Side effects accompanied with SSRIs involve increased risk of bleeding, hyponatremia (manifested as drowsiness, dizziness, confusion) as well as sexual dysfunction (amenorrhea, decreased libido). SSRIs are also associated with the gastrointestinal (Wang *et al*., 2018) and sleep disturbances (Hillhouse & Porter, 2015). SSRIs, while effective in their action on serotonergic neurons, also carry the risk of causing serotonin syndrome characterized by the excessive activation of serotonergic neurons, both centrally and peripherally (Ramachandraih *et al*., 2011). The condition leads to heightened neuromuscular and autonomic activity, hypotension, tremors, agitation, confusion, and alterations in mental state (Rush *et al.*, in 2011). Importantly, serotonin syndrome has the potential to be life-threatening and can occur either due to SSRIs' overdose or interactions with other drugs (Agius & Bonnici, 2017; Bodner *et al*., 1995). The discontinuation of SSRIs can lead to a pronounced discontinuation syndrome. Common symptoms experienced after stopping the use of SSRIs include headaches, sweating, dizziness, fatigue, emotional blunting and anxiety (Agius & Bonnici, 2017; Marazziti *et al.*,2019).

### 2.4.2 Fluoxetine

Fluoxetine (FXT) was the first SSRI produced and marketed to treat MDD and is usually the 1st line of treatment for depressive disorders owing to its relative safety for pregnant women, elderly, children and adolescents (Rossi *et al*., 2004). FXT has a shorter half-life of 1-4 days. After that it breaks down into several metabolites *e.g*.,

Norfluoxetine which has a comparatively greater half-life of 7-10 days. FXT is lipophilic so it can cross BBB where it elicits its therapeutic effects (Caballero *et al*., 2014). Chronic FXT treatment improves anxiety, depression and cognition (Caballero *et al*., 2014). FXT is also prescribed for several other neuropsychiatric disorders such as premenstrual dysphoric disorder, panic attacks, bulimia nervosa and obsessive-compulsive disorder (OCD). FXT decreases inflammation by decreasing proinflammatory cytokines. Studies have shown that chronic FXT use can enhance neuroplasticity and neurogenesis (Almeida *et al*., 2020).

Side effects of using FXT include diarrhoea, insomnia, headaches, nausea, weight changes, sexual dysfunction, agitation, aggression and vomiting. After initial treatment with FXT, cognitive decline and suicidal ideation have been reported (Rossi *et al*., 2004). Another side effect that is rare with other antidepressants is akathisia which is a movement disorder characterized by restlessness and inability to stay still (Boyer & Shannon, 2005). Hyponatremia (low concentration of sodium in serum), platelet dysfunction (Carvalho *et al*., 2016), increased bleeding risk, serotonin syndrome (Dantzer *et al*., 2008) are also the reported side effects of FXT. Frequent and persistent adverse effects require additional treatment. This leads to the increased treatment cost and non-compliance rate and eventually cause discontinuation syndrome. Non-adherence to antidepressants can explain why patients do not achieve full remission (Cuevas *et al.*, 2016) and 50% of them experience recurrence after recovery (Solomon *et al*., 2000). Owing to these side effects, FXT is only prescribed by practitioners when faced with cases of moderate and severe depression. In research studies, it is used as a standard drug against which other antidepressant drugs are assessed.

## 2.5. Ascorbic Acid

AA or vit C is a water-soluble vitamin and a potent anti-oxidant. Organisms can consume vit C as in reduced form, which is identical to AA and occurs in its anion form as ascorbate ion under physiological pH, or in oxidized form which is dehydroascorbic acid (DHA) resulting from the oxidation of AA (Du *et al.,* 2012). It is a hydrophilic molecule. BBB and Blood-Cerebrospinal Fluid barrier restrict the AA entry in brain (Nualart *et al.,* 2014). In body, its uptake is governed by two transporters: the sodium-dependent Vit C transporter type 1 (SVCT1) and type 2 (SVCT2) (Corpe *et al.,* 2010). AA is crucial for the proper nervous system functioning. It plays a vital role in brain because it serves as an antioxidant defense molecule. In addition to its antioxidant function, AA participates in various non-oxidant processes, including the process of collagen synthesis and peptide hormones biosynthesis. An important role is also played by AA in the process of myelin formation. Eldridge *et al.,* 1987 reported another key role of AA in maintenance of neurotransmission and neuronal maturation .Studies have demonstrated that AA can alleviate seizure severity and reduce seizure-induced damage (Warner *et al.,* 2015). Conversely, in premature infants, the disruption of AA transport is a contributing factor to brain damage (Nyborg *et al.,* 2012). Moreover, AA treatment mitigates the memory impairments as well as the neurodegenerative changes. This neuroprotective property was observed in animals exposed to neurotoxins *e.g.* aluminum and colchicine (Olajide *et al.,* 2017; Sil *et al.,* 2016). Vitamin C has an indirect impact on the oligomerization of α-synuclein (α-syn). Posttranslational modifications of α-syn such as oxidation and nitration lead to the α-syn oligomerization which contribute to Parkinson's disease (PD) pathology. In contrast, Vitamin C, acting as an antioxidant, is believed to counteract this effect, preventing the oligomerization of α-syn (Xiang *et al.,*2013; Krauss *et al.,* 2016). AA has shown decreased immobility

in TST in Swiss mice (Binfaré *et al.*,2009) and decreased immobility in FST in restraint stressed mice (Moretti *et al.*, 2013). Together these findings indicate that Ascorbic acid can be used as an antidepressant by targeting different pathologies associated with the disease, so this study will focus on the effects of AA in UCMS-induced depression model particularly targeting neurogenesis and ER stress.

## 2.6 Animal Models of Depression

Animal models offer a vital platform for exploring neural circuitry, as well as molecular and cellular pathways, that play a key role in the progression as well as the onset of depression. While skepticism initially surrounded use of animals as model of depression because of the existence of disparities in cognitive abilities between animals and humans, refinements over time have enabled these models to encompass various facets of depression and cognitive behaviors that mirror human experiences of depressive disorder (Hao *et al.*, 2019; Becker *et al*., 2021). Models used in research include Olfactory bulbectomy, Social defeat model, Learned helplessness, TNF alpha-induced depression, Corticosterone model and UCMS Model (Hao *et al*., 2019).

In 1982, Katz proposed the UCMS rat model of depression which was then modified further by Papp & Wilner (Willner, 2017) also has been validated in different mice strains now (Surget & Belzung, 2008). The Unpredictable Chronic Mild Stress (UCMS) procedure entails subjecting the animal to various mild stressors at unpredictable intervals over several weeks. Various behavioral alterations are produced as a result including anhedonia (the core symptom of depression) and apathy. The observed changes in behavior, along with modifications in specific endocrine and neural factors, bear resemblance to those seen in individuals experiencing MDD. Certainly, the UCMS model is noted for its closer resemblance to the chronic and multidimensional nature of

clinical depression. The application of chronic and unpredictable stressors in this model enhances its capacity to capture various aspects of the complexity observed in human depressive conditions (Mineur *et al*., in 2006). Similar to the clinical condition, these behavioral changes can be reversed using a broad range of antidepressant drugs employing various mechanisms of action (Willner,2017). The UCMS exhibits construct, face, and predictive validity. It is one of the few models where the effectiveness of monoaminergic antidepressant administration is observed in chronic rather than acute conditions (Belzung & Lemoine, 2011; Willner, 2017).

# MATERIAL AND METHODS

## 3.1 Chemicals

Ascorbic Acid was purchased from Sigma Aldrich (Lot#03250). Sold under the brand name PROZAC® , Fluoxetine (Fluoxetine Hydrochloride) was purchased from local pharmacy in Islamabad (manufactured by Eli Lilly, USA). Sucrose (lot#10A0391127D) was purchased from Phytotechnology Laboratories. Chemicals required for RNA extraction and qRT-PCR were obtained from Sigma Aldrich, Merck and ThermoFisher Scientific, USA.

## 3.2 Animals

Male BALB/c mice aged 6 - 8 weeks weighing 25-35 g were procured from and housed in Laboratory Animal House Facility of ASAB. The mice were kept under standard conditions (23-27 ºC) . Maintained on a natural12 hr light/12 hr dark cycle, the subjects were given access to food and water *ad libitum*.

## 3.3 Ethical statement

Institutional Review Board (IRB), ASAB, NUST approved the procedures carried out during the research (IRB number 01-2023-ASAB-01/01). The procedures were carried out ensuring the minimal harm to animal subjects according to the guidelines of Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health USA.

## 3.4 Unpredictable Chronic Mild Stress (UCMS) procedure

For UCMS imposition, mice were subjected to a series of stressors that were mild. To prevent habituation, the presentation of stressors was randomized in terms of both timing and order. This also ensured that the procedure remains unpredictable for the animal subjects. Two stressors were applied each day, five days per week for a time period of 9 weeks (Surget *et al.,*2008; Vargas *et al*., 2008). Stressors included cage tilt at 45˚ with bedding, cage tilt without bedding, substitution of bedding with 1cm high water level, soiled bedding (addition of rat feces to the cage), no bedding (removal of sawdust), overnight illumination, predator sounds (15 minutes), cold swim (5˚C for 2 mins), restraint stress (3-4 hrs) (Chevalier *et al.,* 2020; Alves *et al*., 2018). Mice were provided with unrestricted access to food and water in consideration of ethical principles (Table 3.1).

To evaluate the UCMS-induced depression, coat state was evaluated mid-weekly from seven body parts *i.e.,* head, forepaws, neck, ventral coat, hind paws, dorsal coat and tail. It is a pharmacologically validated index of depressive state in rodents. Mice were scored 0 for a well-groomed smooth fur (shiny fur visible with no spiky patches), 0.5 if the coat was slightly deteriorated (fluffy fur with minor spiky patches) and 1 if a significant deterioration (dirty, unkempt fur with slight staining/yellowing) was observed (Figure 3.1). The cumulative score was obtained by addition of the score of each body part (Farooq *et al.,* 2012).

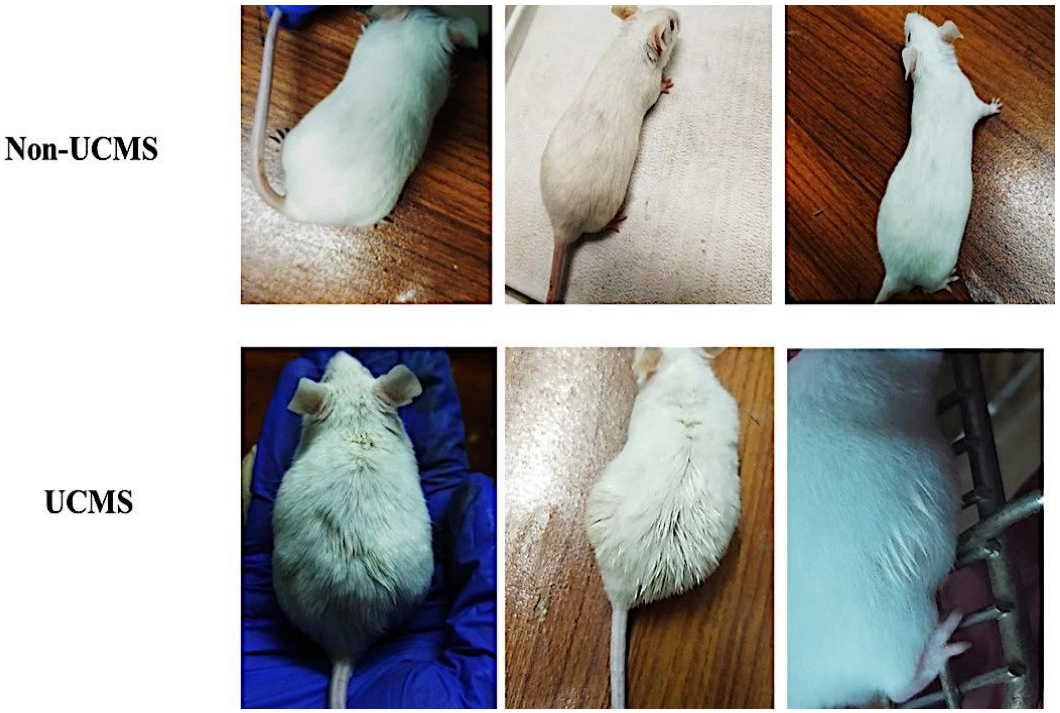


**Figure 3.1 Coat State of Healthy Mice and Mice Exposed to UCMS.** Staining of coat and spiky patches are visible in UCMS exposed mice. (photographs taken at fifth week of UCMS)**.**

**Table 3.1 Schedule for imposition of UCMS protocol**

| | Day 1 | | Day 2 | | Day 3 | | Day 4 | | Day 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| **WEEK 1** | No bedding | Cage tilt with bedding | Cold swim | Substitution of bedding with water | Restraint | Predator sounds | Addition of Rat Droppings | Damp Sawdust | Cage tilt without bedding | Constant illumination |
| **WEEK 2** | Predator sounds | Cold swim | Damp Sawdust | Cage tilt without bedding | Addition of Rat droppings | No bedding | Constant illumination | Substitution of bedding with water | Restraint | Cage tilt with bedding |
| **WEEK 3** | Damp Sawdust | Restraint | No bedding | Addition of Rat droppings | Cage tilt with bedding | Constant illumination | Cage tilt without bedding | Cold swim | Predator sounds | Substitution of bedding with water |
| **WEEK 4** | Cage tilt without bedding | Constant Illumination | Substitution of bedding with water | Predator sounds | Cold swim | Damp sawdust | Restraint | Cage tilt with bedding | No bedding | Addition of Rat droppings |
| **WEEK 5** | Substitution of bedding with water | Addition of Rat droppings | Cage tilt with bedding | Restraint | Constant Illumination | Cage tilt without bedding | Predator sounds | No bedding | Damp sawdust | Cold swim |
| **WEEK 6** | Damp Sawdust | No bedding | Predator sounds | Addition of Rat droppings | Substitution of bedding with water | Cold swim | Cage tilt without bedding | Constant illumination | Cage tilt with bedding | Restraint |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **WEEK 7** | Cage tilt without bedding | Predator sounds | Cage tilt with bedding | Constant illumination | No bedding | Addition of Rat droppings | Restraint | Substitution of bedding with water | Cold swim | Damp sawdust |
| **WEEK 8** | Substitution of bedding with water | Restraint | Damp Sawdust | Addition of Rat droppings | Cage tilt with bedding | Predator sounds | No Bedding | Cold swim | Constant illumination | Cage tilt without bedding |
| **WEEK 9** | Cage tilt with bedding | Addition of Rat droppings | Restraint | Cold swim | Constant illumination | Damp Sawdust | Cage tilt without bedding | Predator sounds | No Bedding | Substitution of bedding with water |

## 3.5 Experimental design

Animals were distributed into eight groups, n=6 for every group. Groups including group II, IV , VI and VIII were imposed with two or three stressors daily, five days a week during 9 weeks of UCMS. From week 7 to 9 (21 days), animals of Groups I and II received Saline (0.9 % NaCl) (10 ml/kg) (Moretti *et al*., 2015), Groups III and IV received FXT (20mg/kg) (Xie *et al.,* 2020), Groups V and VI received AA (10mg/kg) (Binfaré *et al.,* 2009) and Groups VII and VIII received FXT (20mg/kg) + AA (10mg/kg) of body weights respectively. The drugs were dissolved in saline and intraperitoneally administered. Behavior tests and histological examination were carried out afterwards. qRT-PCR was carried out for gene expression analysis.

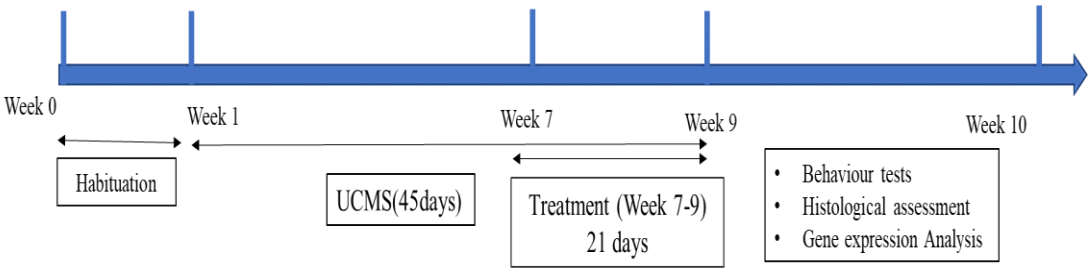


**Figure 3.2 Study design**

**Table 3.2 Experimental groups**

| Sr# | Groups | Treatment<br>Intraperitoneal administration<br>(10ml/kg of body weight) | Number of Animals |
|---|---|---|---|
| I | Saline | 0.9%Saline | 6 |
| II | UCMS | 0.9%Saline | 6 |
| III | FXT | FXT (20mg/kg) | 6 |
| IV | UCMS+FXT | FXT (20mg/kg) | 6 |
| V | AA | AA (10mg/kg) | 6 |
| VI | UCMS+ AA | AA (10mg/kg) | 6 |
| VII | AA+FXT | AA (10mg/kg) +FXT (20mg/kg) | 6 |
| VIII | UCMS +AA+FXT | AA (10mg/kg) +FXT (20mg/kg) | 6 |

## 3.6 Behavior tests

A battery of behavior tests was conducted for the assessment of depression and anxiety like behavior in experimental mice (Figure 3.3).

### 3.6.1. Sucrose Splash Test

It is used to assess motivation towards grooming and selfcare which is observed to be reduced in depressive disorders. Grooming typically commences with a sequence that involves paw-licking and nose-wiping, followed by the cleaning of the eyes and ears. Afterward, animals may proceed to mouth various parts of their torso, starting with the front and ending with the rear sections. The grooming behavior is common among animals and plays a crucial role in their selfcare and hygiene. Mice were placed in an arena (15cm×27cm×41cm). Sucrose solution (10 %) is sprayed onto the dorsal coat of mice 5 - 6 times ensuring equal coverage. Latency to start grooming, duration spent on grooming and the frequency of grooming were recorded under a time frame of 5 minutes (McCready *et al.*, 2023).

### 3.6.2 Novelty Suppressed Feeding Test

This conflict-based test relies on hyponeophagia phenomenon which states that the subject animal that has been deprived of food has to make a choice to approach and consume the food placed in a novel environment or avoid doing so due to anxiety. The reduction in eating behavior induced by exposure to a new environment is often viewed as a manifestation of anxiety-like behavior. This phenomenon is seen as indicative of the motivational aspect related to the desire for food and can be used to evaluate the comorbid anxio-depressive like phenotypes in rodents (Berridge & Kringelbach, 2015). The animals underwent a 24-hour food deprivation period, while water was still provided, before the commencement of the testing procedure. In an open field arena with dimensions (40cm×40cm×40cm), a food pellet was placed inside the box center onto a filter paper of diameter 10 cm. Mouse was placed inside the box at the corner and latency to consume food was observed for a duration of 10 minutes. Feeding was specifically characterized as the act of biting the food, rather than merely involving activities like sniffing or touching the food. The apparatus was cleaned after testing each animal subject (Chevalier *et al.*, 2020).

### 3.6.3 Y-Maze Test

Y-maze test was conducted to assess the short-term memory and spatial memory in mice. The Y-maze apparatus comprises of three rectangular arms usually named as start arm, other arm and the novel arm. Each arm lies at 120$^{o}$ from each other. Three different visual cues were hung at the walls of the maze. The apparatus was thoroughly cleaned after testing each animal. In the habituation phase (10 minutes), the animal explored the maze as it was placed firstly in the start arm. At this time, the novel arm was kept blocked. In the inter-trial phase that lasted for 10 minutes, the mice were put back inside

their home cages. During the trial period, the mice freely explored the three arms of the maze for a duration of 5 minutes as the novel arm was unblocked and the mice activity was recorded. Percentage spontaneous alterations, percentage entries in novel arm along with the cumulative time spent by the animal in novel arm were calculated (Conrad *et al.,* 1996).

$$\%\ Spontaneous\ Alterations = \frac{Number\ of\ spontaeneous\ alternations}{Total\ number\ of\ alternations\ -\ 2} \times 100$$

### 3.6.4 Tail Suspension Test

For the assessment of behavioral despair, helplessness and depression- like behavior in rodents, TST was performed. The protocol was adapted from Steru *et al.,* 1985 that states an animal when subjected to an inescapable stress for short term will try to escape the situation. The apparatus comprised of a 50 cm high wooden stand. With the help of an adhesive tape, the mouse tail was suspended via its tail. The tape was applied 2 cm from the tail tip. The adhesive tape used was strong enough to prevent the animals from falling down but not damaged the tail of the mice. The test lasted for a total of six minutes, but the immobility period was noted during the last 4 minutes as every mouse tries to escape in the initial 2 minutes. State of being complete motionless and passive hanging was considered as immobility period.

### 3.6.5 Forced Swim Test (FST)

This test is used for the assessment of depressive like state in rodents. It is a valid and reliable method for the screening of anti-depressant compounds. The FST is also known as the Porsolt Forced Swim Test (Porsolt *et al.,* 1997). The FST apparatus is basically an open cylindrical-shaped container with dimensions (diameter:10 cm, height :25 cm).

After filling with clean water (25 ºC±2 ºC), mice were forced to swim for a duration of 6 minutes. The water level adjustment was made so that the limbs of the mice do not touch the bottom of the container. The duration of immobility, number of immobile episodes as well as the latency to immobility was documented during the final 4 minutes of the trial. Immobilization of the animal was defined as the cessation of struggling, remaining completely motionless and just floating above water. The increased immobility time indicated the increased depressive like state.

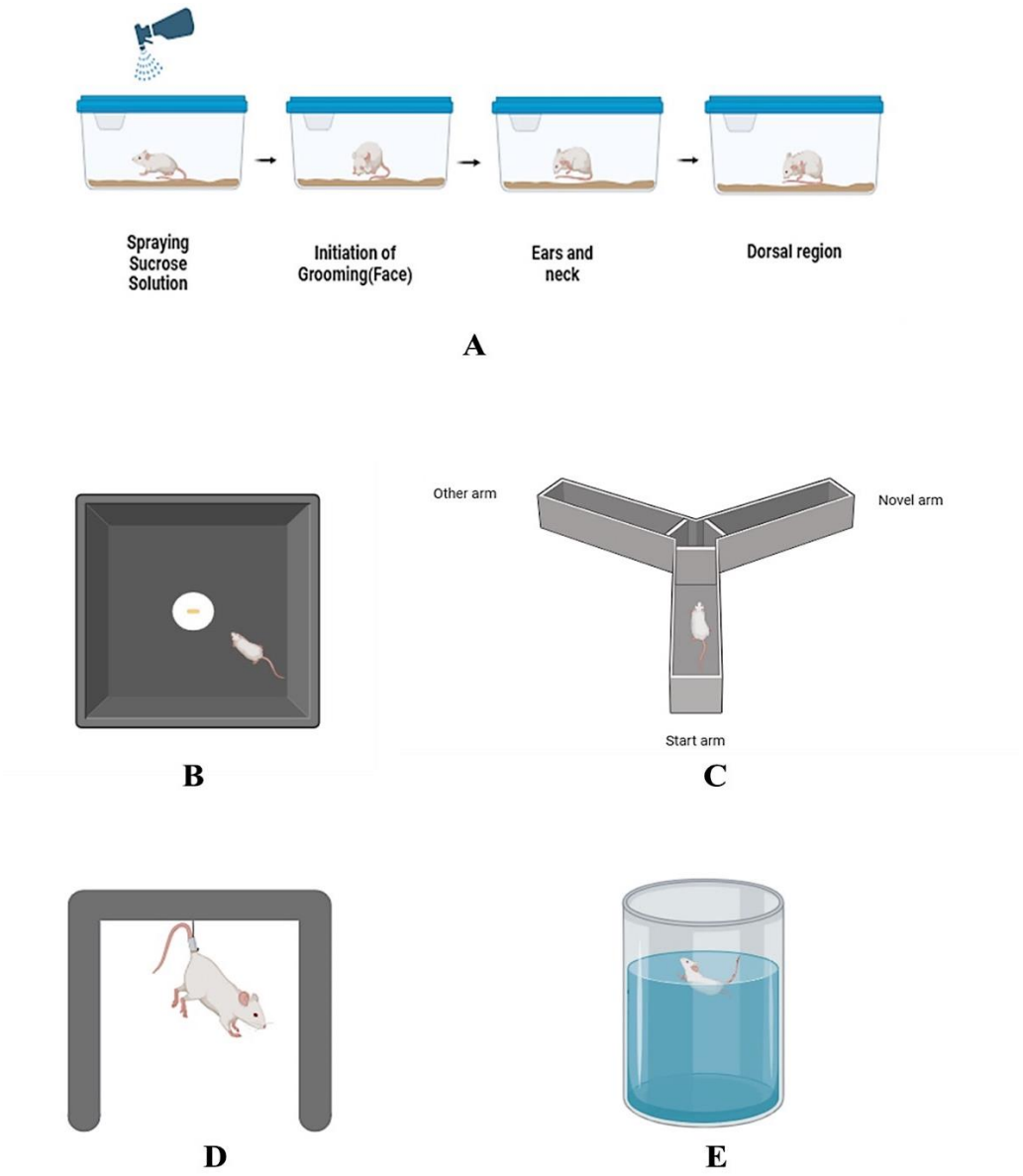


**Figure 3.3 Behavior tests battery (A)** Sucrose Splash Test, **(B)** Novelty Suppressed Feeding Test, **(C)**Y-Maze Test, **(D)** Tail Suspension Test, **(E)** Forced Swim Test, (Created in BioRender.com)

## 3.7 Dissections

The animals were anesthetized with chloroform and euthanized to collect brain. A cut was made behind the ears, and a small incision was then made along the sagittal suture which was then peeled off and brain was harvested without causing any damage. The brain tissue was then washed with pre-chilled 1X Phosphate Buffer Saline (PBS). Olfactory bulbs and cerebellum were removed. The cortical halves were separated mid-section to extract hippocampus. The tissue sections were transferred to labelled microcentrifuge tubes and stored at -80 ºC.

## 3.8 Histological Examination

### 3.8.1 Tissue preparation

For histological examination, whole brain was harvested, washed with 1×PBS and then transferred to 10% Formalin for 24 hours. It was dehydrated by treating it with increasing concentrations of isopropanol *i.e.,* 70 %, 95 % and 100 %, each for 1 hr followed by an incubation in xylene for 4 hours. Molten paraffin was then poured over the tissue for embedding, with temperature of 60 ºC. The embedded tissue was allowed to solidify at 4 ºC in a mold (block formation) before cutting.

### 3.8.2 Hematoxylin and Eosin Staining

For getting a detailed view of neuronal density and morphology, Hematoxylin and Eosin (H&E) staining was done. Tissue were sectioned (5 µm) from each group using SLE Mainz microtome CUT 6062 and then de-paraffinized. After this, tissues were incubated for 8 minutes in Mayer's hematoxylin solution. This was followed by 10

minutes washing in warm water, dipping in 95 % ethanol and then counterstaining with Eosin for 30 seconds.

### 3.8.3 Cell quantification

The prepared slides were visualized under light microscope OPTIKA ® ITALY B-150 LED at magnifications 4X, 10X and 40X. Images were captured using OPTIKA Vision Lite Version 2.11 software. Cells were quantified in the DG (Dentate Gyrus) region of the hippocampus. Three randomly selected areas, each measuring 10,000 $\mu m^2$, were chosen in the DG region, and cell counts were conducted. For each group, average of 6 tissue sections were evaluated.

## 3.9 Gene expression studies

### 3.9.1 RNA Extraction

For RNA extraction, Tri-reagent protocol was followed. Tissues samples were homogenized in 1 ml Trizol (Invitrogen; LOT# 00975289) and then incubated at 4 ºC (15 minutes). Trizol hinders the actions of RNases, preserving RNA integrity. Additionally, during the homogenization step, it disrupts cells and dissolves their components. For phase separation of RNA and proteins in tissue homogenates, 200 µl of Chloroform (Sigma Aldrich) was added, shaken for 15 seconds, and subsequently incubated at 4 ºC for 10 minutes. At a temperature of 4 ºC, the samples were further centrifuged at 12,000rpm for 15 minutes. This led to the formation of three phases: the lower phase (pink), the interphase (phenol-chloroform), and an aqueous colorless upper phase (containing RNA). This upper phase, constituting approximately 60 % of the TRIZOL reagent used per homogenate, was carefully collected without disrupting the interphase and transferred to a new, pre-labeled, pre-chilled Eppendorf tube. For RNA

precipitation, 500µl of ice-cold isopropanol (Sigma Aldrich) was added and then incubated for 10 minutes. Centrifuged was done again at 12,000 rpm (10 minutes). At this stage, RNA pellet was visible at the bottom or side of the Eppendorf. Without disturbing the pellet, the supernatant was discarded carefully. To wash the pellet, 1ml absolute ethanol was added to the samples. At 7500x*g*, centrifugation was done for 5 minutes at 4 ºC. The left-over ethanol was delicately removed after the washing process to prevent any disturbance to the pellet. Subsequently, to the pellet, 30 µl DEPC water was added after allowing it to air dry. RNA was then stored at -80 °C. The RNA concentration was assessed using the nanodrop method utilizing BioPhotometer Plus (Eppendorf, Germany), with the A 260/280 ratio serving as an indicator of the extracted RNA quality. The A 260/280 ratios fell within the range of 1.8-2.0. A ratio below 1.8 suggested the presence of contaminants.

### 3.9.2. Reverse Transcription Polymerase Chain Reaction (RT-PCR) employed for cDNA synthesis

RNA (3 µg) from each sample was utilized. The reaction mixture comprised 2µl of 10mM dNTPs, 4µl of 5X RT buffer, 1µl of 10mM oligodTs (pre-heated at 55 °C for 5 minutes), and 1µl of RevertAID (Thermofisher Scientific) enzyme was added at the end. The total reaction volume was made 25 µl by adding NFW (nuclease free water) in the mixture. The sample mixture was placed inside the PCR machine at temperature profile mentioned in Figure 3.4. The prepared cDNA samples were stored at -20 degrees Celsius.

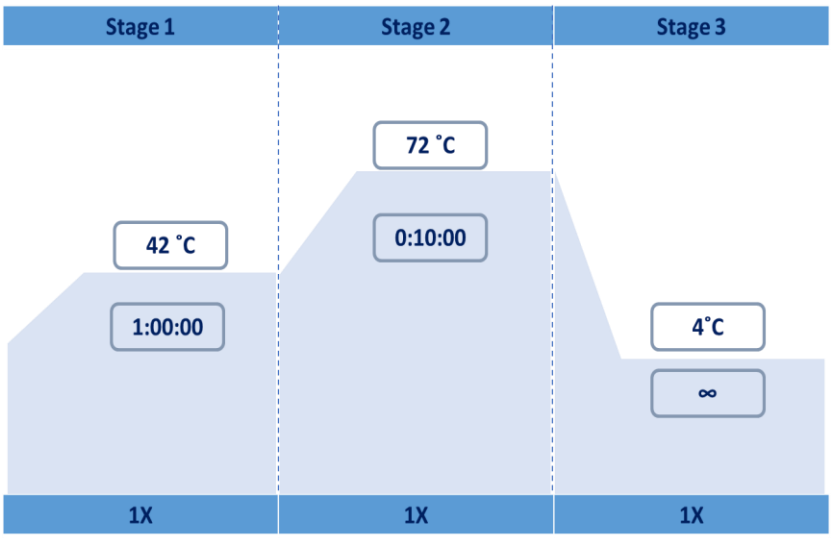


**Figure 3.4 Thermocycling conditions for cDNA synthesis**

### 3.9.3 Gene expression analysis using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Expression analysis was conducted for ATF6 and NeuN in the hippocampus. The normalization was performed using β Actin as the housekeeping gene. For qRT-PCR, the reaction volume was adjusted to 20 ul. A master mix with a ratio of 13:1:1 was prepared using PCR water, forward and reverse primers. Subsequently, a volume of 15 µl of this master mix was added to each vial in the PCR strips. To this, 1 ul of cDNA template and 4 ul of Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher Scientific) were added, followed by a 10-second brief spin. The strips were promptly transferred to the PCR machine, and the profile was configured for real-time amplification. The total reaction time was 1 hr and 56 minutes, as depicted in Figure 3.5. Primer sequences are mentioned in Table 3.2.

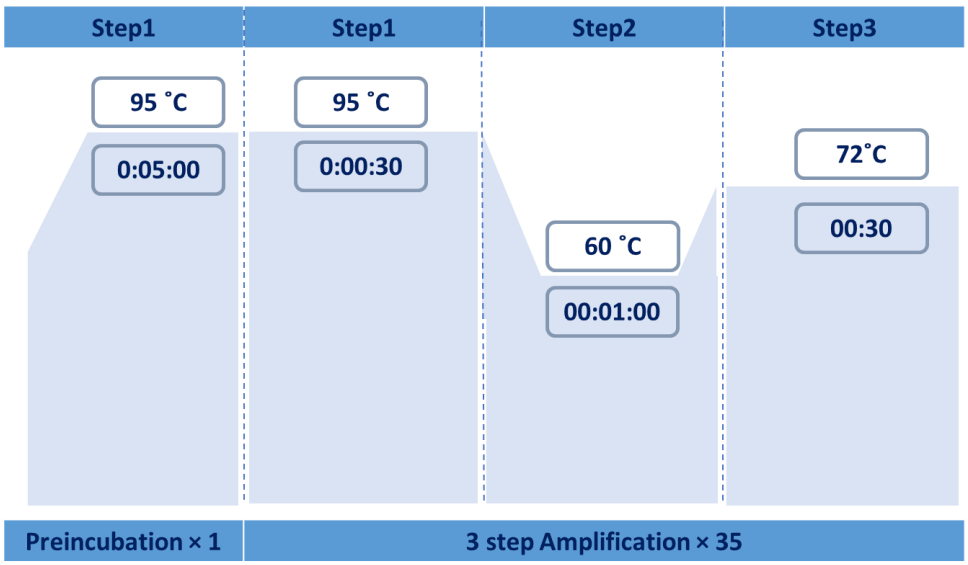


**Figure 3.5 Real Time qPCR profile for gene expression quantification of NeuN and ATF6**

**Table3.2: Primer Sequences**

| Primer | | Sequence |
|---|---|---|
| β-actin | Forward | 5′-GACGGCCAAGTCATCACTATT-3′ |
| | Reverse | 5′-CCACAGGATTCCATACCCAAGA-3′ |
| ATF6 | Forward | 5′-TGCCTTGGGAGTCAGACCTAT-3′ |
| | Reverse | 5′-GCTGAGTTGAAGAACACGAGTC-3′ |
| NeuN | Forward | 5′-GGCAATGGTGGGACTCAAAA-3′ |
| | Reverse | 5′-GGGACCCGCTCCTTCAAC-3′ |

## 3.10 Statistical Analysis

The data were analyzed statistically using Graph Pad Prism 8.0.1. To analyze the statistical significance of the data, one-way and two-way ANOVA were applied accordingly. Bonferroni's multiple comparison test was applied afterwards. A p-value less than 0.05 was deemed statistically significant. Mean±SEM (Standard Error of Mean) represented the data.

## 3.11 *In-silico* Analysis

To analyze the receptor ligand interaction geometrics, molecular docking analysis was performed.

### 3.11.1 Ligand and receptor structure retrieval

PubChem database ( https://pubchem.ncbi.nlm.nih.gov ) was employed for retrieving the 3D conformer ligand structures (Kim *et al.,* 2023) and saved in SDF format, Fluoxetine hydrochloride (CID: 62857) ; AA (CID: 54670067). For obtaining the crystal structures of NeuN (Uniprot ID: A6NFN3) and ATF6 (Uniprot ID: P18850), the respective Uniprot IDs ( http://www.uniprot.org/ ) (Hinz, 2010) were entered in Alphafold protein structure database ( https://alphafold.ebi.ac.uk/ ) and the required structures were downloaded in PDB format (Jumper *et al* ., 2021). Bound ligands and all the non-standards were removed using UCSF Chimera 1.17.3 and the isolated structures were then downloaded in PDB format ( https://www.cgl.ucsf.edu/chimera/ ) (Pettersen *et al*., 2004).

### 3.11.2 Binding site detection

The PDB structures were uploaded on ProteinsPlus ( https://proteins.plus/ ) and DoGSiteScorer (Volkamer *et al.*, 2010) function was used to detect the best binding site of protein based on its structure. The highest drug score was selected for each protein and amino acid residues were recorded for defining the binding pocket.

### 3.11.3. Molecular docking

Molecular docking was performed using PyRx 0.8 ( https://pyrx.sourceforge.io/ ) which uses Autodock for docking (Dallakyan & Olson , 2015). Crystal structures of proteins and ligands were loaded in the PyRx workspace and the binding pockets were defined using grid. Docking was performed, binding affinities were recorded and output files were saved in pdbqt format. The interactions were then visualized in BIOVIA Discovery studio 21.1.0. ( https://www.3ds.com/products/biovia/discovery-studio/visualization ) (Systèmes, 2016). Interaction geometrics, hydrogen bonds and interacting amino acid residues were recorded after the docking.

# RESULTS

## 4.1. Effect of UCMS-induced depression on coat state

Coat scoring was done weekly for the assessment of depression as this parameter is a pharmacologically validated index. The higher score indicated more deterioration of the coat. From week 1 - 3, a non-significant difference between the UCMS and non-UCMS exposed mice was observed. However, a significant difference in coat state was observed from week 4 - 9 between the control and UCMS-exposed group (for week 4 $p<0.001$, for week 5 to 9, $p<0.0001$) (Figure 4.1). The coat score decreased as the treatment commenced, and a noteworthy difference between the UCMS and treated groups was noted in the final week of the treatment. For UCMS+FXT (0.66±0.10) ($p<0.05$), UCMS+AA (0.5±0.10) ($p<0.001$) and for UCMS+FXT+AA (0.500±0.129) ($p<0.001$).

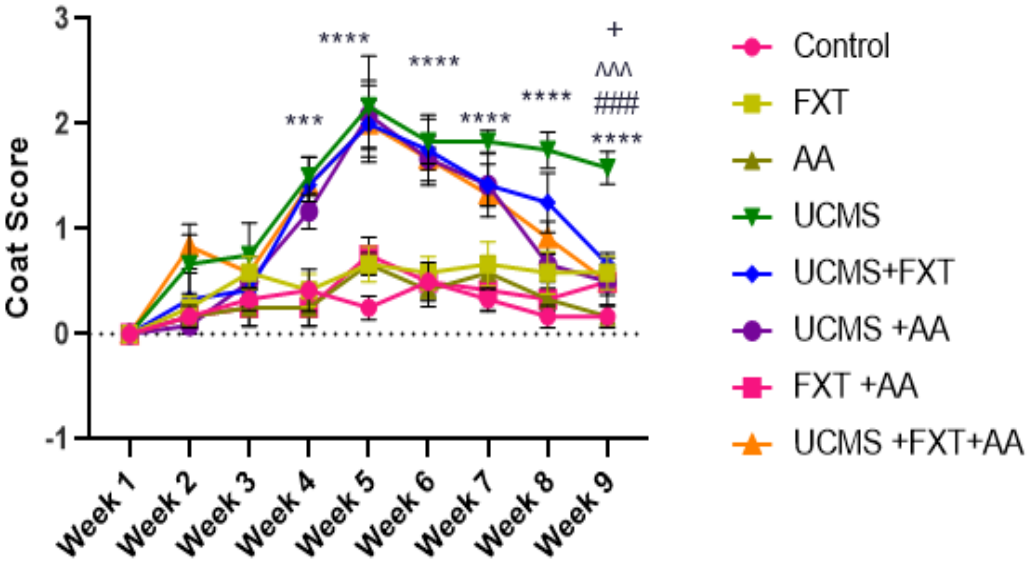


**Figure 4.1 The effect of UCMS on coat state deterioration**. Graphpad Prism version 8.0.1 was utilized for statistical analysis. Mean±SEM represent the error bars. Two-way ANOVA test was applied. *, +, # and ^ represent statistical significance between control versus UCMS, UCMS versus UCMS+ FXT, UCMS versus UCMS+ AA and UCMS versus UCMS+FXT+AA ("n.s =$p > 0.05$, *=$p< 0.05$, **=$p < 0.01$, ***= $p < 0.001$ and **** =$p < 0.0001$").

## 4.2. Behavior analysis

### 4.2.1 Effect of Ascorbic acid on grooming behavior

SST was used for the evaluation of depression-induced anhedonia deficit which is the hallmark symptom of depression. Animals that are more depressed start their grooming later as compared to the healthy mice, do less frequent grooming and spend less time on grooming. UCMS mice showed significantly($p<0.0001$) increased latency (9±2.01s) in comparison to the control group (2±0.51s). UCMS+FXT (3.5±0.88s) ($p<0.01$), UCMS+AA (3±0.77s) ($p<0.001$), and UCMS+FXT+AA (4.66±0.21s) ($p<0.05$) showed significantly reduced latency to groom indicating the reversal of anhedonia in UCMS mice.

Time spent on grooming is another parameter that was recorded which is also an indicative of anhedonia in UCMS mice. UCMS displayed significantly reduced ($p<0.001$) grooming time (85.33±18.61s) as compared to the control group (185.8±9.10s). UCMS mice when treated with FXT spent significantly more ($p<0.05$) time (161±10.99), on grooming. Similarly, UCMS+AA (189.5±19.67s) ($p<0.001$), and the mice that received combinational treatment UCMS+FXT+AA (195.5±15.31s) spent significantly more time on grooming in contrast to the UCMS-exposed mice ($p<0.001$). However, there was no significant difference observed between the UCMS+AA and UCMS+FXT+AA groups in both parameters.

UCMS mice exhibited significantly less frequent grooming (12±1.77) as compared to the control group (23.67±2.140). However, UCMS+FXT, UCMS+AA and UCMS+AA+FXT had no significant impact on the frequency of grooming. UCMS mice exhibited significantly less frequent grooming (12±1.77) as compared to the control group (23.67±2.140). The UCMS+FXT, UCMS+AA and UCMS+AA+FXT had no significant impact on the frequency of grooming (Figure 4.2)

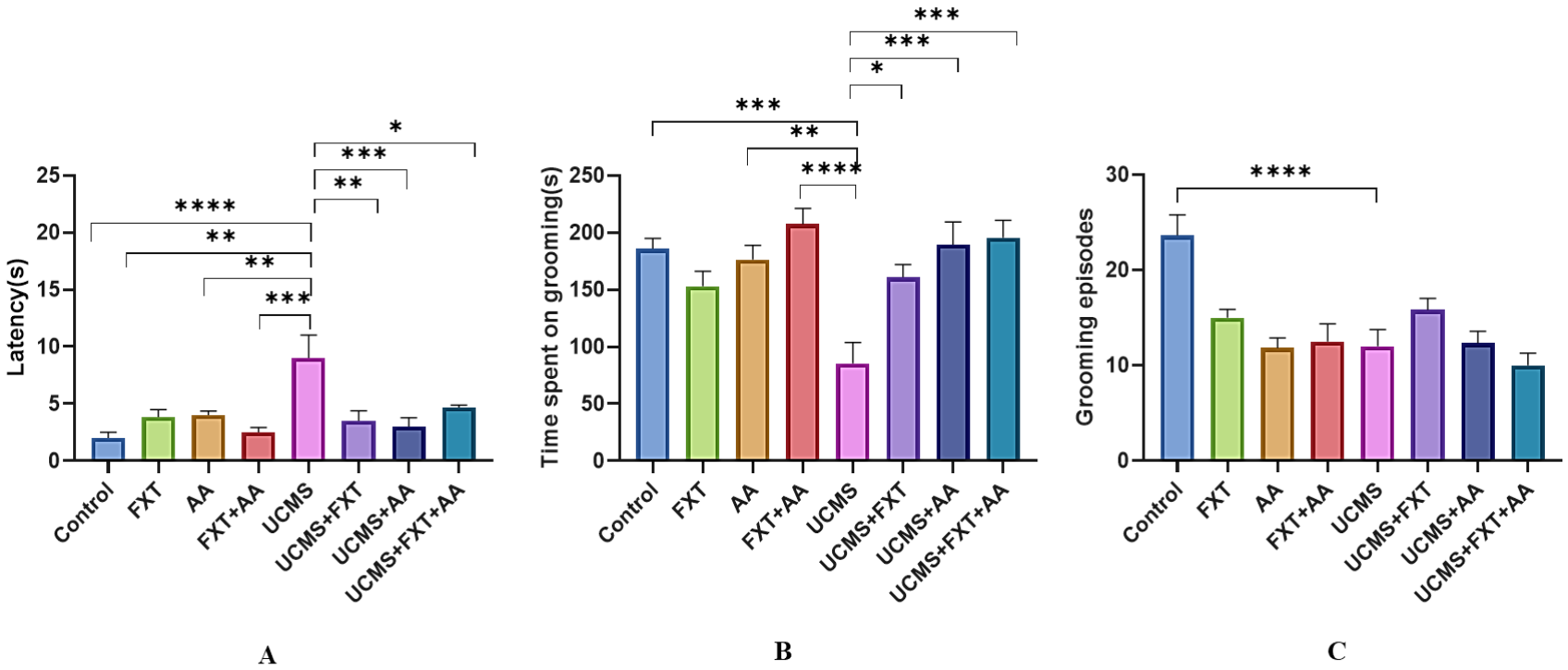


**Figure 4.2: The effect of Ascorbic acid on grooming behavior in Sucrose Splash Test (A)** Latency to Groom**, (B)** Grooming Time, **(C)** Grooming Episodes. Graphpad Prism version 8.0.1 was utilized for statistical analysis. The error bars were presented by Mean±SEM. ("n.s =p > 0.05, *=p< 0.05, **=p < 0.01, ***= p < 0.001 and **** =p < 0.0001"). The data underwent One way ANOVA test and Bonferroni's multiple comparison test.

### 4.2.2 Effect of Ascorbic acid on anxiety like behavior

NSFT was performed to investigate the mice's ability to explore the environment and eat the food pellet by overcoming the anxiety in novel environment. Mice that are more anxious, take more time to approach and eat the food pellet. The results of one-way ANOVA depicted that UCMS mice showed increased latency to eat food (185 ±65.8s) and hence increased anxiety in comparison to the control group (89 ±15.24s). This was a non-significant increase ($p>0.05$). UCMS+FXT (88±25.57s), UCMS+AA (80.33±11.37s) and UCMS+AA+FXT (116.3 ±31.59s) reduced the latency to eat food non significantly ($p>0.05$) elaborative of the fact that treatment reduced the anxiety levels (Figure 4.3).

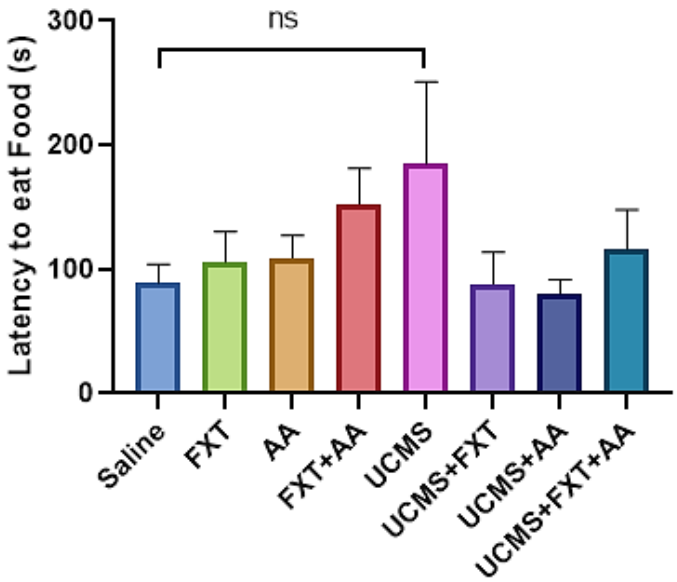


**Figure 4.3 The effect of AA on anxiety in NSFT,** Latency to consume food**.** Graphpad Prism version 8.0.1 was employed for statistical analysis. The error bars were presented by Mean±SEM. The data underwent One way ANOVA test and Bonferroni's multiple comparison test ("n.s =$p > 0.05$, *=$p< 0.05$, **=$p < 0.01$, ***= $p < 0.001$ and **** =$p < 0.0001$")

### 4.2.3 Effect of Ascorbic acid on short-term spatial memory in Y-maze test

To evaluate the spatial working memory as well as the exploratory behavior in UCMS-induced animals, Y-maze test was performed. Rodents tend to explore the novel environments as their innate behavior and in Y maze, an animal should enter the three arms consecutively which will be counted as a spontaneous alteration. Increased number of alterations show increased short term spatial memory.

UCMS mice displayed a non-significant decline in percentage spontaneous alterations (76.78±3.27) in contrast to the control group (88.5±5.31). An increase in percentage spontaneous alterations was exhibited by UCMS+FXT (82.23±4.72), UCMS+AA (92.95±3.58) and UCMS+AA+FXT (95.50 ±2.09) treated mice in a non-significant manner. UCMS-induced mice spent less time in novel arm (86.33±1.89s) as compared to control treated group (112.6±6.17s) but this decrease was non-significant ($p>0.05$). Similarly, less percentage entries in novel arm by UCMS mice (33.55±0.80) as compared to control treated group (36.03±0.97) was observed (Figure 4.4).

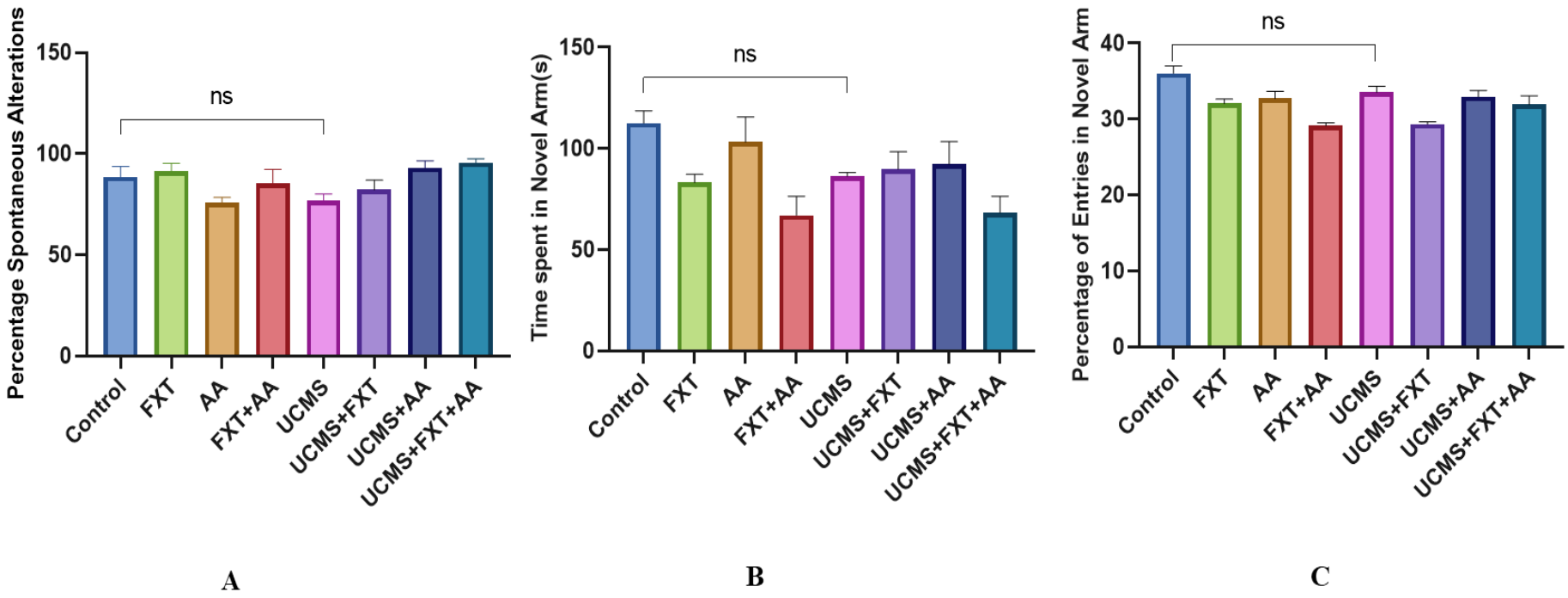


**Figure 4.4: The effect of Ascorbic acid on short-term spatial learning and memory in Y maze test (A)** Percentage Spontaneous alterations **(B)**Time spent in novel arm **(C)** Percentage of entries in novel arm. Graphpad Prism version 8.0.1 was used for statistical analysis. The error bars were presented by Mean±SEM. The data was tested using One way ANOVA test and Bonferroni's multiple comparison test afterwards ("n.s =$p > 0.05$, *=$p < 0.05$, **=$p < 0.01$, ***= $p < 0.001$ and **** =$p < 0.0001$")

### 4.2.4 Effect of Ascorbic acid on behavioral despair in UCMS-induced mice

The TST was conducted for investigation of the despair in mice and observe how mice behave when exposed to an inescapable stress. The more the mice remain immobile and do not struggle to escape, the more depressed they are. The group exposed to UCMS procedure remained immobile for significantly more($p<0.01$) time (187.7±8.56s) as compared to the control group (126.7±15.75s). UCMS+FXT (118±5.98s), UCMS+AA (93.67±3.24s) and UCMS+AA+FXT (127.5 ±4.29s) significantly displayed decrease in the immobility period ( $p<0.001$, $p<0.0001$ and $p<0.01$ respectively). The results indicated that FXT treatment and ascorbic acid treatment alleviate the behavioral despair. The combinational treatment (UCMS+FXT+AA) also significantly decreased the despair but did not potentiate the effects of FXT (UCMS+FXT) and AA (UCMS+AA) treatment (Figure 4.5).

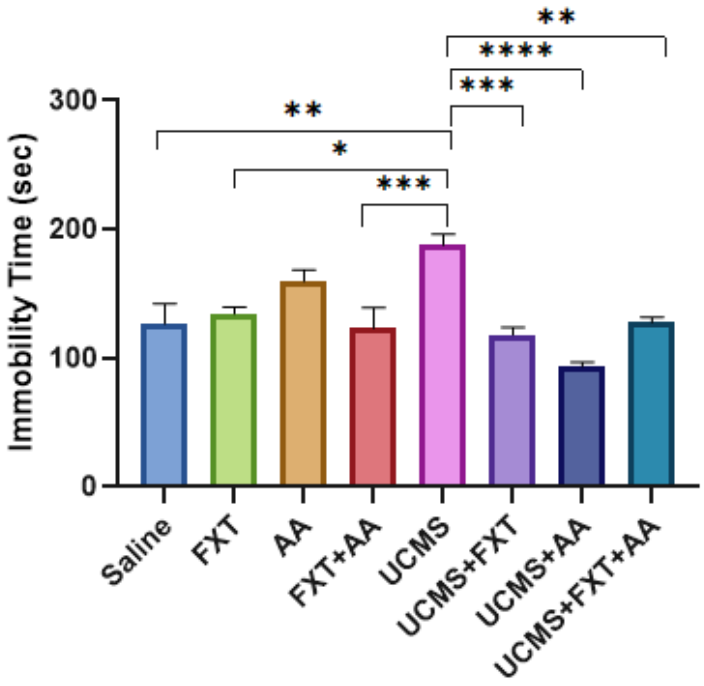


**Figure 4.5: The effect of Ascorbic Acid on Behavioral despair in TST**. Graphpad Prism version 8.0.1 was utilized for statistical analysis. The Error bars were presented by Mean±SEM . One way ANOVA test and Bonferroni's multiple comparison test were employed for the comparison ("n.s =$p > 0.05$, *=$p< 0.05$, **=$p < 0.01$, ***= $p < 0.001$ and **** =$p < 0.0001$")

## 4.5 Anti-depressant effect of Ascorbic Acid in UCMS-induced mice

To estimate the antidepressant effect of our compounds, Forced Swim Test was conducted. In this test, the mouse tends to struggle for swimming because rodents naturally exhibit an aversion to water. However, mice that are more depressed, make minimum or no movements to keep themselves afloat or only keep their head above water. Thus, immobility time, latency to immobility and number of immobile episodes were recorded.

UCMS group showed the least latency (4.66 ±1.33s) to immobility exhibiting the depressive behavior in UCMS mice in contrast to the control group (47±6.62s) ($p<0.01$). Treatment groups significantly alleviated the depression as the mice spent more time struggling to swim and hence the delayed immobility. UCMS+FXT

(46.17±6.81s) ($p<0.01$), UCMS+AA (42.67±8.98s) ($p<0.01$) significantly delayed the latency to immobility but UCMS+FXT+AA (14.83±5.30s) group did not increase the latency to immobility. The duration of immobility was documented as an indicator of depressive behavior. UCMS mice (124.3±10.16s) remained immobile during the swim for significantly longer ("$p<0.0001$") time when observed in comparison to the control group (11±2.43s). UCMS+FXT (38.5±4.16), UCMS+AA (46.67±10.43s) and UCMS+AA+FXT (35.5 ±7.5s) significantly decreased the immobility time, $p<0.001$ for each group.

Likewise, UCMS induced mice became immobile (19.5±1.80) more frequently (5.66±1.05) in comparison to the controls ($p<0.0001$). Groups treated with FXT (UCMS+FXT) (11.8±1.07), AA (UCMS+AA) (12.5±0.95) and UCMS+AA+FXT(8.6±0.84) exhibited the reduction in number of immobile episodes significantly ("$p<0.01$", "$p<0.05$" and "$p<0.0001$" respectively). However, the combination of FXT+AA showed the least number of the immobile episodes (8.66±0.84) in comparison to other treatment groups. No significant difference among the treatment groups was observed. These results indicate that FXT, AA and the combination of AA and FXT reduce the depressive behavior effects in induced via imposition of the UCMS procedure (Figure 4.6).

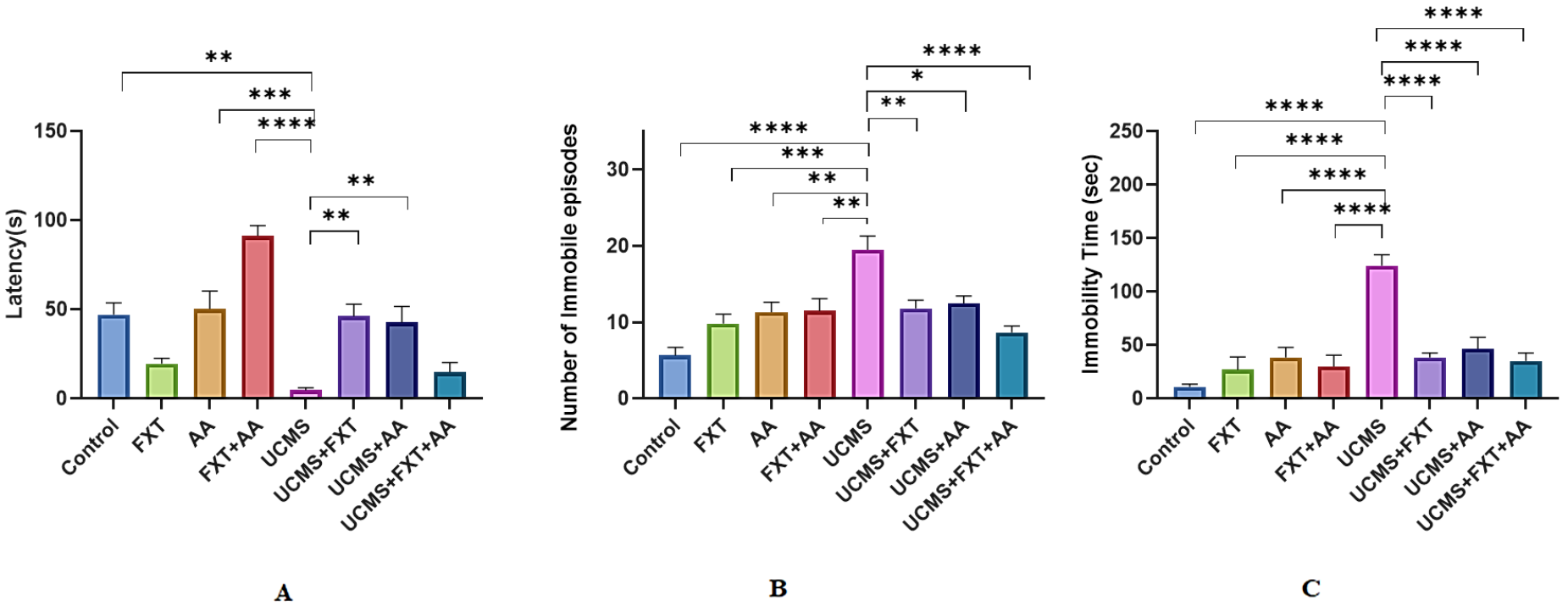


**Figure 4.6: The anti-depressant effect of Ascorbic acid on UCMS mice in FST (A)** Latency to immobility, **(B)** Immobile episodes, **(C)** Immobility time **.** Graphpad Prism version 8.0.1 was employed for statistical analysis. The error bars were presented by Mean±SEM ("n.s =$p > 0.05$, *=$p < 0.05$, **=$p < 0.01$, ***= $p < 0.001$ and **** =$p < 0.0001$"). The data underwent One way ANOVA test and Bonferroni's multiple comparison test afterwards.

### 4.3 Histological assessment of neuronal cell density post exposure to UCMS and treatment with Ascorbic acid

Hematoxylin and Eosin staining was done to assess the neuronal density. Histopathological assessment revealed a substantial loss of neurons in hippocampal DG region as comapred to the controls. Compact layers of cells were observed on 4X, 10X and 40X in the control treated group whereas reduction in cell layers and dispersed cells were observed in UCMS group. Administration of FXT, AA and the combination of AA+FXT showed increased cell layers as compared to UCMS group (Figures 4.8, 4.9 & 4.10) . Cell counting at 10X in the dentate gyrus revealed significant decrease ($p<0.0001$) in cell number in UCMS(8.75±1.01) in comparison to the control group (17.75±0.85). UCMS+FXT (24.5±0.86), UCMS+AA (27.5±1.04) and UCMS+AA+FXT(24±1.08) showed a significantly decreased neuronal loss as compared to UCMS group , $p<0.0001$ for each group (Figure 4.7).

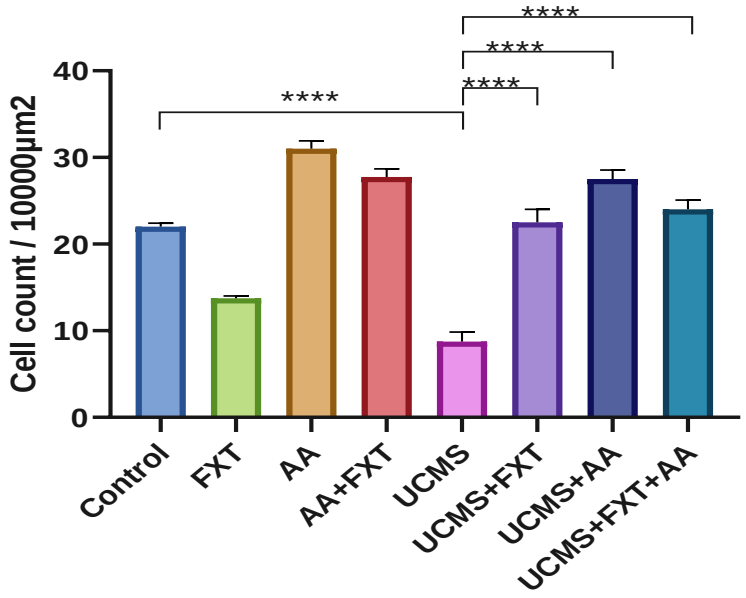


**Figure 4.7 The effect of Ascorbic Acid on neuronal cell count in DG (dentate gyrus) of UCMS-induced mice**. Cell count/10,000 µm$^2$ , Graphpad Prism version 8.0.1 was utilized for statistical analysis. The error bars were presented as Mean±SEM. ("n.s =$p > 0.05$, *=$p< 0.05$, **=$p < 0.01$, ***= $p < 0.001$ and **** =$p < 0.0001$").One way ANOVA test and Bonferroni's multiple comparison test were applied respectively.

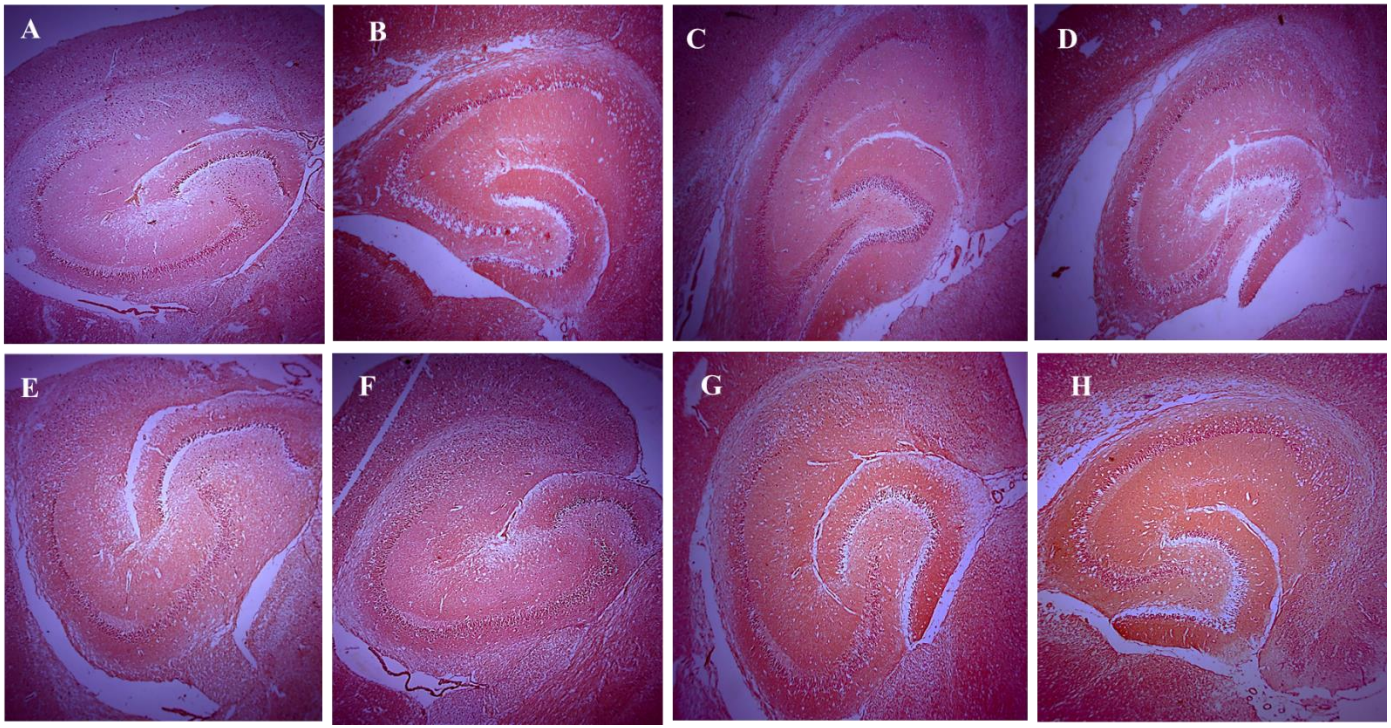


**Figure 4.8 H & E-stained coronal sections of hippocampus**. (A) Control (B) Fluoxetine (C) Ascorbic Acid (D) Ascorbic Acid+Fluoxetine (E) UCMS (F)UCMS+ Fluoxetine (G) UCMS+Ascorbic Acid (H) UCMS+ Ascorbic Acid+Fluoxetine. Magnification 4X.

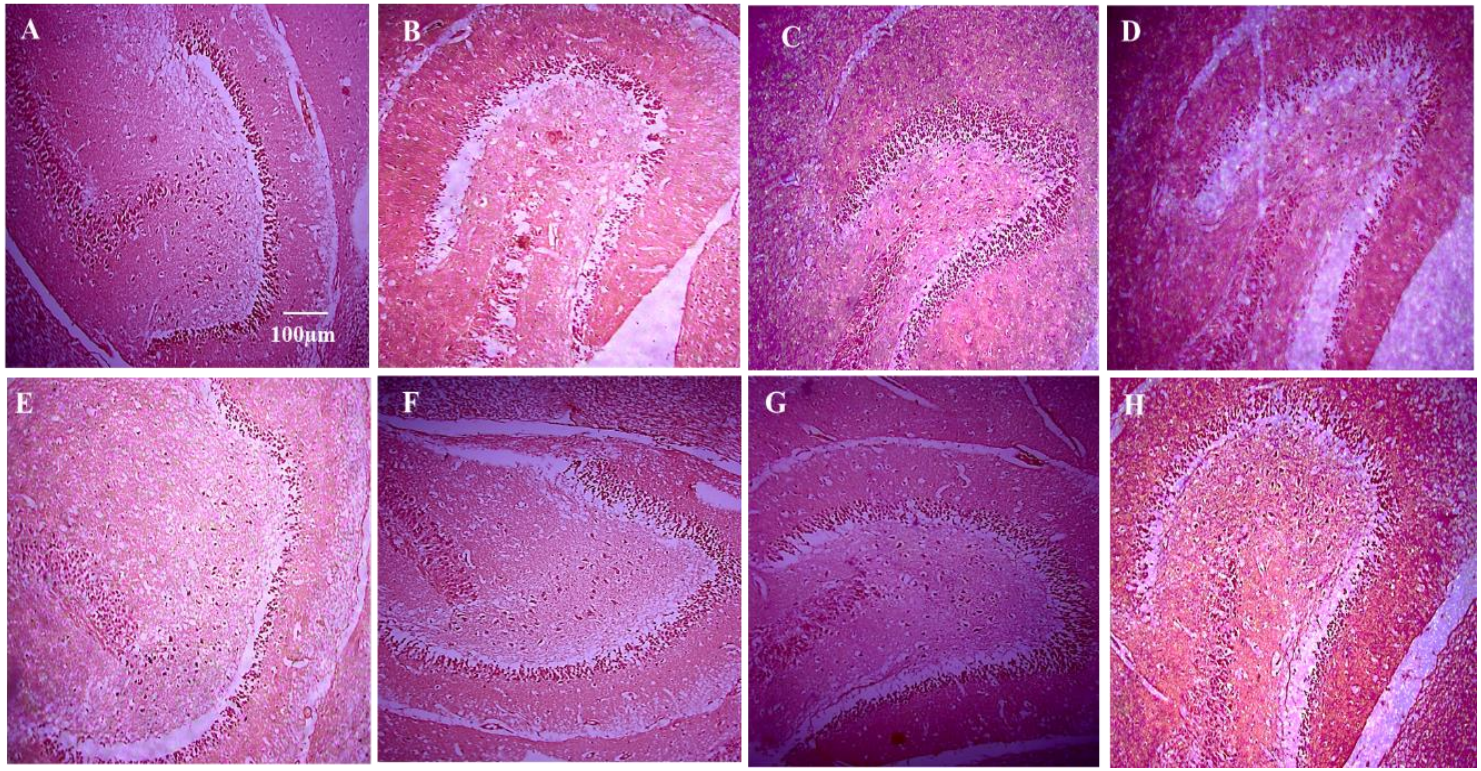


**Figure 4.9 H & E-stained sections of dentate gyrus (DG) region** (A) Control (B) Fluoxetine (C) Ascorbic Acid (D)Ascorbic Acid+Fluoxetine (E)UCMS (F)UCMS+ Fluoxetine (G)UCMS+ Ascorbic Acid (H) UCMS+ Ascorbic Acid+Fluoxetine. Magnification 10X.

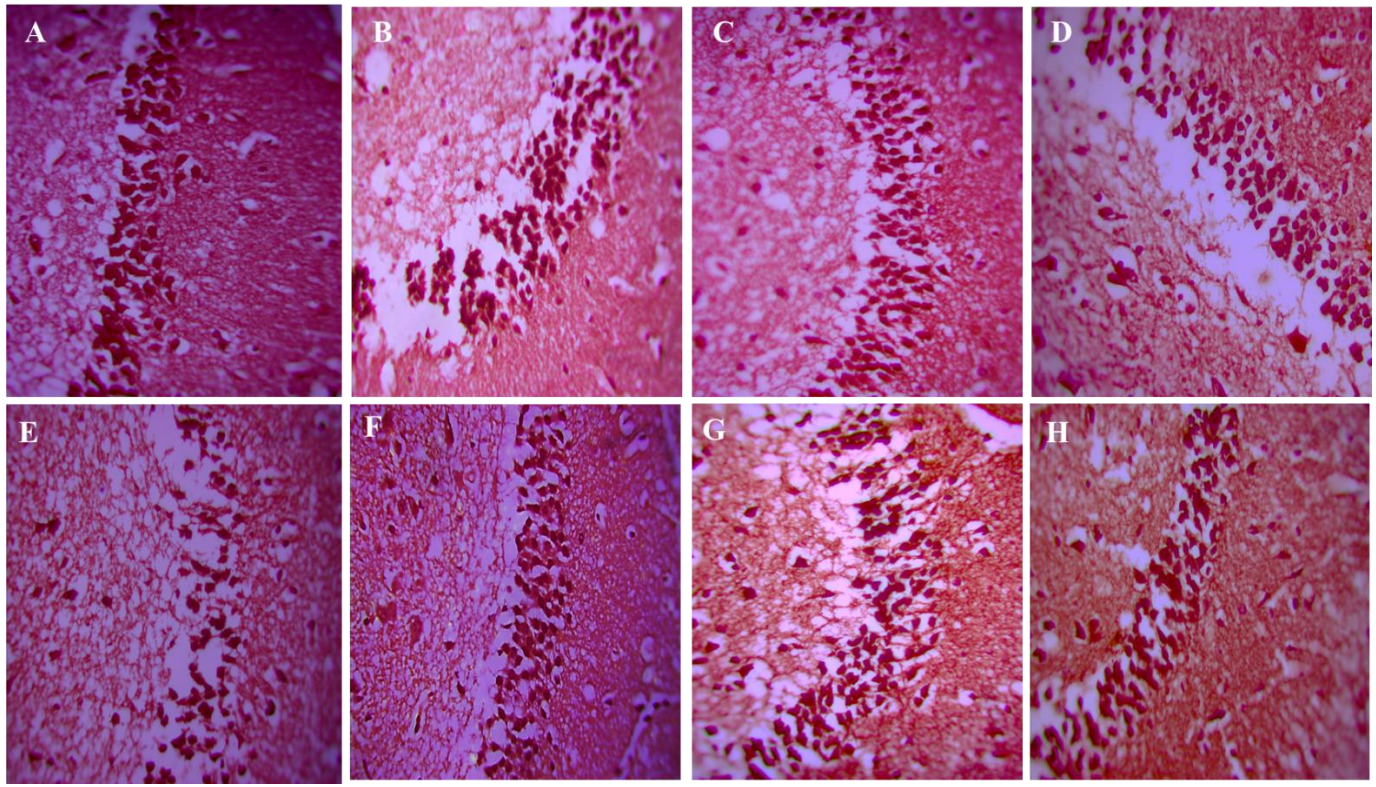


**Figure 4.10 H & E-stained coronal sections of DG region of hippocampus (40X magnification)**. (A) Control (B)Fluoxetine (C)Ascorbic Acid (D)Ascorbic Acid+Fluoxetine (E)UCMS (F)UCMS+ Fluoxetine (G)UCMS+ Ascorbic Acid (H) UCMS+ Ascorbic Acid+Fluoxetine

## 4.4. Molecular docking analysis

To assess whether our target proteins NeuN and ATF6 interact with our therapeutic compounds, molecular docking was performed. Molecular docking analysis revealed that the target protein ATF6 can interact with both FXT and AA (Figure 4.13). Binding energy of ATF6 with FXT was -6.0 Kcal/mol while with Ascorbic acid, it was -4.0 kcal/mol indicating that FXT has lower binding energy and hence stronger affinity with ATF6. However, Ascorbic acid forms one hydrogen bond with ATF6 at Ala558, and two hydrogen bonds with Gln 615 residue whereas FXT form H-bonds with two amino acid residues of ATF6 *i.e.,* Ser551, Tyr552 (Table 4.1).

FXT and AA can interact with NeuN as exhibited by the binding energies. FXT showed lower binding energy of -5.9 Kcal/mol as compared to AA which is -3.8 Kcal/mol with NeuN. Ascorbic acid forms three H-bonds with Asn105, Lys138 and Gly139 amino acid residues whereas FXT forms only one bond with Arg 100.This indicates that FXT binds with ATF6 and NeuN relatively easier but AA has stronger interaction with both ATF6 and NeuN (Figure 4.14).

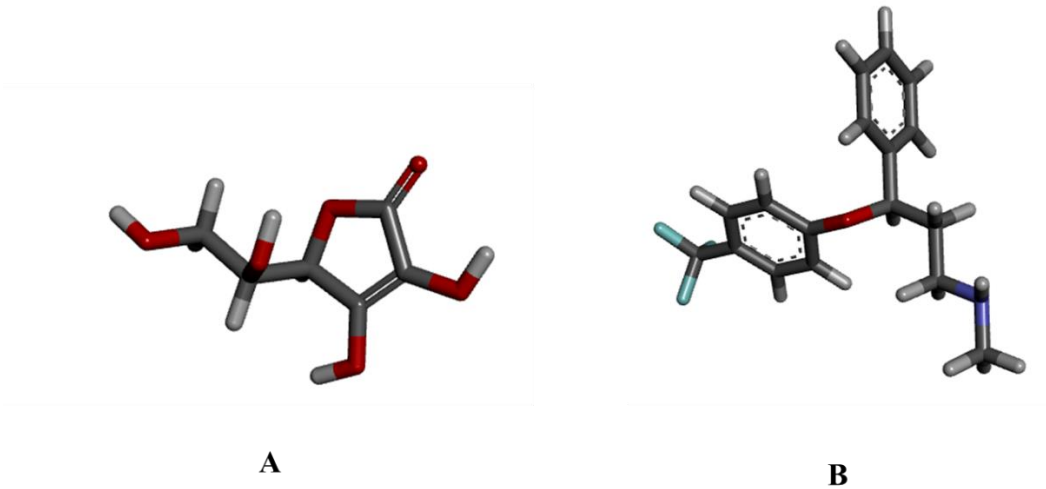


**Figure 4.11 Alphafold Protein Structure Database predicted crystal structures of (A)**ATF6α **(B)** NeuN viewed in BIOVIA Discovery Studio.

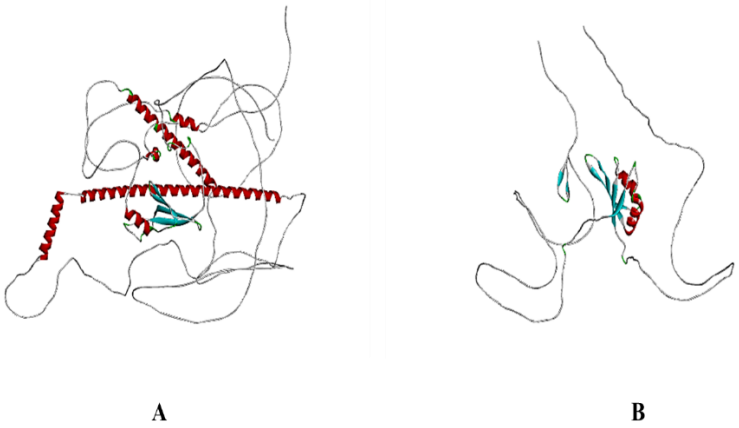


**Figure 4.12. 3D conformer structures of (A)**Ascorbic acid **(B)** Fluoxetine downloaded from PubChem and viewed in BIOVIA Discovery Studio.

**Table 4.1 Binding energies of FXT and AA with target proteins**

| **Proteins** | **Ligand** | **Binding energies (**Kcal/mol) | **Interacting residues** |
|---|---|---|---|
| **ATF6** | Ascorbic Acid | -4.0 | Ala 558, Gln 615<br>H-Bonds: Ala 558, Gln 615, Gln615 |
| | Fluoxetine | -6.0 | Phe555, Phe571, Leu576, Ser551, Tyr552<br>H-Bonds: Ser551, Tyr552 |
| **NeuN** | Ascorbic Acid | -3.8 | Arg166, Asn105, Lys138, Gly139<br>H-Bonds: Asn105, Lys138, Gly139 |
| | Fluoxetine | -5.9 | Arg100, Lys99, Ala175<br>H-Bonds: Arg100 |

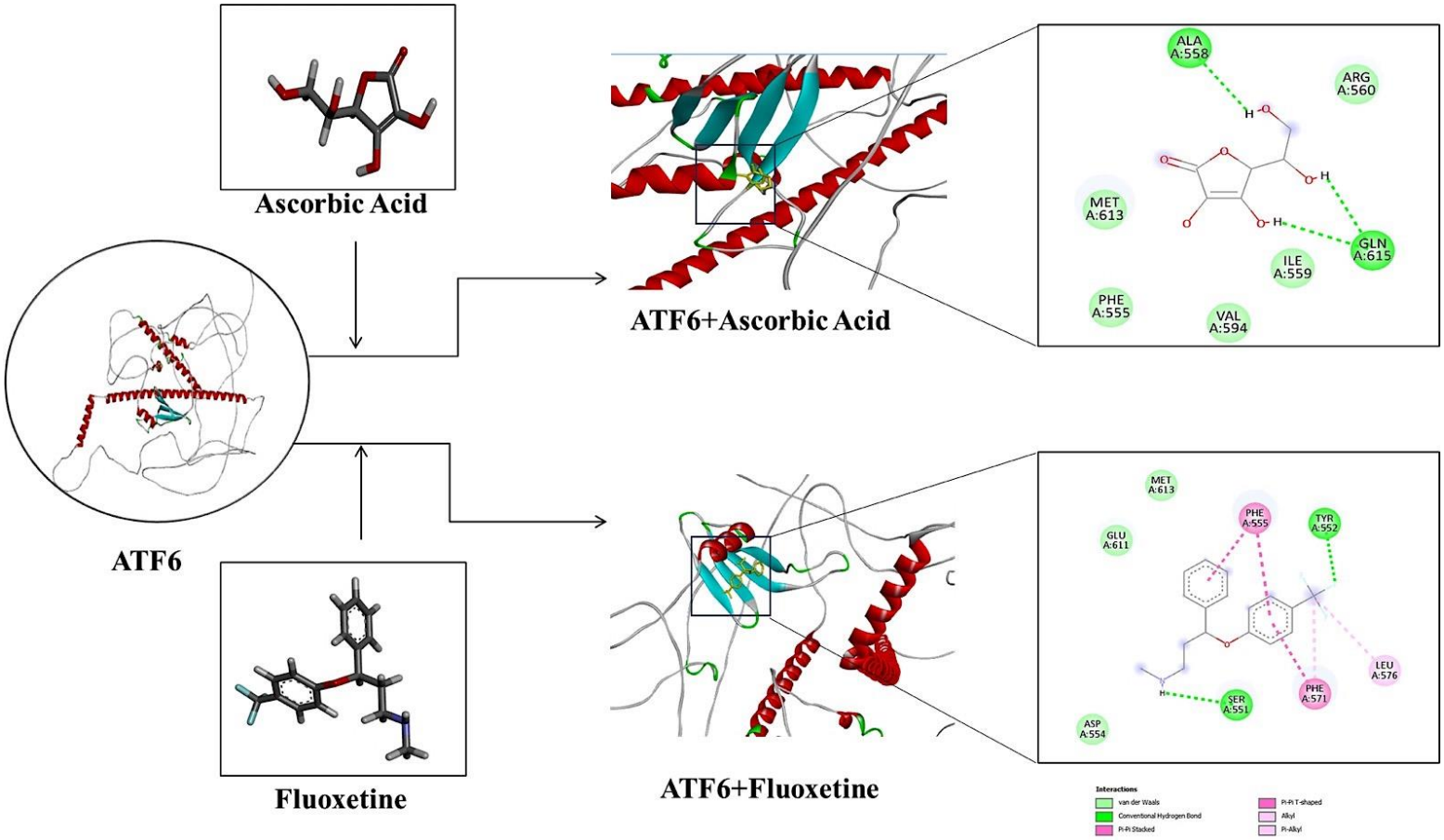


**Figure 4.13 Molecular docking of ATF6 with Ascorbic Acid and Fluoxetine** 3-dimensional binding poses in 3D and 2-dimensional interaction of ligands with target proteins are shown. Interactions were visualized in BIOVIA Discovery Studio.

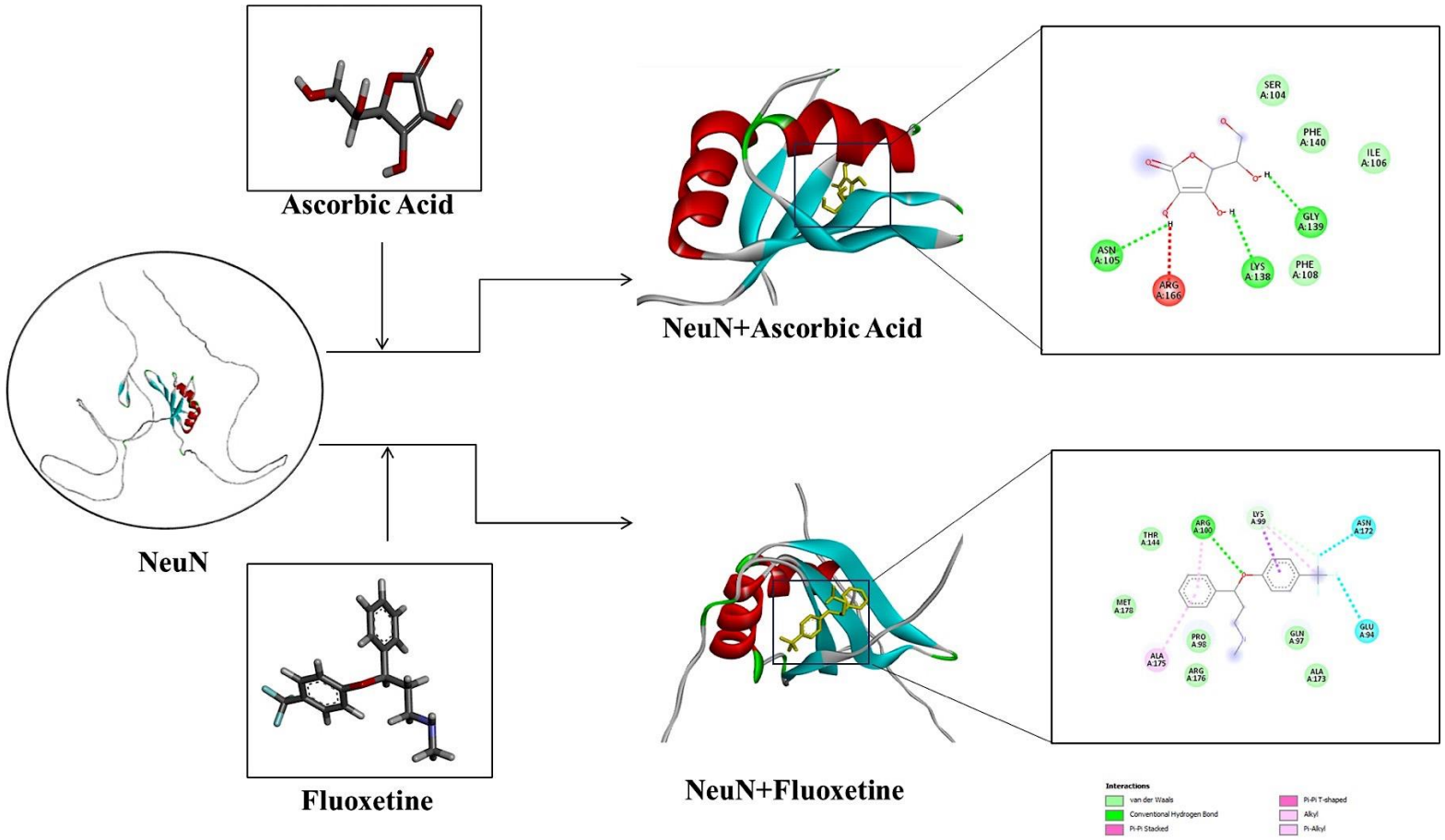


**Figure 4.14 Molecular docking of NeuN with Ascorbic Acid and Fluoxetine.** 3-dimensional binding poses in 3D and 2-dimensional interaction of ligands with target proteins are shown. Interactions were visualized in BIOVIA Discovery Studio

## .4.5. Gene expression analysis through RT-PCR

### 4.5.1 Effect of UCMS and treatment with Ascorbic acid on hippocampal ATF6 expression

ATF6 gene is highly expressed when the cellular endoplasmic reticulum stress levels are higher and Unfolded Protein Response is initiated. RT-PCR was performed for estimation of gene expression. UCMS exposed showed significantly increased($p<0.0001$) levels of ATF6 (5.22±0.09) in comparison to the control group (1.00±0.00). A significant normalization of ATF6 levels was noted ($p<0.0001$) in UCMS+FXT (0.32±0.04) group (Figure 4.15). The combination group UCMS+FXT+AA (0.53±0.03) displayed a similar effect ($p<0.0001$). However, UCMS+AA group exhibited slight normalization of the ATF6 expression in hippocampus ($p>0.05$).

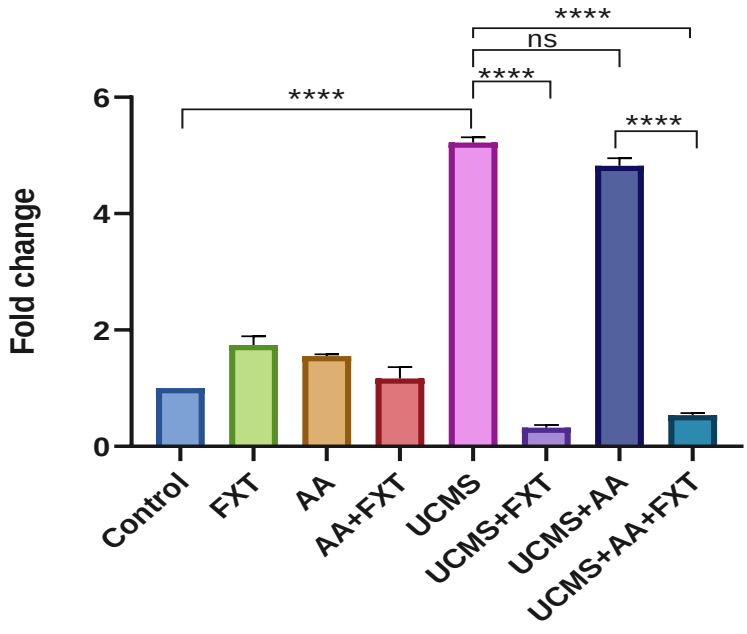


**Figure 4.15 The effect of Ascorbic acid on mRNA expression of ATF6 in hippocampus.** Graphpad Prism version 8.0.1 was utilized for the statistical analysis of data. The error bars were presented by Mean±SEM. The One-way ANOVA test and Bonferroni's multiple comparison test were applied on data. ("n.s =$p > 0.05$, *=$p< 0.05$, **=$p < 0.01$, ***= $p < 0.001$ and **** =$p < 0.0001$").

### 4.5.2 Effect of UCMS and Ascorbic acid treatment on hippocampal NeuN expression

NeuN mRNA expression was found to be significantly decreased ($p<0.05$) in the UCMS exposed mice's hippocampus indicating the neuronal loss. UCMS+FXT (1.4±0.09) and UCMS+AA (1.11±0.05) depicted the significantly elevated expression levels of NeuN ("$p<0.01$" and "$p<0.05$" respectively). The UCMS+FXT+AA (4.2±0.10) group also normalized the NeuN expression level ($p<0.0001$). The combination group UCMS+FXT+AA potentiated the effect of UCMS+FXT and UCMS+AA group significantly ($p<0.0001$) eliciting a synergistic neurogenic effect (Figure 4.16).

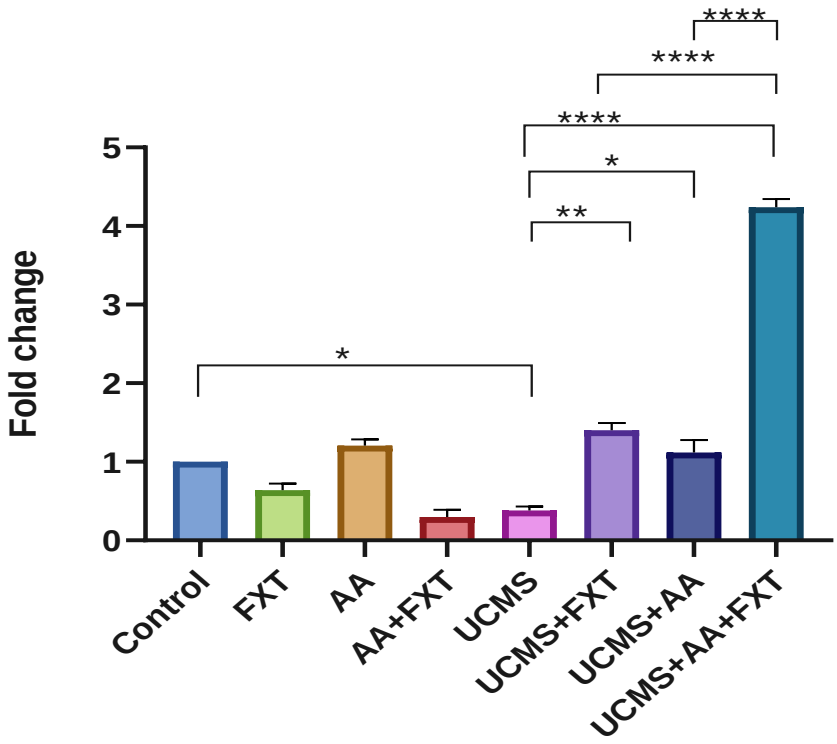


**Figure 4.16 The effect of Ascorbic acid on mRNA expression of neurogenesis marker NeuN in hippocampus.** Graphpad Prism version 8.0.1 was employed for statistical analysis. The error bars were presented by Mean±SEM. Data were tested using One way ANOVA test and Bonferroni's multiple comparison test. ("n.s =$p > 0.05$, *=$p< 0.05$, **=$p < 0.01$, ***= $p < 0.001$ and **** =$p < 0.0001$").

# Discussion

The present study was conducted to elucidate the neuroprotective role of AA, a potent antioxidant in UCMS model of depression. Additionally, the protective effects of AA supplementation with FXT; an SSRI were evaluated in UCMS depression model.

The Unpredictable Chronic Mild Stress (UCMS) model of depression was established to investigate the antidepressant properties of the compounds. Although laborious, time consuming and difficult to reproduce, the choice of UCMS model for this study was based on the construct and face validity, along with the predictive validity making it an appropriate option (Abelaira *et al.,* 2013). The UCMS model successfully produced behavioral deficits of anhedonia and despair as reported by various studies (Chevalier *et al.,* 2020; Dovvombaygi *et al.,* 2021). Our study observed a noteworthy decline in the coat condition of mice subjected to the UCMS protocol from week 4 to 9. Degradation of coat is a pharmacologically validated index of evaluating depression indicative of reduced motivation and is also reported by other studies. A significant coat state degradation was also observed at 7 weeks UCMS in a study that evaluated the effects of *Dacryodes edulis* pulp oil (Miguel *et al.,* 2019). Farooq *et al.,* 2018 demonstrated the effect of antagonism of P2X7 receptor on microglial cells in UCMS (9 weeks) induced BALB/cByJRj mice which is a substrain of BALB/c mice. Female BALB/c mice received 10 mg/kg of FXT for four weeks post exposure to CMS and exhibited that FXT treatment reverted the coat deterioration (Rodríguez *et al.,* 2021). Similarly, in our study, 20 mg/kg intraperitoneal administration of FXT for three weeks significantly reversed the coat state deterioration caused by UCMS procedure. AA (10mg/kg) alone and the combination of AA (10 mg/kg) with FXT(20 mg/kg) when administered , significantly improved the coat state evident in the last week of treatment thus providing evidence of the amelioration of physical indication of disease.

Anhedonia, the core symptom of depression characterized by apathy, loss of pleasure, reduced motivation and reward seeking was assessed by sucrose splash test. In the current study, UCMS-induced anhedonia in the mice evident from significantly reduced time mice took to initiate the grooming, spent significantly less time on grooming and did significantly less frequent grooming. Prolonged treatment with fluoxetine (FXT) at a dosage of 10 mg/kg significantly increased grooming time in a chronic restraint stress model (Misztak *et al.,* 2021). Similarly, our study demonstrated that FXT (20 mg/kg) significantly enhanced the grooming time of mice, and decreased the grooming latency in UCMS mice. A similar effect was noted for AA (10 mg/kg) administration. The effects of coadministration of AA and FXT in UCMS-induced depression model have not been previously evaluated. Our study elucidated that the co administration of FXT and AA can alleviate the UCMS-induced anhedonia significantly but this effect was not synergistic. Another observation was that the administration of FXT, AA, and the combination of AA and FXT did not result in an increment of the frequency of grooming in UCMS group because the mice engaged in grooming for an extended time period, leading to an overall reduced frequency of grooming.

To evaluate the anxiety, latency to consume food in NSFT was evaluated. UCMS exposed mice took more time to eat the food pellet after being deprived of food for 24 hours however this increase was non-significant making it complicated to assess the anxiolytic effects of our compounds of interest. In accordance with our study, C57BL/6 mice exposed to 5 weeks of UCMS did not exhibit induced anxiety, as evidenced by both the EPM and the NSFT (Bansal *et al.,* 2023). Our study demonstrated that FXT, AA and supplementation of AA with FXT exhibited a slight decrease in the latency to eat food thus showing their anti-anxiety efficacy. FXT has shown its benefits as an anti-anxiety compound in streptozotocin-induced mice by attenuating inflammation (Yuan

*et al.,* 2019). Different dosages of AA alleviated anxiety in Female Swiss mice assessed by Open field test (Fraga *et al.,* 2018).

To assess the memory impairment in the UCMS group, Y-maze test was performed. A slight decline in the percentage spontaneous alterations, time duration spent in novel arm and percentage of entries in the novel arm were observed, however this decline was non-significant. This non-significant decline was in accordance with another study where 7 weeks of UCMS imposition to CBA/H, C57BL/6 and DBA/2 mice caused no affect on the percentage spontaneous alterations (Pothion *et al.,* 2004). However, Foyet *et al.,* 2017 demonstrated that 30 days exposure to UCMS can produce spatial memory impairment in Wistar rats quantified by Y maze test. The non-significant memory impairment and anxiolytic effects observed in our findings provide a potential rationale for the limited reproducibility observed in the UCMS model (Cryan & Mombereau, 2004). This non-reproducibility could stem from the difference in the stressors that mice were exposed to in different laboratory settings. Several factors play a crucial role in determining the success and consistency of the UCMS method including stress-related conditions, such as painful stressors, chronic sleep deprivation, variations in sex, age ,age factor, social stressors, diverse stress tolerance as well as susceptibility across various strains and sub-strains. The way animals are handled and the inclusion of a mix of physical and psychological stressors in the protocol also contribute significantly to the results (Markov & Novosadova, 2022).

In TST, the animal exposed to inescapable stress situation, remains in an immobile posture for a significant longer period. UCMS exposure caused a significant elevation in immobility time as reported previously (Gutiérrez *et al.,* 2023). In the present study, a significant decline in the immobility time in UCMS mice by chronic AA treatment as well as chronic FXT administration was evident. Moretti *et al.,* 2015 documented that

in a TNF alpha-induced depression model, an administration of 1 mg/kg AA was effective in reducing the immobility period in TST in adult female Swiss mice . In the current study, the supplementation of AA and FXT lowered the immobility duration in a significant manner and hence alleviated the behavioral despair indicating its potential anti-depressant properties.

UCMS exposure induced the depressive like state assessed by the FST. There was a notable reduction in the latency to immobility, accompanied by an increase in immobility time and the number of immobile episodes. This behavioral deficit produced by UCMS procedure is previously reported (Lee *et al.,* 2019) in many studies (Zhao *et al.,* 2019). FXT, AA as well as AA supplementation with FXT significantly caused a reduction in the immobility time and immobile episodes. The administration of AA at a dosage of 1 mg/kg and FXT at a dose of 10 mg/kg to female Swiss mice undergoing restraint stress prevented the elevation in immobility time in the FST. Also AA was found to restore the levels of SOD and glutathione peroxidase that were altered by repeated stress, suggesting that the anti-depressive properties of AA are due to its anti-oxidant potential (Moretti *et al.,* 2013). The combination of AA and FXT did not improve the latency to immobility, however once the mice started swimming , the least number of immobile episodes were manifested by the group as compared to the AA group and FXT group alone. This indicates that FXT and AA reduced depressive like behavior in a synergistic manner.

A randomized trial conducted on pediatric major depressive order conducted on 24 children suggested that vit C can be used as an adjunct for the mitigation of depressive symptoms (Amr *et al.,* 2013). i.c.v, *p.o and i.p,* administration of AA in a dose dependent manner (0.1-10 mg/kg) in adult Swiss mice produces an antidepressant effect displayed in TST (Binfaré *et al.,* 2009). The authors illustrated that this effect is

contingent upon the monoaminergic system as AA can modulate this pathway by interacting with dopamine D2 receptors. The dose dependent effect was observed as a U shape trend, showing that at higher dosages, AA does not display any anti-depressant property. The subeffective doses of single administration of AA (0.1 mg/kg ) potentiated the effect of FXT (1mg /kg) in the TST. Our study, for the first time evaluated the effect of co administration of FXT and AA in UCMS model of depression. The effective dosages of both the compounds were used in the study. Our study elicited that AA administration does not significantly potentiate the effects of FXT at behavioral level. These varying effects could be caused by the difference of dosages used and the treatment duration followed in both the studies. An alternative explanation might be the limited sample size employed in the present study. However, a synergistic effect evident at the mRNA level compels to investigate further underlying mechanisms. Misztak *et al.,*2022 documented that zinc supplementation in male C57BL/6J mice subjected to restraint stress can significantly potentiate the effects of FXT in SST but not in the TST, thus indicating that the synergistic effects also vary depending on the type of behavioral assessment being performed.

Histopathological examination was done by using H and E staining to quantify the neuronal cell count in hippocampus. UCMS group depicted a significant reduction in cell number and UCMS+ FXT, UCMS+AA and UCMS+FXT+AA treatment exhibited a significant increase in the cell count. The decrease in cellular density aligns with the findings of the study conducted by Ali *et al*., 2017, which demonstrated a decline in the hippocampal granular cell layer thickness in mice exposed to CUMS procedure for four weeks particularly in DG region and CA3 region. Similarly, another study reported that male Swiss mice exposed to UCMS procedure for 21 days exhibited distortions in histomorphology of the prefrontal cortex, amygdala and hippocampus (Okoh *et al.,*

2020). Consistent with the results of our study, FXT (18 mg/kg) administered to Wistar rats for 24 days after exposing them to 6 weeks UCMS regimen significantly enhanced the expression of BrdU positive cells in DG region indicating its neurogenic and neuroplasticity enhancing effects (Zavvari *et al.,* 2020). Male Sprague-Dawley rats receiving FXT for 14 days (10 mg/kg) displayed significantly larger number of Ki67+ve and DCX+ve in the SGZ of hippocampal dentate gyrus and this effect was shown to be modulated by the 5HT3 receptor activation by FXT (Cano *et al.,* 2023). Marcinkute *et al.,* 2019 reported that varying concentrations of fluoxetine hydrochloride (1 nM -100 μM) when used to treat HCT116 +/+ human colorectal cancer cells induced apoptosis by causing DNA fragmentation. Hence literature suggests both anti-proliferative as well as neurogenic potential of FXT.

Similarly, four week administration of AA (150 mg/kg) in C57BL/6 mice substantially prevented the neuronal degeneration as indicated by the increased number of Ki67+ve and DCX+ve cells owing to the anti-inflammatory and anti-oxidative properties of AA observed in D-galactose-induced brain aging (Nam *et al.,* 2019).

Gene expression analysis of ATF-6 and NeuN was done. Before that, molecular docking analysis was performed to check any possible interactions which is a less time and resource consuming process as compared to wet lab experimentation. Both ATF6 and NeuN were docked successfully with AA and FXT and showed comparable binding affinities, with less binding energy for FXT indicating a more favorable interaction. However, AA forms more hydrogen bonds with both ATF6 and NeuN thus implicating a stronger interaction as compared to the FXT.

Increased ER stress is contributive to the pathophysiology of depression. ATF6/CHOP increased levels have been linked with CUMS-induced depression (Tang *et al.,* 2023). Our study demonstrated the significantly increased expression of ATF6 in hippocampus

of UCMS mice. Elevated levels of hippocampal ATF6 were also observed in male Sprague-Dawley rats subjected to chronic restraint stress indicative of increased ER stress (Timberlake *et al.,* 2018). Treatment with AA mitigated cadmium-induced ER stress in germ cells in the testes, and this attenuation was associated with the activation of XBP-1 protein which plays its role in ER stress pathway (Wang *et al.,* 2012). Similarly, gene expression of ATF6 was markedly elevated in the male Holtzman rat's hippocampus subjected to learned helplessness paradigm (Timberlake & Dwivedi, 2016). However, in our study, UCMS+AA group showed a slight decrease in ATF6 expression however it was non-significant. A potential explanation for this statistically non-significant decrease could be attributed to the limited sample size chosen in the present study. This also needs further investigation by evaluating the gene expression of further upstream and downstream genes contributive to ER stress to pinpoint the exact targets of AA in ER stress pathway. UCMS+AA+FXT also significantly decreased the ATF6 expression. FXT has shown its protective effects against PD-associated ER stress by suppressing the XBP1/Caspase3 activation (Peng *et al.,* 2018). Likewise, our results also reflects the significant reduction of ATF6 expression by FXT, hence alleviating the ER stress.

Gene expression of NeuN which is indicative of neurogenesis in neuronal cells was assessed to validate the results of histological assessment. The significantly decreased expression of NeuN in the hippocampus of mice in UCMS group, observed in the study shows the neurodegenerative effect of UCMS. UCMS+FXT and UCMS+AA and UCMS+AA+FXT significantly enhanced the expression of NeuN in hippocampus. A single administration of AA to female Swiss mice (1mg/kg) increased the AKT phosphorylation and decreased hippocampal $p38^{MAPK}$ thus initiating cell survival signaling pathways (Moretti *et al.,* 2016). Another finding reported herein our study

was the significantly increased expression of hippocampal NeuN in UCMS+AA+FXT group as compared to the UCMS+AA and UCMS+FXT groups elucidating the synergistic effect of AA and FXT in enhancing the hippocampal neurogenesis.

# CONCLUSION AND FUTURE PROSPECTS

The administration of AA was effective in alleviation of depression and associated behavioral deficits. Significant modulation of ER stress and neurogenesis evident at transcriptional level indicates that AA can be used as an alternative therapeutic option for the treatment of MDD. This further imposes the need to investigate underlying mechanisms and explore the common and uncommon pathways targeted by both SSRIs and AA. These findings support the notion of using nutraceuticals as an effective therapeutic strategy against depression as well other neuropsychiatric and neurodegenerative diseases.

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Protective Effects of Ascorbic Acid In Mouse Model of Depression

ii

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