Behavioral and Neuro-pharmacological Screening of Phosphodiesterase-4 (PDE4) Inhibitors for Anti-depressant and Anxiolytic Potential

THESIS

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by

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Under the Supervision of

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CERTIFICATE

This is to certify that the thesis entitled **"Behavioral and Neuro-pharmacological Screening of Phosphodiesterase-4 (PDE4) Inhibitors for Anti-depressant and Anxiolytic Potential"** and submitted by **Ankur Jindal, ID No. 2009PHXF405P**, for the award of Ph.D. Degree of the Institute, embodies the original work done by him under my supervision.

Signature in full of the supervisor:

Name in capital block Letters: **Prof. R. MAHESH**

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Date: 20th Aug., 2014

Dedicated

To

My Affectionate Parents & Family

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List of Abbreviations/Symbols

α-AR	α - Adrenergic Receptor
β-AR	β - Adrenergic Receptor
5-HT	5-Hydroxy Tryptamine
5-HTP	5-Hydroxy Tryptophan
p75NTR	p75 Neurotrophin Receptor
AC	Adenyl Cyclase
AD	Anti-depressant
ADs	Anti-depressants
ANOVA	Analysis of Variance
APA	American Psychiatric Association
ATP	Adenosine Triphosphate
BDNF	Brain Derived Neurotrophic Factor
BUP	Bupropion
BZD	Benzodiazepine
Ca^{2+}/CaM	Calcium/Calmodulin
CA1	cornu Ammon 1 region
cAMP	3',5'-Cyclic Adenosine Monophosphate
CAT	Catalase
cGMP	3',5'-Cyclic Guanosine Monophosphate
CNS	Central Nervous System
CORT	Corticosterone
CRE	cAMP Response Element
pCREB	Phosphorylated cAMP Response Element Binding Protein
CUMS	Chronic Unpredictable Mild Stress
DA	Dopamine
DG	Dentate Gyrus
DAT	Dopamine Transporter
DMI	Desipramine
DSM	Diagnostic and Statistical Manual of Mental Disorder
DZM	Diazepam
ECT	Electroconvulsive Therapy

ELISA	Enzyme Linked Immunosorbent Assay
EPM	Elevated Plus Maze
ERK	Extracellular Signal-regulated Kinase
ETZ	Etazolate
FST	Forced Swim Test
FLX	Fluoxetine
GABA	γ- Amino butyric Acid
GAD	Generalized Anxiety Disorder
GCs	Glucocorticoids
GPCR	G-protein Coupled Receptor
GR	Glucocorticoid Receptor
GSH	Reduced Glutathione
HARBS	High Affinity Rolipram Binding State
H&E	Haematoxylin and Eosin
HB	Hole Board
HPA	Hypothalamic-Pituitary-Adrenal Axis
Hr	Hour
HT	Histamine
HTR	Head Twitch Response
i.p.	Intra-peritoneal
KO	Knockout
LARBS	Low Affinity Rolipram Binding State
L/D	Light/Dark
MAO	Monoamine Oxidase
MAOI	Monoamine Oxidase Inhibitor
mBDNF	Mature BDNF
MDA	Malondialdehyde
MDD	Major Depressive Disorder
NA	Nor-adrenaline
NaSSA	Nor-adrenergic and Specific Serotonergic Anti-Depressants
NDRI	Nor-adrenaline and Dopamine Re-Uptake Inhibitor

NE	Nor-epinephrine
NET	Nor-epinephrine Transporter
NMDA	N-methyl-D-aspartate
NRI	Nor-adrenaline Re-uptake Inhibitor
NTs	Neurotransmitters
OAT	Open arm entries
OBX	Olfactory Bulbectomy
OCD	Obsessive Compulsive Disorder
OFT	Open Field Test
PDE	Phosphodiesterase
PDE4	Type 4 Phosphodiesterase/Phosphodiesterase-4
PKA	Protein Kinase A
p.o.	Per-oral
PPD	Post-Partum Depression
ProBDNF	Precursor or Immature BDNF Protein
PTSD	Post Traumatic Stress Disorder
Q-12	QCA-12
Q-21	QCA-21
REC	Receiver
RIH	Reserpine-induced Hypothermia
ROL	Rolipram
ROS	Reactive Oxygen Species
S	Seconds
SAD	Seasonal Affective Disorder
SARI	Serotonin Antagonist and Re-uptake Inhibitors
s.c.	Subcutaneous
SLA	Spontaneous Locomotor Activity
SNRI	Serotonin and Nor-adrenaline Re-uptake Inhibitors
SOD	Superoxide Dismutase
SSRE	Selective Serotonin Re-uptake Enhancers
SSRI	Selective Serotonin Re-uptake Inhibitor
TBARS	Thiobarbituric Acid Reactive Substance

TBI	Traumatic Brain Injury
TCAs	Tricyclic Anti-depressants
TRD	Treatment Refractory depressions
TRI	Triple Re-uptake Inhibitor
Trk	Tyrosine Kinase
TSOA	Time Spent in Open Arm
TST	Tail Suspension Test
UCR	Upstream conserved region
VLA	Venlafaxine

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Abstract

Beyond the receptor level, secondary messenger cyclic adenosine monophosphate (cAMP) has been shown to plays an important role in the mood and emotional behavior regulation. cAMP and its-mediated transduction cascade regulate molecular process, underlying the synaptic plasticity and neuronal survival, a common feature in the patho-physiology of psychiatric disorders. Studies have reported that a decrease level of cAMP has been shown to be involved in the patho-physiology of the depression and anxiety disorders. Type-4 phosphodiesterase (PDE4), an enzyme catalyzes the hydrolysis of cAMP and regulates its-mediated signal transduction. These findings led to the hypothesis for this thesis work that enhancing cAMP-mediated signal transduction by inhibition of PDE4 is known to be beneficial in the treatment of depression and anxiety disorders. Thus, the present study was designed to investigate thoroughly the anti-depressant (AD)- and anxiolytic-like potential of standard PDE4 inhibitors, like rolipram (ROL) and etazolate (ETZ) and in-house synthesized PDE4 inhibitors, such as QCA-21 (Q-21) and QCA-12 (Q-12) using rodent models.

Separate groups of mice, received acute treatment of drugs, such as ROL, ETZ, Q-21 and Q-12, were subjected to spontaneous locomotor activity (SLA) test or AD assays, namely, the forced swim test (FST), tail suspension test (TST), 5-hydroxytryptophan (5-HTP)-induced head twitch response (HTR) and reserpine-induced hypothermia (RIH) response. Acute intra-peritoneal (i.p.) treatment with ETZ (0.25-1 mg/kg), ROL (0.25-1 mg/kg), Q-21 (0.5-2 mg/kg) and Q-12 (0.5-2 mg/kg) exhibited AD-like effects in FST and TST without influencing the baseline locomotion in SLA test. Moreover, ETZ (0.5 & 1 mg/kg, i.p.) and ROL (0.5 & 1 mg/kg, i.p.) increased HTR in mice and antagonized RIH response in rats.

Interaction studies of ETZ and ROL sub-effective doses (0.12 mg/kg, i.p.), were carried out with sub-effective doses of conventional ADs, like fluoxetine (FLX), venlafaxine (VLA) and desipramine (DMI) in FST. ETZ and ROL at sub-effective dose produced synergistic AD-like effects with FLX (5 mg/kg, i.p.), VLA (4 mg/kg, i.p.) and DMI (5 mg/kg, i.p.) in FST. In addition, combined treatment of ETZ, ROL and conventional ADs had no significant effect on baseline locomotion.

Further, to confirm the efficacy of PDE4 inhibitors, the effects were also evaluated in the chronic rodent models such as olfactory bulbectomy (OBX), chronic corticosterone (CORT)-injection, chronic unpredictable mild stress (CUMS) and traumatic brain injury (TBI) models, using behavioral, biochemical and neurobiological test battery.

OBX was performed in anesthetized rats. Post-surgery, drugs [ROL (0.5 & 1 mg/kg), ETZ (0.5 & 1 mg/kg), Q-21 (0.5-2 mg/kg) and Q-12 (0.5-2 mg/kg)] administration and behavioral tests were carried out. OBX rats exhibited hyperactivity (increased ambulation, rearing and defecation) in open field test (OFT), decreased sucrose consumption, which resembling anhedonia, increased hyper-emotionality behavior (emotional anomalies to non-noxious stimuli). Chronic per-oral (p.o.) treatment (14 days) with above mentioned drugs significantly reversed the behavioral anomalies induced by OBX in all the behavioral tests.

The present study focused on neurons morphology in the hippocampal cornu Ammon (CA1) and Dentate gyrus (DG) regions in OBX rats and studied the effect of ETZ and Q-21 on neuronal degeneration. The photomicrographs of the Haematoxylin & Eosin (H&E) stained hippocampal CA1 and DG regions of OBX group showed neuronal death as compared to sham controls that showed healthy neurons. ETZ (0.5 & 1 mg/ kg, p.o.) and Q-21 (1 & 2 mg/kg, p.o.) treatments showed neuronal protection in both the regions of OBX rats.

In addition, the possible underlying mechanism(s) of PDE4 inhibitors in OBX model was also investigated by measuring hypothalamic pituitary adrenal (HPA) axis activity [in terms of serum CORT level], neurotransmitters (NTs) level [serotonin (5-HT), nor-epinephrine (NE) and dopamine (DA)], oxidative stress (lipid peroxidation and nitrite level)/anti-oxidant enzymes markers [reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT)] markers level, cAMP level, cAMP response element binding (CREB) protein level and brain derived neurotrophic factor (BDNF) levels. OBX rats showed a profound elevated serum CORT and reduced cAMP, pCREB and BDNF levels. OBX rats also showed remarkably increased oxidative/nitrosative stress markers, like lipid peroxidation and nitrite levels and decreased anti-oxidant enzymes, such as GSH, SOD and CAT activity. Moreover, OBX also remarkably ($P<0.05$) decreased the 5-HT, NE and DA levels. PDE4 inhibitors markedly ($P<0.05$) reversed the OBX-induced biochemical and cAMP signaling alterations, which may be possible mechanisms for AD-like effect and neuronal survival in OBX model. However, in the present study no effect of test drugs treatments on the brain NTs levels was observed in OBX rats as compared to vehicle treated OBX rats.

In another study, anesthetized rats were subjected to impact accelerated TBI paradigm. Post 10 days of healing, ETZ (0.5 & 1 mg/kg, p.o.) and Q-21 (0.5-2 mg/kg, p.o.) were administered for a period of 14 days in TBI rats. In addition to exploratory hyperactivity in OFT, TBI rats showed emotional anomalies to non-noxious stimuli in hyper-emotionality test and decreased sucrose consumption as compared to sham control rats. These results demonstrated complete neurological deficits following TBI and selective behavioral changes.

Chronic (14 days) ETZ and Q-21 treatment remarkably reversed the behavioral anomalies induced by TBI in modified open field, sucrose preference and hyper-emotionality tests.

The possible underlying mechanism(s) of ETZ and Q-21 in TBI model was also investigated by measuring cAMP signaling cascade markers, such as cAMP, pCREB and BDNF levels. TBI rats had a profound decreased cAMP, pCREB and BDNF levels. Moreover, TBI also showed remarkable ($P<0.05$) increase in oxidative and nitrosative stress markers and decrease in anti-oxidant enzymes activity. ETZ and Q-21 treatment significantly ($P<0.05$) reversed the TBI-induced biochemical and neurobiological markers alterations.

In addition, the present study was designed to investigate, whether, PDE4 enzyme involves in CUMS model of depression and PDE4 inhibitors could affect the CUMS-induced depression-like behavior deficits in mice. Mice were subjected to different stress paradigms daily for a period of 28 days to induce depressive-like behavior. Drugs were administered during the last 21 days (8^{th}-28^{th}) of the CUMS paradigm. The results showed that 4-weeks CUMS exposure produced depression-like behavior in the FST, TST and sucrose consumption test. Chronic treatment with ETZ (0.5 and 1 mg/kg., p.o.) and Q-21 (1 & 2 mg/kg., p.o.) produced significant AD-like behaviors in FST (decreased duration of immobility & increased swimming episodes), TST (decreased duration of immobility) and sucrose consumption test (increased preference and consumption to sweetened solution).

The possible underlying mechanisms of ETZ and Q-21 in CUMS model were investigated by measuring HPA axis activity, NTs levels (5-HT, NE and DA), oxidative-nitrosative stress/antioxidant markers level and cAMP transduction pathway (cAMP/CREB/BDNF). Stressed mice showed a marked increase in serum CORT level and decrease in cAMP, pCREB and BDNF levels. CUMS also remarkably increased oxidative stress markers and decreased anti-oxidant enzymes activity. CUMS also profoundly ($P<0.05$) decreased the 5-HT, NE and DA levels. ETZ and Q-21 treatments reversed the CUMS-induced biochemical alterations, which may be possible mechanisms for AD-like effects in CUMS model. However, in the present study, no effect of the treatments on brain NTs levels was found in stressed mice as compared to vehicle treated stressed mice.

To explore the potential of PDE4 inhibitors in treatment resistant depression (TRD), the chronic CORT injection-induced depression model was standardized in our laboratory. Mice were subcutaneously (s.c.) injected with CORT (30 mg/kg) daily for a period of 21 days to induce depressive-like behavior. Drugs were administered for a period of 21 days along with the CORT injection. The results showed that 21 days CORT-injection produced marked

depression-like behavior in the FST, TST and sucrose consumption test. Chronic treatment with ETZ (0.5 and 1 mg/kg., p.o.) and Q-21 (1 & 2 mg/kg., p.o.) produced significant AD-like behaviors in chronic CORT-injected model.

The effects of chronic CORT-injection and PDE4 inhibitors were also investigated on neurons morphology in hippocampal CA1 and DG regions of CORT-treated mice. The photomicrographs of H&E stained hippocampal CA1 and DG regions showed neuronal death as compared to normal control mice. Chronic ETZ (0.5 and 1 mg/ kg, p.o.) and Q-21 (1 and 2 mg/kg, p.o.) treatment showed neuronal protection in hippocampal CA1 and DG regions of CORT-treated mice.

The possible underlying mechanism(s) of ETZ and Q-21 in chronic CORT-injected model were also investigated by measuring the HPA axis activity and cAMP/pCREB/BDNF levels. CORT-treated mice indicate pronounced increase in serum CORT level and decrease in cAMP, pCREB and BDNF levels. ETZ and Q-21 treatment significantly ($P<0.05$) reversed the chronic CORT-injection induced biochemical and neurobiological alterations, which may be responsible for AD-like effects in this model.

Beside the AD-like potential, preliminary anxiolytic-like effects of PDE4 inhibitors were also investigated. Separate groups of mice received acute treatment of drugs (ROL, ETZ, Q-21 and Q-12) and were subjected to experimental anxiety models [elevated plus maze (EPM), light/dark (L/D) aversion test and hole board (HB) test]. Acute treatment with ETZ (0.25-1 mg/kg, i.p.) and ROL (0.25-1 mg/kg, i.p.) exhibited anxiolytic-like effects in the EPM, L/D and HB tests. In other set, Q-21 (1 & 2 mg/kg, i.p.) and Q-12 (2 mg/kg, i.p.) also exhibited anxiolytic-like effects in the EPM and L/D tests.

In conclusion, these findings strongly support the conclusion that PDE4 enzyme plays a fundamental role in the patho-physiology of depression and anxiety disorders and the inhibitors of PDE4 enzyme produced AD- and anxiolytic-like effects in various acute and chronic rodent models. Further, PDE4 inhibitors, like ETZ and Q-21 showed neuroprotection in DG and hippocampal CA1 regions in OBX and chronic CORT-injected models. The aforementioned results in various animal models indicated the possible mechanism(s) for the PDE4 enzyme in depression patho-physiology and PDE4 inhibitors-mediated AD-like effects and neuronal survival is atleast in part mediated by modulating the cAMP signaling cascade, HPA axis activity and oxidant/anti-oxidant systems.

Chapter 1: Introduction

1. Depression: A Neuropsychiatric Problem

Major depression disorder (MDD) is a common incapacitating and life-threatening psychiatric disorder with lifetime prevalence approaching 21% expressing significant morbidity and mortality rate (Nemeroff, 2007; Maes et al., 2009). It is periodic and recurrent in nature, with partial improvement between episodes. Globally, it ranks fourth among the leading causes of disability (Nemeroff, 2007; Maes et al., 2009). The morbidity and mortality rates for MDD patients are high; two thirds of MDD patients contemplate suicide and 10-15% succeed. World Health Organization has predicted that MDD will be the second largest contributor to global burden of the disease by the year 2020 (Aan het Rot et al., 2009), only behind ischemic heart disease. Therefore, depression represents a major medical and social problem, illustrating the severity and impact of this disorder on society.

1.1. Epidemiology, Prevalence and Social Cost of Depression Disorder

The prevalence of MDD is increasing frequently. The lifetime prevalence of MDD is reported to be as high as 10-25 % for women and 5-12% for men (Kessler et al., 2003). This indicates that women are 2–3 times more susceptible to experience a depressive episode in comparison to men. The prevalence of depression does not depend on the parameters, ethnicity, education, income, or marital status. Several epidemiological reports indicate that severe forms of depression affect nearly about 2-5% of the population worldwide and up to 20% of the population is affected by milder forms (Kessler et al., 2003). Beside these, the death rate among depressed persons has been shown high, indicating that nearly 15% of the depressed patients commit suicide (Gold and Chrousos, 2002).

A person can experience depressive episodes at virtually any period of life time, as a function of genetic and developmental pre-disposition in interaction with unpleasant life-events (Kessler et al., 2003; Ritchie et al., 2004). Studies report that mood disorders tend to appear in the third decade of life, but the first occurrence of MDD can be at any point in life. The onset of depressive episodes commonly occurs between the ages of 20-30 years, with a later peak between 30-40 years (Eaton et al., 1997). Studies address that average age of depression in India is 31.9 years, while in the US it is around 22.7 years. Further, prevalence for one or other forms of depression is reported to be 11.2% for the age group between 13-18 years, while 6.7% for 25-30 years age group (Kessler et al., 2005a). These indicate that near about 50% of patients experience their first depressive episode before the age of 40 and most of those patients (50 to 85%) will experience a second episode. Each subsequent depressive episode raises the possibility of sustained episodes and decreases the possibility of a positive response to treatment (Eaton et al., 2008). Reports indicate that early onset

depression is considered to be a more severe form of MDD and indicative of poorer diagnosis (Hammen and Brennan, 2001). Disturbingly, depressive state is, often identified in adolescents and even children (Ritchie et al., 2004). Further, the occurrence of depressive episode severely disrupts the ability to work, familial relationships, social integration and self-care (Ayuso-Mateos et al., 2001; Evans and Charney, 2003).

The economic costs of this disorder are enormous, estimated to be tens of billions of dollars every year in the US alone (Donohue and Pincus, 2007). Despite a high economic cost, MDD often remains untreated, undertreated, or improperly treated. Several studies have shown that depression is associated with a significant social and economic burden, as indicated by low productivity, the need for continued medical care, early morbidity due to suicide and a widespread susceptibility to other serious problems (Greenberg et al., 2003; Andlin-Sobicki and Wittchen, 2005).

1.2. Symptoms/Clinical Assessment of Depression Disorder

Most experts agree with the statement that MDD should be considered as a syndrome, not a disease. MDD is an affective disorder, whose feature is a negative or depressed mood occurring for an extended period of time. It includes body, mood and thoughts that affect the way, a person eats, the way one feels about oneself and one thinks about things.

As per the recently published reports, depression is a type of neuro-degenerative disorder and characterized by neuronal death in some brain regions, including hippocampus and prefrontal cortex, which are involved in the regulation of mood behavior. The neuro-degeneration in these regions also alters the neuronal circuitry to other brain regions and may induce several mixed type of abnormal behavioral symptoms in an individual.

Depression is characterized as per criteria given by Diagnostic Statistical Manual of Mental Disorders-IV/V (DSM-IV/V). The DSM manuals are a series of official publications of the American Psychiatric Association (APA) that gives a standardized view on the treatment, diagnosis and evaluation of mental disorders. It is an integrated manual that incorporates all aspects of mental disorders in humans, thereby, providing a common language, standardized evaluation, diagnostic and treatment advisory to the global community. According to DSM-IV/V, a MDD manifests with symptoms at different levels, such as psychological, behavioral and physiological levels. As per, DSM-IV/V criteria, **table 1** given below represents the symptoms of the MDD with their scoring pattern on the basis of duration of occurrence (APA, 1994; Hankin, 2006).

Table 1: Symptoms of MDD and Scoring Pattern

S. No.	Symptoms	Not at all	Several days	More than a half day	Nearly every day
1	Depressed or irritable mood	0	1	2	3
2	Decreased interest in pleasure activities	0	1	2	3
3	Significant weight loss or gain	0	1	2	3
4	Insomnia or hypersomnia	0	1	2	3
5	Psychomotor agitation or retardation	0	1	2	3
6	Fatigue or loss of energy	0	1	2	3
7	Feeling of worthlessness	0	1	2	3
8	Diminished ability to think or concentrate	0	1	2	3
9	Recurrent thought of suicide	0	1	2	3

All individuals with depression do not experience the similar symptoms mentioned above. The severity, incidence and duration of depressive symptoms vary, depending on the individual particular illness. The presence of five or more than five symptoms continuously for a period of 2-week, represent a change from normal functioning; atleast one of the symptoms is either (1) depressed mood or (2) anhedonia (decreased interest or pleasure in activities that usually would be enjoyed). The severity of MDD may then be graded according to the overall scores and intensity of the symptoms observed **(table 2)**.

Table 2: MDD Severity on the Basis of Scores

Key for Score	Depression Severity
1-4	Minimal Depression
5-9	Mild Depression
10-14	Moderate Depression
15-19	Moderately severe depression
20-27	Severe depression

The DSM series started with DSM-I (1968) and the latest one is DSM-V (2013). DSM-IV series was structured into a five-part axial system. The first axial system incorporates clinical disorders, while the second axis covers personality disorders and intellectual disabilities. The other remaining axils include medical, psychosocial, environmental and childhood factors,

functionally required to provide diagnostic criteria for health care evaluation. The organizational structure of DSM-IV failed to reflect shared features/or symptoms of associated disorders and diagnostic groups, such as psychotic disorders with bipolar, depression, anxiety and somatic disorders. Thus, DSM-V edition is restructured in 2013, which better reveals these interrelationships, within and across diagnostic chapters. DSM-V has incorporated many radical changes as well as introduced many new definitions and diagnostic criteria for the evaluation of previously unknown or poorly defined mental disorders. A detail of modifications made in DSM-V related to this study has been summarized in **table 3**.

Apart from the DSM-IV/V criteria, the other common symptoms which are not essential in diagnosis include constipation, decreased salivation and diurnal variations in the symptoms (worsening in the morning). The exclusion criteria are occurrence of these symptoms in schizophrenia or other neurological disorders and no evidence of recent death in the family or other traumatic events.

Table 3: The DSM-IV vs. DSM-V – A Critical Evaluation of Modifications

	DSM-IV	**DSM-V**
(A) Bipolar disorder	(1) Bipolar disorder comes under mood disorder category (2) Diagnosis of bipolar I disorder, mixed episode, requires that the individual simultaneously meet full criteria for both mania and major depressive episode	(1) Bipolar disorder is a separate category. (2) To enhance the accuracy of diagnosis and facilitate earlier detection in clinical settings, Criterion A for manic and hypomanic episodes includes an emphasis on changes in activity and energy as well as mood. (3) A new specified criteria, **"with mixed features,"** has been added that can be applied to episodes of mania/hypomania, when depressive features are present.
(B) Other Specified Bipolar and Related Disorder	(1) No specification of particular conditions	(1) Allows specification of particular conditions, like categorization for individuals with a past history of MDD, who meet all criteria for hypomania except the duration criterion. (2) A second condition is that too few symptoms of hypomania are present to meet criteria for the full bipolar II syndrome, although, the duration is sufficient at 4 or more days.

	DSM-IV	DSM-V
(C) Depressive Disorders	(1) Dysthymia is a separate category (2) Disruptive mood dysregulation disorder is not defined. (3) Pre-menstrual dysphoric disorder is not defined.	(1) Now falls under the category of persistent depressive disorder, which includes both chronic MDD & previous dysthymic disorder. (2) This is included to address concerns about potential over diagnosis and overtreatment of bipolar disorder in children up to age 18 years, who exhibit persistent irritability and frequent episodes of extreme behavioral dyscontrol three or more times a week for more than a year. (3) Pre-menstrual dysphoric disorder is now a distinct diagnosis in the Depressive Disorders chapter.
(D) Bereavement Exclusion for Depression	(1) There was an exclusion criterion for a major depressive episode that was applied to depressive symptoms lasting less than 2 months, following the death of a loved one. (2) Not differentiated between grief and depression	(1) This exclusion is omitted in DSM-V to remove the implication that bereavement typically lasts only 2 months when clinicians recognize that the duration is more commonly 1–2 years. (2) Several notes within the text delineating the differences between grief and depression.
(E) Anxiety Disorders	(1) Social Phobia in DSM-IV (2) Obsessive compulsive disorder (OCD) is included under Anxiety disorder. (3) Panic disorder and Agoraphobia are linked. (4) Separation Anxiety disorder and Selective Mutism fall under the chapter, Disorders of Infancy, Childhood or Adolescence.	(1) Social Phobia is now called as Social Anxiety Disorder (2) No longer includes OCD under Anxiety disorder. (3) Panic Disorder and Agoraphobia are unlinked, as many patients experience Agoraphobia without panic symptoms (4) Separation Anxiety Disorder and Selective Mutism now fall under the Anxiety Disorders chapter
(F) Post Traumatic Stress Disorder (PTSD)	(1) PTSD was not present as a new chapter, included under Anxiety disorder.	(1) It is included in a new chapter on Trauma and Stressor Related Disorders. (2) DSM-V pays more attention to the behavioral symptoms that accompany PTSD and proposes four distinct diagnostic clusters instead of three. (3) It will also be more developmentally sensitive for children and adolescents.

	DSM-IV	DSM-V
(G) Trauma and Stress Related Disorders	(1) Criterion A2 regarding the subjective reaction to traumatic event (e.g., "the person's response involved intense fear, helplessness, or horror").	(1) This criterion has been eliminated.
(H) Panic attack	(1) Situationally bound/cued, situationally predisposed and unexpected/uncured are the terms used for describing different type of panic attack.	(1) Expected and unexpected are the new terms to differentiate the type of panic attack. (2) Panic attacks function as a prognostic factor for severity of diagnosis, course and co-morbidity across many anxiety and other disorders and thus, can be listed as a specifier that is applicable to all DSM-V disorders.
(I) Agoraphobia, Specific Phobia and Social anxiety	(1) There is 6 months of duration for diagnosis, only limited to individuals under age of 18.	(1) Changes include deletion of the requirement that individuals over age 18 years recognize that their anxiety is excessive or unreasonable. (2) 6 months of duration is extended for all ages to minimize over diagnosis of transient fear.

1.3. Different Forms of Depression Disorder

As per the spectrum view of mood disorders (Angst and Cassano, 2005), depression disorder is not categorized into independent categories, as in DSM-IV/V. Instead, various depression forms lie along a sequence, which does not has sharp margins between categories, following a dimensional approach. Several forms of depression disorder are defined, mainly on the basis of severity scores and associated features. The most common forms of depression are MDD, dysthymic disorder and bipolar disorder. Each form has it's distinguish feature, yet all share almost same symptoms with different intensities **(table 4).**

1.3.1. Major Depressive Disorder (Unipolar Depression)

MDD or unipolar depression is mainly characterized by a group of symptoms that negatively affect the work ability, sleep, study, eating and enjoy once-pleasurable activities of an individual. It is pervasive, persistent and intense. MDD is disabling and prevents an individual from normal functioning. MDD episode may happen just once in a person's lifetime, but more often, it recurs throughout a person's life (Keller and Nesse, 2005).

1.3.2. Bipolar (Manic-Depressive) Disorder

Bipolar disorder, also known as manic-depressive illness, is not as frequent as MDD or dysthymia. Recently, several modifications have been made in the DSM-V manual for bipolar disorder, as shown in **table 3.** Bipolar disorder involves cycling mood changes—from extreme highs (e.g., mania) to extreme lows (e.g., depression). Patients, who experience both depressive and manic episodes, are classified under this category. Occasionally the mood switches are dramatic and rapid, but most often they are gradual. Near about 25% of the patients with MDD do experience a manic episode. In the depressed phase, people may have some or all the symptoms of a depression (depressed mood, hopelessness, anhedonia, varying sleep disturbances, difficulty in concentrating, psychomotor retardation and often, suicidal ideation), while in the manic phase, people may be experienced some or all the symptoms of mania (hyperactivity, grandiosity, euphoria, racing thoughts, decreased sleep, increased energy, talkativeness, risk taking behavior and insomnia).

Mania generally influences the judgment, thinking and social behavior of a person in ways that cause serious problems and embarrassment. The duration and intensity of bipolar disorder varies tremendously. More than just a brief good or bad mood, the bipolar disorder cycles take place for days, weeks, or months. After an initial episode of euphoria, subsequent episodes of either euphoria or depression are likely to occur (Stahl, 1998). The reasons for bipolar disorder aren't fully understandable, but it regularly occurs in families. The manic episodes have to be controlled with anti-psychotics agents, like lithium, carbamazepine, olanzapine, risperidone, ziprasidone, aripiprazole and depression component can be managed by conventional ADs like tricyclic anti-depressants (TCAs) and selective serotonin re-uptake inhibitors (SSRIs) (Potter and Hollister, 2004). Bipolar disorder comprises of four types of mood episodes, such as mania, hypomania, depression and mixed episodes as shown in **fig. 1**.

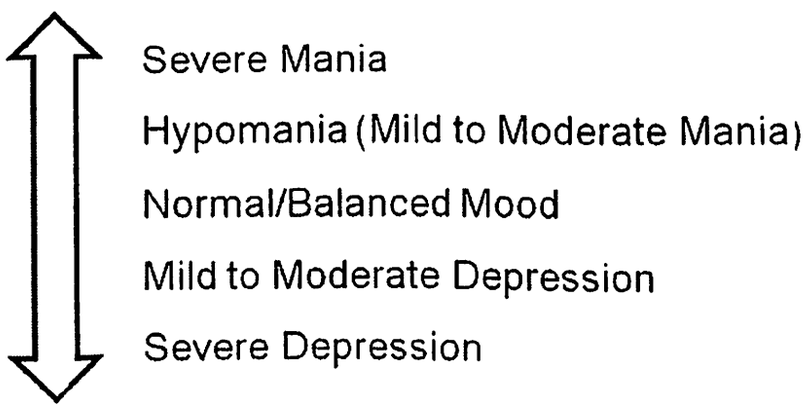


Fig. 1: Representing various stages in bipolar disorder

Table 4: Comparison of Symptoms for Different Types of Depression

Comparison of Specific Major Symptoms of Different Types of Depression Disorders						Common Symptoms of Different Types of Depression
Unipolar or MDD	**Dysthymia**	**Melancholic**	**Bipolar**	**Post-natal**	**Seasonal Affective**	
High agitation	Milder agitation than unipolar	More worsening of symptoms in morning time	Mixed type of symptoms	Miserable feeling especially in morning/evening time	Increased sleep	Feelings of hopelessness
Restlessness	Milder restlessness than unipolar	Excessive guilt	Flight of ideas	More tearful & strange behavior towards baby	Increased appetite particularly crave for carbohydrates	Low energy or fatigue
Irritability	Less irritability than unipolar		Hyperactivity		Weight gain	Poor sleep
Self hate	Milder pleasure loss than unipolar		Increased talkativeness			Poor concentration
Guilt			Increased energy			Thought of death & suicide
						Feeling of worthlessness
						Affects day to day life
						Poor appetite

1.3.3. Dysthymic Disorder

Dysthymic disorder, also known as dysthymia, is a low-level depression and characterized by long-term, such as two years or longer. Recently, according to DSM-V, dysthymia falls under the category of persistent disorder (**table 3).** The symptoms of dysthymia are less severe than MDD, which may not disable an individual, but disrupt the normal functioning or feeling well. The person may still be able to carry out daily responsibilities and obligations. Occasionally, people suffering from dysthymia may also experience one or more episodes of MDD during their lifetime-s. Without adequate treatment, dysthymia is more likely to become chronic (Stahl, 1998). It may represent a quite stable and chronic illness of low-grade depression or may represent a condition of partial recovery from an episode of MDD. Cognitive and psychotherapy are beneficial (Pinquart et al., 2006), though treatment with newer SSRIs have been the best solution (Koran et al., 2007).

1.3.4. Melancholic Depression

As per DSM-IV/V, a major depressive episode with melancholic specifier (melancholic depression) can be present in almost all mood disorders. It commonly occurs in older population and in more severe and psychotic depressions. Accounting for 40-60% of the untreated unipolar depression, the melancholic depression has no apparent external precipitating cause; hence, formerly it was referred as endogenous depression. According to the DSM diagnostic criteria, melancholic depression is characterized by;

(1) Loss of pleasure in activities or lack of reactivity to pleasurable stimuli
(2) Distinct quality of mood
(3) Worsening of symptoms in the mornings
(4) Sleep disturbances
(5) Pronounced psychomotor retardation or agitation
(6) Decreased eating or weight loss

Atleast the presence of any three symptoms, mentioned above indicate melancholic depression. Patients with melancholic depression, often have a history of one or more previous episodes of MDD with recovery (Kandel, 2000). They respond well to electro-convulsive therapy (ECT), TCAs and SSRIs (Potter and Hollister, 2004).

1.3.5. Atypical Depression

DSM-IV/V criteria indicate that a major depressive episode with atypical characteristics (atypical depression) may be observed in almost all types of mood disorders. It is characterized by (1) mood reactivity, (2) weight gain or increase in appetite, (3) hypersomnia

and (4) personality trait interpersonal rejection sensitivity (atleast the presence of any two indicate atypical depression) (Kandel, 2000). Atypical depression accounts for 15 % of patients hospitalised for MDD. Since, the symptoms are opposite to that of melancholic type, it is termed 'atypical' and occurs in earlier part of life (Stahl, 1998). Monoamine oxidase inhibitors (MAOIs) show better efficacy than TCAs in atypical depression (Potter and Hollister, 2004). Various distinguishing characteristics of atypical depression are the following.

(i) It commonly occurs in bipolar disorders (especially bipolar II disorder)
(ii) It commonly occurs in seasonal depression
(iii) It commonly occurs in younger than in older individuals
(iv) It has an early onset in comparison of MDD
(v) It has high prevalence rate in females
(vi) It has high chance of bipolar family history in comparison of MDD

1.3.6. Post-partum Depression (PPD)

PPD or post-natal depression is a form of clinical depression, which mainly occurs in women after child birth, usually in the first few months and may last up to several months or even a year (O'Hara and Swain 1996). Generally, PPD occurs within the first 3 months after delivery. Several studies have reported the prevalence rates of PPD among women from 5% to 25%. Methodological differences among these different studies make the actual prevalence rates unclear (Paulson and Bazemore, 2010). Women have mood changes, feeling of anxiety, irritation, tearfulness and restlessness during pregnancy, especially after delivery. Deficiency of vitamins levels in women are sometimes assumed as a major cause for PPD (Beard et al., 2005). Considerable research has established that alteration in women hormonal levels, during pregnancy, are more likely to cause PPD.

1.3.7. Seasonal Affective Disorder (SAD)

DSM-IV/V indicates that SAD is not a separate disorder, but indicates the major depressive episode of bipolar and depressive disorders. It is also known as seasonal depression, related to day length. Lack of light in winter season (short photoperiod) is sometimes assumed as a main reason for SAD (Terman and Terman, 2005). Symptoms of SAD are often atypical ones, such as hypersomnia and overeating. SAD may be effectively treated with light therapy, but approximately half of the population suffer from SAD and may not respond to light therapy alone. ADs and psychotherapy are more effective to reduce SAD symptoms, either alone or in combination with light therapy (Rohan et al., 2004).

1.3.8. Disease-Induced Depression

Sometimes a physical disease/disorder, such as Parkinson's disorder, heart diseases, stroke and Cushing's syndrome can cause symptoms similar to that of depression (Regier et al., 1993). Another cause may be hypothyroidism, where reduction of thyroid metabolism leads to impaired metabolism and induction of depression disorder (Regier et al., 1993).

1.4. Anxiety Disorders

Anxiety is a response to an unknown, internal, vague, or chronic threat. Anxiety disorders include generalized anxiety disorder (GAD), PTSD, panic disorder, social anxiety disorder, specific phobia and OCD. These disorders are characterized by shortness of breath, chest pain, motor tension, autonomic hyperactivity and increased vigilance. Recently, several guidelines have been revised in DSM-V manual for different types of anxiety disorder, as shown in **table 3.**

1.4.1. Generalized Anxiety Disorder

GAD is characterised as a long period of anxiety and worry. It is diagnosed when a patient experiences six or more months of excessive anxiety and worry accompanied by atleast three additional symptoms, such as restlessness, fatigue, difficulty concentrating, irritability, and muscle tension. Lifetime and 12-month prevalence rates of GAD are 2.8% and 1.2% for men and 5.3% and 2.7% for women, respectively (Vesga-Lopez et al., 2008).

1.4.2. Panic Disorder

Panic disorder is characterized by recurring and persistent panic attacks. A panic attack in panic disorder is a period of acute terror with physical symptoms, including shortness of breath, sweating, irregular heartbeat, dizziness, feelings of unreality and wanting to escape the place, where the attack began (Klerman et al., 1993). Moreover, in between the panic attacks, people suffer from anticipatory anxiety, which finally leads to agoraphobia (Vesga-Lopez et al., 2008). The prevalence rate of panic disorder is about 1-2%, where, women have two-fold chance in comparison to men to develop panic disorder. Panic attacks are generally associated with the features of social phobia, GAD and MDD as well.

1.4.3. Post Traumatic Stress Disorder

It is mainly characterized by persistent avoidance of stimuli associated with trauma and numbing of common awareness to current life events. PTSD also includes several other symptoms, including self-destructive and impulsive behavior, dissociative symptoms,

somatic objections, feelings of in-effectiveness, shame, hopelessness, loss of previously sustained beliefs, hostility and social withdrawal, along with a constant sense of being threatened and changes in personality characteristics. PTSD is diagnosed when exposure to perceived or actual threat of death or serious injury results in intense fear, helplessness, or horror for atleast one month.

1.5. Depression and Anxiety

Depression forms a risk factor for other central nervous system (CNS) disorders, including anxiety. Although, depression and anxiety are treated as distinct disorders, the difference is especially based on whether; the patient individually has a depressed or anxious mood (Kessler et al., 2005a). Studies have reported that approximately 80 to 90% of depressed patients have anxiety symptoms, although, about 30% show severe anxiety. Depression and anxiety are co-morbid conditions and symptomatology reflects affective and cognitive dysregulations, which are observed in depression and anxiety (Kessler et al., 2005a; 2005b).

1.5.1. Major Depression Disorder and Generalized Anxiety Disorder

Among anxiety disorders, GAD and PTSD have the greatest co-morbidity with MDD. Clinical studies have addressed that near about two thirds of population with a lifetime GAD, retrospectively show the presence of MDD, while, only one fifth of population with a lifetime MDD, retrospectively show the presence of GAD (Kessler et al., 2005a; 2005b). Thus, it indicates that GAD tends to come first and finally develops into MDD.

Table 5. DSM-IV Diagnostic Criteria for Mixed Anxiety-Depressive disorder (APA, 2000)

A. Persistent or recurrent dysphoric mood lasting atleast 1 month

B. The dysphoric mood is accompanied by atleast 1 month of 4 (or more) of the following symptoms:

- Difficulty concentrating or mind going blank
- Sleep disturbances (restless, unsatisfying sleep)
- Fatigue or low energy
- Irritability
- Worry and emotional imbalance (Hyper-emotionality)
- Natural Aversion and Social Impairment
- Hopelessness (pervasive pessimism about the future)
- Low self-esteem or feelings of worthlessness

C. The symptoms lead to clinically significant distress or impairment in social, occupational or other important areas of functioning.

Diagnostic co-morbidity can affect treatment planning. In addition, several anxiety symptoms are also frequently observed in depressed patients (Berton and Nestler, 2006). In fact, the symptomatology of depression and anxiety partly overlap, as there are number of symptoms that are common to both anxiety and depression **(table 5).** The attitude of patients with both disorders is more comfortless than for depressed individuals that do not suffer from anxiety.

1.6. Causes or Risk Factors Associated with Development of Depression Disorder

The bio-psychosocial model proposes that there is not a single factor responsible or involved in the progression of MDD. The situation is much more complex. A growing body of factors, namely biological, psychological and social, all play a major role in induction of depression (Caspi et al., 2003). The diathesis–stress model states that depression results, once a previous vulnerability or diathesis is activated by stressful life events. This vulnerability can be either genetically (Haeffel et al., 2008), involve an interaction between nature and nurture, or schematic, resulting from views of the world learned in childhood. Moreover, pre-clinical studies as well as current brain imaging technologies have shown that, during depression neural circuits, which are implicated in the regulation of mood, thought, appetite and behavior, failed to function normally along with dysregulated NTs.

1.6.1. Genetic Factor

MDD is a familial disease and genetic factors play very critical roles in disease progression. Depression, which associates with a person's biology or genetic inheritance, is sometimes referred as endogenous depression (Hamet and Tremblay, 2005). Studies reported that the heritability or genetic risk for depression is nearly 40-50 % (Sullivan et al., 2000; Levinson, 2006). Further, studies reported that heritability seems to be more prominent in women in comparison with men (Marcus et al., 2005), although, genes alone are not predictive for development of affective disorders.

Studies reported that one might hope that an acknowledgement of heightened risk would be accompanied by greater insight into the nature of depression, its recognition and the lowering of the barriers to treatment (Sullivan et al., 2000). Depression repeatedly runs in families. Relatives of depressed patient are more likely to develop the disease than those with no family history, although, there are chances that depression can strike a person with no family history. It is very well accepted today that induction of MDD disorder may result from an interaction between genetic liability and environmental risk factors (Farmer et al., 2005).

1.6.2. Psychological Aspects

Different personality features and their development appear to be essential for the occurrence and persistence of MDD with negative emotionality, as a general precursor (Kanter et al., 2004). Although, depressive episodes are strongly linked with adverse events, a person's trait of dealing may be associated with their resilience. In addition, low self-respect and embarrassing/or distorted thinking may be also related to the induction of depression. It is also predicted that depression is less likely to be observed as well as quicker to diminish among, those who are religious. Moreover, several times it is also not clear that which factors are the causes or which are the effects of depression. However, depressed persons, those have ability to reflect upon and challenge their thinking patterns, often show improved mood and self-respect (Monroe et al., 2007).

1.6.3. Social Factors

Studies have reported that poverty and social isolation in general population may also be major causes for increased risk of mental health problems (Kanter et al., 2004). Child exploitation (physical, emotional, sexual or neglect) is also involved in the development of depressive disorders, later in life (Slavich et al., 2009). Child exploitation by the caretaker is supposed to alter the personality development and produces more chances for depression and many other debilitating mental and emotional states. Moreover, disturbances in family functioning, such as, parental (particularly maternal) depression, severe marital conflict or divorce, death of a parent or other disturbances in parenting are additional major risk factors (Kanter et al., 2004). Clinical reports have shown that unfavourable conditions at work, particularly demanding jobs with little scope for decision-making, are also responsible for the development of depression.

Besides this, during adulthood, the occurrences of stressful life events are strongly associated with the onset of depressive episodes. In this context, life events linked to social rejection appear to be predominantly associated with induction of depression (Vilhjalmsson, 1993). Several researchers addressed that the relationship between stressful life events and social support has been a matter of some debate; the absence of social support may increase the possibility that stressful life events will lead to depression, or may comprise of harmful stimuli that leads to depression directly. Recently, published studies are in support with the hypothesis that stressful events link with an increased susceptibility for depression in a manner that stressful situations, often lead to the onset of illness and are also related to the severity of depression (Holsboer, 2001). Studies showed that a first depressive episode is more likely to be instantly led by stressful life events than the recurrent ones, which is

consistent with the hypothesis that people may become progressively more sensitized to life related stress over successive recurrences of depression (Vilhjalmsson, 1993).

1.6.4. Drugs Related Factors

As per the DSM-IV-TR criteria, drug(s)-induced depression is defined as a persistent disturbance of mood that is observed during the causal use of depression related medication or within 1 month of intoxication or withdrawal of drug therapy (DSM, 2000). Various classes of drugs, including anti-infective agents, cardiovascular agents, CNS drugs, hormonal treatments, immunological agents and chemotherapeutic agents increase the risk of developing MDD (APA, 1994). Various studies have mentioned that drug-induced depression occurs via several mechanism(s), such as a direct alteration of monoamine function, alteration of HPA axis activity, remarkable hormonal alteration and increase production of cytokines. In addition, alcoholism increases the risk of developing MDD (Boden and Fergusson, 2011). The symptoms of drug-induced depression may be similar to those of MDD, but do not have to meet full diagnostic criteria. Few other studies have reported that symptoms associated with drug-induced depression must be severe enough to induce clinically significant distress in social, occupational, or other areas of functioning.

1.6.5. Biological Factors/Patho-physiological Mechanism Involved in Depression

In the early 20th century the description of mental problem changed from a disease of the 'mind' to an appropriate brain dysfunction. Despite of a steady increase in the number of people treated for depression over the past thirty years, the prevalence of disorder remains stable, which may be due to unclear neurobiological understanding of patho-physiology. Many factors may contribute to the progression of depression (**fig. 2**); however, the etiological and pathological mechanisms underlying this disorder are not yet well established. Today, neuroscientists know that, in many cases, psychopathology arises as a result of impaired regulation of particular brain NTs. In continuation, cell and molecular biology have proved that it is helpful to study the mechanisms of information processing, plasticity and neuronal survival, which are involved in mood disorders. Hence, different theories have been postulated to account for the overall patho-physiological state or particular symptoms of depression based on dysfunction of monoamine neurotransmission (Prins et al., 2011), the HPA axis activity (Anacker et al., 2011), circadian rhythms (McClung, 2007) or neuro-immune processes (Miller, 2010) as **shown in fig. 2.**

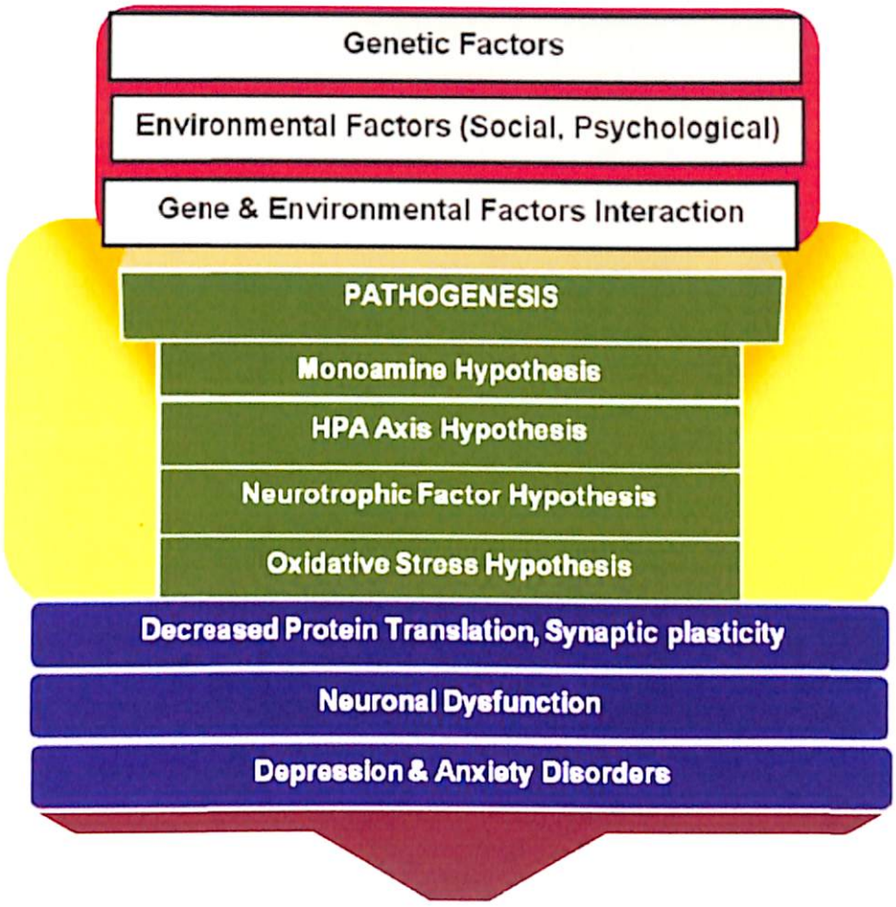


Fig: 2. Etiological and patho-physiological factors for depression disorder

1.6.5.1. Monoamine Hypothesis and Depression Disorder

The earliest description of this mental disorder dates back to the time of Hippocrates around 400 B.C. but the understanding of the pathology of depression is very limited (Nestler et al., 2002a). Brain functions are manifested through release of NTs (monoaminergic), inducing biochemical signaling events and regulation of neuronal plasticity and survival. Derangement in NT levels manifest psychiatric disorders, like depression, anxiety and other co-morbidity. The first biochemical hypothesis (monoamine hypothesis) of depression was formulated in the mid 1960s (Schildkraut, 1965), after the serendipitous discovery of the AD effects of monoamine treatment during the 1950s. In brain, the proper monoaminergic signaling (regulation of NT levels such, as 5-HT, NE and DA) is regarded as one of the key mechanism for the modulation of mood and emotion. There is a direct correlation between the monoaminergic neuronal systems and depression (Tremblay and Blier, 2006). Moreover, the monoamine hypothesis postulates that patho-physiology and/or behavioral manifestations of depression are associated with depletion in monoamine neural transmitters or functional deficiency of monoamine NTs in synaptic cleft (Schildkraut and Kety, 1967; Millan, 2006). The monoamine levels may be altered, as a result of disrupted synthesis, storage and release. Although in several cases, the level of monoamine may be normal, but

the post-synaptic receptors and/or sub-cellular messenger activity may impair. Most current ADs act by increasing the amount of monoamines (5HT or NE released & retained in the synapse) or by restoring the normal functioning of monoamines. Regulations of serotonergic and nor-epinephrinergic circuits are known to indirectly modulate the dopaminergic system, which is implicated in anhedonia behavior (Dunlop and Nemeroff, 2007).

Although, this hypothesis does not elucidate, what structures are affected in brain and how does deficiency in NTs affect the underlying function of these structures. Moreover, the synaptic monoamine levels are quickly augmented by ADs, the therapeutic effects require weeks to months to appear (Andrade and Rao, 2010). It is now believed that increasing monoamine levels in the synapse itself does not attenuate depression; rather, it is the effect of these monoamines, acting to regulate firing activity in multiple, connected, neural systems which, has primary effect in treating depression (Ressler and Nemeroff, 2000). The current research on the pathology and the treatment of depression is centred in finding affected area in brain and identify general mechanism of depression (Nestler et al., 2002a; 2002b).

1.6.5.2. Neuronal Survival/Neurogenesis Hypothesis and Depression Disorder

Recently, an emerging hypothesis suggests that MDD, which has been conceptualized as a neurochemical disorder, is associated with neuronal degeneration and impaired structural plasticity (Manji et al., 2003; Goshen et al., 2008). This hypothesis provides a frame work in which the patho-physiology and pharmacotherapy for depression congregate on information processing within specific neural networks (regulation of neuronal survival and synaptic plasticity), rather than changes in chemical balance (Perez and Tardito, 2001), **as shown in fig. 3.** Neuroplasticity refers to the ability of neurons to change with experience, in response to an intervention, environmental modification, or as a result of learning.

Considerable research has established that the activation of intra-cellular signaling cascades and regulation of genes expression influence structural plasticity and neuronal survival in brain regions. Prefrontal cortex, hippocampus and amygdala are the brain regions, which are mainly involved in mood regulation (Yu and Chen, 2011). Over the past few decades, a large number of evidences have addressed that depression involves a deficit of synaptic plasticity in hippocampus and amygdala that is mediated via intra-cellular cascades (Pittenger and Duman, 2008). Recent researches suggest that adult neurogenesis may have a role in etiology and treatment of MDD (Ehninger and Kempermann, 2008) and be a requirement to achieve the behavioral effects of ADs (Santarelli et al., 2003).

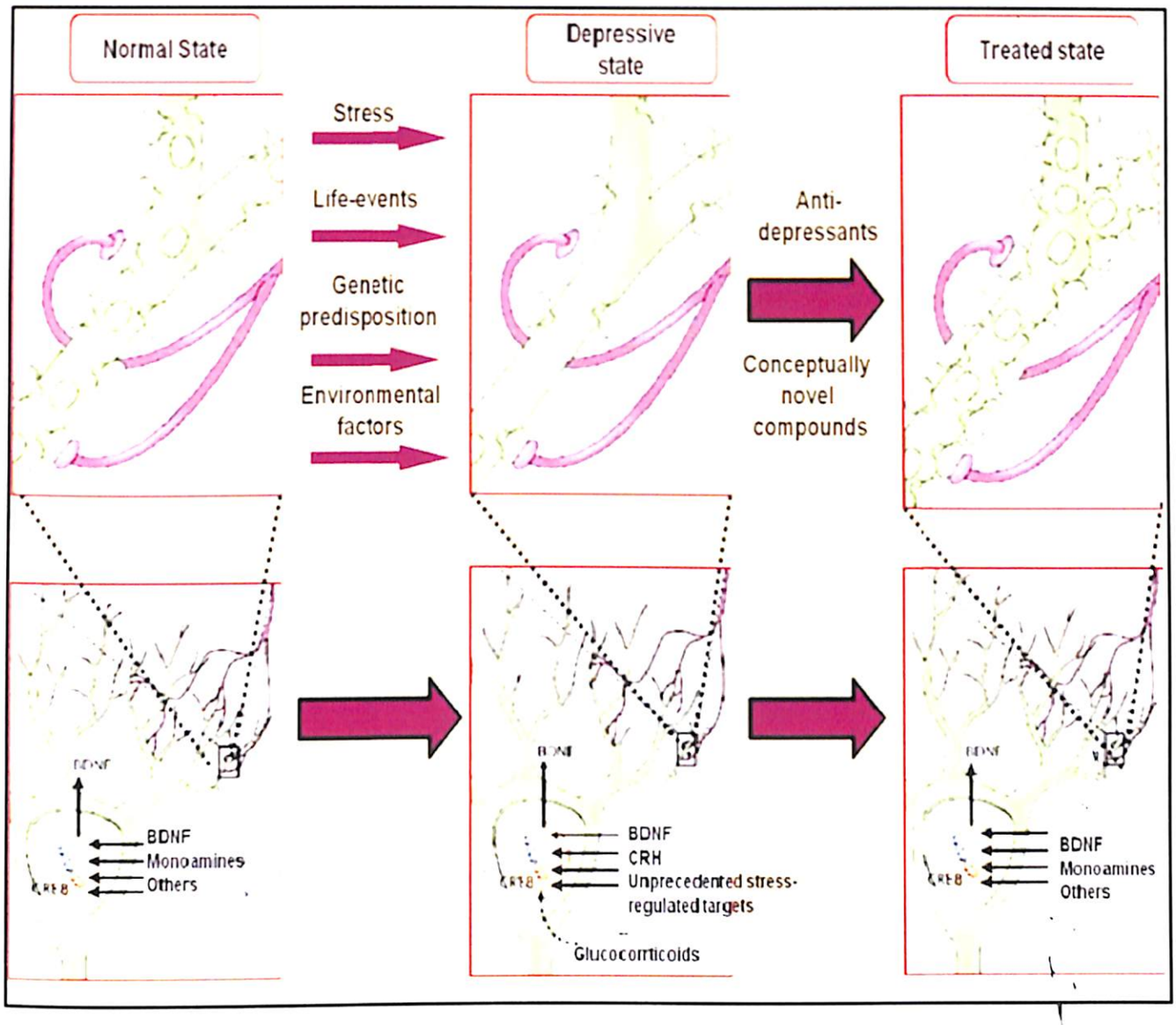


Fig. 3. The intracellular neurotrophic mechanism beyond the receptor level, indicating the mechanism of depression and antidepressants. **Normal state** shows a normal hippocampal pyramidal neuron and its innervations by monoaminergic and other types of neuron. **Depressive state** causes several changes in these neurons, including a reduction in their dendritic arborization and a reduction in BDNF expression, mediated partly by excessive glucocorticoids. **Treated state** shows the opposite effects to those seen in **Depressive state;** they increase dendritic arborization and BDNF expression of these hippocampal neurons. (*Adopted from Olivier and Nestler, 2006*).

1.6.5.3. Hypothalamic–Pituitary–Adrenal Axis Hypothesis and Depression Disorder

Recently, a growing body of clinical data suggests that stressful experiences play an important role in the onset and relapse of depression in humans (Charney and Manji, 2004; De Kloet et al., 2005; Heim et al., 2008). The involvement of stress in the patho-physiology of depression disorder was primarily drawn from the observations of hyperactivity of the HPA axis and increased cortisol levels in depressed patients (Dinan, 1994). Studies reported that there is a causal relationship between the incidences of depression disorder and the hyperactivation of HPA axis (Nikisch et al., 2005; Himmerich et al., 2007).

Numerous pre-clinical and clinical studies evidences suggest that hyper-activation of HPA axis has been observed in depressed patients, who do not respond to currently marketed ADs (De Kloet et al., 2005; McEwen, 2008). The hyperactivation of HPA axis is characterized by increase level of circulating glucocorticoids (GCs) (Murray et al., 2008). High circulating GCs level leads to hippocampal neuronal degeneration (Murray et al., 2008) and induces depressive-like behaviors in rodents and these behaviors, however, are significantly reversed by AD treatments (Johnson et al., 2006; Murray et al., 2008). Studies reported the three types of GCs mechanism for the development of stress disorders:

(a) GCs-induced atrophy: A few weeks' exposure to high GCs level or to stress, causes reversible atrophy of dendrite processes in hippocampus region;

(b) GCs neurotoxicity: Over the course of months, exposure to GCs kills hippocampal neurons and

(c) GCs neuro-endangerment: Elevated levels of GCs at the time of a neurological insult, such as stroke or seizure, which alter the ability of neurons to survive the insult.

1.6.5.4. Neurotrophic Factor Hypothesis and Depression Disorder

Besides this in recent years, a neurotrophic factor hypothesis has postulated that the structural and chemical alterations seen in brain of depressed patients led to investigation of neurotrophic mechanism in brain. This hypothesis supports an important role of neurotrophic factors in mood disorders and in mediating the beneficial effect of ADs.

1.6.5.4.1. Brain Derived Neurotrophic Factor

BDNF is a 14 kDa protein, belongs to the neurotrophin/nerve growth family that also includes other nerve growth factor, namely, neurotrophin-3 and neurotrophin-4. Several molecular cloning and sequencing studies indicate that BDNF is related to nerve growth factor, as evidenced by sequence homology, which was the primary peptidic growth factor discovered with neurotrophic activity (Cohen et al., 1954). The gene encoding BDNF, located on chromosome 11p13, belongs to gene family, which have been identified to be responsible for MDD (Maisonpierre et al., 1990; Levinson, 2006).

1.6.5.4.2. Structure and Types of Brain Derived Neurotrophic Factor

The genomic structure of BDNF protein is relatively complex and its gene expression depends on several regulatory regions. The BDNF gene is composed of 4 distinct non-coding 5` exons with their unique promoters and one common 3` coding exon that produces the mature BDNF (mBDNF) protein (Timmusk et al., 1993). BDNF protein has two forms,

such as un-cleaved precursor or immature BDNF protein (ProBDNF) and mBDNF. Each of the BDNF form shows distinct binding characteristics and biological activity (Teng et al., 2005).

1.6.5.4.3. Synthesis, Secretion and Storage of Brain Derived Neurotrophic Factor

BDNF is predominantly produced by neurons and secreted in response to neuronal activity, mainly through regulated pathway. It is derived from both pre- and post-synaptic sites. Several studies indicate that astrocytes also produce and release BDNF, under the control of neuronal activity (Juric et al., 2006).

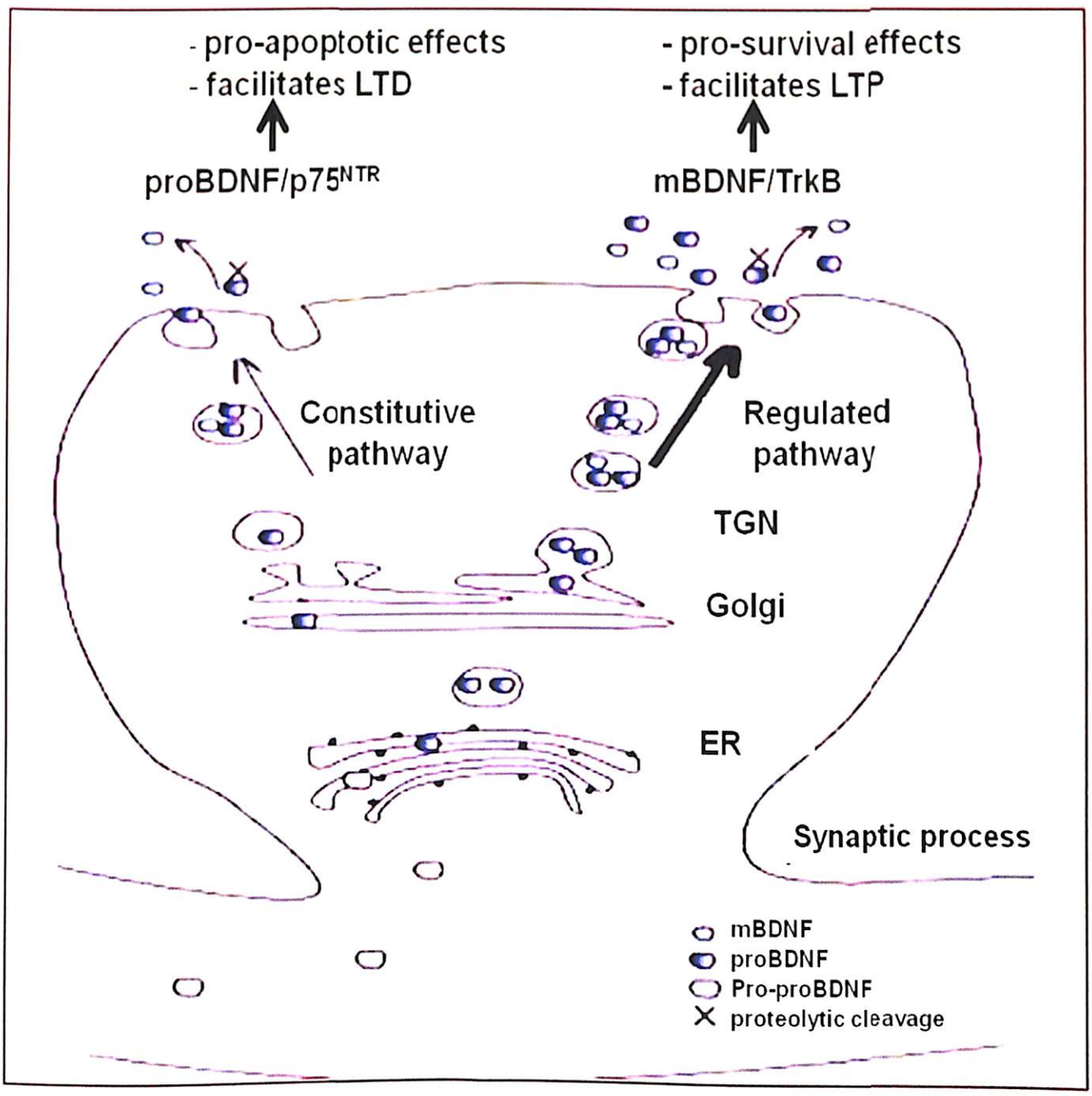


Fig. 4. BDNF processing, packaging and secretion in neurons *(adopted from Cunha et al., 2010)*. ER - Endoplasmic reticulum, TGN - Trans-Golgi Network and mBDNF - Mature BDNF.

BDNF is synthesized as pre-proBDNF, a precursor protein. In endoplasmic reticulum, the pre-sequence of BDNF protein is cleaved off, resulting in a 32-kDa, proBDNF protein **(fig. 4).** The 32-kDa proBDNF protein sequence moves into the trans-Golgi network, through the Golgi apparatus, where two different kinds of secretory vesicles for constitutive secretory (spontaneous release) and regulated pathways (release in response to stimuli & secretion is activity-dependent) are generated. ProBDNF packages in both the vesicles types, is either cleaved intra-cellularly by enzymes like furin/pro-convertases and secreted as 14 kDa mBDNF or secreted as proBDNF and then cleaved by extracellular proteases, such as metalloproteinase and plasmin to mBDNF (Lessmann et al., 2003). The level of intra-cellular and extracellular processing of proBDNF is not exactly clear, but proBDNF is less efficiently processed by intra-cellular proteases. Both proBDNF and mBDNF are preferentially stored in vesicles of regulated pathway (Cunha et al., 2010).

1.6.5.4.4. Polymorphism of Brain Derived Neurotrophic Factor Gene

The dendritic trafficking and synaptic localization of BDNF protein is mainly regulated by its pro-domain. However, polymorphism of BDNF gene affects the trafficking and localization of BDNF. Several studies reported a single nucleotide polymorphism (rs6265) in exon 11 of the BDNF gene, where the 66th amino acid valine converts into methionine (BDNFMet) in the pro-domain of BDNF protein (Schumacher et al., 2005).

1.6.5.4.5. Distribution and Regulation of Brain Derived Neurotrophic Factor

BDNF is the most abundant neurotrophic factor in mammalian brain (Duman et al., 1997). It is extensively expressed in various brain regions, including hippocampus and prefrontal cortex, which are involved in mood regulation (Murakami et al., 2005). In brain, BDNF expression is regulated by various factors, including stress (Nair and Vaidya, 2006), exercise (Berchtold et al., 2005), enriched environment (Rossi et al., 2006) and ischemia (Lee et al., 2004). Moreover, neuronal BDNF expression is affected by γ-amino butyric acid (GABA), glutamate neurotransmission and cell membrane depolarization, via Ca^{+2}-mediated channels (Aid et al., 2007).

1.6.5.4.6. Receptor of Brain Derived Neurotrophic Factor

The specific tyrosine kinase (Trk) that regulates BDNF-mediated signalling is known as TrkB receptor. Both the BDNF forms, namely ProBDNF and mBDNF are activated via two different receptor systems. The cellular actions of both BDNF forms are mediated through TrkB and p75 neurotrophin receptor (p75NTR), a member of tumor necrosis factor receptor super-family (Chao, 2003). ProBDNF form preferentially activates p75NTR to mediate

apoptosis, whereas, mBDNF form selectively activates TrkB receptors to promote survival **(fig. 4).** The majority of BDNF-mediated cellular functions are attributed to the TrkB signaling. The mBDNF form directly binds and dimerizes TrkB receptor, resulting in activation of Trk, present in their cytoplasmic domains. It then leads to the activation of various signaling pathways: MEK–MAPK, phosphatidylinositol-3-kinase and phospholipase C-g (Chao, 2003; Huang and Reichardt, 2003). However, ProBDNF, which preferentially binds to p75NTR, activates a different set of intra-cellular signaling transduction cascades, like nuclear factor-kappa B or c-Jun kinase (Teng et al., 2005). Activation of p75 by proBDNF has been linked to the activation of apoptotic signaling and initiation of N-methyl D-aspartic acid (NMDA) receptor-dependent synaptic depression in brain (Lu et al., 2005).

1.6.5.4.7. Brain Derived Neurotrophic Factor and Depression Disorder

Considerable researches have been addressed that alteration in level/functioning of BDNF is related to the patho-physiology of depression disorder (Murakami et al., 2005; Castren, 2007) and plays an important role in the actions of ADs treatment (Calabrese et al., 2009; Kunugi et al., 2010). Several reports have evidenced that the polymorphic form of BDNF (BDNFMet allele) is associated with poorer cognitive function and reduced hippocampal region activity, both of which are evident in MDD (Egan et al., 2003). Moreover, clinical studies reported that depressive patients have a low serum BDNF level as compared to control subjects (Karege et al., 2005; Aydemir et al., 2007). Further, post-mortem analyses of brain tissue samples from depression patients showed a decreased BDNF level (Castrén et al., 2007), whereas, infusion of BDNF in brain produced AD-like effect in rodents (Shimizu et al., 2003; Başterzi et al., 2009). Studies have found that decreased or altered expression of BDNF is associated with reduced neurogenesis, low synaptic plasticity and neuronal atrophy in hippocampus, result in maladaptive changes in neural networks that underlie the patho-physiology of depression (Kuipers et al., 2003). According to the neurotrophic factor hypothesis, an increase in BDNF level, following AD treatment is thought to play a key role in neuronal development, plasticity and survival and implicated as a possible mediator of AD-like effect (Nibuya et al., 1995; Conti et al., 2002).

1.6.5.5. Oxidative Stress Hypothesis and Depression Disorder

In fact, one of the other recent attempts to explain the etiology of the depression disorder is the oxidative stress hypothesis (Michel et al., 2007). The facts for this theory comprise of high oxidative damage/stress and low anti-oxidant enzymes activity in the CNS (Maes et al., 2009; 2011). In general, there is a balance between the generation of free radicals and anti-oxidant defence system activity. Free radicals are produced constitutively under normal

physiological states. In response to free radical generation, organisms have developed various defence mechanisms namely, various anti-oxidant enzymes and free radical scavengers to protect themselves against injury from free radicals (Reiter, 1995). However, in several conditions, where the formation of free radicals exceeds the capacity of anti-oxidant defence system, oxidative stress may lead to membrane degradation, cellular dysfunction and apoptosis (Niki et al., 1993).

The brain is particularly vulnerable to reactive oxygen species (ROS) production because it metabolizes 20% of total body oxygen and has a limited amount of anti-oxidant capacity. Previous studies reported that stress has a significant impact on ROS formation in brain, which in turn results in oxidative damage in the CNS (Fontella et al., 2005; Lucca et al., 2009). In brain, oxidative and nitrosative stress are well known to linked to neuronal cell injury or death and contribute to neuronal degeneration in the progression of neuro-degenerative problems (Teyssier et al., 2010; Maes et al., 2011). Induction of increased levels of oxidative stress in brain is considered as a major factor for neurotoxicity towards the patho-physiology of depression disorder (Sarandol et al., 2007; Rothman and Mattson, 2010). The relationship between high oxidative stress and depression has been reported in both pre-clinical and clinical studies. Recent studies have consistently reported increase ROS level in serum and plasma of depressed patients, especially with melancholia associated (Maes et al., 2009). Moreover, study addressed the evidences of high oxidative stress in depression, as reflected by increased oxidative stress in frontal regions of depressed patients, compared to those of matched controls (Michel et al., 2007).

In addition, a growing body of data have reported that alteration in brain anti-oxidant defence systems (SOD, CAT and GSH) for scavenging ROS to prevent neuronal damage, induced depressive-like behavior (Ng et al., 2008; Zafir et al., 2009). Pre-clinical reports have addressed that anti-oxidants (radical scavengers) may have AD potential (Eren et al., 2007; Zafir et al., 2009). Hence, it appears realistic to suggest that exogenous anti-oxidants may be effective in treating depression.

1.7. Current Therapeutics Approaches for Depression Disorder

Over the last half century, the research in psychiatric area has undergone a major paradigmatic shift. The treatment of any disease depends on our understanding for the patho-physiology of disease and of the mechanisms by which drugs relieve symptoms of particular problem. Despite the relative lack of adequate understanding of the aetiology and patho-physiology of depression, there are several treatments available for MDD and patients

with MDD showing significant improvement with optimal treatment. The pharmacotherapy available for depression symptoms are based on two chance discoveries (serendipitous findings) that were made more than half a century ago. First, that monoamine depletion with anti-hypertensive agent reserpine causes depression in some patients and second, that anti-tubercular agent isoniazid, which inhibits monoamine oxidase (MAO), an enzyme responsible for degrading monoamine NTs was noted to improve patients' mood (Nestler et al., 2002a; Morilak & Frazer, 2004).

Early theories implicated a direct correlation between the monoamine neuronal systems and depression disorder, where, decreased monoamine availability as a biological substrate of depression (Schildkraut, 1965). Conventional ADs use for depression pharmacotherapy directly affect monoamine turnover in brain and engage in the restoring of normal function of monoamine associated signaling pathway (Schildkraut and Kety, 1967; Millan, 2006). Monoamines currently monopolize the treatment of depressive states in the sense that they are engaged by all currently available AD agents (Millan, 2004). The understanding of the mechanism(s) of action of these available drugs led to the common idea that all effective AD medications produce beneficial effects by increasing the activity of brain's serotonergic or nor-adrenergic systems at post-synaptic monoamine receptors (Holsboer, 2004). Current treatments for depression include various non-pharmacological approaches and pharmacological approaches, such as AD drugs in severe cases. **Table 6** summarizes the information of various classes of conventional pharmacotherapy for depression.

1.7.1. Tricyclic Anti-depressants

In the 1950s, researchers were attempting to create anti-psychotic drugs, created the tricyclic molecule imipramine. Imipramine was demonstrated to possess AD properties (Azima and Vispo, 1958), leading to the development of additional tricyclic compounds as ADs. The therapeutic potential of first-generation tricyclic agents, including tertiary amines, amitriptyline and imipramine, reflect combined inhibition of 5-HT and NA re-uptake in serotonergic or adrenergic nerve endings in brain (Millan et al., 2001; Davis et al., 2002). Secondary amines include: nortriptyline, desipramine, imipramine and amitriptyline. It has since been determined that these drugs block pre-synaptic re-uptake transporters for the NTs such as 5-HT and NE and this is thought to reduce symptoms of depression. However, TCAs also block post-synaptic receptors for histamine (HT), resulting in sedation and for acetylcholine receptors, resulting in blurred vision, tachycardia and cognitive distortion. Further, supplementary antagonist activities of TCAs at different 5-HT receptors (including 5-HT receptor blockade) may contribute to their valuable effects (Palvimaki et al., 1996).

Table 6: ADs and their Common Side-effects (Rajkumar and Mahesh, 2008)

S.No.	Class	Marketed drugs	Common side-effects
1	Tricyclic Anti-depressants	Imipramine, Desipramine, Trimipramine, Amitriptyline, Nortriptyline, Protriptyline, Doxepin	Sedation, tremor, blurred vision, arrhythmias orthostatic hypotension, weight gain, sexual disturbances
2	Tetracyclic Anti-depressants	Amoxepine, Mianserin Maprotiline	Similar to TCAs
3	Monoamine Oxidase Inhibitors	Tranylcypramine, Phenelzine, Moclobemide	Sleep disturbances, weight gain, sexual disturbances
4	Selective 5-HT Re-uptake Inhibitors	Fluoxetine, Sertraline, Paroxetine, Fluvoxamine, Citalopram	Gastro-intestinal disturbance, sexual dysfunction, insomnia
5	Dual 5-HT and NA Re-uptake Inhibitor	Venlafaxine, Duloxetine, Desvenlafaxine	Nausea, sweating, dry mouth, dizziness, hypertension
6	$5\text{-}HT_2$ Antagonist and Re-uptake Inhibitors	Nefazodone, Trazodone	Drowsiness, dizziness, insomnia, nausea, agitation
7	NE and DA Re-uptake Inhibitor	Bupropion	Dizziness, dry mouth, sweating, tremor, agitation, aggravation of psychosis, seizures (High doses)
8	Nor-adrenergic and Specific Serotonergic anti-depressants	Mirtazapine	Somnolence, increased appetite, weight gain, dizziness, sedation
9	NA Specific Re-uptake Inhibitor	Reboxetine	Dry mouth, constipation, headache, drowsiness, dizziness
10	5-HT Re-uptake Enhancer	Tianeptine	Abdominal pain, dry mouth, anorexia, nausea, vomiting

1.7.2. Monoamine Oxidase Inhibitors

MAOIs class agents were among the first drugs to be proposed clinically as ADs, but were mainly overcome by TCAs and other class of ADs, whose clinical efficacies were considered to be better and side-effects generally less than those of MAOIs. MAOIs inhibit the MAO enzyme, a mitochondrial bound enzyme having two iso-forms MAO-A and MAO-B (Bortolato et al., 2008). MAO-A iso-form with high affinity oxidizes 5-HT and NE, while MAO-B iso-form with high affinity oxidizes DA (Johnston, 1968). The reaction mechanism of MAO involves oxidative deamination of primary, secondary and tertiary amines to the corresponding aldehyde and free amine, with the generation of hydrogen peroxide (Youdim and Bakhle, 2006). These NTs are among the most widely used and universally distributed neurochemical systems in brain (Youdim and Bakhle, 2006). The reversible MAO-A inhibitors, like moclobemide have good safety profile and very low incidence of side-effects as compared to irreversible inhibitors, including phenelzine, isocarboxazid and tranylcypromine (Swinkels and de Jonghe, 1995). The side-effect profile of these drugs is a major limiting factor in their usage. Despite the side-effects, even the original MAOIs are still prescribed and can be successful in patients, who failed to respond to SSRIs and TCAs.

1.7.3. Selective Serotonin Re-uptake Inhibitors

The hypothesis which addressed that serotonergic neurotransmission as the key deficit underlying depressive states, prompted the development of well-known SSRIs, drugs that "selectively" interact with 5-HT transporters (Manji et al., 2003). In 1987, FDA approved FLX, first agent of the SSRIs class for MDD. This group of compounds have a selective effect on the pre-synaptic re-uptake of 5-HT and has assumed most commonly prescribed group of ADs in the management of depression. SSRIs were expected to maintain the therapeutic properties of tricyclic agents, while lacking undesirable side-effect profile of TCAs. Thus, SSRIs are definitely more effective, comparatively safe and cost-effective ADs with an improved therapeutic window as compared to TCAs (Barrett et al., 2005).

1.7.4. Nor-adrenaline Re-uptake Inhibitor (NRI)

As with 5-HT, a role for NE in depression was suggested by the depressogenic features of reserpine. Reboxetine was the first drug to be described as an NRI. NRIs work by inhibiting the NE re-uptake and thereby, increase the concentration of NE in synaptic cleft and enhance the α1-adrenergic receptor (AR) stimulation. The drugs of this class are devoid of the receptor-blocking actions of TCAs, at HT and muscarinic receptors. Previous study addressed that, in out-patients with depression, reboxetine was found to be more effective than FLX in improving social functioning. The efficacy included greater improvement on

several parameters, like measuring work, spontaneous activity and the ability to manage with finances. Recently, attention has been directed to the possibility that NE-induced activation of β-AR in hippocampus participates in the mechanisms of synaptic plasticity engaged by NARIs and other ADs with an adrenergic mechanism of action (Crissman and O'Donnell. 2002; Mallick et al., 2005). The selectivity of reboxetine for AR and low side-effect profile than other drugs and placebo (i.e. dry mouth, constipation and sexual dysfunction), makes this agent, as a drug of choice with better tolerability (Wong et al., 2000).

1.7.5. Serotonin Nor-adrenaline Re-uptake Inhibitors (SNRI)

The agents of this class are typically used for depression, anxiety and chronic pain. Unlike SSRIs, SNRIs work by inhibiting the re-uptake of two NTs namely, 5-HT and NE. This leads to an increase in the extracellular levels of both the NTs and, therefore, an increase in neurotransmission. This class drugs differ from TCAs, by their lack of receptor-blocking activity at histaminergic, cholinergic or AR, thereby, avoiding side-effects such as hypotension and sedation. Compared with imipramine, VLA treated patients showed greater reductions in Montgomery Asberg Depression Rating Scale total scores from week 4 until the end of the study, showing a significant difference in favour of VLA at week 4.

1.7.6. Nor-adrenergic and Specific Serotonergic Anti-depressant (NaSSA)

The term NaSSA describes a new class of drugs with complex pharmacology. Currently, mirtazapine is the only available drug in this class, although, mianserin also shows strong similarity. It works by blocking α2-NA autoreceptors, which results in increased release of NE from nor-adrenergic terminals and by blocking α2 heteroreceptors (receptors on 5-HT neurons), which increases 5-HT release from serotonergic terminals (Berendsen and Broekkamp, 1997). Increased NE release also raises the firing of serotonergic neurons by stimulating α1 receptors. In addition, mirtazapine blocks post-synaptic 5-HT_2 and 5-HT_3 (but not 5-HT_{1A}) receptors; it is this action that indicates the explanation 'specific serotonergic'. Mirtazapine also strongly blocks HT_1 receptors, resulting in sedative effect. It also shows slight effect on acetylcholine, DA and α1-ARs. Mirtazapine mediated blockade of 5-HT_2 and 5-HT_3 receptors minimises the side-effects (insomnia, agitation, sexual dysfunction and nausea), that are observed due to the stimulation of these receptors.

1.7.7. Nor-adrenaline and Dopamine Re-uptake Inhibition (NDRI)

The drug falls into NDRI category is Bupropion (BUP). This is the only AD that ignores the 5-HT system and acts selectively on the nor-adrenergic and dopaminergic systems (Cooper et al., 1994). NDRI class drugs act as re-uptake inhibitor for NE and DA NTs, by blocking the

action of nor-epinephrine transporter (NET) and dopamine transporter (DAT), respectively (Stephen, 2009). This leads to increase in extracellular levels of both NE and DA and, therefore, an increase in adrenergic and dopaminergic neurotransmission (Stephen, 2009). BUP is a well known AD with high efficacy, but it is mainly popular as an alternative medication, in the cases, when first-line SSRI are ineffective. It's one of the few ADs that don't cause sexual side-effects. The side-effects associated with this class of drug are mostly due to DA over-stimulation.

1.7.8. Serotonin Antagonist and Re-uptake Inhibitors (SARIs)

5-HT antagonists boost 5-HT by blocking its re-uptake at nerve synapses, much like the SSRIs. The drugs, which belong to this class, are nefazodone and trazodone. The only difference of this class drug from SSRIs is the blockade of 5-HT_2 receptors. Nefazodone and trazodone acts primarily as a potent antagonist of 5-HT_{2A} receptors. Both of these drugs appear to block some of the 5-HT receptor sites on post-synaptic nerve membranes, namely the 5-HT_{2A} and 5-HT_{2C} receptors. While blocking these receptors, trazodone and nefazodone indirectly stimulate another type of 5-HT receptor called the 5-HT_{1A} receptor (http://holisticonline.com/remedies/depression/dep_AD-serotonin-_ntagonists.htm). This pre-synaptic autoreceptor in the raphae nuclei influences neuronal firing, 5-HT synthesis and release. These drugs have negligible affinity for cholinergic and histaminergic receptors. SARIs do not produce some of the side-effects that are produced by SSRIs, such as, short-term increase in anxiety or insomnia and sexual dysfunction (Stahl, 1998).

1.7.9. Triple Re-uptake Inhibitors (TRI)

The monoamines transmitters have been related to depression for more than four decades. The development of TRI was based on the hypothesis that adding a stimulatory dopaminergic component to a dual 5-HT and NE re-uptake inhibitor that might be superior to current pharmacotherapy in one or more dimensions, including rapid onset, efficacy and better side-effect profile (Trivedi et al., 2006). DOV 216,303 inhibits NE, 5-HT and DA re-uptake, in in-vitro studies at concentration of 20, 14 and 78 nM, respectively (Skolnick et al., 2006). Skolnick and Colleagues (2006) showed that DOV 216,303 is well-tolerated in humans and reduced the HAM-D scores of depressed patients, as like SSRIs.

1.7.10. Selective Serotonin Re-uptake Enhancer (SSRE)

SSREs are a class of drugs, which enhance the plasmalemmal re-uptake of 5-HT, leading to a decrease in synaptic concentrations of 5-HT and ultimately, a decrease in serotonergic neurotransmission (Mennini et al., 1987). Tianeptine, a tricyclic compound of

dibenzothiazepine type, is the only known drug of this class. This class of agent has been shown to be as effective as SSRIs against depression, with a faster onset of action (immediate) and better tolerability profile (Defrance et al., 1988). The exact mechanism of action of tianeptine remains unclear. Although, it is speculated that tianeptine might act as an allosteric modulator of serotonin transporter at a separate site that is normally targeted by SSRI class agents. Recent study indicates that tianeptine direct action, is an alteration of AMPA glutamate receptor activity, resulting in enhanced serotonin re-uptake in a downstream mechanism, which seems to involve altered neuroplasticity and release of BDNF. Particularly, in hippocampus, tianeptine prevents stress-induced dendritic atrophy, improves neurogenesis, reduces apoptosis and normalizes metabolite levels and hippocampal volume (Kasper and McEwen, 2008).

Recently, a substantial body of literature provides strong evidence to support that non-monoaminergic system might be an attractive target, when investigating depression-like behavior or resilience in pre-clinical and clinical study. Several reports addressed that a dysfunction of cAMP-mediated signal transduction may be implicated as promising mechanism in the patho-physiology of psychiatric disorders, including depression and anxiety. Impairments of cAMP-mediated signal transduction are responsible for reduced plasticity and neuronal survival in depression and other psychiatric disorders (Manji and Duman, 2001). In this respect, agents such as Phosphodiesterases (PDEs) inhibitors are expected to influence cAMP intracellular cascade may act as a potential strategy to treat depression and anxiety disorder. PDEs are key enzymes in various cellular signaling pathways. They degrade secondary messengers and their inhibition via specific inhibitors proposes distinctive 'receptor-independent' prospects to modulate cellular functions. Of the eleven PDE families, PDE4 is highly specific for cAMP and implicated in the control of mood, neuro-protection, anxiety and dementia expression in CNS. Recent findings support the hypothesis that PDE4 might be associated with anti-depressant and anti-stress effects. Despite increased interest among the clinical neurosciences, information regarding the AD- and anxiolytic-like activity of PDE4 inhibitors is still not explored adequately. Thus, selecting the tests sensitive to AD and anxiolytic drugs, the present study was designed to investigate the AD and anxiolytic potential of PDE4 inhibitor in behavioral models of depression.

Chapter 2: Review of Literature

2. General Information for Cyclic Nucleotide Phosphodiesterases

2.1. Background

Four decades ago, almost immediately after the discovery of secondary messenger cAMP, Sutherland and Rall first reported the hydrolytic activities of cyclic nucleotide PDEs enzymes (Butcher and Sutherland, 1962). PDEs are a super family of metallophosphohydrolases enzymes, which catalyse the hydrolysis of cAMP and GMP to their corresponding inactive 5-monophosphate counterparts (Bender and Beavo, 2006). PDEs hydrolyze cAMP and cyclic guanosine monophosphate (cGMP) by breaking their phosphodiester bond with the corresponding monophosphate. By hydrolyzing cyclic nucleotides, PDEs enzymes play a crucial role in the regulation of various cellular functions. The existence of multiple PDE species, however, was not appreciated until almost a decade after they were described (Thompson and Appleman, 1971).

2.2. Types and General Characteristics of Different Phosphodiesterases Enzyme

PDEs comprise of atleast eleven families of enzymes (PDE1–PDE11) and most of these families have more than one gene product (e.g., PDE4A, PDE4B, PDE4C and PDE4D). Moreover, each gene product may comprise of multiple splice variants, like PDE4B iso-form has 5 splice variants (PDE4B1–PDE4B5). Transcription from different initiation sites and differential splicing of mRNAs in these genes products, results in the generation of about 100 iso-forms of PDE proteins, which are found in all cell types and almost in all sub-cellular compartments (Bender and Beavo, 2006). PDEs are categorized into 11 different families, based on various characteristics, including primary structural similarity (PDEs sequence homology), protein domains and enzymatic properties, including substrate specificity (cAMP or cGMP), kinetic properties, sensitivity to endogenous regulators [e.g. Ca^{+2}/calmodulin (CaM)], iso-forms and pharmacological inhibitors as **showed in table 7**.

In mammals, among the 11 sub-families of PDEs, three of the PDE families (PDEs 4, 7 and 8) specifically regulate intra-cellular levels of cAMP, three families (PDEs 5, 6, and 9) are selective for cGMP and five families (PDEs 1, 2, 3, 10 and 11) show a dual specificity for both cAMP and cGMP, but with variable efficiency (Beavo and Brunton, 2002; Lugnier, 2006). Due to their distinctive features and participation in a variety of cellular mechanisms, alterations in PDEs activities may affect several cellular processes, such as apoptosis, differentiation, lipogenesis, glycogenolysis, gluconeogenesis and muscle contraction etc.

Table 7. General Information of Phosphodiesterases Enzymes Family Types

PDE Type	Substrate Specificity	Iso-form	Sub-family	Localization/Distribution	Species	Drugs	References
PDE1	cAMP/ cGMP	8	PDE1A	Cerebellum, Cortex, Hippocampus, Olfactory bulb, Striatum, Thalamus	Human, Rat Mouse	Vinpocetine, Phenothiazine, Caffeine	Reed et al., 1998; Lal et al., 1999; Cho et al., 2000
			PDE1B	Cortex, Hippocampus, Olfactory bulb, Striatum,	Mouse, Rat		
			PDE1C	Cortex, Hippocampus, Cerebellum, Amygdala	Mouse		
PDE2	cAMP/ cGMP	----	PDE2A	Amygdala, Cortex, Hippocampus, Cerebellum, Forebrain, Midbrain, Thalamus	Human, Rat Mouse	EHNA, Bay 60-7550,	Van Staveren et al., 2003; Reyes et al., 2007
PDE3	cAMP/ cGMP	4	PDE3A	Cerebellum, Forebrain, Brainstem nuclei, Midbrain, Thalamus	Human, Rat	Piroximone, Cilostamide, Milrinone, Inamrinone Enoximone	Bolger et al., 1994; Lakics et al., 2010
			PDE3B	Cerebellum, Forebrain, Brainstem nuclei, Midbrain, Thalamus	Human, Rat		
PDE4	cAMP	35	PDE4A	Amygdala, Cerebellum, Cortex, Hippocampus, forebrain, Striatum, Hypothalamus, Midbrain, Olfactory bulb, Thalamus,	Human, Rat Mouse	Rolipram, Cilostamide, Piomilast, Roflumilast, Piclamilast	Richter et al., 2005; Fujita et al., 2007; McLachlan et al., 2007; Lakics et al., 2010
			PDE4B	Cerebellum, Cortex, Striatum, Hippocampus, Hypothalamus	Human, Rat, Mouse		
			PDE4C	Not expressed in brain region (Expressed peripherally)	Human, Rat, Mouse		
			PDE4D	Cerebellum, Striatum, Cortex, Hippocampus, Hypothalamus	Human, Rat, Mouse		

PDE Type	Substrate Specificity	Iso-form	Sub-family	Localization/Distribution	Species	Drugs	References
PDE5	cGMP	3	PDE5A	Cerebellum, Cortex, Hippocampus	Human, Rat Mouse	Sildenafil, Tadalafil, Vardenafil, Mirodenafil	Van Staveren et al., 2003; Reyes et al., 2007
PDE6	cGMP	----	PDE6A PDE6B PDE6C	Not expressed in brain regions	-----	Zaprinast	Van Staveren et al., 2003; Reyes et al., 2007
PDE7	cAMP	3	PDE7A PDE7B	Cortex, Hippocampus, Striatum, olfactory bulb Cortex, Hippocampus, Striatum, Midbrain	Human, Rat Human, Rat	BRL-50481	Torres et al., 2003; Lakics et al., 2010
PDE8	cAMP	----	PDE8A PDE8B	Brain stem, Cerebellum, Forebrain, Cortex, Hippocampus Cortex, Hippocampus, Striatum, Midbrain, Olfactory bulb	Human, Rat Human, Rat	E4021, Dipyridamole, BRL-50481	Kobayashi et al., 2003; Lakics et al., 2010; Kruse et al., 2011
PDE9	cGMP	4	PDE9A	Amygdala, Striatum, Olfactory bulb, Cerebellum, Substantia nigra, Cortex, Hippocampus	Mouse, Rat	BAY 73-6691	Van Staveren et al., 2003
PDE10	cAMP/ cGMP	2	PDE10A	Caudate nucleus, Mid brain, striatum, Nucleus accumbens, Cortex, Hippocampus	Human, Rat	Papaverine	Seeger et al., 2003; Xie et al., 2006
PDE11	cAMP/ cGMP	4	PDE11A	Hippocampus, Substantia nigra	Human, Mouse	Isobutylmethyl xanthine, Zaprinast	Lakics et al., 2010; Michele et al., 2010

PDE1 Enzyme Family

PDE1 was the first PDE to be discovered (Kakiuchi and Yamazaki, 1970). Three sub-family genes (PDE1A-PDE1C) constitute the PDE1 family. The sensitivity of PDE1 to CaM is unique. Moreover, PDE1 iso-forms encode Ca^2/CaM dependent PDEs for cAMP and cGMP hydrolysis. All PDE1 class enzymes can degrade both the cyclic nucleotide, such as cAMP and cGMP, although, the affinity for each nucleotide varies with iso-form. PDE1A shows high affinity for cGMP (Loughney et al., 1996). PDE1B also prefer to hydrolyze cGMP with faster rate than cAMP (Bender et al., 2005), whereas, PDE1C shows high affinity for both cAMP and cGMP (Loughney et al., 1996).

PDE2 Enzyme Family

PDE2 enzyme belongs to a family of proteins that regulate the intra-cellular levels of both cGMP and cAMP, although, this family enzyme hydrolyses cGMP as preferred substrate, over cAMP (Erneux et al., 1981). There is only single gene encoded for PDE2, namely PDE2A. Three splice variants of PDE2A have been found, such as PDE2A1, PDE2A2 and PDE2A3 (PDE2A2 has only found in rat species). PDE2 has functional roles in brain, heart, adrenal cortex and platelets (Van Staveren et al., 2003).

PDE3 Enzyme Family

PDE3 enzyme hydrolyzes cAMP and cGMP with similar affinity, but the preferred substrate is cAMP. PDE3 family hydrolyzes cAMP at a rate 10 times higher than that of cGMP (Conti and Beavo, 2007). Although, studies reported that cGMP inhibits cAMP hydrolysis by PDE3 enzyme. Therefore, PDE3s are also known as cGMP-inhibited cAMP PDEs. Two genes with splice variants (PDE3A and PDE3B) constitute PDE3 family (Omori and Kotera, 2007). PDE3 family enzymes comprise of a 44-amino acid insert in the catalytic domain, which is a unique characteristic of this family. Another special feature of PDE3 family, is the presence of N-terminal hydrophobic membrane association domains (Wechsler et al., 2002).

PDE4 Enzyme Family

PDE4 family is the most extensive PDE enzyme family at present, which solely degrades cAMP. It comprises of four different genes with various splice variants (Houslay et al., 2005). ROL is a highly selective first generation inhibitor of PDE4 enzyme that has been used for many years as a research tool to investigate the role of PDE4 (Chung, 2006). Transgenic mice models of PDE4 gene emphasize the importance of this family for various psychiatric and neurological problems (Zhang et al., 2008). PDE4 is a target of the prototypical AD,

namely, ROL (Dyke and Montana, 2002). The significance of PDE4 enzyme family in psychiatric diseases patho-physiology will be covered in detail in a later section.

PDE5 Enzyme Family

PDE5 family enzyme exclusively binds to cGMP without being activated by Ca^{+2}/CaM. There is only one gene family coding for PDE5, namely PDE5A. In the N-terminal half, PDE5A comprises of 2 GAF domains (GAF-A and GAF-B), which are preferentially hydrolyzed by cGMP. GAF-A domain is mainly responsible for PDE5 allosteric binding to cGMP (Liu et al., 2002) and therefore, PDE5A is termed as cGMP-binding cGMP-specific PDE. In the N-terminal region, the presence of protein kinase G- and protein kinase A (PKA)-dependent phosphorylation sites are related to the activation of PDE5A enzyme (Corbin et al., 2000). Binding of cGMP to GAF-A domain promotes phosphorylation, which is not only triggered the catalytic activity, but also increased cGMP binding affinity (Zoraghi et al., 2005). PDE5 inhibitor shapes the vasodilatators action by increasing cGMP (Lugnier et al., 1986).

PDE6 Enzyme Family

This family (also called photoreceptor PDEs) includes the iso-enzymes from the cone and rod cell of retina. This sub-family gene controlled the level of cGMP in visual signal transduction by regulating cGMP hydrolysis. Light-activated transducin stimulates PDE6 enzyme activity by removing the inhibitory subunit. PDE6 enzyme family consists of three genes, namely PDE6A, PDE6B and PDE6C. PDE6A and PDE6B are expressed in rods cells, form a holoenzyme, with 2 copies of the smaller inhibitory subunit encoded by PDE6γ. PDE6C is expressed in cones cells, forms a homodimer with cone-specific inhibitory subunits, encoded by PDE6γ. The PDE6γ and PDE6 "subunits" involve in the modulation of the activity and localization of these enzymes. Small inhibitory subunits-mediated regulation of PDE6 activity represents a unique aspect of this family. Sildenafil, vardenafil & udenafil, but not tadalafil, inhibit PDE6 activity with notably lesser affinities than those for PDE5A.

PDE7 Enzyme Family

PDE7 family is encoded as ROL insensitive high-affinity cAMP-specific PDEs. This family has two sub-family genes, PDE7A and PDE7B. There are no known regulatory domains on the N terminus of PDE7 family, which is established for most of the other PDE families, although, in the N-terminus of PDE7A, a PKA pseudo-substrate site is present (Han et al., 1997). The pharmacological functions of this class of enzyme remain largely to be determined. Dipyridamole non-selectively inhibits the PDE7 activity.

PDE8 Enzyme Family

PDE8 contains two sub-family genes, PDE8A and PDE8B, which specifically hydrolyze cAMP (Fisher et al., 1998). PDE8 enzymes are expressed as high-affinity cAMP-specific PDEs and insensitive to ROL. The N-terminal region of PDE8 enzyme contains receiver (REC) and PAS domains. REC domain mainly involves in receiving signals from the sensor component, whereas, PAS domain mainly regulates the small ligands binding and protein–protein interaction. However, REC or PAS domains-mediated regulation of PDE8 enzyme is not explored adequately and endogenous PDE8 activity has not been, yet verified in either tissue or cell extracts.

PDE9 Enzyme Family

PDE9 family shows the highest affinity for cGMP. This family contains one sub-family gene, PDE9A. The activity at this enzyme may contribute to behavioral state regulation and learning behavior (Andreeva et al., 2001). However, there is no single report that adequately addresses the regulation of PDE9A enzyme activity or the presence of endogenous PDE9A enzyme activity in either tissue or cell extracts.

PDE10 Enzyme Family

The PDE10 family enzyme has dual specificity for both cAMP and cGMP. Currently, reports have provided evidence for the potential role of PDE10 enzyme in neuro-degenerative problems, as this enzyme is involved in regulation of long-term potentiation (Hebb et al., 2004). To date, there is only single PDE10A gene in this family. PDE10A enzyme consists of 2 N-terminal GAF domains and hydrolyzes cyclic nucleotides, cAMP and cGMP (Fujishige et al., 1999). The high affinity of PDE10 for cAMP inhibits hydrolysis of cGMP, making this enzyme a cAMP-inhibited dual-substrate PDE. The enzymatic activity of a chimeric construct of PDE10A GAF domain and cyanobacterial adenyl cyclase (AC) enzyme, which is activated by cAMP, indicates a potential allosteric modulation of PDE10A activity by cAMP (Gross-Langenhoff et al., 2006).

PDE11 Enzyme Family

PDE11, the most recently described PDE, is a single gene, but transcripts of multiple splice variants have been detected in various tissues (Fawcett et al., 2000). This enzyme catalyzes both cAMP and cGMP with similar affinity (Fawcett et al., 2000). Till date, only single gene product, namely PDE11A, has been identified with four splice variants (PDE11A1-PDE11A4). A full-length PDE11A4 form consists of one catalytic domain and two GAF domains. Recently, a pharmacogenetic study provided evidence for a possible role of this

family enzyme in depression disorder (Wong et al., 2006). Tadalafil inhibits PDE11A enzyme activity less potently than it inhibits PDE5A.

2.3. Nomenclature of Phosphodiesterases Enzyme

PDE nomenclature includes, an Arabic number classifying the specific gene family and alphabets that represent individual sub-families and splice variants. For example HsPDE1A1, where the first 2 alphabets represents the animal species, whereas, the first Arabic number is followed by PDE, indicating the PDE gene family. This Arabic number is followed by a single capital alphabet representing a different sub-family gene. The Arabic number, placed in the last of nomenclature sequence, indicates a specific splice variant or/ specific transcript, generated from a unique transcription initiation site (http://depts.washington.edu/pde/pde.html) as shown in **fig. 5.**

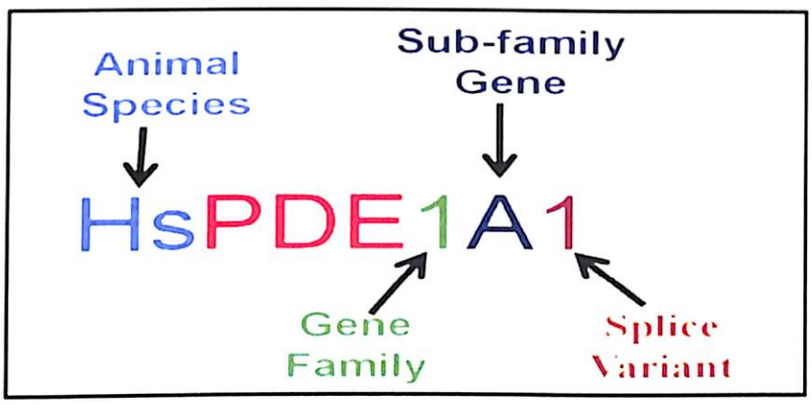


Fig. 5. The nomenclature sequence of human PDEs Family

2.4. Structure of Phosphodiesterase Enzymes

Although, PDEs enzymes are structurally, biochemically and pharmacologically different, but share several common structural features. All PDEs show a multidomain structure composed of three functional domains: a regulatory N-terminal domain, a central (conserved) catalytic domain and a regulatory C-terminus domain as shown in **fig. 6**.

Fig. 6. Common structural features of PDE iso-forms

The catalytic domain with the size of about 300 amino acids has a considerable sequence similarity of atleast ~50% on the amino acid levels, whereas, the C-and N-termini are heterologous (Torphy, 1998). The catalytic domains comprises of family-specific sequences that decide variation in substrate affinities, catalytic properties and sensitivities to specific

effectors and inhibitors, as well as common structural determinants involved in cleavage of cyclic phosphate bonds of secondary messengers, like cAMP and cGMP.

N-termini of PDEs contain unique binding sites for small messenger molecules (Ca^{2+}/CaM, cGMP, or phosphatic acid), motifs for membrane targeting, phosphorylation sites and metal ion binding sites (Bender and Beavo, 2006; Conti and Beavo, 2007). N-termini can therefore determine the activity status of PDEs, their dimerization and cellular compartmentalization. In general, the occupancy of the respective N-terminal binding site or the phosphorylation at the N-terminus mediates activation of the corresponding PDEs (Richter and Conti, 2004).

2.5. Secondary Messenger Cyclic AMP

Since cAMP discovery in 1957 (Berthet et al., 1957; Sutherland and Rall, 1958), it has been shown to play a role as secondary messenger in cells throughout the body (Tasken and Aandahl, 2004). No less than five different Nobel Prizes have been awarded in this field of research and therewith underline the importance of this regulatory system (Beavo and Brunton, 2002). AC, an effector cellular enzyme is responsible for the production of cyclic nucleotide by converting adenosine triphosphate (ATP) to cAMP as shown **in fig. 7.** cAMP, essentially binds and activates PKA and thereby, regulating multiple cellular functions (Colledge and scott, 1999).

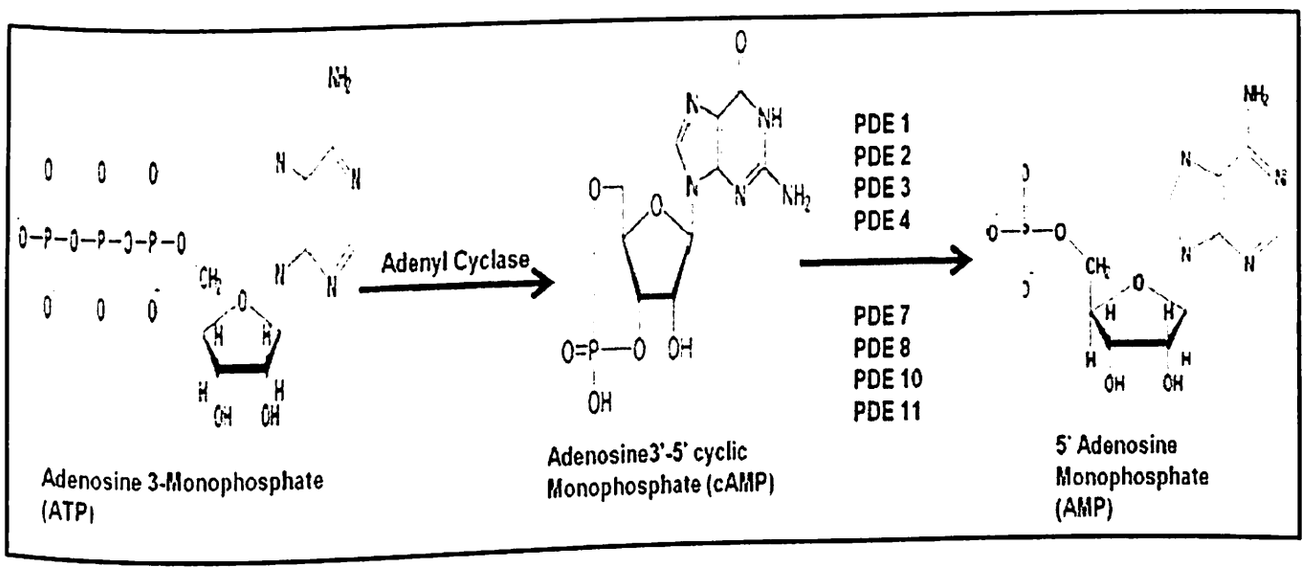


Fig. 7. Schematic representation of cAMP formation and degradation

2.5.1. Regulation of Cyclic AMP Signaling

cAMP formation takes place in response to a wide variety of extracellular signals, including hormones, growth factors and NTs. These agents trigger the cAMP signalling cascade by binding to a large class of receptor, namely, G-protein coupled receptors (GPCR) (Wong et al., 2004). Two G-proteins are implicated in the cAMP formation, namely, Gs and Gi, with the

subscripts indicating "stimulation" or "inhibition", respectively. Briefly, NT binding to the receptor causes the Gαs subunit to exchange its bound GDP for a GTP. This exchange activates the Gα subunit, causing it to dissociate from the Gβ and Gγ subunits. From there, Gα activates AC enzyme, which catalyzes the conversion of ATP into cAMP. cAMP, in turn, activates PKA by binding to the PKA regulatory domain and freeing the catalytic domain, which then acts to phosphorylate multiple downstream target proteins. Activation of cAMP-dependent PKA phosphorylates CREB. Then CREB, a transcription factor initiates transcription of specific genes involve in cellular functions **(fig. 8).**

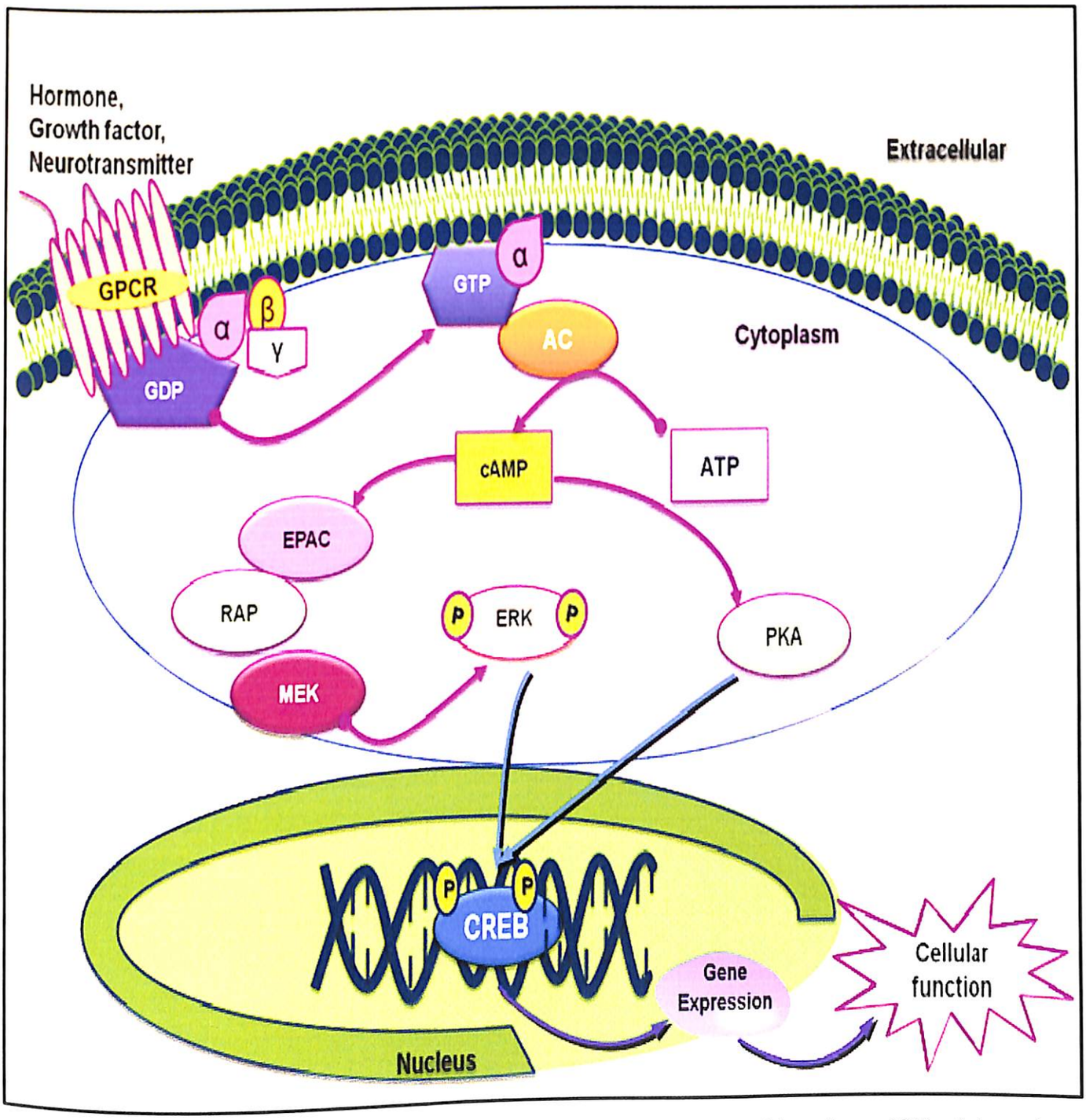


Fig. 8. cAMP and its-mediated signaling by PKA and EPAC. AC - Adenylyl cyclase, ATP - Adenosine triphosphate, GDP - Guanosine diphosphate, GTP- Guanosine triphosphate, cAMP- Cyclic adenosine monophosphate, PKA- Protein kinase A, CREB - Cyclic AMP response element binding Protein, EPAC - Exchange proteins activated by cAMP, ERK - Extracellular signal-regulated kinases.

2.5.2. Cyclic AMP and Depression Disorder

In the brain, beyond the level of monoaminergic receptor signaling, cAMP has been shown to plays a key role in the modulation of mood (Wachtel, 1989). Previous studies reported that altered cAMP level and its signal transduction pathway have been implicated as promising mechanisms of reduced synaptic plasticity and neuronal survival in depression patho-physiology (Manji and Duman, 2001; Shelton, 2007). This hypothesis provides a frame work in which the patho-physiology and pharmacotherapy for depression congregate on cAMP-mediated transduction pathway.

The possibility that the cAMP system is involved in the mechanism of action of ADs is supported by basic and clinical studies of inhibitors of PDE, the enzyme responsible for metabolism of cAMP. Studies have reported that cellular cAMP levels are returned to baseline levels by the activity of PDE4 enzyme, which cleaves cAMP into 5'-AMP. Considerable research has established that inhibition of PDE4 enzyme increases intra-cellular availability of cAMP and influences the cAMP/PKA/CREB signaling cascade (Manji and Duman, 2001). cAMP/PKA/CREB transduction signaling is implicated in the regulation of synaptic plasticity and neuronal survival in depression (Manji and Duman, 2001). Inhibition of PDE4 enzyme has been reported to increase intra-cellular availability of cAMP, which activates PKA and CREB protein. CREB leads the activation of BDNF, which plays a key role in neuronal survival (O'Donnell and Zhang, 2004).

2.6. Phosphodiesterase-4 Functional Anatomy

PDE4 family is introduced, as a low Km, cAMP-selective and ROL-sensitive PDE. PDE4 has a key regulatory role in the regulation of cAMP pathway. The PDE4 family gene was first cloned in rat as the homolog of "dunce", a gene required for the learning and memory in drosophila (Davis et al., 1989). Moreover, in 1980 there was one of the first biochemical characterizations of a PDE enzyme, proteolysed from liver membranes that demonstrated to be cAMP-specific and cGMP insensitive (Marchmont et al., 1980). Of the eleven PDE families, PDE4 family enzymes is particularly important for controlling intra-cellular cAMP concentrations and proven to be of particular importance in neuro-psychopharmacology (O'Donnell and Zhang, 2004). The genetics of PDE4 family enzyme are especially complex, as a number of variants exist for each of the four iso-forms.

2.6.1. Sub-families of Phosphodiesterase-4 Enzyme Family

PDE4 is the extensive family among the eleven PDE families. The PDE4 enzyme family consists of four independently coded sub-types, such as PDE4A–PDE4D (Lugnier, 2006). All sub-types are products of distinct genes and exhibit similar kinetics of cAMP hydrolysis.

PDE4s are located on different chromosomes, like PDE4A on chromosome 19p13.1 (Horton et al., 1995), PDE4C on chromosome 19p13.1 (Sullivan et al., 1999), whereas, PDE4B and PDE4D on chromosome 5q12 (Milatovich et al., 1994). All the four PDE4 genes produce distinct splice variants (iso-forms), like seven variants for PDE4A, five for PDE4B, three for PDE4C and ten for PDE4D (Zhang, 2009), as shown in **fig. 9**.

Each gene has several distinct promoters, 18 or more exons and can code for up to 6 splice variants. The splice variant has an unique N-terminus region, involved in localizing the enzyme to specific sites within the cell (Zhang, 2009). Some PDE4 enzymes also contain additional binding sites allowing macromolecular interactions with anchoring proteins, namely, A-kinase anchoring proteins and beta-arrestins (Houslay et al., 2007). It is believed that through specific localization, PDE4 is able to fine-tune intra-cellular levels of cAMP at specific loci within the cell.

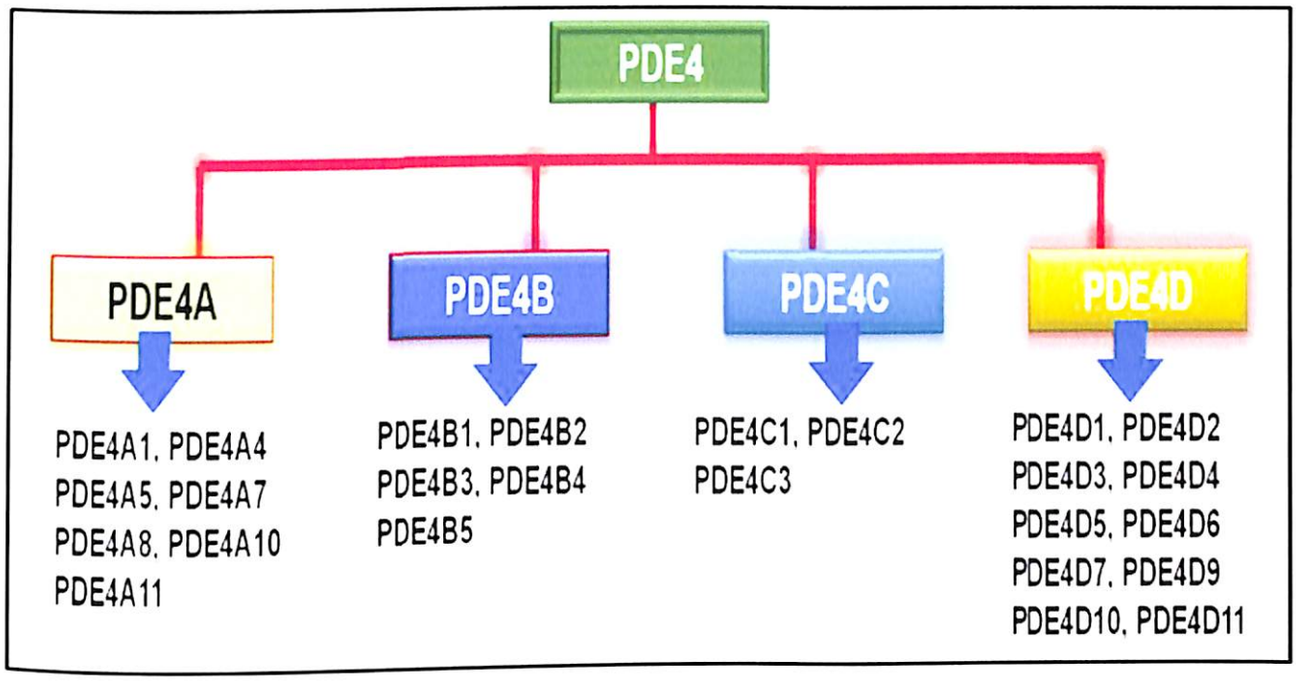


Fig. 9. PDE4 iso-forms with their specific splice variants *(Zhang, 2009)*

2.6.2. Structure of Phosphodiesterase-4 Enzyme Family

Each PDE4 splice variant consists of a highly conserved catalytic domain in sub-type specific C- terminal, which shows 75% sequence identity to any other PDE4 family member. However, dead-short form PDE4 enzymes comprise of truncated catalytic domain at both N- and C-termini. Due to a highly conserved catalytic domain, all PDE4 iso-forms have similar kinetic properties and ion requirements. Furthermore all iso-forms are inhibited by ROL. The catalytic domains of all PDE4 iso-forms, except PDE4A, contain an extracellular signal-regulated kinase (ERK) phosphorylation site, which regulates hydrolytic activity in a variant-

specific manner. PKA phosphorylation in the upstream conserved region 1 (UCR1) domain of long forms opens the PDE4 structure, increase enzyme activity, while ERK is capable of phosphorylating UCR2 in PDE4B, 4C and 4D. The PDE4A iso-form lacks the ERK 44 consensus site.

In general, on the basis of structure, these PDE4 splice variants can be classified into four categories: long form, short form, super-short form and dead-short form PDE4s **(fig. 10).** The long-form PDE4s have two unique and highly conserved N-terminal regions, termed as UCR1 and UCR2. Short form PDE4s (i.e., PDE4B2, PDE4D1 and PDE4D2) consists of an intact UCR2 but lack UCR1, whereas, super-short form PDE4s (PDE4A1, PDE4B5, PDE4D6, PDE4D10 and PDE4D11) only have a portion of UCR2 (i.e., N-terminally truncated UCR2) (Chandrasekaran et al., 2008; Lynex et al., 2008). Both UCR1 and UCR2 regions are absent in dead-short form PDE4 (PDE4A7) (Johnston et al., 2004; Houslay et al., 2007).

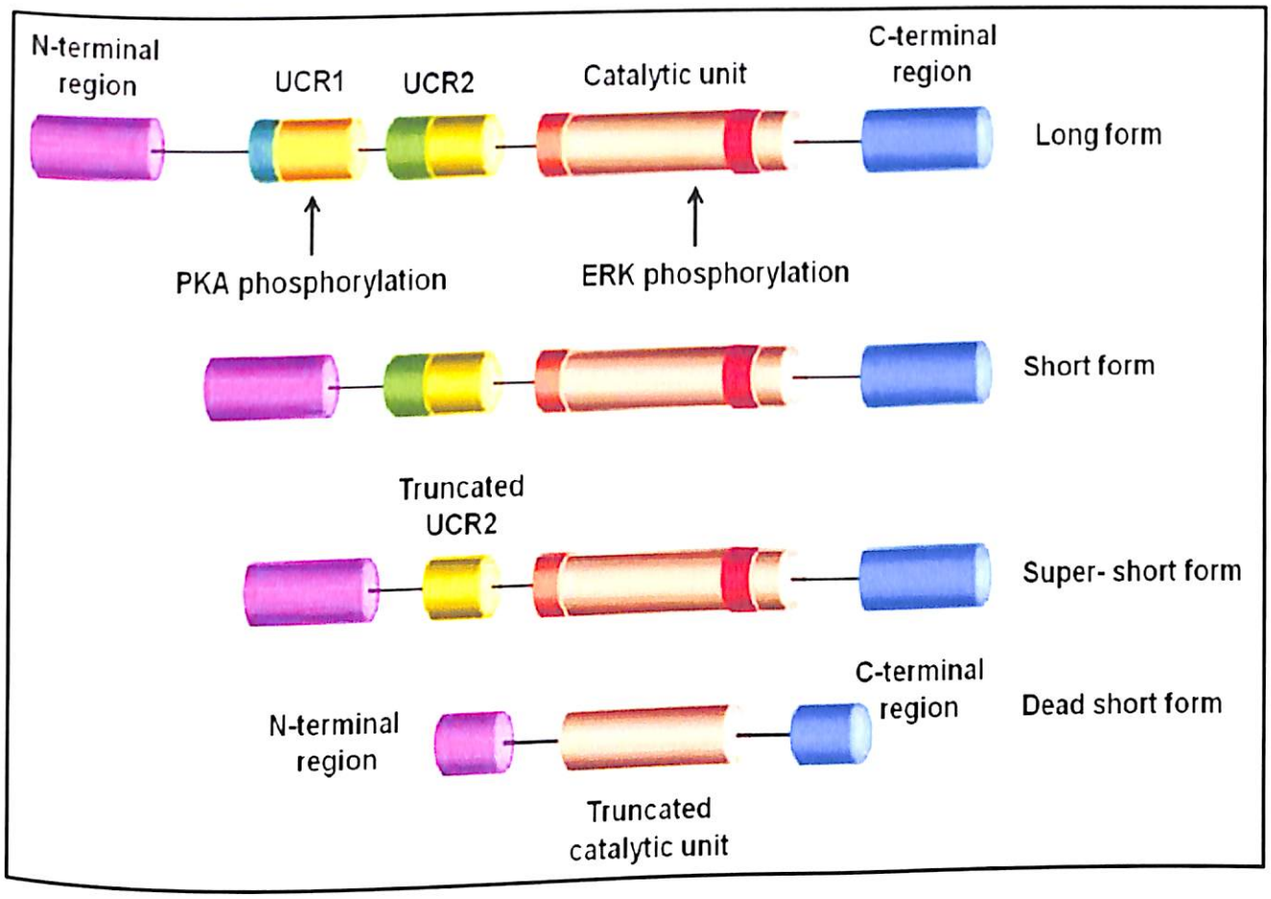


Fig. 10. Structure of various iso-form of PDE4 enzyme (*Adopted from Zhang, 2009*).

The N-terminal region regulates several functions of PDE4 enzyme, including cellular distribution, sub-cellular localization, catalytic activity, inhibitor binding and regulation by phosphorylation (Sette and Conti, 1996). UCR motifs were shown to be involved in, (i) PDE4 dimerization (intra- and intermolecular interactions), (ii) PDE4 activation or inhibition and (iii)

intra-cellular targeting of PDE4 enzyme (posttranslational modification). In addition, studies reported that UCR1 has a conserved phosphorylation site for cAMP-dependent PKA, which regulates hydrolytic activity (MacKenzie et al., 2002). This provides important mechanism(s) of cellular de-sensitization for cAMP signaling by increasing the ability for cAMP degradation (Oki et al., 2000).

2.6.3. Conformers of Phosphodiesterase-4 Enzyme Family

PDE4 enzyme family is characterized by two conformers, the high-affinity ROL binding site (HARBS) and low-affinity ROL binding site (LARBS), based on affinity analysis of ROL binding (Jacobitz et al., 1996). It is confirmed that ROL and other inhibitors bind with low nanomolar affinity (Ki = 1-2 nM) to HARBS, which is approximately 500-fold greater relative to the affinity for LARBS (Jacobitz et al., 1996). The studies in truncated PDE4A mutants have shown that HARBS requires both the N terminal and the catalytic domain, while LABRS requires only the catalytic domain (Jacobitz et al., 1996).

The HARBS conformer is extensively expressed in brain regions, which are involved in AD-like effect, such as hippocampus, frontal cortex and olfactory bulb, whereas, LARBS conformer is expressed in both peripheral and central tissues (Kaulen et al., 1989; Zhao et al., 2003a). Consistent with this, both the conformers of PDE4 enzyme may be differentially contributed to AD activity and other pharmacological functions. The HARBS conformer regulates central functions, such as head twitches, tremor, RIH (Schmiechen et al., 1990) and emetic responses (Hirose et al., 2007), while, LARBS conformer mainly regulates peripheral activity, including suppression of inflammatory reactions (Souness et al., 1997). In addition, DMI and FLX repeated treatments up-regulate HARBS, but not LARBS, in hippocampus and cerebral cortex (Zhao et al., 2003a). In addition, PDE4 inhibitors have high affinity for HARBS, exhibit greater potency for producing an AD-like effect than those with low affinity for HARBS (Zhang et al., 2006).

2.6.4. Phosphodiesterase-4 Enzyme Sub-types and Distribution

PDE4 gene products are expressed extensively and one or more iso-form can be expressed in a plethora of tissues and cell types. Various PDE4 iso-forms and variants show tissue and cell type-specific expression, e.g. all PDE4D splice variants represent different mRNA tissue expression with each splice variant usually distributed in various tissues, but often overlap with multiple variants (Richter et al., 2005). In situ hybridization histo-chemistry indicates that PDE4A, PDE4B and PDE4D iso-forms are extensively expressed in the mouse and rat brain regions, which are associated with reward function and emotional behavior (with the later

two being more rich in brain regions), whereas, PDE4C iso-form is expressed only minimally in mouse and rat brains (Cherry and Davis, 1999; Perez-Torres et al., 2000). The different expression patterns suggest that sub-types may be involved in different functions in rodents.

The PDE4D distribution was found to be different from that of PDE4A and PDE4B in mouse brain immuno-histochemical analyses (Zhang, 2009). PDE4A and PDE4B are highly expressed in anterior olfactory tract, cerebral cortex and cortical-spinal tract (PDE4A) and hypothalamus and ventral striatum (PDE4B). PDE4D is expressed in high concentration in cerebellum, habenula and thalamus and in a number of rat forebrain areas like, cerebral cortex and hippocampus (Chandrasekaran et al., 2008; Mackenzie et al., 2008).

2.6.5. Role of Phosphodiesterase-4 Enzyme in Central Nervous System Problems

PDE4 is highly expressed in brain regions, which are involved in the regulation of psychiatric and neurological process, such as affective behavior, mood and cognition. Considerable research have shown that among eleven PDEs families, the PDE4 is likely to be a key target for the therapeutic intervention of a variety of psychiatric and neurological problems, like anxiety, Alzheimer's, impaired cognition, Parkinson's, Schizophrenia and Huntington's (Zhang and O'Donnell, 2000; Mclachlan et al., 2007; Nakayama et al., 2007; DeMarch et al., 2008). During the last several years, efforts have been made to address problems, which are associated with disruption of PDE4 enzyme. Gene knockout (KO), gene inactivation and genetic studies have addressed the role of PDE4 enzyme in many diseases. Here, a brief overview of PDE4 enzymes role in various CNS problems is presented, followed by a description of their role in depression patho-physiology.

2.6.5.1. Role of Phosphodiesterase 4 Enzyme in Alzheimer's disease

Alzheimer's disease is an age-related neurological disorder, characterized by a progressive loss of cognitive functions (Han, 2005). The cholinergic replacement therapy is a major therapeutic approach for the treatment of cognitive impairment, associated with Alzheimer's disease, since the FDA approved acetylcholinesterase inhibitor tacrine, in 1993. Recently, research has been focused on the ability of PDE4 inhibitors to modulate the cognition and memory function.

ROL and other PDE4 inhibitors have shown improvements in object recognition, spatial reference, working and associative memory behavioral tasks in rodents and monkeys (Monti et al., 2006; Rutten et al., 2007; 2008). ROL inhibits the amnesic effects of NMDA receptor antagonist, namely MK-801 (Zhang and O'Donnell, 2000). In addition, PDE4 inhibitors

reverse memory deficits and restore working and reference memory in several spatial memory tasks, such as water escape and radial arm maze task, following scopolamine, MK-801 and MAPK/ERK inhibitors treatments (Zhang et al., 2005b; Rutten et al., 2006). It is reported that PDE4 inhibitors reverse cognitive impairments (improved working memory and spatial memory) in transgenic mouse models (PS1/PDAPP KO) of Alzheimer's disease in the radial arm water maze (Comery et al., 2005; Costa et al., 2007).

This approach is based on reports that cAMP regulates cell signaling, synapse communication and synaptic plasticity. cAMP has been shown to play a key role in learning, memory and impact cognitive function to some degree (Prickaerts et al., 2004). PDE4 enzyme is extensively expressed in hippocampus and cortex, brain regions that are mainly involved in memory formation, which confers the benefits of targeting PDE4 (Barco et al., 2003). Considerable research have addressed that increase in amyloid-beta peptide levels in rodent models of Alzheimer's disease, leads to the down-regulation of CREB-mediated intra-cellular signalling cascades and alters long-term potentiation of cognitive functions (Puzzo and Arancio, 2006). PDE4 inhibitors block the catabolism of cAMP, thereby, increasing the intensity and duration of cAMP-mediated cAMP/PKA/CREB signalling (Scuvee-Moreau et al., 1987).

2.6.5.2. Role of Phosphodiesterase 4 Enzyme in Parkinson's Disorder

Parkinson's disorder is a chronic and progressive neuro-degenerative disorder of brain that alters motor control, speech and other functions (Jankovic, 2008). Studies have reported that it is the second largest neuro-degenerative disorder, only behind Alzheimer's disease. Clinically, it is characterized by symptoms, including resting tremor, muscular rigidity, bradykinesia and postural instability (Fahn, 2003), although, psychiatric symptoms and cognitive deficits are also present (Aarsland et al., 1999). Several hypotheses have been proposed to explain the etiology of Parkinson's disorder, however, no unifying mechanism(s) have yet been discovered.

Studies reported that motor symptoms can be treated relatively well with pharmacological (L-DOPA, DA agonists, enzyme inhibitors) and non-pharmacological approaches (deep brain stimulation). However, effective therapies for non-motor symptoms, such as dementia are lacking and disease progression cannot be counteracted (David, 2008). During the last few decades significant advancement has been made and out of several intra-cellular mechanisms, transcriptional dysregulation in Parkinson's disorder pathology has been highlighted in considerable studies. Several reports have highlighted a reduction in cAMP

level in patients with Parkinson's disorder and cAMP specific PDE4 may be a good aspect for the pharmacotherapy of Parkinson's disorder. Numerous selective PDE4 inhibitors, Ro-20-1724 and SDZ-MNS 949 have been reported to stimulate the DA uptake and augment intra-cellular DA levels in rat mesencephalonic neurons (Yamashita et al., 1997).

In addition, PDE4 inhibitors demonstrated neuro-protective effect in MPTP mouse model of Parkinson's disorder. Recent study demonstrated that ROL attenuates MPTP-induced DA depletion in striatum and reduces the loss of tyrosine hydroxylase immune-positive neurons in substantia nigra region of C57BL/6 mice (Yang and Calingasan, 2008). This could be attributed as an effect of cAMP action on tyrosine hydroxylase, a rate limiting enzyme in the biosynthesis of DA. Thus, increase in cAMP levels by PDE4 inhibitors could facilitate the induction of tyrosine hydroxylase gene transcription and increased DA synthesis.

The dysregulation of CREB/BDNF-mediated signaling has been reported to be played a main role in Parkinson's pathology (Nishi et al., 2008; Zuccato and Cattaneo, 2009). Studies show that BDNF has protective actions on nigrostriatal dopaminergic neurons in *in-vivo* and *in-vitro* models of Parkinson's disorder (Sun et al., 2005). It is noteworthy, that targeting PDE4 is possibly a good approach to regulate cAMP level and its-mediated signalling in brain, addressing that inhibitors of PDE4 provide a potentially governing approach to manipulate cAMP, involves in Parkinson's disorder. Thus, enhancing cAMP-mediated signaling by PDE4 inhibitors may be helpful to halt/or modify Parkinson's pathology.

2.6.5.3. Role of Phosphodiesterase-4 Enzyme in Schizophrenia Disorder

Schizophrenia is most chronic, debilitating and costly of all mental illnesses. It is affecting one out of every hundred individuals and characterized by positive, negative and cognitive symptoms. DA remains a key NT and strongly associated with the patho-physiology of schizophrenia disorder. It exerts its action through GPCR and modulates the activity of AC and its second messenger cAMP. The link between DA and schizophrenia rests greatly on the fact that all the currently available anti-psychotic drugs with proven clinical efficacy block DA receptors. Despite the significant advancements achieved in the treatment of schizophrenia since last few decades, schizophrenia is observed to respond inefficiently to currently available drug treatments.

Studies addressed that increase in the intra-cellular cAMP levels and its downstream are helpful for the treatment of schizophrenia (Meltzer, 2003). All the currently available anti-psychotic drugs block D2 receptor (Kapur, 2003), resulting in augmented brain cAMP level (Kelly et al., 2007). Concomitant with increase in extracellular cAMP level, results in the

activation of PKA. Further, the PKA activation leads to phosphorylation of CREB and ERK-mediated downstream markers for the activation of neuronal signal transduction that produce therapeutic benefits of anti-psychotic medications (Siuciak et al., 2006). In this respect, agents are expected to influence cAMP intra-cellular cascade, such as PDE4 inhibitors may act as a potential strategy to treat schizophrenia disorder.

Recently, several reports have addressed that PDE4 inhibitors may be used as potential atypical anti-psychotic drugs with a low risk of extrapyramidal symptoms (Jeon et al., 2005). A compensatory increase in PDE4 enzyme activity has been found in an endophenotypic mouse model of schizophrenia (Kelly et al., 2007). Considerable data has shown that ROL antagonises phencyclidine and D-amphetamine-induced hyperactivity, well known animal models of schizophrenia (Siuciak et al., 2007). Moreover, ROL suppressed conditioned avoidance responding behavior and induced catalepsy with potency as like haloperidol, indicating an anti-psychotic-like effect (Kanes et al., 2007). Some studies indicated that PDE4 inhibitors increase baseline prepulse inhibition in a dose-dependent manner and ameliorate amphetamine-induced disruption of prepulse inhibition, the event-related potentials abnormalities and habituation of the acoustic startle response (Maxwell et al., 2004). These data support the statements that PDE4 inhibitors modulate the functioning of dopaminergic and glutamatergic systems, which are two most prominent neurotransmission systems, associated with schizophrenia (Lindsley et al., 2006; Jarskog et al., 2007).

Further, studies using transgenic mice suggest that the anti-psychotic effects of ROL and other PDE4 inhibitors are likely mediated by PDE4B sub-type (Siuciak et al., 2007). Moreover, genetically modified PDE4B mice showed a clear dose-dependent suppression of conditioned avoidance response to ROL. These studies suggest that PDE4B iso-form mediates the anti-psychotic-like effect of PDE4 inhibitors.

2.6.5.4. Role of Phosphodiesterase-4 Enzyme in Huntington's Disorder

Huntington's disorder is an autosomal dominant, progressive neuro-degenerative disorder. It is characterized by uncontrolled choreiform movements, deficits in executive functions, cognitive impairment, loss of motivation, self care and severe neuro-degeneration in basal ganglia region within the neostriatum (Duff et al., 2010). Huntington's disorder is caused by the inheritance of a mutant huntingtin gene containing an expanded trinucleotide repeat region, which codes for an expanded polyglutamine region in the mutant htt protein (Zuccato et al., 2010). Study reported that higher the number of repeats on htt gene, the more severe form of disorder. Although, mutated huntingtin is widely expressed; Huntington's disorder pathology is associated with a specific pattern of neuro-degeneration.

Studies have addressed that neuro-degeneration of the medium spiny neurons of striatum region (caudate/putamen) are mainly observed in the progression of Huntington's disorder (Zuccato et al., 2010). Till date no significant advancement has been made in the therapeutic approach for Huntington. Tetrabenazine, a reversible vesicular monoamine transporter-2 inhibitor is a single drug, approved by FDA for the treatment of this disorder. Although, tetrabenazine causes several severe side-effects, such as suicidality, depression, akathesia and other motor disturbances (Yero and Rey, 2008). Thus, there is an unmet medical need to identify and explore new therapeutic options for Huntington's disorder.

Recently, the research has been focused on the role of cAMP in the patho-physiology of Huntington's disorder. It is reported that mutated htt impairs cAMP level and its downstream CREB transcriptional pathways that has been hypothesized to play a key role in the patho-physiology of Huntington's disorder (Choi et al., 2009). This data in line with the other studies reported that 3-nitropropionic acid and quinolinic acid, which are widely being used to induce symptoms of Huntington's disorder in rodents, also affect cAMP levels and decrease CREB-mediated transcription (DeMarch et al., 2008; Almeida et al., 2010). Moreover, reduced cAMP levels have been observed in cerebral spinal fluid and post-mortem caudate region of Huntington patients (Gines, 2003). These findings suggest that inhibition of PDE4 may represent a valid therapeutic approach to elevate cAMP levels and overcome changes to gene expression, which may contribute to Huntington pathogenesis.

Pre-clinical studies have reported that chronic treatment with ROL leads to decrease striatal neuro-degeneration, along with increase levels of phosphorylated CREB in striatum region of quinolinic acid treated rats. It is reported that direct injection of quinolinic acid in the striatum region leads to striatal lesions and widely used to recapitulate striatal degeneration that observes in Huntington's disorder (DeMarch et al., 2008). Furthermore, PDE4 inhibitor also increased motor activity and time spent in OFT and rota rod test, respectively. Taken together, these studies support a beneficial effect of PDE4 inhibitors in rat chemical-induced lesion and transgenic mouse models of Huntington's disorder.

2.6.5.5. Role of Phosphodiesterase-4 Enzyme in Anxiety Disorder

Anxiety, an aversive emotional state is one of the most frequently occurring mental disease of CNS across the globally. It involves powerful emotional element associated with fearful thoughts and a physiological response. Moreover, study reported that anxiety disorders associate with significant disability and contribute to ever increasing health burden worldwide. Anxiolytic effects of most of the currently available drugs are mediated through an activation of brain NT GABA at the $GABA_A$ receptor complex (Fraser, 1998).

Benzodiazepine (BZD) class agents have widespread therapeutic potentials and generally prescribed medications for the treatment of several forms of anxiety. However, this class of agents have a narrow safety margin and not optimal for long-term monotherapy treatment of anxiety disorders, because more than one third of patients treated with BZDs do not respond properly (Greenblatt et al., 1983; Gerald et al., 1993). Despite a steady increase in the number of people treated for anxiety disorder, the prevalence of the disorder remains stable that could be attributed to the inconsistent efficacy of currently available pharmacological treatment.

Recently, one of the mediators known to play a central role in the patho-physiology of anxiety disorders is cyclic nucleotide PDE4 enzyme. Although, compared with the role of PDE4 in AD activity and other CNS disorder, little is explored about the regulation of PDE4 enzyme in anxiety disorder.

Previous studies have been addressed that rats treated with ROL demonstrate anxiolytic-like effect, as evidenced by increased open arm entries (OAE) and time spent in open arm (TSOA) compared with saline-treated animals in EPM test (Silvestre et al., 1999a; Li et al., 2009). In addition, acute and chronic treatment with ROL and other PDE4 inhibitors produced anxiolytic-like effect in other experimental models of anxiety, such as L/D aversion test, HB test and OFT, sensitive to the proven anxiolytic effect. Contrary, several studies reported the opposite results such as anxiogenic-like behavior of PDE4 inhibitors including ROL in several models of anxiety (Heaslip and Evans, 1995). The reason for discrepancy in the anxiolytic effect of PDE4 inhibitors may be atleast partially, because of sedative property of PDE4 enzyme inhibitors administered acutely (Silvestre et al., 1999b).

The therapeutic response of PDE4 inhibitors in anxiety may be associated with change in cAMP levels. Several pre-clinical and clinical reports provided evidences to support that a dysfunction of cAMP signaling may be implicated as promising mechanism in the patho-physiology of anxiety disorders. It is reported that cAMP/CREB signaling pathway is involved in the regulation of anxiety-like behavior (Pandey et al., 2005; Wand, 2005). In fact, PDE4 enzyme is a critical controller of cAMP signaling pathway and is expected to play a role in cAMP signalling-mediated behavioral effects. Inhibition of PDE4 enzyme is a way to augment the cAMP intra-cellular levels (O'Donnell and Zhang, 2004) and regulates its transduction pathway. cAMP activates PKA-mediated CREB phosphorylation, results activation of intra-cellular signaling, which are implicated in the patho-physiology of anxiety. Studies have reported that CREB KO mice also display anxiety-like effects (Gur et al., 2007).

2.6.6. Type 4 Phosphodiesterase Enzyme and Depression Disorder

Several reports have indicated that PDE4 gene product may be primarily implicated in the mediation of depression-like behaviors and drugs with PDE4 inhibitory activity could be used as an ADs (O'Donnell and Zhang, 2004; Diaboga et al., 2006).

The potential of PDE4 as a target for psycho-pharmacological therapy was started by Helmut Wachtel in the early 1980s. The work illustrated the behavioral effects of ROL and demonstrated its AD-like activity in pre-clinical models sensitive to AD drugs. ROL, a prototype PDE4 inhibitor was introduced as a potential candidate to treat depression and reached to Phase II of Clinical trials in 1983 as an AD.

Helmut Wachtel' and further extensive reports addressed that ROL produces AD-like effect in various pre-clinical tests sensitive to ADs, including reversal of RIH, potentiation of yohimbine-induced lethality, anti-muricidal activity in OBX rats and reduced duration of immobility in FST (Watchel, 1983; Mizokawa et al., 1988; O'Donnell and Frith, 1999). ROL was found to be more potent than other PDE4 inhibitors, like Ro 201724, ICI 63,197, and CP 76,593, in terms of AD-like effects (O'Donnell, 1993). Further, PDE4 KO mice have also revealed that they exhibit AD-like effect in rodent models of depression (Zhang et al., 2002).

Moreover, studies have reported that ROL is also much more potent than classic ADs, such as DMI and imipramine in pre-clinical tests (Wachtel, 1983; Mizokawa et al., 1988; O'Donnell and Frith, 1999). In addition, AD-like effects of PDE4 inhibitors are potentiated by classic ADs (Itoh et al., 2004), SSRIs and β-AR agonists (Zhang et al., 2005a). Besides this, the AD potential of ROL has been confirmed by clinical studies (Zeller et al., 1984; Fleischhacker et al., 1992), although, ROL has not advanced to clinical use due to severe emesis and gastrointestinal disturbances.

2.6.7. Role of Phosphodiesterase-4 Enzyme Iso-forms in Depression

In mammals, all the encoded PDE4 enzymes genes (PDE4A, PDE4B, PDE4C and PDE4D) display identical sensitivity to ROL inhibition (MacKenzie and Houslay, 2000). Due to unavailability of selective inhibitors of all the four individual PDE4 sub-types, the detailed functions of each gene product are not explored adequately. However, the differential distributions of sub-types suggest that these may sub-serve distinct roles and these roles in CNS have only recently, begun to be examined (Ye et al., 2000). Using a gene KO technique, it has been demonstrated that different sub-types have a significant role to play in the patho-physiology of depression. Initial studies with the PDE4D and 4B KO rodents on

CNS function are beginning to be reported. It is addressed that PDE4A, 4B and 4D sub-type genes have been knocked out in mice in the laboratory of Marco Conti (Jin et al., 2005). Currently, comprehensive knowledge on the resulting phenotypes is limited, largely to the PDE4B and 4D mice (Jin and Conti, 2002; Mehats et al., 2003).

Given the potential AD-like activity of ROL (O'Donnell, 1993; O'Donnell and Frith, 1999) and the imperative role of PDE4D sub-type in the regulation of cAMP levels (Keravis et al., 2000), it was thought that this sub-type might be implicated in the mediation of depressive-like behavior and AD-like responsiveness. PDE4D KO mice have revealed that they exhibit an AD-like profile, as evidenced by typical reduction in duration of immobility in the FST and TST relative to wild-type controls (Zhang et al., 2002). This reduction in immobility is consistent to that observed following the administration of a proven AD or PDE4 inhibitor. Moreover, studies addressed that when PDE4D-KO or PDE4D-deficient mice treated with ROL, no additional effect on reduction in immobility was observed.

In contrast, the administration of DMI and FLX to PDE4D-deficient mice was showed an additional reduction in immobility. Further, the potential of ROL to augment β-AR-mediated cAMP formation in cerebral cortex was also reduced in PDE4D-deficient mice (Zhang, 2009). These findings strongly signifying a role for PDE4D sub-family in the mediation of AD-like effects of PDE4 inhibitors and indicate that ROL actions are mediated significantly by PDE4D sub-type, whereas FLX and DMI actions are independent of PDE4D iso-form.

Beside this, very little is explored about the role of PDE4B iso-form in the mediation of AD-like effects. The data obtained using PDE4B-deficient mice suggests a different function for this sub-type (Zhang et al., 2003). Recent studies have shown that PDE4B sub-type expression is increased in the frontal cortex region with repeated AD treatments. Initial results indicate that PDE4B-deficient mice also exhibit an AD-like effect, as evidenced by typical reduction in immobility in the FST relative to wild-type controls and ROL causes no significant additional reduction in duration of immobility (Zhang et al., 2002). However, unlike to PDE4D-deficient mice, administration of DMI in PDE4B-deficient mice produces no further AD-like effects. These reports addressed that PDE4B-deficient mice exhibit reduced sensitivity to ROL as like PDE4D-deficient mice and DMI does not produce any additional effect on the PDE4B-deficient mice behavior in FST, unlike to PDE4D-deficient mice.

These important differences between the AD sensitivity of PDE4D- and PDE4B-deficient mice suggest that these PDE4B and PDE4D sub-types may be differentially distributed in signaling cascades, which are affected by AD drugs. However, it remains to be explored,

whether this difference is observed with 5-HT re-uptake inhibitors. Studies address that reduction in the cAMP catabolic function in a signaling cascade should augment sensitivity to pre-synaptically acting AD drugs. This may be the case for PDE4D, but not for PDE4B, addressing the relative importance of PDE4D sub-family in the mediation of AD-like effects.

Recent studies have demonstrated that PDE4A (PDE4A1 and PDE4A5) sub-type expression is increased in hippocampus and frontal cortex with repeated AD treatment (Ye et al., 2000). Even though the different sub-types are implicated in mediating the AD-like effects of PDE4 inhibitors is by no means resolved, mainly as mice lacking PDE4A gene have not been examined and results found till date, do specify a vital role for PDE4D sub-type.

The aforementioned statements indicate that PDE4 enzyme iso-forms show different selectivity to DMI and FLX treatments. The information regarding the responsiveness of PDE4D iso-form to AD treatment is inconsistent. This statement indicates two important concerns related to the responsiveness of PDE4D iso-form to AD treatment.

First, this concern may result in part from a species difference. Results obtained in rats demonstrated that chronic treatment with various classes ADs, result increases expression of PDE4A and PDE4B sub-type, but not PDE4D (Takahashi et al., 1999; Ye et al., 2000). Whereas, recent study in mice, by Dlaboga and Colleagues (2006), addressed that PDE4D expression is increased in response to chronic treatment with ADs, namely FLX and DMI. Thus, rats and mice may utilize different PDE4 sub-types in particular signaling cascades of AD treatment or the preferential regulation of PDE4D, as opposed to PDE4A and PDE4B.

Second concern is to identify to what extent side-effects, including emesis and sedation are associated with inhibition of PDE4D sub-type, which some extent is more troubling. Studies represented that PDE4D iso-form appears to be highly expressed in an emetic-trigger zone such as area postrema (Cherry and Davis, 1999; Perez-Torres et al., 2000). However, the involvement of PDE4D in the cAMP degradation in this region is still not clear. Thus, this issue remains a grave need for more systematic evaluation of behavioral phenotypes and pharmacological sensitivity, if it is expected to separate the AD-like and emetic effects of PDE4 inhibitors (Ye et al., 2000; Miro et al., 2002).

2.6.8. Phosphodiesterase-4 and Anti-depressant Sensitive Signaling Pathways

2.6.8.1. Nor-adrenergic and Serotonergic Signaling

The potential AD-like activity of ROL and others PDE4 inhibitors, dates back decades to the concept that these agents would be expected to stimulate the NE actions at β-AR, which, at the time, were proposed to partly mediate AD responses. In general, most proven ADs enhance nor-adrenergic-mediated or serotonergic-mediated neuro-transmission, or both, in brain, either by blocking re-uptake catabolism or by blocking inhibitory pre-synaptic α-AR (either autoreceptors or heteroreceptors) (Frazer, 1997). Thus, this issue was of attention to find , whether, PDE4 enzyme is involved in signaling mechanisms that are associated with 5-HT and NE. It has been shown that PDE4 mediates the cAMP hydrolysis, which is formed by stimulation of β-AR, in rat cerebral cortex (Ye and O'Donnell, 1996). Further, PDE4 is found to be involved in cAMP signaling mediated by adrenergic and serotonergic receptors (Ye and O'Donnell, 1996), which are implicated in the mediation of behavioral effects of AD drugs (Crissman and O'Donnell, 2002).

It is observed that PDE4 activity is regulated through changes in NA-mediated activity. In line with this, it has been addressed that ROL inter-relate either additively or synergistically with AD-like effects mediated by β1- or β2-AR (Zhang et al., 2005a). In addition, reducing nor-adrenergic function by 6-hydroxydopamine-induced nor-adrenergic lesions or chronic blockade of β-AR with propranolol inhibits both the PDE4 enzyme activity and expression of PDE4A and PDE4B sub-types. In contrast, increasing nor-adrenergic function by repeated administration of re-uptake inhibitor, like DMI markedly enhances the expression of these two PDE4 sub-types (Farooqui et al., 2000; O'Donnell, 1993). Overall, these findings address that PDE4 is a key regulator of cAMP signaling cascade, mediated by β-AR. Thus, inhibition of PDE4 enzyme might produce AD-like effects in part, by regulating NE-mediated neuro-transmission **(fig. 11).**

The involvement of the PDE4 in 5-HT receptor mediated neuro-transmission is not explored adequately. However, there is evidence for a relationship between PDE4 inhibition and serotonergic neurotransmission (Schoffelmeer et al., 1985; West and Galloway, 1996) as shown in **fig. 11.** Previous studies have provided evidences that AC activators, such as forskolin and ^{8}bromo-cAMP can augment the release of ^{3}H-5-HT (Schoffelmeer et al., 1985). Besides, some 5-HT-receptor sub-types namely, 4, 6 and 7 are coupled positively to AC and indirect evidences indicate that PDE4 inhibitors enhance 5-HT-mediated neuro-transmission which involves cAMP. In line with this, repeated FLX treatment increases the expression of PDE4A and PDE4B in rat cerebral cortex and hippocampus (Ye et al., 2000; Miro et al.,

2002). However, it is not well known, whether, the AD induces, increase in expression of PDE4A and PDE4B, but not in the PDE4D sub-type, which points out that either, PDE4A and PDE4B are mainly implicated in the signaling cascades that regulate the effects of ADs, or PDE4D sub-type expression in brain is less susceptible to regulation **(fig. 11).**

2.6.8.2. N-Methyl D-Aspartate Receptor–Mediated Signaling

A third pathway which may be implicated in AD sensitive signaling is NMDA-receptor-mediated cAMP signaling. The involvement of NMDA receptors in mediating AD-like effects is still not clear. Studies have been shown that NMDA receptor antagonist, such as MK-801 produces AD-like effects (Papp and Moryl, 1994). However, this observation has been questioned (Panconi et al., 1993). Activation of NMDA receptor in rat cerebral cortical neurons primary cultures, results in increased Ca^{+2} entry into neurons, which then activates Ca^{+2}/CaM-dependent cAMP. These neurons express enzymes from different PDE families, but NMDA-receptor stimulation mediated cAMP formed is exclusively hydrolyzed by PDE4 enzyme. Despite of precise nature of the functional role of PDE4 enzyme in NMDA receptor-mediated signaling, it does seem to be a key factor. However, currently, it is not reported which PDE4 sub-types are components of NMDA-receptor-mediated signaling in neurons.

Another signalling mechanism that could explain the role of PDE4 enzyme in the AD-like effect may be related to intra-cellular signaling. PDE4 Inhibitors, leading to an increase in intra-cellular cAMP availability and its-mediated neuronal survival and plasticity.

2.6.8.3. Neurotrophic Factor Signaling

Re-emergence of interest in PDE4 inhibitors recently, as potential ADs has come from the finding that they promote the neurogenesis by inducing BDNF expression in hippocampus (Nibuya et al., 1995; Conti et al., 2002). The features of available AD drugs to induce neurogenesis, seem to be important in mediating late-developing effects on behavior (Santarelli et al., 2003). These effects are mainly regulated by the activation of cAMP pathway, which leads to the activation of transcription factor CREB and to the direct induction of BDNF gene via a cAMP CRE site in its promoter. This mechanism of action of PDE4 inhibitors to alleviate depressive symptoms is thought to be through inhibition of PDE4 enzyme, leading to an increase in cAMP levels **(fig. 11)**. It is evident that administration of PDE4 inhibitors can facilitate mood regulation by enhancing cAMP signaling.

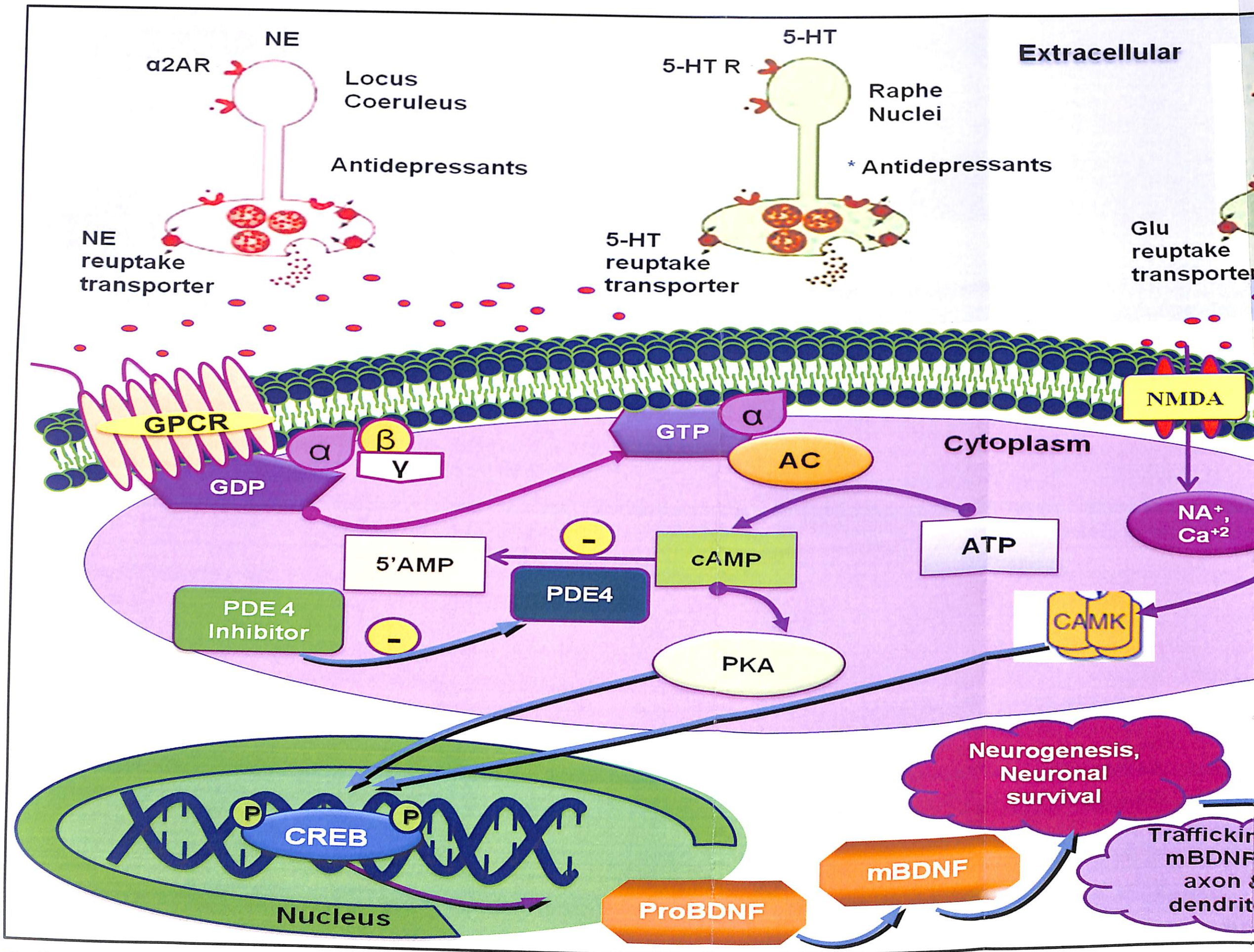


Fig. 11. Possible hypothetical mechanism of PDE4 enzyme in ADs-sensitive signaling pathways. AC - Adenylyl cyclase, ATP - Adenosine triphosphate, GTP - Guanosine triphosphate, cAMP cyclic Guanosine monophosphate, 5'AMP - 5' Adenosine monophosphate, 5'GMP - 5' Guanosine monophosphate, PDE - Phosphodiesterase, PKA - Protein kinase A, CRE - cAMP resp element binding Protein, BDNF - Brain derived neurotrophic factor, NE - Nor-epinephrine, 5-HT - Serotonin, Glu - Glutamate.

The ability of signaling pathways triggered by GPCR mediate the stimulation of AC enzyme. Stimulation of AC regulates the formation of cAMP, but up-regulation of PDE4 enzyme is implicated in the catabolism of cAMP and impairs the cAMP signal transduction. Increased cAMP activates the downstream signaling cascade, including activation of phosphorylating enzyme PKA. PKA has many cellular targets, one of which is the CREB, a transcription factor. Activation of PKA, results in activation of its target transcription factor CREB which culminating in new protein synthesis (Zhang and O'Donnell, 2000). Increased expression of CREB in turn, regulates the transcription of many genes particular, BDNF, which is implicated in neuronal growth and survival. Studies have reported that phosphorylation of CREB at its transcriptional regulatory residue Serine-133, is required to trigger BDNF transcription (Conti et al., 2002). BDNF regulates the synaptic plasticity and neuronal survival in the brain areas, involved in AD-like effect, mainly hippocampus and prefrontal cortex and then produces the ADs-like effect (Shieh and Ghosh, 1999).

2.6.9. Problems and Future Perspectives

The clinical development of first-generation PDE4 enzyme inhibitor, namely ROL, showed promising results but has therapeutic limitations due to their dose-limiting side-effects, such as nausea and emesis. The differential binding of inhibitors to PDE4 sub-types might be important in treating the adverse effects of PDE4 inhibitors. It is generally believed and hypothesized that side-effects of PDE4 inhibitors arise from inhibition of non-targeted PDE families or lack of selectivity against sub-families, such as PDE4A, 4B, 4C and 4D. There is still a huge lack of understanding over the subtle differences in expression pattern, localisation and function of PDE4 iso-forms. In addition, little is presently known about the differential expression of PDE4 in the emetic brain centres compared to that in emotional circuitry of depressed and normal individuals.

The molecular basis for family and sub-family selectivity of PDE4 inhibitors is poorly understood. It is thought that side-effects associated with PDE4 inhibitors are mediated atleast partly through the action on PDE4D iso-form, which is highly expressed in the emesis trigger zones, such as area postrema, nucleus tractus solitarius and locus coeruleus of squirrel monkey (a useful model as rats and mice do not vomit) (Lamontagne et al., 2001).

Another factor, such as relative affinity of PDE4 inhibitors for both conformers, namely HARBS and LARBS **(fig. 12A)** might be involved in the development of side-effects associated with PDE4 inhibitors. Affinity of PDE4 inhibitors for HARBS and LARBS conformers has suggested to be played a fundamental role in terms of tolerability and

efficacy of PDE4 inhibitors. Studies report that marked binding affinity of PDE4 inhibitors at HARBS predominantly is associated with high adverse events profile.

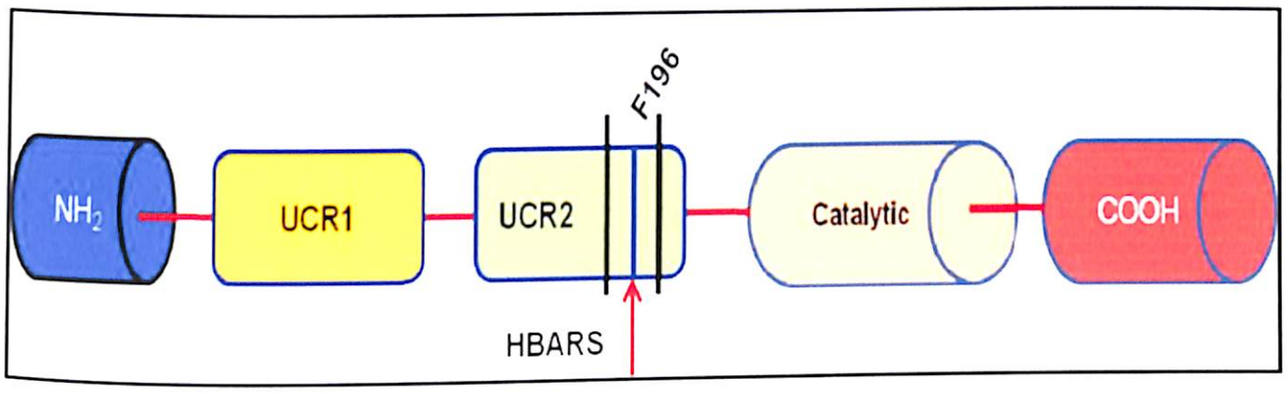


Fig. 12A. Location of HARBS on PDE4 enzyme domain structure

PDE4 inhibitors also have a narrow therapeutic window. The awareness that first generation PDE4 inhibitors have a narrow safety margin, promoted many researchers to evaluate new agents in the hope of identifying new PDE4 inhibitors with better safely profile and less unwanted effects. There are multiple challenges ahead for the search for clinically useful sub-type specific PDE4 inhibitors. Theoretically, due to the diversity of PDE4 sub-types, it may be likely to separate the emetic side-effects of ROL from its clinical efficacy. Nowadays, different strategies are being carried out to improve the side-effect profiles of these drugs.

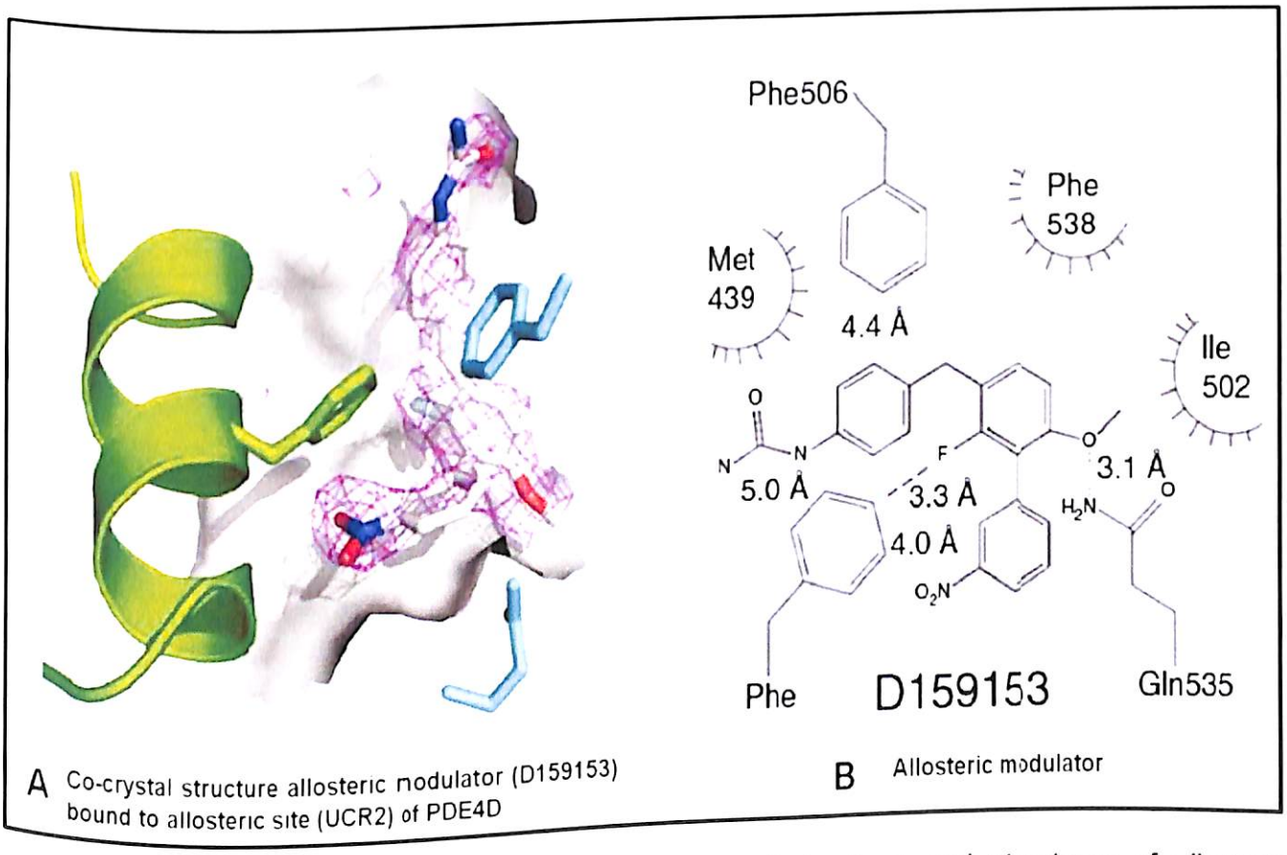


Fig. 12B. Represent the allosteric binding site of PDE4D iso-form and structures of allosteric modulators *(Adopted from Burgin et al., 2010).*

Recently, roflumilast, a PDE4 inhibitor, was found to express a more balanced binding affinity at LARBS and HARBS, which could account for the low incidence of nausea (3.2%), seen with this agent. Moreover, allosteric modulators that target the UCR2 domain of PDE4 have been developed **(fig.12B).** Importantly, this inhibitor was found to be 3000 times less emetic than ROL in beagle dogs and 500 times less emetic in cynomolgus monkeys (Burgin et al., 2010). Whether or not these second- and third- new generation PDE4 inhibitors will be able to overcome the nausea and emetic side-effects of earlier compounds or whether they will be useful for treatment of depression disorder remains to be seen.

2.7. Animal Models

A model is defined as an experimental training (here animal), proposed to study a situation in similar or different species. In the present scenario of drug discovery, experimental animal models have become an invaluable and rapid tool in the analysis of large number of factors, including genetic, environmental or pharmacological that can convey information about symptoms, similar to those of patients with a particular disorder (Shekhar et al., 2001). Although, it is uncertain that any single animal model captures all of the components of the complex expression of depression and anxiety, thus a battery of tests have been used to evaluate the potential of new drug molecules.

McKinney and Bunney, (1969) proposed more than 30 years ago that the minimum needs for depression animal model are:

- ✓ There is a 'reasonable analogy' to the human disorder in its manifestations or symptomatology;
- ✓ A behavioral change that can be examined objectively;
- ✓ Behavioral deficits should be attenuated by same treatments, which are effective clinically; and
- ✓ Animal model should be reproducible among investigators.

Along with these, further research indicates that animal models of neuro-psychiatric disorders should describe some more criteria such as:

- ✓ Stating the hypothesis to be tested
- ✓ Listing the specific features of illness which are supposed to be modeled
- ✓ Stating the validators (e.g., construct, face and predictive) type(s) applied to the model, where construct validity is usually the most convincing and valuable parameter

- ✓ Stating the facts, for and against the model validity in the context of the validators used
- ✓ All reputed animal models should be assessed with behavioral assays in a broadest range possible

Modelling of human neuro-psychiatric disorders in rodents is really a tough task. However, advancements in understanding the patho-physiology and treatment development would be benefited really from better animal models. Over the last 50 years, many models for MDD have been developed on the basis of theoretical aspects (Bourin, 1990). Several slight variations have been applied to each animal model, but the validity of the models needs to be examined closely (Yadid et al., 2000). Animal models of depression can be used to study behavioral and neuro-biological parameters that have been involved in the induction of depression in humans. A large number of validated animal models for affective disorders are available and still growing.

Animal models of depression have the purpose to reproduce some known aspects of depression in selected animal species (e.g. rodents). On this basis, they can be used:

- ✓ as a tool for investigating neurobiology/patho-physiology aspects of depression;
- ✓ as experimental models for studying the mechanism of action of AD drugs;
- ✓ as screening tests for elucidating AD activity.

2.7.1. Validation Criteria for Animal Models

The problem in all animal models and especially models for psychiatric conditions, which are defined via subjective knowledge, is to define clear criteria that permit asserting the validity of the model. Studies have reported that lack of validated animal models is a major problem in depression research. Studies have reported that many symptoms of depression (e.g., depressed mood, feelings of worthlessness and sociality) cannot be easily evaluated in laboratory animals. Willner, (1984) refined McKinney and Bunney, (1969) criteria as mentioned above and proposed different types of validities. Later, the animal models of depression are evaluated for their validity based on three criteria: face validity, construct validity and predictive validity.

Face validity

It generally describes the phenomenological resemblance between the behavior displayed by animal model and the specific symptoms of human condition. This is largely a sensitive criterion of the "reasonableness" of the model, but is neither essential nor adequate to create

a model. A model, which parallels multiple symptoms of human depression, is considered valuable.

Construct Validity

It addresses the theoretical rationale of the model. It is strongly associated with the pathology and symptomatology of disorder and accuracy by which changes in the animal reflects that in human.

Predictive Validity

It concerns the extent to which the model responds appropriately to AD effect as in humans. A valid model should be responsive and specific, that it should respond to effective Ads, but not to non-selective drugs and responses should observe within an appropriate dose range.

2.7.2. Advances and Strategies in Animal Models of Anxiety and Depression

Animal experimental models have many methodological and conceptual problems. Several characteristics of human behavior and cognition cannot be fully reproduced in an animal, indicating the difficulty for possible translation of human symptoms into animal models. Other problems with these animal models are, such as conflicting time-course results, questionable reliability, over-sensitivity to external (environmental, epigenetic) or internal (genetic) factors, as well as their variable reproducibility even within the same laboratory setting (Willner, 1993; 1995). Designing animal models may face "bottleneck" problems, as some features of brain pathogenesis may be restricted to specific phases of development or to a narrow range of cells in brain (Berrios and Markova, 2002). Thus, complete understanding of advantages and drawbacks of the existing animal models' is important for producing valid animal data, to parallel the existing clinical findings (Dedic et al., 2011). To achieve the goal of producing more suitable animal models, it will be required to apply and combine all recent advances in genetics and pharmacology with environmental challenges **(fig. 13).** This will hopefully help to begin the advancement of new treatment modalities, which are based on knowledge and not serendipity.

Moreover, to create more appropriate depression models with strong construct validity criteria, major efforts should be focussed towards the combination of genetic modification and environmental challenges in the same subject (Dedic et al., 2011). This would simulate gene-environment interactions that probably reflect the patho-physiological mechanisms of depression. Such models should show adequate face validity, as evaluated by behavioral and/or physiological parameters and respond to classical or novel drugs (predictive validity).

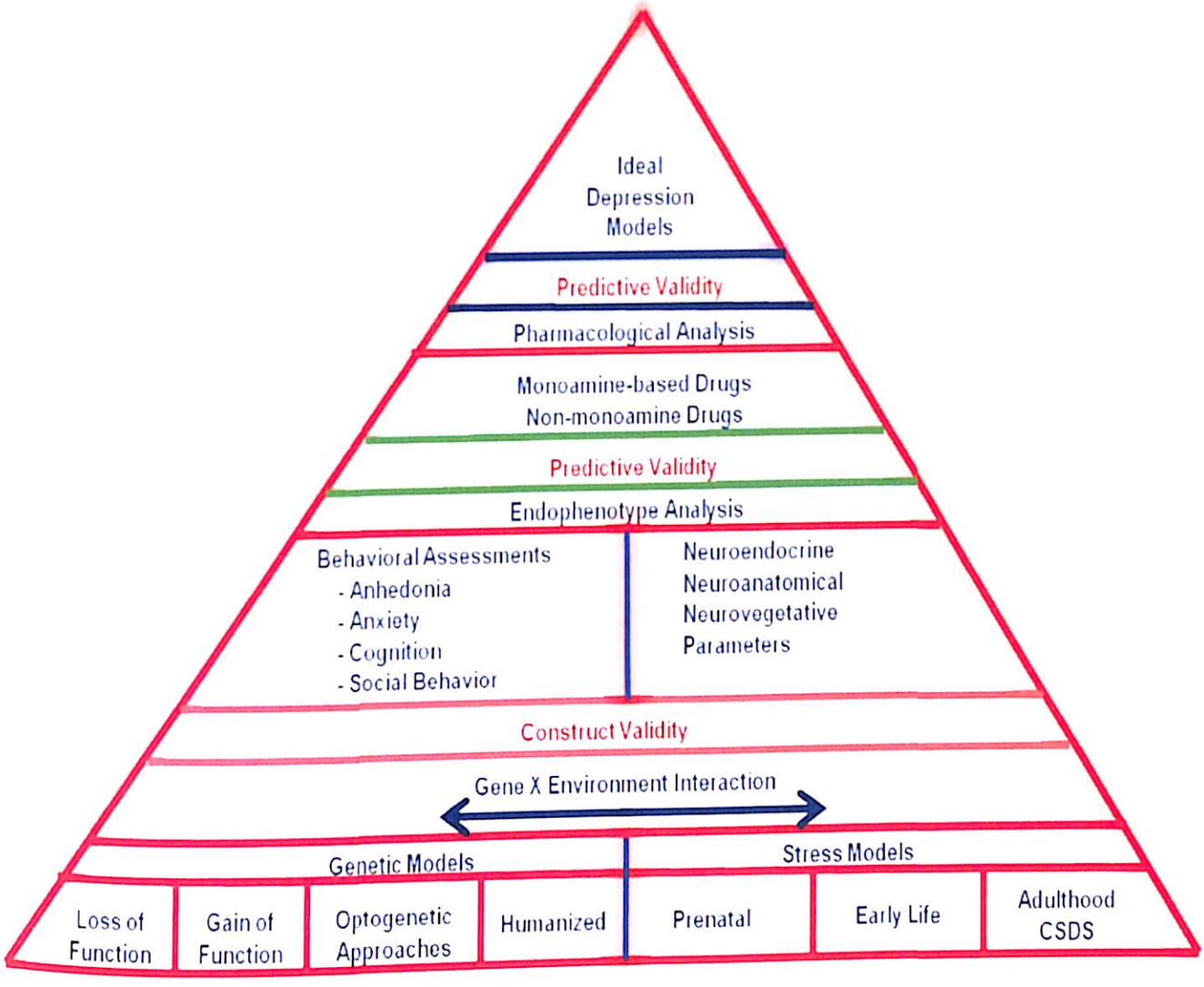


Fig. 13. An ideal animal model of depression *(Dedic et al., 2011)*

Conventional animal models of anxiety include, exploration-based paradigms (e.g., OFT, HB, EPM, L/D aversion, mirrored chamber & social interaction tests) and conditioned or unconditioned threat responses (Griebel et al., 1993; Adamec et al., 2004). Well known experimental models of depression include "despair" paradigms, such as FST, TST and learned helplessness, as well as OBX and CUMS (Willner et al., 1997; Cryan et al., 2005) as shown in **table 8**. With the rising popularity of these models in neuroscience research, drug development and genetic research (File, 2001; Crowley and Lucki, 2005), it is time to re-examine the recent condition with animal models of anxiety and depression **(table 8).**

Table 8: A brief detail of animal Models/Tests and Paradigm Shifts in Depression (D) and Anxiety (A) Research

Model	Common Species	Major NTs	Validity Criteria	Advantages	Disadvantages	Paradigm shift	Reference(s)
FST (Despair-based acute model of depression)	Mice, Rat	5HT and NE	Face and Predictive	Sensitive to acute AD treatment Easy to perform High reproducibility	Does not reliably detect SSRIs Risk of hypothermia or motor dysfunction	Exposure to unavoidable and inescapable stress develop hopelessness	Porsolt et al., 1977
TST (Despair-based acute model of depression)	Mice	DA	Face and Predictive	Sensitive to acute AD treatments Easy to perform Avoids hypothermia or motor dysfunction	limited to only several mice strains that do not tend to climb on their tail	Exposure to unavoidable and inescapable stress develop hopelessness	Steru et al., 1985
Learned Helplessness (Despair-based acute model of depression)	Rat, Mice	5HT and NE	Face and Constructive	Sensitive to short-term AD treatments Found to be reproducible in many species Resemble many aspects of human depression	Not all animals exposed to shock develop helplessness Requires very strong stressors Ethical restrictions	Exposure to unavoidable and inescapable stress develops hopelessness	Millan et al., 2001
OBX (Lesion-based chronic model of depression)	Rat (High priority), Mice	5HT, NE, DA, Ach and GABA	Face, Constructive and Predictive	One of the few tests that mimic the slow onset of ADs action - reported in clinical studies Affords possibility to detect a fast acting AD treatment Used for co-morbid disorder	Behavioral effects evident only following chronic treatment; Invasive in nature, Mechanism of action poorly understood	Disruption of limbic-hypothalamic axis involved in regulation of mood and emotional behaviors	Van Riezen and Leonard, 1990; Kelly et al., 1997)

Model	Common Species	Major NTs	Validity Criteria	Advantages	Disadvantages	Paradigm shift	Reference(s)
CUMS (Stress based chronic model of depression)	Rat, Mice	5HT, NE and DA	Face, Constructive and predictive	Better reflects the human condition characterized more by daily hassles than traumatic events Effects evident after chronic treatment	Reproducibility is poor in behavioral abnormalities and their response to ADs within and between labs Reliability has been questioned repeatedly	Exposure to stressors leads to disruption of neuroendocrine hormonal pathways	Ducottet et al., 2003
CORT-treated (Altered HPA axis based chronic model of depression)	Mice, Rat	5HT, NE and DA	Face, Constructive and Predictive	Sensitive to chronic ADs treatment Used as a model of TRD High reproducibility than CUMS models depression	Painful procedure (Daily CORT administration by s.c. route) Mostly existing ADs are not effective in this model	Alteration of the neuroendocrine system	Koike et al., 2013
TBI (Lesion based chronic model of depression)	Rat	5HT, NE, DA, Ach and GABA	Face and Predictive	Used to explore the role of different brain regions in specific disorder Model for co-morbid disorders	Mechanism of action poorly understood Not specific for ADs	Weight drop on brain regions leads to the alteration of neural circuitry	Foda and Marmarou, 1994; Heath and Vink, 1999
EPM (exploration based acute model of anxiety)	Mice, Rat	5HT, NE and GABA	Predictive	Permits a fast evaluation of anxiety-modulating drugs Investigation of the psycho-logical and neurochemical basis of anxiety	Performance on exploration based locomotor activity can produce a false positive increase or decrease in anxiety-like behavior.	Natural aversion of rodents for elevated open spaces	Handely and Mittani, 1984

Model	Common Species	Major NTs	Validity Criteria	Advantages	Disadvantages	Paradigm shift	Reference(s)
L/D aversion (natural aversion to illumination based model of anxiety	Mice, Rat	5HT, NE and GABA	Predictive	Allow a fast evaluation of anxiety-modulating agents Easy to use, without any prior training to rodents	Different measures and procedures used by different laboratories Contributes to a number of false positive results.	Natural aversion phenomenon of rodents to brightly illuminated areas	Crawley, 2000
5HTP-induced HTR (Pharmacology based model of depression)	Mice, Rat	5-HT	Constructive and Predictive	Sensitive to acute ADs treatment Direct assess the effects of a compound on NT levels.	Limited to face validity	Increased neuro-transmitter release; relief from depressive symptoms	Martin et al., 1989
RIH (Pharmacology based model of depression)	Mice, Rat	5-HT, NE and DA	Constructive and Predictive	Sensitive to acute ADs treatment	Limited to face validity	Increased neuro-transmitter release; relief from depressive symptoms	Askew, 1963; Van Riezen & Delver, 1971

2.8. Gap in Existing Research

MDD is a serious mental health issue that affects the life of most people at any age point during their lifetime. Significant advances have been made in the understanding of depression and anxiety disorders. Despite a constant increase in the advancement in research, the prevalence of these disorder remains stable probably due to unclear neurobiological understanding of patho-physiology/ or the inconsistent efficacy of current pharmacotherapy.

Conventional ADs use for depression and anxiety disorders pharmacotherapy directly affect monoamine (5-HT, NE and DA) turnover in brain and engage in restoring of normal function of monoamine associated signaling pathways (Schildkraut and Kety, 1967; Millan, 2006). However, no single agent has emerged as a gold standard or a first-line treatment in clinical studies and their ability to improve daily performance and productivity is questionable. The current pharmacotherapy of depression has several limitations as mentioned below;

(1) The efficacy of ADs is often unsatisfactory and inconsistent (showed only 51% efficacy) (Thase et al., 2001; Turner et al., 2008).
(2) One of the major issue(s) with the use of ADs is treatment resistant patients; nearly 30% to 50% of individuals treated with a given AD do not show a response (Dodd et al., 2005; Ruhé et al., 2006).
(3) Delayed onset of action with the available drugs poses several challenges in the treatment of depression. The meaningful improvement in depressive symptomatology is seen only after several weeks of ADs treatment, although, they begin to modify brain chemistry with the very first dose. The major problem with the delay in onset of AD action is the tremendous suffering.
(4) High co-morbidity of MDD with anxiety can lead to changes in diagnosis during the course of the illness. Several studies have addressed that depressed patients with co-morbid anxiety; (a) have a poorer long-term course of disorder, (b) do not respond properly to drug treatment and (c) may experience abnormalities in psychosocial functioning, in comparison of depressed patients without co-morbid anxiety.

The above mentioned issues with the current pharmacotherapy are adding urgency to the need of new strategies in order to improve the clinical management of these disorders. So, if ADs effect comes within hours, or even one or two days, it would be great in minimizing the disturbance in the personal, professional life of that individual and in theory, it could be argued that there would be a decrease in the risk of suicide, in the sense that depression symptoms are very rapidly relieved in hours or a couple of days with novel approaches.

Attempts to improve the efficacy of AD treatments have focused on reducing the latency of response and on finding new approaches for treatment-resistant cases. Moreover, development of fast acting drugs or drugs combination for therapy with a scientific and methodological development requires clear understanding of disorders and complexities of depression, as well as its standardization with valid animal models. Some selective studies are required on more specific targets that could ultimately lead to improved and faster activity of existing as well as potentially new drug molecules. This has created opportunities to achieve better efficacy in subgroups with different mechanisms of action.

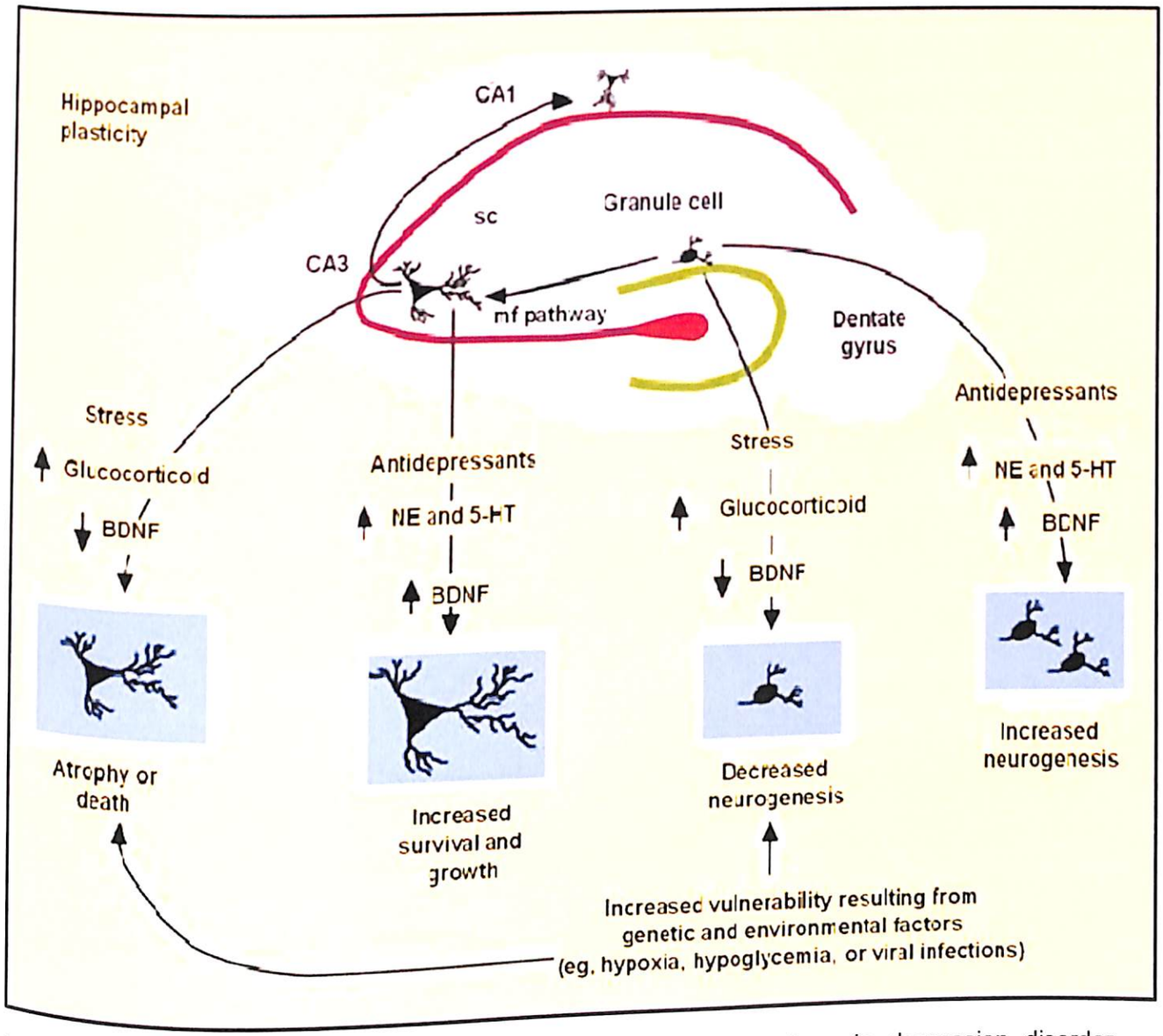


Fig. 14. Model of hippocampal plasticity showing structural alterations in depression disorder *(adopted from Duman, 2004).*

Recently, an emerging hypothesis suggests that MDD is associated with altered structural plasticity and neuronal dysfunction (Manji et al., 2003), as shown in **fig. 14.** Moreover, alteration in the intra-cellular signaling cascade is involved in the patho-physiology of psychiatric disorders, like depression and anxiety. Review of literature has revealed that

cAMP plays a key role in synaptic plasticity and neuronal survival. An increased cAMP-mediated signaling, such as cAMP/CREB/BDNF may contribute in the development, plasticity and survival of neurons and possibly implicated as a mediator in the patho-physiology of depression disorder (Conti et al., 2002) and mechanism of ADs treatment (Nibuya et al., 1995; Fujimaki et al., 2000). PDE4 modulates intra-cellular cAMP signaling pathways and its downstream modulators (CREB and BDNF) that are affected by ADs (Fujimaki et al., 2000; Nakagawa et al., 2002) as shown in **fig. 15**. The aforementioned criteria drew the attention to focus beyond the receptor level and explore intra-cellular signaling pathways to identify novel target(s) for depression.

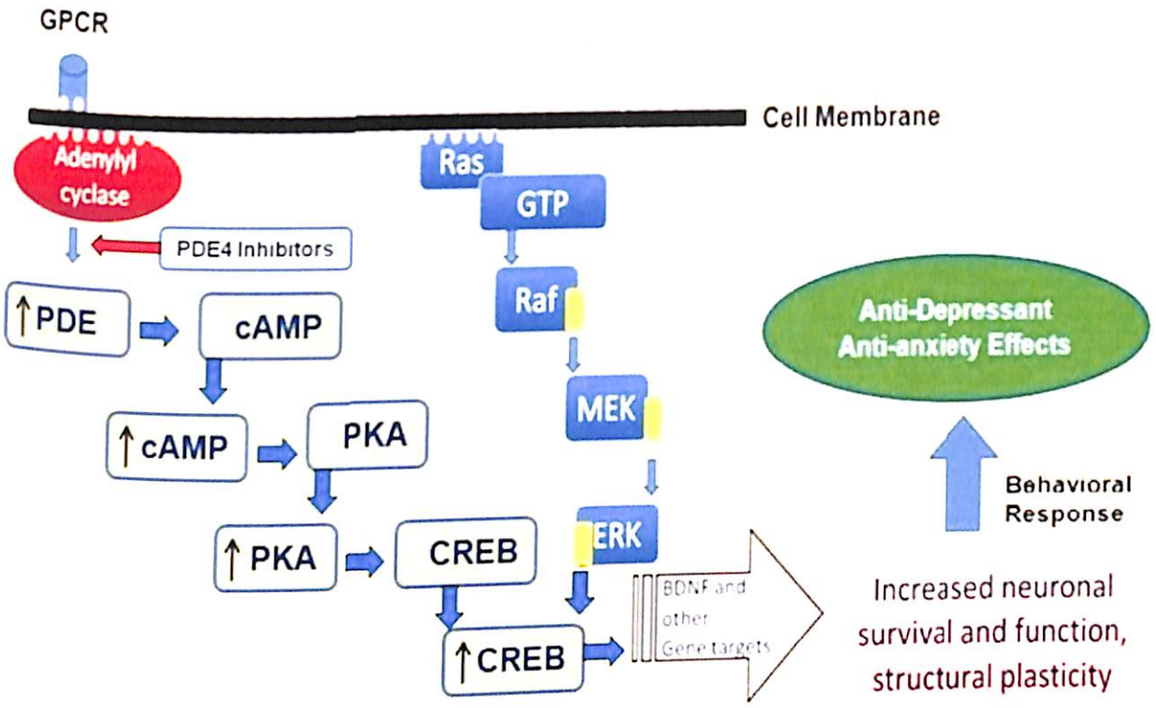


Fig. 15. Proposed hypothetical represenation for behavioral effect of PDE 4 inhibitors. cAMP - cyclic Amino-Mono Phospahte, PDE - Phosphodiesterase, CREB - Cyclic AMP response element binding protein, BDNF- Brain derived Neurotrophic factor, GPCR - G-protein coupled receptor.

Hence, from the pharmacological point of view, exploration of the intra-cellular signaling mechanisms, involve in the regulation of the neuronal survival and plasticity for depression disorders appears to be a reasonable approach to follow outstanding clinical requirements in CNS disorders. There are lot of reports about non-monoamine systems or the intra-cellular signaling mechanism, beyond the monoamine receptor that might contribute to the patho-physiology of depression disorder in rodent models. However, none of these discoveries have so far been translated into a new valid pharmacotherapy for depression. Novel hypotheses are emerging that are changing the approach, pathology of depression and mechanisms that underlie AD drug action.

Chapter 3: Objectives and Plan of work

3.1. Broad Objectives

One of the important reasons to design this study for depression and anxiety disorders beyond the receptor level is that existing ADs have been associated with many limitations. Involvement of cAMP in intracellular signaling beyond the receptor levels has prompted interest in the role of PDE4 class enzyme (an enzyme class hydrolyzes the cAMP) for the treatment of depression and anxiety.

The several aims of the work were:

- To conduct behavioral and Neuro-psychopharmacological investigations on existing and in-house synthesized PDE4 inhibitors, employing *in-vivo* behavioral AD and anxiolytic assays.
- To explore the role of PDE4 enzyme beyond the receptors level in the patho-physiology of depression disorder.
- To explore the relationship between the various hypothetical mechanisms (HPA axis, monoamine system, neurotrophic factor and oxidative stress) in the regulation of neuronal plasticity during depression and anxiety disorders.
- To explore the effect of PDE4 inhibitors on the hypothetical mechanisms in depression and anxiety disorders.

On the paucity of this information, following objectives were set for the current study.

- To design and standardize animal model(s) of depression and anxiety.
- Selection of specific behavioral test battery for designing *in-vivo* AD and anxiolytic assay, suitable to screen PDE4 inhibitors (possible new pharmacological class in relation to depression).
- To design and standardize behavioral symptoms to reflect the patho-physiological and neuro-behavioral aspects in depression and anxiety models.
- To evaluate an AD- and anxiolytic-like potential of existing PDE4 inhibitors (ROL and ETZ) and in-house synthesized molecules (Q-21 and Q-12) following acute and chronic administration, in the battery of standardize *in-vivo* AD and anxiolytic assays.
- To evaluate the effect of PDE4 inhibitors on neuroendocrine hormone (HPA axis activity in term of serum CORT), cAMP signaling (cAMP, CREB & BDNF), NTs (5HT, NE & DA) and oxidative (TBARS & nitrite)/anti-oxidant (SOD, CAT and GSH) markers to find out and explore the possible underlying mechanism(s) of action.

3.2. Plan of Work

In 2010, when the study was planned, the overall aim of the study was to investigate the unclear neurobiological understanding of patho-physiology of depression disorder. It is evident from the literature survey that Neuro-psychopharmacological investigations at different levels (*in-vitro and in-vivo*) show noteworthy evidence on the role of cAMP in the mood regulation. Likewise, possible links between cAMP-mediated transduction pathway and patho-physiology of depression and anxiety disorders have been suggested in pre-clinical studies. Agents are expected to influence cAMP intra-cellular signal transduction cascade may compromise PDE4 enzyme inhibitors. In this respect, inhibition of PDE4 enzyme is a potential approach to augment the cAMP intra-cellular signaling. The quantum of research literature was adequate enough that it provided for necessary and adequate information to support formulation of new hypotheses and to design and develop model(s) to explain the hypotheses in relation to recurrent depression. Hence, the following molecules (**PDE4 Inhibitors**) were screened in rodent behavioral assays of depression and anxiety.

Existing PDE4 Inhibitors:

(1) Rolipram

(2) Etazolate

In-house Synthesized PDE4 Inhibitors:

(1) QCA-21

(2) QCA-12

Based on the literature review, the following steps were outlined to achieve the objectives.

- Standardization of rodent models that can be simulated as model(s) for the screening of AD potential.

1. Chronic corticosterone-Injection model (i) Chronic administration of CORT for 21 days (iii) Behavioral tests	(ii) Drug Treatment (iv) Biochemical assays
2. Olfactory bulbectomy (i) Surgery (iii) Behavioral tests	(ii) Drug Treatment (iv) Biochemical assays
3. Chronic unpredictable mild stress (i) Chronic stress (iii) Behavioral tests	(ii) Drug Treatment (iv) Biochemical assays
4. Traumatic brain injury (i) Injury (iii) Behavioral tests	(ii) Drug Treatment (iv) Biochemical assays

- Standardization of animal model(s) that can be simulated as model(s) for screening of anxiolytic-like potential.

1. Elevated plus maze	
(i) Test drugs treatment	(ii) Behavioral tests
2. Light/dark aversion test	
(i) Test drugs treatment	(ii) Behavioral tests
3. Hole board test	
(i) Test drugs treatment	(ii) Behavioral tests

- Evaluation of neuro-behavioral aspects of depression and anxiety using symptomatological approaches in rodents
- Evaluation of PDE4 inhibitors, like ROL, ETZ, Q-21 and Q-12 in various depression and anxiety model(s)
- Biochemical and neurochemical estimation in brain and serum samples
    - estimation of CORT in serum samples
    - estimation of 5HT, NE and DA in brain samples
    - estimation of cAMP, CREB and BDNF in brain samples
    - estimation of oxidative stress and anti-oxidant markers in brain samples
- Histological analysis of DG and hippocampal CA1 regions of brain samples

3.3. Preliminary Work

3.3.1. Selection of Q-21 and Q-12 as Test Molecules:

Several PDE4 inhibitors were designed and synthesized based on molecular modeling and three point pharmacophore models in Medicinal Chemistry Laboratory. The compounds were tested for their PDE4 inhibitory activity using Enzyme linked immunosorbent assay (ELISA) technique and the percentage inhibition was determined using ROL as standard PDE4 inhibitor. Q-21 and Q-12, which exhibited an optimal percentage inhibition value for PDE4D iso-form, were selected for pre-clinical screening for AD- and anxiolytic-like potential.

The test substances and interacting agents (mentioned later) were subjected to mice SLA test in order to identify their influence on locomotion. Those substances (tested at specific dose levels and schedule), which exhibited non-significant influence on locomotion were short listed for behavioral AD assays.

3.3.2. Dose Response Studies

Preliminary work involved the dose response studies of the selected compounds. The dose response studies, following acute administration of test substances were constructed, using validated animal models of depression viz. FST and TST.

3.3.3. Interaction Studies

Interaction studies with conventional ADs viz. FLX, VLA and DMI were carried out using mice FST.

3.4. Screening of Phosphodiesterase-4 inhibitors in Chronic Models

As we all are aware that depression is a chronic psychiatric disorder and clinically requires chronic treatment of existing ADs to relapse the symptoms. Hence, in this study to explore and identify the potential usefulness for the treatment or prevention of depression disorder, selected PDE4 inhibitors (existing and in-house synthesized) were screened in chronic models of depression, such as OBX, chronic CORT-injection, CUMS and TBI at selected dose levels.

3.4.1. Behavioral Test Procedures and Parameters Measured in Chronic Model (s)

It is important to differentiate between the characteristics of an animal model and how they are measured (animal tests). The characteristic of an animal model is a matter of consensus among experts of the relevant disciplines and measurement is subject to psychological testing theory. Testing procedure is not necessarily the model, instead, rodent exposed to brain insult may be considered as an animal model of behavioral disorder.

(1) Olfactory Bulbectomy and Traumatic Brain Injury in Rats:

- **Open Field Test:** ambulation, rearing and number of fecal pellets
- **Sucrose Consumption Test:** volume of sucrose consumption
- **Hyper-emotionality Test:** bite, struggle, startle and fight responses

(2) Chronic CORT-treated and Chronic Unpredictable Mild Stress in Mice:

- **FST:** duration of immobility and swimming episodes
- **TST:** duration of immobility
- **Sucrose Consumption Test:** volume of sucrose consumption
- **SLA test:** locomotor scores

3.4.2. Biochemical Test Procedures and Parameters Measured in Chronic Model (s)

Finally the various biochemical and neurobiological estimations were performed as mentioned below.

- **Neuroendocrine marker:** HPA axis activity in terms of serum CORT level
- **cAMP signaling cascade:** cAMP, BDNF and pCREB levels
- **Oxidative-nitrosative stress markers:** lipid peroxidation and nitrite levels
- **Anti-oxidant enzymes:** GSH, SOD and CAT levels
- **Neurotransmitters:** 5HT, NE and DA levels

3.5. Preliminary Anxiolytic Studies

The above mentioned all four existing and in-house synthesized PDE4 inhibitors were also tested for their anxiolytic-like potential in experimental models of anxiety.

3.5.1. Behavioral Test Procedures and Parameters Measured in Anxiety Model (s)

- **EPM Test:** percentage of both OAE and TSOA
- **L/D aversion Test:** latency time to leave light compartment, time spent in light chamber and number of transition between the compartment
- **HB Test:** latency time to head dipping, time spent in head dipping and number of head dip.

Chapter 4: Experimental Methodology

4.1. Materials and Methods

4.1.1. Animals

Behavioral experiments were carried out using Swiss Albino mice (22–30 g) and Wistar rats (250–275 g) of either sex, procured from Hissar Agricultural University, Haryana, India. Animals were housed under standard laboratory conditions (temperature 23 ± 2 °C & room humidity 60 ± 10%), maintained on 12:12 h light/dark cycle with free access of standard diet and filtered water. All behavioral experiments were carried out between 09.00 a.m. to 03.00 p.m. Following a quarantine period of three weeks, the animals were randomly assigned to different experimental groups.

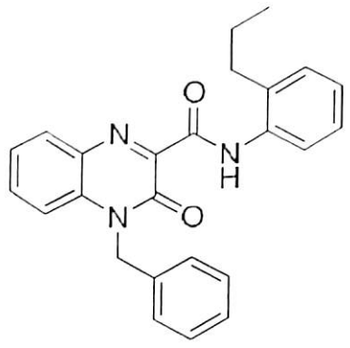
4.1.2. Ethical Approval

The experimental procedures on animals were in compliance with the Institutional Animal Ethics Committee of Birla Institute of Technology & Science, Pilani, India (Protocol No. IAEC/RES/14/03 and IAEC/RES/REV/16/08).

4.2. Drugs and Chemicals

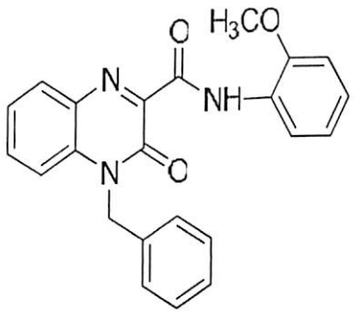
4.2.1. Novel Chemical Entities

Q-12 and Q-21, both of novel PDE4 enzyme inhibitors **(fig. 16),** were synthesized in Medicinal Chemistry Laboratory, Birla Institute of Technology & Science, Pilani.



Chemical Formula: $C_{25}H_{23}N_3O_2$
Molecular Weight: 397.47

Q-12



Chemical Formula: $C_{23}H_{19}N_3O_3$
Molecular Weight: 385.42

Q-21

Fig. 16. Structure of in-house synthesized PDE4 inhibitors

4.2.2. Standard Drugs and Chemicals

ROL, DMI, pargyline, 5-HTP and CORT were purchased from Sigma Chemicals, USA. ETZ and diazepam (DZM) were purchased from Tocris bioscience, UK and Cipla Ltd. India, respectively. FLX was procured from Glenmark Pharmaceuticals Ltd, India, as gift samples.

VLA and BUP were generously gifted by Ranbaxy Research Laboratories, India. In chronic studies, FLX was used as positive control for AD-like action. Reserpine was purchased from Sisco Research Laboratories, India. Ketamine and xylazine were purchased from Indian Immunologicals, India. All the drugs were dissolved freshly before use and administered by i.p. and p.o. routes according to the studies protocols. ROL, ETZ, FLX, VLA, DMI and BUP were dissolved in distilled water, whereas, Q-12 and Q-21 were prepared by triturating with 2-3 drops of the polyethylene glycol and di-methyl sulphoxide and final volume make with distilled water.

4.2.3. Chemicals for Oxidative and Anti-oxidant Markers Assays

Thiobarbituric acid, trichloroacetic acid, N-(1-naphthyl) ethylenediamine dihydrochloride, sulphanilamide, sulfosalicylic acid, 5-dithio-bis (2-nitrobenzoic acid), (−)-epinephrine, potassium dichromate, acetic acid, phosphoric acid, Ethylene di-amine tetra acetic acid and hydrochloric acid were purchased from different companies, such as SD Fine, Hi-Media and Spectrochem Chemicals, India.

4.2.4. Enzyme Linked Immunosorbent Assay Kits

ELISA kits for 5-HT, NE and DA were procured from DLD, Diagnostika, GMBH, Germany. ELISA kit for CORT and BDNF were procured from Immuno-Biological Laboratories, Inc (IBL), USA and Boster Biological Technology Co., LTD, USA, respectively. ELISA kit for cAMP and CREB were procured from Enzo Llife Science Ltd, USA and Wuhan EiAab Sciences Co., Ltd. China, respectively.

4.3. Surgicals

4.3.1. Haemostatic Sponge:

AbGel, Absorbable gelatin sponge USP, Srikrishna Laboratories, Mumbai, India.

4.3.2. Sterile Sutures:

Ethicon 4-0, Non-absorbable surgical sutures USP, Ethicon 4-0, Absorbable surgical sutures, USP (Catgut), Johnson and Johnson, India and Mersilk (Braided silk black).

4.3.3. Surgical Needle:

Curved surgical needles were obtained from Pricon Surgicals, New Delhi, India.

4.4. Equipments Used

- ✓ Automated animal tracking system: Panlab Co., USA
- ✓ Digital EPM: SN Scientific, India
- ✓ Stereotaxic Frame: Inco Ambala, India
- ✓ Centrifuge: Eppendorf refrigerated centrifuge, 5702-R, Eppendorf AG, Germany
- ✓ Elisa Pate reader and washer: Ark Diagnostic, India
- ✓ Autoanalyzer: Ark Diagnostic, India
- ✓ Tissue Homogeniser: Kinematica™ Polytron™ Homogenizers, Germany
- ✓ Spectrophotometer: UV-1800 Shimadzu, Japan
- ✓ Digital Microscope: Optika TCB5, Microscopes, Italy
- ✓ Deep freeze (-70°C): OPR-DFC-300CE, Operon Co. Ltd., Korea.

4.5. Pharmacological Paradigms

4.5.1. General Considerations for Behavioral Studies

Separate sets of animals were used for each experiment to avoid habituation effects with experimental situations. All drugs solutions were freshly prepared and administered p.o./i.p. (as specified) in a constant volume of 10 ml/kg before experiment. The drug/vehicle treated animals were acclimatized to the experimentation room for one hour before testing. Proper care was taken before and after surgical procedure. The doses of all drugs were selected on the basis of preliminary testing or previous studies conducted in our laboratory (Pandey et al., 2010; Jindal et al., 2012).

4.6. Preliminary Behavioral Assay

4.6.1. Dose Selection of ROL, ETZ, Q-21 and Q-12 for Anti-depressant Screening by Spontaneous Locomotor Activity Test

The SLA test was used for the selection of appropriate doses of test drugs to avoid the false positive and false negative AD-like effect. The SLA of mice was assessed using the actophotometer (Boissier and Simon, 1965), which contains a square arena (30 × 30 cm) with walls that are fitted with photocells just above the floor level. The photocells were checked before the beginning of the experiment. The drug/vehicle treated mice were then individually placed in the arena. After a 2 min acclimatization period, the digital locomotor scores were recorded for the next 8 min in a dimly lit room. All the drugs were administered 30 min prior to testing.

4.6.2. Evaluation of Preliminary Anti-depressant Effect of ROL, ETZ, Q-21 and Q-12 Using Mice Forced Swim Test

The procedure reported elsewhere (Porsolt et al., 1977) was adopted with slight modifications in glass cylinder dimension, number of quadrants and water temperature (Pandey et al., 2008). In brief, each mouse was placed, individually in a glass cylinder (diameter: 22.5 cm, height: 30 cm) containing 15 cm of water. The floor of the cylinder was demarcated into four equal quadrants. All mice (vehicle/drug treated) were placed in the water and forced to swim for 6 min. The duration of immobility which reflects the state of depression was recorded during the last 4 min of the 6 min test. A mouse was considered to be immobile, when it stopped struggling and passively moved to remain floating and keep its head above water. Water was changed between trials and temperature was maintained at 23 ± 2°C. In this study, we also measured the number of swimming episode in FST, which was adopted from our group previous studies. The swimming episodes were recorded as number of quadrants (demarcated at the base of the cylinder) crossed. The animal activities were tracked and recorded by an overhead camera and "*Smart*" version 2.5 computer software (Panlab co., USA). This test is based on the hypothesis that depression is also caused by chronic stress. The state of immobility has been named 'behavioral despair' on the statement that rodent has given up hope of 'escaping' a symptom, that reflects clinical feature of depressive disorder.

4.6.3. Evaluation of Preliminary Anti-depressant Effect of ROL, ETZ, Q-21 and Q-12 Using Mice Tail Suspension Test

TST is another most widely used behavior despair model of depression. Behavioral despair was induced by TST protocol of Steru et al., (1985). In brief, the mice were suspended on the edge of a shelf 50 cm above a table top by adhesive tape placed approximately 1 cm from the tip of the tail. The duration of immobility during the 6 min observation period was tracked and recorded using "*Smart*" version 2.5 computer software (Panlab co., USA). The state of immobility has been named 'behavioral despair' on the statement that rodent has given up hope of 'escaping' a symptom, that reflects clinical feature of depressive disorder. Mice were considered immobile only when they were totally motionless. The parameter recorded was duration of immobility.

4.6.4. Experimental Design for ROL, ETZ, Q-21 and Q-12 in Mice FST and TST

In the preliminary investigation (FST & TST), all the animals were divided into different matched groups as given below:

Set: A. This set of FST and TST as performed using ROL

FST		TST	
1. Normal control	= 8	1. Normal control	= 8
2. ROL (0.12 mg/kg)	= 8	2. ROL (0.12 mg/kg)	= 8
3. ROL (0.25 mg/kg)	= 8	3. ROL (0.25 mg/kg)	= 8
4. ROL (0.5 mg/kg)	= 8	4. ROL (0.5 mg/kg)	= 8
5. ROL (1 mg/kg)	= 8	5. ROL (1 mg/kg)	= 8
6. FLX (10 mg/kg)	= 8	6. BUP (20 mg/kg)	= 8

Set: B. This set of FST and TST as performed using ETZ

FST		TST	
1. Normal control	= 8	1. Normal control	= 8
2. ETZ (0.12 mg/kg)	= 8	2. ETZ (0.12 mg/kg)	= 8
3. ETZ (0.25 mg/kg)	= 8	3. ETZ (0.25 mg/kg)	= 8
4. ETZ (0.5 mg/kg)	= 8	4. ETZ (0.5 mg/kg)	= 8
5. ETZ (1 mg/kg)	= 8	5. ETZ (1 mg/kg)	= 8
6. FLX (10 mg/kg)	= 8	6. BUP (20 mg/kg)	= 8

Set: C. This set of FST and TST as performed using Q-21

FST		TST	
1. Normal control	= 8	1. Normal control	= 8
2. Q-21 (0.25 mg/kg)	= 8	2. Q-21 (0.25 mg/kg)	= 8
3. Q-21 (0.5 mg/kg)	= 8	3. Q-21 (0.5 mg/kg)	= 8
4. Q-21 (1 mg/kg)	= 8	4. Q-21 (1 mg/kg)	= 8
5. Q-21 (2 mg/kg)	= 8	5. Q-21 (2 mg/kg)	= 8
6. FLX (10 mg/kg)	= 8	6. BUP (20 mg/kg)	= 8

Set: D. This set of FST and TST as performed using Q-12

FST		TST	
1. Normal control	= 8	1. Normal control	= 8
2. Q-12 (0.25 mg/kg)	= 8	2. Q-12 (0.25 mg/kg)	= 8
3. Q-12 (0.5 mg/kg)	= 8	3. Q-12 (0.5 mg/kg)	= 8
4. Q-12 (1 mg/kg)	= 8	4. Q-12 (1 mg/kg)	= 8
5. Q-12 (2 mg/kg)	= 8	5. Q-12 (2 mg/kg)	= 8
6. FLX (10 mg/kg)	= 8	6. BUP (20 mg/kg)	= 8

4.6.5. Interaction Studies (Combination of Sub-Effective Dose of ROL and ETZ with Conventional Anti-depressants) in Forced Swim Test

Interaction studies were performed to investigate a possible synergistic AD-like effect of the combined administration of sub-effective doses of PDE4 inhibitors (ROL and ETZ) and conventional ADs (FLX, DMI and VLA). For the interaction studies, PDE4 inhibitors and conventional ADs were administered 45 and 30 min (i.p.) prior testing according to the previously described method (Redrobe and Bourin, 1997; Ramamoorthy et al., 2008). Sub-effective dose of ROL (0.12 mg/kg, i.p.) and ETZ (0.12 mg/kg, i.p.) were tested with sub-effective dose of FLX (5 mg/kg, i.p.), a SSRI, VAL (4 mg/kg, i.p.), a SNRI and DMI (5 mg/kg, i.p.), a TCA (present better selectivity for NE re-uptake), in FST. The doses of conventional ADs were selected on the basis of the previous studies conducted in our laboratory and reported not to alter locomotor activity (Pandey et al., 2008; Ramamoorthy et al., 2008). The effects of the combined treatment of conventional ADs with PDE4 inhibitors were also verified in the actophotometer test.

4.6.6. Mechanistic Models

4.6.6.1. Evaluation of ROL and ETZ in 5-HTP-induced Head Twitch Response Model

This test was performed as an indicator of constructive validity criteria, representing the patho-physiology relevance. The method mentioned elsewhere (Martin et al., 1989), was adopted with slight modifications (5-HTP dose and total duration of head twitch count) (Mahesh et al., 2011). Briefly, mice were treated with pargyline (75 mg/kg, i.p.) 30 min before 5-HTP (5 mg/kg, i.p.) treatment. Tested drugs (ROL and ETZ) were injected 15 min prior to 5-HTP administration. 15 min post 5-HTP administration, the numbers of head twitch exhibited by mice during the next 30 min was observed. The observer was blind to the drug treatment. The HTR was characterized by abrupt lateral movements, which may or may not be accompanied by body twitches and hind limb retractions.

4.6.6.2. Evaluation of ROL and ETZ in Reserpine-induced Hypothermia Model

The antagonism of RIH was also performed as an indicator of constructive validity criteria, representing the patho-physiology relevance. The test was performed as described in literature (Askew, 1963; Van Riezen and Delver, 1971; Pandey et al., 2008). Wistar rats were treated with tested drugs (ROL and ETZ) and reserpine (1 mg/kg, i.p.), 45 & 30 min prior to testing, respectively. The animals were gently restrained by hand while inserting the probe rectally with lubrication. On the day preceding the experiments, the rats were individually subjected to temperature recording in order to habituate the animals to the

experimental procedures. The effects of ROL and ETZ on RIH (measured with digital thermometer) were recorded by measuring temperatures at 0, 30, 60, 90 & 120 min following reserpine injection. Hypothermia was measured by calculating temperature difference between 0^{th} & 120^{th} min.

In both the, 5HTP-induced HTR (mice) and RIH (rats) models two higher doses on the basis of FST and TST results were selected and animals were divided into groups as given below:

Set: A. This set of HTR and RIH as performed using ROL

HTR (mice)		RIH (rats)	
1. 5-HTP + Pargyline control	= 8	1. Reserpine control	= 6
2. ROL (0.5 mg/kg)	= 8	2. ROL (0.5 mg/kg)	= 6
3. ROL (1 mg/kg)	= 8	3. ROL (1 mg/kg)	= 6
4. FLX (10 mg/kg)	= 8	4. FLX (10 mg/kg)	= 6

Set: B. This set of head twitch and RIH as performed using ETZ

HTR (mice)		RIH (rats)	
1. 5-HTP + Pargyline control	= 8	1. Reserpine control	= 6
2. ETZ (0.5 mg/kg)	= 8	2. ETZ (0.5 mg/kg)	= 6
3. ETZ (1 mg/kg)	= 8	3. ETZ (1 mg/kg)	= 6
4. FLX (10 mg/kg)	= 8	4. FLX (10 mg/kg)	= 6

4.7. Pharmacological Screening of Test drugs in Chronic Model of Depression

4.7.1. Evaluation of ROL, ETZ, Q-21 and Q-12 in Olfactory Bulbectomy Model of Depression

4.7.1.1. Surgery

18-20 weeks old male rats (an average age to gain 250-275 g weight, appropriate for surgery) were employed for the OBX model. Removal of olfactory bulb(s) in rats is an approach to damage the neuronal circuit leading to neuro-behavioral disorder. Bilateral olfactory bulb ablation was performed according to previously described method (Kelly et al., 1997) with slight modifications (method of bulb suction and drilling) (Ramamoorthy et al., 2008; Jindal et al., 2012). Briefly, rats were anaesthetized with the cocktail of xylazine and ketamine (5 & 75 mg/kg, i.p. respectively). The animals were fixed in a stereotactic frame

(Inco, India) and the skull was exposed by a midline incision. The burr holes (2 mm in diameter) were drilled 8 mm anterior to bregma and 2 mm on either side of the midline at a point corresponding to the posterior margin of the orbit of an eye **(fig. 17).** The olfactory bulbs were removed by suction, the holes were then filled with haemostatic sponge to control excessive bleeding and scalp was sutured. Sham-operated rats were subjected to the same surgical procedure, including piercing of the dura mater but their bulbs were left intact. To prevent post surgical infection, the animals were given Sulprim injection (each ml containing 200 & 40 mg of sulphadiazine and trimethoprim, respectively), intramuscularly (0.2 ml/300 g), once a day for 3 days. The detail procedure for the OBX surgery is shown in **fig. 18**. During the 14 days recovery period, the animals were handled regularly to avoid aggressive behavior, which might have developed otherwise.

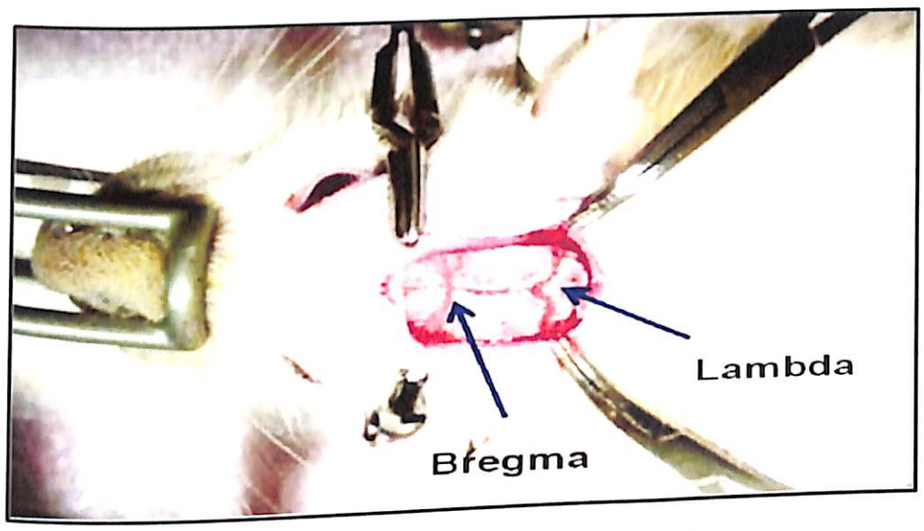


Fig.17. Model representing the bregma and lambda regions in rat brain

Tests for depression-like behavioral deficits were performed in single sets of rats' post-OBX. ROL, ETZ, Q-21 & Q-12 were evaluated, at different dose levels in OBX model.

4.7.1.2. Schedule for Drugs Administration and Behavioral Tests

The administration of drugs or vehicle was started once daily post 14 day of surgery. After the 14 days (15^{th} to 28^{th} day) of drugs treatment the first behavioral test was performed. In order to avoid the acute effects of drug treatment on the behavior, the OBX/sham-operated rats were subjected to the behavioral assessments 20 hrs after the last drug/vehicle administration. Drug treatment was continued till 30^{th} days after the surgery. The surgery, rehabilitation, treatment and behavioral screening of OBX/sham rats were carried out based on the customised schedule reported earlier (Pandey et al., 2008; Rajkumar et al., 2009). The detail of surgical process and treatment schedule is mentioned in the **table 9**.

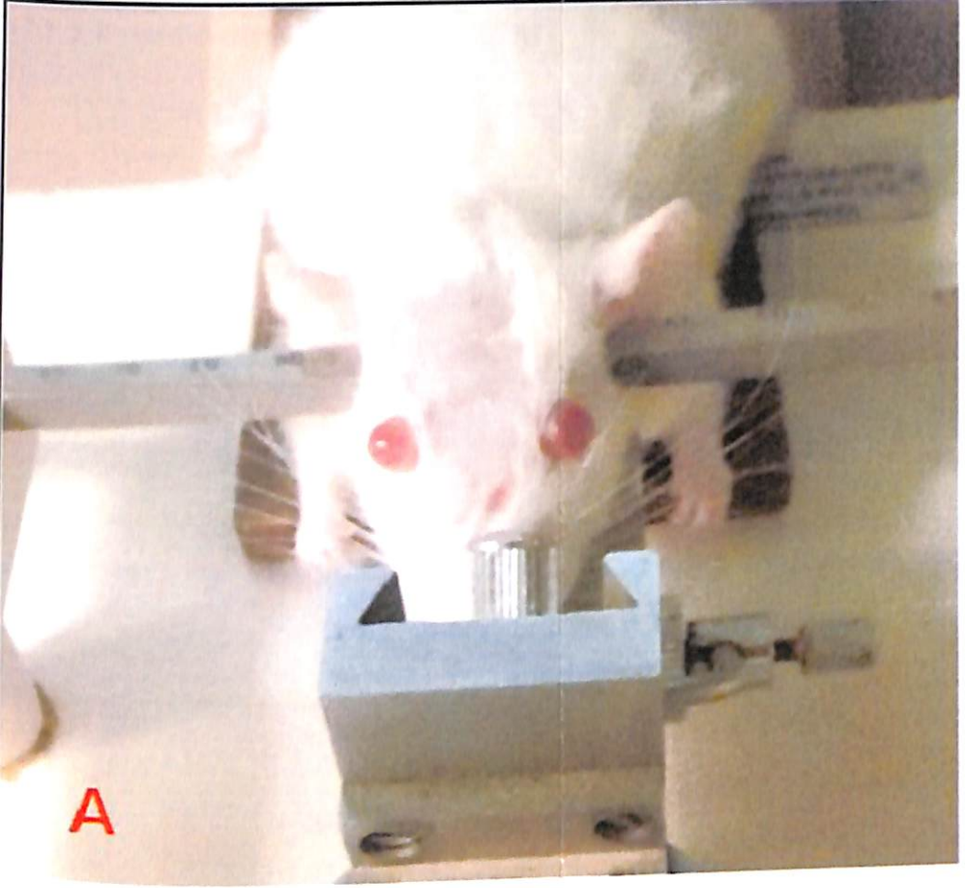
4.7.1.3. Rationale

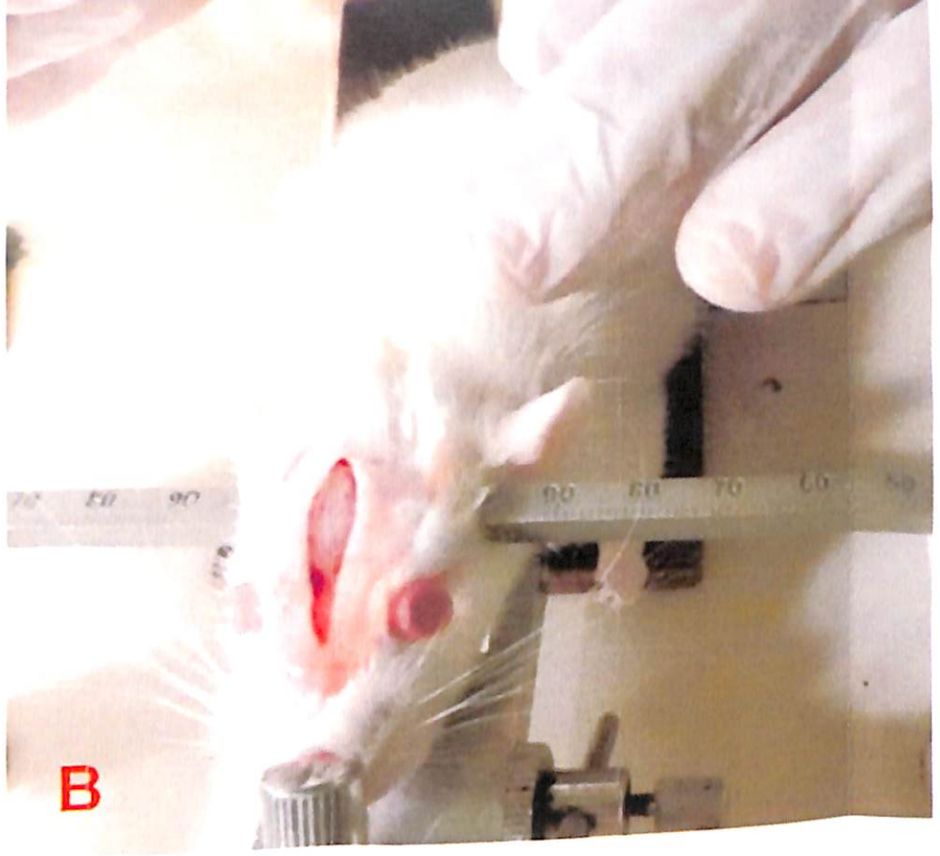
OBX is based on the assumption that depression is caused by neuronal regulatory deficits (Van Riezen and Leonard, 1990; Kelly et al., 1997). Bilateral OBX leads to alter behaviors and NT systems that simulate many of those seen in patients with MDD. A disconnection of the olfactory bulb(s) has been shown to produce deficits in emotional behavior (termed the bulbectomy syndrome) due to a disruption in the homeostatic regulation of signalling mechanism in the limbic system. Hence, it was hypothesized that OBX can be the model of depression and therefore, post-OBX, set of behavioral tests of depression were performed. Olfactory bulb(s) were removed in one or two attempts to avoid severe injury to the brain. Treatment was started 14 days post-surgery to ensure that the animals completely recovered following surgery. Animals were shifted to the observation room 1 hr before the behavioral tests.

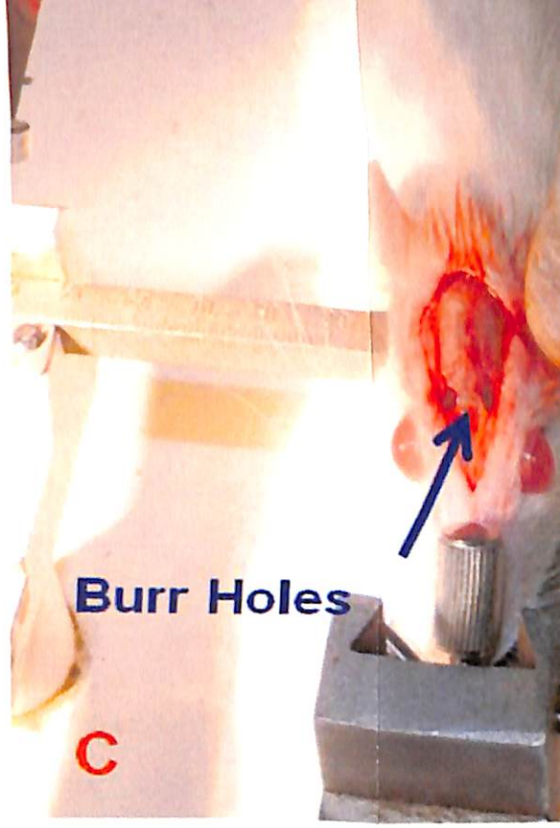
Table 9. Schedule of OBX surgery, drugs treatments, behavioral and biochemical tests

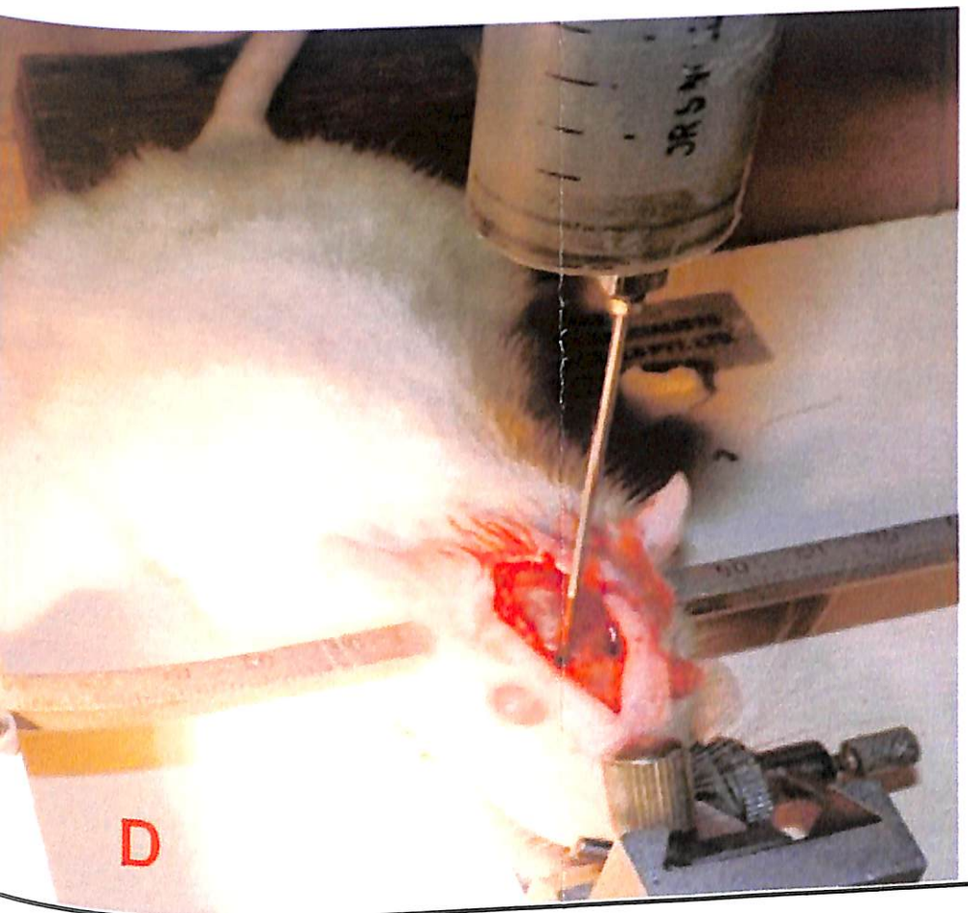
Day 0	0^{th} -1^{st} day	1^{st} -14^{th} day	15^{th} - 28^{th} day	29^{th} -31^{st} day (drug treatment was continued)		
				Behavioral Assessments		
				29^{th}	30^{th}	31^{st}
Surgery	Recovery from surgery (continuous care)	Rehabilitation period (Daily handling and observation)	Drug/vehicle treatment (Once a day p.o. for 14 days)	Modified open field exploration	Hyper-emotionality test	Sucrose consumption test
						After 6 hrs

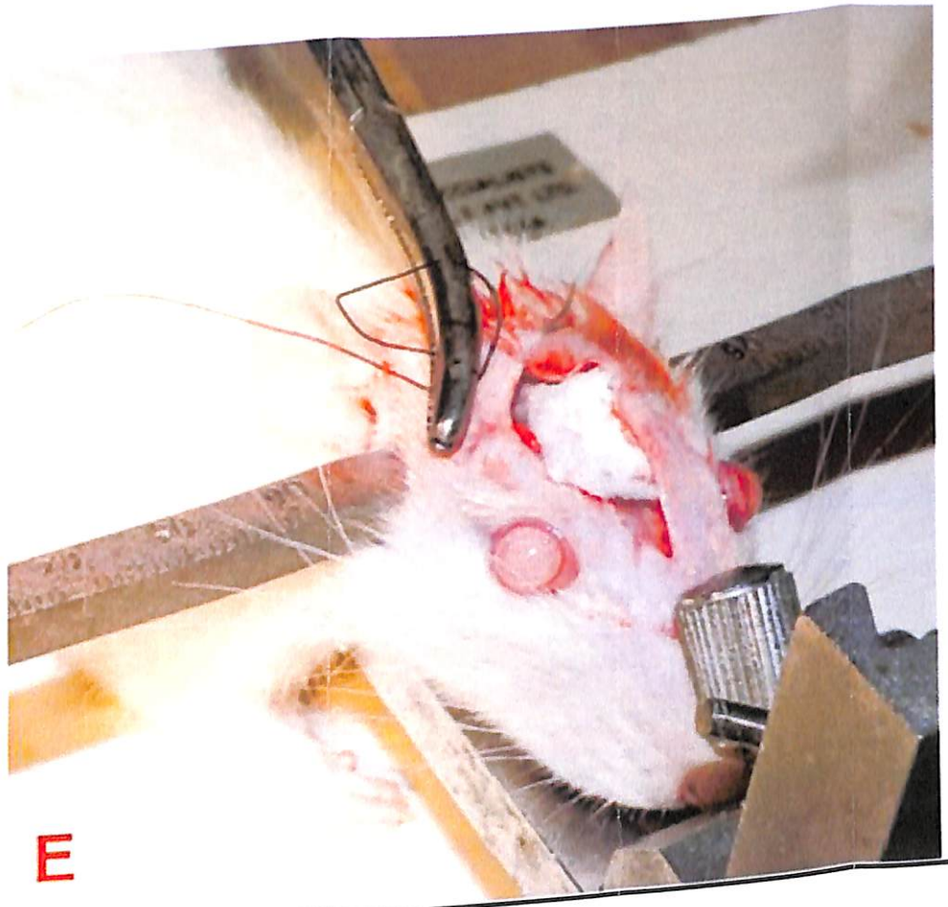
Samples collection for Biochemical, Neurobiological markers and Histo-pathological Analysis

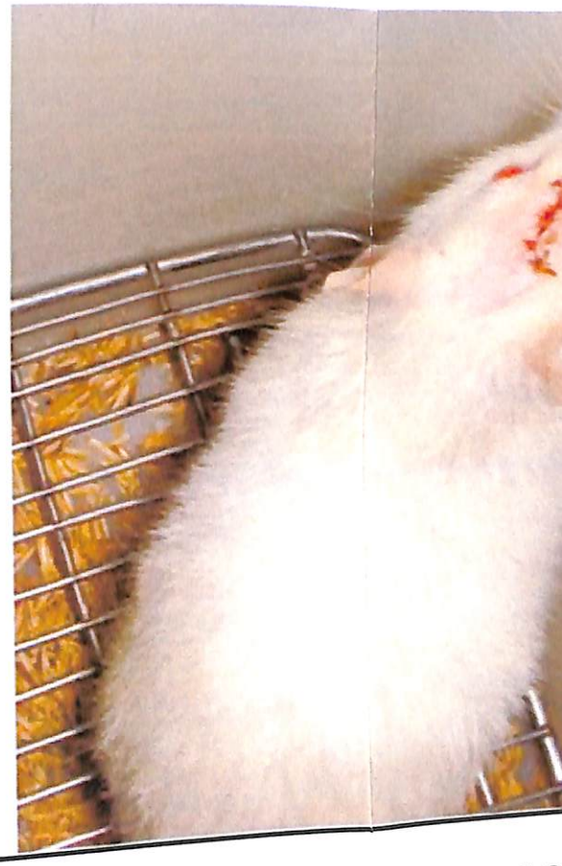












...ᵗʰ Incision. (C) Burr holes on either side of midline, (D) Ablation of the olfactory bulb, (E) S

4.7.1.4. Experimental Design for OBX Model

After 14 post-operative days (recovery period), rats were randomly divided into different groups (six rats in each group), namely the sham control group (no treatment was given); drug treated sham group (sham control rats treated with test drugs) OBX control group and drug treated OBX group (OBX rats treated with test drugs).

Set A. This set of OBX rats was performed using ROL

1. Sham control = 6
2. Sham + ROL (0.5 mg/kg) = 6
3. Sham + ROL (1 mg/kg) = 6
4. Sham + FLX (10 mg/kg) = 6
5. OBX control = 6
6. OBX + ROL (0.5 mg/kg) = 6
7. OBX + ROL (1 mg/kg) = 6
8. OBX + FLX (10 mg/kg) = 6

Set B. This set of OBX rats was performed using ETZ

1. Sham control = 6
2. Sham + ETZ (0.5 mg/kg) = 6
3. Sham + ETZ (1 mg/kg) = 6
4. Sham + FLX (10 mg/kg) = 6
5. OBX control = 6
6. OBX + ETZ (0.5 mg/kg) = 6
7. OBX + ETZ (1 mg/kg) = 6
8. OBX + FLX (10 mg/kg) = 6

Set C. This set of OBX rats was performed using Q-21

1. Sham control = 6
2. Sham + Q-21 (1 mg/kg) = 6
3. Sham + Q-21 (2 mg/kg) = 6
4. Sham + FLX (10 mg/kg) = 6
5. OBX control = 6
6. OBX + Q-21 (0.5 mg/kg) = 6
7. OBX + Q-21 (1 mg/kg) = 6
8. OBX + Q-21 (2 mg/kg) = 6
9. OBX + FLX (10 mg/kg) = 6

Set D. This set of OBX rats was performed using Q-12

1. Sham control = 6
2. Sham + Q-12 (1 mg/kg) = 6
3. Sham + Q-12 (2 mg/kg) = 6
4. Sham + FLX (10 mg/kg) = 6
5. OBX control = 6
6. OBX + Q-12 (0.5 mg/kg) = 6
7. OBX + Q-12 (1 mg/kg) = 6
8. OBX + Q-12 (2 mg/kg) = 6
9. OBX + FLX (10 mg/kg) = 6

4.7.2. Evaluation of ETZ and Q-21 in Traumatic Brain Injury Model of Depression

4.7.2.1. Surgery

TBI was performed based on the method of Foda and Marmarou, (1994) and Heath and Vink, (1999). Rats weighing (250-275 g) were anesthetized using a mixture of ketamine (75 mg/kg i.p.) and xylazine (5 mg/kg i.p.). A 1 cm midline scalp incision was made and the

muscles were retracted to expose the skull. A stainless steel disc (10 mm in diameter and 3 mm in depth) was placed centrally between the lambda and bregma sutures **(fig. 19B).** Injury was then induced using impact acceleration model of TBI. A 420 g metal weight was dropped from a height of 1 m guided through straight pipe (1 m in length and 35 mm in diameter), onto the metal disc placed over the rat's skull **(fig. 19A).** A 10 cm foam bed underneath the animal helped to absorb the impact. After the impact, the metal disc was removed and skin was sutured and Povidone-iodine (10% w/v, Betadine) was applied. During 10 days–rehabilitation period following injury, the rats were housed in standard laboratory cages and separated from the others rats in the housing unit. Wounds were daily inspected to ensure complete healing. **Fig. 20** shows the various steps of TBI procedure. Impact accelerated (weight drop) method was adopted here to minimize the chances of mortality. Sham operated rats were treated in the same way, including mid-line incision except TBI. Neurological outcome was assessed using different functional tests. The disc is used to target regions of interest.

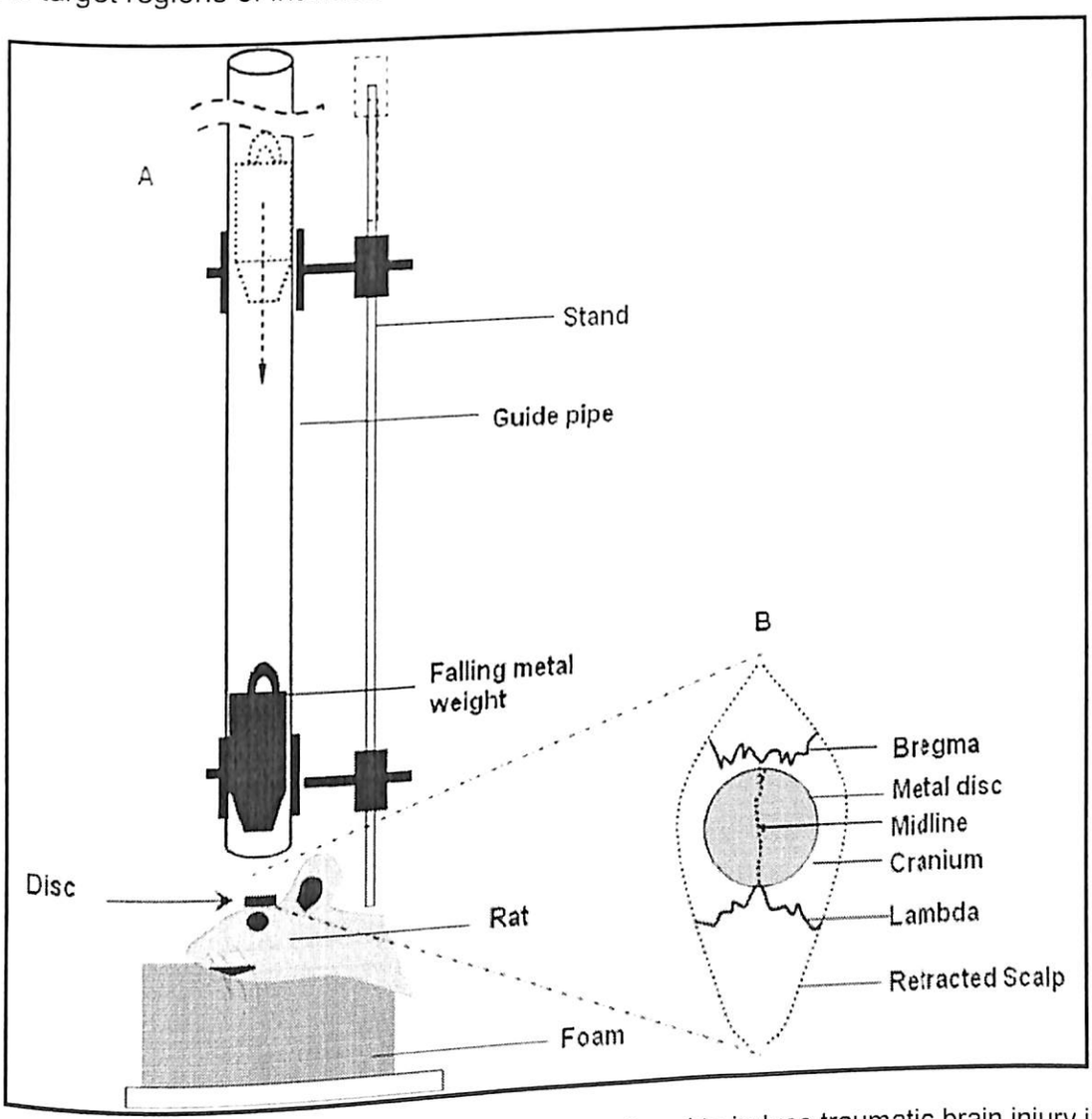


Fig.19. (A) Schematic representation of the method employed to induce traumatic brain injury in rats. **(B)** Dorsal view of the rat cranium showing the positioning of the metallic disc.

Force applied for the injury was calculated by Newton's Law (eq.1)

eq. 1 **F= m x a** F= force, m= mass, a= acceleration

Here, m=420 g and a = 9.8 (acceleration due to gravity)

F= 420 x 9.8

F= 4116 Newton

4.7.2.2. Schedule for Drugs Administration and Behavioral Tests Post-TBI

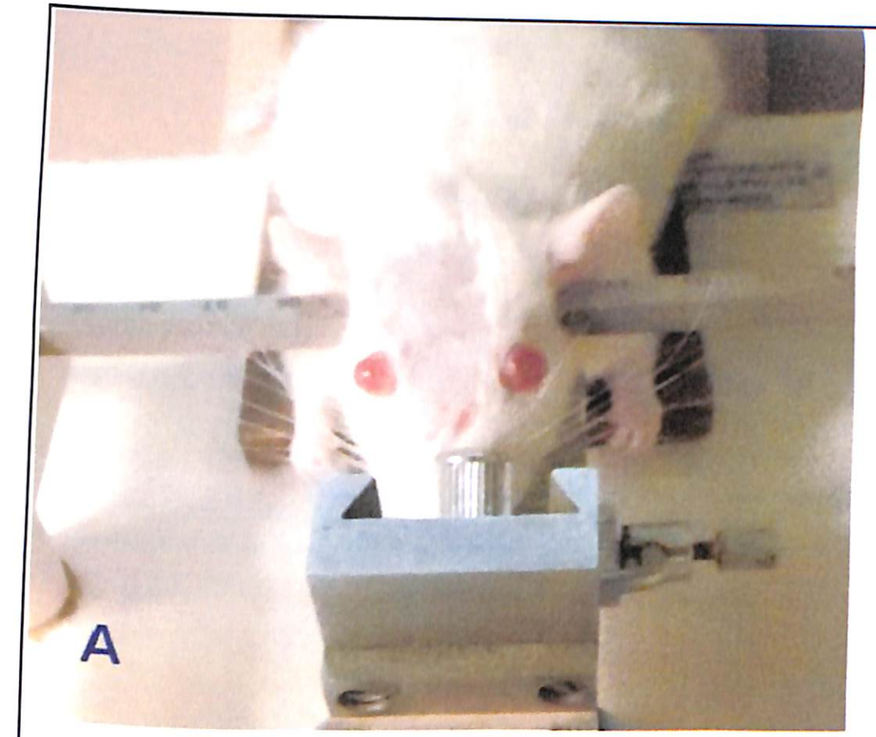
The drug solutions for ETZ and Q-21 were made in similar fashion as discussed in the OBX model. The administration of drugs, daily was started 10 days after the injury and continued for 14 days. After the rats treated with drugs/vehicle for 14 days, the first behavioral test was performed. To avoid the direct effect of drugs on behavior, drug treatment was performed after each behavioral test. Drug treatment was continued till 26th day following injury. The surgery, rehabilitation, treatment and behavioral screening of TBI/sham rats were carried out based on the customized schedule reported earlier (Pandey et al., 2009; Rajkumar et al., 2009). Post-TBI induction a set of depression behavioral tests was performed during three days (25th-27th day following injury) **(table 10).** ***Animals with abnormal behavior and/or diseased condition (0.5 %) were discarded from the study.*** Dosing schedule and behavioral tests were decided based on the time taken for recovery of TBI rats.

Table 10. Schedule of TBI surgery, drugs treatment, behavioral and biochemical tests

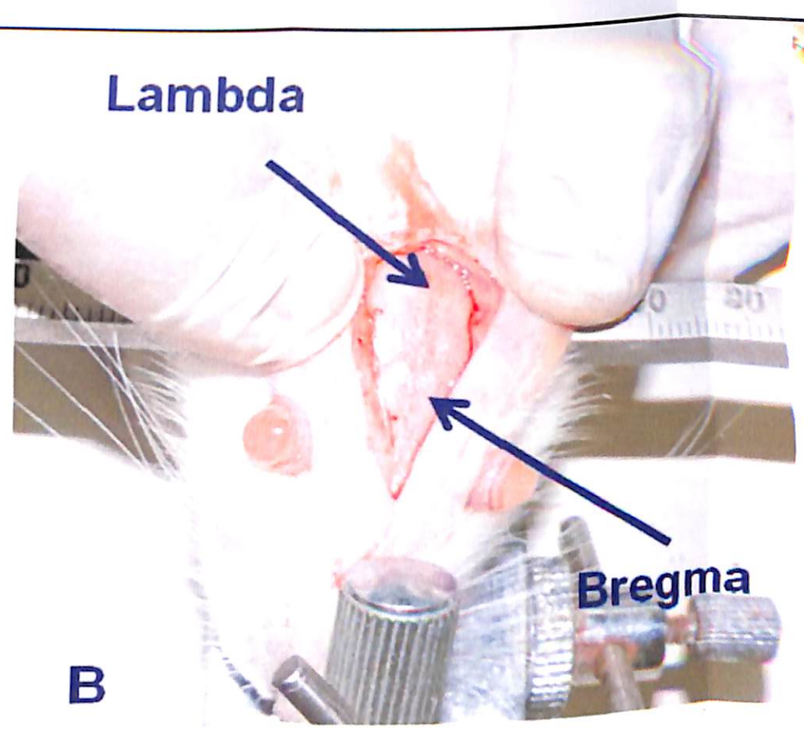
0 Day	**0th -1st Day**	**1st -10th Day**	**11th -24th Day**	**25th-27th day Behavioral assessments**		
				25th	**26th**	**27th**
Surgery	Recovery from surgery	Rehabilitation period (Daily handling and observation)	Drug/ vehicle treatment (Once a day p.o for 14 days	Open field test	Hyper-emotionality	Sucrose consumption test

After 6 h

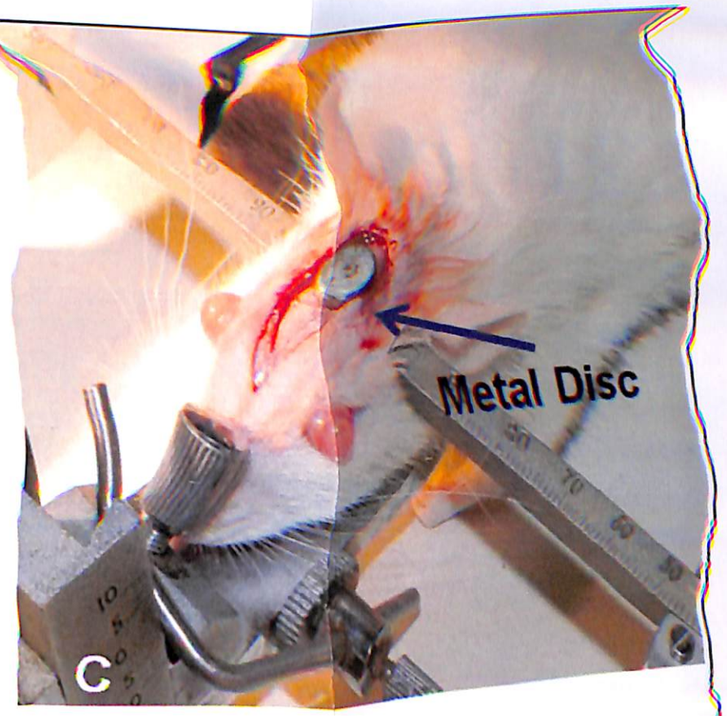
Samples collection for Biochemical and Neurobiological Markers Analysis



A



B



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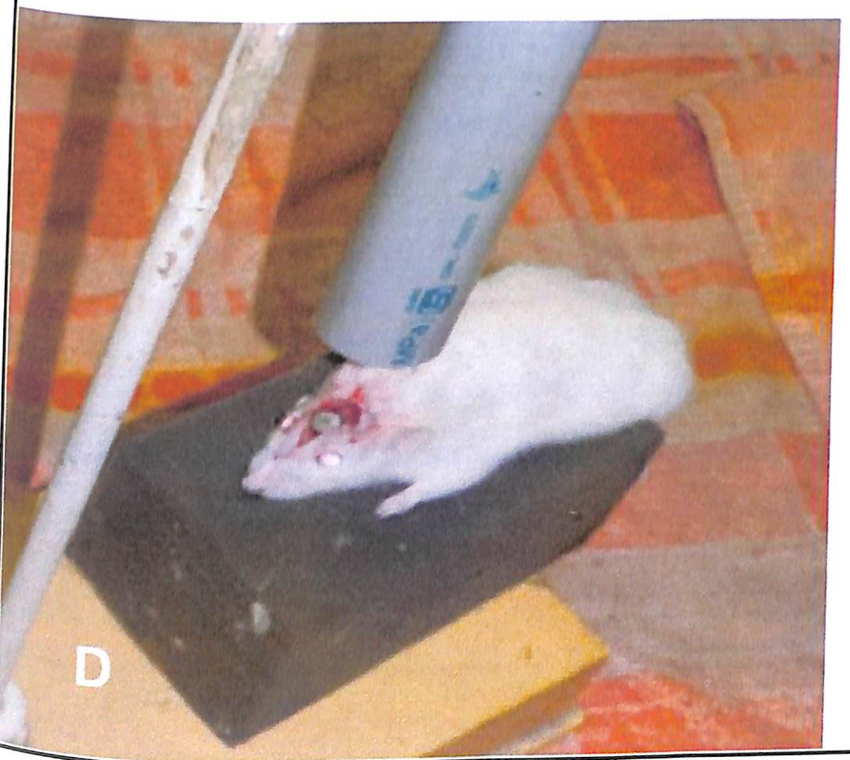

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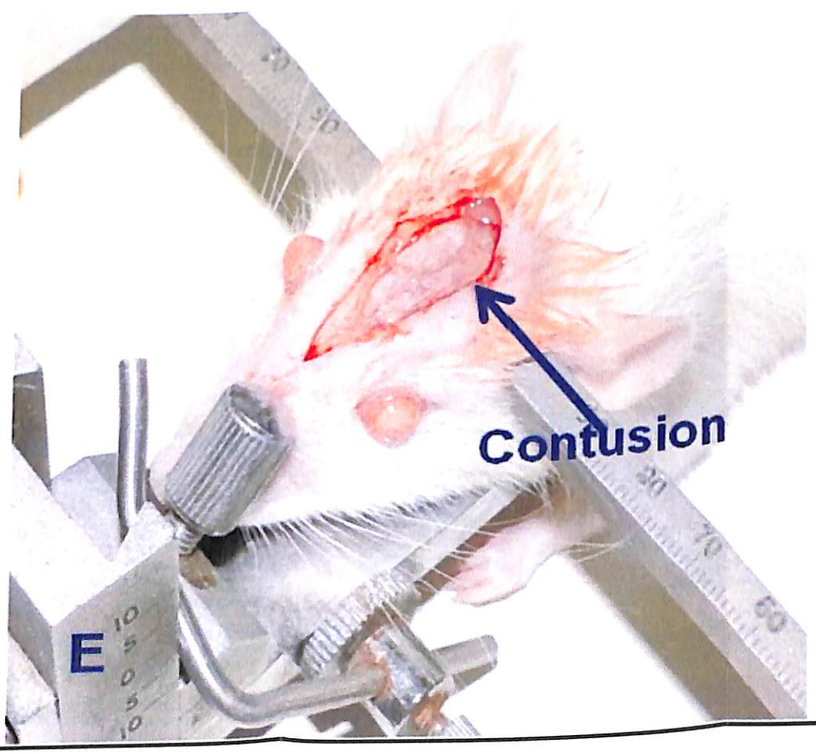

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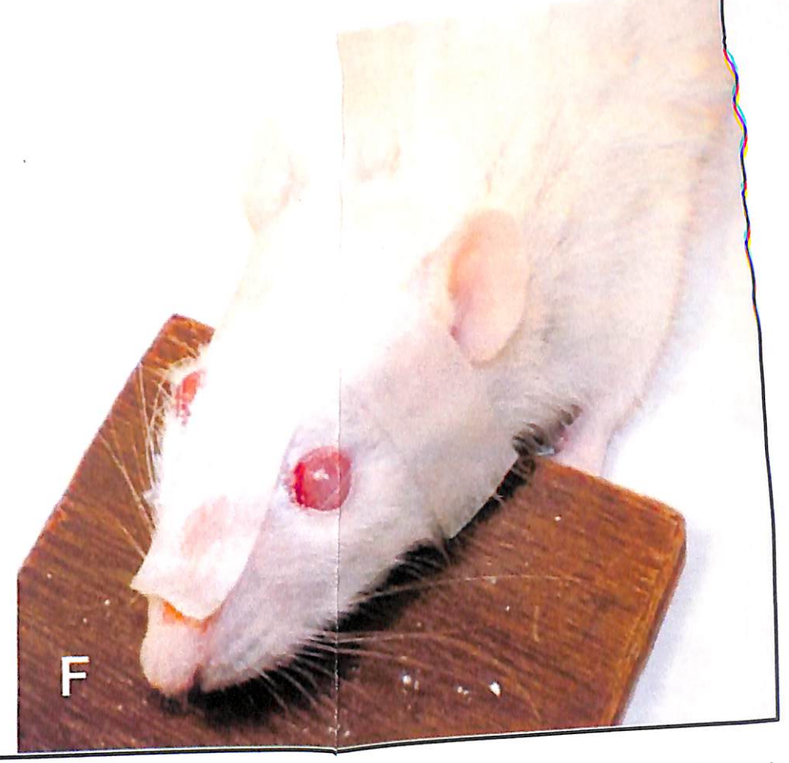


F

head. (E) Contusion post injury and

4.7.2.3. Experimental Design for TBI Procedure

TBI model was standardized in our laboratory, after different attempts with modifications in the height of pipe, weight of the metal bob and dimension of the metal disc. Extra care was taken to prevent skull fracture and bleeding during TBI in rats. After 10 post-operative days (recovery period), the rats (six rats in each group) were randomly divided into different groups, namely the sham control group (no treatment was given); drugs treated sham group (sham control rats treated with test drugs); TBI control group and drug treated TBI group (TBI rats treated with test drugs).

Set A. This set of TBI rats was performed using ETZ

1. Sham control = 6
2. Sham + ETZ (0.5 mg/kg) = 6
3. Sham + ETZ (1 mg/kg) = 6
4. Sham + FLX (10 mg/kg) = 6
5. TBI control = 6
6. TBI + ETZ (0.5 mg/kg) = 6
7. TBI + ETZ (1 mg/kg) = 6
8. TBI + FLX (10 mg/kg) = 6

Set B. This set of TBI rats was performed using Q-21

1. Sham control = 6
2. Sham + Q-21 (1 mg/kg) = 6
3. Sham + Q-21 (2 mg/kg) = 6
4. Sham + FLX (10 mg/kg) = 6
5. TBI control = 6
6. TBI + Q-21 (1 mg/kg) = 6
7. TBI + Q-21 (2 mg/kg) = 6
8. TBI + FLX (10 mg/kg) = 6

4.7.3. Evaluation of ETZ and Q-21 in Chronic Unpredictable Mild Stress Model of Depression

4.7.3.1. CUMS Procedure

The CUMS procedure was performed as described by Ducottet et al., (2003), with slight modifications (foreign object and water temperature in forced swimming stressors) (Jindal et al., 2012; 2013). Exposure to a single severe or repetitive and uncontrollable stressor may trigger or facilitate the development of psychopathologies. This animal model of stress consists of chronic exposure to variable unpredictable stressors, none of which is sufficient alone to induce long-lasting effects. Briefly, CUMS consisted of exposure to a variety of unpredictable stressors (randomly); (1) food deprivation, (2) water deprivation, (3) exposure to a empty bottle, (4) cage tilt (45°), (5) overnight illumination (60 W lamp), (6) soiled cage (200 ml water in 100 gm bedding), (7) forced swimming at 12 °C, (8) physically restraint (placing the mouse in a plastic tube and adjusting it with non allergic plaster tape on the outside, so that the mouse was unable to move) and (9) exposure to a foreign object

(glass marbles; diameter 20 mm). These stressors were randomly scheduled over a 1 week period and repeated throughout the 4 weeks experiment. Details of different stressors and their schedule are given in **table 11**. This procedure was developed to maximize unpredictability; in that the stressors were applied in apparently random order and at varying times during the light phase (09–17 hr). Normal control mice were undisturbed, except for necessary housekeeping procedures. Mice with abnormal behavior were excluded from the study. All the mice subjected to CUMS were given uniform stress throughout the procedure.

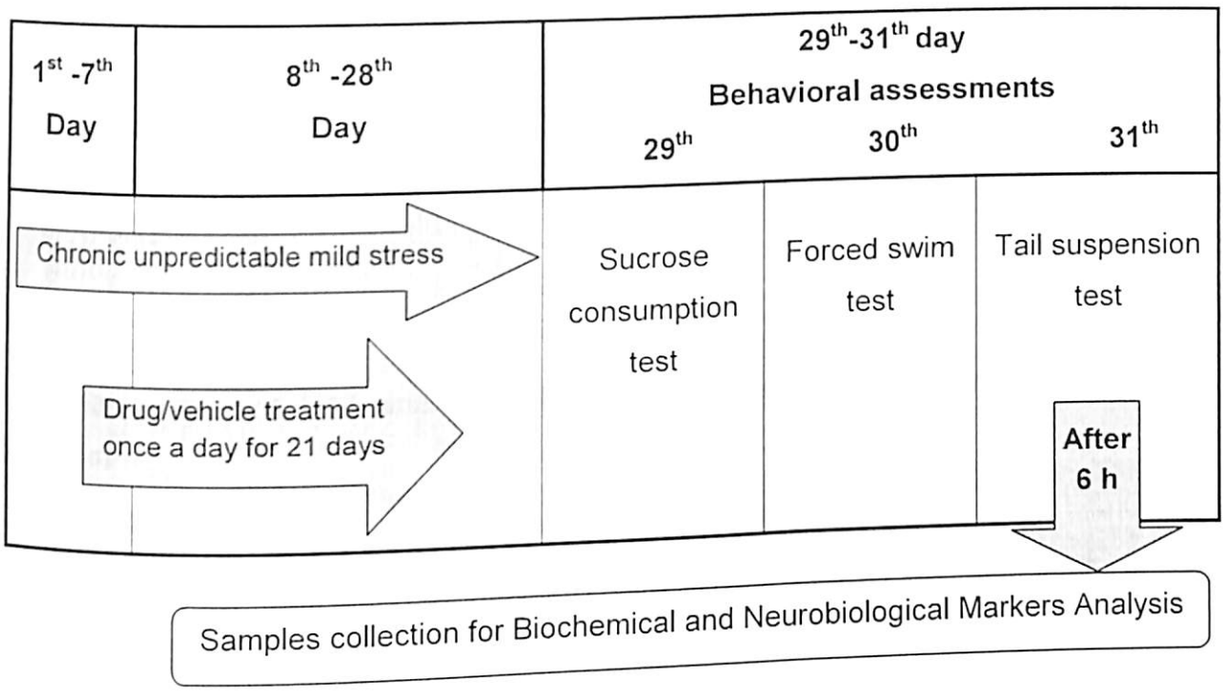
Table 11. Types and duration of stressors used in CUMS model

Stressor	Duration	Day
Food deprivation	24 h	Monday
Water deprivation	24 h	
Empty bottle	1 h	Tuesday
Foreign object (glass marbles)	24 h	
Forced swimming	6 min	Wednesday
Overnight illumination	12 h	
Restraint	2 h	Thursday
Cage tilt	7 h	
Food deprivation	24 h	Friday
Soiled cage	24 h	
Water deprivation	24 h	Saturday
Overnight illumination	12 h	
Empty bottle	1 h	Sunday
Cage tilt	7 h	

4.7.3.2. Schedule for Drugs Administration and Behavioral Tests

ETZ and Q-21 were administered by p.o. route once a day during the last 21 days (8^{th} -28^{th} days) of the CUMS procedure **(table 12)**. After the mice were treated with drugs/vehicle for 21 days, the first behavioral test was performed. The behavioral testing was done atleast 16-18 hrs after the last dose in order to avoid the acute effect of drug treatment. Post stress, set of depression behavioral tests was performed during three days (29^{th}-31^{th}), following CUMS. The doses of ETZ, Q-21 and FLX were selected on the basis of our previous studies or reported elsewhere (Mao et al., 2009; Jindal et al., 2012). Behavioral models, incorporating repeated exposure to stress have been widely used as experimental models for depression (Willner et al., 1997), because stress is thought to play an important role in the etiology of depression.

Table 12. Schedule of CUMS, treatments, behavioral and biochemical tests



4.7.3.3. Rationale

Stress based models have greater etiologic validity compared to those, which rely on brain lesions or monoamine depletion, which is a not a common etiologic factor in clinical depression. Therefore, CUMS model can be considered as an animal model of depression, involving chronic stress as the etiological factor of depression. The model also represents predictive validity criteria, since the reversal of abnormal behavior requires 2-3 weeks of treatment.

4.7.3.4. Experimental Design for CUMS Procedure

The study included normal (unstressed) and CUMS-subjected (stressed) mice. The normal mice were randomly divided into different groups, namely, normal control group (no drugs treatment was given) and drug treated normal control group (normal mice treated with test drugs). CUMS-subjected mice were randomly divided into stressed control and drug treated stressed mice (stressed mice treated with test drugs).

Set A. This set of CUMS-induced mice was performed using ETZ

1. Normal control = 6
2. Control + ETZ (0.5 mg/kg) = 6
3. Control + ETZ (1 mg/kg) = 6
4. Control + FLX (20 mg/kg) = 6
5. CUMS control = 6
6. CUMS + ETZ (0.5 mg/kg) = 6
7. CUMS + ETZ (1 mg/kg) = 6
8. CUMS + FLX (20 mg/kg) = 6

Set B. This set of CUMS-induced mice was performed using Q-21

1. Normal control = 6
2. Control + Q-21 (1 mg/kg) = 6
3. Control + Q-21 (2 mg/kg) = 6
4. Control + FLX (20 mg/kg) = 6
5. CUMS control = 6
6. CUMS + Q-21 (0.5 mg/kg) = 6
7. CUMS + Q-21 (1 mg/kg) = 6
8. CUMS + Q-21 (2 mg/kg) = 6
9. CUMS + FLX (20 mg/kg) = 6

4.7.4. Evaluation of ETZ and Q-21 in Repeated Corticosterone-injection Model of Depression

4.7.4.1. CORT Treatment

The chronic CORT-treated model was first time standardized in our laboratory. Different doses and time period were used to assed the depression type symptoms after chronic CORT injection. This model has been proposed as an animal model of depression that mimics the alteration of HPA axis activity in depression disorder. Chronic CORT administration produces similar behavioral, biochemical and neuronal abnormalities in rodents, those observed in human depression (Gourley and Taylor, 2009; Sterner and Kalynchuk, 2010).

4.7.4.2. Schedule of Drugs Administration and Behavioral Test

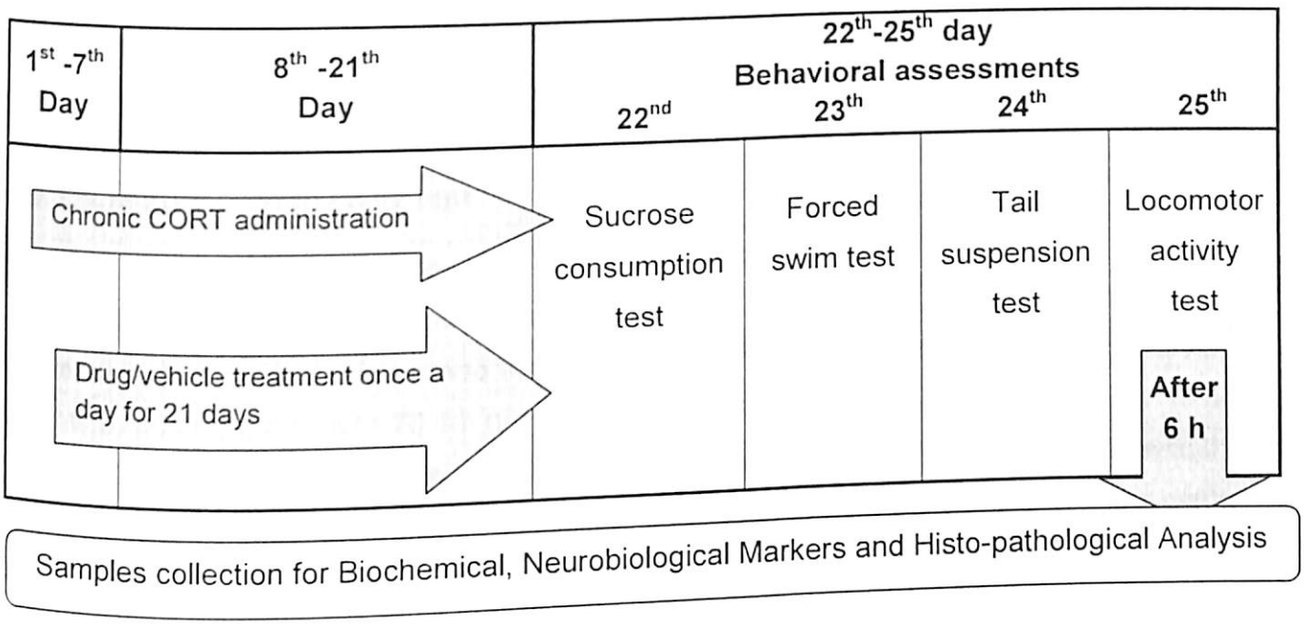
The CORT was suspended in 0.5% (w/v) carboxy methylcellulose in similar fashion as discussed, elsewhere (Ago et al., 2008; Zhao et al., 2008). Mice were injected, once daily for 21 consecutive days (between 10:30 a.m. to 12:00 a.m.) with CORT (30 mg/kg, s.c.) as shown in **table 13.** ETZ and Q-21 were administered by p.o. route once a day for 21 consecutive days, continued till 24th day of the study. After 21 days of drug/vehicle treatment, the first behavioral test was performed. The behavioral testing was done at least 16-18 hrs after the last dose in order to avoid the acute effect of drug treatment. Post CORT-injection, set of behavioral tests was performed during four days (22th-25th).

4.7.4.3. Rationale

The HPA axis, a crossroad between central and peripheral pathways, is also known to play an important role in the pathogenesis of mood disorders (De Kloet et al., 2005). Similarities between features of depression and other disorders associated with high GCs levels have been reported (Popa et al., 2008). On the basis of these findings, chronic exposure to CORT in rodents has been used to induce depression-like changes in behavior, neurochemistry and

brain morphology (Gourley et al., 2008; Murray et al., 2008). Hence, it is hypothesized that chronic CORT-injection to rodents can be used as a model of TRD.

Table 13. Schedule of CORT, drugs treatment, behavioral and biochemical tests



4.7.4.4. Experimental Design for Chronic CORT-treated Model

Chronic CORT-injection model was standardized in our laboratory, using different dose levels and time schedules. The study included normal and CORT-treated mice. The normal mice were randomly divided into different groups, namely the normal control group (no treatment was given) and drugs treated normal group (normal control mice treated with test drugs). CORT-injected mice were randomly divided into CORT control group and drug treated CORT group (CORT-injected mice treated with test drugs).

Set A. This set of CORT-injected mice was performed using ETZ

1. Normal control = 6
2. Control + ETZ (0.5 mg/kg) = 6
3. Control + ETZ (1 mg/kg) = 6
4. CORT control = 6
5. CORT + ETZ (0.5 mg/kg) = 6
6. CORT + ETZ (1 mg/kg) = 6
7. CORT + FLX (20 mg/kg) = 6

Set B. This set of CORT-injected mice was performed using Q-21

1. Normal control = 6
2. Control + Q-21 (1 mg/kg) = 6
3. Control + Q-21 (2 mg/kg) = 6
4. CORT control = 6
5. CORT + Q-21 (0.5 mg/kg) = 6
6. CORT + Q-21 (1 mg/kg) = 6
7. CORT + Q-21 (2 mg/kg) = 6
8. CORT + FLX (20 mg/kg) = 6

4.8. Behavioral Assays

4.8.1. Behavioral Assays Performed Post-OBX and TBI Models

Once the OBX and TBI procedures are over, a series of behavioral tests were carried out. A set of behavioral tests were performed and parameters associated with the tests were designed in such a manner, so as to simulate the depression behavioral symptoms in rats.

4.8.1.1. Modified Open Field Test

The rats were subjected to an open field exploration test on 15th day of chronic drug treatment according to the method described by Kelly et al., (1997) with slight modifications (dimension of the OFT) (Ramamoorthy et al., 2008). The apparatus consisted of a circular (diameter 90 cm) arena with 75 cm high aluminium walls and floor equally divided into 10 cm squares. A 60 W light bulb was positioned 90 cm above the base of the arena, which was the only source of illumination in the testing room. Each rat was individually placed in the centre of the open field and the following parameters were observed for 5 min. Ambulation scores (number of squares crossed) and number of rearing episodes were noted as horizontal and vertical activity, respectively. Crossing of a square was considered only when hind limbs of rats move to the next square. Other parameter observed was defecation. The number of fecal pellets was counted at the end of each 5 min trial. After each test, the apparatus was sprayed with alcohol and wiped thoroughly to eliminate residual odour.

Rationale: Measurement of hyperactivity reflects psychomotor effect.

4.8.1.2. Sucrose Preference/Consumption Test

Sucrose preference test was performed to measure the anhedonia type behavior, a core symptom of depression disorder. The procedure was performed with modification as described by (Willner et al., 1997). Rats had free access to both filtered water and sucrose solution (1%) for 5 days from the commencement of test drugs treatment. The position of 250 mL bottles containing sucrose solution or filtered water was alternated each day to prevent location preference. The sucrose consumption test was performed by presenting both sucrose solution and water in the morning (9:30 am) on the test day. The bottles were weighed after 24 h (next morning). Sucrose preference was calculated by using the following formula: % preference = (sucrose intake/total intake) X 100.

Rationale: Measurement of anhedonia type behavior.

4.8.1.3. Hyper-emotionality Test

Hyper-emotionality was measured with slight modification of the previously reported procedure (Shibata and Watanabe, 1994; Pandey et al., 2008). Hyper-emotionality of rats was measured by scoring responses to the following stimuli:

(1) Startle response: it was induced by a stream of air (using a 10-ml syringe) directed at the dorsum, **(2)** Struggle response: it was induced by handling with a gloved hand, **(3)** Fight response: it was induced by pinching the tail with forceps and **(4)** Bite response: it was induced by presenting a rod 4–5 cm in front of the snout. A trained researcher performed these operations. These responses were graded as follows: 0 - no reaction, 1 - slight, 2 – moderate, 3 – marked and 4 – extreme. All animals in each group were observed in 1 day. The hyper-emotional score for each rat was determined within 5 min of the observed response. The observers were blind with respect to the drug treatment.

Rationale: Loss of emotional component was measured.

4.8.2. Behavioral Assays Performed Post-CUMS and Chronic CORT-treated Models

The behavioral analysis after the CUMS and CORT models consisted of the following test.

4.8.2.1. Spontaneous Locomotor Activity

The procedure for SLA test was followed as mentioned in the section **4.6.1.**

4.8.2.2. Forced Swim Test

The procedure for FST was followed as mentioned in the section **4.6.2.**

4.8.2.3. Tail Suspension Test

The procedure for TST was followed as mentioned in the section **4.6.3.**

4.8.2.4. Sucrose Preference Test

Sucrose preference test in CUMS and CORT-treated mice models was carried out as described earlier (Luo et al., 2008; Mao et al., 2009) with slight modification in the amount of water and sucrose solution provided in bottle. In brief, before the test, mice were trained to adapt sucrose solution (1%, w/v) by placing two bottles of sucrose solution in each cage for a period of 24 h; then one bottle of sucrose solution was replaced with water for 24 h. After the adaptation, mice were deprived of water and food for 24 h. Sucrose preference test was conducted at 9:30 a.m. The mice were housed in individual cages and free to access two

bottles containing 250 ml of sucrose solution (1% w/v) and 100 ml of water, respectively. After 24 h, the volumes of consumed sucrose solution and water were recorded. Then percentage of sucrose consumption was calculated as ratio of the amount of sucrose solution to that of total solution (sucrose & water), ingested within 24 h. In addition, the normal water intake (%) was calculated as ratio of the amount of normal water to that of total solution (sucrose & water).

4.9. Biochemical, Neurochemical and Neurobiological Assays Performed Post-OBX, TBI, CUMS and Chronic CORT-treated Models

All the biochemical, neurochemical and neurobiological estimations were carried out 6 hrs after completion of all the behavioral assessments.

4.9.1. Tissue Extraction

The rats and mice were decapitated and brain was harvested quickly as per procedure mentioned elsewhere (Glowinski and Iversen, 1966) and placed on a petri dish in an ice bath. The homogenate for different estimations were performed as per the ELISA kits instruction or the reported literatures.

4.9.2. Oxidants and Anti-oxidants Assays

4.9.2.1. Brain Homogenate Preparation

Brains were quickly removed and washed with ice-cold sterile saline (0.9%). The whole brain samples were then homogenized with ice-cold 0.1 M phosphate buffer (pH 7.4) 10 times (w/v). The homogenate was centrifuged at 4000 rpm (4 °C) for 20 min to remove cellular debris and aliquots of supernatant were separated and stored at -80 ºC until the oxidative and anti-oxidant assays were carried out.

4.9.2.2. Preparation of Reagents for Oxidant/Anti-oxidant Markers Estimation

4.9.2.2.1. Greiss reagent preparation:

This was prepared by adding 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid.

4.9.2.2.2. Dichromate-acetic acid reagent:

This was prepared by adding 5% potassium dichromate and glacial acetic acid in ratio of 1:3.

4.9.2.3. Estimation of Lipid Peroxidation Level

The malondialdehyde (MDA) content, a quantitative measurement of lipid peroxidation, was assayed in the form of thiobarbituric acid reactive substances (TBARS) by the method of Wills, (1966). In this 0.1 ml of supernatant was incubated with 0.5 ml tris HCL (0.1 M, pH 7.4) for 2 h. To this, 1 ml of trichloroacetic acid (10% w/v) was added and centrifuged at 1000 × g for 10 min. To 1 ml supernatant, 1 ml (0.67% w/v) thiobarbituric acid was added and kept in the boiling water bath for 10 min, cooled and then 1 ml distilled water was added. The amount of lipid peroxidation products was measured by reaction with thiobarbituric acid at 532 nm using the spectrophotometer (UV-1800 Shimadzu, Japan). The values were expressed as nanomole of MDA per milligram of protein.

4.9.2.4. Estimation of Nitrite/Nitrate Level

The accumulation of nitrite in brain supernatant, an indicator of the production of nitric oxide was determined by a colorimetric assay using Greiss reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid) as described by Green et al., (1982). Equal volumes of supernatant and Greiss reagent were mixed, the mixture incubated for 10 min at room temperature in the dark and the absorbance determined at 540 nm using Spectrophotometer (UV-1800 Shimadzu, Japan). The concentration of nitrite in supernatant was determined from sodium nitrite standard curve and expressed as micromole per milligram of protein.

4.9.2.5. Estimation of Reduced Glutathione Level

The test procedure was adopted as indicated elsewhere (Ellman, 1959) with slight modifications (Jindal et al., 2013). In brief, the procedure is as follows. 1 ml supernatant was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested at 4 °C for 1 h. The samples were centrifuged at 1200×g for 15 min at 4 °C. To 1 ml of this supernatant, 2.7 ml of phosphate buffer (0.1 mol/l, pH 8) and 0.2 ml of 5, 5-dithio-bis (2-nitrobenzoic acid) were added. The colour developed was measured immediately at 412 nm using Spectrophotometer (UV-1800, Shimadzu, Japan). Results were expressed as micromole per milligram of protein.

4.9.2.6. Estimation of Superoxide Dismutase Activity

SOD activity was measured by the method of Misra and Fridovich, (1972). Auto-oxidation of epinephrine at pH 10.4 was measured. In this method, supernatant of the tissue was mixed with 0.8 ml of 50 mM glycine buffer, pH 10.4 and the reaction was started by addition of 0.02

ml (−)-epinephrine. After 5 min the absorbance was measured at 480 nm using spectrophotometer (UV-1800, Shimadzu, Japan). The activity of SOD was expressed as % activity of sham control group.

4.9.2.7. Estimation of Catalase Activity

Brain CAT activity was assayed by the method described earlier (Sinha, 1972). The reaction mixture (1.5 ml) contained 1.0 ml of 0.01 mol/l phosphate buffer (pH 7), 0.1 ml of brain homogenate supernatant and 0.4 ml of 2 mol/l hydrogen peroxide. The reaction was stopped by the addition of 2 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in a 1:3 ratio). The absorbance was measured at 620 nm using Spectrophotometer (UV-1800, Shimadzu, Japan) and expressed as micromoles of hydrogen peroxide decomposed/min/milligram protein.

4.9.2.8. Protein Estimation

The protein content was measured in all brain samples by the biuret method using bovine serum albumin as standard (Koller, 1984).

4.9.3. Hormonal Parameter

4.9.3.1. Estimation of Endocrinology Hormone (CORT)

To determine the alterations in the HPA axis, we measured serum CORT level. Measurement of serum CORT was performed using a commercially available ELISA kit (IBL, USA) according to the manufacturer instructions. The Kit was a solid phase ELISA, based on the principle of competitive binding, where an unknown amount of CORT (present in the sample) and a defined amount of CORT (conjugated to horseradish peroxidise) competed for a fixed number of CORT anti-serum binding sites, coated to the wells of a microplate.

4.9.3.1.1. Serum Sample Separation

The blood samples were collected from rats or mice after 6 hrs of the last behavioral assessments as depicted in the schedule of test drugs administration and behavioral assessment sections. Blood samples were collected and allowed to coagulate at room temperature for 30 min and were subsequently centrifuged at 3500 rpm for 20 min. Serum was separated and all the samples were stored at -80 °C until the CORT estimations were carried out.

4.9.3.1.2. Standard Curve

The standard curve for the CORT assay was prepared by using the kit instruction. Already prepared standards (Concentrations: 15 – 50 – 185 – 640 and 2250 ng/ml) provided in the ELISA kit were used to plot a standard curve. A standard curve was constructed by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

4.9.3.1.3. Estimation of Corticosterone Level in Mice and Rats Serum Samples

- ✓ *Briefly, a* sufficient number of microplate wells were prepared to accommodate calibrators and samples in duplicates.
- ✓ 10 µl of each calibrator and sample were dispensed into appropriate wells. Further, 100 µl of incubation buffer dispended into each well.
- ✓ This was followed by 50 µl of enzyme conjugate added into each well and incubated for 2 hrs at room temperature on a microplate mixer (optimal reaction in this assay is markedly dependent on shaking of the microplate).
- ✓ After 2 hrs incubation the plate content was discarder and washed 4 times with diluted wash solution (300 µl per well).
- ✓ After washing 200 µl of substrate solution was added to each well and incubated without shaking for 30 min in the dark.
- ✓ Then reaction stop solution was added and the absorbance was noted at 450 nm.

4.9.4. Molecular Biology Assay

The molecular biology assays were carried out for the estimation of cAMP/CREB/BDNF signaling transduction cascade modulators in rats and mice brain samples.

4.9.4.1. Estimation of cAMP Level

The measurement of cAMP level was performed using a commercially available ELISA kit (Enzo Llife Science Ltd, USA), according to the manufacturer instructions. The Kit was based on the principle of competitive binding, where an anti-Rabbit IgG polyclonal coating antibody adsorbed onto a microtiter plate. Then, an unknown cAMP (present in the sample) or standard competed with peroxidase cAMP tracer for microtiter plate binding, in the presence of rabbit anti-cAMP polyclonal antibody. Any peroxidase cAMP tracer bound to the plate was detected with addition of substrate solution and colour product formed was inversely proportional to the amount of cAMP present in the sample.

4.9.4.1.1. Tissue Homogenate

The brain samples preparation was carried out as per the ELISA kit instruction. The brain tissues were homogenate with the calibration buffer, provided in kit and centrifuged to remove particulates. The supernatant was separated out and stored at -80 °C.

4.9.4.1.2. Standard Curve Preparation

The standard curve for the cAMP assay was prepared by using the kit instruction. The different standards (Concentrations: 20 – 5 – 1.25 – 0.332 and 0.078 pmol/ml) provided in the ELISA kit were used to plot a standard curve.

4.9.4.1.3. Estimation of cAMP Level in Mice and Rats Brain Samples

- ✓ Briefly, a sufficient number of microplate wells were prepared to accommodate standards and samples in duplicates.
- ✓ Then, 100 µl of the appropriate standard diluent (Assay Buffer 2 or non-conditioned culture media) into the NSB (non-specific binding) and Bo (0 pmol/mL standard) wells were dispensed.
- ✓ Further, 50 µl of assay buffer 2 dispended to the NSB well.
- ✓ Then 100 µL of standard concentration and samples were added to the bottom of the appropriate wells.
- ✓ 50 µl of blue conjugate was added into each well except the TA and Blank wells.
- ✓ Then 50 µL of the yellow antibody was added into each well except the Blank, TA, and NSB wells and incubated for 2 hrs at room temperature on a microplate mixer (~500 rpm).
- ✓ After 2 hr incubation the plate content was discarded and washed 3 times with diluted wash solution (400 µl per well).
- ✓ After washing, plate was inverted and blotted against clean paper towels.
- ✓ Then 5 µl of blue conjugate was added to the TA wells
- ✓ Further, 200 µL of the substrate solution was added in each well and incubated for 1 hr without shaking. Then 50 µL reaction stop solution was added and the absorbance of each well was noted at 405 nm.
- ✓ To normalize for protein content, divided the resulting pmol/mL determinations by the total protein concentration (mg/mL) in each sample and expressed as pmol cAMP per mg of total protein.

4.9.4.2. Estimation of Brain Derived Neurotrophic Factor

The measurement of BDNF level was performed using a commercially available ELISA kit (Boster Biological Technology Co., LTD, CA, USA), according to the manufacturer instructions. The Kit was based on the principle of sandwich ELISA assay. The density of yellow colour was directly proportional to the BDNF amount of sample captured in plate.

4.9.4.2.1. Brain Samples Preparation

The brain samples preparation was carried out as per the ELISA kit instruction. The brain tissues were homogenate in the lysis buffer and centrifuged. The supernatant was separated out and stored at -80 °C.

4.9.4.2.2. Standard Curve Preparation

The standard curve was plotted according the instruction of the ELISA kit. The different standards (Concentrations: 1000 – 500 – 250 – 125 - 62.5 - 31.2 pg/ml) provided in ELISA kit were used to plot a standard curve. A standard curve was plotted as the relative absorbance value of each standard solution (Y) vs. the respective concentration of standard solution (X). The rat BDNF concentration of the samples is interpolated from the standard curve.

4.9.4.2.3. Estimation of BDNF Level in Mice and Rats Brain Samples

- ✓ Briefly, sufficient number of microplate wells was prepared to accommodate standards and samples in duplicates.
- ✓ Then wells were coated for 90 min with samples (diluted with sample diluents buffer in a proportion of 1:10) and different standards concentrations (curve ranged from 31.25 to 2000 pg/ml of BDNF).
- ✓ After 90 min the plate content was discarded.
- ✓ Then biotinylated anti-rat BDNF antibody (diluted with antibody diluents buffer in a proportion of 1:100) was added to each well and incubated for 1 hr at room temperature.
- ✓ After 1 hr incubation the plate was washed three times with wash buffer.
- ✓ After washing, an Avidin-Biotin-Peroxidase working solution (diluted with ABC dilution buffer in a proportion of 1:100) was added to each well and incubated at room temperature for 30 min.
- ✓ After 30 min the plate content was washed five times with wash buffer.
- ✓ After washing TMB colour developing agent was added in each well and incubated for 20 min in dark.

- ✓ Then reaction stop solution was added and the amount of BDNF was determined by measuring absorbance at 450 nm.
- ✓ The standard curve demonstrates a direct relationship between optical density and BDNF concentration.

4.9.4.3. Estimation of cAMP Response Element Binding Protein

The measurement of pCREB level was performed using a commercially available sandwich ELISA kit (Wuhan EiAab Sciences Co., Ltd. China), according to the manufacturer instructions.

4.9.4.3.1. Brain Samples Preparation

The brain tissues were rinsed with 1X phosphate buffer saline to remove excess blood, homogenized in 20 mL of 1X PBS and stored overnight at -20 °C. Then two freeze-thaw cycles performed to break the cell membranes and the homogenates were centrifuged for 5 min at 5000 rpm. The supernatant was separated and used immediately or aliquot and stored at -80 °C.

4.9.4.3.2. Standard curve preparation

The standard curve was plotted according the instruction of ELISA kit. The different standards (Concentrations: 10 – 5 – 2.5 – 1.25 – 0.62 – 031 – 0.07 ng/ml) provided in the ELISA kit were used to plot a standard curve. A standard curve was plotted as the relative absorbance value of each standard solution (Y) vs. the respective concentration of the standard solution (X). The pCREB concentration of the samples can be interpolated from the standard curve.

4.9.4.3.3. Estimation of pCREB Level in Mice and Rats Brain Samples

- ✓ Briefly, a sufficient number of microplate wells were prepared to accommodate standards and samples in duplicates.
- ✓ Then, 100 µl of standard, blank, or sample were dispensed into appropriate wells and incubated for 2 hrs at 37 °C after covering with the plate sealer.
- ✓ After 2 hrs the plate content was discarded.
- ✓ After that 100 µl of detection reagent A working solution was added into each wells and incubated for 1 hr at 37 °C after covering with the plate sealer.
- ✓ After 1 hr incubation the plate was washed three times with wash buffer.
- ✓ After washing plate was inverted and blotted against clean paper towels.

- ✓ Then 100 of detection reagent B working solution was added to each well and incubated at room temperature for 1 hr, covering with plate sealer.
- ✓ After 1 hr, the plate content was washed four times with wash buffer.
- ✓ After washing 90 µl of substrate solution was added in each well and incubated for 20-30 min at 37 °C in dark.
- ✓ Then 50 µl of reaction stop solution was added and absorbance was noted at 450 nm.

4.9.5. Neurochemical Estimation

4.9.5.1. Serotonin, Nor-epinephrine and Dopamine levels Estimation

The measurement of NTs level was performed using commercially available sandwich ELISA kit (DLD, Diagnostika, GMBH, Germany), according to the manufacturer instructions. In this, NTs were bound to the solid phase of the microtiter plate and competed for a fixed number of anti-serum binding sites. The antibody bound to the solid phase was detected by anti-rabbit IgG/peroxidase. The amount of antibody bound to the solid phase was inversely proportional to NT concentration of the sample.

4.9.5.1.1 Brain Samples Preparation

The brain samples of rats and mice were homogenized in 10 ml of cold acidified n-butanol. After centrifugation for 5 min at 3000 rpm the supernatant was pipette out.

4.9.5.1.2. Preparation of Reagents for Neurotransmitters Estimation

Acid butanol:

This was prepared by adding 0.85 ml of conc. HCl to n-butanol (volumes make up to 1 litre) (This solution was used to prepare the brain tissue homogenate).

4.9.5.1.3. Standard Curve Preparation

The standard curves for 5-HT, NE and DA were plotted according the instruction of ELISA kit.

4.9.5.1.4. Estimation of Serotonin Level in Mice and Rats Brain Samples

- ✓ Briefly, a sufficient number of microplate wells were prepared to accommodate standards and samples in duplicates.
- ✓ Then, 50 µl of prepared standards, blank and samples were dispensed into appropriate wells and incubated for 15-20 hrs at 2-8 °C after covering with the adhesive foil.

- ✓ After the incubation time, the plate content was discarded and washed 3-4 times with wash buffer (300 µl).
- ✓ After washing, the plate was inverted and blotted against clean paper towels.
- ✓ 100 µl of enzyme conjugate was added into each wells and incubated for 1 hr at room temperature on an orbital shaker.
- ✓ After the incubation time, the plate contents were discarded and washed 3-4 times with wash buffer (300 µl).
- ✓ After 1 hr of incubation, the plate was washed three times with wash buffer.
- ✓ Then 100 µl of substrate solution was added in each well and incubated for 20-30 min at room temperature on an orbital shaker.
- ✓ Then 100 µl of reaction stop solution was added and the absorbance was noted at 450 (reference wavelength between 570 and 650) nm.

4.9.5.1.5. Estimation of Nor-epinephrine and Dopamine Level in Mice and Rats Brain

- ✓ Briefly, sufficient numbers of microplate wells were prepared for NE and DA separately to accommodate standards and samples in duplicates.
- ✓ For NE estimation, 10 µl of prepared standards, blanks and samples were dispensed into appropriate wells (Colour Coded yellow). In case of DA, 50 µl of prepared standards, blanks and samples were dispensed into appropriate wells (Colour Coded green).
- ✓ Then 50 µl NE-Antiserum (colour coded yellow) and 20 µl DA-Antiserum (colour coded green) were added into all wells and incubated for 15-20 hrs at 2-8 °C after covering with adhesive foil.
- ✓ After the incubation time the plate content was discarded and washed 3-4 times with wash buffer (300 µl).
- ✓ After washing, plate was inverted and blotted against clean paper towels.
- ✓ Then 100 µl POD-Conjugate was added into each wells and incubated for 30 min at room temperature on an orbital shaker.
- ✓ After incubation, each plate contents were discarded and washed 3-4 times with wash buffer (300 µl).
- ✓ 100 µl of substrate solution was added in each well and incubated for 20-30 min at room temperature on an orbital shaker.
- ✓ 100 µl of reaction stop solution was added and the absorbance was noted at 450 (reference wavelength between 570 and 650) nm.

4.10. Preliminary Behavioral Evaluation for Anxiolytic-like Activity

4.10.1. Evaluation of Preliminary Anxiolytic-like Effect of ROL, ETZ, Q-21 and Q-12 Using Elevated Plus Maze Test

The test was performed essentially as described elsewhere (Pellow et al., 1985; Lister, 1987). In brief, the apparatus consisted of a wooden maze with two enclosed arms (30 × 5 × 15 cm) and two open arms (30 × 5 × 0.25 cm) that extended from a central platform (5 × 5 cm) to form a plus sign. The plus-maze apparatus was elevated to a height of 45 cm and placed inside a sound-attenuated room. The trial was started by placing a mouse on the central platform of the maze facing its head towards an open arm. The behavioral parameters recorded during a 5 min test period were; percentage of OAE, percentage TSOA and total closed arm entries (Klodzinska et al., 2004). Entry into an arm was considered valid only when all four paws of the mouse were inside that arm (Biala and Kruk, 2008). The animal activities were tracked and recorded via an overhead video camera linked to a monitor with computer software, *"Smart" version* 2.5 (Panlab co., USA). The apparatus was thoroughly cleaned with 70% ethanol after each trial.

4.10.2. Evaluation of Preliminary Anxiolytic-like Effect of ROL, ETZ, Q-21 and Q-12 Using Light/Dark Aversion Test

The L/D apparatus comprised of a box divided into two separate compartments, occupying two-thirds and one-third of the total size, respectively. The larger compartment (light compartment) was illuminated by a 60 W bulb, while the smaller (dark compartment) was entirely black. The L/D compartments were separated by a partition with a tunnel to allow passage from one compartment to the other (Mi et al., 2005). At the beginning of the test, the mouse was placed individually at the centre of the light compartment facing towards the tunnel and was allowed to explore the entire apparatus for 5 min. The behavioral parameters such as latency time to leave the light compartment, total time spent in the light compartment and number of transitions between the L/D compartments were tracked and recorded using computer software, *"Smart" version 2.5* (Panlab co., USA). A compartment entry was considered valid, when all the four paws of animals were inside that chamber. The apparatus was thoroughly cleaned with 70% ethanol after each trial.

4.10.3. Evaluation of Anxiolytic-like Effect of ROL and ETZ, Using Hole Board Test

The HB apparatus consisted of a grey Plexiglas platform (40 × 40 cm) raised to a height of 15 cm from the floor of a gray wooden box (40 × 40 × 40 cm). The grey Plexiglas platform consisted of 16 equivalent square compartments (12 peripheral and 4 central), each featuring

a central circular hole (3 cm diameter). Test session was started by placing each animal in the centre of the HB and allowed to freely explore on the apparatus for 5 min. The behavioral performances, such as number of head dipping, total time spent in head dipping and latency to the first head dipping (Wei et al., 2007) were tracked and recorded using computer software, "*Smart" version 2.5* (Panlab co., USA).

4.10.4. Experimental Design for ROL, ETZ, Q-21 and Q-12 in EPM, L/D and HB tests

In the preliminary anti-anxiety investigation, the mice of either sex were divided into different matched groups namely;

Set: A. This set of EPM, L/D and HB tests as performed using ROL

EPM		L/D		HB	
1. Normal control	= 8	1. Normal control	= 8	1. Normal control	= 8
2. ROL (0.25 mg/kg)	= 8	2. ROL (0.25 mg/kg)	= 8	2. ROL (0.25 mg/kg)	= 8
3. ROL (0.5 mg/kg)	= 8	3. ROL (0.5 mg/kg)	= 8	3. ROL (0.5 mg/kg)	= 8
4. ROL (1 mg/kg)	= 8	4. ROL (1 mg/kg)	= 8	4. ROL (1 mg/kg)	= 8
5. DZM (2 mg/kg)	= 8	5. DZM (2 mg/kg)	= 8	5. DZM (2 mg/kg)	= 8

Set: B. This set of EPM, L/D and HB tests as performed using ETZ

EPM		L/D		HB	
1. Normal control	= 8	1. Normal control	= 8	1. Normal control	= 8
2. ETZ (0.25 mg/kg)	= 8	2. ETZ (0.25 mg/kg)	= 8	2. ETZ (0.25 mg/kg)	= 8
3. ETZ (0.5 mg/kg)	= 8	3. ETZ (0.5 mg/kg)	= 8	3. ETZ (0.5 mg/kg)	= 8
4. ETZ (1 mg/kg)	= 8	4. ETZ (1 mg/kg)	= 8	4. ETZ (1 mg/kg)	= 8
5. DZM (2 mg/kg)	= 8	5. DZM (2 mg/kg)	= 8	5. DZM (2 mg/kg)	= 8

Set: C. This set of EPM and L/D tests as performed using Q-21

EPM		L/D	
1. Normal control	= 8	1. Normal control	= 8
2. Q-21 (0.5 mg/kg)	= 8	2. Q-21 (0.5 mg/kg)	= 8
3. Q-21 (1 mg/kg)	= 8	3. Q-21 (1 mg/kg)	= 8
4. Q-21 (2 mg/kg)	= 8	4. Q-21 (2 mg/kg)	= 8
5. DZM (2 mg/kg)	= 8	5. DZM (2 mg/kg)	= 8

Set: D. This set of EPM and L/D tests as performed using Q-12

EPM		L/D	
1. Normal control	= 8	1. Normal control	= 8
2. Q-12 (0.5 mg/kg)	= 8	2. Q-12 (0.5 mg/kg)	= 8
3. Q-12 (1 mg/kg)	= 8	3. Q-12 (1 mg/kg)	= 8
4. Q-12 (2 mg/kg)	= 8	4. Q-12 (2 mg/kg)	= 8
5. DZM (2 mg/kg)	= 8	5. DZM (2 mg/kg)	= 8

Rationale for Mice and Rats Used as Animal Models

Pre-clinical psychiatric research has clearly favoured rats and mice as animal models (surgical and non-surgical models) of choice for behavioral and brain biochemistry studies. Several animal models of depression and anxiety are well established in rats and mice.

In the current investigation, mice were used in acute models (for the preliminary testing) and chronic non-surgical models of depression and anxiety studies.

The rats were used in acute model (RIH) and chronic models, such as surgical models due to more survival rate after surgery in comparison to mice. Moreover, rats are more specific to behavioral analysis followed by surgery. In addition, the segregation of different brain regions is easier in rats as compared to mice.

A brief summary of rodent models, used in the current investigation for depression and anxiety screening is summarized in table 14.

Table 14. Summary of Rodent Models Used for AD and Anxiolytic Screening

Model of Depression (D) & Anxiety (A)	Species/No. of animals used in each group	Validity criteria	Remarks (Indicating the phenotypes of Depression or Anxiety)
FST (D)	Mouse/8	Face, Predictive	Increase duration of immobility and decrease swimming episode
TST (D)	Mouse/8	Face, Predictive	Increase duration of immobility
5HTP-induced HTR (D)	Mouse/8	Constructive, Predictive	Potentiation of head twitches
RIH (D)	Rat/6	Constructive, Predictive	Mean decrease in temperature
OBX (D)	Rat/6	Face, Constructive, Predictive	Hyperactivity, increase hyper-emotionality behavior and decrease intake of sweetened solution
CUMS (D)	Mouse/6	Face, Constructive, Predictive	Increase immobility in despair tests & decrease intake of sweetened solution
Chronic CORT exposure (D)	Mouse/6	Face, Constructive, Predictive	Increased immobility, decreased intake of sweetened solution & altered locomotor activity
TBI (D)	Rat/6	Face, Predictive	Hyperactivity, increase hyper-emotionality behavior and less intake of sweetened solution
EPM (A)	Mouse/8	Predictive	Decrease % of both TSOA and OAE
L/D (A)	Mouse/8	Predictive	Decrease latency to leave and time spent in light box
HB (A)	Mouse/8	Predictive	Head dipping score and time spent in head dipping

4.11. Neuro-anatomical Studies (Assessment of Histological Changes)

4.11.1. Measurement of Neuronal Morphology and Density in Brain Regions

At the end of experimental period in OBX and chronic CORT-treated models of depression, rats and mice, respectively, were sacrificed by decapitation. The brain samples were rapidly removed and fixed by immersion in 10% formalin. Subsequently, they were embedded in paraffin wax, cut into 5 µm thick sections and stained with H&E staining (Shoham et al., 2003). Hippocampal CA1 and DG regions of brain **(fig. 21)** were examined under bright field illumination using "**Optika TCB5"** microscope (Optika Research Microscope) at total magnifications of 40X (DG region) and100X (hippocampal CA1 region). To examine the cell

density, the neurons were counted in atleast three sections, at each level. Both healthy and pyknotic cells were counted at 3 areas chosen randomly for each sample. Counting of total neuronal number was based on counting nuclei technique.

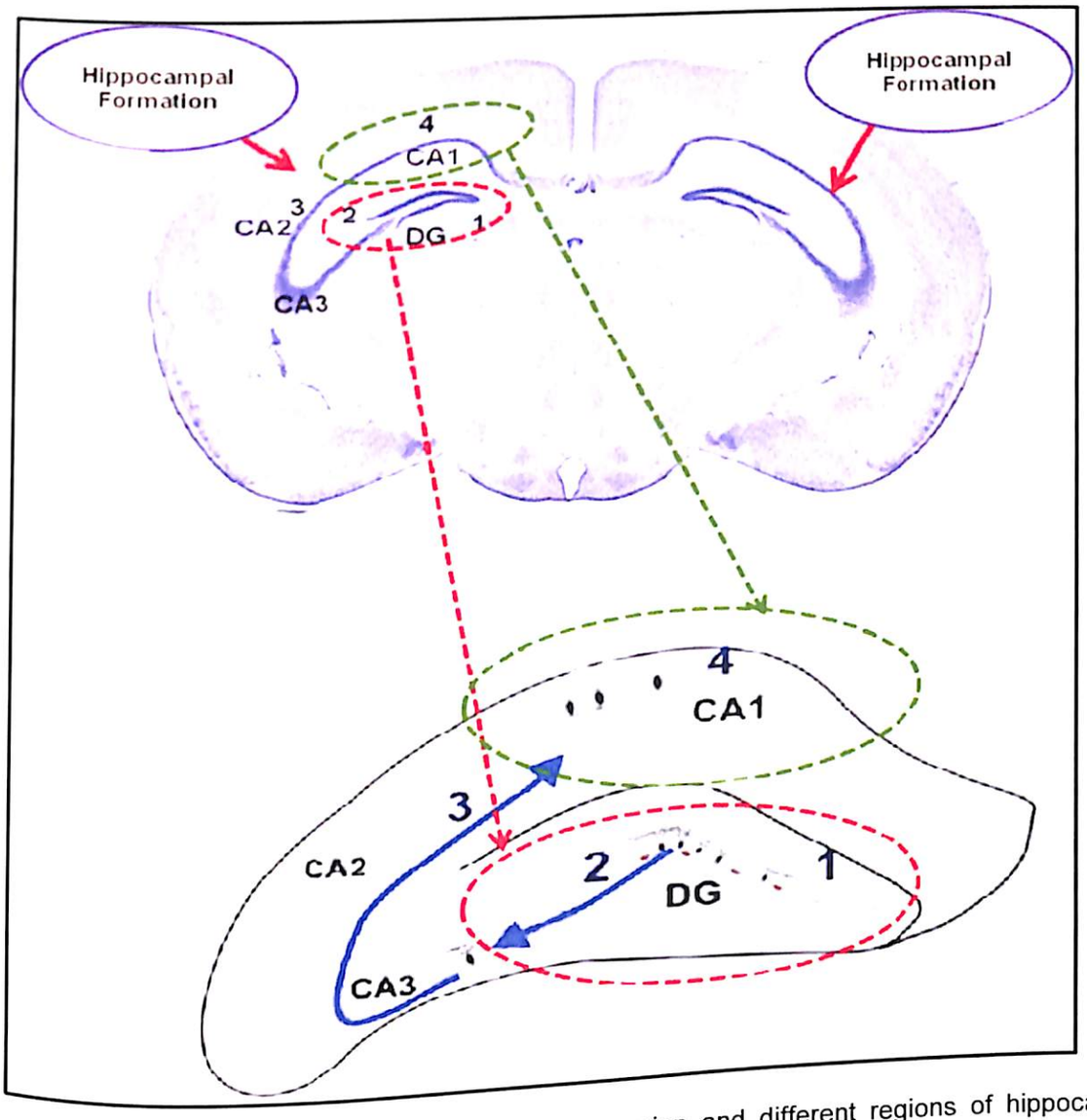


Fig. 21: Brain section representing the hippocampus region and different regions of hippocampal formation. Dotted red circle indicate DG region, whereas, dotted green circle indicate hippocampal CA1 region.

4.12. Statistical Analysis

All data are expressed as mean ± S.E.M. The data obtained from various groups were statistically analyzed using one way analysis of variance (ANOVA) followed by post-hoc Tukey's test. Moreover, the data from interaction studies were analysed using two-way ANOVA followed by Bonferroni test. The value of $P<0.05$ was considered as statistically significant.

5.1. Evaluation of Rolipram for Antidepressant-like Potential in Behavioral and Biochemical Test Battery

5.1.1. Effect of ROL on Locomotor Score in SLA Test

Fig. 22 shows the effect of ROL on locomotor score in mice using SLA test. ROL (0.12-1 mg/kg, i.p.) treatment had no effect on SLA of mice.

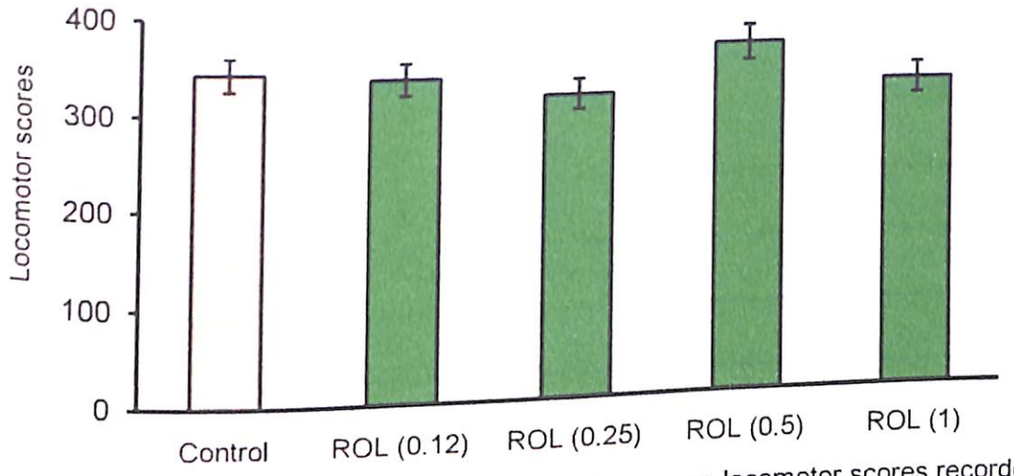
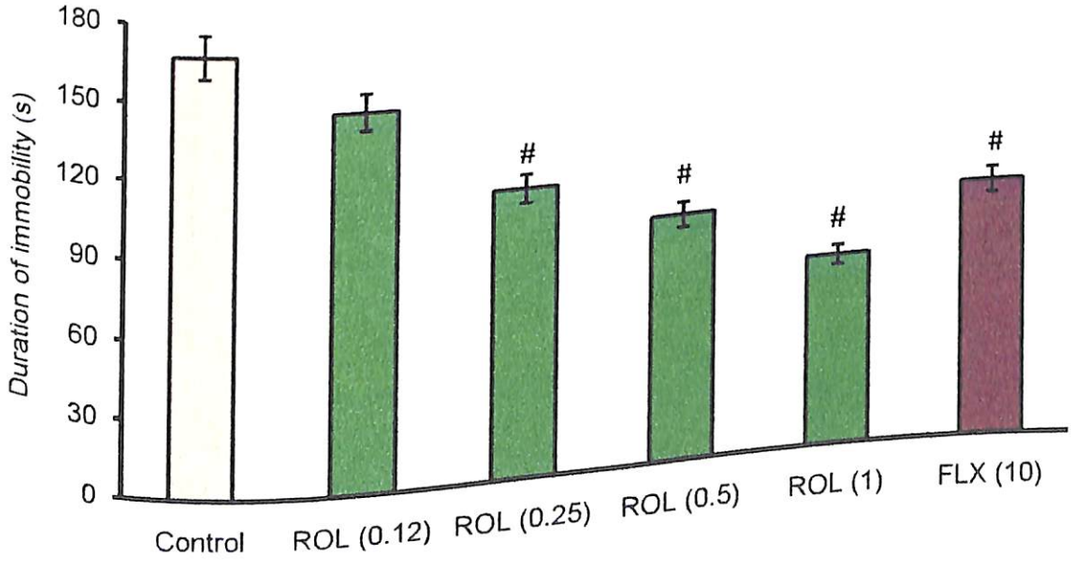


Fig. 22. Effect of ROL on SLA in mice. Each column represents mean locomotor scores recorded in 8 min observation period. The error bars indicate S.E.M; n = 8/group.

5.1.2. Effect of ROL on Mice Behavior in FST

ROL (0.25-1 mg/kg, i.p.) remarkably decreased [$F_{(5,42)} = 33.57$, $P<0.05$] the duration of immobility and increased [$F_{(5,42)} = 28.32$, $P<0.05$] swimming episodes in mice FST as compared to normal control group **(Fig. 23A & B).** ROL at a dose of 0.12 mg/kg failed to reach the level of significance in comparison to control group. FLX (10 mg/kg, i.p.) also decreased the duration of immobility and increased swimming episodes in FST.

(23A) Duration of immobility



(23B) Swimming episode

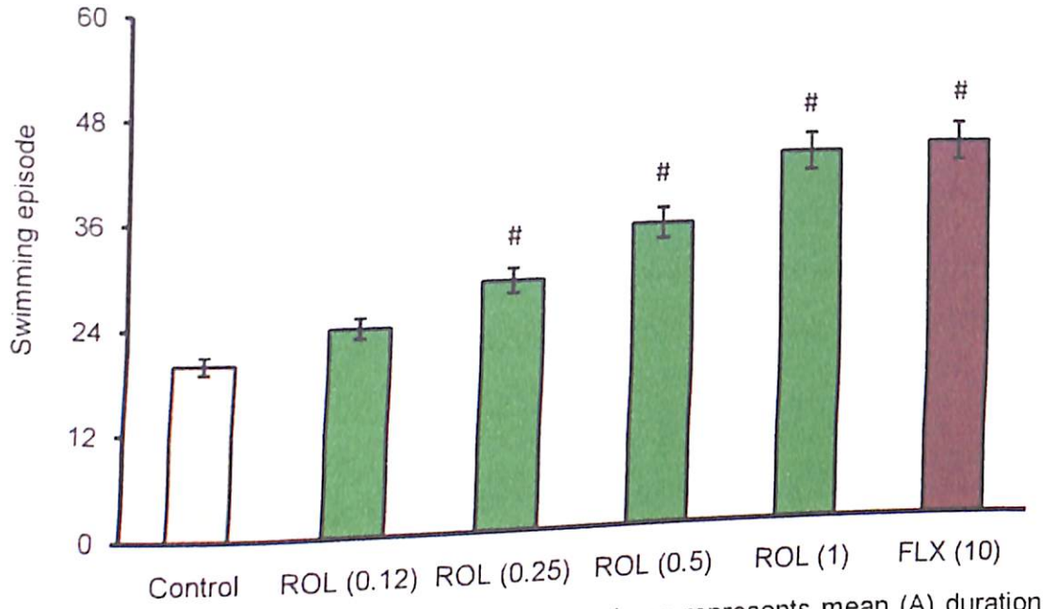


Fig. 23. Effect of ROL on the mice behavior in FST. Each column represents mean (A) duration of immobility (s) and (B) swimming episodes. The error bar indicates S.E.M. #P <0.05 when compared with normal control group; n = 8/group.

5.1.3. Effect of ROL on Mice Behavior in TST

TST is a "behavioral despair" test, which measures the duration of immobility, reflecting behavioral despair. ROL (0.25-1 mg/kg, i.p.) treatment markedly decreased the duration of immobility [$F_{(5,42)} = 18.77$, $P<0.05$] in comparison of control group **(fig. 24).** BUP (20 mg/kg, i.p.), also reduced the duration of immobility as compared to control group.

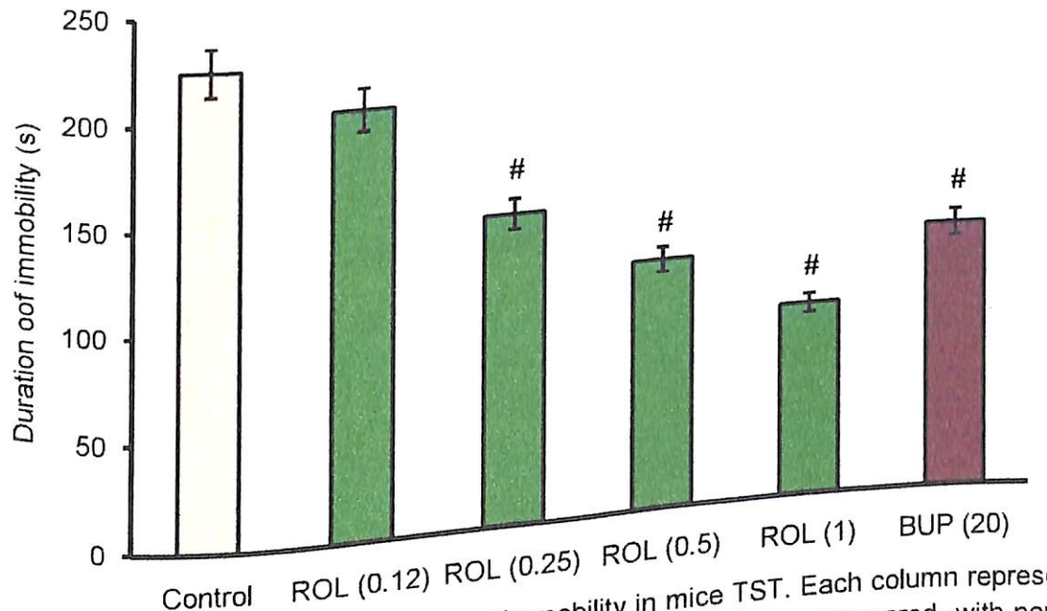


Fig. 24. Effect of ROL treatment on the duration of immobility in mice TST. Each column represents mean duration of immobility (s). The error bar indicates S.E.M. #P<0.05 when compared with normal control group; n = 8/group.

5.1.4. Effect of ROL on 5-HTP-induced HTR

The combination of 5-HTP (5 mg/kg, i.p.) and pargyline (75 mg/kg, i.p.) induced characteristic HTR in mice **(table 15)**. Acute pre-treatment of ROL (0.5 & 1 mg/kg, i.p.) & FLX (10 mg/kg, i.p.) predominantly increased [$F_{(3,28)}$ = 26.39, P<0.05] 5-HTP- and pargyline-induced HTR.

5.1.5. Effect of ROL on RIH Response

Table 15 shows the effect of ROL treatment on RIH response in rats. Reserpine (1 mg/kg, i.p.) markedly lowered the core body temperature of control group rats. Pre-treatment with ROL (0.5 & 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) predominantly [$F_{(3,20)}$ = 19.46, P<0.05] reversed the hypothermic response-induced by reserpine in rats **(table 15).**

Table 15. Effect of ROL treatment on 5-HTP-induced HTR in mice and RIH in rats

5-HTP-induced HTR (mice)		RIH (rats)	
Groups (mg/kg)	**Head twitch**	**Groups (mg/kg)**	**Mean decrease in temp (°F)**
5HTP + Pargyline	52.8±9.71	Reserpine control	3.4±0.4
ROL (0.5)	82.8±5.17[a]	ROL (0.5)	1.88±0.41[a]
ROL (1)	116.1±12.61[a]	ROL (1)	1.48±0.320[a]
FLX (10)	130.0±6.59[a]	FLX (10)	1.12±0.28[a]

The values are expressed as mean ± S.E.M. [a]P<0.05 compared with 5HTP+Pargyline control in 5-HTP induced HTR model and reserpine control in RIH model; n = 8/group (mice) and n = 6/group (rats).

5.1.6. Interaction Study of Sub-effective Doses of ROL and ADs in FST and SLA Test

5.1.6.1. ROL and ADs in FST

The sub-effective doses of ROL (0.12 mg/kg, i.p.), FLX (5 mg/kg, i.p.), VLA (4 mg/kg, i.p.) and DMI (5 mg/kg, i.p.), which did not show notable decrease in immobility duration in FST were selected for the interaction studies. ROL sub-effective dose in combination with sub-effective doses of FLX, VLA and DMI exhibited AD-like effect, by decreasing immobility duration in FST than did either of these drugs alone **(table 16).** A two-way ANOVA showed a profound change for FLX × ROL interaction [$F_{(1,40)}$ =12.63, P<0.05], VLA × ROL interaction [$F_{(1,40)}$ = 12.20, P<0.05] and DMI × ROL interaction [$F_{(1,40)}$ = 10.83, P<0.05].

5.1.6.2. ROL and ADs in SLA test

The effects of interaction study of ROL (0.12 mg/kg, i.p.) with FLX (5 mg/kg, i.p.), VLA (4 mg/kg, i.p.) and DMI (5 mg/kg, i.p.) were also verified in actophotometer test. The results revealed that administration of FLX, VLA and DMI in combination with ROL did not produce any marked change in the locomotor scores **(table 16).**

Table 16. Effect of combination of sub-effective doses of ROL and ADs in SLA and FST

Groups	Dose (mg/kg)	Actophotometer test (Locomotor scores)	FST (Duration of immobility)
Control	0	343.33±26.75	167.33±9.42
ROL	0.12	337.50±11.67	144.67±11.25
FLX	5	350.20±14.11	142.40±8.48
VLA	4	380.19±17.95	168.16±2.99
DMI	5	320.31±15.28	145.40±4.98
FLX + ROL	(5) + (0.12)	344.14±18.14	113.60±12.05[a,b,c]
VLA + ROL	(4) + (0.12)	359.51±15.34	117.50±9.97[a,b,d]
DMI + ROL	(5) + (0.12)	370.50±22.40	109.33±6.68[a,b,e]

The values are expressed as mean ± S.E.M. [a]P<0.05 compared with normal control, [b]P<0.05 compared with ROL alone, [c]P<0.05 compared with FLX alone, [d]P<0.05 when compared with VLA alone and [d]P<0.05 when compared with DMI alone; n = 6/group.

5.1.7. Evaluation of ROL for Anti-depressant-like Potential in OBX Model Using Behavioral and Biochemical Tests

5.1.7.1. Behavioral Analysis

5.1.7.1.1. Effect of ROL and OBX/Sham on Rats Body Weight

Body weight of sham and OBX rats was continuously observed till the behavioral tests were started. Statistical analysis revealed that after the surgery, OBX rats gained lesser weight than sham group **(table 17).** However, there was no prominent difference observed in weight gain after 28 days of surgery between drug treated OBX rats and OBX control rats.

Table 17. Effect of ROL and OBX on rats body weight

Groups	Dose (mg/kg)	Initial weight (0^{th} day)	Final weight (28^{th} day)
Sham control	0	257.50±2.50	294.0±10.09
Sham + ROL	0.5	251.33±2.19	287.67±7.76
Sham + ROL	1	250.00±3.61	288.0±5.15
Sham + FLX	10	251.50±3.50	280.5±10.5
OBX control	0	254.6±7.55	268.5±9.47[a]
OBX + ROL	0.5	255.67±9.03	281.0±5.17
OBX + ROL	1	250.00±5.51	278.33±5.24
OBX + FLX	10	253.67±8.43	280.67±7.76

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]P<0.05 compared with sham control; n = 6/group.

5.1.7.1.2. Effect of ROL Treatment on Behavior of OBX/Sham Rats in OFT

The effects of chronic ROL (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatment on the behavior of OBX/sham rats were analyzed in the OFT paradigm **(table 18).** Removal of olfactory bulbs produced a characteristic hyperactivity behavioral pattern in the OFT, characterized by increase in ambulation scores (square crossed), rearing numbers and fecal pellets count as compared to sham control group. ROL (0.5 & 1 mg/kg, p.o.) remarkably reduced the ambulation scores [$F_{(7,40)} = 19.71$, $P<0.05$], rearing [$F_{(7,40)} = 26.53$, $P<0.05$] and fecal pellets [$F_{(7,40)} = 4.69$, $P<0.05$] in OBX rats as compared to OBX control rats. FLX treatment also notably ($P<0.05$) reversed all behavioral anomalies in OBX rats. The chronic administration of ROL as well as FLX did not show any effect in sham rats **(table 18).**

Table 18. Effect of ROL treatment on behavior of OBX/Sham rats in OFT

Groups	Dose (mg/kg)	Ambulation	Rearing	Fecal Pellet
Sham control	0	109.3±7.75	12.5±1.37	3.0±0.42
Sham + ROL	0.5	103.80±8.87	9.33±1.05	2.33±0.71
Sham + ROL	1	95.67±5.51	8.33±1.15	2.0±0.86
Sham + FLX	10	99.67±9.31	8.67±0.97	2.00±0.70
OBX control	0	179.25±7.70[a]	42.63±2.68[a]	8.0±0.77[a]
OBX + ROL	0.5	136.0±8.59[b]	28.83±2.58[b]	4.0±1.30[b]
OBX + ROL	1	102.86±5.80[b]	15.13±2.71[b]	3.3±0.88[b]
OBX + FLX	10	134.75±10.65[b]	24.57±7.25[b]	2.4±0.68[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control group, [b]$P<0.05$ vs. OBX control group; n = 6/group.

5.1.7.1.3. Effect of ROL on Sucrose Consumption in OBX/Sham Rats

One-way ANOVA revealed a profound effect of the groups for the percentage of sucrose consumption [$F_{(7,40)} = 10.92$, $P<0.05$]. The post-hoc Tukey's test indicated that OBX rats showed a marked reduction in the percentage of sucrose consumption as compared to sham control group **(fig. 25).** Repeated ROL (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatments remarkably ($P<0.05$) increased sucrose consumption in OBX rats as compared to OBX control group. No effect of ROL and FLX treatment was observed on sucrose consumption in drug treated sham groups **(fig. 25).**

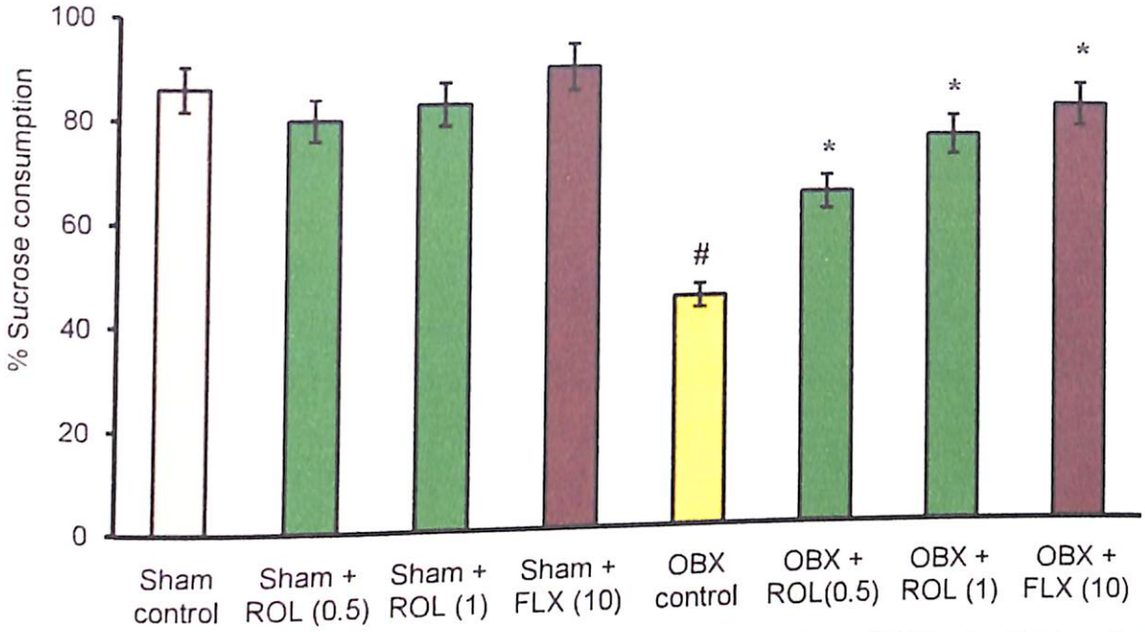


Fig. 25. Effect of ROL and FLX treatment on anhedonia behavior in Sham/OBX rats. Each column represents mean percentage sucrose consumption. The error bar indicates S.E.M. #P<0.05 when compared with sham control; *P<0.05 when compared with OBX control; n = 6/group.

5.1.7.1.4. Effect of ROL on Hyper-emotionality Behavior in OBX/Sham Rats

OBX rats exhibited more pronounced [$F_{(7,40)} = 6.27$, $P<0.05$] increase in emotional behavior (included evaluation of the bite, startle, struggle and fight response) as compared to sham control. Hyper-emotional behavior exhibited by OBX rats was markedly ($P<0.05$) reversed by treatment with ROL (0.5 and 1 mg/kg) and FLX (10 mg/kg) **(fig. 26).**

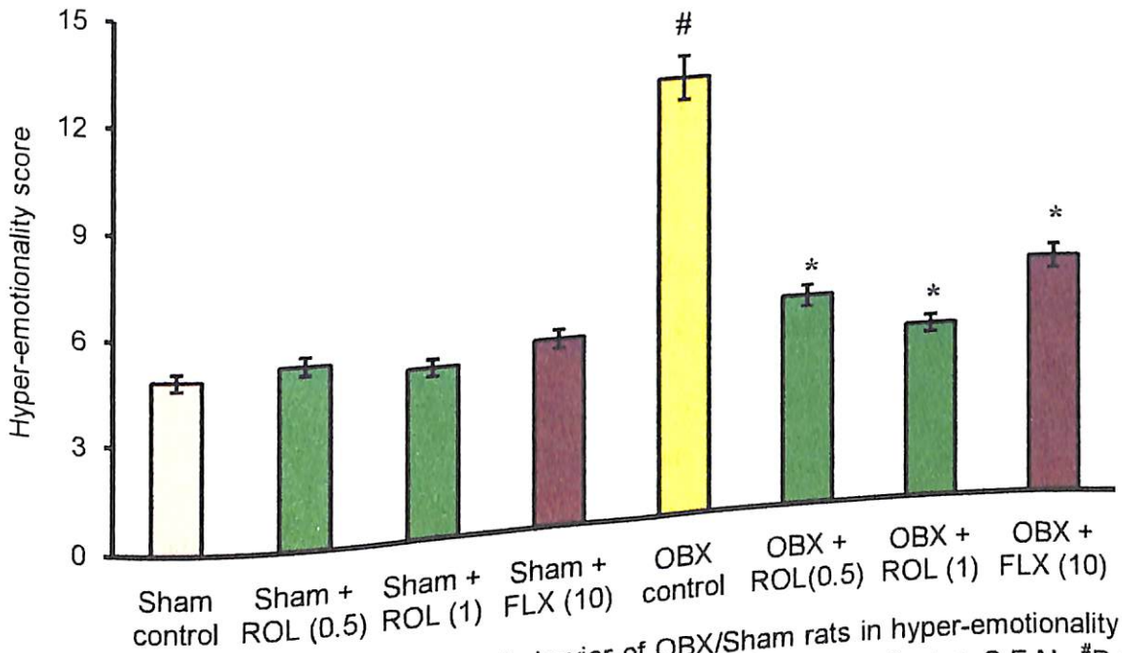


Fig. 26. Effects of ROL and FLX treatment on behavior of OBX/Sham rats in hyper-emotionality test. Each column represents mean hyper-emotionality scores. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with OBX control; n = 6/group.

5.1.7.2. Biochemical Estimation

5.1.7.2.1. Effect of ROL on Neuroendocrine Hormone Level in OBX Model

5.1.7.2.1.1. Effect of ROL on Serum CORT Level in OBX/Sham Rats

Fig. 27 displays serum CORT levels in sham and OBX rats. One-way ANOVA revealed a marked difference among groups for CORT [$F_{(7,40)} = 4.62$, $P<0.05$]. OBX rats showed a marked increase in serum CORT level as compared to sham control group. Two weeks treatment with ROL (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) reverted the increase of serum CORT levels in OBX rats as compared to OBX control group. Sham treatment did not show any significant effect on CORT level **(fig. 27).**

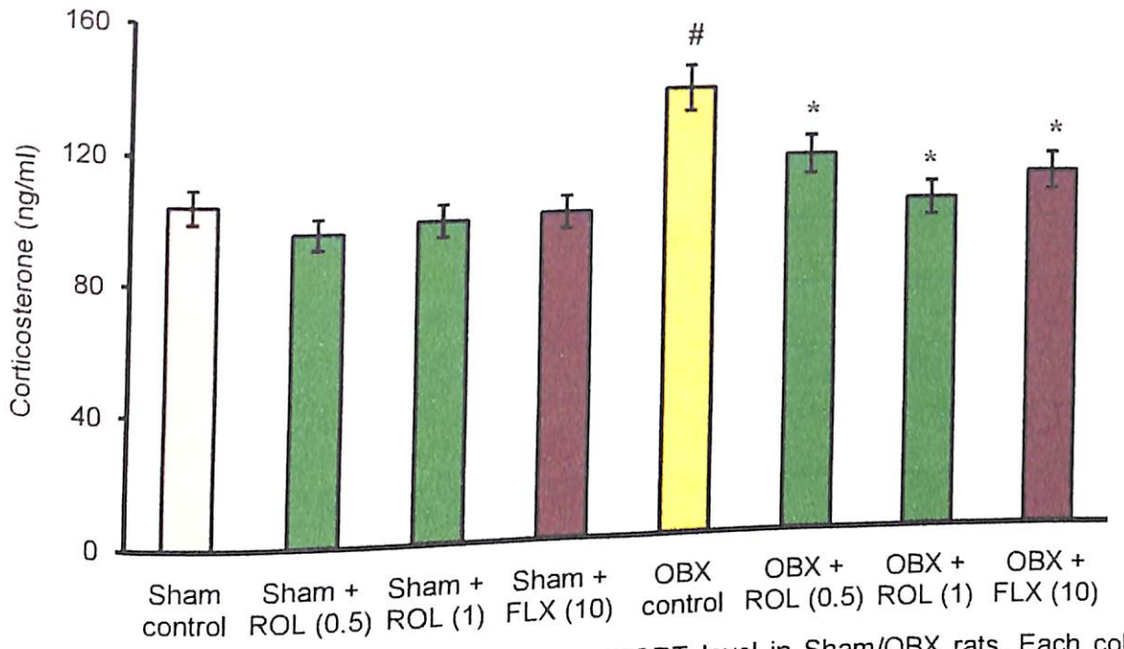


Fig. 27. Effect of ROL and FLX treatment on serum CORT level in Sham/OBX rats. Each column represents mean CORT level. The error bar indicates S.E.M. $^{\#}P<0.05$ when compared with sham control, $^{*}P<0.05$ when compared with OBX control; *n = 6/group.*

5.1.7.2.2. Effect of ROL on cAMP Signaling in OBX Model

5.1.7.2.2.1. Effect of ROL on cAMP, pCREB and BDNF Levels in OBX/Sham Rats

The effects of ROL (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatment on the cAMP, pCREB and BDNF levels in OBX/Sham rats are shown in **table 19.** Profound decrease in cAMP [$F_{(7,40)} = 22.16$, $P<0.05$], pCREB [$F_{(7,40)} = 12.38$, $P<0.05$] and BDNF [$F_{(7,40)} = 29.62$, $P<0.05$] levels were observed in OBX rats as compared to sham rats. Chronic ROL (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatment notably increased the cAMP, pCREB and BDNF levels in OBX rats as compared to OBX control. Two weeks ROL treatment also increased cAMP, pCREB and BDNF levels in sham control group **(table 19).**

Table 19. Effect of ROL treatment on cAMP, pCREB and BDNF in OBX/sham rats

Groups	Dose (mg/kg)	cAMP (pmol/mg protein)	BDNF(ng/mg protein)	pCREB (% of sham control)
Sham control	0	21.3±1.65	82.95±2.37	100.00±4.95
Sham + ROL	0.5	33.07±2.14[a]	107.09±6.33[a]	132.02±9.23[a]
Sham + ROL	1	40.42±2.76[a]	120.82±4.16[a]	142.43±5.39[a]
Sham + FLX	10	24.76±1.33	89.77±6.21	112.18±6.13
OBX control	0	12.86±1.70[a]	31.60±2.68[a]	54.39±4.21[a]
OBX + ROL	0.5	27.76±1.21[b]	61.35±4.65[b]	95.95±3.28[b]
OBX + ROL	1	34.11±3.98[b]	75.70±2.11[b]	110.67±4.13[b]
OBX + FLX	10	19.13±1.27[b]	48.17±7.25[b]	72.90±7.81[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]P<0.05 compared with sham control, [b]P<0.05 compared with OBX control; n = 6/group.

5.2. Evaluation of Rolipram for Anxiolytic-like Potential in Behavioral Test Battery

5.2.1. Effect of ROL on Mice Behavior in EPM Test

EPM was employed for the anxiolytic test in the laboratory set-up. Percentage of OAE and TSOA were measured in the EPM test. In this test, one-way ANOVA revealed a profound effect of groups for the percentage of both OAE [$F_{(4,35)}$ = 3.08, P<0.05] and TSOA [$F_{(4,35)}$ = 5.30, P<0.05]. The post-hoc test indicated that ROL and DZM (2 mg/kg, p.o.) treated mice exhibited a profound (P<0.05) increase in percentage of both OAE and TSOA as compared to normal control group **(table 20)**. Lower dose of ROL (0.25 mg/kg) had no change on the behaviors of mice in EPM.

Table 20: Effect of ROL on behavior of mice in EPM test

Groups	Dose (mg/kg)	No. of entries		% OAE	% TSOA
		open arm	closed arm		
Control	0	2.12±0.61	5.62±0.50	27.62±6.89	26.41±2.71
DZM	2	6.80±1.46[a]	4.40±0.87	60.15±2.56[a]	44.13±3.6[a]
ROL	0.25	2.75±0.86	4.20±1.39	26.64±8.34	22.12±5.13
ROL	0.5	5.0±0.81[a]	3.50±0.57	57.67±3.89[a]	32.44±2.21[a]
ROL	1	5.50±1.05[a]	3.16±0.74	64.72±2.45[a]	38.94±2.13[a]

The values are expressed as mean ± S.E.M. [a]P<0.05 when compared with normal control; n = 8/group.

5.2.2. Effect of ROL on Mice Behavior in HB Test

The results of the HB test are shown in **table 21**. The total number of head dipping [$F_{(4,35)}$ = 6.02, P<0.05] and time spent in head dipping [$F_{(4,35)}$ = 6.24, P<0.05] were remarkably increased by ROL (0.5 & 1 mg/kg, i.p.) and DZM (2 mg/kg, i.p.) treatments as compared to control group **(table 21).** In addition, both the treatments markedly decreased the head dipping latency [$F_{(4,35)}$ = 5.08, P<0.05]. The effect of ROL lower dose (0.25 mg/kg) failed to reach the level of statistical significance.

Table 21: Effect of ROL on behavior of mice in HB test

Groups	Dose (mg/kg)	Head dipping latency	No. of Head dipping	Time spent in head dipping
Control	0	11.89±1.92	15.11±1.98	10.11±2.21
DZM	2	5.86±1.57[a]	36.85±4.38[a]	26.14±2.05[a]
ROL	0.25	10.33±1.24	19.17±2.2	13.0±1.39
ROL	0.5	8.0±1.01[a]	26.0±1.69[a]	16.0±1.64[a]
ROL	1	6.33±1.6[a]	30.67±3.78[a]	24.83±2.08[a]

All values are expressed as mean ± S.E.M. [a]P<0.05 as compared to control; n=8/group.

5.2.3. Effect of ROL on Mice Behavior in L/D Aversion Test

L/D aversion test is based on a conflict between the innate aversions to brightly illuminated areas in response to a novel environment. Acute treatment of ROL (0.5 & 1 mg/kg, i.p.) and DZM (2 mg/kg, i.p.) markedly increased the latency time to leave the light compartment [$F_{(4,35)}$ = 2.70, P<0.05] and time spent in light compartment [$F_{(4,35)}$ = 6.59, P<0.05] compared to control group **(table 22).** Further, DZM also increased the number of crossings [$F_{(4,35)}$ = 12.19, P<0.05] between the compartments. ROL (0.25 mg/kg, i.p.) did not produce profound change in any of the parameters in L/D test.

Table 22. Effect of ROL on behavior of mice in L/D aversion test

Groups	Dose (mg/kg)	Latency to leave light box (s)	No. of crossing	Time spent in light box (s)
Control	0	19.43±2.11	12.67±1.47	80.14±6.34
DZM	2	73.83±5.43[a]	16.18±1.40[a]	164.84±11.06[a]
ROL	0.25	27.17±10.26	13.17±0.51	99.67±12.93
ROL	0.5	58.0±10.38[a]	10.02±1.49	128.0±22.63[a]
ROL	1	70.0±17.22[a]	14.3±2.29	174.14±14.93[a]

All values are expressed as mean ± S.E.M. [a]P<0.05 as compared to control; n = 8/group.

5.3. Evaluation of Etazolate for Anti-depressant-like Potential in Rodent Behavioral and Biochemical Test Battery

5.3.1. Effect of ETZ on Locomotor Score in SLA Test

ETZ (0.12-1 mg/kg i.p.) had no influence on the mice SLA after acute treatment at tested dose levels as compared with normal control group **(fig. 28).** This proves that ETZ had no baseline locomotion effect.

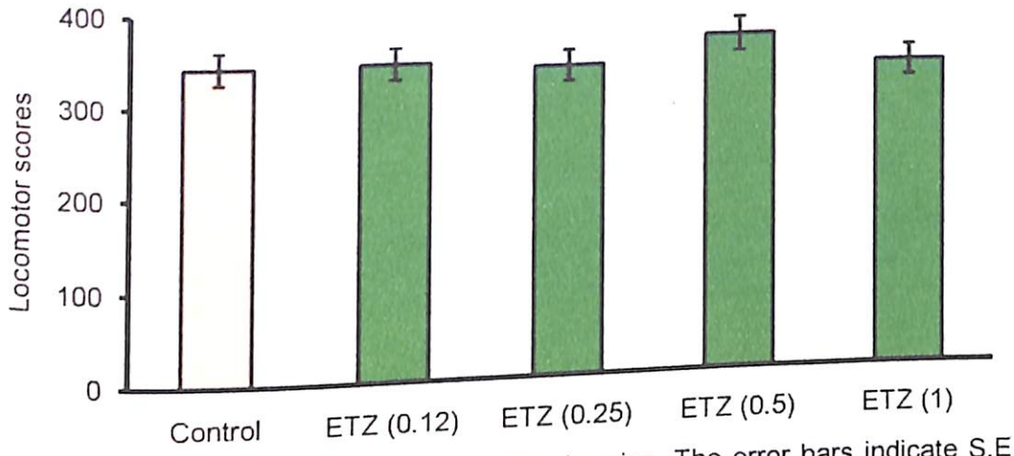
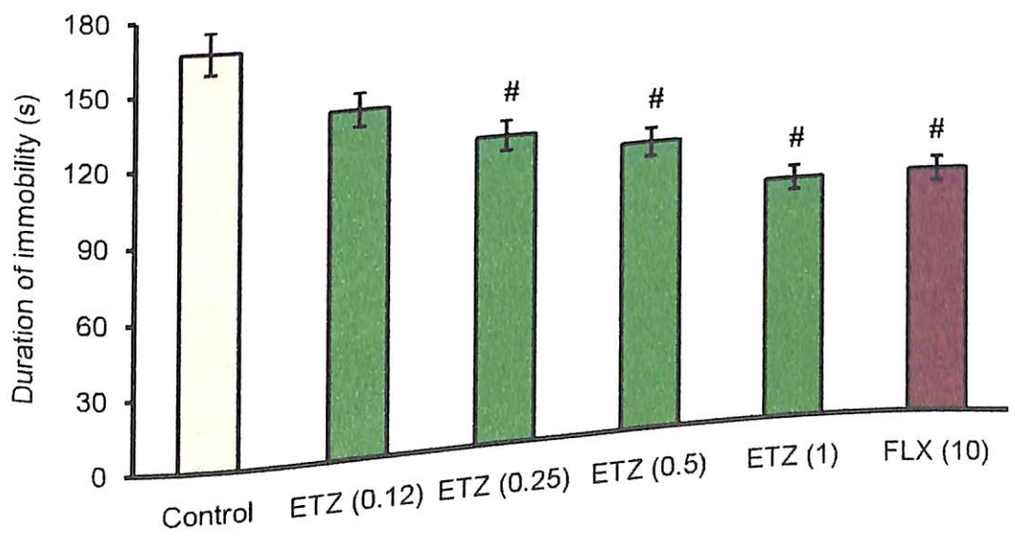


Fig. 28. Effect of ETZ treatment on locomotor scores in mice. The error bars indicate S.E.M. Each column represents mean locomotor scores recorded in 8 min observation period; n = 8/group.

5.3.2. Effect of ETZ on Mice Behavior in FST

Acute treatment with ETZ (0.25-1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) induced a pronounced reduction in immobility time [$F_{(5,42)} = 10.12$, $P<0.05$] and increase in swimming episodes [$F_{(5,42)} = 14.44$, $P<0.05$] as compared to control group **(fig. 29A & B).** Decreased duration of immobility and increased number of swimming episodes indicate the AD-like effect of ETZ in FST.

29A. Duration of immobility



29B. Swimming episode

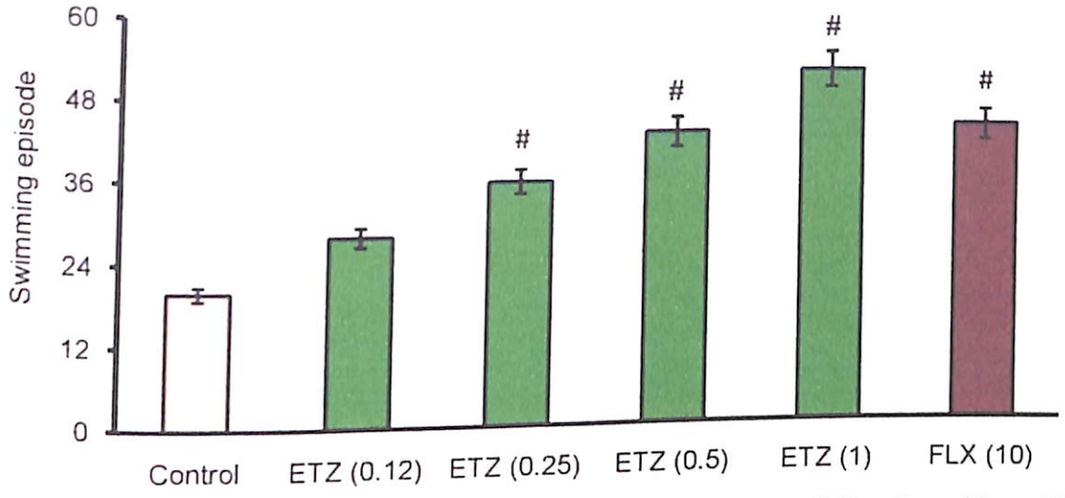


Fig. 29. Effect of ETZ on behavior of mice in FST. Each column represents (a) Duration of immobility and (b) swimming episode. The error bar indicates S.E.M. #P<0.05 when compared with normal control group; n = 8/group.

5.3.3. Effect of ETZ on Mice Behavior in TST

In TST, statistical analysis revealed that acute treatment of ETZ (0.25-1 mg/kg, i.p.) and BUP (20 mg/kg, i.p.) notably [$F_{(5,42)}$ = 12.36, P<0.05] decreased the duration of immobility as compared to the control group **(fig. 30).**

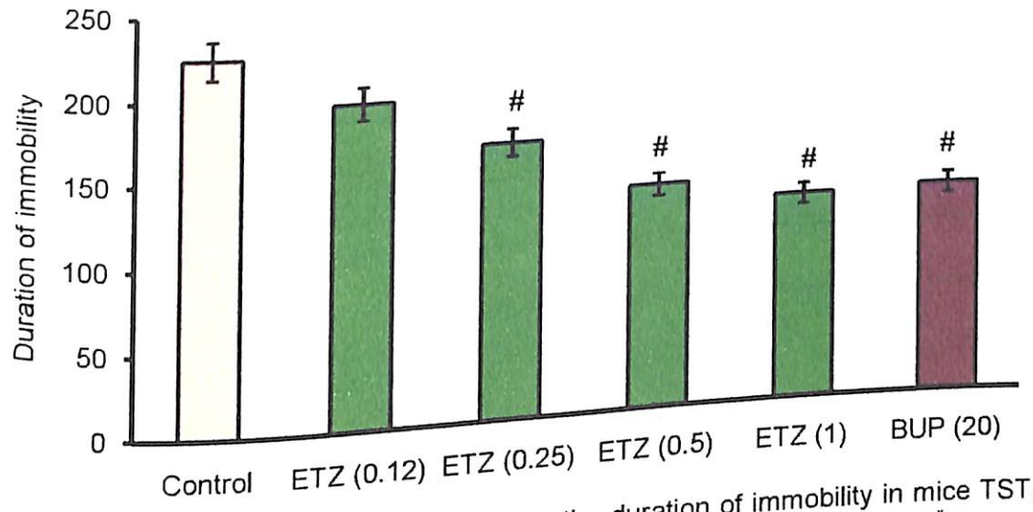


Fig. 30. Effect of ETZ (0.12-1 mg/kg, i.p.) treatment on the duration of immobility in mice TST. Each column represents mean duration of immobility (s). The error bar indicates S.E.M. #P<0.05 when compared with normal control group; n = 8/group.

5.3.4. Effect of ETZ on 5-HTP-induced HTR Response

The co-administration of pargyline and 5-HTP (75 + 5 mg/kg, respectively) induced the characteristic HTR. Pre-treatment with ETZ (0.5 & 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) markedly [$F_{(3,28)}$ = 8.16, P<0.05] increased the HTR as compared to combination of pargyline and 5-HTP alone **(table 23).**

5.3.5. Effect of ETZ on RIH Response

Administration of reserpine (1 mg/kg, i.p.) to rats, elicited a pronounced decrease in core body temperature. This effect was predominantly ($P<0.05$) attenuated by ETZ (0.5 & 1 mg/kg, i.p.). Similarly, positive control FLX (10 mg/kg, i.p.) also reversed the hypothermic effect of reserpine **(table 23).**

Table 23. Effect of ETZ treatment on 5-HTP-induced HTR in mice and RIH in rats

5-HTP-induced HTR (mice)		RIH (rats)	
Groups (mg/kg)	**Head twitch**	**Groups (mg/kg)**	**Mean decrease temp (°F)**
5-HTP + Pargyline	52.8±9.71	Reserpine control	3.4±0.4
ETZ (0.5)	121.4±14.1[a]	ETZ (0.5)	1.44±0.25[a]
ETZ (1)	145.1±12.61[a]	ETZ (1)	1.26±0.10[a]
FLX (10)	130.0±6.59[a]	FLX (10)	1.12±0.28[a]

The values are expressed as mean ± S.E.M. [a]$P<0.05$ compared with 5-HTP+Pargyline in 5-HTP-induced HTR and Reserpine control in RIH models; n = 8/group (mice) and n = 6/group (rats).

5.3.6. Interaction Study of Sub-effective Doses of ETZ and Standard ADs in FST and SLA test

5.3.6.1. ETZ and ADs in FST

The effects of combined administration of ETZ (0.12 mg/kg, i.p.) with FLX (5 mg/kg, i.p.), VLA (4 mg/kg, i.p.) and DMI (5 mg/kg, i.p.) are shown in **table 24**. On the basis of pilot study results in FST, the sub-effective doses of ETZ and all conventional ADs were selected for interaction study. In the interaction study, administration of ETZ sub-effective dose in combination with sub-effective doses of FLX, VLA and DMI exhibited AD-like effect, by decreasing the duration of immobility in mouse FST than did either of these drugs alone **(table 24)**. Statistical analysis two-way ANOVA showed pronounced differences for FLX × ETZ interaction [$F_{(1,40)} = 10.13$, $P<0.05$] and VLA × ETZ interaction [$F_{(1,40)} = 17.19$, $P<0.05$], DMI × ETZ interaction [$F_{(1,40)} = 9.98$, $P<0.05$].

5.3.6.2. ETZ and Standard ADs in SLA Test

The effect of the combined treatment of FLX (5 mg/kg, i.p.), VLA (4 mg/kg, i.p.) and DMI (5 mg/kg, i.p.) were verified with ETZ (0.12 mg/kg, i.p.) in actophotometer test. The results revealed that the administration of FLX, VLA and DMI in combination with ETZ did not produce any significant change in locomotor scores **(table 24).**

Table 24. Effect of combination of sub-effective dose of ETZ and ADs in SLA and FST

Groups	Dose (mg/kg)	Actophotometer (Locomotor scores)	FST (Duration of immobility)
Control	0	343.33±26.75	167.33±9.42
ETZ	0.12	345.0±12.30	140.15±4.93
FLX	5	350.20±14.11	142.40±8.48
VLA	4	380.19±17.95	168.16±2.99
DMI	5	320.31±15.28	145.40±4.98
FLX + ETZ	(5) + (0.12)	399.10±28.10	116.34±9.10[a,b,c]
VLA + ETZ	(4) + (0.12)	320.51±17.23	132.33±9.71[a,b,d]
DMI + ETZ	(5) + (0.12)	368.0±10.12	115.33±4.26[a,b,e]

The values are expressed as mean ± S.E.M. [a]P<0.05 compared with normal control, [b]P<0.05 compared with ETZ alone, [c]P<0.05 compared with FLX alone, [d]P<0.05 as compared to VLA alone and [e]P<0.05 when compared with DMI alone; n = 6/group.

5.3.7. Evaluation of ETZ for Antidepressant-like Potential in OBX Model Using Behavioral and Biochemical Tests

5.3.7.1. Behavior Analysis

5.3.7.1.1. Effect of OBX and ETZ on Rats Body Weight

Two way ANOVA revealed that after the surgery, OBX rats gained lesser body weight as compared to sham rats **(table 25).** Although, there was no significant difference observed in weight gain after 28 days of surgery between drug treated OBX rats and OBX control rats.

Table 25. Effect of ETZ and OBX surgery on body weight of rats

Groups	Dose (mg/kg)	Initial weight (0^{th} day)	Final weight (28^{th} day)
Sham control	0	257.50±2.50	294.0±10.09
Sham + ETZ	0.5	260.50±7.50	292.0±9.00
Sham + ETZ	1	254.00±7.00	283.5±5.50
Sham + FLX	10	251.50±3.50	280.5±10.50
OBX control	0	254.60±7.55	268.5±9.47[a]
OBX + ETZ	0.5	259.67±5.30	281.0±4.67
OBX + ETZ	1	255.00±4.28	279.33±8.40
OBX + FLX	10	260.67±8.43	280.67±7.76

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]P<0.05 compared with sham control; n = 6/group.

5.3.7.1.2. Effect of ETZ treatment on OBX/Sham Rats Behavior in OFT

To validate the OBX model, rats were subjected to the OFT during the post-operative periods. In the OFT, 28 days post-surgery, OBX rats showed the expected hyperactivity in open field arena. OBX rats exhibited a profound increase in ambulation, rearing and fecal pellets as compared to sham operated group. Chronic treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) significantly reduced the ambulation [$F_{(7,40)} = 14.43$, $P<0.05$], rearing [$F_{(7,40)} = 17.38$, $P<0.05$] & fecal pellets [$F_{(7,40)} = 6.37$, $P<0.05$] in OBX rats as compared to the OBX control rats **(table 26).** ETZ and FLX treatment did not show any remarkable effect on the behavior of sham rats in OFT **(table 26).**

Table 26. Effect of ETZ treatment on the behavior of OBX/Sham rats in OFT

Groups	Dose (mg/kg)	Ambulation	Rearing	Fecal Pellet
Sham control	0	109.3±7.75	12.5±1.37	3.0±0.42
Sham + ETZ	0.5	100.20±9.3	13.50±1.02	2.12±0.65
Sham + ETZ	1	108.27±4.84	9.75±2.86	2.71±0.76
Sham + FLX	10	99.67±9.31	8.67±0.97	2.0±0.70
OBX control	0	179.25±7.70[a]	42.63±2.68[a]	8.0±0.77[a]
OBX + ETZ	0.5	119.0±15.64[b]	30.80±3.48[b]	5.80±0.97[b]
OBX + ETZ	1	92.33±12.68[b]	21.0±6.11[b]	3.17±0.44[b]
OBX + FLX	10	134.75±10.65[b]	24.57±7.25[b]	2.40±0.68[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control, [b]$P<0.05$ compared with OBX control; n = 6/group.

5.3.7.1.3. Effect of ETZ on Sucrose Consumption in OBX/Sham Rats

The effects of ETZ (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatments on the percentage of sucrose consumption in OBX/sham rats are presented in **fig. 31.** OBX rats showed a profound reduction in the percentage of sucrose consumption [$F_{(7,40)} = 35.78$, $P<0.05$] as compared to sham control group. Repeated treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX predominantly ($P<0.05$) reversed the decrease in percentage of sucrose consumption in OBX rats compared to OBX control. Administration of ETZ as well as FLX did not show any remarkable effect on sucrose consumption in sham groups **(fig. 31).**

5.3.7.1.4. Effect of ETZ on OBX/Sham Rats Behavior in Hyper-emotionality Test

One-way ANOVA showed a marked difference among groups for the hyper-emotionality scores [$F_{(7,40)} = 16.02$, $P<0.05$) **(fig. 32).** Post-hoc test indicated that OBX rats exhibited increase in emotional behavior as compared to sham control. ETZ (0.5 and 1 mg/kg) and FLX (10 mg/kg) treatment reduced hyper-emotional behavior compared to OBX control.

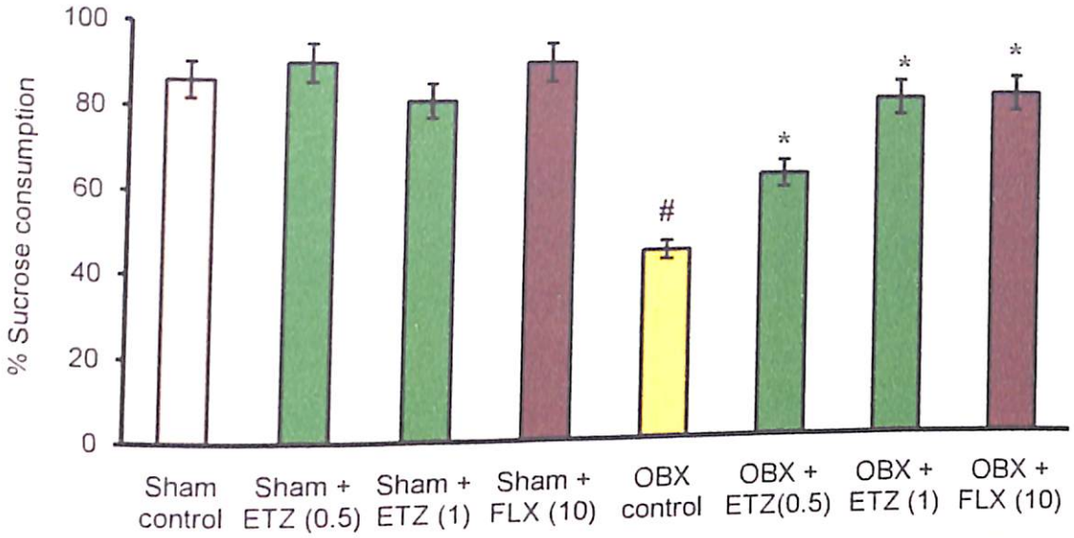


Fig. 31. Effect of ETZ and FLX treatment on anhedonia behavior in OBX/Sham rats. Each column represents mean percentage sucrose consumption. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with OBX control; n = 6/group.

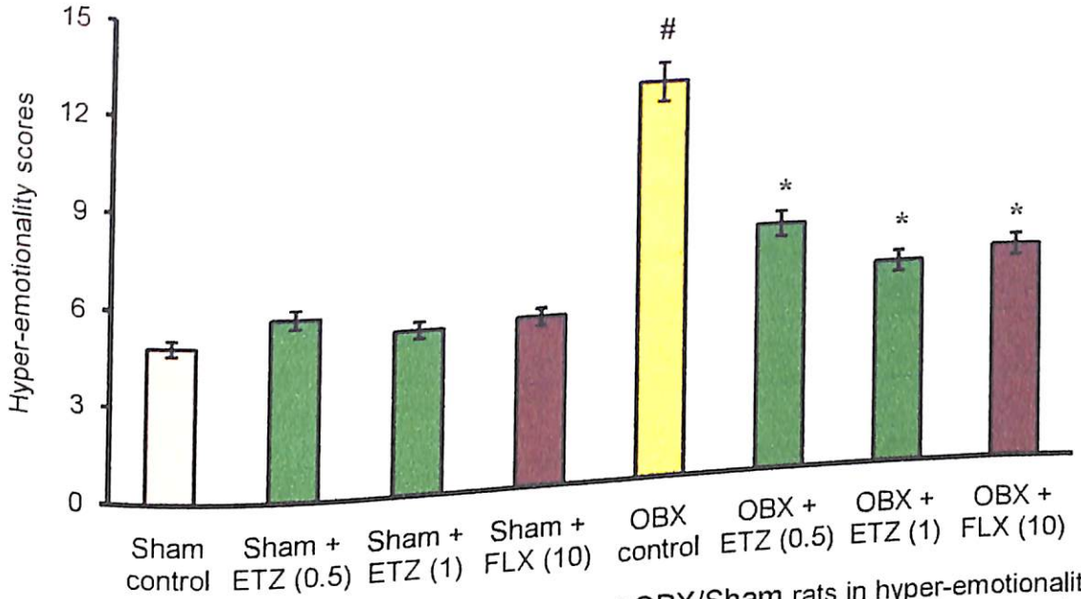


Fig. 32. Effects of ETZ and FLX treatment on behavior of OBX/Sham rats in hyper-emotionality test. Each column represents mean hyper-emotionality scores. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with OBX control; n = 6/group.

5.3.7.2. Biochemical Estimation

5.3.7.2.1. Effect of ETZ on Serum CORT Level in OBX/Sham Rats

The effect of ETZ (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatment on serum CORT level is shown in **fig. 33.** Statistical analysis indicated that OBX rats had a high serum CORT [$F_{(7,40)} = 7.34$, $P<0.05$] level as compared to sham control group. Chronic ETZ (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatment remarkably ($P<0.05$) decreased the serum CORT level as compared to OBX control group **(fig. 33).**

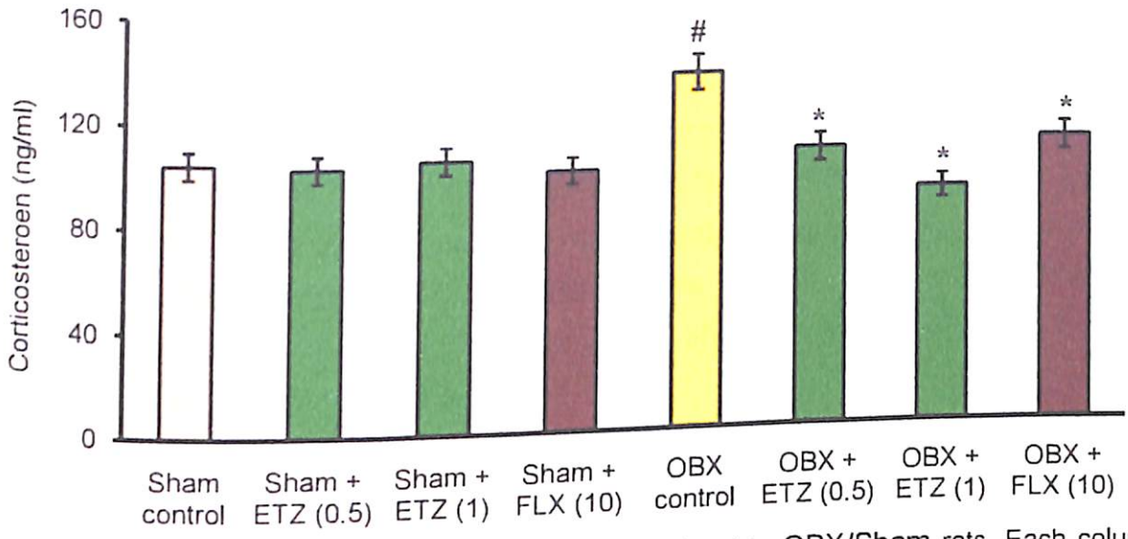


Fig. 33. Effect of ETZ and FLX treatment on serum CORT level in OBX/Sham rats. Each column represents mean CORT level. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with OBX control; n = 6/group.

5.3.7.2.2. Evaluation of ETZ on cAMP Signaling in OBX Model

5.3.7.2.2.1. Effect of ETZ on cAMP, pCREB and BDNF Levels in OBX/Sham Rats

Statistical analysis indicated a remarkable difference among groups for cAMP [$F_{(7,40)}$ = 31.22, P<0.05], pCREB [$F_{(7,40)}$ = 8.12, P<0.05] and BDNF [$F_{(7,40)}$ = 13.56, P<0.05] levels. OBX rats showed pronounced decrease in cAMP, pCREB and BDNF levels as compared to sham rats. Repeated treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) markedly increased the cAMP, pCREB and BDNF levels in OBX rats as compared to OBX control group. Similar effects of ETZ treatment were observed on cAMP, pCREB and BDNF levels in sham rats (**table 27**).

Table 27. Effect of ETZ treatment on cAMP, pCREB and BDNF levels on OBX/sham rats

Groups	Dose (mg/kg)	cAMP (pmol/mg protein)	BDNF (ng/mg protein)	pCREB (% sham control)
Sham control	0	21.3±1.65	82.95±1.37	100.00±4.95
Sham + ETZ	0.5	28.92±0.62[a]	99.32±4.02[a]	124.21±7.21[a]
Sham + ETZ	1	32.22±1.10[a]	111.67±3.86[a]	135.71±5.35[a]
Sham + FLX	10	24.76±1.33	89.77±6.21	112.18±6.13
OBX control	0	12.86±1.70[a]	31.60±2.68[a]	54.39±4.21[a]
OBX + ETZ	0.5	23.24±2.77[b]	53.50±4.12[b]	81.32±5.32[b]
OBX + ETZ	1	29.09±2.01[b]	68.70±2.11[b]	98.43±7.97[b]
OBX + FLX	10	19.13±1.27[b]	48.17±7.25[b]	72.90±7.81[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]P<0.05 compared with sham control, [b]P<0.05 compared with OBX control; n = 6/group.

5.3.7.2.3. Effect of ETZ on Monoamines (5HT, NE and DA) Levels in OBX/Sham Rats

The effects of ETZ (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatment on brain monoamines (5-HT, NE and DA) levels in OBX rats are presented in **table 28.** OBX rats remarkably reduced 5-HT [$F_{(7,40)} = 14.25$, $P<0.05$], NE [$F_{(7,40)} = 22.31$, $P<0.05$] and DA [$F_{(7,40)} = 9.31$, $P<0.05$] levels as compared to sham operated controls. FLX (10 mg/kg, p.o.) treated OBX rats exhibited a significantly higher levels of brain 5-HT and NE levels as compared to OBX control rats (**table 28).** ETZ did not show any profound change in 5-HT, NE and DA levels in OBX and sham control rats.

Table 28. Effect of ETZ treatment on monoamines levels of OBX/Sham rats

Groups	Dose (mg/kg)	5-HT (ng/g)	NE (ng/g)	DA (ng/g)
Sham control	0	540.33±21.67	489.56±11.67	381.38±21.55
Sham + ETZ	0.5	531.90±17.33	473.38±21.04	390.55±18.21
Sham + ETZ	1	550.67±17.21	485.23±16.33	406.21±24.09
Sham + FLX	10	537.23±13.65	496.14±15.98	370.65±17.19
OBX control	0	395.36±18.32[a]	225.33±18.48[a]	307.93±14.16[a]
OBX + ETZ	0.5	390.25±10.45	230.33±12.46	317.33±22.16
OBX + ETZ	1	405.09±22.18	232.67±17.35	320.21±19.78
OBX + FLX	10	499.37±17.45[b]	310.13±15.27[b]	314.23±16.74

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control, [b]$P<0.05$ compared with OBX control; n = 6/group.

5.3.7.2.4. Evaluation of ETZ on Oxidant/Anti-oxidant Markers in OBX Model

5.3.7.2.4.1. Effect of ETZ on Brain TBARS Level in OBX/Sham Rats

Removal of olfactory bulbs caused significant oxidative damage, as evidenced by increase in lipid peroxidation [$F_{(7,40)} = 6.45$, $P<0.05$] as compared to sham group **(table 29).** Repeated treatment with ETZ (0.5 & 1 mg/kg, p.o.) produced a marked ($P<0.05$) decrease in brain TBARS level in OBX rats as compared to OBX control. FLX (10 mg/kg, i.p.) treatment also remarkably reduced brain TBARS level in OBX rats. Administration of ETZ as well as FLX did not show any notable effect on TBARS level in sham control group **(table 29).**

5.3.7.2.4.2. Effect of ETZ on Brain Nitrite Level in OBX/Sham Rats

As depicted in **table 29**, OBX rats increased nitrosative stress as evidenced by high nitrite level [$F_{(7,40)} = 14.93$, $P<0.05$] compared to sham control group. Statistical analysis revealed that chronic treatment with ETZ (0.5 and 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) decreased nitrite levels when compared with OBX control rats **(table 29).**

5.3.7.2.4.3. Effect of ETZ on Brain GSH, SOD and CAT Levels in OBX/Sham Rats

GSH, SOD and CAT enzymes levels are showed in **table 29**. Rats subjected to OBX procedure displayed decrease in brain GSH [$F_{(7,40)} = 12.39$, $P<0.05$], SOD [$F_{(7,40)} = 31.08$, $P<0.05$] and CAT [$F_{(7,40)} = 7.62$, $P<0.05$] levels compared with sham control rats. Treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) reverted the decrease of GSH, SOD and CAT levels compared with OBX control rats **(table 29).**

Table 29. Effect of ETZ on oxidants/anti-oxidants markers in OBX/Sham rats

Groups (mg/kg)	TBARS (nmole/mg protein)	Nitrite/Nitrate (µmole/mg) protein)	GSH (µmole/mg) protein)	CAT (µmole H_2O_2/min/ mg protein)	%SOD activity
Sham control	3.63±1.01	3.71±0.57	0.238±0.08	3.03±0.56	100±4.32
Sham + FLX (10)	3.94±1.1	4.93±0.37	0.243±0.06	3.13±0.68	94.0±5.5
Sham + ETZ (0.5)	3.34±0.90	3.92±1.12	0.274±0.10	3.60±0.99	84.0±5.35
Sham + ETZ (1)	3.53±0.84	4.53±0.73	0.250±0.08	3.40±1.04	90.0±7.32
OBX control	7.41±0.78[a]	5.94±0.37[a]	0.099±0.09[a]	0.75±0.82[a]	26.09±6.49[a]
OBX + FLX (10)	4.20±0.92[b]	3.93±1.04[b]	0.195±0.05[b]	2.10±0.94[b]	57.14±6.43[b]
OBX + ETZ (0.5)	4.78±0.89[b]	4.77±0.91[b]	0.168±0.09[b]	1.46±0.23[b]	52.38±7.43[b]
OBX + ETZ (1)	4.10±0.66[b]	3.83±0.86[b]	0.220±0.06[b]	2.55±0.46[b]	63.64±4.96[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control, [b]$P<0.05$ compared with OBX control; n = 6/group.

5.3.8. Evaluation of ETZ for Anti-depressant-like Potential in TBI Model Using Behavioral and Biochemical Tests

5.3.8.1. Behavioral Analysis

5.3.8.1.1. Effect of ETZ on TBI/Sham Rats Behavior in OFT

The OFT is a behavioral test used to evaluate the hyperactivity in TBI rats as summarized in **table 30**. TBI rats exhibited a characteristic hyperactivity in open field arena, as depicted by pronounced increase in ambulation [$F_{(7,40)}$ =42.15, $P<0.05$], rearing [$F_{(7,40)}$ = 26.51, $P<0.05$] and defecation (number of fecal pellets) [$F_{(7,40)}$ = 9.76, $P<0.05$] as compared to sham rats **(table 30).** Increase frequencies of ambulation, rearing and fecal pellet number were significantly ameliorated by ETZ (0.5 and 1 mg/kg) and FLX (10 mg/kg) treatment. The ambulation, rearing and defecation in sham group were not affected by both the treatments.

Table 30. Effect of ETZ treatment on behavior of TBI/sham rats in OFT

Groups	Dose (mg/kg)	Ambulation	Rearing	Fecal Pellet
Sham control	0	101.17±3.56	15.16±1.50	1.30±0.38
Sham + ETZ	0.5	110.0±8.87	12.33±1.05	1.90±0.71
Sham + ETZ	1	106.17±7.34	14.33±1.15	2.0±0.86
Sham + FLX	10	108.12±5.48	14.67±0.55	2.4±0.58
TBI control	0	194.25±9.89[a]	51.5±4.69[a]	6.6±0.65[a]
TBI + ETZ	0.5	140.67±15.44[b]	35.17±2.46[b]	4.83±0.60[b]
TBI + ETZ	1	124.67±9.94[b]	29.33±2.51[b]	4.20±0.31[b]
TBI + FLX	10	147.12±7.15[b]	35.25±6.06[b]	3.05±0.51[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control, [b]$P<0.05$ vs. TBI control group; n = 6/group.

5.3.8.1.2. Effect of ETZ on Sucrose Consumption in TBI/Sham Rats

As seen in **fig. 34**, TBI resulted in decreased sucrose consumption in rats. Statistical analysis demonstrated that TBI rats had profound reduced sucrose preference [$F_{(7,40)}$ = 14.76, $P<0.05$] than sham rats. Administration of ETZ (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) predominantly increased sucrose consumption in TBI rats compared to TBI control **(fig. 34).**

5.3.8.1.3. Effect of ETZ on Hyper-emotionality Behavior in TBI/Sham Rats

Evaluation of vehicle-treated TBI rats in hyper-emotionality test showed a profound ($P<0.05$) increase in total emotional responses when compared with sham control rats. ETZ (0.5 and 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) significantly [$F_{(7,40)}$ =19.45, $P<0.05$] decreased the

total hyper-emotionality scores in TBI rats as compared to TBI control rats **(fig. 35).** The results indicate that emotional expression of rats, as measured by the scoring scale employed here, dramatically increased following TBI.

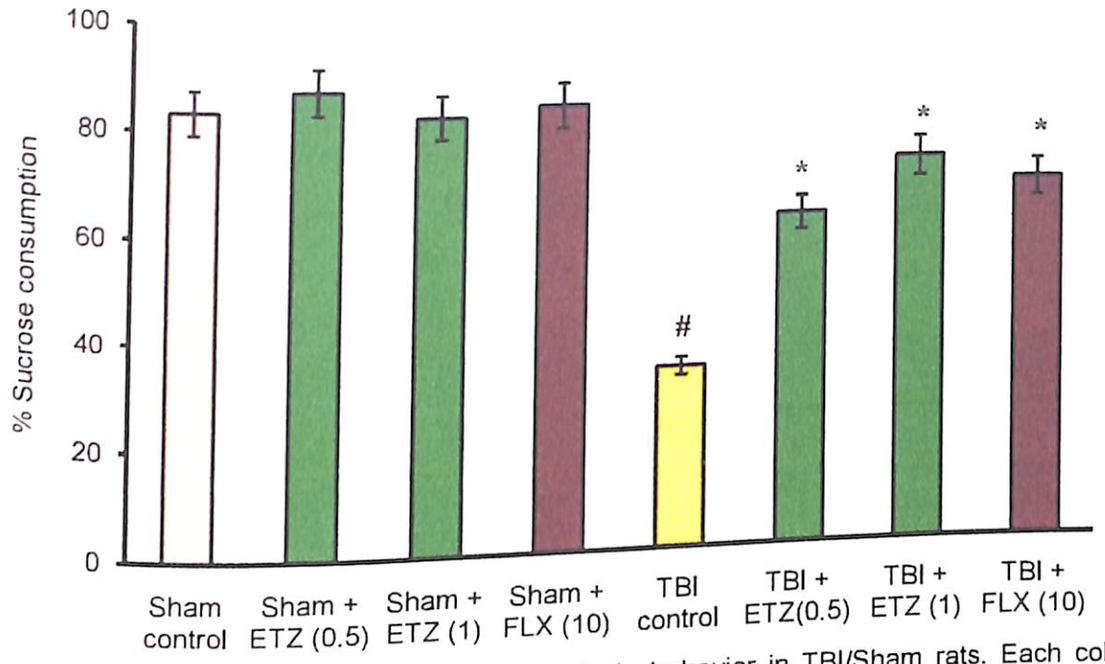


Fig. 34. Effect of ETZ and FLX treatment on anhedonia behavior in TBI/Sham rats. Each column represents mean percentage sucrose consumption. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with TBI control; n = 6/group.

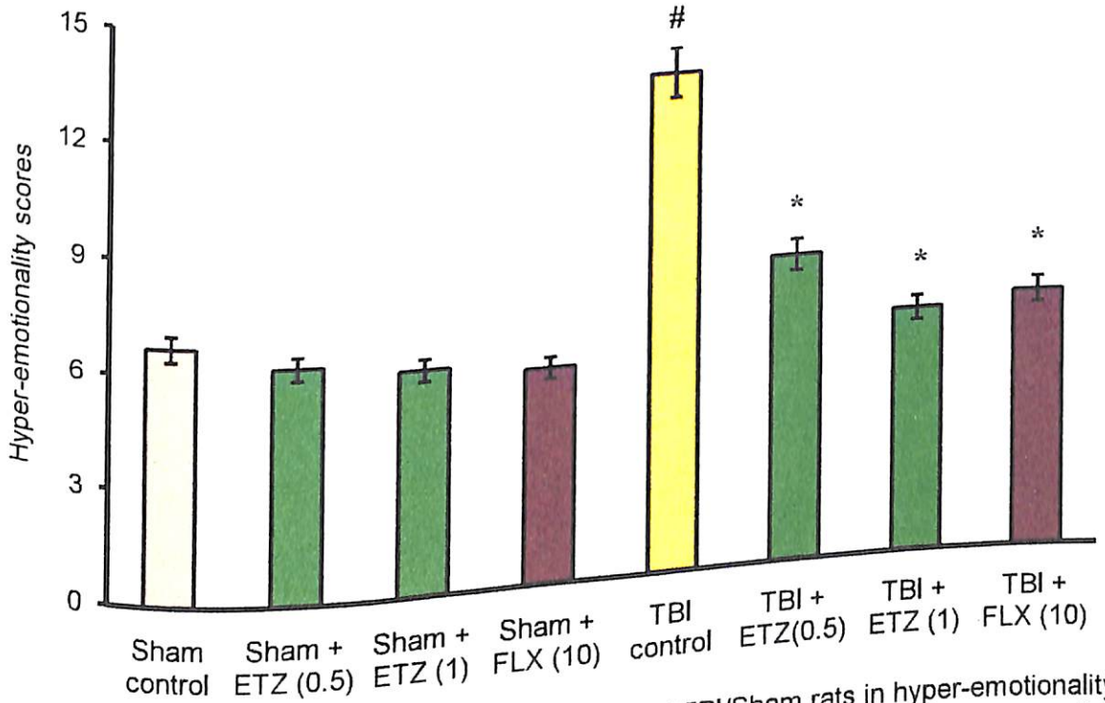


Fig. 35. Effects of ETZ and FLX treatment on the behavior of TBI/Sham rats in hyper-emotionality test. Each column represents mean hyper-emotionality scores. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with TBI control; n = 6/group.

5.3.8.2. Biochemical Estimation

5.3.8.2.1. Effect of ETZ on cAMP, pCREB and BDNF Levels in TBI/Sham Rats

The levels of cAMP, pCREB and BDNF detected in the brain of TBI/sham rats are summarized in **table 31.** TBI rats showed a marked decrease in cAMP [$F_{(7,40)} = 30.16$, $P<0.05$], pCREB [$F_{(7,40)} = 11.60$, $P<0.05$] and BDNF [$F_{(7,40)} = 20.14$, $P<0.05$] levels as compared to sham group. ETZ (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatment showed a pronounced increase in cAMP, pCREB and BDNF levels in TBI rats as compared to TBI control group. Similarly, administration of ETZ also increased the levels of these markers in sham rats **(table 31)**.

Table 31. Effect of ETZ treatment on cAMP, pCREB and BDNF levels of TBI/sham rats

Groups	Dose (mg/kg)	cAMP (pmol/mg protein)	BDNF (ng/mg protein)	pCREB (% sham control)
Sham control	0	18.7±2.43	70.65±5.23	100.00±5.67
Sham + ETZ	0.5	24.67±2.96[a]	116.33±4.67[a]	116.75±7.67[a]
Sham + ETZ	1	27.12±4.67[a]	129.67±7.18[a]	121.96±5.89[a]
Sham + FLX	10	18.35±1.33	81.77±4.08	104.18±6.13
TBI control	0	5.12±0.86[a]	20.72±4.87[a]	38.11±3.26[a]
TBI + ETZ	0.5	14.67±1.16[b]	32.92±2.45[b]	62.88±4.38[b]
TBI + ETZ	1	18.11±1.90[b]	39.52±2.77[b]	79.55±5.04[b]
TBI + FLX	10	14.50±0.66[b]	51.24±3.12[b]	59.58±5.54[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control, [b]$P<0.05$ as compared to TBI control; n = 6/group.

5.3.8.2.2. Effect of ETZ on Oxidant/Anti-oxidant Markers in TBI Model

5.3.8.2.2.1. Effect of ETZ on Brain TBARS and Nitrite Levels in TBI/Sham Rats

The effects of ETZ on the brain oxidative-nitrosative stress markers were also assessed as shown in **table 32**. From the results depicted, TBI rats had high lipid peroxidation [$F_{(7,40)} = 13.87$, $P<0.05$] and nitrite levels [$F_{(7,40)} = 9.72$, $P<0.05$] as compared to sham operated rats. The repeated treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX (10 mg/kg, i.p.) reverted the increase of brain TBARS and nitrite levels in TBI rats as compared to TBI control group.

5.3.8.2.2.2. Effect of ETZ on Brain GSH, SOD and CAT Levels in TBI/Sham Rats

TBI control rats displayed a profound decline in the brain GSH [$F_{(7,40)} = 16.21$, $P<0.05$], SOD [$F_{(7,40)} = 8.32$, $P<0.05$] and CAT [$F_{(7,40)} = 19.39$, $P<0.05$] levels compared with sham control rats. ETZ (0.5 & 1 mg/kg, p.o.) treatment resulted a marked increase in GSH, SOD and CAT levels. Similarly, FLX (10 mg/kg, p.o.) also markedly increased the brain GSH, SOD and CAT levels in TBI rats as compared to TBI control group **(table 32).** There was no marked difference observed between the drug treated sham group and sham control group.

Table 32. Effect of ETZ treatment on oxidant/anti-oxidant markers levels in TBI/Sham rats

Groups (mg/kg)	TBARS (nmole/mg protein)	Nitrite/Nitrate (µmole/mg protein)	GSH (µmole/mg protein)	CAT (µmole H_2O_2/min/mg protein)	%SOD activity
Sham control	1.76±0.61	1.08±0.12	0.278±0.53	5.34±1.12	100±5.67
Sham + FLX (10)	1.72±0.55	1.03±0.37	0.285±0.71	5.20±0.99	96.40±2.77
Sham + ETZ (0.5)	1.81±0.33	1.15±0.17	0.270±0.49	5.41±1.05	93.11±4.89
Sham + ETZ (1)	1.74±0.67	1.00±0.24	0.279±0.56	5.55±0.91	89.44±8.15
TBI control	3.16±0.51[a]	3.85±0.61[a]	0.118±0.18[a]	3.26±0.66[a]	53.57±4.65[a]
TBI + FLX (10)	2.25±0.82[b]	1.44±0.97[b]	0.227±0.33[b]	5.12±1.12[b]	63.12±5.15
TBI + ETZ (0.5)	2.17±0.67[b]	2.36±0.74[b]	0.166±0.11[b]	3.07±0.87[b]	59.30±8.13
TBI + ETZ (1)	1.87±0.43[b]	1.23±0.39[b]	0.253±0.19[b]	5.06±0.65[b]	70.23±4.43

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control, [b]$P<0.05$ vs. TBI control group; n = 6/group.

5.3.9. Evaluation of ETZ for Anti-depressant-like Potential in CUMS Model Using Behavioral and Biochemical Tests

5.3.9.1. Behavioral Analysis

5.3.9.1.1 Effect of Chronic Stress and ETZ on Mice Body Weight

Table 33 summarizes the effects of CUMS and drugs treatments on body weight of mice. No difference in the initial body weight was observed in any experimental group. Two-way ANOVA revealed that CUMS subjected mice after a period of 28 days showed a marked [$F_{(1,40)} = 16.04$, $P<0.05$] decrease in body weight as compared to unstressed mice **(table 33)**. Body weight gained was remarkably higher in ETZ (1 mg/kg) treated stressed mice as compared to stressed control mice. The CUMS-induced decrease in body weight was not markedly affected by ETZ (0.5 mg/kg) and FLX treatments **(table 33)**.

Table 33. Effect of ETZ treatment and CUMS on mice body weight

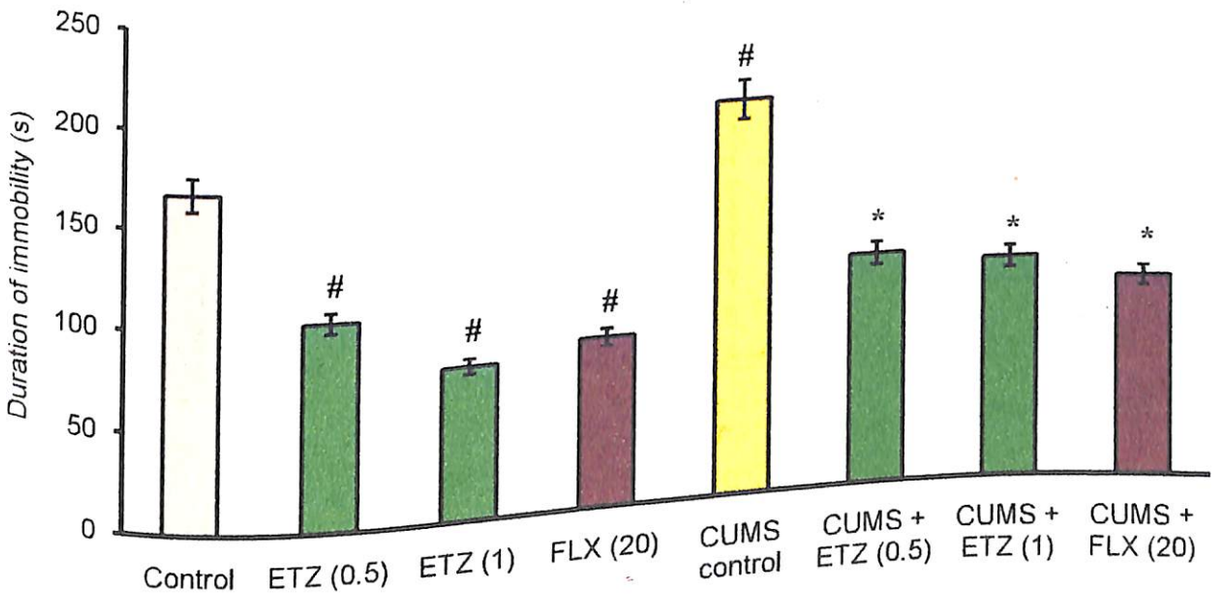
Groups	Dose (mg/kg)	Body weight (g)	
		Initial (0^{th} day)	Final (28^{th} day)
Normal control	0	30.97±0.92	37.20±1.87
Control + ETZ	0.5	29.73±2.05	38.45±1.67
Control + ETZ	1	31.80±1.13	38.20±1.54
Control + FLX	20	30.91±1.08	36.81±0.98
CUMS control	0	31.19±0.99	21.14±2.01[a]
CUMS + ETZ	0.5	31.50±0.57	24.17±1.43
CUMS + ETZ	1	31.27±1.01	28.47±1.16[b]
CUMS + FLX	20	31.31±1.12	24.01±2.20

All values are presented as mean ± S.E.M. [a]P<0.05 vs. normal control group and [b]P<0.05 vs. stress control group; n = 6/group.

5.3.9.1.2. Effect of ETZ on Stressed/Unstressed Mice Behavior in FST

Fig. 36A & B shows the effect of ETZ treatment on the duration of immobility and swimming episodes in stressed mice. CUMS-subjected mice exhibited a pronounced increase in the duration of immobility [$F_{(7,40)}$ = 13.48, P<0.05] and decrease in swimming episodes [$F_{(7,40)}$ = 8.76, P<0.05] compared to normal control mice. Statistical analysis revealed that treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) profoundly decreased the duration of immobility and increased swimming episodes in stressed mice as compared to stressed group **(fig. 36A & B).** Similarly, ETZ and FLX also predominantly (P<0.05) decreased the immobility duration and increased swimming episodes in normal control mice.

36A. Duration of immobility



36B. Swimming Episode

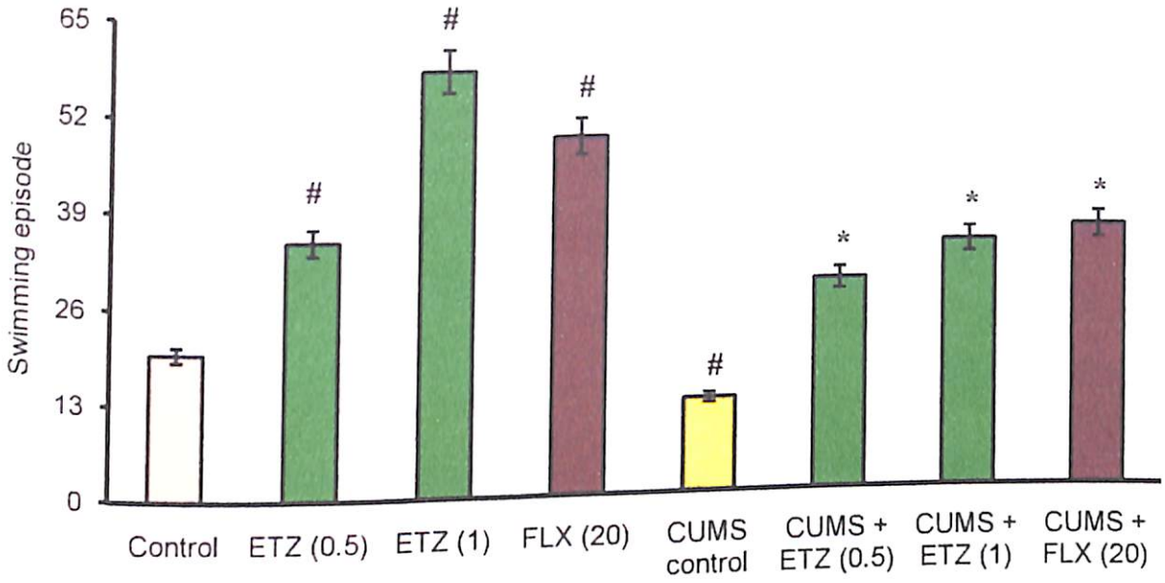


Fig. 36. Effect of ETZ and FLX treatment in mice FST. Each column represents mean (A) duration of immobility(s) & (B) swimming episodes. The error bar indicates S.E.M. #P<0.05 when compared with normal control; *P<0.05 when compared with stressed control group; n = 6/group.

5.3.9.1.3. Effect of ETZ on Stressed/Unstressed Mice Behavior in TST

The stressed mice showed a pronounced (P<0.05) increase in duration of immobility in TST compared to normal control mice. The chronic treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) for a period of 21 days remarkably [$F_{(7,40)} = 19.92$, P<0.05] decreased the immobility duration in stressed mice as compared to stressed control **(fig. 37).** Also, ETZ and FLX treatment markedly decreased the duration of immobility in normal control mice.

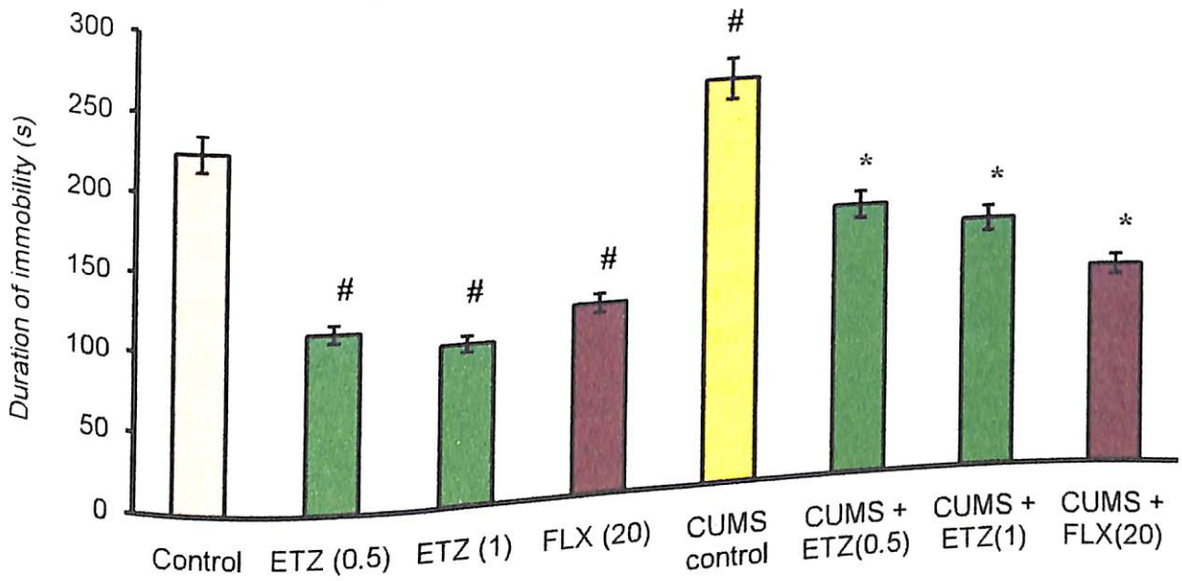


Fig. 37. Effects of ETZ and FLX treatment on duration of immobility in TST. Each column represents mean duration of immobility (s). The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 when compared with stressed control group; n = 6/group.

5.3.9.1.4. Effect of ETZ on Sucrose Consumption in Stressed/Unstressed Mice

The stressed mice showed a notable reduction in the percentage of sucrose consumption, when compared to normal control **(fig. 38).** Administration of ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) markedly [$F_{(7,40)} = 16.75$, $P<0.05$] restored the decrease in percentage of sucrose consumption in stressed mice as compared to stressed control group. Chronic treatment with ETZ and FLX did not affect the sucrose consumption in normal control mice.

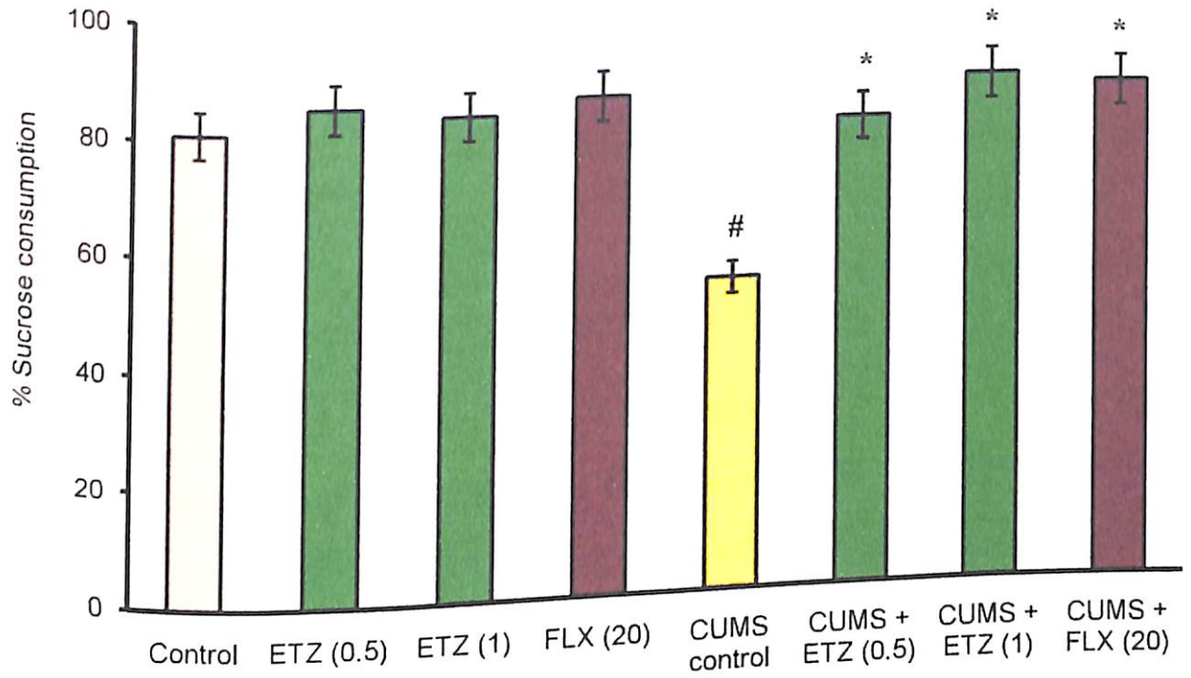


Fig. 38. Effect of ETZ and FLX treatment on anhedonia behavior in stressed mice. Each column represents sucrose preference (%). The error bar indicates S.E.M. #P<0.05 as compared to normal control, *P<0.05 as compared to stress control group; n = 6/group.

5.3.9.2. Biochemical analysis

5.3.9.2.1. Effect of ETZ on Neuroendocrine Hormone Level in CUMS Model

5.3.9.2.1.1. Effect of ETZ on Serum CORT Level in Stressed/Unstressed Mice

To elucidate the molecular basis of behavioral changes, we examined the serum levels of CORT in stressed and unstressed mice. Chronic exposure of mice to CUMS resulted in a marked elevation of serum CORT level as compared to normal control mice. Chronic treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) markedly [$F_{(7,40)} = 9.25$, $P<0.05$] decreased serum CORT level in stressed mice as compared to stressed control. No effect of drug treatment was observed on serum CORT level in normal control mice **(fig. 39).**

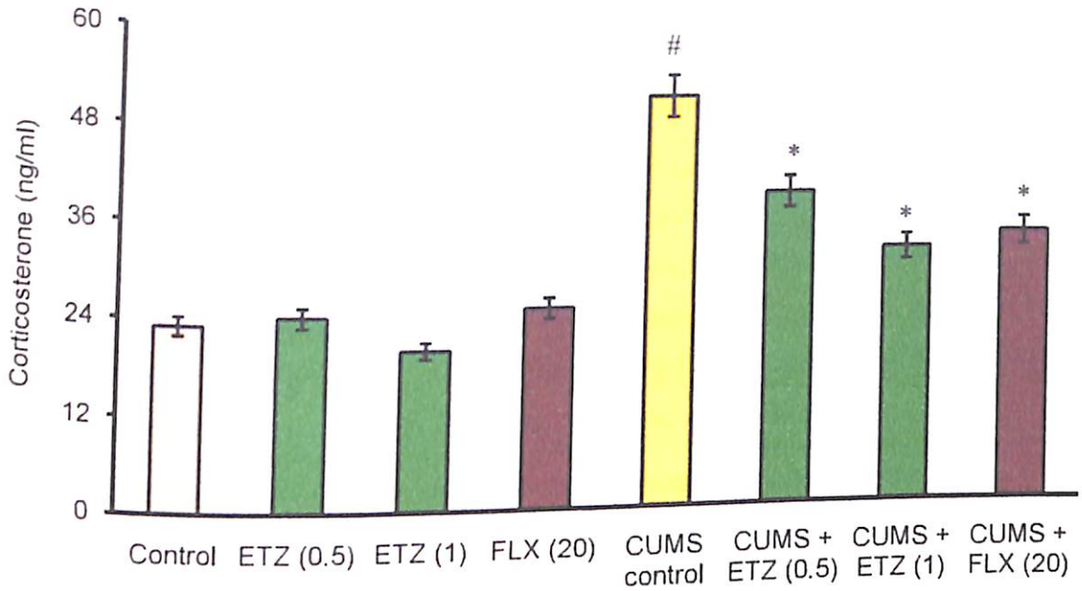


Fig. 39. Effects of ETZ and FLX treatment on serum CORT level. The error bar indicates S.E.M. #P<0.05 as compared to normal control, *P<0.05 as compared to stress control group; n = 6/group.

5.3.9.2.2. Effect of ETZ on cAMP Signaling in CUMS Model

5.3.9.2.2.1. ETZ Effect on cAMP, pCREB and BDNF Levels in Stressed/Unstressed Mice

As depicted in **table 34**, stressed mice showed remarkably reduced cAMP [$F_{(7,40)} = 47.46$, P<0.05], pCREB [$F_{(7,40)} = 17.29$, P<0.05] and BDNF [$F_{(7,40)} = 12.47$, P<0.05] levels as compared to normal control. Post-hoc test indicated that treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) markedly increased the levels of cAMP, pCREB and BDNF in stressed mice as compared to stressed control. Also, ETZ treated normal control mice displayed a profound increase in cAMP, pCREB and BDNF levels **(table 34).**

Table 34. Effect of ETZ on cAMP, pCREB and BDNF levels in stressed/unstressed mice

Groups	Dose (mg/kg)	cAMP (pmol/mg protein)	BDNF (ng/mg protein)	pCREB (% normal control
Normal control	0	103.25±5.87	11.33±0.99	100±3.56
Control + ETZ	0.5	114.20±3.67[a]	18.47±0.36[a]	111.19±4.08[a]
Control + ETZ	1	133.09±5.33[a]	20.36±0.99[a]	126.67±5.32[a]
Control + FLX	20	95.34±12.05	13.12±1.17	101.12±10.13
CUMS control	0	68.43±6.46[a]	3.45.±087[a]	44.76±5.21[a]
CUMS + ETZ	0.5	82.45±7.19[b]	8.79±1.10[b]	60.95±2.07[b]
CUMS + ETZ	1	109.45±5.90[b]	10.25±0.75[b]	86.36±4.52[b]
CUMS + FLX	20	81.22±4.38[b]	6.35±1.04[b]	62.59±3.67[b]

Results are presented as mean ± S.E.M. [a]P<0.05 vs. normal control group, [b]P<0.05 vs. stress control group; n = 6/group.

5.3.9.2.3. Effect of ETZ on Neurotransmitter Levels in CUMS Model

5.3.9.2.3.1. Effect of ETZ on Monoamines Levels in Stressed/Unstressed Mice

The levels of monoamines detected in the brain of stressed and unstressed mice are summarized in **table 35**. CUMS markedly decreased 5-HT [$F_{(7,40)} = 11.54$, $P<0.05$], NE [$F_{(7,40)} = 13.66$, $P<0.05$] and DA [$F_{(7,40)} = 10.12$, $P<0.05$] levels in mice as compared to normal control group. FLX (20 mg/kg, p.o.) profoundly restored the brain 5-HT and NE levels in stressed mice as compared to stressed control mice **(table 35).** No significant change in 5-HT, NE and DA levels was found in ETZ treated stressed and normal mice.

Table 35. Effect of ETZ on monoamines levels of stressed/unstressed mice

Groups	Dose (mg/kg)	5-HT (ng/g)	NE (ng/g)	DA (ng/g)
Normal control	0	410.47±16.67	310.33±15.75	284.12±25.20
Control + ETZ	0.5	400.56±8.93	320.67±10.92	272.56±12.56
Control + ETZ	1	407.02±11.47	315.08±22.18	285.09±19.34
Control + FLX	20	421.90±17.90	302.56±9.87	289.75±21.13
CUMS control	0	252.56±21.77[a]	157.13±16.15[a]	195.11±13.25[a]
CUMS + ETZ	0.5	265.33±15.08	150.67±17.22	204.43±19.78
CUMS + ETZ	1	260.98±16.14	165.89±11.56	200.67±24.95
CUMS + FLX	20	332.19±14.67[b]	182.67±13.87[b]	196.14±24.08

Results are presented as mean ± S.E.M. [a]$P<0.05$ vs. normal control group and [b]$P<0.05$ vs. stress control group; n = 6/group.

5.3.9.2.4. Effect of ETZ on Oxidant/Anti-oxidant Markers in CUMS Model

5.3.9.2.4.1. Effect of ETZ on TBARS and Nitrite Levels in Stressed/Unstressed Mice

Stressed mice showed a pronounced elevation in brain TBARS and nitrite levels as compared to the normal control **(table 36).** ETZ (0.5 & 1 mg/kg, p.o.) treatment notably reduced the brain TBARS [$F_{(7,40)} = 16.52$, $P<0.05$] levels of stressed mice as compared to stressed control group. ETZ also at higher dose (1 mg/kg) produced a marked [$F_{(7,40)} = 9.26$, $P<0.05$] reduction of elevated brain nitrite level in stressed mice. Similarly, FLX (20 mg/kg, p.o.) remarkably reduced TBARS and nitrite levels in the brain of stressed mice **(table 36).**

5.3.9.2.4.2. Effect of ETZ on GSH, SOD and CAT Levels in Stressed/Unstressed Mice

The brain GSH, SOD and CAT levels were found to be markedly depleted in stressed mice compared to normal control **(table 36).** ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) treatment showed a marked increase in brain GSH [$F_{(7,40)}$ = 16.35, P<0.05], SOD [$F_{(7,40)}$ = 8.95, P<0.05] and CAT [$F_{(7,40)}$ = 17.11, P<0.05] levels as compared to stressed control.

Table 36. Effect of ETZ on oxidant/anti-oxidant markers in stressed/unstressed mice

Group (mg/kg)	TBARS (nmole/mg protein)	Nitrite/Nitrate (µmole/mg protein)	GSH (µmole/mg protein)	CAT (µmole H_2O_2/min/ mg protein)	%SOD activity
Normal control	1.63±0.05	12.36±1.32	0.14±0.02	1.34±0.09	100±2.32
Control + ETZ (0.5)	1.70±0.05	10.67±1.57	0.14±0.05	1.40±0.10	94.21±4.33
Control + ETZ (1)	1.49±0.07	12.99±1.18	0.13±0.04	1.29±0.08	89.9±5.87
Control + FLX (20)	1.72±0.03	11.07±2.01	0.14±0.02	1.21±0.06	96.3±4.12
CUMS control	2.96±0.04[a]	19.41±1.82[a]	0.08±0.02[a]	0.27±0.02[a]	25.85±9.19[a]
CUMS + FLX (20)	1.65±0.04[b]	15.52±1.26[b]	0.16±0.05[b]	1.12±0.05[b]	72.18±6.94[b]
CUMS + ETZ (0.5)	2.22±0.03[b]	19.0±2.39	0.17±0.06[b]	1.16±0.08[b]	59.72±3.38[b]
CUMS + ETZ (1)	1.62±0.05[b]	14.93±1.24[b]	0.19±0.07[b]	1.76±0.05[b]	95.77±8.32[b]

Results are presented as mean ± S.E.M. [a]P<0.05 vs. normal control group, [b]P<0.05 vs. stressed control group; n = 8/group.

5.3.10. Evaluation of ETZ for Anti-depressant-like Potential in Chronic CORT-Injection Model Using Behavioral and Biochemical Tests

5.2.10.1. Behavioral Analysis

5.3.10.1.1. Effect of ETZ on Chronic CORT-injected Mice Behavior in FST

The duration of immobility in FST was recorded after chronic exposure of mice to CORT. One-way ANOVA revealed a significant effect of the groups for the duration of immobility [$F_{(6,35)}$ = 48.61, P<0.05]. The post-hoc test indicated that CORT treatment caused a profound (P<0.05) increase in duration of immobility in mice compared with normal control. ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) treatment notably (P<0.05) decreased the duration of immobility of CORT-treated mice as compared to CORT control mice. Also, ETZ (0.5 & 1 mg/kg, p.o.) and FLX showed a markedly decrease in the duration of immobility in normal control mice **(fig. 40).**

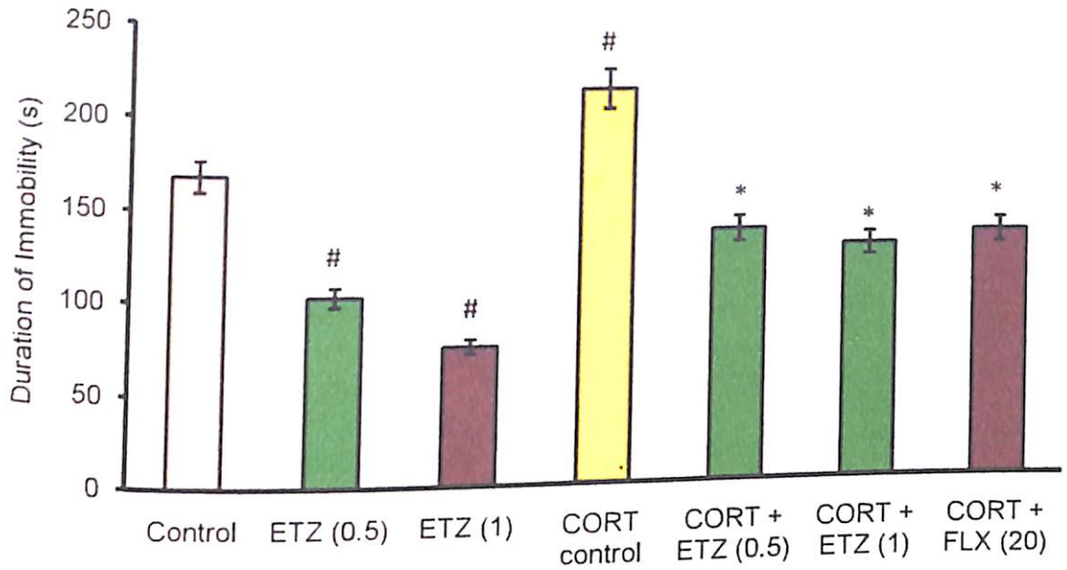


Fig. 40. Effect of ETZ and FLX treatment in mice FST. Each column represents mean duration of immobility (s). The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 vehicle treated CORT-injected control group; n = 6/group.

5.3.10.1.2. Effect of ETZ on Chronic CORT-injected Mice Behavior in TST

The duration of immobility in TST by normal control and CORT-injected mice, cumulated over the 6 min test was displayed in **fig. 41.** Chronic CORT-injected mice showed a remarkably increase in the duration of immobility [$F_{(6,35)} = 31.75$, P<0.05] as compared to normal control. Administration of ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) profoundly (P<0.05) decreased the duration of immobility of CORT-treated mice as compared to CORT control mice. ETZ and FLX treatment also resulted in a significant reduction of the duration of immobility in normal mice **(fig. 41).**

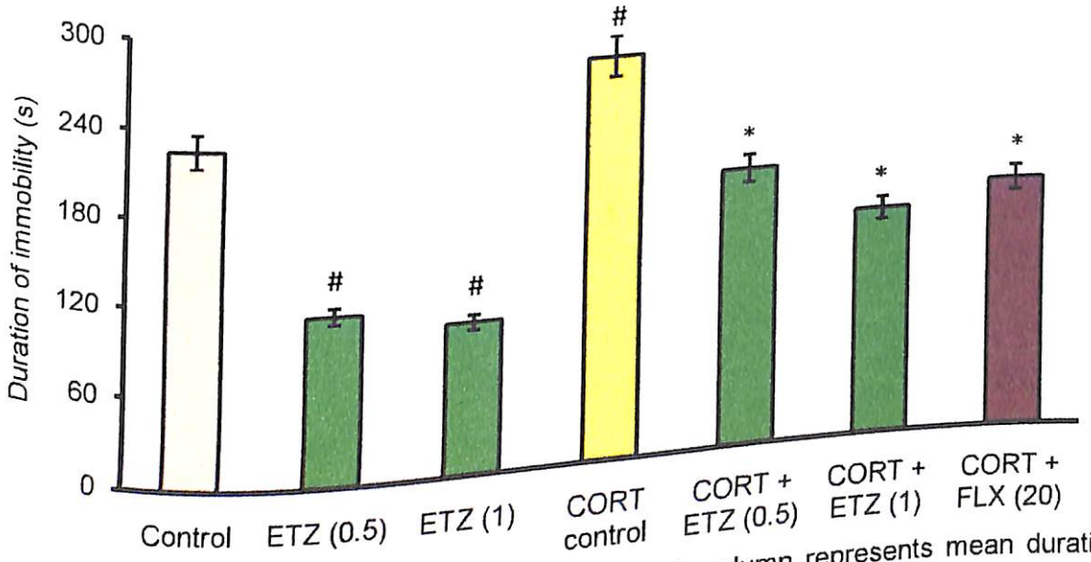


Fig. 41. Effect of ETZ and FLX treatment in mice TST. Each column represents mean duration of immobility (s). The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 compared to CORT-injected control group; n = 6/group.

5.3.10.1.3. Effect of ETZ on Sucrose Consumption in Chronic CORT-injected Mice

The CORT-treated mice showed a profound reduction in the percentage of sucrose consumption as compared to normal control **(fig. 42).** Treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) for a period of 3 weeks predominantly increased the percentage of sucrose consumption [$F_{(6,35)} = 9.68$, $P<0.05$] in CORT-treated mice compared with CORT control mice.

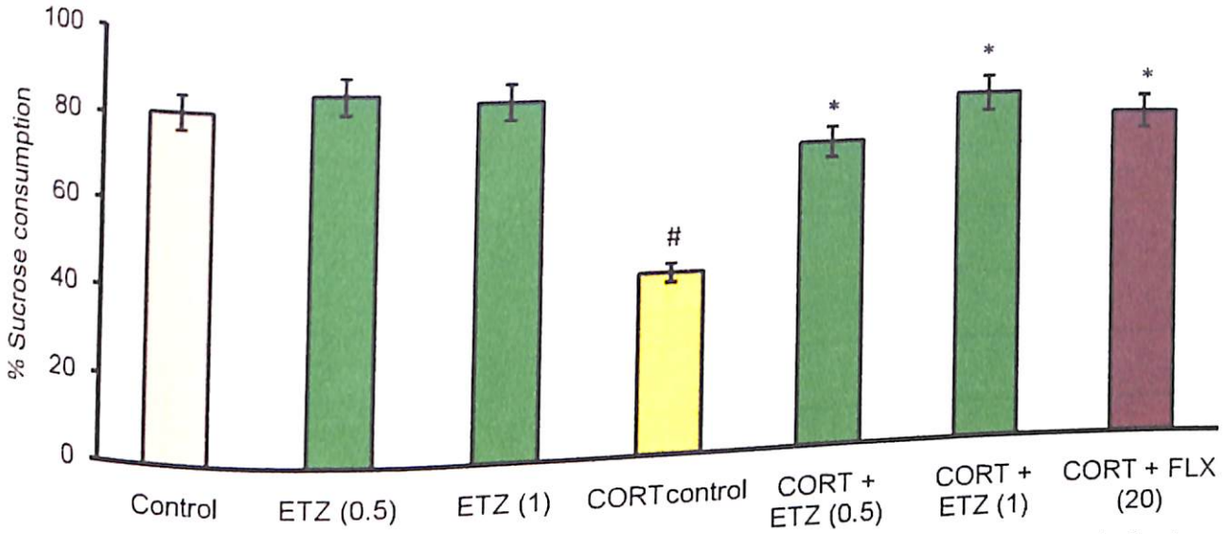


Fig. 42. Effect of ETZ and FLX on anhedonia behavior in CORT-injected mice. The error bar indicates S.E.M. #P<0.05 as compared to normal control, *P<0.05 as compared to CORT control; n = 6/group.

5.3.10.1.4. Effect of ETZ on Locomotor Score in Chronic CORT-injected Mice

In the present study, there was a marked difference observed on the locomotor scores between the CORT-injected and normal control mice **(fig. 43).** In addition, chronic treatment of ETZ (0.5 & 1 mg/kg, p.o.) as well as FLX (20 mg/kg, p.o.) produced a profound change in locomotor scores in CORT-injected mice as compared to CORT control mice **(fig. 43).**

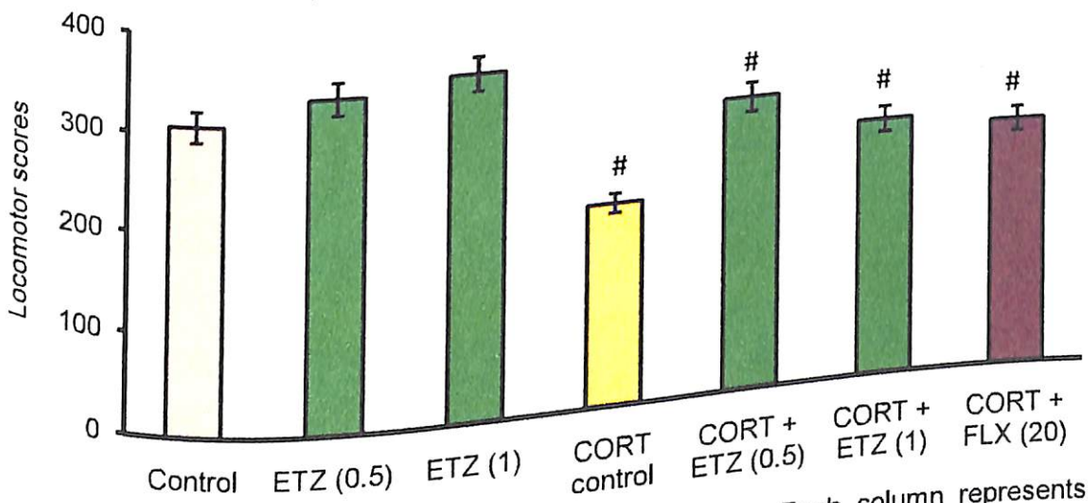


Fig. 43. Effect of ETZ and FLX treatment on locomotor scores. Each column represents mean locomotor scores recorded in 8 min observation period. The error bars indicate S.E.M. #P<0.05 as compared to normal control, *P<0.05 as compared to vehicle treated CORT control; n = 6/group.

5.3.10.2. Biochemical Estimation

5.3.10.2.1. Effect of ETZ on Neuroendocrine Hormone Level in CORT-injected Model

5.3.10.2.1.1. Effect of ETZ on Serum CORT Level in CORT-injected Mice

The effect of ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) treatment on serum CORT level in CORT-treated mice is shown in **fig. 44**. CORT-treated mice were observed to produce a remarkable elevation in serum CORT level as compared to normal control mice. Chronic treatment with ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) resulted, a pronounced decrease in serum CORT [$F_{(6,35)} = 16.39$, $P<0.05$] level in CORT-treated mice as compared to CORT control group.

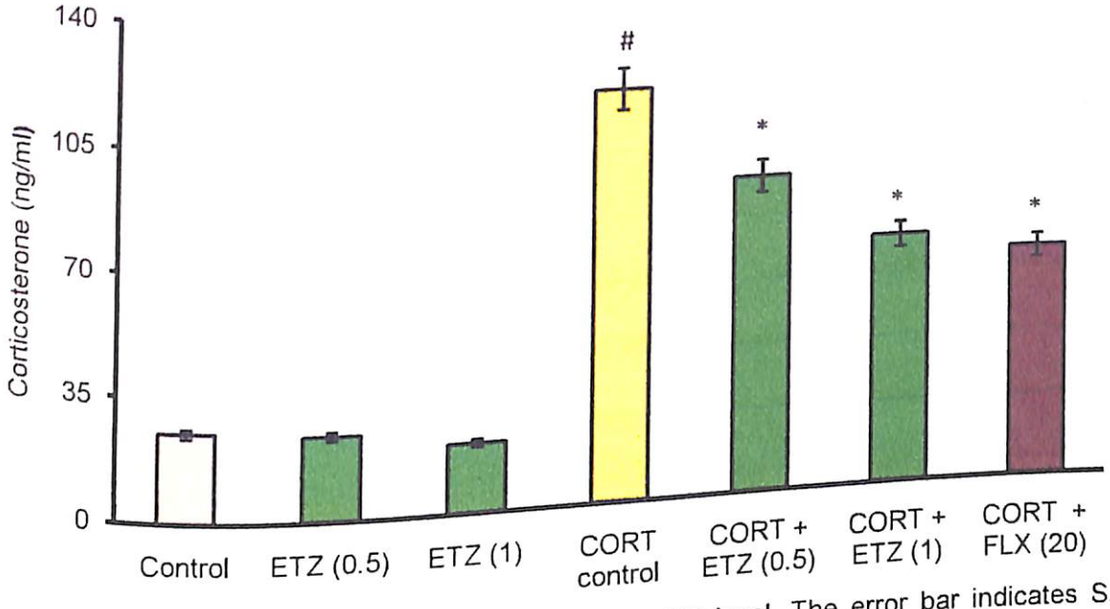


Fig. 44. Effects of ETZ and FLX treatment on serum CORT level. The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 when compared with vehicle treated CORT-injected control group; n = 6/group.

5.3.10.2.2. Effect of ETZ on cAMP Signaling in CORT-injected Model

5.3.10.2.2.1. Effect of ETZ on cAMP, pCREB and BDNF Levels in CORT-injected Mice

To elucidate the molecular basis of behavioral changes in CORT-treated mice, we examined the levels of cAMP, CREB and BDNF. **Table 37** illustrates the effects of ETZ (0.5 & 1 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) treatment on cAMP, pCREB and BDNF levels. Exposure of mice to CORT notably decreased cAMP, pCREB and BDNF levels in brain as compared to normal control. Administration of ETZ (0.5 & 1 mg/kg, p.o.) and FLX remarkably increased cAMP [$F_{(6,35)} = 28.09$, $P<0.05$], pCREB [$F_{(6,35)} = 42.17$, $P<0.05$] and BDNF [$F_{(6,35)} = 20.12$, $P<0.05$] levels in CORT-treated mice as compared to CORT control. Treatment with ETZ also elevated the cAMP, pCREB and BDNF levels in normal control group **(table 37).**

Table 37: Effect of ETZ treatment on cAMP, pCREB and BDNF levels in CORT-treated mice

Groups	Dose (mg/kg)	cAMP (pmol/mg protein)	BDNF (ng/mg protein)	pCREB (% normal control
Normal control	0	103.25±4.87	11.33±0.99	100±3.56
Control + ETZ	0.5	114.20±3.67[a]	18.47±0.36[a]	111.19±4.08[a]
Control + ETZ	1	133.09±5.33[a]	20.36±0.99[a]	126.67±5.32[a]
CORT control	0	46.67±9.34[a]	2.15±0.33[a]	33.33±5.21[a]
CORT + ETZ	0.5	78.42±3.70[b]	6.39±0.44[b]	66.19±5.07[b]
CORT + ETZ	1	91.24±3.39[b]	8.97±1.42[b]	78.21±4.14[b]
CORT + FLX	20	67.23±6.33[b]	5.12±2.11[b]	49.67±6.17[b]

Results are presented as mean ± S.E.M. [a]P<0.05 vs. normal control group, [b]P<0.05 vs. vehicle treated CORT-injected control group; n = 8/group.

5.3.11. Evaluation of Anxiolytic-like Potential of ETZ in Experimental Models

5.3.11.1. Effect of ETZ on Behavior of Mice in EPM test

The OAE and TSOA by normal and drug treated mice, cumulated over the 5-min test is displayed in **table 38.** Acute treatment with ETZ (0.5 & 1 mg/kg, i.p.) and DZM (2 mg/kg, i.p.) predominantly increased the percentage of both OAE [$F_{(4,35)} = 9.27$, P<0.05] and TSOA [$F_{(4,35)} = 10.89$, P<0.05] as compared to normal control. A lower dose of ETZ (0.25 mg/kg) did not show any significant effect in the EPM test **(table 38).**

Table 38: Effect of ETZ on behavior of mice in EPM test

Groups	Dose (mg/kg)	No. of entries		% OAE	% TSOA
		open arm	closed arm		
Control	0	2.12±0.61	5.62±0.50	27.62±6.89	26.41±2.71
DZM	2	6.80±1.46[a]	4.40±0.87	60.15±2.56[a]	44.13±3.6[a]
ETZ	0.25	2.33±0.67	4.50±1.01	25.17±7.51	21.61±4.04
ETZ	0.5	5.29±1.08[a]	5.86±1.03	48.26±5.53[a]	37.05±2.98[a]
ETZ	1	5.14±1.52[a]	4.71±1.26	52.32±3.66[a]	45.67±6.09[a]

All values represent mean ± S.E.M. [a]P<0.05 when compared with control group; n = 8/group.

5.3.11.2. Effect of ETZ on Behavior of Mice in L/D Aversion Test

ETZ (0.5 & 1 mg/kg, i.p.) and DZM (2 mg/kg, i.p.) treatment markedly increased latency time to leave the light compartment [$F_{(4,35)}$ = 10.74, P<0.05] and time spent in light compartment [$F_{(4,35)}$ = 19.25, P<0.05] as compared to control group **(table 39).** In addition, DZM markedly [$F_{(4,35)}$ = 3.25, P<0.05] increased crossings between the compartments, whereas, no effect on crossings was observed in ETZ treatment groups **(table 39)**. ETZ (0.25 mg/kg, i.p.) did not produce notable change in any of the parameters.

Table 39. Effect of ETZ on behavior of mice in L/D aversion Test

Groups	Dose (mg/kg)	Latency to leave light box (s)	No. of crossing	Time spent in light box (s)
Control	0	19.43±2.11	12.67±1.47	80.14±6.34
DZM	2	73.83±5.43[a]	16.18±1.40[a]	164.84±11.06[a]
ETZ	0.25	26.83±14.29	6.0±0.42	66.34±16.41
ETZ	0.5	46.0±10.58[a]	11.0±2.17	141.0±14.67[a]
ETZ	1	117.5±18.47[a]	8.0±1.70	207.0±14.31[a]

All values represent mean ± S.E.M. [a]P<0.05 when compared with control group; n = 8/group.

5.3.11.3. Effect of ETZ on Behavior of Mice in HB Test

Table 40 summarizes the results of the HB test. ETZ (0.5 & 1 mg/kg, i.p.) and DZM (2 mg/kg, i.p.) treatment produced a profound increase in the number of head dipping [$F_{(4,35)}$ = 7.23, P<0.05] and time spent in head dipping [$F_{(4,35)}$ = 38.79, P<0.05] as compared to normal control. ETZ and DZM treatment also markedly decreased the head dipping latency [$F_{(4,35)}$ = 2.77, P<0.05]. The effect of ETZ lower dose (0.25 mg/kg) in HB test was failed to reach the level of significance **(table 40)**.

Table 40. Effect of ETZ on behavior of mice in HB test

Groups	Dose (mg/kg)	Head dipping latency (s)	No. of head dipping	Time spent in head dipping (s)
Control	0	11.89±1.92	15.11±1.98	10.11±2.21
DZM	2	5.86±1.57[a]	36.85±4.38[a]	26.14±2.05[a]
ETZ	0.25	12.67±1.47	21.16.5±4.7	16.34±5.57
ETZ	0.5	6.71±1.35[a]	29.57±6.39[a]	20.43±4.12[a]
ETZ	1	6.16±1.6[a]	34.43±3.89[a]	27.67±4.03[a]

All values represent mean ± S.E.M. [a]P<0.05 when compared with control group; n = 8/group.

5.4. Evaluation of Q-21 for Anti-depressant-like Potential in Behavioral Test Battery

5.4.1. Effect of Q-21 on Mice Behavior in SLA test

Fig. 45 displays the effects of Q-21 on locomotor activity in mice. Tested doses of Q-21 (0.25–2 mg/kg, i.p.) had no influence on locomotor activity as compared to control group.

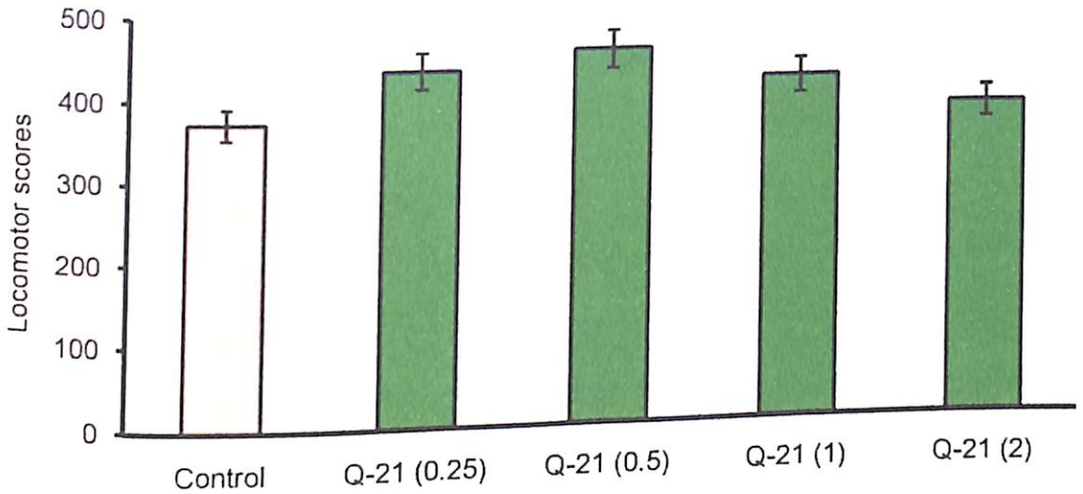
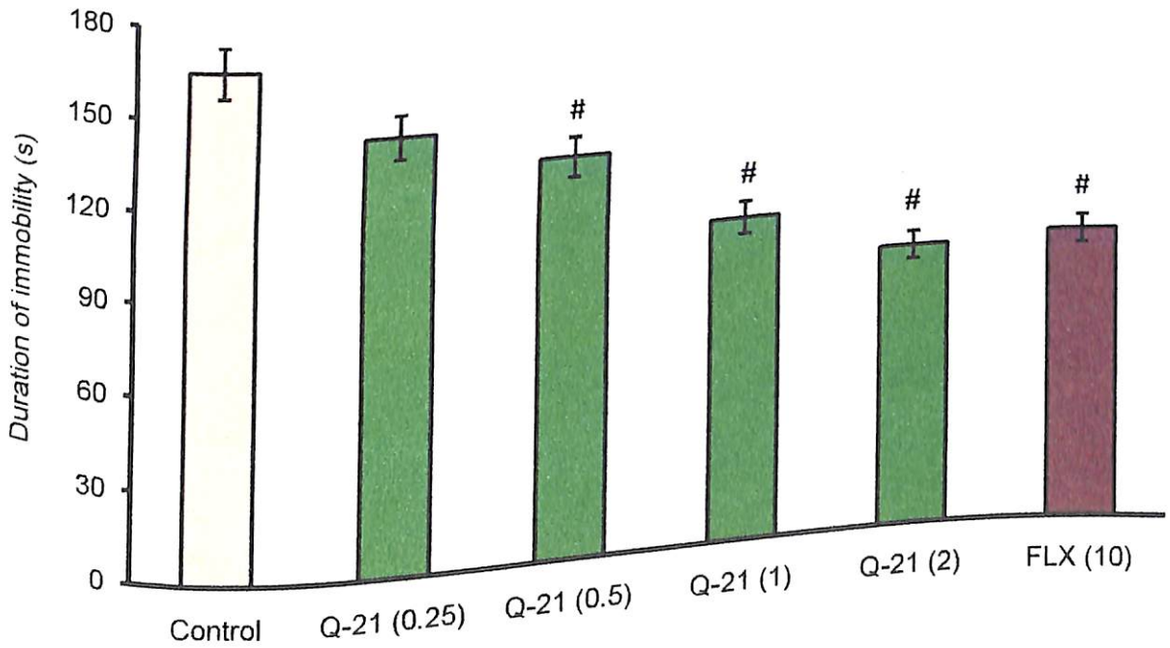


Fig. 45. Effect of Q-21 treatment on SLA in mice. The error bars indicate S.E.M. Each column represents mean locomotor scores recorded in 8 min observation period; n = 8/group.

5.4.2. Effect of Q-21 on Mice Behavior in FST

This test for depressive-like behavior measures the duration of immobility. One-way ANOVA showed that acute treatment with Q-21 (0.25-2 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) induced a marked reduction in immobility time [$F_{(5,42)} = 7.32$, $P<0.05$] and increase in swimming episodes [$F_{(5,42)} = 21.32$, $P<0.05$] as compared to control treatment **(fig. 46A & B).**

46A. Duration of immobility



46B. Swimming episode

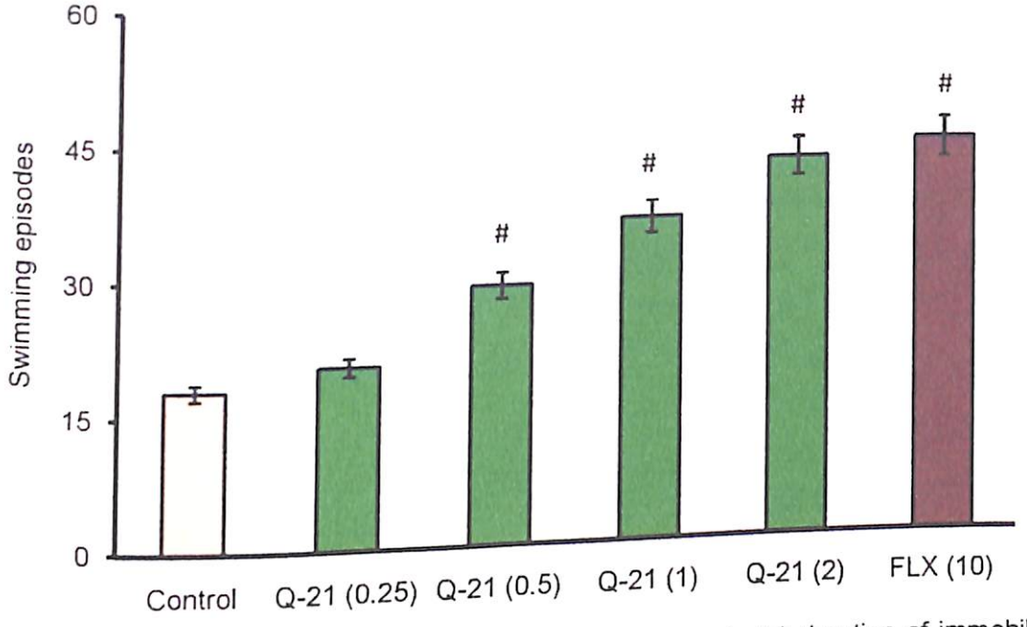


Fig. 46. Effect of Q-21 treatment in mice FST. Each column represents (a) duration of immobility(s) and (b) swimming episode. The error bar indicates S.E.M. #P<0.05 when compared with vehicle-treated normal group; n = 8/group.

5.4.3. Effect of Q-21 on Mice Behavior in TST

Acute treatment with Q-21 (0.5-2 mg/kg, i.p.), resulted in profound decrease in the duration of immobility [$F_{(5,42)} = 9.17$, $P<0.05$] in mice TST as compared to vehicle treated group **(fig. 47).** Similarly, BUP (20 mg/kg, i.p.), also remarkably reduced the duration of immobility.

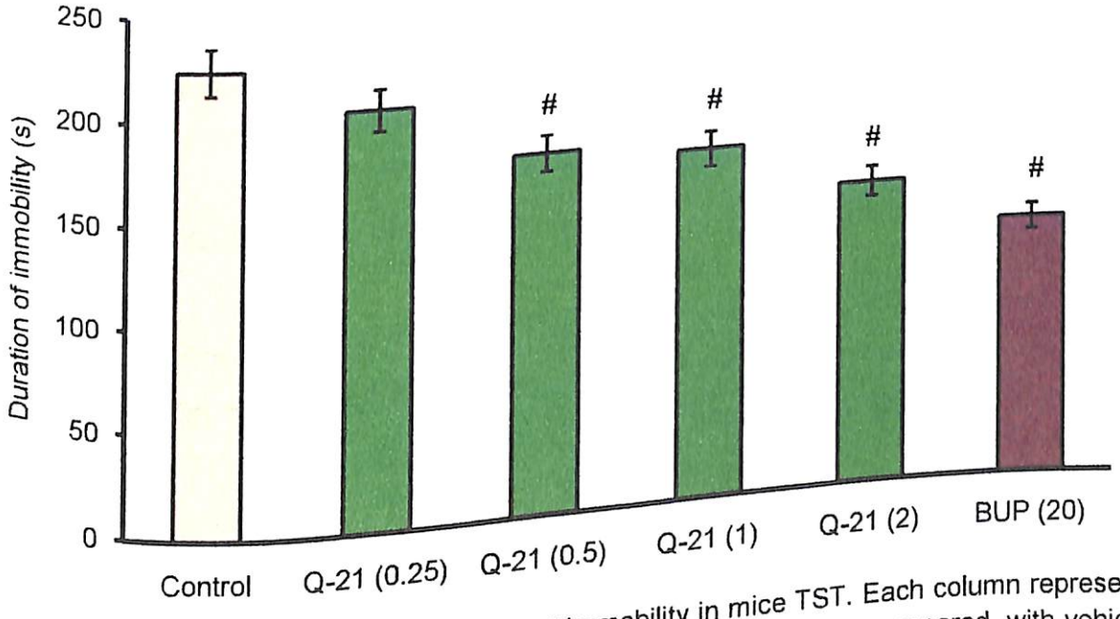


Fig. 47. Effect of Q-21 treatment on the duration of immobility in mice TST. Each column represents mean duration of immobility (s). The error bar indicates S.E.M. #P<0.05 when compared with vehicle-treated control group; n = 8/group.

5.4.4. Evaluation of Q-21 for Antidepressant-like Potential in OBX Rats Using Behavioral and Biochemical Test Battery

5.4.4.1. Behavioral Analysis

5.4.4.1.1. Effect of OBX Surgery and Q-21 on Rats Body Weight

Body weight of sham and OBX rats was continuously observed till the behavioral tests were started. Two way ANOVA revealed that after the surgery, OBX rats gained lesser body weight as compared to sham rats **(table 41).** Although, there was no significant difference observed in weight gain between drug treated OBX rats and OBX control rats.

Table 41. Effect of Q-21 and OBX on body weight

Groups	Dose (mg/kg)	Initial weight (0^{th} day)	Final weight (28^{th} day)
Sham control	0	251.50±6.50	283.0±5.70
Sham + Q-21	1	242.00±9.25	281.0±11.21
Sham + Q-21	2	259.00±9.32	290.50±8.12
Sham + FLX	10	251.50±3.50	280.50±10.5
OBX control	0	247.76±6.77	260.21±9.22[a]
OBX + Q-21	0.5	244.0±7.33	269.32±7.52
OBX + Q-21	1	253.0±5.72	270.0±8.08
OBX + Q-21	2	247.67±8.84	274.33±14.10
OBX + FLX	10	260.67±8.43	280.67±7.76

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control; n = 6/group.

5.4.4.1.2. Effect of Q-21 on OBX/Sham Rats Behavior in OFT

The rats that had undergone OBX procedure exhibited increase in ambulation scores, rearing number and fecal pellet counts compared with sham control rats. **Table 42** shows that the OBX-induced alterations in ambulation [$F_{(8,45)} = 21.78$, $P<0.05$], rearing [$F_{(8,45)} = 18.65$, $P<0.05$] & fecal pellets [$F_{(8,45)} = 8.67$, $P<0.05$] were markedly reversed after 14 days of repeated Q-21 (1 & 2 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) administration. No profound effect of Q-21 at lower dose was observed on the OBX-induced hyperactivity. In addition, repeated Q-21 and FLX did not alter the behavior of sham-operated rats.

Table 42. Effect of Q-21 treatment on behavior of OBX/sham rats in OFT

Groups	Dose (mg/kg)	Ambulation	Rearing	Fecal Pellet
Sham control	0	113.14±7.46	15.8±2.92	2.28±0.24
Sham + Q-21	1	118.27±4.75	11.75±3.16	2.05±0.47
Sham + Q-21	2	109.20±11.7	15.50±1.42	1.30±0.65
Sham + FLX	10	99.67±9.31	8.67±0.97	2.00±0.70
OBX control	0	188.22±12.26[a]	46.37±4.33[a]	7.10±0.44[a]
OBX + Q-21	0.5	168.33±13.64	32.33±2.85	4.33±2.19
OBX + Q-21	1	149.0±4.08[b]	29.0±2.45[b]	5.87±0.22[b]
OBX + Q-21	2	127.67±12.02[b]	22.67±2.96[b]	5.09±0.53[b]
OBX + FLX	10	134.75±10.65[b]	24.57±7.25[b]	2.40±0.68[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]P<0.05 compared with sham control, [b]P<0.05 compared with OBX control; n = 6/group.

5.4.4.1.3. Effect of Q-21 on Sucrose Consumption in OBX/Sham Rats

The sucrose preference test is an indicator of anhedonia-like behavioral change. In this test, we found that OBX rats consumed notably less sucrose solution (P<0.05) compared with sham-operated group. Treatment with Q-21 (1 & 2 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) remarkably increased the percentage of sucrose consumption [$F_{(8,45)}$ = 34.13, P<0.05] in OBX rats as compared to OBX control group. No alteration in sucrose intake was observed in normal control, treated with Q-21 and FLX **(fig. 48).**

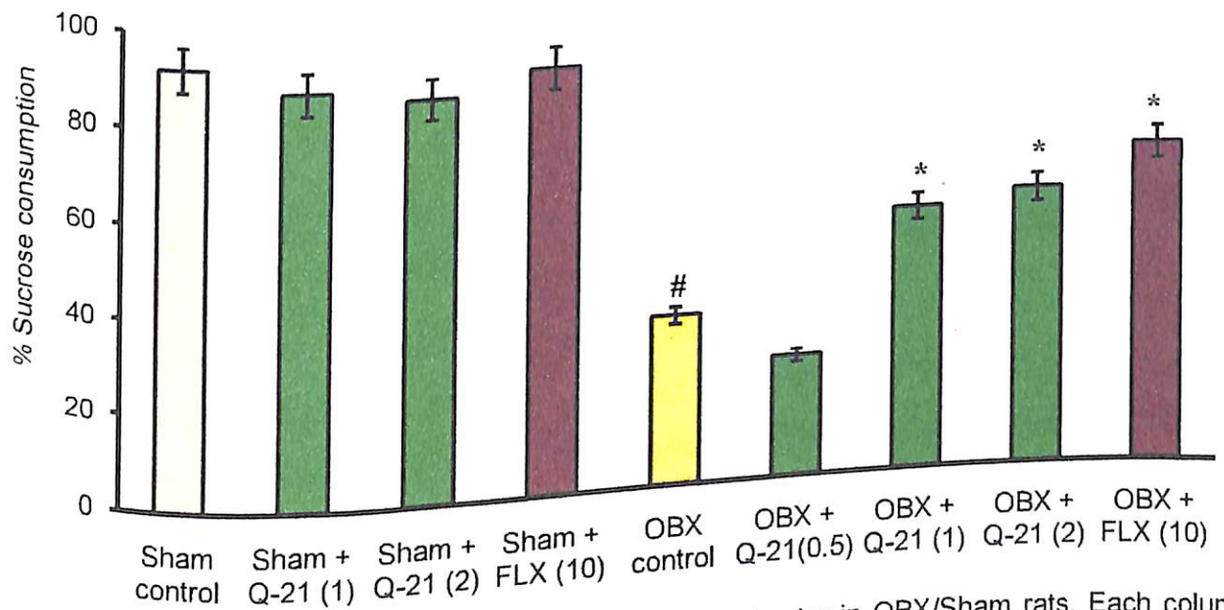


Fig. 48. Effect of Q-21 and FLX treatment on anhedonia behavior in OBX/Sham rats. Each column represents mean percentage sucrose consumption. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with OBX control; n = 6/group.

5.4.4.1.4. Effect of Q-21 on OBX/Sham Rats Behavior in Hyper-emotionality Test

Mean scores for hyper-emotionality in sham and OBX rats are shown in **fig. 49.** OBX rats showed an enhanced emotional behavior as compared to sham-operated rats. Such an effect was markedly [$F_{(8,45)} = 9.35$, $P<0.05$] reduced by chronic Q-21 (1 and 2 mg/kg) and FLX treatment when compared to vehicle treatment **(fig. 49).** The drugs treatment did not notably influence the behavior of sham operated rats.

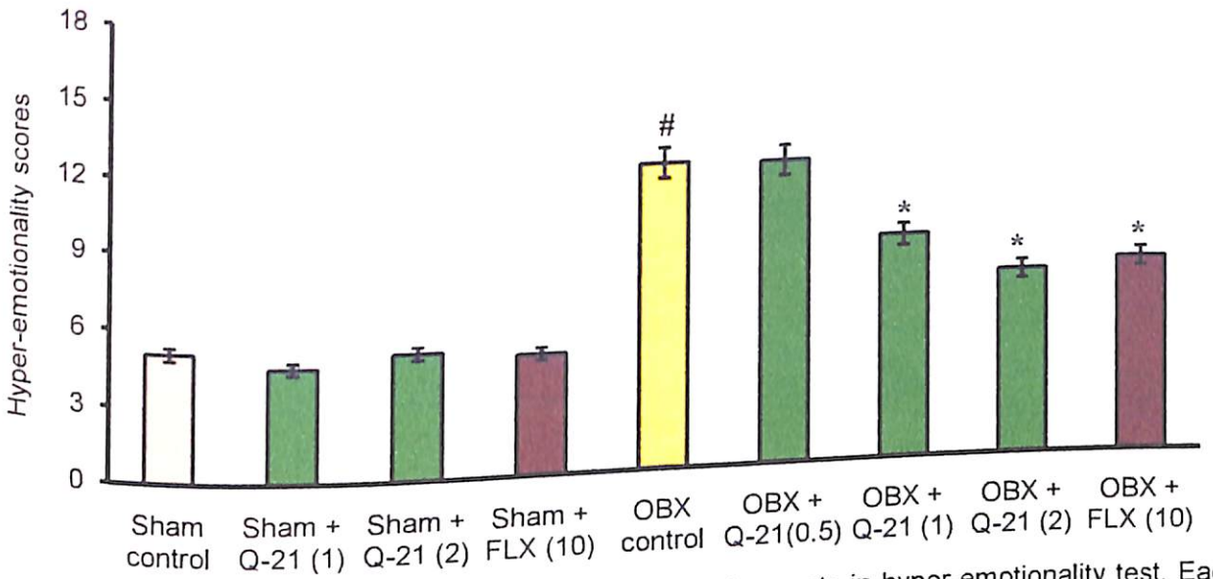


Fig. 49. Effects of Q-21 and FLX on the behavior of OBX/Sham rats in hyper-emotionality test. Each column represents mean hyper-emotionality scores. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with OBX control; n = 6/group.

5.4.4.2. Biochemical Estimation

5.4.4.2.1. Effect of Q-21 on Neuroendocrine Hormone (CORT) Level in OBX Model

A marked elevation in the serum CORT levels in OBX rats was noticed as compared to sham control group. Treatment with Q-21 (1 & 2 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) remarkably attenuated the increase in serum CORT [$F_{(8,45)} = 26.18$, $P<0.05$] level as compared to OBX control. Q-21 (0.5 mg/kg) did not show profound reduction in serum CORT levels **(fig. 50).**

5.4.4.2.2. Effect of Q-21 on cAMP, pCREB and BDNF Levels in OBX/Sham Rats

The effects of Q-21 and FLX treatments on cAMP, pCREB and BDNF levels in OBX/Sham rats are shown in **table 43.** OBX dramatically decreased the levels of cAMP, pCREB and BDNF as compared to sham control rats. Chronic treatment with Q-21 (1 & 2 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) remarkably increased the levels of cAMP [$F_{(8,45)} = 52.18$, $P<0.05$], pCREB [$F_{(8,45)} = 32.16$, $P<0.05$] and BDNF [$F_{(8,45)} = 19.28$, $P<0.05$] in OBX rats compared to OBX control group. Also, Q-21 (2 mg/kg) treatment showed notably increase in cAMP, pCREB and BDNF levels in sham group **(table 43).**

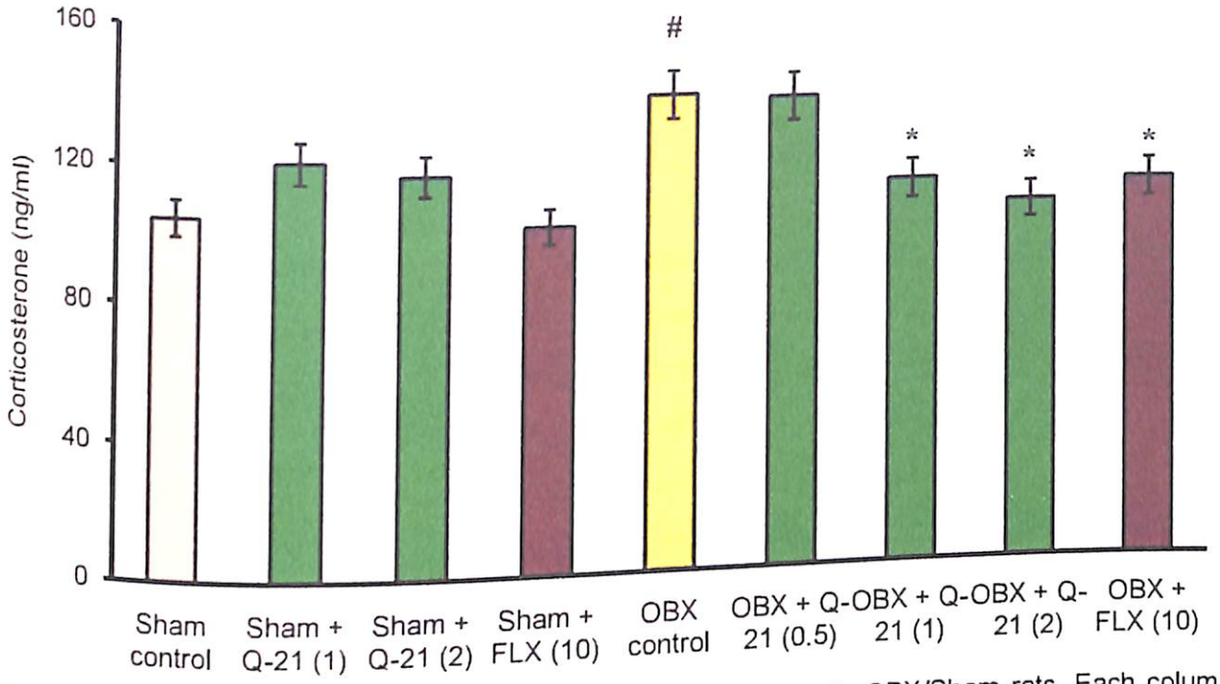


Fig. 50. Effect of Q-21 and FLX treatment on serum CORT level in OBX/Sham rats. Each column represents mean CORT level. The error bar indicates S.E.M, $^{\#}P<0.05$ when compared with sham control, $^{*}P<0.05$ when compared with OBX control; n = 6/group.

Table 43. Effect of Q-21 on cAMP, pCREB and BDNF levels in OBX/Sham rats

Groups	Dose (mg/kg)	cAMP (pmol/mg protein)	BDNF (ng/mg protein)	pCREB (% sham control)
Sham control	0	21.3±1.65	82.95±1.37	100.00±4.95
Sham + Q-21	1	26.32±2.98	90.32±2.14	107.21±7.21
Sham + Q-21	2	29.22±1.10[a]	102.34±5.31[a]	118.31±3.65[a]
Sham + FLX	10	24.76±1.33	89.77±6.21	112.18±6.13
OBX control	0	12.86±1.70[a]	31.60±2.68[a]	54.39±4.21[a]
OBX + Q-21	0.5	15.14±3.42	36.89±4.87	60.33±6.14
OBX + Q-21	1	20.32±1.33[b]	47.39±2.98[b]	69.23±6.37[b]
OBX + Q-21	2	26.88±1.25[b]	56.21±7.20[b]	87.09±5.23[b]
OBX + FLX	10	19.13±1.27[b]	48.17±7.25[b]	72.90±7.81[b]

All values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. $^{a}P<0.05$ compared with sham control, $^{b}P<0.05$ compared with OBX control group; n = 6/group.

5.4.4.2.3. Effect of Q-21 on Brain Neurotransmitters in OBX Model

5.4.4.2.3.1. Effect of Q-21 on 5HT, NE and DA Levels in OBX/Sham Rats

The monoamines levels measured in brain of OBX/sham rats are summarized in **table 44**.OBX markedly reduced the brain 5-HT [$F_{(8,45)} = 9.24$, $P<0.05$], NE [$F_{(8,45)} = 14.86$, $P<0.05$] and DA [$F_{(8,45)} = 20.21$, $P<0.05$] levels compared to sham controls rats. Chronic treatment with FLX notably restored 5-HT and NE levels in OBX rats as compared to OBX control **(table 44).** No changes in 5-HT, NE and DA levels in sham or OBX groups were observed after Q-21 treatment. FLX treatment marginally increased 5-HT levels in sham groups.

Table 44. Effect of Q-21 treatment on monoamines levels in OBX/Sham rats

Groups	Dose (mg/kg)	5HT (ng/g)	NE (ng/g)	DA (ng/g)
Sham control	0	540.33±21.67	489.56±11.67	381.38±21.55
Sham + Q-21	1	551.65±14.23	486.78±17.09	374.26±15.80
Sham + Q-21	2	546.18±18.34	475.67±21.32	369.87±26.53
Sham + FLX	10	557.23±13.65	496.14±15.98	370.65±17.19
OBX control	0	395.36±18.32[a]	225.33±18.48[a]	307.93±14.16[a]
OBX + Q-21	0.5	398.67±11.30	234.12±20.98	300.78±20.54
OBX + Q-21	1	387.67±15.22	226.37±12.45	321.78±20.54
OBX + Q-21	2	390.29±17.67	252.33±15.87	302.47±14.06
OBX + FLX	10	499.37±17.45[b]	310.13±15.27[b]	314.23±16.74

All values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control, [b]$P<0.05$ compared with OBX group; n = 6/group.

5.4.4.2.4. Effect of Q-21 on Oxidant/Anti-oxidant Markers in OBX Model

5.4.4.2.4.1. Effect of Q-21 on TBARS and Nitrite levels in OBX/Sham Rats

The levels of oxidative-nitrosative markers detected in brain of OBX/sham rats are summarized in **table 45**. OBX surgery resulted in a profound increase in TBARS and nitrite levels, as compared with sham controls. Q-21 (1 & 2 mg/kg, p.o.) and FLX (10 mg/kg, i.p.) administration noticeably reduced TBARS [$F_{(8,45)} = 10.34$, $P<0.05$] and nitrite [$F_{(8,45)} = 16.54$, $P<0.05$] levels in OBX rats as compared to vehicle controls. Q-21 at lower dose (0.5 mg/kg) did not notably influence the TBARS and nitrite levels in OBX rats **(table 45).**

5.4.4.2.4.2. Effect of Q-21 on Brain GSH, SOD and CAT Levels in OBX/Sham Rats

The anti-oxidant enzyme status of brain tissue was assessed by measurements of GSH, SOD and CAT levels. Brain GSH, SOD and CAT levels were decreased in OBX rats when compared with sham control group, indicative of impairment in anti-oxidant status. Treatment with Q-21 (1 & 2 mg/kg, p.o.) and FLX restored brain GSH [$F_{(8,45)} = 16.21$, $P<0.05$], SOD [$F_{(8,45)} = 8.32$, $P<0.05$] and CAT [$F_{(8,45)} = 19.39$, $P<0.05$] levels as compared to OBX control group **(table 45)**. Further, Q-21 lower dose (0.5mg/kg, p.o.) did not produce any effect on the anti-oxidant enzyme levels in OBX rats.

Table 45: Effect of Q-21 on oxidants/anti-oxidants markers in OBX/Sham rats

Groups (mg/kg)	TBARS (nmole/mg protein)	Nitrite/Nitrate (µmole/mg protein)	GSH (µmole/mg protein)	CAT (µmole H_2O_2/min/ mg protein)	%SOD activity
Sham control	3.52±0.89	4.01±0.33	0.225±0.05	2.97±0.08	100±7.67
Sham + Q-21 (1)	3.29±0.76	4.10±0.85	0.244±0.10	2.99±0.09	89±6.75
Sham + Q-21 (2)	3.70±0.45	4.44.±0.89	0.236±0.10	3.05±0.06	94.5±4.21
Sham + FLX (10)	3.65±1.10	4.29±0.67	0.229±0.07	3.01±0.06	91.78±5.5
OBX control	6.99±0.34[a]	5.99±0.37[a]	0.104±0.04[a]	0.87±0.04[a]	31.40±4.72[a]
OBX + Q-21 (0.5)	6.73±1.15	5.67±0.33	0.105±0.03	0.94±0.06	32.56±5.90
OBX + Q-21 (1)	4.59±0.69[b]	5.01±0.11[b]	0.136±0.05[b]	1.23±0.05[b]	57.97±2.59[b]
OBX + Q-21 (2)	2.67±1.36[b]	4.67±0.55[b]	0.181±0.07[b]	1.96±0.7[b]	56.47±2.06[b]
OBX + FLX (10)	4.04±0.92[b]	4.46±0.83[b]	0.167±0.08[b]	1.92±0.09[b]	61.05±4.67[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control, [b]$P<0.05$ compared with OBX control group; n = 6/group.

5.4.5. Evaluation of Q-21 for Anti-depressant-like Potential in TBI Model Using Behavioral and Biochemical Tests

5.4.5.1. Behavioral Analysis

5.4.5.1.1. Effect of Q-21 on TBI/Sham Rats Behavior in OFT

As shown in **table 46,** TBI resulted a profound increase in ambulation [$F_{(7,40)} = 34.15$, $P<0.05$], rearing [$F_{(7,40)} = 41.21$, $P<0.05$] and number of fecal pellets [$F_{(7,40)} = 3.61$, $P<0.05$] for 5 min after being put into the open field arena as compared to sham-operated rats **(table 46).** Increased frequencies of ambulation, rearing and fecal pellets were markedly ameliorated by Q-21 (1 and 2 mg/kg) and FLX (10 mg/kg) treatments. The ambulation, rearing and fecal pellets in sham group were not markedly affected by any treatment.

Table 46. Effect of Q-21 treatment on behavior of TBI/Sham rats in OFT

Groups	Dose (mg/kg)	Ambulation	Rearing	Fecal Pellet
Sham control	0	101.17±3.56	15.16±1.50	1.30±0.38
Sham + Q-21	1	108.67± 4.29	12.67±1.48	2.33±0.76
Sham + Q-21	2	105.17±3.90	14.50±1.34	2.08±0.45
Sham + FLX	10	108.12± 5.48	14.67±0.55	2.40± 0.58
TBI control	0	194.25±9.89[a]	51.5±4.69[a]	6.60±0.65[a]
TBI + Q-21	1	153.40±14.34[b]	40.20±3.71[b]	5.10±0.48[b]
TBI + Q-21	2	138.20±5.63[b]	32.20±4.50[b]	3.78±0.72[b]
TBI + FLX	10	147.12±7.15[b]	35.25±6.06[b]	3.05±0.51[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]P<0.05 compared with sham control, [b]P<0.05 vs. TBI control; n = 6/group.

5.4.5.1.2. Effect of Q-21 on Sucrose Consumption in TBI/Sham Rats

As shown in **fig. 51**, OBX rats showed marked reduction in sucrose consumption as compared to sham-treated rats. Repeated Q-21 (1 & 2 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) administration remarkably (P<0.05) increased sucrose consumption [$F_{(7,40)}$ = 14.78, P<0.05] in TBI rats as compared to TBI control group. The amount of sucrose consumption in sham group was not remarkably affected by any of the treatment **(fig. 51).**

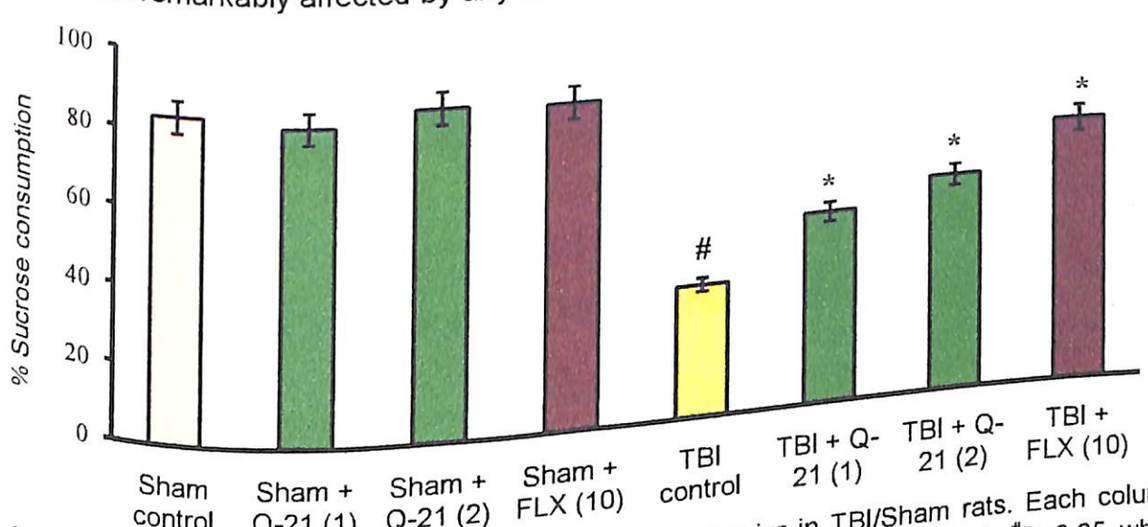


Fig. 51. Effect of Q-21 and FLX treatment on anhedonia behavior in TBI/Sham rats. Each column represents mean percentage sucrose consumption. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with TBI control; n = 6/group.

5.4.5.1.3. Effect of Q-21 on Behavior of TBI/Sham Rats in Hyper-emotionality Test

Removal of olfactory bulbs produced a profound increase in hyper-emotionality scores [$F_{(7,40)}$ =7.67, P<0.05], as compared to sham group **(fig. 52).** Treatment with Q-21 (1 and 2 mg/kg p.o.) and FLX (10 mg/kg p.o.) considerably (P<0.05) decreased the hyper-emotionality score

as compared to vehicle-treated TBI group. No marked difference on hyper-emotionality scores was observed in drug treated sham rats.

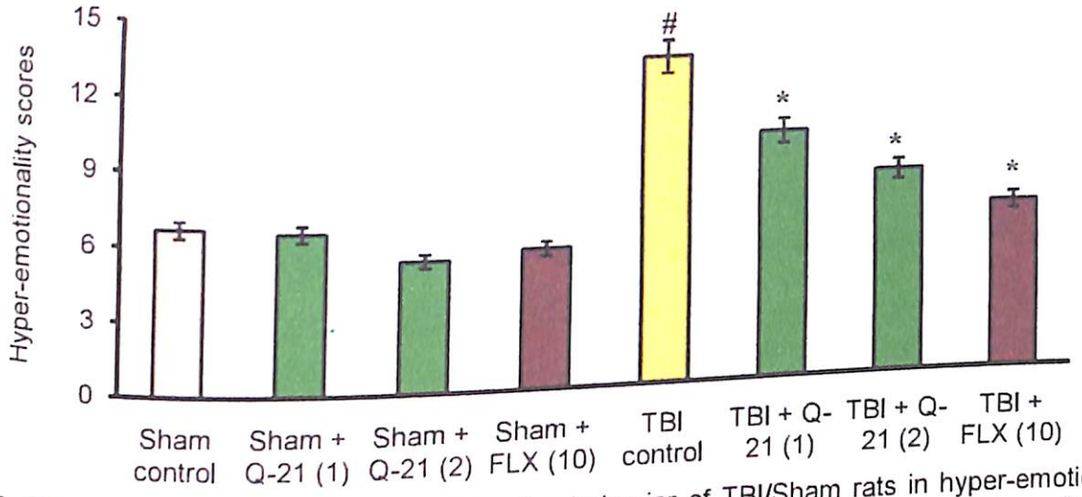


Fig. 52. Effects of Q-21 and FLX treatment on the behavior of TBI/Sham rats in hyper-emotionality test. Each column represents mean hyper-emotionality scores. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with TBI control; n = 6/group.

5.4.5.2. Biochemical Estimations

5.4.5.2.1. Effect of Q-21 on cAMP, pCREB and BDNF Levels in TBI/Sham Rats

TBI rats were observed to elicit a profound (P<0.05) reduction in cAMP, pCREB and BDNF levels as compared to sham control. Chronic Q-21 (1 & 2 mg/kg, p.o.) and FLX (10 mg/kg, p.o) treatment resulted, a pronounced increase in cAMP [$F_{(7,40)} = 22.58$, P<0.05], pCREB [$F_{(7,40)} = 9.33$, P<0.05] and BDNF [$F_{(7,40)} = 8.45$, P<0.05] levels in TBI rats as compared to TBI control group. Q-21 (2 mg/kg) treatment for 2 weeks also markedly increased cAMP, pCREB and BDNF levels in sham rats **(table 47).**

Table 47. Effect of Q-21 on cAMP, pCREB and BDNF levels in TBI/Sham rats

Groups	Dose (mg/kg)	cAMP (pmol/mg protein)	BDNF (ng/mg protein)	pCREB (% sham control)
Sham control	0	18.7±2.43	70.65±5.23	100.00±4.95
Sham + FLX	10	18.35±1.33	81.77±4.08	104.18±6.13
Sham + Q-21	1	21.26±3.67	78.12±3.89	110.09±5.33
Sham + Q-21	2	27.42±3.05[a]	89.23±6.25[a]	141.33±6.09[a]
TBI control	0	5.12±0.86[a]	20.72±4.87[a]	38.11±3.26[a]
TBI + Q-21	1	15.67±3.19[b]	42.15±3.12[b]	63.94±3.08[b]
TBI + Q-21	2	19.11±1.90[b]	57.87±1.65[b]	82.58±6.21[b]
TBI + FLX	10	14.50±0.66[b]	51.24±3.12[b]	59.58±5.54[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]P<0.05 compared with sham control, [b]P<0.05 compared with TBI control; n = 6/ group.

5.4.5.2.2. Effect of Q-21 on Oxidant/Anti-oxidant Markers in TBI Model

5.4.5.2.2.1. Effect of Q-21 on brain TBARS and nitrite levels in TBI/Sham Rats

Statistical analysis exhibited a remarkable difference among groups for lipid peroxidation [$F_{(7,40)} = 7.12$, $P<0.05$] and nitrite levels [$F_{(7,40)} = 4.42$, $P<0.05$]. The TBARS and nitrite levels in brain of TBI rats were predominantly increased, when compared with those of the sham control group (**table 48**). Q-21 (1 & 2 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatment for a period of 2 weeks produced a marked ($P<0.05$) reduction in the brain TBARS and nitrite levels as compared to TBI control group (**table 48**).

5.4.5.2.2.2. Effect of Q-21 on Brain GSH, SOD and CAT Levels in TBI/Sham Rats

TBI procedure resulted impairment in anti-oxidant enzymes activity, as evidenced by decrease in GSH, SOD and CAT levels compared to sham operated group. Administration of Q-21 (1 & 2 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) restored brain GSH [$F_{(7,40)} = 10.92$, $P<0.05$], SOD [$F_{(7,40)} = 14.87$, $P<0.05$] and CAT [$F_{(7,40)} = 19.03$, $P<0.05$] levels as compared to TBI control group. The drug treatments did not influence the anti-oxidant enzymes levels of sham operated rats (**table 48**).

Table 48: Effect of Q-21 treatment on oxidant/anti-oxidant markers in TBI rats

Groups (mg/kg)	TBARS (nmole/mg protein)	Nitrite/ Nitrate (µmole/mg) protein)	GSH (µmole/mg protein)	CAT (µmole H_2O_2/min/ mg protein)	%SOD activity
Sham control	0.76±0.61	1.08±0.12	0.278±0.53	5.34±1.12	100±4.29
Sham + Q-21 (1)	0.80±0.67	0.99±0.18	0.257±0.05	4.88±0.67	87.23±6.35
Sham + Q-21 (2)	0.73±0.91	0.95±0.12	0.271±0.07	4.76±0.81	89.24±4.33
Sham + FLX (10)	0.72±0.55	1.03±0.37	0.285±0.71	5.20±0.99	96.40±2.77
TBI control	2.16±0.51[a]	3.85±0.31[a]	0.118 ±0.18[a]	3.26±0.56[a]	51.57±4.65[a]
TBI + Q-21 (1)	1.62±0.23[b]	2.77±0.39[b]	0.175±0.06[b]	4.19±0.24[b]	61.11±3.23
TBI + Q-21 (2)	1.47±1.03[b]	2.27±0.46	0.215±0.03[b]	5.42±0.47[b]	64.95±3.67[b]
TBI + FLX (10)	1.25±0.12[b]	1.44±0.97[b]	0.227±0.33[b]	5.12±1.12[b]	78.12±5.15[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control, [b]$P<0.05$ vs. TBI control group; n = 6/group.

5.4.6. Evaluation of Q-21 for Antidepressant-like Potential in CUMS Model Using Behavioral and Biochemical Tests

5.4.6.1. Behavioral Analysis

5.4.6.1.1. Effect of CUMS and Q-21 on Mice Body Weight

The effects of CUMS and drugs treatment on body weight are summarized in **table 49.** No difference in the initial body weight was observed in any experimental group. Two-way ANOVA revealed that CUMS subjected mice gained less body weight than non-stressed mice. After a period of 28 days of CUMS pronounced decrease in body weight [$F_{(1,45)}$ = 36.18, $P<0.05$] was found as compared to non-stressed mice. Q-21 (0.5-2 mg/kg) and FLX (20 mg/kg, p.o.) treatments did not produce pronounced difference in body weight as compared to vehicle treated stressed mice **(table 49).**

Table 49. Effect of CUMS and Q-21 on mice body weight

Group	Dose	Body weight (g)	
		Initial (0^{th} day)	Final (28^{th} day)
Normal control	0	22.4±0.24	27.20±0.86
Control + Q-21	1	23.17±0.33	29.21±1.122
Control + Q-21	2	23.17±0.33	29.21±1.122
Control + FLX	20	21.99±0.97	29.34±0.55
CUMS control	0	23.0±0.32	23.00±1.01[a]
CUMS + Q-21	0.5	23.0±0.41	21.00±0.77
CUMS + Q-21	1	25.80±0.53	22.60±2.73
CUMS + Q-21	2	25.0±0.55	24.00±1.58
CUMS + FLX	20	24.0±1.05	25.76±1.38

All values are presented as mean ± S.E.M. [a]$P<0.05$ vs. normal control group; n = 6/group.

5.4.6.1.2. Effect of Q-21 on Stressed/Unstressed Mice Behavior in FST

The mice subjected to CUMS showed profound ($P<0.05$) increase in duration of immobility compared to normal control mice. Treatment of stressed mice with Q-21 (1 & 2 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) for a period of 3 weeks, resulted, a remarkable decrease in immobility duration [$F_{(8,45)}$ = 9.42, $P<0.05$] as compared to stress control **(fig. 53).** No alteration in the duration of immobility was observed in Q-21 (0.5 mg/kg) treated stressed mice. Q-21 (1 & 2 mg/kg) also significantly ($P<0.05$) decreased the duration of immobility in normal control mice.

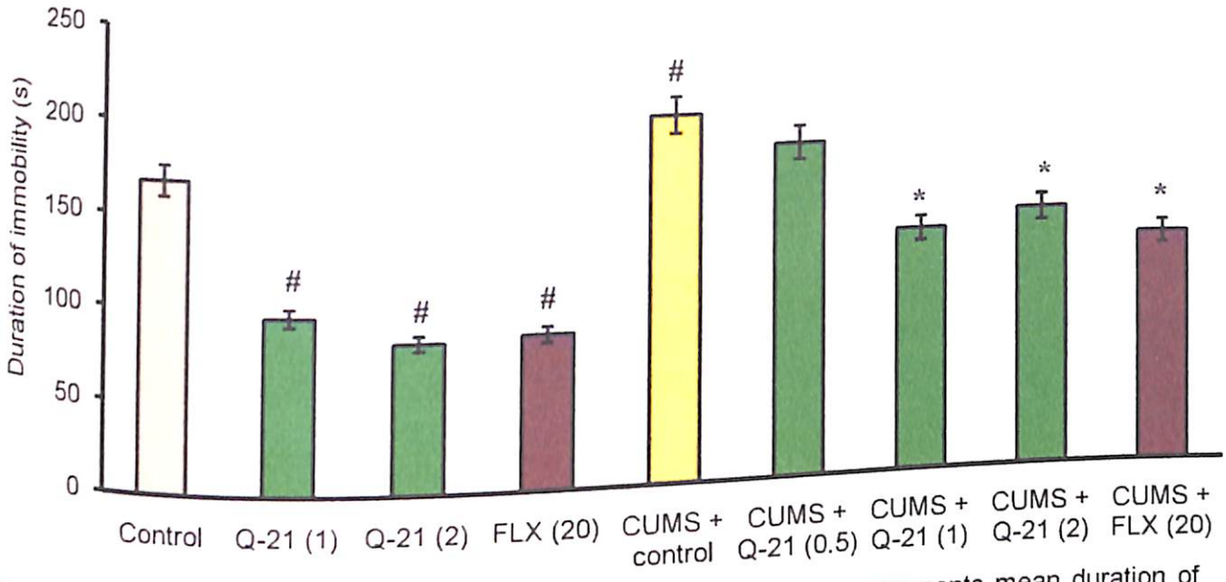


Fig. 53. Effect of Q-21 and FLX treatment in mice FST. Each column represents mean duration of immobility (s). The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 when compared with stress control group; n = 6/group.

5.4.6.1.3. Effect of Q-21 on Stressed/Unstressed Mice Behavior in TST

As depicted in **fig. 54** stressed mice developed a marked behavioral despair in TST, as evidenced by increase in duration of immobility compared to normal control. Treatment with Q-21 (1 & 2 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) decreased the immobility duration [$F_{(8,45)}$ = 29.47, P<0.05] in stressed mice as compared to stress control **(fig. 54).** Similarly, the normal control mice received Q-21 (1 & 2 mg/kg, p.o.) treatment, also exhibited considerably reduction in the duration of immobility. Lower dose of Q-21 (0.5 mg/kg) did not show any profound effect on the duration of immobility in stressed mice.

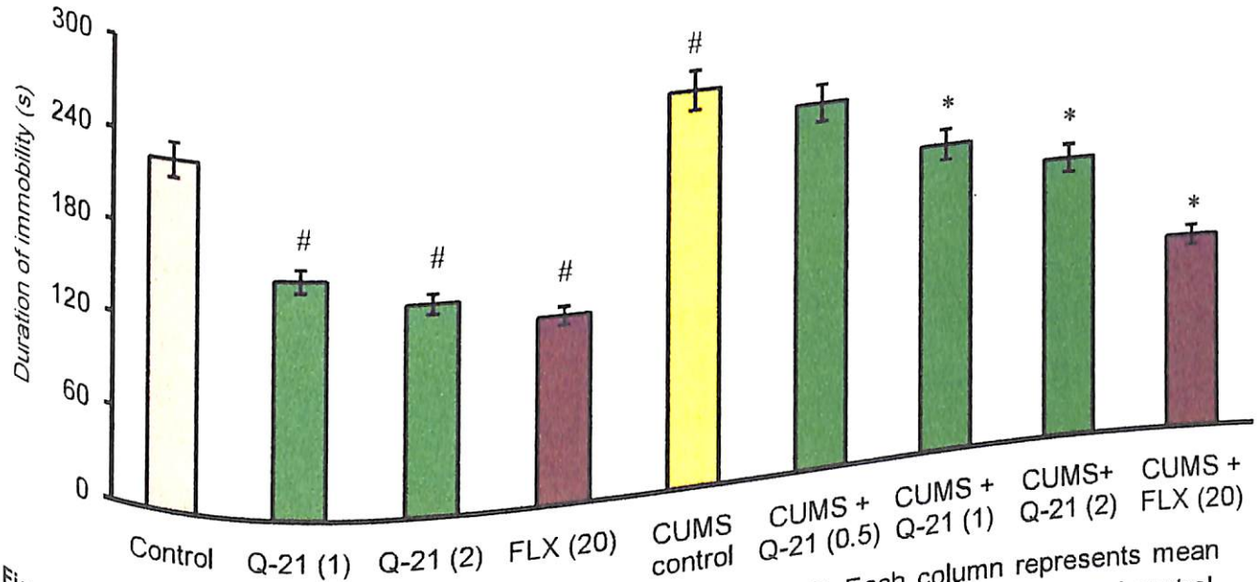


Fig. 54. Effects of Q-21 and FLX on duration of immobility in TST. Each column represents mean duration of immobility (s). The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 when compared with stress control group; n = 6/group.

5.4.6.1.4. Effect of Q-21 on Sucrose Consumption in Stressed/Unstressed Mice

The effect of Q-21 and FLX treatment on sucrose consumption is shown in **fig. 55.** CUMS-subjected mice displayed a noticeable ($P<0.05$) reduction in sucrose consumption than normal control mice. Administration of Q-21 (1 & 2 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) remarkably [$F_{(8,45)} = 17.74$, $P<0.05$] restored the decrease in percentage of sucrose consumption in stressed mice than vehicle treated stressed group. Treatment-induced changes in sucrose consumption were not observed in normal control mice. The lower dose of Q-21 (0.5 mg/kg) was failed to attain the level of significance **(fig. 55).**

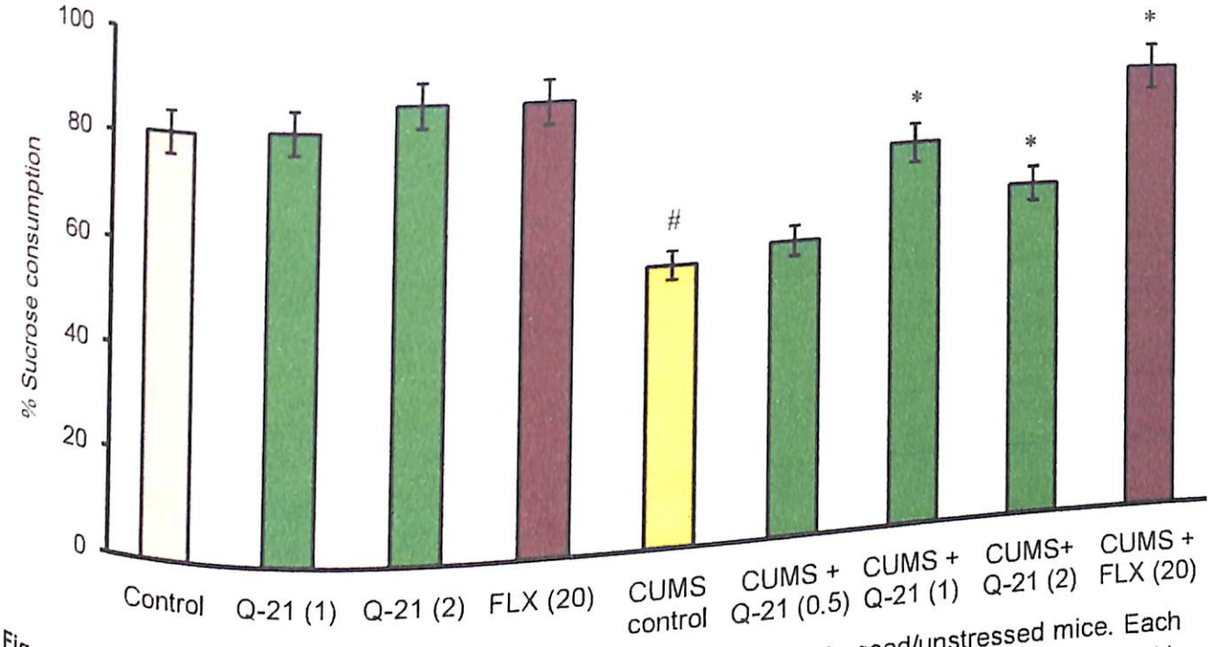


Fig. 55. Effect of Q-21 and FLX treatment on anhedonia behavior in stressed/unstressed mice. Each column represents sucrose preference. The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 when compared with stressed control; *n= 6/group.*

5.4.6.2. Biochemical Analysis

5.4.6.2.1. Effect of Q-21 on Neuroendocrine Hormone Level in CUMS model

5.4.6.2.1.1. Effect of Q-21 on Serum CORT Level in Stressed/Unstressed Mice

One-way ANOVA showed a profound difference among various groups for CORT [$F_{(8,45)} = 24.33$, $P<0.05$] levels. Post-hoc test indicated that stressed mice showed notably ($P<0.05$) elevation in serum CORT level as compared to normal control mice **(fig. 56).** Chronic treatment with Q-21 (1 & 2 mg/kg, p.o.) and FLX remarkably attenuated the increase in serum CORT level as compared to vehicle treated stressed mice. No pronounced change in CORT level of stressed mice was found at lower dose of Q-21.

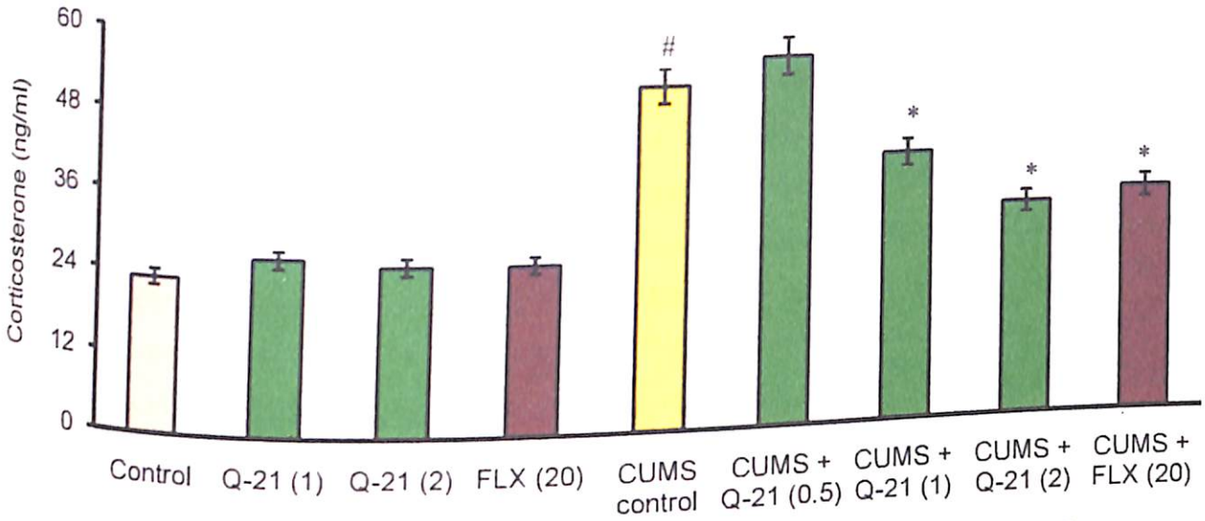


Fig. 56. Effects of Q-21 and FLX on serum CORT level. The error bar indicates S.E.M. #P<0.05 as compared to normal control, *P<0.05 as compared to vehicle treated stresses group; n = 6/group.

5.4.6.2.2. Effect of Q-21 on cAMP Signaling in CUMS model

5.4.6.2.2.1. Q-21 Effect on cAMP, pCREB and BDNF in Stressed/Unstressed Mice

Exposure to CUMS for a period of 28 days dramatically decreased the brain cAMP, CREB and BDNF levels compared to normal control mice **(table 50).** Q-21 (1 & 2 mg/kg, p.o.) and FLX (20 mg/kg, p.o) treated mice, exhibited a notable (P<0.05) elevation of cAMP [$F_{(8,45)}$ = 8.12, P<0.05], pCREB [$F_{(8,45)}$ = 22.90, P<0.05] and BDNF [$F_{(8,45)}$ = 24.46, P<0.05] levels as compared to the stressed control **(table 50).** Q-21 (2 mg/kg) treatment also had notable increased cAMP, pCREB and BDNF levels in normal control groups.

Table 50. Effect of Q-21 on cAMP, pCREB and BDNF levels in stressed/unstressed mice

Groups	Dose (mg/kg)	cAMP (pmol/mg protein)	BDNF (ng/mg protein)	pCREB (% normal control)
Normal control	0	103.25±5.87	11.33±0.99	100.00±3.56
Control + Q-21	1	104.11±4.67	14.91±0.55	109.32±5.99
Control + Q-21	2	119.09±5.33[a]	17.87±0.46[a]	125.07±2.67[a]
Control + FLX	20	95.34±12.05	13.12±1.17	101.12±10.13
CUMS control	0	68.43±6.46[a]	3.45.±087[a]	44.76±5.21[a]
CUMS + Q-21	0.5	71.35±2.77[b]	5.99±0.64[b]	58.43±6.35[b]
CUMS + Q-21	1	79.35±2.77[b]	6.82±0.37[b]	81.43±5.35[b]
CUMS + Q-21	2	81.22±4.38[b]	6.35±1.04[b]	62.59±3.67[b]
CUMS + FLX	10	81.22±4.38[b]	6.35±1.04[b]	62.59±3.67[b]

Results are presented as mean ± S.E.M, [a]P<0.05 vs. normal control group and [b]P<0.05 vs. vehicle treated stress control group; n = 6/group.

5.4.6.2.3. Effect of Q-21 on Neurotransmitter Levels in CUMS Model

5.4.6.2.3.1. Effect of Q-21 on 5-HT, NE and DA Levels in Stressed/Unstressed Mice

Table 51 shows the influence of Q-21 and FLX treatment on the monoamine levels in stressed mice. Stressed mice showed pronounced decrease in 5-HT [$F_{(8,45)} = 22.56$, $P<0.05$], NE [$F_{(8,45)} = 18.47$, $P<0.05$] and DA [$F_{(8,45)} = 32.26$, $P<0.05$] levels as compared to the normal controls. Chronic FLX (10 mg/kg, p.o.) treatment restored decrease in the brain 5-HT and NE levels in stressed mice as compared to vehicle treated stressed mice **(table 51).** Administration of Q-21 (0.5-2 mg/kg, p.o.) did not affect monoamine levels in both stressed and unstressed mice.

Table 51. Effect of Q-21 on monoamines levels of stressed/unstressed mice

Groups	Dose (mg/kg)	5-HT (ng/g)	NE (ng/g)	DA (ng/g)
Normal control	0	410.47±16.67	310.33±15.75	284.12±25.20
Control + Q-21	1	402.96±10.85	311.45±12.44	299.75±11.91
Control + Q-21	2	406.23±09.33	306.59±15.34	280.28±15.67
Control + FLX	20	421.90±17.90	302.56±9.87	289.75±21.13
CUMS control	0	252.56±21.77[a]	157.13±16.15[a]	195.11±13.25[a]
CUMS + Q-21	0.5	244.87±15.23	160.67±13.10	200.77±8.67
CUMS + Q-21	1	245.67±10.78	164.33±9.14	205.54±6.42
CUMS + Q-21	2	258.22±22.90	168.67±19.57	208.33±14.24
CUMS + FLX	10	332.19±14.67[b]	182.67±13.87[b]	196.14±24.08

Results are presented as mean ± S.E.M, [a]$P<0.05$ vs. normal control group, [b]$P<0.05$ vs. vehicle treated stress control group; n = 6/group.

5.4.6.2.4. Effect of Q-21 on Oxidant/Anti-oxidant Markers in CUMS Model

5.4.6.2.4.1. Effect of Q-21 on TBARS and Nitrite Levels in Stressed/Unstressed Mice

The stressed group showed a remarkably increase in lipid peroxidation (measured in terms of TBARS) and nitrite levels compared to normal control **(table 52).** Q-21 (2 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) attenuated the increase in oxidative–nitrosative stress markers, as evidenced by reduce TBARS [$F_{(8,45)} = 67.35$, $P<0.05$] and nitrite [$F_{(8,45)} = 40.23$, $P<0.05$] levels in the stressed mice brain as compared to stress control group. No marked effect of Q-21 (0.5 & 1 mg/kg) was found on TBARS and nitrite levels in stressed mice.

5.4.6.2.4.2. Effect of Q-21 on GSH, SOD and CAT Levels in Stressed/Unstressed Mice

GSH, SOD and CAT levels were found to be predominantly depleted in brain of stressed mice compared to normal control mice **(table 52).** Chronic treatment with Q-21 (2 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) showed a notably increase in brain GSH [$F_{(8,45)} = 18.09$, $P<0.05$], SOD [$F_{(8,45)} = 4.12$, $P<0.05$] and CAT [$F_{(8,45)} = 7.39$, $P<0.05$] levels as compared to vehicle treated stress group. The effects of Q-21 lower doses (0.5 & 1 mg/kg) on anti-oxidant enzyme levels failed to reach the level of significance **(table 52).**

Table 52. Effect of Q-21 on oxidant/anti-oxidant markers in stressed/unstressed mice

Groups (mg/kg)	TBARS (nmole/mg protein)	Nitrite/Nitrate (µmole/mg protein)	GSH (µmole/mg protein)	CAT (µmole H_2O_2/min/ mg protein)	%SOD activity
Normal Control	1.63±0.05	12.36±1.32	0.14±0.02	1.34±0.09	100±2.32
Control + Q-21 (1)	1.50±0.02	13.99±2.33	0.15±0.04	1.27±0.18	95.7±4.42
Control + Q-21 (2)	1.67±0.56	15.27±2.03	0.15±0.05	1.36±0.45	90.7±4.42
Control + FLX (20)	1.72±0.03	11.07±2.01	0.14±0.02	1.21±0.06	96.3±4.12
CUMS control	2.96±0.04[a]	19.41±1.82[a]	0.08±0.02[a]	0.27±0.02[a]	25.85±9.19[a]
CUMS + Q-21 (0.5)	2.37±0.38	18.48±1.81	0.09±0.04	0.59±0.07	35.72±5.38
CUMS + Q-21 (1)	2.02±0.17[a]	15.67±2.67[a]	0.14±0.02[a]	1.01±0.05[a]	59.01±6.92[a]
CUMS + Q-21 (2)	1.33±0.21[b]	13.82±1.33[b]	0.16±0.02[b]	1.09±0.3[b]	69.51±2.44[b]
CUMS + FLX (20)	1.65±0.04[b]	15.52±1.26[b]	0.13±0.05[b]	1.12±0.05[b]	62.18±6.94[b]

Results are presented as mean ± S.E.M. [a]$P<0.05$ vs. normal control group, [b]$P<0.05$ vs. vehicle treated stress control group; n = 8/group.

5.4.7. Evaluation of Q-21 for Anti-depressant-like Potential in Chronic CORT-Injected Model Using Behavioral and Biochemical Tests

5.4.7.1. Behavioral Analysis

5.4.7.1.1. Effect of Q-21 on CORT-injected Mice Behavior in FST

Repeated CORT-injection for a period of 21 days predominantly increased the immobility time in FST [$F_{(7,40)} = 21.30$, $P<0.05$] as compared with normal control **(fig. 57).** Statistical analysis revealed that administration of Q-21 (1 & 2 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) markedly ($P<0.05$) decreased the immobility duration of CORT-treated mice as compared to vehicle treated CORT mice. Also, Q-21 (1 & 2 mg/kg) remarkably decreased the duration of immobility in normal control mice. Q-21 (0.5 mg/kg, p.o.) did not affect the immobility time in CORT-treated mice **(fig. 57).**

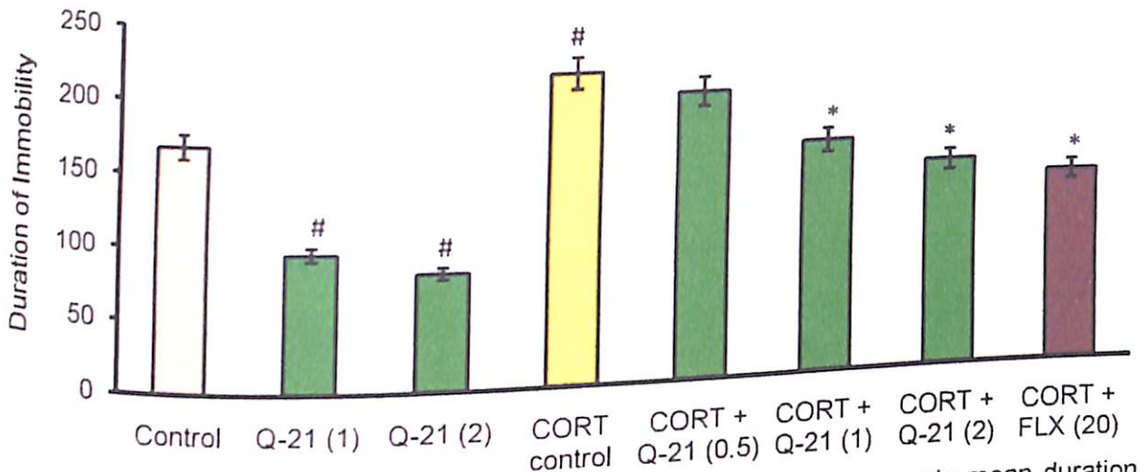


Fig. 57. Effect of Q-21 and FLX treatment in mice FST. Each column represents mean duration of immobility (s). The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 when compared with CORT control group; n = 6/group.

5.4.7.1.2. Effect of Q-21 on CORT-injected Mice Behavior in TST

Chronic CORT exposure produced a notable "depressed" phenotype, characterised by increase in duration of immobility in TST **(fig. 58).** Chronic treatment with Q-21 (1 & 2 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) remarkably (P<0.05) produced the reduction in immobility duration [$F_{(7,40)} = 8.45$, P<0.05] in CORT-injected mice as compared to CORT-control mice. Similarly, Q-21 (1 & 2 mg/kg) showed a pronounced decrease in the duration of immobility in normal control mice. Q-21 (0.5 mg/kg) was ineffective in reducing the duration of immobility in CORT-treated mice **(fig. 58).**

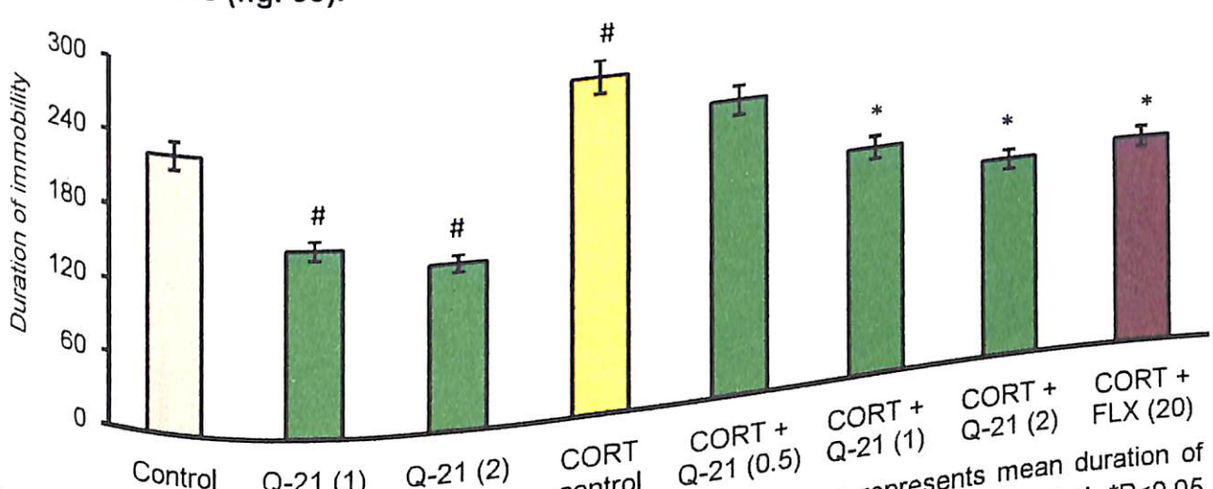


Fig. 58. Effect of Q-21 and FLX treatment in mice TST. Each column represents mean duration of immobility (s). The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 when compared with CORT control group; n = 6/group.

5.4.7.1.3. Effect of Q-21 on Sucrose Consumption in CORT-injected Mice

The effect of Q-21 treatment on the sucrose consumption is shown in **fig. 59.** In sucrose consumption test, we found that CORT-treated mice consumed remarkably (P<0.05) less

sucrose solution as compared with normal control. Chronic treatment with Q-21 (1 & 2 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) reversed the reduction of sucrose consumption [$F_{(7, 40)}$ = 34.87, P<0.05] to the baseline value. Q-21 (0.5 mg/kg) failed to reach the level of significance in sucrose consumption test **(fig. 59).**

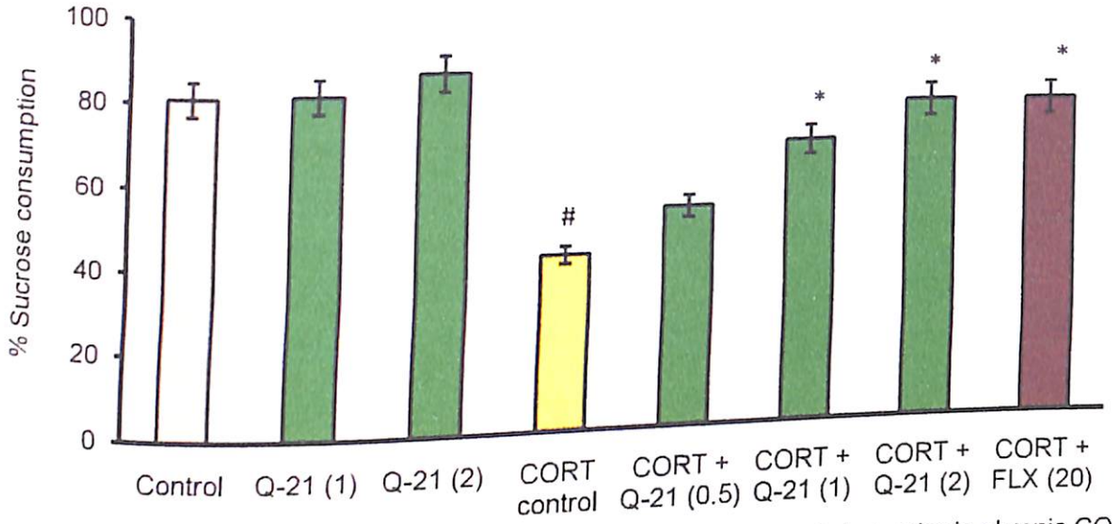


Fig. 59. Effect of Q-21 (0.5-2 mg/kg, p.o.) and FLX treatment on anhedonia behavior in chronic CORT-injected mice. The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 when compared with CORT control group; n = 6/group.

5.4.7.1.4. Effect of Q-21 on CORT-injected Mice Behavior in SLA Test

In the present study, there was a profound difference observed on the locomotor scores [$F_{(7, 40)}$ = 21.47, P<0.05] between the CORT control and normal control mice. Chronic treatment of Q-21 (1 & 2 mg/kg, p.o.) as well as FLX (20 mg/kg, p.o.) produced a marked change in locomotor scores in CORT-treated mice as compared to CORT control group **(fig. 60).**

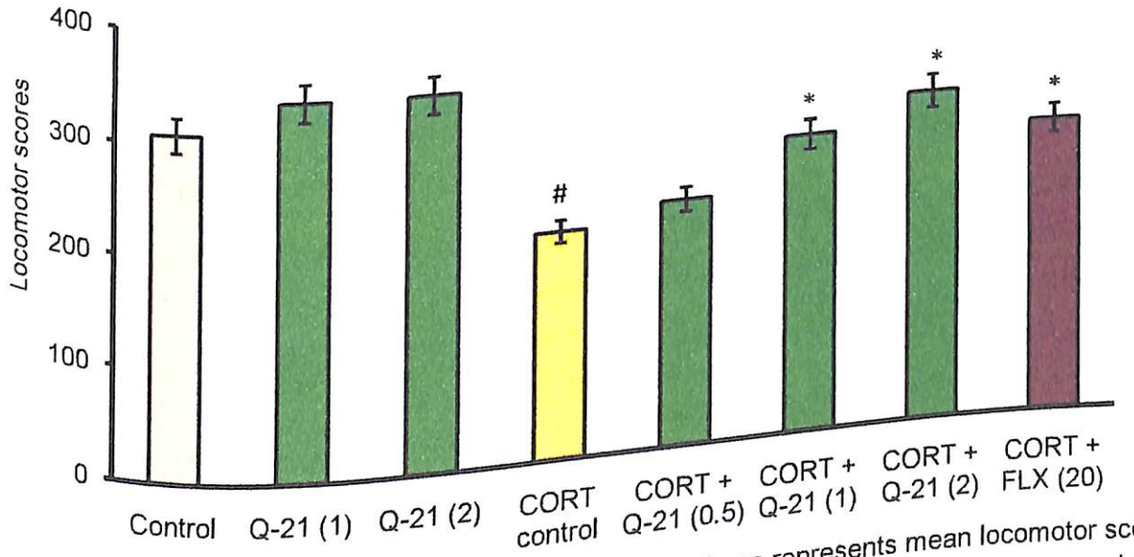


Fig. 60. Effect of Q-21 and FLX on locomotor scores. Each column represents mean locomotor scores recorded in 8 min observation period. The error bars indicate S.E.M. #P<0.05 when compared with normal control, *P<0.05 when compared with CORT control group; n = 6/group.

5.4.7.2. Biochemical Estimation

5.4.7.2.1. Effect of Q-21 on Serum CORT Level in CORT-injected Mice

The chronic CORT treatment caused a profound elevation of basal serum CORT [$F_{(7,40)}$ = 36.19, P<0.05] levels relative to the normal control group **(fig. 61).** Elevation in serum CORT level was significantly reduced in CORT mice that were treated with Q-21 (1 and 2 mg/kg, p.o.) and FLX (20 mg/kg, p.o.). Although, the reductions were robust, they were not sufficient to bring hormone levels down all the way to normal control baseline levels. Q-21 (0.5 mg/kg) did not show any notable effect on serum CORT in CORT-treated mice **(fig. 61).**

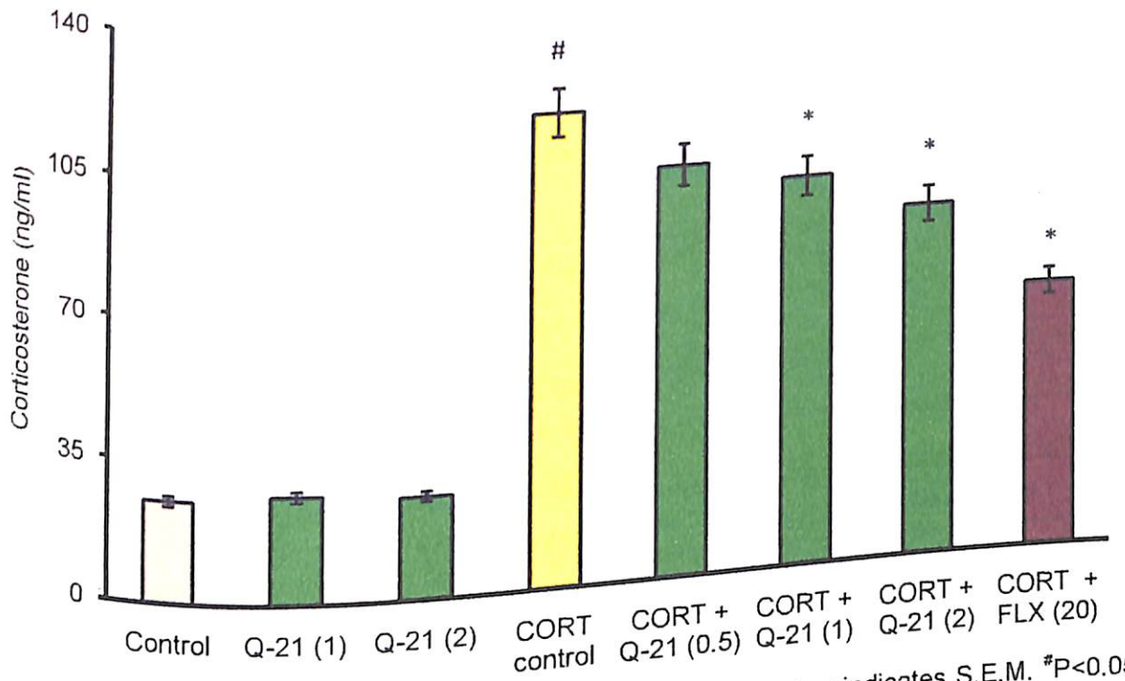


Fig. 61. Effects of Q-21 and FLX on serum CORT level. The error bar indicates S.E.M. #P<0.05 as compared to normal control, *P<0.05 as compared to CORT control group; n = 6/group.

5.4.7.2.2. Effect of Q-21 on cAMP, pCREB and BDNF Levels in CORT-injected Mice

Table 53 shows the effects of Q-21 (0.5-2 mg/kg, p.o.) and FLX (20 mg/kg, p.o.) treatment on the levels of cAMP, pCREB and BDNF in chronic CORT-treated mice. Brain cAMP, pCREB and BDNF levels were reduced following exposure of mice to CORT injection for 21 days. The reduction in cAMP [$F_{(7,40)}$ = 22.21, P<0.05], pCREB [$F_{(7,40)}$ = 7.12, P<0.05] and BDNF [$F_{(7,40)}$ = 18.35, P<0.05] levels was remarkably reversed by Q-21 (1 & 2 mg/kg, p.o.) and FLX treatment, when compared to vehicle treatment. Q-21 (2 mg/kg) also increased cAMP, pCREB and BDNF levels in normal control mice **(table 53).**

Table 53. Effect of Q-21 on cAMP, pCREB and BDNF levels in CORT-treated mice

Groups	Dose (mg/kg)	cAMP (pmol/mg protein)	BDNF (ng/mg protein)	pCREB (% normal control
Normal control	0	103.25±5.87	11.33±0.99	100±3.56
Control + Q-21	1	104.11±4.67	14.91±0.55	109.32±5.99
Control + Q-21	2	119.09±5.33[a]	17.87±0.46[a]	125.07±2.67[a]
CORT control	0	46.67±9.34[a]	2.15±0.33[a]	33.33±5.21[a]
CORT + Q-21	0.5	50.13±9.78	2.97±0.35	40.28±4.82
CORT + Q-21	1	78.42±3.70[b]	4.18±0.29[b]	60.95±2.07[b]
CORT + Q-21	2	91.24±3.39[b]	6.24±0.52[b]	81.36±4.52[b]
CORT + FLX	20	67.23±6.33[b]	5.12±2.11[b]	49.67±6.17[b]

All values represent mean ± S.E.M. [a]P<0.05 when compared with normal control, [b]P<0.05 when compared with vehicle treated CORT-injected group; n = 6/group.

5.4.8. Evaluation of Anxiolytic-like Potential of Q-21 in Experimental Models

5.4.8.1. Effect of Q-21 on Behavior of Mice in EPM Test

Statistical analysis indicated that Q-21 evoked a profound effect on the percentage of both OAE [$F_{(4,35)}$ = 36.12, P<0.05] and TSOA [$F_{(4,35)}$ = 49.20, P<0.05] **(table 54).** Post-hoc comparisons revealed that Q-21 at doses of 1 & 2 mg/kg, but not at the lower dose (0.25 mg/kg) and DZM (2 mg/kg, i.p.), notably increased the percentage of both OAE and TSOA as compared to normal control. Neither, Q-21 nor DZM significantly changed the number of total arm entries, indicating no effect on locomotor activity.

Table 54: Effect of Q-21 on behavior of mice in EPM test

Groups	Dose (mg/kg)	No. of entries		% OAE	% TSOA
		open arm	closed arm		
Control	0	2.12±0.61	5.62±0.50	27.62±6.89	26.41±2.71
DZM	2	6.80±1.46[a]	4.40±0.87	60.15±2.56[a]	44.13±3.6[a]
Q-21	0.5	2.0±0.55	5.6±1.08	26.27±3.77	18.60±4.41
Q-21	1	3.20±0.79[a]	6.0±1.10	34.03±2.62[a]	26.27±2.03[a]
Q-21	2	4.01±0.67[a]	5.87±2.12	41.67±3.15[a]	35.09±4.22[a]

All values represent mean ± S.E.M. [a]P<0.05 when compared with normal control group; n = 6/group.

5.4.8.2. Effect of Q-21 on Behavior of Mice in L/D Aversion Test

Acute administration of Q-21 (1 & 2 mg/kg, i.p.) and DZM (2 mg/kg, i.p.) remarkably increased latency time to leave the light compartment [$F_{(4,35)}$ = 44.13, P<0.05], time spent in

light compartment [$F_{(4,35)}$ = 17.65, P<0.05] and number of crossings between the compartments [$F_{(4,35)}$ = 7.95, P<0.05] as compared to control group **(table 55).** No effect of Q-21 (0.5 mg/kg) was observed on any of the parameters in L/D aversion test.

Table 55: Effect of Q-21 on behavior of mice in L/D aversion test

Groups	Dose (mg/kg)	Latency to leave light box (s)	No. of crossing	Time spent in light box (s)
Control	0	19.43±2.11	12.67±1.47	80.14±6.34
DZM	2	73.83±5.43[a]	16.18±1.40[a]	164.84±11.06[a]
Q-21	0.5	20.60±6.22	14.60±1.12[a]	86.67±11.33
Q-21	1	45.0±6.63[a]	15.0±1.75[a]	101.0±8.94[a]
Q-21	2	57.5±9.15[a]	13.0±2.43[a]	115.40±12.36[a]

All values represent mean ± S.E.M. [a]P<0.05 when compared with normal control group; n = 6/group.

5.5. Evaluation of Q-12 for Antidepressant-like Potential Using rodent's Models

5.5.1. Effect of Q-12 on Mice Locomotor Scores in SLA test

Acute treatment of Q-12 (0.25-2mg/kg i.p.) did not predominantly influence the locomotor scores at tested dose **(fig. 62).**

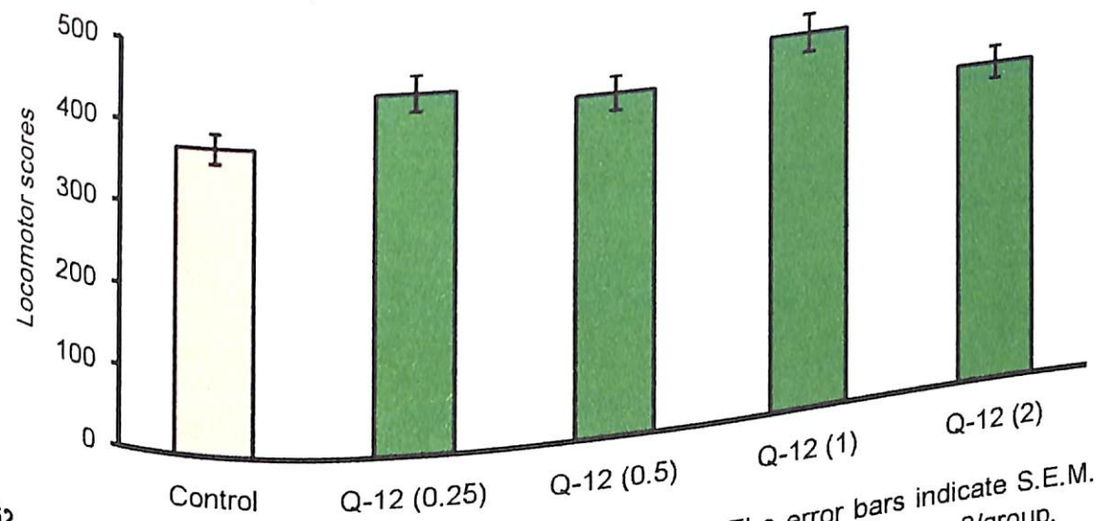
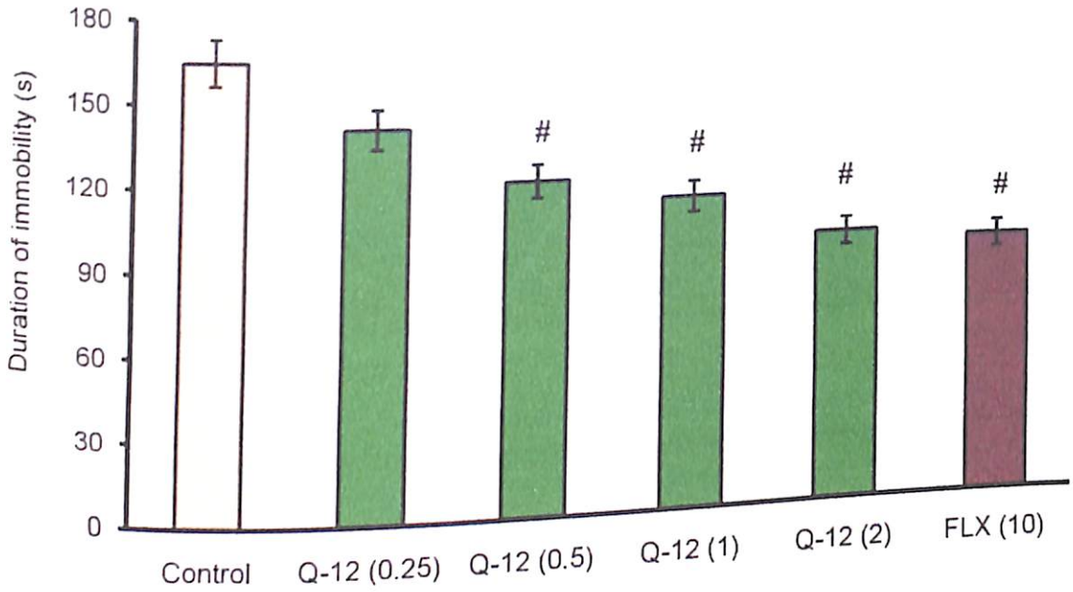


Fig. 62. Effect of Q-12 treatment on locomotor scores in mice. The error bars indicate S.E.M. Each column represents mean locomotor scores recorded in 8 min observation period; n = 8/group.

5.5.2. Effect of Q-12 on Mice Behavior in FST

Acute treatment of Q-12 (0.5-2 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) showed profound decrease in duration of immobility [$F_{(5,42)}$ = 23.67, P<0.05] and increase in swimming episodes [$F_{(5,42)}$ = 40.33, P<0.05] as compared to control group **(fig. 63A & B).** The AD-like effects were not evident at the lower dose level of Q-12 (0.25 mg/kg) in FST.

63A. Duration of immobility



63B. Swimming episode

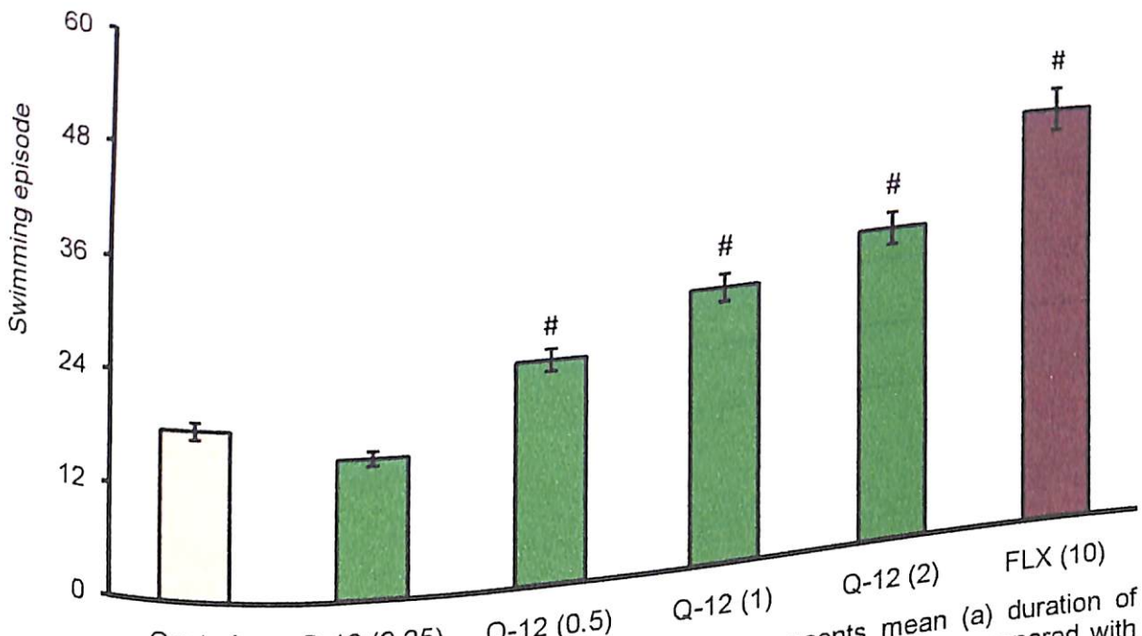


Fig. 63. Effect of Q-12 treatment in mice FST. Each column represents mean (a) duration of immobility and (b) swimming episode. The error bar indicates S.E.M. $^{\#}P<0.05$ when compared with normal control group; n = 8/group.

5.5.3. Effect of Q-12 on Mice Behavior in TST

Following acute administration of Q-12, the duration of immobility was scored in TST **(fig. 64)**. Q-12 (1 & 2 mg/kg, i.p.) and positive control, BUP (20 mg/kg, i.p.) treatments remarkably [$F_{(5,42)} = 29.57$, $P<0.05$] decreased the duration of immobility in mice TST as compared to normal control group **(fig. 64)**. Lower doses of Q-12 (0.25 & 0.5 mg/kg) did not show any statistically profound change in the duration of immobility.

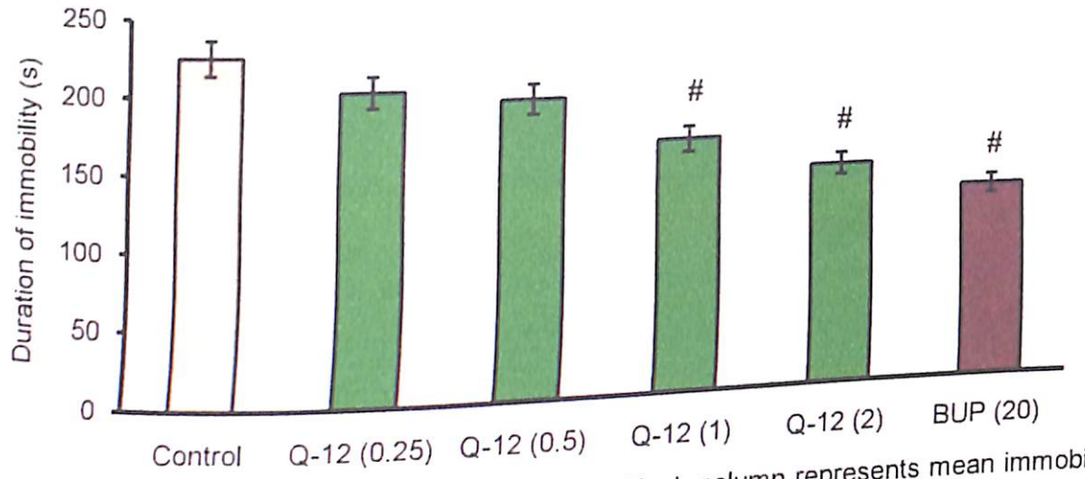


Fig. 64. Effect of Q-12 on immobility duration in mice TST. Each column represents mean immobility duration (s). Error bar indicates S.E.M. $^{\#}P<0.05$ as compared with normal control group; n = 8/group.

5.5.4. Evaluation of Q-12 for Anti-depressant-like Potential in OBX Model Using Behavioral and Biochemical Tests

5.5.4.1. Behavioral Analysis

5.5.4.1.1. Effect of Q-12 on OBX/Sham Rats Behavior in OFT

For screening in OBX model, the dose levels of Q-12 were selected based on data obtained from the FST, TST and pilot study. OBX rats exhibited increase in ambulation, rearing and defecation behaviors for 5 min after being put into open field arena **(table 56).** The results of this study, demonstrated that increased ambulation [$F_{(8,45)} = 31.89$, $P<0.05$], rearing [$F_{(8,45)} = 6.46$, $P<0.05$] and defecation [$F_{(8,45)} = 2.20$, $P<0.05$] behaviors were predominantly reversed by chronic Q-12 (2 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) treatment. The behaviors of OBX rats in open field arena were not significantly affected by Q-12 lower doses (0.5 & 1 mg/kg).

Table 56. Effect of Q-12 on behavior of OBX/Sham rats in OFT

Groups	Dose (mg/kg)	Ambulation	Rearing	Fecal Pellet
Sham control	0	113.14±7.46	15.8±2.92	1.78±0.24
Sham + Q-12	1	110.07± 3.99	12.50 ±2.13	2.10 ±0.56
Sham + Q-12	2	100.20±9.3	16.67±2.27	2.33±0.65
Sham + FLX	10	99.67±9.31	8.67±0.97	2.00±0.70
OBX control	0	188.22±12.26[a]	46.37±4.33[a]	7.10±0.44[a]
OBX + Q-12	0.5	180.50±10.40	39.75±8.64	6.35±1.25
OBX + Q-12	1	169.0±18.19	36.75±4.12	5.00±1.25
OBX + Q-12	2	141.67±13.86[b]	25.33±3.38[b]	3.67±2.03[b]
OBX + FLX	10	134.75±10.65[b]	24.57±7.25[b]	2.40±0.68[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]$P<0.05$ compared with sham control, [b]$P<0.05$ compared with OBX group; n = 6/group.

5.5.4.1.2. Effect of Q-12 on Sucrose Consumption in OBX/Sham Rats

One-way ANOVA revealed a marked effect of groups for the percentage of sucrose consumption [$F_{(8,45)} = 31.70$, $P<0.05$]. OBX rats exhibited a pronounced reduction in percentage of sucrose consumption as compared to sham operated rats **(fig. 65).** Q-12 (2 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) noticeably ($P<0.05$) increased the percentage of sucrose consumption in OBX rats compared to OBX vehicle group. Q-12 (0.5 & 1 mg/kg, p.o.) could not improve the amount of sucrose consumption in OBX rats **(fig. 65).**

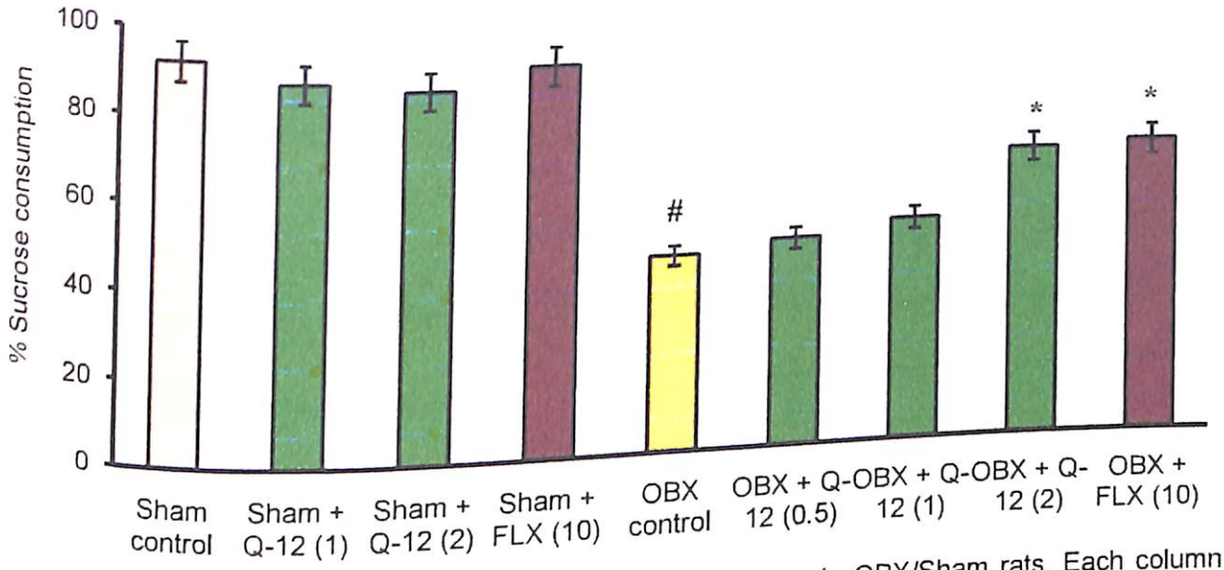


Fig. 65. Effect of Q-12 and FLX treatment on anhedonia behavior in OBX/Sham rats. Each column represents mean percentage sucrose consumption. The error bar indicates S.E.M. *P<0.05 when compared with sham control, #P<0.05 when compared with OBX control; n = 6/group.

5.5.4.1.3. Effect of Q-12 on Behavior of OBX/Sham Rats in Hyper-emotionality Test

One-way ANOVA showed a significant difference among groups for the hyper-emotionality scores [$F_{(8,45)} = 15.92$, $P<0.05$) **(fig. 66).** Post-hoc test indicated that OBX rats exhibited increase in emotional behavior as compared to sham control group. The mean hyper-emotional score was markedly reduced by Q-12 (2 mg/kg) and FLX (10 mg/kg) treatments. No effect on hyper-emotional scores in OBX rats was observed at doses of 0.5 & 1 mg/kg.

5.5.4.2. Biochemical Estimation

5.5.4.2.1. Effect of Q-12 on Serum CORT Level in OBX/Sham Rats

Fig. 67 shows the effect of Q-12 on serum CORT levels in OBX rats. OBX rats were observed to elicit a profound elevation in serum CORT levels compared to sham control. Chronic treatment with Q-12 (2 mg/kg, p.o.) and FLX (10 mg/kg, p.o.) notably ($P<0.05$) decreased serum CORT [$F_{(8,45)} = 22.12$, $P<0.05$] level as compared to OBX control group. No alteration on serum CORT level in OBX rats was observed at doses of 0.5 & 1 mg/kg.

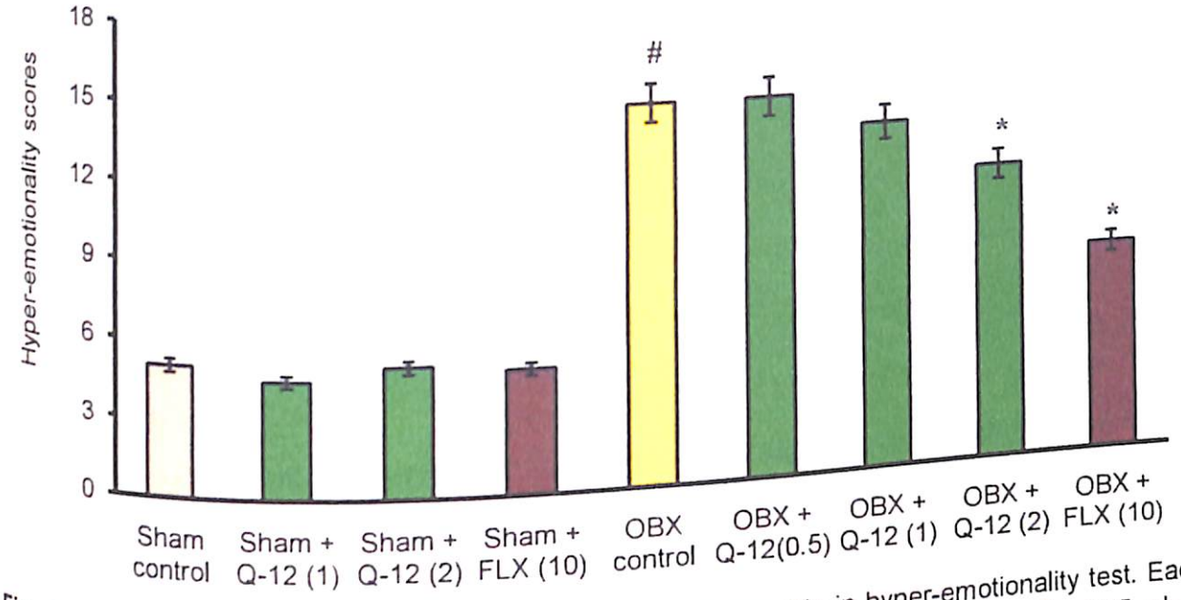


Fig. 66. Effects of Q-12 and FLX on the behavior of OBX/Sham rats in hyper-emotionality test. Each column represents mean hyper-emotionality scores. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with OBX control; n = 6/group.

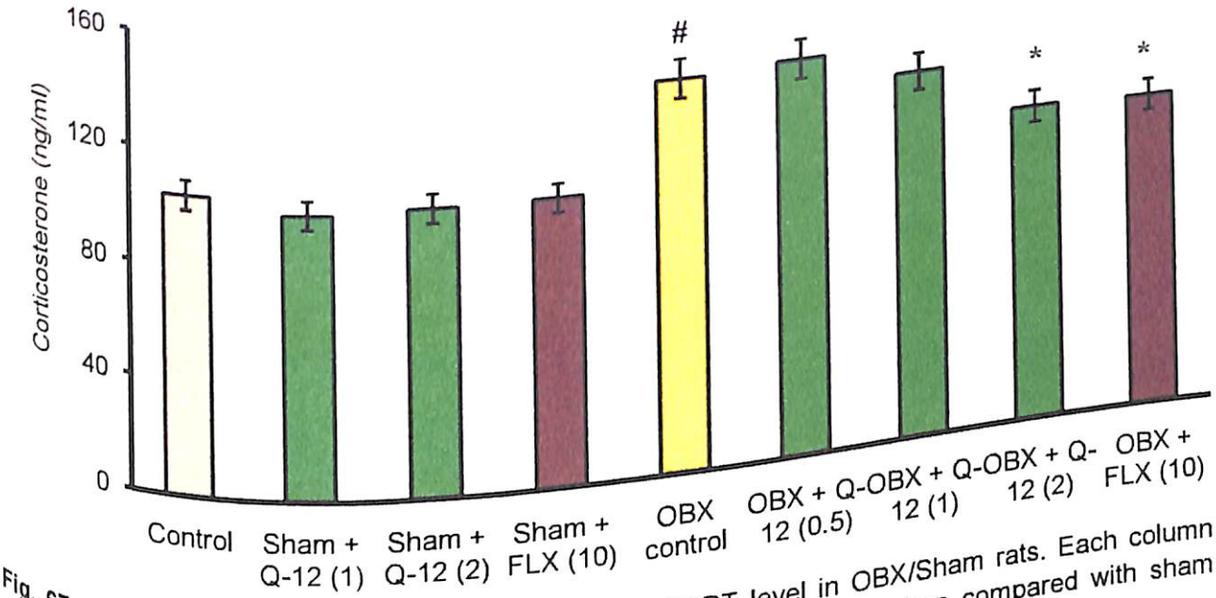


Fig. 67. Effect of Q-12 and FLX treatment on serum CORT level in OBX/Sham rats. Each column represents mean CORT level. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with OBX control; n = 6/group.

5.5.4.2.2. Effect of Q-12 on cAMP, pCREB and BDNF Levels in OBX/Sham Rats

OBX remarkably reduced the cAMP, pCREB and BDNF levels as compared to sham control rats. Q-21 (2 mg/kg, p.o.) and FLX (10 mg/kg) treated OBX rats, exhibited a profound higher levels of cAMP [$F_{(8,45)} = 7.56$, $P<0.05$], pCREB [$F_{(8,45)} = 9.14$, $P<0.05$] and BDNF [$F_{(8,45)} = 22.34$, $P<0.05$] as compared to vehicle treatment. There was no increased in cAMP, pCREB and BDNF levels observed at Q-12 (0.5 and 1 mg/kg) treatment **(table 57)**.

Table 57. Effect of Q-12 on cAMP, pCREB and BDNF levels in OBX/Sham rats

Groups	Dose (mg/kg)	cAMP (pmol/mg protein)	BDNF (ng/mg protein)	pCREB (% sham control)
Sham control	0	21.3±1.65	82.95±1.37	100.00±4.95
Sham + Q-12	1	22.10±1.47	86.50±7.67	103.75±5.34
Sham + Q-12	2	29.65±2.29[a]	97.36 ±6.12[a]	129.97±3.79[a]
Sham + FLX	10	24.76±1.33	89.77±6.21	112.18±6.13
OBX control	0	12.86±1.70[a]	31.60±2.68[a]	54.39±4.21[a]
OBX + Q-12	0.5	13.16±3.11	33.673±2.35	50.26±4.43
OBX + Q-12	1	17.09±2.23	41.45±4.08	58.27±2.17
OBX + Q-12	2	24.27±2.35[b]	59.53±2.12[b]	70.42±3.56[b]
OBX + FLX	10	19.13±1.27[b]	48.17±7.25[b]	72.90±7.81[b]

The values are expressed as mean ± S.E.M. The drug/vehicle treatments were carried out once a day for 14 days. [a]P<0.05 compared with sham control, [b]P<0.05 compared with OBX group; n = 6/group.

5.5.5. Evaluation of Anxiolytic-like Potential of Q-12 in Experimental Models

5.5.5.1. Effect of Q-12 on Behavior of Mice in EPM Test

Administration of Q-12 (2 mg/kg, i.p.) and DZM (2 mg/kg, i.p.) markedly increased the percentage of both OAE [$F_{(4,35)}$ = 6.42, P<0.05] and TSOA [$F_{(4,35)}$ = 7.20, P<0.05] as compared to normal control group **(table 58).** Q-12 (0.5 & 1 mg/kg) effects on the mice behavior in EPM test were failed to reach the level of significance.

Table 58: Effect of Q-12 on behavior of mice in EPM test

Groups	Dose (mg/kg)	No. of entries		% OAE	% TSOA
		open arm	closed arm		
Control	0	2.12±0.61	5.62±0.50	27.62±6.89	26.41±2.71
DZM	2	6.80±1.46[a]	4.40±0.87	60.15±2.56[a]	44.13±3.6[a]
Q-12	0.5	1.60±0.40	5.0±0.84	24.20±3.09	15.13±3.01
Q-12	1	2.40±1.20	4.20±0.86	26.67±9.65	20.40±7.50
Q-12	2	3.10±0.24[a]	6.11±3.13	34.33±4.20[a]	28.21±2.15[a]

All values represent mean ± S.E.M. [a]P<0.05 when compared with normal control group; n = 8/group.

5.5.5.2. Effect of Q-12 on Behavior of Mice in L/D Aversion Test

Q-12 (2 mg/kg, i.p.) and DZM (2 mg/kg, i.p.) treated mice exhibited increase in latency time to leave the light compartment [$F_{(4,35)}$ = 11.25, P<0.05], time spent in light compartment [$F_{(4,35)}$ = 8.42, P<0.05] and number of crossings between the compartments [$F_{(4,35)}$ = 3.86, P<0.05]

as compared to normal control group **(table 59).** Q-12 at doses (0.5 & 1 mg/kg, p.o.) did not produce significant change in any of the parameters compared to normal control group.

Table 59: Effect of Q-12 on behavior of mice in L/D aversion test

Groups	Dose (mg/kg)	Latency to leave light box (s)	No. of crossing	Time spent in light box (s)
Vehicle	0	19.43±2.11	12.67±1.47	80.14±6.34
DZM	2	73.83±5.43[a]	16.18±1.40[a]	164.84±11.06[a]
Q-12	0.5	19.60±3.21	11.20±1.02	74.60±10.76
Q-12	1	23.65±4.30	12.60±1.57	81.29±10.37
Q-12	2	41.33±6.22[a]	18.12±1.67[a]	110.0±12.36[a]

All values represent mean ± S.E.M. [a]P<0.05 when compared with normal control group; n = 8/group.

5.6. Effect of ETZ and Q-21 on Morphological Changes in Hippocampal CA1 and Dentate Gyrus Regions of OBX/Sham Rats

Fig. 69 (Panel A1-A8 and B1-B8) shows the effects of surgery and drug treatments on the neuronal structure in OBX rats. The brain samples of three rats chosen randomly for each group and the morphological study and neurons counting were done in atleast three sections. The H&E stained brain sections of DG and hippocampal CA1 regions showed healthy neurons in sham control **(Fig. 69; Panel A1 & B1)** and ETZ sham treatment group **(Fig. 69; Panel A2 & B2)**. Healthy neurons were robust in shape, had a pale and spherical slightly oval nucleus and a single large nucleolus with clear visible cytoplasm. Neuro-degenerative morphologies are indicated by dark staining of pyknotic neurons. The neurons showed fragmented or no nucleus and some cells were sickle shaped or reduced in size. The photomicrographs from the DG and hippocampal CA1 regions of OBX control group showed the damaged and shrinked neurons **(Fig. 69; Panel A3 & B3)** with marked decrease in total neuronal count, number of healthy neurons and increase in pyknotic cells in DG as well as hippocampal CA1 regions as compared with sham control group **(fig. 70A and 70B).** Chronic treatment with ETZ (0.5 and 1 mg/ kg, p.o.) **(Fig. 69; Panel A4, A5 and B4, B5),** Q-21 (1 and 2 mg/ kg, p.o.) **(Fig. 69; Panel A6 and A7 & B6 and B7)** and FLX (10 mg/kg, p.o.) **(Fig. 69; Panel A8 & B8)** in OBX rats attenuated OBX-induced cell loss and pyknotic cells. Although, at lower doses of ETZ and Q-21 treatments some degenerating cells with changes of morphology were observed. The protective effects of PDE4 inhibitors were also confirmed by healthy and pyknotic neurons counting, where ETZ and Q-21 prevented the decrease in total neurons and healthy neurons and attenuated the increased number of pyknotic cell bodies in the DG and hippocampal CA1 regions. The higher dose of ETZ and Q-21 had displayed

rounded and open nuclei with marked protection from OBX-induced neuro-degenerative morphologies.

5.6.1. Effect of Surgery and Treatments (ETZ and Q-21) on Neuronal Structure in DG Region of OBX/Sham Rats

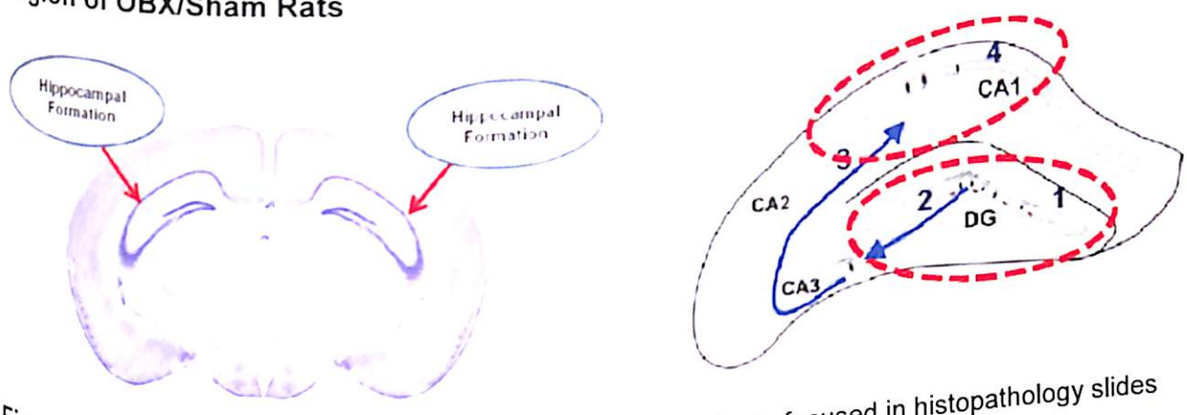
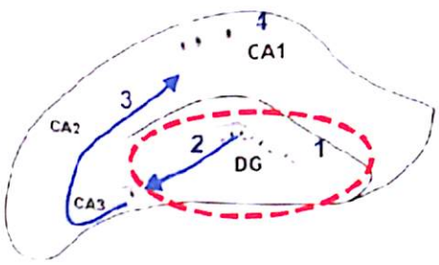


Fig. 68. Red circles indicate the DG & hippocampal CA1 regions, focused in histopathology slides



Represents H&E stained DG region (Fig. 69; Panel A1-A8): **Total magnification = 40** [Eye piece magnification (10) X objective magnification (4)]

Panel A1: Sham Control

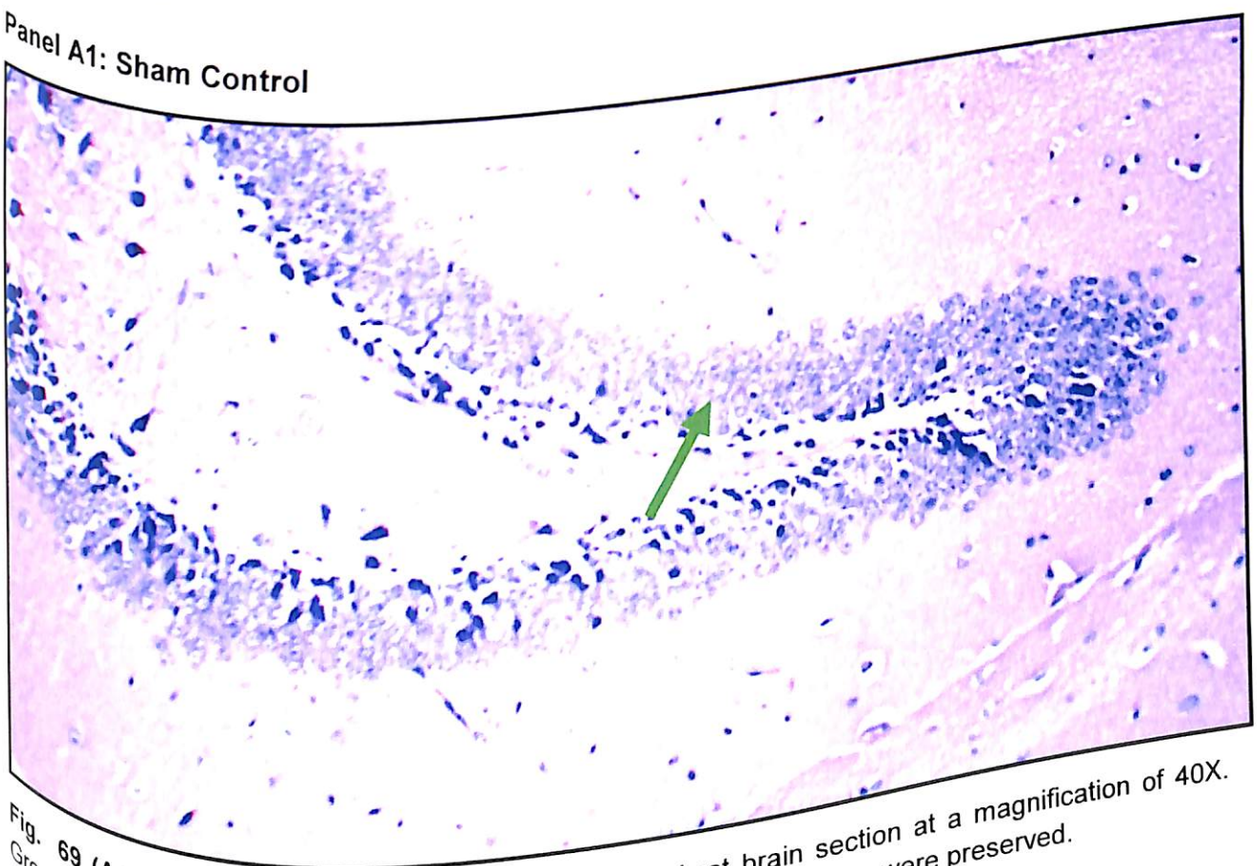


Fig. 69 (A1): H&E stained DG region of Sham control rat brain section at a magnification of 40X. Green arrow indicates the healthy neurons. In sham control rats neurons were preserved.

Panel A2: Sham + ETZ (1 mg/kg)

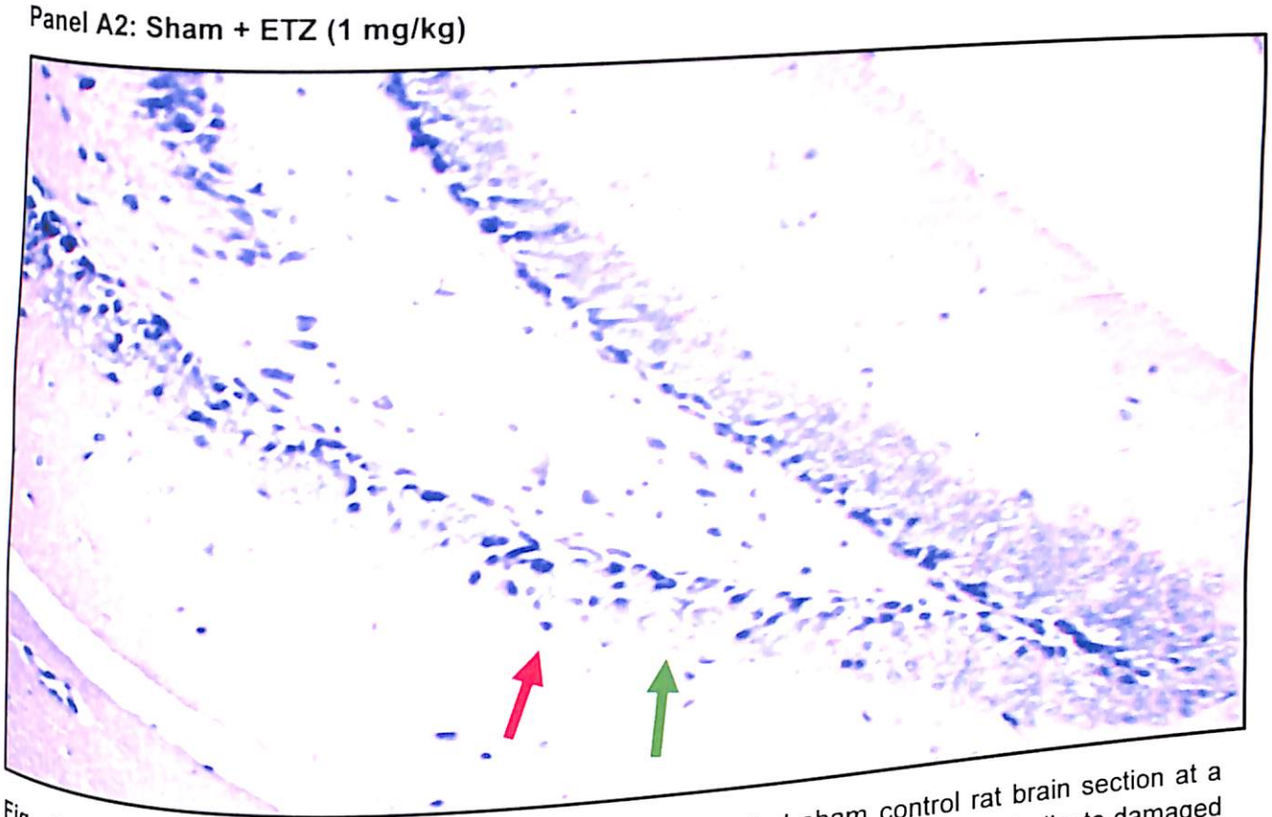


Fig. 69 (A2): H&E stained DG region of ETZ (1 mg/kg) treated sham control rat brain section at a magnification of 40X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. In ETZ (1 mg/kg) treated sham group neurons were preserved.

Panel A3: OBX Control

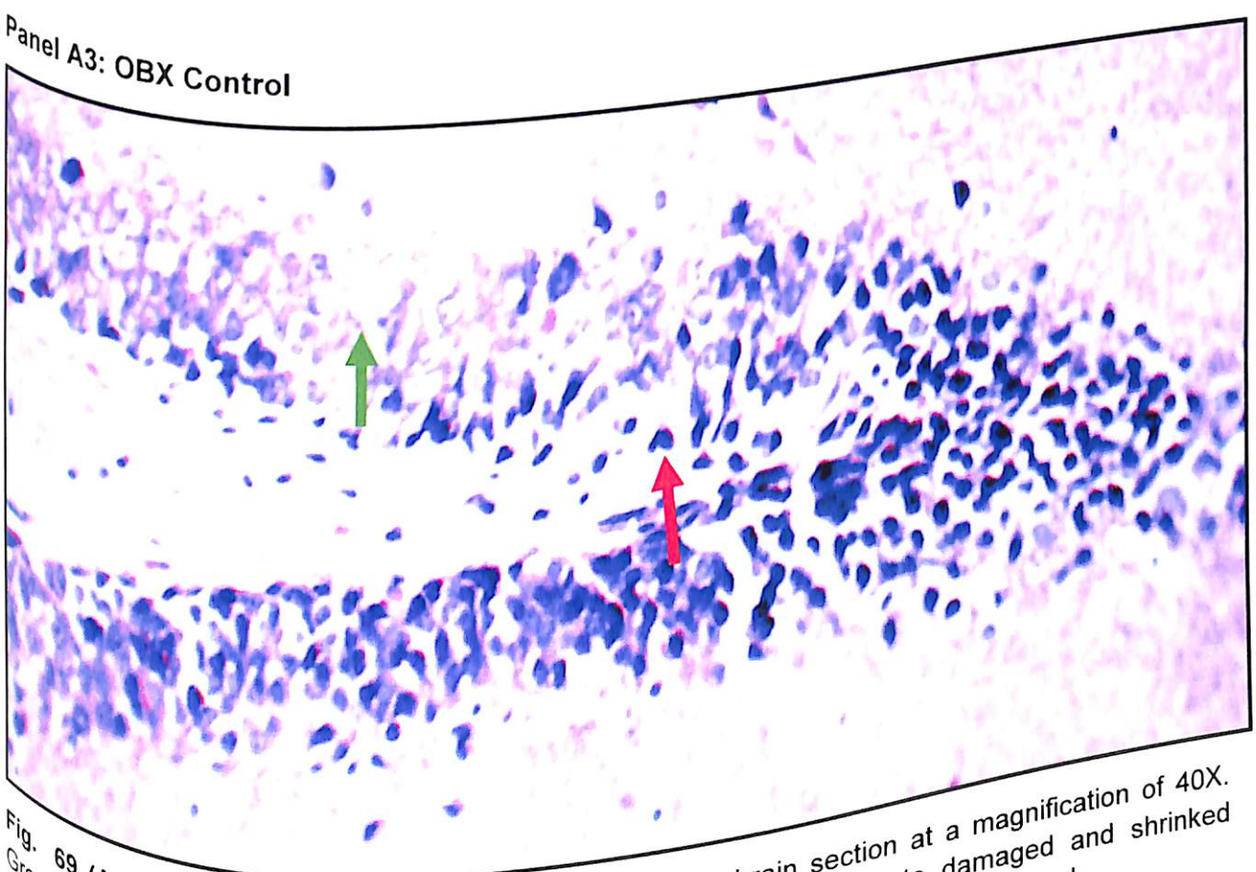


Fig. 69 (A3): H&E stained DG region of OBX control rat brain section at a magnification of 40X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. In OBX control large numbers of damaged and shrinked neurons were observed

Panel A4: OBX + ETZ (0.5)

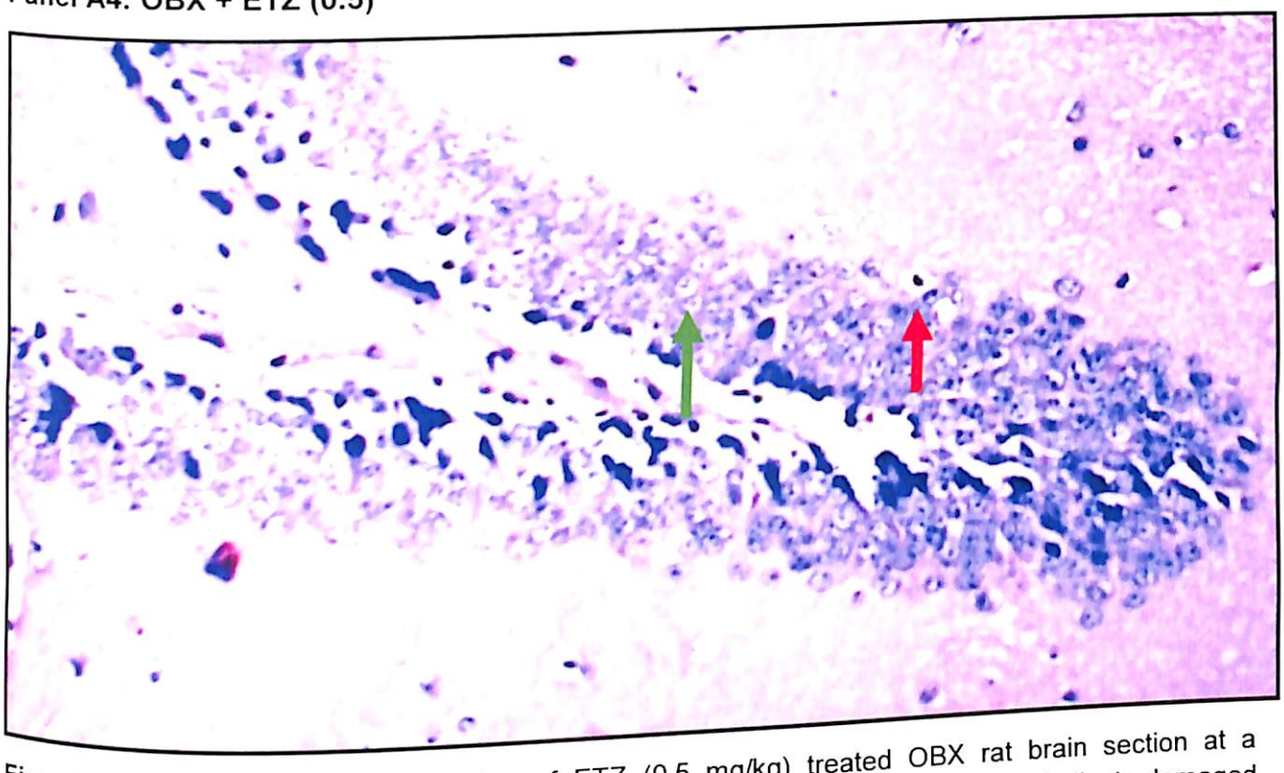


Fig. 69 (A4): H&E stained DG region of ETZ (0.5 mg/kg) treated OBX rat brain section at a magnification of 40X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. ETZ (0.5 mg/kg) showed less numbers of damaged and shrinked neurons and neuroprotection against OBX-induced neurotoxicity.

Panel A5: OBX + ETZ (1)

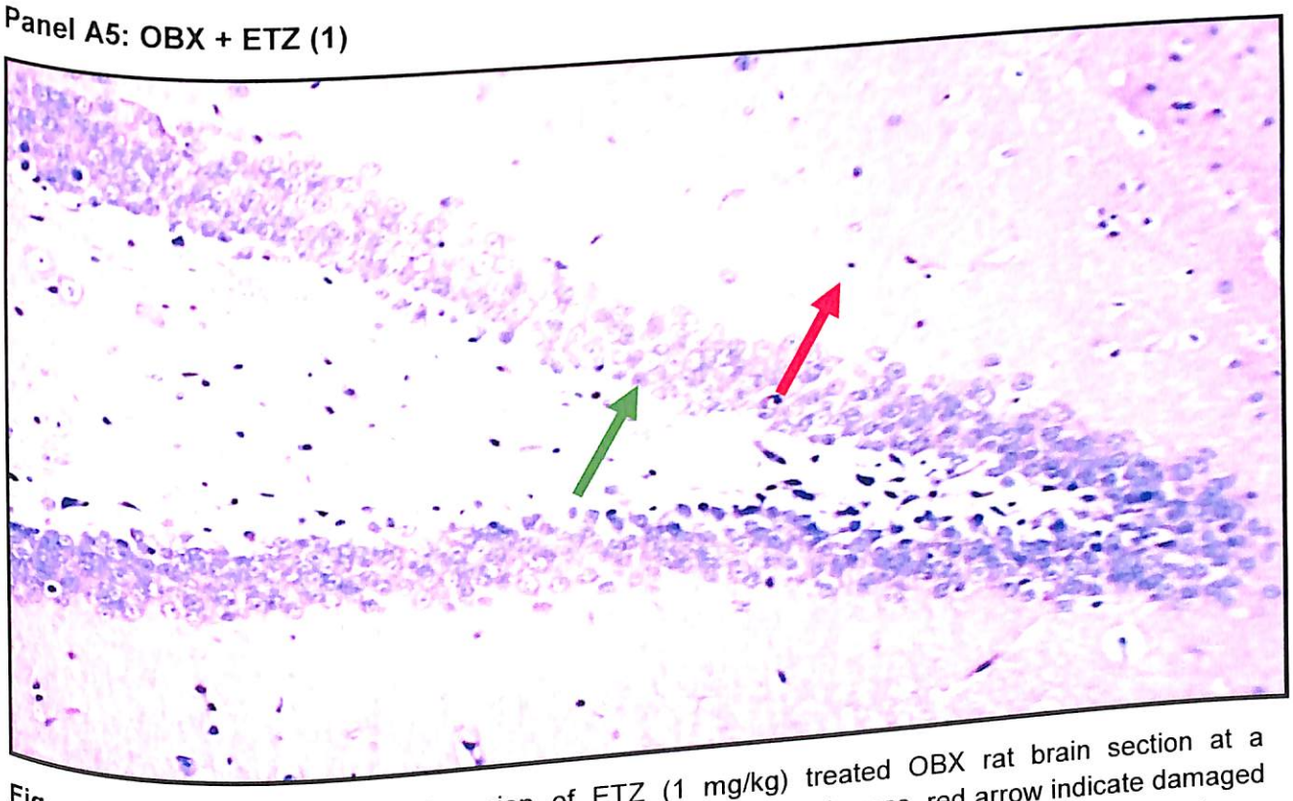


Fig. 69 (A5): H&E stained DG region of ETZ (1 mg/kg) treated OBX rat brain section at a magnification of 40X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. ETZ (1 mg/kg) showed overall appearance similar to the sham group and very little neuronal loss.

Panel A6: OBX + Q-21 (1)

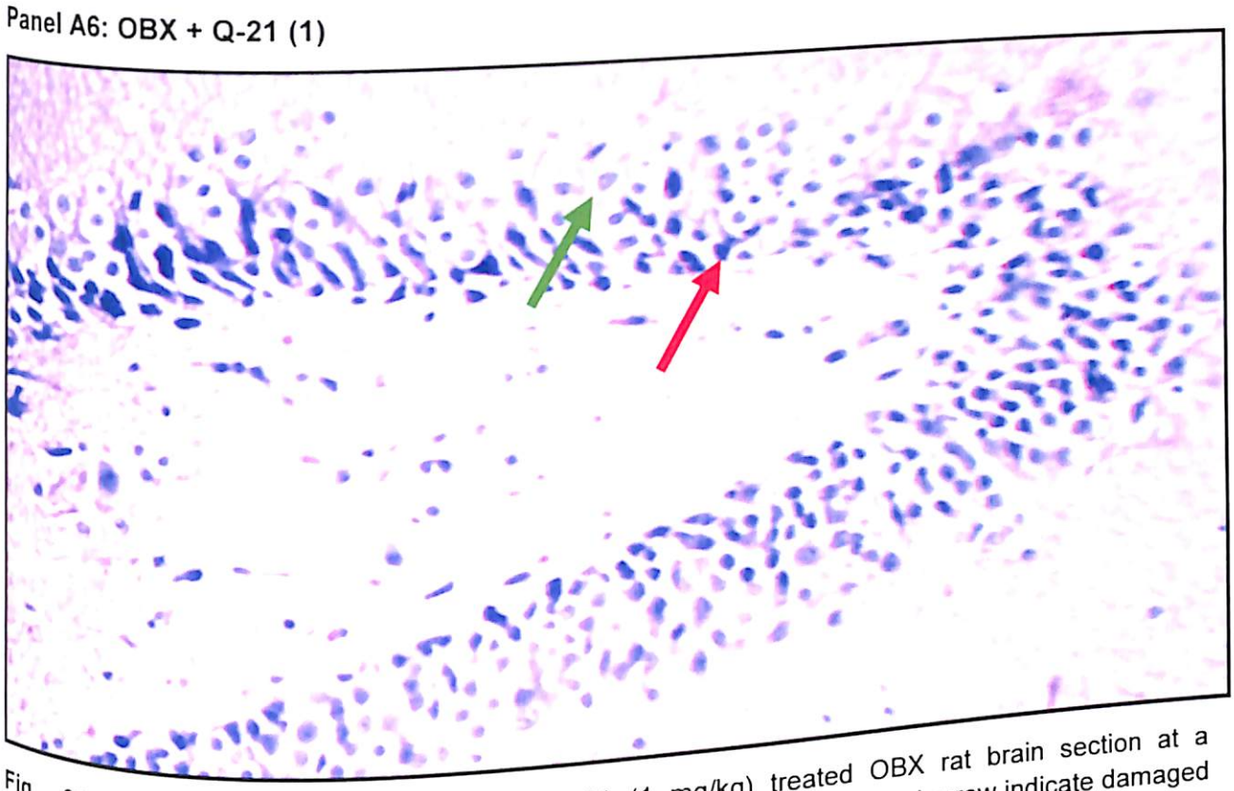


Fig. 69 (A6): H&E stained DG region of Q-21 (1 mg/kg) treated OBX rat brain section at a magnification of 40X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. Q-21 (1 mg/kg) treatment showed mild neuronal damage and shrinked neurons.

Panel A7: OBX + Q-21 (2)

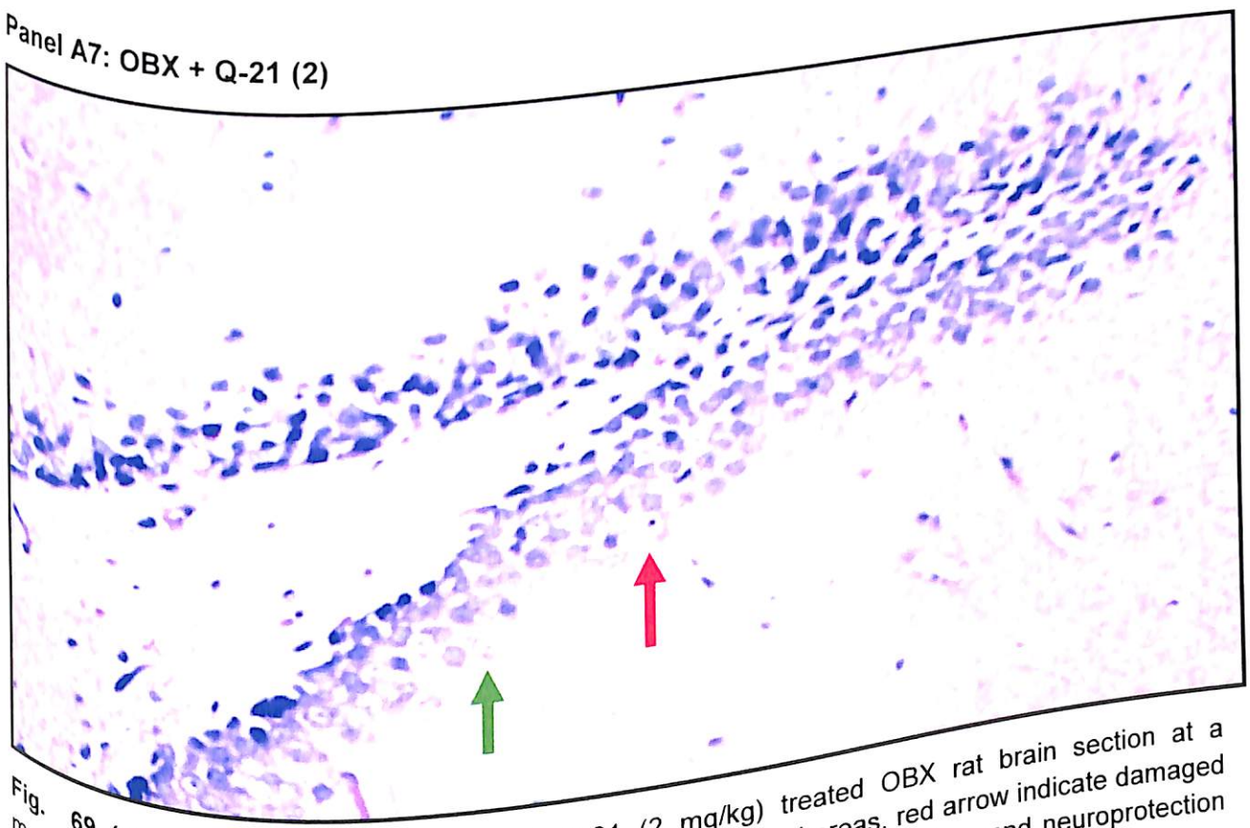


Fig. 69 (A7): H&E stained DG region of Q-21 (2 mg/kg) treated OBX rat brain section at a magnification of 40X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. Q-21 (2 mg/kg) treatment showed minimal neuronal loss and neuroprotection against OBX-induced neurotoxicity.

Panel A8: OBX + FLX (10)

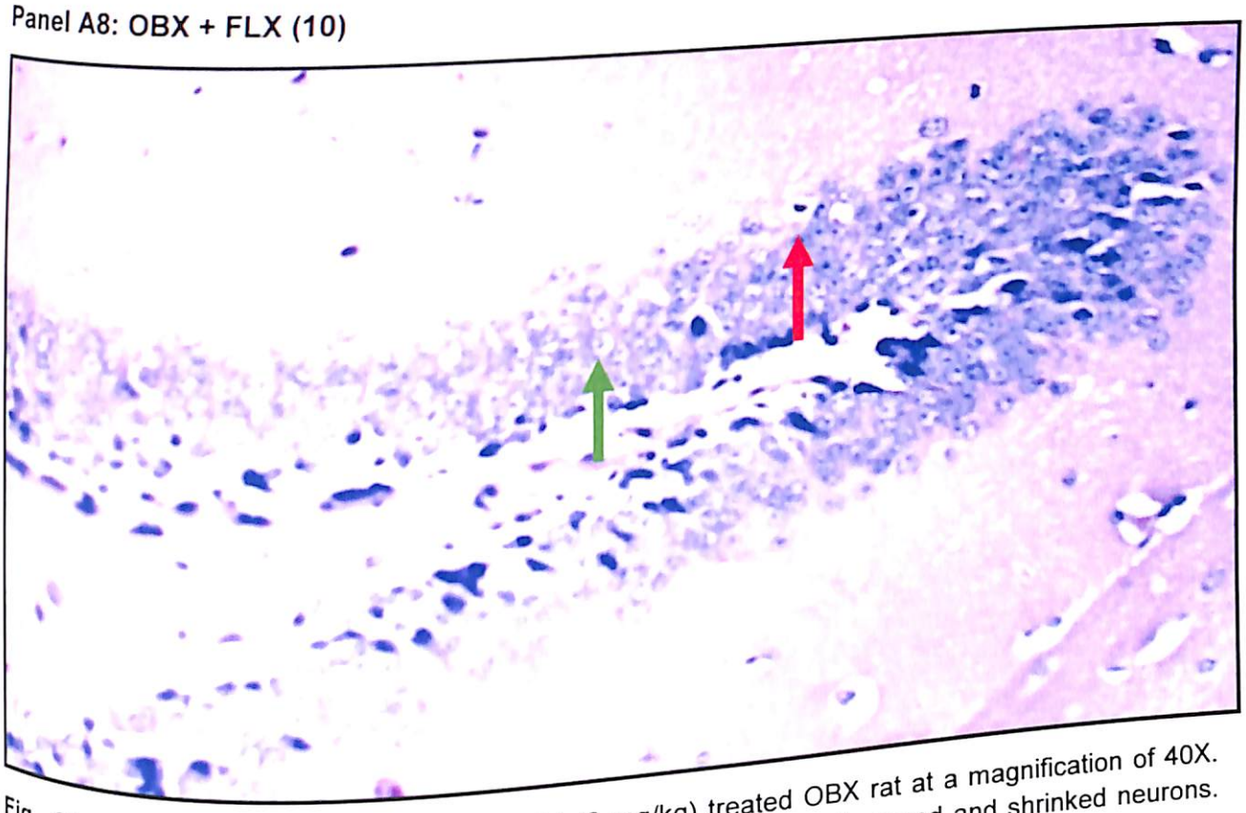
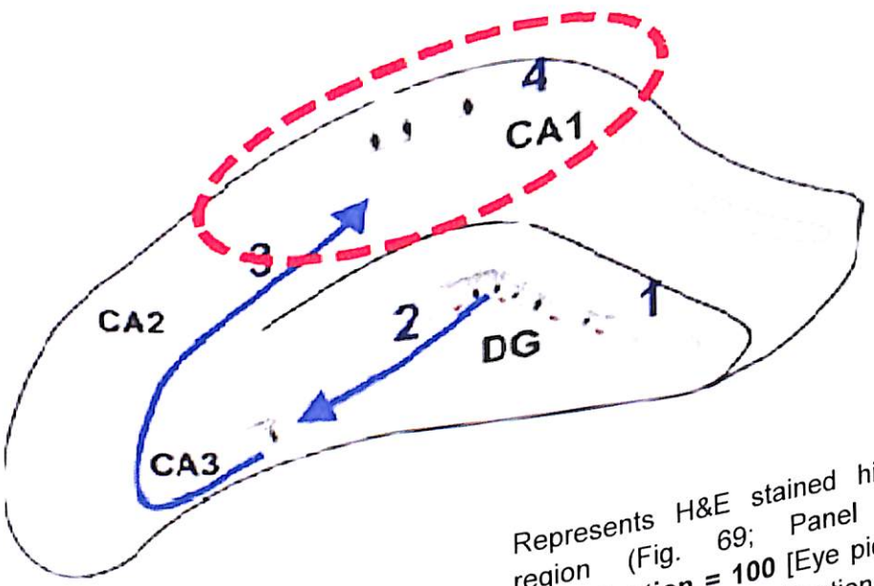


Fig. 69 (A8): H&E stained DG region of Q-21 (2 mg/kg) treated OBX rat at a magnification of 40X. Green arrow indicate healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. Q-21 showed intact neurons with less neuronal loss and overall appearance similar to sham group.

5.6.2. Effect of Surgery and Treatments (ETZ and Q-21) on Neuronal Structure in Hippocampal CA1 Region of OBX/Sham Rats



Represents H&E stained hippocampal CA1 region (Fig. 69; Panel B1-B8): **Total magnification = 100** [Eye piece magnification (10) X objective magnification (10)]

Panel B1: Sham Control

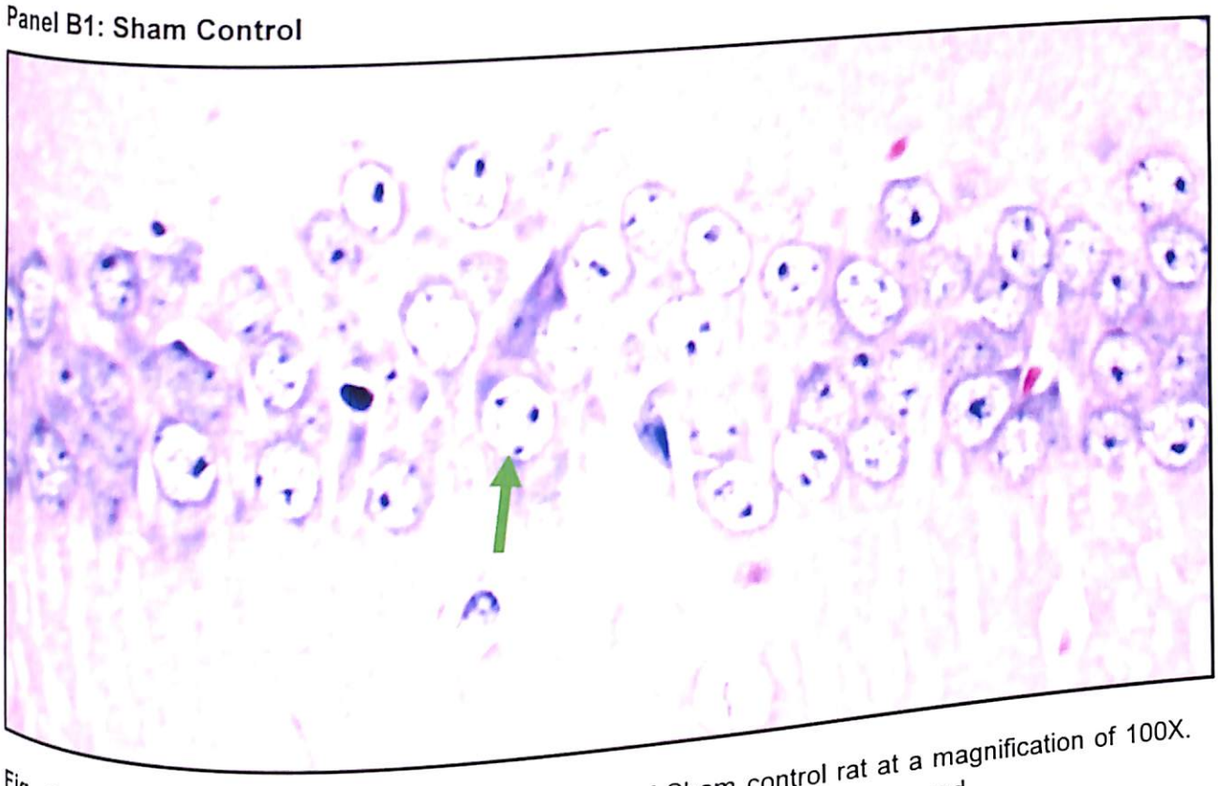


Fig. 69 (B1): H&E stained hippocampal CA1 region of Sham control rat at a magnification of 100X. Green arrow indicates healthy neurons. In sham rats intact neurons were observed.

Panel B2: Sham control + ETZ (1)

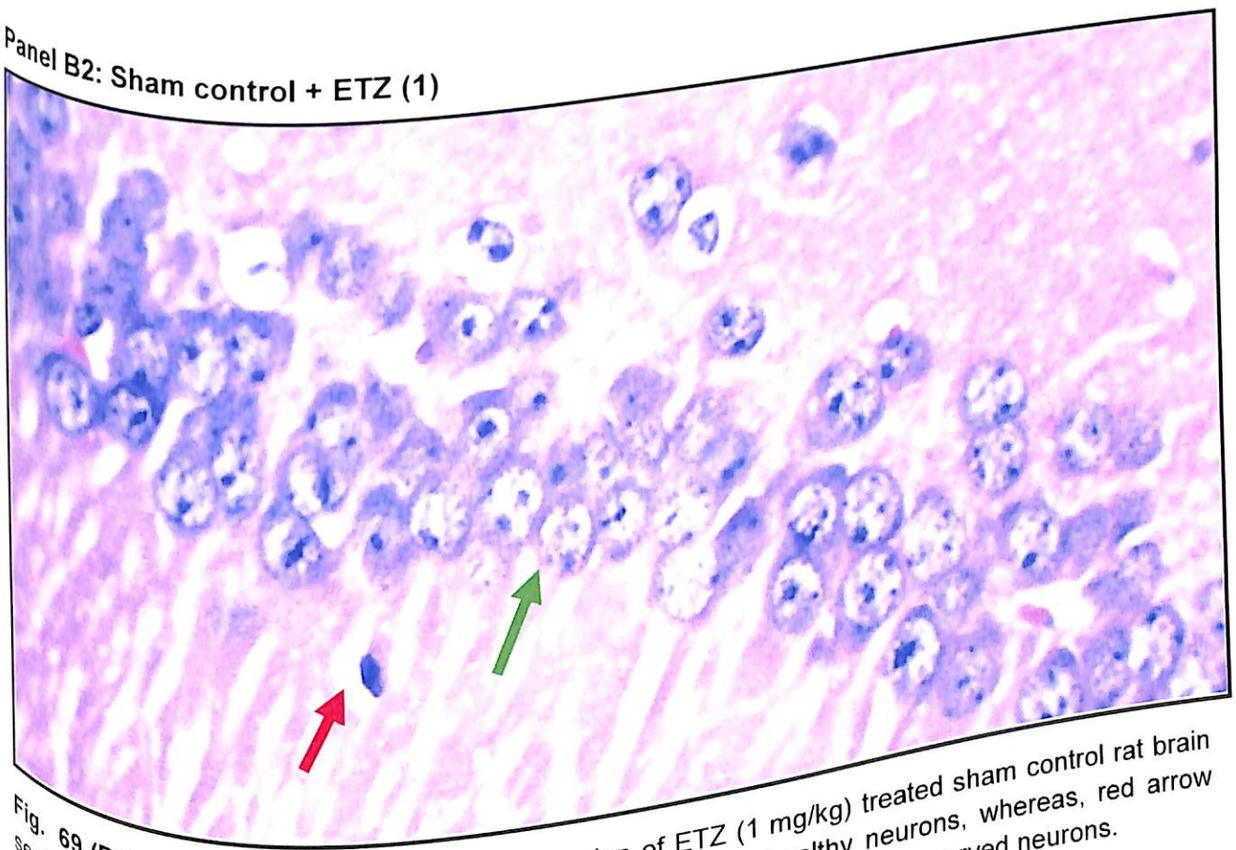


Fig. 69 (B2): H&E stained hippocampal CA1 region of ETZ (1 mg/kg) treated sham control rat brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. ETZ treated sham group showed preserved neurons.

Panel B3: OBX control

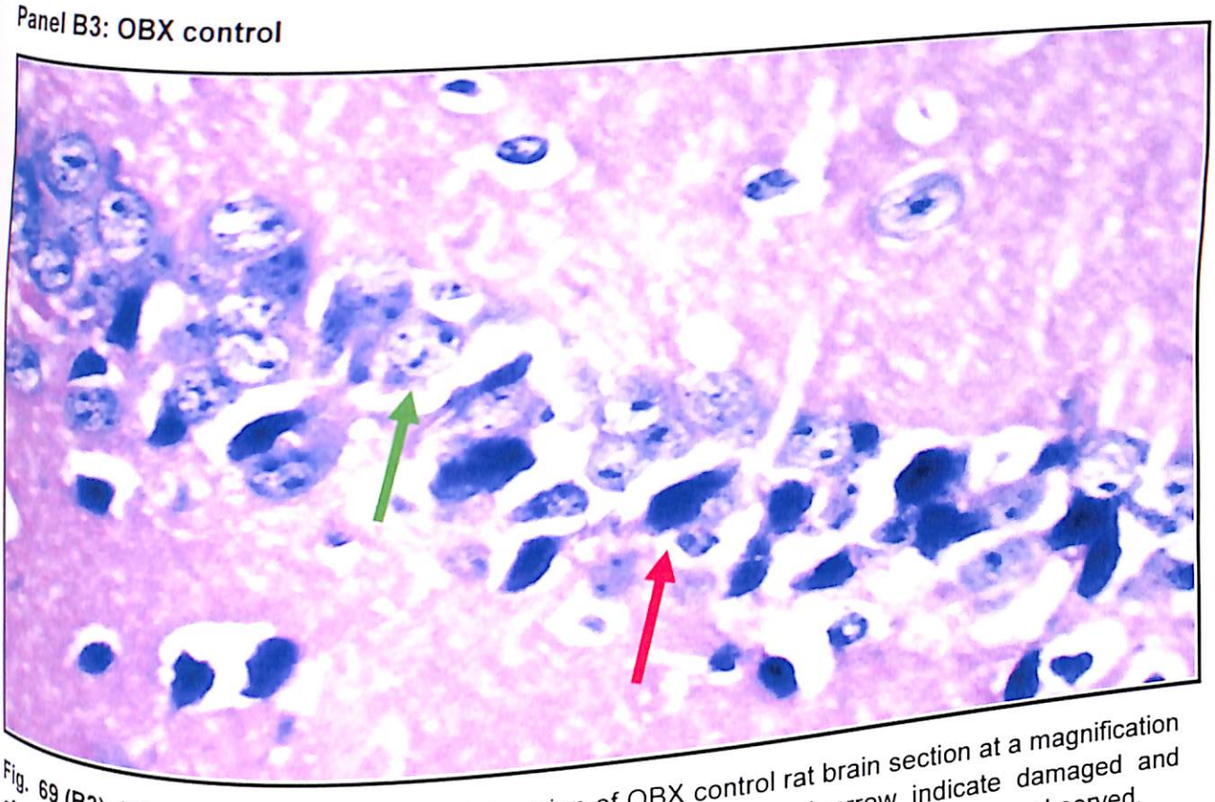


Fig. 69 (B3): H&E stained hippocampal CA1 region of OBX control rat brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. In OBX control large numbers of damaged and shrinked neurons were observed.

Panel B4: OBX + ETZ (0.5)

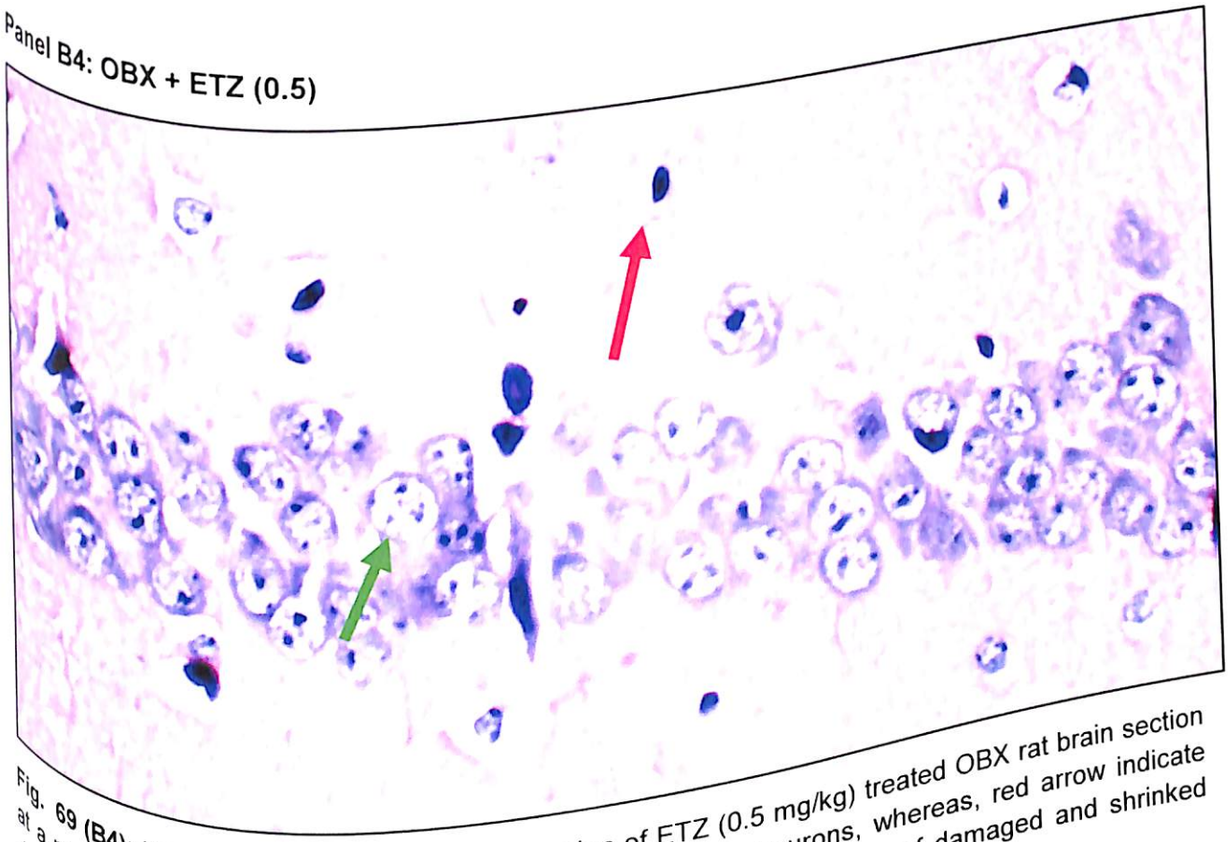


Fig. 69 (B4): H&E stained hippocampal CA1 region of ETZ (0.5 mg/kg) treated OBX rat brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. ETZ (0.5 mg/kg) showed less numbers of damaged and shrinked neurons and neuro-protection against OBX-induced neurotoxicity.

Panel B5: OBX + ETZ (1)

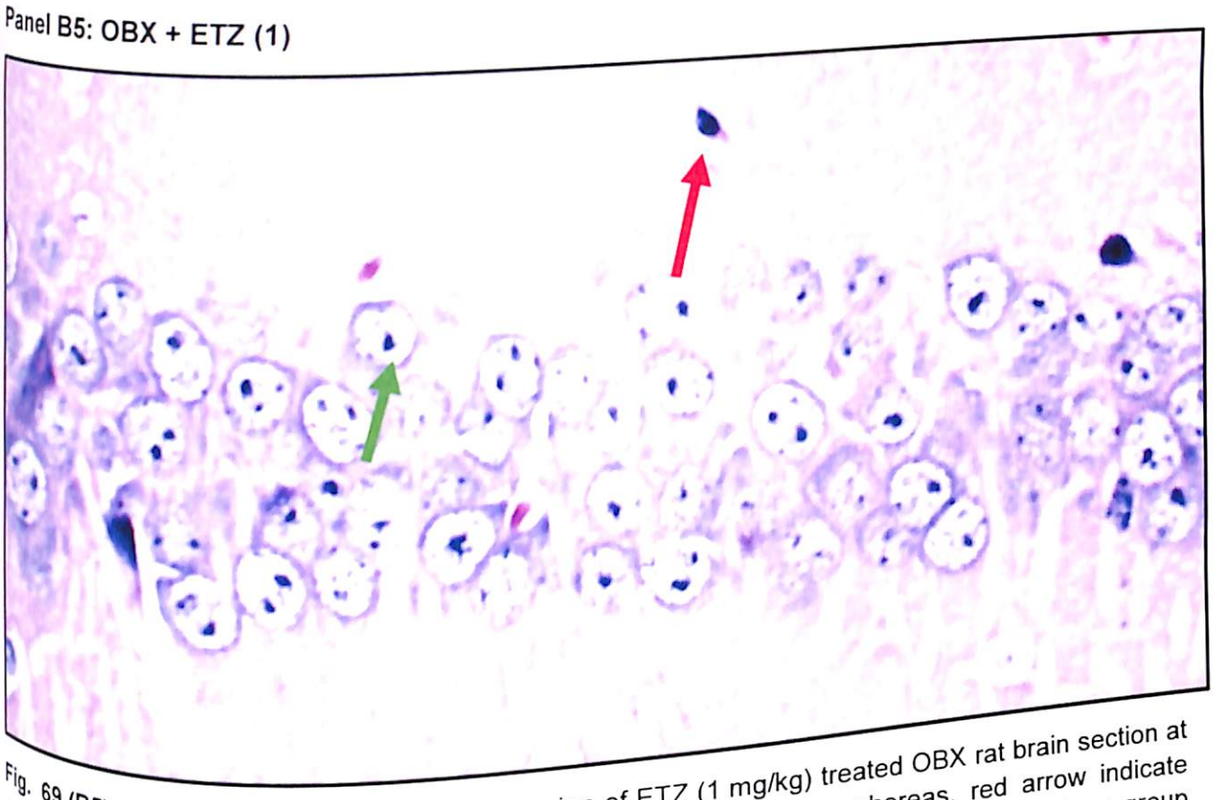


Fig. 69 (B5): H&E stained hippocampal CA1 region of ETZ (1 mg/kg) treated OBX rat brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. ETZ (1 mg/kg) showed overall appearance similar to the sham group and very little neuronal loss.

Panel B6: OBX + Q-21 (1)

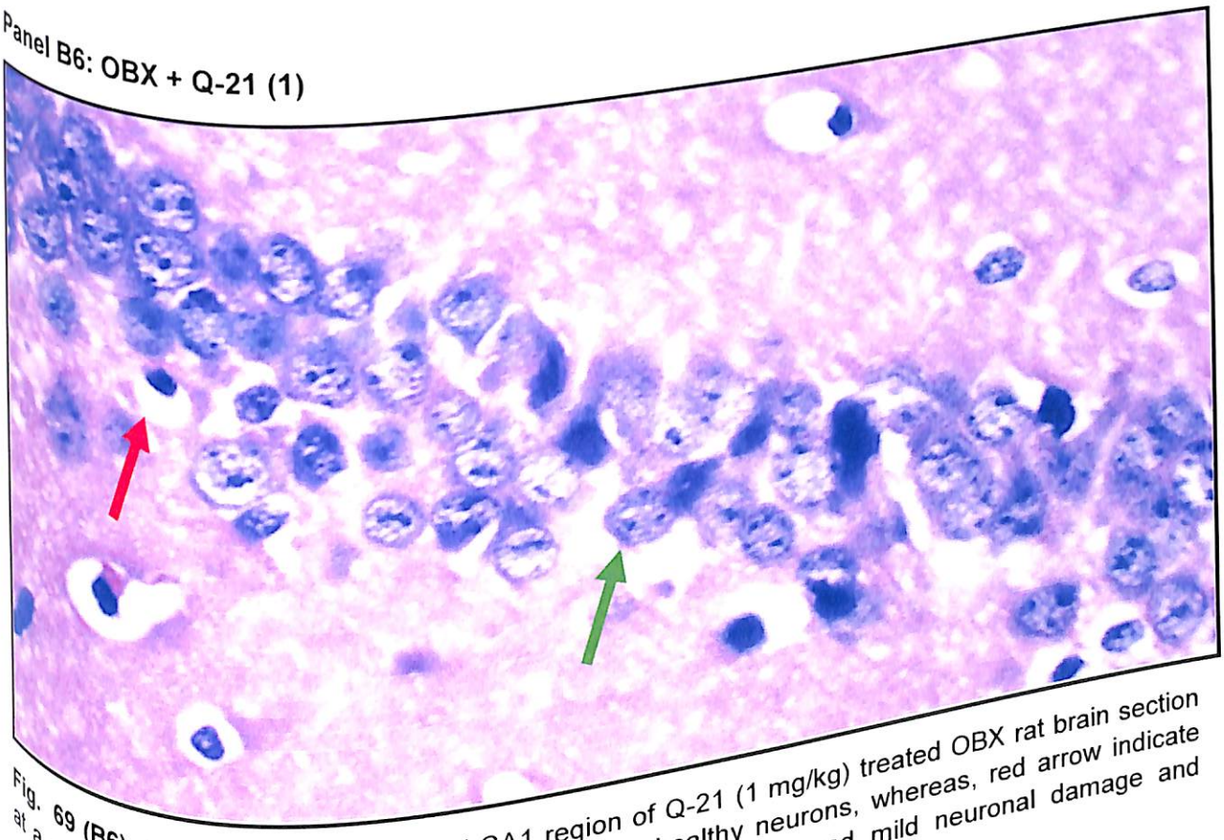


Fig. 69 (B6): H&E stained hippocampal CA1 region of Q-21 (1 mg/kg) treated OBX rat brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. Q-21 (1 mg/kg) treatment showed mild neuronal damage and shrinked neurons.

Panel B7: OBX + Q-21 (2)

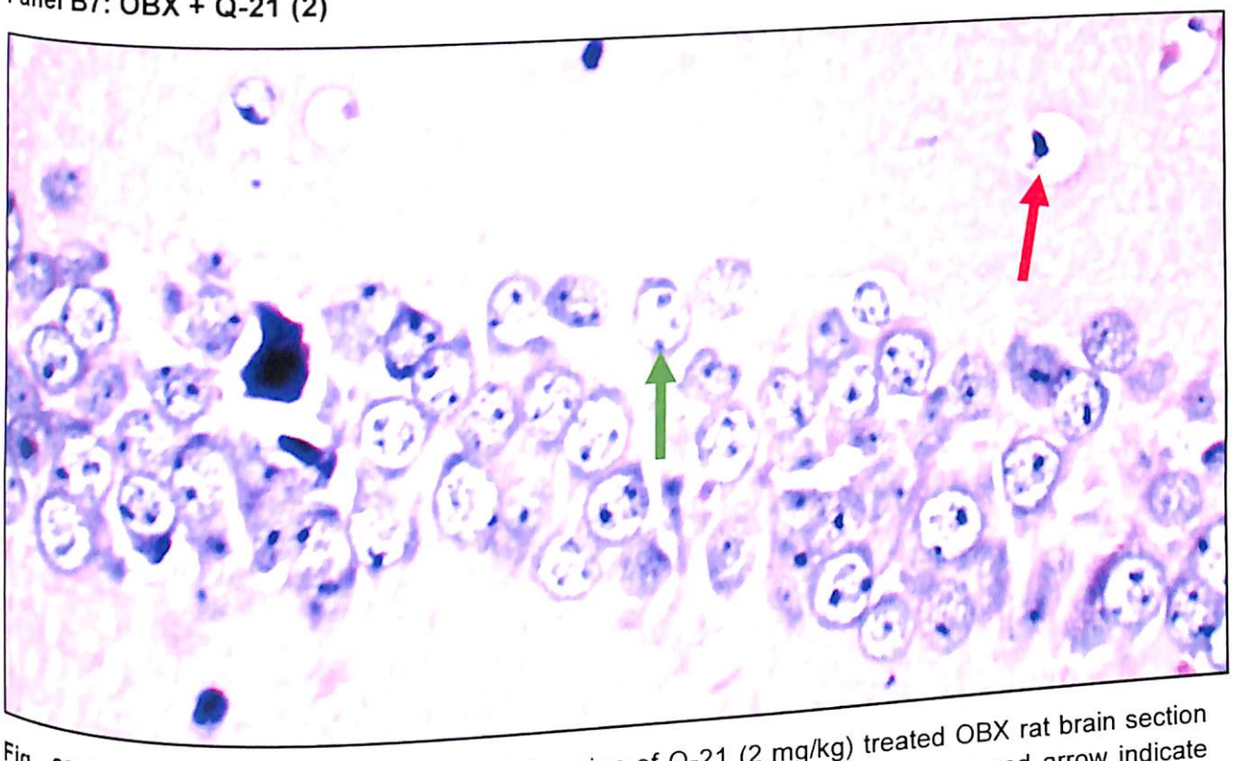


Fig. 69 (B7): H&E stained hippocampal CA1 region of Q-21 (2 mg/kg) treated OBX rat brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. Q-21 (2 mg/kg) treatment showed minimal neuronal loss and neuroprotection against OBX-induced neurotoxicity.

Panel B8: OBX + FLX (10)

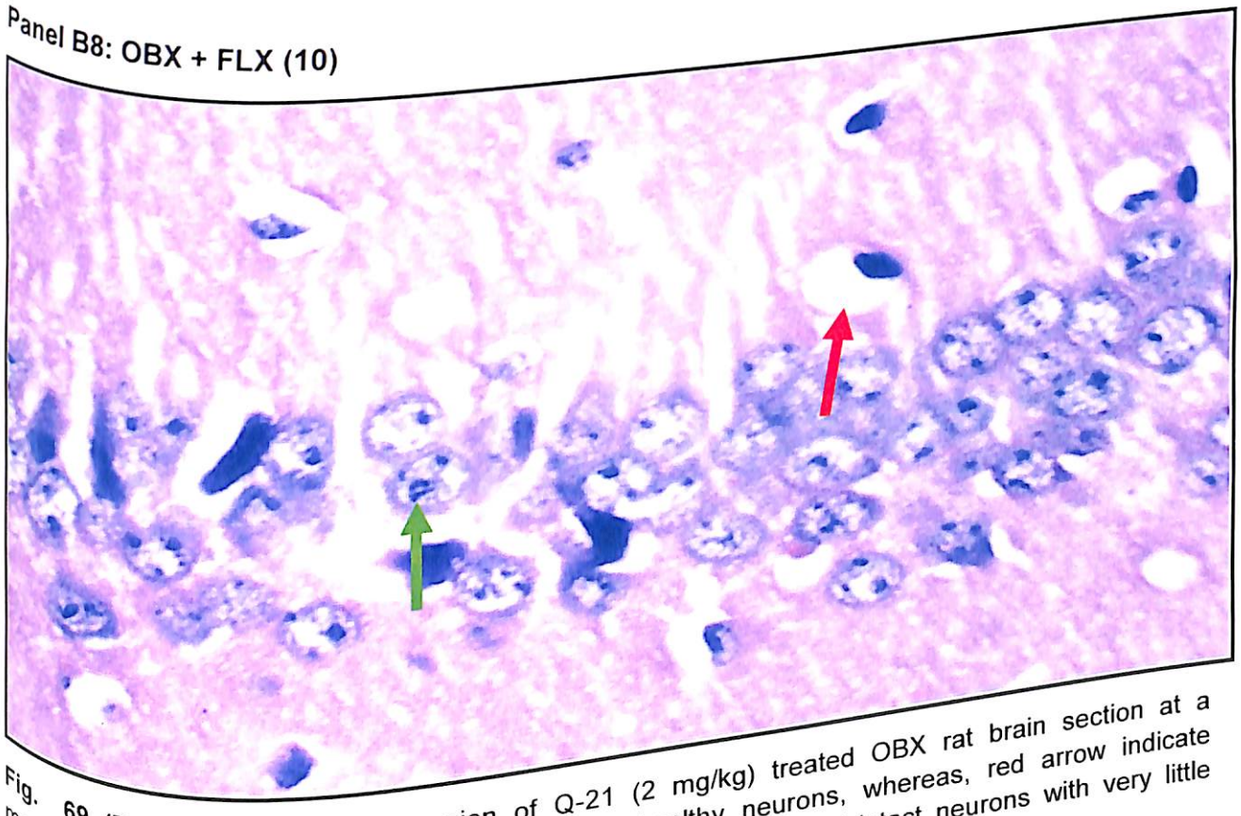


Fig. 69 (B8): H&E stained DG region of Q-21 (2 mg/kg) treated OBX rat brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. Q-21 (2 mg/kg) treatment showed intact neurons with very little neuronal loss and overall appearance similar to the sham group.

Fig. 70 A: Neuronal density in DG region of OBX rats

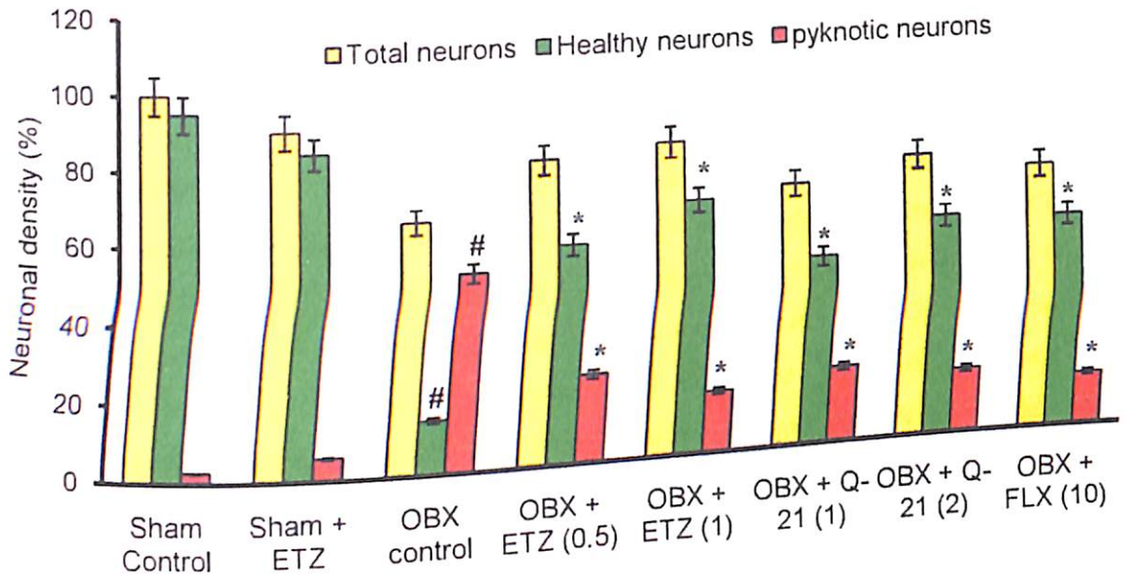


Fig. 70 B: Neuronal density in hippocampal CA1 region of OBX rats

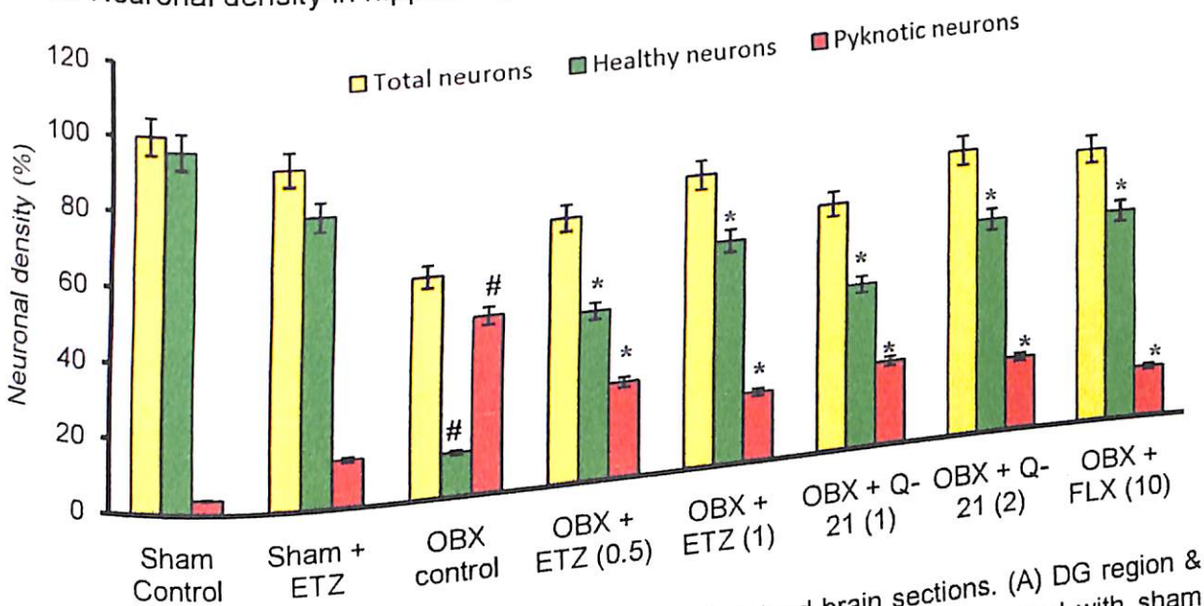


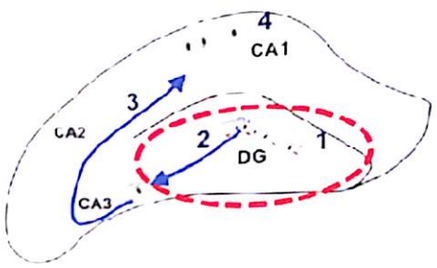
Fig. 70. Effect of ETZ and Q-21 on neuronal density in H&E stained brain sections. (A) DG region & (B) Hippocampal CA1 region. The error bar indicates S.E.M. #P<0.05 when compared with sham control, *P<0.05 when compared with OBX control.

5.7. Effect of ETZ and Q-21 on Histological Changes in CORT-treated Model

Fig. 71 shows the effects of CORT-injection and drug treatments on the neuronal structure of hippocampal CA1 and DG regions in chronic CORT-treated model. **The brain samples of three mice chosen randomly for each group and the morphological study and neurons counting were done in atleast three sections.** The H&E stained brain DG and hippocampal CA1 sections showed healthy neurons in normal control mice **(fig. 71; Panel A1 & B1).** The photomicrographs from the DG and hippocampus CA1 regions of chronic

CORT-injection group showed a marked loss of DG and hippocampal CA1 cell bodies and decreased neuronal density **(fig. 71; Panel A2 & B2).** Further, CORT-treated mice showed markedly decrease in total neuronal count, number of healthy neurons and increase in pyknotic cells in DG as well as hippocampal CA1 regions as compared to normal control group **(fig. 72A and B).** Chronic treatment with ETZ (0.5 and 1 mg/ kg, p.o.) **(fig. 71; Panel A3, A4 and B3, B4)**, Q-21 (1 and 2 mg/ kg, p.o.) **(fig. 71; Panel A5 and A6 & B5 and B6)** and FLX (20 mg/kg, p.o.) **(fig. 71; Panel A7 & B7)** attenuated chronic CORT-injection-induced cell loss and pyknotic cells. Although, at lower doses of ETZ and Q-21 treatments some degenerating cells with changes of morphology were observed. The protective effects of PDE4 inhibitor were also confirmed by normal and abnormal neuron counting, where ETZ and Q-21 prevented the decrease in total neurons and healthy neurons and attenuated the increased number of pyknotic cell bodies in DG and hippocampal CA1 region.

5.7.1. ETZ and Q-21 Effect on Neuronal Structure in DG Region of CORT-treated mice



Represents H&E stained DG region (Fig. 71; Panel A1-A7): **Total magnification = 40** [Eye piece magnification (10) X objective magnification (4)]

Panel A1: Normal control

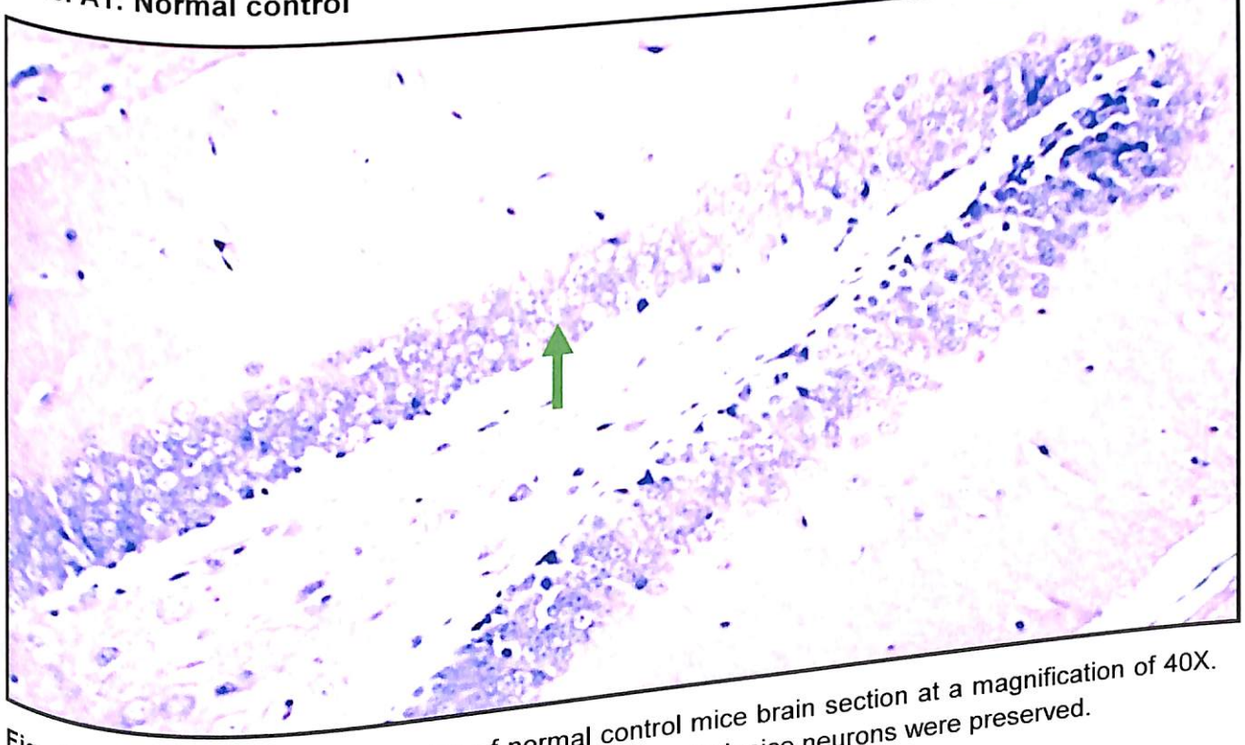


Fig. 71 (A1): H&E stained DG region of normal control mice brain section at a magnification of 40X. Green arrow indicates the healthy neurons. In normal control mice neurons were preserved.

Panel A2: CORT control

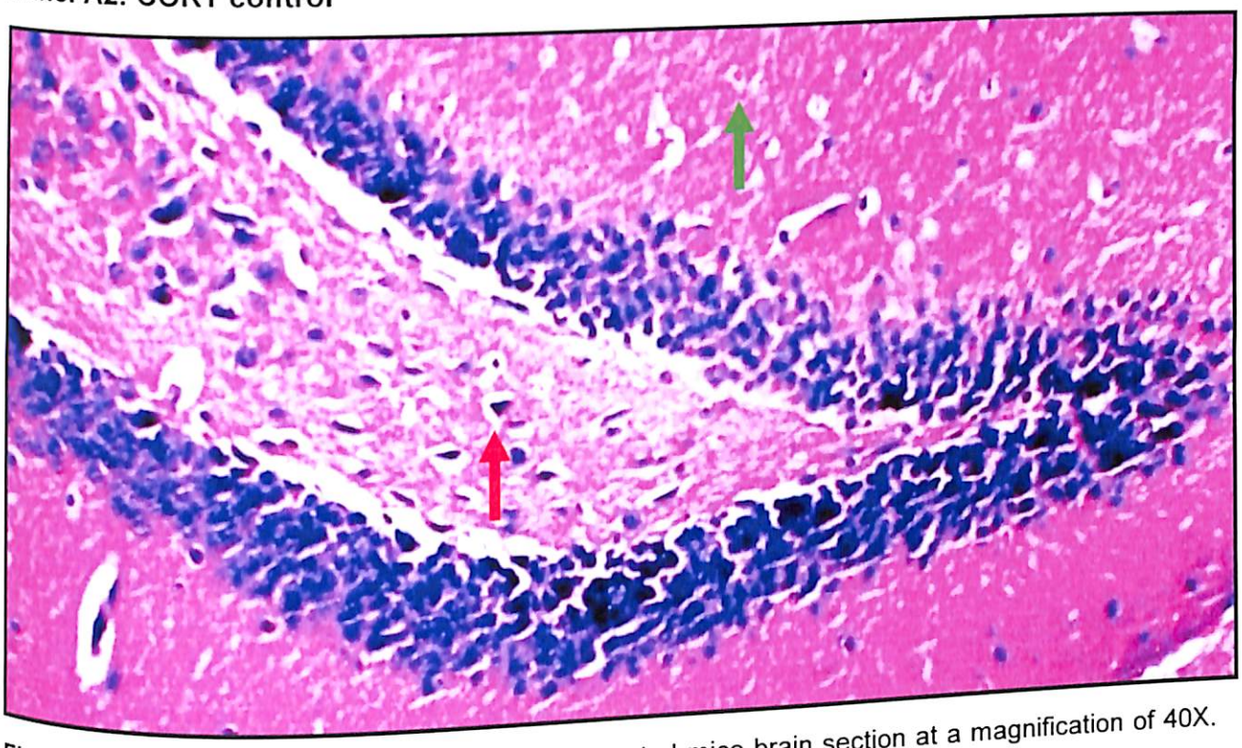


Fig. 71 (A2): H&E stained DG region of CORT-treated mice brain section at a magnification of 40X. Green arrow indicate healthy neurons, whereas, red arrow indicate damaged and shrinked neurons,

Panel A3: CORT + ETZ (0.5)

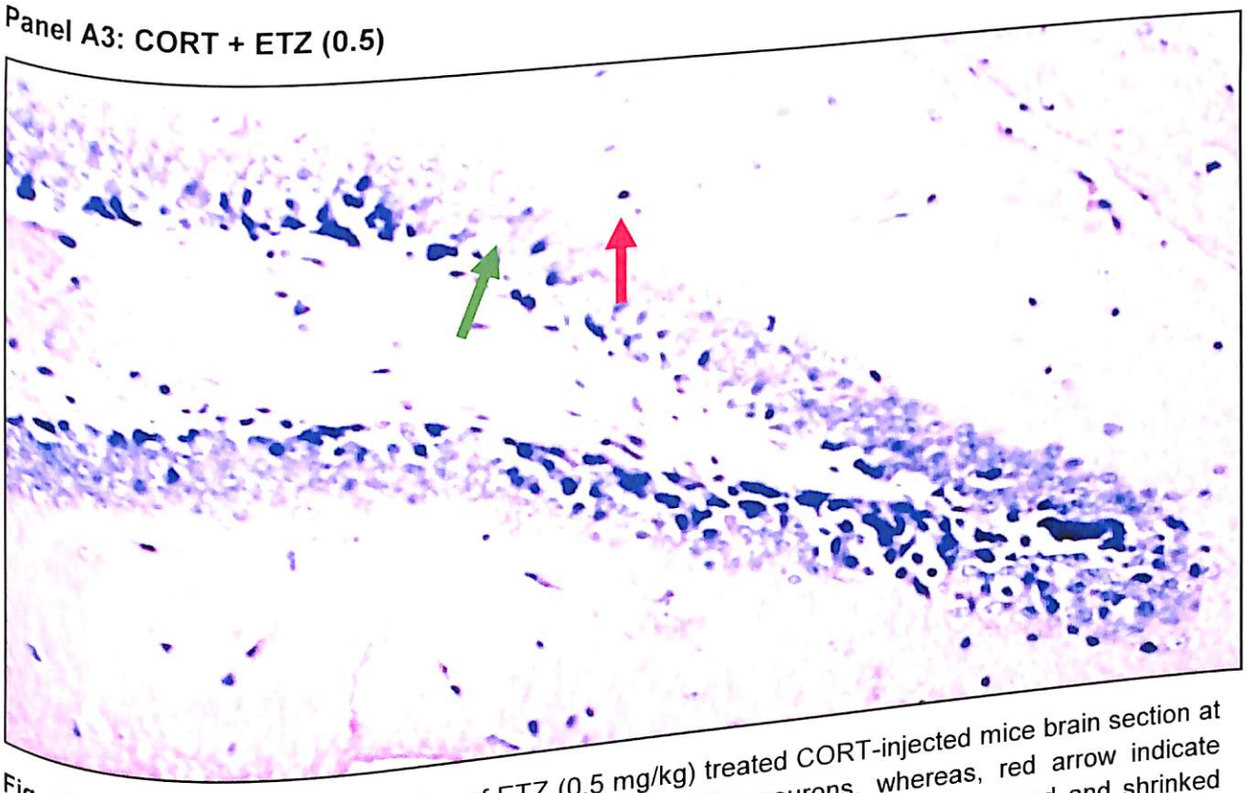


Fig. 71 (A3): H&E stained DG region of ETZ (0.5 mg/kg) treated CORT-injected mice brain section at a magnification of 40X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. ETZ (0.5 mg/kg) showed less numbers of damaged and shrinked neurons and neuro-protection.

Panel A4: CORT + ETZ (1)

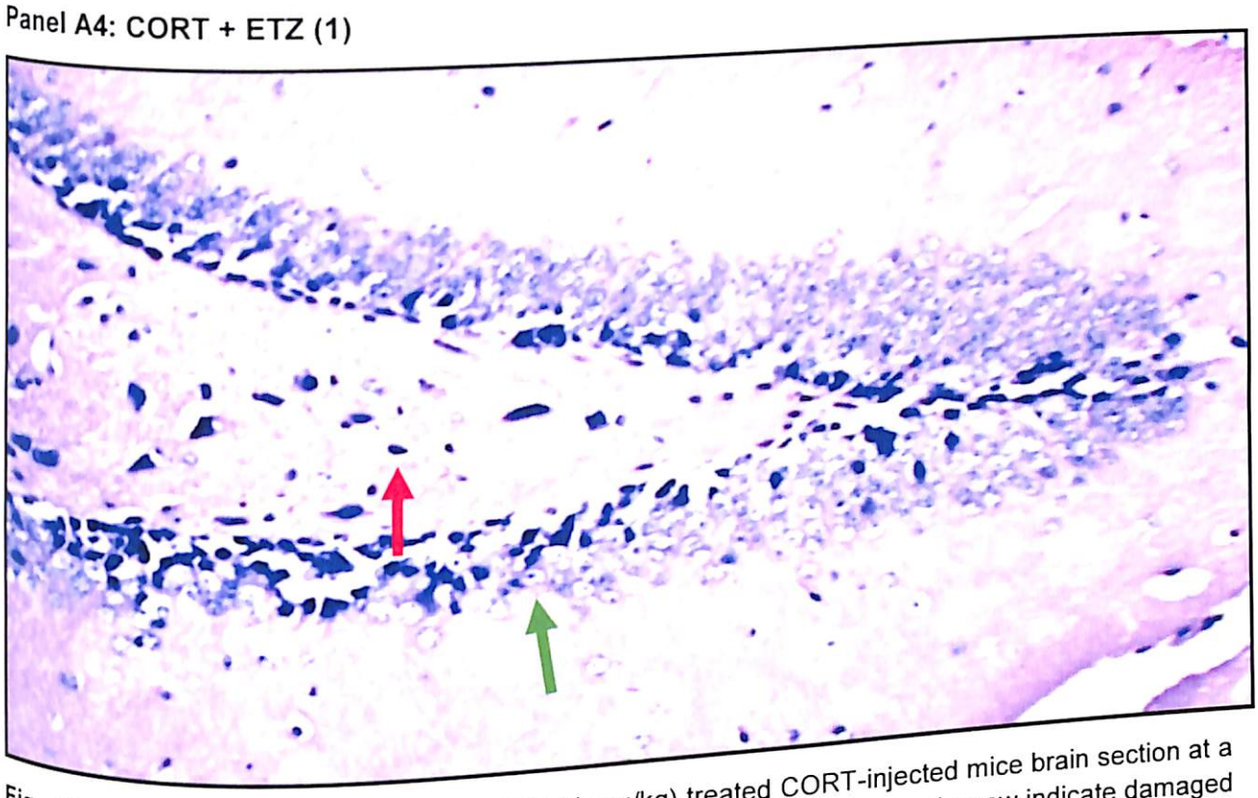


Fig. 71 (A4): H&E stained DG region of ETZ (1 mg/kg) treated CORT-injected mice brain section at a magnification of 40X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. ETZ (1 mg/kg) showed overall appearance similar to the sham group and less neuronal loss.

Panel A5: CORT + Q-21 (1)

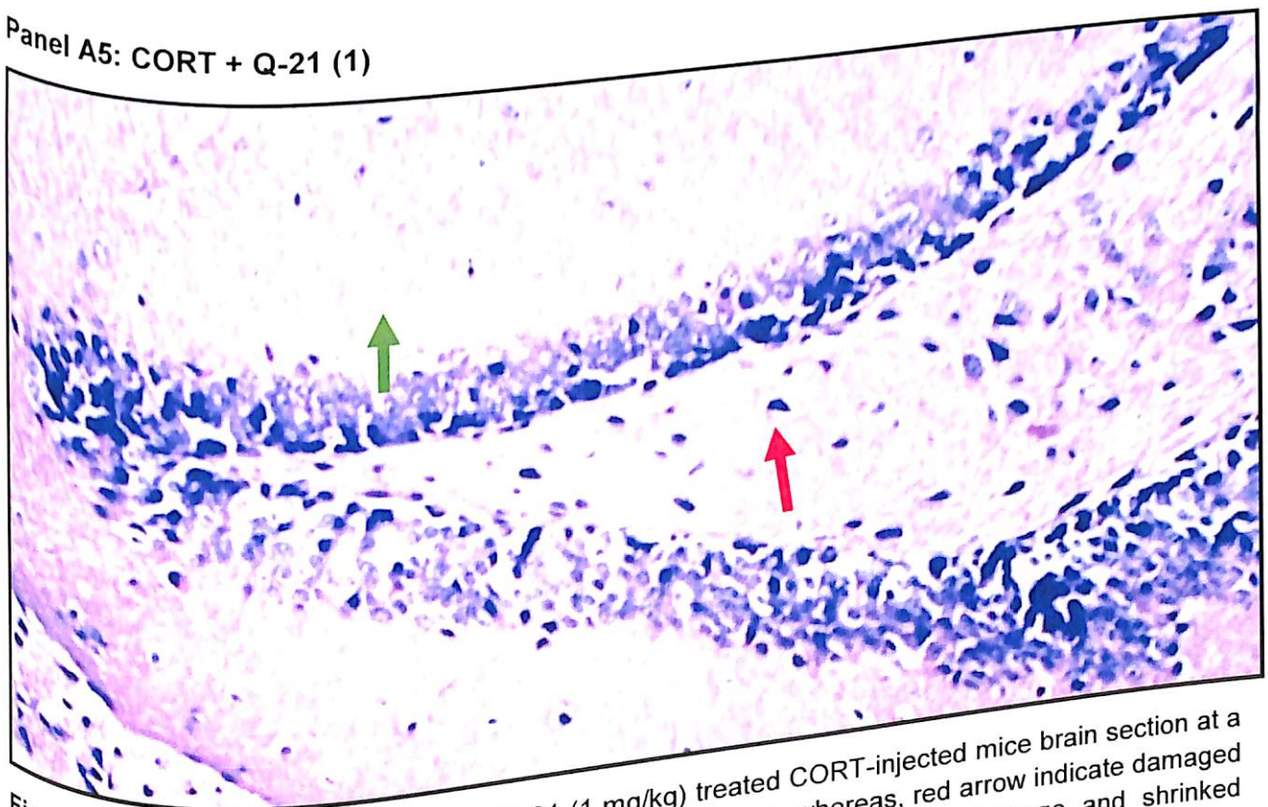


Fig. 71 (A5): H&E stained DG region of Q-21 (1 mg/kg) treated CORT-injected mice brain section at a magnification of 40X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. Q-21 (1 mg/kg) treatment showed mild neuronal damage and shrinked neurons.

Panel A6: CORT + Q-21 (2)

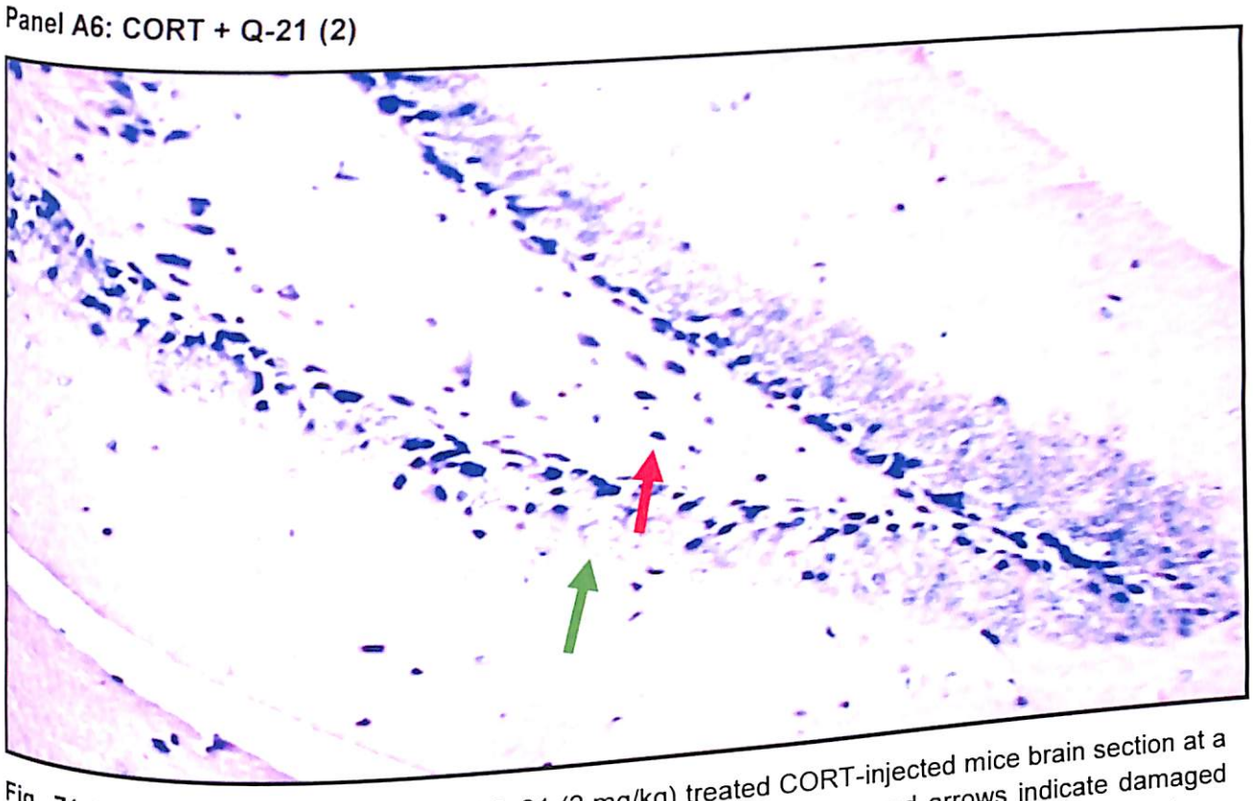


Fig. 71 (A6): H&E stained DG region of Q-21 (2 mg/kg) treated CORT-injected mice brain section at a magnification of 40X. Green arrow indicate healthy neurons, whereas, red arrows indicate damaged and shrinked neurons. Q-21 (2 mg/kg) treatment showed minimal neuronal loss and neuro-protection.

Panel A7: CORT + FLX (20)

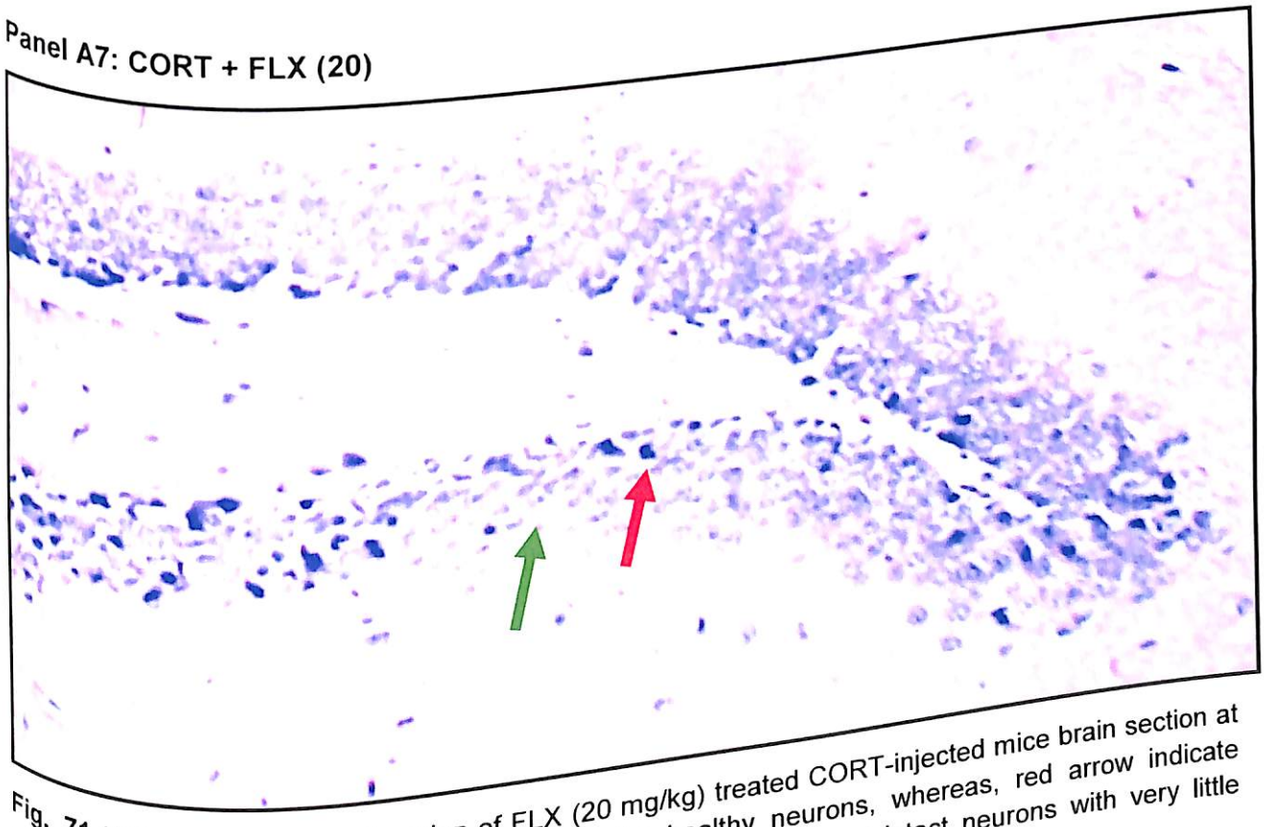
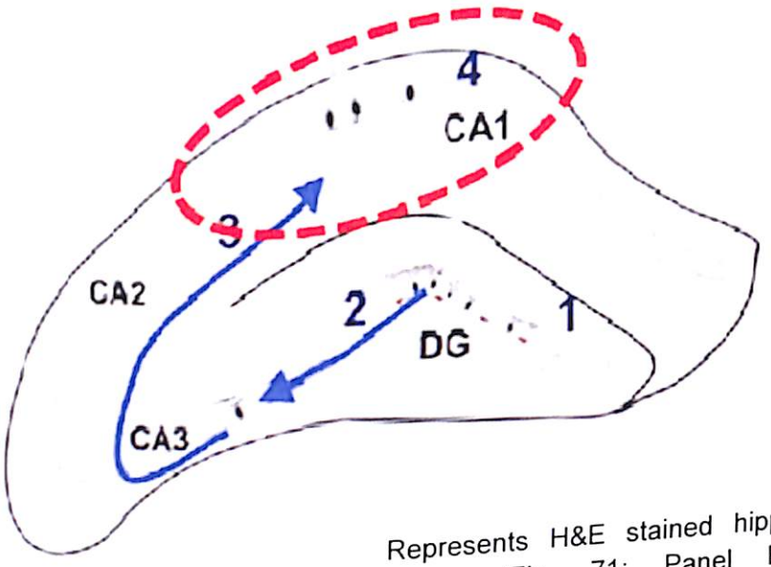


Fig. 71 (A7): H&E stained DG region of FLX (20 mg/kg) treated CORT-injected mice brain section at a magnification of 40X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. FLX (20 mg/kg) treatment showed intact neurons with very little neuronal loss and overall appearance similar to the sham group.

5.7.2. ETZ and Q-21 Effect on Neuronal Structure in Hippocampal CA1 Region of CORT-treated mice



Represents H&E stained hippocampal CA1 region (Fig. 71; Panel B1-B7): **Total magnification = 100** [Eye piece magnification (10) X objective magnification (10)]

Panel B1: Normal Control

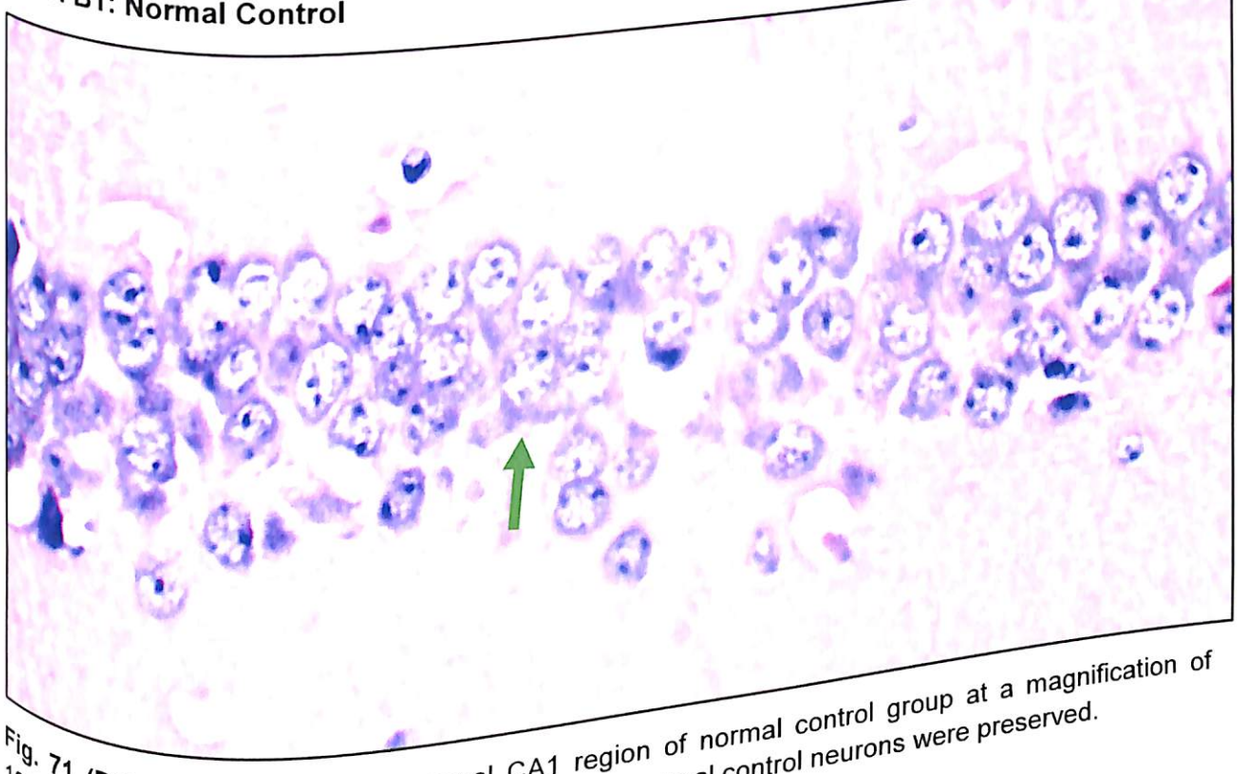


Fig. 71 (B1): H&E stained hippocampal CA1 region of normal control group at a magnification of 100X. Green arrow indicates the healthy neurons. In normal control neurons were preserved.

Panel B2: CORT control

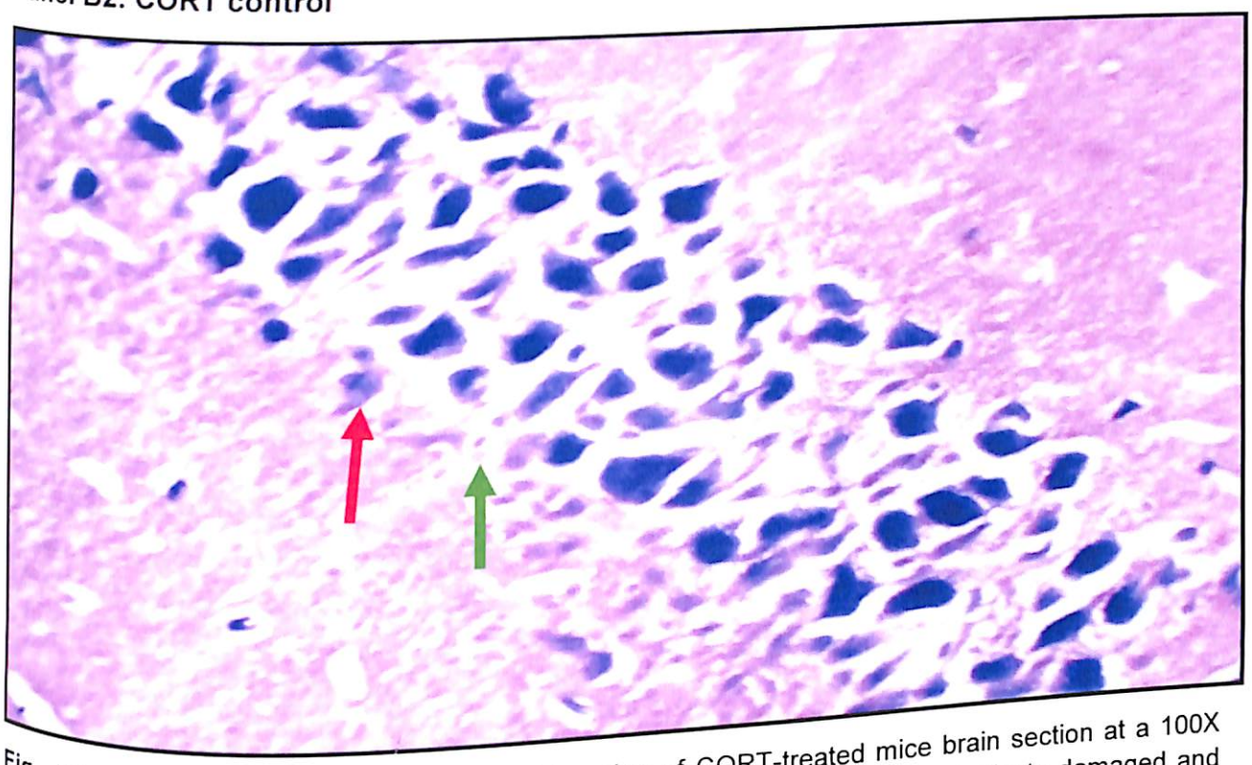


Fig. 71 (B2): H&E stained hippocampal CA1 region of CORT-treated mice brain section at a 100X magnification. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. CORT control group showed large numbers of damaged & shrinked neurons.

Panel B3: CORT + ETZ (0.5)

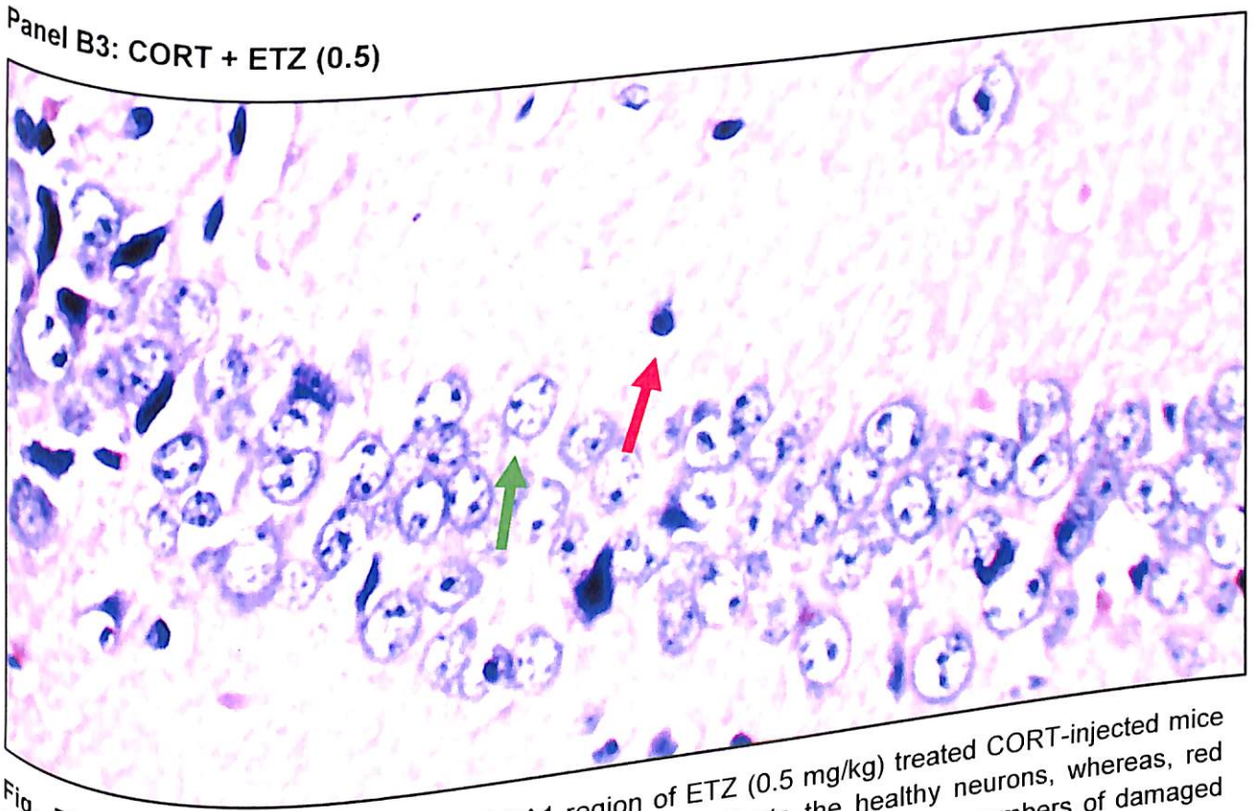


Fig. 71 (B3): H&E stained hippocampal CA1 region of ETZ (0.5 mg/kg) treated CORT-injected mice brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. ETZ (0.5 mg/kg) showed less numbers of damaged and shrinked neurons and neuro-protection.

Panel B4: CORT + ETZ (1)

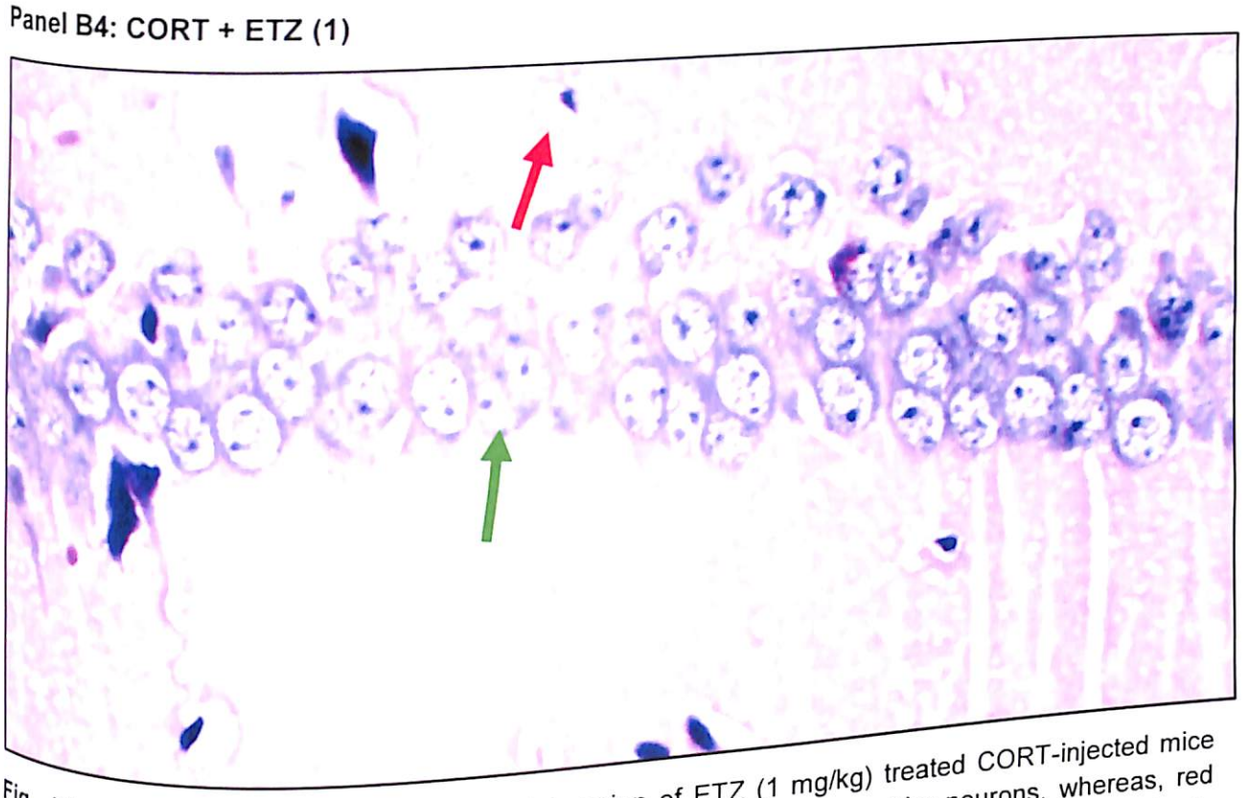


Fig. 71 (B4): H&E stained hippocampal CA1 region of ETZ (1 mg/kg) treated CORT-injected mice brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. ETZ (1 mg/kg) showed overall appearance similar to the normal group and very little neuronal loss.

Panel B5: CORT + Q-21 (1)

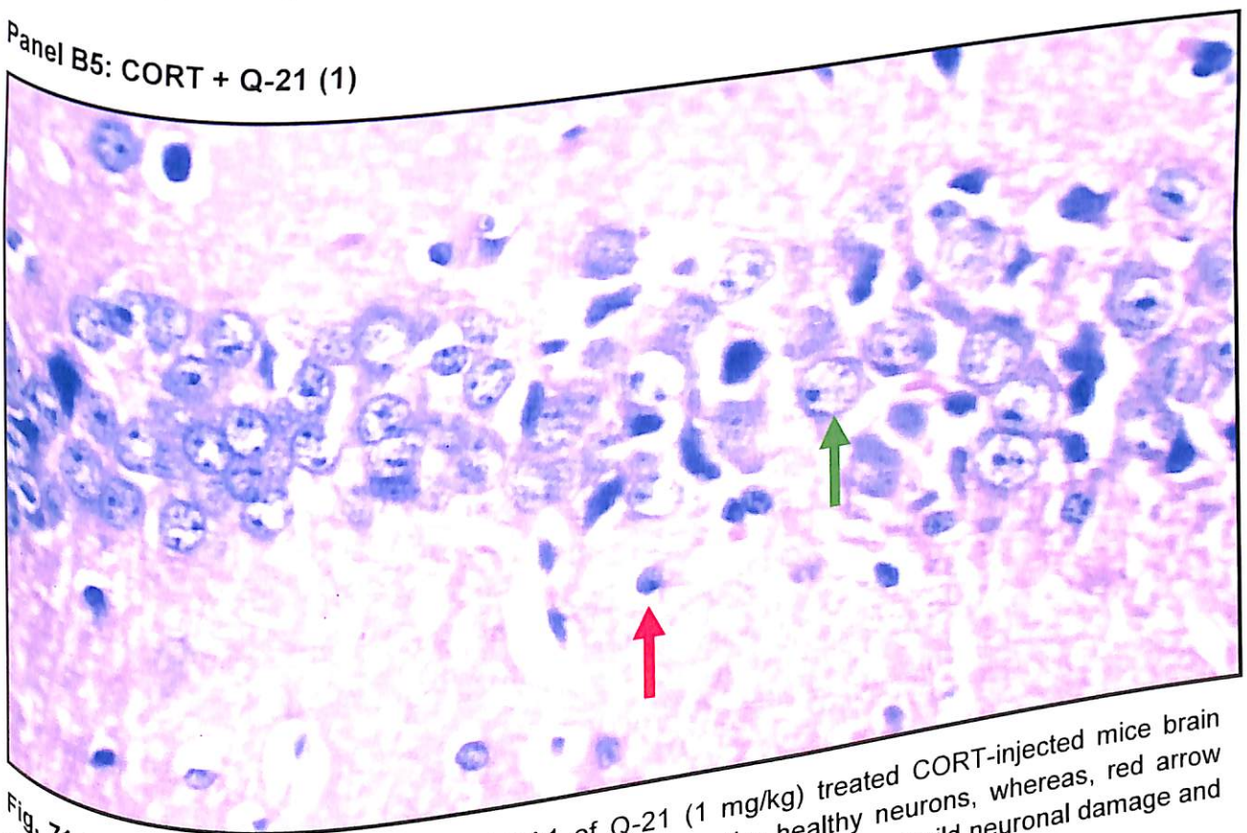


Fig. 71 (B5): H&E stained hippocampal CA1 of Q-21 (1 mg/kg) treated CORT-injected mice brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. Q-21 (1 mg/kg) treatment showed mild neuronal damage and shrinked neurons.

Panel B6: CORT + Q-21 (2)

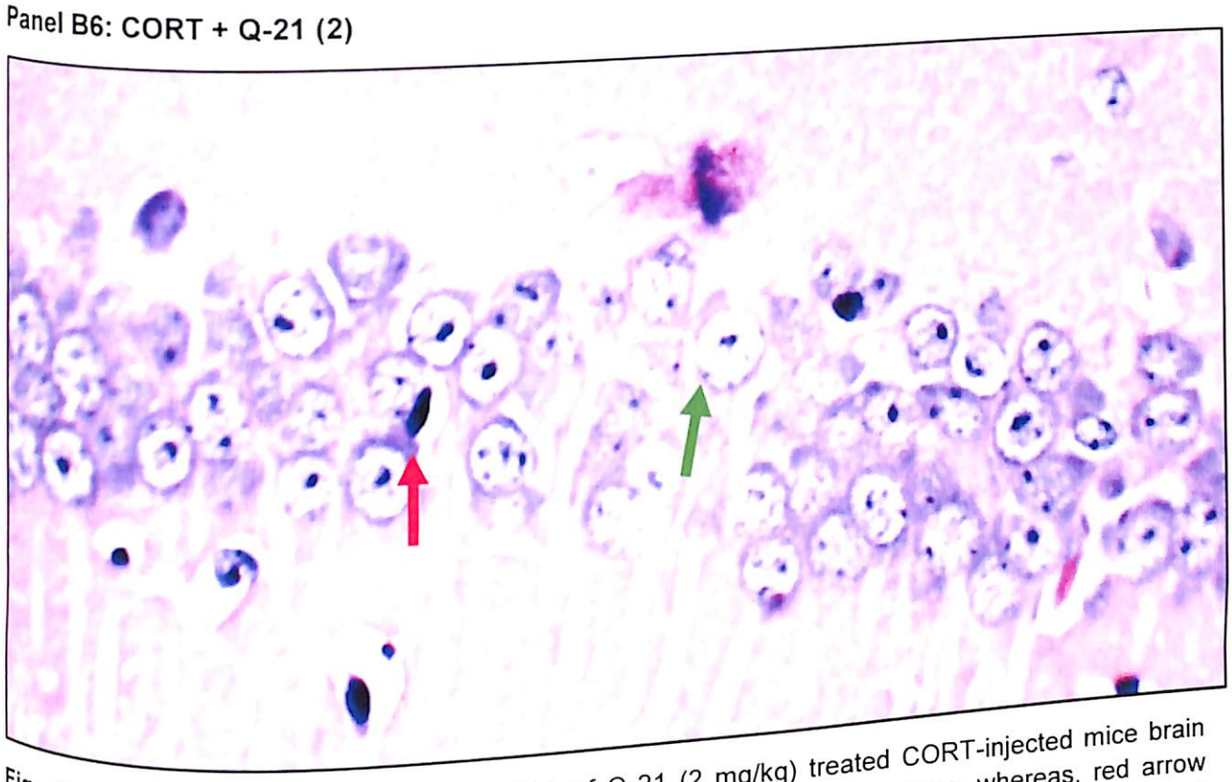


Fig. 71 (B6): H&E stained hippocampal CA1 of Q-21 (2 mg/kg) treated CORT-injected mice brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. Q-21 (2 mg/kg) treatment showed minimal neuronal loss and neuro-protection.

Panel B7: CORT + FLX (20)

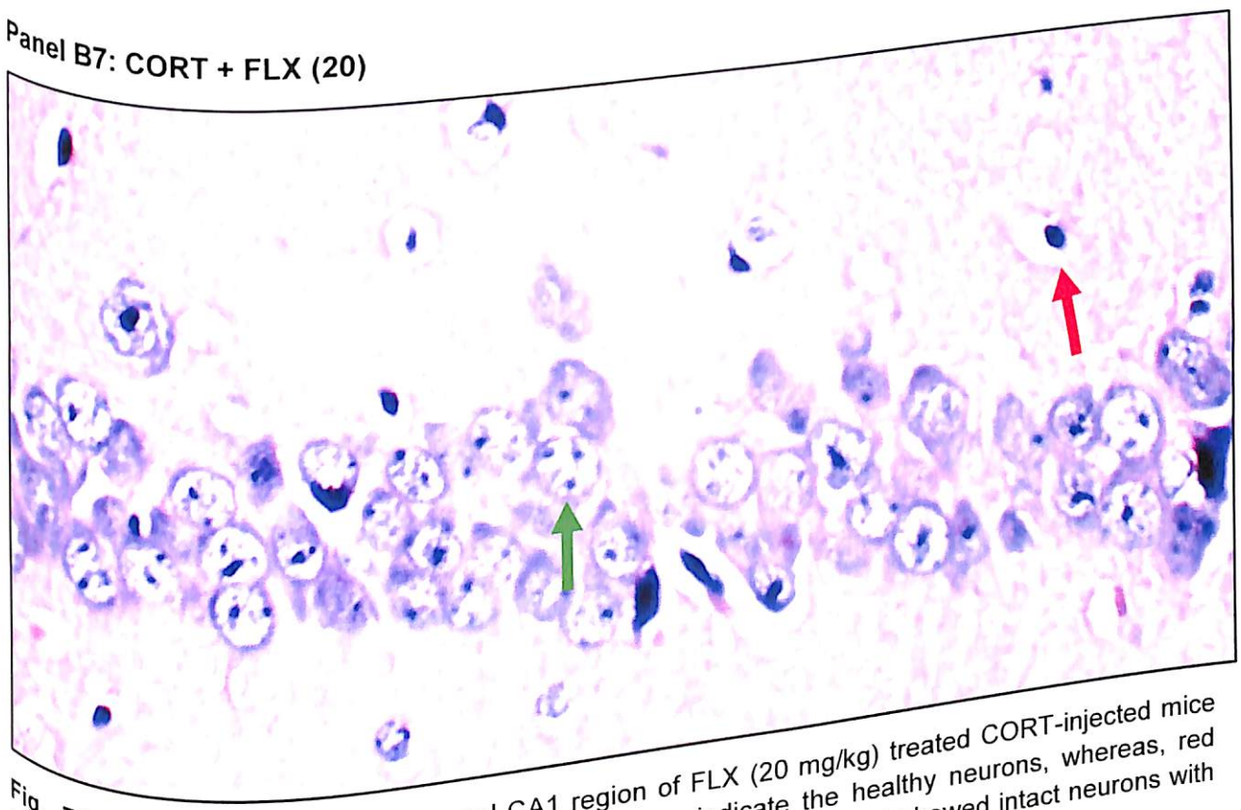


Fig. 71 (B7): H&E stained hippocampal CA1 region of FLX (20 mg/kg) treated CORT-injected mice brain section at a magnification of 100X. Green arrow indicate the healthy neurons, whereas, red arrow indicate damaged and shrinked neurons. FLX (20 mg/kg) treatment showed intact neurons with little neuronal loss.

Fig. 72 A: Neuronal density in DG region of CORT-treated mice

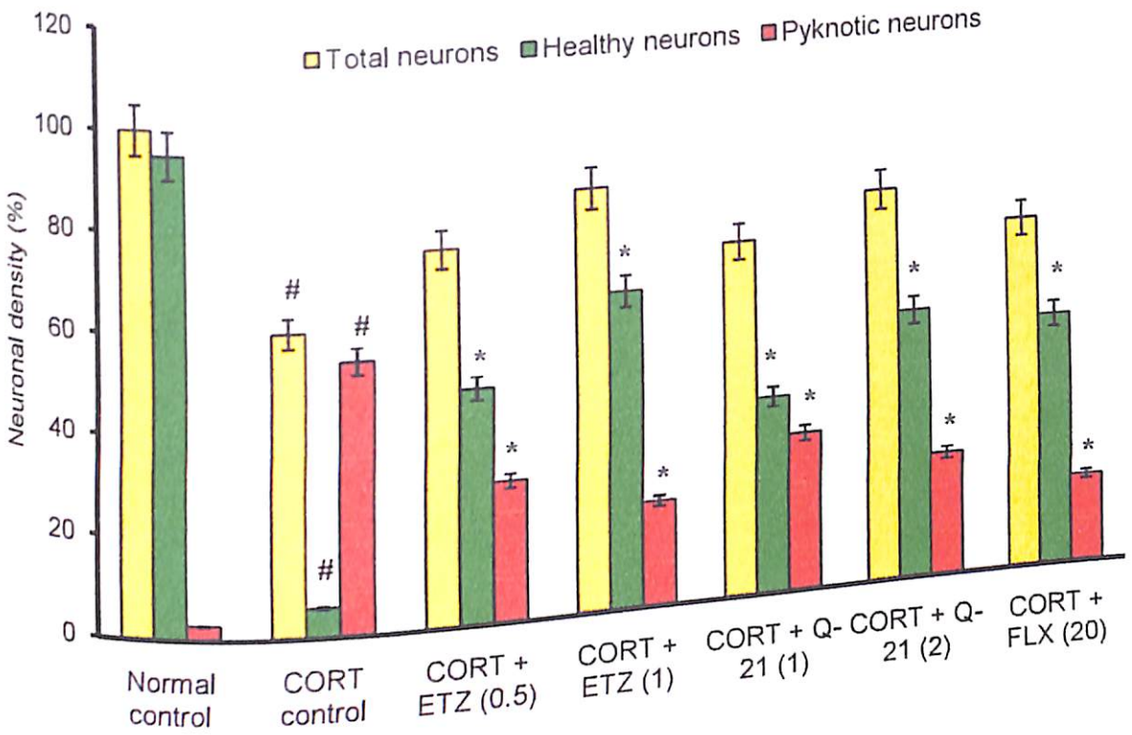


Fig. 72 B: Neuronal density in hippocampal CA1 region of CORT-treated mice

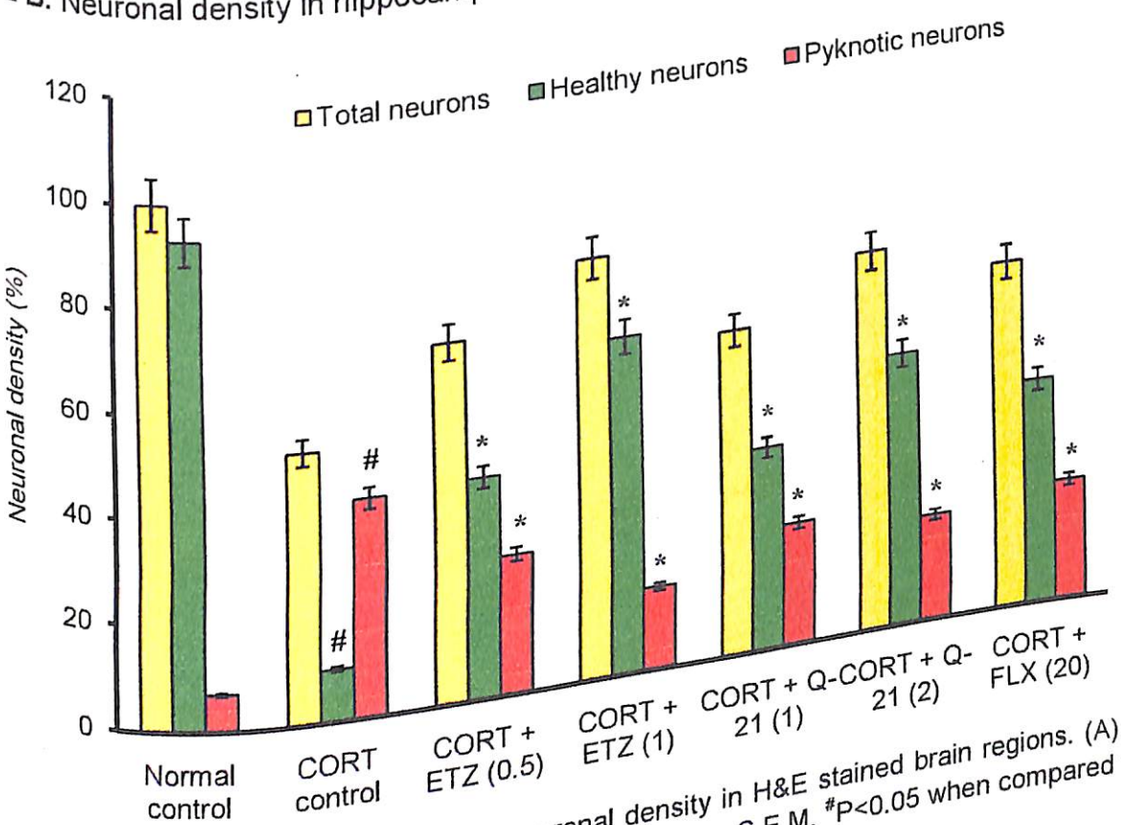


Fig. 72. Effect of selective ETZ and Q-21 on neuronal density in H&E stained brain regions. (A) DG region and (B) Hippocampal CA1 region. The error bar indicates S.E.M. #P<0.05 when compared with normal control, *P<0.05 when compared with CORT-treated control.

Table 60: Effect of PDE4 inhibitors on behavior, biochemical, neurobiological & neurochemical deficits in animal models of depression & anxiety

Test	Parameters	FLX		ETZ (Dose and Net effect)					Q-21 (Dose and Net effect)					ROL (Dose and Net effect)					Q-12 (Dose and Net effect)				
				(Dose and Net effect)				Rem-arks	(Dose and Net effect)				Rem-arks	(Dose and Net effect)				Rem-arks	(Dose and Net effect)				Rem-arks
		10	20	0.12	0.25	0.5	1		0.25	0.5	1	2		0.12	0.25	0.5	1		0.25	0.5	1	2	
FST	Duration of Immobility	S	—	NS	S	S	S	AD-like effect	NS	S	S	S	AD-like effect	NS	S	S	S	AD-like effect	NS	S	S	S	AD-like effect
	Swimming Episodes	S	—	NS	S	S	S		NS	S	S	S		NS	S	S	S		NS	S	S	S	
TST	Duration of Immobility	S	—	NS	S	S	S		NS	S	S	S		NS	S	S	S		NS	NS	S	S	
HTR	Number of Head Twitch	S	—	—	—	S	S		—	—	—	—		—	—	S	S		—	—	—	—	
RIH	Decrease in Temperature	S	—	—	—	S	S		—	—	—	—		—	—	S	S		—	—	—	—	
Behavioral Assessment Performed Post-OBX/TBI																							
OFT	Ambulation, Rearing, Fecal	S	—	—	—	S	S	AD-like effect	—	—	S	S	AD-like effect	—	—	S	S	AD-like effect	—	—	NS	S	AD-like effect
	Sucrose Consumption	S	—	—	—	S	S		—	—	S	S		—	—	S	S		—	—	NS	S	
	Hyper-emotionality Score	S	—	—	—	S	S		—	—	S	S		—	—	S	S		—	—	NS	S	
Behavioral Assessment Performed Post-CUMS/Chronic CORT																							
FST	Duration of Immobility	—	S	—	—	S	S	AD-like effect	—	—	S	S	AD-like effect	—	—	—	—		—	—	—	—	
	Swimming Episodes	—	S	—	—	S	S		—	—	S	S		—	—	—	—		—	—	—	—	
TST	Duration of Immobility	—	S	—	—	S	S		—	—	S	S		—	—	—	—		—	—	—	—	
	Sucrose Consumption	—	S	—	—	S	S		—	—	S	S		—	—	—	—		—	—	—	—	
SLA test	Locomotor Scores	—	S	—	—	S	S		—	—	S	S		—	—	—	—		—	—	—	—	

Neuroendocrine Markers Estimation Performed Post-OBX/CUMS/CORT-treated Models																					
	Serum CORT	S	S	—	—	S	S		—	—	S	S		—	—	S	S		—	—	NS	S
Neurobiological Markers Estimation Performed Post-OBX/TBI/CUMS/CORT-treated Models																					
	cAMP	S	S	—	—	S	S		—	—	S	S		—	—	S	S		—	—	NS	S
	pCREB	S	S	—	—	S	S		—	—	S	S		—	—	S	S		—	—	NS	S
	BDNF	S	S	—	—	S	S		—	—	S	S		—	—	S	S		—	—	NS	S
Biochemical Markers Estimation Performed Post-OBX/TBI/CUMS Models																					
	TBARS	S	S	—	—	S	S		—	—	S	S		—	—	—	—		—	—	—	—
	Nitrite/nitrate	S	S	—	—	S	S		—	—	S	S		—	—	—	—		—	—	—	—
	GSH	S	S	—	—	S	S		—	—	S	S		—	—	—	—		—	—	—	—
	SOD	S	S	—	—	S	S		—	—	S	S		—	—	—	—		—	—	—	—
	CAT	S	S	—	—	S	S		—	—	S	S		—	—	—	—		—	—	—	—
Neurotransmitter Markers Estimation Performed Post-OBX/CUMS Models																					
	5-HT	S	S	—	—	NS	NS		—	—	NS	NS		—	—	—	—		—	—	—	—
	NE	S	S	—	—	NS	NS		—	—	NS	NS		—	—	—	—		—	—	—	—
	DA	NS	NS	—	—	NS	NS		—	—	NS	NS		—	—	—	—		—	—	—	—
		DZM 2						**Behavioral Assessment Performed for Anxiolytic Screening**														
EPM test	% OAE	S		—	NS	S	S	Anti anxiety effect	—	NS	S	S	Anti-anxiety effect	—	NS	S	S	Anti-anxiety effect	—	NS	NS	S	Anti-anxiety effect
	%OAT	S		—	NS	S	S		—	NS	S	S		—	NS	S	S		—	NS	NS	S	
	Closed Arm Entries	NS		—	NS	S	S		—	NS	S	S		—	NS	S	S		—	NS	NS	S	
L/D test	Latency to Leave lit area	S		—	NS	S	S		—	NS	S	S		—	NS	S	S		—	NS	NS	S	
	Total Time Spent in Lit Area	S		—	NS	S	S		—	NS	S	S		—	NS	S	S		—	NS	NS	S	
	Transition Number	S		—	NS	NS	NS		—	NS	S	S		—	NS	NS	NS		—	NS	NS	S	
HB test	Head dipping Number	S		—	NS	S	S		—	—	—	—		—	NS	S	S		—	—	—	—	
	Time spent in Head dipping	S		—	NS	S	S		—	—	—	—		—	NS	S	S		—	—	—	—	
	Latency to Head dipping	S		—	NS	S	S		—	—	—	—		—	NS	S	S		—	—	—	—	

Keys: --: Test drugs/or dose level not tested; **S:** significant effect; **NS:** Non-significant effect

Chapter 6: Discussion

6. Discussion

The results of present neuro-psychopharmacological investigation show the AD-like response of existing (ROL and ETZ) and in-house synthesized (Q-21 and Q-12) PDE4 inhibitors in the acute and chronic rodent models that predicts the efficacy of AD drugs. Acute treatment exhibited AD-like effects in the FST and TST models and anxiolytic-like effect in EPM, L/D and HB tests at selected doses. In addition, pre-treatment with ROL and ETZ potentiated 5-HTP-induced HTR in mice and reversed RIH in rats. Interaction studies of ROL and ETZ with various standard ADs indicated that both of these drugs produced synergistic effect with standard ADs in the FST. Chronic treatment with test drugs reversed OBX- and TBI-induced behavioral deficits as indicated in the OFT, hyper-emotionality test and sucrose preference test. PDE4 inhibitors also reversed the OBX- and TBI-induced biochemical (oxidant/anti-oxidant parameters) and neurobiological alterations (HPA axis activity and cAMP signaling aspect, such as cAMP, pCREB and BDNF). Furthermore, ETZ and Q-21 reversed the CUMS-induced depressive-like symptoms, along with biochemical and neurobiological alterations. ETZ and Q-21 also expressed beneficial effects on chronic CORT-injection-induced depression-like symptoms by reversing behavioral, biochemical and neurobiological parameters.

6.1. Antidepressant-like Potential of Phosphodiesterase-4 Inhibitors in Acute Models

While assessing the AD potential of a test drug, the psychomotor stimulation/sedation can lead to a false positive/negative result in behavioral despair assays, as the psychomotor stimulation/sedation property of a test compound mimics AD/depressant-like behavioral effect of rodents in FST and TST (Porsolt et al., 1977; Steru et al., 1985). Thus, the influence of test compounds on the locomotor status of animal is a governing concern. To eliminate non-specific motor effects of ROL, ETZ, Q-21 and Q-12 that could influence the activity in FST and TST, locomotor activities of all test drugs in mice were evaluated. Interestingly, all the tested drugs did not influence the basal locomotor scores as compared to normal control mice in SLA test at selected dose range. Following doses selection, as per findings from SLA test, all test drugs were tested for their AD- and anxiolytic-like potential.

The preliminary AD-like effects of test drugs were evaluated in the FST, a behavioral despair test. Since 1977, when the original report by Porsolt and Colleagues was published, the FST has been widely used for assessing the effectiveness of candidate ADs, as well as to investigate the underlying mechanisms of action of ADs (Porsolt et al., 1977). Acute treatment with ROL and ETZ markedly decreased the duration of immobility and increased the swimming episodes in mice FST in dose dependent manner. In another set of study, in-

house synthesized PDE4 inhibitors, namely, Q-21 and Q-12 also notably decreased the immobility duration and increased the swimming episodes in mice FST at different doses. Porsolt and Colleagues (1977) have been shown that decrease in the duration of immobility in FST, reflects the AD-like potential of molecules. Moreover, earlier investigations have suggested that PDE4 enzyme involved in the patho-physiology of depression (Wang et al., 1997; Dlaboga et al., 2006) and molecules with PDE4 inhibitory activity could be used for AD-like effect (Horovitz et al., 1972; Halene and Siegel, 2007). Furthermore, PDE4 KO mice displayed a reduction in immobility time in FST by increasing swimming episodes (Zhang et al., 2002).

Effects of PDE4 inhibitors on depression-like behavior were also investigated using TST, another behavioral despair model of depression. TST is well established screening paradigm for AD/depressant-like effect of molecules. The principle for this test is similar to FST, where an animal is placed in inescapable situation and initial attempts to escape are followed by prolonged duration of immobility. Acute treatment with ROL and ETZ significantly decreased the duration of immobility in mice TST. In another set of study, both Q-21 and Q-12 also significantly decreased the duration of immobility in mice TST. Decrease in the duration of immobility in TST is an indicative of AD-like effect (Steru et al., 1985; Lucki, 1997). Zhang and Colleagues (2002) have indicated that PDE4 KO mice exhibited decreased immobility time in the TST, which has positive predictive value for an AD-like effect in humans. The results of FST and TST strongly support the AD-like potential of PDE4 inhibitors. Moreover, the AD-like effects of ROL, ETZ, Q-21 and Q-12 in the FST and TST were not due to hyper-locomotive effects as indicated by the SLA test.

Further, in the present study to identify the involvement of cAMP in AD-like effects of PDE4 inhibitors, 5HTP-induced HTR and RIH models were performed. The 5HTP-induced HTR and RIH models were performed for the constructive validity.

Induction of HTR in rodent is a characteristic behavioral phenomenon of AD-like activity (Wachtel, 1982). The observation of HTR and subsequent administration of a PDE4 inhibitor may indicate AD-like activity with a similar mechanism to prototype PDE4 inhibitor, like ROL (Wachtel, 1989). In the current investigation, pargyline and 5-HTP-induced HTRs were significantly increased by ROL and ETZ pre-treatment. It has been reported earlier that increased brain cAMP availability induce a characteristic HTR in mice (Wachtel, 1982). Inhibition of PDE4 is shown to induce significant rise in cAMP and augments both the intensity and duration of cAMP signaling (Scuvee-Moreau et al., 1987).

Further, antagonism of RIH in rats, another model in rodents was used for AD drugs identification (Askew, 1963; Van Riezen and Delver, 1971). Antagonism to hypothermic effect of reserpine may be associated with ADs-like effect of a molecule (Wachtel and Schneider, 1986). In the present study, ROL and ETZ significantly prevented the hypothermic effect of reserpine indicating AD-like effect in this sensitive model. The results of both the 5-HTP and RIH models strongly support that the AD-like effects of PDE4 inhibitors can be attributed to the increase in cAMP level in brain.

Interaction studies with conventional ADs are essential for conclusive appraisal of AD potential (Cryan et al., 2005). The synergistic effects of combination of ROL and ETZ with existing ADs were carried out in FST. On the basis of preliminary results the sub-effective doses of ROL (0.12 mg/kg) and ETZ (0.12 mg/kg) were selected for the interaction studies. Pilot studies conducted in our laboratory revealed that ETZ and ROL at 0.12 mg/kg, failed to produce any AD-like effects in FST. In the present study, ROL and ETZ pre-treatment at sub-effective dose was found to enhance AD-like effects of conventional ADs in FST.

In fact, extensive studies have been suggested that conventional ADs influence the cAMP signal transduction pathway (Fleischhacker et al., 1992; Zhao et al., 2003b). Moreover, increase in cAMP level might be likely to increase the sensitivity to and efficacy of conventional ADs (Fujimaki et al., 2000). It is well reported that PDE4 inhibitors regulate the intra-cellular concentration of cAMP in brain (Santarelli et al., 2003). In this regard, synergistic AD-like effect of ROL and ETZ with conventional ADs may be modulated through cAMP-mediated signal transduction pathway.

6.2. Anxiolytic-like Potential of Phosphodiesterase-4 Inhibitors in Animal Models

In the present study, anxiolytic effect of standard and in-house synthesized PDE4 inhibitors was also examined in experimental models of anxiety, such as EPM, L/D and HB. The results of the present study substantiate the designed hypothesis that inhibitors of PDE4 enzyme may produce anxiolytic-like effect. Although, it is uncertain that any single animal model captures all of the components of the complex expression of anxiety, thus, a battery of tests have been used to evaluate the potential anxiolytic-like effects.

EPM is considered as one of the well established model for unconditioned anxiety to detect anxiolytic/anxiogenic-like activity by investigating aspects of physiological and pharmacological behavior. In the EPM test, increase in both OAE and TSOA are the most reliable indicators of decrease anxiety or indicating the anxiolytic-like activity of a compound, while anxiogenic substances have the opposite effects (Masood et al., 2008). In the current

study, treatment with ROL and ETZ produced anxiolytic-like effect, as evidenced by increase percentage of both OAE and TSOA. This data is in concurrence with other studies, which suggest that PDE4 inhibitors may be produced anxiolytic activity in EPM test by increasing percentage of both, number of OAE and TSOA (Silvestre et al., 1999a; Li et al., 2011). Another set of studies included Q-21 and Q-12, which were also evaluated for their anxiolytic effects. Q-21 showed potential anxiolytic-like effect, although, Q-12 produced pronounced anxiolytic-like effect only at higher dose in the EPM test.

The L/D test is another widely used animal model for screening anxiolytic/anxiogenic drugs by utilizing the animal's natural preference for dark spaces (Crawley and Goodwin, 1980). This test is designed to exploit the tendency of rodents to explore a novel environment, when confronted with the aversive properties of a brightly lit area (Crawley, 1981). In the present study, we found that ROL, ETZ, Q-21 and Q-12 (higher dose only) treatment markedly increased the time spent in and latency time to leave the light compartment. Considerable data showed that anxiolytic-like effect of a compound in this test may be reflected by an increase in time spent in and latency time to leave light compartment (Bradley et al., 2007).

Review of literature indicates that the number of transitions between the two compartments is a controversial parameter (Crawley and Goodwin, 1980). Some studies have reported that an anxiolytic drug increased the transitions between the two compartments, while others reported no significant changes in the transition number after treatment with an anxiolytic. In the present study, ROL and ETZ treatment failed to influence the number of transitions between the two compartments, whereas, in other set of study Q-21 and Q-12 were observed to influence the transitions between the two compartments.

The anxiolytic-like effect of ROL and ETZ was further confirmed using the HB test. Currently, the HB test is popular as a model of anxiety and offers a simple method for measuring the behavioral response of rodents to an unfamiliar environment (Takeda et al., 1998). The head dipping behavior of a rodent in HB test is sensitive to change in emotional state of animal (Nolan and Parkes, 1973). The results of the present study, revealed the anxiolytic-like activity of ROL and ETZ in HB test, as evidenced by predominantly increase in head dipping number and time spent in head dipping. This effect is in agreement with previous study suggesting that increase in head dipping number and time spent in head dipping reflects the anxiolytic-like activity of a molecule (Hranilovic et al., 2005). Moreover, in this study, a marked decrease in head dipping latency of mice was also observed, indicating the anxiolytic-like activity of ROL and ETZ.

After the confirmation of preliminary AD- and anxiolytic-like potential, the further key purpose of the work was to design/standardize chronic animal model(s) of depression in our laboratory. To simulate human depression, animal models such as OBX, TBI, CUMS and chronic CORT-injection were standardized in the laboratory setting. The standardized chronic models were employed for the screening of AD-like potential of the PDE4 inhibitors, to identify their potential usefulness in the treatment or prevention of depression. The present study, thus, captivates the information of these chronic models and potential effects of PDE4 inhibitors. Post inductions of traumatic insult by various procedures, battery of behavioral tests were performed with slight modification, which can simulate the symptoms of human depression. Treatment schedules were designed for the studies to reverse the behavioral anomalies induced by OBX, TBI, CUMS and chronic CORT-treated models.

6.3. Evaluation of ROL, ETZ, Q-21 and Q-12 in Olfactory Bulbectomy Model

OBX is a well known chronic model of depression (Kelly et al., 1997). This study was conducted to investigate the possible depression-like behavior and the potential effects of PDE4 inhibitors in OBX model through behavioral and biochemical test batteries of depression. A set of depression tests were performed in OBX rats to simulate the symptoms of depression. An impressive number of animal models to assess depression individually are available today; however, the validity criteria between these models and the clinical syndromes of depression are not always clear. Thus, a behavioral test is an important tool to simulate the symptoms of human depression disorder. All test drugs (ROL, ETZ, Q-21 and Q-12) were tested individually for behavioral deficits (depression), biochemical and neurobiological markers alterations-induced by OBX in rats.

6.3.1. Effect of PDE4 inhibitors on Behavioral, Biochemical, Neurobiological Deficits and Neuronal Degeneration in OBX Rats

The rat OBX model (with adequate face and predictive validity) has been validated as a model of depression over the past 20 years (Van Reizen and Leonard, 1990; Song and Leonard, 2005). This is primarily known as a model for predicting AD-like activity, as in an experimental setup its hyperactivity is normalized following chronic, but not acute administration of ADs (Song and Leonard, 2005; Breuer et al., 2007). OBX-induced lesion in rats showed depression-like behavioral anomalies. The potential effects of PDE4 inhibitors in this model were explored, using measurement of open-field activity, sucrose solution preference and hyper-emotionality score. Further, the underlying mechanism(s) were also explored by estimating the HPA axis activity (in terms of serum CORT level), cAMP signaling cascade (cAMP, CREB and BDNF levels), NT levels (5-HT, NE and DA) and oxidative (TBARS and nitrite)/anti-oxidant (GSH, SOD and CAT) markers levels.

In the present study, slight reduction in body weight was observed in the first week post-OBX. This result is in line with previous reports that reduction in body weight is observed initially, after the OBX surgery. However, no difference in the body weight of OBX control and drug treated OBX rats were seen, post-OBX.

OFT is a most widely accepted index of hyperactivity. This is the most widely employed test for emotional investigations in OBX rats (Van Riezen and Leonard, 1990). The design of open field arena may be extremely important. On placement in a novel 'open field' environment, OBX rats exhibit behavioral hyperactivity, which reflect the psychomotor retardation, as one of the symptoms of MDD as per DSM. Several studies have addressed that this effect is not related to anosmia, as peripherally-induced anosmia does not affect the 'open field' behavior in a similar fashion. Precipitation of hyperactive response is generally dependent upon high illumination and reflective walls in the 'open field' and is associated with stress-induced behaviors, like thigmotaxis and defecation (Kelly et al., 1997).

In the present study, OBX rats exhibited increase in ambulation, rearing and defecation as compared to sham control rats in open field arena, in response to stressful environment. Hyperactivity showed by OBX rats in a stressful and novel environment could be attributed to a decrease in activity in competing behaviors (Song and Leonard, 2005). In addition, the rate of habituation to the stressful open field is decreased in lessoned animals in comparison to sham operated controls, which is reflected in increased movements of OBX rats. Furthermore, the "stressfulness" of the task conditions may be a major determinant of hyperactivity, observed in OBX animals (O'Connor and Leonard, 1988). We found that chronic ROL and ETZ treatment significantly reversed OBX-induced behavioral changes, such as increased activity in novel environments (Jindal et al., 2012). In other set, chronic treatment with Q-21 (1 & 2 mg/kg), also significantly reversed the hyperactivity exhibited by OBX rats in OFT. Although, Q-12 did not produce pronounce effect on behavioral anomalies in the OFT.

Literature reports that hyper-emotionality is also another behavioral parameter to evaluate the emotional imbalance in rodents. Hyper-emotionality behavior is reflected by violent attacks or flight response to innocuous stimuli. Moreover, previous studies indicate that these types of behaviors have been observed in rats with lesions in brain areas (King, 1958). Thus, there is a possibility that hyper-emotional behavior showed by OBX rats in response to innocuous stimuli may resemble the behavior of psychomotor agitation, which is a diagnostic criterion for depression (Fukuchi and Kanemoto, 2000). Considering this, hyper-emotional behavior in OBX rats may be useful for evaluating AD-like effects of drugs (Shibata and

Watanabe, 1994). In this test, OBX rats exhibited significantly increased hyper-emotional responses to innocuous stimuli, reflecting psycho-motor retardation. Chronic treatment with ROL, ETZ and Q-21 predominantly decreased the hyper-emotional responses in OBX rats. However, Q-12 showed pronounced effects at higher dose level only. Thus, apart from having a strong theoretical rationale, the OBX model was found to exhibit face and predictive validities.

Sucrose preference test is another valuable and valid behavioral indicator of depression disorder in rodent paradigm (Willner et al., 1997; Willner, 2005). In the present study, the effect of all test drugs on percentage of sucrose consumption was investigated, which is a marker of anhedonia in OBX rats (Willner, 2005; Wang et al., 2009). Anhedonia is one of the characteristic features of endogenous depression, according to DSM-IV/V criteria (APA, 1994; Willner, 2005) and is modelled by inducing a decrease in responsiveness to rewards, reflected by a less sucrose consumption and/or preference of sweetened solutions (Willner et al., 1997; Li et al., 2011). In this study, OBX rats consumed less sucrose solution than sham control rats, reflecting anhedonia symptoms. This result is in accordance with earlier studies conducted in our laboratory that OBX rats have low preference and consume less sucrose solution (Pandey et al., 2010). An olfaction is related to emotion and memory behavior and the deficits in olfactory system may lead to anhedonia. Treatment with ROL, ETZ, Q-21 and Q-12 (higher dose only) markedly reversed the reduction in sucrose consumption, which represented the AD-like effects in OBX model. In our laboratory, we showed that a molecule with AD-like property has an ability to reverse the reduction in sucrose consumption in OBX rats (Pandey et al., 2010).

Numerous studies have reported that ablation of olfactory bulb disturbs the neuronal circuitry in several brain regions, which normally receive projection from bulbs. OBX leads the neuronal degeneration remodelling from olfactory bulb to brain areas, such as prefrontal cortex, hippocampus, dorsal raphe, medial raphe and amygdala. Earlier reports on depression addressed that hippocampus region is mostly affected in depression disorder, hence, in the present study; we mainly focused on neurons morphology in the hippocampal area of OBX rats and studied the role of PDE4 inhibitors on OBX-induced neuronal degeneration. The present study provides direct histo-pathological evidences of neuronal damage in DG and hippocampal CA1 regions of OBX rats.

The results of present study provide direct histo-pathological evidences of neuronal damage in hippocampal CA1 and DG regions of OBX rat brain. In this study, OBX rats showed marked morphological changes and neuronal loss in the hippocampal CA1 and DG regions

as compared to sham controls, which showed healthy neurons. This is supported by earlier studies that OBX leads to neuronal loss and decreases neuronal survival in hippocampus region, which is one of the most accepted mechanism(s), implicated in the induction of depression disorder (Jaako-Movits et al., 2006). Chronic 14 days treatment with ETZ and Q-21 remarkably attenuated OBX-induced neuronal loss and decreased pyknotic cell density.

Beside the behavioral and neuro-anatomical assessments in this study, the underlying mechanism(s) of PDE4 inhibitors in OBX models of depression were investigated. Since animal models and predictive tests represent only one or few of the symptoms of psychiatric disorders, which are frequently revised and their pathogenesis being revisited (APA, 2000; O'Neill and Moore, 2003), it is imperative to conduct biochemical and neurobiological investigations to purport OBX rat as a model of depression. The olfactory bulb extends to various brain regions, including cortex, amygdala and hippocampus; consequently, ablation of olfactory bulb produces neurological deficits in these projections and possibly results in numerous neurobiological, biochemical and behavioral alterations (Song and Leonard, 2005). Hence, the aberrations in biochemical and neurobiological parameters are key components in depression and can serve as markers to evaluate the therapeutic potential of PDE4 inhibitors in depression disorder. Neurobiological aspects such as, involvement of neuroendocrine system (HPA axis activity in terms of CORT level), NT levels (5-HT, NE and DA), cAMP signaling aspects [cAMP, transcription factor (CREB) and neurotrophic factor (BDNF)] and oxidant (TBARS and nitrite)/anti-oxidant systems (GSH, SOD and CAT) were measured in OBX model. Results revealed that behavioral changes in OBX rats when subjected to stressful and neophobic situation may be related to the alterations in numerous neurobiological and biochemical pathways **(fig. 73).**

In the current study, OBX rats displayed a significant high serum CORT level as compared to sham control. This data is in accordance with earlier findings that OBX rats displayed a high serum CORT level than sham controls (Cairncross et al., 1979; Machado et al., 2012). However, the data from this study did not match with several other previous studies which indicate no change in the serum CORT level in OBX rats (Broekkamp et al., 1986). The increased level of CORT has been reported to impair the proliferation and survival of neurons and dendritic spines in the hippocampus, which is accountable for the pathophysiology of depression. Chronic ROL, ETZ, Q-21 and Q-12 (only higher dose) treatment decreased the elevated serum CORT level in OBX rats. This result provided the evidence that OBX may lead to alteration in the HPA axis and that PDE4 inhibition restored the HPA axis activity in OBX rats **(fig. 73).** This may be one of the possible mechanisms for the AD-like effect of PDE4 inhibitors in OBX model.

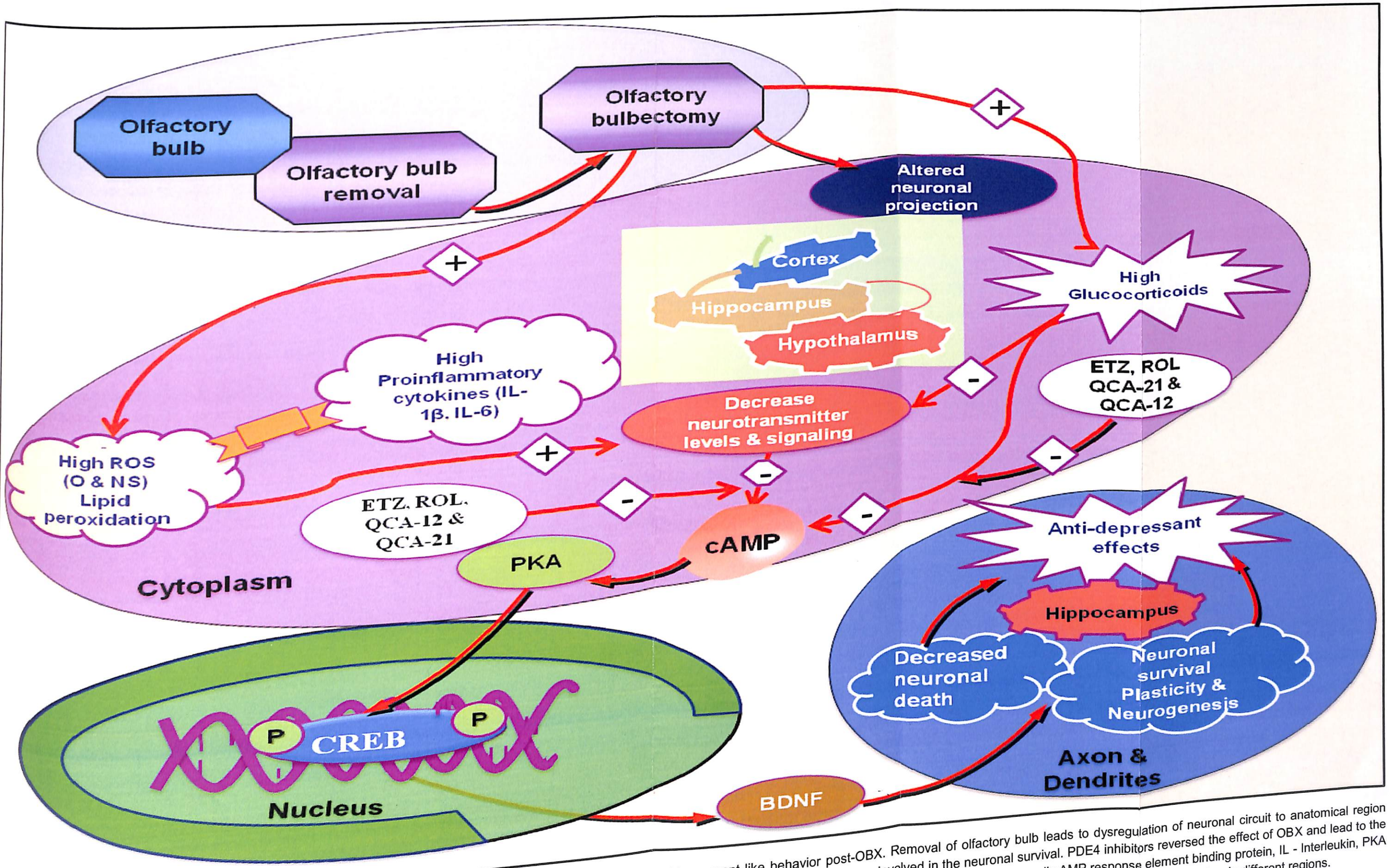


Fig. 73: Proposed hypothetical schematic representation of possible mechanism of PDE4 inhibitors for antidepressant-like behavior post-OBX. Removal of olfactory bulb leads to dysregulation of neuronal circuit to anatomical region involved in modulation of mood. Further dysregulation of neuronal circuit disturbed the biochemical, neurochemical and neurobiological factors involved in the neuronal survival. PDE4 inhibitors reversed the effect of OBX and lead to the neuronal survival by regulating biochemical, neurochemical and neurobiological factors and produced AD-like effects. (BDNF - Brain derived neurotrophic factor, CREB - cyclic AMP response element binding protein, IL - Interleukin, PKA - Protein kinase A, ROS - Reactive oxygen species and NS-Nitrosative stress). Pink (Cytoplasm), green (Nucleus) and blue (Axon & dendrites) colours circles indicating the proposed hypothetical mechanism in different regions.

In addition, this study extends reports from the existing literature regarding the way in which OBX model of depression may influence the expression of cAMP signaling (cAMP, CREB and BDNF). In the current study, marked decrease in cAMP level was observed as compared to sham control rats. The chronic treatment with ROL, ETZ, Q-21 and Q-12 increased cAMP level in OBX and sham control. This data in sham rats is supported by previous studies, which showed high cAMP level, following chronic treatment with PDE4 inhibitors (Xiao et al., 2011). cAMP is the ubiquitous intra-cellular second messenger, which stimulate activities of cAMP-dependent PKA and CREB.

CREB is a nuclear transcription factor that has been implicated in neuro-protection, synaptic transmission, neuronal survival, cell differentiation and axonal growth (Lonze and Ginty, 2002). Neurotrophic factor theory of depression addressed that CREB expression and functions are reduced in animal models of depression and reversed by ADs treatment (Li et al., 2009). In the study, OBX rats showed a pronounced low level of pCREB in brain tissue, indicting the influence of CREB in depression patho-physiology. Chronic treatment with ROL, ETZ, Q-21 and Q-12 significantly increased the pCREB expression, thereby, producing support for the possibility of PDE4 inhibitors, having a beneficial effect in OBX model via changes in CREB-controlled gene expression **(fig. 73).**

It is well known that hippocampal BDNF expression is dependent on CREB activation and this event may be a mediator of the therapeutic responses to ADs (Nair and Vaidya, 2006; Duman, 2009). The role of BDNF in depression disorder and in the mechanism of action of AD drugs is well appreciated (Middeldorp et al., 2010). In the study, OBX rats showed a decreased BDNF levels. This data is in agreement with neurotrophic theory of depression, that BDNF level might be expected to be reduced in rodent models of depression (Nestler et al., 2002a). Chronic ROL, ETZ, Q-21 and Q-12 treatment increased the BDNF level in both sham control and OBX rats. In accordance with these findings, it is proposed that BDNF plays an important role in OBX-induced depression-like behaviors and can serve as a marker to evaluate the therapeutic potential of PDE4 inhibitors in depression **(fig. 73)**.

Oxidative damage to macromolecules, such as lipid, protein and nucleic acids by excessive ROS level (Zhao et al., 2008), leads to neuronal dysfunction, which is associated with the development of depression disorder (Atmaca et al., 2004; Ng et al., 2008). Further, several studies addressed that high anti-oxidant enzyme activity shows an important role in the neurons survival and protection against neuro-degeneration. Thereby, increase anti-oxidant enzymes activity may be an important factor in the patho-physiological mechanism and development of pharmacotherapy for depression disorder.

Finding from current study displays a significant increase in oxidative-nitrosative stress markers as evidenced by high TBARS (lipid peroxidation) and nitrite levels (nitrosative stress) in brain of OBX rats. A very few previous studies have been also shown high oxidative-nitrosative stress in OBX rats as evidenced by an elevated lipid peroxidation and nitrite levels in OBX rats (Tasset et al., 2010). Several clinical studies have suggested an increased MDA (a by-product of lipid peroxidation) level in plasma and serum of depressive subjects as compared to normal control subjects (Khanzode et al., 2003). In the present study, chronic PDE4 inhibitors (ETZ and Q-21) treatment restored the altered lipid peroxidation and nitrosative stress levels in the brain of OBX rats. Although, previous studies have addressed that PDE4 inhibitors significantly reduced the increase TBARS and nitrosative stress level in rats brain (Sharma et al., 2012), but to our best knowledge this is the first report, indicating the potential role of PDE4 inhibitors on oxidative-nitrosative stress markers in OBX model.

In addition, a more high oxidative stress could cause the saturation of anti-oxidant enzymatic systems and decrease of their activity (Tasset et al., 2010). The results of present study show a profound reduction in the anti-oxidant enzymes (GSH, SOD and CAT) activity in brain of OBX rats. This result is in agreement with previous findings that show a marked decrease in SOD and GSH activity in OBX rat's brain (Tasset et al., 2008; Túnez et al., 2010). Thus, OBX paradigms might be linked with more intense oxidative stress, which could cause the saturation of anti-oxidant enzymatic systems and decrease of their activity. In contrast to this, treatment with ETZ and Q-21 restored the normal levels of anti-oxidant enzymes, like GSH, SOD and CAT in brain of OBX rats **(fig. 73).** Based on these findings, it may be concluded that oxidant/anti-oxidant systems also may have a valuable role in depression patho-physiology and in potential effects of PDE4 inhibitors in OBX model.

The olfactory system in rodents forms a part of limbic region in which amygdala and hippocampus contribute to the emotional and memory components of behavior (Kelly et al., 1997). Removal of the olfactory bulbs in rats, results in similarities to brain chemistry seen in depressed humans (Song and Leonard, 2005; Slotkin et al., 2005), such as altered monoamines levels in the brain regions. In our study OBX rats showed a notably decrease in brain 5-HT level. Literature reported that neuro-behavioral changes in OBX rats, when subjected to stressful and neophobic situation were evidenced with the changes in brain 5-HT levels. These changes may mediate the behavioral abnormalities (e.g. hyperactivity and cognitive deficits) that resemble depression symptoms (Van Rijzingen et al., 1995). Along with 5-HT, a marked decrease in NE level in OBX rat brain was observed. This is consistent with other studies that showed a reduced NA brain levels in OBX rats (Van Riezen and

Leonard, 1990; Song and Leonard, 1995) and changes in the α-AR function in OBX rats, similar to those in human depression. OBX rats also showed a notable decrease in DA level, which is in line with previous studies that OBX results in decreased brain DA level (Masini et al., 2004). In the current study, ETZ and Q-21 did not show any effect on OBX-induced decrease in 5-HT, NE and DA levels, indicating that potential effects of PDE4 inhibitors on OBX-induced behavioral anomalies were not dependent on NT mediated signaling.

Argument emphasizing the loss of olfaction claimed that removal of olfactory bulb produced a psychosocial stress (since the sense of smell, the most important sensory modulator by which rat obtains information from the environment was lost following bulb removal). In addition to the behavioral changes, neurochemical alteration and imbalance in NT system occurs as consequences of bulbectomy. There is substantial evidence that abnormal HPA axis activity, cAMP signaling, oxidant/anti-oxidant system and low NT level play a role in the induction of depression. From the discussion of the behavioral, biochemical and neurobiological changes that occur in rat following the bilateral lesion of the olfactory lobes, it is apparent that there is a marked overlap between the functional abnormalities in OBX rat and those changes that have been reported to occur in the patient with MDD.

6.4. Evaluation of ETZ and Q-21 in Traumatic Brain Injury Model

TBI is a highly heterogeneous clinical problem with wide range of pathological behavioral and biochemical consequences (Maas and Menon, 2012). It results in both focal and diffuse brain pathologies that are aggravated by various inflammatory responses. Studies have reported that subsequent progressive injury in brain trauma develops from hours to days after initiating insult, providing an accessible time window for pharmacological therapies.

Considerable research has indicated that TBI induces neurological impairments and patients with TBI may indulge in abnormal goal-directed behaviors, which can further increase their emotional distress and cause social and occupational problems. Hence, the present study was designed to determine general neuro-behavioral impairments and symptomatological correlation of depression disorder following TBI in the laboratory setting. In this study, using impact accelerated TBI; the development of depressive-like behavior in rats was demonstrated. A rodent's behavioral test battery was constructed to evaluate the AD-like effects in the impact accelerated TBI model. Chronic administration of drugs is necessary in order to recover from behavior anomalies post TBI (Burt et al., 1995).

In this study, weight drop (420 g) TBI was standardized in our laboratory to assess the depressive-like symptoms in rats, although, it is not explore adequately that which brain

regions are involved in the behavioral effects. A set of behavioral test battery was used to assess the depression-like behavior in TBI rats. The biochemical, neuroendocrine and cAMP signaling aspects were also measured to explore the possible mechanism(s) of PDE4 inhibitors in the TBI model. ETZ and Q-21 were evaluated for the first time for behavioral deficits (depression) and biochemical alteration-induced by TBI.

6.4.1. Effect of PDE4 Inhibitors on Behavioral, Biochemical and Neurobiological Deficits in TBI Rats

The sequel of disabling neuropsychological deficits ensuing, TBI is manifested as a wide spectrum of psychiatric conditions in the survivors, amongst which depression has been the most prevalent disorders. Depression is common post-TBI and adversely influence the uptake of rehabilitation and psychosocial adjustment (Jorge and Robinson, 2003). Post-TBI, rats were subjected to behavioral tests simulating the core symptoms of human depression.

TBI rats were first tested in OFT, a behavioral test most widely employed to investigate exploratory hyperactivity (Kulkarni, 1977; Ramamoorthy et al., 2008). TBI rats exhibited hyperactivity, resembling the agitated symptom(s) of depressive patients, a psychological state that leads a person to suicidal tendency (Simpson et al., 2007). Post-TBI, suicide risk is higher than general population, may be due to feelings of hopelessness, worthlessness and lack of ability to enjoy life (major symptoms of MDD). In this study, increased frequencies of ambulation, rearing and fecal pellets (reflecting hyperactivity) were observed in TBI rats on exposure to aversive condition. The behavioral deficits as observed in open field arena were predominantly reduced by chronic treatment with ETZ and Q-21. TBI-induced hyperactivity in the OFT, resembled one of the common behavioral symptoms of depression given by DSM.

Neurological damage post-head injury leads to an emotional volatility (intense mood swings or extreme reactions to daily conditions). Such over reactions could be sudden aggression, tears, angry outbursts or laughter (King, 1958). In such conditions, it becomes very important to understand that a person has lost some degree of control over emotional response. Thus, there is possibility that TBI-induced hyper-emotional behavior might resemble emotional imbalance, as one of the diagnostic criterion for depression (King, 1958). It is well reported that irritability and aggression are mainly worrying outcomes, post-TBI and particularly associated with frontal lobe damage (Grafman et al., 1996). On the basis of these findings, it is assumed that hyper-emotional reactivity following lesions of the brain regions could likely be manifested because of emotional imbalance following TBI. In this study, TBI rats showed a high hyper-emotionality score as compared to the sham control rats. TBI-induced hyper-emotional behaviors in rats were effectively reversed by chronic ETZ and Q-21 treatment.

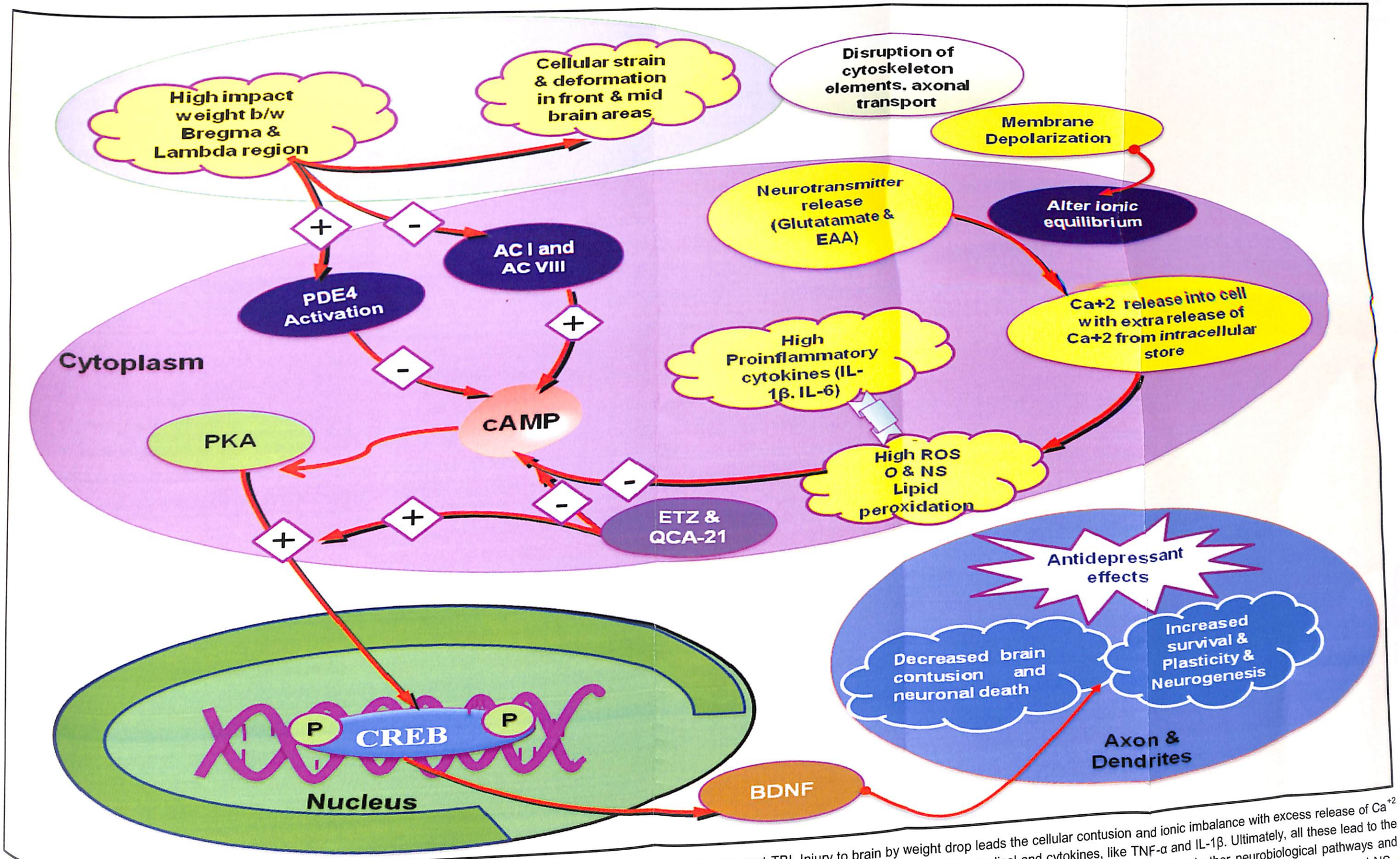


Fig. 74: Proposed hypothetical schematic representation of possible mechanism of PDE4 inhibitors for AD-like behavior post-TBI. Injury to brain by weight drop leads the cellular contusion and ionic imbalance with excess release of Ca^{+2} ions. TBI also leads to the alteration in cAMP signaling by activating PDE4 enzyme and down regulating AC enzyme. Further, TBI causes a generation of free radical and cytokines, like TNF-α and IL-1β. Ultimately, all these lead to the alteration in the intra-cellular signal transduction involve in the regulation of synaptic plasticity and neuronal survival. PDE4 inhibitors lead to the neuronal survival by regulating biochemical and other neurobiological pathways and produced AD-like effects. (AC - Adenyl cyclase, BDNF - Brain derived neurotrophic factor, CREB- Cyclic AMP response element binding protein, IL-1 - Interleukin, PKA - Protein kinase A, ROS - Reactive oxygen species and NS - Nitrosative stress. Pink (Cytoplasm), green (Nucleus) and blue (Axon & dendrites) colours circles indicating proposed hypothetical mechanism in different regions.

Clinical studies have addressed that an individual with neurological damage aftermath head injury may experience decreased interest in pleasure activities or reward functions. This effect could be a result of brain regulation of hormonal activity or an emotional response to the injury. An individual with traumatic events show anhedonia behavior or decreased consumption of sweetened solution (Mary et al., 2000), which resembles loss of interest in reward function or decreased pleasure activity (Edwards et al., 1990). The presence of anhedonia behavior in TBI rats adds to the rationality of using this as a model of depression. It was observed that TBI rats showed a profound decrease in sucrose consumption than sham operated rats. ETZ and Q-21 improved anhedonia behavior in experiments conducted.

Behavioral tests performed post-TBI notably reflects the behavioral deficits, as like human depression. To explore the exact mechanism(s) involved in the behavioral anomalies post-TBI, further studies were carried out. Behavioral anomalies post-TBI may be correlated with alterations of biochemical, neurobiological and neurochemical pathways **(fig. 74).**

Previous studies related to brain injury have investigated the potential role of molecules that modulate cAMP signaling by targeting PDE4 iso-forms for CNS problems in TBI model, although, no such study explored in the area of depression. There is a growing body of evidence that suggests the neurobiology factors, such as cAMP signaling aspects that are related to neuronal survival may be involved in the patho-physiology of depression and in the mechanism of action of ADs (Vidal et al., 2011). Atkins and Colleagues (2007) have reported impairment in cAMP transduction cascade in parasagittal fluid-percussion brain injury model, a clinically relevant model of TBI. Hence, to determine neurobiological mechanism involved in the improvement of depression-like behavioral outcomes post-TBI (accelerated weight drop method to induce brain injury), the levels of cAMP, pCREB and BDNF were measured.

In the study, TBI rats showed a decrease in cAMP signaling components, such as cAMP, pCREB and BDNF level as compared to sham control group. The decrease in cAMP levels after TBI could be either due to an increased PDE4 enzyme activity or decreased AC enzyme activity (Atkins et al., 2007). On the other hand, chronic treatment with ETZ and Q-21 restored cAMP level and its-mediated downstream signaling components, like pCREB and BDNF, in TBI rats as compared to sham rats. Atkins and Colleagues (2007) reported that PDE4 inhibitors, including ROL and others may improve outcome after TBI via regulating cAMP/PKA/CREB signaling pathway. This result indicated that alteration in cAMP signaling aspect may be responsible for the depression-like behavioral deficits post-TBI, in accelerated weight drop method and that ETZ and Q-21 may be showed potential beneficial effect through this mechanism.

The levels of oxidative and anti-oxidant markers were also measured in TBI model. Numerous bodies of evidence have reported that oxidative stress markers play key roles in primary abnormalities related to acute TBI (Ikeda and Long, 1990) and in secondary adverse consequences associated with pro-inflammatory markers (Feuerstein et al., 1997; Juurlink and Paterson, 1998). The current study displayed a significant increase in oxidative-nitrosative stress markers in brain of TBI rats. The result of this study is supported by earlier pre-clinical studies that showed high oxidative stress in FPI model-induced disruption of neuronal homeostasis (Inci et al., 1998). In this study, chronic ETZ and Q-21 treatment restored the altered oxidative-nitrosative stress.

Moreover, the study revealed low anti-oxidant enzyme levels as compared to sham control rats. Earlier studies have also reported a decrease in anti-oxidant enzyme markers levels in several brain regions in FPI model-induced brain injury (Tyurin et al., 2000). Chronic treatment with PDE4 inhibitors restored the decrease level of anti-oxidant enzymes in brain of TBI rats. These findings strongly supported the proposed hypothesis that oxidant and anti-oxidant systems play key roles in the development of depression-like behavioral deficits, post-TBI **(fig. 74).**

With such a strong link between behavioral, biochemical, neurobiological alterations and TBI, it is perhaps surprising that there are few direct diagnostic links for depression disorders in TBI model. Moreover, reversal of behavioral, biochemical and neurobiological alterations by ETZ and Q-21 indicate the AD-like potential in TBI model.

6.5. Evaluation of ETZ and Q-21 in Chronic Unpredictable Mild Stress Model

In the present study, chronic exposure of mice for a period of 28 days to CUMS paradigms showed pronounced depressive-like behaviors. A variety of stress situations have been employed to investigate the consequences of stress and to evaluate the depression-like behaviors in rodents. As such, a feasible and valid model of experimental stress has to include the factors of chronicity and unpredictability of stressors. The experimental design of the current study fulfilled these criteria to induce significant behavioral deficits in mice. The major advantage of repeated stress models is that they offer a best approach to study the neurobiological changes produced by repeated stress exposure (Nestler et al., 2002a).

The CUMS model is attractive because it displays multifaceted stress-induced behavioral and biochemical alterations that reflect the complex nature of depression made up of a variety of emotional, behavioral and cognitive elements. Although, a major drawback of current repeated stress models is that only few models produce consistent and robust

changes in depressive symptomatology (Nestler et al., 2002b). Having a model in which both behavioral deficits and neurobiological mechanisms that could be studied in the same animal, propose substantial benefits for studying the role of stress in depression. Thus, it is important that more attention is given to developing and identifying chronic/repeated stress models, which constantly and reliably produce increase in depression-like behaviors.

In this study, mice exposed to chronic stress exhibited decrease in body weight, which is in accordance with previous studies (Konkle et al., 2003; Lucca et al., 2008). The decrease in body weight in stressed mice is most likely due to the effect of CUMS on physiological parameters. Treatment with ETZ only at 1 mg/kg exhibited an increase in body weight as compared to stressed mice. In another set of study, no effect of Q-21 treatment on body weight of stressed mice was observed. Moreover, FLX did not show any effect on body weight, which is supported by earlier studies that decrease in body weight in CUMS model, may not be prevented by AD treatment (Song et al., 2006; Yalcin et al., 2008).

6.5.1. Effect of ETZ and Q-21 on Behavioral, Biochemical and Neurobiological Alterations in Stressed Mice

CUMS model is a well documented animal model of depression and widely used to understand the patho-physiology of depression, as it has an edge over genetic models in closely mimicking human depression (De Kloet et al., 2005; Willner, 2005). This model was developed in an attempt to resemble a variety of behavioral, neurochemical, neuroendocrine and neuroimmune alterations, similar to those observed in clinical depression (Holsboer, 2001; McEwen, 2005). In addition, CUMS model invites a good interest due to its potential of combining three types of validity criteria, such as face validity, construct validity and predictive validity for animal model of depression (Larsen et al., 2010; Kumar et al., 2011). Hence, this model can be well used to measure the AD-like potential of new drug molecule.

The FST is most frequently used to determine depression/AD-like behavior in rodents after exposure to various stressors (Takeda et al., 2006). Hopelessness is a characteristic behavioral symptom of the depression disorder in humans. The increased duration of immobility in FST reflects the hopelessness of mice to unavoidable and inescapable stress condition. The data from this investigation showed that mice subjected to chronic stress, exhibited increase in duration of immobility and decrease in swimming episodes. The results obtained in FST are supported by previous reports, indicating that mice exposed to chronic stress exhibited increase in duration of immobility (Zhou et al., 2007; Kumar et al., 2011). Chronic administration of ETZ markedly decreased the duration of immobility and increased the swimming episodes in stressed mice, indicating AD-like effect. In another set of study, Q-

21 predominantly decreased the duration of immobility and increased the swimming episodes in stressed mice, at selected dose ranges. In addition, chronic treatment with ETZ and Q-21 notably decreased immobility duration in unstressed mice that could be attributed to the AD-like potential of PDE4 inhibitors (Jindal et al., 2012; 2013). Previous studies from our laboratories have shown that ETZ decreased the duration of immobility and increased the swimming episode in mice FST under normal condition (Jindal et al., 2012). At this juncture, it is worth mentioning that PDE4 inhibitors may be effective for MDD treatment.

AD-like effects of ETZ and Q-21 in CUMS model using mice TST were investigated. The observed results from TST studies indicated that stressed mice, showed an increase in duration of immobility in TST as compared to normal control mice. It is reported earlier, that rodents exposed to chronic stress exhibit depressive-like behavior, as evidenced by increase duration of immobility in TST (Nirmal et al., 2008; Moretti et al., 2012). The possible reason of increased duration of immobility under chronic stress condition may be related to the more prevalent hopelessness behavior in stressed mice. Chronic administration of ETZ and Q-21 significantly decreased the duration of immobility in stressed mice as compared to stressed control mice, indicating the AD-like effect. In addition, chronic treatment with ETZ and Q-21 significantly decreased duration of immobility in unstressed mice, which may be attributed to the AD potential of both drugs. At this juncture, it is worth mentioning that PDE4 inhibitors may be a good approach for pharmacotherapy of MDD (Jindal et al., 2012; 2013).

Sucrose preference test is a valuable and valid behavioral indicator of chronic stress in rodent paradigm. Literature reports that this test is frequently used in conjunction with CUMS (Willner, 2005), for the assessment of depressive-like state in other animal models of depression. The loss of pleasure activity (anhedonia behavior) is defined as a core symptom of depression which was successfully modelled in mouse. The influence of ETZ and Q-21 on sucrose consumption in CUMS model was investigated. Results indicated that stressed mice showed a reduced preference for sucrose solution and also consumed less amount of sucrose solution as compared to unstressed mice. This result is accordance with earlier published findings that stressed mice consumed less sucrose solution as compared to unstressed mice (Holsboer, 2001; Anisman et al., 2009). The decrease in sucrose consumption may be related to alterations in several hormonal and neurochemical systems, however, it has been also suggested that reduction of body weight, following CUMS contributes to a decrease in sucrose intake (Matthews et al., 1995). Chronic treatment with ETZ and Q-21 in different set of studies predominantly reversed this behavioral change, representing the AD-like effect of ETZ and Q-21 in CUMS model of depression. Remarkably, molecules with AD-like potential have an ability to reverse the chronic stress-induced

reduction in sucrose consumption (McEwen, 2005; Kumar et al., 2011). These evidences strongly support the AD-like effects of ETZ and Q-21 in the CUMS model of depression.

Chronic stress can also influence the multiple mechanisms, such as HPA axis activity, oxidant/anti-oxidant balance and neurobiological aspects acting upon multiple levels and depending upon individual coping abilities. Stress and incidences of depression-like behavioral deficits may be linked in a variety of ways as shown in **fig. 75.**

CUMS induced-neurobehavioral changes may be associated with changes in HPA axis activity in terms of high plasma/serum concentration of CORT, as it correlates with the stress effects (Sapolsky, 2001). Stress is characterized by physiological alterations that arise in response to novel or threatening stimuli. These changes comprise a cascade of neuroendocrine events mediated by stress systems, such as HPA axis. In the present investigation, we found a notable increase in serum CORT level in stressed mice, which is in accordance with previous reports (Chen et al., 2007; Garcia et al., 2009). High serum CORT level in stressed condition has been reported to plays a key role in the induction of depressive-like behaviors (McEwen, 2008; Vreeburg et al., 2010). Administration of ETZ and Q-21 predominantly decreased the serum CORT level in stressed mice. Previous findings addressed that chronic treatment with agents having AD-like effect, reduced high CORT level in CUMS model (Buhl et al., 2010; Lenze et al., 2011). In this relationship, altered HPA axis activity may be used to explore the patho-physiological mechanism of depression in animal models and restoration of normal HPA axis function may be linked with therapeutic actions of drugs with AD potential **(fig. 75).**

Many of the neurobiological abnormalities found in chronically stressed rodents parallel, those found in human depressed patients. The regulations of cAMP signaling aspects are considered as a key mechanism in stress-induced depression. In the present study, exposure to chronic stress paradigms markedly decreased the cAMP, pCREB and BDNF levels as compared to normal control mice. This is accordance with earlier pre-clinical studies that exposure of rodents to stress can result in low hippocampal pCREB and BDNF levels (Nibuya et al., 1996; Rasmusson et al., 2002). Conversely, chronic treatment with ETZ and Q-21 ameliorated the reduction of pCREB and BDNF levels. Some ADs, such as FLX was also found to increase BDNF levels and the pCREB/CREB ratio in the hippocampus and frontal cortex brain regions of stressed rodents (Tiraboschi et al., 2004), indicating that pCREB and BDNF implicate in the chronic stress-induced depression **(fig. 75).**

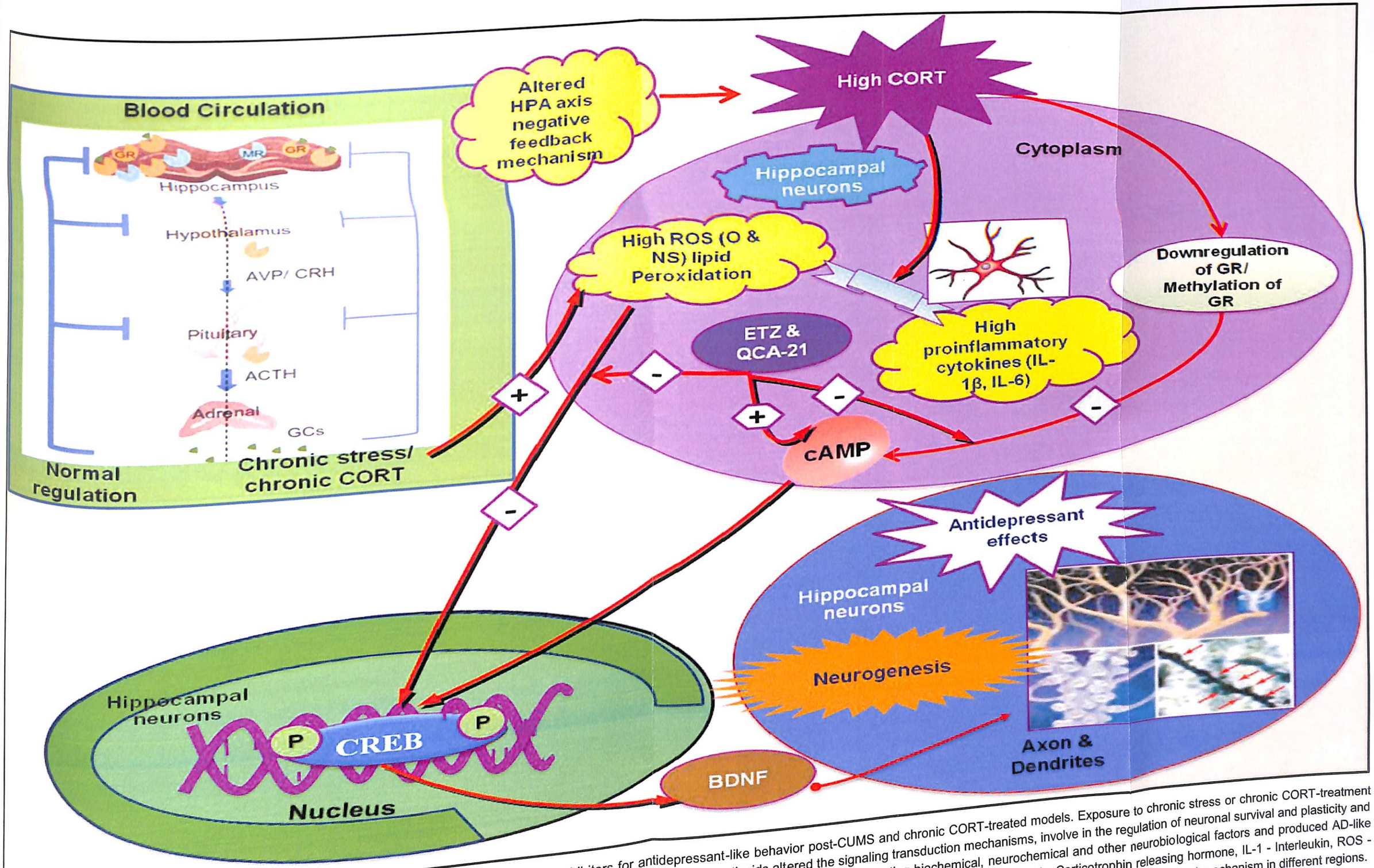


Fig. 75. Proposed hypothetical schematic representation of possible mechanism of PDE4 inhibitors for antidepressant-like behavior post-CUMS and chronic CORT-treated models. Exposure to chronic stress or chronic CORT-treatment altered the HPA axis negative feedback mechanism and increased glucocorticoids levels, through ACTH. High glucocorticoids altered the signaling transduction mechanisms, involve in the regulation of neuronal survival and plasticity and results neuronal degenerations in hippocampus region. ETZ and Q-21 reversed the effect of CUMS and leads to the neuronal survival by regulating biochemical, neurochemical and other neurobiological factors and produced AD-like effects. (ACTH – Adrenocorticotropic hormone, AC - Adenyl cyclase, BDNF - Brain derived neurotrophic factor, CREB - Cyclic AMP response element binding protein, CRH - Corticotrophin releasing hormone, IL-1 - Interleukin, ROS - Reactive oxygen species, TNF - Tumor necrosis factor). Light green (Blood circulation), Pink (Cytoplasm), dark green (Nucleus) and blue (Axon & dendrites) colours circles indicating proposed hypothetical mechanism in different regions.

In this study, the influence of CUMS paradigm on the oxidant and anti-oxidant markers in mice brain were investigated. Recently, it is extensively reported that CUMS impairs the anti-oxidant status of brain tissue, presumably by generating excessive ROS and increasing oxidative stress. In the current investigation, TBARS level that is proportional to lipid peroxidation and nitrosative stress was significantly increased in brain of stressed mice. Studies have addressed that depressive-like behavior induced by chronic stress was paralleled by a significant lipid peroxidation, as evidenced by increasing amount of TBARS in cerebral cortex and hippocampus of mice (Fontella et al., 2005; Moretti et al., 2012). Additionally, 21 days exposure to different stressors resulted in augmented lipid peroxidation in mice brain (Kumar et al., 2011). The stressed mice also showed a nitrosative stress as evidenced by elevated brain nitrite level. Literature data has suggested that depressed patients show elevated nitrite levels (Suzuki et al., 2001). Our results shed light on this theme by showing that repeated administration of ETZ and Q-21, which prevented CUMS-induced depressive-like behavior, also restored the stress-induced lipid peroxidation and nitrosative stress, suggesting a potential relationship between both events **(fig. 75).**

Literature also reports that CUMS paradigm also produces an imbalance in the anti-oxidant enzymes levels that contributes to stress-related depression (Lucca et al., 2009). The finding of this investigation showed that stressed mice have lower brain GSH, SOD and CAT levels indicating an alteration in anti-oxidant-mediated brain defence system in CUMS model of depression. Earlier research reports have shown a decrease in CAT and SOD activities in the prefrontal cortex, hippocampus and striatum regions of stressed mice, indicating an alteration in anti-oxidant defence system in chronic stress model of depression (Eren et al., 2007). In the current study, ETZ and Q-21 restored the GSH, SOD and CAT levels in brain of stressed mice. Extensively studies indicate that ADs significantly normalized the reduced GSH, SOD and CAT levels in rodents brain (Zafir et al., 2009). In the present investigation, CUMS resulted in significant oxidative damage and depletion of anti-oxidant enzymes, however, ETZ and Q-21 reversed the alteration in oxidant and anti-oxidant levels. Thus, this result strengthens the oxidative theory of depression disorder and may form the basis for possible mechanism of the PDE4 inhibitors in CUMS depression model **(fig. 75).**

Further, CUMS-induced behavioral deficits may be associated with the alteration in the brain NT levels. Findings from the experimental study established that stressed mice showed decrease of 5-HT, NE and DA levels in brain. This result is consistent with earlier findings that CUMS, results in altered NTs (5-HT, NE and DA) and their metabolites levels in various brain regions in rodents (Bekris et al., 2005) and these changes may mediate the behavioral abnormalities induced by chronic stress (e.g. hypoactivity, behavioral despair and

anhedonia). However, chronic ETZ and Q-21 administration did not show any effect on the CUMS-induced decrease in 5-HT, NE and DA levels, indicating that the potential effects of PDE4 inhibitors on the CUMS-induced behavioral anomalies was not dependent on NT-mediated signaling.

6.6. Evaluation of ETZ and Q-21 in Chronic Corticosterone-injection model

In order to better understand the relationship among chronic stress, GCs and depression, chronic CORT-injection in rodents was proposed as an animal model of depression that mimics the dysfunction of the HPA axis in depression. In addition, this animal model of depression may serve as a model for TRD. Pre-clinical findings supported that GR antagonists may be a suitable approach for TRD (Murphy et al., 1991). Hence, repeated CORT-injection in rodents may be used to screen for ADs or potential drugs for TRD (Heim et al., 2008; Juruena et al., 2009). Therefore, one of the main objective(s) of this study was to standardize the chronic-CORT injected model in the laboratory setting to address effective treatment for TRD.

A set of behavioral test battery was used to assess the depression-like behavior in chronic CORT-injected mice. Besides this, the biochemical, neurochemical, neuroendocrine and cAMP signaling aspects were measured to explore the possible mechanism(s) of the PDE4 inhibitors in this model. ETZ and Q-21 were evaluated for the first time for depression-like behavioral deficits-induced by chronic CORT treatment.

6.6.1. Effect of ETZ and Q-21 on Behavioral, Biochemical, Neurobiological and Neuronal Morphology Deficits in Chronic CORT-treated Model

The results of this study showed that chronic CORT-treated mice increased the immobility time in the behavioral despair test, such as FST and TST. Studies reported that repeated CORT-injection induced depression-like behavioral deficits, as evidenced by increased duration of immobility in FST (Ago et al., 2008; Gourley and Taylor, 2009; Iijima et al., 2010). Moreover, administration of GC synthesis inhibitors reversed the increase of immobility duration as observed in the FST (Baez and Volosin, 1994). This suggests that exposure to high CORT has an immobility enhancing effect and further this theory is supported by other research, indicating that impairment of GRs via anti-sense treatment, results in decreased immobility duration, which is displayed in behavioral despair tests (Korte et al., 1996). Chronic treatment with the ETZ and Q-21 significantly decreased the duration of immobility in both the tests. This is the first time it was shown that both the drugs produced AD-like effects in the FST and TST in chronic CORT-injection model.

In addition to the FST and TST, the preference to sucrose consumption in chronic CORT-injection model was also assessed. In this study, CORT control mice showed a reduced preference for sucrose solution and consumed less amount of sucrose solution as compared to normal control mice. This result is consistent with earlier findings that CORT-treated mice consumed less sucrose solution as compared to normal mice (Anisman et al., 2008). Chronic treatment with ETZ and Q-21 significantly reversed this behavioral change, which represents the AD-like effect of PDE4 inhibitors.

Despite having several complex biological mechanism(s), clinically depression indicates a general underlying final neural state. Regarding the identification of this state, it is supposed that various emotional and cognitive behaviors are regulated by an impaired neural network of brain structures (Swanson, 2000). Therefore, there is possibility of major alterations in the normal function of this neural network in MDD.

In the present study, histo-pathological evidences of DG and hippocampal CA1 regions in chronic CORT-injected mice were studied. Literature reports that apart from behavioral abnormalities with depression, moderately elevated CORT for a prolonged time is enough to induce morphological and histological changes (altered hippocampal volume) in hippocampus (Sapolsky, 2001), which is a common feature in MDD patients **(fig. 75).** In the present study, there was a neuronal damage in hippocampus region of CORT-injected mice brain as compared to normal controls that showed healthy neurons. This finding is in accordance with the previous studies, which indicate that hyper-secretion of GCs produces morphological changes in hippocampus region (Vyas et al., 2003). ETZ and Q-21 treatment for 21 days remarkably attenuated CORT-induced neuronal loss in hippocampus regions.

In the present investigation, CORT-injected mice showed a significant increase in serum CORT level. This data is in accordance with previous reports that chronic injection of CORT in rodents shows high serum CORT levels (Lee et al., 2009). Chronic administration of ETZ and Q-21 notably decreased the serum CORT level in CORT-injected mice. It is well known that CORT can easily perfuse in brain and bind directly to mineralocorticoid receptors and GR (De Kloet, 1997). These two receptor types have different affinities for CORT hormone. Mineralocorticoid receptors have a 10 fold higher affinity for CORT and almost occupied under normal conditions (Reul and De Kloet, 1985). In contrast, GRs have a low affinity for CORT and become occupied only during stress condition, when circulating CORT level is high. This leads to down-regulation of hippocampal GRs and ultimately impairment of hippocampus GRs ability to control GC negative feedback mechanism (Sapolsky, 2001). The alteration in the negative feedback mechanism leads to a further hyper-secretion of

circulating GCs, which may responsible for the development of depressive type behavioral anomalies **(fig. 75)**.

Further, repeated CORT exposure may influence the expression of proteins, implicated in neuroplasticity and neuronal survival. Chronic GC exposure may impair cAMP/CREB-mediated signaling in limbic and forebrain regions (Jacobsen and Mork, 2006; Gourley et al., 2008), similar to that seen in patients with depression (Karege et al., 2005). In the current study, it was observed that chronic CORT-injected mice showed a pronounced decrease level of cAMP and pCREB. Earlier reports indicate that chronic CORT administration is associated with reduce expression of transcription factor, namely CREB in hippocampus region and induces depression-like behaviors in adult mice (Gourley et al., 2008; Pittenger and Duman, 2008). On the other hand, chronic treatment with ETZ and Q-21 remarkably increased the cAMP and pCREB level in brain of chronic CORT-injected mice. Interestingly, the effect of PDE4 inhibitors on the cAMP and pCREB, support that cAMP/CREB may contribute to the AD-like potential of ETZ and Q-21 in chronic CORT-injected model **(fig. 75).**

BDNF protein was proposed to play an important role in CORT-induced depression in mice (Dwivedi et al., 2006). In our study, the 3-week CORT injections decreased BDNF protein level in the hippocampus region of mice. The data is consistent with previous studies that indicated that administration of CORT causes a pronounced decrease in BDNF mRNA and protein levels in hippocampus and frontal cortex (Jacobsen and Mork, 2006; Gourley et al., 2008). The BDNF gene contains a cAMP response element (CRE), to which phosphorylated CREB binds and thereby enhances transcription. Clinical observations address that high CORT level may interfere indirectly with CREB transcription, when CORT-GR complex binds to CREB, prevent its phosphorylation and therefore blocking the expression of CREB-regulated genes, such as BDNF **(fig. 76).** In this study, ETZ and Q-21 reversed the CORT-induced changes in BDNF expression, further indicating that BDNF may be involved in the AD-like effect of PDE4 inhibitors in chronic CORT-injected model.

The present study contains an extensive data characterizing the effect of chronic exposure of CORT and action of PDE4 inhibitors, namely, ETZ and Q-21, on behavioral consequences in chronic CORT-injected mice. The tests were developed specifically with the goal of redressing the inattention paid to neuro-behavioral symptoms in the assessment of patients with high CORT level. The study showed that depression is a frequent complication with chronic CORT-injection that exerts a deleterious effect on the recovery process and psychological outcomes and that chronic administration of PDE4 attenuates depression following chronic CORT exposure **(fig. 75).**

CORT injection

CORT/GCs

CORT/GCs

Extracellular

Cytoplasm

Cyclic AMP

Stimulates

ETZ, QCA-21

+

Protein kinase A

GR/GC

GR/GC

Nucleus

GRE

ATP

ADP

P

CBP

CREB

CREB

GRE—GR/GC

ETZ, QCA-21

-

CRE

DNA

CRE

BDNF mRNA

BDNF

Transcription is activated

Cyclic AMP response element

Neuronal survival

diated deregulation of CREB and BDNF expression & function. ETZ and Q-21 inhibits the complex formation between CREB and GRE complex and leads to the neuronal
t, CREB - Cyclic AMP response element binding protein, CORT - Corticosterone, GR- Glucocorticoids receptor, GRE - Glucocorticoids receptor

6.7. Integration of Neurobiological Aspects (HPA Axis, Intra-cellular Signaling Transduction Cascade, Neurochemical and Oxidant/Anti-oxidant Systems) on Neuronal Survival in Antidepressant-like Effects of Phosphodiesterase-4 Inhibitors in Various Animal Models

The results of this study emphasized that there is a possible link among all the biochemical neurochemical and neurobiological markers, which may be associated with the behavioral deficits that also alter the neuronal structure in animal models of depression. Neuronal degeneration is a common feature of stress-related psychiatric disorders, such as depression and anxiety. In depressed subjects, neuronal rearrangement has been observed in hippocampus, prefrontal cortex and amygdala regions (Cotter et al., 2005; Hercher et al., 2009). Moreover, various animal models of depression-like behavior mimic these neuronal rearrangements. A large number of biochemical and neurobiological factors (HPA axis activity, cAMP signaling, NTs and oxidant/anti-oxidant markers) may play a key role and contribute to neural adaptations (neuro-protection and adult neurogenesis) that may underlie the neuropathology of depression.

There is a close relationship between the hyper-activation of HPA axis and neurobiological factors of neurogenesis in depression. The results of our study in various animal models (OBX, CUMS and chronic CORT-injection) also indicated a relation between the HPA axis hyper-activation and alter BDNF levels. The result of our study showed an increase in HPA axis activity (high serum CORT level) and decrease in BDNF levels in OBX, CUMS and chronic CORT-injection models of depression. The relationship between the HPA axis hyperactivation and low BDNF level in rodents was found that HPA axis hyper-activation links with low BDNF level (Xu et al., 2006; Zheng et al., 2006) and central administration of BDNF substantially modifies HPA axis activity (Naert et al., 2006). It is well reported that activation of GR can suppress neurogenesis by inhibiting BDNF synthesis (Henn et al., 2004). Recently, studies have showed that an acute or a chronic administration of BDNF, rapidly and frequently modified release and synthesis of CRH (Givalois et al., 2004; Naert et al., 2006), thus, supporting our proposed hypothesis and suggesting that cAMP/CREB/BDNF could be involved in the protective process of PDE4 inhibitors in depression models. The regulation of both HPA axis activity and cAMP signaling, thus, plays an important role in the PDE4 inhibitors-mediated neuronal survival in animal models of depression.

Induction of high oxidative stress in brain is also considered as a major factor for neurotoxicity towards the patho-physiology of depression (Sarandol et al., 2007; Rothman and Mattson, 2010). It is reported that oxidative stress mainly affects the pathways or

modulators involved in neurogenesis or neuronal survival. In fact, the results of this study in various animal models (OBX, TBI and CUMS) also established a relation between the oxidant/anti-oxidant system and neurobiological modulators, involved in neuronal survival. In this study, oxidative stress-mediated neurotoxicity is dependent upon CREB/BDNF signaling and was found to regulate the vitality of neurons and implicated in patho-physiology of depression disorder. BDNF, a major factor involved in the neurogenesis process has also been found to be involved in the modulation of ROS reactions under the influence of ADs (Shelton, 2007). BDNF is highly expressed in hippocampus and cerebral cortex regions, where it contributes to neuronal growth, development, plasticity, survival, neuro-protection and repair (Duman and Monteggia, 2006). Some studies have suggested that depression models-induced hippocampal oxidative stress and oxidant/anti-oxidant system imbalance may cause neuronal impairment or structural changes by decreasing BDNF levels (Joels et al., 2007).

Kwon and Colleagues (2013) have demonstrated a direct correlation between oxidative stress and BDNF signaling in hippocampus region. Moreover, decreased BDNF level and MDD are related to oxidative stress in animal models (Zafir et al., 2009; Tagliari et al., 2011). Hence, BDNF protein is not only protected against oxidative stress, but it is also promoted neurogenesis and synaptic plasticity by inhibiting oxidative stress. Thus, our study result raises an intriguing possibility that decreased BDNF, favours a condition reflective of high oxidative stress and reduced plasticity, all leading to a potentially high depression state.

Several studies have shown that increased levels of monoaminergic NTs, including NA, 5-HT and DA induce BDNF expression in hippocampus and cerebral cortex (Ivy et al., 2003; Juric et al., 2006). Several other studies also have addressed that NTs also potently and rapidly increased BDNF protein level (Miklic et al., 2004; Juric et al., 2006). In addition, the activation of 5-HT receptors, linked with cAMP formation and CREB activation, ultimately induces BDNF gene regulation. Moreover, conventional ADs influence the cAMP signal transduction pathway (Zhao et al., 2003a), which activates the downstream signaling pathway, responsible for the neuronal survival and plasticity. However, in the current study PDE4 inhibitors did not show any significant changes in the NT levels in OBX rats and stressed mice. These results suggested that in the study, conducted the AD-like effects and regulation of neuronal survival in rodent models of depression was not mediated by the central monoaminergic systems.

In fact, the results of this study raise an intriguing possibility that regulation of the neuronal survival, by modifying the various biochemical and neurobiological factors, is involved in the AD-like effect of PDE4 inhibitors in various animal models.

In conclusion, the current study comprises of widespread data distinguishing the effect of OBX, TBI, CUMS, chronic CORT-injection and action of PDE4 inhibitors on behavioral, biochemical, neurobiological and histo-pathological features resembling the severe depression. Behavioral, biochemical, neurochemical and neurobiological markers measured in the present study suggest the development of depression-like symptoms in all the above animal models. The chronic treatment with ROL, ETZ, Q-21 and Q-12 were found to significantly improve the depressive-like behavior anomalies in the aforementioned models of depression, corroborating the notion of the involvement of PDE4 enzyme in the pathophysiology of depression disorder, mainly by altering cAMP level and its mediated signaling cascade. In addition, the results of this study revealed that others biochemical, neurochemical and neurobiological factors may be also responsible for the possible mechanism of AD-like effects of PDE4 inhibitors in rodent models of depression.

Chapter 7: Summary & Conclusion

7. Summary and Conclusion

As a second messenger, cAMP and its-mediated transduction cascade play a crucial role for intra-cellular signalling, involved in the patho-physiology of psychiatric disorders, including depression and anxiety. Hence, PDE4 family became an attractive drug target for the treatment of psychiatric disorder, like depression and anxiety. In fact, PDE4 inhibitors have shown AD- and anxiolytic-like effects and proved efficacy for neurological disorders in several phase of clinical trials. Recent evidences that cAMP-mediated mechanisms underlie depression and anxiety are providing support for the use of newer PDE4 inhibitors. Their use may enable the clinicians to help patients to achieve wellness or full remission, instead of simply experiencing improvement with treatment. In this study, significant advances have been made in the understanding of PDE4 enzyme role in depression and anxiety, along with AD- and anxiolytic-like potential of PDE4 inhibitors in animal models. To best of our knowledge, here, we report for the first time, effects of ETZ and in-housed synthesized Q-21 and Q-12 on the preliminary and chronic depression-like behaviors, following experimental models of traumatic brain insult, like chronic CORT-injection, OBX, TBI and CUMS.

The acute assays using mice, such as SLA (for assessing locomotor status), FST, TST and 5-HTP-induced HTR were used for preliminary AD studies. Besides these, acute assays using mice, such as EPM, L/D and HB tests were used for preliminary anxiolytic studies.

Interaction studies with existing ADs, such as FLX, VLA and DMI were carried out in mice FST. The CUMS and chronic CORT-injection in mice were used as chronic models, to assess the efficacy of chronic treatment of PDE4 inhibitors.

The acute AD assay using rat as test system included in the present study, was RIH. Moreover, other AD assays using rat as test system were: effects on chronic OBX- and TBI-induced behavioral anomalies in OFT, sucrose preference test and hyper-emotionality test.

Beside the behavioral analysis, the current work also reintegrates the role of PDE4 enzyme on the neuronal degeneration to explore the PDE4 involvement in patho-physiology mechanism of depression. MDD has been reported, a type of neuro-degenerative disorder and associated with neuronal degeneration in the brain regions, like hippocampus and prefrontal cortex, which are involved in mood regulation. Based on this, the histopathology analysis of hippocampus brain regions were carried out in animal models of depression.

Alteration in biochemical, neurochemical and neurobiological aspects could possibly be responsible for behavioral and morphological anomalies, post-OBX, TBI, CUMS and chronic

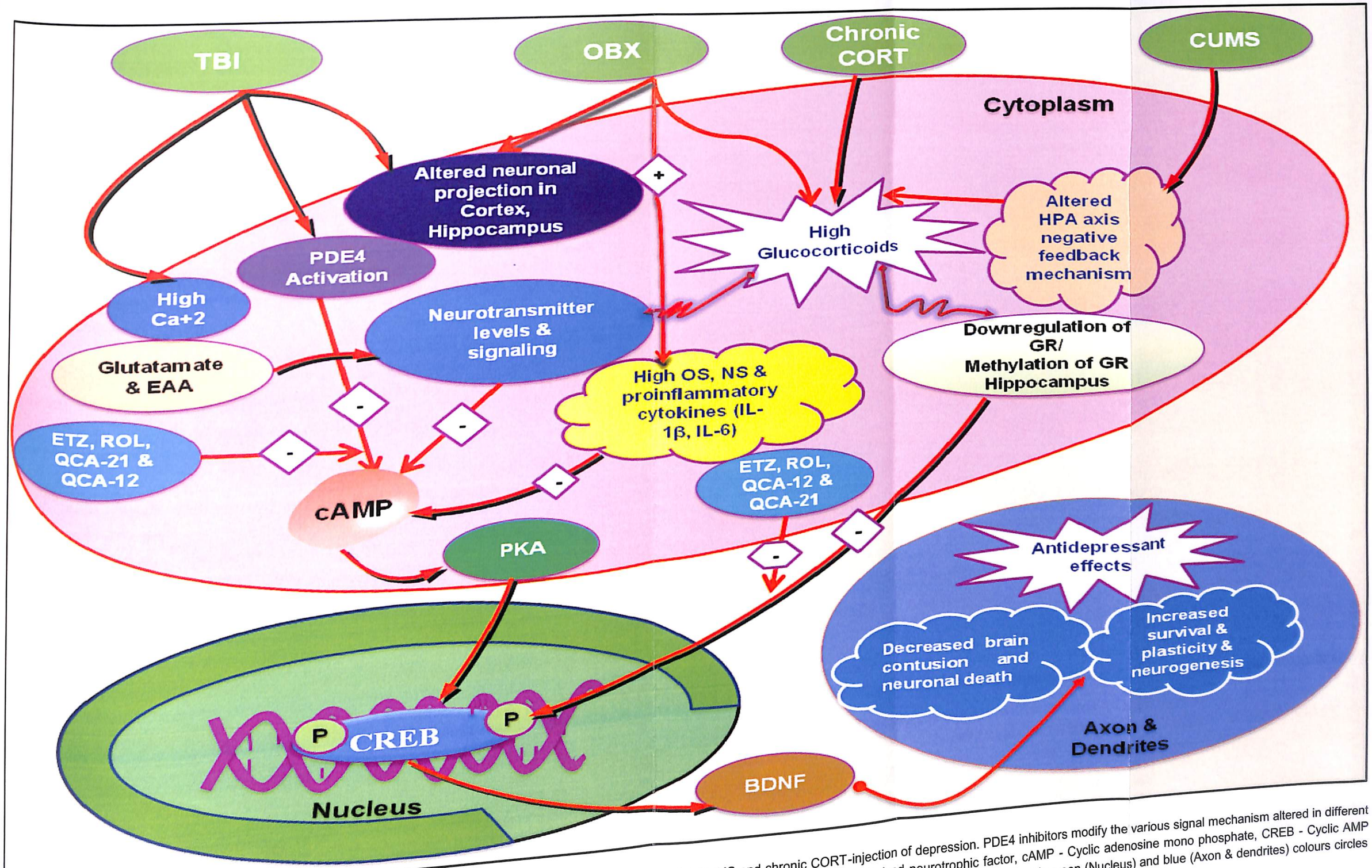


Fig. 77: Proposed hypothetical novel mechanism of PDE4 inhibitors in chronic animal models, such as OBX, TBI, CUMS and chronic CORT-injection of depression. PDE4 inhibitors modify the various signal mechanism altered in different animal models. By modifying the signal mechanism, PDE4 inhibitors regulate neuronal survival and results in AD-like effect. (BDNF - Brain derived neurotrophic factor, cAMP - Cyclic adenosine mono phosphate, CREB - Cyclic AMP response element binding protein, EAA - Excitatory amino acid, IL-1 - Interleukin, PKA - Protein kinase A, OS - Oxidative stress and NS -Nitrosative stress). Pink (Cytoplasm), green (Nucleus) and blue (Axon & dendrites) colours circles indicating proposed hypothetical mechanism in different rodent models of depression.

The CUMS subjected mice showed pronounced depression-like behaviour anomalies, expressing behavioral despair and anhedonia symptoms, which more closely resemble the human depression symptoms. Chronic treatment with ETZ and Q-21 for a period of 21 days was found to markedly reverse the depression-like behavioral deficits in mice, exposed to CUMS paradigm.

In this study, PDE4 inhibitors were observed to reverse the behavioral anomalies induced by chronic experimental models, such as OBX, TBI, CUMS and chronic CORT-injection. This observation has important clinical implications, suggesting that the common occurrence of depression disorders in humans, atleast in part, have a neurobiological basis. Additionally, it also signifies that OBX, weight drop TBI, CUMS and chronic CORT-injection can be used as models for depression disorder.

Along with the behavioral and morphological analysis in various animal models of depression, this work reconstructs the various biochemical and neurobiological aspects that possibly simulate the symptom(s) of depression in human. Alterations in the biochemical, neurochemical and neurobiological aspects were also observed in this study. In the current studies OBX, CUMS, TBI and chronic CORT-treated models showed decreased NT levels and anti-oxidant defence system of brain and increased the HPA axis activity and oxidative stress markers. Moreover, a significant decrease in cAMP, CREB and BDNF levels were also observed in chronic CORT-injection, OBX, CUMS and TBI experiments. Studies revealed that (a) decrease in cAMP, CREB, BDNF levels, (b) decrease in anti-oxidant marker levels, (c) increase in HPA axis activity and (d) increase in oxidative stress markers-mediated signaling, play an important role in neuro-degeneration associated with the development of psychiatric disorder like depression.

OBX, TBI, CUMS and chronic CORT-injection induced behavioral deficits, was found to be associated with altered cAMP signaling cascade, HPA axis activity, level of NTs and oxidant/anti-oxidant markers. Further, the results of the study revealed that HPA axis activity, NTs and oxidant/anti-oxidant system affected the cAMP-mediated downstream signaling transduction cascade, such as CREB and BDNF, involve in various animal models of depression **(fig. 77).** The current study, also reports the role of PDE4 inhibitors on HPA axis activity, oxidant/anti-oxidant system and cAMP-mediated signalling, which could be the rationale for reversal of depression-like behavior induced by aforementioned models.

Thus, alteration in cAMP signaling cascade was found to be played a significant role in the induction of depression. Molecules which selectively target cAMP level and its-mediated signaling beyond the receptor level, such as PDE4 inhibitors can favour the reversal of depression and anxiety-like behavioral anomalies and may be useful as suitable ADs and anxiolytics. Since, ETZ has an excellent safety profile, subjecting ETZ to clinical trials (post-pilot dose-response studies) in patients with various sub-types of depressive disorder is advocated.

Chapter 8: Salient Findings & Future Prospects

8.1. Salient Findings from the Work

- Investigations of AD-like and anxiolytic-like potential of existing and in-house synthesized PDE4 inhibitors were performed.
- Exploration of neurobiological mechanism(s) in the patho-physiology of depression disorder was performed.
- Development and standardization of animal model, such as chronic-CORT model of depression in mice by using chronic injection of CORT in a laboratory set up was perfected.
- Other chronic animal model(s) of depression, like OBX, CUMS and TBI were standardized.
- Behavioral acute tests, such as FST, TST, 5-HTP-induced HTR and RIH were defined as preliminary tests for the assessment of depressive symptoms.
- Experimentally, merging of various paradigms were conducted in a manner that emotional condition of a rodent becomes measurable via various non-exclusive tasks that could contribute to increased consistency, rapidity and completeness of behavioral measurement.
- Along with behavioral tests, the possible involvement of the HPA axis activity, NTs, oxidant/anti-oxidant system and cAMP signaling transduction cascade were also assessed and explored with respect to depression patho-physiology.
- Integrations of all hypotheses (HPA axis hypothesis, monoamine hypothesis, oxidative stress hypothesis and intra-cellular signaling transduction cascade hypothesis) were studied.
- It was observed that PDE4 inhibitors markedly reversed the behavioral deficits induced by TBI, OBX and CUMS models.
- For the first time, AD-like potential of ETZ and Q-21 were tested for TRD, in the laboratory set up using chronic CORT-injection model.
- The potential beneficial effects of ETZ and Q-21 on the neuronal morphology in OBX and chronic CORT-injection models were found.
- The anxiolytic-like potential of PDE4 inhibitors was also explored using EPM, L/D aversion test and HB test.
- PDE4 inhibitors were also found to normalize the altered HPA axis activity, oxidant/anti-oxidant systems and intra-cellular signaling cascade.

8.2. Implications for Future Research

The experimental evidences explained in this thesis are tantalising, but these proof of-concept experiments require further mechanistic elucidation and extra experimentation to assess the true biological significance of these events.

- Further research is needed to ascertain, whether immunological alterations are involved in the pathogenesis of affective disturbance following CORT, TBI, OBX and CUMS models.
- More studies (such as combination therapy, long term behavioral assessment) are needed to give consistency to the animal models and to determine, whether, concurrent treatment of depression and anxiety can reduce somatic symptom burden, medical testing and polypharmacy.
- To develop and study the iso-form selective PDE4 enzyme inhibitors for their potential role in the anxiety and depression disorders.
- Acute and chronic toxicity studies can be conducted, as an extension of the work on in-house synthesized molecules, such as Q-21 and Q-12.
- Role of ETZ in depression and associated co-morbid problems need to be further explored.
- Further, molecular biology investigations are required to strength the proposed hypothesis, such as role of HPA axis, neurotrophic factor, monoamine system, oxidant/anti-oxidant system and their integration with each other in the development of depression.

8.3. Limitation of the study

- In the present study, we could not able to explore the specific PDE4 enzyme iso-forms role in depression and anxiety due to lack of iso-form specific PDE4 inhibitors.
- This study had lack of several advanced molecular biology techniques, like RT-PCR, western blotting and immuno-histochemistry, which could strengthen the proposed hypothesis.

8.4. Inclusion and Exclusion criteria for Mice and Rats

8.4.1. Inclusion Criteria

- Age and weight matched rats (18-20 weeks old/ 250-275g) and mice (10-12 weeks old/ 22-30g) were included for the neuro-psychopharmacology screening.
- For the preliminary AD and anxiolytic screening rodents of either sex were used.
- In the present study, male rats were employed for chronic surgical models (OBX and TBI), whereas, male mice in non-surgical models (CUMS and chronic CORT injection).

8.4.2. Exclusion Criteria

- Mice with abnormal exploratory behavior (5-8%) in actophotometer test were excluded from the study.
- Female rats and mice were excluded from chronic study, because of low recovery rate post-surgery and hormonal alterations could influence the study parameters.
- Post-surgery all rats were examined for recovery and injured rats (1-2 %) with abnormal behavior patterns were excluded.
- Infected mice (2 %) during the study protocol were excluded.

Chapter 9: References

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Appendix

Publications from thesis

1. Jindal A, Mahesh R, Bhatt S. Etazolate rescues behavioral deficits in chronic unpredictable stress model: Modulation of hypothalamic-pituitary-adrenal axis activity and brain-derived neurotrophic factor level. ***Neurochemistry International***, 2013; 63, 465-475.
2. Jindal A, Mahesh R, Bhatt S. Etazolate, a Phosphodiesterase 4 Inhibitor Reverses Chronic Unpredictable Mild Stress-Induced Depression-Like Behavior and Brain Oxidative Damage. ***Pharmacology Biochemistry Behaviour***, 2013; 105, 63–70.
3. Jindal A, Mahesh R, Gautam B, Bhatt S, Pandey D. Antidepressant-like effect of etazolate, a cyclic nucleotide phosphodiesterase 4 inhibitor-an approach using rodent behavioral antidepressant tests battery. ***European Journal of Pharmacology***, 2012; 689, 125-131.
4. Jindal A, Mahesh R, Bhatt S. Anxiolytic-like effect of etazolate, a type 4 phosphodiesterase inhibitor in experimental models of anxiety. ***Indian Journal of Experimental Biology***, 2013, 51, 444-449.
5. Etazolate, a Phosphodiesterase 4 enzyme inhibitor produces antidepressant-like effects by blocking the behavioral, biochemical, neurobiological deficits and histological abnormalities in hippocampus region caused by olfactory bulbectomy. ***Psychopharmacology*** (Accepted).
6. Type 4 Phosphodiesterase Enzyme Inhibitor, Rolipram Rescues Behavioral Deficits in Olfactory Bulbectomy Models of Depression: Involvement of Hypothalamic-Pituitary-Adrenal Axis, Brain-Derived Neurotrophic Factor and Antioxidant Defense System. ***Neuroscience*** *(Under Revision)*

Other Publications

7. Jindal A, Mahesh R, Gautam B. Anxiolytic-like effect of linezolid, an oxazolidinone class antibiotic in experimental mouse models of anxiety. ***Progress in Neuro-Psychopharmacology & Biological Psychiatry***, 2013; 40, 47-53.
8. Mahesh R, Jindal A, Gautam B, Bhatt S, Pandey D. Evaluation of anti-depressant-like activity of linezolid, an oxazolidinone class derivative – An investigation using behavioral tests battery of depression. ***Biochemical & Biophysical Research Communications***, 2011; 409, 723-726.

9. Jindal A, Mahesh R, Singh K, Bhatt S, Gautam, B; Pandey D. Ameliorative Effect of Wortmannin and Rapamycin Treatment on Obesity Markers in High Fat Diet feed rats. ***Indian Journal of Pharmaceutical Education & Research***, 2011; 45, 333-338.

10. Jindal A, Mahesh R, Gautam B, Bhatt S, Pandey D. Evaluation of anti-diabetic activity of methanolic extract from the bark of Atalantia monophylla (Linn.) in alloxan-induced diabetic mice. ***International Journal of Green Pharmacy***, 2012; 6, 133-137.

11. Dhar AK, Mahesh R, Jindal A, Devadoss T, Bhatt S. Design, synthesis and pharmacological evaluation of novel 2-(4-substituted piperazin-1-yl)-1,8-naphthyridine-3-carboxylic acids as 5-HT(3) receptor antagonists for the management of depression. ***Chemical Biology & Drug Design,*** 2014 (Accepted).

12. Bhatt S, Mahesh R, Jindal A, Devadoss T. Protective effects of a novel 5-HT(3) receptor antagonist, N-n-butyl-3-methoxy quinoxaline-2-carboxamide (6o) against chronic unpredictable mild stress-induced behavioral changes and biochemical alterations. ***Pharmacology Biochemistry Behaviour***, 2014; 122, 234-239.

13. Bhatt S, Mahesh R, Jindal A, Devadoss T. Neuropharmacological effect of novel 5-HT3 receptor antagonist, N-n-propyl-3-ethoxyquinoxaline-2-carboxamide (6n) on chronic unpredictable mild stress-induced molecular and cellular response: Behavioural and biochemical evidences. ***Pharmacological Reports,*** 2014; 2014; 66, 804-810.

14. Gautam BK, Jindal A, Dhar AK, Mahesh R. Antidepressant - Like Activity of 2-(4-phenylpiperazin-1-yl)-1, 8-naphthyridine-3-carboxylic acid (7a), a 5 -HT(3) Receptor Antagonist in Behaviour Based Rodent Models: Evidence for the Involvement of Serotonergic System. ***Pharmacology Biochemistry Behaviour***, 2013; 109, 91–97.

15. Mahesh R, Dhar AK, Jindal A, Bhatt S. 2-(4-substituted piperazin-1-yl)-1,8-naphthyridine-3-carboxylic acids: Novel 5-HT3 receptor antagonists with anxiolytic like activity in rodent behavioral models. ***Canadian Journal of Physiology & Pharmacology,*** 2013; 91, 848-54.

16. Mahesh R, Dhar AK, Jindal A, Bhatt S. Design, synthesis and evaluation of anti depressant activity of novel 2-methoxy 1, 8 naphthyridine 3-carboxamides as 5-HT(3) receptor antagonists. ***Chemical Biology & Drug Design***, 2014; 83, 583-591.

17. Mahesh R, Devadoss T, Dhar AK, Venkatesh SM, Mundra S, Pandey DK, Bhatt S, Jindal AK. Ligand-Based Design, Synthesis, and Pharmacological Evaluation of 3-Methoxyquinoxalin-2-carboxamides as Structurally Novel Serotonin Type-3 Receptor Antagonists. ***Arch Pharm***, 2012; 345(9), 687-694.

18. Mahesh R, Kumar B, Jindal A, Bhatt, S, Devadoss T, Pandey DK. Antidepressant-like activity of (4-phenylpiperazin-1-yl) (quinoxalin-3-yl) methanone (4a), a Novel 5 HT3 receptor antagonist: An investigation in behaviour-based rodent models of depression. ***Indian Journal of Pharmacology***, 2012; 44, 560-565.

19. Kumar B, Jindal A, Pandey DK, Bhatt S, Devadoss D, Mahesh R. Antidepressant and anxiolytic-like effects of 4n, a novel 5-HT3 receptor antagonist using behaviour based rodent models. ***Indian Journal of Experimental Biology***, 2012; 50, 625-632.

20. Bhatt S, Mahesh R, Devadoss T, Jindal A. Anti-depressant like activity of N-n-butyl-3-methoxyquinoxaline-2-carboxamide (6o) a 5-HT3 receptor antagonist. ***Indian Journal of Experimental Biology***, 2013, 51, 435-443.

21. Bhatt S, Mahesh R, Jindal A, Devadoss T. Anxiolytic-like effect of N-n-butyl-3-methoxyquinoxaline-2-carboxamide (6o) in experimental mouse models of anxiety. ***Indian Journal of Experimental Biology***, 2013, 51, 510-514.

22. Mahesh R, Bhatt S, Devadoss T, Jindal AK, Gautam BK, Pandey DK. Antidepressant Potential of 5-HT3 Receptor Antagonist, N-n-propyl-3-ethoxyquinoxaline-2-carboxamide (6n). ***Journal of Young Pharmacist***, 2012; 4, 235-244.

23. Bhatt S, Mahesh R, Devadoss D, Jindal A. Anxiolytic-Like Effect of (4-benzylpiperazin-1-yl)(3-methoxyquinoxalin-2-yl)methanone (6g) in Experimental Mouse Models of Anxiety. ***Indian Journal of Pharmacology***, 2013; 45, 248-251.

24. Bhatt S, Mahesh R, Jindal A, Devadoss T. Antidepressant- like effect of novel 5-HT3 receptor antagonist N-n-butyl-3- ethoxyquinoxalin-2-carboxamide (6p)- an approach using rodent behavioral antidepressant tests battery. ***Indian Journal of Pharmacology***, 2013; 45, 348-353.

25. Bhatt S, Mahesh R, Jindal A, Devadoss T, Dhar AK. Neuropharmacological evaluation of a novel 5-HT3 receptor antagonist (6g) on chronic unpredictable mild stress-induced changes in behavioural and brain oxidative stress parameters in mice. ***Indian Journal of Pharmacology***, 2014; 46, 191-196.

PAPER PRESENTATION

1. Mahesh R, **Jindal A**, Bhatt S. Etazolate rescues behavioral deficits in chronic unpredictable stress models of depression by the modulation of HPA axis, BDNF signaling and antioxidant defence system: possible mechanism(s) of neuroprotection. **(International Conference on Recent Advances in Molecular Mechanisms of Neurological Disorders, 21-23rd February 2013, AIIMS New Delhi).**
2. Mahesh R, Bhatt S, Devadoss T, **Jindal A**. Neuropharmacological effect of (4-benzylpiperazin-1-yl) (3-methoxyquinoxalin-2-yl) methanone (qcm-15), a potential 5-ht3 receptor antagonist on chronic unpredictable mild stress-induced changes in behavior and brain oxidative stress in mice. **(International Conference on Recent Advances in Molecular Mechanisms of Neurological Disorders, 21-23rd February 2013, AIIMS New Delhi)**
3. Mahesh R, Dhar AK, **Jindal A**, Bhatt S. 2-(4-substitutedpiperazin-1-yl)-1,8-naphthyridine-3-carboxylic acids: novel 5-HT3 receptor antagonists as potential anxiolytics. **(International Conference on Recent Advances in Molecular Mechanisms of Neurological Disorders, 21-23rd February 2013, AIIMS New Delhi)**
4. Muthu VS, Mahesh R, **Jindal A**. Design, synthesis and pharmacological evaluation of novel Phosphodiesterases-4 inhibitors as potential anti-depressants. **(International Conference on Recent Advances in Molecular Mechanisms of Neurological Disorders, 21-23rd February 2013, AIIMS New Delhi)**
5. Mahesh R, Bhatt S, Panjawani R, Devadoss T, **Jindal A**. Neuro-pharmacological evaluation of novel serotonin type-3 (5-HT3) receptor antagonists qcm-15, qcm-16, qct-16 & qct-22 (quinoxaline derivatives) for their anxiolytic potential. **(Association of Pharmaceutical Teachers in India Conference, 17-18th March 2012, Manipal)**
6. Mahesh R, Dhar AK, **Jindal A**, Muthu VS, Mundra S. Design, synthesis and pharmacological evaluation of Novel 2-(4-substituted piperazine 1-yl)-1,8-naphthyridine 3-carboxylic acids as 5-HT3 receptor antagonists for the management of depression. **(Association of Pharmaceutical Teachers in India Conference, 17-18th March 2012, Manipal)**
7. Mahesh R, Kumar B, **Jindal A**, Pandey D, Bhatt S, Devadoss T. Antidepressant like activity of (4-phenylpiperazin-1-yl) (quinoxalin-3-yl) methanone (4a), a novel 5 HT3 receptor antagonist: An investigation in behaviour-based rodent models. **(Indian**

Pharmacological Society Conference 19-21th December 2011, Manipal) (Oral Presentation).

8. Mahesh R, **Jindal A**, Kumar B, Bhatt S, Pandey D. Rolipram, a phosphodiesterase 4 (PDE4) enzyme inhibitor as a potential anti-depressant: An investigation using behavioral tests battery of depression. **(Indian Pharmacological Society Conference 19-21th December 2011, Manipal)**
9. Mahesh R, Dhar A, Singh S, **Jindal A**, Sharma A, Bhatt S. Evaluation of antianxiety like activity of 2-(-4-phenylpiperazine-1-yl) 1.8-Naphthridine-3-carboxylic acid(7a) A serotonin type-3(5-HT3) receptor antagonist: An investigation using behavioural test battery of anxiety. **(Indian Pharmacological Society Conference 19-21th December 2011, Manipal).**
10. Mahesh R, Dhar A, Sharma A, **Jindal A**, Singh S, Kumar B. Behavioral investigation on antidepressant like activity of 2-(-4-phenylpiperazine-1-yL) 1,8-Naphthlidine-3-carboxylic acid(7a) A novel serotonin type-3(5-HT3) receptor antagonist: for comorbid depression with cancer chemotherapy induced nausea and vomiting(CINV). **(Indian Pharmacological Society Conference 19-21th December 2011, Manipal).**
11. Mahesh R, Srinivasrao B, Devadoss T, **Jindal A**, Bhatt S, Kumar B. Studies on the analgesic and anti-inflammatory activity of n-[2-(1h-indol-3-yl)ethyl]quinoxalin-2-caboxalicacid (qcf-20), a novel serotonin type-3 (5-ht3) receptor antagonist. **(Indian Pharmacological Society Conference 19-21th December 2011, Manipal).**
12. Mahesh R, **Jindal A**, Kurdekar V, Jhadav H, Bhatt S, Gautam BK, Pandey DK, Evaluation of Anti-oxidant, Analgesic and Anti-inflammatory activity of Methanolic Bark extract of Atalantia monophylla. **(Indian Pharmacological Society Conference, 14-16th December 2010, Hyderabad)**
13. Mahesh R, Gumpally C, Bhatt S, Thangaraj Devadoss, Dilip Pandey, Gautam B, **Jindal A**. Evaluation of novel sertonergic type-3 receptor antagonist quinoxalin-2-carboxylic acid derivative (qct-22) for their antidepressant potential. **(Indian Pharmacological Society Conference, 14-16th December 2010, Hyderabad)**
14. Mahesh R, Chaudhary S, Bhatt S, Devadoss T, Pandey D, Gautam B, **Jindal A**. preclinical evaluation of quinoxalin-2-carboxylic acid derivative (qct-16), a novel sertonergic modulator for their anti-depressant potential. **(Indian Pharmacological Society Conference, 14-16th December 2010, Hyderabad)**
15. Mahesh R, Modak N, Bhatt S, Devadoss T, Pandey D, Gautam B, **Jindal A**. Novel Quinoxaline Derivative: A Potential Anti Depressant For Treatment Resistant

Depression. **(Indian Pharmacological Society Conference, 14-16th December 2010, Hyderabad)**

16. Mahesh R, Bhatt S, Devadoss T, Gautam B, **Jindal A**, Pandey D. Qcm-13(n-cyclohexyl-3-methoxyquinoxalin-2carboxamide) a novel antidepressant; pharmacological evaluation of the potential serotonin type 3 (5-ht3) receptor antagonist. **(Indian Pharmacological Society Conference, 14-16th December 2010, Hyderabad).**

SYMPOSIUM/WORKSHOP PARTICIPATED:

1. **"Fourth DST-SERC Training school on System and Cognitive Neurosciences"** held by **National Brain Research Centre**, Manesar Haryana, India (February to March 2011).

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