Behavioral and Neuro-pharmacological Screening of Potential Serotonergic Ligands for the Management of Depression and Anxiety Co-morbid with Type-1 Diabetes Mellitus

THESIS

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by

DEEPALI GUPTA

Under the Supervision of

Prof. R. MAHESH

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Dedicated to My Beloved

Parents,

My Family and

My Friends

BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE, PILANI

CERTIFICATE

This is to certify that the thesis entitled **Behavioral and Neuro-pharmacological Screening of Potential Serotonergic Ligands for the Management of Depression and Anxiety Co-morbid with Type-1 Diabetes Mellitus** and submitted by **Deepali Gupta** ID No **2008PH08411P** for award of Ph.D. degree of the Institute embodies original work done by her under my supervision.

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-Deepali Gupta

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List of Abbreviations and Symbols

5-HIAA	5-Hydroxy Indole Acetic Acid
5-HT	5-Hydroxy Tryptamine
5-HT_1	Serotonin Type-1
5-HT_2	Serotonin Type-2
5-HT_3	Serotonin Type-3
5-HTP	5-Hydroxy Tryptophan
5-HTR	5-HTP induced Head Twitch Response
5-HTT	5-Hydroxy Tryptamine Transporter
8-OH-DPAT	8-Hydroxy Dipropyl Amino Tetralin
Ach	Aetylcholine
ACTH	Adrenocorticotropic Hormone
AD	Alzheimer's disease
ANOVA	Analysis of Variance
APA	American Psychiatric Association
ATP	Adenosine Triphosphate
BB	Bio-Breeding
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
BMI	Body Mass Index
cAMP	Cyclic Adenosine Mono Phosphate
CNS	Central Nervous System
CORT	Corticosterone
CREB	Cyclic AMP Response Elment Binding

CRF	Corticotrophin Releasing Factor
CSF	Cerebrospinal Fluid
CUS	Chronic Unpredictable Stress
DA	Dopamine
DSM	Diagnostic and Statistical Manual of Mental Disorder
DZM	Diazepam
EDTA	Ethylene-diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EPM	Elevated Plus Maze
EXM	Elevated Xero Maze
FST	Forced Swim Test
FLX	Fluoxetine
GABA	γ- Aminobutyric Acid
GAD	Generalized Anxiety Disorder
GC	Glucocorticoids
GLUT	Glucose Transporter
GPCR	G-protein Coupled Receptor
GSH	Reduced Glutathione
HPA	Hypothalamic Pituitary Adrenal Axis
hr	Hour
IAEC	Institutional Animal Ethics Committee
IDO	indoleamine 2,3 dioxygenase
IL	Interleukin
i.p.	Intra-peritoneal
KA	Kynurenic Acid

KO	Knock Out
LDT	Light-Dark Test
LHT	Learned Helplessness Test
MAO	Monoamine Oxidase
MAOI	Monoamine Oxidase Inhibitor
mCPBG	m-chlorophenylbiguanide
MDA	Malondialdehyde
MDD	Major Depressive Disorder
NSF	Novelty Suppressed Feeding
NCEs	New Chemical Entities
NDRI	Nor-epinephrine and Dopamine Re-Uptake Inhibitor
NMDA	N-methyl-D-aspartate
NOD	Non-Obese Diabetic
NRI	Nor-epinephrine Re-uptake Inhibitor
OBX	Olfactory Bulbectomy
OFT	Open Field Test
OND	Ondansetron
PD	Parkinson’s disease
PEG	Poly Ethylene Glycol
RH	Reserpine Induced Hypothermia
ROS	Reactive Oxygen Species
SERT	Serotonin Transporter
SIT	Social Interaction Test
SNRI	Serotonin and Nor-epinephrine Re-uptake Inhibitors
SSRI	Selective Serotonin Re-uptake Inhibitor

STZ	Streptozotocin
T1DM	Type-1 Diabetes Mellitus
T2DM	Type-2 Diabetes Mellitus
TBARS	Thiobarbituric Acid
TCAs	Tricyclic Antidepressants
TDO	Tryptophan Dioxygenase
TNF-α	Tumor Necrosis Factor-α
TP	Tryptophan
TST	Tail Suspension Test
US	United States
WHO	World Health Organization
°C	Degree centigrade
λ_{max}	Wavelength of maximum absorbance
%	Percentage
=	Equal to
<	Less than
>	More than
β	Beta
γ	Gamma
µM	Micro molar
µl	Micro liter
µg/l	Microgram per liter
µg/ml	Microgram per milliliter
L	Liter
Mg	Milligram

mg/Kg	Milligram per kilogram
Min	Minutes
mM	Millimolar
nM	Nanomolar
v/v	Volume by volume
w/v	Weight by volume

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Abstract

Depression is a highly prevalent, severely disabling, mood disorder characterized by sad mood, hopelessness, loss of motivation and disinterest. People with depression often experience symptoms similar to those of an anxiety disorder. There are several causative factors that can lead to depression and anxiety, among which, chronic metabolic illness such as diabetes mellitus (DM) is an important one. Epidemiological data suggests that diabetic patients are almost three times as likely to develop depression as those, who do not have a chronic medical condition.

Despite the presence of a multitudinous pharmacotherapy, depression and anxiety co-morbid with DM (in particular, Type-1; T1DM) remains undertreated. This could, partly be, attributed to the unclear complex pathophysiology, severe and frequent side effects of current drugs (in particular, adverse impact on glucose level, being the most). Hence, there is a grave need to develop novel candidates balanced with as high efficacy and minimum untoward effects as possible to further improve the pharmacotherapy.

5-HT_3 receptors have been identified as potential target for depression and related psychiatric disorders. 5-HT_3 receptor signaling is involved in mood, emotion, cognition, learning and memory, whereas 5-HT_3 receptor antagonists have been reported as potential antidepressant agents. In addition, 5-HT_3 receptor antagonists are suggested to have quick onset of action, specific and dose dependent therapeutic potential and wide safety margin. Interestingly, 5-HT_3 receptor antagonists are shown to have favorable effects on glucose homeostasis.

Therefore, the present work focuses on investigating the potential effect of standard (ondansetron, OND) and novel 5-HT_3 antagonists (synthesized in house in Medicinal Chemistry laboratory of the Department, **4i** and **6z**), to ameliorate depression and anxiety co-morbid with T1DM.

The work was conducted in adherence to three major objectives namely, (1) preliminary screening of antidepressant-like effects of test drug candidates; (2) investigation of the effects of selected drug candidates on depression and anxiety-like behavioral abnormalities co-morbid with T1DM as well as the underlying mechanism(s) of action, and (3) determination of the role of 5-HT_3 receptors in mediating antidepressant response of the tested drugs.

Dose response studies of OND (standard drug), **4i** and **6z** (the novel candidates with 5-HT_3 receptor antagonistic action) were conducted using basal locomotor activity test, followed by preliminary screening of antidepressant-like effects (using forced swim and tail suspension tests) and anti-anxiety-like effects (in elevated plus maze, open field test, social interaction test, light-dark test, hole board test).

Acute treatment with OND (0.5 and 1 mg/kg, i.p.), **4i** (0.5 and 1 mg//kg) and **6z** (0.5, 1 and 2 mg/kg, i.p.), exhibited significant antidepressant and anxiolytic-like effects in these neurobehavioral models, without affecting general locomotion in rodents.

To simulate the clinical time course of drug action, chronic rodent's behavioral studies were carried out, using chronic unpredictable stress (CUS) mouse and olfactory bulbectomy (OBX) rat models. Chronic treatment with OND (0.5 and 1 mg/kg, i.p.), **4i** (0.5 and 1 mg/kg, i.p.) and **6z** (1 and 2 mg/kg, i.p.) significantly reversed the chronic stress or OBX induced depression and anxiety-like behaviors; that corroborate the findings obtained in acute behavioral studies.

Following time course of development of depression and anxiety in STZ-induced type-1 diabetic mice (which was nearly 8-weeks after persistent diabetes), the antidepressant-like effects of OND (0.5 and 1 mg/kg, i.p.), **4i** (0.5 and 1 mg/kg, i.p.) and **6z** (1 and 2 mg/kg, i.p.) were evaluated. Chronic treatment with the test drug candidates, significantly reversed the behavioral anomalies induced by persistent diabetes in mice, indicating the antidepressant-like effects of the OND, **4i** and **6z**, in diabetic condition as well.

Neuro-biochemical, molecular and morphological studies were conducted to investigate the plausible underlying mechanism(s). OND (0.5 and 1 mg/kg, i.p.), **4i** (0.5 and 1 mg/kg, i.p.) and **6z** (1 and 2 mg/kg, i.p.) chronic treatment, significantly reversed the diabetes-induced imbalance in 5-HT levels and GABA levels in discrete brain regions (mid brain, frontal cortex and cerebellum).

In addition, chronic treatment with OND, **4i** and **6z**, inhibited the diabetes-induced hyperactivity of HPA-axis, as indicated by marked reduction in plasma corticosterone levels. Diabetes-induced impairment in neurotrophic factor signaling, measured in terms of BDNF and cAMP levels, in mid brain and frontal cortex, were also reversed by chronic treatment with the above mentioned drugs.

The histopathological evaluation of neuronal and sub-neuronal structures revealed that persistent diabetes induced a significant neurodegeneration, which was attenuated by the chronic treatment of the tested drug candidates.

Subsequently, the role of 5-HT_3 receptors, in mediating antidepressant response of OND (1 mg/kg, i.p.) **4i** (1 mg/kg, i.p.) and **6z** (1 mg/kg, i.p.), was investigated. Concomitant administration of mCPBG (m-chloro-phenyl-biguinide, 10 mg/kg, i.p.), a selective 5-HT_3 receptor agonist, blunted the behavioral and biochemical effects of OND, **4i** and **6z** in diabetic mice. This indicates that 5-HT_3 receptors play a key role, in cellular processes that regulate depression and anxiety-like behavior and antidepressant-like effects of the tested drug candidates.

Interestingly, the effects of OND (0.5 and 1 mg/kg, i.p.) **4i** (0.5 and 1 mg/kg, i.p.) and **6z** (1 and 2 mg/kg, i.p.) on glucose homeostasis were examined using fasting blood glucose and plasma insulin levels, as metabolic markers. OND, **4i** and **6z**, markedly reversed the increase in fasting blood glucose and decrease in plasma insulin levels in mice with chronic diabetes.

Altogether, the findings of the present work strongly suggest that, 5-HT_3 receptors play a key role in mediating cellular signaling pathways that regulate mood and emotional behavior. 5-HT_3 receptor antagonists may be effective therapeutic candidates to ameliorate depression and anxiety, co-morbid with T1DM.

The normalization of diabetes-induced impairment in neurotransmitter dynamics, HPA-axis hyperactivity, neurotrophic support and neural morphological remodeling may be the plausible underlying mechanisms of their antidepressant action; mediated by antagonism of 5-HT_3 receptors. In addition, antagonists at 5-HT_3 receptors may regulate, or at least, not cause aggravation of glycemic control; thereby providing an additional benefit over current antidepressants.

1.1 Depression

Depression is a highly prevalent, severely disabling, mood disorder with a worldwide bearing (Gilbert, 1992; Renneberg et al., 2005). Depression is an emotional condition in which a person feels discouraged, sad, hopeless, unmotivated or disinterested in life, in general. However, when this condition persists for more than two-weeks and when the feelings interfere with daily activities, it likely transforms into a psychological disorder that absolutely requires a medical intervention (APA, 2013).

Depression is often associated with anxiety. Perhaps, symptomatically these mood disorders are interwoven and inseparable. People with depression often experience symptoms similar to those of an anxiety disorder, such as nervousness, irritability, and problems in sleeping and concentrating. Moreover, both depression and anxiety increase the risk of suicidal tendencies (Gilbert, 1992). Many people who develop depression have a history of an anxiety disorder earlier in life. There is no evidence of which disorder causes the other, but there is clear evidence that many people suffer from both disorders. About 85% of patients with depression have significant anxiety, and 90% of patients with anxiety disorder have depression (Tiller, 2013). It also suggests that these disorders may share the common pathophysiology and signaling pathways.

There are several causative factors that can lead to depression and anxiety. These include:
(1) Traumatic life events, upheaval or sad experiences,
(2) Physical causes such as irregular sleep, eating habits and life style,
(3) Genetic factors and family history,
(4) Medications and substance or drug abuse,
(5) Chronic diseased conditions like cancer, heart disease, thyroid and other endocrinal problems, chronic pain, diabetes milletus (DM) and other metabolic disorders (Gilbert, 1992).

Having a chronic illness is one of the strongest risk factors for depression and anxiety. As mentioned above, there are several physical and mental chronic disorders that can gradually lead to depressive symptomology (Gilbert, 1992). Among them, DM is the foremost leading illness that can culminate into depression and anxiety. In fact, depression is one of the most common disorders co-morbid with DM (Katon, 2011). DM, itself is a seventh leading cause of deaths, worldwide (ADA, 2014).

It is a chronic, persistent, progressive and incurable metabolic disorder, which further exacerbates with the development of psychiatric complications such as depression and related conditions. Depression and anxiety co-morbid with DM makes the condition of patients, even worse. The co-morbidity makes the diagnosis of psychological condition difficult, reduces the efficacy of treatment intervention and increases the risk of complications. Therefore, depressed patients associated with DM require more medical attention than that of individual with general depression. Hence, the present study is aimed at investigating the underlying pathophysiology and screening of the novel candidates, for the manamgement of depression and anxiety co-morbid with DM, in particular Type-1 (T1DM), due to the fact that T1DM is more lethal and less studied as compared to the Type-2 DM (T2DM).

1.2 Diabetes Mellitus

Diabetes Mellitus is a chronic metabolic stress disorder characterized by deficiency in insulin secretion or action or both, resulting in hyperglycemia (the elevated circulating glucose levels). The disorder was the first recorded in 1552 B.C., when an Egypt physician diagnosed a patient with polyuria (frequent urination, among the major signs of diabetes mellitus). However, it was in the last century that diabetes mellitus was recognized to exist in two major forms, namely, T1DM (insulin dependent) and T2DM (non-insulin dependent).

1.2.2 Forms of diabetes mellitus: There are two basic forms of diabetes (Fig. 1.1)

1.2.2.1 Insulin dependent diabetes mellitus (Type-1), T1DM

T1DM also previously known as ‘Juvenile diabetes’ or ‘early onset diabetes’ is associated with a significant loss of insulin due to destruction of the insulin-producing β-cells of the islets of Langerhans in the pancreas. The insulin deficiency, leads to the impaired insulin mediated regulation of metabolic glucose, causing hyperglycemic state. The clinical damage of insulin producing β-cells is caused mostly by immune-mediated, in which a T-cell-initiated autoimmune attack leads to the loss of β-cells and thus insulin (Rother, 2007). Besides, T1DM may be caused by a genetic predisposition. T1DM patients constitute, although minority of the overall diabetic population (nearly 10 %), T1DM is the most lethal form of diabetes and is sensitive of inheriting severe complications in chronic state (Rother, 2007).

A number of medical risks are associated with T1DM including neurological and psychiatric disorders. Many of them stem from damage to the nervous system and subsequently impairment to the central nervous system (CNS) functions.

1.2.2.2 Non-insulin dependent diabetes mellitus (Type-2), T2DM

T2DM is the most common type. Among diabetic population, 90 % of the cases are T2DM (Rother, 2007). T2DM also called 'Adult diabetes' occurs due to insulin resistance leading to hyperglycemia. The T2DM patients have normal to elevated levels of circulating insulin. This form of diabetes mellitus, frequently remains undiagnosed at early stages, because the hyperglycemia develops gradually and initially is often not severe enough in patients to notice any of the classic symptoms of diabetes mellitus.

The pathogenesis suggests that the defective responsiveness of body tissues to insulin is believed to involve the insulin receptors. The causes of T2DM are due primarily to lifestyle factors and genetics (Granberg et al., 2005). The long term cases of T2DM also evoke the complications including that related to CNS. Several reports have suggested that persistent T2DM lead to associated behavioral disorders.

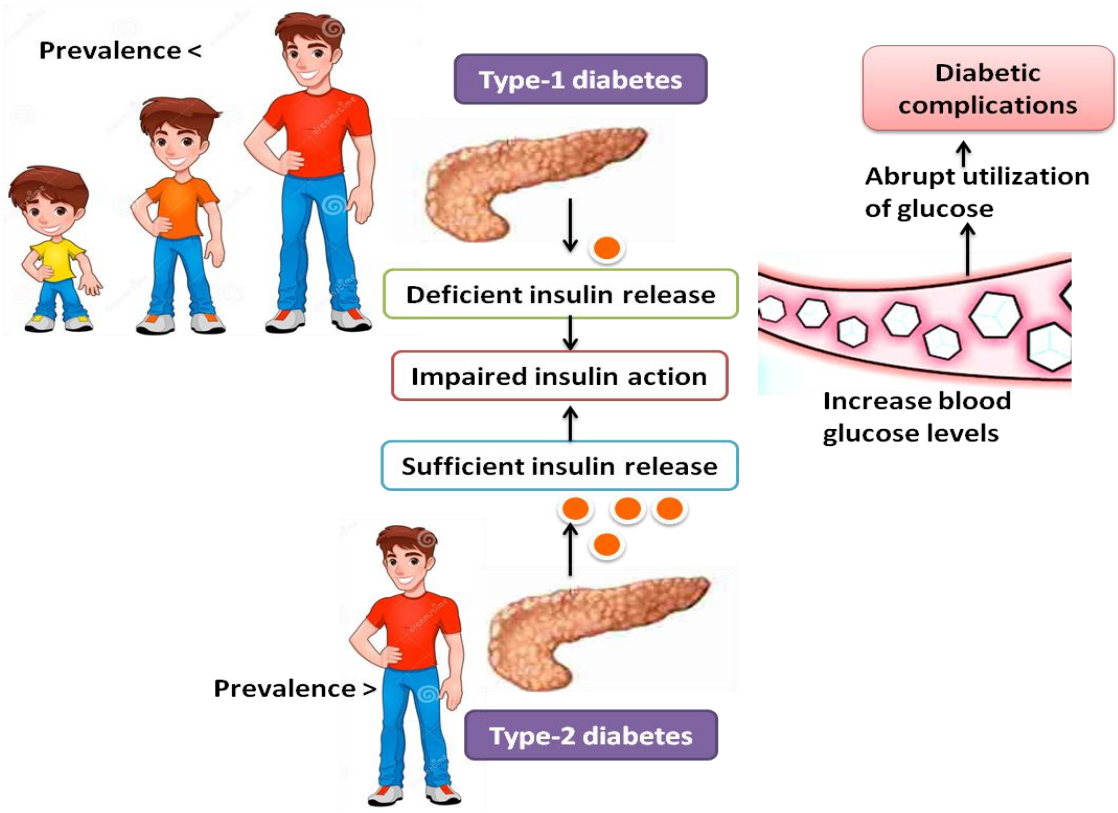


Fig. 1.1 The two forms of diabetes mellitus

1.3 Diabetes mellitus and central nervous system complications

So far, the research core on diabetes mellitus including T1DM has focused on peripheral endocrinology and nervous system. However, the impact of diabetes on the CNS been largely recognized. Among these cognitive and mood related complications have largely been studied in the past investigations. The following section will discuss in detail the effect of persistent diabetes mellitus on these complications (Fig. 1.2).

Fig. 1.2 The different psychiatric complications of diabetes mellitus

1.3.1 Diabetes mellitus and Parkinson's disease

Several epidemiological studies have reported that diabetic patients suffer from a variety of neurological disorders, including Parkinson's disease (PD). Almost 36 % of patients with diabetes have shown an increased risk of PD and often display symptoms of PD (Arvanitakis et al., 2007; Hu et al., 2007; Xu et al., 2011).

PD is a second most common neurodegenerative disorder, demonstrated by the symptoms like muscle rigidity, bradykinesia, resting tremor, and postural instability (Arvanitakis et al., 2004a).

Reports have shown that diabetes and PD share common pathophysiology that may be accompanied by the development of this co-morbidity (for further details please refer the review: Santiago et al., 2013). Impaired insulin signaling and glucose intolerance are two of them. More than 60% of PD patients have reported impaired insulin signaling and glucose intolerance. Recently, insulin resistance was found in 62% of PD patients with dementia, of whom 30% were glucose intolerant (Bosco et al., 2012).

1.3.2 Diabetes mellitus and Alzheimer's disease

Alzheimer's disease (AD) is another chronic neurodegenerative disorder characterized by a major symptom of dementia (Reitz et al., 2011). Vast research have established strong link between diabetes and AD, with a direct correlation between uncontrolled glycemic levels and dementia (Xu et al., 2007). The prevalence of AD has been reported in both types of diabetes (Type-1 and Type-2) with an increased risk of nearly 65 % (Arvanitakis et al., 2004b). To date, attention has largely focused on T2DM with insulin resistance being the primary insults. Conversely, sparse data are available on associations between T1DM and AD. Nevertheless, cognitive deficits, such as impaired learning, memory, problem solving and mental flexibility have been recognized as being more common in T1DM subjects than in the general population (Biessels et al., 2008).

1.3.3 Diabetes mellitus and schizoaffective disorders

Among several contributors, diabetes is one of the etiological factors that have proven to cause schizophrenia in humans. The diagnostic link between diabetes and schizophrenia has been found in early 20^{th} century, however, the recent reports revealing the association has alarmed and revived the interest in this field (Kohen, 2004). The etiology of this co-morbidity is uncertain, but the studies have reported that a combination of genetic, environmental (e.g., side-effects of antipsychotic medications), and lifestyle factors (e.g., sedentary lifestyle, poor diet) are likely play a role in the high prevalence of this co-morbidity (Citrome, 2004; Lamberti et al., 2004).

1.3.4 Diabetes mellitus and mood disorders

In addition to the aforementioned neurological perturbations, mood disorders have been significantly reported in diabetic patients; the main focus of the work reported herein. There are several forms of mood-related disorders defined by the standards (diagnostic and statistical manual (APA, 2013), that can be find in Table 1.1 (Sperry, 2003).

Table 1.1 Criteria for the diagnosis of mood disorders in DSM-IV

DSM-IV Criteria for Mood Disorders
Major Depressive Episode
5 or < symptoms (1 must be either depressed mood or loss of interest), Present for at least 2 weeks
Symptoms: ✓ Depressed mood ✓ Loss of interest in activities ✓ Significant weight loss or gain, or increase or decrease in appetite ✓ Insomnia or hypersomnia ✓ Psychomotor agitation or retardation ✓ Fatigue or loss of energy ✓ Feelings of worthlessness or guilt ✓ Inability to concentration or make decisions ✓ Recurrent thoughts of death or suicide
MAJOR DEPRESSIVE DISORDER
Single episode and Recurrent (2 or more episodes)
DYSTHYMIC DISODER
Depressed mood most of day for most days and 2 or more symptoms Present for at least 2 years (but not a Major Depressive Episode during this time)
Symptoms: ✓ Poor appetite or overeating ✓ Insomnia or hypersomnia ✓ Low energy or fatigue ✓ Low self-esteem ✓ Poor concentration or difficulty making decisions ✓ Feelings of hopelessness
Mixed Episode
Criteria for both Manic Episode and Major Depressive Episode Present for at least 1 week
BIPOLAR DISORDER
SINGLE MANIC EPISODE ✓ No past Major Depressive Episode
MOST RECENT EPISODE HYPOMANIC ✓ At least 1 past Manic or Mixed Episode
MOST RECENT EPISODE MANIC ✓ At least 1 past Major Depressive, Manic, or Mixed Episode
MOST RECENT EPISODE MIXED ✓ At least 1 past Major Depressive, Manic, or Mixed Episode
MOST RECENT EPISODE DEPRESSED ✓ At least 1 past Manic or Mixed Episode
BIPOLAR DISORDER ✓ At least 1 or more Major Depressive Episode ✓ At least 1 Hypomanic Episode ✓ Never a Manic or Mixed Episode
CYCLOTHYMIC DISORDER ✓ Numerous periods with hypomanic symptoms and depressive symptoms ✓ Present for at least 2 years, never without symptoms for more than 2 months ✓ No Major Depressive, Manic, or Mixed Episode

Manic Episode
Abnormally and persistently elevated mood 3 or more symptoms Present for at least 1 week
Symptoms: ✓ Inflated self-esteem or grandiosity ✓ Decreased need for sleep ✓ More talkative than usual ✓ Flight of ideas ✓ Distractibility ✓ Increase in goal-directed activity ✓ Excessive involvement in pleasure with high potential for painful consequences

Among those, depression and anxiety are the two major forms of disorders spread worldwide. Depression is a psychological disorder characterized by the low activity, sad mood and inability to experience pleasure or reward activity, whereas anxiety is associated with fear and hyperactivity. These disorders often exist together and share common symptomatic features such as agitation/retardation and irritability. These forms are discussed in later sections.

1.3.4.1 Diabetes mellitus and depression

Having diabetes increases the chances of developing depression, and depression can make it difficult to manage diabetes effectively.

Epidemiology: The prevalence of depression and related disorders in diabetic subjects is higher than in general population. Almost 20-25 % of T1DM patients report depressive episodes (Egede and Zheng, 2003) in their lifetime, while nearly 40% of patients with T1DM have elevated levels of anxiety (Grigsby et al., 2002) Other reports have indicated that T1DM subjects are associated with severe psychosocial withdrawal (Talbot et al., 1997). Recent evidence indicates that the prevalence psychiatric condition is increasing in populations of T1DM (Katon et al., 2009). There is also evidence to indicate a higher recurrence rate of depression in T1DM (Peyrot and Rubin, 1999). These increased rates of depression among people with T1DM have been confirmed in multiple studies, as well as across different cultural and ethnic groups (Pibernik-Okanovic, 2005). Worldwide estimates of the prevalence of depression among people with diabetes appear to vary by nation (Egede and Ellis, 2008), with prevalence rates ranging from roughly 15 to 40%. Though data is scarce from developing countries, studies report prevalence rates of 30% in rural Bangladesh (Asghar et al., 2007), suggesting that depression in Bangladesh is common and those with diabetes. Similarly high rates were reported in Greece at 33.45 (Sotiropoulos et al., 2008). The prevalence rates in other countries is given in Table 1.2

Table 1.2 Prevalence of depression in diabetic patients across the world

Country	Prevalence	References
India	Nearly one third (29% males, 30% females) of those with diabetes reported clinical depression, compared with 6% of males and 15% of females without diabetes	Asghar et al., 2007
Pakistan	14.7% (6.6-22.8) amongst those with diabetes as opposed to 4.9 (3.7-6.1) amongst those without diabetes	Zahid et al., 2008
Spain	Prevalence rates of 15.4% are reported in older people (over 55) with diabetes, compared with 11% in those without diabetes	de Jonge et al., 2006
America	Indigenous American Indians have higher rates of diabetes and depression compared to the general population Native Americans with T2D–a threefold increased risk of depression	Singh et al., 2004 Asghar et al., 2007
Dutch and Croatian countries	Croatian males have higher prevalence, as compared with Dutch or English men (39% vs 19%), but similar rates in Croatian and English women (34% vs 39%), which were lower in Dutch women (21%)	Pouwer et al., 2005; Lloyd et al., 2009
Iran	Depression was diagnosed in 41.9% of patients in a cross-sectional study of 375 diabetic patients	Larijani et al., 2004
China	A nationally-representative sample of older Chinese found that amongst older persons with diabetes, 26% of them reported elevated levels of depressive symptoms	Chou et al., 2005

1.3.4.1.1 Need for treating depression in diabetes mellitus

High severity of symptoms: Depression co-morbid with T1DM is associated with poor self-care, lack of exercise and non-adherence to dietary or medication routines, leading to inadequate glycemic control and severe symptomology. Several clinical studies have reported the attenuation of self care behavior in diabetic patients with depression. Lin et al. (2004) confirmed this notion by analyzing home based activities in these patients. They showed that depression was mainly associated with patient-initiated behaviors that are difficult to maintain (e.g., exercise, diet, medication adherence). Moreover, depression symptoms were shown to increase alternatively with the self careless behavior (Ciechanowski et al., 2003; McKellar et al., 2003). It has been found that depression impacts subsequent physical symptoms of poor glucose control by influencing ability of patients to adhere to their self-care regimen.

***Increase relapse of symptoms*:** In diabetic patients, the course of depression is chronic and severe and up to 80% of patients with both conditions are expected to experience a relapse of depressive symptoms in a 5-year period (Katon et al., 2005).

Poor medical outcomes: Depression and diabetes have been associated with poor medication adherence (Kilbourne et al., 2005), that is responsible for poor medical outcomes and ineffective therapy. Depression affects the ability to perform tasks, communicate and think clearly, which can interfere with the ability to manage diabetes.

Increase risk of complications: Depression can lead to poor lifestyle decisions, such as unhealthy eating, less exercise, smoking and weight gain, all of which increases risk for diabetes complications. This also leads to poor glycemic control (McKellar et al., 2004) and an increased risk of complications of both disorders (Bruce et al., 2005).

High medical costs: The economic burden of diabetes alone is high, and depression along with diabetes manifests additional health-service costs by 50-75 % (Petrak and Herpertz, 2009). Egede and Ellis, (2008) reported that healthcare expenditures were even larger and costs were 4.5 times greater in patients with depression than those who were not, in the USA.

1.3.4.1.2 Current therapy for depression co-morbid with diabetes mellitus

Depression in diabetic patients follows a chronic and severe course as compared to depressed individuals without diabetes (Lustman et al., 1992). Most patients show recovery (Keller et al., 1982a) or remission from an initial episode of major depression (Mueller and Leon, 1996). However, these patients have a high risk for relapse and experience additional episodes during the course of patient's life (Keller et al., 1982b).

1.3.4.1.2.1 Antidepressant therapy

Based on the assumption that persistent diabetes may lead to neuropathological changes in the brain, similar to those found in depressed patients, several currently existing antidepressants have been presented as therapeutic interventions to treat behavioral complications in diabetes (Lustman et al., 1998).

The monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs): Treatment strategy utilizing MAOIs and TCAs has been used to treat depression in diabetic patients.

Previous reports have shown that, TCAs are effective in relieving depression associated with diabetic subjects. Phenelzine was used to prevent depression in diabetic patients which showed 27 % recovery of symptoms (Goodnick et al., 1995). Lustman et al. (1997) showed that the reduction in depression symptoms was significantly higher in depressed patients received nortriptyline (TCA and a norepinephrine reuptake inhibitor) compared to those given placebo. In another study, imipramine produced 81% response rate in relieving depression in diabetic patients (Himmelhoch et al., 1991). However, TCAs do not have complete remission rate.

Selective serotonin reuptake inhibitors: Selective serotonin reuptake inhibitors are currently the first line drugs to mitigate depression-related behavior in diabetes. These act by inhibiting the pre-synaptic reuptake and enhancing the synaptic serotonergic levels (Goodnick, 2001). Previous reports have shown that fluoxetine (an SSRI) has shown recovery of depression symptoms in patients with diabetes. An 8-week, randomized, placebo-controlled, double-blind study evaluated the efficacy of fluoxetine in 60 patients with type 1 and MDD (Lustman et al., 2000). Fluoxetine effectively reduced the severity of depression in diabetic patients and produced an efficacy of 66.7 % as compared to placebo which showed 37 % recovery of symptoms (Lustman, et al., 2000). In another study, sertraline, another SSRI showed effective antidepressant effect in diabetic subjects (Goodnick et al., 1995). Paroxetine on the other hand did not reveal significant outcome measures. Paile-Hyvarinen et al. found no significant relief of depression symptoms in diabetic subjects as compared to placebo in both single blind and double blind study (Paile-Hyvarinen et al., 2003, 2007). However, Gulseren et al. (2005) found a significant efficacy of depressive symptoms in patients with diabetes treated with paroxetine. Thus, it indicates that SSRIs are effective in preventing depression associated with diabetes.

Serotonin norepinephrine reuptake inhibitors: Milnacipran, a SNRI was also tested for preventing depression in diabetes. In a clinical study, milnacipran was effective in relieving depression symptoms in respondent diabetic patients with a recovery rate of 50 % (Abrahamian et al., 2009). These patients also received metformin, an anti-diabetic drug. However, the treatment dose was higher in respondents than that of non-respondents and the duration of treatment was chosen 6 months to find relief of depressive symptoms. However, the effect produced by the drug was similar to that produced in non-diabetic subjects with depression.

Nor-epinephrine and dopamine reuptake inhibitors: Bupropion, a norepinephrine dopamine reuptake inhibitor (NDRI) exhibited efficacy similar to those of SSRIs in relieving depression associated with diabetes. In a clinical study, bupropion was effective in preventing depression in diabetic subjects with persistently maintaining the treatment schedule (Lustman et al., 2007). Recovery from the symptoms were shown to be observed after acute phase of treatment (10 weeks) and the symptoms remained consistently low in maintenance phase (24 weeks), indicating that bupropion may be an effective alternate to SSRIs. However, bupropion has been reported to improve symptoms only at high and chronic dosing that may lead to dose related adverse effects in patients.

1.3.4.1.3 Limitations of currently existing antidepressants

Although, currently existing antidepressants have shown efficacy in depression in diabetic patients, these are associated with major limitations (Table 1.3).

Table 1.3 Effects of various antidepressants on metabolic parameters

Drug	Effect on metabolic parameter	References
Nortryptaline	Worsen glycemic control	Lustman et al., 1997
s-citalopram	Non-significant reduction in fasting glucose and glycosylated Hb-A1C	Amsterdam et al., 2006
Bupropion	Improved glucose levels on high and chronic dosing	Lustman et al., 2007
Fluoxetine	Induced hyperglycemia	Carvalho et al., 2004
Venlafaxine	No effect on the metabolic parameters	Kunz et al., 2000
Milnacipran	Reduction in HbA1c, fasting blood glucose, body mass index, total and LDL-cholesterol and serum triglyceride levels only in responders	Abrahamian et al., 2009
Mirtazapine	Increased in body weight, body fat mass and leptin concentration	Laimer et al., 2006
Buspirone	No effect on basal plasma glucose and insulin levels	Ojha et al., 2006
Phenalzine	Hypoglycemia and glucose intolerance	Adnitt, 1968, Goodnick, 1995
Mebanazine	Hypoglycemia and glucose intolerance	Adnitt, 1968
Sertraline	Slight improvement in hemoglobin A1c	Goodnick, 1997
Duloxetine	Increased fasting glycemia	Raskin et al., 2005
Imipramine	Increased body mass index and decreased insulin secretion	Moosa et al., 2003

Delayed onset of therapeutic effects: Although SSRIs are the first choice of antidepressants, these are reported to have delayed onset of therapeutic effects. Several studies have shown that SSRIs show improvement of depressive symptomology only after several weeks of treatment (Demyttenaere, 1997; Guadarrama-Cruz et al., 2008). Speed of onset of the antidepressant action is clinically important for several reasons. Delayed onset of therapeutic effects indicates that persistent depressive symptoms, its associated disability, and for some patients the potential risk of suicide continues. Thus, overcoming the slow onset of action is of prime concern.

Low remission rates: Remission from depression is defined as being free or nearly free of symptoms from the current episode. Several reports have shown that current antidepressants have low remission rates. A large proportion of the patients do not respond adequately to the SSRI as first-line therapy. The rate of treatment response from baseline symptoms following SSRIs has shown to be moderate, varying from 40-60 % and remission rates vary from 30-45% (Carvalho et al., 2007). This suggests that a new course of therapy is essentially required.

Frequent relapse of the symptoms: It has been reported that several of currently existing antidepressants show relapse of depressive symptoms after discontinuation of the therapy. Moreover, patients have reported severe symptoms while still on therapy. Up to one third of persons on drug treatment will develop recurrent symptoms of depression and suicidal tendency while taking medication (Souery et al., 2006).

Impairment of glycemic control: Several clinical and preclinical reports have revealed that antidepressants have a significant impact on blood glucose regulation. For example, a preclinical report indicated that central administration of fluoxetine induces hyperglycemia in rats (Carvalho et al., 2004). Amitriptyline, doxepin and imipramine also reported, altered glucose homeostasis in patients with diabetes (McIntyre et al., 2006). Similarly, maprotiline, nortriptyline, mianserin and mirtazapine showed, increased glucose levels in diabetic patients. Based on the analysis it is observed that hyperglycemia is more pronounced with antidepressants having NE reuptake inhibiting property and facilitating 5-HT, 5-HT_{2C} receptor signaling. This could be due to the fact that NE increases the glucose levels in blood stream by directly stimulating glycogenolysis and gluconeogenesis (Larsen et al., 2003).

In addition, central blockade of 5-HT_{2C} receptors stimulates energy intake by increasing appetite with a resultant positive energy balance (Mulder et al., 2006; Tecott et al., 1995; Wirshing et al., 1999) which may result in impaired insulin signaling and increased hyperglycemia.

Increase weight gain and body mass: In addition to increase in glucose levels in diabetics, certain antidepressants have shown changes in energy metabolism and increase in body weight. As discussed above, antidepressants with high affinity on 5-HT_1 and 5-HT_{2C} receptors affect satiety and increase body weights. In a clinical study, mirtazapine was shown to increase body weights and leptin concentration in diabetic subjects (Laimer et al., 2006). Also, imipramine exhibited increased body mass index (BMI) and decreased insulin secretion in depressed patients (Moosa et al., 2003). Amitriptyline also showed an increased BMI in patients (Hinze-Selch et al., 2000). The increase in body weight may affect body energy homeostasis that may lead to impaired insulin action and hyperglycemia. These studies suggest that TCAs are more prone to induce body weight gain as comparative to other class of antidepressants.

Increased risk of developing cardiac and vascular complications: Some clinical studies have reported increased cardiac and vascular complications in patients treated with various antidepressants. There was an increased risk of ischemic heart disease in patients treated with TCAs after diabetes. This was also found in few patients those received SSRIs (Hippisley-Cox et al., 2001).

Drug interactions: Drug interactions are the frequent problems with the current antidepressants due to concomitant use of anti-diabetic drugs (Gumnick and Nemeroff, 2000; Masand, 2003; Robinson, 2003), due to alterations in drug disposition and metabolism.

Poor therapeutic response: It has been found that persistent diabetes may lead to poor therapeutic response of antidepressants. The antidepressant effects of drugs like fluoxetine and of those mediated by 5-HT_{1A} receptor modulation was shown to be attenuated by diabetes in mice (Miyata et al., 2004).

1.4 Summary

Altogether, it is summarized that:

1) Depression and anxiety more commonly occur in diabetes mellitus.
2) Having depression and anxiety co-morbid with diabetes worsens the condition in diabetic patients and increases both depression and diabetes associated complications.
3) Till date current antidepressants though effective, the efficacy is not 100 % and the drugs have a number of severe undesirable effects.
4) Therefore, this remains a crucial need to develop and discover new effective agents with a balance of high efficacy and less adverse effects for improving depression pharmacotherapy in diabetes.

2.1 Serotonin

Serotonin (5-HT), chemically known as 5-hydroxytryptamine is a monoamine that structurally contains one amino group attached to an aromatic ring by a two carbon chain (Fig. 2.1). Biologically, it is a neurotransmitter widely distributed in the periphery and in the central nervous system (CNS), where it plays a key role regulating a number of biological processes.

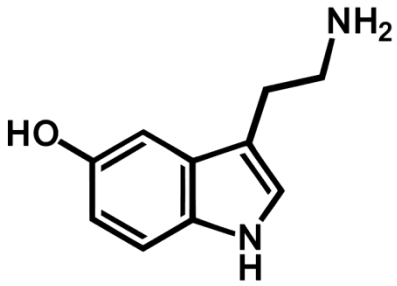


Fig. 2.1 The structure of serotonin (5-Hydroxytryptamine)

2.1.1 Overview

In the neuronal cell, it is synthesized from the precursor L-tryptophan. L-tryptophan is converted into 5-HT by a short biosynthetic pathway involving two enzymatic systems. TP is initially transformed into 5-hydroxy L-tryptophan (5-HTP) by tryptophan hydroxylase (TPH) enzyme (Sakowski et al., 2006; Walther et al., 2003; Zhang et al., 2004). 5-HTP is then converted into 5-HT by 5-HTP decarboxlylase enzyme via decarboxylation process. 5-HT in impermeable to blood brain barrier and hence does not traverse from the periphery to the brain (Fernstrom et al., 2013; Fernstrom and Wurtman, 1971). L-tryptophan enters into the brain by active transport mechanism, after competing with the tyrosine and other branched chain aminoacids for the carrier protein (Fernstrom and Fernstrom, 1995). 5-HT is then stored in pre-synaptic vesicles by vesicular monoamine transporter (VMAT) system, shielding it from degradation and concentrating it into the vesicles for release into synaptic cleft (Tamir and Gershon, 1979). Once inside the vesicles, it remains stored till the signaling stimuli potentiates the release of 5-HT in synaptic cleft. After being released into the cleft, it binds to multitude of the receptors and causes cascading events to further processing of brain.

5-HT action is then terminated by two major mechanisms. One involves the pre-synaptic reuptake and other the metabolic degradation of synaptic 5-HT as pictorially represented in Fig. 2.2.

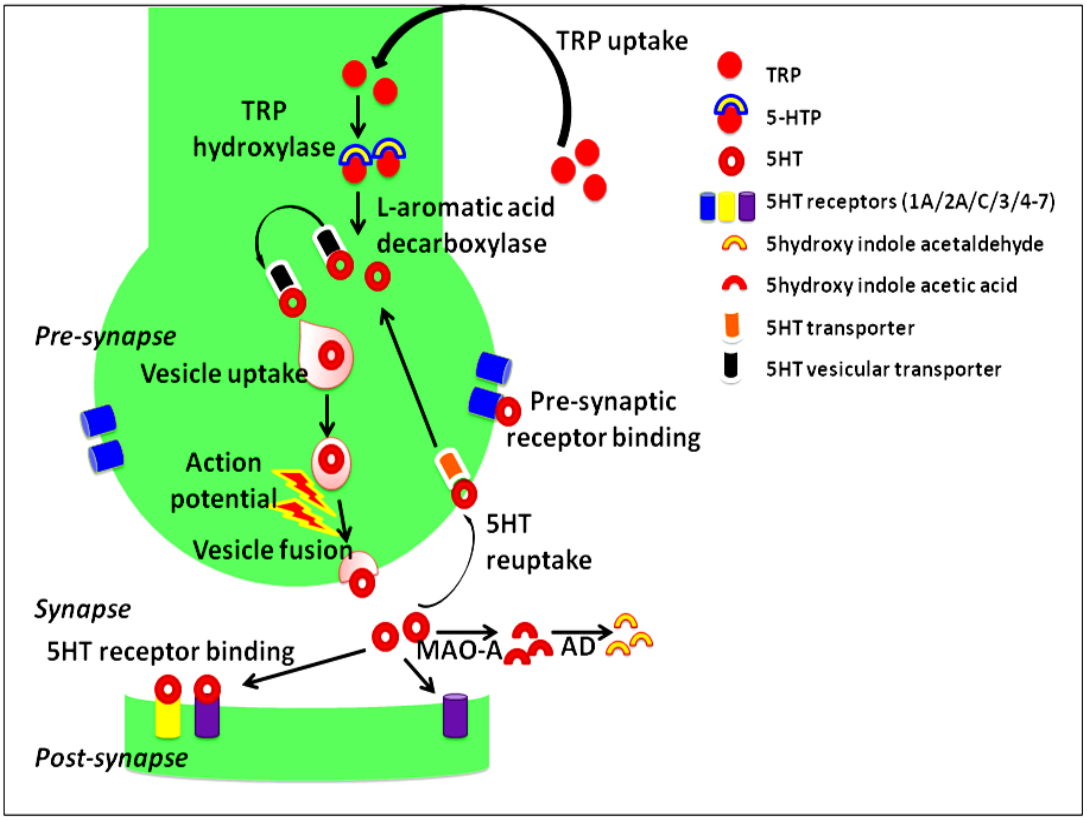


Fig. 2.2 Pictorial representation of the serotonin system

2.1.2 Distribution

Almost 95 % of total body 5-HT is found in periphery, in particular, densely concentrated in the gut where it is released by intestinal enterochromaffin cells (Gershon and Tack, 2007); at the very moment that food enters the gastro-intestinal track. Within the brain, the highest concentration of 5-HT is found in raphe nuclei of the brain stem (Blier and Mansari, 2013). Major amount of the brain 5-HT is synthesized in this region. From raphe nuclei, the serotonergic pathway projects to various regions as indicated in Fig 2.3 (Azmitia et al., 2000).

2.1.3 Functions

5-HT is an important contributor, regulating mood and emotional behavior. In the brain, the signaling cascades utilizing 5-HT neurotransmission affect the psychological status of an individual. Besides, 5-HT also regulates appetite, sleep, cognitive functions, including memory and learning (Mosienko et al., 2015). 5-HT does so by a bunch of its receptor systems.

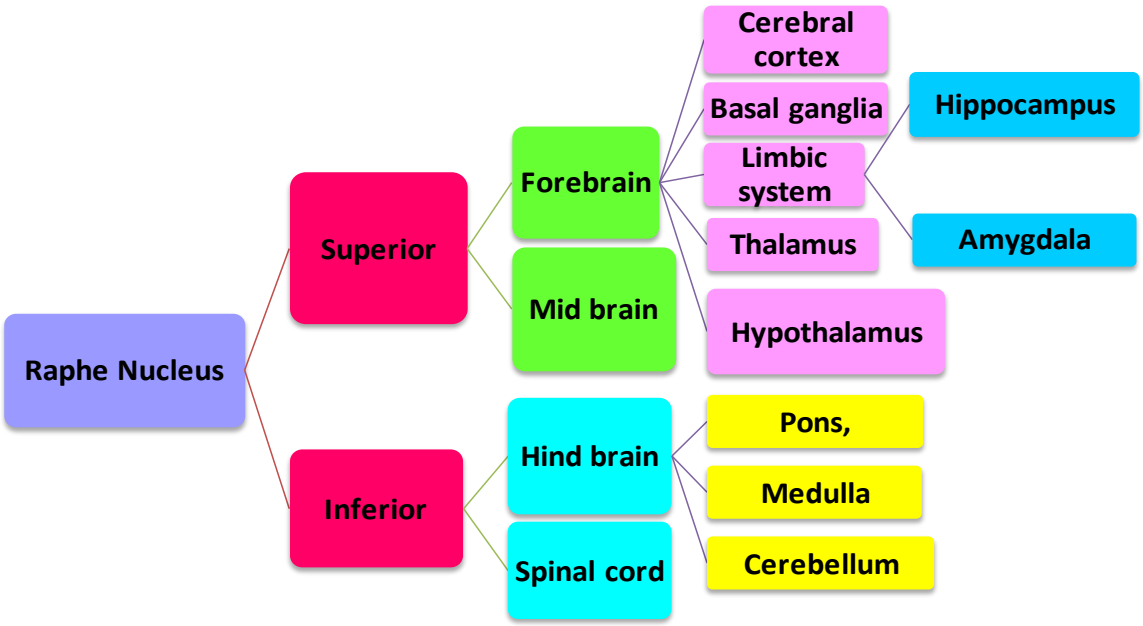


Fig. 2.3 The schematic diagram showing innervations of 5-HT system and firing in brain areas

2.1.4 Serotonin Receptors

There are 14 receptor subtypes of 5-HT, which are all G-protein coupled receptors except 5-HT_3 receptor type, which is a ligand gated ion channel (Lummis, 2012).

2.1.5 5-HT_3 receptors

The term, 5-HT_3 receptors were first proposed by Bradley et al. (1986), although it was a mere renaming of the 5-HT-M receptor discovered by Gaddum and Picarelli (1957). Initially, it was identified in high concentrations in guinea pig intestine, which demonstrated the peripheral action of this receptor, where it mediates depolarization of neurotransmitter release (Rondé and Nichols, 1998). However, until the radioligand-binding studies revealed 5-HT_3 receptors in brain, the role of it, in CNS was less known.

2.1.6 5-HT_3 receptors: the structure

The membrane spanning 'cationotropic' receptors belong to a pentameric neurotransmitter gated Cys-loop receptor class and are responsible for fast excitatory and inhibitory neurotransmission (Fig. 2.4) (Lummis, 2012). To date, five subunits of 5-HT_3 receptors have been identified in humans; 5-HT_{3A}, a homopentameric type and 5-HT_{3B}, a heteropentameric form, and 5-HT_{3D}, 5-HT_{3E}, 5-HT_{3F} that are yet to be elucidated (Niesler et al., 2007).

Conversely, only two subunits namely, 5-HT_{3A} and 5-HT_{3B} have been found in rodents (Karnovsky et al. 2003). This could possibly present the reason why the structures of these subunits remain unknown. Recently, the discovery of x-ray crystallographic structure of the 5-HT_{3A} receptor in mouse has significantly refined our understanding on the full length nanostructure of 5-HT_3 receptor (for details refer: Hassaine et al., 2014). Basically, a single 5-HT_3 receptor unit contains the symmetrically arranged subunits, in five-fold cylindrical pattern, around an aqueous pore with gradual tapering downwards the membrane, forming the tree major domains: extracellular domain, intracellular domain and the transmembrane domain, as shown in Fig. 2.4.

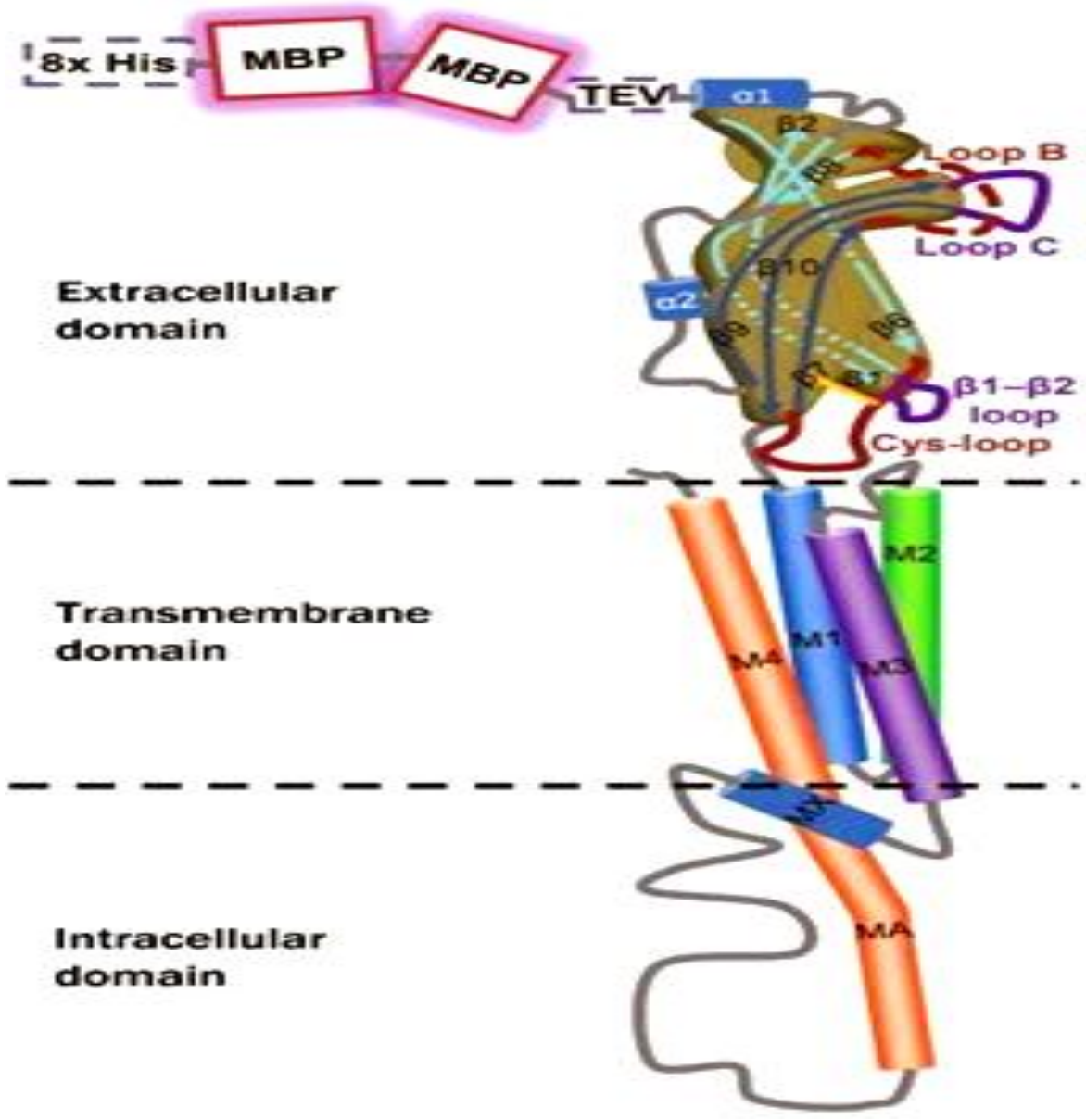


Fig. 2.4 The structure of 5-HT_3 receptor (showing different domains), (Hassaine et al., 2014)

2.1.7 5-HT_3 receptors: the brain distribution

2.1.7.1 Cellular distribution

The current interest in understanding the role of 5-HT_3 receptors in depression is primarily contributed by studies based on radio-ligand binding and electrophysiology that demonstrated a high occupancy of 5-HT_3 receptor binding sites in discrete brain regions, in particular, those that regulate affective behavior (Fig. 2.5). 5-HT_3 receptors are widely distributed in different regions of brain, in rodents, primates and humans.

2.1.7.1.1 5-HT_3 receptors distribution in rat brain

In rats, a 5-HT_3 receptor antagonist (3H-GR65630) binding studies have shown the presence of 5-HT_3 receptors in hippocampus, amygdala, nucleus accumbens, frontal and entorhinal cortex. However, no significant 5-HT_3 receptors binding sites have been found in cerebellum (Kilpatrick et al., 1987; Miller et al., 1992). Similarly, ligand binding experiments with 5-HT_3 receptors antagonists namely 3H-LY278584 and [125I] iodo-zacopride, evidenced high abundance of 5-HT_3 receptors in nucleus of solitary tract, dorsal motor nucleus of vagus and area postrema, limbic areas including hippocampus, the tract of olfactory bulb and to a small extent in dorsal raphe nucleus (DRN), striatum and substantia nigra (Gehlert et al., 1991; Koscielniak et al., 1989; Laporte et al., 1992).

2.1.7.1.2 5-HT_3 receptors: distribution in mice brain

In mice, studies based on the quantitative autoradiographic techniques have shown the presence of 5-HT_3 receptors in cortical and hippocampal regions (Mössner et al., 2004). Among the cortical regions, highest concentration was found in cingulate cortex followed by frontal and parietal cortex. Within hippocampus, maximum density was reported in CA_3 and CA_1 regions with neurons expressing 5-HT_{3A} subunit in high population.

2.1.7.1.3 5-HT_3 receptors: distribution in primate brain

Studies have reported a wide distribution of 5-HT_3 receptors in cortical circuits of primate brain (Jakab and Goldman-Rakic, 2000). Recent findings have revealed the presence of 5-HT_{3A} subunit in hippocampal regions as well (Shukla et al., 2014).

2.1.7.1.4 5-HT_3 receptors: distribution in human brain

In humans, the distribution pattern of 5-HT_3 receptors is well elaborated. Autoradiographic binding studies using 3H-(S)-zacopride, [3H] GR65630 and [3H] LY27858 revealed the variable densities of 5-HT_3 receptors in various regions of brain.

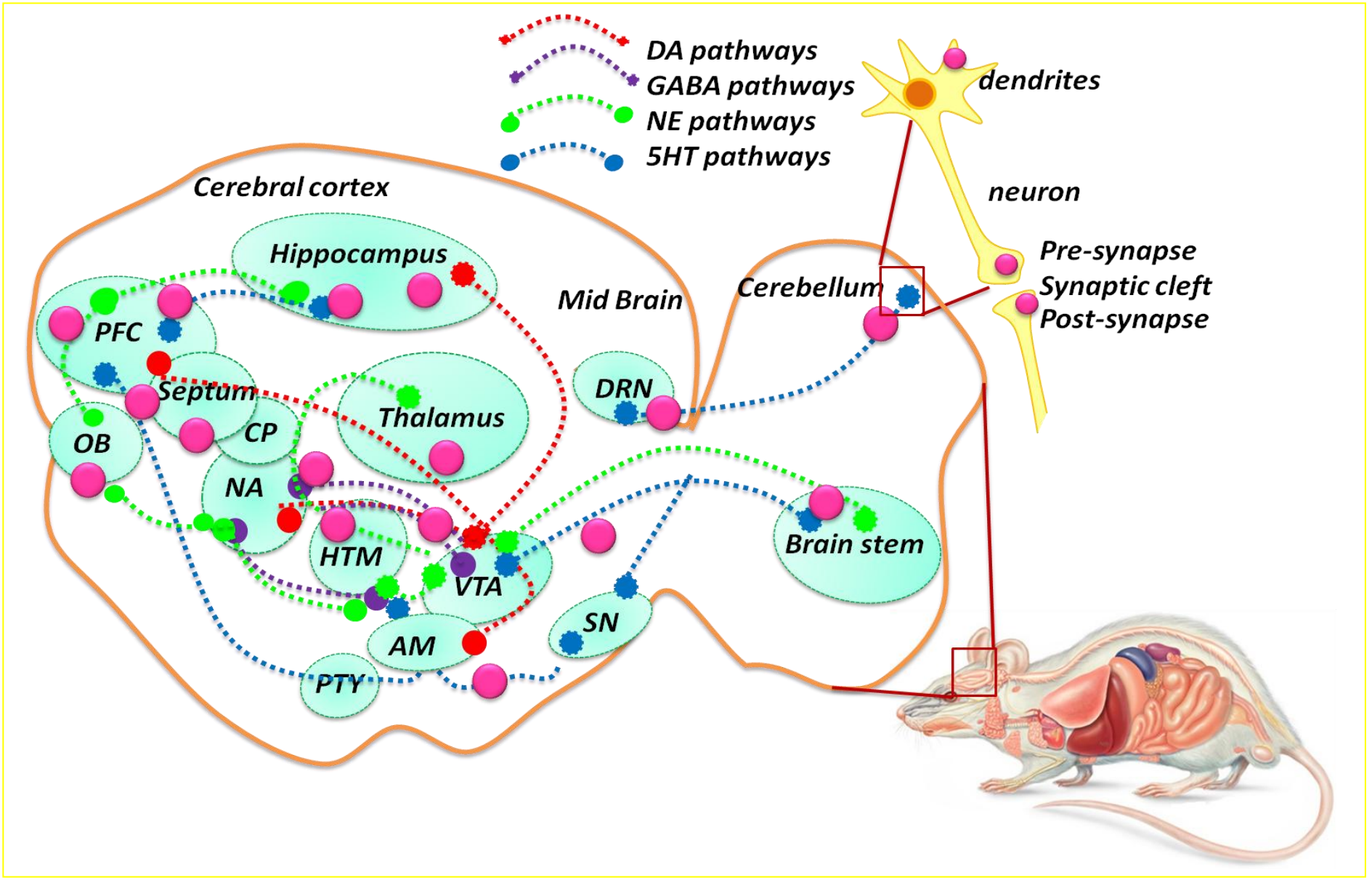


Fig. 2.5 Distribution of 5-HT_3 receptors in the brain

These include brain stem (nucleus tractus solitaries, area postrema, spinal trigeminal nerve nucleus), forebrain (hippocampus, nucleus accumbens, putamen, caudate nucleus), olfactory tract and cortical regions (prefrontal cortex) (Abi-Dargham et al., 1993; Parker et al., 1996; Marazziti et al., 2001). However, negligible amounts were reported in cerebellum (Hammer et al., 2012; Marazziti et al., 2001). The variable distribution and different concentrations of 5-HT_3 receptors in brain, suggests its influence in regulating affective behavior.

2.1.7.2 Subcellular distribution

Within the neuronal circuits, 5-HT_3 receptors occupy interneuron terminals, in addition to the serotonergic projections and within the sub-neuronal sites, 5-HT_3 receptors are located both at the presynaptic and postsynaptic terminals (Choi et al., 2007). Multiple functional studies support the presence of 5-HT_3 (hetero) receptors in presynaptic portals of GABA neurons, where activation of these carries the fast synaptic excitation mediated GABA release (Choi et al., 2007).

Moreover, the functional experiments have confirmed the location of presynaptic 5-HT_3 receptors on a sub-population of GABAergic interneurons in rat forebrain (hippocampus, amygdala and striatum) (Katsurabayashi, 2003; Koyama et al., 2000; Nayak et al., 1999; Rondé and Nichols, 1998), prefrontal cortex (Puig et al., 2004) and telencephlon (Morales et al., 1996).

Recent studies have demonstrated the expression of 5-HT_3 receptors in other neurotransmitter system as well. Oostland et al. (2011) showed abundant expression of 5-HT_3 receptors in glutamate interneurons in mice cerebellum. They reported the presence of 5-HT_3 receptors both at presynaptic and postsynaptic glutaminergic portals.

The presence of 5-HT_3 receptors in dopamine (DA) interneuronal projections is poorly known. However, stimulation of these receptors has been reported to modulate the excitatory effect of compounds acting upstream from DA neurons (Engleman et al., 2008). Consistently, a number of studies reported region specific regulation of 5-HT_3 receptors in DA neurotransmission in many brain areas including mesolimbic, mesocortical and nigrostriatal DA pathways (Allan et al., 2001; Dempsey et al., 2005; Porras et al., 2003; Rammes et al., 2004).

Altogether, these evidences help us come to a consensus on the following points:

1) Within a neuronal domain, 5-HT_3 receptors have both axon terminus and dendrite occupancy

2) Within neuronal circuits, 5-HT_3 receptors are widely distributed both at serotonergic neurons and at inter-neuronal projections of the other neurotransmitters

3) 5-HT_3 receptors control the synaptic neurotransmission of homo (5-HT) and hetero (GABA and glutamate) neurotransmitters with varying degrees of inhibitory and excitatory effects

4) Consequently, modulation of 5-HT_3 receptor activity may be implicated in neurobehavioral effects of these neurotransmitters in brain

2.1.8 5-HT_3 receptors: the neuronal activity

Ionotropic 5-HT_3 receptors, mediate fast neuronal transmission and subsequent neuromodulation in discrete brain areas. Stimulation of 5-HT_3 receptors results in opening of the channel and influx of cations into the cell, leading to rapid membrane depolarization and release of neurotransmitters (Reeves and Lummis, 2002). Although, 5-HT_3 receptors mainly control influx of sodium and potassium ions, studies indicate the channel permeability of calcium ions as well (Reeves and Lummis, 2002). Specifically, reports have demonstrated that 5-HT_3 receptors at neuronal terminals induce calcium ion influx, while those at postsynaptic site infuse sodium and potassium ions (Rondé and Nichols, 1998; Turner et al., 2004).

2.1.9 5-HT_3 receptors and depression

There are few investigations including our findings that indicate the potential antidepressant effect of 5-HT_3 receptor antagonists. The antidepressant response was initially observed in the late 90s, when the 5-HT_3 receptor antagonists in clinical use (such as ondansetron and zacopride) demonstrated a significant reversal of depressive behavior in learn helplessness test in rats (Martin et al., 1992; Thiebot and Martin, 1991). Later, the role of 5-HT_3 receptors in depressive symptomology was also suggested by the findings that 5-HT3R agonists attenuate (Nakagawa et al., 1998), while 5-HT_3 receptor antagonists potentiate the action of clinical antidepressants (Redrobe and Bourin, 1997). The effects of chemically dissimilar 5-HT_3 receptor antagonists were then observed in a number of neurobehavioral animal models (Table 2.1), clearly revealing their antidepressant potential.

Moreover, studies have shown that the antidepressants are functional antagonists of 5-HT_3 receptors (Eisensamer et al., 2003). Similarly, clinical studies have revealed the antidepressant effects of 5-HT_3 receptor antagonists. Piche et al. (2006) and Dimitrov, (2009) have reported that ondansetron prevents depressive symptomology in hepatitis infected patients. Ondansetron has presented beneficial effect in relieving mood disturbance predisposed in alcohol dependent patients (Johnson et al., 2003) and in bulimic patients (Faris et al., 2000).

Table 2.1 The antidepressant activity of various 5-HT_3 receptors antagonists in preclinical investigations.

5-HT_3 receptor antagonist	Behavioral model	Species	Reference(s)
Zacopride	LHT	Rats	Martin et al., 1992
MCL-225	FS	Rats	Eguchi et al., 1997
Tropisetron (ICS205-930)	TST	Male C57BL/6J/ Han mice	Bravo and Maswood, 2006;
Tropisetron (ICS205-930)	FST	Female Wistar rats	Kos et al., 2006
Bemesetron (MDL72222)	TST	Male C57BL/6J/ Han mice	Kos et al., 2006
Ondansetron (GR 38032F)	FST TST OBX	Mice Mice Rat	Ramamoorthy et al., 2008
QCF-3	FST; TST; OBX; 5-HTR; RH	Mice Mice Rats Mice Rats	Devadoss et al., 2010
Vortioxetine (Lu AA21004)	FST	Flinders Line rats	Mork et al., 2012
A6CDQ	TST	Mice	Dukat et al., 2013
7a	FST; TST; OBX	Mice Mice Rats	Gautam et al., 2013
Vortioxetine (Lu AA21004)	FST	BalB/cJ≠RJ Mice	Guilloux et al., 2013
Ondansetron (GR 38032F)	FST	Flinders sensitive Line and Flinders Resistant Line Rats	Bétry et al., 2015

It is interesting to note that since anxiety disorders share many neurobiological substrates of depression and hence antagonism of 5-HT_3 receptors may also have anxiolytic-like pharmacological effect. In line with this contention, several drugs and novel candidates with 5-HT_3 receptor antagonistic action have been evaluated for anxiolytic-like response (Table 2.2).

Table 2.2 Anxiolytic action of 5-HT_3 receptor antagonists in preclinical studies

5-HT_3 receptor antagonist	Behavioral model	Species	Anxiolytic response	Reference(s)
Ondansetron (GR 38032F)	SIT	DBA/2 female mice	+	Cutler, 1991
RS-42358-197	SIT EXM	Rats	++	Costall et al., 1993
Tropisetron (ICS205-930)	EPM	Mice	+	Artaiz et al., 1995
Ondansetron (GR 38032F)	EPM	Mice	-	Artaiz et al., 1995
VA21B7	LDT PDT EPM	Mice	++	Artaiz et al., 1995
WAY-SEC-579	LDT	Mice	++	Middlefell et al.,1996
Ondansetron (GR 38032F)	SIT EPM	Rats	++ +	Eguchi et al., 2001
MCL-225	SIT EPM	Rats	++	Eguchi et al., 2001
DAIZAC	EPM	Mice	+	Zhang et al., 2001
Bemesetron (MDL 72222)	EPM	alcohol-non-preferring rats	+	Hensler et al., 2004
Ondansetron (GR 38032F)	OFT EPM	OBX rats	++	Ramamoorthy et al., 2008
Vortioxetine (Lu AA21004)	OFT	BalB/cJ≠RJ mice	++	Guilloux et al., 2013
Vortioxetine (Lu AA21004)	NSF	129S6/SvEvTac mice	++	Guilloux et al., 2013

Altogether, it is summarized that:

1) 5-HT_3 receptors are substantially involved in regulation of mood and emotional behavior

2) Increased activity of 5-HT_3 receptors increases the risk of depressive episodes

3) Blockade of 5-HT_3 receptor functions at cellular level (antagonizing receptor mediated cascading events) or at genetic level (by inhibiting receptor expression or genetic deletion) has beneficial effects in preventing depression and related disorders

2.2 Animal models of diabetes mellitus

To study the nature of a disease, its initiation, progression, pathophysiology and treatment condition without exposing humans and potentially unethical risks, animal models have been proven an efficient tool. They have historically played a critical role in target identification and in vivo evaluation of novel therapeutic agents.

Animal models have contributed important knowledge regarding the study of diabetes and its complications. In the later section, the different types of animal models used for diabetic research have been categorized and discussed.

2.2.1 Animal models of Type-1 diabetes mellitus

The basic characteristic of Type-1 diabetes mellitus (T1DM) is autoimmune destruction of β-cells leading to a significant decrease in insulin levels (hypoinsulinemia).

There are several ways by which insulin deficiency can be achieved in animal models, ranging from chemical ablation of β-cells to breeding animals that spontaneously develop autoimmune diabetes. Some of the most commonly used models of T1DM are outlined in Table 2.3.

Table 2.3 Animal models of Type-1 diabetes mellitus

Mode of induction	Model	Mechanism(s)
Spontaneous model	NOD mouse	autoimmune process leading β-cell destruction
	BB rat	
	LEW.1AR1/-iddm rat	
Chemically-induced model	Streptozotocin	DNA break by alkylation, β-cell necrosis
	Alloxan	Reactive oxygen species (ROS)-induced β-cell damage, Glucokinase inhibition
Transgenic model	AKITA mouse	mutation in the insulin 2 gene, disrupting insulin biosynthesis from pro-insulin
Virus induced model	Coxsackie B virus Encephalomyocarditis virus Kilham rat virus LCMV under insulin promoter	Virus infection induced β-cell destruction
Surgical model	Pancreatectomy	Surgical removal of pancreas including β-cells.

2.2.1.1 Spontaneous autoimmune animal model of Type-1 diabetes mellitus

Spontaneous models have been derived from inbreeding over many generations by selecting for hyperglycemia. Thus, many genes and phenotypes have been developed that are relevant to pathophysiology of diabetes. It is interesting to note that the main advantage of spontaneous diabetic models is that they can be utilized for studying pre-diabetic state which is impossible in humans. The most widely used spontaneous T1DM models are non-obese diabetic mouse and the Bio-breeding (BB) rat (Yang and Santamaria, 2006).

2.2.1.1.1 Non-obese diabetic mouse model

The NOD mouse was developed at the Shionogi Research Laboratories in Osaka, Japan (Hanafusa et al., 1994) in a study of cataract development. NOD mouse show initial diabetes at an age of 12-15 weeks, with a high incidence in females (approximately 80 % diabetic at week 25) and a more slowly developing phenotype in males with a lower overall incidence of diabetes (Leiter, 1997; Yoon and Jun, 2001). Experimentally, it represents many aspects of human diabetes and is a model that has helped identify many of the genetic and signaling pathways associated with T1DM.

2.2.1.1.2 Bio-breeding (BB) rat model

The model was derived from outbreed Wistar rats. It was first identified by the Bio Breeding Laboratories, Ottawa, in 1974 (Nakhooda et al., 1977). Diabetes develops just after the onset of puberty and has similar incidence with no gender difference (unlike NOD mouse). Almost 90 % of the animals develop diabetes at an age of 8-16 weeks with prominent signs of T1DM diabetes including weight loss, polyuria, polydipsia, hyperglycemia and insulinopenia. Similar to human diabetes, ketoacidosis is severe and fatal and thus these rats require exogenous insulin for survival (Mordes et al., 2001).

2.2.1.1.3 LEW.1AR1/-IDDM rats.

This model of T1DM was first developed by the Institute of Laboratory Animal Science of Hannover Medical School (Ztm) in a colony of congenic Lewis rats with a defined MHC haplotype (LEW.1AR1). Diabetes and insulin deficiency develops at around 8–9 weeks (Lenzen et al., 2001), with an incidence of 60% and equal in both genders (Jorns et al., 2005).

Like NOD mouse bred, these exhibit a pre-diabetic period with islet infiltration one week before the onset of hyperglycemia, which is effective particularly studying the different stages of immune cell infiltration (Jorns et al., 2005), whereas unlike NOD mouse, the LEW-IDDM rat does not exhibit other autoimmune diseases.

2.2.1.2 Experimental models of diabetes mellitus

Experimental diabetic models include the artificial induction of diabetes in laboratory animals either by administration of chemicals, transgenetic technology or immunomanipulation.

2.2.1.2.1 Chemically-induced Type-1 diabetic animal model

There are certain compounds that cause specific destruction to pancreatic β-cells and thus produce T1DM. The degree of endogenous β-cell damage is more in case of chemical destruction and there is little insulin production leading to overt hyperglycemia and weight loss. The advantage of chemically-induced diabetes model is that they are relatively cheap and can be produced in higher animals (Dufrane et al., 2006). Diabetes develops fast only after 3 days with a stable hyperglycemia after 5-7 days. These models are effective in studies, involving complications associated with insulin-dependent type of diabetes. The two most commonly used chemicals to induce diabetes in mice and rats are streptozotocin (STZ) and alloxan. Although, these models include chemical destruction of β-cells unlike humans, in which the damage occurs silently over many years, at clinical condition there is little surviving β-cell mass and the disorder progresses, to absolute insulinopenia and thus, significantly simulates the human condition.

2.2.1.2.1.1 Streptozotocin-induced diabetes model

Streptozotocin, [2-deoxy-2-(3-(methyl-3-nitrosoureido)-D glucopyranose] (Fig. 2.6) derived from *Streptomycetes achromogenes* is a nitrosourea derivative with broad spectrum antibiotic and anti-neoplastic activity (Bono, 1976). It has powerful alkylating property and has been shown to interfere with glucose transport (Wang and Gleichmann, 1998). In STZ, N-methyl-N-nitrosourea (MNU) moiety (Fig. 2.6) is linked to the carbon-2 of a hexose. Usually, nitrosourea compounds are lipophillic in nature; however, due to hexose moiety STZ is less lipophillic and thus has limited plasma membrane permeability (Tjälve et al., 1976).

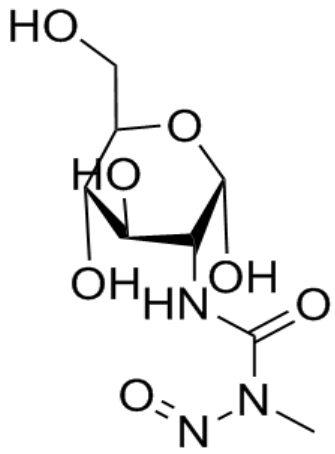


Fig. 2.6 The structure of streptozotocin

It accumulates outside the plasma membrane of pancreatic β-cells and traverses into the cell via an active GLUT2 transporter system (Karunanayake et al., 1976; Ledoux and Wilson, 1984). Once into the cell, it causes alkylation of the DNA molecules and thus, induces multiple DNA strand breaks. Subsequently, in an attempt of DNA repair process that results in activation of poly-(ADP-ribose) polymerase (PARP), STZ diminishes NAD+ and cellular ATP. The depletion of the cellular energy stores ultimately results in β-cell necrosis, leading to inhibition of insulin production (Sandler and Swenne, 1983). In addition, STZ is a source of free radicals that can also contribute to DNA damage and subsequent cell death (Lenzen, 2008). Thus, the diabetogenic effects of STZ occurs by a significant reduction in insulin biosynthesis, glucose-induced insulin secretion and hence glucose metabolism.

Streptozotocin is used in different forms to induce diabetes: A single large dose of STZ is sufficient to induced diabetes in rodents. In mice, a range of 100-200 mg/kg has been reported to induce diabetes with stable hyperglycemia (Srinivasan and Ramarao, 2007; Dekel et al., 2009) depending on the mouse strain (Hayashi et al., 2006), and in rats 35–65 mg/kg (Srinivasan and Ramarao, 2007). In susceptible animals it induces insulinopenic diabetes in which immune destruction plays a role, as in human T1DM. STZ-diabetic animals are hypoinsulinaemic, but do not require insulin treatment to survive.

Alternately, multiple low doses of STZ (20- 40 mg/kg on five consecutive days) are also used to induce diabetes (Lenzen, 2008). In this case, the decrease in number and volume of β-cells are apparent.

2.2.1.2.1.2 Alloxan-induced diabetes model

Alloxan, (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) is a uracil derivative that highly resembles glucose structure (Fig. 2.7) (Lenzen, 2008). Due to glucomimetic effect it is readily taken up by GLUT2 transporter into the pancreatic β-cells. It is therefore not toxic to the cell, which is not expressing this transporter; likely that of the STZ. Once inside the cell, it is rapidly reduced to dialuric acid and then re-oxidized back to alloxan, creating a redox cycle for the generation of superoxide radicals that undergo dismutation to form hydrogen peroxide and thereafter highly reactive hydroxyl radicals that cause fragmentation of β-cell DNA (Szkudelski, 2001). Other mechanism by which it induces diabetogenic effects includes inhibition of glucose-induced insulin secretion through specific inhibition of glucokinase (Gunnarsson and Hellerström, 1973; Lenzen and Panten, 1988).

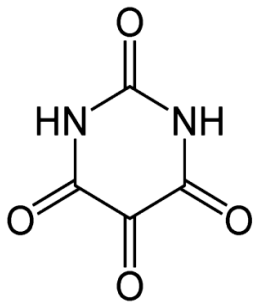


Fig. 2.7 The structure of alloxan

Alloxan doses in mice range from 50 to 200 mg/kg and in rats from 40 to 200 mg/kg, depending on the strain and the route of administration (Szkudelski, 2001). The main disadvantage of this model is that alloxan has a narrow diabetogenic dose, and even light overdosing can cause general toxicity, especially to kidney (Szkudelski, 2001).

2.2.1.3 Transgenic mouse model of T1DM

AKITA mice are genetically engineered T1DM model. It was first derived in Akita, Japan from a C57BL/6NSlc mouse with a spontaneous mutation in the insulin 2 gene, which is involved in the processing of pro-insulin. This results in an overload of misfolded proteins thereby hampering the insulin biosynthesis from pro-insulin. T1DM occurs starting from 3-4 weeks of age. Because of almost complete loss of circulating insulin, the animals have less survival rate unless treated with exogenous insulin. This model has been utilized in studies investigating pancreatic or β-cell transplantation (Mathews et al., 2002).

2.2.1.4 Virus-induced Type-1 diabetic model

Virus are used as a source to induce β-cell destruction that can be achieved by either a direct infection or initiation of an autoimmune response against β-cells (Jun and Yoon, 2003). Viruses used to induce diabetes in animals include, coxsackie B virus (Jaidane et al., 2009), encephalomyocarditis virus (Shimada and Maruyama, 2004) and Kilham rat virus (Ellerman et al., 1996).

2.2.1.5 Surgical model of Type-1 diabetes mellitus

Although, not popularly utilized, pancreatectomy is a surgical model used to induce T1DM in animals. The removal of pancreas results in severe deficiency of insulin levels in blood resulting in insulin-dependent diabetes. However, this is an invasive method and may cause hypoglycemia and pancreatic exocrine deficiency in animal (Lebovitz et al., 2011).

2.2.2 Selection and criteria of an appropriate diabetic model

A variety of animal models for T1DM have been discussed above. There are several factors that should be taken into account while selecting an animal model. Among them, the purpose for which the model is being used is of prime concern. In the present work, STZ-induced diabetes model was chosen because of the several advantages over the other existing models, discussed above.

- Higher incidence rate
- Early onset of diabetes
- Less mortality (compared to alloxan)
- Cheaper as compared to the other experimentally induced diabetes.
- Long term diabetes induction
- Fast excretion rate
- Simulate human T1DM condition
- Presence of wide literature
- Fast availability
- Blood-brain-barrier (BBB) impermeability

Cheaper as compared to the other experimentally induced diabetes: Induction of diabetes using STZ presents an economically viable (cheaper) model as compared to transgenic and other experimentally induced models. Indirect cost is also less, for example, the maintenance of transgenic animal is difficult as well as costlier.

Higher incidence rate: Diabetes induced by STZ has been reported an incidence rate of up to 95 % (Graham et al., 2011). That is, it induces diabetes in most of the mice. In addition, the incidence is same in male and female (independent of gender variation) and in rats and mice (independent of strain variation).

Less mortality (compared to alloxan): It has been reported that diabetic window for STZ is wider and it has low mortality rate as compared to alloxan (another chemical used to induce diabetes) (Szkudelski, 2001). This suggests that it saves the number of animals and also fulfills the requirement of ethical guidelines to conduct research in animals.

Early onset of diabetes: STZ has shown an early onset of diabetes as compared to transgenic or spontaneous models of T1DM, saving the research time duration. With STZ, diabetes develops within 72 hrs of dosing and a stable hyperglycemia occurs after 5-7 days (Lenzen, 2008).

Long term diabetes induction: Unlike alloxan-induced diabetes that shows a rapid regeneration of β-cells and progressive improvement in glycemia, STZ induces a state of diabetes for a long period of time.

This is particularly useful to study the long term complications of diabetes and is one of the major criteria for selecting this model in the present work (Brosky and Logothetopoulos, 1969).

Blood-brain-barrier (BBB) impermeability: STZ is transported across the cell only via GLUT2 transporter. Since, blood-brain junction is lack of this transporter, STZ does not cross BBB (Karunanayake et al., 1976; Ledoux and Wilson, 1984).

Fast excretion rate: After administration of STZ, it is rapidly excreted out from the body (excretion rate: 1.2 hr approximately). It suggests that STZ does not accumulate into the system (Karunanayake et al., 1974), which makes it as an effective tool to study diabetes-related complications.

Simulate human T1DM condition: In animals, STZ induces hyperglycemia by insulinopenia (lack of insulin) in which immune destruction plays a role, as in human T1DM (Lenzen, 2008). In addition, STZ-induced diabetes is associated with weight loss, polyuria and polydipsia, the condition commonly seen in diabetic patients (Szkudelski, 2001). Thus, it seems that STZ induces diabetes that is closely related to the clinical diabetic condition.

Presence of wide literature: A vast literature is available on pharmacodynamics and pharmacokinetics of STZ. In addition, STZ has been utilized to evaluate several diabetes-induced peripheral and central complications. That is particularly needful to support the new findings involving CNS complications of diabetes.

2.3 Animal models of mood disorders

Behavior based models have been widely used to mimic human mood disorder and to evaluate pathophysiology and treatment condition. The diagnosis of psychological state is highly based on the gross behaviors that have imprecise similarity and/or correlation with each other within and between individuals rather than etiology, neurobiology, epidemiology, genetics or intervention response. Thus, it is very difficult to define experimentally, these behaviors in animals.

Animals not only lack the perception of self, self-reflection and thoughtfulness of others but also aspects of the disorder such as sad mood, low sense of worth or suicidality (Deussing, 2007). Moreover, heterogeneous depressive symptomology (e.g., substantial weight gain or loss, insomnia or hypersomnia) makes it difficult to express these converse conditions in animals.

Yet attempts have been made to recapitulate human behavioral trait in animals. Certain endophenotypes of depression has been reproduced developing behavior-based animal models that are being widely used across the laboratories (Table 2.4).

However, a full consensus regarding the prerequisites of animal model to be valid, three criteria are set up by McKinney and Bunney (1969); they include construct validity, face validity, and predictive validity.

Table 2.4 Defining, the models and their relevance

Models	Rationale	Parameters measured
Animal models of depression		
Forced swim test	Forced inescapable exposure in water filled cylinder causes cease of active behavior leading to immobility: reduced by antidepressants	Duration of immobility Swimming behavior: Number of square crossed, Number of climbing
Tail suspension test	Hung with tail, develop a despair behavior: reduced by antidepressants	Duration of immobility
Sucrose preference test	Preference of sweetened solution indicate hedonic behavior: increased by antidepressants	Total volume of sucrose solution consumed over drinking water
Chronic mild stress	Mild stressors in an unpredictable manner induces depressive behavior: reduced by antidepressants	Anhedonia, despair behavior.
Corticosterone-induced depression	HPA-axis hyperactivity induces depression like effects: reversed by antidepressants	Anhedonia and despair behavior
Lipopolysaccharide induced depression	Increased inflammatory response induces depressive effects: prevented by antidepressants	Anhedonia and despair behavior
Animal models of anxiety		
Elevated plus maze	Height and open spaces cause innate aversion to animals: prevented by anxiolytic drugs	Total time spent in open arms Number of entries in open arms
Light-dark test	Bright illumination causes innate aversion: reduced by anxiolytic drugs	Latency to leave light chamber Time spent in light chamber Number of transitions
Open field test	Light and open spaces induce aversive behavior: decreased by anxiolytic drugs	Thigmotaxis (wall seeking behavior) as time spent in center of arena Number of crossings Number of rearings
Hole-board test	Novel environment affect exploratory behavior: degree of exploration is increased by anxiolytic drugs	Number of head dips Duration of head dips Number of crossings Number of rearings
Social interaction test	When introduced with another animal, test animal show social interaction: increased by anxiolytic drugs	Time spent in social interaction Number of social interaction Others: number of fecal pallets Number of crossings Number of rearings

2.3.1 Validity of a model

A model is said to be valid if it fulfills the requirement of presenting relevance of human pathophysiology. There are two basic validities that a model should express to be valid, internal and external validity. Internal validity denotes that consistency of the experimental design such as: reproducibility, inter-observer reliability, randomization, blind experimentation (Campbell and Stanley, 1963). On the other hand, external validity defines the applicability of the results of a test on a sample in an animal model (Mook, 1989). The external validity is further categorized in three major valid criteria.

2.3.1.1 Predictive validity

It represents that the animal model is efficient enough to respond to the pharmacological treatment, which should also correlate with the clinical trials. Hence, it is also known as pharmacological validity. According to Willner (1984) a model should meet the following five criteria to have predictive validity, whether:

- a model correctly identifies antidepressant activity of a drug
- defines antidepressant treatments of pharmacologically diverse types
- makes errors of omission
- makes errors of commission
- potency in the model correlates with clinical potency.

These criteria thus present that this validity seeks only the pharmacological aspects of drugs. The validity is later extended to all kinds of pharmacological intervention (such as electroconvulsive therapy) and thus lack to define the 'specificity' of a model to respond to a particular category or class of drugs or correspond to a specific mode of action.

Later on Koob (2000), further extended the definition for anxiety based model that 'the ability to make consistent predictions about anxiety based on an animal's performance in the model'. However, the models with high predictive validity are also being utilized to evaluate the depression associated with other chronic diseased condition.

2.3.1.2 Construct validity

This validity defines the comparable etiology of a model, i.e. whether the behavior assessed in an animal model is based on the same neurobiological mechanism(s)/process as of the human depression (Nestler and Hyman, 2010).

Alternatively, Wilner (1984) demonstrated the criteria of a model to have a construct validity that:

- should be homologous,
- the behavior in the model and the features of depression being modeled should be unambiguously interpreted,
- whether the feature being modeled should stand in an established empirical and theoretical relationship to depression.

The theoretical aspects include, the nature of human depressive symptomology, the central impact of some states (for example, that helplessness or anhedonia are central symptoms in depression), the dynamic of the disorder (for example, its biphasic course) and its etiology (Wilner (1984)

2.3.1.3 Face validity

It expresses that the model resembles human disorder in several respects specific to depression. Alternatively, the model should not show features that are not present in clinical condition. Thus, face validity encompasses both some treatment features and symptomatic aspects. The model should show homology:

- anatomically and biochemically,
- neuropathologically, or
- behavioral features of a human disease.

In this context, it can be assumed that the more the criteria a model meets, the more compelling it will be (Malkesman et al., 2009). Thus, it can be said that to design a model the challenges that are faced includes:

- constructing a model with similarity in disease progression and symptomology as in clinical condition,
- detecting these phenotypes with the appropriate behavioral tests and
- ameliorating them with treatment modalities that are also effective in humans.

2.3.2 Animal models of depression

Several diagnostic features of depression have been simulated in animal models of depression. These models are developed to understand the pathophysiology of depression-related disorder and to identify the treatment intervention. In recent years, some of these models have been adopted to investigate the etiology, pathophysiology and treatment for co-morbid disorders involving an association of depression with other chronic disorders. Several of these models fulfill validity criteria (Table 2.5).

Table 2.5 The animal models of mood disorders and their validity criteria

Animal Model	Common species used	Validity criteria	Target Disorder	NT involved/ Biochemical pathway	Reference
FST (Despair based model)	Mice, Rats	Predictive Face (poor) Construct (poor)	D	5-HT NE DA	Castagne et al., 2011;
TST (Despair based model)	Mice	Predictive Face and Construct (poor)	D	DA 5-HT NE	Cryan et al., 2005
SPT (Anhedonia behavioral model)	Mice, Rats	Predictive Face Construct (poor)	D	DA	Powell et al., 2012
HBT (exploratory and novelty based model)	Mice	Predictive	A	GABA 5-HT	Brown and Nemes, 2008
EPM (exploration based model)	Mice, Rats	Face Predictive Construct	A	GABA 5-HT	Walf and Frye, 2007
OFT (novelty and exploratory based model)	Mice, Rats	Predictive Construct	A	GABA 5-HT	Royce, 1977 Tachibana, 1980.
LDT (exploratory based model)	Mice	Predictive	A	GABA 5-HT	Rodgers and Shepherd, 1993
CUMS (mild unpredictable stressor based chronic model)	Mice, Rats	Face Predictive Construct	A, D	HPA-axis 5-HT NE DA	Powell et al., 2012; Willner, 1997
LPS (Neuro-inflammatory based chronic model)	Mice, Rats	Face Predictive Construct	A, D	Inflammatory pathway (TNF-ά, IL-6) HPA-axis	Dedic et al., 2011
CORT (Chronic mechanistic model)	Mice, Rats	Face Predictive Construct	A, D	HPA-axis 5-HT	Dedic et al., 2011
OBX (Lesion-based chronic model)	Rats	Face Predictive Construct	A, D	5-HT, NE, DA, GABA, Ach	Harkin et al., 2003

A, Anxiety; D, depression

2.3.2.1 Despair based models

'Despair' is a term that defines a state of hopelessness or helplessness (Porsolt et al., 1977; Slattery and Cryan, 2012). It reveals one of the core symptoms necessary for the diagnosis of depressive episodes in humans (Castagné et al., 2011). These models are based on the rationale that when an animal is exposed to an unavoidable stress condition, after some time it ceases and eventually develops a state of hopelessness. The duration in which it become helpless is a critical parameter and is used to measure depression-like behavior in the despair based models (Slattery and Cryan, 2012). There are basically two kinds of such models, forced swim test and tail suspension test.

2.3.2.1.1 Forced swim test

It was first introduced by Porsolt et al. in 1977 is extremely used since then. In this model the animal in exposed to the enclosed water, after an enormous escape-oriented behavior like swimming, climbing and curling, it stops struggling and shows passive immobile behavior. The immobility is believed to mirror either a failure to persist in escape-directed behavior after stress (i.e., behavioral despair) or the development of passive behavior that disengages the animal from active forms of stress coping. A large array of antidepressants has been tested and has shown the efficacy in FST with a significant reduction in the immobility time. Also, literature suggests that non-pharmacological treatment has shown the efficacy in reversing stress-induced increased immobility duration, whereas, non-antidepressant compounds have shown ineffective activity in FST that demonstrates the specificity of the model to identify the compounds, active as antidepressants (Borsini and Meli, 1988; Cryan et al., 2002, 2005a). The model has been applied to several animal strains such as rats, mice, gerbils and even guinea pig (Petit-Demouliere et al., 2005; Porsolt et al., 1977; Cryan et al., 2002, 2005a; Wicke et al., 2007).

Advantages of FST

- The main advantage of FST is that it is cheap and reliable model,
- Require short time duration to examine the effect of a compound,
- Can be used in rats, unlike tail suspension test,
- Sensitive to pro-depressant effects of a number of diseased conditions,
- It has high predictive validity, that is significantly predicts the antidepressant activity of any compound,

- The test may be appropriate to determine the underlying neurobiological basis of discrete aspects of the etiology of depression. For example altered stress-coping behavior is one of the hallmarks of depression.

Disadvantages of FST

- Not efficiently differentiate between the acute and chronic effects of drugs.
- Lack face validity that is the swimming behavior and the immobility do not correspond to any of the clinical situation causing depression,
- Insignificantly predicts the clinical course of an intervention. For example drugs like SSRIs show delayed onset of action in humans, however, no such therapeutic lag is observed in FST.
- Despite the presence of psychomotor agitation or retardation as a symptom of human depression, the FST does not fulfill an endophenotype approach given the necessity to nullify general locomotor activity.

2.3.2.1.2 Tail suspension test

The test was first demonstrated by Steru et al. (1985). Like FST, TST is based on the principle that when an animal is suspended by its tail, which develops a state of short-term, inescapable stress, the animal ceases and become immobile. In this case, the stressful condition involves the hemodynamic stress of being hung in an uncontrollable fashion by their tail whereas in the FST mice are placed in a cylinder filled with water (Thierry et al., 1986). Several antidepressants have been reported to reduce the immobility time in animal exposed to TST. In addition, similar to that of FST, TST can also predict the pro-depressant activity of the stressor and diseased condition (Cryan et al., 2005b).

Advantages of TST

- In expensive tool to detect antidepressant activity of compounds,
- Methodologically it is unsophisticated,
- The method is easily amenable to automation,
- It has high predictive validity,
- Not associated with any confounds induced by hypothermic exposure as in FST,
- It is relatively rapid test of antidepressant action, and is sensitive to short-term antidepressant effects.

Disadvantages of TST

- Requires an assessment of general locomotor activity like FST to nullify the psycho-stimulant/depressant effect,
- It cannot be adopted for higher animals, because of their heavy weight the sensitivity of the model decreases to detect the antidepressant activity,
- It cannot predict the clinical course of antidepressant drugs and has insignificant fact validity, like in FST
- Tail climbing of the animal is another problem associated with the TST.

2.3.2.2 Sucrose preference test

Another important model designed to evaluate the depressant/antidepressant behavior is sucrose preference test, which mimics one of the diagnostic symptoms of depression in humans as per the criteria defined by DSM-IV: anhedonia (APA, 2000). The term 'anhedonia' refers to the reduced capacity to enjoy or experience pleasure in response to the formerly rewarding stimuli. Several reports have shown a potent relationship between depression and anhedonia. Patients with depression display hedonic deficits in response to pleasurable imagery (Fiorito and Simons, 1994), films (Renneberg et al., 2005), sweetened talk and pictures (Mathews and Barch, 2006) when compared with non-depressed individuals. This behavior has been significantly framed in animals presenting the model of depression. When animals are exposed to sweetened solutions such as of sucrose, they prefer consuming it over the normal drinking water that seems to reflect the hedonic behavior (Rygula et al., 2005). It has been found that animals subjected to chronic stress show low level of consumption of sucrose solution as compared to coeval controls (Rygula et al., 2005). This suggests that animals can also have a tendency to experience pleasure that can be estimated in terms of preference of sucrose solution consumption.

Advantages of sucrose preference test

- Reliable, inexpensive model and not time-intensive,
- Easy to conduct and require less human efforts,
- High throughput and artifacts (for example effects of locomotion as occur in FST or TST) are minimal,
- High predictive, face and construct validity,
- Used in different strains of animals,
- Helpful to assess the chronic effects of antidepressants.

Disadvantages of sucrose preference test

- Preference of sucrose solution depends upon the position of the bottle and experimental room conditions, as observed by some researchers,
- Require a baseline stabilization prior to the test,
- Stress condition such as deprivation of food and water increase consumption sometimes and hence the test may give confounding results due to general consummatory behavior instead of reward deficits.

2.3.2.3 Chronic unpredictable stress model

This is the most prescribed model to detect the pro-depressant or antidepressant like behavior in animals.

It has been demonstrated that exposure of animals to a number of stressors in an unpredictable manner evokes depression-like behavior that closely relates the human depression in several phenomenon including behavioral, neurobiological, etiological and biochemical (Willner, 1997). The chronic stress model was initially developed in rats but was widely adopted for mice (Goshen et al., 2008).

Next, defining the validity of chronic stress model suggests that it has all predictive, construct and face validity (Willner, 1997). A wide variety of stressors are used to induce depression in animals.

Some of the stressors that are applied to the animals are (Moretti et al., 2012)

- Food deprivation
- Wet bedding
- Restraint
- Cage titling
- Light and dark cycle inversion
- Tail suspension
- Predator exposure
- Isolation
- Water deprivation
- Empty water bottles exposure

Several antidepressants currently in market as well as novel candidate with potential antidepressant activity have shown reversal of the chronic stress induced depression (Goshen et al., 2008; Papp, 2012). That indicates the high predictive validity of the model. Moreover, the reversal of chronic stress induced depression-like behavior requires 3-4 weeks of treatment, indicating that it can predict the chronic effects of drugs, which closely resembles the clinical time course of action of antidepressant drugs (Moretti et al., 2012; Willner, 1997).

A second observation is that, as measured by anhedonia, reversal of depression-like behavior by several antidepressants have been reported to occur only in animals exposed to stress protocol, but not in non-stressed one. That denotes the selectivity of the model to predict the specific activity of the compounds in the diseased condition (Goshen et al., 2008). Previous reports have shown that non-antidepressant drugs are ineffective in this model that presents the accuracy and reliability of this model towards examining specific antidepressant compounds (Rygula et al, 2008).

The theoretical rationale of the model simulates anhedonia, which is the core symptom of depression and is one of the features of melancholia (a form of major depression in humans) (APA, 2000). Interestingly, chronic stress model has also demonstrated various signs of depression apart from anhedonia and weight loss. These include impairment in sexual activity, aggressive and investigative behavior, locomotor action and active waking during dark phase, which suggest the simulation with major depressive symptoms (Grønli et al., 2004, 2005; Li et al., 2008; Mineur et al., 2006).

In addition, neurobiological studies have demonstrated that chronic stress model show various signs of altered neurochemical systems that are also present in depressed patients such as altered endocrinal functions (HPA-axis including adrenal hypertrophy and enhanced glucocorticoids secretion) (Li et al., 2008), changes in immune system (Farooq et al., 2012) and brain signaling (such as hippocampal atrophy and altered neurotransmitter functions) (Li et al., 2008). All of these factors indicate the efficacy of the model to mimic the clinical state of depression.

Advantages of chronic stress model

- Effective in evaluating the clinical course of action of the compounds
- Applies to different strains of the animals
- High predictive, construct and face validity
- Inexpensive
- Effective tool to evaluate the neurobiology of stress disorder

Disadvantages of chronic stress model

- Time consuming,
- Weight loss may be a confound that masks the effect of chronic stress,
- Requires comparatively more number of animals.

2.3.3 Animal models of anxiety

The animal models of anxiety are largely utilized to evaluate the anxiolytic activity of several potential candidate as well as to investigate the anxiogenic activity of various stimuli including chronic diseased condition. The different conditioned and unconditioned anxiety tests are presented in Table 2.6 Since the present work used unconditioned based anxiety models, these are elaborated in detail.

Table 2.6 The different forms of anxiety tests

Unconditioned anxiety models	**Conditioned anxiety models**
Exploratory behavior based	**Conflict based**
Elevated plus maze	Vogel conflict test
Open field test	**Avoidance based**
Elevated zero maze	Passive avoidance test
Light/dark transition based	Active avoidance test
Light/dark test	**Condition based**
Novel exposure based	Fear-potentiated behavior test
Hole-board test	
Social behavior based	
Social interaction test	
Anti-predator based	
Mouse defense test battery	
Novelty induced	
Hyponeophagia test	

2.3.3.1 Unconditioned anxiety models

These are also called ethological models and do not require prior training of animals. Therefore, these models are based on the proximate and evolutionary behavior of the animals. These models are based on spontaneous behavior and have been suggested to have high degree of ecological concern in that they rely upon unconditional reactions to potentially threatening novel situations (Ohl and Keck, 2003). Thus, several behavioral models have been devised on this basis.

2.3.3.1.1 Elevated plus maze test

The model was first described by File and co-workers. The elevated plus maze model consisted of the two open and two closed arms. It is based on the principle of exploration that when an animal is placed on the apparatus, the more anxious animal prefers the closed arms. The degree of preference depends upon the conflict between the preference of animal for protected areas (in closed arms) and its innate motivation to explore novel environments (in open arms) (for review see Walf and Frye, 2007). It has been assumed that animal avoid the open arms because of novelty, height, or open space (Albani et al., 2015; Rodgers and Cole, 1993; Treit et al., 1993). This model has high face, predictive and constructs validity.

Advantages of elevated plus maze

- Test is fast and simple,
- Does not involve expensive equipment,
- Based on spontaneous behavior and thereby avoids lengthy training,
- Does not require the use of noxious stimulation,
- Able to identify acute anxiolytic effects of drugs
- Bidirectionally sensitive to manipulations of animal's anxiety-like behavior
- Has high face, predictive and construct validity,
- Useful to predict the neurobiological mechanism(s) of novel compounds,
- Helpful to detect the change in the level of anxiety.

Disadvantages of elevated plus maze

- Parameters are highly influenced by environmental condition such as lighting that may affect the results,
- It artifacts due to alteration in locomotion may affect and thus need to be concomitantly evaluated.
- The center square arena sometime may provide ambiguous measurements.

2.3.3.1.2 Light-dark test

Light-dark test is another ethological based model, widely used to investigate the anxiety behavior in animals and effects of drugs and diseased condition, in anxiety. It is based on the rationale that the bright illumination induces an innate aversion and reduces spontaneous exploratory activity in response to mild stressors that is bright light and novel environment, which reflects a state of anxiety (Bourin and Hascoët, 2003).

The latency to leave light chamber, time spent in light chamber and number of transitions are the most commonly measured parameters in this test (Ohl, 2003). It has been reported that the test has high predictive validity, which is an essential criteria for a behavioral based model to be developed. Several classes of drugs produce significant alteration in the aversive behavior of animals in light-dark test (Bourin and Hascoët, 2003). It seems to have high face and construct validity (Bourin and Hascoët, 2003; Chaouloff et al., 1997).

Advantages of light-dark test

- Easy to conduct, similar to the elevated plus maze test ,
- Inexpensive and does not require much labor.
- Does not require pre-training of animals as with other ethological models,
- Can also be used in the series of behavior testing paradigms.
- Natural stimuli (bright illumination) is used and thus measures innate responses,

Disadvantages of light-dark test

- May give false positive results due to general increase in the activity and thus require measuring the locomotion in animals prior to the test
- Difficult to measure the differential measure between the two compartments
- If the light area in not enough to induce aversion, may give altered effects of the compounds.

2.3.3.1.3 Open field test

Open field test was first demonstrated by Hall in 1934 using rats (Hall, 1934). The test apparatus used was a bright lit circular area surrounded by the walls to protect animal from escaping (Christmas and Maxwell, 1970; Ohl, 2003). The standard procedure employs a forced confrontation with the open field in that the animal is placed in the center of the area (Ohl, 2003). The degree of exploration is measured as number of crossings and number of rearings. It represents the horizontal and vertical exploratory activity, respectively. Crossings refer to the movement by the animal in the squares (of a particular dimension) marked on the floor of arena. Rearings refers to the upright standing of the animal, trying to explore the area outside the walls. The other parameters being measured include defecation and freezing or immobility that reflects innate anxiety like condition of the animal (Choleris et al., 2001; Prut and Belzung, 2003).

There have been several anxiolytic drugs and novel candidates investigated for the anti-anxiety effects using open field test. This suggests that the test has high predictive validity. Moreover, it has been reported that it has high face validity as recommended to present fear responses that simulate the clinical condition (Choleris et al., 2001). However, the construct validity of the compound is questionable, though it may be assumed that the forced confrontation with novelty in open field induces a stressful condition that evokes anxiety, as does in humans (stress develops anxiety condition) the model may also fit construct validity criteria (Prut and Belzung, 2003).

Advantages of open field test

- Inexpensive, simple, reliable and time effective,
- Can be applied in a number of strain and several adaptations are possible,
- Simultaneously measures general and anxiety activity in animal, thus avoiding inter-subject variation,
- As other ethological based models it provides the innate response to anxiety,
- Neurobiological substrates can be easily examined

Disadvantages of open field test

- Exploration and locomotion are often cause ambiguous measurements in open field studies,
- The model is affected by testing condition, which need to be kept constant.

2.3.3.1.4 Hole-board test

The hole-board apparatus appears to provide the more detailed ethological testing, as head dips into holes in the floor is assumed to be a valid measure of the subject's attraction towards novelty. Hole-board test was first introduced by Boissier and coworker (1965) and has largely been used to examine the multiple anxiety parameters as it offers a simple approach to study different behavioral patterns, locomotor activities, level of movement and possibly the effects of neuroactive drugs on anxiety in a single apparatus (Harada et al., 2006; Kliethermes and Crabbe, 2006). The behavioral parameters mostly measured are number and duration of head dips or collectively head dips behavior that represent a valid measure of anxiety in animals. The locomotor activity can be evaluated as number of crossings and rearings. The other anxiety parameters such as defecation and freezing can also be evaluated with this model at the same test period (Ohl et al., 2001).

The model has been reported to have high predictive value with a large variety of drugs being tested and presented high anti-anxiety like effects in this model (Kamei et al., 2004). Apart from the classic anxiolytic drugs, several antidepressants and novel targets of anxiety disorders have been tested (Tsuji et al., 2000). The animal behavior in this model show an increased innate emotionality and more passive stress coping strategies. Extensive studies, including pharmacological validation, have shown that hyper-anxiety in this test represents a robust trait; resembling signs and symptoms seen in psychiatric patients thus it may also have face and construct validity (Ohl et al., 2001).

Advantages of hole-board test

- Helps in determining multiple anxiety behavior measures
- Simple, easy to conduct and inexpensive,
- Time efficient and does not require training,
- Different strains of animals can be used to test,
- The general locomotion and anxiety are simultaneously measured,
- Neurobiological substrates of anxiety can be predicted.

Disadvantages of hole-board test

- The testing condition requires being constant as with other tests as stress condition may affect the activity.

2.3.3.1.5 Social interaction test

Psychosocial withdrawal is one of the important features of human mood disorder. Dysfunctional approach and withdrawal tendencies towards emotional stimuli have been well documented in depressed patients (Derntl et al., 2011). On the other hand, social skill deficits have shown to be among the most restraining symptoms aggravating depressive effect in humans (Kubera et al., 2011) and prolonged social isolation constitutes *per se* a chronic model in rodents have demonstrated to accede the emergence of depressive-like behavior (Takatsu-Coleman et al., 2013). In addition, a large number of researches have shown that social phobia is one of the characteristic of anxiety disorders. This suggests that, altered social activity may represent an overlapping symptom of depression and anxiety in humans and evaluating the altered social activity may provide one of the co-morbid symptoms of depression and anxiety in animals. The test was first introduced by File and Pellow, 1984.

The rationale behind this test is that when an animal is introduced with the other animal of nearly same age gender and strain, it shows social interaction activity such as grooming, mounting and crawling with the other animal. However, the animal with altered mood behavior such as that exposed to chronic stress show reduced social activity in this test. Besides, the depressive features that can be evaluated using this test, majority of the researchers have provided the significance of this test to evaluate the anxiety component (File and Seth, 2003; Patin et al., 2005). It helps determine the valid anxiety measure without the need to introduce aversive and appetitive conditions. Thus, it may be said that similar to hole-board test it gives an inherent behavioral measure of the anxiety in animals (File and Seth, 2003). The model has high predictive, face and constructs validity that can be referred to as the ability of this model to predict the anxiolytic efficacy of test compounds, the simulation with the clinical condition and having an unambiguous theoretical rationale to determine the biological components associated with the anxiety disorders (File and Hyde, 1978).

Advantages of social interaction test

- Provides a simple approach to test anxiety
- Helps determine some aspects of depression-like behavior
- Inexpensive tool to study
- Does not require additional apparatus as can be conducted in open field
- Can be used for different animal strains
- Provide specific (social phobic) trait of anxiety
- Provide to measure the locomotor activity, simultaneously.

Disadvantages of social interaction test

- Confounding results may be present due to locomotion alteration
- The stimuli animal has a significant effect on the test animal activity,
- There is no way of fully automating the scoring.

2.3.3.2 Conditioned models

These are the anxiety models that require a set of training protocol in animals before actual conduct of the study. However, the major concern of these kinds of the tests is that they may show interference with mnemonic and motivational processes (Steimer, 2011).

Advantages of conditioned models

- The conditioned models provide different component of anxiety than the unconditioned models
- These models have minimal effects of testing condition as the stress procedure induces a high threshold of anxiety.

Disadvantages of conditioned models

- The models are based on the extensive stress provoking thus affected by the degree of the anxiety level evoked in the animal
- The models require training of the animal prior to the test.
- Thus it is time consuming
- Require more labor efforts as compared to unconditioned models.

2.3.4 Selecting an ideal model to predict depression and anxiety

Till date there is no single model that can provide all ideal state in screening the effects of drug. However, there are some basic requirements that a model has to fulfill to be effectively utilized. Firstly, an ideal animal model offers an opportunity to understand molecular, genetic and epigenetic factors associated with the disorder. In addition, it should have following requirements.

- The model should have high predictive validity
- It should have reliability
- The model should be simple and easy to conduct
- It should measure, what it purports to be measure
- It should be ethically effective to perform in animals.

2.3.5 Defining preclinical model of depression-related disorders associated with diabetes

Investigational studies in animals can help elucidate mechanisms associated with depression-related disorders in humans with diabetes leading to identifying biomarkers and new treatments. However, studying behavioral models of depression in animals differs greatly from studying clinical depression in humans. The diagnosis of depression in humans requires meeting DSMIV-TR criteria (APA, 2000), whereas, in animals, researchers can only measure the possible symptoms or behaviors that represent animal analogs of depression in humans.

In order to develop a rodent model of predisposition to depression, it is necessary to evaluate the multiple components of depression and anxiety-related behavior. It can be possible only by utilizing a battery of tests. Furthermore, depression and anxiety often reveals overlying symptomatic and pathogenic states, thus it will be effective to conduct the depression and anxiety tests in diabetic animal under the same protocol. Since the present study used STZ-induced diabetic mouse model, the diabetes induced alterations specific to this model, are collectively represented in Table 2.7.

Table 2.7 Collective data presenting the effect of STZ-induced diabetes on the depression and anxiety related behavior in the testing paradigms.

Behavioral model	Response of STZ-induced diabetes	Species	References
FST	Enhanced despair behavior	Mice	Haider et al., 2013
		Rats	Khanam and Pillai, 2006
TST	Enhanced despair behavior	Mice	Ho et al., 2012 Kamei et al., 2003
Sucrose preference test	Anhedonia	Rats	Wang et al., 2009
Intracranial self stimulation test	Anhedonia	Mice	Ho et al., 2012
Open field test	Thigmotaxis	Mice	Damián et al., 2014
		Rats	Ates et al., 2014
EPM	Reduced open arm exploration	Rats	Ramanathan et al., 1998
		Mice	Damián et al., 2014
	No effect	Mice	Ho et al., 2012
Hole-board test	Reduced head dips behavior	Mice	Kamei et al., 2001
Social interaction test	Reduced social interaction activity	Rats	Ramanathan et al., 1998
	No effect	Mice	Hilakivi-Clarke et al., 2003

2.4 Biological pathways linking diabetes and depression

It is generally recognized that the prime causes of the disturbances in hormonal signaling systems in diabetes, apart from hyperglycemia and insulin signaling system dysfunctions, are oxidative stress and changes in HPA-axis function. At the same time, there is supporting evidence that, changes in monoamine- and neuropeptide-regulated brain neurotransmitter systems may also lead to depression-like behavioral disorders.

Since all these signaling systems are tightly interrelated into a single integrative network, changes in one of them inevitably induce changes in other systems, causing eventually a disintegration of the whole network of brain signaling. As a result, a question arises what signaling cascades sustain changes at the initial T1DM stages, how reversible these changes are, and how they affect other signaling systems later on. Alternately, the query remains of what initiates depression in diabetic condition. While some changes in brain signaling are reversible and partly or completely reversed by improvement of T1DM, some are irreversible. It depends not only on the duration and severity of T1DM, but on a specific signaling system type as well. The signaling systems that are altered and may lead to depression and related behavioral disorders include:

- Altered brain neurotransmitter signaling
- Altered HPA-axis functions
- Altered brain oxidative/nitrosative stress
- Altered neurotrophic factor signaling
- Altered brain neuronal morphology

2.4.1 Altered brain neurotransmitter signaling

2.4.1.1 Monoamine neurotransmitters

Monoamine, 5-HT is among the key neurotransmitters, which regulates behavior and emotional activities in brain. The well-coordinated activation and inhibition of this neurotransmitter system in normal brain has been observed to be disrupted in diabetes (Shpakov et al., 2011). In particular, diabetes patients have been reported with a significant decrease in 5-HT levels in brain with persistent depressive behavior and altered mood functions, which has shown to be normalized by chronic treatment with antidepressants (Manjarrez-Gutierrez et al., 2009; Shpakov et al., 2011; for review see: Ho et al., 2013). Similarly, preclinical studies have revealed that STZ-induced diabetic rodents exhibit reduced 5-HT levels in brain (Miyata et al., 2007; Sandrini et al., 1996; Chu et al., 1986). On the other hand, repeated administration of antidepressants (such as fluoxetine) resulted in significant increase in brain content of 5-HT in diabetic rodents (Kolta et al., 1986). However, the alterations in the 5-HT levels in discrete brain areas, in particular, those involved in the regulation of mood and emotional behavior is unknown and calls for further investigations.

2.4.1.2 GABA neurotransmitter

GABA is a principle non-monoaminergic neurotransmitter that controls mood and emotional behavior. Several studies have found alteration in GABA signaling in diabetes (Guyot et al., 2001) that may result in behavioral abnormality. Using brain microdialysis study, Ohtani et al. (1997) have demonstrated an elevated extracellular level of GABA in brain of STZ-induced diabetic rats, which was accompanied by a decline in 5-HT levels. Overall, it may be concluded that diabetes result in a relative elevation of GABA action in brain that may be associated with changes in mood and depression-like disorder.

2.4.2 Altered HPA-axis functions

It has been well-established that depression is linked with dysfunction of the hypothalamic-pituitary-adrenal (HPA)-axis. This manifests itself in two ways: (a) hyperactivation of HPA-axis and (b) blunting of normal diurnal glucocorticoid (GC) (cortisol in humans and corticosterone (CORT) in rodents) profiles (Pariante and Lightman, 2008). CORT is a counter-regulatory hormone which regulates HPA-axis activity. Physiologically, a stimulus (acute stress) triggers hypothalamic corticotrophin releasing factor (CRF) release, which induces adrenocorticotrophic hormone (ACTH) from anterior pituitary. ACTH releases GCs from the adrenal cortex (Fig. 2.8). It has been found that during stress condition, HPA-axis gets activated and triggers the release of CORT (Lanfumey et al., 2008). CORT act on the liver to increase glucose production through gluconeogenesis and glycogenolysis. The high blood glucose is further utilized to meet the energy demand for brain to function (Hasselblatt, 1969). Subsequently, elevated glucose level induces a negative feedback inhibition of GC release. During diabetes, as a result of high cellular stress, this negative feedback mechanism get inhibited and GC levels remains high and elevated glucose repeatedly get metabolized to produce ROS that subsequently culminate into morphological damage of brain tissues (Brownlee, 2001; Chan et al., 2002, 2003; Che et al., 2010; Mastrocola et al., 2005). In addition, during diabetes, a transient reduction in the hippocampal GCs receptors occurs that blocks the negative feedback inhibition causing an increase in GC level. It is now well established that hippocampus including other limbic areas exert neuronal regulation of HPA-axis (Boyle et al., 2005). Electrical stimulation of hippocampus reduces circulating GC levels, consistent with an inhibitory effect of hippocampal activation on HPA-axis (Herman et al., 2003). In line, lesion studies indicate that destruction of hippocampus increases HPA-axis responses to acute stressors (Herman et al., 1998).

This is thought to occur via GC receptors widely spread in hippocampal neuronal structures (Boyle et al., 2005; Furay et al., 2008) Therefore, it may provide an additional explanatory link between depression and diabetes. The disrupted feedback inhibition of HPA-axis via CORT and hippocampal neuronal regulation and subsequent over-activity of HPA-axis may lead to depression-like behavior in diabetes.

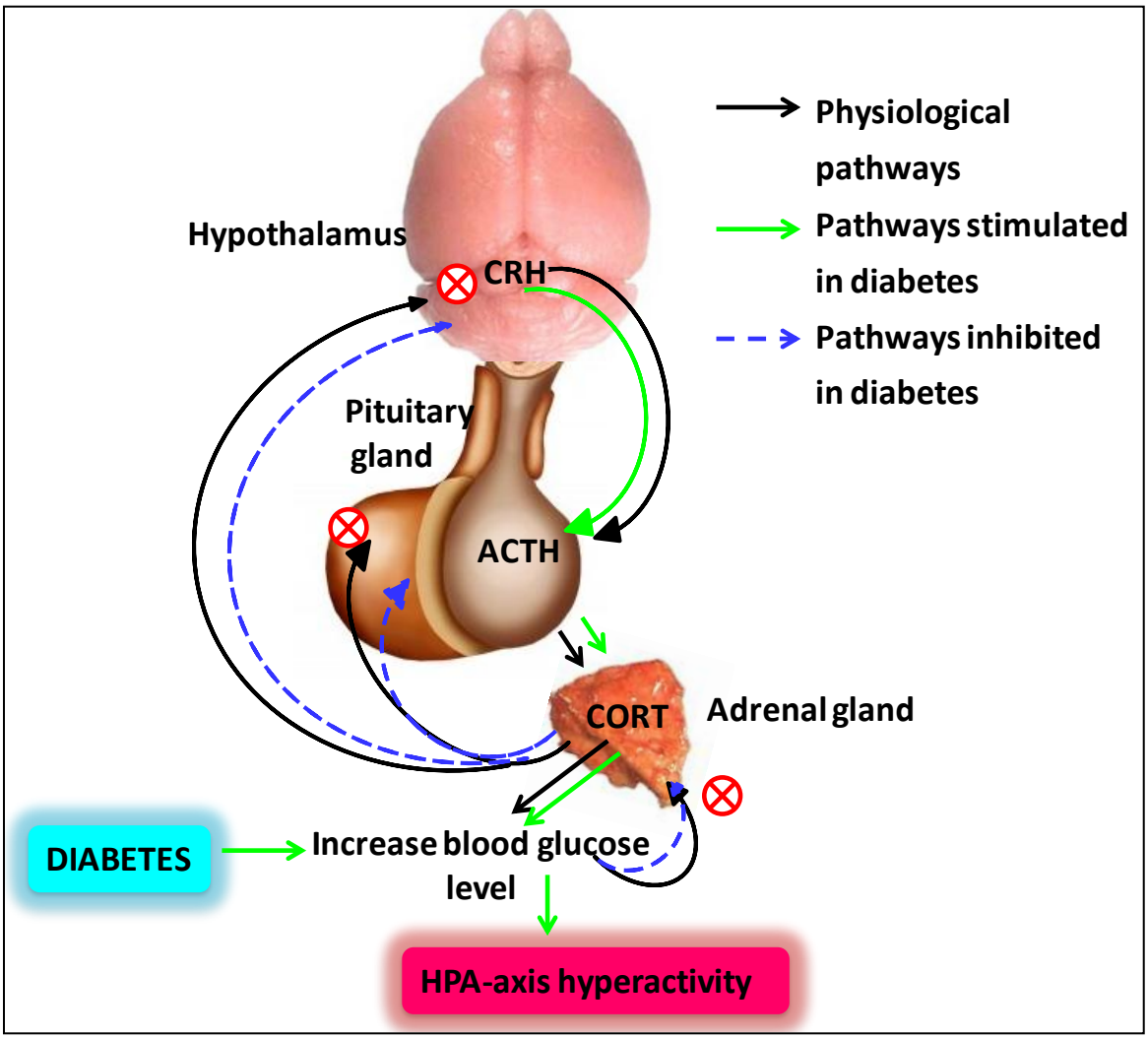


Fig. 2.8 HPA-axis and diabetes

2.4.3 Altered brain oxidative/nitrosative stress

Oxidative stress plays a pivotal role in the development of diabetes complications including neuronal and psychological (Moretti et al., 2012). Brain is an obligate glucose consumer. It has very high energy consumption for its size, mainly due to the energy required to maintain the potential difference across nerve cell membranes, as well as axonal and dendritic transport and tissue repair.

Glucose enters the brain by insulin-insensitive facilitated diffusion across the blood-brain barrier, and enters brain cells mainly via a couple of insulin-insensitive glucose transporters. In brain cells it is metabolized via hexokinase mediated phosphorylation to glucose-6-phosphate. Glucose-6-phosphate is metabolized further, mainly in the glycolytic pathway to produce pyruvate and the pentose phosphate shunt to generate glycogen. A major fraction of the pyruvate transported into brain mitochondria is devoted to the oxidative phosphorylation of ADP to ATP (Lowry and Passonneau, 1964). However, during diabetes a major proportion of glucose is accumulated into brain due to active transportation process. It is then metabolized to generate ATP as well as free oxygen radicals (commonly known as reactive oxygen species ROS). Physiologically, these free radicals are quenched by endogenous antioxidant defense mechanism (antioxidant enzymes such as catalase and SOD). However, during diabetes ROS levels overwhelm the antioxidant enzyme capacity and leads to excessive ROS accumulation (Kishi et al., 1999). ROS then react with the cellular macromolecules such as lipids proteins and nucleic acids and causes cell death, which subsequently lead to the alteration in the morphological and functional integrity of brain (Butterfield et al., 2001). Previous reports have shown that increased oxidative stress to brain injury is associated with increased glucose metabolism, lipid peroxidation and decreased tissue concentrations of low molecular weight antioxidants such as reduced GSH (Reagan et al., 2000; Grillo et al., 2003; Ulusu et al., 2003; Muriach et al., 2006). The alteration in GSH levels may be related to an increased polyol pathway activity as this leads to a depletion of NADPH which is necessary for the enzymatic reduction of oxidized glutathione (Preet et al., 2005) (Fig. 2.9). Mastrocola et al. (2005) have reported that the excessive glucose may lead to increase in brain oxidative and nitrosative stress that may lead to brain damage in rats. Thus, it may be that the increased circulating glucose in T1DM may lead to a series of pathological changes and may cause depression-like behavior.

2.4.4 Altered neurotrophic factor signaling

Neurotrophic factors are critical controllers of the formation and plasticity of neuronal networks in brain (Castrén et al., 2007; Castrén and Rantamäki, 2010). Brain-derived neurotrophic factor (BDNF) is abundant in brain and periphery, and is found in both human and animal, serum and plasma (Martinowich et al., 2007; Shimizu et al., 2003).

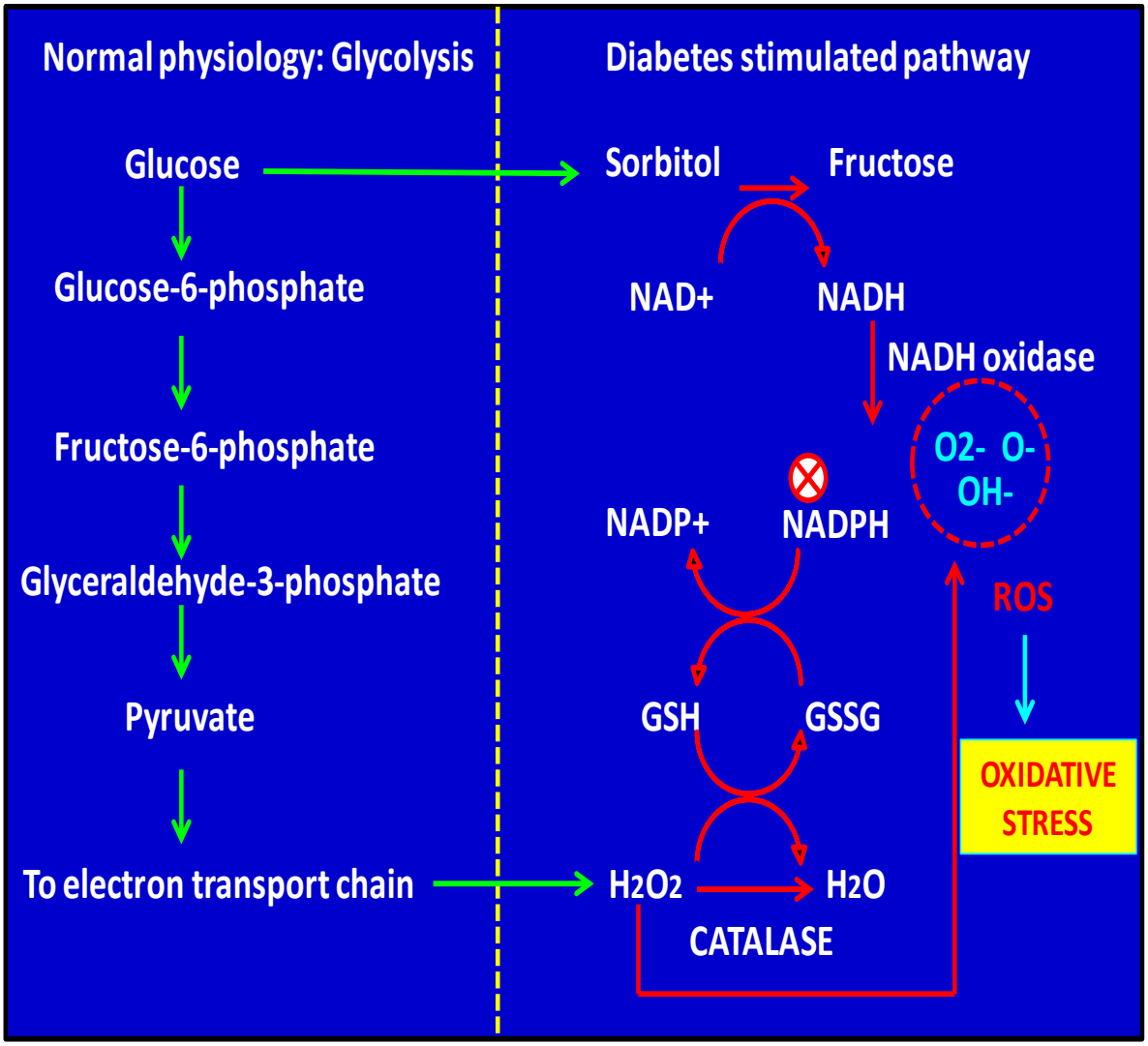


Fig. 2.9 Diabetes induced shift in glucose shunt and generation of reactive oxygen species (ROS), leading to brain oxidative stress

Studies have indicated that animal with depression-like behavior in neurobehavioral models exhibit reduce BDNF level and expression in hippocampus and in serum; the effect which is antagonized by clinical antidepressants (Petit-Demouliere et al., 2005). In line, clinical studies have indicated that serum or plasma BDNF levels are decreased in untreated depressed patients. Antidepressant treatment for at least four weeks can restore this reduction up to the normal value. Therefore, depression is associated with impaired neuronal plasticity. Interestingly, reports have shown that diabetes results in significant decline in BDNF in humans as well as in animal models. Both protein and mRNA levels of BDNF are shown to severely reduced STZ induced diabetic rats (Nitta et al., 2002).

Similarly, reduced serum BDNF levels were found in patients with diabetes (Ola et al., 2013). This indicates that alterations in BDNF levels are due to progressive changes in diabetes.

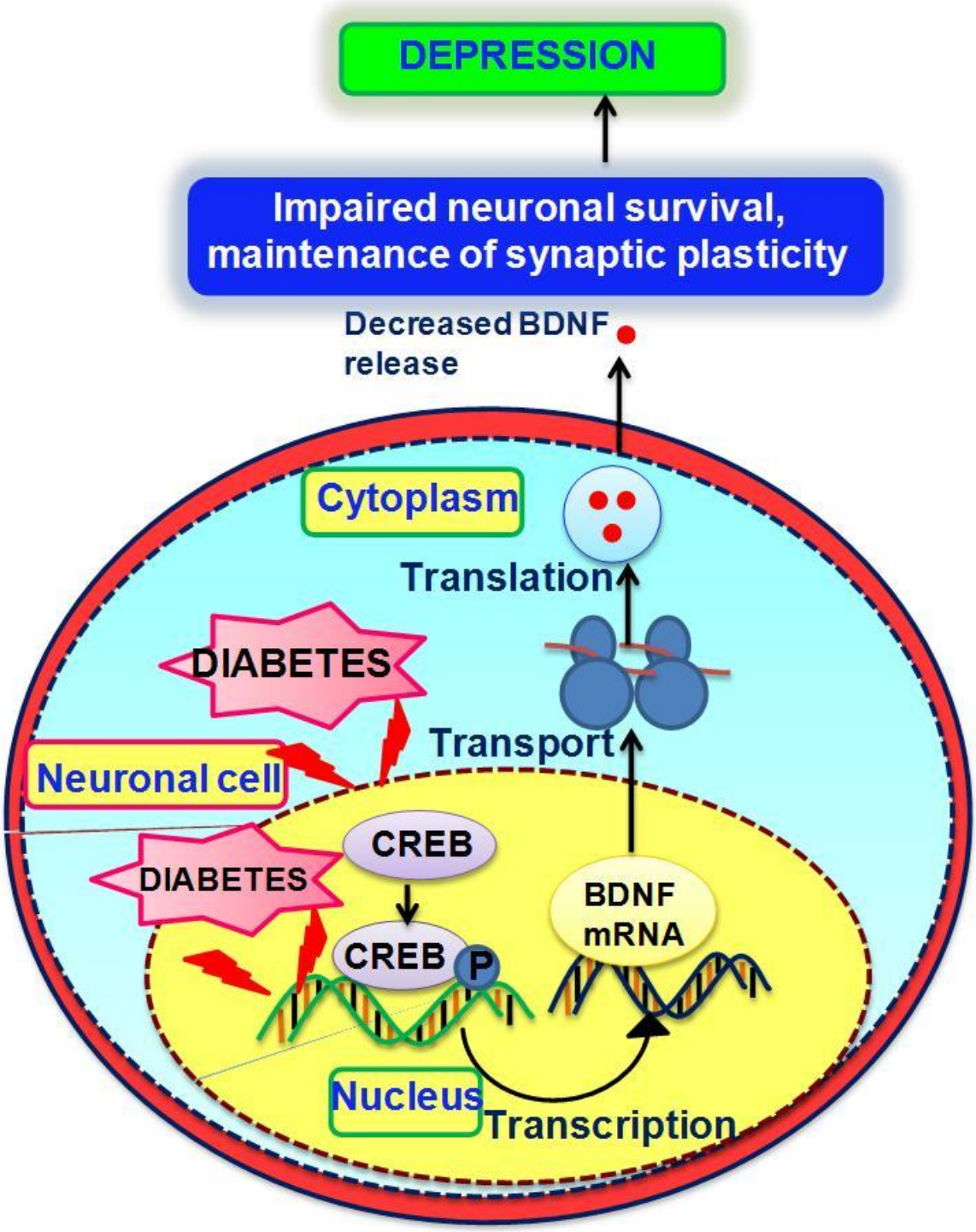


Fig. 2.10 the effect of diabetes on BDNF signaling. CREB, cyclic response element binding protein; CREB-P, phosphorylated cyclic response element binding protein.

2.4.5 Altered brain neuronal morphology

In line with the above neurotrophic factor hypothesis, impairment in brain morphological and structural abnormalities has also been suggested by few histological studies. Light and electron microscope studies have shown degenerative changes of neurons and oligodendrocyte abnormalities (Hernández-Fonseca et al., 2009). In addition, histological studies have shown that STZ-induced diabetic hyperglycemic rats exhibited neuronal damage in brain structures, including cingulate cortex, thalamus nuclei, substantia nigra, pars reticulata, and the hippocampus (Li et al., 1998). In another study indicated that after 2 months of STZ-induced diabetes rats have expressed a decrease in the dendritic length of pyramidal cells in prefrontal cortex and hippocampus (20% to 45%). Furthermore, the density of dendritic spines was decreased in all the pyramidal cells in diabetic animals (36% to 58%). However, the pyramidal neurons of CA1 hippocampus region were the most affected (58%). Besides, the density of dendritic spines was decreased in all the pyramidal cells (Martínez-Tellez et al., 2005). These studies indicate that diabetes condition severely affects the brain morphology. The structural changes are thought to result from long-term passive shunting of excess glucose through alternative metabolic pathways. However, recent studies indicate that apart from diabetes induced ROS generation and subsequent neuronal damage, high GC level, due to HPA-axis hyperactivity, may cause neuronal damage (Frodl and O'Keane, 2003).

2.5 Summary

Overall, it may be suggested that:

1) Changes in the neurochemical, molecular and morphological abnormalities in brain that may culminate into depression and anxiety co-morbid with T1DM.
2) Excessive glucose metabolism and production of ROS occur in diabetes. At high levels that saturate buffering mechanisms, ROS may initiate neuronal damage resulting in psychopathologies like depression and anxiety.
3) ROS creates an environment in brain that fosters insidious elevations of oxidative damage leading to brain abnormalities.
4) Also, changes in neurotransmitter system impairment, HPA-axis hyperactivity, impairment in neurotrophic factors and brain morphology, which may provide additional pathological pathways leading to depression and related disorders such as anxiety co-morbid with T1DM.

3. GAPS IN RESEARCH

Understanding the pathophysiology of depression co-morbid with diabetes milletus (in particular T1DM) is an important step for the identification, discrimination and treatment of such a disorder in diabetic patients. Despite a constant increase in the number of therapeutic agents, the prevalence of this disorder in diabetic patients is progressively increasing, probably due to unclear neurobiological understanding of pathophysiology or the inconsistent efficacy of current pharmacotherapy.

Proper management of diabetes is essential for the prevention of serious psychological complications such as depression. In both forms of diabetes, the preventive goal is to keep blood glucose levels as close to normal as possible in order to prevent or reduce these consequences. Despite this goal, a large percentage of diabetes patients develop this co-morbidity (Egede and Zheng, 2003; Pibernik-Okanovic et al., 2005; Stahl et al., 2008). Therefore, it may be that idiopathic changes in diabetes stimulate a number of pathological systems that eventually lead to depression. The role of different pathological factors, causing depression in diabetes is poorly known; primarily underscores the identification of effective therapeutic candidates and management of such chronic co-morbid disorders. Reports have shown that alteration of brain neurotransmitter (such as 5-HT) activity, has a critical role in the pathogenesis of depression associated with T1DM (for review see: Ho et al., 2013; Manjarrez-Gutierrez et al., 2009; Shpakov et al., 2011), however, little is known about the downstream signaling pathways and specific receptor system involved. Moreover, the complex interplay of multiple pathological systems makes the target identification difficult and ineffective.

Clinically existing antidepressants are ineffective and associated with severe drawbacks (section 1.3.4.1.3.). In brief, several antidepressants have a negative impact on glucose homeostasis and may alter energy metabolism, leading to complications in diabetic patients. Also, biological alterations in cellular system during diabetes milletus make antidepressants, ineffective. Thus, current scenario necessitates, the identification of effective target(s) that can normalize, at least not all, but multiple pathological markers that lead to depression in diabetes, and concomitantly regulate energy metabolism and glucose homeostasis thereby ameliorate diabetic condition.

4. OBJECTIVES AND PLAN OF WORK

4.1 Objectives

As a part of our enduring effort to identify better antidepressants with improved therapeutic effectiveness and minimum untoward effects for managing depression and anxiety co-morbid with type-1 diabetes, the present work investigates the role of "5-HT_3 receptor antagonists" as "potential antidepressants".

Effective usage of clinical antidepressants is restricted by ineffective therapeutic response and aggravating effects on diabetic condition. To minimize the side effects and to improve therapeutic utility, it is important to target specific receptor type that has substantial regulatory effect on both psychological behavior and metabolic system. 5-HT_3 receptors have been implicated in the mediation of mood and emotional behavior, memory, cognition, psychotic and schizophrenic behavior. Furthermore, 5-HT_3 receptors have been shown to have a distinct functional role in metabolic regulation of glucose. Based on the observations that the current antidepressants have poor efficacy and 5-HT_3 receptors antagonists present effective and safety profile, the current work was designed. The following objectives were set:

- To evaluate the antidepressant activity of standard and selected in-house synthesized 5-HT_3 receptor antagonists in depression and anxiety co-morbid with T1DM using validated animal models.
- To investigate the role of 5-HT_3 receptors in the pathogenesis of depression and anxiety co-morbid with T1DM, using in vivo assays.
- To identify the plausible mechanism of antidepressant and anxiolytic action of standard and novel 5-HT_3 receptors antagonists using in vivo assay techniques.

4.2 Plan of work

The work was divided into five phases, as represented in Fig. 4.1.

4.2.1 Development of depression and anxiety models co-morbid with T1DM

Swiss albino mice were selected and rendered diabetic by chemical (STZ) method (the criteria for choosing this model is given in section 2.2.2) for induction of diabetes.

These animals were then subjected to a number of neurobehavioral assays of depression and anxiety. The inclusion and exclusion criteria are given in Table 4.1.

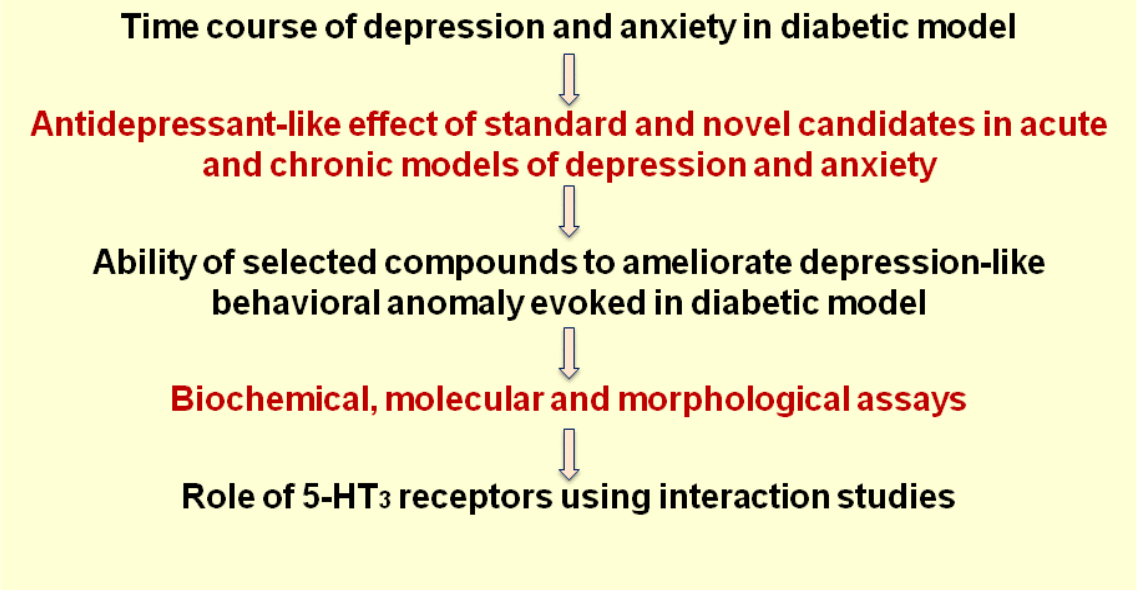


Fig. 4.1 The flow chart showing overall work plan

Table 4.1 The inclusion and exclusion criteria for the diabetic mice model

Inclusion criteria	Exclusion criteria
Animals with fasting blood glucose levels > 200 mg/dl	Presence of any diseased or infectious state
Animals with an age of 8-12 weeks	Animal showing minimal or partial behavioral response
	Animal showing altered locomotor activity
	Animal with week physical health during experimentation

4.2.2 Standardization of neurobehavioral models for depression and anxiety co-morbid with T1DM

Hypothesis 1: progressive changes, rather than acute, in T1DM lead to depression like behavior

The primary goal was to demonstrate that progressive changes in T1DM condition lead to depression and anxiety-like behavior rather than acute diabetic changes that is the study was done to determine the time course of development of depression in T1DM.

To evaluate the time course of diabetic condition that leads to depression and anxiety, STZ-induced diabetic mice were subjected to neurobehavioral models of depression and anxiety periodically (1, 2, 4, 6, 8 weeks) and the development of depression and anxiety-like behaviors were investigated. The neurobehavioral models included in preliminary study are shown in Fig. 4.2.

4.2.3 Validation of neurobehavioral models of depression and anxiety co-morbid with diabetes

Hypothesis 2: clinical antidepressants may prevent depression and anxiety-like behavior in STZ-induced diabetic mice

Modeling depression and anxiety in animals is a complicated challenge compared to the modeling of other disorders as these psychological disorders are defined through subjective experience. To make the model more relevant and homologous to clinical condition, the criteria that needs to be fulfilled is "validity". Validation of the models was carried out using standard antidepressant, fluoxetine (the selective serotonin reuptake inhibitors, SSRI) a prototype drug. The drug was selected based on the literature review that indicates the efficacy of the drug in ameliorating depression in T1DM subjects during clinical trials.

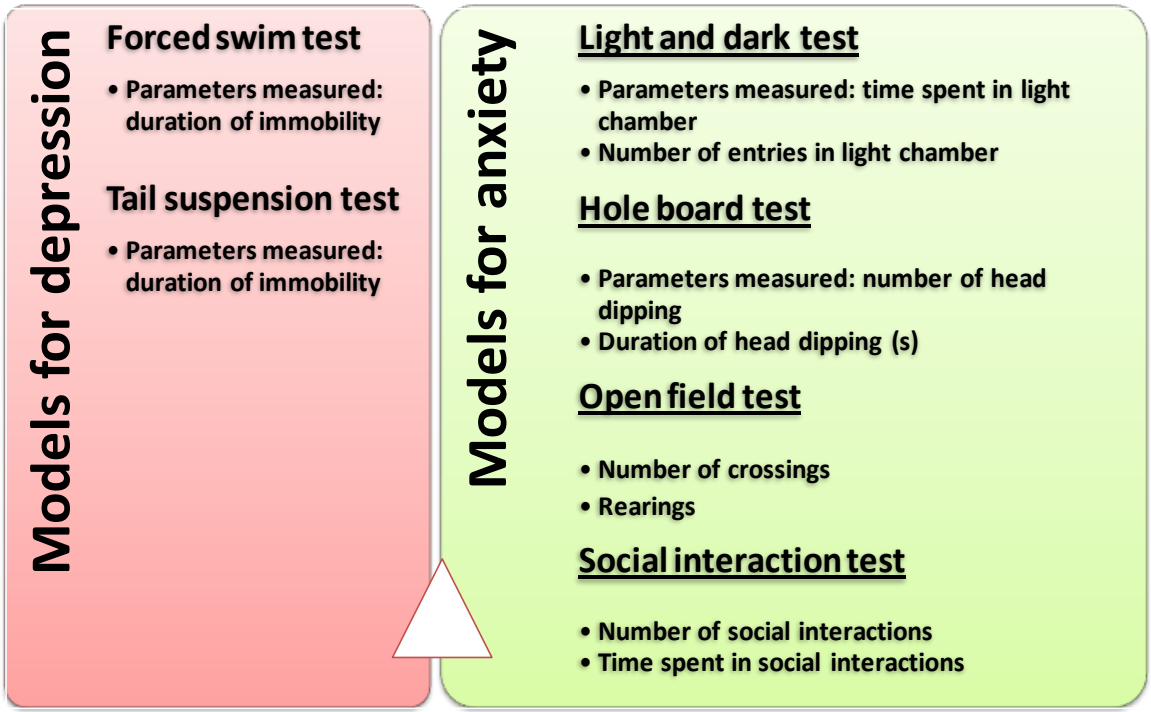


Fig. 4.2 Models for investigating depression and anxiety in diabetic mice

4.2.4 Pharmacological evaluation of 5-HT_3 receptor antagonists in validated animal models of depression and anxiety

Hypothesis 3: 5-HT_3 receptor antagonists may ameliorate depression and anxiety-like behavior in STZ-induced diabetic mice

It was also thought worthwhile to investigate the antidepressant effect of 5-HT_3 receptor antagonists in depression associated with diabetes. Detailed pharmacological investigations of the antidepressant and anti-anxiety-like action of standard and novel 5-HT_3 receptor antagonists were carried out using validated neurobehavioral models of depression and anxiety in STZ-induced diabetic mice.

4.2.4.1 Selection of in-house synthesized 5-HT_3 receptor antagonists

Several novel 5-HT_3 receptor antagonists were designed and synthesized based on the molecular modeling and pharmacophoric model by the Medicinal Chemistry Group of the Demaprtment. The compounds were tested for their 5-HT_3 receptor antagonistic action in vitro. **4i** (*N*-(3-Chloro-2-methylphenyl) quinoxalin-2-carboxamide) and **6z** (N-(Benzo [d] thiazol-2-yl)-3-methoxyquinoxalin-2-carboxamide) demonstrated a significant pA2 values (comparable to that of standard ondansetron) and hence selected for extended pharmacological investigations.

4.2.4.2 Dose response studies

Dose selection for all drugs (ondansetron, **4i** and **6z**) was carried out by spontaneous locomotor activity test in naïve mice. The doses at which the drugs exhibited insignificant responses were taken further, and investigated for preliminary in vivo tests for their antidepressant potential.

4.2.4.3 Preliminary pharmacological screening assay

The drug candidates were initially evaluated for the antidepressant and anxiolytic action in naïve mice, to examine their potential antidepressant and anxiolytic activity.

4.2.5 Screening of selected 5-HT_3 receptor antagonists for depression and anxiety co-morbid with T1DM

The antidepressant and anxiolytic potential of the selected 5-HT_3 receptor antagonists were then examined in diabetic condition. For this, the selected drug candidates at selected doses were given to mice with chronic state of diabetes and subjected to neurobehavioral screening assays for depression and anxiety. The models selected:

For depression:

1) Forced swim test: duration of immobility (s)
2) Tails suspension test: duration of immobility (s)
3) Sucrose preference test: percentage of sucrose solution consumed

For anxiety:

1) Light-dark test: latency (s) and time spent (s) in light chamber
2) Hole-board test: number of head dips and duration of head dips (s)
3) Social interaction test: number and time spent (s) in social interactions

4.2.6 Pharmacological screening to investigate the plausible mechanism(s) of antidepressant action

Hypothesis 5: 5-HT_3 antagonists may prevent diabetes induced neuronal and endocrinal abnormalities.

To investigate the probable mechanism(s) of action of drug candidates, the following biochemical and molecular estimations were carried out.

1) Brain neurotransmitter assay: Estimation of 5-HT and GABA was done in discrete brain regions
2) Endocrine marker: Estimation of HPA-axis abnormality was carried out
3) Brain oxidative stress markers: Lipid peroxidation and nitrite levels were estimated Brain antioxidant abnormality: Estimation of anti-oxidant (such as GSH and catalase activity) in the brain was conducted
4) Neuronal growth markers and neurodegeneration: Estimation of neuronal growth factors such as BDNF and cAMP levels in discrete brain regions and histological examination of neuronal degeneration were performed.

4.2.7 Pharmacological screening to investigate the role of 5-HT_3 receptors in mediating antidepressant and anxiolytic activity of drug candidates

Hypothesis 6: 5-HT_3 receptor antagonism may be involved in the antidepressant and anxiolytic action of test drug candidates.

To examine whether the 5-HT_3 receptor antagonism is involved in the postulated effect of test compounds; concomitant treatment with 5-HT_3 receptor agonist (such as mCPBG) was performed and diabetic mice were subjected to neurobehavioral screening and biochemical assays. This study further provides the role of 5-HT_3 receptors in the pathophysiology of depression and anxiety co-morbid with T1DM.

5.1. Animals

Swiss Albino mice (either sex, 25-30g) and Wistar rats (either sex, 200-250g) (Fig. 5.1) were obtained from Hisar Agricultural University, Hisar, Haryana, India. Animals were group housed in cages and were maintained under standard laboratory conditions with alternating light-dark cycle of 12 h each, temperature 23±2 º C and humidity conditions, 62±5% relative humidity in the housing unit. Animals had free access to standard pellet chow feed (except during estimation of fasting blood glucose levels, where mice were kept on fasting overnight as well as when subjected for the chronic stress protocol) and filtered water *ad libitum*. Behavioral testing was done during the light cycle. Following a quarantine period of three weeks, the animals were randomly assigned to different treatment groups. Mice were housed in laboratory cages [24 x 17 x 14 cm] in groups of 5-6 per cage and rats were housed in laboratory cages [36 x 23 x 17 cm] 3-4 per cage. Autoclaved corn cob was used as the bedding material.

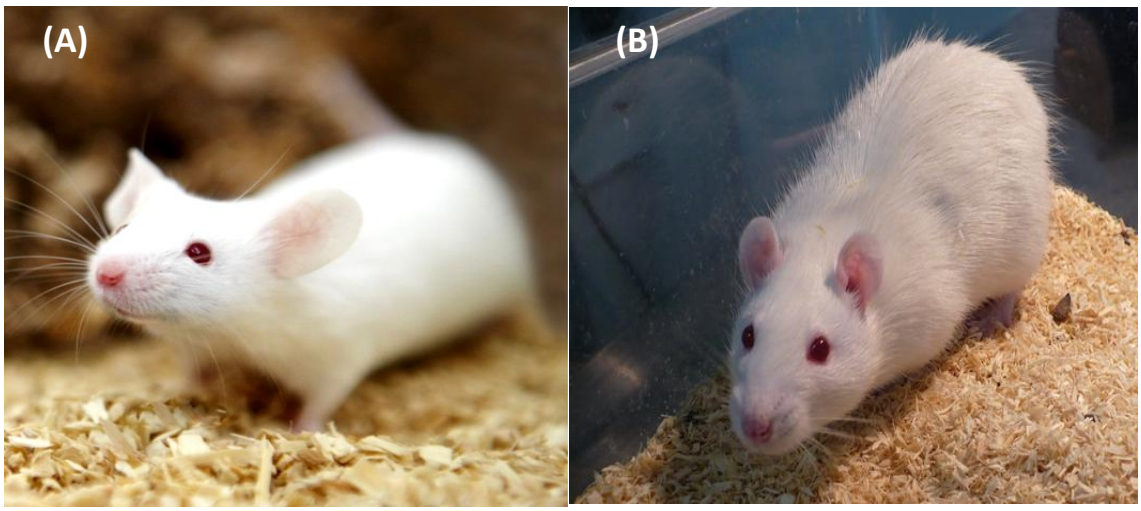


Fig. 5.1 Laboratory animals used in this study. **(A)** Swiss albino mouse, **(B)** Wistar rat

5.2. Ethical approval

Animals were treated according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA, Registration number: 417/01/a/ CPCSEA) and all experiments were conducted in adherence to the approved protocols of the Institutional Animal Ethics Committee (IAEC) of BITS Pilani, Pilani, India (Protocol numbers: IAEC/RES/14/11, September-2011, IAEC/RES/14/11/REV/16/10, July-2012; IAEC/RES/18/19, February, 2014; IAEC/RES/18/19/REV-02/21/13, March-2015). Animals were used only once for the experiment.

5.3. Drugs and Chemicals

5.3.1 Standard antidepressant and anxiolytic

Fluoxetine (FLX), a selective serotonin reuptake inhibitor was used as standard antidepressant. It was obtained from Ranbaxy Research Laboratories, India. Diazepam (DZM) (Cipla Ltd. India) was used as standard anxiolytic agent. FLX and DZM were dissolved in distilled water and given i.p. as per study protocol.

5.3.2 Standard 5-HT_3 receptor antagonist

Ondansetron (OND) was used as a selective 5-HT_3 receptor antagonist for evaluation of the antidepressant and anxiolytic-like effects in preclinical investigations. It was procured from Indian Pharmaceutical Combine Association Labs, India. OND was dissolved in distilled water and administered i.p. route according to the study protocol.

5.3.3 Novel chemical entities

4i and **6z**, both of the novel 5-HT_3 receptor antagonists (Fig. 5.2), were synthesized by our Medicinal Chemistry group, BITS-Pilani. The physicochemical properties of compounds are given below. The two chemical entities were dissolved in distilled water containing 0.1 % v/v Tween 80.

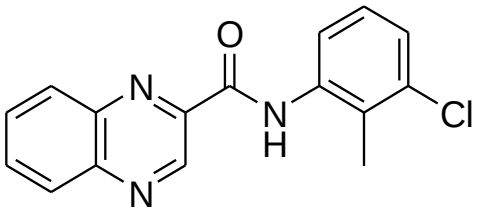


Fig. 5.2 (A) The structure of **4i**.

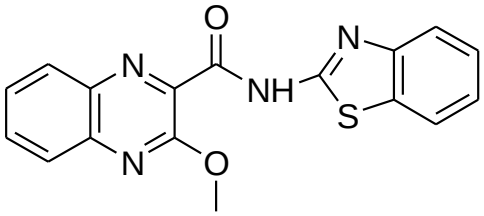


Fig. 5.2 (B) The structure of **6z**.

Table 5.1: The physicochemical parameters of 4i and 6z.

Parameters	**Values for 4i**	**Values for 6z**
IUAC Name	*N*-(3-Chloro-2-methylphenyl) quinoxalin-2-carboxamide	*N*-Benzo[d]thiazol-2-yl)-3-methoxy quinoxalin-2-carboxamide
pA_2 Value	7.6	7.3
log p Value	2.91	2.87
Melting point	148-150° C	150-152° C

5.3.4 Diabetogenic agent

Streptozotocin (STZ) was purchased from the Sisco Research Laboratories Pvt. Ltd. India. STZ was prepared freshly every time before dosing in 5M citrate buffer (sodium citrate and citric acid), pH 4.5. The citrate buffer is prepared by dissolving 1.47 g of sodium citrate in 5 ml of distilled water. STZ is highly photosensitive and hence always kept in dark and dosing was done in dim light within 15 min of preparation of STZ.

5.3.5 Anti-diabetic agent

Insulin was used as anti-diabetic agent for type-1 diabetes mellitus. Insulin glargine (Lantus) was purchased from Sanofi-Aventis Pharma Ltd (Germany).

5.3.6 Chemicals for corticosterone estimation

Corticosterone (CORT) was purchased from Sigma, USA. Methanol, chloroform, sulfuric acid sodium hydroxide were purchased from Sisco research labs, India.

5.3.7 Chemicals for neurotransmitter estimations

5-HT and *o*-Phthalaldehyde (OPT) were purchased from Sigma, USA. n-butanol, hydrochloric acid (HCl), L-cysteine, *n*-heptane, sodium potassium tart rate and copper sulphate were used of analytical grade. GABA was purchased from Sisco research labs, India.

5.3.8 Chemicals for pro-oxidant and anti-oxidant markers estimations

Thiobarbituric acid (TBA), trichloroacetic acid (TCA), N-(1-naphyl) ethelenediamine dihydrochloride (NEDA), sulphosalicylic acid (SSA), dithio bis (2-nitrobenzoic acid) (DTNB), hydrogen peroxide were purchased from companies: SD fine, Hi-Media and Spectrochem-chemicals, India.

5.4 Enzyme Linked Immunosorbent Assay kit

ELISA kit was used for the estimation of insulin in the plasma and the discrete brain regions and was procured from BIOSciences, Millipore, USA. BDNF and cAMP kits were obtained from Boster Biological Technology Co. Ltd, USA.

5.5 Surgicals

Haemostatic sponge: AbGel, Absorbable gelatin sponge USP, Srikrishna Laboratories, India. Sterile sutures: Ethicon 4-0, Non-absorbable surgical sutures USP. Mersilk (Braided silk black).

Ethicon 4-0, Absorbable surgical sutures USP (Catgut), Johnson and Johnson, India. Surgical needles: Curved surgical needles were used from Pricon Surgicals, India.

5.6 Equipments

1) Digital Actophotometer: Inco Ambala, India.
2) Stereotaxic frame: Inco Ambala, India
3) Centrifuge: Eppendorf centrifuge, 5702-R, Eppendorf AG, Germany.
4) ELISA plate reader and washer: ARK Diagnostic, India.
5) Optical Microscope: Optika Microscope, Italy.
6) Spectrofluorophotometer: Shimadzu RF 5301 PC Shimadzu, Japan.
7) Spectrophotometer: UV-1800, Shimadzhu, Japan.
8) Tissue Homogenizer: Kinematica^{TM} Polytron^{TM} Homogenizers, Germany.
9) Deep freeze: Operon deep-freeze, OPR-DFC-300CE, Operon Co.Ltd., Korea, Digital EPM: SN Scientific India, Other apparatuses for FST, TST, Light-dark, Hole-board, Open field test.

5.7 Induction of diabetes and fasting blood glucose monitoring

To induce diabetes, overnight fasted mice/rats were given a single intraperitoneal (i.p.) injection of STZ (200 mg/kg for mice and 40 mg/kg for rats). Fasting blood glucose levels were measured on third day after STZ injection, using a portable Freestyle glucometer (Akkiscan, Zee^{+} Glucose Meter, Nepro Care, India). Blood was obtained via tail snip. Animals with fasting blood glucose values 200 mg/dl or above were included in the diabetic groups. Glucose levels were then measured on a weekly basis, in the morning between 0800 and 1000, until the completion of the study.

5.8 Behavioral assays

5.8.1 Animal model for the evaluation of basal locomotor activity (BLA)

Rationale: The behavioral assays for antidepressant and anxiolytic activity are based on the behavioral despair and degree of exploration principle, respectively. Hence, it may be that the effect of the drug or test compound on the rodent's motor activity may have false positive or negative influence. In order to avoid such effect it is essential to evaluate their effect on the basal locomotor activity.

Procedure: Basal locomotor activity was assessed using actophotometer apparatus consisted of a dark square chamber (30 cm × 30 cm) with inside walls painted black (Ramamoorthy et al., 2008) (Fig. 5.3). Drug or vehicle treated mice were individually placed in the chamber and after an initial 2 min familiarization period, the digital locomotor scores were recorded for the next 8 min period. The chamber was cleaned with dilute (70 % v/v) alcohol and dried between trails.

Fig. 5.3 Pictorial representation of the actophotometer

5.8.2 Animal models used for the evaluation of depression-like behavior

5.8.2.1 Forced swim test

Rationale: When an animal is forced to swim in an unavoidable space, it ceases after some time and becomes static. The immobility represents a state of 'Despair' that simulates one of the two core symptoms of depression (hopelessness) in humans.

Procedure: FST was carried out as described elsewhere with slight modifications (Porsolt et al., 1977). After dosing, mice were dropped individually into a plexiglass cylinder (height: 30 cm, diameter: 22.5 cm) filled with water to a depth of 15 cm and maintained at 23-25 °C (Fig. 5.4). In this test, after an initial vigorous activity of 2 min, mice acquired an immobile posture which was characterized by motionless floating in water and making only those movements necessary to keep the head above the water. The duration of immobility (s), was recorded during the last 4 min of the 6 min test. The mice were subjected to 15 min training session under similar conditions, 24 h before the test.

Fig. 5.4 Pictorial representation of the forced swim test

5.8.2.2 Tail suspension test

Rationale: When the animal is hung through its tail at a height, it struggles initially and then become immobile. The immobility represents a state of 'DESPAIR' that mimic the condition of human depression (hopelessness).

Procedure: Following the dosing, mice were individually suspended by their tail on a horizontal bar (distance from floor was 50 cm) using scotch tape (distance from tip of tail was approximately 1 cm) (Fig. 5.5).

Typically, mice exhibited several escape-oriented behavior interspersed with temporally increasing bouts of immobility (Steru et al., 1984). The duration of immobility (s) during the 6 min test session was recorded.

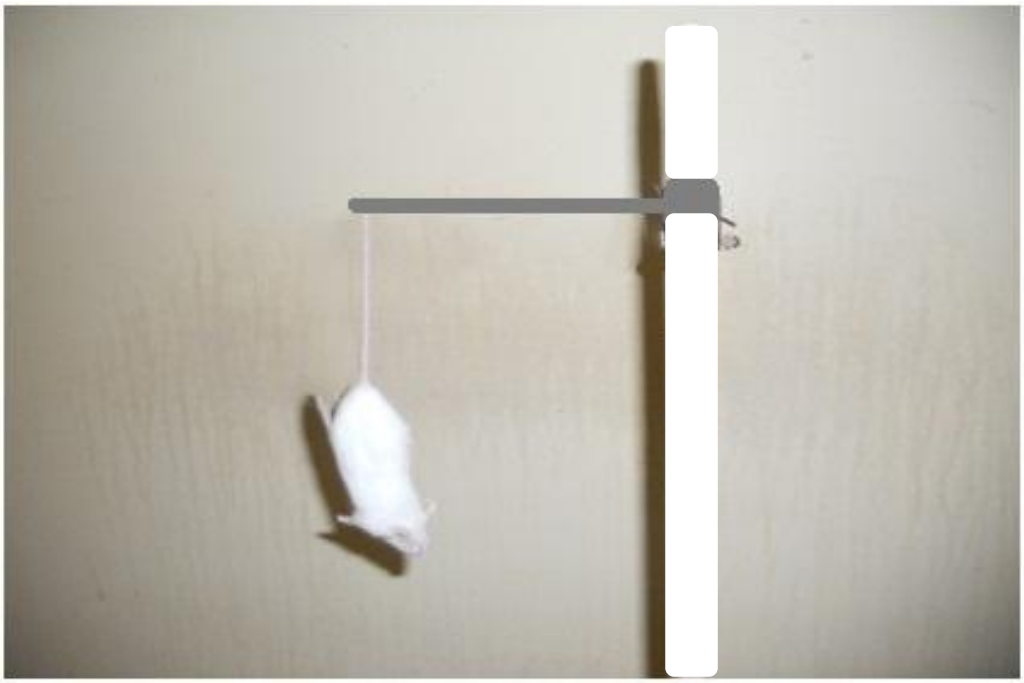


Fig. 5.5 Pictorial representation of the tail suspension test

5.8.2.3 Sucrose preference test

Rationale: Anhedonia or loss of interest in pleasurable activities is one of the two core symptoms required for the diagnosis of a major depressive episode in humans and is a useful endophenotype for modeling depression-related symptom in mice (APA, 2000; Willner et al., 1987). The sucrose preference test is one of the tools to evaluate the anhedonia behavior in rodents and to evaluate the antidepressant-like activity of the novel candidates and standard compounds. A pronounced reduction in percentage of sucrose preference is a putative indicator of anhedonia in this test.

Procedure: Sucrose preference test was carried out as described previously (Willner et al., 1987). It was conducted in three phases as follows: phase: 1-habituation, phase: 2-sucrose preference baseline, and phase: 3-sucrose preference testing. In phase: 1, tap water in the home-cage was replaced with 1% w/v sucrose in tap water for 24 hr to habituate mice to the novel solution.

In phase 2, each mouse was transferred to single cage and was exposed to both tap water and sucrose solution consequently for 3 days to attain the sucrose preference baseline. Sucrose preference was then determined by a two-bottle choice test using standard bottles, one filled with tap water and one with 1% sucrose solution, supplied to mice for 24 hr (phase 3). The locations of water and sucrose (left/right) were counterbalanced across the study. Tap water and sucrose solution intake was quantified by subtracting the final weight of bottles after 24 hr exposure period from their initial weight. The sucrose preference was then calculated as percentage preference = [(sucrose intake/total intake) × 100].

5.8.3 Animal models used for the evaluation of anxiety-like behavior

5.8.3.1 Elevated plus maze test

Rationale: Elevated plus maze has been used as an animal model of anxiety for over the last two decades (Wall and Messier, 2001). The open arm activity reflects the conflict between the preference of animal for protected areas (in closed arms) and its innate motivation to explore novel environments (in open arms) (for review see Walf and Frye, 2007). Increased open arms activity refers to anti-anxiety effects, while a decrease in activity reveals elevated anxiety-like behavior in rodents (Belzung and Griebel, 2001; Carobrez and Bertoglio, 2005).

Procedure: 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 extend from a central platform (5×5 cm) to form a plus sign (Fig. 5.6).

The plus-maze apparatus was elevated to a height of 45 cm and placed inside a room free from noise and disturbances. Each mouse was placed 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 open arm entries and percentage time spent in open arm (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 recorded by a trained observer blind to the treatments. The apparatus was thoroughly cleaned with 70 % v/v ethanol after each trial to remove the residual odor (that could influence the animal behavior under study).

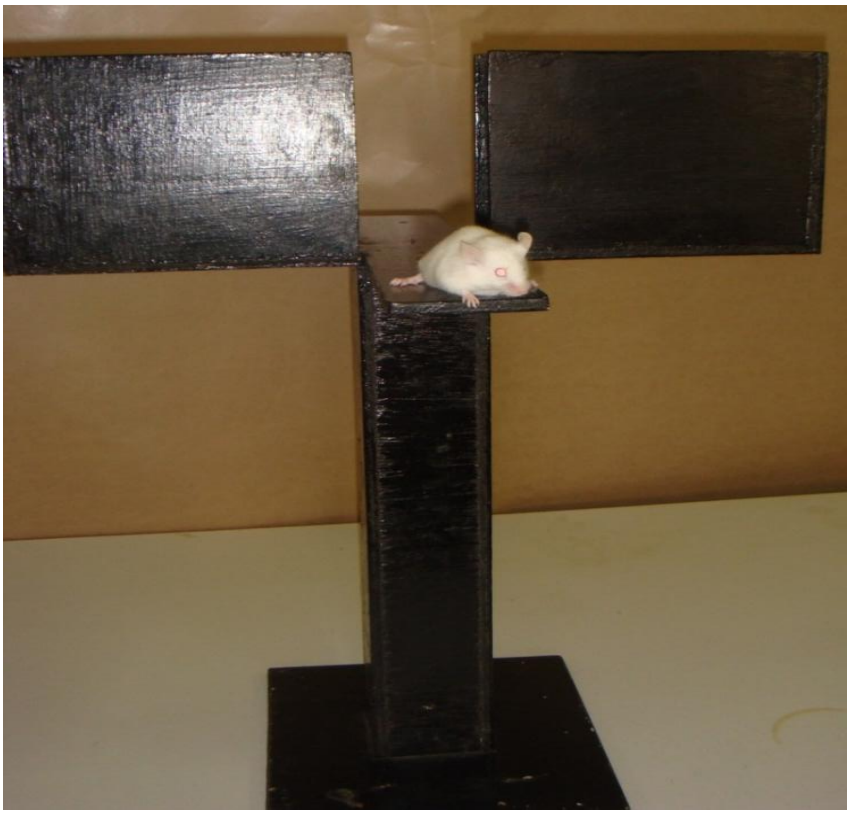


Fig. 5.6 Pictorial representation of the elevated plus maze test

5.8.3.2 Hole-board test

Rationale: Hole-board is another ethological model based on the exploratory behavior of rodents exposed to a novel unfamiliar environment consisting of a number of holes (Lister, 1990). Among the behavioral activities, more frequently measured in hole-board test is head dipping (insertion of head into a hole) represents one of the most studied (Casarrubea et al., 2009). Anxiolytic effect has been demonstrated as increased number of head dips, while reduced activity has been represented as anxiety-like behavior in hole-board (Crawley, 1985; Kliethermes and Crabbe, 2006).

Procedure: The hole-board apparatus consisted of a black painted wooden platform (40 × 40 cm), rose to a height of 15 cm from the floor. The platform consisted of 16 equivalent square compartments (12 peripheral and 4 central), each featuring a central circular hole (3 cm diameter) (Fig. 5.7). Test session starts when each mouse is placed in the center of the hole-board and allowed to freely explore on the apparatus for 5 min.

The behavioral performances such as the number of heads dips and duration of head dips were recorded (Silva et al., 2007; Wei et al., 2007). The apparatus was cleaned in between tests, as mentioned earlier.

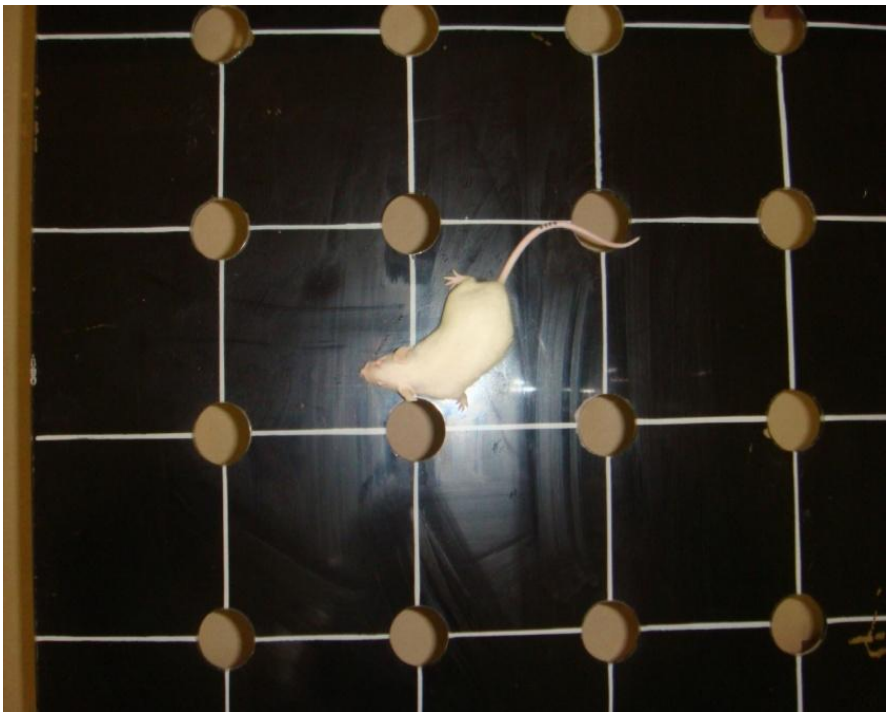


Fig. 5.7 Pictorial representation of the hole-board test

5.8.3.3 Light-dark test

Rationale: Light-dark model is an ethologically based murine model of anxiety, which is based on the innate aversive property of rodents to brightly illuminated area (Crawley and Goodwin, 1980). Exposure to LD apparatus develops a natural conflict situation (the conflict between the tendency to explore and the initial tendency to avoid the unfamiliar high light environment), in mice (Crawley and Goodwin, 1980; Bourin and Hascoet, 2003). Likewise, increased activity in the brightly illuminated light chamber is suggested as an index of anxiolytic activity, while the decreased exploratory behavior in the light chamber reveals anxiety-like condition in rodents (Costall et al., 1989).

Procedure: The 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 chamber), illuminated by a 60-Watt bulb was painted white, while the smaller (dark chamber) one was entirely black and enclosed under a dark cover.

The light-dark chambers were separated by a 13 cm long block having 5 cm high opening to allow passage from one chamber to the other (Mi et al., 2005) (Fig. 5.8). At the beginning of the test, the mice were placed individually in the center of the light chamber facing towards the tunnel and allowed to explore the entire apparatus for 5 min. The behavioral parameters such as latency time to leave the light chamber and total time spent in the light chamber were recorded (Crawley and Goodwin, 1980). A chamber entry was considered valid when all the four paws of animal were inside that chamber. The apparatus was thoroughly cleaned with 70% ethanol after each trial.

Fig. 5.8 Pictorial representation of the light-dark test

5.8.3.4 Open field test

Rationale: the test is based on the degree of exploration. The more the exploration in the center of the arena represents the anxiolytic-like effect as the animal spends more time in exploring the novel environment. The parameters measured in this test are number of crossings and number of rearings (that represent horizontal and vertical activity, respectively).

Procedure: The test was conducted as described by Kelly et al. (1997) with slight modifications. The apparatus consisted of a black wooden square arena with 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 (Fig. 5.9).

Each mouse was placed in the center of the open-field apparatus 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 scored only when the hind limbs of the animal moved to the next square. The apparatus was cleaned with 70 % v/v ethyl alcohol and dried between trials.

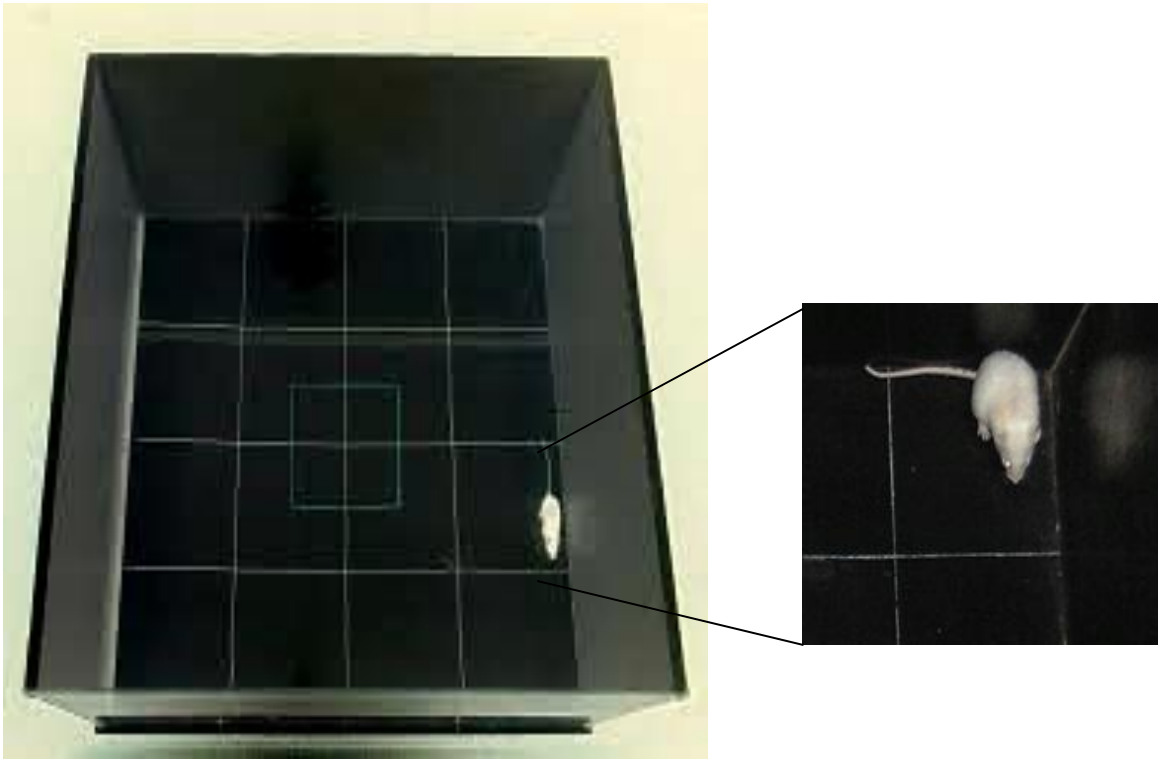


Fig. 5.9 Pictorial representation of the open field test

5.8.3.5 Social interaction test

Rationale: Social activities themselves and their quality, exert profound influences on the mood and emotionally relevant behavioral measures. In humans, positive psychosocial activities can reduce depressive symptoms and improve stress resiliency, while persistent distressed condition can temper emotional reactivity (Derntl et al., 2011). Social-interaction mirrors positive social activity and emotional stability. In this test, increased social activity as presented by increased grooming, clawing and crossing (Fig. 5.10) indicate the anxiolytic like effects or vice versa that can be measured in the form of number of social interaction and cumulative duration of social interaction.

Procedure: Social-interaction test was conducted in the same apparatus that was used for open-field test (File et al., 1985). Two mice (the test mouse and the stimulus mouse of same age and sex, unknown to the test mouse) were put into two different corners of the open-field arena. The social-interaction behavior (grooming, mounting and crawling under the stimulus mouse) including the passive interaction (number of crossing to the stimulus mouse) and cumulative social-interaction time (s) were recorded for 5 min after placement of the mice into the apparatus. This test was adopted particularly to measure the social behavior, in which more 'affected' mice spend less time in social-interaction (Chaudhury et al., 2012). After each test, the apparatus was sprayed with 70% w/v alcohol and wiped thoroughly as stated above.

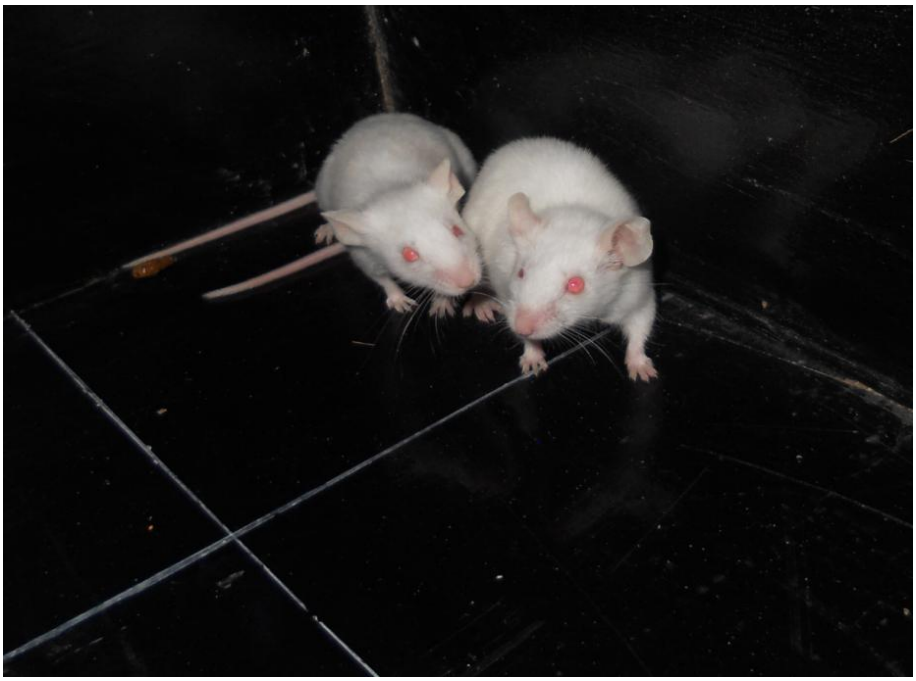


Fig. 5.10 Pictorial representation of the social interaction test

5.8.5 Chronic model of depression

5.8.5.1 Olfactory bulbectomized rat model

Rationale: Bilateral bulbectomy, brings about the alterations in behavioral, neuroanatomical, neurochemical and signaling circuits associated with depression. The behavioral features observed in the OBX rodents are similar to that of the depressed patients. Disruption in the neuronal signaling through limbic system is the prominent factor proposed to be the causative factor of behavioral anomaly in OBX.

Hence, OBX serves as a model for the evaluation of antidepressant activity of several compounds and novel candidates. In addition, the chronic dosing mimics the clinical course of therapeutic intervention, in humans.

Procedure for surgery: Bilateral ablation of olfactory bulbs was performed as described elsewhere (Kelly et al., 1997; Van Riezen and Leonard, 1990). Rats were anesthetized with ketamine/xylazine (75/5 mg/kg, i.p.) and head was shaved.

The rat was then fixed in a stereotaxic frame and the cranium was exposed by a midline sagittal incision. Two 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 the eye (Fig. 5.11) The olfactory bulbs were ablated by suction, avoiding damage to the frontal cortex, and the dead space was filled with haemostatic sponge to prevent excessive bleeding and the scalp was sutured (Fig. 5.12).

To prevent post surgical infection, the rats were given Sulprim injections (each milliliter containing sulfadiazine 200 mg and trimethoprim 40 mg), intramuscularly (0.2 ml/300 g) once a day for 4 days. Sham operation was performed in a similar manner but bulbs were left intact.

During the 14-day recovery period, the animals were handled regularly to avoid aggressive behavior (Leonard and Tuite, 1981), which might have developed otherwise. 15^{th} day after the surgery, drug (**4i** at 0.5 and 1 mg/kg or FLX at 10 mg/kg, i.p.) or vehicle treatment was started and was continued once a day for 14 days. Behavioral assays were performed 24 hr after the last dosing of the drug/vehicle. Only one behavioral test was performed on each day to avoid the residual effects.

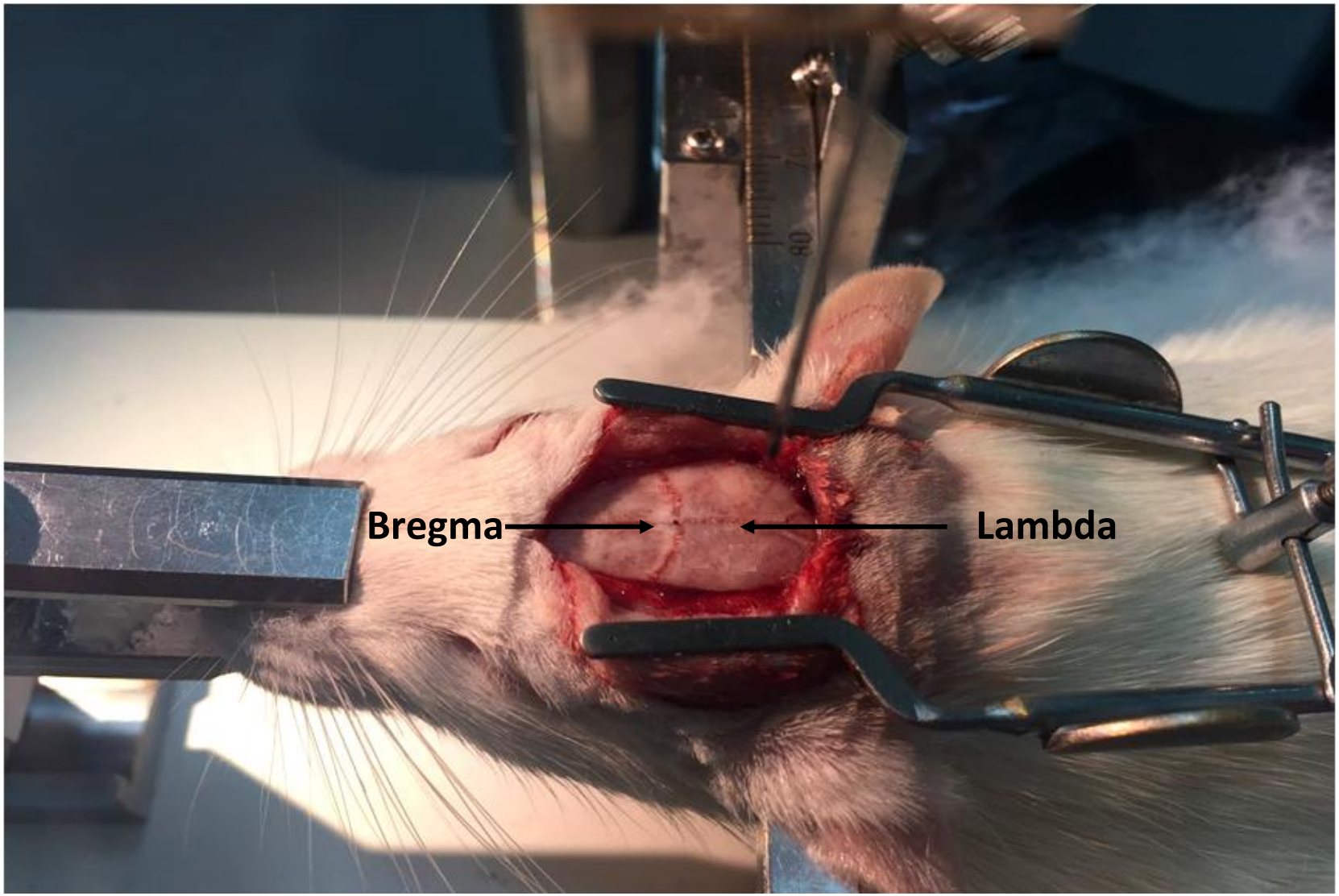


Fig. 5.11 Bregma region for OBX surgery in rat

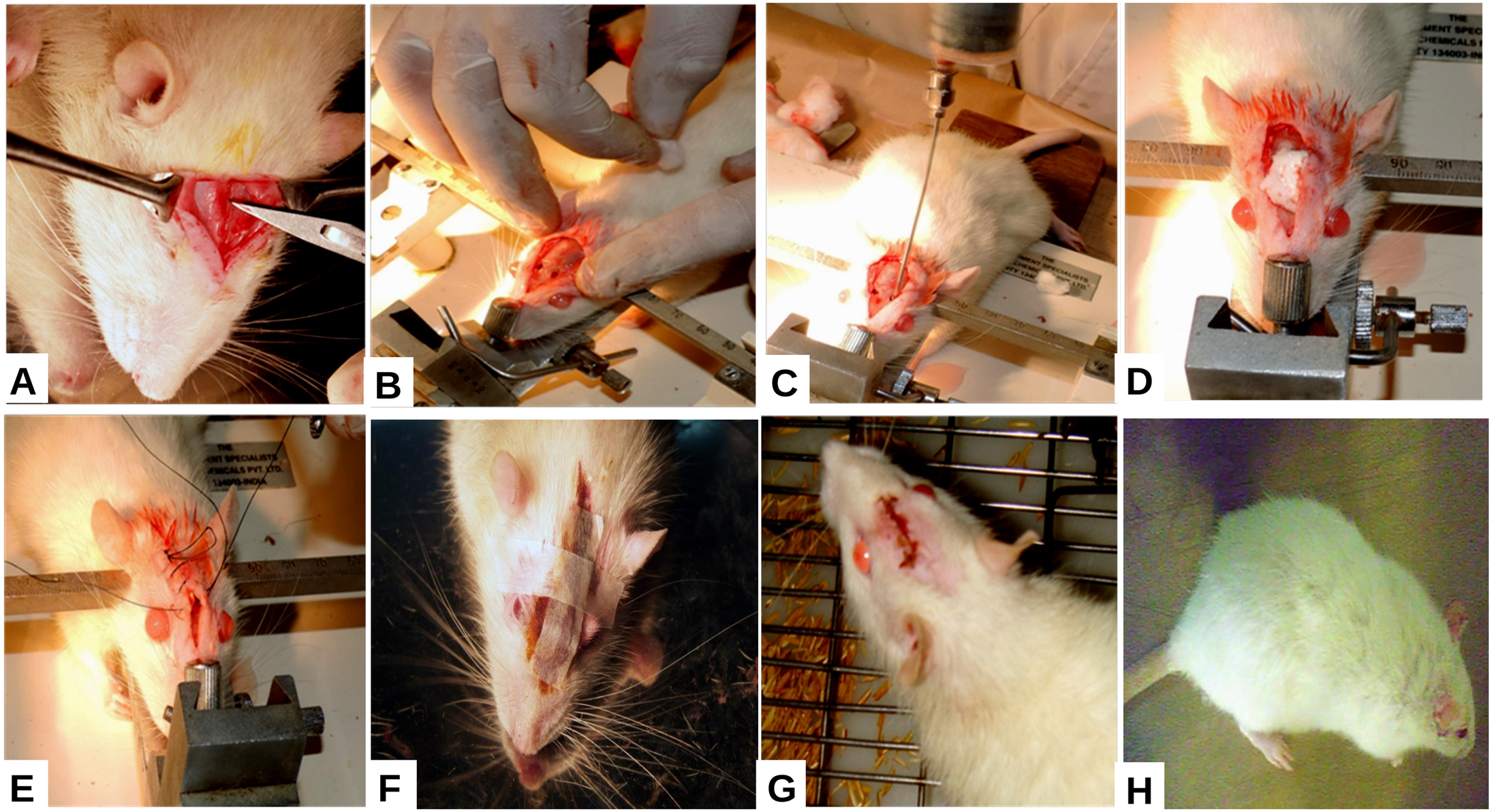


Fig.5.12 Preliminary steps of OBX surgical procedure. **(A)** Positioning of rat in sterotaxic system, **(B)** Midline sagittal incision, **(C)** Burr holes on either side of midline, **(D)** hemostatic sponge, **(E)** Suturing of incision site, **(F)** Post surgical medication **(G)** Healing of the surgical wound on 7^{th} day and **(H)** 14^{th} day.

5.8.5.2 Chronic unpredictable stress

Rationale: In rodents, chronic unpredictable stress (CUS) as a model of depression was developed in an attempt to mimic some of the environmental factors contributing to induction of depression in humans (Moretti et al., 2012; Nollet et al., 2013).

CUS resembles variety of neurochemical, neurobehavioral and neuroendocrine alterations of human depression (Holsboer, 2000). Animals subjected to a variety of stressors such as deprivation of food and water, wet husk bedding exhibited depressive behavior in numerous behavioral testing paradigms (Larsen et al., 2010; Ma et al., 2011; Rasheed et al., 2011).

Stress procedure: Mice in CUS groups were subjected to different types of stressors (Table 5.2), while mice in normal control group were left undisturbed except for the general housekeeping procedure (Strekalova and Steinbusch, 2010). CUS procedure was continued for 4-weeks, followed by behavioral and biochemical assessment. Behavioral assays were performed 24 hrs after the last dosing to avoid the acute effects of the drug with one behavioral test performed on each day to avoid the residual effect of the earlier testing paradigm. Plasma and the brain samples were collected 24 hr after the last behavioral assay to eliminate the effect of acute stress.

5.9 Pharmacological paradigms

5.9.1 General considerations for behavioral assays

In order to avoid habituation effects with experimental conditions, separate sets of randomized animals were used for each experiment. The drugs were freshly prepared before administration. The doses were given in a constant volume of 10ml/kg for mice and 1 ml/kg for rats.

The drug administrations and behavioral screenings were performed between 09:00 and 17:00 hrs. The animals were acclimatized to the experimentation room for one hour before testing. The test apparatus and the work bench were cleaned with ethyl alcohol and dried.

Table 5.2 The protocol employed in chronic unpredictable stress

Day	Stress type	Duration of stress (hrs)
1	Food and water deprivation	24
2	Wet bedding	6
3	Restraint	3
4	Cage titling (45°)	6
5	Inversion of light and dark cycle	12
6	Tail suspension	3
7	Predator exposure	3
8	Food and water deprivation	24
9	Exposure to empty water bottles	12
10	Cage titling (45°)	6
11	Isolation	24
12	Wet bedding	6
13	Inversion of light and dark cycle	12
14	Restraint	3
15	Tail suspension	3
16	Isolation	24
17	Exposure to empty water bottles	24
18	Predator exposure	3
19	Food and water deprivation	24
20	Cage titling (45°)	6
21	Isolation	24
22	Tail suspension	3
23	Inversion of light and dark cycle	12
24	Restraint	3
25	Food and water deprivation	24
26	Predator exposure	3
27	Cage titling (45°)	6
28	Wet bedding	6

5.9.2 Preliminary screening for dose selection using basal locomotor activity test

Groups under the test for BLA: The test was performed using standard 5-HT_3 receptor antagonist, OND and the novel candidates **4i** and **6z** (n = 6 mice per group) using actophotometer. Mice were given vehicle or drugs i.p. and 30 min later the locomotor scores were recorded as per the method described in section 5.8.1.

Group No.	**Set A**	**Set B**	**Set C**
1	Normal control	Normal control	Normal control
2	OND (0.25 mg/kg, i.p.)	**4i** (0.25 mg/kg, i.p.)	**6z** (0.25 mg/kg, i.p.)
3	OND (0.5 mg/kg, i.p.)	**4i** (0.5 mg/kg, i.p.)	**6z** (0.5 mg/kg, i.p.)
4	OND (1 mg/kg, i.p.)	**4i** (1 mg/kg, i.p.)	**6z** (1 mg/kg, i.p.)
5	OND (2 mg/kg, i.p.)	**4i** (2 mg/kg, i.p.)	**6z** (2 mg/kg, i.p.)
6	-	**4i** (4 mg/kg, i.p.)	**6z** (4 mg/kg, i.p.)
7	-	**4i** (8 mg/kg, i.p.)	-
8	FLX (10 mg/kg, i.p.)	FLX (10 mg/kg, i.p.)	FLX (10 mg/kg, i.p.)

5.9.3 Preliminary screening for antidepressant activity of OND, 4i and 6z

Groups under the tests for FST and TST: Mice were given vehicle or drug (i.p.) and 30 min later the duration of immobility (s) was recorded as per the method described in sections 5.8.1 and 5.8.2, respectively.

Set A: FST and TST were performed using standard 5-HT_3 receptor antagonist, OND (n = 6 mice per group).

Group No.	**FST**	**TST**
1	Normal control	Normal control
2	OND (0.5 mg/kg, i.p.)	OND (0.5 mg/kg, i.p.)
3	OND (1 mg/kg, i.p.)	OND (1 mg/kg, i.p.)
4	FLX (10 mg/kg, i.p.)	FLX (10 mg/kg, i.p.)

Set B: The test was performed using novel 5-HT_3 receptor antagonist, **4i** (n = 6 mice per group)

Group No.	**FST**	**TST**
1	Normal control	Normal control
2	**4i** (0.5 mg/kg, i.p.)	**4i** (0.5 mg/kg, i.p.)
3	**4i** (1 mg/kg, i.p.)	**4i** (1 mg/kg, i.p.)
4	FLX (10 mg/kg, i.p.)	FLX (10 mg/kg, i.p.)

Set C: The test was performed using novel 5-HT_3 receptor antagonist, **6z** (n = 7 mice per group)

Group No.	FST	TST
1	Normal control	Normal control
2	**6z** (0.5 mg/kg, i.p.)	**6z** (0.5 mg/kg, i.p.)
3	**6z** (1 mg/kg, i.p.)	**6z** (1 mg/kg, i.p.)
4	**6z** (2 mg/kg, i.p.)	**6z** (2 mg/kg, i.p.)
5	FLX (10 mg/kg, i.p.)	FLX (10 mg/kg, i.p.)

5.9.4 Preliminary screening for anxiolytic activity of OND, 4i and 6z

Groups under the tests for hole-board test and light-dark test: Mice were given vehicle or drug (i.p.) and 30 min later the parameters were recorded as per the method described in sections 5.8.3.2 and 5.8.3.3, respectively.

Set A: Hole-board test and Light-dark test: the tests were performed using standard 5-HT_3 receptor antagonist, OND (n = 6 mice per group).

Group No.	Hole-board test	Light-dark test
1	Normal control	Normal control
2	OND (0.5 mg/kg, i.p.)	OND (0.5 mg/kg, i.p.)
3	OND (1 mg/kg, i.p.)	OND (1 mg/kg, i.p.)
4	FLX (10 mg/kg, i.p.)	FLX (10 mg/kg, i.p.)

Set B: The test was performed using novel 5-HT_3 receptor antagonist, **4i** (n = 6 mice per group).

Group No.	Hole-board test	Light-dark test
1	Normal control	Normal control
2	**4i** (0.5 mg/kg, i.p.)	**4i** (0.5 mg/kg, i.p.)
3	**4i** (1 mg/kg, i.p.)	**4i** (1 mg/kg, i.p.)
4	FLX (10 mg/kg, i.p.)	FLX (10 mg/kg, i.p.)

Set C: The test was performed using novel 5-HT_3 receptor antagonist, **6z** (n = 7 mice per group)

Group No.	Hole-board test	Light-dark test
1	Normal control	Normal control
2	**6z** (0.5 mg/kg, i.p.)	**6z** (0.5 mg/kg, i.p.)
3	**6z** (1 mg/kg, i.p.)	**6z** (1 mg/kg, i.p.)
4	**6z** (2 mg/kg, i.p.)	**6z** (2 mg/kg, i.p.)
5	FLX (10 mg/kg, i.p.)	FLX (10 mg/kg, i.p.)

5.9.5 Pharmacological screening of OND, 4i and 6z using chronic models of depression

5.9.5.1 Evaluation of antidepressant effect in CUS model

Schedule for the drug administration and behavioral testing

After 14 days of chronic stress (explained in Table 5.2), mice were treated with the test or standard drug for next 14 days (i.p.) along with the continuation of stress protocol and then subjected to the FST, TST, sucrose preference test (the measures of depression-like behavior) and open field test, a measure of anxiety-like behavior, in mice.

Set A: CUS assay with OND treatment (n = 6 mice per group).

Group No.	Groups	Groups
1	Non-CUS control	CUS control
2	Non-CUS + OND (0.5 mg/kg, i.p.)	CUS + OND (0.5 mg/kg, i.p.)
3	Non-CUS + OND (1 mg/kg, i.p.)	CUS + OND (1 mg/kg, i.p.)
4	Non-CUS + FLX (10 mg/kg, i.p.)	CUS + FLX (10 mg/kg, i.p.)

Set B: CUS assay with 6z treatment (n = 7 mice per group).

Group No.	Groups	Groups
1	Non-CUS control	CUS control
2	Non-CUS + **6z** (1 mg/kg, i.p.)	CUS + **6z** (1 mg/kg, i.p.)
3	Non-CUS + **6z** (2 mg/kg, i.p.)	CUS + **6z** (2 mg/kg, i.p.)
4	Non-CUS + FLX (10 mg/kg, i.p.)	CUS + FLX (10 mg/kg, i.p.)

5.9.5.2 Evaluation of antidepressant activity using olfactory bulbectomized (OBX) model

Schedule for the drug administration and behavioral testing

The OBX and sham surgeries were conducted in rats. After 14 days of surgery, rats were randomly divided into different groups. Following the dosing of the vehicle or drug (given i.p.), both OBX and sham rats were subjected to sucrose preference test (a measure of depression) and open field test (a measure of anxiety). The details of the protocol are given in Table 5.3.

Table 5.3 The schedule for the OBX study.

Day 0	**Day 1-4**	**Day 4-14**	**Day 15-28**	**Day 29**	**Day 30**
SURGERY	Recovery, continuous care	Rehabilitation, gentle handling, observation	Dosing (drug/vehicle, i.p.) once a day	OFT	SPT

Set A: The OBX was performed using 4i (n = 7 rats/group).

Group No.	**Groups**	**Groups**
1	Sham control	OBX control
2	Sham + **4i** (0.5 mg/kg, i.p.)	OBX + **4i** (0.5 mg/kg, i.p.)
3	Sham + **4i** (1 mg/kg, i.p.)	OBX+ **4i** (1 mg/kg, i.p.)
4	Sham + FLX (10 mg/kg, i.p.)	OBX + FLX (10 mg/kg, i.p.)

5.9.6 Time course of development of depression and anxiety-like behavior in STZ-induced diabetic mice

5.9.6.1 Evaluation of the basal locomotor activity in diabetes using BLA test

Schedule: The basal locomotor activity of STZ-induced diabetic mice was tested before the evaluation of depression and anxiety-like behavior.

.**Set A: Groups used for evaluation of BLA (n = 7 mice per group)**

Group No.	**FST**
1	Control(nDIA)
2	Diabetic control (DIA)

5.9.6.2 Evaluation of the depression-like behavior evoked in diabetic mice using FST and TST

Schedule: STZ-induced diabetic mice were subjected to FST and TST (separate mice for each test) after 1 week of diabetes and subsequently exposed to FST and TST, periodically (alternate week) for depressive behavior, till 8 weeks.

The diabetic mice were subjected to FST and TST (n = 7 mice per group).

Group No.	FST	TST
1	Control(nDIA)	Control(nDIA)
2	Diabetic control (DIA)	Diabetic control (DIA)

Procedure: The detailed protocol for the estimation of despair behavior is given in section 5.8.2.

5.9.6.3 Evaluation of anxiety-like behavioral effects in diabetes using light-dark test, open field test, hole-board test and elevated plus maze test

Schedule for the drug administration and behavioral assays

The diabetic mice were subjected to light-dark model, open field test, hole-board test and EPM after 1 week of induction of diabetes and subsequently every alternate week for the presence of anxiety-like behavior till 8 weeks, n = 7 mice per group.

Group No.	Light-dark test	Open field test	Hole-board test	Elevated plus maze test
1	Normal control	Normal control	Normal control	Normal control
2	Diabetic control	Diabetic control	Diabetic control	Diabetic control

Procedure: The detailed protocol for estimation of anxiety-like behavior using light-dark test, open field, hole-board test and elevated plus maze test is given in section 5.8.3.

5.9.7 Evaluation of antidepressant and anxiolytic-like effects of 5-HT_3 receptor antagonists in depression and anxiety associated with diabetes

Rationale: 5-HT_3 receptor antagonists have proven antidepressant and anxiolytic-like profile in the naïve rodents. The clinical data also suggests that they have antidepressant and anxiolytic-like effects relieving the symptoms of depression in patients suffering from various chronic illnesses.

Furthermore, 5-HT_3 receptor antagonists have profound influence on the regulation of energy metabolism. This suggests that 5-HT_3 receptor antagonists may have beneficial effects in alleviating depression and anxiety-like anomaly co-morbid with diabetes, which is the main objective of this work. The present study focuses on the behavioral screening of the 5-HT_3 receptor antagonists in attenuating depression and anxiety, evoked in diabetic rodents.

Schedule for the drug administration and behavioral assays

Eight weeks after induction of diabetes, mice were randomly divided into different groups, namely: diabetic mice received vehicle (distilled water), (diabetic control); diabetic mice received test drug (at different doses) and diabetic mice received FLX. Another group of mice with normal blood glucose levels were used as control.

The doses and treatment schedule were selected on the basis of preliminary screening and previous studies, performed in our laboratory (Gupta et al., 2014a, b). After chronic dosing (for 28 days), mice were subjected to behavioral and biochemical assays. In the study, which involved the evaluation of effects of OND, the behavioral assays performed were FST and TST (the measure of depression), and light-dark and hole-board tests (as the measure of anxiety). The study, which involved the evaluation of the effects of **4i**, the behavioral assays performed were FST and TST (as the parameter of depression) and open field test and social interaction test (as the measure of anxiety). The behavioral assays performed for the evaluation of the **6z** effects were FST and TST and light-dark test and social interaction test for anxiety, Table 5.4.

Table 5.4 The schematic representation of the study protocol.

Day 0	**Day 3**	**Day 56-83**	**Day 84-87**	**Day 88**
STZ dosing	Fasting blood glucose test	Drug/vehicle dosing (i.p.)	Behavioral assays for depression and anxiety	Collection of brain and blood for biochemical estimations

Set A: Evaluation of the antidepressant-like effects of OND in STZ-induced diabetic mice (n = 6 mice per group).

1. Normal control
2. Diabetic control
3. Diabetic mice + OND (0.5 mg/kg, i.p.)
4. Diabetic mice + OND (1 mg/kg, i.p.)
5. Normal + FLX (10 mg/kg, i.p.)

Set B: Evaluation of the antidepressant-like effects of 4i in STZ-induced diabetic mice (n = 6 mice per group).

1. Normal control
2. Diabetic control
3. Diabetic mice + **4i** (0.5 mg/kg, i.p.)
4. Diabetic mice + **4i** (1 mg/kg, i.p.)
5. Diabetic mice + FLX (10 mg/kg, i.p.)

Set C: Evaluation of the antidepressant-like effects of 6z in STZ-induced diabetic mice (n = 6 mice per group).

1. Normal control
2. Diabetic control
3. Diabetic mice + **6z** (1 mg/kg, i.p.)
4. Diabetic mice + **6z** (2 mg/kg, i.p.)
5. Diabetic mice + FLX (10 mg/kg, i.p.)

5.9.8 Evaluation of the role of 5-HT_3 receptors in the antidepressant-like and anxiolytic-like effects of 5-HT_3 receptor antagonists

Rationale: The goal of this experiment was to evaluate, the role of 5-HT_3 receptors in the behavioral effects of the tested compounds. The concomitant administration of the selective 5-HT_3 receptor agonist should ameliorate the beneficial effects of the 5-HT_3 antagonists. To test this hypothesis, mCPBG, a selective 5-HT_3 receptor agonist was used.

Schedule for the drug administration and behavioral assays

Mice were treated with a selective 5-HT_3 agonist, mCPBG (10 mg/kg, i.p.) and 1hr later administered with 5-HT_3 antagonists (OND / **4i** / **6z**, i.p.) everyday for 28 days (4-weeks). After repeated dosing, mice were subjected to the behavioral and biochemical assays. The type of behavioral tests schedule of the study were used as described in section 5.9.7 for each test drug candidate.

Set A: Study was performed using OND (n = 6 mice per group).

1. Diabetic control
2. Diabetic + OND (1 mg/kg, i.p.)
3. Diabetic + mCPBG (10 mg/kg, i.p.)
4. Diabetic + mCPBG (10 mg/kg, i.p.) + OND (1 mg/kg, i.p.)

Set B: The study was performed using 4i (n = 6 mice per group).

1. Diabetic control
2. Diabetic + **4i** (1 mg/kg, i.p.)
3. Diabetic + mCPBG (10 mg/kg, i.p.)
4. Diabetic + mCPBG (10 mg/kg, i.p.) + **4i** (1 mg/kg, i.p.)

Set C: The study was performed using 6z (n = 6 mice per group).

1. Diabetic control
2. Diabetic + **6z** (1 mg/kg, i.p.)
3. Diabetic + mCPBG (10 mg/kg, i.p.)
4. Diabetic + mCPBG (10 mg/kg, i.p.) + **6z** (1 mg/kg, i.p.)

5.9 Biochemical, neurochemical and neurobiological assays

24 hrs after the behavioral assays in each of the study protocol, the biological samples (blood or tissue) were collected, which were then processed and stored at -80°C until analysis.

5.9.1 Collection of blood and plasma isolation

Blood was collected, in mice, using retro-orbital method through venous sinus. Each mouse was hand restrained, the neck was gently scuffed and the eye was made to bulge. A glass capillary tube/pipette was inserted laterally (Fig. 5.13).

Blood was allowed to flow by capillary action into the eppendorf tubes containing 100 µl of EDTA (10 % w/v) (Luzzi et al., 2005). Nearly, 0.4 ml of total blood was collected in every goal and the blood flow was then stopped by applying gentle pressure on the eye. The tubes were then centrifuged at 12000 rpm, 4°C for 20 min and the supernatant was collected in another set of clean eppendorf tubes.

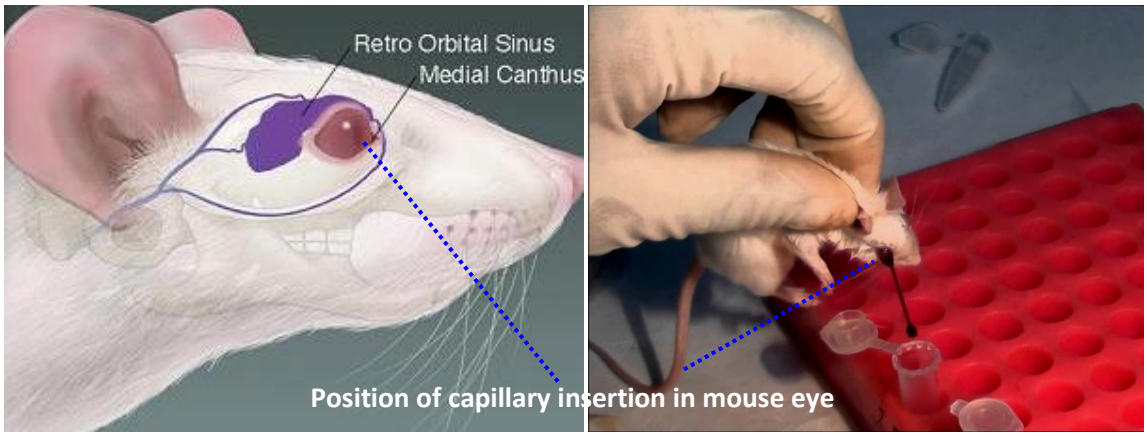


Fig. 5.13 Pictorial representation of the retro-orbital method, **(A)** shows the sinus position, **(B)** represents the capillary action

5.9.2 Collection of brain, dissection and preparation of homogenates

The animals were decapitated and their brains were collected in a petri dish over an ice bath. For the biochemical estimations, each brain was gently cryo-dried and dissected as per the method described elsewhere (Glowinski and Iversen, 1966). The mid brain (containing hippocampus), frontal cortex and cerebellum were collected in separate tubes. The dissected brain samples were once washed with ice cold phosphate buffer saline (PBS) and then homogenized in same. The dissected samples were homogenized in 10 volumes of ice-cold PBS.

5.9.4 Estimation of pro-oxidant markers

5.9.4.1 Lipid peroxidation

Rationale: Oxidation of lipid is one of the important key markers, for the presence of oxidative stress, in the venerable tissues like brain. Malondialdehyde (MDA) is one of several low-molecular-weight end products, formed via the decomposition of certain primary and secondary lipid peroxidation products (Halliwell and Chirico, 1993).

At low pH and elevated temperature (100° C), MDA readily participates in nucleophilic addition reaction with 2-thiobarbituric acid (TBA), generating a red, fluorescent 1:2 MDA:TBA adduct also known as thiobarbituric acid reactive substances (TBARS) and the degree of the lipid peroxidation is estimated by the amount of the MDA present in the tissues (Fig. 5.14).

Procedure: The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reactive substance (TBARS) as per reported method (Wills, 1966). Briefly, 0.1 ml of tissue homogenate and 0.1 ml of Tris–HCl were incubated at 37°C for 2 h. After incubation, 0.5 ml of 10% trichloroacetic acid was added and centrifuged at 1,000 rpm for 10 min. To 0.5 ml of supernatant, 0.5 ml of 0.67% thiobarbituric acid was added and the tubes were kept in boiling water for 10 min. After cooling, 1 ml double distilled water was added and absorbance was measured at 532 nm (UV-1800 spectrophotometer, Shimadzu, Japan). The amount of lipid peroxidation products (TBARS) was quantified using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nanomoles of MDA per milligram of protein.

The reaction can be written as:

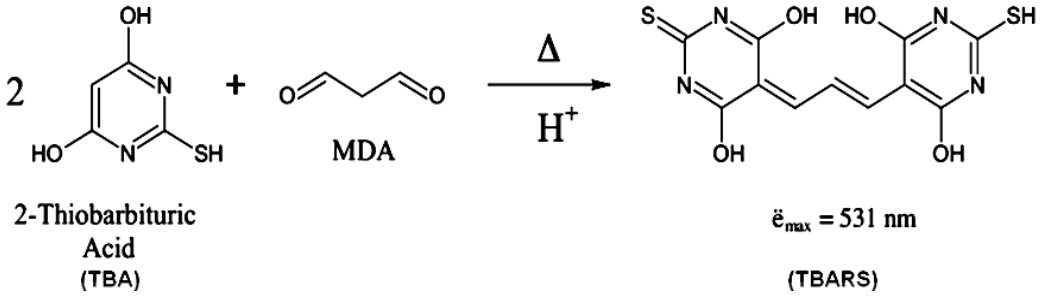


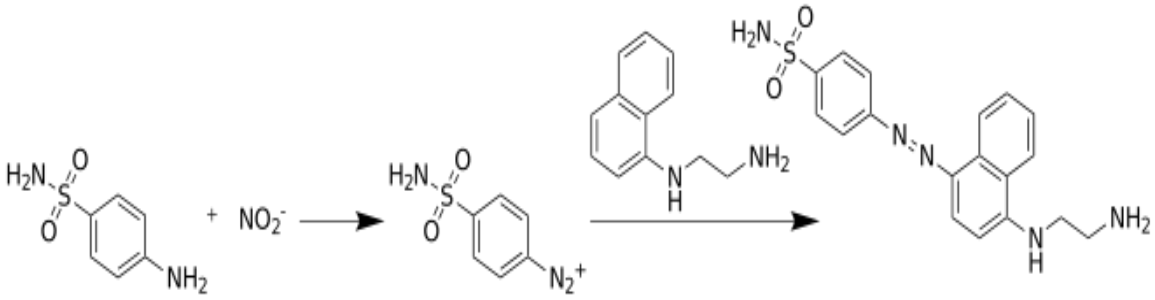
Fig. 5.14 Reaction between 3-thiobarbituric acid and MDA under high temperature. Two molecules of the TBA react with the 1 molecule of MDA to form TBARS, which give red fluorescence that can be read at 531 nm.

5.9.4.2 Nitrite levels

Rationale: Nitrite is another important marker of the oxidative stress. Griess diazotization reaction method is used to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under physiological conditions in the tissues.

Nitrite is detected and analyzed by formation of a red pink color upon treatment of a NO_2^--containing brain sample with the Griess reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthaylamine diamine dihydrochloric acid in water). When sulphanilic acid is added (formed in situ be decomposition of sulphanilamide in acidic condition), the nitrites form a diazonium salt. When the azo dye agent (N-alpha-naphthyl-ethylenediamine) is added, a pink color develops (Fig. 5.15). This diamine is used in place of the simpler and cheaper alpha-naphthaylamine because the latter is a potent carcinogen and moreover the diamine forms a more polar and hence a much more soluble dye in acidic aqueous medium.

The reaction can be written as:



Sulphanilamide nitrite diazonium salt pink color adduct

e = 546 nm

Fig. 5.15 The reaction between nitrite and sulphanilamide to form pink color adduct.

Procedure: Nitrite levels were estimated using Greiss reagent which served as an indicator of nitric oxide production (Green et al., 1982).

A measure of 0.5 ml of Greiss reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% napthaylamine diamine dihydrochloric acid in water) was added to 0.1 ml of tissue homogenate, the mixture was incubated for 10 min at room temperature in dark and absorbance was measured at 546 nm (UV-1800 spectrophotometer, Shimadzu, Japan).

The nitrite levels were calculated using a standard curve for sodium nitrite and expressed as micromoles per milligram of protein.

5.9.5 Estimation of the anti-oxidant markers

5.9.5.1 Catalase activity

Rationale: Catalase, an antioxidant, is a very important enzyme in protecting the cell from oxidative damage by ROS. Reduced level of the enzyme is associated with the oxidative stress. The catalase activity can be detected by a reduction reaction with an oxidizing agent.

It catalyzes the decomposition of hydrogen peroxide to water and oxygen. The presence of catalase in tissue sample can be tested, by adding a known volume of hydrogen peroxide and observing the reaction. Hydrogen peroxide enters the active site and interacts with the amino acids Asn147 (asparagine at position 147) and His74, causing a proton (hydrogen ion) to transfer between the oxygen atoms. The free oxygen atom coordinates, the newly formed water molecule and Fe(IV)=O. Fe(IV)=O reacts with a second hydrogen peroxide molecule to reform Fe(III)-E and produce water and oxygen.

The reaction can be written as:

H_2O_2 + Fe(III)-E → H_2O + O=Fe(IV)-E(+)

H_2O_2 + O=Fe(IV)-E(+) → H_2O + Fe(III)-E + O_2

Fe()-E represents the iron center of the heme group attached to the enzyme. Fe(IV)-E(+) is a mesomeric form of Fe(V)-E, indicating the iron is not completely oxidized to +V, but receives, some supporting electrons from the heme ligand. This heme has to be present as a radical cation (+) as a blood.

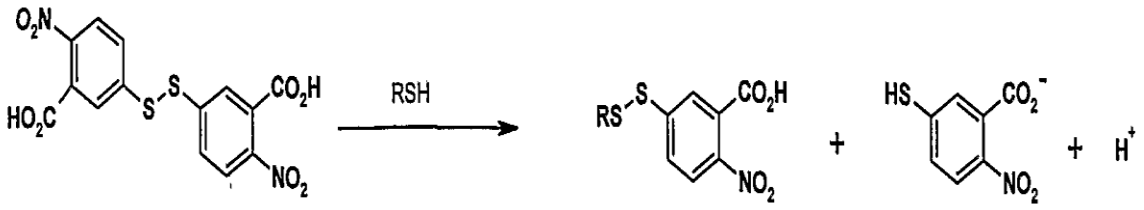
Procedure: Catalase activity was assayed by standard method (Claiborne, 1985). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml tissue homogenate (10%) in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated and expressed as µmoles of hydrogen peroxide, consumed per min per milligram of protein (U/mg protein).

5.9.5.2 Reduced glutathione levels

Rationale: Glutathione (GSH) is an important antioxidant, preventing damage to important cellular components, caused by ROS, such as free radicals and peroxides.

It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and the amine group of cysteine (which is attached by normal peptide linkage to a glycine). GSH reduces disulfide bonds, formed within cytoplasmic proteins to cysteines, by serving as an electron donor. In the process, GSH is converted to its oxidized form, glutathione disulfide (GSSG), also called L-(–)-GSH. Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) is a chemical used to quantify the GSH in the tissue samples by estimating the number or concentration of thiol groups. It was developed by George L. Ellman. Thiols react with the reagent, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (TNB^-), which ionizes to the TNB^{2-} dianion in water at neutral and alkaline pH. This TNB^{2-} ion gives a yellow color (Fig. 5.16).

The reaction can be written as:



Ellman's reagent TNB^- e_{max} = 412 nm

Fig. 5.16 The reaction between GSH and the DTNB

Procedure: Reduced glutathione in the dissected brain sections were estimated according to the method described by Ellman (1959). 0.1 ml supernatant was precipitated with 0.1 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 0.1 ml of supernatant, 0.5 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 color developed was measured immediately at 412 nm (UV-Spectrophotometer). Results are expressed as micromole/milligram protein.

5.10 Estimation of corticosterone levels in the plasma

Rationale: The basic principle involved in the estimation of corticosterone is that it gives blue/green fluorescence, which is enhanced in the presence of sulfuric acid. It is due to the presence of double bond in the first ring, Fig. 5.17.

However, the difficulties arise due to the structural similarities of various steroids. Thus, extraction of a steroid of choice and then treating it with the acid is important. Corticosterone fluoresces at 533 nm emission and 472 nm excitation wavelengths. The other steroids which fluoresce at the same e_{max} are 17-β-estradiol and estrogens. Thus, initially the corticosterone is extracted by liquid-liquid extraction, using chloroform and methanol mixture and then chloroform, followed by removal of hindering steroids by a sodium hydroxide wash. The pure corticosterone in the reaction medium is treated with the acid to develop green fluorescence, which is then measured.

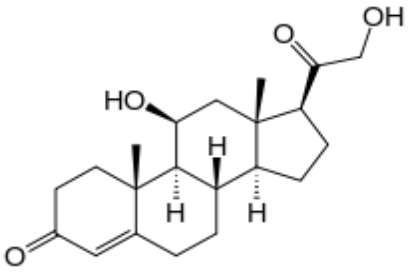


Fig. 5.17 Structure of corticosterone

Standard curve: 1 % w/v of corticosterone was prepared in methanol and the particular stock was diluted to prepare the analytical standards. 5 % v/v of each analytical standard was added in the blank plasma, to prepare the calibration standard concentrations in the range of 10-200 ng/ml (10, 20, 40, 80, 100 and 200 ng/ml), which were then processed by the following procedure. The calibration curve was prepared by plotting the mean fluorescent intensity (FI) obtained from each standard against its concentration, with FI value on the vertical (Y) axis and concentrations on the horizontal (X) axis method was validated and utilized for the estimation of CORT in all samples.

Procedure: Corticosterone assay was performed as per method described by Katyare and Pandya (2005) with slight modifications. Plasma (0.5 ml) was treated with 0.1 ml of freshly prepared chloroform: methanol mixture (2:1 v/v), followed by extraction with 1.5 ml of chloroform. The chloroform extract was treated with 0.15 ml of sodium hydroxide (0.1N) and then with 1.5 ml of 30 N sulfuric acid. The tubes containing the sulfuric acid layer were kept in the dark for 30–60 min and thereafter fluorescence measurements were carried out in an SL-174-spectrofluorometer with excitation and emission wavelengths set at 472 and 533 nm, respectively. The plasma CORT contents were measured and expressed as ng/ml of plasma.

5.11 Estimation of neurotransmitters

5.11.1 Serotonin estimation

Rationale: *o*-Phthalaldehyde (OPT) has been shown to produce colored or fluorescent product, with the indole-amines in acidic media. The 5-HT contents are initially extracted by liquid-liquid extraction method, using heptane and acidified aqueous media (containing cysteine as stabilizer).The extracted 5-HT is reacted with OPT to form the fluorescent adduct.

Preparation of the reagents: *n*-Butanol was acidified by adding 85 µl concentrated HCl in 100 ml of *n*-butanol. 0.004 % w/v OPT in 10N hydrochloric acid (HCl), 0.1 % w/v L-cysteine in 0.1N HCl and standard 5-HT in millipore water were prepared immediately before use.

Standard curve: A primary stock (100 µg/ml) was prepared by dissolving 1 mg of standard 5-HT in 10 ml of acid butanol, which was further diluted and added in blank brain tissue homogenates to prepare the bioanalytical standards. For calibration, curve of 10-100 ng/ml range was prepared. The standards were processed as per method and 5-HT was estimated and expressed as ng/ml. The calibration curve was prepared by plotting mean fluorescent intensity (FI) obtained from each standard against its concentration with FI value on the vertical (Y) axis and concentrations on horizontal (X) axis.

Procedure: Concentrations of 5-HT in different regions of brain like midbrain (along with hippocampus), frontal cortex and cerebellum were measured by spectrophotofluorometric method. 1 ml of cold acidified *n*-butanol was added to the tissue homogenates (0.1 ml). The samples were centrifuged at 3000 rpm for 20 min at 4° C. 500 µl of the supernatant was mixed with 1 ml of *n*-heptane and 80 µl of 0.1N HCl (containing 0.1 % L-cysteine). The resultant mixture was mechanically shaken for 5 min and the phases were separated by centrifugation (3000 rpm, 4° C for 20 min). The 0.1 ml samples of the aqueous phase were pipetted into another centrifuge tubes and 0.6 ml of 0.004 % w/v *o*-phthalaldehyde was added. The tubes were shaken and heated over boiling water bath for 15 min. The tubes were cooled and fluorescence was measured in micro-cuvettes. Activation and fluorescent wavelengths used were 360 and 465 nm, respectively.

The 5-HT levels were then determined using calibration curves of respective brain regions and expressed as the percentage with respect to control group (taking control group values as 100 %) (Curzon and Green, 1970).

5.11.3 Estimation of GABA

Rationale: GABA is an amino acid, which gives a colored adduct, when reacted with the reagent, ninhydrin. The reaction can be written as:

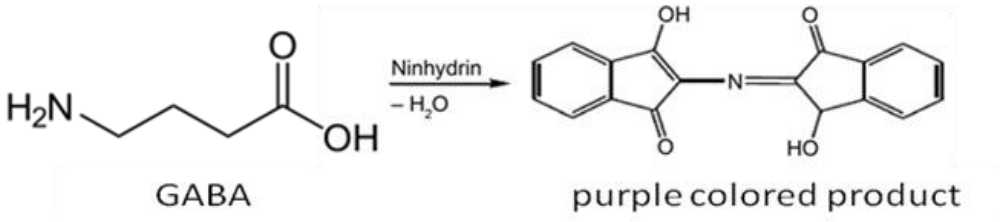


Fig. 5.18 The reaction of ninhydrin with GABA

Standard curve: Primary stock was prepared by dissolving 1 mg of GABA in 10 ml of 10 % w/v trichloroacetic acid to get a final concentration of 100 µg/ml. It was diluted with the trichloroacetic acid to get the concentration range of 50-5000 ng/ml. 5 % of each concentration point was then incorporated in the blank tissue homogenate to get the bioanalytical calibration concentration points in the range of 10-1000 ng/ml (10, 50, 100, 200, 400, 600, 800, 1000 ng/ml). The standards were then processed and GABA content estimated.

The calibration curve was prepared by plotting, the mean fluorescent intensity (FI) obtained from each standard, against its concentration with FI value on the vertical (Y) axis and concentrations on the horizontal (X) axis.

Procedure: 0.1 ml of the tissue homogenate treated with 0.5 ml of the 10 % w/v trichloroacetic acid and vortexed for few minutes. The sample was allowed to stand for stabilization and then 0.5 ml of the supernatant was taken out. It was reacted with ninhydrin reagent (1 ml of 14 mM).

The reaction mixture was heated at 60 °C for 30 min and allowed to cool. The copper tart rate was added (2 ml) to the reaction mixture and incubated at room temperature for 15 minutes. The GABA content was estimated at e_{max} excitation 377 and e_{max} emission 451 nm. The amount of GABA in the discrete brain regions were noted as ng/ml.

5.12 Estimation of neurotrophic factor (BDNF) & cAMP

BDNF and cAMP levels in hippocampus and frontal cortex were estimated, by commercially available ELISA kit (Boster Biological Technology Co., Ltd, USA) according to manufacturer's instructions and expressed as pmoles/ mg protein.

5.13 Protein estimation

The protein content (mg per g of wet tissue) was measured in all tissue samples using commercially available kits, which are based on the method of Lowry et al. (1951).

5.14 Neuro-anatomical studies

5.14.1 Hematoxylin-eosin staining and histochemical quantification

The brain samples were divided into two hemispheres and one hemispheres from each brain sample were fixed in neutral buffered formalin (consisting of 10 % v/v formaldehyde solution in phosphate buffer pH 7.4) for 3 days and embedded in paraffin to form the tissue-paraffin blocks. Multiple 5 µm sections, 200 mm apart from bregma -1.86 to -2.25 were sectioned in the coronal plane with a microtome from the brain (care was taken to carry out uniform sectioning of the brain among different groups).

Sections were stained using hematoxylin-eosin staining and observed using an optical microscope, [(Optika 4083. B5) connected to a digital camera, Optikam-B5 managed by OptikaView 7 Software (Optika Microscopes, Italy)] at ×40 for the quantitative evaluation of the normal and degenerated cells in CA_3 and dentate gyrus (DG) regions of hippocampus (Fig. 5.19) using ImageJ software (US National Institutes of Health, Bethesda, USA). Briefly, using this software, images were separated into individual color channels (hematoxylin counter stain and eosin chromagen), using color deconvolution algorithm (for plugin: http://fiji.sc/Colour_Deconvolution). The two non-overlapping areas (50 μm^2), each from CA_3 and DG regions of hippocampus (from the left half of the brain) were randomly selected and total number of normal and degenerated cells (showing shrunken cell bodies, triangulated and pyknotic nuclei) were counted using cell counter plugin (for plugin: http://fiji.sc/Cell_Counter) of ImageJ software and averaged for two areas (Li, 2012). The degree of neurodegeneration was then expressed as percent degenerated neuronal cells in CA_3 and DG regions of the hippocampus.

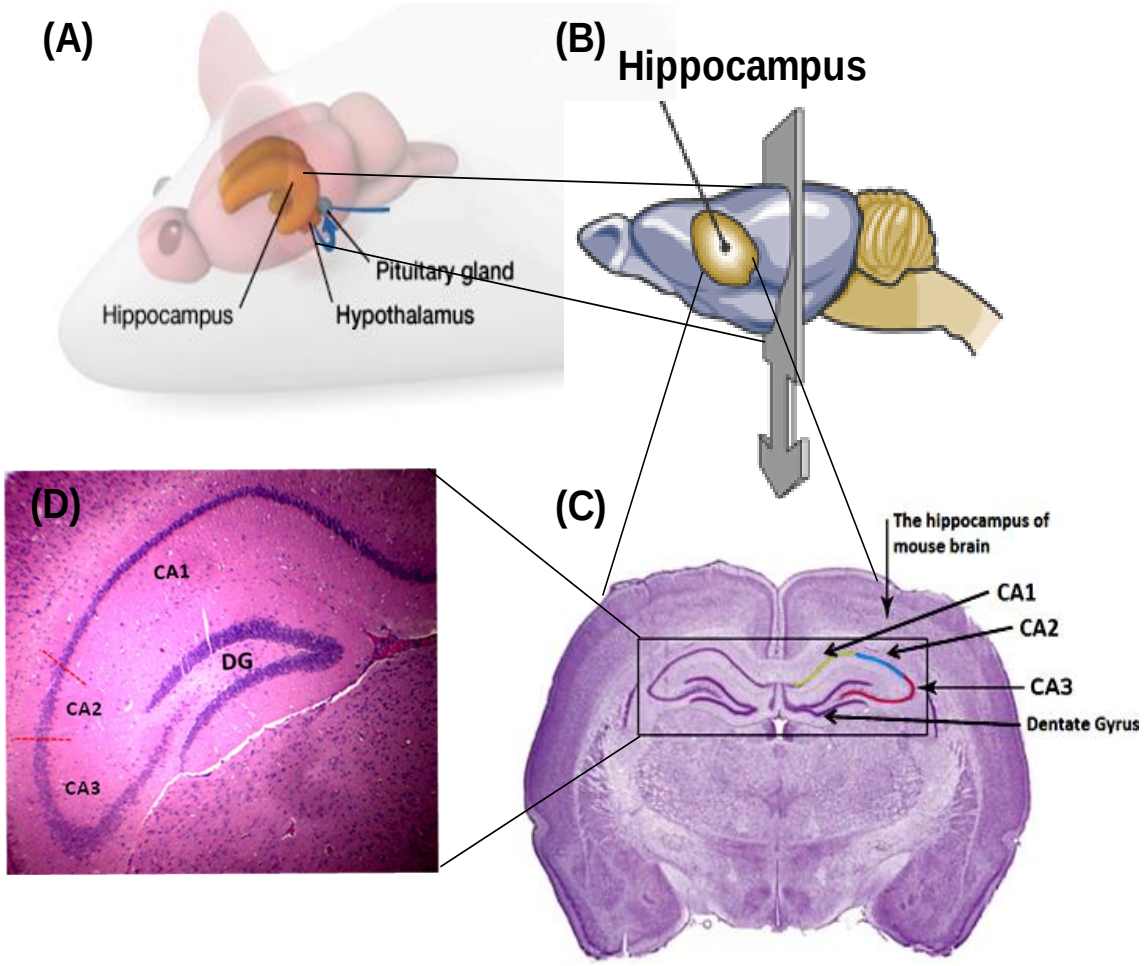


Fig. 5.19 The schematic representation (A) showing the location of hippocampus in mouse brain, (B) the axis of sections taken, (C) the hippocampal regions namely, CA_1 (green colored), CA_2 (blue colored), CA_3 (pink colored) and dentate gyrus (DG, purple colored) and (D) the H&E stained view of a single hemisphere of hippocampus and its different regions.

5.14.2 Golgi-Cox staining

Rest half of the hemispheres were submerged in Golgi-Cox solution for 14 days in dark and embedded in paraffin to form the tissue-paraffin blocks. 50 µm coronal sections were sliced and collected onto gelatin-coated glass slides. The stain was developed in ammonia (3:1 in distilled water) solution for 10 min. Finally sections were rinsed with distilled water, dehydrated and mounted with DPX (Distrene, Plasticiser, and Xylene). Golgi-impregnated pyramidal neurons in CA_3 subfield of hippocampus were studied. Only neurons that were fully impregnated, not obscured by neighboring neurons, and had no obviously truncated dendrites were chosen for analysis.

Morphological characteristics were analyzed using ImageJ software. Briefly, neurons were digitally reconstructed using the NeuronJ plugin from the open source software (Meijering et al., 2004). Cumulative branch lengths for primary, secondary and tertiary dendrites were recorded. Dendrites that extend from the soma are defined as primary dendrites, and those that emanate from primary dendrites are secondary dendrites. Dendrites that emanate from secondary dendrites are tertiary dendrites.

To more closely examine the extent of dendritic branching, Sholl analyses (Ghosh LabWebsite, http://biology.ucsd.edu/labs/ghosh/software/ShollAnalysis.class; Kutzing et al., 2010) was performed, in which a grid of concentric rings spaced 10 µm apart was overlaid onto the neuronal image centered on the soma and the number of ring intersections with dendrites was computed for each neuron as a branching index (Fig. 5.20).

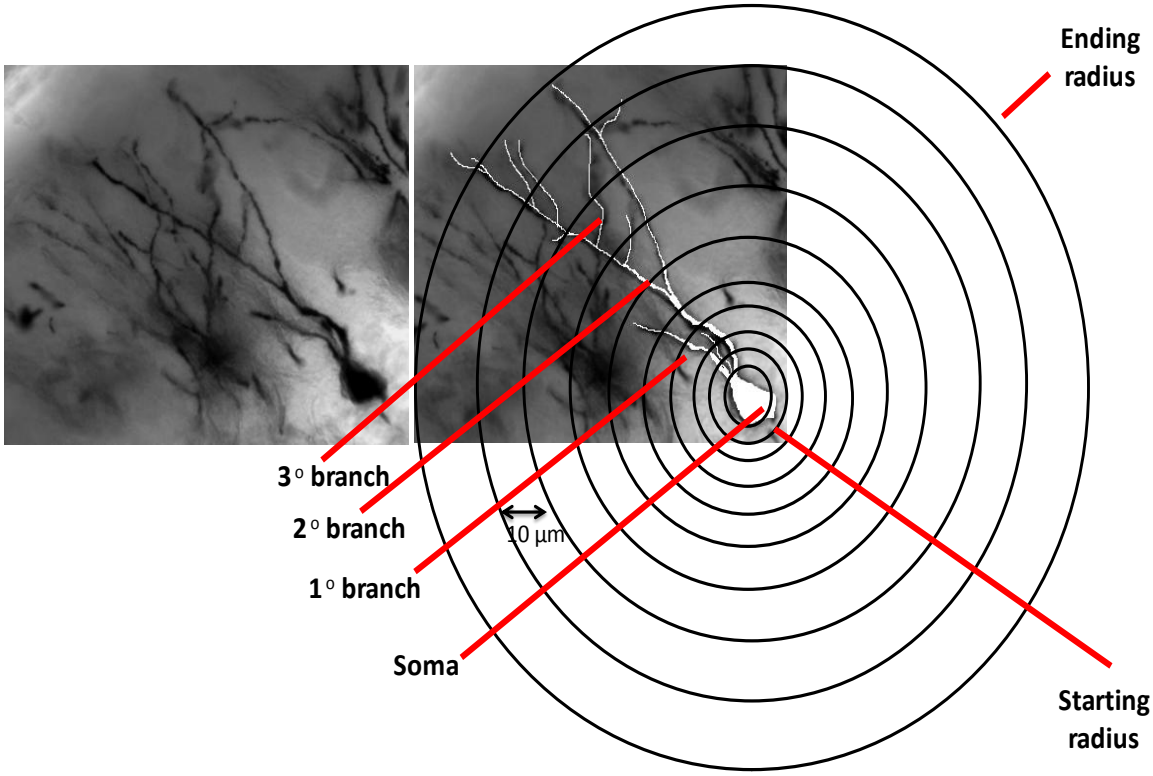


Fig. 5.20 The histological representation showing the neuronal structure, its branching and Sholl counters of neurons in pyramidal cell layer of CA_3 region of the hippocampus.

5.15 Statistical analysis

Data of the present study were analyzed by GraphPad Prism software (version 5.0), USA. All values were expressed as mean ± standard error of mean (SEM). The data that is normally distributed from a single study (having only two groups) were analyzed using 'Student t-test' and for more than two groups were analyzed by one-way-ANOVA followed by Tukey's Multiple comparison test (for FST and TST).

Data from the anxiety test (open field test and social interaction test) were estimated by Kruskal–Wallis test on ranks, followed by post hoc 'Dunn's Multiple Comparison test' to compare groups. In diabetic studies, data normally distributed in the study (for example in FST, TST sucrose preference test and in other biochemical assays) were estimated using two-way ANOVA (drug × diseased (diabetes) condition, as two variables) followed by post hoc Bonferroni's test.

Data obtained from anxiolytic assays such as open field test and social interaction test were non-parametric and hence analyzed by Friedman's two-way ANOVA test for ranks. In all cases, significance of the study was judged $p < 0.05$ (and above).

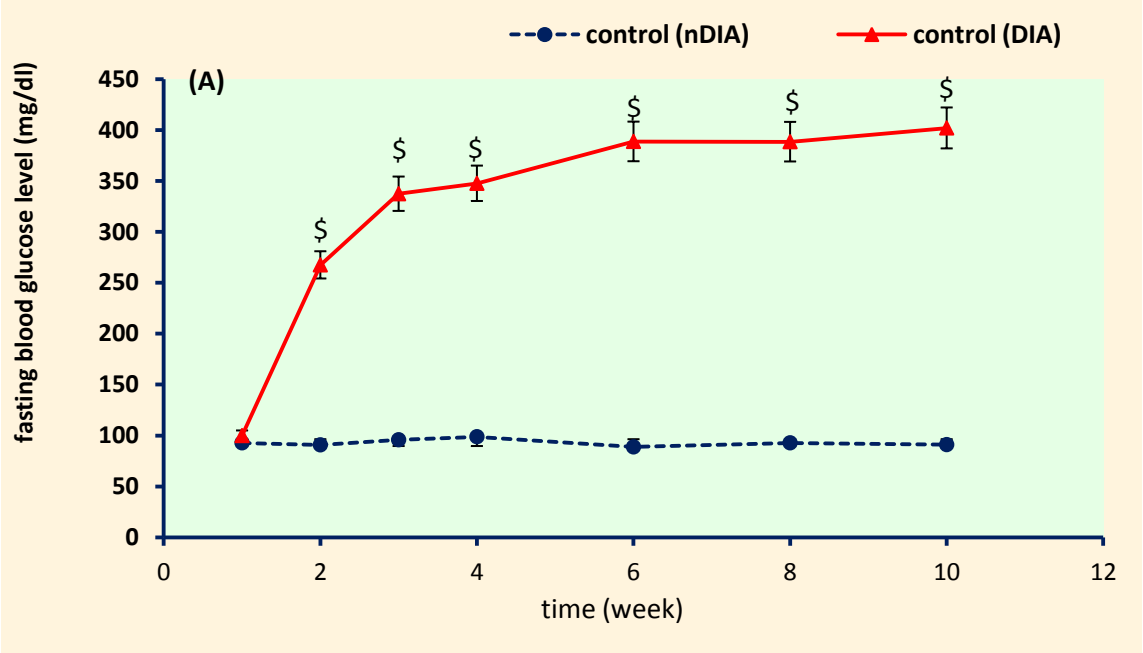
6.1. Development of STZ-induced diabetic mouse model

6.1.1 Fasting blood glucose level, plasma insulin level & body weight changes in diabetic mice

Mice treated with a intraperitoneal injection of STZ (200 mg/kg, i.p.), exhibited significantly elevated fasting blood glucose levels [student t-test, $p < 0.001$], after 3 days of STZ injection and this effect was consistent even after 8-weeks of persistent diabetes, as shown in Fig. 6.1A.

In addition, STZ-induced diabetic mice exhibited a significant decrease in the plasma insulin level as compared to control [student t-test, $p < 0.001$] (Fig. 6.1B), which is the core feature of insulin-dependent diabetes. The reduction in plasma insulin level was found after 3 days of STZ dosing and was consistent with the time course of 8 weeks of diabetes.

It was also found that in the STZ-induced diabetic mice, the body weights were significantly reduced as compared to non-diabetic mice [student t-test, $p < 0.001$] as depicted in Fig. 6.1C. However, a statistically significant decline in body weights in STZ-induced diabetic mice was found only after 8 weeks of diabetes, although a trend of decrease in body weight was observed, consistently after 2-weeks of persistent diabetic condition.



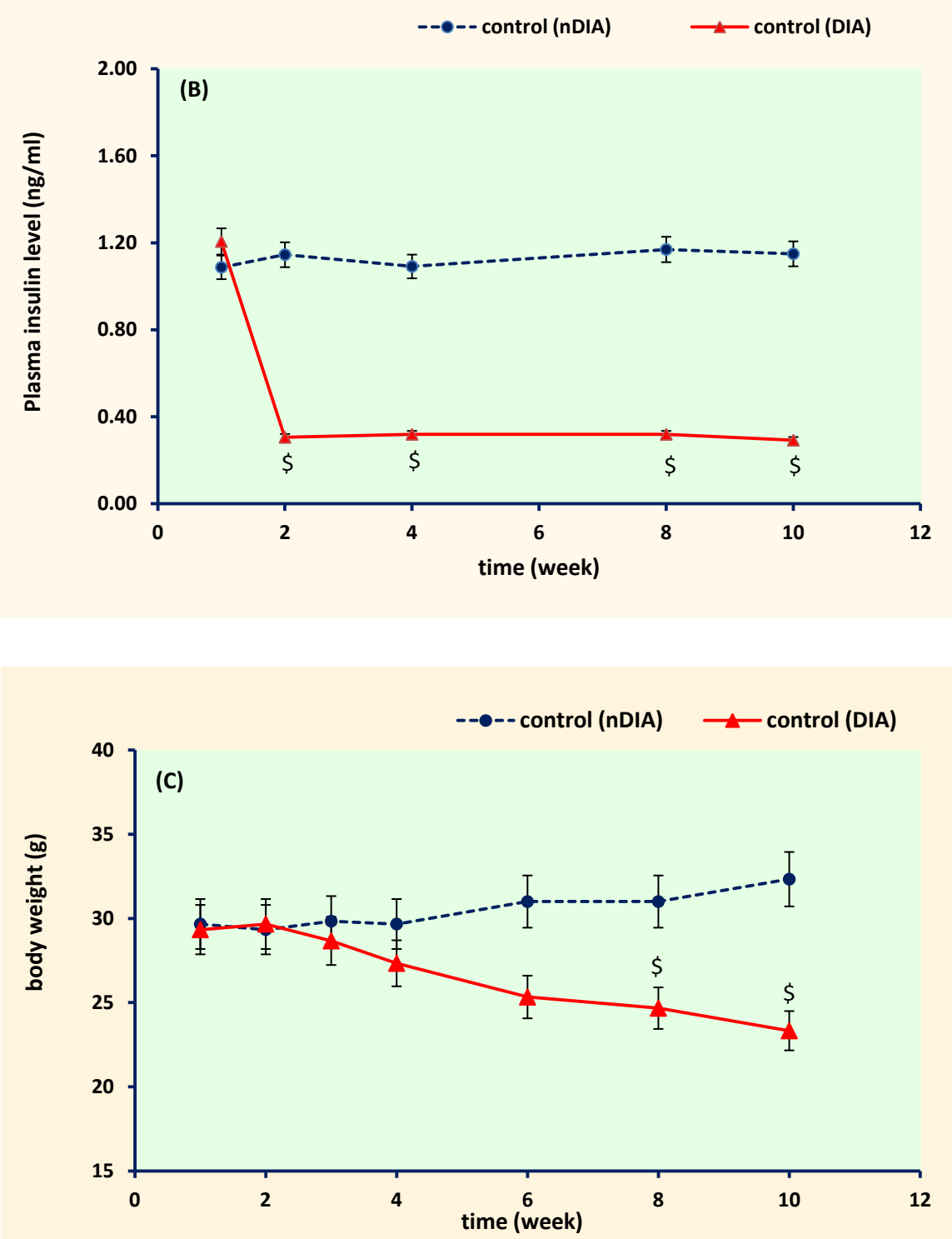
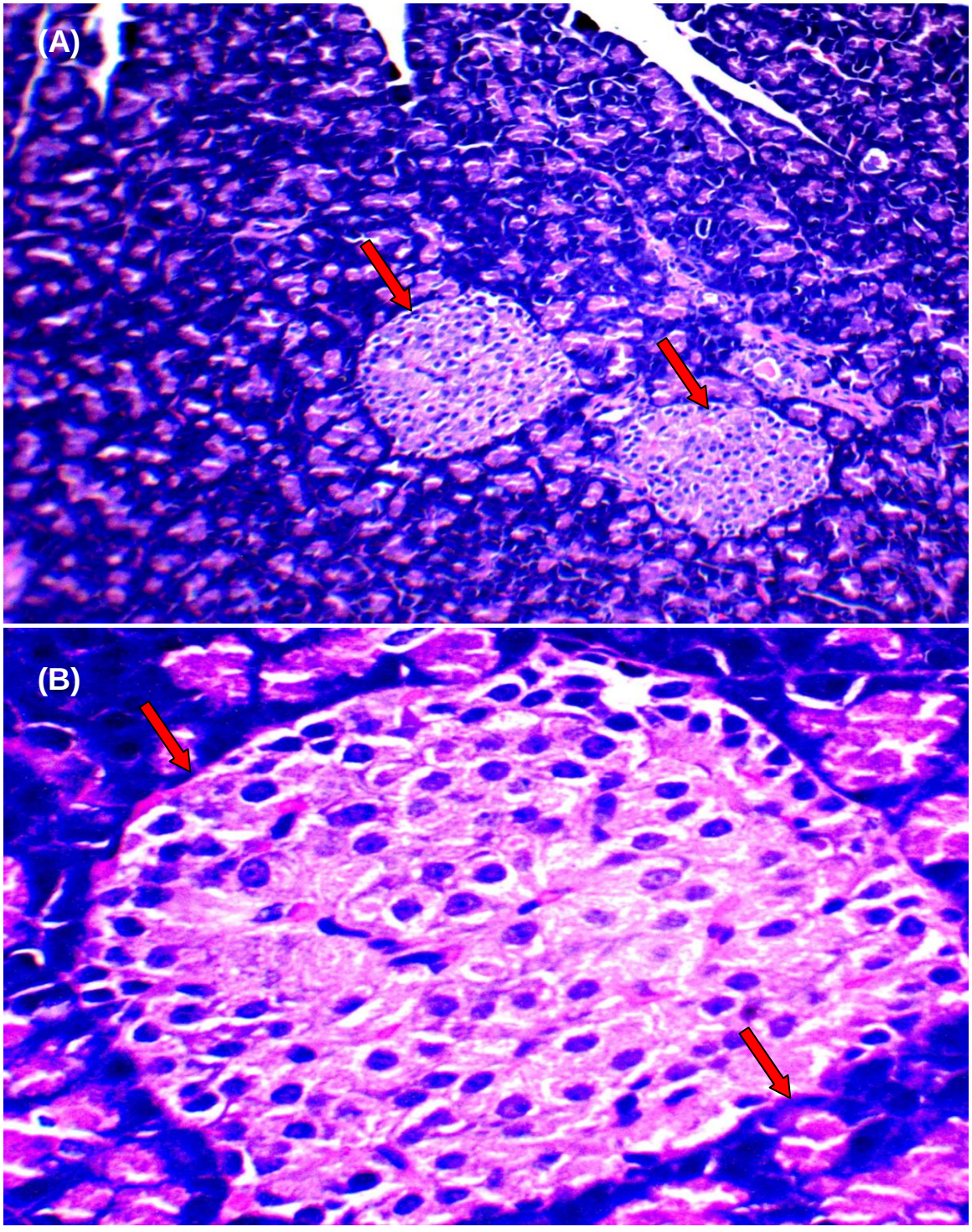


Fig. 6.1 The effect of STZ-induced diabetes on **(A)** fasting blood glucose levels and **(B)** plasma insulin level and **(C)** body weight changes. The marker represents mean values at each time point and error bars indicate S.E.M. **$** $p < 0.001$ compared with control group, n = 6/group.

6.1.2 Pancreatic damage in STZ-induced diabetic mice

The pancreatic damage was observed in STZ-induced diabetic mice after 3 days of injection of STZ, which can be seen in photomicrographs of the pancreatic cells of STZ-induced diabetic mice as compared to control.



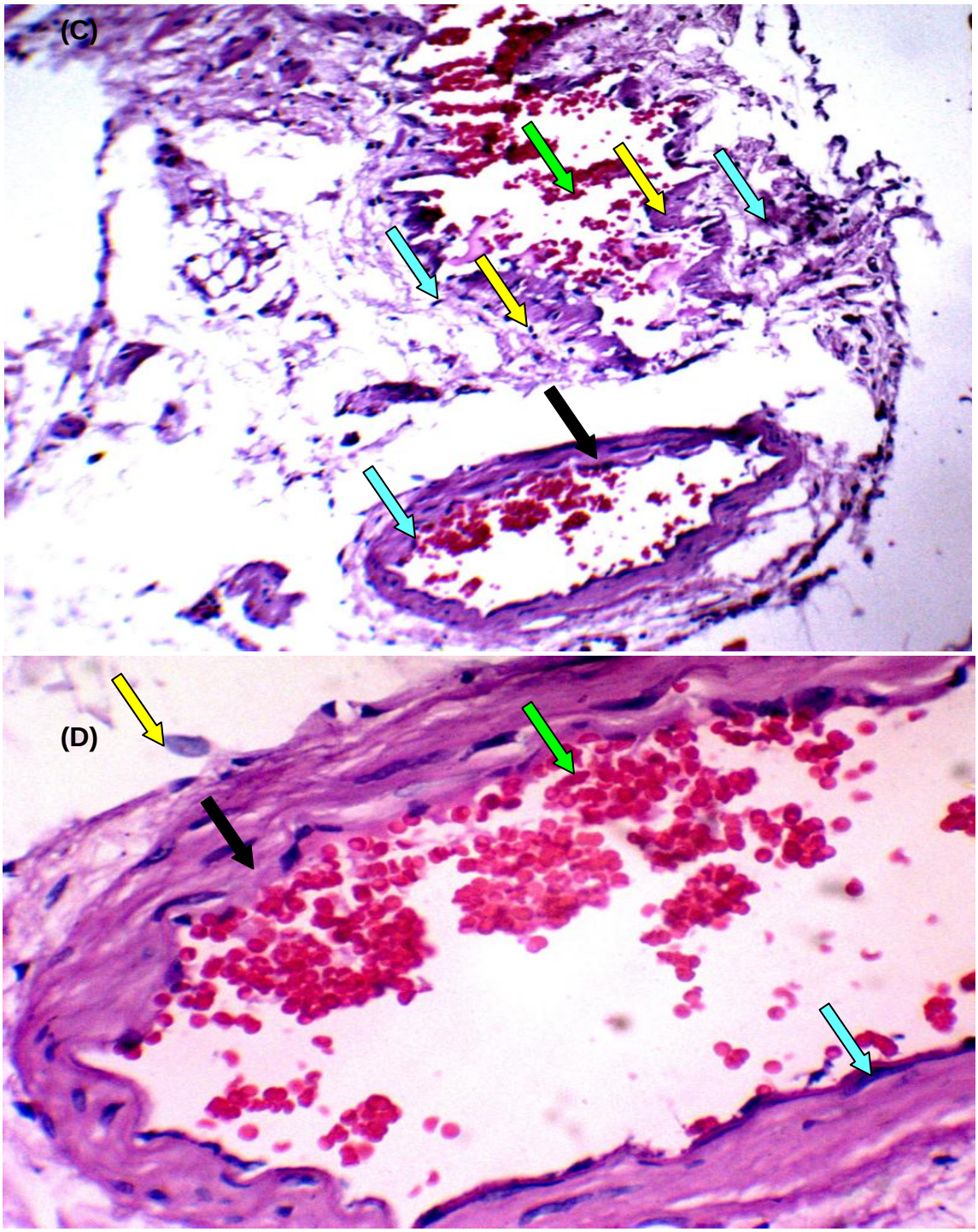


Fig. 6.2 Control (10x) **(A)** and 40x **(B)**, the histological appearance is normal with islets of Langerhans scattered in between acini of well-preserved cytoplasm, and nucleus normal interlobular connective tissue septa (red arrow). Diabetic control 10x **(C)** and 40x **(D)**, the histological appearance of pancreas show congestion (green arrow), inflammation (blue arrow), and tubular desquamation (yellow arrows) and damaged endothelium lining (black arrow). Quantitatively, nearly 62-80 % of β-cell damage was observed in diabetic mice.

6.2 Time course of depression in STZ-induced diabetic mouse model

There was a non-significant trend of developing depression and anxiety-like behavior in diabetic mice, with respect to time. However, after 8-weeks of STZ-induced diabetes, mice exhibited a significant increase in duration of immobility (s) [student t-test, $p < 0.001$] during FST and TST models of depression, as depicted in Fig. 6.3A and B.

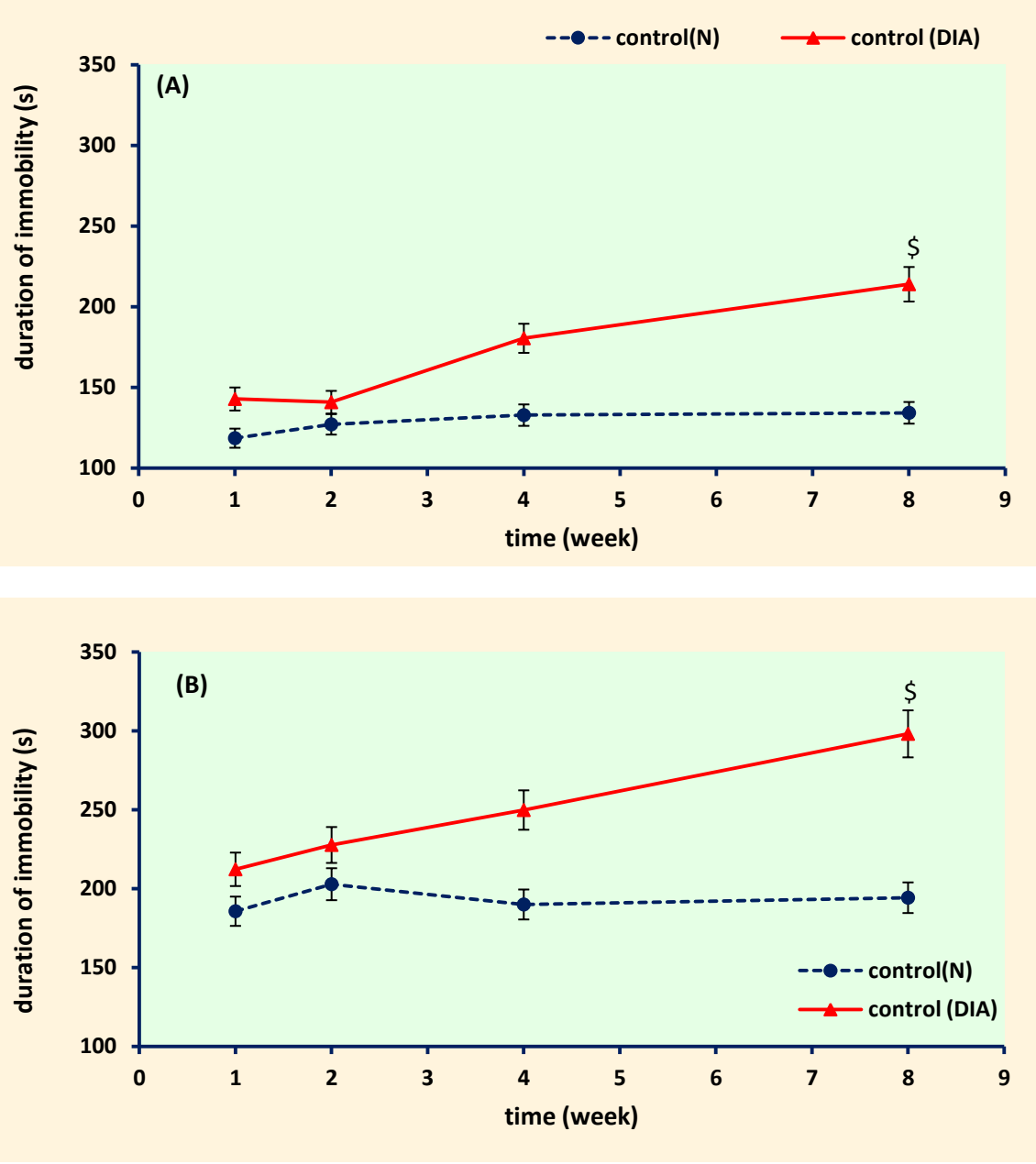


Fig. 6.3 Effect of STZ-induced diabetes on duration of immobility (s) in **(A)** FST and **(B)** TST. The marker represent mean values at each time point and error bars indicate S.E.M. **$** $p < 0.001$ compared with control group, n = 7/group.

6.3 Time course of anxiety-like behavior in STZ-induced diabetic mouse model

STZ-induced diabetic mice exhibited significant changes in anxiety parameters as observed in neurobehavioral models of anxiety, consistently after 8-weeks of persistent diabetes.

In hole-board test, STZ-induced diabetic mice exhibited a significant decrease in the number of head dips [student t-test, $p < 0.05$], after 4-weeks of diabetes, which was consistent at 8-weeks of diabetic state [student t-test, $p < 0.001$]. However, the duration of head dips was statistically reduced [student t-test, $p < 0.001$] only after 8-weeks of diabetes, as depicted in Table 6.1.

In light-dark test, diabetic mice exhibited a statistically significant reduction in latency (time required to initially enter into dark chamber) after 2-week of diabetic state [student t-test, $p < 0.05$] and was consistent even after 8-weeks of diabetes [student t-test, $p < 0.001$]. There was a non-significant trend of decrease in the time spent in light chamber in diabetic mice, initially, after 2-week of diabetes, but the statistical significance was observed only after 8-week of diabetic state [student t-test, $p < 0.001$] as compared with control group, as indicated in Table 6.1.

During open field test, STZ-induced diabetic mice exhibited a significant reduction in number of crossings and rearings, only after 8-weeks of persistent diabetic state [student t-test, $p < 0.001$], when compared with control group, as depicted in Table 6.1.

In EPM, STZ-induced diabetic mice demonstrated a significant decline in the percentage of entries made [student t-test, $p < 0.05$] and time spent in open arms [student t-test, $p < 0.001$] as compared to control group, only after 8-weeks of persistent diabetic condition, as represented in Table 6.1.

Table 6.1 The effect of STZ-induced diabetes on anxiety behavior, in mice

Control (nDIA)										
Week	hole-board test		light-dark test		social interaction test		open field test		EPM	
	No. of head dips	Duration of head dips (s)	Latency (s)	Time spent in light chamber (s)	No. of SI	Time spent in SI (s)	No. of crossing	No. of rearing	% open arm entries	% open arm time
1	25.71 ± 2.39	39.09 ± 4.87	46.14 ± 6.08	86.13 ± 5.09	28.86 ± 2.56	113.60 ± 12.99	121.71 ± 10.78	27.29 ± 4.17	30.15 ± 4.59	20.10 ± 2.42
2	24.43 ± 2.00	43.78 ± 4.75	42.75 ± 6.59	90.19 ± 19.49	26.86 ± 2.06	154.48 ± 10.26	139.14 ± 13.37	23.00 ± 3.31	35.10 ± 2.42	20.86 ± 2.25
4	29.71 ± 2.02	35.80 ± 3.91	58.97 ± 6.86	109.80 ± 17.69	27.57 ± 2.58	147.79 ± 15.00	149.71 ± 7.69	22.43 ± 1.70	36.47 ± 4.86	17.90 ± 2.37
8	29.57 ± 2.68	32.59 ± 2.37	45.63 ± 9.01	106.30 ± 12.85	32.29 ± 3.37	140.31 ± 14.47	137.00 ± 11.33	25.14 ± 3.31	41.29 ± 7.27	22.24 ± 3.17
Diabetic control (DIA)										
Week	hole-board test		light-dark test		social interaction test		open field test		EPM	
	No. of head dips	Duration of head dips (s)	Latency (s)	Time spent in light chamber (s)	No. of SI	Time spent in SI (s)	No. of crossing	No. of rearing	% open arm entries	% open arm time
1	21.00 ± 2.79	41.30 ± 4.75	54.11 ± 10.19	73.50 ± 10.41	31.00 ± 4.21	109.01 ± 18.26	116.57 ± 9.45	22.14 ± 2.51	25.94 ± 4.47	18.10 ± 3.59
2	20.00 ± 4.30	30.96 ± 4.79	20.00 ± 4.30 *	80.26 ± 20.54	20.43 ± 3.49	118.02 ± 20.33	103.29 ± 23.63	23.43 ± 3.81	24.50 ± 4.27	14.52 ± 3.92
4	16.71 ± 3.35 *	23.51 ± 4.28	28.36 ± 7.96 *	83.69 ± 20.29	19.86 ± 6.13 $	102.46 ± 26.38	117.71 ± 18.21	19.43 ± 2.97	22.84 ± 5.34	14.00 ± 3.92
8	8.43 ± 1.88 $	14.67 ± 2.06 $	12.44 ± 2.25 $	42.11 ± 16.21 $	13.29 ± 3.05 $	54.09 ± 22.22 $	42.86 ± 9.49 $	7.43 ± 1.17 $	19.51 ± 5.24 $	10.43 ± 2.10 $

Values represent mean ± S.E.M. * $p < 0.05$, $ $p < 0.001$, when compared with control, n = 7/group.

6.4 Antidepressant-like effects of standard and novel 5-HT_3 receptor antagonists in animal models of depression

6.4.1 Antidepressant-like effect of OND in acute and chronic models of depression

6.4.1. Effect of OND in acute models of depression

6.4.1.1.1 Effect of OND on basal locomotor activity (BLA)

In BLA test, OND (0.25, 0.5, 1 and 2 mg/kg, i.p.) treatment showed significant changes [$F(4, 25) = 2.023$, $p < 0.05$] in locomotor activity of mice. OND, 0.5 and 1 mg/kg had no significant changes in BLA ($p > 0.05$). However, the lowest dose of 0.25 mg/kg and highest dose of OND treatment (2 mg/kg, i.p.) exhibited a significant decrease in BLA, in mice ($p < 0.05$), when compared with those received vehicle only, Fig. 6.4.

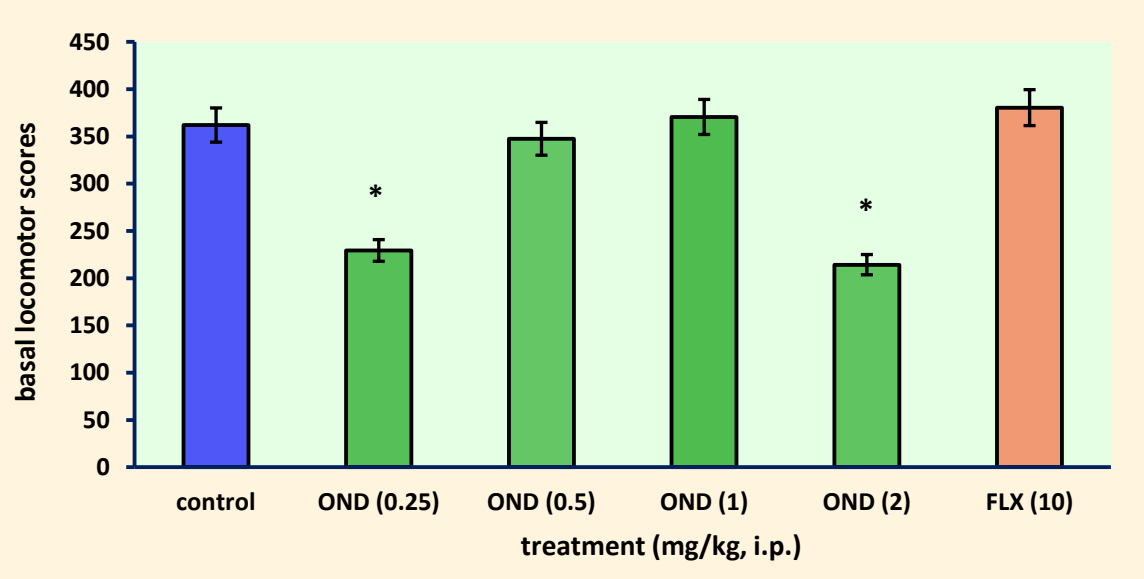


Fig. 6.4 Effect of OND and FLX on the basal locomotor activity, in mice, the columns represent mean basal locomotor scores and error bars indicate S.E.M. ***** $p < 0.05$, significant vs control group, n = 6/group.

6.4.1.1.2 Effect of OND on duration of immobility in FST and TST:

OND (0.5 and 1 mg/kg, i.p.) treatment exhibited a significant decrease in duration of immobility (s), in mice, during FST [$F(3, 20) = 6.593$, $p < 0.001$] and TST [$F(3, 20) = 11.16$, $p < 0.001$].

OND (0.5 mg/kg, i.p.) ($p < 0.05$) and (1 mg/kg, i.p.) ($p < 0.001$) dose dependently reduced duration of immobility, in mice. Similarly, FLX (10 mg/kg, i.p.) reduced the duration of immobility, in mice, when compared with vehicle treated control group ($p < 0.001$), as indicated in Fig. 6.5A and B.

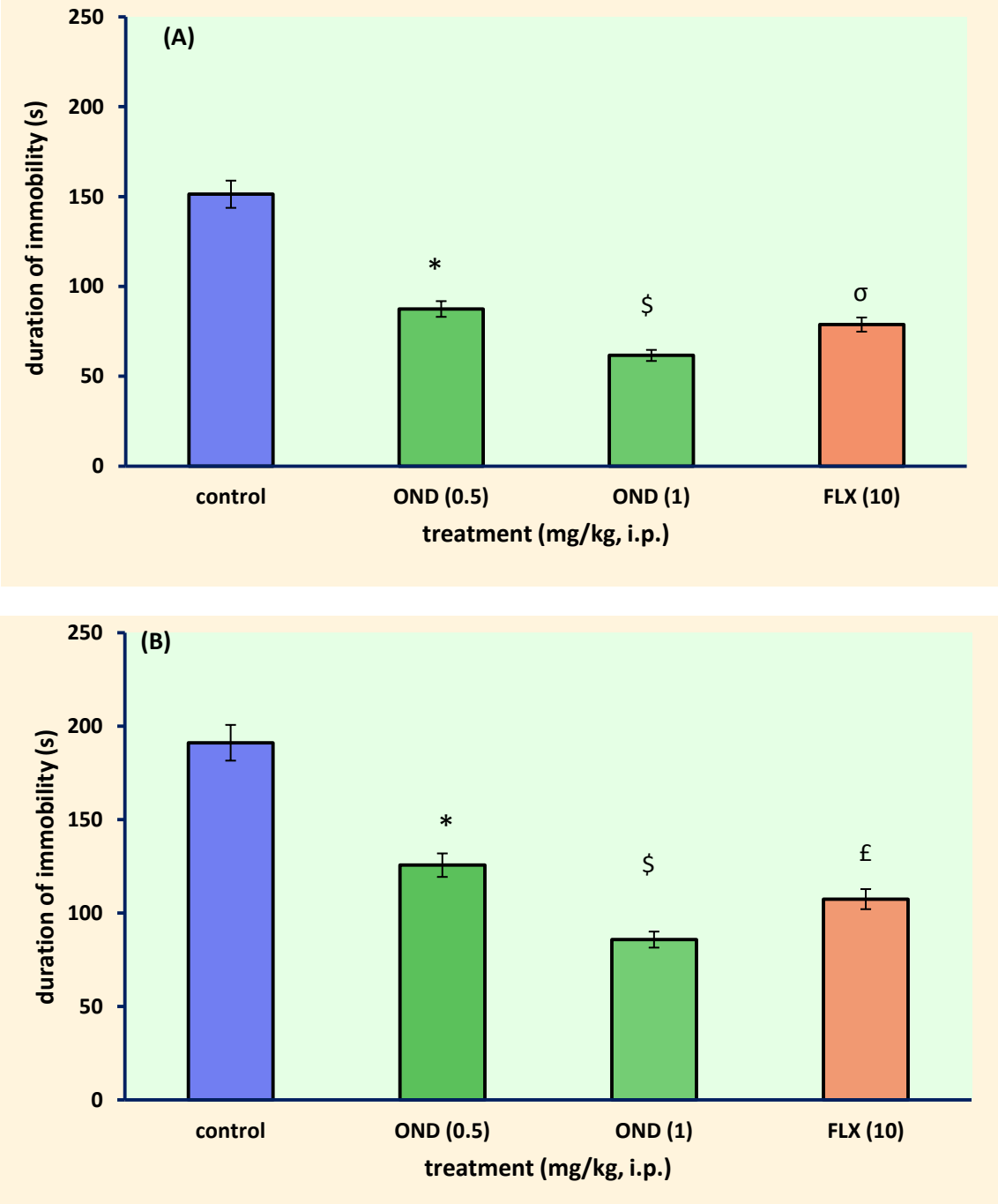


Fig. 6.5 Effect of OND and FLX on the duration of immobility, in mice. The columns represent mean duration of immobility (s) during **(A)** FST and **(B)** TST, and error bars indicate S.E.M. *$p < 0.05$, **$** $p < 0.001$, σ $p < 0.05$, £ $p < 0.001$ significant vs control group, n = 6/group

6.4.1.1.3 Effect of OND on exploratory behavior in hole-board test and light-dark test:

Statistical analysis revealed that, OND (0.5 and 1 mg/kg, i.p.), significantly altered behavioral parameters- namely number of head dips [F (3, 20) = 19.01, p < 0.001] and duration of head dips [F (3, 20) = 6.99, p < 0.01] as well as latency [F (3, 20) = 4.548, p < 0.05] and time spent in light chamber [F (3, 20) = 3.454, p < 0.05], in mice, subjected to hole-board test and light-dark test, respectively.

In hole-board test, OND (0.5 and 1 mg/kg, i.p.) treatment significantly increased the number and duration of head dips, in mice, vs control group. Similarly, diazepam produced a significant increase in the number and duration of head dips, in mice, as compared to control group, Table 6.2.Similarly, in light-dark test, acute OND (0.5 and 1 mg/kg, i.p.) treatment significantly increased the latency (s) and time spent in light chamber (s), in mice, as compared to the mice that received vehicle only. Similarly, treatment with diazepam (the standard drug), significantly increased the latency and time spent in light chamber, in mice, Table 6.2.

Table 6.2 The effect of OND in hole-board test and light-dark test

Treatment (dose, mg/kg, i.p.)	hole-board test		Light-dark test	
	number of head dips	duration of head dips (s)	latency (s)	time spent in light chamber (s)
Control	15.83 ± 0.91	22.61 ± 2.26	26.69 ± 3.90	57.73 ± 8.51
OND (0.5)	20 ± 2.21 *	34.86 ± 4.18 *	45.03 ± 2.83 $	96.65 ± 19.25 *
OND (1)	33 ± 1.65 $	45.49 ± 3.89 $	44.12 ± 3.99 $	88.82 ± 12.68 *
DZM (1)	32.16 ± 2.70 £	40.95 ± 4.32 £	46.66 ± 6.14 £	107.41 ± 15.79 £

Values represent mean ± S.E.M. * p < 0.05, $ p < 0.01, £ p < 0.01 vs control, n = 6/group.

6.4.1.2 Effect of OND on chronic model of depression: CUS model

In this model, mice were subjected to chronic stress and subsequent chronic treatment with either drug or vehicle for 14 days, after which the changes in behavior were predicted using FST and TST models of depression and open field test as a model of anxiety. In FST, there was a significant change in duration of immobility (s), in mice subjected to different treatments [F (4, 30) = 5.746, p < 0.001].

CUS mice exhibited a significant increase in duration of immobility (s) as compared to non-CUS mice ($p < 0.001$). Chronic OND treatment (0.5 and 1 mg/kg, i.p.) significantly reversed CUS induced increase in duration of immobility, in mice ($p < 0.001$). Similarly, FLX (10 mg/kg, i.p.) decreased duration of immobility, in mice subjected to chronic stress ($p < 0.001$), as represented in Fig. 6.6.

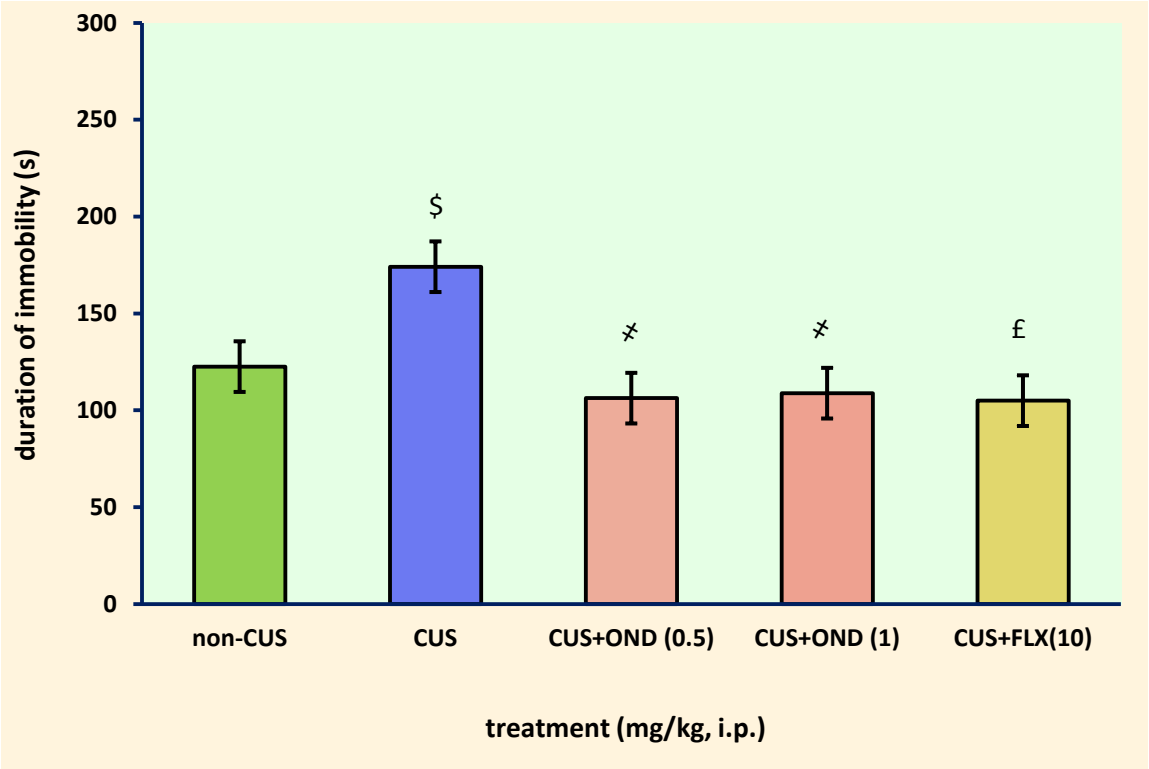


Fig. 6.6 The columns indicate mean duration of immobility (s) during FST and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to non-CUS control group. ≠ $p < 0.001$. £ $p < 0.001$ indicate significant difference vs CUS control group, n = 6/group.

In TST, there was a significant change in duration of immobility, in mice subjected to different treatments [$F (4, 30) = 5.973$, $p < 0.001$]. A significant reduction in duration of immobility (s) was observed in CUS mice ($p < 0.001$) as compared to non-CUS control group. OND (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) chronic treatment, significantly reversed the increase in duration of immobility, in mice exposed to chronic stress ($p < 0.001$), as depicted in Fig. 6.7.

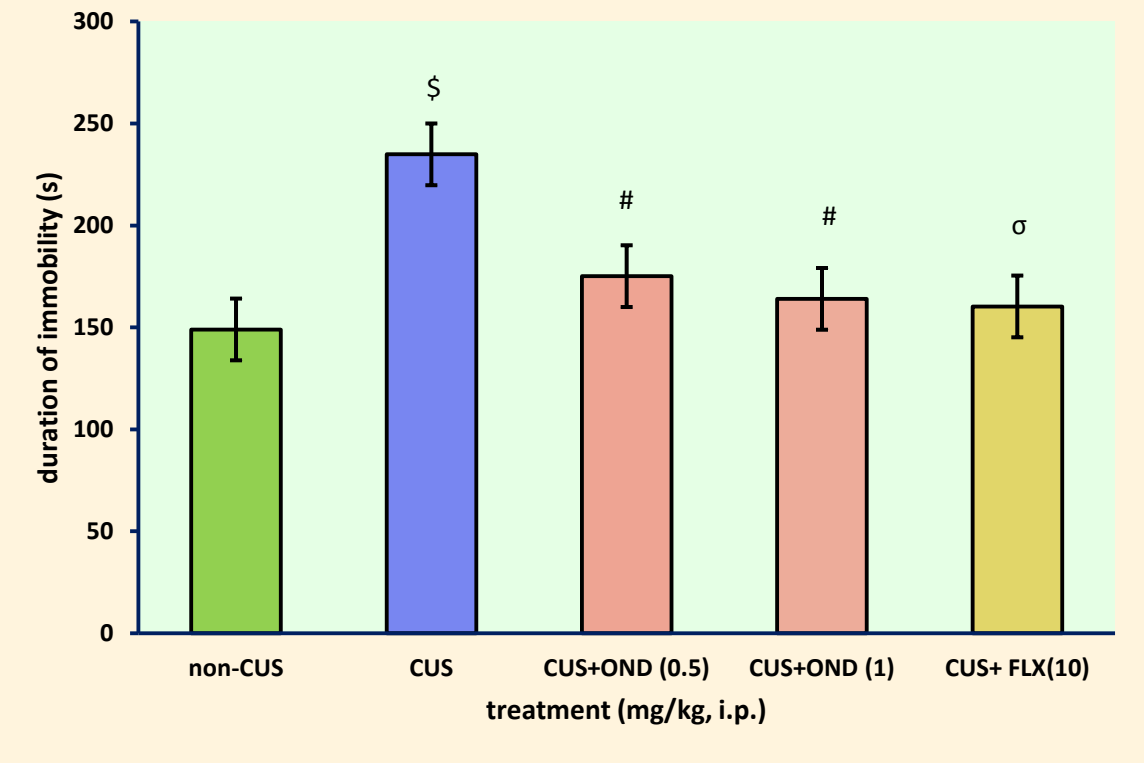


Fig. 6.7 The columns indicate mean duration of immobility (s) during TST, and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to non-CUS control group. # $p < 0.05$, σ $p < 0.05$ indicate significant difference vs CUS control group, n = 6/group.

During sucrose preference test, there was a significant alteration in percentage of sucrose preference among the groups [$F (4, 30) = 8.318$, $p < 0.001$]. CUS mice exhibited a significant decline in percentage of sucrose consumption over normal drinking water ($p < 0.001$) as compared to non-CUS control mice. Chronic treatment with OND (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.), significantly reversed the reduction in percentage of sucrose consumption in CUS mice ($p < 0.001$), as indicated in Fig. 6.8.

In open field test, a significant difference in number of crossings [$F (4, 30) = 7.695$, $p < 0.001$] and rearings [$F (4, 30) = 10.19$, $p < 0.001$] was observed, among the groups. CUS mice exhibited a significant decrease in number of crossings and rearings ($p < 0.001$) as compared to non-CUS control. Chronic treatment with OND (1 mg/kg, i.p.) significantly reversed the decrease in these parameters in CUS mice ($p < 0.001$). However, OND at lower dose (0.5 mg/kg, i.p.) increased only number of crossings in CUS mice ($p < 0.05$), but did not alter number of rearings, significantly ($p > 0.05$).

In addition, FLX (10 mg/kg, i.p.) reversed the decrease in number of crossings and rearings in CUS mice ($p < 0.001$), Fig. 6.9.

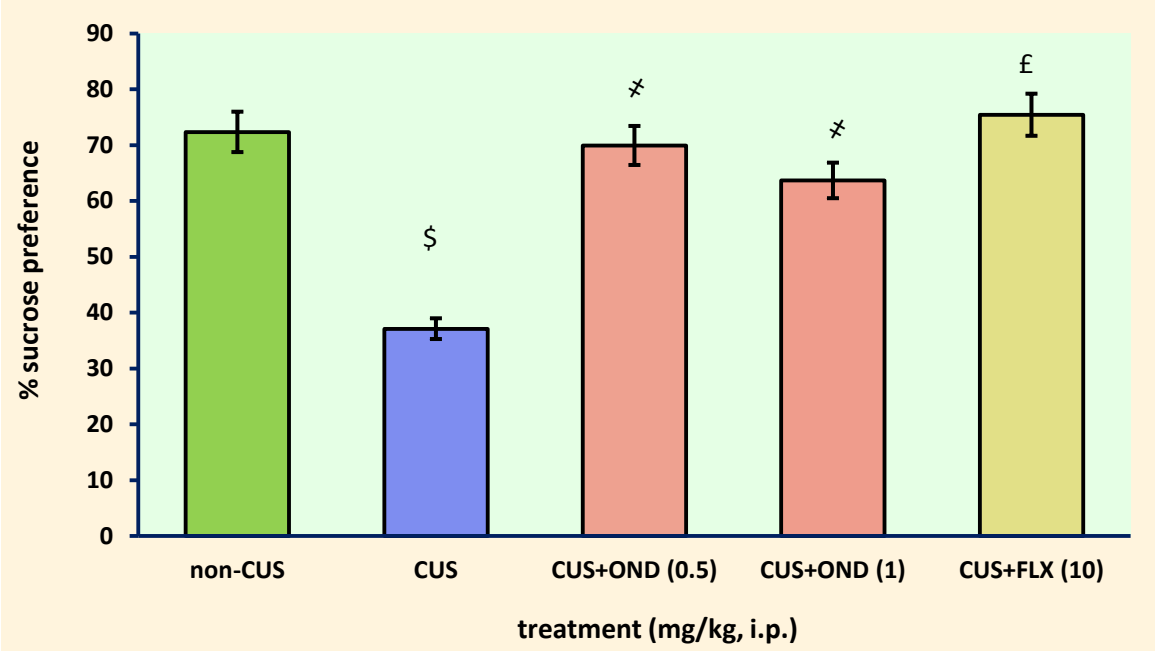
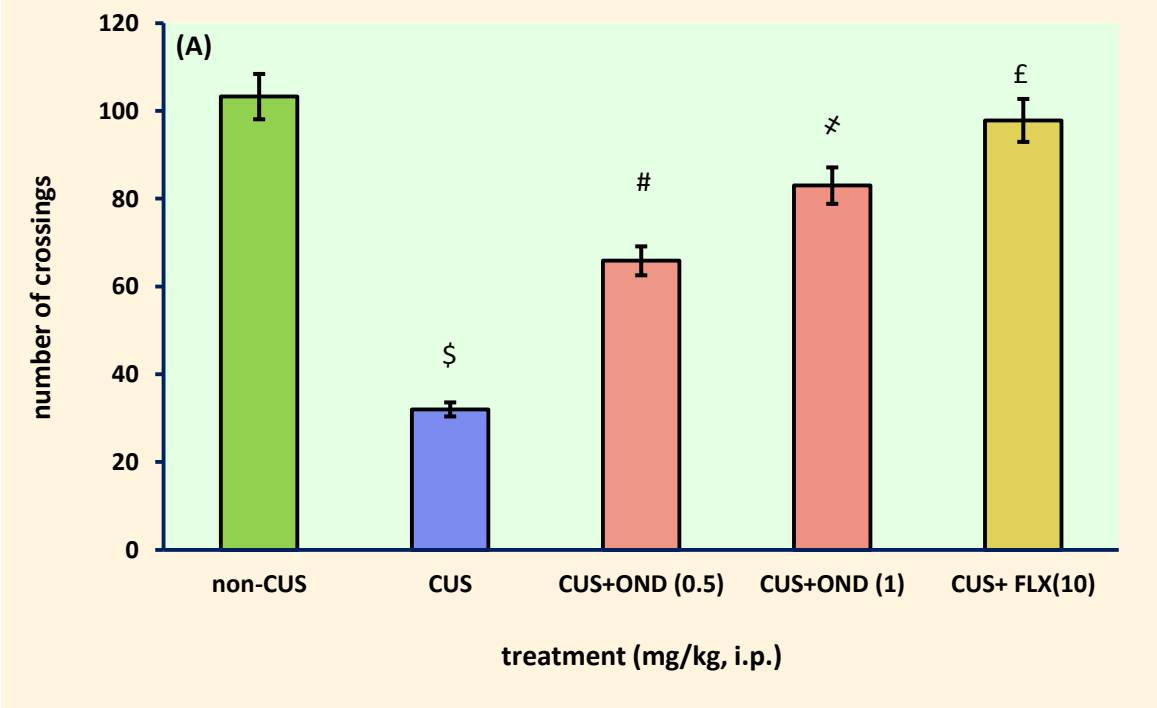


Fig. 6.8 The columns indicate % of sucrose preference and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference vs non-CUS control group. ≠ $p < 0.001$, £ $p < 0.001$ indicate significant difference vs CUS control group, n = 6/group.



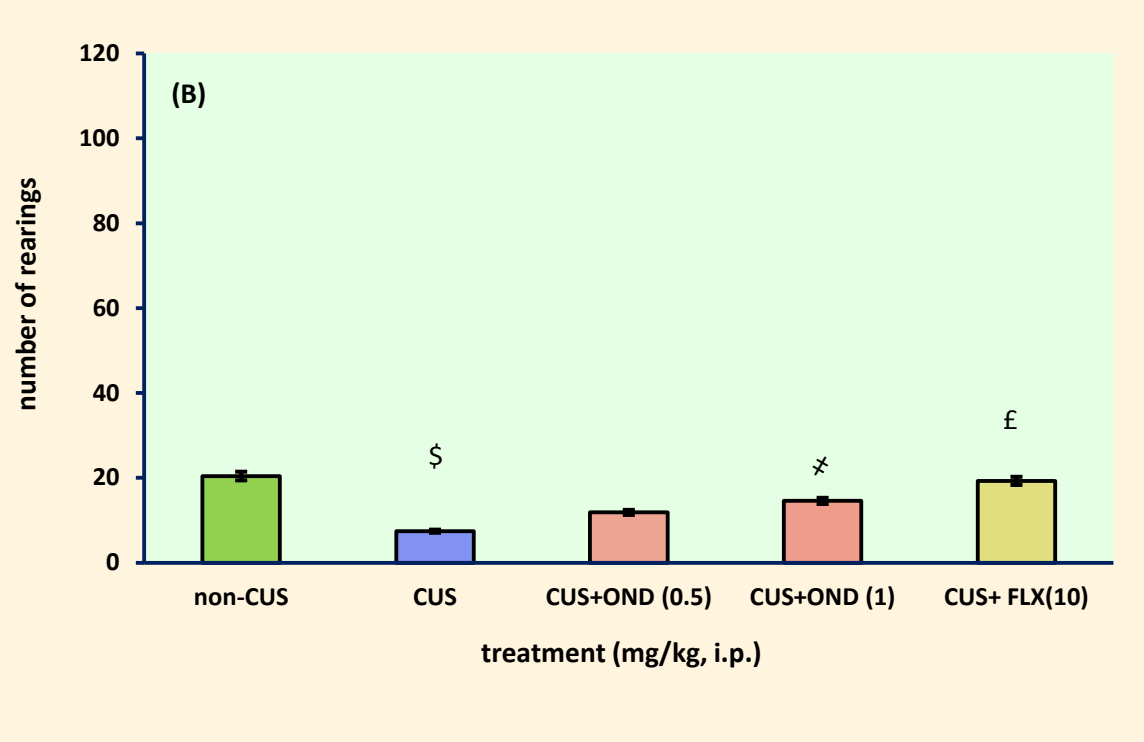


Fig. 6.9 The columns indicate mean values of number of crossings **(A)** and rearings **(B)** in open field test, and error bars show S.E.M. $ p < 0.001, indicate significant difference vs non-CUS control group. # p < 0.05, ≠ p < 0.001, £ p < 0.001 indicate significant difference vs CUS control group, n = 6/group.

6.4.2 Antidepressant-like effect of 4i in acute and chronic models of depression

6.4.2.1 Effect of 4i in acute models of depression

6.4.2.1.1 Effect of 4i on basal locomotor activity (BLA)

Novel 5-HT_3 receptor antagonist **4i** (0.25-8 mg/kg, i.p.) was evaluated in BLA test. There was a significant difference in BLA among the groups [F (6, 42) = 3.48, p < 0.05]. **4i** at 0.5 and 1 m/kg had no significant influence on BLA (p > 0.05). However, **4i** at 0.25 and higher doses of 1 and 2 mg/kg significantly altered the BLA during the test (p < 0.05), as depicted in Fig. 6.10.

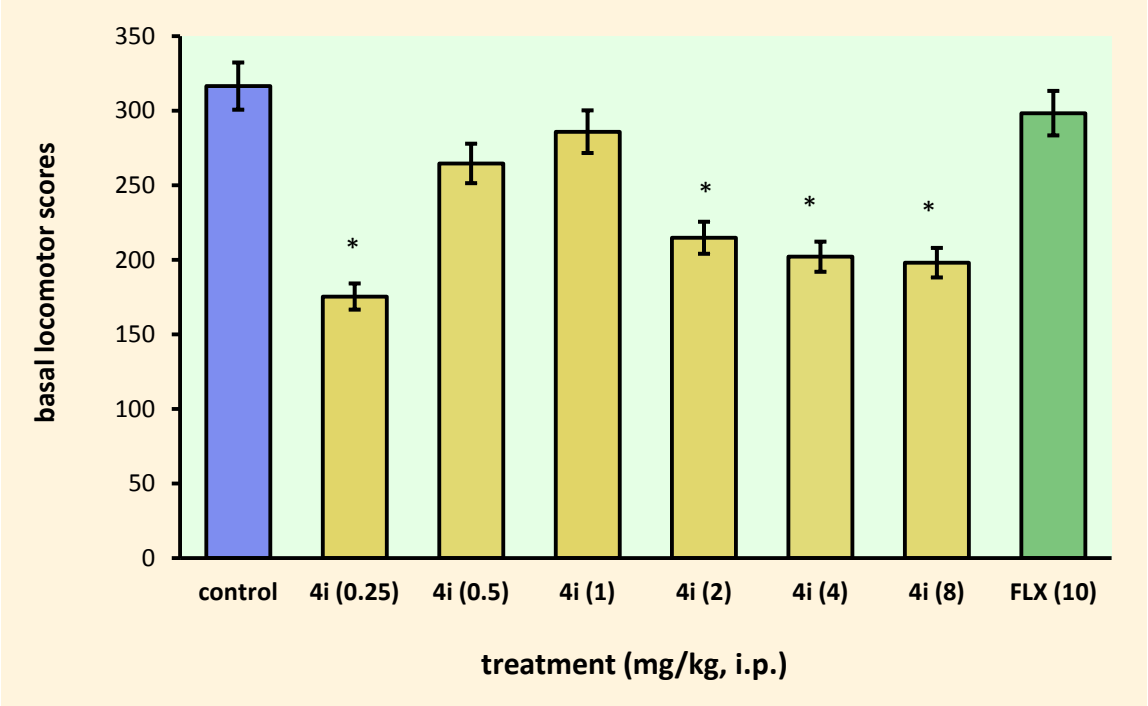


Fig. 6.10 The columns indicate mean values and error bars show S.E.M. * $p < 0.05$ indicates significant difference compared to control group, n = 6/group.

6.4.2.1.2 Effect of 4i on duration of immobility in FST and TST

Acute treatment with**4i** (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.), significantly altered the duration of immobility, in mice, during FST [$F(2, 23) = 28.18$, $p < 0.001$] and TST [$F(2, 23) = 27.63$, $p < 0.001$]. Mice with **4i** treatment, exhibited a significant decrease in duration of immobility in FST ($p < 0.01$) and TST ($p < 0.001$). In addition, FLX (10 mg/kg, i.p.) treatment produced significant reduction in duration of immobility, in mice subjected to FST and TST ($p < 0.001$), when compared with mice that received vehicle only, as indicated in Fig. 6.11.

6.4.2.1.3 Effect of 4i, on exploratory behavior in hole-board test and light-dark test

The effect of **4i**, in anxiety models namely, hole-board test and light-dark test were evaluated. In hole-board test, there was a significant alteration in the number of head dips [$F(2, 23) = 7.499$, $p < 0.001$] and duration of head dips [$F(2, 23) = 6.557$, $p < 0.001$], in mice, subjected to different treatments. Acute **4i** (1 mg/kg, i.p.) treatment, significantly increased the number ($p < 0.001$) as well as duration ($p < 0.001$) of head dips, in mice.

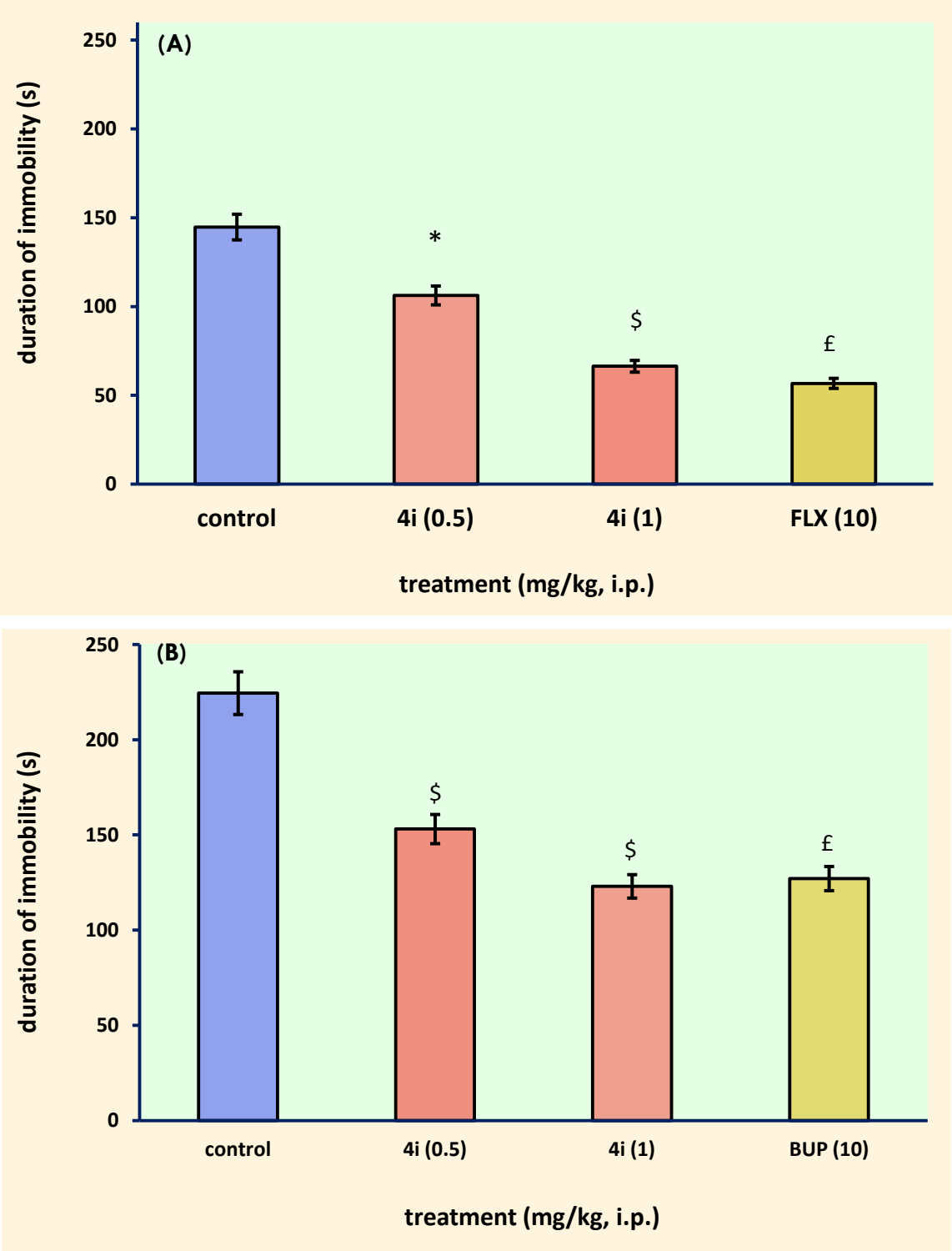


Fig. 6.11 The columns indicate mean values of duration of immobility (s) in **(A)** FST and **(B)** TST, and error bars show S.E.M. * $p < 0.05$, **$** $p < 0.001$, £ $p < 0.001$ indicate significant difference vs control group, n = 6/group.

Similarly, the lower dose (0.5 mg/kg, i.p.) of **4i** increased both the number ($p < 0.05$) and duration ($p < 0.001$) of head dips, in mice. In addition, standard drug diazepam (1 mg/kg, i.p.), increased the number of head dips and duration of head dips, in mice ($p < 0.001$), as illustrated in Table 6.3. In light-dark test, there was a significant difference in latency [$F (2, 23) = 7.313$, $p < 0.001$], and time spent in light chamber [$F (2, 23) = 4.14$, $p < 0.05$], in mice subjected to different treatments. The latency and time spent in light chamber (s) was significantly increased, in mice treated with **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.001$), when compared with mice that received vehicle only. Similarly, diazepam (1 mg/kg, i.p.) treatment significantly increased the latency ($p < 0.001$) and time spent in light chamber ($p < 0.05$), in mice, as compared to control group (Table 6.3).

Table 6.3 The effect of acute treatment of 4i in hole-board and light-dark test

Treatment (dose, mg/kg, i.p.)	hole-board test		light-dark test	
	number of head dips	duration of head dips (s)	Latency (s)	time spent in light chamber (s)
Control	15.83 ± 0.91	22.61 ± 2.26	26.69 ± 3.90	57.73 ± 8.51
4i (0.5)	31.14 ± 2.38 $	39.47± 3.87 $	48.17± 6.48 $	121.13 ± 11.49 $
4i (1)	35.14 ± 3.71 $	43.3 ± 6.13 $	48.21 ± 4.12 $	119.98 ± 3.04 $
DZM (1)	32.16 ± 2.70 £	40.95 ± 4.32 £	46.66 ± 6.14 £	107.41 ± 15.79 σ

Values represent mean ± S.E.M. * $p < 0.05$, $ $p < 0.001$, σ $p < 0.05$, £ $p < 0.001$ vs control group, n = 6/group.

6.4.2.2 Effect of 4i on chronic model of depression: OBX rat model

In OBX model, similar to acute neurobehavioral models, **4i** significantly altered the depression and anxiety-like behavior.

Sucrose preference test was performed to measure anhedonia (loss of pleasure) in OBX model. Percentage of preference of sucrose consumption was tested and was found to be significantly altered [$F (7, 63) = 4.571$, $p < 0.001$] in OBX rats. **4i** (0.5 mg/kg, i.p.) treatment significantly increased the percentage of sucrose preference in OBX rats as compared to the OBX rats that received vehicle only ($p < 0.05$).

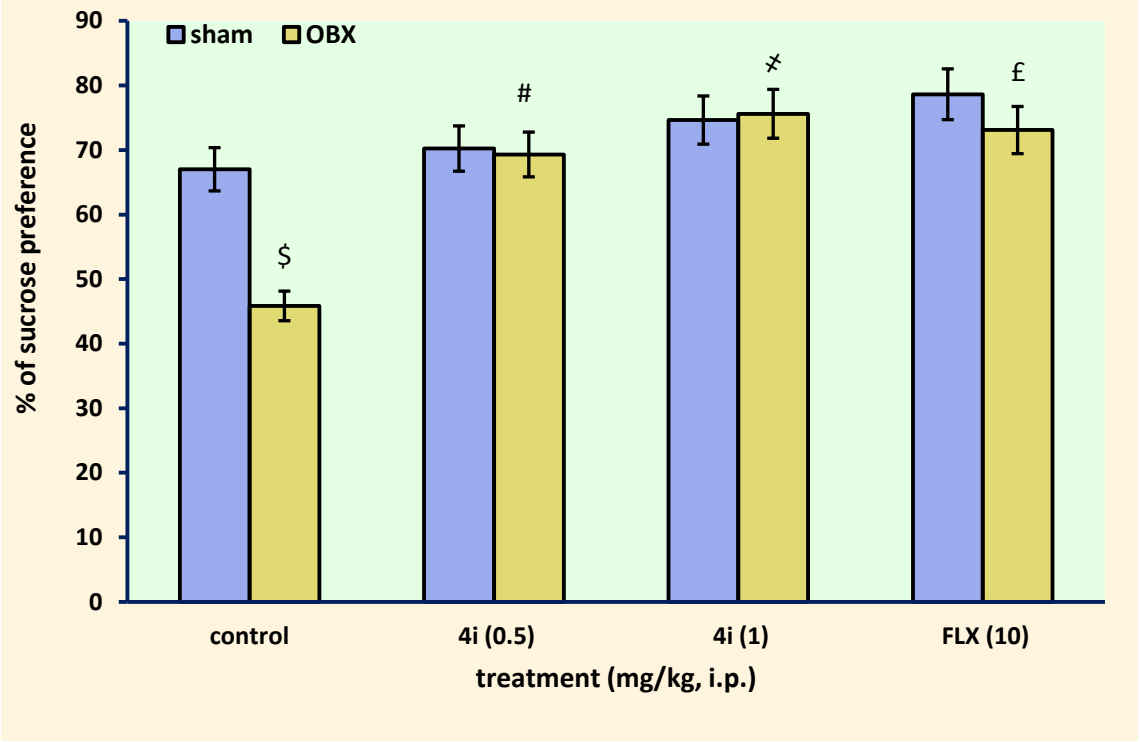


Fig. 6.12 The columns indicate mean values of % sucrose preference and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to sham control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs OBX control group, n = 7/group.

In addition, **4i** at 1 mg/kg and FLX (10 mg/kg, i.p.) treatment significantly increased the percentage of sucrose preference in OBX rats ($p < 0.001$). However, both **4i** (0.5-1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) treatment, had no effect on sham treated rats ($p > 0.05$), as indicated in Fig. 6.12.

24 hrs after the sucrose preference test, open field test was performed as a measure of anxiety-like behavior, in OBX rat model. A significant change in both number of crossings [$F (7, 63) = 6.000$, $p < 0.001$] and number of rearings [$F (7, 63) = 4.390$, $p < 0.001$] was observed in OBX rats. OBX rats treated with **4i** (0.5 and 1 mg/kg, i.p.), exhibited a significant increase in number of crossings and rearings ($p < 0.05$), as compared to those, given vehicle only.

Similarly, OBX rats treated with FLX (10 mg/kg, i.p.), significantly increased the number of crossings ($p < 0.001$) and number of rearings ($p < 0.05$), as compared to OBX rats given only vehicle treatment, as indicated in Fig. 6.13.

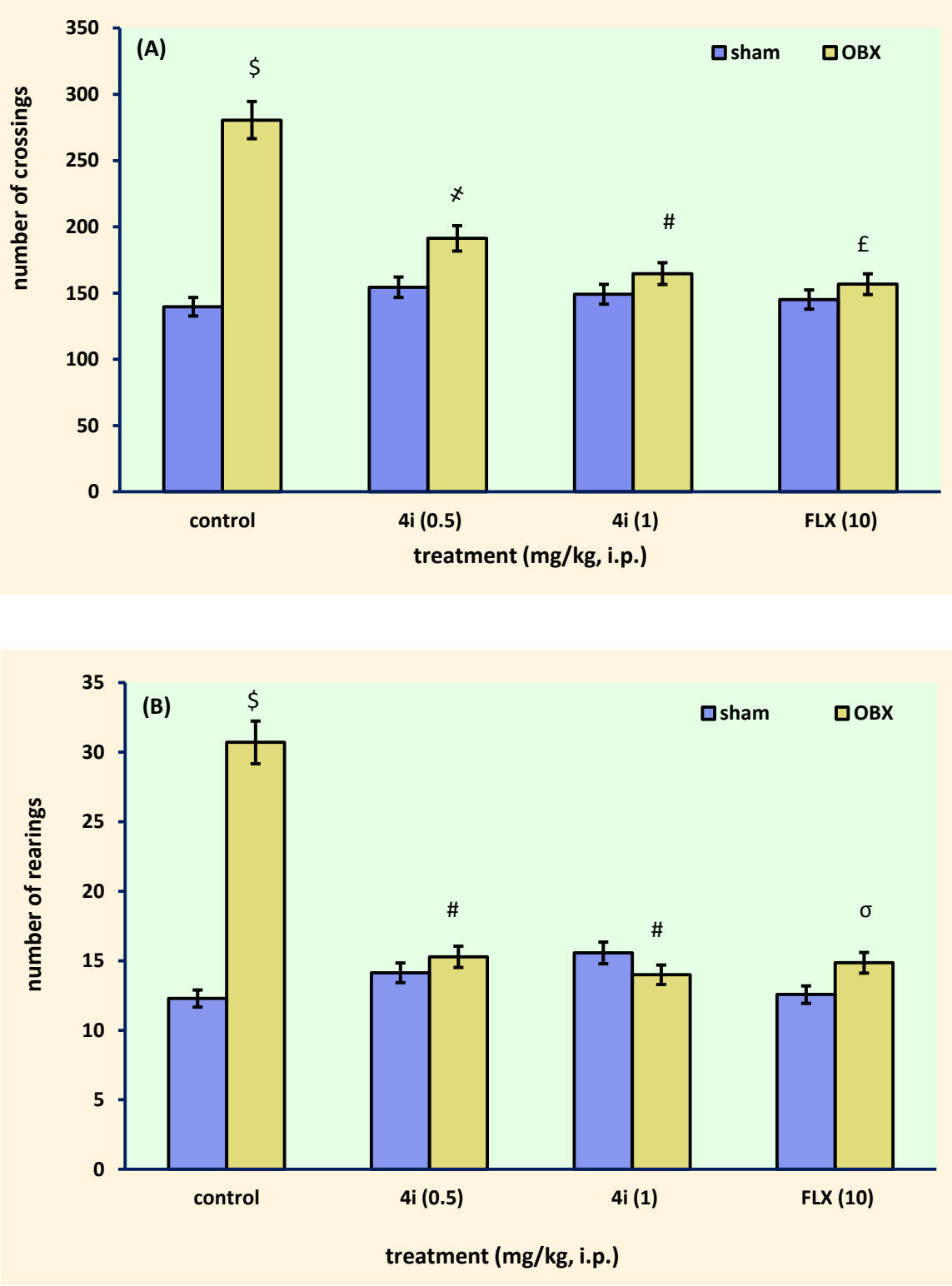


Fig. 6.13 The columns indicate mean values of number of crossings **(A)** and Number of rearings **(B)** and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference vs sham control and # $p < 0.05$, ≠ $p < 0.001$,σ $p < 0.05$, £ $p < 0.001$ vs OBX control group, n = 7/group.

6.4.3 Antidepressant-like effect of 6z in acute and chronic models of depression

6.4.3.1 Effect of 6z in acute models of depression

6.4.3.1.1 Effect of 6z on basal locomotor activity (BLA)

The effect of **6z** (0.5, 1, 2 and 4 mg/kg, i.p.) treatment was evaluated in BLA test. There was no significant effect on BLA, in mice subjected to different treatments [$F(5, 41) = 1.521$, $p > 0.05$]. **6z** at 1 and 2 mg/kg had no significant effect on BLA. However, treatment with 4 mg/kg dose of **6z**, significantly increased the BLA, in mice ($p < 0.05$). Fluoxetine (10 mg/kg, i.p.) had no effect on this parameter ($p > 0.05$), as depicted in Fig. 6.14.

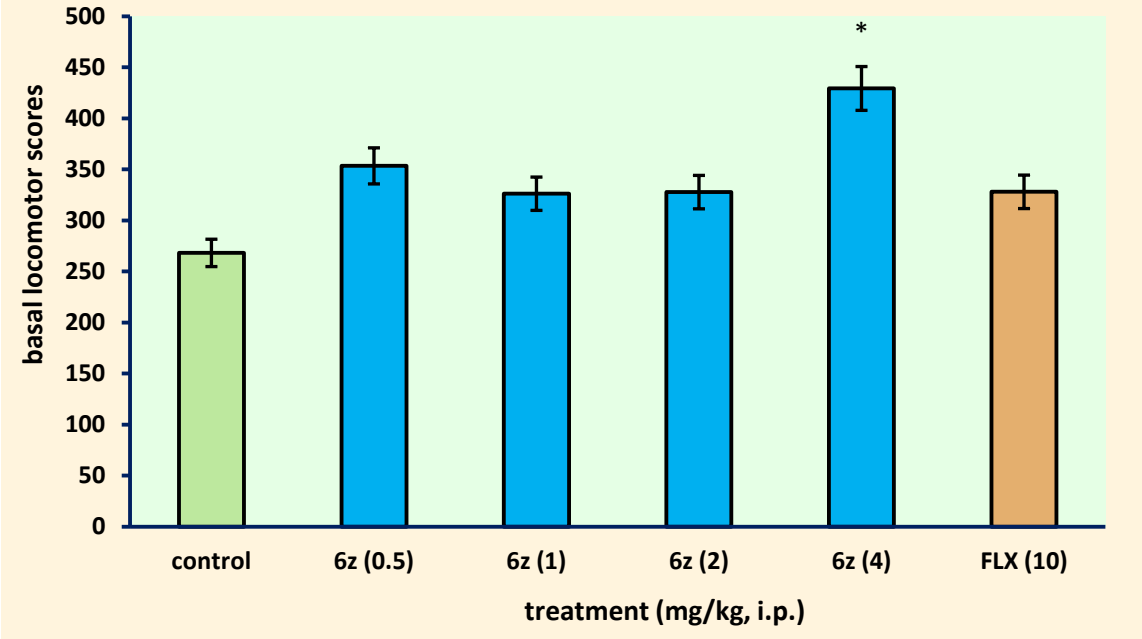


Fig. 6.14 The columns indicate mean values and error bars show S.E.M. * $p < 0.05$ indicate significant difference vs control group, n = 7/group.

6.4.3.1.2 Effect of 6z on duration of immobility in FST and TST

During, FST, there was a significant effect on duration of immobility, in mice subjected to different treatments [$F(4, 28) = 5.016$, $p < 0.001$]. Acute treatment with **6z** (1 and 2 mg/kg, i.p.) had a significant effect and reduced the duration of immobility ($p < 0.001$), in mice, as compared to those that received only vehicle treatment.

However, treatment of **6z** at 0.5 mg/kg had no significant effect ($p > 0.05$). In addition, FLX (10 mg/kg, i.p.) treatment exhibited a significant decrease in duration of immobility, in mice ($p < 0.001$), as illustrated in Fig. 6.15.

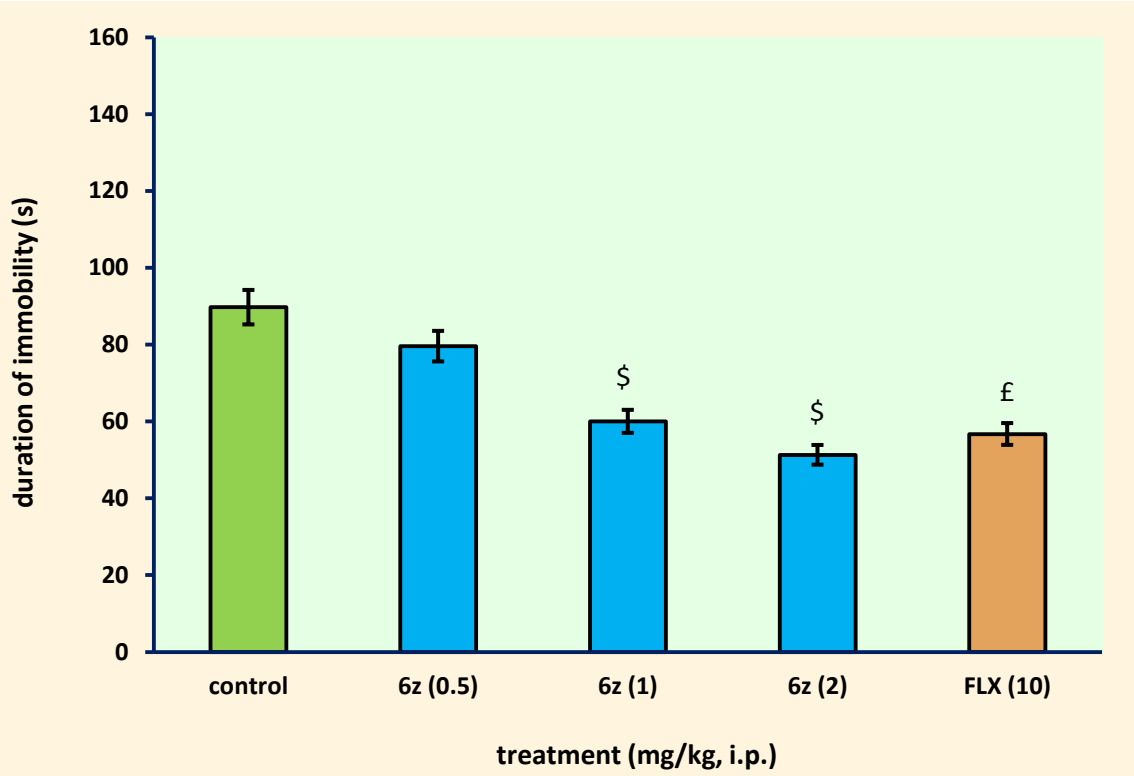


Fig. 6.15 The columns indicate mean values of duration of immobility (s) in FST and error bars show S.E.M. **$** $p < 0.001$, £ $p < 0.001$ indicate significant difference compared to control group, n = 7/group.

When tested in TST, it was found that, the duration of immobility was significantly altered, in mice subjected to different treatments [$F (4, 28) = 4.429$, $p < 0.001$]. Acute **6z** (1 and 2 mg/kg, i.p.) treatment, significantly reduced the duration of immobility ($p < 0.001$), in mice, as compared to those that received only vehicle treatment.

Unlike in FST, **6z** at 0.5 mg/kg treatment significantly reduced the duration of immobility, in mice ($p < 0.05$). In addition, FLX (10 mg/kg, i.p.) treatment exhibited a significant decrease in duration of immobility, in mice ($p < 0.001$), as illustrated in Fig. 6.16.

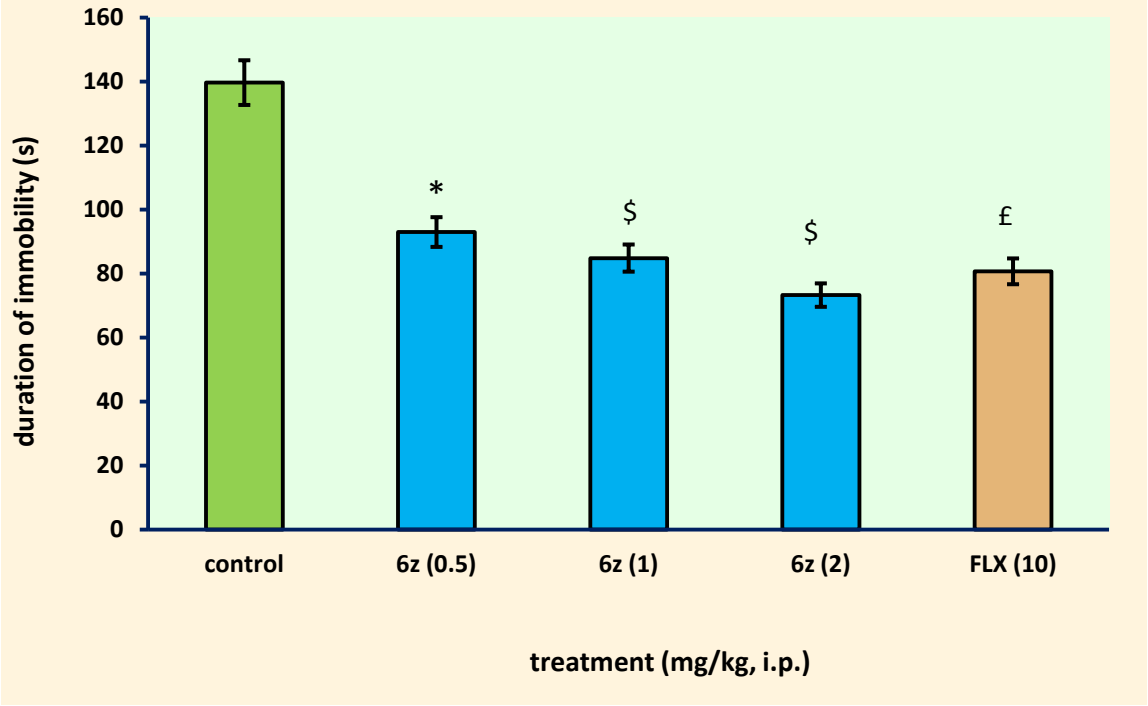


Fig. 6.16 The columns indicate mean values of duration of immobility (s) in TST and error bars show S.E.M. * $p < 0.05$, **$** $p < 0.001$, £ $p < 0.001$ indicate significant difference compared to control group, n = 7/group.

6.4.3.1.3 Effect of 6z on behavior in hole-board and light-dark test

Hole-board test and light-dark test were employed as the models, to evaluate the anxiety-like behavior.

During hole-board test, there was a significant change in number of head dips [$F(4, 34) = 10.050$, $p < 0.001$] and duration of head dips [$F(4, 34) = 8.787$, $p < 0.05$], in mice subjected to different treatments. **6z** (1 and 2 mg/kg, i.p.) treatment significantly increased the number and duration of head dips ($p < 0.05$), in mice, as compared to control group.

Moreover, **6z** at 0.5 mg/kg treatment had no significant effect on these parameters ($p > 0.05$).Diazepam (1 mg/kg, i.p.) treatment, exhibited a significant increase in number and duration of head dips, in mice ($p < 0.001$), as illustrated in Table 6.4. In light-dark test, there was a significant change in latency (s) [$F(4, 34) = 5.767$, $p < 0.05$] and time spent in light chamber (s) [$F(4, 34) = 7.569$, $p < 0.05$]. **6z** (2 mg/kg, i.p.) treatment produced a significant increase in latency ($p < 0.05$) and time spent in light chamber ($p < 0.001$), in mice, as compared to mice that received only vehicle treatment.

On the other hand, **6z** at 0.5 and 1 mg/kg treatments had no significant effect on latency ($p > 0.05$), but **6z** at 1 mg/kg treatment increased the time spent in light chamber, in mice ($p < 0.001$), as compared to vehicle treated group. Diazepam (1 mg/kg, i.p.) treatment exhibited a significant increase in latency ($p < 0.001$) and time spent in light chamber, in mice ($p < 0.05$), as illustrated in Table 6.4.

Table 6.4 Effect of acute treatment of 6z in hole-board and light-dark test

Treatment (dose, mg/kg, i.p.)	hole-board test		light-dark test	
	number of head dips	duration of head dips (s)	Latency (s)	time spent in light chamber (s)
Control	15.83 ± 0.91	2.61 ± 2.26	26.69 ± 3.90	57.73 ± 8.51
6z (0.5)	17.16 ± 1.75	24.25 ± 2.50	35.56 ± 8.80	97.41 ± 9.69
6z (1)	28.50 ± 3.34 *	45.50 ± 6.87 *	45.13 ± 8.15	150.44 ± 12.82 **$**
6z (2)	30.66 ± 2.75 **$**	46.37 ± 5.51 *	53.65 ± 6.33*	132.60 ± 9.15 **$**
DZM (1)	32.16 ± 2.70 £	40.95 ± 4.32 £	6.4.99 ± 5.16 £	107.41 ± 15.79 σ

Values represent mean ± S.E.M. * $p < 0.05$, **$** $p < 0.001$, σ $p < 0.05$, £ $p < 0.001$ vs control group, n = 6/group.

6.4.3.2 Effect of 6z on chronic model of depression: CUS mouse model

CUS model was employed to evaluate the clinical time course of drug action. In this model, mice were treated with either vehicle or **6z** (0.5, 1 and 2 mg/kg, i.p.), during the last two weeks of chronic stress protocol. This was followed by behavioral analysis. There was a significant change in behavioral parameters, in mice subjected to FST and sucrose preference test, as the models of depression and open field test, as a model to evaluate the anxiety related behavior.

In FST, there was a significant change in duration of immobility (s) among the groups subjected to different treatments [$F(5, 41) = 4.022$, $p < 0.001$]. CUS mice exhibited a significant increase in duration of immobility (s) as compared to non-CUS control group ($p < 0.05$). On the other hand, **6z** (0.5, 1 and 2 mg/kg, i.p.) treatment, significantly reversed the CUS-induced increase in duration of immobility, in mice ($p < 0.05$).

In addition, the FLX (10 mg/kg, i.p.) administration, significantly reversed the CUS-induced increase in duration of immobility, in mice ($p < 0.05$), as represented in Fig. 6.17.

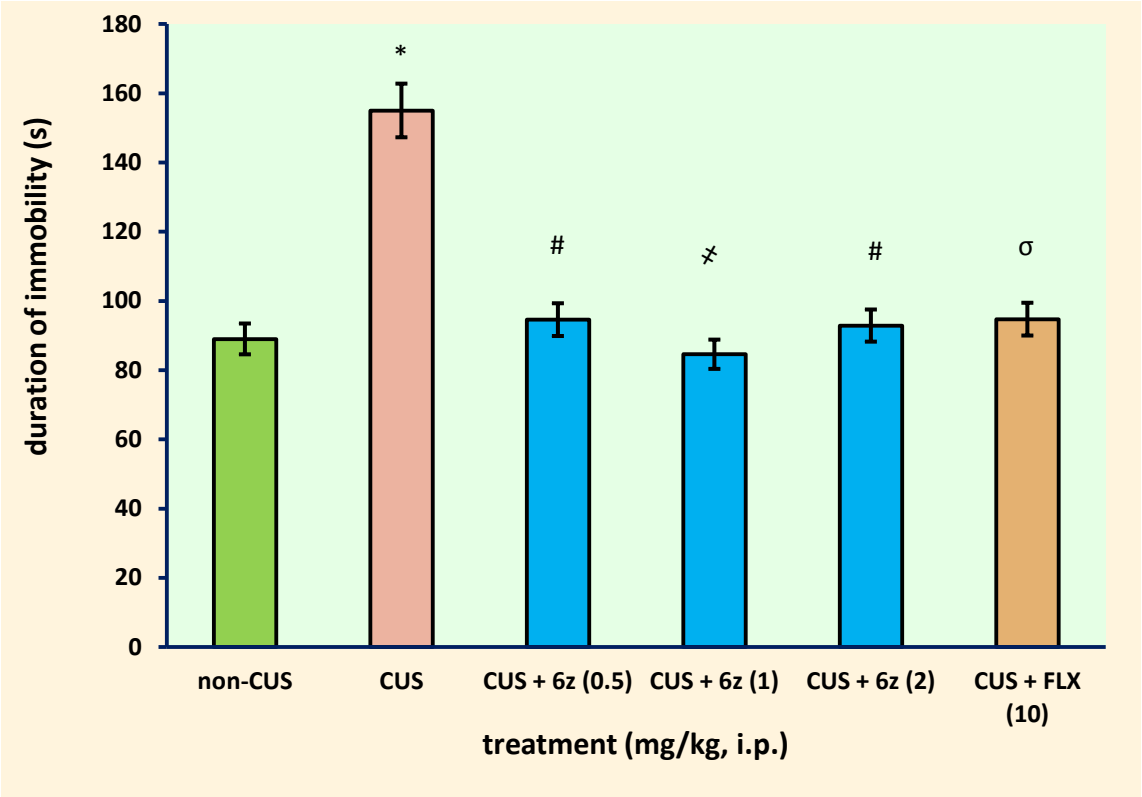


Fig. 6.17 The columns indicate mean values of duration of immobility (s) and error bars show S.E.M.* $p < 0.05$ indicate significant difference compared to non-CUS control and # $p < 0.05$, ≠ $p < 0.001$, σ $p < 0.05$ vs CUS control group, n = 7/group.

In sucrose preference test, there was a significant change in percentage of sucrose preference among the groups subjected to different treatments [$F(5, 41) = 4.549$, $p < 0.05$]. A significant decrease in percentage of sucrose preference was found in CUS mice as compared to non-CUS group ($p < 0.001$).

Interestingly, **6z** (0.5, 1 and 2 mg/kg, i.p.) treatment significantly increased the percentage of sucrose preference in CUS mice as compared to CUS control mice ($p < 0.05$). Similarly, FLX (10 mg/kg, i.p.) treatment significantly elevated the percentage of sucrose preference in CUS mice as compared to CUS control ($p < 0.001$), Fig. 6.18.

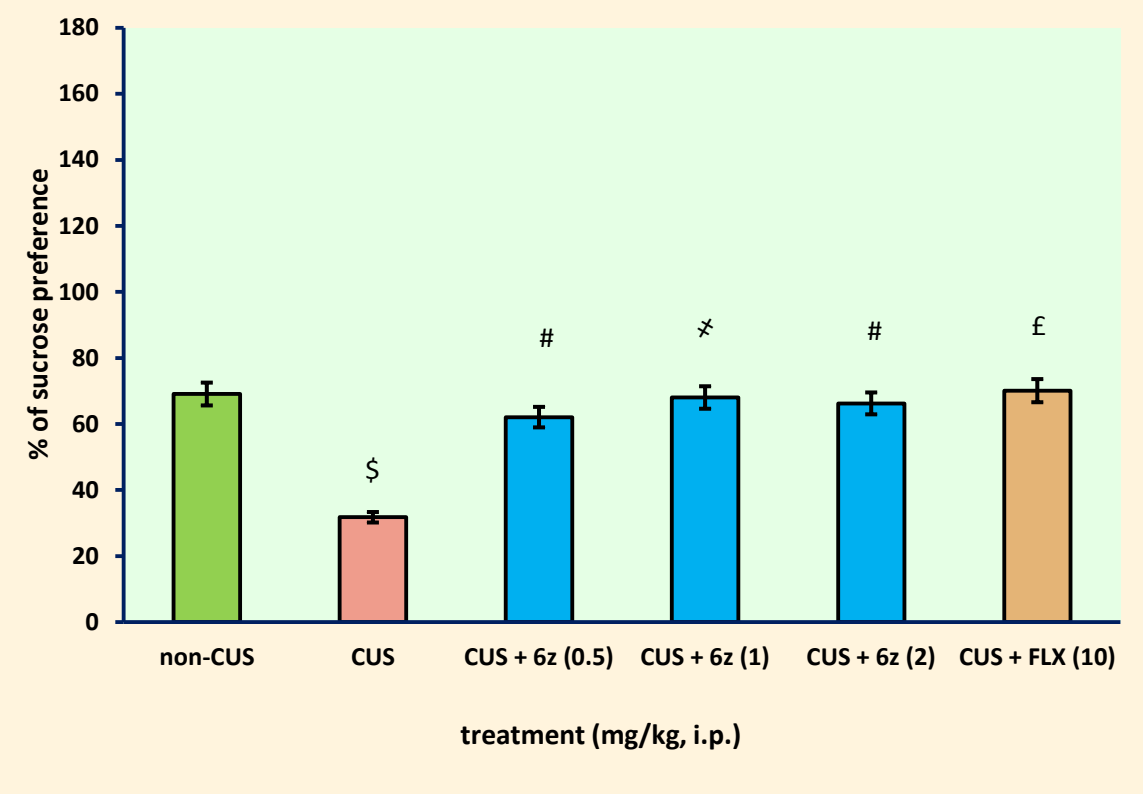


Fig. 6.18 The columns indicate mean values of % sucrose preference and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to non-CUS control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs CUS control group, n = 7/group.

In open field test, statistical analysis revealed that there was a significant change in number of crossings [$F (5, 41) = 4.549$, $p < 0.05$] and rearings [$F (5, 41) = 4.549$, $p < 0.05$] among the groups subjected to different treatment schedule. The number of crossings ($p < 0.001$) and number of rearings ($p < 0.05$) were significantly reduced in CUS mice as compared to non-CUS group. **6z** (0.5 and 1 mg/kg, i.p.), treatment significantly reversed the CUS-induced, decrease in both number of crossings and rearings, in mice ($p < 0.05$).

However, the highest tested dose of **6z** (2 mg/kg, i.p.), reversed only diabetes-induced decrease in number of crossings, in CUS mice ($p < 0.001$), but did not reverse the number of rearings ($p > 0.05$), although a non-significant trend was observed. Similarly, FLX (10 mg/kg, i.p.) treatment significantly increased the number of crossings ($p < 0.05$) and number of rearings ($p < 0.001$) in CUS mice as compared to those that received only vehicle, as represented in Fig. 6.19.

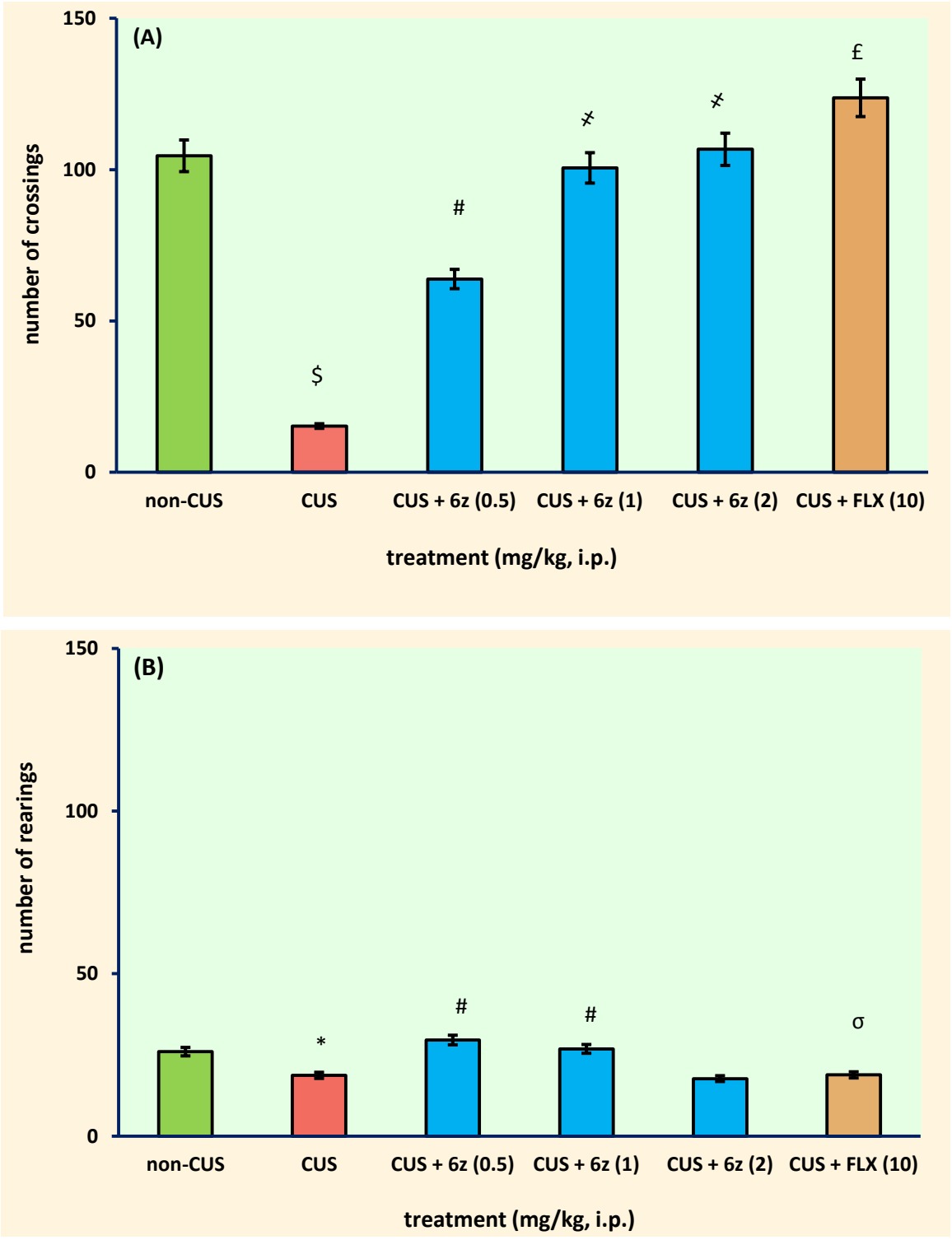


Fig. 6.19 The columns indicate mean values of number of crossings **(A)** and number of rearings **(B)** and error bars show S.E.M. * $p < 0.05$, **$** $p < 0.01$ indicate significant difference vs non-CUS control and # $p < 0.05$, ≠ $p < 0.001$, a $p < 0.05$, ≠ $p < 0.001$, σ $p < 0.05$, £ $p < 0.001$ vs CUS control group, n = 7/group.

6.5 Antidepressant-like effects of standard and novel 5-HT_3 receptor antagonists in STZ-induced diabetes model

6.5.1 Effect of OND on depression-like behavior evoked in STZ-induced diabetic mice

6.5.1.1 Effect of OND on basal locomotor activity (BLA)

In BLA, neither STZ-induced diabetes nor chronic dosing with OND (0.5 and 1 mg/kg, i.p.) had a significant influence on BLA, in mice [$F(4, 29) = 1.465$, $p > 0.05$]. Moreover, chronic treatment with FLX (10 mg/kg, i.p.) had no significant effect on BLA, in STZ-induced diabetic mice, as indicated in Fig. 6.20.

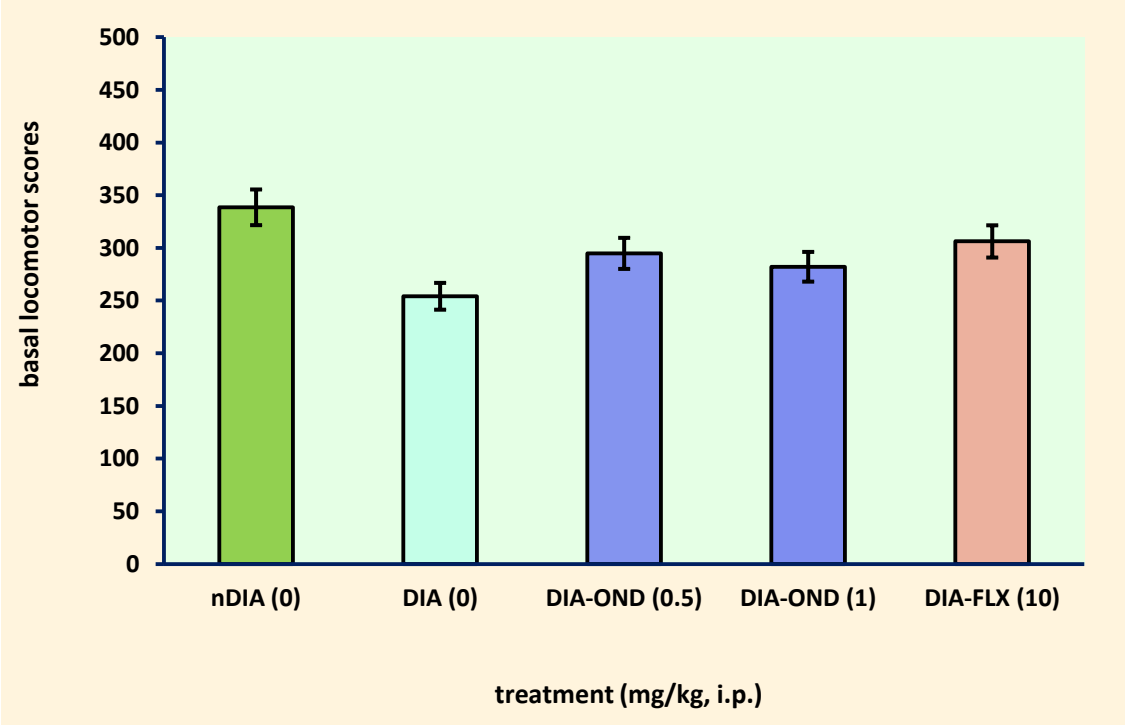


Fig. 6.20 The columns indicate mean values of basal locomotor activity and error bars show S.E.M. No significant difference among the groups was found, n = 6/group.

6.5.1.2 Effect of OND on FST and TST

In FST, statistical analysis indicated that there was a significant alteration in duration of immobility, in mice subjected to different treatments [$F(4, 30) = 7.556$, $p < 0.001$].

The duration of immobility (s) was significantly increased in STZ-induced diabetic mice ($p < 0.05$) as compared to non-diabetic mice. Chronic administration of OND (0.5 and 1 mg/kg, i.p.), significantly reversed the diabetes-induced increase in duration of immobility in mice, as compared to those that received only vehicle treatment ($p < 0.001$). In addition, chronic FLX (10 mg/kg, i.p.), treatment significantly reversed the diabetes-induced increase in duration of immobility, in mice ($p < 0.001$), as illustrated in Fig. 6.21.

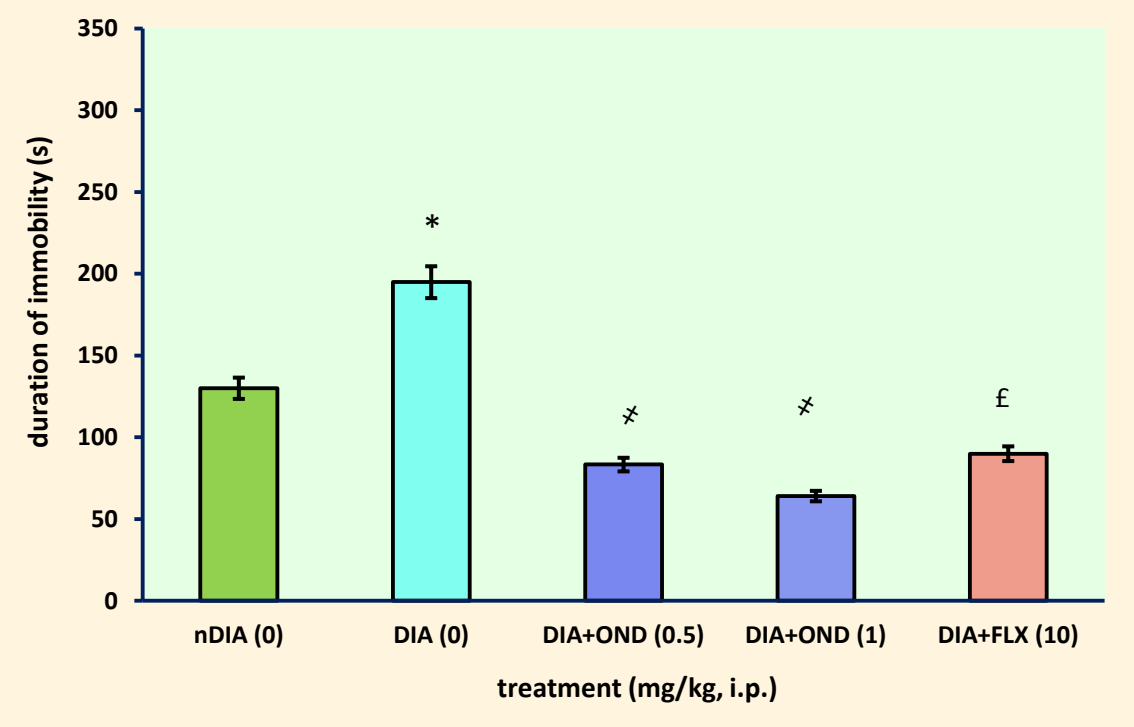


Fig. 6.21 The columns indicate mean values of duration of immobility (s) in FST and error bars show S.E.M. * $p < 0.05$ indicate significant difference compared to control and ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n= 6 /group.

In TST, there was a significant effect on duration of immobility, in mice, subjected to different treatments [$F(4, 30) = 19.41$, $p < 0.001$]. STZ-induced diabetic mice produced a significant increase in the duration of immobility ($p < 0.001$) as compared to non-diabetic mice. Chronic OND (0.5 and 1 mg/kg, i.p.) treatment, significantly reversed the diabetes-induced increase in duration of immobility, in mice, as compared to diabetic mice that received only vehicle treatment ($p < 0.001$). Repeated FLX (10 mg/kg, i.p.) treatment, significantly reversed the diabetes-induced increase in duration of immobility, in mice ($p < 0.001$), as illustrated in Fig. 6.22.

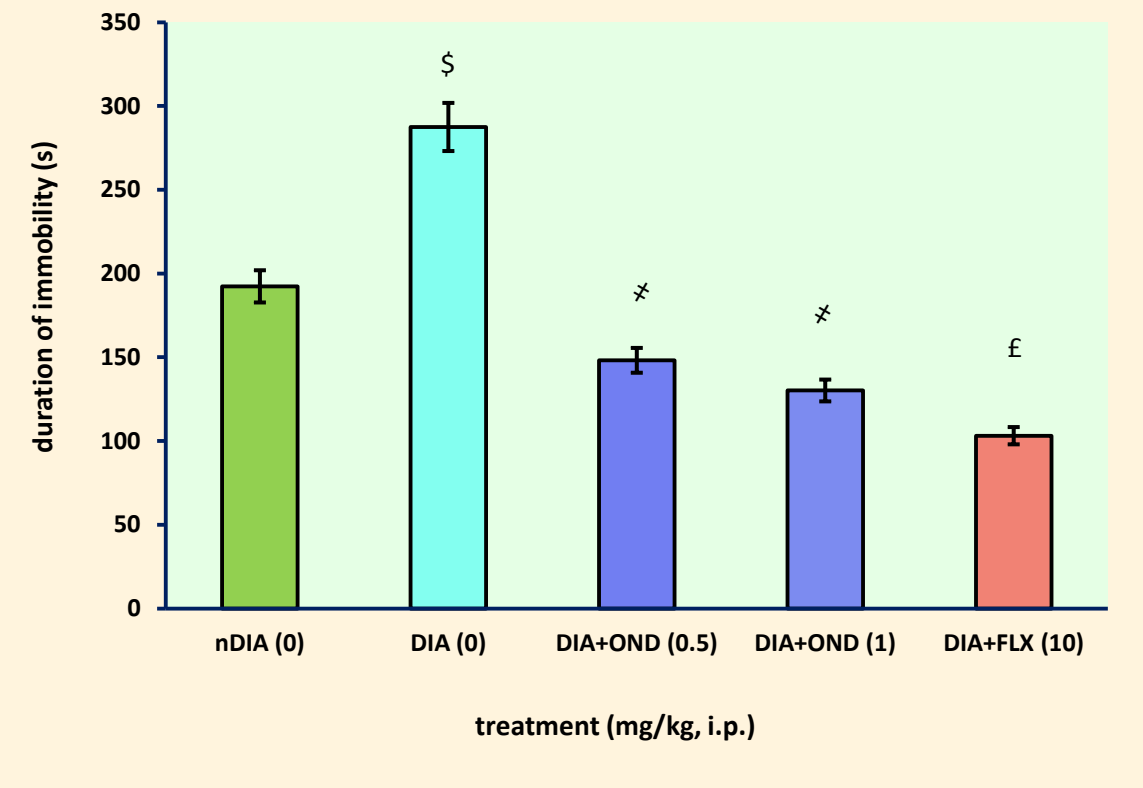


Fig. 6.22 The columns indicate mean values of duration of immobility (s) in TST and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.5.1.3 Effect of OND on hole-board test and light-dark test

The effect of OND on anxiety-like behavior produced in STZ-induced diabetes was evaluated using hole-board test and light-dark test. In hole-board test, there was a significant change in number of head dips [$F (4, 30) = 4.919$, $p < 0.01$] and duration of head dips [$F (4, 30) = 5.450$, $p < 0.01$], in mice. STZ-induced diabetic mice exhibited a significant decrease in the number as well as duration of head dips. However, chronic OND (1 mg/kg, i.p.) treatment reversed diabetes induced decrease in number of head dips ($p < 0.05$) and duration of head dips ($p < 0.001$), in mice. The lower dose of OND (0.5 mg/kg, i.p.) treatment, reversed only decrease in duration of head dips ($p < 0.001$) but did not affect, number of head dips ($p > 0.05$), in STZ-induced diabetic mice. Similarly, chronic treatment with FLX (10 mg/kg, i.p.) reversed diabetes-induced decrease in these parameters, in mice subjected to hole-board test ($p < 0.05$), Fig. 6.23A and B.

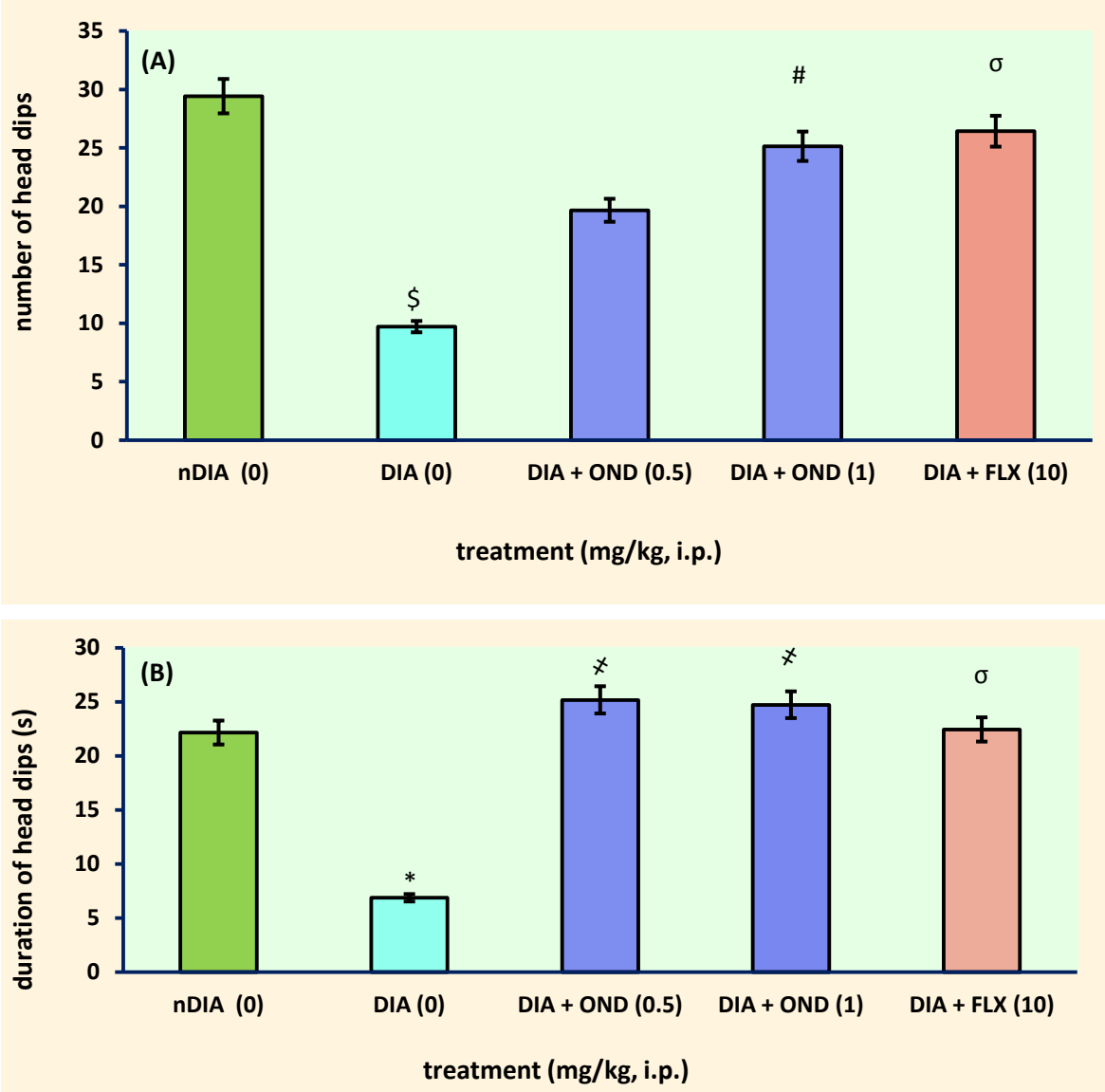


Fig. 6.23 The columns indicate mean values of number **(A)** and duration of head dips **(B)**, in hole-board test and error bars show S.E.M. * $p < 0.05$, **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, σ $p < 0.05$ vs diabetic control group, n = 6/group.

In light-dark test, there was a significant change in latency (to enter dark chamber for the first time, when mouse was kept in light chamber) [$F(4, 30) = 5.166$, $p < 0.01$] and time spent in light chamber [$F(4, 30) = 6.020$, $p < 0.001$], in mice. STZ-induced diabetic mice produced a significant decrease in latency (s) as well as time spent in light chamber(s). Chronic treatment with OND (1 mg/kg, i.p.) reversed diabetes-induced decrease in these parameters ($p < 0.001$), in mice.

In line, the lower dose of OND (0.5 mg/kg, i.p.) treatment significantly reversed these behavioral measures ($p < 0.05$) in STZ-induced diabetic mice. Similarly, FLX (10mg/kg, i.p.) treatment reversed diabetes induced decrease in latency (s) ($p < 0.05$) and time spent in light chamber, in mice ($p < 0.001$), Fig. 6.24A and B.

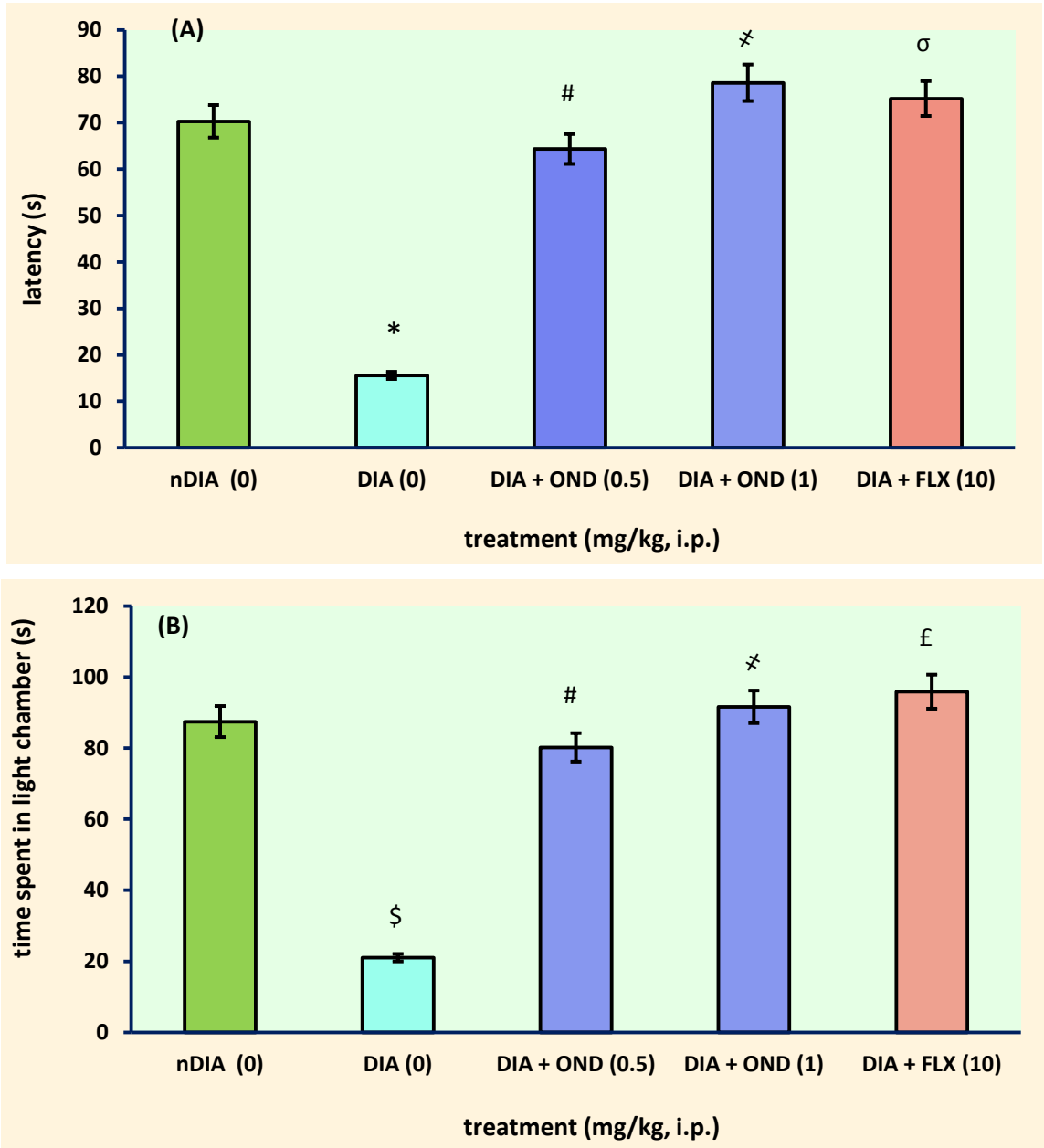


Fig. 6.24 The columns indicate mean values of latency (s) **(A)** and time spent in light chamber **(B)**, in light-dark test and error bars show S.E.M. * $p < 0.05$, $ $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, σ $p < 0.05$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.5.2 Effect of 4i on depression-like behavior evoked in STZ-induced diabetic mice

6.5.2.1 Effect of 4i on basal locomotor activity (BLA)

In BLA, neither STZ-induced diabetes, nor chronic **4i** treatment, had a significant effect on BLA, in mice [F (4, 30) = 0.551, p > 0.05]. In addition, FLX (10 mg/kg, i.p.) treatment had no effect on BLA in STZ-induced diabetic mice (p > 0.05), as indicated in Fig. 6.25.

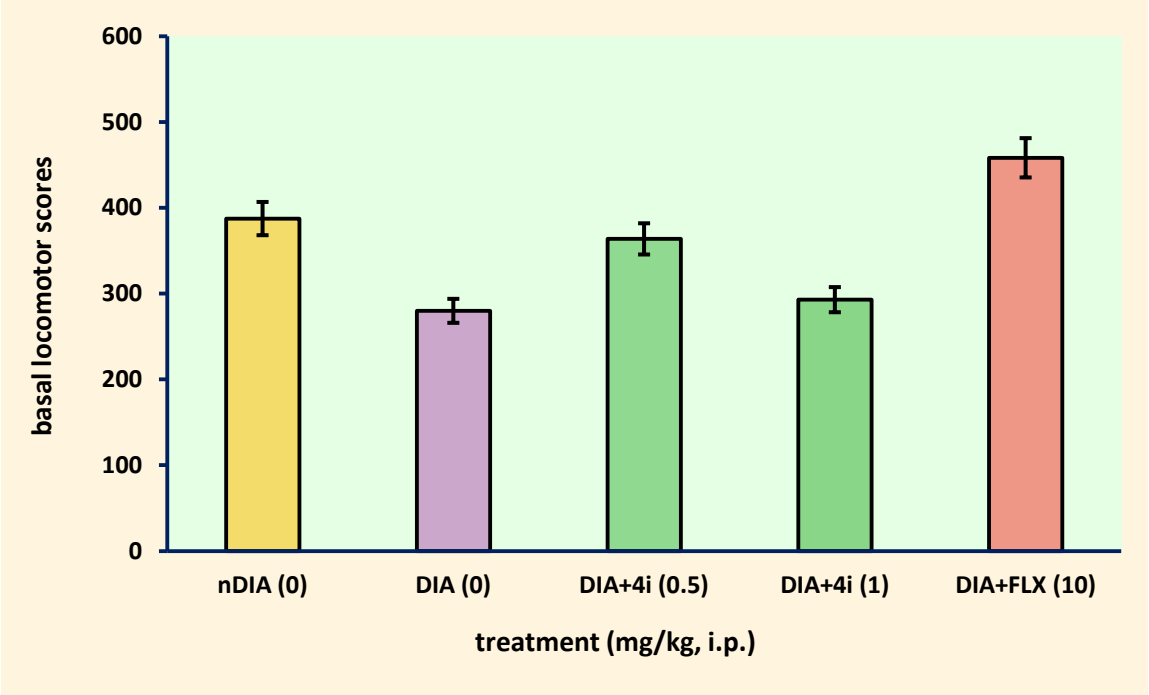


Fig. 6.25 The columns indicate mean values of BLA and error bars show S.E.M. No significant difference found among the groups, n = 6/group.

6.5.2.2 Effect of 4i on FST and TST

The effect of **4i** on depression-like behavior induced in STZ-induced diabetes was evaluated, using FST and TST models of depression. In FST, a significant alteration in duration of immobility was observed, in mice, subjected to different treatments [F (4, 30) = 6.020, p < 0.001]. The duration of immobility was significantly increased in STZ-induced diabetic mice, as compared to control. Chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.), significantly reversed the increase in duration of immobility in STZ-induced mice (p < 0.05).

Similarly, chronic FLX (10 mg/kg, i.p.) administration reversed the increase in duration of immobility (s) in STZ-induced diabetic mice ($p < 0.001$), Fig. 6.26A.

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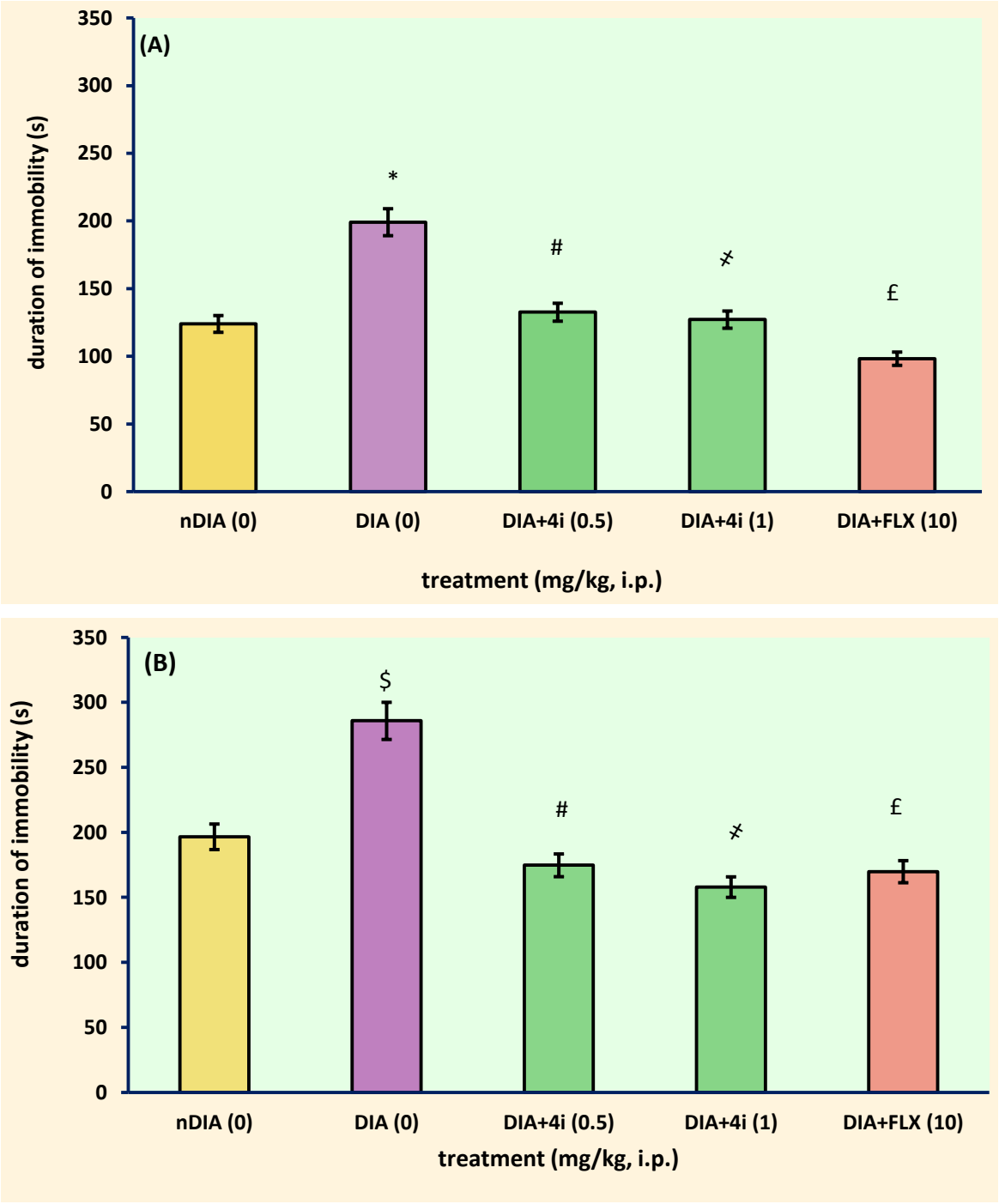


Fig. 6.26 The columns indicate mean values of duration of immobility in FST **(A)** and TST **(B)** and error bars show S.E.M. * $p < 0.05$, **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

Similarly, in TST, there was a significant change in duration of immobility, in mice subjected to various treatments [F (4, 30) = 13.79, p < 0.001]. STZ-induced diabetic mice exhibited a significant increase in duration of immobility in TST (p < 0.001), which was significantly reversed by chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) (p < 0.05) and FLX (10 mg/kg, i.p.). Moreover, the effect of **4i** was dose dependent, as depicted in Fig. 6.26B.

6.5.2.3 Effect of 4i on open field test and social interaction test

In open field test, there was a significant change in number of crossings [F (4, 30) = 18.880, p < 0.001] and rearings [F (4, 30) = 19.170, p < 0.001], in mice. STZ-induced diabetic mice exhibited a significant decrease in number of crossings as well as number of rearings in open field test (p < 0.001).

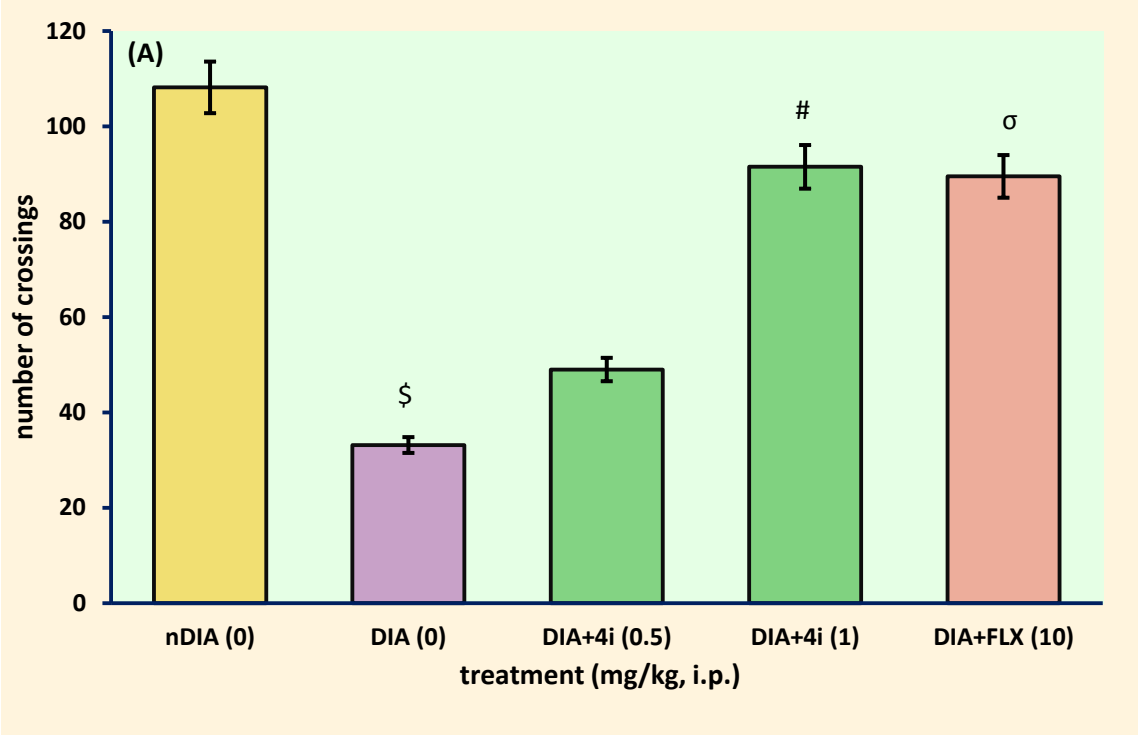
On the other hand, OND (1 mg/kg, i.p.) treatment reversed diabetes induced decrease in these parameters (p < 0.05), in mice. However, the lower dose of OND (0.5 mg/kg, i.p.) had no effect on these parameters (p > 0.05) in STZ-induced diabetic mice.

Fluoxetine (10mg/kg, i.p.) treatment reversed diabetes-induced decrease in number of crossings (p < 0.05) and rearings, in mice (p < 0.001), as indicated in Fig. 6.27A and B.

In social interaction test, there was a significant change in number of social interaction [Kruskal-Wallis statistic = 15.54, p < 0.01] and time spent [Kruskal-Wallis statistic = 25.03, p < 0.001] in social interaction, in mice subjected to different treatments.

STZ-induced diabetic mice exhibited a significant decline in number (p < 0.05) as well as time spent (p < 0.001) in social interaction, as compared to control. Chronic treatment with **4i** (1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.), significantly reversed these parameters in STZ-induced diabetic mice (p < 0.05).

However, the lower dose of **4i** had no significant effect, as observed in open field test (p > 0.05), Fig. 6.28A and B.



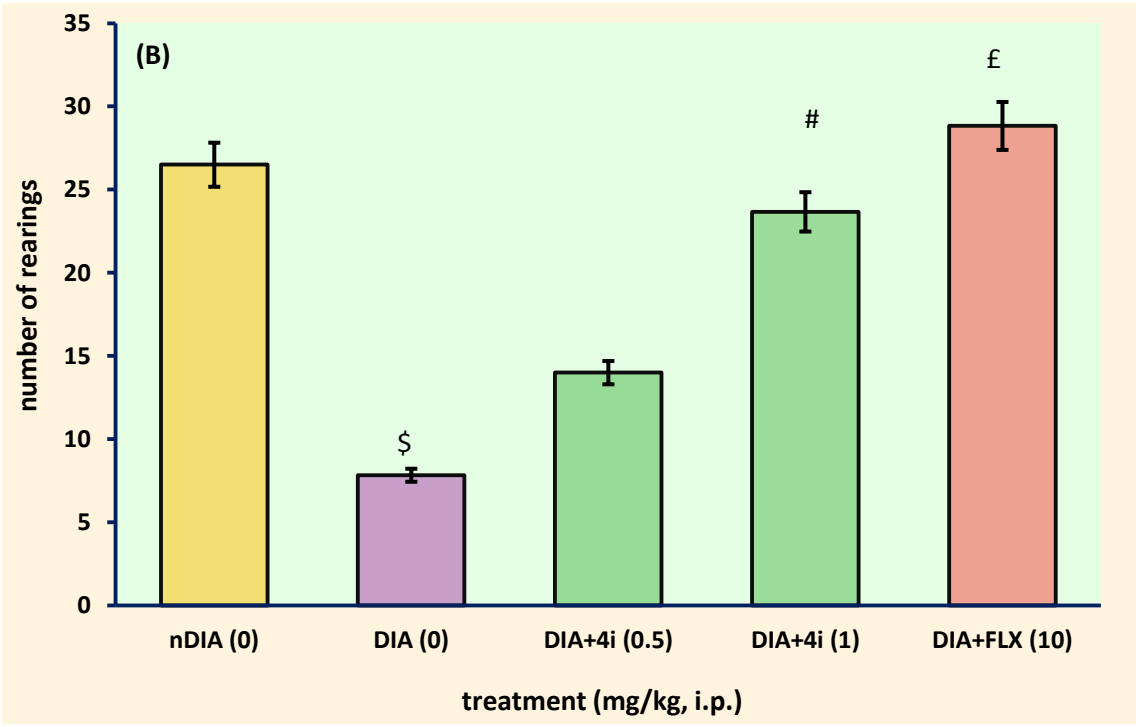


Fig. 6.27 The columns indicate mean values of number of crossings **(A)** and rearings **(B)** in open field test and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, σ $p < 0.05$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

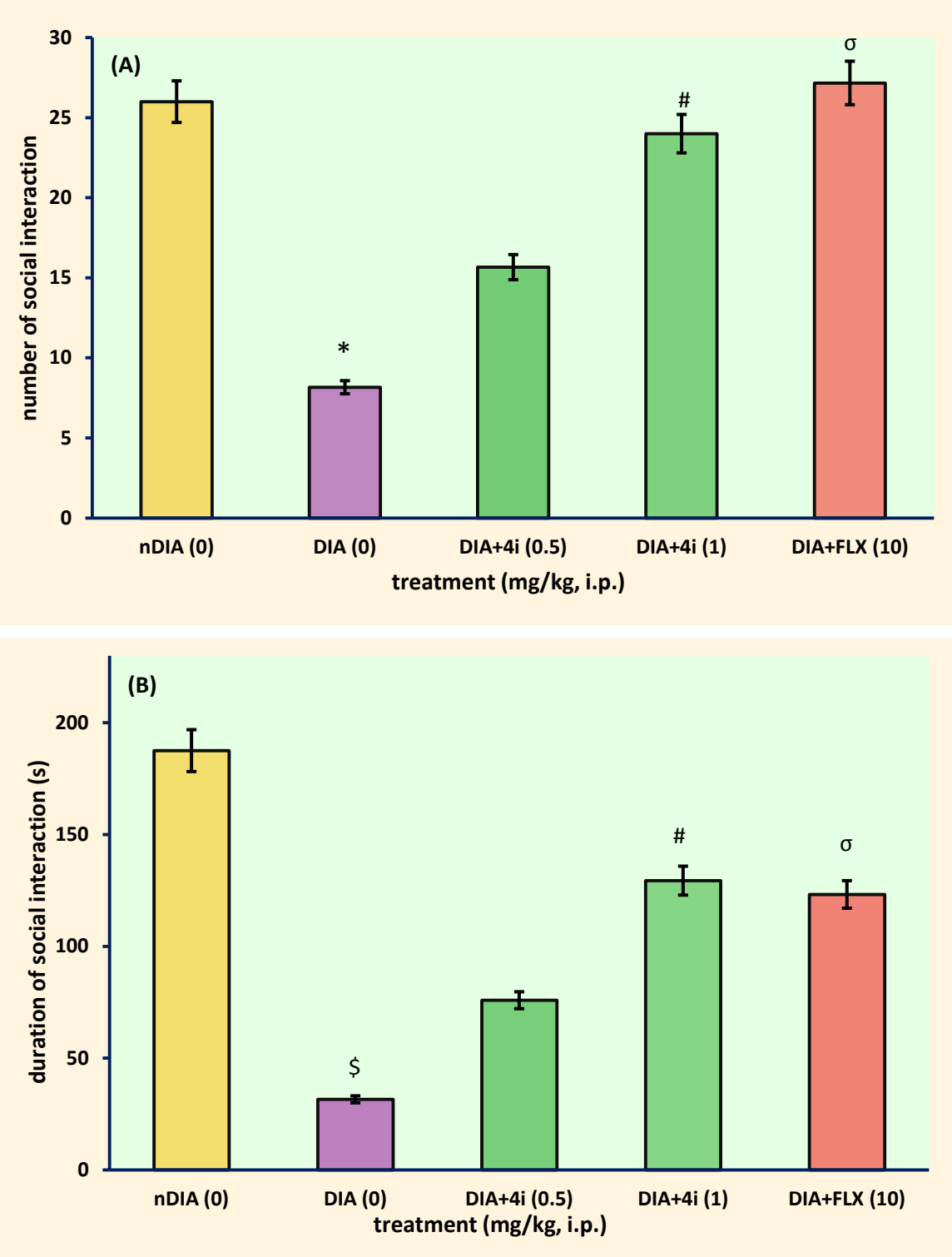


Fig. 6.28 The columns indicate mean values of number of social interaction **(A)** and time spent in social interaction **(B)** in social interaction test and error bars show S.E.M. * $p < 0.05$, **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, σ $p < 0.05$, vs diabetic control group, n = 6/group.

6.5.3 Effect of 6z on depression-like behavior evoked in STZ-induced diabetic mice

6.5.3.1 Effect of 6z on basal locomotor activity (BLA)

In BLA test, there was no significant effect on locomotor activity [F (4, 30) = 1.998, p > 0.05] of mice, subjected to different treatments. STZ-induced diabetes had no effect on the BLA, in mice (p > 0.05). In addition, neither **6z** (1 and 2 mg/kg, i.p.) nor FLX (10 mg/kg, i.p.) had significant effect on BLA in STZ-induced diabetic mice (p > 0.05), Fig. 6.29.

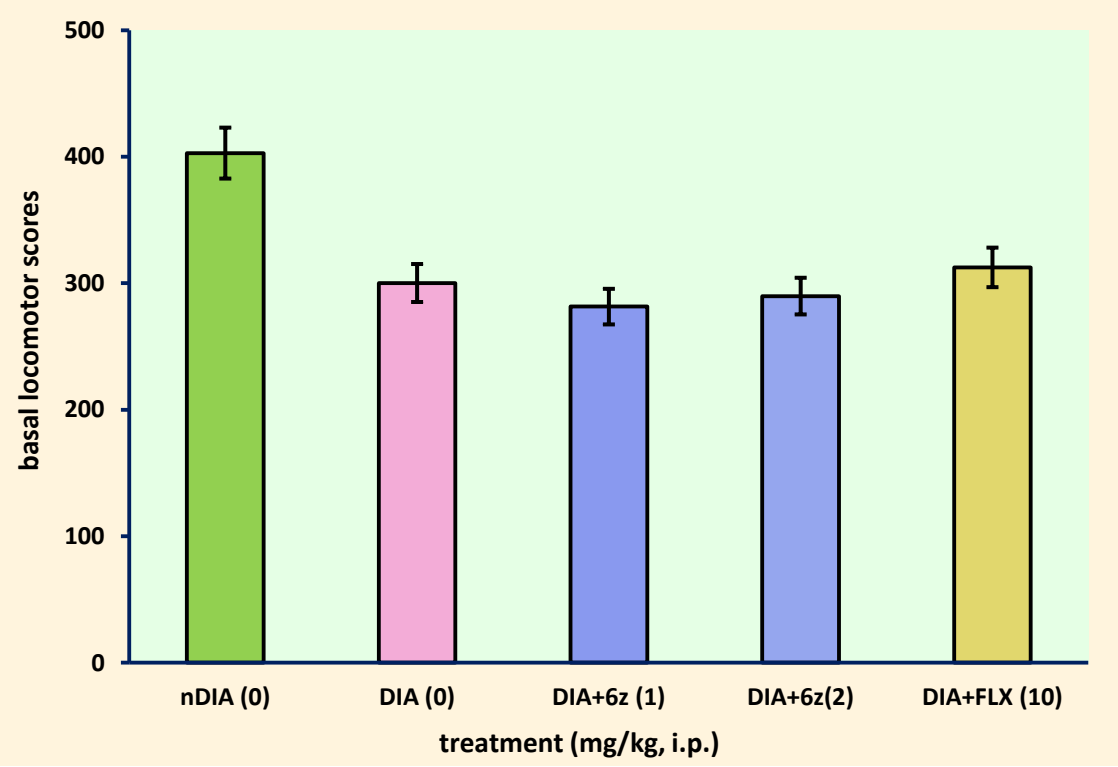


Fig. 6.29 The columns indicate mean values of BLA and error bars show S.E.M. There was no statistical difference among the groups, n = 6/group.

6.5.3.2 Effect of 6z on FST and TST

The effect of **6z**, on the depression-like behavior induced in STZ-induced diabetes, was evaluated using FST and TST models of depression. In FST, there was a significant change in duration of immobility, in mice subjected to different treatments [F (4, 30) = 6.224, p < 0.001].

The duration of immobility, significantly increased in STZ-induced diabetic mice as compared to control ($p < 0.001$). Chronic treatment with **6z** (1 and 2 mg/kg, i.p.) resulted in a significant reduction of the duration of immobility, in STZ-induced mice ($p < 0.05$). Similarly, chronic FLX (10 mg/kg, i.p.) administration reversed the increase in duration of immobility (s) in STZ-induced diabetic mice ($p < 0.001$), as indicated in Fig. 6.30.

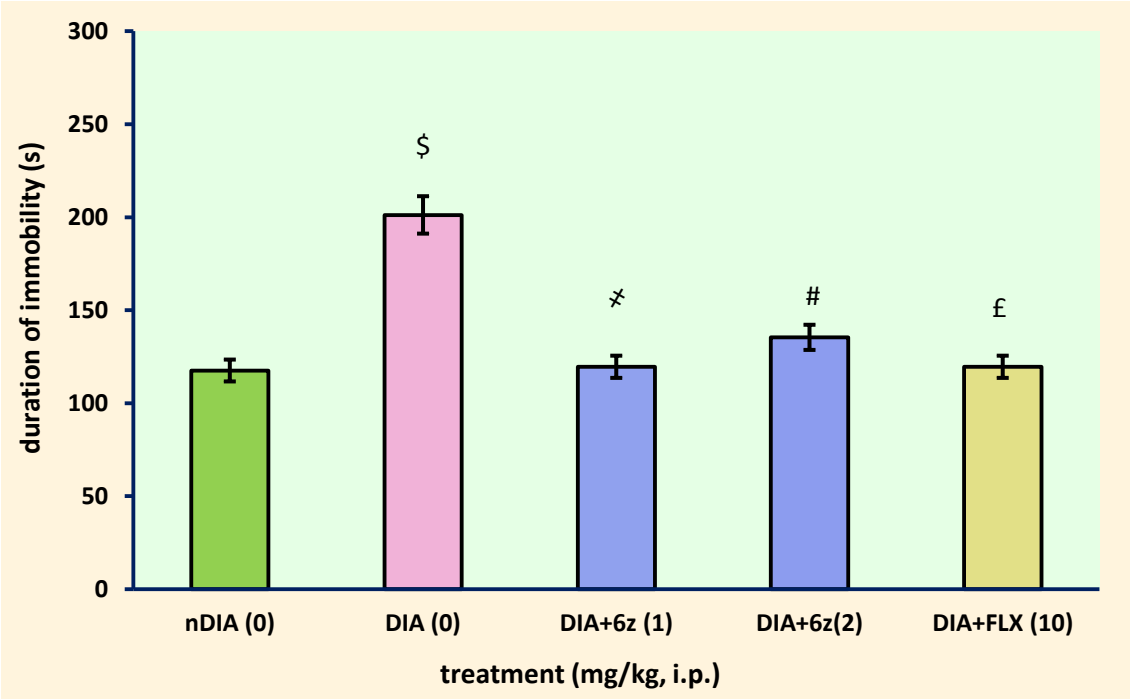


Fig. 6.30 The columns indicate mean values of duration of immobility (s) during FST and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

In TST, statistical analysis indicated that there was a significant change in duration of immobility, in mice, subjected to different treatments [$F (4, 30) = 5.433$, $p < 0.01$]. The duration of immobility was significantly increased in STZ-induced diabetic mice, as compared to control ($p < 0.05$). Chronic treatment with **6z** (1 and 2 mg/kg, i.p.) significantly reversed the increase in duration of immobility in STZ-induced mice ($p < 0.05$).

In addition, chronic FLX (10 mg/kg, i.p.) administration reversed the increase in duration of immobility (s) in STZ-induced diabetic mice ($p < 0.001$), as indicated in Fig. 6.31.

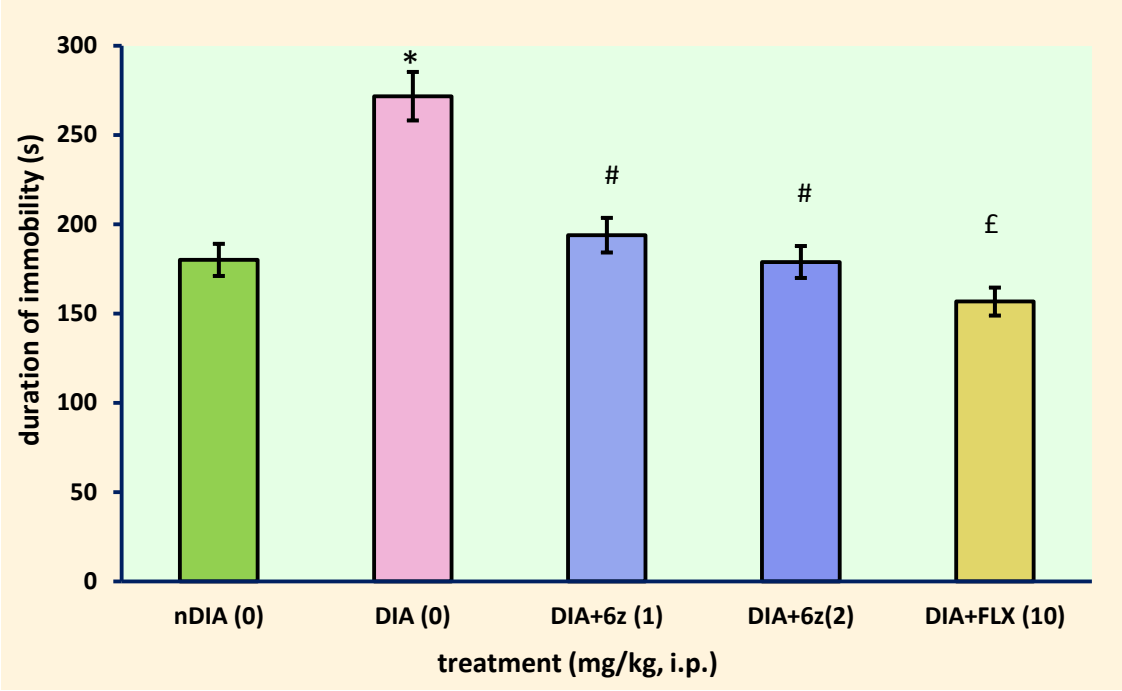
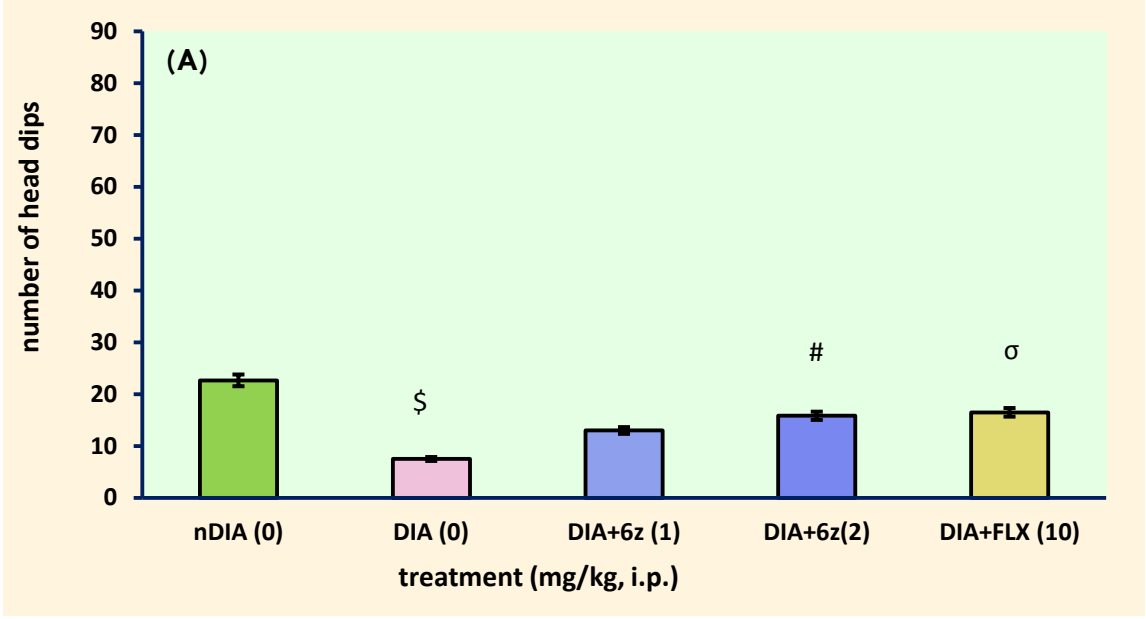


Fig. 6.31 The columns indicate mean values of duration of immobility (s) during TST and error bars show S.E.M.* $p < 0.05$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.5.3.3 Effect of 6z on hole-board test, light-dark test and social interaction test

The effect of **6z** on anxiety-like behavior evoked in STZ-induced diabetes was examined using hole-board test and social interaction test behavioral models. A significant change in number of head dips [$F (4, 30) = 8.511$, $p < 0.001$] and duration of head dips [$F (4, 30) = 9.544$, $p < 0.01$] was observed, in mice, subjected to different treatments. When tested in hole-board test, STZ-induced diabetic mice exhibited a significant decrease in number as well as duration of head dips as compared to control ($p < 0.001$). Chronic treatment with **6z** (2 mg/kg, i.p.) produced a significant reversal in these parameters ($p < 0.05$). On the other hand, **6z** at 1 mg/kg only reversed the duration of head dips in STZ-induced diabetic mice, but had no effect on the number of head dips ($p > 0.05$), as compared with STZ-induced diabetic mice that received vehicle treatment.

Besides, chronic FLX (10 mg/kg, i.p.) treatment, significantly reversed the reduction in both number ($p < 0.05$) as well as duration of head dips ($p < 0.05$) in STZ-induced diabetic mice, as depicted in Fig. 6.32A and B.



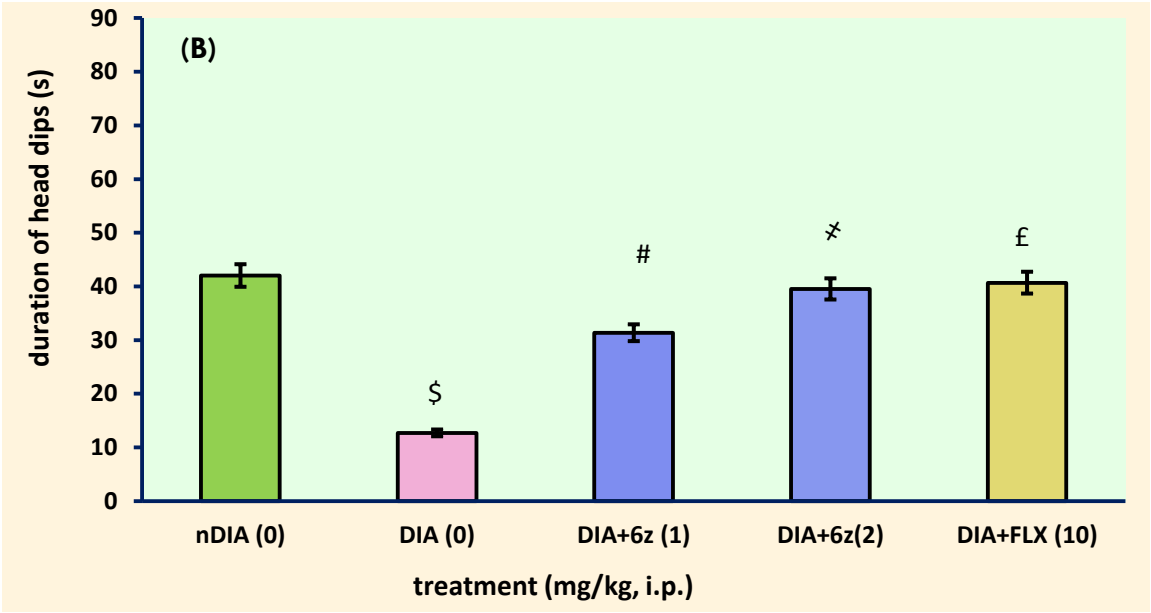


Fig. 6.32 The columns indicate mean values of number of head dips **(A)** and duration of head dips **(B)** in hole-board test and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, σ $p < 0.05$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

In social interaction test, statistical analysis indicated that there was a significant change in number of social interaction [Kruskal-Wallis statistic = 8.017, $p < 0.001$] and time spent in social interaction (s) [Kruskal-Wallis statistic = 7.261, $p < 0.01$], in mice, subjected to different treatments.

The number as well as time spent in social interactions, was significantly reduced in diabetic mice, as compared to control ($p < 0.001$). **6z** (2 mg/kg, i.p.) and FLX treatment significantly reversed the decrease in number and time spent in social interaction (s), in STZ-induced diabetic mice ($p < 0.05$). On the other hand, **6z** at 1 mg/kg, reversed only the time spent in social interaction in STZ-induced diabetic mice ($p < 0.001$), but had no effect on the number of social interaction ($p > 0.05$), as compared with STZ-induced diabetic mice that received vehicle treatment, Fig. 6.33A and B.

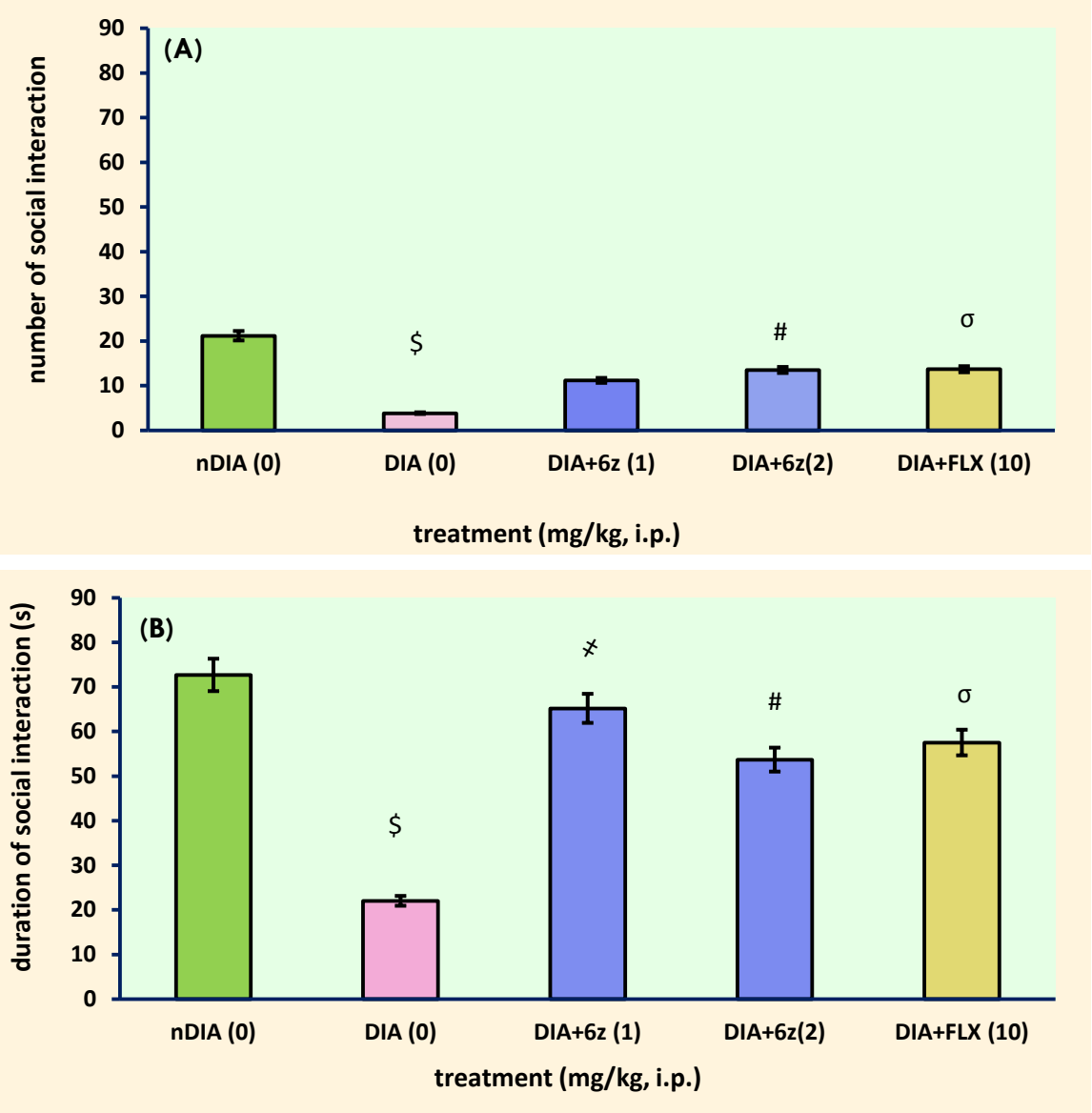


Fig. 6.33 The columns indicate mean values of number of social interaction **(A)** and duration of social interaction **(B)** in social interaction test and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, σ $p < 0.05$ vs diabetic control group, n = 6/group.

6.6 Effect of diabetes and drug treatments on biochemical, neurobiological and histological changes

From the behavioral studies, it may be concluded that STZ-induced diabetic mice exhibited a significant depressive phenotypic behavior, which was reversed by chronic treatment with OND (0.5 and 1 mg/kg, i.p.), **4i** (0.5 and 1 mg/kg, i.p.) and **6z** (1 and 2 mg/kg, i.p.) and hence, the biochemical, neurobiological and histo-morphological studies were carried out, subsequently, to evaluate the underlying cause and possible mechanism of action of tested drug candidates.

6.6.1 Effect on 5-HT levels in discrete brain areas

6.6.1.1 Effect of diabetes and OND

The effect of STZ-induced diabetes and OND (0.5 and 1 mg/kg, i.p.) treatment, was examined on the 5-HT levels in discrete brain regions (midbrain including hippocampus, frontal cortex and cerebellum).

There was a significant change in the percentage of 5-HT level in midbrain (including hippocampus) [$F(4, 30) = 6.375$, $p < 0.001$], frontal cortex [$F(4, 30) = 17.570$, $p < 0.001$], and cerebellum [$F(4, 30) = 6.976$, $p < 0.001$], among the groups subjected to different treatments.

In midbrain, STZ-induced diabetic mice exhibited a significant reduction in 5-HT levels as compared to control group ($p < 0.001$), which was significantly reversed by chronic treatment with OND (0.5 and 1 mg/kg, i.p.) ($p < 0.05$). Similarly, FLX (10 mg/kg, i.p.) treatment significantly reversed the decrease in percentage of 5-HT level in midbrain of STZ-induced diabetic mice ($p < 0.05$). In frontal cortex, there was a significant decrease in percentage of 5-HT level in STZ-induced diabetic mice given vehicle treatment as compared to control mice ($p < 0.001$), whereas OND (1 mg/kg, i.p.) significantly reversed the decrease in percentage of 5-HT level in frontal cortex of STZ-induced diabetic mice ($p < 0.05$) an effect likely produced by chronic FLX (10 mg/kg, i.p.) treatment ($p < 0.001$), Fig. 6.34.

However, the lower dose of OND (0.5 mg/kg, i.p.) had no significant effect on diabetes-induced decrease in 5-HT level in frontal cortex. Besides, a non-significant trend of reversal in STZ-induced diabetic mice was observed, as indicated in Fig. 6.34.

In cerebellum, STZ-induced diabetic mice exhibited a significant decrease in percentage of 5-HT level as compared to non-diabetic mice ($p < 0.001$). Chronic treatment with OND (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) significantly reversed the reduction in percentage of 5-HT level, in cerebellum of STZ-induced diabetic mice ($p < 0.05$), Fig. 6.34.

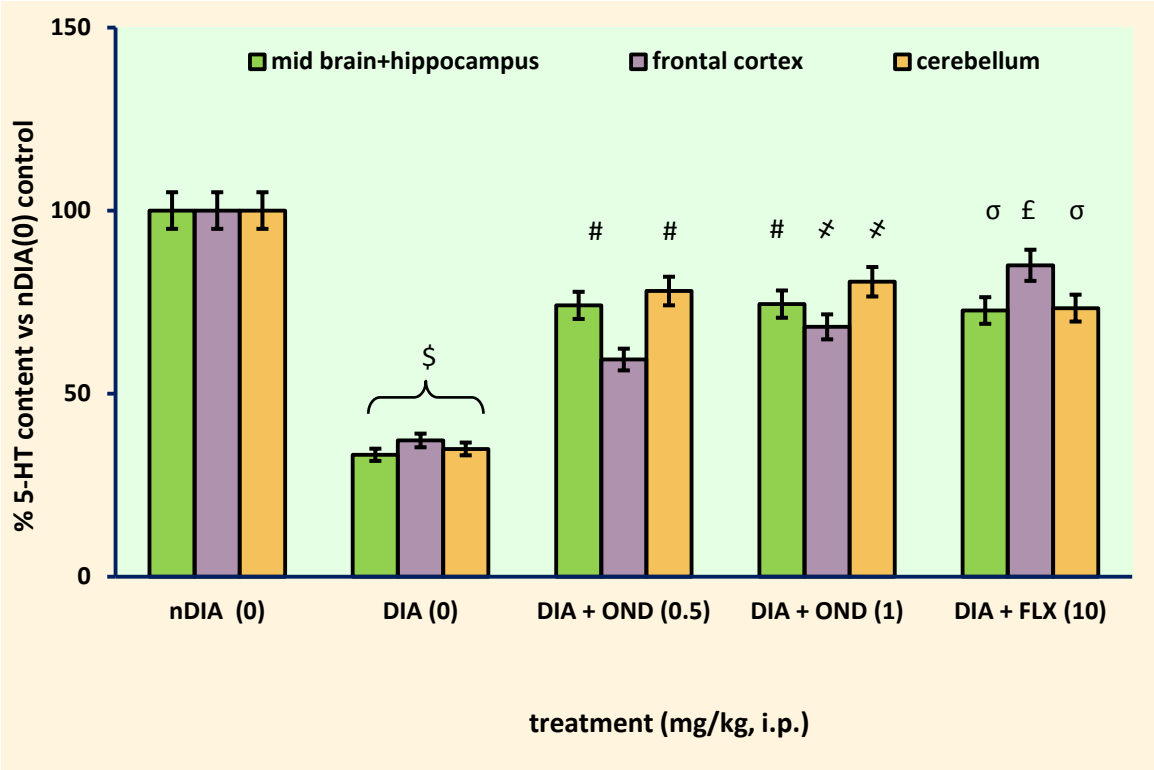


Fig. 6.34 The columns indicate mean values of 5-HT level measured as % with respect to control and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, σ $p < 0.05$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.6.1.2 Effect of diabetes and 4i

The effect of STZ-induced diabetes and **4i** (0.5 and 1 mg/kg, i.p.) treatment was examined on 5-HT levels in midbrain (including hippocampus), frontal cortex and cerebellum.

There was a significant change in the percentage of 5-HT level in midbrain (including hippocampus) [$F (4, 30) = 15.290$, $p < 0.001$], frontal cortex [$F (4, 30) = 13.870$, $p < 0.001$] and cerebellum [$F (4, 30) = 19.62$, $p < 0.001$], among the groups subjected to different treatments.

The decrease in percentage of 5-HT level was markedly reversed by chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.001$). Similarly, FLX (10 mg/kg, i.p.) treatment significantly reversed the decrease in percentage of 5-HT level in midbrain of STZ-induced diabetic mice ($p < 0.001$), as indicated in Fig. 6.35.

In frontal cortex, there was a significant decrease in percentage of 5-HT level in STZ-induced diabetic mice as compared to those without diabetes ($p < 0.001$) which was significantly reversed by chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.05$). Moreover, chronic FLX (10 mg/kg, i.p.) treatment significantly reversed the decrease in percentage of 5-HT level frontal cortex of diabetic mice ($p < 0.001$), Fig. 6.35.

In cerebellum, STZ-induced diabetic mice exhibited a significant decrease in percentage of 5-HT level as compared to non-diabetic mice ($p < 0.001$). Chronic treatment with **4i** (1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.), significantly reversed the reduction in percentage of 5-HT level in cerebellum in STZ-induced diabetic mice ($p < 0.001$). However, the lower dose of **4i** (0.5 mg/kg, i.p.) although, increased the percentage of 5-HT level in cerebellum, the effect did not reach up to a statistically significant level, as represented in Fig. 6.35.

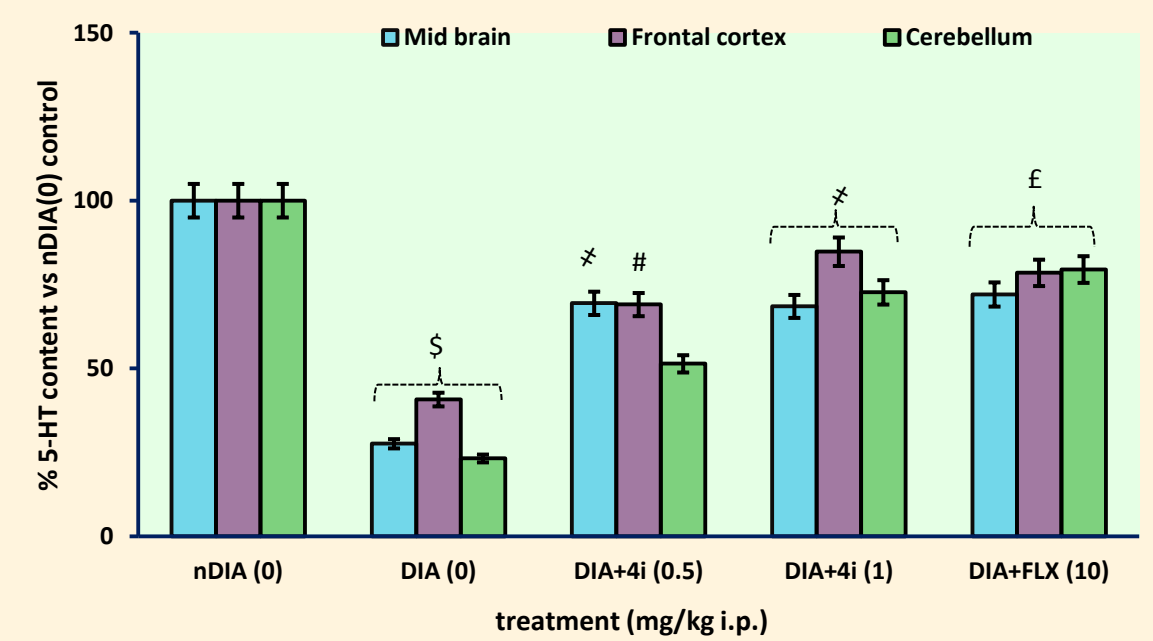


Fig. 6.35 The columns indicate mean values of 5-HT level measured as % with respect to control and error bars show S.E.M. $ $p < 0.001$ indicate significant difference vs control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.6.1.3 Effect of diabetes and 6z

Statistical analysis indicated that there was a significant change in the percentage of 5-HT level in midbrain (including hippocampus) [F (4, 30) = 17.96, p < 0.001], frontal cortex [F (4, 30) = 7.009, p < 0.001] and cerebellum [F (4, 30) = 5.393, p < 0.001], among the groups subjected to different treatments. When measured in midbrain, STZ-induced diabetic mice exhibited a significant reduction in percentageof 5-HT levels as compared to control group (p < 0.001). The decrease in percentage of 5-HT level was significantly reversed by chronic treatment with **6z** (1-2 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) (p < 0.001), Fig. 6.36.

In frontal cortex, there was a significant decrease in percentage of 5-HT level in STZ-induced diabetic mice given vehicle treatment as compared to those without diabetes (p < 0.001). Interestingly, chronic dosing with **6z** (1 and 2 mg/kg, i.p.) or FLX (10 mg/kg, i.p.) significantly reversed the decrease in percentage of 5-HT level in frontal cortex of STZ-induced diabetic mice (p < 0.05), Fig. 6.36.

When estimated in cerebellum, STZ-induced diabetic mice exhibited a significant decrease in percentage of 5-HT level as compared to non-diabetic mice (p < 0.001). Chronic treatment with **6z** (1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) significantly reversed the reduction in percentage of 5-HT level in cerebellum in STZ-induced diabetic mice (p < 0.05). However, the **6z** (2 mg/kg, i.p.) although, increased the percentage of 5-HT level in cerebellum, the effect did not reach up to a statistically significant level, Fig. 6.36.

6.6.2 Effect on GABA levels in discrete brain areas

6.6.2.1 Effect of diabetes and OND

In addition, the effects of STZ-induced diabetes and OND (0.5 and 1 mg/kg, i.p.) treatment on the percentage of GABA levels in discrete brain regions such as midbrain (including hippocampus), frontal cortex and cerebellum were examined. A significant change in the percentage of GABA levels in midbrain [F (4, 30) = 19.782, p < 0.001], frontal cortex [F (4, 30) = 6.129, p < 0.01] and cerebellum [F (4, 30) = 12.903, p < 0.001] was observed among the groups. In midbrain the percentage of GABA levels were significantly increased in STZ-induced diabetic mice as compared to control group (p < 0.001), which was significantly reversed by chronic treatment with OND (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) (p < 0.001), as represented in Fig. 6.37.

In frontal cortex, there was a significant increase in percentage of GABA in level in STZ-induced diabetic mice given vehicle treatment as compared to those without diabetes ($p < 0.001$).

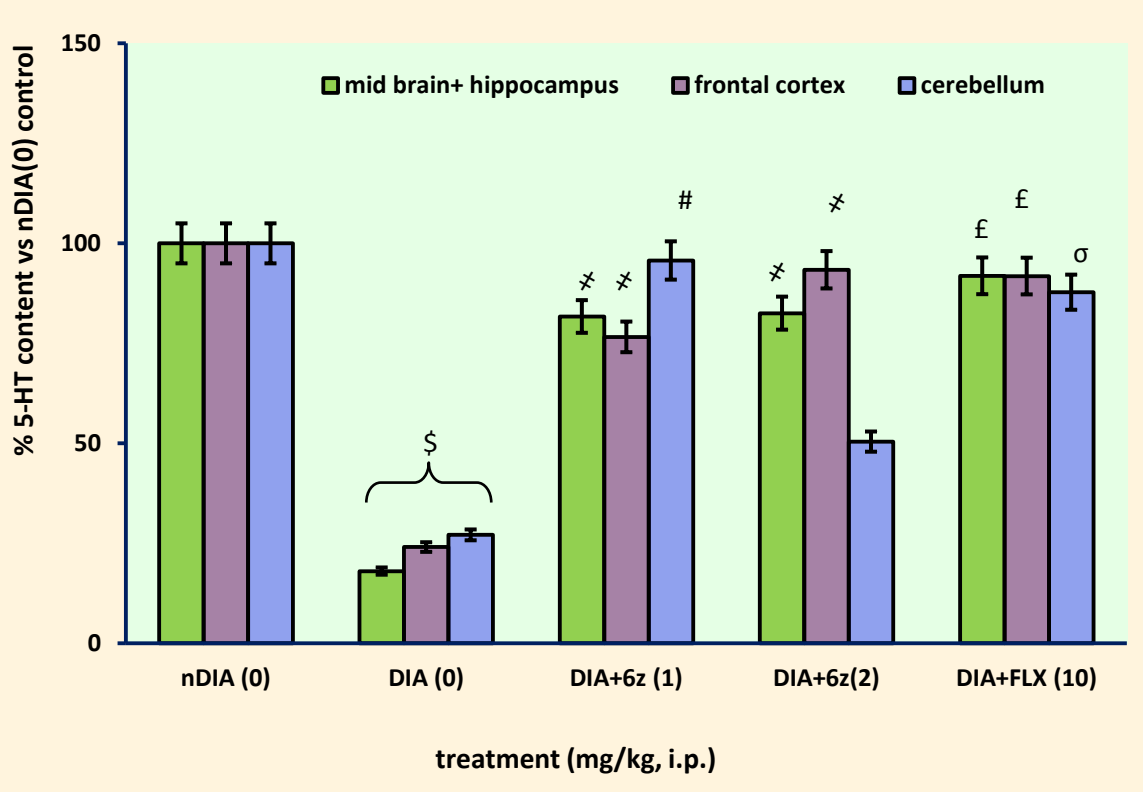


Fig. 6.36 The columns indicate mean values of 5-HT level measured as % with respect to control and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, σ $p < 0.05$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

On the other hand, chronic dosing with OND (0.5 and 1 mg/kg, i.p.) or FLX (10 mg/kg, i.p.) significantly reversed the increase in percentage of GABA level in frontal cortex of STZ-induced diabetic mice ($p < 0.05$), as indicated in Fig. 6.37. A similar pattern was observed in cerebellum.

STZ-induced diabetic mice exhibited a significant increase in percentage of GABA level as compared to non-diabetic mice ($p < 0.001$). Chronic treatment with OND (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) significantly reversed the increase in percentage of GABA level in cerebellum in STZ-induced diabetic mice ($p < 0.05$), as represented in Fig. 6.37.

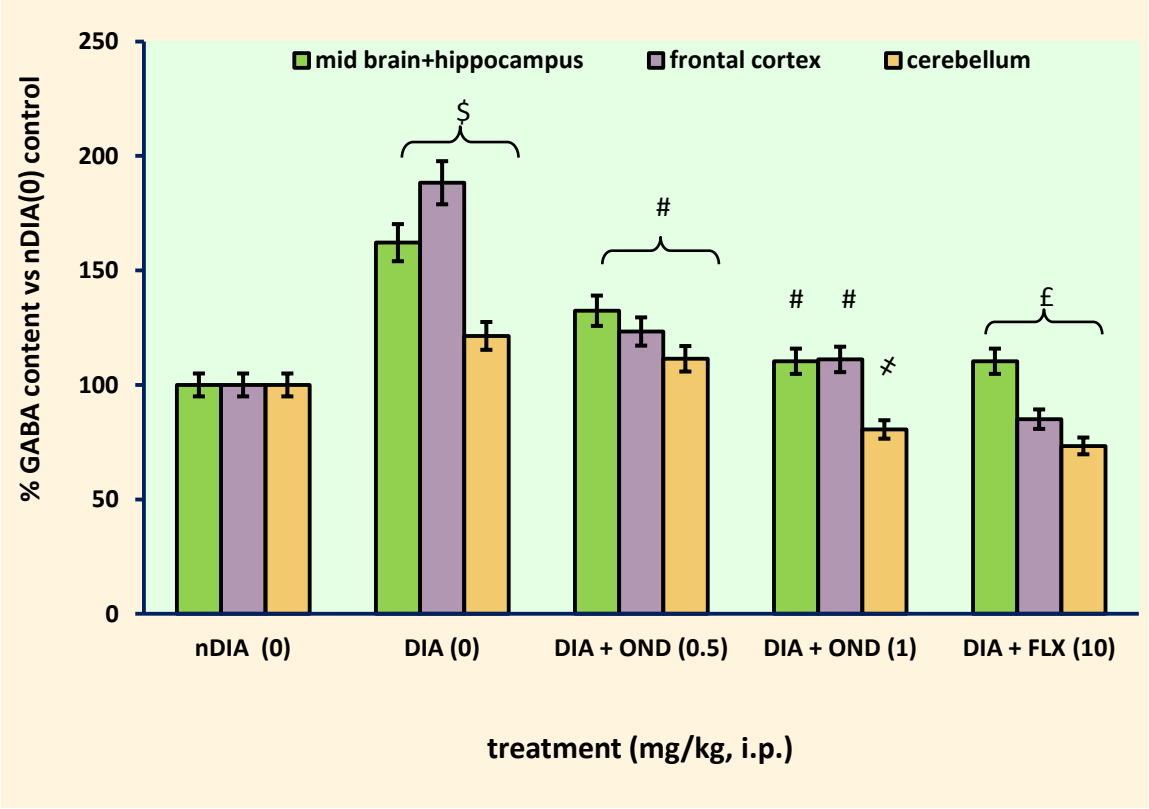


Fig. 6.37 The columns indicate mean values of GABA level measured as % with respect to control and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.6.2.2 Effect of diabetes and 4i

A significant change in the percentage of GABA levels in midbrain (including hippocampus) [$F(4, 30) = 15.913$, $p < 0.001$], frontal cortex [$F(4, 30) = 12.732$, $p < 0.01$] and cerebellum [$F(4, 30) = 17.930$, $p < 0.001$] was observed among the groups. In midbrain, STZ-induced diabetic mice exhibited a significant increase in percentage of GABA level as compared to control group ($p < 0.001$). The elevation in percentage of GABA level was significantly reversed by chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) ($p < 0.001$).

Likewise, in frontal cortex, there was a significant increase in percentage of GABA in level in STZ-induced diabetic mice given vehicle treatment as compared to those without diabetes ($p < 0.001$). On the other hand, chronic dosing with **4i** (0.5 and 1 mg/kg, i.p.) or FLX (10 mg/kg, i.p.) significantly reversed the increase in percentage of GABA level in frontal cortex of STZ-induced diabetic mice ($p < 0.001$).

In cerebellum, STZ-induced diabetic mice exhibited a significant increase in percentage of GABA level as compared to non-diabetic mice ($p < 0.001$), which was significantly reversed by **4i** (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) significantly ($p < 0.001$), Fig. 6.38.

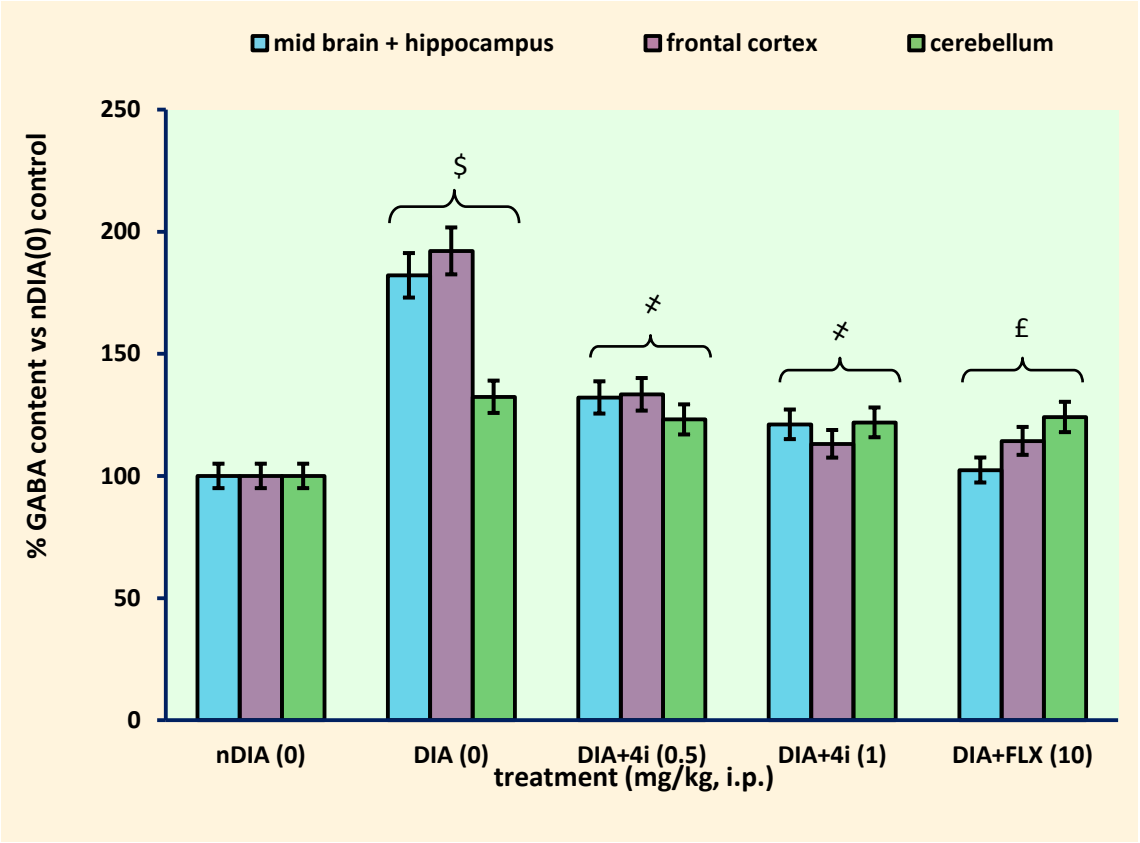


Fig. 6.38 The columns indicate mean values of GABA level measured as % with respect to control and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.6.2.3 Effect of diabetes and 6z

The effect of STZ-induced diabetes and **6z** (1 and 2 mg/kg, i.p.) treatment on the percentage of GABA level in discrete brain regions such as midbrain (including hippocampus), frontal cortex and cerebellum were estimated. There was a significant change in the percentage of GABA level in midbrain (including hippocampus) [$F (4, 30) = 11.007$, $p < 0.001$], frontal cortex [$F (4, 30) = 10.320$, $p < 0.01$] and cerebellum [$F (4, 30) = 6.932$, $p < 0.001$] among the groups subjected to different treatments.

STZ-induced diabetic mice exhibited a significant increase in GABA levels in midbrain as compared to control group ($p < 0.001$). The elevation in percentage of GABA level was significantly reversed by chronic treatment with **6z** (1 and 2 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) ($p< 0.05$), as represented in Fig. 6.39. In frontal cortex, there was a significant increase in percentage of GABA in level in STZ-induced diabetic mice given vehicle treatment as compared to those without diabetes ($p < 0.001$).

Moreover, chronic dosing with **6z** (1 and 2 mg/kg, i.p.) or FLX (10 mg/kg, i.p.) significantly reversed the increase in percentage of GABA level in frontal cortex of STZ-induced diabetic mice ($p < 0.001$), as indicated in Fig. 6.39.

In cerebellum, STZ-induced diabetic mice exhibited a significant increase in percentage of GABA level as compared to non-diabetic mice ($p < 0.05$). **6z** (1 and 2 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) treatments significantly reversed the increase in percentage of GABA level in cerebellum in STZ-induced diabetic mice ($p < 0.05$), Fig. 6.39.

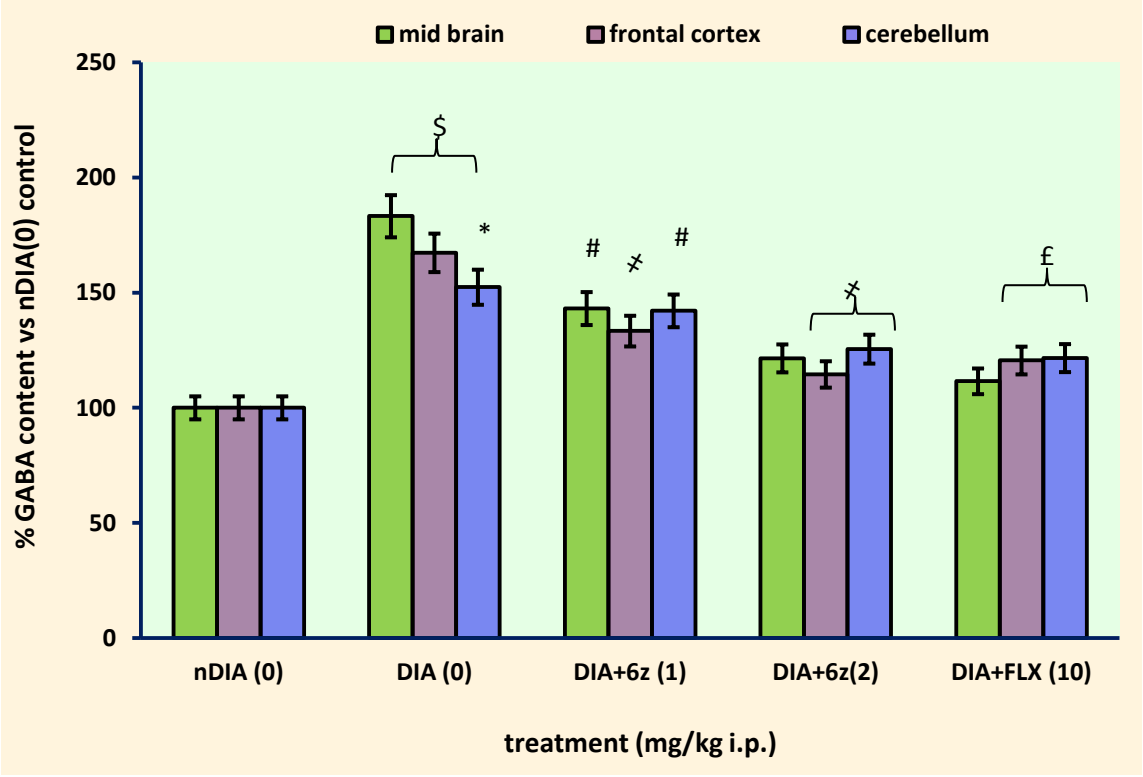


Fig. 6.39 The columns indicate mean values of GABA level measured as % with respect to control and error bars show S.E.M.*$p < 0.05$, **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.6.2 Effect on plasma CORT level

6.6.2.1 Effect of diabetes and OND

There was a significant change in the plasma CORT level, a marker of HPA-axis activity, in mice subjected to different treatments [F (4, 30) = 12.680, p < 0.001]. STZ-induced diabetic mice exhibited a significant increase in plasma CORT level as compared to that of non-diabetic mice (p < 0.001). Chronic OND (0.5 and 1 mg/kg, i.p.) administration significantly reversed the elevation in plasma CORT level in STZ-induced diabetic mice (p < 0.001), and the effect was similar to that of chronic FLX treatment (p < 0.05), as indicated in Fig. 6.40.

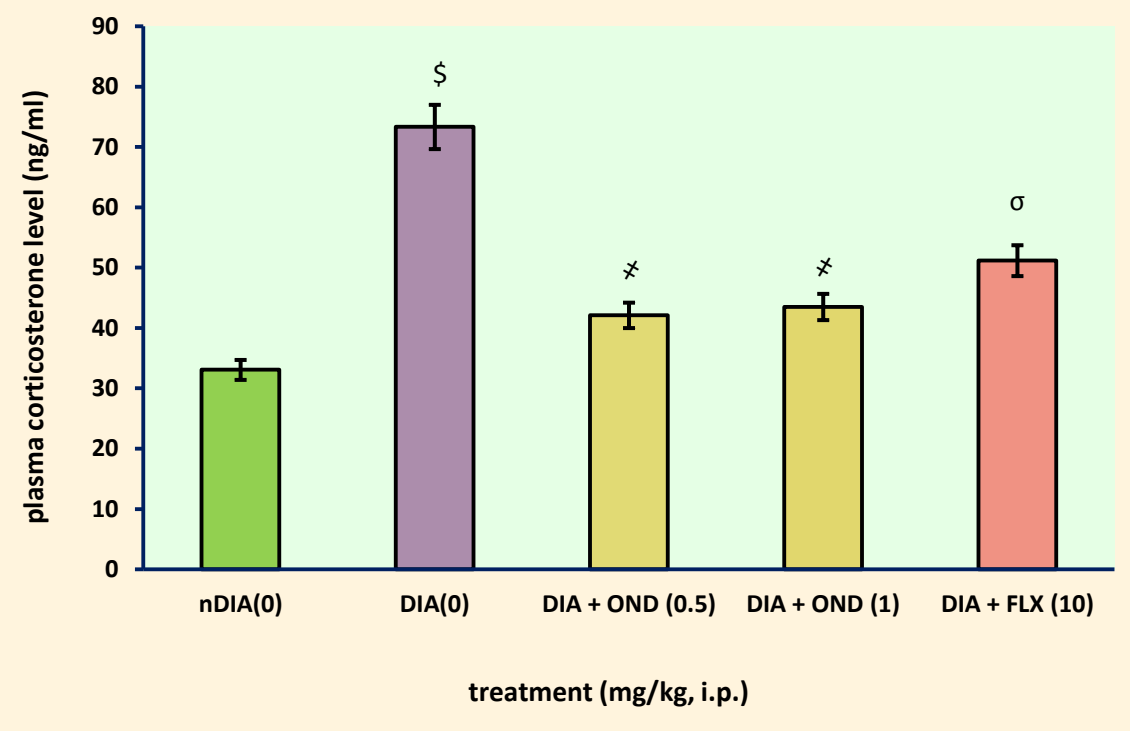


Fig. 6.40 The columns indicate mean values of plasma corticosterone level (ng/ml) and error bars show S.E.M. **$** p < 0.001 indicate significant difference compared to control and # p < 0.05, ≠ p < 0.001, σ p < 0.05 vs diabetic control group, n = 6/group.

6.6.2.2 Effect of diabetes and 4i

Plasma CORT level were significantly differ, in mice subjected to different treatments [F (4, 30) = 11.410, p < 0.001]. There was a significantly increased plasma CORT level in STZ-induced diabetic mice as compared to that of non-diabetic mice (p < 0.001).

Interestingly, chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) significantly reversed the elevation of plasma CORT level in STZ-induced diabetic mice ($p < 0.05$), as indicated in Fig. 6. 41.

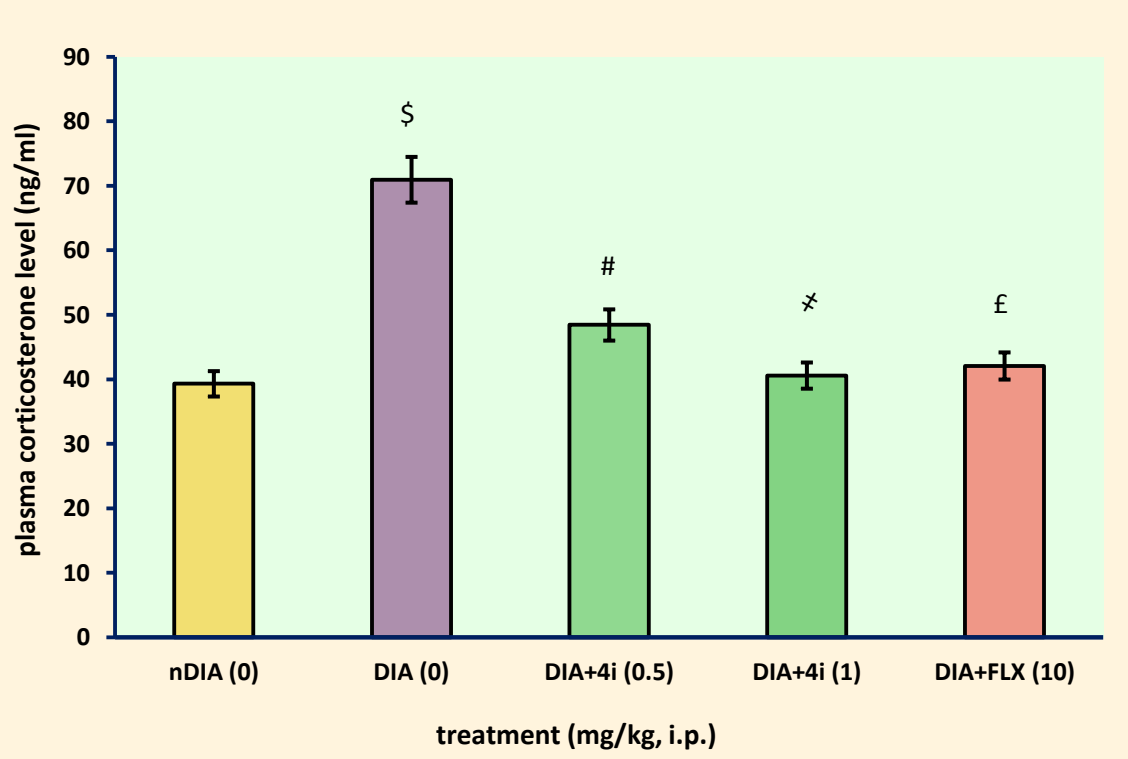


Fig. 6.41 The columns indicate mean values of plasma CORT level (ng/ml) and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6 /group.

6.6.2.3 Effect of diabetes and 6z

A significant change in the plasma CORT level was observed, in mice subjected to different treatments [$F (4, 30) = 10.420$, $p < 0.001$]. STZ-induced diabetic mice exhibited a significant increase in plasma CORT level as compared to that of non-diabetic mice ($p < 0.001$).

Whereas, chronic **6z** (1 and 2 mg/kg, i.p.) administration significantly reversed the elevation of plasma CORT level to normal in STZ-induced diabetic mice ($p < 0.05$). Similarly, chronic FLX treatment ($p < 0.001$) reduced the elevated plasma CORT level in STZ-induced diabetic mice, as indicated in Fig. 6.42.

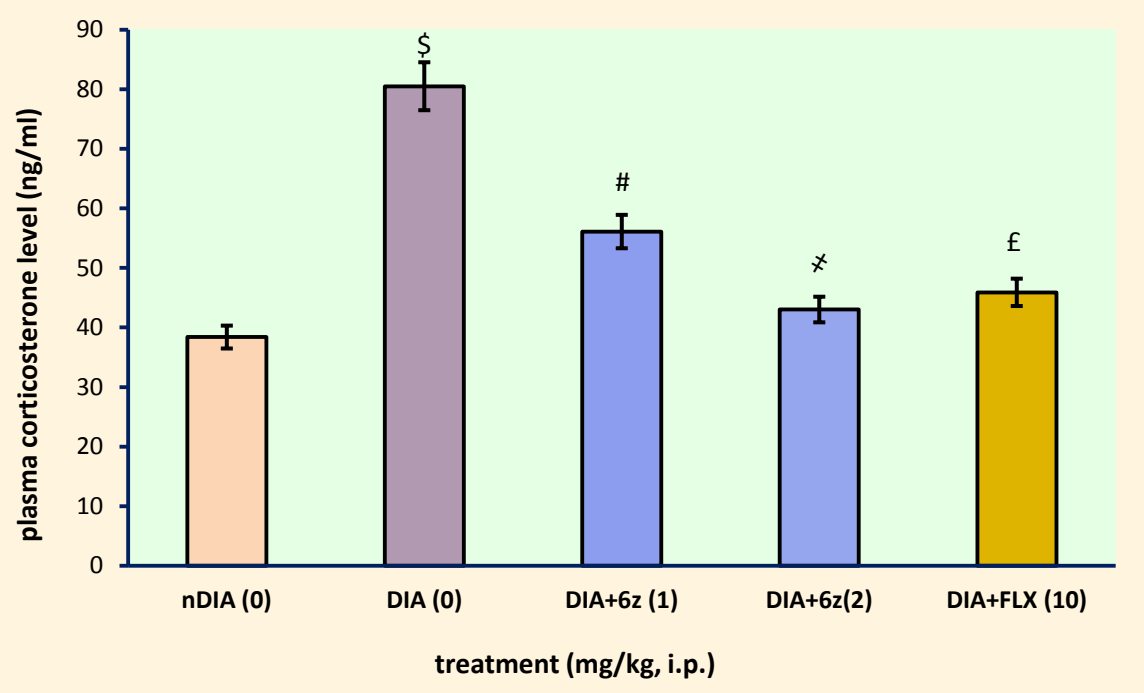


Fig. 6.42 The columns indicate mean values of plasma CORT level (ng/ml) and error bars show S.E.M. $ p < 0.001 indicate significant difference compared to control and # p < 0.05, ≠ p < 0.001, £ p < 0.001 vs diabetic control group, n = 6/group.

6.6.3 Effect on brain oxidative/nitrosative stress markers

The effect of diabetes and 5-HT_3 receptor antagonists on oxidative stress markers were estimated in terms of pro-oxidant markers and anti-oxidant enzyme activity, in discrete brain regions.

6.6.3.1 Effect of diabetes and OND

Lipid peroxidation

The effects of diabetes and OND on lipid peroxidation were estimated as the level of TBARS in midbrain (including hippocampus) and frontal cortex. There was a significant change in TBARS level among the groups subjected to different treatments in midbrain [F (4, 30) = 10.530, p < 0.001] and in frontal cortex [F (4, 30) = 12.156, p < 0.001]. STZ-induced diabetic mice exhibited a significant increase (p < 0.001) in TBARS level in midbrain as well as in frontal cortex and this was significantly and dose dependently reduced by chronic treatment with OND (0.5 and 1 mg/kg, i.p.) (p < 0.05); similar to that of the effect produced by FLX (10 mg/kg, i.p.) (p < 0.001), Table 6.5.

Nitrite level

The effects of diabetes and OND on nitrite level in discrete brain regions were estimated. There was a significant change in the nitrite level among the groups subjected to different treatments in midbrain (including hippocampus) [$F(4, 30) = 9.039$, $p < 0.001$] and frontal cortex [$F(4, 30) = 12.782$, $p < 0.001$].

In midbrain and frontal cortex, nitrite levels were significantly increased in STZ-induced diabetic mice as compared to non-diabetic mice ($p < 0.001$). Whereas, chronic treatment with OND (0.5 and 1 mg/kg, i.p.) ($p < 0.001$) and FLX (10 mg/kg, i.p.) ($p < 0.001$) significantly reversed the increase in nitrite level in midbrain of STZ-induced diabetic mice. In addition, OND (0.5 and 1 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.05$) treatment significantly reversed the increase in nitrite level in frontal cortex of these mice, as given in Table 6.5.

Reduced glutathione

The effects of diabetes and OND on GSH, an antioxidant enzyme activity were estimated. There was a significant effect on the GSH level in midbrain [$F(4, 30) = 5.808$, $p < 0.001$] and in frontal cortex [$F(4, 30) = 6.245$, $p < 0.001$] among the groups subjected to different treatments. STZ-induced diabetic mice exhibited a significant decline ($p < 0.001$) in GSH level in midbrain and frontal cortex; this effect was significantly reversed by chronic treatment with OND (0.5 and 1 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$) in both midbrain and hippocampus, as shown in Table 6.5.

Catalase

The effects of diabetes and OND on catalase activity were estimated and a significant change in the catalase activity in midbrain [$F(4, 30) = 5.217$, $p < 0.001$] and in frontal cortex [$F(4, 30) = 3.157$, $p < 0.01$] was observed among the groups. STZ-induced diabetic mice exhibited a significant decrease in catalase activity in midbrain ($p < 0.05$) and in frontal cortex ($p < 0.001$). Chronic treatment with OND (1 mg/kg, i.p.) ($p < 0.001$) and FLX (10 mg/kg, i.p.) ($p < 0.001$) significantly reversed the decrease in catalase activity in midbrain of STZ-induced diabetic mice, whereas the lower dose of OND (0.5 mg/kg, i.p.) although increased the midbrain catalase activity in STZ-induced diabetic mice, the effect was not statistically significant.

In frontal cortex of STZ-induced diabetic mice, chronic treatment with OND (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) significantly increased the catalase activity, as given in Table 6.5.

Table 6.5 Effect of diabetes and OND treatment on oxidative stress parameters in discrete brain regions

Parameters	TBARS (nmoles/mg pr)	Nitrite levels (µg/mg pr)	Catalase activity (U/mg pr)	GSH level (µmoles/mg pr)
Mid brain (including hippocampus)				
nDIA (0)	1.432 ± 0.193	37.371 ± 5.011	0.867 ± 0.191	0.147 ± 0.033
DIA (0)	6.550 ± 0.932 **$**	117.327 ± 11.741 **$**	0.213 ± 0.051 *	0.016 ± 0.011 **$**
DIA-OND (0.5)	3.020 ± 0.721 #	57.213 ± 10.321 ≠	0.701 ± 0.144	0.139 ± 0.012 #
DIA-OND (1)	2.781 ± 0.872 ≠	50.182 ± 16.861 ≠	0.895 ± 0.171 ≠	0.127 ± 0.014 #
DIA-FLX (10)	2.662 ± 0.601 £	49.140 ± 11.312 £	0.885 ± 0.183 £	0.146 ± 0.013 £
Frontal cortex				
nDIA (0)	2.51 ± 0.891	51.723 ± 4.154	0.912 ± 0.173	0.219 ± 0.035
DIA (0)	8.182 ± 1.243 **$**	103.782 ± 6.901 **$**	0.314 ± 0.164 *	0.058 ± 0.004 **$**
DIA-OND (0.5)	4.154 ± 0.893 #	59.124 ± 7.352 #	0.715 ± 0.190 #	0.191 ± 0.009 #
DIA-OND (1)	3.784 ± 0.673 ≠	51.534 ± 12.895 ≠	0.883 ± 0.192 ≠	0.197 ± 0.012 #
DIA-FLX (10)	3.782 ± 1.242 £	55.352 ± 10.302 σ	0.914 ± 0.140 £	0.167 ± 0.008 £

Values represent mean ± S.E.M. *p < 0.05, **$** p < 0.001 vs control group, # p < 0.05, ≠ p < 0.001, σ p < 0.05, £ p < 0.001 vs diabetic control group, n = 6/group.

6.6.3.2 Effect of diabetes and 4i

Lipid peroxidation

The effects of diabetes and **4i** on TBARS levels were estimated, which is a marker of lipid peroxidation. There was a significant change in the TBARS level in midbrain [F (4, 30) = 14.322, p < 0.001] and in frontal cortex [F (4, 30) = 18.572, p < 0.001] among the groups subjected to different treatments.

STZ-induced diabetic mice exhibited a significant increase in TBARS levels in midbrain ($p < 0.001$) and frontal cortex ($p < 0.001$). This increase in TBARS levels in midbrain as well as in frontal cortex was significantly reduced by chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$), Table 6.6.

Nitrite level

The effects of diabetes and **4i** on nitrite level in discrete brain regions were estimated. It was found that there was a significant change in nitrite level in midbrain [$F (4, 30) = 10.432$, $p < 0.001$] as well as in frontal cortex [$F (4, 30) = 12.151$, $p < 0.001$] among the groups subjected to different treatments.

STZ-induced diabetic mice produced a significant increase ($p < 0.05$) in nitrite level in midbrain and frontal cortex, while, chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$) significantly reversed this effect. Similarly, chronic **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.001$) and FLX (10 mg/kg, i.p.) treatment ($p < 0.001$) significantly reversed the increase in nitrite level in frontal cortex of STZ-induced diabetic mice, Table 6.6.

Reduced glutathione

The effects of diabetes and **4i** on GSH were estimated. A significant change in the GSH level in midbrain [$F (4, 30) = 7.658$, $p < 0.001$] as well as in frontal cortex [$F (4, 30) = 6.211$, $p < 0.001$] was found. In midbrain and frontal cortex, GSH level was significantly low in STZ-induced diabetic mice as compared to control group ($p < 0.001$). This effect was significantly reversed by chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$), Table 6.6.

Catalase

Catalase activity was significantly altered in midbrain [$F (4, 30) = 5.154$, $p < 0.001$] and frontal cortex [$F (4, 30) = 8.531$, $p < 0.001$] among the groups subjected to different treatments. There was a significant reduction in catalase activity in STZ-induced diabetic mice in midbrain ($p < 0.05$) as well as in frontal cortex ($p < 0.05$) as compared to control mice. Chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.), ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$) significantly reversed the decrease in midbrain and frontal cortex catalase activity in STZ-induced diabetic mice, Table 6.6.

Table 6.6 Effect of diabetes and 4i on the brain oxidative stress parameters

Parameters	TBARS (nmoles/mg pr)	Nitrite levels (µg/mg pr)	Catalase activity (U/mg pr)	GSH level (µmoles/mg pr)
Mid brain (including hippocampus)				
nDIA (0)	1.631 ± 0.182	43.831 ± 4.434	0.911 ± 0.100	0.163 ± 0.026
DIA (0)	8.120 ± 0.255 $	98.137 ± 15.343 *	0.191 ± 0.052 *	0.029 ± 0.004 $
DIA-4i (0.5)	2.142 ± 0.541 ≠	50.143 ± 11.72 #	0.614 ± 0.151 #	0.098 ± 0.014 #
DIA-4i (1)	2.011 ± 0.426 ≠	42.253 ± 13.814 #	0.713 ± 0.041 ≠	0.111 ± 0.051 #
DIA-FLX (10)	2.312 ± 0.514 £	41.662 ± 8.147 £	0.862 ± 0.138 £	0.132 ± 0.087 £
Frontal cortex				
nDIA (0)	2.144 ± 0.194	59.153 ± 4.434	1.002 ± 0.131	0.115 ± 0.023
DIA (0)	6.724 ± 0.543 $	113.893 ± 17.824 $	0.242 ± 0.089 $	0.021 ± 0.009 $
DIA-4i (0.5)	3.273 ± 0.352 #	68.452 ± 12.452 ≠	0.592 ± 0.159 #	0.093 ± 0.018 #
DIA-4i (1)	2.932 ± 0.251 ≠	52.145 ± 8.285 ≠	0.536 ± 0.083 ≠	0.098 ± 0.020 #
DIA-FLX (10)	2.419 ± 0.289 £	55.253 ± 7.903 £	0.832 ± 0.211 £	0.094 ± 0.029 £

Values represent mean ± S.E.M. *p < 0.05, $ p < 0.001 when compared with control group, # p < 0.05, ≠ p < 0.001, £ p < 0.001, when compared with diabetic control group, n = 6/group.

6.6.3.3 Effect of diabetes and 6z

Lipid peroxidation

TBARS levels were estimated in midbrain and frontal cortex as a marker of lipid peroxidation. Statistical analysis revealed a significant change in the level of TBARS in midbrain [F (4, 30) = 6.195, p < 0.001] and frontal cortex [F (4, 30) = 8.111, p < 0.001] among the groups.

STZ-induced diabetic mice exhibited a significant increase in TBARS level in midbrain (p < 0.05) as well as in frontal cortex (p < 0.05). This was significantly reversed by chronic treatment with **6z** (1 and 2 mg/kg, i.p.) (p < 0.05) and FLX (10 mg/kg, i.p.) (p < 0.001), Table 6.7.

Nitrite level

The effects of diabetes and **6z** on nitrite level were estimated. There was a significant change in the nitrite level in midbrain [$F(4, 30) = 9.756$, $p < 0.001$] as well as in frontal cortex [$F(4, 30) = 7.897$, $p < 0.001$] among the groups subjected to different treatments.

STZ-induced diabetic mice exhibited a significant increase in nitrite level in midbrain and frontal cortex ($p < 0.001$). This effect was significantly reversed by chronic treatment with **6z** (1 and 2 mg/kg, i.p.), ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$), as shown in Table 6.7.

Reduced glutathione

A significant change in the GSH level in midbrain [$F(4, 30) = 7.235$, $p < 0.001$] as well as in frontal cortex [$F(4, 30) = 6.176$, $p < 0.001$], among the groups subjected to different treatments. STZ-induced diabetic mice produced a significant decrease in GSH level in midbrain as well as in frontal cortex ($p < 0.001$). On the other hand, **6z** (1 and 2 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) chronic treatment ($p < 0.001$) significantly reversed this effect, Table 6.7.

Catalase

The effects of diabetes and **6z** on catalase activity in midbrain and frontal cortex were estimated.

There was a significant difference in midbrain [$F(4, 30) = 11.360$, $p < 0.001$] and frontal cortex [$F(4, 30) = 10.126$, $p < 0.001$] catalase activity among the groups. STZ-induced diabetic mice exhibited a significant decrease in catalase activity in midbrain as well as in frontal cortex ($p < 0.001$).

Chronic treatment with **6z** (1 and 2 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$) significantly reversed the decrease in catalase activity in STZ-induced diabetic mice, as given in Table 6.7.

Table 6.7 Effect of diabetes and 6z on the brain oxidative stress parameters

Parameters	TBARS (nmoles/mg pr)	Nitrite levels (µg/mg pr)	Catalase activity (U/mg pr)	GSH level (µmoles/mg pr)
Mid brain (including hippocampus)				
nDIA (0)	1.362 ± 0.177	47.072 ± 12.172	0.613 ± 0.070	0.247 ± 0.030
DIA (0)	5.201 ± 0.888 *	145.077 ± 33.723 $	0.161 ± 0.010 *	0.085 ± 0.015 $
DIA-6z (1)	4.689 ± 0.303 #	104.716 ± 19.592 ≠	0.335 ± 0.010 #	0.181 ± 0.021 #
DIA-6z (2)	4.561 ± 0.237 ≠	83.104 ± 23.225 ≠	0.440 ± 0.019 ≠	0.216 ± 0.025 ≠
DIA-FLX (10)	3.504 ± 0.247 £	75.708 ± 8.360 £	0.658 ± 0.151 £	0.201 ± 0.019 £
Frontal cortex				
nDIA (0)	1.981 ± 0.389	56.732 ± 3.782	0.699 ± 0.060	0.225 ± 0.015
DIA (0)	7.218 ± 1.324 $	123.142 ± 6.111 $	0.211 ± 0.083 *	0.061 ± 0.003 $
DIA-6z (1)	3.164 ± 0.793 #	61.141 ± 4.522 #	0.514 ± 0.120 #	0.098 ± 0.008 #
DIA-6z (2)	4.771 ± 0.778 ≠	61.174 ± 11.143 ≠	0.661 ± 0.052 ≠	0.135 ± 0.012 #
DIA-FLX (10)	3.824 ± 1.773 £	58.142 ± 6.144 £	0.512 ± 0.170 £	0.168 ± 0.007 £

Values represent mean ± S.E.M. * $p < 0.05$, $ $p < 0.001$ when compared with control group, # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$, when compared with diabetic control group, n = 6/group.

6.6.4 Effect on neurotrophic factor, BDNF in discrete brain regions

The effect of diabetes and test drug treatments on the neurotrophic factor namely BDNF was estimated in discrete brain regions.

6.6.4.1 Effect of diabetes and OND

The effects of diabetes and OND, on BDNF levels in midbrain (including hippocampus) and frontal cortex were estimated. BDNF levels in midbrain (and hippocampus) [$F (4, 30) = 37.050$, $p < 0.001$] and frontal cortex [$F (4, 30) = 12.130$, $p < 0.001$] were significantly different among the groups.

In midbrain, STZ-induced diabetic mice exhibited a significant decrease ($p < 0.001$) in BDNF levels and this effect was significantly reversed by chronic treatment with OND (0.5 and 1 mg/kg, i.p.) ($p < 0.001$) and FLX (10 mg/kg, i.p.) ($p < 0.001$), Fig. 6.43.

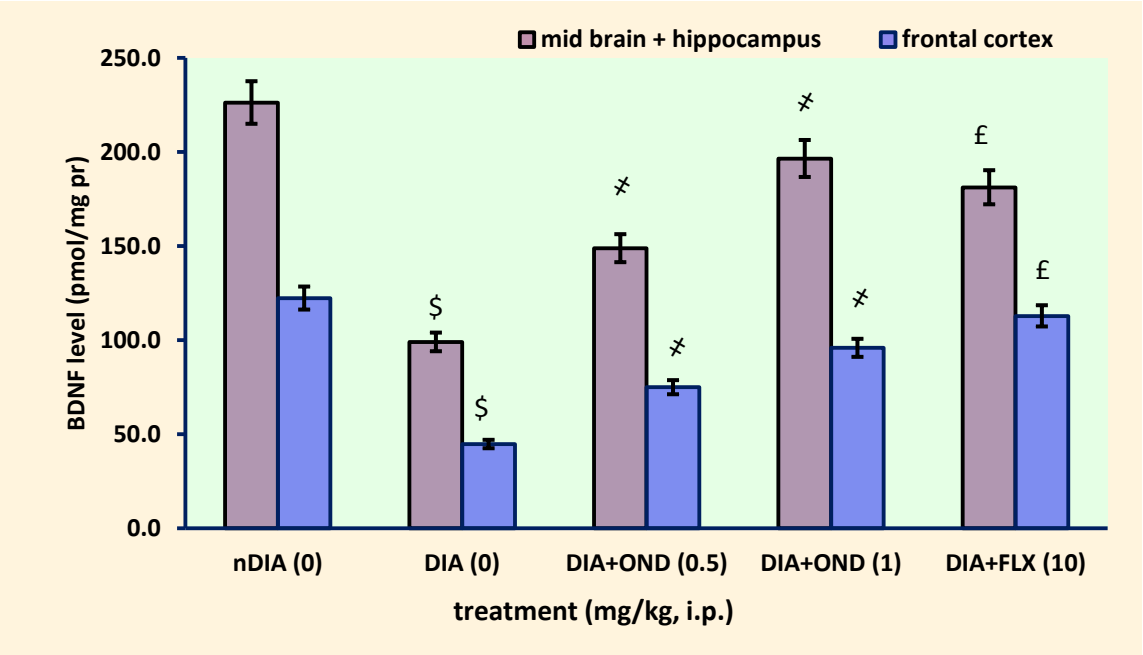


Fig. 6.43 The columns indicate mean values of BDNF (pmol/mg protein) level in midbrain (including hippocampus) and frontal cortex and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference vs control and ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

Similarly, in frontal cortex, STZ-induced diabetic mice exhibited a significant decrease ($p < 0.001$) in BDNF level, which was reversed by chronic treatment with OND (0.5 and 1 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$), Fig. 6.43.

6.6.4.2 Effect of diabetes and 4i

There was a significant change in BDNF level in midbrain (and hippocampus) [$F (4, 30) = 19.010$, $p < 0.001$] and frontal cortex [$F (4, 30) = 10.690$, $p < 0.001$] among the groups subjected to different treatments. In midbrain, BDNF level was significantly reduced in STZ-induced diabetic mice as compared to non-diabetic mice ($p < 0.001$). On the other hand, chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.001$) and FLX (10 mg/kg, i.p.) ($p < 0.001$) significantly reversed the decrease in BDNF level in midbrain of STZ-induced diabetic mice, as shown in Fig. 6.44.

In line with changes observed in mid brain BDNF level, in frontal cortex, STZ-induced diabetic mice exhibited a significant decrease ($p < 0.001$) in BDNF level and this effect was significantly reversed by chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.001$) and FLX (10 mg/kg, i.p.) ($p < 0.001$), as shown in Fig. 6.44.

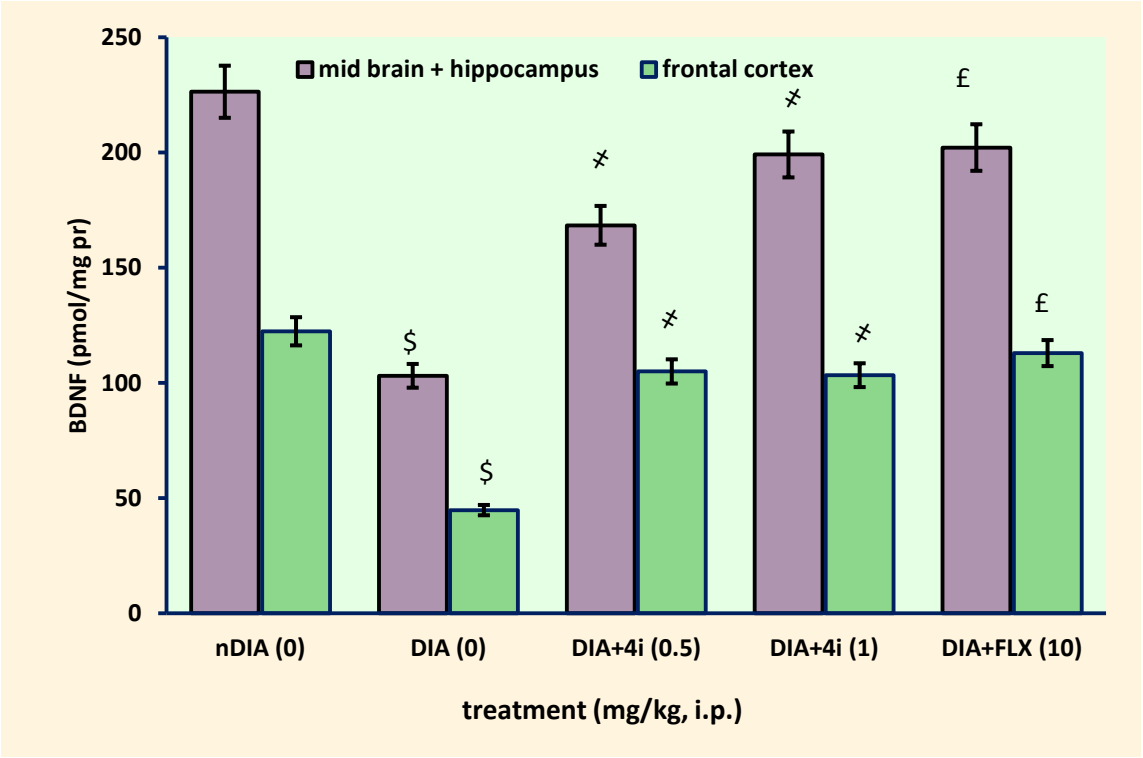


Fig. 6.44 The columns indicate mean values of BDNF (pmol/mg protein) level in midbrain (including hippocampus) and frontal cortex and error bars show S.E.M. $ $p < 0.001$ indicate significant difference vs control and ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.6.4.3 Effect of diabetes and 6z

The effects of diabetes and **6z**, on level of neurotrophic factor, BDNF in midbrain (including hippocampus) and frontal cortex were estimated. There was a significant change in BDNF level in midbrain (and hippocampus) [$F (4, 30) = 4.272$, $p < 0.001$] and frontal cortex [$F (4, 30) = 9.009$, $p < 0.001$] among the groups subjected to different treatments. In midbrain, STZ-induced diabetic mice exhibited a significant decline ($p < 0.05$) in BDNF level, whereas chronic treatment with **6z** (1 and 2 mg/kg, i.p.) ($p < 0.005$) and FLX (10 mg/kg, i.p.) ($p < 0.001$) significantly reversed this effect, as shown in Fig. 6.45.

Similarly in frontal cortex, STZ-induced diabetic mice exhibited a significant decrease ($p < 0.001$) in BDNF level. Interestingly, this effect was significantly reversed by chronic treatment with **6z** (2 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$). However, the lower dose of **6z** (1 mg/kg, i.p.) had no statistically significant effect on this parameter, though a non-significant trend of reversal was there, Fig. 6.45.

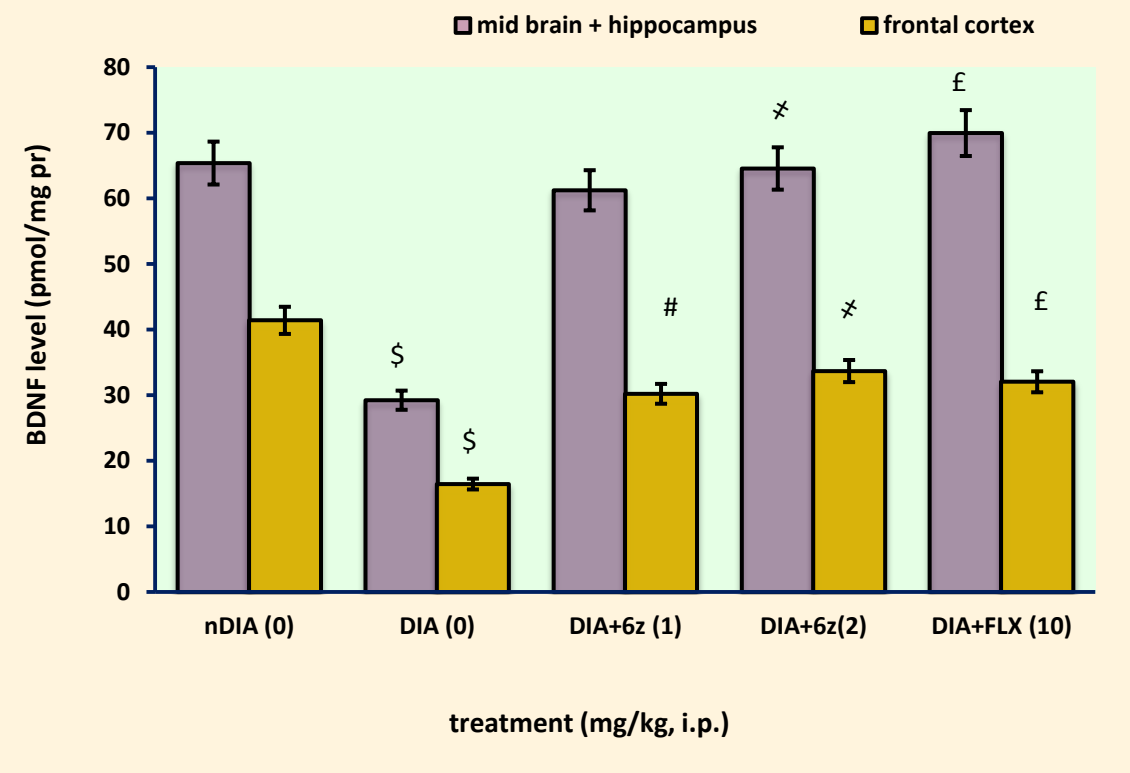


Fig. 6.45 The columns indicate mean values of BDNF (pmol/mg protein) level in **(A)** midbrain (including hippocampus) and **(B)** frontal cortex and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.6.5 Effect on cAMP level in discrete brain regions

6.6.5.1 Effect of diabetes and OND

The effects of diabetes and OND on cAMP level in midbrain (including hippocampus) and frontal cortex were estimated. There was a significant change in cAMP level in midbrain (and hippocampus) [$F (4, 30) = 7.541$, $p < 0.001$] and frontal cortex [$F (4, 30) = 10.330$, $p < 0.001$] among the groups subjected to different treatments.

Statistical analysis indicated that in midbrain, STZ-induced diabetic mice exhibited a significant reduction ($p < 0.001$) in cAMP level and this effect was significantly reversed by chronic treatment with OND (0.5 and 1 mg/kg, i.p.). In addition, chronic FLX (10 mg/kg, i.p.) treatment significantly reversed the decrease in cAMP level in STZ-induced diabetic mice ($p < 0.001$), Fig. 6.46.

In frontal cortex, STZ-induced diabetic mice exhibited a significant decrease ($p < 0.001$) in cAMP level and this effect was significantly reversed by chronic treatment with OND (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) ($p < 0.001$), Fig. 6.46.

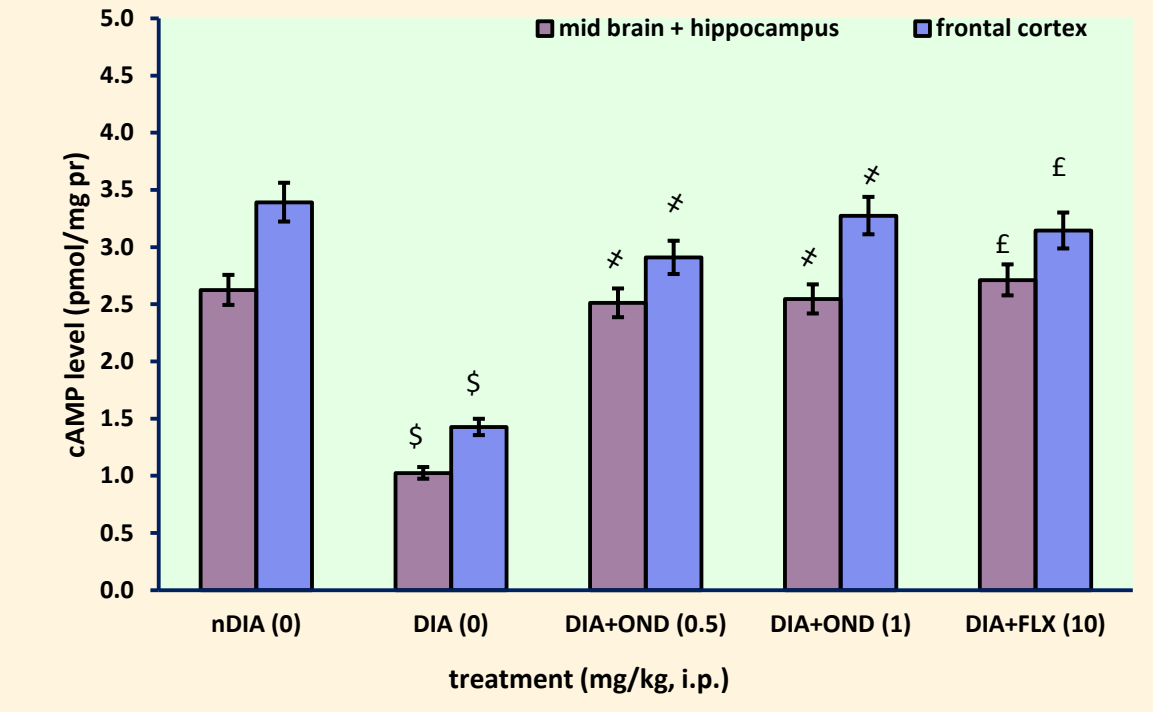


Fig. 6.46 The columns indicate mean values of cAMP (pmol/mg protein) level in midbrain (including hippocampus) and frontal cortex and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.6.4.2 Effect of diabetes and 4i

A significant alteration in cAMP level in midbrain (and hippocampus) [$F (4, 30) = 8.135$, $p < 0.001$] and frontal cortex [$F (4, 30) = 14.610$, $p < 0.001$] was observed among the groups subjected to different treatments. In midbrain, cAMP level was significantly reduced in STZ-induced diabetic mice as compared to control mice ($p < 0.001$).

In contrast, chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$) significantly increased the cAMP level in midbrain of STZ-induced diabetic mice. In frontal cortex, STZ-induced diabetic mice exhibited a significant decrease ($p < 0.001$) in cAMP level and this effect was significantly reversed by chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$), as shown in Fig. 6.47.

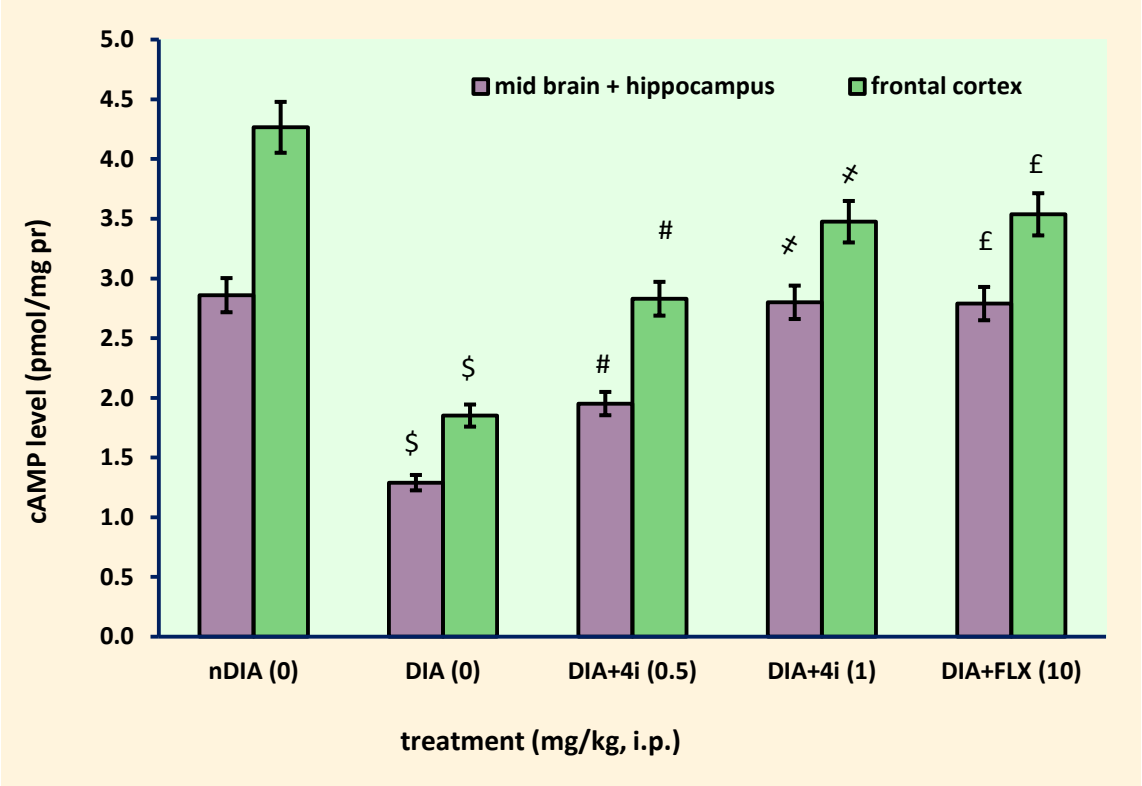


Fig. 6.47 The columns indicate mean values of cAMP (pmol/mg protein) level in midbrain (including hippocampus) and frontal cortex and error bars show S.E.M. **$** $p < 0.001$ indicate significant difference compared to control and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ vs diabetic control group, n = 6/group.

6.6.4.3 Effect of diabetes and 6z

The effects of diabetes and **6z** on cAMP level in midbrain (including hippocampus) and frontal cortex were estimated. There was a significant change in cAMP level in midbrain (and hippocampus) [$F (4, 30) = 40.901$, $p < 0.001$] and frontal cortex [$F (4, 30) = 13.590$, $p < 0.001$] among the groups.

In midbrain, statistical analysis revealed that STZ-induced diabetic mice exhibited a significant decrease ($p < 0.001$) in cAMP level and this effect was significantly reversed by chronic treatment with **6z** (1 and 2 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$).

In frontal cortex, STZ-induced diabetic mice exhibited a significant decrease ($p < 0.001$) in cAMP level and this effect was significantly reversed by chronic treatment with **6z** (1 and 2 mg/kg, i.p.) ($p < 0.05$) and FLX (10 mg/kg, i.p.) ($p < 0.001$), Fig. 6.48.

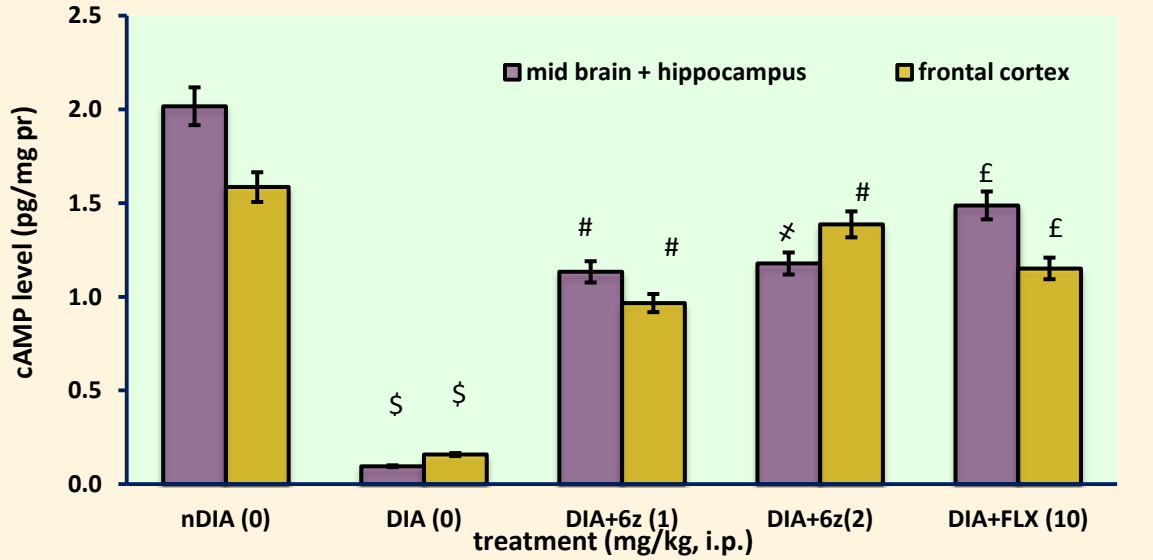


Fig. 6.48 The columns indicate mean values of cAMP (pmol/mg protein) level in midbrain and frontal cortex and error bars show S.E.M. **$** $p < 0.001$ and # $p < 0.05$, ≠ $p < 0.001$, £ $p < 0.001$ indicate significant difference vs normal control and diabetic control group, respectively, n = 6/group.

6.6.6 Effect on histopathological changes

The morphological changes in neuronal structure of hippocampus were estimated using haematoxylin-eosin and Golgi-Cox staining techniques.

6.6.6.1 Effect of diabetes and OND

Haematoxylin-eosin staining: In haematoxylin-eosin staining method the degree of neurodegeneration was evaluated as percentage of damaged neuronal cells stained blue due to haematoxylin. There were a large number of damaged neuronal cells as a consequence of STZ-induced diabetes, in mice that was qualitatively assessed by the photomicrographs, Fig. 6.49.

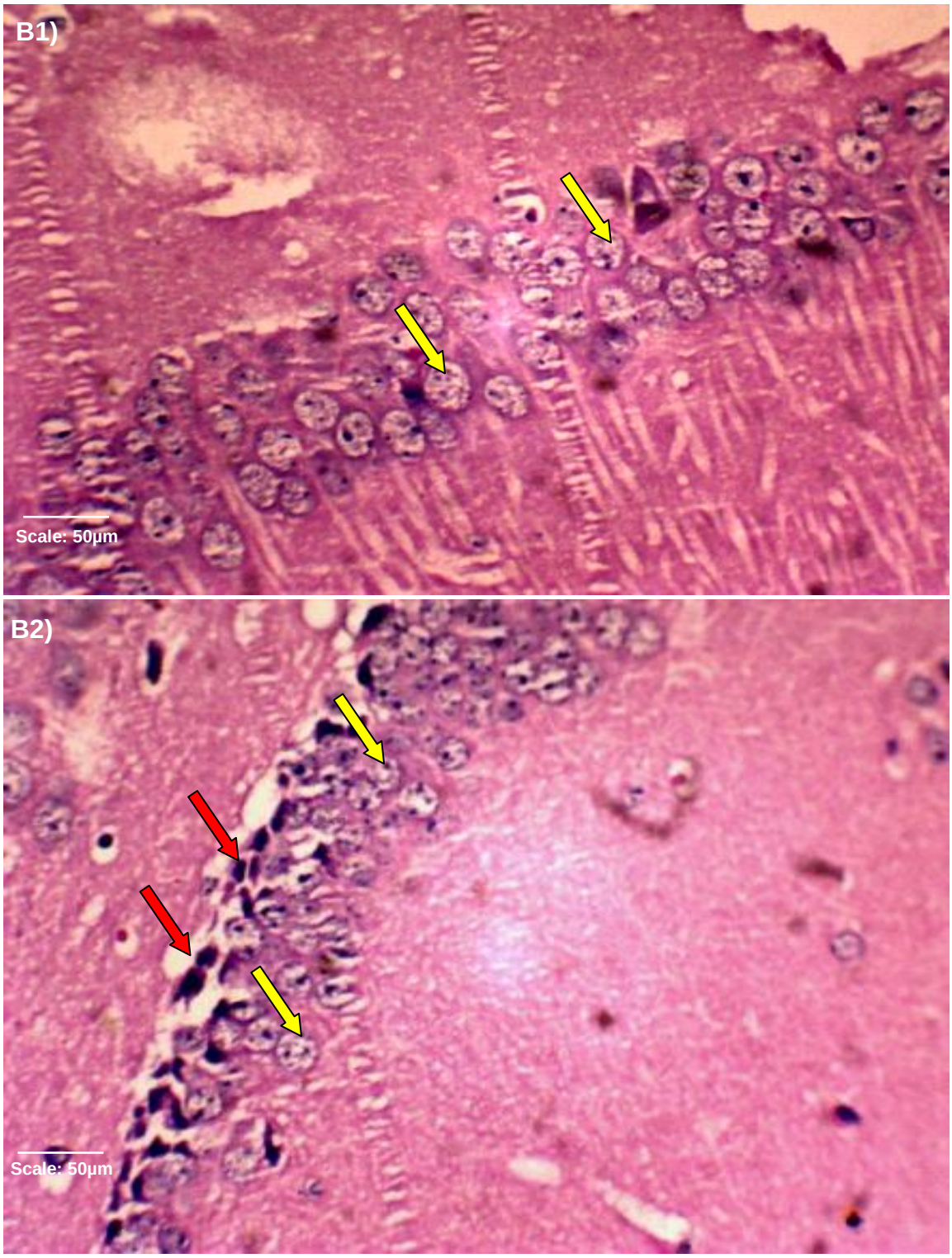
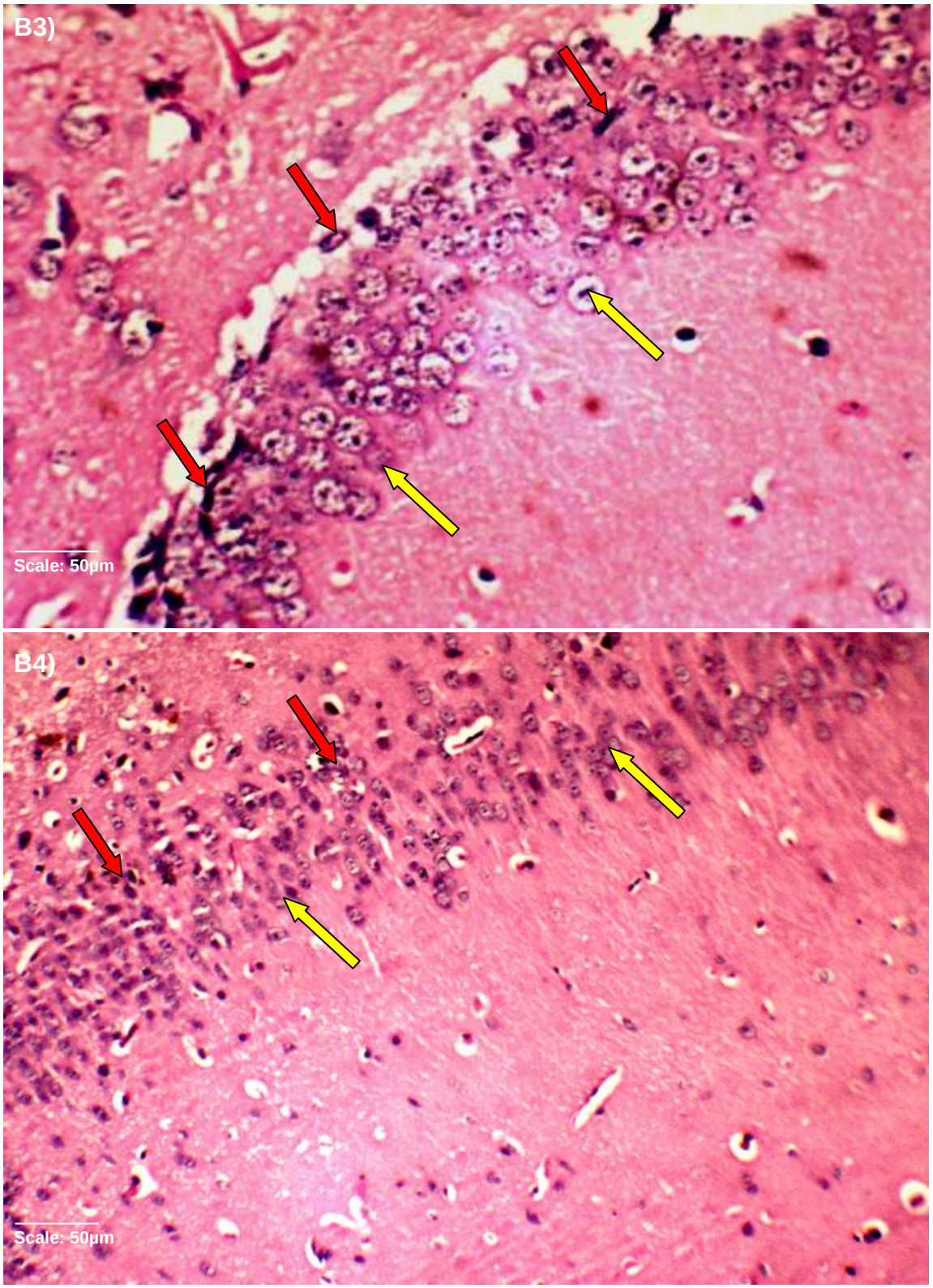
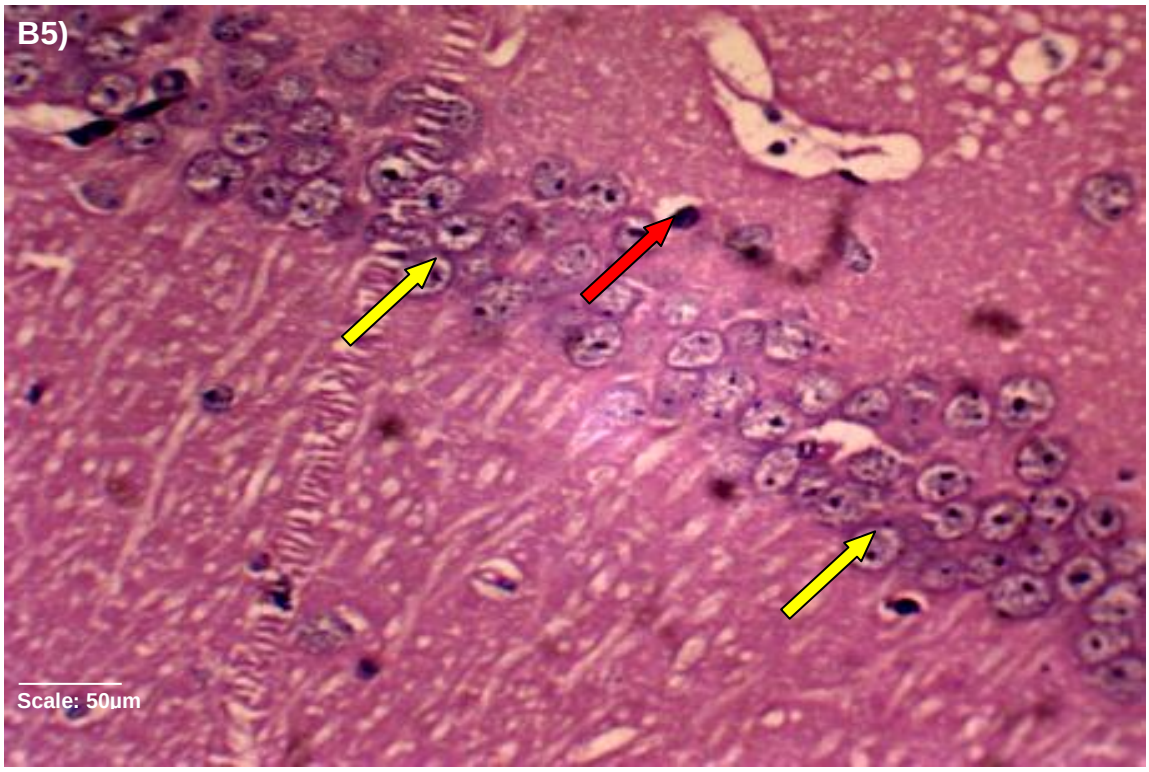


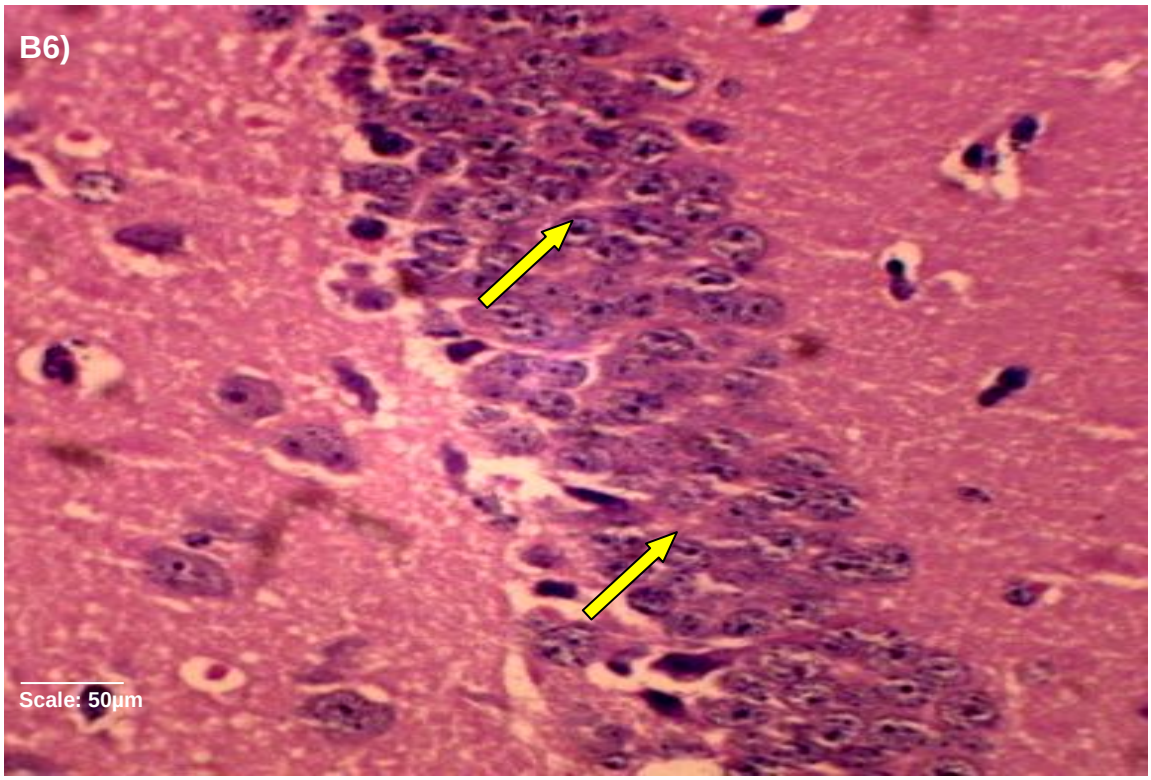
Fig. 6.49 Representative photomicrographs of sections of hippocampal CA_3 regions of mice brain stained with hematoxylin and eosin. **B1,** CA_3 region of control: row of normal nerve cells in the section is seen. **B2,** CA_3 region of diabetic control: among few normal nerve cells (marked by yellow arrows), the dark (deeply stained), irregular and shrunken nerve cells (marked by red arrows) are seen.



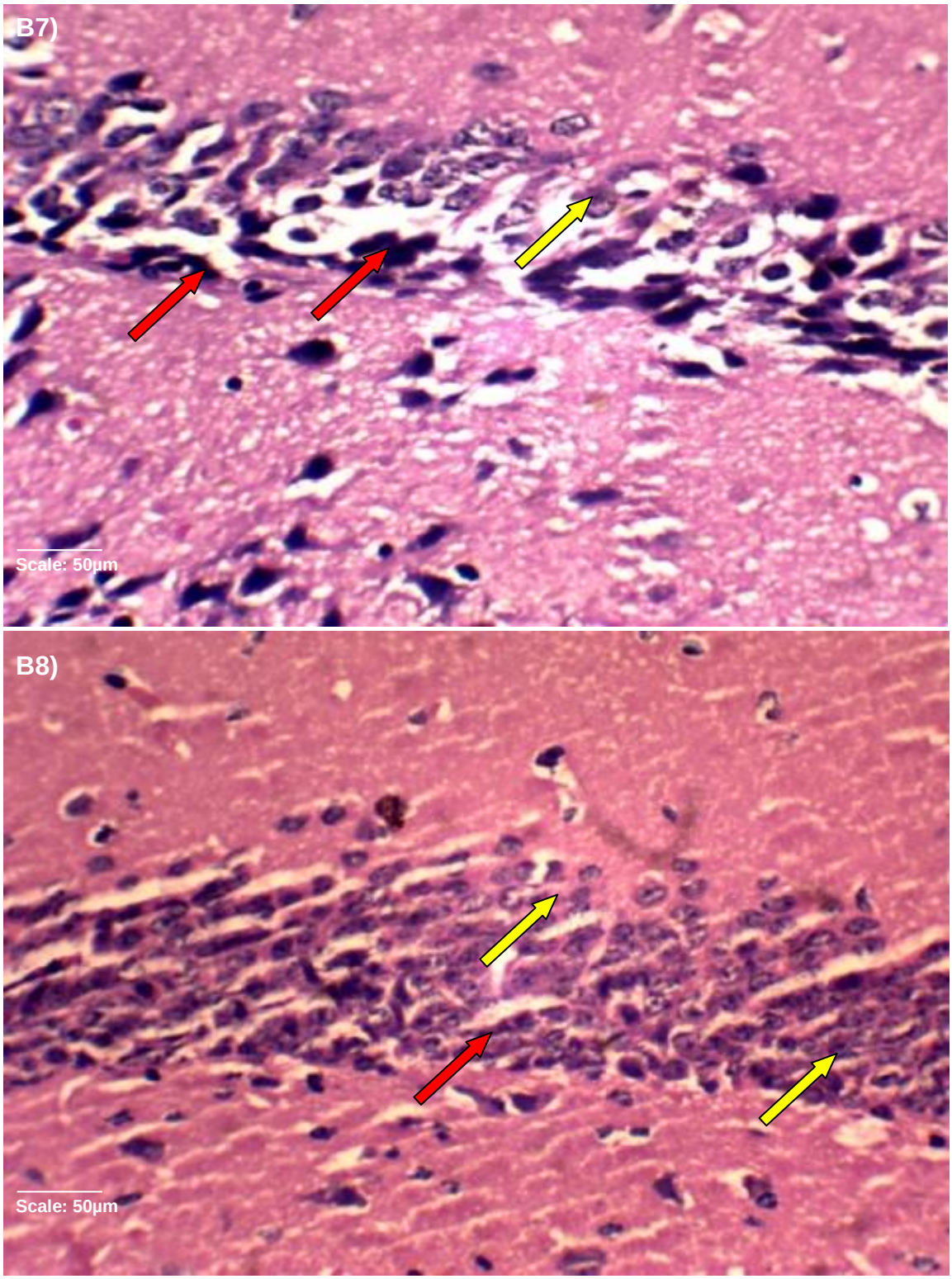
B3 and B4, CA_3 regions of diabetic mice treated with OND (0.5 mg/kg, i.p.) and OND (1 mg/kg, i.p.), respectively: more number of normal cells is seen in each section. Normal cells (marked by yellow arrows) and pyknotic cells (marked by red arrows)



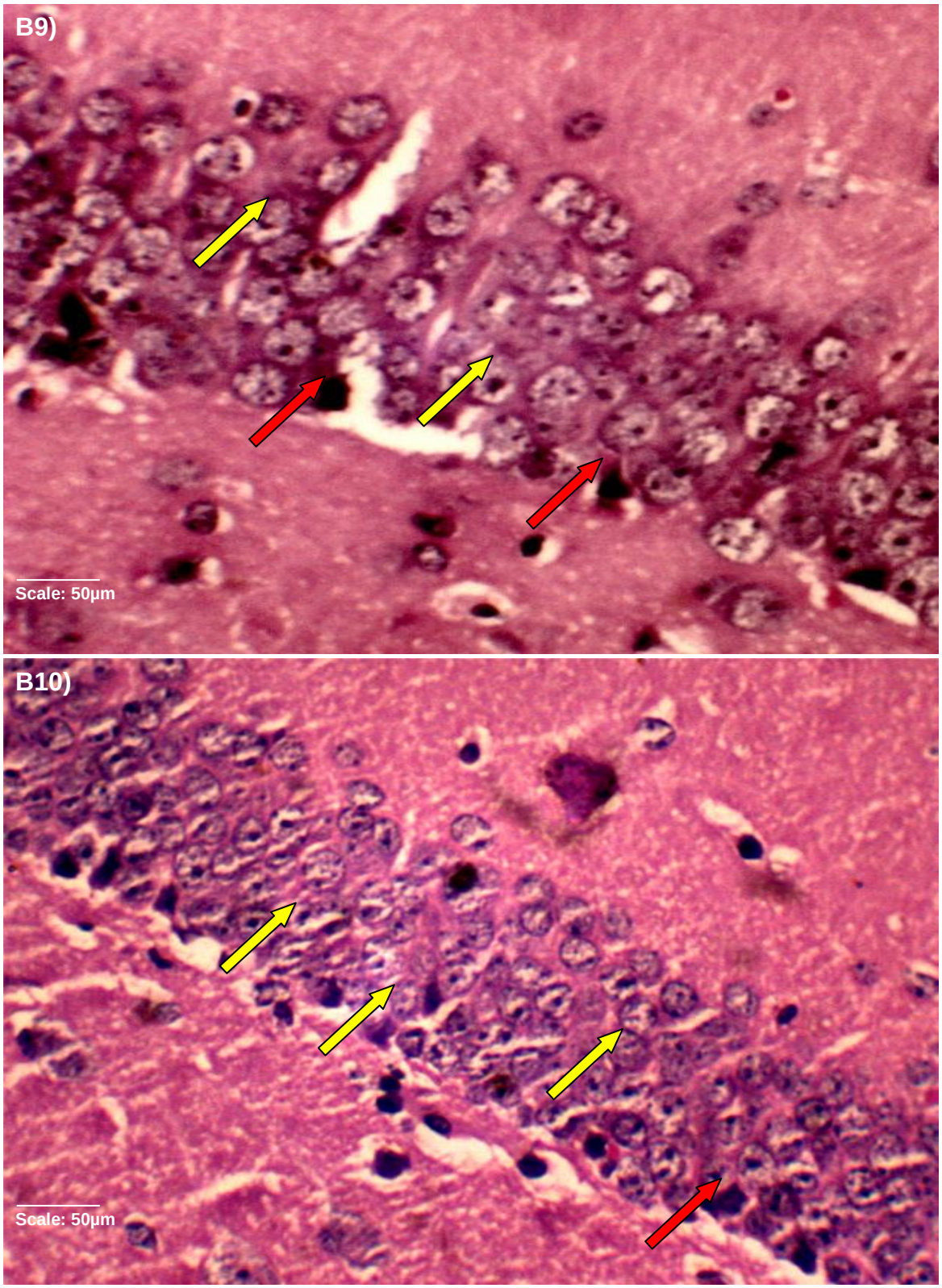
B5, CA_3 region of diabetic mice treated with FLX (10 mg/kg, i.p.): more number of normal cells is seen, normal cells (marked by yellow arrows) and pyknotic cells (marked by red arrows).



B6, dentate gyrus region of control: row of normal nerve cells in the section is seen (marked by yellow arrows).



B7, dentate gyrus region of diabetic control: there are few normal nerve cells (marked by yellow arrows) and more number of dark, irregular and shrunken nerve cells (marked by red arrows) is seen. **B8, dentate gyrus** region of diabetic mice treated with OND (0.5 mg/kg, i.p.): more number of normal cells is seen.



B9 and B10, dentate gyrus regions of diabetic mice treated with OND (1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.), respectively: more number of normal cells is seen in each section. Normal cells (marked by yellow arrows) and pyknotic cells (marked by red arrows)

The degree of neurodegeneration was quantitatively evaluated using 'Cell Counter Plugin' of ImageJ software. There was a significant change in the percentage damaged cells in hippocampal regions of CA3 [F (4, 14) = 13.010, p < 0.001] and dentate gyrus [F (4, 14) = 14.090, p < 0.001], in mice, subjected to different treatments. STZ-induced diabetic mice, exhibited a significant increase in the percentage of damaged cells in CA_3 and dentate gyrus hippocampal regions, as compared to control mice (p <0.001). On the other hand, the percentage of damaged cells were significantly less in STZ-induced diabetic mice treated with OND (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) (p < 0.001), as compared to those that received vehicle only, as shown in Fig. 6.50.

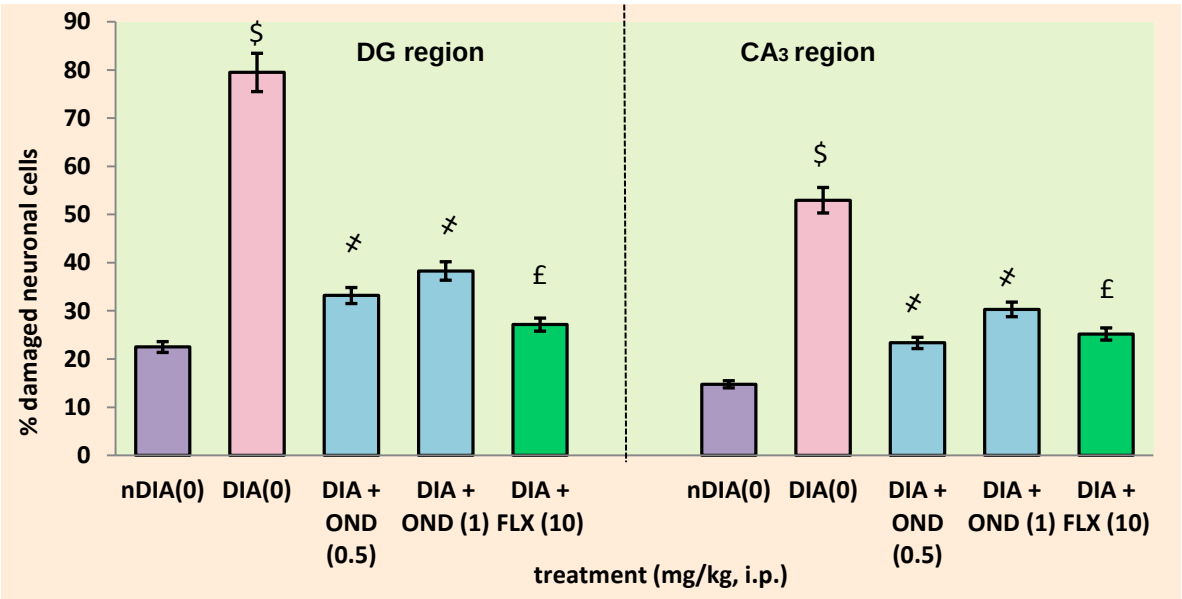


Fig. 6.50 The columns indicate mean values of % of damaged cells and error bars show S.E.M. **$** p < 0.001 indicate significant difference compared to control and ≠ p < 0.001, £ p < 0.001 vs diabetic control group, n = 6/group.

Golgi-Cox staining*:* In Golgi-Cox staining method, the morphological changes were evaluated at sub-neuronal level by estimating the dendritic morphology in the form of branching of dendrites and dendritic length. These two parameters were analyzed using ImageJ software with 'NeuronJ' and 'Sholl analysis' plugins, as shown in Fig. 6.51.

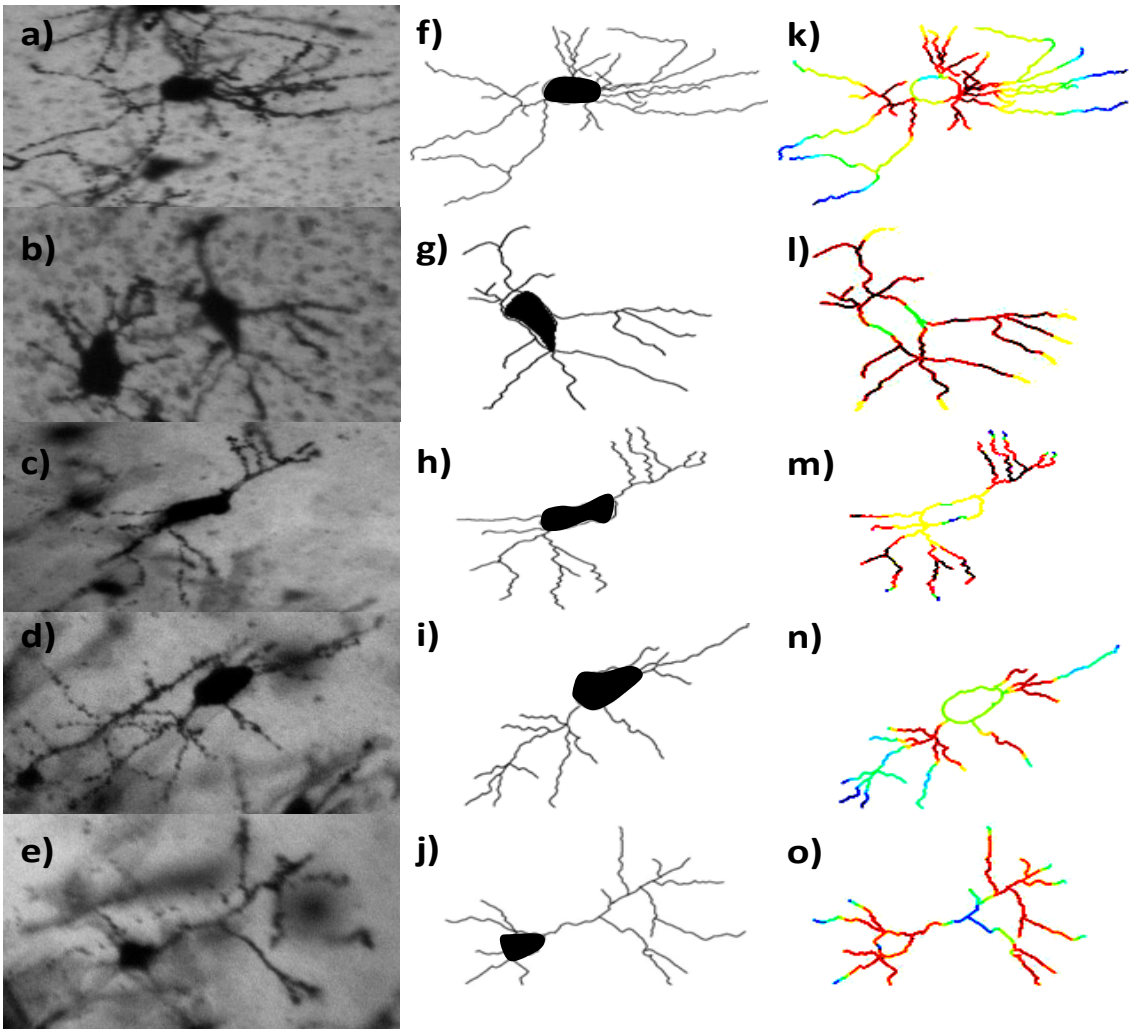


Fig. 6.51 Representative photomicrographs of neurons stained with Golgi-Cox, **(a-e)**, tracing of the neurons, **(f-j)** and Sholl mask of these neurons **(k-o)** corresponding to the experimental groups, DIA (0), nDIA (0), DIA+OND (0.5), DIA+OND (1) and DIA+FLX (10) respectively**.**

There was a significant difference in cumulative branch length (µm) of primary, secondary and tertiary dendrites of pyramidal neurons among the groups [$F(4, 55) = 24.57$, $p < 0.01$]. STZ-induced diabetic mice exhibited a significant reduction in branch length of primary, secondary and tertiary dendrites of pyramidal neurons as compared to non-diabetic mice ($p < 0.01$). This reduction was significantly abolished by chronic treatment with FLX (10 mg/kg, i.p.) ($p < 0.001$). Similarly, chronic OND (0.5 and 1 mg/kg, i.p.) treatment, significantly enhanced the branch length of primary and tertiary dendrites of pyramidal neurons in diabetic mice ($p < 0.01$). However, no effect was observed for secondary dendrites in diabetic mice treated with OND (0.5 and 1 mg/kg, i.p.) ($p > 0.05$), Fig. 6.52.

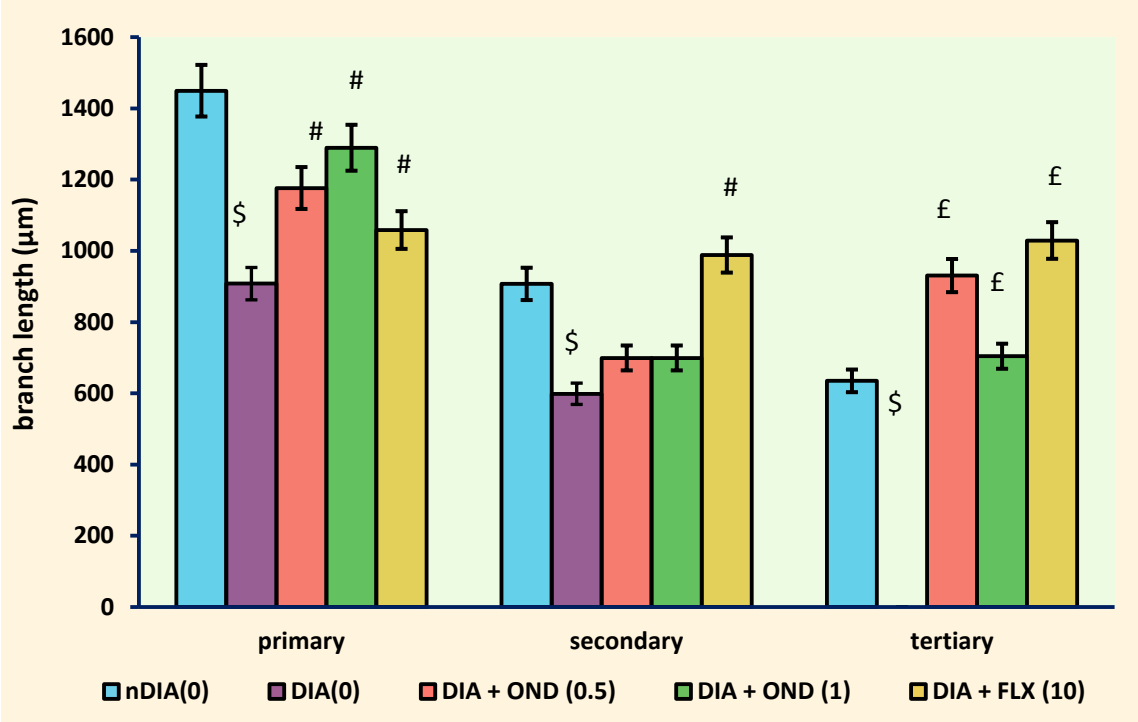


Fig. 6.52 The dendritic morphology is quantified as cumulative branch length (µm) of primary, secondary and tertiary branch points. The columns represent mean values, while error bars show SEM. $ p < 0.01 vs control (nDIA (0)) and # p < 0.05, ≠ p < 0.01, £ p < 0.01 vs diabetic control, n = 3/group.

There was a significant difference in the number of dendritic intersections among the different groups [F (4, 55) = 13.68, p < 0.01]. STZ-induced diabetic mice exhibited significantly fewer dendritic intersections than non-diabetic mice (p < 0.01) at the proximal 10-20 µm, 70-100 µm and above 190 µm regions.

Chronic treatment with FLX (10mg/kg, i.p.) increased the number of intersections in STZ-induced diabetic mice at the 10-20 and above 190 µm regions to levels similar to those found in control mice (p < 0.01).

Similarly, chronic OND (0.5 mg/kg, i.p.) treatment significantly enhanced the number of intersections in diabetic mice at 10-20 and above 130 µm regions (p < 0.05). In addition, OND (1 mg/kg, i.p.) treatment, significantly enhanced the number of intersections in diabetic mice at the proximal 10-20 µm, 70-100 µm and above 190 µm regions (p < 0.05), Fig. 6.53.

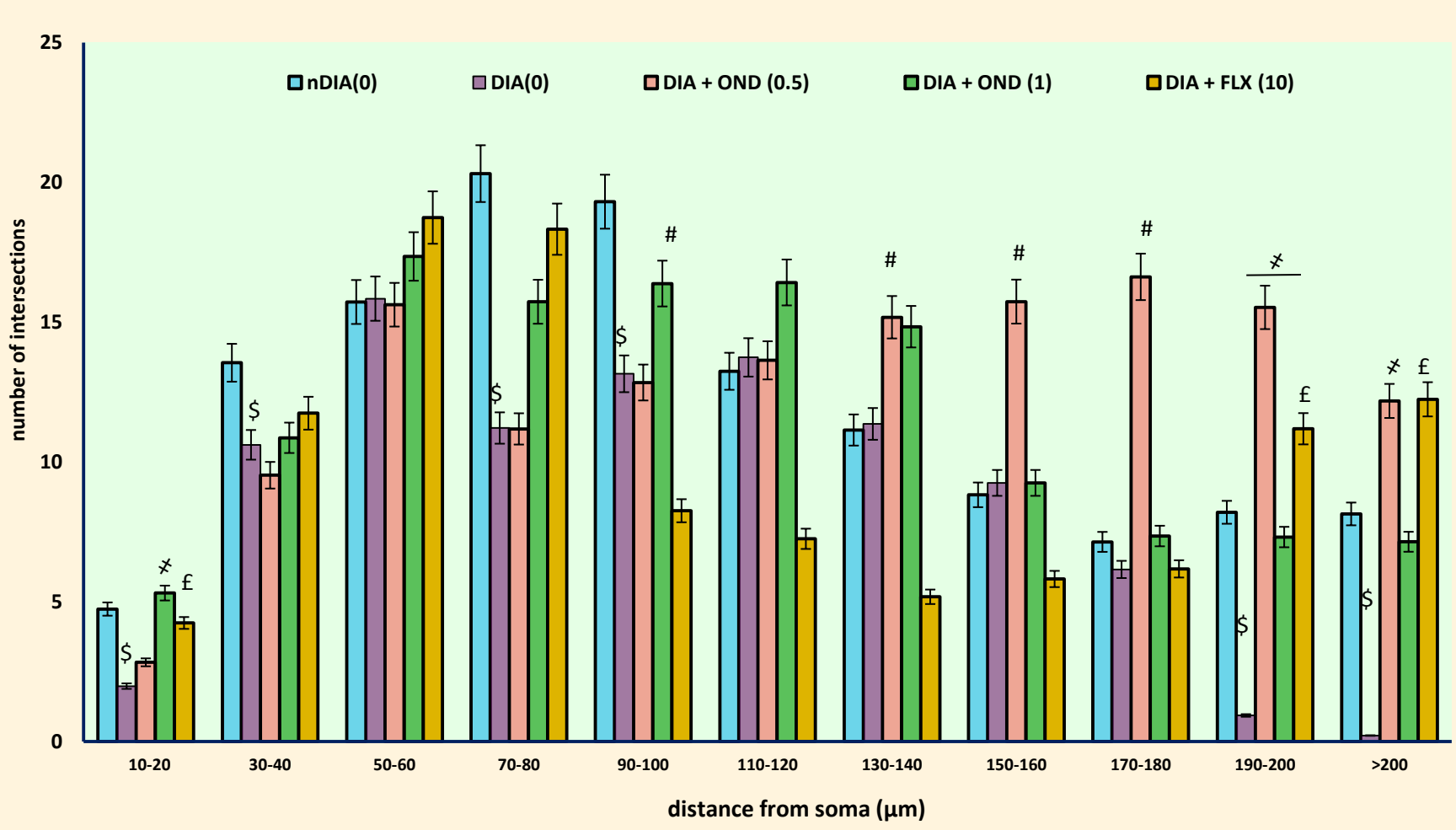


Fig. 6.53 The dendritic morphology is quantified as the number of intersections per circle (positioned at radial intervals of 10 µm**)** of pyramidal neurons in the CA_3 subfield of hippocampus. The columns represent mean values, while error bars show SEM. * $p < 0.01$ vs control (nDIA (0)) and # $p < 0.05$, ≠ $p < 0.01$, £ $p < 0.01$ vs diabetic control, n = 3/group.

6.6.6.2 Effect of diabetes and 4i

Haematoxylin-eosin staining: A significant change in the degree of neurodegeneration was observed in hippocampus of mice subjected to different treatments, as represented in the photomicrographs, Fig. 6.54.

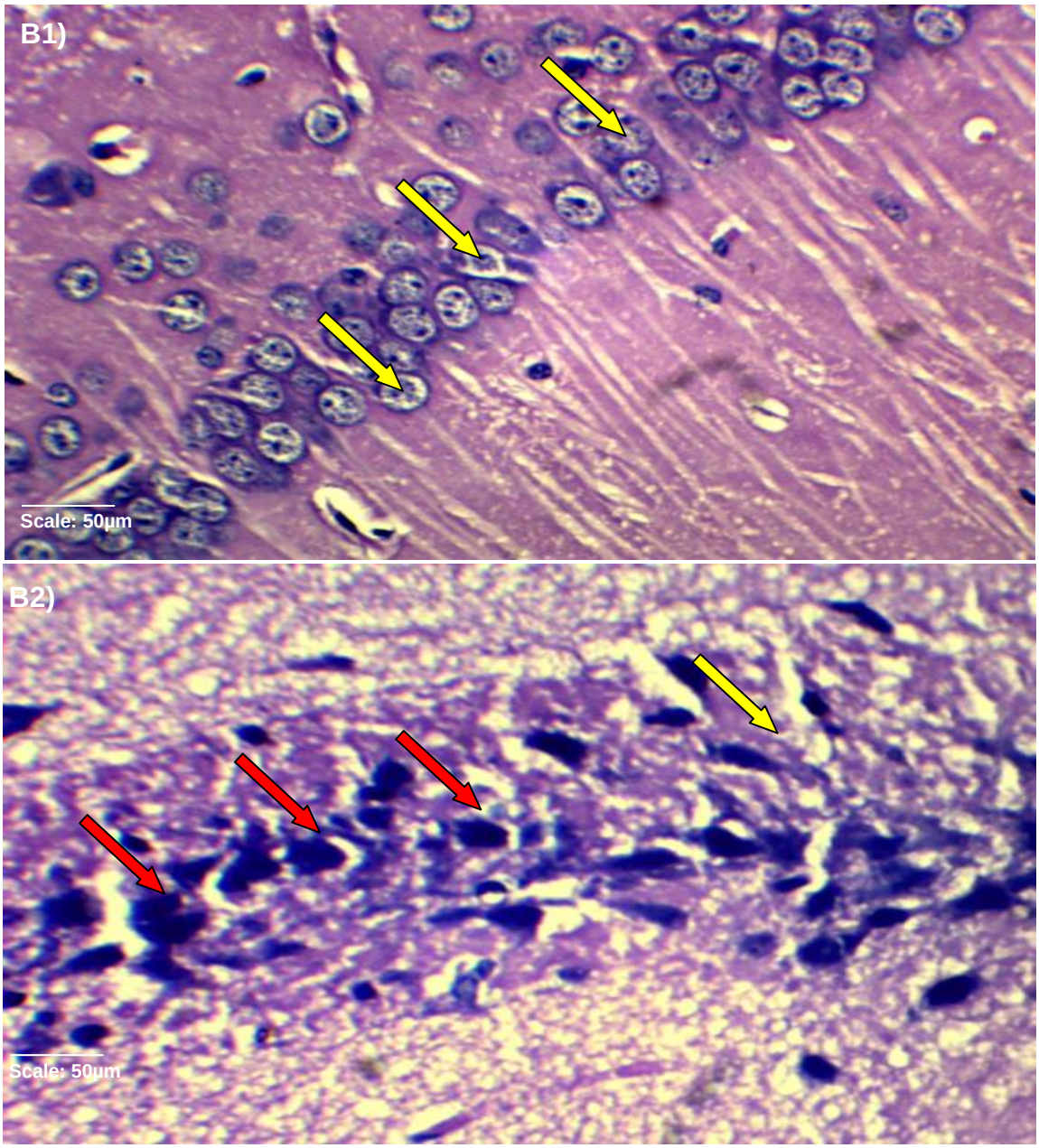
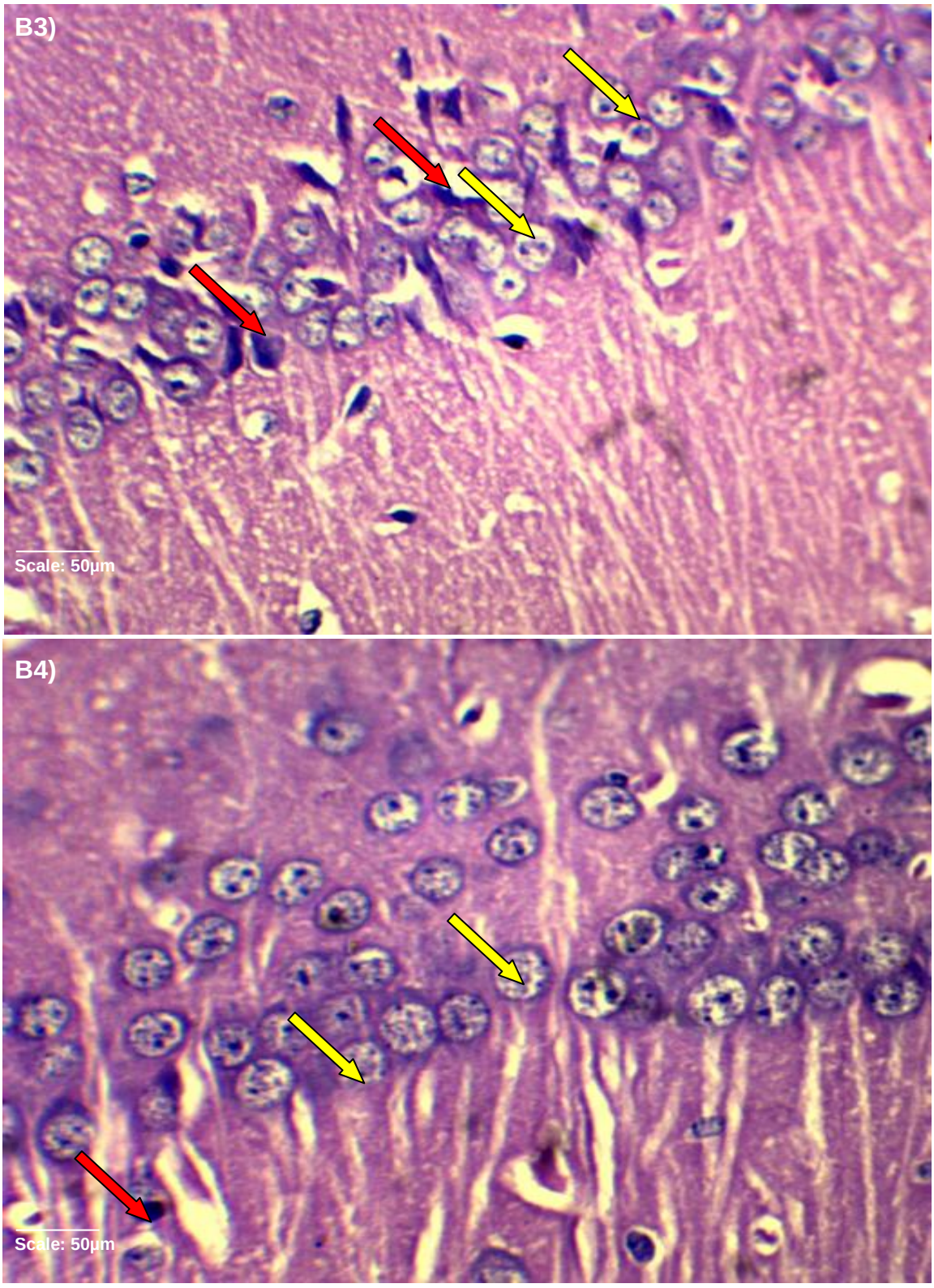
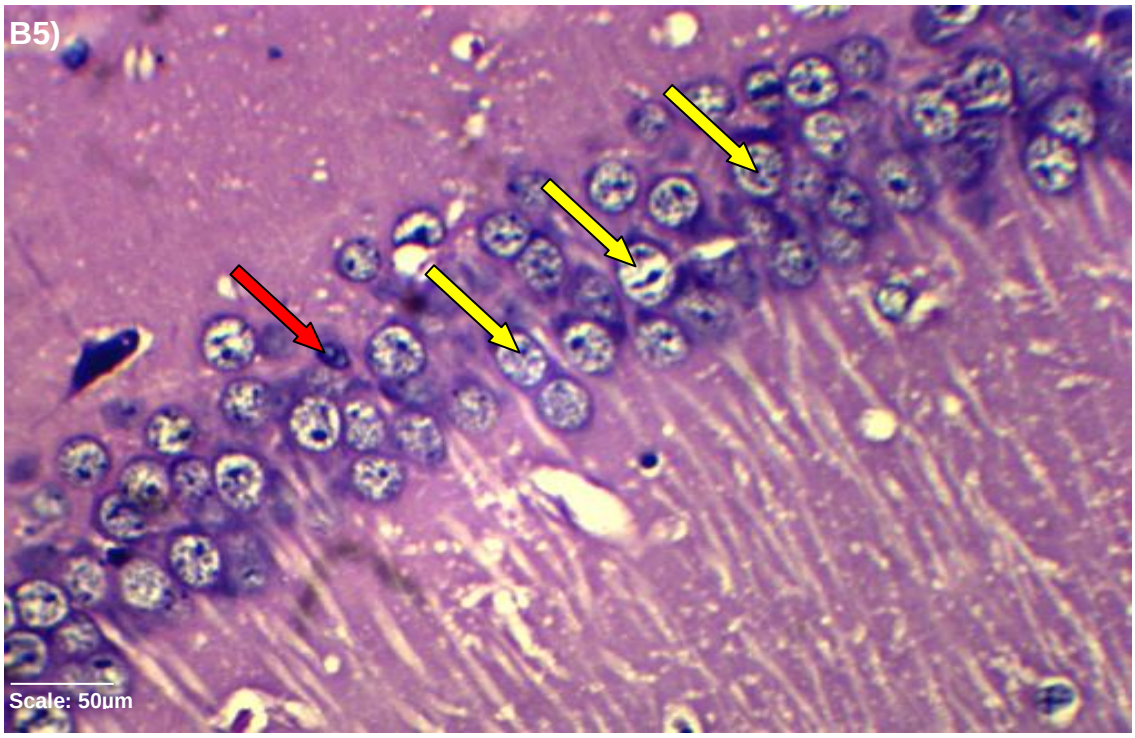


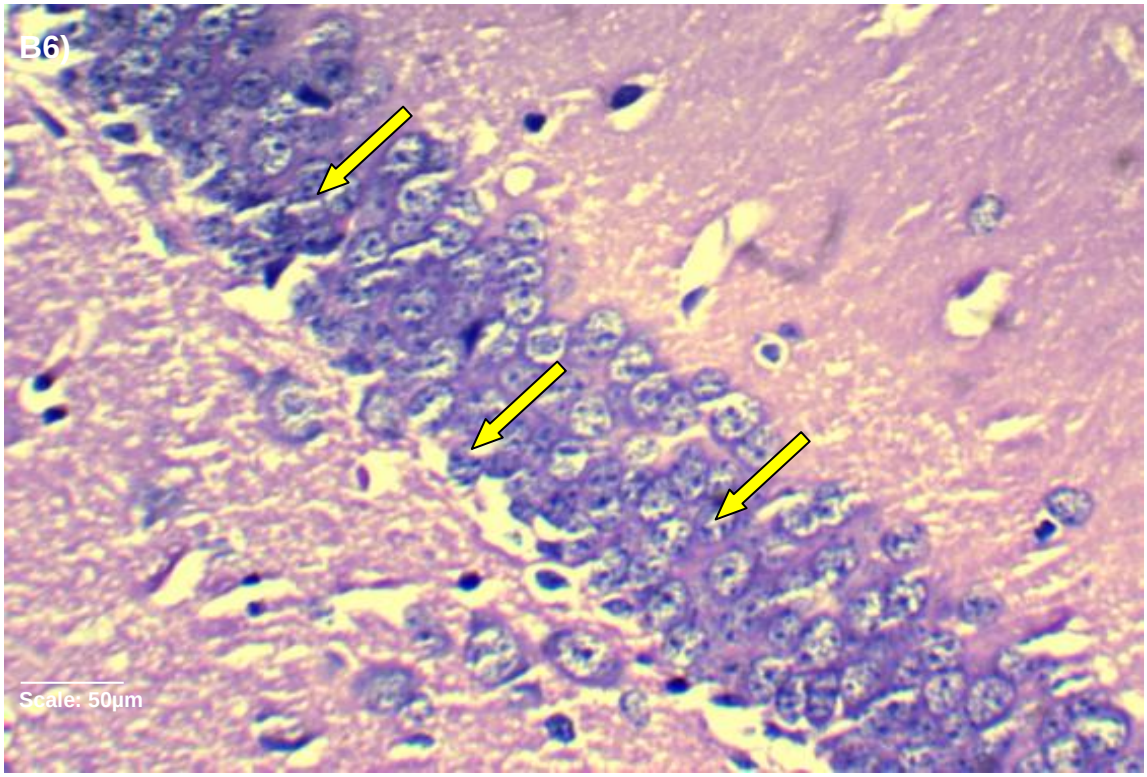
Fig. 6.54 The photomicrographs of sections of hippocampal CA_3 regions of mice brain subjected to hematoxylin and eosin staining. **B1,** CA_3 region of control: row of normal nerve cells with regular shape nucleus in the section, is seen. **B2,** CA_3 region of diabetic control: among very few normal nerve cells (marked by yellow arrows), the dark (deeply stained), irregular and shrunken nerve cells with damaged nucleus (marked by red arrows) are seen.



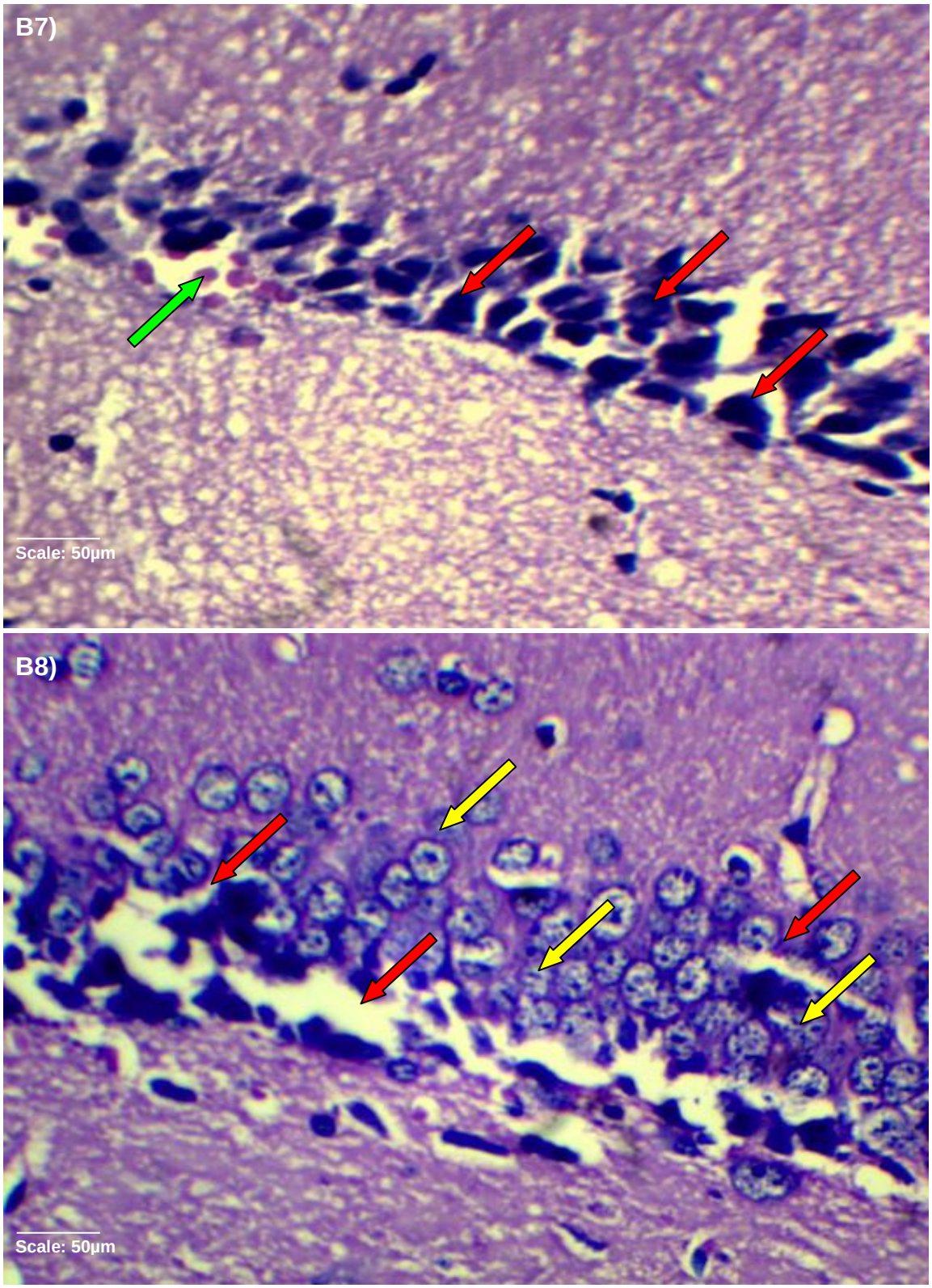
B3 and B4, CA_3 regions of diabetic mice treated with **4i** (0.5 mg/kg, i.p.) and **4i** (1 mg/kg, i.p.), respectively: more number of normal cells is seen, in each section. Normal cells (marked by yellow arrows) and pyknotic cells (marked by red arrows)



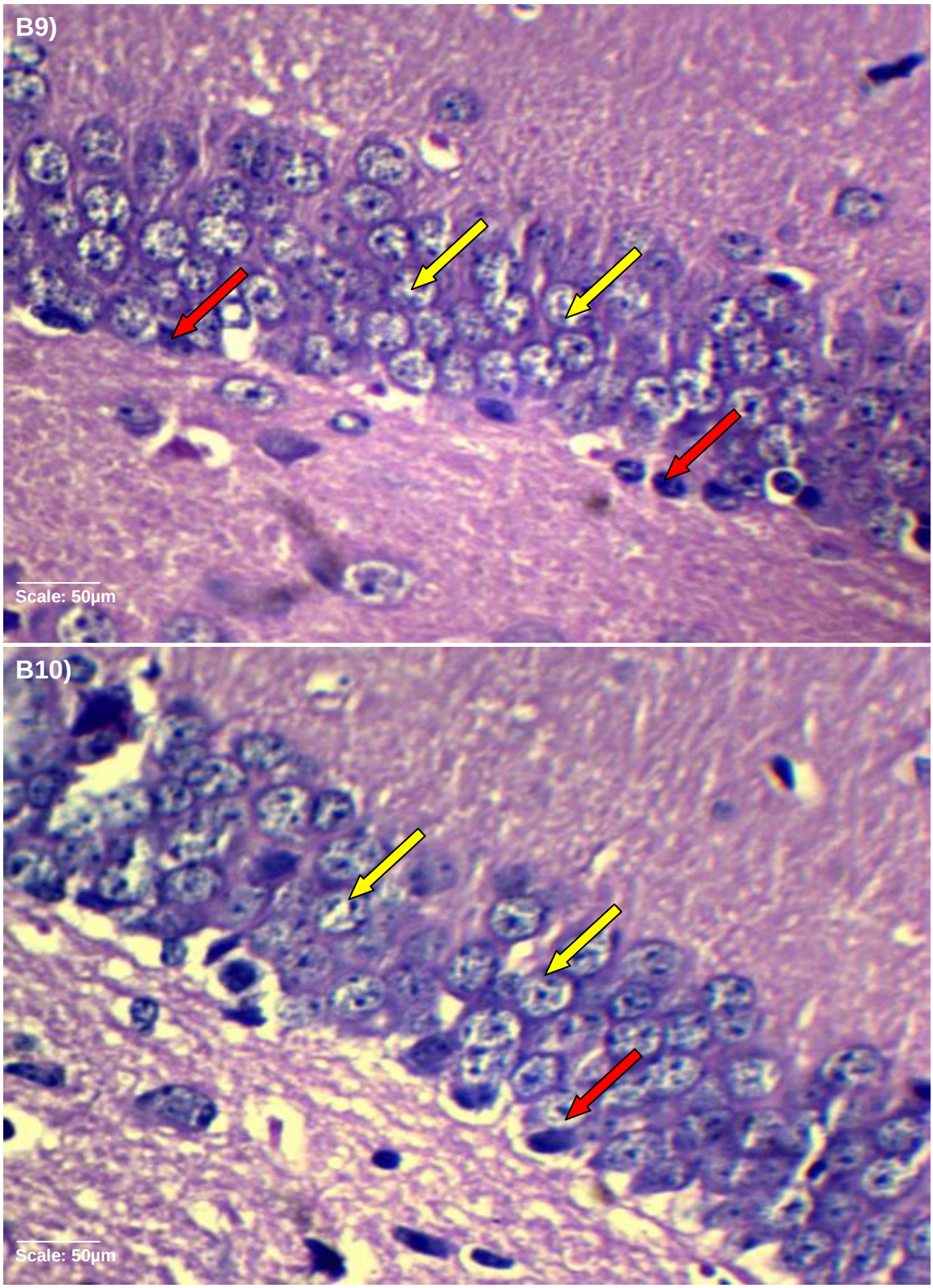
B5, CA_3 region of diabetic mice treated with FLX (10 mg/kg, i.p.): more number of normal cells is seen, normal cells (marked by yellow arrows) and pyknotic cells (marked by red arrows).



B6, dentate gyrus region of control: row of normal nerve cells in the section, is seen (marked by yellow arrows).



B7, dentate gyrus region of diabetic control: almost no normal nerve cells are present markedly dark, irregular and shrunken nerve cells (marked by red arrows) is seen as well as astrocyte filtrations (marked by green arrow) is observed. **B8, dentate gyrus** region of diabetic mice treated with **4i** (0.5 mg/kg, i.p.): more number of normal cells is seen.



B9 and B10, dentate gyrus regions of diabetic mice treated with **4i** (1 mg/kg, i.p.) and FLX (10 mg/kg, i.p.), respectively: more number of normal cells is seen in each section. Normal cells (marked by yellow arrows) and pyknotic cells (marked by red arrows)

The degree of neurodegeneration was quantitatively evaluated using Cell Counter plugin of ImageJ software. A significant change in the percentage of damaged cells in hippocampal regions of CA_3 [F (4, 14) = 18.860, p < 0.001] and dentate gyrus [F (4, 14) = 20.800, p < 0.001] was observed, in mice subjected to different treatments. STZ-induced diabetic mice exhibited a significant increase in the percentage of damaged cells in CA_3 and dentate gyrus hippocampal regions as compared to control mice (p <0.001), whereas the percentage damaged cells were significantly less in STZ-induced diabetic mice treated with **4i** (0.5 and 1 mg/kg, i.p.) and FLX (10 mg/kg) (p < 0.001), as compared to those that received vehicle only, as shown in Fig. 6.55.

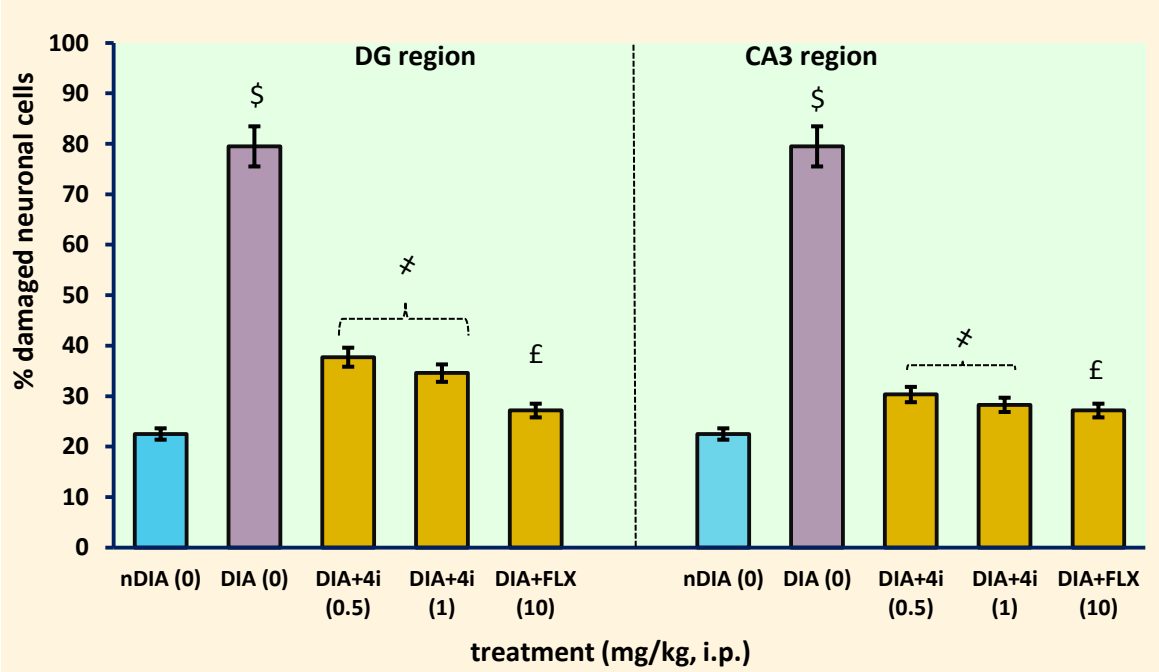


Fig. 6.55 The columns indicate mean values of % of damaged cells and error bars show S.E.M. **$** p < 0.001 indicate significant difference vs control and ≠ p < 0.001, £ p < 0.001 vs diabetic control group, n = 6/group.

Golgi-Cox staining: In Golgi-Cox staining, a marked difference in branching lengths of primary, secondary and tertiary dendrites as well as dendritic branching were observed, in mice subjected to different treatments, as shown in Fig. 6.56.

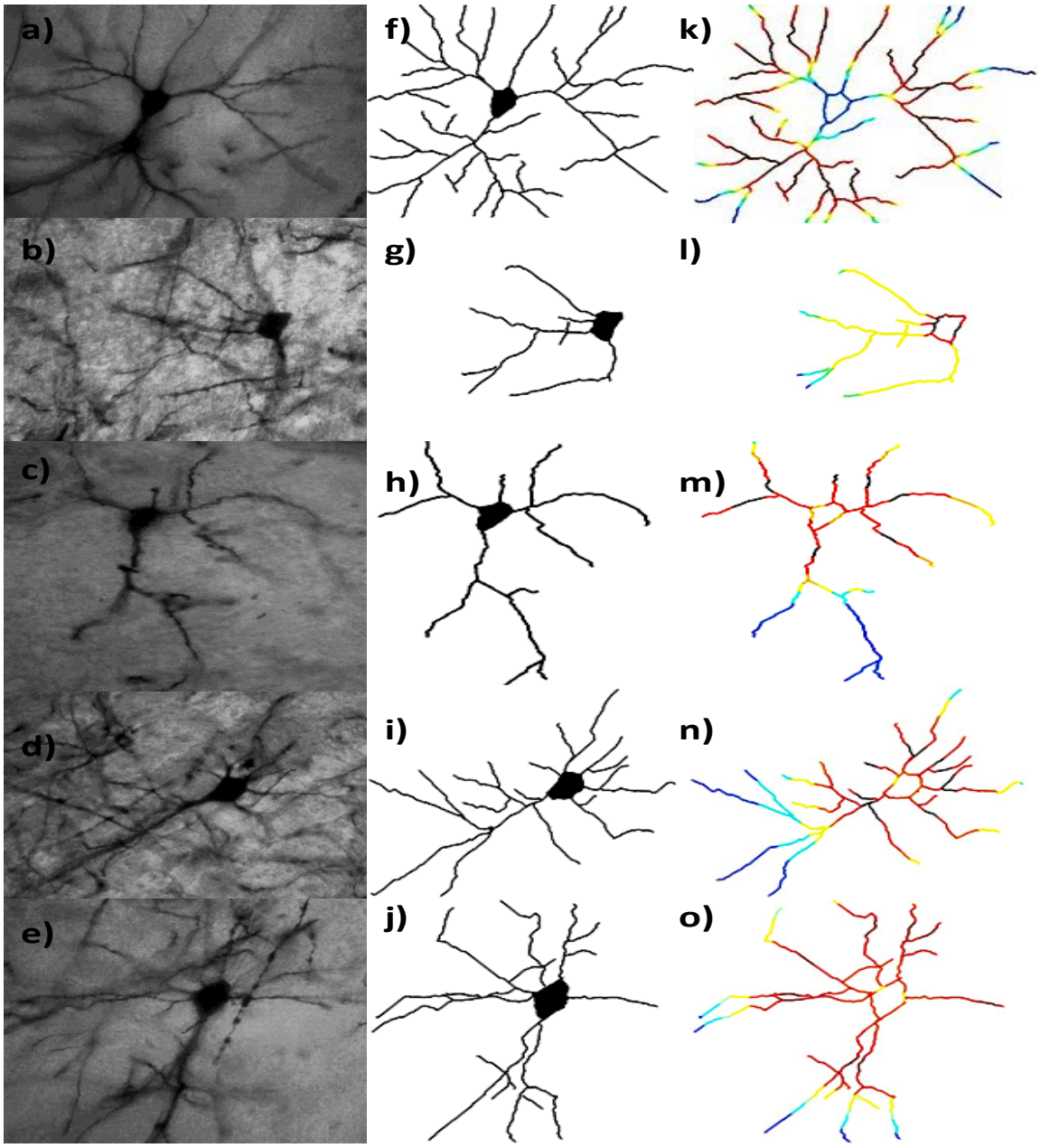


Fig. 6.56 Representative photomicrographs of neurons stained with Golgi-Cox, **(a-e),** tracing of the neurons, **(f-j)** and Sholl mask of these neurons **(k-o)** corresponding to the experimental groups, DIA (0), nDIA (0), DIA+**4i** (0.5), DIA+**4i** (1) and DIA+FLX (10) respectively.

The cumulative branch length (µm) of primary, secondary and tertiary dendrites of pyramidal neurons among the groups [$F(4, 55) = 21.11$, $p < 0.01$] were significantly altered, in mice subjected to different treatments. STZ-induced diabetic mice exhibited a significant reduction in branch length of primary, secondary and tertiary dendrites of pyramidal neurons as compared to non-diabetic mice ($p < 0.001$).

Chronic treatment with FLX (10 mg/kg, i.p.) significantly abolished the decrease in branch length of primary and secondary dendrites ($p < 0.05$). Similarly, chronic **4i** (0.5 and 1 mg/kg, i.p.) treatment significantly enhanced the branch length of primary, secondary and tertiary dendrites of pyramidal neurons in diabetic mice ($p < 0.01$), Fig. 6.57.

In addition a significant difference in the number of dendritic intersections was observed among the different groups [$F(4, 55) = 18.61$, $p < 0.01$] subjected to different treatments. STZ-induced diabetic mice exhibited significantly fewer dendritic intersections than non-diabetic mice ($p < 0.01$), with marked difference at all regions. Chronic treatment with FLX (10mg/kg, i.p.) significantly increased the number of intersections in diabetic mice ($p < 0.01$). Chronic treatment with **4i** (0.5 and 1 mg/kg, i.p.) significantly enhanced the number of intersections in diabetic mice, with more prominent effect at above 130 µm regions ($p < 0.05$), Fig. 6.58.

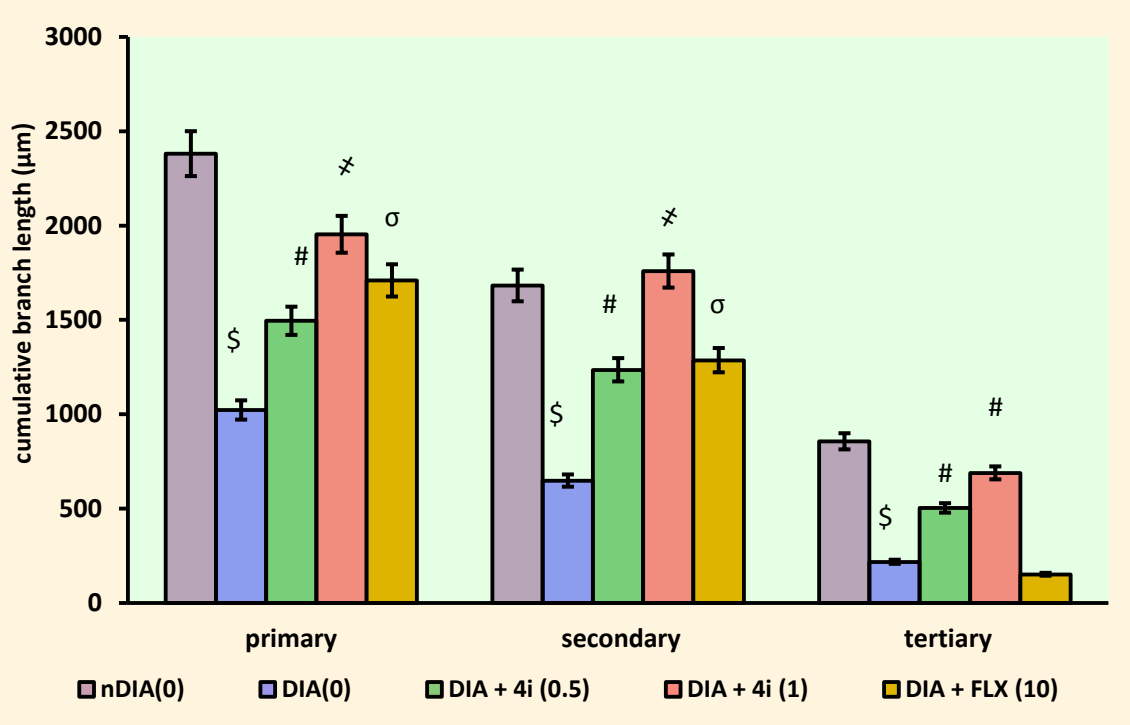


Fig. 6.57 The dendritic morphology is quantified as cumulative branch length (µm) of primary, secondary and tertiary branch points of pyramidal neurons in the CA_3 subfield of hippocampus. The columns represent mean values, while error bars show SEM. **$** $p < 0.01$ vs control (nDIA (0)) and # $p < 0.05$, ≠ $p < 0.01$, σ $p < 0.05$, vs diabetic control (DIA (0)), n = 3/group.

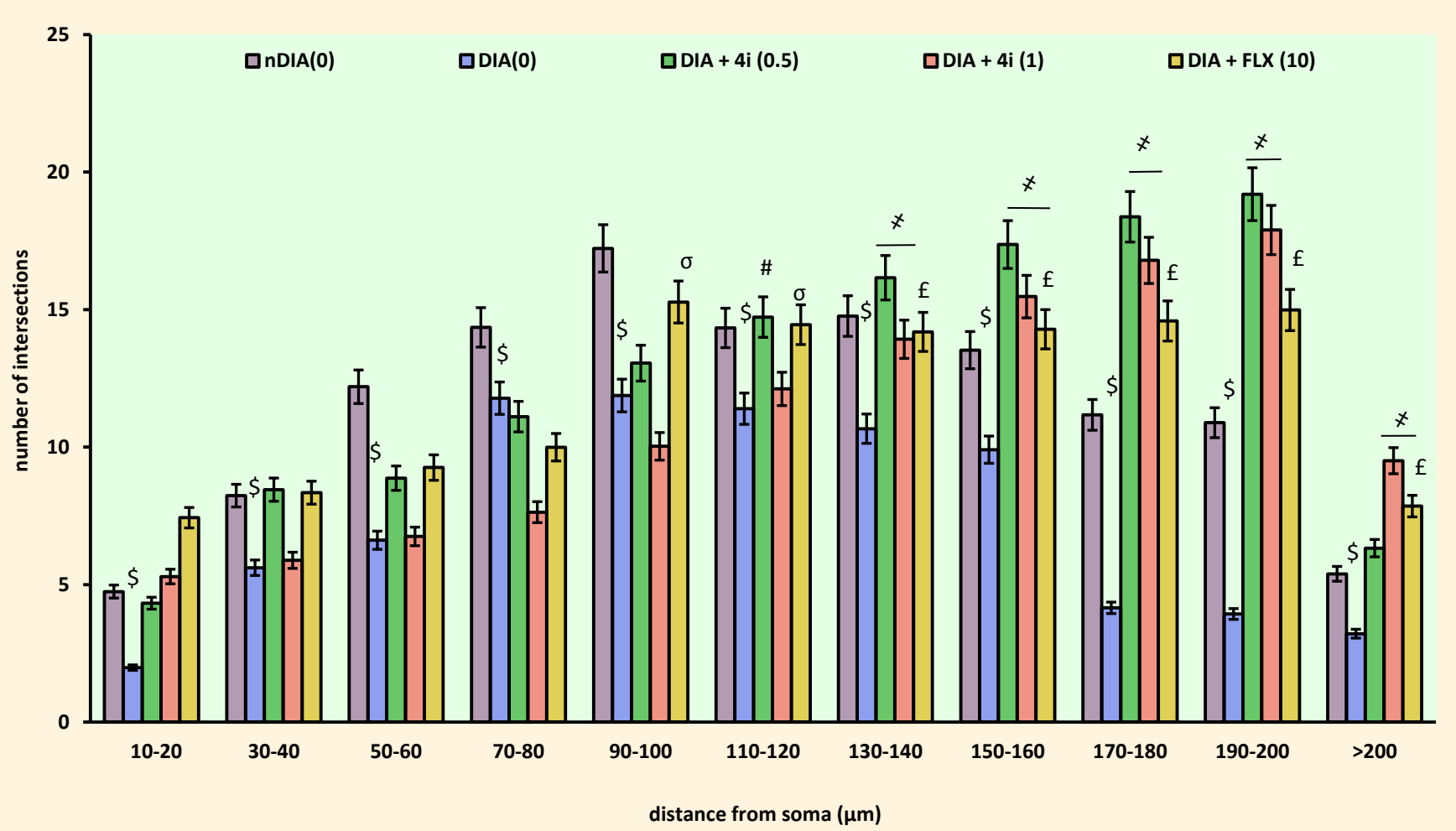


Fig. 6.58 The dendritic morphology is quantified as the number of intersections per circle (positioned at radial intervals of 10 µm) of pyramidal neurons in the CA_3 subfield of hippocampus. The columns represent mean values, while error bars show SEM. $ p < 0.01 vs control (nDIA (0)) and # p < 0.05, ≠ p < 0.01. σ p < 0.05, £ p < 0.01 vs diabetic control (DIA (0)), n = 3/group

6.6.6.3 Effect of diabetes and 6z

Haematoxylin-eosin staining: Qualitative examination of photomicrographs revealed that there was a marked change in number of damaged neuronal cells as a consequence of STZ-induced diabetes and chronic treatment with **6z**, in mice, which was qualitatively assessed by the photomicrographs, Fig. 6.59.

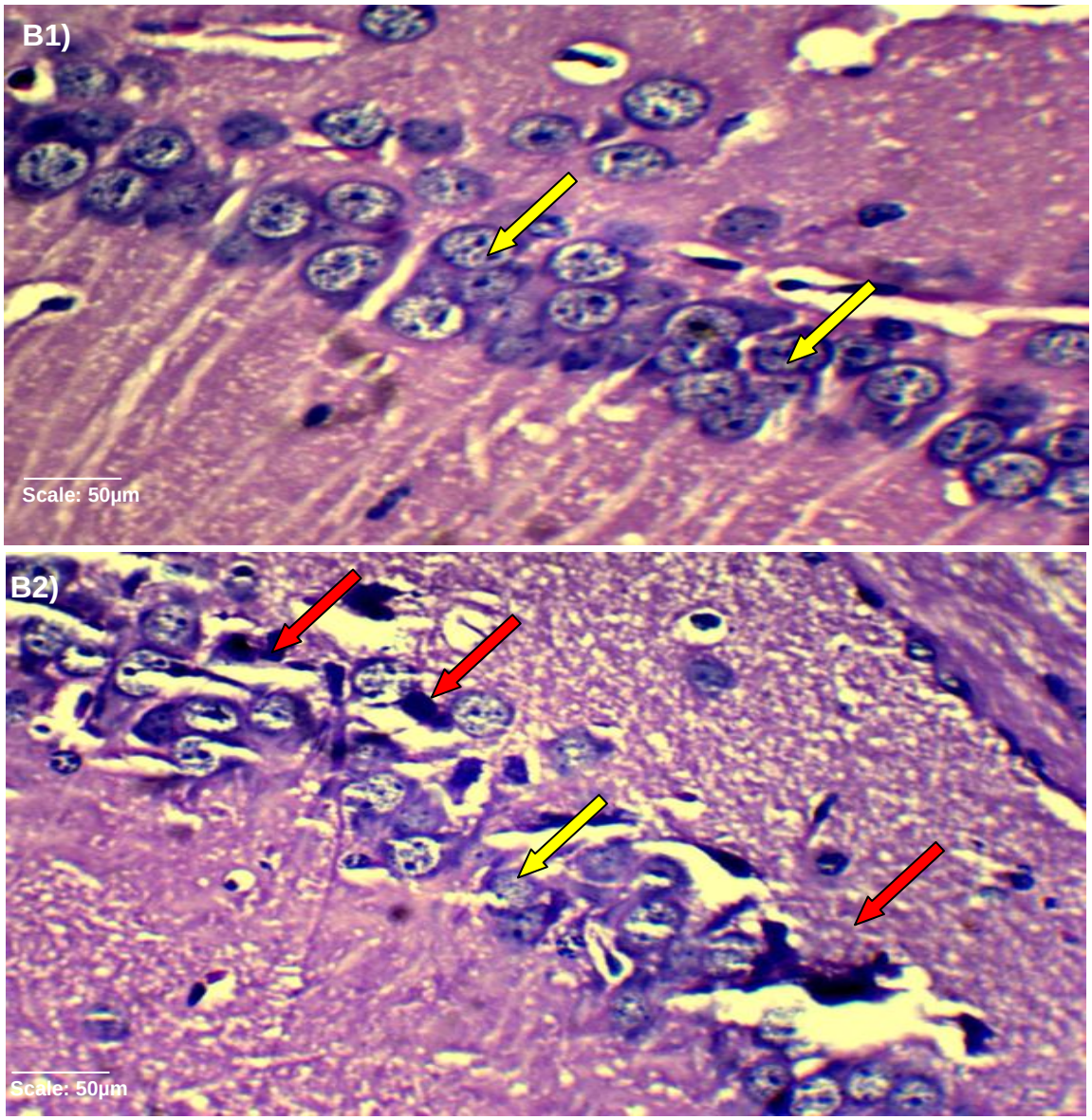
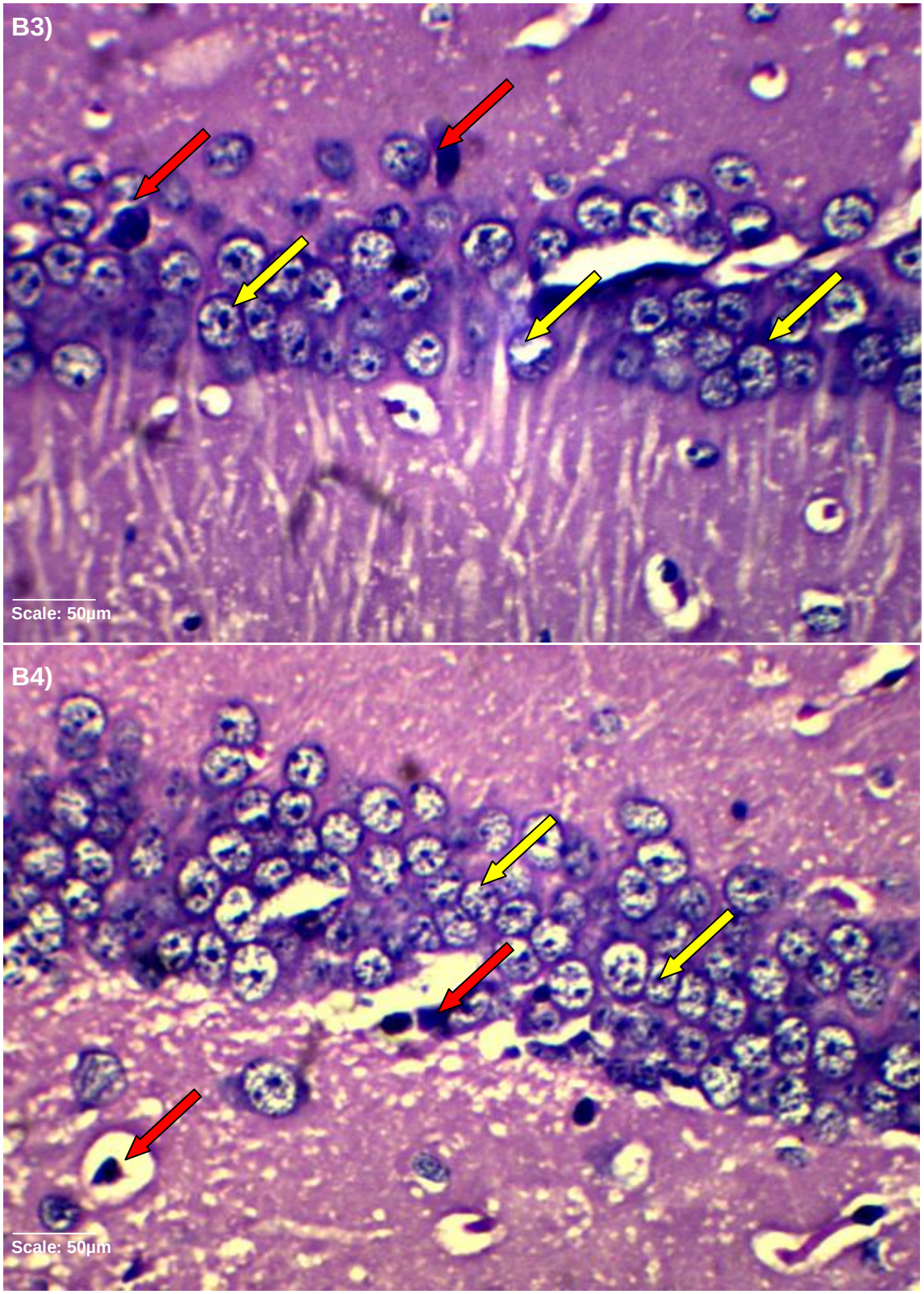
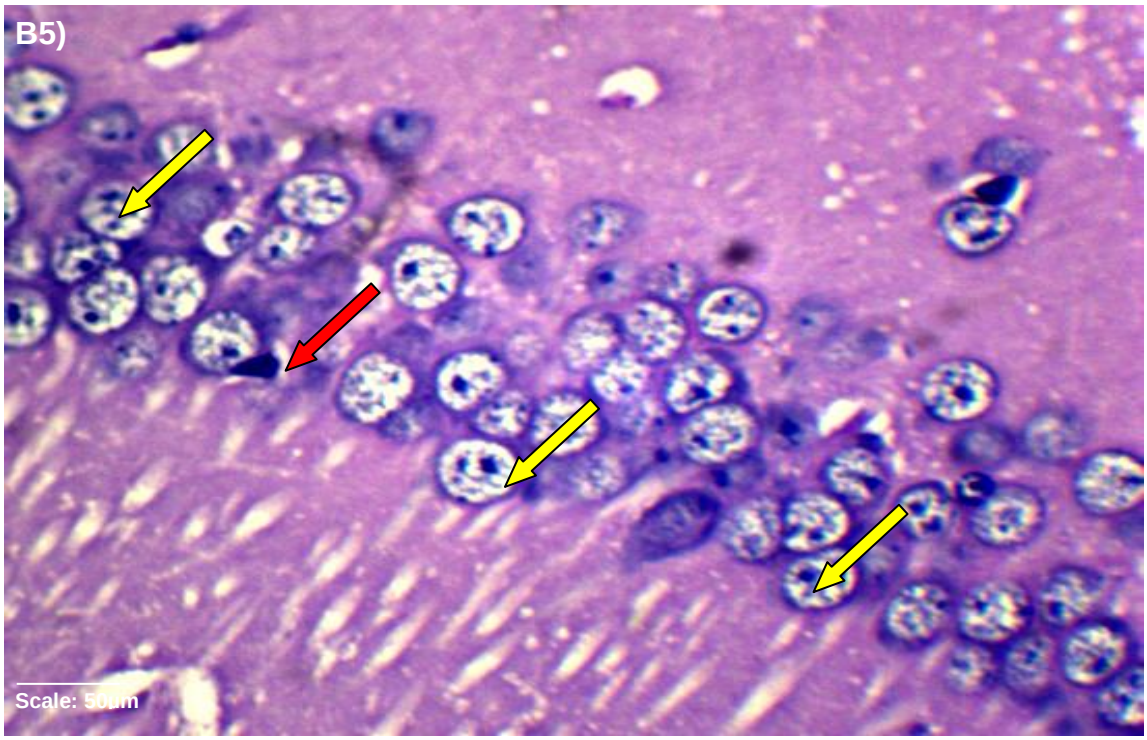


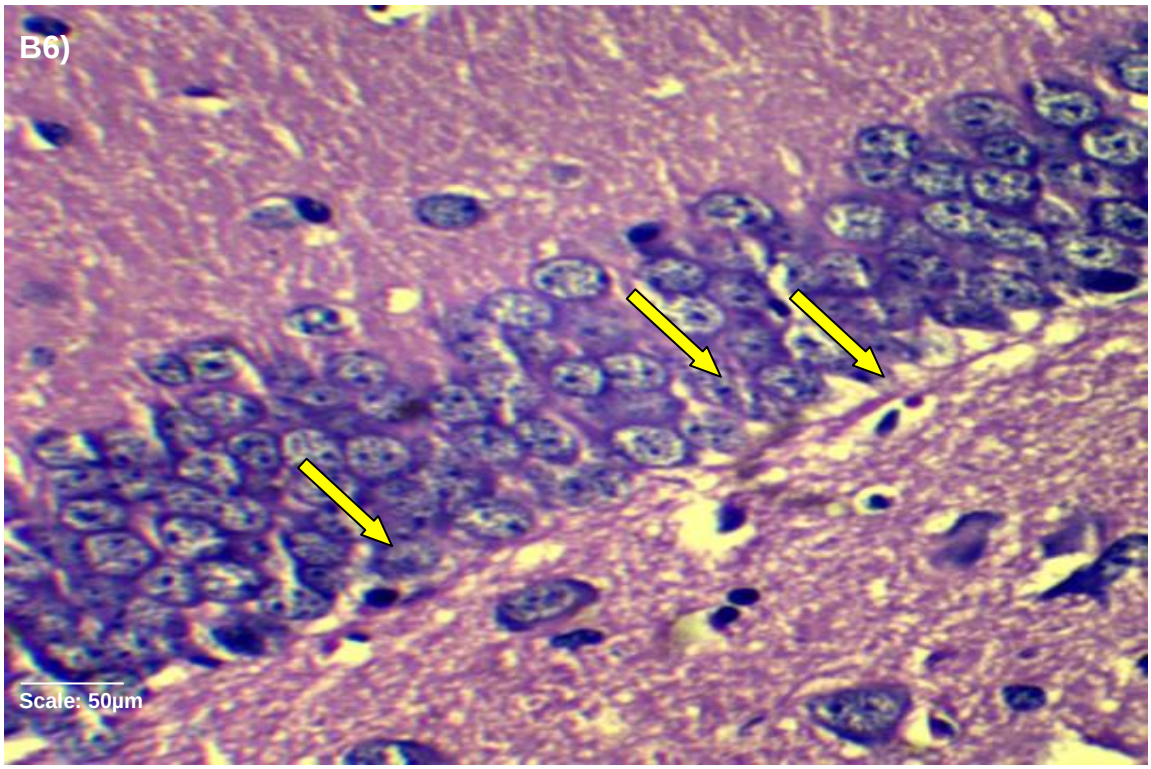
Fig. 6.59 The photomicrographs of histopathological sections of hippocampal CA_3 regions of mice brain subjected to hematoxylin and eosin staining. **B1,** CA_3 region of control: row of normal nerve cells with regular shape nucleus in the section, is seen. **B2,** CA_3 region of diabetic control: there are few normal nerve cells (marked by yellow arrows) with more number of the dark (deeply stained), irregular and shrunken nerve cells with damaged nucleus (marked by red arrows) are seen.



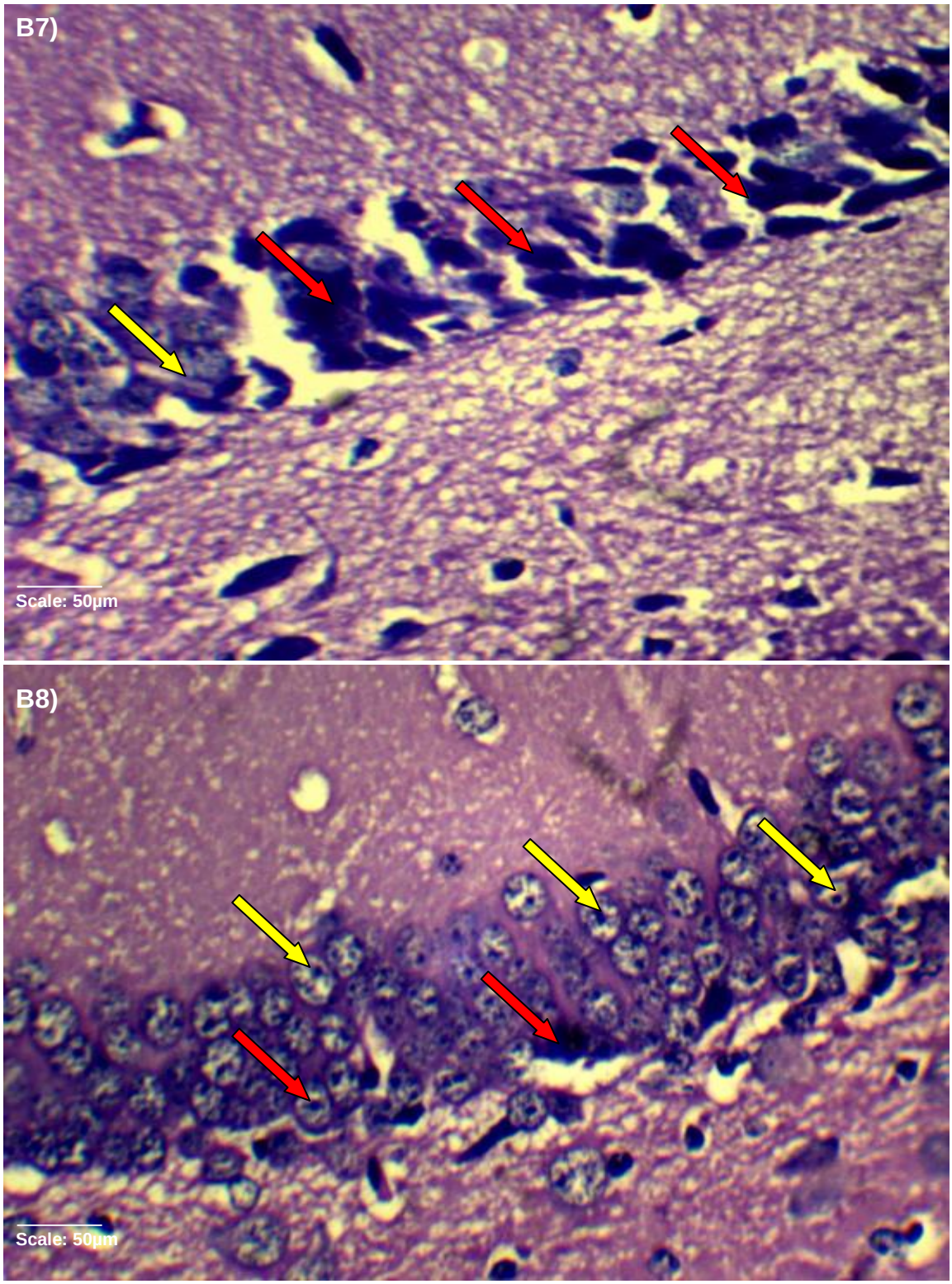
B3 and B4, CA_3 regions of diabetic mice treated with **6z** (1 mg/kg, i.p.) and **6z** (2 mg/kg, i.p.), respectively: more number of normal cells with regular shape nucleus is seen, in each section. Normal cells (marked by yellow arrows) and pyknotic cells (marked by red arrows)



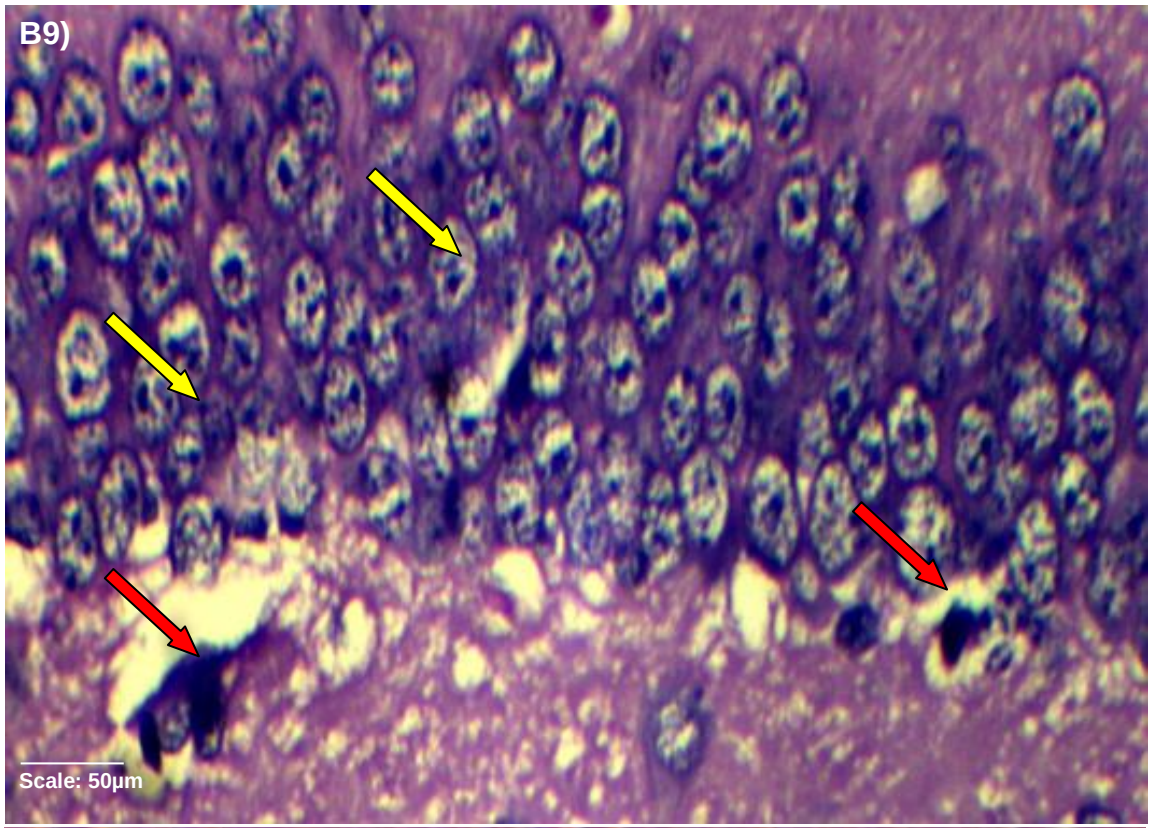
B5, CA_3 region of diabetic mice treated with FLX (10 mg/kg, i.p.): more number of normal cells is seen, normal cells (marked by yellow arrows) and pyknotic cells (marked by red arrows).

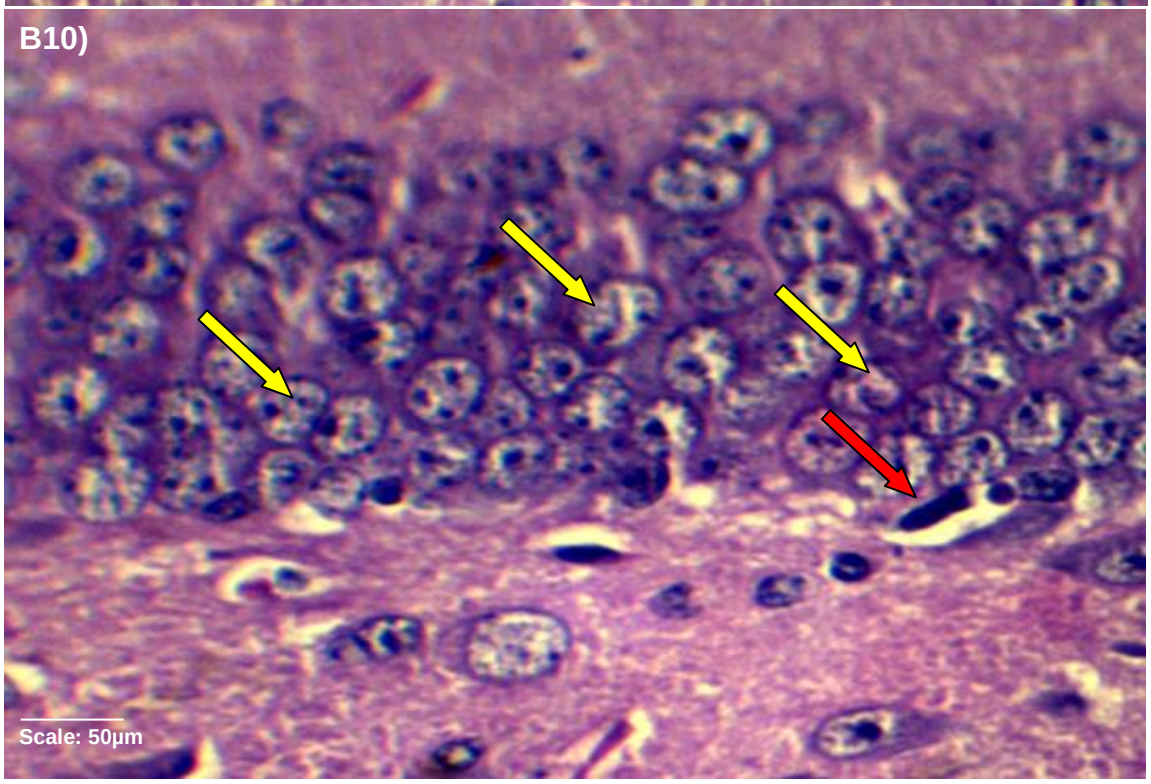


B6, dentate gyrus region of control: row of normal nerve cells in the section, is seen (marked by yellow arrows).



B7, dentate gyrus region of diabetic control: almost no normal nerve cells are present markedly dark, irregular and shrunken nerve cells (marked by red arrows) is seen as well as astrocyte filtrations (marked by green arrow) is observed. **B8, dentate gyrus** region of diabetic mice treated with **6z** (1 mg/kg, i.p.): more number of normal cells is seen.





B9 and B10, dentate gyrus regions of diabetic mice treated with **6z** (2 mg/kg, i.p.) and FLX (10 mg/kg, i.p.), respectively: more number of normal cells is seen in each section. Normal cells (marked by yellow arrows) and pyknotic cells (marked by red arrows).

Quantitative evaluation using 'Cell Counter' plugin indicated that there was a significant change in the percentage damaged cells in hippocampal regions of CA_3 [F (4, 14) = 12.110, p < 0.001] and dentate gyrus [F (4, 14) = 13.892, p < 0.001], in mice subjected to different treatments.

STZ-induced diabetic mice exhibited a significant increase in the percentage of damaged cells in CA_3 and dentate gyrus hippocampal regions, as compared to control mice (p <0.001). Interestingly, the percentage of damaged cells was significantly less in STZ-induced diabetic mice treated with **6z** (1 and 2 mg/kg, i.p.) and FLX (10 mg/kg, i.p.) (p < 0.001), as compared to those received vehicle only, as shown in Fig. 6.60.

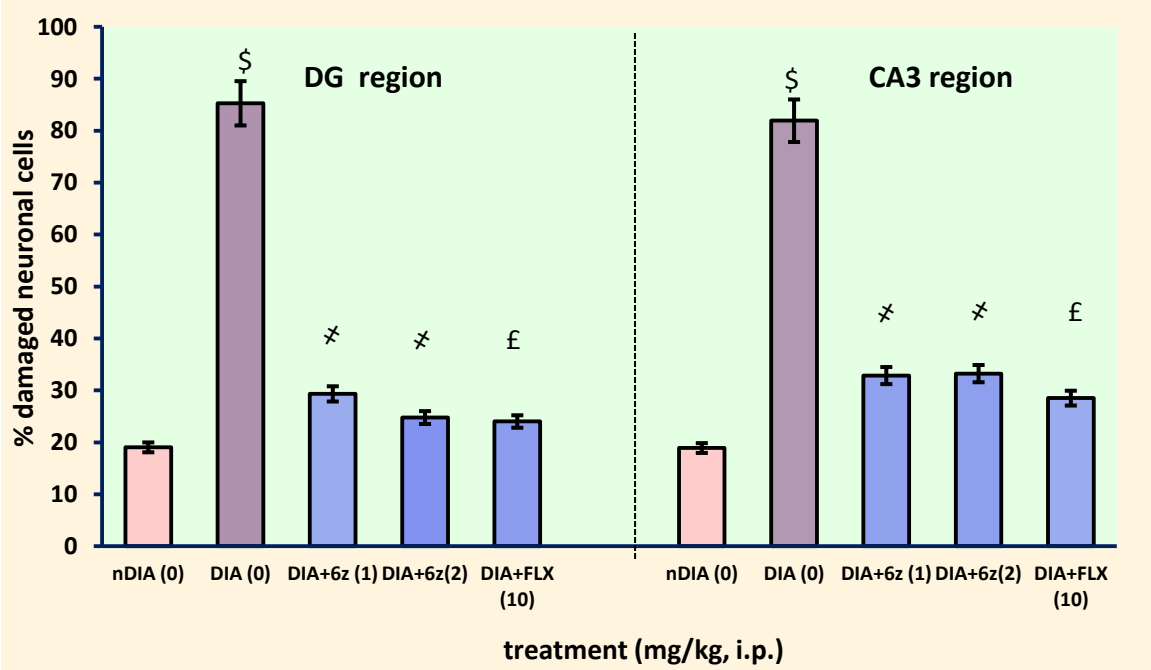


Fig. 6.60 The columns indicate mean values of % of damaged cells and error bars show S.E.M. **$** p < 0.001 indicate significant difference compared to control and ≠ p < 0.001, £ p < 0.001 vs diabetic control group, n = 6/group.

Golgi-Cox staining: In Golgi-Cox staining there was a marked change in dendritic morphology which is qualitatively evaluated in the photomicrographs, Fig. 6.61.

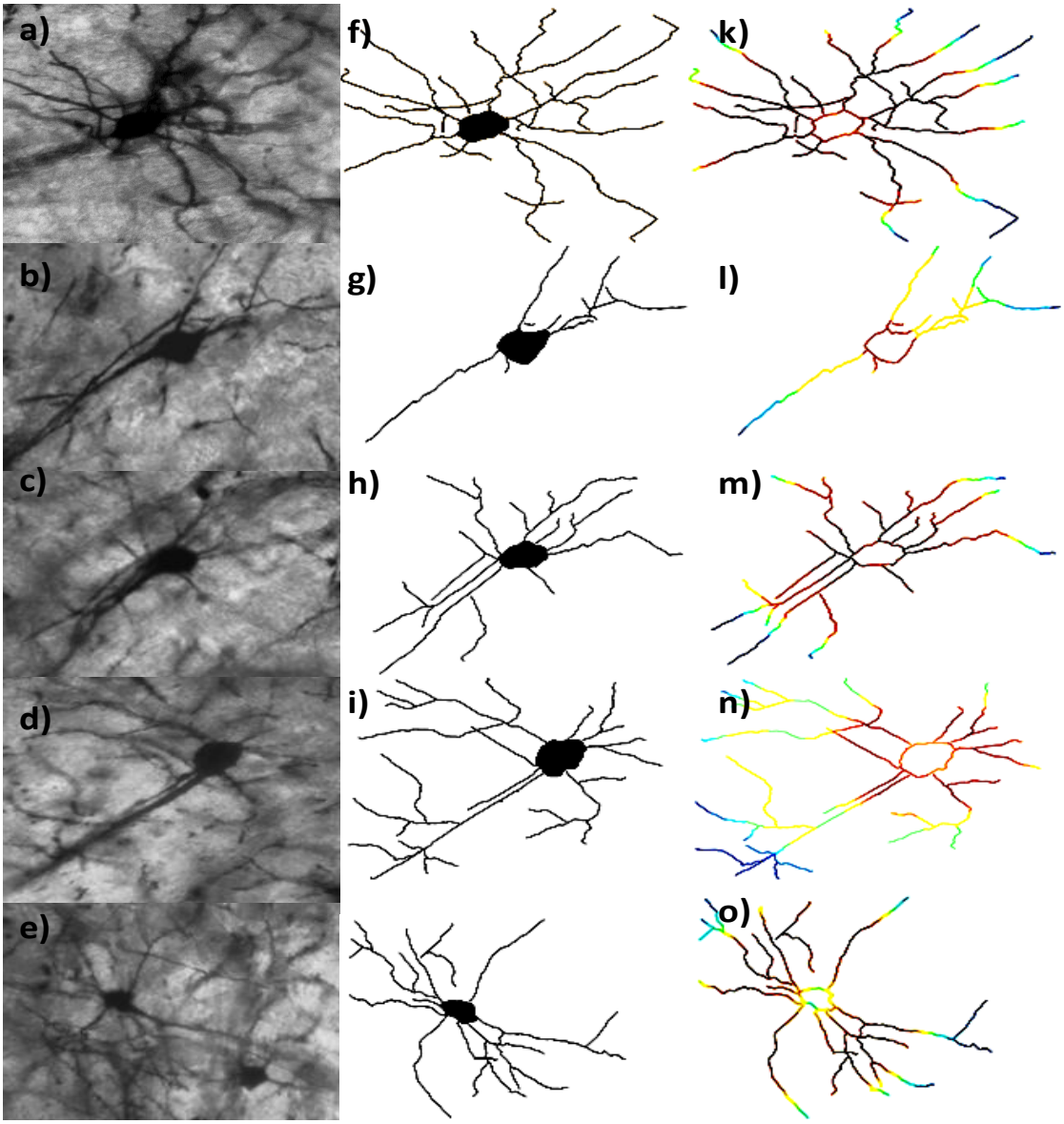


Fig. 6.61 Representative photomicrographs of neurons stained with Golgi-Cox, **(a-e),** tracing of the neurons, **(f-j)** and Sholl mask of these neurons **(k-o)** corresponding to the experimental groups, DIA (0), nDIA (0), DIA+**6z** (1), DIA+**6z** (2) and DIA+FLX (10) respectively.

There was a significant difference in cumulative branch length (µm) of primary, secondary and tertiary dendrites of pyramidal neurons among the groups [$F(4, 55) = 29.11$, $p < 0.01$] subjected to different treatments. STZ-induced diabetic mice exhibited a significant reduction in branch length of primary, secondary and tertiary dendrites of pyramidal neurons as compared to non-diabetic mice ($p < 0.01$). This reduction was significantly abolished by chronic treatment with FLX (10 mg/kg, i.p.) ($p < 0.001$).

Chronic **6z** (1 and 2 mg/kg, i.p.) treatment significantly enhanced the branch length of primary and secondary dendrites of pyramidal neurons in diabetic mice ($p < 0.01$), as shown in Fig. 6.62.

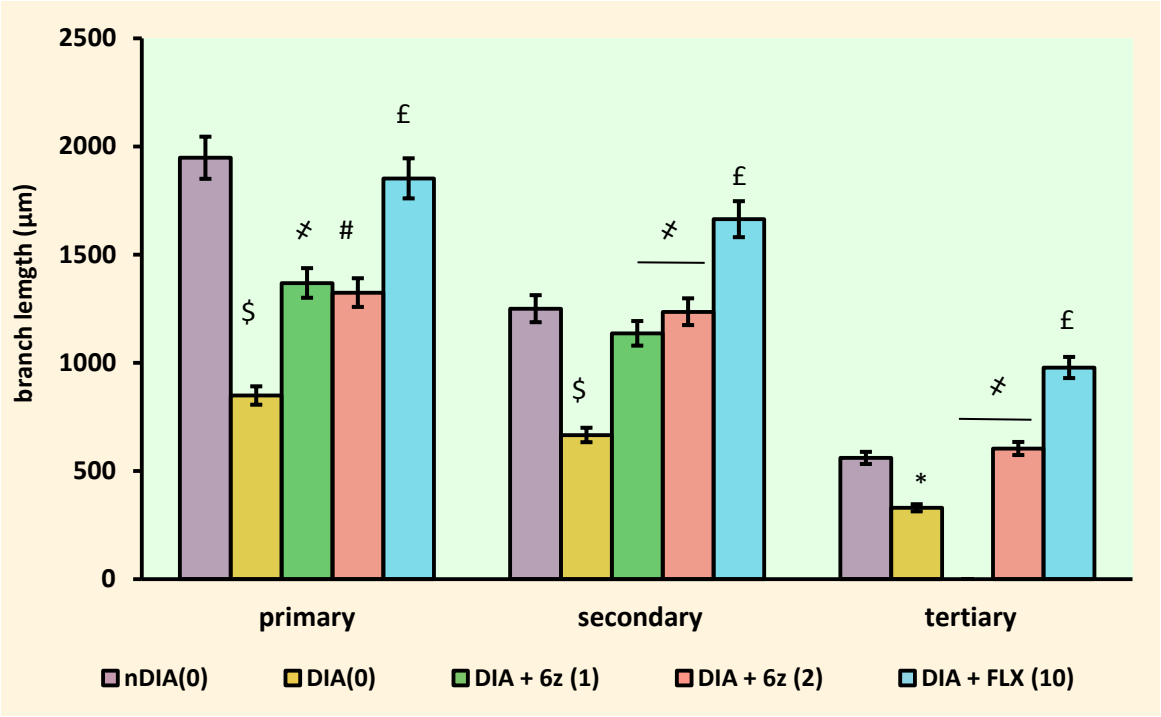


Fig. 6.62 The dendritic morphology is quantified as cumulative branch length (µm) of primary, secondary and tertiary branch points of pyramidal neurons in CA_3 subfield of hippocampus. The columns represent mean values, while error bars show SEM. * $p < 0.05$, $ $p < 0.01$ vs control and # $p < 0.05$, ≠ $p < 0.01$, £ $p < 0.01$ vs diabetic control, n = 3/group.

There was a significant difference in the number of dendritic intersections among the different groups [$F(4, 55) = 11.33$, $p < 0.01$]. STZ-induced diabetic mice exhibited significantly fewer dendritic intersections than non-diabetic mice ($p < 0.001$) at the proximal 10-20 µm, 30-80 µm and above 190 µm regions. Chronic treatment with FLX (10mg/kg, i.p.) increased the number of intersections in diabetic mice at the uniformly at all regions to levels similar to those found in control mice ($p < 0.01$). Chronic **6z** (1 mg/kg, i.p.) treatment significantly enhanced the number of intersections in diabetic mice at 10-80 and above 110 µm regions ($p < 0.05$). **6z** (2 mg/kg, i.p.) treatment significantly enhanced the number of intersections in diabetic mice at these regions ($p < 0.05$), Fig. 6.63.

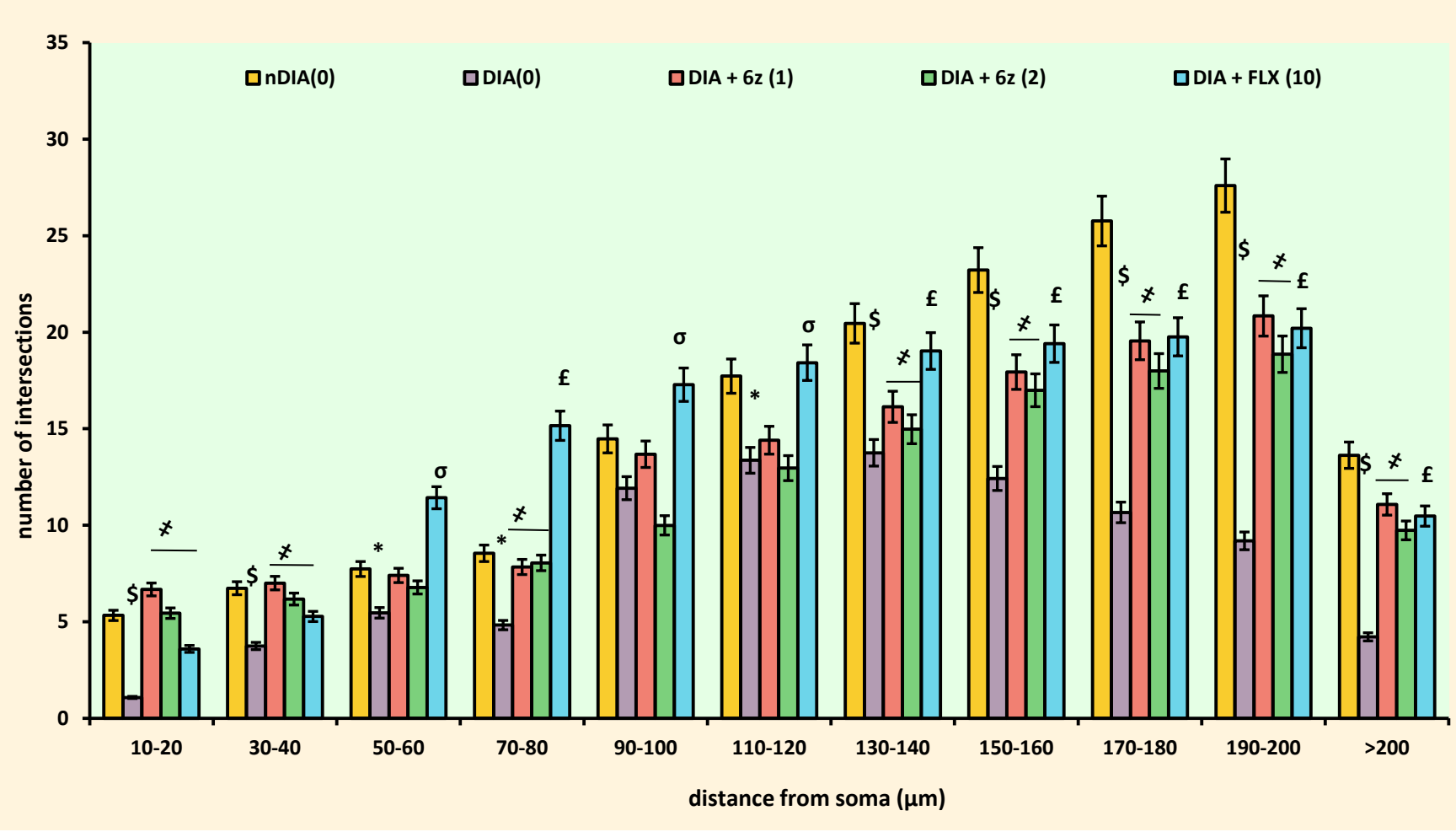


Fig. 6.63 The dendritic morphology is quantified as cumulative the number of intersections per circle (positioned at radial intervals of 10 µm) of pyramidal neurons in the CA_3 subfield of hippocampus. The columns represent mean values, while error bars show SEM. * $p < 0.05$, $ $p < 0.01$ vs control and # $p < 0.05$, ≠ $p < 0.01$, σ $p < 0.05$, £ $p < 0.01$ vs diabetic control, n = 3/group.

6.7 The role of 5-HT_3 receptors in mediating antidepressant-like effect of the test drug candidates

To investigate the role of 5-HT_3 receptors on the antidepressant-like effect of the OND and novel 5-HT_3 receptor antagonists, the effect of concomitant administration of a selective 5-HT_3 receptor agonist, mCPBG was carried out using behavioral test battery and biochemical assays.

6.7.1 Effect of concomitant treatment of mCPBG, a selective 5-HT_3 receptor agonist on antidepressant-like effect of OND

6.7.1.1 Effect on duration of immobility in FST

During FST, pre-treatment with mCPBG blunted the OND (1 mg/kg, i.p.) mediated reduction in duration of immobility (s) [$F(3, 15) = 4.103$, $p < 0.05$] in STZ-induced diabetic mice. In this case, (mCPBG + OND treatment), the duration of immobility (s) in STZ-induced diabetic mice remained significantly elevated after OND (1 mg/kg, i.p.) dosing as compared to the group that received OND (1 mg/kg, i.p.) but not mCPBG (OND treatment alone) ($p < 0.01$). Comparison between STZ-induced diabetic control group and diabetic group received mCPBG + OND represented no significant alteration in duration of immobility ($p > 0.05$), which suggests an impairment in the behavioral effect produced by OND (1 mg/kg, i.p.) due to mCPBG. Furthermore, mCPBG alone had no significant effect on duration of immobility in STZ-induced diabetic mice, subjected to FST ($p > 0.05$), as shown in Fig. 6.64.

6.7.1.2 Effect on duration of immobility in TST

TST, pre-treatment with mCPBG blocked the OND (1 mg/kg, i.p.) mediated reduction in duration of immobility (s) [$F(3, 15) = 7.192$, $p < 0.001$] in STZ-induced diabetic mice. Diabetic mice treated with mCPBG (10 mg/kg, i.p.) + OND (1 mg/kg, i.p.) exhibited elevated duration of immobility (s) as compared to the group that received OND (1 mg/kg, i.p.) but not mCPBG (OND treatment alone) ($p < 0.05$). Comparison between STZ-induced diabetic control group and diabetic group received mCPBG + OND represented no significant alteration in duration of immobility ($p > 0.05$), which suggests an impairment in the behavioral effect elicited by OND (1 mg/kg, i.p.) due to mCPBG, as shown in Fig. 6.65.

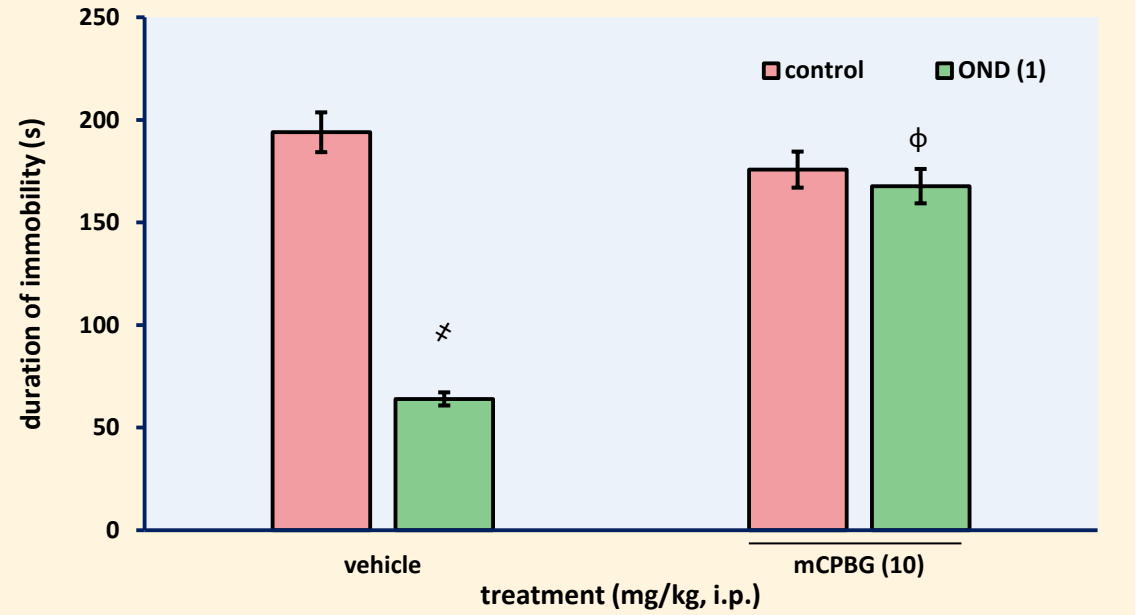


Fig. 6.64 The columns represent mean values of duration of immobility (s), while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$, vs diabetic control, ϕ $p < 0.01$ vs diabetic mice that received OND (1 mg/kg, i.p.), n = 6/ group.

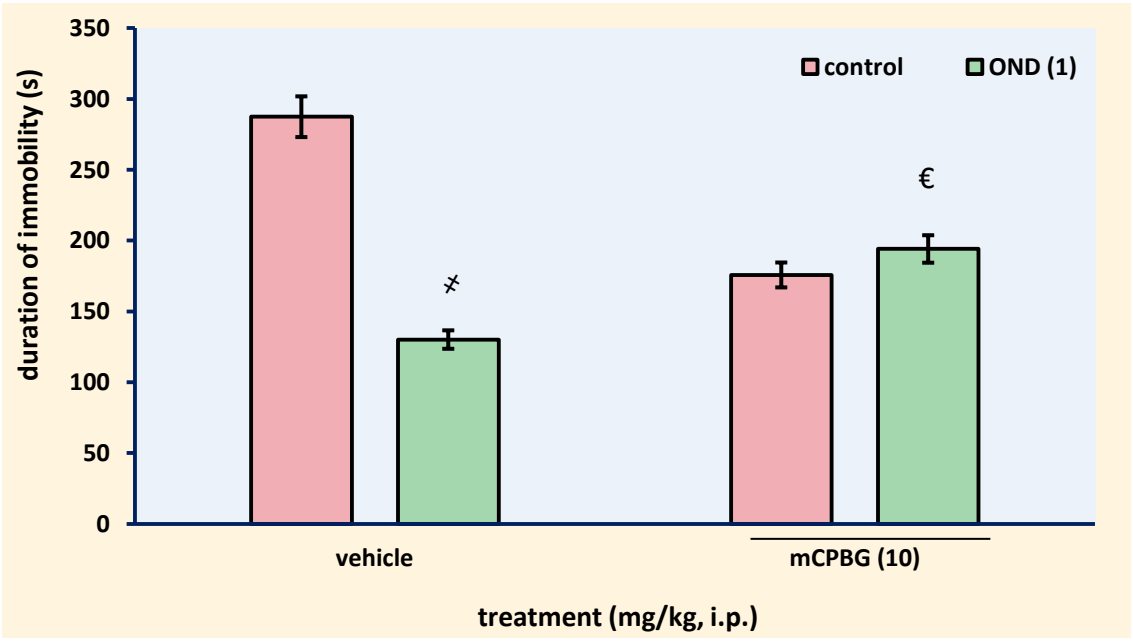


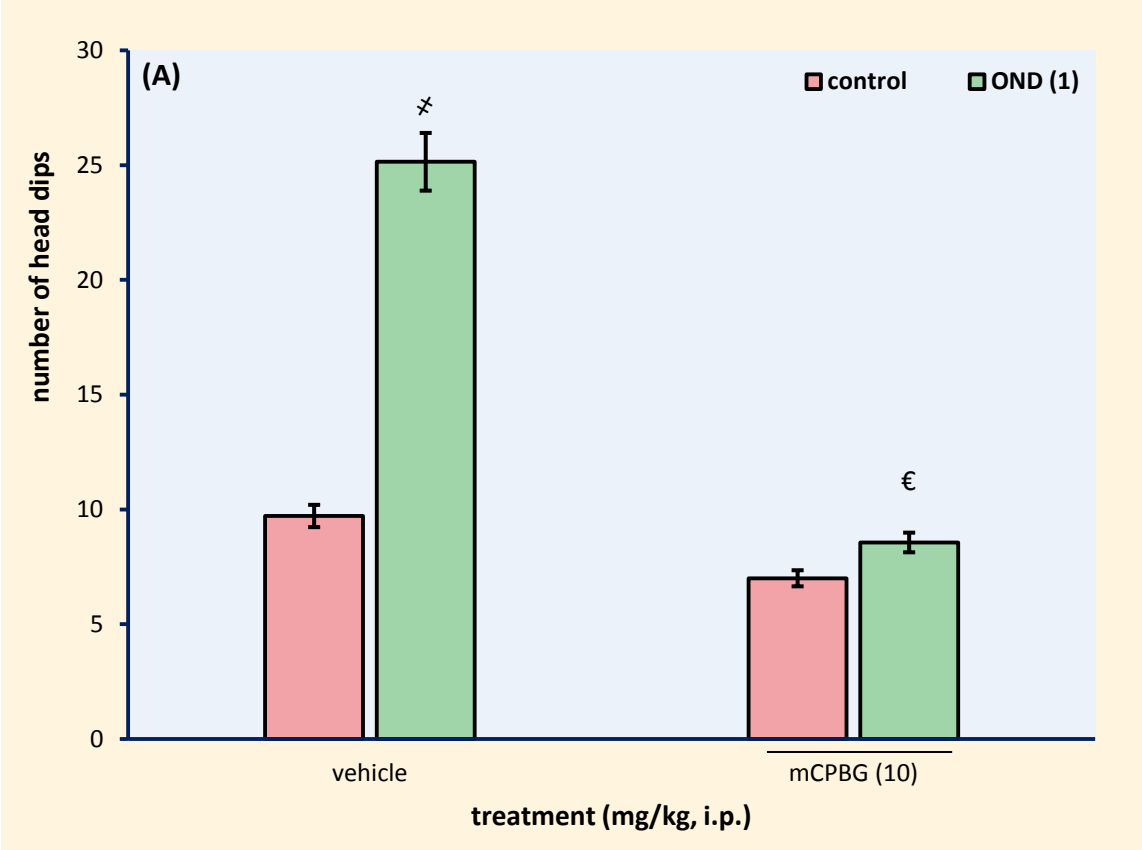
Fig. 6.65 The columns represent mean values of duration of immobility (s), while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$, vs diabetic control, € $p < 0.05$, vs diabetic mice that received OND (1 mg/kg, i.p.), n = 6/group.

6.7.1.3 Effect on exploratory behavior in hole-board test

Similarly, in hole-board test, pre-treatment of mCPBG (10 mg/kg, i.p.) blunted the increase in exploratory behavior, namely: number of head dips [F (3, 15) = 7.832, $p < 0.001$] and duration of head dips [F (3, 15) = 12.902, $p < 0.001$] in STZ-induced diabetic mice given OND (1 mg/kg, i.p.) treatment.

The number of head dips ($p < 0.001$) as well as duration of head dips ($p < 0.001$) remained significantly reduced in STZ-induced diabetic mice that received mCPBG before OND treatment (mCPBG + OND treatment) as compared to the group that did not receive mCPBG before OND (OND treatment alone).

Furthermore, mCPBG alone exhibited no significant effects on these parameters ($p > 0.05$), Fig. 6.66.



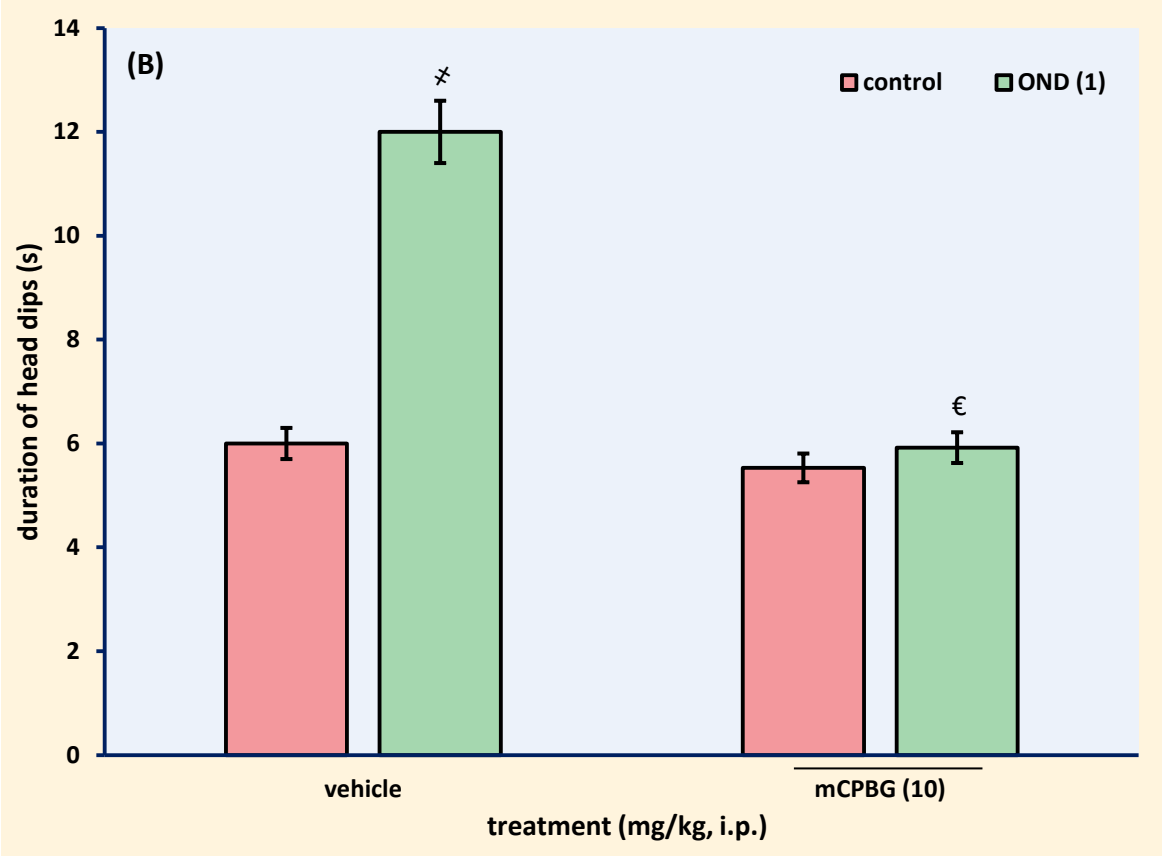


Fig. 6.66 The columns represent mean values of number of head dips **(A)** and duration of head dips (s) **(B)**, while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$, vs diabetic control, € $p < 0.01$ vs diabetic mice that received OND (1 mg/kg, i.p.), n = 6/group.

6.7.1.4 Effect on exploratory behavior in light-dark test

Statistical analysis revealed that in light-dark test, pre-treatment of mCPBG (10 mg/kg, i.p.), inhibited the increase in exploratory behavior namely, latency (s) [$F (3, 15) = 12.894$, $p < 0.001$] and time spent in light chamber [$F (3, 15) = 8.994$, $p < 0.001$] in STZ-induced diabetic mice treated with OND (1 mg/kg, i.p.).

The latency ($p < 0.001$) and time spent in light chamber ($p < 0.001$) remained significantly reduced in diabetic mice that received mCPBG before OND treatment (mCPBG + OND treatment) as compared to the group subjected to OND treatment alone. mCPBG alone, exhibited no significant effects on these parameters ($p > 0.05$), Fig. 6.67A and B.

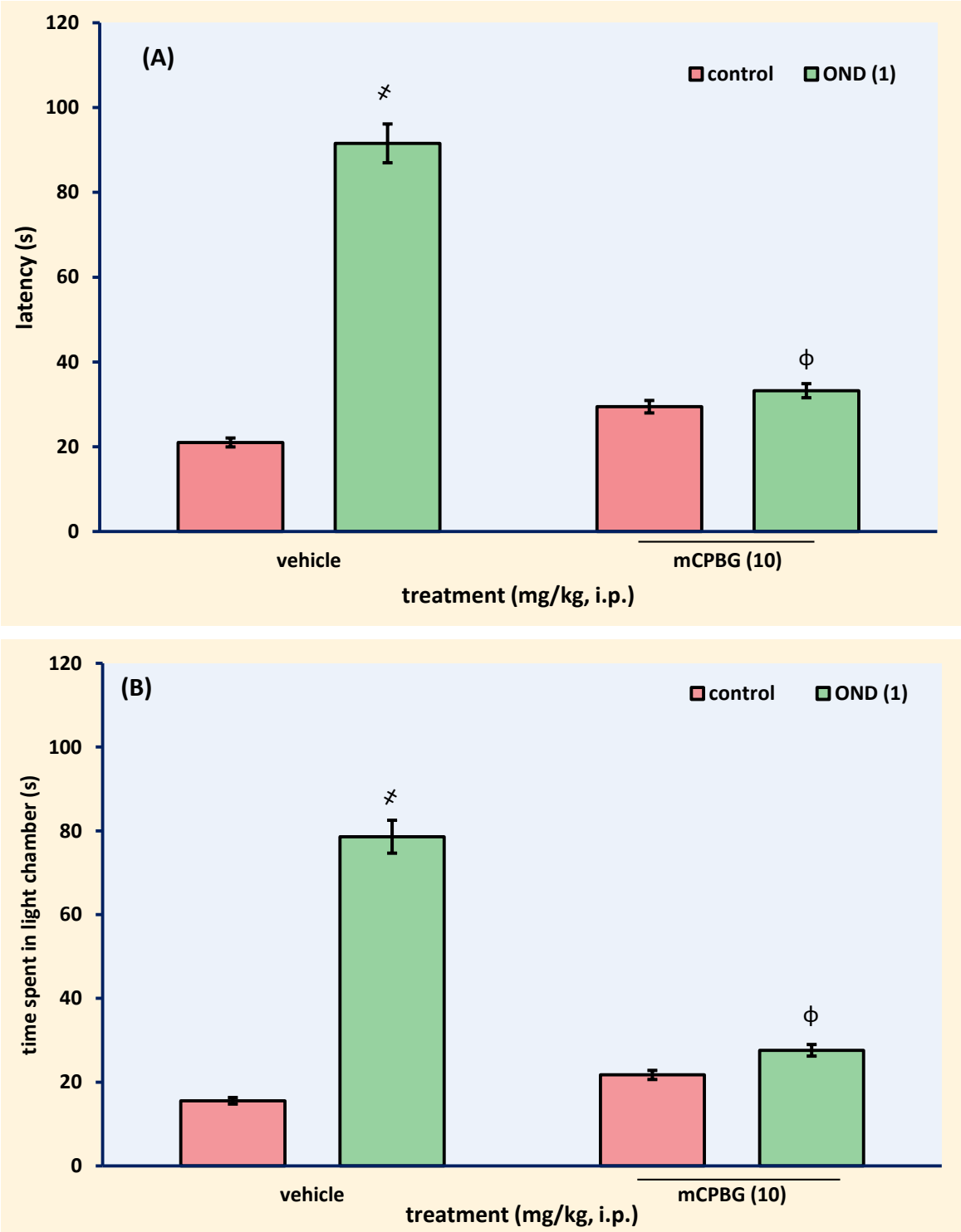


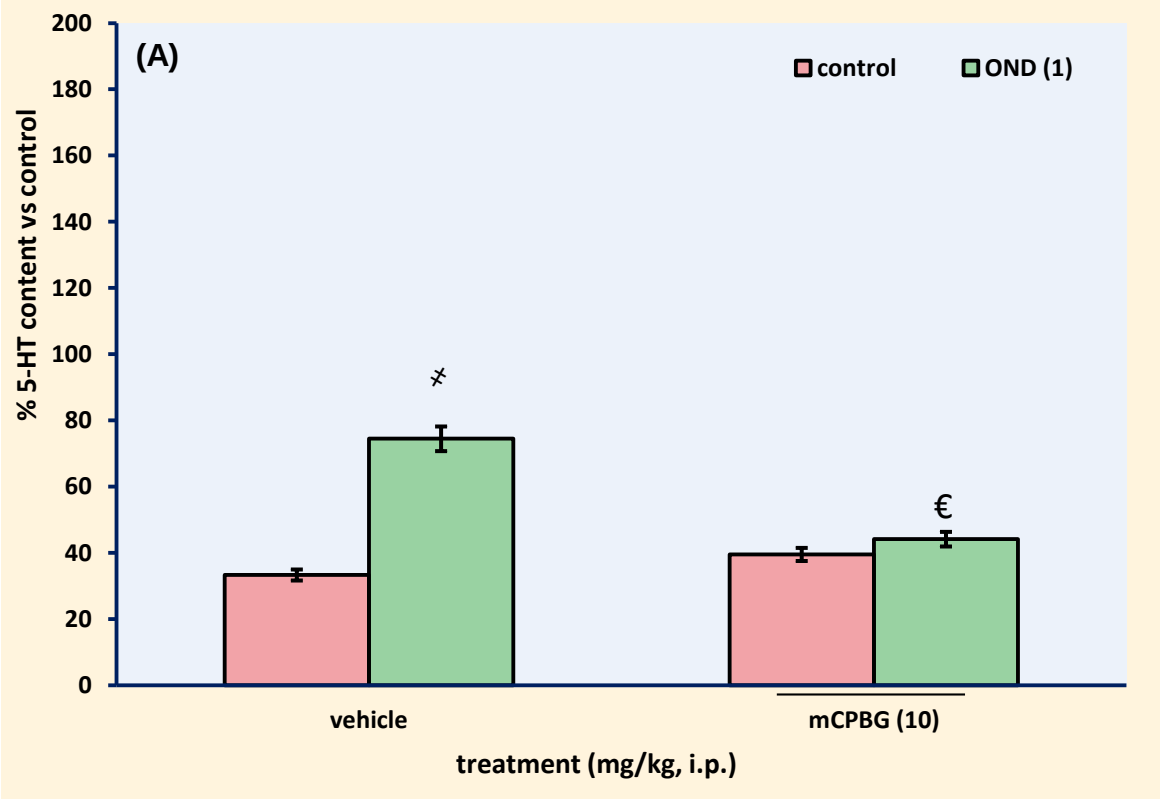
Fig. 6.67 The columns represent mean values of latency (s) **(A)** and time spent in light chamber (s) **(B)** while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$, vs diabetic control, ϕ $p < 0.01$ vs diabetic mice that received OND (1 mg/kg, i.p.), n = 6/group.

6.7.1.5 Effect on 5-HT levels in discrete brain regions

Biochemical assay examining 5-HT level in discrete brain regions also depicted the similar pattern. mCPBG (10 mg/kg, i.p.) treatment significantly diminished OND-mediated increase in 5-HT level, in midbrain (including hippocampus) [$F(3,15) = 12.210$, $p < 0.001$], frontal cortex [$F(3,15) = 4.324$, $p < 0.001$] and cerebellum [$F(3,15) = 12.600$, $p < 0.001$], in STZ-induced diabetic mice.

After OND (1 mg/kg, i.p.) dosing, the 5-HT level remained significantly low in midbrain ($p < 0.05$), frontal cortex ($p < 0.001$) and cerebellum ($p < 0.001$) in STZ-induced diabetic mice pre-treated with mCPBG (10 mg/kg, i.p.) (that is in the group received mCPBG + OND treatment), as compared to those that did not receive mCPBG (OND treatment alone).

Furthermore, no significant alterations in 5-HT level, in these measured brain areas were observed after mCPBG administration alone ($p > 0.05$), as represented in Fig. 6.68A-C, respectively.



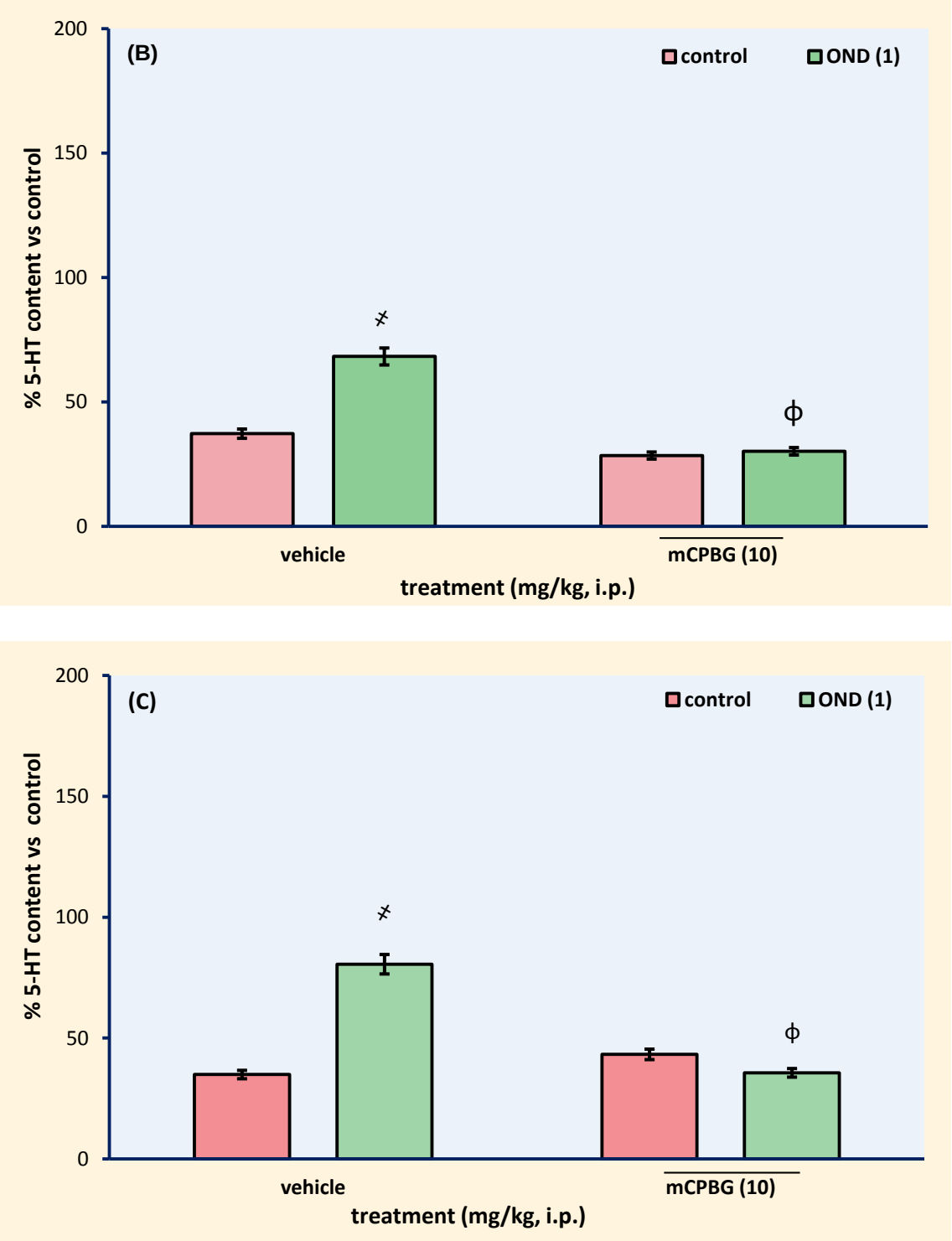


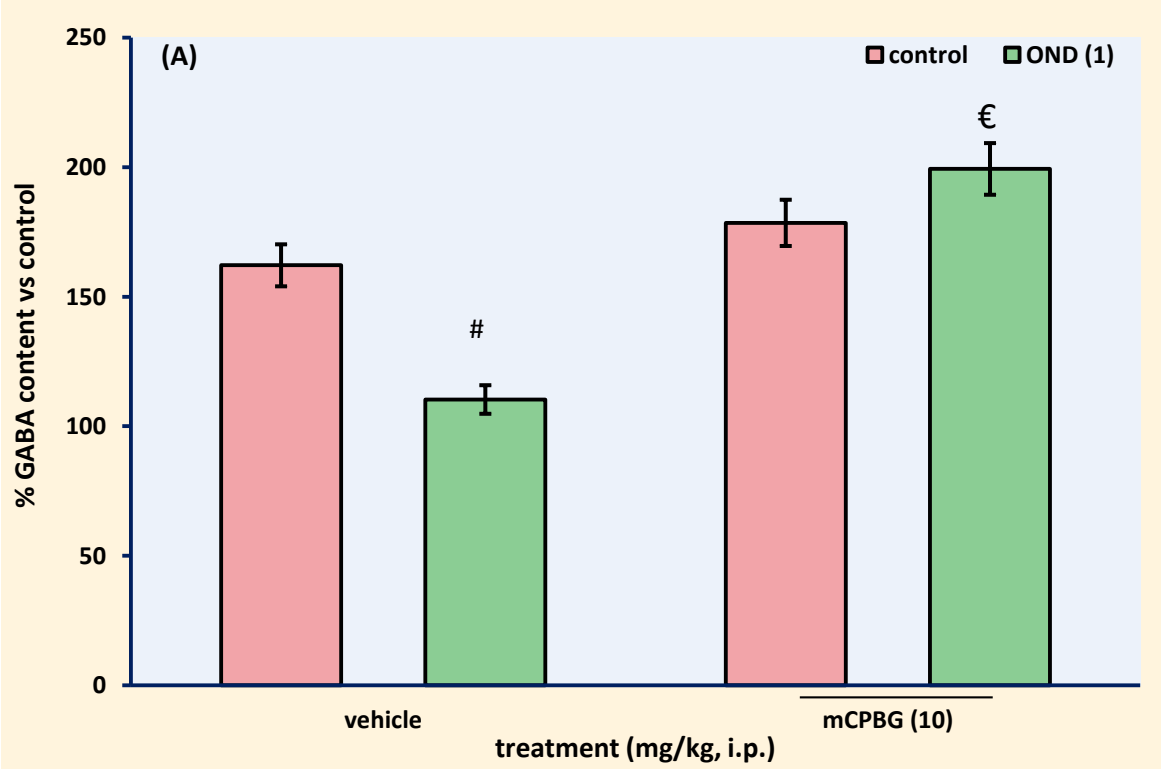
Fig. 6.68 The columns represent mean values of % 5-HT content in **(A)** midbrain (including hippocampus, **(B)** frontal cortex and **(C)** cerebellum, while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$, vs diabetic control, € $p < 0.05$, ϕ $p < 0.01$ vs diabetic mice that received OND (1 mg/kg, i.p.), n = 6/group.

6.7.1.6 Effect on GABA levels in discrete brain regions

A similar pattern, as that of the 5-HT levels, was observed in case of estimation of GABA levels in discrete brain regions. mCPBG (10 mg/kg, i.p.) treatment, significantly diminished OND-mediated decrease in GABA level in midbrain (including hippocampus) [$F(3,15) = 5.760$, $p < 0.05$], frontal cortex [$F(3,15) = 8.120$, $p < 0.05$] and cerebellum [$F(3,15) = 6.680$, $p < 0.05$] in STZ-induced diabetic mice.

After OND (1 mg/kg, i.p.) dosing, the GABA level remained significantly high in midbrain ($p < 0.05$), frontal cortex ($p < 0.05$) and cerebellum ($p < 0.05$) in STZ-induced diabetic mice pre-treated with mCPBG (10 mg/kg, i.p.) (i.e., in the group that received mCPBG + OND treatment) as compared to those that received vehicle instead of mCPBG (OND treatment alone).

Furthermore, no significant alterations in GABA level were observed in these measured brain areas, after mCPBG administration alone ($p > 0.05$), as represented in Fig. 6.69A-C, respectively.



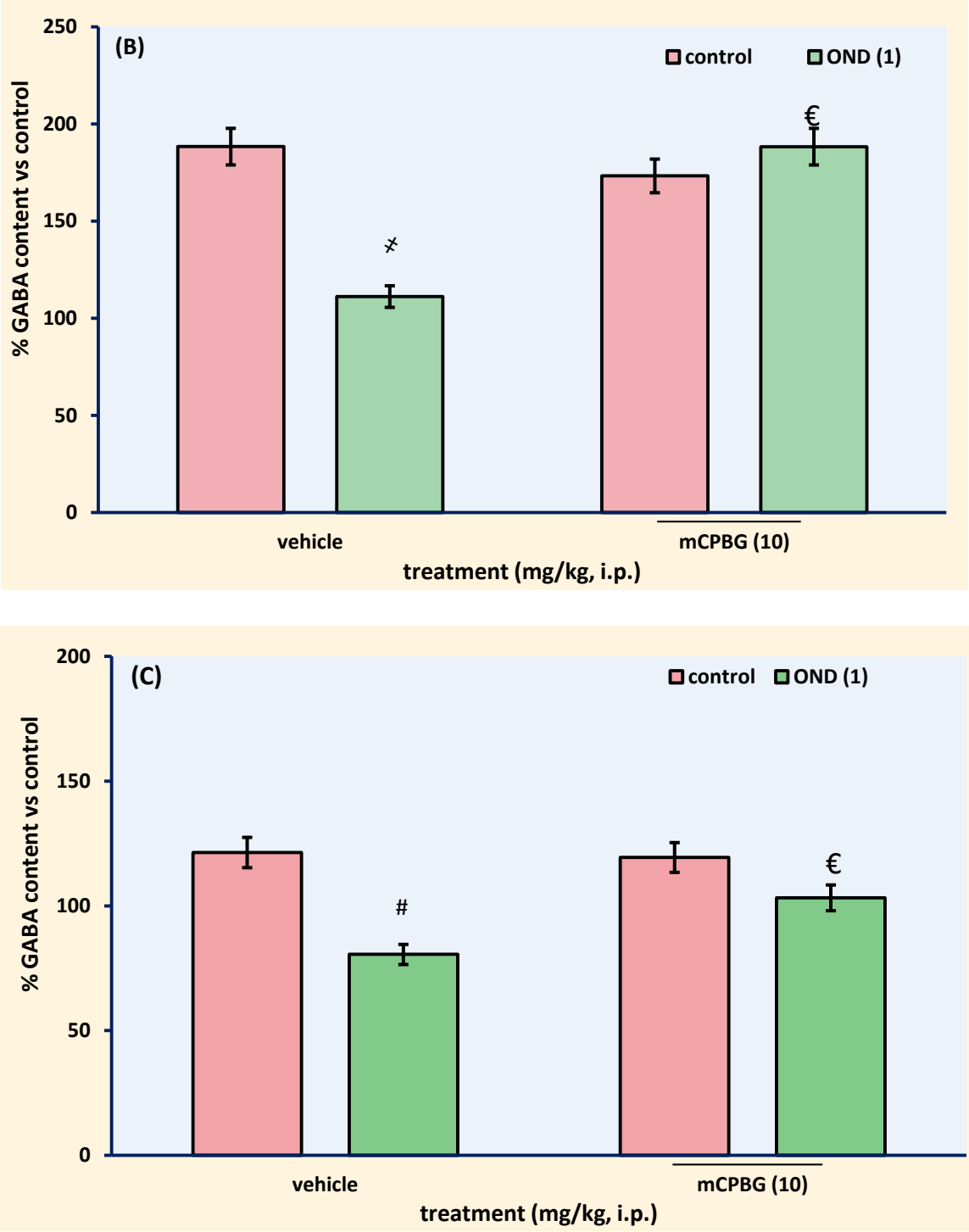


Fig. 6.69 The columns represent mean values of % GABA content in **(A)** midbrain (including hippocampus, **(B)** frontal cortex and **(C)** cerebellum, while error bars show S.E.M. The results from post hoc tests are indicated in the figure, # $p < 0.05$ vs diabetic control, € $p < 0.05$ vs diabetic mice that received OND (1 mg/kg, i.p.), n = 6/group.

6.7.2 Effect of concomitant treatment of mCPBG, a selective 5-HT_3 receptor agonist, on antidepressant-like effect of 4i

6.7.2.1 Effect on duration of immobility in FST

Statistical analysis depicted that in FST, pre-treatment with mCPBG blunted the **4i** (1 mg/kg, i.p.) mediated reduction in duration of immobility (s) [$F(3,15) = 5.709$, $p < 0.001$] in STZ-induced diabetic mice. In this case (mCPBG + **4i** treatment), the duration of immobility (s) in diabetic mice remained significantly elevated after **4i** (1 mg/kg, i.p.) dosing as compared to the group that received **4i** (1 mg/kg, i.p.) but not mCPBG (**4i** treatment alone) ($p < 0.01$). Comparison between diabetic control group and diabetic group that received mCPBG + **4i** exhibited no significant change in duration of immobility ($p > 0.05$), thereby suggesting an impairment in the behavioral effect elicited by **4i** (1 mg/kg, i.p.) due to mCPBG. Interestingly, mCPBG alone had no significant effect on duration of immobility in diabetic mice subjected to FST ($p > 0.05$), Fig. 6.70.

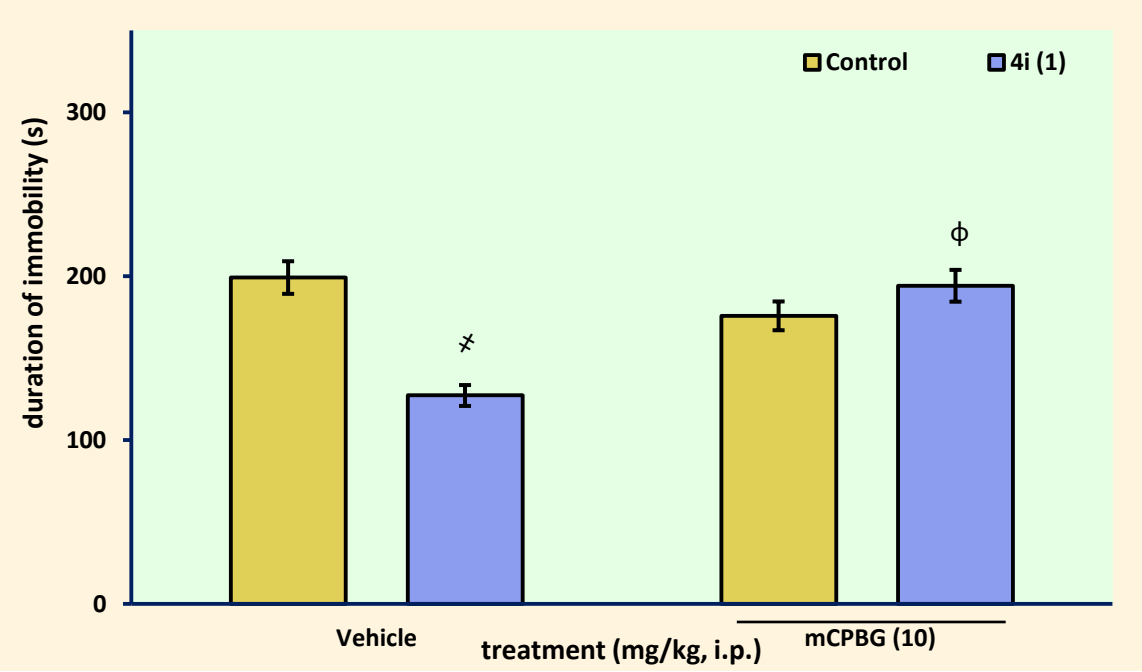


Fig. 6.70 The columns represent mean values of duration of immobility (s) in FST, while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$, vs diabetic control, ϕ $p < 0.01$ vs diabetic mice that received **4i** (1 mg/kg, i.p.), n = 6/ group.

6.7.2.2 Effect on duration of immobility in TST

Similarly, during TST, concomitant administration of mCPBG blocked the **4i** (1 mg/kg, i.p.) mediated reduction in duration of immobility (s) [F (3,15) = 6.809, p < 0.001] in STZ-induced diabetic mice. In diabetic mice that received (mCPBG + **4i** treatment), the duration of immobility (s) remained significantly elevated after **4i** (1 mg/kg, i.p.) dosing as compared to the group that received only **4i** (1 mg/kg, i.p.) (p < 0.05). When compared between diabetic control group and diabetic group that received mCPBG + **4i**, no significant alteration in duration of immobility (p > 0.05) was observed, which suggests an impairment in the behavioral effect elicited by **4i** (1 mg/kg, i.p.) due to mCPBG. In addition, mCPBG alone had no significant effect on duration of immobility in diabetic mice subjected to TST (p > 0.05), Fig. 6.71.

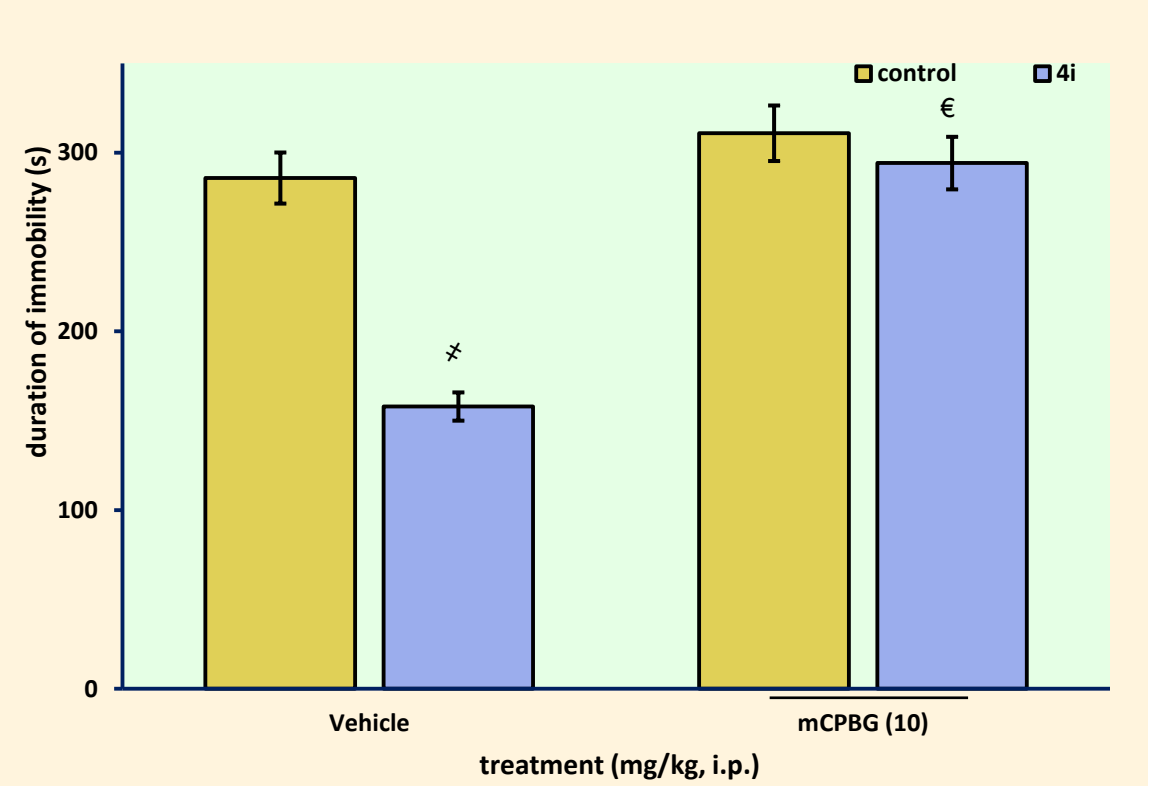


Fig. 6.71 The columns represent mean values of duration of immobility (s) in TST, while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ p < 0.01, vs diabetic control, € p < 0.01 vs diabetic mice that received **4i** (1 mg/kg, i.p.), n = 6/group.

6.7.2.3 Effect on exploratory behavior in open field test

mCPBG (10 mg/kg, i.p.) pre-treatment inhibited the increased exploratory behaviors namely, number of crossings [F (3, 15) = 6.792, p < 0.001] and rearings [F (3,15) = 5.321, p < 0.001] in diabetic mice treated with **4i** (1 mg/kg, i.p.). The number of crossings (p < 0.05) and rearings (p < 0.05) remained significantly reduced in diabetic mice that received mCPBG before **4i** treatment (mCPBG + **4i** treatment), as compared to the group subjected to **4i** treatment alone, Fig. 6.72A and B.

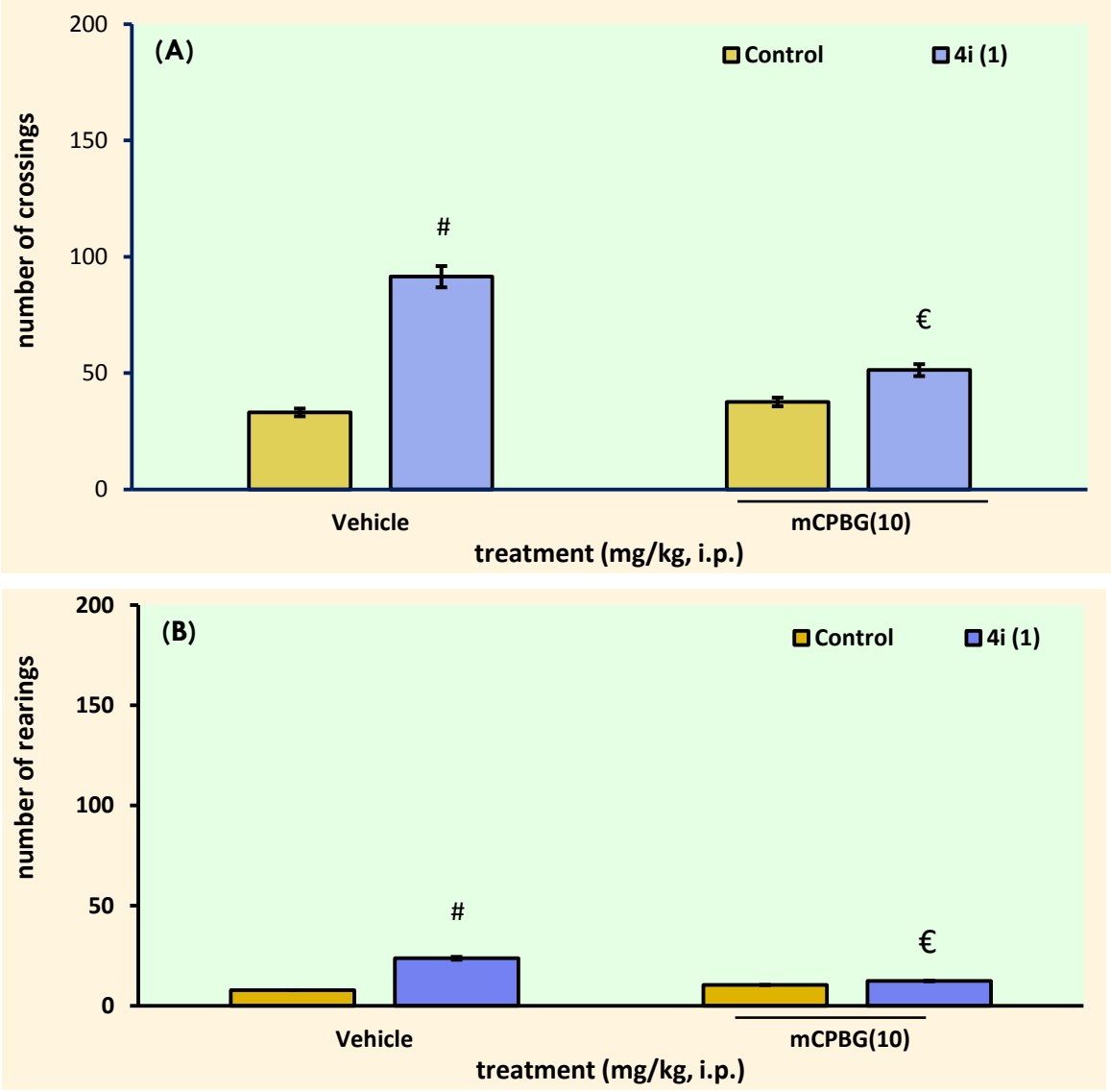


Fig. 6.72 The columns represent mean values of number of crossings **(A)** and number of rearings **(B),** while error bars show S.E.M. The results from post hoc tests are indicated in the figure, # p < 0.05, vs diabetic control, € p < 0.05, vs diabetic mice that received **4i** (1 mg/kg, i.p.), n = 6/ group.

6.7.2.4 Effect on exploratory behavior in social interaction test

When tested in social interaction test for anxiety-like behavior, pre-treatment of mCPBG (10 mg/kg, i.p.) blunted the **4i** response, observed as number of social-interactions [Friedman statistic = 12.56, $p < 0.001$] and duration of social-interactions [Friedman statistic = 11.40, $p < 0.001$], in diabetic mice. The number ($p < 0.05$) and duration of social-interaction ($p < 0.05$) remained significantly decreased after **4i** (1 mg/kg, i.p.) dosing as compared to the group that received **4i** (1 mg/kg, i.p.) alone (**4i** treatment only). The diabetic mice that received mCPBG + **4i** treatment showed no change in number and duration of social-interaction as compared to diabetic control, Fig. 6.73A-B.

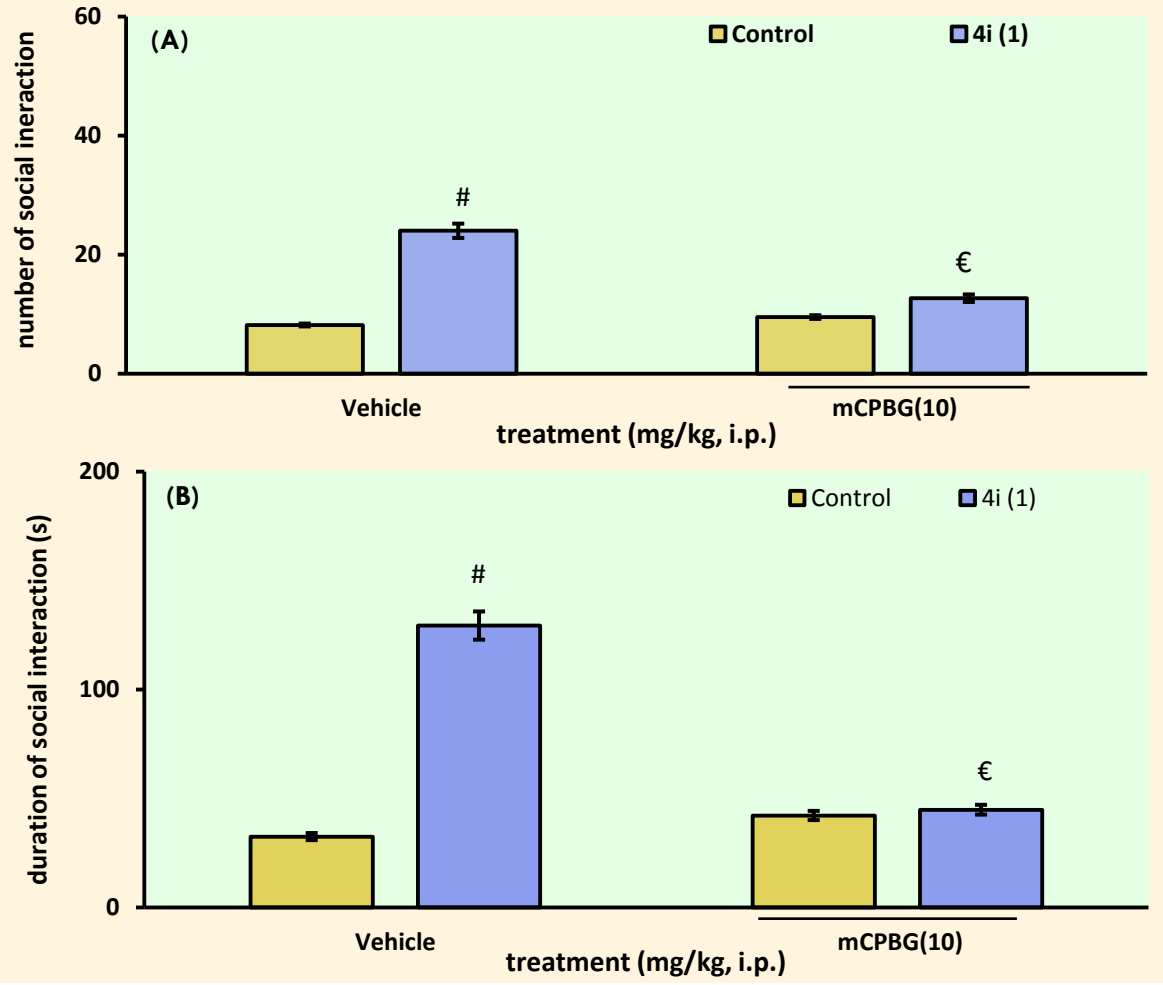
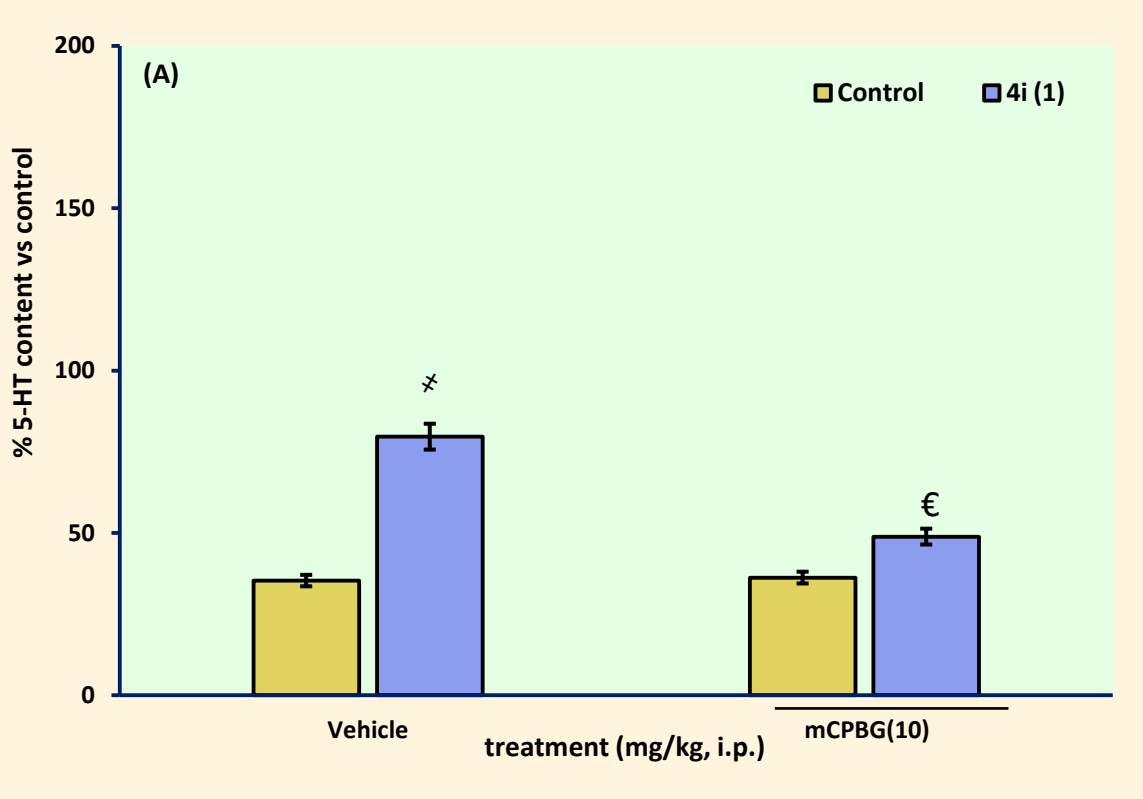
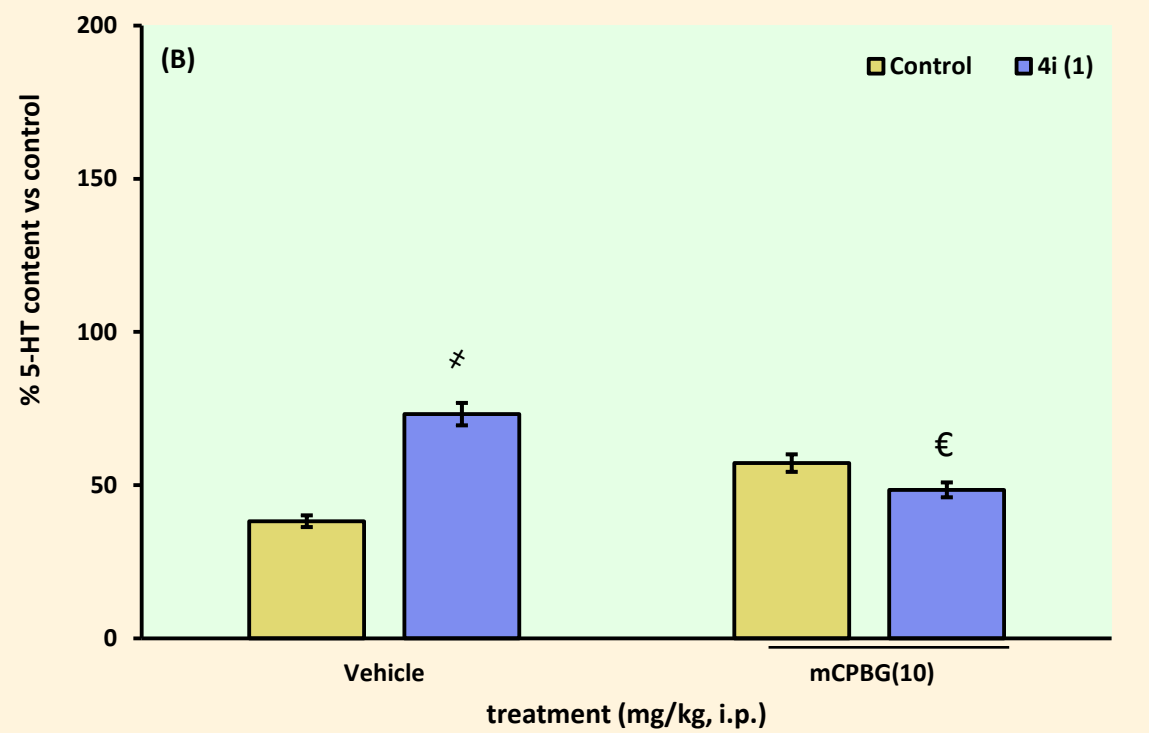


Fig. 6.73 The columns represent mean values of number **(A)** and time spent in social interaction (s) **(B),** while error bars show S.E.M. The results from post hoc tests are indicated in the figure, # $p < 0.05$, vs diabetic control, € $p < 0.05$, vs diabetic mice that received OND (1 mg/kg, i.p.), n = 6/group.

6.7.2.5 Effect on 5-HT levels in discrete brain regions

The effect of mCPBG on **4i** mediated changes in 5-HT levels in discrete brain regions also estimated. Pre-treatment with mCPBG (10 mg/kg, i.p.) significantly diminished **4i**-mediated increase in 5-HT levels in midbrain (including hippocampus) [$F(3,15) = 15.610$, $p < 0.001$], frontal cortex [$F(3,15) = 3.329$, $p < 0.048$] and cerebellum [$F(3,15) = 10.100$, $p < 0.001$], in diabetic mice. After **4i** (1 mg/kg, i.p.) dosing, the 5-HT level remained significantly low, in midbrain ($p < 0.05$), frontal cortex ($p < 0.05$) and cerebellum ($p < 0.05$) in diabetic mice pre-treated with mCPBG (10 mg/kg, i.p.) as compared to those that **4i** treatment alone. No significant alterations in 5-HT levels in these measured brain areas, were observed after mCPBG administration alone ($p > 0.05$), as represented in Fig. 6.74A-C, respectively.





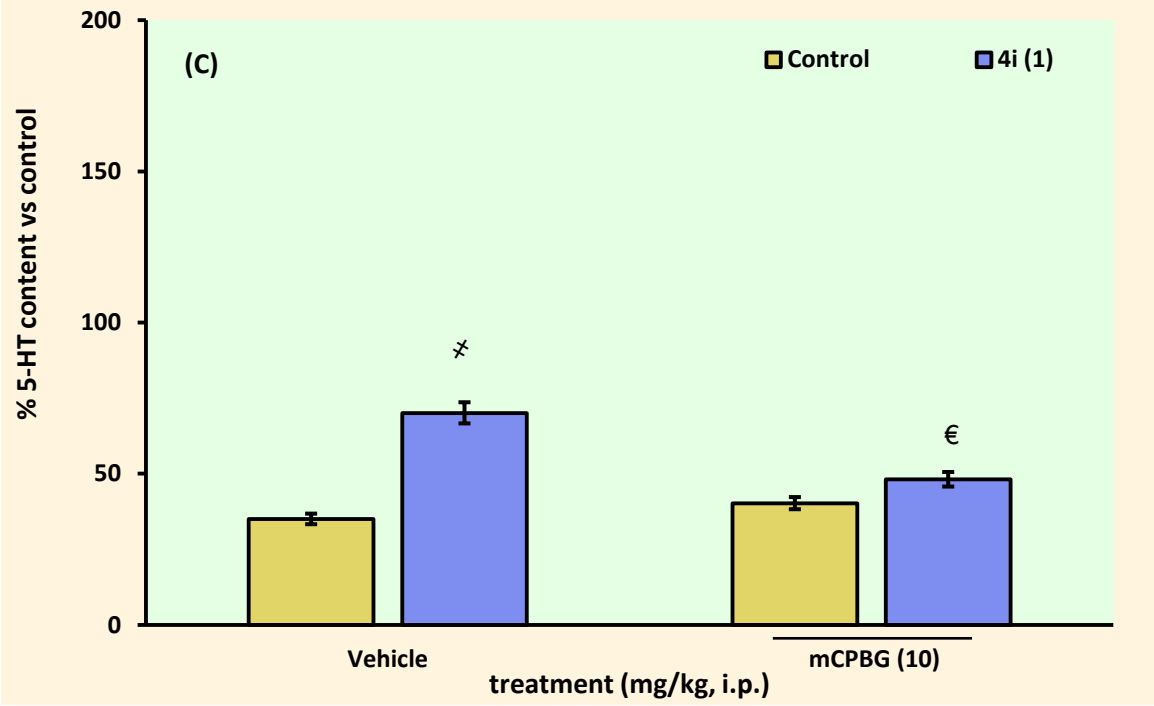
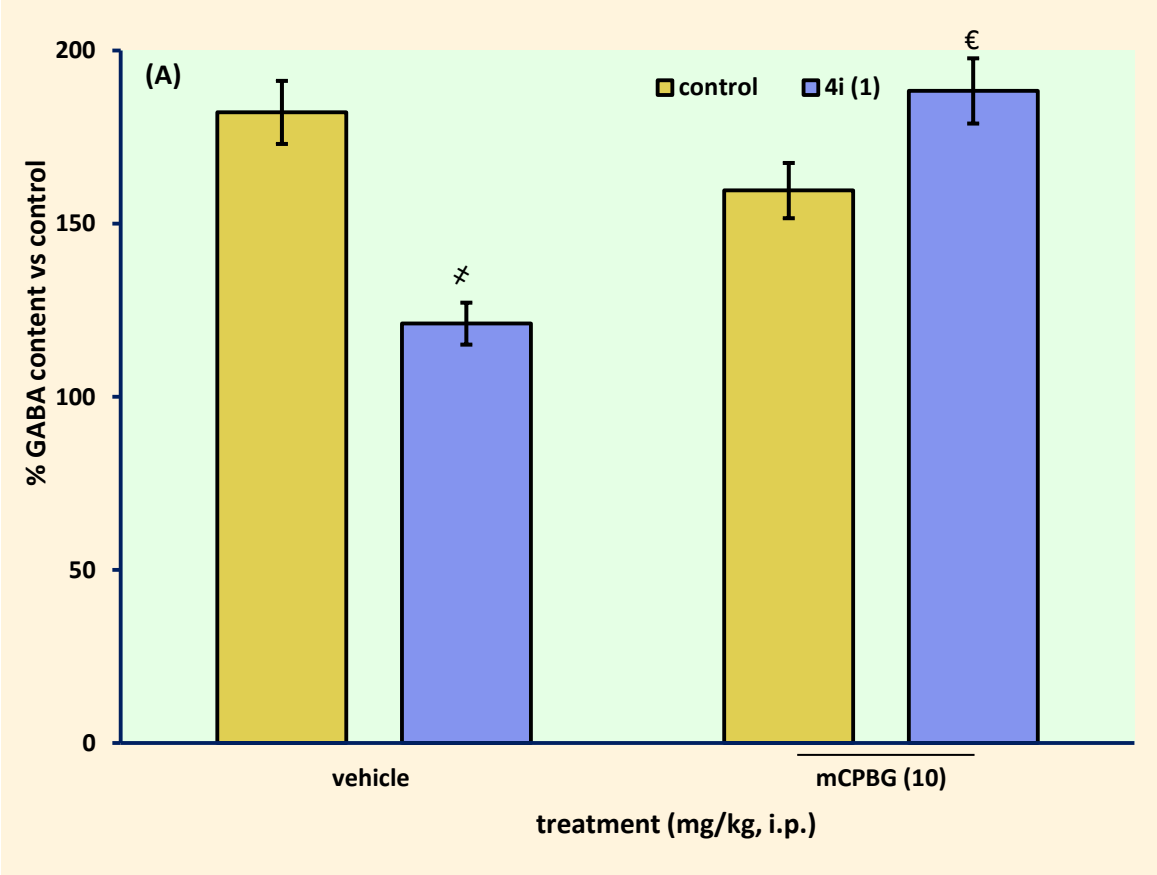


Fig. 6.74 The columns represent mean values of % 5-HT content in **(A)** midbrain (including hippocampus), **(B)** frontal cortex and **(C)** cerebellum, while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$, vs diabetic control, € $p < 0.05$, vs diabetic mice that received **4i** (1 mg/kg, i.p.), n = 6/group.

6.7.2.6 Effect on GABA levels in discrete brain regions

The GABA levels in discrete brain regions had a significant influence of concomitant dosing of mCPBG. In STZ-induced diabetic mice, mCPBG (10 mg/kg, i.p.) treatment significantly diminished **4i**-mediated increase in GABA level in midbrain (including hippocampus) [F (3,15) = 12.531, $p < 0.001$], frontal cortex [F (3,15) = 7.433, $p < 0.048$] and cerebellum [F (3,15) = 2.561, $p > 0.05$]. In diabetic mice chronic **4i** (1 mg/kg, i.p.) dosing had no significant effect on GABA levels in midbrain ($p < 0.05$), frontal cortex ($p < 0.001$) when pre-treated with mCPBG (10 mg/kg, i.p.). However, in cerebellum, neither **4i** nor mCPBG had a significant influence on GABA levels ($p > 0.05$). Furthermore, no significant alterations in the GABA level in these measured brain areas were observed after mCPBG administration alone ($p > 0.05$), as represented in Fig. 6.75A-C, respectively.



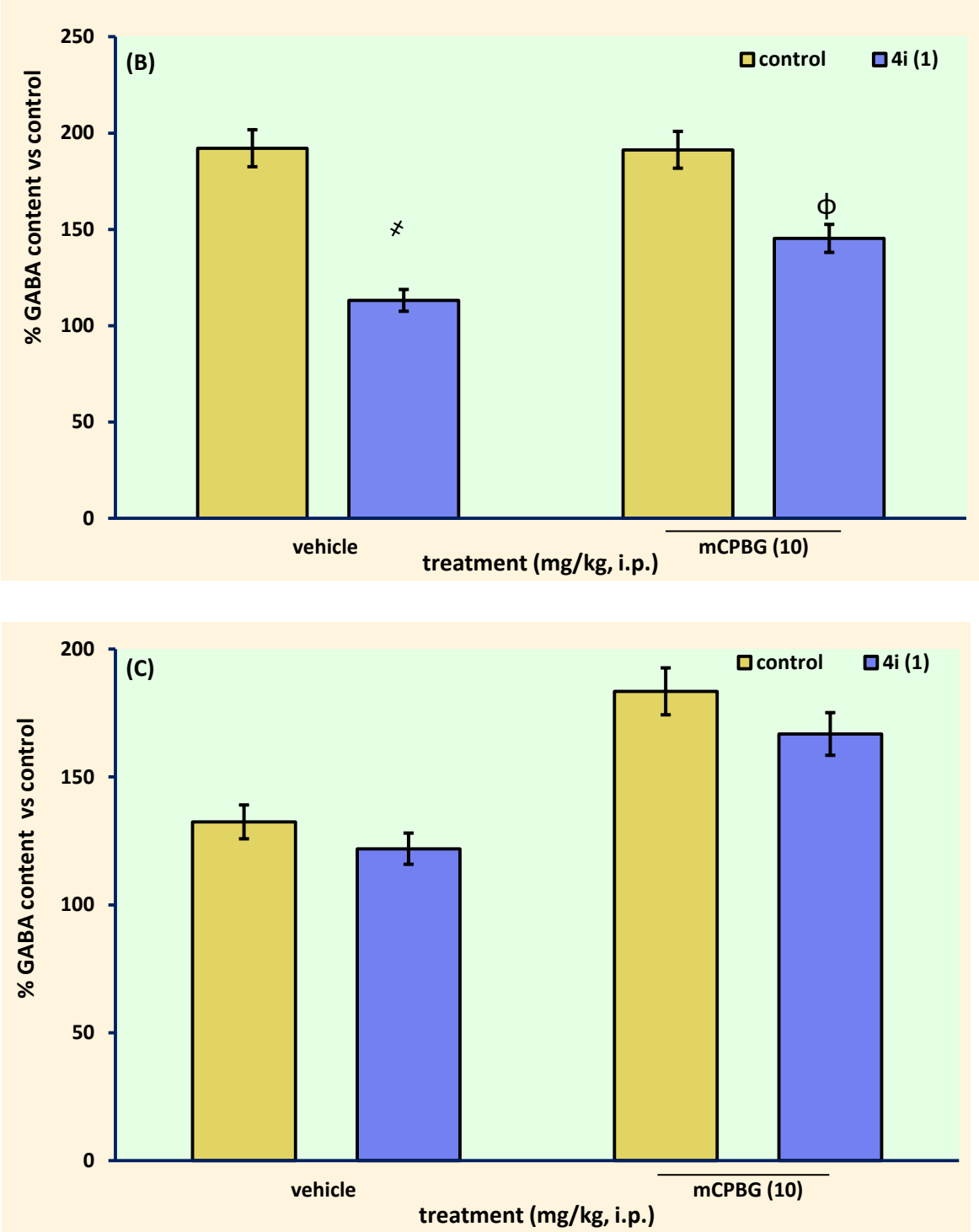


Fig. 6.75 The columns represent mean values of % GABA content in **(A)** midbrain (including hippocampus), **(B)** frontal cortex and **(C)** cerebellum, while error bars show S.E.M. The results from post hoc tests are indicated in the figure ≠ $p < 0.01$, vs diabetic control, € $p < 0.05$, ϕ $p < 0.01$ vs diabetic mice that received **4i** (1 mg/kg, i.p.), n = 6/group.

6.7.3 Effect of concomitant treatment of mCPBG, a selective 5-HT_3 receptor agonist on antidepressant-like effect of 6z

6.7.3.1 Effect on duration of immobility in FST

The effects of mCPBG on **6z** mediated behavioral activity were examined. In FST, concomitant administration of mCPBG blunted the **6z** (1 mg/kg, i.p.) mediated decrease in duration of immobility (s) [$F(3,15) = 12.564$, $p < 0.001$] in STZ-induced diabetic mice. STZ-induced diabetic mice treated with mCPBG prior to**6z** treatment exhibited elevated duration of immobility (s) as compared to the group that received vehicle prior to **6z** (1 mg/kg, i.p.) ($p < 0.05$). Comparison between STZ-induced diabetic control group and diabetic group, which received mCPBG + **6z**, exhibited no alteration in duration of immobility ($p > 0.05$), suggesting an impairment in the behavioral effect elicited by **6z** (1 mg/kg, i.p.) due to mCPBG. Furthermore, mCPBG alone had no significant effects on duration of immobility in diabetic mice subjected to FST ($p > 0.05$), Fig. 6.76.

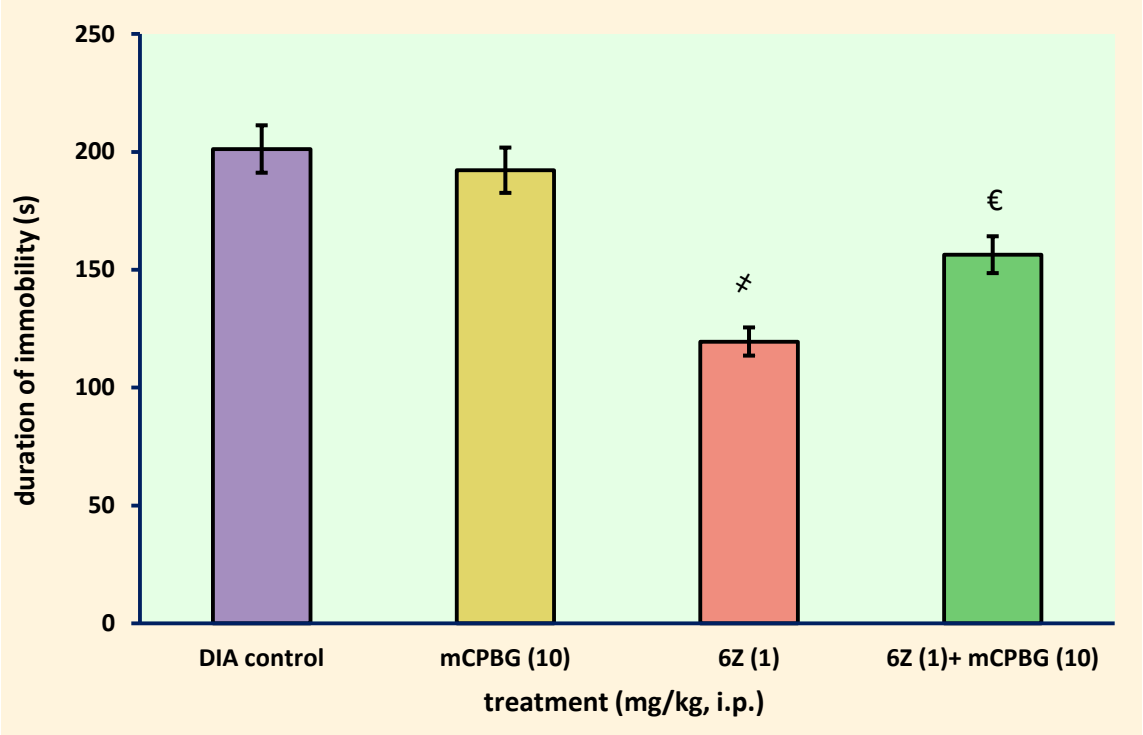


Fig. 6.76 The columns represent mean values of duration of immobility (s), while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$ vs diabetic control, € $p < 0.05$, vs diabetic mice that received **6z** (1 mg/kg, i.p.), n = 6/group.

6.7.3.2 Effect on duration of immobility in TST

A similar effect of mCPBG pre-treatment was observed in TST. Treatment with mCPBG prior to **6z** (1 mg/kg, i.p.) blunted the reduction in duration of immobility (s) [$F(3,15) = 11.673$, $p < 0.001$], in STZ-induced diabetic mice. In this case (mCPBG + **6z)**, the duration of immobility (s) in diabetic mice remained significantly elevated after **6z** (1 mg/kg, i.p.) dosing as compared to the group that received **6z** (1 mg/kg, i.p.) but not mCPBG (**6z** treatment alone) ($p < 0.05$). Interestingly, comparison between diabetic control group and diabetic group received mCPBG + **6z** indicated no significant alteration in duration of immobility ($p > 0.05$), which depicts an impairment in the behavioral effect elicited by **6z** (1 mg/kg, i.p.) due to mCPBG. It was also found that mCPBG alone had no significant effect on duration of immobility in diabetic mice subjected to FST ($p > 0.05$), as shown in Fig. 6.77.

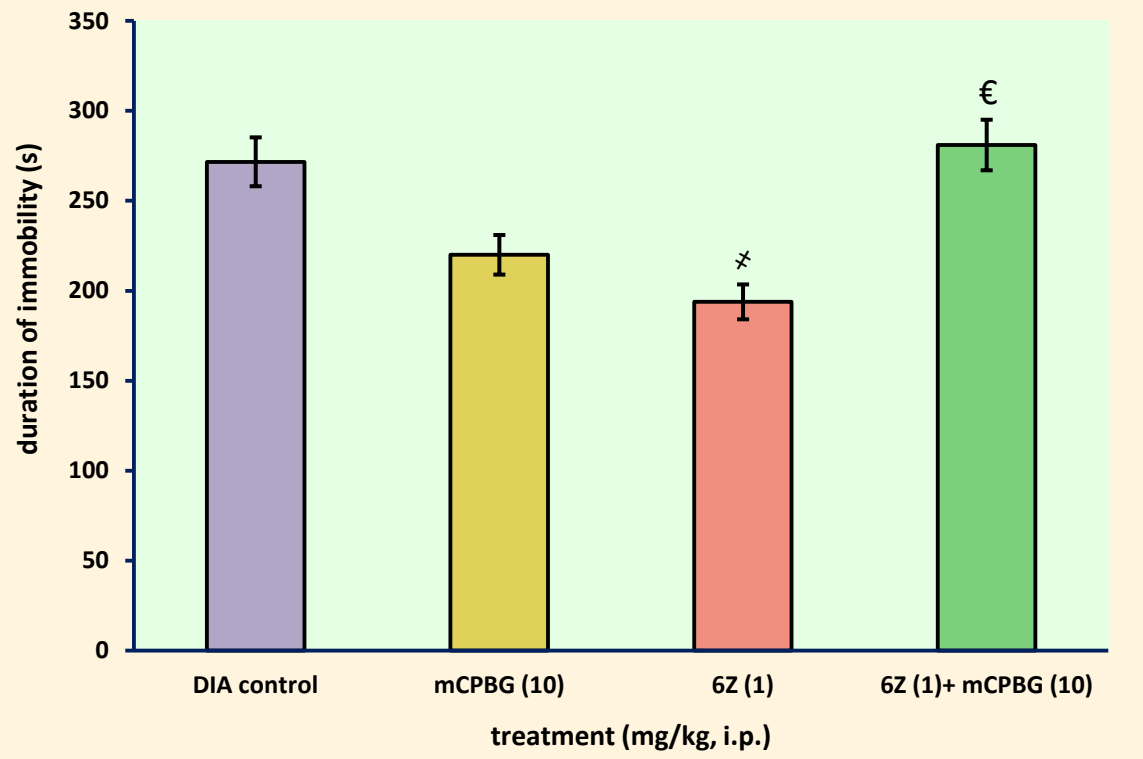


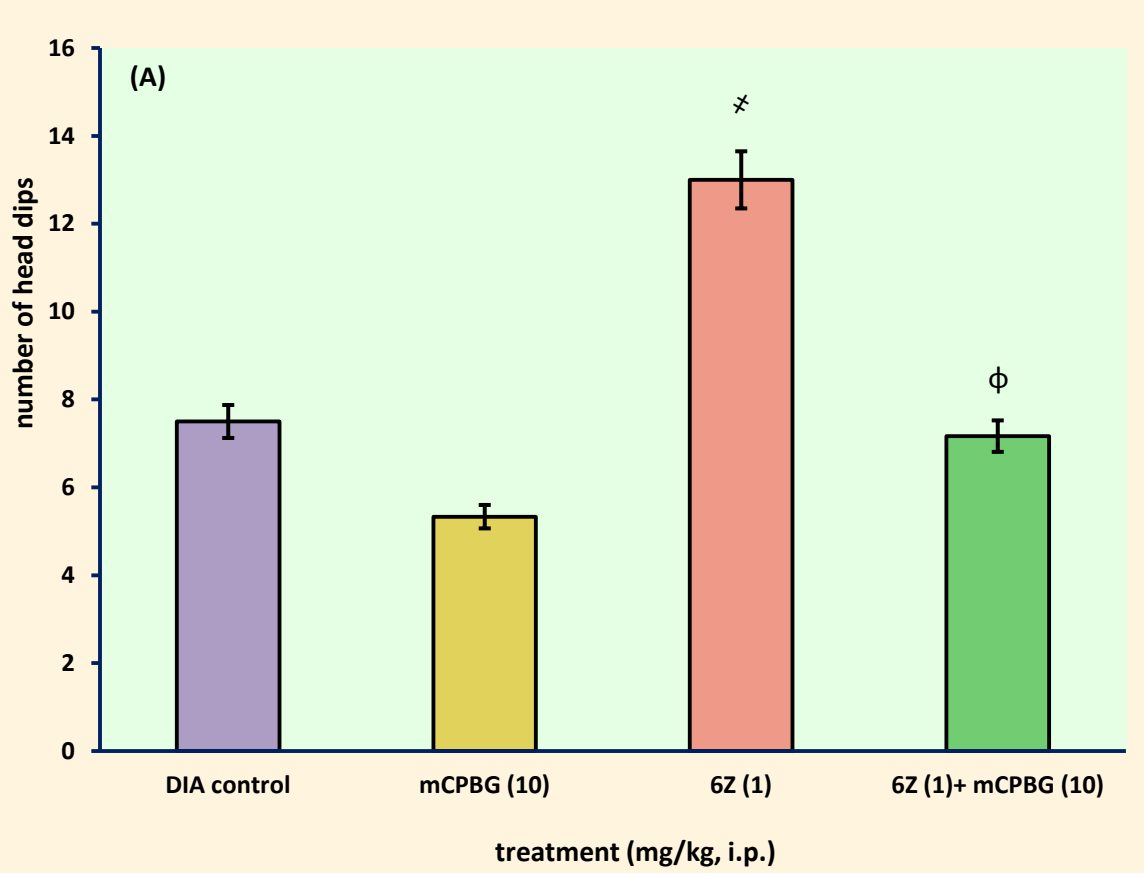
Fig. 6.77 The columns represent mean values of duration of immobility (s), while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$ vs diabetic control, € $p < 0.05$, vs diabetic mice that received **6z** (1 mg/kg, i.p.), n = 6/group.

6.7.3.3 Effect on exploratory behavior in hole-board test

Similarly, in hole-board test, mCPBG (10 mg/kg, i.p.) pre-treatment blocked the increase in exploratory behaviors measured as the number of head dips [F (3, 15) = 4.769, $p < 0.001$] and duration of head dips [F (3, 15) = 10.423, $p < 0.001$], in STZ-induced diabetic mice administered with **6z** (1 mg/kg, i.p.).

The number of head dips ($p < 0.001$) as well as duration of head dips ($p < 0.05$) remained significantly low in STZ-induced diabetic mice that received mCPBG before **6z** treatment (mCPBG + **6z** treatment) as compared to the group that did not receive mCPBG before **6z** (**6z** treatment alone).

Moreover, mCPBG alone exhibited no significant changes on these parameters ($p > 0.05$) as presented in Fig. 6.78A and B, respectively.



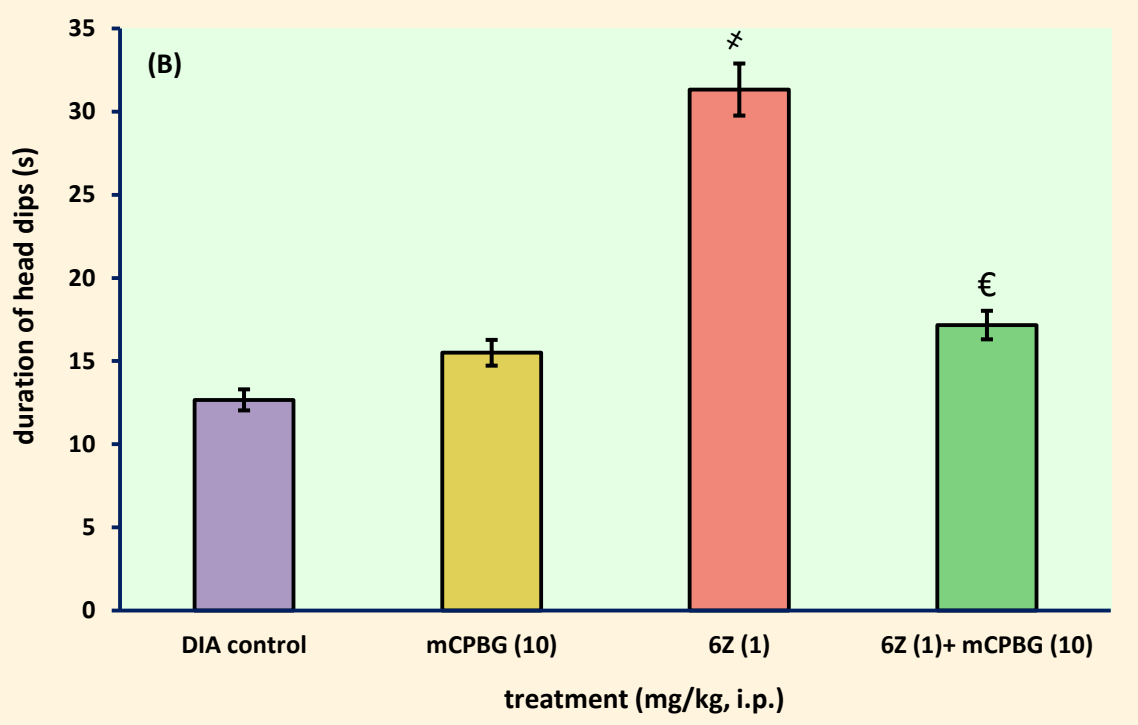


Fig. 6.78 The columns represent mean values of number of head dips **(A)** and duration of head dips (s) **(B),** while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$, vs diabetic control, € $p < 0.05$, ϕ $p < 0.01$ vs diabetic mice that received **6z** (1 mg/kg, i.p.), n = 6/group.

6.7.3.4 Effect on exploratory behavior in social interaction test

During social interaction test, pre-treatment of mCPBG (10 mg/kg, i.p.) blunted the **6z** response, observed as number of social-interactions [Friedman statistic = 11.547, $p < 0.001$] and duration of social-interactions [Friedman statistic = 11.40, $p < 0.001$], in diabetic mice.

The number of social-interactions ($p < 0.05$) and duration of social-interactions ($p < 0.05$) remained significantly reduced after **6z** (1 mg/kg, i.p.) dosing (in the group that received mCPBG + **6z** treatment) as compared to the group received **6z** (1 mg/kg, i.p.) alone (**6z** treatment only).

Diabetic mice that received mCPBG + **6z** treatment demonstrated no significant change in number and duration of social-interactions as compared to diabetic control group ($p > 0.05$), as depicted in Fig. 6.79 A and B, respectively.

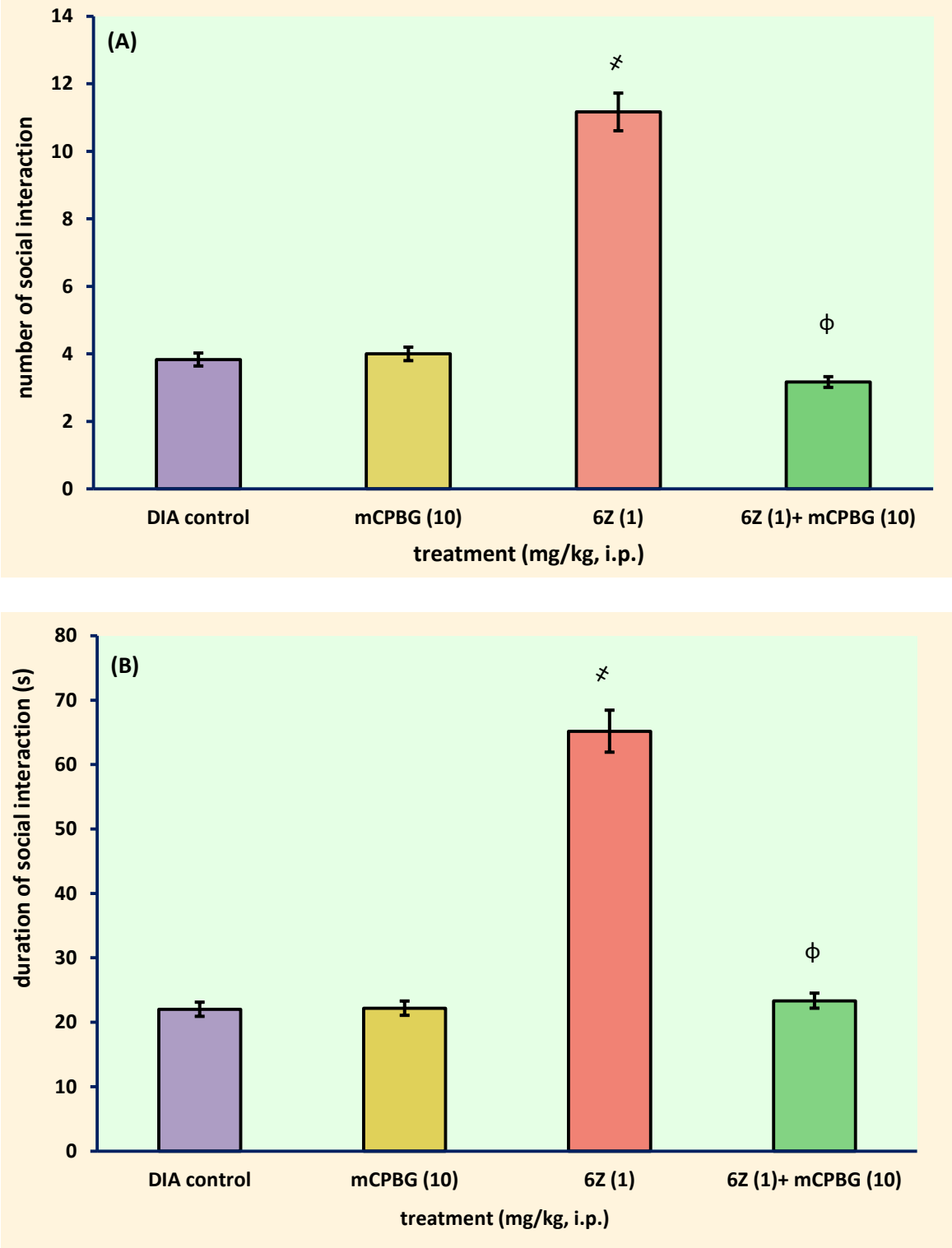


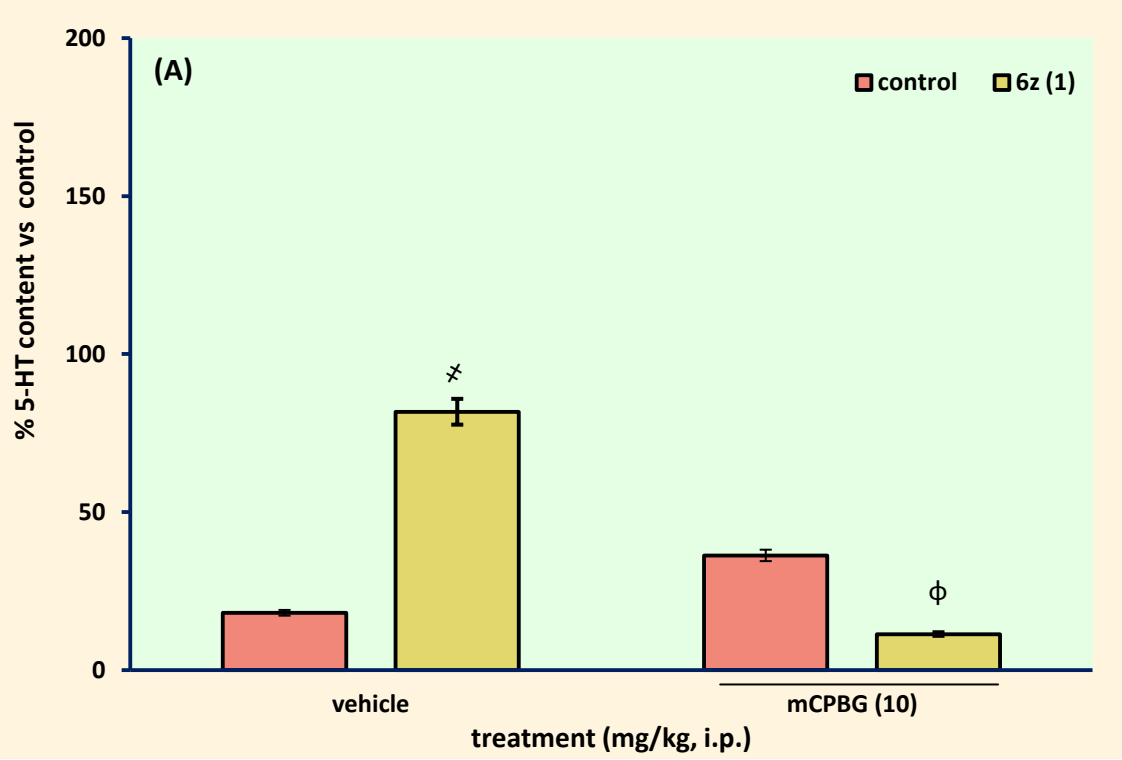
Fig. 6.79 The columns represent mean values of number of social interaction **(A)** and duration of social interaction (s) **(B),** while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$, vs diabetic control, ϕ $p < 0.01$ vs diabetic mice that received **6z** (1 mg/kg, i.p.), n = 6/group.

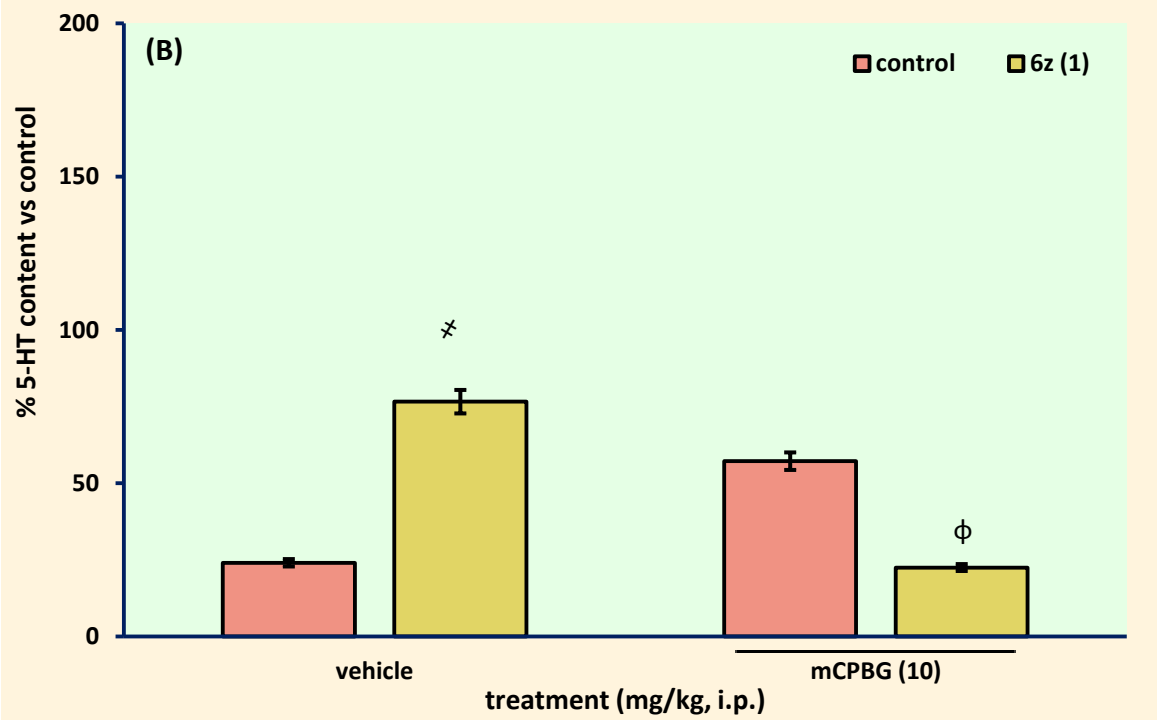
6.7.3.5 Effect on 5-HT levels in discrete brain regions

The effects of mCPBG on **6z** mediated changes in 5--HT levels in discrete brain regions were estimated. It was found that mCPBG (10 mg/kg, i.p.), pre-treatment significantly reduced **6z**-mediated increase in 5-HT levels in midbrain (including hippocampus) [$F(3,15) = 11.032$, $p < 0.001$], frontal cortex [$F(3,15) = 4.352$, $p < 0.048$] and cerebellum [$F(3,15) = 12.917$, $p < 0.001$] in diabetic mice.

After **6z** (1 mg/kg, i.p.) dosing, the 5-HT levels remained significantly low in midbrain ($p < 0.001$), frontal cortex ($p < 0.001$) and cerebellum ($p < 0.001$) in diabetic mice, pre-treated with mCPBG (10 mg/kg, i.p.) (the group that received mCPBG + **6z** treatment) as compared to those, which did not receive mCPBG (**6z** treatment alone).

Furthermore, no significant alterations in 5-HT levels in these measured brain areas were observed after mCPBG administration alone ($p > 0.05$), as represented in Fig. 6.80A-C, respectively.





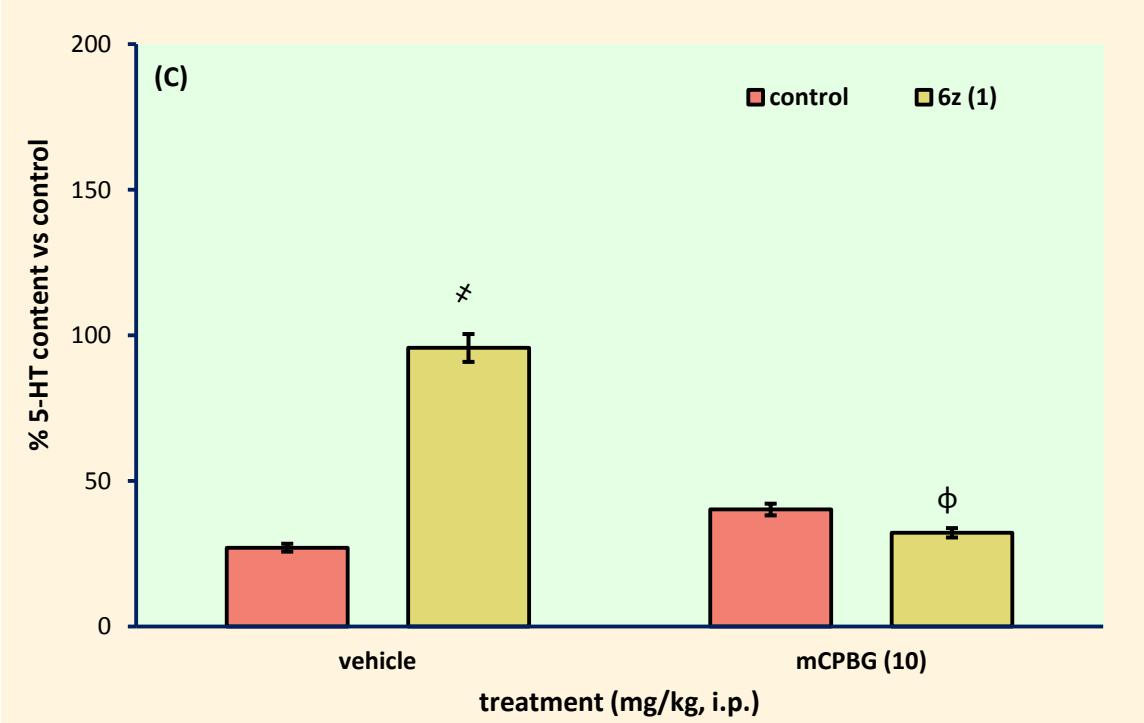


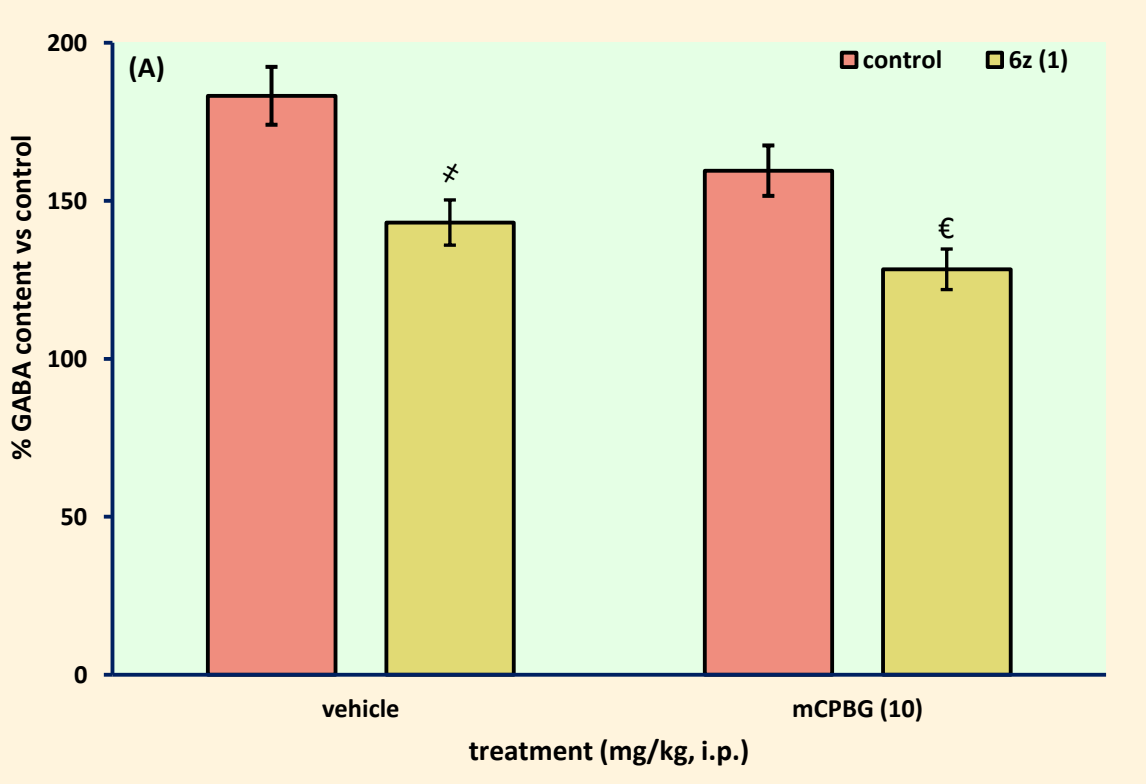
Fig. 6.80 The columns represent mean values of % 5-HT content in **(A)** midbrain (including hippocampus), **(B)** frontal cortex and **(C)** cerebellum while error bars show S.E.M. The results from post hoc tests are indicated in the figure, ≠ $p < 0.01$, vs diabetic control, ϕ $p < 0.01$ vs diabetic mice that received **6z** (1 mg/kg, i.p.), n = 6/group.

6.7.3.6 Effect on GABA levels in discrete brain regions

The GABA levels in discrete brain regions had a significant influence of concomitant dosing of mCPBG. Pre-treatment with mCPBG (10 mg/kg, i.p.) significantly blocked the **6z**-mediated decrease in GABA levels in midbrain (including hippocampus) [$F(3,15) = 12.531$, $p < 0.001$], frontal cortex [$F(3,15) = 8.387$, $p < 0.001$] and cerebellum [$F(3,15) = 1.453$, $p > 0.05$] in STZ-induced diabetic mice.

Chronic administration of**6z** (1 mg/kg, i.p.) had no effect on GABA levels in midbrain ($p < 0.05$) and frontal cortex ($p < 0.05$) in STZ-induced diabetic mice due to pre-treatment with mCPBG (10 mg/kg, i.p.).

However, in cerebellum, neither **6z** nor mCPBG had a significant influence on GABA levels ($p > 0.05$). Interestingly, no significant alterations in the GABA levels in these measured brain areas, were observed after mCPBG administration alone ($p > 0.05$), as represented in Fig. 6.81A-C, respectively.



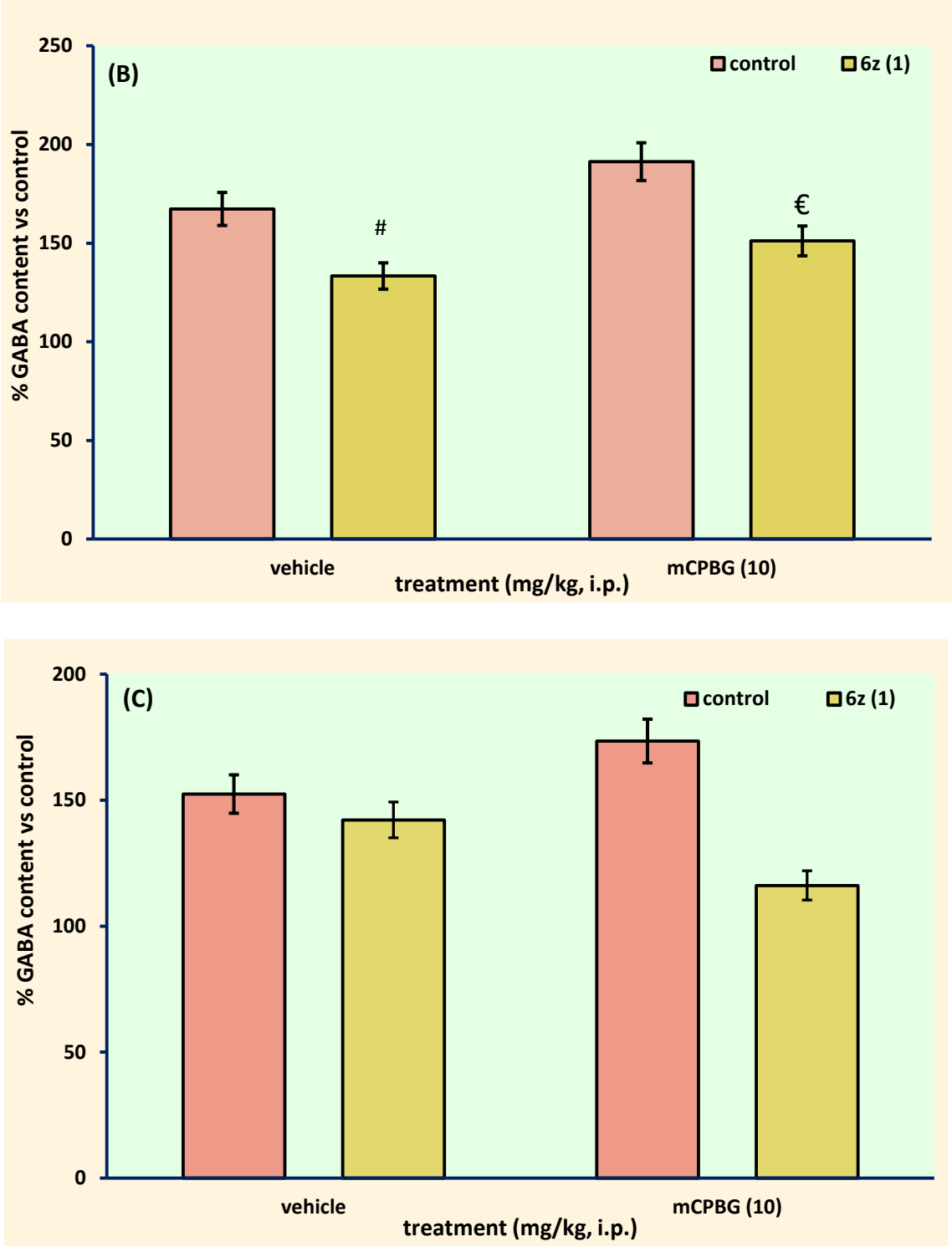


Fig. 6.81 The columns represent mean values of % GABA content in **(A)** midbrain, **(B)** frontal cortex and **(C)** cerebellum, while error bars show S.E.M. The results from post hoc tests are indicated in the figure, # $p < 0.05$, ≠ $p < 0.01$, vs diabetic control, € $p < 0.05$, vs diabetic mice that received **6z** (1 mg/kg, i.p.), n = 6/group.

6.8 Effect of 5-HT_3 receptor antagonists on metabolic parameters

To evaluate whether, the test drug candidates had any influence on metabolic markers such as blood glucose and insulin, the fasting blood glucose level and plasma insulin levels, were estimated.

6.8.1 Effect on fasting blood glucose levels

There was a significant effect on fasting blood glucose levels, in mice subjected to different treatments [F (4, 30) = 19.032, $p < 0.05$]. STZ-induced diabetic mice, exhibited a significant increase in fasting blood glucose levels ($p < 0.001$). Chronic treatment with OND (0.5 and 1 mg/kg, i.p.), significantly reversed the increase in fasting blood glucose levels in STZ-induced diabetic mice ($p < 0.05$).

Similarly, chronic treatment with the novel 5-HT_3 receptor antagonists, **4i** (0.5 and 1 mg/kg, i.p.) and **6z** (1 and 2 mg/kg, i.p.), significantly reversed the increase in fasting blood glucose levels in STZ-induced diabetic mice ($p < 0.05$). However, chronic treatment with FLX (01 mg/kg, i.p.) had no significant effect on this metabolic parameter in STZ-induced diabetic mice ($p > 0.05$), as shown in Table 6.7.

Table 6.8 Effect of STZ-induced diabetes and drug treatments on fasting blood glucose level, in mice

Parameters	Fasting blood glucose levels (mg/dl)
nDIA (0)	99.00 ± 1.83
DIA (0)	456.50 ± 47.18 **$**
DIA-OND (0.5)	208.67 ± 27.72 #
DIA-OND (1)	136.33 ± 14.42 ≠
DIA-4i (0.5)	293.15 ± 9.52 #
DIA-4i (1)	207.13 ± 10.41 ≠
DIA-6z (1)	198.45 ± 12.62 ≠
DIA-6z (2)	244.88 ± 17.69 #
DIA-FLX (10)	390.67 ± 36.00

Values represent mean ± S.E.M. **$** $p < 0.001$ when compared with control group, # $p < 0.05$, ≠ $p < 0.001$ when compared with diabetic control group, n = 6/group.

6.8.2 Effect on plasma insulin levels

In addition the effect of OND and novel drug treatments on plasma insulin levels was estimated. A significant effect on plasma insulin levels, in mice subjected to different treatments [F (4, 30) = 12.011, $p < 0.05$]. It was found that STZ-induced diabetic exhibited a significant decrease in plasma insulin levels ($p < 0.001$).

Interestingly, chronic treatment with OND (0.5 and 1 mg/kg, i.p.) significantly reversed the decrease in plasma insulin levels in STZ-induced diabetic mice ($p < 0.001$). In addition, chronic treatment with the novel 5-HT_3 receptor antagonists, **4i** (0.5 and 1 mg/kg, i.p.) and **6z** (1 and 2 mg/kg, i.p.), significantly reversed the reduction in plasma insulin levels in STZ-induced diabetic mice ($p < 0.05$).

Besides, chronic treatment with FLX (01 mg/kg, i.p.) had no significant effect on plasma insulin levels in STZ-induced diabetic mice ($p > 0.05$), as shown in Table 6.8.

Table 6.9 Effect of STZ-induced diabetes and drug treatments on plasma insulin level, in mice

Parameters	Plasma insulin levels (ng/ml)
nDIA (0)	1.17 ± 0.27
DIA (0)	0.26 ± 0.05 **$**
DIA-OND (0.5)	0.61 ± 0.08 ≠
DIA-OND (1)	0.85 ± 0.08 ≠
DIA-4i (0.5)	0.52 ± 0.04 #
DIA-4i (1)	0.72 ± 0.09 ≠
DIA-6z (1)	0.81 ± 0.09 ≠
DIA-6z (2)	0.54 ± 0.04 #
DIA-FLX (10)	0.24 ± 0.09

Values represent mean ± S.E.M. **$** $p < 0.001$ when compared with control group, # $p<0.05$, ≠ $p<0.001$ when compared with diabetic control group, n = 6/group.

7. Discussion

Treatment of depression and anxiety co-morbid with T1DM represents a major unmet medical need. Although, a number of antidepressants are available, current drugs are only symptomatic and often worsen glycemic control (Goodnick, 2001). Developing a drug that not only ameliorates depression and related disorders (anxiety) in T1DM, but also maintains or at least does not aggravate glycemic control, would meet the significant unmet medical needs of this co-morbidity.

Current work originates from the need to identify effective psychological treatments for depression and anxiety co-morbid with T1DM. It focuses, simultaneously on investigating the antidepressant-like effect of 5-HT_3 receptor antagonists and the role of 5-HT_3 receptors as a novel target, in the pathogenesis of this co-morbidity. The main aim was to investigate the general effectiveness of both standard and novel 5-HT_3 receptor antagonists, as therapeutic interventions, for treating depressive symptoms associated with T1DM. In the following paragraphs, objectives of the study are supplemented with experimental evidence. Briefly, the development of depression-like behavior in persistent diabetes and antidepressant-like effect of 5-HT_3 receptor antagonist in naïve animal models, are discussed. Subsequently, the focus is placed on the antidepressant-like effect of the tested drug candidates on experimentally induced diabetic model and the mechanism(s) underlying their antidepressant action. Then the role of 5-HT_3 receptors in their antidepressant-like effect is discussed. Finally, the effects of the tested drug candidates on metabolic parameters that are related to the diabetic condition are summarized.

7.1 Type-1 diabetic mouse model

To evaluate the effect of persistent diabetes, in culminating depression and anxiety-like behavior, STZ-induced diabetic mouse model was standardized, in the present work. STZ is a nitrosourea analogue, in which the N-methyl-N-nitrosourea (MNU) moiety is linked to carbon-2 of a hexose (Fig. 7.1). It inhibits insulin secretion and causes a state of insulin-dependent diabetes mellitus, which can be attributed to its alkylating potency (Lenzen, 2008). STZ is selectively accumulated in pancreatic β-cells via the low-affinity GLUT2 glucose transporter, in the plasma membrane, after which, it interacts with DNA of β-cells.

The transfer of the methyl group from STZ to the DNA molecule causes damage, which along a defined chain of events (Pieper et al., 1999), results in the fragmentation of the DNA (Yamamoto, et al., 1981).

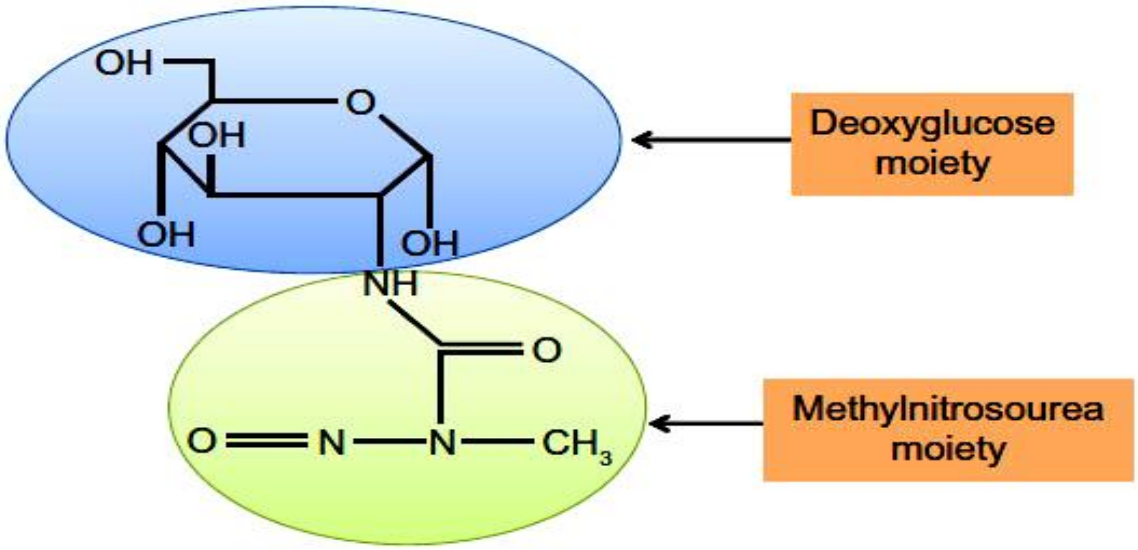


Fig. 7.1 The structure of streptozotocin, indicating deoxyglucose and methylnitrosourea moieties.

In the attempt to repair DNA, poly (ADP-ribose) polymerase (PARP) is over-stimulated. This diminishes cellular NAD+, and subsequently ATP stores (Sandler and Swenne, 1983; Schein and Lopen field testus, 1962; Uchigata et al., 1982; Yamamoto et al., 1981). The depletion of the cellular energy stores, ultimately results in β-cell necrosis and β-cell death (Fig. 7.2).

A single intraperitoneal injection of STZ (dose, 200 mg/kg, i.p.) was administered, in mice. After 72 hrs of injection, mice exhibited a pronounced increase in fasting blood glucose levels and low plasma insulin levels, indicating the development of T1DM, in mice, which remained consistent till the termination of the study (12-weeks of STZ dosing). In addition, diabetic mice exhibited a decline in body weights, in accordance with previous studies (Graham et al., 2011).

It suggests that, a single high dose of STZ produces severe uncontrolled hyperglycemia, hypoinsulinemia and body weight loss; similar to features of T1DM, in humans. During histological examination of pancreas, Islets of Langerhans of STZ-induced diabetic mice showed marked necrotic changes and vacuoles. Karyolysis, the disappearance of nucleus, was also observed.

The pancreatic acini appeared small with dark nuclei, vacuolated cells, lost apical acidophilia, which mostly resulted from decreased zymogen granules congestion, inflammation, tubular desquamation and damaged endothelium lining of beta-cells.

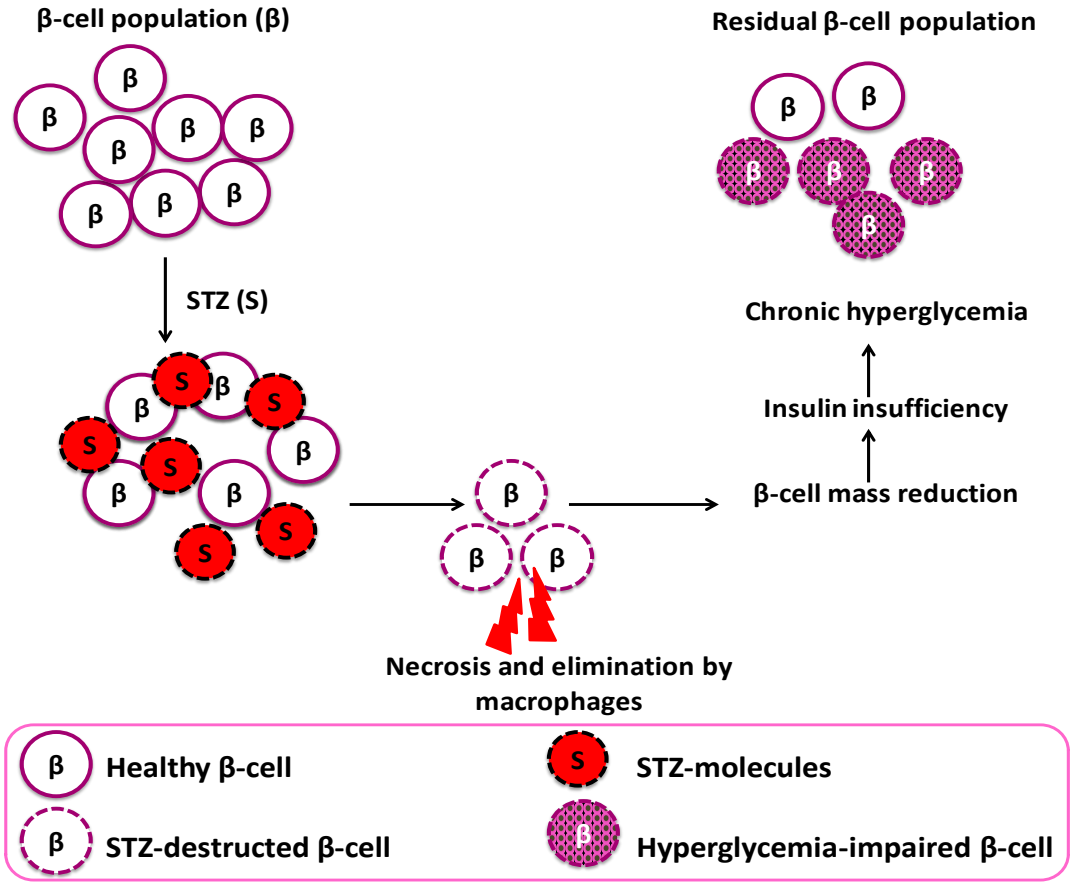


Fig. 7.2 Diagram showing partial destruction of β-cell population by STZ and reduction in β-cell mass that induces insulin insufficiency and chronic hyperglycemia.

7.2 Time course of depression and anxiety in Type-1 diabetes

Depression-like behavior

FST and TST are the most widely utilized models, with high predictive validity to evaluate the depression/antidepressant-like effects (Cryan et al., 2005; Petit-Demouliere et al., 2005). These tests are based on the principle that when a mouse in exposed to an unavoidable stress (like forced to swim in an enclosed space, in FST or hung with its tail to a certain height, in TST), it ceases motility and acquires an immobile position that reflects a state of despair behavior or hopelessness. It represents one of the two core symptoms of depression, in humans.

Although, the underlying principle measuring the lack of active coping behavior is identical in TST and FST, their variability in response to certain antidepressants and substances indicates potentially different substrates and neurochemical pathways, mediating performance in these tests (Bai et al., 2001).

During 1st, 2nd and 4th weeks of persistent T1DM, mice did not present any significant change in the duration of immobility, a marker of despair behavior in FST and TST. However, during 8th week of diabetes, mice, exhibited a significant increase in duration of immobility, suggesting that progressive idiopathic changes in persistent, but not initial T1DM may lead to depression like complications, in mice.

Anxiety-like behavior

Anxiety is the most common behavioral deficits occur in association with depression (Ressler and Nemeroff, 2000). Thus, mice with persistent diabetes were also investigated, for the anxiety-like behavior using hole-board test and open field test.

Hole-board test and open field test are unconditioned exploration based models, widely used to estimate the anxiety/anti-anxiety like behavior, in mice. The increase in exploratory behavior, when a rodent is exposed to a novel environment, indicates anti-anxiety like effects.

In hole-board test, the anxiety-like behavior was partially observed after 4th week of diabetes (as number of head dips was significantly reduced but not the duration of head dips). Similarly, in social interaction test partial anxiety-like behavior was observed after 4th week of diabetes induction. However, in hole-board test and social interaction test, consistent anxiety behavior was observed only after 8th week of diabetes induction.

In another model of anxiety, which is light-dark test, STZ-induced diabetic mice showed anxiety-like behavior after 2nd week of induction of diabetes and become more pronounced after 8th week of diabetic condition. However, in open field test and EPM, STZ-induced diabetic mice exhibited anxiety-like behavior only after 8th week of diabetes induction. It indicates a marked increase in anxiety-like behavior, in mice due to persistent diabetes and that persistent, but not acute diabetes leads to anxiety-like behavior.

7.3 Antidepressant-like effect of OND, 4i and 6z, in naïve depression models

Dose response curve of OND, 4i and 6z

Before screening antidepressant-like effects of OND, **4i** and **6z**, suitable dose selection was carried out using an effect on the general locomotion, in mice. It also ensured that during behavioral measures, as in FST and TST, the effect produced by the drugs were not merely due to change in locomotion. OND was screened at the doses of 0.25, 0.5, 1 and 2 mg/kg for the effect on the BLA, in mice. It was found that 0.5 and 1 mg/kg had no effects on the general locomotion, in mice. Hence, these doses were selected for further screening. Similarly, the novel candidates **4i** and **6z** were evaluated for their effects on the basal locomotor activity, in mice. 0.25, 0.5, 1, 2, 4 and 8 mg/kg doses of **4i** and 0.5, 1, 2 and 4 mg/kg doses of **6z** were tested. It was found that **4i** at the doses of 0.5 and 1 and **6z** at the doses of 0.5, 1 and 2 mg/kg did not alter the BLA, in mice. Thus, these doses of **4i** and **6z** were selected.

OND, 4i and 6z, demonstrated antidepressant-like effects in acute models of depression

Preliminary screening of the antidepressant-like effects of OND, **4i** and **6z**, was carried out, using acute and chronic models of depression and anxiety. OND, a selective 5-HT_3 receptor antagonist, demonstrated a significant antidepressant-like effect in FST and TST models of depression. Acute treatment with OND (0.5 and 1 mg/kg, i.p.) exhibited a marked decrease in the duration of immobility, in mice, during FST and TST. Similarly, the prototype drug FLX, reduced the duration of immobility, in mice subjected to FST and TST. It indicates the high predictive validity of the models. Moreover, the likely effect of OND indicates that it posses antidepressant-like effects, similar to that of FLX. In addition, acute treatment with the novel candidates **4i** (0.5 and 1 mg/kg, i.p.) produced a marked decrease in the duration of immobility, in mice subjected to FST and TST. The effect likely observed for OND and FLX, suggesting the antidepressant-like effects of **4i,** in behavioral models of depression, as well. **6z** was also evaluated for the antidepressant-like effect in FST and TST. The higher doses' of **6z** (1 and 2 mg/kg, i.p.) produced a pronounced antidepressant-like effect with a significant decrease in duration of immobility in FST. However, **6z** had no effect at the lower dose of 0.5 mg/kg.

In contrast, during TST, **6z** at 0.5, 1 and 2 mg/kg produced antidepressant-like effect, in mice, suggesting that **6z** possibly exhibit potential antidepressant-like effects, in mice, a behavioral profile shown by several antidepressants in clinical use (Millan et al., 2001) and several 5-HT_3 receptor antagonists (Bravo and Maswood, 2006; Devadoss et al., 2010; Kos et al., 2006; Ramamoorthy et al., 2008). In addition, the anxiolytic effects of OND, **4i** and **6z**, in mice were evaluated using exploratory based models, namely hole-board test and light-dark test. The models demonstrated high predictive value, as indicated by the increased exploratory behavior, in mice, given DZM (1 mg/kg, i.p.) treatment. In the validated hole-board test, OND, **4i** and **6z**, markedly increased the number and duration of head dips, as compared to vehicle treated mice. In light-dark test, OND, **4i** and **6z**, increased the latency (time to travel from light chamber to dark chamber) and total time spent in light chamber. These results are in agreement with the previous findings that 5-HT_3 receptor antagonists have anti-anxiety like effects in rodents (Costall and Naylor, 1992).

OND, 4i and 6z, demonstrated antidepressant-like effects in chronic model of depression

Although acute behavioral studies substantially demonstrate the pharmacological activity of the compounds, the tested doses, do not correspond to the clinical time course, of their action. Since, chronic models mimic the clinical time course of the antidepressant therapy, it is essential to verify the antidepressant-like effects of the drugs in chronic models. Therefore, the effects of OND, **4i** and **6z**, in chronic behavioral models were evaluated.

Chronic unpredictable stress (CUS) as a chronic model of depression was developed in an attempt to mimic some of the environmental factors contributing to induction of depression in humans (Moretti et al., 2012; Nollet et al., 2013). In addition, several lines of evidences revealed that CUS resembles a variety of neurochemical, neurobehavioral and neuroendocrine alterations of human depressive disorder (Holsboer, 2000; McEwen, 2008). Mice, subjected to a variety of stressors such as deprivation of food and water, wet husk bedding, cage tilting and exposure to inverted light and dark cycle have expressed depression-like behavior in numerous behavioral testing paradigms (Larsen et al., 2010; Ma et al., 2011; Rasheed et al., 2011).

Previous studies have shown that mice exposed to four week stress condition exhibit despair behavior in FST and TST, anhedonia-like behavior in sucrose preference test and anxiety-like effects in anxiety test paradigms (Dang et al., 2009; Larsen et al., 2010; Moretti et al., 2012; Strekalova and Steinbusch, 2010). Therefore, it was suggested that CUS is a validated model of depression that is effective to evaluate the antidepressant-like effects of the new drugs (Moretti et al., 2012). In the present study, OND and **6z** demonstrated a significant decrease in the depression and anxiety-like behavior induced by CUS, in mice. In agreement with the previous reports (Larsen et al., 2010), CUS mice exhibited an increase in the duration of immobility in FST and TST. It revealed a pronounced increase in depression-like behavior, in mice, which was reversed by chronic treatment with OND and **6z**. The effects were similar to that of FLX, which indicates that the OND and **6z** possess antidepressant-like effects.

In addition, anhedonic behavior, in mice, due to CUS was also measured. Anhedonia referred as the diminished capacity to experience pleasure and reward activity; is one of the two core symptoms of depression in humans (APA, 2000). It has been reported that exposure to chronic stress, results in increased anhedonic behavior, in mice, that can be measured using sucrose preference test (Willner, 1987). The test is based on the principle that

(1) Consumption of sucrose solution is a valid measure of reward-related behavior and (2) CUS causes a generalized decrease in reward-related behavior, rather than a specific effect on responses to sweet tastes (Willner, 1987).

Chronic treatment with antidepressants has been reported to reverse this effect (Harkin et al., 2002; Gronli et al., 2005). In accordance with the previous reports, CUS mice exhibited a marked decrease in the percentage of sucrose solution over drinking water, indicating anhedonia in mice, in the current study. Chronic treatment with OND and **6z** reversed the CUS induced increased anhedonia, in mice. In addition, FLX treatment significantly blunted the CUS induced anhedonia, in mice.

CUS mice exhibited a significant increase in anxiety-like behavior in open field test. CUS mice with vehicle treatment demonstrated a reduced exploratory behavior in open arena, as indicated by reduced number of crossings and rearings. The results are in accordance with the previous findings that CUS causes a significant increase in anxiety-like behavior, in mice exposed to open field test (Bowman et al., 2002; Katz et al., 1981).

Interestingly, chronic OND and **6z** treatment significantly reversed the CUS induced anxiety-like behavior in the open field test. Similarly, chronic FLX treatment reversed the CUS induced anxiety, in mice. These results demonstrated that similar to FLX, OND and **6z**, have antidepressant-like effects in chronic model, as well. Moreover, neither CUS exposure nor drug treatments affected the basal locomotor activity, in mice, suggesting that behavioral effects observed in the assays were not due to mere alteration in general locomotion.

Similarly, in OBX model of depression, **4i**, demonstrated a significant antidepressant-like effect. Bilateral ablation of olfactory bulb resulted in several neurobehavioral changes that correspond to both depression and anxiety like symptoms in humans (Glinka et al., 2012; Kelly et al., 1997). In example, hyperactivity is one of the putative indices of agitation like behavior in anxious patients whereas, decreased reward related behavior reflects, anhedonia, which is one of the cardinal signs of depression in humans (Kelly et al., 1997; Song and Leonard, 2005; Wang et al., 2007).

In the current study, after 4-weeks of bulbectomy, OBX rats exhibited abnormal behavioral pattern in sucrose preference test and open field test, used as testing paradigms of depression and anxiety, respectively (Kelly et al., 1997; Wang et al., 2007). Furthermore, FLX, reference drug, reversed the OBX induced behavioral defects, demonstrating high predictive validity of the model (Zueger et al., 2005). In sucrose preference test, OBX rats exhibited marked increase in anhedonia behavior and this effect was significantly reversed by chronic treatment with **4i**. This is in accordance with the previous findings, which showed that antidepressants reverse anhedonia behavior in OBX rats (Song and Leonard, 2005). It demonstrated the potential influence of **4i** in reward related activity.

Since, **4i** had no influence on hedonic behavior of sham rats; it suggested that **4i** effects were specific to the diseased (that is in OBX) condition. In addition, it revealed that the antidepressant-like effect of **4i**, following chronic treatment, was consistent, with that produced with acute dosing (Romeas et al., 2009). Similarly, in open field test, OBX rats exhibited the increased number of ambulation and rearing (the measure of horizontal and vertical locomotor activity, respectively), which showed hyperactive behavior following bulbectomy and revealed anxiety condition in association with depression like behavior in rats (Song and Leonard, 2005; Wang et al., 2007).

Chronic **4i** treatment, on the other hand, reversed the hyperlocomotor performance in OBX rats without any influence on the motor behavior of sham rats, which revealed the specificity of **4i** treatment, in diseased condition. This is in agreement with the activity of several antidepressants, including reference drug, used in the present study and several 5-HT_3 antagonists (Devadoss et al., 2010; Song and Leonard, 2005; Ramamoorthy et al., 2008), indicating, the anti-anxiety like effect of **4i**, on chronic treatment.

Taking all the behavioral results into account, OND, **4i** and **6z**, when administered either acutely or chronically, displayed robust antidepressant and anti-anxiety like effects, in multiple animal models, in mice and rats.

7.4 OND, 4i and 6z reverse Type-1 diabetes-induced depression-like behavior

As stated earlier, chronic diabetic state induced by STZ, for eight weeks, resulted in a significant increase in the depression and anxiety-like effects, in mice. Thus, in order to examine the antidepressant-like effects of the test compounds, after persistent diabetes for eight weeks, mice were treated chronically, with either OND or **4i** or **6z** or FLX, for four-weeks and then assessed for behavioral changes in depression and anxiety models.

During FST, diabetic mice exhibited a significant increase in the duration of immobility indicating depression-like behavior. In addition, chronic FLX administration, reversed the diabetes induced increased duration of immobility, in mice. This is in line with several preclinical reports, confirming the notion that chronic diabetic state, induces a pronounced depressive behavior and chronic antidepressant treatment, may reverse the same (da Silva Haeser et al., 2007; Wayhs et al., 2010). Similar to FLX, OND, **4i** and **6z**, reversed despair effects in diabetic mice, indicating antidepressant-like effects of the test compounds in diabetic state as well.

Interestingly, OND, **4i** and **6z**, demonstrated the significant antidepressant-like effects in non-diabetic mice. It suggests that the effects of the drugs were not altered as a results of diabetes, unlike observed for the clinical antidepressants (Massol et al., 1989). Moreover, these results indicate the efficacy of 5-HT_3 receptor antagonists, in mediating antidepressant-like effect in diabetic condition, as well.

In, TST, diabetic mice exhibited a significant increase in the duration of immobility, which was reversed by chronic treatment with FLX, OND, **4i** and **6z**, indicating that, depression-like behavior induced in mice, as a consequence of persistent diabetes, can be reversed by antidepressant treatment. In addition, it confirms the antidepressant-like effects of OND, **4i** and **6z**, in diabetic state. Moreover, these effects were independent of generalized alteration, in the locomotor activity, confirming the accuracy of the behavioral effects observed in these tests.

7.5 OND, 4i and 6z reverse Type-1 diabetes-induced anxiety-like behavior

Clinical data have shown that patients with diabetes, present behavioral impairment with pronounced anxiety symptoms, in addition to depression (Bystritsky et al., 2014; Lloyd et al., 2000). In line with this contention; preclinical studies have reported that experimentally (STZ) induced diabetic rodents, display anxiety-like condition in various behavioral tests (Aksu et al., 2012; Ramanathan et al., 1998). Hence, the effect of OND, **4i** and **6z**, in ameliorating anxiety-like behavior associated with diabetes, was also investigated. In hole-board test, diabetic mice exhibited a significant decrease in the number and duration of head dips. This is in line with the earlier findings that reflect the anxiogenic effects of persistent diabetic state, in mice. Diabetes induced anxiogenic effect was significantly reversed by chronic FLX treatment, reflecting the high predictive validity of the test. Moreover, OND and **6z**, reversed the diabetes-induced reduced exploratory behavior, in mice subjected to the test. Similarly, as observed in previous findings (Aksu et al., 2012), in this study, during open field test, diabetic mice exhibited a pronounced anxiety-like behavior, as observed by reduced open field activity, which was significantly reversed by chronic **4i** treatment, thereby indicating the anti-anxiety like effect, of the test drug.

Considering the fact that there exists heterogeneity of anxiety disorders with different neurobiological substrates for each and that it is inappropriate to assume that a single model covers all components of the complex expression anxiety (Bourin et al., 2007; Finn et al., 2003; Ramos, 2008), the effect of OND, **4i** and **6z**, was also evaluated in another model of anxiety. In light-dark test, which is based on the natural aversion of animal to brightly illuminated spaces (Crawley and Goodwin, 1980), diabetic mice displayed a pronounced anxiety-like behavior, as observed by decreased activity in light chamber.

Interestingly, treatment with FLX and OND, resulted in a significant reversal of diabetes-induced enhanced anxious behavior in mice, subjected to light-dark test, indicating anti-anxiety like activity of OND, in diabetic condition. Social activities themselves and their quality, exert profound influences on the mood and emotionally relevant behavioral measures. In humans, psychosocial activities can reduce depressive symptoms and improve stress resiliency, while persistent distressed condition can temper emotional reactivity (Derntl et al., 2011). Social-interaction is one of the important parameters that mirror psychotic social activity and emotional stability. In the present study, chronic diabetic state caused a significant impairment in social behavior as indicated by reduced number and time spent in social-interaction (increased social avoidance), which is in agreement with the previous reports (Ramanathan et al., 1998). Conversely, repeated FLX treatment, reversed this diabetes-induced sociability deficit. In addition, chronic administration of **4i** and **6z**, enhanced social activity in diabetic mice, indicating antidepressant and anxiolytic activity of the test drug candidates. Overall, these behavioral effects observed in the tests indicate that OND, **4i** and **6z**, exhibit potential antidepressant-like effects and can reverse diabetes-induced depression and anxiety-like behavior, in mice.

7.6 The mechanism of action

From the previous sections, it is evident that 5-HT_3 receptor antagonists (OND, **4i** and **6z**), posses significant antidepressant-like activity and that they may ameliorate depressive symptomology associated with persistent T1DM as well. However, the mode of their antidepressant efficacy is poorly known. In this section, plausible signaling pathways that may lead to depression in T1DM, role of 5-HT_3 receptors in modulating those and various hypotheses governing the mechanism of antidepressant action of the test drug candidates, are discussed.

7.6.1 OND, 4i and 6z, prevent diabetes-induced altered neurotransmitter levels in discrete brain regions

For more than five decades, the monoamine hypothesis has been a leading theory in the pathogenesis of depression. Schildkraut,(1965) proposed that depression is caused by the under-activity of monoamines, in particular 5-HT in brain, while the elation, on the other hand, might be associated with the antidepressant action of various drug candidates. (Shpakov et al., 2011; Yamato et al., 2004).

Serotonergic projections are highly innervated in discrete brain regions including midbrain, hippocampus and frontal cortex, where these regulate mood and emotional behavior (Ressler and Nemeroff, 2000). The biochemical estimation of 5-HT in diabetic mice brain revealed that diabetes resulted in a significant depletion of 5-HT levels in midbrain (including hippocampus), frontal cortex and cerebellum. Consistent with the view, a clinical study reported that diabetic patients with symptoms of dysregulated mood behavior exhibited impaired 5-HT functions (Manjarrez-Gutierrez et al., 2009). Moreover, few preclinical reports indicated that diabetes resulted in reduced 5-HT activity in brain, which can mediate behavioral abnormalities that resemble depressive and anxiety symptoms in humans (Yamato et al., 2004). On the other hand, correction of 5-HT activity by some classic antidepressants has been reported as a key mechanism to normalize depression and anxiety-like derangements in diabetes (Shpakov et al., 2011).

In the present study, chronic treatment with FLX significantly reversed the diabetes induced reduction in 5-HT content in these brain regions. Similarly, OND, **4i** and **6z** treatment, prevented diabetes induced deficits in 5-HT levels in midbrain (including hippocampus), frontal cortex and cerebellum, which may be correlated with their antidepressant–like effects observed in the behavioral assays.

It has been observed that diabetic state also alters the other non-monoaminergic neurotransmitter functions. Thus, the effect of diabetes on the GABA levels was also estimated. Chronic diabetic state had a pronounced effect on the GABA levels in discrete brain regions. Diabetic mice exhibited a significantly increased GABA content in midbrain, frontal cortex and cerebellum. This is in line with the previous reports demonstrating an elation of GABA content in the brain, as a consequence of chronic diabetic state (Chan et al., 2011). More importantly, chronic treatment with OND, **4i** and **6z**, reversed diabetes-induced increase in the GABA levels, in these brain regions. Besides, it demonstrates that OND, **4i** and **6z**, have significant GABA modulatory effects as well. Previous reports have shown that 5-HT_3 (hetero) receptors are involved in the regulation of other neurotransmitters, (such as GABA, in frontal cortex and hippocampus regions) release.

A number of reports have indicated that activation of 5-HT_3 receptors facilitates, while 5-HT_3 receptors antagonists block extracellular GABA release in the discrete brain regions (Dorostkar and Boehm, 2007; Koyama et al., 2000; Puig et al., 2004;Turner et al., 2004).

It is an important indication of the notion that 5-HT_3 receptor signaling influences mood and emotional behavior associated with persistent diabetic state. It may also be possible that, 5-HT_3 receptor blockade on the GABAergic interneuronal projections, blunted inhibitory control of GABA on the 5-HT release in midbrain and frontal cortex (Fig. 7.3). In support of this speculation, recently, Bétry et al. (2013) demonstrated that blockade of 5-HT_3 receptors by vortioxetine increased 5-HT release, after consistent dosing. Consistently, separate studies have reported that OND, enhanced release of citalopram-induced extracellular 5-HT, in rat frontal cortex (Mork et al., 2012) and prevented paroxetine-induced decrease in 5-HT cell firing in dorsal raphe nucleus thereby subsequently enhancing the extracellular 5-HT release, in rat hippocampus (Bétry et al., 2015).

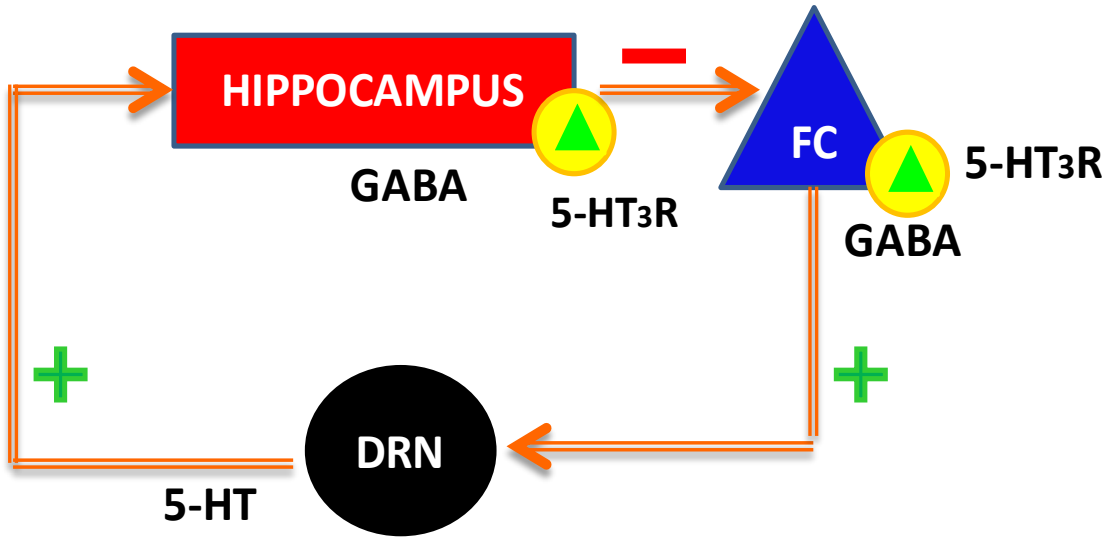


Fig. 7.3 The plausible mechanism of 5-HT_3 receptor antagonist modulating serotonergic and GABAergic neuronal signaling in the discrete brain regions. 5-HT, 5-HT; GABA, gamma amino butyric acid; 5-HT_3R, 5-HT_3 receptors; DRN, dorsal raphe nucleus; FC, frontal cortex; (+) indicates the facilitation and (-) indicates the inhibition of the signaling pathway.

Several, consistent studies suggest that serotonergic transmission exerts powerful control over neurotrophic factor, BDNF expression, and this may be a key mechanism underlying the therapeutic effects of antidepressants act by enhancing 5-HT signaling. Therefore, it may be speculated that facilitation of 5-HT transmission, by the tested drug candidates, would affect BDNF signaling and increase neuronal plasticity and survival, thereby providing long term effect of the drugs, in preventing depression associated with diabetes (Fig. 7.4).

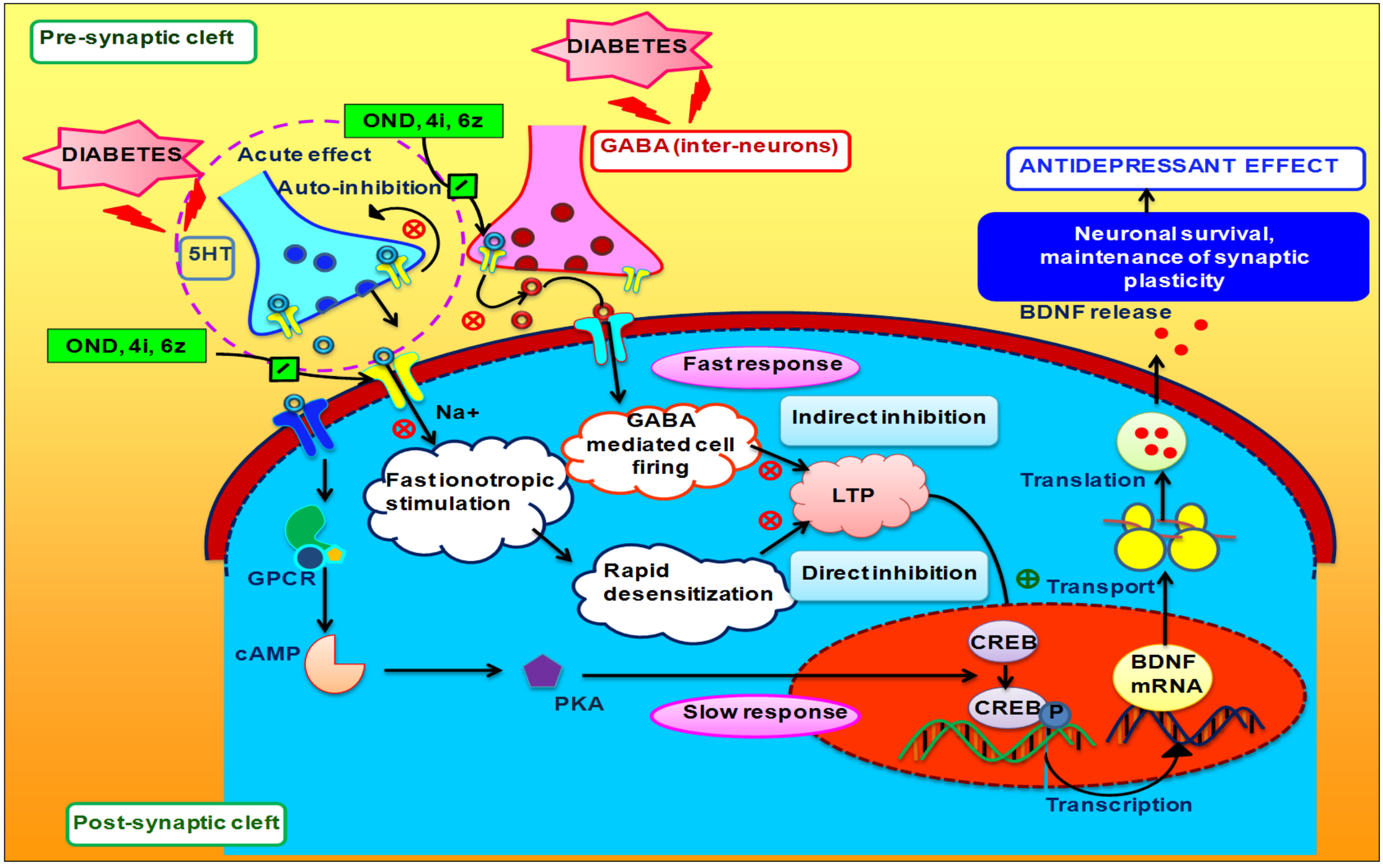


Fig. 7.4 The plausible mechanism of long term effect of 5-HT$_3$ receptor antagonist modulating serotonergic and GABAergic neuronal signaling at the interneuronal projections in the brain. 5-HT, serotonin; GABA, gamma amino butyric acid; GPCR, G-protein coupled receptors; PKA, protein kinase-A; CREB; cyclic response element binding protein; cAMP, cyclic adenosine monophosphate; BDNF, brain derived neurotrophic factor; (×) indicates the inhibition and (-) indicates the blockade of the signaling pathway.

7.6.2 OND, 4i and 6z, prevent diabetes-induced HPA-axis hyperactivity

In addition to the above, the possible mechanisms of depression-like behavior in persistent T1DM induce dysfunction of HPA-axis (Chan et al., 2003; Korczak et al., 2011). Hyperactivity of HPA-axis, characterized by elevated circulating levels of glucocorticoids (cortisol in humans and corticosterone in rodents) is one of the best-replicated findings in the neurobiology of depression (Barden, 2004). Increase in plasma glucocorticoid levels is beneficial, during times of stress; however, prolonged exposure to elevated glucocorticoid levels can have severe pathological complications. HPA-axis is a central promoter of circulating glucose levels. Pathologically activated HPA-axis, as in T1DM, increases circulating glucocorticoid levels, which in turn stimulate glucose production via increased gluconeogenesis, glycogenolysis, and lipolysis (Teague et al. 2007). The enhanced glucose oxidation, subsequently generates ROS and increases oxidative load in brain structures that can cause damage to brain tissues (Höschl and Hajek 2001; Piotrowski et al. 2001; Stranahan et al. 2008).

Another major complication is that elevated glucocorticoid levels, repress BDNF activity (a neurotrophic factor in the brain), by inhibiting its expression. It has been reported that elevated corticosterone block transcriptional activity of the BDNF promoter site, thereby blocking its expression (Herbert, 2013; Schaaf et al., 1998). On the other hand, antidepressants such as FLX, increase BDNF expression, which is prevented by excess corticosterone administration (Kunugi et al., 2010). This may have important implications in neuropathologies associated with depression in T1DM (Korczak et al., 2011).

To clarify the mechanisms mediating antidepressant-like effect of OND, **4i** and **6z**, in diabetes, the level of plasma corticosterone as a marker of HPA-axis activity was measured. Results showed that plasma corticosterone level in STZ-induced diabetic mice was markedly elevated, in accordance with a previous study (Ho et al., 2012), and significantly reversed to normal by chronic administration of OND, **4i** and **6z**. Similarly, chronic FLX treatment normalized the elevated levels of plasma corticosterone in STZ-induced diabetic mice. The mechanism whereby, 5-HT_3 receptor antagonists attenuate HPA-axis hyperactivity, is indicated by the neuroanatomical studies demonstrating direct synaptic connections between serotoninergic terminals and CRH-containing neurons at the paraventricular nucleus (PVN) of the hypothalamus, which support the direct effect of 5-HT on HPA-axis.

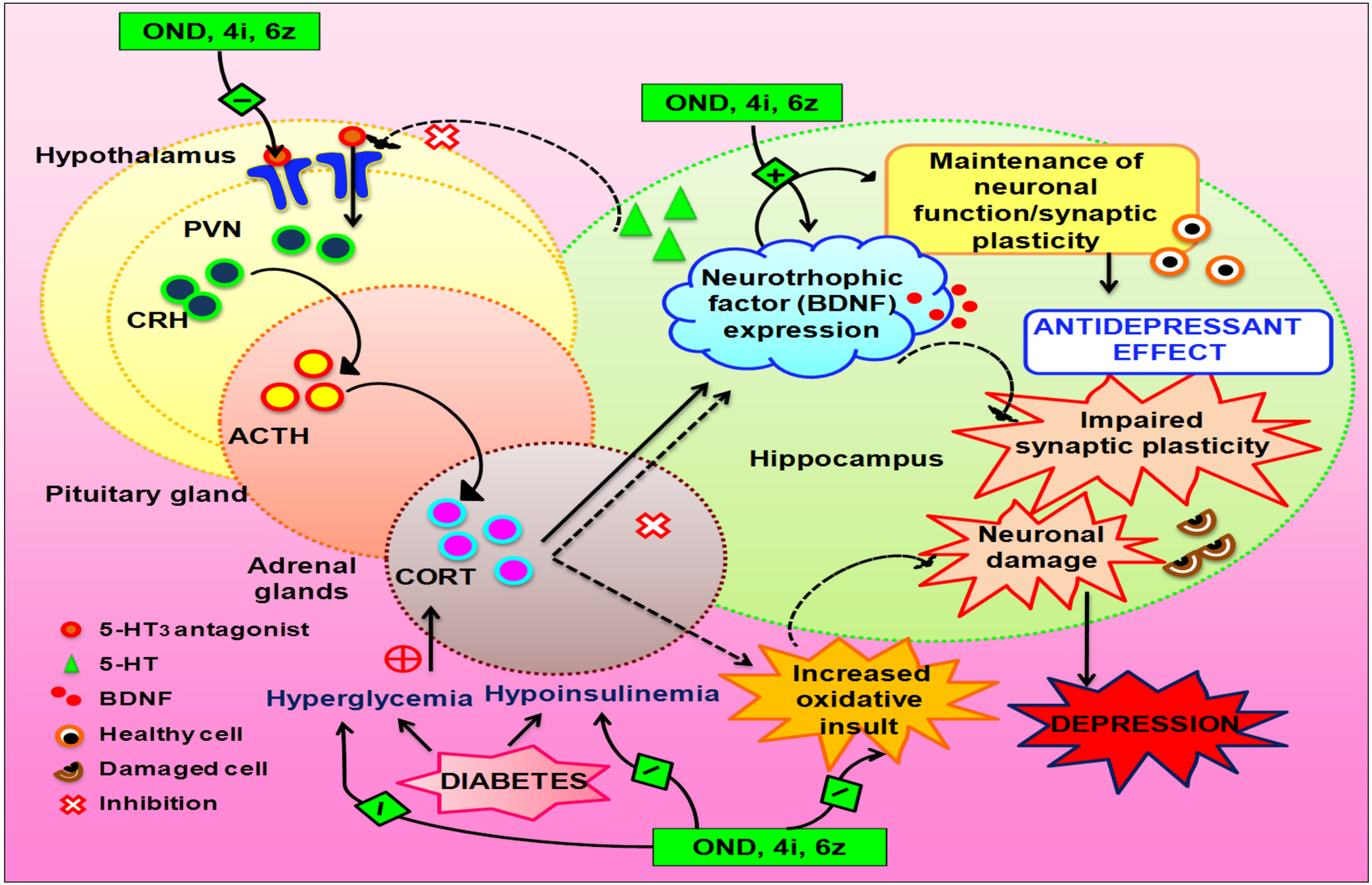


Fig. 7.5 The plausible mechanism of 5-HT_3 receptor antagonists in ameliorating depression-like behavior associated with diabetes. ACTH, Adrenocorticotrophic hormone ; CRH, Corticotrophin-Releasing Hormone; CORT, Corticosterone; OND, Ondansetron; BDNF, Brain Derived Neurotrophic Factor; PVN, Paraventricular Nucleus.

Stimulation of 5-HT_3 receptors, have been shown to enhance an increased neuronal release of CRH into the portal blood, where the increased peptide concentration leads to the increased synthesis of proopiomelanocortin peptide precursor (POMC), leading to an increase cell-secretion of ACTH from anterior pituitary lobe and subsequently, to the secretion of corticosterone or cortisol from adrenal gland.

Therefore, it may be that blockade of 5-HT_3 receptors in PVN region of hypothalamus, attenuates increased HPA-axis function and subsequently downstream signaling (Leff et al., 2010), which may contribute to the mechanism, whereby the tested drug candidates ameliorate depression and anxiety-like behavioral deficits, evoked in diabetic mice (Fig. 7.5). Since, elevated corticosterone level can affect BDNF signaling and enhance oxidative damage in neuronal structures; it is interesting to evaluate the effect of these drug candidates on the oxidative stress and changes in BDNF signaling, as a consequence of diabetes, which are discussed in later sections.

7.6.3 OND, 4i and 6z, prevent diabetes-induced impairment of neurotrophic factor, BDNF signaling in discrete brain regions

BDNF is one of the most abundant neurotrophic factors in the CNS. Clinical studies have shown reduced BDNF levels in the blood of depressive patients (Bhatnagar et al., 2004), whereas antidepressant treatment seemed to normalize BDNF levels (Smit-Rigter et al., 2010).

The antidepressant action of BDNF is closely linked to the induction hippocampal neurogenesis (Schmidt and Duman, 2010). Indeed, it plays an important role in development, maintenance and plasticity of neurons in hippocampus.

BDNF promotes differentiation of neurons from stem cells, enhances neurite outgrowth and synaptogenesis and can prevent programmed cell death/apoptosis (Castrén and Rantamäki, 2010), which might account for its ability to improve depressive symptomology. Therefore, it may be speculated that persistent diabetes may lead to alteration in the BDNF functions in discrete brain regions that may account for the depressive-like behavior, in mice. With regard to the effect on neurotrophic factor, STZ-induced diabetic mice exhibited a significant decline in BDNF level in midbrain (including hippocampus) and frontal cortex, in accordance with the previous findings (Ho et al., 2012; Lenart et al., 2016; Redivo et al., 2016).

This is in line with the clinical findings, which showed that persistent diabetes results in reduction of serum BDNF levels in patients (Ola et al*.,* 2013). In contrast, FLX treatment reversed the diabetes induced reduction in BDNF level in these regions of the brain. In addition, chronic OND, **4i** and **6z** treatment, increased BDNF level in these brain regions in STZ-induced diabetic mice suggesting that OND, **4i** and **6z**, might produce antidepressant-like effect via amelioration of neurotrophic support. Persistent diabetes resulted in a substantial decline of cAMP level (a secondary messenger in BDNF downstream signaling) in midbrain and frontal cortex and this was significantly attenuated, by chronic treatment with FLX.

Furthermore, OND, **4i** and **6z**, treatment attenuated the reduction of cAMP levels, in mice with persistent diabetes, thereby confirming the potential protective role of the 5-HT_3 receptor antagonists, against diabetes induced impaired neurotrophic factor signaling, in brain regions.

Previous studies have shown that, hyperglycemia results in suppression of BNDF expression in vivo (Uchino et al*.,* 1997). Thus, the reduced BDNF levels found in case of diabetic mice could be due to direct consequence of hyperglycemia in diabetes. However, this may not be the case as the FLX reversed the diabetes-induced BDNF levels in diabetic mice without affecting hyperglycemic condition (discussed in later sections). Therefore, alternative mechanism must exist, that leads to changes in BDNF levels in diabetes and its reversal by potential antidepressants. This could be explored in futuristic studies.

7.6.4 OND, 4i and 6z, prevent diabetes-induced increase in oxidative stress in discrete brain regions

Oxidative stress has been implicated in the pathogenesis of diverse disease states, including depression, as the brain has comparatively greater vulnerability to oxidative cellular damage (Gandhi and Abramov, 2012). The altered oxidative status is implicated in various aspects of brain cellular function. During diabetes, the chronically elevated intracellular glucose concentration and its enhanced oxidation resulted in production of large amount of reactive oxygen species (ROS) (Bonnefont-Rousselot, 2002). Persistent hyperglycemia results in accelerated biochemical process of advanced glycation, which leads to production of advanced glycation end products (AGEs) (Ramasamy et al., 2005; Uchino et al., 1997).

AGEs are a complex group of compounds formed via a nonenzymatic reaction between reducing sugars and amine residues on proteins, lipids, or nucleic acids. AGEs are postulated to play a central role in neurodegenerative disorders.

AGEs activate receptor for advanced glycation end products (RAGE) and affect protein kinase C (PKC) activity (Ola et al., 2013). It has been demonstrated that abrupt PKC activity leads to impairment of neuronal cell conduction and signaling, that could lead to the changes in neuronal pathways regulating mood and emotional behavior, Fig. 7.6. ROS is physiologically counteracted by endogenous antioxidant defense system. However, pathologically elevated ROS may overwhelm the antioxidant defenses and lead to damage of major components of the cellular structure, including nucleic acids, proteins, amino acids, and lipids (Valko *et al,* 2007).

These oxidative modifications in diabetes would affect several cell functions, metabolism and gene expression leading to depression-like behavioral deficits (Maes *et al,* 2011). Thus, it is possible that augmentation of *in vivo* antioxidant defenses and attenuation of pro-oxidant stimuli in persistent diabetes could serve as an important mechanism underlying the neuroprotective pharmacological effects of antidepressant drugs, observed clinically in the treatment of various stress disorders. In the present study, persistent diabetes resulted in increased pro-oxidant markers with a substantial decline in the anti-oxidant enzyme activity in midbrain and frontal cortex.

Diabetic mice exhibited an increased level of TBARS (a maker of lipid peroxidation) and nitrite in these brain regions as well as a substantial reduction in the catalase and GSH activity. The results corroborate the previous reports demonstrating that diabetes may lead to increased oxidative stress in the brain (de Morais et al. 2014; Wayhs et al. 2010, 2013). Interestingly, chronic treatment with FLX inhibited the diabetes induced increase in oxidative stress in measured brain regions. In addition, OND, **4i** and **6z**, treatment reversed the diabetes-induced oxidative insults with increased anti-oxidant enzyme activity and reduced pro-oxidant markers in these brain regions of STZ-induced diabetic mice, suggesting the plausible involvement of oxidative stress in the induction of depression-like behavior in diabetic mice, the reversal of which provides one of the key mechanisms of antidepressant action of the tested drug candidates (Fig. 7.6).

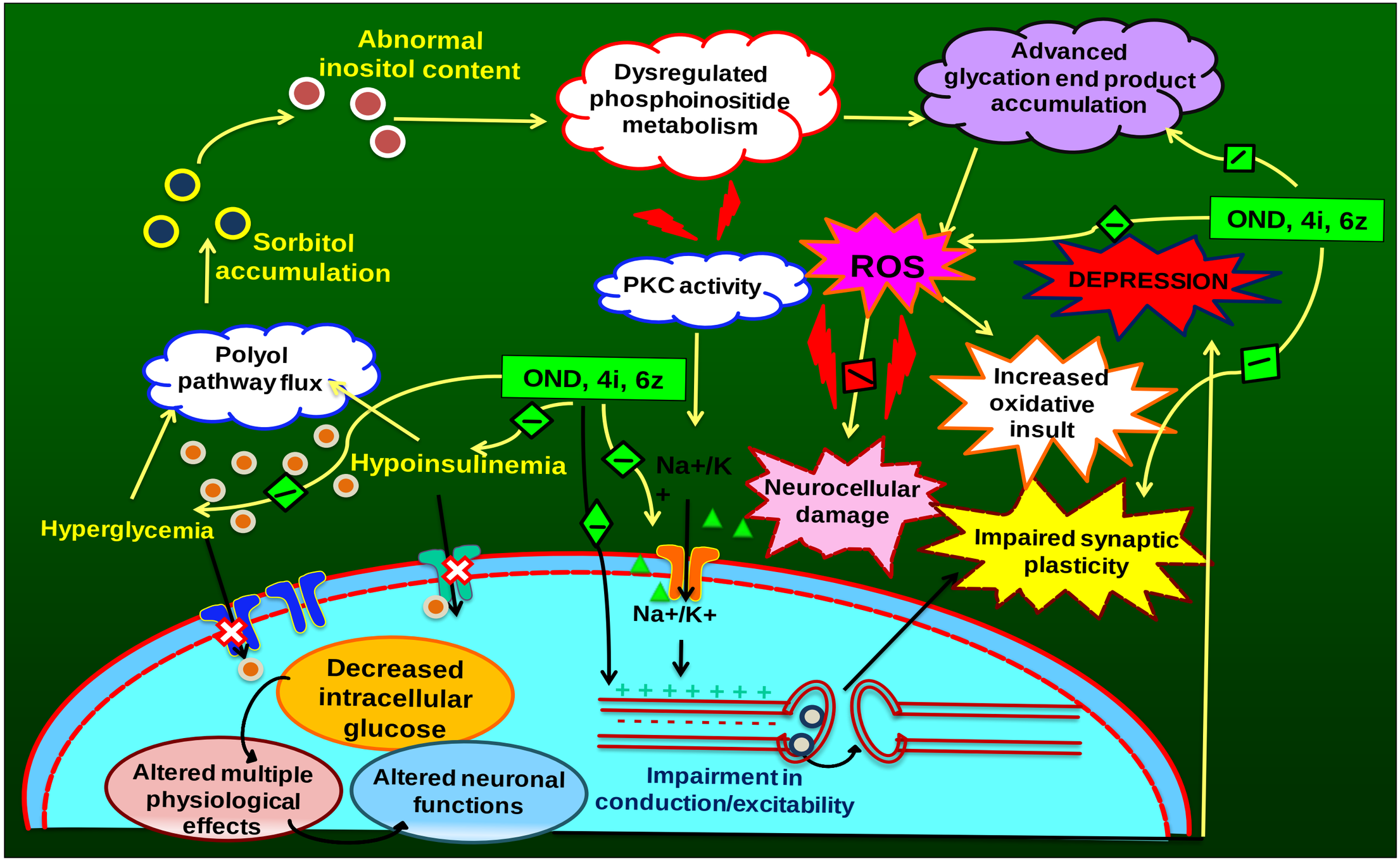


Fig. 7.6 The plausible pathways involving the role of diabetes-induced oxidative stress in mediating depression and anxiety as well as antidepressant-like effect of 5-HT_3 receptor antagonists. The extra-neuronal increase in glucose concentration leads to decrease glucose influx into neuronal cell and shift of glucose metabolism to polyol pathway, leading to abrupt increase in ROS and hence oxidative stress in brain. The reversal of this abnormal activity could be associated with the antidepressant mechanism of action of the test drug candidates. ROS, Reactive Oxygen Species, indicating free radicals; PKC, Protein Kinase-C

7.5 OND, 4i and 6z, prevent diabetes-induced increase in morphological perturbations in hippocampus

Neurological studies have shown that morphological integrity of the brain structures are drastically affected in diabetes (Bessa et al., 2009). The effect of chronic treatment of test drug candidates on the neuronal damage and dendritic morphology, as a consequence of diabetes, was estimated in hippocampal regions (the key brain structure that regulate mood and emotional behavior), using histological techniques namely, H&E and Golgi-Cox staining. At neuronal level using H&E staining, it was found that persistent diabetes, resulted in an increased hippocampal neurodegeneration as indicated by increased percentage of damaged neuronal cell in DG and CA_3 regions of hippocampus. This effect was markedly reversed by chronic treatment with FLX, the standard prototype antidepressant.

Similarly, OND, **4i** and **6z**, notably reversed the diabetes-induced neuronal damage in these regions. This suggests that the tested drug candidates may have potential neuroprotective activity. At sub-neuronal level using Golgi-Cox staining, it was found that persistent diabetes resulted in a pronounced impairment in dendritic morphology, indicated by decrease in branch lengths of primary, secondary and tertiary dendrites and a marked decline in dendritic branching in CA_3 region of hippocampus. All these changes provide a plausible basis for some of the depression-like behavior found in STZ-induced diabetic mice, which were reversed by chronic treatment with FLX. Moreover, OND, **4i** and **6z**, prevented the diabetes-induced altered dendritic remodeling. Interestingly, experimental studies showed that dendritic remodeling is associated with the prolonged activation of HPA-axis, resulting in consistently elevated corticosterone level and can be reversed by chronic antidepressant treatment (Conrad, 2008). Therefore, it is possible that dendritic outgrowth impairment in STZ-induced diabetic mice and its reversal by OND, **4i** and **6z**, observed in the current study, could involve modulation of HPA-axis hyperactivity. Finally, since dendritic outgrowth is regulated by the action of neurotrophic factors including BDNF (Dijkhuizen and Ghosh, 2005; McAllister et al., 1996) and diabetic mice expressed low levels of BDNF, reversed by OND, **4i** and **6z**, treatment, the possibility that the restoration of diabetes-induced low BDNF activity, could be involved in normalization of neuronal structural changes in hippocampus, by OND, **4i** and **6z**, treatment in STZ-induced diabetic mice, cannot be overruled (Fig. 7.7).

Overall, it may be suggested that impaired neurotransmitter dynamics, HPA-axis hyperactivity, enhanced oxidative load, reduced neurotrophic factor signaling and morphological and structural remodeling in discrete brain regions, as a consequence of persistent diabetes, may cause depression-like behavior and a coupled regulation of these events by OND, **4i** and **6z**, may be involved in the antidepressant-like effect of these drug candidates.

7.6 Role of 5-HT_3 receptor antagonism in the activity of OND and 4i

In order to investigate whether, the effects of OND, **4i** and **6z**, involved antagonism of 5-HT_3 receptors; an interaction study with a selective agonist, mCPBG was carried out. Pre-treatment with mCPBG altered the behavioral and biochemical responses of OND, **4i** and **6z**, in STZ-induced diabetic mice. It indicates that the effects of OND, **4i** and **6z**, were regulated by the modulation of 5-HT_3 receptors. During FST, pre-treatment with mCPBG abolished the OND, **4i** and **6z**, mediated decrease, in duration of immobility, in diabetic mice, the effect typically observed in TST.

It is in line with the previous reports, which have shown the antidepressant effects of several drugs with 5-HT_3 receptor antagonistic action in these testing paradigms and blockade of their action by concomitant administration of 5-HT_3 receptor agonists (Fan, 1994; Nakagawa et al., 1998).

In addition, it has been demonstrated that several clinical antidepressant drugs are functional antagonists at 5-HT_3 receptors (Eisensamer et al., 2003). Similar to the results obtained in depression models, in anxiety tests, mCPBG blunted the OND, **4i** and **6z**, mediated anxiolytic behavior in anxiety based behavioral models.

In hole-board test, the head dips activity remained low in STZ-induced diabetic mice given either OND or **6z** but pre-treated with mCPBG instead of vehicle. Similarly, during light-dark test, the exploratory behavior in brightly lit chamber, which is an indicator of reduced anxiety-like behavior, remained significantly low in STZ-induced diabetic mice given either OND or **6z**, but pre-treated with mCPBG instead of vehicle. Likewise, in open field test the open field activity, measured as number of crossings and rearings remained low in diabetic mice given **4i,** but pre-treated with mCPBG instead of vehicle.

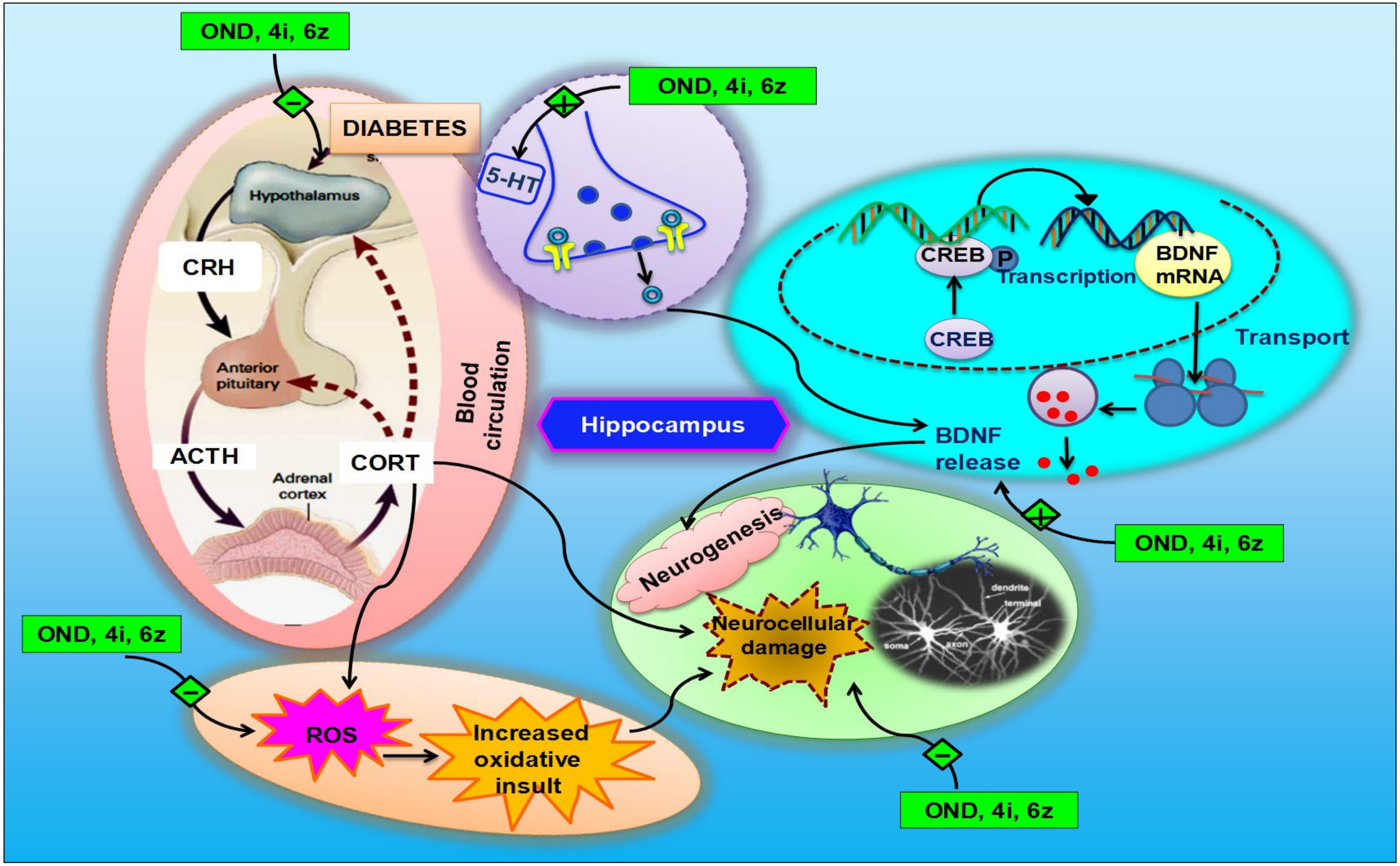


Fig. 7.7 The plausible role of 5-HT_3 receptor antagonist in ameliorating diabetes-induced neuronal damage in hippocampus. 5-HT, serotonin; ROS, reactive oxygen species; CREB, cyclic response element binding protein; BDNF, brain derived neurotrophic factor; CORT, corticosterone; ACTH, Adrenocorticotrophic hormone ; CRH, Corticotrophin-Releasing Hormone

During social interaction test, the number and time spent in social interaction remained significantly low in STZ-induced diabetic mice given **4i**, but pre-treated with mCPBG, instead of vehicle. It suggests that the behavioral effects observed for OND, **4i** and **6z**, in depression and anxiety testing paradigms were mediated by antagonism of 5-HT_3 receptors. In addition, these results suggest that 5-HT_3 receptors may be involved in the pathophysiology of depression associated with diabetes. Furthermore, the effects of OND, **4i** and **6z**, in the biochemical assays, were inhibited by pre-treatment with mCPBG. Pre-treatment with the selective agonist, mCPBG, abolished the OND, **4i** and **6z**, mediated increase in the 5-HT levels in midbrain (including hippocampus), frontal cortex and cerebellum in STZ-induced diabetic mice, confirming the assumption that 5-HT_3 receptor antagonism is involved in the 5-HT facilitatory effects of OND and **4i**.

In addition, the effect of pre-treatment of mCPBG on the modulatory effects of OND, **4i** and **6z**, on GABA, was estimated. It was found that mCPBG blunted the OND, **4i** and **6z**, mediated reduction in GABA levels in midbrain (including hippocampus) and frontal cortex. However, it had no effect on the cerebellum.

These results suggest that the effect of 5-HT_3 receptor mediated GABA modulation is region specific. Thus, it seems that the effect of 5-HT_3 receptors on GABA is more pronounced in midbrain and frontal cortex. It could be due to high receptor expression of 5-HT_3 in these regions as compared to cerebellum. It has been reported that 5-HT_3 receptors are highly expressed in GABAergic interneurons in frontal cortex. Excitation of 5-HT_3 receptor via increased 5-HT level in these neuronal projections activates the inhibitory GABAergic tone. GABA binds with GABAergic receptors ($GABA_A$ and $GABA_B$), which ultimately reduces 5-HT release (Artigas, 2013). It has been observed that mCPBG increased GABA release in isolated rat brain, an effect prevented by specific 5-HT_3 receptor antagonist, tropisetron (Koyama et al., 2000).

Therefore, antagonism of 5-HT_3 receptors may decrease GABA release from the inter-neuronal GABAergic projections and indirectly increase 5-HT release though inhibition of this local negative feedback, mediated by inhibitory GABAergic tone. Thus, a coupled-regulation of 5-HT_3 receptor mediated neurotransmitter (5-HT and GABA) release, may act to finally tune the excitatory and inhibitory response in brain neuro-circuits (affecting the behavioral activity) (Fig. 7.3).

Several lines of research have shown that application of extracellular agonists (including mCPBG) induce a rapid internalization of 5-HT_3 receptors in the plasma membrane, while 5-HT_3 receptor antagonists prevent such effects (Ilegems et al., 2004). Thus, the ineffective neurobehavioral activity of mCPBG in diabetic mice, observed in the present study, could be explained by the fact that chronic treatment with mCPBG may cause a decrease in 5-HT_3 receptor density and lead to the ineffective activation of the 5-HT_3 receptor mediated response. However, if this is the case, then it may be assumed further, that desensitization of 5-HT_3 receptor may inhibit a feedback stimulation of 5-HT release from serotonergic neuronal projections (Martin et al., 1992). However, biochemical estimation of 5-HT content did not reveal a significant change in 5-HT level after mCPBG administration, in discrete brain regions. Therefore, the rapid desensitization of 5-HT_3 receptors on GABAergic interneurons and subsequently enhanced GABA mediated inhibitory control on 5-HT release, seems to be predominantly leading to the inhibited levels of 5-HT in the measured brain regions, after mCPBG treatment.

This predominance is also thought to be associated with the protective effects of OND and **4i** against diabetes-induced affective behavioral impairment, in conjunction with the enhanced 5-HT content in the discrete brain regions, in mice.

7.7 Glucose lowering effects of OND, 4i and 6z

The effect of 5-HT_3 receptor antagonist (OND, **4i** and **6z**) on circulating glucose levels was estimated. It was found that chronic treatment with OND, **4i** and **6z**, reduced fasting blood glucose levels in STZ-induced diabetic mice, suggesting that antagonism of 5-HT_3 receptors may have potential to regulate, blood glucose levels. The results are in line with the previous reports, which showed that 5-HT_3 receptors are involved in the metabolic control of glucose (Carvalho et al., 2004, 2005; Rahimian et al., 2013). Stimulation of 5-HT_3 receptors by chronic administration of selective 5-HT_3 agonists has shown to increase fasting blood glucose levels in non-diabetic mice, the effect reversed by selective antagonist, OND.

The mode of this regulation is poorly known, however, it may occur via an increase in insulin signaling. Previous reports have shown that tropisetron, another 5-HT_3 receptor antagonist stimulated insulin release via insulin secreting β-cell lines, in vitro (Heimes et al., 2009).

Taking this into account, the present work included the studies to explore the effect of chronic treatment of OND, **4i** and **6z**, on plasma insulin levels. It was found that treatment with OND, **4i** and **6z**, significantly increased plasma insulin levels, in STZ-induced diabetic mice.

This may be associated with the reduction in the elevated blood glucose levels (hyperglycemia), in mice. It also revealed the fact that 5-HT partly regulates the metabolic glucose via 5-HT_3 receptor system.

This is in line with a previous study, which demonstrated that central stimulation of 5-HT_3 receptors produces hyperglycemia in mice, which was reversed by central OND treatment (Carvalho et al., 2004).

This is an important advantage over existing antidepressant drugs. It has been clinically reported that current antidepressants, although prevent depressive symptomology in patients with diabetes, worsen glycemic control (Goodnick, 2001).

Therefore, if proven clinically, 5-HT_3 receptor antagonists may have advantage over existing antidepressant drugs, for the management of depression and other mood related disorders, associated with diabetes.

In conclusion, the current study comprises of widespread pharmacological data distinguishing the effect of diabetes, in particular, T1DM and 5-HT_3 receptor antagonists, on behavioral, biochemical, neurobiological and histopathological features resembling the clinical depression.

The effects of potential 5-HT_3 receptor antagonists namely, OND, **4i** and **6z**, treatment were found to ameliorate depression-like behavior, evoked in STZ-induced diabetic mice, corroborating the notion that 5-HT_3 receptors are involved in the pathogenesis of depression associated with diabetes, which is possibly mediated by the modulation of multifaceted signaling cascades involving neurotransmitter dynamics, HPA-axis activity, oxidative stress, neurotrophic factor signaling and morphological remodeling.

In addition, the present study revealed that the drugs with 5-HT_3 receptor antagonism may ameliorate depression symptomology associated with diabetes by normalizing these overlapping molecular and cellular events.

8.1 Summary

Depression and anxiety co-morbid with Type-1 Diabetes Mellitus (T1DM) often leads to worsening of patient's condition. Studies demonstrated the behavioral abnormalities, with persistent and prominent symptoms of depressive disorders, co-morbid with T1DM patients. Although, several clinical antidepressants are currently prescribed for the management of this co-morbid disorder, the frequent and persistent side effects (in particular, impairment of glycemic control being the most prominent), limit their use.

Currently prescribed drugs ameliorate depressive and anxiety symptoms co-morbid with diabetic patients, mainly by elation of 5-HT neurotransmission (for example SSRIs) in discrete brain regions. However, central 5-HT system participates in the regulation of blood glucose homeostasis and non-specific activation of central 5-HT increase blood glucose level via stimulation of 5-HT_3 receptors downstream signaling. Therefore, there are critical unmet needs for new drugs, which exert antidepressant effect with specific mechanism(s) and also maintain or at least not cause aggravation of diabetic condition.

5-HT_3 receptors are involved in signaling processes that regulate learning, cognition and emotion and have been implicated in the pathogenesis of various psychiatric disorders, including depression and anxeity. Interestingly, studies identified the metabolic effects, have found that 5-HT_3 receptors are involved in the regulation of glucose homeostasis. Therefore, it may be hypothesized that 5-HT_3 receptors play an important role in signaling processes, involving regulation of affective behavior and glucose homeostasis, and antagonism of 5-HT_3 receptors may provide an alternative therapeutic strategy to combat depression and anxiety co-morbid with T1DM, with better efficacy and minimum untoward effects. In the present work, initially, the induction of T1DM, in mice using STZ, a diabetogenic agent, was carried out. Diabetes, in mice was successfully induced with mice resulting in hyperglycemia, hypoinsulinemia and damage of insulin secreting β-cells. This was followed by the investigation of time course of development of depression-like behavior, in mice, as a consequence of T1DM. Mice were periodically evaluated for behavioral changes, using neurobehavioral models of depression and anxiety. After 8-weeks of persistent diabetes, mice demonstrated pronounced depression-like behavior in FST and TST as well as anxiety-like behavior in hole-board, light-dark, open field and social interaction testing paradigms, suggesting that persistent but not initial state of diabetic condition, leads to depression and anxiety disorders.

In order to evaluate the antidepressant potential of 5-HT_3 receptor antagonists, preliminary screening in naïve animals was carried out. First, the dose response studies were conducted for the dose selection, using actophotometer. The doses of 0.5 and 1 mg/kg for OND, the standard 5-HT_3 receptor antagonist; 0.5 and 1 mg/kg for **4i** and 1 and 2 mg/kg for **6z**, the novel candidates, were selected as these doses had no significant influence on basal locomotor activity of mice, which could have produced false results during behavioral analysis, otherwise. The antidepressant activity of, OND, **4i** and **6z**, was evaluated in both acute and chronic models of depression. OND, **4i** and **6z** indicated potential antidepressant-like effects in acute behavioral models. Similarly, CUS mouse model and OBX rat model were utilized to evaluate the antidepressant-like effect of the tested drug candidates, in order to simulate the clinical course of drug treatment, in humans. OND, **4i** and **6z**, indicated potential antidepressant-like activity in these models.

Subsequently, the ability of OND, **4i** and **6z**, to ameliorate depression and anxiety-like behavior, evoked in STZ-induced diabetic mice, were investigated. Eight weeks of diabetic mice with pronounced depression and anxiety-like behavior, were given repeated dosing of the tested drug candidates and subjected to a battery of behavioral assays.

The results showed that OND, **4i** and **6z**, significantly reversed diabetes-induced depression-like behavior, in mice. Next, the pathogenesis of depression associated with T1DM and mechanism(s) underlying antidepressant effect of the tested drug candidates were investigated using biochemical, neurobiological, molecular and histopathological assays.

Diabetic mice exhibited a significant alteration in neurotransmitter activity, as indicated by deficits in 5-HT levels and elation in GABA levels in midbrain (including hippocampus), frontal cortex and cerebellum. Moreover, mice with persistent diabetes resulted in hyperactivity of HPA-axis, with increased plasma corticosterone levels. These biochemical alterations appear as key to the pathogenesis of depression in diabetes. Chronic treatment with OND, **4i** and **6z**, markedly reversed diabetes evoked changes in neurotransmitters level and HPA-axis activity, which may be related to their antidepressant-like effects.

Based on the involvement of these neurotransmitters (5-HT and GABA) and HPA-axis on the regulation of neurotrophic factor, BDNF signaling; the effect of persistent diabetes and chronic treatment of OND, **4i** and **6z** on BDNF and cAMP (the secondary messenger of BDNF signaling cascades) were investigated in discrete brain regions.

Data indicated that persistent diabetes resulted in a marked impairment in BDNF signaling, which was prevented by OND, **4i** and **6z** treatment, suggesting that the tested candidate molecules, might produce antidepressant-like effect via amelioration of neurotrophic support. Besides, the effect of persistent diabetes on the oxidative stress parameters in discrete brain regions were investigated, based on the fact that excessive glucose, leads to generation of high free radicals which overwhelm the antioxidant defense system and can lead to increased oxidative stress. As expected, STZ-induced diabetic mice exhibited enhanced oxidative load in midbrain (including hippocampus) and frontal cortex and this perturbation was reversed by the chronic treatment with OND, **4i** and **6z**, which may be suggested as one of the plausible mode of their antidepressant-like action.

Moreover, histopathological studies demonstrated that persistent diabetes resulted in pronounced neuroanatomical damage in hippocampal regions, with high degree of neuronal loss and dendritic remodeling, which was reversed by the chronic treatment of the tested drug candidates. In line with the results obtained, the role of 5-HT_3 receptors in mediating antidepressant-like effects of the drug candidates was investigated. Concomitant administration of a selective agonist, mCPBG blocked the OND, **4i** and **6z**, mediated behavioral and biochemical effects in STZ-induced diabetic mice.

The effect of OND, **4i** and **6z** on the metabolic parameters indicating diabetic condition were examined. The tested drug candidates were effective in reducing fasting blood glucose levels and increasing plasma insulin levels, in diabetic mice, suggesting that chronic administration of OND, **4i** and **6z**, may also reduce or at least not cause aggravation of diabetic state, complimentary to reducing depression-like behavior.

8.2 Conclusions

Altogether, these findings, point to several predictive hypotheses:

- Depression and anxiety-like disorders may occur, co-morbid with T1DM.
- Alterations in neurotransmitter dynamics, HPA-axis hyperactivity, impaired neurotrophic factor signaling, enhanced neuronal oxidative stress and neuro-anatomical damage in discrete brain regions, involved in regulating mood and emotional behavior, may be the underlying causes, that leads to depression and anxiety co-morbid with T1DM.
- 5-HT_3 receptor antagonists have potential to ameliorate the depression-related behavior co-morbid with T1DM, plausibly by normalizing these overlapping and multifaceted set of events.
- 5-HT_3 receptors may play a prominent role in the pathogenesis of depression and anxiety co-morbid with T1DM.
- Molecules with 5-HT_3 receptor antagonism may be potential candidates for the management of depression and other related behavioral abnormalities (anxiety) co-morbid with T1DM.

8.3 Significance of the work

- The present work added evidence for the speculation that depression and related psychiatric disorders such as anxiety may be associated with T1DM.
- This is the first study that depicted the role of 5-HT_3 receptors in the pathogenesis of depression and anxiety co-morbid with T1DM.
- For the very first time, the study signified the ability of 5-HT_3 receptor antagonists in ameliorating depression and anxiety evoked in T1DM.
- The results of the study indicated the potential and benifical efficacy of 5-HT_3 receptor antagonists in the management of depression and anxiety co-morbid with T1DM.

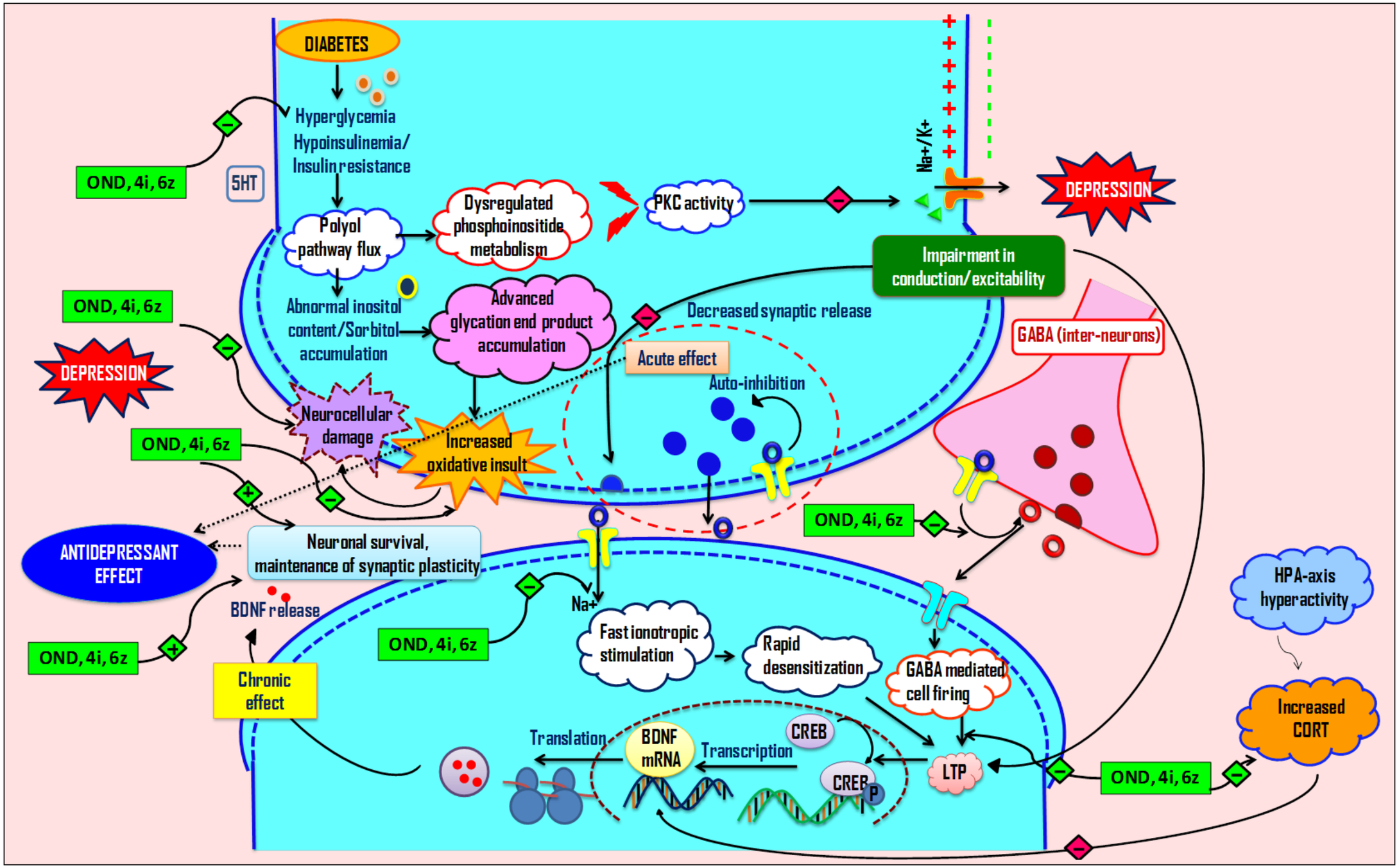


Fig. 8.1 Proposed mechanisms of 5-HT_3 receptor antagonists in depression and anxiety co-morbid with T1DM. 5-HT_3 receptor antagonists modify various signaling pathways altered in T1DM. By modifying the signal mechanisms, 5-HT_3 receptor antagonists prevent behavioral abnormalities evoked in T1DM and produce antidepressant-like effects. BDNF, brain derived neurotrophic factor; CREB, cyclic response element binding protein; CORT, corticosterone; LTP, long term potentiation; PKC, protein kinase-C; 5-HT, serotonin; GABA, gamma amino butyric acid; HPA-axis, hypothalamic-pituitary-adrenal axis

9.1 Salient findings from the work

- The potential ability of selected 5-HT_3 receptor antagonists in ameliorating depression and anxiety-like behavior co-morbid with T1DM, was investigated.
- Development of depression and anxiety, as a consequence of T1DM was confirmed.
- Time course for the development of depression and anxiety in T1DM was established.
- The plausible pathogenic hypothetical pathways, involved in the development of depression and anxiety in T1DM was confirmed.
- Alterations in neurotransmitter dynamics, HPA-axis function, neurotrophic factor signaling, neuronal oxidative stress and neuroanatomical and morphological changes in discrete brain areas, in relevance to the neurobiological changes, accompanied with depression associated with T1DM were studied.
- The effect of chronic treatment of standard 5-HT_3 receptor antagonist, OND and novel candidates synthesized in our medicinal laboratory as 5-HT_3 receptor antagonists, **4i** and **6z**, on the T1DM, induced neurobiological perturbations, were examined, as the underlying mechanism of their antidepressant-like effects.
- The role of 5-HT_3 receptors in pathogenic events leading to depression in T1DM, was investigated.
- The effect of 5-HT_3 receptor antagonists on fasting blood glucose levels and plasma insulin levels, as the metabolic indicators of T1DM were examined.
- From the studies it may be concluded that T1DM may result in pronounced depression and anxiety-like behavioral abnormalities, which increases with the duration of T1DM condition.
- Persistent T1DM, may result in:
    - Significant impairment in neurotransmitter activity in mid brain (including hippocampus), frontal cortex and cerebellum:
    - hyperactivity of HPA-axis with elevated level of circulating corticosterone

- significant impairment in BDNF signaling in mid brain (including hippocampus) and frontal cortex
- enhanced neuronal oxidative load in mid brain (including hippocampus) and frontal cortex
- morphological changes including neuronal damage and dendritic remodeling in hippocampal regions

- 5-HT_3 receptor antagonists exhibit potential ability to ameliorate depression and anxiety associated, with T1DM.
- 5-HT_3 receptor antagonists may normalize T1DM induced neurobiological perturbations, which may underlie the mechanism, of their antidepressant activity.
- 5-HT_3 receptors play a key role in the pathogenic pathways and neuronal signaling cascades, leading to depression in T1DM.
- 5-HT_3 receptors are also involved in the regulation of glucose homeostasis and 5-HT_3 receptor antagonists may attenuate hyperglycemia and hypoinsulinemia in T1DM.

9.2 Implications for future research

The current work analyzed the efficacy of 5-HT_3 receptor antagonists and their plausible underlying mechanisms in ameliorating depression and anxiety associated with T1DM. Based on the findings, future avenues utilizing the evidence obtained in this work will be explored. Some of these include:

- Pharmacological studies investigating the effects of 5-HT_3 receptor antagonists in ameliorating depression and anxiety associated with other forms of diabetes
- Studies investigating the expression of 5-HT_3 receptors in discrete brain regions during T1DM state that will help in strengthening the role of 5-HT_3 receptors in this pathogenesis
- Pharmacological screening of other drug candidates with potential 5-HT_3 receptor antagonistic action in depression associated with T1DM, and other forms of diabetes

- Research investigating the role of specific forms of 5-HT_3 receptors in the pathogenesis of depression associated with T1DM
- Studies investigating safety and toxicity profile of the tested drug candidates
- Experiments identifying other biochemical mechanisms, such as effects of candidates on immunological responses and downstream signaling
- Further studies characterizing cellular signaling cascades, targeted 5-HT_3 receptors, are necessary to better understand the molecular pathways governing the therapeutic action of 5-HT_3 receptor antagonists
- Investigating studies on selective 5-HT_3 receptor antagonists for their potential anti-diabetic activity.

Such knowledge may yield valuable insight into mechanisms underlying recovery from depression and related disorders such as anxiety associated with T1DM, while opening up novel prospects for their treatment and prevention.

9.3 Limitations of current study

- In the present study, the antidepressant and anxiolytic action of drugs acting on specific isoforms of 5-HT_3 receptors was not taken into consideration due to the lack of iso-form specific antagonists.
- Long-term effects of drug candidates on depression and anxiety associated with T1DM as well as toxicity sutides of the tested compounds were not investigated due to time constraints and availability of animals.
- Effect of oral administration of OND, **4i** and **6z** was not assayed (although oral intake of drugs is more convenient), due to studies involving novel candidates.
- Side effects of the test drug candidates were not investigated, even though no obvious untoward effects were detected at tested doses.

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List of Publications and Presentations

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- **Gupta D**, Prabhakar V, Radhakrishnan M. $5HT_3$ receptors: Target for new antidepressant drugs. *Neurosci Biobehav Rev.* 2016;64:311-325. PMID: 26976353.
- **Gupta D,** Thangaraj D, Radhakrishnan M. A novel $5HT_3$ antagonist 4i (N-(3-chloro-2-methylphenyl) quinoxalin-2- carboxamide) prevents diabetes-induced depressive phenotypes in mice: Modulation of serotonergic system. *Behav Brain Res.* 2016;297:41-50. PMID: 26454237
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- **Gupta D**, Radhakrishnan M, Kurhe Y. Antidepressant effects of insulin in streptozotocin induced diabetic mice: modulation of brain serotonin system. *Physiol Behav.* 2014;129:73-78. PMID: 24582678
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- Prabhakar V, **Gupta D,** Kanade P, Radhakrishnan M. Diabetes-associated depression: The serotonergic system as a novel multifunctional target. *Indian J Pharmacol.* 2015;47:4-10. PMID: 25821303

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- **Gupta D**, Radhakrishnan M. 6z, a novel $5HT_3$ receptor antagonist reverses depressive phenotype in diabetic mice by normalizing serotonin deficits and HPA-axis hyperactivity. 14th Meeting on Asian-Pacific Society for Neurochemistry, *Malaysia*, 27-30 August, 2016.
- **Gupta D**, Radhakrishnan M. 25th ISN-APSN Biennial Meeting organized in conjunction with ANS*, Cairns, Australia*, 23-27 August, 2015.
- **Gupta D**, Radhakrishnan M. 10th, The American Association of Pharmaceutical Scientists (*AAPS*) National University of *Singapore* (NUS), NUS, *Singapore,* 8-9 April, 2015.
- **Gupta D**, Radhakrishnan M, Kurhe Y, Thangaraj D. International Conference on Pharmacy and Pharmacology (ICPP), Holiday Inn*, London, UK*. 28-29, September, 2014
- **Gupta D**, Radhakrishnan M. 3rd world Congress on Diabetes and Metabolism. Marriott Convention Center, *Hyderabad, India*. 24-26, September 2012.
- **Gupta D,** Devadoss T, Bhatt S, Mahesh R. Phramnext, AIPER*, M.P., India*. 28-29, April 2012. (Awarded young scientist and Best Poster).

Biography of Prof. R. Mahesh

Prof. Mahesh is currently working as Professor, Department of Pharmacy and Dean, Faculty Affairs in BITS, Pilani. He was awarded PhD (Medicinal Chemistry) in 1997 from BITS, Pilani. He has been involved in teaching and research for past more than two and a half decades. He has vast experience in the field of Molecular Modeling and Drug Design, Medicinal chemistry, Neuropharmacology and Clinical Pharmacy and Therapeutics. He has successfully completed several funded projects and some ongoing projects as Principal Investigator by UGC, DBT, DST and ICMR. He has guided eight PhD students and four students are pursuing. He has guided several postgraduate and undergraduate students on various projects. Several of his projects have won several awards in Academic Exhibitions. He has published several papers in peer reviewed international/national Journals and in conferences of international/national repute. He is life time member of Association of Pharmaceutical Teachers of India, Indian Pharmacological Society and Society of Neurochemistry, India.

Biography

Ms. Deepali Gupta has completed her Bachelor of Pharmacy from V.N.S. Institute of Pharmacy, Bhopal, affiliated to Rajiv Gandhi Technical University, Madhya Pradesh in 2008, Master of Pharmacy from BITS-Pilani, Pilani Campus, Rajasthan in 2011 and enrolled as PhD student in August, 2011. She served as lecturer in Pharmacy College in V.N.S. Institute of Pharmacy. She is the recipient of UGC-BSR fellowships during doctoral program. Her area of interest includes investigating pathophysiology of Psychiatric and Neurodegenerative disorders, and their novel therapeutic interventions. She has published 10 articles as first author from her research work, in well renowned international journals and presented 6 papers in international conferences. She has also received Best Poster Award, Young Scientist Award and several Travel Grants for her doctoral dissertation work. She is a life time member of International Society for Neurochemistry (ISN), International Brain Research Organization (IBRO) Women Committee.