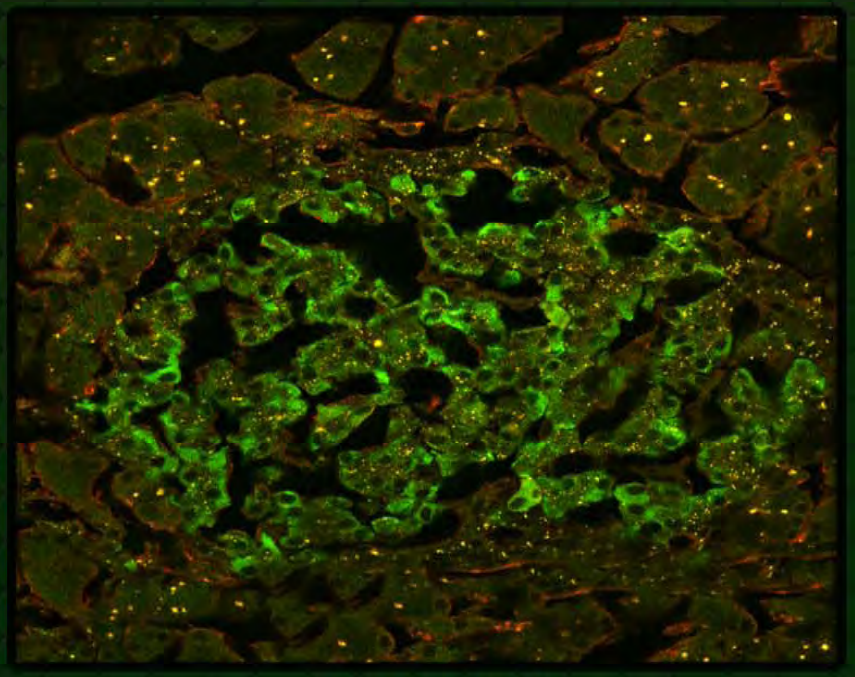
Ph.D. Thesis

Jayanarayanan S

December 2012

Glutamatergic NMDA and AMPA Receptors Functional Regulation in Streptozotocin Induced Diabetic Rats: Effect of Vitamin D_3 and Curcumin Supplementation



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GLUTAMATERGIC NMDA AND AMPA RECEPTORS FUNCTIONAL REGULATION IN STREPTOZOTOCIN INDUCED DIABETIC RATS: EFFECT OF VITAMIN D_3 AND CURCUMIN SUPPLEMENTATION

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CERTIFICATE

This is to certify that the thesis entitled **"Glutamatergic NMDA and AMPA Receptors Functional Regulation in Streptozotocin Induced Diabetic Rats: Effect of Vitamin D_3 and Curcumin Supplementation"** is a bonafide record of the research work carried out by **Mr. Jayanarayanan S,** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

Cochin – 682 022

(C. S. Paulose)

December 21, 2012

DECLARATION

I hereby declare that the thesis entitled **"Glutamatergic NMDA and AMPA Receptors Functional Regulation in Streptozotocin Induced Diabetic Rats: Effect of Vitamin D_3 and Curcumin Supplementation"** is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of Prof. C. S. Paulose, Director, Centre for Neuroscience, Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

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Jayanarayanan.S

Dedicated to my beloved family. . .

ABBREVIATIONS

1,25(OH)2D3	1α,25-dihydroxyvitamin D_3
5-HIAA	5-hydroxy indole - 3 acetic acid
5-HT	5-Hydroxy tryptamine
ACh	Acetylcholine
AD	Alzheimers disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine-5'-triphosphate
Bcl-2	B-cell lymphoma 2
bHLH	basic helix-loop-helix
B_{max}	Maximal binding
BSA	Bovine serum albumin
BAX	Bcl-2-associated X protein
Ca^{2+}	Calcium
CaBP	Calcium binding protein
CAT	Catalase
CICR	Ca^{2+}-induced calcium release
CNS	Central Nervous System
CSF	Cerebrospinal fluid
Ct	Crossing threshold
DA	Dopamine
DAG	Diacyl Glycerol

DEPC	Di ethyl pyro carbonate
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
EAAT2	Excitatory amino-acid transporter
EDTA	Ethylene diamine tetra acetic acid
EPI	Epinephrine
ER	Endoplasmic reticulum
FITC	Florescent isothiocyanate
GABA	Gamma amino butyric acid
GAD	Glutamate decarboxylase
GPCRs	G protein coupled receptors
GPxs	Glutathione peroxidase
GDH	Glutamate dehydrogenase
GLUT2	Glucose transporter type 2
GLUT3	Glucose transporter type 3
GLUT4	Glucose transporter type 4
GSH	Glutathione
HbA_{1c}	Glycated haemoglobin
HBSS	Hang's balanced salt solution
HIV	Human immunodeficiency virus
IDDM	Insulin-dependent diabetes mellitus
IGF	Insulin-like growth factor
iGluRs	Ionotropic Glutamate Receptor

INS	Insulin
KA	Kainate
K_d	Dissociation constant
Km	Michaelis constant
L-DOPA	L-3,4-dihydroxyphenylalanine
LTD	Long term depression
LTP	long-term potentiation
mGluRs	Metabotropic glutamate receptors
mRNA	Messenger ribonucleic acid
NE	Norepinephrine
NIDDM	Non-insulin-dependent diabetes mellitus
NMDA	N-methyl-D-aspartate
NOS	Nitrous Oxide Systems
P	Level of significance
Pdx1	Pancreatic duodenal homeobox 1
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Triton X- 100
PCP	Phencyclidine
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositide 3-kinases
PIP2	phosphatidyl inositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C

PP cells	Pancreatic polypeptide
QNB	Quinuclidinylbenzilate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SEM	Standard error of mean
Ser	Serine
SOD	Superoxide dismutase
STZ	Streptozotocin
Thr	Threonine
Tyr	Tyrosine
VDR	Vitamin D receptor
VGLUTs	vesicular glutamate transporters
WHO	World Health Organization

CONTENTS

Introduction

Diabetes mellitus is a common metabolic disorder characterised by hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (DeFronzo, 2004). According to World Health Organisation (WHO), diabetes is currently growing at a fast rate throughout the world and is the 9th leading cause of global mortality. Increased risk for diabetes is primarily associated with age, ethnicity, family history of diabetes, smoking, obesity and physical inactivity. Diabetes related complications including cardiovascular disease, nephropathy, neuropathy, blindness and lower-extremity amputation are a significant cause of increased morbidity and mortality among people with diabetes.

The most common form of diabetes is type 2 diabetes, in which resistance to insulin is accompanied by an inadequate compensation in the secretion of insulin. Type 1 diabetes is caused by an absolute shortage in the production of insulin due to the destruction of pancreatic β cells (American Diabetes Association, 2002). This type of diabetes, which was previously defined as Insulin Dependent Diabetes Mellitus, is not as common as type 2 diabetes; only 5–10% of the patients with diabetes have type 1 diabetes.

A great number of anatomical, functional and biochemical alterations have been described in the nervous system of diabetic animals (Tomlinson *et al.*, 1992; Ozturk *et al.*, 1996). These variety of alterations (generally named as diabetic neuropathy) affects the brain, spinal cord and peripheral nerves. Diabetic patients have increased risk for developing central nervous system (CNS) dysfunctions like impaired learning and memory, neurodegeneration and loss of synaptic plasticity. In the CNS, diabetes reduces brain weight and neocortical volume, which is associated with a reduction of the number of cortical neurons (Jakobsen *et al.*, 1987).

The CNS neurotransmitters play an important role in the regulation of glucose homeostasis. These neurotransmitters mediate rapid intracellular communications not only within the CNS but also in the peripheral tissues.

Neurotransmitters have been reported to show significant alterations during hyperglycaemia resulting in altered functions causing neuronal degeneration (Bhardwaj *et al.,* 1999). Chronic hyperglycaemia during diabetes mellitus is a major initiator of diabetic micro-vascular complications like retinopathy, neuropathy and nephropathy (Sheetz & King, 2002; Monnier *et al.,* 2009). The autonomic nervous system plays a prominent role in the regulation of insulin secretion. It has been proposed that neuronal afferent signals delivered to the pancreatic β cell through the vagus are responsible for the cephalic phase of insulin secretion (Lausier *et al.,* 2010). In pancreatic β-cells, IP3 mobilizes Ca^{2+} from intracellular stores, resulting in an elevation of the intracellular concentration of Ca^{2+} and allowing activation of Ca^{2+}/calmodulin. Diacylglycerol (DAG) on the other hand, activates Protein kinase C (PKC) (Nishizuka, 1995; Renstrom *et al.,* 1996; Shawl *et al.,* 2009). PKC, like Ca^{2+}/calmodulin, accelerates exocytosis of insulin granules (Nakano *et al.,* 2002). Chronic hyperglycaemia is strongly implicated in the development of vascular complications of diabetes, including gradual damage to the CNS (Brands *et al.,* 2004)

Glutamate is the primary excitatory neurotransmitter of the CNS and glutamate receptors play important roles in many CNS functions, including learning, memory, development and synaptogenesis (Collingridge & Lester 1989). Glutamate receptors have been classified into two major categories: ionotropic receptors (iGluRs) and metabotropic receptors (mGluRs). The ionotropic GluRs possess intrinsic cation-permeable channels and include N-methyl-d-aspartate (NMDA), kainate (KA) and α--amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Excessive stimulation of glutamate receptors can be neurotoxic, a phenomenon known as excitotoxicity that causes neuronal damage (Gill & Pulido, 2001). Glutamic acid decarboxylase (GAD) is the rate limiting enzyme in the decarboxylation of glutamate to GABA; deactivation of GAD can trigger synaptic glutamate overload (Roberts & Kuriyama, 1968). Various studies reported the glutamate excitotoxicity mediated neuronal damage in diabetic

complications like diabetic retinopathy and diabetic neuropathy (Chabot *et al.*, 1997; Delyfer *et al.*, 2005; Gowda *et al.*, 2011).

Clearance of extracellular glutamate from the synaptic cleft is carried out by specific high-affinity sodium-dependent excitatory amino acid transporters (EAAT), which can be modulated by the redox status of the cells (Trotti *et al.*, 1998). Therefore, higher oxidative stress associated with loss of activity of antioxidant enzymes and glutamate transporters aggravate cell damage in the brain. The astrocytic sodium dependent glutamate transporters - glutamate aspartate transporter EAAT1 (GLAST) and EAAT2 (GLT-1) stabilize the concentration of extracellular excitatory amino acids and are responsible for removal of more than 90% of the extracellular glutamate. This buffers the glutamate level, thus avoiding excessive stimulation of neuronal glutamate receptors and protecting neurons from glutamate toxicity (Dunlop, 2006).

Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of diabetes mellitus (Brownlee 2001; Rosen *et al.*, 2001; Bonnefont-Rousselot 2002; Ceriello 2003; Yang *et al.*, 2011). Free radicals are formed disproportionately in diabetes by glucose oxidation, non-enzymatic glycation of proteins and the subsequent oxidative degradation of glycated proteins. The increase in the level of reactive oxygen species (ROS) in diabetes could be due to their increased production and/ or decreased destruction by nonenzymic and enzymic catalase (CAT), reduced glutathione (GSH), and superoxide dismutase (SOD) antioxidants (Chen *et al.*, 2012). Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation and development of insulin resistance (Hansen *et al.*, 1999; Urakawa *et al.*, 2003; Furukawa *et al.*, 2004; Houstis *et al.*, 2006). Therefore, treatment with antioxidants or over expression of antioxidant enzymes can, at least partially, prevent oxidative stress induced insulin resistance. Glutathione peroxidases (GPxs) are members of the family of antioxidant enzymes that scavenge hydrogen peroxide in the presence of reduced GSH (Drevet *et al.*, 2006).

Nutritional therapy is important in preventing diabetes, managing existing diabetes and preventing or at least slowing the rate of development of diabetic complications. Antioxidant agents from diet have a significant therapeutic influence on various neurodegenerative disorders associated with diabetes and oxidative stress (González-Burgos & Gómez-Serranillos, 2012). The significance of Curcuma longa Linn (Turmeric) in health and nutrition has changed considerably since the discovery of the anti-oxidant properties of naturally occurring phenolic compounds. Curcuminoids, the active polyphenols of *C.longa* rhizomes, contain curcumin, demethoxycurcumin and bis-de-methoxycurcumin, which were shown to have a wide spectrum of pharmacological actions (Chattopadhyay *et al.,* 2004). Curcumin, a yellow pigment from *Curcuma longa*, is a major component of turmeric and exhibits powerful anti-oxidant, anti-diabetic, anti-inflammatory and anti-cancer properties (Miller, 2001; Surh *et al.,* 2001; Reddy *et al.,* 2010; Meng *et al.,* 2012). In diabetes, curcumin was shown to perform a multitude of activities including reduction in glycemic level, elevation in antioxidant status of pancreatic β-cells and attenuation of the mechanisms involved in diabetic encephalopathy (Arun & Nalini 2002; Kuhad & Chopra, 2007). A number of experimental studies have demonstrated curcumin's antioxidant and neuroprotective potential (Bala *et al.,* 2006; Kuhad & Chopra, 2007; Huang *et al.,* 2012). Studies from our lab showed that curcumin treatment ameliorates the altered muscarinic expression in the brain regions of diabetic rats (Peeyush *et al.,* 2011).

Vitamin D is made in the epidermis from 7-dehydrocholesterol in response to sunlight exposure and is obtained from the diet (Lips 2001; Mathieu *et al.,* 2005; Holick 2005; Hart 2012). The major dietary sources of vitamin D are oily fish, eggs and meat. Even in countries where certain foods are fortified with vitamin D, dietary intake of vitamin D alone is usually insufficient to maintain adequate serum levels of 25-hydroxyvitamin D (Nowson *et al.,* 2002; Holick *et al.,*2003). The biological actions of Vitamin D_3 are mediated through binding to the vitamin D receptor (VDR), a member of the nuclear steroid hormone receptor family. An increased prevalence of diabetes has been described in vitamin D-

deficient individuals (Chiu *et al.,* 2004). The VDR can be viewed as a master regulator of transcription. VDRs are present in pancreatic β-cells and vitamin D is essential for normal insulin secretion (Jessica *et al.,* 2010). An increased prevalence of type 2 diabetes has been described in vitamin D-deficient individuals (Boucher *et al.,* 1995; Isaia *et al.,* 2001; Chiu *et al.,* 2004).

The present study was designed to investigate the protective effect of curcumin and vitamin D_3 in the functional regulation of glutamatergic NMDA and AMPA receptors in streptozotocin (STZ) induced diabetic rats. Alterations in glutamatergic neurotransmission in the brain were evaluated by analyzing the glutamate content, glutamate receptors - NMDA and AMPA receptors binding parameters and gene expression, GAD and GLAST gene expression. Immunohistochemistry studies using confocal microscope were carried out to confirm receptor density and gene expression results of NMDA and AMPA receptors. The role of glutamatergic receptors in pancreas was studied using the following parameters; glutamate content, GLAST expression, glutamate receptors - NMDA and AMPA receptor binding and gene expression. Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of diabetes. In the present study SOD assay and GPx gene expression were done to evaluate the activity of antioxidant enzymes in the brain regions and pancreas. NeuroD1 and Pdx1 gene expression were performed in pancreas of experimental rats to evaluate pancreatic islet survival. Gene expression profiles of caspase 8, Bax, and Akt in brain regions and pancreas were studied to understand the possible mechanism behind curcumin and vitamin D_3 mediated neuroprotection and islet survival. Gene expression studies of vitamin D_3 receptor localisation in the pancreas was done to understand the mechanism of vitamin D_3 in insulin secretion. Curcumin and vitamin D_3 mediated insulin secretion via Ca^{2+} release were studied using confocal microscope.

OBJECTIVES OF THE PRESENT STUDY

1. To study the anti-hyperglycemic activity of curcumin and vitamin D_3 in STZ-induced diabetic rat model.

2. To measure the circulating insulin concentration of control, diabetic, insulin, curcumin and vitamin D_3 treated diabetic rats.

3. To quantify glutamate content in the cerebral cortex, hippocampus, brain stem, cerebellum and pancreas of experimental rats.

4. To study GAD gene expression in the cerebral cortex, hippocampus, brain stem and cerebellum of experimental rats using Real-Time PCR.

5. To study the transport of glutamate using GLAST gene expression in the cerebral cortex, hippocampus, brain stem, cerebellum and pancreas of experimental rats using Real-Time PCR.

6. To study AMPA and NMDA receptors binding parameters in cerebral cortex, hippocampus, brain stem, cerebellum and pancreas of experimental rats.

7. To study NMDA R1, NMDA 2B, AMPA (GluR2), AMPA (GluR4) receptor subunits gene expression in cerebral cortex, hippocampus, brain stem and cerebellum of experimental rats using Real-Time PCR.

8. To study AMPA (GluR2), AMPA (GluR4) receptor subunits gene expression in pancreas of experimental rats using Real-Time PCR.

9. To measure the second messenger IP3 levels in the cerebral cortex, hippocampus, brain stem, cerebellum and pancreas of experimental rats.

10. To study antioxidant potential of curcumin and vitamin D_3 using SOD assay and GPx gene expression in cerebral cortex, hippocampus, brain stem, cerebellum and pancreas of experimental rats.

11. To study the expression of NMDA R1, NMDA 2B, AMPA (GluR2), AMPA (GluR4) receptor subunits by immunofluorescent specific antibodies in the brain slices of experimental rats using confocal microscope.

12. To study the gene expression of apoptotic factors Bax and caspase 8 in the cerebral cortex, hippocampus, brain stem, cerebellum and pancreas of experimental rats using Real-Time PCR.

13. To study the gene expression of anti-apoptotic factor Akt-1 in the cerebral cortex, hippocampus, brain stem and cerebellum of experimental rats using Real-Time PCR.

14. To study the gene expression of pancreatic β cell survival transcription factors Pdx-1 and NeuroD1 in experimental rats using Real-Time PCR.

15. To study the expression of insulin, AMPA (GluR2), AMPA (GluR4), IP3 receptor and vitamin D_3 receptor in the pancreatic islets of experimental rats using confocal microscope.

16. To study the calcium release in pancreatic β cells of curcumin and vitamin D_3 treated streptozotocin induced diabetic rats.

Literature Review

Diabetes mellitus is the most common endocrine disorder characterized by increased blood glucose levels resulting from defective insulin secretion, resistance to insulin action or both. Diabetes is associated with long term complications and affects eyes, kidneys, blood vessels, heart, and nerves (Gispen & Biessels, 2000; Northam *et al*., 2006; Ametov & Kulidzhanian, 2012.). The latest World Health Organization statistics have estimated that more than 346 million people worldwide have diabetes. This number is likely to more than double by 2030 without intervention. Almost 80% of diabetes deaths occur in low- and middle income countries.

There are two main forms of diabetes (Zimmet *et al*., 2001), type 1 and type 2 diabetes. In type 1 diabetes, the body does not produce insulin, and daily insulin injections are required. Type 1 diabetes is usually diagnosed during childhood or early adolescence and it affects about 1 in every 600 children. Type 2 diabetes is the result of failure to produce sufficient insulin and insulin resistance. Elevated blood glucose levels are managed with reduced food intake, increased physical activity, and eventually oral medications or insulin.

History of Diabetes

The earliest description of diabetes was documented in the writings of Hindu scholars as long as in 1500 BC. They had already described *"a mysterious disease causing thirst, enormous urine output, and wasting away of the body with flies and ants attracted to the urine of people"* The term diabetes was probably coined by Apollonius of Memphis around 250 BC, which literally meant "to go through" or siphon as the disease drained more fluid than a person could consume. Later on, the Latin word "mellitus" was added because it made the urine sweet (MacCracken & Puzzles 1997). Early research linked diabetes to glycogen metabolism, and the islet cells of pancreas were discovered by Paul Langerhans, a

young German medical student. In 1916, Sharpey-Shafer of Edinburgh suggested that a single chemical was missing from the pancreas and proposed its name as “insulin.” The term insulin originates from the word *Insel*, which is German for an islet or island. EL Scott and Nikolae Paulesco were successful in extracting insulin from the pancreas of experimental dogs. The key breakthrough, though, came from the Toronto University with the discovery of insulin in 1921 by FG Banting and CH Best (Bliss 1982).

Epidemiology of Diabetes

The global increase in the prevalence of diabetes is due to population growth, aging, urbanisation and an increase of obesity and physical inactivity. The primary determinants of the epidemic are the rapid epidemiological transition associated with changes in dietary patterns and decreased physical activity (Goldberg & Mather, 2012). The number of people with diabetes is increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity. In globally estimated diabetes prevalence for 2011 is 366 million and is expected to affect 552 million people by 2030. The International Diabetes Federation (IDF) estimates that in 2010 the five countries with the largest numbers of people with diabetes are India, China, the United States, Russia and Brazil. The first national study on the prevalence of type 2 diabetes in India was done between 1972 and 1975 by the Indian Council Medical Research (ICMR, New Delhi) (Ahuja, 1979). India has the world's largest diabetes population, it is estimated that in India 50.8 million people living with diabetes followed by China with 43.2 million. Most familiar to the general public are type 1 diabetes (previously known as juvenile-onset, insulin dependent diabetes mellitus, IDDM), type 2 diabetes (formerly known as maturity-onset diabetes, noninsulin dependent diabetes mellitus, NIDDM) and gestational diabetes. Type 2 diabetes is by far the most common, accounting for 85–95% of all cases of diabetes.

Pancreas

Pancreas is an essential organ important for digestion and glucose homeostasis in higher organism. Malfunction of the pancreas results in several debilitating diseases such as diabetes, pancreatitis and pancreatic cancer. The mature pancreas of higher vertebrates and mammals comprises two major functional units: the exocrine pancreas, which is responsible for the production of digestive enzymes to be secreted into the gut lumen, and the endocrine pancreas, which has its role in the synthesis of several hormones with key regulatory functions in food uptake and metabolism. The exocrine portion constitutes the majority of the mass of the pancreas, and contains only two different ell types, the secretory acinar cells and the ductular cells. An acinar cell produces various digestive enzymes like amylase, proteases nuclease etc and duct cells that transport these enzymes in to the intestine. The endocrine portion, which comprises only 1–2% of the total mass, contains five different cell types, which are organized into mixed functional assemblies referred to as the islets of Langerhans. The endocrine cells produce indispensable hormones such as insulin, glucagon, somatostatin and pancreatic polypeptide, which are crucial to the optimum functioning of body metabolism. The pancreas is well innervated by autonomic nerves rich in different types of neuropeptides including vasoactive intestinal polypeptide and neuropeptide Y; galanin, calcitonin-gene-related-peptide, cholecystokinin and leucine-enkephaline (Adeghate *et al.*, 2001, Joost, 2008). In addition to the presence of neuropeptides, neurotransmitters such as serotonin, GABA or neurotransmitter-regulating enzymes such as tyrosine hydroxylase and dopamine hydroxylase have been identified in the pancreas (Adeghate & Donáth 1990; Adeghate & Ponery 2001).

The islets of Langerhans, which are embedded in the exocrine pancreatic tissue, are known to secrete three hormones: insulin, glucagon and somatostatin. In 1869, a medical student named Paul Langerhans described systems of cells in the pancreas which he thought were lymph glands. Islets constitute about 2% by weight of the adult human pancreas and are multicellular microorgans (Bonner-

Weir, 2005). In descending order of both cell number and cell mass, islet cell types are β (insulin secreting, 70–80%), α (glucagon secreting, 15–20%), PP (pancreatic polypeptide secreting, 15–20%), δ (somatostatin secreting, 5–10%), and the recently discovered ghrelin-secreting cells ghrelin, 1%; (Wierup *et al*., 2002).

Glucagon

Pancreatic α -cells were discovered in 1907 as histologically distinct cells from the β-cells of the islet of Langerhans (Lane 1909). Glucagon-containing cells constitute about 28% of the total number of endocrine cells in a normal pancreatic islet (Adeghate *et al.,* 1997). They are located in the periphery of the islets in normal animals. However, in STZ-diabetic animals many glucagon- positive cells are seen scattered within the central portion of the islets (Adeghate & Ponery 2003).

The history of glucagon begins with that of insulin. In 1921, when F. Banting and C. Best tested their first pancreatic extracts in depancreatized dogs, they observed that insulin-induced hypoglycemia was preceded by a transient, rather mild hyperglycemia, and they thought that this unwanted effect was due to epinephrine release (Best 1972). Murlin *et al.,* 1923 must be credited with the discovery of glucagon, because they suggested that the early hyperglycemic effect of the pancreatic extracts was due to a contaminant with glucogenic properties that they also proposed to call “glucagon” or the mobilizer of glucose. In a classical paper published in 1948. Sutherland and de Duve established the α-cells of the pancreas as being the source of glucagon. Glucagon-containing cells constitute about 28% of the total number of endocrine cells in a normal pancreatic islet (Adeghate & Donáth 1991; Quesada *et al*., 2008).

Glucagon is a 29 amino acid peptide hormone processed from proglucagon. Proglucagon is expressed in various tissues (e.g., brain, pancreas, and intestine) and is proteolytically processed into multiple peptide hormones in a

tissue-specific fashion. Glucagon acts via a seven transmembrane G protein-coupled receptor consisting of 485 amino acids (Jelinek *et al.*, 1993). To date, glucagon binding sites have been identified in multiple tissues, including liver, brain, pancreas, kidney, intestine, and adipose tissues (Burcelin *et al*., 1996; Christophe, 1996). Glucagon is released into the bloodstream when circulating glucose is low. The main physiological role of glucagon is to stimulate hepatic glucose output, thereby leading to increases in glycemia. This provides the major counter regulatory mechanism for insulin in maintaining glucose homeostasis *in vivo*. There is ample evidence suggesting that glucagon plays an important role in initiating and maintaining hyperglycemic conditions in diabetic animals and humans. Insulin and glucagon are the key regulatory hormones for glucose homeostasis. The absolute levels and, even more so, the ratios of the two hormones are tightly regulated *in vivo*, depending on nutritional status. It has been reported that the absolute levels of glucagon or the ratios of glucagon to insulin are often elevated in various forms of diabetes in both animal and human subjects (Burcelin *et al.*, 1996; Semenchenko *et al.*, 2012).

Insulin

Insulin positive cells are the most numerous cell types in the normal pancreas. They are located in both the central and peripheral parts of the islet and account for about 60 - 70% of the total cell population in an islet of Langerhans (Adeghate & Ponery 2003). The pancreatic β-cell secretes insulin in response to elevated glucose levels and also responds to other substances such as glucagon and acetylcholine. Insulin responses to intravenous glucose are time-dependent and referred to as first- and second-phase responses

The endocrine pancreas is richly innervated, but the abundance and organisation of these innervations are highly variable between species (Kobayashi & Fujita, 1969). Most of the nerve fibers enter the pancreas along the arteries (Miller, 1981; Woods & Porte, 1974). Unmyelinated nerve fibers are found in the

neighborhood of all islet cell types at the periphery and within the islet. At some distance from the islets, glial Schwann cells often form a thin sheet around nerve fibers on their travel toward and within the islet. In the vicinity of islet cells, however, it is not rare to see some nerve fibers lacking this glial protection and coming close to or ending blindly 20–30 nm from the endocrine cells (Legg, 1967; Watari, 1968; Kobayashi & Fujita, 1969; Radke & Stach, 1986).

The biological effects of insulin in classical insulin target tissues, such as skeletal muscle, fat and liver are glucose uptake, regulation of cell proliferation, gene expression and the suppression of hepatic glucose production. Insulin decreases hepatic glucose production and increases glucose entry into muscle and fat cells. In the post-absorptive state, the majority of total body glucose disposal takes place in insulin independent tissues. Approximately 50% of all glucose use occurs in the brain, which is insulin-independent (Grill, 1990). Another 25% of glucose disposal occurs in the splanchnic area (liver plus gastrointestinal tissues) which is also insulin independent. The remaining 25% of glucose use in the post-absorptive state takes place in insulin-dependent tissues, primarily muscle and to a lesser extent adipose tissue. Approximately 85% of endogenous glucose production is derived from the liver and the remaining 15% is produced by the kidney (Krysiak *et al.*, 2012).

Insulin is secreted from the pancreatic β cells in response to various stimuli like glucose, arginine, sulphonylureas though physiologically glucose is the major determinant. Various neural, endocrine and pharmacological agents can also exert stimulatory effect. Glucose is taken up by beta cells through GLUT-2 receptors. After entering the beta cell, glucose is oxidized by glucokinase, which acts as a glucose sensor (Ait-Lounis *et al.*, 2010). Glucose concentration below 90 mg/dl does not cause any insulin release. At such sub-stimulatory glucose concentrations, K^+ efflux through open K_{ATP} channels keeps the pancreatic β cell membrane at a negative potential at which voltage-gated Ca^{2+} channels are closed (Saisho *et al.*, 2012). As there is increase in plasma glucose, glucose uptake and

metabolism by the pancreatic β cell is enhanced. Rise in ATP concentration result in closure of K_{ATP} channels, leading to a membrane depolarization, opening of voltage-gated Ca^{2+} channels, Ca^{2+} influx, a rise in intracellular calcium concentration, and ultimately exocytosis of insulin granules (Pinho *et al.,* 2010).

The physiological effects of insulin are mediated by its cell surface receptor, a α2β2 transmembrane glycoprotein with intrinsic protein tyrosine kinase activity (Ebina *et al.*, 1985). Binding of insulin to the extracellular α -chains results in auto-phosphorylation of specific tyrosine residues in the portion of the β chains: two in the juxtamembrane region, three in the kinase (catalytic) domain, and two in the C-terminal tail (Feener *et al.*, 1993; Kohanski, 1993; Saisho *et al.*, 2012). Autophosphorylation of Tyr1158, Tyr1162 and Tyr1163 diphosphate (AMP-PNP) in the activation loop (A-loop) of the kinase domain is critical for stimulation of kinase activity and function (Rosen *et al.*, 1983).

As recently as 10 years ago, the brain was described as "an insulin insensitive organ" in medical textbooks. Evidence for the presence of insulin and its receptors in the CNS has challenged that notion (Ferrannini *et al.,* 1999; Schulingkamp *et al.,* 2000). Insulin is readily transported in to the CNS across the blood–brain barrier by a saturable, insulin receptor-mediated transport process (Baskin *et al.,* 1987; Banks *et al.,* 1997). The raising of the peripheral insulin concentration acutely increases the concentration in the brain and CSF, whereas prolonged peripheral hyperinsulinemia downregulates blood–brain barrier insulin receptors and reduces insulin transport into the brain (Wallum *et al.,* 1987). Insulin receptors are located on the synapse of both neurons and astrocytes (Rulifson *et al.,* 2002). The localisation of insulin receptors in the hippocampus and medial temporal cortex in rats is consistent with evidence that insulin influences memory (Park *et al.,* 2000).

Diabetes and central nervous system

The brain is not usually thought to be a target of chronic diabetes complications, but new research has shown that the disease has particular effects on the CNS. These include impaired learning and memory, neurodegeneration and loss of synaptic plasticity. Most drug discovery efforts aimed at diabetes target insulin action in peripheral tissues. There is evidence that there is substantial overlap between the CNS circuits that regulate energy balance and those that regulate glucose levels, suggesting that their dysregulation could link obesity and diabetes (Myers & Olson, 2012).

CNS complications can include stroke and possibly cognitive impairment (Lin *et al.*, 2010). Many studies both in type I and type II diabetic subjects have found significant impairment of various neurophysiological parameters (Meneilly *et al.,* 1993). These neuropsychological changes are often accompanied with objective electrophysiological evidence of delayed conduction velocity and data processing time in the central nervous system (Khardori *et al* 1986). Persistent blood glucose elevation contributes to atherosclerosis that impairs blood flow to the brain. Individuals with glycosylated haemoglobin (HbA_{1c}) levels (a test that indicates blood glucose levels over the previous 3 months) greater than 7% are nearly three times as likely to have a stroke compared with people who have an HbA_{1c} level less than 5% (Myint *et al.,* 2007). Other CNS complications may result from changes in blood–brain barrier or transport functions of the cerebral microvasculature (Mooradian 1997). Such damage might be associated with vascular dementia. Studies also suggest that diabetics are at greater risk of depression than non-diabetics (Lin *et al.*, 2010).

Brain neurotransmitter changes during diabetes

Neurotransmitters have been reported to show significant alterations during hyperglycemia resulting in altered functions causing neuronal degeneration. A significant increase in the catecholamine contents and activity of

metabolising enzymes has been reported in experimental diabetes (Gupta *et al*., 1992; Donato, 2012). Norepinephrine (NE) has been reported to increase in several brain regions during diabetes (Tassava *et al*., 1992; Chen & Yang, 1991), but a significant decrease in NE has been reported in hypothalamus (Ohtani *et al*., 1997) pons and medulla (Ramakrishna & Namasivayam, 1995). Epinephrine (EPI) levels were significantly increased in the striatum, hippocampus and hypothalamus of diabetic rats and these changes were reversed to normal by insulin treatment (Ramakrishna & Namasivayam, 1995). STZ induced diabetes and acute insulin deficiency were demonstrated to result in increased content of EPI in the suprachiasmatic nucleus. In addition to this, a decreased turnover of dopamine in the ventromedial nucleus in diabetes was found to be reversed by insulin treatment (Oliver *et al*., 1989). These data indicate that experimental diabetes and acute insulin deficiency result in the rapid onset of detectable alterations in epinephrine and dopamine activity in specific hypothalamic nuclei. This leads to development of secondary neuroendocrine abnormalities, known to occur in diabetes. The dopamine content was increased in whole brain, (Lackovic *et al*., 1990; Chen & Yang, 1991) corpus striatum (Chu *et al*., 1986) cerebral cortex and hypothalamus of diabetic rats (Tassava *et al*., 1992; Ohtani *et al*., 1997). The plasma dopamine content was decreased in diabetic rats (Eswar *et al*., 2006). Serotonin (5-HT) content is increased in the brain regions and hypothalamic nuclei (Lackovic *et al*., 1990; Chen & Yang, 1991) but there are reports suggesting a decrease in brain 5-HT content during diabetes (Sandrini *et al*., 1997; Sumiyoshi *et al*., 1997; Jackson & Paulose, 1999). Brain tryptophan was also reduced during diabetes (Jamnicky *et al*., 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al*., 1993).

Role of neurotransmitters in insulin regulation & secretion

Acetylcholine

Acetylcholine (ACh) is one of the principal neurotransmitters of the parasympathetic system. Ach is a major neurotransmitter from autonomic nervous system, regulates the cholinergic stimulation of insulin secretion, through interactions with muscarinic receptors especially through vagal muscarinic and non-vagal muscarinic pathways (Greenberg & Pokol, 1994). Muscarinic M1 receptor subtype antagonist, pirenzepine inhibits cholinergic mediated insulin secretion (Iismaa *et al*., 2000). Acetylcholine agonist, carbachol, at low concentration (10^{-7} M) stimulated insulin secretion at 4 mM and 20 mM concentrations of glucose (Renuka *et al*., 2006).

Dopamine

Dopamine is reported to inhibit glucose stimulated insulin secretion from pancreatic islets (Tabeuchi *et al*., 1990). Eswar *et al*., (2006) reported that dopamine significantly stimulated insulin secretion at a concentration of 10^{-8} M in the presence of high glucose (20mM). Treatment with the dopamine precursor L-DOPA in patients with Parkinson's disease reduces insulin secretion upon oral glucose tolerance test (Rosati *et al.,* 1972). *In vitro* studies performed in isolated pancreatic islets suggested the participation of the D2R in insulin secretion (Rubi *et al.,* 2005). The uptake affinity and velocity of dopamine in synaptosomes decreased significantly during diabetes. The dopamine content was increased in the cerebral cortex and hypothalamus of diabetic rats (Shiimzu, 1991; Tassava *et al*., 1992; Ohtani *et al*., 1997). The altered turnover ratio in the limbic forebrain is reported to cause enhanced spontaneous locomotor activity in diabetic rats (Kamei *et al*., 1994) Isabel *et al.,* 2010 reported that the D2R plays an essential role in β-cell proliferation and insulin secretion adds a novel participant to the list of growth factors and hormones that control the fundamental and multifactorial process of glucose homeostasis. Dopamine D3 receptors are implicated in the control of blood glucose levels (Alster & Hillegaart, 1996). Dopamine D1 receptors have

also been reported to be present on pancreatic β -cells (Tabeuchi *et al.,* 1990). These clearly indicate the role of dopamine in the regulation of pancreatic function.

Gamma-Aminobutyric acid

Gamma-aminobutyric acid (GABA) is a major neurotransmitter in the CNS, where GABA produces fast inhibition in mature neurons primarily by activation of GABA A receptor (GABAAR), a hetero-pentameric Cl^- channel (Luscher & Keller, 2004). A large amount of GABA is also produced in the pancreatic islet (Okada *et al.,* 1976, Sorenson *et al.,* 1991). Pancreatic GABA is primarily produced by the β cell (Vincent *et al.,* 1983), in which GABA is stored in synaptic like micro-vesicles that are distinct from insulin containing large-dense core vesicles (Reetz *et al.,* 1991). In the pancreatic islet, GABA released from β-cells plays a critical role in the regulation of glucagon secretion from α-cells. Specifically, GABA activates $GABA_A$Rs in a-cells, sequentially leading to an influx of Cl^- and membrane hyperpolarization, and hence an inhibition of glucagon secretion. Studies demonstrated that $GABA_A$Rs are also expressed in the primary islet β-cells (Glassmeier *et al.,* 1998; Xu *et al.,* 2006) and insulin-secreting clonal β-cell lines (Dong *et al.,* 2006). Unlike in mature neurons and α cells, stimulation of $GABA_A$Rs in β-cells induces membrane depolarization, enhancing insulin secretion in the presence of physiological concentrations of glucose (Dong *et al.,* 2006; Braun *et al.,* 2010). Recent studies demonstrated that GABA, in cooperation with insulin, enhances the proliferation and survival of the β-cells through activation of the PI3-K/Akt pathway. Remarkably, GABA promotes β-cell regeneration and reverses diabetes in mouse models (Soltani *et al.,* 2011). The brain GABAergic mechanisms also play an important role in glucose homeostasis. Inhibition of central $GABA_A$ receptors increases plasma glucose concentration (Lang, 1995). $GABA_A$ receptors in brainstem have a regulatory role in pancreatic regeneration (Kaimal *et al*., 2007) Thus, any

impairment in the GABAergic mechanism in the CNS and/or in the pancreatic islets is important in the pathogenesis of diabetes.

Serotonin

Serotonin content is increased in the brain regions and hypothalamic nuclei (Lackovic *et al*., 1990; Chen & Yang, 1991), but there are reports suggesting a decrease in brain 5-HT content during diabetes (Sandrini *et al*., 1997; Jackson & Paulose, 1999; Deuschle, 2012). 5-HT is synthesized within β-cells (Richmond *et al.*, 1996), it is stored together with insulin in their secretory β-granules (Ekholm *et al.*, 1971), and it is co-released when pancreatic islets are stimulated with glucose (Smith *et al.*, 1999). Chu *et al*., (1986) has reported lower 5-HT levels in both hypothalamus and brainstem but not in corpus striatum. Insulin treatment brought about an increase in the cerebral concentration of 5-HIAA and accelerated the cerebral 5-HT turnover (Juszkiewicz, 1985). The 5-HIAA concentration was reported to be approximately twice as high as the controls regardless of duration of treatment. Brain tryptophan, the precursor of 5-HT, was also reduced in brain regions during diabetes (Jamnicky *et al*., 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al*., 1993). There was a significant increase in 5-HIAA observed at 2-6 hours after insulin administration (Kwok & Juorio, 1987).

Epinephrine and Norepinephrine

Epinephrine (EPI) and Norepinephrine (NE) are secreted by the adrenal medulla. NE is a principal neurotransmitter of sympathetic nervous system. These hormones inhibit insulin secretion, both *in vivo* and *in vitro* (Porte, 1967; Renstrom *et al*., 1996). Both β2- and α2-adrenoceptors are expressed in the islets. Noradrenaline has been shown to stimulate insulin and glucagon secretion through the β2-adrenergic receptors (Kuo *et al.*, 1973; Ahrén, 2000). At the same time, noradrenaline also interacts with α2-adrenoceptors, which results in the inhibition of insulin secretion and the stimulation of glucagon secretion (Ahrén, 2000).

Therefore, catecholamines may affect insulin secretion both as stimulators through β2-adrenoceptors and as inhibitors through α2-adrenoceptors (Ullrich & Wollheim, 1985). Studies reported that adrenaline did not inhibit insulin secretion in mice with a double knockout of α2A and α2C adrenoceptors, and that the inhibition of insulin secretion by adrenaline was partially reduced in mice with single knockout of these receptors (Peterhoff *et al.,* 2003). Transgenic mice with β-cell-specific overexpression of α2A adrenoceptors displayed reduced glucose-stimulated insulin secretion and impaired glucose tolerance (Devedjian *et al.*, 2000; Champaneri *et al.,* 2012).

Epinephrine exerts opposite effects on peripheral glucose disposal and glucose stimulated insulin secretion (Avogaro *et al*., 1996). NE and EPI - the flight and fright hormones are released in all stress conditions and are the main regulators of glucose turnover in strenuous exercise (Simartirkis *et al*., 1990). In severe insulin-induced hypoglycemia, a 15 to 40 fold increase of epinephrine plays a pivotal role in increasing glucose production independently of glucagon (Gauthier *et al*., 1980). It is already known that, when used in high doses *in vivo* or *in vitro*, EPI reduces the insulin response to stimulators (Malaisse, 1972). *In vitro* studies with yohimbine – α_2-adrenergic receptor antagonist, showed that the insulin secretion from the pancreatic islets increased significantly suggesting that when the alpha 2-adrenergic receptors are blocked, it enhances islet cell proliferation and insulin secretion (Ani *et al*., 2006). EPI and NE have an antagonistic effect on insulin secretion and glucose uptake (Porte *et al*., 1966). They also inhibit insulin stimulated glycogenesis through inactivation of glycogen synthase and activation of phosphorylase with consequent accumulation of glucose-6-phosphate. In addition, it has been reported that epinephrine enhances glycolysis through an increased activation of phospho-fructokinase. In humans, adrenaline stimulates lipolysis, ketogenesis, thermogenesis and glycolysis and raises plasma glucose concentrations by stimulating both glycogenolysis and gluconeogenesis. Adrenaline is, however, known to play a secondary role in the

physiology of glucose counter-regulation. Indeed, it has been shown to play a critical role in one pathophysiological state, the altered glucose counter-regulation in patients with established insulin-dependent diabetes mellitus (Cryer, 1997). The inhibitory effect of EPI upon insulin secretion induced by glucose was reported by Coore & Randle, (1964), who incubated pancreatic tissue from the rabbit. As judged by Malaisse *et al*., (1967) the inhibitory effect of EPI on glucose-induced insulin secretion is mediated through the activation of α-adrenoreceptors.

Glutamate

A growing body of evidence suggests that glutamate, the major excitatory neurotransmitter in the central nervous system, acts as a signaling molecule in peripheral tissues (Gill & Pulido, 2001; Skerry & Genever, 2001; Hinoi *et al.*, 2004). In the cells of the endocrine pancreas, glutamate is stored in glucagon or insulin containing granules (Yamada *et al*., 2001; Hoy *et al*., 2002; Hayashi *et al*., 2003) and, once secreted, might act extracellularly to regulate hormone secretion (Moriyama &Hayashi, 2003; Uehara *et al.*, 2004). In addition, glutamate has been implicated as a putative intracellular messenger coupling glucose metabolism to insulin secretion in cells (Maechler *et al.,* 2002; Becerril Ángeles *et al.,* 2010). The molecular mechanisms underlying the action of glutamate in the endocrine pancreas are only partially elucidated. The role of L-glutamate in insulin secretion has been robustly challenged (MacDonald & Fahien, 2000; Bertr *et al.*, 2002). An increase in intracellular L-glutamate concentration on addition of glucose (16.7 mmol/l) in rat islets was not observed in a key study (MacDonald & Fahien, 2000). Incubation with L glutamine (10 mmol/l) increased the L-glutamate concentration 10-fold but did not stimulate insulin release, leading the authors to cast doubt on the proposed role of L-glutamate. In a separate study, it was demonstrated that, on incubation with glucose, a significant increase in L-glutamate concentration occurred in depolarized mouse and rat islets (Bertr *et al.*, 2002).

Glutamate Receptors

Glutamate is the most prominent neurotransmitter in the body, being present in over 50% of nervous tissue. A large proportion of the glutamate present in the brain is produced by astrocytes through synthesis *de novo* (Hertz *et al*., 1999), but levels of glutamate in glial cells are lower than in neurons, 2–3 mM and 5–6 mM, respectively. During excitatory neurotransmission, glutamate-filled vesicles are docked at a specialized region of the presynaptic plasma membrane known as the active zone. Packaging and storage of glutamate into glutamatergic neuronal vesicles requires Mg^{2+}/ATP-dependent vesicular glutamate uptake systems, which utilize an electrochemical proton gradient as a driving force. Substances that disturb the electrochemical gradient inhibit this glutamate uptake into vesicles. The concentration of glutamate in vesicle reaches as high as 20–100 mM (Nicholls & Attwell, 1990). In brain tissue, low concentrations of glutamate and aspartate perform as neurotransmitters, but at high concentration these amino acids act as neurotoxins.

Glutamate receptors are divided into two main groups: the fast-acting ligand-gated ionotropic channels and the slower-acting metabotropic receptors. The ionotropic receptors are cation-specific ion channels, and are subdivided into three groups: α-amino-3- hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainite (KA) and N-methyl-D-aspartate (NMDA) receptor channels. Metabotropic glutamate (mGlu) receptors are G-protein coupled receptors (GPCRs) that have been subdivided into three groups, based on sequence similarity, pharmacology and intracellular signaling mechanisms. Group I mGlu receptors are coupled to PLC and intracellular calcium signaling, while group II and group III receptors are negatively coupled to adenylyl cyclase. Activation of these receptors is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), which are thought to underlie learning and memory. It appears however, that aspartate aminotransferase and glutaminase account for a majority of glutamate production in brain tissue (McGeer *et al*., 1987).

Glutamate functions as a fast excitatory transmitter in the mammalian brain. Glutamate triggers neuronal death when released in excessive concentrations by over excitation of its receptors (Vizi, 2000). Glutamate receptor activation and excitotoxicity has long been recognized as an upstream event in this cascade (Wieloch, 1985). In brain, glutamate accumulation is reported to cause neuronal degeneration (Berman & Murray, 1996; Budd & Nicholas, 1996; Atlante *et al*., 1997). The excitatory amino acid glutamate is the most prevalent transmitter in the brain; its effect on postsynaptic receptors is limited by uptake process (Erecinska, 1997) and by diffusion of glutamate from the cleft. The cellular uptake of Glu is driven by the electrochemical gradients of Na^+ and K^+ and is accompanied by voltage and pH changes. In nervous tissue, glutamate dehydrogenase (GDH) appears to function in both the synthesis and the catabolism of glutamate and perhaps in ammonia detoxification (Mavrothalassitis *et al*., 1988). The extracellular accumulation of glutamate results in neuronal death by activating ionotropic glutamate receptors sensitive to NMDA or AMPA kainite (Choi, 1988). Hypoglycemia is associated with increased glutamate release (Sandberg *et al*., 1986; Becerril Ángeles *et al.,* 2010) and conversely, glutamate toxicity is augmented by hypoglycemia (Novelli *et al*., 1988).

The majority of excitatory synapses are glutamatergic, in which glutamate transmits the signal through postsynaptic ionotropic NMDA, AMPA, and KA and metabotropic receptors (Bettler & Mulle, 1995). Glutamate is a fast excitatory transmitter in the CNS and has been shown, with GABA, to interact primarily with receptors in the synaptic cleft (Dingledine *et al*., 1999). Studies have shown that both ionotropic glutamate receptors and glutamate transporters are involved in oxygen-glucose deprivation-induced necrotic cell death in hippocampal slice cultures (Bonde *et al.,* 2005). The extracellular accumulation of glutamate results in neuronal death by activating ionotropic glutamate receptors sensitive to NMDA or AMPA–kainate (Choi, 1988). The presence of G protein-coupled glutamate receptors has been described and since 1991 (Conn & Pin, 1997), eight receptors

have been discovered and classified into three groups based on their linkage to second messenger systems and their pharmacology: group I acts *via* the phosphoinositol system and groups II and III inhibit adenylyl cyclase. In addition, the stimulation of receptors of these three groups directly influences voltage-gated Ca^{2+} and K^{+} channels through their G proteins, but their physiological correlate has not yet defined (Rondón *et al.,* 2010).

There are several reports of presynaptic localization of glutamate receptors and their involvement in transmitter release. The fact that NMDA releases glutamate (Pittaluga *et al*., 1996), DA (Kuo *et al*., 1998) and NE (Pittaluga & Raiteri, 1992) from axon terminals indicates that glutamate released is able to facilitate transmitter release via NMDA receptors (Barnes *et al*., 1994; Desai *et al*., 1994). Montague *et al*., (1994) suggested that glutamate and NE release from cortical synaptosomes was in correlation with NMDA induced production of nitric oxide (NO), an endogenous chemical that is able to inhibit basal membrane transporters, thereby increasing the concentration and life-span of transmitters (e.g., glutamate and NE) released into the extracellular space. The inhibition of neuronal NO synthase by 7-nitroindazole protects against NMDA mediated excitotoxic lesions but not against those evoked by AMPA or KA (Schulz *et al*., 1995).

The most consistent age-related change in the glutamatergic system is the loss of glutamate receptors. Significant decreases in the mRNA level of glutamate receptors were found in the aged cerebral cortex (Carpenter *et al*., 1992). Among different glutamate receptors, NMDA receptors are preferentially altered in the aged brain. Decrease in NMDA binding was shown in both rodents and mammalian brain (Wenk *et al*., 1991; Cohen & Muller, 1992). The mRNA level of both NR1 and NR2B subunits of the NMDA receptors have been shown to decrease preferentially in the aged cerebral cortex, whereas no age-related change was observed in the NR2A subunit (Magnusson, 2000). The modification of subunit expression alters the receptor composition of NMDA receptor in the aged

brain and lead to age-related changes in the binding properties of this receptor (Priestley *et al*., 1995; Gallagher *et al*., 1996) and/or physiological properties such as desensitization (Monyer *et al*., 1992). Binding studies revealed significant decrease in NMDA but not AMPA and kainate receptors (Tamaru *et al*., 1991). These findings support a significant loss of postsynaptic glutamatergic receptors, especially the NMDA subtype, in the aged brain.

NMDA receptors

NMDA receptors are excitatory receptors in neurons that play a fundamental role in neuronal development, synaptic transmission, and synaptic plasticity. The discovery of potent and selective agonists and antagonists has resulted in extensive information on the NMDA receptor-channel complex (Wood *et al*., 1990). It consists of four domains:- (1) the transmitter recognition site with which NMDA and L-glutamate interact; (2) a cation binding site located inside the channel where Mg^{2+} can bind and block transmembrane ion fluxes; (3) a PCP binding site that requires agonist binding to the transmitter recognition site, interacts with the cation binding site and at which a number of dissociative anesthetics PCP and ketamine, opiate N-allylnormetazocine (SKF-10047) and MK-801 bind and function as open channel blockers; and (4) a glycine binding site that appears to allosterically modulate the interaction between the transmitter recognition site and the PCP binding site. NMDA is allosterically modulated by glycine, a co-agonist whose presence is an absolute requirement for receptor activation (Russo *et al.,* 2008).

NMDA receptors form heterotetrameric channels containing two obligatory glycine-binding NR1 subunits and two other subunits, either from the NR2 subfamily (2A, 2B, 2C, 2D) or the NR3 subfamily (3A or 3B) (Premkumar & Auerbach 1997; Laube *et al.*,1998; Furukawa *et al.,* 2005; Ulbrich *et al.,* 2007). The non-NR1 subunits confer onto the receptor distinct physiological properties that lead to a diversity of NMDA receptor function (Cull-Candy & Leszkiewicz 2004). NR2 subunits bind glutamate and form NR1/NR2 receptors that require

glycine and glutamate for activation (Johnson & Ascher 1987). NR3 subunits bind glycine and NR1/NR3 receptors are gated by glycine alone; however, excitatory glycine receptors have not yet been observed in neurons expressing the NR3 subunit (Chatterton *et al.*, 2002, Yao & Mayer 2006; Ai *et al.*, 2010). Molecular cloning has identified to date cDNAs encoding NR1 and NR2A, B, C, D subunits of the NMDA receptor, the deduced amino acid sequences of which are 18% belonging to NR1 and NR2, 55% belonging to NR2A and NR2C or 70% belonging to NR2A and NR2B are identical. Site-directed mutagenesis has revealed that the NR2 subunit carries the binding site for glutamate within the N-terminal domain and the extracellular loop between membrane segments M3 and M4; whereas the homologous domains of the NR1 subunit carry the binding site for the co-agonist glycine. . It has been shown that the combination of NR1 with different NR2 subunits results in diverse electrophysiological and pharmacological responses. NR1 and NR2A are ubiquitous, NR2B occurs in the forebrain, NR2C in the cerebellum, with NR2D being the rarest. There is a binding place in the channel pore for Mg2+, and at resting membrane potential, Mg2+ is attached to this binding site, blocking ion flow through the channel (Cull-Candy *et al.*, 2001, Kalia *et al.*, 2008, Paoletti & Neyton, 2007)

During normal development, NMDA receptor-mediated calcium fluxes are necessary for the formation and plasticity of synaptic connections, but also to initiate apoptotic pathways that mediate physiologic synaptic pruning. Disorders at this level have been implicated with cognitive disabilities and mood disorders (Pilpel *et al.*, 2009; Gardiner, 2010) Ongoing NMDA receptor signalling remains critical in adult, and malfunctions underlie many brain disorders: over activation of NMDA receptors can cause excitotoxic neurodegeneration; insufficient activation underlies some forms of psychoses and cognitive deficits; and anomalous plasticity has been implicated in several forms of addiction, neuropathic pain, and behavioral disorders (Pittenger *et al.*, 2007; Kalivas *et al.*, 2009). Normal functioning of the NMDA receptor complex depends on a dynamic

equilibrium among various domain components. Loss of equilibrium during membrane perturbation causes the entire system to malfunction and result in abnormal levels of glutamate in the synaptic cleft (Olney, 1989). An important consequence of NMDA receptor activation is the influx of Ca^{2+} into neurons (MacDermott *et al*., 1986; Murphy & Miller, 1988; Holopainen *et al*., 1989, 1990). Collective evidence suggests that when the membrane is depolarized, the Mg^{2+} block is relieved and the receptor can be activated by glutamate. Activation of the NMDA receptor therefore requires the association of two synaptic events: membrane depolarization and glutamate release. This associative property provides the logic for the role of the NMDA receptor in sensory integration, memory function, coordination and programming of motor activity (Collingridge & Bliss, 1987; Lam *et al.,* 2010) associated with synaptogenesis and synaptic plasticity.

The NMDA channel is blocked in a use and voltage dependent manner by Mg^{2+}. This means that NMDA receptors are activated only after depolarization of the postsynaptic membrane by, for example, AMPA receptor activation, which relieves the voltage dependent blockade by Mg^{2+}. This biophysical property and their high Ca^{2+} permeability render NMDA receptors inherently suitable for their role in mediating synaptic plasticity underlying learning processes and development (Collingridge & Singer, 1990; Danysz *et al*., 1995; Russo *et al*., 2008). Similar to Mg^{2+}, uncompetitive NMDA receptor antagonists such as ketamine, dextromethorphan, memantine, phencyclidine (PCP) and (+) MK-801 [(+) 5-methyl-10, 11-dihydro-5*H*-dibenzocyclohepten-5, 10-imine maleate] block the NMDA channel in the open state, although the blocking kinetics and voltage of this effect depend on the antagonist (Rogawski, 1993; Parsons *et al*., 1998b).

To date, two major subunit families, designated NR1 and NR2, have been cloned. Various heteromeric NMDA receptor channels formed by combinations of NR1 and NR2 subunits are known to differ in gating properties, magnesium sensitivity, and pharmacological profile (Sucher *et al*., 1996; Parsons *et al*.,

1998b). The heteromeric assembly of NR1 and NR2C subunits, for instance, has much lower sensitivity to Mg^{2+} but increased sensitivity to glycine and very restricted distribution in the brain (Lam *et al.,* 2010). *In situ* hybridization has revealed overlapping but different expression profiles for NR2 mRNA. For example, NR2A mRNA is distributed ubiquitously like NR1, with the highest densities occurring in hippocampal regions and NR2B is expressed predominantly in forebrain but not in cerebellum, where NR2C predominates; NR2D is localized mainly in the brainstem (Moriyoshi *et al*., 1991; Monyer *et al*., 1992; Nakanishi, 1992; McBain & Mayer, 1994).

In addition to NR1 and NR2, the NR3A subunit has recently been discovered. This receptor subunit, previously termed chi-1, or NMDAR-L, is a relatively recently identified member of a new class in the ionotropic glutamate receptor family. It attenuates NMDA receptor currents when co-expressed with NR1/NR2 subunits in Xenopus oocytes but has no effect when tested with non-NMDA receptors or when expressed alone (Ciabarra *et al*., 1995; Sucher *et al*., 1995; Das *et al*., 1998; Lam *et al.,* 2010). Highest levels are present in the spinal cord, brainstem, hypothalamus, thalamus, CA1 field of the hippocampus and amygdala and this distribution remains the same throughout life. Genetic knockout of NR3A in mice results in enhanced NMDA responses and increased dendritic spines in early postnatal cortical neurons, suggesting that NR3A is involved in the development of synaptic elements by modulating NMDA receptor activity (Das *et al*., 1998).

AMPA receptors

AMPA receptors are also widely expressed in the mammalian CNS and mediate fast excitatory neurotransmission in response to glutamate binding (Palmer *et al.,* 2005). The cloning of the first AMPA receptor subunit in 1989 enabled the structural analysis of AMPA receptors and a detailed characterization of their physiology and pharmacology (Hollmann *et al*., 1989; Ai *et al.,* 2010). AMPA receptor composed of different combinations of GluR1, GluR2, GluR3,

and GluR4 subunits. These subunits have a modular organization (Mayer, 2006; Sobolevsky *et al*., 2009). There is a large extracellular amino terminal domain that is involved in receptor assembly. All AMPA receptors are tetrameric combinations of the four subunits. While homomeric receptors are functional, native AMPA receptors are believed to be heteromers. Upon forming a tetrameric complex of GluR1-4s, AMPA receptors mediate fast excitatory neurotransmission that can be blocked by specific quinoxalinediones including 6-nitro-7-sulphamobezo (f) quinoxaline-2, 3-dione (NBQX), a potent and selective AMPA receptor antagonist. The AMPA receptors are permeable to Na^+ and K^+ but their Ca^{2+} permeability is variable. GluR2 subunit of AMPARs undergoes RNA editing at Q/R site in second transmembrane domain replacing glutamine to arginine during post transcriptional modification (Van Den Bosch *et al.,* 2000; Van Damme *et al.,* 2002; Duncan, 2009). GluR2 subunit is considered as the determinant of Ca^{2+} permeability of AMPA receptors (Van Damme *et al.,* 2002; Kawahara & Kwak, 2005; Duncan, 2009). It was suggested that the lack of GluR2 subunits or unedited form of GluR2 makes the AMPA receptors permeable to Ca^{2+} ions. Some earlier studies have reported that the motor neurons possess unedited form of GluR2 or completely lack GluR2 subunit which makes the AMPA receptor highly permeable to Ca^{2+} ions thus increasing their vulnerability to excitotoxicity (Williams *et al.,* 1997; Bar-Peled *et al*., 1999). In the mature hippocampus, most AMPARs are composed of GluR1–GluR2 or GluR2–GluR3 combinations *(*Wenthold *et al.,* 1996*),* whereas GluR4-containing AMPARs are expressed mainly in early postnatal development (Zhu *et al.,* 2000).

The release of even small and brief concentrations of glutamate into the synaptic cleft generates robust excitatory postsynaptic potentials (EPSPs). AMPA-mediated currents generate a fast upstroke and rapid current decay while NMDA-receptor activation provides a more prolonged phase of depolarization that can last several hundred milliseconds. EPSP generation is hypothesized to be controlled by AMPA receptor de/activation while the longer pharmacokinetics of NMDA

receptor sensitization provides ample opportunity for spatial and temporal summation at numerous postsynaptic inputs. The higher affinity of glutamate for NMDA-to-AMPA receptors likely explains these pharmacokinetic differences, as prolonged receptor activation is often the result of slower dissociation of agonist and receptor (Ai *et al.,* 2010).

AMPA receptor trafficking has been widely studied, especially its intracellular cycling and its potential physiological sequelae. Like all membrane receptors, AMPA receptors are synthesized in the soma and transported to the cell surface via the secretory pathway involving multiple membrane sorting steps and cytoskeleton transport proteins (Kennedy & Ehlers, 2006; Kapitein *et al.,* 2010). Dendritic AMPA receptor localization to synapses is regulated via two mechanisms: (1) exocytic and endocytic trafficking and recycling, respectively, in the secretory pathway and (2) membrane diffusion from extra-synaptic to synaptic localizations (Groc & Choquet, 2006; Newpher & Ehlers, 2008; Wang *et al.,* 2008; Hoogenraad *et al.,* 2010).

A physiological role for AMPA receptor trafficking and surface diffusion has been hypothesized in learning and memory. An LTP-like strengthening of neocortical synapses occurs after sensory stimulation *in vivo* (Holtmaat & Svoboda, 2009; Kessels & Malinow, 2009), and this process appears dependent on AMPA receptor number, localization and facilitation at synapses (Takahashi *et al.,* 2003). In the brain, soon after birth, most excitatory synapses in the hippocampus (Hsia *et al.,* 1998; Petralia *et al.,* 1999*)* and other brain regions (Wu *et al.,* 1996; Isaac *et al.,* 1997; Losi *et al.,* 2002) contain only NMDARs, whereas the prevalence of AMPARs increases gradually over the course of postnatal development. In fact, the delivery of AMPARs into synapses is a regulated process that depends on NMDAR activation and underlies some forms of synaptic plasticity in early postnatal development (Zhu *et al.,* 2000) and in mature neurons *(*Sheng, & Lee 2001; Song, & Huganir 2002). Synaptic plasticity is thought to underlie higher cognitive functions, such as learning and memory *(*Elgersma *et*

al.,1999, Martin *et al.*, 2000; Ai *et al.*, 2010), and is also critical for neural development *(*Cline *et al.*, 1998*)*. Learning in the hippocampus also appears to be regulated by AMPA receptor dynamics (Whitlock *et al.*, 2006) as evidenced by the recruitment of AMPA receptors to mushroom shaped dendritic spines in the CA1 region of the hippocampus 24 h after fear conditioning (Matsuo *et al.*, 2008). Stress hormones have recently been recognized to play a role in AMPA receptor trafficking (Groc *et al.*, 2008; Krugers *et al.*, 2010; Yuen *et al.*, 2011), and may provide a mechanism for the dose dependent facilitative and suppressive effects of corticosteroid hormones on synaptic plasticity and cognition (Martin *et al.*, 2009). Further complexity in the regulation of ionotropic glutamatergic neurotransmission is provided by molecular variability at the transcriptional and post-transcriptional level. RNA editing of AMPA and kainate receptor subunits (Higuchi *et al.*, 1993) and alternative splicing of mRNA transcripts (Sommer *et al.*, 1990) modulate second messenger cascades critical for downstream intracellular effects.

Glutamate receptors in pancreas

Large evidence shows that pancreatic islets cells, particularly insulin secreting β cells, share with neurones common characteristics; they contain tyrosine hydroxylase (Teitelman & Lee, 1987), neurone specific enolase (Polak *et al.*, 1984), Go protein (Terashima *et al.*, 1987), glutamic acid decarboxylase, high levels of GABA (Okada, 1986) as well as synaptic-like microvesicles (Reetz *et al.*, 1991). Coexpression of kainate KA1 or KA2 receptor subunits with GluR5–7 has not been observed in these cells, which suggests that the expression of functional kainate receptors is negligible (Morley et al., 2000; Molnar *et al.*, 1995; Inagaki *et al.*, 1995). Some laboratories have detected the expression of NMDA receptors in islet cells (Molnar *et al.*, 1995; Inagaki *et al.*, 1995; Ai *et al.*, 2010) although neither NMDA evoked ion transport nor coexpression of NR1 and NR2A–D receptor subunits has been observed by other laboratories (Weaver *et al.*, 1996; Morley *et al.*, 2000) AMPA and kainate, agonists of AMPA receptors, each

stimulate insulin secretion from perfused or isolated islets or clonal islet cells in the presence of high levels of glucose (Bertrand *et al.,* 1992, 1995).

The differential distribution of the GluR subunits in the pancreas has already been described (Molnar *et al.,* 1995; Weaver *et al.,* 1996). Liu *et al.,* (1997) showed that GluR 1 and GluR 4 were mainly localized to insulin-secreting cells in the central mass of the pancreatic islet, it appears that insulin and non–insulin-secreting cells express different AMPA receptor subunits, which may be used to mediate their hormone secretion, as was suggested earlier by Bertrand *et al., (*1992, 1993). Weaver *et al.,* (1996) reported that the AMPA receptors were located in the, α, β and PP cells but were generally absent from the *δ* cells, whereas kainate receptors were expressed in α and *δ* cells although they were not found in β or PP cells. These observations add to the evidence that these receptors may be involved in the regulation of hormone secretion (Barb *et al.,* 1996; Cho *et al.,* 2010). Studies of Weaver *et al.,* (1996) show that glutamate depolarizes islet cells when glutamate serum levels are elevated. Intracellular Ca^{2+} measurements and electrophysiological recordings showed that kainate, AMPA and NMDA elicited increases in Ca^{2+} in single β-pancreatic cells and depolarized them. In addition, kainate and AMPA stimulated the release of insulin whereas NMDA did not (Hollmann & Heinemann, 1994; Rondón *et al.,* 2010). This stimulatory effect was dependent on the glucose concentration: Glutamate stimulated insulin release in the presence of a glucose concentration of 8.3 mM but not in the presence of a low concentration (2.8 mM).

Glutamate transporters

Neuronal and glial glutamate transporters in the mammalian central nervous system remove the neurotransmitter glutamate from the synaptic cleft. After the release glutamate has been released to the synaptic cleft, it can be either inactivated by enzymatic degradation or transport back to the neuron or back to the glial cells by active transport. According to the structure and site of action, glutamate transporters as other neurotransmitter transporters, can be divided in to

two sub families: the plasma membrane transporters (EAATs) and vesicular transporters (VGLUTs) (Li *et al.,* 2005). Excitatory amino acid transporters (EAAT), formerly known as glutamate transporters, belongs to the family of neurotransmitter transporters. The removal of glutamate from the extracellular milieu is achieved by a family of excitatory amino acid transporters 1 to 5 (EAAT1 to 5) that are localized in glial cells and neurons (Rothstein *et al.,* 1994; Chaudhry *et al.,* 1995). They serve to terminate the excitatory neurotransmitter signal by removal of glutamate from the neuronal synapse into glia cells. To date, 5 high-affinity, sodium-dependent glutamate transporters have been cloned from mammalian and human tissue: astrocyte-specific glutamate transporter (GLAST [excitatory amino acid transporter 1 (EAAT1)]), glutamate transporter 1 (GLT-1 [excitatory amino acid transporter 2 (EAAT2)]), excitatory amino acid carrier 1 (EAAC1 [excitatory amino acid transporter 3 (EAAT3)]), excitatory amino acid transporter 4 (EAAT4), and excitatory amino acid transporter 5 (EAAT5) (Arriza *et al.,* 1994, Fairman *et al.,* 1995). Immunohistochemical studies have revealed that EAAT 1 and EAAT 2 are localized primarily in astrocytes (Lehre *et al.,* 1995), while EAAT 3 and EAAT 4 are distributed in neuronal membranes (Kanai & Hediger 1992; Anderson & Swanson, 2000; Li *et al.,* 2005). The neuronal transporters EAAT3 and EAAT4 appear to be localized to plasma membranes in a perisynaptic distribution. The greatest density of these transporter proteins appears to be at the edge of postsynaptic densities, rather than within the synaptic cleft. EAAT5 is only found in the retina where it is principally localised to photoreceptors and bipolar neurons in the retina (Pow & Barnett, 2000; Hawkins, 2009). In rodents, the orthologs for EAAT1-3 are named GLAST, GLT1 and EAAC1 respectively (Shigeri *et al.,* 2004).

EAATs are membrane bound pumps that resemble ion channels (Ganel & Rothstein, 1999). The uptake of one glutamate molecule is coupled to the uptake of three Na^+ ions and one proton, and the extrusion of one K^+ ion (Zerangue & Kavanaugh, 1996; Levy *et al.,* 1998; Hawkins, 2009). The large electrochemical

gradients for Na^+ and K^+ provide the driving force for glutamate uptake against its gradient (Zerangue & Kavanaugh, 1996). The proton has been hypothesized to play a neuroprotective role during ischemia by shutting down reverse uptake of glutamate (Billups *et al.,* 1996), but the exact mechanism by which the proton reduces reverse transport is unclear.

When glutamate is taken up into glia cells by the EAATs, it is not reused directly but converted to glutamine and stored vesicles. Subsequently these vesicle are released from glia cells and glutamine transported back into the presynaptic neuron, converted back into glutamate and store into vesicles by action of the VGLUTs (Pow & Robinson, 1994; Shigeri *et al.,* 2004). This process is named the glutamate-glutamine cycle. Given that glutamate transporters provide the main route by which glutamate is cleared, it is logically predicted that an aberration in transporter expression and function lead to toxic glutamate levels and thus promote neuronal degeneration (Tanaka *et al*., 1997). Recent studies have suggested the involvement of the glutamate transporters in radiation induced neurotoxicity (Martha *et al.,* 2009). Studies in brain autopsy specimens of HIV-1-infected patients have shown that the expression of EAAT-2 by activated microglia exert a compensatory effect that protects neurons from glutamate neurotoxicity (Xing *et al*., 2009; Hawkins, 2009).

Oxidative stress and diabetes

Oxidative stress and oxidative damage to the tissue are common end points of chronic diseases, such as atherosclerosis, diabetes and rheumatoid arthritis (John *et al.,* 1999). Oxidative stress is currently suggested as mechanism underlying diabetes and diabetic complications (Moussa 2008). During diabetes, persistent hyperglycemia causes increased production of free radicals, especially reactive oxygen species (ROS), for all tissues from glucose auto-oxidation and protein glycosylation. The increase in the level of ROS in diabetes could be due to their increased production and/ or decreased destruction by nonenzymic and

enzymic catalase (CAT), reduced glutathione (GSH), and superoxide dismutase (SOD) antioxidants. The level of these antioxidant enzymes critically influences the susceptibility of various tissues to oxidative stress and is associated with the development of complications in diabetes (Lipinski, 2001). Oxidative damage to various brain regions constitutes into the long term complications, morphological abnormalities and memory impairments (Fukui *et al.*, 2001). In the study of Ha & Lee, 2000 it was shown that oxidative stress is one of the important mediators of vascular complications in diabetes including nephropathy. In the central nervous system, oxidative stress signifies an important pathway that leads to the damage of both neuronal and vascular cells (Root-Bernstein *et al.*, 2002).

Oxidants are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. In addition, a number of external agents can trigger ROS production. A sophisticated enzymatic and nonenzymatic antioxidant defense system including catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) counteracts and regulates overall ROS levels to maintain physiological homeostasis. Lowering ROS levels below the homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defence. Similarly, increased ROS may also be detrimental and lead to cell death or to acceleration in ageing and age-related diseases. Because of their ability to directly oxidize and damage DNA, protein, and lipid, ROS are believed to play a key direct role in the pathogenesis of late diabetic complications (Rosen *et al.*, 2001). In addition to these effects, a rise in ROS levels may also constitute a stress signal that activates specific redoxsensitive signalling pathways. Once activated, these diverse signalling pathways may have either damaging or potentially protective functions (Toren & Nikki, 2000). The primary ROS produced in the course of oxygen metabolism is superoxide, which is a highly reactive, cytotoxic ROS. Superoxide is dismutated to a far less reactive product, hydrogen peroxide (H_2O_2), by a family of metalloenzymes known as SOD **(**Brownlee, 2001**).** The ubiquitous

SOD catalyzes the disproportionation of superoxide to molecular oxygen and peroxide and thus is critical for protecting the cell against the toxic products of aerobic respiration.

Recently, pancreatic β-cells emerged as a target of oxidative stress-mediated tissue damage (Evans *et al.,* 2003; Drews *et al.,* 2010). Extracellular hyperglycemia causes intracellular hyperglycemia in β-cells, leading to the induction of ROS in pancreatic islets of diabetic animals. Indeed, it was shown that expression of oxidative stress markers such as 8-hydroxy-2'- deoxyguanosine (8-OHdG) and 4-hydroxy-2, 3-nonenal (4-HNE) are increased in islets under diabetic conditions (Gorogawa *et al.,* 2002). In addition, due to the relatively low expression of antioxidant enzymes such as catalase and glutathione peroxidase (Tiedge *et al.,* 1997) β -cells are rather vulnerable to oxidative stress. Thus, it is likely that oxidative stress plays a major role in β -cell deterioration in type 2 diabetes. There are several sources of ROS productions in cells: the non-enzymatic glycosylation reaction (Matsuoka *et al.,* 1997).

Hyperglycemia, defining established diabetes, can induce oxidative stress by various mechanisms; excessive levels of glucose reaching the mitochondria lead to an overdrive of the electron transport chain, resulting in overproduction of superoxide anions normally scavenged by mitochondrial SOD. When the latter fails oxidative stress develops and it was recently proposed that this mechanism is responsible for the activation of all major pathways underlying the different components of vascular diabetic complications (glycation, PKC activation, sorbitol pathway) (Nishikawa *et al.,* 2000). The *in vitro* supplementation of SOD like drugs corrected most of these defects, supporting the importance of these mechanisms (Yamagishi *et al.,* 2001; Recchioni *et al.,* 2002). It has also been proposed that uncoupling mitochondrial NOS by hyperglycemia would be involved (Brodsky *et al.,* 2002).

Glutamate mediated excitotoxic cell death

Glutamate is the principal excitatory neurotransmitter in the CNS, but it is also a potent neurotoxin that can kill nerve cells. Excessive glutamate signaling can lead to excitotoxicity, a phenomenon whereby overactivation of glutamate receptors (GluRs) initiates cell demise. Excitotoxic cell death was first described during the late 1950s in retinal neurons (Lucas & Newhouse, 1957; Carozzi & Ceresa, 2012) and was found later to occur in virtually all neurons that express GluRs (Olney *et al.,* 1969). Thereafter, glutamate excitotoxicity has been implicated in acute injury to the CNS and in chronic neurodegenerative disorders (Choi, 1988; Lee *et al.,* 1999). Activation of NMDA, AMPA, kainate and metabotrobic receptor subtypes by glutamate (Paoletti, 2011), the most ubiquitous cerebral neurotransmitter, leads to an increase in the levels of free intracellular calcium (Coyle & Puttfarcken, 1993; Pivovarova & Andrews, 2011). Such events can cause prolonged depolarization and subsequent ionic imbalance, ATP depletion and increases in intracellular free calcium levels that together culminate in cerebral edema, raised intracranial pressure (ICP), vascular compression and brain herniation, an often fatal complication of severe head injury (Lau & Tymianski, 2010).

Over activation at NMDA receptors triggers an excessive entry of Ca^{2+}, initiating a series of cytoplasmic and nuclear processes that promote neuronal cell death. For instance, Ca^{2+} activated proteolytic enzymes, like calpains, can degrade essential proteins (Dong *et al*., 2009). Neuronal activity can lead to a marked increase in the concentration of cytosolic Ca^{2+}, which then functions as a second messenger that mediates a wide range of cellular responses (Blaustein 1988; Tymianski & Tator 1996; Mallick *et al.,* 2007). Excessive influx of extracellular Ca^{2+} together with any Ca^{2+} release triggered from intracellular stores can elevate neuronal cytosolic free Ca^{2+} concentrations to levels that exceed the capacity of intracellular Ca^{2+} regulatory mechanisms and can lead to metabolic derangements such as the formation of free radicals and cell death (Choi, 1988; Sattler &

Tymianski, 2000). Although cellular Ca^{2+} overload is unlikely to be a common pathway mediating all forms of neuronal cell death, several lines of evidence support a close relationship between excessive Ca^{2+} influx and neuronal injury in the adult mammalian nervous system *(*Choi 1988; Tymianski & Tator, 1996; Carozzi & Ceresa, 2012).

AMPA receptors are generally heteromers of subunits encoded by four genes, GluR1-4 (GluR-A-D) and exhibit a higher affinity to AMPA and glutamate compared to kainite *(*Hollmann & Heinemann 1994). The mRNAs for GluR1, 2, and 3 are expressed broadly throughout the CNS, whereas GluR4 shows a more restricted spatial and temporal expression pattern *(*Monyer *et al.*, 1991). AMPA receptor subunits have four hydrophobic membrane domains with an extracellular N-terminal domain and a cytoplasmically disposed C-terminal tail (Hollmann & Heinemann 1994). Due to its influence on receptor Ca^{2+} permeability, the GluR2(R) subunit has attracted significant interest in studies of excitotoxicity. GluR2 mRNA is widely expressed in mammalian neurons that are highly vulnerable to excitotoxic damage, such as hippocampal pyramidal and granule neurons as well as cortical neurons (Kondo *et al.,* 1997), rendering the majority of these AMPA receptors Ca^{2+} impermeable. Despite this generally low Ca^{2+} permeability, AMPA receptor toxicity is likely to be, at least in part, mediated by Ca^{2+} ions. Neurons expressing Ca^{2+} permeable AMPA gated channels can be identified by kainite induced Ca^{2+} uptake and have been shown to be present in many regions of the brain, though low levels (8–15%) (Turetsky *et al.,* 1994; Sattler *et al.,* 1998; Carozzi & Ceresa, 2012). This subpopulation of neurons was selectively destroyed in a Ca^{2+} dependent manner after AMPA or kainite exposures *(*Turetsky *et al.,* 1994). In addition, Brorson *et al.,* (1994) reported that Ca^{2+} entry via Ca^{2+} permeable AMPA/KA receptors was sufficient to induce excitotoxicity in cerebellar Purkinje cells. Surprisingly, GluR2 containing AMPA receptors do show low Ca^{2+} and other divalent cation permeability, especially in

cells expressing low levels of GluR2 relative to other AMPA receptor subunits (Jonas *et al.*, 1994; Geiger *et al.*, 1995; Mallick *et al.*, 2007).

In the retina, glutamate is the primary excitatory transmitter in the vertical pathway from photoreceptors to ganglion cells (Lucas & Newhouse, 1957; Choi, 1988; Ng *et al.*, 2004). Several recent studies have shown that a significant increase in glutamate in the retina is associated with the development of diabetic retinopathy (DR), a disease characterized by neurodegeneration and vasculopathy (Li & Puro, 2002; Diederen *et al.*, 2006). Although the detailed mechanism remains unknown, it may be of critical importance for neuroprotection to remove excess glutamate from the extracellular space in the retina.

Diabetes and apoptosis

Apoptosis is a coordinated series of events for the programmed execution of cell death, and plays an important role in the maintenance of tissue homeostasis. A host of physical, chemical and biological factors can trigger apoptotic death by activating complex yet tightly controlled intracellular signal transduction pathways. The extrinsic pathway is activated upon ligation of the cell surface death receptors, which in turn activates downstream effector mechanisms orchestrated by the caspase family of cysteine proteases (Green, 2004).

Cell death can occur by necrosis or apoptosis, with these two mechanisms having distinct histological and biochemical markers (Kanduc *et al.*, 2002; Ueda *et al.*, 2004). In contrast to necrosis, apoptosis involves a cascade of intracellular events that ultimately culminates in cell destruction (Green & Droemer, 2004). This process involves caspases, cysteine-dependent, aspartate-specific proteases, that exist in an inactivated state that, when activated, initiate the death program. The intrinsic apoptotic pathway can be initiated by external signals or internal changes, such as release of cytochrome *c* from mitochondria; indeed, mitochondrion is a very important component of this cascade (Hengartner *et al.*, 2000). Release of apoptogenic factors from mitochondria can be induced by

distinct factors, including members of the Bcl-2 protein family. The balance between proapoptotic and antiapoptotic members of this family has a crucial role in determining the integrity of the mitochondria and, hence, cell death (Kanduc *et al.,* 2002). The extrinsic cell-death pathway involves activation of extracellular death receptors, which belong to the TNF receptor superfamily (Wajant, 2003). Binding of the appropriate ligand to one of these receptors results in receptor aggregation and recruitment of FADD (Fas-associated death domain) and procaspase 8 (also called FLICE or MACH-1). Procaspase 8 can then be activated by self-cleavage or cleavage by another caspase 8 molecule (Kaufmann & Earnshaw, 2000). Activated caspase 8, functioning as an initiator caspase, activates downstream executioner caspases that cleave cell death substrates or directly induces apoptosis (Muzio *et al.,* 1998). Caspases have a pivotal role in the progression of a variety of neurologic disorders. Caspase 8 is the apical protease in the extrinsic apoptotic pathway activated at the plasma membrane by various TNF family death receptors (Ashkenazi & Dixit, 1998; Su *et al.,* 2012).

Caspases are evolutionarily conserved cysteine-aspartyl specific proteases that play a key role in apoptosis. In mammals, there are over 14 caspases, of which some are involved in apoptosis and others in cytokine activation (Woo *et al.,* 2000; Creagh *et al*., 2003; Shi, 2004). The processes that lead to diabetic embryopathy in the embryos of diabetic mothers are not well understood. However, it is clear that caspase-3 and 6 activity and Bax levels are increased, implying alterations in the apoptotic processes (Gareskog *et al.,* 2007; Yang *et al.,* 2008). However, knowledge is limited regarding the processes that account for this increased apoptosis. As a key upstream regulator, caspase 8 is also activated by maternal diabetes (Toder *et al.,* 2002),

Oxidative stress has been cited as another critical mediator of cell death, and may either trigger or modulate apoptosis (Oyarzún *et al.,* 2011). A role for oxidative stress in apoptosis has been shaped by several independent observations. For many years, direct treatment of cells with oxidants such as hydrogen peroxide

or redoxactive quinones was thought to exclusively cause necrosis, but more recent studies have shown that lower doses of these agents can trigger apoptosis (Hampton & Orrenius, 1997).

Inositol 1, 4, 5-trisphosphate (IP3)

IP3 is a ubiquitous second messenger that functions by binding to receptors (IP3Rs) on the ER membrane to cause liberation of sequestered Ca^{2+} (Berridge, 1997, Foskett *et al.,* 2007). The resultant cytosolic Ca^{2+} transients serve numerous signaling functions in neurons, including modulation of membrane excitability synaptic plasticity and gene expression (Fujii *et al.,* 2000; Yamamoto *et al.,* 2002; Stutzmann *et al.,* 2003). Many biological stimuli, such as neurotransmitters, hormones and growth factors, activate the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP2) in the plasma membrane which is hydrolyzed by phospholipase C (PLC) to produce IP3 and diacylglycerol (DAG). The IP3 mediates Ca^{2+} release from intracellular Ca^{2+} stores by binding to IP3R. IP3R are the IP3 gated intracellular Ca^{2+} channels that are mainly present in the endoplasmic reticulum (ER) membrane. The IP3 induced Ca^{2+} signaling plays a crucial role in the control of diverse physiological processes such as contraction, secretion, gene expression and synaptic plasticity (Berridge, 1993). In mammalian cells, there are three IP3R subtypes- IP3R1, IP3R2 and IP3R3 which are expressed to varying degrees in individual cell types (Wojcikiewicz, 1995; Taylor *et al.*, 2002) and form homotetrameric or heterotetrameric channels (Bosanac *et al.*, 2004).

Group I mGluRs (mGluR1/5 subtypes) are also demonstrated to mainly affect intracellular Ca^{2+} mobilization (Bordi & Ugolini, 1999). To sequentially facilitate intracellular Ca^{2+} release, group I receptors activate the membrane bound phospholipase C (PLC), which stimulates phosphoinositide turnover by hydrolyzing PIP2 to IP3 and diacylglycerol. IP3 then causes the release of Ca^{2+} from intracellular Ca^{2+} stores (such as endoplasmic reticulum) by binding to

specific IP3 receptors on the membrane of Ca^{2+} stores. Altered Ca^{2+} levels could then engage in the modulation of broad cellular activities. The neuronal intracellular calcium has an important role in the regulation of synaptic plasticity (Barbara, 2002). Moreover, disruptions in this pathway are implicated in neurodegenerative disorders (Mattson *et al.*, 2000; LaFerla, 2002). Therefore, factors that modulate or disrupt IP3 mediated Ca^{2+} signaling are expected to exert powerful physiological and possibly pathological effects on the nervous system.

Insulin secretion is largely a Ca^{2+} dependent process and restricted increases in intracellular Ca^{2+} have been related to impairment of glucose-stimulated insulin release (Boschero *et al.*, 1990). Several lines of evidence point to IP3 playing an important role in insulin secretion. IP3 was shown to mobilize intracellular Ca^{2+} in permeabilized insulin-secreting cells and IP3 production correlated with Ca^{2+} mobilization in intact cells (Biden *et al.*, 1984; Gromada *et al.*, 1996). In the mouse anx7 (1/2) phenotype, where there is a profound reduction in IP3R expression and Ca^{2+} mobilization in islets, there is also defective insulin secretion (Srivastava *et al.*, 1999; Ye *et al.*, 2011).

Inositol 1, 4, 5-trisphosphate (IP3) and activation of calcium release

Cytosolic Ca^{2+} is a focal point of many signal transduction pathways and modulates a diverse array of cellular activities ranging from fertilization to cell death (Berridge *et al*., 2000). In most cell types, the major internal [Ca^{2+}] stores are the endoplasmic reticulum/sarcoplasmic reticulum (ER/SR). One mechanism for mobilizing such stores involves the phosphoinositide pathway. The binding of many hormones to specific receptors on the plasma membrane leads to the activation of an enzyme (phosphoinositidase C) that catalyses the hydrolysis of phospholipids to produce the intracellular messenger inositol 1,4,5-trisphosphate (IP3) (Luciani *et al.*, 2009). Although derived from a lipid, IP3 is water soluble and diffuses into the cell interior where it encounters IP3 receptors (IP3Rs) on the ER/SR. The binding of IP3 changes the conformation of IP3Rs such that an

integral channel is opened, thus allowing the Ca^{2+} stored at high concentrations in the ER/SR to enter the cytoplasm (Ye *et al.,* 2011). A critical feature of IP3Rs is that their opening is regulated by the cytosolic Ca^{2+}concentration. This sensitivity to cytosolic Ca^{2+} allows them to act as Ca^{2+} induced calcium release channels that promote the rapid amplification of smaller trigger events.

Curcumin

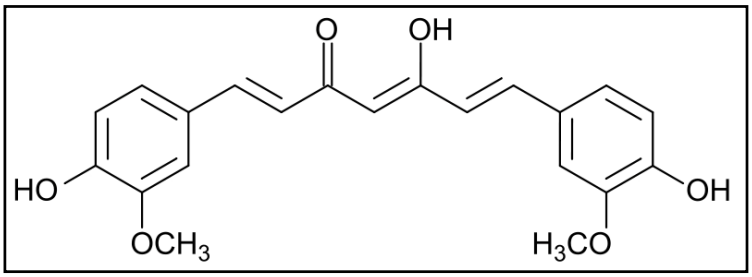
Curcumin is the principal curcuminoid of the popular Indian spice turmeric. Turmeric is a spice which is obtained from rhizomes of plant *Curcuma longa*, which is a member of the ginger family (Zingiberaceae). *C.longa* is a perennial plant having a short stem with large oblong leaves and bears ovate, pyriform or oblong rhizomes, which are often branched and brownish-yellow in colour. The origin of the plant is not certain, but it is thought to be originated from south eastern Asia, most probably from India. The plant is cultivated in all parts of India (Kapoor, 2000). India produces most of the world supply (Leung & Foster, 1996), but turmeric is cultivated also in southern China, Taiwan, Japan, Burma, and Indonesia (Yen, 1992) as well as throughout the African continent (Iwu, 1993; Stefanska, 2012). The commercially available material (i.e. turmeric powder) in Europe is obtained mainly from India and somewhat from other south eastern Asian countries (Murugananthi *et al.,* 2008). *C.longa* is also considered as auspicious and is a part of religious rituals. In old Hindu medicine, it is extensively used for the treatment of sprains and swelling caused by injury. In recent times, traditional Indian medicine uses turmeric powder for the treatment of biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorders, rheumatism and sinusitis (Ammon *et al.,* 1991; Meng *et al.,* 2012).

Turmeric is one of most essential spices all over the world with a long and distinguished human use particularly in the Eastern civilization (Ravindran, 2007). The other two curcuminoids are desmethoxycurcumin and bis-desmethoxycurcumin. The curcuminoids are polyphenols and are responsible for

the yellow colour of turmeric. Turmeric consists of 3-5% curcuminoids. Curcumin is the most important fraction which is responsible for the biological activities of turmeric. The melting point of curcumin is 184^{o}C. It is soluble in ethanol and acetone, but insoluble in water (Joe *et al.,* 2004). Curcumin exists in solution as ketoenol tautomers (Payton *et al.,* 2007). Because of its biological activities, a large number of studies have been presented on curcumin. According to these studies, curcumin exhibits antiinflammatory (Chainani-Wu, 2003; Stefanska, 2012) antioxidant (Masuda *et al.*, 1993, Cohly *et al.*, 1998) anticarcinogenic (Frank *et al.,* 2003) antiviral (Suai *et al.,* 1993) antimicrobial activity (Mahady *et al.*, 2002, Han & Yang 2005) beside these, curcumin has a variety of potentially therapeutic properties, such as antineoplastic, antiapoptotic, antiangiogenic, cytotoxic, immunomodulatory, (Strimpakos & Sharma, 2008; Meng *et al.*, 2012) and antithrombotic, wound healing, antidiabetogenic, antistressor and antilithogenic actions (Chainani-Wu, 2003; Rungseesantivanon *et al.*, 2010; Stefanska, 2012). The colouring principle of turmeric is the main component of this plant and is responsible for the anti inflammatory property. Turmeric was described as *C. longa* by Linnaeus and its taxonomic position is as follows:

Class	Liliopsida
Subclass	Commelinids
Order	Zingiberales
Family	Zingiberaceae
Genus	*Curcuma*
Species	*Curcuma longa*

Structure of curcumin



Chemical Formula: $C_{21}H_{20}O_6$

Molecular Weight: 368.38

Curcumin and neurodegenerative diseases

Curcumin exhibits antioxidant, anti-inflammatory, antirheumatic antimicrobial and anti cancer activities (Ammon & Wahl, 1991; Rao *et al.,* 1995; Ruby *et al.,* 1995; Zhao *et al.,* 2012) as well as nephroprotective activity, therapeutic activity against myocardial infarction, skin diseases and cystic fibrosis (Aggarwal *et al.,* 2003; Limtrakul *et al.,* 1997; Dikshit *et al.,* 1995). Studies revealed that curcumin mediates its anti-inflammatory and antioxidant effects by downregulation of nuclear factor-kB (NF-kB) and modulation of several important molecular targets, including, enzymes COX-2 (cyclooxygenase-2), iNOS (inducible nitric oxide synthases), and cytokines TNF α (Tumor necrosis factoralpha), IL-1b (Interleukin-1 beta), IL-6(Interleukin-6) and chemokines (Hong *et al.,* 2004; Kim *et al.,*, 2007; Kunnumakkara *et al.,* 2008; Moon *et al.,* 2008).

Brain is perhaps the most sensitive organ to oxidative damages (Halliwell, 1992). This organ consumes 20% of the body's oxygen despite accounting for only 2% of the total body weight (Smith *et al.,* 2007). Oxidative stress, which is due to the highly oxidative intracellular environment of the neurons and glial cells, has been shown to increase with both normal brain ageing as well as with brain injuries (Lu *et al.,* 2004; Zhao *et al.,* 2012). Administration of curcumin

significantly reduced the progression of kindling and also attenuated the oxidative stress in mice; therefore it could be a candidate to control development of seizure and oxidative stress during epilepsy (Guangwei *et al.,* 2010). Curcumin have been described to ability for scavenge oxygen derived free radicals that it has been implicated its potential as a neuroprotective agent (Sharma *et al.,* 2009; Rungseesantivanon *et al.,* 2010). Cerebral edema, a cause of increased intracranial pressure after acute brain injury, was significantly controlled by pretreatment as well as post treatment with curcumin (Thiyagarajan & Sharma, 2004). Curcumin has potential to increase the cholinergic activity of neurons in streptozotocin-induced dementia in rats (Awasthi *et al.,* 2010)

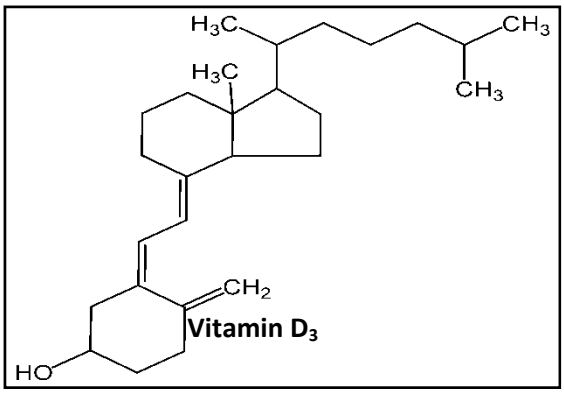
AD is the most common form of progressive neurodegenerative dementia in the elderly population, and after heart disease, cancer and stroke AD is the fourth common cause of death in western countries (Selkoe, 2001; Zhao *et al.,* 2012). AD is induced by different causes including genetics, oxidative stress, head trauma, inflammation and environmental factors (Butterfield, 2005; Zhu *et al.,* 2005). AD is associated with impairment in working memory (Germano & Kinsella, 2005), visuoperception, attention and semantic memory (Bolla, 1992). Oxidative stress and extracellular beta- amyloid (Aβ) deposits is known to contribute to the etiology of AD (Zhu *et al.,* 2005; Hardy & Higgins, 1992). Oxidative damage to lipid and protein can lead to structural and functional disruption of the cell membrane, inactivation of enzymes, and finally caused cell death (Ashok & Ali, 1992; Meng *et al.,* 2012).

Vitamin D_3

Most vertebrates synthesise vitamin D in their skin under the influence of UV light (Holick & Clark, 1978; Holick, 2003). An efficient sun exposure of the face and hands to the sun for 2 h/ week is probably sufficient to maintain normal levels. There are two forms of vitamin D, vitamin D_3 (cholecalciferol), which is produced from the conversion of 7-dehydrocholesterol in the epidermis and

dermis in humans, and vitamin D_2 (ergocalciferol) which is produced in mushrooms and yeast (Langer *et al.,* 2012). The chemical difference between vitamin D2 and D3 is in the side chain; in contrast to vitamin D_3, vitamin D_2 has a double bond between carbons 22 and 23 and a methyl group on carbon 24. Vitamin D can be obtained from dietary sources of vegetable (vitamin D_2, also known as ergocalciferol) or animal origin (vitamin D_3, also known as cholecalciferol). The best food sources are fatty fish or their liver oils; however, small amounts are also found in butter, cream and egg yolk. One of the major biological functions of vitamin D is to maintain calcium homeostasis (Norman *et al.,* 1982; Zitman-Gal *et al.,* 2012) which impacts on cellular metabolic processes and neuromuscular functions. Vitamin D affects intestinal calcium absorption by increasing the expression of the epithelial calcium channel protein, which in turn enhances the transport of calcium through the cytosol and across the basolateral membrane of the enterocyte (Langer *et al.,* 2012).

Structure of Vitamin D_3



Molecular formula $C_{27}H_{44}O$

Molar mass 384.64 g/mol

Vitamin D receptor

1,25(OH)2D3 form of vitamin D is metabolically active, and this molecule exerts its effects by activating the nuclear vitamin D receptor (VDR)

(Buell & Dawson-Hughes, 2008). The VDR is a member of the nuclear receptor super family (Buell & Dawson-Hughes, 2008; Brown & Slatopolsky, 2008) of ligand activated transcription factors, which also includes the thyroid hormone receptor, the retinoic acid receptor and the peroxisome proliferator activated receptor. The VDR regulates gene transcription both positively and negatively by binding to hexameric core binding motifs in the promoter regions of target genes, designated vitamin D response elements (Zitman-Gal *et al.,* 2012). Vitamin D receptors are activated when certain mediator substances, or ligands, dock at them. This ligand function can be exerted not only by vitamin D compounds, but also by steroid hormones, thyroid hormones and Vitamin A1 acid. By binding to the receptor, these ligands regulate the metabolism of Ca^{2+} and phosphate, and thus also of bone and control cell replication and differentiation. This occurs *via* an influence on the synthesis of certain regulatory proteins. When a VDR is activated by binding of a ligand, it exerts its action as a transcription factor. This means that it binds to specific sites on DNA (deoxyribonucleic acid), the molecule in the cell nucleus that bears genetic information and thereby initiates the synthesis of certain regulatory proteins. VDR is expressed in most brain areas. Vitamin D_3 has been detected in the cerebrospinal fluid, and this hormone has been shown to cross the blood-brain barrier (Balabanova *et al.,* 1984). The presence of VDR in the limbic system, cortex, cerebellumof rodents and humans (Musiol *et al.,* 1992; Eyles *et al.,* 2005) support a functional role for Vitamin D_3 in the regulation of behaviour and cognitive functions.

Vitamin D and diabetes

An increased prevalence of type 2 diabetes has been described in vitamin D deficient individuals (Boucher *et al.,* 1995; Chiu *et al.,* 2004), and insulin synthesis and secretion have been shown to be impaired in beta cells from vitamin D deficient animals (Kadowaki & Norman, 1984; Tanaka *et al.,* 1984; Langer *et al.,* 2012). This impairment is primarily caused by the direct effect of

vitamin D deficiency on the beta cell. Glucose tolerance is restored when vitamin D levels return to normal (Brazdilova *et al.,* 2012). β cells of endocrine pancreas are among the nonclassical target tissues for the action of 1α,25(OH)2D3. Studies revealed the presence of receptor protein for 1α, 25(OH)2D3 in chick pancreas(Christakos & Norman,1981; Pike,1981) and the presence of immunoreactive vitamin D-dependent calcium binding protein (CaBP) in pancreas (Morrissey *et al* 1975).

The identification of receptors for 1,25(OH)2D3 in cells of the immune system led to experiments in animal models of type 1 diabetes in which the administration of high doses of 1,25(OH)2D3 was shown to prevent type 1 diabetes (Mathieu *et al*., 1994; Boucher *et al*., 1995; Eichhorn *et al.*, 2012), mainly through immune regulation. It has been demonstrated that 1, 25(OH) 2D3 is one of the most powerful blockers of dendritic cell differentiation and that it directly blocks IL-12 secretion (Ambrosio *et al*., 1998). Evidence suggests that Vitamin D_3 has potential benefits with respect to diabetes. Cholecalciferol has been shown lower blood pressure (Vianna *et al.,* 1992) and may have a role on normal pancreatic function and treatment of diabetes (Bland *et al*., 2004; Magge *et al.*, 2012).

Materials and Methods

Chemicals used and their sources

Biochemicals

AMPA (α-amino-3-hydroxy-5- methylisoxazole-4-propionic acid), (+) MK-801 [(+) 5 - methyl-10, 11-dihydro-5 H-dibenzocyclohepten-5, 10-iminemaleate], ethylene diamine tetra acetic acid (EDTA), Tris HCl, calcium chloride, paraformaldehyde], Streptozotocin, citric acid, Tris HCl, foetal calf serum (heat inactivated), D-glucose, calcium chloride, collagenase type XI, bovine serum albumin fraction V and RPMI-1640 medium were purchased from Sigma Chemical Co., St. Louis, MO. USA). All other reagents were of analytical grade purchased locally.

Radiochemicals

(+)-[^{3}H] MK-801 (Sp. Activity 27.5 Ci/mmol) was purchased from Perkin Elmer Nen Life and Analytical Sciences, Boston, MA, USA. [^{3}H] AMPA (Sp. Activity 43 Ci/mmol) was purchased from American Radiolabelled Chemicals INC, St Louis, Missouri, USA, [^{3}H] IP3 Biotrak Assay Systems was purchased from G.E Healthcare UK Limited, UK. Radioimmunoassay kit for insulin was purchased from Baba Atomic Research Centre (BARC), Mumbai, India.

Molecular Biology Chemicals

Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, USA. ABI PRISM High Capacity cDNA Archive kit, Primers and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, Foster City, CA, USA. GluR4 AMPAR (Rn00568544_m1), GluR2 AMPAR (Rn00568514_m1), NMDAR1 (Rn_00433800), NMDA2B (Rn00561352_m1), GLAST (Rn00570130_m1), Bax (Rn 01480160_g1), GAD (Rn00562748_m1), Akt 1

(Rn00583646_m1), Caspase 8 (Rn00574069_m1), GPx (Rn00577994), Pdx-1 (Rn_00755591), NeuroD1 (Rn_00824571) primers were used for the study.

Confocal Dyes

Rat primary antibody for AMPAR (BD Pharmingen), NMDAR (BD Pharmengen), Insulin (Cell signaling) IP3 Receptor3 (BD Pharmengin) Vitamin D receptor (Pierce antibody) secondary antibody of either FITC (Chemicon), Rhodamine dye (Chemicon), Alexa Fluor 488 (Invitrogen), Alexa Fluor 594 (Invitrogen) and CY5 (Chemicon) were used for the immunohistochemistry studies using confocal microscope.

Animals

Adult male Wistar rats of 180-250g body weight were purchased from Kerala Agriculture University, Mannuthy and Amrita Institute of Medical Sciences, Kochi and were used for all the experiments. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water *ad libitum*. Adequate measures were also taken to minimize pain and discomfort of the animals. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health guidelines and CPCSEA guidelines.

DIABETES INDUCTION

Diabetes was induced in rats by intrafemoral injection of streptozotocin (Sigma chemicals Co., St. Louis, MO, USA.) freshly dissolved in citrate buffer pH 4.5 under anesthesia (Junod *et al.,*1969). Streptozotocin was given at a dose of 55mg/Kg body weight (Arison *et al.,* 1967; Hohenegger & Rudas, 1971).

DETERMINATION OF BLOOD GLUCOSE

The diabetic state of animals was assessed by measuring blood glucose concentrations at 72 hours after streptozotocin treatment. The rats with a blood sugar level above 250 mg/dl were selected as diabetic rats.

DETERMINATION OF ANTI-DIABETIC POTENTIAL OF CURCUMIN AND VITAMIN D_3

Animals used in this study were randomly divided into the following groups. Each group consisted of 6-8 animals.

a) Group 1: Control (given citrate buffer injection)
b) Group 2: Diabetic
c) Group 3: Diabetic rats treated with insulin
d) Group 4: Diabetic rats treated with Curcumin
e) Group 5: Diabetic rats treated with Vitamin D_3

The insulin treated diabetic group (Group 3) received subcutaneous injections (1Unit/kg body weight) of insulin daily during the entire period of the experiment. A mixture of both lente and plain insulin (Abbott India) were given for the better control of glucose (Sasaki & Bunag, 1983). The last injection was given 24 hr before sacrificing the diabetic rats. Curcumin was given orally to the 4^{th} group of diabetic rats in the dosage of 60mg/Kg body weight suspension of curcumin orally at 24 hour intervals. Curcumin was suspended in 0.5% w/v sodium carboxymethylcellulose immediately before administration in constant volume of 5ml/kg body weight (Sharma *et al.,* 2006). Cholecalciferol was given orally to the 5^{th} group of diabetic rats in the dosage of 12 µg/Kg body weight dissolved in 0.3 ml of coconut oil (deSouzaSantos *et al.,* 2005). Blood samples were collected from the tail vein at 0 hours (before the start of the experiment), 3^{rd}, 8^{th}, 12^{th} and 16^{th} day and the glucose levels were estimated. Blood samples were collected 3hrs after the administration of morning dose. Changes in the body weight of animals were monitored on 1^{st} Day (before the start of the experiment), 7^{th} and 15^{th} day.

SACRIFICE AND TISSUE PREPARATION

The animals were then sacrificed on 16th day by decapitation. The cerebral cortex, cerebellum and brain stem were dissected out quickly over ice according to the procedure of Glowinski and Iversen, (1966) and the pancreas was dissected quickly over ice. Hippocampus was dissected according to the procedure of Heffner *et al*., (1980). The blood samples were collected and plasma was separated by centrifugation. The tissue samples and plasma were kept at -80° C until assay. All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

ESTIMATION OF BLOOD GLUCOSE

Blood glucose was estimated using Glucose estimation kit (Merck). The spectrophotometric method using glucose oxidase-peroxidase reactions is as follows:

Principle: Glucose oxidase (GOD) catalyses the oxidation of glucose in accordance with the following equation:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{(GOD)}} \text{Gluconic acid} + H_2O_2.$$

$$H_2O_2 + \text{Phenol} \quad \text{4-aminoantipyrene} \xrightarrow{\text{(Peroxidase)}} \text{Coloured complex} + H_2O$$

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(-4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 510nm in (Shimadzu UV-1700 pharmaSPEC) spectrophotometer.

ESTIMATION OF CIRCULATING INSULIN BY RADIOIMMUNOASSAY

Principle of the assay

The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method is based on the competition of unlabelled insulin in the standard or samples and [^{125}I] insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free insulin were separated by the second antibody-polyethylene glycol (PEG) aided separation method. Measuring the radioactivity associated with bound fraction of sample and standards quantitates insulin concentration of samples.

Assay Protocol

Standards, ranging from 0 to 200 μU/ml, insulin free serum and insulin antiserum (50μl each) were added together and the volume was made up to 250μl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. They were incubated overnight at 2°C. Then [^{125}I] insulin (50μl) was added and incubated at room temperature for 3 hours. The second antibody was added (50μl) along with 500μl of PEG. The tubes were then vortexed and incubated for 20 minutes and they were centrifuged at 1500 x g for 20 minutes. The supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter.

A standard curve was plotted with %B/Bo on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. %B/Bo was calculated as:

$$\frac{\text{Corrected average count of standard or sample}}{\text{Corrected average count of zero standard}} \times 100$$

Insulin concentration in the samples was determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

Quantification of Glutamate

Glutamate content in the brain regions – hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats were quantified by displacement method using modified procedure of Enna and Snyder, (1976). Tissues were homogenized in 20 volumes of 0.32 M sucrose, 10 mM Tris/HCl and 1 mM MgCl2 buffer, pH 7.4, with a polytron homogenizer. The homogenate was centrifuged twice at 27,000 x g for 15 min. The supernatant was pooled and used for the assay. The incubation mixture contained 1 nM [^{3}H] glutamate with and without glutamate at a concentration range of 10^{-9} M to 10^{-4} M. The unknown concentrations were determined from the standard displacement curve using appropriate dilutions and calculated for nmoles/g wt. of the tissue.

Determination of SOD Activity

The brain regions- hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats were homogenized in 0.1M potassium phosphate buffer, pH 7.8 and centrifuged at 100,000 x g for 60 min at 4°C. The supernatant corresponds to the cytosolic fraction containing CuZn-SOD. The pellets were re -suspended in the buffer, freeze-thawed three times and centrifuged at 100,000 x g for 60 min at 4°C. The supernatant, the particulate fraction containing Mn-SOD, was mixed with the cytosolic fraction to obtain the total enzyme fraction. SOD was analyzed after inhibition by SOD of the pyrogallol autoxidation (Marklund & Marklund, 1974) at pH 8.2 in the presence of EDTA. A 3ml assay mixture contained 0.2 mM pyrogallol, 1 mM EDTA and 50 mM. Tris-HCl buffer. Pyrogallol autoxidation was monitored at 420 nm for 3 min in a spectrophotometer (Shimadzu UV-1700) with or without the enzyme. The inhibition of pyrogallol oxidation was linear with the activity of the enzyme present. Fifty percent inhibition/mg protein/min was taken as one unit of the enzyme activity.

GLUTAMATE RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

NMDA receptor binding studies

The membrane fractions were prepared by a modification of the method described by Hoffman *et al*., (1996). The brain regions (cerebral cortex, hippocampus, cerebellum and brain stem) and pancreas were homogenized in a 0.32 M sucrose buffer solution containing 10 mM HEPES, 1 mM EDTA buffer, pH 7.0. The homogenate was centrifuged at 1,000 × g for 10 min and the supernatant was centrifuged at 40,000 × g for 1 h. The pellet was re-suspended and homogenized in 10 mM HEPES buffer containing 1.0 mM EDTA, pH 7.0 and centrifuged at 40,000 × g for 1 h. The final pellet was suspended in 10 mM HEPES, 1 mM EDTA buffer, pH 7.0 and stored at -80°C until binding assays were performed. The [^{3}H] MK-801 binding saturation assay was performed in a concentration range of 0.25 to 50 nM at 23°C in an assay medium containing 10 mM HEPES, pH 7.0, 200 - 250 μg of protein, 100 μM glycine and 100 μM glutamate. After 1 h of incubation, the reaction was stopped by filtration through GF/B filters and washed thrice with HEPES buffer pH 7.0. Specific [^{3}H] MK-801 binding was obtained by subtracting nonspecific binding in the presence of 100 μM unlabeled MK-801 from the total binding. Bound radioactivity was counted with cocktail-T in a Perkin Elmer Tri-Carb 2810 TR liquid scintillation analyser.

AMPA receptor binding studies

The brain regions (cerebral cortex, hippocampus, cerebellum and brain stem) and pancreas of control and experimental rats were homogenized in 25 volumes of cold 50 mM Tris-HCl, 10 mM EDTA, pH 7.1, buffer with a Polytron 10,000rpm for 30 s. The pellet was re-suspended in 50 volumes of 50 mM Tris-HC1 pH 7.1, containing 0.04% Triton X-100. The homogenate was incubated for 30 minutes at 37°C, then washed three times with 50 mM Tris-HCl, pH 7.1,

binding buffer, and centrifuged as above. The final pellet was re-suspended in 50 volumes of binding buffer original wet weight and used as such in the assay. The final concentration of membrane in the assay was 10 mg/ml wet weight. The incubation was performed in the presence of 1, 2.5, 5, 7.5 nM [^{3}H] AMPA respectively, specific activity 45.8 Ci/mmol]. Nonspecific binding was determined in the presence of 1 mM AMPA. After 1 h of incubation at 4°C, the suspension was filtered in Whatman GF/C and washed five times with 3 ml of cold binding buffer. The radioactivity on the filter was measured by liquid scintillation spectrometer. Specific binding was determined by subtracting non-specific binding from the total binding.

Protein determination

Protein was measured by the method of Lowry *et al*., (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein which was read in Spectrophotometer at 660nm.

ANALYSIS OF THE RECEPTOR BINDING DATA

Linear regression analysis for Scatchard plots

The data was analysed according to Scatchard, (1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

GENE EXPRESSION STUDIES IN DIFFERENT BRAIN REGIONS AND PANCREAS OF CONTROL AND EXPERIMENTAL RATS

Isolation of RNA

RNA was isolated from the brain regions (cerebral cortex, hippocampus, cerebellum and brain stem) and pancreas of control and experimental rats using the Tri reagent from Sigma Chemicals Co., St. Louis, MO, U.S.A). 25-50 mg tissue homogenates were made in 0.5 ml Tri Reagent. The homogenate was kept in the room temperature for 5 minutes. 100 µl of chloroform was added to the homogenate, mixed vigorously for 15 seconds kept in the RT for 10-15 minutes and was centrifuged at 12,000xg for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000xg for 10 minutes at 4°C. RNA precipitated as a pellet on the sides and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000xg for 5 minutes at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2 µl of RNA was made up to 1 ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one absorbance A_{260} = 42µg.

REAL-TIME POLYMERASE CHAIN REACTION

cDNA synthesis

Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2ml microfuge tubes. The reaction mixture of 20 µl contained 0.2µg total RNA, 10 X RT buffer, 25 X dNTP mixture, 10 X random primers, MultiScribe RT (50U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 minutes and 37 °C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA designed using Primer Express software version (3.0).

Real-time PCR assays

Real Time PCR assays were performed in 96-well plates in an ABI 7300 Real Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase "polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan probe, designed by Applied Biosystems. All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5' end and a quencher (Minor Groove Binding Protein - MGB) at the 3' end. The Real-Time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20 µl contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and PCR analyses were conducted with gene-specific primers and fluorescently labelled Taqman probes of NMDAR1, NMDAR2, GluR4 AMPAR, GluR2 AMPAR, GLAST, Gpx, GAD, Akt 1, Bax and Caspases 8. Endogenous control (β-actin) was labeled with a reporter dye (VIC). 12.5 µl of TaqMan 2X Universal PCR Master Mix was taken and the volume was made up with RNAse free water. Each run contained both negative (no template) and positive controls.

The thermocycling profile conditions were as follows:

50ºC -- 2 minutes ---- Activation

95ºC -- 10 minutes ---- Initial Denaturation

95ºC -- 15 seconds ---- Denaturation 40 cycles

50ºC -- 30 seconds --- Annealing

60ºC -- 1 minute --- Final Extension

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β- actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{- actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta}C\ T$).

IP3 CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

The brain regions (cerebral cortex, hippocampus, cerebellum and brain stem) and pancreas were homogenised in a polytron homogeniser in 50 mM Tris-HCl buffer, pH.7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15 min and the supernatant was transferred to fresh tubes for IP3 assay using [^{3}H] IP3 Biotrak Assay System kit.

Principle of the assay

The assay was based on competition between [^{3}H] IP3 and unlabelled IP3 in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound IP3 was then separated from the free IP3 by centrifugation. The free IP3 in the supernatant was then discarded by simple decantation, leaving the bound fraction adhering to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled IP3 in the sample to be determined.

Assay Protocol

Standards, ranging from 0.19 to 25 pmoles/tube, [^{3}H] IP3 and binding protein were added together and the volume was made up to 100 µl with assay

buffer. Samples of appropriate concentration from experiments were used for the assay. The tubes were then vortexed and incubated on ice for 15 minutes and they were centrifuged at 2000 x g for 10 minutes at 4°C. The supernatant was aspirated out and the pellet was re-suspended in water and incubated at room temperature for 10 minutes. The tubes were then vortexed and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with %B/Bo on the Y-axis and IP3 concentration (pmoles/tube) on the X-axis of a semi-log graph paper. %B/Bo was calculated as:

$$\frac{\text{(Standard or sample cpm – NSB cpm)}}{\text{(Bo cpm – NSB cpm)}} \times 100$$

NSB- non specific binding and B_0 - zero binding. IP3 concentrations in the samples were determined by interpolation from the plotted standard curve.

NMDA R1, NMDA 2B AND AMPA GluR2 GluR4 RECEPTOR SUBUNIT EXPRESSION STUDIES IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Control and experimental rats were deeply anesthetized with ether. The rats were transcardially perfused with phosphate buffered saline (PBS), pH- 7.4, followed by 4% paraformaldehyde in PBS (Chen *et al*., 2007). After perfusion the brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1 M PBS, pH- 7.0. 10 µm sections were cut using cryostat (Leica, CM1510 S). The sections were treated with PBS. To block unspecific binding the sections were incubated for 1 hour at room temperature with 5% bovine serum albumin in normal goat serum. Brain slices were incubated overnight at 4°C with either rat primary antibody for NMDA

R1 (diluted in Phosphate buffered saline Triton X- 100 (PBST) at 1: 500 dilution), NMDA 2B (diluted in PBST at 1: 500 dilution) and AMPA (GluR2) (diluted in PBST at 1: 500 dilution) and AMPA (GluR4) (diluted in PBST at 1: 500 dilution) After overnight incubation, the brain slices were rinsed with PBST and then incubated with appropriate secondary antibody of either FITC (diluted in PBST at 1: 1000 dilution) or rhodamine dye (Chemicon, diluted in PBST at 1: 1000 dilution). The sections were observed and photographed using confocal imaging system (Leica SP 5).

INSULIN, AMPA GluR4, GluR2, VITAMIN D AND IP3 RECEPTOR EXPRESSION STUDIES IN THE PANCREAS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Anaesthetized rats with chloral hydrate were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). After perfusion the pancreas was dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1M PBS. 10 µm sections were cut using cryostat (Leica, CM1510 S). To block unspecific binding the sections were incubated for 1 hour at room temperature with 5% bovine serum albumin in normal goat serum. Pancreatic sections were incubated overnight at 4°C with either rat primary antibody for IP3 receptor (diluted in PBST at 1: 500 dilution) and Vitamin D receptor (diluted in PBST at 1: 500 dilution. After overnight incubation, the pancreatic sections were rinsed with PBST and then incubated with appropriate secondary antibody of either FITC (diluted in PBST at 1: 1000 dilution) or rhodamine dye (Chemicon, diluted in PBST at 1: 1000 dilution).

For co-label studies after blocking with goat serum the pancreatic sections were incubated overnight at 4°C with primary antibody for AMPA (GluR4) (diluted in PBST at 1: 500 dilution) and AMPA (GluR2) receptor (diluted in PBST at 1: 500 dilution) subunits. After overnight incubation, the pancreatic sections were rinsed with PBST and then incubated with Alexa flour 594 secondary antibody (diluted in PBST at 1: 1000 dilution) for 2 h at room

temperature. At the end of incubation period, sections were washed three times using PBST and incubated overnight with rat primary antibody for Insulin. The slides were washed with PBST after the incubation time and secondary antibody Alexa Fluor 488(diluted in PBST at 1: 1000 dilution) was added and incubated for 2 hours at. The sections were observed and photographed using confocal imaging system (Leica SP 5). After incubation pancreatic slices were thoroughly washed, mounted, observed and photographed using confocal imaging system (Leica SP 5).

ISOLATION OF PANCREATIC ISLETS

Pancreatic islets were isolated from male Wistar rats by standard collagenase digestion procedures using aseptic techniques (Howell & Taylor, 1968). The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers *et al.*, 1985) with the following composition: 137 mM Choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM KH_2PO_4, 14.3 mM $KHCO_3$ and 10 mM HEPES**.** Autoclaved triple distilled water was used in the preparation of the buffer.

The pancreas from the rats were aseptically dissected out into a sterile petridish containing ice cold HBSS and excess fat and blood vessels were removed. The pancreas was cut into small pieces and transferred to a sterile glass vial containing 2 ml collagenase type XI solution (1.5 mg/ml in HBSS, pH 7.4). The collagenase digestion was carried out for 15 minutes at 37°C in an environmental shaker with vigorous shaking (300 rpm/minute). The tissue digest was filtered through 500 μm nylon screen and the filtrate was washed with three successive centrifugations and re-suspensions in cold HBSS. The pancreatic islet preparation having a viability of >90% as assessed by Trypan Blue exclusion which was chosen for experiments.

CALCIUM IMAGING STUDIES USING CONFOCAL MICROSCOPE

Pancreatic islets were prepared from adult rats by collagenase digestion method as mentioned earlier. The isolated islets were incubated for 4 hours at room temperature in 1 ml of calcium free RPMI medium containing 5 μM of Ca^{2+} fluorescent dye, fluo 4-AM (Molecular Probes, Eugene, OR) to monitor the changes in the intracellular Ca^{2+}. Following experimental conditions were given; Control: Pancreatic islet in 4mM glucose Diabetic: Pancreatic islet in 20mM glucose, D+I: Pancreatic islet in 20mM glucose, insulin and D+C Pancreatic islet in 20mM glucose, curcumin (Abdel Aziz *et al.,* 2010) and, D+V Pancreatic islet in 20mM glucose, 10^{-12} M Vitamin D_3 (Bourlon et al., 1999). After incubation cells were washed twice in indicator free RPMI medium to remove excess dye that was non-specifically associated with the cell surface and then incubated for further 30 minutes to allow complete de-esterification of intra- cellular AM esters. The 35 mm plates, containing pancreatic islet cells were placed on the stage of a Leica TCS SP5 laser scanning confocal microscope equipped with a HC PL FLUOTAR 20.0x 0.50 dry objective (NA 0.5). Fluo 4-AM was excited with 514 nm laser lines from an argon laser, with laser intensity set at 38% of available power. For visualization of Fluo 4-AM, the emission window was set at 508.4 nm – 571.5 nm. The images were continuously acquired before and after addition of 10^{-5} M AMPA (Bertrand et al., 1992) at time intervals of 26.35, 104.1 seconds. Time series experiments were performed collecting 512x512 pixel images at 400 Hz. Fluorescence intensity was analysed using the quantitation mode in LAS-AF software from Leica Microsystems, Germany. A region of interest (ROI) was drawn within a field of view. For each ROI, the pixel intensity was calculated for each image in the 600 seconds sequence to analyse the intracellular Ca^{2+} release from the pancreatic islet cells in experimental conditions.

Statistics

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03). Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve fitting procedure (GraphPad PRISM^{TM}, San Diego, USA). Empower software were used for HPLC analysis. Relative Quantification Software was used for analyzing Real-Time PCR results.

Results

BODY WEIGHT

The body weight was significantly decreased ($p<0.001$) in the diabetic rats when compared to control. Body weight of diabetic rats was decreased significantly in 7^{th} day. After insulin treatment, curcumin and vitamin D_3 supplementation for 14 days, the body weight was significantly reversed ($p<0.001$) when compared with diabetic rats. (Figure-1, Table-1).

BLOOD GLUCOSE LEVEL

Blood glucose level of all rats before streptozotocin administration was within the normal range. Streptozotocin administration in rats led to a significant increase ($p<0.001$) in blood glucose level when compared to control group. Insulin curcumin and vitamin D_3 treatments were significantly reversed ($p<0.001$) the increased blood glucose level when compared to diabetic group (Figure-2, Table-2).

CIRCULATING INSULIN LEVEL

There was a significant decrease in the serum insulin level of the diabetic group when compared to control ($p<0.001$). Insulin, curcumin and vitamin D_3 treatment for 14 days significantly increased ($p<0.001$) the serum insulin when compared to diabetic group (Figure-3, Table-3).

CEREBRAL CORTEX

Glutamate content in the cerebral cortex of control and experimental rats

Glutamate content was significantly ($p<0.001$) increased in cerebral cortex of the diabetic rats compared to the control. Treatment using insulin ($p<0.05$) curcumin ($p<0.001$) and vitamin D_3 ($p<0.001$) significantly reversed the glutamate content when compared to diabetic group. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in glutamate content when compared with insulin treatment (Figure -4, Table- 4).

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebral cortex of control and experimental rats

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebral cortex of diabetic rats showed a significant ($p<0.001$) increase in B_{max} compared to control rats. This shows increased NMDA receptor density in the cerebral cortex of diabetic rats. Significant reversal in the B_{max} was observed in treatment groups: insulin ($p<0.01$), curcumin ($p<0.001$) and vitamin D_3 ($p<0.001$). There was no significant change in K_d in all experimental groups of rats. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in B_{max} when compared with insulin treatment (Figure- 5, 6 & Table- 5, 6).

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the cerebral cortex of control and experimental rats

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the cerebral cortex of diabetic rats showed a significant increase in B_{max} ($p< 0.001$) compared to control rats. This result showed increased AMPA receptor density in the cerebral cortex of diabetic rats compared to control. Treatment using insulin ($p<0.01$), curcumin ($p<0.01$) and vitamin D_3 ($p<0.05$) significantly reversed the changes in receptor binding when compared with diabetic group. There was no significant change in K_d in all experimental groups of rats (Figure- 7, 8 & Table- 7, 8).

Real time PCR amplification of NMDA R1 receptor subunit mRNA from the cerebral cortex of control and experimental rats

Gene expression of NMDA R1 receptor subunit mRNA showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. Treatment using insulin, curcumin and vitamin D_3 significantly ($p<0.001$) reversed the altered gene expression when compared with diabetic group (Figure-9, Table-9).

Real time PCR amplification of NMDA 2B receptor subunit mRNA from the cerebral cortex of control and experimental rats

Real-time PCR gene expression of NMDA 2B receptor subunit showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. There was a significant reversal ($p<0.001$) in NMDA 2B receptor subunit gene expression in diabetic rats treated with insulin, curcumin and vitamin D_3 (Figure-10, Table-10).

Real time PCR amplification of GluR4 subunit of AMPA receptor mRNA from the cerebral cortex of control and experimental rats

Real-time PCR gene expression of GluR4 subunit of AMPA receptor subunit showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control rats. There was a significant reversal ($p<0.001$) in AMPA GluR4 subunit gene expression in diabetic rats treated with insulin, curcumin and vitamin D_3 (Figure-11, Table-11).

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the cerebral cortex of control and experimental rats

Gene expression of AMPA GluR2 receptor subunit mRNA showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. Treatment using insulin, curcumin and vitamin D_3

significantly ($p<0.001$) reversed these changes when compared with diabetic group (Figure -12, Table -12).

Real time PCR amplification of GLAST mRNA from cerebral cortex of control and experimental rats

Real-time PCR gene expression of GLAST showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats. There was significant reversal ($p<0.001$) in GLAST gene expression in diabetic rats treated with insulin, curcumin and vitamin D_3. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in GLAST gene expression when compared with insulin treatment (Figure -13, Table -13).

Real time PCR amplification of GAD mRNA from the cerebral cortex of control and experimental rats

Real-time PCR gene expression of GAD showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats. Insulin ($p<0.01$) and curcumin ($p<0.001$) treated diabetic rats showed a significant reversal when compared to diabetic rats. In vitamin D_3 treated rats GAD gene expression was significantly reversed and up regulated ($p<0.001$) when compared with diabetic rats (Figure-14; Table-14). Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in GAD gene expression when compared with insulin treatment.

IP3 content in cerebral cortex of control and experimental rats

IP3 content was significantly increased ($p<0.001$) in the cerebral cortex of diabetic rats when compared to control rats. Insulin ($p<0.001$), curcumin ($p<0.001$) and vitamin D_3 ($p<0.001$) treatment in diabetic rats significantly reversed the IP3 content when compared to diabetic group. Vitamin D_3 treatment showed prominent reversal ($p<0.05$) in IP3 content when compared with insulin treatment (Figure-15; Table-15).

Superoxide dismutase assay in the cerebral cortex of control and experimental rats

There was a significant decrease in SOD activity ($p<0.001$) in cerebral cortex of diabetic rats. Treatment using insulin ($p<0.05$), Curcumin ($p<0.001$) and Vitamin D_3 ($p<0.001$) reversed the activity of SOD enzyme when compared with diabetic rats. Curcumin treatment showed prominent reversal ($p<0.05$) in SOD activity when compared with insulin treatment (Figure-16; Table-16).

Real time PCR amplification of GPx mRNA from the cerebral cortex of control and experimental rats

Real time PCR gene expression of GPx showed significant down regulation ($p<0.001$) in the cerebral cortex of the diabetic rats compared to the control. Treatment using insulin, curcumin and vitamin D_3 significantly reversed ($p<0.001$) the changes when compared with diabetic rats. Curcumin treatment showed prominent reversal ($p<0.001$) in GPx gene expression when compared with insulin treatment (Figure-17; Table-17).

Real time PCR amplification of Akt-1 mRNA from the cerebral cortex of control and experimental rats

Real-time PCR gene expression of Akt-1 showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats. Treatment using insulin and curcumin significantly reversed ($p<0.001$) the altered gene expression when compared with diabetic rats. In vitamin D_3 treated diabetic rats, there was significant ($p<0.001$) reversal and up regulation of Akt-1 gene expression when compared to diabetic and control rats respectively. Vitamin D_3 treatment showed prominent reversal ($p<0.001$) in Akt-1 gene expression when compared with insulin treatment (Figure-18; Table-18).

Real time PCR amplification of Bax mRNA from the cerebral cortex of control and experimental rats

Real-time PCR gene expression of Bax showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control rats. The Bax gene expression was reversed significantly in insulin ($p<0.01$) and curcumin ($p<0.001$) and vitamin D_3 ($p<0.001$) treated rats when compared with diabetic rats. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in Bax gene expression when compared with insulin treatment (Figure-19; Table-19).

Real time PCR amplification of caspase 8 mRNA from the cerebral cortex of control and experimental rats

Real-time PCR gene expression of caspase 8 showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control rats. There was a significant reversal ($p<0.001$) in Caspase 8 gene expression in diabetic rats treated with insulin, curcumin and vitamin D_3 (Figure-20; Table-20).

NMDA R1 receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

NMDA R1 subunit specific antibody staining in the cerebral cortex showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin ($p<0.05$), curcumin ($p<0.001$) and vitamin D_3 ($p<0.01$) treatment in diabetic rats significantly reversed mean pixel value when compared with diabetic rats (Figure-21; Table-21). Curcumin treatment showed prominent reversal when compared with insulin treated rats.

NMDA 2B receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

NMDA 2B subunit specific antibody staining in the cerebral cortex showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin ($p<0.01$), curcumin ($p<0.001$) and vitamin D_3

($p<0.001$) treatment in diabetic rats significantly mean pixel value when compared with diabetic rats (Figure-22; Table-22).

AMPA (GluR4) receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

AMPA (GluR4) receptor subunit specific antibody staining in the cerebral cortex showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin, curcumin and vitamin D_3 treatment in diabetic rats significantly ($p<0.001$) reversed the mean pixel value to near control (Figure-23; Table-23).

AMPA (GluR2) receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

AMPA (GluR2) receptor subunit antibody staining in the cerebral cortex showed a significant decrease ($p<0.001$) in the mean pixel value in diabetic rats compared to control. Insulin ($p<0.05$), curcumin ($p<0.001$) and vitamin D_3 ($p<0.01$) treatment to diabetic rats significantly reversed AMPA (GluR2) receptor subunit expression in the cerebral cortex to near control (Table-24, Figure-24).

HIPPOCAMPUS

Glutamate content in the hippocampus of control and experimental rats

Glutamate content was significantly ($p<0.001$) increased in hippocampus of the diabetic rats compared to the control. Treatment using insulin, curcumin and vitamin D_3 reversed ($p<0.001$) the glutamate content when compared to diabetic group. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.01$) in glutamate content when compared with insulin treatment (Figure -25, Table- 25).

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the hippocampus of control and experimental rats

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the hippocampus of diabetic rats showed a significant ($p<0.001$) increase in B_{max} compared to control rats. Significant reversal ($p<0.001$) in the B_{max} was observed in treatment groups: Insulin, curcumin and vitamin D_3. There was no significant change in K_d in all experimental groups of rats. Vitamin D_3 treatment showed prominent reversal ($p<0.001$) in B_{max} when compared with insulin treatment (Figure- 26, 27 & Table- 26, 27).

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the hippocampus of control and experimental rats

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the hippocampus of diabetic rats showed a significant increase in B_{max} ($p< 0.001$) compared to control rats. Treatment using insulin ($p<0.05$), curcumin ($p<0.001$) and vitamin D_3 ($p<0.001$) significantly reversed the changes in receptor binding when compared with diabetic group. There was no significant change in K_d in all experimental groups of rats. Vitamin D_3 ($p<0.001$) treatment showed prominent reversal in B_{max} when compared with insulin treatment (Figure- 28, 29 & Table- 28, 29).

Real time PCR amplification of NMDA R1 receptor subunit mRNA from the hippocampus of control and experimental rats

Gene expression of NMDA R1 receptor subunit mRNA showed significant up regulation ($p<0.001$) in the hippocampus of diabetic rats compared to control. Treatment using insulin, curcumin and vitamin D_3 significantly reversed ($p<0.001$) the altered gene expression when compared with diabetic group. Curcumin treatment showed prominent reversal ($p<0.001$) in NMDA R1 receptor subunit gene expression when compared with insulin treatment (Figure-30, Table-30).

Real time PCR amplification of NMDA 2B receptor subunit mRNA from the hippocampus of control and experimental rats

Gene expression of NMDA 2B receptor subunit mRNA showed significant up regulation ($p<0.001$) in the hippocampus of diabetic rats compared to control. Treatment using insulin, curcumin and vitamin D_3 significantly ($p<0.001$) reversed these changes when compared with diabetic group. Curcumin treatment showed prominent reversal ($p<0.001$) in NMDA 2B receptor subunit gene expression when compared with insulin treatment (Table-31, Figure-31).

Real time PCR amplification of GluR4 subunit of AMPA receptor mRNA from the hippocampus of control and experimental rats

Real-time PCR gene expression of GluR4 subunit of AMPA receptor subunit showed significant up regulation ($p<0.001$) in the hippocampus of diabetic rats compared to control rats. AMPA GluR4 subunit gene expression was significantly reversed in diabetic rats treated with insulin ($p<0.01$), curcumin and vitamin D_3 ($p<0.001$) when compared with diabetic. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in GluR4 subunit of AMPA receptor subunit gene expression when compared with insulin treatment (Figure-32, Table-32).

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the hippocampus of control and experimental rats

Real-time PCR gene expression of GluR2 subunit of AMPA receptor subunit showed significant down regulation ($p<0.001$) in the hippocampus of diabetic rats compared to control rats. There was a significant reversal ($p<0.001$) in AMPA GluR2 subunit gene expression in diabetic rats treated with insulin, curcumin and vitamin D_3 (Figure-33, Table-33).

Real time PCR amplification of GLAST mRNA from hippocampus of control and experimental rats

Gene expression of GLAST mRNA showed significant down regulation ($p<0.001$) in the hippocampus of diabetic rats compared to control. Treatment using insulin, curcumin and vitamin D_3 significantly reversed ($p<0.001$) the changes to near control. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in GLAST gene expression when compared with insulin treatment (Figure-34, Table-34).

Real time PCR amplification of GAD mRNA from the hippocampus of control and experimental rats

Real-time PCR gene expression of GAD showed significant down regulation ($p<0.001$) in the hippocampus of diabetic rats. Insulin, curcumin and vitamin D_3 treatment showed a significant reversal ($p<0.001$) when compared with diabetic rats. Vitamin D_3 treatment showed prominent reversal ($p<0.001$) in GAD gene expression when compared with insulin treatment (Figure-35; Table-35).

IP3 content in hippocampus of control and experimental rats

IP3 content was significantly increased ($p<0.001$) in the hippocampus of diabetic rats when compared to control rats. Insulin, curcumin and vitamin D_3 treatment in diabetic rats significantly reversed ($p<0.001$) the IP3 content to near control (Figure-36; Table-36).

Superoxide dismutase assay in the hippocampus of control and experimental rats

There was a significant decrease in SOD activity ($p<0.001$) in hippocampus of diabetic rats. Treatment using curcumin ($p<0.01$) and vitamin D_3 ($p<0.05$) reversed the activity of SOD enzyme when compared with diabetes Treatment using insulin did not showed any significant reversal. Curcumin treatment showed prominent reversal ($p<0.01$) in SOD activity when compared with insulin treatment (Figure-37; Table-37).

Real time PCR amplification of GPx mRNA from the hippocampus of control and experimental rats

Real time PCR gene expression of GPx showed significant down regulation ($p<0.001$) in the hippocampus of the diabetic rats compared to the control. Treatment using insulin and vitamin D_3 significantly reversed ($p<0.001$) the changes when compared with diabetic rats. Curcumin treatment significantly reversed and up regulate the GPx gene expression when compared with both control ($p<0.01$) and diabetic ($p<0.001$). Curcumin ($p<0.001$) treatment showed prominent reversal in GPx gene expression when compared with insulin treatment (Figure-38; Table-38).

Real time PCR amplification of Akt-1 mRNA from the hippocampus of control and experimental rats

Real-time PCR gene expression of Akt-1 showed significant down regulation ($p<0.001$) in the hippocampus of diabetic rats. Curcumin and insulin treated diabetic rats showed a significant ($p<0.001$) reversal of Akt-1 gene expression when compared to diabetic rats. Vitamin D_3 treatment showed reversal ($p<0.001$) and up regulation in Akt-1 gene expression when compared with both control and diabetic group. (Figure-39; Table-39).

Real time PCR amplification of Bax mRNA from the hippocampus of control and experimental rats

Real-time PCR gene expression of Bax showed significant up regulation ($p<0.001$) in the hippocampus of diabetic rats compared to control rats. The Bax gene expression was reversed significantly ($p<0.001$) in insulin, curcumin and vitamin D_3 treated rats when compared with diabetic rats. Curcumin ($p<0.001$) treatment showed prominant reversal in Bax gene expression when compared with insulin treatment (Figure-40; Table-40).

Real time PCR amplification of caspase 8 mRNA from the hippocampus of control and experimental rats

Real-time PCR gene expression of caspase 8 showed significant up regulation ($p<0.001$) in the hippocampus of diabetic rats compared to control rats. There was a significant reversal ($p<0.001$) in Caspase 8 gene expression in diabetic rats treated with insulin, curcumin and vitamin D_3. Curcumin ($p<0.001$) treatment showed prominent reversal in caspase 8 gene expression when compared with insulin treatment (Figure-41; Table-41).

NMDA R1 receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

NMDA R1 subunit specific antibody staining in the hippocampus showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin ($p<0.01$), curcumin ($p<0.001$) and vitamin D_3 ($p<0.001$) treatment in diabetic rats significantly reversed the mean pixel value when compared with diabetic group (Figure-42; Table-42).

NMDA 2B receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

NMDA 2B subunit specific antibody staining in the hippocampus showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin, curcumin and vitamin D_3 treatment in diabetic rats

significantly ($p<0.001$) reversed the mean pixel value when compared with diabetic group (Figure-43; Table-43).

AMPA (GluR4) receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

AMPA (GluR4) receptor subunit specific antibody staining in the hippocampus showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin ($p<0.01$), curcumin ($p<0.001$) and vitamin D_3 ($p<0.001$) treatment in diabetic rats significantly reversed the mean pixel value when compared with diabetic group (Figure-44; Table-44).

AMPA (GluR2) receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

AMPA (GluR2) receptor subunit antibody staining in the hippocampus showed a significant decrease ($p<0.01$) in the mean pixel value in diabetic rats compared to control. Insulin ($p<0.05$), curcumin ($p<0.05$) and vitamin D_3 ($p<0.05$) treatment to diabetic rats significantly reversed AMPA (GluR2) receptor subunit expression in the hippocampus when compared with diabetic rats (Table-45, Figure-45).

BRAIN STEM

Glutamate content in the brain stem of control and experimental rats

Glutamate content was significantly ($p<0.001$) increased in brain stem of the Diabetic rats compared to the control The glutamate content was reversed significantly ($p<0.001$) in insulin, curcumin, and vitamin D_3 treated rats when compared with diabetic rats. (Figure -46, Table- 46). Vitamin D_3 treatment showed prominent reversal ($p<0.05$) in glutamate content when compared with insulin treatment.

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the brain stem of control and experimental rats

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the brain stem of diabetic rats showed a significant ($p<0.001$) increase in B_{max} compared to control rats. Significant reversal ($p<0.001$) in the B_{max} was observed in treatment groups: Insulin, curcumin and vitamin D_3. There was no significant change in K_d in all experimental groups of rats. Vitamin D_3 treatment showed prominent reversal ($p<0.001$) in B_{max} when compared with insulin treatment (Figure- 47, 48 & Table- 47, 48).

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the brain stem of control and experimental rats

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the brain stem of diabetic rats showed a significant increase in B_{max} ($p<0.001$) compared to control rats. Treatment using curcumin ($p<0.01$) and vitamin D_3 ($p<0.001$) significantly reversed the changes in receptor binding when compared with diabetic group. There was no significant change in K_d in all experimental groups of rats. Vitamin D_3 ($p<0.001$) treatment showed prominent reversal in B_{max} when compared with insulin treatment (Figure- 49, 50 & Table- 49, 50).

Real time PCR amplification of NMDA R1 receptor subunit mRNA from the brain stem of control and experimental rats

Gene expression of NMDA R1 receptor subunit mRNA showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. There was a significant reversal ($p<0.001$) in NMDA R1 receptor subunit gene expression in diabetic rats treated with insulin, curcumin, and vitamin D_3 (Figure-51, Table-51).

Real time PCR amplification of NMDA 2B receptor subunit mRNA from the brain stem of control and experimental rats

Real-time PCR gene expression of NMDA 2B receptor subunit showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. Treatment using insulin, curcumin and vitamin D_3 significantly reversed ($p<0.001$) the altered expression when compared with diabetic group. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in NMDA 2B gene expression when compared with insulin treatment (Figure-52, Table-52).

Real time PCR amplification of GluR4 subunit of AMPA receptor mRNA from the brain stem of control and experimental rats

Real-time PCR gene expression of GluR4 subunit of AMPA receptor subunit showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control rats. There was a significant reversal ($p<0.001$) in AMPA GluR4 subunit gene expression in diabetic rats treated with curcumin and vitamin D_3. Insulin treatment did not show any significant reversal when compared with diabetes. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in gene expression of GluR4 subunit of AMPA when compared with insulin treatment (Figure-53, Table-53).

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the brain stem of control and experimental rats

Gene expression of AMPA GluR2 subunit receptor mRNA showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. Treatment using insulin, curcumin and vitamin D_3 significantly ($p<0.001$) reversed these changes when compared with diabetic group. Curcumin treatment showed prominent reversal ($p<0.001$) in gene expression of AMPA GluR2 subunit when compared with insulin treatment (Table-54, Figure-54).

Real time PCR amplification of GLAST mRNA from brain stem of control and experimental rats

Real-time PCR Gene expression of GLAST showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats. Treatment using insulin, curcumin and vitamin D_3 significantly reversed ($p<0.001$) the altered expression when compared with diabetic group. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in gene expression of GLAST when compared with insulin treatment (Figure-55, Table-55).

Real time PCR amplification of GAD mRNA from the brain stem of control and experimental rats

Real-time PCR Gene expression of GAD showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats. There was a significant reversal ($p<0.001$) in GAD gene expression in diabetic rats treated with insulin, curcumin and vitamin D_3. Curcumin ($p<0.001$) treatment showed prominent reversal in GAD gene expression when compared with insulin treatment (Figure-56; Table-56).

IP3 content in brain stem of control and experimental rats

IP3 content was significantly increased ($p<0.001$) in the brain stem of diabetic rats when compared to control rats. Curcumin ($p<0.001$) and vitamin D_3 ($p<0.001$) treatment in diabetic rats significantly reversed the IP3 content when

compared with diabetic group. Insulin treated rats did not show any significant reversal when compared to diabetic rats. Curcumin and vitamin D_3 treatment showed significant reversal ($p<0.05$) in IP3 content when compared with insulin treatment (Figure-57; Table-57).

Superoxide dismutase assay in the brain stem of control and experimental rats

There was a significant decrease in SOD activity ($p<0.001$) in brain stem of diabetic rats. Treatment using insulin, curcumin and vitamin D_3 reversed ($p<0.001$) the activity of SOD enzyme when compared with diabetes. Curcumin ($p<0.05$) treatment showed prominent reversal in SOD activity when compared with insulin treatment (Figure-58; Table-58).

Real time PCR amplification of GPx mRNA from the brain stem of control and experimental rats

Real time PCR gene expression of GPx showed significant down regulation ($p<0.001$) in the brain stem of the diabetic rats compared to the control. Insulin, curcumin and vitamin D_3 treatment significantly reversed ($p<0.001$) the changes when compared with diabetes. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in GPx gene expression when compared with insulin treatment (Figure-59; Table-59).

Real time PCR amplification of Akt-1 mRNA from the brain stem of control and experimental rats

Real-time PCR Gene expression of Akt-1 showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats. Curcumin treatment showed significant down regulation when compared with diabetic group. In vitamin D_3 treated diabetic rats, there was prominent ($p<0.001$) reversal and up regulation of Akt-1 gene expression when compared to diabetic, control and insulin treated rats. Insulin treated rats did not show any significant reversal when compared to diabetic rats (Figure-60; Table-60).

Real time PCR amplification of Bax mRNA from the brain stem of control and experimental rats

Real-time PCR Gene expression of Bax showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control rats. There was a significant reversal ($p<0.001$) in Bax gene expression in diabetic rats treated with insulin, curcumin and vitamin D_3. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in Bax gene expression when compared with insulin treatment (Figure-61; Table-61).

Real time PCR amplification of caspase 8 mRNA from the brain stem of control and experimental rats

Real-time PCR Gene expression of caspase 8 showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control rats. Insulin, curcumin and vitamin D_3 treatment significantly reversed ($p<0.001$) the changes when compared with diabetic. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in caspase 8 gene expression when compared with insulin treatment (Figure-62; Table-62).

NMDA R1 receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

NMDA R1 subunit specific antibody staining in the brain stem showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin, curcumin and vitamin D_3 treatment in diabetic rats significantly ($p<0.001$) reversed the mean pixel value to near control (Figure-63; Table-63).

NMDA 2B receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

NMDA 2B subunit specific antibody staining in the brain stem showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin, curcumin and vitamin D_3 treatment in diabetic rats significantly reversed ($p<0.001$) the mean pixel value to near control (Figure-64; Table-64).

AMPA (GluR4) receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

AMPA (GluR4) receptor subunit specific antibody staining in the brain stem showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin, curcumin and vitamin D_3 treatment in diabetic rats significantly ($p<0.05$) reversed the mean pixel value when compared with diabetic group (Figure-65; Table-65).

AMPA (GluR2) receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

AMPA (GluR2) receptor subunit antibody staining in the brain stem showed a significant decrease ($p<0.001$) in the mean pixel value in diabetic rats compared to control. Insulin, curcumin ($p<0.05$) and vitamin D_3 ($p<0.01$) treatment to diabetic rats significantly reversed AMPA (GluR4) receptor subunit expression in the brain stem to near control. Vitamin D_3 treatment showed prominent reversal when compared with insulin (Table-66, Figure-66).

CEREBELLUM

Glutamate content in the cerebellum of control and experimental rats

Glutamate content was significantly ($p<0.001$) increased in cerebellum of the diabetic rats compared to the control. There was significant reversal in glutamate content in Insulin ($p<0.01$) curcumin ($p<0.001$) and vitamin D_3 ($p<0.001$) treated rats. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in glutamate content when compared with insulin treatment. (Figure -67, Table- 67).

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebellum of control and experimental rats

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebellum of diabetic rats showed a significant ($p<0.001$) increase in B_{max} compared to control rats. This shows increased NMDA receptor density in the cerebellum of diabetic rats. Significant reversal in the B_{max} was observed in treatment groups: curcumin ($p<0.001$) and vitamin D_3 ($p<0.001$). There was no significant change in K_d in all experimental groups of rats. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.01$) in B_{max} when compared with insulin treatment (Figure- 68, 69 & Table- 68, 69).

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the cerebellum of control and experimental rats

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the cerebellum of diabetic rats showed a significant increase in B_{max} ($p<0.001$) compared to control rats. This result showed increased AMPA receptor density in the cerebellum of diabetic rats compared to control. Treatment using curcumin and vitamin D_3 significantly reversed ($p<0.01$) the changes in receptor binding when compared with diabetic group. There was no significant change in K_d in all experimental groups of rats. Treatment using vitamin D_3

showed prominent reversal ($p<0.05$) in B_{max} when compared with insulin treatment (Figure- 70, 71 & Table- 70, 71).

Real time PCR amplification of NMDA R1 receptor subunit mRNA from the cerebellum of control and experimental rats

Gene expression of NMDA R1 receptor subunit mRNA showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. Treatment using insulin, curcumin and vitamin D_3 significantly ($p<0.001$) reversed the altered expression when compared with diabetic group (Figure-72, Table-72). Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in NMDA R1 receptor subunit gene expression when compared with insulin treatment.

Real time PCR amplification of NMDA 2B receptor subunit mRNA from the cerebellum of control and experimental rats

Real-time PCR Gene expression of NMDA 2B receptor subunit showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. Treatment using insulin and curcumin reversed ($p<0.001$) the altered expression when compared with diabetic group. Whereas vitamin D_3 treatment significantly ($p<0.001$) reversed the altered NMDA 2B receptor subunit expression to near control. Vitamin D_3 treatment showed prominent reversal ($p<0.001$) in NMDA 2B receptor subunit gene expression when compared with insulin treatment (Figure-73, Table-73).

Real time PCR amplification of GluR4 subunit of AMPA receptor mRNA from the cerebellum of control and experimental rats

Gene expression of AMPA GluR4 subunit receptor mRNA showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. Treatment using curcumin and vitamin D_3 significantly ($p<0.001$) reversed these changes when compared with diabetic group. Insulin treated rats did not show any significant reversal when compared to diabetic rats. Curcumin

and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in AMPA GluR4 receptor subunit gene expression when compared with insulin treatment (Figure-74, Table-74).

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the cerebellum of control and experimental rats

Gene expression of AMPA GluR2 subunit receptor mRNA showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. Treatment using insulin ($p<0.05$) curcumin ($p<0.001$) and vitamin D_3 significantly ($p<0.001$) reversed these changes when compared with diabetic group. Treatment using curcumin and vitamin D_3 showed prominent reversal ($p<0.001$) in AMPA GluR2 receptor subunit gene expression when compared with insulin treatment (Figure-75; Table-75).

Real time PCR amplification of GLAST mRNA from cerebellum of control and experimental rats

Real-time PCR Gene expression of GLAST showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats. Treatment using insulin ($p<0.001$) reversed the altered expression when compared with diabetic group. Whereas vitamin D_3 and curcumin treatment significantly reversed ($p<0.001$) the altered GLAST gene expression to near control. Treatment using curcumin and vitamin D_3 showed prominent reversal ($p<0.001$) in GLAST gene expression when compared with insulin treatment (Figure-76; Table-76).

Real time PCR amplification of GAD mRNA from the cerebellum of control and experimental rats

Real-time PCR gene expression of GAD showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats. Insulin ($p<0.01$) and curcumin ($p<0.001$) treated diabetic rats showed a significant reversal when compared with diabetic rats. In vitamin D_3 treated rats GAD gene expression was significantly reversed ($p<0.001$) to near control. Curcumin and vitamin D_3

treatment showed prominent reversal ($p<0.001$) in GAD gene expression when compared with insulin treatment (Figure-77; Table-77).

IP3 content in cerebellum of control and experimental rats

IP3 content was significantly increased ($p<0.001$) in the cerebellum of diabetic rats when compared to control rats. Insulin treatment in diabetic rats showed a significant reversal ($p<0.05$) when compared with diabetic rats. In curcumin and vitamin D_3 treated rats IP3 content was significantly reversed ($p<0.001$) to near control. Treatment using curcumin and vitamin D_3 showed prominent reversal ($p<0.001$) in IP3 content when compared with insulin treatment (Figure-78; Table-78).

Superoxide dismutase assay in the cerebellum of control and experimental rats

There was a significant decrease ($p<0.001$) in SOD activity in cerebellum of diabetic rats. Treatment using curcumin ($p<0.001$) reversed the activity of SOD enzyme to near control. Insulin and vitamin D_3 did not show any significant reversal when compared to diabetic rats. (Figure-79; Table-79).

Real time PCR amplification of GPx mRNA from the cerebellum of control and experimental rats

Real time PCR gene expression of GPx showed significant down regulation ($p<0.001$) in the cerebellum of the diabetic rats compared to the control. Treatment using insulin and vitamin D_3 significantly reversed ($p<0.001$) the changes when compared with diabetes. In curcumin treated diabetic rats, there was significant ($p<0.001$) reversal and up regulation of GPx gene expression when compared to diabetic and control rats respectively. Treatment using curcumin and vitamin D_3 showed prominent reversal ($p<0.001$) in GPx gene expression when compared with insulin treatment (Figure-80; Table-80).

Real time PCR amplification of Akt-1 mRNA from the cerebellum of control and experimental rats

Real-time PCR gene expression of Akt-1 showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats. Treatment using curcumin showed significant reversal when compared with control. Treatment using vitamin D_3 significantly reversed ($p<0.001$) and up regulated the altered gene expression when compared with both diabetic and control. Treatment using vitamin D_3 showed prominent reversal ($p<0.001$) in Akt-1 gene expression when compared with insulin treatment (Figure-81; Table-81).

Real time PCR amplification of Bax mRNA from the cerebellum of control and experimental rats

Real-time PCR Gene expression of Bax showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control rats. The Bax gene expression was reversed significantly in insulin ($p<0.001$) treated rats when compared with diabetic rats. . In curcumin and vitamin D_3 treated rats Bax Gene expression was significantly reversed ($p<0.001$) to near control. Vitamin D_3 treatment showed prominent reversal ($p<0.01$) in caspase 8 gene expression when compared with insulin treatment (Figure-82; Table-82).

Real time PCR amplification of caspase 8 mRNA from the cerebellum of control and experimental rats

Real-time PCR gene expression of caspase 8 showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control rats. There was a significant reversal ($p<0.001$) in Caspase 8 gene expression in diabetic rats treated with insulin, curcumin and vitamin D_3. Treatment using curcumin and vitamin D_3 showed prominent reversal ($p<0.001$) in caspase 8 gene expression when compared with insulin treatment (Figure-83; Table-83).

NMDA R1 receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

NMDA R1 subunit specific antibody staining in the cerebellum showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin, curcumin and vitamin D_3 treatment in diabetic rats significantly reversed ($p<0.001$) the mean pixel value to near control (Figure-84; Table-84).

NMDA 2B receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

NMDA 2B subunit specific antibody staining in the cerebellum showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin, curcumin and vitamin D_3 treatment in diabetic rats significantly ($p<0.001$) reversed the mean pixel value to near control (Figure-85; Table-85).

AMPA (GluR4) receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

AMPA (GluR4) receptor subunit specific antibody staining in the cerebellum showed a significant increase ($p<0.001$) in mean pixel value in the diabetic rats when compared to control. Insulin, curcumin and vitamin D_3 treatment in diabetic rats significantly ($p<0.001$) reversed the mean pixel value when compared with diabetic (Figure-86; Table-86).

AMPA (GluR2) receptor subunit antibody staining in control and experimental groups of rats using confocal microscope

AMPA (GluR2) receptor subunit antibody staining in the cerebellum showed a significant decrease ($p<0.001$) in the mean pixel value in diabetic rats compared to control. Insulin, curcumin and vitamin D_3 treatment to diabetic rats significantly reversed ($p<0.01$) AMPA (GluR2) receptor subunit expression in the cerebellum to near control (Table-87, Figure-87).

PANCREAS

Glutamate content in the pancreas of control and experimental rats

Glutamate content was significantly ($p<0.001$) increased in pancreas of the Diabetic rats compared to the control. There was significant reversal ($p<0.001$) in glutamate content in curcumin and vitamin D_3 Treated rats. Insulin treated rats did not show any significant reversal when compared to diabetic rats (Figure -88, Table- 88)

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the pancreas of control and experimental rats

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the pancreas of diabetic and treatment groups did not show any significant change when compared with control. There was no significant change in K_d in all experimental groups of rats (Figure- 89, 90 & Table- 89, 90).

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the pancreas of control and experimental rats

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the pancreas of diabetic rats showed a significant increase in B_{max} ($p< 0.01$) compared to control rats. This result showed increased AMPA receptor density in the pancreas of diabetic rats compared to control. Treatment using insulin ($p<0.05$), curcumin ($p<0.05$) and vitamin D_3 ($p<0.01$) significantly reversed the changes in receptor binding when compared with diabetic group. There was no significant change in K_d in all experimental groups of rats (Figure- 91, 92 & Table- 91, 92).

Real time PCR amplification of GluR4 subunit of AMPA receptor mRNA from the pancreas of control and experimental rats

Gene expression of AMPA GluR4 subunit receptor mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to

control. Treatment using insulin ($p<0.01$) curcumin and vitamin D_3 significantly ($p<0.001$) reversed these changes when compared with diabetic group. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in AMPA GluR4 subunit receptor gene expression when compared with insulin treatment (Figure-93, Table-93).

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the pancreas of control and experimental rats

Gene expression of AMPA GluR2 subunit receptor mRNA showed significant up regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. Treatment using insulin, curcumin and vitamin D_3 significantly reversed ($p<0.001$) these changes when compared with diabetic group. Treatment using vitamin D_3 showed prominent reversal ($p<0.001$) in AMPA GluR2 subunit receptor gene expression when compared with insulin treatment (Figure-94; Table-94).

Real time PCR amplification of GLAST mRNA from Pancreas of control and experimental rats

Real-time PCR Gene expression of GLAST showed significant down regulation ($p<0.001$) in the Pancreas of diabetic rats. Whereas vitamin D_3 and curcumin ($p<0.001$) treatment significantly ($p<0.001$) reversed the altered GLAST gene expression when compared to diabetic rats. Insulin treatment did not show any significant reversal when compared to diabetic rats (Figure-95; Table-95).

IP3 content in the pancreas of control and experimental rats

IP3 content was significantly decreased ($p<0.01$) in the Pancreas of diabetic rats when compared to control rats. In curcumin ($p<0.01$) and vitamin D_3 ($p<0.01$) treated diabetic rats IP3 content was significantly reversed when compared with diabetic group. Insulin treatment did not show any significant reversal when compared to diabetic rats (Figure-96; Table-96).

Superoxide dismutase assay in the Pancreas of control and experimental rats

There was a significant decrease in SOD activity ($p<0.001$) in pancreas of diabetic rats. Vitamin D_3 treatment showed a significant reversal ($p<0.001$) when compared with diabetic group. Treatment using curcumin ($p<0.001$) reversed the activity of SOD enzyme to near control. Insulin treatment did not show any significant reversal when compared to diabetic rats (Figure-97; Table-97).

Real time PCR amplification of GPx mRNA from the Pancreas of control and experimental rats

Real time PCR gene expression of GPx showed significant down regulation ($p<0.001$) in the Pancreas of the diabetic rats compared to the control. Treatment using vitamin D_3 ($p<0.001$) significantly reversed the changes when compared with diabetes. In curcumin treated diabetic rats, there was significant reversal ($p<0.001$) and up regulation of GPx gene expression when compared to diabetic and control rats respectively. Insulin treatment did not show any significant reversal when compared to diabetic rats. (Figure-98; Table-98).

Real time PCR amplification of Bax mRNA from the Pancreas of control and experimental rats

Real-time PCR Gene expression of Bax showed significant up regulation ($p<0.001$) in the Pancreas of diabetic rats compared to control rats. The Bax gene expression was reversed significantly ($p<0.001$) in insulin, curcumin and vitamin D_3 treated rats when compared with diabetic rats. Curcumin and vitamin D_3 treatment showed prominent reversal ($p<0.001$) in Bax gene expression when compared with insulin treatment (Figure-99; Table-99).

Real time PCR amplification of caspase 8 mRNA from the Pancreas of control and experimental rats

Real-time PCR Gene expression of caspase 8 showed significant up regulation ($p<0.001$) in the Pancreas of diabetic rats compared to control rats. There was a significant reversal ($p<0.001$) in Caspase 8 gene expression in

diabetic rats treated with insulin, curcumin and vitamin D_3. Treatment using curcumin and vitamin D_3 showed prominent reversal ($p<0.001$) in caspase 8 gene expression when compared with insulin treatment (Figure-100; Table-100).

Real time PCR analysis of NeuroD-1 gene expression in Pancreas of control and experimental rats

Real time PCR gene expression of NeuroD-1 showed significant down regulation ($p<0.001$) in the Pancreas of the diabetic rats compared to the control. In curcumin and vitamin D_3 treated diabetic rats, there was significant reversal ($p<0.001$) and up regulation of NeuroD-1 gene expression when compared to diabetic and control rats respectively. Insulin treated diabetic rats did not show any significant change when compared to diabetic rats. Curcumin and vitamin D_3 showed prominent reversal ($p<0.001$) in NeuroD-1 gene expression when compared with insulin treatment (Figure-101; Table-101).

Real time PCR analysis of Pdx1 gene expression in Pancreas of control and experimental rats

Real time PCR gene expression of Pdx1 showed significant down regulation ($p<0.001$) in the Pancreas of the diabetic rats compared to the control. Treatment using insulin ($p<0.001$) significantly reversed the changes to near control. In curcumin and vitamin D_3 ($p<0.001$) treated diabetic rats, there was significant reversal and up regulation of Pdx1 gene expression when compared to diabetic and control rats respectively. Curcumin and vitamin D_3 showed prominent reversal ($p<0.001$) in Pdx1 gene expression when compared with insulin treatment (Figure-102; Table-102).

Co-labeling studies using Insulin and AMPA (GluR2) receptor subunit specific antibody in the pancreatic islets of control and experimental groups of rats using confocal microscope

Insulin positive cells of pancreatic islets showed a significant increase ($p<0.001$) in the mean pixel value of AMPA (GluR2) receptor subunit antibody

staining in diabetic rats when compared to control. Insulin ($p<0.05$), curcumin ($p<0.01$) and vitamin D_3 ($p<0.001$) treatment to diabetic rats significantly reversed AMPA (GluR2) receptor subunit expression in the insulin positive pancreatic islets to near control (Figure-103, Table-103).

Co-labeling studies using Insulin and AMPA (GluR4) receptor subunit specific antibody in the pancreatic islets of control and experimental groups of rats using confocal microscope

Insulin positive cells of pancreatic islets in diabetic rats showed a significant decrease ($p<0.001$) in the mean pixel value of AMPA (GluR4) receptor subunit antibody staining when compared to control. Insulin ($p<0.01$), curcumin and vitamin D_3 treatment to diabetic rats significantly reversed ($p<0.001$) AMPA (GluR4) receptor subunit expression in the insulin positive pancreatic islets to near control (Table-104, Figure-104).

IP3R3 expression in the pancreatic islets of control and experimental groups of rats using confocal microscope

IP3R3 receptor antibody staining in the pancreatic islets showed a significant increase ($p<0.001$) in the mean pixel value in diabetic rats compared to control. Curcumin and vitamin D_3 treatment to diabetic rats significantly reversed ($p<0.001$) IP3R3 expression in the pancreatic islets to near control. Insulin treatment did not show any significant change when compared with diabetic group (Table-105, Figure-105).

Vitamin D receptor expression in the pancreatic islets of control and experimental groups of rats using confocal microscope

Vitamin D receptor antibody staining in the pancreatic islets showed a significant decrease ($p<0.001$) in the mean pixel value in diabetic rats compared to control. Vitamin D_3 ($p<0.001$) treatment in diabetic rats significantly reversed the mean pixel value when compared with diabetic rat. Insulin and curcumin

treatment did not show any significant change when compared with diabetic rat (Table-106, Figure-106).

Calcium release from pancreatic islets using Fluo-4 AM

The Fluo-4 AM staining showed a significant ($p<0.001$) decrease in mean pixel value indicating reduced calcium release from the pancreatic islets in diabetic rats compared to control. Insulin ($p<0.05$), curcumin ($p<0.05$) and vitamin D_3 ($p<0.001$) treatment in diabetic rats significantly reversed the mean pixel value when compared with diabetic Vitamin D_3 treatment showed more prominent reversal in calcium release when compared to other treatment group (Figure-107; Table-107).

Figure-1

Body weight (g) of Experimental rats

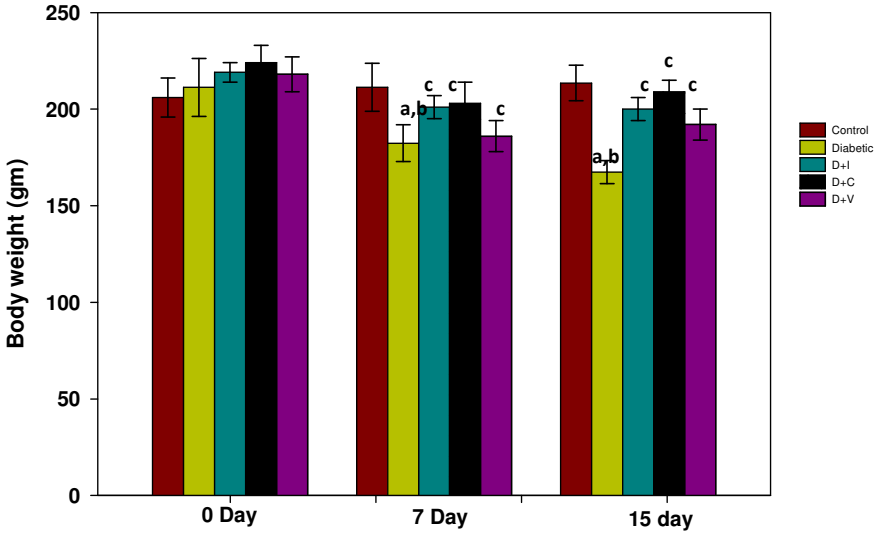


Table-1

Body weight (g) of Experimental rats

Experimental groups	0 Day (Initial)	7th day	15th day
Control	206.0 ± 10.1	211.3 ± 12.5	213.5 ± 9.25
Diabetic	211.2 ± 14.9	182.3± 9.6 [a]	167.3 ± 5.9 [a, b]
D+I	219.6 ± 5.3	201.7 ± 6.5 [c]	200.3 ± 6.9 [c]
D+C	224.7 ± 9.7	203.3 ± 11.8 [c]	209.8 ± 6.9 [c]
D+V	218.3 ± 9.8	186.9 ± 8.9 [a]	192.7 ± 8.9 [c]

Values are mean ± S.E.M of 4-6 rats in each group. [a] $p<0.001$ when compared with control [b] $p<0.001$ when compared with initial weight, [c] $p<0.001$ when compared with diabetic group. D + I- insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats.

Figure-2

Blood glucose (mg/dl) level in Experimental rats

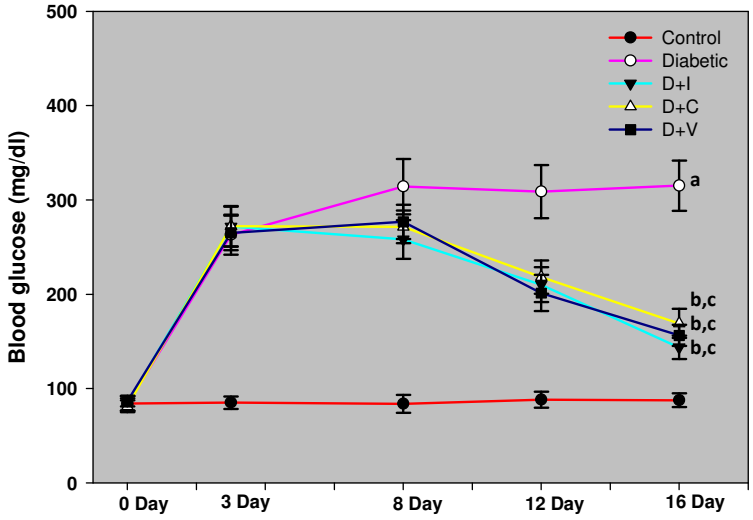


Table-2

Blood glucose (mg/dl) level in Experimental rats

Experimental groups	0 day (Before STZ injection)	3rd day (Initial)	8th day	12th day	16th day (Final)
Control	84.3 ± 8.1	85.1 ± 6.6	83.9 ± 9.5	88.3 ± 8.4	87.7 ± 7.2
Diabetic	83.2 ± 6.3	263.1 ± 21.2 [a]	314.2 ± 29.4 [a]	308.8 ± 28.2 [a]	315.2± 26.5 [a]
D + I	84.2 ± 7.5	271.8 ± 21.2 [a]	258.2 ± 20.5 [a,]	210.2 ± 18.5 [a]	143.8 ± 12.3 [b, c]
D+C	81.2 ± 6.2	272.3 ± 21.3 [a]	271.6 ± 17.2 [a]	218.3 ± 17.5[a]	169.2 ± 15.4 [b, c]
D+V	86.7 ± 5.5	265.2 ± 1.4 [a]	276 .8 ± 18.2 [a]	201.3 ± 19.2 [a]	156.4 ± 11.3 [b, c]

Values are mean ± S.E.M of 4-6 rats in each group. Each group consist of 6-8 rats. **a** P<0.001 when compared to control, **b** P<0.001 when compared to diabetic group, **c** p<0.001 when compared with initial reading. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-3

Circulating insulin level in the plasma of control and experimental rats

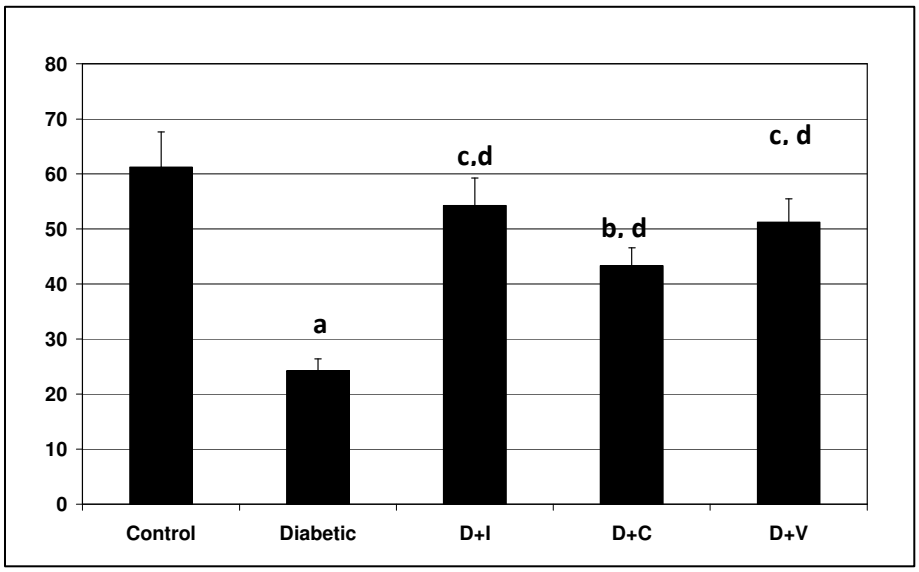


Table-3

Circulating insulin level in the plasma of control and experimental rats

Experimental groups	Insulin Concentration (µU/ml)
Control	61.25 ± 6.35
Diabetic	24.25 ± 2.11 [a]
D + I	54.31 ± 5.02 [c, d]
D+C	43.35 ± 3.21 [b, d]
D+V	51.25 ± 4.25 [c, d]

Values are mean ± S.E.M of 4-6 rats in each group. Each group consist of 6-8 rats. [a] P<0.001, [b] P<0.01, [c] P<0.05 when compared to control, [d] P<0.001 when compared to diabetic group. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-4

Glutamate content in the cerebral cortex of control and experimental rats

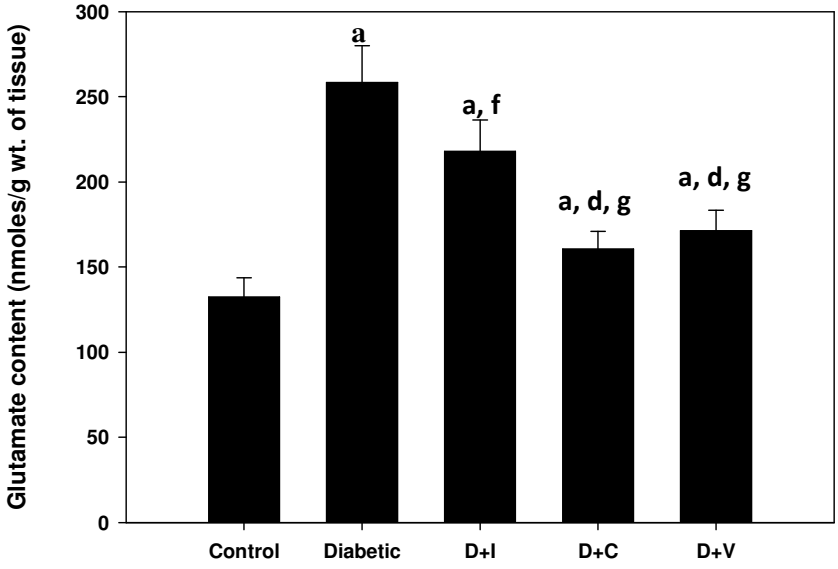


Table-4

Glutamate content in the cerebral cortex of control and experimental rats

Animal Status	Glutamate content (nmoles/g wt. of tissue)
Control	**132.41 ± 11.32**
Diabetic	**258.32 ± 21.62** [a]
D+I	**218.00 ± 18.25** [a, f]
D+C	**160.70± 10.20** [a, d, g]
D+V	**171.25 ± 12.22** [a, d, g]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a] $p<0.001$ when compared with control, [d] $p<0.001$, [f] $p<0.05$ when compared with diabetic, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-5

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebral cortex of control and experimental rats

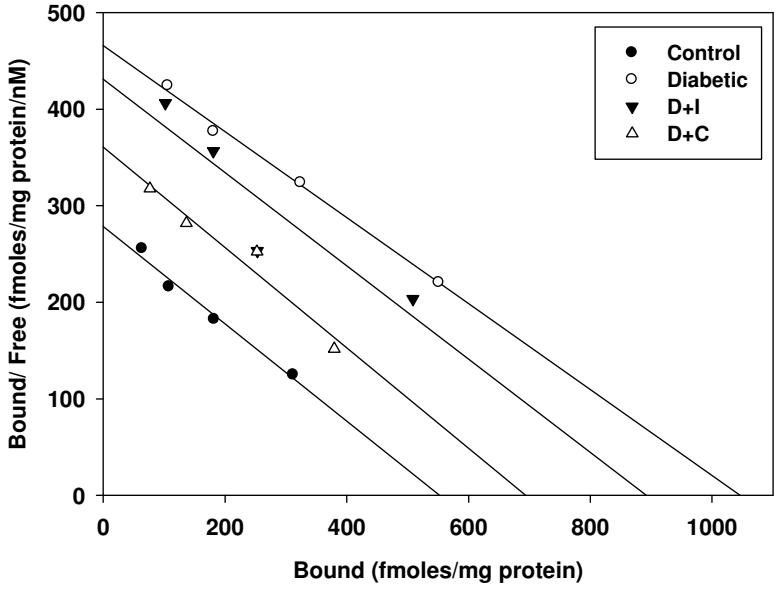


Table-5

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebral cortex of Control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	548 ± 19	1.97 ± 0.25
Diabetic	1036 ± 30 [a]	2.23 ± 0.27
D+ I	888 ± 27 [a, e]	2.06 ± 0.21
D + C	688± 23 [a, d, g]	1.91 ± 0.23

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$when compared to control, [d] $p<0.001$, [e] $p<0.01$ when compared to diabetic group. [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats.

Figure-6

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebral cortex of control and experimental rats

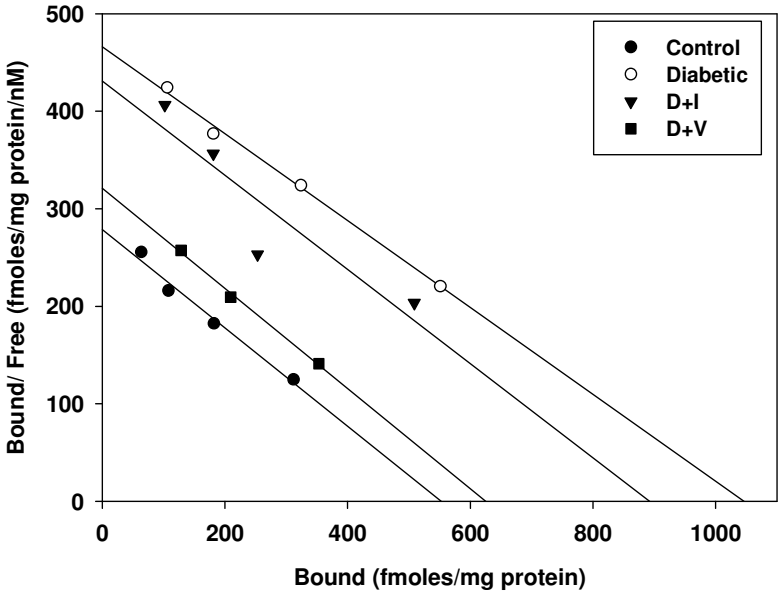


Table-6

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebral cortex of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	548 ± 19	1.97 ± 0.25
Diabetic	1036 ± 30 [a]	2.23 ± 0.27
D+ I	888 ± 27 [a, e]	2.06 ± 0.21
D + V	621± 34 [a, d, g]	1.94 ± 0.29

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats [a] $p<0.001$when compared to control, [d] $p<0.001$, [e] $p<0.01$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+V- vitamin D_3 treated diabetic rats.

Figure-7

Scatchard Analysis AMPA receptors using [^{3}H] AMPA binding against AMPA in the cerebral cortex of control and experimental rats

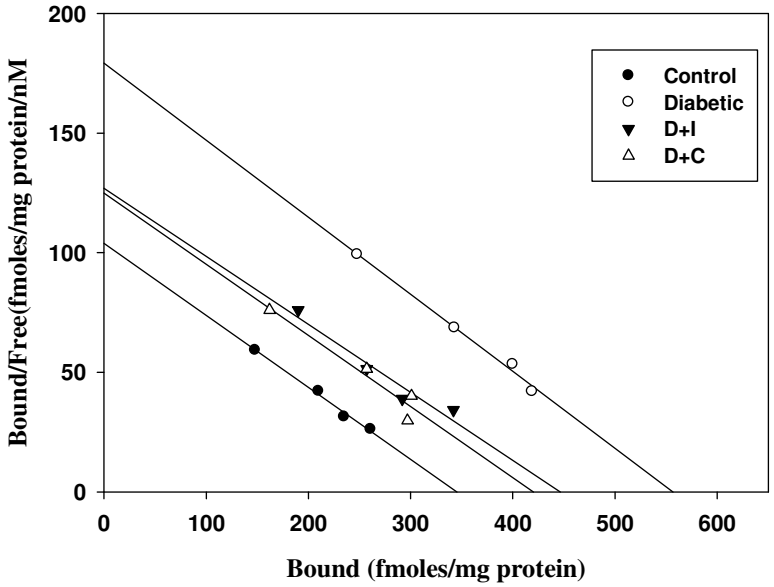


Table-7

Scatchard Analysis AMPA receptors using [^{3}H] AMPA binding against AMPA in the cerebral cortex of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	345 ± 29	3.28 ± 0.44
Diabetic	557 ± 49 [a]	3.07 ± 0.57
D+ I	445 ± 40 [c, e]	3.51 ± 0.54
D + C	416 ± 31 [c, e]	3.36 ± 0.43

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats [a] $p<0.001$, [c] $p<0.05$, when compared to control, [e] $p<0.01$ when compared to diabetic group. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats.

Figure-8

Scatchard Analysis AMPA receptors using [^{3}H] AMPA binding against AMPA in the cerebral cortex of control and experimental rats

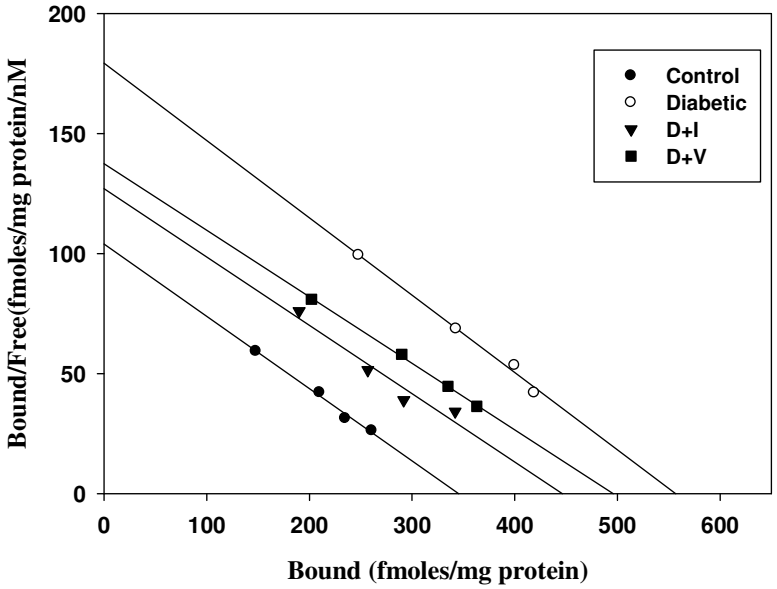


Table-8

Scatchard Analysis AMPA receptors using [^{3}H] AMPA binding against AMPA in the cerebral cortex of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**345 ± 29**	**3.28 ± 0.44**
Diabetic	**557 ± 49** [a]	**3.07 ± 0.57**
D+ I	**445 ± 40** [c, e]	**3.51 ± 0.54**
D + V	**493± 29** [b, f]	**3.63± 0.74**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.005$, when compared to control, [e] $p<0.01$, [f] $p<0.05$ when compared to diabetic group. D + I- insulin treated diabetic rats, D+V- vitamin D_3 treated diabetic rats.

Figure –9

Real time PCR analysis of NMDA R1 Subunit gene expression in cerebral cortex of control and experimental rats

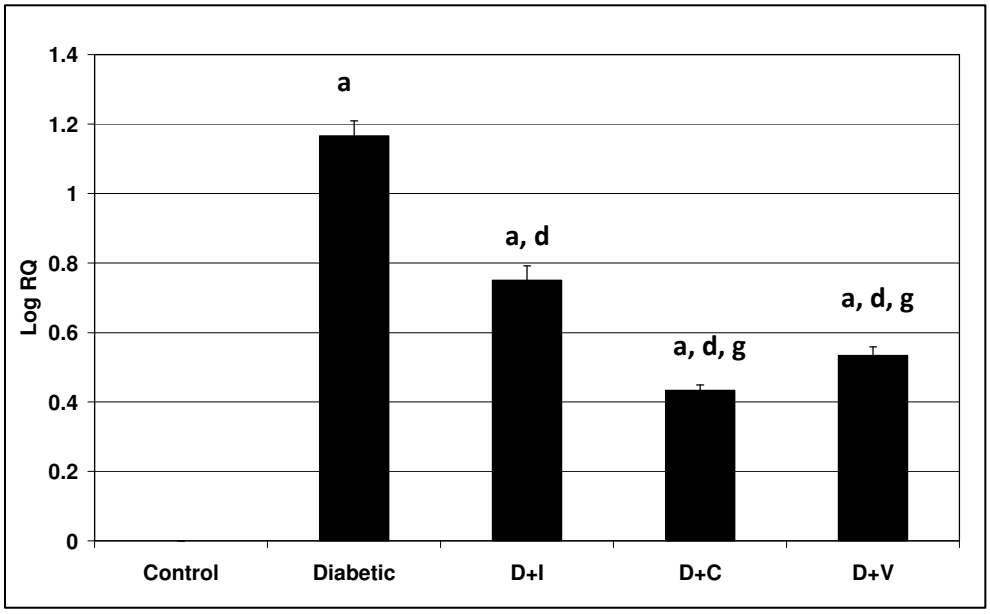


Table-9

Real time PCR analysis of NMDA R1 subunit gene expression in Cerebral Cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.16 ± 0.45 [a]
D + I	0.75 ± 0.08 [a, d]
D+C	0.43 ± 0.04 [a, d, g]
D+V	0.53 ± 0.03 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$when compared to diabetic group. [g] $p<0.001$when compared to insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure –10

Real time PCR analysis of NMDA 2B receptor subunit gene expression in cerebral cortex of control and experimental rats

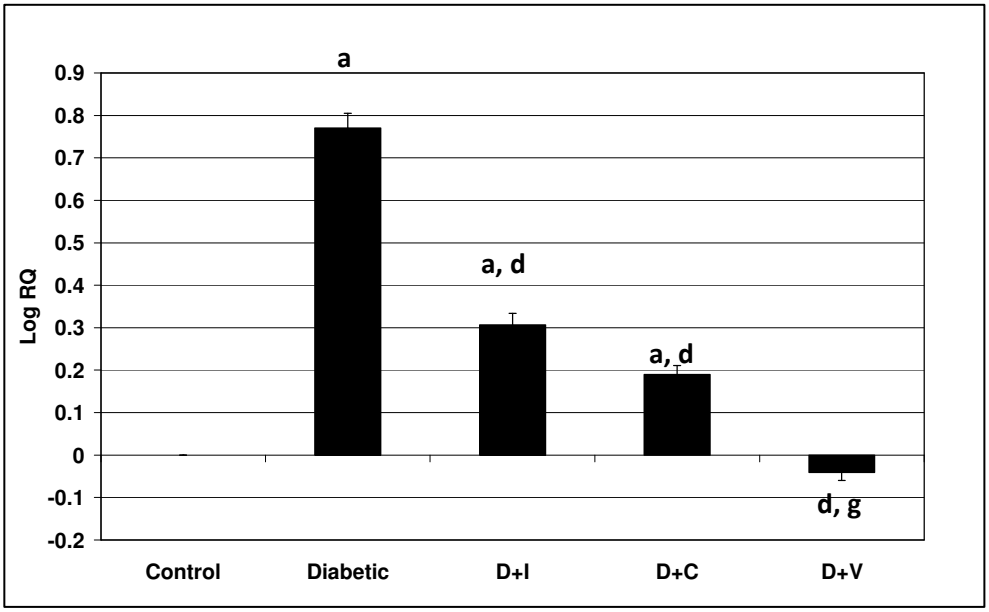


Table-10

Real time PCR analysis of NMDA 2B receptor subunit gene expression in Cerebral Cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.771 ± 0.025 [a]
D + I	0.304± 0.065 [a, d]
D+C	0.186± 0.027 [a, d]
D+V	-0.042 ± 0.007 [d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats.
[a] $p<0.001$ when compared to Control, [d] $p<0.001$, when compared to Diabetic group, [g] $p<0.001$when compared to insulin treated diabetic rats D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats.

Figure – 11

Real time PCR analysis of AMPA (GluR4) receptor subunit gene expression in cerebral cortex of control and experimental rats

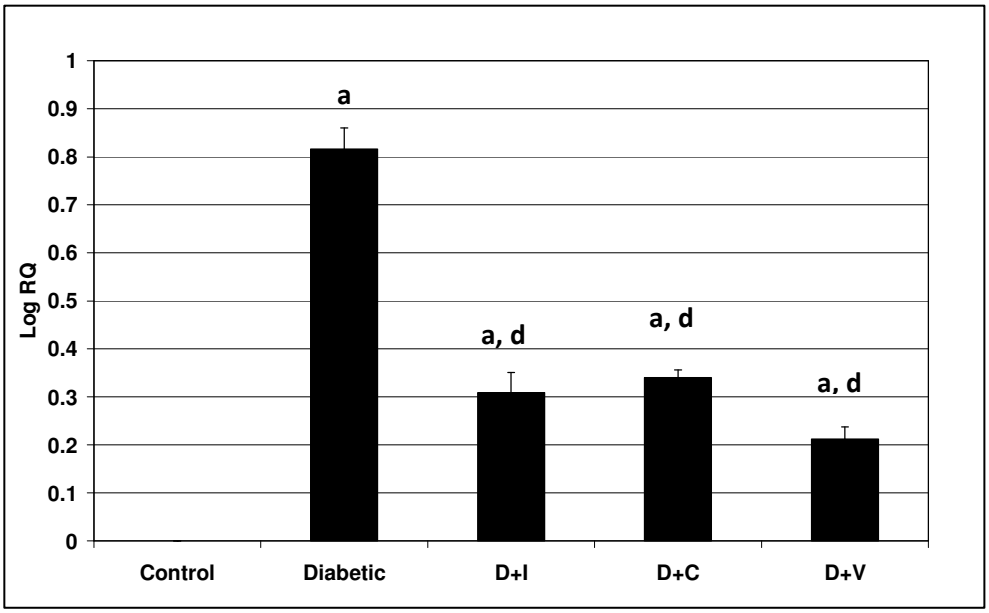


Table-11

Real time PCR analysis of AMPA (GluR4) receptor subunit gene expression in cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.813 ± 0.045 [a]
D + I	0.301 ± 0.053 [a, d]
D+C	0.342± 0.019 [a, d]
D+V	0.214 ± 0.017 [a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats.
[a] $p<0.001$when compared to control, [d] $p<0.001$ when compared to diabetic group. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure –12

Real time PCR analysis of AMPA (GluR2) receptor subunit gene expression in cerebral cortex of control and experimental rats

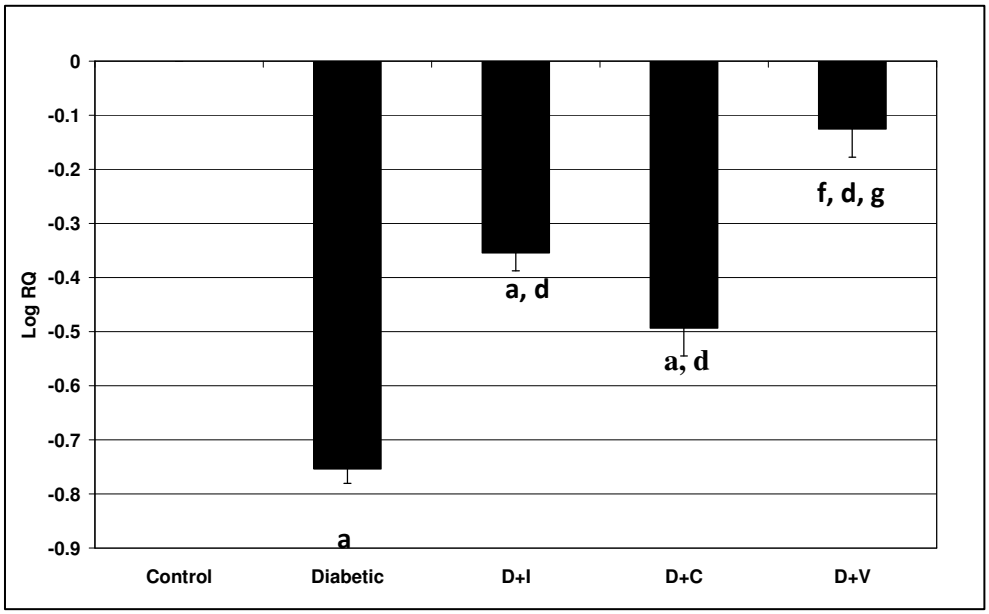


Table-12

Real time PCR analysis of AMPA (GluR2) receptor subunit gene expression in cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.753 ± 0.268 [a]
D + I	-0.354 ± 0.327 [a, d]
D+C	-0.493 ± 0.051 [a, d]
D+V	-0.125 ± 0.051 [f, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats.
[a] $p<0.001$when compared to control, [d] $p<0.001$, [f] $p<0.05$ when compared to diabetic group. [g] $p<0.001$when compared to insulin treated diabetic rats. D + I- Insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-13

Real time PCR analysis of GLAST gene expression in cerebral cortex of control and experimental rats

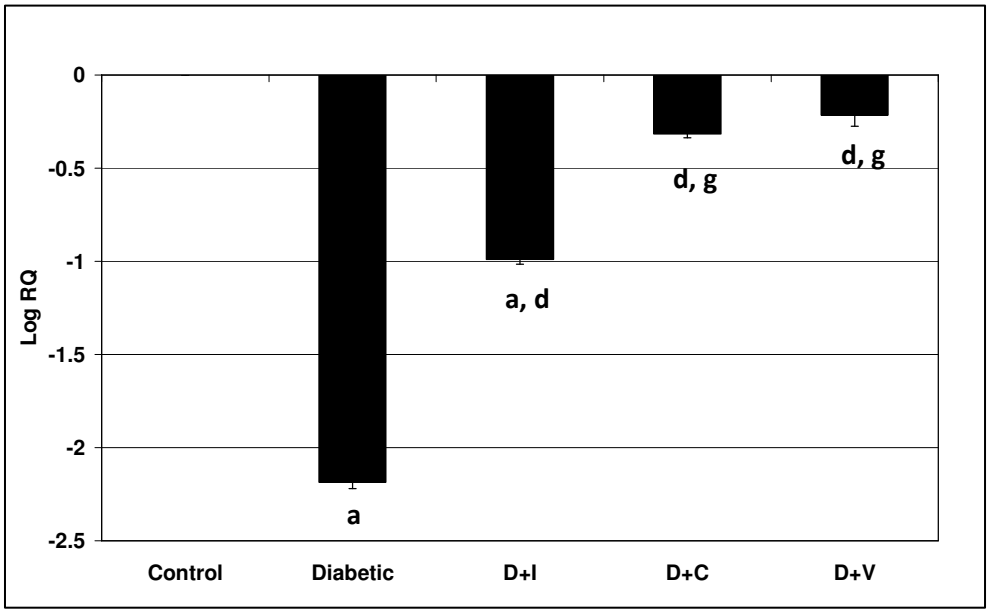


Table-13

Real time PCR analysis of GLAST gene expression in cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-2.18 ± 0.54 [a]
D + I	-0.98± 0.08 [a, d]
D+C	-0.31 ± 0.05 [d, g]
D+V	-0.21± 0.04 [d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to Control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-14

Real time PCR analysis of GAD gene expression in cerebral cortex of control and experimental rats

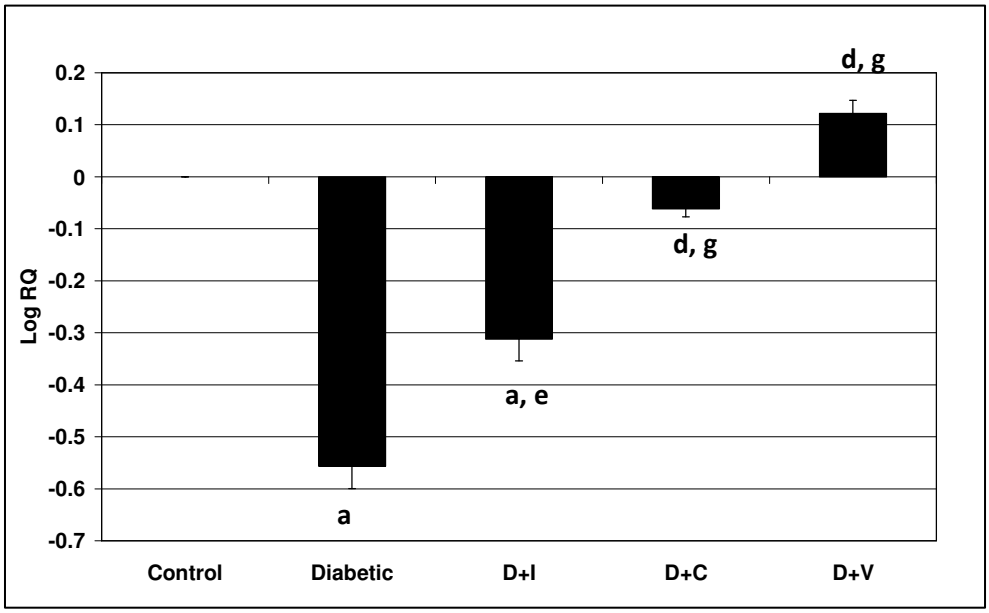


Table-14

Real time PCR analysis of GAD gene expression in cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.553 ± 0.085 [a]
D + I	-0.311 ± 0.065 [a ,e]
D+C	-0.060 ± 0.0054 [d, g]
D+V	0.122 ± 0.045 [d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, when compared to control, [d] $p<0.001$, [e] $p<0.01$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-15

IP3 content in the cerebral cortex of control and experimental rats

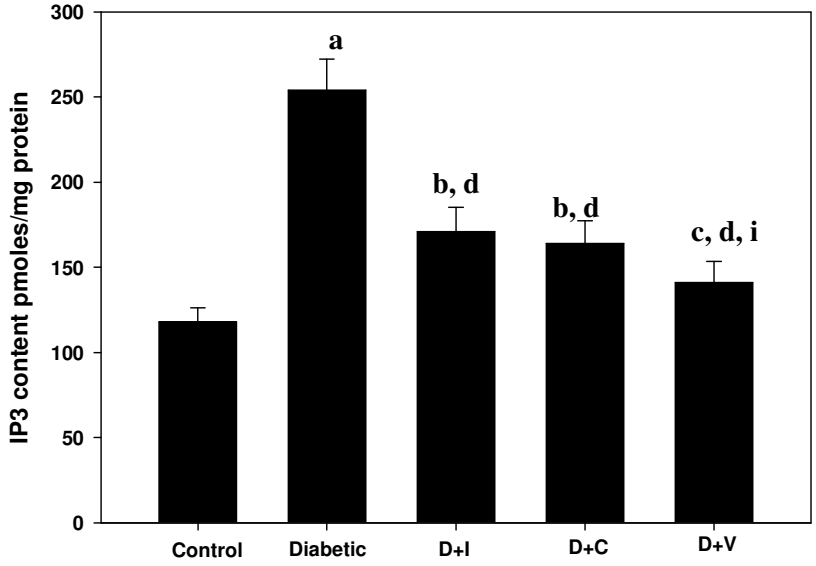


Table-15

IP3 content in the cerebral cortex of control and experimental rats

Animal Status	IP3 Content (pmoles/mg protein)
Control	118.12 ± 8.25
Diabetic	254.32 ± 18.51 [a]
D + I	171.14 ± 14.94 [b, d]
D+C	164.84 ± 13.45 [b, d]
D+V	141.74 ± 12.53 [c, d, i]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control, [d] $p<0.001$ when compared to diabetic group [i] $p<0.05$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-16

Superoxide dismutase assay in the cerebral cortex of control and experimental rats

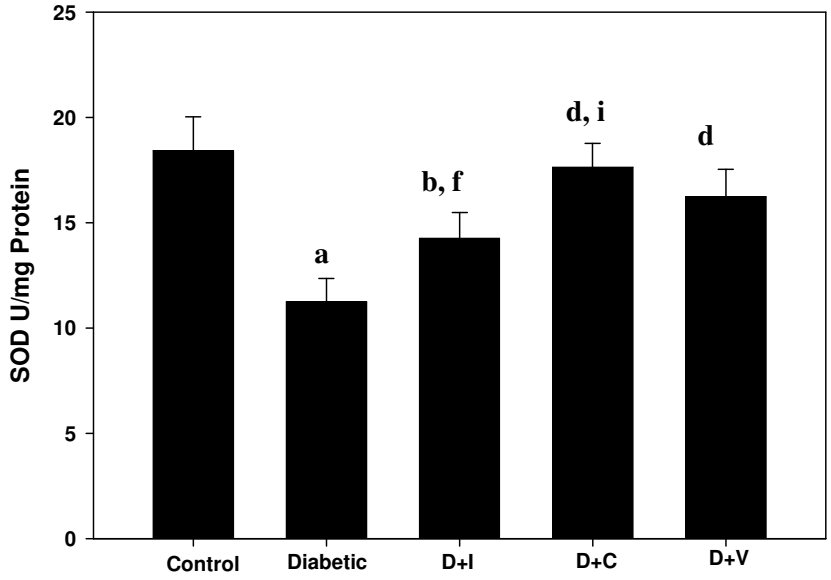


Table-16
Superoxide dismutase assay in the cerebral cortex of control and experimental rats

Animal Status	SOD activity (unit/mg protein)
Control	18.42 ± 1.60
Diabetic	11.25 ± 1.10 [a]
D + I	14.25 ± 1.23 [b, f]
D+C	17.63 ± 1.14 [d, i]
D+V	16.23 ± 1.30 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$, [f] $p<0.05$, when compared to diabetic group, [i] $p<0.05$ when compared with insulin treated diabetic ras. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-17

Real time PCR analysis of GPx gene expression in cerebral cortex of control and experimental rats

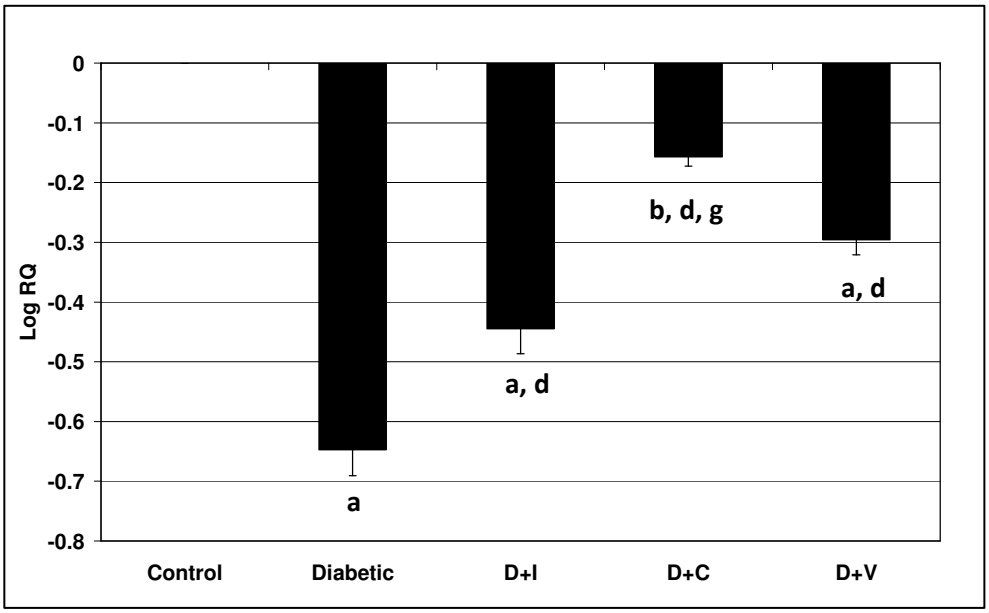


Table-17

Real time PCR analysis of GPx gene expression in cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.641 ± 0.078 [a]
D + I	-0.445 ± 0.084 [a, d]
D+C	-0.154 ± 0.025 [b, d. g]
D+V	-0.291 ± 0.027 [a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic group. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-18

Real time PCR analysis of Akt-1 gene expression in cerebral cortex of control and experimental rats

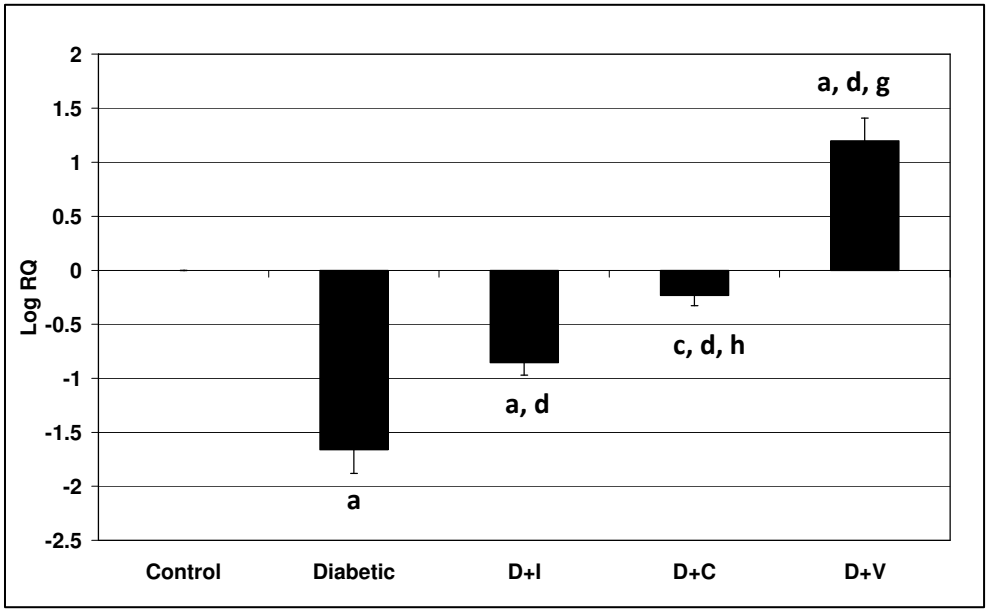


Table-18

Real time PCR analysis of Akt-1 gene expression in Cerebral Cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.660 ± 0.219 [a]
D + I	-0.854 ± 0.115 [a, d]
D+C	-0.232 ± 0.095 [c, d, h]
D+V	1.197 ± 0.209 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.05$, [c] $p<0.01$ when compared to control, [d] $p<0.001$, [f] $p<0.01$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic group. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-19

Real time PCR analysis of Bax gene expression in cerebral cortex of control and experimental rats

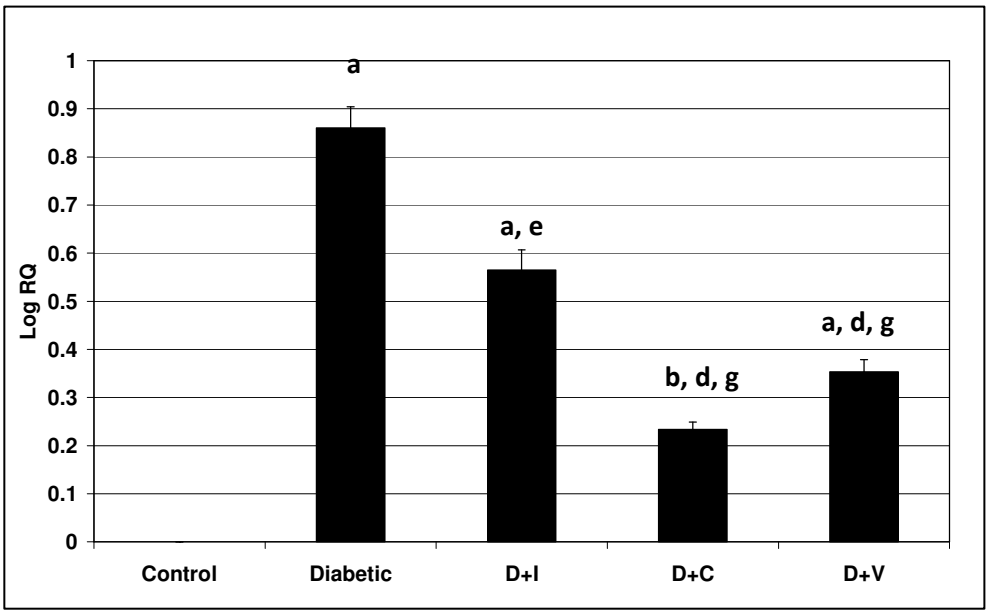


Table-19

Real time PCR analysis of Bax gene expression in cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.862 ± 0.084 [a]
D + I	0.565± 0.052 [a ,e]
D+C	0.233 ± 0.015 [b ,d, g]
D+V	0.353 ± 0.040 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to Control, [d] $p<0.001$, [e] $p<0.01$ when compared to Diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic group. D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats.

Figure-20

Real time PCR analysis of Caspase 8 gene expression in cerebral cortex of control and experimental rats

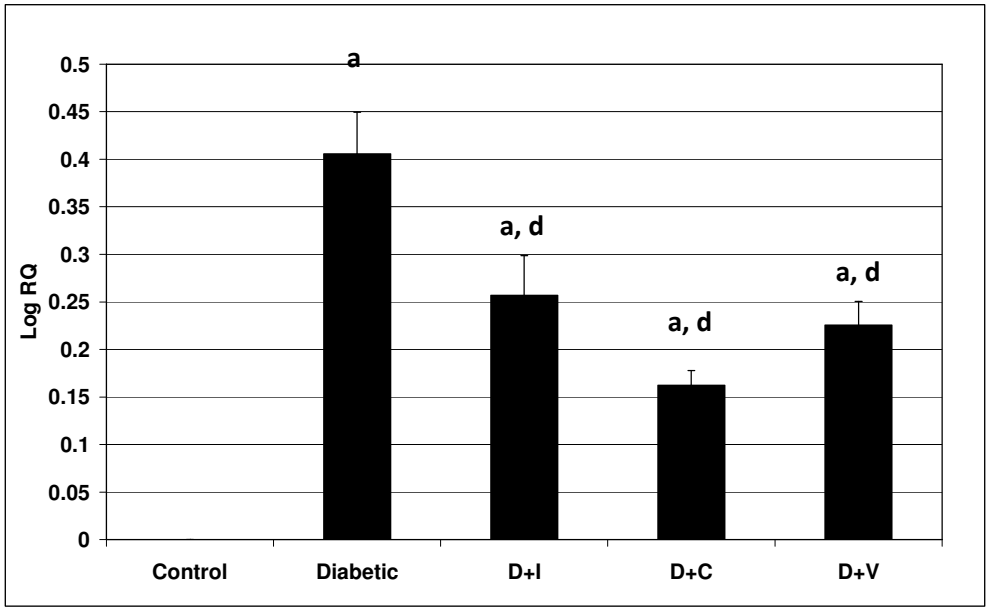


Table-20

Real time PCR analysis of Caspase 8 gene expression in cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.405 ± 0.066 [a]
D + I	0.256 ± 0.021 [a, d]
D+C	0.162 ± 0.026 [a, d]
D+V	0.225 ± 0.018 [a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ when compared to diabetic group. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure- 21
NMDA R1 receptor subunit expression in the cerebral cortex of control and experimental rats

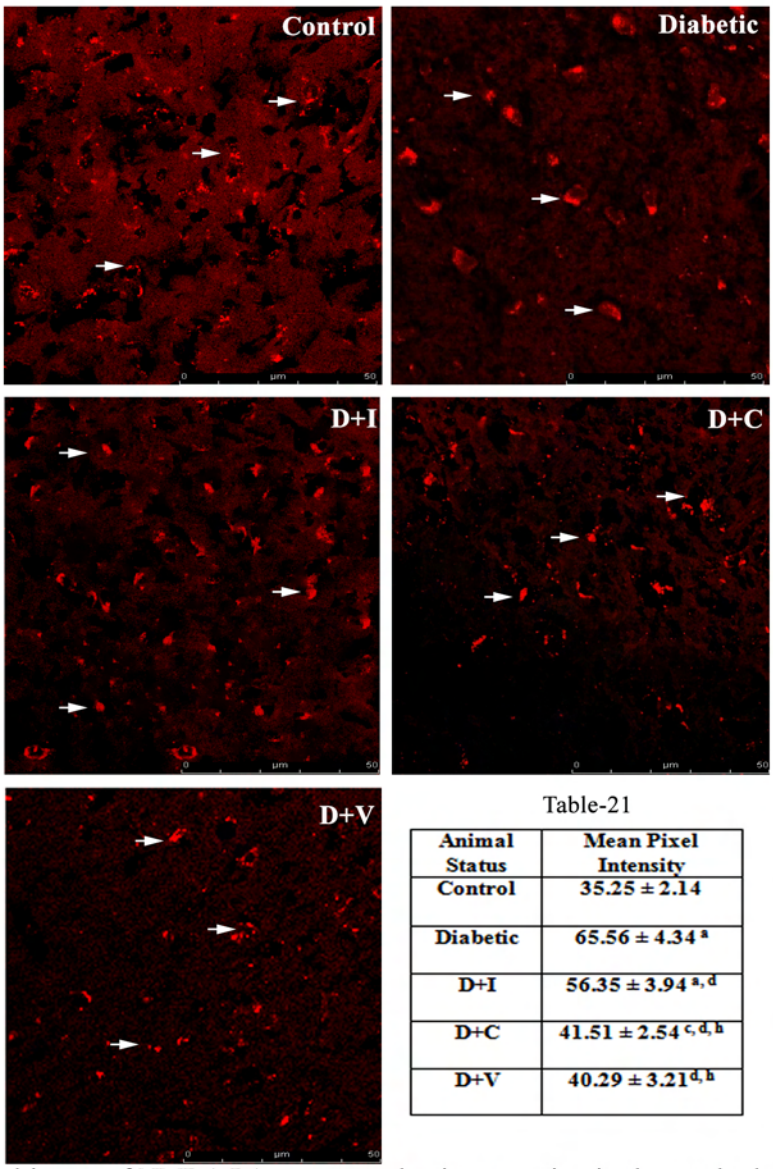


Table-21

Animal Status	Mean Pixel Intensity
Control	35.25 ± 2.14
Diabetic	65.56 ± 4.34 [a]
D+I	56.35 ± 3.94 [a, d]
D+C	41.51 ± 2.54 [c, d, h]
D+V	40.29 ± 3.21 [d, h]

Confocal image of NMDA R1 receptor subunit expression in the cerebral cortex of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent NMDA R1 receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$ when compared to diabetic group; [h] $p<0.01$ when compared to D+I group; (➡) in white shows NMDA receptors. Scale bar = 50 µm

Figure- 22
NMDA 2B receptor subunit expression in the cerebral cortex of control and experimental rats

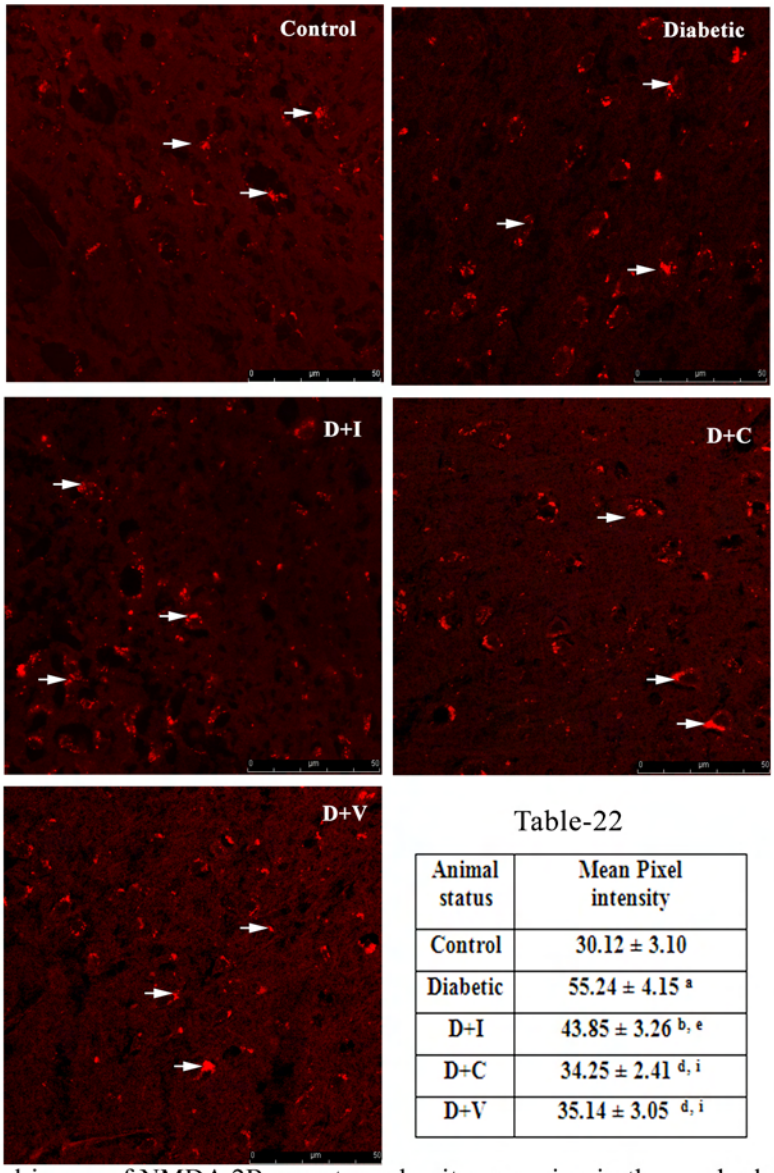


Table-22

Animal status	Mean Pixel intensity
Control	30.12 ± 3.10
Diabetic	55.24 ± 4.15 [a]
D+I	43.85 ± 3.26 [b, e]
D+C	34.25 ± 2.41 [d, i]
D+V	35.14 ± 3.05 [d, i]

Confocal image of NMDA 2B receptor subunit expression in the cerebral cortex of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent NMDA 2B receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, [b] $p<0.01$when compared to control group: [d] $p<0.001$, [e] $p<0.01$when compared to diabetic group; [i] $p<0.05$ when compared to D+I group; (➡) in white shows NMDA receptors. Scale bar = 50 µm

Figure- 23

AMPA (GluR4) receptor subunit expression in the cerebral cortex of control and experimental rats

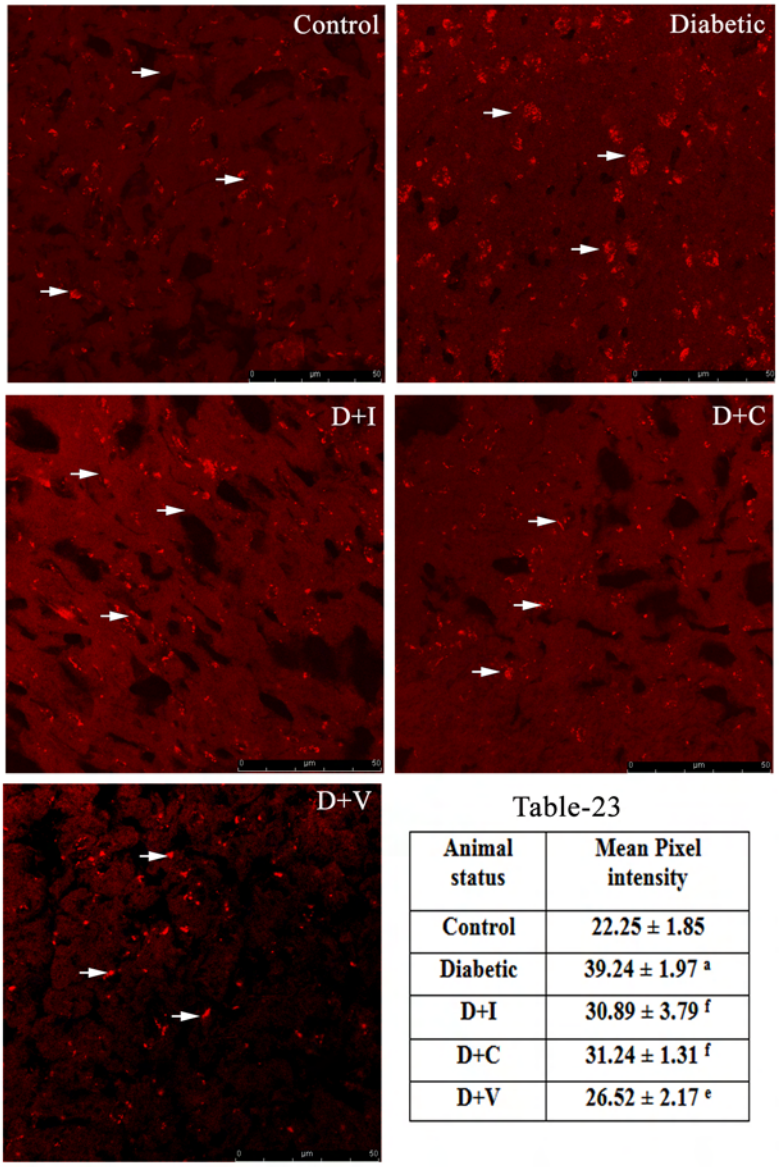


Table-23

Animal status	Mean Pixel intensity
Control	22.25 ± 1.85
Diabetic	39.24 ± 1.97 [a]
D+I	30.89 ± 3.79 [f]
D+C	31.24 ± 1.31 [f]
D+V	26.52 ± 2.17 [e]

Confocal image of AMPA Glur4 receptor subunit expression in the cerebral cortex of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent AMPA Glur4 receptor subunit specific primary antibody and Cy 5 as secondary antibody; a $p<0.001$, [e] $p<0.05$ when compared to control group: [d] $p<0.001$ when compared to diabetic group; (➡) in white shows AMPA receptors. Scale bar = 50 µm

Figure- 24

AMPA (GluR2) receptor subunit expression in the cerebral cortex of control and experimental rats

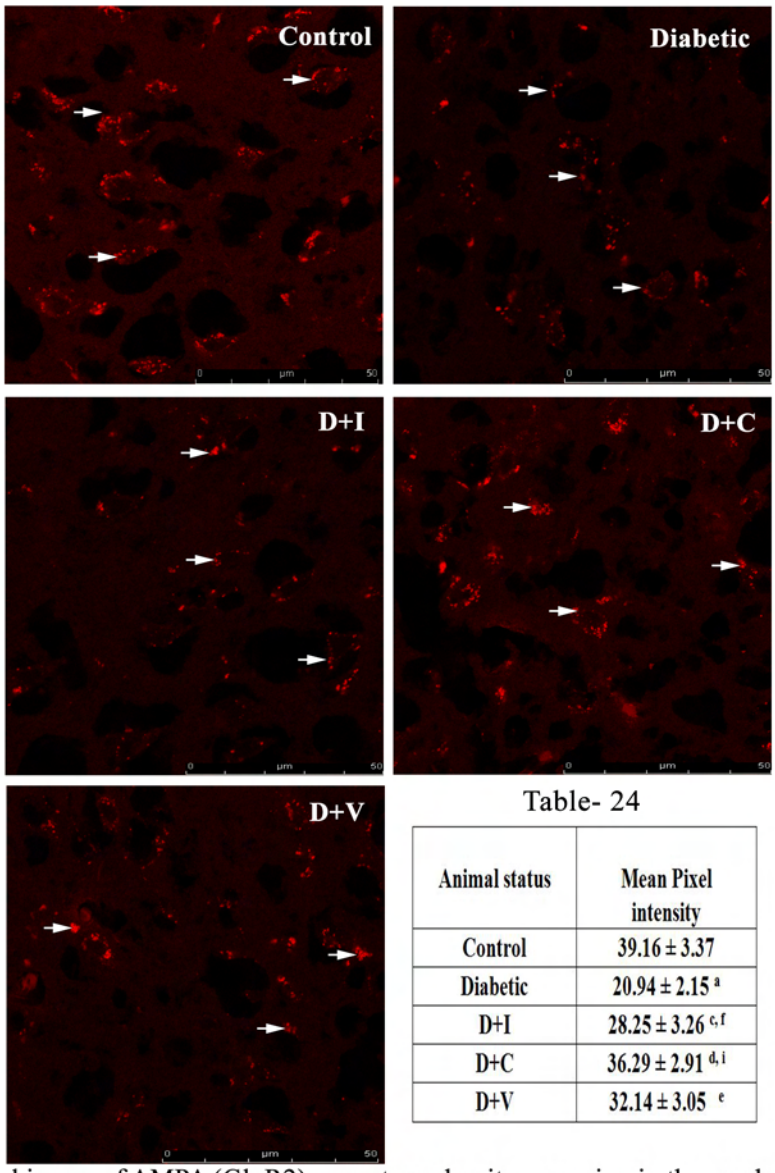


Table- 24

Animal status	Mean Pixel intensity
Control	39.16 ± 3.37
Diabetic	20.94 ± 2.15 [a]
D+I	28.25 ± 3.26 [c, f]
D+C	36.29 ± 2.91 [d, i]
D+V	32.14 ± 3.05 [e]

Confocal image of AMPA (GluR2) receptor subunit expression in the cerebral cortex of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent AMPA GluR2 receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared to Diabetic group; i $p<0.05$ when compared to D+I group; (➡) in white shows AMPA receptors. Scale bar = 50 µm

Figure-25

Glutamate content in the hippocampus of control and experimental rats

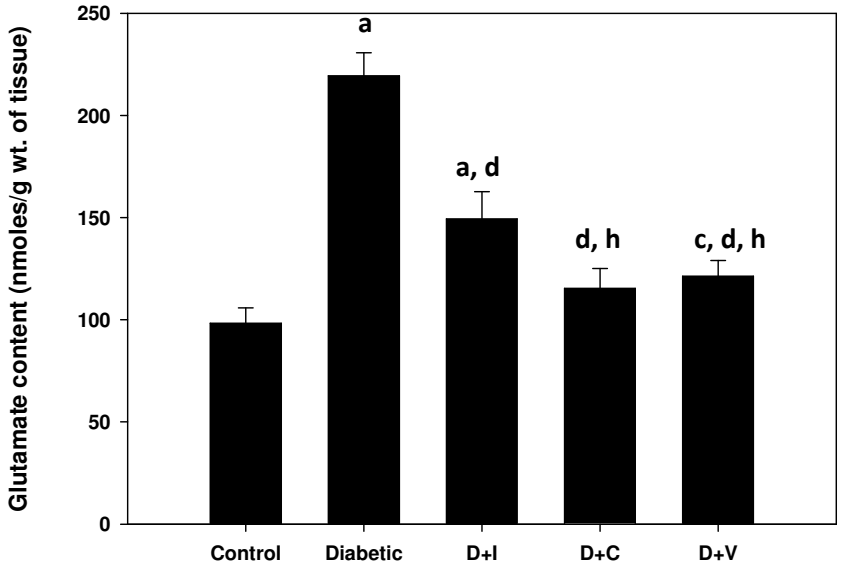


Table-25

Glutamate content in the hippocampus of control and experimental rats

Animal Status	Glutamate content (nmoles/g wt. of tissue)
Control	**98.24 ± 7.59**
Diabetic	**219.35 ± 11.25** [a]
D+I	**149.35 ± 13.36** [a, d]
D+C	**115.35 ± 9.73** [d, h]
D+V	**121.36 ± 7.58** [c, d, h]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a] $p<0.001$, [c] $p<0.05$ when compared with control adult rats, [d] $p<0.001$ when compared with diabetic, [h] $p<0.01$ when compared with insulin treated diabetic group. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-26

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the hippocampus of control and experimental rats

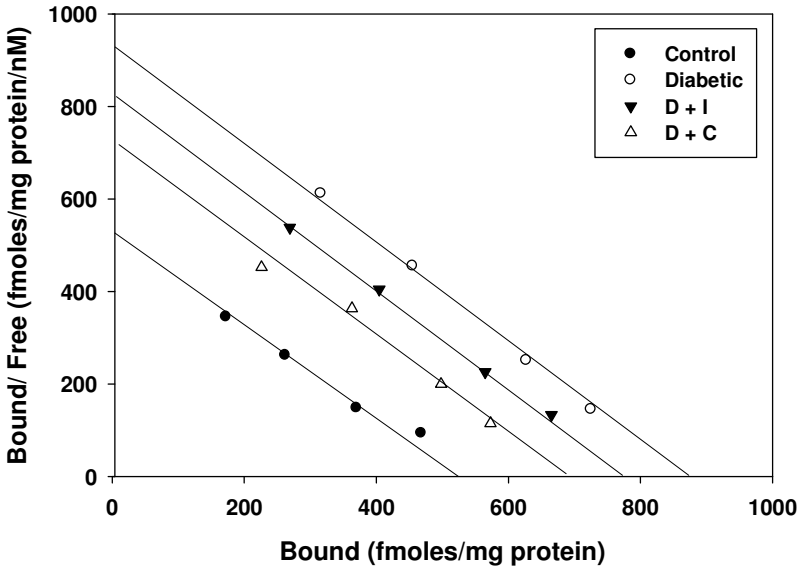


Table-26

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the hippocampus of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	525 ± 20.1	1.01 ± 0.28
Diabetic	873 ± 31.2 [a]	0.94 ± 0.19
D+ I	775 ± 28.4 [a, d]	0.94 ± 0.21
D + C	691 ± 23.55 [a, d, h]	0.84 ± 0.16

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ when compared to diabetic group. [h] $p<0.01$ when compared with insulin treated diabetic rats.D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats.

Figure-27

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the hippocampus of control and experimental rats

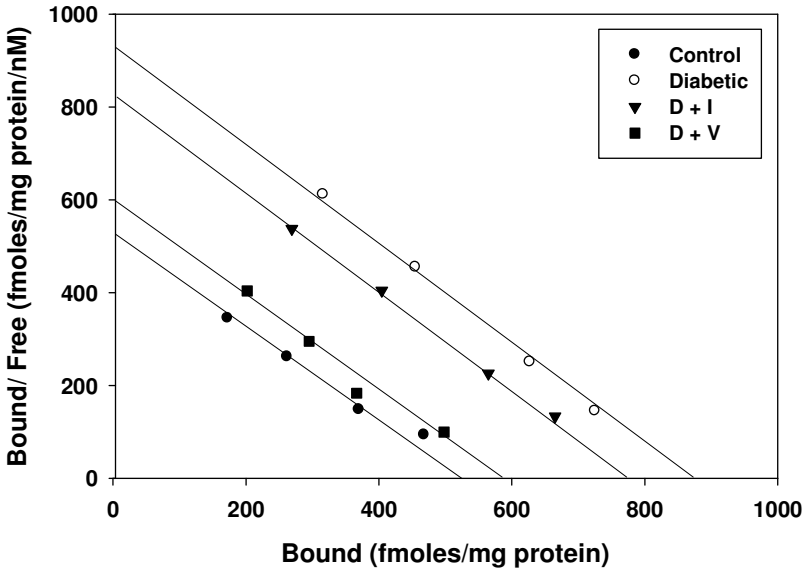


Table-27

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the hippocampus of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	525 ± 20.1	1.01 ± 0.28
Diabetic	873 ± 31.2 [a]	0.94 ± 0.19
D+ I	775 ± 28.4 [a, d]	0.94 ± 0.21
D +V	585 ± 13.55 [c, d, g]	0.98 ± 0.23

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$, [c] $p<0.05$ when compared to control, [d] $p<0.001$ when compared to diabetic group. , [g] $p<0.001$ when compared with insulin treated diabetic rats D + I- insulin treated diabetic rats, D+V- vitamin D_3 treated diabetic rats.

Figure-28

Scatchard Analysis AMPA receptors using [^{3}H] AMPA binding against AMPA in the hippocampus of control and experimental rats

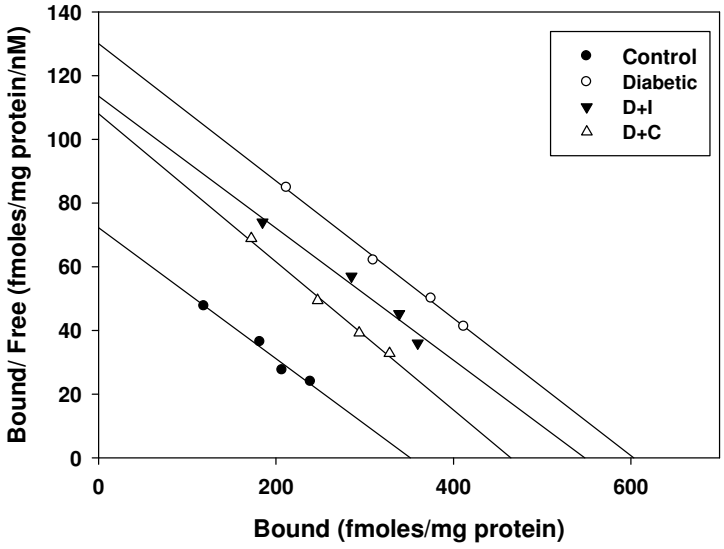


Table-28

Scatchard Analysis AMPA receptors using [^{3}H] AMPA binding against AMPA in the hippocampus of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**346 ± 29.35**	**4.81 ± 0.84**
Diabetic	**601 ± 37.45** [a]	**4.65 ± 0.57**
D+ I	**542 ± 27.82** [a, f]	**4.80 ± 0.93**
D + C	**460 ± 19.45** [a, d, h]	**4.24 ± 0.87**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats.
[a] $p<0.001$ when compared to control, [d] $p<0.001$, [f] $p<0.05$ when compared to diabetic group
[h] $p<0.01$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats.

Figure-29

Scatchard Analysis AMPA receptors using [^{3}H] AMPA binding against AMPA in the hippocampus of control and experimental rats

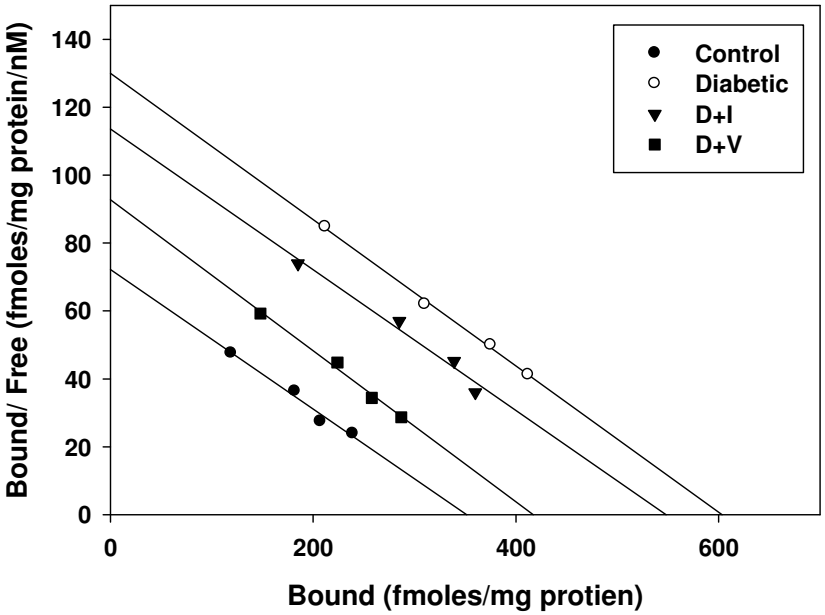


Table-29

Scatchard Analysis AMPA receptors using [^{3}H] AMPA binding against AMPA in the hippocampus of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**346 ± 29.35**	**4.81 ± 0.84**
Diabetic	**601 ± 37.45** [a]	**4.65 ± 0.57**
D+ I	**542 ± 27.82** [a, f]	**4.80 ± 0.93**
D + V	**413 ± 18.82**[c, d, g]	**4.46 ± 0.63**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$, [c] $p<0.05$ when compared to control, [d] $p<0.001$, [f] $p<0.05$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+V- vitamin D_3 treated diabetic rats.

Figure-30

Real time PCR analysis of NMDA R1 subunit gene expression in hippocampus of control and experimental rats

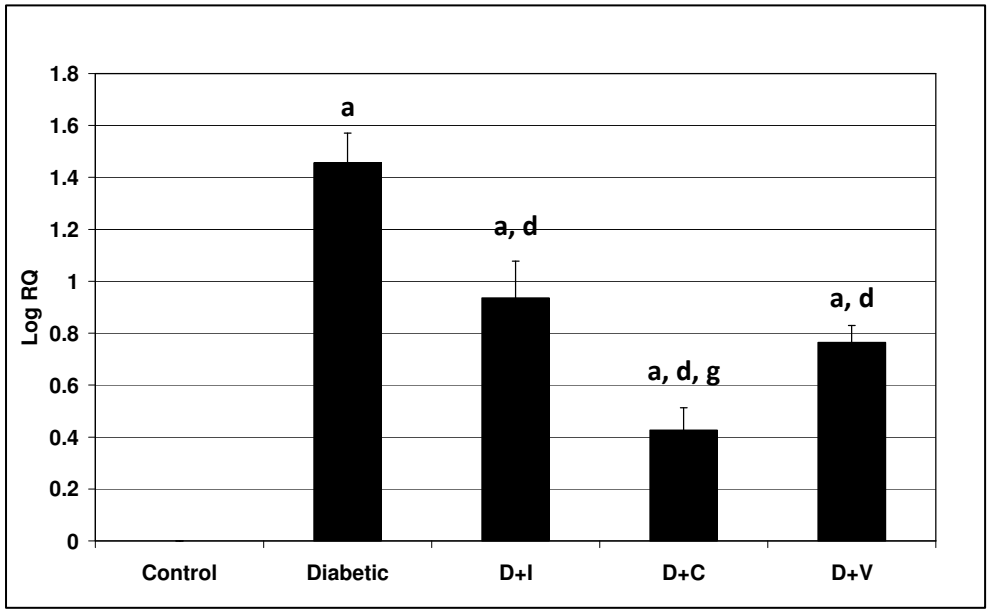


Table-30

Real time PCR analysis of NMDA R1 subunit gene expression in Hippocampus of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.455 ± 0.114 [a]
D + I	0. 935 ± 0.141 [a, d]
D+C	0.426 ± 0.085 [a, d, g]
D+V	0.764 ± 0.065 [a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$when compared to control, [d] $p<0.001$, when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats. D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-31

Real time PCR analysis of NMDA 2B subunit gene expression in hippocampus of control and experimental rats

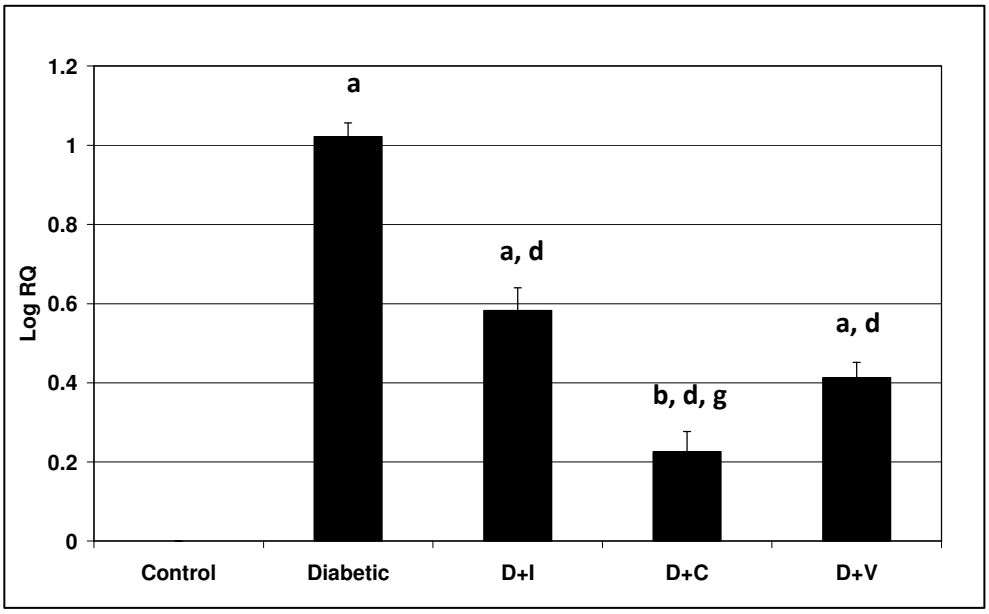


Table-31

Real time PCR analysis of NMDA 2B gene expression in hippocampus of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.021 ± 0.034 [a]
D + I	0.582 ± 0.057 [a, d]
D+C	0.225 ± 0.021 [b, d, g]
D+V	0.412 ± 0.038 [a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$, when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-32

Real time PCR analysis of AMPA (GluR4) Receptor subunit gene expression in hippocampus of control and experimental rats

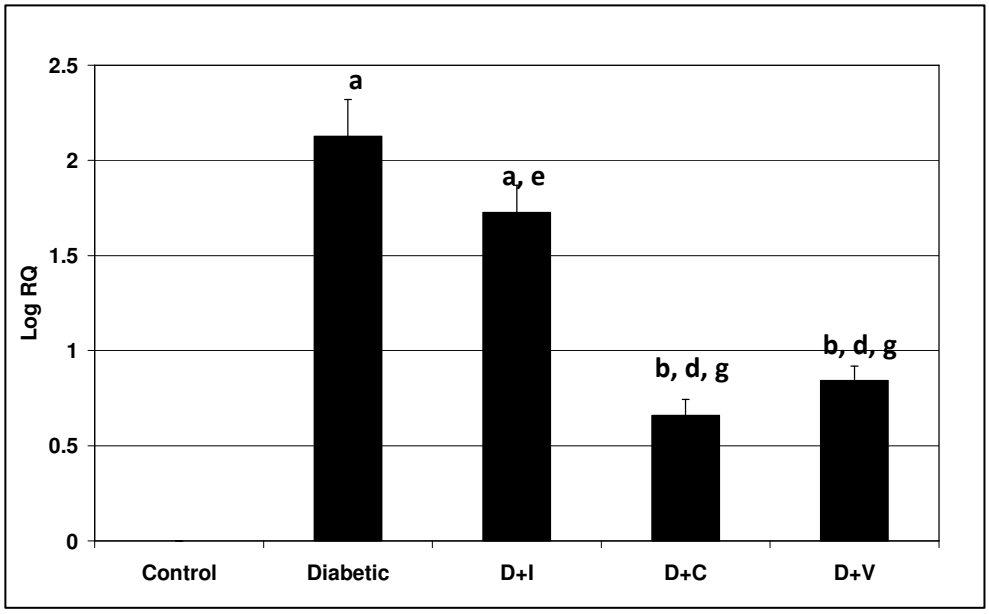


Table-32

Real time PCR analysis of AMPA (GluR4) Receptor subunit gene expression in hippocampus of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	2.125 ± 0.193 [a]
D + I	1.725 ± 0.142 [a, e]
D+C	0.658 ± 0.085 [b, d, g]
D+V	-0.842 ± 0.075 [b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$, [e] $p<0.01$, when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- Insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-33

Real time PCR analysis of AMPA (GluR2) Receptor subunit gene expression in hippocampus of control and experimental rats

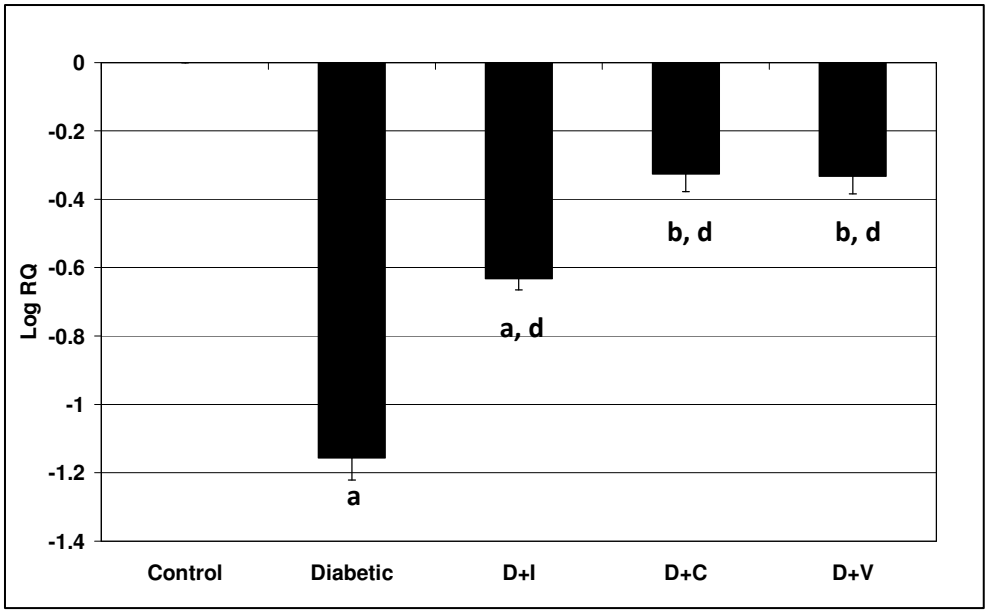


Table-33

Real time PCR analysis of AMPA (GluR2) Receptor subunit gene expression in hippocampus of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.156 ±0.06 [a]
D + I	-0.632 ±0.03 [a, b]
D+C	-0.326 ±0.05 [b, d]
D+V	-0.332 ±0.05 [b, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$ when compared to diabetic group . D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-34

Real time PCR analysis of GLAST gene expression in hippocampus of control and experimental rats

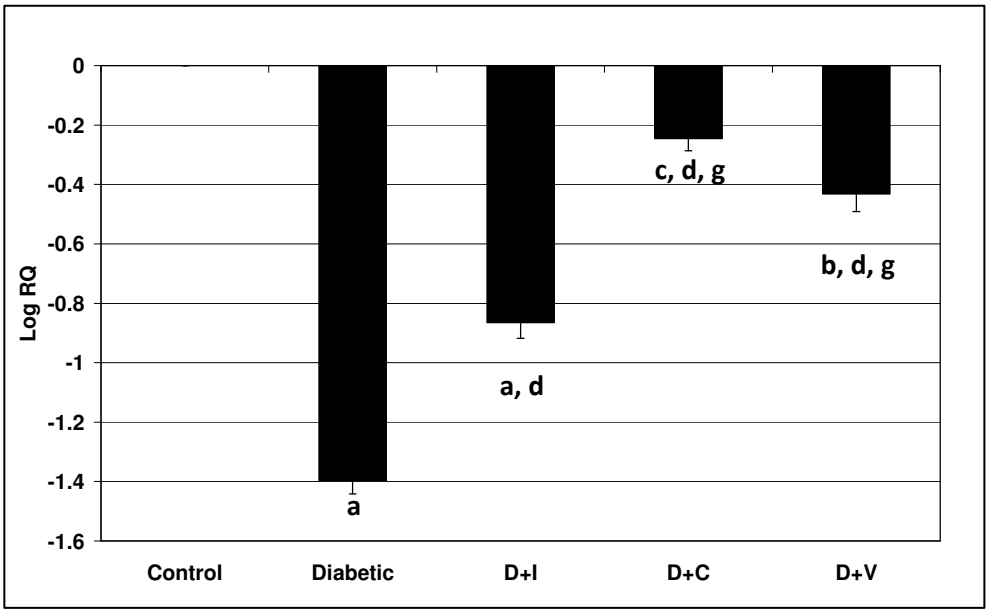


Table-34

Real time PCR analysis of GLAST gene expression in hippocampus of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.396 ± 0.49 [a]
D + I	-0.865 ± 0.07 [a, d]
D+C	-0.245 ± 0.03 [c, d, g]
D+V	-0.43 2 ± 0.09 [b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats . D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-35

Real time PCR analysis of GAD gene expression in hippocampus of control and experimental

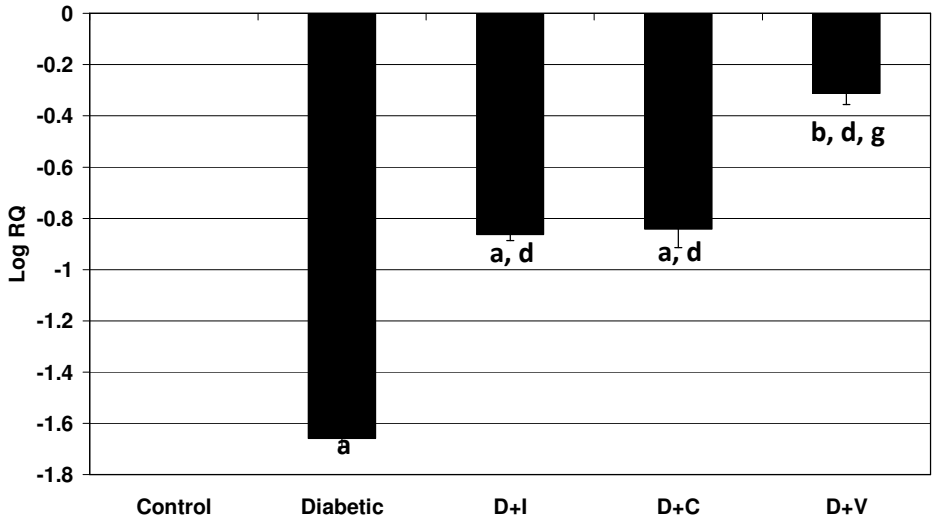


Table-35

Real time PCR analysis of GAD gene expression in hippocampus of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.658 ± 0.033 [a]
D + I	-0.862 ± 0.024 [a ,d]
D+C	-0.842 ± 0.071 [a, d]
D+V	-0.312 ± 0.043 [b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-36

IP3 content in the hippocampus of control and experimental rats

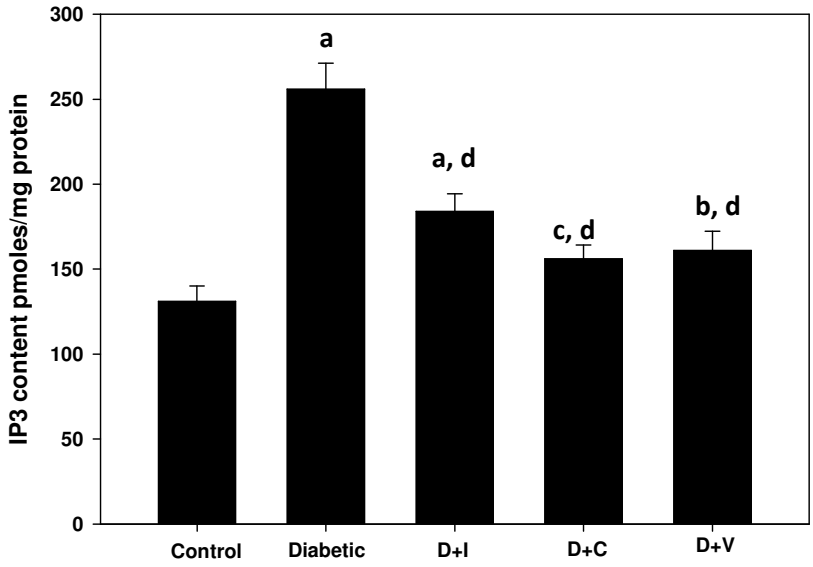


Table-36

IP3 content in the hippocampus of control and experimental rats

Animal Status	IP3 Content
Control	131.15 ± 9.12
Diabetic	256.21 ± 15.24 [a]
D + I	184.42 ± 10.25 [a, d]
D+C	156.15 ± 8.14 [c, d]
D+V	161.51 ± 11.24 [b, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control, [d] $p<0.001$ when compared to diabetic group. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-37

Superoxide dismutase assay in the hippocampus of control and experimental rats

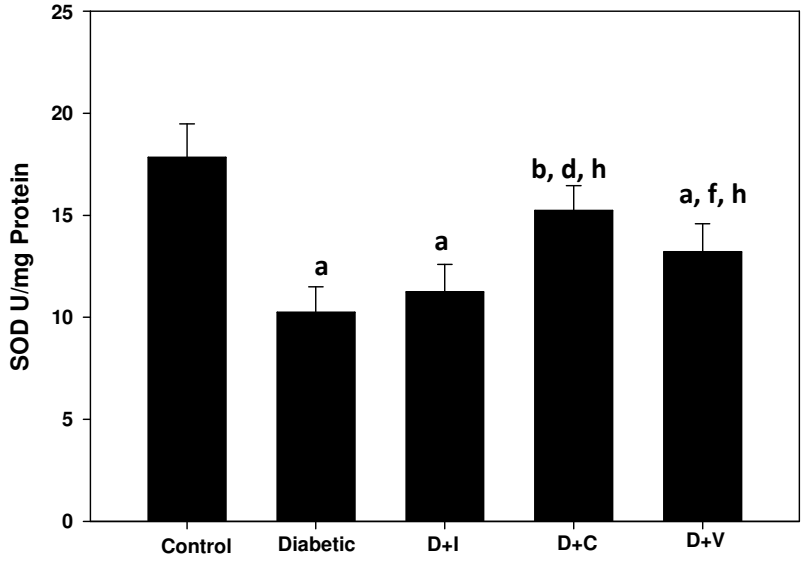


Table-37

Superoxide dismutase assay in the Hippocampus of control and experimental rats

Animal Status	SOD activity (unit/mg protein)
Control	17.84 ±1.64
Diabetic	10.25 ± 1.24 [a]
D + I	11.25 ± 1.34 [a]
D+C	15.24 ± 1.21 [b, d, h]
D+V	13.21 ± 1.37 [a, f, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control [d] $p<0.001$, [f] $p<0.05$ when compared to diabetic group, [h] $p<0.01$ when compared with insulin treated diabetic rats. D + I- Insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-38

Real time PCR analysis of GPx gene expression in hippocampus of control and experimental rats

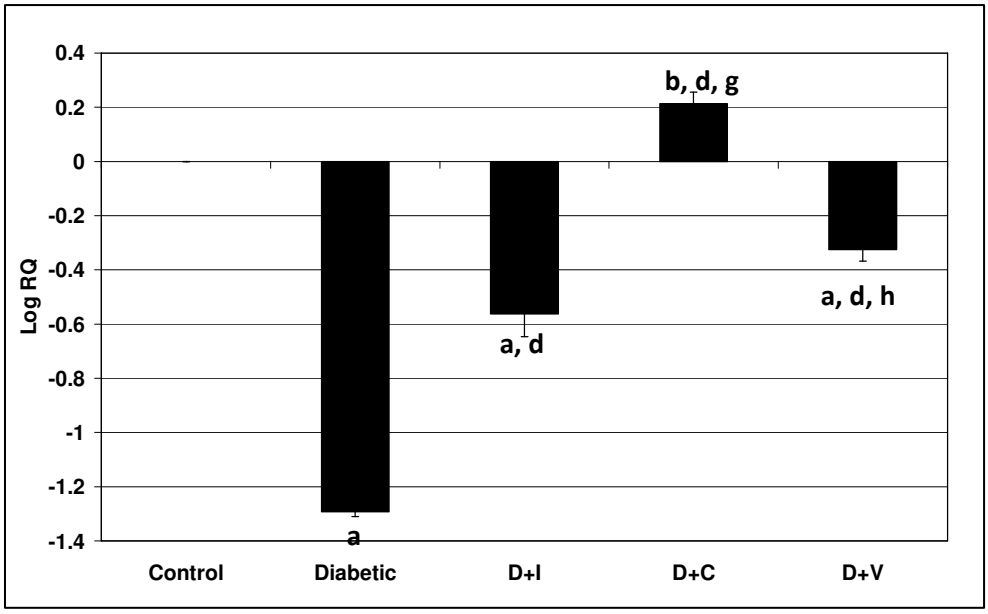


Table-38

Real time PCR analysis of GPx gene expression in hippocampus of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.292 ± 0.172 [a]
D + I	-0.562 ± 0.084 [a, d]
D+C	0.213 ± 0.042 [b, d, g]
D+V	-0.325 ± 0.042 [a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$, when compared to diabetic group, [g] $p<0.001$, [h] $p<0.01$ when compared with insulin. D + I- insulin treated diabetic rats, D+C-curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-39

Real time PCR analysis of Akt-1 gene expression in hippocampus of control and experimental rats

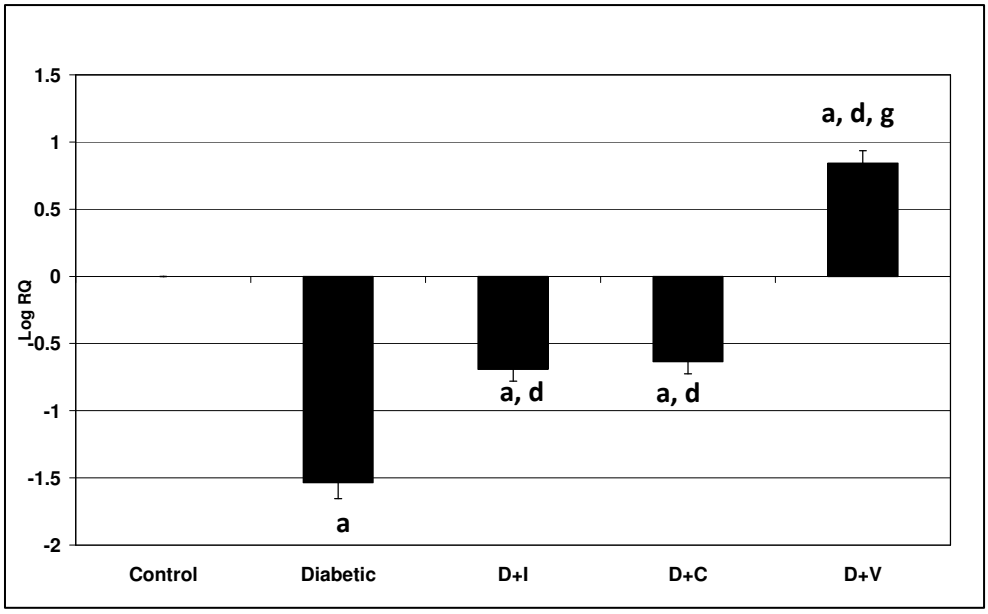


Table-39

Real time PCR analysis of Akt-1 gene expression in hippocampus of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.536± 0.119 [a]
D + I	-0.692 ± 0.089 [a, d]
D+C	-0.635 ± 0.090 [a, d]
D+V	0.842 ± 0.0192 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$when compared with insulin. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-40

Real time PCR analysis of Bax gene expression in hippocampus of control and experimental rats

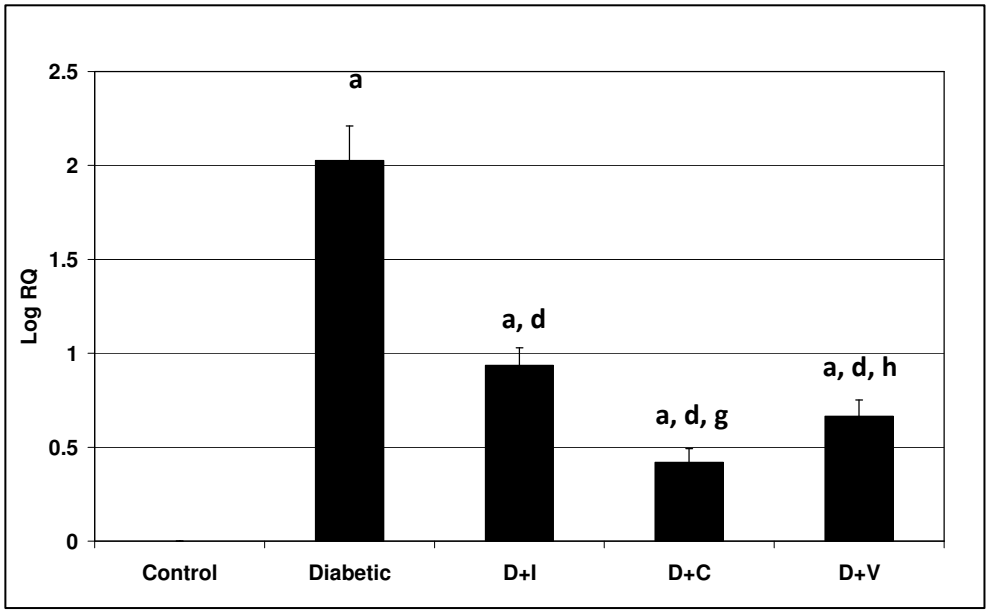


Table-40

Real time PCR analysis of Bax gene expression in hippocampus of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	2.025 ± 0.184 [a]
D + I	0.936 ± 0.092 [a, d]
D+C	0.419 ± 0.073 [a, d, g]
D+V	0.665 ± 0.087 [a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$, [h] $p<0.01$ when compared with insulin treated diabetic rats, D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure –41

Real time PCR analysis of Caspase 8 gene expression in hippocampus of control and experimental rats

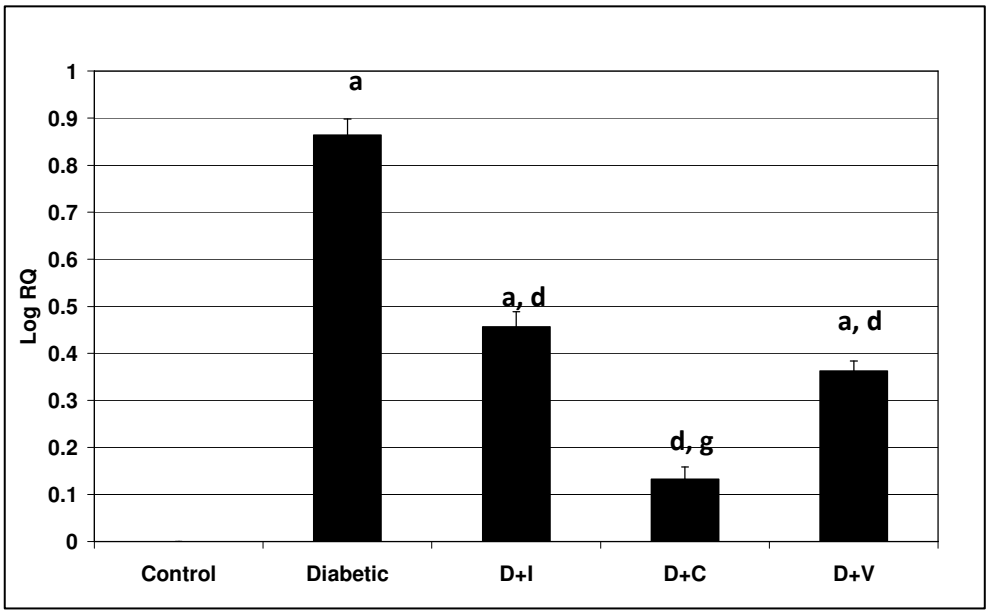


Table-41

Real time PCR analysis of Caspase 8 gene expression in hippocampus of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	0.864 ± 0.033 [a]
D + I	0.456 ± 0.041 [a, d]
D+C	0.132 ± 0.026 [d, g]
D+V	0.362 ± 0.031 [a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure- 42

NMDA R1 receptor subunit expression in the Hippocampus of control and experimental rats

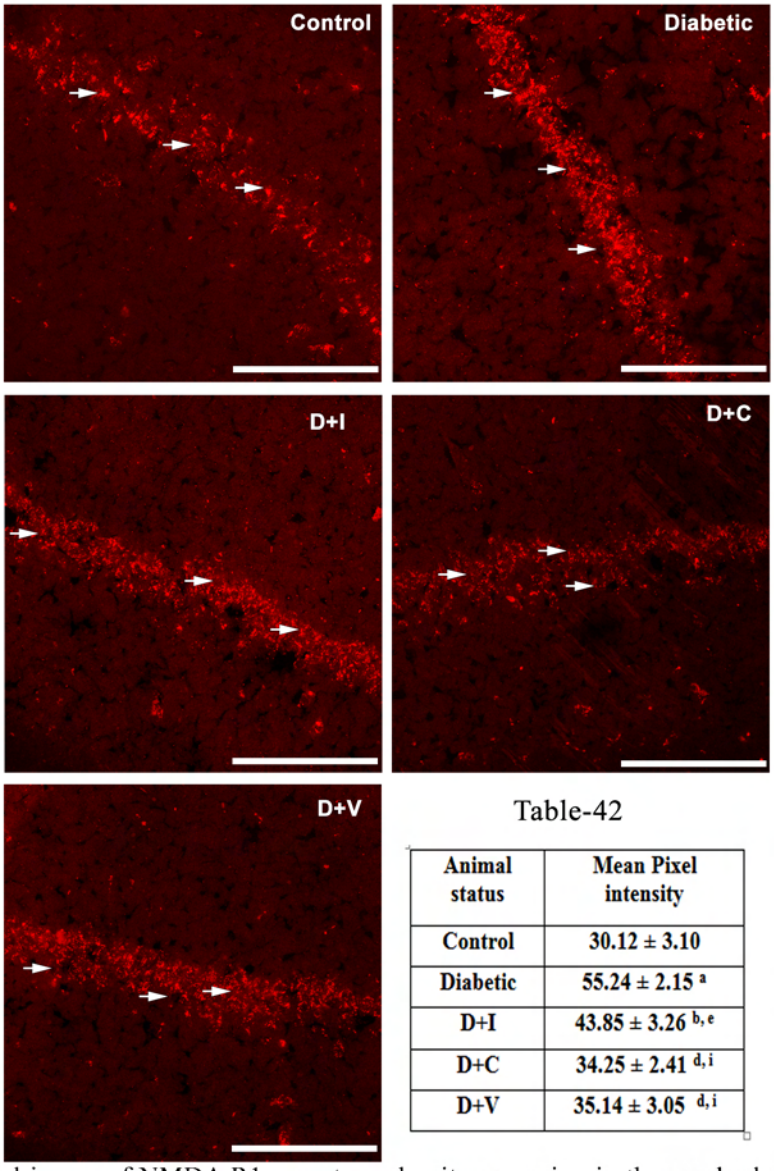


Table-42

Animal status	Mean Pixel intensity
Control	30.12 ± 3.10
Diabetic	55.24 ± 2.15 [a]
D+I	43.85 ± 3.26 [b, e]
D+C	34.25 ± 2.41 [d, i]
D+V	35.14 ± 3.05 [d, i]

Confocal image of NMDA R1 receptor subunit expression in the cerebral cortex of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent NMDA R1 receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, [b] $p<0.01$ when compared to control group: [d] $p<0.001$, [e] $p<0.01$ when compared to diabetic group; [i] $p<0.05$ when compared to D+I group; (➡) in white shows NMDA receptors. Scale bar = 50 µm

Figure- 43

NMDA 2B receptor subunit expression in the hippocampus of control and experimental rats

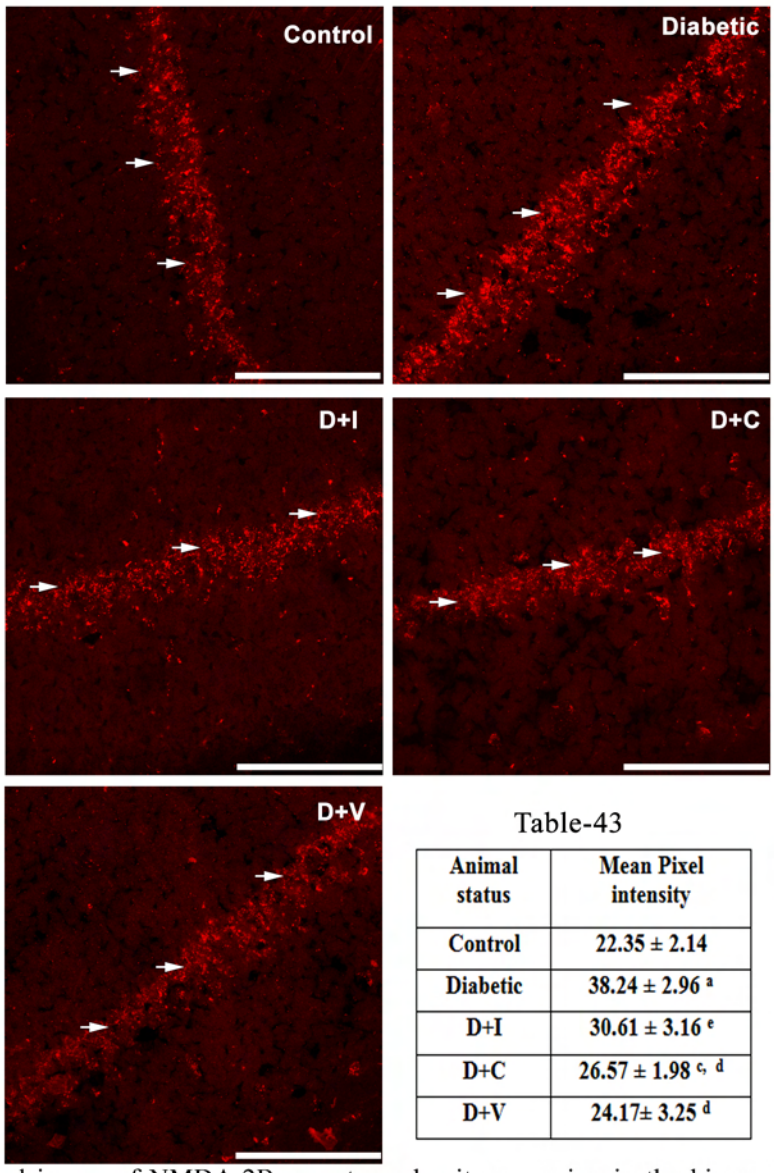


Table-43

Animal status	Mean Pixel intensity
Control	22.35 ± 2.14
Diabetic	38.24 ± 2.96 [a]
D+I	30.61 ± 3.16 [e]
D+C	26.57 ± 1.98 [c, d]
D+V	24.17± 3.25 [d]

Confocal image of NMDA 2B receptor subunit expression in the hippocampus of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent NMDA 2B receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$ when compared to diabetic group; (➡) in white shows NMDA 2B receptor subunit. Scale bar = 50 µm

Figure- 44
AMPA (GluR4) receptor subunit expression in the hippocampus of control and experimental rats

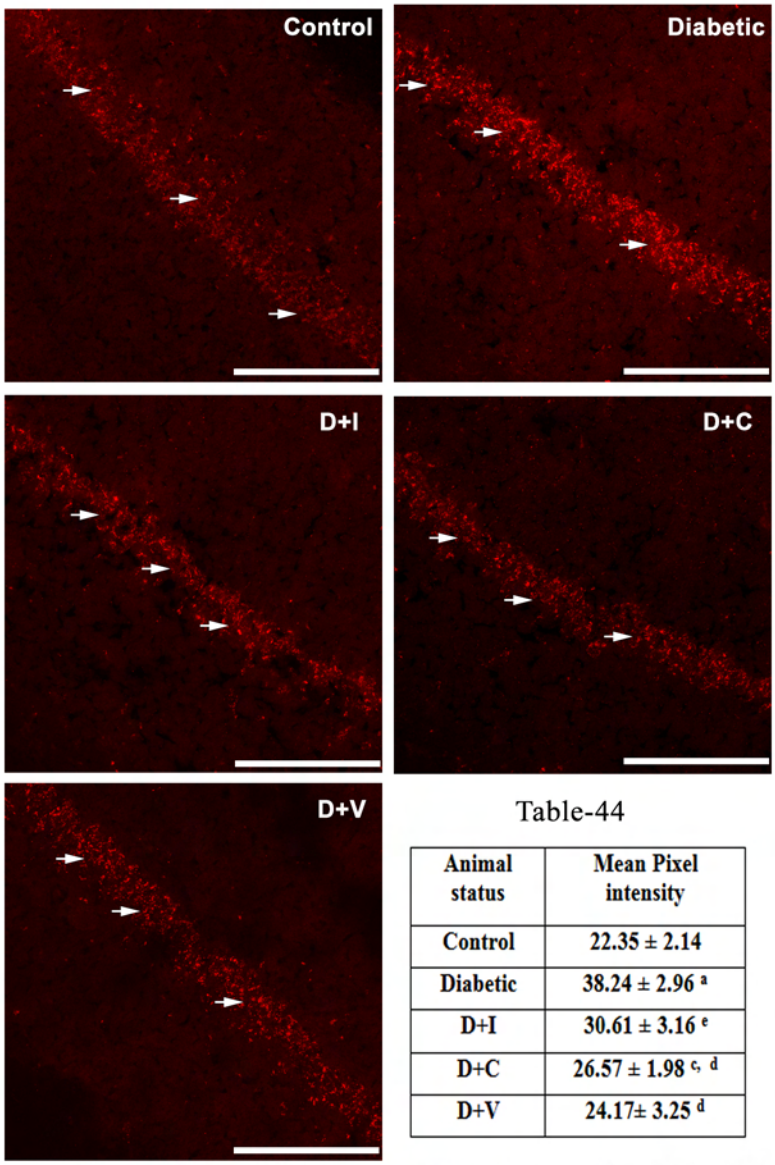


Table-44

Animal status	Mean Pixel intensity
Control	22.35 ± 2.14
Diabetic	38.24 ± 2.96 [a]
D+I	30.61 ± 3.16 [e]
D+C	26.57 ± 1.98 [c, d]
D+V	24.17± 3.25 [d]

Confocal image of AMPA (GluR4) receptor subunit expression in the hippocampus of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent AMPA (GluR4) receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$ when compared to control group: [d] $p<0.001$, [e] $p<0.01$ when compared to diabetic group; (➡) in white shows AMPA (GluR4) receptor subunit. Scale bar = 50 µm

Figure- 45

AMPA (GluR2) receptor subunit expression in the hippocampus of control and experimental rats

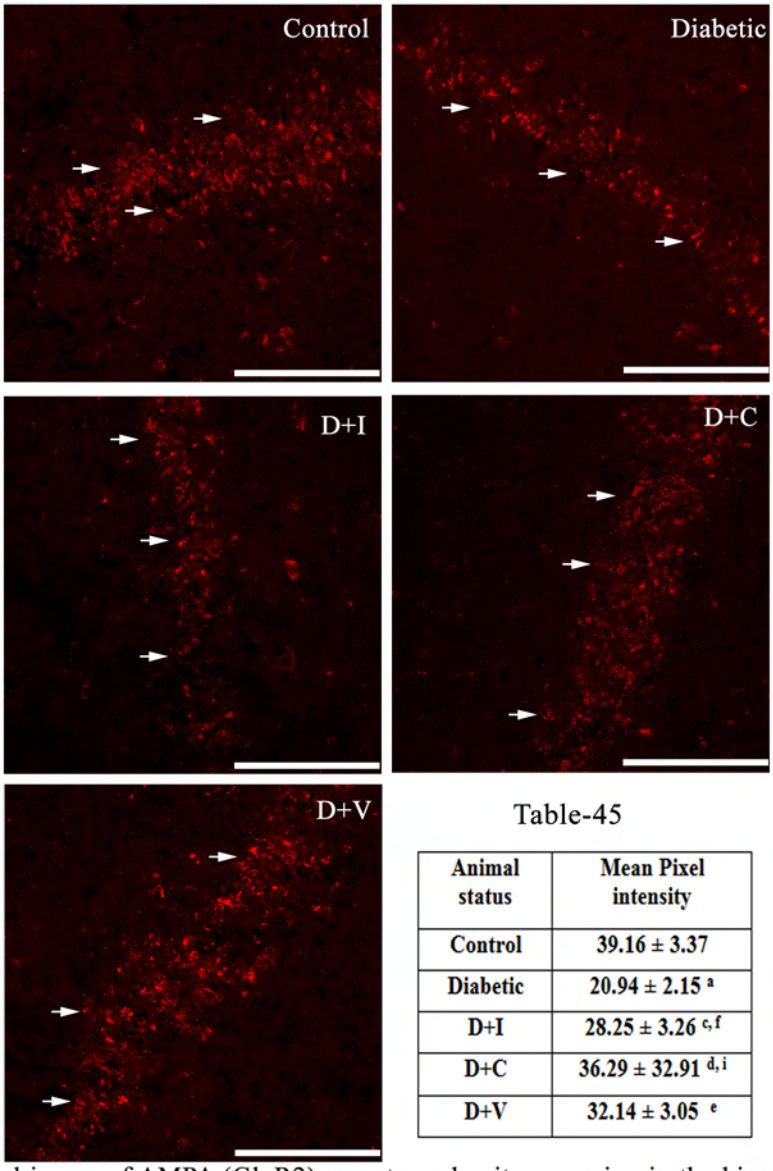


Table-45

Animal status	Mean Pixel intensity
Control	39.16 ± 3.37
Diabetic	20.94 ± 2.15 [a]
D+I	28.25 ± 3.26 [c, f]
D+C	36.29 ± 32.91 [d, i]
D+V	32.14 ± 3.05 [e]

Confocal image of AMPA (GluR2) receptor subunit expression in the hippocampus of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent AMPA (GluR2) receptor subunit specific primary antibody and Cy 5 as secondary antibody; [b] $p<0.01$ when compared to control group: [f] $p<0.05$ when compared to diabetic group; [h] $p<0.01$, [i] $p<0.05$ when compared to D+I group; (➡) in white shows AMPA (GluR2) receptor subunit. Scale bar = 150 µm

Figure-46

Glutamate content in the brain stem of control and experimental rats

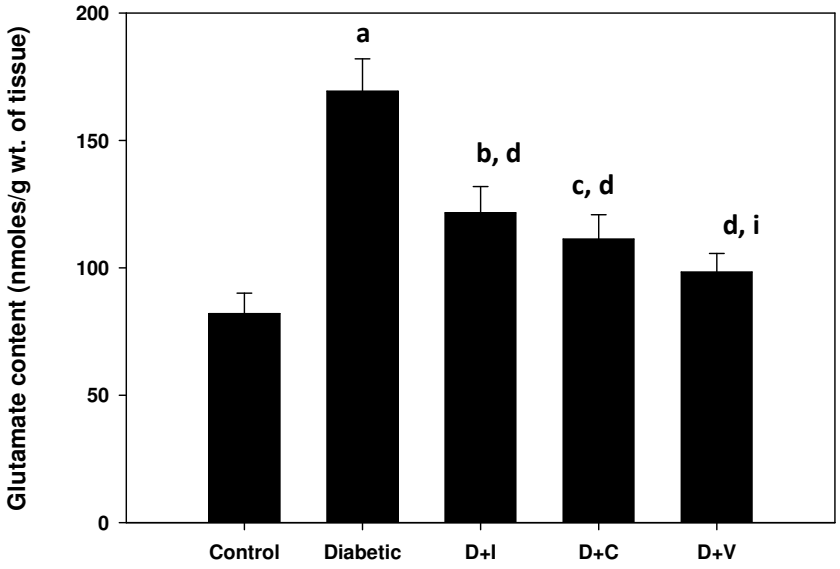


Table-46

Glutamate content in the brain stem of control and experimental rats

Animal Status	Glutamate content (nmoles/g wt. of tissue)
Control	**82.03 ± 8.01**
Diabetic	**169.32 ± 12.62** [a]
D+I	**121.56 ± 10.25** [b, d]
D+C	**111.36 ± 9.54** [c, d]
D+V	**98.35 ± 7.25** [d, i]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [i] $p<0.05$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-47

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the brain stem of control and experimental rats

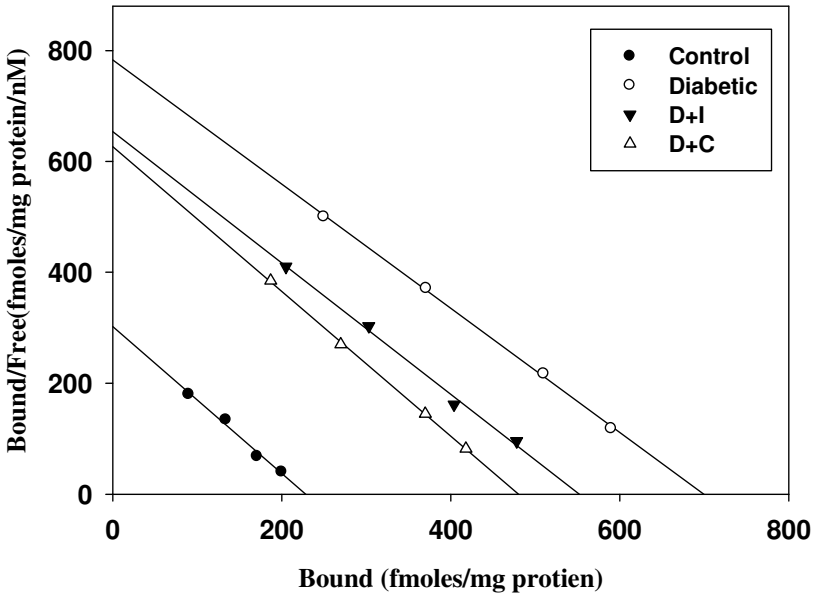


Table-47

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the brain stem of control and experimental rats

Animal status	B_{max}	K_d
Control	226 ± 18	0.73 ± 0.13
Diabetic	695 ± 49 [a]	0.88 ± 0.12
D+ I	546 ± 39 [a, d]	0.83 ± 0.11
D + C	475 ± 25 [a, d]	0.77 ± 0.13

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$when compared to control, [d] $p<0.001$ when compared to diabetic group. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats.

Figure-48

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the brain stem of control and experimental rats

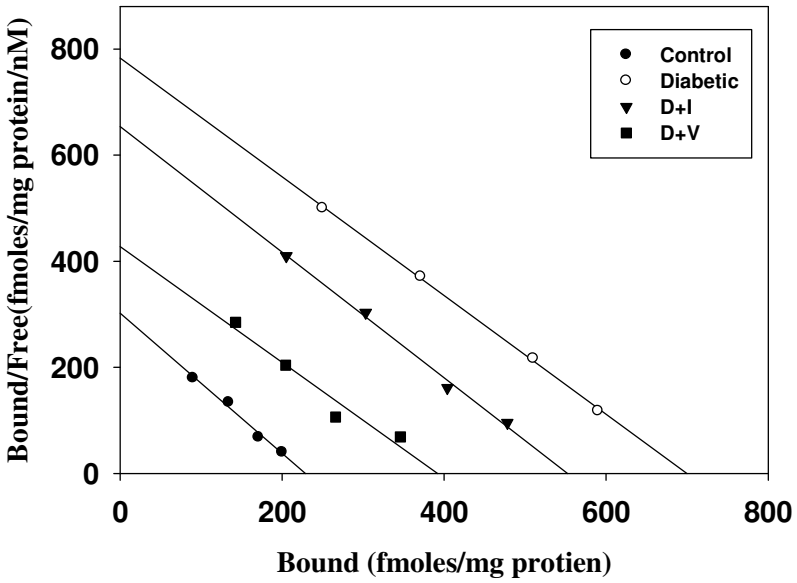


Table-48

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the Brain stem of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	226 ± 18	0.73 ± 0.13
Diabetic	695 ± 49 [a]	0.88 ± 0.12
D+ I	546 ± 39 [a, d]	0.83 ± 0.11
D + V	385 ± 31 [a, d, g]	0.89 ±0.08

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$, when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats.

Figure-49

Scatchard analysis of [^{3}H] AMPA binding against AMPA in brain stem of control and experimental rats

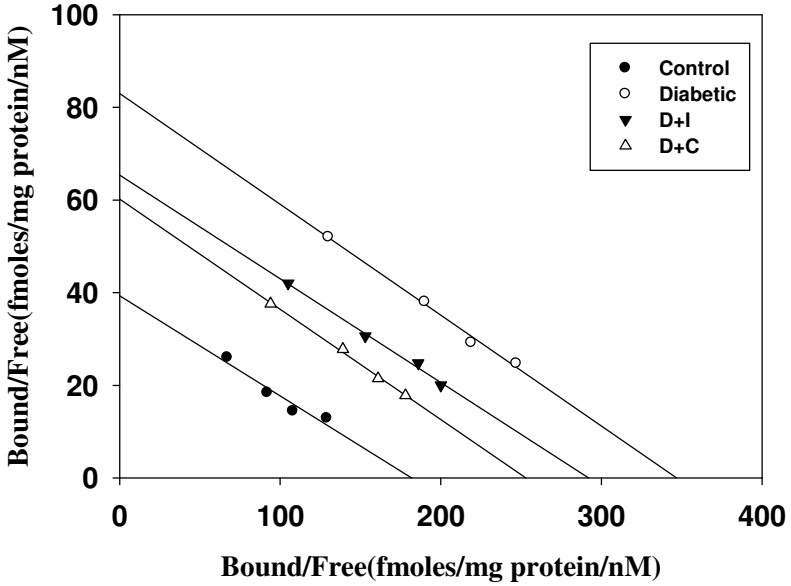


Table-49

Scatchard analysis of [^{3}H] AMPA binding against AMPA in brain stem of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	181 ± 15	4.53 ± 0.95
Diabetic	345 ± 27 [a]	4.13 ± 0.61
D+ I	291 ± 25 [a]	4.54 ± 0.75
D + C	252 ± 21 [a, e, h]	4.21 ± 0.81

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$ when compared to control [d] $p<0.001$, [e] $p<0.01$ when compared to diabetic group, [h] $p<0.01$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats.

Figure-50

Scatchard analysis of [^{3}H] AMPA binding against AMPA in brain stem of control and experimental rats

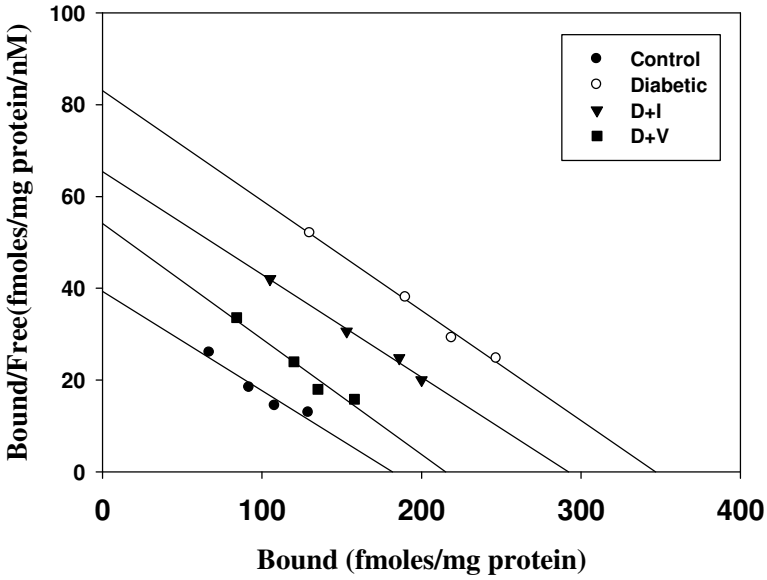


Table-50

Scatchard analysis of [^{3}H] AMPA binding against AMPA in brain stem of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**181 ± 15**	**4.53 ± 0.95**
Diabetic	**345 ± 27** [a]	**4.13 ± 0.61**
D+ I	**291 ± 25** [a]	**4.54 ± 0.75**
D + V	**214 ± 19** [a, d, g]	**3.98 ± 0.96**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] <0.01 when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C-curcumin treated diabetic rats.

Figure-51

Real time PCR analysis of NMDA R1 subunit gene expression in brain stem of control and experimental rats

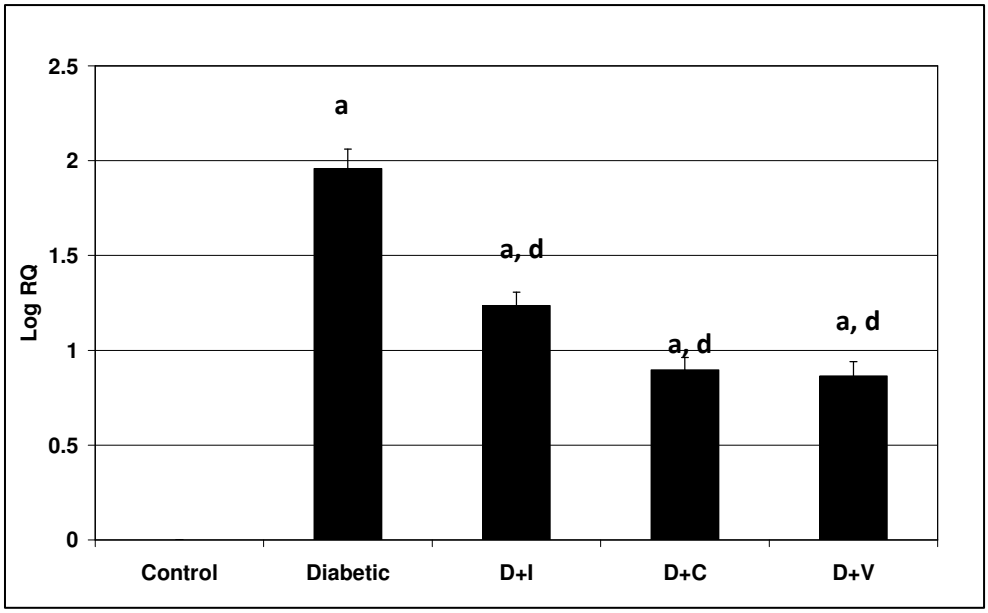


Table-51

Real time PCR analysis of NMDA R1 subunit gene expression in Brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.958 ± 0.103 [a]
D + I	1.235 ± 0.071 [a, d]
D+C	0.896±0.065 [a, d]
D+V	0.864 ± 0.075 [a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$when compared to control, [d] $p<0.001$when compared to diabetic group . D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-52

Real time PCR analysis of NMDA 2B subunit gene expression in brain stem of control and experimental rats

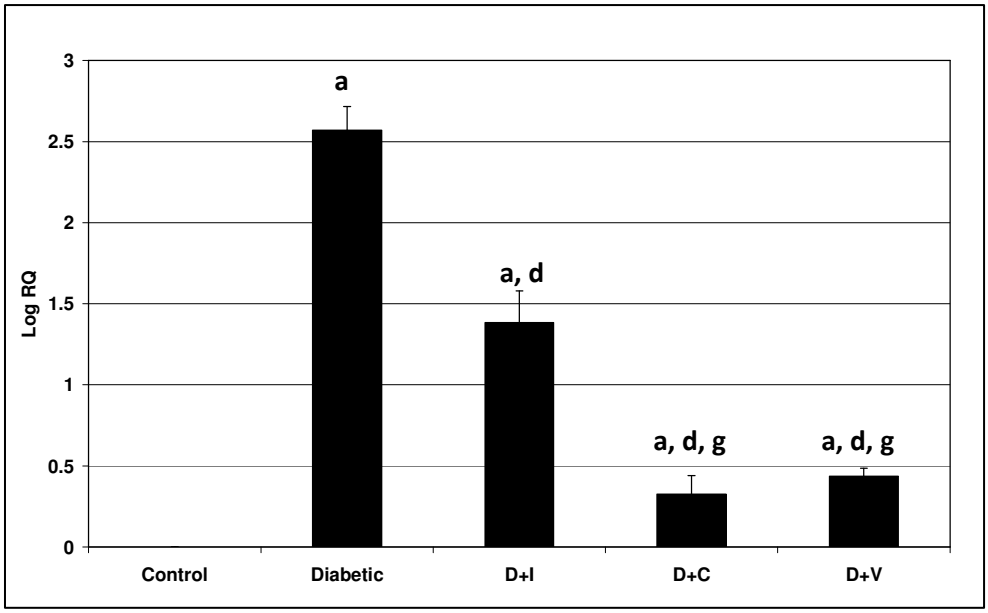


Table-52

Real time PCR analysis of NMDA 2B subunit gene expression in brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	2.569 ± 0.145 [a]
D + I	1.382 ± 0.197 [a, d]
D+C	0.325 ±0.114 [a, d, g]
D+V	0.4356 ± 0.048 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$when compared to control, [d] $p<0.001$,[e] $p<0.01$when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats . D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-53

Real time PCR analysis of AMPA (GluR4) Receptor subunit gene expression in brain stem of control and experimental rats

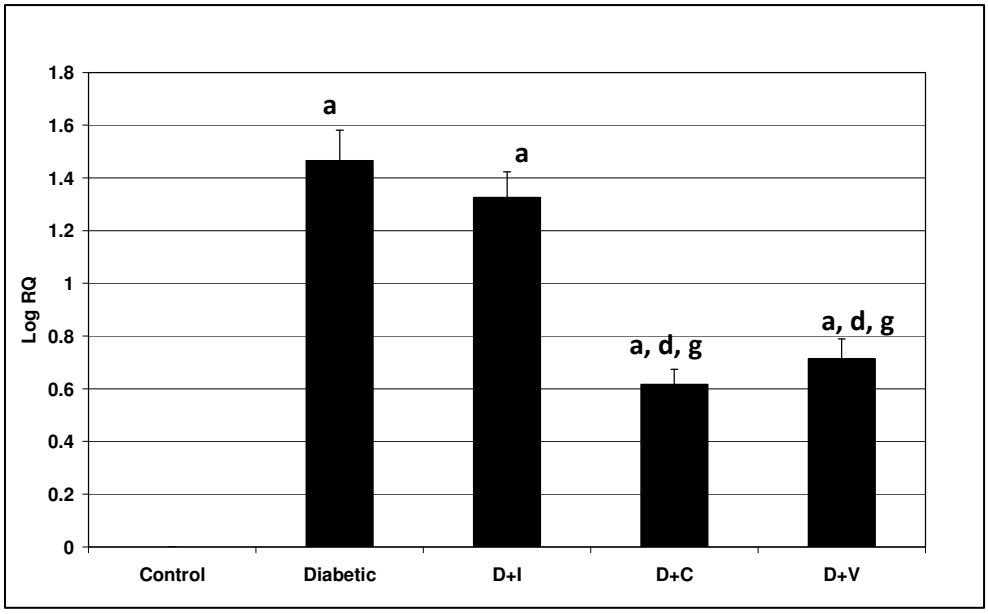


Table-53

Real time PCR analysis of AMPA (GluR4) Receptor subunit gene expression in Brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.465 ± 0.115 [a]
D + I	1.326 ± 0.096 [a]
D+C	0.616 ± 0.057 [a, d, g]
D+V	0.713 ± 0.075 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-54

Real time PCR analysis of AMPA (GluR2) Receptor subunit gene expression in brain stem of control and experimental rats

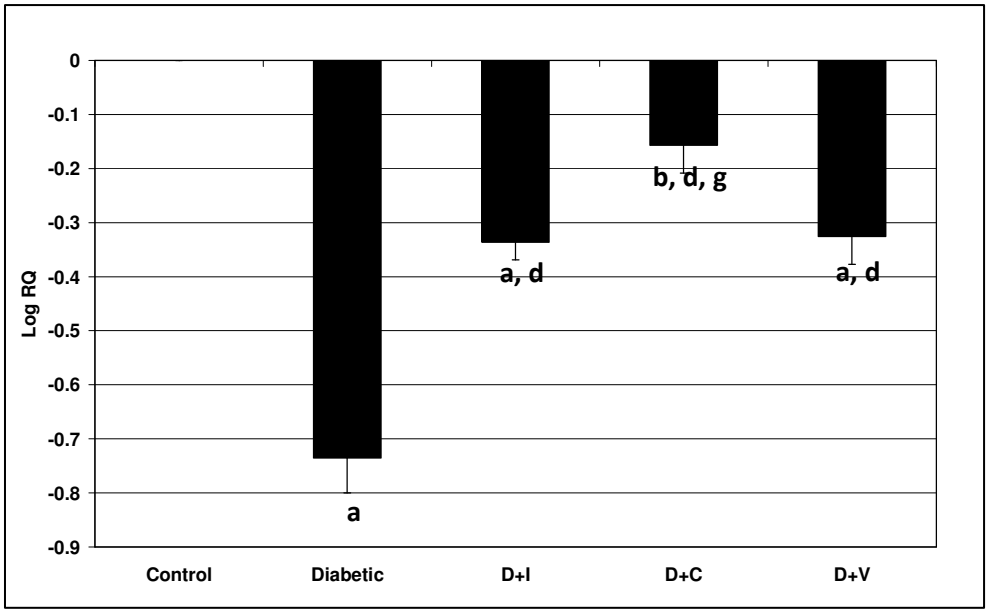


Table-54

Real time PCR analysis of AMPA (GluR2) Receptor subunit gene expression in brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.735 ± 0.064 [a]
D + I	-0.336 ±0.032 [a, d]
D+C	-0.156 ±0.051 [b, d, g]
D+V	-0.1325 ±0.025 [a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$when compared to control, [d] $p<0.001$when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure -55

Real time PCR analysis of GLAST gene expression in brain stem of control and experimental rats

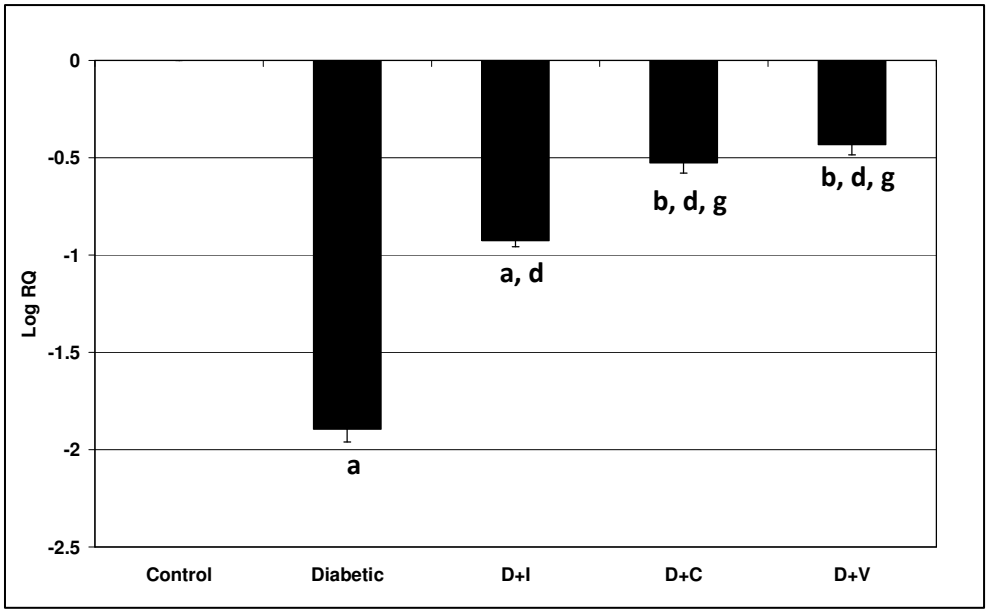


Table-55

Real time PCR analysis of GLAST gene expression in brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.895 ± 0.064 [a]
D + I	-0.925 ± 0.032 [a, d]
D+C	-0.526 ± 0.051 [b, d, g]
D+V	-0.432 ± 0.051 [b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure -56

Real time PCR analysis of GAD gene expression in brain stem of control and experimental rats

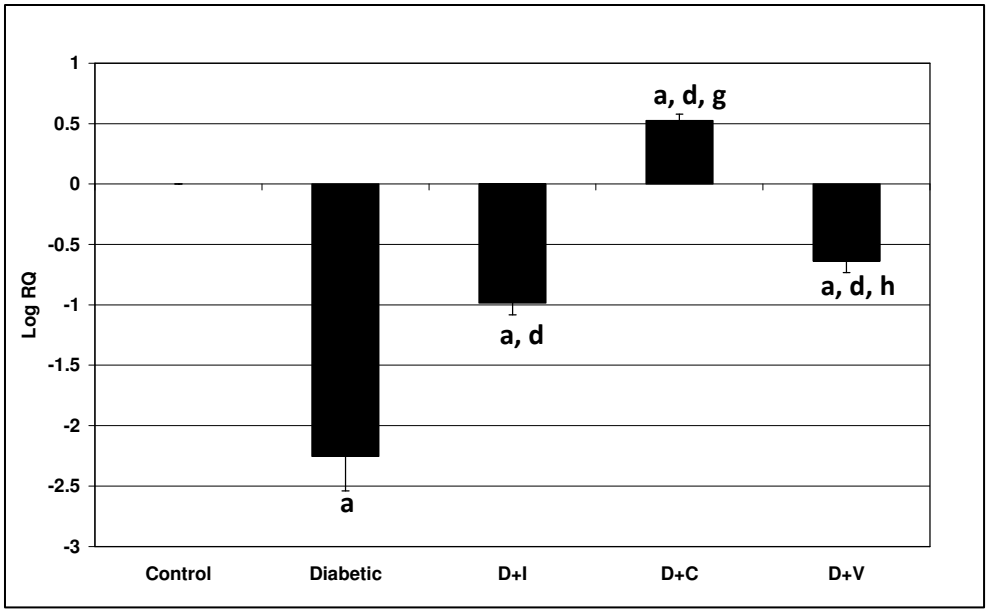


Table-56
Real time PCR analysis of GAD gene expression in brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-2.253 ± 0.285 [a]
D + I	-0.985 ± 0.096 [a, d]
D+C	0.525 ± 0.051 [a, d, g]
D+V	-0.636 ± 0.095 [a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$, [h] $p<0.01$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-57

IP3 content in the brain stem of control and experimental rats

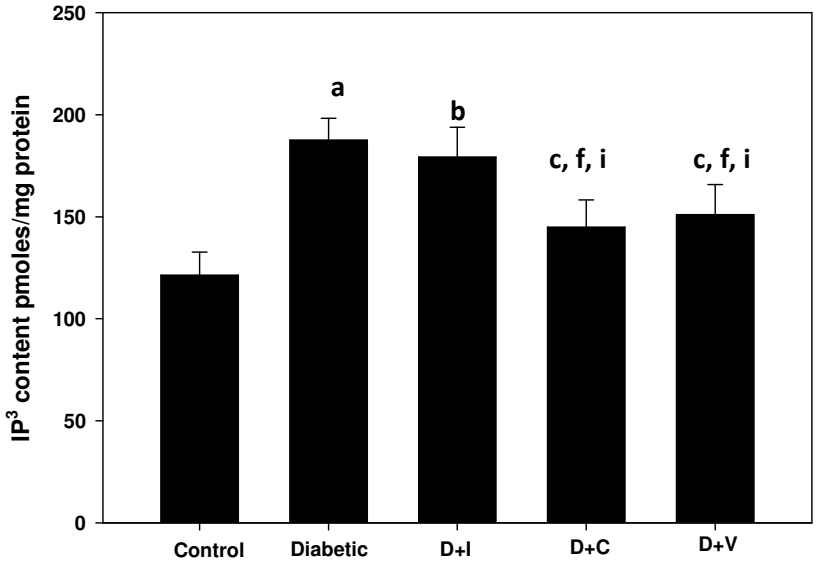


Table-57

IP3 content in the Brain stem of control and experimental rats

Animal Status	IP3 Content (pmoles/mg protein)
Control	121.36 ± 11.36
Diabetic	187.54 ± 10.75 [a]
D + I	179.36 ± 14.56 [b]
D+C	145.00 ± 13.24 [c, f, i]
D+V	151.00 ± 14.82 [c, f, i]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control, [f] $p<0.05$ when compared to diabetic group. [i] $p<0.05$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-58

Superoxide dismutase assay in the brain stem of control and experimental rats

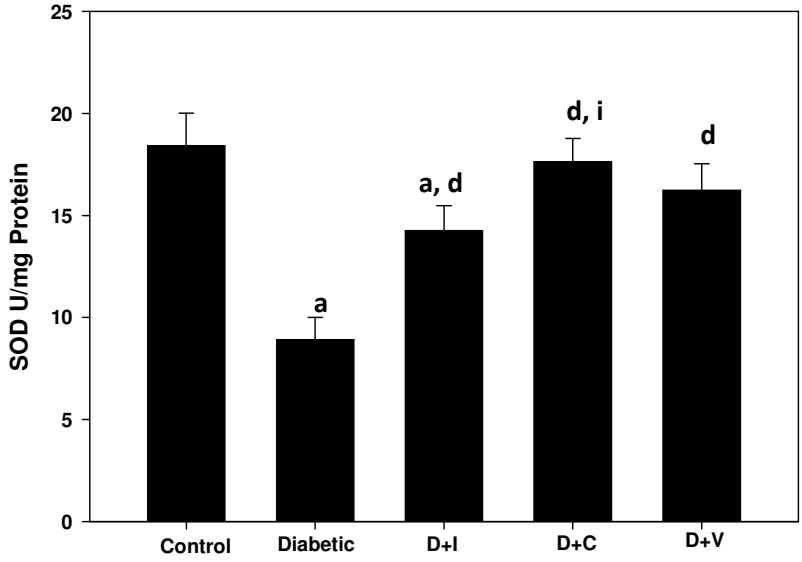


Table-58

Superoxide dismutase assay in the brain stem of control and experimental rats

Animal Status	SOD activity
Control	18.42 ± 1.60
Diabetic	8.91 ± 1.10 [a]
D + I	14.25 ± 1.23 [a, d]
D+C	17.63 ± 1.14 [d, i]
D+V	16.23 ± 1.30 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, when compared to control, [d] $p<0.001$when compared to diabetic group, [i] $p<0.05$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure -59

Real time PCR analysis of GPx gene expression in brain stem of control and experimental rats

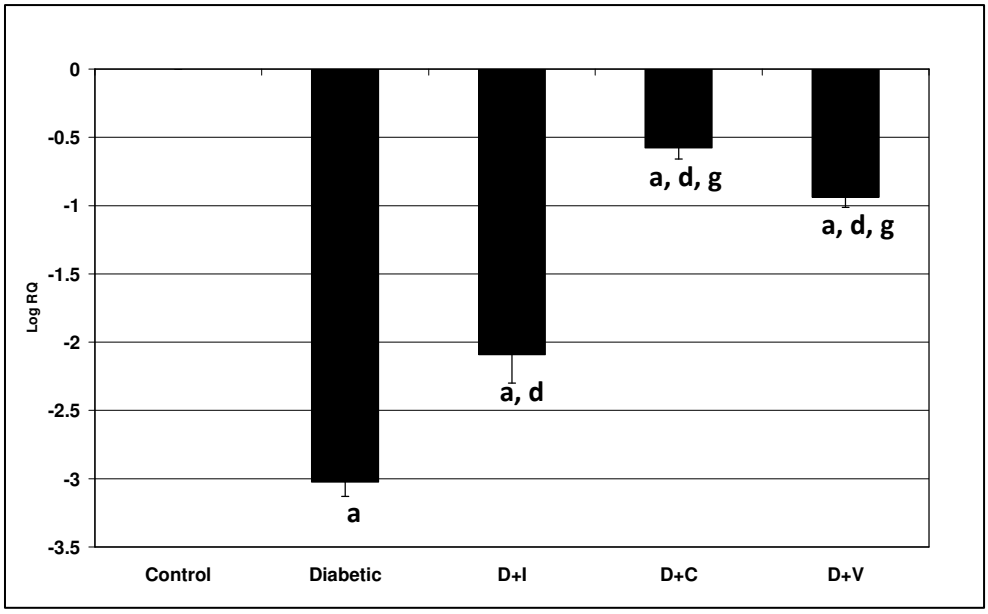


Table-59

Real time PCR analysis of GAD gene expression in brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-3.025± 0.040 [a]
D + I	-2.092± 0.010 [a, d]
D+C	-0.575 ± 0.009 [a, d, g]
D+V	-0.936 ± 0.012 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-60

Real time PCR analysis of Akt-1 gene expression in brain stem of control and experimental rats

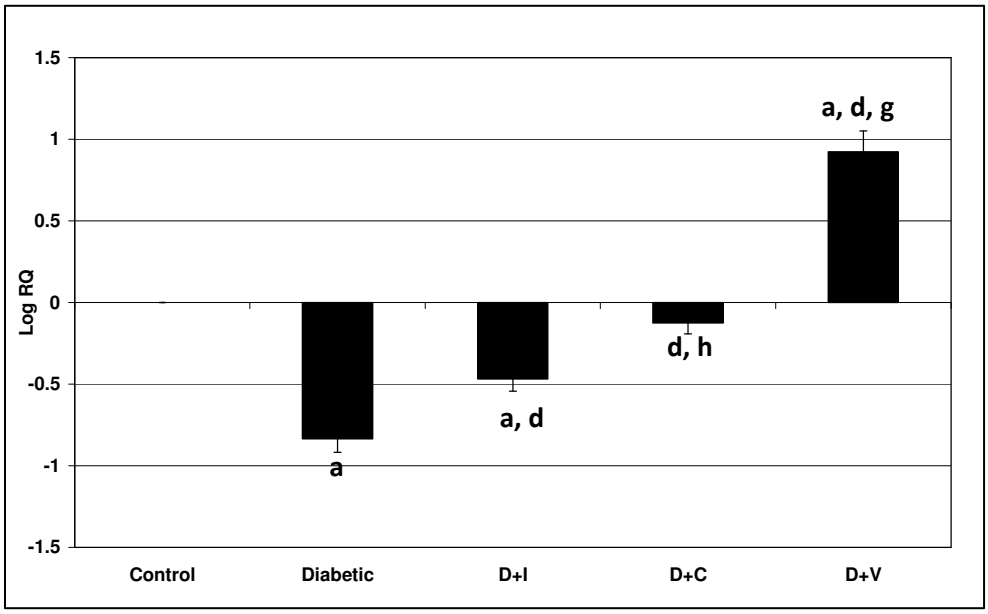


Table-60

Real time PCR analysis of Akt-1 gene expression in Brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.836 ± 0.081 [a]
D + I	-0.468 ± 0.075 [a. d]
D+C	-0.124 ± 0.067 [d, h]
D+V	0.924 ± 0.125 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ [b] $p<0.01$ when compared to Control, [d] $p<0.001$ when compared to Diabetic group. [g] $p<0.001$, [h] $p<0.01$ when compared to Diabetic group D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats.

Figure-61

Real time PCR analysis of Bax gene expression in brain stem of control and experimental rats

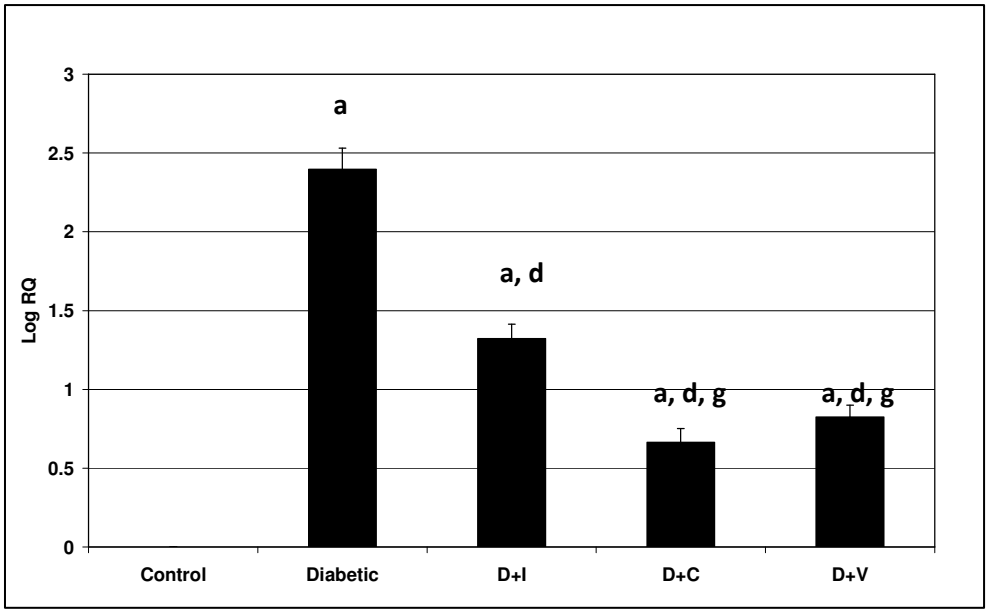


Table –61

Real time PCR analysis of Bax gene expression in Brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	2.396 ± 0.135 [a]
D + I	1.321 ± 0.091 [a, d]
D+C	0.664 ± 0.085 [a, d, g]
D+V	0.825 ± 0.076 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-62

Real time PCR analysis of Caspase 8 gene expression in brain stem of control and experimental rats

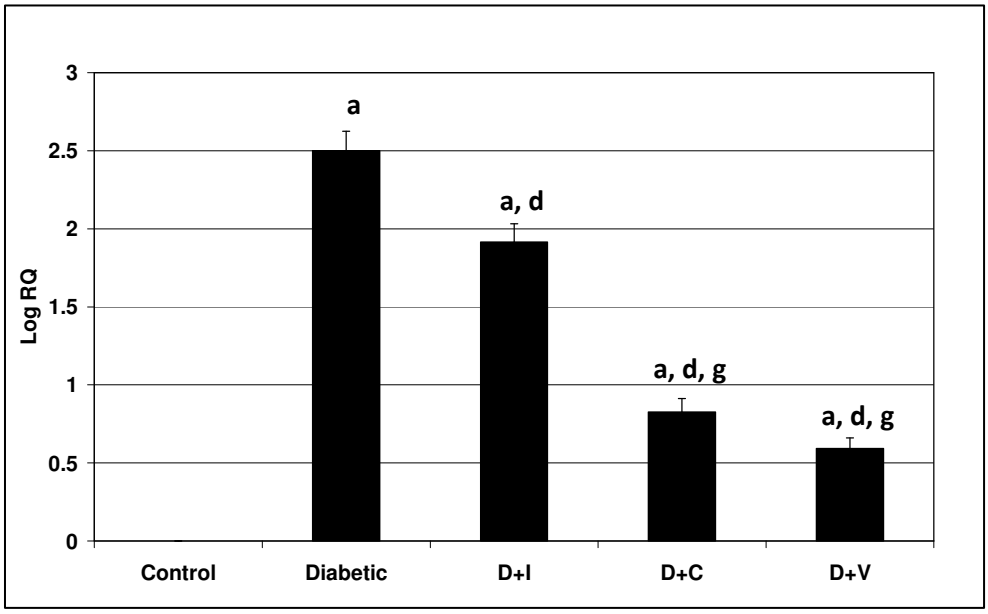


Table –62

Real time PCR analysis of Caspase 8 gene expression in Brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	2.498 ± 0.125 [a]
D + I	1.915 ± 0.116 [a, d]
D+C	0.825 ± 0.861 [a, d, g]
D+V	0.593 ±0.066 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure- 63

NMDA R1 receptor subunit expression in the Brain stem of control and experimental rats

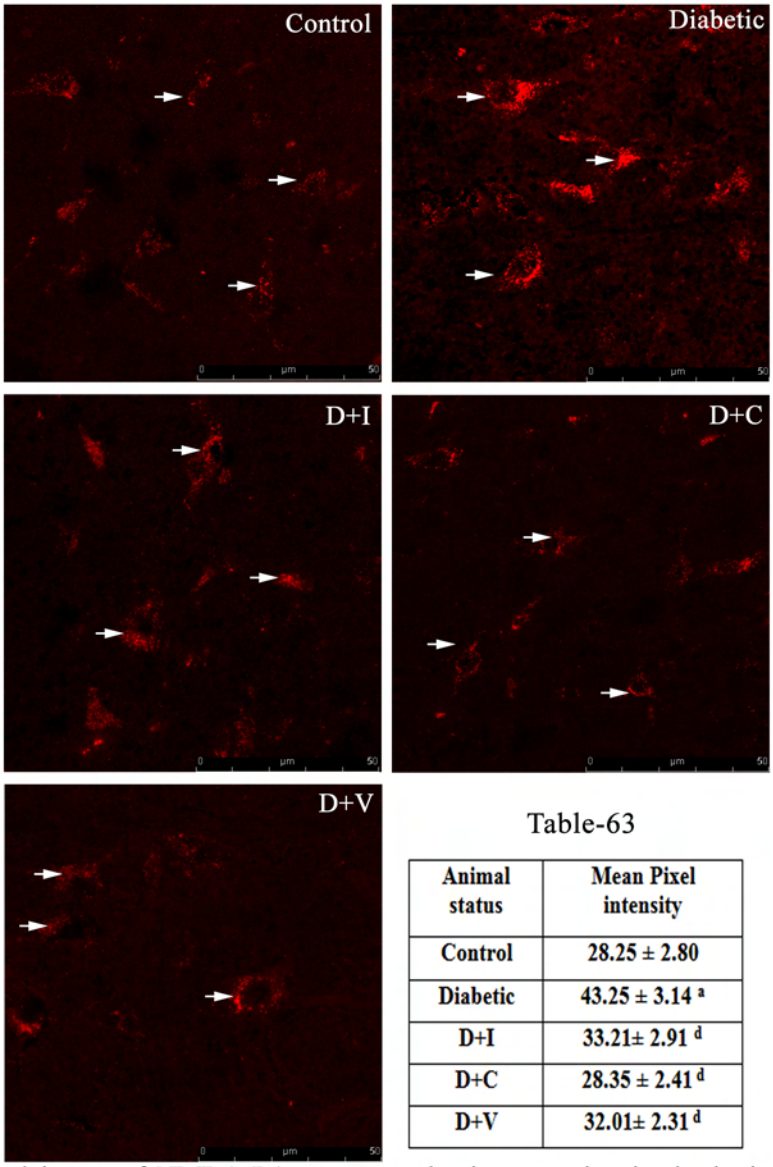


Table-63

Animal status	Mean Pixel intensity
Control	28.25 ± 2.80
Diabetic	43.25 ± 3.14 [a]
D+I	33.21± 2.91 [d]
D+C	28.35 ± 2.41 [d]
D+V	32.01± 2.31 [d]

Confocal image of NMDA R1 receptor subunit expression in the brain stem of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent NMDA R1 receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, when compared to control group: [d] $p<0.001$ when compared to diabetic group; (➡) in white shows NMDA receptors. Scale bar = 50 µm

Figure- 64

NMDA 2B receptor subunit expression in the brain stem of control and experimental rats

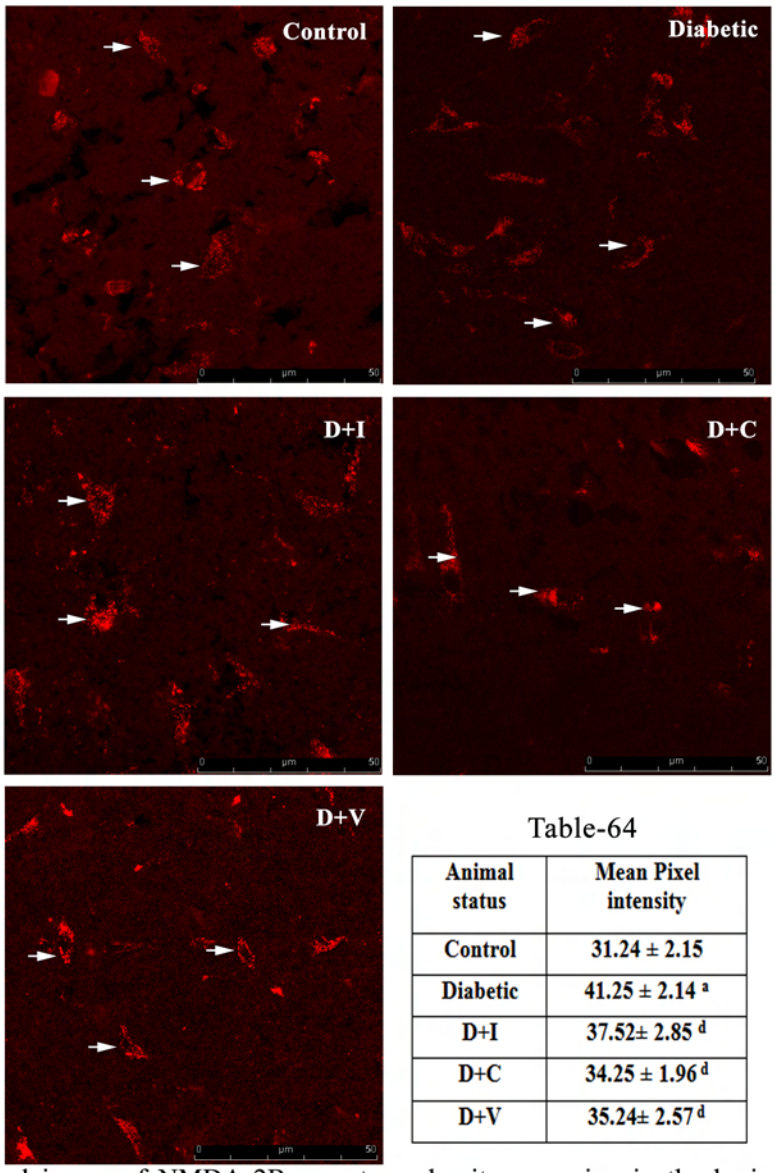


Table-64

Animal status	Mean Pixel intensity
Control	31.24 ± 2.15
Diabetic	41.25 ± 2.14 [a]
D+I	37.52± 2.85 [d]
D+C	34.25 ± 1.96 [d]
D+V	35.24± 2.57 [d]

Confocal image of NMDA 2B receptor subunit expression in the brain stem of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent NMDA 2B receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, when compared to control group: [f] $p<0.05$ when compared to diabetic group; (➡) in white shows NMDA receptors. Scale bar = 50 µm

Figure- 65

AMPA (GluR4) receptor subunit expression in the Brain stem of control and experimental rats

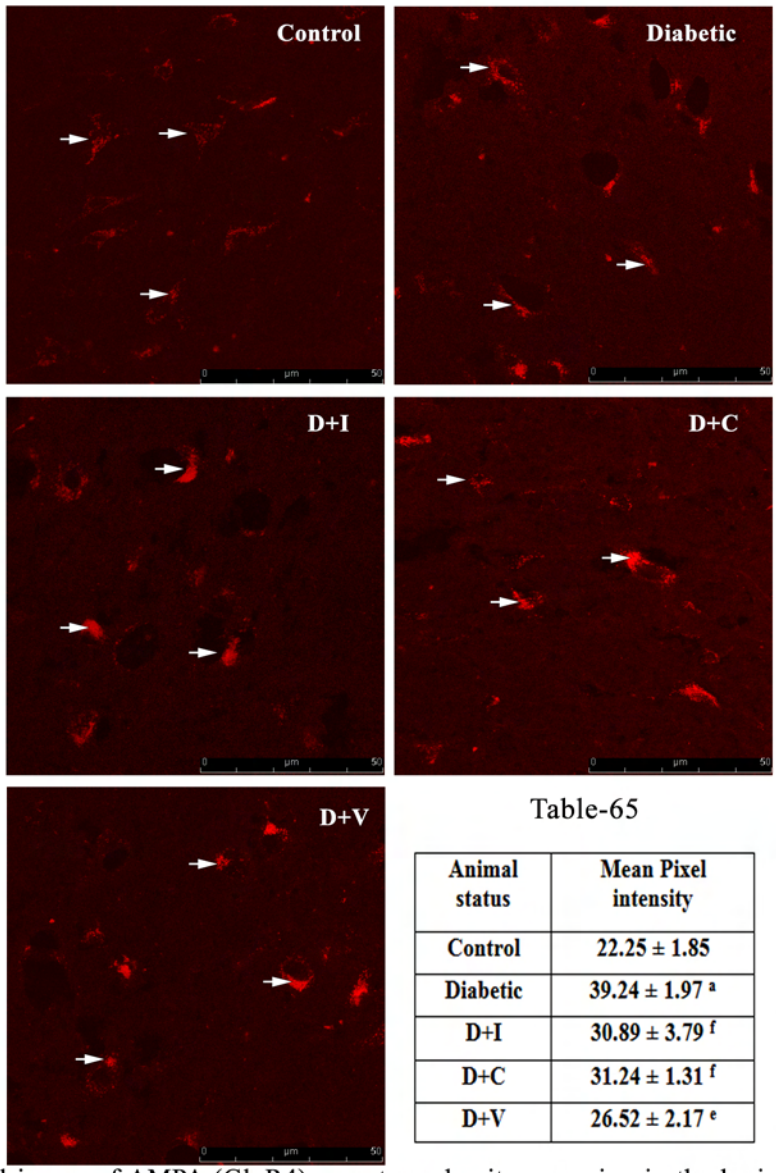


Table-65

Animal status	Mean Pixel intensity
Control	22.25 ± 1.85
Diabetic	39.24 ± 1.97 [a]
D+I	30.89 ± 3.79 [f]
D+C	31.24 ± 1.31 [f]
D+V	26.52 ± 2.17 [e]

Confocal image of AMPA (GluR4) receptor subunit expression in the brain stem of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent AMPA GluR4 receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, when compared to control group: [f] $p<0.05$ when compared to diabetic group; (➡) in white shows AMPAreceptors.Scale bar = 50 µm

Figure- 66

AMPA (GluR2) receptor subunit expression in the brain stem of control and experimental rats

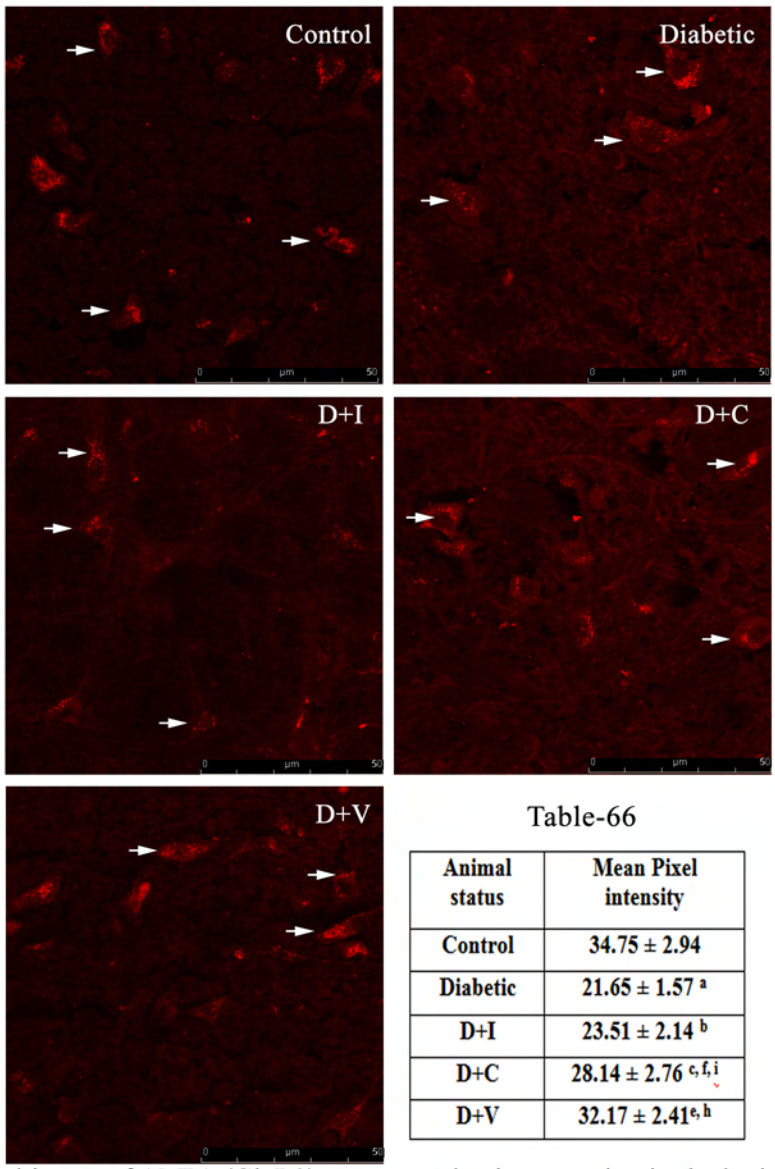


Table-66

Animal status	Mean Pixel intensity
Control	34.75 ± 2.94
Diabetic	21.65 ± 1.57 [a]
D+I	23.51 ± 2.14 [b]
D+C	28.14 ± 2.76 [c, f, i]
D+V	32.17 ± 2.41 [e, h]

Confocal image of AMPA (GluR2) receptor subunit expression in the brain stem of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent AMPA GluR2 receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [e] $p<0.01$, [f] $p<0.05$ when compared to diabetic group; [h] $p<0.01$, [i] $p<0.05$ when compared to D+I group; (➡) in white shows AMPA GluR2 receptors. Scale bar = 50 µm

Figure-67

Glutamate content in the cerebellum of control and experimental rats

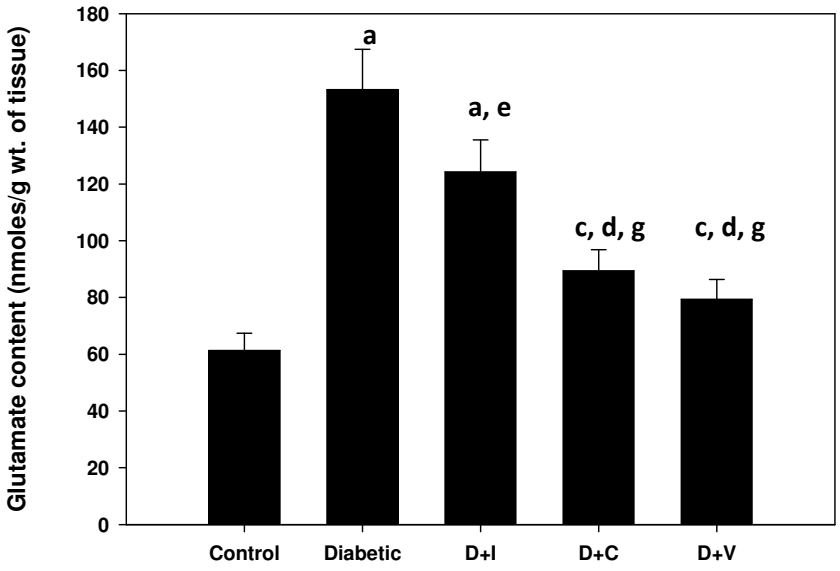


Table-67

Glutamate content in the Cerebellum of control and experimental rats

Animal Status	Glutamate content (nmoles/g wt. of tissue)
Control	**61.25 ± 6.16**
Diabetic	**153.25 ± 14.25** [a]
D+I	**124.25 ± 11.25** [a, e]
D+C	**89.33 ± 7.53** [c, d, g]
D+V	**79.34 ± 6.95** [c, d, g]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a] $p<0.001$, , [c] $p<0.05$ when compared with control adult rats, [d] $p<0.001$, [e] $p<0.01$ when compared with diabetic adult rats, [g] $p<0.001$, when compared with insulin treated diabetic rats. . D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-68
Scatchard Analysis NMDA receptors using [^{3}H] MK-801 binding against MK-801 in the cerebellum of control and experimental rats

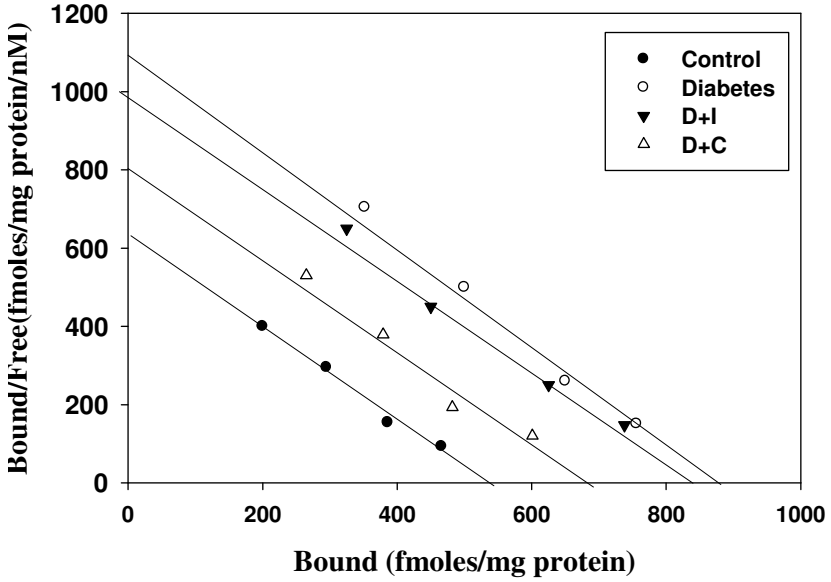


Table-68

Scatchard Analysis NMDA receptors using [^{3}H] MK-801 binding against MK-801 in the cerebellum of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	542 ± 42	0.84 ± 0.14
Diabetic	883 ± 75 [a]	0.79 ± 0.10
D+ I	835 ± 81 [b]	0.85 ± 0.19
D + C	687 ± 56 [d, h]	0.85 ± 0.13

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [h] $p<0.01$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats and D+C- curcumin treated diabetic rats.

Figure-69
Scatchard Analysis NMDA receptors using [^{3}H] MK-801 binding against MK-801 in the cerebellum of control and experimental rats

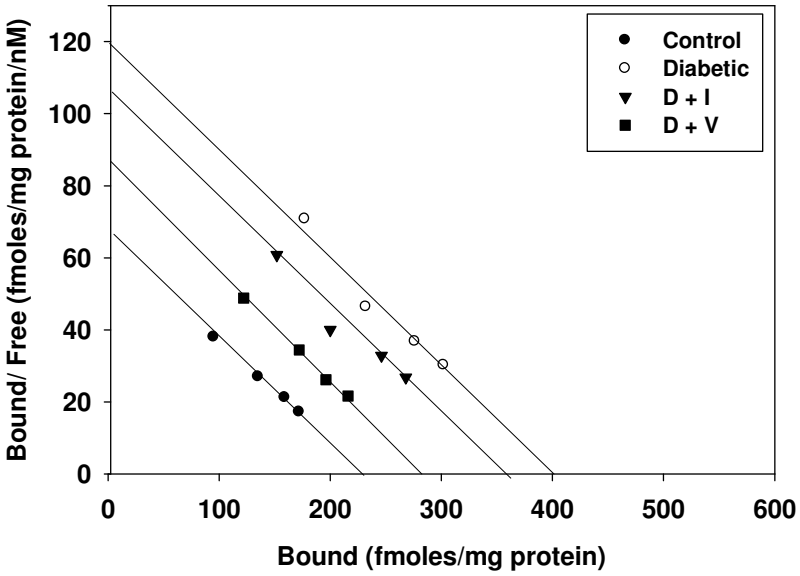


Table-69

Scatchard Analysis NMDA receptors using [^{3}H] MK-801 binding against MK-801 in the cerebellum of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	542 ± 42	0.84 ± 0.14
Diabetic	883 ± 75 [a]	0.79 ± 0.10
D+ I	835 ± 81 [b]	0.85 ± 0.19
D + V	612 ± 57 [d, h]	0.82 ± 0.09

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$ when compared to diabetic Group, [h] $p<0.01$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-70

Scatchard analysis of [^{3}H] AMPA binding against AMPA in cerebellum of control and experimental rats

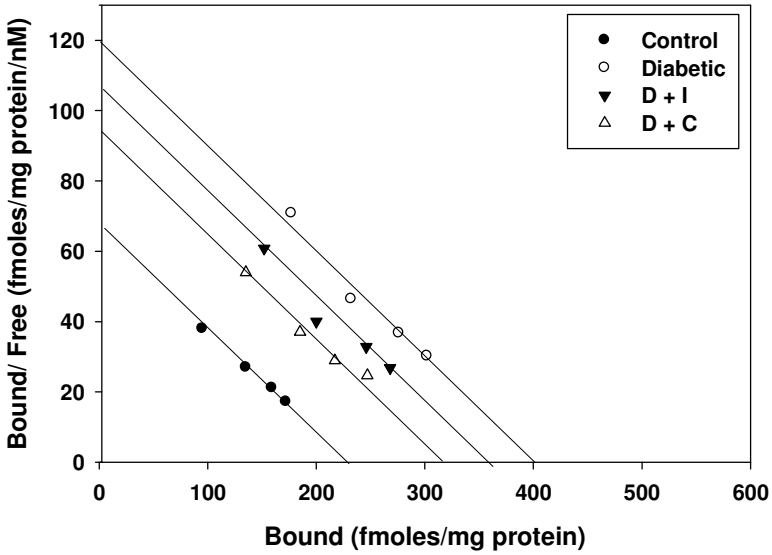


Table-70

Scatchard analysis of [^{3}H] AMPA binding against AMPA in cerebellum of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	227 ± 19	3.31 ± 0.51
Diabetic	399 ± 26 [a]	3.35 ± 0.67
D+ I	354 ± 31 [b]	3.40 ± 0.57
D + C	316 ± 29 [b, e]	3.39 ± 0.42

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [e] $p<0.01$ when compared to diabetic group. D + I- insulin treated diabetic rats and D+C- curcumin treated diabetic rats.

Table-71

Scatchard analysis of [^{3}H] AMPA binding against AMPA in cerebellum of control and experimental rats

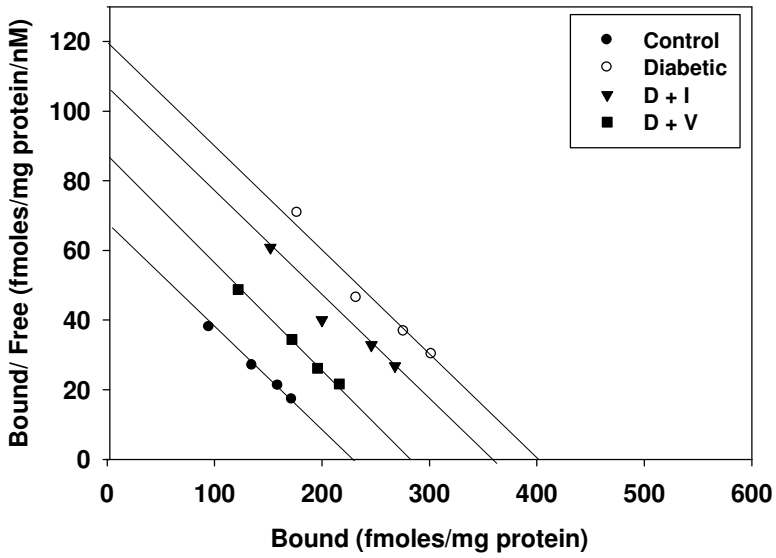


Table-71

Scatchard analysis of [^{3}H] AMPA binding against AMPA in cerebellum of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	227 ± 19	3.31 ± 0.51
Diabetic	399 ± 26 [a]	3.35 ± 0.67
D+ I	354 ± 31 [b]	3.40 ± 0.57
D + V	281 ± 22 [c, e. i]	3.24 ± 0.42

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats. [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control. [e] $p<0.01$ when compared to diabetic Group, [i] $p<0.05$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-72

Real time PCR analysis of NMDA R1 subunit gene expression in cerebellum of control and experimental rats

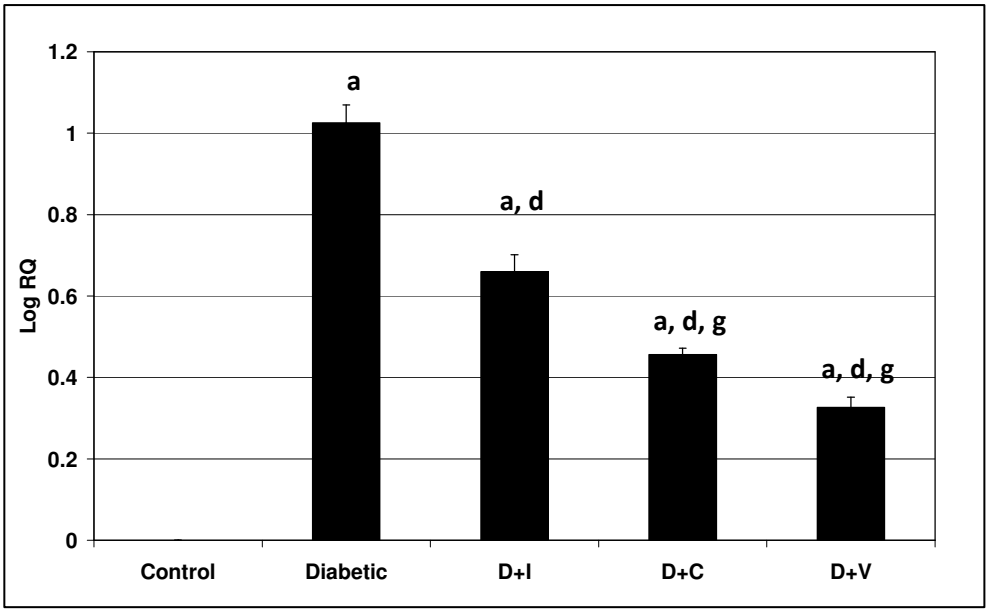


Table-72

Real time PCR analysis of NMDA R1 subunit gene expression in Cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.02 ± 0.04 [a]
D + I	0.65 ± 0.04 [a, d]
D+C	0.45 ± 0.01 [a, d, g]
D+V	0.32 ± 0.02 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-73

Real time PCR analysis of NMDA 2B subunit gene expression in cerebellum of control and experimental rats

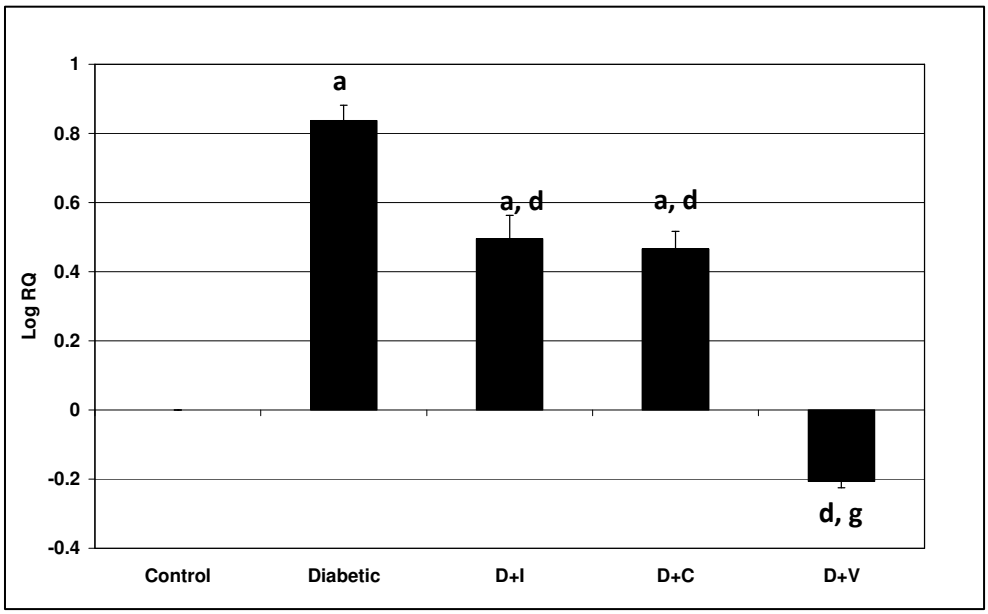


Table-73

Real time PCR analysis of NMDA 2B subunit gene expression in cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	**0**
Diabetic	**0.836 ± 0.044** [a]
D + I	**0.495 ± 0.067** [a, d]
D+C	**0.465 ± 0.051** [a, d]
D+V	**-0.206 ± 0.018** [d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats . D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure- 74

Real time PCR analysis of AMPA (GluR4) Receptor subunit gene expression in cerebellum of control and experimental rats

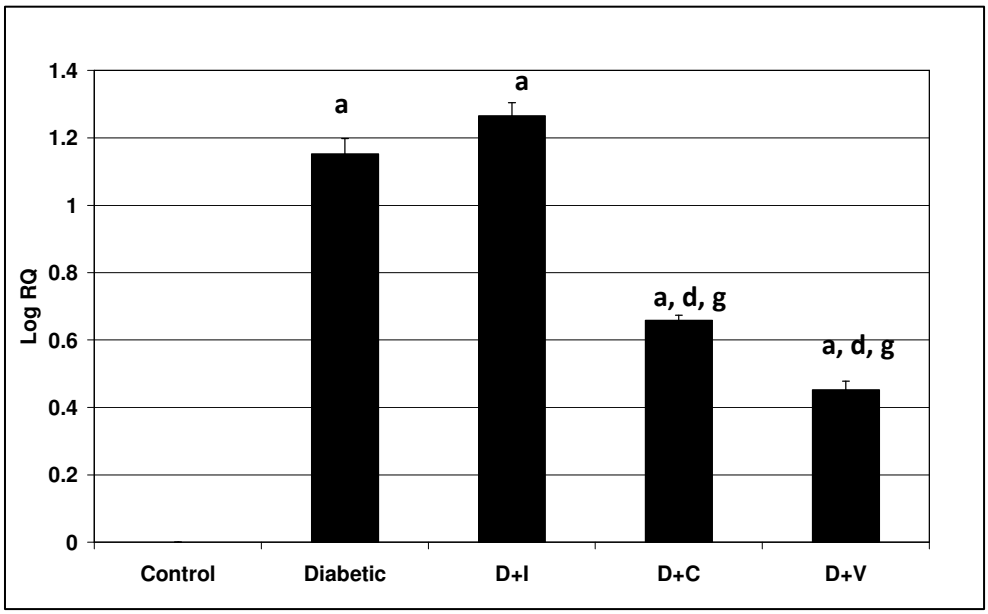


Table-74

Real time PCR analysis of AMPA (GluR4) Receptor subunit gene expression in Cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.152 ± 0.065 [a]
D + I	1.265 ± 0.072 [a]
D+C	0.658 ± 0.015 [a, d. g]
D+V	0.452 ± 0.025 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to Control, [d] $p<0.001$, when compared to Diabetic group. [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats.

Figure-75

Real time PCR analysis of AMPA (GluR2) Receptor subunit gene expression in cerebellum of control and experimental rats

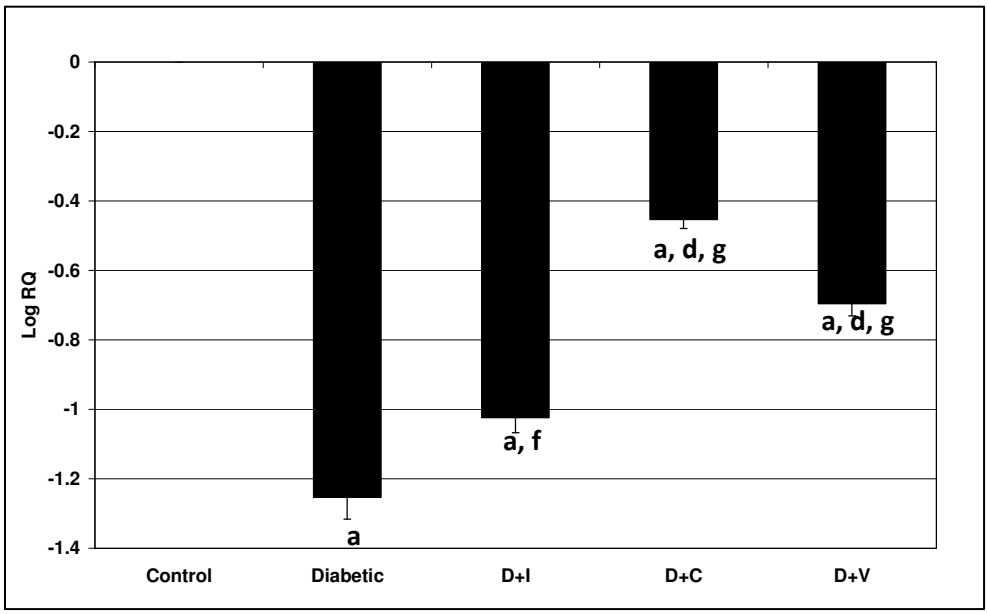


Table-75

Real time PCR analysis of AMPA (GluR2) Receptor subunit gene expression in cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.253 ± 0.083 [a]
D + I	-1.024 ±0.067 [a, f]
D+C	-0.453 ±0.025 [a, d, g]
D+V	-0.695 ±0.036 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$, [f] $p<0.05$, when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure –76

Real time PCR analysis of GLAST gene expression in cerebellum of control and experimental rats

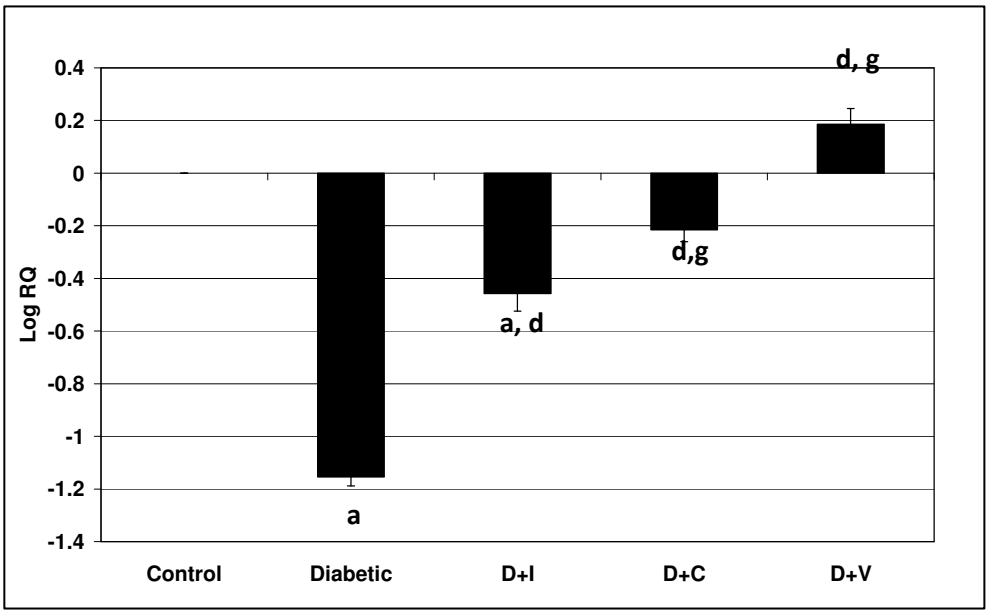


Table -76

Real time PCR analysis of GLAST gene expression in Cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.154 ± 0.078 [a]
D + I	-0.456 ± 0.067 [a, d]
D+C	-0.215 ± 0.047 [d, g]
D+V	0.186 ± 0.057 [d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-77

Real time PCR analysis of GAD gene expression in cerebellum of control and experimental rats

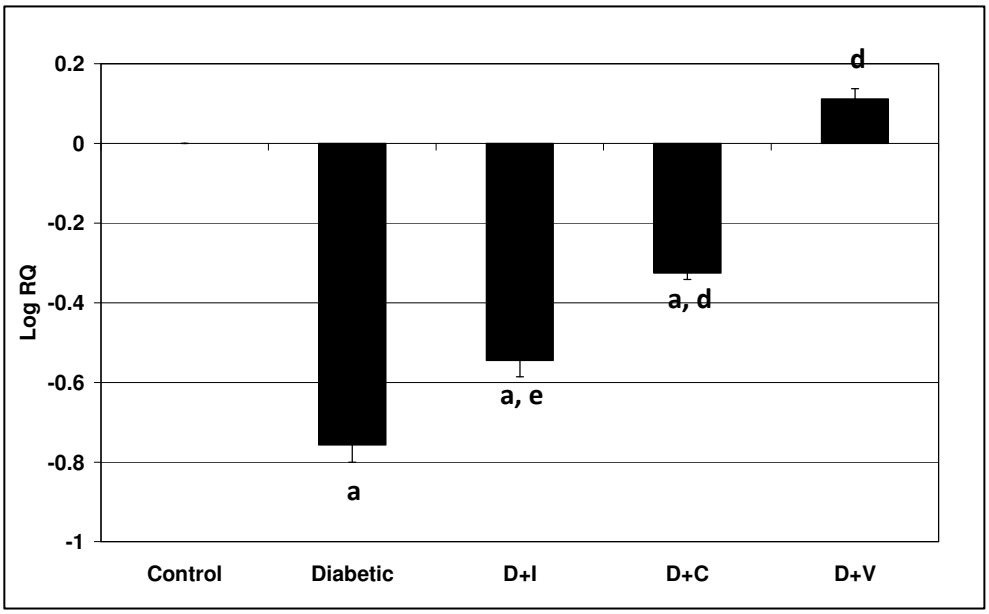


Table -77

Real time PCR analysis of GAD gene expression in Cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.756 ± 0.078 [a]
D + I	-0.544 ± 0.067 [a, e]
D+C	-0.325 ± 0.047 [a, d]
D+V	0.112 ± 0.057 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$when compared to control, [d] $p<0.001$, [e] $p<0.01$ when compared to diabetic group . D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-78

IP3 content in the cerebellum of control and experimental rats

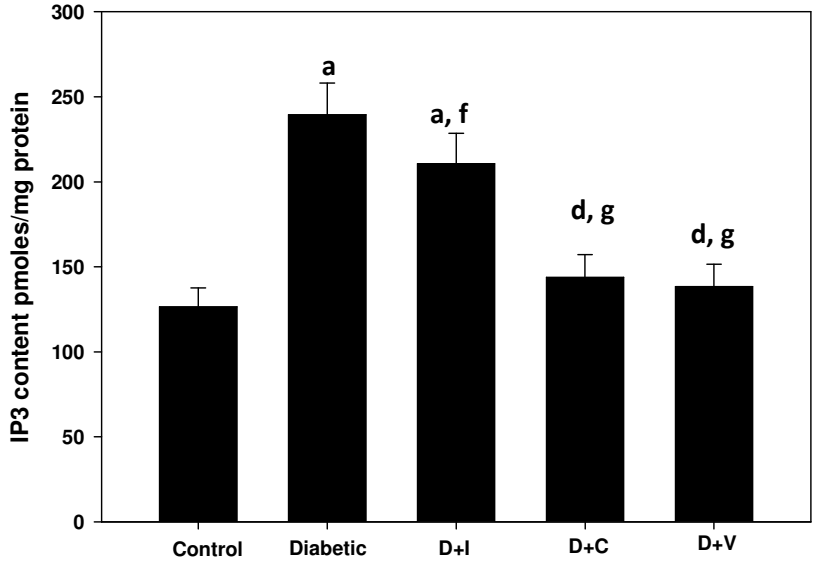


Table-78

IP3 content in the cerebellum of control and experimental rats

Animal Status	IP3 Content (pmoles/mg protein)
Control	126.35 ± 08.25
Diabetic	239.35 ± 17.95 [a]
D + I	210.54 ± 14.65 [a, f]
D+C	143.85 ± 09.73 [d, g]
D+V	138.36 ± 10.74 [d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$, [f] $p<0.05$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-79

Superoxide dismutase assay in the cerebellum of control and experimental rats

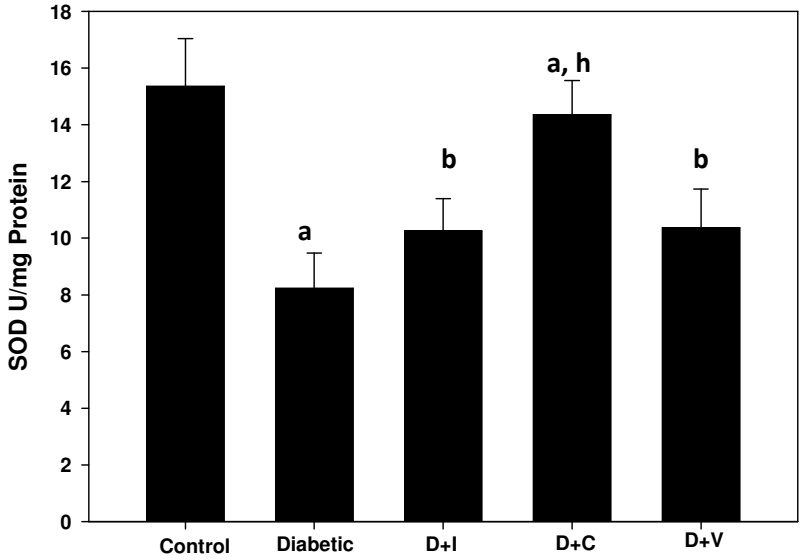


Table-79

Superoxide dismutase assay in the Cerebellum of control and experimental rats

Animal Status	SOD activity (unit/mg protein)
Control	15.35 ± 1.69
Diabetic	8.24 ± 1.04 [a]
D + I	10.25 ± 1.15 [b]
D+C	14.35 ± 1.21 [a, h]
D+V	10.36 ± 1.37 [b]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$, [e] $p<0.01$ when compared to diabetic group, [h] $p<0.01$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-80

Real time PCR analysis of GPx gene expression in cerebellum of control and experimental rats

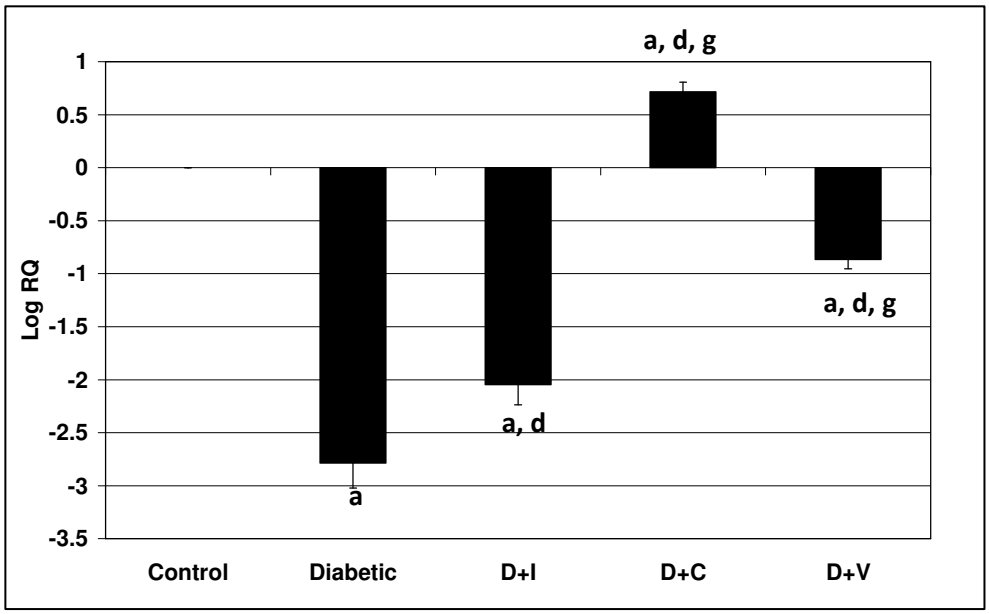


Table -80

Real time PCR analysis of GPx gene expression in cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-2.786 ± 0.235 [a]
D + I	-2.046 ± 0.191 [a, d]
D+C	0.715 ± 0.092 [a, d, g]
D+V	-0.867 ± 0.085 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-81

Real time PCR analysis of Akt-1 gene expression in cerebellum of control and experimental rats

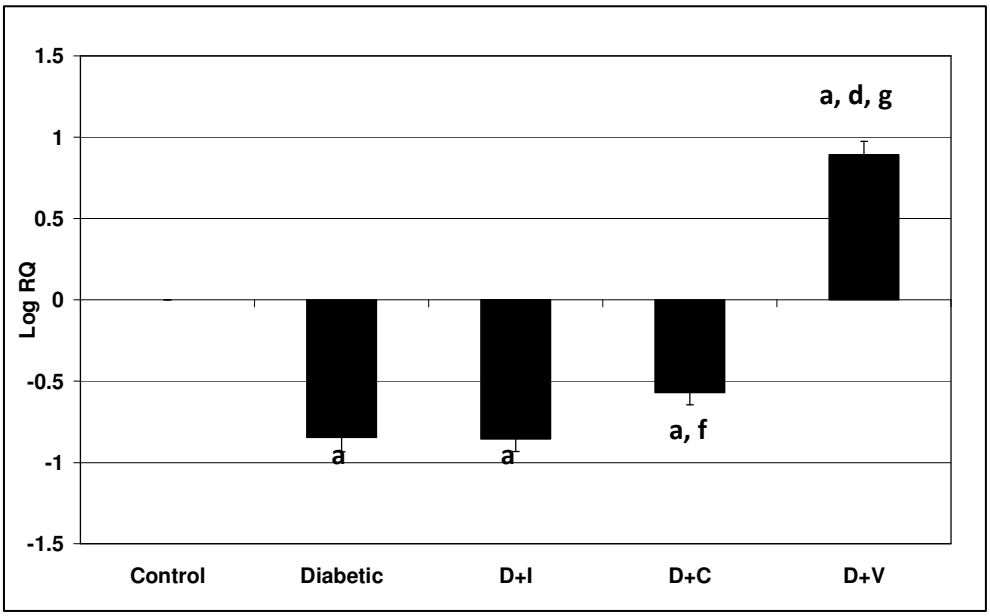


Table -81

Real time PCR analysis of Akt-1 gene expression in Cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-0.846 ± 0.089 [a]
D + I	-0.856 ± 0.075 [a]
D+C	-0.569 ± 0.075 [a, f]
D+V	0.892 ± 0.081 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$when compared to control, [d] $p<0.001$, [f] $p<0.05$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats . D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-82

Real time PCR analysis of Bax gene expression in cerebellum of control and experimental rats

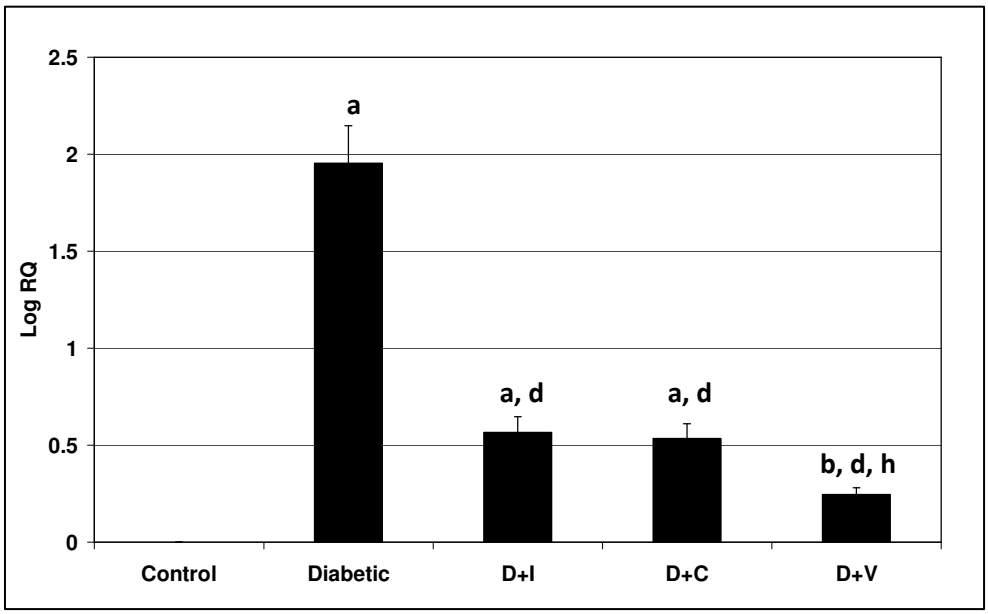


Table-82

Real time PCR analysis of Bax gene expression in Cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.954 ± 0.193 [a]
D + I	0.565 ± 0.081 [a, d]
D+C	0.535 ± 0.075 [a, d]
D+V	0.245 ± 0.035 [b, d. h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [h] $p<0.01$ when compared with insulin treated diabetic rats . D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-83

Real time PCR analysis of Caspase 8 gene expression in cerebellum of control and experimental rats

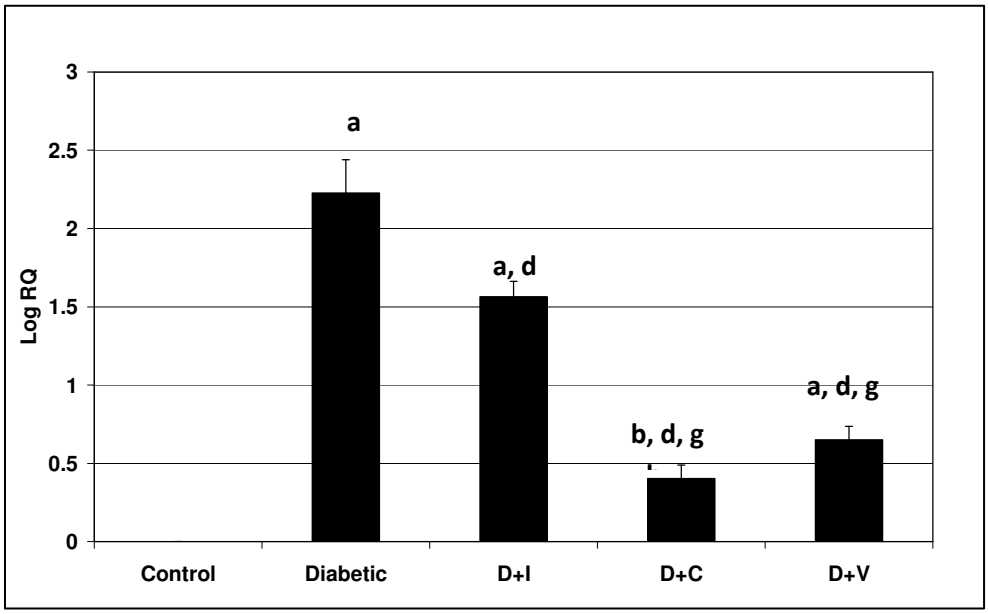


Table-83

Real time PCR analysis of Caspase 8 gene expression in Cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	2.226 ± 0.213 [a]
D + I	1.565 ± 0.096 [a, d]
D+C	0.402 ± 0.086 [b, d, g]
D+V	0.651 ± 0.085 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control, [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure- 84

NMDA R1 receptor subunit expression in the cerebellum of control and experimental rats

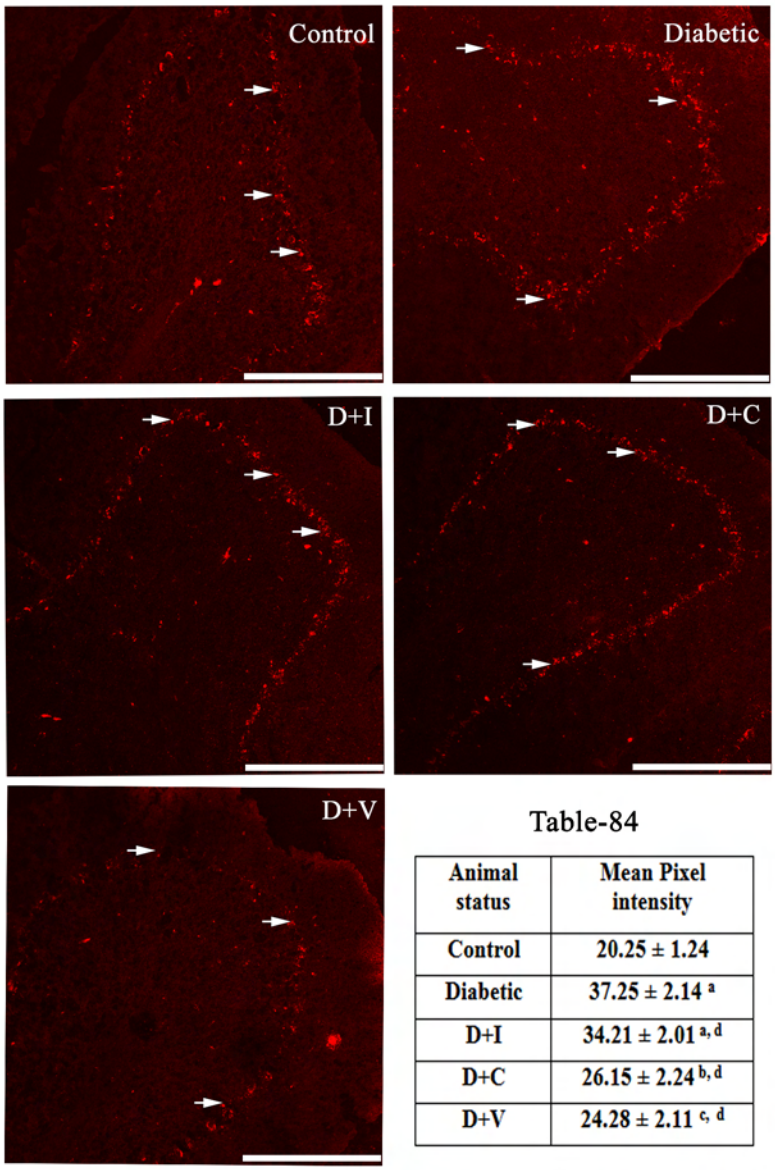


Table-84

Animal status	Mean Pixel intensity
Control	20.25 ± 1.24
Diabetic	37.25 ± 2.14 [a]
D+I	34.21 ± 2.01 [a, d]
D+C	26.15 ± 2.24 [b, d]
D+V	24.28 ± 2.11 [c, d]

Confocal image of NMDA R1 receptor subunit expression in the cerebral cortex of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent NMDA R1 receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$ when compared to diabetic group (➡) in white shows NMDA R1 receptor subunit.Scale bar = 50 µm

Figure- 85

NMDA 2B receptor subunit expression in the cerebellum of control and experimental rats

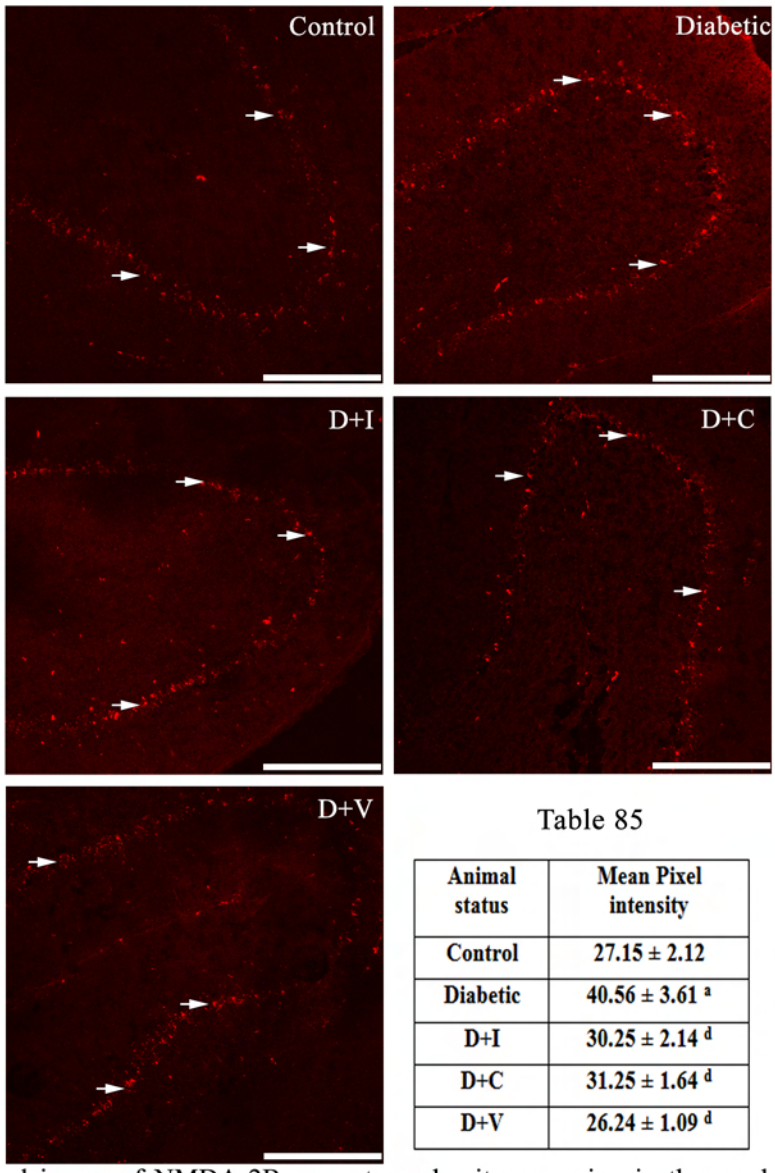


Table 85

Animal status	Mean Pixel intensity
Control	27.15 ± 2.12
Diabetic	40.56 ± 3.61 [a]
D+I	30.25 ± 2.14 [d]
D+C	31.25 ± 1.64 [d]
D+V	26.24 ± 1.09 [d]

Confocal image of NMDA 2B receptor subunit expression in the cerebellum of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent NMDA 2B receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$ when compared to control group: [d] $p<0.001$ when compared to diabetic group; (➡) in white shows NMDA 2B receptor subunit . Scale bar = 200 µm

Figure- 86

AMPA (GluR4) receptor subunit expression in the Cerebellum of control and experimental rats

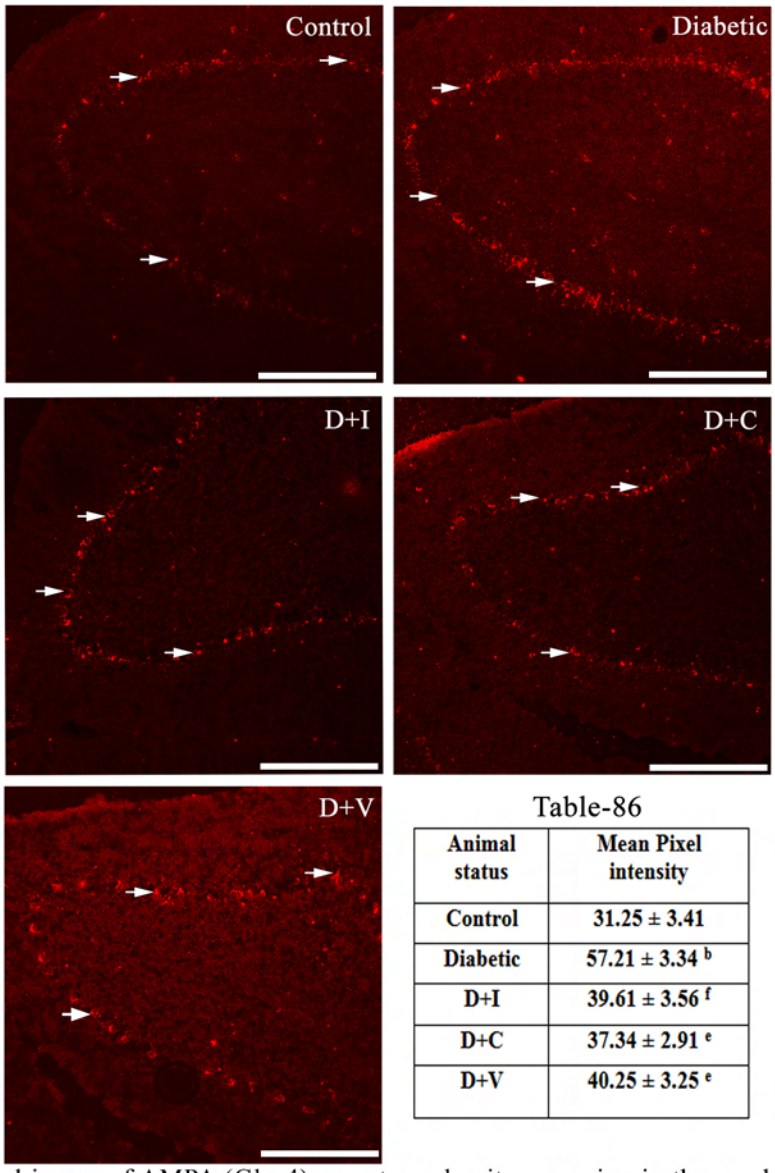


Table-86

Animal status	Mean Pixel intensity
Control	31.25 ± 3.41
Diabetic	57.21 ± 3.34 [b]
D+I	39.61 ± 3.56 [f]
D+C	37.34 ± 2.91 [e]
D+V	40.25 ± 3.25 [e]

Confocal image of AMPA (Glur4) receptor subunit expression in the cerebellum of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent AMPA Glur4 receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$when compared to diabetic group; (➡) in white shows AMPA Glur4 receptor subunit . Scale bar = 50 µm

Figure- 87

AMPA (GluR2) receptor subunit expression in the cerebellum of control and experimental rats

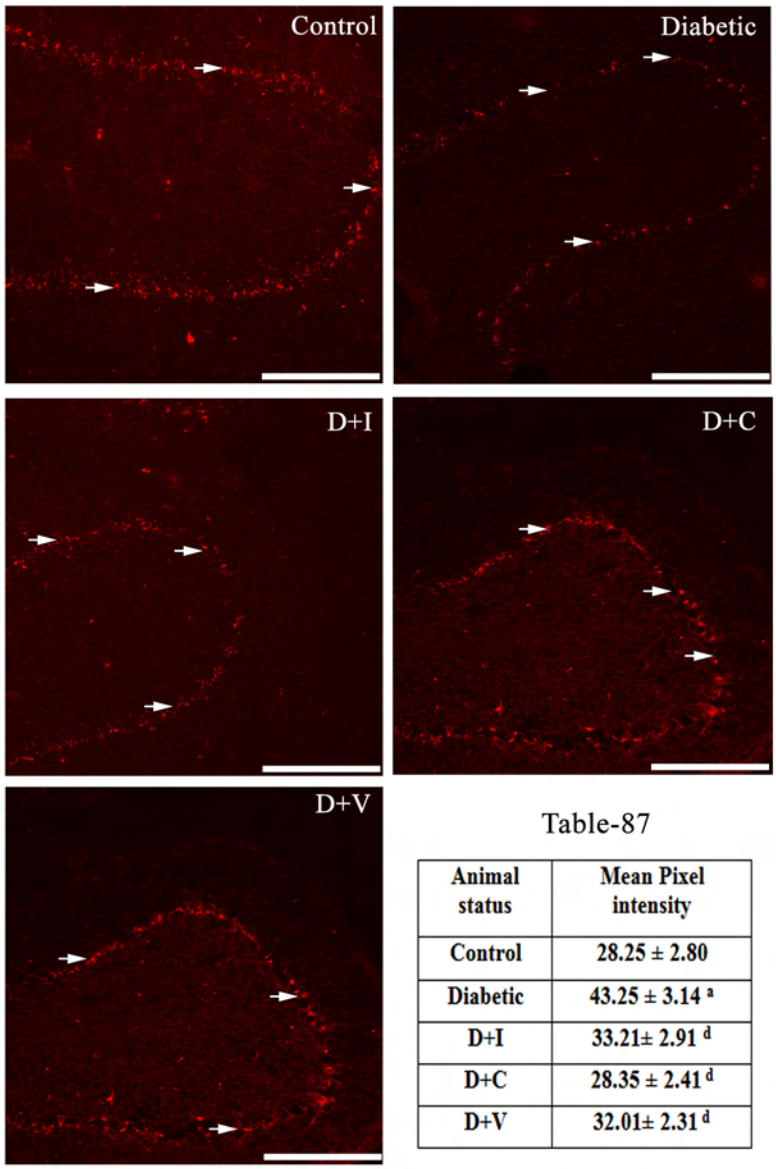


Table-87

Animal status	Mean Pixel intensity
Control	28.25 ± 2.80
Diabetic	43.25 ± 3.14 [a]
D+I	33.21± 2.91 [d]
D+C	28.35 ± 2.41 [d]
D+V	32.01± 2.31 [d]

Confocal image of AMPA (GluR2) receptor subunit expression in the cerebellum of Control, Diabetic, D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats using immunofluorescent AMPA GluR4 receptor subunit specific primary antibody and Cy 5 as secondary antibody; [a] $p<0.001$ when compared to control group: [c] $p<0.01$when compared to diabetic group(➡) in white shows AMPA GluR2 receptor subunit . Scale bar = 200 µm

Figure-88

Glutamate content in the pancreas of control and experimental rats

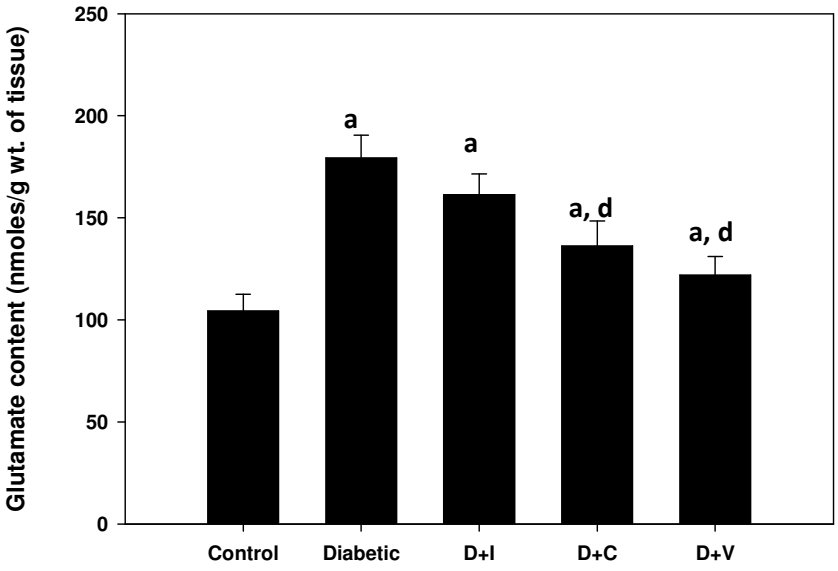


Table- 88

Glutamate content in the pancreas of control and experimental rats

Animal Status	Glutamate content (nmoles/g wt. of tissue)
Control	**104.35 ± 8.24**
Diabetic	**179.25 ± 11.25** [a]
D+I	**161.25 ± 10.25** [a]
D+C	**136.24 ± 12.20** [a, d]
D+V	**121.90 ± 9.12** [a, d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
[a] $p<0.001$when compared with control rats, [d] $p<0.001$when compared with diabetic rats D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-89

Scatchard Analysis NMDA receptors using [^{3}H] MK-801 binding against MK-801 in the pancreas of Control and Experimental rats

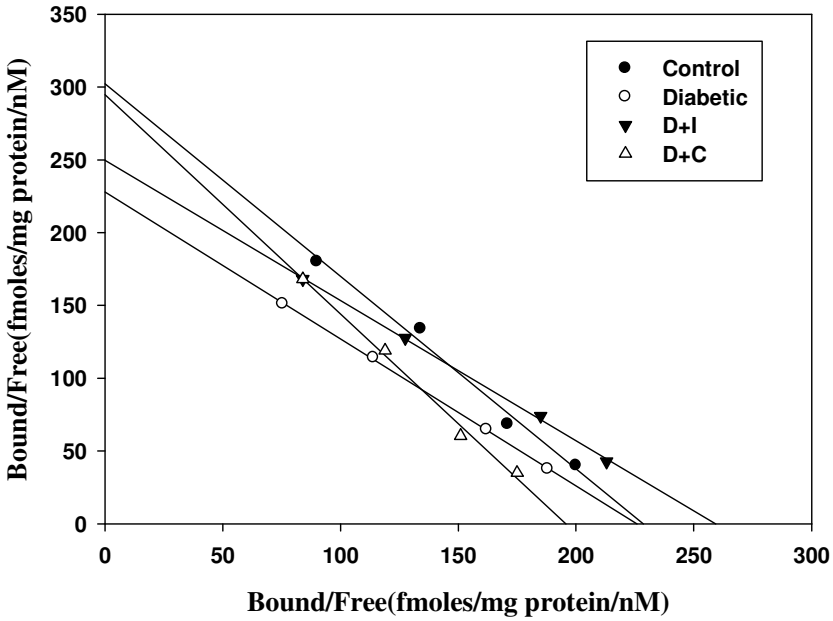


Table-89

Scatchard Analysis NMDA receptors using [^{3}H] MK-801 binding against MK-801 in the pancreas of Control and Experimental rats

Animal status	B_{max}	K_d
Control	227± 22	0.86± 0.08
Diabetic	224 ± 19	0.98 ± 0.10
D+ I	258 ± 24	1.04 ± 0.09
D + C	195 ± 15	0.77 ± 0.10

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats

Figure-90

Scatchard Analysis NMDA receptors using [^{3}H] MK-801 binding against MK-801 in the pancreas of Control and Experimental rats

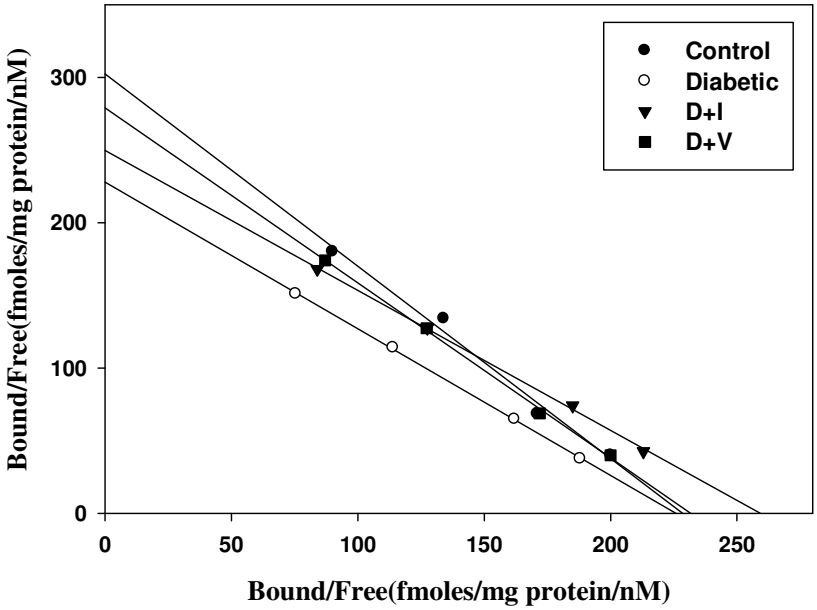


Table-90

Scatchard Analysis NMDA receptors using [^{3}H] MK-801 binding against MK-801 in the pancreas of Control and Experimental rats

Animal status	B_{max}	K_d
Control	**227± 22**	**0.86± 0.08**
Diabetic	**224 ± 19**	**0.98 ± 0.10**
D+ I	**258 ± 24**	**1.04 ± 0.09**
D +V	**226 ± 18**	**0.81 ± 0.07**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D_3 treated diabetic rats

Figure-91

Scatchard Analysis of AMPA receptors using [^{3}H] AMPA binding against AMPA in the pancreas of Control and Experimental rats

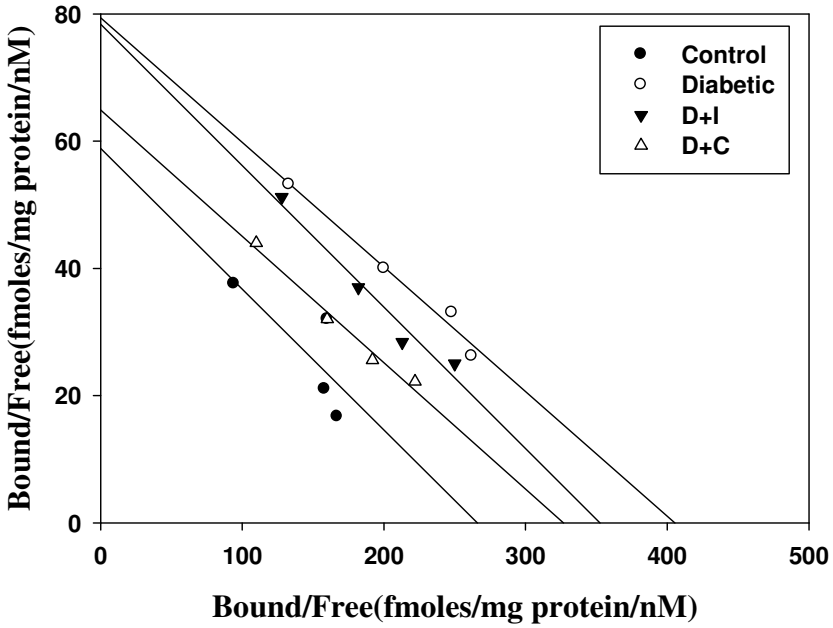


Table-91

Scatchard Analysis of AMPA receptors using [^{3}H] AMPA binding against AMPA in the pancreas of Control and Experimental rats

Animal status	B_{max}	K_d
Control	264 ± 22	4.5 ± 0.44
Diabetic	405 ± 36 [b]	5.1 ± 0.37
D+ I	349 ± 31 [c, f]	4.5 ± 0.54
D + C	325 ± 31 [f]	5.0 ± 0.43

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [b] $p<0.01$, [c] $p<0.05$ when compared to control. [f] $p<0.05$ when compared to diabetic group . D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats

Figure-92

Scatchard Analysis of AMPA receptors using [^{3}H] AMPA binding against AMPA in the pancreas of control and experimental rats

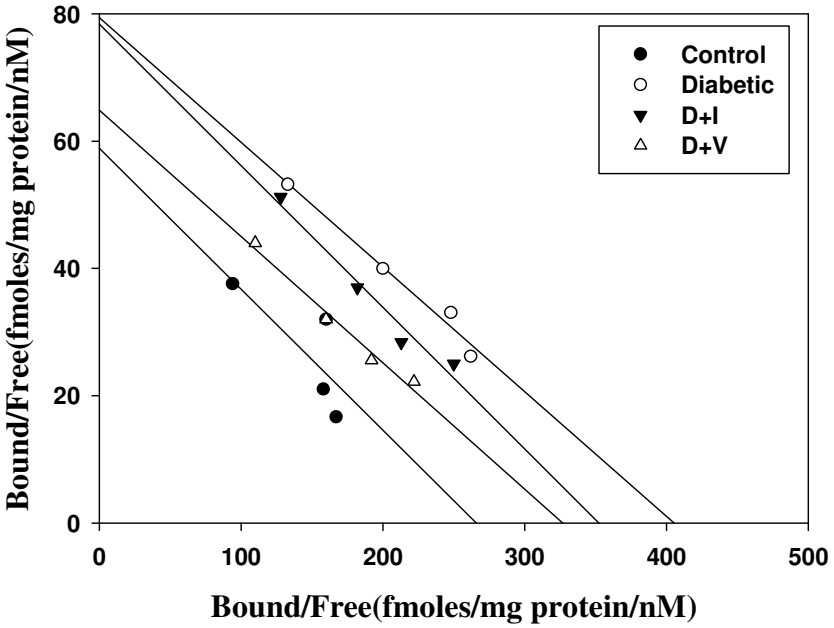


Table-92

Scatchard Analysis of AMPA receptors using [^{3}H] AMPA binding against AMPA in the pancreas of control and experimental rats

Animal status	B_{max}	K_d
Control	264 ± 22	4.5 ± 0.44
Diabetic	405 ± 36 [b]	5.1 ± 0.37
D+ I	349 ± 31 [c, f]	4.5 ± 0.54
D +V	281 ± 16 [e]	4.2 ± 0.71

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [b] $p<0.01$, [c] $p<0.05$ when compared to control [e] $p<0.01$ [f] $p<0.05$ when compared to diabetic group.D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure-93

Real time PCR analysis of AMPA (GluR4) gene expression in pancreas of control and experimental rats

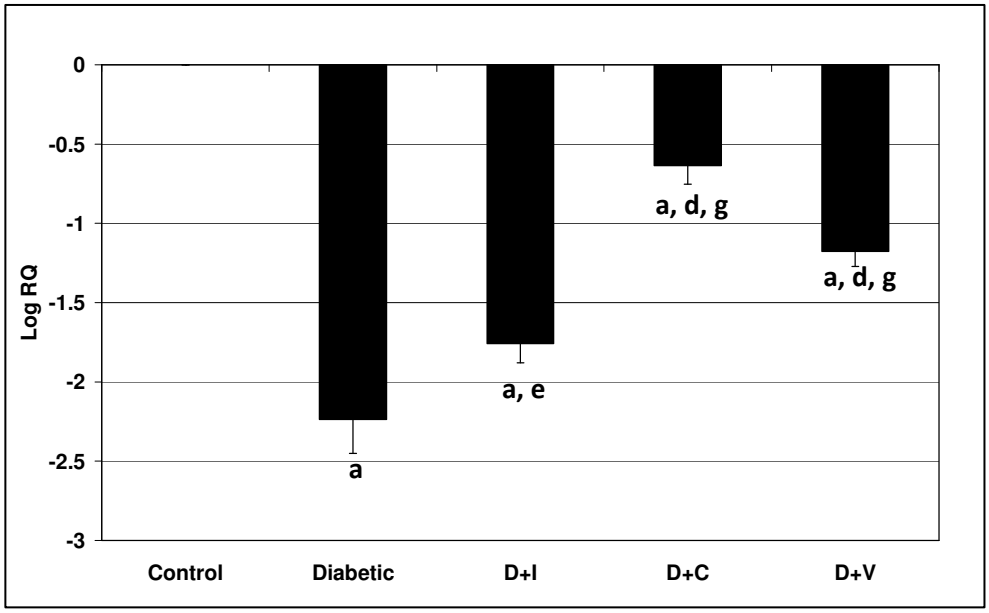


Table-93

Real time PCR analysis of AMPA (GluR4) gene expression in Pancreas of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-2.236 ± 0.213 [a]
D + I	-1.758 ± 0.121 [a, e]
D+C	-0.636 ± 0.115 [a, d, g]
D+V	-1.176 ± 0.095 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control, [d] $p<0.001$ [e] $p<0.01$ when compared to diabetic group. [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats

Figure-94

Real time PCR analysis of AMPA (GluR2) gene expression in pancreas of control and experimental rats

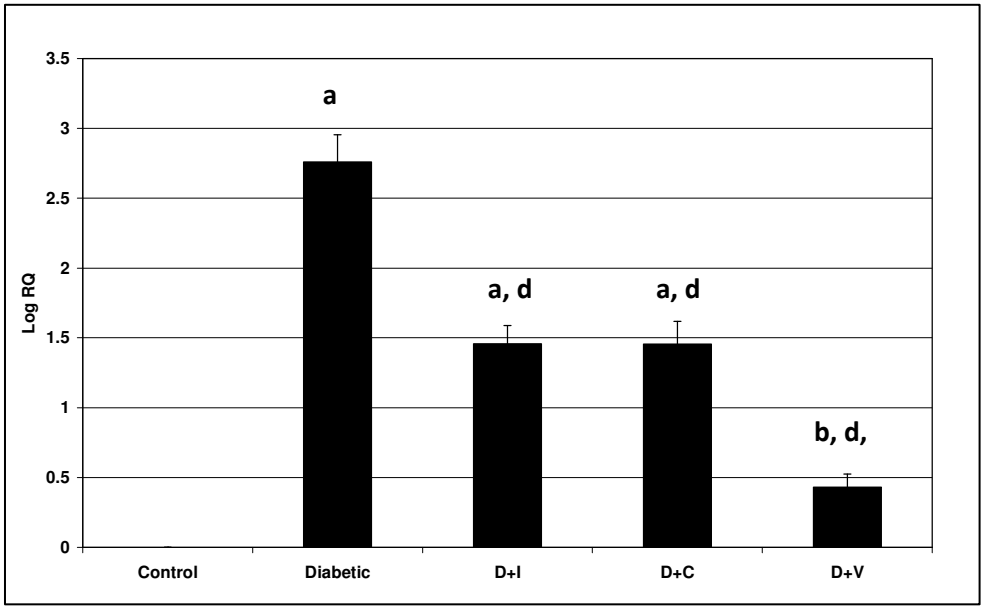


Table-94

Real time PCR analysis of AMPA (GluR2) gene expression in Pancreas of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	2.758 ± 0.195 [a]
D + I	1.456 ± 0.132 [a, d]
D+C	1.159 ±0.161 [a, d]
D+V	0.432 ±0.091 [b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$ when compared to control. [d] $p<0.001$when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- Insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats

Figure-95

Real time PCR analysis of GLAST gene expression in pancreas of control and experimental rats

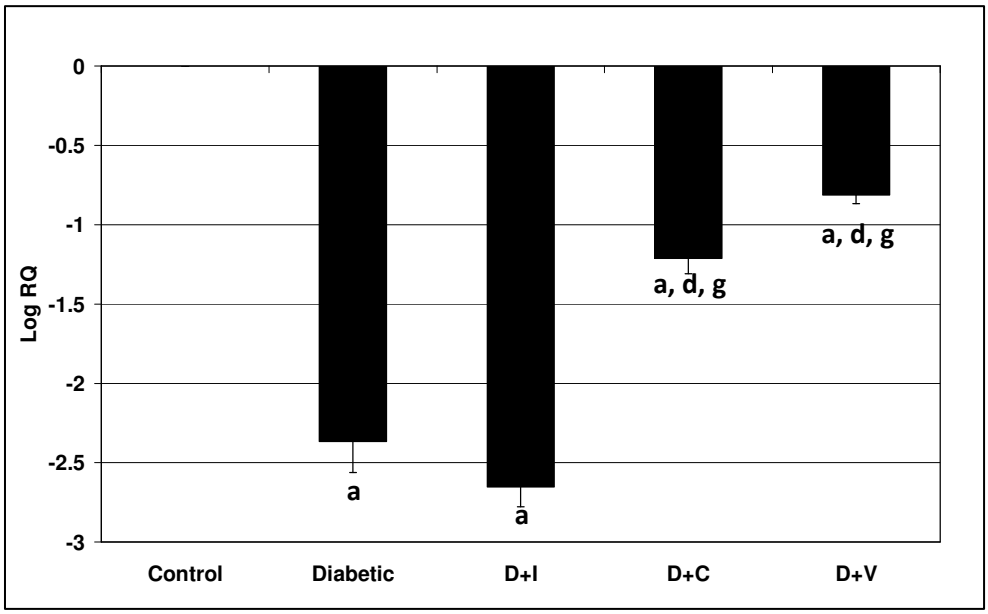


Table-95

Real time PCR analysis of GLAST gene expression in Pancreas of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-2.365 ± 0.195 [a]
D + I	--2.652 ± 0.124 [a]
D+C	-1.212± 0.096 [a, d, g]
D+V	-0.812 ± 0.055 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control. [d] $p<0.001$when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- Insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats

Figure- 96

IP3 content in the pancreas of control and experimental rats

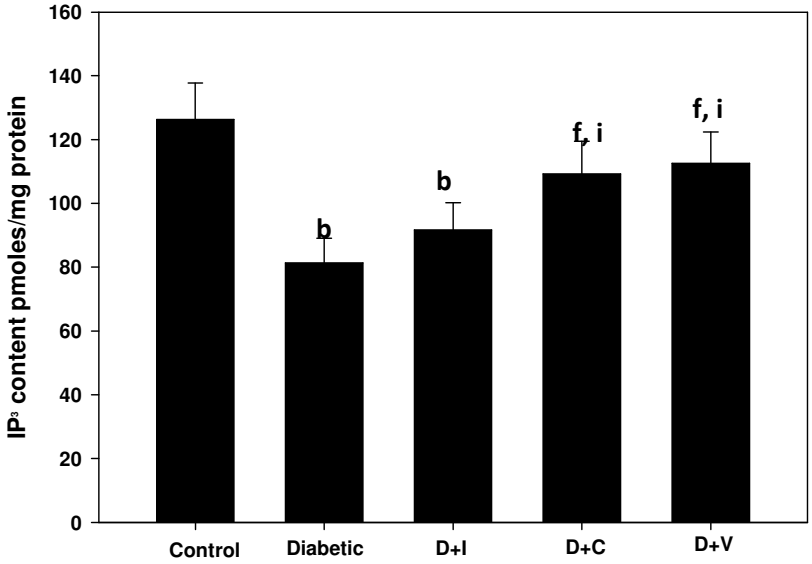


Table-96

IP3 content in the pancreas of control and experimental rats

Animal Status	IP3 Content (pmoles/mg protein)
Control	126.35 ± 11.36
Diabetic	81.35 ± 7.75 [b]
D + I	91.67 ± 8.56 [b]
D+C	109.25 ± 10.24 [f, i]
D+V	112.53 ± 9.82 [f, i]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [b] $p<0.01$ when compared to control, [f] $p<0.05$ when compared to diabetic group, [i] $p<0.05$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats

Figure-97

Superoxide dismutase assay in the pancreas of control and experimental rats

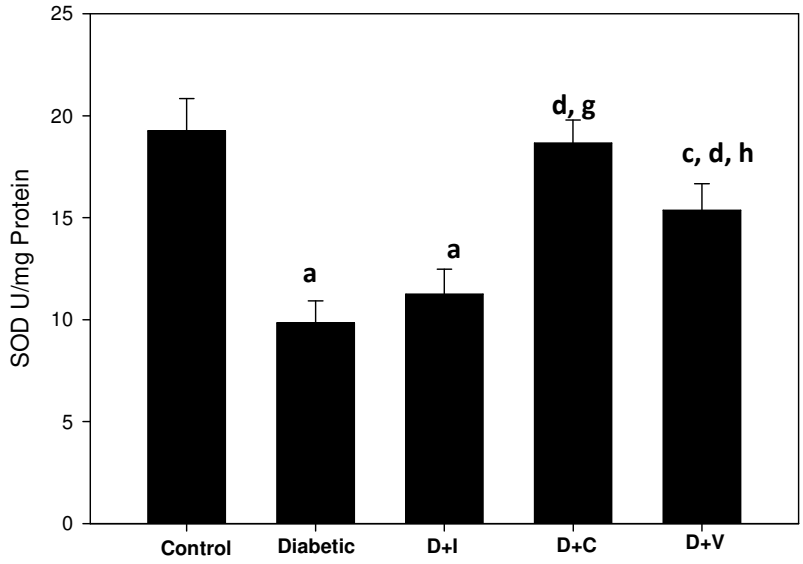


Table-97

Superoxide dismutase assay in the pancreas of control and experimental rats

Animal Status	SOD activity (unit/mg protein)
Control	19.25 ± 1.60
Diabetic	9.83 ± 1.10 [a]
D + I	11.25 ± 1.23 [a]
D+C	18.65 ± 1.14 [d, g]
D+V	15.36 ± 1.30 [c, d. h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [c] $p<0.05$ when compared to control. [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats, [h] $p<0.01$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats

Figure-98

Real time PCR analysis of GPx gene expression in pancreas of control and experimental rats

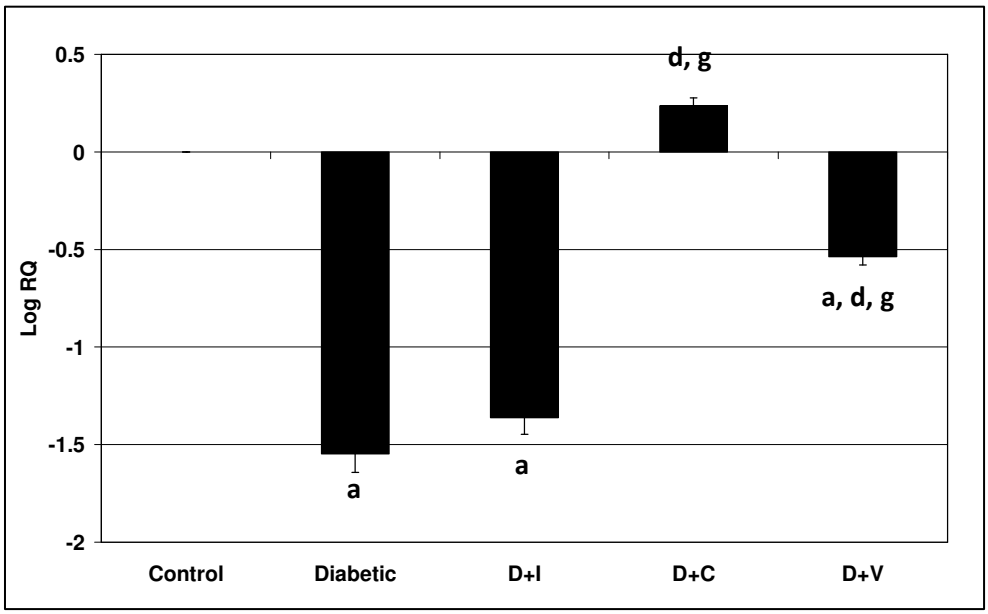


Table-98

Real time PCR analysis of GPx gene expression in Pancreas of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	-1.546 ± 0.047 [a]
D + I	-1.362 ± 0.084 [a]
D+C	0.235 ± 0.042 [d, g]
D+V	-0.535 ± 0.042 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control. [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats

Figure-99

Real time PCR analysis of Bax gene expression in pancreas of control and experimental rats

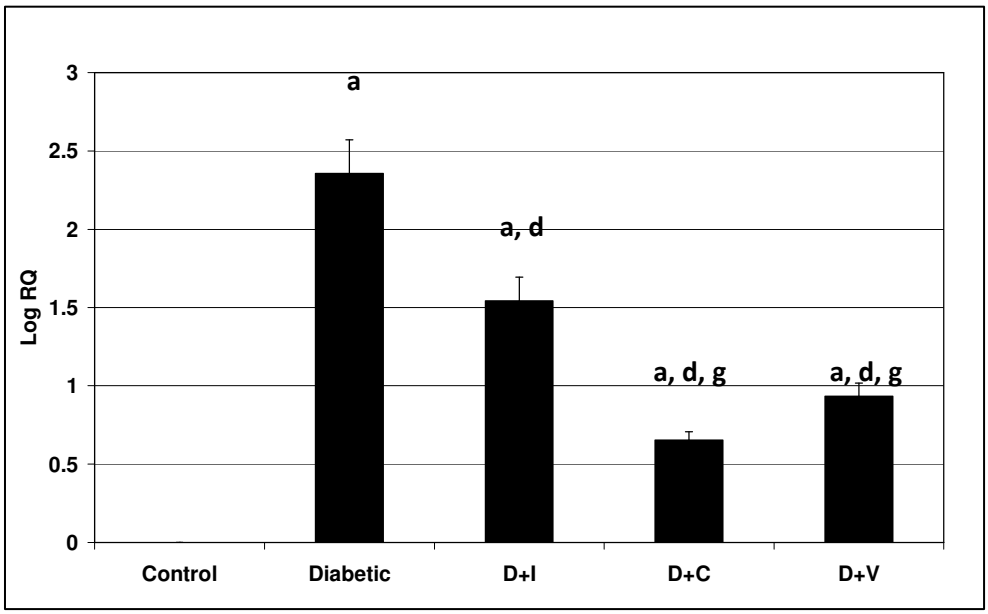


Table-99

Real time PCR analysis of Bax gene expression in Pancreas of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	2.356 ± 0.215 [a]
D + I	1.542 ± 0.152 [a, d]
D+C	0.653 ± 0.053 [a, d, g]
D+V	0.932 ± 0.084 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$, [b] $p<0.01$when compared to control. [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats

Figure- 100

Real time PCR analysis of caspase 8 gene expression in pancreas of control and experimental rats

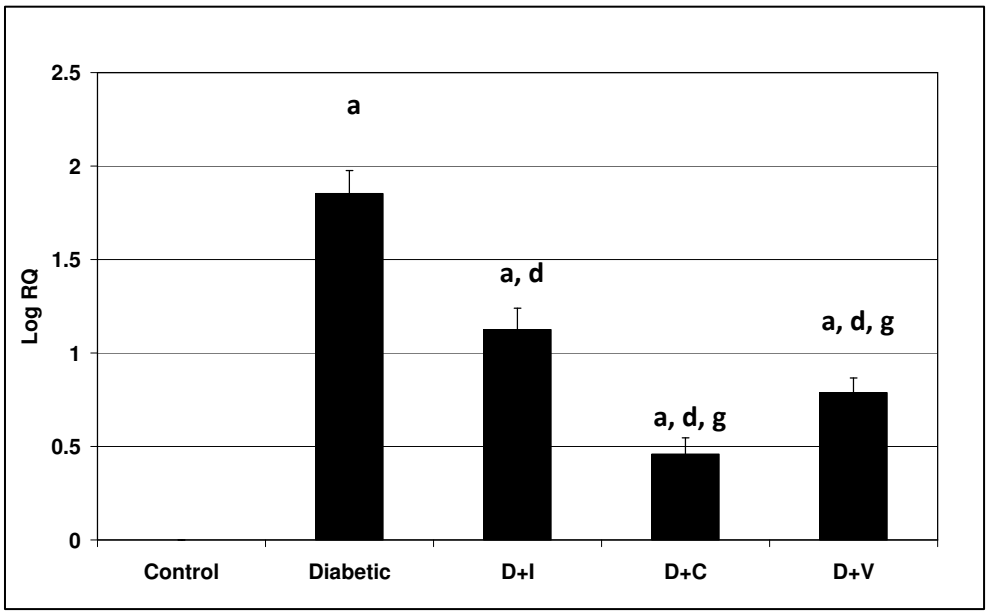


Table- 100

Real time PCR analysis of caspase 8 gene expression in Pancreas of control and experimental rats

Experimental groups	Log RQ
Control	0
Diabetic	1.852 ± 0.123 [a]
D + I	1.125 ± 0.114 [a, d]
D+C	0.458 ± 0.087 [a, d, g]
D+V	0.787 ± 0.078 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to Diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- Insulin treated diabetic rats, D+C- Curcumin treated diabetic rats and D+V- Vitamin D3 treated diabetic rats

Figure- 101

Real time PCR analysis of NeuroD-1 gene expression in pancreas of control and experimental rats

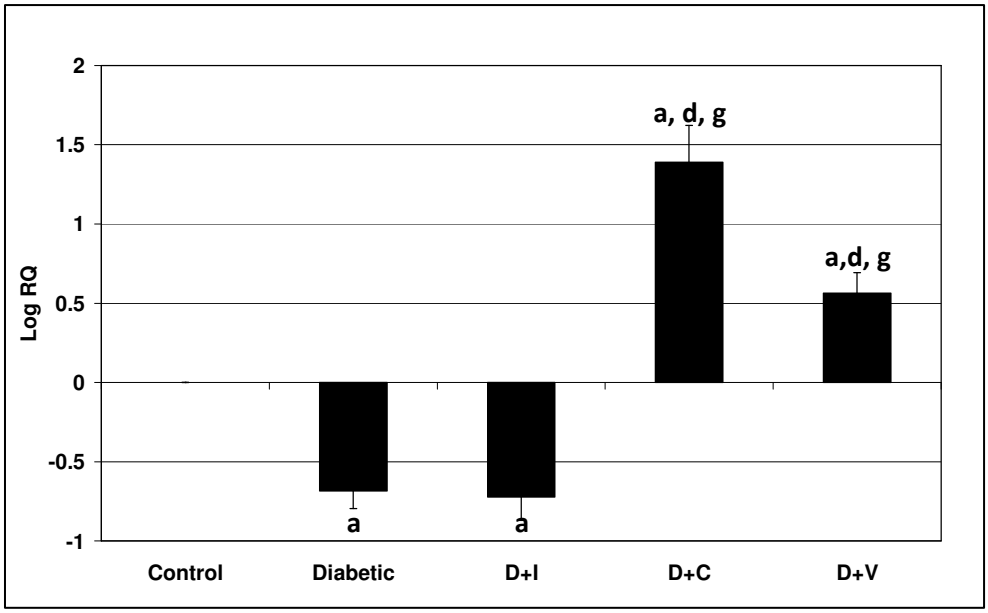


Table- 101

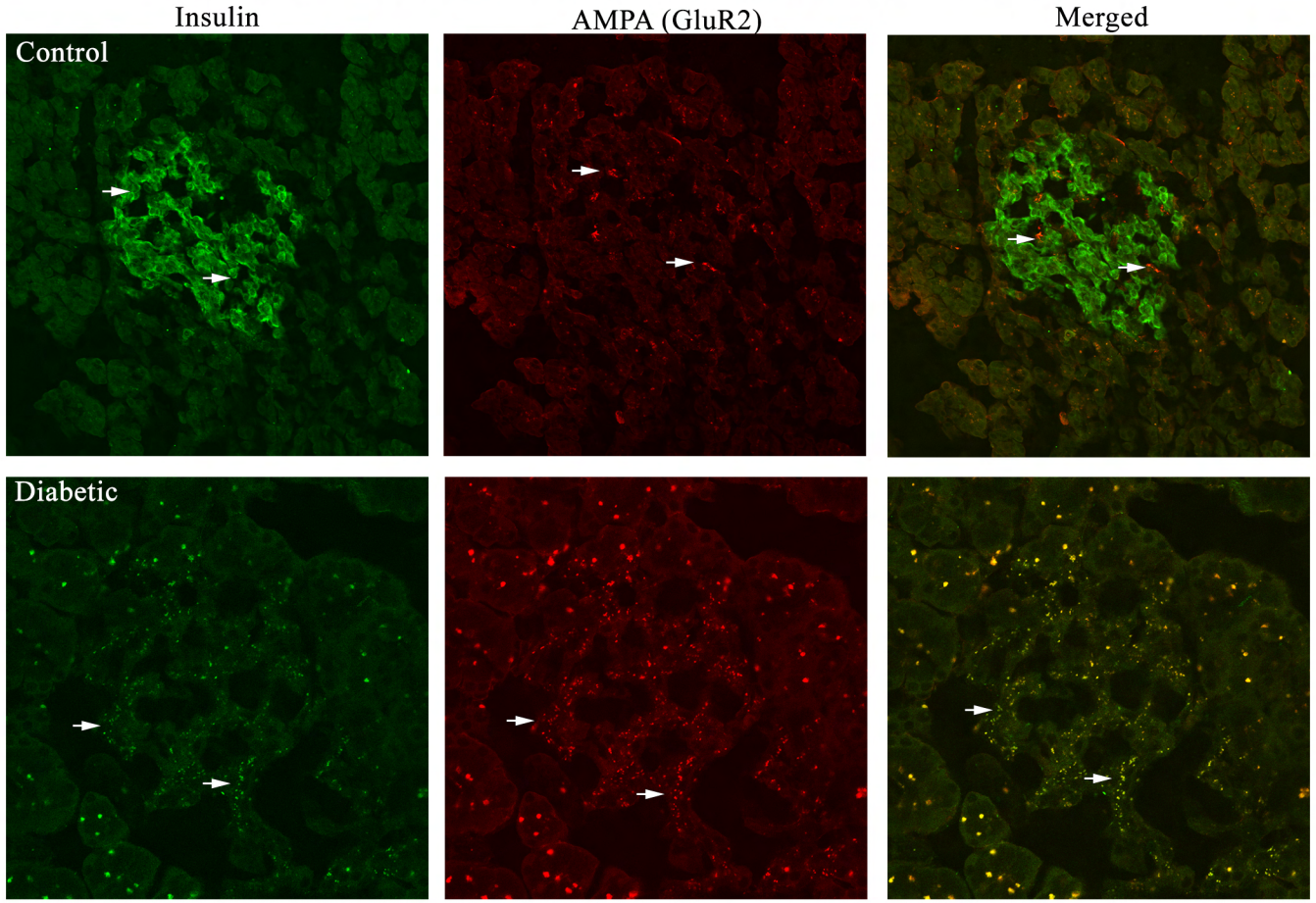
Real time PCR analysis of NeuroD-1 gene expression in Pancreas of control and experimental rats

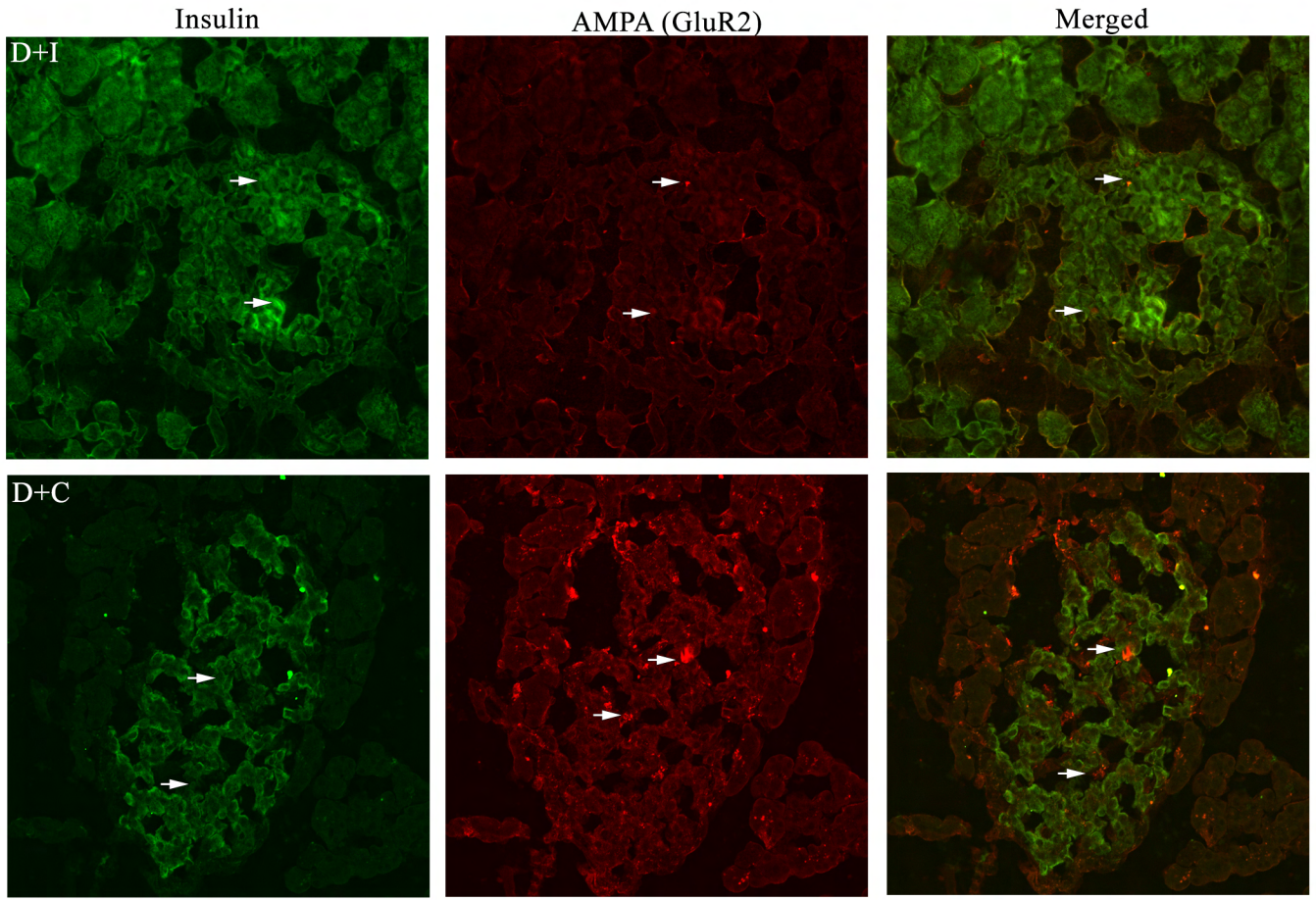
Experimental groups	Log RQ
Control	0
Diabetic	-0.685 ± 0.096 [a]
D + I	-0.726 ± 0.045 [a]
D+C	1.389 ± 0.129 [a, d, g]
D+V	0.287 ±0.068 [a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. [a] $p<0.001$ when compared to control. [d] $p<0.001$ when compared to diabetic group, [g] $p<0.001$ when compared with insulin treated diabetic rats. D + I- insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D_3 treated diabetic rats.

Figure: 103

Co-labeling studies using Insulin and AMPA (GluR2) receptor subunit specific antibody in the pancreatic islets of control and experimental groups of rats using confocal microscope




Insulin
AMPA (GluR2)
Merged
D+I
D+C

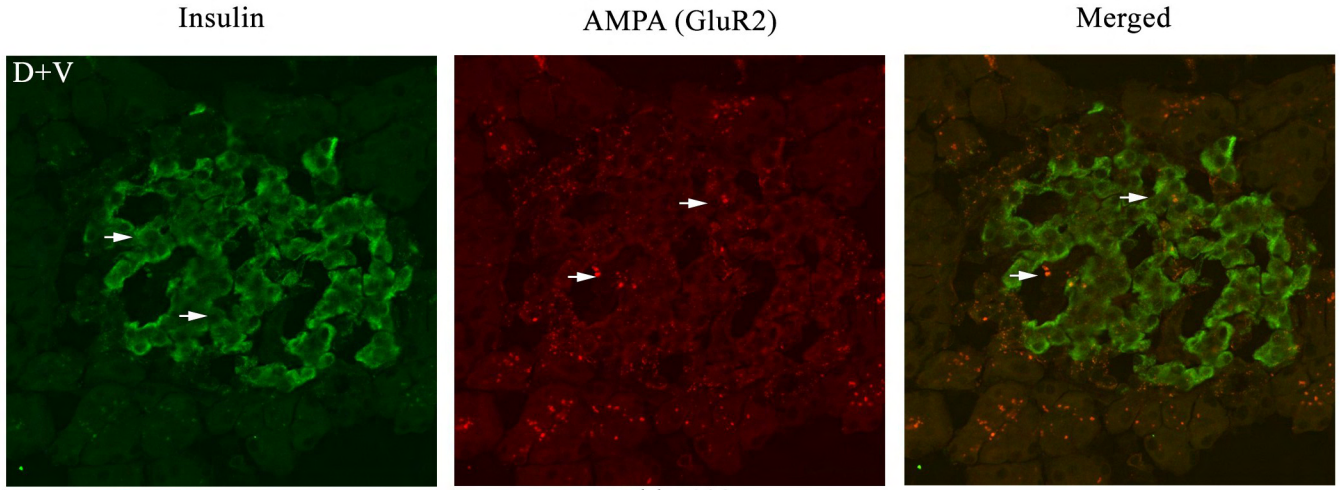


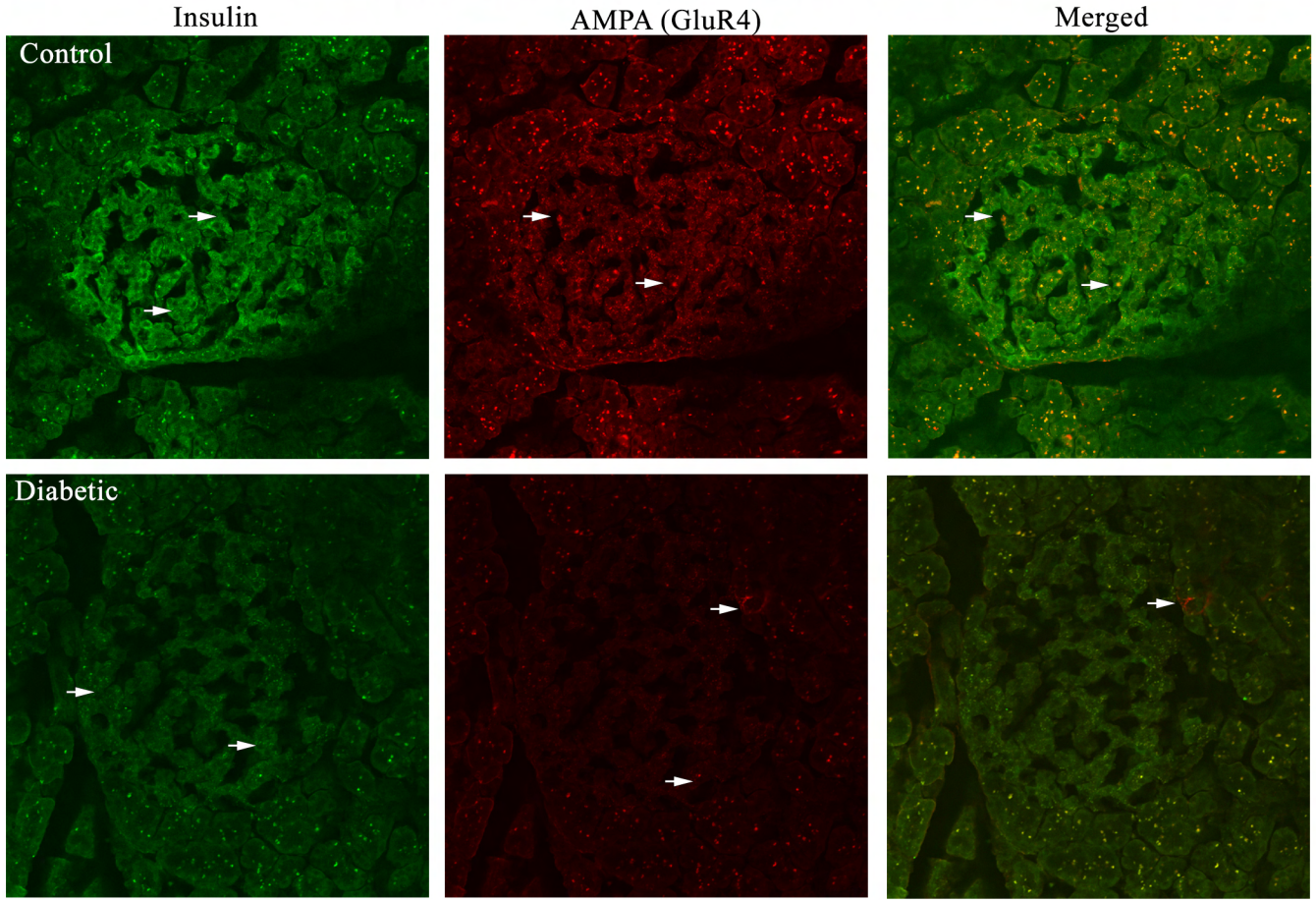
Table-103

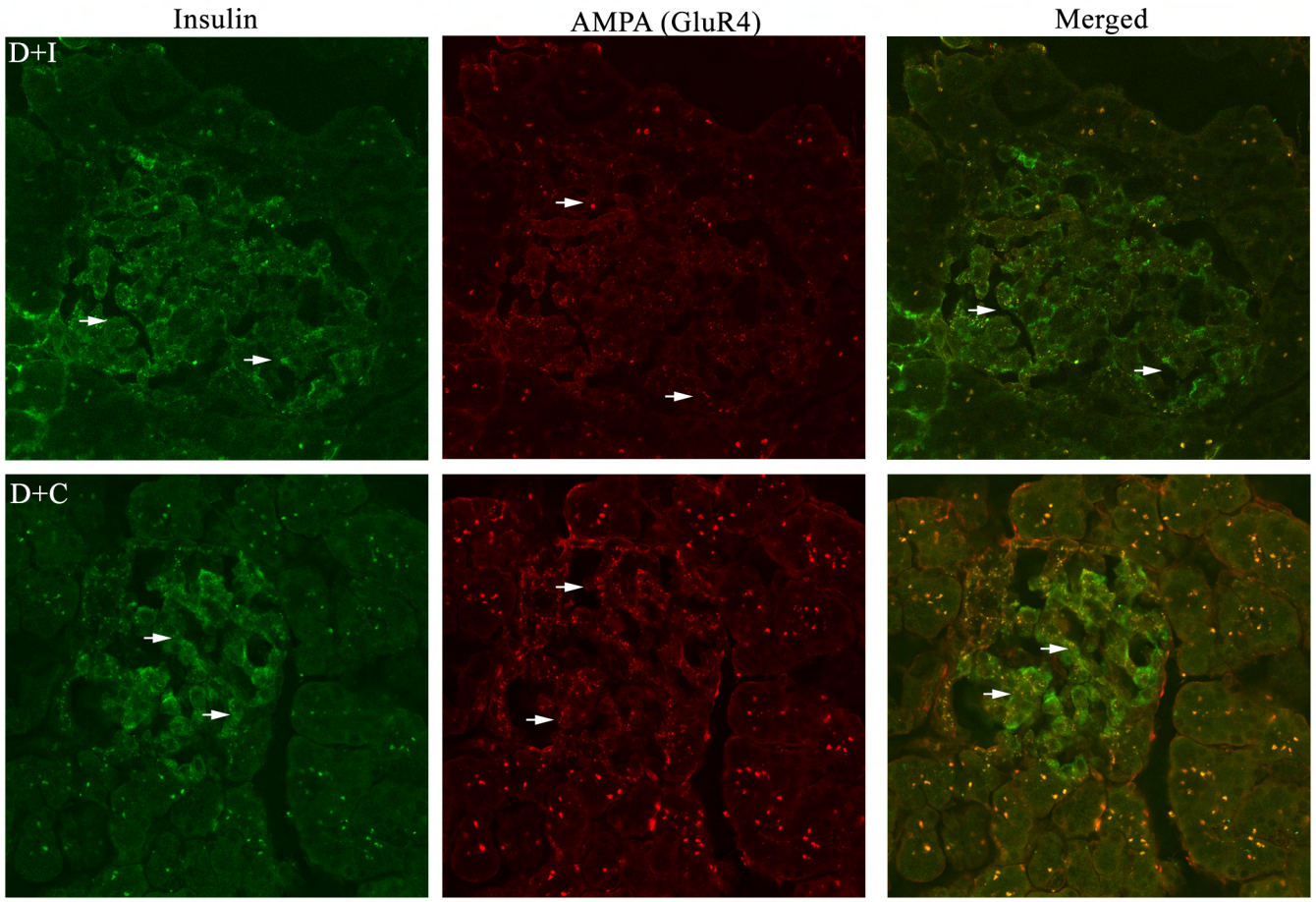
Groups	Mean pixel intensity Insulin	Mean pixel intensity AMPA (GluR2)
Control	52.35 ± 5.21	24.25 ± 2.22
Diabetes	24.25 ± 1.21[a]	42.27 ± 3.24 [a]
D+I	27.24± 3.25 [a, d]	39.15 ± 4.21 [a, f]
D+C	38.24±3.21 [a, d]	30.25 ± 3.11 [b, e]
D+V	44.25 ± 2.53 [b, d]	28.35 ± 2.15 [c, d]

Confocal image of Insulin (green) and AMPA GluR2 (red) receptor subunit expression in the pancreatic islets of control, diabetic, D+I, D+C and D+V rats using immunofluorescent insulin receptor and AMPA (GluR2) receptor subunit specific primary antibody and FITC and CY5 respectively as secondary antibody. C-control, D-Diabetes, D + I- Insulin treated diabetic rats, D+C- Curcumin. [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control, [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared to diabetic group.

Figure: 104

Co-labeling studies using Insulin and AMPA (GluR4) receptor subunit specific antibody in the pancreatic islets of control and experimental groups of rats using confocal microscope




Insulin
AMPA (GluR4)
Merged
D+I
D+C

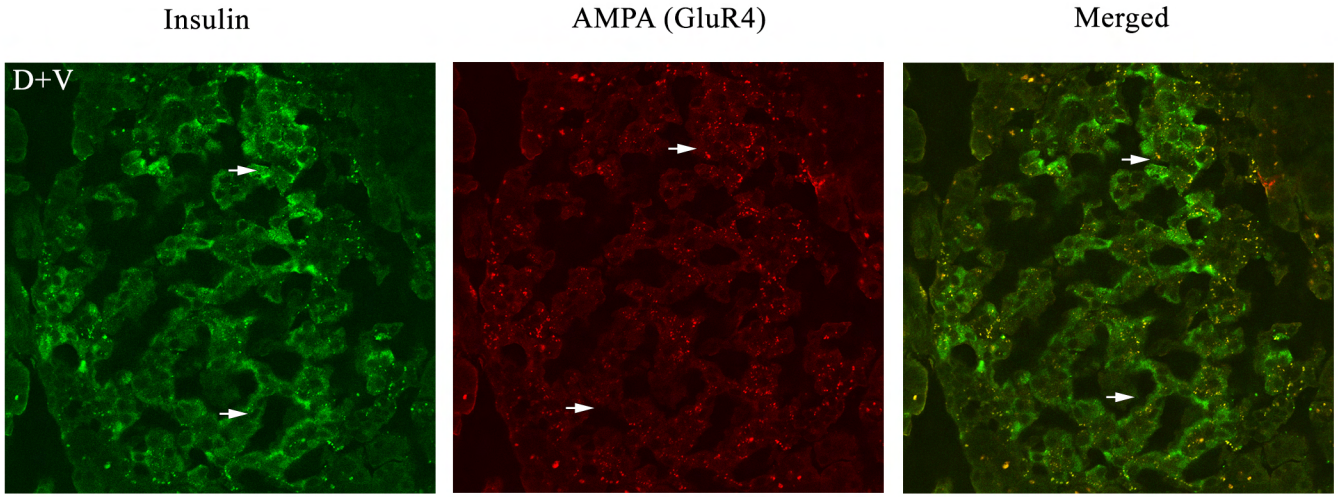


Table-104

Groups	Mean pixel intensity Insulin	Mean pixel intensity AMPA (GluR4)
Control	48.25 ± 3.21	39.54 ± 3.41
Diabetes	25.45 ± 2.21	24.52 ± 2.65 [a]
D+I	30.25 ± 3.25 [a, d]	30.24 ± 2.87 [e]
D+C	36.24 ± 3.71 [a, d]	35.25 ± 3.21 [b, d]
D+V	39.84 ± 1.86 [b, d]	38.23 ± 3.14 [c, d]

Confocal image of Insulin (green) and AMPA GluR4 (red) receptor subunit expression in the pancreatic islets of control, diabetic, D+I, D+C and D+V rats using immunofluorescent insulin receptor and AMPA (GluR4) receptor subunit specific primary antibody and FITC and CY5 respectively as secondary antibody. C-control, D-Diabetes, D + I- Insulin treated diabetic rats, D+C- Curcumin treated with diabetic rats, D+V- Vitamin D3 treated diabetic rats.. [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control, [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared to diabetic group.

Figure- 105
IP3R3 expression in the Pancreas of control and experimental rats

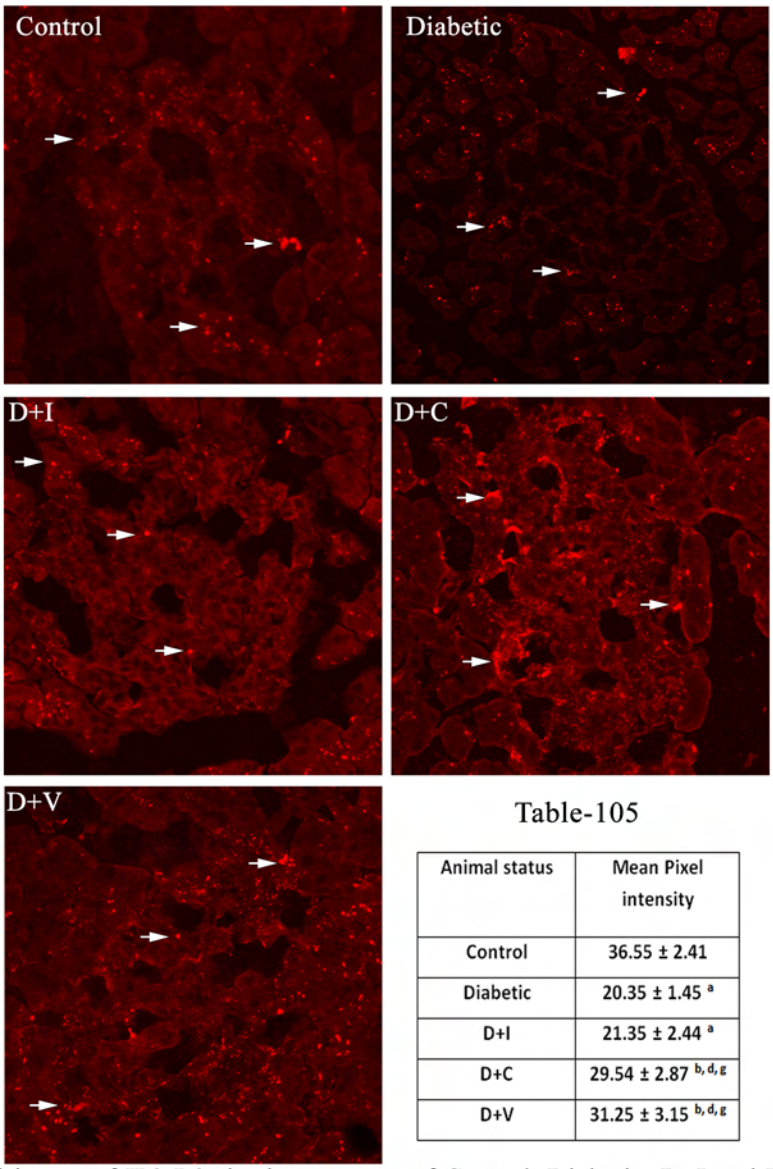


Table-105

Animal status	Mean Pixel intensity
Control	36.55 ± 2.41
Diabetic	20.35 ± 1.45 [a]
D+I	21.35 ± 2.44 [a]
D+C	29.54 ± 2.87 [b,d,g]
D+V	31.25 ± 3.15 [b,d,g]

Confocal image of IP3 R3 in the pancreas of Control, Diabetic, D+I and D+V rats using immunofluorescent IP3 R3 specific primary antibody and Cy5 as secondary antibody. There was an down regulation of IP3 R3 in the Pancreas of experimental rats when compared to control rats. (➡) in white shows receptors. Scale bar = 50 µm. C-control, D-Diabetes, D + I- Insulin treated diabetic rats, D+V- Vitamin D3 treated diabetic rats

Figure- 106
Vitamin D receptor expression in the Pancreas of control and experimental rats

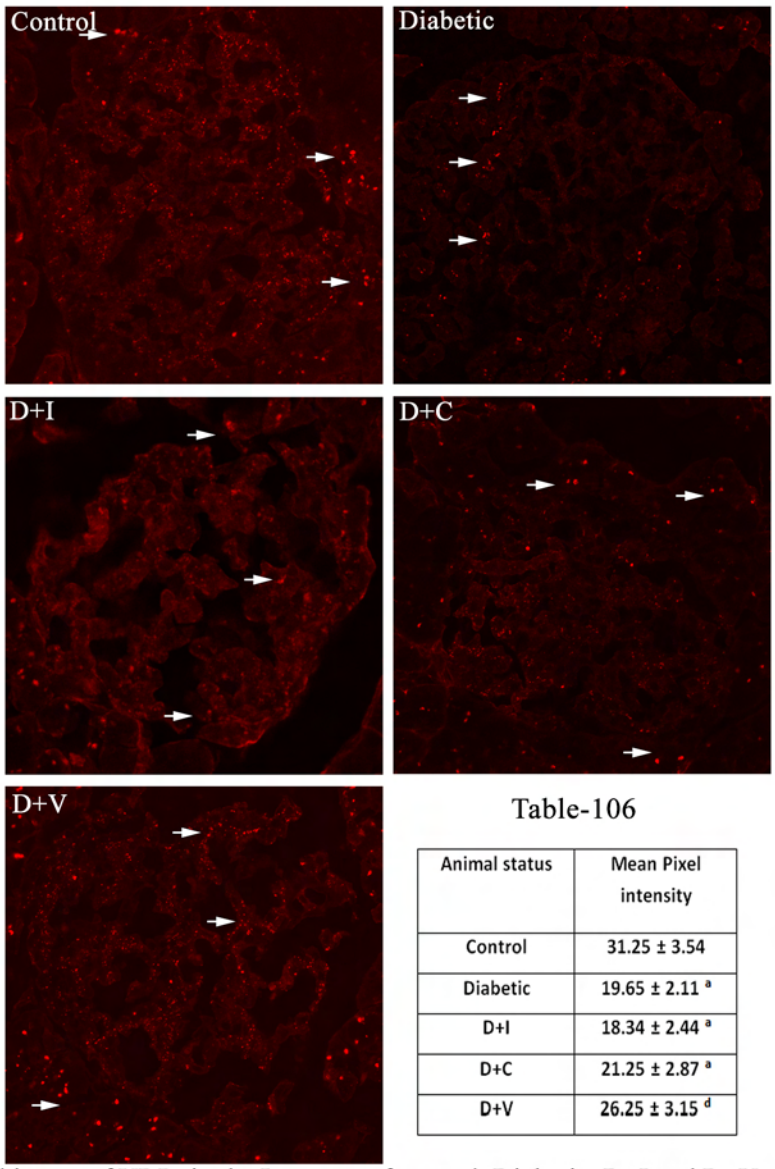
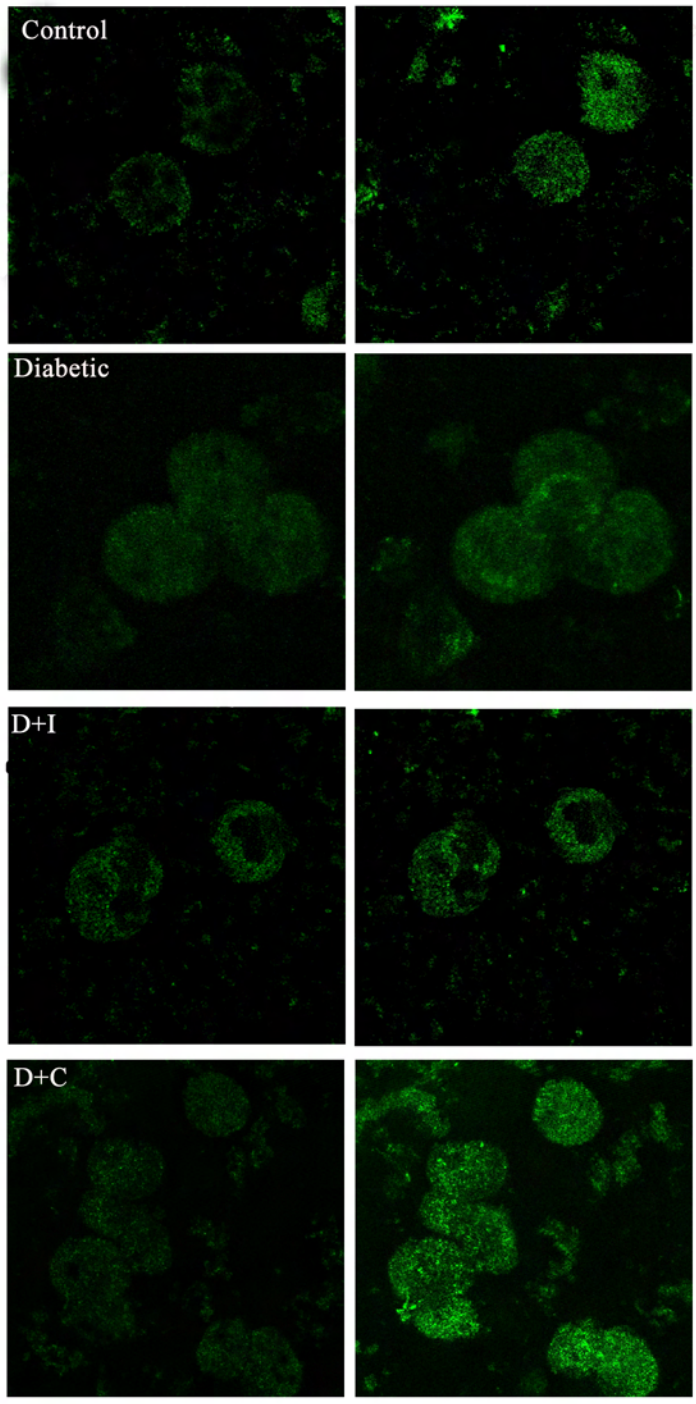


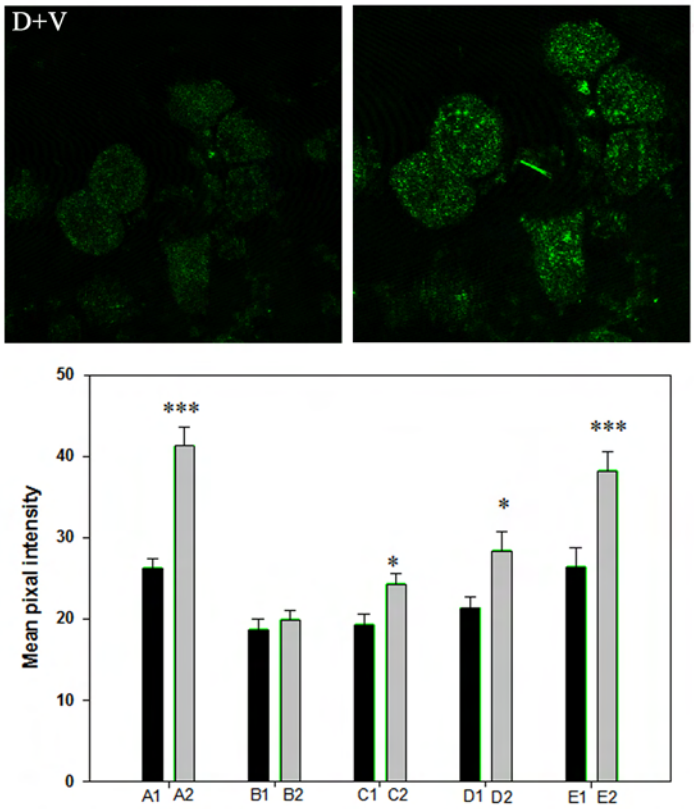
Table-106

Animal status	Mean Pixel intensity
Control	31.25 ± 3.54
Diabetic	19.65 ± 2.11 [a]
D+I	18.34 ± 2.44 [a]
D+C	21.25 ± 2.87 [a]
D+V	26.25 ± 3.15 [d]

Confocal image of VDR in the Pancreas of control, Diabetic, D+I and D+V rats using immunofluorescent VDR specific primary antibody and Cy5 as secondary antibody. There was an down regulation of VDR in the Pancreas of experimental rats when compared to control rats. (➡) in white shows receptors. Scale bar = 50 µm. C-control, D-Diabetes, D + I- Insulin treated diabetic rats, D+V- Vitamin D3 treated diabetic rats

Figure-107
Calcium release from pancreatic islets using Fluo-4 AM





Calcium release from pancreatic islets using Fluo-4 AM in control, diabetic, D + I-insulin treated diabetic rats, D+C- curcumin treated diabetic rats and D+V- vitamin D3 treated diabetic rats; **A1**(control)-4mM glucose, **A2** (control) -4mM glucose +50mM AMPA; **B1**(diabetic)-20mM glucose, **B2** (diabetic)-20mM glucose +50mM AMPA; **C1** (D+I) -20mM glucose+insulin, **C2** (D+I) -20mM glucose+insulin + 50mM AMPA; **D1** (D+C)-20mM glucose+curcumin, **D2** (D+C)-20mM glucose+ curcumin + 50mM AMPA; **E1** (D+V)-20mM glucose+vitamin D3; **E2** (D+V) -20mM glucose+ vitamin D3 + 50mM AMPA, *** $p<0.001$,* $p<0.01$ when compared with pancreatic islet.

Discussion

Diabetes mellitus, a major endocrine disorder, has become a severe health problem in the world. The disease is one of the most severe metabolic disorders in humans characterised by hyperglycaemia due to relative or an absolute lack of insulin or the action of insulin on its target tissue or both. Prolonged exposure to chronic hyperglycaemia in diabetes can lead to vascular disorder, retinopathy, altered immune functions, changes in the intestinal function, peripheral neuropathy and dysfunctions of the CNS (Biessels *et al.,* 2004; McNay & Sherwin 2004). The neurological consequences of diabetes mellitus in the CNS are now receiving greater attention. Studies reported that diabetic patients are vulnerable to neurodegenerative diseases (Gasparini *et al.,* 2002; Matsuzawa *et al.,* 2012). Both type 1 and type 2 diabetes can cause impaired learning, memory, mental flexibility and cognitive functions (Umegaki *et al.,* 2012; McCrimmon *et al.,* 2012; Kalalian-Moghaddam *et al.,* 2012). The various neurotransmitter systems including serotonergic, cholinergic, dopaminergic and GABAergic undergo a significant change in diabetes (Gireesh *et al.,* 2008; Antony *et al.,* 2010; Kumar *et al.,* 2010; Anitha *et al.,* 2012).

BLOOD GLUCOSE, INSULIN LEVEL AND BODY WEIGHT

In the present study STZ-induced rats were used as an experimental model for diabetes, since they provide a relevant example of endogenous chronic oxidative stress due to the resulting hyperglycaemia (Low *et al.,* 1997). The STZ diabetic rat serves as an excellent model to study the molecular, cellular and morphological changes in brain induced by stress during diabetes (Aragno *et al.,* 2000). There was an increase in blood glucose level and a decrease in circulating insulin level in diabetic rats when compared to control group. The increased blood glucose level is due to the decreased circulating insulin level. Decreased circulating insulin level in diabetic rats is a result of marked destruction of insulin secreting pancreatic islet β-cells by STZ (Junod *et al.,* 1969; Ahmadi *et al.,* 2010). Treatment using curcumin, insulin and Vitamin D_3 showed restorative effect on

blood glucose level by increasing the insulin level in the serum. Previous reports showed that curcumin has the potential to protect pancreatic islet cells against STZ-induced death (Meghana *et al.,* 2007) and elevated plasma insulin level in diabetic mice (Seo *et al.,* 2008). Previous studies reported that Vitamin D deficiency in rabbits and mice lead to impaired insulin secretion and supplementation with vitamin D corrects the defect (Cade & Norman, 1986). The increased insulin secretion in curcumin treated rats is due to the activation of β-cell survival factors Pdx-1 and Neuro D in this group. Vitamin D_3 treatment modulates the altered AMPA receptor subunit expression leads to increased intracellular Ca^{2+}, which enhances exocytosis of insulin granules (Fujimoto *et al.,* 1995; Shimono *et al.,* 2005).

Diabetic rats showed a significant decrease in body weight when compared with control. Hyperglycemia and decreased body weight during diabetes are in agreement with the previous reports. (Junod *et al.,* 1969; Kumar *et al.,* 2010; Willsky *et al.,* 2011). The decreased body weight in the diabetic rats is due to the excessive breakdown of tissue proteins (Salahuddin *et al.,* 2010; Poongothai *et al.,* 2011). Treatment of diabetic rats with insulin, curcumin and Vitamin D_3 improved body weight significantly which indicate prevention of muscle tissue damage due to hyperglycemic condition. Evidence has shown that NMDA receptors mediate some aspects of eating and satiety (Duva *et al.,* 2005). It has also been shown that stimulation of eating by intra hypothalamically injected neuropeptide Y is dependent upon NMDA receptor activation (Lee & Stanley, 2005). These findings suggest that several central and peripheral glutamatergic circuits are involved in feed intake regulation. Present study showed an altered NMDA receptor expression and density in the brain regions of diabetic rats. These alterations in NMDA receptor subunit might have affected the feeding habit of diabetic rats. Increased oxidative stress in diabetic rats promotes the skeleton muscle damage leading to weight loss (Aragno *et al.,* 2005). Insulin, curcumin and vitamin D_3 treatment significantly reversed the body weight when compared with the control group. A more prominent reversal in body weight was observed in curcumin treated diabetic rats than the other treatment groups. This can be

explained as an effect of the antioxidant activity of curcumin which helped in preventing skeletal muscle damage and also its ability to ameliorate the altered NMDA receptor expression in brain regions thereby maintaining the feed intake. Previous studies reported that curcumin treatment can suppress body weight loss in diabetic db/db mice (Seo *et al.,* 2008).

GLUTAMATERGIC RECEPTOR ALTERATIONS AND FUNCTIONAL REGULATION IN CONTROL AND EXPERIMENTAL RATS

Diabetes mellitus is a metabolic disorder that not only causes a decrease in efficiency of the pancreatic β-cells to secrete insulin but also is accompanied by altered monoamine levels and their turnover rates in the CNS (Garris, 1990; Lackovic *et al.,* 1990; Bhattacharya & Saraswathi, 1991; Manni *et al.,* 2012). Complications of the peripheral nervous system also are known to be very common in diabetic patients (Dyck *et al.,* 1993; Ametov *et al.,* 2003) and a substantial body of evidence has demonstrated that diabetes have negative impacts on the CNS (Gispen & Biessels, 2000; Ryan & Geckle, 2000; Biessels *et al.,* 2002). People with diabetes, especially older adults, apparently face a greater risk of vascular dementia, with large population studies detecting an association between diabetes mellitus, depression and Alzheimer's disease (Leibson *et al.,* 1997; Ott *et al.,* 1999; Anderson *et al.,* 2001; Gasparini *et al.,* 2002).

Glutamate is involved in most aspects of normal brain function including cognition, memory and learning. Brain tissue contains large amounts of glutamate, around 5-15 mmol per kg depending on the region (Schousboe, 1981; Zaganas *et al.,* 2012). The extracellular concentrations are kept low and are in the order of a few micromolar (Hamberger *et al.,* 1983), or even lower (Herman & Jahr, 2007). The highest glutamate concentrations are found intracellularly in glia cells, nerve terminals and synaptic vesicles (in increasing order) (Ottersen *et al.,* 1992). Glutamate concentration of more than 60 mM was reported inside synaptic vesicles (Shupliakov *et al.,* 1992). Excessive activation of ionotropic glutamate receptors (NMDAR, AMPA-R and Kainate-R) induces a massive Ca^{2+} influx into

the cell which can trigger neuronal death in the CNS. There is strong evidence suggesting the involvement of this glutamate excitotoxicity in acute injury to the CNS and many chronic neurodegenerative disorders

Cerebral cortex

The cerebral cortex is the seat of our highest forms of intelligence. It plays a central role in many complex brain functions including memory, attention, perceptual awareness, thought, language and consciousness. Besides autonomic and peripheral neuropathy, diabetes is also associated with gradually developing end-organ damage in the CNS (Brands *et al.,* 2004) and leads to impairment in cognitive functions and electrophysiological changes (Allen *et al.,* 2004). L-Glutamate is regarded as the major excitatory neurotransmitter in the mammalian CNS. All three ionotropic glutamate receptors exhibit a ubiquitous distribution in the brain, the NMDA receptors being particularly abundant in the forebrain (Ozawa *et al.,* 1998). Although all receptors have pivotal roles in brain functions, the NMDA receptors have received special attention in development and aging. They are involved in cell migration, growth and differentiation in the developing brain (Vallano, 1998; Unezaki *et al.,* 2012).

In addition to being the most important excitatory neurotransmitter in the brain, glutamate is a potent neurotoxin and is considered the primary cause of neuronal death during acute insults to the brain and in neurodegenerative diseases. GAD catalyzes the decarboxylation of glutamate yielding CO_2 and GABA. Our findings reported a decreased gene expression of GAD mRNA in the cerebral cortex of diabetic rat. Decreased gene expression of GAD leads to increased glutamate content in the diabetic rats. The extracellular concentration of the excitatory neurotransmitter L-glutamate in the CNS must be kept low to ensure a high signal to noise ratio during synaptic activation (Katagiri *et al.,* 2001) and to prevent excitotoxicity due to excessive activation of glutamate receptors (Mangano & Schwarcz, 1983; Wang *et al.,* 1998). Curcumin and vitamin D_3 treatment significantly reversed the GAD expression near to control which helps in the decarboxylation of glutamate to GABA and CO_2. This conversion of

glutamate to GABA helps to reduce the glutamate content in the cerebral cortex. Decreased glutamate in the postsynaptic neuron helped in inhibition of hyper excitability of glutamate and reduces glutamate induced excitotoxicity. Over activation of glutamate receptors can damage the neurons leading to impairment in the motor function and co-ordination in hyperglycaemic rats (Anu *et al.,* 2010). Previous reports showed that dysregulation of glutamate signaling in the brain regions leads to neuronal damage and causes a number of neuropsychiatric diseases (Rahn *et al.,* 2012) and neurodegenerative diseases like Alzheimer's disease (Hynd *et al.,* 2004). Population-based study estimated that the risk of Alzheimer's disease increased with diabetes mellitus (Leibson *et al.,* 1997; Exalto *et al.,* 2012). Glutamate excitotoxicity in diabetic brain is a reason for neuronal injury leading to neurodegenerative disorders.

To evaluate the role of curcumin and vitamin D_3 in cortical NMDA receptor kinetics, radio receptor assay was done in the cerebral cortex of control and experimental rats and it was observed that the NMDA receptor number was significantly increased in the diabetic group when compared to control. The gene expression analysis of NMDA R1 and NMDA 2B receptor subunits supported the NMDA receptor binding data. Curcumin and vitamin D_3 treatments significantly reversed the altered NMDA receptor density and gene expression in the cerebral cortex to near control. The increased B_{max} observed showed the increased receptor number whereas the K_d value signifies that the receptor affinity remained unaltered. The immunohisochemical studies using confocal microscope confirmed the binding parameters and gene expression of NMDA receptor subunits in cerebral cortex of control and experimental rats.

AMPARs mediate the majority of the fast excitatory transmission in the CNS of vertebrates. These receptors are concentrated at postsynaptic densities of excitatory synapses, although large pools of AMPARs are also present in the cytoplasm of neuronal somata and dendrites (Petralia, 1997; Beckerman & Glass, 2011). One subtype of glutamate receptor that is thought to play a central role in excitotoxic injury is the AMPA (Beattie *et al.,* 2010). The results from our study suggest that AMPA receptor number significantly increased in the cerebral cortex

of diabetic rats with no change in the receptor affinity. We observed a different pattern of gene expression with AMPA receptor subunits. AMPA GluR4 receptor gene expression showed a significant up regulation while AMPA GluR2 receptor subunit expression was significantly down regulated in the cerebral cortex of diabetic rats. Studies showed that neuronal cells preferentially expressing the GluR4 subunit of AMPA receptors are particularly vulnerable to AMPA-induced toxicity (Page & Everitt, 1995). Homomeric complexes made of the GluR1, GluR3 or GluR4 proteins form channels that are permeable to Ca^{2+}, Mg^{2+} and Ba^{2+} (Hollmann *et al.,* 1991). A key subunit in determining the ion channel properties of AMPA receptors is GluR2 (Lee *et al.,* 2010). When this subunit is present in a receptor complex, the AMPA receptors exhibit a linear relationship between voltage applied to the membrane and the current conducted through the receptor channels (Nakanishi *et al.,* 1990; Hume *et al.,* 1991). These receptor complexes also have very low permeability to Ca^{2+}, i.e. they resemble most native AMPA receptors in CNS neurons, although extrapolations to native receptor structure is imprecise as neuronal receptors with linear conductance are also permeable to Ca^{2+} (Schneggenburger *et al.,* 1993; Li *et al.,* 2012).

The results from our study indicate that the change in AMPA receptor subunit composition in diabetic condition makes AMPA receptor more permeable to Ca^{2+}. The over stimulation of GluR4-containing AMPA receptor leads to excessive calcium accumulation in neurons; ultimately leading to their death (Choi *et al.,* 1988). Treatment using curcumin and vitamin D_3 reversed the receptor number and gene expression in cerebral cortex of the diabetic rats and it could prevent the neuronal death as evident from the decreased apoptotic factors expression in our study. The immunohistochemical studies using confocal microscope confirmed the gene expression of AMPA receptor subunits in cerebral cortex of control and experimental rats. We also reported an increase in intracellular IP3 content in cerebral cortex of diabetic rats. Inositol phosphates are known to regulate AMPA receptor trafficking, intracellular Ca^{2+} homeostasis, particularly the release of stored Ca^{2+} through IP3 receptors (Miyazaki, 1995). This leads to excess Ca^{2+} release through IP3 receptor mediated Ca^{2+} channel

leading to neuronal damage. The treatment with curcumin and vitamin D_3 has resulted in reversal of enhanced IP3 content. Vitamin D_3 treatment showed a prominent reversal in the IP3 content when compared with insulin treated rats.

In diabetes, oxidative stress cause an increased production of free radicals and a sharp reduction in antioxidant defenses (Giugliano *et al.*, 1995). Diabetes also induces an increase in lipid peroxidation products (Sies *et al.*, 1985) and a decrease in SOD, CAT (Wohaieb & Godin, 1987) and GSH levels (Miranda *et al.*, 2006). GPx is a soluble selenoprotein which reduces H_2O_2 and organic peroxides to H_2O and corresponding stable alcohols using reduced GSH as an essential co-substrate thus inhibiting the formation of free radicals. Our study showed a decreased gene expression of GPx and decreased SOD activity in cerebral cortex of diabetic rats compared to control. Decreased SOD activity and GPx gene expression leads to high oxidative stress in diabetic rats. The high oxidative stress in the cerebral cortex resulted in a decreased GLAST gene expression which in turn leads to reduced reuptake of extracellular glutamate. GLAST contains functional cysteine residues that are sensitive to oxidative formation of cysteine bridges leading to inhibition of glutamate flux through the transporters (Trotti *et al.*, 1998). Hydrogen peroxide, nitric oxide, superoxide anion and peroxynitrite anion can inhibit glutamate uptake through GLAST (Zeng *et al.*, 2010). GPx catalyzes the reduction of hydrogen peroxide and hydro peroxides formed from fatty acids, thereby effectively removing toxic peroxides from living cells. It plays the important role of protecting cells from potential damage by free radicals, formed by peroxide decomposition (Mannervik 1985; Ursini 1995). We observed that the treatment using insulin, curcumin and vitamin D_3 increased GPx gene expression and SOD activity thereby reducing the oxidative stress. GLAST gene expression was significantly reversed to control when compared with diabetic rats. Curcumin being a potent antioxidant showed a more prominent reversal in SOD activity and GPx gene expression than the insulin treated rats.

Our results showed that the glutamate content, NMDA and AMPA receptor number in the cerebral cortex of the diabetic rats were increased with decreased GLAST and GAD gene expression. Previous studies reported that

NMDA R1 and GluR4-containing AMPA receptor play a primary role in triggering intracellular cascades that lead to glutamate mediated neuronal apoptosis (Hollmann *et al.,* 1991; Santos *et.al*., 2006). To find out whether these changes in the glutamate pathway cause any neuronal damage due to apoptosis we studied the expression of apoptotic factors like caspase 8 and Bax. Bax is a proapoptotic factor which act as apoptosis executers (Reed, 1998; Cory & Adams, 2002; Polster & Fiskum, 2004; Ward *et al.,* 2004). In the present study, the gene expression of apoptotic factors, caspace 8 and Bax was up regulated in diabetic groups. Suppression of antioxidant enzyme GPx gene expression and reduced SOD activity in diabetic condition could lead to the increased oxidative stress and directly activates apoptotic pathways. The treatment using curcumin and vitamin D_3 reversed the altered GPx gene expression and SOD activity to near control and glutamate mediated excitotoxicity by reversing the altered NMDA and AMPA receptors. Akt-1 gene expression was down regulated in the diabetic group. Akt-1 or serine threonine kinase is a member of an anti-apoptotic cascade of neurons (Endo *et al.,* 2006). The constitutively active Akt-overexpressing neurons could survive potential cellular distresses (Namikawa *et al.,* 2000; Narayanan *et al.,* 2009). In the present study only vitamin D_3 treatment activated the Akt mediated survival pathway.

In conclusion our study showed that curcumin and vitamin D_3 treatment provides neuroprotection by acting as an antioxidant and modulator of glutamatergic neurotransmission. The result of this study has demonstrated that the supplementation of curcumin and vitamin D_3 to STZ-induced diabetic rats has beneficial effects in reducing the alterations in glutamatergic receptors, oxidative stress and imbalanced glutamate metabolism in the cerebral cortex.

Hippocampus

Hippocampus is based on recent or declarative memory and plays important roles in long-term memory and spatial navigation (Squire *et al.,* 1992). Many organs and organ systems are adversely affected by diabetes, including the brain, which undergoes changes that increase the risk of depression and cognitive

decline (Greenwood & Winocur, 2005; Messier, 2005). In the hippocampus excitatory transmission is mediated by glutamate acting on ionotropic NMDA and non-NMDA receptors as well as on metabotropic receptors (Hollmann & Heinemann, 1994). Glutamate receptors are implicated in physiological functions like neuronal plasticity, learning and memory (Ekonomou & Angelatou, 1999; Lynch, 2004). Moderate disturbances of learning and memory and complex information processes have been reported in both type 1 and 2 diabetic patients (Biessels & Gispen 2005, Cukierman *et al*., 2005, Biessels *et al*., 2006; Haider *et al.,* 2012).

Alterations in the hippocampal glutamate receptor can cause impaired cognitive function, learning and memory (Smijin *et al.,* 2012). Our results showed that NMDA and AMPA receptors were increased in diabetic rats compared to control. Gene expression studies showed up regulation of NMDA R1, NMDA 2B and AMPA GluR4 receptor subunits mRNA in the hippocampus of diabetic rats. AMPA GluR2 receptor subunit showed significant down regulation in the diabetic rats when compared to control. Immunohistochemical studies also showed an increased NMDA R1, NMDA 2B, AMPA GluR4 and decreased GluR2 receptor subunits expression in diabetic rats. The altered AMPA subunits expression in the hippocampus of diabetic rats makes AMPA receptor more permeable to Ca^{2+} and causes neuronal damage through the activation of apoptotic factors Bax and caspase 8. Presence of GluR2 subunit determines the Ca^{2+} permeability of the AMPA receptor (Isaac *et al.,* 2007; Liu & Zukin, 2007). NMDA receptor channels are highly permeable to Ca^{2+} ions. Hyper activity of NMDA receptors can lead Ca^{2+} influx and neuronal damage in the hippocampus of diabetic rats (Arundine & Tymianski, 2003). Recent reports suggest that both hypoglycaemia and hyperglycaemia have adverse effects on the brain neuronal structural changes and impaired long-term spatial memory (Malone *et al.,* 2008). Long-term potentiation of neuronal activity in the hippocampus is thought to be a substrate for learning and memory. Gasparova *et al.,* (2008) revealed that prolonged exposure to hypoglycaemic state influenced induction of LTP in the hippocampus and that it had deleterious effects on learning and memory. Insulin, curcumin and vitamin D_3

treatment significantly reversed the altered NMDA and AMPA receptor number and receptor subunits gene expression when compared with diabetic group. Curcumin and vitamin D_3 treatment showed prominent reversal in the NMDA and AMPA receptor subunits when compared with insulin. Immunohistochemical analysis also showed a significant reversal in the NMDA and AMPA receptor subunits expression in the treatment group. While reversing the altered NMDA, AMPA receptors subunit gene expression and receptor number, it prevents the hyperactivation of the receptor leading to excitotoxicity. Second messenger IP3 content was increased in the hippocampus of diabetic rats .The increased levels of IP3 in hippocampus can cause enhanced Ca^{2+} levels resulting in activation of protein kinase C leading to series of events culminating in internalisation of AMPA receptors (Ruiz *et al.,* 2009).

Increased glutamate content was observed in the hippocampus of diabetic rats mainly due to the down regulation of GAD and GLAST mRNA. Decreased GAD gene expression indicates the reduced decarboxylation of glutamate to GABA. GLAST is the glutamate transporter which transports 90% of the glutamate from synapse to glial cells (Kim *et al.,* 2011). The decreased expression of glutamate transporter leads to the impaired clearance of glutamate from the extracellular space and high glutamate content in the hippocampus of diabetic rats compared with control. Up regulation of GAD gene expression and down regulation of glutamate transporter gene expression can lead to altered synaptic glutamate levels (Lyon *et al.,* 2008). Treatment using insulin, curcumin and vitamin D_3 significantly reversed the altered GAD and GLAST gene expression when compared to diabetic group. Curcumin and vitamin D_3 treatment showed prominent reversal in GAD and GLAST gene expression when compared to insulin treatment. This reversed GAD and GLAST gene expression in the curcumin and vitamin D_3 treated rats maintain the glutamate content in the synapse than insulin treatment. The hyperactivation of glutamate receptor can cause increased oxidative stress (Parfenova *et al.,* 2005).

Oxidative stress is a key participant, along with metabolic compromise and excitotoxicity, in apoptotic neurodegenerative process (Alexi *et al.,* 2000).

The brain is particularly vulnerable to oxidative injury because of its high rate of oxygen consumption, intense production of reactive radicals and high levels of transition metals, such as iron, that catalyze the production of reactive radicals. In the animal models of diabetes, several brain alterations have been described, such as increased hippocampal astrocytic reactivity, impaired synaptic plasticity, vascular changes, decreased dendritic complexity and disturbed neurotransmission (Magariños *et al.,* 2000). Many studies reported that excitotoxicity can cause increased oxidative stress (Trudeau *et al.,* 2004; Melo *et al.,* 2011). In the present study, diabetic group showed a significant decrease in the SOD activity and GPx gene expression when compared with control. Decreased antioxidant enzymes in the diabetic group can lead to a state of oxidative stress and the activation of apoptotic factors, Bax and caspase 8. Treatment using curcumin and vitamin D_3 significantly enhances the SOD activity and GPx gene expression when compared with diabetic group. Curcumin treatment showed prominent reversal in antioxidant system when compared to insulin treatment because of its antioxidant activity. We observed a significant up regulation in the Bax and caspase 8 gene expressions in the hippocampus of diabetic group indicating increased apoptosis. Insulin, curcumin and vitamin D_3 treatment significantly reverse the Bax and caspase 8 gene expression by reversing the altered glutamatergic neurotransmission and antioxidant enzyme status.

In conclusion the diabetic rats showed alterations in the glutamatergic transmission in the hippocampus leading to increased oxidative stress and activation of apoptotic factors. The altered glutamate transmission in the hippocampus can lead to impaired cognitive function, learning and memory. Curcumin and vitamin D_3 treatment reversed the altered glutamate transmission and reduced the expression of apoptotic factors than insulin treatment.

Brain Stem

Brain Stem is a part of the brain located beneath the cerebrum and in front of the cerebellum. It connects the spinal cord to the rest of the brain. Brain stem reticular formation has been considered to play an important role in generating

behavioural states as well as in the modulation of pain sensation (Paré & Steriade 1993, Steriade, 1996). Brain stem along with hypothalamus serves as the key centre of the central nervous system regulating the body homeostasis (Araújo & Martel, 2012). Stimulation of the peripheral vagus nerve leads to an increase in circulating insulin levels. Anatomical studies suggest that the origin of these vagal efferent fibres is nucleus ambiguus and dorsal motor nucleus directly innervating pancreas (Bereiter *et al.,* 1981). Brain stem along with hypothalamus serves as one of the key centers of the central nervous system regulating body homeostasis (Araújo & Martel, 2012). Stimulation of the peripheral vagus nerve leads to an increase in circulating insulin levels. The dorsal motor nucleus of the vagus nerve is located in the brain stem. It is connected to the endocrine pancreas exclusively through vagal fibers and has a role in neural mediated insulin release (Costoli *et al.,* 2005). Nucleus ambiguous stimulation has been reported to increase plasma insulin levels in rats (Bereiter *et al.,* 1981; Moreno *et al.,* 2008).

Neurotransmitter alterations are reported in the brain stem of STZ induced diabetic rats (Carndall *et al.,* 1981; Gireesh *et al.,* 2008; Abraham *et al.,* 2010). Present study showed an increase in NMDA and AMPA receptor binding in the brain stem of diabetic rats when compared with control with no change in affinity. Insulin, curcumin and vitamin D_3 treatment significantly reversed the changes when compared with diabetic group. The increased NMDA receptor activity observed in diabetic group from the Scatchard plot was supported by the gene expression study of NMDA R1 and NMDA 2B subunits. AMPA receptor subunits showed differential expression. AMPA GluR4 subunit expression was significantly increased and AMPA GluR2 subunit expression was decreased in diabetic group when compared with control. The immunohistochemical studies using confocal microscope confirmed the binding parameters and gene expression of NMDA and AMPA receptor subunits in brain stem of control and experimental rats. This subunit variation in the diabetic group make AMPA receptors more permeable to Ca^{2+} and leads to glutamate mediated excitotoxicity (Santos *et al.,* 2006). The increased levels of second messenger, IP3 in diabetic rats indicates enhanced Ca^{2+} levels (Miyakawa *et al.,* 1999; Rahman 2012).

Glutamate content in the brain stem was significantly increased in the diabetic rats due to the altered gene expression of GAD mRNA. Decreased GLAST gene expression was observed in the brain stem indicating the altered glutamate transport in the diabetic group. The elevated NMDA and AMPA receptor in the presence of increased glutamate content and altered glutamate transport in diabetic brain leads to the neuronal apoptosis through glutamate mediated excitotoxicity. Apoptotic factors, Bax and caspase 8 were up regulated in the brain stem indicating apoptosis. Insulin curcumin and vitamin D_3 treatment significantly reversed the altered glutamate receptor subunit and GAD gene expression to near control and prevent the Ca^{2+} influx and excitotoxic cell death through the over-activation of NMDA and AMPA receptors. Studies have shown that vitamin D_3 has regulatory benefits in neuronal Ca^{2+} homeostasis and protects neurons from excess Ca^{2+} entry in the brain (Brewer *et al.,* 2001). Insulin treatment was found to alter glutamate receptor activation (Liu *et al.*, 1995) and interact with AMPA receptor trafficking between the plasma membrane and the intracellular compartment in neuronal cell culture (Man *et al.,* 2000) indicating that mechanisms underlying diabetic neuropathies could be initiated in the early stages of the disease, as a consequence of abnormal glutamate receptor properties. This is relevant to the clinical situation because excessive activation of glutamate receptors is a characteristic feature of brain damage during stroke and ischemia (McCall, 1992; La Via *et al.,* 2012).

The antioxidant enzyme GPx gene expression and SOD activity was decreased in the diabetic rats indicating increased state of oxidative stress in the diabetic brain stem. The increased Ca^{2+} influx due to hyper activation of NMDA and AMPA receptors can cause increased oxidative stress (Bondy *et al.,* 1993). This increased state of oxidative stress led to neuronal death which is indicated by enhanced expression of caspase 8 and Bax. The treatment using curcumin and vitamin D_3 restores antioxidant status and reduce the expression of apoptotic factors. Curcumin treatment showed prominent reversal in GPx gene expression and SOD activity due to its antioxidant properties (Menon & Sudheer, 2007). The up regulation of Akt-1 gene expression indicates the activation of neuronal

survival pathway (Brunet *et al.,* 2001) in the brain stem of vitamin D_3 treated rats. Active Akt has vital roles in cell survival, metabolism and neuronal function (Guo *et al.,* 2012).

Cerebellum

Experimental evidence indicate the involvement of cerebellum in a variety of human mental activities including language (Fiez *et al.,* 1996), attention (Allen *et al.,* 1997), cognitive affective syndromes (Schmahmann & Sherman, 1998), fear and anxiety caused by threats of pain (Ploghause *et al.,* 1999), thirst sensation and fear for air, hunger (Parsons *et al.,* 2001) and motor relearning (Stoodley & Schmahmann, 2009; Strick *et al.,* 2009). Cerebellar activity is often detected in neuroimaging studies of pain (Moulton *et al.,* 2010) and other studies evaluating emotional processing (Fusar-Poli *et al.,* 2009), even in the absence of a motor task. The cerebellar vermis integrates and processes the inputs from the vestibular, visual and proprioceptive systems to coordinate muscle timing as a result of which the centre of gravity stays within the limits of stable upright standing (Diener *et al.,* 1989). Damage to the cerebellum, in particular the vermis (Baloh *et al.,* 1998) results in more postural sway than in control subjects (Ho *et al.,* 2004, Marvel *et al.,* 2004). Decreased postural stability would correspond with abnormalities of the vermis observed in autistic subjects (Gowen & Miall, 2005). Unlike explicit memory such as recognition memory and spatial memory, motor learning is characterized by slow development, without the requirement of conscious recall and in general being lifetime-lasting (Llinas & Welsh, 1993; Tulving & Markowitsch, 1998; Eichenbaum, 2000). Based on the role of the cerebellum in motor activities such as fine motor movement and motor coordination as well as the computational network within the neural circuitries, cerebellar motor learning was first postulated by Marr (1969) and Albus (1971). Studies have indicated that the cerebellum is involved in generalized emotional perception (Murphy *et al.,* 2003; Konarski *et al.,* 2005), including aversive picture perception (Lane *et al.,* 1997; Paradiso *et al.,* 1999; Bermpohl *et al.,* 2006). Studies from our laboratory showed that neurotransmitter receptor alterations in cerebellum during diabetes

can cause impaired motor learning and coordination (Joseph *et al.,* 2007; Peeyush *et al.,* 2010)

The present study showed that glutamate content is significantly increased in the cerebellum of diabetic rats with decreased gene expression of GAD and GLAST. The decreased production of GAD resulted in the decreased decarboxylation of glutamate yielding CO_2 and GABA. This blockage in the glutamate content leads to glutamate accumulation in the synapse of diabetic rats. Our previous studies reported that GDH enzyme activity enhanced during diabetes and did not completely reverse even after insulin administration leading to increased glutamate content (Preetha *et al*. 1996; Aswathy *et al*. 1998; Biju & Paulose, 1998). Clearance of extracellular glutamate from the synaptic cleft is carried out by specific high-affinity sodium-dependent excitatory amino acid transporters, GLAST. Glutamate aspartate transporter (EAAT1) stabilizes the concentration of extracellular excitatory amino acids are responsible for removal of more than 90% of the extracellular glutamate. This buffers the glutamate level, thus avoiding excessive stimulation of neuronal glutamate receptors and protecting neurons from glutamate toxicity (Dunlop, 2006). The decreased expression of GAD and GLAST mRNA in the cerebellum result in the elevated extracellular glutamate levels and lead to abnormalities in glutamatergic neurotransmission. The extracellular concentration of the excitatory neurotransmitter L-glutamate in the CNS must be kept low to ensure a high signal to noise ratio during synaptic activation (Katagiri *et al.,* 2001) and to prevent excitotoxicity due to excessive activation of glutamate receptors (Wang *et al.,* 1998). Treatment using insulin, curcumin and vitamin D_3 significantly reversed the altered GAD and GLAST gene expression when compared with diabetic group. Curcumin and vitamin D_3 treatment showed prominent reversal when compared to insulin treated group. The improved GAD and GLAST gene expression in the curcumin and vitamin D_3 treated rats helped in the normal conversion and transportation of glutamate and its helps to maintain the reduced glutamate content in the synapse than insulin treated rats.

AMPARs mediate the majority of the fast excitatory transmission in the CNS of vertebrates. These receptors are concentrated at postsynaptic densities of excitatory synapses, although a large pool of AMPARs is also present in the cytoplasm of neuronal somata and dendrites (Petralia, 1999). One subtype of glutamate receptor that is thought to play a central role in excitotoxic injury is the AMPA. The results from our study showed that AMPA receptor number was significantly increased in the cerebellum with out change in affinity. The elevated AMPA receptor number in the presence of increased glutamate content resulted in hyper activation of AMPA receptor. GluR2 and GluR4 subunits showed a different pattern of gene expression. GlurR2 gene expression was significantly down regulated in the cerebellum of diabetic rats when compared with control. The relative presence of the GluR2 subunit determines the functional properties of AMPA receptors. The GluR2 subunit in most neurons expresses at a high level, which renders these cells impermeable for Ca^{2+} influx through AMPA receptors. Studies showed that reduction of GluR2 subunit levels enhance glutamate excitotoxicity (Friedman *et al.,* 1998; Bogaert *et al.,* 2012). The immunohisochemical studies using confocal microscope confirms the gene expression of GluR2 AMPA receptors in cerebellum of control and experimental rats. GluR4 receptor subunit gene expression was significantly up regulated in the cerebellum of diabetic rats. Studies reported that neuronal cells expressing the GluR4 subunit of AMPA receptors are particularly vulnerable to AMPA-induced toxicity (Page & Everitt, 1995). The over stimulation of GluR4 AMPA receptor leads to excessive Ca^{2+} accumulation in neurons ultimately leading to their death (Choi, 1988). The immunohisochemical studies showed an increased expression of AMPA GluR4 receptor and decreased expression of AMPA GluR2 in diabetic group when compared to control. Treatment using curcumin and vitamin D_3 reversed the altered AMPA receptor number and subunit gene expression when compared to diabetic group. Vitamin D_3 treatment showed prominent reversal in the AMPA receptor number when compared to insulin treatment. Both curcumin and vitamin D_3 treatment showed prominent reversal in AMPA GluR4 and AMPA GluR2 receptor subunit gene expression when compared to insulin treated group.

IP3 content in the cerebellum is significantly increased in the diabetic rats compared to control. Inositol phosphates are known to regulate AMPA receptor trafficking, intracellular Ca^{2+} homeostasis, particularly the release of stored Ca^{2+} through IP3 receptors (Miyazaki, 1995; Bogaert *et al.,* 2012). Treatment using curcumin and vitamin D_3 significantly reversed the IP3 content in the cerebellum when compared to diabetic rats.

Recent studies have shown the involvement of NMDA receptor subunits-NMDAR1, NMDA2B in the cerebellum in motor learning in mouse (Jiao *et al.,* 2008). Receptor binding studies in cerebellum showed an increased B_{max} without change in K_d value in the diabetic rats when compared to control. This increased B_{max} observed indicates the increased receptor number with no change in the affinity of the receptors as shown from the K_d value. Linear regression data by Scatchard plot was supported by the gene expression and immunohistochemical studies of NMDAR1 and NMDA 2B subunits. The elevated NMDA receptor number in the presence of increased glutamate content resulted in the hyper activation of NMDA receptor. NMDA receptors possess several unique properties that distinguish them from other ionotropic glutamate receptors. In addition to monovalent cations, NMDA receptors are also highly permeable to the divalent cation Ca^{2+}, which has numerous important intracellular functions. Over activation of NMDA receptors also result in cellular dysfunction and contribute to the symptoms of many disorders of the nervous system (Rothman & Olney, 1986). Treatment using vitamin D_3 significantly reversed the altered NMDA receptors when compared with diabetic without change in affinity. The reduction in the glutamate content helped to reduce the elevated NMDA receptors in synapse. Treatment using insulin, curcumin and vitamin D_3 reversed the altered NMDA receptor number and subunit gene expression when compared with diabetic group. Curcumin and vitamin D_3 treatment showed prominent reversal in the NMDA receptor number and NMDA R1 when compared to insulin treatment. Vitamin D_3 treatment showed prominent reversal in NMDA 2B receptor subunit gene expression when compared with insulin treated group.

Cerebellum of diabetic rats showed a significant increase in the glutamatergic receptor activity, up regulated glutamatergic activity mediated neurodegeneration through excitotoxicity. Excitotoxicity can cause increased oxidative stress (Nguyen *et al.,* 2011). Our study showed a decreased SOD activity and GPx gene expression in the cerebellum of diabetic rats. Decrease in the antioxidant enzymes SOD and GPx indicates increased oxidative stress in the diabetic rats. Treatment using curcumin showed a significant reversal in the SOD activity but insulin and vitamin D_3 did not show any significant change when compared with diabetic rats. Insulin, curcumin and vitamin D_3 treatment showed a significant reversal in the GPx gene expression when compared with diabetic group. Curcumin treatment showed a significant reversal when compared with insulin treated rats. Curcumin is a potent antioxidant and this antioxidant activity of curcumin helps to protect the cerebellum from further damages. Glutamate mediated excitotoxicity and increased oxidative stress in the diabetic cerebellum can activate the apoptotic factors (Fonfría *et al.,* 2002). In the present study apoptotic factors caspase 8 and Bax gene expression in the cerebellum was significantly up regulated when compared with control rats. Increased gene expression of caspase 8 and Bax indicates increased apoptosis in the diabetic group. Treatment using curcumin and vitamin D_3 reversed caspase 8 and Bax expression and showed neuroprotective effect on diabetic cerebellum through revising altered glutamatergic neurotransmission and antioxidant system.

Pancreas

Plasma glucose levels are regulated by the action of insulin, a hormone that is produced and secreted by the pancreatic islet β-cells in response to nutrients. Diabetes mellitus, which comprises a heterogeneous group of hyperglycaemic disorders, results from inadequate mass and function of β -cells (Prentki & Nolan, 2006). Insulin secretion from the pancreatic islets is controlled by the central nervous system through sympathetic and parasympathetic nerves (Burr *et al.,* 1976; Campfield & Smith, 1980; Ahren, 2000). Studies from our laboratory described the regulatory role of the sympathetic and parasympathetic

systems in pancreatic regeneration (Renuka *et al.,* 2004, 2005; Mohanan *et al.,* 2005). Pancreatic islets receive innervations from both divisions of the autonomic nervous system and pancreatic endocrine secretion is partly controlled by the autonomic nervous system (Liu *et al.,* 2001). Anatomical studies suggest that the vagal efferent fibers originating from the nucleus ambiguus and dorsal motor nucleus of the brain stem directly innervate the pancreas (Bereiter *et al.,* 1981) and have a role in neural mediated insulin release (Azmitia & Gannon, 1986).

Reports suggest that amino acids can, under appropriate conditions, enhance insulin secretion from primary islet cells and β-cell lines (Charles & Henquin 1983; Smith *et al.,* 1997; Brennan *et al.,* 2002; Dixon *et al.,* 2003). L-glutamine release from skeletal muscle modulates glucagon release from pancreatic α-cells (Chang & Goldberg, 1978), which subsequently influence insulin secretion from β -cells. Glutamate, a major excitatory neurotransmitter in the central nervous system, is also found in pancreatic islets (Gonoi *et al.,* 1994; Inagaki *et al.,* 1995; Muroyama *et al.,* 2004) and is released from α cells (Yamada *et al.,* 2001; Hayashi *et al.,* 2003). Various ionotropic glutamate receptors are found in insulinoma cells and pancreatic islets (Inagaki *et al.,* 1995; Muroyama *et al.,* 2004). Studies suggest that cytoplasmic glutamate concentration can influence insulin production (MacDonald & Fahien, 2000; Hoy *et al.,* 2002; Maechler *et al.,* 2002).

In the present study, the glutamate content was significantly increased in diabetic group when compared to control. It was reported that enhanced GDH can produce glutamate, a second messenger of insulin secretion (Anno *et al.,* 2004). The claim that glutamate potentiated insulin secretion (Maechler & Wollheim, 1999; Rubi *et al.,* 2001; Hoy *et al.,* 2002) was based on the observation that a rise in cytoplasmic glutamate concentration correlated with increased insulin release, but this claim was later contradicted (Bertrand *et al.,* 2002) when it was found that insulin release did not always correlate with the total islet glutamate concentration. Curcumin and vitamin D_3 treatments showed a significant reversal in glutamate content of pancreas when compared with diabetic group. Insulin treatment did not show any significant change in the glutamate content. Increased glutamate content

in the pancreas can cause damage to insulin producing β-cells (Choi *et al.,* 2010). Insulin treatment did not show any significant change in the glutamate content while the treatment using curcumin and vitamin D_3 significantly reversed the changes when compared to diabetic rats.

Glutamate acts through two classes of receptors, ligand gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors. Many studies showed that iGluRs expressed in β cells can modulate the level of insulin release (Weaver *et al.,* 1995; Moriyama & Hayashi, 2004). All three ionotropic receptors are present in the pancreas (Inagaki *et al.,* 1995). Our study showed that NMDA receptor number did not show any significant change in the diabetic and treatment group when compared with control. Bertrand *et al.,* (1992) reported that NMDA receptors did not have a significant role in insulin secretion. Various reports suggest that glutamate stimulates insulin release in rat pancreas, by acting on an excitatory amino acid receptor of AMPA subtype (Bertrand *et al.,* 1992; Wu *et al.,* 2012). Our study showed that the AMPA receptor number was significantly increased in the diabetic group when compared with control. AMPA GluR4 receptor subunit showed a significant down regulation and AMPA GluR2 receptor subunit showed a significant up regulation when compared with diabetic rats. This change in subunit variation can change the AMPA receptor function. GluR2 subunit determines the functional properties of AMPA receptors; presence of GluR2 subunit makes cells impermeable for Ca^{2+} influx through AMPA receptors (Friedman *et al.,* 1998). The reduction in the Ca^{2+} influx can affect the insulin secretion (Chen *et al.,* 2010). Treatment using insulin, curcumin and vitamin D_3 significantly reversed the receptor number when compared with diabetic rats. Altered AMPA GluR4 and GluR2 receptor subunit expression was significantly reversed in curcumin and vitamin D_3 treatment. Insulin treatment did not show any significant reversal in the GluR4 receptor subunit when compared with diabetic group. Vitamin D_3 treatment showed more prominent reversal in the AMPA GluR2 receptor subunit gene expression when compared with insulin treated group. To understand the role of AMPA receptor subunits expression in the insulin producing pancreatic islets, the double immunohistochemical analysis was

done. Insulin staining is localized to the insulin granules of the islet β cells, thereby considering it as a specific marker for pancreatic β cells. Insulin-AMPA GluR2 subunit co-labelling study showed that in diabetic condition, AMPA GluR2 receptor expression was significantly increased in the insulin positive cells when compared with control. AMPA GluR4 receptor expression was significantly decreased in the insulin positive cells when compared with control. Increased expression of AMPA GluR2 receptor subunit during diabetes makes AMPA receptor more impermeable to Ca^{2+}. Immunohistochemical analysis showed that curcumin and vitamin D_3 treatment significantly reversed the altered AMPA receptor subunits expression in the pancreatic islets.

Inositol phosphates are known to regulate AMPA receptor trafficking, intracellular Ca^{2+} homeostasis, particularly the release of stored Ca^{2+} through IP3 receptors (Miyazaki, 1995). Our study showed that IP3 content was significantly decreased in the pancreas of the diabetic rats when compared with control rats. Treatment using insulin, curcumin and vitamin D_3 reversed the decreased IP3 content to near control. Immunohistochemical analysis showed a decreased expression of IP3 receptor subtype 3 (IP3R-3) in the pancreatic islets of diabetic rats when compared to control. Decreased IP3R3 in the pancreatic islets can affect the Ca^{2+} release (Wong *et al.,* 2006). Immunolocalization studies indicated that IP3R3 receptor is present in secretory granules of the β cell (Blondel *et al.,* 1994; Ravazzola *et al.,* 1996). Treatment using curcumin and vitamin D_3 reversed the decreased IP3 receptor to near control.

In recent years, several reports suggested that the endocrine pancreas is also a target tissue for the hormonally active form of vitamin D_3, 1,25-(OH)2-D3, along with the classical vitamin D target organs: the intestine, bone and kidney (Norman *et al.,* 1982). The biological actions of vitamin D_3 are mediated through binding to the VDR, a member of the nuclear steroid hormone receptor family (McGrath *et al.,* 2001). Immunohistochemical analysis showed a decreased expression of vitamin D receptor in the pancreatic islets of diabetic rats when compared to control. Decreased expression of vitamin D receptor in the diabetic rats affect the vitamin D_3 mediated pathways. Reduced expression of VDR can

cause vitamin D_3 deficiency. Reports suggest that vitamin D_3 deficiency decreases Ca^{2+} uptake by islets (Chertow *et al.,* 1986) 1, 25(OH) $2D_3$ enhances Ca^{2+} entry into islets (Billaudel *et al.,* 1993). Previous studies have indicated that the pancreas has receptors specific for Vitamin D_3 and that Vitamin D_3 increases insulin secretion in vitamin D-deficient rats (Norman *et al.,* 1980). Treatment using vitamin D_3 reversed the altered expression of VDR to near control. Insulin and curcumin treatment did not show any significant reversal. In our *in vitro* Ca^{2+} release studies in pancreatic β cell using Fluo 4-AM, decreased Ca^{2+} release from pancreatic islets observed in hyperglycemic condition. Elevation of ATP is necessary for the membrane-dependant increase in cytosolic Ca^{2+}, the main trigger of insulin exocytosis (Maechler & Wollheim, 2000). In the presence of reduced Ca^{2+} release in diabetic rats islets fail to produce insulin through exocytosis. Vitamin D_3 treatment showed more prominent increase in Ca^{2+} release in the presence of AMPA when compared with other treatment groups.

Oxidative stress is produced under diabetic conditions and is likely involved in progression of pancreatic β-cell dysfunction found in diabetes (Kajimoto & Kaneto, 2004). In the present study the antioxidant enzyme SOD activity was decreased in the diabetic rats when compared with control group. Reduction in the SOD activity is due to increased oxidative stress (Matsunami *et al.,* 2010). Superoxide dismutase administration showed protection against STZ-induced diabetes (Robbins *et al.,* 1980). Treatment using curcumin and vitamin D_3 showed significant reversal in the SOD activity when compared to diabetic group. Insulin treatment did not show any significant change in the SOD activity. Real-Time PCR amplification studies showed that GPx gene expression in diabetic pancreas was down regulated indicating increased oxidative stress. Insulin, curcumin and vitamin D_3 treatment showed prominent reversal in GPx gene expression when compared to diabetic group. The reduction in the antioxidant enzyme makes pancreas vulnerable to oxidative stress and leads to the destruction of pancreatic β-cells through the activation of apoptotic factors, Bax and caspase 8. We observed that Bax and caspase 8 gene expressions were significantly up regulated in the pancreas of diabetic rats when compared with

control. Treatment using insulin, curcumin and vitamin D_3 showed a significant reversal in the caspase 8 and Bax gene expression when compared to diabetic rats.

Insulin gene transcription is regulated by the cooperation of a group of glucose-sensitive transcription factors expressed in a tissue-restricted manner (Ohneda *et al.,* 2000, Aramata *et al.,* 2005). Among the most important of these transcription factors are NeuroD1 and the homeodomain transcription factor pancreatic duodenal homeobox 1 (Pdx1) which activate the insulin gene promoter synergistically and are essential for glucose-stimulated insulin gene transcription. Pdx-1 is required for pancreas development in mice and in humans (Jonsson *et al.,* 1994; Ahlgren *et al.,* 1996; Offield *et al.,* 1996). Pdx1 plays critical role in insulin gene transcription, insulin secretion as well as β cell survival (Babu *et al.,* 2007). Genetic studies in which Pdx1 is conditionally inactivated in mice suggest that *Pdx1* gene dosage is critical both for development of the endocrine and exocrine pancreas and for the maintenance of adult β cells (Hale *et al.,* 2005; Holland *et al.,* 2005; Fujitani *et al.,* 2006). In the present study Pdx1 gene expression was significantly down regulated in the diabetic rats when compared with control. Animal models suggest that down regulation of Pdx1 expression in the β-cell may underlie the pathogenesis of β-cell failure and type 2 diabetes (Weir *et al.,* 1997). Treatment using curcumin and vitamin D_3 significantly reversed and up regulated the Pdx 1 expression when compared with both diabetic and control. The up regulation of Pdx 1 indicates β-cell survival in treatment group (Claiborn *et al.,* 2010). Report suggests that the Pdx 1 expression is downregulate during chronic oxidative stress (Kaneto *et al.,* 2005). The reduced oxidative stress in the treatment group may favor the expression of Pdx 1 resulting in reversing pancreatic dysfunction.

NeuroD is a basic helix-loop-helix (bHLH) transcription factor that is crucial for development of the pancreas (Naya *et al.,* 1997; Huang *et al.,* 2002; Chae *et al.,* 2004; Chao *et al.,* 2007). NeuroD null mice die of severe diabetes shortly after birth; and their β cells are poorly differentiated, islets fail to form, and the majority of β cells are lost (Naya *et al.,* 1997). In the presence study, NeuroD1 gene expression is significantly down regulated in the diabetic rats when

compared with control. NeuroD has been shown to be critical for insulin gene expression *in vitro* (Naya *et al.,* 1995; Qiu *et al.,* 2002). Islets lacking NeuroD respond poorly to glucose and display glucose metabolic profile similar to immature β cells (Gu *et al.,* 2010). Treatment using curcumin and vitamin D_3 showed significant reversal in NeuroD1 gene expression when compared with diabetic group. Insulin treatment did not show any significant change in the NeuroD1 gene expression. The increased expression of NeuroD1 indicates the β cell survival in the treatment group.

In conclusion, the altered AMPA receptor subunit expression in the pancreas of diabetic rats affects AMPA receptor mediated insulin release. Treatment using curcumin and vitamin D_3 showed beneficial effect through ameliorating the alterations in AMPA receptor and increases the Ca^{2+} release in pancreatic islets leading to restored insulin secretion. The decrease in antioxidant enzymes SOD and GPx indicates oxidative stress in the pancreas of diabetic rats leading to the activation of pro apoptotic factors Bax and caspase 8 resulting in β cell death. The down regulation of transcription factors Pdx1 and NeuroD1 indicates reduced insulin production and β cell function. The treatment using curcumin and vitamin D_3 enhanced the anti oxidant enzyme status leading to a state of abridged oxidative stress thereby restricting the expression of Bax and caspase 8. The increased expression of Pdx1 and NeuroD1 in treatment group indicates β cell survival leading to increased insulin production.

Summary

1. Streptozotocin induced diabetic rat model was used to study the alterations of NMDA and AMPA receptor and their functional regulation by curcumin and vitamin D_3.

2. Body weights were determined and diabetic rats showed a significant reduction in the body weight when compared to control. Curcumin and vitamin D_3 treatment showed a significant reversal in body weight when compared with diabetic group.

3. Antihyperglycemic activity of curcumin and vitamin D_3 were evaluated by measuring the blood glucose and circulating insulin level in experimental rats. Diabetic rats showed increased blood glucose and decreased insulin level. Curcumin and vitamin D_3 supplementation in diabetic rats reversed the blood glucose and circulating insulin, when compared with diabetic group.

4. Oxidative stress and free radical scavenging capability of diabetic and other experimental conditions were evaluated by studying the SOD activity and GPx gene expression. In diabetic condition, SOD activity was decreased and GPx gene expression was down regulated in the cerebral cortex, cerebellum, hippocampus, brain stem and pancreas. Oxidative stress in diabetic brain regions and pancreas was significantly reduced in insulin, curcumin and vitamin D_3 treated diabetic rats by reversing the altered antioxidant enzyme activity and expression. Curcumin treatment showed prominent reversal in the antioxidant enzyme activity when compared with other treatments.

5. Glutamate content increased in the cerebral cortex, cerebellum, hippocampus, brain stem and pancreas of diabetic rats. Treatment with insulin, curcumin and vitamin D_3 reversed these changes when compared to diabetic in brain regions. Insulin treatment did not show any significant reversal in pancreas.

6. NMDA receptor functional status was analysed by Scatchard analysis using [^{3}H] MK801. NMDA receptor number was increased in cerebral cortex, cerebellum, hippocampus and brain stem of diabetic rats compared to control with no significant change in K_d. NMDA receptor number did not show any significant change in the pancreas of control and experimental rats. Treatment with insulin reversed the alteration in NMDA receptors of cerebral cortex, hippocampus and brain stem but Insulin treatment did not show any significant reversal in cerebellum when compared with diabetic group. The treatment with curcumin and vitamin D_3 significantly reversed the alteration in brain NMDA receptors when compared with diabetic group.

7. AMPA receptor functional status was analysed by Scatchard analysis using [^{3}H] AMPA. AMPA receptor number was increased in cerebral cortex, cerebellum, hippocampus, brain stem and pancreas of diabetic rats compared to control with no significant change in K_d. The treatment with insulin, curcumin and vitamin D_3 reversed the alteration in AMPA receptor when compared with diabetic group.

8. Glutamate mediates its action through its receptor subunits – NMDA R1, NMDA 2B, AMPA GluR2, AMPA GluR4. Real Time PCR analysis of NMDA R1, NMDA 2B, AMPA GluR2, AMPA GluR4 receptors subunit gene expression confirmed the receptor binding data. There was a significant up regulation in NMDA R1, NMDA 2B and AMPA GluR4 and down regulation in AMPA GluR2 gene expression in the cerebral

cortex, cerebellum, hippocampus and brain stem of diabetic rats compared to control, indicating receptor function alterations in the brain regions of diabetic rats. The treatment with insulin, curcumin and vitamin D_3 reversed these changes when compared with diabetic rats.

9. Real Time PCR studies in the pancreas showed that AMPA GluR4 receptor subunit expression was down regulated and AMPA GluR2 receptor subunit expression was up regulated leading to altered pancreatic function. The treatment with insulin, curcumin and vitamin D_3 reversed the gene expression to near control.

10. Differential expression of NMDA R1, NMDA 2B, AMPA GluR4 and AMPA GluR2 receptor subunits in the cerebral cortex, cerebellum, hippocampus and brain stem of diabetic was confirmed by immunohistochemical studies using confocal microscope with specific antibodies in the brain slices. The treatment with insulin, curcumin and vitamin D_3 reversed the mean pixel value to near control.

11. Immunohistochemical studies using confocal microscope with specific antibodies of AMPA GluR4, AMPA GluR2 receptor subunits co-labelled with insulin antibody was carried out to detect the expression of AMPA receptor subunit in the pancreatic islets. AMPA GluR4 subunits expression was significantly decreased and AMPA GluR2 receptor subunits expression was significantly increased in the insulin positive cells in pancreatic islets of diabetic rats. The treatment with insulin, curcumin and vitamin D_3 reversed the mean pixel value when compared with diabetic group.

12. To prevent glutamate mediated excitotoxic effects, glutamate should be cleared from the extracellular space by glutamate transporters. The gene expression of Glutamate aspartate transporter (GLAST) was studied in

control and experimental rats. GLAST showed decreased expression in cerebral cortex, cerebellum, hippocampus, brain stem and pancreas of diabetic rats leading to impaired reuptake of extracellular glutamate formed in the diabetic condition. Insulin treatment showed significant reversal only in brain regions. Curcumin and vitamin D_3 showed prominent reversal of GLAST gene expression in the brain regions and pancreas when compared with diabetic rats.

13. Real time PCR gene expression analysis of Glutamate decarboxylase (GAD) was done in cerebral cortex, cerebellum, hippocampus and brain stem of control and experimental rats. A significant down regulation of GAD mRNA was observed in diabetic rat brain. Decreased GAD gene expression in the diabetic rat leads to increased accumulation of glutamate. Treatment using insulin, curcumin and vitamin D_3 reversed these changes when compared with diabetic rats.

14. Second messenger IP3 content was increased significantly in cerebral cortex, cerebellum, hippocampus and brain stem of diabetic rats. The increased levels of IP3 causes enhanced Ca^{2+} levels leading to neurotoxicity in brain regions. The treatment using Insulin, curcumin and vitamin D_3 reversed these changes when compared with diabetic rats.

15. In pancreas, second messenger IP3 content was significantly reduced and immunohistochemical studies showed that inositol trisphosphate receptor (IP3R3) expression was significantly decreased in the pancreatic islet of diabetic rats when compared with control. Treatment using curcumin and vitamin D_3 reversed these changes when compared with diabetic rats. Insulin treatment did not show any significant change when compared with diabetic group.

16. Calcium imaging results showed decreased calcium release from the pancreatic islets in diabetic rats. Treatment using vitamin D_3 showed prominent increase in Ca^{2+} release when compared with diabetic group.

17. Increased expression of pro-apoptotic factors, caspase-8 and Bax was observed in cerebral cortex, cerebellum, hippocampus, brain stem and pancreas of diabetic rats. Treatment using insulin, curcumin and vitamin D_3 reversed these changes when compared with diabetic rats.

18. A significant down regulation of anti-apoptotic factor Akt-1 was observed in cerebral cortex, cerebellum, hippocampus and brain stem of diabetic rats. Curcumin treatment showed significant reversal in the Akt-1 gene expression when compared with diabetic rats. The treatment with Vitamin D_3 resulted in a prominent reversal and up regulation of Akt-1 gene expression when compared with diabetic and insulin treated rats.

19. Pdx1 and NeuroD1 expression was significantly up regulated in the curcumin and vitamin D_3 treated rats indicating pancreatic β cell survival and reduced pancreatic β cell death in the treatment group. Insulin treatment did not show any significant change.

The alterations in the GAD and GLAST mRNA in diabetic rats can lead to increased production of glutamate content. Increased AMPA and NMDA receptors in the presence of increased glutamate resulted in elevated IP3 levels and Ca^{2+} levels leading to a state of oxidative stress. The activated apoptotic factors Bax and caspase 8 lead to neuronal death. The altered AMPA receptor subunits expression along with increased oxidative stress and activation of apoptotic factors in the pancreas results in deceased secretion of insulin. Thus, from our study it is suggested that curcumin and vitamin D_3 has therapeutic role in diabetes management mediated through glutamatergic function.

Conclusion

Diabetes leads to neuronal damage and altered CNS function, making diabetic patients vulnerable to neurodegenerative diseases. The elevated NMDA and AMPA receptors with an alteration in NMDA R1, NMDA 2B, AMPA (GluR2) and AMPA (GluR4) receptor gene expression in the brain regions indicates an impaired glutamatergic receptor function in diabetic condition. The decreased GAD and GLAST mRNA expression in diabetic rats indicates an impairment in glutamate metabolism and transport. The alterations in glutamate metabolism and transport augmented the synaptic glutamate concentration, which resulted in the hyper activation of NMDA and AMPA receptors, there by leading to glutamate mediated excitotoxicity. The elevated IP3 level in the brain regions of diabetic rats accounts for the Ca^{2+} mediated toxicity. SOD activity and GPx gene expression were decreased in the brain regions of diabetic rats leading to a state of oxidative stress. Impaired glutamatergic neurotransmission and oxidative stress up regulated the apoptotic factors Bax and caspase 8 and down regulated the anti-apoptotic factor Akt-1 resulting in neuronal death in the brain regions of diabetic rats. The alterations in glutamatergic receptor function, glutamate transporter, glutamate metabolism, IP3 content, antioxidant enzyme function and associated neuronal death were reversed in the brain regions of curcumin and vitamin D_3 treated diabetic rats. In the pancreas of diabetic rats, even though NMDA receptors were not altered significantly, AMPA receptor density, AMPA GluR2 and GluR4 receptor subunit expression were altered. Lowered IP3 content and decreased IP3 receptor was observed in the pancreas of diabetic rats. There was an enhanced state of oxidative stress observed in pancreas of diabetic rats through reduced SOD activity and GPx gene expression. In the pancreas of curcumin and vitamin D_3 treated rats, the decreased IP3 content and IP3 receptor expression were reversed. This leads to increased cytosolic Ca^{2+} concentration, resulting in restored insulin level. Apart from that, vitamin D_3 treatment in diabetic rats reversed the decreased vitamin D_3 receptor resulting in regulation of Ca^{2+} release in pancreatic β cells. Along with this, the up regulated Pdx1, NeuroD1 and

down regulated Bax and caspase 8 in curcumin and vitamin D_3 treated diabetic rats prevented pancreatic β cell death. The reversed AMPA receptor function and reduced oxidative stress restored insulin secretion. Thus the neuroprotective and anti-diabetic property of curcumin and vitamin D_3 are suggested to have therapeutic role through regulating glutamatergic function in diabetic rats.

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