Discussion

Diabetes mellitus is a major global health problem currently affecting more than 180 million people worldwide. The disease is one of the most severe metabolic disorders in humans and it is characterised by hyperglycaemia as a result of a relative or an absolute lack of insulin or the action of insulin on its target tissue or both. The neurological consequences of diabetes mellitus in the CNS are now receiving greater attention. Prolonged exposure to chronic hyperglycaemia in diabetes can lead to various complications, affecting the neurological, cardiovascular, renal and visual systems (Brownlee, 2001). The utilisation of glucose for cell's energy is critical in the functioning of the organs. Nutritional therapy is a challenging but necessary dimension in the management of diabetes and neurodegenerative changes associated with it.

BLOOD GLUCOSE & BODY WEIGHT

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). 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 facts' that increased blood glucose level and decreased body weight, observed during diabetes, are similar with previous reports as a result of the marked destruction of insulin secreting pancreatic β-cells by STZ (Junod *et al*., 1969). Hyperglycemia occurs as a result of increased glycogenolysis, decreased glycogenesis, increased gluconeogenesis, impaired glucose transport across membranes and almost complete suppression of the conversion of glucose into fatty acids through acetyl-CoA.

Previous reports showed that curcumin has the potential to protect pancreatic islet cells against STZ-induced death dysfunction (Meghana *et al*., 2007) and increase plasma insulin level in diabetic mice (Seo *et al*., 2008). Previous studies showed that pancreatic insulin secretion is inhibited by Vitamin D deficiency (Norman *et al*., 1980). An increased prevalence of diabetes has been associated with Vitamin D-deficient individuals (Chiu *et al*., 2004). The results of this study have demonstrated that curcumin, Vitamin D_3 and insulin treatment to STZ-induced diabetic rats have beneficial effects in reducing blood glucose levels to near control. The results suggest that the mode of action of curcumin and Vitamin D_3 is probably mediated by an enhanced secretion of insulin and enhanced tissue glucose utilization. The decreased body weight in the diabetic rats is due to excessive breakdown of tissue proteins. 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. The central complications of hyperglycaemia also include potentiating of neuronal damage observed following hypoxic/ischemic events, as well as stroke. Glucose utilization is decreased in the brain during diabetes (McCall, 1992), providing a potential mechanism for increased vulnerability to acute pathological events.

CIRCULATING INSULIN LEVEL

There was a significant decrease in the circulating insulin level of diabetic rats when compared to control group. The increase in insulin levels in curcumin and Vitamin D_3 treated diabetic rats attribute to the stimulation of the surviving beta cells by the treatment, which in turn exerts an antihyperglycaemic action. Thus, it is suggested that the curcumin and Vitamin D_3 treatment induced insulin release from pancreas, thereby potentiating its effect. A possible mechanism of action is that the

curcumin and Vitamin D_3 stimulated the residual pancreatic β-cell function or produced the antihyperglycaemia through an extra-pancreatic mechanism, probably increasing peripheral utilization of glucose. This data confirmed the anti hyperglycemic activity of curcumin and Vitamin D_3.

CIRCULATING TRIIODOTHYRONINE (T3) LEVEL

Thyroid hormone is essential for maintaining normal neurological functions both during development and in adult life. Type III-iodothyronine deiodinase (D3) degrades thyroid hormones by converting thyroxine and 3, 3′, 5-triiodo-L-thyroinine (T3) to inactive metabolites. A regional expression of D3 activity has been observed in the human CNS and a critical role for D3 has been suggested in the regulation of local T3 content in concert with other enzymes. The serum T3 levels, basal TSH levels and TSH response to thyrotropin releasing hormone (TRH) is influenced by the glycemic status (Schlienger *et al*., 1982). T3 content in the serum was increased significantly in diabetic groups compared to control. Long term thyrotoxicosis has been shown to cause β cell dysfunction resulting in reduced pancreatic insulin content, poor insulin response to glucose and decreased rate of insulin secretion (Bech *et al*., 1996). Insulin, curcumin and Vitamin D_3 treatment significantly reversed the increased T3 content near to control. A reduced secretion of thyroid hormones with age has been documented in humans and animals with no substantial increase in TSH secretion, which is indicative of an age related impairment of the pituitary sensitivity to the negative control exerted by thyroid hormones (Schlienger *et al*., 1982).

BEHAVIOURAL DEFICITS IN DIABETIC RATS

Several studies have described the effects of diabetes in the central nervous system (CNS) as a series of neurochemical, neurophysiological and structural abnormalities, a condition referred to as diabetic encephalopathy (Biessels *et al*., 2002a; Sima *et al*., 2004). In addition to these abnormalities, impairments in cognitive function have been observed in diabetic patients and also in animal models of diabetes (Strachan *et al*., 2003; Brands *et al*., 2007). These impairments have been characterized mainly by moderate deficits in learning and memory, psychomotor slowing and reduced mental flexibility (Cukierman *et al*., 2005; Brands *et al*., 2007). Furthermore, diabetic patients also seem to double the probability of developing Alzheimer's disease and other dementias (Arvanitakis *et al*., 2004; Biessels *et al*., *2006*).

We evaluated the behavioural response of diabetic rats in Y-maze test and memory enhancing property of curcumin and Vitamin D_3. Y-maze is used to evaluate the spatial learning in different rat models (Murugesan, 2005). Also, motor performance of control and experimental rats on rotarod, beam walk and grid walk test were studied.

The Y-maze test is a classic model behavioral test, with a strong aversive component, utilized for evaluating learning and memory in rats and mice (Katz & Chudler, 1980; Woo et al., 2008). Y-maze performance showed that intensity of derangement in diabetic rats increased. These results are in agreement with other studies that have also verified cognitive impairment in STZ-induced diabetes mellitus (Kamal *et al*., 2000) which is associated with intensification of pathological processes within the cortical and other brain regions engaged in these processes (Artola *et al*., 2005). Furthermore, spatial memory and exploratory activity have an influence on behavioral tests including Y-maze performance. In this regard, the number of novel

arm entries and time spent was significantly lower in STZ-diabetic rats. There are also reports on the involvement of the cholinergic system abnormality in the impaired acquisition and/or retention of passive avoidance learning. In this respect, it has been postulated that the observed behavioral abnormalities consequent on an impairment of cerebral glucose metabolism suggestive of cholinergic dysfunction (Jackson *et al*., 2000). However, when the diabetic rats were treated with insulin, curcumin and Vitamin D_3, the time spent and number of novel arm entry in the Y-maze was similar to that found for rats from the control group. These findings indicate that curcumin and Vitamin D_3 were able to normalize the cholinergic receptor dysfunction which assists in lowering their time for spatial recognition and thus improving the cognitive functions.

Diabetes mellitus has been reported to be accompanied by a number of behavioural and hormonal abnormalities, including reduced locomotor activity (Marshall *et al*., 1976). Rotarod test has been used to examine the Motor in-coordination (Cendelin *et al*., 2008). The rotarod, beam walk and grid walk test experiment demonstrated the impairment of the motor function and coordination in the diabetic rats. Diabetic rats showed lower fall off time from the rotating rod when compared to control and increased number of foot slips in beam and grid walk test and decreased time spent in narrow beam test compared to control, suggesting impairment in their ability to integrate sensory input with appropriate motor commands to balance their posture. At the same time, they adjusted their limb movements on the metallic rod which is indicative of cerebellar dysfunction. The insulin, curcumin and Vitamin D_3 treated diabetic rats showed an improved motor performance in rotarod, beam and grid walk test compared to STZ- induced diabetic rats. Our findings indicate that curcumin and Vitamin D_3 normalizes their alleviated stress level which assists in

lowering their time for spatial recognition and thus helps to maintain their posture during movement on the rod.

CHOLINERGIC ENZYME ALTERATIONS IN BRAIN AND PANCREAS OF CONTROL AND EXPERIMENTAL RATS.

Choline acetyltransferase (ChAT) is the rate-limiting enzyme of generating acetylcholine (Ach), which is synthesized in cholinergic neuronal cell bodies and is often used in the studies of tissue localization and functional activity. The reduction of ChAT is correlated with the severity of dementia and pathologic changes (Rodrigo *et al*., 2004). The elevated activity of insulin could improve the expression of ChAT (Rivera *et al*., 2005). Acetylcholine is the primary neurotransmitter of the cholinergic system and its activity is regulated by acetylcholine esterase (AChE). The termination of nerve impulse transmission is accomplished through the degradation of acetylcholine into choline and acetyl CoA by AChE (Weihua Xie *et al*., 2000). Acetylcholine esterase activity has been used as a marker for cholinergic activity (Ellman *et al*., 1961). It has been well established that there is a marked change in the acetylcholine esterase activity in diabetic condition. Akmayev *et al*., (1978) showed that there is difference in distribution of the enzyme in the neurons of the central vagal nuclei and medulla oblongata in normal and diabetic adult male rats. It is suggested that the changes in the plasma glucose or insulin levels is influenced by the activity of cholinergic neurons. Cholinergic neurons may be regulated by insulin signaling, and require this signaling for repair and survival. Impairment of insulin signaling in cholinergic neurons results in a disorder of energy metabolism and impairs repair and cell survival, thus evoking a series of pathologic changes and corresponding clinical manifestations (Hongjuan *et al*., 2009). Thus central cholinergic activity is implicated in the insulin secretion.

Central cholinergic activity was studied in experimental rats after using ChAT and AChE as marker. Our results showed an increase expression of AChE in cerebral cortex, cerebellum, brainstem, hippocampus and hypothalamus of diabetic rats when compared to control group. In corpus striatum there was a decrease in the expression of AChE in diabetic group when compared to control rats. ChAT shows a decreased expression in cerebral cortex, cerebellum, corpus striatum, hippocampus and hypothalamus. In brain stem ChAT expression was increased. These results are in accordance with Kuhad *et al* (2007) where a significant elevation in AChE activity was observed in cerebral cortex from STZ-induced diabetic rats. AChE activation leads to a fast ACh degradation and a subsequent down regulation of ACh receptors causing undesirable effects on cognitive functions (Appleyard *et al*., 1990). In this context, it is suggested that the increase in AChE activity caused by experimental diabetes leads to a reduction in the efficiency of cholinergic neurotransmission due to a decrease in acetylcholine levels in the synaptic cleft, thus contributing to the progressive cognitive impairment and other neurological dysfunctions seen in diabetic patients (Biessels *et al*., 1994). STZ causes reduced cerebral energy metabolism leading to cognitive dysfunction by inhibiting the synthesis of adenosine triphosphate (ATP) and acetyl CoA which results in cholinergic deficiency supported by reduced ChAT activity in hippocampus (Prickaerts *et al*., 1999) and increased AChE activity in rat brain (Sonkusare *et al*., 2005). The enhancement of cholinergic activity by inhibition of AChE enzyme is the main stay of symptomatic treatment of dementia (Siddiqui & Levey, 1999).

In insulin, curcumin and Vitamin D_3 treated diabetic rats AChE and ChAT expression were reversed to near control. Our result showed that diabetic state influenced the expression of AChE and ChAT enzyme and the reversal of altered expression to near control found in the insulin, curcumin and Vitamin D_3 treated diabetic rat brain regions is a compensatory mechanism to maintain the normoglycemic level. The improvement of cognitive impairment by curcumin is suggestive of diverse mechanisms including increasing cholinergic activity by inhibiting acetylcholine esterase activity. Curcumin has been shown to lower the acetylcholine esterase level in the cerebral cortex and hippocampus of the rat brain (Sharma *et al*., 2009).

Pancreas

The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Several different neurotransmitters are stored within the terminals of these nerves, acetylcholine, noradrenaline and several neuropeptides. Stimulation of the autonomic nerves and treatment with neurotransmitters affect islet hormone secretion. Insulin secretion is stimulated by parasympathetic nerves and inhibited by sympathetic nerves (Ahren, 2000). Acetylcholine mediates insulin release through vagal stimulation. Acetylcholine acts through the activation of Gq-phospholipase C. Expression of muscarinic receptors in rat islets, RINm5F cells and INS-1 cells was established by reverse transcriptase-polymerase chain reaction and quantified by RNase protection. Both methods indicated that M1 and M3 receptors were expressed approximately equally in the various cellular preparations (Lismaa *et al*., 2000). ACh is released from cholinergic synapses on β-cells during the cephalic phase of digestion causing a transient increase in insulin secretion. It has been proposed that ACh activates phospholipid turnover and thereby increases the

intracellular calcium levels. IP_3 mediates Ca^{2+} mobilization from intracellular Ca^{2+} stores and plays an important role in insulin secretion from pancreatic β-cells (Laychock, 1990). Our results showed an increased expression of AchE and decreased expression of ChAT in the pancreas of diabetic rats when compared to control. Treatment with insulin, curcumin and Vitamin D_3 reversed these altered expression to near control. Confocal studies using AchE specific antibodies in isolated pancreatic islets confirmed the results of gene expression studies. Our findings results emphasize the involvement of cholinergic enzyme dysfunction in the pancreas of diabetic animals and point towards the potential of curcumin and Vitamin D_3 as a therapy for treatment of diabetes.

CENTRAL MUSCARINIC RECEPTOR ALTERATIONS

Over the past decade, the role of muscarinic receptors in health was given much importance. Central muscarinic receptors, particularly M1 are involved in higher cognitive processes of learning and memory. Central muscarinic M1 antagonism lead to cognitive dysfunction and other CNS-related adverse events. Muscarinic M1 and M2 knockout mice, both demonstrate cognitive defects (Tzavara *et al*., 2003). The potential therapeutic value of various cholinergic agonists and antagonists has received increasing attention (Zwieten & Doods, 1995; Zwieten *et al*., 1995). Muscarinic receptors are a family of G protein-coupled receptors that have a primary role in central cholinergic neurotransmission. Specific agonists, which activate postsynaptic muscarinic receptors, stimulate cholinergic signaling (Valentin *et al*., 2006). It is known that different parts of the brain, particularly the hypothalamus and the brainstem are important centers involved in the monitoring of glucose status The effect of the cholinergic agonist blocked by the muscarinic antagonist atropine shows the involvement of muscarinic receptors in the central

cholinergic glucose homeostasis. The muscarinic M1 receptor is one of five known muscarinic subtypes in the cholinergic nervous system (Bonner *et al*., 1987; Hulme *et al*., 1990; van Zwieten & Doods, 1995). The muscarinic M1, M2 and M4 subtypes of mAChRs are the predominant receptors in the CNS. These receptors activate a multitude of signaling pathways important for modulating neuronal excitability, synaptic plasticity and feedback regulation of ACh release (Volpivelli *et al*., 2004)

Cerebral cortex

The RT-PCR and HPLC studies revealed that the M1 receptor was present in a relatively high density in the cerebral cortex (Jian *et al*., 1994; Oki *et al*., 2005). It is hypothesized that the cerebral cortex participates in the memory, attention, perceptual awareness, thought, language and consciousness which are necessary for the normal life style. The muscarinic M1, M3 and M5 receptors are located predominantly on postsynaptic nerve terminals and are thought to be responsible for the role of the muscarinic cholinergic system in cognition and long term potentiation in the hippocampus and cortex (Bartus, 2000). Immunoprecipitation and immunofluorescence studies indicate that muscarinic M1 and M3 receptors are expressed in cortex (Levey, 1993).

Binding studies using [^{3}H] QNB and muscarinic general antagonist, atropine revealed that total muscarinic receptors are decreased in the cerebral cortex during diabetic condition. In insulin, curcumin and Vitamin D_3 treated diabetic rats; binding parameters were reversed to near control. In these groups, treatment groups maintained glucose and circulating insulin levels to near control. Central cholinergic neurons participate in the complex neural events responsible for the hyperglycemic response to neurocytoglucopenia and to stressful situations. The hyperglycemia induced by intracerebroventricular 2-deoxyglucose (2-DG) was significantly reduced

by previous intracerebroventricular injection of atropine (Brito *et al*., 2001). Atropine injected into the third cerebral ventricle suppressed epinephrine secretion and dose-dependently inhibited hepatic venous hyperglycemia induced by neostigmine in intact rats (Iguchi *et al*., 1990). The down regulation of muscarinic receptors during diabetes is a compensatory mechanism to facilitate insulin secretion and maintenance of normoglycemia in diabetic rats.

Muscarinic M1 receptor changes during diabetes were studied using subtype specific antagonist, pirenzepine and [^{3}H] QNB. Muscarinic M1 receptors were decreased in diabetic rats, with a decrease in K_d indicating an increase in the affinity of receptors during diabetic state. Also, in STZ- induced diabetes, the mRNA level and binding parameter of muscarinic M3 receptors showed an increase in the cerebral cortex when compared to control. In insulin, curcumin and Vitamin D_3 treated diabetic rats, binding parameters were reversed to near control values. Down regulation of the muscarinic M1 receptor in the central nervous system helps to regulate the NE and EPI secretion which are inhibitory to insulin secretion (Apparsundaram *et al*., 1998). Real Time-PCR analysis also revealed a down regulation of the muscarinic M1 receptor mRNA level during diabetic condition. This is concordant with our receptor binding studies. Immunohistochemistry study using confocal microscope confirmed a similar expression pattern in localization of muscarinic M1 receptor in the cerebral cortex of control and experimental rats. Thus curcumin and Vitamin D_3 treatment contributes to amelioration of progressive cognitive impairment and other neurological dysfunctions associated with cortex seen in diabetes. Earlier reports showed significant alterations in neurotransmitters during hyperglycemia causing degenerative changes in neurons of the CNS (Bhardwaj *et al*., 1990; Garris, 1990). Curcumin and Vitamin D_3 treatment was able to significantly reverse these altered parameters to near the control value. Previous reports showed

that activation of muscarinic M1 cholinergic receptors produced an increase of glucose utilization (Hosey, 1992). Thus, we speculated that curcumin and Vitamin D_3 have ability to modulate muscarinic receptors, thereby ameliorating the impaired cognitive performance shown by STZ- induced diabetes.

Cerebellum

Cerebellum is a region of the brain that plays an important role in the integration of sensory perception, memory consolidation, coordination and motor control. In order to coordinate motor control, there are many neural pathways linking the cerebellum with the cerebral motor cortex and the spinocerebellar tract (Roberta & Peter, 2003). There is currently enough anatomical, physiological and theoretical evidence to support the hypothesis that cerebellum is the region of the brain for learning, basal ganglia for reinforcement learning and cerebral cortex for unsupervised learning (Doya, 1999). The cellular basis of motor learning has been mostly attributed to long term depression (LTD) at excitatory parallel fiber - purkinje cell synapses. LTD is induced when parallel fibers are activated in conjunction with a climbing fiber, the other excitatory input to Purkinje cells. Recently, by using whole-cell patch-clamp recording from Purkinje cells in cerebellar slices, a new form of synaptic plasticity was discovered.

Gene expression studies showed that the mRNA level of muscarinic M1 and M3 receptors in the cerebellum of diabetic rats substantially increased when compared to control. Binding parameters B_{max} of total muscarinic, muscarinic M1 and M3 receptors were increased in diabetic rats compared to control. Earlier reports showed significant alterations in neurotransmitters during hyperglycaemia and causes degenerative changes in neurons of the central nervous system (Garris, 1990; Lackovic *et al*., 1990; Bhardwaj *et al.,* 1999). Cerebellum participates in the learning

and coordination of anticipatory operations which are necessary for the effective and timely directing of cognitive and non-cognitive resources (Allen *et al*., 1997). The current study revealed the modulatory function of insulin, curcumin and Vitamin D_3 on total muscarinic, muscarinic M1 and M3 receptors by normalising the altered receptor gene expression and binding parameters to near control. Immunohistochemical analysis confirmed the result of mRNA expression and binding parameters. The cerebellum has generally been suggested to be involved in the control and integration of motor processes, as well as cognitive functions. In the current study, we observed the neuroprotective effect of curcumin and Vitamin D_3 on muscarinic receptors and muscarinic M1 and M3 receptor subtypes in cerebellum, which is responsible for the coordination of voluntary motor movement, balance and equilibrium and declarative memory.

Brain stem

The 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. The brain stem controls involuntary muscles such as the stomach and the heart. The brain stem also acts as a relay station between the brain and the rest of the body. 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). These reticular functions originate from interacting neuronal groups in the brain stem, including cholinergic, adrenergic and serotoninergic neurons (Steriade, 1996). Brain stem along with hypothalamus serves as the key centre of the central nervous system regulating the body homeostasis. 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).

The total muscarinic receptors of the brainstem are found to be increased during diabetic condition. Muscarinic M1 receptors are decreased and muscarinic M3 receptors are increased during diabetic state. In insulin treated, curcumin and Vitamin D_3 treated diabetic rats, binding parameters were reversed back to near control values.

The dorsal motor nucleus of the vagus nerve is located in the brain stem. It is connected to the endocrine pancreas exclusively *via* vagal fibres and has a role in neurally mediated insulin release. Nucleus ambiguus stimulation reported to increase plasma insulin levels in rats (Bereiter *et al*., 1981). RT-PCR analysis also revealed a down regulation of the muscarinic M1 receptor mRNA level during diabetic condition. This is in accordance with our receptor binding studies. Also confocal studies using specific antibodies of muscarinic M1 and M3 brainstem confirmed the Real time PCR and Scatchard analysis. The brain stem provides the main motor and sensory innervation *via* the cranial nerves. Muscarinic alterations in brainstem during diabetes result in memory problems, difficulty concentrating, difficulty staying focused and physical defects including the inability to walk, remain balanced, and a loss of strength. Our results showed that curcumin and Vitamin D_3 restored the altered muscarinic functions associated with brainstem.

Corpus striatum

Densities of muscarinic M1 receptor subtype were highest in the corpus striatum (Oki *et al*., 2005). The corpus striatum is the largest component of the basal ganglia. Cholinergic terminals within the striatum contain presynaptic muscarinic

receptors that inhibit neurotransmitter release (Chesselet, 1984). Various anatomical, electrophysiological and pathological observations provide evidence that ACh plays a major role in the control of striatal function and in the regulation of motor control (Jabbari *et al*., 1989). Striatal ACh is released from a population of large cholinergic interneurons that establish complex synaptic contacts with dopamine terminals, originating from the substantia nigra and with several striatal neuronal populations (Lehmann & Langer, 1982, 1983; Wainer *et al*., 1984; Phelps *et al*., 1985; Izzo & Bolam, 1988; Vuillet *et al*., 1992). Corpus striatum regulates endocrine functions indirectly through the secretion of other hormones like thyroxine. Scatchard analysis of total muscarinic receptors revealed a decreased B_{max} in corpus striatum during diabetic condition. In insulin, curcumin and Vitamin D_3 treated diabetic rats, binding parameters were reversed to near control values. Muscarinic M1 receptors were increased and muscarinic M3 receptors decreased during diabetic state. Supplementation of insulin, curcumin and Vitamin D_3 to diabetic rats reversed the binding parameters to near control . mRNA level revealed an up regulation of the muscarinic M1 receptor and down regulation of M3 receptor during diabetic condition. The results of confocal studies confirmed the alterations of muscarinic M1 and M3 receptor at protein level. CNS mAChRs regulate a large number of important central functions including cognitive, behavioural, sensory, motor and autonomic processes (Wess, 1996; Felder *et al*., 2000; Eglen, 2005). The present study suggests that drugs that can selectively activate muscarinic receptors are of significant therapeutic benefit in the diabetes management. Thus our results revealed the significance of central muscarinic receptor changes during diabetes and the regulatory role of curcumin and Vitamin D_3 on muscarinic receptors in corpus striatum.

Hippocampus

Uncontrolled diabetes mellitus leads to severe complications of the peripheral and central nervous system. In addition to the well-known peripheral neuropathy, data from epidemiologic studies confirm that diabetes is a risk factor for brain aging, stroke, cerebrovascular diseases and Alzheimer's disease (Gispen & Biessels, 2000; Biessels *et al*., 2002). Deterioration of cognitive functions is also present in humans with type I diabetes (Gold *et al*., 1994). More than 20 neurodegenerative diseases are associated with diabetes mellitus in humans. These associations reflect direct effect of hyperglycemia on the brain, or of the diabetes-associated comorbidities of hypertension, dyslipidemia, or hyperinsulinemia (Makimattila *et al*., 2004). Pronounced pathological changes also characterize the brain of diabetic animals, particularly the hippocampus. There is damage to presynaptic and postsynaptic structures, dysregulation of Ca^{2+} homeostasis, neuronal loss, dendritic atrophy in CA3 neurons, reduced expression of insulin growth factors and their receptors and decreased neurogenesis (Jackson-Guilford *et al*., 2000; Saravia *et al*., 2004). In the hippocampus of diabetic rats, our results showed that total muscarinic, muscarinic M1 receptors binding parameters, B_{max} was decreased and muscarinic M3 receptors were increased in diabetic rats compared to control.

mRNA expression showed down regulation of M1 receptor and up regulation of M3 receptor in the hippocampus of diabetic rats. This suggests an impaired muscarinic receptor function in the hippocampus leading to deficits in cognitive performance and long term memory formation in diabetic rats. In correspondence with the hippocampal neuropathology, diabetic animals showed reduced learning and memory deficits (Gispen & Biessels, 2000). A recent report has pointed out an association between memory alterations of diabetic rodents and the decrease of

neuronal proliferation in the dentate gyrus (Jackson-Guilford *et al*., 2000). In dentate gyrus, as well as the subventricular zone (SVZ), neurogenesis continues throughout adulthood (Taupin & Gage, 2002).

Insulin, curcumin and Vitamin D_3 supplementation reversed the altered parameters to near control. Immunohistochemistry studies using confocal microscope confirmed the results of binding parameters and gene expression. In line with this, we suggest that regulation of muscarinic receptor function by curcumin and Vitamin D_3 contribute consequently to improve the cognitive functions, such as learning and memory.

Hypothalamus

Hypothalamus is the centre involved in the neuroendocrine regulation. It is the region of the central nervous system where the autonomic and endocrine systems are integrated. Hypothalamic paraventricular nucleus serves as the major neuroendocrine and autonomic output centre. Specialized subgroups of hypothalamic neurons exhibit specific excitatory or inhibitory electrical responses to changes in extracellular levels of glucose (Burdakov *et al*., 2005). Hypothalamic centers involved in the regulation of energy balance and endogenous glucose production constantly sense fuel availability by receiving and integrating inputs from circulating nutrients and hormones such as insulin and leptin. In response to these peripheral signals, the hypothalamus sends out efferent impulses that restrain food intake and endogenous glucose production. This promotes energy homeostasis and keeps blood glucose levels in the normal range. Disruption of this intricate neural control is likely to occur in type 2 diabetes and obesity which contribute to defects of glucose homeostasis and insulin resistance common to both diseases (Demuro & Obici, 2006).

The cholinergic glucoregulatory hippocampal activity transmitted to peripheral organs *via* the ventromedial hypothalamus (Iguchi *et al*., 1992). The ventromedial hypothalamus (VMH), lateral hypothalamus, paraventricular hypothalamus and median site of the lateral preoptic area are involved in increasing the plasma glucose and epinephrine levels (Honmura *et al*., 1992). The muscarinic antagonist atropine suppressed the hyperglycemia induced by administration of neostigmine in a dose-dependent manner, suggesting the involvement of muscarinic receptors of the VMH in the glucoregulation (Iguchi *et al*., 1991).

Gene expression of muscarinic M1 receptor was down regulated and muscarinic M3 receptor was up regulated in diabetic rats compared to control. Previous studies demonstrated that the distribution of mRNA of muscarinic receptor generally parallels with the distribution of their protein. These alterations in muscarinic transmission suggested impairing neuroendocrine function which includes disturbed secretion of pituitary hormones, notably growth hormone and cortisol, which, by impairing tissue sensitivity to insulin, contribute to poor metabolic control in diabetes. In insulin, curcumin and Vitamin D_3 treated diabetic rats the altered mRNA levels of muscarinic M1 and M3 receptors were reversed to near control. The ventromedial hypothalamus, lateral hypothalamus, paraventricular hypothalamus, and median site of the lateral-preoptic are involved in increasing the plasma levels of glucose and epinephrine by cholinergic stimulation (Honmura *et al*., 1992). These results unravelled the therapeutic effect of curcumin and Vitamin D3 supplementation on regulating hypothalamus mediated metabolic processes, secretion of neurohormones, secretion of pituitary hormones, control of body temperature, hunger, thirst fatigue and circadian cycles and other activities of the autonomic nervous system.

MUSCARINIC RECEPTORS AND VESICULAR ACETYLCHOLINE TRANSPORTER ALTERATIONS IN THE PANCREAS

The autonomic nervous system plays an important role in the insulin release. Physiological insulin secretion is initiated by glucose and augmented by nervous and humoral systems (Ahren *et al*., 1986). The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Neurotransmitters are stored within the terminals of these nerves, both acetylcholine and noradrenalin and several neuropeptides. Expression of muscarinic receptors in rat islets was established by reverse transcriptase-polymerase chain reaction and quantified by RNase protection. Both methods indicated that muscarinic M1 and M3 receptors were expressed approximately equally in the various cellular preparations (Lismaa *et al*., 2000).

Stimulation of the autonomic nerves and treatment with neurotransmitters affect islet hormone secretion. Insulin secretion is stimulated by parasympathetic nerves and inhibited by sympathetic nerves (Ahren, 2000). Acetylcholine mediates insulin release through vagal stimulation. Acetylcholine acts through the activation of Gq-phospholipase C. It stimulates Ca^{2+} influx through the voltage dependent L-type Ca^{2+} channel that is primarily activated by glucose. Studies showed that muscarinic M1 and M3 are the major muscarinic receptors present in the pancreas (Lismaa *et al*., 2000). During diabetic condition, total muscarinic, muscarinic M1 and M3 receptor binding parameters decreased when compared to control. Gene expression studies also showed the down regulation of muscarinic receptors in diabetic rats. Insulin, curcumin and Vitamin D_3 treatment reversed the binding parameters to near control.

Localization of muscarinic M1, M3 receptors and vesicular acetylcholine transporter using confocal laser scanning microscopy showed a decreased mean pixel value in pancreatic islets of diabetic rats when compared to control. Administration of

choline to rats elevates serum insulin. Pretreatment with a peripheral muscarinic acetylcholine receptor antagonist, atropine methylnitrate blocked the choline-induced increase in blood insulin. The increase in serum insulin elicited by choline was prevented by pretreatment with the M1 antagonist, pirenzepine, or the muscarinic M1 and M3 antagonist, 4-DAMP. Pretreatment with an antagonist of ganglionic nicotinic acetylcholine receptors, hexamethonium, prevented the choline-induced increase in serum insulin. Choline increased the acetylcholine content of the pancreas and enhanced acetylcholine release from minced pancreas, which suggests that choline stimulates insulin secretion indirectly by enhancing acetylcholine synthesis and release (Ilcol *et al*., 2003).

Muscarinic M3 receptors appears to be the predominant subtype expressed by pancreatic β-cells (Gilon & Henquin, 2001; Lismaa *et al*., 2000). Earlier study demonstrated that muscarinic stimulation of pancreatic insulin and glucagon release is mediated by the M3 muscarinic receptor subtype (Duttaroy *et al*., 2004). Immunocytochemistry analysis in pancreas showed an increased expression of muscarinic M3 receptor in insulin, curcumin and Vitamin D_3 treated diabetic rats. An improvement in insulin secretion and response to an intravenous glucose tolerance test has also been seen with Vitamin D_3 replacement in Vitamin D deficient rabbits (Nyomba *et al*., 1984). In individuals with diabetes mellitus, Vitamin D treatment increased insulin secretion and improved glucose tolerance (Rudnicki & Molsted-Pedersen, 1997). Our result showed that Vitamin D_3 supplementation plays a pivotal role in regulating muscarinic M3 receptor expression through the VDR present in the pancreas and thereby enhancing the insulin synthesis and secretion. Thus our results demonstrate a possible mechanism of reducing the neuronal disorders in diabetes with Vitamin D_3 supplementation thereby mediating potential therapeutic effect through muscarinic M3 receptors in pancreas (Peeyush *et al*., 2010). Previous reports showed

that curcumin has the potential to protect pancreatic islets cells against streptozotocin-induced death dysfunction (Meghana *et al*., 2007) and increase plasma insulin level in diabetic mice (Seo *et al*., 2008). Thus it is suggested that curcumin supplementation ameliorated the decreased muscarinic receptor function and acetylcholine transport in pancreatic islets of diabetic rats. In insulin, curcumin and Vitamin D_3 treated diabetic rats, muscarinic M1, M3 receptor function and acetylcholine transport reversed to near control. The present findings showed the potential anti-diabetic effect of curcumin and Vitamin D_3.

α7 nicotinic receptor gene expression in control and experimental rats

The search for potential targets for a treatment of neurodegenerative diseases associated with cholinergic deficits has led to an increasing interest in nicotinic acetylcholine receptors (nAChR). The number of the different nAChR subunits and their possible combinations forming different receptor subtypes explain the individual anatomical distribution, the electrophysiological and pharmacological diversity as well as the variable effects of nAChR agonists and antagonists. Several lines of evidence suggest that the α7 nAChR is an important pharmacological target for the treatment of cognitive deficits. (Levin *et al*., 2006). Furthermore, the α7 nAChR agonist GTS-21 improves attentional function in patients with schizophrenia (Olincy *et al*., 2006; Freedman *et al*., 2008) and α7 nAChR agonists have been shown to improve performance on a variety of cognitive tests related to working, short-term and long- term memory function in animal models (Bitner *et al*., 2007; Boess *et al*., 2007; Hashimoto *et al*., 2008). However, the involvement of the α7 nAChR in the prevention of cognitive deficits in diabetes has not been addressed. The major aim of the study was to further explore whether diabetes is related to α7 nAChR modulation

in brain regions also to learn the neuroprotective role of curcumin and Vitamin D_3 treatment in restoring the changes.

In the brain, nicotinic receptors include several subtypes with differing properties and functions. The abundant presence of α7 nAChR's in the hippocampus, neocortex and basal ganglia (Clarke *et al.,* 1985), in conjunction with the memory-enhancing activity of selective α7 nicotinic agonists such as DMXB (Meyer *et al.,* 1997), suggests a significant role for α7 nAChR's in learning and memory. In addition, the protective action of nicotine is mediated, at least partially, through α7 nACh receptors. Our results showed an increased expression of α7 nAChR in cerebral cortex, cerebellum, brain stem, corpus striatum and decreased expression in hippocampus of diabetic rats compared to control. Confocal studies using specific antibody for α7 nAChR confirmed the mRNA expression in cerebral cortex, cerebellum, brainstem corpus striatum and hippocampus in diabetic rats. The α7 nAChR subunit has been linked to inhibit neuronal function in the hippocampus by several lines of investigation (Frazier *et al.,* 1998; Freedman *et al.,* 1999). In addition to this role in inhibitory neuronal function, the α7 nAChR has also been proposed to have a developmental role. The receptor is expressed by hippocampal neurons as soon as they have formed from the neuroepithelium (Adams *et al*., 1999). Because the receptor admits Ca^{2+} into the neuron (Vijayaraghavan *et al.,* 1992), it has the ability to affect neuronal migration (Komuro & Rakic, 1996) as well as other developmental functions such as apoptosis (Sastry & Rao, 2000). In line with this, α7 nAChR functional difference in diabetes is suggested to be one of the major factor causing memory and behavioral deficit.

Curcumin and Vitamin D_3 treatment significantly reversed the altered changes in the brain regions of diabetic rats to near control while insulin treatment did not show any significant reversal other than in corpus striatum. Neuronal nicotinic

acetylcholine receptors are crucial to acetylcholine neurotransmission in both the CNS and autonomic nervous system. However, in the CNS, these receptors are more often associated with modulation of release of several neurotransmitters including dopamine, norepinephrine, GABA and glutamate (Wonnacott, 1997; Girod and Role, 2001). Thus, these receptors significantly influence the activity within the CNS and deregulation of this activity contribute to diabetes mellitus associated disorders involving the CNS. Abnormalities of nAChR function in the hippocampus lead to cognitive and memory impairments (Levin *et al*., 2002; Green *et al*., 2005) and sensory gating deficits (Adler *et al*., 1998). Curcumin and Vitamin D_3 supplementation was found to be more effective in reversing the altered gene expression to near control stage.

CENTRAL DOPAMINERGIC RECEPTOR ALTERATIONS

Cerebral cortex

Diabetes is considered to be one of the most psychologically demanding chronic medical illnesses and is often associated with several psychiatric disorders (deGroot *et al*., 2001). Although the mechanism responsible for cognitive deficits in stress-related neuropsychiatric disorders has been obscure, prefrontal cortical (PFC) dopaminergic dysfunction is thought to be involved. In animals, the mesoprefrontal dopaminergic system is particularly vulnerable to stress. Prefrontal cortex is a cortical area involved in selecting and retaining information to produce complex behaviours (Arianna *et al*., 2007). Our results showed an up regulation of total dopamine receptors accompanied with decrease in its affinity in the cerebral cortex of diabetic rats. The dopamine neurons projecting to the prefrontal cortex are thought to be involved in various motor and behavioural functions (Tam & Roth, 1997). This

increased number of dopamine receptors could account for the behavioural supersensitivity to dopamine agonist as a result of damage in the dopamine functions (Cresse *et al*., 1977).

Dopamine D_1 receptors are located postsynaptically on the cortical neurons (Tassin *et al*., 1978, 1982) and the decreased dopamine level in the PFC induced by electrolytic lesion up regulates the dopamine D_1 receptor density in the PFC (Tassin *et al*., 1982). The mesoprefrontal dopaminergic system is particularly vulnerable to stress (Abercrombie *et al*., 1989) and that an over stimulation of dopamine D_1 receptor in the PFC impairs the working memory (Zahrt *et al*., 1997). We observed an increase in dopamine D_1 receptors mRNA level in the cerebral cortex of diabetic rats when compared to control. Excessive dopamine D1 receptor stimulation is sufficient to produce marked PFC dysfunction. Stress impairs PFC cognitive function through a hyperdopaminergic mechanism. It is reported that chronic stress induced depressive state is caused by a dopamine D1 receptor mediated hypodopaminergic mechanism in the PFC (Mizoguchi *et al*., 2002). Thus excessive cortical dopamine D1 receptor density with decreased dopamine is suggested to be the cause for cortical dysfunction during diabetes.

However, the finding that DA D1 receptor stimulation alone is sufficient to induce PFC dysfunction does not rule out an additional role for DA D2 receptors. Cognitive deficits induced by either stress exposure or ketamine (Verma & Moghaddam, 1996) is blocked by selective DA D2 receptor antagonists. These findings suggest that both DA D1 and DA D2 receptor families contribute to the detrimental actions of dopamine in the PFC and that the two may synergize to take the PFC "off-line" during stress. We observed that DA D2 receptors also increased significantly in the cerebral cortex of diabetic rats.

Stimulation of DA D_1/D_2 receptors under DA depleted conditions cause a

subtle impairment in spatial working memory performance (Ellis *et al*., *2005*). Dopaminergic neurotransmission is critically involved in many aspects of complex behaviour and cognition beyond reward/reinforcement and motor function. Our results showed that treatment with insulin, curcumin and Vitamin D_3 reversed the increased dopamine receptor expression in diabetic rats to near control. Previous findings suggest the antidepressant-like effects of curcumin involve the central monoaminergic neurotransmitter systems.(Ying Xu *et al*., 2005) Vitamin D help to protect against cognitive deterioration and dementia, specifically, vascular dementia and Alzheimer's disease, through vasculoprotection (Lind *et al*., 1987; Pfeifer *et al*., 2001; Wang *et al*., 2001; Zittermann *et al*., 2003; Wang *et al*., 2008a,b), preservation of neurons (Sutherland *et al*., 1992; Landfield & Cadwallader-Neal, 1998; Brewer *et al*., 2001) and protection against risk factors for cognitive dysfunction (Lind *et al*., 1987; Zittermann *et al*., 2003; Bischoff-Ferrari *et al*., 2004). Thus, curcumin and Vitamin D_3 treatment exerted antidepressant-like effect and reduce stress by normalising the increased expression of dopamine receptors in cerebral cortex.

Cerebellum

Dopamine is the predominant catecholamine neurotransmitter in the mammalian brain, where it controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake and endocrine regulation. This catecholamine also plays multiple roles in the periphery as a modulator of cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function and gastrointestinal motility (Missale *et al*., 1998). Dopamine receptors are reported to be increased in diabetes causing significant alterations in central dopaminergic system (Lozovsky *et al*., 1981). Our results showed that total dopaminergic receptor binding parameters were decreased in the

cerebellum, which is responsible for the coordination of voluntary motor movement, balance, equilibrium and declarative memory. The decreased dopamine receptor density in the cerebellum of diabetic rats when compared to control indicates an imbalance in dopaminergic neural transmission. Furthermore, many behavioral studies have shown evidence that the dopamine system plays an important role in regulating exploratory and locomotor behavior (Fink & Smith, 1979; Funada *et al*., 1994). The current data reveal a significant reversal of this altered binding parameter to near control in insulin, curcumin and Vitamin D_3 treatment. Diabetes mellitus has been reported to be accompanied by a number of behavioural and hormonal abnormalities, including reduced locomotor activity (Marshall *et al*., 1976). The present experiments further revealed the effect of curcumin and Vitamin D_3 to modulate the dopaminergic receptors in the cerebellum by standardising the altered expression near to a normal level.

Dopamine D_1 receptors are highly expressed in basal ganglia followed by cerebral cortex, hypothalamus and thalamus. The gene expression studies of DA D1 receptors showed a decrease in the cerebellum of diabetic rats which confirm and extend our observations of total dopamine receptors. DA D1 receptor seems to mediate important actions of dopamine to control movement, cognitive function and cardiovascular function. The DA D_1 receptors in the brain are linked to episodic memory, emotion, and cognition. Diabetes mellitus has been reported to cause degenerative changes in neurons of the CNS (Bhattacharya & Saraswathi, 1991; Garris, 1990., Lackovic *et al*., 1990). Haloperidol and SCH23390, a selective dopamine D1 receptor antagonist, significantly reduced spontaneous locomotor activity in diabetic mice, but not in nondiabetic mice (Kamei *et al*., 1994). Our study showed that diabetes regulate the expression of DA D1 receptor which reduce the cerebellar function. In our study, insulin, curcumin and Vitamin D_3 increased the

dopamine D1 receptor expression levels in the cerebellum, which suggests that the curcumin supplementation modulated the functional regulation of these receptors to maintain normal dopaminergic function and this is involved as a mechanism for preventing cerebellar dysfunctions. Such interference with the dopaminergic system could explain, at least in part, the ameliorative effect of curcumin and Vitamin D_3 on CNS.

The interest in learning DA D2 receptor expression begins with the hypothesis that DA D2 receptors are involved in the pathophysiology of schizophrenia and in the mechanism of antipsychotic drug action (de Paulis, 2003). Thus, our findings bring attention to the cerebellum as a possible site of dysfunction in diseases like diabetes mellitus. To examine whether DA D2 receptors are altered in diabetes, we examined the expression of DA D2 in the cerebellum, to which dopaminergic neurons project, and are related to memory, attention, perceptual awareness, thought, language, consciousness and motor function. The present study showed that DA D2 receptors expression of cerebellum in diabetic rats were up regulated when compared to control. These results indicate an alteration of the dopaminergic function in diabetes, because it is known that dopamine is a principal modulator of higher functions including attention, working memory [Castellano *et al*., 1999] and motor control (Zhou & Palmiter, 1995). The increase in the central dopaminergic postsynaptic receptors has been related to decrease the locomotor and ambulatory activity in STZ-induced diabetic rats (Kobayashi & Shigeta, 1990; Shimomura *et al*., 1990). It was reported that injection of DA D2 agonist into lobules 9 and 10 of the cerebellum, induced balance and motor coordination disturbances in the rotarod test (Kolasiewicz & Maj 2001). It was observed that insulin, curcumin and vitamin D_3 reversed the adverse effects of diabetes on DA D2 receptors in the cerebellum to near control level.

Brainstem

Brainstem is an important part of the brain in monitoring the glucose status and the regulation of feeding (Guilford *et al*., 2000). When glucose levels were lowered to 2.8 mmol/l, brain function was impaired in nondiabetic rats as well. Our results showed an increased binding of total dopamine receptors with decreased affinity in the brainstem of diabetic rats compared to control. Our previous studies demonstrated adrenergic, serotonergic and DA D_2 receptor function alterations in the brainstem of diabetic rats (Abraham & Paulose, 1999; Padayatti & Paulose, 1999; Paulose *et al*., 1999; Eswar *et al*., 2007). In diabetic condition, DA D_1 receptors gene expression was up regulated in the brain stem. Gene expression studies using Real-Time PCR showed that DA D_2 receptors significantly down regulated in the brainstem of diabetic rats. Treatment with insulin, curcumin and Vitamin D_3 reversed the increased binding parameters of dopamine and altered gene expression of DA D1 and DA D2 in the brain stem. From our data we suggest that there is increased activation of sympathetic stimulation during diabetes as a result of increased NE and EPI (Tassava, *et al.,* 1992; Jackson, *et al*., 1997; Jackson & Paulose,; 1999) is because of decreased dopamine content in the brainstem with an up regulation of DA D_1 receptors and down regulation of DA D_2 receptors. In the brainstem there was a decrease in the expression of DA D_2 receptor mRNA as a result of diabetes. It has been reported that damages in the brain cause alterations in the expression of the DA D_{2L} isoform which is expressed in the *in vivo* condition (Neve *et al*., 1991; Snyder *et al*., 1991).

Modest reductions in plasma glucose to 3mM produce marked alterations in brainstem responses to auditory stimuli. Adverse effects of hyperglycemia on brain function are not limited to higher centers but also involve the brainstem (Jones *et al.*,

1990). We observed an up regulation of DA D_1 receptors and down regulation of DA D_2 receptors in the brainstem of diabetic rats. These results indicate that the dopaminergic activity in the brainstem altered in hyperglycaemic rats impairing dopamine related functions of brainstem. Earlier studies reported that brainstem is universally spared in hypoglycaemic brain damage (Auer, 2004). Our results showed a prominent dopaminergic functional improvement with curcumin and Vitamin D_3 supplementation in the brainstem of diabetic rats.

Corpus striatum

Striatal dopamine receptors were markedly decreased with increased affinity during diabetes with the depletion of dopamine in the striatum and an increased HVA metabolism. Striatal dopamine firing during diabetes is decreased affecting dopaminergic functions (Saller, 1984). The decreased dopamine receptor density during diabetes is related to the decreased locomotor activity in STZ-induced diabetic rats (Kobayashi *et al*., 1990; Shimomura *et al*., 1990). This finding correlates with our present data suggesting that the disturbances in the central dopaminergic receptors during STZ- induced diabetes affects dopamine related functions. The firing of dopamine neurons projecting from the substantia nigra to the striatum is reported to be rapidly suppressed by hyperglycaemia leading to the hypofunction of dopamine receptors (Saller, 1984). There are hypothesis that suggests activities related to the functional capacities of dopamine receptors like stereotypy, ambulation, behaviour are diminished due to hyperglycaemia (Lozovsky *et al*., 1981). Also, a decrease in dopamine receptors during diabetes results in hyporesponsiveness (Saitoh *et al*., 1998). In diabetic rats we observed a significant increase in striatal total dopamine receptors which is a compensatory response to decreased dopamine content. The

insulin, curcumin and Vitamin D_3 supplementation significantly modulates the altered binding parameters of dopamine receptors in the striatum to near control.

Real-Time PCR analysis showed a decreased expression of DA D_1 receptors in the striatum of diabetic rats. This correlates with previous reports that DA D_1 receptor density decreased in the striatum of alloxan induced diabetic rats (Salkovic & Lackovic, 1992). DA D_1 stimulated cAMP production was markedly increased in diabetic rats, whereas ability of DA D_2 receptor action to reduce cAMP formation was almost abolished during diabetes (Abbracchio *et al*., 1989). An imbalance between G_s -proteins and G_i/G_o protein mediated efficacy of G_s activity as a result of the loss of G_i/G_o inhibitory functions has been found in the striatum and other tissues of diabetic animals (Salkovic & Lackovic, 1992). Dopamine through its DA D_1 receptor stimulates adenylyl cyclase and inhibits adenylyl cyclase activity through its DA D_2 receptors. Decreased DA D_1 receptors expression during diabetes that we observed in the striatum is a major cause in affecting dopamine related functions. It has been suggested that curcumin and Vitamin D_3 reversed the effects of diabetes on DA D1 receptors in the brainstem and this is involved as a mechanism of preventing dopamine related functions in brainstem.

Gene expression studies showed that DA D2 receptors up regulated in diabetic rats compared to control. Insulin, curcumin and Vitamin D_3 treatment reversed the increased expression to near control. Previously [^{3}H] spiroperidol binding to DA D_2 receptors have been reported to be increased during diabetes (Trulson & Himmel, 1983). Striatal DA D_2 receptor primarly represents a population of dopamine D_2 sites (Marzella *et al*., 1997). During diabetes it has been documented that the sensitization of these receptors and their increased number results in a decreased locomotory and ambulatory activity (Kobayashi & Shigeta, 1990; Shimomura *et al*., 1990). DA D_2 receptor gene expression increased in the striatum during diabetes as a

result of the decreased transmission of dopamine. Hyperglycaemia depressed the dopaminergic function. Therefore a decreased dopaminergic activity is suggested to increase the DA D_2 receptors. A lesion in the striatum is reported to increase the expression of DA D_{2L} receptor gene (Zhang *et al*., 1994). *In vivo* release of dopamine from mesolimbic and neostriatal dopamine neurons appears to be modulated by DA D_2 but not by DA D_1 receptors, whereas both receptor types modulate dopamine metabolism (Boyar & Altar, 1987). DA D_2 receptors are reported to regulate the release of dopamine from dopaminergic neurons originating in the ventral tegmental area as well as in the substantia nigra (Plantje *et al*., 1987). The two dopamine receptor subtypes interact in a synergistic way to adapt to the alterations in glucose metabolism. The insulin, curcumin and Vitamin D_3 treatment regularise the imbalanced DA receptor functions in the corpus striatum.

Hippocampus

Previous reports suggest that, in both insulin-deficient rats and insulin-resistant mice, diabetes impairs hippocampus-dependent memory and learning, perforant path synaptic plasticity and adult neurogenesis (Alexis *et al*., 2008). The hippocampal formation receives a dopamine input from different midbrain groups and a more prominent dopamine input into the temporal pole of hippocampus (Hortnagl *et al*., 1991). The hippocampus has long been known to be important for memory function. It is reported that profound hypoglycaemia selectively damages CA1 and the dentate gyrus of the hippocampus (Tasker *et al*., 1992). The dopaminergic system is a strong candidate for mediating novelty acquisition and synaptic plasticity in CA1. We observed a significant up regulation of dopamine receptors in the hippocampus of diabetic rats. Our data suggest that the impairment in glucose metabolism caused up regulation of hippocampal dopamine receptors. Treatment using insulin, curcumin and

Vitamin D_3 reversed the increased binding of total dopamine receptor in the hippocampus of diabetic rats to near control.

The characterizations of neuronal populations expressing dopamine receptor subtypes in the hippocampus have shown a prominent labeling of DA D_1 receptors in dentate gyrus and subicular complex (Fremeau *et al*., 1991). Yokoyama (1995) demonstrated widespread distribution of DA D_2 like receptor in the hippocampus. DA D_2 receptors in the ventral hippocampus were shown to have important influences on spatial working memory (Wilkerson & Levin, 1999). DA D_2 receptor plays a role in hippocampal memory function (Hiroshige *et al*., 2005). An intact mesocortical dopaminergic input to the PFC has been reported to be necessary for long-term potentiation to occur at hippocampal-prefrontal cortex synapses. Earlier studies suggest that DA D_1 but not DA D_2 receptors are crucial for the dopamine control of the NMDA receptor-mediated synaptic response on a specific excitatory input to the PFC. The interactions of these receptors play a crucial role in the storage and transfer of hippocampal information in the PFC. Real-Time PCR analysis showed an increased expression of DA D_1 and DA D_2 receptors in the hippocampus of diabetic rats compared to control. The increase in dopamine receptor sensitivity is a compensatory response to diminished firing of dopamine. Insulin curcumin and vitamin D_3 treatment reversed the increased expression to near control. These findings suggest that the neuroprotective effects of curcumin in hippocampus involve the central monoaminergic neurotransmitter systems (Xu *et al*., 2005b). Recently it was shown that the VDR was distributed throughout rat hippocampus (Langub *et al*., 2001). Earlier reports have shown that Vitamin D_3 acts as a potent differentiation agent in rat hippocampal cultures as assessed by a reduction in mitosis and increased neurite outgrowth. In addition, vitamin D_3 induces NGF, a neurotrophin.

Hyperglycemia markedly affects hippocampally dependent spatial working memory task (McNay *et al*., 2006). DA D_1 and D_2 receptors are generally considered to exert opposite effects at the cellular level, but many behavioural studies find an apparent cooperative effect of DA D_1 and DA D_2 receptors in the nucleus accumbens. Opposing influences of DA D_1 and DA D_2 receptor activation on cAMP-dependent signaling have been reported in many studies (Kebabian & Calne, 1979; Missale *et al*., 1998), with DA D_1 receptors acting through the stimulatory G_s-like G_{olf}, and D_2 receptors acting through the inhibitory $G_{i/o}$ proteins. Hopf *et al*., (2003) reported that cooperative action of DA D_1 and DA D_2 receptors in the brain mediate dopamine-dependent behaviours. Recent studies explains that stimulation of DA D_1 and DA D_2 dopamine receptors has the potential to give rise to different intracellular signals depending on whether DA D_1 or DA D_2 receptors are activated alone or together (Pollack, 2004). Thus our results suggest that the co activation of DA D_1 and DA D_2 receptors with dopamine depletion have particular relevance in the impairment of glucose metabolism and dopamine related functions. Also, co-activation of DA D_1 and DA D_2 receptors is reported to enhance glutamate mediated cellular excitation (Hopf *et al*., 2003). The hippocampal cell populations in particular are important for learning and memory and impairment of cognitive abilities and neuronal damage in diabetes is ameliorated by curcumin and Vitamin D_3 treatment.

Hypothalamus

Dopaminergic action is important in the regulation of the hypothalamic-pituitary hormone release. Also, DA and its receptors are implicated in the satiety, hunger and body weight maintenance. The central vagal connection with dopaminergic innervation is reported to reach the pancreatic islets through the parahypothalamic ventricular (PHV) nucleus while aderenergic and serotonergic

innervations reach the pancreas through the brain stem (Smith & Davis, 1983). Altered DA is reported to affect the feeding pattern, as food intake is accompanied by DA release which differs significantly in the hypothalamus of obese and lean Zucker rats. The reduction in DA, NE and EPI levels in the hypothalamus suggests a low metabolism of monoamines (Bellush & Henley, 1990). They are responsible for the development of thermoregulatory deficits when exposed to cold environment (Leu, *et al*., 1986).

Our studies in the hypothalamus suggest that DA D1 receptor expression decreased during diabetes. An alteration in the sensitivity of the receptors during diabetes has been previously reported causing a difference in the modulation of innervating DA systems. Dopamine D1-like but not DA D2-like receptor antagonism in the LH attenuated taste avoidance learning (Fenu *et al*., 2001).The nucleus paraventricularis of the hypothalamus is regarded as an important region of the brain, operating as a neuronal interface between various brain structures and hormonal systems (Hoebel *et al*., 1989; Armstrong, 1995). There are several anatomical and functional evidences that DA and its receptors in the PVN might constitute an important afferent system controlling the activity of PVN neurons and subsequent release of hormones. Specifically, the PVN receives dopaminergic innervation from two major sources: the dorsal periventricular nucleus (group A14) and the zona incerta (group A13) (Cheung *et al*., 1998; Wagner *et al*., 1995). There are also evidences indicating that agonists of dopaminergic receptors might modulate the activity of PVN neurons and subsequent release of hormones such as TSH (Andersson, 1989). DA D1 receptors are localized in PVN neurons, which are activated tonically by neurotransmitters operating *via* receptors that elevate intracellular concentration of cAMP and CA 21 (Gonzalez, 1989), Colocalization of DA D1 receptor protein with pCREB may also, at the anatomical level, suggest that

dopamine, *via* the DA D1 receptors, may control not only the release of PVN hormones, but also the transcriptional activity of their genes. Diabetes associated impaired hypothalamic functions such as neuroendocrine regulation and memory processing through CREB is due to the altered expression of dopaminergic receptors is suggested. Insulin, curcumin and Vitamin D_3 treatment normalized the decreased expression to near control values thus proposing a potential nutritional value in managing diabetes.

Gene expression studies showed that DA D2 receptor down regulated in diabetic rats compared to control. The regional difference in the receptor status is relevant to the role which DA plays during various physiological and behavioural activites. In the intra lateral hypothalamic area (Intra-LHA) blockade of DA D2 receptors by specific antagonist in tumor bearing (TB) and non tumor bearing (NTB) rats increased food intake indicating the involvement of DA D2 receptors in feeding mechanisms (Zhang, *et al*., 2001). Thus during diabetes the decrease in DA D2 receptor expression could disturb hypothalamic functions. Impairment of DA D2 receptor is an important factor that leads to hyperphagic and polydypsic condition as DA participates in regulating meal size (Yang, *et al*., 1997). Dopamine–acetylcholine (DA-ACh) interaction within the lateral hypothalamus (LH) is involved in the regulation of locomotion, feeding behaviour and reinforcement (Baptista, *et al*., 1990; Hoebel, *et al*., 2000). The cholinergic stimulation of these activities is regulated by DA through D2 receptors in the hypothalamus. Thus DA in the hypothalamus is related to sensory input, feeding reflexes, food reward or memory processes (Hernandez & Hoebel, 1988). In the hypothalamus co-administration of dopamine D1 and DA D2 agonists inhibit the feeding effect mediated by the action on neuropeptide Y (NPY) (Kuo, 2002). This is effective in the reduction of food intake in diabetic rats, revealing the efficiency of DA D1/ D2 agonist in the improvement of

hyperphagia in diabetic animals. Decreased DA D2 receptor mRNA expression in diabetes is reversed to near control in insulin, curcumin and Vitamin D_3 treatment. We report an increased expression of DA D2 receptor mRNA during insulin treatment in diabetic rats. Modulated expression during treatment in the hypothalamus normalize the decreased number to control levels suggesting the therapeutic value of curcumin and Vitamin D_3.

PANCREATIC DOPAMINERGIC RECEPTOR EXPRESSION IN CONTROL AND EXPERIMENTAL RATS

DA is a neurotransmitter that plays a critical role in neurological and psychiatric disorders, such as schizophrenia, Parkinson disease, and drug addiction (Callier *et al*., 2003). Increasing evidence also shows implication of dopamine in various physiological functions such as cell proliferation (Hoglinger *et al*., 2004), gastrointestinal protection (Mezey *et al*., 1996) and inhibition of prolactin secretion (Freeman *et al*., 2000). Effects of DA on insulin secretion in general and on pancreatic beta cell function in particular have been poorly studied. Treatment with dopamine precursor L-dopa in humans suffering from Parkinson disease reduces insulin secretion upon oral glucose tolerance test (Rosati *et al*., 1976). In rodents, a single injection with L-dopa results in the accumulation of dopamine in beta cells and inhibition of the insulin secretory responses (Ericson *et al*., 1977; Zern *et al*., 1980). In isolated islets, analogues of DA inhibit glucose-stimulated insulin release (Arneric *et al*., 1984), whereas one study reports potentiation of insulin secretion upon acute DA accumulation (Ahren & Lundquist, 1985). Taken as a whole, previous studies suggest that beta cells are directly responsive to DA. Here, we investigated the molecular mechanisms implicated in beta cell responses to DA receptors action in diabetes and insulin, curcumin and Vitamin D_3 treated diabetic rats.

In particular, the present data demonstrate the up regulation of DA D1 receptors in the pancreas of diabetic rats when compared to control. Greengard *et al.*, (1942) reported that exogenous DA stimulated the pancreatic secretion of water and bicarbonate in anesthetized dogs. This has been confirmed in the isolated, perfused pancreas (Hashimoto *et al*., 1971; Furuta *et al*., 1974; Bastie *et al*., 1977). Receptor binding studies with [^{3}H] DA also demonstrated the presence of specific postsynaptic receptors for DA in the exocrine pancreas of the dog (Vayssette *et al.,* 1986). Previous studies reported that DA-stimulated pancreatic secretion is mediated by DA D1 receptors on the basis of the antagonism by SCH23390, a selective DA D1 receptor antagonist (Horiuchi *et al*., 1989). DA-induced pancreatic exocrine secretion is mediated by activation of DA D1 receptors of the pancreas in dogs (Horiuchi *et al*., 1989). Insulin curcumin and Vitamin D_3 treatment reversed the distorted DA D1 receptor in the pancreas of diabetic rats to near control. Thus our results showed the functional difference in DA D1 receptor in pancreas of diabetic rats contributing to the dysfunction of pancreatic islets. Curcumin and Vitamin D_3 proved a novel therapeutic role in modulating DA D1 receptor in the pancreas of diabetic rats.

Moreover, the inhibitory effects of dopamine are predominantly ascribed to activation of the DA D2-like receptor family members. DA receptors are present in INS-1E beta cells as well as rat, mouse and human islets. Dopamine inhibited glucose-stimulated insulin secretion, an effect reproduced by activation of DA D2-like receptors using the DA D2/D3 receptor agonist quinpirole (Blanca *et al*., 2005). DA D2 receptor expression was confirmed by immunodetection revealing localization on insulin secretory granules of INS-1E and primary rodent and human beta cells. DA (10M) and the D2-like receptor agonist quinpirole (5 M) inhibited glucose stimulated insulin secretion tested in several models, i.e. INS-1E beta cells, fluorescence-activated cell-sorted primary rat beta cells, and pancreatic islets of rat, mouse, and

human origin (Blanca *et al*., 2005). Our data showed an up regulation of DA D2 receptors in the pancreas of diabetic rats compared to control. Thus our findings proved that DA D2 like receptors are expressed in pancreatic beta cells and mediate inhibition of insulin secretion in diabetic rats. The role played by dopamine in glucose homeostasis involve dopamine receptors, expressed in pancreatic beta cells, modulating insulin release. Also, treatment with insulin, curcumin and Vitamin D_3 reversed the increased expression of DA D2 receptor to near control. Therefore, the potential of curcumin and Vitamin D_3 in modulating DA D2 receptor action on beta cells have relevant implications for the better management of diabetes.

INSULIN RECEPTOR ALTERATIONS IN BRAIN AND PANCREAS

Several studies have found high levels of insulin receptors in the CNS at specific locations. The highest concentrations of insulin receptors in the brain are in olfactory bulb, cerebral cortex, hippocampus, cerebellum and hypothalamus (Havrankova *et al*., 1978; Unger *et al*., 1989; 1991). Furthermore, areas with high levels of insulin receptors correspond to the areas with the highest level of extractable insulin (Baskin *et al*., 1983). Most insulin receptor immunoreactivity is on neurons, with very little seen on glial cells (Unger *et al*., 1991; Baskin *et al*., 1993). In the hippocampus, insulin binding is detected in the molecular layer of the dentate gyrus, and in the dendritic fields of CA1 pyramidal cells (stratum oriens and stratum radiatum) (Unger *et al*., 1991 Corp *et al*., 1986). Importantly, insulin binding in the hippocampus is associated with immunocytochemically detectable phosphotyrosine and IRS-1, one of the putative cellular intermediates in insulin action (Baskin *et al*., 1993; 1994).

Our results showed that insulin receptor expression down regulated in cerebral cortex and up regulated in cerebellum, brain stem, corpus striatum,

hippocampus and hypothalamus of diabetic rats when compared to control. An alteration in insulin signaling ability will have a major impact on cellular energy balance by affecting rate of uptake of glucose and other metabolic substrates and also directly by affecting the activity of enzymes involved in carbohydrate metabolism (e.g. glycolysis, glycogen synthesis, gluconeogenesis), lipid metabolism (lipolysis, fatty acid and triacylglycerol synthesis, and protein metabolism (protein synthesis and degradation) (Dimitriadis, 2000). Many or all of the enzymes involved in the mitochondrial tricarboxylic acid cycle, the final common catabolic sequence, appear to be modulated by insulin independently of insulin-stimulated glucose transport (Bessman & Mohan, 1997). Expression of the genes for many enzymes involved in metabolism also appears to be regulated by insulin (O'Brien, 1996). Thus an alteration of insulin signalling in brain regions have a profound effect on cellular energetics and is a contributing factor in the energetic deficit associated with the development of diabetes associated neurodegenerative diseases.

Our results suggest an altered insulin receptor expression in the brain regions of diabetic rats which could elicit cognitive deficits. Experiments have shown the ability of small doses of insulin (0.4–0.8 units/kg) to reverse the amnesia produced by a 2 mg/kg scopolamine injection (Messier & Destrade, 1994; Blanchard & Duncan, 1997) and intra-cerebro-ventricular injection of insulin facilitates memory (Park *et al*., 1968). The wide distribution of insulin and insulin receptors in the brain as well as the presence of insulin-dependent glucose transporters suggest that insulin in the brain participates in several cognitive functions, including learning and memory. An obvious problem that has impeded further research is that exogenous insulin injection can reduce blood glucose and lead to hypoglycaemia which is associated with impaired memory (Santucci *et al*., 1990; Kopf & Baratti, 1995; Kopf *et al*., 1998). Cognitive impairments associated with diabetes mellitus caused by inadequate

insulin/insulin receptor functions have also been documented. In this study, the altered expression of insulin receptor in the brain regions of diabetic rat brought back to near control level by the treatment with insulin, curcumin and Vitamin D_3. Animal model research indicates that insulin deficiency results in impairments in synaptic plasticity and cognitive processes while human studies suggest that insulin insensitivity also affect cognitive processing. These results provide a confirmatory evidence for prevention of insulin receptor dysfunction in brain with insulin, curcumin and Vitamin D_3 treatment and represent a novel possibility for the better management of diabetic mediated neurological complications.

Pancreas

Insulin regulates peripheral energy homeostasis by acting on multiple tissues to control carbohydrate, lipid and protein metabolism (Saltiel, 2001). It has also been demonstrated that insulin receptor and post-receptor signaling mechanisms are required for pancreatic beta cell function (Kulkarni, 2002). Recent studies has shown that the beta cell insulin receptor knock out mice failed to show the growth of islet cells while the control and IGF1knock out mice did exhibit this growth response. Mice with global deletion of insulin receptor substrate (IRS) 2 develop type 2 diabetes due to a combination of insulin resistance and beta cell failure (Withers *et al*., 1998; Kubota, *et al*., 2000). Furthermore, cell-specific gene targeting in mice using Cre/loxP-mediated recombination strategies has shown that beta cell deletion of the insulin receptor reduces first-phase insulin release and beta cell insulin content and causes a progressive deterioration in glucose tolerance (Kulkarni *et al*., 1999). Deletion of the insulin-like growth factor 1 receptor gene (Igf1r) likewise impairs insulin synthesis and secretion and combined deletion of the insulin receptor gene and Igf1r causes marked beta cell failure (Kulkarni *et al*., 2002; Ueki *et al*., 2006). Our

results showed a decreased expression of insulin receptor in the pancreatic islets of diabetic rats and treatment with insulin, curcumin and Vitamin D_3 reverse this decreased expression to near control. Our findings suggest that insulin receptor dependent mechanisms are required for normal growth and function of beta cell and suggest a novel role of curcumin and Vitamin D_3 for maintenance of a normal glucose homeostasis through modulating insulin receptors in pancreatic islets.

VITAMIN D RECEPTOR GENE EXPRESSION IN BRAIN AND PANCREAS

Vitamin D_3 regulate immune function (Deluca & Cantorna, 2001) and cell differentiation (Segaert & Bouillon, 1998). Vitamin D_3 acts *via* a member of the nuclear hormone receptor family to directly regulate gene transcription (Clancy *et al*., 2001). There is now accumulating evidence that Vitamin D_3 plays a role in the central nervous system (Garcion *et al*., 2002). The Vitamin D_3 receptor and key enzymes involved in the metabolism of Vitamin D_3 are expressed in the rat brain (Garcion *et al*., 2002). Both animal and clinical studies strongly support the notion that chronic VD deficiency is harmful to brain development and to adult neural functions. For example, in rodent models, VD deficiency leads to brain malformation and has effects on rodent behaviour. In humans, it is associated with mood disorders, multiple sclerosis, schizophrenia, and epilepsy. The functions of VD are mediated through the nuclear VD receptor (VDR), a member of the nuclear receptors (NR). VDR is widespread in both the developing and adult brain, as well as in the spinal cord, suggesting a potential role for VD and VDR in the brain (Eyles *et al*., 2005).

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 (Gascon-Barre & Huet, 1983). The presence of VDR in the limbic system, cortex, cerebellum of rodents and humans (Musiol *et al*., 1992) support a functional role for

Vitamin D_3 in the regulation of behaviour and cognitive functions. The present study showed an increased expression of VDR in cerebellum, brain stem and hypothalamus and decreased expression in cerebral cortex, corpus striatum and hippocampus of diabetic rats compared to control. This varying expression of VDR in the brain of diabetic rats will confer to altered neuronal activity. Efferents to cerebellar Purkinje cells and the thalamic part of the vestibular system, nucleus ventrolateralis, suggesting that the vestibular system is also a target of VD (Prufer *et al*., 1999). Expression of VDR in motor neurons (Prufer *et al*., 1999) suggests its role in regulation of motor functions. A putative receptor for 1,25(OH)2D has been detected in chick brain (Jia and Nemere, 1999), allowing speculation that 1,25(OH)2D could act like other neuroactive hormones in modulating neuronal activity and neurotransmitter receptors (Zakon, 1998; Rupprecht & Holsboer, 1999).

Our results showed that insulin, curcumin and Vitamin D_3 reversed the altered expression of VDR in the brain regions of diabetic rats. VDR is found in the olfactory, visual and auditory sensory systems (Glaser *et al*., 1999; Prufer *et al*., 1999; Zou *et al*., 2008), suggesting that the somatosensory system is also a target of 1,25(OH)2D. Recent studies showed curcumin a nutritionally-derived ligand of VDR (Bartika *et al*., 2010). Studies have shown that Vitamin D confers regulatory benefits in neuronal Ca^{2+} homeostasis and protects neurons from excess calcium entry in the brain (Brewer *et al*., 2001). Regulation of brain calcium homeostasis occurs *via* down-regulation of the L-type voltage-sensitive Ca^{2+} channels (L-VSCCs) in hippocampal cultured neurons, thus contributing to protection from excitotoxic cell death (Brewer *et al*., 2001). Treatment with 1,25(OH)2D in aged rats restores aging neurons (Brewer *et al*., 2006). These beneficial changes protect neurons during ischemic events or excitotoxic insults. Neuroprotective effect of 1,25(OH)2D also happen through reduction of Ca^{2+} toxicity by stimulation of expression of Ca-binding proteins (de Viragh *et al*., 1989),

thus supporting the idea that Vitamin D regulates the changes in VDR expression in the brain regions of diabetic rats and prevent neuronal degeneration in diabetes.

Pancreas

In recent years, there have appeared several reports which suggest that the endocrine pancreas is also a target tissue for the hormonally active form of vitamin D3, 1,25-(OH)2-D3, along with the classical vitamin D target organs: the intestine, bone and kidney (Norman *et al*., 1982). These observations include: (a) the presence of a cytosol receptor protein for 1,25-(OH)2-D3 in the chick pancreas (Christakos & Norman, 1981; Pike *et al*., 1980; Pike, 1981). 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).

Our results showed a decreased expression of VDR mRNA in the pancreatic islets of diabetic rats Also, treatment with insulin curcumin and vitamin D_3 has reversed this expression to near control. Early *ex vivo* studies by Norman *et al*. (1980) have shown that insulin but not glucagon release after stimulation with glucose and arginine is reduced in the isolated perfused pancreas from vitamin D-deficient rats. Later on, the same group showed that glucose tolerance and insulin secretion are impaired in vitamin D-deficient rats *in vivo* (Cade & Norman, 1986) and that insulin secretion was improved within 3 h after a single administration of 1,25(OH)2D3 to vitamin D-deficient rats (Cade & Norman, 1987). In more recent studies, it was reported that *de novo* insulin synthesis is reduced in isolated islets from vitamin D-deficient rats and that insulin biosynthetic capacity is restored in vitro by addition of 1,25(OH)2D3 (Bourlon *et al*., 1999). It is proposed that curcumin act as an agonist for vitamin D receptors and there by modulating its expression in diabetic pancreas.

GLUT3 EXPRESSION IN BRAIN

Glucose transport into the brain is critical for the maintenance of brain metabolism. Although under basal conditions the rate of glucose transport is not the rate-limiting step for glycolysis in the central nervous system, hypoglycaemia or hyperglycaemia is known to change the glucose transport system in the brain (Devivo *et al*., 1991), suggesting that there is glucose-regulatable mechanisms associated with the transport of glucose.

The expression, regulation and activity of glucose transporters play an essential role in neuronal homeostasis, because glucose represents the primary energy source for the brain (Lund-Anderen, 1979; Pardridge, 1983). Although many isoforms of glucose transporters have been identified in the brain, GLUT-3, the neuron-specific glucose transporter, is solely responsible for the delivery of glucose into neurons in the central nervous system. GLUT-3 mRNA is widely expressed in the brain, including the pyramidal neurons of the hippocampus and the granule neurons of the dentate gyrus (Nagamatsu *et al*., 1992; Nagamatsu *et al*., 1993; McCall *et al*., 1995) and immunohistochemical analysis has demonstrated that GLUT-3 protein expression also exhibits a widespread distribution in the brain (Nagamatsu *et al*., 1993; McCall *et al*., 1994; Zeller *et al*., 1995). In the hippocampus, GLUT3 immunoreactivity has been identified in mossy fibers, the stratum radiatum and stratum oriens of Ammon's horn, and the molecular layer of the dentate gyrus (McCall *et al*., 1994; Gronlund *et al*., 1995). Our study investigated the effect of learning-induced neuronal activation on brain glucose utilization. Our data showed an up regulation of GLUT3 mRNA in the brain regions- cerebral cortex, cerebellum, brain stem, corpus striatum, hippocampus and hypothalamus. Region-specific increased neuronal activity has been shown to be often associated with parallel increases in brain glucose uptake (Sokoloff *et al*., 1977; Sarter *et al*., 1989; Bontempi *et al*., 1996; Barrett *et al*., 2003). A number of studies

investigated the effect of learning-induced neuronal activation on local cerebral glucose utilization (LCGU). Learning and memory processing is usually found to produce increases of glucose metabolism in the hippocampus (Shimada *et al*., 1983; Friedman & Goldman-Rakic, 1988) and cortical brain regions (Matsunami *et al*., 1989; Friedman & Goldman-Rakic, 1994) that are functionally related to memory processing as well as to the sensorimotor task requirements (Matsunami *et al*., 1989; Friedman & Goldman-Rakic, 1994). Our results confirm the alterations in GLUT3 expression, a major glucose transporter in CNS with STZ-induced diabetes.

Also, insulin, curcumin and Vitamin D_3 treatment improved the glucose transport system in brain regions of diabetic rats by regulating the increased GLUT3 expression. Alterations in glucose utilization are known to occur in the important regions of brain connected with learning and memory (van der *et al*., 1992). Learning and memory processing is found to produce increases of glucose metabolism in the cortical brain regions that is functionally related to memory processing as well as to the sensorimotor task requirements (Friedman & Goldman-Rakic, 1994). Our findingS suggest a modulation of GLUT 3 expression in the brain with curcumin and Vitamin D_3 supplementation which consecutively normalise the glucose transport in CNS.

GLUT2 EXPRESSION IN PANCREAS

In previous morphological studies the changes in the pancreatic islets and the destruction of the beta cells during the development of diabetes have been documented (Like *et al*., 1974 a,b; Frankel *et al*., 1987). Together with glucokinase, the low-affinity plasma membrane GLUT2 glucose transporter in the pancreatic beta cell is responsible for recognition of glucose as the signal for glucose-induced insulin secretion (Lenzen, 1992; Matschinsky *et al*., 1993; Lenzen & Tiedge 1994). Rat pancreatic beta cells display a dense immunostaining for GLUT2 in the cell

membrane (Jetton & Magnuson, 1992). Loss of GLUT2 immunoreactivity is an early indicator of beta cell dysfunction and is an element of importance for the deterioration of glucose-induced insulin secretion in diabetic Chinese hamsters. The expression of GLUT2 in pancreatic beta cells has been suggested to be important for the normal glucose sensitivity of these cells (Unger, 1991; Thorens, 1992). Importantly, the expression of this transporter is reduced or suppressed in glucose-unresponsive beta cells from diabetic rats and mice, a phenomenon that participate in the beta cell dysfunctions associated with diabetes (Johnson *et al*., 1990b; Orci *et al*., 1990; Thorens *et al*., 1992). While in most situations decreased expression of GLUT2 correlates with a decrease in its mRNA levels, decreased GLUT2 expression in dexamethasone-treated rats has been reported to be controlled at the translational or posttranslational level (Ogawa *et al*., 1992). Our results showed a decreased mRNA expression of GLUT2 in the pancreatic islets of diabetic rats. Earlier studies reports that curcumin *in vitro* protects pancreatic islets against cytokine-induced death and dysfunction and *in vivo* prevents STZ-induced diabetes (Kanitkar *et al*., 2008). As glucose is absorbed, the process is reversed, Ca^{2+} absorption is down regulated as the apical membrane is repolarized and glucose absorption is down regulated by loss of apical GLUT2. The integration of glucose and Ca^{2+} absorption represents a complex nutrient sensing system, which allows both absorptive pathways to be regulated rapidly and precisely to match dietary intake (Emma *et al*., 2008). It is suggested that Vitamin D_3 through absorption of calcium through VDR regularise the decreased GLUT2 expression in diabetes to control. Our results showed a novel role of curcumin and Vitamin D_3 in reversing the altered expression of GLUT2 in pancreatic islets and thereby eliciting glucose induced insulin secretion.

PHOSPHOLIPASE C EXPRESSION IN BRAIN AND PANCREAS

There is now great interest in the identification of molecules involved in the regulation of both normal neuronal differentiation and its activity-dependent modification. While a variety of transmitter receptors have been implicated in neuronal plasticity, much less is known of the second messenger systems and intracellular signalling pathways that subsequently lead to changes in the structure and functional properties of brain cells. Phospholipase C mediates transduction of neurotransmitter signals across membranes via hydrolysis of phosphatidylinositol-4,5-bisphosphate, leading to generation of second messengers inositol- 1,4,5-trisphosphate and diacylglycerol. In the CNS, neurotransmitter receptor coupling to phospholipase C (PLC) has been extensively documented in [^{3}H] inositol-labeled tissue slices and synaptosomes obtained from animal brains (Fisher & Agranoff, 1987; Stephens & Logan, 1989; Chandler & Crews, 1990). In the present study, we observed diabetes-mediated alterations in phospholipase C expression in the brain regions- cerebral cortex, cerebellum, brain stem, corpus striatum, hippocampus and hypothalamus. Further we extended the studies to phospholipase C regulation with insulin, curcumin and Vitamin D_3 treatment for potential therapeutic drugs which modulate signal transduction pathway thereby contributing the prevention of CNS dysfunction in diabetes. Our results showed a decreased expression of phospholipase C in the cerebral cortex, cerebellum, brain stem, hippocampus and hypothalamus of diabetic rats and an increased expression in corpus striatum when compared to control. The DA D1 receptors showed characteristic ability to stimulate adenylyl cyclase and generated inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol *via* the activation of phospholipase C (Monsma *et al*., 1990; Sibley *et al*., 1993). Muscarinic receptors M1–M3–M5 typically couple *via* α subunits of the Gq/11 family to activate phospholipase C (PLC), stimulating phosphoinositide (PI) hydrolysis (Caulfield & Birdsall, 1998). In

particular, reconstitution experiments with purified muscarinic m1 receptors, G protein subunits, and PLC suggested that the β1 subtype of PLC serves as the primary effector for the muscarinic m1 receptor (Felder, 1995). We considered that the down regulation of the Phospholipase C in rat cerebral cortex and cerebellum during diabetes contribute to the impaired signal transduction of G-protein coupled neurotransmitter receptors. Phospolipase C performs a catalytic mechanism, generating inositol triphosphate (IP3) and diacylglycerol (DAG). Altered phospholipase C expression fails to modulate the activity of downstream proteins important for cellular signaling. Defective expression of phospholipase C results in low levels of IP3 causing the impaired release of Ca^{2+} and bring down the level of intracellular calcium and thus failed to execute the normal neuronal function in cerebral cortex and cerebellum. Previous studies reports that phospholipase C-mediated signaling, initiated by growth factor receptor types, are involved in long-term memory formation, a process that requires gene expression (Paul *et al*., 1999). These evidences led us to propose that the enhancement of diabetes-mediated phospholipase C gene expression could impart damage to the central cognitive functions, which has been effectively protected by curcumin and Vitamin D_3 treatment.

Pancreas

In response to glucose stimulation, a variety of metabolic, ionic, and signal transduction events occur contemporaneously (Hedeskov, 1980; Henquin, 1985; Rasmussen *et al*., 1995; Zawalich, 1996) These events culminate in a rapid biphasic insulin secretory response from the perfused rat pancreas and from freshly isolated perifused rat pancreatic islets (Grodsky, 1972; Gerich *et al*., 1974; Grill *et al*., 1978; Bolaffi *et al*., 1986; Zawalich *et al*.,1989a; Zawalich, 1990). These events include

not only the cation Ca^{2+}, which gains access to the β-cell via the opening of voltage-regulated channels, but also cyclic adenosine monophosphate and phosphoinositide derived second-messenger molecules, generated as a consequence of PLC activation. The underlying explanation for impaired insulin secretion in diabetes resides, at least in part, in the inability of glucose to activate information flow in the phospholipase C/protein kinase C (PLC/PKC) signal transduction system to the same quantitative extent in mouse islets as it does in rat and, presumably, human islets as well. Our study showed a decreased expression of phospholipase C expression in the pancreatic islets of diabetic rats. Treatment using insulin, curcumin and Vitamin D_3 normalized these change in expression to near control. Stimulation of muscarinic M1 and M3 receptor activate PLC–phosphoinositide 3-kinase (PI3K) pathway to increase glucose uptake (Biddlecome *et al*., 1996; Elmendorf, 2002; Hutchinson & Bengtsson, 2005). Thus, we conclude that curcumin and Vitamin D_3 has a regulatory role on phospholipase C expression and thereby controlling insulin synthesis and release from the pancreas at the second messenger level.

CREB EXPRESSION IN BRAIN

The CREB plays a pivotal role in dopamine receptor-mediated nuclear signaling and neuroplasticity (Finkbeiner, 2000). Here we demonstrated the significance of CREB gene expression in the brain regions- cerebral cortex, cerebellum, brain stem, corpus striatum, hippocampus and hypothalamus of STZ-induced diabetes rats. CREB-responsive transcription plays a central role in the formation of long-term memory in *Drosophila, Aplysia* and mice (Alcino *et al*., 1998). Agents that disrupt the activity of CREB specifically block the formation of long-term memory, whereas agents that increase the amount or activity of the transcription factor accelerate the process.

CREB plays a pivotal role in dopamine receptor-mediated nuclear signaling and neuroplasticity (Finkbeiner, 2000). Our findings showed a significant down regulation of CREB in cerebral cortex, cerebellum, brain stem, hippocampus and hypothalamus and up regulation in corpus striatum of diabetic rats, when compared to control. Electrophysiological studies with hippocampal slices suggest that cAMP-dependent transcription is required for the maintenance of LTP (Frey *et al*., 1990; 1993; Huang & Kandel 1994; Impey *et al*., 1996). CREB activation is detected in cultured hippocampal neurons using an antibody (Ginty *et al*., 1993) specific to phosphorylated CREB proteins (Ser133 of CREB) (Deisseroth *et al*., 1996). The study of the cholinergic and dopamine receptors expression in relation with CREB phosphorylation in diabetes is an important step toward elucidating the relationship between molecular adaptations and behavioural consequences. CREB proteins in neurons are thought to be involved in the formation of long-term memories; this has been shown in the marine snail Aplysia, the fruit fly *Drosophila melanogaster*, and in rats. CREB is necessary for the late stage of long-term potentiation. CREB also has an important role in the development of drug addiction (Mayr & Montminy, 2001**).** It is therefore important to identify the elements that elicit phosphorylation of CREB and thereby its expression in the nucleus.

Our results showed that curcumin and Vitamin D_3 treatment reversed the decreased expression of CREB in diabetes to near control. The curcumin and Vitamin D_3 supplementation significantly modulated the altered gene expression of CREB in the brain regions of diabetic rats to near control. Insulin treatment did not show any significant effect in the CREB expression of diabetic rats in cerebral cortex, brain stem and hippocampus whereas cerebellum, corpus striatum and hypothalamus showed a significant reversal. This study demonstrated that curcumin and Vitamin D_3 possess regulatory effect in the transcription factor CREB expression, which is crucial

in maintaining the normal neuronal function and better management in diabetes. The DA D1 signal transduction pathway, activation of the transcription factor CREB and dopamine-mediated gene expression are critically involved in memory processing, behavioural responses and drug addiction (Nestler, 2001). Interruption of this pathway interferes with important cognitive performance and behavioural aspects associated with CNS. The effect of curcumin and Vitamin D_3 in interacting with the cholinergic, dopaminergic receptor and CREB in STZ-induced diabetes proved its potential in managing CNS disorders in diabetes.

SUPEROXIDE DISMUTASE EXPRESSION IN BRAIN AND PANCREAS

Glucose utilization is decreased in the brain of diabetic patients (McCall, 1992) providing a potential mechanism for increased vulnerability to acute pathological events. Since glucose is the main brain energy supply for the maintenance of the nervous system, the deficiency of glucose in the cell trigger neuronal injury (Seo *et al*., 1999). Impaired energy metabolism in neurons induce production of increased amount of free radicals (Coyl & Puttfarcken, 1993) and initiate excitotoxic neuronal cell damage (Simon *et al*, 1984; Monyer *et al*., 1989). The increased oxidative stress in diabetes (Baynes 1991; Wolff, 1993; Travero, 1998) and immobilization of stress produces oxidative damage in many regions of rat brain including the hippocampus (Liu *et a.l*., 1996). Furthermore, oxidative damage in rat brain is increased by experimentally induced hyperglycemia (Aragno, 1997). Oxidative damage to various brain regions constitute into the long term complications, morphological abnormalities and memory impairments (Aksenov et al., 2001; Bunsey et al., 1996; Eichenbaum et al., 1992; Regan et al, 2001; Suzuki & Clayton, 2000). Protection of brain cells from degeneration should be an effective strategy to prevent

or to slow the progression of disease. Compounds that prevent oxidative damage increase the resistance of neuronal cells to degeneration.

It has been suggested that free radical species responsible for STZ toxicity is the hydroxyl radical. The destruction of superoxide radical or H_2O_2 by SOD or CAT would ameliorate STZ toxicity, as would substances able to scavenge the hydroxyl radical (Walling, 1975; Lubec, 1996). Vulnerability of brain to oxidative stress induced by oxygen free radicals seems to be due to the fact that, on one hand, the brain utilizes about one fifth of the total oxygen demand of the body and on the other, that it is not particularly enriched, when compared with other organs, in any of the antioxidant enzymes. Relatively low levels of these enzymes are responsible in part for the vulnerability of this tissue (Baynes & Thrope, 1999). Our results showed a decreased expression of SOD in diabetic rats compared to control in cerebral cortex, cerebellum and hippocampus and an increased expression in brain stem, corpus striatum and hypothalamus. The decreased SOD activity in organs suggests that the accumulation of superoxide anion radical is responsible for increased lipid peroxidation. The inactivity of the antioxidant enzymes, SOD in the diabetes-induced groups was attributed to peroxidative damage to the tissues caused by administering STZ (Kwag, 2001). The decreased activities of SOD is a response to increased production of H_2O_2 and O_2 by the autoxidation of glucose and non-enzymatic glycation (Aragno *et al*., 2000). This alteration of SOD represents one of the important factors for the vulnerability of the brain against oxygen free radicals or is relevant to the pathophysiology of diabetes in Wistar rats. Treatment with insulin, curcumin and Vitamin D_3 ameliorated the expression of enzyme and helps to control free radicals in brain regions. Curcumin has been shown to have a broad spectrum of biological activities such as anti-inflammatory, anti-neoplastic, antimutagenic and antioxidant (Naik *et al*., 2004). Research has shown curcumin to be a powerful

scavenger of the superoxide anion, the hydroxyl radical and nitrogen dioxide (Daniel *et al*., 2004). Previous studies report that Vitamin D_3 exhibit membrane anti oxidant property and an ability to inhibit iron-dependent lipid peroxidation in liposomes (Wiseman, 1993). Our data proved the anti oxidant property of curcumin and Vitamin D_3 in the brain regions, which could exert a beneficial action against numerous morphological and functional alterations during diabetes caused by the presence of free radicals in STZ diabetes.

Pancreas

In the past, numerous studies established a crucial role of reactive oxygen species in the pathogenesis of acute and chronic pancreatitis (Guyan *et al*, 1990; Schoen *et al*, 1992; Antosiewicz *et al.*, 1995). The damage of pancreatic acinar cells by oxidative stress leads to an uncontrolled release of digestive enzymes from the zymogen granula, which then causes the destruction of the surrounding tissue. Mechanisms have been detected resulting in an increase of oxygen radicals in pancreatic tissue (Uden *et al.*, 1988). Recently, Kishimoto et al. successfully detected superoxide production in rat pancreas using the well-established model ofceruleininduced pancreatitis (Kishimoto *et al.*, 1995; Ito *et al.*, 1996*).* SOD is implicated in the pathophysiology of various disease states including diabetes mellitus. Oxygen free radicals exert their cytotoxic effect by peroxidation of membrane phospholipids leading to change in permeability and loss of membrane integrity (Meerson *et al*., 1982). Pancreatic β-cell death underlies the pathogenesis of Type I (insulin-dependent) diabetes mellitus and liver is an important organ which offers an adequate site for various metabolic functions. Oxygen free radicals have been implicated in both β cell destruction as well as in liver injury (Roza *et al.*, 1985; Poli *et al*., 1989; Hunt *et al*., 1990; Robinovitch *et al*., 1992). Our results showed a

decreased expression of SOD in the pancreas of diabetic rats when compared to control.

The treatment using insulin, curcumin and vitamin D_3 reversed the pancreatic SOD expression in diabetes to near control. It is known that pancreatic β -cells contain very low levels of antioxidant enzymes which render them more susceptible to reactive oxygen species-induced toxicity as compared to other cell types (Tiedge *et al*., 1997). Hence, curcumin and Vitamin D_3 showed a prominent anti oxidant activity by normalizing the SOD expression to near control.

Thus our results showed that uncontrolled hyperglycaemia, deficiencies of central insulin, or both contributes to CNS disorders mediated through cholinergic, dopaminergic, insulin and Vitamin D receptor. Also, gene expression of cholinergic enzymes, glucose transporter GLUT3/2, transcription factor CREB, second messenger enzyme phospholipase C and anti oxidant enzyme, superoxide dismutase is found to be altered in the CNS of diabetic rats. Nutritional therapy using curcumin and Vitamin D_3 exhibited a potential effect in improving glucose homeostasis and reversing the altered functional regulation of receptors and enzymes of STZ induced diabetic rats to near normal. These results provide a confirmatory evidence for neuroprotective role of curcumin and Vitamin D_3 and represent a novel therapeutic possibility for the better management of diabetic mediated neurological complications.