Study on the cognitive changes in STZ-induced diabetes with special reference to glial changes and immune activation in rat hippocampus

A thesis submitted to
Jiwaji University

For the award of the degree of
Doctor of Philosophy in Neuroscience

By
Aarti Nagayach

Under the supervision of
Prof. I. K. Patro

School of Studies in Neuroscience,
Jiwaji University, Gwalior (M.P.)
2015
Acknowledgements

At this moment of accomplishment I wish to express my sincere acknowledgements to all those who have contributed to this thesis and supported me in one way or the other during this amazing journey.

First and foremost, I would like to thank my supervisor Prof. I. K. Patro, School of Studies in Neuroscience/ Zoology, Jiwaji University, Gwalior for encouraging my research instincts and allowing me to grow as a research scientist. His treasured advice, practical criticism and discussions helped me a lot in developing a researcher perspective inside me. During the most difficult times of my thesis writing, he gave me the support and freedom I needed to move on. I appreciate all his contributions of timing and ideas to make my Ph.D. experience productive and stimulating.

My profound gratitude are reserved for Dr. Nisha Patro, Scientist, School of Studies in Neuroscience, Jiwaji University, Gwalior. Her invaluable suggestions in immunohistochemical technique have aided me well. I owe her my wholehearted appreciation.

I express my gratitude to Hon’ble Prof. Sangeeta Shukla, Vice-chancellor, Jiwaji University, Gwalior for providing all the necessary help and encouragement to our department.

My deep sense of respect is due for Prof. P.K. Tiwari, Coordinator-Head, School of Studies in Neuroscience, Jiwjai University, Gwalior for providing me the moral support and encouragement during this tenure.

This work would not be possible without the fellowship of Indian Council of Medical Research-Senior Research Fellowship (ICMR-SRF), New Delhi. Facilities and support provided by Department of Biotechnology-Bioinformatics Information Facility (DBT-BIF) and Department of Biotechnology-Human Resource Development (DBT-HRD), Govt. of India, New Delhi is acknowledged.

I take this opportunity to genuinely acknowledge all my seniors who guided and supported me from the early days of my research. Dr. Sarika Kuswaha, Dr. Kapil Saxena, Dr. Meghna Saxena, Dr. Surya Awasthi, Dr. Amit Awasthi, Dr. Arpita Sharma, Dr. Kamendra Kumar and Shashank sir, were among those whom friendly attitude and elderly guidance kept me going at the beginning. I am extremely thankful to Dr. Vinay Lomash, whose distinguished helping nature and ingenious suggestions helped me immensely while doing histopathological studies and research paper writing.

Appreciations are due to my juniors Kavita, Brijendra, Aijaz, Deepika, Shrestha, Nisar and Priya for being the most delightful lab-mates, providing a great working environment and for their ‘anytime’ help, discussions and pleasing chats.

I am also indebted to my hostel friends (Samta di, Rohinika, Hrillekha, Preeti, Aaliya, Shilpi, Manvi, Pooja and Preeto), not only for all their support, love and concern but also for being there to listen whenever I needed an ear (amazingly
untiring listeners!!). My friends in hostel and other parts of the world (Trapti, Leena, Naveen, Bhaskar, MAK, Arojit, Varun, Deepak and Abhilasha) were the sources of laughter, joy and esteem support during the tough times of Ph.D. pursuit. EXTENDED, HUGE and WARM thanks to you all!

I am thankful to Mr. Vishnu, Mr. Mahesh, Mrs. Seema and Mrs. Kavita our departmental administrative staff members, who have always been kind enough to advise and help me at the office front.

I am also thankful to our animal house keepers, Udaybhan and Awadesh for routinely maintaining and taking care of the animals, I worked upon. I would also wish to acknowledge Sureshji, for always keeping the working place clean and tidy, undeniably needed for the good experimentation.

Last but not the least, I warmly thank and appreciate my family for their love and encouragement. My deep and lasting gratitude is due to my ma and pa for their unconditional love, faith and support that encouraged me to work hard and to continue pursuing a research career. Their support without any complaint or regret has enabled me to complete this Ph.D. tenure. I owe you my eternal appreciation and affection. They are the reason I thrive to be better.

I must offer my thankfulness to the experimental rats who sacrificed their lives for the accomplishment of this work.

Utmost of all, I praise the God, the Almighty for providing me this opportunity and granting me the capability, indispensable to proceed with and complete this work successfully.

Lastly, I would like to thank all the people, who are somehow, were involved in this work and provided me with the necessary help and made it possible for me to write this thesis.

Aarti Nagayach
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced Glycation End products</td>
</tr>
<tr>
<td>ApoR3</td>
<td>apolipoprotein receptor-3</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri phosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BDV</td>
<td>Borna disease virus</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis 1</td>
</tr>
<tr>
<td>CA2</td>
<td>Cornu Ammonis 2</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu Ammonis 3</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Cystein aspartate protease-3</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>DAB</td>
<td>3’3- diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’6,- diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate Gyrus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPX</td>
<td>Distrene tricresyl phosphate xylene</td>
</tr>
<tr>
<td>EGL</td>
<td>External granular layer</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocynate</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GLT-1</td>
<td>Glutamate transporter-1</td>
</tr>
<tr>
<td>GLUT-2</td>
<td>Glucose transporter-2</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>i.p</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Iba-1</td>
<td>Ionized calcium binding adaptor molecule-1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IGIF</td>
<td>Interferon Gamma Inducing Factor</td>
</tr>
<tr>
<td>IGL</td>
<td>Internal granular layer</td>
</tr>
<tr>
<td>IL-10/12/15/18</td>
<td>Interleukin-10/12/15/18</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-3/6</td>
<td>Interleukin-3/6</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP-10</td>
<td>IFN-γ-inducible protein-10</td>
</tr>
<tr>
<td>Jak/Stat</td>
<td>Janus kinase/ Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTD</td>
<td>Long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potential</td>
</tr>
<tr>
<td>mAChR</td>
<td>Muscarinic acetylcholine receptors</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony – stimulating factor</td>
</tr>
<tr>
<td>MGlur2</td>
<td>Metabotropic glutamate receptor 2</td>
</tr>
<tr>
<td>MHC class-I/II</td>
<td>Major histocompatibility complex I/II</td>
</tr>
<tr>
<td>MIP-1α/β</td>
<td>Macrophage-Inflammatory Protein-1α/β</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Macrophage-Inflammatory Protein-2</td>
</tr>
<tr>
<td>ML</td>
<td>Molecular layer</td>
</tr>
<tr>
<td>NF-kβ</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth factor</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NMDA</td>
<td>N- methyl D- aspartate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NO</td>
<td>Nitrous Oxide</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase 2</td>
</tr>
<tr>
<td>NOX1/2</td>
<td>NADPH oxidase 1/2</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>RAC1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffer saline</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetra methyl rhodamine isothiocynate</td>
</tr>
<tr>
<td>TrkA</td>
<td>Tropomyosin receptor kinase A</td>
</tr>
<tr>
<td>Symbol</td>
<td>Unit Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>hr</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>m</td>
<td>Metre</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μM</td>
<td>Micomolar (mole/litre)</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliters</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeters</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar (mmole/litre)</td>
</tr>
<tr>
<td>M</td>
<td>Molar (mole/litre)</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>sec</td>
<td>Second(s)</td>
</tr>
<tr>
<td>wt.</td>
<td>Weight</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
</tbody>
</table>
CONTENTS

Introduction 1-4

Review of Literature 5-34

Materials and Methods 35-60

Results 61-86

Discussion 87-106

Summary and conclusions 107-118

Photomicrographs & graphs 119-181

References 182-223

Appendix I 224-226

Publications & presentations 227-228
INTRODUCTION

Diabetes is the most prevalent heterogeneous endocrine disorder characterized by hyperglycemia due to the absolute and relative insulin deficiency that results from inadequacies in insulin secretion or action or both. According to the world health organization (WHO) 2014 fact sheets, diabetes mellitus is one of the non-communicable diseases that will be the 7th leading cause of death in 2030 globally. The ramification of diabetes associated impediments is not just limited to the insulin dependent organs. Irrespective of its two major forms, i.e., type 1 and type 2, diabetes mellitus is associated with several comorbid complications including structural and functional dysfunctions in central nervous system (McCall, 1992; Biessels et al., 1994; Coleman et al., 2004; Guven et al., 2009) and end organ damage via many diverse mechanisms. In so far, the cellular mechanisms affecting the brain functioning during diabetic state are still not well understood. Accordingly, the diagnostic approaches or therapeutic tools to comprehend and treat the cognitive and behavioural deficits in diabetes are in vain.

Studies have depicted that the prevalence of cognitive deficits and emotional alterations are two times more in diabetic patients than in the non-diabetic population. Cognitive and behavioural dysfunctions are being the most apparent CNS impairments well-reported in both humans (Petrofsky et al., 2005; Kodl and Seaquist, 2008; Alvarez et al., 2009) and animal models of diabetes (Biessels, 1994; Coleman et al., 2004; Guven et al., 2009). These dysfunctions were resulted in impaired cognitive and psychiatric ability, dementia and alterations in learning and memory (Gispen and Biessels, 2000; Popović et al., 2001; Allen et al., 2004; Arvanitakis et al., 2006; Zavoreo et al., 2014; Nagayach et al., 2014a). Patients with diabetes and hyperglycemia also reported with poor motor coordination and reduced motor activity (Daneman, 2001; Petrofsky et al., 2005; Cox et al., 2005; Nagayach et al., 2014b).
The causative factors for these alterations are presumed as dysregulated glucose utilization via glucose transporters (Duelli et al., 2000), oxidative stress (Sharma et al., 2010; de M Bandeira et al., 2013) and deposition of advanced glycated end products (Wang et al., 2009) etc.

The hippocampal formation, an elemental hub for the various aspects of cognition and behavioural processes is particularly sensitive to the glucose homeostatic alterations (Routh, 2002; Huang et al., 2007). Hyperglycemia supplies additional substrate for anaerobic glycolysis in the brain, which further results in lactic acidosis and enhanced damage to both glial and neuronal cells (Biessels et al., 1994). Concomitantly hyperglycemia also augment the formation of oxygen free radicals following lipid peroxidation in the brain tissues which later contributes to increased neuronal death (Hawkins and Davis, 2001; Hernández-Fonseca et al., 2009; Guven et al., 2009) and astrocytic glial reaction (Barber et al., 2000). Diabetes not only affects the cell proliferation associated neurogenesis but also restricts the synaptic plasticity in hippocampal neurons (Saravia et al., 2004; Zhang et al., 2008; Alvarez et al., 2009; Mardirossian et al., 2009) which possibly leads to the deterioration of hippocampal functioning.

In the nervous system, neurons do not exist in isolation; they are accompanied by a heterogeneous group of cells known as glial cells. Several researchers have evidenced that glial cells were endowed with a wide range of indispensable regulatory and immune functions in the CNS. Glia (astroglia and microglia) have a wide range of tasks in CNS ranging from the regulation of innate immunity, scavenging dead cell debris, scaffolding and protecting neurons to the regulation and maintenance of synaptic transmission and putative integration of information with neurons. Following any brain insult or immune breaching, glial cells get activated and exhibit morphological transformations from resting to activated, increase in their cell population and secrete an array of inflammatory molecules to combat the baleful condition (Patro et al., 2005; Luo and Chen, 2012; Boche et al., 2013; Verkhratsky et al.,
Glial activation based on the progression of insult and severity of stimulus, imposes contrasting features of brain curator (Morgan et al., 2004; Luo and Chen, 2012; Wang et al., 2013; Peng et al., 2014) and/or executioner (Heneka et al., 2010; Verkhratsky et al., 2014a,c; Verkhratsky and Parpura, 2014). Conclusively, the multifarious attribute of glial cells had made them an ideal target for therapeutic interventions during various neurodegenerative diseases and disorders.

Despite of universal acceptance, of glial participation in cerebral health and disease, the information regarding functional and structural stature of glial cells and allied changes following diabetes are still ineffectual. Thus, a pertinent research is needed to clarify the impact of diabetes on glial cells per se or their rejoinder effects on neuronal cells with special reference on associated brain functions. Concomitantly, the neuro-immunological aspects of neurodegeneration and neuroregulation following diabetes also require specific attention. Evidences are also instigating an insight on possible role of oxidative stress following diabetes in perpetuation of glial activation, neuronal cell death and associated immune response. Thus, an associative reciprocity between metabolic stress, cell death and gliosis in hippocampus also deserve consideration as they have never been studied and will provide an effective window to understand the underlying cellular mechanism following diabetes in brain.

Thus, the present study was intended to elucidate the scenario of glial activation, cellular degeneration and associated immune response either neuroprotective/ degenerative in consort with subsequent behavioural and cognitive alterations during experimentally induced diabetes in rats. This information will provide an insight which not only help in understanding the aura of consequent complications of diabetes but also in establishing a concrete strategic therapeutic approach for developing the treatment of diabetes associated cognitive and behaviour deficits.
Therefore, the main objectives of the present study are:

a) To study the glial response following STZ-induced diabetes state and associated neuronal damage with special reference to neuroimmunological changes.

b) To record the phenotype changes in microglia and astrocytes following STZ-induced diabetes.

c) To correlate the cognitive changes following induced hyperglycemia to the allied glial response in terms of immune activity.
REVIEW OF LITERATURE

1. Diabetes mellitus: Global health havoc

Diabetes mellitus is a global health problem swiftly attaining the status of a potential epidemic. According to a report of world health organization (WHO), there are 347 million people worldwide suffering from diabetes (Danaei et al., 2011), and this number is estimated to double by 2030 (WHO, 2013). In India, about 62 million people are facing the prevalent of diabetes, and presumably this number will become 79.4 million in the next fifteen years (Kaveeshwar and Cornwall, 2014).

Diabetes mellitus is a heterogeneous endocrine disorder mainly categorized in two forms, i.e., type 1 and type 2. Type 1 diabetes is an autoimmune disorder resulting from the absolute or relative destruction of the pancreatic beta cells leading to depletion in insulin production. It is also defined as insulin-dependent diabetes mellitus (IDDM). Type 2 diabetes is non-insulin-dependent diabetes mellitus (NIDDM) characterized by the reduced insulin sensitivity or relative insulin deficiency. Irrespective of its form, diabetes mellitus associated chronic hyperglycemia trails various micro- and macro-vascular complications. Diabetes alters the blood-glucose homeostasis which further affects the end-organs like kidney, eyes, brain, etc. In addition to this, diabetes has also been associated with many structural and functional complications in both central and peripheral nervous system (Biessels, 1994; Baydas et al., 2005; Guven et al., 2009). The underlying mechanism(s) causing these alterations are not fully understood. The presumed causative factors are (i) variations in the cerebral energy homeostasis and metabolism possibly through fluctuation in blood-glucose level (Horani and Mooradian, 2003; Brands et al., 2004; Scherbakov et al., 2012); (ii) changes in osmolar gradients in hyperglycemia (Stevens et al., 1993; Wang et al., 2012); (iii) dysregulated glucose utilization via glucose transporters (Duelli et al., 2000; Lacombe, 2014); (iv) acute and/ or chronic metabolic and vascular impairment, such as deficits in cerebral
blood flow, changes in osmolytes (Biessels et al., 2002; Brands et al., 2004) as well as oxidative stress (Sharma et al., 2010; Wang et al., 2011; Soares et al., 2012; de M Bandeira et al., 2013), deposition of advanced glycated end products (Yan et al., 2008; Wang et al., 2009; Vlassara and Striker, 2013) and the altered levels of ketone bodies (Felig, 1974; Balasse and Fery, 1989; Umpierrez et al., 2002) may cause functional and structural cerebral changes in diabetic patients (Biessels et al., 2002; Brands et al., 2004).

1.1. Type 1 diabetes

Type 1 diabetes is a common and chronic disorder with notable incidence in children and adolescents. It is due to the auto-immune destruction of pancreatic beta cells leading to an absolute loss of endogenous insulin production (Atkinson, 2001; Bluestone et al., 2010). This results in elevation of blood-glucose level (hyperglycemia) trailing the characteristic symptoms of type-1 diabetes i.e., polydipsia, polyuria and body weight loss. In a diabetic state as body run out of the insulin, the energy extraction from blood glucose gets depleted and then frequent need of energy causes the breakdown of fats (through lipolysis and release glycerol and free fatty acids) and other stored energy reservoir which further results in sudden body weight loss. Furthermore, the increased level of free fatty acids get converted into ketones and resulted in elevated ketone level and decreased hydrogen ion concentration (pH) in the body fluids. The accretion of ketones in body fluids, reduced pH and electrolyte level and dehydration from excessive urination, and alterations in the bicarbonate buffer system result in diabetic ketoacidosis (DKA). Uncontrolled severe hyperglycemia is prone to develop DKA that can result in coma or death. Similarly, the increased glucose content in blood also causes osmotic effect and starts pulling out water from the body to dilute the excess of glucose that causes the excessive thirst or polydipsia and subsequently resulted in the state of polyuria in order to dampen the superfluous glucose water in form of urine. The abrupt body weight loss due to low energy conversion and conservation by the cells leads to hunger state
and resulted in polyphagia to compensate the energy loss required for normal body functioning and maintenance.

1.1.1. Pathophysiological mechanism

Diabetes-related impediments are not just limited to the insulin dependent organs like muscle and adipose tissue but also extended upto the other vital organs, i.e., kidney, liver, eyes and brain. These organs are directly affected by the glucose toxicity allied hyperglycemia during the diabetes mellitus.

Hyperglycemia dramatically alters the function of various cell types and their extracellular matrix causing changes in structure and functions of the affected tissue. Hyperglycemia trailed diabetes causes glycation of proteins, fats and nucleic acids. The advanced glycation end products (AGEs) accumulate in renal glomerulus, endoneurial areas, microvasculature of the retina and at the walls of the larger blood vessels.

The invariant critical participation of various cellular pathways generated and/ or stimulated by diabetes allied hyperglycemia is well known in reinforcing the possible reasons of diabetic complications (Fig. 1; Brownlee, 2001; Vlassara and Palace, 2002). During diabetic state excess of glucose get converted into sorbitol and fructose through polyol pathway and generate a milieu of osmotic stress inside the cell which further resulted in the oxidative stress and cell death. Similarly formation of advanced glycation end products (AGEs) and increased glucose shunting in the hexosamine pathway stimulate the inflammatory signals which later on ensues the diabetic complications. Furthermore superfluous AGEs production increases the level of circulating AGEs and affects the normal protein functions of the cell which ultimately causes the alterations in renal, vascular and connective tissues. Stress signalling via MAPK, Jak/Stat, p38 and NF-kB pathway is another marked consequent of hyperglycemia resulting in elevation of cytokines and iNOS level developing the state of vascular damage and cell death. Hyperglycemia also leads to the
mitochondrial dysfunctions leading to the activation of protein kinase C isoforms which further leads to oxidative stress allied, alterations of structural proteins and damage the relative organ via causing alterations in gene expression and enzyme functions. Thus, type 1 diabetes resulted in long-term complications, which develop gradually and even increase the risk of other life-threatening and disabling syndromes.

1.2. Effect of diabetes on brain

Diabetes associated hyperglycemia induces multiplying changes in vascular and neuronal cells, as well documented in both animal model of diabetes and diabetic patients. The changes at both cellular and functional level following diabetes are pleotypic in nature as the flow of glucose and its metabolites is well-known to affect several cellular pathways. As glucose is the prime source of energy in brain, and its consistent functioning is reliant on the continuous supply of glucose (Clarke and Sokoloff, 1999; Mergenthaler et al., 2013), so any alteration in glucose content or supply would possibly affect the cerebral functions (Ryan et al., 2004; Oddo et al., 2008). The well-recognized diabetic impediments in CNS are listed as:
impaired cognition (Gispen and Biessels, 2000; Allen et al., 2004; Arvanitakis et al., 2006; Biessels et al., 2008; Kodl and Seaquist 2008; Nagayach et al., 2014a; Mayeda et al., 2015), dementia (Biessels et al., 2002; Wessels et al., 2008; Cheng et al., 2012; Crane et al., 2013), altered learning and memory processes (Popovic et al., 2001; Baydas et al., 2003; Choi et al., 2009; Capiotti et al., 2014), stroke (Tiehuis et al., 2008; Eriksson et al., 2012; Hägg et al., 2014), cerebrovascular disorders (Mankovsky et al., 1996; Yan et al., 2013) and Alzheimer’s disease (Ahtiluoto et al., 2010; Kim et al., 2013; Wang et al., 2015).

1.2.1. Mechanism and consequent effect of cellular damage following diabetes in brain

During diabetes increased level of glucose reacts with oxygen and generates a series of oxidative radicals. The state of oxidative stress caused by these free radicals increase the polyol pathway activation, formation of advanced glycation end products (AGEs), enhance the activation of protein kinase C and glucose shunting in the hexosamine pathway that could ultimately damage the cell constituents and resulted in cell death (Yamagishi et al., 2003; Van Harten et al., 2006). Hyperglycemia instigated through reduced level of insulin provides substrate for the anaerobic glycolysis that resulted in brain lactic acidosis and dehydration which further involved in diminished cerebral blood flow and ischemia (de Kloet et al., 1998; Chan et al., 2003).

Long term studies on STZ-induced rodent model characterized the diabetic encephalopathy in relevant regions of the brain i.e., cerebral cortex, cerebellum and hypothalamus. Diabetes trigger various morphological changes in brain cells like increased area of myelinated neurons, disarrangement of myelin sheath, perivascular and mitochondrial swelling, presynaptic vesicle dispersion in swollen axonal boutons and fragmentation of neurofilaments (Hernández-Fonseca et al, 2009). Interestingly the oxidative and nitrosative stress milieu in the brain of STZ-induced diabetes rats was imposed by the mitochondrial
swelling and vacuolation (Mastrocola et al., 2005) caused by increased intracellular glucose level or via other damaging inducers following diabetes (Hernández-Fonseca et al, 2009).

Among various brain regions hippocampus is more susceptible to diabetes. Previous studies conducted on both spontaneous model of autoimmune T1D (NOD mice) and STZ-induced model depicted the deleterious effect of diabetes. Diabetes dramatically decreases the cell proliferation (Saravia et al., 2004; Revsin et al., 2009; Piazza et al., 2011), altered the neurogenesis (Beauquis et al., 2006, 2008; Bachor and Suburo, 2012) and causes neuronal apoptosis in hippocampus (Beauquis et al., 2006, 2008; Nagayach et al., 2014a) and damaged the dendrites and synaptic structures of CA3 neurons (Jackson-Guilford et al., 2000; Li et al., 2002; Zhang et al., 2008). Diabetes also reduced the dendritic growth of newly formed neurons in dentate gyrus that severely affect the neurogenesis (Alvarez et al., 2009; Choi et al., 2009). The deleterious effects of diabetes not only limited to the malfunctioning of the hippocampal cells but also affecting the synaptic plasticity (Mardirossian et al., 2009; Detka et al., 2013) and associated learning memory (Stranahan et al., 2008; Revsin et al., 2009; Piazza et al., 2011). The above discussed consequent of diabetes on hippocampus provides an iota of explanation for the cognitive impairment following diabetes.

2. Experimental animal models of diabetes mellitus

For the advancement of information and to explore the facts, several animal models have been established to study the diabetes mellitus and related consequences more precisely. These models are not just providing evidence regarding pathogenesis of the disease but also helping to a great extent in developing therapeutic preventive strategies to combat the diabetic state and associated complications. These animal models were developed using chemical, genetic, surgical (pancreatectomy) and other techniques. Success of these animal models relies on their depiction of various clinical features and related phenotypes of diabetes mellitus.
Chemicals used to create the diabetic animal models are aimed to selectively destroy the pancreatic beta cells partially or completely and retain the hyperglycemic state for a sustainable duration without causing unsolicited syndromes other than diabetes. Studies over the past 70 years considered, alloxan and Streptozotocin (STZ) as the most used chemical substances for developing successful animal models for both Type 1 and Type 2 diabetes mellitus (McNeill, 1999; Rees and Alcolado, 2005; Ventura-Sobrevilla et al., 2011). The universal proclaim regarding alterations in pancreatic beta cells as well as in the animal physiology following alloxan (Lenzen and Patten, 1988; Kikumoto et al., 2010) and streptozotocin (Rakieten et al., 1963; Balamurugan et al., 2003; Eleazu et al., 2013) induced diabetes made them a well-accepted diabetogenic drug even today. The metabolic disturbances in rats caused by these diabetogenic drugs (Szkudelski et al., 1998, 2013) and their mechanisms of cytotoxic action on the pancreas are thoroughly illustrated by various studies (Lenzen and Patten, 1988; Szkudelski, 2001; Hayashi et al., 2006; Lenzen, 2008). Streptozotocin and alloxan exert their effect via administrating parentally (intravenous, intraperitoneal or subcutaneous) at specific dose depending upon the animal species and nutritional status of the animal (Federiuk et al., 2004; Etuk and Muhammed, 2010).

Intriguingly, besides having similar diabetogenic action, streptozotocin is widely opted over alloxan for developing the animal model for diabetes (Islas-Andrade et al., 2000; Lee et al., 2010; Eleazu et al., 2013). Streptozotocin is not only capable of inducing an irreversible long-term effect of diabetes (Gaulton et al., 1985; Méndez and Ramos, 1993; Eleazu et al., 2013) but also has a low mortality rates in comparison to alloxan (Mansford and Opie, 1968; Lenzen, 2008; Chatzigeorgiou et al., 2009; Deeds et al., 2011).

3. A brief account on Streptozotocin: A diabetogenic drug

In 1963, Rakieten and his co-workers claimed that streptozotocin is a diabetogenic drug. It is a naturally occurring broad-spectrum, cyto-toxic chemical isolated from a mould
Streptozotocin is popularly used to induce both insulin-dependent and non-insulin dependent diabetes mellitus.

**Chemical name**: 2-deoxy-2-([(methylnitrosoamine) carbonyl] amino)-D glucopyranose

**Structure**:

![Chemical Structure of Streptozotocin](image)

*Cytotoxic methylnitrosourea moiety (N-methyl-N-N-Nitrosourea) attached to the glucose (2-deoxyglucose) molecule.*

**Mechanism of Action**

Streptozotocin induces diabetes in mammals via inhibiting insulin production in pancreas by killing beta cells.

*Streptozotocin (through i.p. or i.v. injection)*

![Mechanism Diagram](image)

Streptozotocin selectively accumulates in the pancreatic beta cells through low affinity GLUT-2 transporters (Tjälve et al., 1976). The DNA alkylating methylnitrosourea moiety (especially at o6 position of guanine) of STZ causes DNA fragmentation (Bennett and Pegg, 1981; Murata et al., 1999; Szkudelski, 2001) along with a defined chain of damaging events
like protein methylation (Bennett and Pegg, 1981; Wilson et al., 1988), nitric oxide (NO) generation that inhibits aconite activity (Kröncke et al., 1995) and reduced level of cellular NAD$^+$ and ATP (Uchigata et al., 1982) that conclusively causes necrotic cell death and hamper the insulin biosynthesis and secretion (Yamamoto et al., 1981; Uchigata et al., 1982). Furthermore, the diabetogenic toxicity of streptozotocin is also accompanied and accelerated by the resultant oxidative stress caused due to a low level generation of ROS, counting superoxide and hydroxyl radical generation from hydrogen peroxide dismutation during hypoxanthine metabolism following enhanced ATP dephosphorylation.

4. The brain cells

Cellular organization of central nervous system (CNS) is mainly constituted of about 10% neurons and 90% of glial cells. Despite of dissimilarity in cellular excitability the reciprocal communication between neurons and glial cells, accomplish the appropriate functioning and maintenance of CNS.

4.1. Neuroglia

One hundred and fifty years ago i.e., on 3rd April 1858, the whole neuroscience world came to know about the term ‘neuroglia’ (nerve glue) when a German pathologist, Rudolf Ludwig Karl Virchow described them. For Virchow, neuroglia was a connective material around the neuron just binding them together. Although these glial elements were also recognized even before Virchow’s proclaim, but Virchow’s nimble description made them a confrontational entity to study and explore in the brain. Soon after, many different forms of glial cells were described and images published by Otto Deiters (1850s), Jacob Henle (1869), Camillo Golgi (1898), Gustav Retzius (1894) and many others.

There are different types of glial cells, i.e., astrocytes, microglia, oligodendrocytes, Schwann cells and radial glial cells. Each of these glial cell types has its own specific attributes, tasks and characteristics. It was speculated that there are at least 10 glial cells for
every single neuron. As their size is much smaller than neurons, they occupy about 50% of the total volume of nerve tissue. Glia were involved in several functions ranging from scavenging debris, scaffolding and protecting neurons, facilitating the synaptic transmission, to putatively integrate the information with neurons. Thus, there have been many groundbreaking studies that are devoted to the structure and functions of glial cells and establish their important and beneficial roles in brain functioning and disorders (Verkhratsky et al., 2014a). An article in Forbes Magazine (July 13, 2009) asserted that glial cells may even be more imperative than neurons for treating neurodegenerative diseases.

4.1.1. Astrocyte

Astrocytes (also known as astroglia) are characteristic star-shaped glial cells in the nervous system. The term ‘astrocyte’ a greek word stands for the star-shaped cell (‘Astar’ means star and ‘cyte’ means cell), which was first proposed by Michael von Lenhossek in 1893, and subsequently, it gained universal acceptance. Astrocytes have been observed as an intricate community of cells. True and the largest group of astrocytes share a similar stellate-shaped architecture, multiple extended thin processes, interface connection with blood vessels and close communications with both neighbouring neurons and mesenchymal elements of the brain (Volterra and Meldolesi, 2005).

Astrocytes are the most abundant macroglial cells in the CNS, having numerous projections. Astrocyte is a parasol term for a heterogeneous population of cells that is highly vulnerable to its local environment. In addition to their vastly different territorial organization across brain regions and species, astrocytes also differ in their physiological properties such as membrane potential, potassium conductance, glutamate transporter, receptor expression and the expression of proteins such as glial fibrillary acidic protein (GFAP; Kalman, 1998; Doetsch, 2003; Oberheim et al., 2012).
Astrocyte functions are grouped into three main categories: guidance and support of neuronal migration during development (Peretto et al., 2005; Bozoyan et al., 2012), maintenance of the neural microenvironment (Stitt et al., 1991) and modulation of immune reactions by serving as antigen-presenting cells (Dong and Benveniste, 2001; Girvin et al., 2002; Barcia et al., 2013). The star-shaped cells are regarded as crucial homeostatic cellular element in the brain as it controls the brain environment by regulating the volume and composition of extracellular space (Kettenmann and Verkhratsky, 2008) and controls the extracellular ion- metabolite- and neurotransmitter homeostasis (Haydon and Carmignoto, 2006; Verkhratsky and Butt, 2007; Verkhratsky and Parpura, 2010). They also play an important role in secretion or absorption of neural transmitters (Perea et al., 2014) as well as in the maintenance of the blood-brain barrier (Bundgaard and Abbott, 2008; Alvarez et al., 2013). Both in vivo and in vitro studies revealed that astrocytes mediate neurotrophic signals and ardently promote the neuronal survival in the CNS (Wagner et al., 2006). The astrocytes express, both ligand-gated and voltage-dependent ion channels (Sontheimer, 1994; Porter and McCarthy, 1997; Verkhratsky and Steinhauser, 2000), and are proposed to have the general function of clearing neurotransmitters and ions away from the synapse (Henn and Hamberger, 1971; Perdan et al., 2009). Astrocytes interact with essentially all other cellular elements of the brain. They contain high affinity glutamate transporters that are critical in maintaining the extracellular glutamate concentration at sub-excitoxic levels and thereby preventing neuronal cell death (Rothstein et al., 1994, 1996; Anderson and Swanson, 2000; Verkhratsky et al., 2014b). Furthermore, astrocytes also play an active role in modulating the neuronal activity and behaviour (Perea and Araque, 2007; Navarrete and Araque, 2008; Halassa and Haydon, 2010). Metabolically, astrocyte supports the neurons by providing them lactate as a nutrient (Cakir et al., 2007; Brown and Ransom, 2007; Magistretti, 2008). Experimentally
activated astrocytes express some metabolic enzymes and transporters, which supports in boosting the resistance towards metabolic injuries and facilitates the neuronal survival (Escartin et al., 2007). This plethora of functions makes these cells as an ideal concierge of neurons.

4.1.1.2. Astrogliosis

Astrogliosis is another popular term used for the astrocytic activation. Astrocytes’ customary functioning may be enhanced by its activation via pharmacological agents or induction of astrocyte reactivity in various pathological conditions. On activation, astrocytes exhibit discernible morphological and functional changes (Figure 2). In response to any stress, injury or pathological conditions, quiescent astrocyte having fine processes transform into activated state that comprises thick hypertrophic processes and soma with the upregulation of cytoskeletal components like glial fibrillary astrocytic protein (GFAP), vimentin, nestin and S100β (Pekny and Nilsson, 2005; Buffo et al., 2010). Activated astrocytes migrate towards the infected region and demarcate it from the healthy part by forming glial scar, a type of anisomorphic gliosis (Kalman, 2004). Astrogliosis as an emblem of neuroinflammation generally considered as a pathological reaction that further instigate a negative influence on the brain pathology. In contrast to this general view, customized astrocyte activation based on the progression of insult and severity of stimulus, may also imposes a defensive and beneficial effect upon the brain cells (Sofroniew and Vinters 2010; Verkhratsky et al. 2012; Colangelo et al., 2014).
Furthermore, astrocytes secrete several inflammatory molecules that directly or indirectly help in the general improvement of brain cell functions like enhanced glutamate uptake, reorganization of metabolic pathways, modulation of synaptic transmission, etc. (Liberto et al., 2004; Sofroniew, 2005; Vesce et al., 2007; Pekny et al., 2007; Claycomb et al., 2013). Astrogliosis has also been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, inflammatory demyelinating disease, cerebral ischemia and Prion-associated spongiform encephalopathy (Mena and García, 2008; Takano et al., 2009; Sofroniew and Vinters 2010; Verkhratsky et al., 2012; Verkhratsky et al., 2014c). This intrinsic ability of

---

**Fig. 2:** Morphology and functions of astrocyte after its transformation from normal to reactive astrocytes under different adverse conditions.
astrocytes makes them important therapeutic targets as they stringently retort to every sort of brain injury and act as saviour via developing a broad range of defence mechanisms against injury or insult.

4.1.2. Microglia

Microglia were first recognized by Nissl in 1880 and thoroughly portrayed by a Spanish neuroanatomist, Pio-del Rio-Hortega as resting ramified cells by light microscopy and silver staining methods. Microglial cells constitute about 5-20% of glial population in central nervous system. As immunocompetent nomadic cells of the brain, microglia continuously survey the CNS microenvironment with their highly motile extensions for any kind of brain insult or injury. As resident immunocompetent phagocytic cells, they perform functions similar to those of tissue macrophages present in other organs and comprise the first line of defence against any baleful invasion. The well-accepted notion of microglia structural plasticity in response to the adverse conditions and its dual action following various pathological conditions, were vastly studied in both in vivo and in vitro models of animal and humans. Moreover, in addition to the defensive role, microglia also takes part in the maintenance and regulation of various vital processes of brain functions throughout the lifespan, which have been neglected so far.
In normal healthy brain ‘resting (ramified) surveillent’ microglia resides at strategic locations throughout the entire brain and spinal cord. The rationale of this state is to maintain a constant level of available microglia to detect and fight the infections (Aloisi, 2001; Christensen et al., 2006). In the resting or ramified state, microglial cells release neurotrophic growth factors to promote neuronal survival and enhance the neurogenesis (Kim and de Vellis, 2005; Ekdahl et al., 2009; Shigemoto-Mogami et al., 2014; Sierra et al., 2014). During various neurodegenerative diseases and brain insults resident/ resting microglia turn into activated or reactive microglia (Streit et al., 1999; Ling et al., 2001; Minghetti et al., 2005; Lull and Block, 2010). Under adverse circumstances microglia release myriad of inflammatory molecules, growth factors, matrix proteins, chemokines, prostanoids and reactive free radicals (Fig. 3) which contribute in dual form i.e., either impose neuronal dysfunction and cell death or provide support in the healing of damaged tissues (Block et al., 2007; Colton, 2009; Gomes-Leal, 2012; Hellwig et al., 2013). The biphasic (detrimental or beneficial) role of microglia depends upon the progression of insult and severity of the stimulus.
4.1.2.1. Microglial structural plasticity

Morphologically, three major types of microglial forms appear: amoeboid, ramified or resting and reactive or activated. These different transition stages make them effectively perform varied functions in the brain (summarized in Table 1).

Table 1: Functions of microglia during different transition phases

<table>
<thead>
<tr>
<th>Amoeboid microglia</th>
<th>Ramified microglia</th>
<th>Activated microglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulate gliogenesis (Giulian et al., 1988).</td>
<td>Vigilant surveyor of function and integrity of brain (Davalos et al., 2005).</td>
<td>Major source of pleiotropic cytokines (Tambuyzer et al., 2009).</td>
</tr>
<tr>
<td>Phagocyte the degenerating cells (Ling et al., 2001).</td>
<td>Precursors for active macrophages (Davis et al., 1994).</td>
<td>Helps in regeneration (Rapalino et al., 1998).</td>
</tr>
<tr>
<td>Tissue histogenesis via removing inappropriate and superfluous axons (Marín–Teva et al., 2004).</td>
<td>Mitigate excitotoxicity-induced degeneration (Vinet et al., 2012).</td>
<td>Secrete neurotrophic factors (Streit, 2002).</td>
</tr>
<tr>
<td>Astroglial proliferation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Amoeboid microglial cells are round or irregular in shape and are prevalent during development and rewiring of the brain. Morphologically, they closely resemble with the macrophages. Amoeboid microglia seeds via primitive myeloid/haematopoietic progenitor cells during the embryonic and perinatal stage and sustain up to the early postnatal stages (Ginhoux et al., 2010; Prinz and Mildner, 2011; Kierdorf et al., 2013). Later at postnatal stages of development these amoeboid microglia transform into ramified (resting) microglia (Kaur et al., 1985; Kaur and Ling, 1991).

Ramified or resting microglial cells are the monitoring immune cells of the adult CNS. Ramified microglia have a small cell body with short, wispy and fine processes extending into the brain microenvironment and creating a matrix like structure to better perceive the CNS milieu. The term resting is a misnomer as these microglial cells in a healthy brain continuously survey the CNS for any damage or insult, so they are not resting but active.
(Ransohoff and Cardona, 2010; Kettenmann et al., 2011; Aguzzi et al., 2013; Kettenmann et al., 2013). In vivo time-lapse video microscopy in mouse has confirmed that the fine processes and protrusions of ramified or resting microglia cells constantly sample the CNS microenvironment (Nimmerjahn et al., 2005). As such they are never in a resting state in the brain.

**Reactive Microglia**

In response to any unfavourable stimuli ramified microglia are transformed into a reactive or activated state characterized by thick and retracted processes with a large and irregular shaped cell body (Gehrmann, 1995). Reactive microglial cells during the disparaged condition starts proliferating to better screen and fight back against the sabotage (Niquet et al., 1994; Huttmann et al., 2003). The population increment and morphological transformation of such reactive cells in affected site is considered as the hallmark of microglial activation (Perry et al., 1985; Castano et al., 1996).

The intricacy to identify the microglial function is eclipsed by the differential phenotypes of the cells, as microglial activation generally acts in two ways either help in efficacious restoration of injured brain cells or generates a threatening environment which causes subsequent brain alterations depending upon the stimulus and progression of the diseased state. To overcome the situation, an activation spectrum was exemplified to characterize the activity-dependent microglial activation. The spectrum was developed on the basis of cytoactive factors released by the reactive microglia.

The “classical activation (popularly known as M1 phase)” is the initial innate immune response induced by TLR ligand and IFN-γ followed by the generation of pro-inflammatory cytokines (Luo and Chen, 2012; Boche et al., 2013). This state of microglial activation promote the protection of damaged tissue via defending against the pathogens by releasing a plethora of pro-inflammatory molecules (like TNF-α, IL-6, IL-1β, IL-12; Benoit et al., 2008),
superoxide anions, nitric oxide synthase and proteases etc.), redox (NOX2, NOX1, RAC1, iNOS, NOS2 etc.) and excitotoxic molecules (MGlur2, GLT-1, P2X7-R etc.). Another phase of microglial activation is “M2 or alternate activation” phase. This is considered as a protective phase as it is characterized by the production of anti-inflammatory molecules such as TGF-β, IL-10, scavenging receptors and extracellular matrix. The cytoactive molecules of this phase down-regulate the generation of pro-inflammatory molecules and accelerate the process of wound healing and damaged tissue repair (Martinez et al., 2008). The third or subtype of M2 phase is termed as “acquired deactivation”. It is associated with deactivation of glial inflammation and uptake of apoptotic cells or oxidized lipids via the release of anti-inflammatory cytokines (Elenkov and Chrousos, 2002; Luo and Chen, 2012; Boche et al., 2013) and glucocorticoid hormones (Mantovani et al., 2004).

5. Role of glial activation in CNS

In the central nervous system, glial cells as a heterogeneous group of cells perform varied functions, i.e., to shape the microarchitecture of the brain, provide support and protection, control extracellular ion- metabolic- and neurotransmitter homeostasis, form myelin, destroy and remove the carcasses of dead neurons, and help in signal transmission, etc. Several studies have elucidated the functions performed by activated microglial and astroglial cells following stringent pathological conditions, injuries and stress in the brain. Glial cells are the dynamic entities that exhibit morphological changes reliant upon their cellular microenvironment. The morphological alterations in both astroglia and microglia are considered as a reliable marker of their activated state, which further trailed by the severe influence on brain cells and associated functions.

5.1. Microglial activation

Microglial cells being resident and as immunocompetent phagocytic cells perform functions similar to those of tissue macrophages present in other organs and constituting the
first line of defence against invading pathogens (Streit, 2002; Vilhardt, 2005). In response to neurodegenerative stimuli either via any infection (Vooend et al., 2010; Mutnal et al., 2011; Tripathi et al., 2014), injury (Galiano et al., 2001; Patro et al., 2005, 2010b), inflammation (Riazi et al., 2008; Patro et al., 2004; Patro et al., 2010a), blood brain barrier (BBB) damage (Davies et al., 1998; Stoll and Jander, 1999) or metabolic disorder (Nagayach et al., a,b), microglia get activated and release an incredible array of immunocompetent molecules, which comprise numerous chemokines like KC, MIP-1α, MIP-1β, MIP-2, MCP-1, RANTES, IP-10, and IL-8 (Hanisch, 2002) and interleukins like IL-1α/β (Peterson et al., 1997; Meda et al., 1999; Nagai et al., 2001), IL-3, IL-6 (Pulliam et al., 1995; Nagai et al., 2001), IL-10 (Ledeboer et al., 2002), IL-12 (Stalder et al., 1997), IL-15, IL-18 (Suzumura, 2002), tumour necrosis factor α (TNF-α; Chabot et al., 1997), interferon gamma inducing factor (IGIF), inflammatory proteins, transforming growth factor (TGF-β; Pratt and McPherson, 1997) etc. Collectively, these molecules not only control the inflammatory processes but also help in the regulation of immune response of the brain (Glabinski and Ransohoff, 1999) and contribute to neuropathogenesis in CNS inflammation. Concurrently activated microglia also phagocytose the debris of apoptotic cells (Reichert and Rotshenker, 2003; Djukic et al., 2006; Neher et al., 2012) that helps in facilitating the reorganization of neuronal circuits and triggers the repair mechanism (Kim and Vallis, 2005; Hanisch and Kettenmann, 2007; Neumann et al., 2009; Salter and Beggs, 2014). In conclusion, microglial cells as immune regulators of the nervous system secrete several types of molecules or express various receptors that facilitate the integration of microglial response towards the changing microenvironment.

5.2. Astrocytic activation

Similarly, astrocytes are being increasingly believed to act as immunocompetent cells within the brain (Shrikant and Benveniste, 1996; Kettenmann and Ransom, 2005). They rapidly react to various neurodegenerative insults leading to vigorous astrogliosis. Such
reactive gliosis is associated with alteration in morphology and structure of activated astrocytes along with their functional characteristics (Mucke and Eddleston, 1993; Ridet et al., 1997). Astrocytes being the predominant “building blocks” of the blood-brain barrier (BBB) play a crucial role in sealing the barrier upon brain injury (Bush et al., 1999; Bundgard and Abbott, 2008) and infections (Barres et al., 2008). Upon activation, astroglia not only release a plethora of factors (cytokines, chemokines, prostanoids, etc.) mediating the tissue inflammatory response but also recapitulate stem cell/ progenitor features after damage and prove as a promising target for reparative therapies following brain damage (Buffo et al., 2010; Verkhratsky et al., 2012; Verkhratsky et al., 2014c).

Recently, there have been many groundbreaking studies that are devoted to the structure and functions of activated glial cells and established their imperative and beneficial role in various brain disorders. Given their functions, glial activation following brain damage represents targets for therapeutic interventions.

6. Gliarial changes following STZ–induced diabetes

The imperious role of glial cells in brain disorders was remarkably evident in the diabetic state, which further underpins the possible reasons of brain dysfunction following diabetes. Diabetes causes the generation of free radicals which actively assault macromolecules within neuron and glial cells, resulting in structural and functional changes in proteins. Various studies revealed that oxidative stress following diabetes on brain cells, causes a selective decrease in astrocyte GFAP levels in the cerebral cortex, cerebellum, hippocampus (Coleman et al., 2004; Hernández-Fonseca et al., 2009; Guven et al., 2009) and spinal cord (Renno et al., 2008) with apparent neurodegeneration at 4-8 weeks of diabetic duration. The diabetic allied decreased GFAP levels, without altering the relative astrocyte number, may be linked to insulin secretion, which essentially takes part in the modification of phenotypic appearance of astrocytes and increases the expression of GFAP mRNA and
protein (Toren-Allerand et al., 1991). A study depicted that microglial activation in the optic nerve in diabetic retinopathy might be an additional pathogenic factor in diabetic optic neuropathy (Zeng et al., 2008). Likewise, there is only one report in the literature demonstrating the response of microglia to STZ-induced diabetes by assessing the reduction in the number of Iba-1 positive microglia in the dorsal horn of the spinal cord following gabapentin treatment in diabetic neuropathy (Wodarski et al., 2008). The impact of hyperglycemia on glial cells per se or their response to neuronal damage remains to be studied in detail. Particular attention needs to be taken on neuro-immunological aspects of neurodegeneration and neuroregulation under diabetic conditions.

Studies on animal models have also shown that diabetes induced GFAP alteration has been linked to other central nervous system disturbances like distorted learning and memory processes and further increased risk of dementia and cognitive dysfunction. With regard to the functional importance of microglial cells in brain following any stress or degenerative changes, it would be quite interesting to study the microglial activation following diabetes in the brain regions, responsible for behaviour and cognition. On activation, microglia secretes plethora of cytokines and chemokines initiating neuroinflammation, which in turn influence neuronal functions and even some of them also play a critical role in learning, memory (Goshen et al., 2007; McAfoose et al., 2009) and motor activity (Patro et al., 2010a). Such microglial activation and related neuroinflammation have never been studied in the diabetic brain hippocampus, neither their expression nor the immune response developed by these cells. Glial cell research is actively addressed today on various aspects of their involvement in neuronal health and disease, but their response to diabetic states has not been studied in large details and thus requires precise investigation.

7. Brain immune response: Neuroinflammation
Our central nervous system and immune system share a bi-directional collaborative relationship, assumed to be imperative in establishing a pertinent physiological, behavioural and immunological response against any injury or disorder. Central nervous system has been considered as “immune privileged” due to the lack of humoral immune response constituting draining lymphatics as seen in the periphery (Barker and Billingham, 1977; Carson et al., 2006). Immunologically insulated and protected through blood-brain barrier (BBB), CNS innate immune system as first line of defence constitutes macrophages, resident microglia and antigen presenting cells. Cellular molecules of innate immune system of CNS in response to any insult or damage, get activated, mobilize towards the site of insult, phagocytose the detrimental factors, present antigens, release and synthesize a varied range of inflammatory molecules such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 that serve as major mediators of the immune response. Interestingly, immune privileged nature of CNS is still intricate and under relevant exploration (Wekerle, 2006; Galea et al., 2007; Harris et al., 2014).

The secretion of inflammatory molecules regarded as “double-edged sword” because of its potential to both protect and harm the tissue. Glial cells as foremost cell population and functionally concierge to the CNS intensely contribute in neuroinflammation (summarized in section 2.2.1 and 3). Neuroinflammation is a dynamic process in which both microglia and astroglia may migrate, proliferate, release potentially harmful factors (i.e. cytokines and reactive oxygen species), display different surface proteins (i.e., MHC-I/II etc.) and bind in functions such as antigen presentation and phagocytosis in response to signals such as protein aggregates, neuronal degeneration and glial products (i.e. colony stimulating factor and cytokines etc.). Under normal conditions, neuroinflammatory response remains transient and non-damaging at both tissue and functional level. But if sustain for prolong duration it probably becomes damaging extended to both tissue and region-specific functions. Several
studies have reported that following pathological conditions the protective role of inflammation switches to the detrimental phase resulted in synaptic impairment, cell death and neurotoxicity that finally become the possible perpetrators of neuronal damage within the brain (Cunningham et al., 1996; Aktas et al., 2007; Frank-Cannon et al., 2009; Bjorkqvist et al., 2009; Lyman et al., 2014).

7.1. Effect of neuroinflammation on behaviour and cognition

Neuroinflammatory mechanism is comprised of an organized set of interaction between varied mediators like cytokines, chemokines and prostaglandins, etc. Rather than pathological conditions the secretion of inflammatory molecules is highly influenced by the glial activation also. In response to any injury, insult or disparaged conditions the apparent activation of glial cells (both microglia and astroglia) trigger the secretion of inflammatory molecules that circumstantially become superfluous at chronic glial activation (Block et al., 2007). Various studies found that excessive expression of cytokines mainly IL-1β, TNF-α and IL-6 induce non-specific symptoms of diseases like lethargy, low activity and reduced social interaction (Kelly et al., 2003). Even elevation in TNF-α and IL-1β level found to be involved in the cognitive impairment and dementia in AD patients (Magaki et al., 2007; Holmes et al., 2009). Studies have shown that exogenous administration of IL-1β or TNF-α to rodents induces the full spectrum of behavioural signs of sickness in a dose- and time-dependent manner while IL-6 could cause fever like symptoms (Dantzer, 2001; Dantzer et al., 2008). Likewise, IL-1β is also capable in directly affecting the hippocampal neurons causing impairment in long term potentiation (LTP; Schneider et al., 1998; O'Connor and Coogan, 1999; Vereker et al., 2000; Lynch, 2014). Experiments showed that prolong exposure of IL-1β reduces the BDNF and TrK B concentration in hippocampus and up-regulates TrK A and p75 expression. Enhanced expression of p75 receptors in cholinergic neurons causes neuronal apoptosis and degeneration of hippocampal cells leading to cognitive deficits (Barrientos et al., 2004;
Coulson et al., 2009). Excessive cytokine secretion as exaggerated immune response is believed to be involved in several neuropsychiatric conditions like depression (Howren et al., 2009; Dowlati et al., 2010; Blume et al., 2011), autism (Ashwood et al., 2011; Depino, 2013) and anxiety behaviour (Pitsavos et al., 2006; Arranz et al., 2007; Vogelzangs et al., 2013).

It had been shown that neuroinflammatory mediators are capable in causing cognitive alterations via various mechanisms that could possibly affect the neuronal properties and cell survival. Several studies are conducted to conceptualize the possible cause and link between neuroinflammation and behavioural and cognitive impairments. Such studies proposed that an associative interplay between cellular excitotoxicity, neuronal cell death, chronic glial activation and oxidative stress trigger the state of cognitive and behavioural deficits. During pathological condition, cellular damage causes activation of glial cells which initiate and perpetuate the state of excitotoxicity and oxidative stress within the brain. Furthermore, oxidative stress leads to the excessive dicarbonyl glycation which further activates the calpain expression and degrades the brain-derived neurotrophic factor (BDNF). This contributes to retard the process of neurogenesis and synaptic plasticity and stimulates NFkB-dependent inflammation and secretion of inflammatory molecules. Recurrent increment in cytokine levels increases the permeability of BBB to peripheral immune molecules and prolongs the central immune inflammatory response that generates an inordinate environment of oxidative and inflammatory stresses, accelerating CNS oxidative damage and elicits adverse behavioural and cognitive consequences.

8. Cortical zone of cognition and behaviour: Hippocampus

Hippocampus is an anatomical structure forming a part of limbic system in CNS. It is mainly responsible for the collection, consolidation and retrieval of various aspects of learning, memory and spatial navigation (Scoville and Milner, 1957; O’Keefe and Nadel, 1978; Rolls, 1996). The term hippocampus arises from the Greek words *hippos* mean horse,
and kampos means sea monster. From 1729, onwards, considering its structural resemblance the name of this structure was wavered between seahorse and silkworm (German anatomist Duvernoy). Later, in 1932, a Danish group proposed the term "Ram's horn" or "cornu Ammonis" (horn of ancient Egyptian God Amun). Anatomically, it is a bilobule structure extended in both right and left lobes of the cerebral cortex of both human and other vertebrates. Through both septotemporal (longitudinal) and transversal axis, hippocampus is mainly divided into two divisions i.e., dentate gyrus (DG) and three sub regions of Cornu Ammonis (CA1, CA2 and CA3). The Cornu Ammonis region is also known as the hippocampus proper having pyramidal neurons as the prime neurons that comprises 90% of the neuron population in CA region of the hippocampus formation where the DG region is mainly consisted of granule cell and molecular cells.

8.1. Hippocampal circuitry and processing

Hippocampus is an elemental hub for the cognition and behavioural processes (Sweatt, 2004). Purview of these functions depends upon the conjunctive relationship between the hippocampal sub-regions, i.e., CA1, CA2, CA3 and DG and their synaptic connections with other cortical areas. Hippocampus receives all the prime subcortical inputs from perirhinal, parahippocampal, posterior cingulate, insular, orbitofrontal and superior temporal cortices (Insausti et al., 1987; Amaral and Witter, 1995; Pikkarainen et al., 1999).
Hippocampal circuitry forms a trisynaptic loop that receives and transmits the cortical information throughout the CNS (Figure 4). Highly processed cortical inputs from the entorhinal cortex (EC) enter into the dentate gyrus through the perforant path projection. Granule cells dendrites in DG make excitatory synaptic contact with perforant path axons. The proximal apical dendrites of CA3 pyramidal neurons receive input through the mossy fibres of granule neuronal cells, which in turn, through Schaffer collaterals pathway projected to the ipsilateral CA1 pyramidal neurons and to contralateral CA3 and CA1 pyramidal neurons via commissural connections. CA1 and CA3 pyramidal neurons also receive direct input from the entorhinal cortex via layer III and layer II cells of entorhinal cortex respectively. Deep layers of EC receive the exclusive output from the hippocampus. Besides EC, some other additional outputs from the hippocampus are also received by other cortical areas like the prefrontal cortex and lateral septal area.

Accordingly, the possible effect of oxidative stress following diabetes on neuronal and glial cell death and the interconnected feedback loop between oxidative stress, cell death and gliosis have never been studied in the hippocampus. Therefore, a detailed study on glial...
activation and immune response either neuroprotective/degenerative, produced by glial cells following diabetic exposure along with subsequent behavioural and cognitive impairment with respect to gliosis needs to be carried out in a more organized manner. Thus, the present study will help in assessing the effect of diabetes on: glial activation, cell death and the possible immune response produced by microglial and astroglial cells along with their effects on behaviour and cognition changes in a rat model.
MATERIALS & METHODS

1. Animals

Wistar rats bred and raised in the animal house facility of School of Studies in Neuroscience, Jiwaji University, Gwalior were used in this study. The animals were maintained at controlled temperature of 25±2°C and 50-65% humidity with a fixed 12:12 hour light dark cycle. Animals were housed, in pairs, in polypropylene cages (52cm x 28cm x 22cm) with clean dust free husk bedding. Water and standard rat pellet diet (Ashirwad Industries, Chandigarh, India) were provided ad libitum. Animal house maintenance practices were carried out as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), and all the procedures were pre-approved by Institutional Animal Ethics Committee, Jiwaji University, Gwalior (M. P.).

2. Diabetes Induction

A pool of healthy rats weighing between 180-230 g and blood-glucose level of 90-140 mg/dl was selected for developing type 1 diabetes animal model (McNeill, 1999). Animals were fasted overnight, but had free access to drinking water prior to STZ-injection. Type 1 diabetes was induced by a single intraperitoneal (i.p.) injection of Streptozotocin (STZ; Sigma) at a dose of 45 mg/ kg body weight, dissolved in 0.1M citrate buffer (pH=4, preparation discussed in Appendix I; Stevens et al., 2007; Lebed et al., 2008; Waer and Helmy, 2012). Rats injected with vehicle alone served as control. Blood-glucose level (non-fasting) was determined at 72hrs post STZ injection, through tail snipping (Kamal et al.,2000; Lechuga-Sancho et al., 2006) using AccuChek Sensor Comfort (Roche Diagnostics, Berlin, Germany). Animals having the blood-glucose level of 250 mg/dl or above were considered as diabetic.

Diabetic animals were randomly divided into following groups, i.e., 2nd week post diabetic confirmation (2nd week), 4th week post diabetic confirmation (4th week), 6th week post
diabetic confirmation (6th week), 8th week post diabetic confirmation (8th week), 10th week post diabetic confirmation (10th week) and 12th week post diabetic confirmation (12th week). Blood glucose level (non-fasting) and body weight were checked once a week throughout the study duration to ensure the diabetic stature. Animal’s food and water consumption was also measured daily to further confirm the diabetic symptoms like polyphagia and polydipsia.

3. Behavioural and Cognitive Assessment

The behavioural and cognitive assessments were done at 2nd, 4th, 6th, 8th, 10th and 12th week post diabetes confirmation (n=8/group) with their respective control groups. A wide range of behavioural and cognitive studies were performed to analyse the spatial cognition, working memory, locomotor activity, motor coordination and muscular strength of the animals following STZ-induced diabetes.

Notably, the experimental room conditions (light, temperature, humidity and noise intensity) and timing (9:00am to 2:00pm) were consistent and similar to the animal house environment in all the behavioural and cognitive studies to avoid any procedural anomalies. To minimize the novel-environment associated variance, animals were taken into the experimental room one hour before the test.

3.1. Open field test

3.1.1. Principle

Open field test is popularly used to assess the locomotor activity of the rodents and serve as a good preliminary test to determine the motor deficits (Qian et al., 2010; Hong et al., 2014). It is also used as an indicator of unconditioned emotionality in response to the novel environment with limited complexity (Hall and Ballechey, 1932; Henderson, 1970). The basic outcome of the study is based on ‘subject’s movement’ which is influenced by the motor output, novelty fear or freezing and exploration, etc.

3.1.2. Instrumentation
The test was performed on a computerized Optavarimax Autotrack activity monitoring system (Columbus Instruments, Columbus, OH, USA). A testing chamber of the transparent acrylic box with an area of 43cm x 43cm x 17cm and preinstalled software Auto-track version 4.41 was used to record and measure the animal’s locomotor and non-locomotor activity. The testing chamber has two arrays of infrared beams perpendicular to each other to measure both horizontal and vertical activity of the animal. Instrument is based on photobeam interruption principle. An arrangement of 30 infrared photocells (15 per row) were located every 2.5cm from side-to-side and 4cm above the arena to record the horizontal activity. On the other side, an array of similarly distributed 16 infrared photocells were placed 10 cm above the arena floor to measure the vertical activity.

3.1.3. Experimental procedure

Test animals were provided with a prior exposure of 5 min of the test chamber to minimize novel-environment fear in the animal. With the help of the software (Auto-track version 4.41) behaviour variables were recorded for 20 min continuously for each animal. Each animal was tested once. After every trial, the chamber was cleaned with 10% alcohol to remove the animal odour from the chamber. Notably, lethargic or abnormally behaved animals were not included in the study and eliminated as outliers. The measured variables were as follows:

![Fig. 3.1: Autotrack activity monitoring system and animal track report.](image)
1. Distance travelled (cm): Total distance covered by the animal after moving from the centre position.

2. Resting time (sec): Total time when the animal is not moving in the testing device.

3. Ambulatory time (sec): Total time taken by the animal to change its position in the testing area either by walking, circling or rearing.

4. Stereotypic time (sec): Time taken by the animal to perform non-locomotor activities like grooming, scratching, etc.

All the pre-described variables were recorded by the sensors and displayed on the screen with their tracking plots and hence was saved for further data analysis (section 4).

3.2. Motor coordination test

3.2.1. Principle

Dependable assessments of motor function essentially require the evaluation of the motor disability in the rodent model of disease (Brooks and Dunnett, 2009). Rotarod is the most popular and widely used test to study the motor coordination as motor function in rodents (Jones and Roberts, 1968; Rustayet al., 2003). Initially, rodents are trained or acclimatized to walk on the rotating rod at a certain speed for specific time duration and after training, animal’s motor coordination is assessed on the basis of their ability to coordinate the balance and movement on the rotating rod with exceeding speed. Animals which are unable to cope with the faster moving rotating rod will drop off early are testified as having impaired motor coordination. The assessment is based on the animals’ falling latency, recorded when the animal fall off and breaks the photo-beam detection over the time.

3.2.2. Instrumentation

Rotamex 5 (Columbus Instruments, Columbus, OH, USA), a motor-driven treadmill was used to evaluate the effects of diabetes on the motor coordination of the rats. Rotarod apparatus has a plastic semi-enclosed box shaped chamber having row of infrared photo-cells (diameter
3cm, width 5cm) on two parallel facing sides. A rotating spindle at the middle of the apparatus between the beams is suspended at a height of 35cm above the floor. The rotating ridged spindle provides optimal grip to the animal. The apparatus is provided with round plates on either side of the separated section to prevent animal from escaping. The latency to fall was recorded automatically by the photocells as the total length of time spent by the animal on the rotating rod with the help of software Rotamex version 1.2.3 installed with the apparatus.

3.2.3. Experimental procedure

Animals from each group were acclimatized for 3 days consecutively at start acceleration speed minimum of 2 rpm to maximum of 8 rpm for a total duration of 100 sec, three trials for each animal. Final reading was taken post 24 hour of last acclimatization, at a start acceleration speed of minimum 2 rpm to maximum of 40 rpm for a total duration of 420 sec. An animal fall is detected by infrared photocells, which cross the space just above the spindle. Once the photocells loose the detection of the animal, the falling latency of that animal was recorded. Test animals were given 3 trials each, with a resting time interval of 5 min was given between each successive trial. If any animal behaved abnormally (i.e., data generated

Fig. 3.2: Rotarod (Rotamex 5)
varied dramatically from the animals of similar states), then it was considered as outlier. Data compilation and analysis is described in section 4.

3.3. Neuromuscular strength test

3.3.1. Principle

Grip strength meter is popularly used to measure the neuromuscular strength of the rodents (Meyer et al., 1979). It determines the peak force value applied by the rodent to hold the grip. The device works under the principle of compression and tension. For the forelimb muscular strength assessment, the tension mode was considered.

3.3.2. Instrumentation

Grip strength meter (Columbus Instruments, Columbus, OH, USA) consisted of a force gauge digital display (sensor range: 0-5kg) is connected with a specially designed forelimb grasping pull bar assemblies (76mm x 50mm) made of steel wire. The values of grip strength were recorded automatically via a RS-232 interface connected to the computer and a software Grip strength version 1.19. The unit of measurement of the sensor is delivered in grams mode.

3.3.3. Experimental procedure

For measuring the grip strength the test animal was placed over the metallic grid and allowed to hold the grid through its forelimbs. Through a gentle force, the animal was pulled back carefully by the tail until its grip was released. Each animal was retested after three successive readings followed by 2 min interval, total six readings per animal were recorded.
The maximum strength value animal takes to release the grid was recorded by the sensors and displayed on the screen and hence was saved for further data analysis (section 4). Notably, lethargic or abnormally behaved animals were not included in the study and eliminated as outliers.

3.4. Test for spatial memory

3.4.1. Principle

Barnes circular maze is a spatial memory task for rodents that involves the learning of the position of a hole that can be used to escape from the open surface of the maze (Barnes, 1979). The task relies on subject’s learning and memory to locate the escape box using spatial reference points that either is fixed in relation to the maze (extra-maze cues) or are on the maze itself fixed in relation to the escape hole (proximal cues).

3.4.2. Instrumentation

Barnes circular maze is consisted of a black circular platform of 120 cm in diameter with 18 holes of 10 cm diameter evenly spaced around the circumference. An escape box (20x10x10 cm) was placed under one of the holes. For recording the test, a camera connected to the
computer interface with preinstalled Any Maze software version 4.82 (Stoelting, USA) was mounted directly over the maze.

![Escape box computer interface with preinstalled Any Maze software version 4.82 (Stoelting, USA) was mounted directly over the maze.](image)

**Fig. 3.4:** Barnes maze and animal track report.

### 3.4.3. Experimental procedure

Test animals were acclimatized for 3 consecutive days to locate the escape box prior to the final reading. Experimental animals those behaved abnormally and were unable to locate the escape box in the provided time duration were excluded from the study as outliers. For the final test, animal was placed in the middle of the platform away from the experimenter face and allowed to explore the maze. Timing of the session ended when the animal found the escape box or after 120s had elapsed. Variables like duration to find the escape box, path efficiency and speed were assessed from the final 3 trials test reading for each animal. Final data as a mean value per animal was statistically calculated and analysed (described in section 4). After each trial, the maze was cleaned with 10% ethanol to remove possible biasing effects due to odour cues left by previous rats.

### 3.5. Test for anxiety and depression

#### 3.5.1. Principle

Elevated plus maze is a widely used test for measuring the fear and anxiety-like behaviour in rodents (Pellow et al., 1985; Walf and Frye, 2007). The test is based on the animal’s unconditioned preference between a comparatively safe and comfortable environment (the
closed arms) and a risky environment (elevated open spaces) and their innate behaviour to explore the novel environment. An anxious animal avoids the entry in open space (Pellow et al., 1985; Walf and Frye, 2007; Mizunoya et al., 2013) and present higher anxiety index, an integrated value of total number of entries in the arms (open and closed) and number of entries in open arms (Cohen et al., 2013; Contreras et al., 2014).

3.5.2. Instrumentation

Elevated plus maze is a metallic plus “+” shaped apparatus, elevated 75 cm above the floor supported with four metallic stands. The apparatus is consisting of two open and two closed arms of same dimensions, i.e., 50 cm long and 10 cm wide. The closed arms were covered by the 40 cm long metallic sheet walls. At the conjunction of four arms, a central square platform (10 x 10 cm) was present. A camera setup mounted just over the maze and connected to the computer interface with preinstalled Any Maze software version 4.82 (Stoelting, USA) was used to record the animal tracking.

3.5.3. Experimental procedure

Prior to the final test, animals were acclimatized for 3 consecutive days on elevated plus maze for 2 min each. Final trial was conducted 24 hour after the last acclimatization session. Test animal was placed at the square platform and allowed to roam inside the maze for upto 120s. Three trials were tested for each animal for the number of entries and time spent in the closed or open arm.

Fig. 3.5: Elevated plus maze and animal track report.
To analyse the data following were calculated: \( \text{percentage of open arm entries} = \left( \frac{\text{open entries}}{\text{total entries}} \right) \times 100 \), \( \text{percentage of time spend in open arms} = \left( \frac{\text{time in the open arms}}{\text{time in the open arms} + \text{time in the closed arms}} \right) \times 100 \) and \( \text{anxiety index} = 1 - \left( \frac{\left( \frac{\text{Open arm time}}{\text{Test duration}} + \frac{\text{Open arms entries}}{\text{Total number of entries}} \right)}{2} \right) \) were calculated (Walf and Frye, 2007; Mizunoya et al., 2013; Cohen et al., 2013; Contreras et al., 2014). Data collection and analysis is described in section 4.

3.6. Test for working memory

3.6.1. Principle

T maze test is a well-known task for studying the working memory of the rodents (Wenk, 2001). In this task, animal learn to find the baited arm (arm having eatable reward) based on their memory of previously visited arms (Nasuti et al., 2013).

3.6.2. Instrumentation

The maze is a ‘T’ shaped structure consisted of black metallic one start arm (60 x 10 x 10 cm) joined to two identical reward arms (45 x 10 x 10 cm). A camera connected to the computer interface with preinstalled Any Maze software version 4.82 (Stoelting, USA) help in recording the track details.

![T maze and animal track report.

Fig. 3.5: T maze and animal track report.

3.6.3. Experimental procedure
Test animals were acclimatized for 3 consecutive days 3 trials per day to learn the baited arm. The trial was completed when the animal reached the baited arm or after 5 min had elapsed. Animals those failed to explore the baited arm in the provided time duration (5 min) or behaved abnormally were excluded from the study as outliers. On the final test day, animals were tested for 6 trials successively to assess the time duration animal takes to choose the rewarded arm and path efficiency to reach this arm. Trials were carried out using 10-min resting intervals. Data assessment and evaluation for the difference between the path efficiency and test duration of diabetic animals in comparison to controls is explained in section 4.

4. Data collection and statistical analysis

For every cognitive and behavioural assessment, final readings of each study were calculated for a single mean value per animal on an excel file, and from the mean of readings of every animal group, standard error was calculated within the mean of different animals (Coolidge, 2002). SigmaStat software (version 3.5; Systat Software, Inc., Point Richmond, CA, USA) was used to analyse the significance between the various experimental groups, assessment was done by one-way ANOVA followed by the Tukey’s posthoc test. The threshold for statistical significance was set at p≤0.05.

5. Tissue collection and processing

Animals (n=6/group) from both diabetic and control groups were sacrificed on 2nd, 4th, 6th, 8th, 10th and 12th week and animal tissues were preserved and processed for further analysis.

5.1. Perfusion-Fixation

5.1.1. Principle

The aim of whole-body perfusion-fixation is to use the vascular system of a deeply anesthetized animal to deliver the fixatives to the tissues of interest (Jonkers et al., 1984). This is an ideal method of tissue preservation as it fixed the tissues before autolysis begins and
retains the tissue in life-like condition. Perfused tissues are less susceptible to the damage caused by handling.

5.1.2. Procedure

A well-designed and approved perfusion set-up was used to achieve the purpose (Figure 1). Animal was first anesthetized with diethyl ether vapours inside a glass desiccator with closed lid. After proper deep anaesthesia, animal was placed dorsally downward over the dissecting tray. An incision of 4-5 cm was made through the integument and abdominal wall just beneath the rib cage. Then with the help of a curved and blunt scissor another small incision was made in the diaphragm and continued along the entire length of the rib cage to expose the heart. Parallel cuts were made on either side of the rib cage up to the collarbone. Sternum was lifted upwardly towards the animal’s head with the help of artery forceps and tissues attached with the heart were carefully removed by a blunt scissor for the better view of heart. Subsequently, a 15 gauge blunt butterfly needle running with buffered saline flow (PBS; 0.01M; pH=7.4, Appendix I) was inserted into the left ventricle of the heart and successively a small nick was made on the right auricle with the help of scissor to allow the flushing of whole-body blood via circulatory system. When the blood was completely flushed out from the body and the fluid exiting the auricle was clear, the fixative valve was opened to uninterruptedly replace the saline flow with buffered fixative (Appendix I), i.e., 2% paraformaldehyde (for immunohistochemical study) and 10% buffered formalin (for histological study). The fixative was allowed to run until the rat’s body became stiff or fixed properly.
5.2. Tissue processing

5.2.1. Principle

Tissue processing is achieved to provide the enough solid medium support to the tissue that offers sufficient rigidity to enable the thin sections to be cut. It also prevents the distortion while performing the sectioning. The processing method not only preserves the morphological integrity but also retains the cellular changes of the tissue. According to the sectioning method directed, i.e., cryotomy or microtomy different procedures of tissue processing were applied.

5.3. Tissue processing for cryotomy

5.3.1. Procedure

After perfusion-fixation cerebral cortex through occipito-temporal region (to expose the hippocampal area) and cerebellum were dissected out carefully, weighed and post-fixed in the same fixative overnight at 4°C. The tissues were then cryoprotected in Phosphate buffered-Sucrose gradients, i.e., 10%, 20% and 30% at 4°C until tissue settled at the bottom.

After processing the coronal sections of hippocampus and sagittal sections of cerebellum were sequentially cut using cryostat (Microm HM 525, Thermo Scientific), at a thickness of 14µm. The sections were collected serially on chromalum-gelatin coated slides and stored at -20°C till they were used for immunocytochemical studies.
5.4. Tissue processing for microtomy

5.4.1. Procedure

Histological studies were done for hippocampus, cerebellum, kidney, liver and pancreas. Following method was employed for the paraffin tissue processing:

Post-fixation: Following perfusion-fixation, tissues were kept in same fixative for 48 hrs at room temperature for the efficient fixation of tissues.

Washing: Washing was done thrice with distilled water for 1½ hour each to remove the excess of fixative from the tissue.

Dehydration: Through a graded series of alcohol, i.e., 30%, 50%, 70%, 90%, 100% I and 100% II sequentially for 1 hour each at room temperature, tissues were dehydrated to extract out the water molecules that would hamper the paraffin impregnation and embedding.

Clearing: Tissues were cleared in 2 changes of toluene for ± 45 min each (until the tissues became transparent in appearance). The purpose of clearing is to remove the dehydrating solution from the tissue and making the tissue permissible for the subsequent paraffin impregnation.

Wax impregnation: Cleared tissues were kept in paraffin wax twice for ± 2hr each in hot oven (56-58°C) for the proper impregnation of wax inside the tissues. After paraffin impregnation, tissues were embedded in wax and paraffin tissue blocks were made with the help of Leukhart’s ‘L’ pieces.

The sectioning was performed on a microtome (Leica Rotary Micotome, RM 2135). Serial sections of cerebrum (10µm thickness), pancreas, liver and kidney (8µm thickness) were collected on chromalum-gelatin coated slides, stretched in hot water bath (50°C), dried on hot air oven (37°C) and stored at room temperature for further histological studies.

6. Immunohistochemical study

6.1. Principle
Immunohistochemistry is a technique to identify or localize the specific protein antigen through antigen-antibody interaction that can be visualized by some specific markers like, fluorescent dye, enzyme or colloidal gold. Immunohistochemical study using enzyme-substrate reaction producing a specific colour to label the antigen-antibody interaction is termed as immunoenzymatic labeling. In this study the immunohistochemical labeling was employed to assess the stature of various brain proteins following STZ-induced diabetes.

6.2. Procedure

Cryocut sections were brought to room temperature and air dried for 1 hour at 37°C. Afterwards, sections were washed thrice with washing buffer (as shown in Table 2) for 5 min each, and then permeabilised with 1% Triton X-100 for 30 min at room temperature followed by three washings (5 min each) with buffer again. To quench the endogenous peroxidase activity sections were treated with 1% hydrogen peroxide for 30 min at room temperature. Then the sections were preblocked with 1% normal serum (prepared in washing buffer) of same species as secondary antibody, for 90 min in a humid chamber. Next to blocking the sections were incubated with primary antibody (Table 2) overnight at 4°C. Next day after three buffer washes, the sections were then incubated with secondary antibody (Table 2), for 90 min at room temperature. Afterward, rinsed in buffer, sections were subsequently incubated in streptavidin-biotin-peroxidase complex (Table 2) for 90 min at room temperature. Following buffer wash the colour was developed with chromogen solution containing 3, 3′-diaminobenzidine tetrahydrochloride (0.025%) and hydrogen peroxide (0.006%) dissolved in the buffer for 20 min at room temperature. Later on trailed by distilled water wash, slides were dried at 37°C, dehydrated in 100% alcohol, cleared in xylene and mounted with DPX. To ensure comparable immunostaining, sections were processed together at the same time in the same conditions. Omission of primary antibodies served as negative control.
Table 2. Antibody Details:

<table>
<thead>
<tr>
<th>Antibody/Source</th>
<th>Specificity</th>
<th>Raised in/Concentration</th>
<th>Secondary(Biotinylated, Sigma)/Concentration</th>
<th>Tertiary(SABC,Amersham)/Concentration</th>
<th>ColourVisualization</th>
<th>WashingBuffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP (Dako, Denmark)</td>
<td>For labeling the astroglia</td>
<td>Rabbit Polyclonal/1:2000</td>
<td>Anti-rabbit (1:100)</td>
<td>1: 200</td>
<td>DAB</td>
<td>PBS</td>
</tr>
<tr>
<td>Iba-1 (Wako, Japan)</td>
<td>For labeling the microglia</td>
<td>Rabbit Polyclonal/1:1500</td>
<td>Anti-rabbit (1:100)</td>
<td>1: 200</td>
<td>DAB</td>
<td>PBS</td>
</tr>
<tr>
<td>Caspase-3 (R&amp;D Systems)</td>
<td>Detection of cell death</td>
<td>Rabbit Polyclonal/1:1000</td>
<td>Anti-rabbit (1:150)</td>
<td>1: 200</td>
<td>DAB + NiSo₄</td>
<td>TBS</td>
</tr>
<tr>
<td>OX-6 (Serotec)</td>
<td>MHC-II presenting cells</td>
<td>Mouse Monoclonal/1:100</td>
<td>Anti-mouse (1:100)</td>
<td>1: 200</td>
<td>DAB + NiSo₄</td>
<td>TBS</td>
</tr>
<tr>
<td>S-100β (Sigma)</td>
<td>Astrogliosis</td>
<td>Rabbit Polyclonal/1:200</td>
<td>Anti-rabbit (1:200)</td>
<td>1: 200</td>
<td>DAB</td>
<td>PBS</td>
</tr>
<tr>
<td>OX-42 (Serotec)</td>
<td>Complement receptor-3 activation</td>
<td>Mouse Monoclonal/1:100</td>
<td>Anti-mouse (1:100)</td>
<td>1: 200</td>
<td>DAB + NiSo₄</td>
<td>TBS</td>
</tr>
<tr>
<td>ED-2 (Serotec)</td>
<td>Macrophagic marker</td>
<td>Mouse Monoclonal/1:100</td>
<td>Anti-mouse (1:100)</td>
<td>1: 200</td>
<td>DAB + NiSo₄</td>
<td>TBS</td>
</tr>
<tr>
<td>TNF-α (R&amp;D Systems)</td>
<td>Pro-inflammatory cytokine</td>
<td>Goat Polyclonal/1:200</td>
<td>1:100, Biotin labelled (VectastainElite ABC Kit, Universal)</td>
<td>1: 200</td>
<td>DAB</td>
<td>PBS</td>
</tr>
<tr>
<td>IL-1β (R&amp;D Systems)</td>
<td>Pro-inflammatory cytokine</td>
<td>Goat Polyclonal/1:200</td>
<td>1:100, Biotin labelled (VectastainElite ABC Kit, Universal)</td>
<td>1: 200</td>
<td>DAB</td>
<td>PBS</td>
</tr>
</tbody>
</table>

Note: All the antibodies were diluted in 1% BSA dissolved in washing buffer.
7. Histological staining

7.1. Principle

Histology is aimed to study the cellular organization and structure of the tissue and cell. It also helps to understand the physiological importance and relationship between cellular structures under various conditions. Histological staining is the eminent and prime tool of the histological study based on the principle of ion-ion and acid-base interactions.

Haematoxylin and Eosin staining is used to study the histopathological changes in both nuclei and the cytoplasm of the cell. Haematoxylin is a cationic dye; that stains the nuclei in blue-black colour while eosin stains the cytoplasm in pink colour.

7.2. Procedure

Paraffin sections were first deparaffinised with two changes of xylene for 15 min each. Then sections were rehydrated through descending series of alcohol i.e., 100%, 90%, 70%, 50%, 30% sequentially for 10 min each followed by 2 changes of distilled water for 5 min each. Afterwards, sections were placed in the Delafield’s haematoxylin (Fisher Scientific, India) stain for 4 min. This was followed by washing under running tap water for 20 min for removing the excess of stain. Differentiation was done with 0.5% HCl (1-2 dips), and the slides were again washed in running tap water for 20 min to remove the acid. After a 5 min wash in distilled water sections were placed in ascending series of graded alcohol, i.e., 30%, 50% and 70% for 5 min each to dehydrate the sections. Then the sections were kept in the 1% eosin stain prepared in 90% alcohol for 2 min. This was followed by 1-2 dips in 90% alcohol for differentiation, and then the sections were placed in 100% alcohol for 20 min with 2 changes (10 min each) trailed by clearing in xylene for 15 min. And finally, the sections were permanently mounted with DPX.

8. Morphological analysis
Images were captured using Leica DM 6000 microscope equipped with Leica DFC 310 RX digital camera and Leica Application Suite (LAS) software in order to have magnified images showing fine structural details for more demarcated morphological analysis.

9. Cell quantification and analysis

9.1 Cell counting using Leica Qwin software

Cell quantification was done on the sections stained through standard HRP-conjugated immunohistochemistry method. The cell population was estimated in CA1, CA2, CA3 and DG regions of the hippocampus. The hippocampal regions for quantification were delineated as twelve frames per area in the two sections per animal in all the groups. In order to count equal areas (mm$^2$) in every subject, a 21670.9µm$^2$ frame comprising exclusively the anatomical areas of interest was applied to ensure that counts were representative of the analysed areas. Photomicrographs of the regions located between bregma 3.8 mm and intraural 5.2 mm (Paxinos and Watson, 1982) were photographed with Leica DM6000 microscope fitted with digital camera (DFC 420C, Leica, Germany). Only tissue sections corresponding to these coordinate were included in the quantification, ensuring that the regions of interest were equivalent among animals and experiments. Cell counting was performed on the digital pictures using Leica Qwin software (version 3.1) and interactive measurement application tool and presented as the total number of cells/ mm$^2$. 
Immunolabelled image was opened through **Open** option in main tool bar. Image was opened and ‘Interactive’ mode was chosen in the ‘Measure’ option panel of the main menu tool bar.

After opening the ‘Interactive Measurement’ box, cell counting was done manually by clicking on the immunolabelled cells.

As the manual cell counting precedes, the data was recorded and displayed in ‘Manual Results’ box as cells present in per frame area of 21670.9μm². Values were copied in separate excel sheet and converted to mm² and averaged for a single mean value.

9.2. **Quantification of area fraction using Image J software:** "Image J" is a freely available java-based public-domain image processing and analysis program developed at the National Institute of Health (NIH). This program is a reliable, well-accepted and
accessible laboratory tool for the scientific image analysis for a very long time (Girish and Vijayalakshmi, 2004; Schneider et al., 2012). In the present study Image J was used to study the area fraction (percentage of staining) of the specific immunolabelling. Images of the same magnification were used for the cell counting to ensure the consistency of the data.

Through **File** option in main tool bar new image was opened. Measurement scale was calibrated by drawing a line over the scale bar with straight line tool and **Set Scale** option from **Analyze** tool bar was selected. In Set Scale window, entries were made as value 100 in ‘Known distance’ box, µm in unit of measurement box and Global option was marked.

**Analyze**  ➔ **Set Scale**

Image was converted into grayscale through following path:

**Image**  ➔ **Type**  ➔ **8-bit**
Adjust option was selected from the main tool bar Image section and threshold was adjusted as:

Image ➔ Adjust ➔ Threshold

Image background and particles were converted into white and black colour respectively via following path:

Process ➔ Binary ➔ Make Binary

Analyze Particles option was selected from Analyze panel of menu tool bar as:

Analyze ➔ Analyze Particles
In Analyze Particles window, 100 value was selected as the minimum particle size and options ‘Display Results’, ‘Summarize’, and ‘Record States’ was opted.

Area fraction was counted from the Summary window. The Summary window also enlisted the count, total area and average size for every image. Provided data was copied to the excel sheet for further statistical analysis.

10. Statistical analysis

Results were statistically analysed with SigmaStat software (version 3.5; Systat Software, Inc., Point Richmond, CA, USA) and were reported as mean ± SEM. In experiments, to assess the significance between the multiple groups one-way ANOVA followed by the Tukey’s posthoc test was done. The threshold for the statistical significance level was set at p≤0.05.

11. Biochemical study

Biochemical study was done to evaluate the level of oxidative stress in rat following diabetes.
Lipid peroxidation (LPO): Lipid peroxidation (LPO) was assessed by measuring malondialdehyde (MDA) levels based on the reaction of MDA with thiobarbituric acid using Buege and Aust (1978) method. The reaction of malondialdehyde (MDA), a degradation product of peroxidized lipids with thiobarbituric acid (TBA) to produce TBA malondialdehyde chromophores has been taken as the index of lipid peroxidation. LPO estimation serves as preliminary tests to determine the oxidative stress in diabetic rats.

Reagents:

- Potassium chloride (0.15 M): 1.1184 g was dissolved in 100 ml of D.H2O.
- Trichloroacetic acid (30%): 30 g TCA was dissolved in 100 ml of D.H2O.
- Thiobarbituric acid (0.8%): 0.8 g was dissolved in 100 ml of D.H2O.

11.1. Procedure

For LPO estimation, animals (n=6) from both diabetic and control groups were sacrificed and tissue of interest i.e., kidney, liver and hippocampus were surgically removed and a 10% (w/v) tissue homogenate was prepared in 0.15M KCl solution. Then, 0.5 ml of 30% TCA and 0.5ml of 0.8% TBA was added sequentially into the homogenate with 2min vortex after the TCA addition. After the reagent addition, test tubes mouth was covered with parafilm and aluminium foil to prevent the spillage. The tubes were then kept in boiling-water bath for 30 min. After cooling the tubes, the homogenate was centrifuged at 3000rpm for 10min and afterwards, the optical density was read at λ 535 nm. The value of absorbance was converted as MDAµmol/gm of tissue with the following formula:

\[
\text{MDAµmol/gm of tissue} = \text{Absorbance} \times 10^{0.156}
\]

12. Statistical analysis

Computation of the statistical analysis was carried out with the help of the Microsoft Excel program (Microsoft Office XP Professional, Microsoft Corporation, USA) and
SigmaStat software (version 3.5.; Systat Software, Inc., Point Richmond, CA, USA). One-way analysis of variance (ANOVA) followed by the Tukey’s posthoc test was done to compare the mean levels of various experimental groups. The threshold for the statistical significance level was set at $p \leq 0.05$. 
RESULTS

Streptozotocin (STZ)-induced diabetic animal model was used in this study as streptozotocin is not only capable of inducing an irreversible long-term effect of diabetes (Gaulton et al., 1985; Méndez and Ramos, 1993; Eleazu et al., 2013) but also has low mortality rate as compared to the other diabetogenic agents (Mansford and Opie, 1968; Lenzen, 2008; Chatzigeorgiou et al., 2009; Deeds et al., 2011). The effect of STZ in experimental animals was checked post 72 hrs of STZ-injection and animals having blood glucose level of 250mg/dl or above were considered as diabetic. Further diabetes associated effects (cognitive, behavioural, biochemical and cellular) were assessed from 2nd to 12th week post diabetes confirmation (PDC). The model was validated through the assessment of cardinal signs of diabetes mellitus i.e., high blood glucose level, body weight loss, polydipsia, polyphagia, absolute pancreatic beta cell loss and acute degenerative changes in vital organs like kidney and liver.

1. STZ-induction causing symptomatic diabetes

Streptozotocin (STZ)-injected animals exhibited characteristic signs of diabetes with blood glucose level consistently high at 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week (p≤0.001) of post injection diabetic durations (post-diabetes confirmation; PDC) as compared to their respective controls (Fig. 1/i). Furthermore, the water intake of diabetic animals was also increased significantly at all the PDCs i.e., 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week (p≤0.001) as compared to their respective controls (Fig. 1/ii). Similarly, the food consumption of the animals also increased at 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week PDC (p≤0.001) as compared to their respective controls (Fig. 1/iii). The increment in food and water intake in STZ-induced
animals also confirmed the symptomatic signs like polyphagia and polydipsia observed in type 1 diabetes.

2. Diabetes induced reduction in body and brain weight

Body weight of diabetic rats reduced significantly after 2nd week (p≤0.001) and continued to remain low till the end of the experiment i.e., 4th week (p≤0.002), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week PDC (p≤0.001) of diabetic animals (Fig. 2/i). Similarly, the brain weight of diabetic animals was also decreased significantly after 2nd week (p≤0.001) and continued to remain low till the end of the experiment i.e., 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.002), 10th week (p≤0.008) and 12th week PDC (p≤0.013) as compared to the respective controls (Fig. 2/ii). A cumulative resultant of reduced body and brain weight, the brain weight/body weight ratio of diabetic animals was escalating at all the diabetic time points i.e., 2nd week (p≤0.005), 4th week (p≤0.024), 6th week (p≤0.003), 8th week (p≤0.008), 10th week (p≤0.013) and 12th week PDC (p≤0.008) as compared to their age-matched controls (Fig. 2/iii).

3. Histological changes in kidney, liver and pancreas following STZ-induced diabetes

3.1. Pancreas

In controls the pancreatic tissue was observed with normal architecture having compact arrangement of beta cells and non-beta cells. The pancreas of control rats showed a clear demarcation of lobes by the means of fibrous connective tissue (Fig. 13). It consisted of exocrine region consisting acinar cells lined by cuboidal epithelium with eosinophilic secretions in the lumen and endocrine region illustrated by the presence of groups of cells lying within each lobule and these cells are known as the island of islets of Langerhans (Fig. 13/a). In the pancreatic tissue of diabetic rats degenerative changes were clearly evident (Fig. 13/b-g). The insulin producing beta cells were successively decreasing both
in numbers and volume with the advancing diabetic state confirming the severe permanent insulin deficiency following STZ-injection. The insulin deficiency is in correlation with the increased blood glucose level of the animals which is gradually increasing from 2\textsuperscript{nd} to 6\textsuperscript{th} week PDC and remained at higher than normal level afterwards. Islet cells were also atrophied (Fig. 13/ b-d; red arrowhead) and their number was reduced from 2\textsuperscript{nd} to 8\textsuperscript{th} week of diabetes. However, a gradual and complete loss of islet cells was evidenced from 6\textsuperscript{th} week post diabetes (Fig. 13/d-g). From 10\textsuperscript{th} week onwards the vacant island of islets of Langerhans was started surrounding by fibrous tissue that clearly observable by 12\textsuperscript{th} week PDC (Fig. 13/d-g). There was no sign of any regenerative changes upto the 12\textsuperscript{th} week of diabetes. Marked necrosis of the exocrine pancreas was also noticed. Severity of necrosis was increased with the time duration and was evidently depicted by the presence of eosinophilic acinar cells showing karyolysis and atrophy (Fig. 13/d-g; red arrow). Histopathological investigation of pancreas reveals persistent loss of beta cells and tissue damage with almost no regenerative changes as observed following STZ-induction, validating the type 1 diabetes animal model. The sustained severity of diabetes upto the 12\textsuperscript{th} week of study was also confirmed. Details of histopathological findings are presented, in the summary incidence table 1.

3.2. Kidney

The kidney of control rat showed the normal architecture upto the 12\textsuperscript{th} week of study (Fig. 14/a). The histopathology of kidney presented normal glomerulus, Bowman’s space and renal parenchyma supported with proximal and distal convoluted tubules and Vasa recta. Renal tubules in the parenchyma showed clear lumen and were lined with cuboidal cells. Following STZ-injection the degenerative changes were clearly evident in the renal tubules as depicted by necrosis of cuboidal cells (Fig. 14/b-g; black arrow) and severity of tubular necrosis from 2\textsuperscript{nd} to 12\textsuperscript{th} week post STZ induction (Fig. 14/b-g). In the later diabetic stage there was complete loss of renal parenchyma leading to the formation of
cyst (Fig. 14/d-g; black arrowhead). Renal parenchyma also showed wide spread glomerular atrophy and its severity was evidenced at 2\textsuperscript{nd} week PDC and continued till the end of the experiment. Additionally, few focal areas showing hyperplasia of mesengial cells in the glomerulus were also noticed in kidney upto the 12\textsuperscript{th} week of diabetes. Furthermore, transmigration of inflammatory cells was also evident at 2\textsuperscript{nd} week of diabetes. However severity of inflammatory foci was reduced at later time points. Details of histopathological findings are presented, in the summary incidence table 1.

3.3. Liver
Liver of control rats showed a normal lobular architecture with hepatocytes arranged in cord like pattern radiating from the central canal. Hepatic cords were lined circumferentially by endothelial cells to form hepatic sinusoids that showed a normal pattern in control rats (Fig. 15/a). In the diabetic rats hepatocytes were found to be hypertrophied, that resulted in narrowing the sinusoidal space. Focal area of necrosis and mild hepatocellular degenerative changes were also observed from 2\textsuperscript{nd} week PDC onwards (Fig. 15/b-g). Additionally, hepatocytes also showed the increasing severity of these lesions from 2\textsuperscript{nd} to 12\textsuperscript{th} week of diabetic duration. Foci of inflammatory cell migration surrounding necrotic hepatocytes were also noticed with hypertrophied kupffer cells in sinusoidal space in diabetic liver throughout the study (Fig. 14; hollow arrow) depicting a consistent severe alteration in liver following STZ-induced diabetes. Details of histopathological findings are presented, in the summary incidence (Table 1).
Effect of diabetes on cognitive ability

4.1. Diabetes resulted in reduced spatial learning and memory

Barnes maze was used to assess the spatial learning and memory of the animals. The test is based on the time duration and path efficiency taken by the animal to locate the escape box that can be used to escape from the open surface of the maze. Diabetic animals presented reduced spatial cognitive ability as compared to the controls. They took more time to reach the escape box in barnes maze test (Fig. 4). The increment in test duration was significant at 2nd week (p≤0.023), 4th week (p≤0.006), 6th week (p≤0.001), 8th week (p≤0.018), 10th week (p≤0.037) and 12th week PDC (p≤0.047) of diabetic duration (Fig. 4).

<table>
<thead>
<tr>
<th>Organs</th>
<th>Pathological lesions</th>
<th>Control</th>
<th>2nd week</th>
<th>4th week</th>
<th>6th week</th>
<th>8th week</th>
<th>10th week</th>
<th>12th week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinar degeneration</td>
<td></td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Atrophy</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Necrosis/ apoptosis</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Inflammation foci</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glomerular atrophy</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Inflammatory foci</td>
<td></td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cyst</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Tubular degeneration</td>
<td></td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Necrosis/ apoptosis</td>
<td></td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Liver</td>
<td>Hepatocellular degeneration</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Necrosis/ apoptosis</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Kupffer cell hyperplasia</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Narrowed Sinusoids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Inflammatory foci</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

- Nil (less than 3%); +, minimal (3-10%); ++, mild (10-20%); ++++, moderate (20-30%); ++++, severe (more than 30%).
The average speed of the diabetic animals was also found to be reduced at 2\textsuperscript{nd} week (p≤0.023), 4\textsuperscript{th} week (p≤0.010), 6\textsuperscript{th} week (p≤0.001), and 8\textsuperscript{th} week (p≤0.041) and 10\textsuperscript{th} week PDC (p≤0.001) as compared to their respective controls (Fig. 3/ii). Additionally, following STZ-induction animals also presented poor path efficiency in locating the escape box at all the diabetic time points (Fig. 3/iii) i.e., 2\textsuperscript{nd} week (p≤0.002), 4\textsuperscript{th} week (p≤0.006), 6\textsuperscript{th} week (p≤0.001), 8\textsuperscript{th} week (p≤0.011), 10\textsuperscript{th} week (p≤0.001) and 12\textsuperscript{th} week PDC (p≤0.001) as compared to their respective controls depicting a continual and persistent spatial cognitive deficit in diabetic animals.

4.2. Diabetes caused alteration in working memory

The working memory of the experimental animals was tested on T-maze by assessing the test duration and path efficiency taken by each animal in exploring the baited arm. STZ-injected animals performed poor while exploring the baited arm (Fig. 6) as the test duration was significantly higher and consistent upto the 12\textsuperscript{th} week of diabetes as compared to their age-matched control animals (Fig. 5/i) i.e., 2\textsuperscript{nd} week (p≤0.001), 4\textsuperscript{th} week (p≤0.001), 6\textsuperscript{th} week (p≤0.001), 8\textsuperscript{th} week (p≤0.001), 10\textsuperscript{th} week (p≤0.001) and 12\textsuperscript{th} week PDC (p≤0.001). Diabetic animals were also presented poor path efficiency in reaching the baited arm at 2\textsuperscript{nd} week (p≤0.001), 4\textsuperscript{th} week (p≤0.001), 6\textsuperscript{th} week (p≤0.001), 8\textsuperscript{th} week (p≤0.001), 10\textsuperscript{th} week (p≤0.001) and 12\textsuperscript{th} week PDC (p≤0.001) as compared to the respective age-matched controls (Fig. 5/ii) confirming impaired spatial working memory in diabetic conditions.

5. Diabetes caused anxiety like behaviour in animals

Elevated plus maze was used to assess the anxiety like behaviour. The test relies upon the rat’s preference toward enclosed, dark space (approach) and an unconditioned fear of heights and/or open spaces (avoidance). Diabetic animals from the 2\textsuperscript{nd} week PDC were presenting anxiety-like behaviour as compared to the age-matched controls (Fig. 8). The percentage of time spend in open arm of diabetic rats were significant reduced in 2\textsuperscript{nd}
week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week PDC (p≤0.001) as compared to their respective controls (Fig. 7/i). Simultaneously, the percentage of open arms entries was also significantly reduced at all the diabetic time points (Fig. 7/ii) i.e., 2nd week (p≤0.01), 4th week (p≤0.01), 6th week (p≤0.01), 8th week (p≤0.01), 10th week (p≤0.01) and 12th week PDC (p≤0.01) as compared to their controls. Furthermore, the anxiety index was significantly increased in diabetic animals (Fig. 7/iii) i.e., at 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week PDC (p≤0.001) as compared to their age-matched controls indicating the diverse effect of diabetes on the emotionality of the animals.

6. Decreased motor coordination following STZ-induced diabetes

Motor coordination was assessed as total length of time spent by the animals on the rotating rod. The test measures the riding time of the animal on the accelerating rod as falling latency. The falling latency of diabetic animals were significantly decreased at 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week PDC (p≤0.001) as compared to their respective controls (Fig. 9/i). Therefore, it is evident that diabetes resulted in reduced motor coordination and this alteration was sustained for a longer duration.

7. Altered neuromuscular strength following diabetes

Forelimb muscular strength was measured by the grip strength meter to assess the effect of diabetes on the neuromuscular strength of the animals. A significant reduction was observed in the grip strength of diabetic animals at 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.002) and 12th week PDC (p≤0.002) as compared to their respective controls (Fig. 9/ii) indicating the adverse and long term effect of diabetes in terms of reduced muscular strength.

8. Diabetic animals presented altered motor activity
Animal’s locomotor activity in open field test is explained in terms of motor output, freezing, grooming, rearing and exploration behaviour of the animal at novel environment. Diabetic animals presented hyperactivity with significantly increased distance travelled consistently at all the PDCs as compared to their respective controls (Fig. 10/i, 11) i.e., 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.002) and 12th week PDC (p≤0.002).

The resting time duration of the diabetic animal as a resultant of hyperactivity was significantly reduced at 2nd week (p≤0.005), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.002) and 12th week (p≤0.002) of diabetes as compared to the ir controls (Fig. 10/ii).

On exposure to the novel environment diabetic animals were doing more non-locomotor activities (Stereotypic movements) like grooming, scratching, etc. Thus, the stereotypic time was significantly increasing from the 2nd week onwards i.e., 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week (p≤0.001) PDCs (Fig. 10/iii) as compared to their age-matched control animals.

Similar trend of increment was also observed in the ambulatory time of the diabetic animals they were doing more ambulatory movements like walking, circling or rearing behaviour in the testing area (novel environment). Accordingly, STZ-induced animals presented significantly increased ambulatory time at 2nd week (p≤0.017), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week (p≤0.001) PDCs as compared to their controls (Fig. 10/iv).

9. Diabetes causing oxidative stress in various vital organs

Assessment of oxidative stress not only explains the level of cytotoxicity in terms of free radicals generation but also reveal the dysregulation of essential cell membrane functions. Following STZ-induced diabetes the tissue lipid peroxidation (LPO) end product concentration i.e., malondialdehyde (MDA) was significantly higher (15-20%) in
all the vital tissues i.e., hippocampus, kidney and liver as compared to the respective controls. The stature of oxidative stress in hippocampus was persistent throughout the 12th week of diabetic duration (Fig. 12/i). At 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p=0.001), 10th week (p≤0.01) and 12th week PDC (p≤0.001) the level of MDA is significantly higher as compared to their respective controls. Furthermore in kidney also the tissue MDA concentration was significantly higher in all the diabetic time points i.e., at 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week PDC (p≤0.001) as compared to their respective controls (Fig. 12/iii). Similar effect of diabetes was also observed in liver as the MDA tissue concentration was increased at 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week (p≤0.001) of diabetes as compared to their respective controls (Fig. 12/ii).

10. Histoarchitectural alterations in brain following diabetes

Neuronal integrity was assessed in the CA1, CA2, CA3 and DG sub regions of hippocampus and cerebellum of control and diabetic rat using haematoxylin and eosin staining.

10.1. Histoarchitectural alterations in hippocampus

Control rats showed regular arrangement of neurons and various types of glial cells in the cell layers of all the hippocampal subfields (Fig. 16-19/a). However, in diabetic hippocampus neurons and glial cells showed various degenerative changes noted at different time points in all the hippocampal subfields i.e., CA1 (Fig. 16/b-g), CA2 (Fig. 17/b-g), CA3 (Fig. 18/b-g), and DG (Fig. 19/b-g). From 2nd weeks onwards diabetic rat hippocampus showed minimal to mild degenerative changes characterized by the ballooning of neuronal and glial cells in all the hippocampal subfields. Focal area showing a very few necrotic and pyknotic (dark cells) neurons in stratum pyramidale layer of CA1, CA2 and CA3 (Fig. 16-18/b) and in granule cell layer of DG region (Fig.
19/b) were also clearly evident at 2nd week PDC. A substantial number of neurons showed necrosis/ apoptosis and severity of this episode got increased with time and was at the peak during 12th week PDC as observed in all the hippocampal subfields. Mild to moderate chromatolysis with cellular acidophilia was observed in the neurons in 4th to 6th week PDC (Fig. 16-19/c, d) and its severity got exacerbated at 8th to 12th week following diabetes (Fig. 16-19/c, d). At this stage (8th to 12th week PDC) hippocampal parenchyma showed vacuolation due to increase in the number of lost cells. An extensive gliosis was also evidenced in all the hippocampal subfields. Incidence of gliosis was mainly observed around the necrotic and apoptotic cells. Details of histopathological findings are presented, in the summary incidence table (Table 2).
### 10.2. Histoarchitectural changes in the cerebellum during diabetes:

Cerebellum of control rats stained with haematoxylin and eosin showed intact arrangement of molecular, Purkinje and granular cell layers with normal distribution of glial cells (Fig. 20/a). Purkinje cell monolayer was continuous with a centrally placed nucleus in the cell body. STZ-induced diabetic rats also showed intact arrangement of all the three layers. In contrast to control most of the Purkinje cells of the diabetic cerebellum were swollen, showing chromatolysis and vacuolation, suggesting necrotic cell death. This focal area of necrosis was surrounded by activated glial cells depicting the typical histomorphology of neuronophagia (Fig. 20/b-g). In addition, some Purkinje...
neurons presented shrunken and pyknotic appearance suggestive of apoptotic cell death (Fig. 20/b-g). Severity and incidence of dying or degenerating cerebellar cells increased with the advancing stage of diabetes. Activation of astroglia and microglia was evident from 2\textsuperscript{nd} week and remained consistent till 12\textsuperscript{th} week of the diabetic rat cerebellum. In the later stages activated, glial cells were circumscribing the necrotic/ apoptotic neurons (Fig. 20/c-g). Interestingly, a discontinuity in the Purkinje cells monolayer indicates a correlation with the severity of degenerative histoarchitectural alterations following diabetes. Details of histopathological findings are presented, in the summary incidence table (Table 3).

<table>
<thead>
<tr>
<th>Table 3: Summary incidence table of histopathological lesions in cerebellum of control and STZ-induced diabetic rat.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 2\textsuperscript{nd} week 4\textsuperscript{th} week 6\textsuperscript{th} week 8\textsuperscript{th} week 10\textsuperscript{th} week 12\textsuperscript{th} week</td>
</tr>
<tr>
<td>Ballooning of Purkinje cell</td>
</tr>
<tr>
<td>Chromatolysis</td>
</tr>
<tr>
<td>Vacuolation</td>
</tr>
<tr>
<td>Necrosis/apoptosis</td>
</tr>
<tr>
<td>Pyknosis/ dark Purkinje cell</td>
</tr>
<tr>
<td>Gliosis</td>
</tr>
<tr>
<td>Neuronophagia</td>
</tr>
</tbody>
</table>

-, Nil (less than 5%); +, minimal (5–10%); ++, mild (10–20%); ++++, moderate (20–30%); +++, severe (more than 30%).

11. Cell death following diabetes

To investigate the diabetes associated apoptotic cell death, active caspase-3 immunolabelling was performed in both hippocampus and cerebellum. Caspase-3 has been accepted to be the major executioner of the cell death mechanism. Any brain injury or disorder activates the caspase-3 and its profuse expression validates the severity of the brain disorder.
11.1. Cellular death in hippocampus during STZ-induced diabetes

A very few cells were presented caspase-3 immunopositivity in controls (Fig. 21/a.i-iv). The apoptotic cells were more pronounced in DG region than in CA1, CA2 and CA3 regions of the diabetic rat hippocampus (Fig. 21/a.g.iv). While arbitrating the morphology of the stained cells, the active caspase-3 expression was mainly present in the cytoplasm and processes of the degenerating cells. On 2nd week PDC onwards the dying caspase-3 positive cells were more pronounced in stratum radiatum and stratum pyramidal cell layer of CA1, CA2 and CA3 region. While in DG region the apoptotic cells were scattered in both granular and polymorphic layer in all the diabetic time points. Quantitatively, the apoptotic cell population was increasing in all the diabetic time points consistently in all the hippocampal fields (Fig. 22/i) i.e., in CA1 (p≤0.001), CA2 (p≤0.001), CA3 (p≤0.001) and DG region (p≤0.001) as compared to the respective controls suggesting a severe cellular degeneration in the hippocampal tissue following diabetes. Volumetric fraction (area fraction) assessment also showed a significant increment in the percentage of active caspase-3 expression in CA1, CA2, CA3 and DG region at all the diabetic time points as compared to the respective controls (Fig. 22/ii).

11.2. Cell death in cerebellum following diabetes:

Similar pattern of cellular damage was also observed in cerebellum as a reflective cell death was observed following STZ-induced diabetes upto 12 weeks from active caspase-3 immunolabelling. Active caspase-3 positive cells were clearly evident in molecular layer, Purkinje cell layer and granule cell layer of the diabetic cerebellum (Fig. 23/b-g) as compared to the controls having minimal presence of active caspase-3 labelled cells (Fig. 23/a). Interestingly, active caspase-3 expression was more prominent in the nuclei of Purkinje cells and in surrounding Bergmann glial cell bodies in comparison to the molecular and granule cell layer (Fig. 23/b-g). The inclination in Purkinje and Bergmann cell degeneration was constant at all the diabetic time points as compared to the controls.
Quantitatively, caspase-3 positive cells were significantly increasing with advancing diabetic state i.e., 2nd week ($p \leq 0.001$), 4th week ($p \leq 0.001$), 6th week ($p \leq 0.001$), 8th week ($p \leq 0.001$), 10th week ($p \leq 0.001$) and 12th week PDC ($p \leq 0.001$) as compared to their respective controls (Fig. 24/i) suggesting a severe cellular degeneration in the cerebellar tissue. Percentage of area fraction of caspase-3 expression was also showing the similar trend. The increment of caspase-3 expression was consistent at all the diabetic time points i.e., 2nd week ($p \leq 0.001$), 4th week ($p \leq 0.001$), 6th week ($p \leq 0.001$), 8th week ($p \leq 0.001$), 10th week ($p \leq 0.001$) and 12th week PDC ($p \leq 0.001$) as compared to their respective controls (Fig. 24/ii).

12. Glial activation and proliferation following STZ-induced diabetes

To examine the glial activation, we gauged and compared the distribution of cells immunoreactive for the astrocyte (GFAP) and microglial (Iba-1) markers in the hippocampus of STZ-induced diabetic and age-matched control rats. The activated state was judged by phenotypic changes, increase in cell number and glial cell scoring. The activation stature was further confirmed by the immunoexpression of OX-6 and OX-42 for microglia and S100β for astrocyte. Glial activation assessment was also done in cerebellar tissue to investigate and compare the further effect of diabetes on other brain regions.

12.1. Glial phenotypic switching

12.1.1. Astroglial activation following diabetes

GFAP (Glial fibrillary astrocytic protein) is an intermediate filament protein in astroglia, popularly used to study the astroglial structure and associated functions.

12.1.1.1. Hippocampus: Immunohistochemical analysis showed an elevated expression of GFAP protein following diabetes in hippocampus alongwith an alteration in the morphology of GFAP-labelled astroglia in CA1, CA2, CA3 and DG regions of hippocampus from 2nd week onwards (Fig. 26/b-g.i-iv). Resting astroglia in controls
presented thin, fine, intact processes with small cell body (Fig. 24/a. i-iv). The presence of resting astroglia was consistent in all the control groups up to the 12th week of diabetic duration.

At 2nd and 4th week of diabetes the GFAP immunolabelling was more intense and hypertrophied astroglia with activated phenotype, i.e., thick, dense, bushy and extensively overlapping processes with enlarged darkly stained cell body were present (Fig. 25/b-d). Such cells were more common in CA1, CA2, CA3 and DG regions of the hippocampus (Fig. 26/b, c. i-iv). Interestingly, hypertrophied astroglial cells at 4th week of diabetes were also having fragmented processes (Fig. 25/c,d; 26/c.i-iv). From 6th week onwards, the unevenly distributed reactive astroglia having thicker, fragmented and overlapped processes, with deeply stained cell body, were clearly observed indicating the astroglial dystrophy in all the aforementioned hippocampal regions (Fig. 26/d, g. i-iv). Interestingly, the scenario of astroglial activation is regular in all the neuronal cell layers of the hippocampus suggesting a long term effect of STZ-induced diabetes on hippocampus astroglial population.

12.1.1.2. Cerebellum: An increased GFAP expression in the diabetic cerebellum up to 12th week of diabetes was immunohistochemically observed. The GFAP labelled Bergmann glial fibres in controls were presented intact, thin and erect morphology (Fig. 28/a) while in diabetic animals Bergmann glial fibres became hypertrophied, fragmented and disorganised at all the time points (Fig. 28/b-g). The astroglia in the white matter and granule cell layer also presented activated morphology having thick, dense and fragmented processes with the darkly stained cell body (Fig. 29/b-g) as compared to the resting astroglia with thin processes and lightly stained cell body in controls (Fig. 29/a).

12.1.2. Microglial response during diabetes

12.1.2.1. Hippocampus: Microglial alterations in hippocampus during diabetes were studied with the immunoeexpression of Iba-1 (Ionized calcium binding adaptor molecule-
1. It is a calcium binding protein that not only participates in membrane ruffling but also regulates the functioning of microglia. In brain, Iba-1 protein expression is specifically restricted to the microglial cells.

Following STZ-induced diabetes, Iba-1+ cells in the entire hippocampus were presenting activated morphology as compared to the controls (Fig. 31, 32). The microglial cells were showing phenotypic transformation, i.e., from ramified to activated states bearing retracted, thick processes and large irregular cell body (Fig. 31/b-d; Fig. 32/b-g). In controls resting microglia cells processes exhibited ramified wispy appearance with round lightly stained cell body (Fig. 31/a) throughout the hippocampus subfields upto the 12th week PDC (Fig. 32/a.i-iv).

In diabetic hippocampus, by 2nd week PDC the resting/ramified microglia get transformed into intermediate activated state having thick processes with enlarged cell body mainly in CA1 and CA3 regions of the hippocampus (Fig. 32/b.i, iii) while in CA2 and DG regions microglial cells were showing mild activation (Fig. 32/b.ii, iv). Fourth week onwards, the intermediate activated microglial cells became fully activated with intense labeling, thicker and retracted processes and larger irregular or rod shaped cell body undergoing hypertrophy that was preceded by hyperplasia (Fig. 32/c-g). The activation state of microglia was consistent in CA1, CA2, CA3 and DG regions of the rat hippocampus upto the 12th week PDC depicting an austere and long term response of microglial cells, as first line of defense following diabetes.

12.1.2.2. Cerebellum: STZ-induced diabetes resulted in intense microglial activation and morphological transformation to activated phenotype in the cerebellum as shown by Iba-1 immunolabelling. In controls, microglia were seen with resting morphology having small, thin, multiple processes and round small cell body (Fig. 34/a). Following diabetes, an increase in Iba-1 expression was clearly evident in the cerebellar microglia. With the advancing diabetic duration, the resting microglia got transformed into activated state
exhibiting thick and fewer numbers of processes with darkly stained Iba-1 positive irregular cell body (Fig. 34/b-g).

12.2. Glial proliferation in response to STZ-induced diabetes

12.2.1. Astrocyte proliferation during diabetes

12.2.1.1. Hippocampus: Cell proliferation and associated glial activation in all the regions of hippocampus was not only prominent but also accentuated on successive time duration following diabetes. GFAP labelled cell quantification showed that there was a considerably steep increment in the number of astroglia in all the hippocampal subfields. The population of reactive astroglia was significantly dense in CA1 (p≤0.001), CA2 (p≤0.05), CA3 (p≤0.001) and DG (p≤0.001) region as compared to the respective controls (Fig. 27/i). Volumetric fraction (area fraction) assessment also showed a significant increment in the percentage of GFAP expression in CA1, CA2, CA3 and DG region during the 2nd (p≤0.001), 4th (p≤0.001), 6th (p≤0.001), 8th (p≤0.001), 10th (p≤0.001) and 12th (p≤0.001) week of diabetic duration as compared to the respective controls (Fig. 27/ii).

12.2.1.2. Cerebellum: Quantification of GFAP positive cells showed a significant gradual increment (p≤0.001) in astroglia population at all the diabetic points i.e., 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week (p≤0.001) as compared to their respective controls. A progressive escalation was also observed in the cerebellar astroglial population following diabetes (Fig. 30/i). Volumetric fraction (area fraction) assessment also showed a significant increment in the percentage of GFAP expression at all the diabetic points i.e., 2nd week (p≤0.001), 4th week (p≤0.001), 6th week (p≤0.001), 8th week (p≤0.001), 10th week (p≤0.001) and 12th week (p≤0.001) as compared to their respective controls (Fig. 30/ii).
12.2.2. Diabetes leads to the microglial proliferation

12.2.2.1. Hippocampus: Cell quantification of Iba-1 immunopositive cells revealed a gradual increase in microglial population indicative of cell proliferation in brain following STZ-induced diabetes. There was a significant ($p \leq 0.001$) increment in microglial population throughout the hippocampus i.e., in CA1, CA2, CA3 and DG region on every subsequent diabetic time point i.e., at 2$^{nd}$ ($p \leq 0.01$), 4$^{th}$ ($p \leq 0.01$), 6$^{th}$ ($p \leq 0.01$), 8$^{th}$ ($p \leq 0.01$), 10$^{th}$ ($p \leq 0.01$) and 12$^{th}$ week ($p \leq 0.01$) PDCs as compared to their respective controls (Fig. 33/i). Volumetric fraction (area fraction) assessment also showed a significant increment in the percentage of Iba-1 protein expression in CA1, CA2, CA3 and DG region during the 2$^{nd}$ ($p \leq 0.01$), 4$^{th}$ ($p \leq 0.01$), 6$^{th}$ ($p \leq 0.01$), 8$^{th}$ ($p \leq 0.01$), 10$^{th}$ ($p \leq 0.01$) and 12$^{th}$ ($p \leq 0.01$) week of diabetic duration as compared to their respective controls (Fig. 33/ii).

12.2.2.2. Cerebellum: A significant increase was observed in microglial population in the diabetic rat cerebellum at all the time point studied i.e., 2$^{nd}$ week ($p \leq 0.001$), 4$^{th}$ week ($p \leq 0.001$), 6$^{th}$ week ($p \leq 0.001$), 8$^{th}$ week ($p \leq 0.001$), 10$^{th}$ week ($p \leq 0.001$) and 12$^{th}$ week PDC ($p \leq 0.001$) as compared to their respective controls indicating microgliosis in response to the alterations following diabetes (Fig. 35/i). Percentage of Iba-1 expression as area fraction in diabetic cerebellum was also significantly increasing throughout the study i.e., at 2$^{nd}$ week ($p=0.044$), 4$^{th}$ week ($p=0.002$), 6$^{th}$ week ($p=0.001$), 8$^{th}$ week ($p=0.001$), 10$^{th}$ week ($p \leq 0.001$) and 12$^{th}$ week ($p \leq 0.001$) of diabetic cerebellum as compared to their respective controls (Fig. 35/ii).

13. S100β positive cells in diabetic rat hippocampus

S-100β, a calcium binding protein is well-accepted astrocytosis marker that particularly associated with astrocytic activated states and functions. S100β immunolabelling was performed to further verify the astroglial activation.
Immunolabelling confirmed the presence of S100β positive cells in all the hippocampal regions (Fig. 36/b-g) following diabetes. Hippocampal cells immunolabelled with S100β had round or irregular shaped cell body with thick processes. These S100β+ cells in diabetic hippocampus were showing stellate morphology having large cell body and thick processes similar to the activated astrocytes. Such S100β+ astrocytes were common in all the hippocampal regions i.e., CA1, CA2, CA3 and DG throughout the 12th week PDC (Fig. 36/b-g. i-iv). While in controls very mild S100β positivity was recorded in all the hippocampal subfields (Fig. 36/a.i-iv). The S100β labelled cell population was gradually increasing following diabetes and consistent upto the 12th week of diabetic duration displaying the adverse effect of increased blood glucose level as astrogliosis in hippocampus.

14. Antigen presenting cells in diabetic hippocampus

OX-6 (MHC-II) immunolabelling was performed to detect the antigen presenting cells in hippocampus following STZ-induced diabetes. In brain, microglial cells are the primary mediators of MHC-II antigen presentation and processing. OX-6 is also regarded as an activation-specific microglial marker.

Immunolabelling revealed a profound expression of OX-6 positive cells in all the subregions of the diabetic hippocampus from 2nd week PDC (Fig. 37/b-g). The OX-6 expressing cells were common in both pyramidal and granular layer of all three hippocampal subregions. In controls the expression was very low in all the hippocampal subfields (Fig. 37/a.i-iv). Cell quantitation results revealed a significant and gradual elevation in MHC-II expressing cells in the entire hippocampus upto the 12th week PDC, i.e., in CA1 (p≤0.001), CA2 (p≤0.001), CA3 (p≤0.001) and DG region (p≤0.001) as compared to their respective controls (Fig. 38/i). Volumetric fraction (area fraction) assessment also showed a significant increment in the percentage of OX-6 expression in CA1, CA2, CA3 and DG region during the 2nd (p≤0.01), 4th (p≤0.01), 6th (p≤0.01), 8th
(p≤0.01), 10\textsuperscript{th} (p≤0.01) and 12\textsuperscript{th} (p≤0.01) week of diabetic duration as compared to their respective controls (Fig. 38/ii). The significant expression of OX-6 not only depicted the activation of microglial cells but also evidenced the state of inflammation following diabetes in hippocampus as MHC-II signalling plays an imperative role in inflammation retorting to both endogenous and exogenous antigenic proteins.

15. Effect of diabetes on OX-42 expressing cells in hippocampus

OX-42 recognizes the type 3 complement receptors (CR3) in mononuclear phagocytes. Thus, OX-42 immunolabelling was performed to assess the microglia/macrophages activation following diabetes.

A robust microglia/macrophages activation was observed at 2\textsuperscript{nd} week PDC till the 12\textsuperscript{th} week of diabetic state, the aftermost time point investigated in our study (Fig. 39/b-g). In controls, however resting/ ramified microglia morphology having small, thin processes and small round cell bodies expressing CR3 (OX-42 positive) were seen (Fig. 39/a.i-iv). Such resting/ramified microglial cells were homogenously distributed in all the hippocampal subfields.

During diabetic state OX-42 positive microglial cells were intensely labelled, thicker and possessed retracted processes with larger irregular or rod shaped cell body indicating austere microglial activation in hippocampus (Fig. 39/b-g.i-iv). These activated cells were mainly present in stratum radiatum and stratum oriens while fewer in stratum pyramidale. Accumulation of activated microglia near pyramidal neurons indicated the moniker protecting approach of microglia cells via enfolding the degenerating neuronal cells. The scenario of microglial activation was consistent upto 12\textsuperscript{th} week PDC. Quantitatively, the population of OX-42 immunolabelled cells was significantly dense and sequentially increasing in CA1 (p≤0.001), CA2 (p≤0.001), CA3 (p≤0.001) and DG (p≤0.001) region as compared to their respective controls (Fig. 40/i). Volumetric fraction (area fraction) assessment also showed a significant increment in the
percentage of OX-42 expression in CA1, CA2, CA3 and DG region during the 2\textsuperscript{nd} (\(p \leq 0.001\)), 4\textsuperscript{th} (\(p \leq 0.001\)), 6\textsuperscript{th} (\(p \leq 0.001\)), 8\textsuperscript{th} (\(p \leq 0.001\)), 10\textsuperscript{th} (\(p \leq 0.001\)) and 12\textsuperscript{th} (\(p \leq 0.001\)) week of diabetic duration as compared to their respective controls (Fig. 40/ii). The plethoric presence of OX-42 (CR-3) positive activated microglial cells in hippocampus confirmed the incidence of neuroinflammation-related brain disorder following diabetes.

16. Macrophagic cell stature in hippocampus following diabetes

ED-2 or CD-163 is a glycoprotein of class B of the scavenger receptor cysteine rich superfamily exclusively expressed on the perivascular (PV) and meningeal macrophages. It is a phagocytic marker which also performs immunoregulatory functions. Immunoexpression of CD-163 is associated with the resolution phase of inflammation and the alternative activation of macrophages. Thus, it is useful in the assessment of prolific microglial activation (express more in perivascular microglia, than in parenchymal microglia) and resulted inflammation following diabetes.

The ED-2 immunolabelling revealed an increased presence of macrophagic cell population in hippocampus following STZ-induced diabetes (Fig. 41). In controls, the immunoexpression was weak in terms of both labelling and quantification indicating the negligible incidence of phagocytic activity and inflammation following microglial activation (Fig. 41/a.i-iv). From 2\textsuperscript{nd} week PDC the presence of ED-2 positive cells increased sequentially upto the 6\textsuperscript{th} week PDC (Fig. 41/b-d.i-iv). Afterwards, a decrement was observed from 8\textsuperscript{th} week PDC, but significantly higher than the respective controls upto the 12\textsuperscript{th} week PDC and remained steady (Fig. 41/e-g.i-iv). Quantitatively, the population of ED-2 positive cells were consistent and significantly higher in all the hippocampal fields i.e., in CA1 (\(p \leq 0.001\)), CA2 (\(p \leq 0.001\)), CA3 (\(p \leq 0.001\)) and DG region (\(p \leq 0.01\)) as compared to their respective controls (Fig. 42/i). Volumetric fraction (area fraction) assessment also presented a significant increment in the percentage of ED-2 positive cells in CA1, CA2,
CA3 and DG region during the 2\textsuperscript{nd} (p≤0.01), 4\textsuperscript{th} (p≤0.01), 6\textsuperscript{th} (p≤0.01), 8\textsuperscript{th} (p≤0.01), 10\textsuperscript{th} (p≤0.01) and 12\textsuperscript{th} (p≤0.01) week PDC as compared to their respective controls (Fig. 42/ii).

17. Neuroinflammation in hippocampus following diabetes

To assess whether oxidative stress, cell death and glial activation following STZ-induced diabetes promotes the neuroinflammation, we have evaluated the expression levels of pro-inflammatory cytokines, i.e., IL-1β and TNF-α in the hippocampus.

17.1. IL-1β expression in hippocampus following diabetes

An increased and pronounced immunoexpression of IL-1β was observed in hippocampus following diabetes (Fig. 43). The IL-1β positive cells were clearly observed in stratum radiatum and stratum oriens layer of CA1 and CA3 region of the diabetic hippocampus (Fig. 43/b-g. i,iii), while in CA2 region the IL-1β positive cells were more in stratum pyramidale as compared to the other neuronal layers (Fig. 43/b-g. ii). In DG region the expression was consistent in both granular and polymorphic layer (Fig. 43/b-g. iv). Similar stature of IL-1β expressing cells was observed throughout the 12\textsuperscript{th} week PDC. However, in controls the population of IL-1β positive cells was very low in all the hippocampal subfields throughout the study (Fig. 43/a.i-iv). Cell quantification also presented a significant and regular appearance of IL-1β positive cells in the entire hippocampus upto the 12\textsuperscript{th} week of diabetes i.e., in CA1 (p≤0.001), CA2 (p≤0.001), CA3 (p≤0.001) and DG region (p≤0.001) as compared to their respective controls (Fig. 44/i). Volumetric fraction (area fraction) assessment also showed a significant increment in the percentage of IL-1β expression in CA1, CA2, CA3 and DG region during the 2\textsuperscript{nd} (p≤0.01), 4\textsuperscript{th} (p≤0.01), 6\textsuperscript{th} (p≤0.01), 8\textsuperscript{th} (p≤0.01), 10\textsuperscript{th} (p≤0.01) and 12\textsuperscript{th} (p≤0.01) week of diabetic duration as compared to their respective controls (Fig. 44/ii). The data indicated the occurrence and persistent stature of neuroinflammation in hippocampus for a long duration following STZ-induced diabetes.
17.2. TNF-α expression in hippocampus following diabetes

Tumor-necrosis factor-α (TNF-α) is a pro-inflammatory cytokine with potent stimulatory actions in immune and vascular responses. It plays a major role in stimulation and propagation of neuroinflammation following brain insults.

Throughout the study the TNF-α immunolabelling in controls was negligible both in terms of expression (Fig. 45/a.i-iv) and quantification (Fig. 46/i,ii). In diabetic hippocampus the immunoexpression of TNF-α positive cells was significantly higher and consistent in all the hippocampal subfields up to the 12th week PDC (Fig. 45/b-g.i-iv). The TNF-α expressing cells were more in stratum oriens and stratum radiatum neuronal layers than in stratum pyramidale layer of CA1, CA2 and CA3 region of hippocampus (Fig. 45/b-g.i-iii). Similarly, in DG region the expression of TNF-α positive cells was regular in both granular and polymorphic layer (Fig. 45/b-g.iv). Quantitatively, the population of TNF-α immunolabelled cells was significantly dense and increased in CA1 (p≤0.001), CA2 (p≤0.01), CA3 (p≤0.001) and DG (p≤0.001) region as compared to their respective controls (Fig. 46/i). Interestingly, the TNF-α expressing cells sequentially increased from 2nd week PDC onwards. The significant presence of pro-inflammatory cytokine TNF-α, directs towards the long term neuroinflammatory state in hippocampus following diabetes. Volumetric fraction (area fraction) assessment also showed a significant increment in the percentage of TNF-α positive cells in CA1, CA2, CA3 and DG region during the 2nd (p≤0.001), 4th (p≤0.001), 6th (p≤0.001), 8th (p≤0.001), 10th (p≤0.001) and 12th (p≤0.001) week PDC as compared to their respective controls (Fig. 46/ii).
DISCUSSION

Diabetes mellitus (DM) is one of the most severe metabolic disorders in humans characterized by the hyperglycemia due to relative/an absolute lack of insulin or the action of insulin on its target tissue or both. It is now a global health concern with a affected population of 347 million worldwide (IDF, 2014). Diabetic patients seem to have more risk of developing Alzheimer’s disease and other dementias as such DM associated alterations in CNS and resulted neurological consequences have become priorities in neuroscience research (Biessels et al., 1994, 2008; Arvanitakis et al., 2004; Northam et al., 2006; Kawamura et al., 2012; Cardoso et al., 2013; Wang et al., 2014). Impairments of cognitive deficits (Popović et al., 2001; Baydas et al., 2003a; Biessels et al., 2008; Alvarez et al., 2009; Choi et al., 2009; Zavoreo et al., 2014; Nagayach et al., 2014a), reduced muscular strength (Andersen et al., 2005; Nagayach et al., 2014b), behavioural deficits (Sweetnam et al., 2012; Nagayach et al., 2014b), anxiety (Lustman and Clouse, 1990; Grigsby et al., 2002; Herzer and Hood, 2010) and depression (Talbot and Nouwen, 2000; Egede and Ellis, 2010) are well documented in both type 1 and type 2 diabetes mellitus. Besides, information on the systematic mechanisms explaining the associative reciprocity among the various brain regions and cells to DM is deficient till date. There are only few studies explaining the diabetic encephalopathy in terms of cognitive and behavioural dysfunctions with limitations (Mc Call, 1992; Biessels et al., 1994; Coleman et al., 2004; Kodl and Seaquist, 2008; Guven et al., 2009; Choi et al., 2009; Alvarez et al., 2009). The pathophysiological and/or cellular mechanisms explaining diabetes allied impediments is limited to the causative factors like poor glycemic control, insulin dependency and oxidative stress (Egede, 2006; Liu et al., 2008; Gupta et al., 2014). Diabetes associated alterations range from structural, cellular to neurochemical changes leading to direct neuronal damage, oxidative stress, cell death
and loss of information processing (Thorré et al., 1997; Alvarez et al., 2009; Nagayach et al., 2014a, b).

The structure and functions of glial cells in healthy and diseased brain is always been a matter of debate and deliberate research. Glial cells are now recognized as an elementary contributor in the pathogenesis of various neurological disease and disorders (Heneka et al., 2010; Parpura et al., 2012; Verkhratsky et al., 2014). Understanding of the imperative and multitasking attribute of glial cells in brain, its stature and response following diabetes allied cognitive and behavioural impairments deserves pertinent investigation. The present study is an approach to fulfil the lacunae on the role of glial cells and associated immune response in cognitive and behavioural deficits in experimentally induced diabetes models.

The hippocampus plays major role in several cognitive aspects linked to emotional, learning, memory and sensorimotor functions (Bast, 2007). Furthermore, cerebellum is associated with emotion, cognition and behaviour (Rapoport et al., 2000; Schmahmann and Caplan, 2006) and any alterations to the cerebellum lead to motor deficits, dementia, schizophrenia and other psychiatric disorders (Baldaçara et al., 2008; Sui and Zhang, 2012). Hence we have studied these two tissues.

**Long term physiological changes as a consequent of increased blood glucose level following STZ-induction**

The consequences of type 1 diabetes are well studied in the STZ-induced experimental animal models. Similar to the previous studies (Choi et al., 2009; Coleman et al., 2004, 2010) the STZ-induced animals were showing increase in blood glucose level, food and water intake, brain-body weight ratio and decreased body weight, directing towards the marked destruction of insulin secreting pancreatic β-cells by STZ (Junod et al., 1969). During diabetes, the glucose utilization in the brain gets decreased (McCall, 1992), due
to reduction in BBB glucose transporters (Choi et al., 1989; Pardridge et al., 1990), which further makes the brain more vulnerable to the critical pathological events. The consequence of diabetes was well manifested by the decreased brain weight which might be due to the increased cell death (Jakobsen et al., 1987) in brain has also been evidenced in this study.

**Pancreatic and other vital organs damage grounded the confirmation for STZ-induced diabetic model**

In our study we have observed a persistent damage in pancreas, kidney and liver upto the 12\textsuperscript{th} week PDC. In both STZ and alloxan-induced type -1 diabetes models there is an irreversible pancreatic damage that includes decrease of secretory granules, fusion of some granules and pyknotic nuclei, adipose tissue infiltration and localized lymphocytic cell accumulations are reported (Li et al., 2000; Jelodar et al., 2005; Abdel Aziz et al., 2013). Similar irreversible degeneration was also observed in the histopathological evaluation of pancreas. This was initiated from the 2\textsuperscript{nd} week PDC and sustained upto the 12\textsuperscript{th} week PDC. Enhanced AGEs production, oxidative stress and increased level of cytokines following type 1 diabetes, resulting in exocrine pancreatic atrophy that includes the severe loss of the islet of Langerhans in exocrine pancreatic tissue (Gepts, 1965), swollen, necrotic and vacuolated beta cells having condensed nucleus with loss of cytoplasmic granularity has been observed (Mir et al., 2008). Such changes have also been recorded in the present investigation.

Effect of STZ-induced diabetes was clearly evidenced in kidney and upto the 12\textsuperscript{th} week PDC the alterations in renal structure and function was consistent and severe. Most presumptive symptoms of diabetes include polyuria (excess of urination) that in uncontrolled diabetic state resulted in severe kidney damage or diabetic nephropathy (Mauer et al., 1984; Makino et al., 1996). Diabetes associated hyperglycemia trigger
multiple mechanisms that instigates not only the development but also extend the outcomes of nephropathy (Ziyadeh, 2004). Hyperglycemia activates vasoactive systems (renin–angiotensin–aldosterone and endothelin systems) which further interact with metabolic system and genetic predisposition and aggravate the kidney damage (Dronavalli et al., 2008). We observed similar alterations in experimentally induced diabetic kidney that resulted in functional and structural changes of nephron like glomerular infiltration and hyperfusion, thickening of the glomerular basement membrane, glomerular hypertrophy, decreasing the width of Bowman’s space, thickening of the parietal layer of Bowman’s capsule, hyalinisation, glomerulosclerosis and mesangial expansion, etc. This is in agreement with the findings of Şen et al. (2002), Öztürk et al. (2005), and Ozdemir et al. (2009).

Furthermore, the necessary function of balancing the blood glucose homeostasis makes the liver more vulnerable to any blood glucose fluctuation. Thus, during diabetes the increment in blood glucose level resulted in imbalance of oxidation-reduction reactions in hepatocytes, which further triggers the generation of AGEs and lead to the state of oxidative stress. Histopathologically, the accumulation of fatty molecules or hyperlipidemia is a most apparent observation in type 1 diabetic liver (Ohno et al., 2000; Zafar et al., 2009). Alike previous studies there were many pathological changes also observed in our study of diabetic liver including dilated sinusoids, periportal fatty infiltration, degenerated or necrotic hepatocytes with polymorphic nuclei, hyperplasia of bile duct, etc. (Cameron et al., 2005; Zafar et al., 2009; Ozdemir et al., 2009).

The irreversible pancreatic damage (Abdel Aziz et al., 2013), renal (Finne et al., 2005; Reutens, 2013) and liver (Martin and Tomlinson, 2014) pathogenesis are considered as major complications of type 1 diabetes. The severe destructions of insulin producing pancreatic beta cells upto the 12th week PDC validates the increased blood glucose level following STZ-induction. Furthermore, renal and liver damage as observed
in the study not only confirms the incidence of polyuria, body weight loss and subsequent polyphagia to reimburse the energy loss during diabetes.

**Diabetes resulted in cognitive and behavioural alterations**

Manifestations of diabetes related cellular changes in brain believed to cause reduced cognitive ability (Alvarez et al., 2009) and high susceptibility for dementia and Alzheimer’s disease (Biessels et al., 1994; Ristow, 2004; Northam et al., 2006). The degenerative changes in neurons (Suh et al., 2007) and declined neurogenesis (Choi et al., 2009; Alvarez et al., 2009) resulted in significant cognitive impairment. In coincidence with these reports we found that STZ-induced animals were performing poorly on Barnes maze and T maze tasks revealing a reduced spatial learning and working memory. Similar alterations were also observed in the motor behaviour and muscle strength of the diabetic animals as they were showing reduced falling latency on Rotarod and poor or weak muscular strength in grip strength meter indicating impaired motor/ muscle activity upto the 12th week PDC. Alterations in motor behaviour following diabetes was previously well reported both in human and animal models with underlying causes of increased blood glucose level associated micro- or macrovascular complications (Demirbüken et al., 2012) and altered neurotransmission (Sherin et al., 2012). On elevated plus maze STZ-injected diabetic animals present anxiety behaviour as they had higher and consistent anxiety index than the controls upto the 12th week PDC. The anxiety index of the diabetic animals coalesces various variables (like percentage of entries in the open arm and total number of entries in both the arms) assessed during exploration in the elevated plus maze and specifies an inclusive tendency of emotionality of the animal (Cohen et al., 2013). A similar anxiety-like behaviour in type 1 diabetic patients in relation to the poor glycemic control has been evidenced (Collins et al., 2009; Herzer and Hood, 2010).
Open field test was generally intended as a test of exploration, locomotor activity and anxiety (Walsh and Cummins, 1976; Prut and Belzung, 2003; Ramos et al., 2008). In open field test diabetic animals presented increased locomotor activity, stereotyped time (grooming, rearing etc.) and ambulatory time indicating the altered motor activity and aberrant exploratory behaviour in the novel environment as has been reported in earlier studies (Ramanathan et al., 1998; Belviranli et al., 2012). Belviranli et al. (2012) have shown that altered grooming behaviour is an index of anxiolytic activity that is frequently performed by the rodents either to escape, resolve or to normalize the anxiety condition (Gispen and Isaacson, 1981; Moody et al., 1988; Kalueff and Tuohimaa, 2004). Additionally, the movement of diabetic animals were more in the corners of the open field test apparatus as they were avoiding the arena centre. In the studies following anxiolytic drug administration it was observed that an anxious animal usually avoids the arena centre as it assumed to be threatening for the rodents than its periphery (Treit and Fundytus, 1988; Simon et al., 1994). The pathophysiological and/or cellular mechanisms related to the above listed motor deficits and anxiety behaviour following diabetes is still limited to the causative factors like poor glycemic control, insulin dependency and oxidative stress (Egede, 2006; Liu et al., 2008; Ho et al., 2012; Gupta et al., 2014).

**Persistent cell death in brain due to STZ-induced diabetes**

It was previously reported that increased blood glucose level could lead to the elevation in brain glucose level within 2-3 days of STZ induction (Mäkimattila et al., 2004; Criego et al., 2005; Heikkilä et al., 2009) and that it persists for months was recorded by Ruderman et al. (1974), Folbergrová et al. (1992), and Shram et al. (1997). Such altered brain glucose level causes increased glycogenolysis, impaired glucose transport across the membranes, reduced glycogenesis and excessive cell death. This has also been well
documented in case of hypoglycemia that altered glucose homeostasis triggers the neuronal death in CNS (Singh et al., 2004).

In our study we found a significant cellular degeneration and/or cell loss in both hippocampus and cerebellum of STZ-induced diabetic animals with immunohistochemical and histological studies consistently up to the 12th week of diabetic duration. Histological study revealed marked cell destruction both in hippocampus and cerebellum following diabetes. Cardinal signs of cell degeneration like, necrosis/apoptosis, vacuolation, chromatolysis, nonalignment of cells, neuronophagia and gliosis were clearly visible in all the subfields of hippocampus and cerebellar layers. The incidence of continual cell degeneration and/or loss is in accordance with the increased level of oxidative stress (level of LPO) in hippocampus up to the 12th week PDC. Reactive oxygen and nitrogen species get reacted with polyunsaturated fatty acids present in cell membranes and initiate the cascade of events leading to lipid peroxidation (LPO). LPO via generation of reactive aldehydes either directly or indirectly cause damage to the cell component. These cellular alterations cause oxidation of low density lipoprotein (LDL) particles, fluctuations in permeability, alterations in ion flux and other toxic constituents, damage to the cell matrix with consequent DNA injury, which ultimately ensue the irreversible injury and finally cell death (Lima and Abdalla, 2001). Additionally, higher oxygen consumption and abundant lipid content makes the brain extremely vulnerable to the oxidative stress. A common notion regarding the pathogenesis of brain dysfunction in diabetes, relates the cerebral cell damage to the free radicals mediated oxidative stress (Kumar and Menon, 1993; Giacco and Brownlee, 2010; Muriach et al., 2014). Thus, oxidative stress reported in the present study is might be a possible cause of cell death in hippocampus and cerebellum during diabetic state.

An increased and conspicuous active caspase-3 activity was evident in hippocampal fields and cerebellar cell layers of the diabetic rats. The caspase-3 positivity
was significantly higher in degenerating neuronal cells and their processes. Active caspase-3 expressing cells were more in stratum pyramidale and radiatum layers of CA regions of hippocampus, while in the DG the cell death was more prominent in granule cell layer suggestive of impaired information circuitry in hippocampus following diabetes. Likewise, active caspase-3 positive cells were also successively increasing in all the three layers of the cerebellum. Furthermore, the population of apoptotic (active caspase-3 positive) cells were also successively rising in the subsequent weeks post diabetes confirmation in both the brain tissues (hippocampus and cerebellum). Caspases play an essential role in neuronal apoptosis (Bredesen, 2000; Yakovlev and Faden, 2001), and caspase-3 has been accepted as a major executioner of the cell death mechanism. Any brain injury or disorder activates the caspase-3 and its profuse expression validates the severity of the brain disorder (Yuan and Yankner, 2000). The deleterious effect of diabetes and hyperglycemia as cell death was previously well reported with underlying mechanisms of activation of p53 transcription factor, intrinsic cell death pathway and decreased IGF-I level due to increased blood glucose (Yamaguchi et al., 2001; Muranyi et al., 2003; Lechuga-Sancho et al., 2006). Muranyi et al. (2003) showed that in transient focal ischemia, diabetes activates the cell death pathway which might be due to caspase-3 activation via binding of initiator caspase-9 with Apaf-1 and mitochondrial matrix component cytochrome c to form an apoptosome which further trigger the activation of the downstream caspase cascade resulting in the cell death. Additionally, studies suggested that p53 transcription factor is involved in the activation of caspase-3, 9 and 6 (Culmsee and Mattson, 2005) and also takes part in some forms of cerebellar cell death (Inamura et al., 2000). In the present study an increase in caspase-3 expression and number of caspase-3 positive cells in hippocampus following diabetes further confirms the excessive cell death as a possible consequence of high blood glucose level noted post diabetes confirmation. Similarly increased blood-glucose level as observed in this study
is expected to reduce the IGF-I (Insulin like growth factor) level in cerebellar granule neurons (Linseman et al., 2002). Constitutively, the reduced IGF-I level due to high blood-glucose level or activation of p53 transcription factors or intrinsic cell death pathway might be the potential cause behind the sequential apoptotic cell death following diabetes as reported in this study.

**Long term glial (astroglia and microglia) activation and proliferation in brain account for the severity of experimentally-induced diabetes**

STZ-induced diabetes instigated a severe astroglial and microglial activation in both hippocampus and cerebellum. During any brain insult microglial and astroglial cells get activated through a paracrine signalling mechanism. Ample of studies have shown that activated microglia and astroglia release various inflammatory molecules and secretory proteins that not only instigate and promote the activation of each other but also take part in the modulation of their activation via gliotransmission, generation of immune molecules and cell-derived soluble factors, etc. (Davalos et al., 2005; Shih et al., 2006; Liu et al., 2011; Pascual et al., 2012; Shinozaki et al., 2014). Our data directed a similar inclination of glial activation in both hippocampus and cerebellum following STZ-induced diabetes that continued upto the 12th week PDC.

**Persistent astroglial activation and proliferation grounds the deleterious effect of diabetes in brain**

Diverse features of glial activation like phenotypic switching, proliferation and expression of activation cell surface markers to measure the extent of activation of astroglia and microglia have been well reported in various models of injury (Pěkný and Nilson, 2005; Patro et al., 2005, 2010b), inflammation (Patro et al., 2010a; Cerbai et al., 2012) and disease (Heneka et al., 2010; Verkhratsky et al., 2012; Peng et al., 2014) in both central and peripheral nervous system. Here, in response to STZ-induced elevated
blood glucose level and subsequent cell death, significant morphological heterogeneity was observed in both type of glial cells i.e., astroglia and microglia in the hippocampus and cerebellum.

Increased level of the astrocyte intermediate filament cytoskeletal protein i.e., GFAP with phenotypic transformation is a considerable marker of astroglial activation caused by CNS injury, aging and neurodegeneration (Pěkný and Nilsson, 2005; Sofroniew and Vinters, 2010; Burda and Sofroniew, 2014). However, astroglial response during diabetic state varies according to the severity and duration of the diabetes (Saravia et al., 2002; Lechuga-Sancho et al., 2006). Previous studies have revealed the selective decrease in astrocyte GFAP levels in the cerebral cortex, cerebellum, hippocampus (Coleman et al., 2004; Guven et al., 2009) and in spinal cord (Renno et al., 2008) with least neurodegeneration at 4-8\textsuperscript{th} weeks of diabetic duration. Differing to the prior reports of decrease GFAP level (Coleman et al., 2004; Hernández-Fonseca et al., 2009; Guven et al., 2009), we noted a progressive hyperactivation of astroglial cells upto the 12\textsuperscript{th} weeks of diabetes in both hippocampus and cerebellum with profound cell death has been recorded. The increased level of GFAP recorded is consistent with the findings of Saravia et al. (2002) and Baydas et al. (2003b). Morphology of classically activated astroglia with bushy, crumpled, extensively overlapping, deeply stained bold projections and large cell nuclei, transformed from the resting astrocytes were present in all the hippocampal subfields and cerebellar layers. Several mechanisms have been proposed to explain the astroglial response in STZ-induced diabetes such as oxidative stress, protein glycation, increases in the polyol pathway and altered calcium homeostasis (Brownlee, 2001). Excessive free radical generation following diabetes have been clearly documented with the altered levels of the GFAP (Baydas et al., 2003b). In our study, stature of oxidative stress in hippocampus upto the 12\textsuperscript{th} week of diabetes is comparable with the altered astroglial response indicative of astroglial reactivity caused by diabetes-
induced oxidative stress. Additionally, many astrocytes were also presenting fragmented processes from 4th week of diabetes onwards. Astrocyte confer a protective role in blood brain barrier by encapsulating the brain capillaries with their long cytoplasmic processes. They also play an imperative role in antioxidant defence producing antioxidant molecules. Thus, fragmentation of the astrocytic processes could possibly compromise functioning of the blood brain barrier (Huber et al., 2006) and oxidative stress (Mastrocola et al., 2005) in brain following diabetes. As suggested that astroglia play an imperative role in controlling the glucose supply and its metabolites to neurons, thus any alterations in brain glucose level could foremostly affects the astroglia. Therefore, this activation might be a customized approach to modulate the effect of excessive cell death following diabetes as in response to adverse state glial cells transform their morphology and proliferate to combat the baleful condition (Patro et al., 2005; Verkhratsky et al., 2012) as documented in this study.

S100β is a reliable predictor of astrocytosis (Woods et al., 2010) and clinical marker of brain damage (Netto et al., 2006; Patro et al., 2009). Thus, S100β immunolabelling was performed to further verify the astroglia activation. Affirmatively, S100β is involved in various intracellular and extracellular regulatory activities including protein phosphorylation, transcription, Ca(2+) homeostasis, stimulation of neurite outgrowth and astrocyte proliferation etc. (Zimmer et al., 1995, 2003; Sorci et al., 2013), but the overexpression of this protein in brain is considered as a biomarker of astrocytic damage in disorders like AD and Down’s syndrome (Griffin et al., 1989; Zimmer et al., 1995; Mrak and Griffin, 2001). As calcium binding protein, S100β responds to the cell signalling induced calcium stimulus. Thus, in activated astroglia it might be possible that the enormously increased calcium influx induces the overexpression or secretion of S100β protein via astrocytes as evidently shown in previous studies of neurodegenerative disorders (Steiner et al., 2011). Likewise in the present study, we have also observed the
pronounced expression of S100β cells in all the hippocampal fields following diabetes. These S100β+ cells were showing stellate morphology having large cell body and thick processes similar to the activated astrocyte. The augmented synthesis and overexpression of S100β protein from activated astrocytes has been involved in the excessive growth of dystrophic neurites in neuritic plaques contributing in the progression of AD (Sheng et al., 1994; Mrak RE, Griffin, 2001). Thus, the incidence of astrocyte activation and overexpression of S100β in diabetic hippocampus possibly explains the vulnerability of diabetic brain more towards the pathogenesis of AD. Additionally, the elevated concentration of S100β in serum or CSF has deleterious consequences like activating the innate immune response in brain consequently causing inflammation (Koppal et al., 2001; Sorci et al., 2011) and cell death (Hu et al., 1997; Rothermundt et al., 2003; Steiner et al., 2011). Therefore, in conclusion the STZ-induced diabetes in hippocampus causes astrogliosis alongwith fragmented processes, ensuing the release of S100β, which possibly in turn, influence or exaggerate the process of cell death and inflammation.

**Heteromorphic microglial activation and proliferation for the longer duration intensifies the damaging magnitudes of diabetes in brain**

Concomitant to the astroglial activation, the intense microglial expression was also observed following diabetes in all the hippocampal fields (CA1, CA2, CA3, and DG) and cerebellar cell layers upto the 12th week PDC. Previously, microglial expression and proliferation was also detected in diabetic retinopathy (Zeng et al., 2000), acute cerebral infarction in presence of diabetes mellitus (Li et al., 2011) and in diabetic hyperalgesic rat spinal cord (Daulhac et al., 2006). Similarly, our data showed an evident activation directed morphological transformation in microglial cells i.e., from resting to reactive phenotype consistently in all the hippocampal fields and cerebellar cell layers following diabetes. During 2nd and 4th week of diabetes the microglial cells are in their intermediate
activated state and from 6\textsuperscript{th} week onwards the presence of highly activated hypertrophied microglial cells were clearly observed in both Iba-1 and OX-42 immunolabelling. Quantitative data of both hippocampus and cerebellum also showed the marked proliferation of microglial cell population following diabetes.

Microglia as affiliate of brain defence system, on any immune breaching or insult become activated as well reported in our previous studies following peripheral nerve injury (Saxena et al., 2007; Patro et al., 2010b) and in hippocampus (Patro et al., 2010a; Patro et al., 2013; Nagayach et al., 2014). Through various pattern-recognition receptors [like Toll-like receptors (TLRs), NOD-like receptors (NLRs), receptors of cell wall components or DNA/RNA of pathogens), purinergic receptors, advanced glycation endproducts (RAGE) receptors], scavenger receptors, microglia receive signals from injured and dying cells and vascular damage (Block et al., 2007; Brown and Neher, 2010). Connexions of these receptors initiate a microglial activation cascade with the expression of various proteins, comprising CD-45, COX-II, iNOS, MHC-II and various co-stimulatory molecules etc. These molecules facilitate the microglial expression of antigens to T cells, entered through the damaged BBB during neuroinflammation (Gehrmann et al., 1995; Aloisi, 2001; Carson, 2005; Gertig and Hanisch, 2014). On activation these immune cells get rapidly shifted into the reactive phenotype and slack their highly ramified morphology not only to protect but also to repair the damaged tissue via removing the dying cell debris and facilitating the healing process (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011). On the contrary, microglial activation is also responsible in aggravating the neurodegeneration (Block and Hong, 2005; Venero et al., 2011). Thus, microglia activation following diabetes in the hippocampus and cerebellum as shown in this study might be in response of cell death, oxidative stress and astroglial activation for providing the potential damage control or possibly microglial
activation was itself inducing the cell death and astroglial activation via generating various immune mediators.

Additionally, the expression and sequential population rise of antigen surface marker OX-6 (MHC-II) positive cells in hippocampal fields following diabetes further reflect the persistent activation of microglial cells and its macrophagic state. MHC-II (OX-6) labelling was undertaken to determine the extent at which diabetic condition can increase the antigen presentation in brain. Under normal healthy conditions the expression of these antigens on microglia is either low or absent (Sedgwick et al., 1993; Bulloch et al., 2008) but can be dramatically augmented in adverse brain conditions. Activated microglia in detrimental phase induces MHC-II expression (O’Keefe et al., 1999; Pabon et al., 2011). Furthermore the expression of ED-2 also accounts for the incidence of alternate activation of macrophages and prolific perivascular microglial activation (Balabanov et al., 1996) in diabetic hippocampus upto the 12th week PDC. ED-2 as a well-recognized marker not only used to define the macrophages and but also play a major role in stimulating the generation of inflammatory molecules like TNFα, IL-1β and IL-6 (Polfliet et al., 2006; Kowal et al., 2011). A recent study on AD has also shown that the ED-2 positive cells were present in higher density around the compromised blood vessels (Pey et al., 2014) might be indicating the BBB breakdown. This information supports the functional aspect of ED-2 expression in the resolution phase of inflammation (Zwadlo et al., 1987; Polfliet et al., 2006) due infiltration of inflammatory molecules following BBB damage (Perry, 2004). Conclusively, it might also be possible that intense expression was aggravated by activated microglia to further facilitate its role in initiating the cascade of events of immune system. Similarly, presence of ED-2 positive cells was also supporting the phagocytic role of activated microglia and associated inflammation following diabetes in hippocampus.
Neuroinflammation as potent immune response resulted from the cell death, oxidative stress and glial activation following STZ-induced diabetes

Neuroinflammation is an essential biological progression that stands as the foreground of various acute and chronic neuropathological conditions. Any alteration in brain’s cellular and functional integrity grounds the incidence of neuroinflammation. Neuroinflammation as a defending responder aims to refurbish the tissue homeostasis via inducing several repair processes (Goldszmid and Trinchieri, 2012). However, if the regulation of this mechanism remains uncontrolled then the initial inflammatory response amplifies exceedingly and the protective mode shifts towards the collateral destruction that would further result in severe disease progression.

The state of neuroinflammation is well reported in various studies on diabetes (Mohamed et al., 1999; Patel and Santani, 2009; Srodulski et al., 2014) and other neurodegenerative disorders (Pacher et al., 2007; Frank-Cannon et al., 2009; Najjar et al., 2013). Analogous to these studies we have also observed the state the neuroinflammation in diabetic hippocampus via profound immunoexpression of pro-inflammatory cytokines (IL-1β and TNF-α) upto the 12th week PDC. Secretion of pro-inflammatory cytokines following glial (astroglia and microglia) activation is the classic theory of neuroinflammation recognized and reviewed widely (Carson et al., 2006; Luo and Chen, 2012; Boche et al., 2013). Likewise, in this study consistent astroglial and microglial activation in hippocampus might be considered as the possible perpetuator of the neuroinflammation. Glial activation is generally accompanied by the proliferation of cells, mobilization towards the damaged or dying cell, and the expression and secretion of pro-inflammatory cytokines, like IL-6, TNFα and IL-1β. Later on, these cytokines stimulate other astroglia and microglia leading to the exacerbation of glial (microglia and astroglia) activation. Furthermore alterations in cytokines expression are a result of stimulation of the transcription factor NF-κB (nuclear factor kappa enhancer of B cells)
via phosphorylation-induced activation of IκB kinase (Brown and Neher, 2010). Additionally the oxidative stress and cell death are also contributing in the generation and propagation of pro-inflammatory cytokines as documented in earlier studies of diabetic neuropathy (Shi et al., 2013; Sandireddy et al., 2014; Muriach et al., 2014). Oxidative stress activate the NF-κB and speciality protein-1 (SP-1) that further results in neuroinflammation and vascular deficits (Cameron and Cotter, 2008; Sandireddy et al., 2014). Similar pattern of immune response regarding inflammation, cell death and oxidative stress was also observed in this study. The incidence of neuroinflammation in hippocampus following STZ-induced diabetes was started from the 2nd week PDC parallel to the cell death and oxidative stress and sustained up to the 12th week PDC depicting a direct involvement of oxidative stress related cell death or vice versa. Intriguingly, reciprocal relationship between neuroinflammation, cell death and glial activation is popularly considered as a prerequisite for the onset and pathogenesis of various psychiatric disorders like AD, PD, dementia and bipolar disorder etc. (Hojo et al., 2004; Enciu and Popescu, 2013; Najjar et al., 2013; Watkins et al., 2014). Accordingly, the stature of glial activation, cell death and neuroinflammation for a longer duration as reported in this study also provides a cue on the vulnerability of diabetic brain more towards the pathogenesis of severe psychiatric disorders.

Cytokine signalling is actively involved in the regulation of various brain functions like synaptic signalling modulation, neurotransmission, neuroendocrine functions and neural circuitry of behaviour and cognition (Camacho-Arroyo et al., 2009; del Rey et al., 2013; Aprile-Garcia et al., 2013; Cuartas and Jorge, 2014). Therefore, it is relatively apparent to presume an altered behavioural and cognitive outcome as a consequence of a dysregulation in cytokine signalling which might be resulted in depression, anxiety, behavioural deficits and cognitive dysfunction as observed in this study as well. Reportedly, IL-1β and TNF-α is involved in the processing of learning and
memory (Lynch et al., 2002; Bains and Oliet, 2007; Baune et al., 2008; McAfoose and Baune, 2009) and any alterations in the level of these cytokines in hippocampus lead to AD, dementia and cognitive impairment as observed in various cross-sectional and prospective population studies (Dik et al., 2005; Magaki et al., 2007; Holmes et al., 2009; Brosseron et al., 2014). In concurrence with this we have observed alterations in the spatial cognition, working memory, motor coordination, muscle strength locomotor activity and emotionality (anxiety-like behaviour) in the animals following STZ-induced diabetes that initiated from the 2nd week PDC and remain affected upto the 12th week of diabetes. These deficits might be the consequences of increased level of pro-inflammatory cytokines in hippocampus following diabetes. The present data revealing longer term cognitive impairment parallel to the inflammation and glial activation concrete the possibility of the advance susceptibility of diabetic patients to develop other psychiatric illness than the non-diabetic population.

**Counteraction between oxidative stress, cell death, glial activation and neuroinflammation embark cognitive and behavioural decline following diabetes**

The role of hippocampus was well elucidated in both cognition and mood regulation. Several studies have proposed the involvement of hippocampus in spatial learning, memory functions, anxiety and defensive behaviours (Ferbinteanu and McDonald, 2001; Pentkowski et al., 2006; Czerniawski et al., 2009; Fanselow and Dong, 2010). Additionally, cerebellum is also involved in the control and execution of several aspects of cognition, emotional behaviour and motor functions, comprising coordination (Horne and Butler, 1995), muscle tone (Manto, 2010) and locomotion (Morton and Bastian, 2004) which is possibly controlled and maintained by the neuron-glia shuttle. Thus, alterations in hippocampal and cerebellar cells are possibly affecting the homeostasis of the neuronal circuitry that further impairs the brain ability to acclimatize
and restructure the crucial behavioural and cognitive functions. Previous reports suggested that in brain a proficient level of glucose is required to perform the hippocampal-dependent cognitive tasks (McNay et al., 2000). Thus, any alteration in blood glucose level necessarily affects the hippocampal function as well documented in previous cognitive and behavioural studies.

This provides us an outline to consider the reciprocal relationship between various hippocampal and cerebellar cells and associated mechanisms. Our data presenting various glial and neuropathological changes resulting in alterations of hippocampal and cerebellar function account for their contribution in the brain symptoms of diabetes-linked impediments by deteriorating the regulation of hypothalamic-pituitary-axis, maintenance of learning and memory, motor coordination and control of emotional expression.

Conclusively, present data depicted the scenario of glial activation, cellular degeneration and associated immune response in consort with subsequent behavioural and cognitive alterations during experimentally induced diabetes in rats. The gliosis and allied neuroinflammation in hippocampal region following diabetes unremittingly induces the alteration in motor coordination, anxiety and reduce cognitive ability in animals that sustained for a longer duration. Consideration of the associative reciprocity between metabolic stress, cell death and gliosis in hippocampus will not only provide an effective window to understand the underlying cellular mechanism following diabetes in brain but also help us in developing the ideal targets for therapeutic interventions.
SUMMARY & CONCLUSIONS

Diabetes mellitus is a heterogeneous endocrine disorder resulting from defects in insulin secretion or action or both and characterized by the hyperglycemia linked to absolute and relative insulin deficiency. In addition to others, diabetes mellitus increases the risk of various cerebral disorders resulting in many structural and functional impediments related to central and peripheral nervous system (Biessels, 1994; Baydas et al., 2005; Guven et al., 2009). These complications are listed as impairment of cognitive functions, dementia in older peoples, alterations in learning and memory (Gispen and Biessels, 2000; Popovic et al., 2001; Allen et al., 2004; Arvanitakis et al., 2006) and mood disorders (Herzer and Hood, 2010; Egede and Ellis, 2010) etc. Diabetes leads to neurochemical and structural abnormalities as well as degenerative changes in brain that could be related to hyperglycemia-induced oxidative stress (Baydas et al., 2003a; McCall, 2004; Mastrocola et al., 2005), poor glycemic control and insulin dependency (Liu et al., 2008; Gupta et al., 2014). Hyperglycemia supplies additional substrate for anaerobic glycosis in the brain, which further results in lactic acidosis and enhanced damage to both glial and neuronal cells (Biessels et al., 1994). Concomitantly hyperglycemia augment the formation of oxygen free radicals following lipid peroxidation in the brain tissues which actively assault macromolecules within neuron and glial cells that later causes structural and functional changes in proteins and subsequent cell death (Hawkins and Davis, 2001; Hernández-Fonseca et al., 2009; Guven et al., 2009) and astrocytic glial reaction (Barber et al., 2000; Kaneko et al., 2002).

Various studies revealed that oxidative stress following diabetes on brain cells, causes a selective decrease in astrocyte GFAP levels in the cerebral cortex, cerebellum, hippocampus (Coleman et al., 2004; Hernández-Fonseca et al., 2009; Guven et al., 2009) and spinal cord (Renno et al., 2008) with apparent neurodegeneration at 4-8 weeks of
diabetic duration. The diabetic allied decreased GFAP levels, without altering relative astrocyte number, may be linked to insulin secretion, which essentially takes part in the modification of phenotypic appearance of astrocytes and increases the expression of GFAP mRNA and protein (Toran-Allerand et al., 1991). The impact of hyperglycemia on glial cells (both astroglia and microglia) per se or their response to neuronal damage remains to be studied in detail. Particular attention needs to be taken on neuro-immunological aspects of neurodegeneration and neuroregulation under diabetic conditions.

In the central nervous system, a heterogeneous group of cells known as glial cells accompany the neuronal population. The major glial population in brain i.e., astroglia and microglia shape the microarchitecture of the brain, provide support and protection, control extracellular ion- metabolic- and neurotransmitter homeostasis, destroy and remove the carcasses of dead neurons and help in signal transmission. Several studies elucidate the affirmative functions of microglial and astroglial cells following stringent pathological conditions, injuries and stress in brain. Microglial cells being resident and as immunocompetent phagocytic cells perform functions similar to those of tissue macrophages present in other organs and constituting the first line of defence against invading pathogens (Streit, 2002; Vilhardt, 2005). In response to any detrimental condition microglial cells become activated and release several cytokines (Hanisch, 2002), trophic factors (da Cunha et al., 1997; Nakajima and Kohsaka, 2004) and anti/pro-inflammatory molecules (Nadeau and Rivest, 2000; Perry, 2004) and concurrently phagocytose the debris of apoptotic cells (Reichert and Rotshenker, 2003; Makranz et al., 2004; Djukic et al., 2006). This activity helps in facilitating the reorganization of neuronal circuits and triggers the repair mechanism (Kim and Vallis, 2005; Hanisch and Kettenmann, 2007; Neumann et al., 2009). Similarly, reactive astroglia not only release a plethora of factors mediating the tissue inflammatory response but also recapitulate stem
cell/progenitor features after damage and prove as a promising target for reparative therapies following brain damage (Buffo et al., 2010). On the contrary, glial (astroglia and microglia) activation is also responsible in aggravating the neurodegeneration via releasing several inflammatory molecules (Venero et al., 2011; Verkhratsky et al., 2014a). Depending upon the stimulus and progression of the diseased state glial cell activation generally acts in two ways either help in efficacious restoration of injured brain cells or generate a threatening environment that further results in subsequent brain alterations. Recently, there have been many groundbreaking studies that are devoted to the structure and functions of glial cells and establish their dual (beneficial/detrimental) role in various brain disorders.

Studies on animal models have also depicted that diabetes induced GFAP alteration has been linked to other central nervous system (CNS) disturbances like distorted learning and memory processes and further increased risk of dementia and cognitive dysfunction (McCall et al., 1996; Kamal et al., 2000). There are several reports depicting that microglial activation in the optic nerve in diabetic retinopathy might be an additional pathogenic factor in diabetic optic neuropathy (Zeng et al., 2008; Grigsby et al., 2014). Likewise, literature also demonstrate the response of microglia in STZ-induced diabetes by assessing the reduction in the number of Iba1 positive microglia in the dorsal horn of the spinal cord following gabapentin treatment in diabetic neuropathy (Wodarski et al., 2008; Daulhac et al., 2011). Furthermore with regard to the functional importance of microglial cells in brain following any stress or degenerative changes, it would be quite interesting to study the microglial activation following diabetes in the brain regions, responsible for behaviour and cognition. On activation microglia secretes plethora of cytokines and chemokines initiating neuroinflammation, which in turn influence neuronal functions and even some of them also play critical role in learning, memory (Goshen et al., 2007; McAfoose et al., 2009) and motor activity (Patro et al.,
Such microglial activation and related neuroinflammation have never been studied in diabetic brain hippocampus, neither their expression nor the immune response developed by these cells.

The functional ramification of glial activation (astroglia and microglia) following brain damage and associated immune response embodies targets for therapeutic interventions. Glial cell research being actively addressed today on various aspects of their involvement in neuronal health and disease, their response to diabetic states has not been studied in large details. Consequently, this study is expected to facilitate in getting information on the possible influence of glial cells associated neuroinflammation and degeneration in the hippocampus following diabetes in the CNS and the subsequent effects on behavioural and cognitive abilities. The possible effect of oxidative stress following diabetes on neuronal and glial cell death and the interconnected feedback loop between oxidative stress, neuronal death, gliosis and neuroinflammation have never been studied in the hippocampus. Therefore, a detailed study on glial activation and immune response either neuroprotective/degenerative, produced by glial cells following diabetic exposure along with subsequent behavioural and cognitive impairment with respect to gliosis needs to be carried out in a more organized manner. Thus, present study will also help us in assessing the effect of oxidative stress following diabetes on: glial activation vice-versa and the possible immune response produced by microglial and astroglial cells along with their effects on behaviour and cognition changes in a rat model. We analysed the hippocampus tissue due to its role in several cognitive aspects linked to emotional, learning, memory and sensorimotor functions (Bast, 2007). Additionally, we have also studied the cerebellum following diabetes as cerebellum is associated with emotion, cognition and behaviour (Rapoport et al., 2000; Schmahmann and Caplan, 2006) and any alterations to the cerebellum lead to motor deficits, dementia, schizophrenia and other psychiatric disorders (Baldaçara et al., 2008; Sui and Zhang, 2012).
For developing diabetic animal model, a group of healthy male, adult rats having normal blood glucose level (between 90-144 mg/dl) were kept on fasting overnight. Next morning diabetes was induced by a single intraperitoneal (i.p.) injection of Streptozotocin (STZ) at a dose of 45 mg/kg body weight, dissolved in 0.1M citrate buffer (pH=4). The diabetic state was confirmed by measurement of non-fasting blood glucose level, 72 hrs post STZ injection with the help of glucometer (Accu-Chek, Roche Diagnostics, Germany). Blood drop sample was obtained from tail tip bleed. Animals having blood glucose level above 250mg/dl were selected for diabetic study. Diabetic animals were randomly divided into following groups i.e., 2nd, 4th, 6th, 8th, 10th and 12th week post diabetes confirmation (PDC). Subsequently, animals’ body weight, consumed food and water were measured to confirm the diabetic symptoms like polyphagia, polydipsia and weight loss.

Furthermore, all the experimental animals (diabetic and age-matched controls) were subjected to the various behavioural and cognitive assessments at 2nd, 4th, 6th, 8th, 10th and 12th week post diabetes confirmation. A wide range of cognitive and behavioural studies were performed to analyse the spatial cognition (Barnes maze), working memory (T maze), anxiety-like behaviour (elevated plus maze), locomotor functions (open-field test), motor coordination (Rotarod) and muscular strength (grip strength meter).

Afterwards, all the experimental animals were sacrificed via transcardial perfusion (using 2% Paraformaldehyde and 10% formaldehyde as fixative; Nagayach et al., a,b) and the tissues like cerebral cortex (occipito-temporal), cerebellum, liver and pancreas, kidney were dissected out carefully and weighed. The brain tissues for immunohistochemical study were post-fixed in the same fixative (2% paraformaldehyde) overnight at 4°C and then cryoprotected in Phosphate buffered-Sucrose gradients i.e. 10%, 20% and 30% at 4°C until tissues settled at the bottom. After processing the coronal sections of cerebral cortex through occipito-temporal region and sagittal sections of
cerebellum were sequentially cut using cryostat (Microm HM 525, Thermo Scientific), at a thickness of 14µm. The sections were collected serially on chromalum-gelatin coated slides and stored at -20°C till they were used for immunohistochemical studies. For histological study a separate set of animals from each group were perfused and processed for paraffin sectioning as per previously described method (Kumar et al., 2013; Nagayach et al., 2014b). Briefly, after perfusion-fixation, tissues were thoroughly washed with water and dehydrated with graded series of ethyl alcohol. Then, the tissues were cleared in toluene and infiltrated in Paraplast (Sigma, m. p. 56–58°C) for proper impregnation of wax. Tissue blocks were made in paraffin and serial coronal sections of cerebellum were cut at a thickness of 6µm using Leica RM2135 microtome. To observe the histological changes, tissue sections were stained with 0.1 % cresyl violet acetate (Sigma certified stain, C-5042), prepared in acetate buffer (pH 3.5). For oxidative stress assessment Lipid peroxidation (LPO) was assessed by measuring malondialdehyde (MDA) levels based on the reaction of MDA with thiobarbituric acid using Buege and Aust (1978) method.

STZ-induced animals exhibited the characteristic signs of diabetes as their blood glucose level was consistently higher throughout the study as compared to their respective controls and this plateau was maintained upto the 12th week of diabetic duration. Similarly, the food consumption and water intake were also significantly increased in the diabetic animals presenting the symptoms of polyphagia and polydipsia. Furthermore, the body weight and brain weight were found to be significantly reduced during diabetic state. A cumulative resultant of reduced body and brain weight, the brain weight/ body weight ratio of diabetic animals was also escalating at all the diabetic time points as compared to their respective controls. The biochemical assessment of the level of lipid peroxidation (LPO) in hippocampus, kidney and liver revealed a persistent state
of oxidative stress in all the respective tissues successively upto the 12th week PDC as compared to their respective controls.

Behaviour data indicated the reduced motor coordination and poor or weak muscular strength of diabetic animals on rotarod and in grip strength meter respectively indicating towards the impaired motor/muscle activity significantly comparable to their age-matched controls. Similar altered locomotor activity was also observed in open field test as diabetic animals were showing restless motion with increased distance travelled, stereotypic time and ambulatory time and decreased resting time. Additionally, the spatial cognitive ability of diabetic animals was also seem to be affected as in barnes maze diabetic animals were taking more time to find the escape box with consequent reduced path efficiency and average speed as compared to the controls. On T-maze task also diabetic animals performed poorly while exploring the baited arm. The time duration of diabetic animals on T maze was significantly increased and path efficiency was reduced as compared to the controls showing altered effect of diabetes on spatial working memory. STZ-induced diabetic animals were also showing the anxiety behaviour on elevated plus maze as they had significantly increased anxiety index than the controls upto the 12th week PDC. The percentage of time spent and total number entries of diabetic animals were less in open arms than in closed arms. The presence of anxiety behaviour is in the support of the restless motion of diabetic animals in open field test.

Histopathological study revealed a persistent cellular damage in pancreas, kidney and liver upto the 12th week PDC. The irreversible severe destructions of insulin producing pancreatic beta cells upto the 12th week PDC validates the increased blood glucose level following STZ-induction. Furthermore, renal and liver damage as observed in the study not only confirms the incidence of polyuria, body weight loss and subsequent polyphagia to reimburse the energy loss during diabetes.
A significant cellular degeneration and/or cell loss was observed in both hippocampus and cerebellum of STZ-induced diabetic animals with immunohistochemical and histological studies consistently up to the 12th week of diabetic duration. Histological study revealed marked cell destruction both in hippocampus and cerebellum following diabetes. Cardinal signs of cell degeneration like, necrosis/apoptosis, vacuolation, chromatolysis, nonalignment of cells, neuronophagia and gliosis were clearly visible in all the subfields of hippocampus and cerebellar layers. The incidence of continual cell degeneration and/or loss is in accordance with the increased level of oxidative stress (level of LPO) in hippocampus up to the 12th week PDC. The active Caspase-3 immunolabelling results were clearly depicting the apoptotic neurons in both hippocampal regions and cerebellar layers following diabetes in rat. The level of active caspase-3 expression was consistent and increasing in all the hippocampal fields i.e., CA1, CA2, CA3 and DG regions and cerebellar layers in terms of both quantification and immunoreactivity. This suggests a severe and long term cellular degeneration in the hippocampal and cerebellar tissue following diabetes. Diabetes caused an alteration in the morphology of GFAP-labelled astrocytes in CA1, CA2, CA3 and DG regions and cerebellar layers in hippocampus and in cerebellum by the 2nd week PDC onwards. Resting astrocytes in controls presented thin, fine, intact processes with small cell body. While during diabetes the GFAP immunolabelling was more intense and hypertrophied astrocytes with activated phenotype, i.e., thick, dense, bushy and extensively overlapping, fragmented processes with enlarged darkly stained cell body. Such cells were common all the hippocampal subregions and purkinje cell layer of the cerebellum. Quantification also revealed a pronounced proliferation of astroglial cells following diabetes in both hippocampus and cerebellum as compared to their respective controls. Furthermore, the S100β immunopositivity in hippocampus up to the 12th week PDC also confirmed adverse effect of increased blood glucose level as astrogliosis consistently for the long duration.
Similarly, the Iba-1+ microglial cells in diabetic hippocampus and cerebellum were also presenting activated morphology as compared to their respective controls. The microglial cells were showing phenotypic transformation, i.e., from ramified to activated states bearing retracted, thick processes and large irregular cell body. In controls resting microglia cells processes exhibited ramified wispy appearance with round lightly stained cell body throughout the hippocampus subregions upto the 12th week PDC. Cell quantification and area fraction of Iba-1 immunopositive cells also revealed a gradual increase in microglial population indicative of microglial proliferation in brain following STZ-induced diabetes. Additionally, the expression and sequential population rise of antigen surface marker OX-42 positive cells in hippocampal fields following diabetes further reflects the persistent activation of microglial cells and its macrophagic state. The significant escalation in expression of OX-6 (MHC-II) both in terms of cell population and immunoeexpression not only depicted the activation of microglial cells but also manifested the state of inflammation following diabetes in hippocampus as MHC-II signalling plays an imperative role in inflammation retorting to both endogenous and exogenous antigenic proteins. The ED-2 immunolabelling revealed an increased expression of macrophagic cell population in hippocampus following STZ-induced diabetes. In controls, the immunoeexpression of ED-2 positive cells was weak in terms of both expression and quantification indicating the negligible incidence of phagocytic activity and inflammation following microglial activation. While assessing the effect of cell death and glial activation following STZ-induced diabetes we observed an increased and pronounced expression of pro-inflammatory cytokines i.e., IL-1β and TNF-α in hippocampus following diabetes. The IL-1β and TNF-α positive cells were equally distributed in all the subfields of the diabetic hippocampus as compared to their respective controls. Quantitatively, the population of pro-inflammatory cytokines i.e., IL-1β and TNF-α immunolabelled cells was also significantly dense and increasing upto the
12th week PDC directing towards the long term neuroinflammatory state in hippocampus following diabetes. In conclusion the consistent stature of oxidative stress trailed increased cell death in hippocampus following diabetes might be activating the astroglial and microglial cells. Furthermore the long term persistent oxidative stress and cell death as reported upto the 12th week of diabetes possibly exaggerating the glial activation angling phenotype and associated neuroinflammation that further instigate the significant hippocampal and cerebellar atrophy followed by cognitive and behavioural impairments.

The study has elucidated the impact of diabetes on glial cells per se and/or their rejoinder effects on neuronal cells with special reference on associated brain functions. Evidences are also instigating an insight on possible role of diabetes allied oxidative stress in perpetuation of glial activation, neuronal cell death and associated neuro-immunological aspects of neurodegeneration and neuroregulation. Thus, this information will provide an insight which not only help in understanding the aura of consequent complications of diabetes but also in establishing a concrete strategic therapeutic approach for developing the treatment of diabetes associated cognitive and behaviour deficits.

In conclusion:

- STZ-induced diabetes model was established with symptomatic signs of diabetes including irreversible histopathological damage in liver, kidney, pancreas, hippocampus and cerebellum.
- An increased level of oxidative stress was noted in hippocampus following STZ-induced diabetes.
- Diabetes caused severe cell death in both hippocampus and cerebellum including a decrease in brain weight.
• STZ-induced diabetic animals showed reduced motor coordination, muscle strength, spatial cognition, working memory, altered motor activity and anxiety behaviour.

• Both in hippocampus and cerebellum diabetes resulted in persistent glial (astroglia and microglia) activation. Glial activation was the consequent effect of profound cell death and oxidative stress. It was also resulted from the paracrine signalling between both astroglia and microglia.

• STZ-induced diabetes caused astrogliosis alongwith fragmented processes, which further stimulated the release and overexpression of S100β in hippocampus, which possibly in turn, exaggerated the process of cell death and inflammation.

• Activated microglia with macrophagic properties expressing antigen surface markers, i.e., OX-6 (MHC-II) and OX-42 (CR-3) and a cascade of immune response molecules (IL-1β and TNF-α) were prevalent in the hippocampus of diabetic rats.

• IL-1β and TNF-α are well known for their austere impact on cognition and behaviour. Thus, increased and prolonged expression of these cytokines following diabetes was deleteriously affecting the cognitive and behavioural abilities of the animal as observed.

• Collectively, the sequential loss of neurons and activated glial reaction associated neuroinflammation as reported in this study could be a contributing hinge to the altered cognitive ability and behaviour following diabetes. From therapeutic standpoint, efforts are required to better elucidate the pathophysiological changes that underpin the progression and severity of brain disorders following diabetes to develop the better treatments.
REFERENCES


Lynch MA (2002) Interleukin-1 beta exerts a myriad of effects in the brain and in particular in the hippocampus: analysis of some of these actions. Vitam Horm 64:185–219.


Wenk GL (2001) Assessment of spatial memory using the T maze. Curr Protoc Neurosci Chapter 8:Unit 8.5B.


APPENDIX I

Preparation of buffers, fixatives and stain

1. Citrate buffer (0.1M/pH=4)
   
   **Reagents (for 20ml of solution):**
   
   Sodium citrate ................................................. 0.5882gm
   Citric acid.............................................................for maintaining the pH

   **Method:**
   
   Calculated amount of sodium citrate was added in 20ml of sterilised distilled water on a magnetic stirrer. Finally, with the help of Citric acid, pH of the solution was maintained up to 4.

2. Phosphate buffer saline (PBS; 0.01M/pH=7.2-7.4)
   
   **Reagents (For 1 litre solution):**
   
   Di sodium hydrogen phosphate (anhydrous)......... 1.149gm
   Sodium phosphate Monobasic (anhydrous)......... 0.296gm
   Sodium chloride..................................................... 8.5gm

   **Method:**
   
   All the reagents were sequentially added in 800ml of distilled water on a magnetic stirrer. Finally the solution was made up to 1 litre with distilled water.

3. 2% Para formaldehyde (0.01M/pH=7.4)
   
   **Reagents (For 1 litre solution):**
   
   Di sodium hydrogen phosphate (anhydrous)......... 1.149gm
   Sodium phosphate Monobasic (anhydrous)......... 0.296gm
   Paraformaldehyde.................................................... 20gm

   **Method:**
   
   All the reagents were sequentially added in 800ml of warm distilled water on a magnetic stirrer. Crystals of Sodium hydroxide was added in the solution, if the solution was not clear. Finally the solution was made up to 1 litre with distilled water and filtered properly. Then, the solution was kept for cooling at 4°C in a tight cap bottle for further use. (Fresh solution was prepared every time for the perfusion)

4. 10% Buffered formalin (0.01M/pH=7.4)
   
   **Reagents (For 1 litre solution):**
   
   Di sodium hydrogen phosphate (anhydrous)......... 1.149gm
   Sodium phosphate Monobasic (anhydrous)......... 0.296gm
   Formaldehyde.......................................................100ml

   **Method:**
Both the reagent salts were added in 900ml of distilled water on a magnetic stirrer and then 100ml of formaldehyde solution added and mixed. The solution was stored at room temperature.

5. **Tris buffer saline (TBS; 0.05M/pH=7.4-7.6)**

*Reagents (For 1 litre solution):*
- Sodium chloride: 8.766gm
- Tris base: 6.057gm

*Method:*
All the reagents were sequentially added in 800ml of distilled water on a magnetic stirrer. Finally the solution was made up to 1 litre with distilled water.

6. **Tris-HCl (0.05M/pH=7.4-7.6)**

*Reagents (For 100ml solution):*
- Tris base: 0.6057gm

*Method:*
Tris base was added in 80ml of distilled water on a magnetic stirrer and finally made up to 100ml with distilled water.

7. **Cresyl violet stain**

*Reagents (for 100 ml of working solution):*
- Cresyl violet: 0.5 g
- Distilled water: 100 ml
- 10% Glacial acetic acid: 0.8ml

*Method:*
- a. Cresyl violet stock solution: 0.5 g cresyl violet was added in 100ml distilled water and mixed well.
- b. Working solution: For working solution 0.8 ml of 10% acetic acid was added to 100 ml of stock cresyl violet solution. Then it was filtered properly for use.
PUBLICATIONS & PRESENTATIONS

PUBLICATIONS:

Research article


Book Chapter


Review article


Abstract

PRESENTATIONS (ORAL & POSTER):


- Oral Presentation on “Microglia type cells exist in DRG and upregulate Iba1 when activated following sciatic nerve injury: A novel finding” in 26th M.P. Young Scientist Congress, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur during February 28 - March 01, 2011.

- Poster entitled “Existence of Iba1 expressing cells in dorsal root ganglion” presented in International Conference on Advances in Neuroscience and 26th Annual Meeting of Indian Academy of Neuroscience at Cochin University, Cochin (India) from 12th-14th Dec. 2008.