Investigating the Neuroprotective Impacts of Chenodeoxycholic Acid in STZ-Induced Diabetic Neuropathy and Cognitive Impairment in BALB/c Mice

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A thesis submitted to the National University of Sciences and Technology, Islamabad, in partial fulfilment of the requirements for the degree of

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Supervisor: Dr. Aneeqa Noor

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No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the School of Mechanical and Manufacturing Engineering in partial fulfilment of the requirements for the degree of Master of Science in the Field of Biomedical Sciences. Department of Biomedical Engineering and Science National University of Sciences and Technology, Islamabad.

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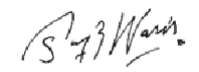
Name of HOD: Dr. Asim Warris Signature: 

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I dedicate this work to my extraordinary parents and siblings with heartfelt respect, love and gratitude. This thesis reflects your unwavering belief in me, every prayer and the immense patience that has guided me through each challenge. It stands not only as a representation of my journey but also as a tribute to your love, dedication and endless support.

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I am deeply thankful to Almighty Allah (SWT) for His endless blessings. I can never thank my parents enough for their unconditional love, prayers, and constant support. To my siblings, your love, patience, and encouragement have been a source of motivation for me. My heartfelt thanks go to my supervisor, Dr. Aneeqa Noor. Your wisdom, encouragement, and constant belief in my abilities have guided me every step of the way. I am also incredibly grateful to my friends and seniors, the encouragement during tough times, and your unwavering support have made this journey so much easier Thank you all for standing by me.

Maria Bano

Table of Contents

LIST OF FIGURES

LIST OF ABBREVIATIONS

CDCA	Chenodeoxycholic acid
DN	Diabetic Neuropathy
STZ	Streptozotocin
NF-κB	Nuclear Factor-kappa Beta
FXR	Farensoid X-receptor
CA	Cholic Acid
TUDCA	Tauroursodeoxycholic acid
AD	Alzheimer's disease
TGR5	Takeda G-protein-coupled bile acid receptor 5
T1DM	Type 1 Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
DPN	Diabetic Peripheral Neuropathy
BDNF	Brain-derived neurotrophic factor
BA	Bile Acid
IDDM	Insulin Dependent Diabetes Mellitus
NIDDM	Non- Insulin Dependent Diabetes Mellitus
LCA	lithocholic acid
CREB	cAMP-response element-binding protein
AGEs	Advanced Glycation End Products
IGT	Impaired Glucose Tolerance
CYP7A1	cholesterol 7α-Hydroxylase
CYP8B1	Sterol 12α-Hydroxylase
$A\beta_{42}$	Amyloid-beta 42

ABSTRACT

Diabetic neuropathy and cognitive impairment are common complications of diabetes, significantly affecting the lives of millions of people. Finding effective treatments for these issues remains a critical challenge. In this study, we investigated whether chenodeoxycholic acid, a naturally occurring bile acid known for its neuroprotective properties, could help alleviate these complications. We utilized a mouse model of diabetes induced by streptozotocin. The mice were categorized into three groups; a healthy control, a diabetic group and a diabetic group that receive treatment with CDCA. The diabetic mice displayed typical signs of nerve pain, anxiety-like behaviour, and memory problems. However, those treated with CDCA showed remarkable improvements in all these areas. They experienced less pain in the hot plate analgesia, exhibited reduced anxiety levels in the open field test, and demonstrated better memory and cognitive function in the test of Y-maze. Beyond behaviour, CDCA also had profound effects on the brain. It preserves the structure of neurons in critical areas like the hippocampus and cortex, which are often affected by diabetic neuropathy. At a molecular level, CDCA may reduce inflammation by decreasing nuclear factor kappa B levels, a key marker of inflammation and cell damage. The brain-derived neurotrophic factor is also increased, a protein essential for nerve growth and repair in the brain, suggesting that CDCA supports the brain's natural ability to heal. These results provide a promising glimpse into the potential of CDCA as a treatment for diabetes-related nerve and cognitive problems. While more research is needed, the ability of CDCA to protect neurons, reduce inflammation, and improve cognitive and behavioural outcomes makes it a promising drug for future therapies aimed at improving the lives of people with diabetes.

Keywords: Diabetic Neuropathy**,** Streptozotocin, Chenodeoxycholic acid, Brain-derived neurotrophic factor, nuclear factor kappa B.

Chapter 1: INTRODUCTION

1.1 Bile Acids

The liver synthesizes bile acids (BA), which are amphipathic molecules that facilitate the process of digestion by acting as natural detergents. These BA are stored in the gallbladder and released into the intestinal lumen following food consumption. Their main role is to solubilise dietary fats and fat-soluble vitamins, enhancing their absorption. Most of the BAs are reabsorbed throughout the digestive tract and return to the liver for recycling through enterohepatic circulation, with only a small fraction being extracted as waste. Beyond their digestive function, BA functions as steroid hormones and powerful signalling molecules.

They influence metabolic processes through nuclear receptors like Farensoid X-receptor FXR and Takeda G-protein-coupled BA 5-receptor (TGR5) and sphingosine-1-phosphate receptor 2 which are membrane-bound receptors (Zwicker & Agellon, 2013). New research suggests that BA signalling may also affect the brain's normal and abnormal functions. Research on BA therapy in clinical trials may offer treatment for several neurological illnesses; this is supported by a Phase III trial using UDCA and continuing research evaluating the long-term effectiveness of CDCA (Chenodeoxycholic acid) (Mondelli et al., 2011).

1.1.1 *Classification of Bile Acids*

BA consists of a saturated cyclopentanoperhydrophenanthrene skeleton consisting of three six-membered rings labelled A, B, and C along with one five-membered ring labelled D, which makes up the stiff steroid nucleus. These are amphiphilic molecules. The core features an aliphatic acidic side chain which is flexible and has a hydrophilic hydroxyl group attached. Primary BA is produced in the liver, whereas secondary BA, such as CDCA and cholic acid (CA), are modified in the gut via 7α-dihydroxylation and deconjugation. Two prominent secondary BA are lithocholic acid and deoxycholic acid. Cystic fibrosis, digestive tract disorders, Primary biliary cirrhosis (PBC), cholangitis, gallstones, cystic fibrosis, basic sclerosing cholangitis also certain malignancies are among the ailments for which BA may have therapeutic uses. Currently, the FDA has only approved UDCA as a BA for treating PBC (Russell, 2003). For almost fifty years, liver and biliary problems have been treated using BA, which are non-traditional steroids made in the liver from cholesterol.

Gallstones containing cholesterol have been successfully dissolved since the early 1970s by UDCA, a secondary BA obtained from CDCA, a primary BA. But with laparoscopic cholecystectomy on the horizon, combined with CDCA's poor efficacy and side effects, its

clinical utility for this reason has declined. The discovery that BA activate particular receptors marked a major advance at the start of the twenty-first century. At the nexus of intestinal microbiota and human physiology, these receptors control vital enterohepatic and entero-pancreatic signalling pathways, inducing a "BA renaissance". Like other steroids, BA binds to and activates nuclear and cell surface receptors, such as the TGR5 and FXR BA receptors. It has been determined that FXR and GPBAR1 are both druggable targets, and new developments have produced several strong, nonselective, and selective ligands for both receptors (Chiang, 2013).

1.1.2 Synthesis of Bile Acids

In humans, CA and CDCA are the sole BA that are formed. BA synthesis occurs through two primary pathways: the classic and the alternative or acidic pathways. One pathway is the classic during which, cholesterol is transformed by 7α-hydroxylase (Cyp7A1) into 7α-hydroxycholesterol within the hepatocytes of the liver. This process leads to the formation of the primary BA through further conversions by 12α-hydroxylase sterol, and CDCA through sterol 27-hydroxylase. During the alternative route, it is converted into 27-hydroxycholesterol by mitochondrial CYP27A1 in peripheral tissues. Oxysterol Cyp7A1 provide help in this pathway, and the end products eventually returning in the liver, presented by arrowheads linking it to earlier pathways (Chiang & Ferrell, 2018).

1.1.3 Mechanism of Action of Bile Acids

Beginning with cholesterol enzymes catalyse the oxidation process of the side-chain of steroid to produce CA and CDCA, and taurine joined these. Cyp8b1 Sterol 12α-Hydroxylase facilitates the production of CA in the liver. CDCA is mostly synthesized via a different pathway that is started by Cyp27a1 and oxysterol Cyp7A1. To enhance the metabolism of lipids and glucose, the is activated FXR/SHP in liver cells, which results in the inhibition of cholesterol enzymes and the promotion of Cyp7b1 and Cyp27a1 expression. INT-767 activates FXR in intestinal L cells, which then promotes the expression of prohormones convertase 1/3 and TGR5. Additionally, FGF15 is induced by FXR, and this improves brown adipocytes' energy metabolism. Hepatic FGFR4/βKlotho signalling is activated by fibroblast growth factor 15, and this inhibits Cyp7a1 by way of the JNK/ERK1/2 pathway shown in figure 1.1. Adenylyl cyclase increases cAMP in response to TGR5 activation, which in turn stimulates protein kinase A. This results in the phosphorylation and activation of cAMP-response element-binding protein (CREB), hence increasing the transcription of PC1/3.

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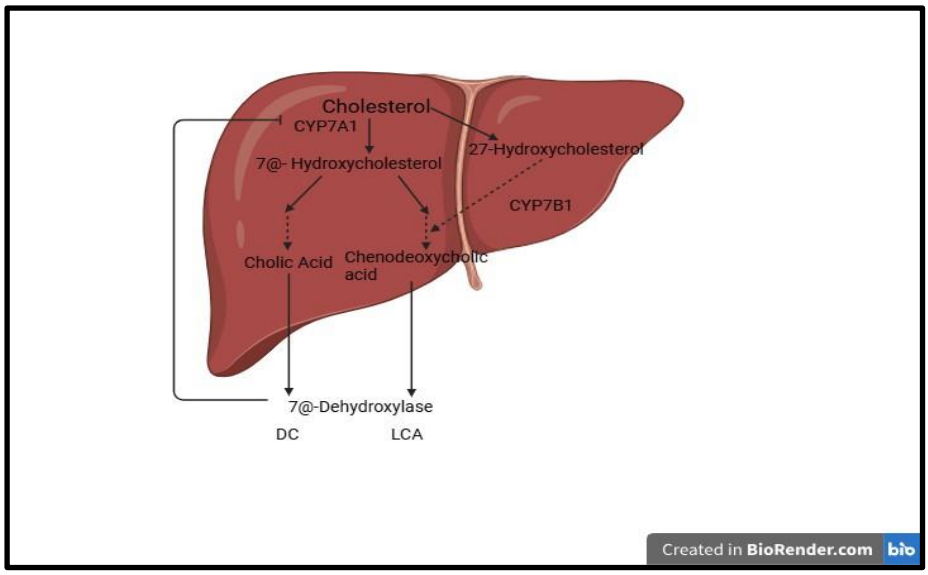

Figure 1.1: Bile Acid Metabolism. CYP7A1 catalyzes the rate-limiting step in BA synthesis, while CYP8B1hydroxylates cholesterol to produce CA. Without CYP8B1, CDCA is the main product. CYP27A1 oxidizes the steroid side chain and can initiate Biosynthesis by converting cholesterol to CDCA.

Pre-proglucagon is converted into Glucagon-like peptide (GLP-1) by PC1/3. INT-767 enhances Ca^{2+} level in the cell and function cAMP. It also activates FXR, which results in depolarization of the potential of the membrane by blocking the sensitivity of the Adenosine triphosphate and the channels of the potassium, promoting Ca^{2+} uptake through Ca^{2+} channels, and causing L cells to secrete GLP-1. FXR and GLP-1 both encourage the production and release of insulin from pancreatic β cells. In this research, a crucial pathway is discovered by which FXR activation enhances glucose sensitivity, sensitivity of insulin, and metabolism of lipids. Firstly, it was reported double FXR and TGR5 opposite INT-767 release the expression of genes, while also enhancing the Ca^{2+} level in the cell and the activity of cAMP.

In contrast, the obeticholic acid did not activate the activity of calcium and cyclic AMP, and the TGR5 agonist INT-777 increased the activity of cAMP without affecting Ca^{2+}. Of these, INT-767 was most effective in enhancing tolerance of glucose and signalling of insulin in the liver, whereas OCA primarily improved hepatic lipid metabolism. Previous studies have shown that T-CDCA causes depolarization of membrane potential, resulting in elevated levels of Ca^{2+} and stimulating the secretion of insulin from cells of the pancreas (Pathak et al., 2017). FXR activation results in the closure of the KATP channel which is ABCC8,

causing membrane depolarization and increased Ca^{2+}. The current data reveal that an L-type Ca^{2+} channel inhibitor decreases glucose-stimulated secretion of GLP-1 from the cells of Glutag. Additionally, FXR activation promotes AKT phosphorylation, which enhances the movement of glucose transporter 2 to the plasma membrane, thereby stimulating insulin secretion in β cells. An identical procedure may be present in FXR-induced secretion of GLP-1 in l-cells of endocrine (He et al., 2015).

1.1.4 Role of Bile Acids in Neurodegenerative Diseases

Numerous causes contribute to neurodegenerative illnesses, which impact millions of people worldwide. These diseases differ in their specific processes of pathogenesis, but they are all characterized by the build-up of mutant or misfolded proteins and disturbed endoplasmic reticulum stress pathways. Increased malfunction, extensive loss of neurons, and brain atrophy result from this. BA signalling and its potential therapeutic benefits for these severe illnesses have come to light recently (Figure 1.2).

1.1.4.1 Bile Acids in the Treatment of AD

Changes in BA profiles provide fresh information in the developing field of BA studies in AD. Plasma samples from thirty healthy form control, twenty people having less cognitive impairment, and thirty people suffering from AD were examined during a recent clinical trial. According to the findings, people suffering from AD had considerably greater amounts of lithocholic acid than controls. Furthermore, in AD patients increased amounts of glycol-CDCA, glycol-deoxycholic acid, and glycol-lithocholic acid than MCI patients were recorded. These results imply that these glycine-conjugated BA and LCA could be helpful biomarkers for disease diagnosis (Marksteiner et al., 2018). Through the stimulation of the CREB and pathway of brain-derived neurotrophic factor (BDNF), FXR overexpression triggered neuronal death in an experimental investigation employing a human neuroblastoma cell line model cured with Aβ1-42 to simulate AD (Chen et al., 2019).

1.1.4.2 Bile acid for the treatment of PD

Studies conducted *in vivo* on the mouse treated with MPTP have validated the neuroprotective effect of TUDCA. TUDCA increased the level of Nrf2 as well as its resulting cytoprotective enzymes, such as glutathione peroxidase and heme oxygenase-1, and reversed MPTP-induced ROS generation (Moreira et al., 2017). A series of TUDCA injections given before and following the injection of MPTP in a mouse model of Parkinson's disease (PD)resulted in enhanced motor abilities in the group treated with MPTP and groups with

TUDCA relative to mice only treated with MPTP. This included fewer tremors and improved movement start. Although MPTP-treated animals had lower levels of Parkin, a ubiquitin ligase known as E3 linked with biogenesis of mitochondria, this decrease was somewhat offset by TUDCA treatment before MPTP administration (Rosa et al., 2018).

1.1.4.3 Role of Bile acids in treating anxiety and depression

Anxiety disorder is a prevalent neuropsychiatric condition that affects between 7.3% and 28.0% of the global population (Szuhany & Simon, 2022). Growing evidence from clinical and preclinical studies suggests that BA dysregulation may contribute to mental health disorders such as anxiety. Studies have found a direct link between BA levels and anxiety symptoms, and BA also appears to affect anxiety-like behaviours in patients with irritable bowel syndrome and other gastrointestinal conditions (Feng et al., 2022). BAs and their signalling pathways are increasingly linked to anxiety, with various BA receptors involved in key metabolic and immune functions that impact organs like the brain, liver, and intestines.

Recent research has highlighted BAs' influence on brain function, suggesting new research directions. BA can cross the barrier of blood and brain and directly join with the receptors in the brain, triggering anxiety-like behaviours. They may also circulate systemically through the enterohepatic pathway, binding to hormone receptors in the brain to induce anxiety. Anxiety's mechanisms are complex, extending beyond brain pathology alone, and targeting BA pathways offers a promising angle for studying anxiety's underlying causes. Although only certain Biopathways have been linked to anxiety, this research may deepen our understanding and support new treatment strategies (Chen et al., 2023).

1.1.4.4 Role of Bile Acids in Huntington's Disease

Huntington's disease (HD) is a hereditary neurological disease characterized by uncontrollable choreiform movements, decline in cognition, and symptoms of psychiatry (MacDonald et al., 1993). Studies on BAs in HD suggest neuroprotective effects, particularly from TUDCA in HD models. CDCA, extracted from 24-hydoxycholestrol, may stimulate protective pathways as a result of damage to neurons. shifting BA synthesis toward alternative pathways. This shift, marked by higher CDCA/CA ratios, may reflect a reaction of damage to the membrane in pre-symptomatic HD. Additionally, increased neuroprotective BAs such as UDCA, GUDCA, and TUDCA and decreased primary-to-secondary BA conversion may help counteract neurodegeneration in the early stages. However, this

protective response diminishes as HD progresses, as shown by reduced plasma 24OHC levels that correlate with caudate atrophy (Chiang et al., 2024).

1.2 Chenodeoxycholic Acid

One of the two main BAs synthesized in the human liver is CDCA. When combined with taurine or glycine, it typically accounts for around one-third of the total amount of biliary BA. The hepatocyte's BA flow and the relative composition of bile control the normal synthesis of 3 linked on BA has significantly improved our knowledge of their production pathways and related pathophysiology within the last 20 years. The main route for the degradation of cholesterol in humans is represented by the formation of BA, which are metabolic products obtained from cholesterol. The average human liver produces 0.2 to 0.6 grammes of BA each day. The liver uses several intricate metabolic processes to convert cholesterol before starting the process of BA production.

Multiple enzymatic processes are involved in this conversion, which results in the synthesis of primary BA, including CDCA and CA. Following their release into the bile, these major BAs are either secreted straight into the gut or retained in the gallbladder. The liver uses several intricate metabolic processes to convert cholesterol before starting the process of BA production. Multiple enzymatic processes are involved in this conversion, which synthesizes BAs, including CA as well as CDCA. Following their release into the bile, these major BAs are either secreted straight into the gut or retained in the gallbladder (Fiorucci & Distrutti, 2019).

1.2.1 Classical Pathway of CDCA Synthesis

Ninety per cent of all BAs are produced via the most significant biosynthetic process, which is the neutral or classic pathway of the BA synthesis cascade. In this manner, nearly equal amounts of CA and CDCA were produced. One important enzyme during the catabolic route that controls the amount of BA is Cyp7A1 cholesterol, which catalysis the conversion of cholesterol to 7α-hydroxycholesterol. Before the aliphatic side chain's oxidative shortening, the steroid ring is modified (figure 1.4). 3β-hydroxy-Δ5-C27-oxidoreductase is the enzyme that changes 7α-hydroxycholesterol into 3-oxo-Δ4. Then, the Δ4 double bond is reduced by Δ4-3-oxosteroid-5β-reductase, which forms the structure of 5β-hydrogen. The last change in the ring structure is caused by the dehydrogenase enzyme 3α-hydroxysteroid, which reduces the 3-oxo group to a 3α-hydroxyl group. CA is generated if hydroxylation at the C12 position

by sterol 12α-hydroxylase takes place; CDCA is created if hydroxylation at this site is absent (Li et al., 2012).

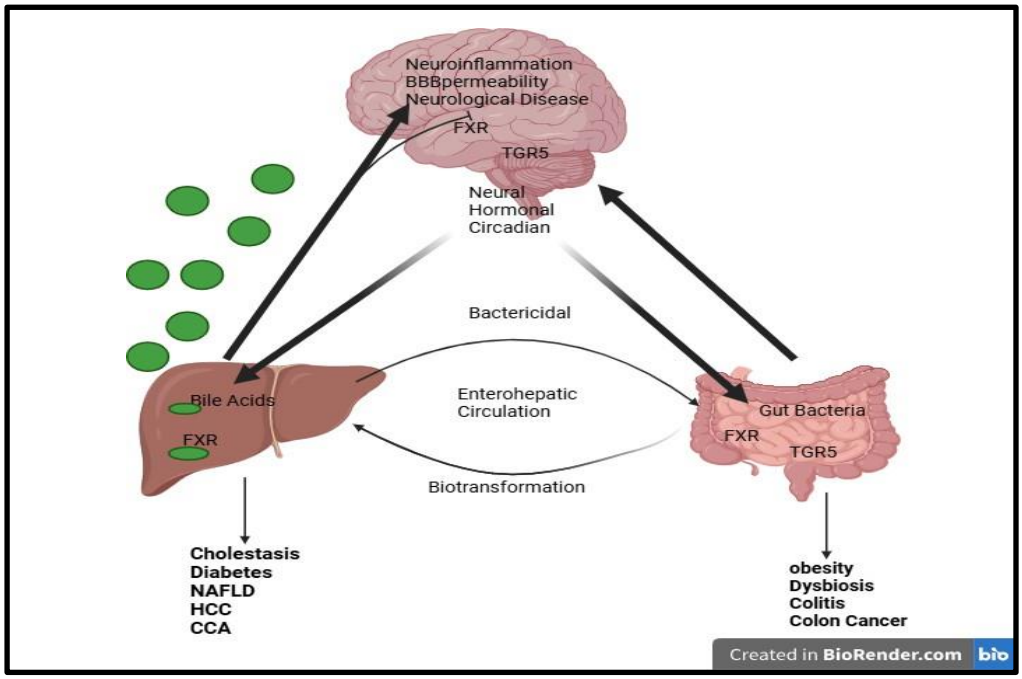

Figure 1.2: Bile acid Signaling in Neuromodulation. BAs are integral to the liver-gut-brain axis, where they facilitate communication between the liver and gut while being regulated by the brain's neural, hormonal, and circadian mechanisms.

1.2.2 The Alternative Pathway of CDCA Synthesis

An alternate process that converts C27 BA and oxysterols produced in different cell types is known as the acidic pathway because it produces acidic intermediates shown in Figure 1.4. After arriving in the liver, these intermediates undergo further metabolism to produce BA. Important enzymes in this process are microsomal oxysterol Cyp7A1 and mitochondrial CYP27A1, which aid in the side chain shortening of C24 BA and the 7α-hydroxylation of hepatocytes, increasing the amount of CDCA (Kevresan et al., 2006).

1.2.3 Therapeutic Role of CDCA

1.2.3.1 Role in the treatment of gallstone

CDCA can dissolve cholesterol gallstones by decreasing the saturation of cholesterol in bile when administered at therapeutic doses. The success rates of CDCA in removing gallstones vary greatly, ranging from roughly one-third of patients to as high as 80–90% in instances that are properly chosen. Radiolucent gallstones and a healthy gallbladder are necessary for the procedure to work. (Iser & Sali, 1981). Chronic inflammation of the intrahepatic and

extrahepatic bile ducts is a characteristic of sclerosing cholangitis, and it can cause fibrosis and possible bile duct damage. BA is used in conjunction with surgical techniques like cholecystectomy and laparoscopic cholecystectomy to remove gallstones. By blocking the essential enzyme that controls the synthesis of cholesterol, HMG-CoA reductase CDCA assists. An important aspect in deciding how well BAs work as a therapy is the size of the gallstones. Gallstones were observed to be destroyed in patients receiving UDCA after two years of treatment.

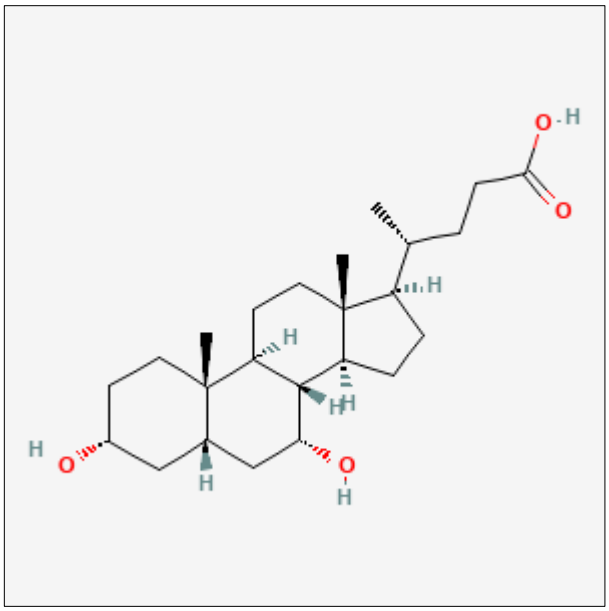

Figure 1.3: Chemical structure of CDCA. The crystal structure of CDCA has a hexagonal arrangement, specifically in the P6(5) symmetry. In this structure, CDCA hydrogen bonds at three key sites: two are the hydroxyl (OH) groups located at positions O3 and O7, and the third is the carboxylic acid group on its side chain, involving atoms O24a, O24b, and an attached hydrogen. (PubChem, 2024).

Hepatolithiasis and choledocholithiasis linked to Caroli syndrome can both be effectively treated with UDCA. It has been shown that UDCA and CDCA can both cause malignant cells to undergo apoptosis and have ant-proliferative properties (Mikov et al., 2007). Obeticholic acid functions as a natural ligand for the FXR and is derived from CDCA, a main BA in humans. OCA decreases BA reabsorption by downregulating the apical sodium-dependent BA transporter and upregulating the production of fibroblast growth factor 19 in the ileum by activating FXR. By blocking CYP7A1, FGF19 further decreases the synthesis of BA in the

liver. OCA may also be able to reduce portal hypertension and have antifibrotic qualities (Mikov et al., 2007).

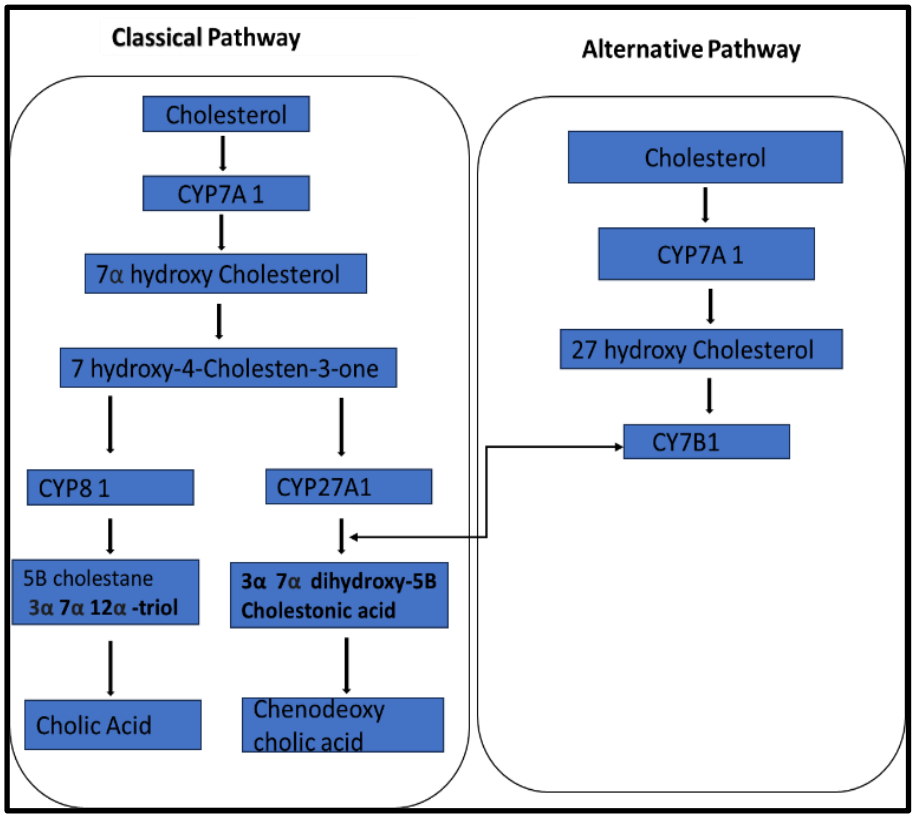

Figure 1.4: Classical and Alternative Pathway of CDCA Synthesis. In the human liver, the primary BAs produced are CA and CDCA, which are regulated by key enzymes in their respective synthesis pathways.

1.2.3.2 Role in the treatment of metabolic disorders related to the liver

6-Ethyl-chenodeoxycholic acid is an agonistic, ligand that is presently being developed to treat metabolic disorders related to the liver, such as non-alcoholic steatohepatitis and non-alcoholic fatty liver disease, as well as cholestatic liver diseases like PBC. FXR is a BA sensor that controls vital elements of BA intake, metabolism, and excretion by interacting with other nuclear receptors. Preclinical models of diet-induced atherosclerosis, liver fibrosis, and cholestasis have all been examined with CDCA. CDCA decreased alkaline phosphatase levels, which was the main objective of a phase II clinical trial involving PBC patients. However, safety data showed that the drug made pruritus worse, which is a common symptom of PBC, suggesting that 6E-CDCA or FXR may be involved in mediating pruritus in humans. Treatment with CDCA increased insulin sensitivity in patients with diabetes and liver steatosis.

However, there was a modest decrease in high-density lipids and an increase in low-density lipid levels. Preclinical research predicted these adverse effects on BA and lipid metabolism, indicating that although powerful FXR ligands appear promising, their possible side effects may restrict their development (Fiorucci et al., 2011). CDCA increases the frequency, eases passage, softens the consistency, and accelerates colonic transit in both healthy volunteers and patients with constipation-predominant IBS (Odunsi–Shiyanbad et al., 2010). This work demonstrates that the FXR agonist CDCA reduces blood vessel constriction and improves blood vessel relaxation in hypertensive rats, thereby lowering blood pressure. Modifications in certain enzymes and decreased vascular inflammation could be the cause of the benefits. This provides clarification on the relationship between metabolic problems and elevated blood pressure and implies that FXR may be a viable target for the treatment of hypertension (Li et al., 2015).

1.2.3.3 Neuroprotective Effects of CDCA

Insulin resistance (IR) is a major risk factor for AD. This study explored whether CDCA could improve insulin signalling and aid in AD treatment. Adult male Wistar rats were divided into three groups and treated for six weeks: an AlCl3-treated AD-model group, a CDCA-treated group of ADS, and a control group. CDCA improved cognitive function, maintained normal tissue structure, and reduced amyloid- β 42 ($A\beta_{42}$) and BACE1 levels in the hippocampus. It also decreased phosphorylation of insulin receptor substrate-1 while enhancing the activation of Akt, GLUT4, PPARγ, and GLP-1. CDCA also increased BDNF and activated CREB. These results suggest that CDCA enhances insulin sensitivity in the hippocampus and may have potential for AD treatment. Reductions in the levels suggest that CDCA also suppressed the formation of amyloids in the hippocampus. Hematoxylin and eosin staining were used to examine morphologically, and the results demonstrated that CDCA had a neuroprotective effect. Specifically, the control and Alzheimer's groups treated with CDCA showed less neuronal degeneration than the Alzheimer's group, which showed severe damage (Bazzari et al., 2019)

1.2.4 Mechanism of Action of CDCA

Mutations in the CYP27A1 gene cause cerebrotendinous xanthomatosis, an autosomal recessive lipid storage condition that results in abnormal BA metabolism. A variety of progressive symptoms, such as ataxia, dementia, epilepsy, tendon xanthomas, and cataracts, are present in this illness. The mutation leads to decreased levels of CDCA and increased

levels of cholesterol 7α-hydroxylase, which in turn increases levels of 7α-hydroxy-4-cholesten-3-one, cholestanol, and bile alcohol in the urine and serum. Patients with CTX benefit from BA replacement therapy, especially when combined with CDCA, since it effectively reduces both neurological and non-neurological symptoms (Nie et al., 2014).

1.3 Overview of Diabetes Mellitus

The term 'diabetes mellitus' (DM) refers to a broad range of conditions marked by elevated blood sugar levels brought on by a relative or absolute lack of insulin synthesis or function. Diabetes mellitus-related chronic hyperglycemia has been related to organ damage, malfunction, and eventual failure, including the kidneys, heart, brain system, retina, and blood vessels. The International Diabetes Federation projects that by 2030, there will be 552 million people worldwide with DM, up from 366 million in 2011 (Alam et al., 2014). Diabetes is predicted to impact 693 million persons worldwide by 2045, making it one of the most common diseases in the world. Severe complications from the condition can include microvascular neuropathy, retinopathy, and cardiovascular disease, which can lead to greater death rates, kidney failure, blindness, and a markedly lower quality of life for people affected (Cole & Florez, 2020).DM is a metabolic disease marked by abnormalities in insulin action, secretion, or both. These abnormalities can result in abnormalities in the metabolism of proteins, fats, and carbohydrates as well as persistent hyperglycemia.

According to predictions, the prevalence of DM is expected to rise from over 200 million cases worldwide in 2010 to 300 million cases by 2025, making it the most common endocrine illness (Mathers & Loncar, 2006). As the condition worsens, it may damage blood vessels and tissues, leading to serious side effects including ulceration, retinopathy, neuropathy, and nephropathy.DM comprises a variety of diverse disorders, primarily classified as type 1 and type 2. Drugs are primarily used to treat diabetes to prolong life and reduce symptoms; long-term problems are avoided, and risk factors are decreased to promote longevity. Insulin replacement therapy is crucial for people with type 1 diabetes, whereas dietary and lifestyle changes are important for patients with type 2 diabetes (Bastaki, 2005).

Insulin replacement therapy is crucial for people with type 1 diabetes, whereas dietary and lifestyle changes are important for patients with type 2 diabetes. However, in type 2 DM, insulin becomes required when diet, exercise, weight loss, and oral medicines are insufficient to manage blood glucose levels. In addition to recommending suitable diet and lifestyle changes, medications should be recommended. These tactics are designed to help people lose weight, improve their glycemic control, and minimize their risk of cardiovascular

complications—which account for 70–80% of fatalities in people with diabetes. Diabetes can be effectively managed with food and exercise alone (non-pharmacological) or in conjunction with insulin (pharmacological), oral hypoglycemic medications, or herbal therapies (Franz et al., 2010).

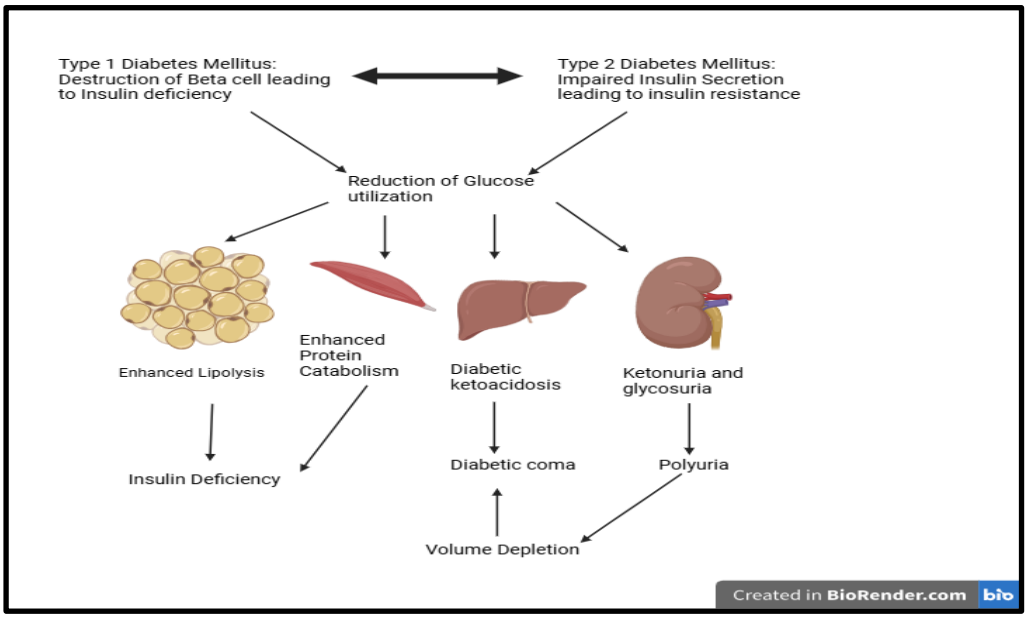

Figure 1.5: Pathophysiology of Diabetes mellitus. The brain, which depends significantly on blood glucose levels for proper functioning, is supported by the insulin released from β-cells of the pancreas when glucose concentration is high in the blood.

Type 2 diabetes pathogenesis is primarily influenced by β-cell dysfunction and IR in the liver and muscles. In people who fall into the higher range,80% of their β-cell does not function and are either completely or almost completely insulin resistant. In addition to the triumvirate of muscle, liver, and β-cells, important in developing intolerance of glucose in diabetics type 2 include fat cells, the tract of the gastrointestinal (unclear decrease/resistance), α-cells (hyperglucagonemia), the kidneys (high re-uptake of glucose), and the insulin resistance in the brain (DeFronzo, 2009).

1.3.1 Prevalence of Diabetes Mellitus in Asia

By 2030, 9% of people worldwide are expected to have diabetes (Hu, 2011). Based on the 8.5 billion individuals that are expected to live in the world, 690 million of them likely have type 2 diabetes. Compared to non-diabetic populations, these people have a higher death rate, which includes coronary heart disease which is causing a rise of 28% in overall death. Asia has had a sharp rise in diabetes cases because of dietary modifications, urbanization, and economic development. Asia is home to 60% of the world's diabetic population. The

prevalence of diabetes soared in China from less than 1% in 1980 to almost 10% by 2008, surpassing India to become the global epicentre of the diabetes epidemic. About 20% of people in southern India's cities are reported to have diabetes. Asians have lower obesity rates, a tendency to develop diabetes at earlier ages, and a higher risk of diabetes in pregnancy, which elevates the chance of diabetes type 2 in children (Zimmet et al., 2001).

1.3.2 Diabetes prevalence in Pakistan

The occurrence of this disease in rural areas was 25.3% and 28.3% in urban areas, with Sindh and Punjab having the highest rates. 14.4% of people had pre-diabetes, with 15.5% of people living in cities and 13.9% in rural regions. The prevalence of total glycemic dysregulation, which includes pre-diabetes and diabetes, was 39.2% in rural areas and 43.8% in urban areas. Pre-diabetes was less prevalent than diabetes; age- and gender-weighted data show that pre-diabetes is more common in younger people, but that the prevalence of diabetes rises dramatically beyond the age of thirty. In some provinces, the transition from pre-diabetes to diabetes occurs quite quickly. On the other hand, Baluchistan has a diabetes-to-pre-diabetes ratio of almost 1:2, indicating that a considerable proportion of the population has a high chance of acquiring diabetes type 2 (Basit et al., 2018).

Women affected with diabetes are about 3.5 per cent and men are about six per cent in cities regions 2.5 per cent of females and 3.3 per cent of males in villages. In urban regions, IGT was found in 14.2% of women and 6.3% of men, while in the countryside, it was found in 10.9% of women and 6.9% of men (Shera et al., 2007). Over the past century, sedentary behaviours, poor diet, and obesity have all contributed to a rise in type 2 diabetes due to lifestyle changes. The ensuing health crisis, which includes complications and untimely fatalities, must be addressed with an international, integrated strategy. Lifestyle changes may be able to prevent or postpone type diabetes, according to early epidemiological and clinical trial studies. There is clear proof that small lifestyle modifications, such as reducing 5–7% of the weight of the body and physical activity for 150 minutes weekly, can successfully avert the onset of disease, according to the survey by the Finnish Diabetes Prevention Study and the Diabetes Prevention Programs (Franz, 2007).

To accomplish their objectives, these studies used intense intervention techniques in conjunction with ongoing follow-up and support. Other smaller trials reported similar results. It has also been demonstrated that bariatric surgery and many drugs, including metformin, acarbose, pioglitazone, troglitazone, orlistat, and rosiglitazone, are beneficial in avoiding or postponing diabetes. Studies on nutritional variables have also revealed evidence linking

excessive consumption of dietary fat, especially saturated fat, to the onset of diabetes, whereas higher consumption of whole grains, fibre, and alcohol is linked to a lower risk. The American Diabetes Association concluded after reviewing the available data that adopting a balanced diet and engaging in regular physical activity should be the primary methods of avoiding or postponing diabetes, rather than using medication therapy (Editors of Encyclopaedia Britannica, 2024).

1.3.3 Type of Diabetes

DM is a macromolecule-related metabolic disease marked by the body's improper synthesis or response to insulin, which makes it difficult to maintain healthy blood sugar (glucose) levels.

1.3.3.1 Hormone Dependent Diabetes (Type 1 DM)

The immune system destroys the cells of the endocrine pancreas β cells are destroyed by the immune system, leading to diabetes type 1 mellitus (T1DM). The genetic, environmental, and immunologic variables that cause β cells to be destroyed and insulin insufficiency to follow in this scenario. In genetically predisposed people, this autoimmune process develops and is frequently brought on by one or more environmental causes. The destruction usually occurs for months or years, during which time the affected individuals test positive for particular autoantibodies but remain asymptomatic and maintain normal blood glucose levels.

Hyperglycemia and overt diabetes symptoms do not manifest until after a protracted latent phase, following a substantial loss of β cells (Katsarou et al., 2017). Insulin replacement is necessary for people with T1DM, a disease of the immune system in which the pancreatic β cells become unable to produce insulin, resulting in hyperglycemia and ketosis. Usually starting during adolescence or the early stages of adulthood, it can happen at any age. In addition to immediate problems like diabetic ketoacidosis and long-term dangers including microvascular and macrovascular disorders, symptoms include increased urination, thirst, and weight loss. Psychosocial difficulties and other autoimmune diseases are also more prevalent in patients having diabetes type 1. Optimizing glucose regulation is the cornerstone of effective management as it guards against both short-term and long-term issues (Paschou et al., 2018)

1.3.3.2 Non-Insulin Dependent Polygenic Disorder Mellitus (Type 2 DM)

Long-term metabolic disease known as is typified by deficiencies in the secretion and activity of insulin. Despite elevated insulin levels, a higher rate of basal hepatic glucose synthesis is

the major reason for hyperglycemia due to fasting. Postprandial hyperglycemia after meals is caused by a combination of decreased muscle glucose absorption and the liver's weakened capacity to inhibit glucose synthesis in response to insulin (DeFronzo, 1999). Abnormally increased levels of glucose are due to abnormalities in the loops that provide feedback between the action of insulin and secretion in the causes of the disease. Insulin release is decreased when β-cell malfunction arises, which makes it harder for the body to keep glucose levels within normal ranges. Conversely, IR results in low uptake of glucose by the liver, muscle, and tissues of fats and high glucose production in the liver.

β-cell dysfunction is usually more significant than IR, even though both conditions appear at the start of the disease's development and also aid in its progression. But when IR and β-cell dysfunction coexist, hyperglycemia worsens and T2DM advances more quickly (Cerf, 2013). Some variables, including ageing populations, rising obesity rates, expanding urbanization, and declining levels of physical exercise, are contributing to the global rise in T2DM (Ginter & Simko, 2013). A rising global health concern, T2DM is joined to the epidemic of obesity. Individuals with T2DM are more vulnerable to macrovascular complications like heart diseases and microvascular complications due to hyperglycemia and also due to IR including affecting eyes, kidneys and nerves (DeFronzo et al., 2015).

1.4 Diabetic Neuropathy and Cognitive Impairment

1.4.1 Cognitive Impairments

The global rise in prediabetic and diabetic patients has triggered a surge in related complications, with neuropathy being the most prevalent. Distal symmetric polyneuropathy, commonly known as DN, is particularly widespread. DN involves a diminished function of senses that begins with the legs and is often followed by aches and fewer moments. With time, a minimum of half of individuals with diabetes witness this condition. While control of glucose levels can slow the development of disease in people suffering from type 1 diabetes, it is not very effective in type 2 diabetes (Feldman et al., 2019). Cognitive impairment and peripheral diabetic neuropathy (PDN) are two consequences of DM that seem to have similar underlying causes. DN can cause PDN among other clinical manifestations since it is characterized by a gradual loss of nerve function (figure 1.5). It has been acknowledged in recent years that cognitive impairment is a consequence of (T2DM).

Patients with diabetes typically exhibit worse cognitive function than non-diabetics, and cognitive deterioration is typically even more pronounced in those with PDN. Microvascular injury, inflammation, oxidative stress, and dyslipidemia are among the factors that have been

linked to the etiology of these two disorders (Moreira et al., 2015), Numerous theories have been put out to explain how diabetes and cognitive impairment are related. Potential causes of cognitive impairment include disturbances in insulin action, such as insulin shortage and insulin resistance, and abnormalities in glucose metabolism, such as hyperglycemia and hypoglycemia (Kawamura, Umemura, & Hotta, 2012).

1.4.2 Peripheral Diabetic Neuropathy:

After other possible causes of peripheral nerve dysfunction have been ruled out, DN, a common illness, is characterized indication of dysfunctional peripheral nerve in people having DM (Bansal, Kalita, & Misra, 2006). A substantial contributing factor to silent myocardial infarction, diabetic autonomic neuropathy can shorten life expectancy and cause mortality in about half of the patients within a few years of diagnosis. It is estimated that either subclinical or clinical neuropathy affects two-thirds of diabetic people. Subclinical DN diagnosis is made with quantitative sensory and autonomic measures in addition to electrodiagnostic testing. People with IDDM, non-insulin-dependent diabetes mellitus (NIDDM), and secondary are all susceptible to neuropathy. The longer diabetes has been present, the greater the chance of neuropathy (Figure 1.6).

According to one study, after 25 years of follow-up, the incidence of neuropathy increased from 7.5% at the time of admission to 50% (Brown & Asbury, 1984). Significant late-stage consequences of DM include peripheral nerve problems. A condition known as polyneuropathy is believed to be the consequence of persistent hyperglycemia-induced metabolic imbalances that impair sensory, motor, and autonomic fibres to differing degrees. Similar to "diabetic amyotrophic," symmetrical proximal motor neuropathy may also have a metabolic cause. Diabetes-related mononeuropathies are frequently linked to compressive or ischemic causes.

Our knowledge of the metabolic pathways driving diabetic polyneuropathy has improved recently. Until more focused treatments are created, the goal of managing symptomatic DN should be to achieve long-term blood glucose normalization (Sima & Kamiya, 2006). DPN begins with an early, reversible metabolic phase that eventually gives way to structural degenerative alterations and nerve fibre loss. In type 1 diabetes, this late structural phase is more prominent, leading to increased axonal shrinkage and loss. Furthermore, paranotal degenerative alterations that are missing in type 2 DPN are indicative of type 1 DPN. Differences in the action of insulin and signal transformation, which influence the expression of neurotrophic factors and their receptors in type 1 diabetes, may be the result of these

variations. The more pronounced pathology observed in type 1 DPN is a result of downstream effects on intraskeletal and sticky proteins, as well as their post-translational modifications and nociceptive peptides (Said, 2013).

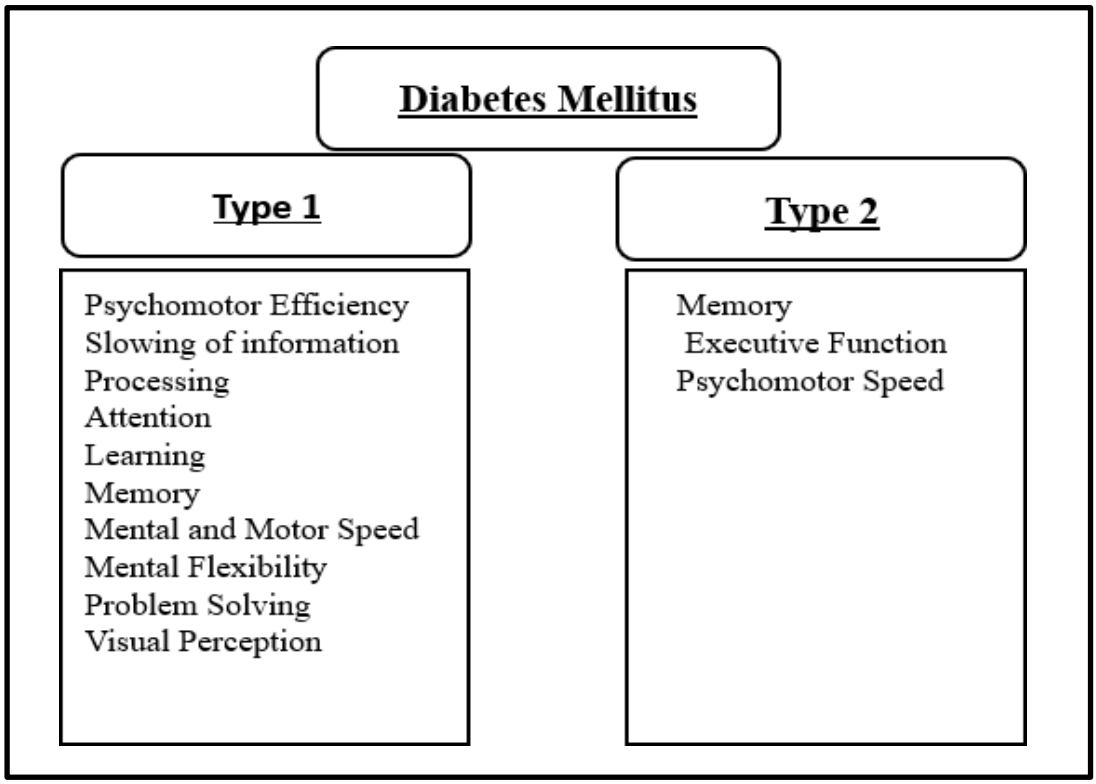

Figure 1.6: Cognitive impairments associated with diabetes. Key cognitive domains shown to be negatively impacted in these include memory, attention, and processing speed.

1.4.3 Length-dependent diabetic polyneuropathy

Although it can potentially indicate NIDDM in older persons, length-dependent diabetic polyneuropathy usually appears years after the development of IDDM. Numbness, scorching feet, tingling, and sharp sensations are among the initial symptoms, which frequently get worse at night or after contact. Sometimes sensory neuropathy is asymptomatic and can only be identified by a foot neurological examination. Silent neuropathy can result in burns, trophic alterations such as plantar ulcers, or painless traumas. The loss of sensation can occur anywhere from the toes to the region above the knees. Strict diabetes management can lower the likelihood of additional neurological impairment, even if many patients have pain, trophic changes, and autonomic dysfunction (Allen et al., 2014).

1.4.4 Focal and Multifocal Diabetic Neuropathies

Nerve biopsies obtained from afflicted areas in patients with multifocal diabetic neuropathy show asymmetrical axonal destruction associated with vasculitis, which is frequently necrotizing, in the perineurial and endoneuria blood vessels. Perivascular mononuclear cell infiltrates and endoneuria red blood cell seepage are observed in the majority of nerve specimens. MDN claims that in older diabetic individuals, these nerve lesions are associated with precapillary blood vessel loss, which sets off a subsequent hemorrhagic and inflammatory response. Why these lesions mostly affect the lumbar plexus, lower spinal roots, and lower limb nerves is still a mystery. Similar to other forms of DN, patients usually get alleviation in a few months, while long-term consequences are common. (Bilbao & Schmidt, 2014).

1.4.5 Distal symmetrical diabetic neuropathy

Axonal degeneration, primary demyelination brought on by Schwann cell failure, and secondary segmental demyelination resulting from compromised axonal regulation of myelination are among the documented anomalies in DN. Other findings include demyelination, atrophy of enervated Schwann cell bands, hypertrophy of the basal lamina, and the formation of onion-bulb formations. "Dying-back" fibres and distal sprouting from the proximal stump after distal axon degeneration are also observed in length-dependent diabetic neuropathy. It has been demonstrated in nerve biopsies that axon loss is more noticeable distally and that there is no connection between demyelination and axon loss.

1.5 Biomarkers of Diabetic Neuropathy and Cognitive Impairment

The US FDA and European Medicines Agency define biomarker categories such as susceptibility, diagnostic, prognostic, predictive, enrichment, monitoring, and surrogate endpoints, with an ideal biomarker being scalable, user-friendly, affordable, and minimally invasive, possessing high predictive power, specificity, and sensitivity (Marshall et al., 2021). Cognitive impairment in diabetes is influenced by insulin signalling defects, autonomic dysfunction, neuroinflammation, and mitochondrial metabolism, with diabetes duration and glycemic control further affecting severity (Zilliox et al., 2016; Figure 1.7).

Oxidative stress, AGE buildup, decreased nitric oxide, elevated protein kinase C activity, and decreased neurotrophic peptides are the causes of DN (Dewanjee et al., 2018). Through AGE production, fructose leads to type 2 diabetes, metabolic syndrome, and nonalcoholic fatty liver disease, which may harm neurons and result in vascular problems. Elevated fructose

levels in diabetic tissues may also trigger gut inflammation and inhibit hepatic AMP-activated protein kinase, linking it to conditions like asthma, arthritis, fatty liver, and insulin resistance (Gugliucci, 2017). Protein kinase C (PKC), which controls protein activation at serine and threonine sites crucial for cellular homeostasis, is activated by hyperglycemia-induced DAG formation. (Vincent et al., 2004).

1.5.1 Nuclear Factor Kappa Beta

Diabetes induces chronic inflammation through NF-κβ activation, leading to DN by producing inflammatory cytokines that damage tissues. Natural compounds like plant phenols activate Nrf2, counteracting NF-κβ and protecting nerves. Curcumin reduces inflammation in diabetes by modulating the NF-κβ pathway and targeting TNF-α and IL-6 (Shaibani & Rafieirad, 2024). CDCA also exhibits anti-inflammatory effects by reducing NF-κ β, Iκ β α, and Akt phosphorylation, inhibiting NF-κB nuclear translocation, and alleviating LPS-induced inflammation through TGR5 activation and Akt/NF-κB pathway inhibition (Zamanian et al., 2024). A study using BAY 11-7082, an NF-κB phosphorylation inhibitor, found that in diabetic animals, increased proinflammatory mediators, elevated nitric oxide levels, and upregulation of iNOS, AGE receptors, and NF-κB contribute to DN pathophysiology (Kumar et al., 2012).

1.5.2 Brain Derive Neurotrophic Factors

The decreased BDNF levels observed in the diabetic hippocampus in this study may contribute to neuronal dysfunction, such as cognitive impairment. However, this dysfunction was prevented by administering a stimulator that promotes BDNF synthesis (Nitta et al., 2002). The study suggests that DHM alleviates the symptoms of diabetic neuropathic pain and diabetic depression by inhibiting the BDNF/TrkB pathway and reducing proinflammatory factors. This indicates that the BDNF/TrkB pathway could be a promising therapeutic target for managing comorbid DNP and DP. BDNF plays a crucial role in the microglia-neuron signalling pathway and is considered a potential therapeutic target for neuropathic pain. However, studies indicate that depression caused by diabetes can reduce BDNF expression in the hippocampus (Ge et al., 2019).

1.5.3 MicroRNAs (miRNAs)

Because of their function in controlling gene expression, miRNAs, or short non-coding RNAs, are being investigated as possible biomarkers. MiRNAs inhibit translation or encourage mRNA degradation by attaching to complementary sequences in the target

mRNAs' 3' untranslated region (Kato et al., 2013). Hyperglycemia in diabetic patients raises IRAK1 and TRAF6 levels while decreasing miR-146a expression in dorsal root ganglia neurons (Wang et al., 2014). Patients with diabetes DN had higher levels of several miRNAs, including miR-125a-5p, miR-145-3p, miR-99b-5p, and miR-873-5p, according to Massaro et al. (Massaro et al., 2019). Furthermore, patients with diabetic peripheral neuropathy had greater serum levels of miR-518d-3p and miR-618, indicating that miRNAs may be useful biomarkers for DN. (Santos-Bezerra et al., 2019).

1.5.4 Nerve Growth Factor

Neurotrophic factors, which promote neuron survival and proliferation, are reduced in diabetic mice, contributing to neuropathy (Apfel et al., 1994). DN is associated with decreased tissue expression of substances such as nerve growth factor, and exogenous NGF injection has been demonstrated to reduce DN symptoms. (Sun et al., 2018). Thus, neurotrophic factors are considered potential biomarkers for DN.

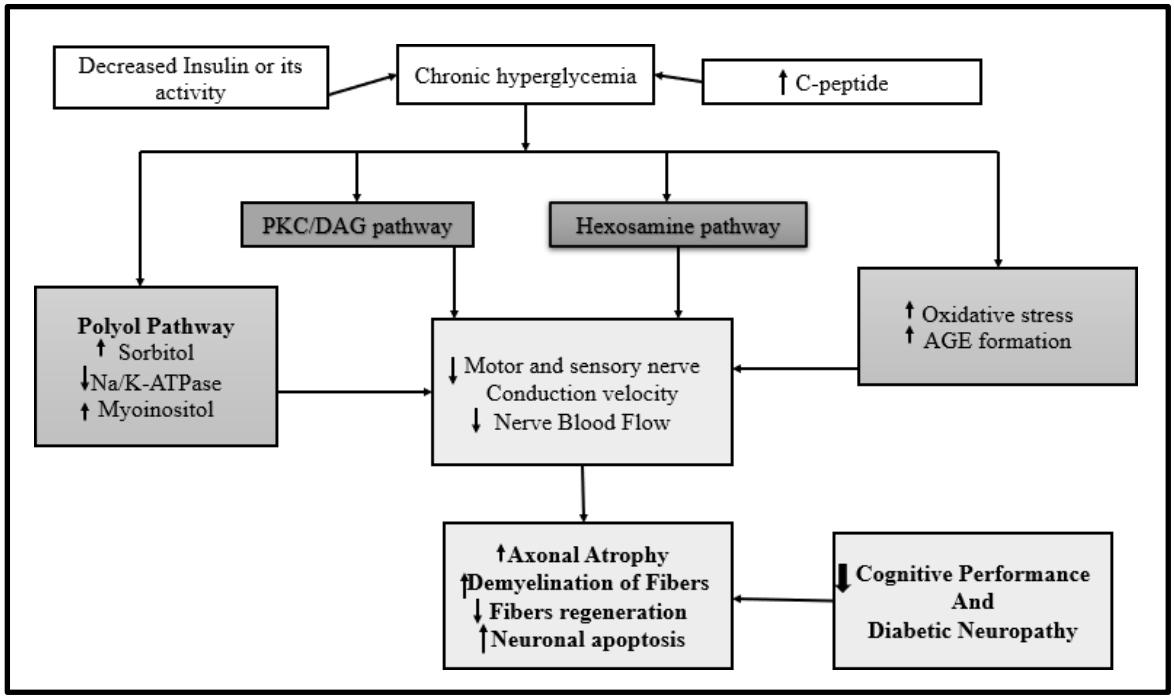

Figure 1.7: Biomarkers of Diabetic Neuropathy. In diabetic conditions, the brain undergoes several changes, including neuronal and dendritic atrophy, altered synapse formation, reduced neurotransmitter release, and electrophysiological impairments.

1.6 Objectives of Research:

The specific mechanisms through which CDCA provides neuroprotection in DN and cognitive impairment are not yet fully understood, and further research is needed to establish

its enduring security and effectiveness in clinical settings. CDCA, with its neuroprotective properties, offers potential as a treatment for DN and cognitive impairment, particularly in STZ-induced animal models. The current study is focused on evaluating whether CDCA can enhance cognitive function, support synaptic plasticity and neurogenesis, decrease neurodegeneration, and improve neurotrophic factors linked to DN and cognitive impairment. Preclinical studies on CDCA's role in treating DN may open doors to clinical trials, and improve the quality of life for people dealing with these challenges, thereby enhancing patient outcomes in DN and cognitive impairment management. The objectives are as follows:

1- Evaluate the neuroprotective effects of CDCA in diabetic mice from developing neuropathy brought on by the disease using STZ.
2- Studying the effect of CDCA through behavioural analysis to quantify hyperalgesia.
3- Examine histological and structural changes in specific brain regions of mice using H&E staining.
4- Analyze whether CDCA influences biochemical markers associated with DN and provides neuroprotective effects

Chapter 2: MATERIALS AND METHOD

2.1 Approval from Ethical Committee

The National University of Sciences and Technology, Islamabad's NUST Institutional Review Board Research Ethics Committee authorized all the protocols utilized in this study. The purpose of granting this approval was to guarantee the rights, security, respect, and welfare of the research subjects.

2.2 Timeline of the Experiment

In this study, we took a detailed approach to examine the effects of CDCA on Diabetic Neuropathy and Cognitive Impairment using animal models. The experimental stages are shown in Figure 2.1. First, three groups of animals were made, after that, a model that mimics DN was established. Once the model was established, the animals were treated following the study protocol. After the treatment period, we carried out behavioural tests to evaluate how the treatment might have affected their functioning. In the final step, the animals were humanely euthanized, and their tissues were collected for further analysis, including histopathology.

2.3 Induction of Type 1 Diabetes

The eighteen male blab/c mice in good health, weighing between 35 and 40 grams a piece, were chosen at the age of eight to twelve weeks taken from the National Institute of Health and given a week to acclimatize. These mice were divided into three groups, each containing 6 mice. The majority of studies involving STZ-induced diabetes are conducted on male mice since female mice are less vulnerable to this islet-cell toxin (Kolb, 1987). Following the period of acclimatization, their body weight was measured. Subsequently, the mice received five days of intraperitoneal injections of STZ, except for those in the control group. Using 40 mg/kg of STZ (Macklin China, CAS), diabetes was developed in the experimental animal (Figure 2.2). Four milligrams of STZ were prepared for each container, which was injected into three mice. The dose was properly weighed and put into a 1.5-millilitre vial that was covered with aluminium foil to keep STZ safe from light as it is light-sensitive. To achieve a final concentration of 4 mg/ml, the STZ was dissolved immediately before injection in a 50 mM sodium citrate buffer with a pH of 4.5.

After dissolving in the citrate solution, STZ degrades in 15 to 20 minutes., freshly made solutions must be given out within 5 minutes of dissolution. Intraperitoneal injection of the STZ solution was performed with a 1 mL syringe fitted with 25 G needles (Furman, 2015).

On the last day of the STZ administration, a 10% sugar solution was added in place of the normal water. The mice's blood glucose levels were assessed on the ninth day after the final STZ injection. Then, a blood sample is drawn from the tail vein to determine the blood glucose levels through the glucometer. Their blood glucose levels were statistically higher than those of control mice and exceeded 150 mg/dL (8.3 mmol/L). (Donovan & Brown, 2006). Subsequently, the animals were allowed to develop the symptoms of DN for almost one month.

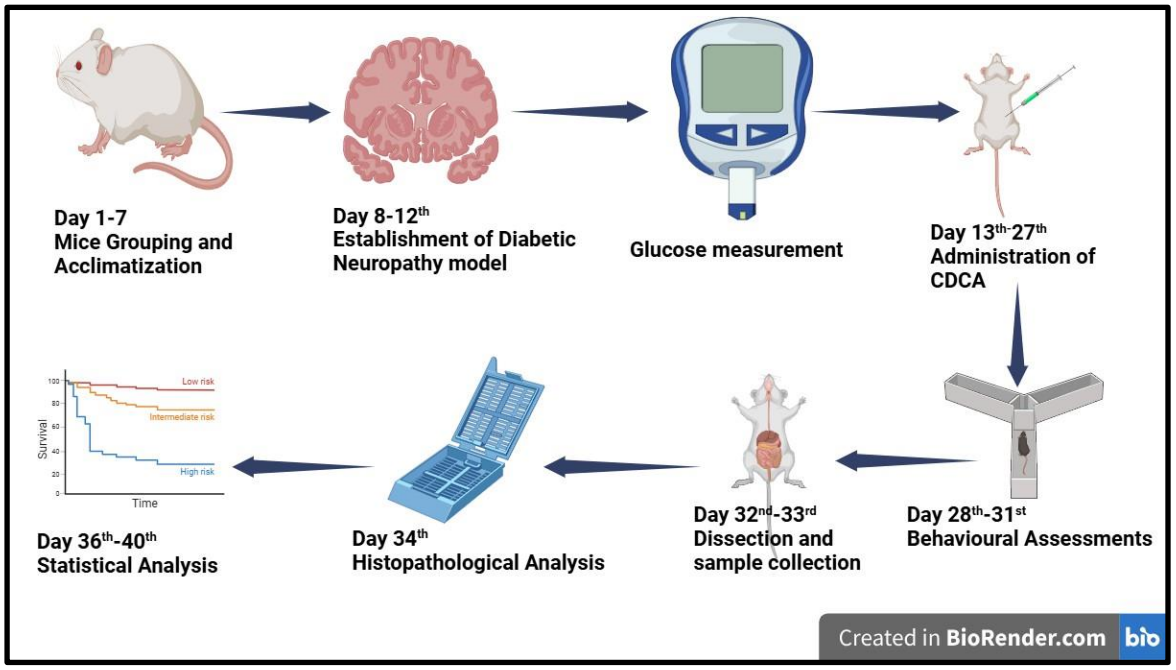

Figure 2.1: Timeline of Experiment. Detailed the sequence of key procedures, including the induction of diabetes, acclimatization of the mice, behavioural testing phases, and data collection milestones.

2.4 Administration of CDCA

90 mg/kg of CDCA (Macklin China, identification No. CAS 474-25-9) was given daily. An 8.4% CDCA suspension in $NaHCO_3$ was made (Sigma Aldrich USA, CAS 144-55-8). Injection volumes were adjusted based on the mice's body weight. The development of Diabetic Neuropathy, followed by the start of CDCA administration via daily intraperitoneal injections for 15 days. Behavioural tests were then performed after the final CDCA dose (Figure 2.3).

2.5 Behavioral Tests

Behavioural tests were conducted after both MPTP and CDCA administration. Following CDCA administration, behavioural assessments were performed to study the effects dose on the diabetic mice.

Figure 2.2: Administration of STZ. The induction of diabetes in a mouse through STZ administration.

2.4.1 Water Morris Maze test

This test was performed according to the protocol established by Richard G. Morris in 1984 (Nunez, 2008). This test is closely linked to hippocampal synaptic plasticity and NMDA receptor activity (Vorhees & Williams, 2006). The amount of time spent on the quadrant where the platform was previously located is used to gauge the animal's memory strength. (Tomás Pereira & Burwell, 2015). During pre-training, the mouse underwent trials for five days to learn to locate a visible platform in the water (Figure 2.3). On the day of the experiment, the platform was taken away once the mice had mastered the job, and the amount of time it took them to locate it was noted. To assess memory, the animal's frequency of crossing the platform's previous location was also counted. After each trial, the mouse is dried and the pool is drained to prepare for the next animal. To assess memory, the animal's

frequency of crossing the platform's previous location was also counted. After each trial, the mouse is dried and the pool is drained to prepare for the next animal.

2.4.2 Novel Object Recognition Test

This test was developed in 1988 as a means of evaluating rats' non-spatial memory without the use of conventional reinforcers (Ennaceur & Delacour, 1988). The NOR test is a common and effective way to assess memory and cognitive function in animals. A mouse was positioned in the middle of the box with two identical items in the corners for this test. The mouse was allowed to explore for five minutes, and a stopwatch was used to record how long it spent with each object. One of the items was swapped out for the new one depicted in Figure 2.4 following a 15-minute pause. After then, the mouse was given another chance to investigate, and the amount of time spent with the new and familiar objects was noted. After every test, the box and items are cleaned with 70% ethanol to guarantee the test's objectivity.

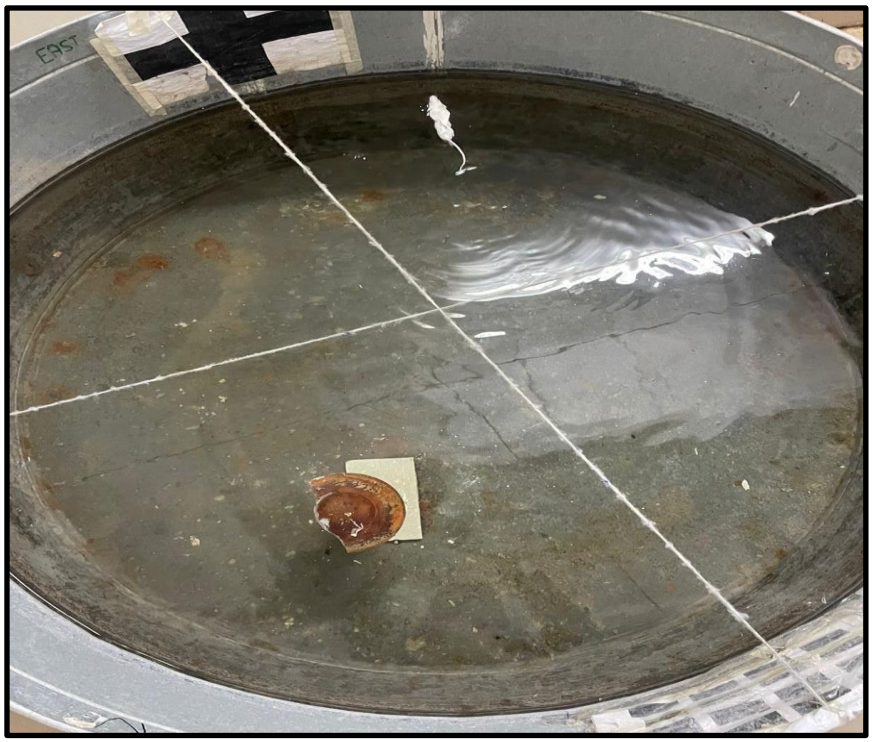

Figure 2.3: Water Morris Maze Test. Anxiety-like behaviour, memory, and spatial learning are evaluated using a mouse in a water maze apparatus.

2.4.3 Y-maze Test

This is an efficient method for evaluating mice's short-term memory despite its straightforward design (Lalonde, 2002). A Y-shaped maze was made from a wooden box wrapped in a black sheet, with two familiar arms and one novel arm. According to Figure 2.5, the arms are arranged at a 120-degree angle to one another and measure 30 cm in length, 8 cm in width, and 15 cm in height. While the novel arm was closed off, the mice were placed in the two familiar arms for the duration of the 10-minute training phase. Following a one-hour interval, the mice were given the opportunity to investigate the novel arm during the 5-minute testing phase. After filming the session, we examined the video to see how frequently the mice switched between the arms. Using the formula [(number of alternations)/ (total number of arm entries − 2)] × 100, the percentage of spontaneous alternations was determined. This procedure was performed for all the mice in each group after thoroughly cleaning the maze with 70% ethanol.

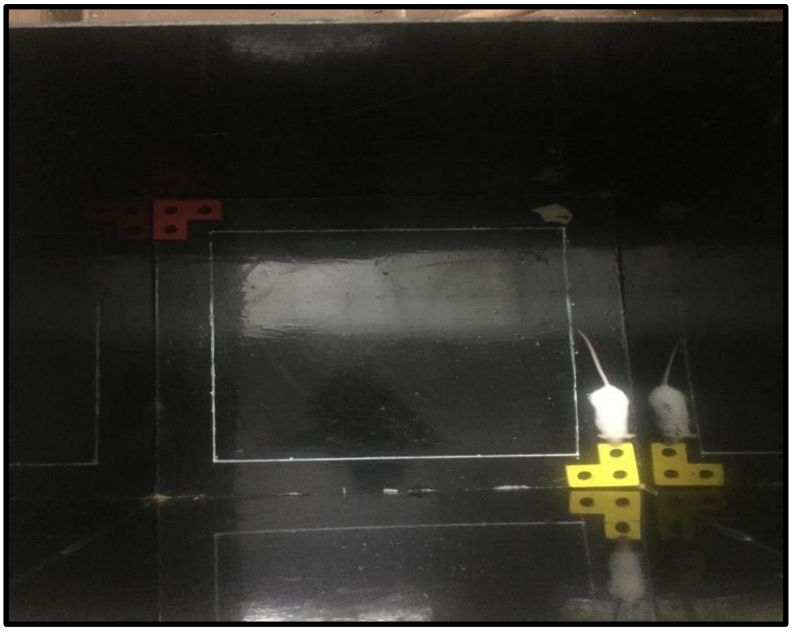

Figure 2.4: Novel Object Recognition Test. A mouse in a NOR test setup is used to assess recognition memory and exploratory behaviour by evaluating the mouse's preference for a new object over a familiar one.

2.4.4 Open Field Test

An extensively used technique for evaluating rats' exploratory behaviours and general activity is an open field test (OFT), which measures the amount and quality of the animals' movements. For the most part, Hall is credited for introducing the OFT first (Hall & Ballachey, 1932). The setup consists of a 60 × 60 cm white box with 60 cm high walls, divided into 16 equal squares (Figure 2.6). After each mouse is carefully positioned in the middle of the box, the timer begins, allowing the mouse to wander for five minutes. The session is recorded on video, and later, the footage was evaluated to see how much time the mouse spends in the center versus the edges and how often it enters these areas. To guarantee a new beginning for the following mouse, the box is cleaned with 70% ethanol after every trial.

Figure 2.5: The Y-maze test. A mouse in a Y Maze Test device was used to measure anxiety-like behaviour and locomotor activity.

2.4.5 Hot Plate Analgesia Test

These techniques are specially made to identify different pain modalities by adjusting for variables including stimulus kind, duration, intensity, and placement (Franklin & Abbott, 1989) Furthermore, when the same treatment is evaluated using several testing methods, the nociceptive testing method can indicate opposing effects on pain reactivity, such as hyperalgesia or hypoalgesia (Authier et al., 2000). The hot plate test is a simple and effective way to measure pain sensitivity in mice. To perform this test, the mouse was placed on a heated plate and covered with a transparent beaker, which was set at around 51–55℃ (Figure 2.7). As soon as the mouse was placed on the plate, the timer was started. The time taken for the mouse to show a pain response, such as licking its paws or jumping was recorded. After each trial, the hot plate was cleaned with ethanol to ensure the next mouse has a fresh start. Each session was recorded on video and then analyzed the time each mouse took for the mouse to show a pain response, such as licking its paws or jumping, was used to assess its pain sensitivity.

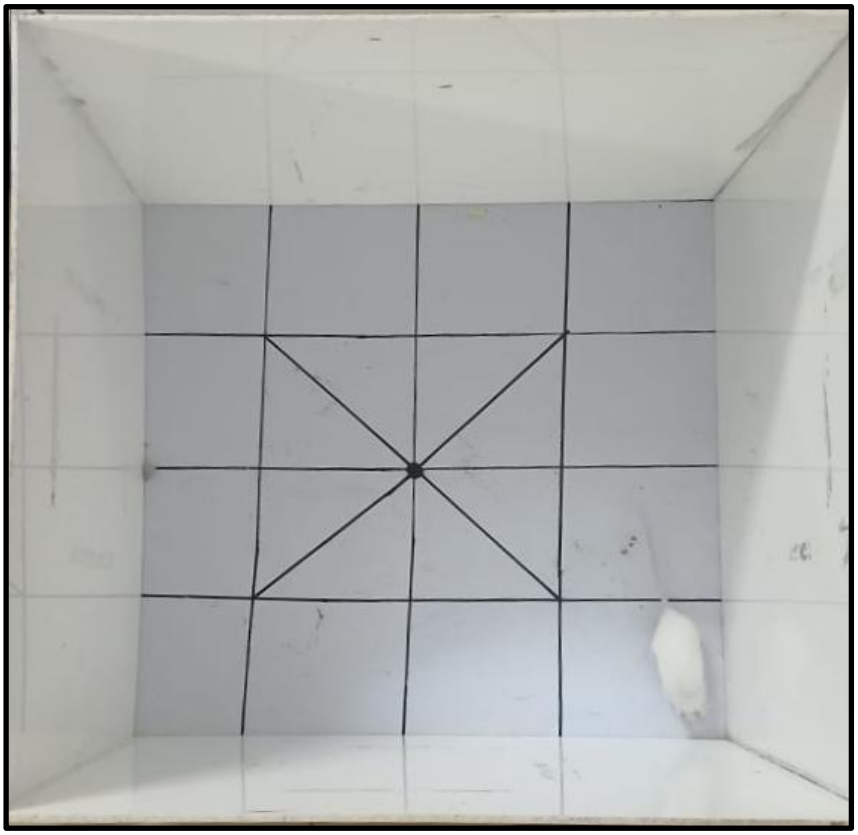

Figure 2.6: Open Field Test. A mouse's locomotor activity and anxiety-like behaviour are measured using open-field equipment.

2.4.6 Tail flick test

By administering a high-intensity thermal stimulus to a mouse or rat's tail, this test evaluates acute nociception (Bannon & Malmberg, 2007). Another popular technique for determining a mouse's sensitivity to pain is the tail-flick test, which frequently makes use of the same hot plate arrangement. For this test, the mouse tail was placed gently over a heated surface, set at around 50–55°C. The timer was started as soon as the tail touched the hot plate. And the mouse's reaction time is recorded via videotape. The maximum time for the test was set to 15-20 seconds to prevent injury. The session was video recorded, and the reaction time was analyzed manually by noticing how much time the mouse took to flick its tail away from the hot plate thus, evaluating the mouse's pain sensitivity. After each trial, the hot plate is cleaned to ensure the next test is conducted in a fresh environment.

Figure 2.7: Hot Plate Test. Pain sensitivity and thermal Nociception were assessed in a mouse by recording response times to heat stimuli such as jumping using a Hot plate apparatus.

2.4.7 Elevated Plus Maze Test

A well-known technique for assessing rodent anxiety reactions is the elevated plus maze (EPM), which was initially presented by File and associates. Rodents may be less worried or exhibit anti-anxiety behaviour if they spend a longer time or enter the maze's wide arms more frequently. Each mouse was only tested in the maze once in the lab, and it took roughly five minutes for each animal to be observed and findings collected (Walf & Frye, 2007). Mice that exhibit anxious behaviour are frequently evaluated using the EPM test.

To ensure objectivity, the apparatus, which is a plus-shaped platform with two open arms and two enclosed arms raised several feet above the ground, was cleaned. The mouse was given a certain length of time (usually five minutes) to explore the maze after being carefully positioned in the middle of it, facing one of the open arms (Figure 2.8). As soon as the mouse was released, the timer was set, and the mouse's motions in both its open and closed arms were noted. A manual analysis of the time spent in the open and enclosed arms was later conducted. The maze was cleaned and prepared for the following mouse by sanitising it with 70% ethanol at the conclusion of each session.

Figure 2.8: Elevate plus maze test. Anxiety-like behaviour in mice was evaluated using an EPM apparatus, which measured the amount of time spent in open versus closed arms.

2.5 Histopathological Analysis

Histopathological analysis was performed to investigate the morphological changes and tissue patterns in the brain's cellular structures at a microscopic level.

2.5.1. Preparation of tissue

The day after the behavioural experiments, tissue dissection and fixation were performed. Mice were anaesthetized by using chloroform before the histological analysis. Brain fixation was accomplished by injecting a fixative solution into one of the arterial blood vessels supplying the brain after exposing the abdominal cavity by making an incision. This was followed by an injection of 100 ml fixative solution with 4% paraformaldehyde in 0.1 M phosphate-buffered saline at pH 7.4 into an arterial blood vessel supplying the brain, and brain fixation was accomplished. The brain was removed carefully and washed with the same saline buffer to wash away all kinds of impurities. The sample was then stored 4% paraformaldehyde that serves as a fixative agent.

2.5.2 Hematoxylin and Eosin Staining and Slide Preparation

Slides from tissue were prepared and fixed with 4% paraformaldehyde. To begin, the tissue was dehydrated by placing it in ethanol solutions of increasing concentration (70%, 95%, and 100%) to remove any remaining water. The tissue was cleaned later using xylene to replace the ethanol and make it transparent. Using a microtome, the tissue was cut into thin slices (3-5 µm) and then rehydrated by submerging it in ethanol at progressively lower concentrations (100%, 95%, and 70%). For staining, the rehydrated slices were placed in a hematoxylin solution for several minutes, followed by a rinse to remove excess stain (Figure 2.9). To stabilize the hematoxylin, place the slides in a mild alkaline solution (ammonium water) before another rinse. Then, eosin was applied for contrast and the sample was rinsed again. After staining, we dehydrated slides with progressively stronger ethanol solutions (70%, 95%, and 100%) and cleared them once more with xylene. Lastly, we added a mounting medium and secured a cover slip over the sections to preserve the tissue for microscopic examination, keeping the samples intact and ready for further study.

2.5.3 Microscopy

The slides were examined under a light microscope version Optica B-150, Italy at 10X and 40X magnifications to assess tissue structure, cell count, and cellular patterns. Photomicrographs were taken of the hippocampus and cortex, to document and compare structural changes across the three groups After that, ImageJ software, version 8, was used to analyse the recorded images. The goal of this microscopy analysis was to observe cellular alterations resulting from the induction of STZ.

2.6 *In Silico* Analysis

To gain insight into molecular markers involved in CDCA-mediated neuroprotection, an *in-silico* analysis was conducted using the 3D structures of BDNF and NF-κB (1nfk), and the chemical structure of CDCA (PubChem CID: 10133) obtained from PDB and PubChem databases. These structures were cleaned up using BIOVIA Discovery Studio Visualizer 3.0. To better understand how NF-κB, BDNF, and CDCA interact, the PyRx 0.8 integrated Vina Wizard tool was used for docking simulations and calculations of their binding affinities. Finally, we visualized the interactions between NF-κB-CDCA and BDNF-CDCA using Discovery Studio, which helped in the identification of key patterns in their interactions.

2.7 Statistical Analysis

Before conducting statistical analyses, the normality of all data sets was assessed. The study aimed to compare differences among three groups: the control, the STZ-induced group, and the STZ-treated group with CDCA. Tukey's post-hoc comparison was performed for groupwise analysis after a one-way ANOVA was used to find significant differences between these groups. GraphPad Prism version 10.0 was used to construct the graphs, and $p < 0.05$ was used for statistical significance. The average and standard error of the mean (SEM) was used to display the data and findings.

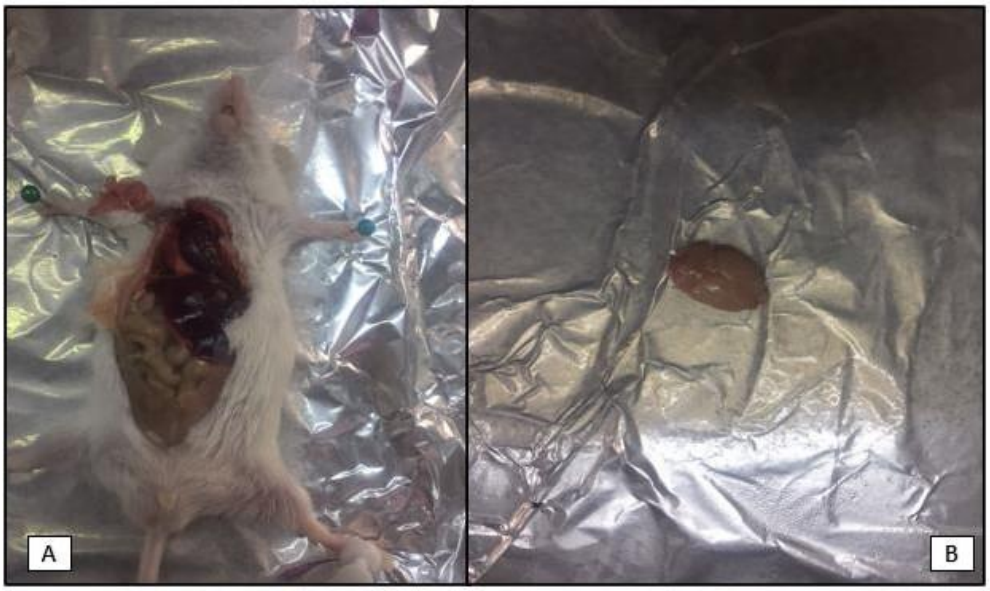

Figure 2.9: Dissection of Mouse. The dissection of a mouse showcases the internal anatomy and organs. B-Brain of mouse.

Chapter 3: RESULTS

3.1 Results of Behavioural Assessments

3.1.1 CDCA improves anxiety-like and exploratory behaviours in a Diabetic neuropathy mouse model.

The EPM test is a frequently employed technique for assessing behavioural changes after STZ treatment. When compared to healthy controls, diseased mice typically spend more time in the closed arms and enter the open arms less frequently. The findings (Figure 3.1) show how the mice's inquisitive and anxiety-like behaviours are affected by the administration of STZ and CDCA. While mice treated with CDCA showed a considerable reduction in anxiety levels, mice in the STZ group displayed increased anxiety and decreased exploratory activity in the open arms. Overall, the EPM test shows that CDCA can help treated mice regain their usual behaviour and lessen the behavioural abnormalities brought on by STZ.

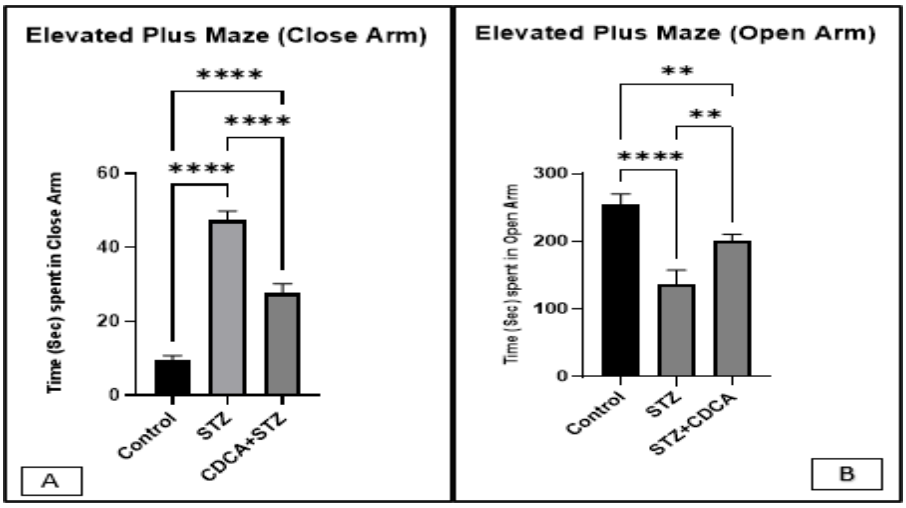

Figure 3.1: Elevate Plus Maze Test. A- Time (sec) spent in closed arm in EPM Test. This Graph shows the time(sec) mice spend in closed arms treated with the drug is significantly less among all the groups. **B**- Time (sec) spent in closed arm in EPM Test. This graph demonstrates that mice given the medicine spend noticeably longer time in open arms (in seconds) than the diseased group. For statistical analysis, a one-way ANOVA was employed, followed by Tukey's multiple comparison test. Error bars depict SEM, ** show $p<0.01$ and **** indicate $p<0.0001$.

3.1.2 CDCA improves **deficits in spatial learning and memory** *in a Diabetic Neuropathy disease mouse model*

Another frequently used tool for evaluating behavioural changes that occur after STZ treatment is the MWM test. Diseased mice generally take longer to locate the hidden platform

and have poorer memory recall in future trials than healthy controls. The results demonstrate how the administration of STZ and CDCA affects the mice's memory and spatial learning. While mice in the STZ group showed deficits in spatial learning and memory retention, mice treated with CDCA significantly improved memory function (Figure 3.2 and 3.3). Overall, the results of the MWM test show that CDCA can help treated mice regain their regular cognitive function and lessen the memory impairments brought on by STZ.

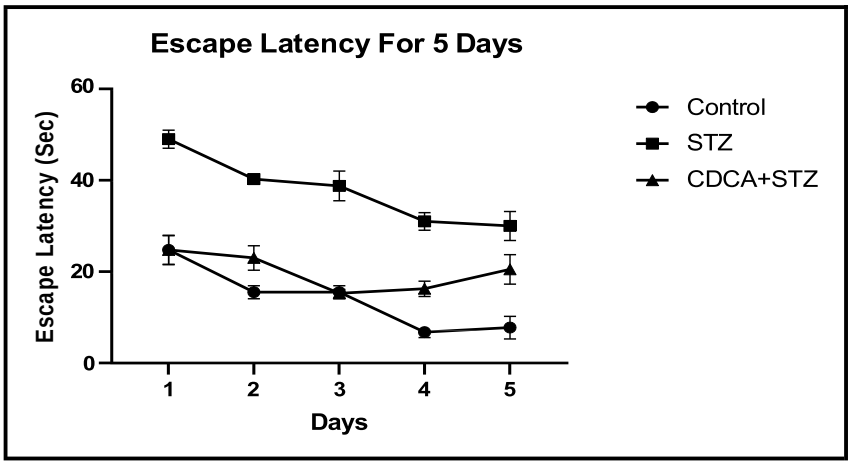

Figure 3.2. Escape latency Graph. Control mice gradually take less time to find the platform, showing normal learning ability. In contrast, STZ-treated mice take longer, reflecting difficulties with memory and learning. Mice treated with CDCA improve significantly, suggesting that CDCA may help protect against these cognitive challenges.

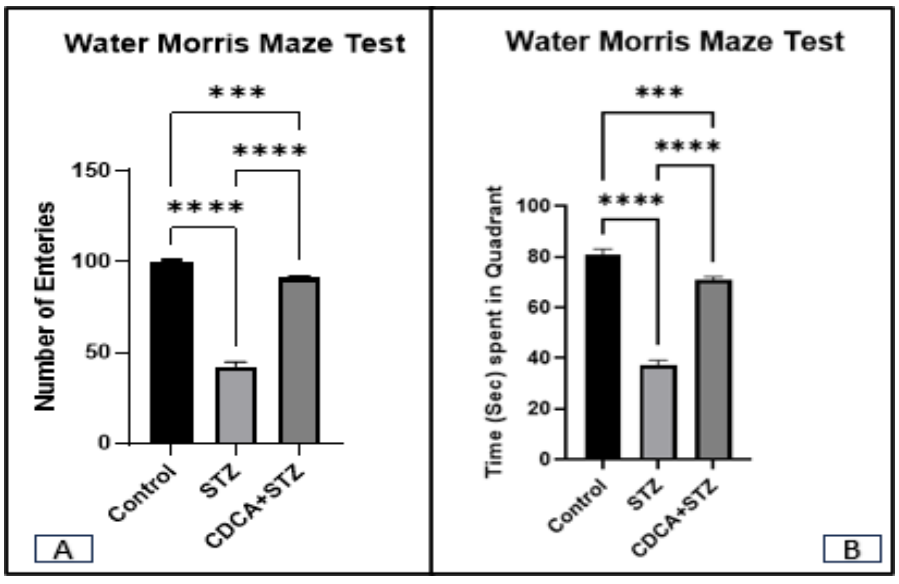

Figure 3.3: The Morris Water Maze Test. A: Duration in MWM quadrant - The drug-treated mice produced the most entries in the target quadrant, according to this graph. **B**: The number of entries in the target quadrant in MWM; the drug-treated mice crossed the most

platforms. Error bars depict SEM and significance is indicated by ***p < 0.001 and ****p < 0.0001.

3.1.3 CDCA improves working memory and increases exploratory behaviour in a Diabetic neuropathy disease mouse model.

Mice affected by the disease typically show lower rates of spontaneous alternation and reduced exploratory behaviour compared to healthy controls. The results indicate how STZ and CDCA treatment influence the mice's working memory and exploratory activity. While the STZ group exhibited deficits in working memory and exploration, mice treated with CDCA demonstrated a significant improvement in spontaneous alternation and increased exploratory behavior (Figure 3.4). Overall, the Y-Maze test results suggest that CDCA can help restore normal cognitive function in treated mice and reduce the working memory impairments caused by STZ.

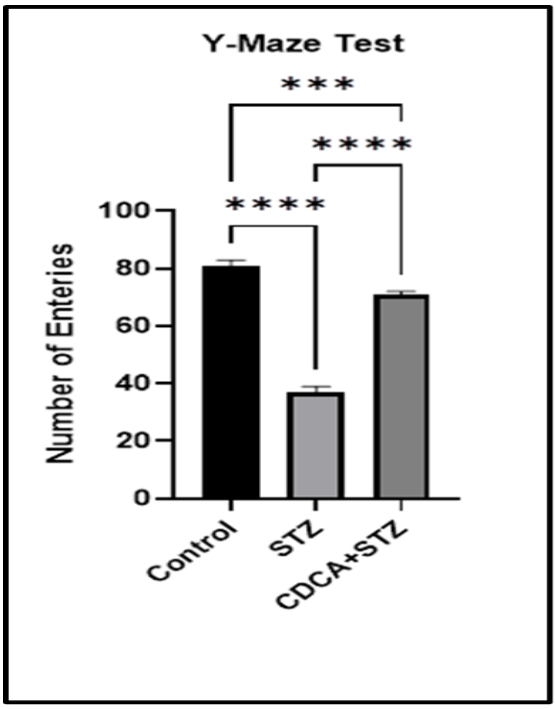

Figure 3.4: The Y-maze Test. The mice in the control group actively switched between the maze's arms, indicating intact and healthy memory and cognitive function. *The* STZ-treated mice showed a clear decline in this alternating behaviour, The mice who received CDCA treatment demonstrated a noticeable improvement, resembling those of the healthy control group. Error bars depict SEM, *** = p < 0.001, and **** = p < 0.0001.

3.1.4 CDCA Promotes Increased Exploration and Cognitive Abilities in Diabetic Neuropathy Models

Generally, compared to healthy controls, mice with STZ-induced diabetes tend to spend more time around the arena's perimeter and enter the centre less frequently. The outcomes show how the mice's inquisitive and anxiety-like behaviours are affected by the STZ and CDCA treatments. Mice treated with CDCA significantly reduced anxiety levels, whereas those in the STZ group showed elevated anxiety and decreased exploratory activity in the central region (Figure 3.5). Overall, the open-field test shows that CDCA can help treated mice regain normal behaviour by reducing the behavioural abnormalities linked to STZ-induced DN.

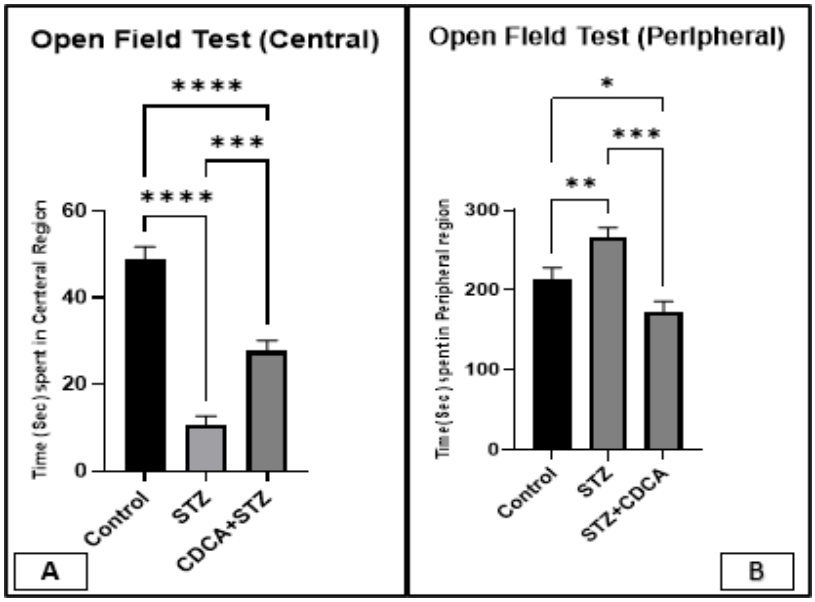

Figure 3.5: The Open Field Test. A- Number of entries in the central area. This Graph shows the mice treated with the drug entered the central area the maximum number of times. B- Number of entries in the peripheral area. This Graph shows the mice treated with drug entered the central area the minimum number of times. Error bars show SEM while *p<0.01 **p<0.01 ***p < 0.001 and ****p < 0.0001 indicate significance.

3.1.5 CDCA reduces pain sensitivity and improves pain response in a Diabetic neuropathy disease mouse model

Using the Hot Plate analgesia Test, the nociceptive responses of mice were evaluated across control, STZ-treated, and CDCA-treated groups. The main measurement taken was the latency to withdraw the paw from the heated surface. As illustrated in Figure 3.6, the control

group displayed significantly shorter withdrawal latencies compared to the group treated with STZ, indicating heightened pain sensitivity in the diabetic mice. Furthermore, the group treated with CDCA showed a marked increase in withdrawal latency compared to the STZ-treated group, suggesting a reduction in pain sensitivity.

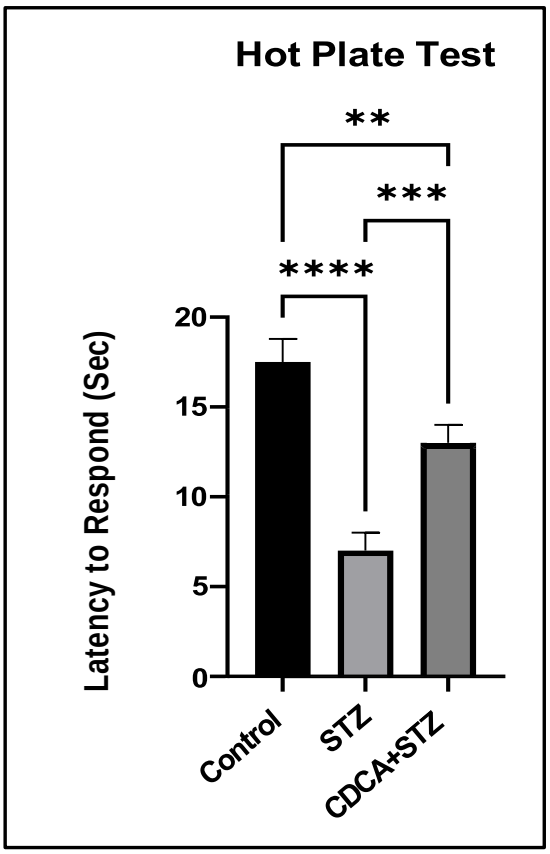

Figure 3.6: Hot Plate Test. Increased hot plate latencies were seen in the hot plate hyperalgesia test outcomes as compared to the disease control group. SEM is represented by error bars, and significance is indicated by **p<0.01, ***p<0.001, and ****p<0.0001.

3.1.6 CDCA decreases pain sensitivity and enhances nociceptive responses in a Diabetic Neuropathy disease mouse model

The nociceptive responses of mice were examined in the control, STZ-treated, and CDCA-treated groups using the Tail Flick Test. The primary metric was the latency to extract the tail from an uncomfortable heat source. The diabetic mice displayed increased pain sensitivity, as seen by the considerably shorter tail withdrawal latencies of the control group compared to the STZ-treated group (Figure 3.7). In addition, there was a discernible increase in withdrawal latency in the group treated with CDCA as opposed to the group treated with STZ, suggesting a reduction in pain sensitivity.

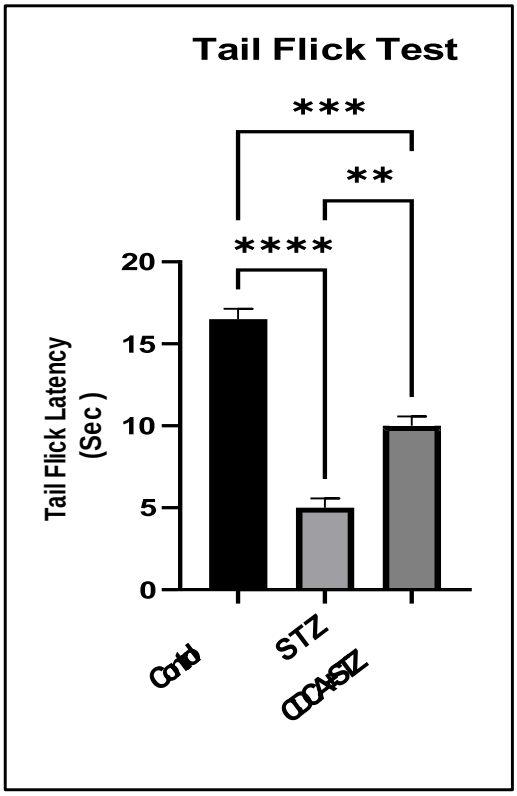

Figure 3.7: The Tail Flick Test. Tail withdrawal latency in the treatment groups was longer than that of the disease control group. SEM is represented by error bars, and significance is indicated by **p<0.01, ***p<0.001, and ****p<0.0001.

3.1.7 CDCA decreases pain sensitivity and enhances nociceptive responses in a Diabetic Neuropathy disease mouse model

The cognitive abilities of mice were assessed in the control, STZ-treated, and CDCA-treated groups using the NOR test. This test measures how much time the mice spend exploring a new object compared to one they've seen before. Diabetic mice, represented by the STZ-treated group, showed signs of memory impairment, as they didn't spend more time with the new item as compared to the control group (Figure 3.8). Interestingly, the CDCA-treated mice group spent a greater amount of time than the STZ-treated group investigating the new object, suggesting a potential improvement in their memory and recognition abilities.

3.2 CDCA Provides Neuroprotection Against STZ-Induced Neurodegenerative Damage

In the control group, the cortex and hippocampus histological structures appear completely normal. However, when the mice were exposed to STZ, significant neuronal degeneration occurred in these brain regions. Furthermore, the group treated with CDCA showed improvements in their histological features. In the areas affected by STZ, the cells in the

cortex and hippocampus were shrunken, formed clusters in some spots, and had an irregular, elongated shape. In contrast, the cells from the control group appeared round and uniform. Due to CDCA treatment, this damage was largely prevented. The number of cells in the STZ-treated group was significantly lower in terms of cell counts. However, CDCA treatment helped maintain cell integrity, highlighting its potential neuroprotective properties.

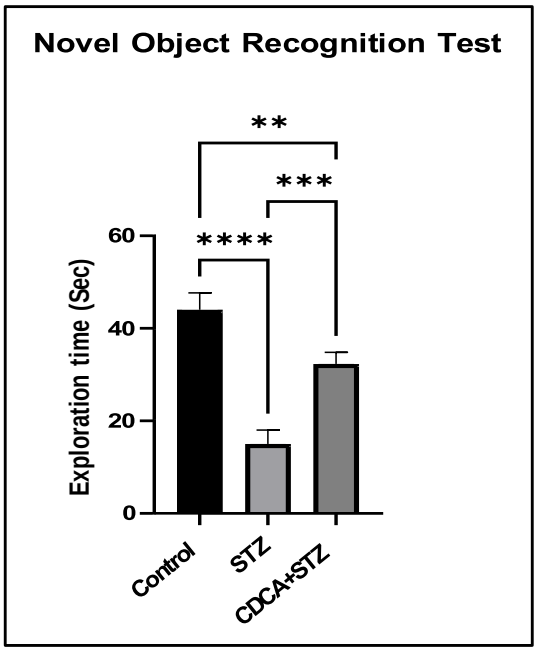

Figure 3.8: Novel Object Recognition Test. The treatment groups' exploring times were noticeably longer than those of the disease control group. SEM is shown by error bars and significance is expressed as **p<0.01, ***p<0.001, and ****p<0.0001.

3.2.1 Cortex

The cortex of the control mice displayed normal histological architecture, with neurons appearing healthy under the microscope. In contrast, the cells in the STZ-treated group showed significant signs of deterioration, including enlarged intracellular gaps, irregular shapes, and disorganized structures. Additionally, blood vessel congestion and cell shrinkage were observed in these animals due to STZ exposure (Figure 3.9).

When compared the mice treated with CDCA exhibited typical histological characteristics in their cortex, featuring a greater number of round cells and very few degenerated cells. Histological scoring, conducted using GraphPad Prism, revealed that the STZ-treated group had a lower overall cell count due to decreased cell density and increased intercellular spacing. In contrast, the CDCA-treated group demonstrated higher cell density, indicating a greater number of healthy cells.

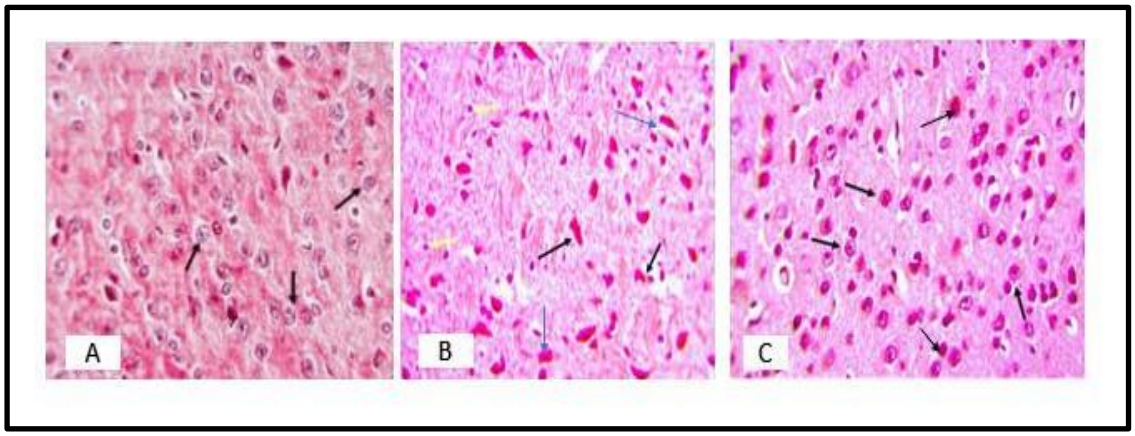

Figure 3.9: The Section of Cortex stained with H&E stained (40X). (A) shows that there are no histopathological changes in the Cortex of the Control group. (B) Black, blue and yellow arrows indicate irregularly shaped cells and shrunken neurons respectively in STZ-treated mice. (C) CDCA-treated cortex shows reduced shrunken neurons in the cortex.

3.2.2 Hippocampus

Figure 3.10 shows a section of the brain stained with H&E, revealing the histological features of the hippocampal tissue. In the control group, the tissue appeared healthy and well-preserved, with no signs of damage, and the cells had a normal shape. In contrast, the hippocampus of the STZ-treated group exhibited shrunken pyramidal cells, indicating significant deterioration. On a positive note, the hippocampal pyramidal layer in the CDCA-treated group showed normal, healthy neurons. Furthermore, when looking at the number of pyramidal cells, it was clear that the STZ-treated group had significantly fewer cells compared to those that received CDCA treatment.

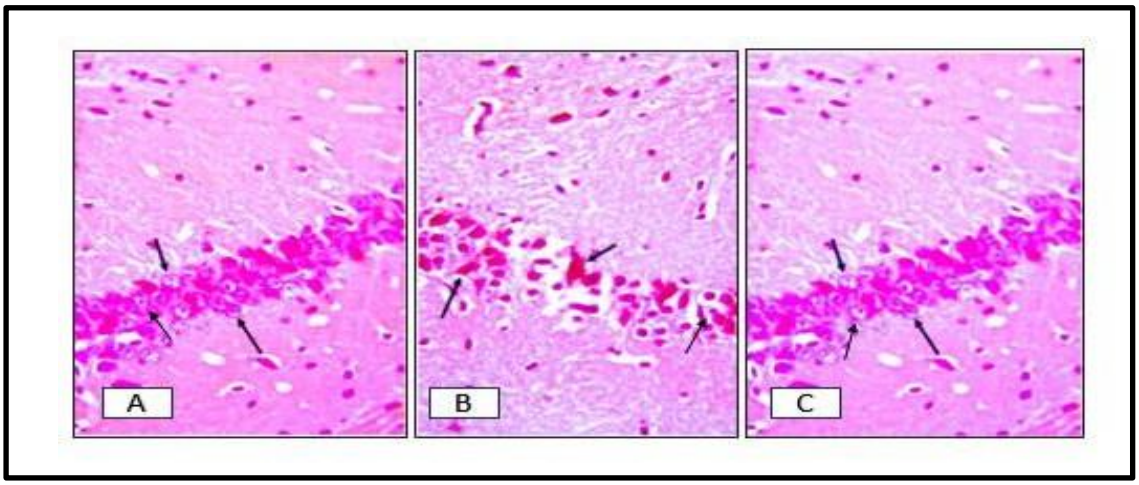

Figure 3.10: The Section of Hippocampus stained with H&E stained (40X). (A)Shows a normal hippocampus with no changes in the cells. (B) The hippocampus of STZ-treated mice presented degenerative neurons. (C) Changes in the treated group and neurons appeared more normal.

3.2.3 Histopathological Scoring of Cortex and Hippocampus

Figure 3.11 shows the total cell counts in the hippocampus and cortex for control, STZ-treated, and CDCA-treated mice. Cell numbers declined in both brain regions after STZ treatment, but CDCA treatment appeared to restore cell counts to levels similar to those in the control group. This confirms the neuroprotective effects of CDCA in diabetic Neuropathy and cognitive Impairment.

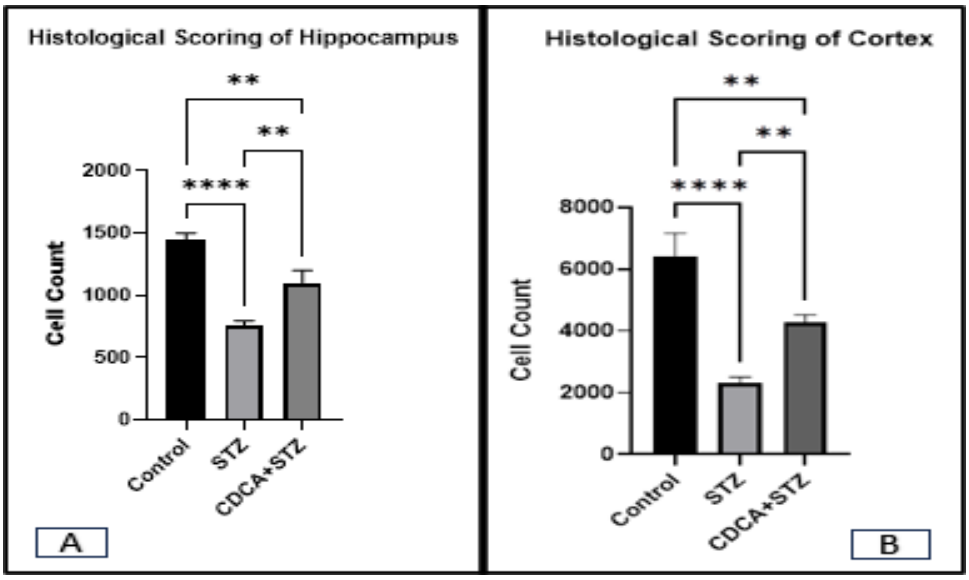

Figure 3.11: The Histological Scoring of Cortex and hippocampus. The graphs in this figure show the cell count of three groups in (A) Cortex and (B)Hippocampus. Tukey's post hoc test was used after a one-way ANOVA to compare these three groups. ****$p<0.0001$ and **$p<0.01$. Error bars present SEM.

3.3 *In Silico* Analysis

3.3.1 Structure of Proteins and Ligand

The protein structures of Nf-kβ and BDNF were downloaded in PDB format from AlphaFold. The chemical structure of the ligand, CDCA, was obtained from PubChem in SDF format, providing details about its composition and conformation. The structures of both, the proteins

and ligands, shown below in Figure 3.12, were then refined and set to publication quality in Discovery Studio Visualizer. These structures are used for further analysis.

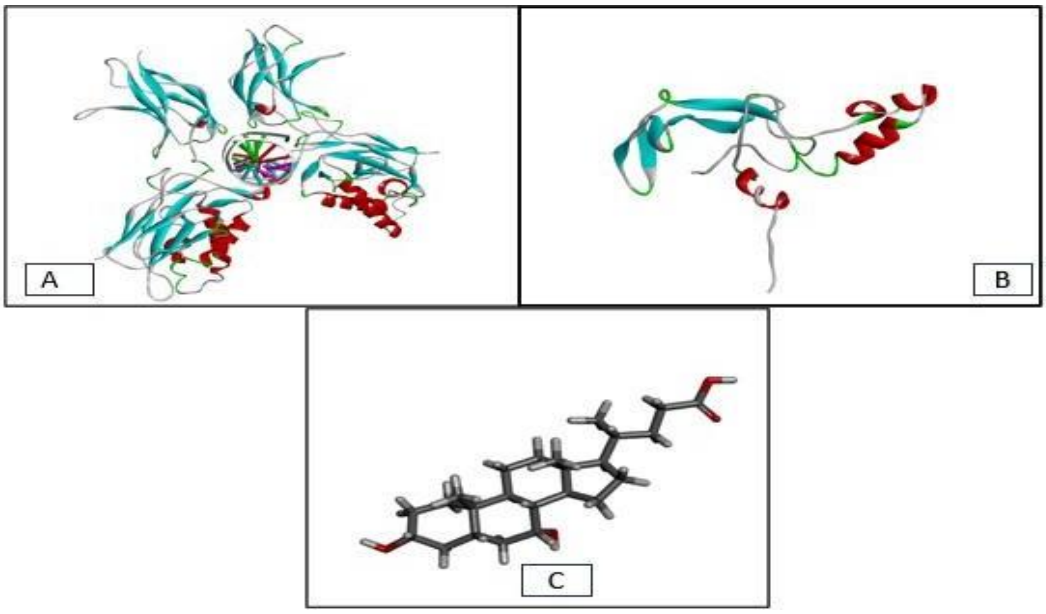

Figure 3.12: 3D Structures of NF-κB (A), BDNF (B), and (C) ligand, CDCA.

3.9.2 Molecular Docking Analysis

Based on a literature review, the target proteins BDNF and NF-κB (1nfk) were selected, along with ligands from CDCA (figure 3.13). Molecular docking was performed using PyRx, and the resulting binding energies are presented in the accompanying table. Using Discovery Studio, 2D visualizations of the binding interactions were generated. The interaction between CDCA and BDNF and NF-κB (1nfk) made it a strong target to select these biomarkers for studying the effect of CDCA on neuroinflammation in the brain regions.

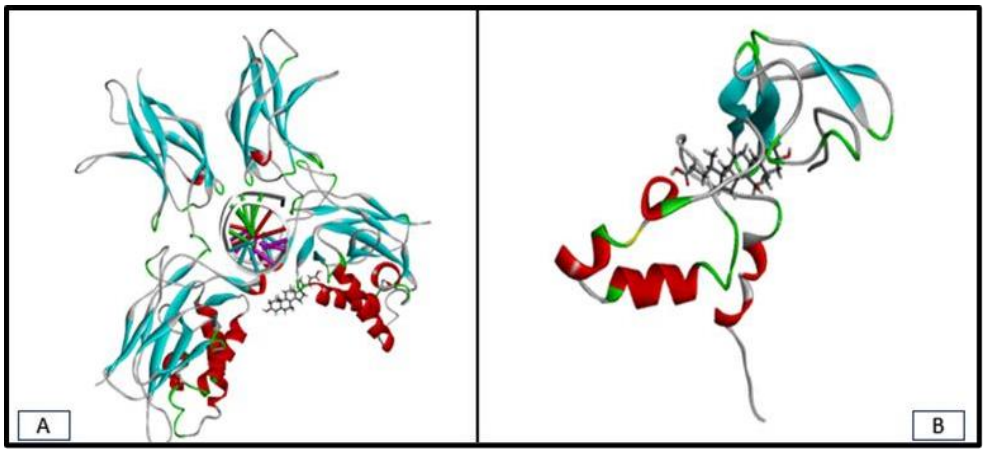

Figure 3.13: Representation of Docking Interactions of CDCA, NF-κB and BDNF.A) computational representation of the interaction between CDCA and NF-κB. B) computational representation of the interaction between CDCA and BDNF.

3.3.3 Binding Affinity

The graphs in Figure 3.14 illustrate how well CDCA binds to the target proteins NF-κB and BDNF. The vertical axis represents the binding energy in units like kcal/mol, which helps us see how strong the interaction is. Each point on the graphs comes from a different computer simulation of the docking or binding process. Lower values on the y-axis mean CDCA binds strongly to the proteins, signalling a good interaction. On the other hand, higher values suggest weaker binding. This analysis gives us valuable insights into how CDCA might work as a ligand for NF-κB and BDNF, revealing not only the stability of the ligand-protein complex but also possible biological implications, like its role in key cellular processes and potential therapeutic effects.

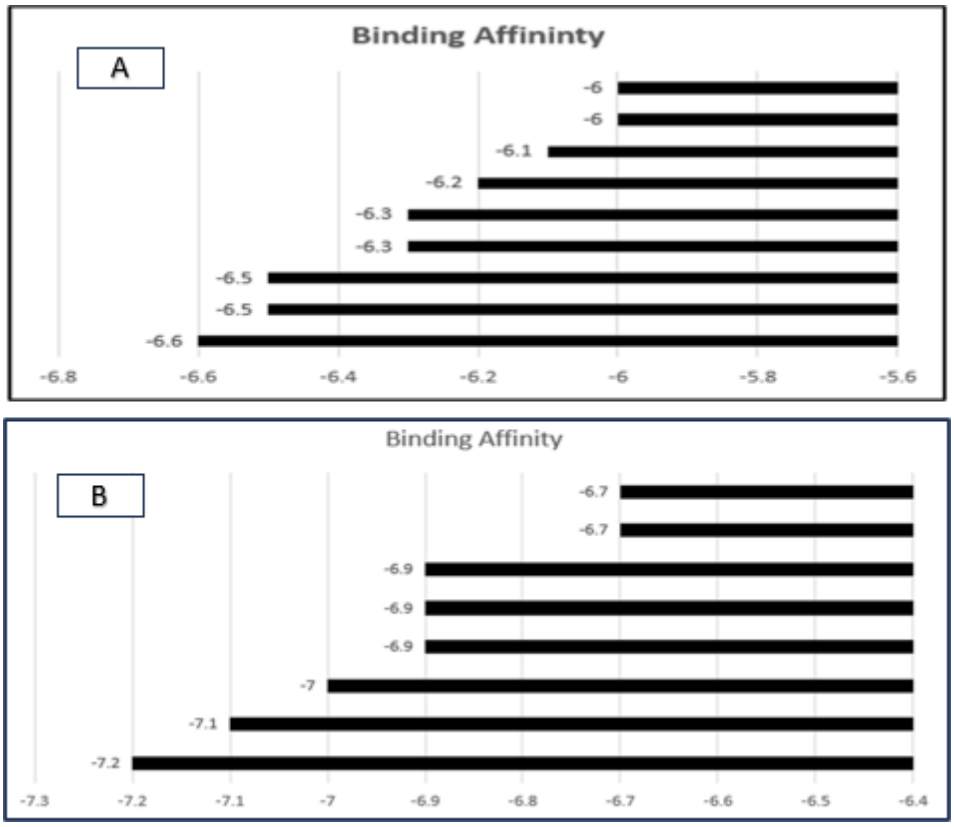

Figure 3.14: Binding Energies from Docking simulations. *In silico* analysis, A) shows *the* interaction of CDCA with NF-κB, with binding energies ranging between -6 and -6.6

kcal/mol and an average binding energy of -6.2 kcal/mol. B) The interaction of CDCA with BDNF, having binding energies that range from -6.7 to -7.2 kcal/mol and an average binding energy of -6.95 kcal/mol.

3.9.4 Molecular Interaction Analysis

PyMOL generated a 3D visualization of the protein-ligand complex (Figure 3.15), while Discovery Studio produced detailed 2D diagrams that illustrated the interactions and bonding patterns between the protein structure and the ligand.

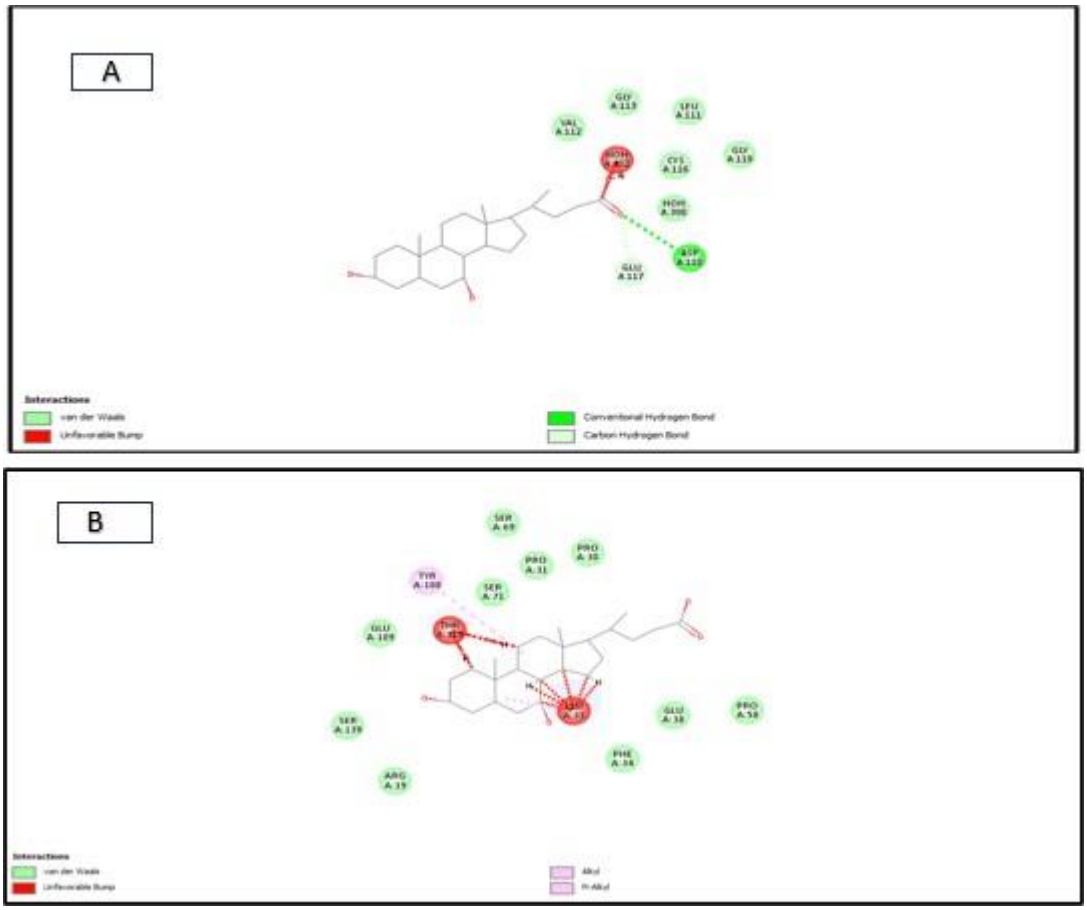

Figure 3.15: Binding sites on NF-κB and BDNF. A- shows the amino acid residues ASP of NF-κB engage in Van Der Waals interactions, while GLU forms a Carbon hydrogen bond. B- the amino acid TYR of BDNF shows Alkyl bonding with CDCA

Chapter 4: DISCUSSION

Diabetes mellitus often leads to complications such as neuropathies, conditions like insulin resistance, hypertension, and obesity are commonly observed in individuals with neuropathy. Despite limited direct treatments for DN, emerging therapies such as neurotrophic factors, gene therapy, and immunotherapy, show promise for promoting nerve repair (Mahmood et al., 2009). Early detection of DN and exploration of biomarkers can improve diagnosis, reveal disease mechanisms, and guide the development of effective treatments. For over 30 years, STZ-treated animals have been used to investigate diabetogenic mechanisms and preclinical evaluations of new therapies (Goud et al., 2015). Studies have highlighted the potential role of BA particularly of CDCA, a primary BA, in promoting neurotrophic factors, mitigating neurodegeneration, and reducing apoptosis. The study explored CDCA's therapeutic potential by focusing on key biomarkers like NF-κB and BDNF in DN pathophysiology, with molecular docking revealing strong interactions, suggesting therapeutic benefits (Shaibani & Rafieirad, 2024).

CDCA effectively inhibits LPS-induced inflammation in BV2 cells, potentially through the activation of TGR5, which suppresses the Akt/NF-κB signalling pathway (Zhu, 2020). BDNF influences neuroprotection and neurodegeneration in AD models, linking gut microbiome changes, inflammation, and amyloid-β-related alterations in the CNS (Hecking et al., 2022). CDCA stimulate the CREBP and increases BDNF levels. As a result, it improves the sensitivity of insulin in the hippocampi of rats treated with $AlCl_3$.Overall, the beneficial effects of CDCA in reducing insulin resistance and Alzheimer's disease-related signs and symptoms were attained by its ability to regulate pathways such as IRS-1/Akt/GLUT4, GLP-1/Akt/GLUT4, PPARγ/GLUT4, BDNF/CREB, and BACE1/Aβ42 (Bazzari et al., 2019). Furthermore, CDCA notably alleviates cognitive dysfunction and spatial deficits caused by $AlCl_3$ in a rat model.

Improvements in cognition were demonstrated through the WMM and test of Y-maze, along with the preservation of normal histological structures. Both CDCA and synthetic FXR ligands have the strength to mitigate resistance to insulin linked to different disorders (Zhang et al., 2006). This study has revealed that prolonged diabetes impairs spatial memory, affecting both the attaining and revival of processes, as evidenced by performance in the MWM. These findings align with previous research (Wang et al., 2012). In diabetes type 1 animals models, a reduction in performing difficult activities, such as the MWM or learning of spatial learning tasks, was observed too (Tomás Pereira & Burwell, 2015). The results of

this study demonstrate the impact of STZ and CDCA treatment on the mice's spatial learning and memory. Mice in the STZ group exhibited impairments in spatial learning and memory retention, while those treated with CDCA showed a notable improvement in memory function.

Overall, the findings from the MWM test suggest that CDCA helps restore normal cognitive function in the treated mice and alleviates the memory deficits caused by STZ. The Y-maze is a test that relies on the hippocampus and is used to observe how willing rodents are to explore new surroundings. The percentage of rats' spontaneous alterations and total arm entries decreased significantly during the Y-maze test, in contrast to the control group in the AlCl3 group. However, CDCA treatment helped alleviate AlCl3's detrimental effects on the rats' spatial working memory and general activity, as demonstrated by a discernible improvement in both %SAP and total arm entries compared to the $AlCl_3$ group (Bazzari et al., 2019). This study has revealed that while the STZ group struggled with working memory and exploration, mice treated with CDCA showed a clear improvement, exploring more and performing better in tasks that measured their memory. Recent reports indicate hypoalgesia and a decrease in the tail nerve's nerve conduction velocity after five weeks of STZ injection ((Murakami et al., 2013).

In mouse models of DPN, Hot plate analgesia and tail-flick tests were employed based on the previous studies and assessed how the mice responded to pain using the Hot Plate Test (Authier et al., 2000), where measurements were made to know how long it took for the mice to pull their paw away from a heated surface. The control group reacted much quicker, pulling their paw away sooner compared to the STZ-treated group, which showed higher pain sensitivity due to diabetes. On the other hand, the CDCA-treated mice took longer to withdraw their paw, indicating that the treatment helped reduce pain sensitivity and improve their pain response. Insulin's capacity to cause chronic depression in the CA1 hippocampus subregion is compromised, and this is correlated with peripheral IR. (Jaggi et al., 2011). Behavioural analysis shows that CDCA improved locomotion and reduced anxiety-like behaviour. The results indicate that diseased mice spent more time in the peripheral area and less time in the central region, suggesting heightened anxiety and reduced exploratory behaviour due to STZ exposure.

In contrast, the CDCA-treated group exhibited behaviours more Comparable to the control group, this suggests that the treatment was effective in lowering anxiety and improving motor

skills. Hyperglycemia in diabetics causes pericytes, astrocytes, and endothelial cells to increase their mitochondrial respiration, which raises the production of reactive oxygen species. This overabundance of reactive oxygen species can exacerbate the inflammatory process by causing neurovascular injury and blood-brain barrier disruption. The activation of certain signalling pathways (PI3K/AKT/NF-κB), a response from microglial cells in the cortex, and elevated levels of proinflammatory cytokines in the serum and cortex are all indicators of increased neuroinflammation (Weng et al., 2024). The spinal dorsal horn's second-order neurons exhibit persistently elevated excitability due in part to the pronociceptive function of the BDNF–TrkB pathway. Neuropathic pain (NP) and central sensitization are characterized by allodynia, hyperalgesia, spontaneous pain, and causalgia, all of which are mostly caused by this heightened excitability (Biggs et al., 2010).

Mice's cognitive memory, short-term memory, and long-term memory were evaluated using the NOR test (Wang et al., 2023). A two-trial cognitive paradigm called the NOR test is used to evaluate recognition memory. It has been demonstrated that NOR may be used to investigate the recognition memory problems linked to schizophrenia and Alzheimer's disease, two conditions that have been extensively studied. It also applies to illnesses like autism spectrum disorders and Parkinson's disease. STZ-induced diabetes is a useful model for evaluating the neurobehavioral effects of diabetes in mice because it dramatically increases anxiety-like behaviour. It can also be used to screen for the effects of different pharmacological compounds on diabetes-related anxiety-related impairments. Prior research has demonstrated that diabetes brought on by STZ causes anxiety-like symptoms in different preclinical models. More time spent in the EPM's closed arms and less time spent in the open field test's central arena serve as indicators of this. (Aksu et al., 2012).

Molecular docking simulations indicate that CDCA binds in multiple ways, each with a unique binding affinity, likely due to different conformations and orientations of CDCA within the binding sites of these proteins. Research indicates a strong interaction between CDCA and both NF-κB and BDNF, as shown by their optimal binding sites and significant negative binding affinities. Specifically, the binding energy between CDCA and NF-κB and BDNF ranges from -6 and -6.6 kcal/mol, and – 6.7 to – 7.2 kcal/mol respectively, demonstrating a stable and efficient molecular interaction. Given that reduced NF-κB levels are linked to neurodegenerative diseases like Diabetic neuropathy, and NF-κB plays a critical role in neuronal survival, growth, and plasticity, this strong binding affinity suggests CDCA could negatively influence NF-κB activation, amplifying its neuroprotective effects. This

interaction could enhance neuronal survival and reduce neuroinflammation in the brain, both essential for combating the neurodegenerative processes involved in DN.

STZ causes selective necrosis of pancreatic β-cells, making it the preferred agent for inducing diabetes in animal models. Due to its low lipophilicity, STZ has specific impacts on glucose and insulin regulation, reflecting toxin-induced disruptions in β-cells cell function. First, it affects glucose metabolism, glucose-stimulated insulin release, and insulin biosynthesis, which impacts oxygen consumption and glucose oxidation. However, STZ does not immediately inhibit glucose transport or glucose phosphorylation via glucokinase. Over time, however, as cell function deteriorates, deficits in gene expression and protein synthesis further disrupt glucose transport and metabolism, highlighting the progressive nature of β-cells damage (Mahmood et al., 2009). CDCA treatment helped protect against the damage caused by $AlCl_3$ by activating CREB and boosting both BDNF and insulin signalling, which ultimately improved cognitive function. NF-κB p65 activation can trigger the release of pro-inflammatory cytokines, such as IL-1β, which contribute to memory loss brought on by neuropathic pain. (Gui et al., 2016).

Consequently, previous studies have indicated that in metabolic disturbances, microglia-mediated neuroinflammation may play a role in the neurodegenerative process by promoting cytokines release and chemokines, for example TNF-α (Biessels et al., 1998). Studies have depicted that in STZ-induced diabetic rats, diabetes triggers microglial activation and elevated GFAP expression in the hippocampus after just 2 weeks. By 6 weeks, there is a marked increase in caspase-3 activity, leading to significant cell death. The activation of microglia and astrocytes coincides with the cell damage caused by caspase-3. According to the authors, these linked occurrences exacerbate and advance brain diseases, which eventually result in cognitive and behavioural deficits. (Pratchayasakul et al., 2011). CDCA administration significantly mitigated the reduction of neurons in the hippocampal region caused by STZ (Hwang et al., 2014). Chronic hyperglycemia leads to decreased extracellular amounts of glutamate as well as GABA in the brain.

Additionally, in STZ-induced diabetic animals, the Downregulation of GABA receptors may be a factor in memory impairment and changed inhibitory function. Excitatory and inhibitory neurotransmission imbalances cause neurodegeneration and can alter cognition. (Guyot et al., 2001). Axonal swellings are a common problem in CTX neurons, so we wanted to see if CDCA treatment could help. After treating the neurons with CDCA, we noticed a clear

improvement, with a significant reduction in the number of axonal swellings (Mou et al., 2023). These findings indicate that CDCA effectively mitigates neurodegeneration while providing positive neuroprotective effects in the brain.

Consistent with previous research, our results show that STZ-treated animals exhibited significantly low levels of NF-κB and BDNF, a marker of neurotoxicity. Notably, CDCA treatment led to a marked decrease in NF-κB expression, indicating a possible neuroprotective effect. This benefit may be attributed to CDCA's ability to promote neuronal survival and modulate neuroinflammatory pathways, offering a promising therapeutic approach for DN. Thus, the results strongly suggest that CDCA has a direct neuroprotective impact, as dopaminergic neurons were preserved and motor impairments reduced. Additionally, decreased NF-κB mRNA levels and a significant rise in BDNF levels in the DN model further confirm CDCA's neuroprotective and neurotrophic potential.

Limitations

1- Our study does have some limitations. First, in DN models created with STZ, nerve damage happens through various pathways, which means there may be additional pathways involved in how CDCA provides its protective effects. However, further research is needed to understand the precise molecular mechanisms behind CDCA's neuroprotective abilities in DN.

2- It's also essential to explore how CDCA affects neuroinflammatory processes, as these play a significant role in DN's progression and impact.

3- Moreover, none of the current DN models can fully mirror the actual disease and symptoms seen in diabetic patients. The slow nerve damage occurring in diabetes is likely different from the more rapid progression of cell damage seen in the STZ-induced DN model. It is still not known how CDCA will impact DN in real clinical settings, so more research is needed to clarify its potential.

Chapter 5: SUMMARY OF RESEARCH WORK

In this study, an STZ-induced model of mice was used to explore the potent neuroprotective effects of CDCA. DN has the characteristics of the gradual damage of neurons, often caused by inflammation and oxidative stress. To better understand how CDCA might help, molecular docking studies were used to examine how CDCA interacts with BDNF and NF-κB, two key proteins involved in inflammation and neurodegeneration. The results showed strong binding between CDCA and BDNF and NF-κB, suggesting that CDCA could play a role in reducing inflammation and protecting neurons in DN. Behavioural tests, namely the hot Plate Analgesia and test of the tail flick, showed that CDCA treatment significantly improved motor and sensory function in diabetic mice.

The CDCA-treated mice performed better in several behavioural assessments, including the forced WMM test, open field test, and Y-maze, indicating improvements not only in motor coordination but also in cognitive function. These results suggest that CDCA can help address the behavioural issues commonly associated with DN. Histological analysis using H&E staining revealed less neuronal damage in key regions of the brain in the CDCA-treated mice compared to the controls. These findings indicate that CDCA may enhance neuroprotection by interacting with BDNF and help reduce inflammation by modulating NF-κB. In conclusion, this study highlights the potential of CDCA to protect against DN by regulating key inflammatory pathways like BDNF and NF-κB. While these findings are promising, extensive research is required to study the long-lasting impacts and safety caused by CDCA in clinical settings.

Chapter 6: Conclusion and Future Perspective

Diabetic neuropathy and cognitive impairment are severe complications of diabetes that greatly affect the quality of life for those affected. These conditions result from a combination of neuronal damage and chronic inflammation, making them difficult to treat. Effective management requires therapies that can both protect nerve cells from any harm and promote their recovery. This study highlights CDCA as a potential therapeutic option for these challenges. Using an STZ-induced diabetic mouse model, we found that diabetes significantly elevated NF-κB levels, a key driver of inflammation, neuronal dysfunction, and degeneration in critical regions such as the hippocampus, and cortex. CDCA treatment provides remarkable results, reducing NF-κB levels and increasing BDNF, a protein crucial for nerve repair and regeneration. These molecular improvements translated into better behavioural and cognitive outcomes in the treated mice. These findings suggest that CDCA prevents neuronal damage. This neuroprotective effect underscores the potential of CDCA as a promising treatment for diabetes-related neurological complications, providing hope for improving the lives of people.

6.1 Future Perspective

1. Future research could investigate how CDCA influences key proteins like BDNF and NF-κB. Understanding these mechanisms may help to find new ways to treat diabetic neuropathy and cognitive impairment more effectively.
2. Identifying additional biomarkers linked to CDCA's protective effects could make it easier to detect diabetic neuropathy early, monitor its progression, and evaluate the success of treatment.
3. Beyond physical symptoms, diabetic patients often struggle with cognitive and emotional challenges. Exploring how CDCA helps manage these issues could lead to more holistic treatments that improve overall quality of life.
4. The insights gained from this study could be applied to other diabetes-related neurological complications opening the door to broader therapeutic possibilities.
5. Using advanced models, like patient-derived stem cells, could help researchers better understand how CDCA works at the cellular level and pave the way for more targeted, personalized therapies.

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