Role of Nucleobindin 1 in Clozapine-Induced Neuroprotection in MPTP-Treated Mice Models

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Dedication

Dedicated to my exceptional parents.

Abstract

A possible candidate for neuroprotection has recently been identified as NUCB1, a multifunctional protein involved in calcium homeostasis and stress response. In this work, the effect of co-administering clozapine and MPTP on the survival of dopaminergic neurons was assessed. To determine the degree of neuroprotection, histological analyses and behavioral evaluations were done. The expression and distribution of NUCB1 in the central nervous system of mice with a particular focus on the brain by using RT-PCR was analyzed. Our results showed that mice treated with MPTP had considerably different levels of NUCB1 expression in the brain than mice treated with clozapine. These findings provide interesting directions for future neuroprotective approaches in the setting of MPTP-induced neuronal injury and shed light on the possible therapeutic implications of NUCB1 in reducing neurodegeneration in Parkinson's disease.

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LIST OF ABBREVIATIONS:

NUCB1	Nucleobindin-1
SLE	Systemic lupus erythematous
AD	Alzheimer's disease
APP	Amyloid Precursor Protein
PD	Parkinson's disease
SNpc	Substantia Nigra pars Compacta
DBS	Deep brain stimiulation
CBT	Cognitive behavioural therapy
MPTP	1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
MPP+	1-Methyl-4-phenylpyridinium
FST	Forced Swim Test
H&E	Haematoxylin and Eosin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
cDNA	Complementary DNA
RT-PCR	Real-time Polymerase chain reaction
qPCR	Quantitative Polymerase chain reaction
LRRK2	Leucine-rich repeat kinase 2
PINK1	PTEN-induced kinase 1
ROS	Reactive oxygen species
MAO B	Monoamine oxidase B

COMT Catechol-O-methyltransferase

GPi Globus pallidus interna

CHAPTER 1: INTRODUCTION

1.1. NUCB1

NUCB1 (Calnuc, NUCB1) is a DNA and calcium-binding eukaryotic protein with several domains.(Mikhaylina *et al.*, 2023). With values of 3.8µg calnuc/mg Golgi protein, NUCB1 is one of the prevalent Golgi proteins. The 461 amino acid human calnuc protein has a signal peptide at the N-terminus. It was initially discovered to share about 30% of its sequence with calreticulin, another Ca21-binding protein. It is a multidomain protein that has a leucine zipper domain towards the C-terminus as well as an N-terminal signal peptide sequence, a basic DNA-binding area, an acidic region in between the two EF-hand motifs, and a basic DNA-binding region (Aradhyam *et al.*, 2010). It was first discovered to be a soluble factor released by KML1-7 cells derived from lupus-prone MRL/l mice and it increases MRL1 mice's susceptibility to develop autoimmune disorders (Yoshiyuki Kanai and Sei-ichi Tanuma, 1992). Additionally, regular injections of NUCB1 into animals predisposed to SLE increased the generation of rheumatoid factor, anti-U1RNP antibodies, and anti-dsDNA autoantibodies. As a result, one might speculate that NUCB1 plays a role in autoimmune processes (Mikhaylina *et al.*, 2023). Furthermore, it enhanced growth activity, which led to the hypothesis that it (a 55-KDa protein) might function as a growth factor (Aradhyam *et al.*, 2010). NUCB1 is a protein that has been linked to numerous biological processes and is widely expressed in many tissues, including the brain (Tulke *et al.*, 2016). Several studies have documented multiple interaction partners for NUCB1 as well as varied regions of localization within the cell. In addition to being engaged in stress response and trafficking, it interacts with significant molecules like DNA, G protein, COX, and APP among others. The vast possibilities (of the numerous activities this protein might play) suggest a promising future in physiology and medicine. Additionally, preliminary research raises the possibility that NUCB1 may play a role in various human disorders (Chen *et al.*, 2007a). NUCB1 is expressed at very high levels in the cytoplasm and Golgi apparatus of cells (Weiss *et al.*, 2001). It has been demonstrated that NUCB1 interacts with G proteins and cyclooxygenases and is essential for maintaining Ca2+ homeostasis (Ramesh, Mohan and Unniappan, 2015).

1.1.1. Role of NUCB1 in human diseases

It is hypothesized that NUCB1 plays an important role in preserving the physical balance of an organism given its numerous interacting partners and different localizations. Thus, NUCB1 promises to be a crucial core molecule, and any deviation from normal in the protein's folding or function will reveal us to a variety of disease pathogenesis (Aradhyam *et al.*, 2010). In contrast to merely 10% of individuals with cancer not associated with lymph node metastasis, it was found that 56% of individuals with lymph-node metastatic tumors expressed NUCB1. Although more investigation is necessary to fully understand the NUCB1's fundamental biochemistry in this illness, it may serve as a possible marker for identifying stomach tumors linked to lymph node metastases (Chen *et al.*, 2007b). Interestingly, patients with "non-Hodgkin's lymphoma" have higher expression levels of NUCB1. NUCB1 may be able to serve as a diagnostic tool for the early detection of the malignancy stage of non-Hodgkin's lymphoma as more information about the biological role it plays in this disease comes to light (Kubota *et al.*, 1998). The APP and calnuc actively interact, and calnuc binds to the amyloid precursor protein's C-terminal region in a Ca21-sensitive manner. Additionally, Alzheimer's patients have far lower amounts of NUCB1 in their brains than non-Alzheimer patients who are age-matched. This demonstrates the significant impact that NUCB1 may have in controlling the beginning of AD by regulating APP quantity and plaque development (Aradhyam *et al.*, 2010). Studying the expression and function of NUCB1 in the context of PD may provide light on the pathophysiology of the condition and suggest new treatment targets (Bonito-Oliva *et al.*, 2017).

1.2. Parkinson's disease

1.2.1. Background

PD is a severe kind of age-related neurodegenerative disease in which the loss of nigrostriatal dopaminergic neurons occurs (Dekker, 2003). PD is thought to primarily affect the dopamine (DA) neurons of the SNpc (von Campenhausen *et al.*, 2005). This chronic, progressive illness, was first identified by James Parkinson in 1817 (Fahn, 2015). Millions of people worldwide are affected by PD and it is one of the most common and severe neurodegenerative conditions. It is the most prevalent neurological condition affecting millions of individuals globally (Pringsheim *et al.*, 2014). The disorder is progressive, complicated, and diverse and has a wide range of

symptoms. Early-onset PD refers to patients who were 20 to 50 years old when they received their PD diagnosis and patients are classified as having late-onset PD after 50 years (Berg *et al.*, 2014). Dopamine, a crucial neurotransmitter required for regulating numerous cognitive and affective functions as well as movement coordination, is reduced as a result of the pathogenesis of PD, marked by a selective loss of dopaminergic neurons in the substantia nigra area of the brain (Savitt, Dawson and Dawson, 2006a). A "mitochondrial senescence disease" is another term for PD from a pathological perspective (Prasad and Hung, 2020). The actual cause of PD, which involves intricate connections between genetic predisposition, environmental variables, and cellular dysfunction, is still unknown despite intensive research efforts (Gasser, Hardy and Mizuno, 2011). PD affects one million persons in Pakistan, and it is predicted that this number will rise to 1,200,000 by the year 2030. (Tufail, 2020).

PD has no known cure, and only a few of its symptoms may usually be controlled with drugs, surgical procedures, and other treatments. Unfortunately, these treatment options only significantly reduce early symptoms for a short time and do not stop the disease from progressing. (Savitt, Dawson and Dawson, 2006b). As a result of variable and non-specific treatment procedures, the therapeutic approaches for PD are still a challenge.

As the population ages, the prevalence of PD is increasing, indicating the critical need for innovative therapies and a deeper comprehension of the underlying mechanisms causing this crippling condition.

1.2.2. Clinical presentation and symptoms

1.2.2.1. Motor and Non-motor symptoms

PD is characterized by a variety of movement symptoms. These motor symptoms, which are a defining feature of PD, can have a serious influence on a person's mobility and ability to carry out daily tasks (Gökçal *et al.*, 2017). PD's main motor symptoms include the following:

1. The most recognizable sign of PD is tremor, which is frequently described as a rhythmic shaking or trembling of a bodily part (typically a hand or fingers) (Thenganatt and Louis, 2012).

2. Bradykinesia is the medical term for sluggish movement. PD patients frequently struggle to start and carry out voluntary motions. Simple actions like walking or buttoning a shirt might become laborious and slow (Moustafa *et al.*, 2016).
3. Rigidity is characterized by stiffness or an increase in muscle tone. Postural instability is the ability to lose balance and trip over, especially when turning or getting out of a sitting position. It is a typical aspect of severe PD and raises the chance of falling (Goetz *et al.*, 2005).
4. Shorter steps, a smaller arm swing, and shuffling feet are typical characteristics of the altered gait that people with PD frequently develop (Chaudhuri *et al.*, 2010).

PD non-motor symptoms include a wide variety of symptoms that do not primarily involve movement but that can have a major effect on a person's quality of life. These non-motor symptoms frequently begin or coexist with motor symptoms, and they can occasionally be more incapacitating than the typical motor symptoms of PD (Goetz, 2011). Here are a few typical PD non-motor symptoms:

1. Depression and anxiety are typical mood problems in PD. These symptoms may be influenced by alterations in brain chemistry and the emotional toll of living with a chronic illness (Grinberg *et al.*, 2010).
2. Some persons with PD develop cognitive impairment, which can range from moderate cognitive impairment to more severe dementia. Decision-making, memory, and attention may all be impacted by this (Tibar *et al.*, 2018).
3. PD can cause sleep patterns to be disturbed. Insomnia, numerous nighttime awakenings, or excessive daytime sleepiness are common symptoms of PD (Arnulf, Leu and Oudiette, 2008).
4. Hallucinations and delusions are hallmarks of psychosis, which can happen in people with PD. It frequently has to do with adverse drug reactions or alterations in brain chemistry (Arnulf *et al.*, 2000).
5. Different types of pain, such as musculoskeletal pain, pain associated with dystonia, and central pain syndromes, can be brought on by PD (Müller *et al.*, 2013).

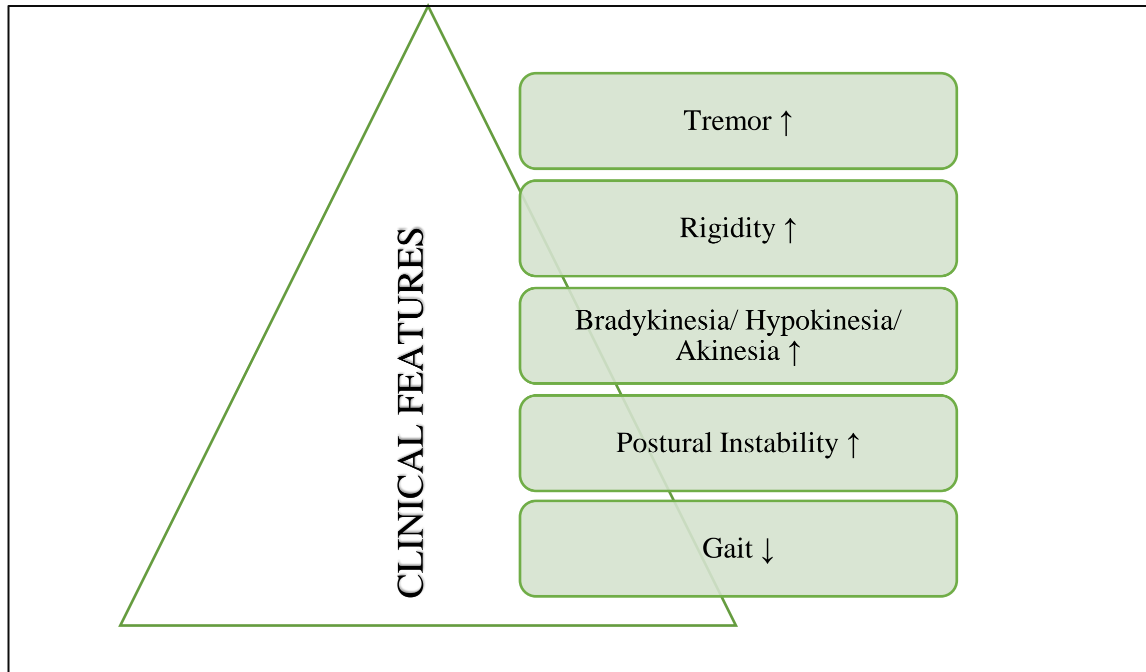

Figure 1: Symptoms of PD. The figure illustrates the clinical features exhibited by patients suffering from PD.

1.1.3. Pathophysiology

1.1.3.1. Dopaminergic system

A key component of PD pathogenesis is the dopaminergic system. This system, which is comprised of neurons that interact between brain regions via the neurotransmitter dopamine, is essential for coordinating movement and a range of cognitive activities (Wakabayashi *et al.*, 2013). The dopaminergic system undergoes severe damage in PD, which results in the disease's hallmark motor symptoms and other signs. The depigmentation of the SNpc and loss of dopaminergic neurons are the primary pathogenic aspects of PD (Balestrino and Schapira, 2020a)

1.1.3.2. Lewy bodies and Alpha-synuclein aggregation

In the pathogenesis of PD, Lewy bodies are very important. Their primary constituents are synuclein and ubiquitin (Balestrino and Schapira, 2020b). Alpha-synuclein is the main protein found in Lewy bodies (Spillantini *et al.*, 1997). It experiences abnormal folding and aggregation in PD, which causes insoluble protein clumps to accumulate inside neurons. These aggregates

slowly grow, inhibiting cellular activity (Prasad and Hung, 2020). The aggregation also causes oxidative stress, inflammation, and disruption of normal protein trafficking, all of which contribute to cellular malfunction and ultimately lead to cell death (Balestrino and Schapira, 2020a).

1.1.3.3. Neuroinflammation

One of the most significant processes connected to the pathophysiology of PD is neuroinflammation (Prasad and Hung, 2020). The brain's resident immune cells, known as microglia, are in charge of immunological surveillance as well as pathogen and injury defense (Gelders, Baekelandt and Van der Perren, 2018). In PD, abnormal protein clumps, such as alpha-synuclein, which form Lewy bodies within neurons, cause microglia to become activated. Activated microglia release cytokines, which are pro-inflammatory chemicals. The presence of these inflammatory chemicals can cause additional harm to neurons by causing local inflammation. The production of ROS and other harmful chemicals is associated with neuroinflammation (Wang, Liu and Zhou, 2015). These oxidative stress agents can harm proteins and lipids in cells, which will cause additional malfunction and eventual cell death (Prasad and Hung, 2020).

1.1.3.4. Mitochondrial dysfunction

The cellular organelles known as mitochondria are in charge of producing energy. In dopaminergic neurons, in particular, dysfunction in the mitochondria has been linked to the pathophysiology of PD (Lin *et al.*, 2020). Defects in the mitochondria can increase oxidative stress and disrupt cellular energy metabolism, which can harm or kill neurons (Prasad and Hung, 2020). The fact that several known genes causing familial PD impact the homeostasis of mitochondria is another significant indicator of the function of mitochondria in the development of PD (Park, Davis and Sue, 2018).

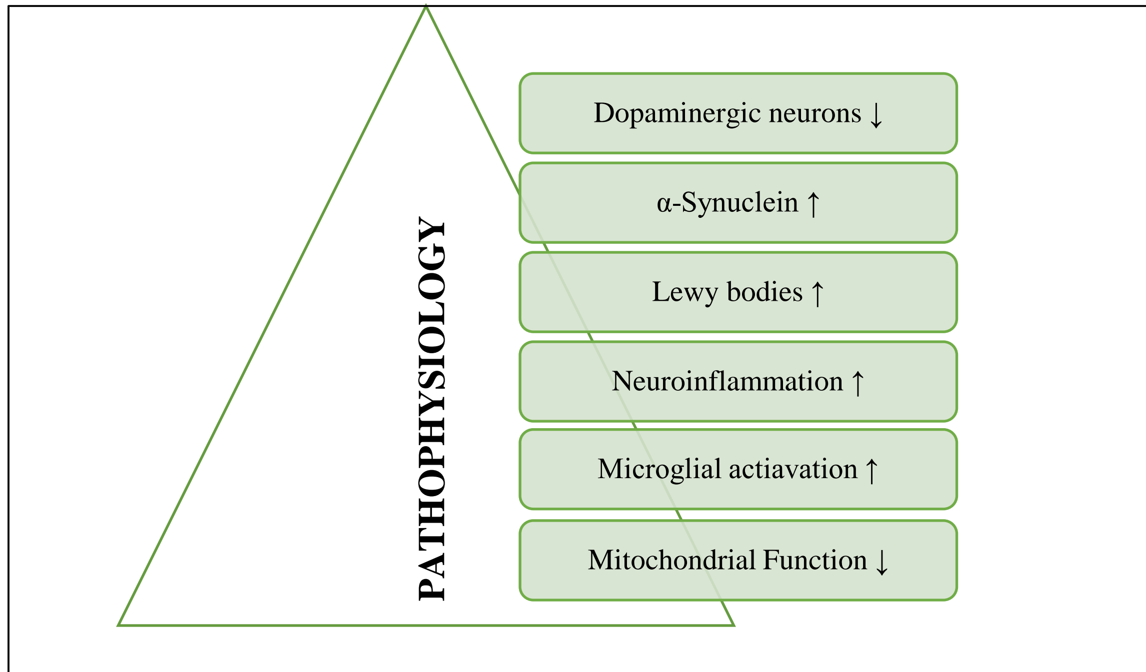

Figure 2: Pathophysiology of PD. The figure illustrates the neurodegenerative changes leading to the Pathogenesis of PD.

1.1.4. Etiology

PD has an etiology that involves a complex interplay of genetic, environmental, and cellular variables. Even though the precise cause of PD is still unknown, research has shed important light on the numerous elements that contribute to the onset and course of the illness. The following are the main factors in PD etiology:

1. **Genetic factors:** PD comes in both familial and sporadic forms, and both are influenced by genetic susceptibility. About 5 to 10% of PD cases are thought to be familial, the result of particular genetic mutations that are passed down from one generation to the next. Numerous genes, including SNCA, LRRK2, PARKIN, PINK1, and DJ-1, as well as mutations in these genes have been linked to familial PD. Genetic risk factors, such as polymorphisms in particular genes, may raise the probability of developing the disease in sporadic cases, although they do not guarantee it (Savitt, Dawson and Dawson, 2006b).
2. **Environmental factors:** Exposure to particular environmental factors has been linked to an increased risk of PD. Paraquat and rotenone are two pesticides and herbicides that have been linked to an increased risk of developing PD (Warner and Schapira, 2003)

Heavy metals, industrial pollutants, and contamination in well water are some additional potential environmental risk factors

3. **Aging:** The likelihood of developing PD rises with age, and the aging-related loss in cellular repair systems may be a factor in the buildup of cellular damage (Savitt, Dawson and Dawson, 2006a).

1.1.5. Diagnosis

As there is no conclusive test to establish PD, the diagnosis is mostly dependent on clinical criteria. The diagnosis is frequently reached following a thorough evaluation of the patient's medical history, a physical examination, and confirmation of the presence of certain clinical symptoms (Rao, Hofmann and Shakil, 2006). The most used diagnostic standard is the "UK Brain Bank Criteria" (Postuma *et al.*, 2015). These requirements include the following characteristics:

- Bradykinesia is a defining feature of PD and refers to the slowness of movement. Patients with PD frequently have trouble starting their motions, and their speed and amplitude may gradually decrease.(Postuma *et al.*, 2015)
- A PD-defining symptom is a resting tremor. Though it can affect other body parts, it most frequently affects the hands and usually happens while you're at rest (Postuma *et al.*, 2015)
- Rigidity is described as stiffness or resistance to the limbs moving passively. Due to the typical "ratchety" sensation experienced when moving the limb, it is a prevalent PD trait and is frequently referred to as "cogwheel" stiffness (Li *et al.*, 2017).

In addition to these motor symptoms, the following characteristics may help with the PD diagnosis:

- PD frequently exhibits asymmetrical motor symptoms, which means that one side of the body is more severely afflicted than the other (Rao, Hofmann and Shakil, 2006)
- Atypical features, such as strong cerebellar symptoms, early and severe autonomic dysfunction, or early and persistent dementia, would lead one to suspect an atypical Parkinsonism condition rather than a typical PD (Postuma *et al.*, 2015).

1.1.6. Current management strategies for PD

A multidisciplinary approach to treating PD is to control the disease's motor symptoms, enhance quality of life, and deal with non-motor symptoms. The following are some of the current PD management techniques:

1.1.6.1. Medications

Table 1: Approved medications for PD. The table shows the approved medications, its adverse effects, and the indications of the disease (Goetz *et al.*, 2005).

	MEDICATIONS	ADVERSE EFFECTS	INDICATIONS
1.	**LEVODOPA/ CARBIDOPA**	Hallucinations, feeling dizzy, fatigue, weakness, hypertension	The best drug for treating PD symptoms is levodopa, which is still used as the major therapy.
2.	**DOPAMINE AGONISTS**	Nausea, dizziness, hypotension, fibrosis of cardiac valves	Useful as a complement therapy for people receiving levodopa and for the initial treatment of Parkinsonism
3.	**MAO-B INHIBITORS** Selegiline	Insomnia, nausea, weakness	Beneficial for the mild to moderate regulation of PD symptoms and as an additional treatment for those with motor fluctuations
	Rasagiline	Dry mouth, low blood pressure, and loss of weight	
4.	**COMT-INHIBITORS** Entacapone	diarrhea; increases the side effects of levodopa; and brightly coloured urine	Useful for minimizing the "wearing-off" impact on levodopa patients' motor seizures
	Tolcapone		

1.1.6.2. Deep brain stimulations

DBS is a surgical process in which sensors are implanted in specific regions of the brain, such as the GPi or subthalamic nucleus. The electrical impulses that these devices transmit modify abnormal brain activity and decrease motor problems. Patients who are experiencing motor

difficulties and who respond to levodopa are typically candidates for DBS (Rao, Hofmann and Shakil, 2006)

1.1.6.3. Non-pharmacologic interventions

Non-pharmacologic treatments help patients retain their overall well-being even while they do not effect on the key symptoms of PD. Counseling and CBT are often used to treat non-motor symptoms including anxiety and depression. Exercises for flexibility, strength, and balance may enhance gait speed, balance, and participation in daily activities. Vocal training specifically can be used to treat voice and speech issues (Rao, Hofmann and Shakil, 2006).

1.2. Neurotoxins used to induce PD invivo

To investigate the pathogenesis of PD and test possible treatments, several neurotoxins are employed to cause the disease in animal models. These neurotoxins specifically target and damage dopaminergic neurons, resulting in motor and non-motor symptoms similar to those experienced by individuals with PD (Prasad and Hung, 2020). Common neurotoxins used to cause PD-like disease in animal models include:

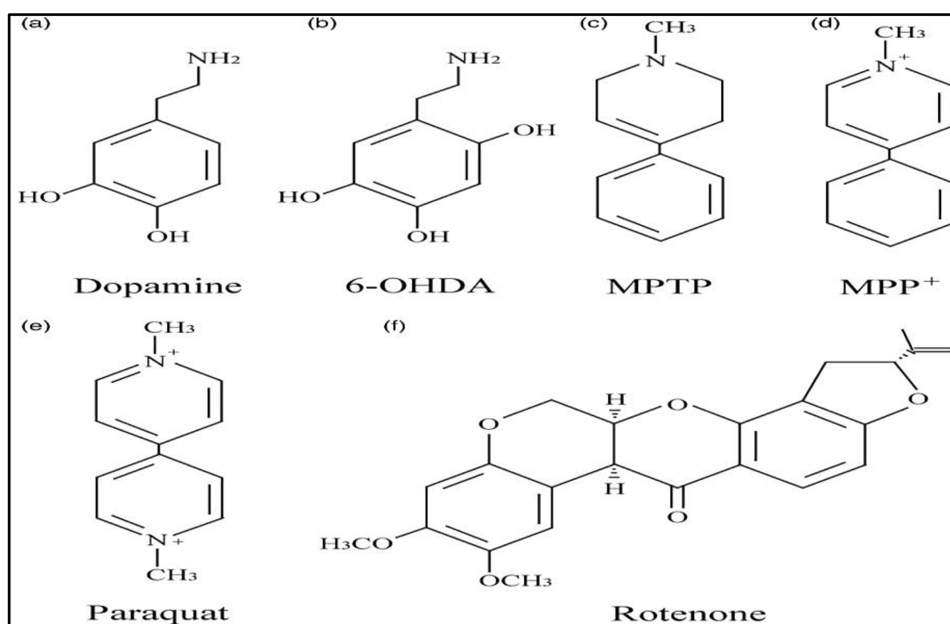

Figure 3: Neurotoxins with specific chemical structures that cause PD in models of animals. The neurotoxins to cause PD, including dopamine, 6-hydroxydopamine (6-OHDA), MPTP, MPP+, Paraquat, and Rotenone,.(Zeng, Geng and Jia, 2018).

1.2.1. 1-Methyl-4-phenyl-1, 2, 3, 6-tetra hydropyridine (MPTP):

Accidentally, it was found that the strong neurotoxic MPTP led to Parkinsonism in drug addicts. After using MPTP on their own, seven young people developed severe PD, in 1972. According to Ballard et al. (1985), MPTP in synthetic heroin induces the dopaminergic neurons in the nigrostriatal pathway to be specifically damaged, which causes PD symptoms in humans and other animals (Prasad and Hung, 2020). It causes the degradation of dopaminergic neurons in the brain by being transformed into the poisonous chemical (MPP+). The mechanism of action of MPTP is shown in Figure 4.

1.2.2. MPTP mechanism of action in the progression of PD

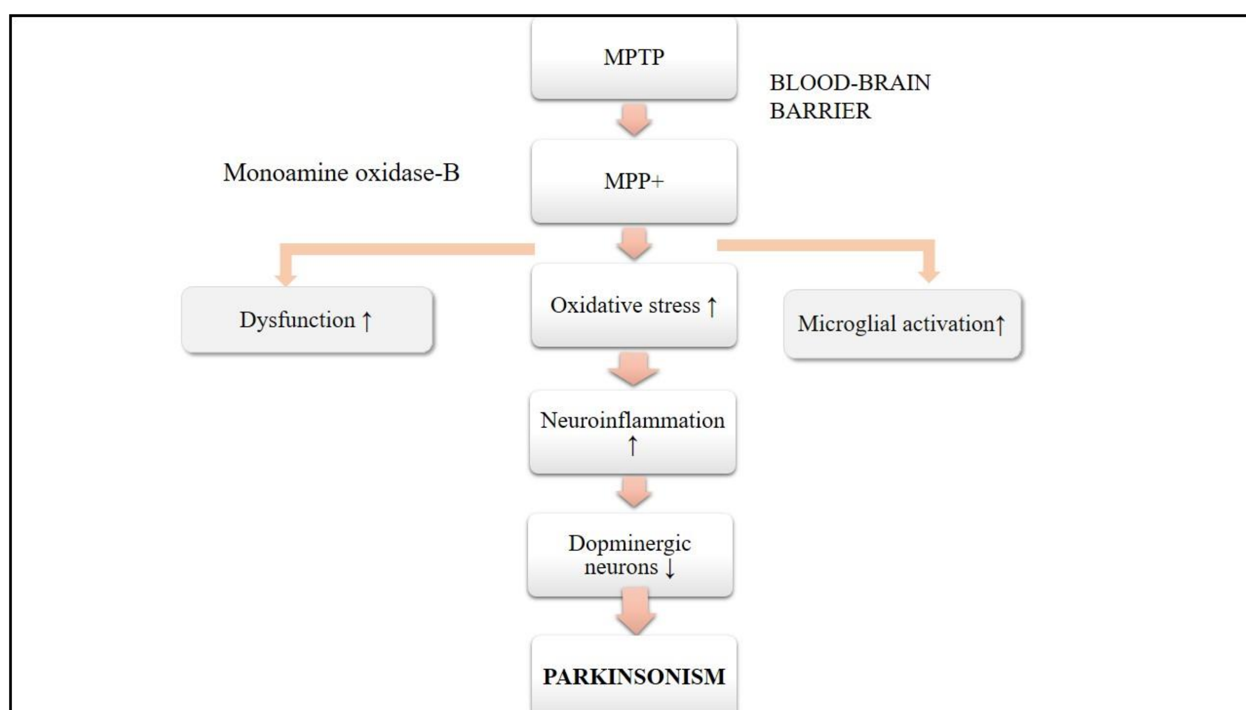

Figure 4: Mechanism of action of MPTP and MPP+ in the progression of PD.

The figure shows that the poisonous metabolite of MPP+, is quickly produced by monoamine oxidase-B from the lipophilic molecule MPTP, which can cross the blood-brain barrier and enter the brain. The mitochondrial electron transport chain's complex I is then selectively inhibited by MPP+ after being selectively taken up by dopaminergic neurons via dopamine transporters. This results in PD.

1.3. Aims and Objectives

1. The primary aim is the establishment of MPTP-induced PD model.
2. The second objective is to assess the interactions between NUCB1 and clozapine through in silico analysis.
3. The third objective of this study is the evaluation of the effects of clozapine using behavioral tests.
4. The fourth objective is the assessment of histological and morphological changes in the brain regions through H&E staining.
5. The ultimate aim is to consolidate the findings by quantifying the levels of NUCB1 through RT-PCR and provide evaluations regarding the role of NUCB1 in mediating the neuroprotective effects of clozapine in the mice models of PD.

CHAPTER 2: MATERIALS AND METHODOLOGY

This chapter discusses the complete materials and methodology used in the entire course of the research study.

2.1. Animals

Male BALB/c adult mice (age 7-8 weeks) were used in this study. The mice were kept in plastic cages with open access to water and rodent food until the time of sacrifice. Prior to starting the experimentation, mice were acclimated to the laboratory environment. The mice were assigned to three different experimental groups: Control group, Disease group (MPTP-treated), and Treatment group (MPTP-treated with clozapine treatment).

2.2. Experimental design

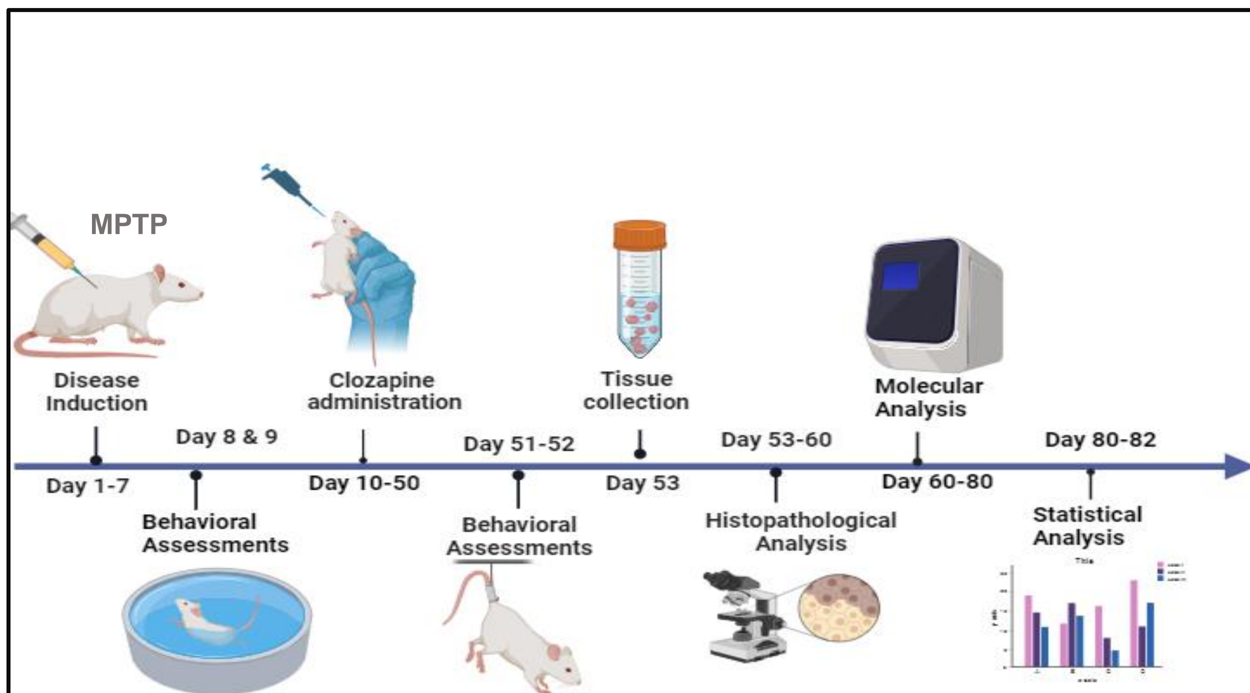

Figure 5: Experimental design. The figure illustrates the timeline and experimental design throughout the research study.

2.3. Ethical considerations

The project was reviewed by the NUST Institutional Review Board (IRB) prior to starting the experimentation and received approval. The study followed all ethical criteria and was administered by the Institutional Animal Care guidelines.

2.4. Grouping of animals for treatment and drug schedule

The following medications were utilized in the present study: MPTP and Clozapine.

Clozapine has been suspended in distilled water and given orally, whereas MPTP was dissolved in distilled water and administered intraperitoneally. The present study used a total of three groups, each with five animals as shown in Figure 6.

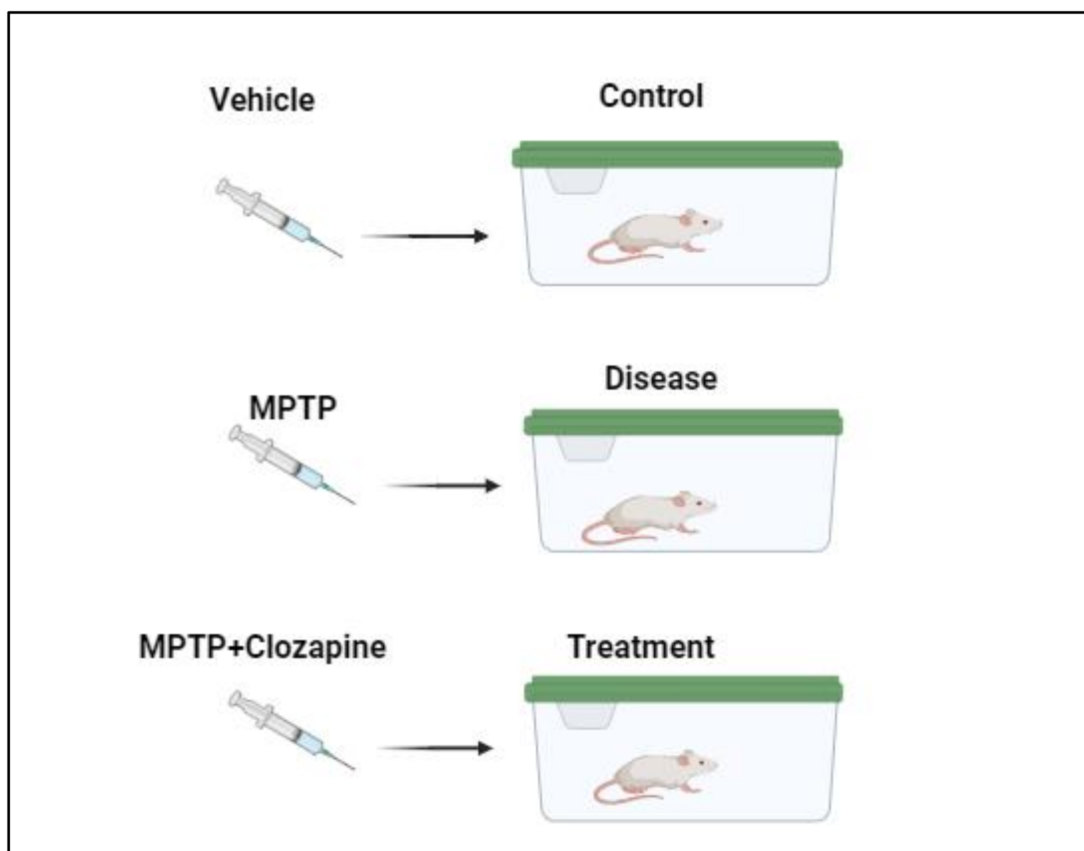

Figure 6: Group design for drug and treatment administration. The figure shows the corresponding groups with their specific drug and treatment.

2.5. *In silico* analysis

Before staring the experimentation *in silico* analysis was carried out using different softwares and computational methods to gain insights and predict the hypothesis before conducting them physically. For that the three-dimensional structure of nucleobindin-1 (PDB: 1SNL) was downloaded from RCSB Protein Data Bank. The chemical structure of clozapine (PubChem CID: 135398737) was obtained from the PubChem compound database. These structures were then cleaned by using the software Discovery Studio Visualizer 3.0, respectively. The docking was then carried out using the software of PyRx to comprehend the structural basis of nucleobindin-1 and clozapine selectivity and to calculate the binding affinity of the nucleobindin-1 (target) with clozapine (ligand). The nucleobindin-1 and clozapine interactions emphasizing key interaction patterns were then visualized using the software of Discovery Studio Visualizer 3.0.

2.6. Induction of the PD model

MPTP (Catalogue #23007-85-4, Sigma Macklin, China) was used to create PD-like pathology. The mice were acclimatized one week before receiving the MPTP injection. One day before administering MPTP, the mice were weighed, grouped, and numbered. For three days, mice in the disease and treatment groups received intraperitoneal injections of MPTP as shown in Figure 7.

Figure 7: Disease induction. The figure shows the injections of MPTP given intraperitoneally to the mice for disease induction and causing neurodegeneration.

2.6.1. MPTP injection (Dose preparation)

The total volume of MPTP solution needed for the experiment was calculated and this was derived by measuring the weights (in grams) of all the mice that will get MPTP injections. An injection for approximately 10µl per 1 gram of body weight (i.e. 0.01ml for 1 gram) was measured and injected accordingly.

2.7. Behavioral assessments

Behavioral tests were conducted following the introduction of PD in animal models assessing symptom development and evaluating treatment efficacy. After the MPTP treatment phase, behavioral evaluations to assess motor deficits were carried out. To assess the motor functions two behavior tests were carried out such as tail suspension and forced swim test:

2.7.1. Forced swim test

The FST was potentially used to assess behavioral changes in animal models treated with MPTP to elicit symptoms resembling those of PD. To assess depressive-like behavior and motor functions, the forced swim test was applied (Taylor, Greene and Miller, 2010). Mice were kept in a water-filled cylinder at 24°C as shown in Figure 8. The water level was maintained in such a way that the mice were able to maintain their heads above water and their feet and tail would not touch the bottom of the cylinder. A video camera was used to capture the test, and the time it took to stop and immobility duration were calculated. A six-minute session was analyzed and videotaped (Rial *et al.*, 2014). After the test phase, the behavioral parameters were measured by analyzing the recorded videos.

The total time that the mice exhibited little to no movement, only making the small movements required to maintain their head above water was calculated. An increase in the period of immobility and a decrease in the time it takes to get to immobility were used to characterize depressive-like states (Campos *et al.*, 2013). To examine the behavioral variations between the MPTP-treated and control groups, a control group of mice who were not given the MPTP treatment were included and the behavior differences were compared.

Figure 8: FST. The figure shows the FST where a mouse is kept in a water-filled cylinder for 6 minutes to assess the behavior changes.

2.7.2. Tail suspension test

The tail suspension test was done to evaluate motor functions and access depressive-like behavior in the mice treated with MPTP. The tail suspension test was conducted by suspending each MPTP-treated mouse individually by its tail which was attached by an adhesive tape to a horizontal stand bar as shown in Figure 9. The basic idea was the observation of the mice who would become immobile when they experience the short-term, inescapable stress of being suspended by their tails. The length of time that the tail suspension test caused immobility overall was measured (Steru *et al.*, 1985). A six-minute session was analyzed and videotaped. After the test phase, the behavioral parameters were measured by analyzing the recorded videos. The total duration during which the mice treated with MPTP remained immobile or motionless while suspended was calculated (Rial *et al.*, 2014). To examine the behavioral variations between the MPTP-treated and control groups, a control group of mice who were not given the MPTP treatment were included and the behavior differences were compared.

Figure 9: Tail Suspension Test. The figure shows the tail suspension test where a mouse is hanged by tail to a horizontal stand bar using adhesive tape

2.8. Clozapine treatment protocol

A Clozapine treatment regimen was designed for MPTP-treated mice models taking mice weights and desired dosage into consideration. The dosage was administered orally once in a day for a period of 40 days as shown in Figure 10. The clozapine treatment regimen for MPTP-treated mice was based on their weights keeping a standard dosage of 2.5mg/kg.

Figure 10: Treatment with clozapine. The figure shows the oral administration of clozapine to the MPTP-treated mice.

2.9. Histopathological analysis

Histopathological analysis was done to examine the tissues at a microscopic level and study the morphological changes and histological patterns in the cellular structures of the brain.

2.9.1. Tissue fixation and dissection

The mice were euthanized under deep chloroform inhalation. For histopathological analysis, the transcardial perfusion was performed by using the fixative solution of 4% paraformaldehyde flushing through the circulatory system. By flushing through the bloodstream and displacing blood, the fixative ensured complete tissue fixation. After that the mice were carefully decapitated. The skull was then removed using scissors and a scalpel along the midline to expose the brain. By using little forceps the brain from the skull was removed gently. To remove extra fixative and blood from the sample, the tissues were then washed with PBS. The brain tissue was then carefully immersed in the fixative solution of 4% paraformaldehyde.

2.9.2. H&E staining

For H&E staining the brain was then dehydrated by immersing the perfusion-fixed brain in ethanol at progressively higher concentration of 100%. Then transferred to a clearing agent of xylene to

remove ethanol. Then thin sections of the tissue were cut down. The sections were then stained with H&E dye to visualize the cellular structures.

2.9.3. Microscopic examination

The stained sections of the brain were then examined under the light microscope and the tissue morphology, cell count, and cellular patterns were analyzed. The photomicrographs of the cerebellum and spinal cord were taken to analyze the changes between the three groups and to understand the effects of the treatment and disease processes.

2.10. Gene expression analysis

2.10.1. Reverse transcription Polymerase Chain Reaction

2.10.1.1. Dissection

The mice were deeply anesthetized under chloroform inhalation. After that the mice were carefully decapitated by using sharp scissors. The skull was then cut using fine scissors and a scalpel along the midline to expose the brain. By using little forceps the brain from the skull was removed gently snap-frozen on dry ice, and kept at -80 °C for later processing.

2.10.1.2. RNA extraction

The total RNA from the tissues was isolated using the TRIzol isolation reagent (Catalog No: FTR-100, Fine Biotech Life Sciences, China).

In this first step, 1000µl trizol reagent was added to the sample and then homogenized followed by centrifugation at 12000rpm for 10 minutes at 4˚C. After centrifugation, the supernatant was transferred to a new tube and 200µl of chloroform was added to the sample. After that, the tube was vigorously shaken for 30 seconds and then centrifuged at 12000rpm for 10 minutes at 4˚C. Then carefully transfer the aqueous phase (containing RNA) to a new tube and add 500µl of isopropanol to it, mix well and, incubate at room temperature for 10 minutes. Then again centrifugation was done at 12000rpm for 10 minutes at 4˚C which was followed by removing the supernatant. The next step was to wash the pellet with 75% ethanol and then centrifuge at 12000rpm for 2 minutes at 4˚C. The ethanol was discarded carefully and then the pellet was air dried for 5-10 minutes followed by resuspending the pellet with 20-50µl nuclease-free water.

2.10.1.3. Assessment of RNA quality and quantity

The extracted RNA's quality and quantity were assessed using Colibri NanoDrop (TitertekBerthold, Germany).

2.10.1.4. cDNA synthesis (Reverse transcription)

The RNA extraction was then followed by cDNA transcription using RevertAid Reverse Transcriptase (Catalog #: EP0441, Thermo Fisher Scientific, Lithuania). The reaction mixture was then prepared including the reaction buffer, dNTPs, reverse transcriptase, oligodts, diathiothreitol (DTT) and, RNA sample. The thermal cycler was then used to incubate the reaction mix under specified conditions of 42˚C for 60 minutes.

2.11. Polymerase Chain Reaction (PCR)

2.11.1. Primer designing

The primers were selected from the published literature. Then primer BLAST was done in NCBI (National Center for Biotechnology and Information) to verify the specificity and accuracy of the selected primer with the target shown in Figure 11 and 12 before using them for Polymerase Chain Reaction (PCR). The primers had the calculated annealing temperature of 42.5. The primers were ordered from Bionics (Islamabad, Pakistan).

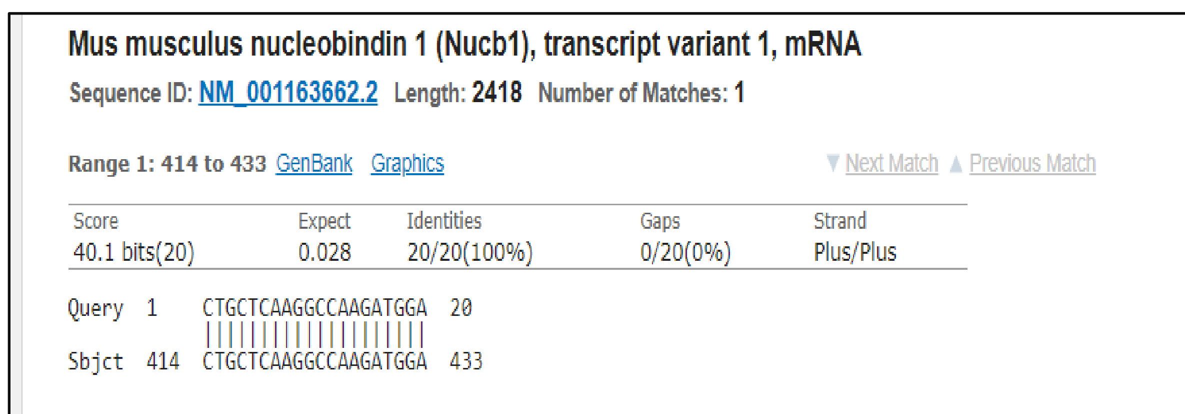

Figure 11: BLAST FOR NUCB1 FORWARD PRIMER. The details of the primer BLAST were done in NCBI to verify the specificity of the forward primer.

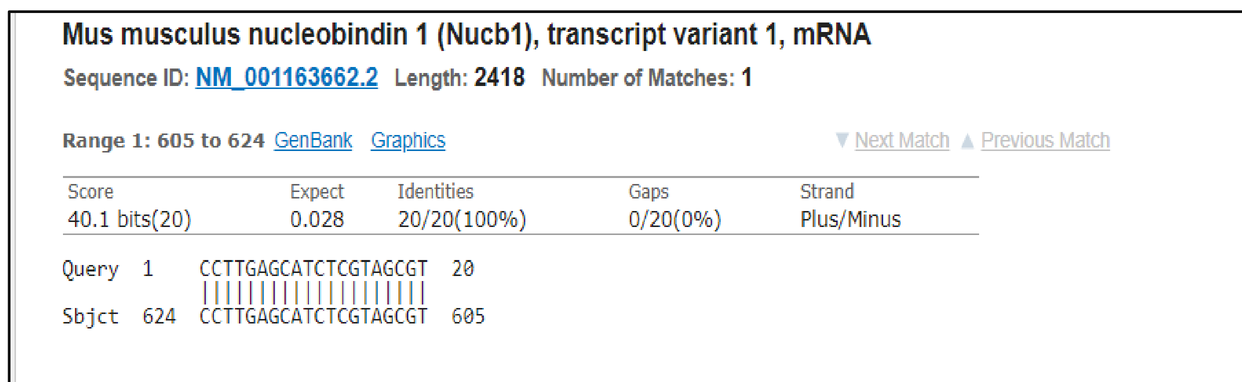

Figure 12: BLAST FOR NUCB1 REVERSE PRIMER. The details of the primer BLAST were done in NCBI to verify the specificity of the reverse primer.

Table 2: Primer characteristics. The table shows the primers used with their specific length, sequence and optimized annealing temperature.

GENE	DIRECTION	LENGTH	SEQUENCE (5' to 3')	ANNEALING TEMP (°C)
Beta-actin	Forward	20	GCCTTCCTTCTTG GGTATGG	61.5
Beta-actin	Reverse	19	CAGCTCAGTAAC AGTCCGC	
NUCB1	Forward	20	CTGCTCAAGGCC AAGATGGA	
NUCB1	Reverse	20	CCTTGAGCATCT CGTAGCGT	42.5

2.11.2. Gradient PCR

Using gradient PCR, a sample was prepared for primer optimization to determine the annealing temperature. Gradient PCR profile is as follows. A 3-minute initial denaturation step at 94 °C, followed by 35 cycles at 94 °C for 30 seconds, and an annealing step at temperatures between 42.5 and

52.5 °C for 30 seconds. Gradient temperatures were then followed by an extension step lasting 45 seconds at 72 °C and a final extension lasting 7 minutes at 72 °C. After the PCR, the resultant product was analyzed for bands on a gel electrophoresis.

Table 3: Gradient temperatures. The table displays the range of annealing temperatures used for gradient PCR.

GRADIENT TEMPERATURES					
42.5˚C	44.5˚C	46.5˚C	48.5˚C	50.5˚C	52.5˚C

2.11.2.1. Reaction mixture:

The PCR tube was filled to a total capacity of 25µl with 12.5µl of PCR master mix (Wizbio Solutions, catalog no: W1401-2, South Korea), 8.5µl of Nuclease-free water, 1µl of forward primer, 1µl of reverse primer, and 2µl of cDNA template.

Table 4: List of PCR ingredients. The table shows the components along with their quantities to make 25µl PCR mix.

	COMPONENTS	QUANTITY (µl)
1.	PCR Master mix	12.5
2.	Nuclease free water	8.5
3.	Forward primer	1.0
4.	Reverse primer	1.0
5.	cDNA template	2.0

2.11.3. Agarose gel electrophoresis

To validate whether annealing had occurred at the appropriate temperatures or not, gel electrophoresis was performed by using 2% of agarose (Sigma Aldrich, catalog no: 39346, USA) and 10X TBE buffer (catalog no: T1051, Solarbio, China). The bands' locations were compared to the DNA ladder (ranging from 100 to 1500bp) to determine whether annealing had occurred or not. The gels were then analyzed using a Benchtop 2UV transilluminator (LM-20 | P/N 95044902, UVP Co., USA).

2.11.4. Real-time PCR

Real-TimePCR also known as the qPCR was used to measure the NUCB1 expression levels in brain tissues on a real-time PCR detection system (Biorad) using NUCB1 primers (sense 5′- ACT ACA TCA GTA ACT CAG CAC AG-3′ and anti-sense 5′- ACA AGT GTC CGT TTC AAA TCT TG-3′) , by using the cycling parameters described in fig.12. Mouse beta actin (control) qPCR was also conducted employing the primers (sense 5′- GCC TTC CTT GGG TAT GG-3′ and sense 5′- CAG CTC AGT AAC AGT CCG C -3′) Denaturation at 94 °C for 30 seconds, annealing at 61.5 °C for 30 seconds, and elongation at 72 °C for 30 seconds. 35 cycles. The reaction mixture was made using WizPure™ qPCR Master (SYBR) (Catalogue No. W1711, Wizbio, Korea). The PCR reaction mix consists of a cDNA template, nuclease-free water, forward primer, reverse primer, and SYBR green master mix making a total of 20µl of the reaction mixture as described in table 5. To assess the quality of the PCR product, amplification curves, and agarose gel electrophoresis were employed. The values obtained from these trials were analyzed about gene expression using their ΔCt values after all values were normalized to those obtained for ß-actin.

Table 5: qPCR master mix preparation. The table shows the components of qPCR master mix preparation along with their quantities to make 20µl of PCR mix.

	COMPONENTS	**QUANTITY(µl)**
1.	cDNA template	1.0
2.	Forward primer	1.0
3.	Reverse primer	1.0
4.	SYBR green master mix	4.0
5.	Nuclease free water	13.0
	Total reaction volume	20µl

2.11.4.1. Cycling parameters for Real-time PCR

Figure 13 displays the Real-time PCR cycling parameters. PCR circumstances: 35 cycles of denaturation at 94 °C (3 min), annealing at 42.5 °C (30 s), and elongation at 72 °C (45 s).

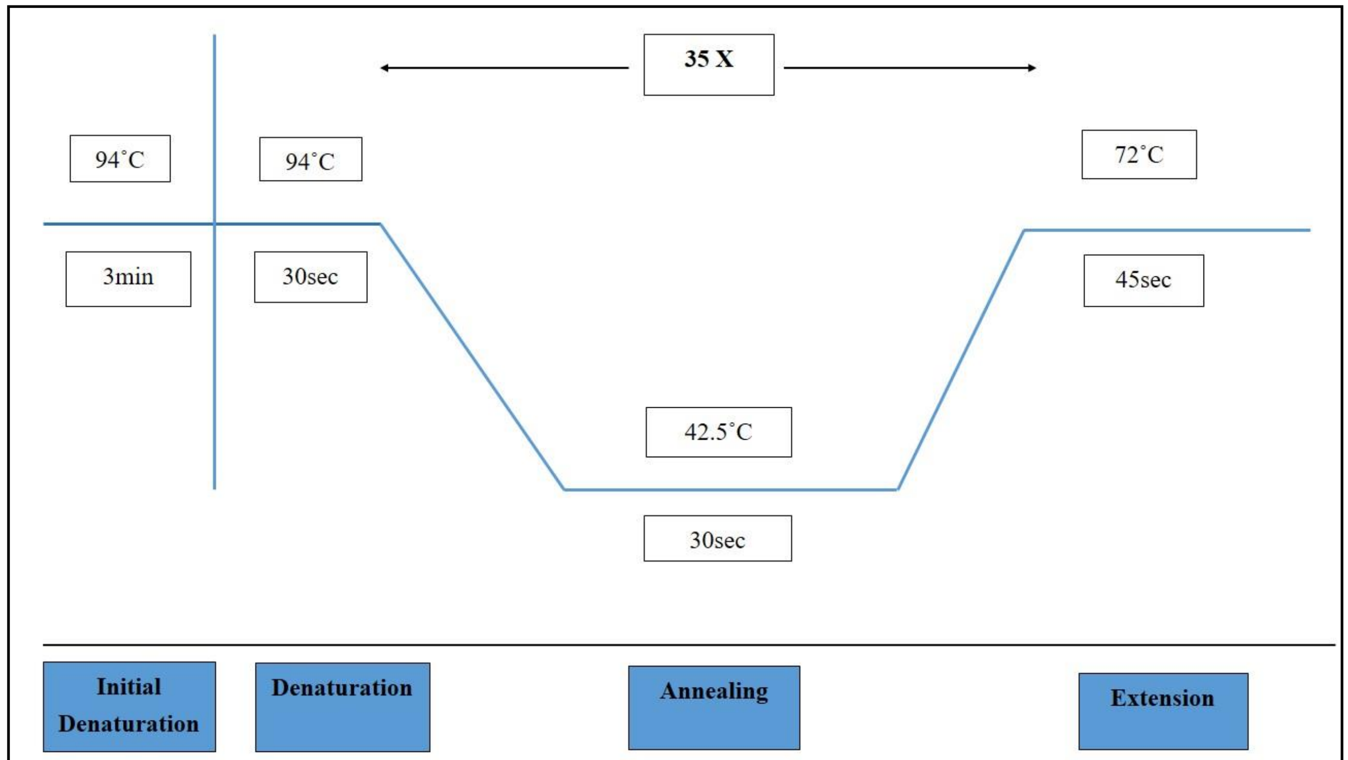

Figure 13 : Cycling parameters for qPCR. The figure shows the thermal cycling profile for NUCB1.

2.12. Statistical analysis

The distribution of all data sets was evaluated for normality before any statistical analysis. Statistical analysis was performed to compare the differences between the control, MPTP-treated, and the clozapine-treated group. To ascertain whether there were any significant differences between the groups, statistical tests such as the T-test and one-way ANOVA were utilized, followed by Tukey's test. The graphs were created using Graph Pad Prism version 10.0, and significance was set at $P < 0.05$. The data and outcomes were expressed using the standard error of the mean, or SEM.

CHAPTER 3: RESULTS

3.1. In silico results

3.1.1. Protein and ligand structures

Protein structure of NUCB1 in PDB format, giving a thorough illustration of the protein's spatial organisation. A ligand of interest, clozapine, also had its chemical structure obtained in SDF format from PubChem providing comprehensive details on its molecular make-up and conformation as shown in Figure 14. The ensuing *in silico* analyses, such as molecular docking simulations, are built on top of these molecular architectures.

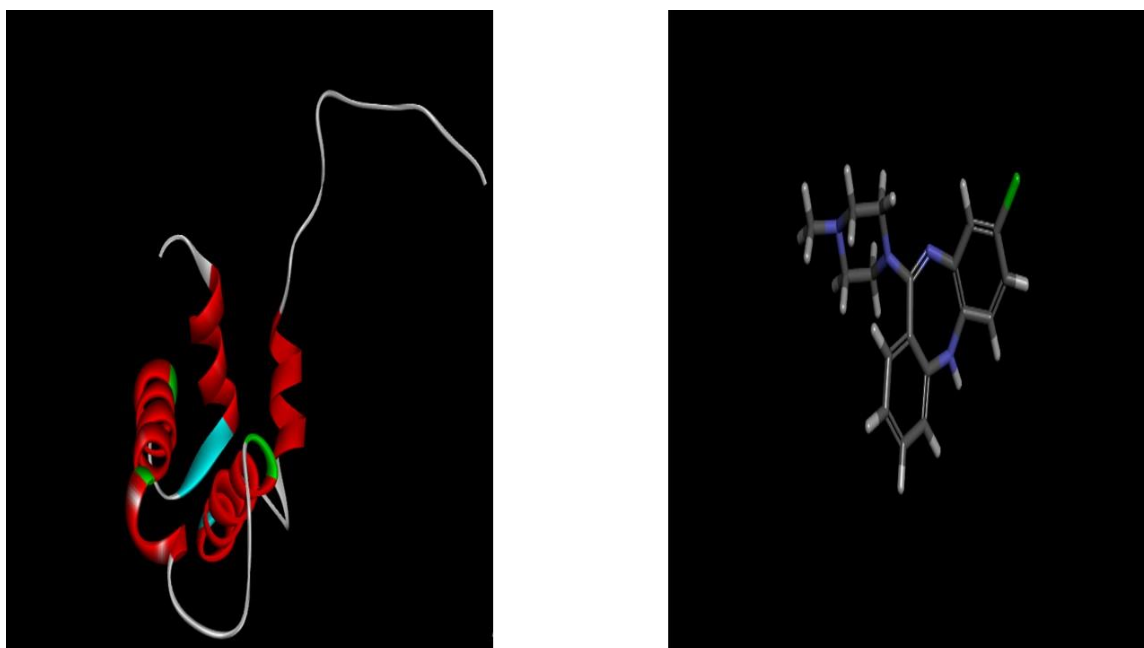

Figure 14: 3D structures of NUCB1 and Clozapine. Protein structure of NUCB1 in PDB format from the Protein data bank and structure of Clozapine in SDF format from PubChem showing comprehensive details on its molecular make-up and conformation.

3.1.2. Molecular docking analysis

Structural complexes of the NUCB1 (target) with Clozapine (ligand) have been analyzed using a ligand-target docking technique and are shown in Figure 15. The results of this docking analysis reveal useful structural and energetic details regarding the potential affinities and binding mechanisms of clozapine with NUCB1. The information will be useful in figuring out how

clozapine interacts with NUCB1 at the molecular level, illuminating its function in neuroprotection pathways, and providing prospective directions for further study.

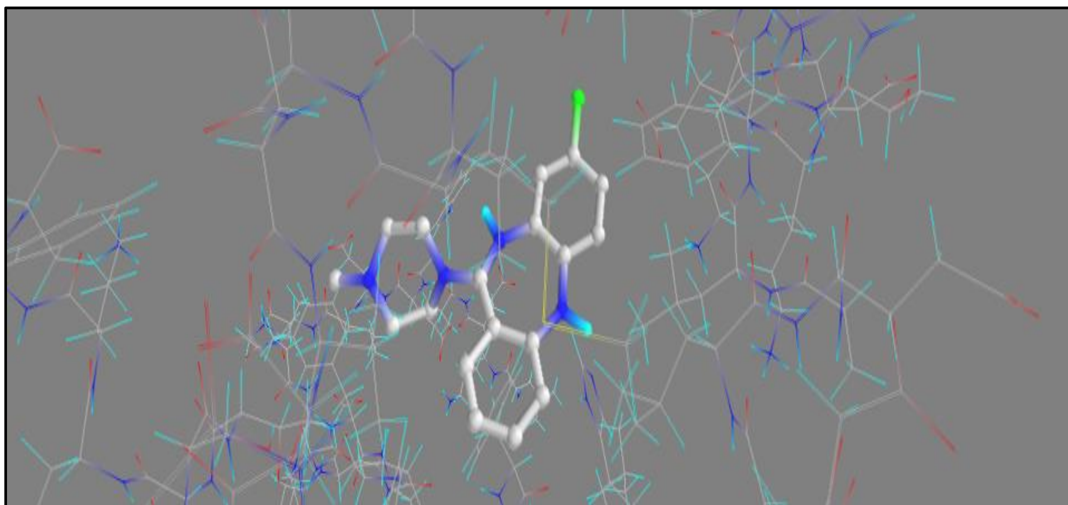

Figure 15: Visuals of docking interactions of clozapine and NUCB1. It shows a computationally projected snapshot of the interactions between clozapine and NUCB1 provided by the docking analysis carried out with PyRx.

3.1.3. Binding affinity

The minimal binding energy as shown in Figure 16 shows that the target protein i.e. NUCB1, was successfully docked with clozapine. This graph represents the specific target protein binding affinities of the ligand, clozapine. The binding affinities, which are shown on the vertical axis, represent the degree of interaction between clozapine and the protein and are commonly expressed in energy units (for example, kcal/mol). It also evaluates clozapine's potential as a ligand for NUCB1, in order to comprehend the stability of the ligand-protein complex and, eventually, its biological consequences in the context of neuroprotection. It has been demonstrated in visual representation of Figure 17, how clozapine may bind to NUCB1 active sites. NUCB1 protein residues Val 77, Val 85, Val 73, and Met 69 form alkyl and pi-alkyl interactions and Leu 41 form pi-sigma interactions.

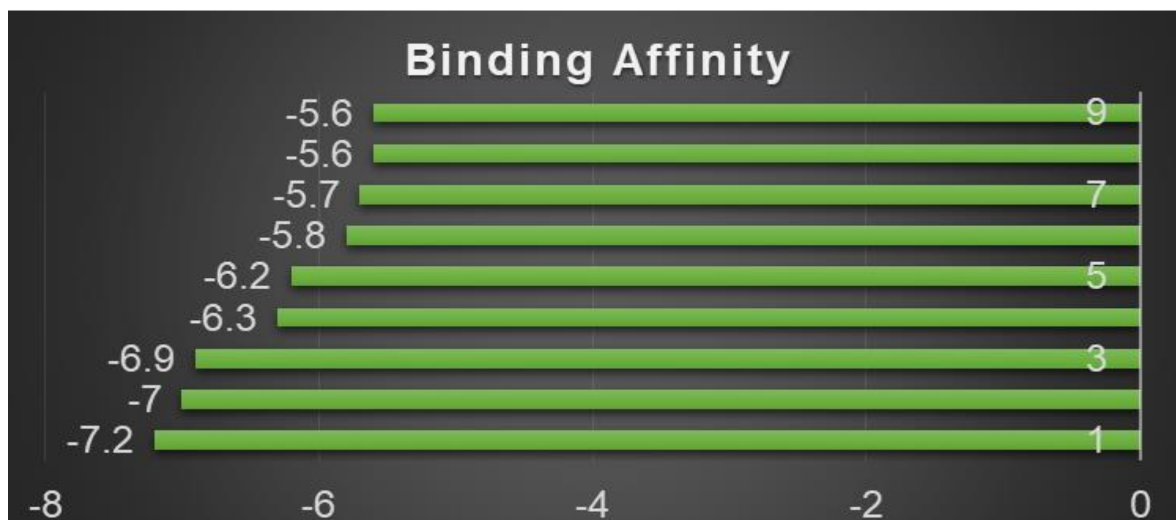

Figure 16: *In silico* analysis showing the interaction of clozapine with NUCB1, with binding energies that range between – 5.6 to – 7.2 kcal/mol. The graph shows how effectively the ligand, clozapine, binds to its target protein, clozapine. Energy values used to express binding affinities give information about how strongly the two molecules interact. Each data point on the graph represents a particular computational docking or binding simulation experiment. Lower values on the y-axis in the graph signal that clozapine has a better affinity for binding to the target protein, which may indicate a positive interaction. Higher values, on the other hand, can suggest weaker binding.

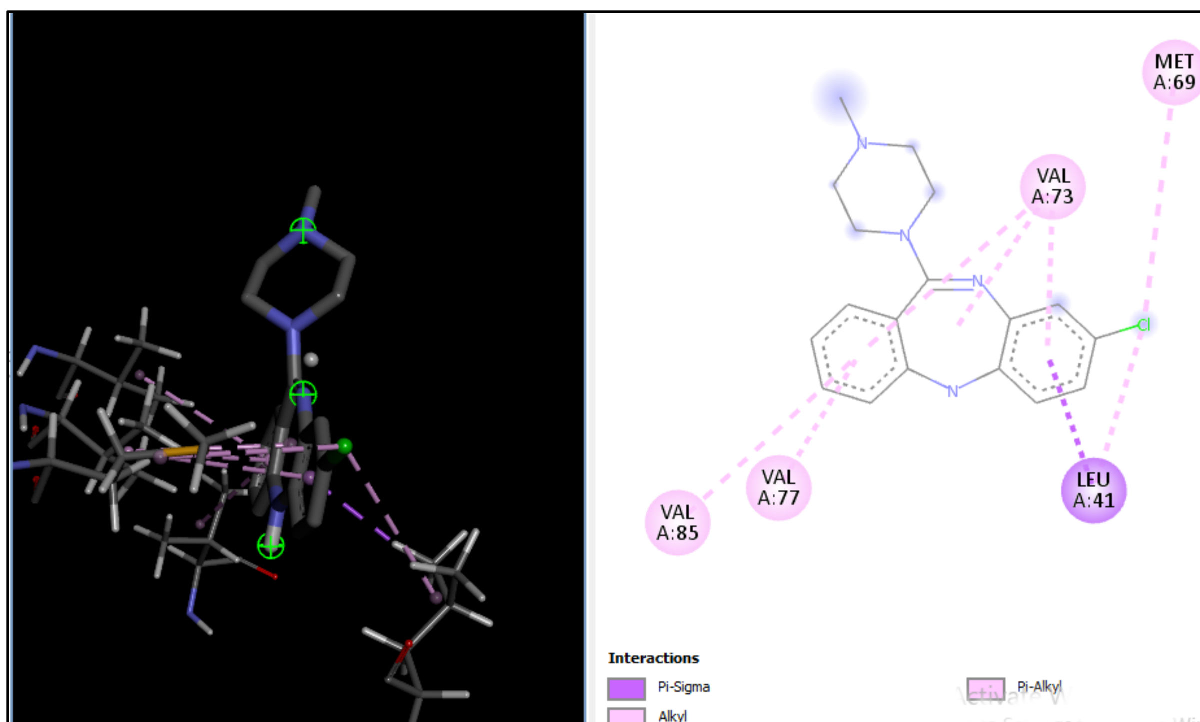

Figure 17: Potential binding sites of NUCB1: VAL A: 77, VAL A: 85, LEU A: 41, VAL A: 73, MET A: 69. NUCB1 protein residues Val 77, Val 85, Val 73, and Met 69 forms alkyl and pi-alkyl interactions and Leu 41 forms pi-sigma interactions.

3.2. Behavioral assessment results after disease induction with MPTP

3.2.1. Forced swim test

The effects of MPTP on the forced swim test are shown in Figure 18. Five mice were included in each group i.e. n=5. The parameters measured during this test were immobility time. The T-test was used, and the results showed significant differences between group I (Control) and group II (Disease), which revealed that the time of immobility in group II treated with MPTP increased.

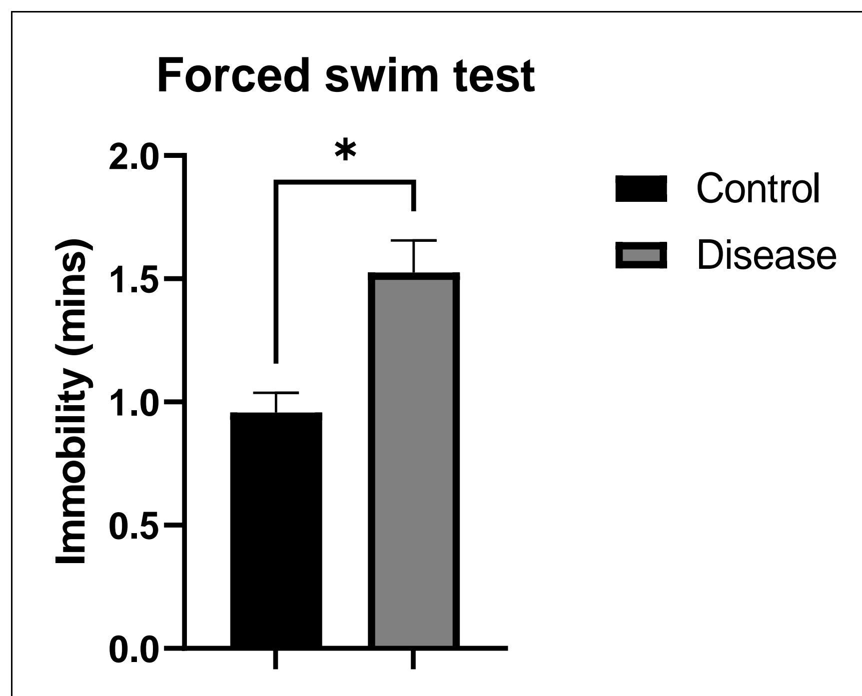

Figure 18: The effects of MPTP in FST. This graph shows the MPTP effects and the duration of immobility in the mice in the FST. Comparison with an appropriate control group using t-test: $p<0.05$. Values are presented as ±SEM.

3.2.2. Tail suspension test

The effects of MPTP on the tail suspension test are shown in Figure 19. Five mice were included in each group i.e. n=5. The immobility time was one of the test's parameters. When the T-test was used, it was found that there were significant differences between group I (Control) and group II (Disease), and it revealed that the time of immobility in group II treated with MPTP increased.

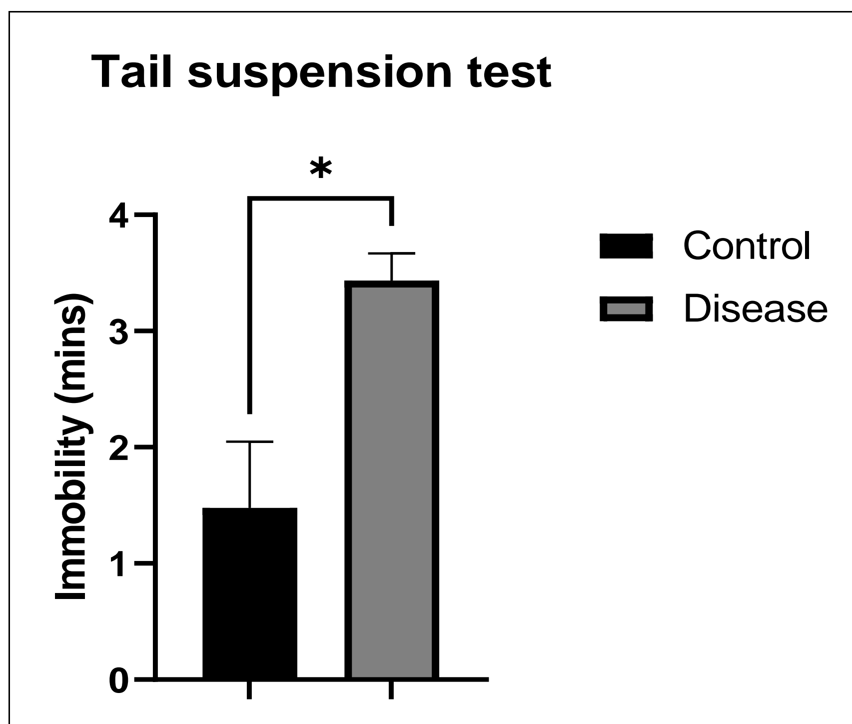

Figure 19: The effects of MPTP in tail suspension test. This graph shows the effects of MPTP and the duration of immobility in the mice in the Tail suspension test. Comparison with an appropriate control group using t-test: $p<0.05$. Values are presented as ±SEM.

3.3. Behavioral assessment results after treatment with clozapine

3.3.1. Forced swim test

The effects of clozapine on the forced swim test are shown in Figure 20. Five mice were included in each group i.e. n=5. One-way ANOVA was applied and it showed significant differences between the three groups. The test revealed that the time of immobility increased in the treatment group as compared to the control group. Interestingly, the time of immobility decreased in the treatment group as compared to the disease group.

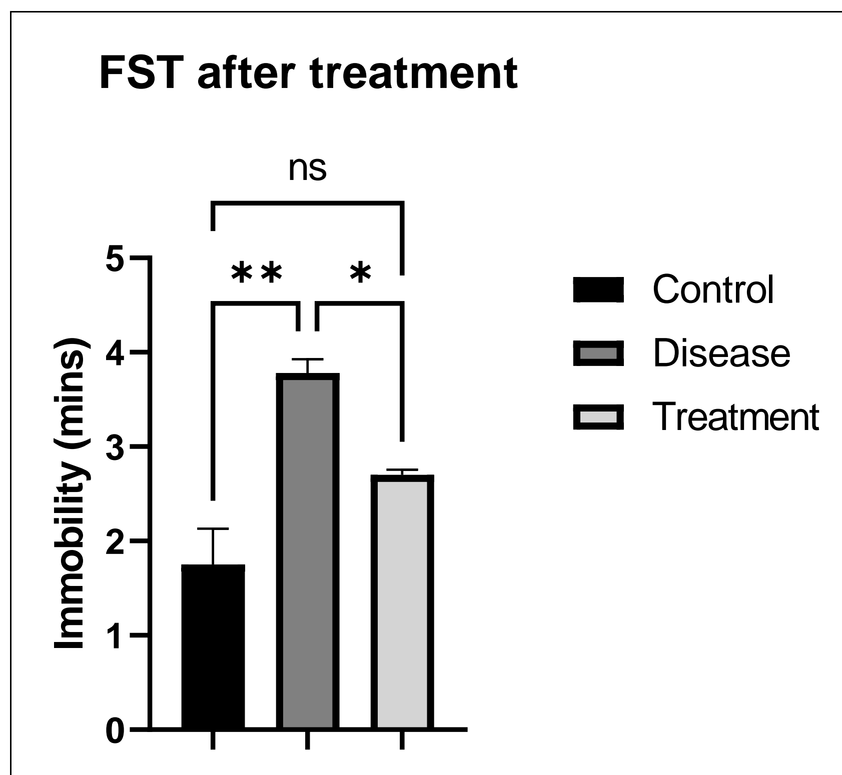

Figure 20: The effects of clozapine in FST. This graph shows the effects of clozapine and the duration of immobility in the mice in the FST. Comparison with disease and control group using one-way ANOVA test; n = 5 mice per group, *p<0.05 and **p<0.01.

3.3.2. Tail suspension test

The effects of clozapine on the tail suspension test are shown in Figure 21. Five mice were included in each group i.e. n=5. One-way ANOVA test was applied and it showed significant differences between the three groups. The test revealed that the time of immobility increased in the treatment group as compared to the control group. On the other hand, the time of immobility decreased in the treatment group as compared to the disease group.

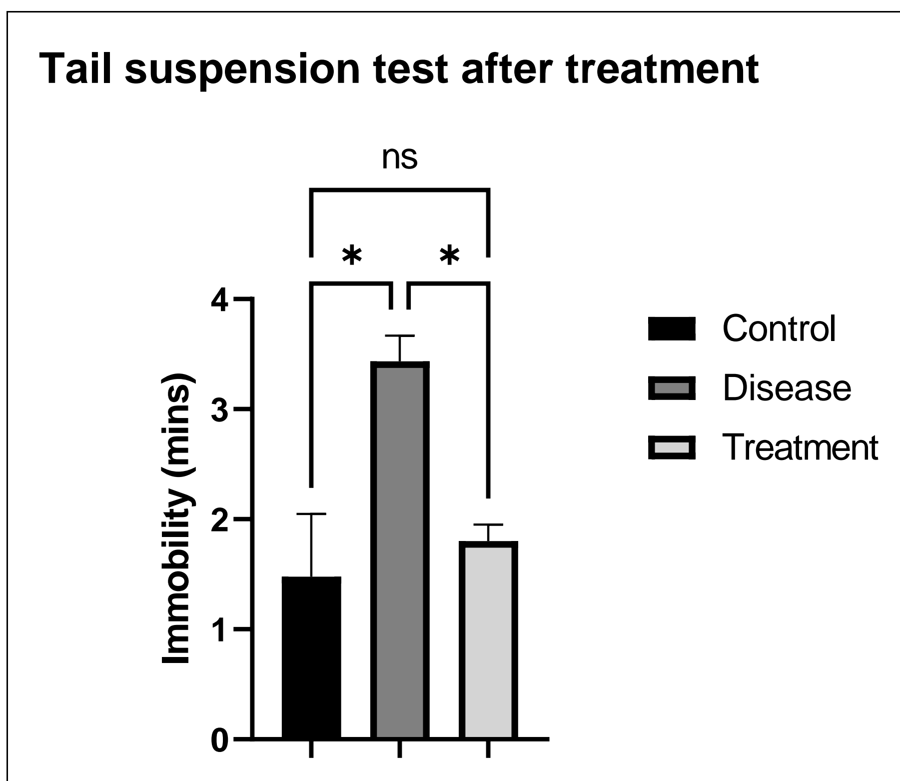

Figure 21: The effects of clozapine in tail suspension test. This graph shows the effects of clozapine and the duration of immobility in the mice in the tail suspension test. Comparison with disease and control group using one-way ANOVA test; with n = 5 mice per group and *p<0.05.

3.4. Histopathological results

The section of the cerebellum was examined in all the groups.

3.4.1. Effects of MPTP and clozapine on histology

3.4.1.1. Cerebellum

The effects of clozapine on the cerebellum of the MPTP-treated mice were stained with H&E and evaluated. The histological findings are presented in Figure 22.

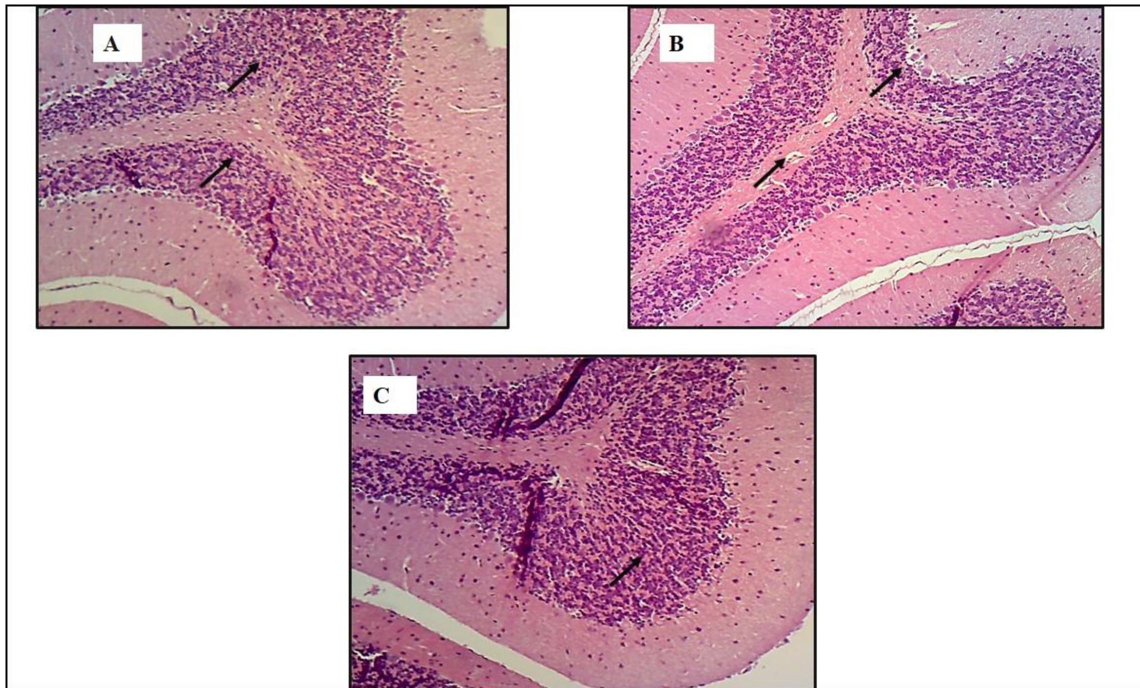

Figure 22: The section of the cerebellum stained with H&E. A) Control mice showed neurons with normal organization. **B)** The diseased group (MPTP-treated) exhibited strong neuronal loss as well as swelling of neurons (arrowhead). **C)** The treatment group (clozapine treated), showed that most neurons were similar to those in the control group and there were fewer swollen neurons.

3.4.1.2. Morphometric results

3.4.1.2.1. H&E neuronal cell count in the cerebellum

Using Image J's software, the cells in the digital photomicrographs were counted, and the results are shown in Figure 23. The diseased group (MPTP-treated) had fewer neurons in their cerebellum than the control group (vehicle-treated) or treatment group (clozapine-treated).

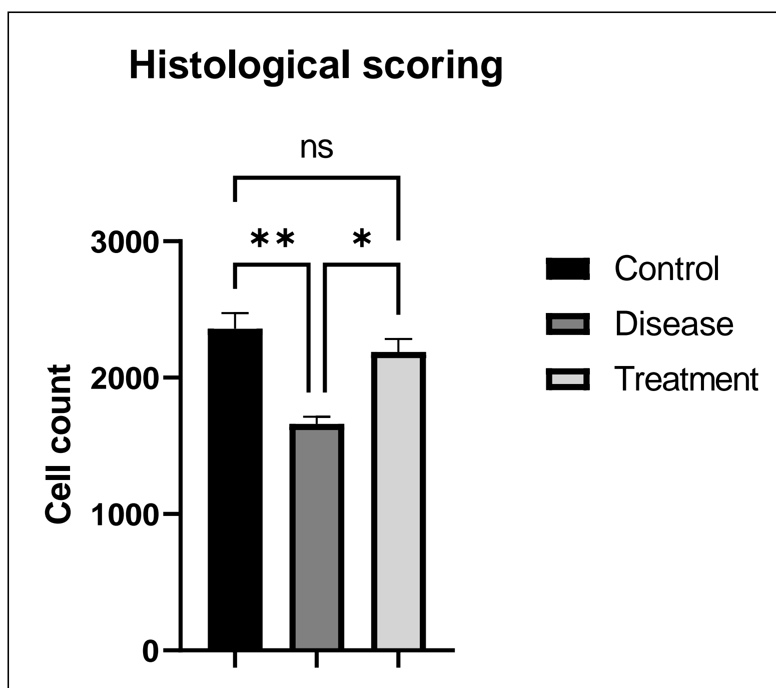

Figure 23: Effect of MPTP and clozapine on the cerebellum histology (H&E stained tissue sections). Data is represented as mean SEM, n = 3 mice per group. *p 0.05, **p 0.01; Ns = non-significant.

3.5. PCR results

3.5.1. Gradient PCR result

The representative gel in Figure 24 shows that using primers specific for NUCB1 produced a single band in the area of expected size of 210 bp, indicating that NUCB1 mRNA is widely expressed in the brain.

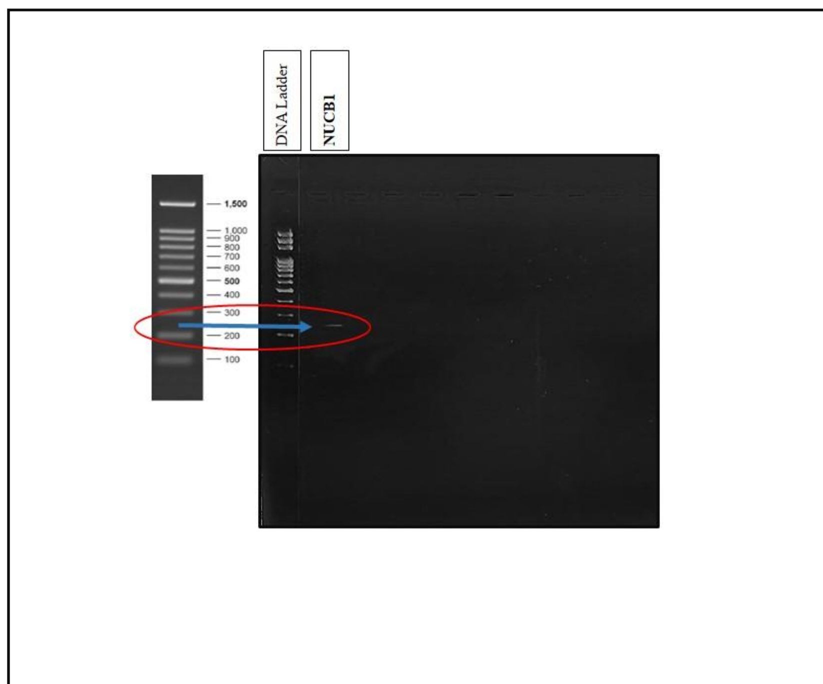

Figure 24: Gel Electrophoresis results for optimization. PCR analysis of NUCB1 expression in the mice brain. Note a single band at approximately 210 bp at 42.5˚C.

3.5.2. Real-time PCR result

The relative expression of NUCB1 is shown in Figure 25. The relative mRNA expression of genes of interest was measured and normalized to the expression of beta-actin as a housekeeping gene. The results showed that the NUCB1 mRNA expression was up-regulated in the mice treated with MPTP and down-regulated in the mice treated with clozapine.

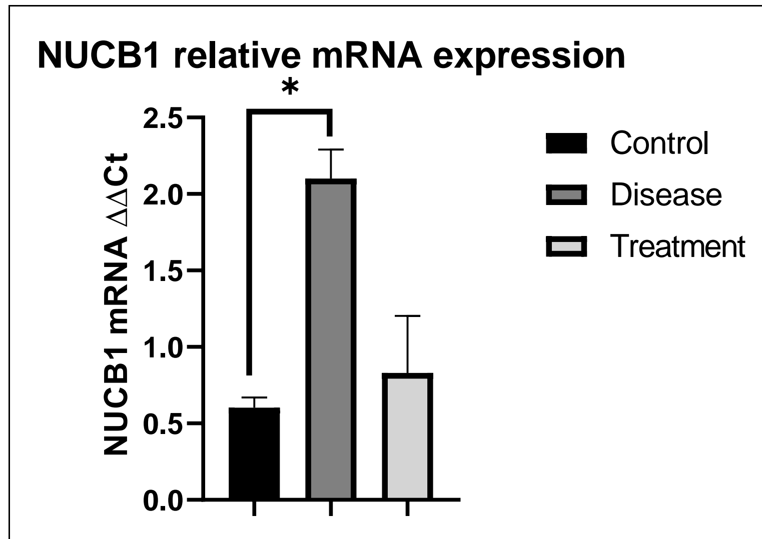

Figure 25: NUCB1 mRNA relative expression (Normalized to Beta-actin). NUCB1 mRNA expression in the mice treated with MPTP for seven days showed up-regulation in contrast to the treatment with clozapine for 40 days which showed down-regulation. Data is shown as ±SEM. For statistical analysis, the non-parametric one-way ANOVA was used, followed by the Tukey multiple comparison test.

CHAPTER 4: DISCUSSION

A number of immune-modulatory strategies have been evaluated for their effectiveness in MPTP-induced PD-like alterations in mice models; however, no single immune-modulatory medication has yet been approved for use as a treatment. Although many theories have been tested to explain the complex pathophysiology and pathogenesis of PD. (Alshammari *et al.*, 2022). Additionally, the therapeutic options that are currently available involve extrapyramidal side effects; as a result, another goal of the current study was to maximize therapeutic efficacy while minimizing adverse events (Alshammari *et al.*, 2022). Therefore, the objective of the current work was to investigate the complex interactions between NUCB1 protein and clozapine drug in the context of neuroprotection in MPTP-treated mouse models. The putative molecular processes underlying the neuroprotective effects of clozapine are usefully illuminated by the *in silico* investigation of the interaction between NUCB1 and clozapine. Through molecular docking simulations, it was discovered that clozapine can attach to NUCB1 in a variety of ways, each with a unique binding affinity. The various conformations and orientations of clozapine within the binding pocket can be blamed for the discrepancies in binding affinities. A substantial interaction between clozapine and NUCB1 is suggested by the most advantageous binding position, which is indicated by the highest negative binding affinity. The significant binding affinity between clozapine and NUCB1 raises the possibility that NUCB1 has a part in determining the neuroprotective effects of clozapine. It is well recognized that NUCB1 participates in calcium control, stress reactions, and neuroprotection pathways (Tulke *et al.*, 2016). These functions may be altered by clozapine's binding to NUCB1, resulting in improved neuroprotection. The structure of this binding mechanism sheds light on potential interactions between clozapine and NUCB1 and the findings highlighted the possible importance of NUCB1 as a vital component in mediating the neuroprotective effects of clozapine.

Modeling the PD pathogenesis is a challenging problem. Several animal models have been created to comprehend the pathophysiology and test potential new drugs against PD (Dovonou *et al.*, 2023). Rodents and non-human primates are the main sources used in PD research (Potashkin, Blume and Runkle, 2010). But rats and mice are more often utilized (Kin *et al.*, 2019). However,

developing PD animal models is vital for evaluating innovative therapy approaches and neuroprotective drugs (Prasad and Hung, 2020).

It is possible to model PD in rodents, particularly mice, using MPTP. In this study, MPTP is used as a neurotoxin to induce PD in the mice. MPTP may cause energy failure, oxidative stress, and ROS, which are factors in neuronal death (Zeng, Geng and Jia, 2018). For examining the biology of PD and assessing prospective treatment strategies, the injection of MPTP in the study served as a vital model. Modeling the motor symptoms and neurodegeneration associated with PD is made possible by the substantia nigra's selective sensitivity to MPTP-induced toxicity (Prasad and Hung, 2020). There are several ways to deliver MPTP, including intraperitoneal or intravenous systemic injection, intracerebral injection, or oral administration (in the prodrug form, MPTP-HCl) (Jackson-Lewis and Przedborski, 2007). As soon as it is administered, MPTP is transformed into its active metabolite, MPP+ which is then specifically absorbed by dopaminergic neurons via the dopamine transporter (DAT). MPP+ interferes with mitochondrial activity inside the neurons, causing oxidative stress and neuronal death (Davis *et al.*, 1979). In animal models, this mechanism causes behavioural and motor impairments similar to bradykinesia, tremors, and stiffness in PD patients. After injecting MPTP which specifically damages dopaminergic neurons in the nigrostriatal pathway (Langston, 2017), behaviour tests such as tail suspension test and force swim test were assessed in MPTP-induced animal models to evaluate the motor functions. The FST and tail suspension tests are quick assessments to determine whether mice exhibit depressive-like behaviour, which is a key symptom of PD (Rial *et al.*, 2014). The FST and tail suspension test can be a useful tool for determining how dopaminergic neuron degeneration affects emotional and motivational states in the context of mouse models treated with MPTP (Rial *et al.*, 2014). The FST and tail suspension test results in this study showed that MPTP-treated mice had longer periods of immobility than control groups. It's common to view this extended immobility as a sign of depressive-like behaviour. The decreased escape-oriented behaviours seen in mice treated with MPTP may point to changes in the brain circuits and neurotransmitter systems related to mood regulation. Surprisingly, mice who were co-treated with clozapine showed a decrease in immobility activity. The observed neuroprotection was connected with this behavioural improvement.

On the other hand, animal pharmacological models are great resources for researching the histopathological causes of PD (Fikry, Saleh and Abdel Gawad, 2022). H&E staining is a key method used in histopathological investigation to evaluate structural changes and pathological abnormalities in tissue section (Alshammari *et al.*, 2022). H&E staining offers significant insights

into the histological features of brain tissue in the context of this investigation studying clozapine-induced neuroprotection in MPTP-treated mouse models. In this study, we examined the protective effects of clozapine on MPTP-induced PD in mouse cerebellum. The preservation of neuronal structure in the clozapine-treated group as compared to the MPTP-only group is one of the study's main findings. This data suggests that clozapine may have neuroprotective properties. Clozapine may be able to counteract the neurodegenerative processes brought on by MPTP, as seen by the treatment group's decreased neuronal loss, maintenance of cellular organisation, and reduced signs of neuroinflammation. This preservation may be attributable to the effect of clozapine on NUCB1 expression, which has been linked to cellular stress responses and preservation of neuronal viability

Quantitative real-time polymerase chain reaction (qRT-PCR) gene expression analysis is a potent method in molecular biology that sheds light on the transcriptional control of particular genes (Valasek and Repa, 2005). In this study, variations in NUCB1 expression under specified experimental settings were evaluated by examining the relative expression of NUCB1 mRNA normalised to the housekeeping gene beta-actin. In qRT-PCR investigations, beta-actin is frequently used as a housekeeping gene or reference for normalisation. Beta-actin is normally expressed at very consistent levels across a variety of cell types and situations and is involved in key cellular activities. The precision and dependability of gene expression measurements are ensured by normalisation with beta-actin, which allows for the adjustment of any changes in RNA input and cDNA synthesis effectiveness (Ruan and Lai, 2007). It is enlightening to compare the relative expression of NUCB1 mRNA in various experimental groups or settings. It enabled to spot up regulation or down regulation patterns and understand how particular therapies or interventions affect NUCB1 expression. These results can help us understand the molecular processes that underlie the observed effects better. Under the investigated experimental conditions, a substantial change in NUCB1 mRNA expression was found. The transcription of NUCB1 may have changed as a result of a number of events, including drug therapies, disease conditions, or

experimental manipulations. It is crucial to comprehend how NUCB1 expression is regulated because this gene has been linked to cellular functions and neuroprotection. While it was found that clozapine suppress the NUCB1 mRNA expression in contrast to the MPTP-treated. NUCB1

has been connected to several cellular functions, such as controlling calcium homeostasis, ER stress responses, and anti-apoptotic signaling (Tulke *et al.*, 2016). This study uncovered several

significant features of NUCB1 and clozapine biology. The reported variations in NUCB1 expression are biologically significant and might have effects on the field of study. For instance, if the focus of this research is on NUCB1's function in neuroprotection, changes in NUCB1 expression may indicate that this protein is involved in the cellular response to neuroprotective drugs, such as clozapine in the context of PD. Future research will need to address significant issues brought up by the variations in NUCB1 mRNA expression found in this study. A natural next step would be to look into how changed NUCB1 expression affects cellular pathways, protein levels, and ultimately cellular function. Additionally, comprehension of the regulatory components that control NUCB1 expression can reveal clues about possible therapeutic targets.

Future Prospects:

1. The molecular pathways by which NUCB1 interacts with clozapine to cause neuroprotection can be studied in greater detail in future studies. Specific targets for drug development can be found by understanding the exact signalling pathways involved.
2. Finding other biomarkers linked to NUCB1-induced neuroprotection may be the main goal of future research. These biomarkers might help with early neurodegenerative disease diagnosis and surveillance.
3. Non-motor symptoms that are frequently present with PD have an important impact on patients' quality of life. Future studies may examine the effects of NUCB1-based therapy on these non-motor symptoms.

Limitations:

- It is important to recognize the inherent limitations of this study. It is still unclear how exactly clozapine affects NUCB1 expression and subsequently, neuroprotection works.
- Additionally, due to the multifactorial connections that are a part of the complicated PD pathophysiology, more investigation is required to determine exactly how NUCB1 fits into this complex network.

- Future research should investigate the downstream molecular pathways that clozapine therapy stimulates in NUCB1. A more thorough understanding of the possible therapeutic effects might result from validating these result.

CONCLUSION:

In this study, NUCB1's role in providing neuroprotection was thoroughly investigated using a mouse model treated with MPTP, with a focus on the neuroprotective potential brought on by clozapine. The research has provided insightful information about the complex interactions between NUCB1, clozapine, and the neurotoxicity caused by MPTP that mimics some symptoms of PD. It has been established through careful research and analysis that NUCB1, a multifunctional protein previously implicated in maintaining calcium balance and responding to cellular stress, does play a role in neuroprotection in this specific situation. By altering crucial pathways linked to neuronal survival, the antipsychotic drug clozapine, which is administered, further strengthens this neuroprotection.

According to evidence from our study, clozapine's facilitation of the decrease in NUCB1 levels considerably lessens the neurotoxic impacts of MPTP. The maintenance of dopaminergic neurons in the brain regions and the improvement of motor and cognitive impairments seen in behavioral tests are both examples of this impact. Additionally, the mechanisms behind NUCB1's neuroprotective function merit study since they may one day lead to treatment approaches that delay or stop the progression of PD.

While we commend these encouraging results, it is critical to acknowledge the challenges currently associated with PD development and the diverse function of NUCB1. Even if it is insightful, our study advances knowledge in a rapidly developing area of study. It prompts new questions, urges additional research into NUCB1's intricate molecular structure, and advocates for the conversion of these findings into clinical uses.

In conclusion, the potential of NUCB1 as a focal point in the search for neuroprotection against PD is highlighted by our investigation in this research thesis. Looking ahead, we predict that

further research will reveal greater aspects of NUCB1's participation, potentially resulting in ground-breaking therapy strategies that offer hope to people suffering from neurodegenerative

illnesses like Parkinson's. This study advances our knowledge of NUCB1, but it also demonstrates the continued search for ground-breaking treatments for neuroprotection and neurodegenerative disease.

5. APPENDICES

5.1. Appendix A

Calculations of MPTP doses:

Table 6: Dose preparation. The table shows the dosing regimen for MPTP administration (Jackson-Lewis and Przedborski, 2007).

	MOUSE WEIGHT (GRAMS)	NO.OF INJECTIONS	TOTAL INJECTION VOLUME (ml)	MPTP CONCENTRATION (mg)
1.	26	4	0.26	1.04
2	27	4	0.27	1.08
3.	22	4	0.22	0.88
4.	26	4	0.26	1.04
5.	25	4	0.25	1
6.	30	4	0.30	1.2
7.	43	4	0.43	1.72
8.	23	4	0.23	0.92
9.	34	4	0.34	1.36
10.	36	4	0.36	1.44

Dosing is calculated by using the following formulas:

Total Volume of solution = Weight of mice×10µl (i.e. 0.01ml for 1 gram) × number of injections

Example,

MPTP concentration

For 26g = 26×0.01ml×4 = 1.04mg

Total MPTP Concentration (mg/ml) = Total volume of Solution × the desired concentration of MPTP per 10ml

For Example, if the total amount of MPTP is 1.04mg then the total amount of solution will be calculated as follows:

Total amount of MPTP = (1.04ml×23.4mg)/10ml= 2.4ml

5.2. Appendix B

Calculations for clozapine treatment

The clozapine treatment regimen for MPTP-treated mice based on their weights and a standard dosage of 2.5mg/kg is as follows:

Table 7: Clozapine treatment regimen.

	MOUSE WEIGHT (kg)	CLOZAPINE DOSAGE (mg/kg)	CLOZAPINE DOSAGE (mg)	TOTAL STOCK SOLUTION (ml)	CLOZAPINE DOSAGE (µl)	TREATMENT FREQUENCY
1.	0.028	2.5	0.07	0.007	7	Once daily
2.	0.031	2.5	0.0775	0.00775	7.75	Once daily
3.	0.026	2.5	0.065	0.0065	6.5	Once daily
4.	0.034	2.5	0.085	0.0085	8.5	Once daily
5.	0.029	2.5	0.0725	0.00725	7.25	Once daily

The calculations done for the dosing of clozapine treatment are as follows:

1. Calculate Clozapine Dosage:
   weight of mouse × standard dosage(i.e. 2.5mg/kg)
2. Prepare Clozapine stock solution:
   Dissolving the calculated clozapine dosage in distilled water to give the stock solution of 10mg/ml.

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