Characterization of the Role of Neurexin-1 in MPTP Induced Parkinsonism in Mice Model.

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Acknowledgments

I am thankful to my Creator Allah Subhana-Wattala for guiding me throughout this work at every step. Everything that I have achieved so far, is because of the countless blessings of every help that came either through people or through ideas in my mind was Your will, so indeed none be worthy of praise but You.

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Abstract

The second-most prevalent neurological disorder in the world, Parkinson's disease (PD) affected roughly 6 million people as of 2016. The death and loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) is the primary pathogenic characteristic of PD. Motor abnormalities like limb stiffness, tremors, and bradykinesia are the major symptoms of PD. Parkinson's disease remains an unsolved clinical problem, as currently, authorized PD therapies offer relatively modest therapeutic benefits. In this study, mice models are utilized for PD induction by MPTP neurotoxin for functional characterization of proteomic factor Neurexin 1. The effect of co-administering clozapine drugs in MPTP mice models was analyzed through histological analyses, behavioural evaluations, and RT-PCR. Results showed that mice treated with clozapine had considerably up-regulated the expression of NRXN-1 in the brain than mice treated with MPTP. These findings provide interesting directions for future approaches in establishing MPTP-induced neuronal injury and shed light on the possible therapeutic implications of NRXN1 in reducing neurodegeneration in Parkinson's disease along with studying the coherent expression of all three Neurexin genes.

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List Abbreviations

PD	*Parkinson's disease*
MPTP	*1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine*
MPP+	*1-methyl-4-phenylpyridinium*
NRXN	*Neurexin*
LB	*LEWY bodies*
RBD	*Rapid Eye Movements Behaviour Disorder*
MSA	*Multi-System Atrophy*
AAN	*American Academy of Neurology*
MAO-B	*Monoamine oxidase B*
COMT	*Catechol-O-methyl transferase*
NMDA	*N-methyl-D-aspartate*
DBS	*Deep Brain Stimulations*
ROS	*Reactive Oxygen Species*
LRRK2	*Leucine-rich repeat kinase 2*
SNpc	*Substantia Niagara Pars Compacta*
DALY	*Disability-adjusted life years*
PD-D	*Parkinson's Disease-Demetia*

CHAPTER 1: INTRODUCTION

1.1 Parkinson's Disease (PD)

PD is a neurodegenerative disorder of the brain that causes several non-motor upsets, including cognitive impairment, mental health challenges, pain, and other sensory problems, in addition to motor symptoms like slow movements, tremors, stiffness, and walking instability. Speech and mobility problems as well as agonizing involuntary contractions of the muscles (dystonia) are all caused by motor deficiencies, which also cause constraints in many other aspects of life. As these symptoms and consequences progress, functioning and quality of life are significantly reduced, which leads to a high rate of physical impairment. Many of the readily apparent symptoms of PD are caused by nerve cell damage or extinction in the basal ganglia, a part of the brain responsible for regulating movements. Dopamine, an essential brain neurotransmitter, is routinely produced by these types of nerve cells, or neurons. Movement problems associated with PD are the consequence of decreased levels of dopamine followed by neuronal degeneration. Patients with PD exhibit Lewy bodies, which are abnormal clumps of a protein called α-synuclein, in many of their brain cells. PD is characterized by Lewy bodies or Lewy neurites, and it also involves losing neurons in the area known as the substantia nigra alongside other areas of the brain. Given that Lewy bodies mainly consist of aggregating or misfolded α-synuclein species, PD is also classified as α-synuclein disorder.

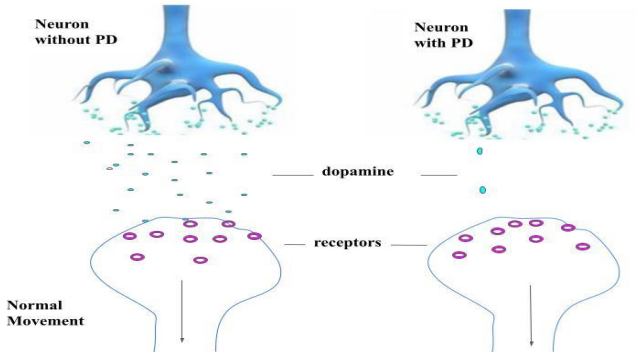

Figure 1 Healthy and Affected Neuron. A healthy neuron without PD can pass healthy synapses. A healthy neuron releases ample dopamine for the normal functioning of impulses however, an unhealthy neuron that is neuron with PD is not able to release dopamine for normal functioning.

The hallmark of PD histopathology is the development of fibrillar aggregates called Lewy bodies (LBs) that are made up of the protein α-synuclein. Pale bodies, which are LBs' precursors, may provide a source of material for LBs to continue to grow (Wakabayashi et al., 2012). As presented in Fig 1, the level of dopamine falls considerably in PD-affected neurons as compared to the healthy neurons which affects the efficient flow of impulses.

1.2 Current Interventions to Manage PD

Medical diagnostic standards which are meant to increase the precision of PD's diagnosis have been thoroughly determined throughout the past 5 years. Because the tests or biomarkers, along with the symptoms of this disorder may coincide with the characteristics of other types of neurodegenerative disorders, an accurate identification is difficult to determine in the initial phases of PD. As an outcome, clinical diagnosis precision is still subpar even after the disease has reached a completely evolved clinical stage (Tolosa et al., 2021).

The medical definition of PD includes bradykinesia (slowed movements due to impaired motor functions) alongside a minimum of one additional indicator, such as muscle rigidity, tremor, or faulty posture. The disease's asymmetry persists along with the unilateral onset of motor symptoms. The majority of those who are impacted report non-motor symptoms. Long before the start of the primary motor symptoms, several of the aforementioned non-motor symptoms can occur (Guerra et al., 2022).

Along with pain, such as non-motor symptoms additionally involve tiredness throughout the day, mental retardation, and disorders of mood, in addition to sleep disturbances involving rambling especially trouble sleeping with rapid eye movements behaviour disorder (RBD). Additionally, symptoms involve constipation, a reduced sense of smell, and abnormalities in autonomous areas for example postural hypotension, and urogenital tract malfunction. The Sydney Multicenter study on PD found that 71% of those who have been suffering from PD for over two decades also had dementia (83%), hallucinosis (74%), symptomatic hypotension (48%), constipation (40%), and urinary incontinence (20%). Furthermore, 81%, 87%, and 48% of those undergoing treatment showed signs of tumbling down, struggling to breathe, and also slowed down movement patterns, respectively (Seppi et al., 2019).

Almost 90% of PD patients report non-motor symptoms during their journey with the disease, followed by dopamine medications rarely helping these symptoms. The likelihood of developing PD later in life is almost increased fourfold by constipation and symptoms of depression. There exists a high likelihood that PD along with other synuclein-related symptoms will appear in patients with idiopathic RBD. Between the onset of RBD and the onset of Parkinsonian motor symptoms, a typical period between twelve to fourteen years will pass (Rocha et al., 2022)

The autonomic symptoms are all elevated with progressing age, disease severity, and higher dopaminergic medication dosages. Expulsion storage problems occur more frequently than voiding problems. Expulsion symptoms are prevalent and appear sooner in multisystem atrophy (MSA) than they do in PD. The acute sensory symptoms that two-thirds of those with PD suffer are thought to be the result of improper nociceptive transmission (Rektorova, 2019).

Dementia, usually developed after the progression of the disease, is approximately six times more likely to occur in people with PD. As many as sixty percent of PD patients develop dementia around 12 years after being diagnosed. Hyposmia affects 90% of persons with the early stages of PD, and it usually shows up years before the characteristic motor symptoms. Olfactory screening may help differentiate PD from various other Parkinsonian disorders, and hyposmia may signal a higher risk of PD progression (Zesiewicz, 2019).

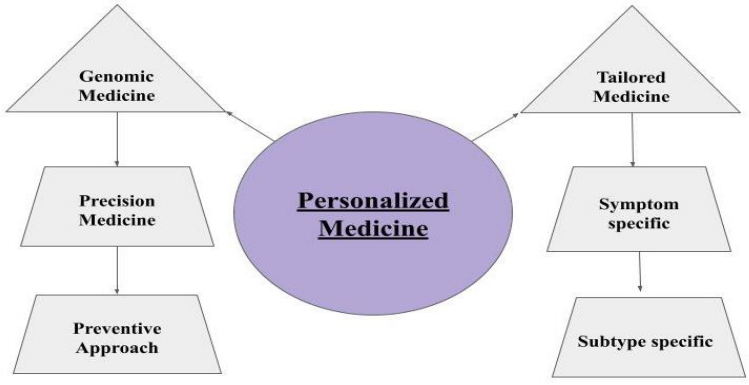

Figure 2 Possible methods for PD-relevant customized medication. In today's world customisation is providing a great help for dealing with the symptomatic control of PD while considering different factors concerning the patient's underlying conditions.

The fundamental objective of PD research is to stimulate the discovery of disease-alternative treatments that may hinder or possibly slow down neurodegenerative processes. Presently there is no clear disease-modifying drug that can achieve this goal. There are many studies in the present time that indicate the development of therapies that are based on customized or précised medicine which can comprehensively target the PD-affected individual as presented in Fig 2.

1.2.1 Dopaminergic Therapy

The American Academy of Neurology (AAN) mentions that patients begin following medication schedules as soon as they begin to exhibit impaired functioning. Of the therapeutic procedures that may be used for the management of PD are levodopa/carbidopa, the agonists of dopamine, monoamine oxidase-B (MAO-B) inhibitors, catechol-O-methyltransferase (COMT) inhibitors, N-methyl-D-aspartate (NMDA) receptor inhibitors, and anticholinergic. During the later phases of PD, additional drug delivery methods, such as intrajejunal injections, transdermal patches, and subcutaneous injections, may be used to supplement oral medication dosing. If the patient's dyskinesias and motor abnormalities continue, deep brain stimulation (DBS) should be considered (Rektorova, 2019).

1.2.2 Dopaminergic effects

Dopaminergic therapy is substantially more effective at curing bradykinesia as well as rigidity than monoamine MAO B inhibitors. Antagonists of dopamine and levodopa delay the progression of disease and disability. Trihexyphenidyl is an anticholinergic drug that can lessen shaking, even though the effect of dopamine substitute therapy on shaking is limited and unpredictable (Feng et al., 2020).

1.2.3 Other Interventions:

The best treatment for treating PD's neurological signs remains levodopa. It aids in reducing symptoms associated with movement by converting to dopamine inside the brain. To increase performance as well as lessen adverse outcomes, it is sometimes coupled alongside carbidopa. Drugs called dopamine agonists imitate the way dopamine functions within the brain. They can be both effective separately and alongside levodopa. By delaying the degradation of dopamine across

the brain, MAO-B inhibitors like selegiline and rasagiline may prolong the effectiveness of levodopa's effects (Jankovic, 2008).

1.3 Proteomic Factors Involved in the Pathology of PD

The degeneration of the cells that manufacture dopamine within the brain, especially around the substantia nigra area, is a hallmark of PD, a form of neurodegenerative disease. Additionally, there exists proof to show that several proteome components may contribute to the pathophysiology of this medical condition, even though the precise origins of PD are still not entirely known (Li & Cookson, 2019). One of the main proteome variables that have been connected is listed below:

- **α-synuclein:** The accumulation of α-synuclein protein is a hallmark of PD. In PD patients, α-synuclein forms insoluble aggregates termed Lewy bodies, which are found in the brain cells. These aggregates are thought to contribute to neuronal dysfunction and cell death.
- **Ubiquitin-proteasome system dysfunction:** The ubiquitin-proteasome system is responsible for protein degradation and maintenance of cellular protein homeostasis. Dysfunction of this system has been observed in PD, contributing to the accumulation of misfolded or damaged proteins. Impaired proteasome function may contribute to the build-up of toxic protein aggregates in the brain.
- **Mitochondrial dysfunction:** Mitochondrial dysfunction is another key feature of PD. Literature shows that defects in mitochondrial function and energy metabolism contribute to neuronal degeneration. Proteomic studies have identified alterations in proteins involved in mitochondrial function, oxidative stress response, and energy metabolism in PD.
- **Oxidative stress and antioxidant systems:** Oxidative stress, regarded as an imbalance among the reactive oxygen species (ROS) production and antioxidant defense mechanisms, is implicated in PD pathology. Proteomic studies have revealed alterations in the activity of the enzymes that function as antioxidants, which may contribute to increased oxidative damage in PD.
- **Protein folding and chaperones:** Proper protein folding is essential for their normal function. Misfolding of protein and decreased activity of chaperones, which assist in protein folding, have been observed in PD. Proteomic studies have identified alterations in chaperone proteins, such as heat shock proteins, in PD, suggesting their involvement in disease pathology.

1.3.1 Neurexins

Neurexins are essential to the nervous system, especially for the generation as well as the functioning of the synapses. The specialized connections between neurons known as synapses are where information is transmitted. The side of the synaptic connection that releases neurotransmitters, known as the presynaptic side, is where neurexins are most frequently found Fig 3. They are trans-membrane proteins, which have portions on both the extracellular and intracellular sides of the cell membrane. There are three main genes for neurexins: NRXN1, NRXN2, and NRXN3. The specificity and intricacy of synaptic connections in the brain are aided by this variety of isoforms.

At the postsynaptic side of the synapse, neuroligins alongside other synaptic proteins, such as neurexins, interact with each other. For facilitating the binding between presynaptic and postsynaptic membranes and for aiding in the growth and stabilization of synapses, neurexins bind to neuroligins. Neurexins play a variety of roles in synaptic function in addition to their binding activity. They control synaptic plasticity which is the capacity of synapses to alter strength and form by the activity and experience as well as regulating the neurotransmitter release.

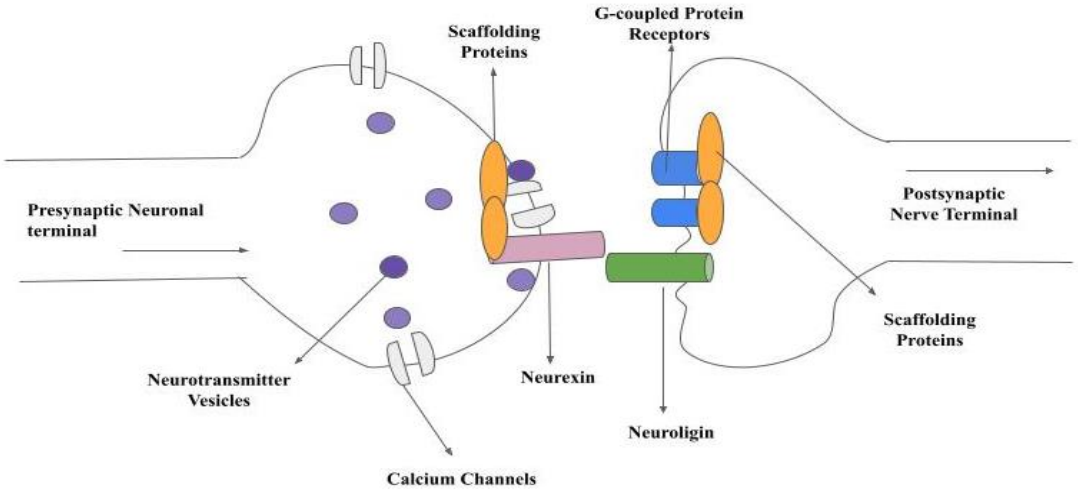

Figure 3 Neurexins and Neuroligins complex at the synapse. At the postsynaptic side of the synapse, neuroligins alongside other synaptic proteins, such as neurexins, interact with each other.

To facilitate the binding between presynaptic and postsynaptic membranes and to aid in the development and stabilization of synapses, neurexins bind to neuroligins.

Trans-membrane proteins known as neurexins serve predominantly on the cellular surface of neurons. Neurexin isoforms have roles in the creation and differentiation of distinct types of excitatory and inhibitory synapses from the central and peripheral nervous systems, where they are required for Ca2+-dependent transmission (Chowdhury, Watters, & Biederer, 2021). Trans-synaptic complexes at excitatory and inhibitory synapses, for instance, can be formed by neurexin isoforms bound to neuroligins and play a role in synapse arrangement, establishment, maturation, and plasticity. Importantly, the instant where mutation takes place between neurexin and neuroligin complex leads to results in a disproportion of excitatory to inhibitory activity (Sudhof, 2008). An outstanding example of a highly organized tissue with many specialized cells arranged into a complex structure is the nervous system. Neuronal connection develops during several developmental processes, involving cell specification, migration, focused growth, synapse creation, as well as remodelling. Components of neural wiring are organized significantly by spontaneous activity and sensory experience conveyed through the growing networks. But regardless of the lack of neurotransmission, numerous basic phases of neuronal morphogenesis and synapse creation continue to take place regularly (Lu et al., 2013).

- **Alpha-synuclein interaction:** α-synuclein, a key protein associated with PD, has been shown to interact with neurexins. α-synuclein can bind to neurexin-containing presynaptic terminals, potentially disrupting synaptic function and neurotransmitter release. This interaction may contribute to the spread of pathological α-synuclein aggregates between neurons that have been linked with the PD disease progression.

- **Synaptic dysfunction:** PD is characterized by the damage of dopaminergic neurons and impaired neurotransmission in the brain. Neurexins are involved in synaptic formation, maintenance, and function. Studies have suggested that alterations in neurexin expression or function could disrupt synaptic connectivity and neurotransmitter release, contributing to the synaptic dysfunction observed in PD.

- **Genetic associations:** Although the precise genetic reasons causing PD are complicated and diverse, certain research works into genetics have established links between the

likelihood of acquiring PD and genes for neurexins (such as NRXN1). Despite the exact processes through which neurexins play a role in PD are still not completely understood, these relationships imply that polymorphisms in neurexin genes may affect vulnerability to the condition.

- **Neuronal survival and neuroprotection:** Neurexins have been experimentally linked to improving neuronal protection as well as viability. Neurexins may play a part in controlling how susceptible dopaminergic neurons are to apoptosis in PD, according to certain research. The mechanisms explaining this putative neuroprotective action of neurexins, however, still require more study.

1.4 PD and Genetics

PD is influenced by genetics; however, most cases are sporadic, meaning that inherited genetic changes might not be the basic cause. The risk of getting PD can, however, be increased by specific genetic variables. Here are some crucial elements of PD genetics.

1.4.1 Genetic changes

An increased chance of getting PD has been linked to mutations in specific genes. These mutations are more frequently discovered in familial forms of the illness, which impact several family members. The following genes have been linked to familial PD:

- SNCA: Lewy bodies, the pathological hallmark of PD, are made up primarily of unusual forms of the protein alpha-synuclein, which is produced when the alpha-synuclein gene (SNCA) is mutated (Singleton et al., 2003).
- LRRK2: Leucine-rich repeat kinase 2 (LRRK2) gene mutations are among the common genetic roots of PD, both in familial and sporadic forms. Alpha-synuclein build-up and other degenerative alterations in the brain may be caused by abnormal LRRK2 protein function.
- DJ-1, PINK1, and PARKIN PD autosomal recessive variants are linked to mutations in these genes. These genes are involved in cellular functions such as oxidative stress response, protein quality regulation, and mitochondrial activity.

A higher chance of acquiring PD is linked to several genetic variants in addition to specific mutations. These variations, which are more prevalent in the general population, increase a person's vulnerability to the illness. For instance, changes in the lysosomal-related GBA gene indicate a higher risk of PD (Ibanez et al., 2017).

1.5 Molecular Hallmarks

Dopaminergic neurons are destroyed or degraded within the SNpc in PD, with abnormal cellular aggregates containing α-synuclein along with ubiquitin build-up in Lewy bodies. Around 60 to 70% of the brain cells or neurons in the SNpc are damaged when abnormalities appear. The findings suggest that the inflammation that occurs in PD impacts both the peripheral as well as central nervous systems in addition to the SNpc sections containing dopaminergic neurons. Lewy body abnormalities, typically commence in neurons from various parts of the brainstem that contain cholinergic neurons in addition to neurons from the olfactory system, affecting limbic regions in addition to neocortical brain regions once the disease starts advancing (Bloem et al., 2020). As discussed earlier the most basic and common hallmark of PD is the formation as well as agglomeration of a protein named α-synuclein in shape as Lewy bodies or Lewy neurites. These clumped proteins damage healthy cellular processes as well as become harmful to neurons (Antony & Balling, 2013). The incapacity of the human body to adequately detox ROS leads to higher levels of oxidative stress, a phenomenon that has been suspected of being a factor in neuronal degeneration in PD. Degradation of cells from oxidative stress might include damaged DNA, oxidative damage to proteins, as well as lipid peroxidation. Another further important molecular indicator of PD is reduced mitochondrial activity. Energy production among cells is carried out by mitochondria, and problems associated with such structures may end up in oxidative stress as well as energy shortages.

Cellular function depends on the regular removal of misfolded or malfunctioning proteins. The breakdown of protein processes, such as the autophagy-lysosome pathway along with the ubiquitin-proteasome system, is dysfunctional in PD, which is susceptible to an excessive build-up of hazardous protein complexes. PD also progresses due to neuroinflammation, and persistent inflammation affecting the brain. The medical condition is aggravated by the secretion of pro-inflammatory cytokines along with ROS by inflamed microglia as well as astrocytes. The majority

of PD occurrences are sporadic, but family variants of the illness are sometimes caused by genetic abnormalities as mentioned under the r the heading 1.3.

1.6 Epidemiology

Globally, neurodegenerative diseases are now the main cause of disability, similarly, PD and other neurodegenerative disorders are becoming more prevalent in old age. In 2016, a total of six million individuals globally suffered from PD, compared to two million in 1990. According to (Dorsey et al., 2018), more than three million disability-adjusted life years (DALYs) and 211296 deaths were linked to PD in 2016. As you get older, your chances of developing PD increase. In line with forecasts provided by the PD Foundation, approximately one percent of adults older than sixty are believed to have been diagnosed with the condition (Tysnes & Storstein, 2017).

Approximately 8.5 million people globally are estimated to have PD in the year 2019. This is an estimated worldwide prevalence that has risen in the previous 25 years. More people end up disabled and dying from PD than from any other neurodegenerative disorder. According to current estimations, 329,000 fatalities occurred due to PD in 2019, a rise of over 100% since 2000, additionally 5.8 million disability-adjusted life years, and an 81% inflation since 2000 (WHO, 2022). The prevalence of PD is 1-2 per thousand individuals at any specific time. PD prevalence rises with age and affects 1% of those over the age of 80 Fig 4.

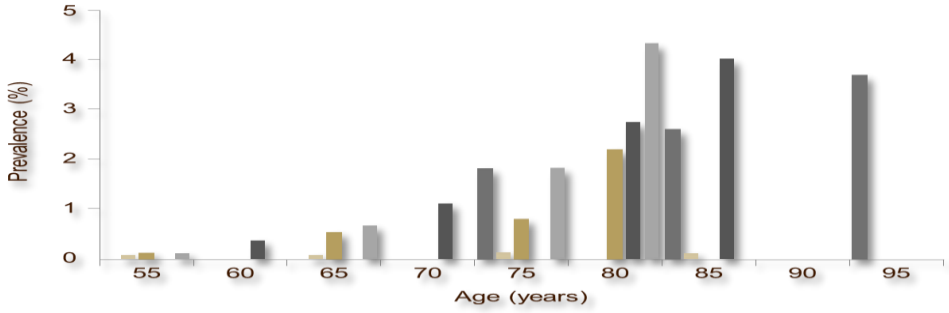

Figure 4 Prevalence rate of PD with age. PD prevalence rate is higher in older individuals than it is in adults or younger people. The highest occurrence percentage of approximately 46% is found between the ages of 80 to 85 years.

1.6 MPTP as a Neurotoxin

Numerous renowned contaminants from the environment, like herbicides such as paraquat or the fish toxin/garden pesticide rotenone, are both believed to result in neuronal degeneration of dopamine (DA) and exhibit basic structural characteristics with the toxin MPTP. When trying to create a synthetic version of heroin, a chemistry student in 1976 accidentally created MPTP, which kills dopaminergic (DAergic) neurons. In the early 1980s, others who were heroin addicts made the same error and experienced severe PD-like symptoms (Mustapha & Taib, 2021). The toxin potential of developing a reliable disease model was acknowledged by Dr Langston, who cared for many of these patients. As soon as MPTP was administered to non-human primates, he and his colleagues were able to pinpoint its effects and explain the side effects that mimicked idiopathic PD's motor deficits. Sonsalla and Heikkila demonstrated in 1986 that mice might experience many of the same effects from MPTP (Bhurtel et al., 2019).

It is universally agreed that MPTP into its toxic form via glial cells into 1-methyl-4-phenylpyridinium (MPP+) ion. Blood-brain barrier is crossed by MPT because of its high lipophilicity, where it primarily adheres to astrocyte lysosomes. (Ferrucci & Fornai, 2021a). MPP+'s polarity prevents it from quickly crossing the blood-brain barrier, therefore systemic therapy has no negative effects on central DAergic neurons. However, its immediate absorption throughout the brain largely destroys the DAergic nigrostriatal pathway Fig 5. MPP+ prefers DAergic neurons, thus clarifying the reason why the dopamine transporter (DAT) prefers it. Even though it is yet unclear how MPP+ causes cell death (Ferrucci & Fornai, 2021b). As an outcome, the MPTP-induced neurotoxicity-prone brain regions of SNpc quickly decline in adenosine triphosphate (ATP) content. It's important to notice that complex I inhibition by 25% can cause a sizable ATP shortage. Depending on the protocol, both necrosis and apoptosis can be the cause of death for DA neurons.

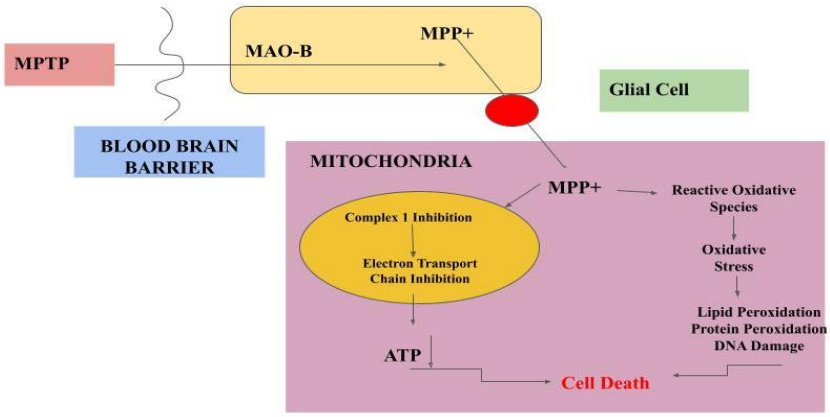

Figure 5 Stages of MPTP-induced dopaminergic toxicity. MPTP is converted to MPP+ by monoamine oxidase-B (MOAB) in glial cells, followed by DAT (dopamine transporter) activity, and MPP+ builds up in SNpc DAergic neurons.

Following the administration of MPTP or MPP+, the brain is typically freed of hydropyridine or perhaps its metabolite over a period of 12 hours, and the ATP deficit will not persist longer than twenty-four hours before being noticeable. Nevertheless, it seems that actual neuronal loss takes longer (Bourque et al., 2016). These results support the hypothesis that MPTP triggers further occurrences that ultimately result in neurotoxicity.

1.6.1 Oxidative Stress Pathway for MPTP

MPTP-induced neurotoxicity is exacerbated by oxidative stress. Because MPTP is converted to MPP+ by MAO-B in glial cells, followed by DAT (dopamine transporter) activity, MPP+ builds up in SNpc DAergic neurons. The mitochondria create ROS, such as nitric oxide (NO), superoxide anion (O2), hydrogen peroxide (H2O2), and hydroxyl radicals (•OH) as a result of this accumulation in DAergic neurons. When PP+ is present DA is delivered more quickly. Because of increased auto-oxidation of both extracellular and intracellular DA, harmful phenolics and strong oxidizing •OH are generated (Gelders et al., 2018). Excessive creation of •OH which reacts at its site of synthesis *in vivo* may be toxic to cells due to several chain events, including membrane lipid peroxidation, modifications in membrane permeability, protein inter, and DNA damage triggered by base pair mutations. Therefore, a contributing role in the degradation of DAergic

neurons is increased •OH formation which may overcome cellular antioxidant defense mechanisms (Marogianni et al., 2020).

Some theories contend that the mitochondrial apoptotic cascade plays a major role in the MPTP-induced DAergic neurotoxicity. The increase of cytochrome c & and caspase-9 in the striatum after MPTP therapy lends credence to this idea (Mestre et al., 2021). The apoptotic cascade in mitochondria is mediated by these enzymes. Additionally, overexpression produced via neuronal proteins Bcl-2, an anti-apoptotic protein, and p35, a caspase inhibitor, offered protection from the neurotoxicity brought on by MPTP. The mitochondrial apoptotic mechanism requires both the release of cytochrome C from the mitochondria and the activation of the mitochondrial transition pore. Significantly, MPP+ blocks complex I and creates Radicals to induce the activation of the mitochondrial transition pore (Emamzadeh & Surguchov, 2018). MPTP medication prevented the cytochrome c release from being released and reduced the mortality of DAergic neuronal tissue in the SNpc in null mice (Hamadjida et al., 2019).

1.6.2 Excitotoxicity Pathway

Data suggest that MPTP-induced DAergic neurodegeneration involves excitotoxicity. In this scenario, the blockage of complexes I of the electron carriers' cycle in the mitochondria causes cellular ATP to be depleted, which various capacities the membrane permeability of SNpc neurons and increases external glutamine concentrations, which in turn triggers NMDA synapses on DAergic neurons (Fleisher et al., 2020). Extracellular glutamate was discovered to have tripled in vivo utilizing micro dialysis after receiving prolonged MPTP treatment. The affinity of the SNpc glutamine transporters for glutamate is also increased by the toxin treatment. The glutamate transporters in the SNpc had a higher affinity for glutamate after receiving the toxin treatment (Binvignat & Olloquequi, 2020).

Even though the glutamatergic supplies causing these elevated levels are not known, they might include near-the-area glia, enhanced as well as a sharing with the receptor activation cotransporter, which transfers receptor activation from the mitochondria of nerve endings even after seeming to be calcium (Ca2+) insensitive. The second theory, however, is still up for debate (Iovino et al., 2020)

1.6.3 Vulnerability of different strains

It has been established that MPTP is neurotoxic to humans, other primates, animals, rabbits, and some rodents. Only some mouse strains are sensitive to MPTP in mice, indicating that genetics may play a role in how MPTP works. There are differences across mouse strains in the degree of ventral striatum DA depletion, the loss of striatum DA neurons, and behavioral deficits in response to MPTP. According to the percentage of lost SNpc neurons, different mouse strains can be categorized as "sensitive" (i.e., >50 percent SNpc lost) or "resistant" (i.e., 25% SNpc loss). The reasons for the variations in MPTP susceptibility amongst mouse strains are unknown (A Anandhan, 2017).

The phenomena have been the basis of several hypotheses, yet none of them fully explain it. The enzyme MAO-B catalyzes the reduction of such MPTP protoxin to the dihdropyrididinium intermediate 1-methyl-4-phenyl 2,3-dihydro pyridinium species (MPDP+), which is ultimately oxidised to the lethal MPP+, and its activity affects the neurotoxicity of MPTP (Darweesh et al., 2018a). It has been proposed that shifts in brain MAO-B activity could account for differences in genus and strain vulnerability to MPTP. It has been proposed that differences in MAO activity are the reason why MPTP does not make rats toxic (SS Ahmed, 2009).

The only mice strains that demonstrated that brain MAOB activity was greater than hepatic MAOB activity were the C57BL/6 and Bulb/C strains, which are the species most susceptible to MPTP. Therefore, this mouse strain's heightened susceptibility to MPTP may be caused by the liver MAO-restricted, B's systemic detoxification of MPTP (Zahoor et al., 2018a). Another possibility for the variability in vulnerability to MPTP across different strains is that they have different thresholds for oxidative changes. Although free radical generation is believed to play a substantial role in MPTP-induced cell death, little is known about the different oxidation states of distinct mouse strains, particularly the SNpc (Bhurtel et al., 2019b).

The Swiss-Weber strain, which is resistant to MPTP, and the C57BL/6 and Bulb/C strains, which are susceptible to MPTP, did not differ in their ability to produce reactive oxygen species in the striatum, suggesting that the free radical production by itself is insufficient to account for the variability in strain susceptibility (de Bie et al., 2020).

Diverse DAT uptake, divergent DAT kinetics, divergent glutamate transporter function in astrocytes, divergent regulatory oversight of Calcium ions+ flow into SN neurons, and divergent properly functioning variability in the electron transportation chain proteins have also been investigated as additional possible reasons for discrepancy sensitivity to MPTP. Sadly, none of them can pinpoint the specific contributions of this research to MPTP-induced neurotoxicity (Abhilash et al., 2021).

According to a theory, different mouse strains have different levels of sensitivity, with pigmentation variants being much more susceptible than albino ones. Behavioural impairments, DA depletion in the stria, and neuron death in the SNpc brought on by MPTP were not prevented in mice with a minor mutation in the gene responsible for tyrosine hydroxylase, the enzyme that catalyses the first two steps of pigment formation, located on chromosome 7. Melanin pigments are inadequate, which causes albinism. If a susceptibility gene is present within the same chromosomal area as the greater MPTP sensitivity observed in pigmented mice, further investigation is required to confirm this (Pathania et al., 2021).

1.6.4 Merits and Demerits of MPTP Mice Models

It is fundamentally assumed that the MPTP mouse model adequately simulates naturally occurring neurodegeneration. It is without a doubt true that MPP+ is a potent complex I inhibitor inside the midbrains of mice and PD patients. The decrease of Dopamine in the striatum is also a result of the axonal degradation and death of DAergic neurons (Baggio et al., 2015). Therefore, ventral striatum DA depletion should indicate the demise of SN DAergic cells, just like it does in PD patients.

In addition, the topographical distribution of DAergic loss of neurons in PD patients and the mouse midbrain is comparable. In other words, neuronal loss is concentrated on the lateral layer and laterally SNpc neuron as well as the posterior locations, bypassing more the anterior and medial cells (Centner, 2021). But it's still not clear if it precisely mimics PD. The mouse model of PD may be used to study mitochondrial malfunction. The model should take into account the gradual progression and behavioural features of PD, which is a neurodegenerative disease like other neurodegenerative diseases. The reduction of substantia nigra DA does not considerably advance with Rademacher/Rodents MPTP Modelling MPTP treatments, although DAergic neurons quickly

die (Pasquini et al., 2018). One of the most advanced MPTP treatments involves the continued delivery of the neurotoxin over several weeks since neurons continue to die long after the poison has ceased being given (Klemann et al., 2017).

The toxin was initially administered in a regimen involving a series of infusions either of 10 mg/kg or 20 mg/kg over the duration of a day at intervals of at least an hour. Tyrosine hydroxylase (TH) reactivity demonstrates that MPTP can kill DAergic neurons for a prolonged period after killing them initially. It is well known that now the scheduling of MPTP treatments in mice can result in wildly different results. Injections of MPTP are frequently given intravenously (i.v) or subcutaneously (s.c), the intravenous injections that are no longer used are injections (Bourque et al., 2016). The surviving DAergic neurons have not been shown to have inclusion bodies, and the neurons don't seem to die naturally by apoptosis or any other process. However, mouse mortality might vary from 50% to higher (Darweesh et al., 2018).

1.7 Clozapine

One antipsychotic drug called clozapine is used mainly in the management of schizophrenia, especially when other antipsychotic drugs have failed to provide relief. One of the most important things to note regarding clozapine and the way it is used for the management of schizophrenia is the fact that it is frequently used only to treat people with schizophrenia who are immune to other treatments. This indicates that additional antipsychotic drugs, which are normally given initially, have not had the desired effect on them. It functions by physically inhibiting the central nervous system's neurotransmitters serotonin and dopamine receptors, specifically. When compared with numerous different antipsychotic medications, it has a wider spectrum of receptor binding. Because there is a chance that clozapine will have major adverse effects, patients must be closely watched. White blood cell counts must be monitored regularly since clozapine can impair the immune system. It can occasionally result in agranulocytosis, an uncommon but potentially fatal illness. Clozapine has a variety of negative effects in addition to its potential for being very successful. Weight gain, sedation, drooling, constipation, and metabolic abnormalities are typical adverse effects. It may also have an impact on the cardiovascular system, necessitating routine monitoring of heart health. The choice to use clozapine is frequently made after carefully weighing

the potential advantages in decreasing severe schizophrenia symptoms against the dangers of side effects.

People with schizophrenia frequently receive advantages from psychological therapies that involve psychotherapy, peer support, as well as skill development alongside their medication. Such therapies can aid people in controlling their medical conditions and enhancing their general state of life. Since schizophrenia is frequently a chronic disorder, prolonged therapy with clozapine or other antipsychotic drugs may be necessary for maintaining stabilization while avoiding relapses.

1.8 Aims and Objectives

This study aims to quantify the levels of Neurexin-1 to relate its effect to PD. The objectives of this study are as follows:

1. Establishment of MPTP-induced Parkinson's disease model.
2. *In silico* analysis to find complex formation between protein of interest and drug.
3. Analysing the effect of clozapine through behavioural tests.
4. Assessment of histological and morphological changes in affected brain regions through histopathology (H&E).
5. Quantifying the levels of neurexin 1 through RT-PCR.

CHAPTER 2: METHODOLOGY

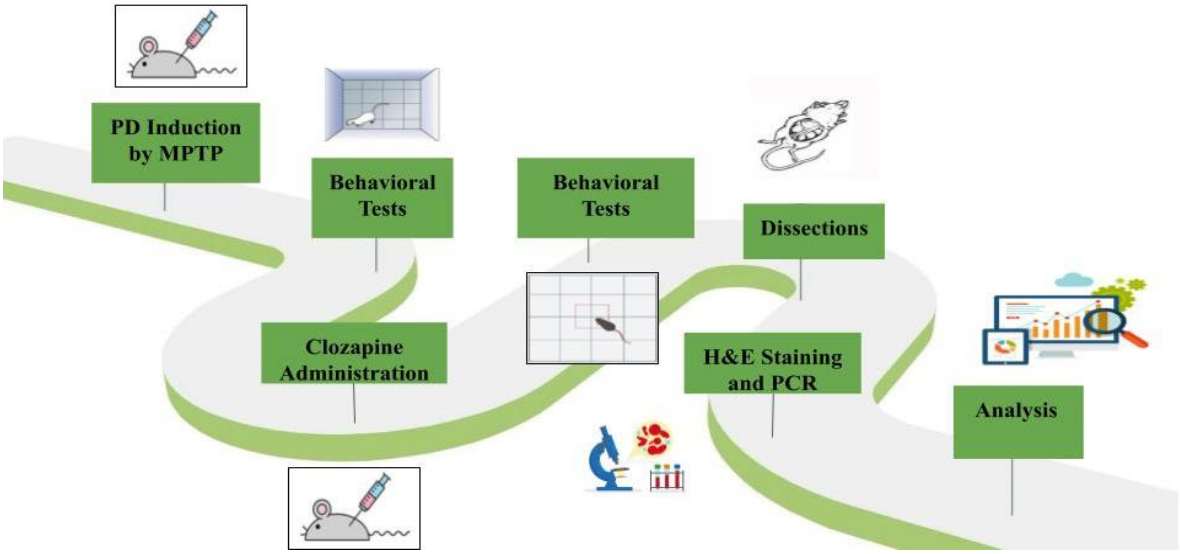

Figure 6 A Roadmap of the Steps Followed in the Research. This diagram presents the methodology followed by each step in the process.

2.1 Ethical approval (IRB)

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

2.2 *In silico* Analysis

The three-dimensional structure of Neurexin 1 (PDB: 3BOD) was downloaded from RCSB Protein Data Bank Fig 7. The chemical structure of clozapine was obtained from the PubChem compound database (PubChem CID 135398737) Fig 8. 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 neurexin-1 and clozapine selectivity and to calculate the binding affinity of the neurexin-1 (target) with clozapine (ligand). The neurexin-1 and clozapine interactions were visualized while emphasizing key interaction patterns (such as Pi-alkyl, Pi-sigma, and alkyl interactions) using the software Discovery Studio Visualizer 3.0.

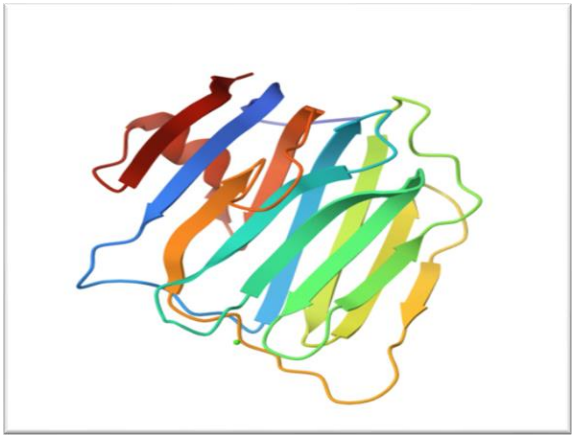

Figure 7 Two-Dimensional Structure of NRXN 1 Protein Acquired Via Protein Data Bank. This is a two-dimensional structure of the target protein Neurexin 1.

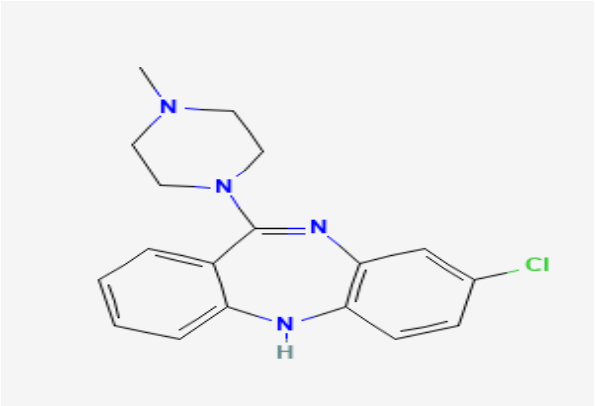

Figure 8 Structure of Clozapine (Ligand) Acquired Via PubChem. This is a two-dimensional structure of Drug clozapine which will bind with target protein.

2.3 Experimental Design

A total of 20 BALB/C male mice were used in this study. Mice were split into three groups of 5 each. The first group of 5 mice served as the control. One group of 5 mice got a dosage of 20 mg/kg MPTP intraperitoneally 4 times within a single day. The doses were administered at a gap of 2 hours. Tremors were observed visually for 45 minutes following the second MPTP injection. Open field and tail suspension tests were performed on the seventh day. Clozapine was administered orally from the eighth day. Behavior tests were performed after 45 days. Animals were dissected on the 46th day. PCR samples were stored at -80 degrees. H&E samples were fixed with PFA and stored at 4 degrees Fig 6.

2.4 MPTP Dose Preparation

The entire batch of mice was acclimatized a week before MPTP (Cat # 2300, Sigma Macklin, China) administration. Mice were weighed, sorted, and coded the day before MPTP disease induction. An acute dosage of 20 mg/kg free base MPTP was administered i.p. to male mice aged eight weeks, and toxicity tests were performed on day 8. A dosage of 20 mg/kg was administered every two hours for a total of four times throughout eight hours in one day shown in Fig 9.

Figure 9 I.P Administration of MPTP to Induce PD. MPTP was administered through i.p injections with respect to the body weights. The doses were designed keeping in view the standard that is 4 doses for a day with a 2-hour gap.

2.5 Doses preparation (Clozapine)

A Clozapine treatment regimen was designed for MPTP-treated mice models taking mice weights and desired dosage into consideration. The dosage was designed for each mouse separately respective of their weights which is 2.5 mg/kg Fig 10. The dosage was administered orally once a day for a period of 45 days.

Figure 10 Oral Administration of the Drug Clozapine in PD Induced MPTP treated Mice. Clozapine was given orally concerning the body weights for a period of 45 days.

2.6 Behaviour Assessment

2.6.1 Open Field Test

The Open Field Test is a widely used method to assess an animal's ability to recognize items or stimuli, which in turn can serve as a measure of memory. In the context of rodent models of CNS diseases, the Open Field Task represents a straightforward sensorimotor test aimed at evaluating general activity levels, gross locomotor activity, and exploratory preferences. The test utilizes a square configuration for assessment purposes (Gould et al., 2009). The test subjects, mice in this case, are placed in one of the corners of the square, and their behaviour is observed for a period of five minutes shown in Fig 11. The number of squares filled in, as well as the animal's exploration of the outer squares near the wall and the inner squares, are counted separately.

Figure 11 Open Field Test to Access the Behaviour of the Mice. The figure shows the animal in a designed open-field test box where two sections are separated by a thin white line.

2.7 Dissection

2.7.1 Histopathological Dissection

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 by using a pair of sharp scissors. The skull was then cut using fine scissors and a scalpel along the midline to expose the brain. By using forceps the brain from the skull was removed gently. After dissection, immediately brain tissue was dipped in the cold PBS. To remove all the blood from the sample, PBS was used to wash it off from the tissue. 10% formalin fixative was used to fix the tissue by placing the tissue in a fixative solution.

2.7.1.1 Slides preparation

The brain tissues collected earlier were fixed using 4% formaldehyde. To prepare the slides the tissue sample was dehydrated using 100% ethanol to immerse the already fixed tissue. The third step followed the removal of ethanol which was done by using xylene. To prepare the final slides, thin slices of tissues were cut with a thickness maintained around 3-10 µm for appropriate slide preparation. A total of 3 impressions were obtained of each tissue. For visualization of the cell structure hematoxylin and eosin stains were used.

2.7.1.2 Microscopy of Cortex and Hippocampus

The stained sections of the brain's hippocampus and cortex were then examined under the light microscope with 10X resolution and the tissue morphology, cell count, and cellular patterns were analysed. The photomicrographs of the cortex were captured to analyse the changes between the three groups and to understand the effects of the treatment and disease processes. The microscopy was performed to view how cell chemistry has changed because of MPTP induction and what effects were shown by clozapine to hinder the damage caused by MPTP.

2.7.1.3 Fresh Tissue Dissection

After that the mice were carefully decapitated by using a pair of 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 and stored at -80°C.

2.8 RNA Extraction

The total RNA from the tissues was isolated using the TRIzol isolation reagent (Cat #: FTR-100 Fine Biotech Life Sciences). To ensure the preservation of RNA's structural integrity, the process of RNA extraction is carried out. A segment of the cerebral cortex was taken and subsequently subjected to the addition of 1000µl of Trizol solution. The process of cell lysis necessitates the homogenization of the sample, which was then left to incubate at room temperature for an interval of five minutes. Following this, the sample was centrifugated at 12000 rpm for a duration of ten minutes at a temperature of 4℃, after which the resulting supernatant was carefully transferred into a fresh container. To facilitate the phase separation process, 200µl of chloroform was introduced into the solution, which was then subjected to thirty seconds of vigorous shaking before undergoing a second round of centrifugation under identical conditions. The extract was subsequently eliminated, and the nucleotides and RNA were isolated through the utilization of 500µl of isopropanol.

The specimen underwent an incubation process lasting ten minutes, at room temperature. Subsequently, the samples were subjected to another centrifugation, under the same conditions. Following the elimination of the supernatant, the sediment was resuspended and underwent a wash of 100µl with 75% ethanol. Vortexing the sample for a minute was then carried out. The sample

was again subjected to centrifugation, this time for only two minutes under identical circumstances. The pellet was air-dried after discarding the supernatant, taking between 5-10 minutes. To prevent damage to the enzyme, 50µl of nuclease-free water was added, and the sample was stored at a temperature of -80°C until further processing.

2.9 Primer Designing

Neurexin-1 plays a huge role in the synaptic functions therefore it holds a great relevance to the brain and its proper functioning. The correct biding temperatures as well as base pair (bp) length is necessary for significant binding and accurate results. are necessary. The binding temperatures ad length of bp is shown in table 1.

Table 1 A list of all the primers. The table shows all the primers used in this study including forward and reverse primers and their binding temperatures for both β-actin as well as NRXN1.

NAME	PRIMER SEQUENCE	LENGTH (BP)	OPTIMIZED ANNEALING TEMPERATURE $^{\circ}C$
β-actin Forward	GCCTTCCTTCTTGGGTATGG	358	61.5
β-actin Reverse	CAGCTCAGTAACAGTCCGC		
NRXN 1 Forward	ACTACATCAGTAACTCAGCACAG	141	47.8
NRXN 1 Reverse	ACAAGTGTCCGTTTCAAATCTTG		

2.10 Amplicon Size

The total length of a certain DNA or RNA segment that was amplified via the PCR or other techniques is referred to by the term amplicon size. For numerous applications, such as genetic analysis, gene expression investigations, or DNA sequencing, PCR is frequently used to produce

duplicates of a certain DNA or RNA code. The selection of PCR primers—short DNA sequences intended to bind to particular areas of what is being targeted DNA or RNA—determines the length of the amplicon.

Range of forward primer = 5735 to 5757

Range of reverse primer = 5854 to 5876

Amplicon Size = 5876 – 5735

Amplicon Size: 141bp

2.10.1 Forward Primer

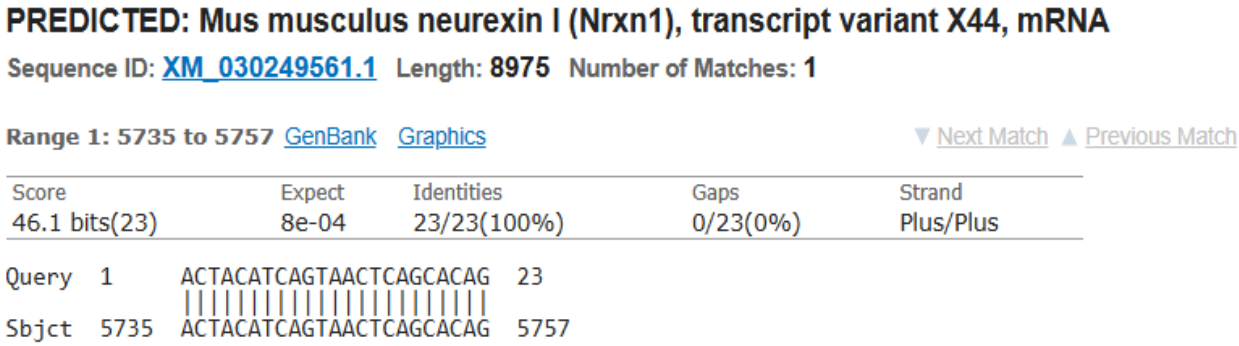

Figure 12 Nucleotide Blast of NRXN1 Forward Primer for Checking Specificity in *Mus musculus*. The figure shows a nucleotide blast where the selected Forward primer of NRXN1 is binding with the sequence of Mus musculus.

2.10.2 Reverse primer

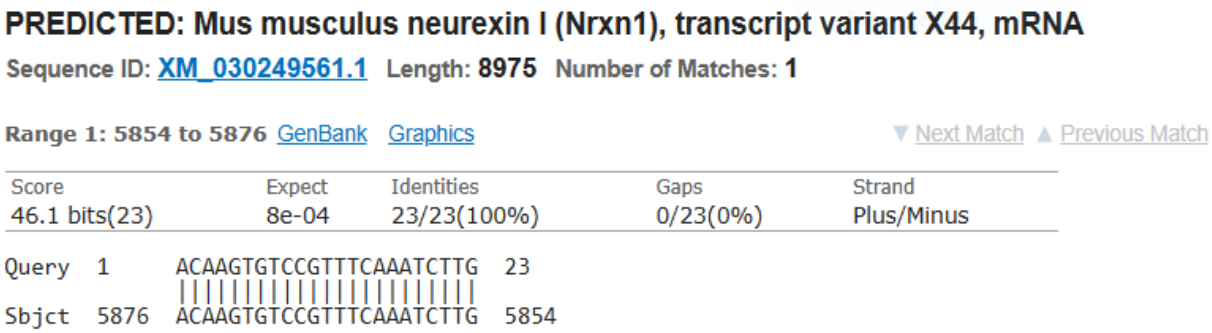

Figure 13 Nucleotide Blast of NRXN 1 Reverse Primer for Checking Specificity in *Mus musculus*. The figure shows a nucleotide blast where the selected Reverse primer of NRXN1 is binding with the sequence of *Mus musculus*.

2.11 cDNA

The RNA that was extracted endured quantification using the Nanodrop 2000 instrument (Thermo Scientific, USA). An equivalent amount of RNA (2ug) was transcribed into cDNA. To do so, 2µl of RNA, 4.5µl of 10 mM dNTPs, and 4.5µl of 5 mM oligo dts were utilized. The mixture was then incubated at 55°C for a duration of five minutes. The next step entailed adding 12ul of RT buffer, 6ul of DTT, and 3ul of RT enzyme, along with 14.5ul of nuclease-free water.

2.12 Gradient PCR optimisation

The annealing temperature conditions were set to 46˚C, 47.9˚C, 49˚C, 50˚C, 52˚C and 54˚C for the six blocks of the Gradient PCR Fig 14 (b). The PCR cycles included the initial denaturation temperature being set to 95 degrees for 30 seconds, second denaturation was set at the same conditions that is 95 degrees for 30 seconds. Annealing temperatures were set at 46 for 40 cycles for each temperature Fig 14 (a).

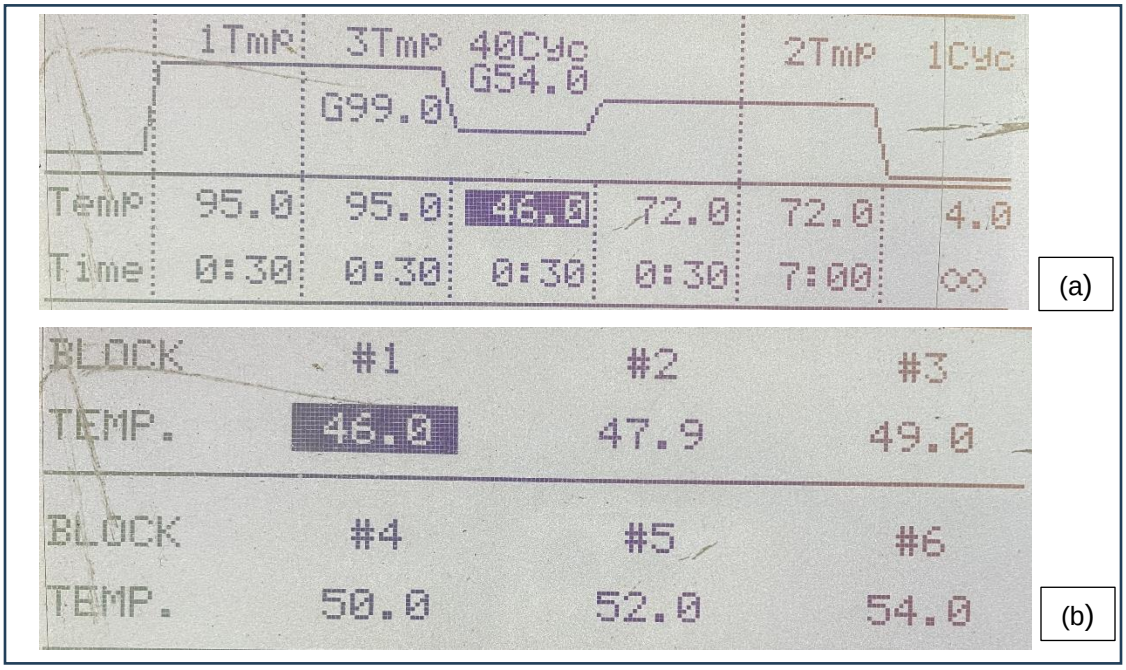

Figure 14 PCR Optimization Conditions. (a) This figure illustrates the PCR conditions used for primer optimization. (b)This figure depicts a range of temperatures for optimizing the effective binding of NRXN1 to the cDNA samples.

2.13 RT-PCR

Real-time polymerase chain reaction was executed utilizing the ABI Prism 7300 Sequence Detection System (Applied Biosystems, 7300). Following the preparation of a reaction mixture comprising 4ul of WizPure^{TM} qPCR Master (SYBR), 1ul of specific forward and reverse primers (Table 1), and 1ul of cDNA template, the volume was increased to 20µl using DNase-free water. The thermo cycling settings consisted of initial denaturation for thirty seconds at 95°C, second denaturation for thirty secondsat 95°C, 40 cycles of thirty seconds at 46°C, thirty seconds at 72°C, and seven minutes at 72°C Fig 15. The values obtained were analyzed about gene expression using their ΔC_t values after all values were normalized to those obtained for ß-actin.

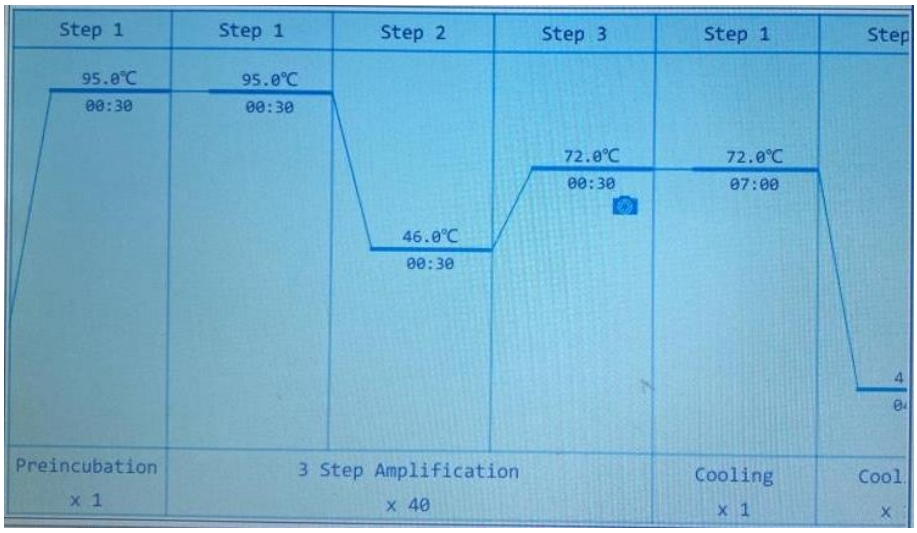

Figure 15 RT-PCR Conditions. This figure represents RT-PCR conditions used to find to expression of NRXN1.

2.14 Statistical Analysis

The results are presented as the mean ± S.D. Data were analysed by the statistical analysis system (SAS) program. Comparison between the control and treated groups were analysed by mean SEM and their significance was established by ANOVA variance analysis with Tukey's multiple comparison test. Differences of $P < 0.05$ were considered statistically significant.

CHAPTER 3: RESULTS

3.1 *In silico* Analysis

The results suggest that the proteins under study that is Neurexin 1 and clozapine are binding when a program is run in Pyrex to dock the respective molecules. These findings validate that there is bonding present between the selected target protein as well as the drug therefore, RT-PCR analysis can be used for quantifying the expression of NRXN1 in PD and its relative expression that has been altered because of drug clozapine Fig 16.

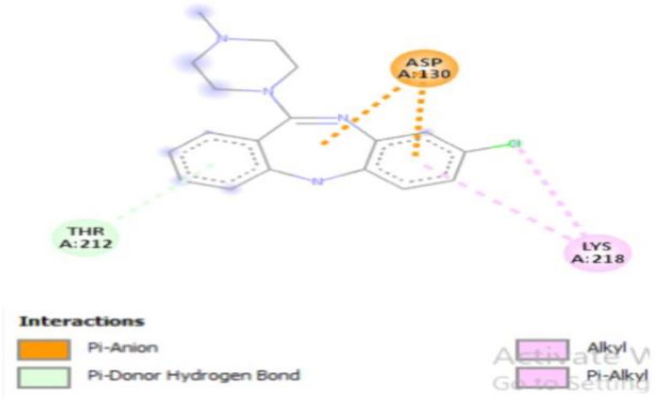

Figure 16 Binding of NRXN 1 with Clozapine. The Discovery Studio Software was used to dock both clozapine as well as neurexin 1 and find their bonds.

The table 2 below shows the different binding energies of the clozapine and neurexin complexes.

Table 2 Bindig Energies. The different binding energies of the complex formed between NRXN1 and Clozapine.

No.	COMPLEX	BINDING AFFINITY
1	NRXN1-CLZ	-6.7
2	NRXN1-CLZ	-6.4
3	NRXN1-CLZ	-6.1
4	NRXN1-CLZ	-6

5	NRXN1-CLZ	-5.9
6	NRXN1-CLZ	-5.9
7	NRXN1-CLZ	-5.8
8	NRXN1-CLZ	-5.8
9	NRXN1-CLZ	-5.7

3.2 Histopathology

3.2.1 Cortex

The H&E-stained section of the cortex in the control group revealed normal-looking cortical neurons have rounded vesicular nuclei. Mice given MPTP displayed shrinking, degenerating neurons. Clozapine-treated mice showed improvement with less degeneration and shrinkage of neurons Fig 17.

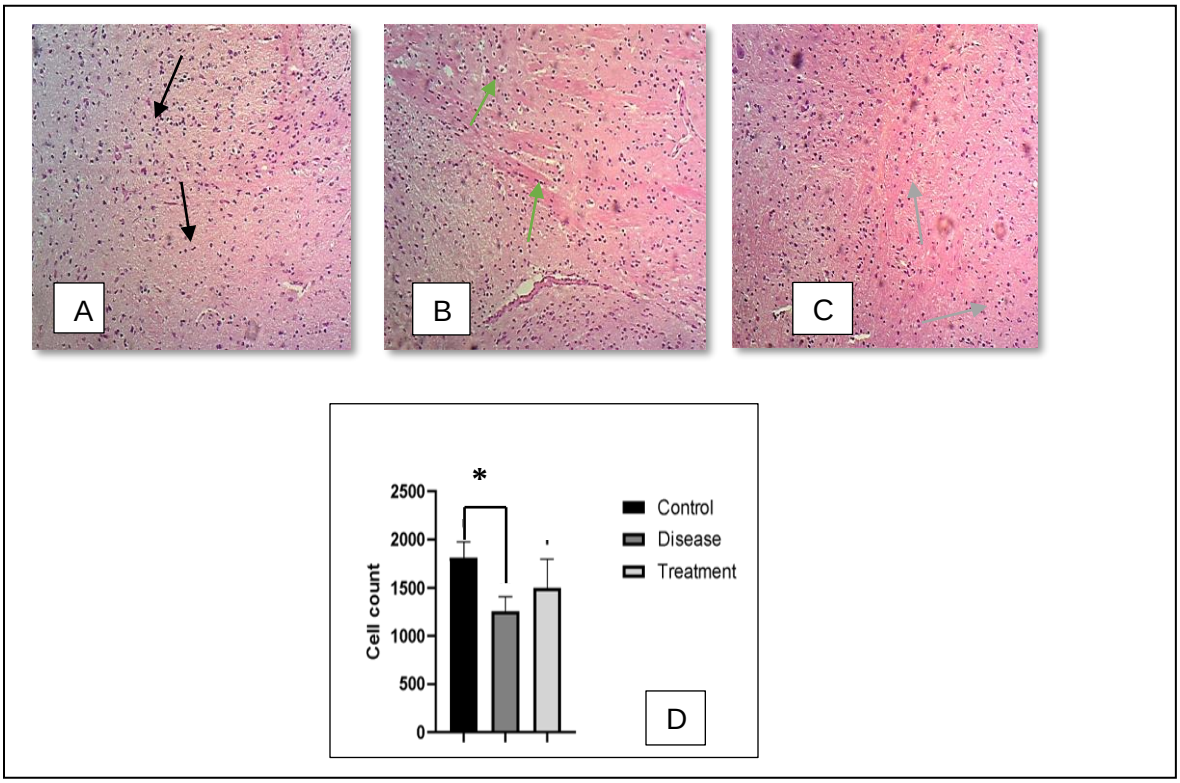

Figure 17 The section of the cortex stained with H&E and viewed at the magnification of 10X. The section of the cortex was stained with H&E and viewed at a magnification of 10X. A) Control mice showed the Cortical Neurons with rounded nuclei shown with black arrows ↑. B) Diseased mice treated with MPTP showed degenerated shrunken neurons↑, the shrunken nuclei leave their actual spaces in the brain↑. C) The treatment group of mice treated with clozapine showed comparatively less degeneration and shrinkage of neurons. D) The morphometric results showed that cell count in the cortex decreased in the diseased group (MPTP-treated) as compared to the control group (vehicle-treated) and treatment group (clozapine-treated).

3.2.2 Hippocampus

The hippocampus sections of the control mice showed that they retain their cellular form as well as nuclear shape however the MPTP-treated hippocampal regions of the mice showed that they lost their regular round cellular. Moreover, there is cell shrinkage evident too. The Clozapine treated hippocampal sections of the mice showed that there is maintenance of the cellular form and rather lesser shrinkage Fig 18.

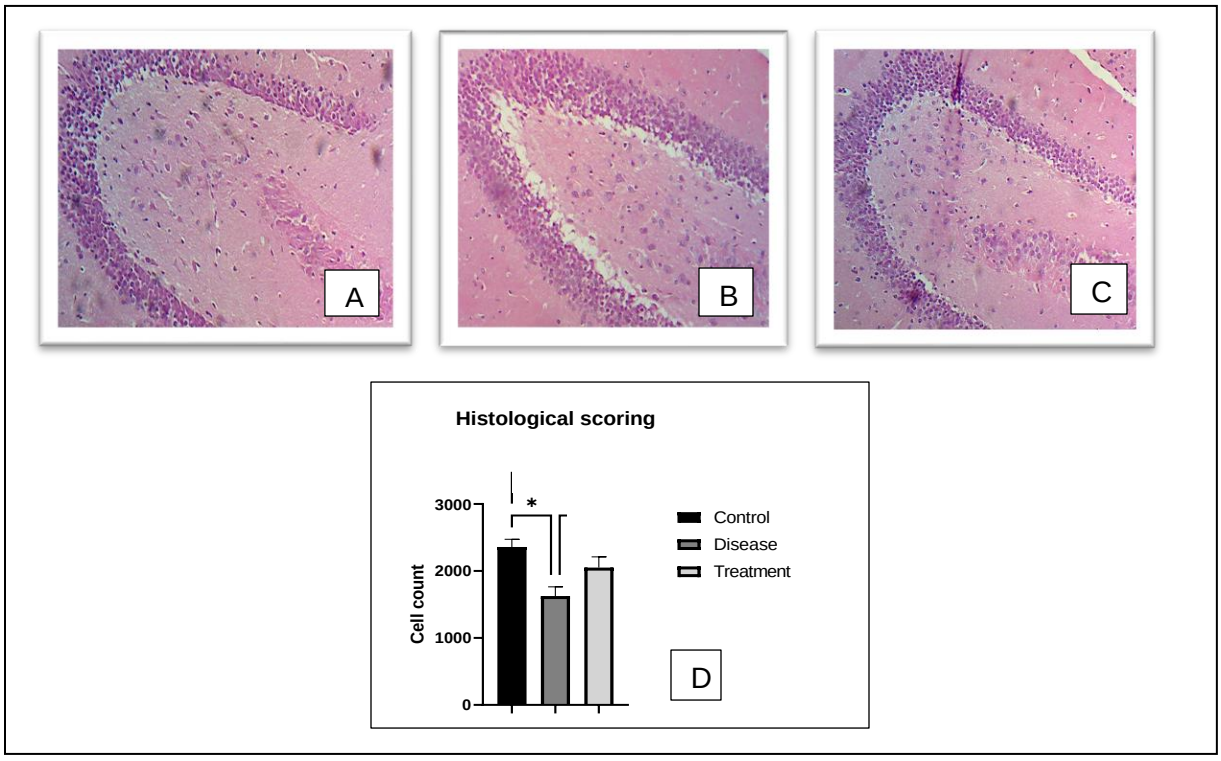

Figure 18 The section of the hippocampus stained with H&E and viewed at the magnification of 10X. A) The hippocampus of the mice brain from the control group shows round nuclei at the inner corner. B) The hippocampal sections of the disease mouse brain show shrinkage and white spaces around the inner corner. C) The treatment group's hippocampus shows fewer white spaces or shrinkage. D) A statistical analysis graph showing the difference between all three groups where control and treatment have similar trends.

3.3 Behaviour Tests Analysis

3.3.1 Open Field Test

In the open field test, both mouse groups engaged in autonomous exploratory behaviour and activity. Mice in the normal or control group were more active than those in the PD-induced group in terms of total distance travelled ($P < 0.001$).

3.3.1.1 Central and Peripheral Open Field Test

The results of anxiety level and exploratory activity level depend on how much time a mouse spends in the central or peripheral area. A significantly increased amount of time was observed control group as compared to a diseased group within the central area. It confirms reduced anxiety level and increased exploratory activity level in the control group whereas increased anxiety and exploratory activity level were observed in the diseased group as represented by the p-value <0.01 Fig 19.

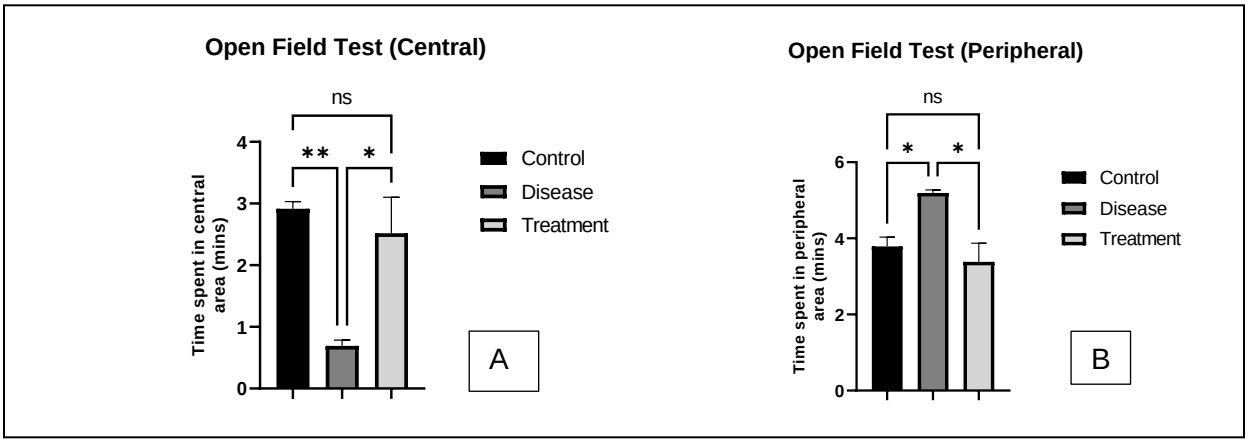

Figure 19 Open Field Test after the Administration of Clozapine. This graph shows the effects of clozapine and the time duration spent in the central area vs. the peripheral area in the open field test. Comparison with disease and control group using one-way ANOVA test. Data is presented as presented as mean ± SEM, ns = non-significant; *p<0.05, **p<0.01.

3.4 Gradient PCR Optimisation

The Gradient PCR showed bands at 46˚C with 141 base pairs as shown in the figure below. A range of temperatures was used for the optimisation including 44 to 52˚C. The best and most prominent band was visible at the temperature of 46 degrees Fig 20.

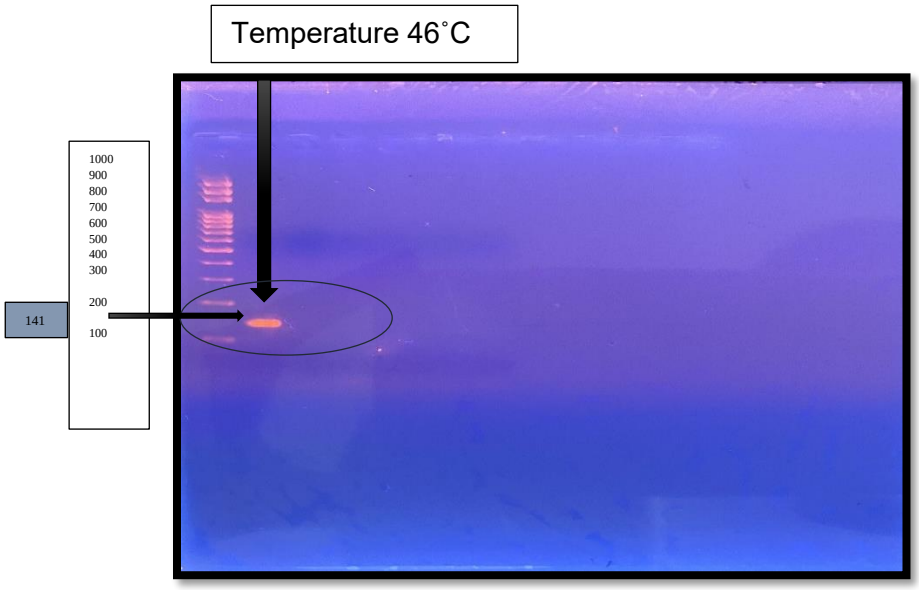

Figure 20 Band shown at 46 degrees during optimisation. The gel shows a dense band of 141bp at 46°C which was found to be the best.

3.5 RT-PCR:

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 NRXN1 mRNA expression was downregulated in the mice treated with MPTP and upregulated in the mice treated with clozapine Fig 21.

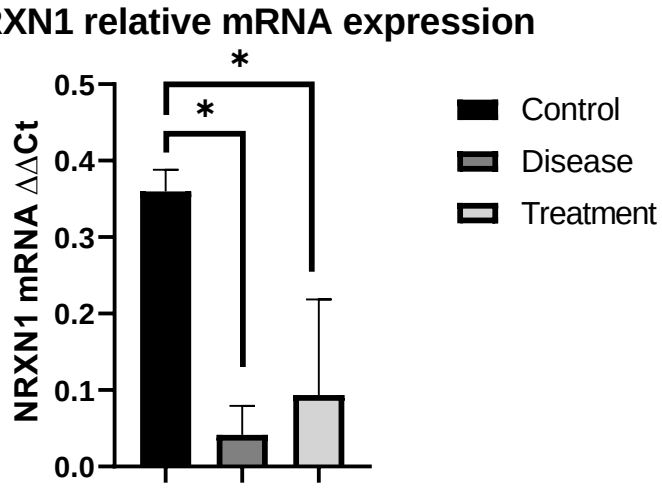

Figure 21 Relative Expression of NRXN1 mRNA. NRXN1 mRNA expression in the mice treated with MPTP for seven days showed downregulation as compared to the treatment with clozapine for 45 days which showed upregulation. Data is presented as mean +SEM. The non-parametric one-way ANOVA was employed for statistical analysis, followed by Tukey's multiple comparison test.

CHAPTER 4: DISCUSSION

Mouse models are used the most to study about PD induced through MPTP. Despite the fact that it might result in PD-like characteristics in mice, its efficacy could differ depending on the kind of animal or even on individual variants among the same species. Mice have a tendency to exhibit greater resilience as well as resembling responses to MPTP. The amount administered along with the period of exposure (20 mg/kg in this study) affect MPTP's efficacy. I.P. injections were used to produce MPTP. The neurotoxicity as well as its spread and intensity can be affected by the route used.

PD is not caused by MPTP directly; rather, it is converted to its final toxic form that is 1-methyl-4-phenylpyridinium ion (MPP+) within the glial cells of the brain, a substance that is lethal to dopaminergic neurons. An animal's vulnerability to this toxin may depend on how effectively it converts MPTP into MPP+. Age and genetic makeup of the animals can affect how susceptible they are to MPTP-induced toxicity. Animals who have certain genetic predispositions or who are older could be more vulnerable therefore a literature reviewed age of 6-8 weeks of mice was used for effective PD induction.

NRXN1 RT-PCR study after of MPTP induction for PD in a mice model provides information on the molecular processes behind this neurodegenerative condition. Presynaptic adhesion of NRX1 receives special focus because of its importance during the formation of synapses as well as maintenance, two procedures essential for typical brain function. The outcomes obtained from the RT-PCR tests showed that MPTP treatment significantly changed the expression of the protein NRXN1. NRXN1 mRNA expression was downregulated within the striatum as well as substantia nigra of mice who received MPTP, suggesting that it may play an integral part in causing PD. This result coincides with line with earlier studies that have shown synaptic disruption to be a distinctive feature of PD.

Additionally, it's possible that these variations in NRXN1 expression will result in larger effects on dopaminergic neurotransmission as well as neuroinflammation. Numerous presynaptic and postsynaptic proteins, particularly neuroligins along with other neurotransmitter receptors, have

been demonstrated for their interactions with NRXN1. NRXN1 expression fluctuations may interfere to these processes by impairing the plasticity of synapses or the production of neurotransmitters, two key factors in the motor and cognitive impairments that accompany PD. NRXN1 might exhibit indirect synaptic activities along with the potential neuroinflammatory modulation. The course of PD may be correlated with neuroinflammation, according to the latest research. The alterations in NRXN1 expression may help activate microglia along with generating proinflammatory cytokines, which would exacerbate the neurodegenerative cascade. It is necessary to conduct more research to determine the specific procedures that occur when NRXN1 plays a role in PD pathogenesis. NRXN1 as well as its related pathways could be targeted as a potential treatment approach to slow the onset of PD and lessen its crippling symptoms.

4.1 Neuronal Loss

According to Aarsland et al. (2017), cognitive dysfunction is a common and severe non-motor impact of PD. Approximately eighty percent of people with PD are likely to acquire Parkinson's disease dementia (PD-D) within the course of the illness, according to studies of the overall incidence of mild cognitive impairment, or MCI, in the disease, which ranges from 27% to 40%. It is widely accepted via neuroimaging studies that cognitive deterioration in PD is related to atrophy in frontal, temporoparietal, and occipital brain areas, notably the hippocampus as well as the basal ganglia. The hippocampus is where α-synuclein first causes cognitive problems; from there, it spreads across the cortex and causes dementia (Villar-Conde, Astillero-Lopez, & Gonzalez-Rodriguez, 2021).

The basal ganglia, a group of brain areas responsible for coordinating activity, are the primary target in PD. Even though the basal ganglia serve as an essential part of PD, the cortex, the brain's outer layer, is also connected to various aspects of the condition. Whilst the brain additionally serves a role in controlling movement, the basal ganglia are assumed to be the primary location of motor failure in PD. The planning, beginning, and execution of voluntary acts are aided by the signals that the basal ganglia send to the brain in the form of feedback. Impairment of this communication among the brain as well as basal ganglia may result in the classic motor symptoms of PD, such as tremors, stiffness, and bradykinesia. In addition to the typical motor symptoms of PD, cognitive and non-motor symptoms are also possible. The cortical portions of the brain are in

charge of higher-order cognitive functions like memory, attention, decision-making, and executive function. Cognitive abnormalities in PD patients often include memory and executive functioning problems. These cognitive deficits are linked to changes in cortical regions, especially in areas like the prefrontal cortex.

The degeneration of neurons that produce dopamine in the substantia nigra, a section of the basal ganglia, is a defining characteristic of PD. However, the dopamine system also enters the cortex. Cognitive processes and emotional control are influenced by the dopamine levels in the cortex. Numerous parts of the brain provide dopaminergic signals to the cortex. PD causes a dopamine shortage in the brain and basal ganglia, which contributes to the disease's wide range of motor and non-motor symptoms. In PD, the brain accumulates abnormal protein clumps like α-synuclein. These aggregates can go from the basal ganglia to the cortex and other regions of the brain. It is believed that this dissemination speeds up the development of cognitive and non-motor symptoms in the later stages of the illness. The cortex has an impact on the brain's ability to modify and rearrange its neural connections in response to environmental stimuli. The brain changes in PD to compensate for dopamine depletion and other abnormalities. These changes may affect how the brain connects and functions.

4.2 Improvement in Memory

Open Field Test is frequently utilised in psychological and neuroscience examinations to evaluate multiple elements of mouse behaviours, notably anxiety, exploration, locomotion, and the overall amount of engagement. Motor dysfunction, particularly bradykinesia along with reduced voluntary muscular movement, constitutes one of the main signs of PD. By analysing mice's movements, Open Field Test depicts the overall functioning of motor symptoms. PD mice move less freely, have less coordination, and encounter trouble beginning movements. Motor deficits resembling those reported in PD patients in humans are frequently seen in animal models of the disease. The Open Field Test can be used to measure modifications in locomotor activity, such as slowed movement, altered movement patterns, and less exploration. Anxiety is one of the non-motor symptoms of PD. By examining how much time mice spend in the center vs. the periphery of the open field box, researchers may gauge anxiety-like behaviour in rodents. Increased anxiety-like behaviour may point to a link between brain changes brought on by PD and emotional responses. PD might affect a person's curiosity about new environments. Variations in

the amount of rearing (standing on the back legs to scan the area) and the distance travelled during exploration in the Open Field Test can shed light on variations in exploratory behaviour. The Open Field Test can be used to assess the efficacy of possible PD treatments. To evaluate if experimental medications or interventions improve motor impairments, anxiety-like behaviours, or other related symptoms, researchers can administer them.

4.3 Neurexin Expression

A growing body of research indicates that synaptic dysfunction occurs during neurological psychiatric conditions like schizophrenia, bipolar disorder, as well as autism spectrum disorders as well as neurodegenerative disorders like PD, AD, and Huntington's disease. The pathophysiology of many different brain disorders involves the synapse in such a prominent way that the word "synaptopathies" was created to describe them. Indeed, it has been proposed that synaptopathy, which occurs before a neuronal loss in the case of PD, is a primary and fundamental event in the etiology of the disease. Mutations that change the structure and operation of synaptic components or aberrant levels of expression of a synaptic protein can lead to synaptic dysfunction. Diseases that change the form and operation of synaptic components or aberrant levels of expression of a synaptic protein can lead to synaptic disorders. The cell adhesion proteins that link the presynaptic and post-synaptic compartments are one type of synaptic proteins that are crucial to their biology. One class of synaptic cell adhesion molecule that has recently attracted greater pathogenic attention is neurexins. Therefore, the expression levels observed through RT-PCR suggest that neurexin 1 is downregulated in the PD models while it is upregulated when treated with an antipsychotic drug clozapine.

Understanding the controls on neurexin expression can help explain the true cause of PD. The downregulated neurexin expression linked to PD can offer insight into the pathology of synaptic failure in the disorder. The expression of Neurexin as well as control values may act as PD biomarkers. Regarding early identification of disease, disease tracking, including analysing the effectiveness of therapy, and biomarkers are helpful. Neurexin isoforms or expression patterns that are consistently linked to PD may be employed as prognostic or diagnostic indicators.

The regulation of neurexin expression can help in the formulation of personalised medicine techniques. Therapies can be personalised for each person with PD if they have certain neurexin-

related genetic or expression profiles, which might improve the results of treatment. For instance, altering neurexin activity in PD mice has helped us better understand how neurexin malfunction is associated with PD. Neuroprotective benefits in PD can originate from treatments that improve or restore neurexin expression. These treatments could stop future dopaminergic neuron loss and reduce the disease's progression.

Conclusion:

According to research, MPTP is a neurotoxin that specifically damages dopaminergic neurons in the substantia nigra region, where it causes PD symptoms that are permanent. A mouse disease model has been developed. The MPTP-induced PD mouse model's behavioural and motor activity showed some significant alterations. This study unveils the critical role of NRXN1 in synaptic activity via MPTP-induced PD mice models while keeping in view the neuroprotective effect of clozapine. The study design reveals that clozapine treatment on MPTP-induced mice helps in the up-regulation of the protein NRXN1. The maintenance of dopaminergic neurons in the brain regions and the improvement of motor and cognitive impairments seen in behavioural tests are both examples of the impact brought about by the antipsychotic drug. Neurexin serves as a potential protein for future perspective in terms of PD treatment.

In the last couple of decades, it has become obvious that there is a connection involving synaptic disruption with both neurodegenerative and neuropsychiatric illnesses. The literature-based analyses turned up several papers that connected neurexin depletion or altered expression to various diseases. The scientific proof is strongest for neurexins' contribution to neuropsychiatric diseases, especially when it comes to neurexin 1. Neurexins may play a role in these illnesses, according to various experiments, but additional research findings remain necessary before any firm conclusions can be made at this time. Additional focused research on the numerous illnesses involving these genes and the proteins they encode is necessary.

Future Prospects:

- NRXN1's function in PD is now well understood, opening up new treatment possibilities. Treatments that alter NRXN1 activity along with how it interacts with additional synaptic

proteins can be studied further. Neurexin-1-targeting medications or gene treatments may provide fresh opportunities to reduce or perhaps stop PD growth.

- In future the distinction between different subcategories of neurexin in PD patients will be easier as our knowledge of NRXN1's role in PD expands. This insight might result in tailored therapies that enable more efficient and patient-specific therapy.
- Findings on NRXN1 might be included into a combination approach for addressing PD. NRXN1-targeted strategies could prove synergistically beneficial in enhancing the results for patients when combined with already-effective therapies like dopaminergic drugs or deep brain stimulation.
- NRXN1 changes might be used as an indicator/biomarker to diagnose PD, track its course, or gauge how well the therapy is working if they are always related to the condition. NRXN1 analyses conducted using blood or CSF may prove to be useful in clinical practice.

APPENDICES

Appendix A
Calculations of MPTP doses:

Table 3: 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 = 1.04 mg

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 1.04 mg then the total amount of solution will be calculated as follows:

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

6.2. 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 4: Clozapine treatment regimen.

	MOUSE WEIGHT (kg)	**CLOZAPINE DOSAGE (mg/kg)**	**CLOZAPINE DOSAGE (mg)**	**TOTAL STOCK SOLUTION (ml)**	**CLOZAPIN E 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., 2.5 mg/kg)

2. Prepare Clozapine stock solution:

Dissolving the calculated clozapine dosage in distilled water to give the stock solution of 10 mg/ml.

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