Chapter 5

Neuroprotective effect and mechanism evaluation of a top-screened chaperone in 6-OHDA-induced rat PD Model

5 Introduction

Glucocerebrosidase (GCase) enzyme paucity is the unavoidable peril factor for developing Parkinson's disease (PD). PD is a profound and intricate neurodegenerative disorder characterized by the progressive loss of nigral dopaminergic neurons (SNc), resulting in a debilitating array of motor symptoms, including rigidity, tremor, and bradykinesia. This irreversible condition also entails a reduction in striatal dopamine (DA) levels and the presence of α-synuclein aggregates known as Lewy bodies, which further contribute to the pathogenesis of the disease (Michel, Tadros et al. 2022, Motawi, Al-Kady et al. 2022). There are currently no approved medicines for GCase-associated PD. Efforts to impede the progression of PD have shown promise through the use of medications that can effectively prevent or reduce the course of the disease (Kalia, Kalia et al. 2015). A potential breakthrough in this area is the development of a disease-modifying treatment known as Ambroxol (AMB), which acts as a chaperone for glucocerebrosidase (GCase) (ClinicalTrials.gov Identifier: NCT02914366) (Silveira, MacKinley et al. 2019) . This treatment is targeted towards PD patients with dementia. Sub-chronic treatment involving AMB exhibits disease-modifying effects by enhancing GCase activity in a rat model of PD induced by 6-hydroxydopamine hydrochloride (6-OHDA), a neurotoxic compound (Mishra and Krishnamurthy 2020). Furthermore, previous studies have already established the neuroprotective properties of AMB (Bhardwaj, Arunachalam et al. 2016). It is important to note that these beneficial effects were observed primarily at higher doses of AMB (ranging from 800 to 1260 mg/kg, orally administered), potentially attributable to limited penetration through the blood-brain barrier. Previous studies revealed that the novel compound, GC466, increased GCase activity in the PD cell line model, albeit its in *vivo* anti-PD effects are yet unknown.

Hence, there is a huge unmet need for focused research to comprehend the molecular mechanisms behind GCase-associated PD pathology and to establish new therapeutic approaches that can aid in the prevention and treatment of PD pathogenesis.

Endoplasmic reticulum stress (ERS), a major cause of GCase-associated PD pathogeneses (Gómez-Suaga, Bravo-San Pedro et al. 2018). **Chapter I** described the details of GCase-ER associated PD pathogenesis. Increased levels of ERS markers have been found in PD brains with unstable GCase. Moreover, GCase deficit *drosophila* resulted in increased levels of the ERS markers including other PD markers such as dopaminergic neuronal loss and locomotors defect (Sanchez-Martinez, Beavan et al. 2016). However, the molecular mechanisms by which GBA deficiency increases the risk of PD due to ERS are still largely unknown; however, inhibiting the ER stress by stabilizing or enhancing GCase activity may be a crucially effective approach for PD treatment. Thus, GC466's *in vivo* anti-PD effects on 6-OHDA-induced PD in rats were assessed in this work, along with how ERS-GCase signaling dysfunction is associated with PD and how GC466 modulates it.

Therefore, our primary objective was to introduce a novel compound called GC466, which acts on glucocerebrosidase (GCase), and for the first time, evaluate its potential as an anti-PD agent using a comprehensive molecular mechanism in a rat model induced by 6-hydroxydopamine (6-OHDA). To evaluate the motor abnormalities associated with PD, we employed a range of behavioural tests to assess the impact of GC466. Additionally, we quantified the expression levels of two key proteins, namely oligomeric α-synuclein (pS129 α-syn) and tyrosine hydroxylase (TH). Furthermore, we measured the severity of neurochemical changes in PD by analyzing striatal dopamine levels. To investigate the loss of dopaminergic cells in the substantia nigra, we performed analyses involving TH-positive staining as well as nissl's staining of neurons. Moreover, we explored the effects

of GC466 on GCase activity and examined the markers associated with endoplasmic reticulum stress (ERS)-mediated apoptosis. **Figure 5.1** presents a graphical representation depicting the schematic diagram outlining the proposed hypothesis concerning the molecular mechanism underlying the pathogenesis of PD associated with GCase-ERS in a rat model induced by 6-OHDA (6-hydroxydopamine). The experimental design, including the specific procedures and methodology employed, is illustrated in **Figure 5.2,** providing further details regarding the study.

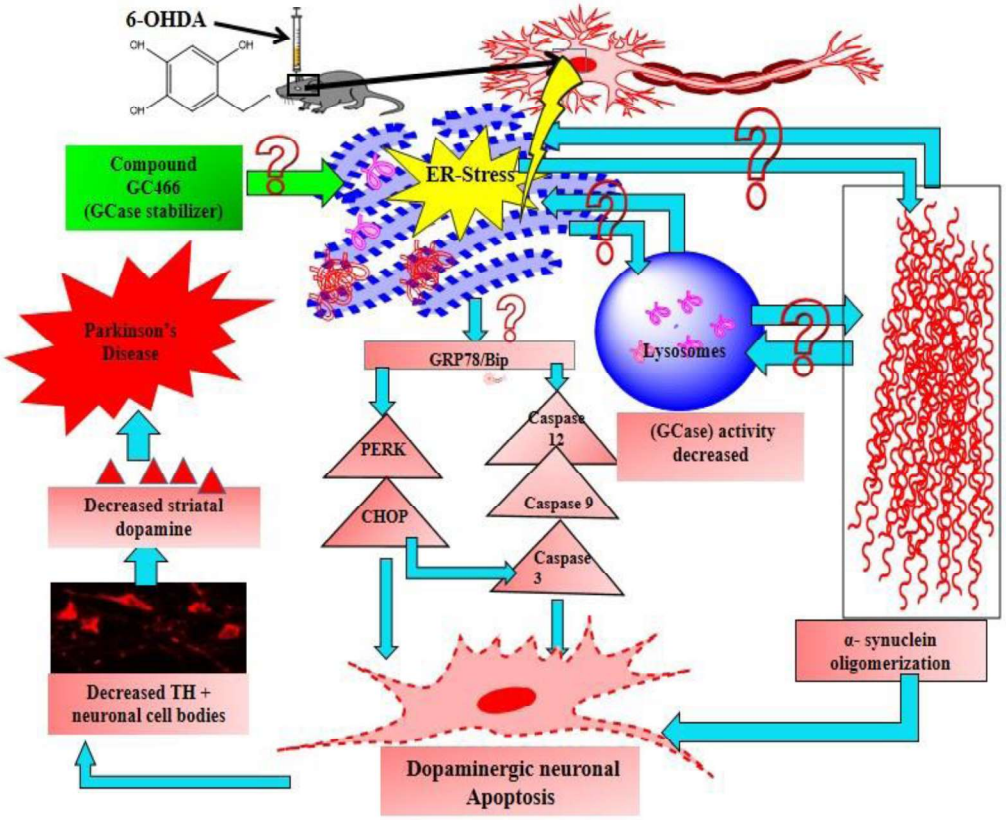

Figure 5.1 presents a graphical representation depicting the schematic diagram outlining the proposed hypothesis concerning the molecular mechanism underlying the pathogenesis of PD associated with GCase-ERS in a rat model induced by 6-OHDA. The impact of 6-OHDA on GCase enzymatic activity may be attributed to the involvement of 6-OHDA-induced ER-stress dysfunction and α-synuclein aggregation. GC466 due to its GCase-stimulating activity may act against 6-OHDA-induced toxicity by attenuating ER-

stress mediated apoptosis, α-synuclein pathology, loss of nigral dopaminergic cell, and reducing motor impairment, and concurrently enhancing the levels of striatal DA content.

5.1 Materials and methods

5.1.1 Experimental animals and their ethical statement

Male albino adult rats weighing approximately 230 ± 20 g, were sourced from the Central Animal House of the Department of Zoology, Banaras Hindu University. These rats were carefully maintained in the same facility throughout the acclimation and experimental periods, ensuring a constant temperature of 25 ± 2 °C and a humidity level of 60 ± 10%. To eliminate gender differences, only male rats are used in the experiment. The \institutional animal ethical committee of Banaras Hindu University conducted a comprehensive review of all animal care and experimentation protocols, granting approval for the study under the reference number BHU/DoZ/IAEC/2021-2022/017. In line with internationally recognized standards, the National Institutes of Health's guide for the care and use of laboratory animals, specifically NIH Publications No. 8023 (revised in 1978), was strictly followed in the execution of every experiment. These guidelines ensure the welfare and appropriate handling of laboratory animals. The experiments were carried out within a specific timeframe, from 08:00 to 17:00 h, to maintain consistency and control for any potential time-dependent factors.

5.1.2 Materials

The GCase-acting compound GC466 (MolPort-029-998-466) was sourced from the reliable Molport database https://www.molport.com/shop/index. The 6-OHDA used in the experiment was acquired from Cayman Chemicals. For the study, Dopamine (Catalog No: KLR0219) and the Caspase-12 ELISA Kit (No: ab65664) were specifically purchased from Krishgen Biosystems and Abcam Biotechnology, respectively. To carry

out the research, various high-quality reagents were employed. TRI reagent, 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG), and cresyl violet acetate were obtained from Sigma-Aldrich (St. Louis, MO, USA), ensuring their analytical grade. The cDNA Synthesis Kit and Powerup SYBR Green mastermix were procured from Thermo Fisher Scientific. Additionally, all the primer sequences (**detailed in Table 5.1**) were sourced from Eurofins Scientific. To facilitate the experiments, primary antibodies were purchased from reputable suppliers. Tyrosine hydroxylase (TH) (No: E-AB-70077) rabbit primary antibodies, caspase 3 (No: E-CK-A311, ELISA Kit), goat anti-rabbit IgG (HRP) (No: E-AB-1003), and goat anti-rabbit IgG (H&L) (Rhodamine/TRITC conjugated) (No: E-AB-1053) secondary antibodies were acquired from Elabscience Biotechnology Co., Ltd., based in the USA. However, the phospho-α-synuclein (pS129 α-syn) rabbit primary antibody (No: 23706T) was obtained from Cell Signaling Technology.

5.1.3 Animal surgical operation for 6-OHDA administration

The rats were carefully anesthetized using a combination of Ketamine and Xylazine (100/10 mg/kg, i.p.) prior to the necessary preparations for the experiment. First, the fur on their heads was gently trimmed, and then they were securely positioned on a stereotaxic frame. To precisely locate the bregma, the skin covering the skull was delicately removed, and a guide cannula was employed to target specific coordinates within the striatum, which were identified as 1.0 mm anterior, 3.0 mm lateral, and 5.0 mm ventral (A/P + 1.0, L/M + 3.0, D/V - 5.0) in relation to the bregma and ventral from the dura, according to Paxinos and Watson's reference guide (GeW 1998, Paxinos and Watson 2006). For inducing unilateral degeneration of dopamine (DA) in the striatum, a hole of 1.5 mm depth was carefully created, followed by the administration of 20 µg of 6-OHDA (4 µl of a freshly prepared solution containing 5 µg/µl in normal saline with 0.2 mg/ml ascorbic acid) into the left striatum. This was done using a 5 µl Hamilton syringe

connected to a polyethylene tube. To ensure the proper diffusion of 6-OHDA, the injection rate was set at 1 µl/min, and the needle was left in place for an additional 5 minutes. To perform these precise microinjections, a reliable stereotaxic injector from Stoelting (USA) was utilized. Following the surgical procedure, once the skin had been sutured, the animals were gently removed from the stereotaxic device and placed under a heat lamp along with a thermal blanket for 30 minutes. This careful measure was taken to maintain their body temperature at 37 ± 2°C before returning them to their respective cages (Mishra, Chandravanshi et al. 2018, Mishra and Krishnamurthy 2020).

5.1.4 Experimental design

For each of the five time points, namely D-0, D-7, D-14, D-21, and D-28, a randomized assignment of animals into seven groups was conducted, with each group consisting of 16 animals (n = 16). The grouping was as follows(1) Control group; (2) Sham group; (3) 6-OHDA group; (4) 6-OHDA + Selegiline 10 mg/kg *p.o* group (SEL; standard drug).; (5) 6-OHDA + GC466 6.25 mg/kg (GC-6.25, lower doses) *p.o.* group (6) 6-OHDA + GC466 12.5 mg/kg (GC-12.5, median doses) *p.o.* group; and (7) 6-OHDA+ GC466 25 mg/kg (GC-25, higher doses) *p.o.* group. Our prior experiment served as the basis for selecting the standard medication, and size was decided upon using G*Power analysis as previously described (Cohen 1988). The choice of standard drug and group sizes was informed by our previous experiment utilizing different drugs in the same model (Mishra, Chandravanshi et al. 2018, Mishra and Krishnamurthy 2020). The GCase-acting compound GC466 was freshly prepared daily in fresh water and administered at a maximum volume of 2 ml/kg. The sham group underwent the same procedures as the 6-OHDA group, except for receiving injections of saline with 0.2 mg/ml ascorbic acid instead of the 6-OHDA solution. The initial day (D-1) for the animals marked the day when they received the intrastriatal unilateral injection of 6-OHDA. The drugs were

administered to the corresponding groups continuously for 25 consecutive days, from D - 4 to D - 28 of the experimental design, commencing on D-4 when behavioral deficits were first observed. Behavioral assessments were conducted on D-0, D-7, D-14, D-21, and D-28, with the exception of apomorphine-induced rotating behavior, which was measured on D-4 as well (**D - 4 data provided in appendices, Figure 5.1**). Video cameras were used by observers who were unaware of the study methodology to record the behavioural parameters. On D-28, following the behavioural experiments, all animals were euthanized by decapitation, and the SNc and striatal tissues from the ipsilateral hemispheres were micro-dissected. The dissected tissues were immediately stored at -80 °C until further research. **Figure 5.2** depicts the experimental setup and provides information on the number of animals used for each testing parameter.

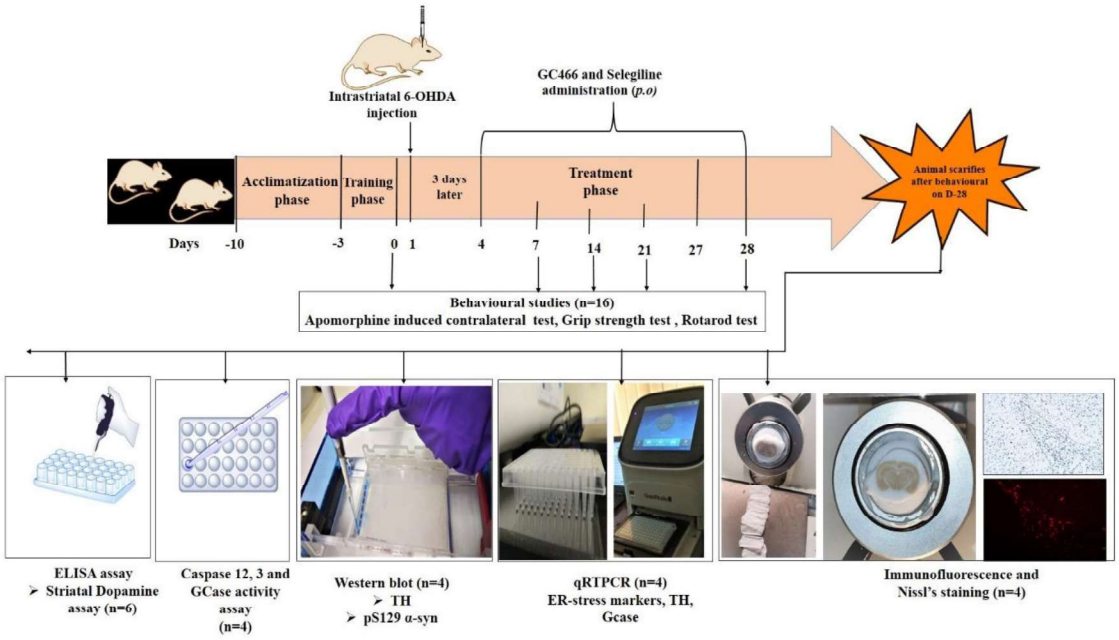

Figure 5.2 The research's experimental layout. The number "n" specifies how many animals from each group were employed.

5.1.5 Neurobehavioral studies:

5.1.5.1 Rotational behaviour test

The apomorphine-induced rotational behavior serves as a reliable physiological indicator of dopamine (DA) depletion and asymmetric DA receptor stimulation. To assess this behavior, an intraperitoneal injection of apomorphine hydrochloride (1 mg/kg) is administered. The rotational behavior is then observed and recorded for a duration of five minutes, specifically focusing on net rotations towards the opposite side (contralateral rotations). Prior to the surgical procedure, the rats are carefully monitored to establish a baseline for contralateral rotations. This ensures accurate comparisons and evaluations during subsequent testing sessions. The rotational behavior test is initially conducted on Day - 4, and subsequently repeated at the conclusion of each week over a period of four weeks (Mishra, Chandravanshi et al. 2018, Mishra and Krishnamurthy 2020).

5.1.5.2 Grip Strength Test

In order to obtain an accurate assessment of an animal's neuromuscular strength, a specific method was employed, as previously described (Mishra, Chandravanshi et al. 2018, Mishra and Krishnamurthy 2020). The animal was suspended by its front paws at the midpoint of a 90-cm-long metal wire with a diameter of 1 mm. This wire was securely fastened 50 cm above the ground. During the experiment, control animals demonstrated their agility by successfully climbing up the wire. However, the animals in the PD group encountered difficulties or were unable to maintain their balance on the wire. A cut-off time of 60 seconds was applied to prevent prolonged exposure. Careful observations were conducted, and the following ratings were assigned to each animal's performance:

Rating 0: The animal fell off the wire.

Rating 1: The animal clung onto the wire using its two forepaws.

Rating 2: Similar to Rating 1, but the animal also made attempts to climb further along the wire.

Rating 3: The animal clung onto the wire using its two forepaws as well as one or both hind paws.

Rating 4: The animal held onto the wire using all four paws, with its tail wrapped around the wire for added support.

Rating 5: The animal managed to escape from the apparatus, resulting in a fall onto a flat surface.

5.1.5.3 Rotarod test

Motor coordination skills were evaluated using the Rotarod, a specialized instrument manufactured by IKON Instrument in New Delhi, India. The rats were trained to walk on the Rotarod for two consecutive days, twice daily, with a consistent rotation speed of 8 revolutions per minute (rpm). This training aimed to ensure stable performances from the rats on the Rotarod. On the day of the test trial session, the rotational speed was adjusted to 15 rpm. Each animal underwent three trials on the rotating rod, and the duration it took for each rat to maintain balance was recorded. The average duration of the three trials was considered the final score, and the data were presented as retention times (Mishra, Chandravanshi et al. 2018, Mishra and Krishnamurthy 2020).

5.1.6 Measurement of striatal rat Dopamine level

The quantification of dopamine in the striatal tissues of rats was conducted using state-of-the-art Enzyme-linked immunosorbent assay (ELISA) plate readers and commercially available ELISA kits (KLR0219). Following a well-established protocol (Bradford 1976), the protein concentration was determined, and the findings were subsequently reported as the concentration of dopamine in ng/mg protein.

5.1.7 Immunoblotting

To extract the nigral protein lysate for immunoblotting, the following protocol was followed. The tissue was first homogenized at a concentration of 10% in RIPA buffer, which consisted of 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 2 mM EDTA, 10 mM sodium deoxycholate, and 0.1 mM PMSF. After homogenization, the mixture was centrifuged at 12,000 g for 20 minutes at 4 °C. To determine the protein concentration, the supernatant was collected in a microcentrifuge tube and the Bradford technique (Bradford 1976)was employed. Following this, the obtained supernatant from each group was denatured by boiling water. Subsequently, equal amounts (10 μg) of proteins were resolved using SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was then blocked for two hours with 5% non-fat skim milk prepared in Tris-buffered saline with 0.1% Tween 20 (1×TBST). For the identification of specific molecular weights, protein molecular weight markers (ladders) were utilized. These markers assisted in determining the molecular weight of proteins of interest, such as TH (~55 kDa) and pS129 α-syn (~15 kDa), and also facilitated tracking the progress of electrophoretic separation or transfer in the western blotting process. The blot was incubated overnight at 4 °C with the respective primary antibodies: rabbit tyrosine hydroxylase (TH, 1:1000, E-AB-70077, Elabscience), pS129 α-syn rabbit primary antibody (1:1000, #23706, Cell Signalling Technologies), and β-actin (1:1000; Santa Cruz Inc., USA). Following the primary antibody incubation, the blots were washed thrice with 1×TBST for 5 minutes each, and subsequently incubated with the corresponding HRP-conjugated goat anti-rabbit secondary antibodies (1:2000, Genei Laboratories Private Limited, India) for 3 hours. The blots were then washed again with 1×TBST three times for 5 minutes each. To detect the signal, X-ray film and ECL reagents (Western brilliant ECL; Advansta, USA) were employed. For

normalization, the same blot was utilized after being incubated for 4 hours with an anti-β-actin HRP-conjugated antibody (1:20,000, Sigma-Aldrich #A3854). Immunoreactivity was measured using the alpha imager 2200 program (Mishra and Thakur 2022).

5.1.8 Glucocerebrosidase (GCase) enzyme activity assay

The GCase-enriched fractions (GEF) were obtained and their activity was evaluated using a spectrofluorophotometric method with slight modifications, as described in previous studies [11, 40, 41]. In summary, approximately 5 mg of nigral tissue was homogenized in a solution containing 0.25 M sucrose and 1 mM ethylene diamine tetra acetic acid (EDTA). The homogenate was then centrifuged at 5000g for 10 minutes. Subsequently, to obtain the GEF, the resulting supernatant underwent an additional centrifugation step at 17,000g for 15 minutes at 4°C. For the enzymatic assay, 10 µl of the GEF was added to a mixture containing 75 µl of the 2.5 mM 4-MUG substrate. The substrate mixture also contained 0.5% Triton X-100 and 0.6% sodium taurocholate, all in a 50 mM phosphate-citrate buffer at pH 5.0. The combination was incubated at 37°C for 60 minutes. To halt the reaction, 200 µl of stop solution (0.3 M glycine/0.2 M sodium carbonate, pH 10.7) was added, and the resulting fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Enzyme activity was calculated in terms of nanomoles per hour per milligram protein (nmoles/hr/mg protein), following the established method described in previous publications (Migdalska-Richards, Daly et al. 2016, Tripathi, Ganeshpurkar et al. 2022)

5.1.9 Quantitative reverse transcription PCR (qRTPCR)

In adherence to the MIQE recommendations (Bustin, Benes et al. 2009), all relevant guidelines for quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) were meticulously followed. Frozen nigral tissue from each experimental group was processed by homogenizing it in 1 ml of cold Trizol reagent (Sigma Aldrich) using an

ultrasonic processor (Cole-Parmer, Vernon Hills, IL). The resulting RNA was then dissolved in 50 µl of nuclease-free water after vacuum drying. To determine the concentration and purity of the RNA, absorbance at 260 nM was measured using a Nanodrop spectrophotometer (Bio Spectrometer, Eppendorf). The integrity of the RNA was assessed using an agarose gel-based band detection system and an electrophoresis station (GeNei Mini Horizontal Gel Unit). For cDNA synthesis, the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was employed, using 5 µg of RNA. Subsequently, qPCR was performed using the PowerUp SYBR Green master mix (Thermo Fisher Scientific, USA) and 1 µl of the generated cDNA as templates. The QuantStudioTM 5 Real-Time PCR System was used for qPCR amplification of both Endoplasmic Reticulum Stress (ERS) specific marker genes and other genes of interest. The thermal cycling conditions involved an initial denaturation at 95°C for 15 s, followed by annealing at 60°C for 30 s, and extension at 72°C. This cycle was repeated for 40 cycles after an initial denaturation at 95°C for 15 min. The specific primer sequences used for PCR amplification are listed in **Table 5.1**. As an internal control for normalization, a primer for β-actin was included and quantified in parallel reaction samples. The fold change in gene expression of the target genes was determined using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008, Mishra and Thakur 2022), where ΔCT represents the difference in threshold cycles between the target gene and the β-actin reference gene, and ΔΔCT is the difference in ΔCT values between the experimental and control groups.

Table 5.1 Primer sequences used for qRTPCR

Name	**Primer sequences**
GRP78/Bip	F - 5'-CACTTGGTATTGAAACTGTGGG-3' R- 5'-TGTTACGGTGGGCTGATTAT-3'
Caspase 12	F - 5′-GGCCGTCCAGAGCACCAGT-3′

	R- 5'-CAGTGGCTATCCCTTTGCTTG-3′;
Caspase 9	F - 5'-AGTTCCCGGGTGCTGTCTAT-3'
	R - 5'-GCCATGGTCTTTCTGCTCAC-3'
Caspase 3	F - 5'-GAATGTCAGCTC GCAATGGTA C 3'
	R - 5'-AGTAGTCGCCTCTGAAGAAACTAG-3'
PERK	F- 5′- TCCTGTCTTGGTTGGGTCTG-3′
	R - 5′-TGCGTGCTCCGCTTATTC-3′
CHOP	F- 5′-GTCACAAGCACCTCCCAAAG-3′
	R- 5′-TCCTGCTCCTTCTCCTTCAT-3′
TH	F - 5'- GGGGAGCTGAAGGCTTATGG-3'
	R- 5'- GGACACAAAGTACACAGGCT-3'
GCase	F- 5'-TGCTGCTCTCAACATCCTTGC C-3'
	R - 5'-TAGGTGCGGATGGAG AAGTCAA-3'
β-actin	F- 5'-CCACAGCTGAGAGGGAAATC-3'
	R-5' AAGGAAGGCTGGAAAAGAGC-3'

5.1.10 Assessment of nigral caspase 12 and caspase 3 activities

Caspase-3 (No:E-CK-A311, Elabscience Biotechnology, USA) and Caspase-12 ELISA Kit (No: ab65664, Abcam) were employed in this study to quantitatively assess the levels of caspase activities, following the respective manufacturer's instructions. The protein concentrations were determined using the Bradford method (Bradford 1976).

5.1.11 Nissl's staining

To investigate the density of neuronal cells in the nigral region of each group, the four rats from each group were anesthetized using Ketamine/Xylazine (100/10 mg/kg, *i.p.*). Then perfused transcardially with 200 ml of precooled 0.01 mol/L phosphate-buffered

saline (PBS; pH 7.4), followed by 200 ml of a precooled fixative solution containing 4 % paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Freezing cryostat (Leica Microsystems GmbH, Wetzlar, Germany) was used to cut 30 μm thick coronal sections after being embedded in an optimal cutting temperature (OCT) solution. These sections were then put on precoated poly-L-lysine slides and stored at - 80 ^{0}C. Sections were dehydrated for 5 hours on the day of the experiment in 95 % ethyl alcohol, followed by 5 minutes of dehydration in 75 % ethyl alcohol at room temperature, and 5 minutes of rinsing in triple-distilled water. Thereafter, all of the sections were nissl-stained for 20 minutes with 0.125 % cresyl violet acetate (warmed at 50–60 °C), washed in water for 5 minutes, and then dehydrated with 75% ethyl alcohol for a few seconds, 95% alcohol for 2 minutes, 100% ethyl alcohol for 2 minutes, and finally cleared in xylene for 2–3 min. The slides were then coated with DPX, and Nissl's stained neurons were seen using a bright-field microscope (Leica, Germany) with a magnification of around 10 times and a scale bar set at 100 μM. NIH ImageJ software (NIH, USA) cell's counter tool was used to analyse the Nissl'slabeled nigral neurons [60]. Data were presented as a percentage of control rats (Mishra, Chandravanshi et al. 2018).

5.1.12 Immunofluorescence

Immunofluorescence labeling of dopamine (DA) neurons in the nigral area, specifically those positive for tyrosine hydroxylase (TH) as previously described (Sanz, Quintana et al. 2009, Goswami, Gupta et al. 2016), was performed using a standard technique (Gorbatyuk et al., 2008). In accordance with the methods outlined in section 5.1.11, dissection, perfusion, fixation, and preparation of coronal slices (30 μm) were carried out. On the day of the experiment, the sections were cleansed three times with phosphate-buffered saline (PBS) for five minutes each. Subsequently, they were subjected to blocking with 10% goat serum in 0.1% PBST for 2 hours at room temperature. Following

this, the sections were incubated overnight at 4°C with the primary antibody, specifically rabbit tyrosine hydroxylase (TH) (1:100, E-AB-70077, Elabscience). Additionally, a 2-hour incubation with the secondary antibody, goat anti-rabbit IgG (H&L) (Rhodamine/TRITC conjugated, 1:100), was carried out. After three washes with 1X PBS, the sections were mounted using fluoro shield mounting media (Sigma-Aldrich). Fluorescent images were captured using a Leica fluorescent microscope (Nikon, Japan) at approximately 10x magnification (scale bar = 100 μm). The analysis of TH-positive cells was conducted using the Image J program (NIH, USA).

5.2 Statistical Analysis

The mean ± standard deviation (SD) were used to represent the experimental data, and statistical analysis was conducted using Graph Pad prism 5.1 version software. To determine statistically significant differences in behavior parameters, repeated measures of two-way ANOVA were employed, followed by the Bonferroni multiple comparison posthoc tests. Western blot analyses for TH and pS129 α-syn were quantified using the Spot densitometry tool of AlphaEaseFC software (Alpha Innotech Corp, San Jose, CA, USA). The signal intensity (integrated density value, IDV) of these two markers was then normalized against the IDV of the endogenous control β-actin, and the resulting values were plotted as percent relative density values (RDV) in graphs. For other datasets, one-way ANOVA was performed, followed by the Tukey post hoc test. Differences were considered statistically significant when the P value $p < 0.05$ in the overall data analysis.

5.2.1 Results

5.2.2 Neurobehavioral studies

5.2.2.1 Effect of GC466 on apomorphine-induced rotational behaviour in 6-OHDA-induced PD rats

The rotational behavior induced by apomorphine was utilized as a means to verify the unilateral degradation of the dopaminergic system in rats. A thorough analysis using repeated measures of a two-way ANOVA demonstrated significant differences in rotational behavior across various groups [$F(6, 525) = 246.8$; $p < 0.05$], time periods [$F(4, 525) = 138.5$; $p < 0.05$], and the interaction between group and time [$F(24, 525) = 54.3$; $p < 0.05$]. Results presented in **Table 5.2** revealed no significant distinctions between the control and sham groups. Rats injected with unilateral 6-OHDA on D-4 (refer to **Figure 5.1 in the appendices**) exhibited a substantial depletion of the ipsilateral dopaminergic nigrostriatal pathway, as evidenced by a 37% increase in contralateral turns compared to the sham group on D-7. Over time, these turns gradually increased in the 6-OHDA rats, reaching 63.27% on D-14 and further escalating to 74.01% on D-28. However, the introduction of higher doses of GC466 treatment resulted in a remarkable reduction of the 6-OHDA-induced increase in rotational behavior by 27.75% after only 11 days of treatment (on D-14). This reduction became even more pronounced after 18 days of treatment (on D-21), with a decrease of 61.58% compared to the 6-OHDA group. Notably, these effects followed a progressive pattern similar to the sham group on D-28. In contrast, the administration of median doses after 18 days of treatment (on D-21) led to a decrease in rotational behavior by only 42.08% compared to the 6-OHDA group. No significant effects on rotational behavior were observed with lower doses compared to the 6-OHDA group.

5.2.2.2 Effect of GC466 on 6-OHDA-induced impairments in grip strength and motor coordination

The effects of GC466 on neuromuscular strength regulation and coordination were assessed using specific tests, namely the grip strength scores and rotarod tests. These tests were conducted after 4 (D-7), 11 (D-14), 18 (D-21), or 25 days (D-28) of GC466 treatment. Statistical analysis using repeated measures of two-way ANOVA revealed significant differences in grip strength scores and rotarod retention time among the different groups [$F(6, 525) = 219.5$, $p < 0.05$; $F(6, 525) = 396.8$, $p < 0.05$, respectively], as well as over time [$F(4, 525) = 147.0$, $p < 0.05$; $F(4, 525) = 333.8$, $p < 0.05$, respectively]. Additionally, an interaction between group and time was observed [$F(24, 525) = 22.75$, $p < 0.05$; $F(24, 525) = 42.28$, $p < 0.05$, respectively], as shown in **Table 5.2**. The control and sham groups did not exhibit significant differences according to post hoc analysis. However, in the 6-OHDA group (GSS: 70.09%; RRT: 55.22%), there was a decline in grip strength scores and rotarod retention time starting from D-7 compared to the sham group, indicating the presence of motor deficits associated with PD symptoms. It is important to note that this decline did not occur gradually, which could be attributed to the rapid degeneration of the nigrostriatal circuit. Lower doses of GC466 did not show any improvement in motor deficits. However, administration of higher doses significantly restored motor function, resulting in an increase in both grip strength scores (59.93%) and rotarod retention time (42.55%) compared to the 6-OHDA injected rats. This restorative effect was observed after 11 days of treatment with higher doses (D-14). In contrast, median doses showed a delayed onset of action after 18 days of treatment (from D-21) with only 41.18% and 32.58% increments in GGS and RRT parameters, respectively. These effects continued to increase progressively up to D-28. On D-28, no

significant differences were observed between the higher dose-treated group and the sham group in terms of both behavioral parameters.

Table 5.2 Dose-dependent effect of compound GC466 on 6-OHDA-induced alternation in motor functions as analyzed by apomorphine- induced rotations, neuromuscular strength score and motor coordination ability tests.

Groups	Contralateral Rotations test	Neuromuscular strength score	Motor coordination ability test
D-0			
Control	5.75 ± 1.57	4.68 ± 0.70	174.68 ± 11.41
Sham	6.06 ± 1.87	4.56 ± 0.51	173.31 ± 13.43
6-OHDA	5.06 ± 1.23	4.81 ± 0.54	175.25 ± 7.83
6-OHDA + SEL	6.06 ± 1.69	4.75 ± 0.77	171.87 ± 12.12
6-OHDA + GC-6.25	6.00 ± 1.03	4.50 ± 0.73	174.50 ± 10.76
6-OHDA + GC-12.5	5.93 ± 1.12	4.81 ± 0.83	176.31 ± 11.60
6-OHDA + GC-25	5.18 ± 1.10	4.62 ± 0.50	171.25 ± 10.05
D-7			
Control	5.31 ± 1.25	4.75 ± 0.57	173.00 ± 16.43
Sham	5.43 ± 1.15	4.31 ± 0.87	173.25 ± 11.86
6-OHDA	8.56 ± 2.30[a]	1.43 ± 0.51[a]	78.37 ± 9.77[a]
6-OHDA + SEL	9.43 ± 1.70[a]	1.37 ± 0.72[a]	97.18 ± 9.24[a]
6-OHDA + GC-6.25	8.44 ± 1.67[a]	1.56 ± 0.51[a]	79.93 ± 12.20[a]
6-OHDA + GC-12.5	9.81 ± 2.22[a]	1.68 ± 0.47[a]	89.87 ± 14.84[a]
6-OHDA + GC-25	9.50 ± 1.96[a]	1.81 ± 0.54[a]	84.25 ± 15.27[a]
D-14			
Control	5.75 ± 1.94	4.50 ± 0.63	176.0 ± 8.08
Sham	5.37 ± 0.95	4.43 ± 0.72	174.18 ± 7.60
6-OHDA	15.43 ± 5.15[a]	1.18 ± 0.66[a]	74.62 ± 9.37[a]
6-OHDA + SEL	12.81 ± 2.37[a,b]	3.12 ± 1.20[a,b]	121.56 ± 14.85[a,b]
6-OHDA + GC-6.25	16.25 ± 3.29[a,c]	1.25 ± 0.44[a,c]	75.62 ± 13.52[a,c]
6-OHDA + GC-12.5	15.18 ± 3.95[a,c]	1.75 ± 0.57[a,c]	92.31 ± 9.53[a,c]
6-OHDA + GC-25	11.25 ± 2.79[a,b,d,e]	2.93 ± 0.85[a,b,d,e]	129.12 ± 13.98[a,b,d,e]
D-21			
Control	5.62 ± 1.36	4.75 ± 0.44	172.93 ± 10.23
Sham	5.68 ± 1.44	4.62 ± 0.50	171.06 ± 12.75
6-OHDA	20.13 ± 3.94[a]	1.31 ± 0.47[a]	82.00 ± 8.43[a]
6-OHDA + SEL	11.06 ± 2.17[a,b]	3.93 ± 0.77[a,b]	152.56 ± 15.62[a,b]
6-OHDA + GC-6.25	21.00 ± 4.11[a,c]	1.18 ± 0.75[a,c]	88.31 ± 9.27[a,c]
6-OHDA + GC-12.5	14.62 ± 3.84[a,b,c,d]	2.50 ± 0.51[a,b,c,d]	120.94 ± 18.97[a,b,c,d]
6-OHDA + GC-25	9.81 ± 1.79[a,b,d,e]	4.12 ± 1.08[b,d,e]	162.75 ± 7.06[b,d,e,]
D-28			
Control	5.56 ± 1.31	4.56 ± 0.51	171.75 ± 14.84
Sham	5.93 ± 1.43	4.68 ± 0.60	172.25 ± 13.19
6-OHDA	21.75 ± 5.13[a]	1.13 ± 0.61[a]	78.43 ± 8.95[a]
6-OHDA + SEL	8.18 ± 1.68[b]	4.37 ± 0.88[b]	163.25 ± 10.79[b]

6-OHDA + GC-6.25	$20.62 \pm 3.36^{a,c}$	$1.06 \pm 0.44^{a,c}$	$84.87 \pm 9.77^{a,c}$
6-OHDA + GC-12.5	$12.12 \pm 2.15^{a,b,c,d}$	$2.81 \pm 0.83^{a,b,c,d}$	$135.83 \pm 14.17^{a,b,c,d}$
6-OHDA + GC-25	$8.56 \pm 1.63^{b,d,e}$	$4.37 \pm 0.61^{b,d,e}$	$165.81 \pm 10.62^{b,d,e}$

Results are expressed as mean + SD; (n = 16). $^{a}p< 0.05$ compared to sham, $^{b}p< 0.05$ compared to 6-OHDA, $^{c}p< 0.05$ compared to 6-OHDA + SEL, $^{d}p< 0.05$ compared to 6-OHDA + GC-6.25, and $^{e}p< 0.05$ compared to 6-OHDA + GC-12.5 [Repeated measures of two-way ANOVA followed by Bonferroni multiple comparison test, Graph Pad Prism 5.1 Software, Inc].

5.2.3 Effect of GC466 on striatal rat Dopamine (DA) level

The motor symptoms of PD are closely linked to the levels of dopamine (DA) in the striatum (Goswami, Gupta et al. 2016). Thus, our investigation aimed to determine if the administration of 6-OHDA led to a reduction in DA content within the striatum and whether this decline could be counteracted by GC466. To analyze the DA levels and identify significant differences among the groups, we employed a one-way ANOVA [$F(6, 41) = 31.63$; $p < 0.05$] using the striatal tissues of rats [**Figure 5.3**]. Notably, there were no observable distinctions between the control and sham groups. However, the group that received 6-OHDA exhibited a substantial decrease in striatal DA levels, with a reduction of $71.35 \pm 8.63\%$ compared to the sham group.

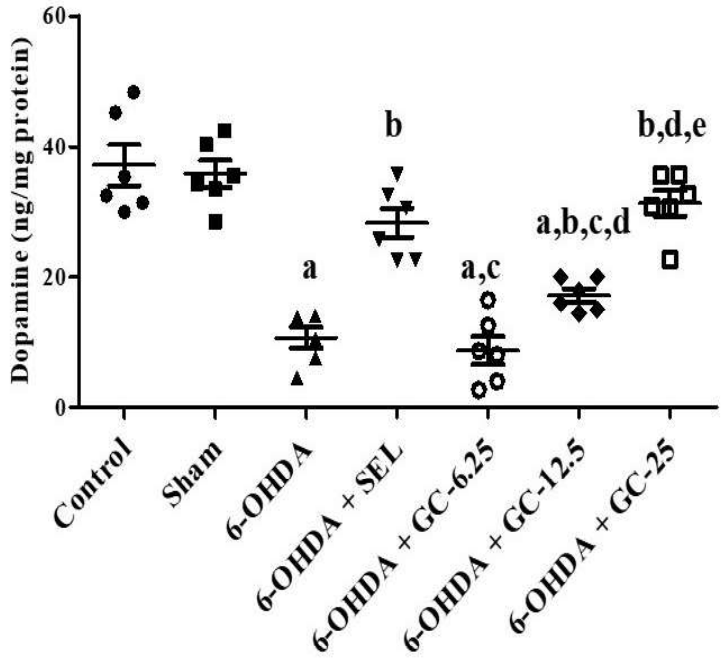

Figure 5.3 The effect of GC466 on 6-OHDA-induced changes in striatal dopamine levels. All values are presented as mean ± SD; n = 6; $^{a}p< 0.05$ compared to sham, $^{b}p< 0.05$ compared to 6-OHDA and $^{c}p< 0.05$ compared to 6-OHDA + SEL, $^{d}p< 0.05$ compared to 6-OHDA + GC-6.25 and $^{e}p< 0.05$ compared to 6-OHDA + GC-12.5 [One-way ANOVA followed by Tukey post-hoc test, Graph Pad Prism 5.1 Software, Inc].

Nevertheless, following 24 days of treatment with GC466, we observed a noteworthy dose-dependent increase in dopamine levels, effectively mitigating the previously lowered levels. Lower doses of GC466 proved to be ineffective in this regard. However, rats infused with 6-OHDA and treated with median and higher doses of GC466 experienced a considerable increase in DA levels of 46.35 ± 9.44% and 75.43 ± 15.25%, respectively. Furthermore, the group that received SEL in addition to 6-OHDA exhibited a significant elevation in dopamine levels, with an increase of 76.21 ± 19.23%.

5.2.4 Effects of GC466 on 6-OHDA-induced alterations in nigral proteins (TH and pS129 α-syn expression)

The administration of 6-OHDA in this study simulated the demise of neurons in the nigrostriatal dopamine pathway, which is the underlying cause of PD. This effect was achieved by interfering with the expression of the TH protein (Tong, Wu et al. 2016)Through the utilization of a one-way ANOVA analysis, notable distinctions were observed between various groups in terms of TH protein expression [$F(6, 27) = 8.15$; $p < 0.05$] within the substantia nigra pars compacta (SNc) **[Figure 5.4a and b].** Interestingly, no significant difference was observed between the control group and the sham group. In rats exposed to 6-OHDA, there was a substantial reduction (68.35% ± 6.87%) in the levels of TH protein compared to the sham group. However, higher doses exhibited an enhanced expression of TH protein when compared to rats infused with 6-OHDA, following a dose-dependent pattern (57.55% ± 13.07% and 79.85% ± 18.82% for median and higher doses, respectively). Similar outcomes were observed with SEL treatment, which also resulted in an elevation (74.84% ± 15.38%) of TH expression in PD-afflicted rats. Another critical aspect associated with the development of PD is the presence of α-Syn phosphorylated at serine 129 (pS129 α-syn) (Migdalska-Richards, Daly et al. 2016). This protein plays a pivotal role in the formation of Lewy bodies and serves as a marker for oligomerization, as assessed in this investigation. Analysis utilizing a one-way ANOVA indicated significant variations among groups in terms of pS129 α-syn expression [$F(6, 27) = 12.66$; $p < 0.05$] within the nigral region **[Figure 5.4a and c].**

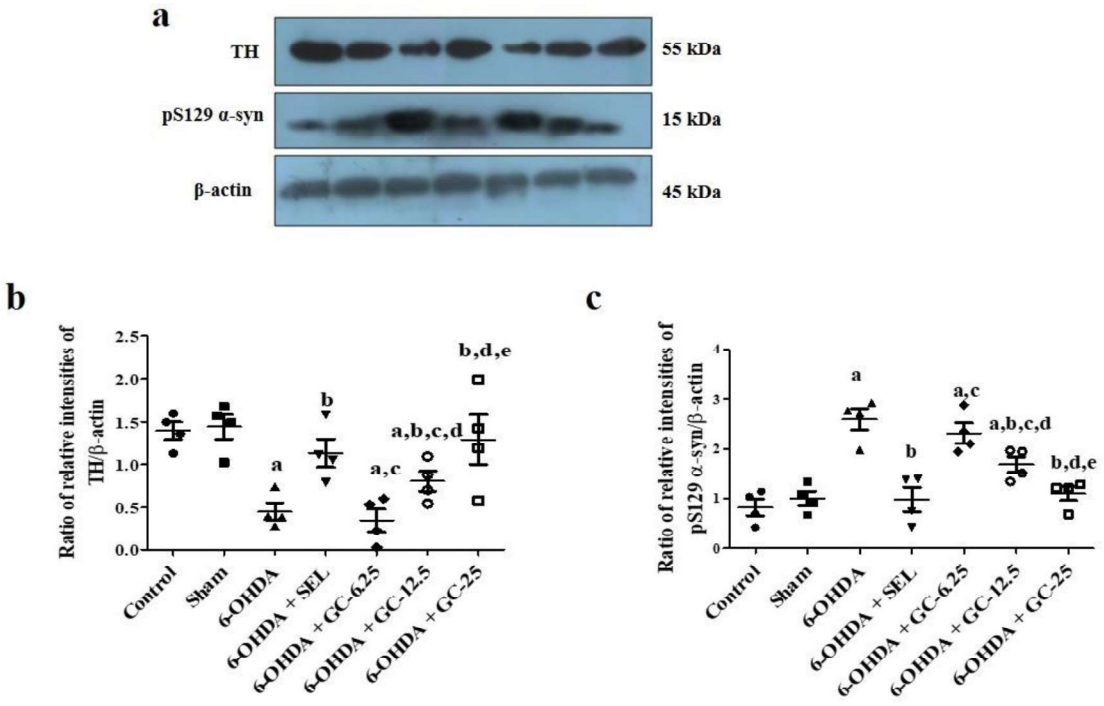

Figure 5.4 The effect of GC466 on 6-OHDA-induced changes in nigral protein expressions. (a) Representative immunoblot images demonstrating the expression of tyrosine hydroxylase (TH) and phospho-synuclein (pS129-syn) following GC466 treatment in 6-OHDA-induced PD rats. Quantification of TH (b) and pS129-syn (c) protein levels following GC466 treatment in 6-OHDA-induced PD rats. β-actin is representing as a loading control. All values are presented as mean ± SD (n = 4); $^{a}p< 0.05$ compared to sham, $^{b}p< 0.05$ compared to 6-OHDA and $^{c}p< 0.05$ compared to 6-OHDA + SEL, $^{d}p< 0.05$ compared to 6-OHDA + GC-6.25 and $^{e}p< 0.05$ compared to 6-OHDA + GC-12.5 [One-way ANOVA followed by Tukey post-hoc test, Graph Pad Prism 5.1 Software, Inc].

Post hoc analysis revealed that 6-OHDA substantially increased the expression of pS129 α-syn protein (71.35% ± 18.02%) compared to the sham group, but this elevation was mitigated by GC466 after 25 days of treatment in a dose-dependent manner (46.54% ±

14.27%, 66.04% ± 15.01% for median and higher doses, respectively). Furthermore, SEL treatment led to a decrease in 6-OHDA-induced pS129 α-syn expression.

5.2.5 Effects of GC466 on 6-OHDA-induced alterations in nigral GCase activity

The enzymatic activity of GCase displayed significant differences among groups, as determined by a one-way analysis of variance (ANOVA) [$F(6, 27) = 12.39$, $p < 0.05$) [**Figure 5.5**]. The sham group exhibited no significant difference compared to the control group. The induction of 6-OHDA resulted in a substantial reduction of GCase activity, measuring 79.88% ± 17.54% compared to the sham group.

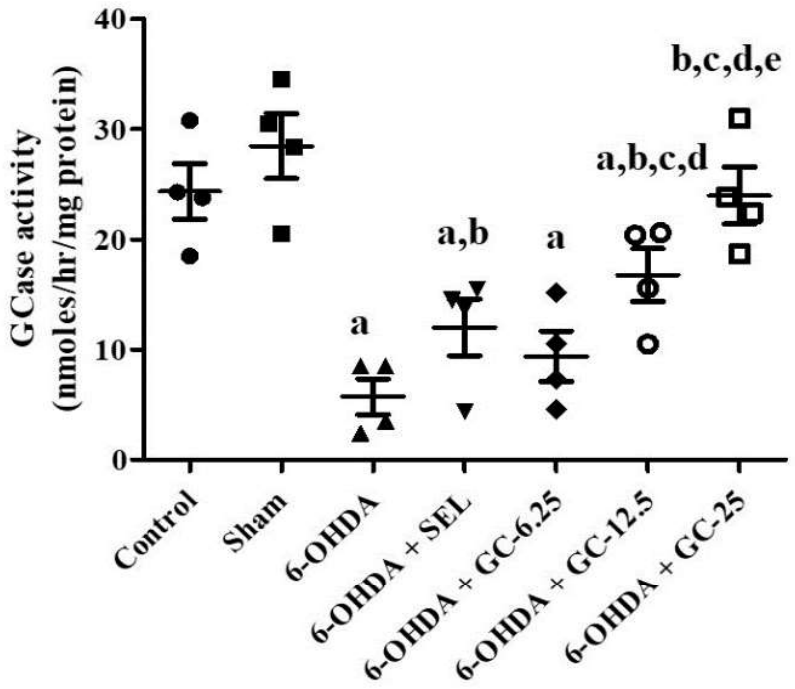

Figure 5.5 Effect of GC466 on 6-OHDA-induced alteration in nigral glucocerebrosidase enzyme activity. All values are shown as mean ± SD; n = 4; [a]$p<0.05$ compared to sham; [b]$p< 0.05$ compared to 6-OHDA, and [c]$p< 0.05$ compared to 6-OHDA + SEL, [d]$p< 0.05$ compared to 6-OHDA + GC-6.25 and [e]$p< 0.05$ compared to 6-OHDA + GC-12.5 [One-way ANOVA followed by Tukey post-hoc test, Graph Pad Prism 5.1 Software, Inc].

Conversely, treatment with moderate and high doses in the 6-OHDA-lesioned groups for a duration of 24 days led to a notable increase in GCase activity, measuring 54.65% ± 10.45% and 75.88% ± 16.27%, respectively, compared to the sham group. However, when compared to the control group, the standard SEL only demonstrated a growth of 34.07% ± 8.43%.

5.2.6 Effects of GC466 on 6-OHDA-induced alterations in nigral gene expression of ER stress markers (GRP78, CHOP, PARK, caspase 12, 9 and 3), TH and GCase

The mRNA gene expression levels of several endoplasmic reticulum (ER) markers, namely GRP78, CHOP, PARK, and Caspases (12, 9, and 3), were measured in the substantia nigra pars compacta (SNPc) of rats with PD. The objective was to investigate whether the anti-PD action of GC466 is mediated through apoptotic pathways related to ER stress (ERS). Gene markers associated with ERS, including GRP78 **(Figure 5.6a),** CHOP **(Figure 5.6b),** PARK **(Figure 5.6c),** caspases 9 **(Figure 5.6d),** caspase 12 **(Figure 5.7a),** and caspase 3 **(Figure 5.7b),** were expressed, and significant differences were observed among different groups. One-way ANOVA revealed significant variations in GRP78 [$F(6, 27) = 11.71$; $p < 0.05$], CHOP [$F(6, 27) = 30.73$; $p < 0.05$], PARK [$F(6, 27) = 27.44$; $p < 0.05$], caspase-12 [$F(6, 27) = 22.22$; $p < 0.05$], caspase 9 [$F(6, 27) = 10.82$; $p < 0.05$], and caspase 3 [$F(6, 27) = 21.16$; $p < 0.05$]. The mRNA expression levels of GRP78, CHOP, PARK, and caspases 12, 9, and 3 were significantly upregulated in the 6-OHDA-exposed PD rats compared to the sham group.

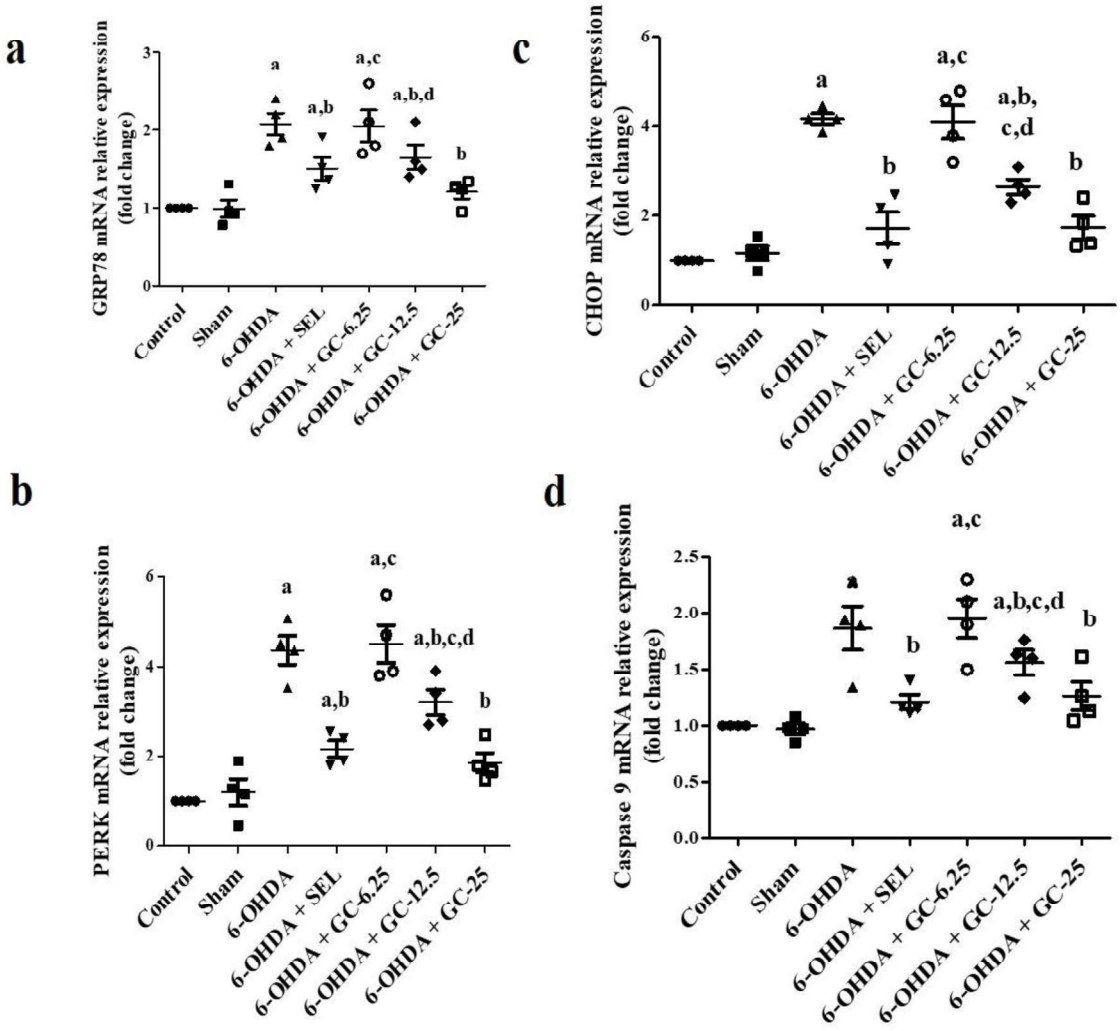

Figure 5.6 Effect of GC466 on 6-OHDA-induced alteration in nigral GRP78/Bip (a), PERK (b), CHOP (c), and Caspase 9 (d) mRNA levels. Results are expressed as mean ± SD; n = 4; $^{a}p< 0.05$ compared to sham, $^{b}p< 0.05$ compared to 6-OHDA and $^{c}p< 0.05$ compared to 6-OHDA + SEL, $^{d}p< 0.05$ compared to 6-OHDA + GC-6.25 and $^{e}p< 0.05$ compared to 6-OHDA + GC-12.5 [One-way ANOVA followed by Tukey post-hoc test, Graph Pad Prism 5.1 Software, Inc].

Specifically, there was a 2.07-fold increase in GRP78, a 4.16-fold increase in CHOP, a 4.36-fold increase in PARK, a 3.76-fold increase in caspase 12, a 1.79-fold increase in caspase 9, and a 3.25-fold increase in caspase 3. However, treatment with GC466 resulted

in a dose-dependent decrease in the expression of these ERS-related genes in PD rats, effectively reversing the upregulation caused by 6-OHDA. In rats infused with 6-OHDA, both medium and high doses of GC466 showed efficacy in reducing ERS markers, with the high dose demonstrating the most pronounced effect. Similarly, SEL treatment also significantly decreased the expression levels of ERS-related genes.

To validate the potential correlation between GCase enzyme activity, protein expression in the nigral region, and the mRNA expression of TH and GCase, additional measurements were conducted. Specifically, TH mRNA expression (**as depicted in Figure 5.2 in the Appendices**) and GCase mRNA expression (**as shown in Figure 5.3 in the Appendices**) were assessed. One-way ANOVA analysis revealed significant differences among the various groups for both TH mRNA expression ($F(6, 27) = 28.11$, $p < 0.05$) and GCase mRNA expression ($F(6, 27) = 19.25$, $p < 0.05$) genes. Notably, no noticeable distinction was observed between the control group and the sham group. Comparing the sham group to the 6-OHDA lesioning group, a substantial reduction of 3.85-fold and 2.94-fold was observed in the mRNA expression of the TH and nigral GCase genes, respectively. Following the administration of GC466 therapy for a duration of 25 days, a dose-dependent increase in the nigral TH and GCase gene expression was observed in 6-OHDA-induced PD rats when compared to the 6-OHDA group. The higher doses of GC466 therapy were found to be the most effective, resulting in a reduction of gene expression levels by 3.15-fold (for the TH gene) and 2.52-fold (for the GCase gene), respectively, in rats infused with 6-OHDA. Conversely, the SEL administration in the 6-OHDA group exhibited less enhancement of GCase activity (1.34 times) compared to the effects observed with GC466.

5.2.7 Effect of GC466 against 6-OHDA-induced alteration in nigral caspase-12 and caspase-3 activity

Caspase-12 overexpression ultimately causes ERS-induced cell death ultimately by acting on the apoptotic protein caspase 3 (Goswami, Gupta et al. 2016, Wang, Dou et al. 2020). When analyzed using One-way ANOVA, significant differences in the activities of caspase-12 [$F(6, 27) = 23.82$, $p < 0.05$] and caspase-3 [$F(6, 27) = 22.06$, $p < 0.05$] were observed among the different groups. However, there were no statistically significant differences between the control and sham groups. Further analysis through post hoc comparisons revealed that rats with 6-OHDA-induced lesions exhibited a significant increase in caspase-12 (3.1-fold, **Figure. 5.7c**) and caspase-3 (2.44-fold, **Figure 5.7d**) activities compared to the sham group.

Interestingly, rats treated with GC466 and SEL were able to prevent the increase in caspase-12 and caspase-3 activities caused by 6-OHDA in a dose-dependent manner. Among the treatments, higher doses of GC466 (25 mg/kg) were the most effective, reducing the activation of caspase-12 and caspase-3 by 2.33-fold and 2.03-fold, respectively, in rats receiving 6-OHDA injections.

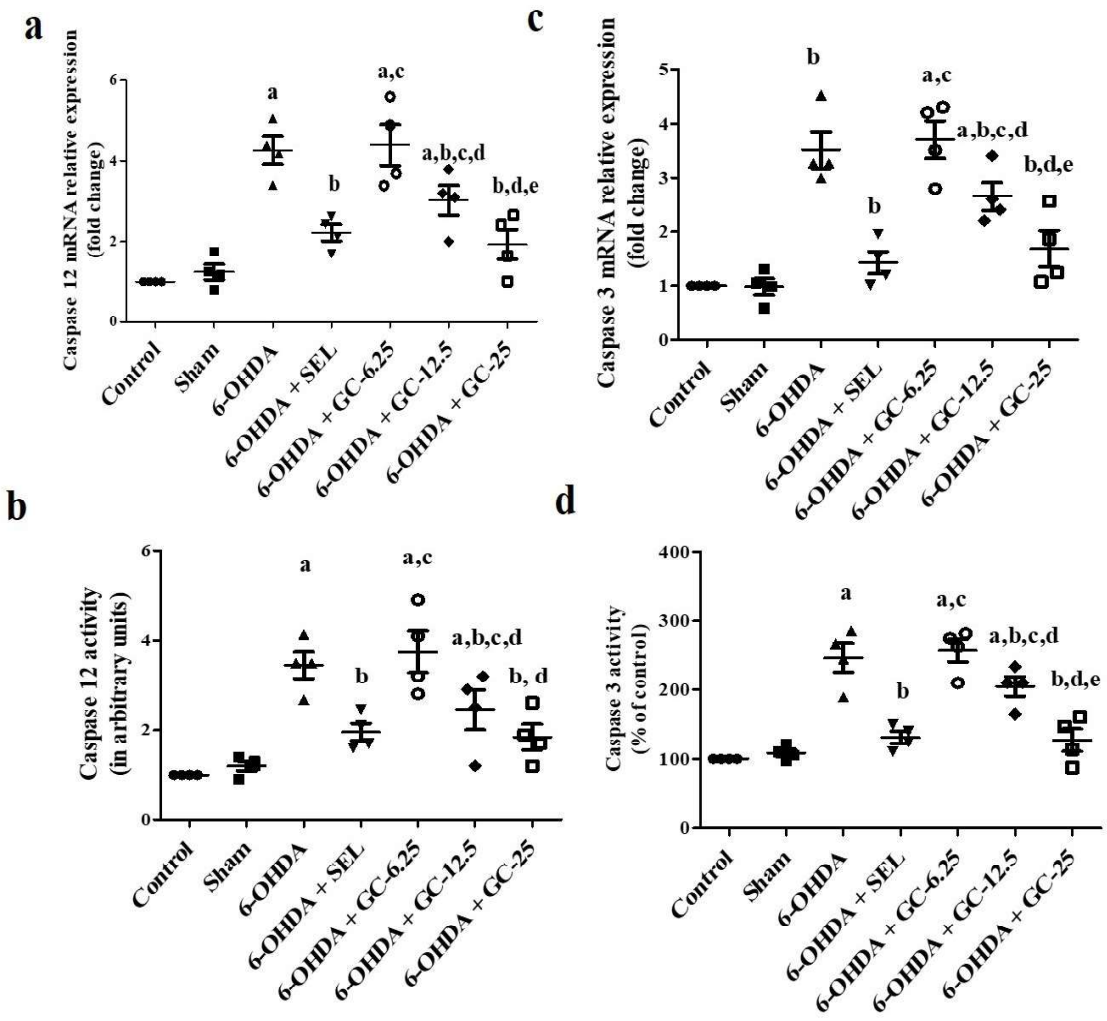

Figure 5.7 Effect of GC466 on 6-OHDA-induced alterations in the mRNA levels (a and c) and activities (b and d) of nigral caspase 12 and caspase 3, respectively. Data are reported as mean ± SD; n = 4; $^{a}p< 0.05$ compared to sham, $^{b}p< 0.05$ compared to 6-OHDA, $^{c}p< 0.05$ compared to 6-OHDA + SEL, $^{d}p< 0.05$ compared to 6-OHDA + GC-6.25 and $^{e}p< 0.05$ compared to 6-OHDA + GC-12.5 [One-way ANOVA followed by Tukey post-hoc test, Graph Pad Prism 5.1 Software, Inc].

5.2.8 Effect of GC466 against 6-OHDA-induced DA neuronal loss in SNpc region

Intrastriatal injection of 6-OHDA leads to a retrograde degeneration of dopamine (DA) neurons, causing the death of neuronal cell bodies in the substantia nigra pars compacta

(SNc) that are identifiable through Nissl staining and TH (tyrosine hydroxylase) immunoreactivity (Berger, Przedborski et al. 1991, Goswami, Gupta et al. 2016) . Statistical analysis using the one-way ANOVA approach demonstrated significant differences in the percentage of Nissl-positive cell bodies [F (6, 27) = 12.26, $p < 0.05$, **Figure 5.8**] and TH-positive cell bodies [F (6, 27) = 21.77, $p < 0.05$, Fig. 9] among different experimental groups.

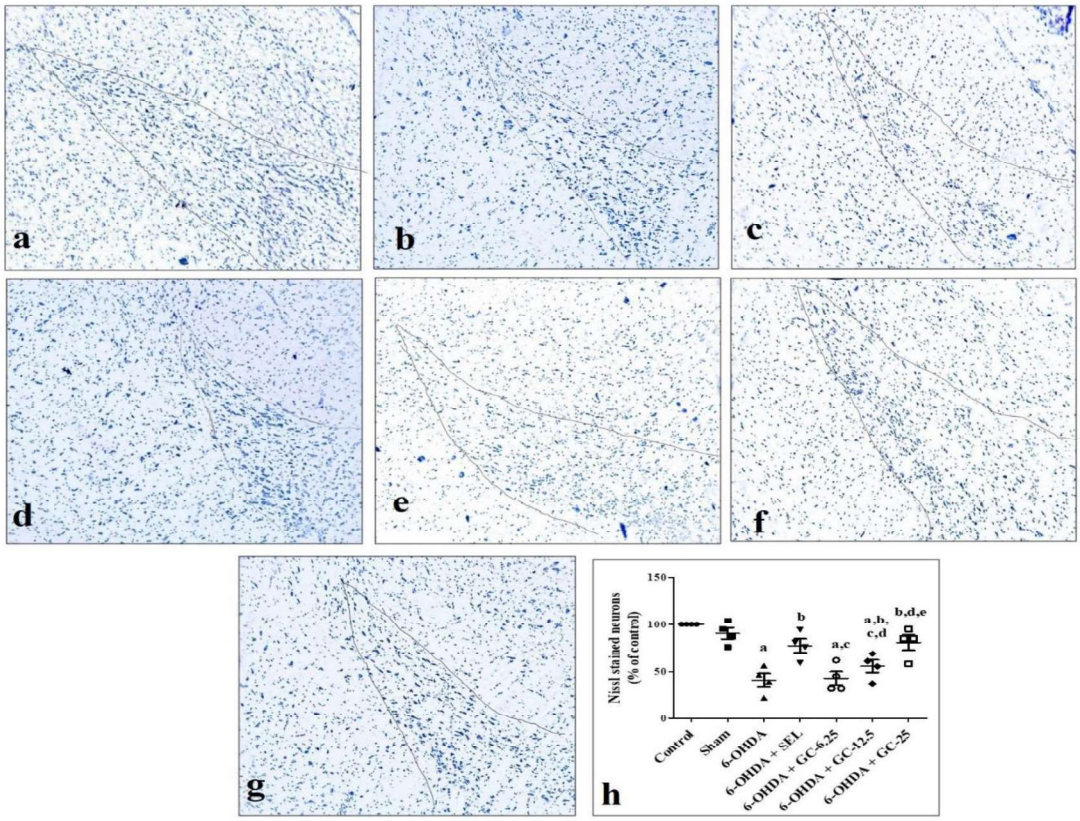

Figure 5.8 Rat SNc on D-28 stained with Nissl's dye (cresyl violet). The scale bar was set at 100 µM with 10X magnification. Control (a); Sham (b); 6-OHDA (c); 6-OHDA + SEL (d); 6-OHDA + GC-6.25 (e); 6-OHDA + GC-12.5 and (f); 6-OHDA + GC-25. Data of counting cells (in percentage, %, Figure g). Results are reported as mean ± SD; n = 4; $^{a}p< 0.05$ compared to sham, $^{b}p< 0.05$ compared to 6-OHDA and $^{c}p< 0.05$ compared to 6-OHDA + SEL, $^{d}p< 0.05$ compared to 6-OHDA + GC-6.25 and $^{e}p< 0.05$ compared to 6-

OHDA + GC-12.5 [One-way ANOVA followed by Tukey post-hoc test, Graph Pad Prism 5.1 Software, Inc].

In animals treated with 6-OHDA, there was a noticeable decrease in the percentage of Nissl-positive and TH-positive neuronal cell bodies compared to the sham group, with only 31.02% ± 12.14% and 38.75% ± 9.18% remaining, respectively. However, treatment with GC466 in a dose-dependent manner significantly reversed this decline in Nissl-positive and TH-positive neuronal cell bodies. In rats infused with 6-OHDA, the median dose of GC466 restored Nissl-positive and TH-positive neuronal cells by up to 53.22% ± 11.45% and 59.34% ± 12.26%, respectively. Higher doses resulted in even greater restoration, reaching up to 75.23% ± 13.60% for Nissl-positive cell bodies and 72.25% ± 18.03% for TH-positive cell bodies. Lower doses, however, did not produce the same effect.

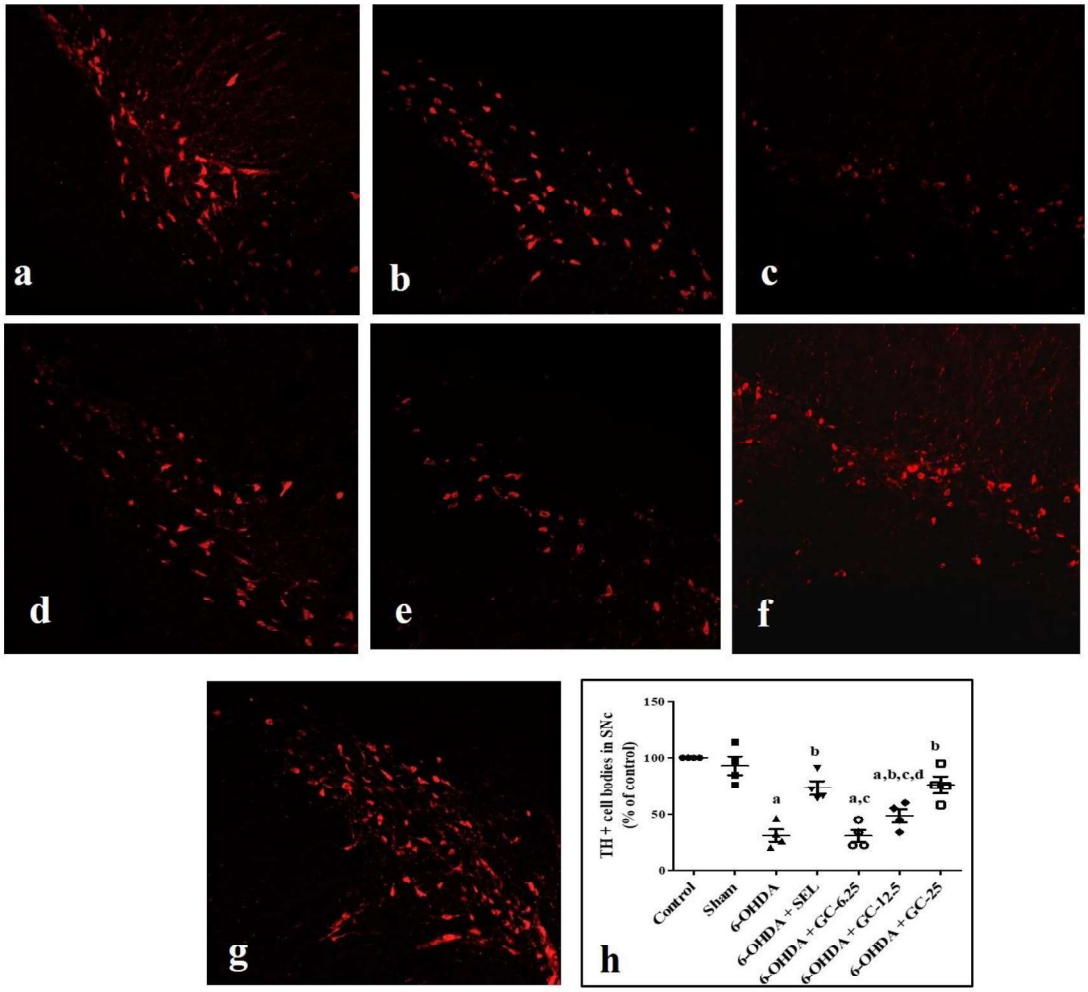

Figure 5.9 Effect of GC466 on 6-OHDA-induced alterations in TH +tive cell bodies in nigra area. Scale bar was set at 100 µM with 10X magnification. Control (a); Sham (b); 6-OHDA (c); 6-OHDA + SEL (d); 6-OHDA + GC-6.25 (e); 6-OHDA + GC-12.5 (f); 6-OHDA + GC-25. Data of countingTH +tive cell bodies in substantia nigra (in percentage, %, Fig. g). Results are reported as mean ± SD; n = 4; $^{a}p< 0.05$ compared to sham, $^{b}p< 0.05$ compared to 6-OHDA and $^{c}p< 0.05$ compared to 6-OHDA + SEL, $^{d}p< 0.05$ compared to 6-OHDA + GC-6.25 and $^{e}p< 0.05$ compared to 6-OHDA + GC-12.5 [One-way ANOVA followed by Tukey post-hoc test, Graph Pad Prism 5.1 Software, Inc].

5.3 Discussion

This study highlights the remarkable characteristics of GC466, demonstrating its potential as an anti-PD agent and its ability to enhance GCase activity in a 6-OHDA animal model. Moreover, GC466 exhibited a beneficial effect on the apoptotic pathway mediated by endoplasmic reticulum stress (ERS) in experimental rats. Given that a deficiency in the GCase enzyme is a significant risk factor in PD, the augmentation of this enzyme holds promise for exploring new therapeutic avenues in PD treatment.

PD is characterized by distinctive motor deficits, degeneration of nigral dopaminergic neurons resulting in reduced dopamine release in the striatum, and the accumulation of the oligomeric form of α-synuclein (Foltynie and Kahan 2013, Gatto, Da Prat et al. 2019). In this study, the administration of 6-OHDA induced motor impairment in rats, observed until day 28. The rats exhibited decreased neuromuscular strength, as indicated by reduced grip strength, and impaired motor coordination, as assessed by the rotarod test. Additionally, there was an increase in head rotational behavior, indicating unilateral dopamine depletion. These behavioral changes were evident in the 6-OHDA rats from day 7 onwards. GC466 treatment effectively reduced head rotations and restored gross motor deficits in a dose-dependent manner, starting from day 14. The decrease in rotational behavior suggests that GC466 may have facilitated the recovery of dopamine neurons at an early stage, leading to the improvement in gross motor deficits. Both median and higher doses of GC466 showed effectiveness, while lower doses failed to ameliorate the gross motor impairments.

Motor impairments have been closely linked to damage in a specific group of neurons called dopaminergic neurons in the substantia nigra pars compacta (SNc). Various clinical and preclinical studies have provided strong evidence supporting this correlation (Eo, Huh et al. 2019). The underlying mechanism involves a decrease in the release of

dopamine in the striatum, a region of the brain associated with motor control. The reduction in the number of dopaminergic cells in the SNc is directly associated with the inhibition of an enzyme called tyrosine hydroxylase (TH), which plays a crucial role in dopamine production (Zhu, Zhang et al. 2012, Michel, Tadros et al. 2022). In experimental models using 6-hydroxydopamine (6-OHDA), a compound known to induce PD-like symptoms, it has been observed that the administration of 6-OHDA reduces the expression of TH mRNA and protein in the substantia nigra. This reduction in TH activity leads to a decrease in the levels of dopamine in the striatum (Agrawal, Kumari et al. 2022). However, when animals were treated with GC466, a potential therapeutic agent, there was a dose-dependent protection against the loss of dopaminergic cells in the substantia nigra and a preservation of striatal dopamine levels in the 6-OHDA-induced Parkinson's disease model. This protective effect was confirmed by an increase in the number of TH-positive cell bodies in the substantia nigra, indicating the preservation or regeneration of dopaminergic neurons (Motawi, Al-Kady et al. 2022).

Dopaminergic cell loss in PD patients is primarily attributed to two key factors: the accumulation of oligomeric α-synuclein aggregates and dysfunction of the GCase enzyme. Upon analyzing multiple studies on the pathogenesis of PD, it becomes evident that the loss of GCase activity can directly contribute to the formation of α-synuclein aggregates (Gegg, Burke et al. 2012, Liu, Chen et al. 2015, Gegg, Menozzi et al. 2022). GCase, an enzyme localized in the lysosomes, plays a crucial role in metabolizing its substrate, glucocerebroside (GC). GCase inhibition has the consequence of accumulating GC within the substantia nigra pars compacta (SNc). This accumulation of GC serves as a framework or platform that promotes the stabilization of α-synuclein oligomers. As a result, toxic forms of α-synuclein can accumulate within dopaminergic neurons, hastening the degeneration of these cells. The presence of α-synuclein oligomeric

aggregates in post-mortem PD brains with GCase deficiency provides evidence for their involvement in the progression of the disease (Mullin, Smith et al. 2020). In studies involving rats, the administration of 6-OHDA led to GCase activity inhibition, reduced mRNA levels, and increased aggregation of α-synuclein protein within SNc tissues. However, when administered at median and higher doses, GC466 exhibited the remarkable capacity to restore the activity of nigral GCase while effectively restraining the aggregation of α-synuclein in a dose-dependent fashion. It is worth noting that GC466's potential in thwarting the formation of oligomeric aggregates of α-synuclein, as highlighted earlier, might be attributed to its remarkable ability to stimulate GCase activity.

Multiple studies have provided evidence connecting the activation of endoplasmic reticulum stress (ERS)-mediated cell death to PD (Goswami, Gupta et al. 2016, Motawi, Al-Kady et al. 2022). Examination of brain tissue from PD patients has indeed shown the activation of the unfolded protein response (UPR), which confirms the involvement of ERS (Hoozemans, Van Haastert et al. 2007). Given that the endoplasmic reticulum (ER) is the sole site of GCase production (Bendikov-Bar, Maor et al. 2013, Chatterjee and Krainc 2023), dysfunction of GCase may act as an initial trigger for ERS-related PD pathogenesis. Elevated ERS markers have been observed in both GBA mutant human cells and fly model lines (Sanchez-Martinez, Beavan et al. 2016). ERS was assessed through the signaling regulator GRP78/BiP, as described previously (Zhang, Long et al. 2017). Inducing 6-OHDA resulted in ERS within SNc tissues, as indicated by increased mRNA expression of GRP78/BiP, as reported in earlier studies (Selvaraj, Sun et al. 2012, Zhang, Long et al. 2017). However, this effect was progressively reduced in a dose-dependent manner following a 25-day treatment with GC466. Impaired endoplasmic reticulum (ER) function in the substantia nigra pars compacta (SNc) region of post-

mortem brain samples from patients with PD plays a crucial role in promoting apoptotic pathways mediated by CHOP and caspases (Hetz, Russelakis-Carneiro et al. 2003, Selvaraj, Sun et al. 2012). Activation of these pathways ultimately leads to neuronal apoptosis and subsequent cell death.. Previous studies have shown that the neurotoxin 6-hydroxydopamine (6-OHDA) induces ER stress, as evidenced by a significant increase in mRNA levels of ER apoptotic markers such as PERK, CHOP, and caspases (12, 9, and 3) in the nigral area (Gaballah, Zakaria et al. 2016, Peng, Liu et al. 2018). Consistent with earlier research (Hetz, Russelakis-Carneiro et al. 2003, Hitomi, Katayama et al. 2004, Tong, Wu et al. 2016), our findings also revealed elevated caspase-12 and caspase-3 activity in response to 6-OHDA administration, indicating a correlation between mRNA levels of these caspases and their enzymatic activities. However, when the compound GC466 was administered in a dose-dependent manner, it successfully alleviated the inhibition of the caspase-12/caspase-3 and PERK/CHOP apoptotic pathways, which had been activated by 6-OHDA. These compelling results strongly suggest that GC466 exerts a noteworthy anti-apoptotic effect, mitigating the detrimental consequences of ER stress-induced apoptosis.

In the context of GCase production in the endoplasmic reticulum (ER), the inhibition of GCase or the continuous generation of non-functional GCase has been implicated as a potential cause for the activation of apoptosis mediated by ER stress (ERS) (Gegg and Schapira 2018). A decrease in GCase mRNA levels observed in the 6-OHDA-induced PD model supports this notion. Previous studies have also arrived at similar conclusions, suggesting that the extended accumulation of non-functional proteins primarily induces ER-mediated apoptosis (Rao and Bredesen 2004, Gaballah, Zakaria et al. 2016). In light of these findings, it can be inferred that the suppression of ERS and the anti-apoptotic effects observed might largely stem from GC466's capacity to restore GCase activity.

Another contributing aspect might be that GC466 bind to the rat GCase active site residues Asp146, Trp198, Tyr263, Phe265, Tyr331, His329, Glu358, and Trp399 in the ER and stabilize it before it transforms into a non-functional GCase, as was previously discovered. ERS triggers the oligomerization of α-synuclein, leading to neuronal cell death. Therefore, the stimulation of GCase activity by GC466 likely inhibits ERS-mediated apoptotic pathways, prevents α-synuclein oligomerization, and promotes the survival of dopaminergic cells in the substantia nigra, as demonstrated in this study.

5.4 Conclusion

To conclude, the current findings establish that GC466 exhibits significant effect in improving the characteristic motor impairments observed in rats with 6-OHDA-induced PD. Notably, GC466 demonstrates potent suppression of caspase-12/caspase-3 and PERK/CHOP apoptotic pathways mediated by endoplasmic reticulum stress (ERS), effectively inhibiting α-synuclein oligomerization and enhancing GCase activity. These actions contribute to the protection of dopaminergic neurons against cell death in the 6-OHDA-induced rat model of PD. Notably, these effects exhibit a dose-dependent relationship, with 25 mg/kg proving to be the most efficacious dose. However, in order to confirm its ability to cross the blood-brain barrier and reach the central nervous system (CNS), further investigation through a pharmacokinetics study in a rat model would be imperative. In conclusion, these findings provide compelling evidence supporting the role of GC466 as a neuroprotective drug that stimulates GCase activity, thus potentially impeding GCase-associated PD pathogenesis.

5.5 Summary

- GCase dysfunction may triggers PD through ERS-induced apoptotic pathways.

- Caspases-12/3 and PERK/CHOP are the apoptotic events leading to nigral cell death.
- GC466 blocks apoptotic cell death and improves impaired GCase activity in PD rats.
- It improves behavioural deficit, GCase dysfunction and inhibits α-synuclein aggregation.
- It has neuroprotective action in PD rats by increase in nigral TH-positive cell bodies.
- GC466 recognize as a promising candidate for GCase-associated PD pathogenesis management.

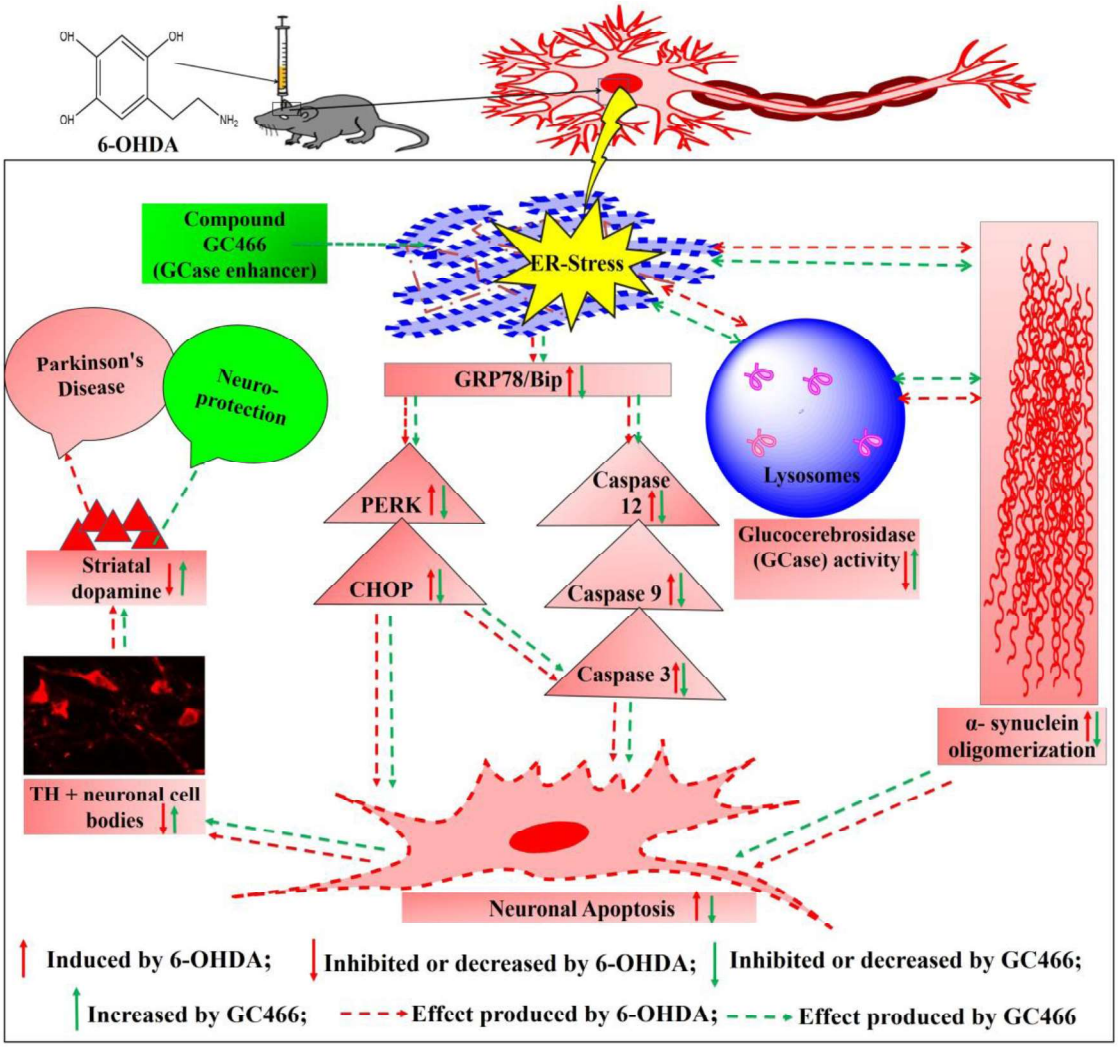

Figure 5.10 showcases the specific objective's outcome in exploring the molecular mechanism behind Parkinson's disease (PD) associated with Glucocerebrosidase Endoplasmic Reticulum Stress (GCase-ERS) in a 6-OHDA induced rat PD model. It suggests that the inhibition of GCase enzymatic activity in the presence of 6-OHDA may be attributed to ER-stress and α-synuclein aggregation. However, the administration of compound GC466 appears to stimulate GCase activity, leading to a reduction in ER-stress-induced apoptosis, α-synuclein pathology, loss of nigral dopaminergic cells, motor impairment, and an increase in striatal dopamine content. These findings highlight the potential of GC466 as a therapeutic intervention against 6-OHDA-induced PD models.