Chapter 8

Evaluation of sub-chronic administration of rebamipide for disease-modifying effects against 6-OHDA-induced model of PD in rats

8.1. Introduction

The concept of "disease modification" involves interfering with disease progression which ranges from slowing the undergoing neuronal degeneration to regenerating vanished neurons. Due to unavailability of medical intervention to alter the disease-course by slowing and arresting its progression, the progressive death of dopaminergic neurons in substantia nigra pars compacta (SNc) leads to two to three times increased mortality within 20 years from the onset of disease compared to general community. Despite making various attempts, "disease modification" could not be achieved till date (Dauer and Przedborski, 2003).

Pathogeneis of PD involves multiple neurotoxic pathways. α-synuclein pathology, GCase enzymatic deficiency, oxidative stress and mitochondrial dysfunction are some of the factors (Gegg et al. 2012; Moore et al. 2005; Olanow et al. 2009). GCase reduction increases the aggregation of α-synuclein oligomers (Mazzulli et al., 2011) and also causes mitochondrial dysfunction (Cleeter et al., 2013) leading to death of nigral cells (Moore et al. 2005). Brain DA synthesis requires involvement of TH, which is decreased in PD (Haavik and Toska, 1998; Zhu et al., 2012). Presynaptic function of DA is regulated by DAT which keep synaptic DA levels constant (Gainetdinov et al., 1998; Sossi et al., 2007). Intervening with mitochondrial dysfunction by rebamipide, may also arrest the downstream toxic events, such as GCase deficiency, followed by α-synuclein pathology. Ultimately, nigral and TH-containg dopaminergic cells may be restored alongwith recovery of motor functions.

Intrastriatal 6-OHDA injection takes 2-3 weeks to show maximum nigral cell loss (Sauer and Oertel, 1994). Therefore, in continuation with the earlier studies in

Chapter 5, where rebamipide administration was initiated before the full development of 6-OHDA induced motor deficits in rats, current study is focused on the neurorestorative effects of rebamipide on the dopaminergic system for the first time. Drug treatment was started after the disease progresses to full development of motor symptoms i.e., 4 weeks after 6-OHDA intrastriatal injection to indicate the disease-modifying property of rebamipide as shown in **Figure 8.1**. Effects of rebamipide were observed on recovery of motor and mitochondrial function, as shown by behavioral tests and mitochondrial complex-I activity respectively. Neurorestorative potential of rebamipide against 6-OHDA-induced dopaminergic toxicity in rats was evaluated by DAT levels, GCase enzymatic activity, α-synuclein concentration, TH concentration and number of Nissl bodies.

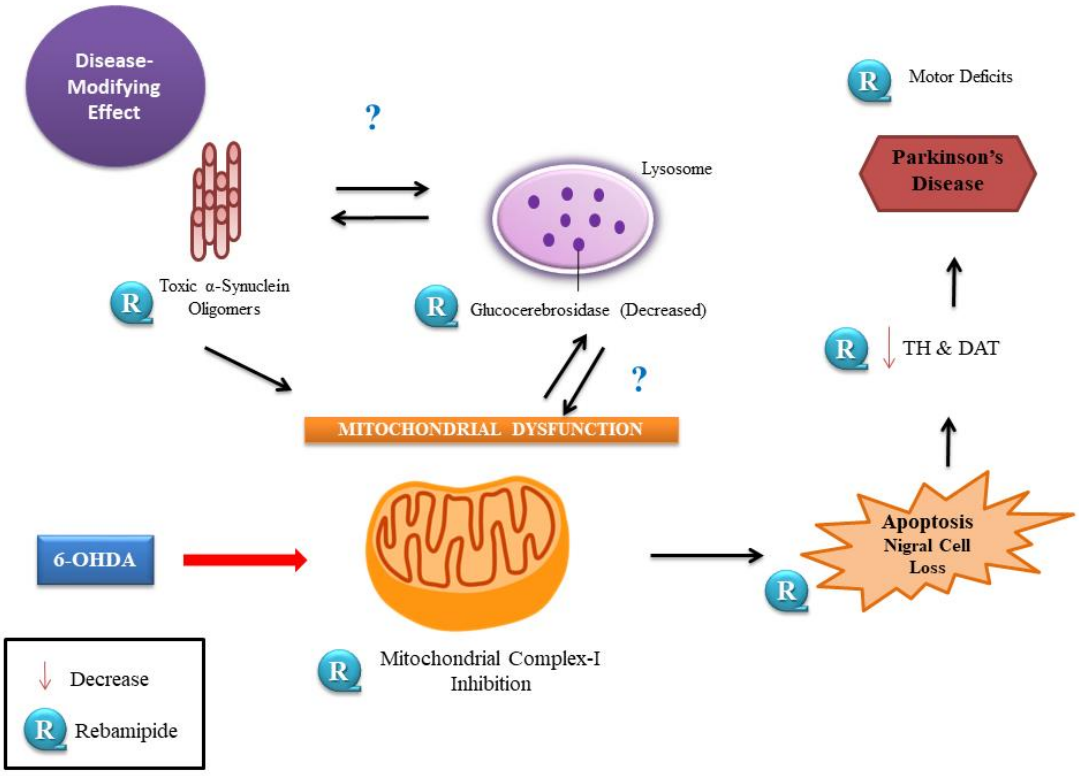

Figure 8.1 The schematic diagram of hypothesis for the evaluation of sub-chronic administration of rebamipide for disease-modifying effects against 6-OHDA-induced model of PD in rats. Rebamipide is administered after the full development of 6-OHDA induced motor deficits to observe its disease-modifying potential. Rebamipide may inhibit mitochondrial dysfunction and α-synuclein pathology, which may be followed by increase in GCase activity, number of Nissl bodies, levels of TH and DAT along with recovery of motor behavior.

8.2. Materials and Methods

8.2.1. Animals

Charles-Foster strain of adult albino rats male (260 ± 20 g) was procured from Central Animal House; Institute of Medical Sciences, Banaras Hindu University (IMS-BHU) and acclimatized at a temperature of 25 ± 1^0C and 45-55% relative

humidity with light/dark cycle of 12:12h by keeping them in polypropylene cages. Commercial food pellets (Doodhdhara Pashu Ahar, India) and water was made available *ad libitum.* No experiments were performed for one week in order to let the animals adapt to the laboratory conditions. All the experimental procedures were carried out in compliance with the principles of laboratory animal care [National Institutes of Health guide for the care and use of Laboratory animals (NIH Publication No. 8023, revised 1978)] guidelines and approved by the Institutional animal ethical committee, BHU (Dean/2016/CAEC/33). The experiments were performed between 9:00h and 16:00h.

8.2.2. Materials

For the source of used materials, please refer Chapter 4 (page 29), Chapter 5 (page 68) and Chapter 6 (page 102).

8.2.3. Stereotaxic surgery

Please refer Chapter 4 (page 30).

8.2.4. Experimental Design

Animals were divided into four groups, namely control, sham, 6-OHDA, and 6-OHDA+R-80 (rebamipide at 80 mg/kg twice a day). The detailed experimental design is illustrated in **Figure 8.2.** Fifteen animals were taken in each group. 6-OHDA intrastriatal injection was given on D-1 except for control and sham groups, as discussed in Chapter 4 (page 31). Stereotaxic surgery was performed on sham group also but the animals received 4 µL of ascorbic acid-normal saline solution only, as discussed in Chapter 4 (page 31). Behavioral parameters were performed on

D-0 and continued at every week up to D-70. Training sessions for behavioral parameters were performed according to Chapter 7 (page 142-143). On the basis of Chapter 5 (page 69-70) and Chapter 6 (Page 103), 80 mg/kg dose of rebamipide was selected and administered to rats by oral gavage at every 12h two times a day from D-28. Rebamipide suspension was prepared in 0.5% CMC (Kim and Hong, 1995; Ohashi et al., 2009). Control group was administered *p.o* with 0.5% CMC suspension. Rebamipide was administered from D-28 as nigral cell loss is reported to be maximum during this period (Blandini et al., 2007; Duty and Jenner, 2011; Przedbroski et al., 1995; Sauer and Oertel, 1994) and motor deficits are fully developed. The drug-treatment schedule was continued up to D-70 after 6-OHDA injection in rats (Coulombe et al., 2016; Lindholm et al., 2007; Voutilainen et al., 2017). ANY-MAZE behavioral tracker version 4.72 (USA) was used to record the observations of open field parameters and rest of the behavioral tests were recorded with a video camera by observers blind to the study protocol. On D-71, 24h after the last drug dosing, three animals from each group were randomly selected for Nissl's staining (n = 3) and remaining animals were killed by decapitation. Brains were isolated, ipsilateral striatum and SNc were micro dissected on ice and stored immediately at -80^0C. SNc tissues were used to assess GCase activity, α-synuclein concentration, TH levels and mitochondrial complex-I (n = 6). DAT levels were estimated in striatal tissues (n = 6).

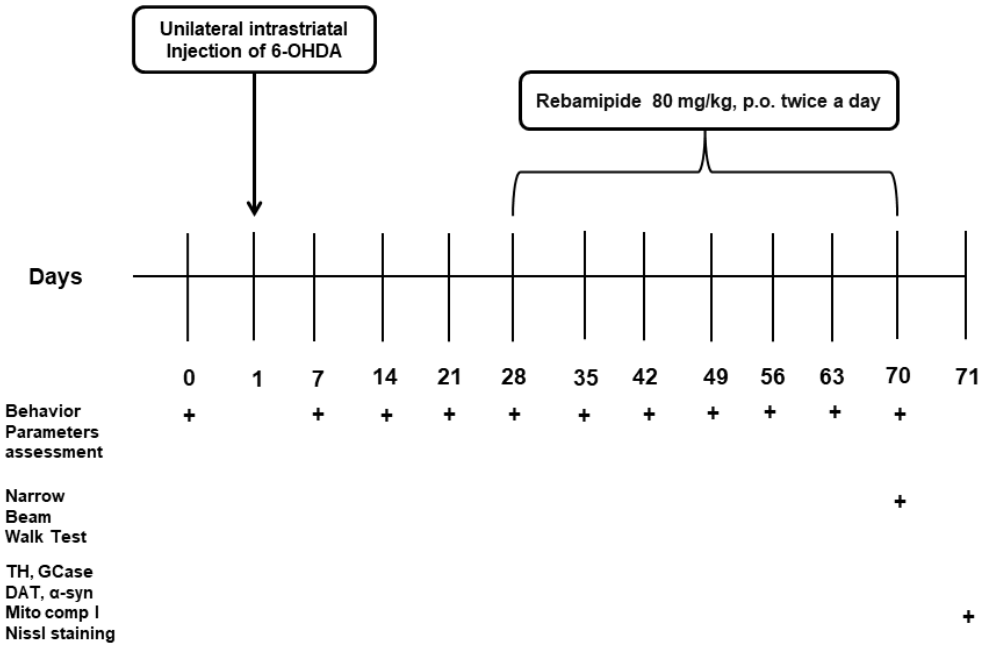

Figure 8.2 The experimental design of study for the evaluation of sub-chronic administration of rebamipide for disease-modifying effects against 6-OHDA-induced model of PD in rats. "+" indicates the days at which parameters were performed.

8.2.5. Behavioral Parameters

8.2.5.1. Apomorphine-induced rotations

Please refer Chapter 7 (page 144).

8.2.5.2. Cataleptic behavior

Please refer Chapter 7 (page 144).

8.2.5.3. Rotarod test

Please refer Chapter 7 (page 144).

8.2.5.4. Grip strength

Please refer Chapter 7 (page 144).

8.2.5.5. Open field behavior

Please refer Chapter 7 (page 143).

8.2.5.6. Narrow beam walk

Please refer Chapter 7 (page 144).

8.2.6. Estimation of TH levels, soluble α-synuclein concentration and DAT levels

Please refer Chapter 4 (page 37) for the estimation of soluble α-synuclein concentration and Chapter 6 (page 106) for the assessment of TH and DAT levels in nigral tissues.

8.2.7. Estimation of mitochondrial respiratory complex-I and GCase activities in nigral tissues

Please refer Chapter 4 (page 36-37) for the estimation of GCase activity and isolation of mitochondria from the ipsilateral nigral tissues of rats. Refer Chapter 5 (page 72) for the estimation of mitochondrial complex-I activity.

8.2.8. Nissl's staining

Please refer Chapter 4 (page 38).

8.2.9. Statistical Analysis

Results were expressed as mean ± SD. Behavioral parameters were analyzed by repeated measures of two-way ANOVA, followed by Bonferroni post-hoc test, except

for narrow beam walk test. All the other parameters and the data of narrow beam walk test were analyzed by one-way ANOVA followed by Student Newman-Keuls post-hoc test. $p < 0.05$ was considered statistically significant.

8.3. Results

8.3.1. Behavior Parameters

8.3.1.1. Behavioral recovery following rebamipide administration against 6-OHDA-induced alterations in bar catalepsy and apomorphine-induced rotation test

Due to degeneration of ipsilateral dopaminergic neurons (Kirik et al., 1998), 6-OHDA showed apomorphine-induced rotational behavior from D-4 (data not shown). Progressive increase was observed in apomorphine induced rotational behavior up to D-21 and was maintained up to D-70. Cataleptic behavior was significantly increased by 6-OHDA from D-14. Cataleptic behavior attained plateau at D-21. That is maximum 6-OHDA-induced cataleptic activity observed on D-21. Repeated measures of two-way ANOVA revealed significant differences in rotational and cataleptic behavior in rats among groups ([$F (3, 616) = 852.4$; $p < 0.05$], [$F (3, 616) = 516.7$; $p < 0.05$] respectively), time ([$F (10, 616) = 44.55$; $p < 0.05$], [$F (10, 616) = 39.06$; $p < 0.05$] respectively) and an interaction ([$F (30, 616) = 26.76$; $p < 0.05$], [$F (30, 616) = 20.41$; $p < 0.05$] respectively) between group and time **(Table 8.1).** Rebamipide when given from D-28 after 6-OHDA intrastriatal injection significantly decreased the rotational behavior progressively from D-49 to D-70. However,

cataleptic behavior was gradually decreased by rebamipide from D-56 to D-70. No significant difference was observed between control and sham groups.

8.3.1.2. Rebamipide attenuated 6-OHDA-induced changes in rotarod retention time and grip strength score

Retention time on rotarod and grip strength were significantly decreased by 6-OHDA from D-7. Statistical analysis by repeated measures of two-way ANOVA indicated that there were significant differences in rotarod retention time and grip strength scores among groups ([$F(3, 616) = 328.4$; $p < 0.05$], [$F(3, 616) = 504.8$; $p < 0.05$] respectively), time ([$F(10, 616) = 17.73$; $p < 0.05$], [$F(10, 616) = 25.47$; $p < 0.05$] respectively) and an interaction between group and time ([$F(30, 616) = 10.43$; $p < 0.05$], [$F(30, 616) = 14.26$; $p < 0.05$] respectively) as shown in **Table 8.1.** Control and sham groups were not found to be significantly different. Rebamipide increased the rotarod retention time from D-42 (after 14 days of oral dosing) and grip strength from D-49 up to D-70 in a progressive manner against 6-OHDA group significantly.

8.3.1.3. Rebamipide inhibited 6-OHDA-induced changes in the parameters of open field test

Repeated measures of two-way ANOVA showed that there were significant differences in number of central squares crossed, ambulation, grooming and rearing behavior among groups ([$F(3, 616) = 438.3$; $p < 0.05$], [$F(3, 616) = 1092$; $p < 0.05$], [$F(3, 616) = 181.7$; $p < 0.05$], [$F(3, 616) = 306.6$; $p < 0.05$] respectively), time ([$F(10, 616) = 33.99$; $p < 0.05$], [$F(10, 616) = 36.67$; $p < 0.05$], [$F(10, 616) = 6.584$; $p < 0.05$], [$F(10, 616) = 10.22$; $p < 0.05$] respectively) and an interaction ([$F(30, 616) = 12.43$; $p < 0.05$], [$F(30, 616) = 14.95$; $p < 0.05$], [$F(30, 616) = 4.738$; $p < 0.05$], [F

(30, 616) = 4.792; $p < 0.05$] respectively) between group and time in open field test **(Table 8.2)**. 6-OHDA significantly decreased ambulation, grooming and rearing from D-7 and increased the number of central squares crossed from D-14 probably due to more time taken to explore the open field (Lamprea et al., 2003). Rebamipide significantly increased the number of central squares crossed progressively from D-56 in 6-OHDA group while remaining of the parameters was significantly increased from D-49. There was no significant difference found between control and sham groups.

8.3.1.4. Rebamipide improved performance against 6-OHDA-induced deficits in narrow beam walk test

Akinesia, bradykinesia, postural instability and balance are consequences of the degeneration of dopaminergic neurons in PD (Moore et al., 2005; Schwab et al., 1959), which are assessed by narrow beam walk test as discussed in Chapter 7 (Allbutt and Henderson, 2007; Geed et al., 2014). No significant difference was observed in both the parameters when sham group was compared with control rats. One way ANOVA showed significant differences among groups in the latency to begin the task [$F(3, 56) = 96.35$; $p < 0.05$] and total time taken to cross the beam [$F(3, 56) = 38.71$; $p < 0.05$] as shown in **Figure 8.3.** 6-OHDA significantly increased both the latency and total time taken by animals to cross the beam compared to sham groups on D-70. Rebamipide significantly decreased the same against 6-OHDA rats.

Table 8.1 Rebamipide (R-80)-induced recovery of behavior symptoms as evaluated by apomorphine-induced rotations, cataleptic behavior, grip strength score and rotarod retention time against 6-OHDA-induced motor deficits in rats

Groups	Apomorphine-induced rotations (Counts/5 min)	Cataleptic Behavior (Sec)	Grip Strength Score	Retention Time In Rotarod Test (Sec)
DAY 0				
Control	5.95 ± 0.90	1.91 ± 0.61	4.41 ± 0.96	181.20 ± 33.38
Sham	5.72 ± 0.87	1.84 ± 0.42	4.49 ± 0.90	180.50 ± 25.64
6-OHDA	5.97 ± 1.13	1.70 ± 0.41	4.53 ± 1.02	181.40 ± 20.04
6-OHDA+R-80	6.04 ± 0.94	1.92 ± 0.33	4.55 ± 0.81	178.20 ± 31.42
DAY 7				
Control	6.02 ± 1.39	1.86 ± 0.55	4.48 ± 1.28	182.80 ± 29.98
Sham	5.56 ± 0.92	1.98 ± 0.54	4.32 ± 1.12	166.60 ± 28.94
6-OHDA	9.40 ± 0.83[a]	2.01 ± 0.23	1.17 ± 0.22[a]	88.20 ± 27.85[a]
6-OHDA+R-80	8.58 ± 1.30[a]	1.96 ± 0.31	1.25 ± 0.28[a]	85.24 ± 24.61[a]
DAY 14				
Control	5.88 ± 1.42	1.61 ± 0.31	4.22 ± 0.96	180.80 ± 31.45
Sham	5.69 ± 0.87	1.71 ± 0.28	4.29 ± 0.81	164.90 ± 26.00
6-OHDA	12.31 ± 1.84[a]	4.13 ± 1.25[a]	1.09 ± 0.20[a]	80.26 ± 16.63[a]
6-OHDA+R-80	13.23 ± 1.92[a]	3.77 ± 0.74[a]	1.22 ± 0.67[a]	84.14 ± 22.81[a]
DAY 21				
Control	5.92 ± 1.81	1.85 ± 0.53	4.38 ± 1.31	180.20 ± 35.09
Sham	5.86 ± 0.92	1.92 ± 0.47	4.26 ± 0.96	165.20 ± 23.44
6-OHDA	15.09± 1.96[a]	6.10 ± 1.33[a]	1.32 ± 0.34[a]	93.58 ± 19.58[a]
6-OHDA+R-80	15.07 ± 2.46[a]	5.90 ± 1.49[a]	1.42 ± 0.37[a]	90.64 ± 21.47[a]
DAY 28				
Control	6.06 ± 1.16	1.93 ± 0.41	4.32 ± 0.82	181.40 ± 25.87
Sham	6.29 ± 0.95	1.87 ± 0.46	4.27 ± 0.95	170.40 ± 31.63
6-OHDA	16.23 ± 2.81[a]	5.98 ± 1.77[a]	1.20 ± 0.27[a]	86.14 ± 23.54[a]
6-OHDA+R-80	15.48 ± 3.15[a]	6.12 ± 1.36[a]	1.17 ± 0.34[a]	93.89 ± 22.71[a]
DAY 35				
Control	5.33 ± 1.59	1.78 ± 0.65	4.53 ± 1.33	184.38 ± 33.59
Sham	5.49 ± 0.87	1.69 ± 0.40	4.49 ± 1.30	178.65 ± 37.61
6-OHDA	15.44 ± 3.39[a]	6.13 ± 1.80[a]	1.18 ± 0.22[a]	92.19 ± 15.95[a]
6-OHDA+R-80	15.09 ± 2.82[a]	5.92 ± 1.29[a]	1.22 ± 0.43[a]	109.48 ± 24.38[a]
DAY 42				
Control	6.16 ± 1.43	1.83 ± 0.35	4.28 ± 0.96	182.13 ± 30.25
Sham	6.32 ± 1.27	1.55 ± 0.57	4.49 ± 1.12	176.09 ± 36.40
6-OHDA	15.64 ± 2.79[a]	6.39 ± 2.00[a]	1.10 ± 0.25[a]	88.03 ± 18.43[a]
6-OHDA+R-80	14.95 ± 2.48[a]	5.97 ± 1.35[a]	1.48 ± 0.57[a]	145.56 ± 28.39[a,b]
DAY 49				
Control	6.22 ± 1.29	1.92 ± 0.67	4.27 ± 0.99	184.38 ± 36.01
Sham	5.97 ± 0.86	1.78 ± 0.49	4.53 ± 1.05	180.43 ± 33.86
6-OHDA	16.74 ± 2.42[a]	6.39 ± 1.44[a]	1.10 ± 0.36[a]	86.66 ± 21.29[a]
6-OHDA+R-80	14.57 ± 3.06[a,b]	5.89 ± 1.05[a]	2.48 ± 0.68[a,b]	160.68 ± 32.44[b]
DAY 56				
Control	5.38 ± 1.52	1.88 ± 0.81	4.54 ± 1.10	183.28 ± 40.17
Sham	5.49 ± 0.81	1.61 ± 0.44	4.47 ± 1.24	179.35 ± 32.47
6-OHDA	15.92 ± 2.76[a]	6.41 ± 2.29[a]	1.08 ± 0.42[a]	82.48 ± 15.53[a]
6-OHDA+R-80	10.01 ± 1.98[a,b]	4.08 ± 1.45[a,b]	2.82 ± 0.62[a,b]	171.79 ± 35.82[b]
DAY 63				
Control	5.33 ± 1.46	1.90 ± 0.23	4.21 ± 1.11	180.83 ± 34.65
Sham	5.51 ± 1.14	1.57 ± 0.26	4.27 ± 0.93	177.45 ± 25.69
6-OHDA	17.69 ± 2.65[a]	6.41 ± 1.52[a]	1.07 ± 0.39[a]	80.17 ± 19.48[a]
6-OHDA+R-80	6.94 ± 1.22[b]	2.59 ± 0.48[b]	4.39 ± 0.81[b]	185.05 ± 31.36[b]
DAY 70				
Control	6.12 ± 1.03	1.77 ± 0.48	4.51 ± 0.83	180.57 ± 40.48
Sham	5.82 ± 1.20	1.85 ± 0.34	4.58 ± 1.04	177.01 ± 32.45
6-OHDA	16.61 ± 3.25[a]	6.40 ± 1.57[a]	1.06 ± 0.34[a]	80.05 ± 19.48[a]
6-OHDA+R-80	6.15 ± 1.38[b]	2.22 ± 0.59[b]	4.69 ± 1.02[b]	178.07 ± 24.39[b]

Rebamipide-administration was initiated from D-28 and continued up to D-70. All values are mean ± SD; n = 15; [a]$p < 0.05$ compared to sham and [b]$p < 0.05$ compared to 6-OHDA [Repeated measures of two-way ANOVA followed by Bonferroni test].

Table 8.2 Rebamipide-induced recovery of behavioral symptoms as evaluated by the number of central squares crossed, ambulation, rearing and grooming in open field test against 6-OHDA-induced motor deficits in rats

Groups	Central Squares crossed (numbers)	Ambulation (numbers)	Rearing (numbers)	Grooming (numbers)
DAY 0				
Control	4.56 ± 0.79	45.88 ± 8.56	15.18 ± 3.85	6.11 ± 1.66
Sham	4.55 ± 0.88	45.25 ± 7.74	14.66 ± 3.09	5.81 ± 1.49
6-OHDA	4.49 ± 0.78	45.94 ± 7.10	15.34 ± 4.46	6.20 ± 1.21
6-OHDA+R-80	4.64 ± 0.62	46.71 ± 9.83	14.97 ± 5.88	6.10 ± 2.00
DAY 7				
Control	4.61 ± 0.80	46.33 ± 9.04	14.93 ± 3.72	6.03 ± 1.61
Sham	4.47 ± 0.73	45.71 ± 9.22	15.24 ± 4.42	5.73 ± 1.47
6-OHDA	4.43 ± 0.83	8.07 ± 1.80[a]	6.14 ± 2.09[a]	3.35 ± 1.07[a]
6-OHDA+R-80	4.76 ± 0.91	9.19 ± 3.53[a]	6.03 ± 2.45[a]	3.26 ± 0.96[a]
DAY 14				
Control	4.43 ± 0.71	46.86 ± 9.32	15.16 ± 3.82	6.34 ± 1.88
Sham	4.56 ± 0.92	44.72 ± 10.89	15.03 ± 5.80	5.92 ± 1.35
6-OHDA	1.93 ± 0.81[a]	6.20 ± 1.70[a]	5.97 ± 1.26[a]	3.06± 0.83[a]
6-OHDA+R-80	2.02 ± 0.65[a]	6.85 ± 2.82[a]	6.39 ± 1.59[a]	2.87 ± 1.59[a]
DAY 21				
Control	4.35 ± 0.84	46.52 ± 10.38	14.81 ± 3.09	6.07 ± 1.32
Sham	4.51 ± 0.86	44.40 ± 9.92	15.02 ± 3.81	5.79 ± 1.35
6-OHDA	1.97 ± 0.33[a]	7.15 ± 2.16[a]	5.71 ± 1.50[a]	3.06 ± 1.23[a]
6-OHDA+R-80	1.84± 0.25[a]	9.06 ± 2.84[a]	5.63 ± 1.92[a]	3.03 ± 0.91[a]
DAY 28				
Control	4.32 ± 0.80	44.95 ± 9.24	14.65 ± 3.24	5.97 ± 1.70
Sham	4.86 ± 0.77	44.21 ± 9.95	14.86 ± 3.77	6.21 ± 1.88
6-OHDA	1.87 ± 0.25[a]	6.43 ± 1.50[a]	6.03 ± 1.81[a]	2.93± 1.25[a]
6-OHDA+R-80	2.10 ± 0.34[a]	6.97 ± 2.38[a]	6.09 ± 1.42[a]	2.83 ± 0.84[a]
DAY 35				
Control	4.53 ± 0.70	45.36 ± 10.75	15.38 ± 2.90	6.29 ±1.99
Sham	4.58 ± 0.93	46.27 ± 9.42	14.74 ± 3.40	6.02 ± 1.56
6-OHDA	1.81 ± 0.44[a]	6.20 ± 3.87[a]	5.48 ± 1.47[a]	2.96 ± 1.49[a]
6-OHDA+R-80	2.10 ± 0.70[a]	6.29 ± 2.30[a]	5.97 ± 1.59[a]	3.28 ± 1.27[a]
DAY 42				
Control	4.35 ± 0.33	45.76 ± 10.84	14.97 ± 3.38	5.97 ± 1.55
Sham	4.60 ± 0.23	46.06 ± 9.85	15.46 ± 4.06	6.14 ± 1.52
6-OHDA	1.73 ± 0.38[a]	5.92 ± 4.05[a]	6.02 ± 1.40[a]	2.76 ± 1.49[a]
6-OHDA+R-80	1.98 ± 0.21[a]	5.78 ± 2.88[a]	6.08 ± 1.74[a]	3.22 ± 0.58[a]
DAY 49				
Control	4.41 ± 0.93	46.68 ± 9.25	15.38 ± 3.88	6.31 ± 1.32
Sham	4.32 ± 0.80	44.76 ± 9.63	14.77 ± 4.27	6.19 ± 1.86
6-OHDA	1.79 ± 0.51[a]	5.66 ± 2.38[a]	5.37 ± 1.74[a]	2.09± 0.92[a]
6-OHDA+R-80	1.94 ± 0.41[a]	13.98 ± 2.12[a,b]	8.86 ± 1.89[a,b]	3.98 ± 0.47[a,b]
DAY 56				
Control	4.55 ± 0.84	45.17 ± 10.74	14.59 ± 4.30	5.88 ± 1.41
Sham	4.59 ± 0.96	46.21 ± 8.45	14.84 ± 4.75	6.10 ± 1.95
6-OHDA	1.72 ± 0.32[a]	5.78 ± 2.89[a]	5.01 ± 1.74[a]	2.47 ± 1.39[a]
6-OHDA+R-80	2.66 ± 0.57[a,b]	17.74 ± 1.91[a,b]	9.09 ± 2.57[a,b]	4.33 ± 0.55[a,b]
DAY 63				
Control	4.60 ± 0.93	45.67 ± 9.83	14.75 ± 4.19	5.57 ± 1.23
Sham	4.39 ± 1.08	46.05 ± 10.75	15.08 ± 3.86	6.00 ± 1.67
6-OHDA	1.70 ± 0.42[a]	5.76 ± 3.12[a]	5.63 ± 1.36[a]	2.76 ± 1.57[a]
6-OHDA+R-80	3.12 ± 0.73[a,b]	20.84 ± 3.52[a,b]	10.13 ± 2.73[a,b]	5.39 ± 0.33[b]
DAY 70				
Control	4.54 ± 0.84	44.86 ± 9.74	14.68 ± 3.70	5.89 ± 1.59
Sham	4.46 ± 0.90	45.42 ± 11.18	15.21 ± 4.19	6.11 ± 1.70
6-OHDA	1.80 ± 0.31[a]	5.78 ± 3.94[a]	5.30 ± 1.28[a]	2.68 ± 1.47[a]
6-OHDA+R-80	3.58 ± 0.79[a,b]	27.40 ± 4.66[a,b]	10.85 ± 3.07[a,b]	5.77 ± 0.45[b]

Rebamipide-administration was initiated from D-28 and continued up to D-70. All values are mean ± SD; n = 15; [a]$p < 0.05$ compared to sham and [b]$p < 0.05$ compared to 6-OHDA [Repeated measures of two-way ANOVA followed by Bonferroni test].

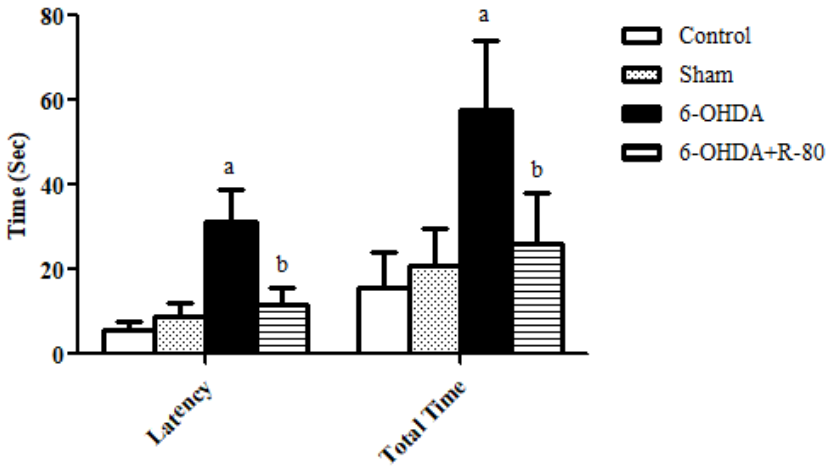

Figure 8.3 Effect of rebamipide on 6-OHDA-induced alterations in beam performance as assessed by latency to begin the task and total time taken to cross the beam in narrow beam walk test in rats. All values are mean ± SD; n = 15; $^{a}p < 0.05$ compared to sham and $^{b}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

8.3.2. Rebamipide attenuated 6-OHDA-induced loss of nigral TH levels

One way ANOVA showed significant differences among groups in the levels of TH [$F (3, 20) = 41.95$; $p < 0.05$] in nigral tissues. Control and sham groups were not found to be significantly different. 6-OHDA significantly decreased TH levels up to 72% in ipsilateral nigral tissues compared to sham rats, which was increased by rebamipide up to 49% compared to 6-OHDA-infused rats as shown in **Figure 8.4.**

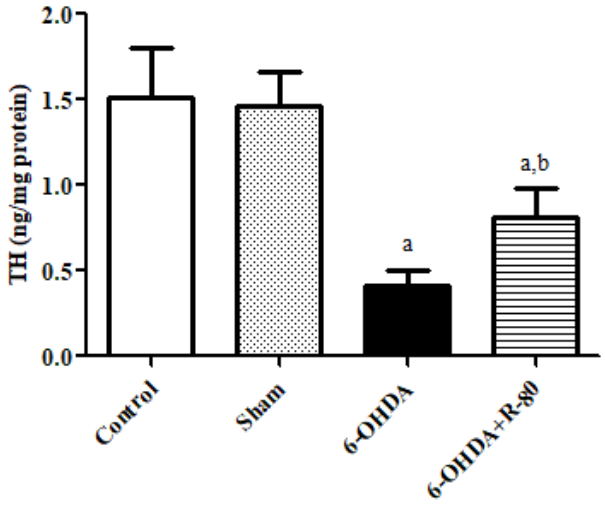

Figure 8.4 Effect of rebamipide on 6-OHDA-induced changes in the TH levels in ipsilateral nigral tissues of rats. All values are mean ± SD; n = 6; $^{a}p < 0.05$ compared to sham and $^{b}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

8.3.3. Rebamipide has restorative effects on 6-OHDA-induced loss of striatal DAT levels

One way ANOVA showed significant differences among groups in the levels of DAT [$F (3, 20) = 7.933$; $p < 0.05$] in striatal tissues as shown in **Figure 8.5.** 6-OHDA significantly decreased (32%) the levels of DAT in ipsilateral striatal tissues of rats which were restored by rebamipide. No significant difference was observed between control and sham groups.

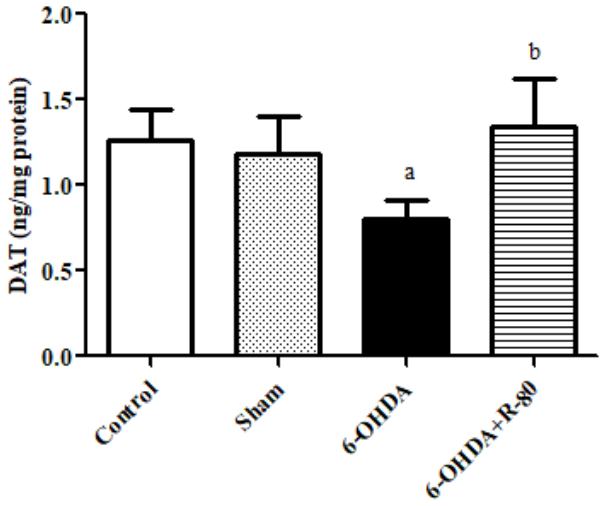

Figure 8.5 Effect of rebamipide on 6-OHDA-induced changes in the DAT levels in ipsilateral striatal tissues of rats. All values are mean ± SD; n = 6; $^{a}p < 0.05$ compared to sham and $^{b}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

8.3.4. Rebamipide attenuated 6-OHDA-induced changes in mitochondrial complex-I, GCase enzymatic activity and α-synuclein concentration in rat nigral tissues

Mitochondrial respiratory complexes (Guo et al., 2013), soluble α-synuclein concentration (Di Maio et al., 2016) and GCase enzymatic activity (Rocha et al., 2015a) play an important role in pathogenesis of PD. 6-OHDA significantly decreased mitochondrial complex-I (66%) and GCase enzymatic activity (74%) compared to sham groups which was significantly increased by rebamipide up to 50% and 61% respectively compared to 6-OHDA-infused rats. No significant difference was found between control and sham groups. One-way ANOVA showed significant differences in mitochondrial complex-I activity [$F(3, 20) = 15.49$; $p < 0.05$], GCase

enzymatic activity [$F(3, 20) = 19.94$; $p < 0.05$] and soluble α-synuclein concentration [$F(3, 20) = 8.724$; $p < 0.05$] among groups as shown in **Figure 8.6**. 6-OHDA also significantly decreased the soluble α-synuclein concentration up to 79% compared to sham group, indicating the aggregation of its insoluble form, as discussed in chapter 4 and 5 (Budi et al., 2012; Mishra and Krishnamurthy, 2019). Rebamipide significantly increased the concentration of soluble α-synuclein up to 71% against 6-OHDA group.

8.3.5. Rebamipide inhibited 6-OHDA-induced nigral cell loss

PD occurs due to degeneration of dopaminergic neurons in SNc region of brain (Kalia and Lang, 2015). The same is achieved by intrastriatal injection of 6-OHDA in the present study, which was estimated by using Nissl's staining. There were significant differences in the percentage of Nissl bodies in nigral tissues among groups [$F(3, 8) = 30.40$; $p < 0.05$], as shown by one-way ANOVA **[Figure 8.7]**. Control and sham groups were not found to be significantly different. 6-OHDA decreased the Nissl-stained cell bodies up to 50% compared to control groups. Rebamipide recovered up to 73% of Nissl-positive cells compared to 6-OHDA-infused animals.

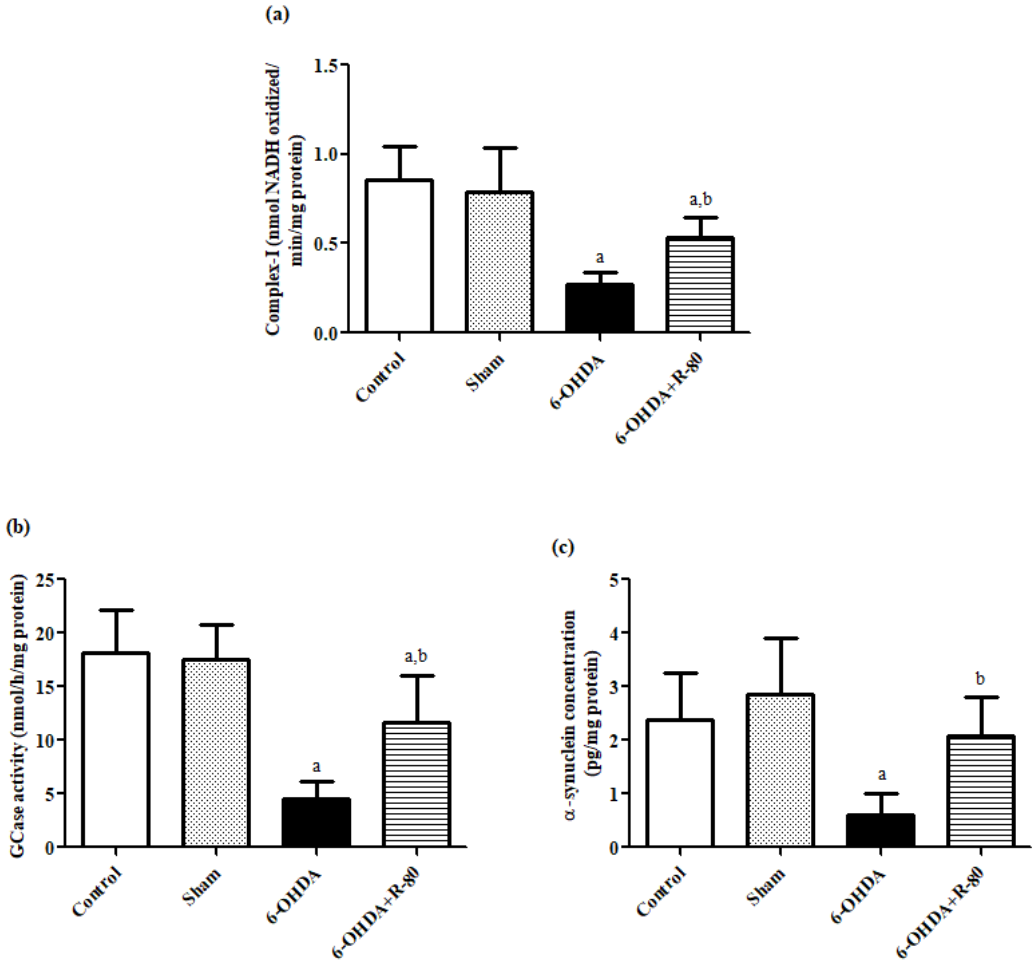

Figure 8.6 Effect of rebamipide on 6-OHDA-induced alterations in mitochondrial complex-I (a), GCase enzymatic activity (b) and α-synuclein protein concentration (c) in ipsilateral nigral tissues of rats. All values are mean ± SD; n = 6; $^{a}p < 0.05$ compared to sham and $^{b}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

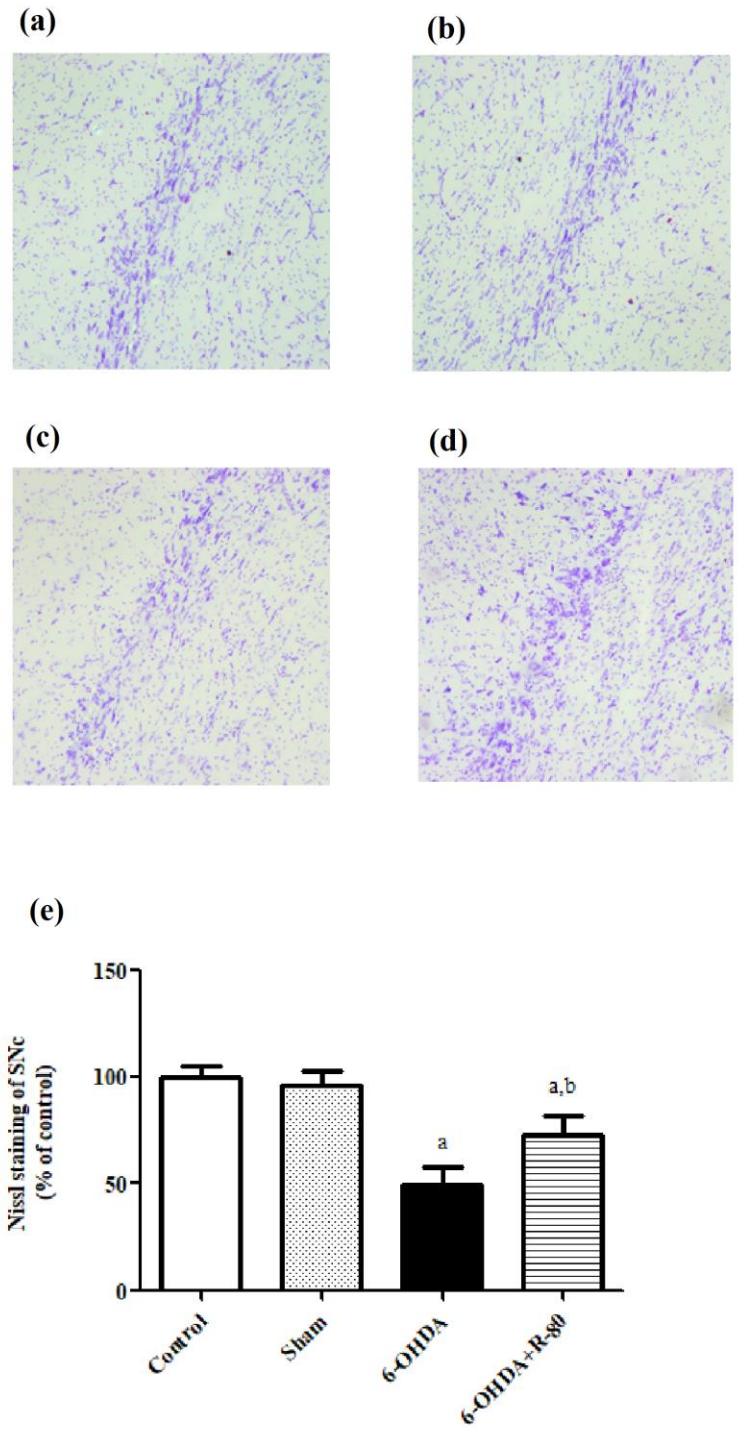

Figure 8.7 Nissl's staining of SNc region of brain in rats. Control (a); Sham (b); 6-OHDA (c); 6-OHDA+R-80 (d); Data of counting cells (e). All values are mean ± SD; n = 3; $^{a}p < 0.05$ compared to sham and $^{b}p < 0.05$ compared to 6-OHDA [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].

8.4. Discussion

The primary finding of current study is the neurorestorative effect of rebamipide on the rat dopaminergic system following 6-OHDA lesion. Rebamipide showed its restorative potential by increasing TH and DAT levels as well as normalizing Nissl bodies in nigrostriatal dopaminergic system in 6-OHDA-infused rats. GCase enzymatic and mitochondrial complex-I activity was increased and α-synuclein pathology was decreased. It also recovered motor functions in 6-OHDA-infused rats.

PD is considered as TH-deficiency disorder due to its involvement in biosynthesis of DA, which become deficient in PD (Haavik and Toska, 1998; Zhu et al., 2012). Therefore, TH is considered as significant dopaminergic cell marker (Voutilainen et al., 2017). In present study, significant decrease in nigral TH levels was observed on D-70 after 6-OHDA intrastriatal injection as reported earlier (Lindholm et al., 2007; Voutilainen et al., 2017). TH deficiency leads to inadequacy of DA, which regulates motor behavior (Joshua et al., 2009). Rebamipide-induced increase of TH indicates attenuation of DA inadequacy against 6-OHDA toxicity and thereby suggests its neurorestorative potential on dopaminergic system. Apart from TH, DA is also controlled by DAT, which is reported to be reduced up to 50-70% in severe PD cases (Nutt et al., 2004). DAT levels have been used in previous studies to measure the viability of dopaminergic cell (Gainetdinov et al., 1998). 6-OHDA decreased striatal DAT levels post ten weeks of 6-OHDA lesion as reported earlier (Coulombe et al., 2016; Voutilainen et al., 2017). Rebamipide-induced restoration of DAT levels in rat striatal tissues indicates the increased viability of dopaminergic cells, as discussed in Chapter 7.

Clinically, PD mainly comprises of motor deficits such as bradykinesia, tremor, postural instability and muscular rigidity (Carrozzino et al., 2018). In the present study, rebamipide attenuated 6-OHDA-induced motor symptoms from D-49, except for the number of central squares crossed and bar catalepsy behavior which was recovered from D-56 and rotarod retention time was decreased from D-42. Exploration of open field is indicated by the number of central squares crossed (Lamprea et al., 2003). Cataleptic behavior shows fine motor control (Walther and Strik, 2012; Whishaw et al., 1990) whereas rotarod test characterizes gross motor skills (Qian et al., 2010; Rozas et al., 1997). Therefore, the difference in onset of action of behavioral parameters may be due to the comparatively less efficiency of rebamipide to affect fine motor control, whereas gross motor skill was recovered earlier. The recovery of 6-OHDA-induced motor symptoms by rebamipide was progressive in all the parameters. Rebamipide also decreased 6-OHDA-induced aggravation of bradykinesia, akinesia and postural instability as assessed by narrow beam walk test (Allbutt and Henderson, 2007), indicating its effectiveness in restoring motor functions.

Mitochondrial complex-I deficiency is observed in SNc region of PD patients (Schapira et al., 1990). In the present study, 6-OHDA decreased mitochondrial complex-I activity which was increased by rebamipide when administered for 42 days after 4 weeks of 6-OHDA lesion, indicating the attenuation of 6-OHDA-induced mitochondrial dysfunction. Mitochondrial function share bidirectional relationship with GCase enzyme (Cleeter et al., 2013; Gegg et al., 2012), which is an integral part of lysosomes (Migdalska-Richards and Schapira, 2016). Mitochondrial dysfunction is found to decrease GCase activity in brain regions of PD patients (Gegg et al., 2012),

which leads to accumulation of dysfunctional mitochondria (Cleeter et al., 2013; Osellame et al., 2013). In the present study, 6-OHDA decreased GCase enzymatic activity in nigral tissues of rats (Mishra et al., 2018). Rebamipide is reported to increase the same after 4 weeks of 6-OHDA lesion in previous Chapter 5 (Mishra and Krishnamurthy, 2019). However, this is the first study showing the restorative effects of rebamipide on 6-OHDA-induced GCase enzymatic deficiency 10 weeks post 6-OHDA lesion. This may be the downstream effect of amelioration of mitochondrial dysfunction. GCase and α-synuclein oligomers affect each other in a negative manner, as discussed in previous chapters (Cleeter et al., 2013; Mazzulli et al., 2011). 6-OHDA-induced reduction in water-soluble concentration of α-synuclein in the ipsilateral nigral tissues measured in the present study indicates the increase in aggregates of α-synuclein toxic oligomers, as explained in previous chapters (Budi et al., 2012; Coulombe et al., 2016; Mishra et al., 2018). 6-OHDA-induced aggregation of α-synuclein oligomers has been reported previously in rat model of PD (Gu et al., 2016). Rebamipide restored the soluble α-synuclein concentration against 6-OHDA-infused rats, indicating the decline of toxic α-synuclein oligomeric aggregates. This may be due to the GCase-stimulating effect of rebamipide. Therefore, increased soluble α-synuclein concentration, GCase enzymatic activity and mitochondrial function in the dopaminergic system indicate the involvement of these factors behind the neurorestorative potential of rebamipide.

α-synuclein is predominantly observed at presynaptic terminals and takes part in regulation of presynaptic function (Chandra et al., 2004; Fortin et al., 2005). Soluble α-synuclein concentration restored by rebamipide in the present study indicates terminal integrity restoration of dopaminergic neurons in nigral region as

discussed in Chapter 7. α-synuclein oligomers also cause mitochondrial dysfunction (Di Maio et al., 2016), which leads to apoptosis (Elmore, 2007). Nissl's staining in SNc indicates the density of dopaminergic neurons, as discussed in earlier chapters (Domesick et al., 1983; Zaitone et al., 2012). Nissl-stained cell bodies were decreased up to 50% by 6-OHDA (Voutilainen et al., 2017) and rebamipide rescued up to 73% of it, indicating the rescue of dopaminergic neurons in SNc tissues. TH neurons-counterstaining with Nissl is better measure of dopaminergic neurons. Rebamipide was previously reported to decrease apoptosis in hepatic ischemia model in rats (Gendy et al., 2017), also decreased indomethacin-induced apoptosis in gastric epithelial cells (Nagano et al., 2005). Hence, in the present study rebamipide-induced normalization of dopaminergic neuronal density in nigral region against 6-OHDA toxicity indicates its neurorestorative potential. Therefore, repeated administration of rebamipide shows neurorestorative effects against 6-OHDA-induced model of PD in rats.

8.5. Conclusions

The current study serves as the first preclinical evidence for the neurorestorative potential of rebamipide in the dopaminergic system using experimental PD model. Rebamipide on administration after development of full motor deficits (due to 6-OHDA) in rats, not only increased DAT and TH, the constituents of intact dopaminergic system but also enhanced GCase enzymatic activity and decreased α-synuclein pathology, which are involved in the regulation of PD pathogenesis. Increased concentration of soluble α-synuclein by rebamipide indicates restoration of terminal integrity of nigral dopaminergic neurons. Mitochondrial function was balanced and total numbers of Nissl-positive cells were also normalized. Overall,

dopaminergic transmission may be increased in nigrostriatal neurons as indicated by rebamipide-induced restoration of DAT levels. This leads to recovery of the motor functions in rats, which dominate the clinical picture of PD. Considering the urgent need for the disease-modifying agent in the current scenario, the present results support the disease-modifying property of rebamipide. Mitochondrial function, α-synuclein concentration and GCase enzymatic activity serve as the responsible factors, as depicted in **Figure 8.8**.

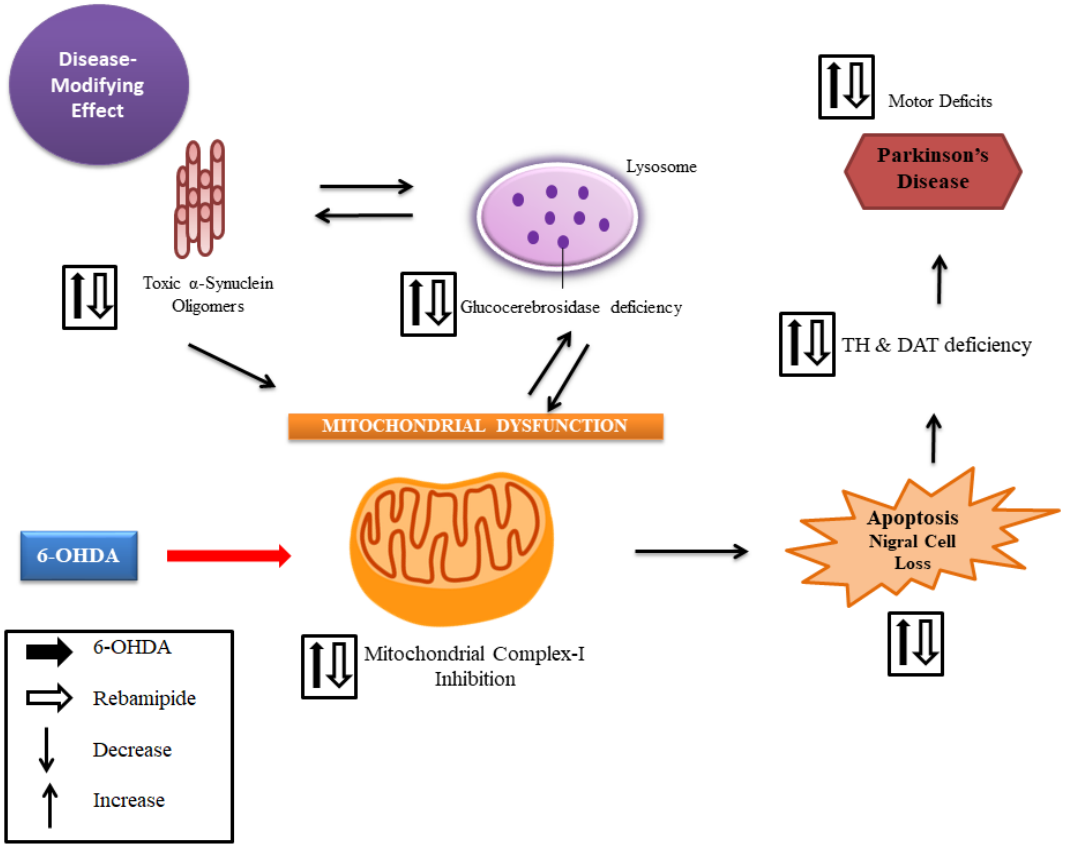

Figure 8.8 The outcome of specific objective for the evaluation of sub-chronic administration of rebamipide for disease-modifying effects against 6-OHDA-induced model of PD in rats. Rebamipide inhibits mitochondrial dysfunction and α-synuclein pathology, which is followed by increase in GCase activity, number of Nissl bodies, levels of TH and DAT along with recovery of motor behavior. Rebamipide is administered after the full development of 6-OHDA induced motor deficits, indicating its disease-modifying potential.