Chapter 3
Development of 6-OHDA-induced Rat Model
3A Introduction

PD is a progressive neurodegenerative disorder characterized by the gradual loss of dopaminergic neurons in the nigro-striatal pathway. Mostly, PD is sporadic in origin. Significant strides have been made in understanding the etiology and pathogenesis of PD in clinical and postmortem studies, however, there is no proven therapy that can prevent cell death or slow its progression. Thus, interventions that can slow or halt the progression of PD remain a crucial challenge (Blesa et al. 2012; Jackson-Lewis et al. 2012; Tieu 2011). Animal models have been important tool in the unraveling the mysteries of PD pathogenesis and discovery of novel treatments for motor symptoms of PD. An ideal model of PD consider pathological and clinical features dopaminergic and nondopaminergic systems, the central and peripheral nervous systems, motor and nonmotor symptoms in addition to the age-dependent onset and progressive nature of PD (Blesa et al. 2012; Tieu 2011). Animal models of PD involves, vertebrate (including rat, mouse, zebrafish) and invertebrate (includes Drosophila melanogaster, Caenorhabditis elegans) (NIH 2013). In vivo expression of PD related mutations (genetic) or those using environmental or synthetic neurotoxins are available, however none of the models display all features of PD (Table 1).

The identification of different genetic mutations (α-synuclein, parkin, LRKK2, PINK1, DJ-1) has led to the development of genetic models of PD but these models are in limited use as only ~10% of total cases of PD are reported to be of genetic origin. However, these animal models may reveal specific molecular events that leading to death of the DA neurons and potential therapeutic targets (Blesa et al. 2012).

Among the environmental or synthetic neurotoxins models, compounds that produce both reversible (reserpine) and irreversible (MPTP, 6-OHDA, paraquat, rotenone) effects have been used effectively. Among these models 6-OHDA causes oxidative stress; MPTP/MPP⁺, paraquat and rotenone inhibits mitochondrial complex I, PSI (proteosomal inbhibitor) and epoximycin through proteasomal inhibition and lipopolysaccharide (LPS) through glial cell activation. These toxins have been used to address the process of neurodegereation and screening of neuroprotective agents (Figure 1).
Table 1: Advantages and disadvantages of animal models used and their potential roles in revealing the mechanisms for PD pathogenesis.

<table>
<thead>
<tr>
<th>Model</th>
<th>Symptoms</th>
<th>Histopathology</th>
<th>Pathogenic relevance</th>
<th>Applications</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reserpine</td>
<td>Akinesia, catalepsy</td>
<td>None</td>
<td>Pharmacological dopamine depletion</td>
<td>Preclinical testing of therapies to improve symptoms</td>
<td>Non-specific liberation of monoamine transmitters; hypothermia</td>
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<tr>
<td>Methamphetamine</td>
<td>No clear parkinsonian symptoms</td>
<td>At very high doses of TH in striatum; loss of dopamine cells in SNc</td>
<td>Dopamine-related oxidative stress</td>
<td>Screen antioxidant therapies to protect dopamine cells</td>
<td>Acute; limited histopathological change</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>Unilateral; rotation after amphetamine; Bilateral; akinesia</td>
<td>Decreased striatal TH-immunoreactivity; degeneration of TH-immunoreactive neurons in SNc</td>
<td>Oxidative stress</td>
<td>Preclinical testing of therapies to improve symptoms; screen pharmacological and genetic therapies designed to protect dopamine cells</td>
<td>Acute; usually unilateral (hemiparkinsonian); intrastratal injection may produce more chronic degeneration; requires intracerebral injection</td>
</tr>
<tr>
<td>MPTP</td>
<td>Akinesia, rigidity, tremor in some species</td>
<td>Decreased striatal TH-immunoreactivity; degeneration of TH-immunoreactive neurons in SNc; some loss of locus ceruleus neurons; α-synuclein aggregation</td>
<td>'Environmental' toxin; oxidative stress; inhibition of mitochondrial complex I</td>
<td>Preclinical testing of therapies to improve symptoms; screen pharmacological and genetic therapies designed to protect dopamine cells</td>
<td>Generally acute; non-progressive or reversible; inclusion bodies are rare</td>
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<td>Paraquat-Maneb</td>
<td>Decreased locomotor activity</td>
<td>Decreased striatal TH-immunoreactivity</td>
<td>Multiple environmental toxins/pesticide exposure; 'oxidative stress'</td>
<td>Screen pharmacological and genetic therapies designed to protect dopamine cells</td>
<td>Not yet extensively investigated/described</td>
</tr>
<tr>
<td>Rotenone</td>
<td>Akinesia, rigidity, tremor, flexed posture, piorocedion</td>
<td>Decreased striatal TH-immunoreactivity; degeneration of TH-immunoreactive neurons in SNc; some loss of locus ceruleus neurons; inclusion remnants of Lewy bodies</td>
<td>Chronic 'environmental' toxin; chronic oxidative stress; chronic inhibition of mitochondrial complex I</td>
<td>Screen pharmacological and genetic therapies designed to protect dopamine cells</td>
<td>Labor- and time-intensive; substantial morbidity and mortality</td>
</tr>
<tr>
<td>3-Nitrotyrosine</td>
<td>Amphetamine-induced rotation</td>
<td>Decreased striatal TH-immunoreactivity; degeneration of TH-immunoreactive neurons in SNc</td>
<td>Oxidative stress</td>
<td>Screen antioxidant therapies to protect dopamine cells</td>
<td>Not yet extensively investigated/described; requires intracerebral injection</td>
</tr>
<tr>
<td>Transgenic α-Synuclein</td>
<td>Reduced or abnormal motor activity</td>
<td>α-Synuclein-positive intraneuronal inclusions; degeneration of TH-immunoreactive neurons observed in flies; modest decrease in striatal TH-immunoreactivity in mice</td>
<td>Known pathogenic mutations</td>
<td>Screen pharmacological and genetic therapies designed to protect dopamine cells</td>
<td>Expensive and time-consuming; mice do not have characteristic PD pathology or phenotype</td>
</tr>
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</table>

MPTP and 6-OHDA are the two most commonly used inducing agents for PD. MPTP is highly lipophilic and after systemic administration rapidly crosses the blood-brain barrier. Once in the brain, MPTP enters astrocytes and metabolizes into MPP+, its active metabolite, by monoamine oxidase-B (MAO-B). MPP+ stored in vesicles is thought to expels DA out into the intercellular space and metabolizes into a number of toxic metabolites, such as DOPAL, superoxide radical (5-cysteinyl-DA) and hydroxyl radical (6-OHDA) (Blesia et al. 2012). Susceptibility to MPTP varies across the species.
and strain of animal used. Rats are resistant to MPTP while mice strains vary in the sensitivity and usually administered as intravenous, intraperitoneal, intra muscular and subcutaneous (Betarbet et al. 2002). MPTP administration do not require skilled personnel, however, standard operating procedure (SOP) is required as unmetabolised MPTP secreted in urine increases the exposure risk to the user (Przedborski et al. 2001; Yang et al. 1988).

Figure 1: Schematic representation of a dopaminergic SNpc neuron showing the molecular targets for the various agents used to induce animal models of PD that exhibit nigro-striatal tract degeneration.

6-OHDA is widely used PD inducing agent and requires skilled personnel to directly inject it into substantia nigra, medial forebrain bundle (MFB), or the striatum. Structure of 6-OHDA is similar to that of dopamine except additional hydroxyl group which makes it toxic to dopaminergic neurons. 6-OHDA is produced endogenously in human brain and urine samples (Bezard et al. 2013). 6-OHDA is used for both in vitro (human neuroblastoma or carcinoma cell lines - PC12, SH-SY5Y SK-N-SH MN9D and NTera-2), immortalised human mesencephalic cell lines - Lund human mesencephalic [LUHMES]) (Schüle 2011) and in vivo models such as Mice, cats, dogs, and monkeys; however it is used much more frequently in rats. 6-OHDA exhibits a high affinity for several catecholaminergic transporters such as the dopamine transporter (DAT) and norepinephrine transporter (NET), therefore it is used in conjunction with a selective norepinephrine reuptake inhibitor such as desipramine in order to spare the
noradrenergic neurons from damage in animal models of PD (Betarbet et al. 2002; Bezard et al. 2013).

6-OHDA is used to induce both unilateral and bilateral lesion in animals. Unilateral model is profoundly used due to its quantifiable circling motor abnormality in animals leading to be used extensively as a preclinical model to assess the antiparkinsonian effects and screening of neuroprotecting agents (Tieu 2011). Bilateral model mimics the human disease more closely as PD affects the brain bilaterally, and also there is no intact site which can partly compensate for the affected site like the case in unilateral model. The bilateral model offers the possibility to assess higher cognitive tasks such as a choice reaction-time task (Deumens et al. 2002).

Oxidative stress has been considered as major causative agent of PD. Nitric oxide contribute it by nitrosative stress due to its overproduction resulting in neurotoxicity, thereby leading to the emergence of nNOS inhibitors as potential therapeutic targets in PD (Adrian J. Hobbs 1999; Wang and Michaelis 2010). 7-Nitroindazole (7-NI) is commonly used as selective inhibitor of nNOS with negligible peripheral side effect and is under investigation as a possible protective agent against nerve damage caused by excitotoxicity or neurodegenerative diseases, cerebral palsy, cerebral ischemia, hypoxia and epilepsy in other than 6-OHDA rat model of PD (Cui et al. 2012; Hrncic et al. 2012; Joung et al. 2012; Kapoor 2012; Komsuoglu-Celikyurt et al. 2011; Luszczki et al. 2011; Madathil et al. 2013; Orzelska et al. 2013; Yu et al. 2011).

Since the prevalence of PD is more in men than in women leading to a lot of research on male animal model of PD to evaluate the role of 7-NI in PD pathology (Di Matteo et al. 2009; Komsuoglu-Celikyurt et al. 2011; Miller and Cronin-Golomb 2010; Padovan-Neto et al. 2009; Thomas et al. 2008; Volke et al. 1997; Yildiz Akar et al. 2007; Yuste et al. 2012). However, the physiology of Nitric oxide in female brain is quite different, due to the physio-anatomical differences, leading to the deviation in the behavioural studies and providing neuroprotection to the females in animal model against the toxicity of 6-OHDA and emergence of female hormone estrogen as the new neuroprotective agent in clinical trials (Dykens et al. 2005; Nicoletti et al. 2007). Majority of workers uses preoperative and acute administration of 7-NI to assess its
neuroprotective ability which does not mimics the actual pathological condition. In the present work, the post-operative effect of subchronic administration of 7-NI has been investigated in the 6-OHDA induced bilateral female rat model of PD.

3A.1 Objectives

Development of bilateral 6-OHDA induced female rat model of PD to study the neuroprotective effect of 7-NI and NO mediated protein expression in 6-OHDA and 7-ni treated rats.

1. Development of 6-OHDA induced rat model to study neuroprotective effect of 7-NI
   (i) Behavioural studies: emotional (depression), motor and cognitive deficits.
   (ii) Immunohistostaning by tyrosine hydroxylase.
   (iii) Estimation of dopamine concentration by HPLC.
   (iv) Antioxidant effects – lipid peroxidation, Glutathione reduced, Catalase, and SOD.
2. NO mediated alterations in the total protein expression compared to s-nitrosylated protein expression in 6-OHDA induced and 7-NI treated rats
   (i) Study the total protein expression profile by two-dimension gel electrophoresis.
3. Identification of novel interacting protein partner/s of nNOS by the virtue of its PDZ domain.

3A.2 Material and Methods

3A.2.1 Animals

The work was carried in the Animal house Dr. B.R. Ambedkar Center for Biomedical research, University of Delhi, Delhi. All the procedures used were approved by the Institutional animal ethical committee of (ACBR*). 35 female rats weighing (200-350 gm) were procured from national brain research center Manesar, Haryana. Rats were homed in standard cages (maximum of 4 animals) at 23 degree celsius under 12 hour light and dark cycle with free access to food and water. The rats were divided into 4 groups:
Group 1: Control (n=8)

The vehicle was administered surgically into the substantia nigra of 4 animals, and remaining 4 animals as untreated control.

Group 2: 7-NI (n=8)

The animals were administered with 7-NI intraperitoneally (I.P) for three consecutive days. The 7-NI used was first dissolved in 0.005 % of DMSO and then mixed with ground.

Group 3: 6-OHDA (n=11)

6-OHDA (10.5 µg) was administered in substantia nigra surgically.

Group 4: 6-OHDA+7-NI (n=8)

The animals of this group were treated with 7-NI (30 mg/Kg body weight) for three consecutive days post surgery.

3A.2.3 Surgery and 6-OHDA Lesion

All surgical procedures were conducted under aseptic conditions. To produce a PD model with incomplete destruction of nigrostriatal DA cells, 6-OHDA (10.5 µg per injection, diluted in 0.9% NaCl, supplemented with 0.02% ascorbic acid, injection volume 1.0µl at the rate of 0.2µl/min; Sigma Chemical, St. Louis, MO, USA) was injected over 5 min bilaterally into the substantia nigral area through a blurr hole using stereotaxic head frame. Stereotaxic injection followed the coordinates of the Paxinos and Watson (29) atlas: AP: -5.0 mm, ML: ±2.0mm and DV: -7.5 mm from bregma, using a Hamilton 10µl syringe with a 26-gauge needle. Following injection, the needle was left in place for 5 min before being retracted, to allow complete diffusion of the drug. All animals were treated with i.p. injection of 25 mg/kg desipramine (Sigma) 30 min before surgery, in order to protect noradrenergic terminals from 6-OHDA toxicity. The stereotaxic surgery was performed under ketamine (75 mg/kg)/xylazine (5 mg/kg) anesthesia. Sham-operated rats followed the same protocol except for the fact that vehicle was injected instead of 6-OHDA. Three animals from 6-OHDA group died before three week after surgery. The behavioral studies were conducted between 4 and 6 weeks after the surgery. Animals were sacrificed after the studies under anesthesia.
3A.2.4 Behavioral Tests

(a) Tests for Assessment of Emotional Parameters

(i) Forced-swimming test: The procedure used was previously described by Porsolt et al. (1977). With slight modification in the apparatus. The rats were placed in individual plexiglass vertical box (17 x 17 x 40) containing water (water depth was 30 cm; 25 °C) (Figure. 2). Two swimming sessions were conducted (an initial 15-min pretest followed 24 h later by a 3-min test). The experiment was recorded using Anymaze software, Stoelting, USA. The total duration of test was recorded continuously for a 3-min period. Following parameters were taken into account during data acquisition: the rat was regarded as immobile when floating motionless or making only those movements necessary to keep its head above the water. Total time immobile was observed.

(b) Tests for Assessment of Cognitive Performance

(i) Olfactory discrimination task: To assess the possible impairment in the olfactory function of the rat models, the olfactory discrimination task was performed 4th, 5th and 6th week after surgery. The test consists of placing each rat for 5 min in a cage, which was divided into two identical compartments (30x30x20 cm) separated by an open door, where it could choose between one compartment with fresh sawdust and another with unchanged sawdust (familiar compartment) that the same rat had occupied for 48 h before the test. Each animal was initially placed in the center of the non-familiar compartment and the time spent by the rat in familiar compartment was recorded by Anymaze software. Usually, mature rats are able to discriminate between the familiar and the non-familiar compartments, spending much more time in the familiar compartment since they significantly prefer their own odor to no odor at all.

(ii) Elevated plus maze: The elevated plus maze apparatus consisted of two open arms (50x10 cm) and two enclosed arms (50x10x40 cm), arranged such that two pairs of identical arms were opposite to each other. Arms emerged from a central platform (10x10 cm), and the entire apparatus was raised to a height of 50 cm above floor level. At the beginning of the test, the rat was placed on the central platform facing an open arm. After each 5 min test, the maze was carefully cleaned up with a spirit wet cloth to
avoid any olfactory clue of previous animal. Anxiety was evaluated through the following behavioral parameters during 5th and 6th week: percent time in open arms, its measures is inversely related to the anxiety level in rodents that can be confounded by changes in motor activity. Operational criterion for entry was center of body which was recorded by Anymaze software.

(c). Tests for Assessment of Motor Function Performance

(i) Assessment of spontaneous locomotor activity: Open field test: In order to evaluate the exploratory behaviour as an index of motor activity, open-field test was performed 4th and 6th weeks after surgery or without it (7-NI group and control). The apparatus, made of wood, had a black floor of 100 X 100 cm (divided by imaginary lines (lines were drawn in the software protocol) into 25 squares of 20 X 20 cm) and white walls, 40 cm high. Whole apparatus was divided into four zones: the walls were included in rearing zone, the peripheral squares were termed as peripheral zone, the squares inner to the peripheral zone were termed as central zone and the center square was termed as center zone. The droppings of each animal were removed after the test for the same is over to avoid the olfactory clue interfering in locomotion. Each rat was placed in the center of the open field, and the number of squares crossed, time spent, and mobility in zone were registered for 5 min by Anymaze software.

(ii) Rota rod test: To assess the fine motor coordination and balance, Rota rod test was performed with all animals with the modification in the method described by Dekundy et al. The animals were given vigours pre-test training till the animal was able to maintain its balance walking on top of the rod on a two compartment Rota rod instrument (kritij instruments pvt. India.) 24 hrs before the actual test at fixed speed of 25 rpm. 24 hrs later animals were subjected to rotarod test for maximum of 180 sec and five trials were given for each animal except for those which completed 180 sec in first three trials. Time spent on rotarod was noted. If the animal falls during the trial then the trial was considered over and animal was given next trial. The Rota-rod performance is expressed as the mean of the total time spent per minute on Rota rod.
3A.2.5 **TH-immunohistopathology:** Six weeks after 6-OHDA treatment animals were intracardially perfused with saline and brains were quickly dissected and stored at -80°C. Serial sections (5µM) were made using cryostat (leica biosystems pvt ltd) spanning the substantia nigra and fixing the sections with methanol which also deactivates endogenous peroxidase, all the sections were stored at -20°C till the use. Tyrosine hydroxylase immunohistology was assessed using the anti-mouse Tyrosine Hydroxylase, (from Santa Cruz Biotechnology INC, USA (catalogue no. of antibodies used: sc-47708, sc-2005). High temperature antigen retrieval was performed by keeping the slides covered with 10 mM trisodium citrate buffer pH 6.0 for 10 mins in microwave. The nonspecific binding was blocked by incubating sections for 1 h with 5 % BSA diluted in PBS. After overnight incubation at 4°C with primary antibodies, the slides were washed with PBS and incubated with the secondary antibody for 1 h at room temperature. The sections were washed in PBS, and the visualized by DAB (3, 3’-diaminobenzidine) (Sigmaaldrich co, India) in PBS solution. Control and experimental tissues were placed on the same slide and processed under the same conditions. Digital images were acquired by NIKON digital camera connected to a light microscope Eclipse-80i (Nikon, Melville, NY, USA). Settings for image acquisition were identical for control and experimental tissues. Digitized images were transferred to a computer, and the average pixel intensity of immunostaining for TH was calculated using imaging software.

3A.2.6 **Estimation of Dopamine Concentration by HPLC**

For determining DA contents in the brain, the rats were killed during anesthesia after 6th weeks after 6-OHDA administration. Brains were removed immediately and the substantia nigra was dissected from the preparation, frozen in liquid nitrogen and stored at -80°C. The concentration of DA was assayed by C-18 reverse-phase high performance liquid chromatography (HPLC) with Photo diode array (PDA). The tissue samples of each group were pooled and homogenized with an ultrasonic cell disrupter (Sonics) in chilled 0.1 M perchloric acid containing 0.05% EDTA. After centrifugation at 10,000 x g for 30 min, 4°C, 10µl of the supernatant was injected into the chromatograph. The mobile phase consists of 0.1M acetate–citrate buffer (pH 4.5) and 10 % methanol at λ max 254 nm), used at a flow rate of 1 ml/min. Dopamine concentration was determined from the standard curve obtained using dopamine.
3A.2.7 Antioxidant Effects

(a) Lipid Peroxidation

Method by (Utley GH 1967) was used to estimate lipid peroxidation. Briefly, The absorbance of each aliquot was measured at 535 nm. The rate of lipid peroxidation was expressed as nmol of thiobarbituric acid reactive substance (TBARS) formed per hour per milligram of protein using a molar extinction coefficient of $1.56 \text{ M}^{-1} \text{ cm}^{-1}$.

(b) Catalase

Catalase activity was measured by the method. The reaction mixture consisted of 2 mL phosphate buffer (0.1 M, pH 7.4), 0.95 mL hydrogen peroxide (0.019 M) and 0.05 mL PMS in a final volume of 3 mL. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H$_2$O$_2$ consumed min$^{-1}$ mg$^{-1}$ protein.

(c) Superoxide Dismutase

The superoxide dismutase was assayed according to the method described by Mice brains were homogenized in phosphate buffer (pH 7.8, 0.1M) and centrifuged at 38,000 rpm for 10 min at 4 °C. The supernatant containing Mn–SOD complex was used as total enzyme fraction. SOD level was determined from inhibition of pyrogallol auto-oxidation by SOD at pH 8.2. The reaction mixture (1ml) contained 0.2mM Pyrogallol, 1mM EDTA and 50mM Tris–HCl buffer. Pyrogallol autooxidation was monitored at 420nm for 3 min using spectroflourimeter (Perkin Elmer; USA) with or without the enzyme protein. 50% inhibition/mg protein/min was taken as one unit of enzyme activity.

(d) Reduced Glutathione

Reduced glutathione was estimated by the method described by Jollow et al. Brain tissue was homogenized in 0.1M potassium phosphate buffer (pH 7.8), centrifuged at 38,000 rpm for 10 min and supernatant were combined. The protein was removed by precipitation with 4% sulfosalicylic acid and centrifuged at 7500×g for 10 min and the
supernatant containing reduced glutathione was collected. Glutathione was estimated by assaying the development of yellow by (4mg/ml) DTNB (5,5’-dithiobis-(2-nitrobenzoic acid/ Elman’s reagent) in 10% w/v sodium phosphate buffer, pH 8.0 and with the yellow colour developed was read immediately at 412 nm.

3A.3. Data Analysis and Statistics
Statistical analyses were performed either with the computer software GraphPad Prism 6.0 (GraphPad Software, Inc.) or by the software itself (ANYMAZE). All data are expressed as mean±S.E.M. Following significant ANOVAs, post-hoc analysis was done by the Bonferroni test. A $P$ value of less than 0.05 was considered significant.

3A.4 Results
3A.4.1 Effects of 7-NI Administration on the 6-OHDA Induced Emotional Behaviour in Rat Model of PD
(a) Effect of 7-NI Administration on 6-OHDA Induced Depressive Behavior in Forced-swimming Test: The forced-swimming test was used to monitor 6-OHDA induced depressive-like behavior after three week post-surgery. Animals were observed for forced swim test for three consecutive weeks: 4th, 5th and, 6th week after surgery. Group 1(control) remained active throughout the study, (45.69±3.18; 39.26±8.55;34.44±9.94 secs for 4th, 5th and, 6th respectively ). Group 2, 7-NI treated rats alone among the groups (except 6-OHDA) do not differ significantly with time duration (37.71±11.24; 32.92±8.12 59.65±23.65 secs for 4th, 5th and, 6th respectively) and creating the impression that 7-NI is not having any effect and is itself exerting the depressive behavior. Group 3(6-OHDA) treated animals showed the longest immobility time (71.60±7.26; 71.86±13.70; 73.84±3.92 secs for 4th, 5th and, 6th respectively). Group 4 (6-OHDA+7-NI) treated animals shows (69.14±15.14; 32.2±4.58; 70.76±26.96; secs for 4th, 5th and, 6th respectively) the similar behaviour with 7-NI (Figure 2).
Figure 2: Effect of 7-NI on total immobile time observed for all treatment groups during 4th to 6th week. Animals (n=8/group) (group 6-OHDA+7NI and 7NI) received 7-NI (30 mg/kg) (i.p.) or 6-OHDA (10.5µg intracranially)/vehicle per injection (group 6-OHDA and CONTROL). The total time immobile in the swimming apparatus was observed for 180s. Bars represent the means±SEM. * represent significant difference from 6-OHDA (ANOVA followed by the bonferroni, \( P<0.05 \) refer the text). The 6-OHDA treated animals were significantly immobile in comparison to control animals with p value less than 0.5. Immobility remained significant during test duration (every week) among the 6-OHDA treated animal in comparison to control animals with p value less than 0.5.

3A.4.2 Effect of 7-NI administration on 6-OHDA induced cognitive behavior

(i) Effect of 7-NI Administration on 6-OHDA Induced Olfactory Deficits in 6-OHDA Treated Animals for the Cognitive Performance in Rats Models of PD

In order to assess any deleterious effect of 6-OHDA on the olfactory discrimination ability of rats, the olfactory discrimination task was performed to choose the preference for familiar and unfamiliar husk zone for 5 min. After three weeks of surgery. Animals were observed for olfactory discrimination test for three consecutive weeks: 4th, 5th and, 6th. Olfactory discrimination was evaluated percent time spent by the animals in the familiar compartments. The results indicates that the control group animals preferred familiar zone during all three weeks. 6-OHDA treated animals spent less time in the familiar zone in three week’s compared to rest of the animal groups. The 7-NI treated animals significantly shows the increased time spent in the familiar zone behaving similarly to control animals. The 6-OHDA +7-NI treated animal shows the
increased entries in familiar zones with respect to 6-OHDA animals suggesting the neuro protection exerted by 7- NI. Conclusively 7-NI has significantly exerted neuro protected effect in 6-OHDA induced toxicity. Two way ANOVA p value < 0.0001 and < 0.0001 followed 6-OHDA (Figure 3).

![Percent time in familiar zone](image)

**Figure 3:** Effect of 7-NI on percent time spent in familiar zone observed for all treatment groups during 5th and 6th week. Key: 1- 5th week, 2- 6th week. Animals (n=8/group) (group 6-OHDA+7NI and 7NI ) received 7-NI (30 mg/kg) (i.p.) or 6-OHDA (10.5µg intracranially)/vehicle per injection (group 6-OHDA and CONTROL). The total time spent in open arm of elevated plus maze was observed for 300s. Bars represent the means±SEM. * represent significant difference from 6-OHDA (ANOVA followed by the bonferroni, \( P < 0.05 \) refer the text). The 6-OHDA treated animals were significantly spending less time in familiar zone in comparison to control animals with p value less than 0.5. Time spent by 6-OHDA animals in familiar zone, remained significantly low during test duration (every week) among the 6-OHDA treated animal in comparison to control animals with p value less than 0.5.

(ii) Effect of 7-NI Administration on 6-OHDA Induced Anxiety Behavior in 6-OHDA Treated Animals in the Elevated Plus Maze Test in Rat Models of PD

In line with previous data (Branchi et al. 2008), no main effect of treatment has been found for behavioral parameters relevant for the anxiety-like response within all animal group along with time, as no significant changes were observed in percent time observed in open arm of elevated plus maze (Figure 4).
Figure 4: Percent time in open arm observed for all treatment groups during 5th and 6th week. Key: 1-5th week and 2-6th week observation. No significant difference was found in percent time spent in open arm of elevated plus maze by all animal groups.

3A.4.3 Effect of 7-NI Administration on 6-OHDA Induced Impairment of the Spontaneous Locomotion in 6-OHDA Treated Animals in the Open Field Test in Rat Models of PD

The spontaneous locomotor activity was evaluated by open field test. The exploratory behavior evaluated by number of line crossed by the animals in open field apparatus. Animals were observed for spontaneous locomotor activity for three consecutive weeks: 4th, 5th and, 6th week after surgery. The results indicates that 6-OHDA treated animals significantly less active for the spontaneous locomotion during all the three week’s observation time as well as among the animals treatment groups indicated by two way ANOVA p value < 0.0001 and < 0.0001 respectively owing to the motor impairments elicited by the 6-OHDA (Figure 5).
Figure 5: Number of line crossings observed for all treatment groups during 4th and 5th week. Animals \( (n=8\)/group) \( (\) group 6-OHDA+7NI and 7NI \( ) \) received 7-NI (30 mg/kg) \( (\) i.p.) or 6-OHDA \( (10.5\mu g \) intracranially)/vehicle per injection \( (\) group 6-OHDA and CONTROL). The numbers of line crossings were observed for 300s in the open field test. Bars represent the means±SEM. * represent significant difference from 6-OHDA \( (\) ANOVA followed by the bonferroni, \( P<0.05 \) refer the text). The 6-OHDA treated animals were significantly crossing less line in comparison to control animals with p value less than 0.5.

3A.4.4 Effect of 7-NI Administration on 6-OHDA Induced Impairment of the Spontaneous Locomotion in 6-OHDA Treated Animals in the Rota Rod in Rat Models of PD

Rota Rod test was performed to evaluate the fine motor skills among the treatment groups. The animals treated with 7-NI performed better than the 6-OHDA in which the motor activity of the animals was effected by the toxicity. By the data presented apparently shows the decrease in the motor activity after five weeks in the 7-NI treated animals. However the clinical observation revealed that the animals treated by 7-NI had learned to escape from the apparatus and do not have motor impairment. Even in 6-OHDA + 7-NI, the gradual improvement per week was observed which is in consistence with the results of 7-NI treated animals. Similar behavior was observed in the forced swim test. The 6-OHDA treated animals were unable to stay on the Rota rod shown by both all the three week’s observation time as well as the difference among the groups taking 6-OHDA by two way ANOVA p value \( < 0.0001 \) and \( < 0.0001 \) respectively owing to the motor impairments elicited by the 6-OHDA (Figure 6).
Figure 6: Rota rod performances observed for all treatment groups during 4th to 6th week. Animals (n=8/group) (group 6-OHDA+7NI and 7NI) received 7-NI (30 mg/kg) (i.p.) or 6-OHDA (10.5µg intracranially)/vehicle per injection (group 6-OHDA and CONTROL). The average time duration of the animal on the Rota rod was observed. Bars represent the means±SEM. * represent significant difference from 6-OHDA (ANOVA followed by the bonferroni, \( P < 0.05 \) refer the text). The 6-OHDA treated animals were significantly unable to stay on the Rota rod in comparison to control animals with p value less than 0.5.

3A.5 Analysis of Neurochemical Alterations Induced by 6-OHDA Administration

The neurochemical alterations were measured by immunohistostaining of dopaminergic neurons by tyrosine hydroxylase expressed in dopaminergic neurones after 45 days week post lesion. The results indicates the significant preservations of neurons (indicated by relative optical density of DAB stained brain tissue sections) by the administration of 7-NI in comparison to 6-OHDA treated animals. Results given are mean ± S.E.M, n=3, (\( P \leq 0.0001 \) vs CONTROL ; ordinary One-way ANOVA and \( P \leq 0.05 \) vs. 6-OHDA; Dunnett’s multiple comparisons test). The relationship between the behavioral alterations induced by 6-OHDA in rats and neurochemical alterations in the levels of DA was measured 45 days after post lesion. It was observed that the dopamine concentration was lowest in 6-OHDA treated group and highest in control group which indicates the significant neuroprotection conferred by 7-NI. The immunohistopathology by tyrosine hydroxylase positive neurons revealed the significant protection of by 7-NI treatment measured after 45 days week post lesion (Figure 7).
Figure 7: A. Photomicrograph showing the neuroprotective effect of post-operative sub chronic treatment of 7-NI (30mg/kg body weight) in the substantia nigra of 6-OHDA treated animal’s sacrificed in about ~ 45 days post lesion. The arrow marks the substantia nigra in all treatment groups (magnification 11x). B. Relative optic density of tyrosine hydroxylase positive cells (TH+). subchronic treatment of 7-NI has significantly protected the neurons of substantia nigra evident by the relative optical density in all treatment group in camparision with control. C. Effect of subchronic treatment of 7-NI on the dopamine concentration in Rat brain substantia nigra tissue. 7-NI has the significant effect on the dopamine concentration on 6-OHDA treated animal group when compared to 6-OHDA. The dopamine concentration is expressed in ng/mg of brain tissue, measured after 45 days post lesion.

3A.6 Antioxidant assays

(a)Lipid Peroxidation

TBARS level are indicator of lipid peroxidation in the brain tissue. Lipid peroxidation was estimated in substantia nigra tissue of rat brain after 45 days post lesion. TBARS
reactive substance formed per mg tissue was significantly high in 6-OHDA animal when compared with other groups, indicating an increase in oxidative stress in the brain tissue. Results given are mean ± S.E.M, n=3 (P≤0.0072 CONTROL vs. 6-OHDA; ordinary One-way ANOVA; and P≤0.05 vs. 6-OHDA; Dunnett’s multiple comparisons test) Figure 8 A.

(b) Glutathione Estimation

Glutathione is an endogenous neuro protective agent that acts as anti-oxidant by reacting with the reactive oxygen and nitrogen species. Its low level indicates the oxidative stress in the brain tissue. Glutathione was estimated in substantia nigra tissue of rat brain after 45 days after post lesion. GSH concentration in tissue was significantly high in 6-OHDA animal when compared with other groups which indicates the neuro protection by 7-NI. Results given are mean ± S.E.M, n=3, (P≤0.0001 CONTROL vs. 6-OHDA; ordinary One-way ANOVA; P≤0.05 vs. 6-OHDA; Dunnett’s multiple comparisons test) Figure 8.

Figure 8: A. Lipid peroxidation estimated in all treatment group and significantly observed highest in 6-OHDA treated group and lowest in 7-NI treated group (p value significant). B. Glutathione reduced was estimated in all treatment group and significantly observed highest in control group and lowest in 6-OHDA group (p value significant).
Figure 9: A. Catalase activity estimated in all treatment group and significantly observed lowest in 6-OHDA treated group and highest in control group (p value significant). B. Superoxide dismutase activity was estimated in all treatment group and significantly observed highest in control group and lowest in 6-OHDA group (p value significant).

(c) Catalase

Catalase activity is also the indicator of oxidative stress. Catalase activity was measured in substantia nigra tissue of rat brain after 45 days after post lesion. Catalase activity in tissue was significantly low in 6-OHDA animal when compared with other groups. Results given are mean ± S.E.M, n=3, (P≤0.0012 CONTROL vs. 6-OHDA; ordinary One-way ANOVA; and P≤ 0.05 vs. 6-OHDA; Dunnett’s multiple comparisons test ) figure 8 (A).

(d) Superoxide Dismutase Activity (SOD)

SOD activity was measured in substantia nigra tissue of rat brain after 45 days after post lesion. SOD activity in tissue was significantly low in 6-OHDA animal when compared with other groups. Results given are mean ± S.E.M, n=3, (P≤0.0001 CONTROL vs. 6-OHDA; ordinary One-way ANOVA; and P≤ 0.05 vs. 6-OHDA; Dunnett’s multiple comparisons test ) figure 15 (B).
3A.7 Discussion

In last two decades, neuroprotective effect of 7-NI has attracted the attention of many workers to explore it in several neurodegenerative disorders including PD (Bostanci and Bagirici 2007; Joung et al. 2012; Kapoor 2012; Luszczki et al. 2011; Nanri et al. 1998; O’Neill et al. 1996; Smith et al. 1996). MPTP-induced increase in the striatal or nigral SOD and catalase activities were significantly attenuated with the treatment of 7-NI treatment(Thomas et al. 2008). The result suggested that 7-NI possessed potent antioxidant and neuroprotective effects against MPTP-induced neurotoxicity. In another study (Haik et al. 2008), the rats were administered intraperitoneal injections of 50 mg/kg 7NI (or vehicle) just before receiving bilateral, intrastratal injections of the DA-toxin, 6-hydroxydopamine (6-OHDA). The rats were then given a various motor tasks to assess their learning ability using a spatial reversal task in a water-T maze. Results indicated that 7NI treatments attenuated 6-OHDA-induced spatial learning deficits and protected against DA cell loss in the substantia nigra (SNc), suggesting that 7NI may have potential in early, presymptomatic pharmacotherapy for PD. Most of the neuroprotective effect of 7-NI has been explored as pre-operative acute administration in Bi/unilateral rodent model of PD (Haik et al. 2008; Madathil et al. 2013; Novaretti et al. 2010). A few reports suggested the sex differences influenced the 6-OHDA induced neurotoxicity in rat model of PD (Pienaar et al. 2007); however, none of the study showed the neuroprotective effect of 7-NI in 6-OHDA induced bilateral female rat modsl of PD.

In the present study, the neuroprotective effects of 7-NI (30 mg/kg) was demonstrated in post operative subchronic bilateral administration of 6-OHDA in SNc of female rat model. Behavioural results indicated that 7-NI ameliorate the toxic effect of 6-OHDA.

Forced swim test(FST) has been widely used for the evaluation of anti-depressant like effect. Nitric oxide synthase (NOS) inhibitor have been tested study FST using mice model an antidepressant-like effect (Doucet et al. 2013; Harkin et al. 1999; Inan et al. 2004; Karolewicz et al. 1999; Volke et al. 2003). Recently, (Ferreira et al. 2012) has investigated the 7-NI mediated effect on the global gene and protein expression in the hippocampus of rats submitted to forced swimming test (FST) and suggested that changes in oxidative stress and neuroplastic processes could be involved in the
antidepressant-like effects induced by nNOS inhibition. It is common observation that estrogen act as anti-depressant (Shaukat et al. 2005; Walf and Frye 2010; Walf et al. 2009). The neuro chemical differences in the female brain with respect to male leads to the deviation in many behavioral studies in the female. Therefore, female animal models are not used in anti-depressant studies. The present study demonstrates that 7-NI exhibit anti-depressant effect in female rat model of PD observed by the significant changes in the immobility time of 7-NI and 7-NI treated 6-OHDA group in comparision to control group with time. Also, (Yildiz et al. 2000) reported that the immobility decreases in the male rats with increase in 7-NI dose and suggested that 7NI might be acting like NMDA receptor antagonist or serotonin reuptake inhibitor and this is supported later by (Volke et al. 2003) and (Joung et al. 2012). However the reverse was observed in the present study with female rats where 7-NI treated groups (6-OHDA+ 7NI and 7NI group) showed gradually increase in immobility with time in comparision with control and 6-OHDA treated animal groups. The result suggests that 7-NI is provides neuroprotection by NOS inhibition and other pathways to be involved. One argument to support the above observation is the action of endogenously circulating estrogen, which regulates the glutamate level by inhibiting the extracellular mGlu receptor 1 (metabotropic glutamate receptor type 1) while 7-NI inhibits NMDA receptor mediated retrograde glutamate release, 7-NI also acts on mGlu receptor 5 not on mGlu receptor 1 (Kipp et al. 2006; Nishi et al. 2005). In accordance to previous results on the spontaneous locomotor activity by open field activity, the present study also suggested that 7-NI did not alter the locomotor activity in the open field at 30 mg/kg (Yildiz et al. 2000)indicating that the effect is not gender biased. (Yildiz Akar et al. 2007) in the modified elevated plus maze (mEPM) study reported that 7-NI significantly prolonged the transfer latency on retention session in a mEPM test in the male rats. In present study even though transfer latency is not reported but the parameters used are in accordance with the anti-depressant effect of 7-NI. For motor coordination, rota rod test is commonly employed, (Spolidorio et al. 2007; Talarek et al. 2008). Administration of 7-NI alone had no significant effect on the motor performance measured by the rotarod in rats and male mice respectively. In agreement to these results, Rota rod performance of bilateral female rats in the current study showed the enhanced motor coordination skill as during the repeated test for three consecutive
weeks not only the animal quickly adapted to the training (shown by first two week results) but also learned to escape from the rotarod instrument resulting in the low scoring in the final week. It has been reported that females learn to escape without having the effect of any stress (Dalla et al. 2008; Dalla and Shors 2009). It is well documented that 7-NI protects dopaminergic neurons from the toxic effect of 6-OHDA which is in agreement to the observed immunostaining by tyrosine hydroxylase and dopamine estimation. The result showed the significant protection of dopaminergic neurons of substantia nigra in 7-NI treated animal group (20 ng) in comparison to 6-OHDA (6.2 ng) lesioned animals. The neuroprotection effect exerted by 7-NI for 6-OHDA is reported to be mediated by the antioxidant action evaluated through reduced glutathione (GSH), superoxide dismutase (SOD) and catalase activities in substantia nigra (Thomas et al. 2008). In consistent to this report, attenuation of 6-OHDA-induced GSH depletion by 7-NI treatment in SNc provides further credibility to the notion that 7-NI is an antioxidant. This is also reflected in the reversal by 7-NI of OHDA-induced increases in SOD activities in the SNc, but different trend was observed with catalase and lipid peroxidation in which the 6-OHDA lesioned animals have lower lipid peroxidation and catalase activity, the reason for such inconsistency is the homeostasis maintained by these enzymes till the animals were sacrificed for data collection.

3A.8 Conclusion

Based on quantitative behaviroural, biochemical and histopathological analyses, the present study provides in vivo evidence for the neuroprotective effect of 7-NI on 6-OHDA induced neurotoxicity in the female rat model of PD. Biochemical and immunostaining suggested the involvement of anti-oxidative and anti-inflammatory effects of 7-NI.
References


Chapter 3  Development of 6-OHDA-induced Rat Model


3B NO mediated alterations in the total protein expressions compared to S-nitrosylated protein expression in 6-OHDA induced and 7-NI treated rats

3B.1 Introduction

Proteomics, the study of proteins to understand their function and structure has emerged as a powerful tool to explore the new avenues in the pathology of disease. The term proteome refers to the expression pattern of the proteins resolved in a 2-dimensional gel. There are relatively few proteomic studies of neurodegenerative diseases like Alzheimer’s and Parkinson’s disease (Zhang et al. 2008). The major foci of these investigations have been analysis of protein from autopsy samples in studies of pathogenesis and from cerebrospinal fluid (CSF) in the pursuit of biomarkers (Hastings 2009; Kitsou et al. 2008; Shiozaki et al. 2004).

Parkinson’s disease occurs as a result of the progressive loss of the structure or function of neurons, including the death of neurons with the hallmark of increased oxidative and nitrosative stress due to overproduction of highly reactive free radicals known as reactive oxygen species (ROS) and reactive nitrogen species (RNS). These molecules are normally removed by cellular antioxidant systems (Surendran and Rajasankar 2010). Under physiological conditions, ROS/RNS are present at low levels, mediating several neurotrophic and neuroprotective signaling pathways. In contrast, under pathological conditions, there is a pronounced increase in ROS/RNS generation, impairing normal neurological function due to the harmful signaling by S-nitrosylation (Nakamura and Lipton 2011; Nikolova 2012; Shahani and Sawa 2012; Tsang and Chung 2009). The addition of NO group to Cys thiol forms an S-nitrosoprotein (SNO-protein) product. S-nitrosylation regulates a broad spectrum of proteins like protein disulfide isomerase (PDI), the E3 ubiquitin ligase parkin, mitochondrial fission protein dynamin-related protein 1 (Drp1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and have been implicated in the pathology of many neurodegenerative diseases including PD (Akhtar et al. 2012)
The complex ranges of chemical modifications, that occur to proteins after translation including S-nitrosylation, are involved in the study of protein interaction by proteomics to understand the pathology of the disease. The method used for the quantification of S-nitrosylated proteins is, biotin-switch technique (BST) described by, S-nitrosylated cysteines are converted into more stable biotinylated forms. The BST method involves three steps: 1) free cysteine thiols are blocked with S-methylmethanethiosulfonate, 2) S-nitrosylated cysteines are reduced by ascorbate without the reduction of disulfide bonds or other oxidative cysteine post translational modifications, and 3) newly exposed cysteine thiols are alkylated by biotin- N-[6-(Biotinamido)hexyl]-3’-(2’-pyridyldithio)propionamide (Biotin-HPDP). The BST method has certain pitfalls like, large amount of false positive results, high background of endogenously S-nitrosylated proteins in the protein gel.(Forrester et al. 2009; Forrester et al. 2007).

Santhanam et al. (2008) has developed a new method called as Selective Fluorescent Labeling Of S-nitrosothiols (S-FLOS) to resolve the limitations of previous method.
(Figure 1) eliminating the need for Western blot and allow direct comparison of the S-nitrosation state between two samples in the same gel lane or on the same 2D gel with a low background and a high signal to noise ratio by labeling the reduced cysteine residues sites with Cy dye.

Cy dyes belong to the cyanine family of dyes, one of the commercially available form is CyDye DIGE Fluor saturation dyes having maleimide reactive group that forms a covalent bond with the thiol group of cysteine residues on proteins via a thioether linkage and have net charge neutral, ensuring that the pI of the protein does not significantly alter on labeling. The extent of the mass shift of a labeled protein depends on the cysteine content of the protein and the accessibility of the cysteine residues to dye in the labeling reaction. Two dyes are available for saturation labeling, CyDye DIGE Fluor Cy3 and Cy5. CyDye DIGE Fluor Cy3 and Cy5 saturation dyes are migration-matched so identical proteins labeled with each of the two CyDye DIGE Fluor saturation dyes will migrate to the same position on a 2–D gel. The ability to multiplex permits the inclusion of both sample and internal standard (internal reference) in every gel. The use of an internal standard within each gel helps to limit system variation, which ultimately provides more accurate quantitation of relative protein abundance (Healthcare 2003).

In the present work detection and identification of the nNOS mediated effect in the protein expression profile of the 6-OHDA induced rat model of PD was carried using 2-DIGE study its role in the molecular pathology.

3B.2 Objectives

1. Total protein expression profiling by 2-Dimension electrophoresis.
2. Standardization of the S-Flos method with bovine serum albumin protein (BSA).
   a. In vitro S-nitrosylation of BSA by sodium nitrite.
   b. Titration of Cy dye
3. Labeling of proteins isolated from the animal model of PD by modified S-Flos method.
4. Analysis of the proteins by 2-Dimensional Difference Gel Electrophoresis (2-DIGE).
5. Identification of the s-nitrosylated proteins by Maldi-toff analysis.
6. Exploration of S-nitrosylated GAPDH as mediator of neuronal cell death.

3B.3 Methodology

Material
Protease inhibitor- leupeptin, pepstatin, phenylmethanesulfonylfluoride (PMSF), sodium fluoride, antipain and other chemicals: acetone, CHAPS (Nondenaturing zwitterionic detergent), Tris-HCl, Glycine, SDS, Tris base from Biobasic Incorporation Pvt Ltd.; urea, thiourea, Dithiothreitol (DTT), Iodoacetamide, Bio-Lyte (ampholyte) and Ready IPG strips from Bio-Rad; Bradford from Bangalore Genei; glycerol (Merck); Bromophenol Blue, neocuprione, MMTS (sigma). The source of chemicals not mentioned here id written alongwith them in the text.

Methods

1. Total Protein Expression Profiling by 2-Dimension Electrophoresis

(a) Sample Preparation
Protein samples (30 mg of brain tissue) prepared from the brain samples of 6-OHDA induced animal models from four treatment group were homogenized in buffer A (50mM Tris Cl, pH 7.5, protease inhibitor- leupeptin 2ng/ml, pepstatin 5ng/ml, PMSF 1 mM, sodium fluoride 2mM, antipain 5ng/ml) with 10-15 strokes of a dounce homgeniser fitted with Teflon probe attached to IKA overhead stirrer at 2000 rpm/min. The homogenate was centrifuged at 3, 000 rpm for 10 mins, at 4°C. supernatant was sonicated at 80% power with 3 cycles of 30 seconds each and again centrifuged at 10,000 rpm for 15 min at 4°C. Supernatant was collected and subjected to acetone precipitation with four volumes of ice cold acetone for atleast 1hr or overnight at -20°C. precipitated protein was dissolved in rehydration buffer (8M urea, 2M Thiourea, 2%
CHAPS, 1% DTT and 1% (w/v) Bio-Lyte 3-10 pH ampholytes) and protein estimation was done using Bradford Assay according to manufacturer’s guidelines (Bangalore Genei). 200µg of protein in maximum 250µl of volume of rehydration buffer (with 0.0001% v/v bromophenol blue) was used to passively rehydrate the Ready IPG strips (pH 3/10, 11 cm; Bio-Rad) overnight at room temperature.

(b) Isoelectric Focusing (IEF)

The first dimension of two-dimensional electrophoresis was done on a Protean IEF System (Bio-Rad) with Ready IPG strips (pH 3/10, 11 cm; Bio-Rad) rehydrated with 200 µl of protein sample and subsequently subjected to high voltages at 20°C with the following electric focusing conditions – Step 1: 250 V for 40 minutes (linear), Step 2: 8,000 V for 2 hours and 30 minutes (linear) and Step 3: 30,000 volt hours (rapid) and a final step of 500V for 30 mins (slow).

(c) Equilibration and SDS PAGE

Isoelectrically focused IPG strips were equilibrated in equilibration buffer I containing 6 M urea (Bio-Rad), 2% SDS (Bio-Rad), 0.375M Tris-HCl pH 8.8, 20% glycerol (Merck), 1% (w/v) DTT (Bio-Rad) and Equilibration buffer II containing 6 M urea, 2% SDS (Bio-Rad), 375 M Tris-HCl pH 8.8, 20% glycerol (Merck) and 2.5% (w/v) Iodoacetamide (Bio-Rad), for 10 min each at room temperature. The IPG strips were removed from the equilibration tray, dipped briefly in 1X Tris Glycine SDS (72.06 g/L Glycine, 5g/L SDS, 15.15 g/L Tris) running buffer and placed on the back plate of the dual Midi Assembly (banglore genei) on the well. Using forceps, the IPG strips were gently pushed into the well, taking care not to trap any air bubble beneath the IPG strip. The agarose solution (0.05% Agarose in 1X running buffer with traces of Bromophenol Blue) was introduced into the IPG well of the gel on top of the strips. After the solidification of agarose the gel was placed in the electrophoresis cell and the second dimension run was started, using the following conditions - 100V, 60 min; 120 V, constant run. The gels were subsequently stained with staining solution (5 % Coomassie Brilliant Blue G250 in 50% methanol, 5% glacial acetic acid, Merck). The gels were destained in destaining solution (50% methanol, 5% Glacial acetic acid) and scanned using a Gel Documentation System (LAS 4000, GE Amersham).
(d) Protein Sequencing

The protein expression profile differences between the total protein from the four animal study group of PD: (i) 6-OHDA group (ii) a sham group (iii) 6-OHDA and 7-NI treated group and (iv) 7-NI control group samples were elucidated by analysing images from each sample using Bio-Rad’s PD Quest software basic version. Samples were then picked and sent to be analysed and identified on 4800 MALDI TOF/TOF Analyser. (Applied Biosystems Instrument).

(e) Database Queries and Protein Identifications

The acquired tandem mass spectral data were queried against protein database using the MASCOT search engine (version 1.8, Matrix Science Ltd., U.K.) with a mass tolerance of 100 ppm and one trypsin miscleavage. Carbamidomethylation of cysteine was set as variable amino acid modifications. For each given sequence, the EST analyzer searched NCBI non redundant protein database to identify a homologous protein (the best hit of BLASTX search), then used the homologous protein to annotate query sequence. Proteins with MOWSE scores equal to the accepted significant threshold (determined at 60% confidence level as calculated by MASCOT) were reported in this study.

2. Standardization of the S-Flos Method with Bovine Serum Albumin Protein (BSA)

In vitro S-nitrosylation of BSA by Sodium Nitrite

Bovine Serum Albumin (BSA) is a ~66.5KDa protein commonly used as protein standard in the research laboratories. BSA has 34 disulfide linked cysteines with one free thiol and all of them are S-nitrosylated in vitro by reacting BSA (1mg) with acidified 0.1 M sodium nitrate at room temperature for 1 h in the dark. The s-nitrosylated BSA was acetone precipitated three times and the s-nitrosylation of BSA dissolved in sterile MiliQ water was verified visually through the violet color developed after the addition of Griess reagent.

Titration of Cy Dye

To optimize Cy-dye labeling (Cy-Dye DIGE Fluor for Scarce Samples, GE Healthcare,), Cy-dye (both Cy 3 and Cy 5) was titrated with 40, 80 and 100 pmol dye
Chapter 3  Development of 6-OHDA-induced Rat Model

101

per 5µg S-nitrosylated BSA protein. Throughout the study the protein was kept in dark. The free thiol site in SNO-BSA was blocked in 100 µl of blocking buffer (50mM Tris HCl, pH 7.5, 10µM neocuprione (prevent the chelation of NO from the thiols), 1% SDS, 20 mM MMTS (methylating agent)) at 50 °C for 1 h followed by the removal of excess MMTS by acetone precipitation. The SNO-BSA was reduced by dissolving the protein pellet in 100 µl of Reducing Buffer (50 mM Tris–HCl pH 7.5, 4% CHAPS, 5 mM ascorbate) at 37 °C for 1 hr and acetone precipitated. Pellet obtained was dissolved into 100 µl Labeling Buffer (50 mM Tris–HCl pH 7.0, 7 M Urea, 4% CHAPS) and labeled with different concentration of Cy dyes at at 37 °C for 30 min. The labeling was stop by adding freshly prepared 2x sample buffer (7M urea, 2M Thiourea, 4% CHAPS, and 1% (w/v) Bio-Lyte 3-10 pH ampholytes and 130 mM DTT) and the sample were loaded on 12% acrylamide gel.

3. Sample Preparation for 2D PAGE by S-Flos Method

Protein samples were prepared from the brain samples of 6-OHDA induced animal models from four treatment groups as mention above according to the reported method. The entire assay, through scanning, was performed in the dark room. Brain tissue was homogenized in Blocking buffer (50mM Tris HCl, pH 7.5, 10µM neocuprione, 1% SDS, 20 mM MMTS) to the final concentration of 1mg/ml with 10-15 strokes of a dounce homogeniser fitted with Teflon probe attached to IKA overhead stirrer at 2000 rpm/min. The homogenate was centrifuged at 3, 000 rpm for 10 mins, at 4 °C, supernatant was sonicated at 80% power with 3 cycles of 30 seconds each and again centrifuged at 10,000 rpm for 15 min at 4°C. Supernatant was collected and subjected to acetone precipitation with four volumes of ice cold acetone for atleast 1hr or overnight at -20°C. the pellet obtained was dissolved in blocking buffer and 1 mg/ml of the protein samples were blocked in 100 µl of 20 mM methyl methanethiosulfonate (MMTS) in 50 mM Tris–HCl, pH 7.5, 10 µM neocuprione and 1% SDS at 50 °C for 1 h. Excess MMTS were removed by precipitation with four volumes of cold acetone for 1 h. Proteins were redissolved in 100 µl Reducing Buffer (50 mM Tris–HCl pH 7.5, 4% CHAPS, 5 mM ascorbate) and incubated at 37 °C for 1 h. The reduced proteins were precipitation with four volumes of cold acetone for 1 h and pellet obtained was dissolved into 100 µl Labeling Buffer (50 mM Tris–HCl pH 7.0, 7 M Urea, 4% CHAPS) and quantify the protein by Nanodrop spectrophotometer (thermo fisher). Finally, 15 µg of total protein was then labeled with
40 pmol of either Cy3 or Cy5-maleimide at 37 °C for 30 min. The labeling was stop by adding freshly prepared 2x sample buffer (7M urea, 2M Thiourea, 4% CHAPS, and 1% (w/v) Bio-Lyte 3-10 pH ampholytes and 130 mM DTT). One biological internal pool (BIP) of Cy3 dye was made by mixing of 5 µg of proteins from each sample group. The IEF condition and method is same as mentioned above.

4. Two Dimensional Differential Gel Electrophoresis

The Cy3 and Cy5-labeled proteins were mixed (sample protein from each group Cy3 labelled and BIP Cy5 labelled) and diluted with freshly prepared 1x rehydration buffer (7M urea, 2M Thiourea, 4% CHAPS, and 1% (w/v) Bio-Lyte 3-10 pH ampholytes and 13 mM DTT) to make up the 200µl of total volume per sample and subsequently subjected to IEF. Isoelectrically focused IPG strips were equilibrated in Equilibration buffer I containing 6 M urea (Bio-Rad), 2% SDS (Bio-Rad), 0.375M Tris-HCl pH 8.8 (Merck), 20% glycerol (Merck), 0.5% (w/v) DTT (Bio-Rad) for 10 mins and resolved using 2D gel electrophoresis by the above described method taking care of the mandatory dark condition to be maintained throughout. Fluorescence images were acquired on a Typhoon trio variable mode (GE Healthcare) scanning at 500 photo multiplier tube (PMT) with excitation/emission filters for Cy3 or Cy5. ImageQuant (version 5.2, GE Healthcare) was used to determine relative fluorescence intensities. Gels were post stained with colloidal coomassie blue.

5. Protein Sequencing

The protein expression profile differences between the total protein from the four animal study group of PD: (i) 6-OHDA group (ii) a sham group (iii) 6- OHDA and 7-NI treated group and (iv) 7-NI control group samples were elucidated by analysing images from each sample using Bio-Rad’s PD Quest software basic version. Samples were then picked and sent to be analysed and identified on 4800 MALDI TOF/TOF Analyser. (Applied Biosystems Instrument) to the commercial source (SANDOR).

6. Database Queries and Protein Identifications

The acquired tandem mass spectral data were queried against protein database using the MASCOT search engine (version 1.8, Matrix Science Ltd., U.K.) with a mass tolerance
of 100 ppm and one trypsin miscleavage. Carbamidomethylation of cysteine was set as variable amino acid modifications. For a given sequence, the EST analyzer searched NCBI non redundant protein database to identify a homologous protein (the best hit of BLASTX search), then used the homologous protein to annotate query sequence. Proteins with MOWSE scores equal to the accepted significant threshold (determined at 60 % confidence level as calculated by MASCOT) were reported in this study.

7. Exploration of S-nitrosylated GAPDH as Mediator of Neuronal Cell Death

GAPDH is a very important house keeping protein. Under normal condition condition the GAPDH is majorly found in cytoplasm but in oxidative stress conditions GAPDH migrates to nucleus and trigger cell death. To evaluate the role of GAPDH in 6-OHDA mediated neurotoxicity in female rat model of PD, cytoplasmic and nuclear proteins were isolated by differential centrifugation at 4,000 rpm for 10 min and 13,000 rpm for 30 min respectively at 4 °C in buffer containing 50mM Tris Cl, pH 7.5. protease inhibitor- leupeptin 2ng/ml, pepstatin 5ng/ml, PMSF 1 mM, sodium fluoride 2mM, antipain 5ng/ml. The isolated Protein samples were loaded on SDS –PAGE, transferred on nitrocellulose membrane and probed with primary mouse anti-GAPDH antibody (1: 4000 dilution) and secondary goat anti-mouse Ig antibody (1:4000 dilution). The detection was done by DAB method as described in previous chapters for cytoplasmic sample and by Enhanced chemiluminescence kit (GE Healthcare) for nuclear samples used according to manufacturer’s instructions.

3B.4 Results

1. Total Protein Expression Profiling by 2-Dimension Electrophoresis

Minor variation(p Value > 0.05) of total protein expression profile showed in 2-dimension gel the expression of total protein in control group (2),7-NI treated group(4) 6-OHDA – induced group(1), 6-OHDA+7-NI group with minor variation in the expression level of the proteins. However, the protein level of 6-OHDA treated Figure 1(Group 1) and 6OHDA+7NI Figure 1(Group 4) decrease slightly as compared to the control (Figure 1 Group 1, 2, 3, and 4).
Major alteration in the protein expression were observed at 35-40 kDa (pH 8-9), 36-75 kDa (pH 3.5 -6.0) and 25 kDa – 30kDa (pH 4-5).

**Figure 1:** Total proteins expression profile of the protein isolated from 6-OHDA induced animal model of PD. 200µg of protein from each treatment group was loaded on 11cm IPG strips. The conserved expression profile is highlighted by the red enclosures. Key: (a) control group, (b) 7-NI treated group, (c) 6-OHDA treated group, (d) 6-OHDA+7-NI

**Standardization of the S-Flos Method with Bovine Serum Albumin Protein (BSA)**

Since the percentage of cysteine residues vary in proteins, the labeling of protein with Cy dye requires the optimization for the saturation labeling. To optimize the labeling of Cy dyes, in-vitro S-nitrosylation was carried and labeled proteins were validated by the
SDS PAGE. Figure 2 shows that 80 pmole, 100 pmole stained dark due to saturation but 40 pmole does not shows saturation in signals.

![Figure 2: Titration of SNO-BSA with Cy dyes. Cy dyes were titrated with 40 pmole (c,c’), 80pmole (b,b’) and 100pmole (c,c’). key: Cy 3 (green, a’,b’,c’) and Cy 5 (red, a, b, c).](image)

2. Labeling and Analysis of Proteins Isolated from the Animal Model of PD by Modified S-Flos Method

After optimization of Cy dyes, proteins (15µg) isolated from each group of the animal model of PD namely (I) 6-OHDA group (II) a centre group (III) 6- OHDA and 7-NI treated group and (IV) 7-NI control group, were labeled by the 40 pmol of each Cy dye and analysed by 2-D DIGE analysis reveals that total 14 proteins were differentially S-nitrosylated Figure 3. Interestingly, the overall total expression level of proteins 25–35kDa(pH 4-5), 30–40kDa(pH 4-6), 40–60kDa(pH 8-9) did not vary much (Figure 1). However 14 samples were selected for the Maldi-toff analysis to the identify of the new proteins (Figure 4). The results revealed the lowest level of S-nitrosylation in the 7-NI group while the highest in 6-OHDA treated animal model group (Table 2).

3. Database Queries and Protein Identifications

The Maldi-toff analysis with the MASCOT score greater than 60 shows the proteins GAPDH, Actin, ATP synthase, calreticulin, cyclic nucleotide phosphodiesterase, 14-3-3 gamma, creatine kinase type-B, heat shock cognate 71 kDa protein, 78 kDa glucose-regulated protein precursor, and Neurofilament light polypeptide. Majority of the proteins have been identified previously in MPTP mice model of PD (Sowell et al. 2009). S-nitrosylation of the proteins induced modulation in expression of GAPDH, Actin, and creatine kinase type-B has been reported. However S-nitrosylation induced modulation in expression of the protein such as Actin, beta, isoform; Creatine kinase B-
type; 14-3-3 protein gama; Glyceraldehyde-3 phosphate (Hara et al. 2006; Komatsubara et al. 2012; Lu et al. 2009) (Table 1 and 2).

Figure 3: Cy Dye labelled 2-D DIGE of the S-nitrosylated proteins of rat model of PD. Cy 3 (green) labelled interanal pool and Cy 5 (red) sample. 12.5 microgram of each protein sample were loaded on 3-10pH 11cm strip. Arrow indicates GAPDH protein position. Key: (a) 7-NI treated animal group; (b) control animal group; (c) 6-OHDA+7NI treated animal group; and (d) 6-OHDA treated animal group.
4. Exploration of S-nitrosylated GAPDH as Mediator of Neuronal Cell Death

Recently, (Hara et al. 2006) and (Li et al. 2012) has shown the the inhibition of GAPDH by deprenyl and 7-nitroindazole respectively showed neuroprotective effect. To explore the similar possibility with 7-nitroindazole in 6-OHDA induced animal model of PD, the GAPDH was probed in the cytosolic and nuclear proteins. The differential expression level of GAPDH in cytosolic proteins was observed the presence of GAPDH in the 6-OHDA and 6-OHDA + 7NI treated protein samples indicated that 7-NI might be conferring the neuroprotection similar to Creatin kinase B-type (Figure 5 and 6) (Hara et al. 2006; Lu et al. 2009; Van Laar et al. 2009). The role of GAPDH in PD has been explored using MAO-B inbhitor (Hara et al. 2006).
Table 1: List of the proteins identified by the Maldi-off analysis with their biological functions.

<table>
<thead>
<tr>
<th>S.No./Spot no.</th>
<th>Name of the protein</th>
<th>Molecular weight (kDa)</th>
<th>pI</th>
<th>Mascot score (above 60 significant)</th>
<th>Function</th>
<th>Previously reported for PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Actin, beta, isoform CRA_b</td>
<td>41.0</td>
<td>5.29</td>
<td>71</td>
<td>Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>Creatine kinase B-type</td>
<td>42.7</td>
<td>5.39</td>
<td>243</td>
<td>Reversibly catalyzes the transfer of phosphate between ATP and play a central role in energy transduction in tissues with large, fluctuating energy demands, such as skeletal muscle, heart, brain and spermatozoa</td>
<td>no</td>
</tr>
<tr>
<td>3</td>
<td>gamma-Enolase</td>
<td>47.2</td>
<td>5.03</td>
<td>176</td>
<td>Carbohydrate Metabolism - glycolysis and gluconeogenesis, response to estradiol stimulus</td>
<td>yes</td>
</tr>
<tr>
<td>4</td>
<td>Tubulin beta-4B chain</td>
<td>49.8</td>
<td>4.79</td>
<td>100</td>
<td>Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain</td>
<td>no</td>
</tr>
<tr>
<td>5</td>
<td>ATP synthase beta subunit</td>
<td>51.2</td>
<td>4.92</td>
<td>135</td>
<td>ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain</td>
<td>no</td>
</tr>
<tr>
<td>6</td>
<td>Calreticulin</td>
<td>47.9</td>
<td>4.33</td>
<td>72</td>
<td>Molecular calcium binding chaperone promoting folding, oligomeric assembly and quality control in the ER via the calreticulin/calnexin cycle. Interacts with the DNA-binding domain of NR3C1 and mediates its nuclear export</td>
<td>yes</td>
</tr>
<tr>
<td>7</td>
<td>2',3'-cyclic-nucleotide 3'-phosphodiesterase</td>
<td>46.2</td>
<td>5.46</td>
<td>70</td>
<td>High affinity cAMP and cGMP calmodulin-dependent phosphodiesterase. May play an essential role in the rapid termination of the odorant-induced cAMP signal</td>
<td>no</td>
</tr>
<tr>
<td>8</td>
<td>14-3-3 protein gamma</td>
<td>27.3</td>
<td>4.80</td>
<td>121</td>
<td>Adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathway. Binds to a large number of partners, usually by recognition of a phosphoserine or phosphothreonine motif. Binding generally results in the modulation of the activity of the binding partner</td>
<td>yes</td>
</tr>
<tr>
<td>S.No./Spot no.</td>
<td>Name of the protein</td>
<td>Molecular weight (kDa)</td>
<td>pI</td>
<td>Mascot score (above 60 significant)</td>
<td>Function</td>
<td>Previously reported for PD</td>
</tr>
<tr>
<td>---------------</td>
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<td>------------------------</td>
<td>----</td>
<td>------------------------------------</td>
<td>----------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>9</td>
<td>14-3-3 protein epsilon</td>
<td>28.0</td>
<td>4.55</td>
<td>69</td>
<td>Same as above</td>
<td>yes</td>
</tr>
<tr>
<td>10</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>35.8</td>
<td>8.14</td>
<td>45</td>
<td>A key glycolytic enzyme that catalyzes the first step of the pathway by converting D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate and participates in apoptosis, membrane trafficking, iron metabolism, nuclear activities and receptor mediated cell signaling</td>
<td>yes</td>
</tr>
<tr>
<td>11</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>35.8</td>
<td>7.26</td>
<td>141</td>
<td>Same as above</td>
<td>In MPTP mouse model and cerebral ischemia</td>
</tr>
<tr>
<td>12</td>
<td>Heat shock cognate 71 kDa</td>
<td>70.8</td>
<td>5.37</td>
<td>118</td>
<td>Acts as a repressor of transcriptional activation. Inhibits the transcriptional coactivator activity of CITED1 on Smad-mediated transcription. Chaperone. regulation of cell cycle; RNA splicing; response to stress</td>
<td>yes</td>
</tr>
<tr>
<td>13</td>
<td>78 kDa glucose-regulated protein precursor</td>
<td>78.0</td>
<td>5.07</td>
<td>109</td>
<td>A member of the HSP family of molecular chaperones required for endoplasmic reticulum integrity and stress-induced autophagy. Plays a central role in regulating the unfolded protein response (UPR), and is an obligatory component of autophagy in mammalian cells</td>
<td>yes</td>
</tr>
<tr>
<td>14</td>
<td>Neurofilament light polypeptide</td>
<td>61.3</td>
<td>4.63</td>
<td>157</td>
<td>Protein polymerization; response to peptide hormone stimulus; response to toxin; intermediate filament organization; microtubule cytoskeleton organization and biogenesis; response to organic nitrogen; retrograde axon cargo transport</td>
<td>yes</td>
</tr>
</tbody>
</table>
Table 2: S-nitrosylation of the proteins identified by the Maldi-topp analysis.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of protein</th>
<th>Predicted/known cysteine modified</th>
<th>Affect on protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Actin, beta, isoform</td>
<td>Not known</td>
<td>Nitrosylated actin is found to polymerize less efficiently</td>
</tr>
<tr>
<td>2.</td>
<td>Creatine kinase B-type</td>
<td>Cys283</td>
<td>Inhibition of ckb</td>
</tr>
<tr>
<td>3.</td>
<td>gamma-Enolase</td>
<td>Not known</td>
<td>Loss in function</td>
</tr>
<tr>
<td>4.</td>
<td>Tubulin beta-4B chain</td>
<td>Not known</td>
<td>Not known</td>
</tr>
<tr>
<td>5.</td>
<td>ATP synthase beta subunit</td>
<td>Not known</td>
<td>Not known</td>
</tr>
<tr>
<td>6.</td>
<td>calreticulin</td>
<td>Not known</td>
<td>Not known</td>
</tr>
<tr>
<td>7.</td>
<td>2’,3’-cyclic nucleotide -3’ phosphodiesterase</td>
<td>Not known</td>
<td>Not known</td>
</tr>
<tr>
<td>8.</td>
<td>14-3-3 protein gamma</td>
<td>Reported</td>
<td>Not known</td>
</tr>
<tr>
<td>9.</td>
<td>14-3-3 protein epsilon</td>
<td>Reported</td>
<td>Not known</td>
</tr>
<tr>
<td>10.</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Cys150, Cys154</td>
<td>Not known</td>
</tr>
<tr>
<td>11.</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase-like</td>
<td>Same as above</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Heat shock cognate 71 kDa</td>
<td>Not known</td>
<td>Not known</td>
</tr>
<tr>
<td>13.</td>
<td>78 kDa glucose-regulated protein precursor</td>
<td>Not known</td>
<td>Not known</td>
</tr>
<tr>
<td>14.</td>
<td>Neurofilament light polypeptide</td>
<td>Not known</td>
<td>Not known</td>
</tr>
</tbody>
</table>
Figure 5: Western blot analysis of GAPDH protein in cytosolic and nuclear proteins isolated from the treatment A(6-OHDA(1)), B(Center(2)), C(6-OHDA+7-NI(3)), D(7-NI(4)), M(Marker).

Figure 6: Possible mechanism of S-nitrosylated GAPDH (SNO-GAPDH) contributing to neuronal cell damage or death (Nakamura and Lipton 2007).
5. Discussion

Protein S-nitrosylation conveys a large influence of nitric oxide mediated cellular signal transduction. In the present study, the role of nNOS in the pathology of PD was evaluated by comparing the modulation of total protein expression with the changes in the level of S-nitrosylation of the proteins isolated from all treatment animal model groups. The neuroprotective effect of 7-NI was evaluated through the decrease in S-nitrosylation of the proteins by 2-D DIGE. The obtained results suggest that 7-nitroindazole successfully confers the neuroprotection by decrease in the levels of S-nitrosylation. Interestingly, those proteins whose expression in the total protein expression profile of all group remained similar were the same proteins whose S-nitrosylation level varied. The identified proteins: Actin, ATP synthase, calreticulin, cyclic nucleotide phosphodiesterase, 14-3-3 gamma, creatine kinase type-B, heat shock cognate 71 kDa protein, 78 kDa glucose-regulated protein precursor, and Neurofilament light polypeptide plays vital role in the cytoskeleton maintainence, axonal transportation, synaptic vesicle formation, bioenergetics, cell survival, cell signaling and cellular stress management (Tristan et al. 2011). Recently it has been shown that, S-nitrosylation of actin in vitro and in vivo and nitrosylated cysteine moieties in the carboxyl terminal area of actin, a region that is important for actin polymerization and for binding several proteins that modify behavior of the molecule S-nitrosylation attenuated dopamine release by decrease in F-actin content in PC12 cells. Among all the identified proteins, GAPDH is the most important protein due to its “house keeping” role in the cell. The multifarious role of GAPDH includes DNA repair, tRNA export, membrane fusion and transport, cytoskeletal dynamics and cell death (Tristan et al. 2011). However, stress/toxic insults elicits S-nitrosylation of GAPDH, which triggers binding to Siah1 (an E3 ubiquitin ligase), whose nuclear localization signal mediates translocation of GAPDH leading to apoptosis. Later, it was shown that In the nucleus, the GAPDH/Siah1 protein complex activates ubiquitination and thus degradation of several nuclear proteins, including nuclear receptor corepressor (N-COR); which leads to cell death while this casade of protein interactions are inhibited by MAO-B inhibitor, l-deprenyl (Hara et al. 2005; Hara et al. 2006; Sawicki and Jugdutt 2007). Quite recently, (Li et al. 2012) have also reported similar work by preoperative administration
of 7-nitroindazole as neuroprotective agent in cerebral ischemia. Present study first time reports the post-operative neuroprotective effect of 7-nitroindazole mediated by denitrosylation of GAPDH and also provided some important new targets like Actin, ATP synthase, calreticulin, cyclic nucleotide phosphodiesterase, 14-3-3 gamma, creatine kinase type-B, heat shock cognate 71 kDa protein, 78 kDa glucose-regulated protein precursor, and Neurofilament light polypeptide for the further exploration in the molecular mechanism of the neuroprotection exerted by 7-NI.

In conclusion, the toxicity by nitrosative stress caused by nNOS, triggered due to 6-OHDA has been eliminated by 7-NI by reducing the levels of S-nitrosylation.
References


