

**BONE MARROW CELLS DIFFERENTIATION TO NEURONS IN THE RAT
BRAIN USING SEROTONIN AND GABA: THEIR ROLE ON GLUTAMATE
RECEPTORS, IP3, cAMP AND cGMP FUNCTIONAL REGULATION IN
UNILATERAL PARKINSONISM INDUCED BY
6-HYDROXYDOPAMINE**

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CERTIFICATE

This is to certify that the thesis entitled “**Bone marrow cells differentiation to neurons in the rat brain using Serotonin and GABA: Their role on glutamate receptors, IP3, cAMP and cGMP functional regulation in unilateral Parkinsonism induced by 6-hydroxydopamine**” is a bonafide record of the research work carried out by Mr. Nandhu M.S., under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

Cochin - 682 022

(C. S. Paulose)

23-03-2011

DECLARATION

I hereby declare that the thesis entitled “**Bone marrow cells differentiation to neurons in the rat brain using Serotonin and GABA: Their role on glutamate receptors, IP3, cAMP and cGMP functional regulation in unilateral Parkinsonism induced by 6-hydroxydopamine**” is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of Dr. C. S. Paulose, Director, Centre for Neuroscience, Professor, Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

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The woods are lovely, dark and deep.

But I have promises to keep, and miles to go before I sleep

Robert Frost

In all, the journey to my Ph.D has been a long and challenging road. And I am thrilled it is done. But most of all, I am excited for what the future holds....

Nandhu MS

Dedicated To My Beloved Parents. . .

ABBREVIATIONS

5-HT	5-Hydroxy tryptamine
6-OHDA	6-hydroxydopamine
A2A	alpha-2 adrenoceptors
AC	Adenylate cyclase
AD	Alzheimer's disease
ACh	Acetylcholine
AChR	Acetylcholine receptor
AIF	Apoptosis inducing factor
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ALS	Amyotrophic lateral sclerosis
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
B_{max}	Maximal binding
BMC	Bone marrow cells
CB	Cerebellum
CC	Cerebral cortex
cAMP	Cyclic adenosine monophosphate
cAPK	cAMP-dependent protein kinase
cDNA	Complementary deoxy ribonucleic acid
cGMP	Cyclic guanosine monophosphate
CNS	Central Nervous System
CREB	cAMP regulatory element binding protein

CSF	Cerebrospinal fluid
CT	Crossing threshold
DA	Dopamine
DARRP-32	Dopamine-receptor-associated protein
DAG	Diacylglycerol
DEPC	Di ethyl pyro carbonate
DHPG	3,5-dihydroxyphenylglycine
DNA	Deoxy ribonucleic acid
EAA	Excitatory amino acids
EAAT	Excitatory amino acids transporter
EDTA	Ethylene diamine tetra acetic acid
EPI	Epinephrine
ER	Endoplasmic reticulum
GABA	Gamma amino butyric acid
GAD	Glutamic acid decarboxylase
GAP	GTPase-activating protein
GDH	Glutamate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate/aspartate transporter
GLT-1	Glutamate transporter-1
GPCR	G-protein-coupled receptors
GPi	Globus pallidus internus
GPM	Medial globus pallidus
GTP	Guanosine triphosphate
HEPES	[n' (2-hydroxy ethyl)] piperazine-n'-[2-ethanesulfonic acid]
HFS	High frequency stimulation

iGluRs	Ionotropic glutamate receptors
IL	Interleukin
IP3	Inositol 1,4,5-triphosphate
IP3R	IP3 receptors
KA	Kainate
K _d	Dissociation constant
LBD	Lewy body diseases
LC	Locus coeruleus
L-DOPA	L-3,4-dihydroxyphenylalanine
LIDs	Levodopa-induced dyskinesia
LTD	Long term depression
LTP	Long term potentiation
mGLU	Metabotropic Glutamate
MHPG	3-methoxy-4-hydroxyphenylglycol
(+) MK-801	(+)5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP+	(1-methyl-4-phenylpyridinium)
mRNA	Messenger Ribonucleic acid
NE	Norepinephrine
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NOS	Nitric-oxide synthase
NPY	Neuropeptide Y
NSB	Non specific binding
O.D.	Optical density
p	Level of significance
PBS	Phosphate buffered saline

PBST	Phosphate buffered saline Triton X- 100
PCP	Phencyclidine
PCPA	Parachlorophenylalanine
PD	Parkinson's Disease
PDE2	phosphodiesterase 2
PFC	Prefrontal cortex
Pi	Inorganic phosphate
PIP2	Phosphatidyl inositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PPN	Pedunculo pontine nucleus
ROS	Reactive oxygen species
RT-PCR	Reverse-transcription-polymerase chain reaction
S.E.M	Standard error of mean
Smac	second mitochondrion-derived activator of caspase
SMC	Smooth muscle cells
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulate
SOD	Superoxide dismutase
STN	Subthalamic nucleus
TM	Transmembrane
TH	Tyrosine hydroxylase
TNF- α	Tumor necrosis factor- α
VGLUTs	Vesicular glutamate transporters
VTA	Ventral tegmental area

CONTENTS

INTRODUCTION	1
OBJECTIVES OF THE PRESENT STUDY	6
LITERATURE REVIEW	7
Pathology, aetiology and pathogenesis	8
Problems associated with current treatments of Parkinson's disease	9
The unilateral 6-Hydroxydopamine Lesion Model	11
Role of neurotransmitters in PD	12
Dopamine	12
Acetylcholine	16
Epinephrine and Norepinephrine	17
Serotonin	18
Serotonin as co-mitogen	19
GABA	20
GABA as co-mitogen	21
Glutamate	22
Metabotropic glutamate receptor	25
Ionotropic Receptors - NMDA Receptors	28
Glutamate mediated excitotoxic cell death	31
Glutamate transporters	33
Signal transduction through Second Messengers	34
Inositol 1, 4, 5-trisphosphate (IP3)	34
Cyclic Adenosine Monophosphate (cAMP)	35
Cyclic Guanosine Monophosphate (cGMP)	36
cAMP-response element binding protein	36
Mode of cell death in PD	38
α -Synuclein modifications, aggregation and fibril formation	39

Tumor necrosis factor- α in PD	40
Cellular transplantation to the rescue	41
Bone marrow cells	43
Role of Astrocytes in PD	44
MATERIALS AND METHODS	47
Chemicals used and their sources	47
Biochemicals	47
Radiochemicals	47
Molecular Biology Chemicals	47
Confocal Dyes	48
Animals	48
Experimental design	48
Rotational behaviour	49
Treatment	49
Tissue preparation	50
Behavioural studies	50
Forelimb use asymmetry test (cylinder test).	50
Rotarod Test	51
Swim-test	51
Y-Maze Test	51
Radial arm maze Test	52
Quantification of Glutamate	53
Quantification of Dopamine	54
Glutamate Receptor Binding Studies Using [^3H] Radioligands in Different Brain Regions of Control and Experimental rats	54
<i>Glutamate Receptor Binding Studies Using [^3H]Glutamate</i>	54
NMDA Receptor Binding Studies Using [^3H] Radioligands in Different Brain Regions of Control and Experimental rats.	55
<i>NMDA Receptor Binding Studies Using [^3H] MK-801</i>	55

Protein determination	56
Analysis of the Receptor Binding Data	56
<i>Linear regression analysis for Scatchard plots</i>	56
Gene Expression Studies in Different Brain Regions of Control and Experimental rats.	57
<i>Preparation of RNA</i>	57
<i>Isolation of RNA</i>	57
<i>cDNA Synthesis</i>	58
<i>Real -Time PCR Assay</i>	58
IP3 Content in Different Brain Regions of Control and Experimental rats.	59
<i>Principle of the assay</i>	59
<i>Assay Protocol</i>	60
cAMP Content in Different Brain Regions of Control and Experimental rats.	60
<i>Principle of the assay</i>	61
<i>Assay Protocol</i>	61
cGMP Content in Different Brain Regions of Control and Experimental rats.	62
<i>Principle of the assay</i>	62
<i>Assay Protocol</i>	63
Bone marrow cell differentiation pattern studies using PKH2GL cell linker dye.	63
NMDAR1, NMDA2B, mGluR5 and tyrosine hydroxylase Expression Studies in Different Brain Regions of Control and Experimental rats Using Confocal Microscope	64
Statistics	64
RESULTS	66
Body Weight of Control and Experimental Rats	66
Apomorphine induced rotational behaviour in control and experimental rats	66
Limb use asymmetry test in control experimental rats	66
Rotarod performance of control and experimental rats	66
Behavioural response of control and experimental rats in swim test	67

Behavioral response of control and experimental rats on number of visit to novel arm in y maze	67
Behavioral response of control and experimental rats on criterion performance in radial arm maze	67
Behavioral response of control and experimental rats on reference errors in radial arm maze	68
Behavioral response of control and experimental rats on working errors in radial arm maze	68
<i>Corpus Striatum</i>	
Scatchard analysis of glutamate receptors using [³ H]Glutamate against glutamate	69
Scatchard analysis of NMDA receptors using [³ H]MK801 against MK801	69
Glutamate content in the Corpus Striatum of control and experimental rats	69
Real-Time PCR analysis of mGluR5 receptors	70
Real-Time PCR analysis of NMDAR1 receptors	70
Real-Time PCR analysis of NMDA2B receptors	70
Real-Time PCR analysis of GLAST glutamate transporter	70
Real-Time PCR analysis of Bax	71
Real-Time PCR analysis of TNF- α	71
Real-Time PCR analysis of α - synuclein	71
Real-Time PCR analysis of CREB	72
IP3, cAMP and cGMP content in the control and experimental rats	72
mGluR5 receptor antibody staining in control and experimental groups of rats	72
NMDAR1 receptor antibody staining in control and experimental groups of rats	73
NMDA2B receptor antibody staining in control and experimental groups of rats	73
<i>Substantia nigra pars compacta</i>	
Real-Time PCR analysis of mGluR5 receptors	74
Real-Time PCR analysis of NMDAR1 receptors	74
Real-Time PCR analysis of NMDA2B receptors	74
Real-Time PCR analysis of GLAST glutamate transporter	74
Real-Time PCR analysis of Bax	75
Real-Time PCR analysis of TNF- α	75
Real-Time PCR analysis of α - synuclein	75
Real-Time PCR analysis of CREB	76
Real-Time PCR analysis of Nestin	76
Real-Time PCR analysis of GFAP	76
Real-Time PCR analysis of tyrosine hydroxylase	76
Dopamine content analysis	77
Morphological changes of bone marrow cells after the injection into Substantia nigra	77
In vivo expression studies of bone marrow cells and Nestin in the	

Substantia nigra of experimental rats	77
In vivo expression studies of bone marrow cells and GFAP in the Substantia nigra of experimental rats	77
Tyrosine hydroxylase antibody staining in control and experimental groups of rats	78

Cerebral cortex

Scatchard analysis of glutamate receptors using [3H]Glutamate against glutamate	79
Scatchard analysis of NMDA receptors using [3H]MK801 against MK801	79
Glutamate content in the Corpus Striatum of control and experimental rats	79
Real-Time PCR analysis of mGluR5 receptors	80
Real-Time PCR analysis of NMDAR1 receptors	80
Real-Time PCR analysis of NMDA2B receptors	80
Real-Time PCR analysis of GLAST glutamate transporter	80
Real-Time PCR analysis of Bax	81
Real-Time PCR analysis of TNF- α	81
Real-Time PCR analysis of α - synuclein	81
Real-Time PCR analysis of CREB	82
IP3, cAMP and cGMP content in the control and experimental rats	82
mGluR5 receptor antibody staining in control and experimental groups of rats	82
NMDAR1 receptor antibody staining in control and experimental groups of rats	83
NMDA2B receptor antibody staining in control and experimental groups of rats	83

Hippocampus

Scatchard analysis of glutamate receptors using [3H]Glutamate against glutamate	84
Scatchard analysis of NMDA receptors using [3H]MK801 against MK801	84
Glutamate content in the Corpus Striatum of control and experimental rats	84
Real-Time PCR analysis of mGluR5 receptors	85
Real-Time PCR analysis of NMDAR1 receptors	85
Real-Time PCR analysis of NMDA2B receptors	85
Real-Time PCR analysis of GLAST glutamate transporter	85
Real-Time PCR analysis of Bax	86
Real-Time PCR analysis of TNF- α	86
Real-Time PCR analysis of α - synuclein	86
Real-Time PCR analysis of CREB	87
IP3, cAMP and cGMP content in the control and experimental rats	87
mGluR5 receptor antibody staining in control and experimental groups of rats	87
NMDAR1 receptor antibody staining in control and experimental groups of rats	88
NMDA2B receptor antibody staining in control and experimental groups of rats	88

Cerebellum

Scatchard analysis of glutamate receptors using [3H]Glutamate against glutamate	89
---	----

Scatchard analysis of NMDA receptors using [3H]MK801 against MK801	89
Glutamate content in the Corpus Striatum of control and experimental rats	89
Real-Time PCR analysis of mGluR5 receptors	90
Real-Time PCR analysis of NMDAR1 receptors	90
Real-Time PCR analysis of NMDA2B receptors	90
Real-Time PCR analysis of GLAST glutamate transporter	90
Real-Time PCR analysis of Bax	91
Real-Time PCR analysis of TNF- α	91
Real-Time PCR analysis of α - synuclein	91
Real-Time PCR analysis of CREB	92
IP3, cAMP and cGMP content in the control and experimental rats	92
mGluR5 receptor antibody staining in control and experimental groups of rats	92
NMDAR1 receptor antibody staining in control and experimental groups of rats	93
NMDA2B receptor antibody staining in control and experimental groups of rats	93
<i>Brain stem</i>	
Scatchard analysis of glutamate receptors using [3H]Glutamate against glutamate	94
Scatchard analysis of NMDA receptors using [3H]MK801 against MK801	94
Glutamate content in the Corpus Striatum of control and experimental rats	94
Real-Time PCR analysis of mGluR5 receptors	95
Real-Time PCR analysis of NMDAR1 receptors	95
Real-Time PCR analysis of NMDA2B receptors	95
Real-Time PCR analysis of GLAST glutamate transporter	95
Real-Time PCR analysis of Bax	96
Real-Time PCR analysis of TNF- α	96
Real-Time PCR analysis of α - synuclein	96
Real-Time PCR analysis of CREB	97
IP3, cAMP and cGMP content in the control and experimental rats	97
mGluR5 receptor antibody staining in control and experimental groups of rats	97
NMDAR1 receptor antibody staining in control and experimental groups of rats	98
NMDA2B receptor antibody staining in control and experimental groups of rats	98
DISCUSSION	99
SUMMARY	130
CONCLUSION	136
REFERENCES	
LIST OF PUBLICATIONS, AWARDS, ABSTRACTS PRESENTED	

Introduction

Parkinson's disease (PD) is the most common neurodegenerative disorders in the elderly that impairs the sufferer's motor skills and cognitive processes (Parkinson, 1817). PD is affecting approximately 1-2% of people over the age of 65 World wide. While there is widespread degeneration in the central and peripheral nervous systems in PD, the hallmark pathology remains the dopaminergic striatal insufficiency secondary to degeneration of dopaminergic neurons in the substantia nigra (SN). Most of PD are sporadic and age related and only approximately 5% is a familial disease (Stewart & William, 2008). With the progressive loss of the nigrostriatal dopaminergic neurons, there is a corresponding decrease of dopamine (DA) content in both the SN and striatum. It is the loss of these DA-producing projections that is thought to account for the majority of physical and motor deficits seen in PD. Although subject to intensive research, the etiology of PD is still enigmatic and the treatment is basically symptomatic. Many factors are speculated to operate in the mechanism of cell death of the nigrostriatal dopaminergic neurons in PD, including oxidative stress and cytotoxicity of reactive oxygen species (ROS), disturbances of intracellular calcium homeostasis, exogenous and endogenous toxins and mitochondrial dysfunction (Lev *et al.*, 2003).

PD is a progressive neurodegenerative disorder clinically characterized by the cardinal symptoms of cogwheel rigidity, resting tremor, bradykinesia, stooped posture and shuffling gait (Thomas & Beal, 2007; Wu *et al.*, 2011). As stated above, there is a loss of dopaminergic cells in the substantia nigra pars compacta (SNpc) that results in insufficient DA innervation of the basal ganglia and subsequent increased inhibition of excitatory thalamo-cortical connections. Lewy bodies, intracellular inclusions principally containing α -synuclein, are also found in the remaining nigral neurons of PD patients (Schlossmacher, 2007; Eller & Williams, 2011). The ultimate result of cell loss and cell dysfunction in the SN is the depletion of the neurotransmitter DA in

the basal ganglia. This insufficient DA innervation is principally localized to the postcommissural putamen and results in the overdrive of globus pallidus and subthalamic nuclear outputs. The resulting inhibition of thalamocortical function results in the characteristic bradykinesia experienced by PD patients (Soderstrom *et al.*, 2009).

Lesions with the neurotoxin, 6-hydroxydopamine (6-OHDA) have provided an important tool to study DA neurons in the brain. The most common version of such lesions is the unilateral one where the toxin is placed in the area of dopaminergic cell bodies in the SN (Schwartz & Huston, 1996). The DA analog, 6-OHDA, because of its similarity in molecular structure can be taken up into dopaminergic terminals through the DA transporter. Once inside the cell, it is metabolized, resulting in the production of hydrogen peroxide and free radicals. Ultimately these toxic molecules induce neuronal death through mitochondrial dysfunction (Soderstrom *et al.*, 2009). This lesion model has been used to investigate the behavioural functions of the basal ganglia and to examine the brain's ability to compensate for specific neurochemical depletions. 6-OHDA lesions model have served as an experimental basis to develop new antiparkinsonian drugs and treatment strategies, or surgical approaches, including transplantation of neural tissue.

The nigrostriatal dopaminergic and corticostriatal glutamatergic systems are anatomically and functionally connected playing antagonistic roles in the basal ganglia controlling spontaneous motor behaviour (Smith & Bolam, 1990; Ossowska *et al.*, 1994; Danysz *et al.*, 1995; Schmidt & Kretschmer, 1997). A DA-glutamate imbalance in the basal ganglia has been hypothesised to underlie the pathophysiology of parkinsonism (Greenamyre & O'Brien, 1991; Mitchell & Carroll, 1997; Starr *et al.*, 1997). It is suspected that because neurons that are most vulnerable in PD are those that also receive strong input from glutamate pathways that glutamate must play some role in the events that lead to neuronal damage during PD. If this is the case,

then cell degeneration or death is the result of a cumulative process of neurotoxicity produced by glutamate (Coyle & Puttfarcken, 1993). In fact, pharmacological treatments that reduce NMDA receptors activity limit the extent of nigro-striatal damage (Sonsalla *et al.*, 1998), improve motor symptoms of PD (Chase & Oh, 2000) & prevent or reduce levodopa-induced dyskinesia (LIDs) (Papa & Chase, 1996; Blanchet *et al.*, 1999; Hadj Tahar *et al.*, 2004) in animal models of PD.

Cell transplantation to replace lost neurons is a novel approach to the treatment of progressive neurodegenerative diseases. Replacement of dopaminergic neurons in patients with PD has spearheaded the development of this approach and was the first transplantation therapy to be tested in the clinic (Björklund *et al.*, 2003). The success of cell replacement for the treatment of PD is based on two hypotheses: first, the predominant symptoms of PD are dependent on the dysfunction or loss of the dopaminergic neurons in the nigrostriatal pathway; and second, dopaminergic neurons grafted into the DA-deficient striatum can replace those neurons lost as a result of the disease process and can reverse, at least in part, the major symptoms of the disease. Cells are commonly grafted to the striatum because DA is required in the striatum and it is unlikely that the cells implanted into the adult degenerating SN will physically re-establish the long nigrostriatal pathway to innervate the striatum and supply it with DA (Alexi *et al.*, 2000). The fetal brain tissue used in clinical transplantation studies is ethically challenging to obtain (Lindvall, 2001). Also, while under normal conditions the CNS immune response can mount a well-organized innate immune reaction in response to allogeneic antigens (Boulangier & Shatz, 2004; Arias-Carrión & Yuan, 2009). Number of reports claim that Bone marrow cells (BMC) can also generate endoderm and ectoderm derivatives including neural cells (Jiang *et al.*, 2002; Kim *et al.*, 2002). Hematopoietic system can be used as a source of progenitor cells for the CNS and it also has the property to differentiate into both microglia and macroglia when injected directly to the brain of adult mice (Martin & 'Eva, 1997).

Autologous BMC to treat neurological disorders offers several unique advantages over other cell replacement therapies. Immunological reactions are avoided and it also bypasses ethical issues in the use of embryonic cells.

Alterations in the brain monoamines DA, serotonin (5-HT) and gamma amino butyric acid (GABA) have been implicated in the etiology and/or pharmacotherapy of PD. Most of the effects of 5-HT and GABA on DA neurons are indirect, mediated through actions on complex neuronal circuitry, rather than direct effects on DA terminals (Poewe, 2009). Since the different 5-HT receptor subtypes are differently distributed in dopaminergic brain regions, it is possible to specifically “target” individual brain regions with serotonergic ligands and thereby affect dopaminergic function selectively in these areas (Muñoz *et al.*, 2008). As GABA helps "quiet" excessive neuronal firing and has been deficient in patients in the advanced stages of PD, directly targeting GABA production rather than DA replacement is an effective way of improving brain function in late-stage PD which also avoids the known therapeutic limitations and complications associated with the over-production of DA. GABA supplementation can help decrease the overstimulation of neurotransmitters such as acetylcholine and can possibly be used in Parkinson's disease help to inhibit acetylcholine (Nandhu *et al.*, 2010). 5-HT and Gamma aminobutyric acid (GABA) can be also used as agents for cell proliferation and differentiation. Our earlier studies showed that 5HT and GABA acting through specific receptor subtypes 5HT₂ (Sudha & Paulose, 1998) and GABA_B (Biju *et al.*, 2002) respectively, control cell proliferation and act as co-mitogens.

In the present study a detailed investigation on the alterations of glutamate and its receptors in the brain regions of unilateral 6-OHDA infused rats were carried out. Glutamate receptor subtypes- NMDAR1, NMDA2B, mGluR5 and GLAST glutamate transporter gene expression in the 6-OHDA infused rats were also studied. In addition to that the possible linkage between the 6-OHDA induced changes in IP3,

Introduction

cAMP and cGMP functional regulation and Bax, α -synuclein, TNF- α and CREB gene expression in the brain regions of PD rats has been elucidated. We also demonstrated the autologous differentiation of BMC to neurons using mitogenic 5-HT and GABA by confocal studies with PKH2GL cell membrane tracker dye, Nestin and GFAP. Our present study on 5-HT, GABA and BMC dependent regulation of glutamatergic receptors in the brain will certainly enlighten novel therapeutic possibilities for PD management.

OBJECTIVES OF THE PRESENT STUDY

1. To induce Parkinson's disease model in rats using unilateral 6-OHDA infusion and to study the effect of 5-HT, GABA and BMC treatment individually and in combinations.
2. To investigate the behavioural changes in control and experimental rats using apomorphine induced rotational analysis, limb use asymmetry test, rotarod test, swim test, Y-maze and radial arm maze.
3. To analyse glutamate content, total glutamate and NMDA receptors alterations in the brain regions of control and experimental rats and also to study DA content in the SNpc of control and experimental rats.
4. To study the NMDAR1, NMDA2B, mGluR5 glutamate receptor subtypes and GLAST glutamate transporter gene expression in the brain regions of control and experimental rats using real time PCR.
5. To study the IP3, cGMP and cAMP content and gene expression status of Bax, α -synuclein, TNF- α and CREB in the brain regions of control and experimental rats
6. To examine the bone marrow cell differentiation pattern using PKH2GL cell linker dye, Nestin and GFAP.
7. To study the localisation and expression status of NMDAR1, NMDA2B, mGluR5 and Tyrosine hydroxylase using confocal microscope by immunofluorescent specific antibodies in the brain slices of control and experimental rats using Confocal microscope.

Literature Review

Parkinson's disease was first described by James Parkinson in a monograph published in 1817 (Parkinson, 1817). It is a degenerative neurological condition and the major neurological manifestations of this disease are: tremor, rigidity, bradykinesia (slowness of movement) and postural instability. PD primarily affects people over the age of 50 years and prevalence and incidence rates increase with age. Therefore, aging of the general population is likely to result in a dramatic increase in the number of people diagnosed with PD. One study projected that by the year 2030, the number of people over the age of 50 and consequently the number of persons with PD will double, resulting in an estimated 9 million persons with PD worldwide (Dorsey *et al.*, 2007; Pahwa & Lyons, 2010). Such an increase will place a significant burden on healthcare systems and caregivers given the progressive nature of PD, associated disability and significant caregiving required in the later stages of the disease. With the expected increase in PD prevalence, it can be anticipated that the disease will continue to exact a significant direct and indirect economic cost.

The primary pathology of PD is the degeneration of dopaminergic neurons in the SNpc with subsequent depletion of nigrostriatal DA and the development of Lewy bodies, proteinaceous intracytoplasmic inclusions (Forno, 1996; Choi *et al.*, 2011). Recent studies have shown that PD is also associated with extensive non-dopaminergic pathology involving noradrenergic neurons in the locus coeruleus, cholinergic neurons in the nucleus basalis of Meynert, serotonergic neurons in the midline raphe and neurons of the autonomic nervous system. Braak *et al* (2003) has demonstrated that the pathological changes in PD occur in a relatively predictable, topographically distinct sequence of events beginning with the olfactory structures and medulla oblongata, spreading to the SN and eventually affecting neocortical structures. A great deal of the brain, especially the regions beneath the cortex, is heavily involved with movement regulation. Such areas include the connected set of

basal ganglia, portions of the thalamus and the cerebellum (Schiff, 2010). In PD, there is degeneration of neurons that use DA as a neurotransmitter, which have their cell bodies in the SN at the upper edge of the midbrain. The decrease in neural output from the SN causes a disturbance in the network balance of excitation and inhibition. The result is a net increase in inhibition from the globus pallidus internus (GPi) to thalamus (Obeso *et al.*, 2008).

Pathology, aetiology and pathogenesis

The hallmark of PD is the cell loss within the SN particularly affecting the ventral component of the pars compacta. By the time of death, this region of the brain has lost 50–70% of its neurons compared with the same region in unaffected individuals. The earliest documented pathological changes in PD (Braak *et al.*, 2006) have been observed in the medulla oblongata/pontine tegmentum and olfactory bulb. In these early stages Braak stages 1 and 2 patients are pre-symptomatic. As the disease advances Braak stages 3 and 4 the SN, areas of the midbrain and basal forebrain become involved. Finally, the pathological changes appear in the neocortex. This pathological staging is based on the distribution of lewy bodies. Lewy bodies are the pathological hallmark of PD. They are α -synuclein-immunoreactive inclusions made up of a number of neurofilament proteins together with proteins responsible for proteolysis. These include ubiquitin, a heat shock protein which plays an important role in targeting other proteins for breakdown. Mutations in the α -synuclein gene are responsible for some familial forms of PD in which lewy bodies are also seen. Mutations in the parkin protein produce a Parkinsonian syndrome without lewy bodies in juvenile cases suggesting that the parkin protein plays an important role in the development of the lewy body. It has been shown that parkin facilitates the binding of ubiquitin (ubiquitination) to other proteins such as the α -synuclein interacting protein synphilin-1 leading to the formation of lewy bodies (Chung *et al.*, 2001). Lewy bodies

are found in PD and Dementia with lewy bodies (DLB), but are not a pathological hallmark of any other neurodegenerative disease.

Identifying environmental factors that predispose to the development of PD has proved elusive. Living in a rural environment appears to confer an increased risk of PD, and perhaps causally linked to this some but not all epidemiological studies have shown a correlation between exposure to pesticide use and wood preservatives (Dick, 2006). Despite intensive research efforts during recent years, fundamental questions regarding the etiology and pathogenesis of the disease are still unresolved (Beal, 1995; Calne & Takahashi, 1991; Youdim & Riederer, 1997). With the progression of the disease, there are a number of non-motor complications in PD like sleep disorders (Frucht *et al.*, 1999; Vendette, *et al.*, 2007), cognitive impairment (Emre *et al.*, 2004; Ravina *et al.*, 2005), dementia (McKeith 2005; McKeith 2007), Mood disturbance (Richard *et al.*, 2004), Psychosis and confusion (Naimark *et al.*, 1996) that are often seen. In many cases, these are not directly related to involvement of dopaminergic pathways and therefore develop even in patients where motor symptoms are well controlled.

Problems associated with current treatments of Parkinson's disease

The understanding of DA receptor function has expanded enormously since the recognition of their existence in brain and the realization of their importance in PD in the early 1970s. But for patients with PD, the pharmacological treatment options have not really changed since then. Recent years have witnessed a number of choices for the therapy of PD. At this time, no therapy has been firmly established to have a neuroprotective role. Preliminary data suggest that high doses (at least 1200 mg/day) of Coenzyme Q10 is associated with slower deterioration of motor disability (Shults *et al.*, 2002), but this finding awaits further confirmation. The propargylamine MAO-B inhibitor, rasagiline was recently reported to have a modest symptomatic effect in patients with early Parkinson's (Parkinson Study Group, 2002), but whether it has an

effect on rate of progression has not been established. A trial of the sodium-dependent glutamate release inhibitor riluzole was terminated early based on futility analysis and trials of antiapoptotic agents, including non-MAO-inhibitor propargylamines and jun kinase inhibitors, are still under way. Although very preliminary results have suggested symptomatic benefit from glial cell line-derived neurotrophic factor (GDNF) (Gill *et al.*, 2003), there is to date no evidence that GDNF or other trophic factors that interfere with disease progression. Thus, for the time being, the pharmacological management of PD is based entirely on symptom control and is in general instituted only when justified by disability.

Since the introduction of L-3,4-dihydroxyphenylalanine (L-DOPA) to treat PD over 40 years ago, numerous studies have examined the status of DA receptors in brain in an attempt to understand the mechanisms that underlie the decline in the efficacy of L-DOPA and the increase in the adverse effects of L-DOPA treatment (Péchevis *et al.*, 2005, Hollingworth *et al.*, 2011). In the early stages of PD treatment with L-DOPA or/and DA receptor agonists provides effective relief from the motor symptoms. After 4–6 years of treatment, 40% of patients experience motor side effects. The motor side effects increase with time so that following 10 years of L-DOPA and/or DA agonist treatment most individuals (95% in some studies) will exhibit some treatment-induced motor complications (Ahlskog & Muentner, 2001). In addition to the systemic side effects (nausea, vomiting and postural hypotension) produced by acute treatment with L-DOPA and DA agonists, chronic administration can result in the development of more serious adverse effects, namely, fluctuations in motor control (end of dose deterioration, on–off phenomenon) and dyskinesias (chorea, dystonia, athetosis). The debilitating motor side effects are compounded by treatment induced psychiatric disturbances such as, psychosis, mania or delirium (Schrag, 2004). Motor side effects were caused by alterations in DA receptor expression due to progression of the disease process and/or adaptive responses to the

drug treatment (Crossman, 1990). The psychotic effects presumably stop from actions on DA receptors in limbic or cortical regions of the brain. The consequence of these motor complications is that the dose of L-DOPA has to be reduced to levels which do not provide the desired reversal of Parkinsonian symptoms.

The Unilateral 6-Hydroxydopamine Lesion Model

All commonly accepted models of PD, like the actual disease itself, are thought to involve oxidative processes at the heart of dopaminergic injury. Examples of such models include neuronal death induced by exposure to the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Cadet & Brannock, 1998), methamphetamine (O'Dell *et al.*, 1991) and 6-OHDA. The DA analog 6-OHDA, because of its similarity in molecular structure, can be taken up into dopaminergic terminals through the DA transporter. Once inside the cell, it is metabolized, resulting in the production of hydrogen peroxide and free radicals. Ultimately these toxic molecules induce neuronal death through mitochondrial dysfunction.

Like DA itself, 6-OHDA is not able to cross the blood–brain barrier and therefore must be delivered directly to the brain of experimental animals through stereotaxic surgery. In 1968, Ungerstedt and colleagues demonstrated the utility of 6-OHDA lesions as animal models of PD. In their study, 6-OHDA was unilaterally injected into the medial forebrain bundle, extensively depleting the nigrostriatal pathway on one side. Ungerstedt noted that lesioned animals rotated toward the side of their lesions spontaneously as well as after administration of the dopaminergic drug d-amphetamine. Conversely, apomorphine, a drug that acts upon up regulated DA receptors on the side ipsilateral to the lesion, induces rotations contralateral to the lesion. The number of rotations performed by a lesioned animal can be quantified to serve as an index of the integrity of nigrostriatal function. Experimental therapeutic strategies, such as neural or stem cell transplantation and gene therapy, can use the number of rotations an animal performs as an index of the intervention's efficacy.

Using this model, neuronal loss is detected as soon as 12 h postinjection and peaks at 48 h. In addition, striatal fibers are found to degenerate between 1 and 7 days after 6-OHDA delivery, ultimately resulting in more than 90% striatal DA depletion. This provides an ideal environment to evaluate cellular replacement strategies. Alternatively, 6-OHDA delivery to the striatum can result in levels of DA depletion more representative of early-stage PD. Kirik *et al.*, (2001) demonstrated the location of striatal injections, either ‘terminal’ (within the caudate–putamen) or ‘preterminal’ (at the caudate–putamen boundary), greatly affected the resulting lesion, with preterminal injections creating greater levels of DA depletion. In addition, they found variable reductions in tyrosine hydroxylase-positive (TH+) fiber densities and TH+ SN neurons after either single or multiple 6-OHDA intrastriatal injections.

Role of neurotransmitters in PD

Dopamine

DA is the predominant catecholamine neurotransmitter in the mammalian brain, where it controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake and endocrine regulation. This catecholamine also plays multiple roles in the periphery as a modulator of cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function and gastrointestinal motility (Missale *et al.*, 1998). DA containing neurons arise mainly from DA cell bodies in the SN and ventral tegmental area in mid-brain region (Carlsson, 1993; Lookingland *et al.*, 1995; Tepper *et al.*, 1997; Tarazi *et al.*, 1997 a, b, 1998, 2001). Dopaminergic system is organized into four major subsystems (i) the *nigrostriatal* system involving neurons projecting from the SNpc to the caudate-putamen of the basal ganglia. This is the major DA system in the brain as it accounts for about 70% of the total DA in the brain and its degeneration makes a major contribution to the pathophysiology of PD; (ii) *the mesolimbic system* that originates in the midbrain tegmentum and projects to the nucleus accumbens septi

and lateral septal nuclei of the basal forebrain as well as the amygdala, hippocampus and the entorhinal cortex. They are all considered components of the limbic system and hence of particular interest for the pathophysiology of idiopathic psychiatric disorders; (iii) the *mesocortical* system, which also arises from neuronal cell bodies in the tegmentum which project their axons to the cerebral cortex, particularly the medial prefrontal regions; (iv) the *tuberinfundibular* pathway, which is a neuroendocrinological pathway arising from the arcuate and other nuclei of the hypothalamus and ending in the median eminence of the inferior hypothalamus.

There are 5 types of DA receptor, which can be subdivided into DA D₁-like (D₁, D₅) and D₂-like (D₂, D₃, D₄), based on their sequence homologies, pharmacology and functional properties (Sokoloff & Schwartz, 1995). In the striatum, DA D₁ and D₂ receptors are mainly present on dendrites of GABAergic striatopallidal neurons which receive input from afferent DA neurons. DA D₁ receptors are also found on the terminals of glutamatergic projections from the cortex and thalamus. Expression of each receptor subtype is enriched on subpopulations of striatopallidal neurons. DA D₁ receptors are more highly expressed on GABAergic neurons which innervate the internal segment of the globus pallidus and SNpr (the direct pathway) and co-localize with substance P and dynorphin, while DA D₂ receptors have higher levels of expression on GABAergic neurons which innervate the external segment of the globus pallidus (the indirect pathway) and co-localize with enkephalin (Gerfen *et al.*, 1995; Le Moine & Bloch, 1995; Aubert *et al.*, 2000). However, there is a degree of overlap, with co-expression of each receptor subtype on most striatal GABAergic neurons, such that the division of striatal neurons should be based on the relative levels of DA D₁ or D₂ receptors, rather than the presence or absence of a particular receptor subtype (Surmeier *et al.*, 1993; Aizman *et al.*, 2000). DA D₂ receptors are also present on the terminals of DA neurons and therefore also function as autoreceptors. Cholinergic interneurons express DA D₂ receptor mRNA, indicating that a proportion of DA D₂

receptors found in the striatum is present on these neurons. DA D₃ receptors have a similar distribution to DA D₂ receptors, except that their density is very low in the CN and putamen, with higher levels only found in the islands of Calleja and ventral areas of the striatum. DA D₃ receptors co-localize with either DA D₁ or D₂ receptors in up to a quarter of ventral striatal neurons (Le Moine & Bloch, 1996). DA D₅ receptors, like the DA D₃ receptor, are found at highest densities in the ventral striatum, but unlike the DA D₂ and D₃ receptors, they are not located on DArgic neuron terminals, but are found on cholinergic interneurons (Bergson *et al.*, 1995). DA D₄ receptors have a very low level of expression in the striatum. The significance of DA D₄ and D₅ receptors in the symptoms or treatment of PD is unknown.

The function of DA receptors in PD is altered not only by the disease but also as a consequence of drug treatment. Alterations in the abundance of receptor density contribute to the complications of treatment. But, for the DA D₂ receptor in particular, there is no temporal correlation between the alterations in expression levels and the occurrence of motor complications of treatment. It is increasingly recognised however that DA receptor signaling cascades are altered both as a consequence of the denervation occurring in PD and as a result of the dopaminergic drug treatment used to treat the disorder. The functional response of DA receptors can therefore change despite no alteration in their expression level by virtue of changes in their coupling to second messengers. Before the cloning and definitive demonstration of 5 DA receptor subtypes, DA D₁ receptors were defined as being positively linked to adenylate cyclase, while DA D₂ receptors had negative coupling to the enzyme (Kebabian & Calne, 1979). The number of signaling cascades that DA receptors are known to interact with has grown considerably since then and has been extensively reviewed by Neve *et al.* (2004). The majority of data was derived from studies using transfected cells or animal models since the experimental techniques can not be used in post-mortem human tissue or living human subjects. However, it is reasonable to assume

that DA receptors couple to a similar repertoire of second messengers in human brain. At the molecular level DA receptors can have opposing actions, even though the final cellular response is similar. For example, in cell lines, arachidonate release was increased by both the DA D₂ and D₄ receptor subtypes, but required activation of protein kinase A for the DA D₂ receptor and protein kinase C for the DA D₄ receptor (Di Marzo *et al.*, 1993; Chio *et al.*, 1994; Lee *et al.*, 2004). It has also recently been demonstrated that different DA receptor subtypes (i.e. D₁ and D₂) can form hetero-oligomers in cells and can cross phosphorylate each other (Lee *et al.*, 2004; So *et al.*, 2005). This means a DA D₁ receptor agonist can elicit a DA D₂ receptor-mediated cellular response and vice versa. Evidently, caution must be observed when extrapolating data derived from such studies to how the native receptors function in brain. But such interactions at the molecular level explain the synergy found between, for example, DA D₁ and D₃ receptors and the dysfunction of such interactions observed in animal models of PD (Ridray *et al.*, 1998; Guigoni *et al.*, 2005).

Receptor supersensitivity, leading to imbalance between the direct and indirect striatal output pathways, is believed to underlie some of the motor complications that occur following chronic treatment with L-DOPA or DA agonists (Obeso *et al.*, 2000). DA D₂ receptor mediated effects in PD and animal models of the disorder can be explained, at least in part, by the increase in receptor DA D₂ receptor density which occurs following dopaminergic denervation of the striatum. In the absence of consistent alterations in the levels of receptor expression, altered functional responses of DA D₁ receptors results from changes in signaling mechanisms. DA receptors present on DA neuron perikarya and dopaminergic projections to areas other than the striatum are also affected by the neurodegeneration which occurs in PD. Also, chronic stimulation of extrastriatal DA receptors by L-DOPA-derived DA or dopaminergic drugs alters extrastriatal DA receptor expression (Hurley & Jenner, 2006).

Acetylcholine

Acetylcholine (ACh) is one of the principal neurotransmitters of the parasympathetic system. Extensive evidence supports the view that cholinergic mechanisms modulate learning and memory formation. Evidence for cholinergic regulation of multiple memory systems, noting that manipulations of cholinergic functions in many neural systems enhance or impair memory for tasks generally associated with those neural systems. The magnitude of ACh release in different neural systems regulates the relative contributions of these systems to learning. ACh is the neurotransmitter that is released by stimulation of the vagus nerve, which alters heart muscle contractions. It is important for the movement of other muscles as well. ACh induces movement by the locomotion of an impulse across a nerve that causes it to release neurotransmitter molecules onto the surface of the neighbouring cell. ACh is critical for an adequately functioning memory. Studies of ACh release, obtained with *in vivo* microdialysis samples during training, together with direct injections of cholinergic drugs into different neural systems, provide evidence that release of ACh is important in engaging these systems during learning and the extent to which the systems are engaged is associated with individual differences in learning and memory (Gold, 2003). Acetylcholine influences striatal DA release predominantly through an action at nicotinic acetylcholine receptors (nAChRs) (Exley *et al.*, 2008), and also muscarinic receptors to a lesser extent (Grilli *et al.*, 2008). These interactions of acetylcholine at the cellular level most likely have important behavioural consequences. Extensive work shows that nicotine, which acts at nAChRs, protects against nigrostriatal damage (Picciotto *et al.*, 2008). In addition, recent studies demonstrate that nicotine administration reduces a major side effect of L-dopa, the primary treatment for PD (Bordia *et al.*, 2008).

Epinephrine and Norepinephrine

Nondopaminergic mechanisms are also responsible for some of the sensory symptoms in patients with PD. In PD, the level of NE is reduced in the locus ceruleus (Zweig *et al.*, 1993) and this is associated with a loss of pigmented neurons and the formation of Lewy body inclusions. Moreover, NE concentrations in the neocortex, nucleus accumbens, amygdala and hippocampus are 40% to 70% lower than normal. In limbic regions, the level of the major metabolite of NE, 3-methoxy-4-hydroxyphenylglycol (MHPG), is also reduced (Riederer *et al.*, 1977) and depressive features commonly observed in PD patients is related to a central NE deficiency (Mayeux *et al.*, 1984). These changes taken together suggest that the dorsal NE system degenerates in PD. Noradrenergic projections from the locus coeruleus to the dorsal horn of the spinal cord, along with direct and indirect noradrenergic fibers from A5/A7 groups in the pontine tegmentum, reportedly inhibit ascending nociceptive pathways (Buzas & Max, 2004). There appears to be changes in both central and peripheral adrenergic receptors in PD. Studies have shown that α -2 receptors are decreased in number in the cerebral cortex (Cash *et al.*, 1984). Other studies have noted a decrease in α -2 adrenoceptors (A2A) and decreased yohimbine-binding sites in platelets of untreated PD patients (Villeneuve *et al.*, 1985). Bernal and coworkers (Bernal *et al.*, 1989) suggest that untreated PD is associated with a significant reduction in A2A sensitivity. It is possible that patients with PD are more vulnerable to panic attacks because they have an alteration of A2A receptors. A2A receptor is thought to result in a decrease in the stimulation of GABA-enkephalin output neurons by striatal cholinergic interneurons and an increase in the GABA-mediated recurrent inhibition of these neurons. Antagonist activity at adenosine A2A receptors in the striatum might effectively compensate for the lack of DA-mediated inhibition of these neurons in PD (Richardson *et al.*, 1997).

Serotonin

The 5-HT systems are widespread throughout the brain, with most of the cell bodies of serotonergic neurons located in the raphe nuclei of the midline brain stem (Palacios *et al.*, 1990). The largest collections of 5-HT neurons are in the dorsal and median raphe nuclei of the caudal midbrain (Jacobs & Azmitia, 1992). The neurons of these nuclei project widely over the thalamus, hypothalamus, basal ganglia, basal forebrain and the entire neocortex. Interestingly, these 5-HT neurons also provide a dense subependymal plexus throughout the lateral and third ventricles. Activation of these innervations result in 5-HT release into the cerebrospinal fluid (CSF) and measurement of 5-HT content in CSF in disease states will largely reflect this pool (Chan-Palay, 1976).

Over the past four decades there have been numerous reports describing the involvement of serotonergic and dopaminergic systems in the mechanism of action of antiparkinsonian agents. Recent advances in our understanding of 5-HT receptor subtypes and their putative role in the control of movement suggest possible novel intervention strategies for modulating dopaminergic and non-dopaminergic systems in PD patients (Thomas, 2004). Post-mortem analysis reveals reductions in the number of 5-HT₁ and/or 5-HT₂ brain binding sites in patients having suffered from various neurodegenerative disorders including PD (Cross, 1988). Based on the distribution, localization and function in the basal ganglia, 5-HT_{1B/D} and 5-HT_{2A} and 5-HT_{2C} receptors are clearly linked with modulation of the nigrostriatal pathway (Barnes *et al.*, 1999). Serotonergic terminals have been reported to make synaptic contacts with both DA-containing and non-DA containing GABA interneurons in the SNpc, substantia nigra pars reticulata (SNpr), striatum and ventral tegmental area (VTA) (Herve *et al.* 1987; Moukhels *et al.* 1997; Di Matteo *et al.*, 2001). These brain areas contain the highest concentration of 5-HT, with the SNr receiving the greatest input. Raphe' projections also innervate terminal areas to which the SNc and VTA project

to, the striatum and nucleus accumbens (Azmita & Segal, 1978). Therefore, receptor subtype-specific serotonergic drugs can act at several sites within the extrapyramidal system to modify DA activity.

Advances in the production of DA neurons from stem or precursor cells for transplantation in PD patients have clearly established an intimacy between 5-HT-DA cells, to the extent that elimination of 5-HT cells induces a marked increase in the generation of DA neurones from mesencephalic precursors cells (Rodriguez-Pallares *et al.* 2003). Thus, scientific rationale strongly suggests that therapeutic strategies that target 5-HT-dopaminergic systems, such as drugs acting on 5-HT transporters, 5-HT_{1A}, 5-HT_{1B/D} and 5-HT_{2C} receptor subtypes, can fulfill the medical need for the symptomatic treatment of PD and the motor fluctuations associated with long-term L-Dopa therapy (Barnes *et al.*, 1999; Jones & Blackburn, 2002). Realistically, only with the use of these selective serotonergic agents will be able to unravel the complex monoaminergic circuitry in the basal ganglia and relate this to the pathophysiology of PD and drug-induced dyskinesias.

Serotonin as co-mitogen

In rats, 5-HT neurons in the brain stem raphe are among the first neurons to differentiate in the brain and play a key role in regulating neurogenesis (Kligman & Marshak, 1985). Lauder and Krebs (1978) reported that parachlorophenylalanine (PCPA), a 5-HT synthesis inhibitor, retarded neuronal maturation, while mild stress, a releaser of hormones, accelerated neuronal differentiation. These workers defined differentiation as the cessation of cell division measured by incorporation of ³H-thymidine. Since then, many other workers have shown a role for 5-HT in neuronal differentiation (Marois & Croll, 1992; Hernandez, 1994). The effects of 5-HT on morphology have long been known. For more than 50 years, 5-HT has been known to constrict blood vessels (indeed, this is the origin of the name) (Page, 1968) and induce shape changes in skeletal muscle (at both the light and electron microscope level)

(O'Steen, 1967), platelets (Leven *et al.*, 1983), endothelial cells (Welles *et al.*, 1985), and fibroblast (Boswell *et al.*, 1992). In the periphery, 5-HT originates largely from mast cells, which can produce, release and re-uptake 5-HT. The released 5-HT, then act as a chemotactic, increase vascular permeability, vasodilatation, and smooth muscle spasm (Metcalf *et al.*, 1981). In addition to its role in morphological changes, 5-HT also has been shown to play a role in cell proliferation. In cultured rat pulmonary artery smooth muscle cells (SMC), 5-HT induces DNA synthesis and potentiates the mitogenic effect of platelet-derived growth factor-BB (Eddahibi *et al.*, 1999). 5-HT effects on cell proliferation are involved with phosphorylation of GTPase-activating protein (GAP), an intermediate signal in 5-HT -induced mitogenesis of SMC (Lee *et al.*, 1997). Earlier studies of from our laboratory showed that 5HT acting through specific receptor subtypes 5HT₂ (Sudha & Paulose, 1998) control cell proliferation and act as co-mitogens. Thus, there is evidence that 5-HT is involved in a variety of cellular processes involved in regulating metabolism, proliferation and morphology.

GABA

The inputs to the basal ganglia portion of the motor circuit are focused principally on the putamen, whereas the caudate nucleus (CN) and the nucleus accumbens are the principal input sites of the limbic circuit depicts a simplified scheme of the 'motor circuit' (Albin *et al.*, 1989). This postulates that in the normal brain there exists a balance between direct inhibitory input (GABA, co-localised with substance P) and indirect excitatory input (aspartate/glutamate) to the medial globus pallidus (GPm), which in turn controls thalamocortical activation. The deprivation of DA-ergic nigrostriatal input, as in PD, reduces the positive feedback through the direct system, and increases the negative feedback through the indirect system (Gerlach *et al.*, 1996). The critical consequences are an overactivity of the

subthalamic nucleus (STN), GPM and SNpr, with the resulting inhibition of thalamocortical drive.

Because specific neural pathways are involved, one might propose that degeneration of nigrostriatal neurons in patients who had manifested a Parkinsonian syndrome causes a characteristic pathologic pattern of neurotransmission in the motor circuit. In fact, electrophysiological, neurochemical and pharmacological studies using experimental models of Parkinsonism have shown a secondary increase of glutamatergic neurotransmission in the STN, the GPM and the SNr, due to a decreased GABAergic input from the lateral GPi (Ossowska, 1994). On the other hand, these assumptions have been confirmed by studies using post mortem tissue. For example, Griffiths *et al.* (Griffiths *et al.*, 1990) found decreased binding of flunitrazepam (a ligand to the GABA/benzodiazepine receptor) in the GPi of PD brains. Assuming that there is a simple relationship between increased pre-synaptic neural activity and post-synaptic receptor down-regulation and vice versa as in peripheral tissues, these data suggest that the GABA-ergic striatal neurons projecting to the GPi would be overactive in PD (Gerlach *et al.*, 1996). As GABA helps "quiet" excessive neuronal firing and has been deficient in patients in the advanced stages of PD. So directly targeting GABA production rather than DA replacement is more effective way of improving brain function in late-stage PD, this also avoids the known therapeutic limitations and complications associated with the over-production of DA. GABA supplementation can help to decrease the over stimulation of neurotransmitters such as acetylcholine and can possibly be used in Parkinson's help to inhibit acetylcholine.

GABA as co-mitogen

GABA, the main inhibitory neurotransmitter in the mature CNS, was recently implicated in playing a complex role during neurogenesis (Ben-Ari *et al.*, 1989; Baher *et al.*, 1996; Behar *et al.*, 2000; Haydar *et al.*, 2000). Through embryonic development, GABA was demonstrated as acting as a chemo-attractant and being

involved in the regulation of progenitor cell proliferation. For example, GABA induces migration and motility of acutely dissociated embryonic cortical neurons (Baher *et al.*, 1996; Behar *et al.*, 2000). In addition, the neurotransmitters GABA and glutamate reportedly reduce the number of proliferating cells in dissociated or organotypic cultures of neocortex (LoTurco *et al.*, 1995). In contrast, GABA was shown to promote cell proliferation in cultures of cerebellar progenitors (Fiszman *et al.*, 1999). GABA also dramatically increases proliferation in the ventricular zone of the embryonic cerebrum in organotypic cultures by shortening the cell cycle. However, a reverse effect was observed in the subventricular zone (Haydar *et al.*, 2000). Thus, during embryonic neurogenesis, GABA emerges as an important signal for cell proliferation and migration, but its precise regulation is depend on the region and cell type affected. Cellular response to GABA is mediated through its known receptors and the intracellular signals associated with them. The contribution of GABA_A-R to both chemo-attraction (Behar *et al.*, 2000) and cell proliferation (Haydar *et al.*, 2000) was indicated. However, in some aspects of cell motility there is an apparent involvement of GABA dependent G protein indicating a role of GABA_B-R (Behar *et al.*, 2000). GABA acts as a trophic factor not solely during prenatal neurogenesis but also in the postnatal period in injured tissue. The effect of GABA involves stimulation of cell proliferation and Nerve growth factor (NGF) secretion (Ben-Yaakov & Golan, 2003). We have previously shown that GABA acting through specific receptor subtypes GABA_B (Biju *et al.*, 2002) control cell proliferation and act as comitogens.

Glutamate

Glutamate is the most prominent neurotransmitter in the body, being present in over 50% of nervous tissue. A large proportion of the glutamate present in the brain is produced by astrocytes through synthesis *de novo* (Hertz *et al.*, 1999), but levels of glutamate in glial cells are lower than in neurons, 2–3 mM and 5–6 mM, respectively.

During excitatory neurotransmission, glutamate-filled vesicles are docked at a specialized region of the presynaptic plasma membrane known as the active zone. Packaging and storage of glutamate into glutamatergic neuronal vesicles requires Mg^{2+} /ATP-dependent vesicular glutamate uptake systems, which utilize an electrochemical proton gradient as a driving force. Substances that disturb the electrochemical gradient inhibit this glutamate uptake into vesicles. The concentration of glutamate in vesicle reaches as high as 20–100 mM (Nicholls & Attwell, 1990). In brain tissue, low concentrations of glutamate and aspartate perform as neurotransmitters, but at high concentration these amino acids act as neurotoxins.

There are two broad categories of glutamate receptors, the ion channel-forming or "ionotropic" receptors and the "metabotropic" receptors, those coupled to GTP-binding proteins (G proteins) and linked to the activation of phospholipase C (PLC) or the inhibition or activation of adenylyl cyclases (AC). The ionotropic receptors are further subdivided into three populations, those activated by N-methyl-D-aspartate (NMDA), those that respond to kainic acid (KA) and those sensitive to α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Activation of these receptors is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), which are thought to underlie learning and memory. It appears however, that aspartate aminotransferase and glutaminase account for a majority of glutamate production in brain tissue (McGeer *et al.*, 1987).

The ionotropic receptors themselves are ligand gated ion channels, i.e. on binding glutamate that has been released from a companion cell, charged ions such as Na^+ and Ca^{2+} pass through a channel in the centre of the receptor complex. This flow of ions results in a depolarisation of the plasma membrane and the generation of an electrical current that is propagated down the processes (dendrites and axons) of the neuron to the next in line. Metabotropic glutamate (mGlu) receptors are G-protein

coupled receptors (GPCR) that have been subdivided into three groups, based on sequence similarity, pharmacology and intracellular signalling mechanisms. Group I mGlu receptors are coupled to PLC and intracellular Ca^{2+} signalling, while group II and group III receptors are negatively coupled to adenylyl cyclase (Michaelis, 1998).

Glutamate functions as a fast excitatory transmitter in the mammalian brain. Glutamate triggers neuronal death when released in excessive concentrations by over excitation of its receptors (Vizi, 2000). Glutamate receptor activation and excitotoxicity has long been recognized as an upstream event in this cascade (Wieloch, 1985). In brain, glutamate accumulation is reported to cause neuronal degeneration (Berman & Murray, 1996; Budd & Nicholas, 1996; Atlante *et al.*, 1997). The excitatory amino acid glutamate is the most prevalent transmitter in the brain; its effect on postsynaptic receptors is limited by uptake process (Erecinska, 1997) and by diffusion of glutamate from the cleft. The extracellular accumulation of glutamate results in neuronal death by activating ionotropic glutamate receptors sensitive to NMDA or AMPA–kainate (Choi, 1988). The presence of G protein-coupled glutamate receptors (metabotropic Glu receptors) has been described and since 1991 (Conn & Pin, 1997), eight receptors have been discovered and classified into three groups based on their linkage to second messenger systems and their pharmacology: group I acts through the phosphoinositol system and groups II and III inhibit adenylyl cyclase. In addition, the stimulation of receptors of these three groups directly influences voltage-gated Ca^{2+} and K^{+} channels through their G proteins, but their physiological correlate has not yet defined. The most consistent age-related change in the glutamatergic system is the loss of glutamate receptors. Significant decreases in the mRNA level of glutamate receptors were found in the aged cerebral cortex (Carpenter *et al.*, 1992). Among different glutamate receptors, NMDA receptors are preferentially altered in the aged brain. Decrease in NMDA binding was shown in both rodents and mammalian brain (Cohen & Muller, 1992; Wenk *et al.*, 1991).

mRNA level of both NMDAR1 and NMDA2B subunits of the NMDA receptors have been shown to decrease preferentially in the aged cerebral cortex, whereas no age-related change was observed in the NMDA2A subunit (Magnusson, 2000).

Neurons are expected to be more vulnerable to oxidative stress because of their high rate of metabolism, the presence of high amounts of lipids that is oxidized to form peroxides and the relatively low levels of some anti-oxidants when compared with other tissues. It is suspected that because neurons that are most vulnerable in PD are those that also receive strong input from glutamate pathways, for example striatum, that glutamate must play some role in the events that lead to neuronal damage during aging or PD. If this is the case, then cell degeneration or death is the result of a cumulative process of neurotoxicity produced by glutamate (Coyle & Puttfarcken, 1993).

Metabotropic glutamate receptor

Recent data on the development of motor dysfunctions in PD and related L-Dopa therapy suggest a critical involvement of enhanced glutamatergic transmission in the basal ganglia nuclei (Calabresi *et al.*, 2000; Calon *et al.*, 2003). In fact, pharmacological treatments that reduce NMDA receptors activity limit the extent of nigro-striatal damage (Sonsalla *et al.*, 1998), improve motor symptoms of PD (Chase & Oh, 2000) and prevent or reduce L-Dopa induced dyskinesias (LIDs) (Blanchet *et al.*, 1999; Hadj Tahar *et al.*, 2004; Papa & Chase, 1996) in animal models of PD. However, alleviation of motor deficits in PD through the blockade of ionotropic glutamate receptors revealed limitations to its clinical use because of considerable side effects (hallucinations, cognitive perturbations, postural imbalance) (Andine *et al.*, 1999; Lee *et al.*, 1999).

On the basis of these considerations, combined with the rich distribution and diverse physiological roles of mGluRs within the basal ganglia structures (Marino *et al.*, 2002), recent attention has been placed on these receptors as an alternative targets

to modulate glutamate hyperactivity in PD. Eight subtypes of mGluRs have been cloned and they are classified into three subgroups based on their sequence similarity, preferred signal transduction mechanisms and relative pharmacology. Group I receptors (mGluR1 and mGluR5) are coupled to the activation of phospholipase C and generally mediate postsynaptic excitatory effects. Group II (mGluR2 and mGluR3) and group III (mGluR4, 6, 7 and 8) receptors are negatively coupled to adenylyl cyclase and inhibit cAMP formation (Ferraguti & Shigemoto, 2006). Recent studies in animal models indicate that antagonists of group I mGluRs, especially of mGluR5, could be considered as a suitable therapeutic approach in PD (Battaglia *et al.*, 2004; Breyse *et al.*, 2003, 2002; Ossowska *et al.*, 2005; Oueslati *et al.*, 2005; Dekundy *et al.*, 2006). Moreover, strong expression of mGluR5 in the striatum, by medium spiny GABAergic neurons as well as by all interneurons and other basal ganglia nuclei including STN, SN and globus pallidus (Marino & Conn, 2002; Ferraguti & Shigemoto, 2006) indicates that mGluR5 has a specific modulatory control of glutamatergic transmission through the basal ganglia circuit and is involved in the development of LIDs.

Eight different types of mGluRs, labeled mGluR1 to mGluR8 are divided into groups I, II, and III (Chu & Hablitz, 2000; Hinoi *et al.*, 2001; Endoh, 2004; Bonsi *et al.*, 2005). Receptor types are grouped based on receptor structure and physiological activity (Ohashi *et al.*, 2002). The mGluRs are further divided into subtypes, such as mGluR7a and mGluR7b. The mGluRs in group I, including mGluR1 and mGluR5, are stimulated strongly by the excitatory amino acid analog L-glutamic acid (Chu & Hablitz, 2000; Bates *et al.*, 2002) Stimulating the receptors causes the associated enzyme phospholipase C to hydrolyze phosphoinositide phospholipids in the cell's plasma membrane (Chu & Hablitz, 2000; Endoh, 2004; Bonsi *et al.*, 2005). This leads to the formation of inositol 1, 4, 5-trisphosphate (IP3) and diacyl glycerol. Due to its hydrophilic character IP3 can travel to the endoplasmic reticulum where it induces,

through fixation on its receptor, the opening of Ca^{2+} channels increasing in this way the cytosolic Ca^{2+} concentrations. The lipophilic diacylglycerol remains in the membrane acting as a cofactor for the activation of protein kinase C. These receptors are also associated with Na^+ and K^+ channels. Their action can be excitatory, increasing conductance, causing more glutamate to be released from the presynaptic cell, but they also increase inhibitory postsynaptic potentials (Chu & Hablitz, 2000). They can also inhibit glutamate release and can modulate voltage-dependent Ca^{2+} channels (Endoh, 2004). Group I mGluRs, but not other groups, are activated by 3,5-dihydroxyphenylglycine (DHPG) (Shigemoto *et al.*, 1997) a fact which is useful to experimenters because it allows them to isolate and identify them. The receptors in group II, including mGluRs 2 and 3 and group III, including mGluRs 4, 6, 7, and 8 prevent the formation of cyclic adenosine monophosphate, or cAMP, by activating a G protein that inhibits the enzyme adenylyl cyclase, which forms cAMP from ATP (Chu & Hablitz, 2000; Hinoi *et al.*, 2001; Bonsi *et al.*, 2005). These receptors are involved in presynaptic inhibition (Endoh, 2004) and do not appear to affect postsynaptic membrane potential by themselves. Receptors in groups II and III reduce the activity of postsynaptic potentials, both excitatory and inhibitory, in the cortex (Chu & Hablitz, 2000). Different types of mGluRs are distributed differently in cells. One study found that Group I mGluRs are mostly located on postsynaptic parts of cells while groups II and III are mostly located on presynaptic elements (Shigemoto *et al.*, 1997), though they have been found on both pre- and postsynaptic membranes (Endoh, 2004). Also, different mGluR subtypes are found predominantly in different parts of the body. mGluR4 is located only in the brain, in locations such as the thalamus, hypothalamus and CN (InterPro, 2008). All mGluRs except mGluR6 are thought to exist in the hippocampus and entorhinal cortex (Shigemoto *et al.*, 1997). Like other glutamate receptors, mGluRs have been shown to be involved in synaptic plasticity (Endoh, 2004; Bonsi *et al.*, 2005) and in neurotoxicity and neuroprotection.

They participate in long term potentiation and long term depression and they are removed from the synaptic membrane in response to agonist binding (Siliprandi *et al.*, 1992; Baskys *et al.*, 2005).

Ionotropic Receptors - NMDA Receptors

The discovery of potent and selective agonists and antagonists has resulted in extensive information on the NMDA receptor-channel complex (Wood *et al.*, 1990). It consists of four domains:- (1) the transmitter recognition site with which NMDA and L-glutamate interact; (2) a cation binding site located inside the channel where Mg^{2+} can bind and block transmembrane ion fluxes; (3) a phencyclidine (PCP) binding site that requires agonist binding to the transmitter recognition site, interacts with the cation binding site and at which a number of dissociative anesthetics PCP and ketamine, opiate N-allylnormetazocine (SKF-10047) and MK-801 bind and function as open channel blockers; and (4) a glycine binding site that appears to allosterically modulate the interaction between the transmitter recognition site and the PCP binding site (Fagg & Baud, 1988). NMDA is allosterically modulated by glycine, a co-agonist whose presence is an absolute requirement for receptor activation. Molecular cloning has identified to date cDNAs encoding NMDAR₁ and NMDAR2A, B, C, D subunits of the NMDA receptor, the deduced amino acid sequences of which are 18% belonging to NMDAR1 and NMDAR2, 55% belonging to NMDA2A and NMDA2C or 70% belonging to NMDA2A and NMDA2B are identical. Site-directed mutagenesis has revealed that the NMDAR2 subunit carries the binding site for glutamate within the N-terminal domain and the extracellular loop between membrane segments M3 and M4; whereas the homologous domains of the NMDAR1 subunit carry the binding site for the co-agonist glycine.

Normal functioning of the NMDA receptor complex depends on a dynamic equilibrium among various domain components. Loss of equilibrium during membrane perturbation causes the entire system to malfunction and result in abnormal

levels of glutamate in the synaptic cleft (Olney, 1989). An important consequence of NMDA receptor activation is the influx of Ca^{2+} into neurons (MacDermott *et al.*, 1986; Murphy & Miller, 1988; Holopainen *et al.*, 1989, 1990). Collective evidence suggests that when the membrane is depolarized, the Mg^{2+} block is relieved and the receptor can be activated by glutamate. Activation of the NMDA receptor therefore requires the association of two synaptic events: membrane depolarization and glutamate release. This associative property provides the logic for the role of the NMDA receptor in sensory integration, memory function, coordination and programming of motor activity (Collingridge & Bliss, 1987) associated with synaptogenesis and synaptic plasticity. Ca^{2+} flux through NMDARs is thought to play a critical role in synaptic plasticity, a cellular mechanism for learning and memory. The NMDA receptor is distinct in that it is both ligand-gated and voltage-dependent. NMDA sensitive ionotropic glutamate receptors probably consist of tetrameric and heteromeric subunit assemblies that have different physiological and pharmacological properties. They are differentially distributed throughout the CNS (Seeburg, 1993; Hollmann & Heinemann, 1994; McBain & Mayer, 1994; Danysz *et al.*, 1995; Parsons *et al.*, 1998a).

To date, two major subunit families, designated NMDAR1 and NMDAR2, have been cloned. Various heteromeric NMDA receptor channels formed by combinations of NMDAR1 and NMDAR2 subunits are known to differ in gating properties, Mg^{2+} sensitivity and pharmacological profile (Sucher *et al.*, 1996; Parsons *et al.*, 1998b). The heteromeric assembly of NMDAR1 and NMDA2C subunits, for instance, has much lower sensitivity to Mg^{2+} but increased sensitivity to glycine and very restricted distribution in the brain. *In situ* hybridization has revealed overlapping but different expression profiles for NMDAR2 mRNA. For example, NMDA2A mRNA is distributed ubiquitously like NMDAR1, with the highest densities occurring in hippocampal regions and NMDA2B is expressed predominantly in forebrain, in

cerebellum NMDA2C predominates; NMDA2D is localized mainly in the brain stem (Moriyoshi *et al.*, 1991; Monyer *et al.*, 1992; Nakanishi, 1992; McBain & Mayer, 1994). The NMDA receptor antagonists have potential therapeutic applications. NMDA receptors are involved in learning and other forms of plasticity, such as drug dependence and addiction, chronic pain and CNS development, as well as in normal or disturbed synaptic transmission in some areas of the CNS. Activation of NMDA receptors depends not only on the level of synaptic activity but also on other factors, such as agonist affinity, gating kinetics and Mg^{2+} sensitivity. The role of NMDA receptors in various processes depends on the subtype composition and area of the CNS involved. In animals, most NMDA receptor antagonists produce impairment of learning when given at sufficiently high doses before the association phase but not when administered after this phase or during retrieval (Rogawski, 1993; Leeson & Iversen, 1994; Danysz *et al.*, 1995; Avenet *et al.*, 1996).

Antagonists of NMDA receptors have been shown to inhibit neurodegeneration of the DA in SN system induced by MPP+ and methamphetamine (Sonsaila *et al.*, 1989; Greenamyre & O'Brien, 1991; Turski *et al.*, 1991). NMDA receptor antagonists in general and aminoadamantanes in particular, have been suggested as potential neuroprotective therapies in PD (Kornhuber *et al.*, 1994, Mizuno *et al.*, 1994). In fact amantadine, an NMDA receptor antagonist increases life expectancy in Parkinson's patients, an effect attributed to neuroprotective activity of this agent (Uitti *et al.*, 1996). An early exposition of CNS to increased Glutamate concentrations appear to modify, at least, the gene expression of NMDA-R subunits, it could represent changes in the neuronal connectivity as well as an increase in the neurodegeneration susceptibility the cerebral regions. NMDA glutamate receptors play a particularly important role in the function of the striatum. NMDA binding sites are very abundant in this region (Albin *et al.*, 1992). Striatal NMDA receptors are involved in the regulation of GABA, acetylcholine, neuropeptide and glutamate

release and NMDA activation causes striatal neurons to dephosphorylate the DA-receptor-associated protein DARPP-32 (Damsma *et al.*, 1991, Bustos *et al.*, 1992, Morari *et al.*, 1993, Young *et al.*, 1993). The pharmacological properties of the receptors mediating some of these actions are distinct, suggesting segregation of different kinds of NMDA receptors among the types of striatal neurons (Nicolas *et al.*, 1994). In addition to direct effects on striatal targets, NMDA receptors modulate the effect of DA on striatal function, an interaction is relevant to the therapy of PD (Starr *et al.*, 1993; Kaur *et al.*, 1997).

Glutamate mediated excitotoxic cell death

Excitotoxicity is the pathological process by which nerve cells are damaged and killed by glutamate and similar substances (Ashpole & Hudmon, 2011). Evidence is gathering that excitatory amino acid (EAA) neurotransmission contribute to neuronal ischemic injury during conditions of metabolic stress (Olney *et al.*, 1973; Choi, 1988). Excessive synaptic accumulation of glutamate can cause neuronal over activation, precipitating a cascade of cellular events that lead ultimately to cell death, a phenomenon termed glutamate excitotoxicity. This occurs when receptors for the excitatory neurotransmitter glutamate such as the NMDA receptor and AMPA receptor are over activated. Glutamate is a prime example of an excitotoxin in the brain and it is also the major excitatory neurotransmitter in the mammalian CNS (Temple *et al.*, 2001). During normal conditions, glutamate concentration can be increased up to 1mM in the synaptic cleft, which is rapidly decreased in the lapse of milliseconds. When the glutamate concentration around the synaptic cleft cannot be decreased or reaches higher levels, the neuron kills itself by a process called apoptosis. Glutamate receptors, including the NMDA subtype and several non-NMDA subtypes, are transiently overexpressed in neonates and infants, in as much as EAAs play a critical role in the development of the central nervous system (McDonald *et al.*, 1990). Hardingham *et al.*, (2002) noted that extrasynaptic NMDA receptor activation,

triggered by both glutamate exposure or hypoxic/ischemic conditions, activate a CREB (cAMP response element binding protein) shut-off, which in turn, caused loss of mitochondrial membrane potential and apoptosis. Excitotoxins like NMDA and kainic acid which bind to these receptors, as well as pathologically high levels of glutamate, cause excitotoxicity by allowing high levels of Ca^{2+} (Manev *et al.*, 1989) to enter the cell. Ca^{2+} influx into cells activates a number of enzymes, including phospholipases, endonucleases, and proteases such as calpain. These enzymes go on to damage cell structures such as components of the cytoskeleton, membrane and DNA. Reports suggest that Ca^{2+} influx through NMDA receptors is involved in ROS production and neuronal damage resulting from moderate energy depletion (Hernández-Fonseca *et al.*, 2008). Excitotoxicity is involved in spinal cord injury, stroke, traumatic brain injury and neurodegenerative diseases of the central nervous system such as Multiple sclerosis, Alzheimer's disease, Amyotrophic lateral sclerosis (ALS), PD, Alcoholism and Huntington's disease (Kim *et al.*, 2002) and neurological disorders such as ischemia, cerebral trauma and some chronic neurodegenerative diseases. An excess of glutamate release, or a deficiency in its clearance from the synaptic cleft, which depends mainly on its transport by high affinity carriers, are potential sources for the accumulation of extracellular glutamate. The SN is a target for extensive glutamatergic inputs from the cortex and the subthalamic nucleus. More importantly, secondary or 'weak' excitotoxicity is a consequence of neuronal depolarization as it might ensue from a defect of cellular energy metabolism after MPTP. The activation of NMDA receptors allows Ca^{2+} influx into the cell with possible detrimental consequences such as NOS activation or free radical cytotoxicity. Secondary excitotoxicity involving NMDA receptors was implied in MPP⁺-induced cell death. *In vivo*, NMDA antagonists were effective against MPTP toxicity. Decortication (i.e. cutting the cortical glutamatergic output, especially to the basal ganglia) alleviated MPP⁺ toxicity in rats (Srivastava *et al.*, 1993).

Glutamate Transporter

Glutamate transport is the major mechanism controlling extracellular glutamate levels, preventing excitotoxicity and averting neural damage associated with PD (McBean & Roberts, 1985; Robinson *et al.*, 1993; Tanaka *et al.*, 1997, Miller *et al.*, 2011). Glutamate transporters are localized to the membranes of synaptic terminals and astroglial processes that ensheath synaptic complex (Conti *et al.*, 1998). GLAST for glutamate–aspartate transporter, (EAAT-1) for excitatory amino acid transporter-1 (Arriza *et al.*, 1994) and GLT-1 for glutamate transporter-1, EAAT-2 (Pines *et al.*, 1992) are astroglial glutamate transporters and EAAC1 for excitatory amino acid carrier-1, EAAT-3 (Eskandari *et al.*, 2000), EAAT-4 (Fairman *et al.*, 1995) and EAAT-5 (Arriza *et al.*, 1997) are neuronal proteins.

The concentration of glutamate is regulated to ensure neurotransmission with a high temporal and local resolution. Neuronal damage is associated with excitotoxicity, a type of cell death triggered by the over activation of glutamate receptors and the loss of Ca^{2+} homeostasis. The removal of glutamate from the extracellular fluid occurs by uptake and by diffusion (Tong & Jahr, 1994). Failure of glutamate clearance leads to neuronal damage, named excitotoxic damage, due to the prolonged activation of glutamate receptors. Extracellular glutamate must be cleared quickly, perhaps within 1ms, to maintain glutamate below toxic levels (Trotti *et al.*, 1998). Glutamate transporters represent the only significant mechanism for the uptake of extracellular glutamate and their importance for the long-term maintenance of low non-toxic glutamate concentrations is well documented (Danbolt, 2001). When glutamate is taken up into glial cells by the EAATs, it is not reused directly but converted to glutamine and stored vesicles. Subsequently these vesicle are released from Glia cells and glutamine transported back into the presynaptic neuron, converted back into glutamate and store into vesicles by action of the vesicular glutamate transporter (VGLUTs) (Shigeri *et al.*, 2004). This process is named the glutamate-

glutamine cycle. Given that glutamate transporters provide the main route by which glutamate is cleared, it is logically predicted that an aberration in transporter expression and function lead to toxic glutamate levels and thus promote neuronal degeneration (Tanaka *et al.*, 1997). Studies have suggested the involvement of the glutamate transporters in radiation induced neurotoxicity (Martha *et al.*, 2009).

Signal transduction through Second Messengers

Inositol 1,4,5-trisphosphate (IP3)

Many biological stimuli, such as neurotransmitters, hormones and growth factors, activate the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP₂) in the plasma membrane which is hydrolyzed by phospholipase C (PLC) to produce IP₃ and diacylglycerol (DAG). The IP₃ mediates Ca²⁺ release from intracellular Ca²⁺ stores by binding to IP₃ receptors (IP₃R). IP₃R are the IP₃ gated intracellular Ca²⁺ channels that are mainly present in the endoplasmic reticulum (ER) membrane. The IP₃ induced Ca²⁺ signaling plays a crucial role in the control of diverse physiological processes such as contraction, secretion, gene expression and synaptic plasticity (Berridge, 1987; Berridge, 1993). Furthermore, Morita *et al.*, (2004) demonstrated that the expressed GFP-IP₃R3 acts as a functional IP₃-induced Ca²⁺ channel. Frequently, IP₃R are not uniformly distributed over the membrane but rather form discrete clusters (Bootman *et al.*, 1997). The clustered distribution of IP₃R has been predicted to be important in controlling elementary Ca²⁺ release events, such as Ca²⁺ puffs and blips, which act as triggers to induce the spatiotemporal patterns of global Ca²⁺ signals, such as waves and oscillations (Thomas *et al.*, 1998; Swillens *et al.*, 1999; Shuai & Jung, 2003). The binding of IP₃ changes the conformation of IP₃R such that an integral channel is opened, thus allowing the Ca²⁺ stored at high concentrations in the ER/SR to enter the cytoplasm. A critical feature of IP₃R is that their opening is regulated by the cytosolic Ca²⁺ concentration.

Group I mGluRs (mGluR1/5 subtypes) are also demonstrated to mainly affect intracellular Ca^{2+} mobilization (Conn & Pin, 1997; Bordi & Ugolini, 2000). To sequentially facilitate intracellular Ca^{2+} release, group I receptors activate the membrane-bound PLC, which stimulates phosphoinositide turnover by hydrolyzing PIP₂ to IP₃ and diacylglycerol. IP₃ then causes the release of Ca^{2+} from intracellular Ca^{2+} stores (such as endoplasmic reticulum) by binding to specific IP₃ receptors on the membrane of Ca^{2+} stores (Berridge, 1993). Altered Ca^{2+} levels could then engage in the modulation of broad cellular activities. Mitochondrial cytochrome c release and IP₃R-mediated Ca^{2+} release from the endoplasmic reticulum mediate apoptosis in response to specific stimuli (Boehning *et al.* 2003). This is serving as a key event in glutamate mediated neurodegeneration in PD.

Cyclic Adenosine Monophosphate (cAMP)

cAMP is produced from ATP adenyl cyclase (AC) in response to a variety of extracellular signals such as hormones, growth factors and neurotransmitters. The second messenger concept of signaling was born with the discovery of cAMP and its ability to influence metabolism, cell shape and gene transcription (Sutherland, 1972) through reversible protein phosphorylations. . Elevated levels of cAMP in the cell lead to activation of different cAMP targets. It was long thought that the only target of cAMP was the cAMP-dependent protein kinase (cAPK), which has become a model of protein kinase structure and regulation (Francis & Corbin, 1999; Canaves & Taylor, 2002). It has become clear that not all effects of cAMP are mediated by a general activation of cAPK (Dremier *et al.*, 1997). Several cAMP binding proteins have been described: cAPK (Walsh *et al.*, 1968), the cAMP receptor of *Dictyostelium discoideum*, which participates in the regulation of development (Klein *et al.*, 1997), cyclic nucleotide gated channels involved in transduction of olfactory and visual signals (Kaupp *et al.*, 1989; Goulding *et al.*, 1992) and the cAMP-activated guanine

exchange factors Epac 1, 2 which specifically activate the monomeric G protein Rap (Kawasaki *et al.*, 1998).

Cyclic Guanosine Monophosphate (cGMP)

The most extensively studied cGMP signal transduction pathway is that triggered by nitric oxide (NO) (Bredt & Snyder, 1990). cGMP effects are primarily mediated by the activation of cGMP-dependent protein kinases (PKGs). cGMP generation has been associated with neurotransmission (Hofmann *et al.*, 2000), vascular smooth muscle relaxation (Fiscus *et al.*, 1985) and inhibition of aldosterone release from adrenal glomerulosa cell suspension (Matsuoka *et al.*, 1985).

Cyclic nucleotide pathways can cross talk to modulate each other's synthesis, degradation and actions. Increased cGMP can increase the activity of cGMP stimulated phosphodiesterase 2 (PDE2) to enhance hydrolysis of cAMP, or it can inhibit the PDE3 family and decrease the hydrolysis of cAMP (Pelligrino & Wang, 1998). cAMP and cGMP are involved in NMDA receptor-mediated signaling in cerebral cortical and hippocampal neuronal cultures. The influx of Ca^{2+} through the NMDA receptor stimulates Ca^{2+} /calmodulin dependent adenylyl cyclase, leading to production of cAMP. This increase in cAMP seems to be tightly regulated by PDE4. The Ca^{2+} influx also stimulates the production of NO and subsequent activation of guanylyl cyclase, leading to cGMP production (Suvarna & O'Donnell, 2002).

cAMP-response element binding protein

cAMP-response element binding protein (CREB) belongs to a family of transcription factors that have been implicated in many important neuronal functions (Walton & Dragunow 2000, Finkbeiner 2000; Shimamura *et al.* 2000). For example, CREB-dependent gene expression has been reported to play a role in such diverse processes as cell survival, plasticity, growth and development and most recently, cell death. In neurons and other cells, CREB and its family members function as effector molecules that bring about changes within a cell in response to a wide range of

signals. Diverse extracellular stimuli such as growth factors, hormones, membrane depolarization and Ca^{2+} influx can all cause activation of CREB and the multiple different signaling cascades all converge to phosphorylate a critical CREB residue-Serine 133 (Mayr & Montminy, 2001). cAMP accumulates in the cytoplasm in response to stimulation of membrane (G)-protein coupled receptors and stimulates the dissociation of the protein kinase A (PKA) heterotetramer, which consists of a pair of regulatory (R) and a pair of catalytic (C) subunits. Once liberated, the catalytic subunits are free to enter the nucleus by passive diffusion where they phosphorylate CREB on its Ser-133 residue and gene expression can then be induced. CREB controls neuronal survival, in part, by controlling transcription of neuroprotective genes. For example, the promoter regions for both Brain Derived Neurotrophic Factor (BDNF) and the anti-apoptotic protein, B-cell lymphoma 2 (Bcl2), each contain CRE sites (Mayr & Montminy, 2001) and both of these gene products have been shown to play an important role in neuronal survival. Additionally, transgenic mice that overexpress Bcl2 are protected from naturally occurring neuronal loss as well as experimentally-induced ischemia (Martinou *et al.*, 1994). BDNF is also known to affect neuronal survival, as this neurotrophic factor protect nigrostriatal dopaminergic neurons from neurotoxins in rodent and monkey models of PD (Sun *et al.* 2005). Interestingly, BDNF has also been shown to be able to stimulate proliferation of neuronal precursors and the possible generation of new dopaminergic neurons in the striatum and SN in the unilateral 6-OHDA lesion rat model of PD (Mohapel *et al.*, 2005). Taken together, these results confirm that CREB, together with its down stream gene products, play an important role in the regulation of neuronal survival throughout the life of a neuron. Activation during development, as well as during times of stress is critical for determining neuronal fate, opening up the possibility that disruption of this important signaling pathway would have detrimental consequences.

Mode of cell death in PD

Defects in several cellular systems have been implicated as early triggers that start cells down the road towards neuronal death. These include abnormal protein accumulation, particularly of α -synuclein; altered protein degradation through multiple pathways; mitochondrial dysfunction; oxidative stress, neuroinflammation and dysregulated kinase signaling (Izumi *et al.*, 2011). As dysfunction in these systems mounts, pathways that are more explicitly involved in cell death become recruited. These include JNK signaling, p53 activation, cell cycle re-activation, and signaling through Bcl-2 family proteins (Levy *et al.*, 2009). Using DNA nick-end labeling, Mochizuki, *et al.* (1996) found in some patients intense nuclear staining indicative of apoptosis. In addition, Anglade *et al.* (1997) found the typical features of apoptosis in the SNpc of patients with PD, using ultrastructural analysis, and fragments of melanized neurons were found in glial cells. Using fluorescent probes specific for both DNA cleavage and chromatin clumping, Tatton *et al.* (1998) were able to confirm, under a light microscope level, the positive staining of melanized neurons in the SNpc. It is likely that a pro-apoptotic transduction pathway dependent on tumor necrosis factor α (TNF- α)-receptor activation is induced in PD, supporting the notion of apoptotic death. Furthermore, caspase-3 is one of the key players in apoptosis and was found to be activated in experimental models of PD (Oo, *et al.* 2002). Caspase-8 is a proximal effector protein of the TNF-receptor-family death pathway and a significantly higher percentage of dopaminergic neurons displaying caspase-8 activation were observed in PD patients than in controls (Green, 1998). In addition, using *in situ* hybridization, the level of B-cell lymphoma-extra large (Bcl-xL) mRNA expression per dopaminergic neuron in PD patients was shown to be almost double that of controls, possibly reflecting the activation of a defense mechanism in the dopaminergic cells (Hartmann *et al.*, 2002).

Bcl-2 family members include pro-apoptotic molecules (Bax, Bak, Bok, Bad, Bid, Bik, Blk, Hrk, BNIP3 and BimL) and anti-apoptotic molecules (Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1). Bcl-2 family proteins participate in the modulation and execution of cell death (Deigner *et al.*, 2000; Marino & Piantelli, 2011) and can preserve or disrupt mitochondrial integrity by regulating the release of cytochrome c/second mitochondrion-derived activator of caspase (Smac)/apoptosis inducing factor (AIF)/endonuclease G (Danial & Korsmeyer, 2004). Cytosolic Bax translocates to mitochondria upon death stimulus, promoting cytochrome c release (Gross *et al.*, 1998). Besides the involvement of the Fas/Caspase-8/Bid cascade, Bid also mediates cytochrome c release while binding to both pro-apoptotic members (e.g. Bax) and anti-apoptotic members Bcl-2 and Bcl-xL. Moreover, cleavage of Bid by caspase-8 and caspase-1 mediates the mitochondrial damage (Guegan *et al.*, 2002). Bax mediates cell death relates with mitochondrial permeability transition (Jin & El-Deiry, 2005).

α -Synuclein modifications, aggregation and fibril formation

α -Synuclein is natively unfolded under physiological conditions *in vitro*, but it is very sensitive to environmental and intrinsic factors that can modify conformation (i.e. configuration of β -sheet species) and facilitate dimer formation, aggregation of soluble oligomers (protofibrillar species) and assembly into insoluble amorphous and fibril aggregates (Uversky, 2003, 2007; Gorbatyuk *et al.*, 2008; Mollenhauer *et al.*, 2011; Winner *et al.*, 2011). Oxidative dimer formation represents the initial step in fibrillogenesis (Krishnan *et al.*, 2003). High levels of α -synuclein, α -synuclein mutations, metal cations and oxidative stress favor α -synuclein aggregation *in vitro* (Narhi *et al.*, 1999; Hashimoto *et al.*, 1999; Paik *et al.*, 2000). Protein tau and tubulin also facilitate α -synuclein aggregation (Giasson *et al.*, 2002; Alim *et al.*, 2002). The solubility of α -synuclein is altered as well, and the protein is prone to the formation of aggregates in Lewy body diseases (LBDs) and related transgenic models (Baba *et*

al., 1998; El-Agnaf *et al.*, 1998; Kahle *et al.*, 2001; Li *et al.*, 2001; Iwatsubo, 2003; Dalfo' *et al.*, 2004).

In Parkinsonian SN, α -synuclein is also modified by acrolein and accumulates in dopaminergic neurons; excess acrolein reduces proteasomal activity *in vitro*, thereby suggesting that acrolein accumulation in Lewy bodies compromises the ubiquitin–proteasome system in dopaminergic neurons (Shamoto-Nagai *et al.*, 2007). Finally, modifications in α -synuclein have effects in other PD related proteins. α -Synuclein aggregates interfere with parkin and tubulin solubility and result in parkin insolubility and cytoskeletal alterations (Kawahara *et al.*, 2008).

Tumor necrosis factor- α in PD

TNF- α is a potent pro-inflammatory molecule, which upon engagement with its cognate receptors on target cells, triggers down stream signaling cascades that control a number of cellular processes related to cell viability, gene expression, ion homeostasis and synaptic integrity (Park & Bowers, 2010). The correlative presence of inflammatory cytokines in the cerebrospinal fluid (CSF) of PD patients was described by Mogi *et al.*, (1994) who found that TNF- α levels were enhanced in CSF of those afflicted with the disease. The same group also observed enhanced TNF- α levels in the striatum of patients that had succumbed to PD. They have also reported that TNF-RI levels are elevated in the SN of PD patients (Mogi *et al.*, 2000), further suggesting altered TNF- α signaling is participating in or a result of PD-related pathogenesis. Infusion of either the toxin MPTP or 6-OHDA induces degeneration of the nigrostriatal pathway and concomitantly enhances the levels of TNF- α within the striatum and SN (Sriram *et al.*, 2002). Experimental ablation of the TNF- α receptors protects against MPTP-induced dopaminergic neurotoxicity. Other studies have focused on a possible role for TNF- α signaling in the microglial activation observed in PD pathogenesis. The administration of MPTP or 6-OHDA activates these brain-resident immune cells and if TNF- α receptor expression is genetically suppressed,

microglial activation is absent and MPTP-induced neurotoxicity is significantly blunted (Sriram *et al.*, 2006). These studies not only suggest that modulation of TNF- α expression enhances one's risk for the development of PD, but further implicate dysfunctional TNF- α signaling in neurodegeneration.

Cellular transplantation to the rescue

Pioneering work by Elizabeth Dunn in 1904 showed that transplanted fetal tissue can survive in the brain of another animal. For many years, fetal tissue has been used for treatment of human disorders, including fetal pancreatic transplants to treat diabetes mellitus and fetal thymic transplants to treat lymphogenic immunological deficiency. The defining basic science research that opened investigations on fetal tissue and brain transplantation was undertaken by Olson and Seiger (1972). They showed that fetal tissue grafted in the immunoprivileged anterior chamber of the eye has the capacity to integrate with the host target neurons and that these graft-host connections were functional. The proof of principle providing evidence that fetal tissue transplantation exerts efficacious benefits against neurodegeneration came from research in PD.

A large variety of cell replacement strategies are under investigation in animal models of PD, which began with the success of transplanted fetal neurons in reconstructing the lesioned nigrostriatal pathway and ameliorating behavioural impairments (Bjorklund & Stenevi, 1979; Perlow *et al.*, 1979). Various types of cells have been tested, such as cells from the embryonic ventral mesencephalon which contains the primordial SN, neuronal stem or progenitor cells, dopaminergic cell lines, non-neuronal cells (usually fibroblasts or astrocytes) engineered to secrete DA or neurotrophic factors, adrenal medullary cells which naturally synthesize DA, testis-derived Sertoli cells which are rich in trophic factors and more recently, carotid body epithelial glomus cells which synthesize DA and co-grafting cells with fetal kidney cells which are rich in neurotrophic factors (Koutouzis *et al.*, 1994; Dunnett, 1995;

Martinez-Serrano & Bjorklund, 1997; Raymon *et al.*, 1997; Rosenthal, 1998). Implanted cells are encapsulated in permselective polymer matrices or seeded on microcarrier beads (Borlongan *et al.*, 1996). Combining various cell types in co-grafts has often resulted in improvements (Meyer *et al.*, 1995; Takeyama *et al.*, 1995; Costantini & Snyder-Keller, 1997; Sautter *et al.*, 1998). Pretreating cells to be transplanted with trophic factors, antioxidants, or anti-apoptotic factors also improve graft survival and supplement behavioural recovery of the animal. Recently, treatment of ventral mesencephalic cells prior to transplantation with an inhibitor of the proapoptotic enzymes, the caspases, dramatically improved not only the survival of grafted dopaminergic neurons, but also the volume of the graft in 6-OHDA lesioned rats (Schierle *et al.*, 1999). Indeed, treated grafts became so robust that they caused an over abundance of dopaminergic activity on the grafted side of the brain which led to an imbalance and turning behaviour in the opposite direction. Many of these approaches have proven successful in ameliorating dopaminergic deficits and/or behavioural impairments in rodent or primate animal models of PD. Several of these techniques have progressed to clinical application.

Cells are commonly grafted ectopically to striatum (which is the target tissue for dopaminergic nigral neurons) because DA is required in the striatum and neuronal or non-neuronal cells implanted into the adult degenerating SN will physically re-establish the long nigrostriatal pathway to innervate the striatum and supply it with DA. Fetal ventral mesencephalic cells transplanted into the striatum 2 weeks after 6-OHDA lesioning in rats have been found to survive out to a full 2 years with many TH neurons remaining and forming functional synaptic connections with host striatum, improving DA content and successfully eliminating methamphetamine-induced rotations (Nishino *et al.*, 1990). Grafting fetal ventral mesencephalic tissue into the SN rather than the striatum has surprisingly also proven successful (Nikkhah *et al.*, 1994). Grafted 6-OHDA lesioned rats showed a reduction of apomorphine-induced

rotations that correlated with the number of TH cells. In addition, grafted dopaminergic neurons integrated into the host SN. However, amphetamine-induced rotations were not affected by the intranigral grafts, which is due to the differing roles of the striatum and SN in rodent drug-induced turning asymmetry. Bridging grafts created by injecting mesencephalic cells at multiple sites to lay down a tract from SN to striatum (Zhou *et al.*, 1996) have shown success in physically re-establishing the nigrostriatal pathway including reciprocal functional synapses, increased DA release and near full reduction of amphetamine-induced turning asymmetry (Zhou *et al.*, 1996). In addition, a bridging tract can be laid with fetal ventral mesencephalic cells along with neurotrophic factor-secreting substrate cells (providing both a physical growing substrate and a trophic factor) resulting in an improved survival of TH fibers along the nigrostriatal bridge and a reduction in amphetamine-induced rotations (Brecknell *et al.*, 1996). A large variety of cell types and strategies have evolved for creating bridging grafts (Olson, 1997).

However, major obstacles remain. The fetal brain tissue used in clinical transplantation studies is difficult or ethically challenging to obtain (Lindvall, 2001). Also, while under normal conditions the CNS immune response is limited (Boulanger & Shatz, 2004), the CNS is continuously patrolled by the immune system and mount a well-organized innate immune reaction in response to allogeneic antigens and cerebral injury (Tambur, 2004). Finally, striatal transplants are difficult to “tune” for appropriate dopaminergic output, causing side effects such as dyskinesia (Carlsson *et al.*, 2006), thereby emphasizing the necessity to restore the complexity of nigrostriatal neuronal circuitry.

Bone marrow cells

Stem cells have been detected in multiple organs in the adult, leading to the emerging concept of stem cell plasticity. These cells exhibit the classical traits of self-renewal and multipotentiality. In addition to well known stem cells of the adult

marrow lymphohematopoietic and stromal mesenchymal lineages (Krause *et al.*, 2001), stem cells have been provisionally identified in liver, muscle, CNS and skin (Toma *et al.*, 2001). Bone marrow cells (BMC), offer an alternative source of cells for treatment of neurodegenerative diseases and CNS injury. These cells normally differentiate into bone, cartilage and adipose tissue (Pittenger *et al.*, 1999), but can be experimentally induced to differentiate into cells with surface markers characteristic of neurons (Sanchez-Ramos *et al.*, 2000). The cells assumed characteristic neuronal forms and expressed a variety of neuron-specific genes and proteins, including neuronspecific enolase, tau, neurofilament M, NeuN (neuronal-specific nuclear protein), β -III-tubulin, and synaptophysin. When injected into the brain or administered systemically, BMC migrate to sites of injury, proliferate, and engraft (Chen *et al.*, 2001). These cells offer several advantages over other sources of stem-like precursor cells as therapy for PD: they are easily harvested, isolated and purified, can be produced in large quantities, and their use does not pose ethical concerns (Munoz-Elias *et al.*, 2004). Potential roles for BMC in treatment of PD include their use as vectors for delivery of gene products to sites of tissue injury (Ye *et al.*, 2007), facilitation of recovery from neuronal damage by replacing injured and/or lost cells (Levy *et al.*, 2008), and production of trophic factors promoting survival and regeneration of host tissue (Crigler *et al.*, 2006). In support of these therapeutic concepts, modest improvements in neurological function have been reported following BMC administration in animal models of PD, stroke, and acute CNS injury (Li *et al.*, 2001; Mahmood *et al.*, 2004; Himes *et al.*, 2006).

Role of Astrocytes in PD

Until relatively recently, astrocytes, along with other cells of the glial lineage such as oligodendrocytes and microglia, were believed to be structural cells, the main function of which was to hold neurons together. It is now known, however, that astrocytes serve many housekeeping functions, including maintenance of the extra

cellular environment and stabilization of cell–cell communications in the CNS. The function of astrocytes in regulating cerebral blood flow and maintaining synaptic function is becoming increasingly recognized as being of paramount importance in the maintenance of the neuronal environment. Astrocytes are also central to the maintenance of neuronal metabolism and neurotransmitter synthesis. The studies that have been carried out to date appear to support a neuroprotective role for astrocytes in PD. From pathological examinations, an increase in the number of astrocytes as well as in GFAP expression is observed in PD, as with other neurodegenerative disorders (Forno *et al.* 1992). The pathological evidence indirectly indicates that antioxidant pathways contribute to this neuroprotective effect, because in control brains the density of glutathione-peroxidase-positive cells was higher in the vicinity of the dopaminergic cell groups known to be resistant to the pathological process of PD. The increase in glutathione peroxidase containing cells was inversely correlated with the severity of dopaminergic cell loss in patients with PD. The quantity of glutathione peroxidase containing cells, therefore, might be critical for a protective effect against oxidative stress (Damier *et al.* 1993) Conversely, the presence of synuclein-positive astrocytes in pathological samples has been shown to correlate with nigral neuronal cell death (Wakabayashi *et al.* 2000).

In a PD model generated by lesioning the brain with the neurotoxin MPTP, it appears that astrocytosis occurs after the death of dopaminergic neurons, and that this response remains elevated even after most dopaminergic neurons have died (Teismann & Schulz, 2004). A more rapid response consisting of increased GFAP immuno reactivity as early as 1 hour following the injection of 6-OHDA into the nigrostriatal DA bundle has been observed, indicating a more direct effect of this compound on astrocytosis. Several pathways for this neuroprotection have been implicated, including the increased activation of astrocytes and neuroprotection in 6-OHDA models following infusion of interleukin-1 β (a cytokine released by activated

microglia) into the SN (Saura *et al.* 2003). Astrocytes contribute to homeostasis in the brain by providing neurons with energy and substrates for neurotransmission. They remove excess neurotransmitter molecules from the extracellular space, allowing discrete and precise encoding of synaptic signals and neurotransmission (Nicola & Ben, 2009). The role of astrocytes in PD and related syndromes is sparsely investigated and poorly understood (McGeer & McGeer, 2008). Astrocytes respond to all forms of CNS insults through a process referred to as reactive astrogliosis, which has become a pathological hallmark of CNS structural lesions (Sofroniew & Vinters, 2010). The discovery of endogenous stem cells that can generate neural tissue has raised new possibilities for repairing the nervous system. Glial progenitors, provides a reservoir for astrocyte that migrates to sites of traumatic, infectious or degenerative brain damage. But, the failure of these migrated cells in brain repair is due to the remarkable resistance to accept such cells into a mature neuronal network (Pasko 2004).

Materials and Methods

Chemicals used and their sources

Biochemicals used in the present study were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally.

Biochemicals

6-hydroxydopamine, serotonin, γ -aminobutyric acid, glutamate, (+)MK-801[(+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate, apomorphine, amphetamine, bovine serum albumin fraction V, ethylene diamine tetra acetic acid (EDTA), Tris HCl, sucrose, Magnesium chloride, calcium chloride, HEPES - [n' (2-hydroxy ethyl)] piperazine-n'-[2-ethanesulfonic acid], glycine, ascorbic acid, sodium dodecyl sulfate and paraformaldehyde were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally from SRL, India.

Radiochemicals

L-[G-³H]Glutamic acid (Sp. Activity 49.0 Ci/mmol) was purchased from Amersham Life Science, UK. (+)-[3-³H] MK-801 (Sp. Activity 27.5 Ci/mmol) was purchased from Perkin Elmer NEN Life and Analytical Sciences, Boston, MA, USA. The [³H] IP3, [³H] cGMP and [³H] cAMP Biotrak Assay Systems were purchased from G.E Healthcare UK Limited, UK.

Molecular Biology Chemicals

Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, USA. ABI PRISM High Capacity cDNA Archive kit, Primers and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, Foster City, CA, USA. NMDAR1 (Rn_00433800), NMDA2B (Rn00561352_m1), mGluR5 (Rn00566628_m1), GLAST (Rn00570130_m1), Bax (Rn 01480160_g1), CREB (Rn 00578826_m1), tumor necrosis factor- α (Rn99999017_m1), α -synuclein (Rn00569821_m1), tyrosine hydroxylase (Rn00562500_m1), Nestin

(Rn00564394_m1), and Glial fibrillary acidic protein (Rn00566603_m1) primers were used for the gene expression studies using real time PCR.

Confocal Dyes

Rat primary antibody for NMDAR1 (No: 556308, BD Pharmingen™), NMDA2B (No: 610416, BD Pharmingen™), mGluR5 (No: AB7130F, Chemicon), Nestin (No. MAB353, Chemicon), Glial fibrillary acidic protein (No. MAB360, Chemicon), tyrosine hydroxylase (No. 106K4865, Chemicon) and secondary antibody of either FITC (No: AB7130F, Chemicon), Rhodamine dye (No: AP307R Chemicon) and CY5 (No: AP124S, Chemicon) were used for the immunohistochemistry studies using confocal microscope. PKH2GL cell linker kit (No. 019K0671) from Sigma Chemical Co., St. Louis, USA used for tagging the bone marrow cells.

Animals

Adult male Wistar rats of 250-300g body weight were purchased from Kerala Agriculture University, Mannuthy and Amrita Institute of Medical Sciences, Kochi and used for all experiments. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water *ad libitum*. Adequate measures were also taken to minimize pain and discomfort of the animals. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health guidelines and CPCSEA guidelines.

Experimental design

The experimental rats were divided into the following groups i) Control ii) 6-OHDA infused (6-OHDA) iii) 6-OHDA infused supplemented with Serotonin (6-OHDA + 5-HT) and iv) 6-OHDA infused supplemented with GABA (6-OHDA + GABA) v) 6-OHDA infused supplemented with BMC (isolated from rats on femur) (6-OHDA + BMC) vi) 6-OHDA infused supplemented with 5-HT and BMC (6-OHDA+5-HT+BMC) vii) 6-OHDA infused supplemented with GABA and BMC (6-OHDA+ GABA+BMC) viii) 6-OHDA infused supplemented with 5-HT, GABA and BMC (6-OHDA+5-HT+GABA+BMC). Each group consisted of 6-8 animals.

Materials and Methods

Rats were anesthetized with Chloryl Hydrate (400 mg/kg body weight. i.p.). The animal was placed in the flat skull position on a cotton bed on a stereotaxic frame (Benchmark™, USA) with incisor bar fixed at 3.5 mm below the interaural line and the coordinates of the striatum (Paxinos & Watson, 1982) were measured accurately as anteroposterior -4.68mm, lateral 3.10mm and dorsoventral 7.8mm relative to bregma and ventral from dura with the tooth bar set at 0 mm. 6-OHDA, 8µg in 1µl in 0.2% ascorbic acid, was infused into the right SNpc at a flow rate of 0.2µl/min. After stopping the infusion of the 6-OHDA, the probe was kept in the same position for a further 5 min for complete diffusion of the drug and then slowly retracted. All the groups except Control group were infused with 6-OHDA and in control animals, 1 µl of the vehicle, 0.2% ascorbic acid, was infused into the right SNpc. Proper postoperative care was provided till the animals recovered completely.

Rotational behaviour

Amphetamine-induced (5 mg/kg, i.p.) rotational behaviour was assessed as described earlier (Ungerstedt, 1971). Rats were tested with amphetamine on the 14th day after intranigral injection of 6-OHDA and with apomorphine (1 mg/kg, s.c.) on the 16th day. Animals that had completed a 360° circle towards the intact (contralateral) and the lesioned (ipsilateral) sides were counted for 60 min continuously and recorded separately. Animals that showed no significant contralateral rotations were excluded from the study.

Treatment

On the 18th day and Stereotaxic single dose of 1µl of 5-HT (10µg/µl), GABA(10µg/µl) and 10µl of Bone marrow cell (BMC) (10⁶ Cells/10 µl) suspension individually and in combinations were infused into the right SNpc at a flow rate of 0.2 µl/min into the respective groups. Bone marrow cells were collected from femurs with saline using a syringe with a No. 18 G needle. Cells were disaggregated by gentle pipetting several times. Cells were passed through 30-µm nylon mesh to remove

remaining clumps of tissue. Cells were washed by adding fresh saline, centrifuging for 10 min at 200g and removing supernatant. The cell pellet was resuspended in 1 ml of saline. Cell counting was done using haemocytometer.

Tissue preparation

All the control and experimental rats were sacrificed on the 30th day by decapitation. The brain regions – corpus striatum, cerebral cortex, cerebellum and brain stem were dissected out quickly over ice according to the procedure of Glowinski and Iversen, (1966) and SNpc were micropunched according to Palkovits and Brownstein (1983). Hippocampus was dissected out quickly over ice according to the procedure of Heffner *et al.*, (1980). The tissues were stored at -80°C for various experiments.

Behavioural studies

Animals were observed everyday for any overt abnormal activity. Body weight was recorded on the 18th day before the treatment and on the 30th day before decapitation. Apomorphine-induced (1 mg/kg, s.c.) rotational behaviour was accessed once again on the 30th day.

Forelimb use asymmetry test (cylinder test).

The animals were evaluated in the forelimb use asymmetry test (cylinder test) (Schallert *et al.* 2000) 12 days after treatment. At each time point, the animals were placed in a Plexiglas cylinder (20-cm diameter × 30-cm high) elevated on a glass plate for a 3-minute period on 2 consecutive days. Testing was done during the dark phase of the cycle and under red lighting. The trials were videotaped from below and scored at a later date by an investigator blind to the animal's treatment. Forelimb placements on the walls of the cylinder were categorized as left independent, right independent or simultaneous movements and a forelimb use asymmetry score was calculated as:

$$\frac{\text{ipsi} + 1/2 \text{ both}}{\text{ipsi} + \text{contra} + \text{both}} \times 100$$

Where ipsi and contra refer to the forelimbs, ipsilateral and contralateral refer to the 6-OHDA-induced lesion, respectively. Animals had to make greater than 20 movements at any given time point for their data to be included in the analysis.

Rotarod Test

Rotarod has been used to evaluate motor coordination by testing the ability of rats to remain on revolving rod (Dunham and Miya, 1957). The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. The rod was placed at a height of 50 cm to discourage the animals to jump from the rotating rod. The rate of rotation was adjusted in such a manner that it allowed the normal rats to stay on it for five minutes. Each rat was given five trials before the actual reading was taken. The readings were taken at 10, 15 and 25 rpm after 12 days of treatment in all groups of rats.

Swim-test

Swim-test was carried on the 30th day in water tubs (40 cm length×25 cm width×16 cm height). The depth of water was kept at 12 cm and the temperature was maintained at 27±2°C. The animals were wiped dry immediately after the experiment using a dry towel and returned to cages kept at 27±2°C. Swim-score scales were: 0, hind part sinks with head floating; 1, occasional swimming using hind limbs while floating on one side; 2, occasional floating/swimming only; 3, continuous swimming (Haobam *et al.*, 2005).

Y-Maze Test

The Y-maze was made of grey wood, covered with black paper and consisted of three arms with an angle of 120 degrees between each of the arms. Each arm was 8 cm width ×30 cm length ×15 cm height. The three identical arms were randomly designated: Start arm, in which the rat started to explore (always open); Novel arm, which was blocked at the 1st trial, but open at the 2nd trial; and the other arm (always

open). The maze was placed in a separate room with enough light. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed on the walls of the maze.

The Y-maze test consisted of two trials separated by an inter-trial interval (ITI). The first trial (training) was of 10 minutes duration and allowed the rat to explore only two arms (start arm and the other arm) of the maze, with the third arm (novel arm) blocked. After a 1 hour ITI (Ma *et al.*, 2007), the second trial (retention) was conducted, during which all three arms were accessible and novelty *vs* familiarity was analyzed through comparing behaviour in all three arms. For the second trial, the rat was placed back in the maze in the same starting arm, with free access to all three arms for 5 minutes. Data was calculated according to the number of visit to the novel arm during the five minutes of test (Akwa *et al.*, 2001).

Radial arm maze Test

Radial maze behavioural testing was conducted under normal room lighting using an eight armed radial maze elevated 100 cm from the floor. Each arm of the maze (11.5 cm wide) extended 68.5cm from an octagonally shaped central platform (40 cm across). Black Plexiglas walls (11.5 cm high) were present for the first 20 cm of each arm to prevent the rat crossing from one arm to another without returning to the central platform. Circular food wells (1.3 cm deep, 3.2 cm diameter) were located 2.5cm from the end of each arm. The maze was centered in an enclosed room where lighting and spatial cues (e.g., posters, door, and boxes) remained constant throughout the course of the experiment. Arms were baited by placing one raisin in each food well.

Rats were placed on the maze 3 days prior to the start of formal acquisition testing in order to habituate them to the apparatus. On the first day of habituation, 4 food pellets were scattered along the length of each arm. The rats were then systematically confined to each arm for 1 min to ensure their exposure to the entire

maze. On the second day of habituation, the previous day's procedure was repeated except that the animals were not confined to each arm following 5 min of exploration. On the third day, one food pellet was placed in the food well at the end of each arm and a second was placed halfway down each arm. Once the rats were habituated to the maze, testing began. Trials began by placing a single rat in the center of the maze facing away from the experimenter. The trial ended when the rat had obtained all 4 pellets or 5min had elapsed, whichever occurred first. Rats were run until they achieved criterion performance for task acquisition. Criterion was attained when the rat collected 3 out of the 4 food pellets within their first 4 arm entries within a trial (while still completing the trial) with this level of performance being maintained for 5 consecutive criterion performance. The number of trials up to and including the last of these 5 criterion performance formed the "number of trials to criterion" measure. Experimental subjects were tested under blind conditions. The time of testing was consistent from day to day for each subject but testing of the various treatment groups was distributed randomly throughout the day. Performance was recorded during daily behavioural trials according to the terminology in previous studies (Leung *et al.*, 1990). Entry into an unbaited arm was scored as a reference error and reentry into a baited arm was scored as a working error.

Quantification of Glutamate

Glutamate content in the brain regions – corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem of control and experimental rats were quantified by displacement method using modified procedure of Enna and Snyder, (1976). Tissues were homogenized in 20 volumes of 0.32 M sucrose, 10 mM Tris/HCl and 1 mM MgCl₂ buffer, pH 7.4, with a polytron homogenizer. The homogenate was centrifuged twice at 27,000 x g for 15 min. The supernatant was pooled and used for the assay. The incubation mixture contained 1 nM [³H] glutamate with and without glutamate at a concentration range of 10⁻⁹ M to 10⁻⁴ M. The unknown concentrations

were determined from the standard displacement curve using appropriate dilutions and calculated for nmoles/g wt. of the tissue.

Quantification of Dopamine

The monoamines were assayed according to the modified procedure of Paulose *et al.*, (1988). The SNpc of experimental groups of rats was homogenised in 0.4N perchloric acid. The homogenate was then centrifuged at 5000 x g for 10 minutes at 4⁰C in a Sigma 3K30 refrigerated centrifuge and the clear supernatant was filtered through 0.22µm HPLC grade filters and used for HPLC analysis.

DA contents was determined in high performance liquid chromatography (HPLC) with electrochemical detector (ECD) (Waters, USA) fitted with CLC-ODS reverse phase column of 5 µm particle size. The mobile phase consisted of 50mM sodium phosphate dibasic, 0.03M citric acid, 0.1mM EDTA, 0.6mM sodium octyl sulfonate, 15% methanol. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.22µm filter (Millipore) and degassed. A Waters (model 515, Milford, USA) pump was used to deliver the solvent at a rate of 1 ml/minute. The neurotransmitters and their metabolites were identified by amperometric detection using an electrochemical detector (Waters, model 2465) with a reduction potential of +0.80 V. Twenty microlitre aliquots of the acidified supernatant were injected into the system for detection. The peaks were identified by relative retention times compared with external standards and quantitatively estimated using an integrator (Empower software) interfaced with the detector.

GLUTAMATE RECEPTOR BINDING STUDIES USING [³H] RADIOLIGANDS IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Glutamate Receptor Binding Studies Using [³H]Glutamate

Membranes were prepared according to the modified method of Timothy *et al.*, (1984). The brain regions - corpus striatum, cerebral cortex, hippocampus,

Materials and Methods

cerebellum and brain stem were homogenized in 20 volumes of 0.32 M sucrose, 10 mM Tris-HCl and 1 mM MgCl₂ buffer, pH 7.4, with a polytron homogenizer. The homogenate was centrifuged twice at 1,000 x g for 15 min at 4°C and the pellets were discarded. The supernatants were pooled and centrifuged at 27,000 x g for 15 min. The resulting pellet was lysed in a 10 mM Tris-HCl buffer, pH 7.4, for 30 min and centrifuged at 27,000 x g for 15 min. The resultant pellet was washed three times in 10 mM Tris/HCl buffer, pH 7.4, and centrifuged at 27,000 x g for 15 min. All steps were carried out at 4°C.

Membranes were incubated in 0.25 ml reaction mixture containing 25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂ and 20 nM to 350 nM of [³H]Glutamate containing 0.2 mg to 0.3 mg protein concentrations. Incubation was carried out at 30°C for 15 min and the reaction was stopped by centrifugation at 27,000 x g for 15 min. The pellet and the wall of the tube were quickly and carefully washed with ice-cold distilled water. 0.1% SDS and scintillation fluid were added to the dry pellet and radioactivity incorporated was determined with a Wallac scintillation counter. All the assays were carried out in triplicate. Nonspecific binding was determined by adding 350 μM nonradioactive glutamate to the reaction mixture in a parallel assay. Specific binding was considered to be the difference between total and nonspecific binding.

NMDA RECEPTOR BINDING STUDIES USING [³H] RADIOLIGANDS IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

NMDA Receptor Binding Studies Using [³H] MK-801

The membrane fractions were prepared by a modification of the method described by Hoffman *et al.*, (1996). The brain regions - corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem were homogenized in a 0.32 M sucrose buffer solution containing 10 mM HEPES, 1 mM EDTA buffer, pH 7.0. The homogenate was centrifuged at 1,000 × g for 10 min and the supernatant was centrifuged at 40,000 × g for 1 h. The pellet was resuspended and homogenized in 10

mM HEPES buffer containing 1.0 mM EDTA, pH 7.0 and centrifuged at $40,000 \times g$ for 1 h. The final pellet was suspended in 10 mM HEPES, 1 mM EDTA buffer, pH 7.0 and stored at -80°C until binding assays were performed. The [^3H] MK-801 binding saturation assay was performed in a concentration range of 0.25 to 50 nM at 23°C in an assay medium containing 10 mM HEPES, pH 7.0, 200 - 250 μg of protein, 100 μM glycine and 100 μM glutamate. After 1 h of incubation, the reaction was stopped by filtration through GF/B filters and washed thrice with HEPES buffer pH 7.0. Specific [^3H] MK-801 binding was obtained by subtracting nonspecific binding in the presence of 100 μM unlabeled MK-801 from the total binding. Bound radioactivity was counted with cocktail-T in a Wallac 1409 a liquid scintillation counter

Protein determination

Protein was measured by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein which was read in Spectrophotometer at 660nm.

ANALYSIS OF THE RECEPTOR BINDING DATA

Linear regression analysis for Scatchard plots

The data was analysed according to Scatchard, (1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

GENE EXPRESSION STUDIES IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Preparation of RNA

RNA was isolated from the different brain regions - corpus striatum, cerebral cortex, hippocampus, cerebellum, brain stem and SNpc of control and experimental rats using the Tri reagent from Sigma Chemical Co., St. Louis, USA.

Isolation of RNA

Tissue (25-50 mg) homogenates were made in 0.5 ml Tri Reagent and was centrifuged at 12,000 x g for 10 minutes at 4°C. The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5 minutes. 100µl of chloroform was added to it, mixed vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. The tubes were then centrifuged at 12,000 x g for 15 minutes at 4°C. Three distinct phases appear after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and a colourless upper aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh tube and 250 µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000 x g for 10 min at 4°C. RNA precipitated as a pellet on the sides and bottom of the tube. The supernatants were removed and the RNA pellet was washed with 500 µl of 75% ethanol, vortexed and centrifuged at 12,000 x g for 5 min at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2 µl of RNA was made up to 1 ml and absorbance was measured at 260 nm and 280 nm in spectrophotometer (Shimadzu UV-1700 pharmaSPEC). For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7 . The concentration of RNA was calculated as 1 OD at 260 = 42µg.

cDNA Synthesis

Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit in 0.2 ml microfuge tubes. The reaction mixture of 20 μ l contained 0.2 μ g total RNA, 10X RT buffer, 25X dNTP mixture, 10X Random primers, MultiScribe RT (50U/ μ l) and RNase free water. The cDNA synthesis reactions were carried out at 25°C for 10 minutes and 37°C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA designed using Primer Express Software Version (3.0).

Real-Time PCR Assay

Real Time PCR assays were performed in 96-well plates in ABI 7300 Real Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan probe (designed by Applied Biosystems). Endogenous control, β -actin, was labelled with a reporter dye (VIC). All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5' end and a quencher (Minor Groove Binding Protein - MGB) at the 3' end. The real-time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20 μ l contained 25ng of total RNA-derived cDNAs, 200nM each of the forward primer, reverse primer and TaqMan probes, endogenous control (β -actin) and 12.5 μ l of TaqMan 2X Universal PCR MasterMIX (Applied Biosystems). The volume was made up with RNase free water. Each run contained both negative (no template) and positive controls.

The thermocycling profile conditions were as follows:

50°C -- 2 minutes ---- Activation
95°C -- 10 minutes ---- Initial Denaturation
95°C -- 15 seconds ---- Denaturation 40 cycles
50°C -- 30 seconds --- Annealing
60°C -- 1 minutes --- Final Extension

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The $\Delta\Delta CT$ method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β -actin in the same samples ($\Delta CT = CT_{\text{Target}} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{\text{Control}}$). The fold change in expression was then obtained ($2^{-\Delta\Delta CT}$).

IP3 CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

The brain regions - corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem were homogenised in a polytron homogeniser in 50 mM Tris-HCl buffer, pH.7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15 min and the supernatant was transferred to fresh tubes for IP3 assay using [³H]IP3 Biotrak Assay System kit.

Principle of the assay

The assay was based on competition between [³H]IP3 and unlabelled IP3 in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound IP3 was then separated from the free IP3 by centrifugation. The free IP3 in the supernatant was then discarded by simple decantation, leaving the

bound fraction adhering to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled IP3 in the sample to be determined.

Assay Protocol

Standards, ranging from 0.19 to 25 pmoles/tube, [³H]IP3 and binding protein were added together and the volume was made up to 100 µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The tubes were then vortexed and incubated on ice for 15 minutes and they were centrifuged at 2000 x g for 10 minutes at 4°C. The supernatant was aspirated out and the pellet was resuspended in water and incubated at room temperature for 10 minutes. The tubes were then vortexed and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with %B/B₀ on the Y-axis and IP3 concentration (pmoles/tube) on the X-axis of a semi-log graph paper. %B/B₀ was calculated as:

$$\frac{(\text{Standard or sample cpm} - \text{NSB cpm})}{(\text{B}_0 \text{ cpm} - \text{NSB cpm})} \times 100$$

NSB- non specific binding and B₀ - zero binding. IP3 concentrations in the samples were determined by interpolation from the plotted standard curve.

cAMP CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

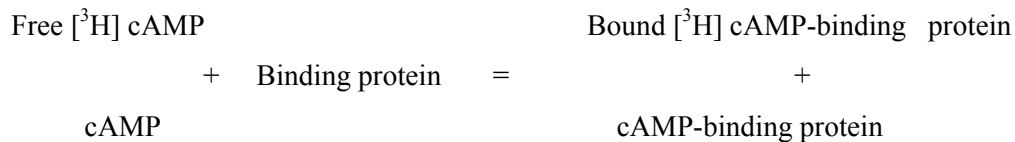
The brain regions - corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem were homogenised in a polytron homogeniser with cold 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA to obtain a 15% homogenate. The

Materials and Methods

homogenate was then centrifuged at 40,000 x g for 15min and the supernatant was transferred to fresh tubes for cAMP assay using [³H]cAMP Biotrak Assay System kit.

Principle of the assay

cAMP assay kit was used. The assay was based on the competition between unlabelled cAMP and a fixed quantity of tritium labeled compound for binding to a protein which has a high specificity and affinity for cAMP. The amount of labeled protein - cAMP complex formed was inversely related to the amount of unlabelled cAMP present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated.



Separation of the protein bound cAMP from unbound nucleotide was achieved by adsorption of the free nucleotide on to a coated charcoal followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the samples were then determined from a linear standard curve.

Assay Protocol

The tubes were placed on a water bath at 0°C. The assay mixture consisted of different concentrations of standard, [³H]cAMP and binding protein in case of standards; buffer, [³H]cAMP and binding protein for zero blank and unknown samples, [³H]cAMP and binding protein for determination of unknown samples. The mixture was incubated at 2°C for 2 hours. Cold charcoal reagent was added to the tubes and the tubes were immediately centrifuged at 12,000 x g for 2min at 2°C.

Aliquots of the supernatant was immediately transferred to scintillation vials and mixed with cocktail-T and counted in a liquid scintillation counter (Wallac, 1409).

C_0/C_x is plotted on the Y-axis against picomoles of inactive cAMP on the X-axis of a linear graph paper, where C_0 is the counts per minute bound in the absence of unlabelled cAMP and C_x is the counts per minute bound in the presence of standard or unknown unlabelled cAMP. From the C_0/C_x value for the sample, the number of picomoles of unknown cAMP was calculated.

cGMP CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

The brain regions - corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem were homogenised in a polytron homogeniser with cold 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15 min and the supernatant was transferred to fresh tubes for cGMP assay using [³H]cGMP Biotrak Assay System kit.

Principle of the assay

The assay was based on the competition between unlabelled cGMP and a fixed quantity of the [³H]cGMP for binding to an antiserum, which has a high specificity and affinity for cGMP. The amount of [³H]cGMP bound to the antiserum is inversely related to the amount of cGMP present in the assay sample. Measurement of the antibody bound radioactivity enables the amount of unlabelled cGMP in the sample to be calculated. Separation of the antibody bound cGMP from the unbound nucleotide was done by ammonium sulphate precipitation, followed by centrifugation. The precipitate which contains the antibody bound complex was resuspended in water and its activity was determined by liquid scintillation counting. The concentration of unlabelled cGMP in the sample was determined from a linear standard curve.

Assay Protocol

Standards ranging from 0.5 to 4.0 pmoles/tube and [³H]cGMP were added together and the volume was made up to 100 µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The antiserum was added to all the assay tubes and then vortexed. The tubes were incubated for 90 minutes at 2 - 8°C. Ammonium sulphate was added to all tubes, mixed and allowed to stand for 5 minutes in ice bath. The tubes were centrifuged at 12000 x g for 2 minutes at room temperature. The supernatant was aspirated out and the pellet was resuspended in water and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with Co/Cx on the Y-axis and cGMP concentration (pmoles/tube) on the X-axis of a linear graph paper. Co - the cpm bound in the absence of unlabelled cGMP; Cx - the cpm bound in the presence of standard/unknown cGMP. cGMP concentration in the samples were determined by interpolation from the plotted standard curve.

Bone marrow cell differentiation pattern studies using PKH2GL cell linker dye.

BMC were tagged with PKH2GL cell linker dye according to the kit protocol Sigma Chemical Co., St. Louis, USA. Tagged BMC (10⁶ Cells/ 10µl) suspension was infused individually and in combination stereotactically into the right SNpc at a flow rate 0.2 µl/min to the respective groups. 10 µm brain sections were cut using Cryostat (Leica, CM1510 S). Brain slices were incubated overnight at 4°C with primary antibody for nestin (No. MAB353, Chemicon, diluted in PBST at 1: 500 dilution) and Glial fibrillary acidic protein (No. MAB360, Chemicon, diluted in PBST at 1: 500 dilution). After overnight incubation brain slices were incubated with the secondary antibody with CY5 (No: AP124S, Chemicon), diluted in PBST at 1: 1000 dilution). The sections were observed and photographed using confocal imaging system (Leica SP 5).

NMDAR1, NMDA2B, mGluR5 AND TYROSINE HYDROXYLASE EXPRESSION STUDIES IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Control and experimental rats were deeply anesthetized with ether. The rat was transcardially perfused with Phosphate buffered saline (PBS), pH- 7.4, followed by 4% paraformaldehyde in PBS (Chen *et al.*, 2007). After perfusion the brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1 M PBS, pH- 7.0. 20 µm sections were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBS. To block unspecific binding the sections were incubated for 1 hour at room temperature with 5% bovine serum albumin in normal goat serum. Brain slices were incubated overnight at 4°C with either rat primary antibody for NMDAR1 (No: 556308 BD Pharmengin™, diluted in Phosphate buffered saline Triton X- 100 (PBST) at 1: 500 dilution), NMDA2B (No: 610416 BD Pharmengin™, diluted in PBST at 1: 500 dilution), mGluR5 (No: AB7130F, Chemicon, diluted in PBST at 1: 500 dilution) and tyrosine hydroxylase (No. 106K4865, Chemicon, diluted in PBST at 1: 500). After overnight incubation, the brain slices were rinsed with PBST and then incubated with appropriate secondary antibody of either FITC (No: AB7130F, Chemicon, diluted in PBST at 1: 1000 dilution) or Rhodamine dye (No:AP307R Chemicon, diluted in PBST at 1: 1000 dilution). The sections were observed and photographed using confocal imaging system (Leica SP 5).

Statistics

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT, Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve-fitting

Materials and Methods

procedure (GraphPad PRISM™, San Diego, USA). Relative Quantification Software was used for analyzing Real-Time PCR results.

Results

Body weight of control and experimental rats

6-OHDA infusion into rats showed a significant ($p < 0.001$) decrease in body weight after 18 days compared to control. 12 days after the treatment 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT, GABA and BMC in combination significantly ($p < 0.001$) regained the body weight near to control compared to the 6-OHDA infused group. Meanwhile 5-HT, GABA and BMC supplemented alone showed no significant reversal in the body weight towards the control (Table-1).

Behavioural studies

Apomorphine induced rotational behaviour in control and experimental rats

Apomorphine induced rotational behaviour showed a significant ($p < 0.001$) increase in rotation/10min compared to control. 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT, GABA and BMC in combination significantly ($p < 0.001$) reversed the rotational behaviour near to control. But 5-HT, GABA and BMC treated alone showed no significant reversal in the rotation towards the control (Figure-1).

Limb use asymmetry test in control experimental rats

There was a significant ($p < 0.001$) increase in the use of unimpaired forelimb in 6-OHDA infused rats compared to control. A significant reversal in the asymmetry score was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in the asymmetry score towards the control (Figure-2).

Rotarod performance of control and experimental rats

Rotarod experiment showed a significant ($p < 0.001$) decrease in the retention time on the rotating rod in the 6-OHDA infused rats at 10, 15 and 25 rpm when compared to control. Treatment groups significantly reversed the retention time: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT,

GABA and BMC ($p < 0.001$) near to control. BMC treated alone showed no significant reversal in the retention time towards the control (Figure-3).

Behavioural response of control and experimental rats in swim test

6-OHDA infusion into rats showed significant ($p < 0.001$) decrease in swim score compared to control. A significant reversal in the swim score was observed in the treatment groups: 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). 5-HT, GABA, BMC treated alone showed no significant reversal in the swim score compared to 6-OHDA infused rats (Figure-4).

Behavioural response of control and experimental rats on number of visit to novel arm (count/5 minutes) in y maze

6-OHDA infusion into rats showed significant ($p < 0.001$) decrease in the number of visit to novel arm compared to control. A significant reversal in the number of visit to novel arm was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). BMC treated alone did not show any significant reversal compared to 6-OHDA infused rats (Figure 5).

Behavioural response of control and experimental rats on criterion performance in radial arm maze

6-OHDA infusion into rats showed significant ($p < 0.001$) increase in the mean number of trials to achieve the criteria compared to control. A significant reversal in the number of trials to achieve the criteria was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone did not show any significant reversal compared to 6-OHDA infused rats (Figure 6).

Behavioural response of control and experimental rats on reference errors in radial arm maze

The mean reference memory error in all the groups of rats decreased over trial from first to the fourth trial. A significant ($p < 0.001$) increase in mean reference memory error over trial was observed in 6-OHDA infused rats compared to control. A significant reversal in the mean reference memory error was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone did not show any significant reversal towards the control rats (Figure 7).

Behavioural response of control and experimental rats on working errors in radial arm maze

The mean working memory error in all the groups of rats decreased over trial from first to the fourth trial. A significant increase in mean working memory error over trial was observed in 6-OHDA infused rats compared to control. A significant reversal in the mean working memory error was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). BMC treated alone did not show any significant reversal towards the control rats (Figure 8).

Corpus Striatum

Scatchard analysis of glutamate receptors using [³H]Glutamate against glutamate

Scatchard analysis of [³H]glutamate against glutamate in the Corpus Striatum of 6-OHDA infused rats showed a significant ($p < 0.001$) increase in B_{max} compared to control rats. All the treatment groups significantly reversed the receptor number to near control: 5-HT ($p < 0.01$), GABA ($p < 0.01$), BMC ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). There was no significant change in K_d in all experimental groups of rats (Figure 9, 10; Table 2, 3).

Scatchard analysis of NMDA receptors using [³H]MK801 against MK801

Scatchard analysis NMDA receptors using [³H]MK801 against MK801 in the Corpus Striatum of 6-OHDA infused rats showed a significant ($p < 0.001$) increase in B_{max} compared to control rats. Significant reversal in the B_{max} was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in B_{max} compared to 6-OHDA infused rats. There was no significant change in K_d in all experimental groups of rats (Figure 11, 12; Table 4, 5).

Glutamate content in the Corpus Striatum of control and experimental rats

Glutamate content in the Corpus Striatum showed a significant ($p < 0.001$) increase in 6-OHDA infused rats compared to control rats. A significant reversal in the glutamate content was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). BMC treated alone did not show any significant reversal in glutamate content compared to 6-OHDA infused rats (Figure 13; Table 6).

Real-Time PCR analysis of mGluR5 receptors

The gene expression studies using real-time PCR was done in corpus striatum to confirm the receptor analysis which showed a significant ($p < 0.001$) up regulation in mGluR5 receptor expression in 6-OHDA infused rats compared to control rats. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 14; Table 7).

Real-Time PCR analysis of NMDAR1 receptors

The Real-Time PCR analysis of NMDAR1 receptors in the Corpus Striatum showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control rats. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 15; Table 8).

Real-Time PCR analysis of NMDA2B receptors

The Real-Time PCR analysis of NMDA2B receptors in the Corpus Striatum showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control rats. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 16; Table 9).

Real-Time PCR analysis of GLAST glutamate transporter

The Real-Time PCR analysis of GLAST glutamate transporter in the Corpus Striatum showed a significant ($p < 0.001$) down regulation in 6-OHDA infused rats

compared to control rats. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+ GABA+BMC ($p<0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 17; Table 10).

Real-Time PCR analysis of Bax mRNA in the control and experimental rats

Gene expression of Bax mRNA showed significant up regulation ($p<0.001$) in the Corpus Striatum of 6-OHDA infused rats compared to control. Treatments with 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$) reversed the alteration. BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 18; Table 11).

Real-Time PCR analysis of tumor necrosis factor- α in the control and experimental rats

Gene expression of tumor necrosis factor- α mRNA showed significant up regulation ($p<0.001$) in the Corpus Striatum of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.001$), GABA+BMC ($p<0.001$) and 5-HT+ GABA+BMC ($p<0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 19; Table 12).

Real-Time PCR analysis of α - synuclein in the control and experimental rats

Gene expression of α - synuclein mRNA showed significant up regulation ($p<0.001$) in the Corpus Striatum of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-

HT+ GABA+BMC ($p<0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 20; Table 13).

Real-Time PCR analysis of CREB in the control and experimental rats

Gene expression of CREB mRNA showed significant down regulation ($p<0.001$) in the Corpus Striatum of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.001$), GABA+BMC ($p<0.001$) and 5-HT+ GABA+BMC ($p<0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 21; Table 14).

IP3, cAMP and cGMP content in the Corpus Striatum of control and experimental rats

The IP3 and cAMP contents in the Corpus Striatum was significantly ($p<0.001$) increased in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$) reversed the alteration. BMC treated alone showed no significant reversal in IP3 and cAMP contents compared to 6-OHDA infused rats (Figure 22, 23; Table 15, 16).

The cGMP content in the Corpus Striatum was significantly ($p<0.001$) decreased in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$) reversed the alteration. BMC treated alone showed no significant reversal in cGMP content compared to 6-OHDA infused rats (Figure 24; Table 17)

mGluR5 receptor antibody staining in control and experimental groups of rats

mGluR5 receptor antibody staining was carried out to confirm the receptor and gene expression studies. The mGluR5 receptor antibody staining in the Corpus Striatum showed significant ($p<0.001$) increase in the mean pixel value in 6-OHDA

infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 25; Table 18).

NMDAR1 receptor antibody staining in control and experimental groups of rats

The NMDAR1 receptor antibody staining in the Corpus Striatum showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Individual treatment with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 26; Table 19).

NMDA2B receptor antibody staining in control and experimental groups of rats

NMDA2B receptor antibody staining was carried out to confirm the receptor and gene expression studies. The NMDA2B receptor antibody staining in the Corpus Striatum showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 27; Table 20).

Substantia nigra pars compacta**Real-Time PCR analysis of mGluR5 receptors**

The gene expression studies of mGluR5 receptors using real-time PCR in the Substantia nigra showed a significant ($p < 0.001$) up regulation in the expression in 6-OHDA infused rats compared to control rats. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 28; Table 21).

Real-Time PCR analysis of NMDAR1 receptors

The Real-Time PCR analysis of NMDAR1 receptors in the Substantia nigra showed a significant ($p < 0.001$) up regulation in the gene expression in 6-OHDA infused rats compared to control. Treatment groups significantly reversed gene expression near to control: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 29; Table 22).

Real-Time PCR analysis of NMDA2B receptors

The Real-Time PCR analysis of NMDA2B receptors in the Substantia nigra showed a significant ($p < 0.001$) up regulation in the gene expression in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 30; Table 23).

Real-Time PCR analysis of GLAST glutamate transporter

Gene expression study of GLAST glutamate transporter in the Substantia nigra showed a significant ($p < 0.001$) down regulation in 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the

treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+ GABA+BMC ($p<0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 31; Table 24).

Real-Time PCR analysis of Bax mRNA in the control and experimental rats

Gene expression of Bax mRNA showed significant ($p<0.001$) up regulation in the Substantia nigra of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+ GABA+BMC ($p<0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 32; Table 25).

Real-Time PCR analysis of tumor necrosis factor- α in the control and experimental rats

Gene expression of tumor necrosis factor- α mRNA showed significant up regulation ($p<0.001$) in the Substantia nigra of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.001$), GABA+BMC ($p<0.001$) and 5-HT+ GABA+BMC ($p<0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats. (Figure 33; Table 26).

Real-Time PCR analysis of α - synuclein in the control and experimental rats

Gene expression of α - synuclein mRNA showed significant up regulation ($p<0.001$) in the Substantia nigra of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$), and 5-HT+ GABA+BMC ($p<0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 34; Table 27).

Real-Time PCR analysis of CREB in the control and experimental rats

Gene expression of CREB mRNA showed significant down regulation ($p < 0.001$) in the Substantia nigra of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.001$), GABA+BMC ($p < 0.001$), and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 35; Table 28).

Real-Time PCR analysis of nestin

Gene expression study of nestin in the Substantia nigra showed a significant ($p < 0.05$) up regulation in 6-OHDA infused rats and the rats individually treated with 5-HT and GABA compared to control rats. BMC treated alone ($p < 0.01$) and along with 5-HT ($p < 0.01$) and GABA ($p < 0.01$) further enhanced the nestin gene expression compared to control. Prominent significant ($p < 0.001$) expression was observed in the rats treated with 5-HT, GABA and BMC in combination (Figure 36; Table 29).

Real-Time PCR analysis of GFAP

Gene expression study of GFAP in the Substantia nigra showed a significant ($p < 0.01$) up regulation in 6-OHDA infused rats and the rats individually treated with 5-HT and GABA compared to control rats. Prominent significant ($p < 0.001$) expression of GFAP was observed in the rats treated with individual BMC treated group ($p < 0.001$), 5-HT + BMC ($p < 0.001$), GABA+BMC ($p < 0.001$) and 5-HT + GABA + BMC ($p < 0.001$) treated groups (Figure 37; Table 30).

Real-Time PCR analysis of tyrosine hydroxylase

Gene expression study of tyrosine hydroxylase in the Substantia nigra showed a significant ($p < 0.001$) down regulation in 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-

HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$), and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 38; Table 31).

Dopamine content analysis

6-OHDA infusion in to the SNpc resulted in a significant ($p < 0.001$) decrease in DA content in the PD rats compared to control. A significant reversal in the DA content was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$), and 5-HT+ GABA+BMC ($p < 0.001$). BMC treatment alone did not reversed DA content (Table-32).

Morphological changes of bone marrow cells after the injection into Substantia nigra

In vitro and *in vivo* imaging of BMC tagged with PKH2GL was done. Cellular morphology was changed once the BMC was injected in to Substantia nigra (Figure 39).

***In vivo* expression studies of bone marrow cells and Nestin in the Substantia nigra of experimental rats**

Our results proved that BMC differentiate to neuronal cells once the proper conditions are given. When autologous BMC treatment was given to SNpc, they differentiated to neuronal cell types. PKH2GL tagged BMC when injected into the brain it started expressing nestin. The BMC division was increased in 5-HT, GABA and BMC in combination later differentiating to neurons *in vivo*. Maximum mean pixel value was observed in the rats treated with 5-HT, GABA and BMC in combination (Figure 40, 41; Table 33).

***In vivo* expression studies of bone marrow cells and GFAP in the Substantia nigra of experimental rats**

PKH2GL tagged BMC injection into the brain leads to its differentiation and within 12 days it started expressing GFAP. Also we observed a marked activation of

Results

astrocytes in the SNPc. In the Confocal analysis, the mean pixel value of GFAP in the Substantia nigra showed a significant ($p < 0.01$) increase in 6-OHDA infused rats and the rats individually treated with 5-HT and GABA compared to control rats. Prominent significance ($p < 0.001$) in expression of GFAP was observed in the rats treated with individual BMC treated group and combinational 5-HT, GABA and BMC treated groups. Activated astrocytes made connections with the transplanted BMC and helped its differentiation. Confocal analysis confirmed astrocytes migration into the SNPc region after BMC injection (Figure 42, 43, 44, 45; Table 34).

Tyrosine hydroxylase antibody staining in control and experimental groups of rats

Tyrosine hydroxylase antibody staining in the Substantia nigra showed significant ($p < 0.001$) decrease in the mean pixel value in 6-OHDA infused rats compared to control. A significant reversal in the mean pixel value was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone did not reverse the alteration compared to other groups (Figure 46; Table 35).

Cerebral cortex

Scatchard analysis of glutamate receptors using [³H]Glutamate against glutamate

Scatchard analysis of total glutamate receptors using [³H] glutamate against glutamate in the cerebral cortex of 6-OHDA infused rats showed a significant ($p<0.001$) increase in B_{max} compared to control rats. All the treatment groups significantly reversed the receptor number to near control: 5-HT ($p<0.01$), GABA ($p<0.01$), BMC ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$). There was no significant change in K_d in all experimental groups of rats (Figure 47, 48; Table 36, 37).

Scatchard analysis of NMDA receptors using [³H]MK801 against MK801

The total muscarinic receptor status was assayed using the specific ligand, [³H] MK801 and NMDA receptor antagonist MK801. Scatchard analysis NMDA receptors using [³H] MK801 against MK801 in the cerebral cortex of 6-OHDA infused rats showed a significant ($p<0.001$) increase in B_{max} compared to control rats. All the treatment groups significantly reversed the receptor number to near control: 5-HT ($p<0.01$), GABA ($p<0.01$), BMC ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$). There was no significant change in K_d in all experimental groups of rats (Figure 49, 50; Table 38, 39).

Glutamate content in the cerebral cortex of control and experimental rats

Glutamate content in the cerebral cortex showed a significant ($p<0.001$) increase in 6-OHDA infused rats compared to control. Significant reversal in the glutamate content was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$). BMC treated alone did not show any significant reversal in glutamate content compared to 6-OHDA infused rats (Figure 51; Table 40).

Real-Time PCR analysis of mGluR5 receptors

The gene expression studies of mGluR5 receptor was done using real-time PCR in cerebral cortex to confirm the receptor analysis which showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 52; Table 41).

Real-Time PCR analysis of NMDAR1 receptors

The Real-Time PCR analysis of NMDAR1 receptors in the cerebral cortex showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control rats. Treatment groups significantly reversed gene expression near to control: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 53; Table 42).

Real-Time PCR analysis of NMDA2B receptors

The Real-Time PCR analysis of NMDA2B receptors in the cerebral cortex showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 54; Table 43).

Real-Time PCR analysis of GLAST glutamate transporter

The Real-Time PCR analysis of GLAST glutamate transporter in the cerebral cortex showed a significant ($p < 0.001$) down regulation in gene expression in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$)

reversed the alteration. BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 55; Table 44).

Real-Time PCR analysis of Bax mRNA in the control and experimental rats

Gene expression of Bax mRNA showed significant up regulation ($p < 0.001$) in the cerebral cortex of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 56; Table 45).

Real-Time PCR analysis of tumor necrosis factor- α in the control and experimental rats

Gene expression of tumor necrosis factor- α mRNA showed significant up regulation ($p < 0.001$) in the cerebral cortex of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.001$), GABA+BMC ($p < 0.001$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 57; Table 46).

Real-Time PCR analysis of α - synuclein in the control and experimental rats

Gene expression of α - synuclein mRNA showed significant up regulation ($p < 0.001$) in the cerebral cortex of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.01$), GABA ($p < 0.01$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 58; Table 47).

Real-Time PCR analysis of CREB in the control and experimental rats

Gene expression of CREB mRNA showed significant down regulation ($p < 0.001$) in the cerebral cortex of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 59; Table 48).

IP3, cAMP and cGMP content in control and experimental rats

The IP3 and cAMP content in the cerebral cortex was significantly ($p < 0.001$) increased in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone showed no significant reversal in IP3 and cAMP content compared to 6-OHDA infused rats (Figure 60, 61; Table 49, 50).

The cGMP content in the cerebral cortex was significantly ($p < 0.001$) decreased in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone showed no significant reversal in cGMP content compared to 6-OHDA infused rats (Figure 62; Table 51)

mGluR5 receptor antibody staining in control and experimental groups of rats

mGluR5 receptor antibody staining was carried out to confirm the receptor and gene expression studies. The mGluR5 receptor antibody staining in the cerebral cortex showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC

($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 63; Table 52).

NMDAR1 receptor antibody staining in control and experimental groups of rats

The NMDAR1 receptor antibody staining in the cerebral cortex showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 64; Table 53).

NMDA2B receptor antibody staining in control and experimental groups of rats

NMDA2B receptor antibody staining was carried out to confirm the receptor and gene expression studies. The NMDA2B receptor antibody staining in the cerebral cortex showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 65; Table 54).

Hippocampus

Scatchard analysis of glutamate receptors using [³H]Glutamate against glutamate

The total glutamate receptors status was assayed using [³H] glutamate against glutamate. Scatchard analysis of [³H]glutamate against glutamate in the hippocampus of 6-OHDA infused rats showed a significant ($p<0.001$) increase in B_{max} compared to control rats. All the treatment groups significantly reversed the receptor number to near control: 5-HT ($p<0.01$), GABA ($p<0.01$), BMC ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$). There was no significant change in K_d in all experimental groups of rats (Figure 66, 67; Table 55, 56).

Scatchard analysis of NMDA receptors using [³H]MK801 against MK801

The total muscarinic receptor status was assayed using the specific ligand, [³H]MK801 and NMDA receptor antagonist MK801. Scatchard analysis NMDA receptors using [³H]MK801 against MK801 in the hippocampus of 6-OHDA infused rats showed a significant ($p<0.001$) increase in B_{max} compared to control rats. All the treatment groups significantly reversed the receptor number to near control: 5-HT ($p<0.01$), GABA ($p<0.01$), BMC ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$). There was no significant change in K_d in all experimental groups of rats (Figure 68, 69; Table 57, 58).

Glutamate content in the Hippocampus of control and experimental rats

Glutamate content in the hippocampus showed a significant ($p<0.001$) increase in 6-OHDA infused rats compared to control rats. A Significant reversal in the glutamate content was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$). BMC treated alone did not show any significant reversal in glutamate content compared to 6-OHDA infused rats (Figure 70; Table 59).

Real-Time PCR analysis of mGluR5 receptors

The gene expression studies of mGluR5 receptors using real-time PCR analysis in the hippocampus showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 71; Table 60).

Real-Time PCR analysis of NMDAR1 receptors

The Real-Time PCR analysis of NMDAR1 receptors in the Hippocampus showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control rats. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 72; Table 61).

Real-Time PCR analysis of NMDA2B receptors

The Real-Time PCR analysis of NMDA2B receptors in the Hippocampus showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 73; Table 62).

Real-Time PCR analysis of GLAST glutamate transporter

The Real-Time PCR analysis of GLAST glutamate transporter in the Hippocampus showed a significant ($p < 0.001$) down regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed

the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 74; Table 63).

Real-Time PCR analysis of Bax mRNA in the control and experimental rats

Gene expression of Bax mRNA showed significant up regulation ($p < 0.001$) in the Hippocampus of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 75; Table 64).

Real-Time PCR analysis of tumor necrosis factor- α in the control and experimental rats

Gene expression of tumor necrosis factor- α mRNA showed significant up regulation ($p < 0.001$) in the Hippocampus of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.001$), GABA+BMC ($p < 0.001$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 76; Table 65).

Real-Time PCR analysis of α - synuclein in the control and experimental rats

Gene expression of α - synuclein mRNA showed significant up regulation ($p < 0.001$) in the Hippocampus of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 77; Table 66).

Real-Time PCR analysis of CREB in the control and experimental rats

Gene expression of CREB mRNA showed significant down regulation ($p < 0.001$) in the Hippocampus of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.001$), GABA+BMC ($p < 0.001$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 78; Table 67).

IP3, cAMP and cGMP content in control and experimental rats

The IP3 and cAMP content in the Hippocampus was significantly ($p < 0.001$) increased in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone showed no significant reversal in IP3 and cAMP content compared to 6-OHDA infused rats (Figure 79, 80; Table 68, 69).

The cGMP content in the Hippocampus was significantly ($p < 0.001$) decreased in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone showed no significant reversal in cGMP content compared to 6-OHDA infused rats (Figure 81; Table 70)

mGluR5 receptor antibody staining in control and experimental groups of rats

mGluR5 receptor antibody staining was carried out to confirm the receptor and gene expression studies. The mGluR5 receptor antibody staining in the Hippocampus showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC

treated alone did not reverse the alteration compared to 6-OHDA group (Figure 82; Table 71).

NMDAR1 receptor antibody staining in control and experimental groups of rats

The NMDAR1 receptor antibody staining in the Hippocampus showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 83; Table 72).

NMDA2B receptor antibody staining in control and experimental groups of rats

NMDA2B receptor antibody staining was carried out to confirm the receptor and gene expression studies. The NMDA2B receptor antibody staining in the Hippocampus showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 84; Table 73).

Cerebellum

Scatchard analysis of glutamate receptors using [³H]Glutamate against glutamate

The total glutamate receptors status was assayed using [³H] Glutamate and glutamate. Scatchard analysis of [³H]glutamate against glutamate in the cerebellum of 6-OHDA infused rats showed a significant ($p<0.001$) increase in B_{max} compared to control rats. All the treatment groups significantly reversed the receptor number to near control: 5-HT ($p<0.01$), GABA ($p<0.01$), BMC ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$). There was no significant change in K_d in all experimental groups of rats (Figure 85, 86; Table 74, 75).

Scatchard analysis of NMDA receptors using [³H]MK801 against MK801

The total muscarinic receptor status was assayed using the specific ligand, [³H]MK801 and NMDA receptor antagonist MK801. Scatchard analysis NMDA receptors using [³H]MK801 against MK801 in the cerebellum of 6-OHDA infused rats showed a significant ($p<0.001$) increase in B_{max} compared to control rats. All the treatment groups significantly reversed the receptor number to near control: 5-HT ($p<0.01$), GABA ($p<0.01$), BMC ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$). There was no significant change in K_d in all experimental groups of rats (Figure 87, 88; Table 76, 77).

Glutamate content in the cerebellum of control and experimental rats

Glutamate content in the cerebellum showed a significant ($p<0.001$) increase in 6-OHDA infused rats compared to control rats. A significant reversal in the glutamate content was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$). BMC treated alone did not show any significant reversal in glutamate content compared to 6-OHDA infused rats (Figure 89; Table 78).

Real-Time PCR analysis of mGluR5 receptors

The gene expression studies using real-time PCR was done in cerebellum to confirm the receptor analysis which showed a significant ($p<0.001$) up regulation in mGluR5 receptor expression in 6-OHDA infused rats compared to control rats. Treatments with 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 90; Table 79).

Real-Time PCR analysis of NMDAR1 receptors

The Real-Time PCR analysis of NMDAR1 receptors in the cerebellum showed a significant ($p<0.001$) up regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 91; Table 80).

Real-Time PCR analysis of NMDA2B receptors

The Real-Time PCR analysis of NMDA2B receptors in the cerebellum showed a significant ($p<0.001$) up regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 92; Table 81).

Real-Time PCR analysis of GLAST glutamate transporter

The Real-Time PCR analysis of GLAST glutamate transporter in the cerebellum showed a significant ($p<0.001$) down regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC

($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 93; Table 82).

Real-Time PCR analysis of Bax mRNA in the control and experimental rats

Gene expression of Bax mRNA showed significant up regulation ($p < 0.001$) in the cerebellum of 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 94; Table 83).

Real-Time PCR analysis of tumor necrosis factor- α in the control and experimental rats

Gene expression of tumor necrosis factor- α mRNA showed significant up regulation ($p < 0.001$) in the cerebellum of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.001$), GABA+BMC ($p < 0.001$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 95; Table 84).

Real-Time PCR analysis of α - synuclein in the control and experimental rats

Gene expression of α - synuclein mRNA showed significant up regulation ($p < 0.001$) in the cerebellum of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 96; Table 85).

Real-Time PCR analysis of CREB in the control and experimental rats

Gene expression of CREB mRNA showed significant down regulation ($p < 0.001$) in the cerebellum of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.001$), GABA+BMC ($p < 0.001$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 97; Table 86).

IP3, cAMP and cGMP content in control and experimental rats

The IP3 and cAMP content in the cerebellum was significantly ($p < 0.001$) increased in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone showed no significant reversal in IP3 and cAMP content compared to 6-OHDA infused rats (Figure 98, 99; Table 87, 88).

The cGMP content in the cerebellum was significantly ($p < 0.001$) decreased in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone showed no significant reversal in cGMP content compared to 6-OHDA infused rats (Figure 100; Table 89).

mGluR5 receptor antibody staining in control and experimental groups of rats

mGluR5 receptor antibody staining was carried out to confirm the receptor and gene expression studies. The mGluR5 receptor antibody staining in the cerebellum showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 101; Table 90).

NMDAR1 receptor antibody staining in control and experimental groups of rats

The NMDAR1 receptor antibody staining in the cerebellum showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 102; Table 91).

NMDA2B receptor antibody staining in control and experimental groups of rats

NMDA2B receptor antibody staining was carried out to confirm the receptor and gene expression studies. The NMDA2B receptor antibody staining in the cerebellum showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 103; Table 92).

Brain stem

Scatchard analysis of glutamate receptors using [³H]Glutamate against glutamate

The total glutamate receptors status was assayed using [³H] Glutamate and glutamate. Scatchard analysis of [³H]glutamate against glutamate in the brain stem of 6-OHDA infused rats showed a significant ($p < 0.001$) increase in B_{max} compared to control rats. All the treatment groups significantly reversed the receptor number to near control: 5-HT ($p < 0.01$), GABA ($p < 0.01$), BMC ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). There was no significant change in K_d in all experimental groups of rats (Figure 104, 105; Table 93, 94).

Scatchard analysis of NMDA receptors using [³H]MK801 against MK801

The total muscarinic receptor status was assayed using the specific ligand, [3H]MK801 and NMDA receptor antagonist MK801. Scatchard analysis NMDA receptors using [³H]MK801 against MK801 in the brain stem of 6-OHDA infused rats showed a significant ($p < 0.001$) increase in B_{max} compared to control rats. Significant reversal in the B_{max} was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in B_{max} compared to 6-OHDA infused rats. There was no significant change in K_d in all experimental groups of rats (Figure 106, 107; Table 95, 96).

Glutamate content in the brain stem of control and experimental rats

Glutamate content in the brain stem showed a significant ($p < 0.001$) increase in 6-OHDA infused rats compared to control. Significant reversal in the glutamate content was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$). BMC

treated alone did not show any significant reversal in glutamate content compared to 6-OHDA infused rats (Figure 108; Table 97).

Real-Time PCR analysis of mGluR5 receptors

The gene expression studies using real-time PCR analysis were done in brain stem to confirm the receptor analysis which showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 109; Table 98).

Real-Time PCR analysis of NMDAR1 receptors

The Real-Time PCR analysis of NMDAR1 receptors in the brain stem showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 110; Table 99).

Real-Time PCR analysis of NMDA2B receptors

The Real-Time PCR analysis of NMDA2B receptors in the brain stem showed a significant ($p < 0.001$) up regulation in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 111; Table 100).

Real-Time PCR analysis of GLAST glutamate transporter

The Real-Time PCR analysis of GLAST glutamate transporter in the brain stem showed a significant ($p < 0.001$) down regulation in 6-OHDA infused rats

compared to control. Treatments with 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 112; Table 101).

Real-Time PCR analysis of Bax mRNA in the control and experimental rats

Gene expression of Bax mRNA showed significant up regulation ($p<0.001$) in the brain stem of 6-OHDA infused rats compared to control. Treatments with 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+GABA+BMC ($p<0.001$) reversed the alteration. BMC treated alone did not show any significant reversal in gene expression compared to 6-OHDA infused rats (Figure 113; Table 102).

Real-Time PCR analysis of tumor necrosis factor- α in the control and experimental rats

Gene expression of tumor necrosis factor- α mRNA showed significant up regulation ($p<0.001$) in the brain stem of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.001$), GABA+BMC ($p<0.001$) and 5-HT+ GABA+BMC ($p<0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 114; Table 103).

Real-Time PCR analysis of α - synuclein in the control and experimental rats

Gene expression of α - synuclein mRNA showed significant up regulation ($p<0.001$) in the brain stem of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p<0.05$), GABA ($p<0.05$), 5-HT + BMC ($p<0.01$), GABA+BMC ($p<0.01$) and 5-HT+ GABA+BMC ($p<0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 115; Table 104).

Real-Time PCR analysis of CREB in the control and experimental rats

Gene expression of CREB mRNA showed significant down regulation ($p < 0.001$) in the brain stem of 6-OHDA infused rats compared to control. A significant reversal in the gene expression was observed in the treatment groups: 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.001$), GABA+BMC ($p < 0.001$) and 5-HT+ GABA+BMC ($p < 0.001$). BMC treated alone showed no significant reversal in gene expression compared to 6-OHDA infused rats (Figure 116; Table 105).

IP3, cAMP and cGMP content in control and experimental rats

The IP3 and cAMP content in the brain stem was significantly ($p < 0.001$) increased in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$) and GABA+BMC ($p < 0.01$) 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone showed no significant reversal in IP3 and cAMP content compared to 6-OHDA infused rats (Figure 117, 118; Table 106, 107).

The cGMP content in the brain stem was significantly ($p < 0.001$) decreased in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$), 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) reversed the alteration. BMC treated alone showed no significant reversal in cGMP content compared to 6-OHDA infused rats (Figure 119; Table 108)

mGluR5 receptor antibody staining in control and experimental groups of rats

mGluR5 receptor antibody staining was carried out to confirm the receptor and gene expression studies. The mGluR5 receptor antibody staining in the brain stem showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 120; Table 109).

NMDAR1 receptor antibody staining in control and experimental groups of rats

The NMDAR1 receptor antibody staining in the brain stem showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 121; Table 110).

NMDA2B receptor antibody staining in control and experimental groups of rats

NMDA2B receptor antibody staining was carried out to confirm the receptor and gene expression studies. The NMDA2B receptor antibody staining in the brain stem showed significant ($p < 0.001$) increase in the mean pixel value in 6-OHDA infused rats compared to control. Treatments with 5-HT ($p < 0.05$), GABA ($p < 0.05$) and 5-HT + BMC ($p < 0.01$), GABA+BMC ($p < 0.01$) and 5-HT+GABA+BMC ($p < 0.001$) significantly reversed the mean pixel value. BMC treated alone did not reverse the alteration compared to 6-OHDA group (Figure 122; Table 111).

Discussion

Parkinson's disease (PD) is a devastating neurodegenerative disorder that results in a wide range of motor and non-motor deficits that includes tremors, bradykinesia, rigidity, cardiovascular and gastrointestinal abnormalities, cognitive dysfunction and depression (Springer & Kahle, 2011). It is the second most prevalent neurodegenerative disease and affects an estimated 6 million people worldwide, with projections suggesting a two fold increase within 25 years (Richard & Serge, 2008). PD is largely characterized by the irreversible loss of DA neurons, although it is becoming increasingly clear that other neurotransmitter systems are likely involved in the pathogenesis. The great majority of cases of PD are sporadic, without any other family members being affected. One of the great advances in the last decade is that several gene mutations have been discovered to cause PD. But these monogenetic causes do not explain the great majority of sporadic cases (Dawson *et al.*, 2010).

Weight loss in Parkinson's disease

Patients with PD frequently loose weight. The frequency of weight loss among such patients is 52 % as reported by Abbott *et al.*, (1992) and 65% by Moroo *et al.*, (1994). Weight loss is more prominent in women (average body weight loss 8.5 %) than in men (4.3 %) and becomes marked in patients with advanced disabilities. Energy expenditure decreases due to motor impairment but increase in parallel with worsening of muscle rigidity and the development of dopa-induced dyskinesias. Moreover, disturbed motility and absorption of the gastrointestinal tract impair energy intake. Dysphagia occurs in the advanced stage of PD, and anorexia caused by depression also could cause disturbed energy intake. Anti-Parkinsonian drugs accelerate anorexia and dysfunction of the gastrointestinal tract. Medical complications such as pneumonia, bone fracture and malignancy cause additional weight loss. Moreover, weight loss is also associated with insufficient nutrition, precipitating infection and decubitis and increasing the mortality rate (Kashihara

2006). We observed a decreased body weight in the 6-OHDA rats compared to the control which is due to a prolonged disequilibrium between intake, digestion and absorption of energy from nutrients on the one hand, and energy expenditure on the other hand. It is a clinically relevant problem since weight loss can contribute substantially to both morbidity and mortality. Treatment of 6-OHDA rats with 5-HT, GABA and BMC in combination improved body weight significantly which indicate prevention of muscle tissue damage.

Behavioural deficits in Parkinson's induced rats

PD is often complicated by a variety of cognitive symptoms that range from isolated memory and thinking problems to severe dementia. While the motor symptoms of PD are well-known (tremor, rigidity, slowness of movement, imbalance), the commonly seen deficits in memory, attention and problem-solving are less understood. Studies have shown that over 50% of people with PD experience some form of cognitive impairment. About 20% have more substantial cognitive impairment (Harish *et al.*, 2010). 6-OHDA induces toxicity through intra- or extracellular auto-oxidation, hydrogen peroxide formation induced by monoamine oxidase-B (MAO-B) activity, or direct inhibition of the mitochondrial respiratory chain and consequent oxidative stress (Shim *et al.*, 2009). New Parkinsonian rat models have been developed with 6-OHDA injected directly into the SN to induce selective and moderate neurodegeneration of DA nerve terminals. Similarly, in PD the progressive degeneration of nigral dopaminergic neurons results in motor deficits only after 80% of the nigrostriatal system has degenerated. Therefore behavioural studies preferentially involve unilateral destruction of the nigrostriatal pathway with 6-OHDA to avoid the debilitating consequences of a bilateral lesion (Hritcu *et al.*, 2008). Depending on the dose and the site of infusion into the brain, unilateral 6-OHDA SNpc-lesioned rats present an almost complete loss of dopaminergic neurons in the SNpc, a proportional depletion of striatal DA and gross motor disturbances, like

Discussion

turning behaviour (e.g. after a challenge with DA receptor agonists) and reduced locomotion. Ungerstedt (1968) reported preliminary findings that injection of the neurotoxin 6-OHDA into one nigrostriatal pathway of the rat produced an animal with loss of catecholamine histochemical fluorescence from the ipsilateral striatum and with marked motor asymmetry, turning spontaneously towards the side of the 6-OHDA injection. Unilateral injection of 6-OHDA into the SN caused degeneration of the ipsilateral nigrostriatal pathway and loss of DA from the ipsilateral striatum. Following injection of 6-OHDA into the nigrostriatal pathway, a rat exhibits rotational behaviour or a body asymmetry towards the operated side. This circling behaviour is exaggerated with systemic administration of amphetamine, which stimulates catecholamine release. Within a few weeks after 6-OHDA treatment, striatal denervation hypersensitivity to DA develops; it is demonstrated by circling behaviour in the opposite, away from the side of the injection site direction with administration of the DA agonist apomorphine. Our studies with apomorphine showed a reversal in the rotational behaviour in rats treated with 5-HT, GABA and BMC compared to 6-OHDA infused rats. This indicates the reduction of DA receptor hypersensitivity after the BMC transplantation with 5-HT and GABA.

We also investigated the ability of 5-HT, GABA and BMC to restore skilled forelimb performances in the PD rat model. Skilled forelimb use is dependent on an intact dopaminergic neurotransmission and is substantially impaired in animals with unilateral 6-OHDA lesions. The asymmetry score is calculated as the number of “ipsi” observations plus 1/2 the number of “both” observations, divided by the total number of observations (ipsi plus contra plus both). This provides an overall asymmetry percentage score, where 50% indicates an animal that explores symmetrically with both limbs, higher scores (> 50%) indicate a greater reliance on the ipsilesional limb, and lower scores (< 50%) indicate a greater reliance on the contralesional limb. In our study 6-OHDA infused rats showed higher scores in the forelimb use asymmetry test

indicates its greater reliance on the ipsilesional limb. BMC treated alone didn't reverse the condition. The higher scores seen also in the groups treated individually with 5-HT and GABA. Prominent reversal in the asymmetry score towards the control was observed in rats treated with 5-HT, GABA and BMC in combination. Previous studies by Vergara-Aragon *et al.*, (2003) showed that severe unilateral DA depletion caused moderate impairments in skilled reaching of the "good" forelimb, which were improved by focused skilled reach training for 5 days/week for 45 days, beginning well after 6-OHDA infusions. Impairments in the "bad" forelimb could not be significantly rehabilitated.

PD has been reported to be accompanied by a number of behavioural and hormonal abnormalities, including reduced locomotor activity. Thus, for understanding the relationship between behavioural expression and underlying neuropathology in the 6-OHDA model, one should take into account that the behaviour is depend on the sensitivity of a test to measure striatal DA loss on the one hand, and the extent to which non-dopaminergic and extra-striatal neurotransmission are affected by the regimen on the other. To provide a behavioural assessment of the 6-OHDA-induced lesion, a rotarod test was done to determine the ability to perform well-coordinated locomotor activity (Cendelin *et al.*, 2008). The rotarod test experiment demonstrated the impairment of the motor function and coordination in the 6-OHDA infused rats. 6-OHDA infused rats showed lower fall off time from the rotating rod when compared to control, suggesting impairment in their ability to integrate sensory input with appropriate motor commands to balance their posture. Moreover, the 6-OHDA infused rats showed clear signs of deficiency in fine motor control as indicated by a reduced tendency to turn around and walk forward on the rotarod. At the same time, they were unable to adjust their limb movements on the metallic rod which is indicative of cerebellar dysfunction. The 5-HT, GABA and BMC treated rats showed an improved motor performance in rotarod, compared to

Discussion

6OHDA induced PD rats. Our findings indicate that 5-HT, GABA and BMC in combination reverses the motor abnormalities which assists in lowering their time for spatial recognition and thus helps to maintain their posture during movement on the rod.

Swim test, is an efficient technique to investigate the overall manifestation of motor dysfunction in PD is imminent for identifying relationship between the loss of dopaminergic neurons and behavioural changes observed in animal models of this disease. While tremor and akinesia are acute behavioural manifestations that are visible, swimming is latent and manifested only when tested in a new environment. Tremor and akinetic responses are short lasting, whereas swim deficit could be setting in slowly, along with depletion of striatal DA. The objective of the present study was to evaluate the suitability of swim endurance (as revealed by swim-score), for assessing motor deficit in rats with striatal DA depletion, following 6-OHDA administration. Two major criticisms for the applicability of swim-test are: (i) in highly akinetic state, the animals may drown and (ii) swimming in cold water may induce stress and interfere with the performance while they swim (Sedelis, 2001). We can always overcome the cold induced stress by maintaining the water temperature at the ambient level. Here, in our study, we have maintained the temperature at 27 ± 2 °C. Swim-test is mostly used for animal models with bilateral lesions and with partial DA depletion. Here we tried to use this in the unilateral model along with the rotational analysis to observe the motor behaviour. In the present study 6-OHDA infused rat showed significant decrease in swim score compared to the control. This indicates a direct relationship between DA depletion and motor impairment in 6-OHDA infused Parkinsonian rat. Rats treated with 5-HT, GABA and BMC showed a considerable recovery in their swim-score as compared with the 6-OHDA infused rats.

Whether spatial deficits are one of the major characteristics of neuropsychological alterations in PD has been discussed for many years. A number of

studies however have demonstrated that spatial deficits are indeed associated with the motor component of the task (Lezak, 1995) or with the task's speed component (Ogden *et al.*, 1990). This pattern of results has been related to a gradually emerging dopaminergic deficit. Spatial deficits which are regularly seen in PD patients are often understood as deficits in the ability to sequence chains of motor responses, to plan these sequences in advance and to perform behavioural acts in multiple steps. The Y-maze test is a classic model behavioural test, with a strong aversive component, utilized for evaluating learning and memory in rats (Woo *et al.*, 2008). Y-maze performance showed that intensity of derangement in 6-OHDA infused rats increased. Furthermore, spatial memory and exploratory activity have an influence on behavioural tests including Y-maze performance. In this regard, the number of novel arm entries was significantly lower in 6-OHDA infused PD rats. There was a prominent reversal towards the control in number of novel arm entry when 6-OHDA infused PD rats were treated with 5-HT, GABA and BMC in combination. These findings indicate that transplantation of BMC along with 5-HT and GABA were able to normalize the dopaminergic and glutaminergic receptor dysfunction which assists in lowering their time for spatial recognition and thus improving the cognitive functions.

DA, glutamate and ACh systems seem to have important interactions with regard to cognitive function. A variety of previous studies have found that DA ligands have significant interactions with ACh agonist and antagonist effects on memory performance (Levin *et al.*, 1992). Alterations such as inhibition of DA neurotransmitter release, changes in pre- or postsynaptic receptor binding, and neurotransmitter transports contributes to the impairment in the brain blood flow on neurons or glial cells and consequently interfere with the information encoding processes. We evaluated the spatial memory and learning by radial arm maze to evaluate the memory deficit in the 6-OHDA infused rats. In radial arm maze, memory

Discussion

errors like working memory error and reference memory error were scored along with the number of trials needed to attain the criterion. Working memory is a transient form of memory that maintains task relevant information during conditions of competing demands (Baddeley, 1986). Working memory can be operationalized as task accuracy in situations where information (or the appropriate response based on that information) changes frequently (i.e., from trial to trial), whereas reference memory reflects task accuracy when information (or the appropriate response based on that information) remains constant indefinitely (i.e. across trials and/or sessions) (Olton *et al.*, 1979). The number of trials to attain five consecutive criterion performances increased significantly in the 6-OHDA infused rats. Increased numbers of trials to criterion performance indicates the learning and memory deficit in 6-OHDA infused rats. A significant increase in memory errors was also scored in 6-OHDA infused rats indicating the impairment in coordination of tasks. High reference memory error in 6-OHDA infused rats points to the impairment with respect to procedural or reference memory i.e., what to do with the information they have. A significant reversal towards the control was observed in the treatment groups: 5-HT, GABA, 5-HT + BMC, GABA+BMC. BMC treated alone did not show any significant reversal compared to 6-OHDA infused rats. Most prominent reversal in the number of trials to attain the criterion performance and memory errors was observed when rats treated with 5-HT, GABA and BMC in combination. The impairment in spatial learning, memory and task management was also reversed by BMC supplementation to 6-OHDA infused rats, in combination with 5-HT and GABA.

CENTRAL NERVOUS SYSTEM ALTERATIONS DURING PD

Corpus striatum

The corpus striatum is the largest component of the basal ganglia. Various anatomical, electrophysiological and pathological observations provide evidence that DA plays a major role in the control of striatal function and in the regulation of motor

control (Avale *et al.*, 2008). Loss of dopaminergic neurons in the SN and nigrostriatal pathway disrupts the physiologic activity of the striatum (Penney & Young, 1983) and compromises the functioning of subcortical-cortical functional-anatomic loops. DA levels in the corpus striatum including the nucleus accumbens and Ventral tegmental area projection sites decreases significantly in 6-OHDA infused rats. The dopaminergic neurons in the SNpc extend its arm to the corpus striatum and hence are directly affected in PD. In the normal brain there exists a balance between direct inhibitory input through GABA and indirect excitatory input through glutamate to the lateral GPi, which in turn controls thalamocortical activation. The deprivation of dopaminergic nigrostriatal input, as in PD, reduces the positive feedback through the direct system and increases the negative feedback through the indirect system (Gerlach *et al.*, 1996). Thus, the low level of DA, in Parkinson's, leads to enhanced glutamate function. Scatchard analysis of glutamate receptor in the corpus striatum of 6-OHDA infused rats showed a significant increase in B_{max} compared to control rats. Increased glutamate content in the 6-OHDA infused rats leads to the up regulation of total glutamate receptors. There was no significant change in K_d in all experimental groups. Significant reversal was seen in all treatment groups except the rats treated BMC alone. More prominent reversal was seen in rats supplemented with 5-HT and GABA treated with BMC. A key event in this process glutamate mediated neurotoxicity is the enhancement of the NMDA receptor-channel complex and a subsequent influx of Ca^{2+} . We observed an increase in NMDA receptors function in the corpus striatum of the 6-OHDA infused rats with no significant change in K_d . The increased receptor activity observed from the Scatchard plot was supported by the gene expression studies of NMDAR1, NMDA2B and mGluR5 glutamate receptor subtypes. Glutamate reuptake into neurons and glia cells is important for termination of glutamatergic transmission. Glutamate transporters are essential for the maintenance of low extracellular levels of glutamate. Our results showed a reduced

Discussion

expression of GLAST glutamate transporter that indicates the reduced reuptake of the extracellular glutamate which is activated through glutamate receptor subtypes-NMDAR1, NMDA2B and mGluR5. The decreased glutamate transporter GLAST expression reduces the reuptake of the extracellular glutamate. Thus the results showed evidence for the dysfunction of the corpus striatum that is a reflection for manifestation of abnormal behavioural patterns. Combination treatment restored the impairment near to control.

NO-cGMP- Protein kinase G (PKG) signaling pathway plays an essential role in the neuroprotection through activating B-cell lymphoma 2 (Bcl-2), Mn Superoxide dismutase (MnSOD) and brain derived neurotrophic factor (BDNF) gene expression which reduces oxidative stress (Andoh *et al.*, 2002). 6-OHDA induced oxidative damage and neurodegeneration leads to a decreased cGMP level in the corpus striatum. At the same time we obtained an increased production of IP3, cAMP in 6-OHDA infused rats which are mediated through the enhanced glutamate receptors. This will trigger the release of Ca^{2+} from the endoplasmic reticulum. IP3-mediated Ca^{2+} release in turn increase mitochondrial Ca^{2+} and consequently, increase respiration and ATP production (Hajnoczky *et al.*, 2000). This causes metabolic stress on mitochondria that leads to excessive oxidative phosphorylation and increased production of reactive oxygen species. Excessive stimulation of glutamate receptor/ion channel complexes triggers Ca^{2+} flooding and a cascade of intracellular events that results in apoptosis (Johnston, 2005). Up regulation of pro-apoptotic Bax protein expression in the corpus striatum indicates the mitochondria mediated apoptosis in 6-OHDA infused rats. Enhanced glutamatergic, IP3 and cAMP activity leads to oxidative stress in the striatum. A plausible source of oxidative stress in striatum neurons is the redox reactions that specifically involve DA and produce various toxic molecules, i.e., free radicals and quinone species. We observed an increased expression of the α -Synuclein in the striatum which advances the

neurodegeneration. Our findings showed a significant down regulation of CREB in the corpus striatum of 6-OHDA infused rats. Even though cAMP level was increased, the CREB expression was decreased. Enhanced activation of the glutamate receptors leads to the production of second messengers. But its acute and prolonged action triggers the cell death pathways by activating pro apoptotic genes like Bax, bad and destabilizing jun- fos complex. The activation of apoptotic pathways down regulates the CREB expression thereby blocking the cAMP signaling cascade in PD rats. These findings suggest that decreased CREB expression is the result of cell loss.

Astrocytes and microglia are thought to play a major role in brain inflammatory responses. Both of these cell types exhibit a reactive phenotype in association with neurodegenerative diseases as well as in response to neurotoxic insults. Cytokines as participants in the pathological processes underlying both neuronal and glial responses associated with PD. TNF- α is one of the most strongly implicated cytokines associated with PD. TNF- α is known to induce generation of reactive oxygen intermediates associated with necrotic cell death (Goossens *et al.*, 1995), and it also induces changes in mitochondrial ultrastructure and function (Larrick & Wright, 1990). Up regulation of TNF- α in 6-OHDA infused rat confirms the mitochondrial impairment in the striatum area. We have demonstrated that enhanced expression of TNF- α is associated with the earliest stages of damage in the 6-OHDA model of dopaminergic neurotoxicity. Moreover, using 5-HT, GABA and BMC in combination we showed complete protection against 6-OHDA-induced neurotoxicity. The early onset of TNF- α expression after 6-OHDA and the neuroprotective effect afforded to dopaminergic neurons by 5-HT, GABA and BMC in combination implicate that this has a potential upstream effect in reducing the neurodegenerative processes underlying PD.

Substantia nigra pars compacta

One division of the basal ganglia, the SN, consists of two major components, the SNpr and SNpc. The SNpr contains one of the populations of basal ganglia output neurons and the SNpc contains the dopaminergic nigrostriatal neurons which are involved in the modulation of the flow of cortical information through the basal ganglia. SNpc is one of the main output nuclei of the basal ganglia structures and as such plays an important role in the motor activity. DA neuronal systems, originating in the SNpc constitute one of the main actors of such an important role (Campusano *et al.*, 2002). Neuropathologic studies of PD suggested that patients with the earliest signs of disease have already lost as much as 50% of the pigmented dopaminergic neurons in the SN (Marsden, 1990). The principal pathological characteristic of PD is the progressive death of the pigmented neurons of the SNpc, the nigrostriatal DA neurons. Anatomical, behavioural, biochemical and electrophysiological studies have demonstrated an interaction between glutamatergic and dopaminergic systems at different levels of the SNpc. The SNpc represents the main output site of the basal ganglia and receives excitatory glutamatergic afferents mainly from the SN, cerebral cortex and pedunculo-pontine nucleus (Iribe *et al.*, 1999). Glutamate receptor-mediated excitotoxicity has been suggested to be a contributory factor in the degeneration of dopaminergic neurons in PD (Blandini *et al.*, 1996). The effects of glutamate in the SNpc are mediated by the two principal types of glutamate receptors, NMDA and metabotropic receptors (Hollmann & Heinemann, 1994). Fast synaptic transmission is proposed to be mediated by NMDA receptors and physiological evidence suggests that the stimulation of NMDA receptors is considered as a mechanism to modulate fast excitatory postsynaptic potentials (Standaert *et al.*, 1994). Our observation in the mgluR5, NMDAR1 and NMDA2B gene expression showed an up regulation in the SNpc, this is due to an imbalance between excitation and inhibition which leads to excessive activation of excitatory amino acid receptors, leading to various types of

neuronal damage. Previous studies demonstrate that GABA counteracts such neuronal hyperactivity (Globus *et al.*, 1991). We proved that GABA along with 5-HT and BMC reverse these abnormalities to near control level. This confirms that over activity of glutamatergic neurotransmission in the basal ganglia leads to the onset and pathogenesis of PD. Glutamate homeostasis around glutamatergic synapses is tightly regulated by two groups of glutamate transporters: glial glutamate transporters GLT1 (EAAT2) and GLAST (EAAT1) and neuronal glutamate transporter EAAC1. The present results indicate that reduction of GLAST which impair glutamate homeostasis around glutamatergic synapses in the SNpc and contribute to over-spills of glutamate in the system.

Glutamate receptor over activation in the SNpc results mitochondrial dysfunction and oxidative stress which “reset” the threshold for activation of apoptotic pathways in response to Bax and similar signals. Our analysis in the Bax gene expression gave an enhanced expression level which substantiates our observation. Previous studies on the role for Bax dependent pathway comes from the demonstration by Vila and colleagues that mice deficient in Bax are protected from the induction of apoptosis and loss of SN DA neurons in the MPTP model (Vila *et al.*, 2001). From our study we can confirm that glutamate mediated neuronal damage occurring in the SNpc of 6-OHDA lesioned rats is Bax dependent. Inflammatory processes that involve a host of cytokines have been shown to be associated with ongoing neuronal degeneration seen in several neurodegenerative diseases, including PD. Proinflammatory cytokines are known to play a role in mitochondrial impairment and oxidative stress; therefore, an inflammatory response serve as an integral feature of the mechanistic underpinnings related to the pathogenesis of PD (Hunot *et al.*, 2001). Previous studies showed an enhanced expression of the proinflammatory cytokine, TNF- α in association with glial cells in the SN of patients with PD (Krishnan *et al.*, 2002). Our results also support the earlier studies. We got an

increased TNF- α expression in the SNpc which confirms the role of inflammatory process in the PD progression.

CREB is a transcription factor which has various roles in development, learning, memory, plasticity, promotion and regulation of neuronal survival. CREB activated (phosphorylated) by a variety of signaling pathways. The most common and best elucidated is the cAMP-PKA pathway. Extracellular signals (ex: Hormones and neurotransmitters) activate heterotrimeric G-proteins, that directly stimulate adenylyl cyclase, which can then catalyze the production of cAMP. cAMP then leads to the activation of PKA, which dissociates into active catalytic subunits which diffuse into the nucleus and phosphorylate CREB (Stewart & William, 2008). In our study increased ROS production and apoptotic pathways in the 6-OHDA infused rats caused a reduced CREB expression level in the SNpc which promote the neuronal damage. Abnormal folding and aggregation of neuronal proteins in the brain has been extensively investigated as one of the central mechanisms leading to neurodegeneration in PD. Accumulation of misfolded α -synuclein has been proposed to be centrally involved in the disease (Masliah & Hashimoto, 2002). 6-OHDA infusion to the SNpc increased the gene expression of the α -synuclein in the region which leads to the misfolding and aggregation.

There is increasing interest in the transplantation of stem cells as a means of recovering function in individuals with neurodegenerative disease. Although substantial improvements result from the systemic administration of L-dopa or DA agonists, such pharmacological interventions do not address the aetiology of the disease, provide a permanent remedy or prevent progression of the degenerative process (Snyder & Olanow, 2005). Implantation of stem cells will provide a more constitutive and relevant solution. This realisation has prompted a renewed interest in stem cells, which serves as a replenishable source of cells for the treatment of neurodegenerative disorders. The success of the cell transplantation will depend on

the ability of the cells to replace those neurons lost as a result of the disease process in the DA-deficient striatum and reverse, at least in part, the major symptoms of the disease. Previous studies on PD animal models have examined functional recovery after cell implantation, employing behavioural testing followed by post-mortem histology to establish cellular efficacy (Lu *et al.*, 2005). A more recent study using MRI alone in PD rats, demonstrated the visualisation of implanted neural stem cells for several weeks with improved post-transplantation behavioural rotation (Yang *et al.*, 2006). Other reports have shown that implanted mesenchymal stem cells tracked by MRI for 50 days in a rodent model of stroke (Jendelova *et al.*, 2004) and oligodendrocyte progenitors were tracked for six weeks in a rodent model of demyelination (Bulte *et al.*, 2002). A clinically relevant strategy is to implant BMC that are constitutively capable of neural differentiation and cytokine secretion. Allowing the cells to develop within the PD-affected brains, yields cells whose phenotypes, numbers, locations and regulation are determined by the interplay of donor elements and the local host milieu. A consequence of such donor–host interaction would result in a more pertinent homeostasis. We hypothesized that the BMC-based approach might better mitigate some of the limitations of previous strategies, where pre-programmed partially differentiated cells did not provide functional recovery (Brederlau *et al.*, 2006). Furthermore, Autologous BMC to treat neurological disorders offers several unique advantages over other cell replacement therapies. Immunological reactions are avoided and it also bypasses ethical issues in the use of embryonic cells. They are also relatively easy to harvest in the clinic, with procedures used routinely in bone marrow donations (Jackson *et al.*, 2009).

The success of this approach is hindered by the absence of methods that not only allow us to follow the fate of transplanted stem cells, but to monitor their efficacy non-invasively in the same subject. *In vivo* cell tracking techniques provide the most appropriate methodologies to achieve this goal in rats. Horan and Slezak

Discussion

(1989) developed the Paul Karl Horan (PKH dyes) which are lipophilic, fluorescent membrane intercalating to provide improved cell tracking capabilities and to allow a better understanding of various disease processes. Three different PKH dyes are used for labeling cells PKH2, PKH26 and PKH67. Several authors have described the use of PKH2GL dyes to study proliferation (Traktuev *et al.*, 2008). BMC were tagged with PKH2GL cell linker dye and it was infused individually and in combination stereotactically into the right SNpc. BMC differentiation analysis using primary antibody for nestin and GFAP proved that BMC differentiate to neuronal cells once the proper conditions are given. When autologous combination treatment was given to SNpc, they differentiated to both neuronal and glial cell types. PKH2GL tagged BMC when injected into the brain it started expressing both nestin and GFAP. The BMC observed *in vitro* changed its morphology once administered to SNpc within 12 days. The BMC differentiation was increased when it was administered along with 5-HT and GABA later differentiating to neurons *in vivo*. This was confirmed with the nestin gene expression analysis in the SNpc region in which it showed a maximum expression in the combinational groups compared to control rats. Our results confirmed the 5-HT and GABA comitogenic effect in proliferation and differentiation of the BMC to neurons in the brain by confocal studies using PKH2GL, Nestin and GFAP. 5-HT and GABA are involved in a variety of cellular processes involved in regulating metabolism, proliferation and morphology. The fine integration of these dynamic events appears to involve multiple receptor action.

Studies by Li *et al.*, (Li *et al.*, 2005) showed that neural transplants into the CNS lead to a marked activation of astrocytes. GFAP is an intermediate filament protein that is known to be localized to astrocytes (Maragakis & Rothstein, 2006). Up regulation of GFAP is the hallmark of reactive astrocytes (Eddleston & Mucke, 1993). We demonstrated here that the transplantation of BMC activate the migration of astrocytes to the SNpc. Our study using PKH2GL and GFAP confirmed the astrocyte

migration in all the experimental groups. This was further proved by gene expression studies of GFAP which also showed an increased expression in 6-OHDA infused rats and the expression were further enhanced when the rats treated with BMC individually and in combination with 5-HT and GABA. In addition to providing structural and trophic supports for neurons, astrocytes are known to modulate the local environment around neural stem cells (Agulhon *et al.*, 2010). Recent studies proved that these cells also release signalling molecules that helps in neuronal communication (Doetsch, 2003). Transplantation of adult neural progenitor cells into different brain regions has revealed neurogenic and non-neurogenic niches, underscoring the influence of environment on the fate of grafted cells (Dziewczapolski *et al.*, 2003). Astrocytes from neurogenic regions of adult brain provide permissive or inductive environmental cues directing differentiation of neural progenitor cells (Song *et al.*, 2002). Migrated astrocytes in our study made connections with the transplanted cells and helped its differentiation and proliferation. Our findings firmly support that the BMC infusion into the SNpc creates an environment which leads to the migration of astrocytes to the SNpc. These astrocytes are playing a crucial role in the differentiation and development of the transplanted BMC.

For confirming whether the transplanted cells can reverse back the normal DA production we checked the DA content analysis using HPLC, Tyrosine hydroxylase (TH) activity using immunohistochemistry and gene expression. A consistent neurochemical abnormality in PD is degeneration of dopaminergic neurons in SN, leading to a reduction of striatal DA levels. In our study 6-OHDA infusion into the SNpc region leads to a reduced DA content in the PD rats. TH catalyses the formation of L-DOPA, the rate-limiting step in the biosynthesis of DA, the disease can be considered as a TH-deficiency syndrome of the striatum. Like other cellular proteins, TH is also a possible target for damaging alterations induced by ROS (Haavik & Toska, 1998). 6-OHDA infusion severely reduced the number of TH-immunopositive

neurons in the SNpc compared to the control group. Confocal analysis confirmed with the gene expression study which showed a reduction in the TH expression in 6-OHDA infused rats. 5-HT, GABA along with BMC treatment reversed this alteration which indicates that the differentiated BMC is able to synthesise DA.

Cerebral cortex

Changes in personality and moderate or mild cognitive debilitation are found in PD. Cerebral glucose metabolism is reduced in the cerebral cortex in PD patients suffering from cognitive impairment (Yong *et al.*, 2007). Metabolic and neuroimaging observations have recently documented decreased prefrontal and parietal 18F-fluorodeoxyglycose uptake in PD cases with mild cognitive deficits (Huang *et al.*, 2007, 2009). Studies have demonstrated that complex I deficiency and abnormal ATP synthase and inner protein membrane prohibiting expression levels in the frontal cortex in PD (Parker *et al.*, 2008). The cerebral cortex receives widespread inputs from subcortical areas involved in sensorimotor and limbic functions. The integration of these glutamatergic inputs is essential for the cerebral cortex role in executive functions and goal-directed behaviour (Miller, 2000). It is also well known that cerebral cortex activity is shaped by a number of neuromodulators, most notably monoamines. Among these, DA stands out as having an important role in cerebral cortex cognitive functions, including working memory, reward, and attention (Schultz, 2002). Several reports have highlighted the need of DA–glutamate coactivation for a number of cortical functions (Gurden *et al.*, 1999, Baldwin *et al.*, 2002).

Glutamate neurotransmission plays an integral role in basal ganglia functioning especially in the striatum, where the balance of glutamate and DA is critical but also in the SN which receives glutamatergic input from the subthalamic nucleus and cortex. Glutamatergic pathways also play a leading role in the structural and functional organization of the cortico-basocortical loops involved in PD (Hirsch *et al.*, 2000). In addition, using morphological criteria, an 88% increase in glutamatergic

perforated synapses was reported in the putamen of PD patients (Anglade *et al.*, 1996). Increased glutamate content in the cerebral cortex of 6-OHDA infused rats leads to the up regulation of total glutamate and NMDA receptors. This was confirmed by the gene expression studies of mGluR5, NMDAR1 and NMDA2B, where it showed an up regulation in 6-OHDA infused rats compared to control. The extracellular concentration of the glutamate in the CNS must be kept low to ensure a high signal to noise ratio during synaptic activation and to prevent excitotoxicity due to excessive activation of glutamate receptors (Katagiri *et al.*, 2001). Glutamate uptake into neurons and glial cells is important for the termination of glutamatergic transmission. They are essential for the maintenance of low extracellular levels of glutamate. We observed a reduced expression of GLAST in 6-OHDA infused rats. The decreased glutamate transporter GLAST expression reduces the reuptake of the extracellular glutamate.

Nitric oxide synthesis can affect guanylyl cyclases and thus exert control on cGMP production. It has also been shown that it down regulates NMDA channels and thus reduces Ca^{2+} flow into the cytosol. We obtained decreased cGMP content in the cerebral cortex of 6-OHDA infused rats which is due to NMDA receptor activation and intracellular Ca^{2+} accumulation. All of glutamate receptors couple positively to phospholipase C through guanine nucleotide binding proteins whereby they stimulate phosphoinositide hydrolysis generating a second messenger cascade consisting of diacylglycerol and inositol 1,4,5 trisphosphate (Berridge, 1987). Jo *et al.*, (2008) demonstrated that NMDA and mGluR receptors mediate Ca^{2+} release by stimulating IP3 and PKC. β_1 -adrenoceptors are highly expressed in PD which induced the up-regulation of cAMP/PKA signaling (Hara *et al.*, 2010). In our studies we observed an elevated cAMP and IP3 level in the cerebral cortex of 6-OHDA induced rats. The elevated IP3 level causes extra cellular release of Ca^{2+} , which in turn enhanced metabolic stress on mitochondria that leads to excessive oxidative phosphorylation

Discussion

and increased production of reactive oxygen species. If the matrix Ca^{2+} level rises too high, then deleterious changes in mitochondrial structure occur. In particular, mitochondria can swell and rupture or undergo permeability transition, thereby releasing several pro-apoptotic factors into the cytoplasm, such as cytochrome C, second mitochondrial activator of caspases (SMAC/Diablo) or apoptosis-inducing factor (AIF) (Orrenius *et al.*, 2003). Our study showed an increased activity of Bax gene expression in the cerebral cortex of the 6-OHDA infused rats which indicated the ROS mediated neurodegeneration in the cerebral cortex. Bax, one of the major pro-apoptotic family members, exerts its effects by compromising the membrane integrity leading to leakage of apoptogenic factors such as cytochrome c into the cytosol, resulting in caspase-3 activation and demise of the cell (Shacka & Roth, 2005). At the same time the CREB gene expression was down regulated in the 6-OHDA rats. This indicates the interruption of the cAMP signalling cascade in the PD rats due to enhanced pro apoptotic factors which further enhances the neuronal death.

Abnormal brain mitochondrial function; oxidative damage on DNA, RNA and proteins; abnormal stress responses; modifications of crucial PD related proteins; and subcellular redistribution of proteins flow into one another and potentiate metabolic damage. Thus abnormal mitochondria function is a source of reactive oxygen species and oxidative stress, at the same time that they promote oxidative damage of glycolysis and energy metabolism-related proteins, mitochondrial proteins such as parkin and DJ1 and proteins involved in stress responses such as SOD1 and SOD2. Enhanced of α -synuclein in the cerebral cortex are the seed of abnormal folding, oligomerization and aggregation. In the central nervous system, TNF- α is produced by brain-resident astrocytes, microglia, and neurons in response to numerous intrinsic and extrinsic stimuli. TNF- α induces neuronal apoptosis though an excitotoxic mechanism mediated through glutamate receptors especially AMPA receptors (Gelbard *et al.*, 1993) and activates microglia, which leads to the propagation of

various neurological diseases (Hanisch *et al.*, 2002). Gene expression studies of TNF- α in 6-OHDA infused rats showed an increased production which resulted in significant enhancement of Ca²⁺ signals downstream of glutamatergic stimulation. An increase in IP3 content positively correlated with this alteration in Ca²⁺ homeostasis. Modulation of Ca²⁺ responses arising from these receptor subtypes and its downstream effectors have exact significant consequences on neuronal function and underlie the compromise in neuronal activity observed in the setting of chronic neuroinflammation associated with PD. Based on these observations we confirm that irregular mood and behaviour and cognitive deficits observed even at early stages PD is the result of these molecular abnormalities (Nandhu *et al.*, 2011).

Hippocampus

DA containing neurons participate in the regulation of certain cognitive processes (Prediger *et al.*, 2011). Cognitive impairments are observed in Parkinson's disease patients, especially on measures of memory, verbal fluency and other executive functions (McPherson & Cummings, 1996). Research suggests that the behavioural, motor and cognitive impairments found in Parkinson's disease patients reflect dysfunction of hippocampal neural circuitry (Saint-Cyr, 2003). Additionally, cognitive and motor problems contribute to the existence of depression in Parkinson's disease and conversely, symptoms of depression impact cognitive and motor deficits (Robinson *et al.*, 2000). The hippocampal formation contains a rich glutamatergic and GABA-ergic input, GABA-ergic interneurons containing peptide co-transmitters and the glutamatergic perforant pathway interconnects with entorhinal cortex, subiculum, CA1, CA3 fields and dentate gyrus (Ottersen & Storm-Mathisen, 1984). Potentiation, defined as an increase in synaptic efficacy, is readily induced by high frequency stimulation (HFS) of the synapses between the Schaffer collaterals and the pyramidal cells in the hippocampus CA1 area (Malenka & Nicoll, 1999). The excitatory synapse

in the stratum radiatum of the CA1 area of the hippocampus has a number of features that have been attributed to various aspects of memory encoding (Martin *et al.*, 2000).

Heightened responsiveness to the excitatory neurotransmitter glutamate and associated excitotoxicity has been implicated in the pathogenesis of PD (Dawson & Dawson, 2003). In the CNS, glutamate is an important factor for maintaining Ca^{2+} homeostasis; it is the most abundant excitatory neurotransmitter and it is widely distributed. Glutamate is associated with various brain functions, such as synaptic plasticity, learning and long-term potentiation (Collingridge & Singer, 1990; Groth *et al.*, 2011). In this study, we focused on the glutamate receptor, which is abundantly expressed throughout the hippocampal formation which showed an increased glutamate content in the hippocampus of 6-OHDA rats compared to control. Our findings also report an increase in total glutamate and NMDA receptors function in the hippocampus with no significant change in K_d . This increased B_{max} observed shows the increased number of receptors with no change in the affinity of the receptors which was shown from the K_d . The increased receptor activity observed from the Scatchard plot was supported by the gene expression studies of NMDAR1, NMDA2B and mGluR5 glutamate receptor subtypes. Based on extensive supportive experimental data, the release of high levels of glutamate by neurons is thought to be the underlying mechanism for the initiation of neurodegeneration. The immunohistochemistry experiments in the present work supported the gene expression studies of NMDAR1, NMDA2B and mGluR5 receptors. This up regulation will increase the glutamate receptor activity and molecular cascades inside the cells. Other studies showed that mGluR5 blockade ameliorates motor abnormalities induced by lesions of the nigrostriatal dopaminergic system, or by dopaminergic receptor antagonists in animal models of PD (Dekundy *et al.*, 2006). The major issue in the treatment of PD has been the occurrence of abnormal involuntary movements (AIMs) resulting from chronic L-dopa treatment of Parkinsonian humans. The AIMs,

occurring in up to 80% of chronically l-DOPA-treated Parkinson's disease patients, are commonly referred to as l-DOPA-induced dyskinesia. Evidences suggest that mGluR5-mediated neurotransmission is involved in the pathogenesis of this disorder (Lundblad *et al.*, 2002). Our experiments also support the same. 5-HT and GABA are playing a direct role in decreasing the activated glutamate receptors. We also obtained a decreased expression of GLAST glutamate transporter in the hippocampus of experimental rats compared to control. This decreased expression of glutamate transporter will lead to the decreased clearance of glutamate from the extracellular space and we report in our present study that glutamate content is high in the hippocampus of experimental group compared to control. Up regulation of NMDA receptor and down regulation of glutamate transporter expression suggests a response to altered synaptic glutamate levels (Lyon *et al.*, 2008). It was found that GLAST glutamate transporter down regulation is involved in cell swelling in hippocampus (Ouyang *et al.*, 2007).

The cyclic nucleotides cAMP and cGMP are involved in a number of intracellular processes such as signal transduction, gene transcription, activation of kinases, and regulation of channel function (Burns *et al.*, 1996). Investigations of the cAMP content in the hippocampus of 6-OHDA rats revealed a significant increase when compared to control. At the same time the cGMP content was down regulated in the 6-OHDA rats. This rise in cAMP could be mainly due to the influence of increased mGLU5 receptors as is seen during PD rats. Treatment with 5-HT, GABA and BMC in combination reversed these alterations to near control. NMDA receptors mediate their function through the IP3 release. In our study the IP3 content increased in the brain stem of 6-OHDA infused rats. IP3 receptor activation leads to excessive Ca²⁺ overload in cells leading to apoptosis. Bax is a pro-apoptotic protein allowing apoptosis to occur through the intrinsic, damage-induced pathway and amplifying that one occurring through the extrinsic, receptor mediated pathway. Bax is present in

Discussion

viable cells and activated by pro-apoptotic stimuli. Bax has multiple functions: it releases different mitochondrial factors such as cytochrome c, SMAC/diablo; it regulates mitochondrial fission, the mitochondrial permeability transition pore; it promotes Ca^{2+} leakage through ER membrane (Ghibelli & Diederich, 2010). The expression of proapoptotic protein Bax can be taken as an index of cell death. Increased Bax gene expression in the 6-OHDA infused rats confirmed the hippocampal neuronal damage. α -synuclein has been identified as the main protein constituent of Lewy bodies. Our study in 6-OHDA infused rats showed increased expression of α -synuclein which makes it more prone to aggregation. Caspase-8 is a proximal effector protein of the TNF-receptor-family death pathway and a significantly higher percentage of dopaminergic neurons displaying caspase-8 activation were observed in PD patients than in controls (Hartmann *et al.*, 2001). Our studies also support the earlier reports. We obtained an increased TNF- α expression which further enhanced the neuronal damage.

CREB is activated in response to cAMP and this mechanism of activation is well-characterized. cAMP accumulates in the cytoplasm in response to stimulation of membrane (G)-protein coupled receptors and stimulates the dissociation of the protein kinase A (PKA) heterotetramer, which consists of a pair of regulatory and a pair of catalytic subunits. Once liberated, the catalytic subunits are free to enter the nucleus by passive diffusion where they phosphorylate CREB on its Ser-133 residue and gene expression is then induced. But in our study increased cAMP content didn't influence on the CREB which showed a decreased expression. It is interesting to note that down regulation of CREB gene expression observed in 6-OHDA infused rats supporting a potential role for impaired nuclear import of phosphorylated signaling proteins in neuronal injury processes. Our evaluation in the hippocampus proves that glutamate over activity as a consequence of striatal DA depletion has a prominent role in the cognitive deficits in PD rats; in addition to motor dysfunction. 5-HT and GABA along

with BMC acting through its receptors ameliorating hippocampal dysfunction occurs in 6-OHDA rats.

Cerebellum

The basal ganglia and cerebellum are two groups of subcortical nuclei that have classically been regarded as motor structures. Damage to these brain regions produces well-described alterations in motor function. Cerebellar output abnormalities affects not only in the primary motor cortex but also subdivisions of premotor, oculomotor, prefrontal and infero temporal areas of cortex. In PD patients, increased activity was seen in the cerebellar vermis. Two distinct motivational systems in the brain have been described: the DA-dependent cortico-striatal-thalamocortical circuits and the DA independent brain stem pedunclopontine tegmental nucleus, which has strong interconnections to the cerebellum. Parkinson's patients utilize the latter circuit when the former has been damaged (Weintraub & Potenza, 2006).

Glutamate is the major excitatory neurotransmitter in vertebrate CNS, but inadequate regulation of extracellular glutamate and glutamate receptor agonists cause toxicity in the nervous system (Greene & Greenamyre, 1996). Glutamate neurotransmission is a paradox in that it is vital for essentially all excitatory synaptic transmission in the CNS, but excessive stimulation of glutamate receptors is toxic to neurons. This phenomenon, known as excitotoxicity, has been intensely studied and implicated as a potential pathogenic factor in a number of neurologic diseases, including PD. Studies have shown the involvement of NMDA receptor subunits-NMDAR1, NMDA2B in the cerebellum in motor learning of the mouse (Jiao *et al.*, 2008). Experimental evidence indicate the involvement of the cerebellum in variety of human mental activities including language (Fiez *et al.*, 1996), attention (Allen *et al.*, 1997), cognitive affective syndromes (Schmahmann & Sherman, 1998), fear and anxiety caused by threats of pain, thirst sensation and fear for air hunger and motor relearning (Imazumi *et al.*, 2004, Marvel *et al.*, 2004).

Discussion

Increased glutamate content in the 6-OHDA infused rats leads to the up regulation of total glutamate receptors. There was no significant change in K_d in all experimental groups. Glutamate regulates neuronal activity by acting on ionotropic and mGluRs. The mGluR5 receptors, in particular, have received considerable attention in PD research, being major players, for example, in the excitatory drive to the SN from glutamatergic afferents. We observed an increase in total glutamate and NMDA receptors function in the cerebellum of 6-OHDA infused rats. This was supported by the real time PCR analysis which showed an increased receptor gene expression of NMDAR1, NMDA2B and mGluR5 in the PD rats. The extracellular concentration of the glutamate in the CNS must be kept low to ensure a high signal to noise ratio during synaptic activation and to prevent excitotoxicity due to excessive activation of glutamate receptors. Excess activation of NMDA receptors by glutamate increases cytoplasmic concentrations of Na^{2+} and Ca^{2+} to levels that exceed the capacity of neuronal homeostatic mechanisms, thereby altering transmembrane ion gradients. As with most classic neurotransmitters, glutamate is specifically concentrated into synaptic vesicles in nerve terminals, released during depolarization in a Ca^{2+} -dependent manner and inactivated by reuptake. However, unlike classic transmitters, glutamate uptake by glial cells is significant. Within glia, glutamate is transaminated by glutamine synthetase to form glutamine, which diffuses into nerve terminals and is converted. As seen in the other brain regions we observed a decreased gene expression of GLAST glutamate transporter in the cerebellum of 6-OHDA infused rats compared to control. This decreased expression of glutamate transporter will lead to the decreased clearance of glutamate from the extracellular space.

The activation of mGluR5 receptors leads to the potentiation of NMDA currents, possibly through the activation of protein kinase C and the subsequent increase in intracellular Ca^{2+} , thereby acting as an indirect agonist of NMDA receptors. NMDA receptor activation in the cerebellum leads to an increase in the

Ca²⁺ also through IP3 receptors. Glutamate stimulates adenylyl cyclase through a G protein to increase cAMP formation and the activity of cAMP-dependent protein kinase (protein kinase A, PKA) leads to phosphorylation of DARPP-32 on a single threonine residue. Our results also showed an increase in the IP3 and cAMP content and decreased cGMP content in the cerebellum of 6-OHDA infused rats. Group I mGluRs couple positively to phospholipase C, the activation of which leads to stimulation of protein kinase C and release of intracellular Ca²⁺, or to adenylyl cyclase, activation of which stimulates cAMP formation. Over activation of NMDA receptors causes excessive influx of Ca²⁺, and consequent production of damaging free radicals together with activation of proteolytic processes that contribute to neuronal injury and cell death. Up regulation in the glutamate receptor, IP3 and cAMP activity increased the intracellular Ca²⁺ which caused enhanced metabolic stress on mitochondria that leads to excessive oxidative phosphorylation and increased production of ROS. Low levels of ROS are important for many life-sustaining processes of cells and tissues, but they induce cell damage and death at higher levels. Our study showed an increased activity of Bax gene expression in the cerebellum of the 6-OHDA infused rats which indicated the ROS mediated neurodegeneration in the cerebellum. CREB-dependent gene expression has been reported to play a role in such diverse processes as cell survival, plasticity, growth and development, and most recently, cell death. CREB is controlling neuronal survival, in part, by controlling transcription of neuroprotective genes. For example, the promoter regions for both BDNF and the anti-apoptotic protein, Bcl2, each contain CRE sites and both of these gene products have been shown to play an important role in neuronal survival. In the present study the gene expression of CREB was down regulated in cerebellum of 6-OHDA compared to control. Even though cAMP level was increased, the CREB expression was decreased.

TNF- α plays an important role in the neurodegeneration of PD this is supported by the finding previous finding that that there is an increase in the number of melanized SN neurons expressing the activated form of caspase-8, demonstrated with an antibody specific for the cleaved form, in PD brains as compared to age-matched controls (Hartmann *et al.*, 2001). Our experiment showed an increased gene expression of TNF- α which supports the earlier reports. Neurons that degenerate in PD accumulate cytoplasmic inclusion bodies composed of α -synuclein referred to as Lewy bodies. Increased α -synuclein gene expression proves that 6-OHDA infusion will lead to massive cell loss, particularly neuronal loss, along with other pathologic processes accompanying neurodegeneration that will influence its expression. Enhanced activation of the glutamate receptors leads to the production of second messengers. The activation of apoptotic pathways down regulates the CREB expression thereby blocking the cAMP signaling cascade in PD rats. Activation during development, as well as during times of stress is critical for determining neuronal fate, opening up the possibility that disruption of this important signaling pathway would have detrimental consequences. Thus the up regulation of glutamate receptor activity in the cerebellum caused the increase in second messengers which mediates the Ca^{2+} overload in the cells, leading to neuronal damage. The receptor analysis and gene expression studies along with the behavioural data implicate a role for glutamate, NMDA and mGluR5 receptors in the modulation of neuronal network excitability through changes in IP3 and cAMP. These neurofunctional deficits are one of the key contributors to motor abnormalities associated with PD. 5-HT and GABA along with BMC reversed these effects by functional recovery (Nandhu *et al.*, 2011b).

Brain stem

There have been changes in our understanding of the pathology of the disease, where it begins in the nervous system and how it progresses. Indeed, there is now evidence to suggest that the PD does not affect the dopaminergic SNpc at the earliest

stages but instead begins in the lower brain stem, olfactory bulb and anterior olfactory nucleus. The brain stem is a part of the brain located beneath the cerebrum and in front of the cerebellum. It connects the spinal cord to the rest of the brain. The brain stem controls involuntary muscles such as the stomach and the heart. The brain stem also acts as a relay station between the brain and the rest of the body. Previous studies on deep brain stimulation at pedunculopontine nucleus (PPN) in the upper brain stem to the patients having PD with gait disturbance showed a significant improvement in gait, over 50%, which opens a whole new approach of the involvement of brain stem in PD (Plaha & Gill, 2005). Sections of the brain stem usually reveal loss of the normally dark black pigment in the locus ceruleus (LC), pigmentation that correlates with neuronal cytoplasmic neuromelanin pigment that accumulates in an age-related manner. Loss of pigment correlates with neuronal loss and with the duration of Parkinsonism. In our study total glutamate and NMDA receptors of the brain stem are found to be increased in 6-OHDA infused rats. 5-HT and GABA along with BMC treated PD rats, binding parameters were reversed back to near control values. The up regulation in the receptor expression is due to the increased Glutamate content which we observed in the PD rats. Depletion in the DA content in the SNpc will increase the glutamate firing rate into the brain stem. Neurophysiological studies have implicated overactivity at brain stem, due to altered glutamatergic neurotransmission, as one underlying mechanism for the development of motor impairment and L-dopa related motor complications in PD (Soares *et al* ., 2004; Wichmann & Soares, 2006). Receptor binding analysis was confirmed with immunohistochemistry and real time PCR analysis which showed an up regulation in m-GluR5, NMDAR1 and NMDA2B expression. Reduced gene expression of the transporter GLAST indicates the accumulation of glutamate in the region.

The signaling from the neurotransmitters is carried to the cell nucleus by second messengers like cAMP, cGMP and IP3. Their expression and changes play a

Discussion

major role in the signaling cascade. The glutamatergic receptor stimulation leads to activation of PLC, which in turn hydrolyses phosphatidylinositol 4, 5-bisphosphate (PIP₂) to produce IP₃ and diacylglycerol (DAG). Increased IP₃ and cAMP content in the brain stem is due to the over activation of these receptors. The elevated IP₃ and cAMP level causes intra cellular release of Ca²⁺, which in turn results in the activation of apoptotic pathways. cGMP mediates physiological effects in the cardiovascular, endocrinological, and immunological systems as well as in CNS. In the CNS, activation of the NMDA receptor induces Ca²⁺-dependent Nitric-oxide synthase (NOS) and Nitric-oxide release, which then activates soluble guanylate cyclase for the synthesis of cGMP. Both compounds appear to be important mediators in long-term potentiation and long-term depression and thus play an important role in the mechanisms of learning and memory. Although we obtained an increased NMDA expression, the cGMP level in the brain stem was significant reduced in 6-OHDA infused rats. Impaired energy metabolism resulting from mitochondrial dysfunction has been proposed to render cells vulnerable to excitotoxicity. Mitochondrial dysfunction could therefore potentially result in a lowering in the threshold for excitotoxic injury. This excitotoxic injury increases free radical generation and add to cellular injury. Mitochondria also play an integral role in the apoptotic cell death pathway. When the outer mitochondrial membrane is permeabilized by action of “death agonists” such as Bax, cytochrome c is released into the cytosol, leading to caspase activation and apoptosis. Up regulated Bax expression in the brain stem confirms the neuronal damage in the brain stem after the 6-OHDA infusion.

6-OHDA treatment results in impaired nuclear import of CREB which accumulate in the cytoplasm. Given the increasing reports of signaling proteins and transcription factors accumulating in the cytoplasm of degenerating neurons, strategies to bypass potential translocation deficits will aid in the development of new strategies for PD treatment. CREB gene expression studies in the brain stem showed a

decreased activity in the 6-OHDA infused rats. Treatment with 5-HT, GABA and BMC in combination reversed this alteration which indicates its ability to improve the translocation deficits occurs in the PD rats. Microglia-mediated neuroinflammation has been hypothesized to play an important role in the pathogenesis of PD, primarily based on findings from postmortem studies and animal experiments (McGeer *et al.*, 2004). Consistently, concentrations of proinflammatory cytokines such as interleukin (IL)-1b, IL-2, IL-6 and TNF- α were elevated in the brain and cerebral spinal fluid of PD patients (Nagatsu *et al.*, 2005). Increased TNF- α expression as we got in 6-OHDA rats will directly influence the further enhancement of neuronal damage. At the ultra structural level Lewy bodies are composed of dense granular material and straight filaments approximately 10 to 15 nm in diameter (Galloway *et al.*, 1992). Similar filaments can be created in the test tube with recombinant α -synuclein, which is normally an unfolded and structure less protein (Crowther *et al.*, 2000). This fact, as well as the immunolocalization of α -synuclein to the filaments in tissue sections subjected to electron microscopy, indicates that the filaments in Lewy bodies are almost certainly derived from aggregates of α -synuclein that have an abnormal conformation. The presence of α -synuclein in cytoplasmic inclusions represents aberrant cytologic localization, since it is normally a protein enriched in presynaptic terminals. We obtained α -synuclein over expression in the 6-OHDA rats supporting the earlier studies. Overall observation in the brain stem comes to a conclusion that 5-HT and GABA along with BMC is directly helping in reversing back the glutamate receptor mediated abnormalities in PD and its antiglutamatergic action is useful in reducing the severity of the disease.

In conclusion, the present study demonstrated that unilateral lesion using 6-OHDA in to SNpc induces the glutamate receptor activity. Our molecular and behavioural results showed that 5-HT and GABA along with BMC potentiates a restorative effect by reversing the alterations in glutamate receptor binding and gene

Discussion

expression that occur during PD. 5-HT and GABA co-mitogenically induced BMC proliferation and differentiation to neurons. Thus, it is evident that 5-HT and GABA along with BMC to 6-OHDA infused rats renders protection against oxidative, related motor and cognitive deficits which makes them clinically significant for functional reestablishment and recovering from PD symptoms.

Summary

1. 6-OHDA infused unilateral Parkinson's disease rats were used as models to study the alterations in brain glutamatergic and NMDA receptors; second messengers - IP3, cAMP and cGMP; apoptotic factors - Bax, TNF- α ; intercellular protein - α -synuclein; transcription factor - CREB and their regulation by 5-HT, GABA and BMC individually and in combinations.
2. The body weight was analyzed to study the changes in body weight in 6-OHDA infused rats compared to control. Parkinson's disease induction in rats caused a reduction in the body weight and treatment combinations with 5-HT, GABA and BMC regained the body weight near to control and 5-HT, GABA and BMC supplemented alone showed no significant reversal in the body weight.
3. Behavioural studies: apomorphine induced rotational analysis, limb use asymmetry test, rotarod test, swim test, Y maze and radial arm test were conducted to assess the motor learning and memory in control and experimental rats. 6-OHDA infused rats showed a significant deficit in cognition, memory and motor learning. Rats treated with 5-HT, GABA and BMC in combinations reversed the behavioural response to near control. BMC treated alone showed no significant reversal in the behavioural deficits towards the control.
4. Glutamate content increased in the corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem of 6-OHDA infused rats compared to control. Individual treatment with 5-HT, GABA and 5-HT, GABA and BMC in combinations functionally reversed the alteration to near control. BMC alone treated group did not show any significant reversal to control.

5. Glutamatergic receptor functional status was analysed by Scatchard analysis using [³H]glutamate. The total glutamate receptors in corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem of 6-OHDA infused rats were increased compared to control with no significant change in the K_d representing the affinity. Treatment with 5-HT, GABA individually and 5-HT, GABA and BMC in combinations restored the total glutamatergic receptors in brain regions near to control. There was no significant reversal in BMC alone treated rats.
6. NMDA receptor functional status was analysed by Scatchard analysis using [³H]MK801. The NMDA receptors in corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem of 6-OHDA infused rats were increased compared to control with no significant change in the K_d. Individual treatment with 5-HT, GABA and 5-HT, GABA and BMC in combinations functionally reversed the NMDA receptors to near control. BMC alone treated rats did not show any significant reversal to control.
7. Glutamate mediates its action through its receptor subunits – NMDAR1, NMDA2B, mGluR5. NMDA receptor binding parameters were confirmed by studying the mRNA status of the corresponding receptor using Real-Time PCR. NMDAR1, NMDA2B, mGluR5 receptors showed an increased expression in corpus striatum, cerebral cortex, hippocampus, cerebellum, brain stem and SNpc of 6-OHDA infused rats compared to control. The results showed a co-actvation of NMDA receptors subunits that affect glutamate mediated functions. This enhanced activity of NMDA receptors produce intracellular signals through activation of signaling pathways. Treatment with 5-HT, GABA individually and 5-HT, GABA and BMC in combinations reversed the receptor gene expression status to control. There was no significant reversal in BMC alone treated rats.

Summary

8. To prevent glutamate mediated excitotoxic effects it should be cleared from the extracellular space by the glutamate transporters. The gene expression of GLAST glutamate transporter was studied in control and experimental rats. GLAST glutamate transporter showed decreased expression in corpus striatum, cerebral cortex, hippocampus, cerebellum, brain stem and SNpc of 6-OHDA infused rats compared to control. The results showed less reuptake of extracellular glutamate formed in the diseased condition. Individual treatment with 5-HT, GABA and 5-HT, GABA and BMC in combinations functionally reversed the alteration in GLAST glutamate transporter gene expression to near control. BMC alone treated rats did not show any significant reversal to control.

9. Second messengers IP3, cAMP contents were increased and cGMP content was decreased significantly in corpus striatum, cerebral cortex, hippocampus, cerebellum, and brain stem of 6-OHDA infused rats compared to control. Individual treatment with 5-HT, GABA and 5-HT, GABA and BMC in combinations functionally reversed the alteration in Second messengers to near control. There was no significant reversal in BMC alone treated rats.

10. A significant up regulation of pro-apoptotic factors Bax and TNF- α was observed in the corpus striatum, cerebral cortex, hippocampus, cerebellum, brain stem and SNpc which indicated the mitochondrial dysfunction and apoptosis in 6-OHDA infused rats. Individual treatment with 5-HT, GABA and 5-HT, GABA and BMC in combinations reversed the gene expression to near control. BMC alone treated group did not show any significant reversal to control.

11. Increased gene expression of the α -Synuclein in the corpus striatum, cerebral cortex, hippocampus, cerebellum, brain stem and SNpc of 6-OHDA infused rats

advanced the neurodegeneration. Treatment with 5-HT, GABA individually and 5-HT, GABA and BMC in combination reversed α -Synuclein gene expression near to control. There was no significant reversal in BMC alone treated rats.

12. Transcription factor, CREB expression in the brain regions - corpus striatum, cerebral cortex, hippocampus, cerebellum, brain stem and SNpc showed a significant decrease in expression in 6-OHDA infused rats. Treatment with 5-HT, GABA individually and 5-HT, GABA and BMC in combinations reversed CREB gene expression status towards control values. There was no significant reversal in BMC alone treated rats.
13. The increased expression of NMDAR1, NMDA2B and mGluR5 receptors in 6-OHDA infused rats observed from the receptors analysis and Real-time PCR was confirmed by confocal studies using receptor specific antibodies in the brain slices. Treatment with 5-HT, GABA individually and 5-HT, GABA and BMC in combination reversed the mean pixel value towards the control. BMC alone treated group did not show any significant reversal to control.
14. We demonstrated using specific fluorescent cell tracking dye - PKH2GL to bone marrow cells and Nestin to premature neurons – the autologous differentiation of bone marrow cells to neurons. PKH2GL tagged BMC when injected into the SNpc started expressing nestin later differentiating to neurons *in vivo*. The BMC division and differentiation was increased when it was infused along with 5-HT and GABA. The prominent expression was seen in rats treated with 5-HT, GABA and BMC in combination. The Confocal studies were confirmed with the gene expression analysis of nestin in the SNpc in which the maximum expression was observed in the rats treated with 5-HT, GABA and BMC in combination.

Summary

15. We observed a marked activation of astrocytes in the SNpc. In the Confocal analysis the mean pixel value of GFAP in the SN showed an increase in 6-OHDA infused rats and the rats individually treated with 5-HT and GABA compared to control. Maximum expression of GFAP was observed in the rats treated with individual BMC treated group and combinational 5-HT, GABA and BMC treated groups. Activated astrocytes made connections with the transplanted BMC and helped its differentiation. Confocal analysis confirmed astrocytes migration into the SNpc region after BMC infusion. This was confirmed with the Real-time PCR analysis of GFAP which also showed the similar pattern of gene expression.

16. DA quantification, tyrosine hydroxylase analysis using confocal and Real-time PCR was done in the SNpc to confirm the DA production after BMC differentiation. 6-OHDA infusion reduced DA production and Tyrosine hydroxylase expression in SNpc. Treatment with 5-HT, GABA individually and 5-HT, GABA and BMC in combinations reversed DA content and Tyrosine hydroxylase expression near to control. BMC alone treated group did not show any significant reversal to control.

Our results showed that glutamate and NMDA receptor functional balance plays a major role in Parkinson's disease management. Gene expression studies of NMDAR1, NMDA2B, mGluR5 receptor subunits and GLAST glutamate transporter showed a prominent glutamatergic functional disturbance in brain regions of 6-OHDA infused rats. These findings have important implications for understanding the molecular mechanisms underlying motor, memory and cognitive impairment by second messengers, pro-apoptotic factors, intercellular proteins and transcription factors due to 6-OHDA infusion. The enhanced receptor activity and the second messenger cascades will lead to Ca^{2+} overload and thereby excitotoxic

neurodegeneration which affect the cognitive, memory and motor ability of the PD rats. Our results proved that the autologous BMC differentiate to neurons and glial cells when they are infused with 5-HT and GABA. The BMC transformed to neurons and glial cells with 5-HT and GABA, was confirmed with PKH2GL, nestin and GFAP. These newly formed neurons have functional significance in the therapeutic recovery of Parkinson's disease.

Conclusion

Parkinson's disease is a chronic progressive neurodegenerative movement disorder characterized by a profound and selective loss of nigrostriatal dopaminergic neurons. Our findings demonstrated that glutamatergic system is impaired during PD. The evaluations of these damages have important implications in understanding the molecular mechanism underlying motor, cognitive and memory deficits in PD. Our results showed a significant increase of glutamate content in the brain regions of 6-OHDA infused rat compared to control. This increased glutamate content caused an increase in glutamatergic and NMDA receptors function. Glutamate receptor subtypes- NMDAR1, NMDA2B and mGluR5 have differential regulatory role in different brain regions during PD. The second messenger studies confirmed that the changes in the receptor levels alter the IP₃, cAMP and cGMP content. The alteration in the second messengers level increased the expression of pro-apoptotic factors - Bax and TNF- α , intercellular protein - α -synuclein and reduced the expression of transcription factor - CREB. These neurofunctional variations are the key contributors to motor and cognitive abnormalities associated with PD. Nestin and GFAP expression study confirmed that 5-HT and GABA induced the differentiation and proliferation of the BMC to neurons and glial cells in the SNpc of rats. We also observed that activated astrocytes are playing a crucial role in the proliferation of transplanted BMC which makes them significant for stem cell-based therapy. Our molecular and behavioural results showed that 5-HT and GABA along with BMC potentiates a restorative effect by reversing the alterations in glutamate receptor binding, gene expression and behaviour abnormality that occur during PD. The therapeutic significance in Parkinson's disease is of prominence.

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Papers Published

- 1.** **Nandhu M S**, Jes Paul, Korah P Kuruvila, Pretty M Abraham, Sherin Antony and C. S. Paulose. Glutamate and NMDA receptors activation leads to Cerebellar dysfunction and impaired motor coordination in unilateral 6-hydroxydopamine lesioned Parkinson's rat: Functional recovery with bone marrow cells, serotonin and GABA. *Mol Cel Biochem* 2011 [Epub ahead of print].
- 2.** **Nandhu M S**, Jes Paul, Korah P Kuruvilla, Anitha Malat, Chinthu Romeo and C. S. Paulose. Enhanced glutamate, IP3 and cAMP activity in the cerebral cortex of Unilateral 6-hydroxydopamine induced Parkinson's rats: Effect of 5-HT, GABA and bone marrow cell supplementation. *J Biomed Sci* 2011. [Epub ahead of print]
- 3.** Anju T R, **Nandhu M S**, Jes P, Paulose C S. Insulin and triiodothyronine regulation of neonatal hypoxia: Role of glucose, oxygen and epinephrine supplementation. *Fetal and Ped Pathol* 2011. [Epub ahead of print]
- 4.** Sherin A, Peeyush KT, Najjil G, **Nandhu MS**, Jayanarayanan S, Jes P, Paulose CS. The effects of abnormalities of glucose homeostasis on the expression and binding of muscarinic receptors in cerebral cortex of rats. *Eur J Pharmacol* 2011 [Epub ahead of print]
- 5.** **Nandhu M S**, Najjil George, Smijin Soman, Jayanarayanan S and C. S. Paulose. Opioid system functional regulation in neurological disease management. *J Neurosci Res* 2010 88(15):3215-21.

6. Anitha M, **Nandhu MS**, Anju TR, Jes P, Paulose CS. Targeting glutamate mediated excitotoxicity in huntington's disease: Neural progenitors and partial glutamate antagonist - Memantine. *Med Hypotheses* 2010 76(1):138-40.
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8. Peeyush T Kumar, Sherin Antony, **Nandhu M S**, Jayanarayanan S, Naijil George, C. S. Paulose Vitamin D3 Restores Altered Cholinergic and Insulin Receptor expression in the Cerebral Cortex and Muscarinic M3 receptor expression in Pancreatic Islets of Streptozotocin Induced Diabetic Rats. *J Nutr Biochem* 2010 [Epub ahead of print]
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11. Joseph B, **Nandhu MS**, Paulose CS. Dopamine D(1) and D(2) receptor functional down regulation in the cerebellum of hypoxic neonatal

rats: Neuroprotective role of glucose and oxygen, epinephrine resuscitation. *Pharmacol Res* 2010 61(2):136-41.

12. Mathew J, Paul J, **Nandhu MS**, Paulose CS. Increased excitability and metabolism in pilocarpine induced epileptic rats: Effect of *Bacopa monnieri*. *Fitoterapia* 2010 81(6):546-51.
13. **Nandhu MS**, Fabia ET, Paulose CS. Dopamine D(1) Receptor Gene Expression Studies in Unilateral 6-Hydroxydopamine-Lesioned Parkinson's Rat: Effect of 5-HT, GABA, and Bone Marrow Cell Supplementation. *J Mol Neurosci* 2010 41(1):1-11.
14. Mathew J, Paul J, **Nandhu MS**, Paulose CS. *Bacopa monnieri* and Bacoside - A for ameliorating epilepsy associated behavioral deficits. *Fitoterapia* 2009 81(5):315-22.
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16. C S Paulose, P S John, Sreekanth R, Mathew Philip, Padmarag Mohan C, Jobin Mathew, Peeyush Kumar T, Jes Paul, Pretty Mary Abraham, Sherin Antony, Binoy Joseph, Anu Joseph, Ameer Krishnakumar, Anju T R, Reas Khan S, Santhosh Thomas K and **Nandhu M S**. Spinal Cord Regeneration and Functional Recovery: Neurotransmitter's Combination and Bone Marrow Cells Supplementation. *Current Sci.* 2009 25: 546-549.
17. Shilpa Joy, Najil George, **Nandhu M. S**, Jes Paul, C. S. Paulose GABA - Chitosan Nanoparticles Induced Hepatocyte Proliferation in Partially Hepatectomised Rats : Inositol

1,4,5 trisphosphate, Phospholipase C and Nuclear Factor κ B Functional Regulation. Small (Under review).

18. **Nandhu M S**, Jes Paul, Korah P Kuruvilla, P.S. John and C. S. Paulose. Bone marrow cells differentiation using neurotransmitter combination in unilateral 6-hydroxydopamine lesioned Parkinson's rat. J Neurochem (Under review).
19. **Nandhu M S**, Jes Paul, Korah P Kuruvilla, P.S. John and C. S. Paulose. Autologous Bone marrow cells transplantation promotes Astrocytes migration in unilateral 6-hydroxydopamine infused Parkinson's rat. Cel Mol Neurobiol (Under review).
20. **Nandhu M S**, Jes Paul, Korah P Kuruvilla, Anju T.R. and Shilpa Joy and C.S. Paulose. Glutamate receptor functional regulation: learning and memory deficit reversed in unilateral 6-hydroxydopamine induced parkinson's rat. Life Sciences (Under review).
21. Chinthu R, **Nandhu MS**, Korah PK, Jayanarayanan S and Paulose CS. Cholinergic receptor subtypes decreased regulation in cerebellum of spinal cord injured rats: Functional recovery with Serotonin, GABA and Bone marrow cells. Mol Cellular Neurosci (Under review).
22. Jayanarayanan.S, **Nandhu M.S**, Peeyush Kumar T, Naigil George & Paulose C.S. Enhanced NMDA receptor activity in the cerebral cortex of streptozotocin induced diabetic rats: effect of Vitamin D3 supplementation. Diabetologia (Under review).

Awards

1. **IBRO Travel Award** for Participating in 10th IBRO School of Neuroscience, in Indian Institute of Chemical Biology, Kolkata. (December 2008).
2. **IBRO Travel Award** for Participating in 4th Canadian School of Neuroscience, in University of Ottawa, Canada. (May 2010).

Abstracts Presented

1. **Nandhu. M. S**, Jes Paul, Korah P Kuruvilla and C S Paulose Enhanced NMDAR1, NMDA2B and mGluR5 receptors gene expression in corpus striatum of unilateral 6-hydroxy dopamine rat model: effect of serotonin, gamma amino butyric acid and bone marrow cell supplementation. Canadian Neuroscience meeting, Ottawa (May 2010).
2. **Nandhu. M. S**, Jes Paul, Korah P Kuruvilla and C S Paulose. Glutamate Receptor Up Regulation In cerebellum of Unilateral 6-Hydroxy Dopamine Rat Model: Effect Serotonin, Gamma Amino Butyric Acid And Bone Marrow Cell Supplementation. International Conference on Neurosciences updates & ISN, APSN, IBRO & SNCI school, Cochin (December 2009).
3. **Nandhu. M. S**, Jes Paul, Korah P Kuruvilla and C S Paulose. Dopamine D1 Receptor up Regulation in Cerebellum and Brain Stem in Unilateral 6-Hydroxy Dopamine Rat Model: Antagonism By Serotonin And Gamma Amino Butyric Acid. Conference of Association of Clinical Biochemists of India, Amritha University, Cochin (November 2009)

4. C.S. Paulose, Jes paul, and **Nandhu M.S** Dopamine D1 Receptor Down Regulation In Corpus Striatum And Upregulation In Cerebral Cortex In Unilateral 6-Hydroxy Dopamine Rat Model: Antagonism By Serotonin And Gamma Amino Butyric Acid. Annual meeting of Society for Neuroscience, Chicago (October 2009).
5. **Nandhu.M.S** and C.S Paulose. Down regulation of GABA receptor in the cerebellum of spinal cord injured rats: Treatment with Neurotransmitters combination and Bone marrow cells. 10th IBRO School of Neuroscience, Kolkata (December 2008).
6. Sherin Antony, **Nandhu.M.S** and C.S Paulose, Altered Muscarinic M1 and M3 Receptor Gene Expression In Corpus Striatum of Diabetic and Insulin induced Hypoglycemic Rats, International Conference on Advances in neuroscience &XXXVI Annual Meeting of Indian academy of Neuroscience. Cochin (December 2008)
7. Akash K.G, **Nandhu.M.S** and C.S Paulose, Increased binding [³H]YM-09151-2 to DA D2 and decreased ALDH function in the cerebellum of ethanol treated rats, International Conference on Advances in neuroscience &XXXVI Annual Meeting of Indian academy of Neuroscience. Cochin (December 2008)
8. Peeyush Kumar T, **Nandhu.M.S** and C.S Paulose, Altered Muscarinic M3 Receptor Gene Expression In Cerebral Cortex of Diabetic Rats: Supplementation of Vitamin D3. Annual meeting of Society of Biotechnologist India, Chennai (October 2008).