Discussion

Parkinson’s disease (PD) is a progressive motor system disorder characterized by selective degeneration of dopaminergic neurons in substantia nigra pars compacta (SNpc) leading to marked reduction of dopamine (DA) levels in the striatum. Mitochondrial dysfunction in the brain leads to oxidative stress within neurons and cause conditions conducive to their degeneration, culminating in neurodegenerative diseases such as PD. Studies on post-mortem brain substantia nigra pars compacta (SNpc) region and platelets from the patients have suggested a general, significant impairment of mitochondrial function in PD (Mizuno, et al., 1989; Schapira, et al., 1989; Sheehan, et al., 1997). Administration of rotenone, a well-characterized, high-affinity, specific inhibitor of complex-I of the inner mitochondrial membrane involved in oxidative phosphorylation (Schuler & Casida, 2001; Venda et al., 2011) has been demonstrated convincingly to produce nigrostriatal dopaminergic neurodegeneration, as well as behavioural and neuropathological hallmarks of PD in rodents. Intrajugular and subcutaneous infusion of rotenone resulted in the selective destruction of the nigrostriatal dopaminergic system, formation of cytoplasmic inclusions in nigral neurons and induction of hypokinesia and rigidity in rats (Betarbet et al., 2000; Sherer et al., 2003). Rotenone-induced selective toxicity to dopaminergic neurons in SN has been attributed to the inhibition of the complex-I activity in the mitochondrial respiratory chain and the unique vulnerability of dopaminergic neurons to oxidative damage as a result of mitochondrial enzyme inhibition (Greenamyre et al., 1999; Jenner, 2001).

Chronic intraperitoneal injections and acute intranigral or median forebrain bundle infusion of rotenone reproduced most of the neurochemical, neuropathological and behavioural features of PD in rats (Heikkila et al., 1985; Alam & Schmidt, 2002; Alam et al., 2004; Sindhu et al., 2005; Saravanan et al.,
Studies have shown that rotenone can affect other basal ganglia structures in addition to striatal neurons (Ferrante et al., 1997; Hoglinger et al., 2003). This model reproduced several of the neurochemical and neuropathological symptoms of PD and indicated that there is involvement of oxidative stress in the specific dopaminergic neuronal degeneration. ROS production following rotenone exposure has been demonstrated in cell lines and organotypic cultures (Li et al., 2003; Sherer et al., 2003a; Testa et al., 2005). Chronic rotenone administration shown to increase nitric oxide levels and lipid peroxidation (Bashkatova et al., 2004). The potent parkinsonian neurotoxin, MPP+ has also been shown to inhibit complex-I (Nicklas et al., 1985; Ramsay et al., 1986; Gluck et al., 1994), generating free radicals including superoxide anions, nitric oxide and •OH (Zang & Mishra, 1992; Jenner et al., 1992; Wu et al., 1993; Mohanakumar & Steinbusch, 1998; Fabre et al., 1999; Cassarino and Bennett, 1999; Mohanakumar et al., 2002) and damage the endogenous antioxidant machinery in the brain (Ferraro et al., 1986; Yong et al., 1986; Muralikrishnan & Mohanakumar, 1998; Thomas & Mohanakumar, 2003).

Studies on post-mortem brains of PD patients suggest that dopaminergic neurons in SNpc region experience a delicate state of oxidative stress, as indicated by an increase in the content of oxidized proteins, DNA and lipids (Dexter et al., 1991; Alam et al., 1997). The inhibition in complex-I activity has been shown to cause marked increases in reactive oxygen species (ROS) production (Turrens & Boveris, 1980; Meloni & Vasak et al., 2011), which is responsible for the oxidative damage generated in dopamine (DA) metabolism or oxidative phosphorylation (Lotharius & O’Malley, 2000).

The classic anti-parkinsonian drugs L-deprenyl and melatonin has been demonstrated to provide neuroprotection against rotenone-induced neuronal damage in rat by means of its •OH scavenging action (Saravanan et al., 2006; Saravanan et al., 2007). 5-HT and L-DOPA have been reported to exhibit protective effects on oxidative tissue damages (Ham et al., 1999; Xu et al., 2011).
5-HT depresses lipid peroxidation of microsomes by Fe$^{3+}$ ADP and NADPH system (Tse et al., 1991). N-Acetylserotonin decreases the peroxidation of linoleic acid induced by 2, 2' -azobis (2-amidinopropane) (Longoni et al., 1997). 5-HT is reported to scavenge superoxide anion and hypochlorous acid (HOCl). L-DOPA is currently used in symptomatic treatment of Parkinson's disease. However, it induces apoptosis in cultured postmitotic chick sympathetic neurons (Ziv et al., 1997). Additionally, 5-HT metabolises to the potential neuroprotective antioxidants, normelatonin and melatonin, which also helps to prevent oxidative damage caused as a result of rotenone administration. N-acetyl-serotonin (normelatonin) and melatonin protect neurons against oxidative challenges and suppress the activity of the transcription factor NF-kappaB (Lezoualc'h et al., 1998).

**Dopamine Content**

In PD, the central pathologic process is a rather selective degeneration of the dopaminergic neurons in the pars compacta of the substantia nigra, leading to anterograde loss of the ascending nigrostriatal projections and their nerve endings resulting in the depletion of dopamine (DA) in the other regions of central nervous system (CNS) (Sang et al., 2003; Lew et al., 2007). Substantia nigra appears darker than neighbouring areas due to high levels of melanin in dopaminergic neurons. Parkinson's disease is caused by the death of dopaminergic neurons in the substantia nigra pars compacta. The substantia nigra consists of two parts with different connections and functions, the *pars compacta* and *pars reticulata*. The *pars compacta* serves mainly as an input to the basal ganglia circuit, supplying the striatum with dopamine. The *pars reticulata*, on the other hand, serves mainly as an output, conveying signals from the basal ganglia to numerous other brain structures.

The substantia nigra sends out fibers to the corpus striatum, grey and white bands of tissue in the caudate nucleus and putamen where the dopamine is
released. The transmission of dopamine and its release into the corpus striatum is necessary for smooth, coordinated muscle movement (Richard, 2009).

The basal ganglia represent parts of corticosubcortical circuits involved in a large variety of motor as well as non motor functions. Parkinsonism emerges as a complex disorder in which striatal dopamine depletion results in an increased and disordered discharge in motor areas of basal ganglia. Parkinsonian motor signs are caused by distinct abnormalities in the basal ganglia discharge and by involvement of subcircuits related to distinct cortical targets.

The main input to the SNr derives from the striatum. It comes by two routes, known as the direct and indirect pathways. The direct pathway consists of axons from medium spiny cells in the striatum which project directly to nigra. The indirect pathway consists of three links, first a projection from striatal medium spiny cells to the external part of the globus pallidus (GPe); second a GABAergic projection from GPe to the subthalamic nucleus (STN); third a glutameric projection from STN to SNr. (Nauta & Cole 1978; Huang et al., 2010). Thus, striatal activity exerts an excitatory (or rather disinhibitory) effect on SNr neurons via the direct pathway, but an inhibitory effect via the indirect pathway. The direct and indirect pathways originate from different subsets of striatal medium spiny cells: they are tightly intermingled but express different types of dopamine receptors, as well as showing other neurochemical differences. There are significant projections to the thalamus (ventral lateral and ventral anterior nuclei), superior colliculus and other caudal nuclei from the pars reticulata (the nigrothalamic pathway). (Carpenter et al., 1972) These neurons use GABA as their neurotransmitter. In addition, these neurons form up to five collaterals which branch within both the pars compacta and pars reticulata, likely modulating dopaminergic activity in the pars compacta (Deniau et al., 1992).
Substantia nigra pars compacta

The SN has a well defined system of dopamine neurones giving rise to the nigrostriatal pathway and is known to have an important role in motor behaviour in animals and extra-pyramidal disorders such as Parkinsonism. One of the major outputs from the striatum appears to project to the SN through the striatonigral pathway. The cell bodies of this nigrothalamic pathway predominantly in the lateral and central regions of the zona reticulata of the SN and their axons project to the ventromedial nucleus (VM) and to a lesser extent to the Centromedianum-Parafascicular nuclear complex (CM-PF) of the ipsilateral thalamus in the rat (Clavier, 1976; Faull, 1978; Gjedde & Geday, 2009).

DA produced by neurons of the SNpc plays a key role in the regulation of GABA neurotransmission in the basal ganglia, including the striatum and substantia nigra pars reticulata. The destruction of these neurons leads to a downstream deficiency in GABA signaling in areas of the brain that regulate movement. GABA controls the activity of the DA containing cells of the substantia nigra and loss of GABA and its synthesizing enzyme glutamic acid decarboxylase have been observed in the basal ganglia of patients dying from Parkinson’s disease (Precht & Yoshida 1971). As GABA helps “quiet” excessive neuronal firing and has been deficient in patients in the advanced stages of Parkinson’s disease directly targeting GABA production rather than dopamine replacement is a more effective way of improving brain function in late-stage Parkinson’s disease while also avoiding the known therapeutic limitations and complications associated with the over-production of dopamine.

Alterations in the brain monoamines DA and 5-HT have been implicated in the etiology and/or pharmacotherapy of multiple mental disorders. Most of the effects of 5-HT on DA neurons is indirect, mediated via actions on complex neuronal circuitry, rather than direct effects on DA terminals (Poewe et al., 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. Dopamine released from 5-HT neurons is responsible for L-DOPA-induced dyskinesia in rotenone-lesioned rats (Munoz et al., 2008). This is important therapeutically, since an individual patient has a range of symptoms that reflect dopaminergic dysfunctions in some brain areas but not others. Thus, the clinical efficacy of psychotherapeutic drugs that act on 5-HT systems is due in part to their effects on DA systems.

Endogenous progenitor cells are harnessed to replace neurons lost in neurodegenerative diseases but require the development of methods to stimulate their proliferation and differentiation. Researchers are also exploring a process called trans-differentiation—“tricking” cells of the bone marrow to produce brain cells or muscle cells.

5-HT and GABA as therapeutic agents for cell proliferation and differentiation is a novel approach. Our earlier studies showed that 5HT and GABA acting through specific receptor subtypes 5HT2 (Sudha & Paulose, 1998) and GABA B (Biju et al., 2001) respectively, control cell proliferation and act as a co-mitogens. Also, it plays a major role in spinal cord regeneration and functional recovery by re-establishing the connections along with BMC (Paulose et al., 2009). Our experiments on PD done using different neurotransmitters – 5-HT and GABA with and without pluripotent bone marrow cells extracted from the same individual given to the site of damage re-established the connection and the functional recovery was observed.

5-HT and GABA has a functional role in the corpus striatum this leads to reversal of DA receptors in the substantia nigra region of PD rats (Jes et al., 2010; Nandhu et al., 2009). In our present study we demonstrated using specific fluorescent dyes - PKH2GL to bone marrow cells and nestin to premature neurons -the autologous differentiation of bone marrow cells to neurons (O'Sullivan et al., 2010). Our results proved that BMC differentiate to neuronal cells 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 division and differentiation was observed with 5-HT and GABA.

In the present work, the effects of 5-HT, GABA and bone marrow cell supplementation intranigrally to the substantia nigra and individually on unilateral Rotenone infused PD rats were analyzed. Real-time polymerase chain reaction work showed significant down-regulation in gene expression of Dopamine D_{1} and Dopamine D_{2} in the substantia nigra of Parkinsonism induced rats and these have been confirmed using immunofluorescent antibodies specific to Dopamine D_{1} and Dopamine D_{2} receptors. 5-HT, GABA and Bone Marrow Cells in combination functionally reversed in dopamine receptors in rotenone induced Hemiparkinsonism rats.

5-HT and GABA could potentially regulate the function of DA neurons through actions on midbrain DA cell bodies and/or DA terminals. Several subtypes of 5-HT receptors, including the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{3} and 5-HT_{4} receptors, act to facilitate DA release (Giovanni et al., 2001; Matteo et al., 2001). Dysfunction of DA, 5-HT and GABA neurotransmission underlie the pathophysiology of most of the neuropsychiatric disorders, including PD (Esposito et al., 2008). 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 and Nestin. 5-HT and GABA are involved in a variety of cellular processes involved in regulating metabolism, proliferation and morphology (Efrain, 2001). The fine integration of these dynamic events appears to involve multiple receptor action. Gene expression of DA D_{1} and DA D_{2} showed significantly increased expression on rotenone infused rats compared to control. This increase in activity is due to the damage of dopaminergic neurons which produces dopamine in the normal individuals. Absence of dopamine resulted in decreased number of DA receptors subtypes in the region. However, the
antioxidant and comitogenic property of 5-HT and GABA resulted in a reversal to near control value in the combinational treatment groups.

**Corpus Striatum**

Serotonergic terminals have been reported to make synaptic contacts with both DA containing and non-DA containing GABA interneurones in the SNc, SNr, striatum and ventral tegmental area (VTA) (Moukhels *et al.*, 1997; Azmita and Segal 1978). 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.

Striatum is anatomically the most prominent nucleus of the basal ganglia and many of the proposed functions of the basal ganglia have been linked to synaptic processing among cells in the striatum (Mallet *et al.*, 2005; Ouyang *et al.*, 2007). All classes of striatal neurons receive prominent inhibitory GABAergic inputs. These inhibitory interactions are likely to be essential for striatal processing (Fujiyama, *et al.*, 2000; Waiddvogel *et al.*, 2004).

Bone marrow stromal cells (BMSC) normally give rise to bone, cartilage, and mesenchymal cells. Recently, bone marrow cells have been shown to have the capacity to differentiate into myocytes, hepatocytes and glial cells (Sanchez-Ramos *et al.*, 2000). The central finding is that cells with major characteristics of NSCs can be efficiently generated. Bone marrow stromal cells (MSC), the non-hematopoietic precursor cells (i.e. mesenchymal stem and progenitor cells) in bone marrow, offer an alternative source of cells for treatment of neurodegenerative diseases and central nervous system (CNS) injury. These cells normally differentiate into bone, cartilage and adipose tissue (Pittenger MF, 1999) but can be experimentally induced to differentiate into cells with surface markers characteristic of neurons (Woodbury *et al.*, 2000).
The dopaminergic neurons in the SNpc extend its arm to the CS and hence are directly affected in PD. CS is mainly associated with motor coordination. We investigated Parkinson’s disease damage to dopaminergic function in CS of control and experimental groups of rats. In the CS, DA D_1 and D_2 receptor showed significantly decreased activity in Rotenone-infused rats and on supplementation of BMC compared to control. This decrease in activity is due to the damage of dopaminergic neurons which produces dopamine in normal individuals. Damage of dopaminergic neurons resulted in less number of DA D_1 and D_2 receptors in the region. However, the 5-HT and GABA-supplemented group resulted in a reversal to near control value. Real-time PCR studies confirm the data obtained in receptor-binding studies and these have been confirmed using immunofluorescent antibodies specific to DA D_1 and D_2 receptors in our study.

Our experimental findings also demonstrate an increase in intracellular IP3 and cAMP content in the Parkinson induced rats. This will trigger the release of Ca^{2+} from the endoplasmic reticulum. IP3-mediated Ca^{2+} release can in turn increase mitochondrial Ca^{2+} and consequently, increase respiration and ATP production (Hajnoczky et al., 2000). Excessive stimulation of dopamine 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 Rotenone infused rats which in turn indicates the ROS mediated neurodegeneration in the striatum. 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). 5-HT, GABA and Bone marrow cell in combination reversed the increased DA receptors and thus the IP3, cAMP content and pro-apoptotic Bax protein was down regulated when compared to the Rotenone infused rats.
α-Synuclien is localized in neuronal mitochondria. α-synuclein is highly expressed in the mitochondria in olfactory bulb, hippocampus, striatum and thalamus. (Zhang et al., 2008; Liu et al., 2009) The hypothesis generated is that over expression of wild-type α-synuclein protein is sufficient to cause Parkinsonism. The true function of α-synuclein protein remains elusive although a number of putative roles have been postulated in vesicle dynamics, through phospholipase D$_2$ and tyrosine hydroxylase inhibition in Parkinson’s induced rats. (Farrer, 2006). Up regulation of neuronal protein α-synuclein expression in the corpus striatum indicates the mitochondria mediated cell death in Rotenone infused rats. 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 (Walton & Dragunow 2000, Finkbeiner 2000; Shimamura et al., 2000). CREB is controlling 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, Bcl$_2$, each contain CRE sites (Mayr & Montminy 2001) 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 corpus striatum of Rotenone rats compared to control. cGMP signal transduction pathway is triggered by Dopamine production. Decreased dopamine in Parkinsonism leads to the reduction in cGMP content and neuronal survival. cGMP activates a protein-kinase, modulating diverse biochemical events through the phosphorylation of specific substrate protein (Scott, 1991). It has been suggested that the state of phosphorylation of the protein DARPP-32, mediated by the nitric oxide/cGMP pathway, represents an important mechanism of information arriving at striatonigral neurons (Tsou et al., 1993). In addition, the nitric oxide/cGMP pathway modulates striatal release of several neurotransmitters, including DA and excitatory amino acids (Guevara-Guzmán et al., 1994).
PD is characterized by a gradual degeneration of midbrain dopaminergic neurons and the accumulation of ubiquitin in cytoplasmic inclusions (Lewy bodies) (Dauer & Przedborski 2003; Hardy & Gwinn-Hardy 1998). The role of the Lewy bodies for the pathological manifestations of PD remains enigmatic. Major components of Lewy bodies are ubiquitin and ubiquitinated proteins. (Alves-Rodrigues et al., 1998) In general, ubiquitination of proteins is critical for protein degradation. Proteins that are conjugated with a chain of ubiquitin moieties are targeted to the ubiquitin-proteasome system (UPS) complex, where they undergo proteolytic degradation. Polyubiquitinlated proteins are enzymatically degraded to peptides, and the ubiquitin moieties released intact. (Hendil & Hartmann-Petersen, 2004). The high levels of ubiquitin and ubiquitinated proteins in Lewy bodies therefore indicate that protein degradation is impaired in PD. Our experimental findings also demonstrate an increase in ubiquitin carboxy-terminal hydroxylase in the Parkinson induced rats. Our treatment showed that 5-HT and GABA along with BMC antagonized these effects maximally which have immense clinical significance in the management of PD.

Our results confirmed the 5-HT and GABA comitogenic effect in proliferation and differentiation of the BMC to neurons in the brain. 5-HT and GABA are involved in a variety of cellular processes involved in regulating metabolism, proliferation and morphology (Azmitia 2001).

Cerebral Cortex

The cerebral cortex is critical to speech, emotion, reasoning, memory, movement and integration of information. However, dopaminergic and glutamergic pathways play a leading role in the structural and functional organization of the cortico-basocortical loops involved in PD (Hirsch et al., 2000). 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 2007). Metabolic and neuroimaging
observations have recently documented decreased prefrontal and parietal 18F-fluorodeoxyglucose uptake in PD cases with mild cognitive deficits (Huang et al., 2007; Huang et al 2008). Recent observations have demonstrated complex I deficiency (Parker, 2008) and abnormal ATP synthase and inner protein membrane prohibition expression levels (Ferrer 2007) in the frontal cortex in PD. Several reports have highlighted the need of dopamine–glutamate coactivation for a number of cortical functions (Gurden 1999; Baldwin 2002).

Parkinson's disease (PD) has been considered a paradigm of degenerative diseases of the nervous system characterized by motor impairment (Parkinsonism) due to malfunction and loss of dopaminergic neurons of the substantia nigra pars compacta. However, PD is a systemic disease of the nervous system with variegated clinical symptoms appearing before Parkinsonism and due to the involvement of selected nuclei of the medulla oblongata, pons, autonomic nervous system and olfactory structures, among others. Furthermore, recent clinical data have shown modification in behaviour, personality changes and cognitive impairment leading to dementia. Lewy pathology, hallmark of PD, in the cerebral cortex does not correlate with cognitive impairment. However, recent studies have shown abnormal mitochondria content and function and increased oxidative stress and oxidative responses in the cerebral cortex in PD (Ferrer, 2009). It has been previously reported that disorders in PD largely occur due to the imbalance of inhibitory and excitatory processes in cortical and subcortical neuronal circuits (Elena 2010).

In our studies we observed an elevated cAMP and IP3 level in the cerebral cortex of Rotenone infused rats. The elevated IP3 level causes extra cellular release of Ca\(^{2+}\), which in turn enhance metabolic stress on mitochondria that leads to excessive oxidative phosphorylation 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
Discussion

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 Rotenone infused rats which indicated the ROS mediated neurodegeneration in the cerebral cortex. Apoptosis whether caspase-dependent or caspase-independent, has been implicated as one of the important mechanisms leading to the death of dopaminergic neurons in the substantia nigra of Parkinson’s disease (Schulz 2006). CREB is a transcription factor that plays an important role in neuronal survival, in part by controlling the transcription of neuroprotective genes (Finkbeiner 2000). The promoter regions of the genes for brain-derived neurotrophic factor (BDNF) and the pro-survival protein Bcl-2 contain cAMP response elements (CREs) (Mayr 2001). Rotenone administration causes a decrease in transactivation of the CRE promotor, resulting in reduced expression of downstream CREB-regulated genes (Chalovich 2006). In the present study the gene expression of CREB was down regulated in cerebral cortex of Rotenone compared to control. Even though cAMP level was increased, the CREB expression was decreased. Enhanced activation of the DA 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 signalling cascade in PD rats. Down regulation of CREB is a consequence of apoptotic pathway activation and down regulation of DA receptor function. These findings suggest that decreased CREB expression is the result of cell loss. BMC administration along with the 5-HT and GABA reversed the expression of Bax and CREB to near control.

PD increased oxidative damage, abnormal α-synuclein solubility and aggregation and increased α-synuclein nitration in the cortex (Gomez & Ferrer 2010). Normally an unstructured soluble protein, alpha-synuclein aggregates in
the form of Lewy bodies and Lewy neurites in the frontal cortex in PD (Ferrer 2007; Arima et al., 1998). High concentrations of Rotenone results in neuronal death accompanied by a decrease of the monomeric form of α-synuclein, leading to both decreased synthesis of the protein and its increased mono-ubiquitination accompanied by nuclear translocation (Monti et al., 2007). Studies by Pierson et al., (2007) showed an increased level of unconjugated ubiquitin in the dorsal striatum of the dopamine depleted hemisphere. Normal α-synuclein expression is essential for the viability of primary neurons. Gene expression studies of alpha-synuclein in the cerebral cortex showed a significant down regulation in the Rotenone induced rats compared to control. This indicates the reduced expression of normal α-synuclein in the PD rats. Up regulation of ubiquitin carboxy-terminal hydrolase gene expression in cerebral cortex confirmed the increased level of unconjugated ubiquitin in the Rotenone infused rats. 5HT, GABA and BMC combinational treatment significantly reversed these changes back to control.

We investigated the Parkinson’s disease damage to dopaminergic functional regulation in CC of control and experimental groups of rats. The increased expression of DA D₁ and D₂ receptors in the cerebral cortex due to the supersensitivity of the DA D₁ and D₂ receptor to DA owing to loss of dopaminergic neurons in the substantia nigra as a result of administration of rotenone. Absence of dopamine results in less number of DA receptors in the region. 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). In the absence of consistent alterations in the levels of receptor expression, altered functional responses of DA D₁ and D₂ receptors result from changes in signaling mechanisms (Corvol et al., 2004). However the antioxidant and co-mitogenic property of 5-HT and GABA resulted in a reversal to near control value in the 5-HT, GABA and Bone marrow cells supplemented groups. Real-time polymerase chain reaction (PCR) results showed decrease in the
gene expression of DA D_1 and DA D_2 receptors in the CC of Parkinsonism induced rats and these have been confirmed using immunofluorescent antibodies specific to DA D_1 and DA D_2 receptors in our study.

Autologous BMC to treat neurological disorders offers several unique advantages over other cell replacement therapies. For one, immunological reactions are avoided and it also bypasses many of the ethical issues that surround the use of embryonic cells. Our study demonstrated that BMC administration alone cannot reverse the above said molecular changes occurring during PD. 5-HT, GABA and BMC in combination potentiates a restorative effect by reversing the alterations in DA receptor binding and gene expression that occur during Parkinson’s disease.

Cerebellum

The cerebellum is the battery of the brain. Parkinson’s disease is a progressive neurodegenerative disorder characterized by selective degeneration of dopaminergic neurons in substantia nigra pars compacta leading to marked reduction of dopamine levels in the cerebellum.

5-HT is widely expressed within the central nervous system, where it is thought to play a major role in the regulation of neuronal network excitability. In rats, 5HT-containing neurons originating from the dorsal and median raphe nuclei innervate forebrain dopamine-containing areas. However, this interaction between brain DA and 5HT-containing neuronal systems is complex, and the effect produced appears to dependent on the relative activity of each system (Jenner et al., 1983; Berg et al., 2008).

In the cerebellum, DA receptor sub types showed significantly increased activity in PD rats compared to control rats whereas its activity reversed to near control in the 5-HT, GABA and Bone marrow cells supplemented groups. Real time PCR studies were conducted to evaluate the DA functional regulation at the mRNA level during PD and supplementation with 5-HT, GABA and Bone
marrow cells. We obtained an up regulation in the DA receptor subtypes mRNA during PD. 5-HT, GABA and Bone marrow cells supplemented groups reversed the DA to near control.

Our experimental findings also demonstrate an increase in intracellular IP3 and cAMP content in the cerebral cortex of Parkinsonism induced rats. Inositol phosphates are known to regulate membrane trafficking, glucose metabolism, cytoskeletal organisation and intracellular Ca\(^{2+}\) homeostasis—particularly the release of stored Ca\(^{2+}\) via IP3 receptors. Excessive Ca\(^{2+}\) overload in cells have been reported to cause apoptosis. Boehning and co-workers (2003) demonstrated a small amount of cytochrome C released from mitochondria can bind to and promote Ca\(^{2+}\) conductance through IP3 in the endoplamic reticulum membrane. The released Ca\(^{2+}\) further triggers mass exodus of cytochrome C from all mitochondria in the cell and thus activating the caspase and nuclease enzyme of the apoptotic process.

The 5-HT and GABA facilitates neural differentiation and regenerative processes of the neurons (Nandhu et al., 2011; Jes et al., 2010), causing the DA neurons to secrete DA although not efficient as in the control group, thus leading to a reversal of the receptor gene expression to control level owing to decreased super sensitivity of the DA receptor subtypes in the cerebellum. The increased expression of DA receptor subtypes in the cerebellum of PD is due to the supersensitivity of the DA receptor subtypes. It is because of the loss of dopaminergic neurons in the substantia nigra as a result of rotenone administration. The Bone marrow cells alone treated group did not show any reversal compared to the other groups.

Our experimental results thus showed that GABA and 5-HT play important role in the differentiation of bone marrow cells in to neurons re-establishes the connections and functional recovery of Parkinson’s disease. All our studies including behavioural and Real time PCR support the above statement. We conclude from our studies that 5-HT and GABA treatment potentiates a
therapeutic effect by reversing the alterations in DA receptor subtypes binding and gene expression that occur in cerebellum during Parkinson’s disease.

**Brain stem**

The destruction of dopaminergic neurons in the substantia nigra constitutes an intermediate step in a broader neurodegenerative process rather than a unique feature of Parkinson’s disease, as a consistent pattern of progression would exist, originating from the medulla oblongata/pontine tegmentum. However, if such a regular neurodegenerative pattern were to exist, consistent damages would be found in the brain stem (Thomas 2009).

The present work was carried out to study the changes in DA receptor subunits gene expression in the brain stem of control and Parkinsonism induced rats and to evaluate the role of 5-HT, GABA and Bone marrow cells supplementation. 5-HT and GABA as therapeutic agents for cell proliferation and differentiation is a novel approach. 5-HT and GABA acting through specific receptor subtypes 5-HT$_2$ and GABA$_B$ respectively, control cell proliferation and act as co-mitogens. In our present study we demonstrated the autologous differentiation of BMC to neurons using combinations of 5-HT and GABA. DA receptors activity in rotenone infused unilateral Parkinsonism induced rats. But the BMC treated group of our studies did not show significant change as compared to the other groups which is due to slow division and differentiation of BMC when it is administrated alone.

An increased production of IP3 and cAMP in rotenone infused rats which are mediated through the enhanced DA receptors. This will trigger the release of Ca$^{2+}$ from the endoplasmic reticulum. IP3-mediated Ca$^{2+}$ release can in turn increase mitochondrial Ca$^{2+}$ and consequently, increase respiration and ATP production (Hajnoczky et al., 2000). Excessive stimulation of DA 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 brain stem indicates the mitochondria mediated apoptosis in rotenone infused rats. 5-HT, GABA and BMC in combination reversed the increased DA receptors compared to the rotenone infused rats.

**Hippocampus**

In rats, 5-HT neurons in the Hippocampus raphe are among the first neurons to differentiate in the brain and play a key role in regulating neurogenesis (Kligman & Marshak 1985) 5-HT and GABA has a direct role in neuronal maturation and accelerate its differentiation (Marois & Croll 1992; Rodriguez 1994). 5-HT and GABA could potentially regulate the function of DA neurons through actions on midbrain DA cell bodies and/or DA terminals. 5-HT and GABA has a direct role in neuronal maturation and accelerate its differentiation (Rodriguez 1994). The present study focused on the hippocampal dysfunction occurring in the PD and the effectiveness of 5-HT, GABA and autologous bone marrow cells transplantation in these alterations.

Up regulation in the IP3 activity increased the intracellular Ca\(^{2+}\) which caused enhanced metabolic stress on mitochondria that leads to excessive oxidative phosphorylation and increased production of reactive oxygen species. Our study showed an increased activity of Bax gene expression in the hippocampus of the rotenone infused rats which indicated the ROS mediated neurodegeneration in the hippocampus. 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).

CREB is a transcription factor that plays an important role in neuronal survival, in part by controlling the transcription of neuroprotective genes (Finkbeiner, 2000). Agents that disrupt the activity of CREB specifically block the formation of long-term memory, whereas agents that increase the amount or activity of the transcription factors accelerate the process (Alcino et al., 1998).
The study of the DA receptors expression in relation with CREB phosphorylation in PD is an important step towards elucidating the relationship between molecular adaptations and behavioural consequences. Our findings showed a significant down regulation of CREB in the hippocampus of rotenone infused rats. Even though cAMP level was increased, the CREB expression was decreased. Enhanced activation of the DA 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. These findings suggest that decreased CREB expression is the result of cell loss.

High concentrations of rotenone results in neuronal death accompanied by a decrease of the monomeric form of α-synuclein, leading to both decreased synthesis of the protein and its increased mono-ubiquitination accompanied by nuclear translocation (Monti et al., 2007). α-Synuclein and β Synuclein positive lesions predominantly localized to abnormal aggregates in the mossy fiber terminals that synapse on hilar neurons, these abnormal processes impair synaptic transmission in hippocampal perforant pathway projections critical to memory and behavior (Galvin et al. 1999). Normal α-synuclein expression is essential for the viability of primary neurons. Gene expression studies of α-synuclein in the hippocampus showed a significant down regulation in the rotenone induced rats compared to control. This indicates the reduced expression of normal α-synuclein in the PD rats. Most of the effects of 5-HT and GABA on DA neurons are indirect, mediated via actions on complex neuronal circuitry, rather than direct effects on DA terminals (Poewe 2009). Since 5-HT and GABA receptor subtypes are differently distributed in dopaminergic brain regions, it is possible to specifically “target” individual brain regions with serotonergic ligands and thereby affecting dopaminergic function selectively in these areas.

We conclude from our studies that 5-HT and GABA along with BMC potentiates a restorative effect by reversing the alterations in DA receptors binding and gene expression that occur during Parkinson’s disease. Thus, it is evident that
5-HT and GABA along with BMC to Rotenone infused rats renders protection against oxidative, related motor and cognitive deficits which makes them clinically significant for functional reestablishment and recovering from PD symptoms.

**Behavioural Deficits in Parkinsonism induced Rats**

We have evaluated the behavioural response of control, Parkinsonism induced and experimental groups of rats which includes rotaroad test, social interaction test, narrow beam test and grid walk test. The rotarod experiment demonstrated the impairment in the motor function and co-ordination in the Parkinsonism induced rats. Parkinsonism induced 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 and at the same time adjust their limb movements on the metallic rod which is indicative of cerebellar dysfunction. Many other brain regions have been associated with timing tasks including the dorsal lateral premotor cortex, inferior parietal lobe, supplementary motor area, superior temporal gyrus, caudal putamen, ventrolateral thalamus and inferior frontal gyrus (Rao et al., 1997; Jancke et al., 2000; Lewis & Miall 2003). Abnormalities of some of these areas, such as the inferior frontal gyrus and superior temporal gyrus (Abell et al., 1999; Castelli et al., 2002) have been reported in autistic subjects rendering it difficult to isolate the cerebellum in this task. However, increased timing variance has been observed in patients with cerebellar disorders (Ivry et al., 1988). Loss of coordination of motor movement, inability to judge distance and timing, incapacity to perform rapid alternating movements and hypotonia have been reported during cerebellar damage (Gowen & Miall, 2005). This study demonstrates the treatment of 5-HT, GABA and bone marrow cells, has modulating effect on the Parkinson’s associated motor defects. The treatment of 5-HT, GABA and Bone marrow cells
to Parkinsonism induced rats increased the fall off time from the rod when compared to control rats.

Behaviour in rodents is determined by the conflict between the drive to explore the unknown area/object and the motivation to avoid potential danger. In Elevated plus Maze Test, the Parkinsonism induced rats exhibit significant alterations in its behavioural response due to damage to the cortical neurons. The PD rats remained for longer period in closed arms of elevated plus-maze which is characteristic to anxio-depressive traits. It has been demonstrated that the preference shown for the closed arms reflects an aversion toward the open arms, caused by fear or anxiety induced by the open space in the elevated plus maze test (de Souza et al., 2007). The head dipping attempt, stretched attend posture and grooming attempts were also greatly reduced supporting the anxiogenic condition as a result of Parkinson’s disease stress. The 5-HT, GABA and bone marrow cells treatment during PD rats reversed the behavioural abnormalities to control. 5-HT, GABA and Bone Marrow Cells in combination treatment to PD rats reversed anxiety effects, as indicated by increase of the time spent in the open arm and increase in head dipping attempt, stretched attend posture and grooming attempts compared to PD rats.

In the social interaction test, PD rats spent less time in active interactions in the novel environment. Attempts at allogrooming, sniffing the partner, following were reduced when compared to control rats and 5-HT, GABA and BMC in combination treatment to control rats. Administration of combinational treatment to Parkinson’s rats resulted in an increase in the time spent in social interaction to near control values.

PD rats showed increased number of foot slips in grid walk test and decreased time spent in narrow beam test compared to control. This indicated the motor dysfunction in the PD rats. Moreover 5-HT, GABA and BMC in combination treatment improved the motor performance of the PD rats.
In the present work, the effects of 5-HT, GABA and BMC supplementation intranigrally to substantia nigra as treatment individually and in combination on rotenone induced Hemi-parkinson’s disease in rats were analyzed. Dopaminergic binding parameters investigated its role in the regulation of dopamine receptor subtypes in the brain regions of the experimental rats. Gene expression analysis of receptor specific probes for DA D$_1$, DA D$_2$, pro apoptotic protein Bax, transcription factor – CREB, α-synuclein and ubiquitin in the brain regions of PD rats and treatment groups were studied. Confocal studies with specific antibodies in brain slices were done to confirm the binding studies and gene expression analysis using specific probes. Behavioural response in rotaroad, social interaction, Elevated plus maze, Grid walking, Narrow beam test was carried out to assess the motor learning and cognition deficit in rotenone induced PD rats.

We conclude from our studies that 5-HT and GABA along with BMC potentiates a restorative effect by reversing the alterations in DA receptors binding and gene expression that occur during Parkinson’s disease. Thus, it is evident that 5-HT and GABA along with BMC to rotenone infused rats renders protection against oxidative, related motor and cognitive deficits which makes functional re-establishment and recovery from PD symptoms. Our results confirmed the 5-HT and GABA co-mitogenic effect in proliferation and differentiation of BMC to neurons which has immense therapeutic significance in the management of PD.