ENVIRONMENTAL TOXINS AND DOPAMINERGIC NEUROTOXICITY:

NOVEL NEUROPROTECTIVE STRATEGIES

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ENVIRONMENTAL TOXINS AND DOPAMINERGIC NEUROTOXICITY: NOVEL NEUROPROTECTIVE STRATEGIES

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VITA

Senthilkumar S. Karuppagounder son of Mr. Shanmugam Karuppagounder and Mrs.Tamilarasi Shanmugam was born on May 28th 1975 in Salem, a small city in the southern part of Tamilnadu, India. He is the second son of Mr. and Mrs.Shanmugam with two other siblings, one elder brother Dr. Saravanan Karuppagounder and one younger brother Mr. Ilayaraja Karuppagounder. Senthil is married to Dr. R. V. Ranjeni who is basically a dentist. He did his Bachelor's degree in Pharmacy in The Tamilnadu Dr.MGR Medical University, Chennai, Tamilnadu, INDIA from 1997 TO 2001. He did his Masters in Bio-Medical engineering in Jadavpur University, Kolkata, INDIA from 2002 to 2004. After completing his Masters degree, he also worked as a Research assistant in Indian Institute of Chemical Biology in the Department of Cell Biology & Physiology. In May 2006 he joined the Auburn University Doctoral program in Pharmacal sciences where he was funded as a graduate teaching and graduate research assistant. During his stay in Auburn University, he has received many awards and prizes. He has also received the outstanding graduate student award in Pharmacal sciences 2008 and Who's Who among the students in American Universities Award in 2009. He has also received several travel awards as a graduate student.

DISSERTATION ABSTRACT

ENVIRONMENTAL TOXINS AND DOPAMINERGIC NEUROTOXICITY:

NOVEL NEUROPROTECTIVE STRATEGIES

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Parkinson's disease (PD) is a progressive degeneration of dopaminergic neurons in the substantia nigra, leading to massive loss of dopamine in the striatum. Environmental and endogenous neurotoxins are implicated the etiopathology and progression of nigral neurodegeneration in PD. Animal models are an important aid to study pathogenic mechanisms, nature of behavioral abnormalities with respect to neurotransmitters changes in the brain and also provides a better understanding to design novel therapeutic drugs and devise neuroprotective strategies. Diquat is a herbicide, which structurally resembles MPTP/paraquat. Dopamine-derived endogenous neurotoxin salsolinol is also involved in the pathogenesis of PD. We evaluated the effect of diquat and/or salsolinol (chronic exposure) on the nigrostriatal dopaminergic system in mice. However, diquat and/or salsolinol affected the behavior but did not induce any dopamine depletion in the

striatum. 1-methyl-4-pheneyl-1,2,3,6-terahydropriydine (MPTP) is a potent neurotoxin that causes selective loss of dopaminergic neurons and causing PD like symptoms. PD animal model was induced in mice by systemic injection of MPTP and then we investigated the behavioral, neurochemical, biochemical and neuropathological hallmarks of the disease. Inflammation, oxidative stress, mitochondrial dysfunction, and altered GABA levels are associated with progressive nigrostriatal neurodegeneration. Adenosine receptor and peroxisome proliferator-activated receptors (PPAR) play an important role in the modulation of inflammation and GABA. Adenosine receptor antagonist, PPAR-modulators and anti-inflammatory drugs have shown to decrease inflammation which can lead to neuroprotection. However, the effect of these drugs on GABA and its role in neuroprotection is not clear. Thus in this study we investigated the various neuroprotective mechanisms (antioxidant activity, effect on GABA and mitochondrial energy enhancing properties) of Caffeine (adenosine A2 receptor antagonist), Dexamethasone (Anti-inflammatory), and GW501516 (PPAR agonist), against MPTP-induced neurotoxicity. Caffeine and dexamethasone significantly improved the behavioral defect induced by MPTP, scavenged the free radical and altered the major endogenous antioxidant molecules and striatal GABA levels leading to neuroprotection. However, GW501516 prevented the development of motor impairment, decreased oxidative stress, without altering GABA attenuated MPTP-induced dopamine depletion. Thus, in the present study we investigated the various neuroprotective mechanisms of drugs with anti-inflammatory effect against MPTP-induced neurotoxicity.

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I would like to dedicate my work to my parents and my brothers. It's with their encouragement, love and inspiration, I have been able to quench my desire for higher studies and strive for excellence in research. I express my sincere gratitude to my co-chairs Dr. Muralikrishnan Dhanasekaran and Dr. Vishnu Suppiramaniam for all their support and assistance. I am also thankful to my committee members Dr. Muralikrishnan Dhanasekaran, Dr. Vishnu Suppiramaniam, Dr. Daniel Parsons, and Dr. Kevin Huggins for extending timely help and guidance throughout my doctoral studies. I would like to extend my appreciation to Dr. Forrest Smith for providing the PPAR agonist for my study. I would like to extend my appreciation and gratitude to all the faculty members, staff and fellow students at Dept. of Pharmacal sciences, Dept. of animal health, Auburn University. I would like to express my heartfelt gratitude to my colleagues and friends Dr. Subramanian Uthayathas, Dr. Kodeeshwaran Parameshwaran, Dr. Brian Shonesy, Mr. Kariharan Thiruchelvam, and Mr. Sateeshkumar Sathigari for their encouragement and personal friendship. I would like to personally thank Dr. Bobby Thomas, Dr. Rajesh, and Dr. Navneet for all their scientific help and guidance. Finally, I would like to personally thank my wife Dr. R.V. Ranjeni for her love and encouragement and my brother Dr. Saravanan Karuppagounder and my sister-in-law Mrs. Vennila Saravanan, and all my family members for their moral help and support throughout my graduate studies.

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1. INTRODUCTION

Parkinson's disease (synonym - paralysis agitans) is an age-related neurodegenerative disease that is prevalent in United States of America (USA) and throughout the world. An unknown disease originally depicted as "shaking Palsy" in 1817, was never expected to become the most studied neurological disorder of 20th century, which unlocked the door for the enormous understanding and development in neurosciences. The extensive study of this neurological disorder is attributable to its cosmic prevalence in elderly people in whole world. Just in the United States of America (USA) alone, at least 500,000 people are believed to suffer from Parkinson's disease, and about 50,000 new cases are reported annually. It is estimated that USA holds a prevalence rate of 100-300 per 100,000 of population. With an increase in life expectancy also comes an increase in the number of people at risk for developing this neurodegenerative disorder. No proven preventive or long-term disease slowing treatment strategies are available as yet for this progressive neurodegenerative disease. One major current research focus in Parkinson's disease is the development of neuroprotective drugs that slow or halt the disease progression resulting in many more years of productive life. With regard to the current study, *Neuroprotection* is the ability of a drug to prevent or block the behavioral or biochemical toxic insults induced by neurotoxins.

Parkinson's disease is a major neurodegenerative disorder that results from progressive damage to the dopaminergic neurons in the substantia nigra (Cornford et al., 1995; Ramsden et al., 2001; Liang et al., 2007; Dhanasekaran et al., 2008). Damage or loss of dopaminergic neurons in this brain region results in the depletion of dopamine from terminals in the striatum/nucleus caudatus putamen involved in coordinating smooth movement. Parkinson's disease is associated predominantly with loss of antioxidants or increase in proxidant levels and appearance of Lewy bodies (Larsen et al., 1994; Spillantini et al., 1997). Parkinson's disease is still considered as a disorder of unknown etiology even after 150 years after its first description, although the evidence for its existence could be found in various ancient medical literatures. Recent data shows that a variety of neurotoxins arising from environmental, dietary and lifestyle factors or from normal metabolism could initiate degeneration in dopamine neurons in genetically predisposed individuals. Environmental toxicants, specifically the pesticides, insecticides, fungicides and herbicides have been implicated as potential risk factors in Parkinson's disease (Rajput and Uitti, 1987; Rajput et al., 1987; Golbe, 1990; Golbe et al., 1990; Tanner et al., 1999; Thiruchelvam et al., 2000; Di Monte, 2003; Thrash et al., 2007). Similarly, there are numerous endogenous substances that can induce dopaminergic neurodegeneration (Collins et al., 1987; Antkiewicz-Michaluk, 2002; Kotake, 2002; Maruyama and Naoi, 2002; Abe et al., 2005). Commonly used toxins may have breakdown products, derivatives or contaminants that can severely damage dopaminergic neurons specifically (Javitch and Snyder, 1984). It has been repeatedly suggested that the presence of these environmental toxins, may be partly responsible for idiopathic

Parkinson's disease (Kopin, 1987; Langston, 1987; Langston et al., 1987; Di Monte, 2003).

Various factors have been attributed to development of nigral degeneration such as inflammation, astroglial activation, oxidative stress, mitochondrial dysfunction, genetic factors and exposure to environmental toxins (Kohutnicka et al., 1998; Mohanakumar and Steinbusch, 1998; Ebadi et al., 2000; Hald and Lotharius, 2005; McGeer and McGeer, 2008; Miller et al., 2009). Inflammation response involves the macrophage activation, pro-inflammatory chemical messengers release from specific cells, activation of immunoreactive cells such as glial cells in the brain, and ultimately the removal of infected cells, tissue or the debris (McGeer and McGeer, 2004; Hirsch et al., 2005; Nagatsu and Sawada, 2005; Sawada et al., 2006; McGeer and McGeer, 2008). Oxidative stress involves the generation of reactive oxygen species leading to the imbalance between the proxidant and antioxidant ratio which results increased proxidant contents (Mohanakumar and Steinbusch, 1998; Hald and Lotharius, 2005; Nagatsu and Sawada, 2006). Postmortem examination of the brains of the parkinsonian patients has revealed an extensive loss of dopaminergic neurons in the substantia nigra associated with oxidative stress, mitochondrial dysfunction, and a massive astrogliosis along with the presence of activated microglial cells (Nagatsu and Sawada, 2007; Reynolds et al., 2008). Even after a long time of immune or reactive oxygen species insult it has been observed that the disease continue to progress, suggesting that toxic substances released by the glial and other immunologically active cells may be involved in the propagation

and perpetuation of neuronal degeneration. This cause the activation of microglia (brain immune cells) leading to release of deleterious compounds such as pro-inflammatory cytokines (TNF-alpha, Il-1beta, IFN-gamma), which may act by stimulating nitric oxide and reactive oxygen species production which may exert a more direct deleterious effect on dopaminergic neurons (Mohanakumar and Steinbusch, 1998; Knott et al., 2000; Liu and Hong, 2003; Nagatsu and Sawada, 2006, 2007).

Modulation of the striatal synaptic neurotransmission has been associated with several motor disorders involving the basal ganglia, such as Parkinson's disease and Huntington's disease. Striatal GABAergic microcircuits modulate cortical responses and movement execution in part by controlling the activity of medium spiny neurons. In rotenone-treated animal model of Parkinson's disease, blockade of $D_{(2)}$-like but not $D_{(1)}$-like dopamine receptors Further, Carbamazepine (antiepileptic drug) modulate multiple transmitter systems to exert its neuroprotective effects against rotenone-induced striatal neuronal dysfunction (Costa et al., 2008). Hence, differential targeting of GABAergic transmission may represent a possible therapeutic strategy against basal ganglia neurodegenerative disorders involving mitochondrial complex I dysfunction. Recent studies have suggesting that it could represent an interesting target for selective pharmacological intervention in movement disorders involving basal ganglia circuitry.

The present study is an attempt to discern the various toxins associated with nigral neurotoxicity and to investigate the new/novel interventions against this neurological

disease. Thus in this study we evaluated the effect of diquat and /or salsolinol on the nigrostriatal tract. Diquat and/or salsolinol did not have significant effect on the striatal dopamine content as compared to the effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is an exogenous toxin which is metabolized into the toxic cation 1-methyl-4-phenylpyridinium (MPP_+) by the enzyme MAO-B of glial cells. MPP_+ is then majorly taken by dopamine-producing neurons by a selective uptake mechanism and ultimately results in striatal dopamine depletion (Javitch and Snyder, 1984; Dhanasekaran et al., 2008).

Figure-1

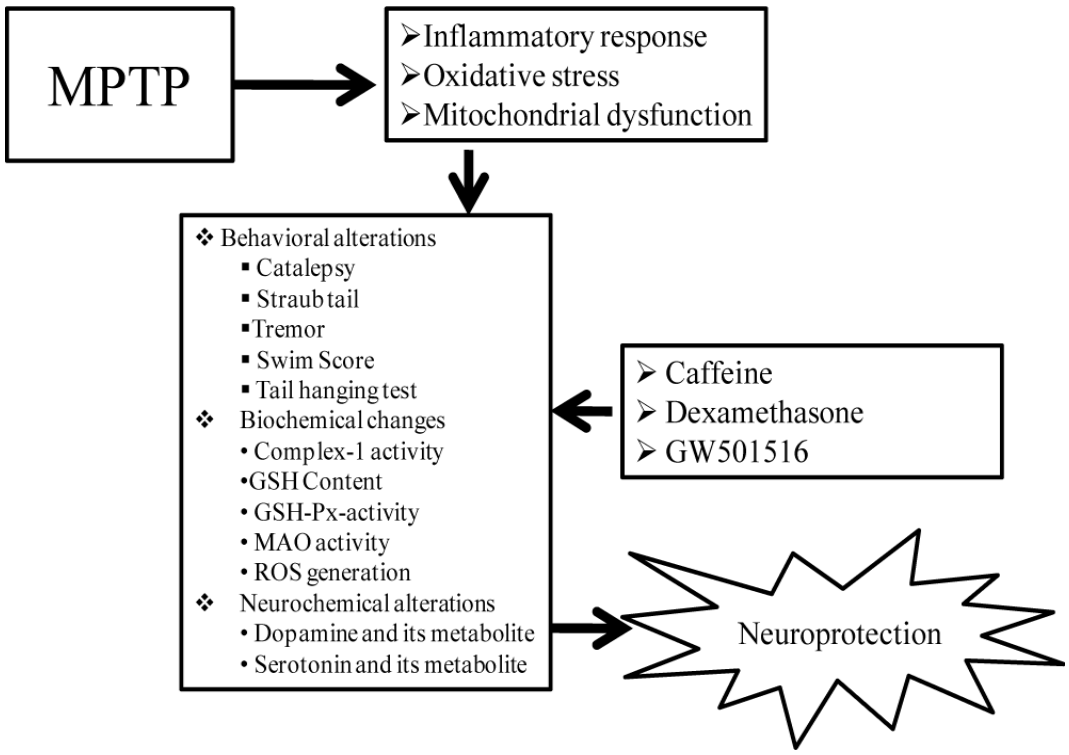

Figure-1: Schematic diagram of MPTP induced neurotoxicity and behavioral, biochemical and neurochemical alterations.

Apropos to this, in the present study Glial fibrillary acidic protein (GFAP) was found to be significantly altered in MPTP treated mouse as compared to the control (Czlonkowska et al., 1996; McGeer and McGeer, 2008). Inflammation, modulation of GABAergic transmission and oxidative stress may be are the major causative factors for neurotoxicity (Muralikrishnan and Mohanakumar, 1998; Kurkowska-Jastrzebska et al., 1999; Thomas and Mohanakumar, 2004). There is involvement of purinergic receptors in regulating inflammation associated with MPTP in the brain. In this study, we used MPTP treated mice as an animal model to investigate the neuroprotective effect/mechanism of various drugs such as Caffeine, Dexamethasone, and GW501516

Caffeine is a xanthine alkaloid and also is a psychoactive stimulant drug. Adenosine (2A) receptor antagonism-mediated neuroprotection has been associated with stroke, excitotoxicity and mitochondrial toxins recently. Therefore, rigorous explorations are currently conducted to dissect out common cellular mechanisms which may underlie the broad spectrum of neuroprotection by A (2A) receptor inactivation in brain. Thus, caffeine a known adenosine-2A receptor antagonist was used in the present study. Caffeine (adenosine receptor antagonist) prevented the GABA increase and blocked dopamine depletion induced by MPTP. Dexamethasone is lipophilic steroidal molecule which acts through steroidal cytoplasmic receptors and regulate the pro-inflammatory cytokines expression. Dexamethasone interferes with many of the features characterizing pro-inflammatory glial activation, prevented the induction of iNOS, loss of catecholamine content, tyrosine hydroxylase activity and TH immunostaining induced by

lipopolysaccharide (Castano et al., 2002; Arimoto and Bing, 2003). Peroxisome proliferator-activated receptors (PPARs) have been implicated originally in lipid and glucose homeostasis, but lately they have been found as regulators of inflammatory responses. It was first observed in PPAR null mice, the inflammatory responses were prolonged which implicated that there is involvement of PPAR in inflammation. The activation of PPAR receptors leads to repression of NF-κB signaling and inflammatory cytokine production. PPAR-delta receptor agonist proved as a newer intervention which can be clinically exploited against the Parkinson's disease since it blocked MPTP-induced dopamine depletion without affecting the monoamine oxidase-b activity.

Primarily, we selected an appropriate animal model (by using various neurotoxins such as Diquat &/ or Salsolinol, and MPTP) to investigate the neuroprotective effect of various substances (Chapter-3). Since only MPTP-induced dopamine depletion and altered GABAergic transmission, we have chosen MPTP treated mice as a Parkinsonian animal model to investigate the neuroprotective effect of various drugs (Chapter-3). Neuroprotective effect of Caffeine, Dexamethasone and GW501516 was evaluated against MPTP-induced neurotoxicity (Chapter-4, Chapter-5, and Chapter-6).

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2. REVIEW OF LITERATURE

Parkinson's disease (PD) is a late onset, progressive neurodegenerative disorder characterized by selective degeneration of nigrostriatal dopaminergic neurons in A_9 substantia nigra pars compacta (SNpc) region. Parkinsonian syndrome, has four cardinal clinical features consisting of tremor, rigidity, bradykinesia and characteristic disturbance of gait and posture. The disease syndrome was first described in detail in a monograph "An essay on the Shaking Palsy" by a London physician, Dr *James Parkinson* in 1817 (Langston, 2002; Parkinson, 2002). Dr. Parkinson described it as "*involuntary tremulous motion with lessened muscular power, in parts not in action and even supported with propensity to bend the trunk forward and to pass from walking to a running pace; the senses and intellect being unimpaired"*. In 1892, a French neurologist Dr. Charcot, suggested that "paralysis agitans or shaking palsy" is not the appropriate term to define this disorder in which "*muscular strength was well maintained until the late stage and shaking was not considered wholly appropriate, as disease may manifest in severe form without tremor"*.

Parkinson's disease is the second most common neurodegenerative disorder and it is a gerontological disorder, affecting more than 1% of adult population over the age of 45 years. One study reported the occurrence of parkinsonian signs in 15% of those who

are in the age group of 65 to 74, nearly 30% in 75 to 84, and over 50% in those older than 85 (Bennett et al., 1996). It may be lower in Africans and Asians (Marttila, 1983). The other symptoms of the disease are difficulty in initiating movement, cog-wheeling, micrographia, loss of finger dexterity, masked face, reduced blink rate, widened palpebral fissure, limitation of upward gaze, drooling and difficulty in swallowing. Dementia is found in approximately 20% of all patients typically starting with slow processing of thought, progressing to difficulty in organizing thoughts. The appearance of dementia is always an ominous sign, associated with poor prognosis and higher mortality rate. Although the neuronal circuitry involved in coordinated movement is complex, the disabling symptoms of Parkinson's disease are predominantly due to the demise of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Forno et al., 1993). The symptoms result from the gradual loss of dopaminergic neurons from SN. The movement disturbances can be separated into positive symptoms (behaviors that do not likely occur in healthy people) and negative symptoms.

In 1894, a woman was diagnosed with the symptom of parkinsonian tremor with unilateral tumor in the SN, which also impinged on the cerebral and cerebellar peduncles, and it was suggested that SN was culpable for this symptom (Cornford et al., 1995). The cause of Parkinson's disease was the loss of pigment from SN, which is normally colored due to the presence of neuromelanin. Later the pathology of idiopathic Parkinson's disease was found to be due to progressive loss of neurons from SNpc region, which contains the neurotransmitter, dopamine (3,4-dihydroxyphenylethylamine or 3-

hydroxytyramine, DA). Since these neurons project their axons to the striatum and utilize dopamine as their neurotransmitter (Dahlstroem and Fuxe, 1964), a profound reduction in striatal dopamine represents the primary neurochemical alteration in PD. In addition to dopamine, there is a loss of dopamine metabolites homovanillic acid and 3,4-dihydroxyphenylacetate and an increase in dopamine receptor sites (Hornykiewicz, 1966; Bernheimer et al., 1973; Lee et al., 1978). The symptoms usually begin when 80% of dopamine in the brain has been lost. The level of dopamine will continue to fall over time worsening the symptoms.

Tretiakoff (1919) was the first to describe the characteristic lesions of the substantia nigra (SN) as the cause of dopamine. It was first found that reserpinized animals showed parkinsonian symptoms, which were due to a depletion of central monoamines (Bein, 1956). L-3,4-Dihydroxyphenylalanine (L-DOPA) was found to reverse the reserpine effects (Carlsson et al., 1957). At that time, dopamine was believed to be an intermediate substance in the metabolism of norepinephrine, but it was soon discovered that it played its own role as a neurotransmitter in the brain (Carlsson, 1959). The final clarification about the link between the anatomical findings of SN lesion and the chemical depletion in the striatum came when striatal dopamine was found to be localized in innervating neurites and not in striatal cell bodies (Carlsson et al., 1962). Nigral cells were found to contain dopamine and to project from the SN to the striatum (Anden et al., 1964; Dahlstroem and Fuxe, 1964). A hallmark in the Parkinson's disease investigation came in 1966 when Hornykiewicz identified severe loss of dopamine

accompanied with deficiency in its metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), in Parkinson's disease patients. These observations threw light on the molecular mechanism underlying pathophysiology of Parkinson's disease.

Parkinson's disease is associated predominantly with two areas of the brain, SN and nucleus caudate putamen (NCP). The SN (meaning "black substance") derives its name from its many pigmented cells. The SN neurons project to caudate nucleus and putamen region. Dopamine a chemical neurotransmitter synthesized in neurons in SN, is the one which governs the motor activity such as movement, balance, and walking. Paralleling the degree of cell loss in the SN is a loss of dopamine in the striatum. Loss of approximately 80% of the SN pigmented cells and 80% of the striatal dopamine content results in the appearance of the Parkinson's disease symptoms.

Another important chemical neurotransmitter, acetylcholine is also found in the striatum. A balance between dopamine and acetylcholine is necessary for the normal function of the brain. As a result of dopamine deficiency in Parkinson's disease, the dopamine/acetylcholine balance is disturbed. This disturbance probably further aggravated the symptoms of the disease. In Parkinson's disease there may also be a deficiency of another chemical transmitter, norepinephrine, which is found mainly in two regions of the brain. These two regions (the locus cerulus and the lateral tegmental area)

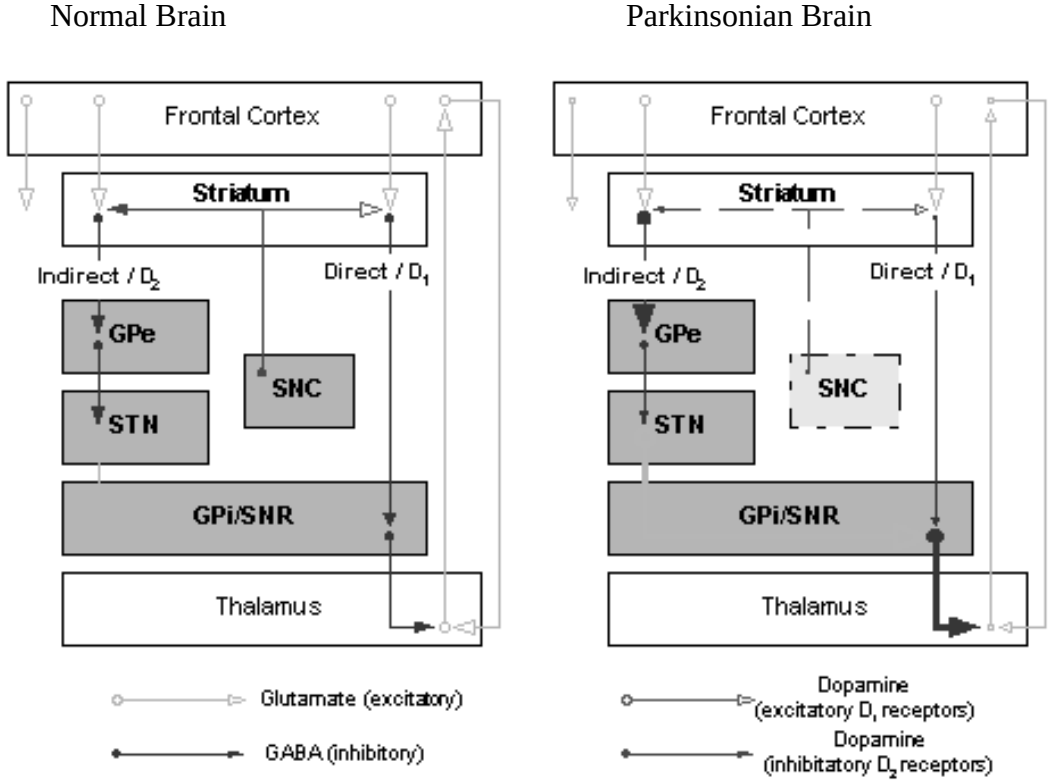
are involved in governing the involuntary autonomic nervous system. The deficiencies of other neurotransmitters may contribute to some of the secondary symptoms.

The formation of Lewy bodies in surviving dopaminergic neurons is the hallmark pathological finding in Parkinson's disease. In Parkinson's disease, degenerating pigmented neurons contain eosinophilic inclusion Lewy bodies, which are characteristic of the disease. These cells contain neuromelanin, a pigment that resembles melanin (the pigment found in skin). Lewy bodies are present in the basal ganglia, brain stem, spinal cord, and sympathetic ganglia (Spillantini et al., 1997). Lewy bodies are intracytoplasmic eosinophilic inclusions composed predominantly of ubiquitin and α-synuclein (Schlossmacher et al., 2002) and, recently, parkin has also been detected (Tanner et al., 1999).

A greater understanding of the neuro-pathophysiologic circuitry involved in parkinsonism is largely responsible for the resurgence of functional neurosurgical procedures in the treatment of this disease. In the parkinsonian brain, increased activity of the subthalamic nucleus (STN) neuron causes the medial (internal) portion of the globus pallidus (GPi) and SN pars reticulata (SNpr) neurons to become hyperactive. Excessive activity of neurons, in these two structures, which exert inhibitory influences on thalamic and brainstem nuclei, leads to a reduction of thalamic and brainstem activity and a reduction of thalamocortical activity (Figure.-2). In addition to the loss of nerve cells in the pigmented SNpc and locus coeruleus in the midbrain, neuronal loss also occurs in the

globus pallidus and caudate putamen. SNpc neurons provide dopaminergic input to the striatum which, is an integral part of the basal ganglia. These dopaminergic neurons and also cholinergic striatal interneurons, modulate a monosynaptic gamma-aminobutyric acid (GABA-ergic) inhibitory output to the globus pallidus interna. Substantia nigra pars reticulata (SNpr) neurons, which protect in turn by GABA-ergic inhibitory pathway to the ventroanterior and ventrolateral nuclei of the thalamus. Stimulation of this "direct" pathway in the striatum disinhibits these thalamic nuclei so that their excitatory output of the motor region of the cerebral cortex is increased.

Figure-2. Schematic diagram of motor control in brain



An alternative polysynaptic ("indirect") pathway from the striatum reduces the excitatory output from these thalamic nuclei to the motor cortex. This later pathway

involves striatal GABA-ergic inhibitory neurons that project to the globus pallidus externa, which has inhibitory effect on the subthalamic nucleus (STN). This nucleus has excitatory glutamatergic connection with the globus pallidus interna and reticulata of the substantia nigra.

The experimental therapies for Parkinson's disease that are under investigation at present assure improvement on the limitations of existing treatments. The prospect of progress in understanding the pathogenesis of the disorder will improve the development of novel molecules and treatments that will retard, or revert, the currently inexorable progressive course of Parkinson's disease. Parkinson's disease is a chronic, progressive, unremitting neurological disorder. Nevertheless, the symptoms found at clinical onset, those that develop throughout the course of the illness, responsiveness to pharmacologic therapy, and disease progression are highly variable (Koller, 1992). For years, physicians have observed that patients with tremor as the primary manifestation appear to have a better prognosis and possibly respond better to L-DOPA than those with prominent postural instability and gait difficulty (Rajput et al., 1993). Despite advances in pharmacotherapy that have improved quality of life for these patients, the mortality rate remains largely unchanged. Existing drugs are symptomatic and temporarily ameliorate the symptoms of Parkinson's disease. Nevertheless, symptom progression can be slowed and quality of life improved with current methods of treatment. Pharmacotherapy for Parkinson's disease includes L-DOPA, dopamine agonists, monoamine oxidase (MAO) inhibitors, anti-cholinergics and most recently, Catechol-O-methyltransferase (COMT)

inhibitors (Olanow and Koller, 1998). The most effective drug for relieving the symptoms of Parkinson's disease is L-DOPA. A combination of L-DOPA and carbidopa is widely used. The carbidopa reduces L-DOPA's peripheral conversion to dopamine by inhibiting DOPA decarboxylase. This in turn reduces side effects and increases the amount of L-DOPA available for uptake into the central nervous system (CNS). Long-term L-DOPA therapy is associated with motor complications that can be as disabling as the disease itself. DA agonists hold promise because of more sustained stimulation of DA receptors. This class of drugs includes bromocriptine mesylate, pergolide mesylate, cabergoline, pramipexole, lisuride and ropinirole hydrochloride. These drugs stimulate dopamine receptors and some of them have been used for many years in the treatment of Parkinson's disease. Recently, they have been gaining popularity for several other reasons. For example, they are longer acting, which creates more sustained stimulation of DA receptors and less pulsativity. In theory, this should reduce the progression of motor complications. Another group of drugs, which is promising and most widely used is, the selective inhibitors of MAO-B. Selegiline hydrochloride does not stop the progression of PD but has been shown in controlled trials to be neuroprotective, delaying the need for L-DOPA therapy. Many of this class of drugs have antioxidant action in Parkinson study group, (1989). Selegiline is generally used early in the illness because of its putative neuroprotection and because it has only a minimal effect on symptoms. It has few side effects, but like other MAO-B inhibitors, it cannot be used in a patient taking selective serotonin reuptake inhibitors. The other MAO-B inhibitors such as rasagiline, lazabemide, entacapone, and moclobemide are under clinical trial. Anticholinergic

synthetic preparations like benztropine mesylate and trihexylphenidyl were prescribed for specifically controlling tremor and rigidity. Anticholinergics were not preferred due to their side effects like dry mouth, decline of cognitive functions, dizziness and urinary retention. COMT inhibitors are the newest class of drugs, which includes entacapone and tolcapone. Other novel therapeutic candidate molecules under clinical trials identified by Committee to Identify Neuroprotective Agents for Parkinson's (CINAPS) include remacimide, acamprostate and topiramate (glutamate antagonist), riluzole (N-methyl-D-aspartate (NMDA) antagonist), caffeine and theophylline (adenosine antagonists), antioxidants such as coenzyme Q_{10}, ropinirole and trophic factors such as GM_1 ganglioside, GPI 1485 which are currently under investigation (Ravina et al., 2003).

The concept of surgical treatment was first reported by Cooper, who found that ligation of anterior choroidal artery abolished parkinsonian tremor and rigidity. This led to further intensive research in the area and established clinical criteria for acceptance of surgery to alleviate tremor or rigidity (Cooper, 1965). The target of such procedures is the disrupted activities of the motor thalamus, Gpi or STN. The introduction of Magnetic Resonance Imaging (MRI) (Zhu et al., 2002), Positron Emission Tomography scan (PET) with [^{18}F]-fluorodeoxyglucose (Carbon and Eidelberg, 2002), and the use of microelectrode recording techniques have improved the safety and accuracy of functional neurosurgical procedures. Despite reduced surgical morbidity, ablative procedures are still associated with a risk of permanent complications, especially when bilateral lesions are created. The pallidotomy and deep brain stimulation of the GPi or STN cause

activation of premotor and supplementary motor areas associated with the reversal of parkinsonism (Davis et al., 1997; Samuel et al., 1997). Allogeneic transplantation of fetal mesencephalon tissue rich in dopamine neurons is currently being studied, and initial reports show promising results (Lindvall, 2001).

HYPOTHESES POSTULATED IN THE PATHOGENESIS OF PD

Several hypotheses have been postulated to explain the pathophysiology of Parkinson's disease. However, none of these are full proof, and could not be established as the major causative event in this neurodegenerative disease.

Radical hypothesis

Oxidative stress has received more attention in Parkinson's disease because of the oxidative metabolism of dopamine to yield hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) (Olanow, 1993). A variety of markers and indices in Parkinson's disease patients and animal models indicate involvement of oxygen-free radicals and oxidative stress in the pathogenesis of Parkinson's disease. These include lipid peroxidation (Dexter et al., 1986), GSH depletion (Sian et al., 1994), increased levels of iron and reduction of ferritin concentrations in the SNpc of Parkinson's disease (Dexter et al., 1990; Jellinger et al., 1993). The vulnerability of nigral neurons to the toxic insult of ROS is primarily due to the high content of transition metals in SN (Poirier et al., 1985). Oxidative stress could lead to cell death under circumstances in which there is (a) increased dopamine turnover, resulting in excess peroxide formation; (b) excessive enzymatic oxidation of dopamine in the synaptic cleft; (c) a deficiency in glutathione

(GSH), thereby diminishing the brain's capacity to clear H_2O_2.; (d) an increase in reactive iron, which can promote **.**OH formation; (e) a decrease in mitochondrial complex-I activity. Most of the toxicity observed following superoxide anion generation is believed to be due to the reaction of the superoxide anion with other ROS such as nitric oxide (**.**NO). Reaction of the superoxide anion with **.**NO produces the potent oxidant peroxynitrite (**.**ONOO), which has been implicated in a number of neurotoxic and neuropathological events (Crow and Beckman, 1995). The peroxynitrite formed triggers the activation of poly ADP-ribose polymerase (PARP) that is a highly energy dependent process and leads to the cleavage of NAD^+ into ADP-ribose and nicotinamide. PARP activation rapidly depletes NAD^+ stores, thereby impairing mitochondrial function, glycolysis, and ATP synthesis (Ying et al., 2001).

Mitochondrial dysfunction

Malfunction in mitochondrial energy metabolism plays a critical role in neuronal homeostasis. It has been reported that mitochondrial complex-I (NADH: ubiquinone oxidoreductase) is inhibited in SNpc of Parkinson's disease patients (Mizuno et al., 1989; Schapira, 1993). Other studies have reported a reduced complex-I activity in the platelets(Bindoff et al., 1989; Parker et al., 1989) and in skeletal muscle (Blin et al., 1994) in PD patients. There are reports suggesting that in Parkinson's disease patients there has been selective loss of GSH in SN. GSH depletion potentiates oxidant-induced loss of mitochondrial functions, oxidative stress and there is oxidative damage to DNA, lipids, and protein (Dexter et al., 1989).

Excitotoxicity

The excitotoxicity is also a known cause that has been implicated in Parkinson's disease. It involves toxicity resulting from increased glutamate formation. SNpc dopaminergic neurons are rich in glutamate receptors, that receive extensive glutamate from the cortex and the STN, and demonstrate a pattern of burst firing in response to exogenously administered glutamate (Johnson et al., 1992). Glutamatergic cortical input also exists in NCP. Glutamatergic pathways project from basal ganglia to thalamus and back to cortex. There are three types of glutamatergic receptors - (i) NMDA, (ii) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and (iii) kainate. The excitatory amino acids become more cytotoxic if released in excess, or if the mechanism of inactivation is impaired. Another excitotoxic mechanism is due to a reduction in energy metabolism due to a defect in mitochondrial function, resulting in loss of the ATP-dependent Mg-blockade of NMDA receptors, causing physiological concentrations of glutamate to mediate a calcium influx into the cell. Excitotoxic damage is also mediated at least in part, via $^{\bullet}$NO. A glutamate-mediated rise in cytosolic calcium results in activation of nitric oxide synthase (NOS) with increased $^{\bullet}$NO production. $^{\bullet}$NO reacts with superoxide radical to form peroxynitrite and hydroxyl radical, both powerful oxidizing agents (Dawson et al., 1991).

Calcium involvement

In idiopathic Parkinson's disease, an altered level of calcium-dependent proteases has been implicated to play a role in differential vulnerability of the dopaminergic neurons (Hirsch et al., 1997). An increased activity of calcium-stimulated phospholipase-

A_2 in putamen was observed, which was inferred to be due to either decreased dopaminergic input in striatum or to a DA nerve terminal degenerative process (Ross et al., 2001). Overexpression of calpain-II, a calcium-dependent protease has been reported in the parkinsonian SN suggesting a rise in intracellular calcium concentrations to be involved in the mechanism leading to cell death in Parkinson's disease (Mouatt-Prigent et al., 1996). The involvement of the different calcium–binding proteins in idiopathic Parkinson's disease has been extensively explored to assess the role of these proteins in buffering calcium load. MPTP causes a rapid depletion of mitochondrial calcium pool followed by a marked and sustained elevation of cytosolic free calcium (Mohanakumar et al., 2002).

In Parkinson's disease, several groups have reported that neurons that contain calcium-binding proteins appear to be less vulnerable than the neurons that lack it, suggesting that calcium-binding proteins might protect these neurons from degeneration by preventing excessive increase in cytosolic calcium concentrations. Involvement of calcium channels, if any, in idiopathic Parkinson's disease has been investigated. Calcium channel antagonists such as nimodipine and flunarizine have been reported to improve nigral graft survival in neural transplantation studies (Brundin et al., 2000). Evidence for the involvement of calcium is obtained from patch-clamp studies on dorsal root ganglia neurons, where MPP^+ has been shown to have a profound and irreversible effect of total as well as K^+-activated currents (Mohanakumar et al., 2002). Calcium

antagonist nicardipine, but not BAY K 8644, the calcium agonist, protects against MPTP-induced dopamine depletion in the striatum (Samantaray et al., 2003).

Apoptosis

Apoptosis or programmed cell death has long been inferred to confer the slow, progressive death of nigral neurons in Parkinson's disease. There has been increasing interest in the notion that cell death in Parkinson's disease occurs by way of apoptosis, rather than necrosis. Necrosis is a rapid form of cell death that is characterized by massive ionic influx, cellular swelling with disruption of subcellular organelles by rupture of plasma membrane, but without extensive damage to nuclear DNA. In contrast, apoptosis is a gradual form of cell death that is characterized by marked cell shrinkage, fragmentation of nuclear DNA by endonucleases, and chromatin condensation with the formation of nuclear or "apoptotic" bodies. In neuronal cell apoptosis, the family of Bax/Bcl-2, interleukin 1β converting enzyme (ICE) and caspases have received particular attention. Increased expression of Bax or caspases promotes apoptosis, whereas increased expression of Bcl-2 or Bcl-xL, promotes survival. It has been shown that c-jun is transiently expressed in the early stages of neuronal apoptosis. Antisense oligonucleotides that block the translation of *c-jun* mRNA and overexpression of a negative *c-jun* mutant reduced apoptosis, and facilitated neuronal survival (Schlingensiepen et al., 1994). In contrast, overexpression of c-jun increases apoptosis (Ham et al., 1995). There are reports that suggest *p53* gene knock-out transgenic mice are resistant to MPTP neurotoxicity (Trimmer et al., 1996) and Bcl-2 overexpression protected catecholaminergic cells against MPTP neurotoxicity (Yang et al., 1998). MPTP

caused an upregulation in caspase 9, 3, 8 and 1 activities (Viswanath et al., 2000; Viswanath et al., 2001).

Genetic basis

The majority of cases of Parkinson's disease appear to be sporadic in nature; however, there may be genetic risk factors that increase the probability of developing Parkinson's disease. Familial Parkinson's disease with specific genetic defects may account for fewer than 10% of all cases of Parkinson's disease (Gasser, 2001); however, identification of these rare genes and their functions has provided tremendous insight into the pathogenesis of Parkinson's disease and opened up new areas of investigation. There are four genes found to be clearly associated to Parkinson's disease, and a number of other genes or genetic linkages have been identified that may cause PD. The first "Parkinson's disease gene", PARK1, was identified as mutant forms of the gene encoding the presynaptic protein α-synuclein. In 1996, Reports showed the involvement of genetic cause for Parkinson's disease, by identifying an alanine to threonine substitution at codon 53 (A53T) in α-synuclein in a large Italian–American kindred and in three Greek kindreds (Polymeropoulos et al., 1997). Later, Kruger and colleagues reported a new mutation in a small German Parkinson's disease pedigree caused by an alanine to proline substitution at codon 30 (A30P) (Kruger et al., 1998). Affected individuals with α - synuclein mutation have typical idiopathic Parkinson's disease, including responsiveness to L-dopa therapy, and Lewy body inclusion formation. The second Parkinson's disease gene, PARK2, is characterized by mutations in the gene for parkin. Deletion and point mutations in the parkin gene have been described in a number of Japanese families with

an early onset, Lewy body–negative parkinsonism and autosomal recessive inheritance, and it leads to autosomal recessive juvenile parkinsonism (AR-JP) (Kitada et al., 1998). The third Parkinson's disease gene, PARK7, results from mutations in DJ-1 (Bonifati et al., 2003). Mutations in α-synuclein, parkin, and DJ-1 definitely cause Parkinson's disease. A mutation (PARK5) in the gene encoding ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) in two family members of a small German kindred with autosomal dominant PD has been described (Polymeropoulos et al., 1997).

Environmental factors

The etiology of idiopathic Parkinson's disease remains unknown, and is reported to be multifactorial, although there is growing evidence implicating environmental risk factors including rural living, consumption of well water and pesticide exposure (Tanner and Langston, 1990). Epidemiological, case control studies have implicated pesticide exposure as a potential risk factor for PD (Gorell et al., 1998; Ritz and Yu, 2000; Priyadarshi et al., 2001; Di Monte et al., 2002). Interestingly, an association between the pesticides and Parkinson's disease is further suggested by clinical and experimental evidence, which has also implicated rotenoid and organophosphate compounds. Paraquat administration was found to induce up-regulation and aggregation of α-synuclein, providing an intriguing model of interactions between this protein and environmental toxicants (Manning-Bog et al., 2002). The finding of synergistic dopaminergic toxicity when mice are exposed to paraquat together with the fungicide maneb further challenges the view that paraquat is a chemical innocuous to the nigrostriatal system (Thiruchelvam et al., 2000).

EXPERIMENTAL ANIMAL MODELS OF PARKINSON'S DISEASE

An ideal animal model of Parkinson's disease should reproduce the progressive, selective nigrostriatal dopaminergic neurodegeneration and recapitulate most of the features of Parkinson's disease. It should cause a preferential loss of dopaminergic neurons within the SNpc, leading to motor dysfunction. In addition to the loss of nigrostriatal dopamine neurons, a key determinant that differentiates this disorder from other neurodegenerative diseases is the Lewy bodies inclusion formation.

6-Hydroxydopamine (6-OHDA) model

6-Hydroxydopamine was the first chemical agent discovered that has specific neurotoxic effect on catecholaminergic pathways (Ungerstedt, 1971; Sachs and Jonsson, 1975). 6-OHDA is a hydroxylated analogue of dopamine and thus uses the same catecholamine transport system. This produces specific degeneration of catecholaminergic neurons. Systemically administrated 6-OHDA is unable to cross the blood–brain barrier. Stereotaxically, 6-OHDA injected into SNpc or the ascending medial fore brain (MFB) or the striatum for the specific target to nigrostriatal dopaminergic pathway. Following 6-OHDA injection, dopaminergic neurons start degenerating within 24 hours and takes 2-4 weeks to induce striatal dopamine depletion. The magnitude of the lesion is dependent on the amount of 6-OHDA injected and the site of injection. Injection of 6-OHDA directly to the striatum causes a retrograde degeneration of the nigrostriatal system over a period of weeks and has been used to mimic the slow progressive nature of PD (Przedborski et al., 1995). Unilateral 6-OHDA injection leads to asymmetric rotational motor behavior after administration of dopaminergic drugs like amphetamine

or apomorphine, due to physiologic imbalance between the lesioned and the unlesioned striatum. This can be quantified and correlated with degree of neuronal lesion (Ungerstedt, 1971). Drawback of the 6-OHDA model is that it differs from progressive degeneration of the dopaminergic nigral neurons in PD. The 6-OHDA model lesion has been used to ascertain the efficacy of antiparkinsonian compounds (Schwarting and Huston, 1996).

Reserpine model

Systemic administration of reserpine causes depletion of brain catecholamines, leading to an akinetic state, in rabbits (Carlsson et al., 1957). The movement deficiency is due to loss of dopamine storage capacity in the intracellular vesicles (Bernheimer et al., 1973). The limitations of this model are that reserpine induced changes are transient and do not show any morphological changes in the dopaminergic neurons. Above all, it is not specific for dopamine, but other biogenic amines also get affected.

Methamphetamine model

The amphetamines are psychostimulatory drugs with addictive potential. Their activity is primarily associated to their dopamine-releasing mechanism (Seiden et al., 1976). At very high doses, it has neurotoxic effects on rodents and non-human primates (Seiden et al., 1976). Like reserpine, methamphetamine administration result in dopamine depletion at the level of dopaminergic nerve terminals (striatum) with minimal effect in the nigral cell bodies (Fibiger and Mogeer, 1971). Methamphetamine animal model has been used extensively for biochemical and physiological studies of the dopamine depleted striatum. Major drawback of this animal model is the lack of histological

changes of Parkinson's disease, including degeneration of dopaminergic neurons and presence of intracellular inclusions.

MPTP model

Remarkable clinical symptoms similar to sporadic Parkinson's disease in humans result after injection of MPTP (Langston et al., 1984). After administration, MPTP crosses the blood brain barrier and is metabolized in astrocytes to it active metabolite 1-methyl-4-phenylpyridinium ion (MPP^+), by monoamine oxidase-B (MAO-B). MPP^+ is selectively taken up by dopaminergic neurons due to its affinity for the dopamine transporter and this results in selective toxicity to dopaminergic neurons (Javitch et al., 1985). Exposure to MPTP results in nigrostriatal dopaminergic pathway with 50% to 93% cell loss in the substantia nigra pars compacta and more than 99% loss of dopamine in the striatum (Hantraye et al., 1993). Rats are resistance to MPTP toxicity and mouse strains vary widely for sensitivity to the toxin. Neurochemical changes following MPTP exposure include decreased level of dopamine and its metabolites in the striatum, increased oxidative damage as evidenced by increased lipid peroxidation, increased 3-nitrotyrosine levels and diminished concentration of antioxidants, such as glutathione (GSH) and superoxide dismutase (SOD), etc. Limitations of MPTP model are its failure to mimic progressive nature of Parkinson's disease. Additionally, the MPTP model does not produce lewy bodies in rodents.

MPP^+ model

Infusion of MPP^+ into the median forebrain bundle in rats caused significant loss of dopaminergic neurons in the SNpc with ensuring behavioral, neurochemical and

biochemical changes characteristic of the lesion (Heikkila et al., 1985). Unilateral intranigral administration of MPP^+ produced dose-dependent depletion of dopamine in the ipsilateral striatum of rats following two weeks of infusion (Sun et al., 1988). Intrastriatal infusion of MPP^+ in rats caused severe biochemical lesion and behavioral symptoms and also helped in studying the neuroprotective action of non-steroidal anti-inflammatory drugs in MPP^+ induced neurotoxicity (Sairam et al., 2003).

Rotenone model

This novel model of Parkinson's disease is based on chronic systemic exposure of rats to rotenone, a pesticide and complex I inhibitor (Betarbet et al., 2000). Rotenone is a lipophilic compound that easily crosses the blood brain barrier. The rotenone model appears to be an accurate model, since chronic exposure to rotenone resulted in uniform and selective dopaminergic neuronal damage, selective striatal oxidative damage, and formation of ubiquitin and α-synuclein-positive inclusions in nigral cells. These were similar to the Lewy bodies observed in human PD. The major problem associated with this animal model is its nature of variability, with only some animals showing lesions.

Genetic model

Genetic defects cases Parkinson's disease in a small percentage of populations. Mutation in three different gene, including α-synuclein, have been associated with familial Parkinson's disease (Polymeropoulos et al., 1997). Since α-synuclein is a major component of lewy bodies, and mutations in α-synuclein may result in nigrostriatal dopaminergic degeneration in familial Parkinson's disease, animal model have been developed to investigate the role of α-synuclein in the etiology of Parkinson's disease.

This model system focused on the use of transgenic mice or drosophila, which express the wild type or mutated α-synuclein. Transgenic mice over expressing human α-synuclein demonstrated a number of features of Parkinson's disease, including loss of nigrostriatal dopaminergic nerve terminal in striatum, development of α-synuclein and ubiqulitin-positive cytoplasmic inclusion, and motor impairments (Masliah et al., 2000).

NEUROPROTECTIVE EFFECT OF THE DRUGS CAFFEINE, DEXAMETHASONE AND GW501516

Caffeine

A(2A) antagonists may be expected to have other beneficial effects in the central nervous system (e.g., on mood and sleep) and in the periphery with regard to the immune and inflammatory processes (Xu et al., 2005). Pharmacological activity of adenosine receptor antagonists includes the regulation of sleep, arousal, neuroprotection, modulation of seizure susceptibility, locomotor effects, and analgesia (Dunwiddie and Masino, 2001). The adenosine A(2A) receptor has recently emerged as a leading non-dopaminergic therapeutic target for Parkinson's disease, largely due to the restricted distribution of the receptor in the striatum and the profound interaction between adenosine and dopamine receptors in brain (Kalda et al., 2006). Pretreatment with A2A antagonist, 8-(3-chlorostyryl) caffeine (CSC) showed partially protection in rats with striatal 6-hydroxydopamine lesion (Bove et al., 2005). Few studies have investigated the neuroprotective effects of caffeine. In methamphetamine induced toxicity, caffeine has

shown to protect the striatal dopamine content and the Th-activity (Delle Donne and Sonsalla, 1994). Caffeine have also shown to play a potential role in the treatment of cerebral ischaemia (Rudolphi et al., 1992; Evans et al., 1999, Strong et al., 2000; Aronowski et al., 2003; Piriyawat et al., 2003). In several toxic models of Parkinson's disease, dopaminergic neurons are protected by A2A receptors (Schwarzschild et al., 2003, Zhao et al., 2005). Neuroprotection by caffeine and adenosine A2A receptor has a beneficial effect in blocking the beta-amyloid neurotoxicity (Dall'Igna et al., 2003). However, the effect on GABAergic transmission in the striatum has not been elucidated.

Dexamethasone

Microglial and astroglial activation with enhanced expression of cytokines can induce severe to neuronal injury due to inflammation (Czlonkowska and Kurkowska-Jastrzebska, 2001). Glucocorticoids are potent anti-inflammatory agents that act by inhibiting various enzymes and proteins (Unlap and Jope, 1995). Glucocorticoids have a protective effect in the nervous system. Neuroprotection against cerebral hypoxic-ischemic damage is observed with dexamethasone (Tuor et al., 1999). Inhibition of inflammatory process is an important protective feature of dexamethasone in MPTP induced mouse model (Kurkowska-Jastrzebska et al., 2004). Neonatal rats with dexamethasone is shown to provide protection against hypoxic-ischemic brain damage by decreasing basal metabolic energy requirements and/or increasing the availability of energy substrates (Tuor, 1997). Dexamethasone modulatate the neuroinflammatory signaling pathway in the dopaminergic neurons directing to neuroprotection (Herber et

al., 2007). The role of astroglia and its pro-/anti-inflammatory mechanisms are involved in the dexamethasone mediated neuroprotection (Previti et al., 2006; Herber et al., 2007). Focal cerebral ischemia produces elevated levels of tumor necrosis factor (TNF) alpha in the brain region. TNF levels increase after brain infarction, but reduced by neuroprotective agents, such as MK 801 and DEX, which act on different cellular levels(Bertorelli et al., 1998). Dexamethasone down regulates chemokine receptor CXCR4 and exerts neuroprotection against hypoxia/ischemia-induced brain injury in neonatal rats (Felszeghy et al., 2004). However, the effect of dexamethasone on mitochondrial complex-I activity and GABA content has not been well established in the MPTP-induced neurotoxicity. In our present study, we examined the various neuroprotective mechanisms of dexamethasone.

GW501516

Peroxisome proliferator-activated receptor (PPAR), a nuclear receptor regulates inflammation, mitochondrial function, and glucose metabolism (Bordet et al., 2006; Ciana et al., 2007). PPARs are activated by fatty acids and fatty acid metabolites (Sprecher, 2007). PPAR is comprised of at least three subtypes, including α, γ, and δ. Recently, several PPAR agonists were shown to exert neuroprotective activity against oxidative damage, inflammation and apoptosis in several neurodegenerative disorders including Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis and multiple sclerosis (Bordet et al., 2006; van Neerven et al., 2008). Indomethacin and ibuprofen activate PPAR and reduce the progression of neurodegenerative disorders. The

PPAR δ is expressed in tissues, such as the brain, (Kliewer et al., 1994; Schoonjans et al., 1996b; Schoonjans et al., 1996a). PPAR δ agonist have neuroprotective effect and also helps in preventing ischemic acute renal failure (Letavernier et al., 2005; Iwashita et al., 2007). There are studies on the role of the PPAR δ in cerebral ischemia or Parkinson's disease. (Iwashita et al., 2007), some reports show that the PPAR δ agonist can protect cultured neurons from cell death (Berger et al., 1999). GW501516 [2-methyl4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-triazol-5-yl)-methylsulfanyl) phenoxy acetic acid] is the most potent PPAR δ agonist and shows a potent neuroprotective effect against various neurotoxic compound (Iwashita et al., 2007).

In summary of the literature review, It is likely that more than one etiological factor contributes to the pathogenesis of idiopathic PD. These factors may include increased genetic susceptibility and exposure to environmental factors. However, the existing animal model will undoubtedly continue to advance our understanding of the pathophysiology of PD. Such models of PD also offer the possibility to test and develop rational therapeutic strategies that will hopefully benefit patients with this progressive neurodegenerative disease.

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3. EVALUATION OF PARKINSONIAN ANIMAL MODEL FOR DRUG SCREENING

INTRODUCTION:

Animal models are an important aid to study pathogenic mechanisms and therapeutic strategies in human disease. Animal models are valuable to the extent to which they accurately simulate the pathogenic, histological, biochemical or clinical features of Parkinson's disease. Animal models help in understanding the pathophysiology of neurodegeneration in Parkinson's disease, and would be an ideal animal model for studying the molecular mechanisms of Parkinson's disease. Such models of Parkinson's disease also offer the possibility to test and develop rational therapeutic strategies that will hopefully benefit patients with this progressive neurodegenerative disease. Parkinson's disease is one of the major neurodegenerative disorders caused by endogenous and exogenous neurotoxins. Chronic exposures to the pesticide and herbicide have been shown to induce neurotoxic effects or results in accumulation of various toxic metabolic by-products, which have the relevant ability to cause symptoms of Parkinson's disease (Gorell et al., 1998; Kamel and Hoppin, 2004). MPTP, rotenone, paraquat, maneb, and dieldrin can play an important role in increasing the risk for oxidative stress, apoptosis and mitochondrial dysfunction leading to

neurodegeneration (Gorell et al., 1998; Menegon et al., 1998; Olanow and Tatton, 1999; Sherer et al., 2002; Wright and Keller-Byrne, 2005). Parkinson's disease is mainly characterized by tremor, bradykinesia, rigidity and postural instability that results primarily from loss of dopaminergic neurons of nigrostriatal pathway. The cardinal pathological feature of the disease is the selective loss of neurons at the SN compacta, resulting in severe depletion of dopamine in the striatum (Cornford et al., 1995; Lang and Lozano, 1998; Olanow and Tatton, 1999; Cookson, 2005; Dhanasekaran et al., 2008b). Degeneration of basal ganglia structures is associated with motor dysfunction. Neurodegeneration of nigrostriatal system produce akinesia, rigidity and catalepsy (Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Muralikrishnan et al., 2003). Understanding the degree and nature of behavioral abnormalities with respect to neurotransmitters' changes in the brain provides a better background to design novel drugs and devise neuroprotective strategies. An efficient technique to investigate the overall manifestation of motor dysfunction in Parkinson's disease is imminent for identifying relationship between the loss of dopaminergic neurons and behavioral changes observed in animal models of this disease (Tillerson et al., 2002). Several methods like rotorod, tail hanging, gait analysis, open-field test, pole test, rigidity, swim-test, etc. are available to measure the degree of motor impairment in animal models (Tsai et al., 1991; Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Muralikrishnan et al., 2003; Bjarkam et al., 2008; Guillot et al., 2008; Luchtman et al., 2009). Methods to study symptoms like akinesia and catalepsy and tremor in animal models of Parkinson's disease are in regular use (Muralikrishnan and Ebadi, 2001;

Uthayathas et al., 2007). Only a few studies exist where swim-test has been employed to examine the motor damage (Muralikrishnan and Ebadi, 2001; Uthayathas et al., 2007).

Diquat (DQ) (1, 1'-ethylene-2, 2'-bipyridilium) is a non-selective contact herbicide that is widely used in USA and other parts of the world (Anton et al., 1998; Jones and Vale, 2000; Xu et al., 2007). Diquat is a quaternary ammonium compound (Ariffin and Anderson, 2006) and structurally resembles other neurotoxins such as MPTP and Paraquat (Anton et al., 1998; Nieto et al., 2006). Environmental neurotoxins such as paraquat (Beligni and Lamattina, 1999; Thiruchelvam et al., 2000; Broussolle and Thobois, 2002; Yumino et al., 2002; Bonneh-Barkay et al., 2005b; Dinis-Oliveira et al., 2006), rotenone (Saravanan et al., 2005; Sindhu et al., 2005; Saravanan et al., 2006; Saravanan et al., 2007; Borland et al., 2008) and MPTP (Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Muralikrishnan and Ebadi, 2001; Muralikrishnan et al., 2003) have shown to play a significant role in the etiopathology and progression of nigral dopaminergic neuronal insult. Chronic exposure to pesticides and/or herbicides can result in nigral neuronal damage and cause parkinsonian symptoms (Sechi et al., 1992; Thiruchelvam et al., 2000; Kamel and Hoppin, 2004; Bonneh-Barkay et al., 2005a; Bonneh-Barkay et al., 2005b; Dinis-Oliveira et al., 2006; Thrash et al., 2007). Diquat is a non-electrophilic redox cycling compound and a potent *in vivo* pro-oxidant. With regard to its pro-oxidant activity, diquat reacts with free radicals and oxygen to generate intracellular superoxide anion and other redox products leading to oxidative damage of lipids (Bonneh-Barkay et al., 2005a; Bonneh-Barkay et al., 2005b).

Thus, acute and chronic exposure to diquat induces acute hepatic and renal toxicity leading to death in rodents (Awad et al., 1994; Yumino et al., 2002; Zhang et al., 2006; Higuchi et al., 2007; Xu et al., 2007). The endogenous neurotoxin, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol), has been considered a potential causative factor for the pathogenesis of Parkinson's disease. Elevated salsolinol, an endogenous metabolite of dopamine may contribute to the pathology of Parkinson's disease. Salsolinol can up regulate the levels of c-Jun and phosphorylated c-Jun leading to degradation of IkappaBalpha and translocation of the active NF-kappaB into the nucleus. Furthermore, salsolinol decreased the levels of the anti-apoptotic protein Bcl-2, and increased pro-apoptotic protein Bax, while enhancing the release of cytochrome-c from mitochondria. Mitochondrial complex-I activity was significantly decreased and reactive oxygen species were increased in salsolinol treated cells. These results partly suggest that salsolinol-induced JNK and NF-kappaB signaling pathways may be involved in induction of apoptosis in human dopaminergic neurons, as seen in Parkinson's disease (Wanpen et al., 2007). However, the effect of diquat and/or salsolinol on the striatal dopamine is unknown. Different endogenous (salsolinol) and/or exogenous toxin (diquat) were evaluated for their nigral neurotoxicity. The aim was to investigate the effect of these toxins on nigrostriatal dopaminergic pathway and there by mimic deficiency observed in Parkinson's disease. As noted, animal model may be useful for studying pathogenic mechanisms, for testing therapeutic strategies, or both. As such, no single model is likely to be suitable for all studies. In this chapter, we also treated the mice with MPTP and compared the neurotoxic effects The present chapter validates MPTP treated

mice as an animal model of Parkinson's disease and thus this model can be used to investigate the neuroprotective effects of various drugs.

MATERIALS AND METHODS:

Animals and chemicals: C57/BL-6 mice (body weight, 20–25 g; age, 8–10 weeks) were purchased from Harlem and were housed with a 12 h day/night light cycle with free access to food and water. All procedures involving animals were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee. The following chemicals/drugs were purchased from Sigma Chemical Company (St. Louis, MO., USA): 3-hydroxytyramine hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxytryptamine creatinine sulphate (5-HT), norepinephrine (NE), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), 1-heptane sulfonic acid (HSA) sodium salt, HPLC-grade acetonitrile (ACN), HPLC-grade methanol, Triton-X 100, 4-hydroxyquinoline (4-HQ), bovine serum albumin (BSA), kynuramine, ethylenediaminetetraacetic acid (EDTA) sodium salt, O-phthaldialdehyde (OPT), Tris buffered saline tablet, Phosphate buffered saline tablet, Reduced glutathione (GSH), Dimethyl sulphoxide (DMSO), hydrogen peroxide (H_2O_2) and 3,3 -N-diaminobenzidine tetrahydrochloride (DAB). Anti-rabbit tyrosine hydroxylase polyclonal, Anti-GFAP and anti-rabbit IgG-conjugated horseradish peroxidase antibodies were purchased from Chemicon Reverse osmosis water was used in all experiments. For

HPLC analysis, double–distilled water was filtered and deionized using Milli-Q system (Waters, Milford, MA).

Experiment design and Treatments: C57/BL-6 mice were intraperitoneally administered with saline or with diquat (10mg/kg) and/or salsolinol (10mg/kg) (twice a weeks for 6weeks), MPTP (30 mg/kg, i.p., twice 16 h apart).

BEHAVIORAL STUDY:

Body weight and general observation: The body weights of the animals were recorded in an interval of 3 days by using digital weighing scale. The general observations included tremor, piloerection, salivation and increased respiration after every injection

Straub tail: 3hr after final saline and MPTP injections, the observation for straub tail was conducted. Straub tail is defined as elevation of tail to more than 45° with a curvature of the tail. The results are expressed as straub tail phenomenon was detected or not detected and (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2002).

Akinesia: Akinesia was measured by noting the latency of the animals to move all four limbs in unit time seconds(s) and the test was terminated if the latency exceeded 180s. The animals were initially acclimatized in akinesia cages which consisted of a wooden platform with ventilated perspex hoods. (Muralikrishnan and Mohanakumar, 1998).

Catalepsy: The term catalepsy implies to correct the externally imposed posture in an animal. In the saline and MPTP treated mice catalepsy was measured by placing the animals on a flat horizontal surface with both the hind limbs on a square wooden block (3 cm, high) and the latency in seconds was measured to move the hind limbs from the block to the ground. The animals were initially acclimatized on the wooden platform (Muralikrishnan and Mohanakumar, 1998)

Swim test: Swimming ability was carried out on 4^{th} day after the saline or MPTP injections; in brief mice were placed in water tubs (40 l x 25 w x 12 h, in cm). The depth of water was kept at 8 cm and the temperature was maintained at 27 ± 2° C. The animals were acclimatized for 10 min one day prior to experimentation. Swim score scales were given from range of 1 to 4 based on their swimming pattern. Swim score scales were: 1 = hind part sinks with head floating, 2 = occasional swimming using hind limbs while floating on one side, 3 = occasional floating/swimming only, 4= continuous swimming. (Muralikrishnan and Mohanakumar, 1998)

Tail Hanging Test: The saline and MPTP treated mice were suspended by holding the tail and recorded for one min and the observers were blind to the treatment conditions. The record made by counting the number of attempts made to reach the tail by bending the body and crawling up towards its tail. The tail hanging was measured for one min and results are expressed as number of attempts/ minutes (Jiao et al., 2005).

Open-field: The open-field apparatus consist of a box with clear plexi-glass walls and floor. The effects of saline and MPTP on motor activity was evaluated in the open-field. An automated sensor system, consisting of 16 photo beams per side measured the total ambulation, fine movement and rearing. Each mouse will remain in the field for a total of 5 minutes. (Holcomb et al., 2006).

Rotorod: Saline and or MPTP treated mice were analyzed for motor coordination using the rotorod tests (Columbus Instruments, Columbus, OH). Mice were initially aclamatized for a period consisting of 3 training trials on the rotorod with a 10-minute interval. The mice will be given a 10 minute resting time in between each testing run. The rotation speed of the rod will accelerate gradually from 2.5 to 20 rpm over the first 5 minutes and be held at 20 rpm for the final minute of testing. The time (seconds) spent on the rod (fall latency) will be recorded for each animal and used as a measure of motor function.

Gait analysis: Briefly, saline and or MPTP mice were made to walk on an inclined gangway (100 cm (l) × 12 cm (w) × 10 cm (h) with 30° inclination) leading to a darkened enclosure. The gangway was lined with white paper and the fore- and hind-paws of the mice were dipped in two different non-toxic watercolors to record the footprints and allow the animals to climb the gangway. After the experiment the limbs were cleaned and dried before placing them back in the cage. The recorded footprints were analyzed for stride length, and stride width.

BIOCHEMICAL ASSAY:

Tissue Preparation for biochemical assays Animals were sacrificed by decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes, and other antioxidant molecules. The brain tissue was homogenized in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon homogenizer. Tissue homogenates were centrifuged at 10,000 g for 60 min at 4°C

NADH Ubiquinone oxidoreductase (Complex - I) assay: The decrease in the absorbance due to oxidation of NADH at 340 nm was monitored. The reaction mixture contained potassium phosphate buffer pH 7.4, coenzyme Q_0 and crude homogenate from saline and MPTP treated mice. The reaction was initiated by the addition of NADH and the rate of decrease in the absorbance was monitored at 340 nm for 2 min. A standard curve for NADH was obtained at 340 nm by using known quantities of NADH. The complex-I activity is expressed as the amount of NADH oxidized/mg protein (Muralikrishnan and Mohanakumar, 1998).

Monoamine oxidase (MAO) assay: This enzyme is responsible for the oxidation of biogenic amines. Fluorimetric assay procedure was used for the estimation of MAO (EC 1.4.3.4) activity. The amount of 4-hydroxyquinoline formed due to oxidation of kynuramine by MAO represents the enzyme activity. Incubation mixture contains crude homogenate, potassium phosphate buffer, pH 7.4 and the reaction was initiated by the addition of kynuramine. The reaction was terminated by addition of ice-cold 0.4N

perchloric acid after 1hour. The mixture was centrifuged (14, 000 x g for 5 minutes) and 2 ml of 1N NaOH was added to one ml supernatant of the reaction mixture. Fluorescence intensity was measured at activation/emission wavelengths of 315/380 nm. The specific enzyme activity is expressed as nmol of 4-hydroxyquinoline formed/h/mg protein. A standard curve was obtained for commercially available 4-hydroxyquinoline.(Muralikrishnan and Mohanakumar, 1998)

GSH estimation: GSH was measured fluorimetrically following the methods of (Muralikrishnan and Mohanakumar, 1998). This method employs OPT condensation reaction with GSH to yield a fluorescent product at pH 8.0. Readings were taken at activation and emission wavelengths of 340 and 420 nm respectively. A standard curve was prepared for commercially obtained GSH

GSH-Peroxidase (GPx) Assay: GPx activity was measured spectrophotometrically at 340 nm as described (Roveri et al., 1994) in with minor modifications. The test mixture contained supernatant, GSH, glutathione disulfide reductase (GSH-Rx), phosphate buffered saline, t-butyl peroxide, and finally the reaction was started by the addition of added NADPH. The NADPH oxidation rate was recorded for 3 minute and activity was calculated from the rate of NADPH oxidation.

ROS generation: Conversion of non-fluorescent chloromethyl-DCF-DA (2′,7′ -dichlorofluorescin diacetate) to fluorescent DCF was used to monitor ROS production

spectrofluorometerically using an excitation wavelength of 492 nm and an emission wavelength of 527 nm. DCFH-DA and crude homogenate were incubated with for 45 min at 37 C. DCFH reacted with ROS to form the fluorescent product DCF. Intensity was analyzed by a fluorescent micro-plate. The generation of ROS was measured and reported as relative fluorescence intensity/mg protein. (Muralikrishnan and Ebadi, 2001)

HPLC ANALYSIS:

Measurement of biogenic amines: The neurotransmitters were estimated following the method described previously employing HPLC-electrochemistry (Muralikrishnan and Mohanakumar, 1998). Briefly, an HPLC system (shimadzu) consisting of shimadzu SCL 10-Avp system controller, LC-10AS pump, LECD-6A electrochemical detector, degasser, Rheodyne injector with 20 μl injection loop, and C_{18}, ion pair, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 μm and pore size of 80 Å were used for the assay of neurotransmitters and their metabolites. The flow rate was 0.7 ml/min and the electrochemical detection was performed at + 0.74 V for the analyses of biogenic amines and their metabolites. The composition of mobile phase was 8.65 mM heptane sulphonic acid, 0.27 mM EDTA, 13% acetonitrile, 0.4-0.45% triethylamine and 0.32 - 0.35% phosphoric acid (v/v). A standard solution containing 4 pmol of all the biogenic amines was run immediately prior to and following sample injections every day. Results are presented as pmol/mg fresh tissue.

Measurement of Glutamate and GABA: The amino acids were estimated following the method described previously employing HPLC-electrochemistry (Clarke et al., 2007). Briefly, an HPLC system (shimadzu) consisting of shimadzu SCL 10-Avp system controller, LC-10AS pump, LECD-6A electrochemical detector, degasser, Rheodyne injector with 20 µl injection loop, and C_{18}, ion pair, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 µm and pore size of 80 Å. The flow rate was 0.65 ml/min and the electrodetection was performed at + 0.8 V for the analyses of glutamate and GABA. **Derivatisation protocol:** Briefly, 10µl of either standard mix or sample supernatant, 90 µl of borate buffer (0.1 M, pH 9.5), 10 µl of potassium cyanide (10 mM) and 10 µl of NDA (6 mM) were added to a single reaction tube, vortex mixed and the reaction was allowed to proceed at ambient temperatures in the absence of light. A 20 µl of the derivative was injected onto the appropriate HPLC system. The composition mobile phase was 0.1M di-sodium hydrogen orthophosphate/50 µM EDTA (pH 5.6, 1 M OPA) and HPLC grade methanol (35:65). Mobile phase was filtered through 0.45 µm and vacuum degassed prior to use. A standard solution containing glutamate and GABA was run immediately prior to and following sample injections. Results are presented as pmol/mg tissue.

IMMUNOHISTOCHEMISTRY STUDIES:

The animals were anaesthetized with (ketamine and xylazine), and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed, and kept in 4% paraformaldehyde fixative for one hour,

and cryoprotected in 20% sucrose in PBS for 24 h at 4°C. Serial coronal sections (20 μm thickness), spanning the substantia nigra and the striatum, were cut on a cryostat, collected free-floating in PBS, and processed as described below. The sections were rinsed 3 times for 10 minutes with 0.1 M PBS. The sections were then incubated in 1% H_2O_2 in PBS for 5 min, and permeabilized with 0.4% Triton X-100 for 30 min and further blocked by incubating for 60 min in 2% BSA, 0.2% Triton X-100 in PBS. Sections were then incubated with the primary antibody anti-rabbit TH (1:1000), Anti-rabit-GFAP (1:1000) and anti-DARPP-32 in PBS, pH 7.4, containing 1% BSA and 0.2% Triton X-100, for 12 h at 4 °C. After rinsing in PBS containing 1% BSA, sections were incubated with secondary anti-rabbit IgG-conjugated horseradish peroxidase antibody (1:1000) in PBS, pH 7.4, and containing 1% BSA for 60 min at room temperature. Visualization was performed by incubation in 1 mg/2 ml DAB containing of 1% H_2O_2 for 5 minutes. All sections were then washed for 10 min in PBS, mounted on slides, dried, dehydrated in graded ethanol, cleared in xylene, and mounted with DPX and cover-slipped. To test the specificity of the immunostaining, control sections were processed in an identical manner but with the primary or secondary antibody (Saravanan et al., 2006).

Protein Estimation: Protein was assayed using the coomassie plus protein assay reagent kit.

Statistical analysis: Statistics was performed using the Sigmastat-version 2.03 software and results were expressed as mean ± SE. Differences were considered significant at $p < 0.05$.

RESULTS:

General observations

The animals were aphagic and adipsic for many hours following the administration of MPTP. Autonomic responses included piloerection, salivation and increased respiration started after 10 minutes and lasted for about 30minutes after the MPTP injection. The administration of MPTP (30mg/kg, twice, 16 hr apart) did not significantly reduce the body weight (Table-3-1, n=8). All the animals treated with MPTP exhibited Straub tail phenomenon (elevation of the tail is kept above 45°) after the neurotoxin injection. MPTP treated mice showed sialorrhea (increased salivation) and hyperpnoea (increased respiration). Few animals showed convulsions following the first MPTP injection. We also observed a small number of mortality (up to 5%) in the MPTP experimental group after the first injection.

EFFECT OF MPTP ON BEHAVIOR:

Tremor: C57BL/6 mice injected with MPTP showed toxin-induced tremor. The tremor response was observed at approximately 10 min after the MPTP injection and lasted for a maximum period of 30 min. Control animals exhibited no tremor (Table-3-1, n=8). The central nervous system (CNS) responses included increased motor activity, many of the

features of 5-HT syndromes and akinesia and catalepsy, which started around 3-5 minutes and lasted for about 30-45 minutes.

Catalepsy is knows as rigidity or inability to correct an externally induced posture of the hind limbs when placed on 2 inch wooden block. Catalepsy was observed in the mice treated with MPTP (Table-3-1, n=8, $p < 0.05$).

Akinesia: MPTP administration caused akinesia (impaired ability to initiate movements) and there by causes significant increase in the latency in seconds (akinetic effect), as compared to the control animals (Table-3-1, n=8, $p < 0.05$).

Tail hanging test: MPTP treated mice significantly decreased the number of attempts during tail hanging (Table-3-1, n=8, $p < 0.05$).

Effect of MPTP on rotorod in mice: The Rotorod apparatus is widely used to measure coordinated motor skills in the MPTP mouse model. All mice were trained on the rotating rod at a speed of 2.5 to 20 rpm for 300 s. MPTP treated mice exhibited significant decrease in the retention time on the rod (less time spent on the rotating rod, indicating a loss of motor coordination) as compared with the control group (Figure-3-1-A, n=8, $p < 0.05$).

Effect of MPTP in Open field: We further recorded the locomotor behaviors in the open field for 5 minutes. Administration of MPTP significantly reduced spontaneous locomotor activity and exploratory behavior in mice. The MPTP mice showed significant decrease in ambulation, fine movements, rearing and total movement when compared to control mice (Figure-3-1-B, n=8, $p < 0.05$).

Effect of MPTP on Effect of MPTP on Swim test: The animals injected with MPTP showed significant long-lasting motor impairment as shown by significant decrease in swim scores (Figure-3-1-C, n=8, $p < 0.05$). **Gait analysis:** Controls and MPTP treated mice were observed for gait abnormalities on 4th day of the final MPTP injections. The animal stride length and gait pattern was consistent in the control, but the stride length was significantly decreased in the animals that received MPTP (Figure-3-1-D, n=8, $p < 0.05$).

EFFECT OF MPTP ON BIOCHEMICAL ASSAY:

Effect of MPTP on ROS: MPTP-induced a marked significant increase in the ROS generation as compared to control animals (Figure-3-2-A, n=8, $p < 0.05$).

EFFECT OF MPTP ON GSH: GSH content were analyzed in both normal and MPTP treated mice. MPTP treated mice shows a significant decrease in GSH content (Figure-3-2-B, n=8, $p < 0.05$).

Effect of MPTP on GSH-px activity: GSH-Px activity in MPTP-treated mice was significantly increased as compared with control group (Figure-3-2-C, n=8, $p < 0.05$).

MAO ACTIVITY: MPTP had no effect on the total MAO activity (Figure-3-2-D, n=8, $p < 0.05$).

Effect of MPTP on complex-I activity: Systemic injection of MPTP exhibited significant inhibition of complex-I activity in the crude whole brain homogenate. (Figure-3-2-E, n=8, $p < 0.05$).

EFFECT OF MPTP ON NEUROTRANSMITTERS:

Administration of MPTP caused a significant decrease in the concentration of dopamine in the striatum (Figure-3-3-A, n=8, $p < 0.05$). Dopamine, DOPAC and HVA turnover was significantly altered in striatum (Table-3-2-B, n=8, $p < 0.05$). MPTP also affected levels of other striatal neurotransmitters (NE, 5-HT, 5HIAA) and dopamine metabolites (DOPAC, HVA and 3-MT) as shown in table-3-2. Diquat and/or salsolinol did not deplete/decrease the striatal dopamine content significantly. However, MPTP caused significant dopamine depletion in the striatum (Figure-3-3-C). We also measured the GABA and glutamate levels in the striatum. MPTP significantly increased the striatal GABA levels (Figure-3-3-D, n=8, $p < 0.05$).

Effect of MPTP on TH-IR and GFAP:

Representative microphotographs of TH immunostaining in the substantia nigra and striatum are shown in Figure-3-4. Tyrosine hydroxylase (TH) immunoreactivity was studied in the cell body region, SN and the terminal region, NCP. MPTP injected SN region showed significantly decrease in TH positive neurons, Figure-3-4. MPTP injected animals showed a significant loss in the TH-immunoreactivity GFAP: Representative microphotographs of GFAP immunostaining in the striatum are shown in figure 3-4. GFAP-positive astrocytes were evident in the substantia nigra MPTP-treated mice. Following the MPTP-treatment, the reaction of astrocytes was increase in the cell number in these regions. This effect was more pronounced in the striatum (maximal increase compared to control) than in the SN (Figure-3-4).

DISCUSSIONS:

Diquat is used commonly by farmers and home gardeners and its systemic adverse effects are well-known. It is shown to induce oral mucosal responses, dermatitis, hepatic and renal failure in humans (Adams et al., 2009, Tanen et al., 1999). Administration of diquat to mice causes liver and kidney damage as seen by lipid peroxidation, altered levels of blood urea nitrogen and activity of serum alanine aminotransferase. Similarly, salsolinol also has shown to induce various neurotoxic effects (in vitro). However, the diquat and/or salsolinol did not decrease the dopamine content in the striatum. However, MPTP caused significant dopamine depletion. Hence, MPTP treated mice were considered as an valid animal for Parkinson's disease in the present study.

MPTP is well known to damage the nigrostriatal dopaminergic pathway as seen in Parkinson's disease. MPTP is a potent neurotoxin that produces parkinsonian syndromes secondary to nigral dopaminergic neuronal damage (Davis et al., 1979; Burns et al., 1983; Ballard et al., 1985; Mohanakumar et al., 2000; McGeer and McGeer, 2004; Watanabe et al., 2005; Dhanasekaran et al., 2008a). MPTP has been shown to cause severe depletion of striatal DA in rodents (Chiueh et al., 1984a; Chiueh et al., 1984b; Heikkila et al., 1984b; Heikkila et al., 1984a; Heikkila et al., 1984c; Mitra et al., 1992, 1994; Muralikrishnan and Ebadi, 2001; Dhanasekaran et al., 2008b). MPTP (non-toxic form) is converted to its active metabolite MPP^+ (toxic form) by (MAO-B) monoamine oxidase-B. This MPP^+ is then selectively taken up by the dopaminergic terminals at the

striatum and exerts its neurotoxic effect (Javitch and Snyder, 1984). This shows that activity of MAO-B in the brain is critical for the neurotoxicity of MPTP (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2003). MPP^+ is shown to produce oxidative stress and it also inhibits NADH ubiquinone reductase in the mitochondria leading to ATP loss (Nicklas et al., 1985; Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2003). MPP^+ involve generation of reactive oxygen species, and free radicals are implicated in the mechanism of MPTP-induced neurotoxicity in animals (Chiueh, 1994; Chiueh et al., 1994; Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2003). Especially generation of .OH *in vivo* in the striatum of rats has been shown following MPP^+ administration (Chiueh et al., 1994; Sairam et al., 2003; Banerjee et al., 2006; Banerjee et al., 2008). The major antioxidant molecules in the brain are GSH, CAT and SOD. The dismutation of superoxide anions to hydrogen peroxide by the action of CAT or by the oxidation of GSH in presence of GSH-Px. Induction of ROS such as superoxide anion or .OH or H_2O_2 result in enhanced synthesis of antioxidant molecules as a defense mechanism. Elevation of ROS is the basic molecular mechanism underlying the pathophysiology of Parkinson's disease, and thus the postmortem brain exhibited changes in the antioxidant molecules (Yokoyama et al., 2008). Tyrosine hydroxylase a marker of the nigrostriatal dopaminergic pathway and TH-immunohistochemical study indicated that TH immunoreactive neuron of substantia nigra and neostriatum were markedly reduced after MPTP treatment (Sundstrom et al., 1987; Mori et al., 1988; Kurkowska-Jastrzebska et al., 1999). MPTP treatment also causes a

large increase in the astrocyte protein, glial fibrillary acidic protein (GFAP) in the striatum after the MPTP treatment.

Behavioral manifestation in the MPTP mouse model, such as changes in general locomotor activity such as catalepsy, akinesia, tail hanging display deficits as behavioral parameters that are significantly correlated with the loss of striatal dopamine (Muralikrishnan et al., 2004; Ghorayeb et al., 2005; Luchtman et al., 2009). In addition, these behavioral measures are correlated to dopamine transporter, vesicular monoamine transporter, and tyrosine hydroxylase expression (Chiueh et al., 1984a; Tillerson et al., 2002). The acute MPTP regimen shows impaired rotorod performance, swimming ability, abnormal foot print patterns and decreased open field activity which was in contrast with the earlier reported impairment after MPTP treatment due to dopamine loss (Mitra et al., 1992; Mohanakumar et al., 2000). Following MPTP administration the antioxidant molecules have also been shown to be altered significantly due to MPTP toxicity, GSH-Px activity being increased and GSH being decreased.

Oxidative stress is a one of the major molecular mechanisms underlying in the MPTP-induced dopaminergic neurodegeneration. In the brain, MPTP is converted to its active metabolite MPP^+ by MAO-B and this is taken up by the dopamine transporter into neurons and exerts its neurotoxic effect. Due to this MAO-B inhibitor can abolish MPTP induced neurodegeneration (Kinemuchi and Arai, 1986; Perez et al., 2000; Muralikrishnan et al., 2003). The specificity to dopaminergic neurotoxicity of MPP^+ is

believed to be due to the selective uptake of MPP^+ by dopaminergic neuron in the SN (Heikkila et al., 1984a; Heikkila et al., 1984c; Langston et al., 1984) *via* a dopamine uptake mechanism (Javitch and Snyder, 1984; Dhanasekaran et al., 2008b). MPP^+ inhibiting the NADH ubiquinone reductase in the mitochondria and the ATP synthesis cause in resulting in loss of membrane potential leading to energy crisis and neurodegeneration (Nicklas et al., 1987; Tipton and Singer, 1993; Nakamura et al., 2000).

In the present study MPTP induced generation of ROS following systemic administration of MPTP. This clearly establishes the involvement of ROS in the neurotoxicity caused by MPTP (Maruyama, 2001; Przedborski and Ischiropoulos, 2005). Free radicals are implicated in the pathophysiological action of MPTP, and involved in generation of reactive oxygen species (ROS) including superoxide anion, H_2O_2, as well as .OH (Thomas et al., 2000; Muralikrishnan et al., 2003; Przedborski and Ischiropoulos, 2005; Banerjee et al., 2008). The enzymatic conversion of MPTP to MPP^+ has been shown to generate O_2^- and .OH in mitochondria (Zang and Misra, 1992; Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2003). These very reactive oxyradicals are all capable of damaging a number of biological molecules such as DNA, RNA, fatty acids, proteins, *etc.* Thus, brain is equipped with its own defense mechanism involving antioxidant enzymes (SOD, CAT, GSH-Px, *etc.*) and scavenging molecules (GSH, ascorbate, α-tocopherol, *etc.*) which act in concerts to prevent the excessive accumulation of these potential oxyradicals. Any change, that is decrease in antioxidant molecule or increase in free radicals, overwhelm the antioxidant defense mechanism leading to chaos

resulting in oxidative stress. We report here alterations of a number of antioxidant molecules in the whole brain region of the dopaminergic pathway in the brain as a consequence of MPTP treatment.

Mitochondrial glutathione is known to protect this organelle from oxidative insult (Dhanasekaran et al., 2006). GSH play an important role in the defense against endogenous membrane peroxidation and subsequent changes by reducing H_2O_2 *via* GSH-Px. In our study, MPTP caused a significant decrease of GSH. This may represent the neurotoxicity caused by the neurotoxin and indicate a direct role of GSH in neurotoxicity by MPTP. The present study, clearly establishes a direct involvement of ROS and related antioxidant molecules in the neurotoxic action of MPTP, referring to experimental parkinsonism in C57BL/6 mice. We also report here that MPTP does not inhibit the total MAO activity in crude mitochondrial fraction following systemic administration in the whole brain regions.

In the present study, MPTP treatment caused significant DA depletion and DA turnover was significantly increased in the striatum and DOPAC level and HVA decreased in MPTP treated mice indicating severe DA-ergic toxicity. The decrease in DA and DOPAC levels as observed following MPTP is similar to reported in literature (Chiueh et al., 1984a; Chiueh et al., 1984b; Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Uthayathas et al., 2007). In the present study, similar to earlier reports the turnover of DA increased in NCP (Heikkila et al., 1984b; Heikkila et

al., 1984a; Sundstrom et al., 1987). HVA has shown to be affected following MPTP. These changes concurred with increased DOPAC/DA and HVA/DA ratios. The increased metabolite to DA ratios suggested that more DA was released from surviving nigrostriatal terminals and utilized (turned-over), a common compensatory mechanism observed in PD patients and MPTP treated mice. DA turnover was most strongly increased in the MPTP treatment. 5-HT turnover (5-HIAA/5-HT) was also increased, which was in line with previous findings in mice (Rozas et al., 1998), suggesting that this could be a compensatory mechanism in the striatum as well (Gaspar et al., 1993; Rozas et al., 1998). Striatal 5-HT was decreased as well, possibly as a result of increased 5-HT turnover, as metabolite levels (5HIAA).

GABA neurons are abundant in the nigrostriatal tract as the dopaminergic neuron. In normal mice, the striatal neurons activity is controlled by the inhibition of nigra dopaminergic neurons. The balance in basal ganglia-thalamo-cortical circuitry is broken and too much inhibition from the thalamus and cortex circuit cause expression of parkinsonian syndrome. MPTP-treatment increases GABA release within the globus pallidus. Evidence indicates that abnormal GABA neurotransmission may be implicated in the pathophysiology of Parkinson's disease and dopaminomimetic-induced dyskinesias (Calon et al., 2000). Glutamate decarboxylase (GAD65 and GAD67) are the two isoforms of the GABA-synthesizing enzyme. GAD67 mRNA was increased in the nucleus caudatus putamen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys (Soghomonian et al., 1996). Earlier studies have shown decrease of

dopamine-activity and increase GABA activity in the striatal neurons. The results give insight as to how dopamine and GABA function within the striatum with respect to the development of neuronal abnormalities(Yamamoto and Kawana, 1991; Otto and Unsicker, 1993). In monkey, MPTP also causes striatal dopamine denervation, decreased D1, elevated D2 and GABAA receptors, as well as the decrease of the D1/D2 receptor ratio in the posterior striatum (Gagnon et al., 1993).

Tyrosine hydroxylase a marker of the nigrostriatal dopaminergic pathway and TH-immunohistochemical study indicated that TH immunoreactive neuron of substantia nigra and neostriatum were markedly reduced after MPTP treatment (Sundstrom et al., 1987; Mori et al., 1988; Kurkowska-Jastrzebska et al., 1999). MPTP also cause a large increase in the astrocyte protein, glial fibrillary acidic protein (GFAP). GFAP-positive astrocytes were evident in the striatum after the MPTP treatment. These results provide valuable information for the pathogenesis of acute stage of Parkinson's disease (O'Callaghan et al., 1990; Francis et al., 1995). Recent evidence shows that glial-related response plays a key role in the MPTP neurotoxic process and the blockade of glial activation may be a new therapeutic approach, which has applicability for Parkinson's disease (Yokoyama et al., 2008; Schintu et al., 2009). GFAP-positive astrocytes exhibited a ramified form with many fine processes in the striatum and markedly increased in this region. These results suggest that increases in GFAP staining produced by MPTP in the striatum are linked to decrements in TH staining which also suggest that factors originating in the damaged dopamine neurons initiated the astrocyte reaction to MPTP.

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LEGENDS:

Table-3-1: Effects of MPTP on the behavior: C57BL/6 was treated with MPTP (30 mg/kg). Saline treated animals served as control. Eight animals were treated in each group. MPTP treated animals differed significantly (*) from the control (One way ANOVA). [A] Body weight was recorded in interval of 3 days, [B] 5min after MPTP, [C] Following three and half hour after MPTP and [D]on 4th after final injection. ND = not detected. Tremor was visually monitored in a double blind study. MPTP caused a good tremor of approximately 3 in 5min. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ vs. control). ND= not detected.

Table-3-2: Animals were sacrificed on 5th day and nigrostriatal monoamine, were analyzed using HPLC-ECD. *$p \leq 0.05$, significant change as compared to the control. Values represent pmol/mg tissue. Mean ± SEM (n = 8).

Figure: 3-1-A: Effect of MPTP on rotorod in mice: Animals were tested on the rotorod 3rd day after the last MPTP injection. In the testing session, animals were tested on 3 successive trials Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$; vs. control).

Figure-3-1-B: Effect of MPTP on open field in mice: Animals were tested on the open field on 3rd day after the last MPTP injection. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$; vs. control).

Figure-3-1-C: Effect of MPTP on swim test: Effect of swimming ability in the saline/ or MPTP mice was tested in warm water (27 ± 2 °C) on the 4th day following the final injection of MPTP (30 mg/kg, i.p., twice, 16 h apart). Swim-scores were recorded on a performance intensity scale of 1–4 for all the animals for 10 min. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$; vs. control).

Figure-3-1-D: Effect of MPTP on gait analysis: Gait abnormalities were analyzed on 4th day of treatment.The stride length was measure between hind and fore limbs. Stride length was measured in cm. Results given are mean ± S.E.M., $n = 8$ ($p \leq 0.05$; vs. control).

Figure-3-2-A: Effect of MPTP on ROS: Effects of MPTP on production of intracellular ROS in crude brain homogenate. Values given are relative fluorescent per milligram tissue and are represented as mean ± S.E.M. *$P \leq 0.05$ as control groups compared with MPTP treated group (n=6).

Figure-3-2-B: Effect of MPTP on GSH: Mice were treated with MPTP (30mg/kg, i.p.) twice 16 hr apart. Animals were sacrificed on the fifth day and reduced glutathione (GSH) was estimated in the whole brain by a sensitive fluorimetric procedure. Values given are nanogram per milligram tissue and are represented as mean ± S.E.M. *$P \leq 0.05$ as control groups compared with MPTP treated group (n=6).

Figure-3-2-C: Effect of MPTP on GSH-Px: Mice were treated with MPTP (30mg/kg, i.p.) twice 16 hr apart. Animals were sacrificed on the fifth day and GSH-Px activity was estimated. Values given are GSH-Px activity per milligram tissue and are represented as mean ± S.E.M. *$P \leq 0.05$ as control groups compared with MPTP treated group (n=6).

Figure-3-2-D: Effect of MPTP on MAO activity: Mice were treated with MPTP (30mg/kg, i.p.) twice 16h apart and sacrificed on 5^{th} day following the second injection. Total MAO activities were assayed and are expressed as in nmol 4-HQ/mg protein/h in enzyme activity and are represented as mean ± S.E.M. *$P \leq 0.05$ as control groups compared with MPTP treated group (n=6).

Figure-3-2-E: Effect of MPTP on complex-I activity: Mice were injected with saline/ or MPTP (30mg/kg, i.p.) twice 16 hr apart, sacrificed 5^{th} day and analyzed for complex-I activity. The specific activities of the enzyme are given as nanomole of NADH oxidized/min/mg protein and are represented as mean ± SEM. *$P \leq 0.05$ significantly different as compared to control side, which received vehicle ($n = 6$ in each group).

Figure-3-3-A&B: Animals were sacrificed on 5^{th} day and nigrostriatal dopamine, were analyzed using HPLC-ECD. (DOPAC+HVA)/DA indicated the turnover of DA.

Figure-3-3-C: Effect of diquat, diquat + salsolinol on striatal dopamine content: Animals were sacrificed on 5th day and nigrostriatal dopamine, were analyzed using HPLC-ECD. *$p \leq 0.05$, significant change as compared to the control. Values represent pmol/mg tissue. Mean ± SEM (n = 8).

Figure-3-3-D: Effect of MPTP on Glutamate and GABA content: Animals were sacrificed on 5th day and nigrostriatal glutamate and GABA, were analyzed using HPLC-ECD. *$p \leq 0.05$, significant change as compared to the control. Values represent pmol/mg tissue. Mean ± SEM (n = 8).

Figure-3-4: Effect of MPTP on TH and GFAP: Cryosection coronal sections from substantia nigra region were process for tyrosine hydroxylase activity. Photomicrographs images shows coronal sections through the SN were stained for TH, immunoreactivity and GFAP.

TABLE AND FIGURES:

TABLE-3-1:

Behavioral Parameters	CONTROL	MPTP
Body weight (in gms)[A]	25 ± 0.42	23.8 ± 0.55
Straub tail[B]	ND	Detected
Tremor[B]	ND	Detected
Akinesia (in sec)[C]	1.7 ± 0.21	13.6 ± 1.62*
Catalepsy (in sec)[C]	1.9 ± 0.23	4.8 ± 0.63*
Tail Hanging (in min)[D]	24.7 ± 0.73	14.5 ± 1.01*

Table-3-2:

Neurotransmitters	Control	MPTP
DOPAC Turnover	1.50 ± 0.19	1.94 ± 0.13*
HVA Turnover	0.56 ± 0.03	1.02 ± 0.10*
NE	13.09 ±1.15	3.23 ± 0.34*
5-HIAA	6.84 ± 0.45	5.63 ± 0.20
5-HT	3.10 ± 0.32	2.24 ± 0.14
3-MT	9.57 ± 1.34	2.28 ± 0.22*

Figure: 3-1

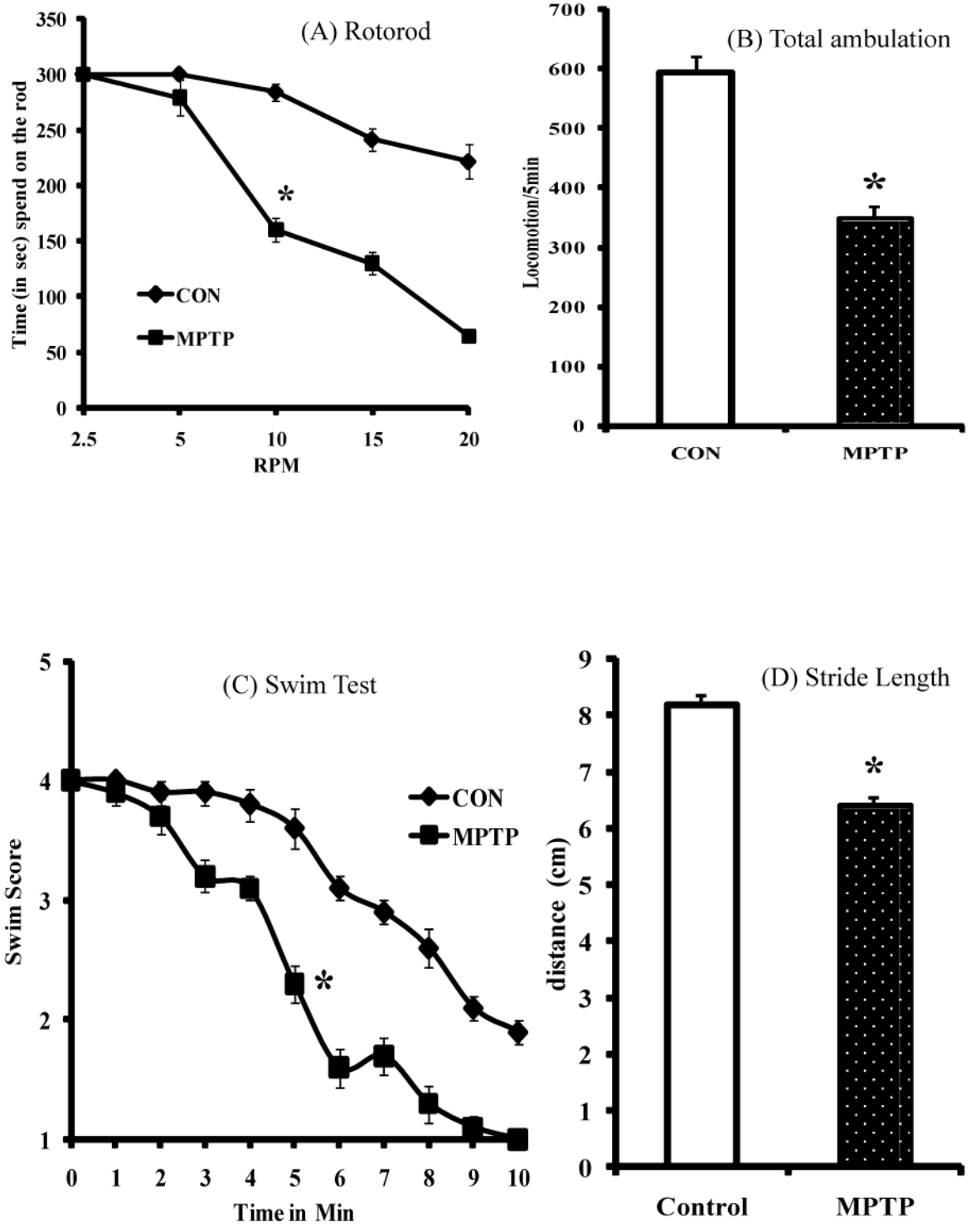
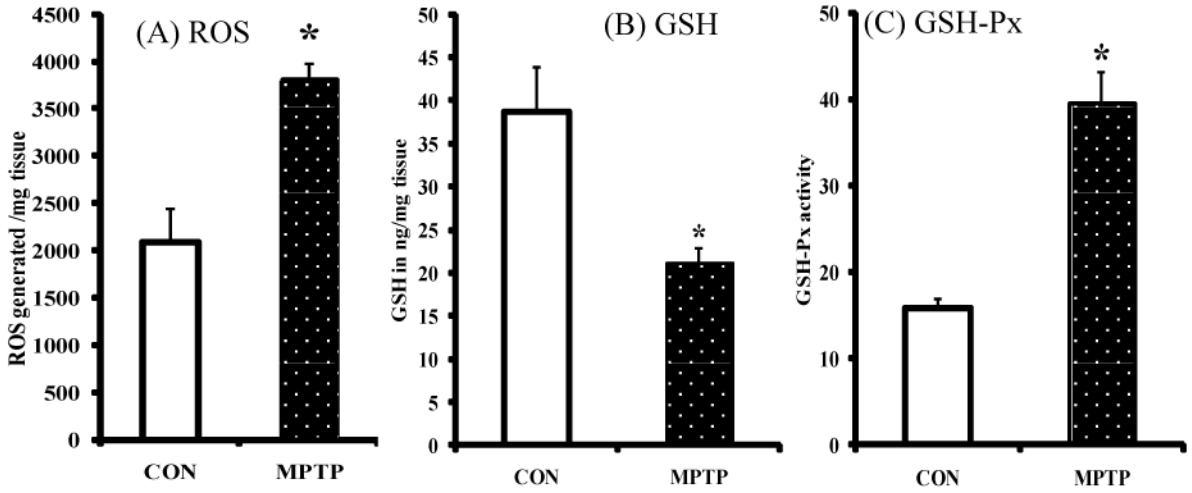

(A) Rotorod
Time (in sec) spend on the rod
350
300
250
200
150
100
50
0
2.5
5
10
15
20
RPM
CON
MPTP
*
(B) Total ambulation
Locomotion/5min
700
600
500
400
300
200
100
0
CON
MPTP
*
(C) Swim Test
Swim Score
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
Time in Min
CON
MPTP
*
(D) Stride Length
distance (cm)
9
8
7
6
5
4
3
2
1
0
Control
MPTP
*

Figure-3-2


(A) ROS
ROS generated /mg tissue
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CON
MPTP
*
(B) GSH
GSH in ng/mg tissue
50
45
40
35
30
25
20
15
10
5
0
CON
MPTP
*
(C) GSH-Px
GSH-Px activity
50
40
30
20
10
0
CON
MPTP
*

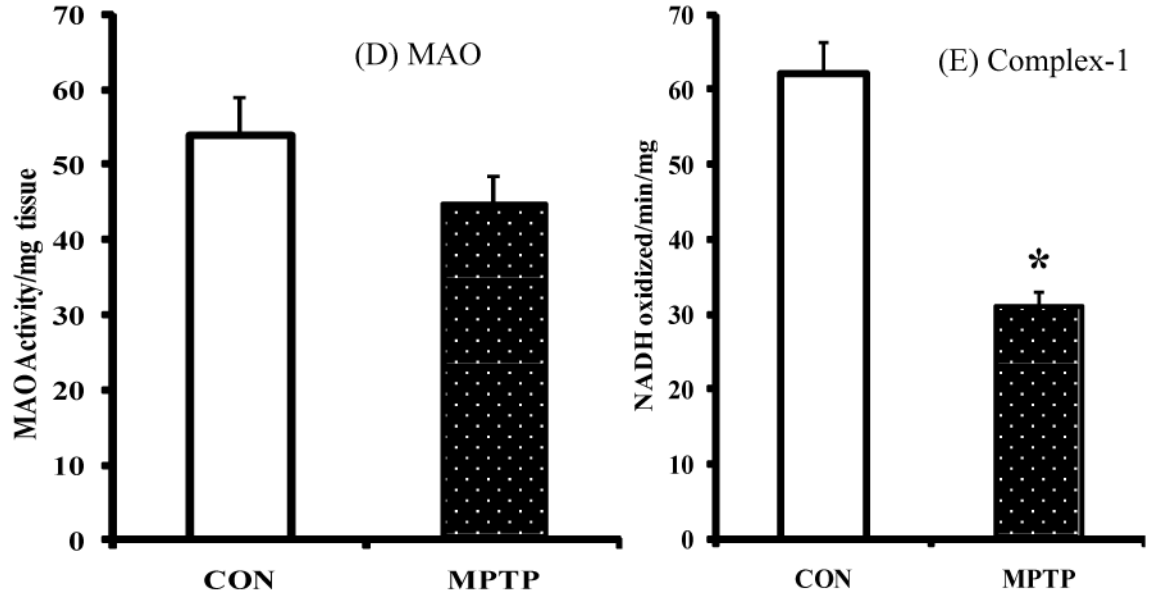
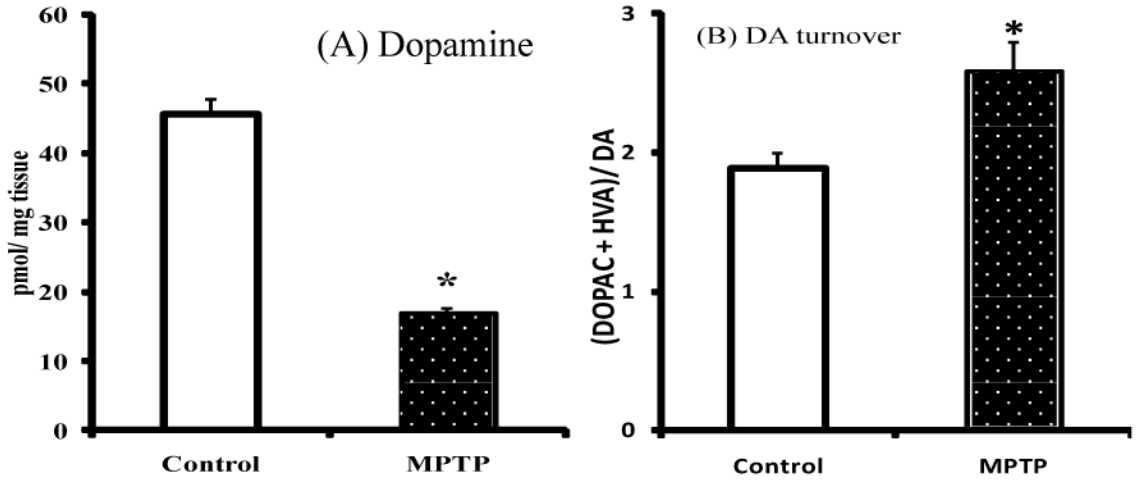

(D) MAO
MAO Activity/mg tissue
70
60
50
40
30
20
10
0
CON
MPTP
(E) Complex-1
NADH oxidized/min/mg
70
60
50
40
30
20
10
0
CON
MPTP
*

Figure-3-3


(A) Dopamine
pmol/ mg tissue
60
50
40
30
20
10
0
*
Control
MPTP
(B) DA turnover
(DOPAC+ HVA)/ DA
3
2
1
0
*
Control
MPTP

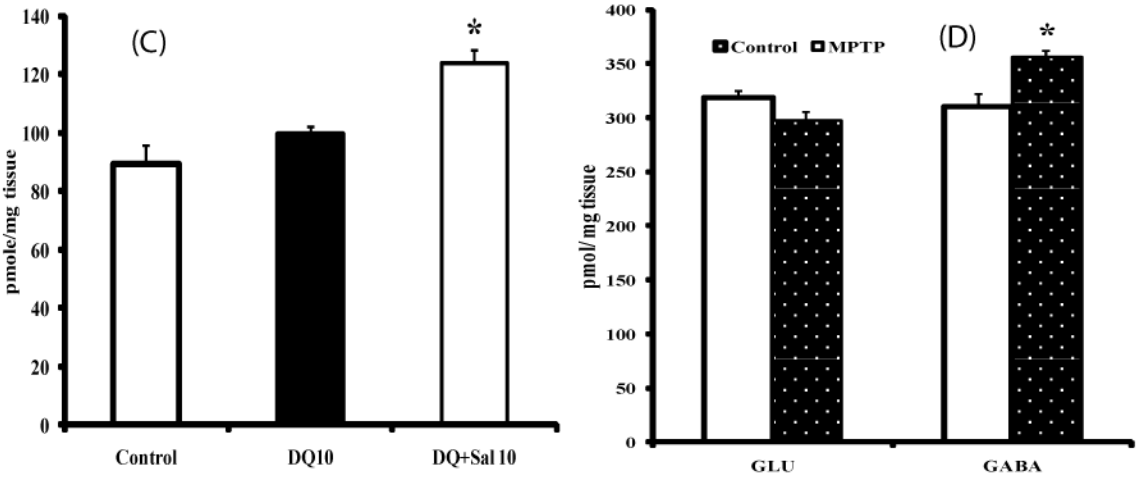
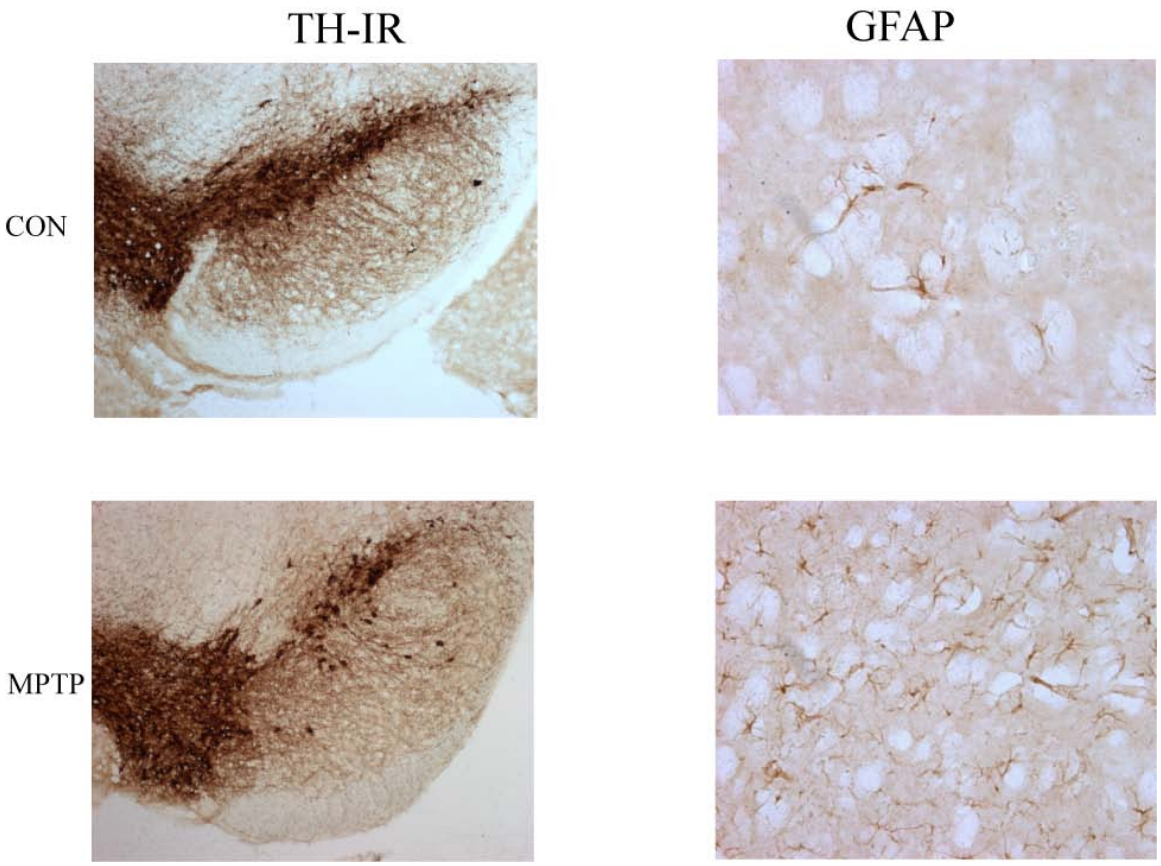

(C)
pmole/mg tissue
140
120
100
80
60
40
20
0
*
Control
DQ10
DQ+Sal 10
Control MPTP
(D)
pmol/ mg tissue
400
350
300
250
200
150
100
50
0
*
GLU
GABA

Figure-3-4



4. INVESTIGATE THE NEUROPROTECTIVE MECHANISMS OF CAFFEINE AGAINST MPTP-INDUCED NEUROTOXICITY

INTRODUCTION:

Accumulating pre-clinical and clinical studies suggests that adenosine A_{2A} receptors (A_{2A} R) may be a potential non-dopaminergic therapeutic target for Parkinson's disease (Chen et al., 2001a). Adenosine receptors are class of purinergic receptors, that belongs to G-protein coupled receptors (Kalda et al., 2006). Currently, there are four subtypes that has been characterized namely, A1, A2A, A2B and A3. A1 and A2A receptors have high affinity for the ligand adenosine and thus, these receptors are responsible for the neurobiological function of adenosine in brain. The A1 receptors subtypes are widely distributed in the brain and peripheral organs, while A2A receptors are abundantly present in striatum. caffeine (1,3,7-trimethylxanthine), a major component of coffee, is a psychoactive drug due to its stimulant properties (George et al., 1999; Fisone et al., 2004) which contains polyphenolic antioxidants (Nettleton et al., 2009). Humans consume moderate amounts of caffeine which increases alertness, reduces fatigue, improves performance on vigilance tasks, complex tasks and in better mental functioning(Smith, 2002). Over the past decade, numerous epidemiological and

experimental evidences support the beneficial effects of A2A receptor antagonists in Parkinson's disease.

MPTP is a potent neurotoxin, an analogue of the narcotic mepiridine (Burns et al., 1983; Langston et al., 1983; Langston et al., 1984) that causes selective nigral dopaminergicergic lesions resulting in irreversible parkinsonism in humans and monkeys. It also causes depletion of dopamine when administered to rodents (Heikkila et al., 1984b; Langston et al., 1984; Muralikrishnan and Ebadi, 2001; Muralikrishnan et al., 2002; Muralikrishnan et al., 2003,Forno et al., 1993). Once inside the brain MPTP is converted to 1-methyl-4-phenyl-pyridinium (MPP^+) by glial cells, and MPP^+ is taken inside the neurons through DA and serotonin transporters (Javitch et al., 1985; Bezard et al., 1999). Inside the cells, MPP^+ concentrates in mitochondria by a mechanism that relies on the mitochondrial transmembrane potential (Ramsay and Singer, 1986; Tipton and Singer, 1993). Within the mitochondria, it inhibits complex-I of the electron transport chain leading to ATP depletion (Tipton and Singer, 1993). Reactive oxygen species (ROS) is also implicated in dopaminergic toxicity caused by iron (Mohanakumar et al., 1994; Mohanakumar and Steinbusch, 1998) which also includes ROS production and subsequent cell death (Gonzalez-Polo et al., 2003; Gonzalez-Polo et al., 2004). MPTP administration into mice has been shown to produce clear apoptotic morphology in SNpc (Tatton and Kish, 1997). The major antioxidant molecules in the brain are GSH, CAT and SOD. Induction of ROS such as superoxide anion or .OH or H_2O_2 results in enhanced synthesis of antioxidant molecules act as a defense mechanism. Tyrosine hydroxylase a

marker of the nigrostriatal dopaminergic pathway and TH-immunohistochemical study indicated that TH immunoreactive neuron of substantia nigra and neostriatum were markedly reduced after MPTP treatment (Sundstrom et al., 1987; Mori et al., 1988; Kurkowska-Jastrzebska et al., 1999). MPTP treatment also causes a large increase in the astrocyte protein, glial fibrillary acidic protein (GFAP).in the striatum after the MPTP treatment. Thus, MPTP animal model have shown to reproduce, both symptomatic, biochemical, neurochemical features of Parkinson's disease. Motor dysfunction is associated with degeneration of basal ganglia. Neurodegeneration of nigrostriatal system produce akinesia, rigidity and catalepsy (Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Muralikrishnan et al., 2003). Understanding behavioral abnormalities and neurotransmitters changes in the brain provides a better background to design novel drugs and neuroprotective strategies. We investigated the different neuroprotective mechanisms of Caffeine against MPTP-induced neurotoxicity.

MATERIALS AND METHODS:

Animals and chemicals: C57/BL-6 mice (body weight, 20–25 g; age, 8–10 weeks) were purchased from Harlem and were housed with a 12 h day/night light cycle with free access to food and water. All procedures involving animals were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee. The following chemicals/drugs were purchased from Sigma Chemical Company (St. Louis, MO., USA): Caffeine, 3-hydroxytyramine hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA), 5-

hydroxytryptamine creatinine sulphate (5-HT), norepinephrine (NE), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), 1-heptane sulfonic acid (HSA) sodium salt, HPLC-grade acetonitrile (ACN), HPLC-grade methanol, Triton-X 100, 4-hydroxyquinoline (4-HQ), bovine serum albumin (BSA), kynuramine, ethylenediaminetetraacetic acid (EDTA) sodium salt, O-phthaldialdehyde (OPT), Tris buffered saline tablet, Phosphate buffered saline tablet, Reduced glutathione (GSH), Dimethyl sulphoxide (DMSO), hydrogen peroxide (H_2O_2) and 3,3 -N-diaminobenzidine tetrahydrochloride (DAB). Anti-rabbit tyrosine hydroxylase polyclonal, Anti-GFAP and anti-rabbit IgG-conjugated horseradish peroxidase antibodies were purchased from Chemicon. Reverse osmosis water was used in all experiments. For HPLC analysis, double–distilled water was filtered and deionized using Milli-Q system (Waters, Milford, MA).

Experiment design and Treatments: C57/BL-6 mice were intraperitoneally administered with [i] Control (Saline) [ii] MPTP. (30 mg/kg, i.p., twice 16 h apart), [iii] Caffeine (20mg/kg), [iv] MPTP+ Caffeine (20mg/kg) and [v] MPTP+ Caffeine (10mg/kg). Based on the group, saline or Caffeine was pretreated for 7 days and followed by two dose of MPTP (30 mg/kg, i.p., twice 16 h apart). Caffeine was dissolved in sterile water.

BEHAVIORAL STUDY:

Body weight and general observation: The body weights of the animals were recorded in an interval of 3 days by using digital weighing scale. The general observations included tremor, piloerection, salivation and increased respiration after every injection

Straub tail: 3hr Straub tail is defined as elevation of tail to more than 45° with a curvature of the tail. The results are expressed as either straub tail phenomenon detected or not detected. (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2002).

Akinesia: Akinesia was measured by noting the latency of the animals to move all four limbs in unit time seconds(s) and the test was terminated if the latency exceeded 180s. The animals were initially acclimatized in akinesia cages which consisted of a wooden platform with ventilated perspex hoods. (Muralikrishnan and Mohanakumar, 1998).

Catalepsy: The term catalepsy implies to correct the externally imposed posture in an animal. In the saline and MPTP treated mice catalepsy was measured by placing the animals on a flat horizontal surface with both the hind limbs on a square wooden block (3 cm, high) and the latency in seconds was measured to move the hind limbs from the block to the ground. The animals were initially acclimatized on the wooden platform (Muralikrishnan and Mohanakumar, 1998)

Swim test: Swimming ability was carried out on 4th day after the saline or MPTP injections. In brief, the mice were placed in water tubs (40 l x 25 w x 12 h, in cm) and the depth of water was kept at 8 cm with temperature maintained at 27 ± 2° C. The animals were acclimatized for 10 min one day prior to experimentation. Swim score scales were given from range of 1 to 4 based on their swimming pattern. Swim score scales were: 1 = hind part sinks with head floating, 2 = occasional swimming using hind limbs while floating on one side, 3 = occasional floating/swimming only, 4= continuous swimming. (Muralikrishnan and Mohanakumar, 1998)

Tail Hanging Test: The saline and MPTP treated mice were suspended by holding the tail and recorded for one minute and the observers were blind to the treatment conditions. The record was made by counting the number of attempts made to reach the tail by bending the body and crawling up towards its tail. The tail hanging was measured for one minutes and results are expressed as number of attempts/ minutes (Jiao et al., 2005).

Open-field: The open-field apparatus consist of a box with clear plexi-glass walls and floor. The effects of saline and MPTP on motor activity was evaluated in the open-field. An automated sensor system, consisting of 16 photo beams per side measured the total ambulation, fine movement and rearing. Each mouse will remain in the field for a total of 5 minutes. (Holcomb et al., 2006).

Rotorod: Saline and or MPTP treated mice were analyzed for motor coordination using the rotorod tests (Columbus Instruments, Columbus, OH). Mice were initially adapted for a period consisting of 3 training trials on the rotorod with a 10-minute interval. The mice will be given a 10 minute resting time in between each testing run. The rotation speed of the rod accelerated gradually from 2.5 to 20 rpm over the first 5 minutes and was held at 20 rpm for the final minute of testing. The time (seconds) spent on the rod (fall latency) will be recorded for each animal and used as a measure of motor function.

Gait analysis: Briefly, saline and or MPTP mice were made to walk on an inclined pathway (100 cm (l) × 12 cm (w) × 10 cm (h) with 30° inclination) leading to a darkened enclosure. The pathway was lined with white paper and the fore- and hind-paws of the mice were dipped in two different non-toxic watercolors to record the footprints and then the animals were allowed to climb the gangway. After the experiment the limbs were cleaned and dried before placing them back in the cage. The recorded footprints were analyzed for stride length, and stride width.(Fernagut et al., 2002)

BIOCHEMICAL ASSAY:

Tissue Preparation for biochemical assays: Animals were sacrificed by decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes, and other antioxidant molecules. The brain tissue was homogenized in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon homogenizer. Tissue homogenates were centrifuged at 10,000 g for 60 min at 4°C

NADH Ubiquinone oxidoreductase (Complex - I) assay: The decrease in the absorbance due to oxidation of NADH at 340 nm was monitored. The reaction mixture contained potassium phosphate buffer pH 7.4, coenzyme Q_0 and crude homogenate from saline and MPTP treated mice. The reaction was initiated by the addition of NADH and the rate of decrease in the absorbance was monitored at 340 nm. A standard curve for NADH was obtained at 340 nm by using known quantities of NADH. The complex-I activity is expressed as the amount of NADH oxidized/mg protein (Muralikrishnan and Mohanakumar, 1998).

Monoamine oxidase (MAO) assay: This enzyme is responsible for the oxidation of biogenic amines. Fluorimetric assay procedure was used for the estimation of MAO (EC 1.4.3.4) activity. The amount of 4-hydroxyquinoline formed due to oxidation of kynuramine by MAO represents the enzyme activity. Incubation mixture contains crude homogenate, potassium phosphate buffer, pH 7.4 and the reaction was initiated by the addition of kynuramine. The reaction was then terminated by addition of ice-cold 0.4N perchloric acid after 1hour. The mixture was centrifuged (14, 000 x g for 5 min) and 2 ml of 1 N NaOH was added to one ml supernatant of the reaction mixture. Fluorescence intensity was measured at activation/emission wavelengths of 315/380 nm. The specific enzyme activity is expressed as nmol of 4-hydroxyquinoline formed/h/mg protein. A standard curve was obtained for commercially available 4-hydroxyquinoline. (Muralikrishnan and Mohanakumar, 1998)

GSH estimation: GSH were measured fluorimetrically (Muralikrishnan and Mohanakumar, 1998). This method employs OPT condensation reaction with GSH to yield a fluorescent product at pH 8.0. Readings were taken at activation and emission wavelengths of 340 and 420 nm respectively. A standard curve was prepared for commercially obtained GSH

GSH Peroxidase Assay: GPx activity was measured spectrophotometrically at 340 nm (Roveri et al., 1994) with minor modifications. The test mixture contained supernatant, GSH, glutathione disulfide reductase (GSH-Rx), phosphate buffered saline, t-butyl peroxide, and finally the reaction was started by the addition of NADPH. The NADPH oxidation rate was recorded for 3 minutes and activity was calculated from the rate of NADPH oxidation.

ROS generation: Conversion of nonfluorescent chloromethyl-DCF-DA (2′7′ - dichlorofluorescin diacetate) to fluorescent DCF is used to monitor ROS production spectrofluorometerically using an excitation wavelength of 492 nm and an emission wavelength of 527 nm. DCFH-DA and homogenate obtained fromcontrol and drug treated animals were incubated for 45 minutes at 37°C. DCFH reacted with ROS to form the fluorescent product DCF. Intensity was analyzed by a fluorescent micro-plate. The generation of ROS was measured and reported as relative fluorescence intensity/mg protein.

HPLC ANALYSIS:

Tissue preparation: Animals were sacrificed by cervical dislocation in the morning before 11.00 AM in order to avoid any diurnal variations of the endogenous amines, enzymes and other antioxidant molecules. For the analysis of neurotransmitters, the whole brains were dissected out within thirty seconds, rinsed in ice cold normal saline, blotted dry on ash-free filter paper and each left and right striata were dissected out separately. Each striatum was deproteinized following sonication (50 Hz for 30 s) in 0.1 M $HClO_4$ (1 mg of tissue/10 µl of $HClO_4$) containing EDTA (0.01%). The samples were centrifuged at 14,000 x g for 10 min and the supernatant was injected into the HPLC system.

Measurement of biogenic amines: The neurotransmitters were estimated by employing HPLC-electrochemistry (Muralikrishnan and Mohanakumar, 1998). Briefly, an HPLC system (shimadzu) consists of shimadzu SCL 10-Avp system controller, LC-10AS pump, LECD-6A electrochemical detector, degasser, Rheodyne injector with 20 µl injection loop, and C_{18}, ion pair, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 µm and pore size of 80 Å were used for the assay of neurotransmitters and their metabolites. The flow rate was 0.7 ml/min and the electrodetection was performed at + 0.74 V for the analyses of biogenic amines and their metabolites. The composition of mobile phase was 8.65 mM heptane sulphonic acid, 0.27 mM EDTA, 13% acetonitrile, 0.4-0.45% triethylamine and 0.32 - 0.35% phosphoric acid

(v/v). A standard solution containing 4 pmol of all the biogenic amines was run immediately prior to and following sample injections every day. Results are presented as pmol/mg fresh tissue.

Measurement of Glutamate and GABA: The amino acids were estimated by employing HPLC-electrochemistry (Clarke et al., 2007). Briefly, an HPLC system (shimadzu) consisting of shimadzu SCL 10-Avp system controller, LC-10AS pump, LECD-6A electrochemical detector, degasser, Rheodyne injector with 20 µl injection loop, and C_{18}, ion pair, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 µm and pore size of 80 Å. The flow rate was 0.65 ml/min and the electrodetection was performed at + 0.8 V for the analyses of glutamate and GABA.

Derivatisation protocol: Briefly, 10µl of either standard mix or sample supernatant, 90 µl of borate buffer (0.1 M, pH 9.5), 10 µl of potassium cyanide (10 mM) and 10 µl of NDA (6 mM) were added to a single reaction tube, vortex mixed and the reaction was allowed to proceed at ambient temperatures in the absence of light. A 20 µl of the derivative was injected into the appropriate HPLC system. The composition mobile phase was 0.1M di-sodium hydrogen orthophosphate/50 µM EDTA (pH 5.6, 1 M OPA) and HPLC grade methanol (35:65). Mobile phase was filtered through 0.45 µm and vacuum degassed prior to use. A standard solution containing glutamate and GABA was run

immediately prior to and following sample injections. Results are presented as pmol/mg tissue.

IMMUNOHISTOCHEMISTRY STUDIES:

The animals were anaesthetized with ketamine and xylazine, and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed, and kept in 4% paraformaldehyde fixative for one hour, and cryoprotected in 20% sucrose in PBS for 24 h at 4°C. Serial coronal sections (20 μm thickness), spanning the substantia nigra and the striatum, were cut on a cryostat, collected free-floating in PBS, and processed as described below. The sections were rinsed 3 times for 10 min with 0.1 M PBS. The sections were then incubated in 1% H_2O_2 in PBS for 5 min, and permeabilized with 0.4% Triton X-100 for 30 min and further blocked by incubating for 60 min in 2% BSA, 0.2% Triton X-100 in PBS. Sections were then incubated with the primary antibody anti-rabbit TH (1:1000), and Anti-rabit-GFAP (1:1000) in PBS, pH 7.4, containing 1% BSA and 0.2% Triton X-100, for 12 h at 4 °C. After rinsing in PBS containing 1% BSA, sections were incubated with secondary anti-rabbit IgG-conjugated horseradish peroxidase antibody (1:1000) in PBS, pH 7.4, and containing 1% BSA for 60 min at room temperature. Visualization was performed by incubating in 1 mg/2 ml DAB containing of 1% H_2O_2 for 2-5 min. All sections were then washed for 10 min in PBS, mounted on slides, dried, dehydrated in graded ethanol, cleared in xylene, and mounted with DPX and cover-slip. To test the specificity of the immunostaining, control sections were processed in an identical manner but with the omission of primary or secondary antibody (Haycock, 1987; Saravanan et al., 2006).

Protein Estimation: Protein was assayed using the coomassie plus protein assay reagent kit.

Statistical analysis: Statistics was performed using the Sigmastat-version 2.03 software and results were expressed as mean ± SE. The experimental data were analyzed by using Student's *t*-test for the comparison between two groups. Differences were considered significant at $p < 0.05$.

RESULTS:

General observations

Caffeine and/or MPTP injected mice showed autonomic responses which included piloerection, salivation and increased respiration. Few animals showed convulsions following the first MPTP injection.

EFFECT OF CAFFINE ON MPTP-INDUCED BEHAVIORAL CHANGES:

Body weight: The administration of saline, caffeine and/or MPTP did not significantly affect the body weight (Table-4-1, n=8, $p < 0.05$).

Straub tail: Animals treated with MPTP exhibited straub tail, however, this was not observed in the caffeine treated animals. MPTP-induced tremor was partially decreased by caffeine (both low and high dose) (Table-4-1, n=8, $p < 0.05$).

Akinesia and catalepsy: MPTP administered animals showed significant increase in the latency in seconds (akinetic effect) to move the limbs. The low and high doses of caffeine exhibited a complete reduction in akinetic effects induced by MPTP (Table-4-1, n=8, $p < 0.05$). Catalepsy was observed in the mice treated with MPTP and this effect was not observed in the caffeine (both dose) treated mice (Table-4-1, n=8, $p < 0.05$). In the tail hanging test, MPTP treated mice showed significant decrease in the number of attempts during tail hanging. Pretreatment of caffeine (both doses) significantly improved the number of attempts (Table-4-1, n=8, $p < 0.05$).

R**otorod:** MPTP treated mice showed significant decrease in the retention time on the rod as compared with the control group (Figure-4-1-A, n=8, $p < 0.05$). MPTP associated decrease in the retention time was completely abolished by pretreatment of caffeine (high and low).

Open field: Administration of MPTP showed significant decrease in total ambulatory movement. Pre-treatment with caffeine significantly increased the ambulation ascompare to the MPTP treatment (Figure-4-1-B, n=8, $p < 0.05$).

Swim test: MPTP group showed significant decrease in swim scores as compared to the saline treated mice. This effect was reversed by caffeine (both dose) (Figure-4-1-C, n=8, $p < 0.05$).

Gait analysis: The animals stride length was consistent in the control and caffeine group, but the stride length was significantly decreased in the animals treated with MPTP. Pre-treatment with caffeine (20mg/kg, ip) significantly reversed the MPTP induced stride length (Figure-4-1-D, n=8, $p < 0.05$).

EFFECT OF CAFFEINE IN MPTP- INDUCED BIOCHEMICAL CHANGES:

ROS: ROS generation was significantly increased in the MPTP treated animals as compared to the control (saline treated) mice. MPTP induced ROS generation was significantly decreased by both doses of caffeine (Figure-4-2-A, n=8, $p < 0.05$).

GSH: GSH content was analyzed in control, caffeine and/ or MPTP treated mice. MPTP caused a significant decrease in GSH content as compared to the control mice. Caffeine (10mg/kg) blocked the MPTP–induced GSH depletion (Figure-4-2-B, n=8, $p < 0.05$).

GSH-px activity: GSH-Px activity was significantly increased in the MPTP-treated mice as compared to the control group (Figure-4-2-C, n=8, $p < 0.05$).. Caffeine had no effect on the GSH-Px activity

MAO ACTIVITY: Following saline, caffeine and/ or MPTP, the MAO activity was studied in the whole brain. The enzyme activity was not affected following MPTP treatment. Similarly, caffeine also had no effect on the MAO activity. (Figure-4-2-D, n=8).

C**omplex-I activity:** Systemic injection of MPTP exhibited significant inhibition of complex-I activity (Figure-4-2-E, n=8, $p < 0.05$). Caffeine treatment had no effect on the complex-1 activity.

EFFECT OF CAFFEINE AND/OR MPTP ON NEUROTRANSMITTERS:

Administration of MPTP-induces neurodegeneration in substantia nigra and leads to significant decrease in the dopamine level in the striatum which caused significant DA depletion (Figure-4-3-A, n=6, $p < 0.05$) and also DA turnover was increased in the NCP, which was restored by caffeine (low dose) pretreatment (Figure-4-3-B, n=6, $p < 0.05$).

DOPAC, and HVA turnover was increased in MPTP treated mice, which was decreased by caffeine pretreatment (Table-4-2, n=6, $p < 0.05$). MPTP also affected levels of other monoamines (NE, 5-HT, 5HIAA) and dopamine metabolites (DOPAC, HVA and 3-MT) in the striatum (Table-4-2, n=6, $p < 0.05$). We also measured the GABA and glutamate levels in the striatum. Levels of glutamate were decreased in caffeine (drug alone) group, but unchanged in the MPTP group when compared to control (Figure-4-3-C, n=6, $p < 0.05$). In the striatum, GABA levels were significantly increased in MPTP treatment but caffeine (both dose) decreased the GABA level in the striatum (Figure-4-3-D, n=8, $p < 0.05$).

EFFECT OF GW501516 AND/OR MPTP ON TH-IR AND GFAP:

Representative microphotographs of TH immunostaining in the substantia nigra are shown in Figure-4-4. MPTP injected SN region showed a decrease in TH positive neurons compared to the Saline and caffeine animals. TH immunostaining of pretreated caffeine followed by MPTP treatment showed minimum loss of neurons in the SN, when compared to MPTP animals **GFAP:** Photographs of GFAP immunostaining of striatum. Control mice exhibited a small number of astrocytes in the striatum (NCP) (Figure-4-4). In MPTP-treatment, the reaction of astrocytes was increased in the NCP. The reduction of GFAP was more evident in the NCP of caffeine (both dose) pretreated animals when compared to MPTP group (Figure-4-4).

DISCUSSIONS:

Caffeine is an antioxidant and radioprotector against the oxic pathway of radiation damage in a wide range of cells and organisms (George et al., 1999). Due to high levels of unsaturated fatty acids and the increased capacity of oxygen consumption, brain is extremely susceptible to oxidative damage. Caffeine inhibits formation of TBARS and restores the depleted levels of SOD and protein thiols (Devasagayam et al., 1996). The observed antioxidant ability of caffeine may be due to its ability to scavenge hydroxyl radical and singlet oxygen (Devasagayam and Kesavan, 1996). Caffeine was an effective drug to inhibit the formation of lipid peroxidation against all the three reactive species. The extent of inhibition was high against peroxidation induced by 'OH, In general, the antioxidant ability of caffeine was similar to that of the established biological antioxidant glutathione and significantly higher than ascorbic acid (Devasagayam et al., 1996). The antioxidant activity is generally associated with its content of indigenous phenolic compounds, including mallard reaction products (del Castillo et al., 2002; Gomez-Ruiz et al., 2007). Oxidative stress is the major molecular mechanisms underlying in the MPTP-induced neurodegeneration. Free radicals are implicated in the mechanism of MPTP, and involved in generation of reactive oxygen species (ROS) including superoxide anion, H_2O_2, as well as .OH (Thomas et al., 2000; Muralikrishnan et al., 2003; Przedborski and Ischiropoulos, 2005; Banerjee et al., 2008). ROS and hydrogen peroxide (H_2O_2), a by-product of oxidative stress, have been reported to induce apoptosis in various cell types. MPTP shows generation of ROS following systemic administration and establishes the involvement of ROS in the MPTP neurotoxicity (Maruyama, 2001; Przedborski and

Ischiropoulos, 2005). Regulation of intracellular ROS and modification of the apoptotic cascade may control apoptosis and provide new strategies for prevention and treatment of Parkinson's disease. Our present study shows that caffeine has significant antioxidant abilities in protecting membranes against oxidative damage induced by three of the major reactive oxygen species of biological significance, and that its ability to quench these species may account for the observed antioxidant ability. Caffeine may be modulating antioxidant responses in the brain, and A_{2A} receptor antagonists might be beneficial in neurodegenerative diseases. In this chapter, we have reported the consequences of MPTP treatment, in which a number of antioxidant molecules are altered. The antioxidant molecules have also been shown to be altered significantly due to toxicity, GSH being decreased and GSH-Px activity increased (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2003; Dhanasekaran et al., 2006; Zhou et al., 2008).

The present study was the assessment of neuroprotective properties of caffeine in a validated experimental Parkinson's disease animal model. In this model, caffeine prevented the development of motor impairment induced by MPTP, as measured by the behavioral test. Behavioral manifestation in MPTP mouse model of Parkinson's disease, such as changes in general locomotor activity are correlated with the loss of striatal dopamine (Muralikrishnan et al., 2004; Ghorayeb et al., 2005; Luchtman et al., 2009, Xu et al., 2002). Adenosine (2A) receptors elicit behavioral and cellular responses despite either the genetic deficiency or pharmacological blockade of dopamine D(2) receptors (Chen et al., 2001b). Adenosine is now widely accepted as the major inhibitory

neuromodulators in the central nervous system besides GABA. Both, agonists of adenosine A(1) and A(2) receptors and the antagonists of A(2A) receptors are known to protect against neuronal damage caused by toxins as well as they can also protect against the cell damage inflicted by reactive oxygen species (Bishnoi et al., 2006). A2A receptor antagonist in the basal ganglia suggests that modulation of adenosine A2A receptors may have a profound influence on motor functions. The selective adenosine A2A receptor antagonist KW-6002 exhibits anti-parkinsonism. KW-6002 also ameliorated the hypolocomotion induced by nigral dopaminergic dysfunction with MPTP (Shiozaki et al., 1999). MPTP-induced a loss of nigral dopaminergic neurons, and the severity of motor impairment depends upon the extent of DA neurons degeneration, we used a validated behavioral task highly sensitive for motor deficits associated with subtle alterations of the nigrostriatal pathway.

Adenosine A2A receptors, are predominantly expressed in striatum, the major area of the basal ganglia (Schwarzschild et al., 2003). Activation of A2A receptors in the striatum interferes other neurotransmitters (Schwarzschild et al., 2003). The inhibitory action of A2A receptors may be a possible target for future development of drugs for treatment of Parkinson's disease. The role of striatal A2A receptors has increased dramatically over the last few years. In the present chapter summarized. that peripherally administered caffeine may be involved in the preventing the motor impairment caused by ethanol (Dar, 1988). Caffeine exposure enhances the motor stimulant effects elicited by dopamine agonists by a preferential sensitization of dopamine D(1) receptors. (Cauli et

al., 2005) A_{2A} antagonists, inhibit MPTP induced dopaminergic degeneration and gliosis at the level of striatum and possible neuroprotective mechanism for A_{2A} receptor antagonists mediated via microglia and astrocytes. (Brambilla et al., 2003; Pierri et al., 2005).

MPTP treatment caused significant dopamine depletion and its metabolites were significantly decreased in the striatum indicating MPTP induced dopaminergic toxicity. The decrease in dopamine and DOPAC levels are as observed and reported in literature (Chiueh et al., 1984b; Chiueh et al., 1984a; Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Uthayathas et al., 2007). Similar to earlier reports the turnover of dopamine, DOPAC and HVA are increased in NCP (Heikkila et al., 1984b; Heikkila et al., 1984a; Sundstrom et al., 1987). The increased metabolite to dopamine ratios suggested that more dopamine was released from surviving nigrostriatal terminals and utilized (turned-over), a common compensatory mechanism observed in Parkinson disease patients and MPTP treated mice. Dopamine turnover was most strongly increased in the MPTP treatment. 5-HT turnover (5-HIAA/5-HT) was also increased, which was in line with previous findings in mice (Rozas et al., 1998), suggesting that this could be a compensatory mechanism in the striatum (Gaspar et al., 1993; Rozas et al., 1998). Striatal 5-HT was decreased as well, possibly as a result of increased 5-HT turnover, as metabolite levels (5HIAA). In normal mice, the striatal neurons activity is controlled by the inhibition of nigra dopaminergic neurons by GABA neurons which are abundant in the SN as the dopaminergic neuron. The balance in basal ganglia-thalamo-cortical

circuitry is broken and too much inhibition from the thalamus and cortex circuit causes expression of parkinsonian syndrome. MPTP-treatment increases GABA release within the globus pallidus and indicates that abnormal GABA neurotransmission may be implicated in the pathophysiology of Parkinson's disease (Calon et al., 2000). Earlier studies have shown decrease of dopamine-activity and increase in GABA activity in the striatal neurons. The results give insight as to how dopamine and GABA function within the striatum with respect to the development of neuronal abnormalities (Yamamoto and Kawana, 1991; Otto and Unsicker, 1993).

Tyrosine hydroxylase a marker of the nigrostriatal dopaminergic pathway and TH-immunohistochemical study indicated that TH immunoreactive neuron of substantia nigra and neostriatum were markedly reduced after MPTP treatment (Sundstrom et al., 1987; Mori et al., 1988; Kurkowska-Jastrzebska et al., 1999). MPTP also cause a large increase in the astrocyte protein, glial fibrillary acidic protein (GFAP). GFAP-positive astrocytes were evident in the striatum after the MPTP treatment. These results provide valuable information for the pathogenesis of acute stage of Parkinson’s disease (O'Callaghan et al., 1990; Francis et al., 1995). Recent evidence shows that glial-related response plays a key role in the MPTP neurotoxic process and the blockade of glial activation may be a new therapeutic approach, which has applicability for Parkinson's disease (Yokoyama et al., 2008; Schintu et al., 2009). GFAP-positive astrocytes exhibited a ramified form with many fine processes in the striatum and markedly increased in this region. These results suggest that increases in GFAP staining produced by MPTP in the

striatum are linked to decrements in TH staining which also suggest that factors originating in the damaged dopamine neurons initiated the astrocyte reaction to MPTP. This study revealed the neuroprotective mechanism of caffeine against MPTP-induced neurotoxicity. Neuroprotective mechanisms of caffeine may be through the modulation of antioxidant and GABAergic neurotransmission.

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LEGENDS:

Table-4-1: EFFECT OF CAFFEINE AGAINST MPTP-INDUCED BEHAVIOR CHANGES: [A] Body weight was recorded in interval of 3 days, [B] 5minutes after MPTP, [C] Following three and half hour after MPTP and [D]on 4th day after final injection. ND = not detected. Tremor was visually monitored in a double blind study. MPTP induced significant tremor. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). ND= not detected.

Table-4-2: EFFECT OF CAFFEINE AND/OR MPTP ON NEUROTRANSMITTERS: Animals were sacrificed on 5th day and striatal monoamines and their metabolites were analyzed using HPLC-ECD. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). Values represent pmol/mg tissue. Mean ± SEM (n = 8).

Figure-4-1-A: EFFECT OF CAFFEINE AND /OR MPTP ON ROTOROD: All treatment group were tested on the rotorod on 3rd day after the last MPTP injection. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group).

Figure-4-1-B: EFFECT OF CAFFEINE AND /OR MPTP IN OPEN FIELD: Animals were tested on the open field on 3rd day after the last MPTP injection. Results

given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group).

Figure-4-1-C: EFFECT OF CAFFEINE AND /OR MPTP ON SWIM TEST: Effect of swimming ability in the saline/ or MPTP mice was tested in warm water (27 ± 2 °C) on the 4th day following the final injection of MPTP (30 mg/kg, i.p., twice, 16 h apart). Swim-scores were recorded on a performance intensity scale of 1–4 for all the animals for 10 minutes. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group).

Figure-4-1-D: EFFECT OF CAFFEINE AND /OR MPTP ON GAIT ANALYSIS: Gait abnormalities were analyzed on 4th day after MPTP treatment. The stride length was measured between hind and fore limbs. Stride length was measured in cm. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group).

Figure-4-2-A: EFFECT OF CAFFEINE AND /OR MPTP ON ROS CONTENT: Effects of MPTP on production of intracellular ROS in crude brain homogenate. Values given are relative fluorescent per milligram tissue and are represented as mean ± S.E.M. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group).

Figure-4-2-B: EFFECT OF CAFFEINE AND /OR MPTP ON GSH CONTNET: Mice were treated with MPTP (30mg/kg, i.p.) twice, 16 hr apart. Animals were sacrificed on the fifth day and reduced glutathione (GSH) was estimated in the whole brain by a sensitive fluorimetric procedure. Values given are nanogram per milligram tissue and are represented as mean ± S.E.M. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). (n=6).

Figure-4-2-C: EFFECT OF CAFFEINE AND /OR MPTP ON GSH-PX: Mice were treated with MPTP (30mg/kg, i.p.) twice 16 hr apart. Animals were sacrificed on the fifth day and GSH-Px activity was estimated. Values given are GSH-Px activity per milligram tissue and are represented as mean ± S.E.M. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). (n=6).

Figure-4-2-D: EFFECT OF CAFFEINE AND /OR MPTP ON MAO ACTIVITY: Mice were treated with MPTP (30mg/kg, i.p.) twice 16h apart and sacrificed on 5^{th} day following the second injection. Total MAO activities were assayed and are expressed as in nmol 4-HQ/mg protein/h in enzyme activity and are represented as mean ± S.E.M. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group).

Figure-4-2-E: EFFECT OF CAFFEINE AND /OR MPTP ON COMPLEX-I ACTIVITY: Mice were injected with saline/ or MPTP (30mg/kg, i.p.) twice 16 hr apart,

sacrificed 5th day and analyzed for complex-I activity. The specific activities of the enzyme are given as nanomole of NADH oxidized/min/mg protein and are represented as mean ± SEM. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). ($n = 6$ in each group).

Figure-4-3-A: EFFECT OF CAFFEINE AND/OR MPTP ON NEUROTRANSMITTERS: Animals were sacrificed on 5th day and nigrostriatal dopamine, were analyzed using HPLC-ECD. (DOPAC+HVA)/DA ratio indicated the turnover of DA. Glutamate and GABA were analyzed using HPLC-ECD. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). Values represent pmol/mg tissue. Mean ± SEM (n = 8).

Figure-4-4: TH, immunoreactivity: Cryosected coronal sections from SN were processed for tyrosine hydroxylase activity. **GFAP:** Coronal sections from NCP were processed for GFAP. Photomicrographs images shows coronal sections through the NCP which were stained for GFAP.

Tables and Figures

Table-4-1

Behavioural	Control	MPTP	CAF20mg	MCAF20mg	MCAF10mg
Body weight (in gms)[A]	24.4 ± 0.67	23.66 ± 0.80	24.6 ± 0.40	22.5 ± 0.42	23.33 ± 0.66
Straub tail[B]	ND	Detected	ND	ND	ND
Tremor[B]	ND	Detected	ND	ND	ND
Akinesia (in sec)[C]	1.4 ± 0.194	12.5 ± 1.842*	1 ± 0.00	1.55 ± 0.257#	1.33 ± 0.177#
Catalepsy (in sec)[C]	2.2 ± 0.2	4.875 ± 0.789*	1.2 ± 0.224	1.5 ± 0.22#	1.3 ± 0.152#
Tail Hanging (in min)[D]	22.8 ± 1.176	16.66 ± 1.358 *	27 ± 0.44	21.16 ± 0.47#	22.5 ± 0.718#

Table-4-2

Neurotransimittors	Control	MPTP	CAF20mg	MCAF20mg	MCAF10mg
DOPAC Tunover	1.44 ± 0.126	1.72 ± 0.155*	1.29 ± 0.054	1.65 ± 0.089	1.42 ± 0.14
HVA Tunover	0.536 ± 0.047	1.047 ± 0.113*	0.375 ± 0.015	0.492 ± 0.035	0.538 ± 0.094
NE	17.06 ± 1.79	10.02 ± 0.79*	9.75 ± 1.18*	10.34 ± 0.88*	10.34 ± 0.35*
5-HIAA	7.06 ± 0.54	5.47 ± 0.35	4.52 ± 0.40*	5.11 ± 0.54	6.03 ± 0.80
5-HT	3.50 ± 0.29	2.89 ± 0.26	2.62 ± 0.05	2.97 ± 0.23	3.10 ± 0.25
Serotonin turnover	2.03 ± 0.29	2.02 ± 0.26	2.18 ± 0.04	1.73 ± 0.23	1.95 ± 0.25
3-MT	11.54 ± 1.40	5.28 ± 0.85*	6.33 ± 0.37	6.81 ± 0.51	6.54 ± 0.63

Figure-4-1

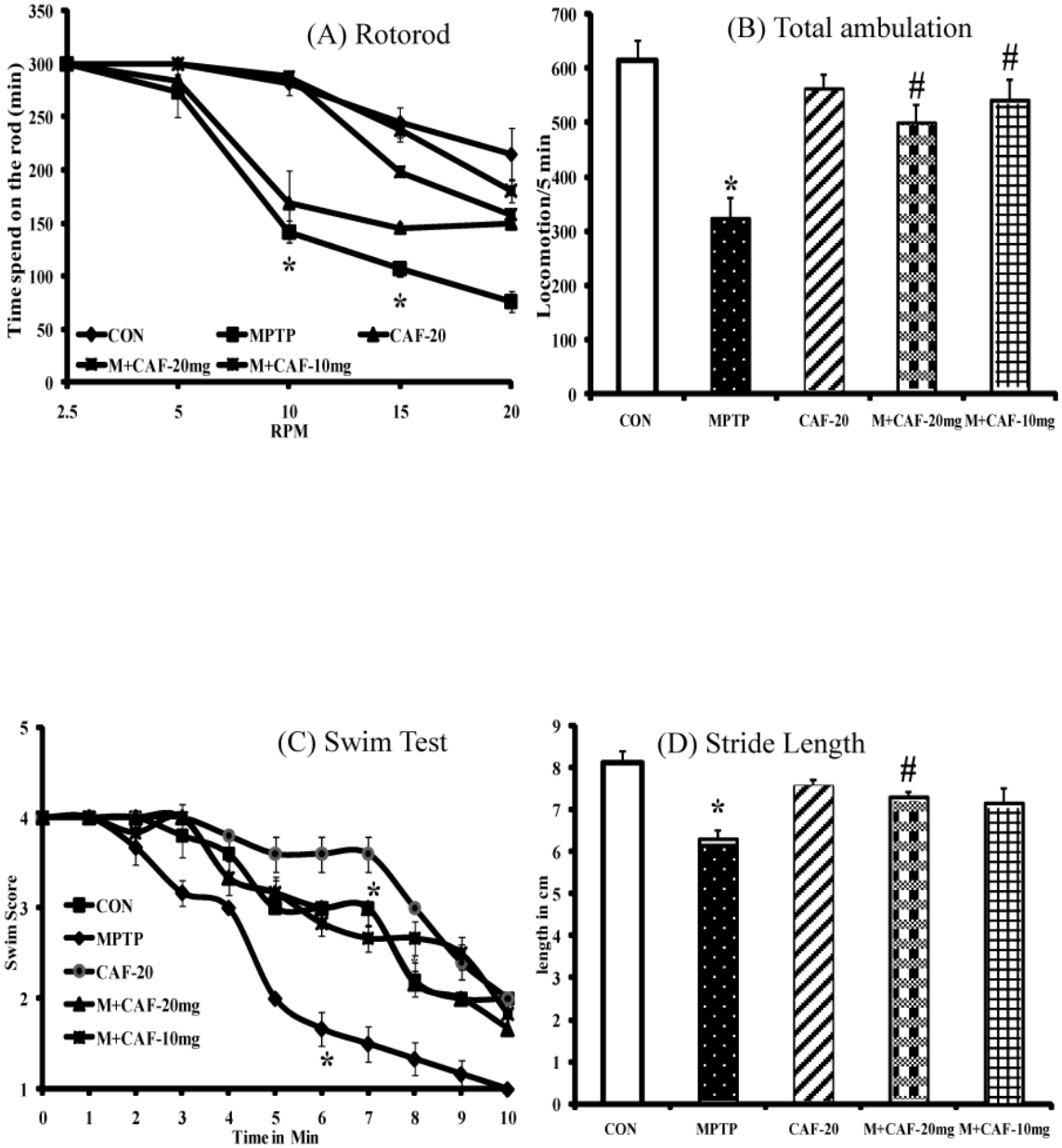

(A) Rotorod
Time spend on the rod (min)
RPM
CON
MPTP
CAF-20
M+CAF-20mg
M+CAF-10mg
(B) Total ambulation
Locomotion/5 min
(C) Swim Test
Swim Score
Time in Min
(D) Stride Length
length in cm

Figure-4-2

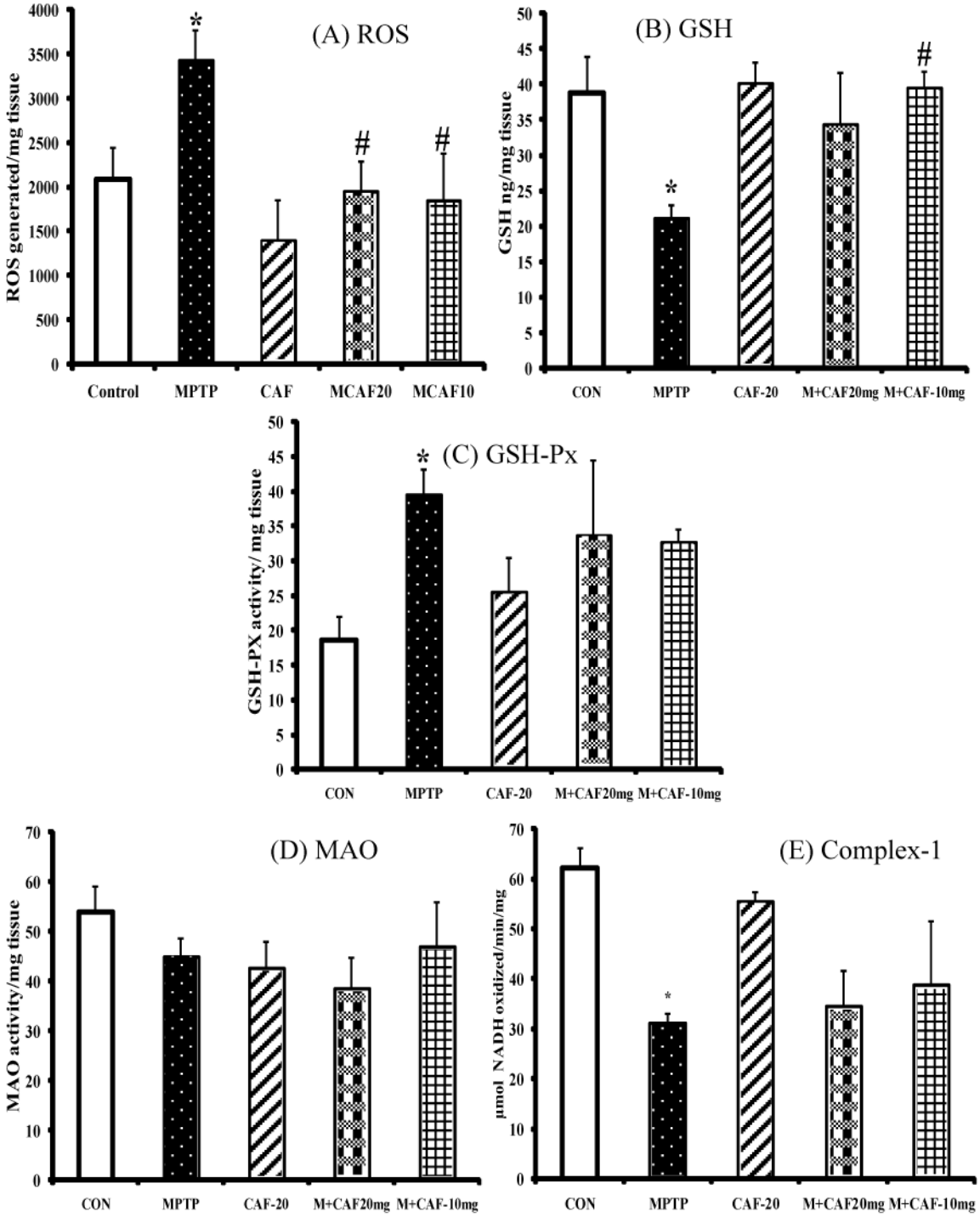

(A) ROS
ROS generated/mg tissue
4000
3500
3000
2500
2000
1500
1000
500
0
Control
MPTP
CAF
MCAF20
MCAF10
(B) GSH
GSH ng/mg tissue
50
45
40
35
30
25
20
15
10
5
0
CON
MPTP
CAF-20
M+CAF20mg
M+CAF-10mg
(C) GSH-Px
GSH-PX activity/ mg tissue
(D) MAO
MAO activity/mg tissue
70
60
50
40
30
20
10
0
(E) Complex-1
µmol NADH oxidized/min/mg

Figure-4-3

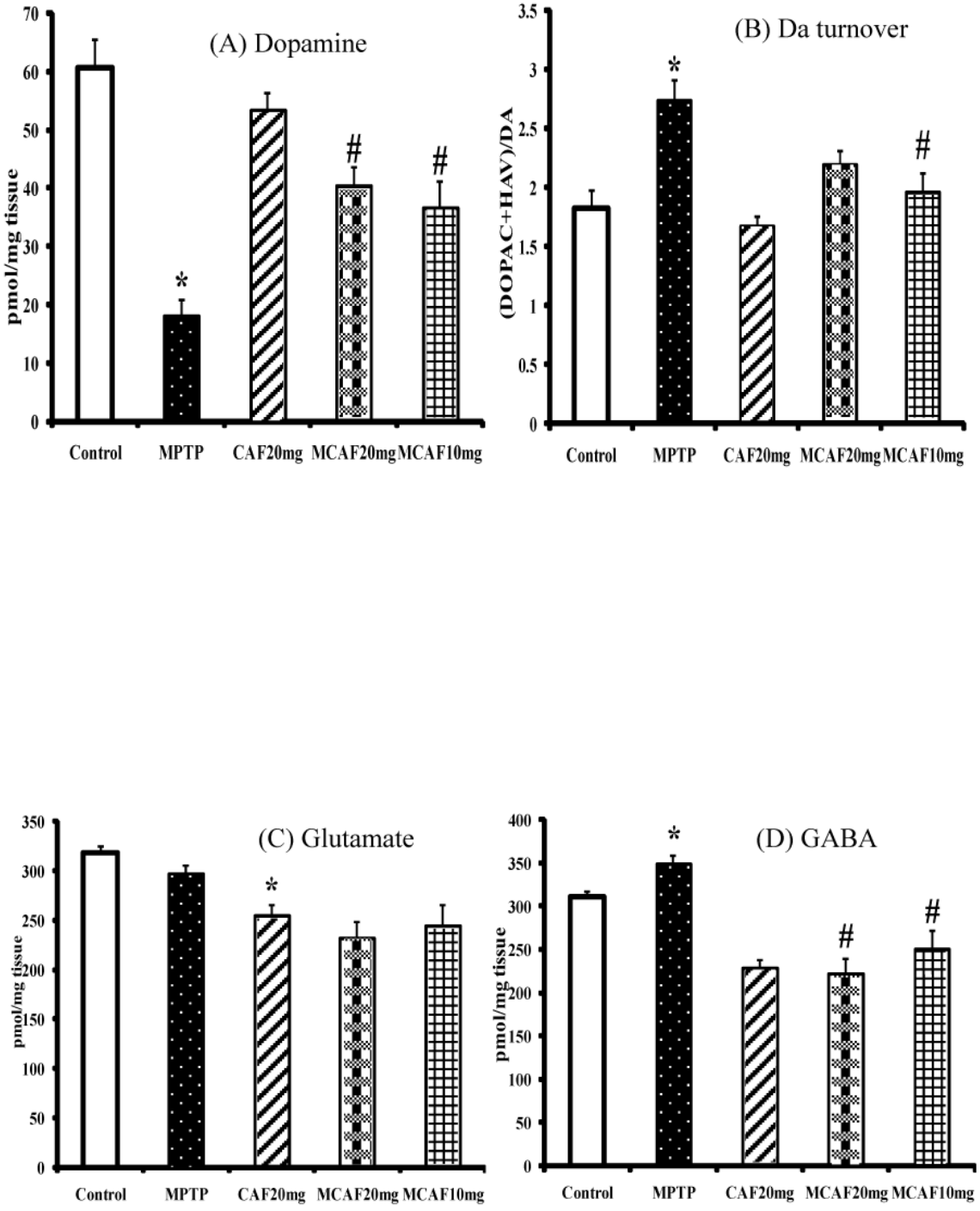
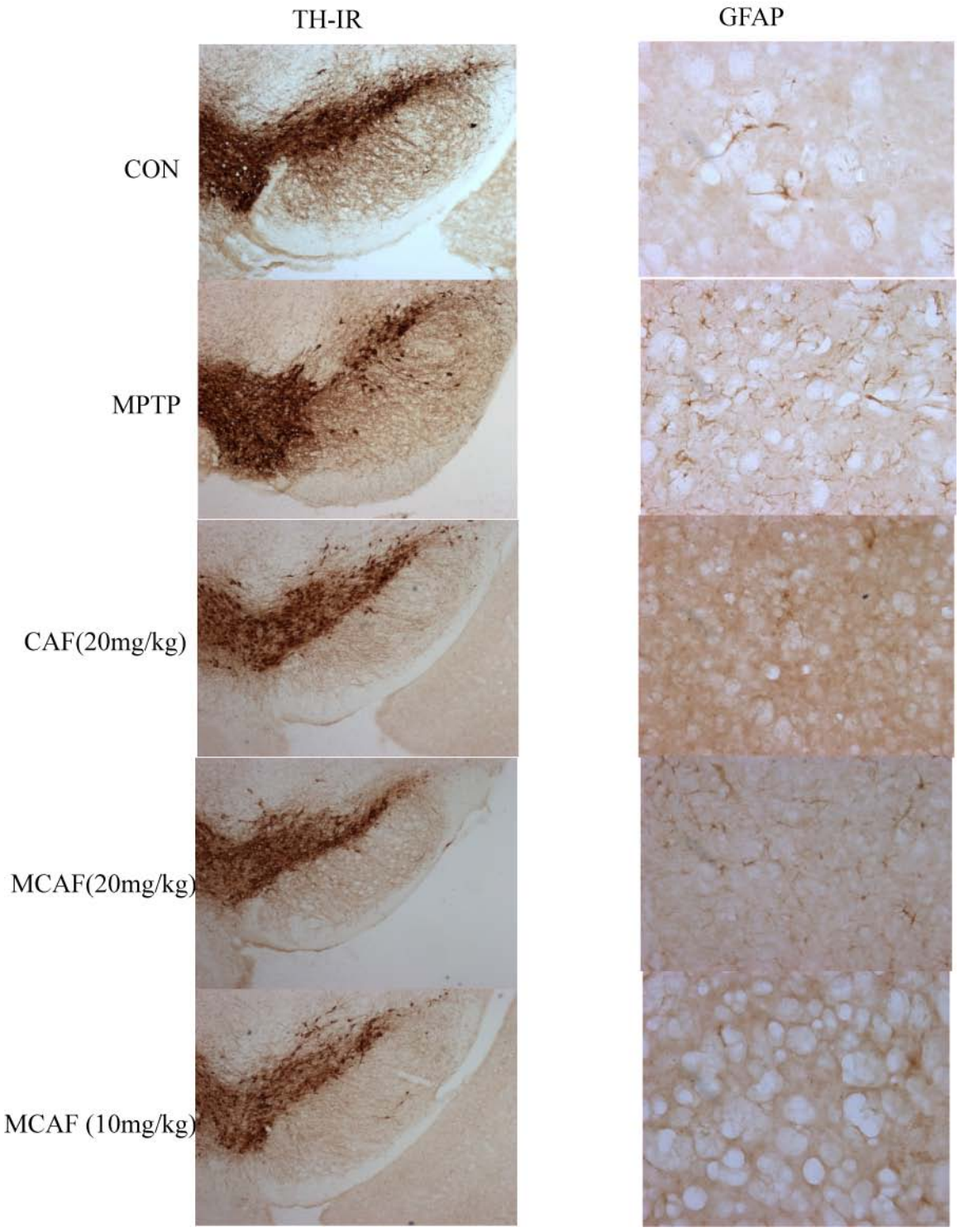

(A) Dopamine
pmol/mg tissue
70
60
50
40
30
20
10
0
*
#
#
Control
MPTP
CAF20mg
MCAF20mg
MCAF10mg
(B) Da turnover
(DOPAC+HAV)/DA
3.5
3
2.5
2
1.5
1
0.5
0
*
#
Control
MPTP
CAF20mg
MCAF20mg
MCAF10mg
(C) Glutamate
pmol/mg tissue
350
300
250
200
150
100
50
0
*
Control
MPTP
CAF20mg
MCAF20mg
MCAF10mg
(D) GABA
pmol/mg tissue
400
350
300
250
200
150
100
50
0
*
#
#
Control
MPTP
CAF20mg
MCAF20mg
MCAF10mg

Figure-4-4



5. EVALUATE THE NEUROPROTECTIVE EFFECTS OF DEXAMETHASONE AGAINST MPTP-INDUCED NEUROTOXICITY

INTRODUCTION:

Glucocorticoids are potent anti-inflammatory agents that act by inhibiting the activatory protein-1 (AP-1) (Unlap and Jope, 1995). The pathological process of neurodegeneration, observed in the Parkinson's diseases is accompanied by an inflammatory reaction (Kurkowska-Jastrzebska et al., 2004a). Inflammation consists of microglial and astroglial activation and results in the over expression of cytokines, and MHC antigens, which leads to neuroinflammation and potentiate neurotoxicity (Czlonkowska and Kurkowska-Jastrzebska, 2001). Nitric oxide has a role in inflammation mediated microglial activation in the substantia nigra (SN) which are found in all animal models of Parkinson's disease and patients with the illness (Arimoto and Bing, 2003; Godoy et al., 2008). The systemic injection of MPTP can cause inflammation and may be involved as a major risk factor for the progression of Parkinson's disease. Neuroinflammation may play an important role in the pathogenesis of Parkinson's disease (Vroon et al., 2007). The inflammatory reaction cause increased synthesis of interleukins (for example IL-1β, IL-6, IL-8) interleukin-1-beta (IL-1β) signaling pathways, tumor necrosis factor (TNF) signaling pathways as well as increase expressions of cytokines and chemokines (TNFα, TGFβ, MIP-1α), and there by activate microglial and astroglial

cells (Floyd et al., 1999; Kurkowska-Jastrzebska et al., 2004b; Tansey et al., 2007). Dexamethasone is a synthetic steroid (glucocorticoids) that is similar to endogenous cortisol. Dexamethasone modulates the neuroinflammatory signaling pathway in the dopaminergic neurons directing to neuroprotection (Herber et al., 2007). The role of astroglia and its pro-/anti-inflammatory mechanisms are involved in the dexamethasone mediated neuroprotection (Previti et al., 2006; Herber et al., 2007). The astrocyte and microglia response to MPTP injury vary according to the dexamethasone with the consequences for dopaminergic neuron survival, recovery and repair. MPTP animal model have shown to reproduce, both symptomatic, biochemical, neurochemical features of Parkinson'sdisease. Motor dysfunction is associated with degeneration of basal ganglia. Neurodegeneration of nigrostriatal system produce akinesia, rigidity and catalepsy (Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Muralikrishnan et al., 2003). Understanding behavioral abnormalities and neurotransmitters changes in the brain provides a better background to design novel drugs and neuroprotective strategies. We evaluated the neuroprotective mechanisms of dexamethasone against MPTP-induced neurotoxicity

MATERIALS AND METHODS:

Animals and chemicals: C57/BL-6 mice (body weight, 20–25 g; age, 8–10 weeks) were purchased from Harlem and were housed with a 12 h day/night light cycle with free access to food and water. All procedures involving animals were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee. The following

chemicals/drugs were purchased from Sigma Chemical Company (St. Louis, MO., USA): Dexamethasone, 3-hydroxytyramine hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxytryptamine creatinine sulphate (5-HT), norepinephrine (NE), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), 1-heptane sulfonic acid (HSA) sodium salt, HPLC-grade acetonitrile (ACN), HPLC-grade methanol, Triton-X 100, 4-hydroxyquinoline (4-HQ), bovine serum albumin (BSA), kynuramine, ethylenediaminetetraacetic acid (EDTA) sodium salt, O-phthaldialdehyde (OPT), Tris buffered saline tablet, Phosphate buffered saline tablet, Reduced glutathione (GSH), Dimethyl sulphoxide (DMSO), hydrogen peroxide (H_2O_2) and 3,3 -N-diaminobenzidine tetrahydrochloride (DAB). Anti-rabbit tyrosine hydroxylase polyclonal, Anti-GFAP and anti-rabbit IgG-conjugated horseradish peroxidase antibodies were purchased from Chemicon. Reverse osmosis water was used in all experiments. For HPLC analysis, double–distilled water was filtered and deionized using Milli-Q system (Waters, Milford, MA).

Experiment design and Treatments: C57/BL-6 mice were intraperitoneally administered with [i] Control (Saline) [ii] MPTP. (30 mg/kg, i.p., twice 16 h apart), [iii] Dexamethasone (2.5mg/kg, i.p), [iv] MPTP+ Dexamethasone (2.5mg/kg, i.p) and [v] MPTP+ Dexamethasone (1mg/kg, i.p). Based on the group, saline or dexamethasone was pretreated for 7 days and followed by two dose of MPTP (30 mg/kg, i.p., and twice 16 h apart). Dexamethasone was dissolved in sterile water.

BEHAVIORAL STUDY:

Body weight and general observation: The body weights of the animals were recorded in an interval of 3 days by using digital weighing scale. The general observations included tremor, piloerection, salivation and increased respiration after every injection

Straub tail: 3hr after final saline and MPTP injections, the straub tail was conducted. Straub tail is defined as elevation of tail to more than 45° with a curvature of the tail. The results are expressed as either straub tail phenomenon detected or not detected. (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2002).

Akinesia: Akinesia was measured by noting the latency of the animals to move all four limbs in unit time seconds(s) and the test was terminated if the latency exceeded 180s. The animals were initially acclimatized in akinesia cages which consisted of a wooden platform with ventilated perspex hoods. (Muralikrishnan and Mohanakumar, 1998).

Catalepsy: The term catalepsy implies to correct the externally imposed posture in an animal. In the saline and MPTP treated mice catalepsy was measured by placing the animals on a flat horizontal surface with both the hind limbs on a square wooden block (3 cm, high) and the latency in seconds was measured to move the hind limbs from the block to the ground. The animals were initially acclimatized on the wooden platform (Muralikrishnan and Mohanakumar, 1998)

Swim test: Swimming ability was carried out on 4th day after the saline or MPTP injections. In brief, the mice were placed in water tubs (40 l x 25 w x 12 h, in cm) with a depth of 8 cm and temperature maintained at 27 ± 2° C. The animals were acclimatized for 10 min one day prior to experimentation. Swim score scales were given from range of 1 to 4 based on their swimming pattern. Swim score scales were: 1 = hind part sinks with head floating, 2 = occasional swimming using hind limbs while floating on one side, 3 = occasional floating/swimming only, 4= continuous swimming. (Muralikrishnan and Mohanakumar, 1998)

Tail Hanging Test: The saline and MPTP treated mice were suspended by holding the tail and recorded for one min and the observers were blind to the treatment conditions. The record made by counting the number of attempts made to reach the tail by bending the body and crawling up towards its tail. The tail hanging was measured for one min and results are expressed as number of attempts/ minutes (Jiao et al., 2005).

Open-field: The open-field apparatus consist of a box with clear plexi-glass walls and floor. The effects of saline and MPTP on motor activity was evaluated in the open-field. An automated sensor system, consisting of 16 photo beams per side measured the total ambulation, fine movement and rearing. Each mouse will remain in the field for a total of 5 minutes. (Holcomb et al., 2006).

Rotorod: Saline and or MPTP treated mice were analyzed for motor coordination using the rotorod tests (Columbus Instruments, Columbus, OH). Mice were initialy adapted for a period consisting of 3 training trials on the rotorod with a 10-minute interval. The mice will be given a 10 minute resting time in between each testing run. The rotation speed of the rod will accelerate gradually from 2.5 to 20 rpm over the first 5 minutes and will be held at 20 rpm for the final minute of testing. The time (seconds) spent on the rod (fall latency) will be recorded for each animal and used as a measure of motor function (Holcomb et al., 2006).

Gait analysis: Briefly, saline and or MPTP mice were made to walk on an inclined gangway (100 cm (l) × 12 cm (w) × 10 cm (h) with 30° inclination) leading to a darkened enclosure. The gangway was lined with white paper and the fore- and hind-paws of the mice were dipped in two different non-toxic watercolors to record the footprints and then the animals were allowed to climb the gangway. After the experiment the limbs were cleaned and dried before placing them back in the cage. The recorded footprints were analyzed for stride length, and stride width.(Fernagut et al., 2002)

BIOCHEMICAL ASSAY:

Tissue Preparation for biochemical assays: Animals were sacrificed by decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes, and other antioxidant molecules. The brain tissue was homogenized in 0.1 M phosphate buffer (pH

7.8), using a glass-Teflon homogenizer. Tissue homogenates were centrifuged at 10,000 g for 60 min at 4°C

NADH Ubiquinone oxidoreductase (Complex - I) assay: The decrease in the absorbance due to oxidation of NADH at 340 nm is monitored. The reaction mixture contained potassium phosphate buffer pH 7.4, coenzyme Q_0 and crude homogenate from saline and MPTP treated mice. The reaction was initiated by the addition of NADH and the rate of decrease in the absorbance was monitored at 340 nm for 2 min. A standard curve for NADH was obtained at 340 nm by using known quantities of NADH. The complex-I activity is expressed as the amount of NADH oxidized/mg protein (Muralikrishnan and Mohanakumar, 1998).

Monoamine oxidase (MAO) assay: This enzyme is responsible for the oxidation of biogenic amines. Fluorimetric assay procedure was used for the estimation of MAO (EC 1.4.3.4) activity. The amount of 4-hydroxyquinoline formed due to oxidation of kynuramine by MAO represents the enzyme activity. Incubation mixture contains crude homogenate, potassium phosphate buffer, pH 7.4 and the reaction was initiated by addition of kynuramine. The reaction was then terminated by addition of ice-cold 0.4 N perchloric acid after 1h. The mixture was centrifuged (14, 000 x g for 5 min) and 2 ml of 1 N NaOH was added to one ml supernatant of the reaction mixture. Fluorescence intensity was measured at activation/emission wavelengths of 315/380 nm. The specific enzyme activity is expressed as nmol of 4-hydroxyquinoline formed/h/mg protein. A

standard curve was obtained for commercially available 4-hydroxyquinoline. (Muralikrishnan and Mohanakumar, 1998)

GSH estimation: GSH were measured fluorimetrically (Muralikrishnan and Mohanakumar, 1998). This method employs OPT condensation reaction with GSH to yield a fluorescent product at pH 8.0. Readings were taken at activation and emission wavelengths of 340 and 420 nm respectively. A standard curve was prepared for commercially obtained GSH

GSH Peroxidase Assay: GPx activity was measured spectrophotometrically at 340 nm (Roveri et al., 1994) with minor modifications. The test mixture contained supernatant, GSH, glutathione disulfide reductase (GSH-Rx), phosphate buffered saline, t-butyl peroxide, and finally the reaction was started by the addition of NADPH. The NADPH oxidation rate was recorded for 3 min and activity was calculated from the rate of NADPH oxidation.

ROS generation: Conversion of nonfluorescent chloromethyl-DCF-DA (2′7′ - dichlorofluorescin diacetate) to fluorescent DCF is used to monitor ROS production spectrofluorometerically using an excitation wavelength of 492 nm and an emission wavelength of 527 nm. DCFH-DA and crude homogenate were incubated for 45 min at 37 C. DCFH reacted with ROS to form the fluorescent product DCF. Intensity was

analyzed by a fluorescent micro-plate. The generation of ROS was measured and reported as relative fluorescence intensity/mg protein. (Muralikrishnan and Ebadi, 2001)

HPLC ANALYSIS:

Tissue preparation: Animals were sacrificed by cervical dislocation in the morning before 11.00 AM in order to avoid any diurnal variations of the endogenous amines, enzymes and other antioxidant molecules. For the analysis of neurotransmitters, the whole brains were dissected out within thirty seconds, rinsed in ice cold normal saline, blotted dry on ash-free filter paper and each left and right striata were dissected out separately. Each striatum was deproteinized following sonication (50 Hz for 30 s) in 0.1 M $HClO_4$ (1 mg of tissue/10 µl of $HClO_4$) containing EDTA (0.01%). The samples were centrifuged at 14,000 x g for 10 min and the supernatant was injected into the HPLC system.

Measurement of biogenic amines: The neurotransmitters were estimated by employing HPLC-electrochemistry (Muralikrishnan and Mohanakumar, 1998). Briefly, an HPLC system (shimadzu) consists of shimadzu SCL 10-Avp system controller, LC-10AS pump, LECD-6A electrochemical detector, degasser, Rheodyne injector with 20 µl injection loop, and C_{18}, ion pair, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 µm and pore size of 80 Å were used for the assay of neurotransmitters and their metabolites. The flow rate was 0.7 ml/min and the

electrodetection was performed at + 0.74 V for the analyses of biogenic amines and their metabolites. The composition of mobile phase was 8.65 mM heptane sulphonic acid, 0.27 mM EDTA, 13% acetonitrile, 0.4-0.45% triethylamine and 0.32 - 0.35% phosphoric acid (v/v). A standard solution containing 4 pmol of all the biogenic amines was run immediately prior to and following sample injections every day. Results are presented as pmol/mg fresh tissue.

Measurement of Glutamate and GABA: The amino acids were estimated by employing HPLC-electrochemistry (Clarke et al., 2007). Briefly, an HPLC system (shimadzu) consists of shimadzu SCL 10-Avp system controller, LC-10AS pump, LECD-6A electrochemical detector, degasser, Rheodyne injector with 20 µl injection loop, and C_{18}, ion pair, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 µm and pore size of 80 Å. The flow rate was 0.65 ml/min and the electrodetection was performed at + 0.8 V for the analyses of glutamate and GABA. **Derivatisation protocol:** Briefly, 10µl of either standard mix or sample supernatant, 90 µl of borate buffer (0.1 M, pH 9.5), 10 µl of potassium cyanide (10 mM) and 10 µl of NDA (6 mM) were added to a single reaction tube, vortex mixed and the reaction was allowed to proceed at ambient temperatures in the absence of light. A 20 µl of the derivative was injected into the appropriate HPLC system. The composition of mobile phase was 0.1M di-sodium hydrogen orthophosphate/50 µM EDTA (pH 5.6, 1 M OPA) and HPLC grade methanol (35:65). Mobile phase was filtered through 0.45 µm and vacuum degassed prior to use. A standard solution containing glutamate and GABA was

run immediately prior to and following sample injections. Results are presented as pmol/mg tissue.

IMMUNOHISTOCHEMISTRY STUDIES:

The animals were anaesthetized with ketamine and xylazine, and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed, and kept in 4% paraformaldehyde fixative for one hour, and cryoprotected in 20% sucrose in PBS for 24 h at 4°C. Serial coronal sections (20 μm thickness), spanning the substantia nigra and the striatum, were cut on a cryostat, collected free-floating in PBS, and processed as described below. The sections were rinsed 3 times for 10 min with 0.1 M PBS. The sections were then incubated in 1% H_2O_2 in PBS for 5 min, and permeabilized with 0.4% Triton X-100 for 30 min and further blocked by incubating for 60 min in 2% BSA, 0.2% Triton X-100 in PBS. Sections were then incubated with the primary antibody anti-rabbit TH (1:1000), and Anti-rabit-GFAP (1:1000) in PBS, pH 7.4, containing 1% BSA and 0.2% Triton X-100, for 12 h at 4 °C. After rinsing in PBS containing 1% BSA, sections were incubated with secondary anti-rabbit IgG-conjugated horseradish peroxidase antibody (1:1000) in PBS, pH 7.4, and containing 1% BSA for 60 min at room temperature. Visualization was performed by incubating in 1 mg/2 ml DAB containing of 1% H_2O_2 for 2-5 min. All sections were then washed for 10 min in PBS, mounted on slides, dried, dehydrated in graded ethanol, cleared in xylene, and mounted with DPX and cover-slip. To test the specificity of the

immunostaining, control sections were processed in an identical manner but with the omission of primary or secondary antibody (Saravanan et al., 2006).

Protein Estimation: Protein was assayed using the coomassie plus protein assay reagent kit.

Statistical analysis: Statistics was performed using the Sigmastat-version 2.03 software and results were expressed as mean ± SE. The experimental data were analyzed by using Student's *t*-test for the comparison between two groups. Differences were considered significant at $p < 0.05$.

RESULTS:

EFFECT OF DEXAMETHASONE IN MPTP-INDUCED BEHAVIOR CHANGES:

No change in the body weight was observed (Table-5-1, n=8, $p < 0.05$). MPTP-induced tremor was decreased by dexamethasone (both the low and high dose), however dexamethasone alone did not induce tremor (Table-5-1, n=8, $p < 0.05$). Akinesia was observed in the MPTP administered animals and showed significant increase in the latency in seconds, as compared to the control animal. Dexamethasone had a partial to complete reduction in the akinetic effects-induced by MPTP (Table-5-1, n=8, $p < 0.05$). Catalepsy-was not noticed in the dexamethasone (both dose) pretreatment followed by MPTP, but observed in the mice treated with MPTP, when compared to control and dexamethasone animals. (Table-5-1, n=8, $p < 0.05$). Interestingly, in the tail hanging test,

MPTP treated mice showed significant decrease in the number of attempts. Pretreatment with dexamethasone (both doses) did not improve the number of attempts (Table-5-1, n=8, $p < 0.05$).

R**otorod:** MPTP treated mice showed significant decrease in the retention time on the rod as compared with the control (Figure-5-1-A, n=8, $p < 0.05$). MPTP associated decrease in the retention time was significantly increased by dexamethasone treatment.

Open field: Total ambulatory movement was significantly decreased after MPTP administration. Pre-treatment with dexamethasone (both dose) showed a significant increase in ambulation (Figure-5-1-B, n=8, $p < 0.05$).

Swim test: MPTP induced significant decrease in swim scores as compared to the saline treatment. Pretreatment of dexamethasone reversed the MPTP-induced decrease in the swim scores (Figure-5-1-C, n=8, $p < 0.05$).

Gait analysis Pre-treatments with dexamethasone (both dose) did not reverse the MPTP caused decrease in stride length (Figure-5-1-D, n=8, $p < 0.05$).

EFFECT OF DEXAMETHASONE ON MPTP- INDUCED BIOCHEMICAL CHANGES:

ROS: MPTP induced ROS generation was significantly decreased by both doses of dexamethasone (Figure-5-2-A, n=6, $p < 0.05$).

GSH: Dexamethasone (2.5mg/kg, ip) blocked the MPTP-induced GSH depletion (Figure-5-2-B, n=6, $p < 0.05$).

GSH-px activity: Pre-treatment of dexamethasone (1mg/kg, i.p) followed by MPTP showed significant decrease in GSH-Px activity (Figure-5-2-C, n=6, $p < 0.05$).

MAO ACTIVITY: Dexamethasone alone or with MPTP did not affect total MAO activity (Figure-5-2-D, n=6, $p < 0.05$).

C**omplex-I activity:** Saline and dexamethasone treated mice did not show any change in complex-I activity. Dexamethasone did block the effect of MPTP on the complex-1 activity (Figure-5-2-E, n=6, $p < 0.05$).

EFFECT OF DEXAMETHASONE AND/OR MPTP ON NEUROTRANSMITTERS:

Administration of MPTP-induces significant decrease in the dopamine level in the striatum (Figure-5-3-A, n=6, $p < 0.05$) and dopamine turnover was increased in the NCP, which were restored by dexamethasone (both dose) (Figure-5-3-B, n=6, $p < 0.05$). DOPAC, and HVA turnover was increased in MPTP treated mice, which was decreased by dexamethasone pretreatment followed with MPTP (Table-5-2, n=6, $p < 0.05$). MPTP also affected levels of other monoamines (NE, 5-HT, 5HIAA) and dopamine metabolites (DOPAC, HVA and 3-MT) in the striatum (Table-5-2, n=6, $p < 0.05$). We also measured the GABA and glutamate levels in the striatum. Glutamate levels were not affected by dexamethasone and/or MPTP treatment (Figure-5-3-C, n=6, $p < 0.05$). In the striatum, GABA levels were significantly increased by the MPTP treatment. examethasone significantly decreased the GABA level in the striatum (Figure-5-3-D, n=6, $p < 0.05$).

EFFECT OF DEXAMETHASONE AND/OR MPTP ON TH-IR AND GFAP:

Dexamethasone showed minimum loss of neurons in the SN, as compared to MPTP animals (Figure-5-4). **GFAP:** The reaction of astrocytes was increased in the NCP (Figure-5-4). The reduction of GFAP was significant in the NCP of dexamethasone pretreated animals as compared to the MPTP group (Figure-5-4).

DISCUSSION:

The systemic injection of MPTP can cause inflammation and this may be involved as a major risk factor for the progression of Parkinson's disease. Neuroinflammation may play a important role in the pathogenesis of Parkinson's disease (Vroon et al., 2007). The inflammatory reaction cause increase in synthesis of interleukins (for example IL-1β, IL-6, IL-8) interleukin-1-beta (IL-1β) signaling pathways, tumor necrosis factor (TNF) signaling pathways as well as increase in expressions of cytokines and chemokines (TNFα, TGFβ, MIP-1α), and there by activate microglial and astroglial cells (Floyd et al., 1999; Kurkowska-Jastrzebska et al., 2004b; Tansey et al., 2007). High levels of ROS generation may activate microglia and astrocytes through arachidonic acid signaling via the activation of cyclooxygenase (COX) and lipoxygenase (LOX) pathways (Tansey et al., 2007). Dexamethasone modulates the neuroinflammatory signaling pathway in the dopaminergic neurons and the cardinal role of glia-neuron crosstalk in directing neuroprotection (Herber et al., 2007). Dexamethasone plays an important role in astroglia and its pro-/anti-inflammatory mechanisms in neuroprotection (Previti et al., 2006; Herber et al., 2007). This study

shows that astrocyte and microglia response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine injury which vary according to the dexamethasone with the consequences for dopaminergic neuron survival, recovery and repair. Our results show that MPTP can aggravate neurodegeneration in the SN and cause the behavioral symptoms in our MPTP mouse model of Parkinson's disease.

The present study is the assessment of the neuroprotective effect of dexamethasone in a validated experimental PD animal model. In this model, dexamethasone prevented the motor impairment induced by MPTP. Behavioral manifestation in MPTP mouse model of PD, such as changes in general locomotor activity are correlated with the loss of striatal dopamine (Muralikrishnan et al., 2004; Ghorayeb et al., 2005; Luchtman et al., 2009) MPTP-induced a loss of nigral dopaminergic neurons, and the severity of motor impairment depends upon the extent of DA neurons degeneration, we used a validated behavioral task highly sensitive for motor deficits associated with subtle alterations of the nigrostriatal pathway. MPTP treatment caused significant dopamine depletion and its metabolites in the striatum indicating MPTP induced DA-ergic toxicity. The decrease in dopamine and DOPAC levels are as observed and reported in literature (Chiueh et al., 1984a; Chiueh et al., 1984b; Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Uthayathas et al., 2007).

TNF-α depletes intracellular GSH, with an increase in oxidised glutathione levels in the cells (Rahman, 2000). Due to high levels of unsaturated fatty acids and the increased capacity of oxygen consumption, brain is extremely susceptible to oxidative damage.. Oxidative stress is the major molecular mechanisms underlying the MPTP-induced neurodegeneration. Free radicals are implicated in the mechanism of MPTP, and involved in generation of reactive oxygen species (ROS) including superoxide anion, H_2O_2, as well as .OH (Thomas et al., 2000; Muralikrishnan et al., 2003; Przedborski and Ischiropoulos, 2005; Banerjee et al., 2008). MPTP shows generation of ROS following systemic administration and establishes the involvement of ROS in the MPTP neurotoxicity. In this chapter, we have reported as consequences of MPTP of treatment a number of antioxidant molecules are altered. The antioxidant molecules have also been shown to be altered significantly due to toxicity, GSH being decreased and GSH-Px activity increased (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2003; Dhanasekaran et al., 2006; Zhou et al., 2008). Dexamethasone has shown to modulate TNF-alpha and this may be a key mechanism involved in the neuroprotective effect against MPTP-induced neurotoxicity.

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LEGENDS:

Table-5-1: EFFECT OF DEXAMETHASONE IN MPTP-INDUCED BEHAVIOR CHANGES: [A] Body weight was recorded regularly (every 3 days), [B] 5minutes after MPTP, [C] Following three and half hour after MPTP and [D]on 4th day after final injection. ND = not detected. Tremor was visually monitored in a double blind study. MPTP caused tremor of approximately at 3 to 5 minutes. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). ND= not detected.

Table-5-2: EFFECT OF DEXAMETHASONE AND/OR MPTP ON NEUROTRANSMITTERS: Animals were sacrificed on 5th day and nigrostriatal monoamine, were analyzed using HPLC-ECD. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). Values represent pmol/mg tissue. Mean ± SEM (n = 8).

Figure-5-1-A: EFFECT OF DEXAMETHASONE AND /OR MPTP ON ROTOROD IN MICE: All treatment groups were tested on the rotorod on 3rd day after the last MPTP injection. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group).

Figure-5-1-B: EFFECT OF DEXAMETHASONE AND /OR MPTP ON OPEN FIELD IN MICE: Animals were tested on the open field on 3rd day after the last MPTP

injection. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group).

Figure-5-1-C: EFFECT OF DEXAMETHASONE AND /OR MPTP ON SWIM TEST: Effect of swimming ability in the saline/ or MPTP mice was tested in warm water (27 ± 2 °C) on the 4th day following the final injection of MPTP (30 mg/kg, i.p., twice, 16 h apart). Swim-scores were recorded on a performance intensity scale of 1–4 for all the animals for 10 min. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group).

Figure-5-1-D: EFFECT OF DEXAMETHASONE AND /OR MPTP ON GAIT ANALYSIS: Gait abnormalities were analyzed on 4th day after MPTP treatment. The stride length was measured between hind and fore limbs. Stride length was measured in cm. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group).

Figure-5-2-A: EFFECT OF DEXAMETHASONE AND /OR MPTP ON ROS: Effects of MPTP on production of intracellular ROS in crude brain homogenate. Values given are relative fluorescent per milligram tissue and are represented as mean ± S.E.M. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). (n=6).

Figure-5-2-B: EFFECT OF DEXAMETHASONE AND /OR MPTP ON GSH: Mice were treated with MPTP (30mg/kg, i.p.) twice, 16 hr apart. Animals were sacrificed on the fifth day and reduced glutathione (GSH) was estimated in the whole brain by a sensitive fluorimetric procedure. Values given are nanogram per milligram tissue and are represented as mean ± S.E.M. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). (n=6).

Figure-5-2-C: EFFECT OF DEXAMETHASONE AND /OR MPTP ON GSH-PX: Mice were treated with MPTP (30mg/kg, i.p.) twice 16 hr apart. Animals were sacrificed on the fifth day and GSH-Px activity was estimated. Values given are GSH-Px activity per milligram tissue and are represented as mean ± S.E.M. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). (n=6).

Figure-5-2-D: EFFECT OF DEXAMETHASONE AND /OR MPTP ON MAO ACTIVITY: Mice were treated with MPTP (30mg/kg, i.p.) twice 16h apart and sacrificed on 5^{th} day following the second injection. Total MAO activities were assayed and are expressed as in nmol 4-HQ/mg protein/h in enzyme activity and are represented as mean ± S.E.M. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). (n=6).

Figure-5-2-E: EFFECT OF DEXAMETHASONE AND /OR MPTP ON COMPLEX-I ACTIVITY: Mice were injected with saline/ or MPTP (30mg/kg, i.p.) twice 16 hr apart, sacrificed 5^{th} day and analyzed for complex-I activity. The specific activities of the enzyme are given as nanomole of NADH oxidized/min/mg protein and are represented as mean ± SEM. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). (n = 6 in each group).

Figure-5-3-A: EFFECT OF DEXAMETHASONE AND/OR MPTP ON NEUROTRANSMITTERS: Animals were sacrificed on 5^{th} day and nigrostriatal dopamine, were analyzed using HPLC-ECD. (DOPAC+HVA)/DA ratio indicated the turnover of DA. Glutamate and GABA were analyzed using HPLC-ECD. ($^{*}p \leq 0.05$ as compared with the control (saline treated) group), ($^{\#}p \leq 0.05$ as compared with the MPTP treated group). Values represent pmol/mg tissue. Mean ± SEM (n = 8).

Figure-5-4: TH, immunoreactivity: Cryosected coronal sections from SN were processed for tyrosine hydroxylase activity. **GFAP:** Coronal sections from NCP were processed for GFAP. Photomicrographs images shows coronal sections through the NCP which were stained for GFAP.

Tables and Figures

Table-5-1

Behavioural	CON	MPTP	DEX 2.5mg	MDEX 2.5mg	MDEX 1 mg
Body weight (in gms)[A]	23.66 ± 1.20	22 ± 0.32	22.4 ± 0.74	22.33 ± 0.61	22.33 ± 0.33
Straub tail[B]	ND	Detected	ND	ND	ND
Tremor[B]	ND	Detected	ND	ND	ND
Akinesia (in sec)[C]	1.4 ± 0.244	12.5 ± 1.84	1.8 ± 0.37	2.3 ± 0.21#	1.8 ± 0.32#
Catalepsy (in sec)[C]	2.2 ± 0.2	4.875 ± 0.789*	1.6 ± 0.22	2.22 ± 0.14#	2.33 ± 0.47#
Tail Hanging (in min)[D]	22.8 ± 1.17	16.66 ± 1.35 *	20.2 ± 1.42	17.16 ± 0.90	18.16 ± 1.16

Table-5-2

Neurotransimitters	CONTROL	MPTP	DEX 2.5mg	MDEX 2.5mg	MDEX 1 mg
DOPAC Turnover	1.31 ± 0.11	1.74 ± 0.09*	1.05 ± 0.12	1.24 ±0.05#	1.18 ± 0.14#
HVA Turnover	0.50 ± 0.04	1.00 ± 0.08*	0.30 ± 0.02	0.44 ± 0.02#	0.45 ± 0.03#
NE	17.06 ± 1.79	10.02 ± 0.79*	10.91 ± 0.89*	11.61 ± 0.40*	10.29 ± 0.93*
5-HIAA	7.06 ± 0.54	5.47 ± 0.35	5.46 ± 0.87	6.71 ± 0.51	5.42 ± 0.62
5-HT	3.50 ± 0.29	2.89 ± 0.26	3.39 ± 0.22	3.44 ± 0.27	3.33 ± 0.15
3-MT	11.54 ± 1.40	5.28 ± 0.85*	9.39 ± 1.22	8.13 ± 0.30	6.27 ± 0.32*

Figure-5-1

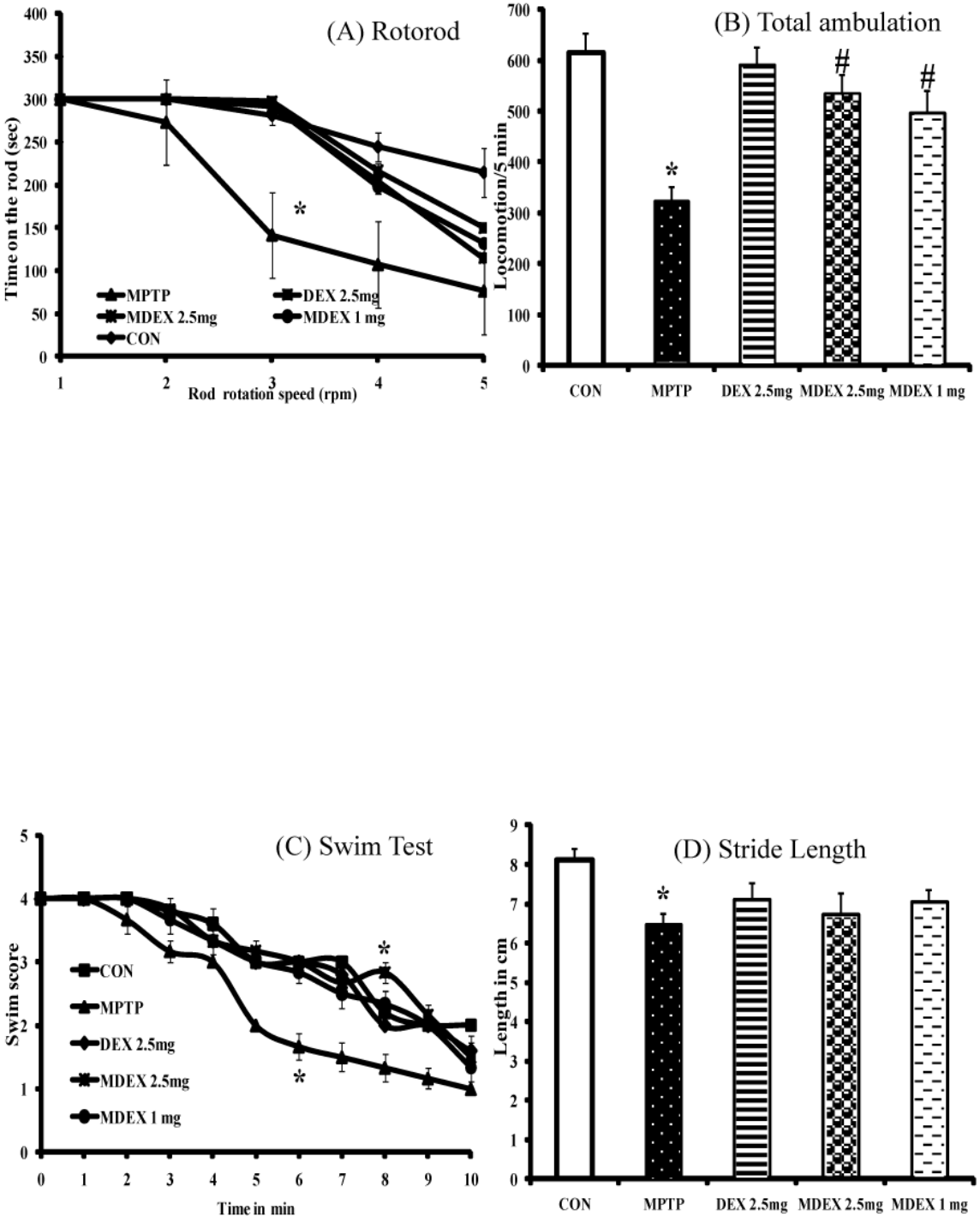

(A) Rotorod
Time on the rod (sec)
Rod rotation speed (rpm)
MPTP
DEX 2.5mg
MDEX 2.5mg
MDEX 1 mg
CON
(B) Total ambulation
Locomotion/5 min
CON
MPTP
DEX 2.5mg
MDEX 2.5mg
MDEX 1 mg
(C) Swim Test
Swim score
Time in min
(D) Stride Length
Length in cm

Figure-5-2

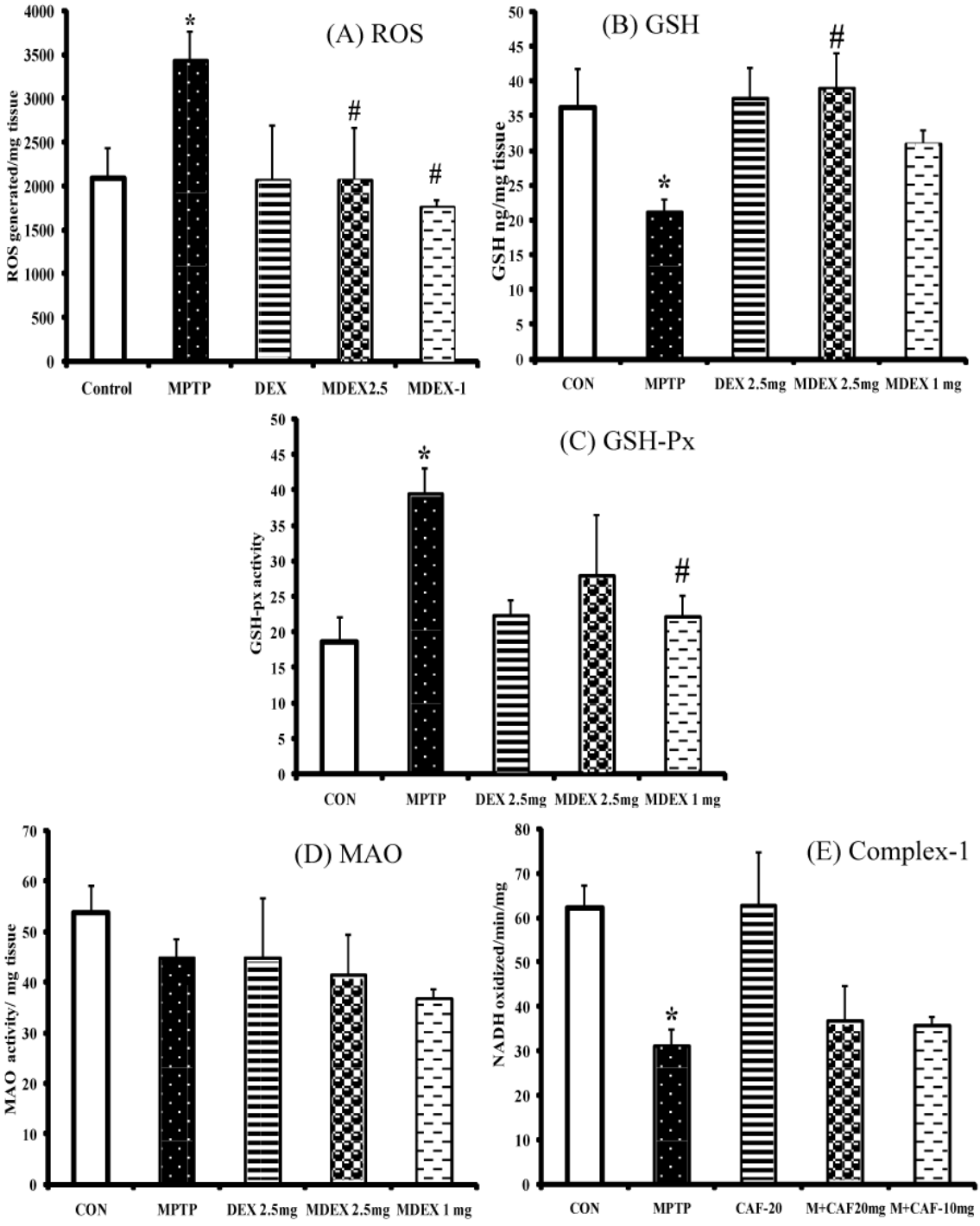

(A) ROS
ROS generated/mg tissue
4000
3500
3000
2500
2000
1500
1000
500
0
Control
MPTP
DEX
MDEX2.5
MDEX-1
(B) GSH
GSH ng/mg tissue
50
45
40
35
30
25
20
15
10
5
0
CON
MPTP
DEX 2.5mg
MDEX 2.5mg
MDEX 1 mg
(C) GSH-Px
GSH-px activity
CON
MPTP
DEX 2.5mg
MDEX 2.5mg
MDEX 1 mg
(D) MAO
MAO activity/ mg tissue
70
60
50
40
30
20
10
0
CON
MPTP
DEX 2.5mg
MDEX 2.5mg
MDEX 1 mg
(E) Complex-1
NADH oxidized/min/mg
80
70
60
50
40
30
20
10
0
CON
MPTP
CAF-20
M+CAF20mg
M+CAF-10mg

Figure-5-3

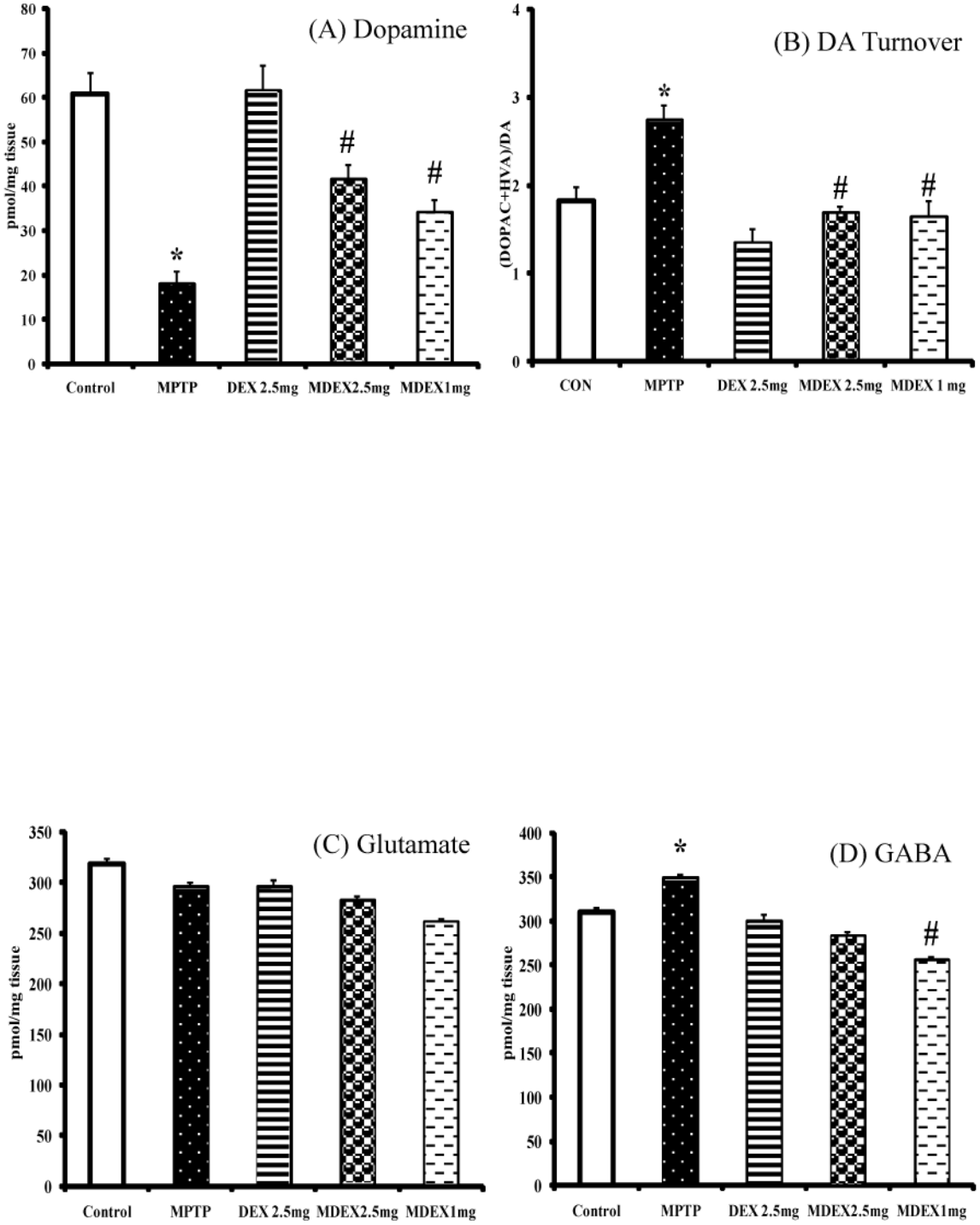
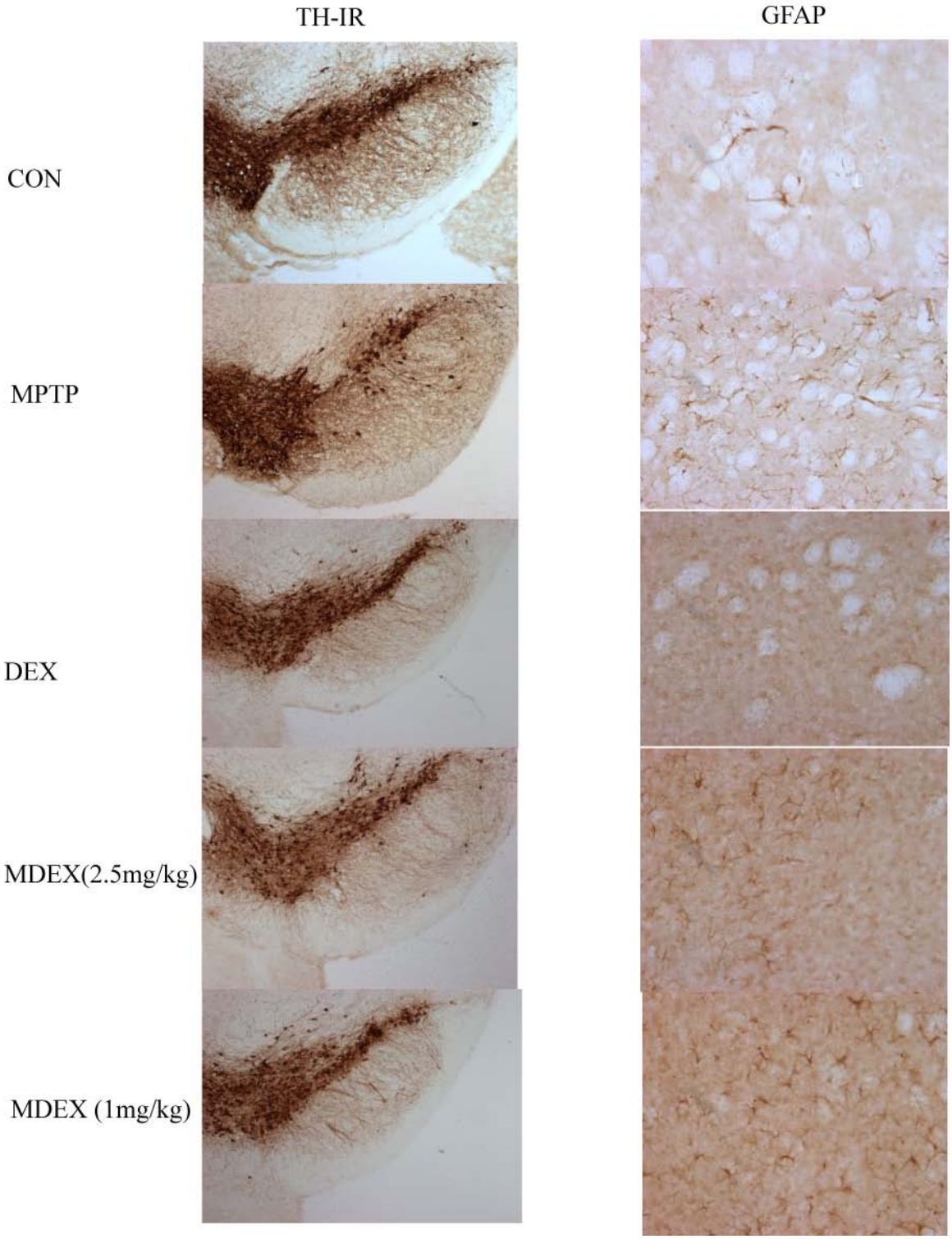

(A) Dopamine
pmol/mg tissue
80
70
60
50
40
30
20
10
0
*
#
#
Control
MPTP
DEX 2.5mg
MDEX2.5mg
MDEX1mg
(B) DA Turnover
(DOPAC+HVA)/DA
4
3
2
1
0
*
#
#
CON
MPTP
DEX 2.5mg
MDEX 2.5mg
MDEX 1 mg
(C) Glutamate
pmol/mg tissue
350
300
250
200
150
100
50
0
Control
MPTP
DEX 2.5mg
MDEX2.5mg
MDEX1mg
(D) GABA
pmol/mg tissue
400
350
300
250
200
150
100
50
0
*
#
Control
MPTP
DEX 2.5mg
MDEX2.5mg
MDEX1mg

Figure-5-4



6. ESTABLISH THE NEUROPROTECTIVE MECHANISMS OF GW591516 AGAINST MPTP-INDUCED NEUROTOXICITY

INTRODUCTION:

Parkinson's disease is one of the major neurodegenerative disorders and is mainly characterized by major symptoms such as tremor, bradykinesia, rigidity and postural instability due to the selective loss of neurons at the SN pars compacta, resulting in severe depletion of dopamine in the striatum (Cornford et al., 1995; Lang and Lozano, 1998; Olanow and Tatton, 1999; Cookson, 2005; Dhanasekaran et al., 2008a). MPTP damages the nigrostriatal dopaminergic pathway and cause dopamine depletion in the striatum (Davis et al., 1979; Burns et al., 1983; Ballard et al., 1985; Mohanakumar et al., 2000; McGeer and McGeer, 2004; Watanabe et al., 2005; Dhanasekaran et al., 2008b). Peroxisome proliferator-activated receptor (PPAR), a member of nuclear receptor superfamily, regulates development, tissue differentiation, inflammation, mitochondrial function, wound healing, lipid metabolism and glucose metabolism (Bordet et al., 2006; Ciana et al., 2007). PPARs are members of the nuclear receptor gene family that are activated by fatty acids and fatty acid metabolites (Sprecher, 2007). Designated PPAR-α (NR1C1), PPAR-δ/β (NR1C2), and PPAR-γ (NR1C3), serve as transcription factors, (Issemann and Green, 1990; Kliewer et al., 1994; Bordet et al., 2006; Sprecher, 2007)

binding to peroxisome proliferating response elements and modulating a cascade of gene expressions associated with fat metabolism (Wahli et al., 1995; Sprecher, 2007). Recently, several PPAR agonists were shown to exert neuroprotective activity against oxidative damage, inflammation and apoptosis in several neurodegenerative disorders including Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis and multiple sclerosis (Bordet et al., 2006; van Neerven et al., 2008). Non-steroidal anti-inflammatory drugs such as indomethacin and ibuprofen activate PPAR and reduce the progression of neurodegenerative disorders. Peroxisome proliferator-activated receptor (PPAR) is comprised of at least three subtypes, including α, γ, and δ . The PPAR δ is expressed in tissues, such as the brain, liver, kidney, spleen, fat skeletal muscle, digestive tube, skin, and placenta (Kliewer et al., 1994; Schoonjans et al., 1996b; Schoonjans et al., 1996a). PPAR δ selective agonist L-165041 is highly protective in neuroprotective and ischemic acute renal failure (Letavernier et al., 2005; Iwashita et al., 2007). In the brain, there is considerable expression of PPAR δ mRNA and protein, with expression at the cellular level in oligodendrocytes and neuron (Woods et al., 2003), There are studies on the role of the PPAR δ in cerebral ischemia or Parkinson's disease. (Iwashita et al., 2007), some reports show that the PPAR δ agonist can protect cultured neurons from cell death (Berger et al., 1999). GW501516 [2-methyl4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-triazol-5-yl)-methylsulfanyl) phenoxy acetic acid] is the most potent PPAR δ agonist and shows a potent neuroprotective effect against various neurotoxic compound (Iwashita et al., 2007).

The chronic MPTP paradigm used here has been shown to reproduce several features of Parkinson's disease, allowing the evaluation of changes in both symptomatic and biochemical Parkinson's disease parameters. Degeneration of basal ganglia structures is associated with motor dysfunction. Neurodegeneration of nigrostriatal system producing akinesia, rigidity and catalepsy (Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Muralikrishnan et al., 2003). Understanding behavioral abnormalities with respect to neurotransmitters' changes in the brain provides a better background to design novel drugs and devise neuroprotective strategies. An efficient technique to investigate the motor dysfunction in PD is imminent for identifying relationship between the loss of dopaminergic neurons and behavioral changes observed in animal models of this disease (Tillerson et al., 2002). Several methods like rotorod, tail hanging, gait analysis, open-field test, pole test, rigidity, swim-test, etc. are available to measure the degree of motor impairment in animal models (Tsai et al., 1991; Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000; Muralikrishnan et al., 2003; Bjarkam et al., 2008; Guillot et al., 2008; Luchtman et al., 2009). Methods to study symptoms like akinesia and catalepsy and tremor in animal models of Parkinson's disease are in regular use (Muralikrishnan and Ebadi, 2001; Uthayathas et al., 2007). Only a few studies exist where swim-test has been employed to examine the motor damage (Muralikrishnan and Ebadi, 2001; Uthayathas et al., 2007).

MPP^+ induces generation of reactive oxygen species, and free radicals which are implicated in the mechanism of MPTP-induced neurotoxicity in animals (Chiueh, 1994;

Chiueh et al., 1994; Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2003). The major antioxidant molecules in the brain are GSH, CAT and SOD. Induction of ROS such as superoxide anion or .OH or H_2O_2 result in enhanced synthesis of antioxidant molecules as a defense mechanism. Elevation of ROS is the basic molecular mechanism underlying the pathophysiology of Parkinson's disease, and thus the postmortem brain exhibited changes in the antioxidant molecules (Yokoyama et al., 2008). Tyrosine hydroxylase a marker of the nigrostriatal dopaminergic pathway and TH-immunohistochemical study indicated that TH immunoreactive neuron of substantia nigra and neostriatum were markedly reduced after MPTP treatment (Sundstrom et al., 1987; Mori et al., 1988; Kurkowska-Jastrzebska et al., 1999). MPTP treatment also causes a large increase in the astrocyte protein, glial fibrillary acidic protein (GFAP) in the striatum after the MPTP treatment. We studied the neuroprotective effect of PPAR delta agonists in MPTP models of Parkinson's disease. In the present chapter, we used a Parkinson's disease mouse model to assess the therapeutic efficacy of GW501516 on behavioral impairment, biochemical, neurochemical and immunohistochemical aspects of nigral neurotoxicity.

MATERIALS AND METHODS:

Animals and chemicals: C57/BL-6 mice (body weight, 20–25 g; age, 8–10 weeks) were purchased from Harlem and were housed with a 12 h day/night light cycle with free access to food and water. All procedures involving animals were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee. The following

chemicals/drugs were purchased from Sigma Chemical Company (St. Louis, MO., USA): 3-hydroxytyramine hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxytryptamine creatinine sulphate (5-HT), norepinephrine (NE), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), 1-heptane sulfonic acid (HSA) sodium salt, HPLC-grade acetonitrile (ACN), HPLC-grade methanol, Triton-X 100, 4-hydroxyquinoline (4-HQ), bovine serum albumin (BSA), kynuramine, ethylenediaminetetraacetic acid (EDTA) sodium salt, O-phthaldialdehyde (OPT), Tris buffered saline tablet, Phosphate buffered saline tablet, Reduced glutathione (GSH), Dimethyl sulphoxide (DMSO), hydrogen peroxide (H_2O_2) and 3,3 -N-diaminobenzidine tetrahydrochloride (DAB). Anti-rabbit tyrosine hydroxylase polyclonal, Anti-GFAP and anti-rabbit IgG-conjugated horseradish peroxidase antibodies were purchased from Chemicon Reverse osmosis water was used in all experiments. For HPLC analysis, double–distilled water was filtered and deionized using Milli-Q system (Waters, Milford, MA). GW501516 was

Experiment design and Treatments: C57/BL-6 mice were intraperitoneally administered with [i] Control (Saline) [ii] MPTP. (30 mg/kg, i.p., twice 16 h apart), [iii] GW501516 (1mg/kg), [iv] MPTP+GW501516 (1mg/kg) and [v] MPTP+GW501516 (0.5mg/kg). Based on the group, saline or GW501516 was pretreated for 7 days and followed by two dose of MPTP (30 mg/kg, i.p., twice 16 h apart). GW501516 were dissolved in 50% polyethylene glycol 400.

BEHAVIORAL STUDY:

Body weight and general observation: The body weights of the animals were recorded regularly in an interval of 3 days by using digital weighing scale. The general observations included tremor, piloerection, salivation and increased respiration after every injection

Straub tail: The straub tail was evaluated by the blind examiners. Straub tail is defined as elevation of tail to more than 45° with a curvature of the tail. The results are expressed as either straub tail phenomenon detected or not detected. (Muralikrishnan and Mohanakumar, 1998; Muralikrishnan et al., 2002).

Akinesia: Akinesia was measured by noting the latency of the animals to move all four limbs in unit time seconds(s) and the test was terminated if the latency exceeded 180s. The animals were initially acclimatized in akinesia cages which consisted of a wooden platform with ventilated perspex hoods. (Muralikrishnan and Mohanakumar, 1998).

Catalepsy: The term catalepsy implies to correct the externally imposed posture in an animal. In the saline and MPTP treated mice catalepsy was measured by placing the animals on a flat horizontal surface with both the hind limbs on a square wooden block (3 cm, high) and the latency in seconds was measured to move the hind limbs from the

block to the ground. The animals were initially acclimatized on the wooden platform (Muralikrishnan and Mohanakumar, 1998)

Swim test: Swimming ability was carried out on 4^{th} day after the saline or MPTP injections. In brief, the mice were placed in water tubs (40 l x 25 w x 12 h, in cm) and the depth of water was kept at 8 cm with temperature maintained at 27 ± 2° C. The animals were acclimatized for 10 min one day prior to experimentation. Swim score scales were given from range of 1 to 4 based on their swimming pattern. Swim score scales were: 1 = hind part sinks with head floating, 2 = occasional swimming using hind limbs while floating on one side, 3 = occasional floating/swimming only, 4= continuous swimming. (Muralikrishnan and Mohanakumar, 1998)

Tail Hanging Test: The saline and MPTP treated mice were suspended by holding the tail and recorded for one min and the observers were blind to the treatment conditions. The record made by counting the number of attempts made to reach the tail by bending the body and crawling up towards its tail. The tail hanging was measured for one min and results are expressed as number of attempts/ minutes (Jiao et al., 2005).

Open-field: The open-field apparatus consist of a box with clear plexi-glass walls and floor. The effects of saline and MPTP on motor activity was evaluated in the open-field. An automated sensor system, consisting of 16 photo beams per side measured the total

ambulation, fine movement and rearing. Each mouse will remain in the field for a total of 5 minutes. (Holcomb et al., 2006).

Rotorod: Saline and or MPTP treated mice were analyzed for motor coordination using the rotorod tests (Columbus Instruments, Columbus, OH). Mice were initialy adapted for a period consisting of 3 training trials on the rotorod with a 10-minute interval. The mice will be given a 10 minute resting time in between each testing run. The rotation speed of the rod will accelerate gradually from 2.5 to 20 rpm over the first 5 minutes and will be held at 20 rpm for the final minute of testing. The time (seconds) spent on the rod (fall latency) will be recorded for each animal and used as a measure of motor function (Holcomb et al., 2006).

Gait analysis: Briefly, saline and or MPTP mice were made to walk on an inclined gangway (100 cm (l) × 12 cm (w) × 10 cm (h) with 30° inclination) leading to a darkened enclosure. The gangway was lined with white paper and the fore- and hind-paws of the mice were dipped in two different non-toxic watercolors to record the footprints and allowed the animals to climb the gangway. After the experiment the limbs were cleaned and dried before placing them back in the cage. The recorded footprints were analyzed for stride length, and stride width.(Fernagut et al., 2002)

BIOCHEMICAL ASSAY:

Tissue Preparation for biochemical assays: Animals were sacrificed by decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes, and other antioxidant molecules. The brain tissue was homogenized in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon homogenizer. Tissue homogenates were centrifuged at 10,000 g for 60 min at 4°C

NADH Ubiquinone oxidoreductase (Complex - I) assay: The decrease in the absorbance due to oxidation of NADH at 340 nm is monitored. The reaction mixture contained potassium phosphate buffer pH 7.4, coenzyme Q_0 and crude homogenate from saline and MPTP treated mice. The reaction was initiated by the addition of NADH and the rate of decrease in the absorbance was monitored at 340 nm for 2 min. A standard curve for NADH was obtained at 340 nm by using known quantities of NADH. The complex-I activity is expressed as the amount of NADH oxidized/mg protein (Muralikrishnan and Mohanakumar, 1998).

Monoamine oxidase (MAO) assay: This enzyme is responsible for the oxidation of biogenic amines. Fluorimetric assay procedure was used for the estimation of MAO (EC 1.4.3.4) activity. The amount of 4-hydroxyquinoline formed due to oxidation of kynuramine by MAO represents the enzyme activity. Incubation mixture contains crude homogenate, potassium phosphate buffer, pH 7.4 and the reaction was initiated by addition of kynuramine. The reaction was then terminated by addition of ice-cold 0.4 N

perchloric acid after 1h. The mixture was centrifuged (14, 000 x g for 5 min) and 2 ml of 1 N NaOH was added to one ml supernatant of the reaction mixture. Fluorescence intensity was measured at activation/emission wavelengths of 315/380 nm. The specific enzyme activity is expressed as nmol of 4-hydroxyquinoline formed/h/mg protein. A standard curve was obtained for commercially available 4-hydroxyquinoline. (Muralikrishnan and Mohanakumar, 1998)

GSH estimation: GSH were measured fluorimetrically (Muralikrishnan and Mohanakumar, 1998). This method employs OPT condensation reaction with GSH to yield a fluorescent product at pH 8.0. Readings were taken at activation and emission wavelengths of 340 and 420 nm respectively. A standard curve was prepared for commercially obtained GSH

GSH Peroxidase Assay: GPx activity was measured spectrophotometrically at 340 nm (Roveri et al., 1994) with minor modifications. The test mixture contained supernatant, GSH, glutathione disulfide reductase (GSH-Rx), phosphate buffered saline, t-butyl peroxide, and finally the reaction was started by the addition of NADPH. The NADPH oxidation rate was recorded for 3 min and activity was calculated from the rate of NADPH oxidation.

ROS generation: Conversion of nonfluorescent chloromethyl-DCF-DA (2′,7′-dichlorofluorescin diacetate) to fluorescent DCF is used to monitor ROS production

spectrofluorometerically using an excitation wavelength of 492 nm and an emission wavelength of 527 nm. DCFH-DA and crude homogenate were incubated for 45 min at 37 C. DCFH reacted with ROS to form the fluorescent product DCF. Intensity was analyzed by a fluorescent micro-plate. The generation of ROS was measured and reported as relative fluorescence intensity/mg protein. (Muralikrishnan and Ebadi, 2001)

HPLC ANALYSIS:

Tissue preparation: Animals were sacrificed by cervical dislocation in the morning before 11.00 AM in order to avoid any diurnal variations of the endogenous amines, enzymes and other antioxidant molecules. For the analysis of neurotransmitters, the whole brains were dissected out within thirty seconds, rinsed in ice cold normal saline, blotted dry on ash-free filter paper and each left and right striata were dissected out separately. Each striatum was deproteinized following sonication (50 Hz for 30 s) in 0.1 M $HClO_4$ (1 mg of tissue/10 µl of $HClO_4$) containing EDTA (0.01%). The samples were centrifuged at 14,000 x g for 10 min and the supernatant was injected into the HPLC system.

Measurement of biogenic amines: The neurotransmitters were estimated by employing HPLC-electrochemistry (Muralikrishnan and Mohanakumar, 1998). Briefly, an HPLC system (shimadzu) consists of shimadzu SCL 10-Avp system controller, LC-10AS pump, LECD-6A electrochemical detector, degasser, Rheodyne injector with 20 µl injection

loop, and C_{18}, ion pair, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 μm and pore size of 80 Å were used for the assay of neurotransmitters and their metabolites. The flow rate was 0.7 ml/min and the electrodetection was performed at + 0.74 V for the analyses of biogenic amines and their metabolites. The composition of mobile phase was 8.65 mM heptane sulphonic acid, 0.27 mM EDTA, 13% acetonitrile, 0.4-0.45% triethylamine and 0.32 - 0.35% phosphoric acid (v/v). A standard solution containing 4 pmol of all the biogenic amines was run immediately prior to and following sample injections every day. Results are presented as pmol/mg fresh tissue.

Measurement of Glutamate and GABA: The amino acids were estimated by employing HPLC-electrochemistry (Clarke et al., 2007). Briefly, an HPLC system (shimadzu) consisting of shimadzu SCL 10-Avp system controller, LC-10AS pump, LECD-6A electrochemical detector, degasser, Rheodyne injector with 20 μl injection loop, and C_{18}, ion pair, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 μm and pore size of 80 Å. The flow rate was 0.65 ml/min and the electrodetection was performed at + 0.8 V for the analyses of glutamate and GABA. **Derivatisation protocol:** Briefly, 10μl of either standard mix or sample supernatant, 90 μl of borate buffer (0.1 M, pH 9.5), 10 μl of potassium cyanide (10 mM) and 10 μl of NDA (6 mM) were added to a single reaction tube, vortex mixed and the reaction was allowed to proceed at ambient temperatures in the absence of light. A 20 μl of the derivative was injected into the appropriate HPLC system. The composition mobile

phase was 0.1M di-sodium hydrogen orthophosphate/50 μM EDTA (pH 5.6, 1 M OPA) and HPLC grade methanol (35:65). Mobile phase was filtered through 0.45 μm and vacuum degassed prior to use. A standard solution containing glutamate and GABA was run immediately prior to and following sample injections. Results are presented as pmol/mg tissue.

IMMUNOHISTOCHEMISTRY STUDIES:

The animals were anaesthetized with ketamine and xylazine, and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed, and kept in 4% paraformaldehyde fixative for one hour, and cryoprotected in 20% sucrose in PBS for 24 h at 4°C. Serial coronal sections (20 μm thickness), spanning the substantia nigra and the striatum, were cut on a cryostat, collected free-floating in PBS, and processed as described below. The sections were rinsed 3 times for 10 min with 0.1 M PBS. The sections were then incubated in 1% H_2O_2 in PBS for 5 min, and permeabilized with 0.4% Triton X-100 for 30 min and further blocked by incubating for 60 min in 2% BSA, 0.2% Triton X-100 in PBS. Sections were then incubated with the primary antibody anti-rabbit TH (1:1000), and Anti-rabit-GFAP (1:1000) in PBS, pH 7.4, containing 1% BSA and 0.2% Triton X-100, for 12 h at 4 °C. After rinsing in PBS containing 1% BSA, sections were incubated with secondary anti-rabbit IgG-conjugated horseradish peroxidase antibody (1:1000) in PBS, pH 7.4, and containing 1% BSA for 60 min at room temperature. Visualization was performed by

incubating in 1 mg/2 ml DAB containing of 1% H_2O_2 for 2-5 min. All sections were then washed for 10 min in PBS, mounted on slides, dried, dehydrated in graded ethanol, cleared in xylene, and mounted with DPX and cover-slip. To test the specificity of the immunostaining, control sections were processed in an identical manner but with the omission of primary or secondary antibody (Haycock, 1987; Saravanan et al., 2006).

Protein Estimation: Protein was assayed using the coomassie plus protein assay reagent kit.

Statistical analysis: Statistics was performed using the Sigmastat-version 2.03 software and results were expressed as mean ± SE. Differences were considered significant at $p < 0.05$.

RESULTS:

General observations

GW501516 and/or MPTP injected mice showed autonomic responses which included piloerection, salivation and increased respiration. Few animals showed convulsions following the first MPTP injection.

EFFECT OF GW501516 IN MPTP-INDUCED BEHAVIOR CHANGES:

Body weight: The administration of GW501516, MPTP and/ or GW501516 did not affect the body weight (Table-6-1, n=8).

Straub tail: Animals treated with MPTP exhibited straub tail and tremor. GW501516 (both dose), reduced the intensity of tremor significantly (Table-6-1, n=8, $p < 0.05$,as compared to the MPTP treated mice).

Akinesia and catalepsy: GW501516 decreased the akinesia and catalepsy significantly (Table-6-1, n=8, $p < 0.05$, as compared to the MPTP treatment).

Tail hanging: GW501516 (both doses) significantly improved the number of attempts in the tail hanging (Table-6-1, n=8, $p < 0.05$, compared to the MPTP treatment).

Rotorod: MPTP treated mice showed significant decrease in the retention time on the rod as compared with the control (Figure-6-1-A, n=8, $p < 0.05$). MPTP associated decrease in the retention time was completely abolished by pretreatment of GW501516 (high and low, Figure-6-1-A, n=8, $p < 0.05$, as compared to the MPTP treatment).

Open field: GW501516 (1mg) significantly increased the ambulation (Figure-6-1-B, n=8, $p < 0.05$, as compared to the MPTP group).

Swim test: MPTP induced significant long-lasting motor impairment with a prominent decrease in swim scores (Figure-6-1-C, n=8, $p < 0.05$, as en compared to saline treatment). This effect was reversed in mice that were treated with a combination of MPTP and 1mg/kg dose of GW501516 (Figure-6-1-C, n=8, $p < 0.05$).

Gait analysis: Stride length was significantly decreased in the animals that received MPTP (Figure-6-1-D, n=8, $p < 0.05$, as compared to the control). The decrease in stride length by MPTP were significantly reversed by both high and low doses of GW501516 pretreatments (Figure-6-1-D, n=8, $p < 0.05$, as compared to MPTP group).

EFFECT OF GW501516 IN MPTP- INDUCED BIOCHEMICAL CHANGES:

ROS: MPTP-induced a marked significant increase in the ROS generation as compared to control (saline treated) animals (Figure-6-2-A, n=8, $p < 0.05$, as compared to the control). MPTP induced ROS generation was significantly reduced by both doses of GW501516 (Figure-6-2-A, n=8, $p < 0.05$, as compared to MPTP).

GSH: GSH content was analyzed in control, GW501516 and/ or MPTP treated mice. MPTP caused GSH depletion (Figure-6-2-B, n=8, $p < 0.05$, as compared to controls). Low dose (0.5mg/kg) of GW501516 blocked the GSH depletion induced by MPTP (Figure-6-2-B, n=8, $p < 0.05$, as compared to MPTP treatment).

GSH-px activity: GSH-Px activity were significantly increased in MPTP-treated mice . (Figure-6-2-C, n=8, $p < 0.05$,as compared to control). Low dose (0.5mg/kg) of GW501516 blocked the MPTP-induced change in the GSH-Px activity (Figure-6-2-C, n=8, $p < 0.05$, as compared to MPTP treatment).

MAO ACTIVITY: GW501516 alone or with MPTP had no effect on the MAO activity (Figure-6-2-D, n=8).

C**omplex-I activity:** GW501516 had no effect on the complex-1 activity (Figure-6-2-E, n=8).

EFFECT OF GW501516 AND/OR MPTP ON NEUROTRANSMITTERS:

MPTP caused a significant decrease in the concentration of DA. MPTP-induced DA depletion (Figure-6-3-A&, n=6, $p < 0.05$) and DA turnover was increased on the 5^{th} day in the nerve terminal region, NCP, which were restored by GW501516 pretreatment

(Figure-6-3-B, n=6, $p < 0.05$). DOPAC and HVA turnover was increased in MPTP, which was restored by GW501516 pretreatment (Table-6-2, n=6, $p < 0.05$). MPTP also affected levels of other striatal neurotransmitters (NE, 5-HT, 5HIAA) and dopamine metabolites (DOPAC, HVA and 3-MT) (Table-6-2, n=6, $p < 0.05$). We also measured the GABA and glutamate levels in the striatum. Levels of glutamate were unchanged after the GW501516 and/ or MPTP injection when compared with the control (Figure-6-3-C, n=8, $p < 0.05$). However in the striatum, GABA levels were significantly increased in MPTP as well as in GW501516 group (Figure-6-3-D, n=8, $p < 0.05$).

EFFECT OF GW501516 AND/OR MPTP ON TH-IR AND GFAP:

TH immunostaining: GW501516 treatment showed minimum loss of neurons in the SN, s compared to MPTP treated mice (Figure-6-4). **GFAP:** Representative microphotographs of GFAP immunostaining in the NCP are shown in figure 6-4. Following the MPTP-treatment, the reaction of astrocytes were increased in the cell number in the NCP regions. This reduction was more pronounced in the NCP of GW501516 pretreated animals (Figure-6-4).

DISCUSSIONS:

The present study investigated the neuroprotective properties of GW501516 in a validated experimental Parkinson's disease animal model. In this model, GW501516 prevented the development of motor impairment induced by MPTP, as measured by the

behavioral test. We used a validated behavioral task which is highly sensitive for motor deficits associated with subtle alterations of the nigrostriatal pathway. GW501516 is a selective agonistic for the peroxisome proliferator-activated receptor-delta (PPAR-δ). PPAR-δ can modulate inflammatory responses in the brain, and agonists might be beneficial in neurodegenerative diseases.

Peroxisome proliferator-activated receptor (PPAR), a nuclear receptor regulates inflammation, mitochondrial function, and glucose metabolism (Bordet et al., 2006; Ciana et al., 2007). PPARs are activated by fatty acids and fatty acid metabolites (Sprecher, 2007). PPAR is comprised of at least three subtypes, including α, γ, and δ. Recently, several PPAR agonists were shown to exert neuroprotective activity against oxidative damage, inflammation and apoptosis in several neurodegenerative disorders including Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis and multiple sclerosis (Bordet et al., 2006; van Neerven et al., 2008). Indomethacin and ibuprofen activate PPAR and reduce the progression of neurodegenerative disorders. The PPAR δ is expressed in tissues, such as the brain, (Kliewer et al., 1994; Schoonjans et al., 1996b; Schoonjans et al., 1996a). PPAR δ agonist have neuroprotective effect and also helps in preventing ischemic acute renal failure (Letavernier et al., 2005; Iwashita et al., 2007). There are studies on the role of the PPAR δ in cerebral ischemia or Parkinson's disease. (Iwashita et al., 2007), some reports show that the PPAR δ agonist can protect cultured neurons from cell death (Berger et al., 1999). GW501516 [2-methyl4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-triazol-5-yl)-methylsulfanyl) phenoxy acetic acid

is the most potent PPAR δ agonist and shows a potent neuroprotective effect against various neurotoxic compound (Iwashita et al., 2007).

PPARs have been implicated originally in lipid and glucose homeostasis, but lately they have been found as regulators of inflammatory responses. It was first observed in PPAR null mice, the inflammatory responses were prolonged which implicated that there is involvement of PPAR in inflammation. The activation of PPAR receptors leads to repression of NF-κB signaling and inflammatory cytokine production. PPAR-delta receptor agonist proved as a newer intervention which can be clinically exploited against the Parkinson's disease since it blocked MPTP-induced dopamine depletion without affecting the monoamine oxidase-b activity.

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LEGENDS:

Table-6-1: EFFECT OF GW501516 IN MPTP-INDUCED BEHAVIOR CHANGES: Table shows the different behavioral parameter studied after MPTP treatments. [A] Body weight was recorded in interval of 3 days, [B] 5min after MPTP, [C] Following three and half hour after MPTP and [D]on 4th day after final injection. ND = not detected. Tremor was visually monitored in a double blind study. MPTP caused a good tremor of approximately 3 in 5min. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$ vs. control), ($^{\#}p \leq 0.05$; vs. MPTP). ND= not detected.

Table-6-2: EFFECT OF GW501516 AND/OR MPTP ON NEUROTRANSMITTERS: Animals were sacrificed on 5th day and nigrostriatal monoamine, were analyzed using HPLC-ECD. *$p \leq 0.05$, significant change as compared to the control and $^{\#}p \leq 0.05$; vs. MPTP. Values represent pmol/mg tissue. Mean ± SEM (n = 8).

Figure-6-1-A: EFFECT OF GW501516 AND /OR MPTP ON ROTOROD IN MICE: All treatment group were tested on the rotorod on 3rd day after the last MPTP injection. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$; vs. control) ($^{\#}p \leq 0.05$; vs. MPTP).

Figure-6-1-B: EFFECT OF GW501516 AND /OR MPTP ON OPEN FIELD IN MICE: Animals were tested on the open field on 3rd day after the last MPTP injection. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$; vs. control) ($^{\#}p \leq 0.05$; vs. MPTP).

Figure-6-1-C: EFFECT OF GW501516 AND /OR MPTP ON SWIM TEST: Effect of swimming ability in the saline/ or MPTP mice was tested in warm water (27 ± 2 °C) on the 4th day following the final injection of MPTP (30 mg/kg, i.p., twice, 16 h apart). Swim-scores were recorded on a performance intensity scale of 1–4 for all the animals for 10 min. Results given are mean ± S.E.M., $n = 8$ ($^{*}p \leq 0.05$; vs. control) ($^{\#}p \leq 0.05$; vs. MPTP).

Figure-6-1-D: EFFECT OF GW501516 AND /OR MPTP ON GAIT ANALYSIS: Gait abnormalities were analyzed on 4th day after MPTP treatment. The stride length was measure between hind and fore limbs. Stride length was measured in cm. Results given are mean ± S.E.M., $n = 8$ ($p \leq 0.05$; vs. control) ($^{\#}p \leq 0.05$; vs. MPTP).

Figure-6-2-A: EFFECT OF GW501516 AND /OR MPTP ON ROS: Effects of MPTP on production of intracellular ROS in crude brain homogenate. Values given are relative fluorescence per milligram tissue and are represented as mean ± S.E.M. $^{*}P \leq 0.05$ as control groups compared with MPTP treated group and $^{\#}p \leq 0.05$; vs. MPTP treatment group (n=6).

Figure-6-2-B: EFFECT OF GW501516 AND /OR MPTP ON GSH: Mice were treated with MPTP (30mg/kg, i.p.) twice, 16 hr apart. Animals were sacrificed on the fifth day and reduced glutathione (GSH) was estimated in the whole brain by a sensitive fluorimetric procedure. Values given are nanogram per milligram tissue and are represented as mean ± S.E.M. *$P \leq 0.05$ as control groups compared with MPTP treated group and $^{\#}p \leq 0.05$; vs. MPTP treatment group (n=6).

Figure-6-2-C: EFFECT OF GW501516 AND /OR MPTP ON GSH-PX: Mice were treated with MPTP (30mg/kg, i.p.) twice 16 hr apart. Animals were sacrificed on the fifth day and GSH-Px activity was estimated. Values given are GSH-Px activity per milligram tissue and are represented as mean ± S.E.M. *$P \leq 0.05$ as control groups compared with MPTP treated group and $^{\#}p \leq 0.05$; vs. MPTP treatment group (n=6).

Figure-6-2-D: EFFECT OF GW501516 AND /OR MPTP ON MAO ACTIVITY: Mice were treated with MPTP (30mg/kg, i.p.) twice 16h apart and sacrificed on 5th day following the second injection. Total MAO activities were assayed and are expressed as in nmol 4-HQ/mg protein/h in enzyme activity and are represented as mean ± S.E.M. *$P \leq 0.05$ as control groups compared with MPTP treated group (n=6).

Figure-6-2-E: EFFECT OF GW501516 AND /OR MPTP ON COMPLEX-I ACTIVITY: Mice were injected with saline/ or MPTP (30mg/kg, i.p.) twice 16 hr apart, sacrificed 5th day and analyzed for complex-I activity. The specific activities of the

enzyme are given as nanomole of NADH oxidized/min/mg protein and are represented as mean ± SEM. $*P \leq 0.05$ significantly different as compared to control side, which received vehicle (n = 6 in each group).

Figure-6-3-A: EFFECT OF GW501516 AND/OR MPTP ON NEUROTRANSMITTERS: Animals were sacrificed on 5^{th} day and nigrostriatal dopamine, were analyzed using HPLC-ECD. (DOPAC+HVA)/DA ratio indicated the turnover of DA. Glutamate and GABA were analyzed using HPLC-ECD. $*p \leq 0.05$, significant change as compared to the control and $^{\#}p \leq 0.05$; vs. MPTP treatment group. Values represent pmol/mg tissue. Mean ± SEM (n = 8).

Figure-6-4: TH, immunoreactivity: Coronal sections from SN were processed for tyrosine hydroxylase activity. **GFAP:** coronal sections from NCP were processed for GFAP. Photomicrographs images shows coronal sections passing through the NCP which were stained for GFAP.

Table and Figures

Table-6-1

Behavioural	CONTROL	MPTP	GW-1mg	MGW-1mg	MGW-0.5mg
Body weight (in gms)[A]	26 ± 0,44	24.33 ± 0.53	27.33 ± 0.36	24.8 ± 0.57	25 ± 0.38
Straub tail[B]	ND	Detected	ND	ND	ND
Tremor[B]	ND	Detected	ND	ND	ND
Akinesia (in sec)[C]	2 ± 0.25	14.25 ± 1.39*	2 ± 0.18#	4.2 ± 0.42#	5.7 ± 0.55#
Catalepsy (in sec)[C]	1.6 ± 0.32	5.12 ± 48 *	1.77 ± 0.16#	2.11 ± 0.12#	1.66 ± 0.18#
Tail Hanging (in min)[D]	24 ± 0.94	14 ± 1.39 *	23.16 ± 1.13#	22.5 ± 2.04#	20 ± 0.89#

Table-6-2

Neurotransmittors	Control	MPTP	GW1mg	MGW-1mg	MGW-0.5mg
DOPAC Turnover	1.04 ± 0.09	1.66 ± 0.07*	1.02 ± 0.67	1.13 ± 0.09#	1.47 ± 0.042#
HVA Turnover	0.44 ± 0.03	0.98 ± 0.06*	0.35 ± 0.03	0.44 ± 0.5#	0.42 ± 0.02#
NE	3.06 ± 0.68	3.70 ± 0.58	3.75 ± 1.00	3.62 ± 1.33	3.09 ± 0.92
5-HIAA	2.67 ± 0.51	3.14 ± 0.44	2.17 ± 0.57	2.36 ± 0.80	1.67 ± 0.62
5-HT	2.50 ± 0.29	2.76 ± 0.06	2.62 ± 0.05	2.67 ± 0.23	2.38 ± 0.25
3-MT	10.24 ± 1.21	4.18 ± 0.65*	8.33 ± 0.47	6.51 ± 0.33	6.59 ± 0.23

Figure-6-1

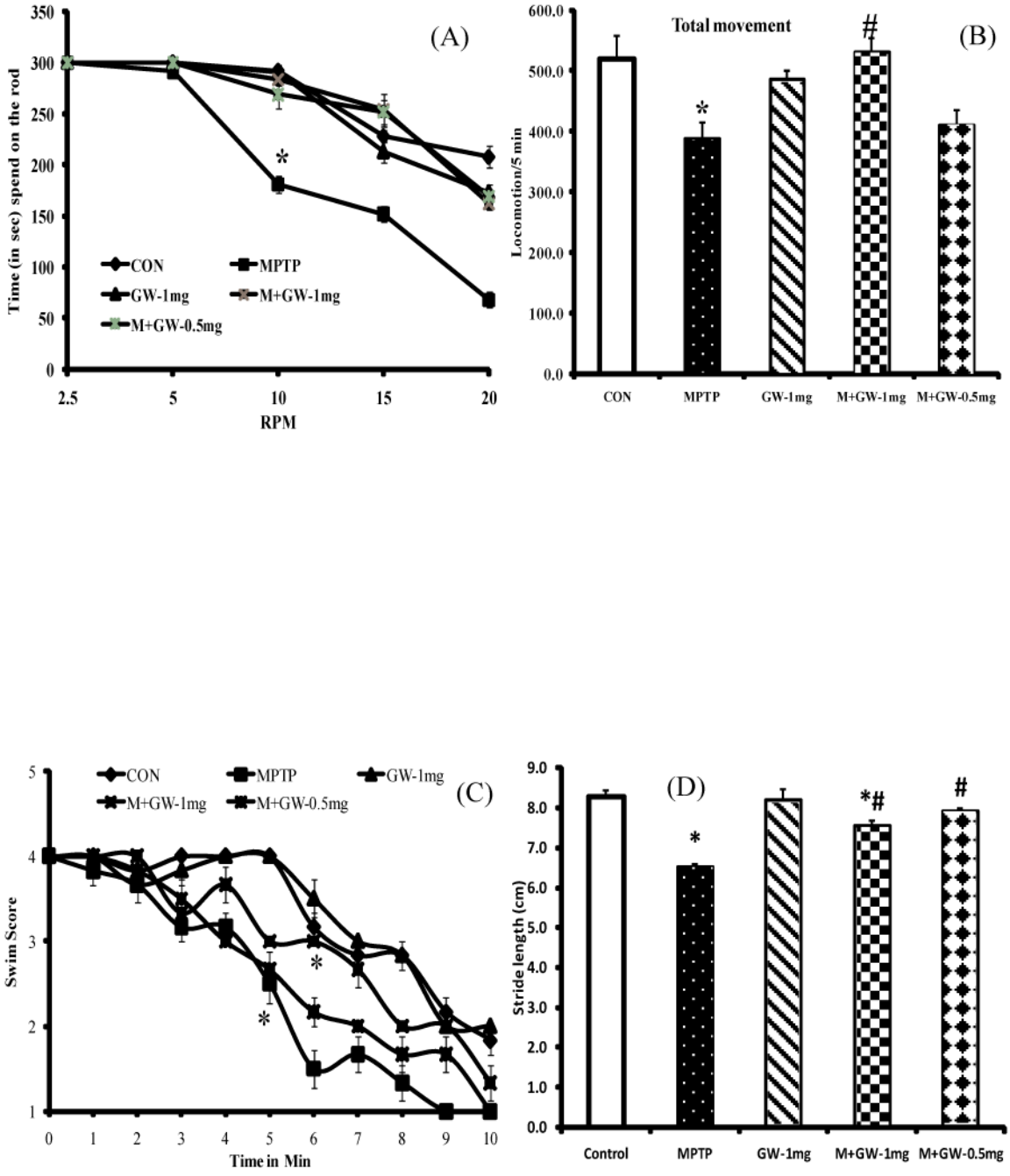

(A)
Time (in sec) spend on the rod
RPM
CON
MPTP
GW-1mg
M+GW-1mg
M+GW-0.5mg
(B)
Total movement
Locomotion/5 min
CON
MPTP
GW-1mg
M+GW-1mg
M+GW-0.5mg
(C)
Swim Score
Time in Min
(D)
Stride length (cm)
Control
MPTP
GW-1mg
M+GW-1mg
M+GW-0.5mg

Figure-6-2

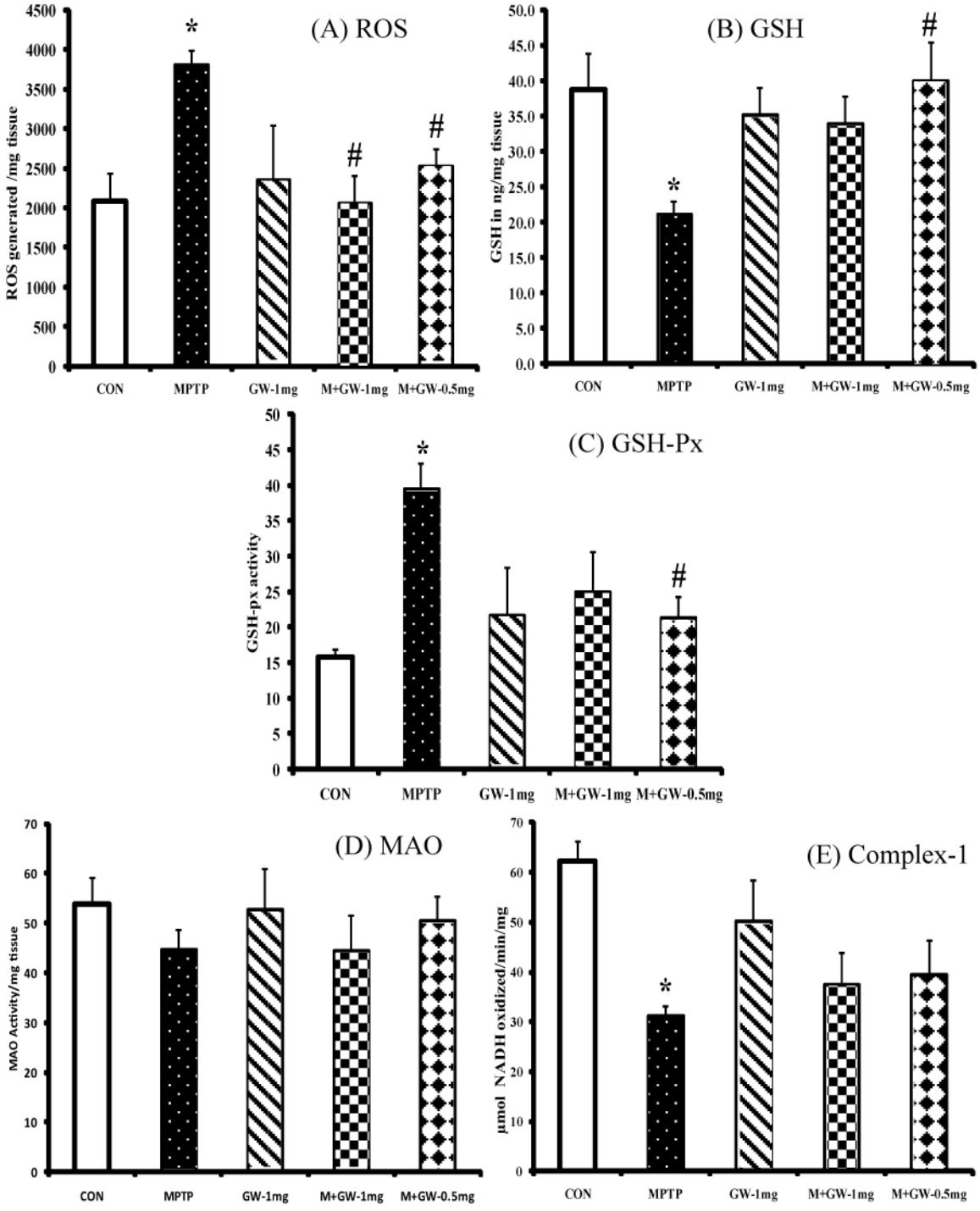

(A) ROS
ROS generated /mg tissue
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
#
#
CON
MPTP
GW-1mg
M+GW-1mg
M+GW-0.5mg
(B) GSH
GSH in ng/mg tissue
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
#
CON
MPTP
GW-1mg
M+GW-1mg
M+GW-0.5mg
(C) GSH-Px
GSH-px activity
50
45
40
35
30
25
20
15
10
5
0
*
#
CON
MPTP
GW-1mg
M+GW-1mg
M+GW-0.5mg
(D) MAO
MAO Activity/mg tissue
70
60
50
40
30
20
10
0
CON
MPTP
GW-1mg
M+GW-1mg
M+GW-0.5mg
(E) Complex-1
µmol NADH oxidized/min/mg
70
60
50
40
30
20
10
0
*
CON
MPTP
GW-1mg
M+GW-1mg
M+GW-0.5mg

Figure-6-3

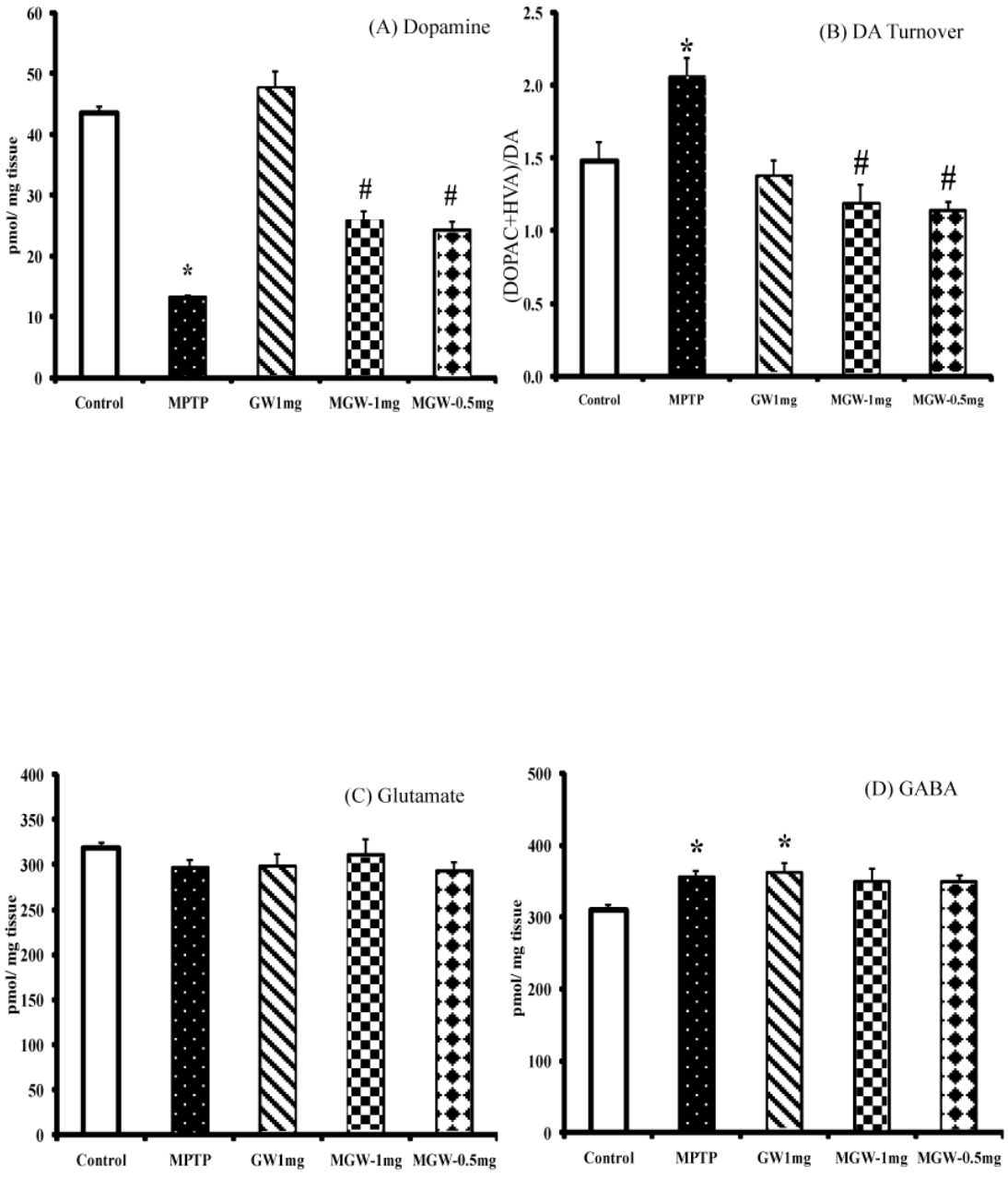
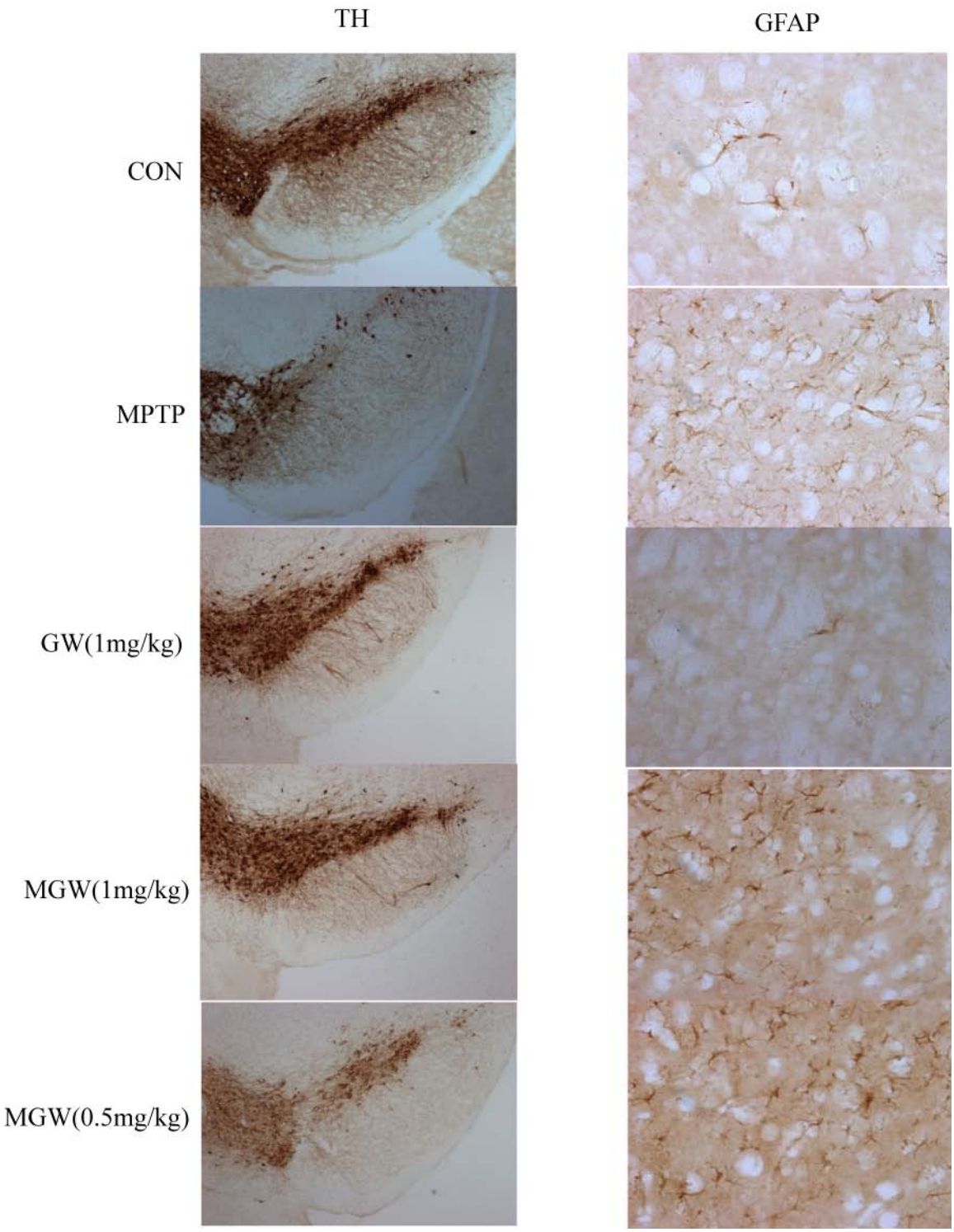

(A) Dopamine
pmol/ mg tissue
60
50
40
30
20
10
0
*
#
#
Control
MPTP
GW1mg
MGW-1mg
MGW-0.5mg
(B) DA Turnover
(DOPAC+HVA)/DA
2.5
2.0
1.5
1.0
0.5
0.0
*
#
#
Control
MPTP
GW1mg
MGW-1mg
MGW-0.5mg
(C) Glutamate
pmol/ mg tissue
400
350
300
250
200
150
100
50
0
Control
MPTP
GW1mg
MGW-1mg
MGW-0.5mg
(D) GABA
pmol/ mg tissue
500
400
300
200
100
0
*
*
Control
MPTP
GW1mg
MGW-1mg
MGW-0.5mg

Figure-6-4



7. SUMMARY AND CONCLUSIONS

PD is a late-onset, progressive neurological disorder characterized by selective nigrostriatal dopaminergic degeneration in A_9 SNpc neurons. The pathophysiology of PD is still unclear, but considerable evidences suggest multi-factorial involvement, including genetic aberration, mitochondrial dysfunction, oxidative stress, excitotoxicity, calcium-mediated cytotoxicity, and apoptosis. Chronic exposures to the pesticide and herbicide have been shown to induce neurotoxic effects. Environmental neurotoxins such as paraquat, and rotenone have shown to play a significant role in the etiopathology and progression of Parkinson's disease. Hence, these toxins are used as an animal model to study the etiopathology of PD. However in our study diquat and/or salsolinol did not show any neurotoxicity in the mouse nigrostriatal tract. MPTP is a potent neurotoxin that causes selective loss of dopaminergic neurons and causing Parkinson's disease like symptoms in humans as well as in animal models. In our study, MPTP has shown to induce oxidative stress (increased ROS generation), decreased tyrosine hydroxylase activity, increase inflammation (increased GFAP) and mitochondrial dysfunction leading to dopamine depletion and increased GABA in the striatum. This acute model resulting in chronic pathology also has the advantage of exhibiting a defined, acceptable motor abnormality along with definite biochemical lesions. This novel model will help in

understanding the pathophysiology of neurodegeneration in PD, and would be an ideal animal model for studying the neuroprotective therapy.

The pretreatment of caffeine (adenosine A2 receptor antagonist), dexamethasone (anti-inflammatory), and GW501516 (PPAR-delta agonist) in MPTP induced animal showed behavioral recovery and protected against MPTP-induced dopamine depletion in the striatum. Pretreatment of these drugs has effect on gabaergic and glutamatergic system. Caffeine, dexamethasone and GW501516 exhibited antioxidant activity but had no effect on the mitochondrial function. These results suggest that these drugs have potential roles in the management of patients with neurodegenerative disease. In the present study, we investigated some of the modern medicines used in the treatment of various disease and have revealed inherent antioxidant and anti-inflammatory actions of these molecules which rendered neuroprotection against dopaminergic neurotoxicity.