CAN EXERCISE AMELIORATE THE SYMPTOMS OF PARKINSON’S DISEASE?

MODES AND MECHANISMS

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DECLARATION

I certify that all material in thesis which is not my own has been identified and that no material has previously been submitted and approved for the award of a degree for this university or any other university.
DEDICATION

This thesis is dedicated to the memory of my father, Professor Dr., Hamdy Ahmed Ali and to my mother, Fatima.

أهدي هذه الأطروحة إلى روح أبي الأستاذ الدكتور حمدى أحمد و إلى أمي السيدة الفاضلة فاطمة نجاتي
ABSTRACT

Movement disorders are the hallmark of Parkinson’s disease (PD) and can severely compromise an individual’s ability to perform well-learned motor skills such as walking, writing, turning around and transferring in and out of bed. The first symptoms of PD typically do not appear until a critical threshold of 70-80% loss of the striatal neurotransmitter called Dopamine (DA) is exceeded. The loss of DA compromises the connection between the striatum and the Substania Nigra (SN); this connection is essential for the control of body movement. The lifelong management of individuals with PD needs a multidisciplinary approach, which includes coordination of pharmacological and non-pharmacological interventions.

The use of prescribed exercise as a non-invasive PD symptom management tool is well recognized. What needs further research and development is an evidence-base for the type, frequency, intensity, duration etc. of exercise bouts. It is however ethically, socially and morally challenging to put unknown physical demands on PD sufferers, therefore in vivo and in vitro studies will be essential in delineating and targeting appropriate interventions. Additionally, in order to establish whether the various interventions are effective will also require a simple measure, preferably one that can be detected following exercise. Ca^{2+} plays an important role in the synthesis of DA via the Ca^{2+} calmodulin system and its increase in exercise coincidences with the reported positive effects of exercise on dopamenirgic neuron activity.

The aim of this thesis was therefore to use in vivo, in vitro and human methodologies to establish a role for physical exercise in the amelioration of the symptoms of PD.
The *in vivo* study comprised of four groups of experimental animals (rats): a control group (C), a training exercise group (E), a group in which Parkinson’s was induced via systemic injections of PD toxin MPTP (PD) and a group where PD-induced animals were trained/exercised (PDE). (E) and (PDE) groups were trained with 8 weeks of endurance exercise at 90% of the lactate threshold (LT), 5 times a week with each bout lasting for 45 min using a custom-built rodent treadmill. After 8 weeks, all animals were sacrificed and brain samples were collected for immunohistochemistry and western blot analysis. Ca$^{2+}$ calmodulin kinases I (CaMK-1) and IV (CaMK-4) were investigated as indicators of the activity of the Ca$^{2+}$ calmodulin pathway.

Immunohistochemical analysis of SN region indicated that in the PD group, CaMK-1 and CaMK-4 expression was suppressed when compared with control (C) animals. This phenotype was apparently rescued by endurance exercise as those animals. The western blot results also showed quantitative differences in CaMK-1 and CaMK-4 proteins in the studied brain regions in the (PDE) and (E) groups compared with the PD group. It was concluded from this data that endurance exercise could up regulate the expression of both CaMK-1 and CaMK-4 in the brain of PD sufferers. It was postulated that changes in Ca$^{2+}$ levels might therefore drive the neuroprotective effect of exercise.

The *in vitro* study was designed to test the hypothesis generated from the *in vivo* work that Ca$^{2+}$ is a main effector of the neuroprotective effect of exercise. The SH-SY5Y human neuroblastoma cell line is used as a model of DA neurons as it has DA activity and can synthesize DA. PD was simulated in these cells by exposure to the toxin 6-OHDA whilst
addition of Ca\textsuperscript{2+} was used as an “exercise mimic”. Results showed differences in the survival of SH-SY5Y cells after exposure to specific concentration of Ca\textsuperscript{2+} following treatment with 6-OHDA.

Finally, in order to assess the importance of this data to the clinical population and to further develop the concept that Ca\textsuperscript{2+} is a major effector of the positive effect of exercise, the effect of moderate-level exercise on the levels of blood Ca\textsuperscript{2+} in subjects with PD was investigated. Measures of cardiovascular physiology and blood biochemistry (total blood Ca\textsuperscript{2+}) were obtained during cycling exercise at an intensity of 90% of the lactate threshold. Results indicated exercise to be beneficial in alleviating motor symptoms of PD.
ACKNOWLEDGEMENTS

This thesis represents the most important four years in my life, throughout these years I have been through all kinds of feelings, from highest degree of happiness to the deepest stage of sorrow. At this moment, I remember how much I have changed and learned. It is something I am incredibly proud of, and that I can call my own. However, I do not think I could have done this without the help and support of a few extremely important people.

I would like to express my deep and sincere gratitude to my supervisor, Professor Mark Lewis, Ph.D, Director of Institute for Sport and Physical Activity Research (ISPAR), University of Bedfordshire. Head of Department of Sport and Exercise Sciences, University of Bedfordshire. Mark, your vast knowledge, endless patience and faultless guidance have made the completion of this thesis possible. From presentation skills to research study advices and confidence boosts, everything you have done for me is now part of my character and is greatly appreciated.

I am deeply grateful to my supervisor Dr Paul Castle, Ph.D. Paul, there has been no limits for your encouragement, patience and support. Joining you in human study is a remarkable experience in my life. From preparing ethical application, testing strategy, and scientific discussions, to collect data and writing up. It was pleasure working with you in this thesis.

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I would like to present special thanks to members of PD society (Bedford and District) for their involvement in the study. Their participation with time and efforts is priceless. They were a great motivation for me to decide about my future research interests. Thanks for Bill Brady, Chairman of the society, for his great role in encouraging society members to join this study and for his interest for the whole project.

I cannot forget to present my very special thanks for my Fiancée Akgul, thank you so much for all the support through working on this thesis. Without you, that work would not has been finished.

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<th>Description</th>
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<tbody>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>AADC</td>
<td>aromatic amino acid decarboxylase</td>
</tr>
<tr>
<td>ADL</td>
<td>Activities of daily living</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>Catecholamine</td>
</tr>
<tr>
<td>CaMK-1</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase I</td>
</tr>
<tr>
<td>CaMK-4</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase IV</td>
</tr>
<tr>
<td>CaMKs</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinases</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO(^2)</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COF</td>
<td>Centre of Force</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’- diaminobenzidine</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>DBS</td>
<td>Deep brain stimulation</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tertra-acetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>H &amp; Y</td>
<td>Hohen and Yahr scale</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LD-50</td>
<td>Lethal Dose test</td>
</tr>
<tr>
<td>M</td>
<td>Metre, measurement</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LT</td>
<td>Lactate Threshold</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>Mg</td>
<td>Milligrams, weight</td>
</tr>
<tr>
<td>Min</td>
<td>Minute, time</td>
</tr>
<tr>
<td>MI</td>
<td>Millilitre, volume</td>
</tr>
<tr>
<td>ML</td>
<td>Mandibular length</td>
</tr>
<tr>
<td>Mm</td>
<td>Millimetres, length</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-d-aspartate</td>
</tr>
<tr>
<td>PAR-Q</td>
<td>Physical activity readiness</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDE</td>
<td>PD + exercise</td>
</tr>
<tr>
<td>PDQ-39</td>
<td>PD questionnaire</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate</td>
</tr>
<tr>
<td>Sec</td>
<td>Second, time</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Human neuroblastoma cell line</td>
</tr>
<tr>
<td>SN</td>
<td>Substania nigra</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substania nigra pars compacta</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TUG</td>
<td>Timed up-and-go test</td>
</tr>
<tr>
<td>UPDRS</td>
<td>Unified parkinson’s disease rating scale</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>VM</td>
<td>Ventral mesencephalon</td>
</tr>
<tr>
<td><strong>VMAT2</strong></td>
<td>vesicular monoamine transporter</td>
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<tr>
<td>---</td>
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<tr>
<td>°C</td>
<td>Degrees celsius, temperature</td>
</tr>
<tr>
<td>µg</td>
<td>Micrograms, weight</td>
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<tr>
<td>µl</td>
<td>Microlitres, volume</td>
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<tr>
<td>Figure 1.1</td>
<td>The substantia nigra</td>
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CHAPTER ONE: REVIEW OF THE LITERATURE

1.1 PARKINSON’S DISEASE

Parkinson’s disease (PD) was first formally described in modern times by a London physician named James Parkinson (1755-1824), Parkinson’s study of this disease was communicated in his “An Essay on the Shaking Palsy” where he wrote:

“Case VI: The gentleman is 72 years of age. About 11 or 12 years ago first perceived weakness in the left hand and arm, and soon after found trembling to commence. In about 3 years afterwards the right arm became affected in a similar manner: and soon afterwards the convulsive motions affected the whole body and began to interrupt speech. In about 3 years from that time, the legs became affected. Of late years the action of the bowels had been very much retarded”.

Quoted in article by James Parkinson’s 1817

PD is a chronic, progressive, neurodegenerative disorder that strikes 1-2% of the world population over 60 years of age (Marttila and Rinne, 1991). The prevalence of PD rises from 0.3% years in the United States population to 1% to 2% in persons 65 years of age or older; some data indicate a prevalence of 4% to 5% in individuals >75 years. The usual age of onset is the early 60s, although up to 10% of those affected are 45 years of age or younger (Weintraub et al., 2008a).
1.1.1 Causes of PD

Most of PD cases are idiopathic with no clear reasons. However, current theories regarding the cause or causes of Parkinson's disease include:

1) Genetic origin, at least 6 gene mutations with more than 11 different links have been seen more frequently in patients with PD (Weidong et al., 2009).

2) Combination of environmental and toxic factors showed a relevant connection with some PD cases (for example working with solvents, exposure to pesticides and herbicides). However, some opinions considered this as higher risk factors of PD more than a direct cause of PD (Schapira and Jenner, 2011).

3) Sequelae of central nerve system infection where toxins and certain vascular injuries are labeled with the progressive of some cases of PD (Lee et al., 2007).

However, many researchers believe that a combination of these three mechanisms may ultimately be proven as the Parkinson's disease cause (Lev et al., 2003).

1.1.2 Motor symptoms of PD

PD can be characterized by four cardinal features: Tremor, which is defined as an involuntary rhythmical movement of small amplitude, muscular rigidity, slowness and poverty of
movement (bradykinesia or hypokinesia), and postural instability. Table (1.1) shows the four symptoms in details.

Movement disorders are the hallmark of PD as it relates to direct daily moving tasks for the patient, which, in the late and the most advanced stages of the disease, leads to a decrease in an individual’s ability to perform well-learned motor skills such as walking, writing, turning around and transferring in and out of bed (Morris et al., 2001a).

These symptoms are generally first noticed by patients when they report difficulties in performing fine movement by hand, such as fastening buttons, accompanied by changes in handwriting (micrographia: abnormally small and cramped) (Bryant et al., 2010). However, these early symptoms may vary from patient to patient. Bradykinesia is the most disabling movement disorder in PD. It directly affects activities of daily living (ADL) such as getting out and in bed, balance, rising from a chair. Moreover, when combined with rigidity, the risk of falls increases.
<table>
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| **Resting tremor**   | - 70%-90% of patients  
                        - More involving the hands  
                        - May be observed as patients rest hands in lap  
                        - May have postural component  
                        - Slow vertical jaw or tongue tremor may be evident, or leg tremor at rest.        |
| **Bradykinesia**     | - 80%-90% of patients  
                        - Slowness in movement  
                        - Most disabling symptom of PD  
                        - May have difficulty turning over in bed or arising from a chair  
                        - Extreme manifestation is (Akinesia)                                           |
| **Rigidity**         | - >90% of patients  
                        - Resistance to the passive movement  
                        - occurring in both flexor and extensor muscles throughout entire range of motion  
                        - May be “cogwheel” (resistance fluctuates in intensity while limb is passively moved) or “lead-pipe” (continuously rigid) |
| **Postural instability** | - Last symptom to appear and reflects progression to advanced stages of PD  
                              - Predisposes to falls and injuries  
                              - Early onset is atypical for PD and suggests other cause of parkinsonism  
                              - Poor or no response to DA therapy                                               |

**Table1.1 The four major motor features of** (Baatile et al., 2000, Chen, 2010, Weintraub et al., 2008a, Weintraub et al., 2008b).
1.1.3 Progression of PD

PD considered as a chronic progressive disease, both motor and non-motor symptoms develop by time (Diaz and Bronstein, 2005). So far, there is no effective therapy, which can stop the progression of the disease (Nutt and Wooten, 2005). However, PD has not been considered as a “fatal” disease, but among the general community, the mortality rate in PD is higher than the general population. Death, normally is caused by other complications such as stroke or cancer (Chen, 2010).

The first symptoms of PD typically do not appear until a critical threshold of 70–80% loss of the neurotransmitter Dopamine (DA) is exceeded (Dauer and Przedborski, 2003). The selective degeneration of DA neurons in the SN is one of the principal features of the pathogenesis of PD (Figure 1.1). The mechanisms of neuronal degeneration in this disease are not fully understood (Grealish et al., 2010). In the brain, DA regulates the striatum, the pigmented cells in the striatum, which is responsible for movement, walking and balance (Baatile et al., 2000). Consequently, lack of DA aggravates the symptoms of the disease. PD starts by Trauma and finishes with incapability to accomplish the simple tasks of daily living (Chen, 2010).
Figure 1.1 The Substantia Nigra (SN). SN contains DA neurons that project to the basal ganglia, including the striatum. It was observed that DA levels in the striatum were reduced to less than 10% of normal in the brains of Parkinsonian patients studied at autopsy (Ehringer and Hornykiewicz, 1960).

The loss of DA neurons accounts for most of the movement disorder symptoms in PD. Treatment with L-3,4-dihydroxyphenylalanine (L-DOPA), the immediate precursor of DA, improves some symptoms in PD but, on the other hand, after treatment with (L-DOPA) for a definite period of time, patients do not maintain benefit or cannot tolerate side effects (Foster and Hoffer, 2004, Bouhaddi et al., 2004). Therefore, researchers have started to investigate the possibility of rectifying the symptoms of PD by other non-pharmacological therapies. Physical exercise has been considered as one of these potential non-pharmacological treatments.
1.1.4 Pathological features of PD (DA depletion)

The hallmark of PD is the degeneration of DA neurons in the SN, which causes a depletion of DA neurons (Figure 1.2). The loss of these neurons produces the gross neuropathological finding of SN depigmentation and is consistent with the finding that depletion of DA is most pronounced in the dorsolateral putamen, the main site of projection for these neurons (Bernheimer, Birkmayer et al. 1973). At the onset of symptoms, putamenal DA is depleted and between 70%- 80% of SN’s dopamine neurons have already been lost (Dauer and Przedborski, 2003).

Many factors are speculated to operate in the mechanism of cell death of nigrostriatal DA neurons in PD, including oxidative stress, disturbances of intracellular Ca^{2+} homeostasis, exogenous and endogenous toxins, and mitochondrial dysfunction. An endogenous neurotransmitter DA is thought to be a major source of oxidative stress to these neural cells (Lev et al., 2003). However, the depletion of DA neurons is not limited only with the SN, it also occurs in that regions on the brain which may cause other non-motor PD symptoms (Schapira and Jenner, 2011).
Figure 1.2 Neuropathology of PD. Diagram (A) represents the normal nigrostriatal pathway (in red). It is composed of DA neurons whose cell bodies are located in the SN. These neurons project (thick solid red lines) to the basal ganglia and synapse in the striatum. The photograph demonstrates the normal pigmentation of the SN, produced by the DA neurons. Diagram (B) represents the diseased nigrostriatal pathway (in red). In PD, the nigrostriatal pathway degenerates. There is a marked loss of DA neurons that project to the putamen (dashed line) and a much more modest loss of those that project to the caudate (thin red solid line). The photograph demonstrates depigmentation (i.e., loss of dark-brown pigment neuromelanin; arrows) of the SN due to the marked loss of DA neurons (Dauer and Przedborski, 2003).

1.1.5 DA neurons in the brain

DA is a neurotransmitter employed by specific neurons in the central nervous system (CNS). DA neurons are involved in neural processes as diverse as neuroendocrine hormonal
release, cognition, emotion, reward and initiation of motor responses (Brown et al., 2005). The neuropathology of PD is a gradual loss of midbrain DA neurons and their innervations of the basal ganglia in the ventral forebrain (Lang and Lozano, 1998).

The DA neurons in the SN have predominantly been utilities for attempts to replace the lost dopaminergic transmission in the basal ganglia by transplantation of immature DA neuroblasts from fetal tissue. This has proved to be a viable approach, in animal models of PD but also in PD patients (Lindvall et al., 1990, Winkler et al., 2005). Since cell replacement therapy requires large numbers of transplantable DA neurons and the yield from fetal tissue is limited, stem cells have been suggested and explored (Arsenijevic and Weiss, 1998) as an option to meet this need.

1.1.6 Location of DA neurons in the brain

The DA neurons in the brain are organized into ten nuclei ranging from the caudalmost cell group A8, the retro-rubral field, to the rostralmost A17, a group of amacrine interneurons in the retina (Bernheimer et al., 1973, Blum et al., 2001). Small groups of DA neurons are located in the olfactory bulb and the diencephalon (part of forebrain). However, the vast majority of DA neurons, around 75%, reside in nuclei in the ventral mesencephalon (VM): the substantia nigra pars compacta (SN, A9), the ventral tegmental area (VTA, A10) and the retro-rubral field (Melamed et al., 1980, Grealish et al., 2010). The DA neurons of the mesencephalic nuclei (the DA neurons) are often investigated together since they develop from the same progenitor location (Grealish et al., 2010).
1.1.7 Projections and functions of midbrain DA neurons

The DA neurons of the midbrain nuclei have distinct functions in the brain and consequently innervate separate structures. The DA neurons whose cell bodies are located in the SN, and which are the neurons most affected in PD, project to the dorso-lateral striatum and caudate putamen forming the so-called nigrostriatal pathway (Grealish et al., 2010). The nigrostriatal pathway modulates the output of these basal ganglia structures that, together with cortical areas control initiation of voluntary movement and control of posture, for example. Since the nigrostriatal innervation is lost in PD patients, they have characteristic symptoms of motor dysfunction such as rigidity and slowness of movements that is accompanied by tremor (Lang and Lozano, 1998). The VTA neurons innervate limbic areas in the ventro-medial striatum (mesolimbic system) and pre-frontal cortex (mesocortical system) and are involved in emotion, cognitive processes and reward behaviours (Chalimoniuk et al., 2004, Fisher et al., 2008).

1.1.8 Identification of DA neurons

DA neurons are generally identified by their expression of the rate-limiting enzyme in the DA pathway, tyrosine hydroxylase (TH). TH modifies the amino acid tyrosine to DA precursor L-DOPA, which is in turn, is converted to DA by aromatic amino acid decarboxylase (AADC). DA neurons share expression of proteins involved in production, storage and release of DA, such as TH, AADC, DA transporter (DAT) and vesicular monoamine transporter (VMAT2). Other proteins are expressed in different DA nuclei. These proteins are likely to play a role in the function of the particular DA neuron subtype but are also used simply as markers to distinguish
them. The DA neurons have very similar expression profiles, more so during development than at adult stages. They can be identified by location and projections in intact adult tissue and their morphologies differ slightly. At adult stages, DA neurons in SN and VTA can also be distinguished by presence of the markers Girk-2 and calbindin, respectively (Thompson L, 2005).

1.1.9 Limitations of the pharmacological treatments of PD

At present, the objectives of the current treatments are to provide symptomatic relief from the motor and the non-motor symptoms. Therefore, the objectives of PD treatments are to enhance quality of life and reduce total costs of healthcare. However, the most effective medication for motor symptoms of PD is Levodopa. Levodopa is a precursor of DA, crosses the blood brain barrier and is decarboxylated to DA in the nigrostriatal pathways. Levodopa is usually given accompanied with Carbidopa, a peripheral dopa-decarboxylase inhibitor, which prevents peripheral metabolism of Levodopa and allows a higher percentage of the dose to cross the Blood brain barrier (Marttila and Rinne, 1991). Combined use of Levodopa with Carbidopa also minimizes the adverse effects of peripheral DA, such as nausea and hypotension (Rao et al., 2006).

Investigations reported a 5 year “honeymoon period” within which the small doses of Levodopa given 3 times a day could be significantly effective (Foster and Hoffer, 2004). However, Levodopa has been reported to cause significant body functional disability and has been linked directly to developing dyskinesias. PD therapists suggest starting the therapy with other drugs than Levodopa before starting with Levodopa (Foster and Hoffer, 2004).
1.1.10 Limitations of the surgical treatments of PD

In recent years, neurosurgery has re-emerged as an important strategy in treating certain symptoms of PD. Advances in basic science have also provided researchers with a fundamental rationale for surgical approaches as well. Surgery does not cure the disease; however, it is a way of setting the clock back on the disease.

Deep Brain Stimulation (DBS) involves placing an electrode in various parts of the brain to deliver continuous high-frequency electrical stimulation to control movements. This stimulation is thought to suppress the abnormal pattern of excessive activity in these brain areas and returns them closer to normal, although the exact mechanism of the DBS effect is still not fully understood (Vergani et al., 2010). DBS can be performed on both sides of the brain or in a combination of different targets. The selection of different DBS targets depends on the symptoms or the disease to treat and may include thalamus, globus pallidus, or the subthalamic nucleus (Lozano et al., 2002).

DBS has shown significant improvement in patient’s moving ability and a significant reduction in bradykineasia. In addition, this treatment allows for significant reduction in increasing quality of daily life movements as well for patients who have been treated with DPS. Recent studies have shown a 55.9% reduction in daily Levodopa equivalent dosage. These studies also showed a 69.1% reduction in dyskinesias, a 68.25% reduction in off periods, and a 34.5% improvement in quality of life (Rao et al., 2006). However, as with any other surgical procedures, the DBS surgery also has some complications including lesioning involves destruction of a part of the brain, risk of infection, hardware failure, loss memory and stroke (Vergani et al., 2010).
1.2 CONTRIBUTION OF EXPERIMENTAL NEUROTOXINS IN PD STUDIES

Research in PD has been progressed dramatically after the discovery of PD neurotoxins, which provided the opportunity to develop PD models and study causes and the different effects of potential medications. The following section highlights the most commonly used neurotoxins in PD research. Rodents and nonhuman primates are used most frequently in PD research, because when a Parkinsonian state is induced, they mimic many aspects of idiopathic PD (Potashkin et al., 2010). For the nonhuman primates, according to the high cost and ethical issues, this model is most often used as a final study of a test compound’s efficacy before testing on humans. Cynomolgus, rhesus, or African green monkeys are the most used non-human primate species in PD and exercise projects, following systemic neurotoxin injections, the animal’s motor and cognitive functions are impaired, as in humans. In particular the animals display muscle rigidity, akinesia, and tremors, along with impairment on a common memory task (Smith and Zigmond, 2003). In this thesis, the neurotoxins MPTP and 6-OHDA have been used to mimic the symptoms of PD. The following section will describe and explain the effect of these neurotoxins in the experimental research of PD.

1.2.1 MPTP neurotoxicity

1-methyl-4-phenyl-4-propionpiperidine (MPTP) was first discovered after a number of adult people showed PD symptoms in a Californian hospital in the early 1980s. These were unusual symptoms for diagnosis as PD symptoms usually start to appear at the age of 60 (Calne et al., 1985). However, it had been discovered that these groups of people were under the effect
of homemade heroin, this early case were led to the identification of symptoms that responded positively to (L-DOPA) treatment. This accidental clinical discovery identified this neurotoxin tool that has been generalized in the past 30 years in animal models and in vitro models of PD. It provided a deep insight into progressive PD. Sershen et al. (1985) demonstrated that MPTP is metabolized to the neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺) by the enzyme MAO-B, which is expressed in neurons. This exhibits striatal DA reductions, SN neuron losses and behavioural impairments. It selectively kills brain tissue in the SN and causes PD symptoms. Figure (1.3) shows the routes of how MPP⁺ (the metabolite of MPTP) can cause the DA damage: (1) concentration into mitochondria through an active process (toxic); (2) interaction with cytosolic enzymes (toxic); (3) sequestration into synaptic vesicles via the vesicular monoamine transporters (VMAT; protective). Within the mitochondria, MPP⁺ blocks complex I (X), which interrupts the transfer of electrons from complex I to ubiquinone (Q). This perturbation enhances the production of reactive oxygen species (not shown) and decreases the synthesis of ATP, without affecting other brain regional monoamine neurotransmitters, paving the way for extensive biochemical investigations on the mechanisms of MPTP neurotoxicity. Nevertheless, MPTP effects in rodents varied with dose, route, number and injecting times, as well as gender and age (Jarvis and Wagner, 1990). This created uncertainty until species appropriate dosing and the range of injecting steps in MPTP toxic effects and metabolism were clarified (Figure 1.3).
Figure 1.3 Diagram of MPP⁺ intracellular pathways inside DA neurons. Inside DA neurons, MPP⁺ can follow one of three routes: (1) concentration into mitochondria through an active process (toxic); (2) interaction with cytosolic enzymes (toxic); (3) sequestration into synaptic vesicles via the vesicular monoamine transporters (VMAT; protective). Within the mitochondria, MPP⁺ blocks complex I (X), which interrupts the transfer of electrons from complex I to ubiquinone (Q). This perturbation enhances the production of reactive oxygen species (not shown) and decreases the synthesis of ATP.

1.2.2 (6-OHDA) neurotoxicity

Blum et al. (2001) demonstrated that 6-Hydroxydopamine (6-OHDA) is one of the most common neurotoxins used to experimentally model nigral degeneration in vitro as well as in vivo. 6-OHDA is a hydroxylated analogue of the natural DA neurotransmitter. Porter et al. (1963) who showed that 6-OHDA induces noradrenaline depletion in the autonomic nervous system of the heart first demonstrated its biological effects. Shortly thereafter, several studies
demonstrated its ability to destroy nerve cell endings of sympathetic neurons (Thoenen and Tranzer, 1968, Tranzer and Thoenen, 1968). Those results were obtained after systemic injection of the neurotoxin. However, 6-OHDA is unable to cross the blood–brain barrier; therefore, production of central neuronal lesions can be achieved only after direct intracerebral administration (directly into the brain parenchyma). Application of 6-OHDA into lateral ventricles was previously shown to produce central catecholamine depletion (Urestsky, 1970).

In experimental models of PD, 6-OHDA is preferably injected into the striatum, the SN or the ascending medial forebrain bundle. It acts to destroy nigral DA neurons and to deplete the striatum DA neurotransmitter (Blum et al., 2001). Hypothetically, the mechanism of how 6-OHDA toxicity could induce catecholaminergic cell death could be summarized by two main mechanisms:

1. Reactive oxygen species generated by intra or extracellular auto-oxidation hydrogen peroxide formation induced.

2. Direct inhibition of the mitochondrial respiratory chain.

These events lead to strong oxidative stress amplified by cytoplasmic free Ca\(^{2+}\) and to a decrease in cellular ATP viability, both leading to cell death (Glinka, 1995).

Several studies have confirmed that 6-OHDA produces oxidative stress \textit{in vivo} (Kumar et al., 1995) as well as \textit{in vitro} (Choi and Cheon, 1999). This explains the protective
effects afforded by antioxidants against 6-OHDA toxicity and the observation of decreased cell death in transgenic mice over expressing superoxide dismutase and glutathion peroxidase (Asanuma et al., 1998). In (6-OHDA) model, the injection targets the medial forebrain bundle at the level of the lateral hypothalamus, that aims to produce an extensive loss of neostriatal DA (Blum et al., 2001).

1.2.3 Limitations of pharmacological models used to induce PD symptoms in rats

Throughout the years of PD research, rodents have been widely used to study the disease because they are readily available and relatively low cost when compared to larger animals, there are several studies that have used dogs, cats and nonhuman primates for PD studies, but the ethical concerns and costs of such studies have limited their usefulness. Therefore, rodents have become the most common models in PD research and they were used in two experimental chapters in this thesis.

Although there is a great deal of similarity between the physiology of rodents and humans, it is clear that significant differences exist. The following section will discuss the limitations in using animal models in PD research. The aim of this section is to explain the limitation in a way that might help other research projects to translate the effect of exercise in animal models into humans. The standard models for PD are designed to produce nigrostriatal dopaminergic lesions usually with 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or, rotenone (Borland et al., 2008). Most of these models inhibit mitochondrial function and/or create reactive oxygen species. However, none of them
completely reproduces the clinical symptoms and pathology of PD seen in humans (Potashkin et al., 2010).

First concern in the limitations is that PD is an irregular disease, which means that the exact symptoms are hard to repeat between one individual and another, when translate that to animal models; there might be differences between the animals especially that each animal needed to be handled individually. In addition, PD occurs most frequently in elderly patients, usually around the age of 60 or older. Unfortunately, most rodent models do not use older animals because of the inconvenience and cost of housing the animals for an extended period of time (Potashkin et al., 2010).

In addition to the differences between the same model animals, there is another problem regarding the assays used to assess changes between the healthy and diseased state. PD patients experience many motor symptoms including akinesia, bradykinesia, muscular rigidity, dystonia, resting tremors, gait abnormalities and postural instability due to progressive DA neuron loss and dysregulation of DA modulated pathways in the basal ganglia (Blum et al., 2001). When assessing behavioural changes in rodent models, it is important to keep in mind that although the neuroanatomical components underlying motor control may be similar for humans and rodents, the manifestation of these motor deficits may be expressed differently between species.

Another important limitation is the degree of DA loss, the timing and dose of the toxin injections, the time between injections and the behavioural testing and genetic manipulations will all affect the results of the behavioural study. As mentioned previously, PD symptoms do not
appear until 80% of DA was destroyed in the brain. However, in animal models, if this percentage is destroyed in the rat brain, it will either die or lose its ability to move completely (Calne et al., 1985).

These limitations might prevent the results of PD studies using animal models to be translated to clinic application unless further research work to decrease these limitations. However, animal models provide valuable understanding for PD and other diseases.

As described previously, the discovery of neurotoxins MPTP and 6-OHDA, which can selectively kill DA neurons, allowed researchers to induce PD in different animal models, which, in sequence, provided a closer look into the molecular and genetic causes in PD neurons. There are two major techniques for producing in vivo models of PD; unilateral lesioning with (6-OHDA) and systemic injection of 1-methyl 4 phenyltetrahydropyridine (MPTP) (Blum et al., 2001). Using one of the previous models, early interventions with exercise training in vivo studies reported vital information about the histological changes in the neurons after different types of exercise. The choices of exercise type are limited in vivo studies; rodent running on a special treadmill was the major exercise intervention (Enevoldsen et al., 2001, Smith and Zigmond, 2003, Langfort et al., 2006).

After the availability of PD models, described previously, the question of “would a program of motor exercise therapy retard the neurodegenerative process that occurs in PD” becomes more able to be investigated. Research strategies were divided into two directions, first, motor exercise before PD condition. Secondly, motor exercise after PD condition. Pre-exercise lesion was
targeted to investigate whether there is any protective effect of exercise against PD toxins. While post-exercise reviews aimed to test the hypothesis that exercise targeting PD models affected by degeneration of nigrostriatal DA neurons will have a beneficial effect. The correlation between exercise and PD has been investigated previously in three different levels, human level, in vivo level and in vitro level; in the following section the three levels of previous investigation will be highlighted.

1.3 EXERCISE AND PD

Because PD is a chronic progressive disorder, it is probable that sustained exercise is necessary to maintain benefits. Indeed, follow-up data from a number of human exercise interventions have demonstrated a gradual return to baseline abilities after the supervised intervention is finished. Results from previous research projects (including both animal models and information from humans) support the importance of exercise for people with PD and raise the question of whether such exercise might play a neuroprotective role. For example, Tillerson et al. (2003) showed that motorized treadmill running twice daily for 10 days enhanced motor performance and brain neurochemistry in 2 different rat models of PD. Likewise, Dobrossy and Dunnett (2006) reported that rats that received motor training after striatal lesions or striatal grafts showed some recovery in spontaneous movements and skilled motor performance.

Data from Fisher et al. (2008) indicate central effects can occur with exercise for people in the early stages of PD. Finally, Thacker and colleagues examined the impact of recreational physical activity on future risk of developing PD. Data were examined from 143325 participants
who were followed for 8 years. The authors identified a reduced relative risk of developing the
disease for those individuals who had reported moderate to vigorous activity at baseline (Thacker
et al., 2008). Although it is not yet clear whether exercise has a neuroprotective effect for people
with PD, at a minimum, exercise does assist people to maintain functional ability.

Taking all of the results together, we advocate the importance of enabling people with PD
to make long-term adaptations to integrate physical activity into their daily lives. Furthermore,
we advocate that vigorous exercise should begin immediately on diagnosis, if possible, and
continue throughout the course of the disease for as long as the individual is able to exercise.

1.3.1 Exercise and the risk of falls for PD patients

As the disease progresses, falls frequently accompany gait disorders in PD. Thus,
minimization of falls is a key goal of exercise therapy for patients with locomotor dysfunction
like PD (Bloem et al., 2001). Between 50% and 70% people with PD experience one or more
falls over a 12-month period, which is much higher than the 30% fall rate reported for
community-dwelling older people (Bloem et al., 2001, Ashburn et al., 2007b). Self-reporting is
known to markedly underestimate the true fall rate in a range of conditions; thus, there is a need
for therapists to look for other indicators of falls such as gait deviations, injuries, increased use of
professional services, hospitalization, and reduced participation in societal roles.

Many of the falls in PD occur when people attempt to perform multiple tasks or long or
complex movement sequences (Bloem et al., 2001). Turning during walking is particularly
problematic, as is carrying trays and other objects or walking at the same time as talking. Moreover, evidence is emerging that patients who experience freezing (an episodic inability to initiate or continue stepping) are particularly at risk of falls (Campbell et al., 2005). This group is susceptible to multiple falls, with some individuals falling many times each week despite the best attempts to optimize medication (Canning et al., 2009). Health promotion activities that educate patients and their families about fall risk factors and how to prevent slips, trips, and falls, therefore, are integral to comprehensive physical exercise management of people with PD. Long-term adaptations by the patient are needed to integrate fall prevention strategies into their daily lives. It is important for the clinician to know when, where, and during what tasks the falls occur, as well as the direction (Gillespie et al., 2005, Canning et al., 2009).

Although population-based data provide a good guide, the PD researchers still need to know the specific experience for each individual. Dibble et al. (2006) conducted a systematic review of exercise-based interventions to improve balance in PD and determined that although there is moderate evidence to support the efficacy of exercise in improving postural instability and balance task performance, it remains unclear which specific types and dosages of exercise are optimal for the management of balance disorders in people with PD. However, with exercise interventions as diverse as (Cakit et al., 2007), training spinal training (Schenkman et al., 1998), muscle strengthening (Dibble et al., 2006), Tai Chi (Hackney and Earhart, 2008) and dancing (Thacker et al., 2008), each proving to be of some benefit to people with PD, it appears that an exercise program tailored to the individual's balance impairment, fall history, lifestyle, and personal interests may be preferable to a “one size fits all” approach and that was a main principle of this thesis.
Exercise and balance for PD patients was not the only area of interest for PD researchers, there were also evidences supporting the efficacy of exercise therapy for other movement disorders. Kwakkel et al. (2007) published a subsequent critical review of the literature on exercise therapy for PD, the review identified 23 randomized controlled trials investigating the effects of physical therapy on function in people with PD. Only 3 of these studies targeted gait disorders (Morris et al., 2001b, Herman et al., 2007, Fisher et al., 2008).

An additional 6 studies measured gait- and mobility-related outcomes from programs directed toward improving posture and balance (Schenkman et al., 1998, Ashburn et al., 2007b). These studies were of moderate methodological quality and demonstrated some benefits of exercise therapy for gait and mobility. The interventions tested and outcome measures used varied markedly, making between-study comparisons difficult. Interventions included cueing, mental rehearsal, exercises and cycling.

The quality of exercise therapy research in PD has improved in the last decade, yet gaps in the evidence base for specific interventions remain. More recently, a number of alternative treatment modalities, such as Tai Chi, treadmill training, and whole-body vibration have been proposed for the management of gait disorders in people with PD (Fisher et al., 2008, Hackney and Earhart, 2008). Other treatments modalities were identified with additional 5 randomized controlled trials exercise therapy for PD patients (Table 1.2). Three of these studies investigated the effects of treadmill training, including high-intensity body-weight–supported training (Fisher et al., 2008) and speed-dependent treadmill training, demonstrating positive results in the small samples tested (Cakit et al., 2007). Similarly, whole-body vibration therapy (Ebersbach et al.,
2008) and Tai Chi training (Hackney and Earhart, 2008) have been reported to produce modest improvements in the gait and balance performance of people with PD. Although all of these studies included a comparison group, none provided control interventions founded on evidence-based “best practice” such as strategy training, cueing, or the management of musculoskeletal sequelae. Table 1.2 summarizes the exercise overview and the progression based on the literature review of some research reports.
<table>
<thead>
<tr>
<th>Exercise</th>
<th>Action</th>
<th>Progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Tai chi: Increase limits of stability,</td>
<td>● Prayer wheel: anterior-posterior slow, rhythmical weight shifts</td>
<td>● Learn one action per week, starting with weight shifting and leg</td>
</tr>
<tr>
<td>improve perception of posture and</td>
<td>coordinated with large arm circles</td>
<td>placement and progressing to coordinated arm, neck, and torso motion</td>
</tr>
<tr>
<td>coordination of arms and legs and</td>
<td>● Cat walk: slow and purposeful steps, with diagonal weight shifts</td>
<td></td>
</tr>
<tr>
<td>backward and lateral large steps (King and</td>
<td>● Cloud hands: slow lateral steps, with trunk vertical.</td>
<td></td>
</tr>
<tr>
<td>Horak, 2009, Hackney and Earhart, 2009).</td>
<td>● Part the wild horse’s mane: coordination of arms and legs while</td>
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<td></td>
<td>● Repulsing the monkey: deliberate slow, beginning walking, with</td>
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<tr>
<td></td>
<td>● Learn one action per week, starting with weight shifting and leg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>placement and progressing to coordinated arm, neck, and torso motion</td>
<td></td>
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</tr>
<tr>
<td>II. Kayaking: Trunk rotation,</td>
<td>● Kayaking stroke: diagonal trunk rotation, with reciprocal forward arm</td>
<td>● Speed, surface, resistance, vision, dual task</td>
</tr>
<tr>
<td>Segmental coordination, speed (King and</td>
<td>extension and backward arm retraction</td>
<td></td>
</tr>
<tr>
<td>Horak, 2009).</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III. Agility course: Agility,</td>
<td>● High knees: high-amplitude stepping, with hand slapping knees</td>
<td>● Speed, dual task, quick change in directions, tight and cluttered spaces,</td>
</tr>
<tr>
<td>multisegmental coordination, quick changes</td>
<td>● Lateral shuffle: quick, lateral steps</td>
<td>vision</td>
</tr>
<tr>
<td>in direction, and mobility in tight spaces</td>
<td>● Tire course: wide-based, quick and high steps, with</td>
<td></td>
</tr>
<tr>
<td>(Ouchi et al., 2002).</td>
<td>Turn</td>
<td></td>
</tr>
<tr>
<td></td>
<td>● Grapevine cross: over coordinated steps</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>IV. Anticipatory postural adjustments,</td>
<td>● Jab: short, straight punch from shoulder</td>
<td>● Speed, dual task, walking forward, walking backward, turns, remembered</td>
</tr>
<tr>
<td>postural corrections, fast arm and foot</td>
<td>● Cross: power punch, with trunk rotation, leading arm crosses midline</td>
<td>sequences of action</td>
</tr>
<tr>
<td>motions, backward walking, timing,</td>
<td>● Hook: short, lateral punch, with elbow bent and wrist twisted</td>
<td></td>
</tr>
<tr>
<td>sequencing actions (Fox et al., 2012).</td>
<td>inward, trunk rotation</td>
<td></td>
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<tr>
<td></td>
<td>● Combinations: 2 or more punches delivered quickly after one another</td>
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<tr>
<td>V. Lungenes: Big steps, stepping for</td>
<td>● Postural correction: lean until center of mass is outside base of</td>
<td>● Surface (up and down stool), external cues, vision, resistance, dual</td>
</tr>
<tr>
<td>postural correction, limits of stability,</td>
<td>mass is outside base of support, requiring a step; all directions</td>
<td>task (add arm movements or cognitive task)</td>
</tr>
<tr>
<td>quick changes in direction, internal</td>
<td>● Single multidirectional steps (clock stepping)</td>
<td></td>
</tr>
<tr>
<td>representation of body (Protas et al., 2005b).</td>
<td>● Dynamic multidirectional lunge walking</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 Representative agility exercise program and its progression (King and Horak, 2009).
1.3.2 Exercise and PD progression: Human studies

The effect of physical exercise on individuals with PD has advanced considerably over the past decades. Numerous approaches investigated the effect of different types of exercise on different movement disorder with PD patients. Table 1.3 shows examples of the previous work on exercise and PD. It has been indicated that an exercise program would lead to significant improvements in cognitive skills, activities of daily living (ADL), motor function, and quality of life (Herman et al., 2007, Fisher et al., 2008). Cakit et al. (2007) suggested that specific exercise programmes using incremental speed-dependent treadmill training may improve mobility, reduce postural instability and fear of falling in patients with PD. Other clinical studies have shown overall improvement in muscle strength, balance, daily activities, motor performance, and ambulation in PD patients (Table1.3). Although clinical findings suggest that lack of motivation and regular physical activities in PD patients cause further motor deterioration and that adequate exercise training benefits their motor performance, especially during early disease stages (Kaminsky et al., 2007).

There is an increased number of reports which has been investigated the role of exercise in prevention the onset of PD and in slowing down its progression. Table 1.3 shows examples of these research papers.
<table>
<thead>
<tr>
<th>Title</th>
<th>Number of participants and PD condition</th>
<th>Duration of the training program</th>
<th>Types of exercise</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Six Weeks of Intensive Treadmill Training Improves Gait and Quality</td>
<td>9 patients of PD, mild to moderate stage (Hoehn and Yahr stage range, 1.5-3). Measures: (PDQ-39), (UPDRS), gait speed, stride time variability, swing time variability, and the Short Physical Performance Battery.</td>
<td>The training program consisted of sessions of 30 min each, 4 sessions a week, for 6 weeks (a total of 24 sessions).</td>
<td>Running in the Treadmill.</td>
<td>Results show the potential to enhance gait rhythmicity in patients with PD and suggest that a progressive and intensive treadmill training program can be used to minimize impairments in gait, reduce fall risk, and increase QOL in these patients.</td>
</tr>
<tr>
<td>Patients With PD: A Pilot Study (Herman et al., 2007)</td>
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<tr>
<td>The Effect of Exercise Training in Improving Motor Performance and</td>
<td>Thirty people with PD, within 3 years of diagnosis with Hoehn and Yahr stage 1 or 2.</td>
<td>Subjects were randomized to high-intensity exercise using body weight–supported treadmill training, low intensity exercise, or a zero-intensity education group. Subjects in the 2 exercise groups completed 24 exercise sessions over 8 weeks. Subjects in the zero-intensity group completed 6 education classes over 8 weeks.</td>
<td>(UPDRS), biomechanic analysis of self-selected and fast walking and sit-to-stand tasks; corticomotor excitability was assessed with cortical silent period (CSP) durations in response to single-pulse TMS.</td>
<td>A small improvement in total and motor UPDRS was observed in all groups. High-intensity group subjects showed postexercise increases in gait speed, step and stride length, and hip and ankle joint excursion during self-selected and fast gait and improved weight distribution during sit-to-stand tasks. Improvements in gait and sit-to-stand measures were not consistently observed in low- and zero-intensity groups. The high-intensity group showed lengthening in CSP</td>
</tr>
<tr>
<td>Corticomotor Excitability in People With Early PD (Fisher et al.,</td>
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<td></td>
<td></td>
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<tr>
<td>2008)</td>
<td></td>
<td></td>
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</tbody>
</table>
The effects of incremental speed-dependent treadmill training on postural instability and fear of falling in PD (Cakit et al., 2007)

Fifty-four patients with idiopathic PD in stage 2 or 3 of the Hoehn Yahr staging entered, and 31 patients (21 training, 10 control) had outcome data. Postural instability of patients with PD was assessed using the motor component of the Unified PD Rating Scale (UPDRS), Berg Balance Test, Dynamic Gait Index and Falls Efficacy Scale.

Twenty-one patients with PD participated in an eight-week exercise program using incremental speed-dependent treadmill training. Before and after the training program, balance, gait, fear of falling and walking distance and speed on treadmill were assessed in both PD groups. Initial total walking was progressively increased after 16 training session. Tolerated maximum speed of the training group on treadmill at baseline was improved. Berg Balance Test, Dynamic Gait Index and Falls Efficacy Scale scores of the training group were improved significantly after the training program. There was no significant improvement in any of the outcome measurements in the control group.

Efficiency of a multidisciplinary treatment program on one-year outcomes of individuals with PD (Carne et al., 2005)

43 consecutive individuals with PD were examined. (UPDRS) was employed to assess disease progression. Changes between initial and one-year follow-up. UPDRS motor functioning (Part III) scores were compared to expected disease progression from prior research. This paper just investigates the progress of the disease, no training tasks. In this cohort, thirty patients (69.8%) had improved, 2 were unchanged (4.7%) and 11 patients (25.6%) had worsened at the mean 12.2-month follow-up period.
| Virtual cues and functional mobility of people with PD: A single-subject pilot study (Kaminsky et al., 2007) | Six adults with PD, akinesia, and stage III or IV Hoehn and Yahr rating scale status | Use virtual cueing spectacles VCS in their homes and communities for a week or more. | Main outcome measures included participant counts of losses of balance and freezes, pre /postintervention completion of the PD Questionnaire-39, observation of baseline and intervention gait, and an interview regarding user satisfaction with VCS. We also assessed participants’ preuse baseline and return to baseline. Use of VCS decreased length of freezes as well as number of freezes for some participants | All participants expressed satisfaction with VCS. VCS shows promise in simulating kinesia paradoxa to improve the gait of some adults with PD in the home and community. |

**Table 1.3 Example of previous articles about exercise and PD.** Table 1.3 shows a negative correlation between incidence of PD and lifetime level of physical activity. Also, physical exercise has been shown to improve movement initiation, quality of life and balance.
Exercise is a planned, structured physical activity which aims to improve one or more aspects of physical fitness. Current models of rehabilitation often use compensatory strategies as the basis of therapeutic management. However, there is a growing body of evidence regarding the impact of exercise in terms of neuroplasticity and the ability of the brain to self-repair. Animal models have found that exercise has protective benefits against the onset of symptoms in Parkinson’s disease (PD). This appears to be due to the release of neurotrophic factors, and greater cerebral oxygenation, which together promote new cell growth and cell survival, it has been found that exercise stimulates DA synthesis in remaining DA cells and thus reducing symptoms (Ashburn et al., 2007a).

There are two main theories tried to explain how exercise might affect brain health. First theory suggested that neurotrophic factors have most of the properties that could underlie such beneficial effects of physical exercise (Cotman and Berchtold, 2002). Among the neurotrophic factors, brain-derived neurotrophic factor (BDNF) was the main interest because it supports the survival and growth of many neuronal subtypes, including glutamatergic neurons subsequently, as the neurotrophin field evolved, BDNF emerged as a key mediator of synaptic efficacy, neuronal connectivity and use-dependent plasticity (Schinder and Poo, 2000). These results encouraged other researchers to investigate roles of other types of neurotrophic factors. (Yasuhara et al., 2007) examined this hypothesis in another Glial cell-derived neurotrophic factor (GDNF) and confirmed that a form of forced exercise caused a small but significant increase in GDNF in the ipsilateral striatum that peaked at 3 days and had returned to normal by 7 days. However, this hypothesis has been examined only with animal models and cell culture models of PD but it has not been widely examined in PD patients.
Sutoo and Akiyama (2003) on the other hand, investigated the role of $\text{Ca}^{2+}$ ions in brain function and plasticity. They demonstrated that acute exercise leads to an increase in the serum $\text{Ca}^{2+}$ levels and subsequently an increase in the brain $\text{Ca}^{2+}$ level in normal mice.

$\text{Ca}^{2+}$/Calmodulin-dependent protein Kinase (CaMKs) is a family of $\text{Ca}^{2+}$ binding proteins, they mediate many neurotransmitters and hormones commonly exert their effects through the regulation of protein phosphorylation (Anderson and Kane, 1998, Hudmon and Schulman, 2002). In PD, neurotransmitters regulation is one of the functions which are believed to be activated by (CaMKs) (Goldberg et al., 1996).

It has been well documented that physical activity levels decline with advancing age and these reductions contribute to functional decline. People with PD have been shown to reduce levels of physical activity more quickly than their healthy peers and have lower levels of strength and functional ability (Carne et al., 2005). However, the observation of muscle weakness is not simply a secondary consequence of ageing and inactivity, but also a primary symptom of PD. This is due to impaired basal ganglia having an inadequate effect on the cortical motor centres that in turn lead to less activation of motor neurones and therefore muscle weakness. This mechanism also contributes to impaired balance, falls, and disability. People with PD are three times more likely to sustain a hip fracture because of a fall when compared to those without the condition (Protas et al., 2005a).

Although these clinical findings are encouraging, they have several shortcomings. For example, the definition of the term “Exercise” is not completely clear and most of the prospective studies have been brief in providing exercise details. In addition, there is a mix between physiotherapy and physical exercise.
1.3.3 Exercise and PD progression: *in vivo* studies

Using the rat model of Parkinsonism induced by 6-OHDA, either voluntary running or treadmill paced exercise attenuated DA loss in the striatum with or without significant recovery of behavioural deficits (Al-Jarrah et al., 2007). The neuronal recovery in 6-hydroxydopamine-treated rats triggered by exercise is associated with an increase of the striatal glial cell line-derived neurotrophic factor (Cohen et al., 2003). In an acute mouse model of Parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), treadmill exercise ameliorates behavioural deficits and reverses several striatal DA indices including the loss of DA, tyrosine hydroxylase (TH), and DA transporter levels compared with sedentary Parkinsonian animals (Petzinger et al., 2007). In another study, Dishman et al. (2006) demonstrated that the high-intensity treadmill exercise in acute MPTP-treated mice leads to behavioural recovery; however, the striatal expression of DA transporter is downregulated and the expression of TH is not changed (Petzinger et al., 2007). Differences in the exercise results obtained from animal models of Parkinsonism could be due to experimental variables such as the age and species of the animal, the method and severity of the induced nigrostriatal lesion, and the type and intensity of the exercise regimen (Blum et al., 2001).

Exercise is neuroprotective after brain damage constraining the non-impaired upper extremity in stroke patients, thereby forcing use of the affected limb, has been shown to improve motor function after stroke (Tajiri et al., 2010). Additionally, constraint induced therapy appears to induce reorganization in brain regions affected by ischemic brain damage (Langfort et al., 2006). Constraint therapy has also been used to improve functional outcome and expand neurophysiologically mapped limb representation areas in primate models and in
humans after motor injury (Langfort et al., 2006). Exercise intervention has been shown to protect in several models of brain damage of PD and has been shown to be neuroprotective in animals subjected to a variety of neurotoxic conditions, including ischemia (Langfort et al., 2006) and genetic modifications associated with PD (Strom et al., 2005).

The neuroprotective effects of physical exercise before exposure to PD neurotoxins have been investigated in several literatures. For example, Gerecke et al. (2010) reported that an exercised PD rat group over 3 months showed better behavioural recovery in cylinder test and significant decrease in the number of amphetamine-induced rotations, compared to the non-exercise PD induced group. Correspondingly, significant preservation of tyrosine hydroxylase (TH)-positive fibres in the striatum and TH positive neurons in the substantia nigra was demonstrated. These results agree with Tajiri et al. (2010), they reported similar results but the exercise program duration was different (4 weeks) running on the treadmill.

On the second direction of research strategies, exercise interventions were induced after the animal models had been exposed to PD. That direction of investigation aimed to look in the neuroplasticity effect of exercise and the ability to generate new DA cells. Smith and Zigmond (2003) reported that the adult male rats that received injections of 6-OHDA were divided into two groups. The first group did not receive any physical therapy, this group showed an extreme motor asymmetry in their use of limbs. The second group of animals were forced to use their contralateral forelimb for 7 days immediately after the administration of 6-OHDA. This exercised group showed no such asymmetry and were not different from intact animals in the limb-use asymmetry task.
Tillerson et al. (2003) showed similar results, where they reported that exposure to the DA toxins (6-OHDA or MPTP) resulted in permanent behavioural and neurochemical loss in two rodent models of PD, 6-OHDA and MPTP. In contrast, when lesioned animals were exposed to treadmill activity two times a day for the first 10 days post-lesion they displayed no behavioural deficits across testing days and had significant recovery of striatal DA, its metabolites, TH, vesicular monoamine transporter, and DA transporter levels compared to sedentary animals. This data demonstrated that exercise following nigrostriatal damage improves related motor symptoms and neurochemical deficits in rodent models of PD.

In similar procedures, Yoon et al. (2007) demonstrated that Spargue Dawley rats undergoing treadmill exercise showed a significant reduction of rotational asymmetry. Analysis via immunohistochemistry for TH expression revealed a substantial loss of cell bodies in the SN to the lesion following 6-OHDA injection into the striatum. They recommended the hypothesis that treadmill running enhanced the survival of DA neurons in the SN and their fibres projecting into the striatum (Yoon et al., 2007).

Following that line of investigations, Pothakos et al. (2009) reported that severe chronic PD mice showed significant deficits in their gait pattern consistency. Their performances on the behavioural tests were considerably attenuated, suggesting a lack of balance and motor coordination. The behavioural deficits in the chronic PD lasted for at least 8 weeks after MPTP treatment. When the chronic PD mice were exercise-trained on a motorized treadmill 1 week before, 5 weeks during, and 8–12 weeks after MPTP, the behavioural deficits in gait pattern and balance performance were reversed; whereas neuronal loss and impairment in cognitive skill and motor coordination were not altered when compared to the sedentary chronic PD animals (Pothakos et al., 2009).
Differences in the exercise results obtained from animal models of PD could be due to experimental variables such as the age and species of the animal, the method and severity of the induced nigrostriatal lesion, and the type and intensity of the exercise regime (Al-Jarrah et al., 2007).

1.3.4 Exercise and PD; *in vitro* studies

This extraordinary complexity of living organisms is a great barrier to the identification of individual components and the exploration of their basic biological functions. Thus, the primary advantage of *in vitro* work is that it permits an enormous level of simplification of the system under study, so that the investigator can focus on a small number of components. Logically, the ideal *in vitro* PD cell model should contain human DA neuronal cells. However, there are great ethical difficulties in obtaining sufficient and appropriate human primary neurons. Therefore, researchers were encouraged to produce DA cell culture models that could be used to address questions regarding the selective loss of DA neurons in the SN. In addition, there was an increasing need to study the effect of pharmacological and non-pharmacological therapies on *in vitro* stage of PD.

*In vitro* model of selective DA neuron loss provided a great benefit in attempts to understand the basis of cell death that can be triggered by oxidative stress and to develop strategies for promoting cell survival. The effect of exercise on DA neurons was investigated in literature throughout different strategies. As there is no well known method to physically exercise cells in this level, strategies mainly focused on adding what has been reported to be enhanced after exposure to exercise. These proteins were isolated and induced into the
culture, that strategy provided a chance to prevent DA neuronal cell death using certain proteins as a mimic of exercise.

A number of cell lines were reported to produce DA neurons and mimic their behaviour against neurotoxins and. Therefore, they were widely used as PD models. Among this number of cell lines there are two widely used, MN9D and SH-SY5Y cell lines. These two cell lines provide an unlimited supply of cells of human origin with the similar biochemical characteristics to human DA neurons. (Borland et al., 2008, Shavali and Sens, 2008).

Cotman and Berchtold (2002) focused initially on brain-derived neurotrophic factor (BDNF) because it supports the survival and growth of many neuronal subtypes, including glutamatergic neurons. BDNF emerged as a key mediator of synaptic efficacy, neuronal connectivity and use-dependent plasticity. They predicted that a neurotrophin-mediated response to exercise would probably be restricted to motor–sensory systems of the brain, such as the cerebellum, primary cortical areas or basal ganglia. The findings were surprising: several days of voluntary wheel-running increased levels of BDNF in the hippocampus. However, neurotrophic factors were the only proteins that have been used as a mimic of exercise in PD model (Ding et al., 2004). Thus, further investigations about the effect of other exercise outcomes substances in PD in culture model still need further investigations.
1.4 CA$^{2+}$ CALMODULIN DEPENDANT PROTEINS AND PD

Calmodulin (Cam) is the major Ca$^{2+}$ binding protein that works as a Ca$^{2+}$ sensor and its binding to Ca$^{2+}$ produces several conformational protein changes. This protein plays an important role in response to changes of Ca$^{2+}$ concentration of neuronal cells (Sola et al., 2001). The Ca$^{2+}$/CaM binding proteins complex initiates many signalling cascades that culminate with the alteration of many cell’s function (Hook and Means, 2001). CaMKs have been found in presynaptic and postsynaptic neuronal cells, which work, in a broad spectrum of neural activities. It has also been found in non neural tissue. The CaMK is a big multifactor family which is formed by CaMK-1, CaMK-2, CaMK-3 and CaMK–4 (Tokuda et al., 1997, Hook and Means, 2001).

The Ca$^{2+}$ influx on neural cells (for example the cells of striatum) as a consequence of synapses, produces CaMKs activation. CaMKs are holoenzymes that exhibit broad substrate specificity (Merrill et al., 2005). CaMK consists of twelve functional subunits that are grouped into two clusters of six. Each subunit contains three different domains: the catalytic, the autoregulatory and the association domain. The last one is the one that binds with Ca$^{2+}$. When this binding takes place, Ca$^{2+}$ relieves the inhibition from the autoregulatory domain allowing the autophosphorylation of the catalytic domain. The autophosphorylation outcome changes of the whole complex (Hudmon and Schulman, 2002). This activation entails multiple functions as neurotransmitters synthesis, neurotransmitters release, module ion channels and receptor functions, Ca$^{2+}$ homeostasis, cytoeskeletal functions, gene expression, synaptic plasticity or learning and memory (long term potentiation) (Colbran, 1992). Therefore, if Ca$^{2+}$ influx increases more than normal inside the cells more Ca$^{2+}$ will bind to CaMKs, increasing the levels of CaMKs autophosphorylated in the cells of striatum in
Parkinsonian brain. Recent studies demonstrated the increase of CaMK-2 autophosphorylated in PD (Brown et al., 2005). It is believed that an excess of CaMK-2 would modify the density of the glutaminergic receptors which will contribute also to the overstimulation of glutaminergic in put of striatum and the excitotoxicity (Oh et al., 1998).

1.4.1 Calcium-Calmodulin Kinases 1 and 4.

Calcium (Ca\(^{2+}\)) is a major second messenger in all cell types. One mechanism by which calcium ions exert their effects is by binding to a 17 kDa protein named calmodulin (CaM). The binding of four calcium ions to calmodulin changes its conformation and promotes its interaction with a number of other proteins, including several classes of protein kinases that are activated by the calcium/CaM complex (Ahmed et al., 2000). A practical way of classifying the calcium/CaM-dependent protein kinases is based on their substrate specificity: some of these enzymes have only one substrate, and are designated as ‘dedicated’ calcium/CaM dependent protein kinases, while others have broad substrate specificity and are termed ‘multifunctional’ kinases (Tokuda et al., 1997, Ahmed et al., 2000).

Multifunctional calcium/CaM-dependent protein kinases comprise three enzymes referred to as CaM-kinases 1, 2 and 4. CaMK-2 is an oligomer of probably 12 subunits which has unique properties and is also the most extensively studied (Colbran, 1992). It is a ubiquitously distributed enzyme that is very highly enriched in neurons, especially in postsynaptic densities (Merrill et al., 2005). As is the case of other CaM-kinases, the activity of CaMK-2 is inhibited by an autoinhibitory domain, this inhibition is alleviated by binding of calcium/CaM that allows autophosphorylation of the autoinhibitory domain. Once autophosphorylation has occurred, the presence of calcium/CaM is no longer necessary and
the enzyme becomes calcium/CaM-independent. Interestingly, the oligomeric structure of CaMK-2 and the fact that autophosphorylation is a ‘trans’ reaction between different subunits of the oligomer has important consequences (Merrill et al., 2005). Autophosphorylation promotes calcium/CaM trapping and occurs only when two adjacent subunits are bound to calcium/CaM. Thus, CaMK-2 is sensitive to the duration and frequency of calcium transients, and therefore is capable of decoding the frequency of calcium spikes. CaMK-2 may also remain active for some time while calcium levels return to normal, thereby maintaining a transient ‘memory’ of neuronal activation. Its unusual properties, in particular its abundance in synaptic regions and its actions on many proteins including ion channels, make CaMK-2 a very important contributor to the processes of synaptic plasticity and LTP induction (Ahmed et al., 2000).

Ahmed et al. (2000) demonstrated that the precise intracellular localization of CaMK-2 also appears to be regulated and recent work has demonstrated the ability of the ionotropic glutamate receptor agonist NMDA (N-Methyl-D-Aspartate) to induce translocation of CAMK-2 from actin filaments to postsynaptic densities. On the other hand, CaMK-1 and CaMK-4 are monomeric enzymes that share the common property of being activated by calcium/CaM binding and by phosphorylation by a CaM-kinase-kinase (CaMKK). Thus, together these kinases are organized as a calcium/CaM-dependent protein kinase cascade. CaMK-1 is a ubiquitously expressed cytosolic enzyme which phosphorylates many substrates, including synapsin. In contrast, CaMK-4 is located in the nucleus and is predominantly expressed in neurons, testis and T-cells (Figure 1.4). CaMK-4 phosphorylates transcription factors, including cAMP responsive element binding protein (CREB) and the associated CREB-binding protein (CBP), and thus plays a major role in calcium-regulated gene transcription. CaMKK controls the activity of both CaMK-1 and CaMK-4. There are two isoforms of
CaMKK, α and β, enriched in the cytoplasm and the nucleus, respectively, CaMKK is also able to phosphorylate and activate PKB, and thus exert anti-apoptotic effects. Recently, a family of pro-apoptotic serine/threonine protein kinases has been identified and termed Death Associated Protein Kinases (DAP-kinases). Two of these DAP-kinases possess a CaM-binding domain and are activated by calcium-CaM (Tokuda et al., 1997, Ahmed et al., 2000).

It was hypothesized that exercise may affect brain function through action of calcium and biogenic amines such as dopamine. This thesis was carried out to test this hypothesis, the isoforms CamK-1 and 4 were chosen as indicators for Ca\(^{2+}\) that has not been investigated in PD and exercise area of research. Such aberrant, physical exercise may contribute significant to symptoms of PD and represent a novel target for the development of new therapeutics.
Figure 1.4 Cellular Signaling by CaMKs. Elevated intracellular Ca\textsuperscript{2+}/CaM bind to and activate CaMK-2, CaMKK, CaMK-1, and CaMK-4. CaMK-2 autophosphorylates to generate significant autonomous activity. Primary targets of CaMKK are CaMK-1 (cytosolic) and CaMK-4 (nuclear) these require binding of Ca\textsuperscript{2+}/CaM to both CaMKK and CaMK-1/ CaMK-4. Secondary substrates of CaMKK, which are phosphorylated at much slower rates than CaMK-1 and CaMK-4 are PKB/Akt and members of the AMP-kinase family, including SAD-B. Crosstalk between adenylyl cyclase (A.C.)/PKA to inhibit CaMKK and between CaMK-1 to activate MEK/Erk are illustrated.

However, there is not sufficient literature about the effect of exercise on levels of CaMK-1 and CaMK-4 in models of PD. Therefore, in this thesis it is investigated (in chapters
three and four) whether exercise could regulate the levels of CaMK-1 and CaMK-4 in Parkinsonian brain. That may support the evidence for the role of exercise in regulating levels of Ca\(^{2+}\) affects and a good additional therapy in delaying PD symptoms.

1.5 THE THESIS

Treatments for PD are generally limited and these treatments do not appear to reduce the progression of the disease. Moreover, side effects of these treatments gradually emerge and efficacy is usually reduced. Thus, developing strategies for slowing or preventing ongoing degeneration or even reversing the neurodegenerative process in PD is of paramount importance. I therefore set out in the broad aims of the dissertation to provide evidence for the following hypotheses:

*Exercise can reduce the vulnerability of DA neurones, via an increase in calcium calmodulin Kinases 1 and calcium calmodulin Kinases 4.*

1.6 AIMS OF THE THESIS

The overall aim of the thesis is to fill the gap in literature in the surrounding endurance exercise and PD. Specifically, to investigate the evidence of whether exercise could be a remedy to PD especially in the early stages. This aim will use three different models of PD (Animal model, cell culture model and human subjects) Thus, the overall aims of the thesis are:
- To study the effect of 8 weeks of endurance training on expression of CaMK-1 and CaMK-4 synthesis in striatum and hippocampus regions in PD rat brain.

- To measure the amount of CaMK-1 and CaMK-4 synthesis in striatum and hippocampus regions after 8 weeks of endurance exercise.

- To investigate the neuroprotective effect of Ca$^{2+}$ on *in vitro* model of DA cells induced with PD neurotoxin 6-OHDA.

- Effect of submaximal exercise on balance, gait and blood Ca$^{2+}$ concentration in patients with PD.
CHAPTER TWO: GENERAL METHODS

The following sections describe the materials and methods used in every experimental chapter. According to the different models used as PD models in this thesis, this chapter is divided into three sections. Section one covers the techniques which have been used for the rat experimental work *in vivo*; section two covers experimental procedures and techniques used in *in vitro* experimental cell culture work and section three covers the experimental procedures for the human work chapter. If additional materials or modified methods were used, details are provided in the relevant section within the respective chapter.

2.1 ETHICAL APPROVAL

Prior to the commencement of each study in this thesis ethical approval was obtained from the responsible institutional Ethical Committee. Animal work experiments (studies one and two) were under an obligation to follow common EU regulations regarding the ethics of experiments in animals. All experimental procedures were approved by a local ethical committee for animal’s experiments, in the Nencki Institute, Warsaw, Poland and local ethics committee, Bialystok, Mossakowski Medical Research Centre, Poland.

Approval of the human work chapter (study four) were obtained from Ethical Committee of Institute of Research in the Applied Natural Sciences (LIRANS), University of Bedfordshire, United Kingdom.
The experimental work was carried out in the following laboratories:

- Preparation of rat models (housing, pre-exercise preparation and exercise program (which has been used in studies one and two) took place in the Department of Applied Physiology Laboratories, Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland.

- The rat models analytical techniques (immunohistochemistry and western blot) took place in Neuroscience Laboratory, Division of Science, Park Square, Luton, UK and in the Institute of Research in Applied Natural Sciences Laboratory (LIRANS), Butterfield, University of Bedfordshire, UK.

- The cell culture experiments (study three) took place in Muscle Cellular and Molecular physiology Research Laboratory, Institute of Sport and Physical Activity Research (ISPAR), University of Bedfordshire, Polhill Avenue, Bedford, UK.

- The human experimental and analytical work (study four) was conducted in The Exercise Physiology Laboratories, Department of Exercise Sciences, Bedford, University of Bedfordshire, Polhill Avenue, Bedford, UK.
2.2 SECTION ONE: IN VIVO STUDIES

2.2.1 Experimental design for studies one and two (in vivo studies)

The in vivo studies comprised four groups of experimental rats: a control group (C), a training exercise group (E), a group in which PD was induced via systemic injections of PD toxin MPTP (PD) and a group where PD-induced animals were trained/exercised (PDE). E and PDE groups were trained with 8 weeks of endurance exercise at 90% of the Lactate Threshold (LT) level, 5 times a week with each for a 45 min bout using a custom-built rat treadmill. After 8 weeks, all rats were sacrificed and rat brains were collected for immunohistochemistry and western blot analysis. Ca^{2+} calmodulin kinases I (CaMK-1) and IV (CaMK-4) were investigated as indicators of the activity of the Ca^{2+} calmodulin pathway in the four groups.

2.2.2 Preliminary procedures to determine exercise intensity (for the exercise-trained groups)

Rats were used as experimental models in studies one and two, in each of these studies, 24 female Sprague Dawley rats were used. Rats were divided into four groups: (1) sedentary controls (C), (2) MPTP induced group (PD), (3) MPTP + exercise-trained group (PDE) and finally exercise-trained only group (E). Rats were selected randomly and each group consisted of 6 rats (N=6).
Rats were then accommodated separately in their cages. Each cage was labelled with information indicating rat type, age, weight and group name. Before starting the experimental procedures, the rat’s feasibility for exercising was determined by using a simple running test. All rats were exposed to 15 min running (at the treadmill speed 13m.min\(^{-1}\)) repeated three times with 1-min intervals. Results showed that 22 rat were able to run without difficulties. Two rats were not able to run at that speed and were triggered by the electrical stimulation (figure 2.1), these two rats were replaced from the general animal house, and the replaced rats showed ability to run on the treadmill without electrical stimulation. By the end of this test, all groups were ready for the main experimental procedures, which are described in the following section.

Groups (PDE) and (E) only were trained for 8 weeks of endurance exercise using a special rat treadmill. The effectiveness of the training was evaluated by measuring lactate threshold (LT) of the rats (see 3.2/ 3.3). Animals that underwent the exercise program, (PDE) group and (E) group, were subjected to running 40-60 min, 5 days a week for 8 weeks, running time was 40 min/day over the first 4 weeks, and then increased by 5 min to reach 1 h, the inclination was 0°.
Figure 2.1 Two photos of the motorized 10 lane rat Treadmill used for the exercise program in studies one and two. The rat treadmill is designed to exercise and monitor rats at a variety of speeds (6-100 m.min$^{-1}$). The treadmill consists of ten isolated chambers that allow ten rodents to use the treadmill simultaneously while stimulating them independently, supplied with a stimulus assembly, an electrical stimulus system composed of six grids, each with individual on/off switches to motivate rats to exercise. The treadmill also contains isolated chambers for housing the rat pre and post exercise, in addition to performing any other application (etc. taking blood samples for lactate threshold test). This treadmill is provided with a small screen on the end of each lane to monitor speed and the incline of the running lane.
2.2.3 Lactate threshold test to determine exercise intensity for rats

In order to determine the exercise intensity, the lactate threshold test (LT) was performed for the (PDE) group (N=6) in two following days. Testing procedures consisted of 7 stages of incremental running on a special rodent treadmill. Individually, each rat was transferred from its cage to the isolated chamber section of the treadmill 5 min before the test (Figure 2.1). The aim of this early transfer was to familiarize the animal with the testing environment, the rat tail was cut and 50 ul of blood were taken for the lactate test, immediately after taking the blood sample, the cut tail was treated with Heparin. The rat was accommodated in the running wheel section of the treadmill Figure and started to run at the speed of 13 m.min$^{-1}$ at 0° inclinations for 3 min, after these 3 min the rat had 30 sec rest to allow collecting of a blood sample. A more detailed description of the LT test procedures was given previously by (Langfort et al., 2006).

The LT was calculated as the exercise speed corresponding to the individual breaking point of blood lactate curve using the two-segmental linear regression (log LA vs. log running speed), according to Beaver et al. (1985). Blood lactate concentration was measured enzymatically using commercial kits (Boehringer Diagnostica, Mannheim, Germany).

The 90% of the speed to elicit lactate threshold was used as an exercise intensity parameter to be compatible with the exercise intensity which is recommended by the American College of Sport Science for PD suffers (ACSM, 1998). The same intensity has been used in the submaximal exercise session for PD participants in study 4 in this thesis. This process was repeated 6 times at the following speeds 17, 21, 25, 29, 34 and 37 m.min$^{-1}$. Finally, the rat was
taken back to the cage. After 4 weeks of endurance exercise, another LT test was conducted for each rat, the aim of the second test was to check whether there was an improvement in LT level which in turn will change the given dose of exercise intensity.

2.2.4 Procedures of MPTP injection to induce PD during the exercise program

Procedures of MPTP injection were followed according to the methods described in Langfort et al. (2006); this method was chosen as it allows determining the amount of the desired lesion in the rat brain, which reflects the percentage of required symptoms, from light to severe. In studies one and two, the experimental design required the rats to be exercise-trained for 4 weeks before the MPTP injection procedures start. Therefore, the amount of lesion was selected to cause PD symptoms, meanwhile, maintain the rat’s ability to run on the treadmill. As mentioned previously in chapter one, PD symptoms do no start before the damage of 80% of DA. However, this lesion will cause death or total disability in the experimental rats (Dauer and Przedborski, 2003). Therefore, 50% lesion was selected in order to allow rats to develop PD symptoms without losing their mobility totally.

One day before the experiment, all animals were weighed and coded before starting the initial exercise session, as previously described. After 4 weeks of exercise, (necessary to induce the neuro-protective effect that allows the MPTP model of PD to be used), the experimental rats (PD and PDE groups) were injected with the first of 10 doses of pre-prepared sterile MPTP. During each injection, rats were placed in the fume hood and 12.5 mg/kg MPTP, in saline, was
injected into the internal carotid artery. During this procedure, the rat was held in position, but not so tightly to avoid any backflow of the injected MPTP from the peritoneum (Yang et al., 1988). At the end of the injection schedule, the remaining MPTP solution was destroyed with an equivalent volume of 1% bleach solution.

During the remaining 4 weeks of the 8-week exercise programme, the injection procedure was repeated every 3 days. Therefore, the total dose of MPTP was 125 mg/kg administered throughout the last 4 weeks of exercise. This dose was determined according to the age, type and the percentage of the required lesions (Dauer and Przedborski, 2003). The required lesions were established to induce symptoms of PD, but to allow functional mobility to complete the exercise program. As such, a maximum of 50% of DA was destroyed by the MPTP (Dauer and Przedborski, 2003). Higher percentage of DA damage would result in complete disability to the animals. Control groups were treated with saline with the same volume doses as the neurotoxin MPTP.

2.2.5 Cylinder test

The Cylinder test is designed to evaluate locomotor asymmetry in rat models of central nerve system disorders (Schallert and Tillerson, 1999). As the rat moves within an open-top, clear plastic cylinder, its forelimb activity while rearing against the wall of the arena is recorded. Forelimb use is defined by placement of the whole palm on the wall of the arena, which indicates its use for body support. Forelimb contacts while rearing are scored with a total of 20 contacts recorded for each rat. The number of impaired and non-impaired forelimb contacts is calculated.
as a percentage of total contacts. This test is used to phenotype strains of transgenic rat and evaluates novel chemical entities for their effects on motor performance (Pothakos et al., 2009).

In studies one and two, the cylinder test was performed to quantify forelimb use. The rats were videotaped as they moved freely in a 20 cm wide clear glass cylinder. Contacts made by each forepaw with the cylinder wall were scored from the videotapes by an observer blinded to the animals’ identities. A total of 20 contacts were recorded for each animal and presented as impaired (left) forelimb contacts as a percentage of totals formed the actual dependent variable.

2.2.6 Sacrificing procedures

Rat brains begin to deteriorate as soon as its blood supply is interrupted, the deterioration is rapid, and it was, therefore, important to arrest the deterioration as quickly as possible. The process of preserving the brain is called "fixation", it took place in the rat surgical stage (Figure 2.2). Langfort et al. (2006) recommended that the best neurohistologic results can be delivered when optimum procedures are followed for tissue preparation. Therefore, rats were killed immediately after the last bout of exercise of the training process (N=24). Prior to sacrifice, all the rats from all groups were deeply anaesthetized with sodium pentobarbital (Vetbutal, Polfa, Poland, 60 mg/kg), the rats were then sacrificed by decapitation and the brains were removed, post fixed in the same fixative for 4 days and then cryoprotected in a phosphate buffered saline containing sucrose 30%. After isolating the brain carefully and fixing it to preserve it from losing
its chemical components (Langfort et al., 2006), brains were ready for freezing until the analysis procedure with either immunohistochemistry or western blot.

![Image](image.png)

**Figure 2.2 Rat surgical stages used in studies one and two**

### 2.2.7 Immunohistochemistry fixation procedures

The aim of the brain fixation, after isolation, was to preserve the brain structure. Fixative brains were freshly depolymerized with 4% paraformaldehyde in 0.1 mM medium phosphate buffer (pH 7.4) (Figure 2.4). According to the method described in Langfort et al. (2006), brains were kept for 4 days in that fixative and then samples were stored in -80°C freezer in PBS containing sucrose 30%.
Figure 2.3 Fixation process for immunohistochemistry analysis. After brains were isolated, then they were accommodated in special plates covered with Frozen Tissue embedding media (Fisher, UK). In each plate the group name and group type were written on the plate side, samples were then placed in dry ice until they were frozen. The brain sample placement was determined using the rat brain atlas (Paxinos and Watson, 1998). Samples were covered with parafilm, placed in a plastic container and finally kept in -80°C freezer until the immunohistochemical and western blot techniques took place.

2.2.8 Sectioning procedures for immunohistochemistry.

Brains were removed from the -80°C freezers on dry ice and sliced using microtome (5030 Bright, UK). Brains were sectioned into 30µm frontal sections in -20°C, sections and were placed in Gluten coated microscope slides (three sections per slide). During the mechanical cut process using the microtome, freezing chamber temperature should not drop below -15°C or the brain will not be suitable to cut. Therefore, when the microtome temperature decrease below -
15°C, the glass isolating door was closed up and left for 10-15 min till the microtome temperature reaches -20°C again. Thickness of sections was adjusted from a small circle pointer connected to the razor blade inside the freezing chamber. Sections were collected on coated glass slides, labelled with group name, brain number, section number and date. Three brain sections were collected on each glass slide. Glass slides contain sections which were put on a slide holder and kept in -20°C freezer till use. When complete, slides were kept in a -20°C freezer.

The rat brain is oriented slightly different than the human brain due to the fact that rats are quadrupeds (walk on all four paws) and humans are bipeds (walk on two legs). In order to determine the geography of the brain for surgical and experimental procedures, researchers use two key points in the rat’s skull anatomy to locate the appropriate brain structures that lie beneath. The first anatomical landmark on the rat’s skull is referred to as “Bregma” (Figure 2.4). (Paxinos and Watson, 1998).

Bregma is the place on the skull where the coronal bones and parietal bones intersect (Figure 2.4). In chapter three and four in this thesis, Bregma measurements were identified (from a rat atlas) to estimate where the studied structures of the brain are located. In the case of anterior-posterior (A-P), structures located anterior to Bregma (towards the nose) are considered to have a positive orientation. Structures located posterior to Bregma (towards the back of the skull) are considered to have a negative orientation (Langfort et al., 2006).
Figure 2.4 Anatomical landmark on the rat’s skull (Bregma point)

2.2.9 Immunohistochemistry procedures

At room temperature, sections were prepared to immunohistochmeical procedures washed in TBS for 5 min then washed with TBS containing 10% H$_2$O$_2$ for another 10 min. Slides were circled with grease a pen (Dako, Denmark) to concentrate the solutions on the top of each section. Then slides were incubated in Normal goat serum for one hour. Anti Ca$^{2+}$/calmodulin-dependent protein kinases CaMK-1 and CaMK-4 primary antibodies were used as primary antibody to detect the presence and localization of CaMK-1 and CaMK-4 antigens in the rat brain cells (Santa Cruz inc, USA). Dilutions were 1:1000 and 1:500 respectively. Sections were incubated with the primary antibody overnight in a 37°C humidified chamber. Sample were then washed 3 times in TBS. Sections were then incubated with the secondary antibody (Vectastain ABC Kit, Vector Laboratories, UK) and horseradish peroxidase (HRP)-conjugated streptavidin (Vector Laboratories, UK). Following further washing in TBS three times for 5 min each,
sections were incubated for 30 min in 50% DAB (Vector Labs, UK), then the reaction terminated by washing by TBS three time for five min each, and the sections were then air dried overnight, dehydrated through washing in a series of alcohol concentrations (50, 60, 70, 80, 90, 95 and 100%) for five min each then dipped in Histoclear for 10 min and cover-slipped. The last step was to use the light microscope (Leica DM2500, Germany) to see the slides.

2.2.10 Image Preprocessing

To obtain accurate images for the studied regions, brain slices where agreed with 2 independent investigators. Image volumes passed through a number of preprocessing steps. First, image volumes were corrected for signal intensity inhomogeneities and transformed into ICBM 305 stereotaxic space using an automatic nine-parameter linear transformation (Tranzer and Thoenen, 1968). Using signal intensity information a spherical mesh surface was created and continuously deformed to fit a threshold intensity value. Image volumes in coronal, axial and sagittal planes were available simultaneously to aid decisions when the brain anatomy was ambiguous. Detailed anatomic protocols for delineating cortical anatomy are available in the rat brain atlas (Paxinos and Watson, 1998).

2.2.11 Image analysis

The regions localizations (from bregma in mm) of the analyzed areas were (A.P: 5.2) for SN and from bregma and (A.P: 2.30 to 5.20 mm) for Hippocampus (Figure 2.4). Labeling of
CaMK-1 and CaMK-4 positive cells/fibers in SN and hippocampus were assessed by measuring optical density of the entire area of the structure. Background (corpus callosum) was subtracted from all subsequent measurements. Results are presented as the percentage of the optical density over the area (in mm\(^2\)) of the ipsilateral side compared to the contralateral one. The number of CaMK-1 and CaMK-4 positive neurons in the SN and Hippocampus were measured digitally using Image J software and results are expressed as the number of positive stained neurons per mm\(^2\) of the structure. For each selected area, quantifications were carried out using four sections per animal, separated by 125 \(\mu\)m approximately. Quantifications were performed blindly, bilaterally, using a light microscope (Leica DM2500, Germany) equipped with a video camera (Leica DFC420). Neuroanatomical sites were identified using the atlas of Paxinos and Watson (1998).

2.3 PROCEDURES OF WESTERN BLOT ANALYSIS

Western blot is an analytical method that involves the immobilization of proteins on membranes before detection using monoclonal or polyclonal antibodies. This technique was used in study two in this thesis to determine specifications of protein antigen using a cell sample, especially the quantity of antigen. Subsequent polyacrylamide gel electrophoresis allows separation of individual proteins by molecular weight. The separated proteins are transferred to a Polyvinylidene fluoride embrane (blot) for detection with anti CaMK-1 and CaMK-4 antibodies.
2.3.1 Preparation of samples

After brain isolation, two brain regions (hippocampus and cortex) were isolated from the whole brain samples using surgical forceps and knife. Afterwards, each region was placed in a glass tube surrounded by a glass beaker full of ice then homogenized using tissue homogenizer (Omni THP115, Canada) for 30 seconds, then samples were transferred to centrifuge tubes and centrifuged at 100,000 ×g for 1h at 4°C (MR23i Bench top High Speed Centrifuge, Thermo Scientific, USA). The resulting supernatant was filtered through 0.45 μm Millipore filter paper. The samples were then stored at −80°C until assayed.

2.3.2 Determination of protein concentration

Once samples were homogenized, a protein assay using BCA kit (PIERCE-USA) was carried out to ensure that equal volumes of proteins were added to each sample, while running the western blot. BCA kits contain two reagents. Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and reagent B (4% cupric sulfate) these two reagents together produce working reagent. It is based on the technique of (Smith et al., 1985).

The BCA Protein Assay method is based on the reaction of protein with alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation. There are two steps that leads to that colour development, the first step is the chelation of copper with
protein in an alkaline environment to form a light blue complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a coloured chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate.

In the second step of the colour development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The reaction that leads to BCA colour formation is strongly influenced by four amino acid residues (cysteine or cystine, tyrosine, and tryptophan) in the amino acid sequence of the protein.

A standard solution of 2 mg/ml BSA was prepared following a guide table from the manufacturer, WR was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B), and then diluted to create eight protein standards (2000, 1500, 1000, 750, 500, 250, 125, 25). 0.1ml of sample and standards were added to clean labelled wells of a 96 well plate. 2.0ml of working reagent (reagent A+ reagent B) were then added to each well using multichannel pipette. Plates were covered and incubated in 37°C for 30 min. After incubation the absorbance of each well was read at 562 nm with the spectrophotometer.

A standard curve was then prepared by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in μg/ml. With the absorbance of the standards proteins and its known concentration a regression line was drawn. This line gives a
regression equation that permits the calculation of the concentration of protein in each sample through its absorbance.

2.3.3 Determination of loading contents

The amount of sample loaded into the eppendorf tubes was according to the results of the protein concentration test. 1 µl of DTT was added to reduce the disulfide bonds of the proteins and prevent disulfide bonds forming. Loading buffer was added as 1/3 of the volume of sample, for example, if the amount of original sample in the eppendorf was 17.65 µl, loading buffer volume would be 5.88µl. 20µl of Molecular Markers (MM) were added to the sample before the western blot procedures. Vials containing samples and MM were centrifuged for 2.5 min at 1000 rmp/s then were put on a hot plate (100º C) for 5 min then were placed in cold water for 5 min to cool samples.

2.3.4 SDS-Polyacrylamide Gel Electrophoresis of Proteins

The gel casting chamber and spacers were assembled and clipped in the casting stand, then filled with deionized H2O to ensure that there were no leaks. 5 ml of 12% gelatin-substrate acrylamide resolving gel (Appendix A) was carefully poured in the mould until it fills two-thirds, the gel was covered with 1ml propanol and left for 30 min to polymerize.

Following polymerization the propanol was poured off and the gel was rinsed with deionised H2O. 2 ml of 5% acrylamide stacking gel was poured in the mould till it covered the top, then the comb was placed on the top of the mould, gel was left to polymerize for 30 min at
room temperature. Once the gel has performed, the clamps were transferred from the casting stand to the electrode assembly and clipped in place. 1x of running buffer was loaded till it half filled the chamber. The comb was removed carefully from the top of the gel sandwich.

The prepared samples and the molecular marker were then loaded into the wells using a micropipette tip, the identity of each sample and its location on the gel was recorded. Running buffer then was poured into the tank to cover the electrode assembly and completes the circuit. The voltage was adjusted to 50 v constant, once the dye has completely crossed the resolving gel; the voltage was increased to 100 v. Electrophoresis step was completed when the dye had migrated to about 2 cm from the bottom of the gel. That process took between 80-120 min.

2.3.5 Gel transfer to the membrane

Nitrocellulose membrane and blot paper were cut to the same size as the resolving gel, and then they were submerged in transfer buffer for 15 min. The transfer unit was prepared and blot module was washed with methanol, sponges were wet in transfer buffer and were placed on the cathode plate. Placing order of the gel is shown in figure 2.5.

The chamber of the blot module was filled until the top with transfer buffer. The external part of the blot module was filled with cold water to the top. The module was connected to power supply at 190 mA constantly for 90 min. Once transfer had finished, the gel and the membrane were removed. The upper left corner of the gel was cut to know the orientation of the
gel with respect to the membrane. Gel was immersed in Coomasie blue for 15 min on a shaker. Gel was placed in destain solution. The area, which contained the enzyme, was clear against the blue/purple background image. The membrane was marked in the side that had been in contact with the gel, it was placed in ponceau stain solution (Appendix E), and it was swirled during 15 min. Then the membrane was washed with TBS (PH 7.7) into a blot membrane and filter paper sandwich in the transfer step. Gel was transferred on top of the blot paper. Wet PVDF membrane was placed on top of the gel. Another blot paper was put on top of the nitrocellulose membrane.

![Diagram showing the placement of gel, PVDF membrane, and filter paper](image)

**Figure 2.5** placing order of gel, PVDF membrane and filter paper are preassembled

### 2.3.6 Immunodetection of Antigens

After protein transfer, the membrane was removed from the blot cassette to a small container. Blocking buffer (Appendix E) was added to the container to block the non occupied protein binding sites. It was swirled for 1 hour on a shaker. The blocking buffer was poured off.
40 μl primary antibody, either (Anti-CaMK-1 goat polyclonal dilution 1:1000) or (Anti-CaMK-4 rabbit polyclonal antibody dilution 1: 500) were added to the small membrane in the container. Then the container with membrane and primary anti-body was left on the shaker for 1 hour. Membrane was washed with TBS for 5 min on the shaker (repeated 3 times). After that the membrane was left in 40μl of secondary antibody (Donkey anti-goat IgG-HRP diluted 1:5000) or (goat anti-rabbit IgG-HRP, dilution 1:10000) on a shaker for a further 1 hour. Secondary antibody was poured off and membrane was washed with TBS (repeated 3 times). The container was covered with parafilm to prevent antibodies from evaporation.

2.3.7 Chemiluminiscent Western Detection.

Enhance Chemiluminescence (ECL) is a light non-radioactive method for detection of immobilised specific antigens conjugated directly into specific proteins. ECL method allows exposing photographic film with a permanent record of the western blot detection. A strong light emission occurs when an oxidation reaction takes place between HRP molecules and ECL reagent.

Procedures start with cut 20 cm × 20 cm cling film and spread it in the bench without air bubbles. Membrane was soaked with HRP solution (1.5 ml of ECL Detection reagent A and 1.5 ml ECL detection Reagent B) using a pipette. The excess HRP solution was kept out by holding the membrane with forceps and putting it in the shaker for 5 min. Membrane was placed facing up on the cling film. A second cling film was put on the membrane avoiding air bubbles. Cling
film was folded. Membrane was put into a cassette facing up the proteins and it was fixed with tape so as to not let the membrane move.

The cassette was transferred to a dark room with safe red light. Then the film was put on top of the membrane. The cassette was closed and left closed for 30 s for exposure. The film was then taken out and it was developed by immersing for 5 min in developer diluted with water (red tray), 30 s in water and 8 min in fixer diluted with water. The X-ray films were washed with tap water then left for 15 min to dry. The final step was to scan the films with densitometer (Biorad, UK).
2.4 SECTION TWO: CELL CULTURE STUDY (*IN VITRO*)

2.4.1 Experimental Design

In chapter five, an *in vitro* study was designed to test the hypothesis generated from the *in vivo* work about the role of Ca\(^{2+}\) in the neuroprotective effect of exercise. The SH-SY5Y human neuroblastoma cell line was used as a model of DA neurons as it has DA activity and can synthesize DA. PD was simulated in these cells by exposure to the toxin 6-OHDA in a systemic method whilst addition of Ca\(^{2+}\) was used as an “exercise mimic”. The survival of DA neurons after adding both 6-OHDA and Ca\(^{2+}\) was statistically analysed.

Human neuroblastoma SH-SY5Y cells were obtained from Health Protection Agency Culture Collections (ECACC). SH-SY5Y has DA-hydroxylase activity and has been used widely as a model of DA containing neurons (Borland et al., 2008, Riveles et al., 2008, Shavali and Sens, 2008). Cells were grown in DMEM medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 g/ml streptomycin. Cells were maintained in a CO\(_2\) incubator (Thermo Scientific, UK) at 37\(^\circ\) C. SH-SY5Y were seeded at 1x 10,000 cells/cm\(^2\), cells were fed every 4 days with fresh medium. Differentiation was taking place when the density reaches 70-80% (Lewis et al., 2000).
2.4.2 Determination of cell density

Cell density was determined using a haemocytometer viewed under light microscopy at 10 x. Cells were trypsinised and resuspended in 10 ml DMEM. 20µl of the sample was taken from the sample, 20 µl of Trypan Blue and mixed. Cells mixed with trypan blue were then transferred to the haemocytometer and performed in both sides to fill both chambers. The cells were then located within the four main squares where they were been counted, cells located outside the lower and right hand boundary was excluded from the counting. Total numbers of cells in the primary squares were then totalled, averaged and the cell number was calculated according to the following formula:

\[ C = \frac{n}{v} \]

\( C \) = cell concentration (Cells/ml), \( n \) = number of counted cells, and \( v \) = volumes (ml). In the type of slide, the total volume of the chamber is \( 1 \times 10^4 \). According to that volume, the formula then becomes \( c = n \times 10 \).
Figure 2.6 The Haemocytometer. The two semi-reflective rectangles are the counting chamber. Image (A) shows sample position, counted squares and the cover glass. While (B) shows cells distribution over the main squares and those which are counted.

2.4.3 Cryopreservation of Cells

After growing cells, frozen cells were stored in liquid nitrogen (-196° C) in order to preserve cells without losing their capacity to differentiate, allowing replication of experiment
and comparative studies. Cells were counted after they were trypsinised and centrifuged at 1000 rpm for 5 min, then cells were re-suspended at a concentration of $1 \times 10^6$ cells/ml in freezing medium (70% growth medium, 20% FCS, 10% DMSO, Appendix E). The 1ml solution was then transferred to a cyroprotective vial, details of the cells (cell line name, passage number and differentiation time) were written in the vial and the transferred to a styrofoam insulated container, containing isopropanol and then transferred to -80° C freezer for overnight for gradual freezing. The vials were then transferred to the liquid nitrogen tank for storage, location of the vials in the liquid nitrogen tank was recorded in the lab book with date and the number of vials indicated.

### 2.4.4 Re-suspension of Frozen Cells

The vials containing frozen cells were taken out of the liquid nitrogen tank and thawed in a 37°C water bath, then sprayed with 70% ethanol to remove any contaminants. Then the contents were transferred to a 15 ml falcon tube and 10 ml of DMEM were added to the cells. Tubes were then centrifuged at 500 g for 5 min. after centrifuging, the supernatant was removed from the tube and pellets were resuspended in culture medium. Cells were finally plated in 75 cm² flasks.

### 2.4.5 Ca²⁺ measurement

In the cell culture experimental chapter, Ca²⁺ doses were added to each well as a mimic of exercise. These doses were calculated according to the Ca²⁺ homeostasis in the human body (2.2-2.6 mmol/L) or (9-10.5 mg/dL). The molecular weight formula was used in order to covert the
amount of Ca\textsuperscript{2+} in the body to the cell culture. These calculated doses were added to the already existing Ca\textsuperscript{2+} in the cells.

2.4.6 Cell viability assay

The CellTiter 96 proliferation assay method was used to determine cell numbers (Promega, USA). This is a colorimetric method for determining the number of viable cells in a cytotoxicity assays (MPTP in this study). Kits contained a tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS (a)] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. This solution is bioreduced by mitochondrial activity into a colored formazan product that is soluble in tissue culture medium. CellTiter reagent was thawed for 5 min in a water bath at 37°C, then 20 μl of CellTiter were pipetted into each well of the 96-well plates containing the Cells in 100μl of DMEM. Plates were incubated for two h in a humidified, 5% CO\textsubscript{2} atmosphere (Riveles et al., 2008).
2.5 SECTION THREE: HUMAN STUDY

2.5.1 Participants

Patients from the PD society (Bedford and District Branch, UK) were recruited in study four in this thesis. The recruitment process started with the ethical approval procedures then with a special arrangement with the chairman of PD society in Bedford Mr Bill Brady, the chairman provided the researcher with an opportunity to present the project plan and aims at the society’s monthly meeting. After the presentation, participants were encouraged to ask about the expected outcomes of this study and any other questions related to PD and exercise. Seven participants volunteered after this presentation and the following meeting. Participants were provided with an information handout that described participation requirements and testing procedures. Participants were fully aware that they were free to withdraw from the study at any time without giving reason. Volunteers visited The University of Bedfordshire Exercise Physiology laboratories on two occasions (visits).

2.5.2 Experimental design for human study

The aim of the first visit was to assess each subject’s stage of PD, quality of life, anthropometric measurements and the lactate threshold test for each individual, that test was used to set the exercise intensity utilised in the subsequent visit. The second visit was for collecting data using 5 m walk test, timed up and go test and balance test before and after a submaximal cycling protocol. The cycling required subject’s to cycle at an intensity set to elicit 90%
of LT. Pre and post-venous blood samples were obtained from an antecubital vein for the assessment of calcium concentration. Details about each step of those testing procedures are described in the following section.

2.5.3 Assessment of PD stage and quality of life

Upon arrival to the laboratory in visit one, participants completed questionnaires with assistance from their partners/spouse where appropriate (due to an inability to read or write legibly) to assess PD Stage and quality of life. Staging of PD disease progression was assessed with the Hoehn and Yahr questionnaire; this questionnaire classifies PD into five stages (Appendix D). The first stage is the mildest stage of the disease and stage five is the most extreme stage of disease progression. This assessment has been widely used as an accurate scale for motor symptoms of PD (Fisher et al., 2008, Yousefi et al., 2009).

The PD Questionnaire 39 (PDQ-39) is the most widely used PD specific measure of health status. It contains thirty-nine questions, covering eight aspects of quality of life. The PDQ-39 has been specifically designed for use with individuals with PD and has been shown to be valid and reliable (Baatile et al., 2000, Hackney and Earhart, 2009) (Appendix D). PDQ-39 assesses quality of life in the following subdivisions: mobility, ADL, emotion, social support, communication and bodily discomfort. All questions on the (PDQ-39) are coded/answered via how often the subject experiences a problem with each question in the last 7 days, with options of: Never, Occasionally, Sometimes, Often and Always. The scoring method is:
• Never = 0
• Occasionally = 1
• Sometimes = 2
• Often = 3
• Always = 4

The total score for (PDQ-39) is 156 points. Zero point means there is no effect of PD, 156 points means most severely affected.

The Unified PD Rating Scale (UPDRS) is a rating scale that was used to follow the longitudinal course of PD. The UPDRS has long been the major rating scale that has been used to assess severity of symptoms of PD (Fisher et al., 2008, Ridgel et al., 2009). UPDRS consists of four parts, parts I, II and III contain 44 questions each measured on a 5-point scale (0-4).

Part I. Behaviour and mood: intellectual impairment, thought disorder, motivation/initiative, depression

Part II. Daily activities, speech, salivation, swallowing, handwriting, cutting food, dressing, hygiene, turning in bed, falling, freezing, walking, tremor, sensory complaints

Part III. Motor examination: speech, facial expression, tremor at rest, action tremor, rigidity, finger taps, hand movements, hand pronation and supination, leg agility, arising from chair, posture, gait, postural stability and body bradykinesia.

The UPDRS score in the human study chapter in this thesis is the points of part III score only: zero (not affected) to 56 (most severely affected). Part three was chosen as it covers this study’s area of interest (Motor examination) as mentioned above.

2.5.4 Anthropometrics measurements

Measurement of height was performed using a Stadiometer (Holtain scale Ltd.UK). Body mass, body fat %, total body water %, muscle mass, physique rating, BMR/Metabolic Age, bone mass and visceral fat level measurements took place using radio wireless body composition platform analyzing system (Innerscan, Tanita, UK).

2.5.5 Lactate Threshold test (LT)

Lactate threshold (LT) is the exercise intensity that is associated with a substantial increase in blood lactate during an incremental exercise test. LT is probably the term most commonly used in the literature in association with estimates of the anaerobic threshold, and in most cases the use of this term is appropriate. The specific criteria used to detect the substantial
increase have become important parameters of the definition, and this has led to specific terms according to the criteria for detection of this threshold (i.e., OBLA, or individual anaerobic threshold). For example, the substantial increase may be detected as an increase by a fixed amount above resting blood lactate levels (i.e., +1 mM), or by the first intensity at which a given absolute level of blood lactate is detected (i.e., 2 mM or 4 mM). Several tests have been developed to determine the intensity of exercise associated with LT: maximal lactate steady state, lactate minimum test, lactate threshold, OBLA, individual anaerobic threshold, and ventilatory threshold. Each approach permits an estimate of the intensity of exercise associated with LT, but also has consistent and predictable error depending on protocol and the criteria used to identify the appropriate intensity of exercise. These tests are valuable, but when used to predict LT, the term that describes the approach taken should be used to refer to the intensity that has been identified, rather than to refer to this intensity as the LT considering its individuality, objectivity, sensitivity and validity (Morton et al., 2012).

One of the methods that show individuality and objectivity is the D-max method put forward by Cheng et al. (1992). It is concluded that the D-max appears to be a reliable method for defining the individual physiological responses to exercise tests, with the advantage of objectivity. However, there is lack of evidence to support the theory that the exercise intensity defined by the D-max method is superior to that defined by other methods to prescribe training intensity or predict aerobic performance for athletes. Furthermore, validation of this method for cyclists and elders has not been undertaken; Zhou and Weston (1997) demonstrated that no significant differences were found in the mean values of the variables between the D-max method and typical LT tests. On the other hand, determination of LT by typical LT submaximal
test has been confirmed to be a safe, accurate, and objective method to measure aerobic capacity in patients and in normal subjects (Matsumura et al., 1983). Therefore, the typical LT test was taken as a valid indicator of LT in response to submaximal cycling exercise for PD participants.

The definition that has been used in study four is: LT is the exercise intensity that is associated with a substantial increase in blood lactate during an incremental exercise.

During the first laboratory visit and after the subject’s anthropometric variables were assessed, a cycling LT test was used to establish the resistance required to elicit the intensity of 90% of LT to be used during the sub-maximal cycling session in the second laboratory visit. The 90% intensity was suggested by the American College of Sports Medicine (ACSM) as cycling guideline for exercise programs for PD patients (ACSM, 1998).

Starting workload was set at a calculated power output of 35 watts (W). Workload was increased by 10 W every 3 min until the LT was reached, determined by an increase in lactate concentration by 1 mM or more between stages. Whole blood fingertip samples were taken during the last 30 s of each three min stage for blood lactate analysis (Analox-Lactate analyser LM 5, Analis- Belgium). Once the LT had been reached, no further blood samples were taken and the subjects stopped pedalling Oxygen uptake was recorded every 3 min using (Cortex Metalyzer 11R; Cortex, Lepzig, Germany), as was heart rate (Polar sports tester, Polar Electro Inc, Finland).
The LT was subsequently determined as a sudden and sustained increase in blood lactate concentration above resting levels from visual inspection of individual plots of blood lactate concentration vs. workload by independent analysis from two experimenters (Jones, et al. 1999). It was emphasized to all participants that the cycle ergometer test is submaximal and they may expect to exercise to a rating of 6 out of 10 for feelings of effort, and that they will not exercise to exhaustion. Verbal encouragement was used throughout to encourage participants to continue cycling until they felt unable to maintain the workload, wanted to stop for any reason when the LT was reached, or the experimenters feel that the participant should stop. Recovery will be monitored and refreshments available.

2.5.6 Balance test

Maintaining postural stability is a problem for individuals with PD especially in moderate and late stages of the disease. Therefore, balance ability and number of falls (due to losing balance) were considered indicators of a patient’s quality of life (Baatile et al., 2000, Goetz et al., 2004). Stability measurement was performed using the Footscan system (RSscan Lab Ltd-UK). Footscan consists of a floor based (2D plate 0.5 m) that records and analyses the pressure and force distribution of the foot at a rate of up to 500 frames of data per second (Figure 2.10). Participants were asked to stand still on the footscan stability sheet for 10 seconds with shoes off, straight back and eyes open. Patient’s foot pressures on the sheet were recorded for 10 seconds. Data were transferred immediately to the software and saved until analysis.
The footscan system works by measuring vertical force over a number of sensors (8192 sensors on a 1 m plate). This allows the pressure to be calculated by knowing the area that the force is being applied over. The system measures the vertical force that is applied by the body through the foot to the ground during stationary standing to assess balance. By dividing this contact area into different zones of the foot, the system can analyse the maximum pressures/forces applied in these different areas, and the timing of this application of force. This allows a detailed analysis of when/where force was applied during posture stability. If there is an imbalance occurring in this phase, the centre of pressure (COP) will migrate more than the normal stability phase measured by millimetres. Participants performed this assessment once before a cycling session and once after a cycling session.

**Figure 2.7 Footscan stability measuring System.** Used in balance test, the system consists of a sensitive sheet (left) connected with analysing software (right).
2.5.7 Blood samples for Ca\textsuperscript{2+} concentration level

Blood samples (4 ml) obtained through a venepuncture of an antecubital vein at rest and immediately upon completion the cycling session in the main session. Blood collecting procedures were carried out in a designated area (blood trolley figure 2.17), which was labelled “Designated Area– for Work with Human Blood”. This station was provided with disposal gloves, blood collection tubes, syringes, needles, hazardous disposal container and needles waste container.

Blood was drawn from a vein, specifically from the inside of the elbow. The site was cleaned with germ-killing medicine (antiseptic), an elastic band was wrapped around the upper arm to apply pressure to the area and make the vein swell with blood. Next, a needle was inserted into the vein. The blood was collected into an airtight tube attached to the needle. Then the elastic band was removed from the arm. Venepuncture was carried out by staff trained and certified for these procedures (Dr Paul Castle). All blood collection procedures (sampling, handling and disposal) were carried out according to University of Bedfordshire generic ethics for ISPAR.

Directly after blood was collected, samples were given a code to keep the confidentiality, centrifuged at 5000 rpm for 10 min then kept in a -20°C freezer. Batches of samples were preserved in dry ice to transport to the Blood Sciences Laboratory, Luton and Dunstable Hospital, NHS Foundation Trust, Luton for measuring Ca\textsuperscript{2+} levels. In the blood collecting procedures, Li-Heparin LH tubes (Sarstedt Ltd. UK) were used as syringe and centrifuge tube,
these tubes and syringes were a gift from the Blood Sciences laboratory Luton and Dunstable Hospital. Luton, UK.

### 2.5.8 Heart rate monitoring and oxygen consumption

During the LT, Heart rate was monitored with short-distance telemetry (Polar, Kempele, Finland), and oxygen consumption will be measured via a facemask linked to Metamax portable metabolic test system (Cortex Biophysik, Leipzig, Germany). At the end of each increment, participants were asked to provide their subjective rating of perceived exertion scale range, 6 –20 (Borg, 1994).

### 2.5.9 Visit Two

At least 1 week after the LT assessment, participants will return to the laboratory for the main exercise session. The following tests took place pre and post the submaximal cycling protocol.

### 2.5.10 (5-m) walk test

For gait speed, the (5-m walk test) was used because it has shown high reliability in neurological patients including PD (Haas et al., 2004). The objective of the test is to ask the patient to walk 5 ms as fast as possible whilst remaining safe. Tape was placed on the floor to
mark the start and end of 5 m with an additional 2 m at the beginning and end to permit acceleration and deceleration. A bright orange pylon placed at the stopping mark provided an easily visualized goal.

Instructions to walk had been standardized as following; “I am going to measure your fastest walking speed. When I say: go, walk as quickly as possible, whilst maintaining control and that you are safe at all time”. The evaluator walked beside the patient and began timing with a digital stopwatch when the subject’s first foot crosses the start line. Timing stopped when the first foot crosses the stop line. No rest period was provided between the 4 trials.

2.5.11 Timed up-and-go test (TUG)

Timed up and go test is an important tool to evaluate balance and mobility speed, it records the time taken to rise from an armchair, walk a distance of 3 m, turn and return to the chair in a seated position. The number of walking steps is also counted. Higher values of time and number of steps represent a higher risk of falls (Baatile et al., 2000, Nordin et al., 2006).

2.5.12 Sub-maximal cycling session

The duration of cycling exercise was dependent upon each person’s exercise capacity, but should be at least 15 min but no more than 30 min. The intensity of exercise was set at 90% of the LT to ensure a sub maximal intensity throughout. Measures of heart rate and RPE was
performed at 5 min intervals during the cycle. Blood Ca\textsuperscript{2+} levels were obtained prior to and immediately upon cessation of exercise as described by (Deogenov et al., 2009).

Figure 2.8 Monarch cycle ergometry (Ergomedic 874E). This cycle ergometry used to estimate the LT level in session one and to exercise in session two, seat height was adjusted according to each participant’s comfort. Intensity of exercise was raising up using different weight added to the front flywheel. Digital screen shows pedal-turns per min (RPM), heart rate in beats per min (HR) and cycling-time in min and seconds (TIME). Metamax portable metabolic system (Cortex Biophysik, Leipzig, Germany) was connected to the participant to assess gas exchange and volumes.

2.5.13 Heart rate monitoring and oxygen consumption
During the LT, heart rate was monitored with short-distance telemetry (Polar, Kempele, Finland). Oxygen consumption, gas exchange and volumes were measured via a facemask linked to Metamax portable metabolic test system (Cortex Biophysik, Leipzig, Germany). At the end of each increment, participants were asked to provide their subjective rating of perceived exertion scale (range, 6–20).

![Borg's RPE Scale](image)

**Figure 2.9 Borg's perceived exertion and pain scales (Borg, 1994).**

Before cycling, participants were asked to wear a heart rate monitor and facemask linked to Metamax portable metabolic test system (Cortex Biophysik, Leipzig, Germany) and perform some light cycling at an intensity they feel comfortable at. This was to familiarize the participant
with the action of cycling and for the experimenters to gauge the participant’s cardiovascular response to exercise.

The rating perceived exertion (RPE) scale was used in this study to gauge participant’s level of intensity during LT test and the cycling session. Each participant was asked to point to the number they feel reflects their physical condition every 2-3 min (Borg, 1970) (Appendix E).

2.6 STATISTICAL ANALYSIS

Statistics are described and explained in individual chapters according to the experimental design and the aims of each study. All statistical analyses were performed using SPSS 17.0 software.
CHAPTER THREE: RESULTS ONE

IMMUNHISTOCHEMICAL LOCALIZATION OF Ca\textsuperscript{2+} CALMODULIN-DEPENDENT PROTEIN KINASES 1 and 4 IN PARKINSONIAN RAT BRAIN AFTER 8 WEEKS OF ENDURANCE EXERCISE

3.1 INTRODUCTION

Numerous investigators have reported, based on the results of experimental, clinical and epidemiological studies, that exercise modifies brain functions as several molecular systems could potentially participate in the benefits of exercise on the brain (Akiyama and Sutoo, 1999, Sutoo and Akiyama, 2003, Zigmond et al., 2009). Cotman and Berchtold. (2002) demonstrated that neurotrophic factors have most of the properties that could underlie such beneficial effects, especially brain-derived neurotrophic factor (BDNF) because it supports the survival and growth of many neuronal subtypes, including glutamatergic neurons (Dishman et al., 2006). On the other hand, Sutoo et al. (2003) have suggested another theory that might provide an explanation on the mechanism of the effect of exercise; this mechanism is the role of Ca\textsuperscript{2+} calmodulin-dopamine pathway in the brain in enhancing the locomotors. Animal studies indicate that exercise increases serum calcium levels, and serum calcium is transported to the brain where it activates tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, which in turn stimulates DA synthesis through a calcium/CaM-dependent system. Subsequently, the
increased DA levels induce behavioural and physiologic changes (Sutoo and Akiyama, 2001). However, this has not been tested with PD models; therefore, this chapter is investigating this theory.

As mentioned previously in the literature review chapter CaMK-1 and CaMK-4 are Ca\(^{2+}\) binding enzymes with a molecular mass of 400-600 KD, these two enzymes are considered one of the most abundant families of kinases in the mammalian brain (Hook and Means, 2001). Because of their broad substrate specificity, CaMKs play an important role in various cellular events. They have been implicated in modification of neurotransmitter synthesis, receptor function and gene expression (Sugiura et al., 1992, Soderling and Stull, 2001).

The rise in intracellular Ca\(^{2+}\) levels upon synaptic activity triggers the activation of several kinases critical for the induction and expression of the calcium-calmodulin dopamine pathway. These include the Ca\(^{2+}\)/CaM kinases CaMK-1 and CaMK-4. Therefore, the aim of this chapter is to investigate whether there are changes in CaMK-1 and CaMK-4 proteins in the substantia nigra region as an indication of the levels of Ca\(^{2+}\) in the PD brain after 8 weeks of endurance exercise.

The primary symptoms of PD stem from the deterioration of the part of the brain that controls motor functioning. This region is the Substantia Nigra (SN) which is found deep within the brain stem and contains neuromelanin, pigment cells, which synapse to cells of the striatum. The striatum is responsible for balance, control of movements, and walking (Morris et al., 2001a, Weintraub et al., 2008a). DA, produced in the SN, passes messages between the
striatum and the SN, when the cells of the SN deteriorate, as in the case of PD, there is a corresponding decrease in the amount of DA produced between these cells. The decreased levels of DA cause the neurons of the striatum to fire uncontrollably, preventing the patient from being able to direct motor function (Brown et al., 2005).

SN lies in the midbrain immediately dorsal to the cerebral peduncles. This nucleus is a motor centre that plays an important role in the motor function. SN cells projects to the caudate and putamen, two nuclei of the basal ganglia that together comprise what is called the Striatum. These nigrostriatal cells utilize the neurotransmitter DA. SN is thought to be the lesion site in PD (Weidong et al., 2009). The most consistent pathological finding in PD is degeneration of the melanin-containing cells in the SN (melanin is an inert by-product of the synthesis of dopamine). As mentioned before, cells within the SN produce DA normally. This substance passes—via axoplasmic flow to the nerve terminals in the striatum (caudate nucleus and putamen), where it is released as a transmitter. It is the absence of this transmitter that produces PD symptoms (Weintraub et al., 2008a).

The hippocampus plays a critical role in formation and retrieval of many forms of memory and its related movements (e.g. spatial learning and episodic memory) (Cotman and Berchtold, 2002, Cechetti et al., 2008). Hippocampus is made up of two parts located symmetrically on each side of the medial temporal lobe of the brain (Andersen et al., 2007). It is divided into several regions, the main areas being: cornu ammonis 1, cornu ammonis 3 (CA1, CA3) and dentate gyrus. The hippocampus is also connected to other parts of the brain, such as the hypothalamus and thalamus, through the fornix, a major pathway that leads the signals in a longitudinal direction with respect to the hippocampus this important role of hippocampus in the
learning of movements was the reason of choosing this region to study in this chapter beside striatum.

The aim of this chapter is to study the effect of 8 weeks of endurance training on expression of CaMK-1 and 4 synthesis in striatum and hippocampus regions in PD rat brain.

3.2 EXPERIMENTAL DESIGN AND EXERCISE PROCEDURES

24 Adult rats were housed individually in a 12/12 h light/dark cycle. Rats were randomly divided between four groups: (1) sedentary controls (C) (N=6), (2) MPTP induced group (PD) (N=6), (3) MPTP +exercise-trained group (PDE) (N=6), (4) Exercise only group (E) (N=6). Rats to be exercise-trained were subjected to running exercise 5 days a week for 8 weeks. Figure 3.1 shows the experimental design procedures and the experimental groups used in this study.

Rats to be exercise-trained (PDE) and (E) groups were transferred as one group from their cages to the special rodent treadmill (see 2.2, 2.3). Treadmill speed initially increased gradually then was maintained at 25 m.min$^{-1}$. Animals maintained running at that speed for the first 4 weeks. Then the speed was increased by 5 m.min$^{-1}$ to be 30 m.min$^{-1}$ for the remaining four weeks of the endurance training. The running time was 40 min/day over the first 4 weeks. In week 5, the running duration was increased by 5 min to reach 1 h then maintained at 1-hour by the end of the eight weeks. The other groups (C and PD) were put in the treadmill (Speed 10 m.min$^{-1}$) for 10 min everyday at 10 m.min$^{-1}$ in order to remain active and to mimic daily life
activities. The relevant experimental groups (PD and PDE) were injected with 10 doses of MPTP (12.5 mg/kg in) into the internal carotid artery. Doses were injected after 4 weeks of exercise training on a 4-week timetable, 3-5 days between the doses. Therefore, the total weeks of training were 8 weeks. MPTP doses were scheduled to potentiate its neurotoxin effect by impeding the renal excretion and neuronal clearance of MPTP and its toxic metabolites. (C) and (E) groups were injected with equal doses of saline. The injecting procedures were followed according to (Langfort et al., 2006). After 8 weeks of exercise training all rats were sacrificed. Procedures are described in detail in Chapter Two (see 2.3.6).

General procedures of exercise program, immunohistochemistry protocol and image processing are described in Chapter Two (see 2.3.8, 2.3.9). Brains were placed in special plate covered with frozen tissue embedding media (Fisher, UK) the orientation and the labelling were determined and recorded. Then the samples kept in -80°C freezers where they were covered with parafilm. Figure 3.1 summarizes the experimental design of this chapter.
24 Sprague Dawley adult rats

Control group (C)
- N=6
- No MPTP
- No Exercise

MPTP group (PD)
- N=6
- MPTP induced (10) doses.
- No Exercise

MPTP + Exercise group (PDE)
- N=6
- Exercised for 4 weeks then MPTP induced + Exercise (4 weeks)

Exercise only group (E)
- N=6
- No MPTP
- Exercise (8 weeks)

MPTP injections

Physical Exercise

IMMUNOHISTOCHEMISTRY ANALYSIS

Figure 3.1 Description of experimental procedures in study one
3.3 RESULTS

Photographs of the area of interest were taken using 10 X magnifications with a Leica DM IL microscope connected to a (Progress CT3) digital camera (Jenoptick-Germany). The selected boundaries for evaluation of the areas of interest were established using the rat brain atlas of Paxinos and Watson (1998). The rat brain atlas shows layers of brain sections in 56 plates. With the aid of this reference, photos were taken in plates 30-32 using the same light intensity and threshold conditions.

3.3.1 Lactate threshold test

In order to determine the endurance exercise intensity, lactate threshold tests (LT) were performed for the (PDE) group (N=6) in two days. Testing procedures consisted of seven stages of incremental running on a special rodent treadmill. Each stage is 3 min. Individually, rats were transferred from its cage to the special rodent treadmill min before the test. The aim of this early transfer was to familiarize the animal with the testing environment. After 10 min the end of the rat-tail was cut and (50 ul) of blood were taken for the lactate test, immediately after taking the blood sample, the cut tail was treated with Heparin. Animals were accommodated in the treadmill runway and started to run at the speed of 13 m.min⁻¹ at 0° inclination for 3 min, after these three min the rat had 3 min to allow the collection of a blood sample.
This process was repeated 6 times at the following speeds 17, 21, 25, 29, 34 m.min\(^{-1}\) and the final speed was 37 m.min\(^{-1}\). Finally, animal was taken back to the cage. To determine the Lactate threshold speed, it can be seen that the lactate level increased rapidly when the running speed was above 22 m.min\(^{-1}\) (Figure 3.1). Therefore, the speed of 25 m.min\(^{-1}\) was chosen as the start point for the endurance exercise intensity in this study.

![Figure 3.2 Lactate threshold level for the first Lactate test.](image)

The average LT level appears when the running speed was 25m/m. The running speed was maintained at 25 m.min\(^{-1}\) for the first 4 weeks then was increased by 5 m.min\(^{-1}\) to be 30 m.min\(^{-1}\) for the remaining four weeks of the endurance training. The running time was 40 min/day over the first 4 weeks. In week 5, the running duration increased by 5 min to reach 1 h then was maintained at 1-hour until the end of the 8 weeks. The other groups (C and PD) were put on the treadmill (speed 10 m.min\(^{-1}\)) for 10 min everyday in 10 m.min\(^{-1}\) to remain active and to mimic the daily activities.
After 4 weeks of endurance exercise, another LT test was conducted for each individual rat (Figure 3.2), the aim of the second test was to check whether there were improvements in LT level, which in turn might require a change in the given dose of exercise intensity.

![Lactate Threshold level for the second Lactate test after four weeks of Exercise. LT levels were raised when the running speed was 30 m.min\(^{-1}\). That LT speed was set as the running speed for the last 4 weeks of endurance exercise.](image)

After 8 weeks of exercise training the (PDE) and (E) groups were sacrificed immediately after the last bout of exercise. On the other hand, control group (C) and (PD) group were left undisturbed for 48 h then sacrificed. Prior to sacrifice, all the rats were deeply anaesthetized with sodium pentobarbital (60 mg/kg) and were perfused transcardially with 0.1 M sodium phosphate buffer pH 7.4, followed by fixative. Rats were then sacrificed by decapitation and the brains
were removed (Figure 2.4), post fixed in the same fixative for 4 days and than cryoprotected in PBS containing sucrose 30%.

Brain sections were agreed with two independent investigators based on two principles, following the studied regions.

Figure 3.4 Section 39 of rat brain where the Striatum and the Hippocampus (lies inside the red shapes) (Paxinos and Watson, 1998).
CaMK-1 expression- Striatum region

Figure 3.5 Representative light microscope photomicrographs illustrating CaMK-1 immunoreactivity under low power (144X, panels A–C) in the four treatment: Control (C), MPTP induced (PD), MPTP induced plus 8 weeks exercise-trained (PDE) and 8 week exercise trained only (E). It is noticeable that the density of Striatum CaMK-1 immunoreactivity MPTP induced (PD) was reduced when compared to the control animal (C). Striatum CaMK-1 immunoreactivity increased in (PDE) rat following 8 weeks of exercise training when compared to chronic group (PD). Exercise training for 8 weeks in the chronic PDE led to a prominent recovery of the Striatum CaMK-1 neurons. Exercise-trained only animal for 8 weeks (E) shows
higher detection than the normal control animal (C). Group (E) did not receive any MPTP.

Inject

![CaMK-1 expression - Striatum region](image)

**Figure 3.6 Quantitative analysis of the change in amount of CaMK-1 in the nuclei and cytosol of striatum cells for the four experimental treatments.** The number of immunolabelled brown particles in the unit area (1 cm², under 10,000 X magnification pictures) was measured in nuclear and cytosolic areas as described in chapter two. Increased immunoreactivity of CaMK-1 was found in C and PD and E groups as compared to other groups. The PD group was significantly decreased in the expression of CaMK-1 compared with control group there were significant differences between the PD group and the PDE group in the expression of CaMK-1 after the 8 weeks of exercise. The numbers of particles were counted in randomly chosen 40 pictures from the immuno-electron micrograph from the four groups using imageJ software, and were analyzed using one way A-NOVA test *P < 0.005.
Figure 3.7 Representative light microscope photomicrographs illustrating CaMK-4 immunoreactivity under low power (144 X, panels A–C) in the four treatments. Control (C). MPTP induced (PD), MPTP induced plus 8 weeks exercise-trained (PDE) and 8 week exercise trained only (E). It is noticeable that the density of Striatum CaMK-4 immunoreactivity MPTP induced (PD) was reduced when compared to the control animal (C). Striatum CaMK-4 immunoreactivity increased in (PDE) rats following 8 weeks of exercise training when compared to chronic group (PD). Exercise training for 8 weeks in the chronic PDE led to a prominent recovery of the Striatum CaMK-4 neurons. Exercise-trained only animals for 8 weeks (E) shows
higher detection than the normal control animal (C). Group (E) did not receive any MPTP injections.
Figure 3.8 Quantitative analysis of the change in amount of CaMK-4 in the nuclei and cytosol of striatum cells for the four experimental treatments. The number of immunolabelled brown particles in the unit area (1 cm², under 10 000X magnification pictures) was measured in nuclear and cytosolic areas as described in chapter two. Increased immunoreactivity of CaMK-4 was found in C and E groups as compared to other groups. PD group was significantly decreased in the expression of CaMK-4 compared with control group. There were significant differences between the PD group and the PDE group in the expression of CaMK-4 after the 8 weeks of exercise. The numbers of particles were counted in randomly chosen 40 pictures from the immunoelectron micrograph from the four groups using imageJ software, and were analyzed using one way A-NOVA test *P< 0:005.
Figure 3.9 Electronmicrographs of groups C, PD, PDE and E induced CA1 neuronal cells in the rat brain hippocampus immunolabelled with CaMK-1 antibody. Image (A) represent controls, (B) represents PD group, (C) represents PD+E group while (D) represents exercise only group (E). CA1-neuronal cells stained with anti-CaMK-1 antibody. Immunoreactivity was seen in the four treatments. Increase of brown Perticles was found in nucleoli or heterochromatin of the nuclei (arrows), under the experimental condition used in this study. Scale bar = 500 nm.
Figure 3.10 Quantitative analysis of the change in amount of CaMK-1 in the nuclei and cytosol of striatum cells for the four experimental treatments. The number of immunolabelled brown particles in the unit area (1 cm$^2$, under 10,000 X magnification pictures) was counted in nuclear and cytosolic areas as described in chapter two. Increased immunoreactivity of CaMK-1 was found in C and E groups as compared to the other groups. Only E group was not significantly increased in the cytosol compared to C group. PD and PDE groups were significantly decreased in the expression of CaMK-4 compared with control group. There were no significant differences between the PD group and the PDE group in the expression of CaMK-1. The numbers of particles were counted in randomly chosen 40 pictures from the immunoelectron micrograph from the four groups, and were analyzed using one way ANOVA test *P< 0.005.
CaMK-4 expression- Hippocampus region

Figure 3.11 Electronmicrographs of groups C, PD, PDE and E induced CA1 neuronal cells, immunolabelled with CaMK-4 antibody. (E) represents the control, (F) represents PD group, (G) represents PD+ E group while (H) represents exercise only group (E). CA1-neuronal cells were stained with anti-CaMK-4 antibody. immunoreactivity was seen in the four treatments. Increase of brown particles was found in nucleoli or heterochromatin of the nuclei (arrows), under the experimental condition used in this study. Scale bar = 500 nm.
Figure 3.12 Quantitative analysis of the change in amount of CaMK-4 in the nuclei and cytosol of CA1 cells in the hippocampus region for the whole experimental treatments. The number of immunolabelled brown particles in the unit area (1 cm², under 10,000 X magnification pictures) was counted in nuclear and cytosolic areas as described in chapter two. Increased immunoreactivity of CaMK-4 was found in C and E groups as compared to other groups. Only the E group was not significantly increased in the cytosol compared to C group. PD and PDE groups were significantly decreased in the expression of CaMK-4 compared to control group. While there were no significant differences between PD group and PDE group in the expression of CaMK-4. The numbers of particles were counted in randomly chosen 40 pictures from the immunoelectron micrograph from the four groups, and were analyzed using one way ANOVA test *P< 0.005.
3.4 RESULTS SUMMARY

After 8 weeks of endurance exercise for PDE and E groups, MPTP injection for PD and PDE groups, Immunhistochemistry analysis has shown the following:

- Higher expression of CaMK-1 in the striatum region in PDE animals compared to PD animals
- CaMK-1 level has decreased in PD group compared to C group
- No significant differences between the four treatments in the Hippocampus region in the level of CaMK-1
- Higher expression of CaMK-4 in the striatum region in PDE animals compared to PD animals
- Higher expression of CaMK-4 in the striatum region in E animals compared to C animals
- CaMK-4 level has decreased in PD group compared to C group
- No significant differences between the four treatments in the Hippocampus region in the level of CaMK-4

3.5 IMPLICATIONS OF RESULTS.

Immunohistochemical analysis shows a qualitative increase in CaMK-1 and CaMK-4 expression in the PDE group compared to the PD group in Substania nigra. In addition, increase in CaMK-1 and CaMK-4 expression in PDE group compared to PD group in Substania nigra in E group compared to C group. These findings may imply a role of CaMK-1 and CaMK-4 in
neuroprotection effect of both proteins against MPTP. However, these findings needed another quantitative analysis in order to determine whether this higher expression of CaMK-1 and CaMK-4 are significant or not. That is the aim of Chapter Four.

3.6 DISCUSSION

In results one, the whole 24 rat brains have been sliced and put on microscopy slides (three sections on each slide), our area of interest were between plates number 37-39 where the SN was obvious, determining this region (RSc) was performed using rat brain atlas (Paxinos and Watson, 1998). Following the experiment and manufacturer’s protocol, anti CaMK-1 and CaMK-4 labeled cell cytoplasm and neuropil regions in the substantia nigra. After the whole slides were treated with the Immunohistochemicaly (IHC) protocol, slides were checked under the microscope.

The main findings from chapter one and two have shown that the rat models with PD which had been treated with endurance exercise for 8 weeks as described displayed higher amounts of CaMK-1 and 4 proteins in the SN more than the same regions in the Parkinsonian rat brain group. Also it has been noticed that the localization of both CaMK-1 and CaMK-4 antibodies is higher in control group than the PD and PDE groups.

These findings agree with (Cechetti et al., 2008) who demonstrated that daily moderate intensity exercise, 2 weeks of 20 min/day of training reduces damage in hippocampal slices from
Wistar rats submitted to *in vitro* ischemia. Exercise demonstrates neuroprotective effects on improving the survival of DA cells in the SN In both the 6-hydroxyDA (6-OHDA) lesioned rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned mouse (Smith and Zigmond, 2003, Dishman et al., 2006).

These results combined with previous studies indicate: (1) CaMKs regulates various functions in the brain and findings suggest that Ca$^{2+}$ activates DA synthesis in addition to the CaMKs-dependent system, and even through CaMK in specific brain regions. These results lead us to highlight the important role of intracellular Ca$^{2+}$ in the neuronal death and survival mechanisms. Rapid and severe elevation of intracellular Ca$^{2+}$ induces neuronal cell death in both matured and established neurons, whereas homeostasis and moderate elevation of Ca$^{2+}$ concentration supports neuronal survival in some immature and developing neurons (Koike et al., 1989). The Ca$^{2+}$/calmodulin dependent protein kinases (CaM-kinase) family which contains CaM-kinases I, II, IV, and CaM-kinase kinase (CaM-KK) are the major proteins regulated by intracellular Ca$^{2+}$ concentration. CaM-kinase II is directly activated by Ca$^{2+}$/calmodulin, whereas CaMK-1 and CaMK-4 are regulated by CaMKK (Yano et al., 2005). In previous investigations sutoo and Akiyama. (2003) confirmed that the activity of CaMK-1 was decreased after MPTP injection in rat CA1 cells. In this study, we documented the role of CaMK-1 and CaMK-4 in the Ca$^{2+}$ regulated neuronal survival in MPTP induce rat brain and localized the distribution of both kinases after 8 weeks of endurance exercise.

Ca$^{2+}$ has many physiological functions in the human body such like, modulating in muscle stimulation, transmission of action potential in neurons, blood clotting, enzyme activation
and fluid transport across membranes. The bones act as a reservoir of Ca\textsuperscript{2+} where it is stored in combination with phosphate. When exercise occurs, a larger amount of Ca\textsuperscript{2+} is needed by the muscles to achieve the required concentration (Mcardle, 2006). This then produces a fall in the levels of Ca\textsuperscript{2+} in the blood. As a physiological response, the parathyroid glands of the body secrete parathyroid hormone (PTH) which activates the bone cells. These cells then start to recycle producing a release of stored Ca\textsuperscript{2+} ions in the bone. Finally, this produces an increased of Ca\textsuperscript{2+} levels in the bloodstream (Merrill et al., 2005). The increase of Ca\textsuperscript{2+} levels will also arrive in the neuronal cells of the brain. As more Ca\textsuperscript{2+} arises at the neuronal cells, it might be expected that there will be higher levels of the Ca\textsuperscript{2+} binding protein. However, Ca\textsuperscript{2+} is an ion that needs to have a strong homeostasis regulation as small variations (above or below normal levels) can cause serious physiological problems (Akiyama et al., 1990, Merrill et al., 2005).

Microscopy photographs agreed with the previous findings that both CaMK-1 and CaMK-4 regulated by CaMKK, and mainly localized in the cytoplasm, which probably refer to the \textit{Y} isoform, which can associate as a lipid modified with Glogi and plasma membranes. Waymanet et al. (2006) demonstrated the link between that \textit{Y} isoform and the RAS/ERK pathway.

To understand the complex network with different pathways, this crosstalk can occur at several levels there can be direct phosphorylation between protein kinases or regulatory phosphorylation of either an upstream or a downstream effect of another kinase. There have been several explanations and communication mechanisms between CaMKs and brain function. In this study, the most important communications are between the CaMK cascade and the Mek/Erk...
pathway. Schmitt et al. (2004) demonstrated that elevated intracellular Ca\textsuperscript{2+} triggers numerous signalling pathways including protein kinases such as the calmodulin-dependent kinases (CaMKs) and the extracellular signal-regulated kinases (ERKs) upon depolarization of the neuroblastoma cell line (Schmitt et al., 2004).

Smith and Zigmond. (2003) demonstrated other evidence on the role of ERK in neurosurvival, volunteer exercise increased neurotrophic factor such as GDNF and BDNF which protect DA neurons from lesions of 6-OHDA. It has been suggested that activation of ERK contributes to basal DA cell survival. Although activation of the ERK5 pathway also provided some neuroprotection from acute 6-OHDA toxicity in MN9D cells (Smith and Zigmond, 2003). Other studies are being conducted to explore the effect of the ERK pathway neuronal survival following chronic exposure to a neurotoxin. ERK were implicated in the protection of cells from injury in several model systems (Engedal and Blomhoff, 2003).

Our immunohistochemical findings show higher expression of CaMK-1 and CaMK-4 in the striatum, side to side with the hypothesis that ERK kinases important for DA neuronal survival might provide an explanation on the neuroprotective effect of CaMKs. It is possible that these pathways activate common targets in DA neuronal cells to promote cell survival, and maximal activation of these transcription factors may require both ERK pathways. Knowledge of these signalling mechanisms in DA neurons should greatly aid in the development of new therapeutic approaches for PD. Results from this study might provide new information on the CaMKs/ ERK signaling pathways that play a role in the neuroprotection and basal survival of DA neurons.
Ca\textsuperscript{2+} has two separate roles in the regulation of blood pressure through the central and peripheral systems; calcium ions reduce blood pressure through a central, calcium calmodulin-dependent DA synthesizing system and increase blood pressure through an intracellular, calcium-dependent mechanism in the peripheral vasculature. Serum calcium increases blood pressure directly as a result of action on the vasculature, but some serum calcium is transported to the brain and reduces blood pressure.
CHAPTER FOUR: RESULTS TWO

EFFECT OF ENDURANCE EXERCISE ON CaMK-1 AND CaMK-4 IN TWO REGIONS IN PARKINSONIAN RAT BRAIN AFTER 8 WEEKS OF ENDURANCE EXERCISE (WESTERN BLOT ANALYSIS)

4.1 INTRODUCTION

Several molecular systems could potentially participate in transfer of exercise outcomes to the brain through the blood brain barrier (Dishman et al., 2006). However, there are two main theories that provide hypothesizes of how exercise effect transfers to the brain. The first hypothesis suggested that neurotrophic factors have most of the properties that could underlie such beneficial effects of physical exercise (Cotman and Berchtold, 2002). Among the neurotrophic factors, brain-derived neurotrophic factor (BDNF) was the main interest because it supports the survival and growth of many neuronal subtypes, including glutamatergic neurons. Subsequently, as the neurotrophin field evolved, BDNF emerged as a key mediator of synaptic efficacy, neuronal connectivity and use-dependent plasticity (Schinder and Poo, 2000). These results encouraged other researchers to investigate roles of other types of neurotrophic factors. Yasuhara et al. (2007) examined this hypothesis in another glial cell-derived neurotrophic factor (GDNF) and confirmed that a form of forced exercise caused a small but significant increase in GDNF in the ipsilateral striatum that peaked at 3 days and had returned to normal by 7 days. However, this hypothesis has been examined only with animal models and cell culture models of PD but it has not been widely examined in PD patients.
On the other hand, Sutoo and Akiyama. (2003) investigated the role of Ca\(^{2+}\) ions in brain function and plasticity. They demonstrated that acute exercise leads to an increase in the serum Ca\(^{2+}\) level and subsequently an increase in the brain Ca\(^{2+}\) level in normal mice. However, this hypothesis also has not yet been investigated with PD brains. In chapter three, this hypothesis was examined with one analytical technique, which required to be confirmed with another analytical technique in order to evaluate the results from chapter three. Therefore, the aim of this chapter is to examine the effect of exercise program in the levels of Ca\(^{2+}\) levels in the rat brain via CaMK-1 and CaMK-4 isoforms as Ca\(^{2+}\) indicators with western blot analysis.

The sensitivity of the CaMK-1 and CaMK-4 enzymes to Ca\(^{2+}\) and calmodulin is governed by the variable and self-associative domains. This sensitivity level of CaMK-1 and CaMK-4 also modulates the different states of activation for the enzyme. Initially, the enzyme is activated; however, autophosphorylation does not occur because there is not enough Ca\(^{2+}\) or calmodulin present to bind to neighboring subunits. As greater amounts of Ca\(^{2+}\) and calmodulin accumulate, autophosphorylation occurs leading to persistent activation of the CaMK-1 and CaMK-4 enzymes for a short period of time. However, the Threonine 286 residue eventually becomes dephosphorylated, leading to inactivation of CaMK-1 and CaMK-4.

Results from study one have shown that 8 weeks of endurance exercise enhanced the expression of CaMK-1 and CaMK-4 in SN and hippocampus regions in the exercise-trained groups of rats compared to control and PD induced groups. Therefore, the aim of this chapter was to confirm results from chapter three with another analytical technique. The aim is to investigate whether the immunohistochemical results in study one could indicate significant
quantitative raises in the amounts of CaMK-1 and CaMK-4 in the studied regions in the four experimental treatments. In this chapter, western blot analyses was carried out to quantify the Ca$^{2+}$ calmodulin dependent protein kinases 1 and 4 levels in the four treatment groups of PD.

Striatum and hippocampus regions were the areas of investigation in this chapter. The involvement of these two regions has been explained in chapters one and three. Briefly, these two regions were chosen because they play a critical role in formation and retrieval of many forms of movement functions and directly related to PD symptoms (Ahmed et al., 2000, Cechetti et al., 2008). Hippocampus is made up of two parts located symmetrically on each side of the medial temporal lobe of the brain (Aberg et al., 2000). It is divided into several regions, the main areas being: cornu ammonis 1, cornu ammonis 3 (CA1, CA3) and dentate gyrus. The term “hippocampus proper” generally refers to the pyramidal cell regions CA1 and CA3 whereas “the hippocampal formation” refers to the hippocampus proper plus the granule cells of DG and the subiculum. The subiculum is located at the end of the CA1 region (Yoshimura et al., 2001). On the other hand, the striatum consists of the caudate nucleus and the putamen, and is the main input station for the basal ganglia. Functionally, the striatum is involved in planning, modulation of movement pathways, and cognitive processes involving executive function. It is activated by rewarding, aversive, novel, unexpected, and intense stimuli. The SN is supplying the striatum with dopamine which is the main cause of PD symptoms (Lev et al., 2003, Grealish et al., 2010).
4.2 Experimental design and Exercise procedures

The experimental procedures in this chapter were as described in the methods chapter (see 2.2). Briefly, table 4.1 shows the experimental procedure in this chapter. Only the sacrifice procedures were different in order to suit western blot technique.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>intervention</th>
<th>Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>6</td>
<td>No MPTP induced No exercise</td>
<td>sacrificed immediately after 8 weeks</td>
</tr>
<tr>
<td>MPTP induced group(PD)</td>
<td>6</td>
<td>10 doses MPTP in 4 weeks(Total 40 mg/kg) No Exercise</td>
<td>sacrificed immediately after 8 weeks</td>
</tr>
<tr>
<td>MPTP induced + Exercise group (PDE)</td>
<td>6</td>
<td>10 doses of MPTP after 4 weeks of exercise (Total 40mg/kg) 4 weeks of exercise during the injections, 5 days a week, 45-60 min per session.</td>
<td>Sacrificed immediately after final bout of exercise</td>
</tr>
<tr>
<td>Exercise only group (E)</td>
<td>6</td>
<td>8 weeks of endurance exercise, 5 days a week, 45-60 min per session No MPTP</td>
<td>Sacrificed immediately after final bout of exercise</td>
</tr>
</tbody>
</table>

Table 4.1 description of the four treatments
4.2.1 Sacrificing procedures

- After 8 week of exercise training all rats were sacrificed. Procedures are described in Chapter Two (see 2.3.6). Brains were taken off and each brain was cut by surgical blade and forceps. Hippocampus and Striatum regions were isolated as following:

![Figure 4.1 Isolation of brain regions for western blot technique](image)

The two regions were quickly isolated on an ice-cold glass Petri dish. Samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.
24 Sprague Dawley Adult Rats

Control group (C)
- N=6
- No MPTP
- No Exercise

MPTP group (PD) group
- N=6
- MPTP induced (10) doses.
- No Exercise

MPTP+ Exercise group (PDE) group
- N=6
- Exercised for 4 weeks then MPTP induced + Exercise (4 weeks)

Exercise only group (E) group
- N=6
- No MPTP
- Exercise 8 weeks

MPTP injections after week 4 then another 4 weeks of exercise

Exercise

SACRIFICE THEN WESTERN BLOT

Figure 4.2 Experimental design and exercise procedures
4.3 STATISTICAL ANALYSIS

The results were expressed as means ± SEM. Differences among means were analyzed using one-way ANOVA, the different parts of brain and training groups were used as independent factors. When ANOVA showed significant differences, the group means were compared using LSD post hoc test. In all experiments, statistical analyses were performed with SPSS software (SPSS Inc).

4.4 RESULTS

After MPTP injection and 8 weeks of endurance exercise training for groups (PDE) and (E), total CaMK-1 and CaMK-4 protein levels were determined in the Striatum and the Hippocampus for the four treatments, Using polyclonal CaMK-1 and polyclonal CaMK-4 antibodies, one-way ANOVA analysis between groups has shown significant differences between treatments as following:

In the Striatum, CaMK-1 was decreased significantly in the PD treatment compared to the control group. In addition, there was significant increase in CaMK-1 expression in the PDE treatment when compared with PD treatment. (PDE) and (E) groups expressed higher expression of CaMK-1 after 8 weeks of exercise compared with PD group.
CaMK-1 in the hippocampus has not shown any significant differences between groups after 8 weeks of endurance exercise.

In CaMK-4 expression values in the striatum region in the four treatments rat brain, there was a significant decrease in CaMK-4 amounts in the PD group compared with the C group. Moreover, a significant increase in CaMK-4 amounts in the PDE group compared with the PD group. There was also significant increase in CaMK-4 amounts in the E group compared with the PD group. Finally, there were no differences between group E and Control group C.

CaMK-4 amounts in the hippocampus region have shown no significant differences between the four treatments after the exercise program. Expression of CaMK-4 was mean SD.
Figure 4.3 Effect of endurance exercise and MPTP treatment on CaMK-1 amounts in the Striatum. In the four treatments, the photographic insets show western blot analyses representative of four separate experiments. Protein amounts were normalized by the intensity of β-actin. ANOVA test has shown that CaMK-1 levels in PDE and E groups were increased (P < 0.05) compared to PD group. # = CaMK-1 levels were decreased (P < 0.05) in the PD group compared with the control group after 8 weeks of the experiment.
Figure 4.4 The effect of endurance exercise and MPTP treatment on CaMK-1 expression in the brain region Hippocampus in rat brain. The photographic insets show western blot analyses representative of four separate experiments. Protein levels were normalized by the intensity of β-actin, One-way ANOVA has not shown any significant differences, * = (P < 0.05), between the four groups in Hippocampus region after 8 week of the experiment.
Figure 4.5 The effect of endurance exercise and MPTP treatment on CaMK-4 expression in the striatum in rat brain. In the four treatments, the photographic insets show western blot analyses representative of four separate experiments. Protein levels were normalized by the intensity of β-actin. ANOVA test has shown that, * = CaMK-4 levels in PDE and E groups were increased (P < 0.05) compared to PD group. # = CaMK-4 levels were decreased (P < 0.05) compared to Control group after 8 week of the experiment.
Figure 4.6 The effect of endurance exercise and MPTP treatment on CaMK-1 expression in Hippocampus. The photographic insets show western blot analyses representative of four separate experiments. Protein levels were normalized by the intensity of β-actin. One-way ANOVA has not shown any significant differences (P < 0.05) between the four groups in hippocampus region after 8 weeks of the experiment.
4.5 RESULTS SUMMARY

After 8 weeks of endurance exercise for PDE and E groups, MPTP injection for PD and PDE groups, western blot analysis has shown the following:

- Higher expression of CaMK-1 in the striatum region in PDE animals compared to PD animals
- CaMK-1 level has decreased in the PD group compared to the C group
- No significant differences between the four treatments in the Hippocampus region in the level of CaMK-1
- Higher expression of CaMK-4 in the striatum region in PDE animals compared to PD animals
- Higher expression of CaMK-4 in the striatum region in E animals compared to C animals
- CaMK-4 level has decreased in the PD group compared to the C group
- No significant differences between the four treatments in the Hippocampus region in the level of CaMK-4

4.6 IMPLICATIONS OF RESULTS

The main implications of the findings in this chapter is that rat models with PD which had been treated with endurance exercise displayed higher levels of CaMK-1 and CaMK-4 in the brain Striatum rather than the same brain regions in the PD rat brain. These findings may provide
evidence about the involvement of CaMK-1 and CaMK-4 and the neuroprotective effect of exercise in the brain against PD toxins.

4.7 DISCUSSION

In results one, a comparison took place between the four experimental treatments using immunohistochemistry techniques. In results two, another quantitative analysis was carried out using western blot technique to evaluate the amount of CaMK-1 and CaMK-4 in the studied regions.

The main findings from results one and two have shown that rat models with PD which had been treated with endurance exercise for 8 weeks as described displayed higher amount of CaMK-1 and CaMK-4 proteins in the SN more than in the same regions in the Parkinsonian rat brain group. In addition, it has been noticed that the localization of both CaMK-1 and CaMK-4 antibodies is higher in control group than the PD and PDE groups.

These findings agree with Cechetti et al. (2008) who demonstrated that daily moderate intensity exercise, 2 weeks of 20 min/day of training reduces damage in hippocampal slices from Wistar rats submitted to in vitro ischemia. Exercise demonstrates neuroprotective effects on improving the survival of DA cells in the SN In both the 6-hydroxyDA (6-OHDA) lesioned rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned mouse (Smith and Zigmond, 2003, Dishman et al., 2006).
These results combined with previous work indicate: (1) CaMKs regulates various functions in the brain findings suggest that Ca\(^{2+}\) activates DA synthesis in addition to the CaMKs-dependent system, and even through CaMK in specific brain regions. These results highlight the important role of intracellular Ca\(^{2+}\) in the neuronal death and survival mechanisms. Rapid and severe elevation of intracellular Ca\(^{2+}\) concentration is reported to induce neuronal cell death in both mature and established neurons, whereas homeostasis and moderate elevation of Ca\(^{2+}\) concentration supports neuronal survival in some immature and developing neurons (Koike et al., 1989). The Ca\(^{2+}\)/calmodulin dependent protein kinases (CaM-kinase) family which contains CaM-kinases I, II, IV, and CaM-kinase kinase (CaM-KK) are the major proteins regulated by intracellular Ca\(^{2+}\)concentration (figure 4.7). CaM-kinase II is directly activated by Ca\(^{2+}\)/calmodulin, whereas CaMK-1 and CaMK-4 are regulated by CaMKK (Yano et al., 2005). In previous investigations Sutoo and Akiyama (2003) confirmed that the activity of CaMK-2 was decreased after MPTP injection in rat CA1 cells. In this study, we documented the role of CaMK-1 and CaMK-4 the Ca\(^{2+}\) regulated neuronal survival in MPTP induced rat brain and localized the distribution of both kinases after 8 weeks of endurance exercise.
Figure 4.7 A working model for the action of Exercise and its neuroprotection effect against MPTP, we are proposing that Ca\(^{2+}\) activates Ca\(^{2+}\)/Cam which in turn regulates CaMK-1 and CaMK-4 through the suppression of CaMK-K which activates MEK/ERK and exert its neuroprotecticton effect.
Restored Ca\(^{2+}\) is the bones act as a reservoir of Ca\(^{2+}\) where it is stored in combination with phosphate. When exercise occurs, a larger amount of Ca\(^{2+}\) is needed by the muscles to achieve the required concentration (Mcardle, 2006). This then produces a fall in the levels of Ca\(^{2+}\) in blood. As a physiological response, the parathyroid glands of the body secrete parathyroid hormone (PTH) which activates the bone cells. These cells start then to recycle producing a release of stored Ca\(^{2+}\) ions in the bone. Finally, this produces an increase of Ca\(^{2+}\) levels in the bloodstream (Merrill et al., 2005). The increase of Ca\(^{2+}\) levels will also arrive to the neuronal cells of the brain. As more Ca\(^{2+}\) arises at the neuronal cells, it might be expected that this will be lead to higher levels of the Ca\(^{2+}\) binding protein. However, Ca\(^{2+}\) is an ion that needs to have a strong homeostasis regulation as small variations (above or below normal levels) can cause serious physiological problems (Akiyama et al., 1990, Merrill et al., 2005).

Microscopy photos agreed with the previous findings that both CaMK-1 and CaMK-4 regulated by CaMKK, and mainly localized in the cytoplasm (which probably refer to the Y isoform) can associate as a lipid modified with Glogi and plasma membranes. Waymanet et al. (2006) demonstrated the link between the Y isoform and RAS/ERK pathway. To understand the complex network with different pathways, this crosstalk can occur at several levels—there can be direct phosphorylation between protein kinases or regulatory phosphorylation of either an upstream or a downstream effect of another kinase. There have been several explanations and communication mechanisms between CaMKs and brain function. In this study, the most important communications between the CaMK cascade and the Mek/Erk pathway. Schmitt et al. (2004) demonstrated that elevated intracellular Ca\(^{2+}\) triggers numerous signalling pathways including protein kinases such as the calmodulin-dependent kinases (CaMKs) and the
extracellular signal-regulated kinases (ERKs) upon depolarization of the neuroblastoma cell line (Schmitt et al., 2004).

Smith and Zigmond (2003) demonstrated further evidence on the role of ERK in neurosurvival, volunteer exercise increased neurotrophic factor such as GDNF and BDNF which protect DA neurons from lesions of 6-OHDA. It has been suggested that activation of ERK contributes to basal DA cell survival. Although activation of the ERK5 pathway also provided some neuroprotection from acute 6-OHDA toxicity in MN9D cells (Smith and Zigmond, 2003). Other studies are being conducted to explore the effect of the ERK pathway neuronal survival following chronic exposure to a neurotoxin. ERK were implicated in the protection of cells from injury in several model systems (Engedal and Blomhoff, 2003).

Our immunohistochemical findings show higher expression of CaMK-1 and CaMK-4 in the striatum, side to side with the hypothesis that ERK kinases important for DA neuronal survival might provide an explanation on the neuroprotective effect of CaMKs. It is possible that these pathways activate common targets in DA neuronal cells to promote cell survival, and maximal activation of these transcription factors may require both ERK pathways. Knowledge of these signaling mechanisms in DA neurons should greatly aid in the development of new therapeutic approaches for PD. Results from this study might provide new information on the CaMKs/ERK signaling pathways that play a role in the neuroprotection and basal survival of DA neurons.
CHAPTER FIVE: RESULTS THREE

EFFECTS OF 6-HYDROXYDA ON SH-SY5Y CULTURES: SPECIFIC DAMAGE TO DA NEURONS AND THE IMPACT OF CA\textsuperscript{2+}

5.1 INTRODUCTION

Parkinson’s disease is characterized by the progressive degeneration of dopamine (DA) neurons in the substantia nigra (SN). Although the disease appears to involve cell loss in multiple brain, protecting DA neurons alone would significantly ameliorate many of the symptoms areas (Zigmond et al., 2009). Cells can often be protected against insults through prior exposure to sub-lethal stress, a phenomenon referred to as ‘preconditioning’ or ‘tolerance’. Beginning with studies of rapid preconditioning in cardiac tissue Murray et al. (1986), and delayed preconditioning in brain (Kitagawa et al. 1990), the majority of evidence for the phenomenon comes from animal models (Cotman and Berchtold, 2002, Lev et al., 2003).

Ca\textsuperscript{2+} regulates the activation of the Ca\textsuperscript{2+} calmodulin Kinase family (CaMKs), neuronal activity induces a moderate increase in intracellular Ca\textsuperscript{2+} concentration, which promotes cellular growth, differentiation, and survival effect on different neuronal populations (Koike et al., 1989). Several intracellular pathways that are activated by Ca\textsuperscript{2+} mediate this biological effect, and the protein (CaM) mainly mediates this activation (Krebs, 1998). Together these observations indicate a significant role of Ca\textsuperscript{2+} and CaM in the regulation and activation of intra-cellular
pathways related to neuronal survival it also mediate responses of many agonists such as hormones, growth factors, and neurotransmitters (Hudmon and Schulman, 2002).

In the previous experimental chapters, there were histochemical and quantitative evidences that CaMK-1 and CaMK-4 have increased in the striatum region in PD rat brain after 8 weeks of endurance exercise. However, those in vivo findings require in vitro verification in order to examine the hypothesis of the Ca$^{2+}$/Calmodulin Dopamine pathway in in vitro model. In vitro studies have therefore been required for initial proof-of-principle demonstrations, they permit detailed mechanistic studies at a fast pace and at far less cost than in the whole animal.

In this chapter, in vitro study was designed to test the hypothesis generated from the in vivo work in studies one and two, to show that Ca$^{2+}$ is a major effector of the neuroprotective effect of exercise via Ca$^{2+}$/calmodulin DA pathway. It is important to investigate whether Ca$^{2+}$ would exert a protective effect in a cell culture model of delayed and progressive nigral cell death more closely related to the cell death seen in human PD. The aim of this chapter is, therefore, to investigate the in vitro behaviour of human neuroblastoma cells SH-SY5Y after exposure to different doses of PD induced 6-OHDA. Then the effect of adding Ca$^{2+}$ as an “exercise mimic” to the culture, and investigate whether it will enhance the neuroprotection ability of SH-SY5Y culture against DA damaged caused by 6-OHDA.

Unlike the MPTP injection used to produce PD symptoms in the in vivo model in studies one and two, 6-hydroxydopamine (6-OHDA) has been used to damage DA neurons in vitro instead of MPTP, which refers to the high risk precautions of using MPTP in the cell culture
laboratory while other cell experiments took place. However, using 6-OHDA as an alternative to MPTP refers to previous evidence that MTPT and 6-OHDA represent equivalent cellular insults (Blum et al., 2001, Ding et al., 2004).

Therefore, 6-OHDA has been chosen as a safer alternative with the same effect of MPTP of killing DA neurons selectively, thereby producing a cell culture model of certain key aspects of PD with fewer opportunities for side effects on other research projects in the cell culture laboratory.

5.2 EXPERIMENTAL DESIGN

Exponential growing human neuroblastoma cell line, obtained from Health Protection Agency Culture Collections UK (HPACC), was used in this study as DA contains neurons cells. This cell line has been widely used in PD research as these cells exhibit moderate levels of dopamine (Shavali and Sens, 2008). SH-SY5Y cells were cultured, fixed, stained, cryoprotected and counted as described previously in Chapter Two (see 2.5.1 and 2.5.3).

The first step in this study was to determine the lethal dose of 6-OHDA to kill the specific percentage of PD neurons in the SH-SY5Y culture to mimic DA condition in the human PD brain, as mentioned before; symptoms of PD do not appear unless 75-80 % of DA is dead. The lethal Dose test (LD-50) was performed to determine the lethal dose of 6-OHDA on SH-SY5Y cells. In (LD-50), the effect of 6-OHDA was determined by exposing SH-SY5Y cells to
different concentrations (40 µM, 60 µM, 100 µM, 200 µM, 400 µM) of 6-OHDA induced in a 6-well plate containing SH-SY5Y culture in the 5th day after differentiation. 6-OHDA was induced in culture for 24 h. Then the cells were counted using light microscopy and photos for the whole field were taken. Cell-counting process took place using Image J cell counter software.

Initially, different doses of calcium were calculated according to calcium homeostasis in the human body, then there were converted to the volume of the 96 well plate for 24h before expose to 6-OHDA for further 24h. Figure 5.1 summarizes the experimental design.
After 7 days in culture, the cells were seeded into 96-well plates at $1 \times 10^4$ viable cells per well and left to attach to the plates for 24 h. After 24h cultures were washed three times with
PBS then 100 µ fresh medium (serum free) was added to the culture. Pre-calculated doses of Ca\textsuperscript{2+} were dissolved in the serum free medium and added to the culture then left for 24 h. The total volume was 200 µl. Then, a predetermined amount of 6-OHDA was added to the cultures. After another 24 h, the medium was removed and cultures were washed gently with PBS. Ca\textsuperscript{2+} dosage was calculated according to their homeostasis levels in the human body. Doses of Ca\textsuperscript{2+} were determined according to the homeostasis level in the human body (Mutch and Banister, 1983; Clarkson and Haymes, 1995).

Image J (1.43) software was used to determine the number of living cells in a multistep process. 6-OHDA in different concentrations was used to induce apoptosis in cultured SH-SY5Y cells. Visual morphology analysis of SH-SY5Y cells was used to discriminate apoptotic cells and cells suspected to be undergoing apoptosis from control cells based on parameters such as nuclear area, circularity and perimeter and nuclear area factor. Cell count and cell image analysis were reviewed with two independent observers.
<table>
<thead>
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<th>Column (1)</th>
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<tr>
<td>(A) Ca(^{2+}): 0 mM 6-OHDA: 0 µM</td>
<td>Ca(^{2+}): 0.7 mM 6-OHDA: 0 µM</td>
<td>Ca(^{2+}): 1.4 mM 6-OHDA: 0 µM</td>
<td>Ca(^{2+}): 2.1 mM 6-OHDA: 0 µM</td>
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<td>(B) Ca(^{2+}): 0 mM 6-OHDA: 20 µM</td>
<td>Ca(^{2+}): 0.7 mM 6-OHDA: 20 µM</td>
<td>Ca(^{2+}): 1.4 mM 6-OHDA: 20 µM</td>
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<td>(C) Ca(^{2+}): 0 mM 6-OHDA: 40 µM</td>
<td>Ca(^{2+}): 0.7 mM 6-OHDA: 40 µM</td>
<td>Ca(^{2+}): 1.4 mM 6-OHDA: 40 µM</td>
<td>Ca(^{2+}): 2.1 mM 6-OHDA: 40 µM</td>
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<tr>
<td>(D) Ca(^{2+}): 0 mM 6-OHDA: 60 µM</td>
<td>Ca(^{2+}): 0.7 mM 6-OHDA: 60 µM</td>
<td>Ca(^{2+}): 1.4 mM 6-OHDA: 60 µM</td>
<td>Ca(^{2+}): 2.1 mM 6-OHDA: 60 µM</td>
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<tr>
<td>(E) Ca(^{2+}): 0 mM 6-OHDA: 80 µM</td>
<td>Ca(^{2+}): 0.7 mM 6-OHDA: 80 µM</td>
<td>Ca(^{2+}): 1.4 mM 6-OHDA: 80 µM</td>
<td>Ca(^{2+}): 2.1 mM 6-OHDA: 80 µM</td>
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<tr>
<td>(F) Ca(^{2+}): 0 mM 6-OHDA: 100 µM</td>
<td>Ca(^{2+}): 0.7 mM 6-OHDA: 100 µM</td>
<td>Ca(^{2+}): 1.4 mM 6-OHDA: 100 µM</td>
<td>Ca(^{2+}): 2.1 mM 6-OHDA: 100 µM</td>
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Table 5.1 Experimental design for cultures exposure for Ca\(^{2+}\) and 6-OHDA. In 96 - well plate, total volume of solution in each well was 200 µl. For example: (A1) well was exposed to 0 mM Ca\(^{2+}\) and 0 mM 6-OHDA. (A2) exposed to 0.7 mM Ca\(^{2+}\) and 0µM 6-OHDA. 10.000 cells were loaded in each well. Exposure time for both Ca\(^{2+}\) and 6-OHDA was 24 h.
5.3 RESULTS

5.3.1 Lethal dose test (LD-50)

LD-50 showed no obvious dead cells in the control treatment (Figure 5.2A). However, percentage of dead cells was 35% when the culture was exposed to 40 µM for 24 h figure (5.2B), that percentage increased to 50% when the 6-OHDA dose was increased to 60 µM (Figure 5.3C). A 100 µM dose increased the number of dead cells to 85% of the total number of cells (Figure 5.3D), a 200 µM dose caused 88% of cell death (Figure 5.3 E). And finally a 400 µM dose of 6-OHDA killed 100% of the total number of SH-SY5Y cells (Figure 5.4). According to the pathological features of PD, PD symptoms appear after the degeneration of 85% of DA cells. LD-50 test determined that 100 µM dose of 6-OHDA causes the degeneration of 85% of DA neurons in SH-SY5Y cells in 24 h time, therefore, 100 µM dose was realistic PD induced dosage in SH-SY5Y cells.
Figure 5.2 Cell morphology images after exposure to 6-OHDA. Image (A) shows SH-SY5Y cells after 7 days in culture, culture left to grow undisturbed in DMEM media 20% FCS. Image (B) shows SH-SY5Y culture after exposure to 40 µM 6-OHDA for 24 h in day 6. 6-OHDA were added to the growth media. Comparison between images has been conducted via Image J (1.43) cell counter software using two independent observers. Black arrows point to number of living cells which have been counted.
Figure 5.3 Cell morphology images after exposure to 6-OHDA. Microscopy image (C) shows SH-SY5Y culture after 7 days of culture and 24 h of exposure to 60 µM of 6-OHDA. Image (D) shows the same culture after the same period (7 days in culture and 24 h of 6-OHDA) but with 100 µM dose of 6-OHDA. Comparison between images has been conducted via Image J (1.43) cell counter software using two independent observers. Black arrows point to number of living cells which have been counted.
Figure 5.4 Cell morphology images after exposure to 6-OHDA. Microscopy image (E) shows SH-SY5Y cells after 6 days of culture and 24 h of exposure to 200 µM of 6-OHDA. Image (F) shows the same culture after the same time frame (7 days in culture and 24 h of 6-OHDA) but with 400 µM 6-OHDA diluted in the growth media DMEM. Comparison between images has been conducted via Image J (1.43) cell counter software using two independent observers. Black arrows point to number of living cells which have been counted.
Figure 5.5 Percentage of apoptosis cells in LD-50 test. SH-SY5Y cells after 24 hour exposure to different doses of 6-OHDA. No dead cells were noticed in control treatment. However, percentage of dead cells was 35% when the cultured exposed to 40 µM for 24 h. that percentage increased up to 50% when the 6-OHDA dose increased to 60 µM. while 100 µM dose increased number of dead cells to 85% of the total number of cells, 200 µM has caused 88% cell death and finally a 400 µM dose of 6-OHDA killed 100% of the total number of SH-SY5Y cells. According to the pathological features of PD, PD symptoms appear after the degeneration of 85% of DA cells. LD-50 test showed that a 100 µM dose of 6-OHDA caused the degeneration of 85% of DA neurons in SH-SY5Y cells, therefore 100 µM dose is a realistic PD induced dosage.
5.3.2 SH-SY5Y exposure for Ca\(^{2+}\) and 6-OHDA

In a 96-well plate, 24 cultures were exposed to different doses of Ca\(^{2+}\) and 6-OHDA (Table 5.1). Briefly, Ca\(^{2+}\) doses were dissolved in the serum free medium, this was added to cultures and left for 24 h. Then, freshly made doses of 6-OHDA were added to the cultures. After another 24 h, medium removed and cultures were washed gently with PBS. The final step was cell viability using MTS assay kits. One-way ANOVA confirmed the effect of treatments in some cultures (P < 0.001). The highest viability was seen for A1 culture (0.0 mM Ca\(^{2+}\), 0 µM 6-OHDA), F3 (1.4 mM Ca\(^{2+}\), 1 µM 6-OHDA) culture and F4 culture (2.1 mM Ca\(^{2+}\), 1 µM 6-OHDA) (Figure 5.7). These cultures were increased significantly – confirmed by Tukey’s pairwise comparison of the means (Table 5.3).
Figure 5.6 Results of Tukey’s pairwise comparison of the means on cell viability after exposure to (0.0, 0.7, 1.4 and 2.4 mM) of Ca\(^{2+}\) and 0.0 µM 6-OHDA. The cultures were analysed by cell viability assay kits after exposure to Ca\(^{2+}\) as an “exercise mimic”. Doses of Ca\(^{2+}\) were dissolved in the serum free medium and left for 24 h. Then the medium was removed and cultures were washed gently with PBS. The final step was to count cells using MTS assay kits. Error bars represent standard error of the means. No significant differences were demonstrated among conditions.
Figure 5.7 Results of Tukey’s pairwise comparison of the means on cell viability after exposure to (0.0, 0.7, 1.4 and 2.4 mM) of Ca$^{2+}$ and (6-OHDA). Cultures were analysed by cell viability assay kits (CellTiter) after exposure to Ca$^{2+}$ as an “exercise mimic” and 6-OHDA. Doses of Ca$^{2+}$ were dissolved in the serum-free medium and were left in the medium for 24 h. Then, 20 µM of 6-OHDA was added to the cultures. After another 24 h, the medium was removed and cultures were washed gently with PBS. The final step was to count cells using MTS assay kits. Error bars represent the standard error of the means. No significant differences were demonstrated among the conditions.
Figure 5.8 Results of Tukey’s pairwise comparison of the means on cell viability after exposure to (0.0, 0.7, 1.4 and 2.4 mM) of Ca\textsuperscript{2+} and 40 µM (6-OHDA). Cultures were analysed by cell viability assay kits (CellTiter) after exposure to Ca\textsuperscript{2+} as an “exercise mimic” and 6-OHDA. Doses of Ca\textsuperscript{2+} were dissolved in the serum-free medium and were left in the medium for 24 h. Then, 40 µM of 6-OHDA was added to the cultures. After another 24 h, the medium was removed and the cultures were washed gently with PBS. The final step was to count the cells using MTS assay kits. Error bars represent the standard error of the means. No significant differences were demonstrated among the conditions.
Figure 5.9 Results of Tukey’s pairwise comparison of the means on cell viability after exposure to (0.0, 0.7, 1.4 and 2.4 mM) of Ca$^{2+}$ and 60 µM (6-OHDA). Cultures were analysed by cell viability assay kits (CellTiter) after exposure to Ca$^{2+}$ as “exercise mimic” and 6-OHDA. Doses of Ca$^{2+}$ were dissolved in the serum free medium and were left in the medium for 24 h. Then, 60 µM of 6-OHDA was added to the cultures. After another 24 h, the medium was removed and the cultures were washed gently with PBS. The final step was to count the cells using MTS assay kits. Error bars represent the standard error of the means. The figure shows that cell viability was increased significantly when the cultures were treated with 1.4 and 2.1 mM of Ca$^{2+}$ for 24 h before exposure to 6-OHDA compared with the cultures exposed to only 0.0 and 0.7 mM Ca$^{2+}$. * Indicates a significant difference between trials (p < 0.05).
Figure 5.10 Results of Tukey’s pairwise comparison of the means on cell viability after exposure to (0.0, 0.7, 1.4 and 2.4 mM) of Ca\(^{2+}\) 80 µM (6-OHDA). Cultures were analysed by cell viability assay kits (CellTiter) after exposure to Ca\(^{2+}\) as an “exercise mimic” and 6-OHDA. Doses of Ca\(^{2+}\) were dissolved in the serum-free medium and were left in the medium for 24 h. Then, 80 µM of 6-OHDA was added to the cultures. After another 24 h, the medium was removed and the cultures were washed gently with PBS. The final step was to count cells using MTS assay kits. Error bars represent the standard error of the means. The figure shows that cell viability was increased significantly when the cultures were treated with 1.4 and 2.1 mM of Ca\(^{2+}\) for 24 h before exposure to 6-OHDA compared with the cultures exposed to only 0.0 and 0.7 mM Ca\(^{2+}\). * Indicates a significant difference between trials (p < 0.05).
Figure 5.11 Results of Tukey’s pairwise comparison of the means on cell viability after exposure to (0.0, 0.7, 1.4 and 2.4 mM) of Ca\(^{2+}\) and 100 µM (6-OHDA). Cultures were analysed by cell viability assay kits (CellTiter) after exposure to Ca\(^{2+}\) as an “exercise mimic” and 6-OHDA. Doses of Ca\(^{2+}\) were dissolved in the serum-free medium and were left in the medium for 24 h. Then, 100 µM of 6-OHDA was added to the cultures. After another 24 h, the medium was removed and the cultures were washed gently with PBS. The final step was to count cells using MTS assay kits. Error bars represent the standard error of the means. The figure shows that cell viability was increased significantly when the cultures were treated with 1.4 and 2.1 mM of Ca\(^{2+}\) for 24 h before exposure to 6-OHDA compared with the cultures exposed to only 0.0 and 0.7 mM Ca\(^{2+}\). * Indicates a significant difference between trials (p < 0.05).
5.4 IMPLICATIONS OF RESULTS

The significant increase in cell viability in cultures with high doses of Ca\(^{2+}\) after exposure to lethal doses of 6-OHDA may provide evidence of the neuroprotective role of Ca\(^{2+}\) in the brain against PD toxins. The results from Chapter Five have clearly demonstrated Ca\(^{2+}\) activity in cultures of human neuroplastoma cells. Moreover, this expression was found to be inhibit the effect of 6-OHDA in the cultures. This action protects the cells from apoptosis and, therefore, delays PD symptoms. If, as suggested here, Ca\(^{2+}\) has a functional role in the neuroprotection process against PD, \textit{in vitro}, this has potential implications during development of non-pharmacological therapies of PD. Furthermore, there is a possibility that the adaptive capacity of plasticity of DA neurons, could be related to the roles of physical exercise and Ca\(^{2+}\), and this requires investigation. The focus of the next chapter was, therefore, to establish if these changes in total blood calcium after exercise, were present in human subjects and the effect of exercise in Ca\(^{2+}\) concentration level, ADL and moving ability.

5.5 DISCUSSION

5.5.1 Effects of 6-OHDA on SH-SY5Y cultures: specific damage to DA neurons and the impact of Ca\(^{2+}\)

The loss of striatal DA accounts for most of the symptoms in PD, and treatment with L-3,4-dihydroxyphenylalanine (L-DOPA), the immediate precursor of DA, improves some symptoms
in PD (Kandel et al., 1991). Therefore, some symptoms of PD might be rectified by exercise through increased calcium/CaM dependent DA synthesis. Studies one and two in this thesis have examined the effect of exercise on amounts of CaMK-1 and CaMK-4 and its involvement with neurologic disorders in PD, and the experimental findings support this hypothesis. In mild to moderate PD rat models, various exercises significantly improved motor performance and activities of daily living (Dobrossy and Dunnett, 2003, Cechetti et al., 2008, Gerecke et al., 2010).

DA neuron loss is the pathological feature of PD. The aim of this study was to investigate whether Ca$^{2+}$ exerted protective effects on in vitro cellular PD models induced with 6-OHDA. Ca$^{2+}$, as it is responsible for muscle stimulation, transmission of action potential in neurons, was used here as an “exercise mimic”, since it had been shown to achieve neuroprotective effect in the models of PD in vivo (chapters Three and Four).

The present results show that (1.4 mM and 2.1 mM) doses of Ca$^{2+}$ protect against 6-OHDA induced damage in cultured SH-SY5Y cells, a cell line used as a model for DA neurons (Lev et al., 2003, Lee et al., 2007, Riveles et al., 2008). Protection against toxicity was obtained with Ca$^{2+}$ at doses (1.4, 2.1 mM) above those already presented in the human body. The normal concentration of Ca$^{2+}$ homeostasis is (2.1-2.5 mmol/L) (Koike et al., 1989, Clarkson and Haymes, 1995). These combined findings suggest that beneficial effects against 6-OHDA-induced toxicity may be attributed, at least in part, to the presence of Ca$^{2+}$ in the DMEM solution. The finding that Ca$^{2+}$ is a component in DMEM that may be involved in protection against toxic insults is consistent with previous work in numerous experimental models with
different components. For example, exposure of primary mesencephalic cultures to $10^{-7}$ and $10^{-6}$ M nicotine partially protected against DA neuron toxicity induced by MPTP (Riveles et al., 2008). Some experimental studies reported that estrogen improves the survival rates of PC12 cells during MPP$^+$ induced cell injury. Estrogen treatment leads to elevated density in the TH positive cells and outgrowth of neurites. It also brings the density in the MPP$^+$ treated cells back to the control level (Cotman and Berchtold, 2002). Following the same line of investigation, Zigmond et al. (2003) demonstrated that Glial cell line-derived neurotrophic factor (GDNF) treatment increased TH expression after 6-OHDA exposure to DA cell lines. SH-SY5Y cells were selected for the present studies because they are derived from human neuroblastoma cells, are of CNS origin and have a catecholaminergic phenotype (Lee et al., 2007, Shavali and Sens, 2008, Zhao et al., 2008).

Previous studies have shown that differentiated cells express TH, the dopamine transporter, a dopamine uptake system, dopamine receptors, as well as other catecholaminergic markers. In addition, they are susceptible to the toxic effects of DA neurotoxins that damage the nigrostriatal pathway, a major site of pathology in PD (Smith and Zigmond, 2003, Zhao et al., 2008). However, the expression of Ca$^{2+}$ has not been investigated before in SH-SY5Y cells. Therefore, this may have been due to the fact of the lack of knowledge regarding calcium-calmodulin dopamine and PD symptoms.

Catecholaminergic SH-SY5Y cells were exposed to different doses of Ca$^{2+}$ as an “exercise mimic” before being exposed to different doses of 6-OHDA. The addition of Ca$^{2+}$ significantly increased SH-SY5Y cell viability in a concentration-dependent manner. 6-OHDA
induced cellular damage was also observed by estimating the number of survived cells using (LD-50) test. (LD-50) test demonstrated that 100 μM of 6-OHDA caused 80% loss in SH-SY5Y culture. These data corresponded to the morphological changes of SH-SY5Y cells after being exposed to 6-OHDA reported previously by (Blum et al., 2001, Riveles et al., 2008).

In 96-well plates, SH-SY5Y cultures were treated with Ca\(^{2+}\) and 6-OHDA in two steps. The first step involved exposure to Ca\(^{2+}\) doses for 24 h then exposed to 6-OHDA doses for another 24 h. The experiment composed of 24 different treatments (Figure 5.1). As described in chapter five, a number of cultures showed significant increase in cell viability in the following treatments, cultures that exposed to (1.4 and 2.1 mM) doses of Ca\(^{2+}\) in comparison with culture exposed to (0.0 and 0.7 mM) doses of Ca\(^{2+}\). These results occurred when 6-OHDA doses were (0.8 and 1 μM). In contrast, cultures exposed to no or low doses of Ca\(^{2+}\) (0.0 mM and 1.4 mM) and then exposed to (0.4, 0.6, 0.8 and 1 μM 6-OHDA) reported the lowest viability of cells. Cultures exposed to (0.2 and 0.4 μM) 6-OHDA doses did not report significant differences in cell viability despite exposure to different doses of Ca\(^{2+}\) compared with the cultures with no treatment. No significant differences found between cultures exposed to (0.7, 1.4 and 2.1 μM) Ca\(^{2+}\) without 6-OHDA and cultures were treated with (0.7, 1.4 and 2.1 μM) Ca\(^{2+}\) then exposed to (0.8 and 1 μM) 6-OHDA. This indicates that the effect of Ca\(^{2+}\) as an “exercise mimic” can protect SH-SY5Y cells against 6-OHDA-induced damage in cultures.

These results show that when Ca\(^{2+}\) doses were (1.4 mM) or above, cultures presented high resistance against 6-OHDA induced damage in cultured SH-SY5Y cells. There were no significant differences between cultures which were exposed to high doses of Ca\(^{2+}\) without
expose to 6-OHDA. And cultures which exposed to high doses of Ca\textsuperscript{2+} then high doses of 6-OHDA, might indicate that the presence of Ca\textsuperscript{2+} in cultures for 24 h before 6-OHDA provided SH-SY5Y cells with intracellular resistance against 6-OHDA. In contrast, cultures which have not been exposed to Ca\textsuperscript{2+} in normal or high doses, do not present any significant resistance to the toxin and as a sequence, were significantly decreased in cell viability.

It is interesting to note that protection against toxicity was obtained when Ca\textsuperscript{2+} doses were similar to those present in the homeostasis Ca\textsuperscript{2+} level in the blood Ca\textsuperscript{2+} after exercise (1.4 and 2.1 mM) (Maimoun and Sultan, 2009). These combined findings suggest that beneficial effects against 6-OHDA-induced toxicity may be attributed. The finding that Ca\textsuperscript{2+} used as a culture supplement may be involved in protection against toxic insults is consistent with previous work with in viv\textit{o} models.

Based on the data above, the role of Ca\textsuperscript{2+} could be clearer when we explain the link between Ca\textsuperscript{2+} and NMDA receptor in brain function. In studies one and two, results have suggest that CaMK-1 and CaMK-4 expressions were higher after 8 weeks of endurance exercise. Therefore, it is hypothesized that CaMK-1 and-4 are activated via the Ca\textsuperscript{2+}calmodulin synthesis pathway. Ca\textsuperscript{2+}/calmodulin-dependent kinases (CaMKS) are a family of ubiquitous Ca\textsuperscript{2+} binding proteins with broad substrate specificity that CaMKS at central to the mechanism of hippocampal NMDA receptor-dependent and long term potentiation (LTP) (Colbran, 1992, Anderson and Kane, 1998, Hook and Means, 2001).
At the molecular level, it has become increasingly evident that the NMDA receptor complex is a dynamic structure that is intimately involved in the regulation of corticostriatal long-term potentiation (LTP) which is altered in experimental parkinsonism (Tokuda et al., 1997). Reduction of CaMKs activity by pharmacological or genetic means impairs LTP, whereas injecting or overexpressing CaMKs increases synaptic strength, which undergoes rapid autophosphorylation following NMDA receptor-mediated Ca\(^{2+}\) influx at a specific residue in its autoregulatory domain (Thr286 in the Alfa isoform of CaMK-1 and CaMK-4). This autophosphorylation renders the kinase Ca\(^{2+}\) independent and has been proposed as a form of molecular memory (Merrill et al., 2005).

The results from this study suggest that Ca\(^{2+}\) can protect SH-SY5Y cells against 6-OHDA-induced cell damage and is worth further research to determine its therapeutic potential.

### 5.6 STUDY LIMITATIONS

In this study, the aim of the experimental procedures was to add different concentrations of Ca\(^{2+}\) to DMEM medium after different doses of 6-OHDA. However, the normal level of calcium in the SH SY-5Y neuroplastoma cells was isolated as the cells were from the same type, source and differentiation number. Therefore, the focus of this chapter is to calculate the number of living cells after the extra doses of Ca\(^{2+}\), which should be mentioned as a limitation in this study.
CHAPTER SIX: RESULTS FOUR

EFFECT OF SUBMAXIMAL EXERCISE ON BALANCE, GAIT AND BLOOD CALCIUM CONCENTRATION IN PATIENTS WITH PD

6.1 INTRODUCTION

The benefits of exercise to the brain and its cognitive functions are voluminous, and have been thoroughly reviewed by PD researchers. In PD patients, recent controlled trials have shown that exercise could in fact help to slow disease progression both directly and indirectly (Baatile et al., 2000). It is postulated that exercise could help to protect DA cells from the toxic effect of MPTP or other PD toxics via exercise calcium calmodulin production in the brain (Sutoo and Akiyama, 2003, Petzinger et al., 2007, Gerecke et al., 2010).

With the progression of PD, a significant proportion (50-70 %) of the cells in the substantia nigra (SN) has already been destroyed. This degeneration progresses until, within a few years, most of the cells have died. It is speculated that with exercise, this may also help to protect and activate the other 20% of the DA cells for PD (Smith and Zigmond, 2003, Poulton and Muir, 2005, Yoon et al., 2007).

In PD patients, exercise could also alter the course of the disease (Chen, 2010). Exercise could help to improve postural instability, balance (Cakit et al., 2007, Canning et al., 2009), and tremor (Falvo et al., 2008). It could also help to overcome musculoskeletal deficiencies of
reactive oxygen and nitrogen species in PD patients (Inkster et al., 2003). Exercise could also possibly alleviate depression or negative mood often associated with PD (Weintraub et al., 2008c). It may also maintain cerebral activity in the retirees thereby preventing cognitive decline (Ouchi et al., 2002). It is also possible that exercise induced growth hormone changes may play a role in promoting neuroregeneration by increasing neural stem cells (Arsenijevic and Weiss, 1998).

Pertinent to studies 1, 2 and 3 of this thesis, there is some evidences from animal and cell culture PD models that exercise could help to alter the biochemical status in the brain under certain circumstances. Collectively, this could well alleviate symptoms or even delay the progression of PD. However, even with all this scientific knowledge, there is still no agreement concerning the optimal exercise intensity and prescription strategy for patients suffering from PD. Furthermore, there is still no standardization for optimal outcomes, such as duration of exercise, intensity, point of intervention and whether exercise could improve motor symptoms of the disease after onset.

Patients with PD (especially in late stages) experience serious difficulties while performing the activity of daily living (ADL), which affects their quality of life. Meanwhile, individuals with early and moderate stages of PD face less serious symptoms, which do not prevent them from participating in physical exercise activities. Therefore, over the past 15 years, there has been a considerable increase in research investigating the benefits of exercise and physical therapy in individuals with PD to improve quality of life (Haas et al., 2004). In general, exercise programs and physical therapy have been shown to be effective in improving strength,
balance, range of motions and various functional measures (Haas et al., 2004, Poulton and Muir, 2005, Fisher et al., 2008). However, the term “Exercise” was not consistently defined in a number of these reports. In other words, it is still not clear what exercise level can be of benefit to PD patients, and what is the exact intensity at which exercise could be beneficial to delay PD progress.

Results from studies one, two and three suggest exercise to be beneficial in alleviating symptoms of PD through the Ca\(^{2+}\) Calmodulin DA pathway. In this chapter, in order to begin to assess the importance of this data to a clinical population and to further develop the concept that Ca\(^{2+}\) is a major effector of the positive effect of exercise, the effect of moderate exercise on blood Ca\(^{2+}\) concentration in subjects with PD is further investigated. We hypothesize that exercise benefits for PD patients may be manifest through the exercise-induced production of Ca\(^{2+}\) and its transfer to the brain.

This study aims to assess the effect of endurance exercise on (ADL) and changes in blood Ca\(^{2+}\) concentration with patients with PD to investigate whether it has a positive effect in reducing symptoms of PD.
6.2 PARTICIPANTS

Five male patients (mean age ± SD: 66.6 ± 3.97) with idiopathic PD volunteered from the PD society (Bedford and District Branch, UK). Table (6.1) shows the general characteristics of the patients. Participants were recruited through two advertising presentations at the society’s monthly meetings, followed by question and answer session to discuss aims and the potential benefits of the project. All participants were required to sign informed consent documents approved by the ethical committee of the University Of Bedfordshire, UK.

Initially, the study sample size was six, but one participant was withdrawn after he mentioned a cardiac history incident in his medical history questionnaire. During the recruitment presentation, all participants were encouraged to ask any questions regarding the study. After the question and answer session, participants were aware of the testing procedures.
Table 6.1 Participants’ characteristics. All subjects were clinically stable (without fluctuations in response to Levodopa or dyskinesias) and classified in Hoehn and Yahr PD stages 2 or 3 (early and moderate stages). The mean (± SD) PD duration was 7.2 ± 2.4 years. Data for this table were collected in visit one, directly after signing the consent forms.
6.3 STUDY PROCEDURES

After the recruitment process, volunteers were asked to visit the Exercise Physiology Labs, University of Bedfordshire, UK, on two occasions. Visiting times were chosen at peak medication period.

6.3.1 Visit one (familiarization session)

The first visit had four aims, the first aim was to interview the volunteers and evaluate their ability to participate in the study. Medical history questionnaires were filled using Physical Activity Readiness (PAR-Q) (Appendix B). After the questionnaire, the assessment level move to evaluate PD stage using Hoehn and Yahr scale (Appendix B). PD severity was assessed using the Unified PD Rating Scale (UPDRS-Part III, motor examination section) (Appendix B). The PD questionnaire (PDQ-39) was used to assess the quality of life (Appendix B). All patients were able to ambulate independently.

Participants were asked to perform specific movement tasks to evaluate their movement function. For example, tap thumb with index finger, open and close hands in rapid succession to examine rigidity tremor of hands, arising from chair and postural stability. Those tasks, parallel with the physical activity readiness questionnaire (PAR-Q) allowed the researchers to accept or not accept the participant to continue for the study. Collected data from 5 participants showed their availability for the testing procedures and one participant’s data showed severe condition
and a history of stroke. This volunteer was excluded from the study. Figure (6.1) describes the first visit procedures.

The second aim of the first visit was to collect essential data about the participant’s, anthropometric measurements and body fat, these measurement were described previously in Chapter Two (see 2.6.4). The third aim for the first visit was to determine lactate threshold level for each participant. LT test is described in chapter two. Fourth aim of the first visit was to familiarize participants with the 5-m walk test, up and go timing test and stability test.

Procedures and purposes of these tests were described in detail for participants and they performed each test 3 times as a practice. Sequence of first visit procedures is summarized in figure 6.1.

The LT test was established during an incremental cycle ergometer test on a Monarch 620 cycle ergometer (Monarch, Sweden) participants were asked to complete 4-6 submaximal stages of 3-min duration of cycling. Before the LT test, participants were asked to wear a heart rate monitor and face mask linked to a (Metamax) portable metabolic test system (Cortex Biophysik, Leipzig, Germany). Seat height was adjusted according to participant’s comfort. Participants performed light cycling (35 watt) for 5 min to familiarize with the action of cycling and for the experimenters to gauge the participant’s cardiovascular response to exercise.

After 5 min rest, participants were asked to pedal at 35 watt for the first stage. In the following stages intensity was increased by 10 Watts (ACSM, 1998, Haas et al., 2004), by the end of each
stage fingertip capillary blood sampling (20 ul) for blood lactate assessment was taken from the participant’s finger. Blood lactate concentration was measured with the lactate analysis system (Analox-UK) immediately after each stage. A definition from Carter et al. (2000) was used to define the lactate threshold as “the exercise intensity before the first sudden and sustained increase in blood lactate above baseline level”. Verbal encouragement was used to encourage participants, but once the LT level been observed, the test was stopped and 90% of LT was calculated and considered as the workload intensity for the second visit.
Figure 6.1 Summary of testing strategy for visit one. Shows the sequence of first visit procedures, in this visit, privacy was required as PD questionnaire included direct questions related to PD assessment and quality of life. First visit procedures consumed approximately for 2 h.
6.3.2 Visit two (main session)

- Pre-Exercise tests

After one week, participants visited the laboratory for Visit Two. The testing procedures started with stability test (see 2.6.5). Participants removed their shoes and stood on the Footscan sensory sheet for stability measurement. Stability test took 10 seconds and was repeated two times. Participants were encouraged to stand normally and look straight ahead. After the stability test was performed data was recorded. Figure 6.2 shows stability data screen for the 10 seconds stability recording.
Figure 6.2 Stability measurement screen. Participants were asked to stand on the footscan plate with both feet in a normal stand up position for 10 s. The manufacturer (RSscan International, Belgium) in order to allow the software to analyze the foot scan recommended this period. In the middle of the screen, the magnitude of pressure distribution is shown based on a colour scale. Blue colour represents the lowest pressure; red represents the highest pressure while black represents absence of pressure. The dotted vertical line (between feet) shows the displacement of the centre of force (COF) during the measurement. The table on the right side of the screen shows numerical (COF) information on the measurement. The subject’s feet should be centralized on the Active sensor area (Figure 2.15). This test was performed two times pre-exercise and two times post-exercise.
Second pre-exercise test was the 5-m walk test. Participants were asked to perform the 5-m walk test as described in Chapter Two. Briefly, participants were asked to walk 5-m as fast as possible between two visible lines on the lab floor, time was recorded from the moment the first foot crossed the first line till the first foot cross the second line.

The third pre-exercise test was timed up and go (TUG). TUG test was performed after the 5-m walk test, participant was asked to sit down on two armed medium height chairs and wait for the signal (Siggeirsdottir et al., 2002). Once the signal was heard the participant was required to stand up and walk for 3 m as fast as possible, then turn around and walk the 3 m again and sit down. Figure 6.3 shows TUG testing procedures.
**Figure 6.3 Timed Up Go Test procedures.** Photographs show steps of TUG test, starting from sitting down on armed chair, photo (A), arm chair height was recommended to be approximately 40 cm, (Siggeirsdottir et al., 2002). Then after the examiner gives the start signal, the participant walks for 3 m (distance is marked with a black tape on the floor) photo (B), then turn around and walk back to the chair and sit down (photos C and D). Timing starts when the start signal was given until the participant sits down normally. Time and number of steps were recorded for this test. TUG was repeated 4 times pre-exercise and 4 times post-exercise. Verbal encouragement was used between trials to motivate participants.
6.4 EXERCISE INTERVENTION IN VISIT TWO

6.4.1 Submaximal cycling session

After the determination of LT level in Visit One, 90% of the LT speed was calculated and the participant was asked to pedal at 90% of the LT speed for 20 min (Figure 6.4). 90% of LT speed was chosen as it has been recommended as the guide exercise intensity by the American College of Sports Medicine (ACSM) for PD patients (ACSM, 1998).

6.4.2 Blood Samples

4 ml blood sample were taken from the participants through a venepuncture of an antecubital vein, before and after the exercise session. Blood samples were collected in Li-Heparin LH tubes (Sarstedt Ltd. UK) (see 2.6.7), the pathology lab recommended these tubes as they contain gel which separates the plasma from the cells, as freezing whole blood lyses all the cells, thereby contaminating the sample. Samples were centrifuged for 10 min at 5000 rpm then the plasma was removed from the cells using a pipette. Then they were kept in -20°C freezer until sent to the pathology lab (Luton and Dunstable Hospital). Figure 6.5 summarizes Visit Two procedures.
Figure 6.4 Photograph shows the submaximal exercise session using cycle ergometer. Participants cycled at 90% of their LT threshold intensity. Participants were required to pedal consistently to maintain workload at that intensity. The display screen on the cycle ergometer shows power output (rpm/Watt) to assist participants to maintain their pedalling with the required workload task. Oxygen consumption was measured via a face mask linked to a Metamax portable metabolic test system (Cortex, Biophysik, Germany). Heart rate was measured with short-distance telemetry (Polar, Finland) which was positioned around the participant’s chest. Multiple data outcomes were recorded every 3 min. A metronome was used to help participants maintain the appropriate cycle cadence. Verbal encouragement was used to encourage participants to continue cycling until they felt unable to maintain the workload. Every 3 min, participants were asked to rate their level of exertion using the Borg rating of perceived exertion scale (range 6–20) (See 2.16).
Figure 6.5 Summary of Visit Two testing and exercise procedures. Estimated time for this visit was 1.5-2 h.
6.4.3 Statistical analysis

Participant anthropometric measurements, PD severity, heart rate monitoring, oxygen uptake and perception of fatigue were analyzed descriptively as means and standard deviations. The effects of submaximal endurance exercise sessions on ADL, stability test and Ca$^{2+}$ concentration pre-exercise and post-exercise were compared using a paired t-test.

All statistical analyses were performed using SPSS (17.0) software. A P value of (0.05) or less was considered statistically significant. Pre and post exercise and ADL were compared using a paired t-test.
6.5 RESULTS

6.5.1 Patient characteristics of PD stages and Mean± SD values for clinical features during the submaximal cycling session

<table>
<thead>
<tr>
<th>PATIENT’S CODE</th>
<th>H and Y</th>
<th>PDQ 39</th>
<th>UPDRS III (MOTOR)</th>
<th>Cycling output (W)</th>
<th>Max HR bpm</th>
<th>VO2 ml/min/kg</th>
<th>RER</th>
<th>RPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>99</td>
<td>18</td>
<td>60</td>
<td>105.3±16.5</td>
<td>22.17±1.9</td>
<td>0.75±0.08</td>
<td>13.5±2.9</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>66</td>
<td>26</td>
<td>35</td>
<td>98.9±8</td>
<td>16.3±5</td>
<td>0.8±1</td>
<td>14±2</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>18</td>
<td>14</td>
<td>60</td>
<td>110.3±14.1</td>
<td>16.1±3.7</td>
<td>0.77±0.02</td>
<td>12.7±3.1</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>38</td>
<td>25</td>
<td>50</td>
<td>114±13.3</td>
<td>17±3.1</td>
<td>0.82±0.07</td>
<td>13.4±2.2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>51</td>
<td>18</td>
<td>50</td>
<td>117±10</td>
<td>17±5</td>
<td>0.83±0.5</td>
<td>14±1.5</td>
</tr>
<tr>
<td>*MEAN±SD</td>
<td>2.2±0.44</td>
<td>54.4±30.5</td>
<td>20.2±5.1</td>
<td>51±10.2</td>
<td>109.1±7.18</td>
<td>17.71±2.52</td>
<td>0.8±0.3</td>
<td>13.5±.53</td>
</tr>
</tbody>
</table>

Table 6.2 Patient characteristics of PD stages and Mean± SD values for clinical features during the submaximal session in visit two. Values are mean ±SD; H and Y score; PDQ-39, UPDRS (motor section); LT Speed workload, HR l/m, VO2 ml/min/kg, RER, Respiratory Exchange Ratio; VE l/min, Pulmonary Ventilation; RPE, Rating Perceived Exertion, (Borg Scale).
6.5.2 Feasibility of exercise

Submaximal exercise load/intensity was designed individually according to each participant’s performance in LT test (Figure 6.1). All five participants with mild to moderate PD completed the cycling-task session as prescribed. During the session, participants were asked to warm up their muscles for 5 min and to choose their comfortable seat height while cycling. Data collection process started once the mouthpiece of the gas analyzer (Metalizer) was connected to the participant’s mask. During the exercise session (20 min), participant’s HR was recorded (mean 109 ±7.1). Participants pedalled at (mean 51± 10.2) watts. Participants reported RPE was (mean 13.5±0.5 ) in RPE is categorised as between “Somewhat Hard and Hard” (Borg, 1970). Throughout the exercise session, adverse effects of testing and training were monitored and none occurred.
- Adjusted workload (90% of LT value) for each participant (Visit Two)

Figure 6.6 The calculated 90% of LT workload. The figure shows the calculated workload/watts, which is the 90% of the speed where the patients achieved their LT value. Participants were asked to cycle at these workloads (individually) in order to adjust the exercise intensity. This intensity is recommended by the (ACSM, 1998) as an exercise guidelines for PD patients. This exercise session took place in visit two.
- 5-m Walking Test pre and post exercise

Figure 6.7 Pre- and post-exercise measures of 5-m walk test (in seconds). The figure shows that the walking speed was significantly decreased post exercise compared with pre exercise. * Indicates a significant difference between trials (p < 0.05).
(TUG) Test pre and post exercise

Figure 6.8 Pre and post-exercise measures of TUG walk test. The figure shows the walking speed pre and post exercise session. No significant differences were demonstrated among conditions.
- Number of steps in TUG test pre and post-exercise

Figure 6.9 Pre and post-exercise measures of TUG walk test (number of steps).

The figure shows that the number of steps was decreased significantly post exercise compared to pre exercise. * Indicates a significant difference between trials (p < 0.05).
• Stability test pre and post-exercise

Figure 6.10 Pre and post-exercise measures of stability test (in millimetres). Values describe the distance of body movement away from the Centre of Force (COF) while the patient stands still on the footscan sensitive sheet for 10 seconds. COF travelling distance was decreased significantly post exercise compared with pre exercise. * Indicates a significant difference between trials ($p < 0.05$).
• \( \text{Ca}^{2+} \) concentration pre and post exercise

![Graph showing pre and post-exercise measures of total blood \( \text{Ca}^{2+} \) concentrations (mg/dL). Blood samples were collected in rest and immediately after the exercise session. Figure shows that the blood \( \text{Ca}^{2+} \) concentration was significantly increased post-exercise compared with blood \( \text{Ca}^{2+} \) concentration pre-exercise. * Indicates a significant difference between trials (\( p < 0.05 \)).]
6.6 RESULTS SUMMARY

Results show that the speed of the (5 m) walking test has increased after the submaximal cycling session to become (mean 4±2 s), compared with the same test speed pre-exercise (mean 5±2.4 s). Paired sample t-test analyzed that difference as significant (p=0.013). On the other hand, results of Up and Go speed were maintained without significant differences between pre-exercise measurement (mean 11.15±4.8 s) and post-exercise measurement (mean 8.65±2.7) (P=0.057). However, in TUG there was a difference with a decrease in number of steps post-exercise (mean 11.35 ± 3.2) compared with pre-exercise number of steps (mean 16.4 ± 5.52). Paired sample t-test showed significance in this difference (p=0.001). There was no significant main effect of the submaximal exercise session on the stability measurement between pre-exercise test and post-exercise test (p<0.05).

6.6.1 Conclusions of the results:

- In 5-m test, walking speed was significantly decreased post exercise compared to pre exercise.
- In TUG, Pre- and post-exercise measures of TUG walk test (number of steps) no significant differences were demonstrated among conditions in walking speed.
- In TUG, Pre- and post-exercise measures of TUG walk test (number of steps) the number of steps was decreased significantly post exercise compared to pre exercise.
- In stability test, (COF) travelling distance was decreased significantly post exercise compared to pre-exercise.
In Ca\(^{2+}\) concentration level, pre and post-exercise measures have shown that the blood Ca\(^{2+}\) concentration was significantly increased post-exercise compared to blood Ca\(^{2+}\) concentration pre-exercise.

6.7 DISCUSSION

The purpose of this study was to ascertain whether a single bout of submaximal exercise session would improve some ADL such as gait speed, number of steps and balance, in addition to elevating the concentration of blood Ca\(^{2+}\) in PD patients. It was hypothesized from *in vivo* and *in vitro* studies that Ca\(^{2+}\) plays a major role to transfer the effect of exercise to the brain through the blood brain barrier. In this study, PD participants performed 20 min of submaximal exercise session at 90% of their LT. Data collected pre and post-exercise showed a significant improvement in gait speed and significant decrease in number of steps in TUP test post-exercise when compared with pre-exercise. Results also shown significant increase in blood Ca\(^{2+}\) concentrations post-exercise when compared with pre-exercise. Moreover, when participants were evaluated with the footscan stability test, there was a significant change in COF measurement pre-exercise and post-exercise.

In the exercise session, submaximal cycling exercise was feasible for participants with mild to moderate PD (stages II and III in H&Y scale). All participants attended and complied with the testing and exercise session and no adverse effects were reported. In addition, the participants reported feeling confident during the session and perceived that the submaximal exercise task was relatively comfortable, shown by RPE of (± 13). Moreover, subjective data
derived from participants reported low levels of physical and mental fatigue despite the physical and cognitive intensity of training.

Not only was submaximal exercise feasible, it also has the potential to be effective in increasing walking speed in people with mild to moderate PD symptoms. Participants were able to increase walking speed by 0.2 m/s. The increase in 5-m walking speed found in the current study agrees with Rochester et al. (2005) who shows that after only four practice trials in a single session, they reported an immediate 0.07 m/s increase in walking task speed in participants with an initial single-task speed of 0.70 m/s. This finding also agreed with Protas et al. (2005b), they monitored that gait speed increased in the trained group from \((1.28 \pm 0.33)\) m/s to \((1.45 \pm 0.37)\) m/sec. However, the main difference between this study’s findings and that of Protas et al. (2005b) is that their exercise intervention was a walking program maintained for 8 weeks while the exercise intervention in this chapter is a single bout of submaximal exercise. In the current study, results indicate a greater increase in the speed than has previously been reported despite no training program being used (Rochester et al., 2005, Protas et al., 2005b).

Participants did not show the same improvement in the TUG test, which might refer to the nature of TUG test which requires more sophisticated movements. To be able to rise from a sitting position to a standing position requires both strength and technique. Walking a path for 3 m includes both acceleration and deceleration as participants prepare for a turn. The turning sequence is challenging for older people with balance disorders like PD (Nocera et al., 2009). Finally, turning around to sit down, this sequence of movement tasks might require a consent-training program; the single session of submaximal exercise is probably not enough to allow participants to improve their gait speed, unlike the 5-m walk test. However,
this stability of TUG test pre and post-exercise, surprisingly, was accompanied by a significant change in the number of steps between pre and post exercise session. In other words, participants after exercise did not perform the TUG test faster. However, they decreased the number of the required steps to perform the test. In other words, this result might indicate that the submaximal exercise session caused better gait performance and assisted PD participants to walk in wider steps. The number of unnecessary steps was discarded after exercise and gates become more efficient.

Gait impairment is an important clinical manifestation of PD and is considered as one of the most disabling aspects of this disease (Giladi et al., 2000). Gait related mobility problems have a negative impact on quality of life and well being of individuals with PD (Hackney and Earhart, 2009). Despite advances in medical therapy and surgical techniques, gait dysfunctions are observed throughout the disease with limited improvement of symptoms (Kaminsky et al., 2007). From an exercise standpoint, several studies have emphasized the contribution of specific exercises and intervention strategies to improve gait in individuals with PD. Treadmill training, use of external cues and specific task training have been investigated and different parameters of gait and quality of life of these individuals (ACSM, 1998, Baatile et al., 2000).

Results have shown a significant increase in Ca\(^{2+}\) concentration level pre-post exercise session for all participants. In addition to the findings from animal models and cell culture model, this might be an evidence of the important role of Ca\(^{2+}\) in physiological functions in humans, as it is responsible for muscle stimulation, transmission of action potential in neurons, blood clotting, enzymes activator and fluid transport across the membranes (Picconi et al., 2004, Schmitt et al., 2004). The bones act as a reservoir of Ca\(^{2+}\)
where it is interacting with phosphate producing a strong material that generates the bones and teeth. When exercise is done, a larger amount of Ca$^{2+}$ is needed by the muscles to get the stimulation. That produces a fall in the levels Ca$^{2+}$ in blood. As a physiological answer, the parathyroid glands of the body secrete parathyroid hormone (PTH) which activates the bone cells. These cells start to make mineral recycling which produces a release of the stored Ca$^{2+}$ ions of the bone. Finally this produces an increased Ca$^{2+}$ level in the bloodstream (Martini et. al., 1998). The increase of Ca$^{2+}$ levels will also arrive at the neuronal cells of the brain. As more Ca$^{2+}$ arises to the neuronal cells, it would be expected to find as well higher levels of the Ca$^{2+}$ binding protein CaMKs after exercise stimulation. However, Ca$^{2+}$ is an ion that needs to have a strong homeostasis regulation as small variations (above or below normal levels) can cause serious physiological problems (Martini et. al. 1998).

In previous chapters, the role of Ca$^{2+}$ CaMK-1 and CaMK-4 in brain function was investigated; results demonstrated that exercise activated the expression CaMK-1 and CaMK-4 in the brain through the Ca$^{2+}$ calmodulin-dependent pathway. Exercise increases DA synthesis in the remaining DA neuronal cells in the neostriatum through the Ca$^{2+}$/CaM-dependent system and eases some symptoms of PD. The whole mechanism is summarized in figure (7.2), which shows that exercise increases blood Ca$^{2+}$ level via the Ca$^{2+}$ calmodulin dependent protein pathways. This in turn, increases Tyrosine hydroxylase (TH) concentration in the brain, which activates Catecholamine production which then increases DA synthesis. Therefore, exercise could increase DA via Ca$^{2+}$ calmodulin dependent pathway. We hypothesize that this is the mechanism by which exercise modifies brain function in PD. Moreover, this mechanism might underlie the rectifying effect of convulsions on brain function disorders in epilepsy, and the rectifying effect of exercise on hypertension. This
mechanism might also underlie the curing effect of exercise on the symptoms in PD or senile dementia, because DA function is abnormally reduced in these diseases.

On the other hand, physiologically important changes in ionized calcium can be produced without change in the total calcium concentration by altering the affinity of albumin for calcium (Sola et al., 2001). Two factors can act by this mechanism to change the amount of calcium bound: the extracellular pH and parathyroid hormone (PTH). Respiratory alkalosis — An elevation in extracellular pH increases the binding of calcium to albumin, thereby lowering the plasma ionized calcium concentration (Sutoo and Akiyama, 2001). The fall in ionized calcium with acute respiratory alkalosis is approximately 0.16 mg/dL (0.04 mmol/L or 0.08 meq/L) for each 0.1 unit increase in pH. Thus, acute respiratory alkalosis, as in the hyperventilation syndrome, can induce symptoms of hypocalcemia, including cramps, paresthesias, tetany, and seizures. The alkaline pH may also contribute to these symptoms (Smith et al., 1985). However, that might require further investigation.

There is also a significant fall in the ionized calcium concentration in chronic respiratory alkalosis. However, this abnormality is not due to increased calcium binding, since the renal adaptation lowers the plasma bicarbonate concentration and minimizes the rise in extracellular pH. The hypocalcemia in this setting is due both to relative hypoparathyroidism and to renal resistance to PTH, with resultant hypercalciuria (Schmitt et al., 2004). Why these changes occur is not well understood (Clarkson and Haymes, 1995).
As mentioned previously, individuals with PD are confronted with at least two major impediments: one is the degenerative depletion of DA and the other is the resulting physical impairments and disabilities (Diaz and Bronstein, 2005). In order to improve neurodegeneration and develop motor performance and quality of life, more recent research steered towards exercise therapy in order to control these disorders. Numerous investigators reported, based on the results of experimental and clinical studies that exercise effects positively on motor performance, straightening-up process and postural instability (Baatile et al., 2000, Cakit et al., 2007, Herman et al., 2007). However, large clinical trials demonstrating evidence-based outcomes of exercise on PD rehabilitation are still limited. Furthermore, neurorestorative potential of exercise in PD has been hypothesized, but results have been highly debated (Dauer and Przedborski, 2003, Carne et al., 2005).

As described previously in chapters one and two, the first symptoms of PD typically do not appear until a critical threshold of 70–80% loss of the striatal neurotransmitter DA terminal is exceeded. The main role of the DA neurotransmitter is regulating substantia nigra and striatum, the pigmented cells in the SN synapse with other cells located in the striatum, which is responsible for movement control, emotional response, walking, postural stability and ability to experience pleasure and pain (Dauer and Przedborski, 2003). Consequently, lack of DA aggravates the symptoms of the disease, which started by trauma and finished with incapability to accomplish simple activities of daily living. Therefore, regulation of DA plays the main objective of PD medication to help relieve the symptoms.

The low values of VO2 (17.71±2.52) obtained in this study are in agreement with those reported in previous studies at about two-thirds of those of age-matched control subjects (Lucia et al., 1997). Lactate levels at peak exercise, on the other hand, were also
similar to those reported by other authors (Matsumura et al., 1983, Hopker et al., 2011). Finally, mean values of peak HR (109.1±7.18 beats/min) were similar to those reported in other studies (Ridgel et al., 2009, Sales et al., 2011). Some authors, however, have reported higher values of VO2 and HR in younger PD patients (mean age younger than 50 years) (Kjaer et al., 2000, Stisen et al., 2006). In addition, our protocol did not include a sufficient warm-up period. During warm-up periods, indeed, circulating catecholamine levels might increase before the beginning of testing, leading to higher HRs during a test.

According to Snijders et al. (2011), cycling training can promote a more stable and dynamic gait pattern in individuals with PD. Furthermore, some studies suggested that cycling training is more effective in improving gait than other traditional approaches (Snijders et al., 2011). It is possible that this intervention is beneficial because the participant is induced to maintain a steady rate with regular and uniform speed through the generation of rhythmic gait cycles due to periodic somatosensory and vestibular receptor stimulation (Morton et al., 2012). Thus, stimuli are transferred to neural circuits modulating gait in different central nervous system levels with rhythmic steps. Therefore, training on a cycling ergometer can be seen as a kind of external cue to trigger the motor activity to be performed (Carter et al., 2000). A recent review suggested that training on a treadmill can be performed in combination with physiotherapy at a frequency of three times per week, for about 20-30 m (Herman et al., 2009). For these authors, long-term treadmill training without weight-bearing is a safe and economical method to increase gait speed, restore gait rhythm and improve the quality of life of individuals with PD. Moreover, these effects may last for several weeks after the end of training (Herman et al., 2009). In this study, the treadmill was replaced by a cycling ergometer for the following reasons, cycling allows the participants to follow the power-metre so they can adjust the working load according to their specific health condition.
and fitness. In addition, it has been proved that the auditory rhythmic of the ergometer cues is an important feature in the treatment of PD, although not widely used in clinical practice. Studies have shown improvement in electromyographic and spatio-temporal parameters of gait in Parkinson’s patients undergoing gait training (Morris et al., 2001a, McGough et al., 2011).

The importance of this chapter’s results are related to the importance of ADL in PD patients’ life. Despite medical treatment, individuals with PD fall frequently with devastating consequences (Bloem et al., 2001). Approximately 66% of individuals with PD will suffer falls and 46% will experience recurrent falls (Cakit et al., 2007). Therefore, there is a critical need of therapies which can improve ADL, which in turn provide a less risky life for PD patients. Factors such as gait freezing, muscular weakness and balance disorders were found as causes of falls in individuals with PD (Bloem et al., 2001, Lord et al., 2003, Campbell et al., 2005, Sherrington et al., 2008). Accordingly, different research attempts have used external cues for gait training, balance exercises, and strength training programs finding improvement in each of these factors (Protas et al., 2005b, Sherrington et al., 2008).

After the submaximal training session, participants were asked to explain their health status after the session, all of the participants indicated that they believed exercise might keep them stronger, functional and fit. This belief seemed to come primarily from each person’s own experiences of exercising. Participants indicated that they are aware of the importance of exercise. However, they had a lack of knowledge about appropriate disease specific exercises. They stated that they had not received or been provided with information about specific exercise programs for his condition. In addition, participants generally felt that they were capable of going to a gym to exercise; several felt that joining a group exercise would be more motivating than home exercise. Participants indicated that motivation was one of the
The findings of this study suggest that there are barriers to initiating and maintaining an independent exercise program, and that these barriers must be taken into account when therapists design and implement such programs. Participants with PD in this experimental chapter perceived exercise to be beneficial in managing the disease process; however, a lack of available information about disease-specific exercises and the optimum intensity to perform the exercise session were reported by the participants as an exercise barrier. Thus, although the research is still limited, it appears that PD patients (mild to moderate stages) can effectively participate in exercise programs, and that this exercise may be beneficial in improving various measures of impairment, activities and participation, despite the presence of a degenerative neurological disease. In this experimental chapter, the intensity of exercise was determined according to the LT level of each participant.

As a conclusion, PD is a chronic and progressive disease that affects elderly people as their reparation and protection systems start to fail (Canning et al., 2006). However, new PD cases are starting to be developed in younger people because of their life style. In the last stages of PD individuals can develop different problems such as chocking, pneumonia and
falls, which can lead to death. Therefore, most of the patients suffering from PD have a lower average life expectancy. Actual research is focused on delaying the progression of PD as an alternative solution would be cell transplants, which so far have not given good results (Dauer and Przedborski, 2003, Weintraub et al., 2008b). Results of this chapter suggest that exercise is a good therapy to delay PD symptoms as the Ca$^{2+}$ levels can be re-established.

6.8 STUDY LIMITATIONS

This study was conducted in a relatively limited geographical area (Bedford district, Bedfordshire, UK) with a relatively small number of participants with mild to moderate stages of PD. This introduces potential bias to the study in that the results by the participants may not be transferable to other PD patients with advanced PD stages.

Due to study limitations, from the current results it is not possible to clarify the differences between gaits, balance and Ca$^{2+}$ concentration improvements between PD patients and control people from the same age. In this study, a pre-post design has been used to measure participants’ level of performance before the intervention took place (pre), and that collected after the intervention took place (post). This design was the best recommended way to be sure that exercise intervention had a causal effect. However, this study did not use a control group to ensure that the exercise effect occurred as a result of participating in the exercise session and that other factors not related to the intervention may have been involved, this may have impacted the ability to detect differences between groups, and thus, limited any insight into how the submaxiaml exercise session might effect on Gait, balance and Ca$^{2+}$ concentration in control group. It also limits the ability to generalize the current results to the
significant number of patients that suffer from movements symptoms of PD. Therefore, further studies should be done to investigate the influence of exercise on gait, balance and Ca\textsuperscript{2+} concentration in elder people.

A third limitation is that participants were tested after they had taken their normal doses of PD medication. Therefore, future studies need to include PD patients both ON medication (ideally after a supramaximal Levodopa dose, to ensure an optimal and consistent ON phase throughout the experiment) and OFF medication, in order to ascertain the Ca\textsuperscript{2+} concentration role in mediating movement-related changes in (ADL). Another limitation of the study was that subjects were excluded on the basis of having a history of heart disease. The exclusion of those with heart disorders was justified on ethical grounds, to avoid placing patients in threatening conditions that could potentially threat their lives.

As a conclusion, the preliminary observation might have some important consequences in designing exercise protocols for patients with PD. A randomized, controlled investigation on a larger group of subjects needs to be carried out to sustain this hypothesis. Repeating this study with differences in testing times according to on and off medication periods, would be valuable in further investigations. In addition, pre-exercise testing time might be another aim for further investigations. For example 2, 6, 12, 24 hour post-exercise measurements might provide valuable information about the period of time where the body can maintain the effect of exercise.

Irrespectively, there are still questions regarding whether subjects with more severe motor deficits would show greater differences in (ADL) after exercise session, and whether they may respond differently after exercise session
7. CONCLUSIONS

The data presented in this thesis has contributed to the understanding of the processes involved in neuroprotective effect of exercise in vivo, in vitro and human subject models of PD. This is paramount in the field of exercise and neurodegenerative disorders like PD. The aim of this thesis was to investigate the neuroprotective effects of exercise against PD.

The following views concerning the effect of exercise on CaMK-1 and CaMK-4 synthesis in the brain have been developed based on animal experiments: (1) immuno histochemical analysis of the expressions of CaMK-1 and-4 levels are increased in the SN and striatum regions in PD induced rat brain. (2) Quantitative amounts of CaMK-1 and CaMK-4 levels in the Striatum were increased as a result of 8 weeks of endurance exercise program, and this effect of Ca$^{2+}$ is abolished by a CaM antagonist. These findings have shown that exercise protocol alters Ca$^{2+}$/calmodulin DA activities. Therefore, the modulation on Ca$^{2+}$ calmodulin DA activities might be related to the neuroprotective effect of exercise.

(3) In vitro study demonstrated that high levels of resistance against 6-OHDA as a result for SH SY-5Y cell culture supported by Ca$^{2+}$ compared with cultures without Ca$^{2+}$. These findings indicate that Ca$^{2+}$ is transported to the brain via the blood, thereby enhancing CaMKs activity, and the CaMKs dependent system subsequently increases DA synthesis in specific brain regions through the phosphorylation of TH by CaMK-1 and CaMK-4. The movement of Ca$^{2+}$ and the phenomena related to Ca$^{2+}$/CaM-dependent DA synthesis. Figure 7.1 summarize the hypothesis which this thesis recommends as an explanation of the effect of exercise on PD patients.
(4) Finally, human study with PD patients has been shown to improve their gait speed and walking economy after single bout of submaximal exercise. The findings of the human study form a foundation for additional research to further explore the relationship between different types of exercise and PD. Exercise stimulates $\text{Ca}^{2+}$ metabolic hormone and induces increased blood $\text{Ca}^{2+}$ levels, thereby increasing DA synthesis in the brain. Subsequently, increased DA levels regulate various brain functions. This thesis suggests that this is the mechanism by which exercise modifies brain function. In addition, this mechanism might underlie the rectifying effect of convulsions on brain function disorders in PD.

It is clear that much additional work will be required before a complete understanding, such as molecular biological, immunological, and pharmacological and non-pharmacological approaches. The future potential strategies might be used to determine the role of other proteins and enzymes in delaying PD symptoms. The detailed mechanism by which exercise effects might be involved on neuroprotective activity of exercise remains a subject for further investigation. Other models must be used to investigate and better characterize the modulation on nucleotidase activities induced by exercise, including \textit{in vivo} models of PD.

In this thesis, the improvements of PD symptoms were significant irrespective of cycling and treadmill exercises. However, other types of exercise would be recommended as a subject for further studies using such as swimming, martial arts (karate, Judo etc), weight training and walking in the mountains. In addition, the impact of exercise looked for changes immediately after the exercise session. This thesis recommended further studies to examine the effect of exercise months or years later. We need to know how long the effects last and
whether there is a "priming effect," whereby prior exercise might potentiate the impact of later exercise.

This thesis demonstrated that exercise exerts neuroprotective effects on the DA system in PD models, and enhances the neuronal migration at least partly through the modulation of Ca\(^{2+}\)/calmodulin DA. Exercise also might exert neuroprotective/neurogenic effects on PD patients, as well as contribute to the functional recovery by other mechanisms including gait speed, walk economy and blood Ca\(^{2+}\) concentration, although further exploration about the mechanisms should be needed.

In this thesis, results have demonstrated that treadmill/exercise for rat models of PD and submaximal cycling session for PD patients exert a protective effect against MPTP/6-OHDA induced nigrostriatal DA neuronal cell loss, and could be suggested that submaximal cycling exercise may provide good preventive value for the PD patients.
Exercise stimulates Ca\(^{2+}\) metabolic hormone and induces increased blood Ca\(^{2+}\) levels, thereby increasing DA synthesis in the brain. Subsequently, increased DA levels regulate various brain functions. This is a mechanism by which exercise might modify brain function. In addition, this mechanism might underlie the rectifying effect of convulsions on...
brain function disorders in epilepsy, and the rectifying effect of exercise on hypertension. This mechanism might also underlie the rectifying effect of exercise on the symptoms in PD or senile dementia, because DA function is abnormally reduced in PD.
APPENDIX A

WESTERN BLOT DATA SHEET
# Western Blot Data Sheet

## Gel 1

<table>
<thead>
<tr>
<th>Well</th>
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<td>4</td>
<td></td>
<td></td>
<td>Secondary AB:</td>
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<td></td>
<td>Dilution:</td>
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## Gel 2:

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**Running Time:**

**Vol:**

219
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<th>Time Category</th>
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<tr>
<td>Transfer Time:</td>
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<tr>
<td>Blocking Time:</td>
<td></td>
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<td>Primary AB incubating Time:</td>
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</tr>
<tr>
<td>Secondary AB incubating Time:</td>
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<td>Exposure Time:</td>
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<td>End Date:</td>
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<td>Time:</td>
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</table>
APPENDIX B

PARTICIPANT INFORMATION SHEET AND CONSENT FORMS
PARTICIPANT INFORMATION SHEET

(General arrangements)

Project title:

Effect of Endurance Exercise on Blood Ca2+ levels and other movement functions in patients with PD

Principle investigators

Professor Mark Lewis. 07939 824453 (Office). Mark.lewis@beds.ac.uk
Dr. Paul Castle 01234 793385 (Office). Paul.Castle@beds.ac.uk

Researcher and team member

Hossam Ali - 07852 518918 (mobile), 01234 793433(Office). Hossam.ali@bed.ac.uk

Secretary of UOB ethical Committee

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Overview of the study

This study aims to investigate the effect of endurance exercise on levels of blood Ca$^{2+}$ which may have a positive effect in reducing symptoms of PD (PD). Our objective is to measure the current physical condition of PD patients and its correlation to their movement ability before and after cycling exercise.

Why are we conducting this study?

As you know, movement disorders are the hallmark of PD (PD); patients with PD (especially in late stages) have difficulties during activities of daily life, which may affect their life style. Meanwhile, individuals of early and moderate stages of PD are facing less serious symptoms which do not prevent them from participating in more vigorous activities / physical exercise. Over the past 15 years, there has been a considerable increase in research investigating the benefits of exercise and physical therapy in individuals with PD. In general, exercise programs and physical therapy have been shown to be effective in improving strength, range of motion and various functional measures. However, it is still unknown which exercise level can be of benefit to patients and the how the exercise affects the brain. Our previous work indicated exercise may be beneficial in alleviating symptoms of PD through changes in Ca$^{2+}$. This study will assess the effect of an exercise bout on the movement abilities for patients with PD and also assess the
blood Ca\(^{2+}\) change as a result of exercise. We think that exercise may benefit PD patients via exercise induced production of Ca\(^{2+}\). Yet this has never been studied.

This research proposal might provide an opportunity for volunteers to enhance their moving condition and their general health.

**Who can be a potential candidate to join this study?**

You could be a candidate to join this study if you meet the following criteria:

- If you are suffering from PD, able to do basic level of physical activity like walking and cycling either on or off medication periods, living in the Bedford district and able to visit University of Bedfordshire, Polhill Avenue, Bedford for 2 times

- You are in general good health (no heart or circulatory problems etc)

You are welcome to visit us for a familiarization session to have a discussion about this study and to ask any question that you might have. Then you will have the opportunity to try balance, walking and cycling to measure how comfortable you are with the testing procedures. Once you feel comfortable with the procedures you can then join the study.

You can withdraw anytime if you do not feel comfortable with any of the procedures without needing to provide a reason.

**What are the procedures of this study?**
You will be asked to visit University of Bedfordshire exercise physiology labs between 1-3 occasions, during these visits you will go through balance and walking tests (specifically designed for PD patients) before and after a light cycling exercise. Also blood samples will be taken before and after the cycling session. Details of those sessions will be in the following information sheets. You will be required to abstain from caffeine, alcohol and exercise for 24 h prior to testing.

Prior to the participating you will be asked to complete a medical questionnaire, a physical readiness questionnaire (PAR-Q), a blood analysis participant screening form and sign an informed consent form to confirm you are physically capable of participating and to demonstrate you have read and understood what you are taking part in and the reasons why.

**What if you do not feel comfortable with the testing procedures?**

You are free to withdraw from this study at anytime without giving reason, you can also contact any of the project members listed above if you have any enquiry regarding any issue

**What are the Potential Benefits of joining this project?**

The session might allow you to know about the best load of exercise which you can do in order to enhance your movement abilities and quality of life. It will also help to quantity your movement ability that you could always monitor yourself at home in the future. By taking part our understanding of exercise as a therapy to improve quality of life of those with PD will be
improved. We aim to use this data to apply for a large scale fund to conduct further research into this topic. By publishing our results the wider population may benefit from our findings.

**What are the Potential Risks?**

During the sampling of blood there is a slight risk of infection, the risk of infection will be kept to a minimum by all sampling taking place in a designated clinical investigation suite and being performed using sterile techniques. The level of discomfort will be minimised during blood sampling through the use of an experienced phlebotomist. The cycling exercise may cause a degree of discomfort. However this will be minimised by ensuring you are of an appropriate level of comfort while you are cycling, are thoroughly familiarised with exercise and through continuous observation during testing by the research project to ensure you do not overexert yourself.

**Confidentiality**

All collected data will be kept in a locked filling cabinet in the principle investigator’s office, Polhill Avenue, Bedford. Data collecting softcopy document will be encrypted to remain confidential. Participants will remain anonymous in any presentation of data and publication of findings. Participants will be able to obtain results upon publication of the findings. All data will be deleted and all documents will be shredder after the data is published. Only principle investigators will have access to all participants’ personal data while the researcher (Hossam Ali)
will have access to the participant’s contact details (phone numbers and e-mails) and semi-anonymous datasheet if they accepted. Data will be used for scientific reasons only.

Once you agree to join the project, your data collecting sheets will have two separate covers with the same bar-code, the first cover, which contains your personal data, will be kept in a locked filling cabinet in the principle investigator’s office, and only principle investigators will have access to that document for scientific reasons only. The second cover will be semi-anonymous (with bar-code only). Before the testing session, principle investigator will replace your personal datasheet cover with the semi-anonymous cover. Data collecting team will have access to the semi-anonymous documents only. Your semi-anonymous datasheet will be returned to principle investigator’s office after each testing session.
General arrangements Consent form

(CONFIDENTIAL)

Please tick box

1. I confirm that I have read participant datasheet (General arrangements) and understand the information sheet dated............... for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily by a member of the research team

[ ]

2. I understand that my participation is voluntary and that I can stop taking part at any time without giving any reason, without my medical care or legal rights being affected.

[ ]

3. I agree to give blood sample (4 ml) before and after the sessions for testing purposes

[ ]

4. I agree to take part in the above study.

[ ]

Name of Participant ___________________________________________

Date ____________________________________________

228
Familiarisation Session Consent form

(Confidential)

- Please **tick** the boxes

1. I confirm that I have read participant datasheet (Familiarization Session) and understand the information sheet dated.................... for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily by a member of the research team

2. I understand that my participation is voluntary and that I can stop taking part at any time without giving any reason, without my medical care or legal rights being affected.

3. I agree to give 80-120 ul (4-6 drops) of finger prick blood samples during the Lactate threshold (cycling) test

3. I agree to take part in the above session.
Main Session Consent form

(CONFIDENTIAL)

Please tick box

1. I confirm that I have read participant datasheet (Visit number 2, 3, 4 and 5) and understand the information sheet dated................. For the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily by a member of the research team

2. I understand that my participation is voluntary and that I can stop taking part at any time without giving any reason, without my medical care or legal rights being affected.

3. I agree to give 4ml of blood before and after the session for testing purposes.

4. I agree to take part in the above study.
APPENDIX C

DATA COLLECTION SHEETS FOR HUMAN STUDY
5-M Walk test

Participant’s code:

Visit number:

Date:

<table>
<thead>
<tr>
<th></th>
<th>Time/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Exercise</td>
<td></td>
</tr>
<tr>
<td>Post-Exercise</td>
<td></td>
</tr>
</tbody>
</table>
## PDand EXERCISE PROJECT

### UP and GO Test

**Participant’s code:**

**Visit number:**

**Date:**

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Number of steps</th>
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</thead>
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<tr>
<td>Pre-Exercise</td>
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<td></td>
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<td>3</td>
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<td></td>
<td>4</td>
<td></td>
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<tr>
<td>Post-Exercise</td>
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<td></td>
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<td></td>
<td>2</td>
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<td></td>
<td>3</td>
<td></td>
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<td></td>
<td>4</td>
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</table>
### Modified Hoehn and Yahr staging

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No signs of disease</td>
</tr>
<tr>
<td>1</td>
<td>Unilateral disease</td>
</tr>
<tr>
<td>1.5</td>
<td>Unilateral plus axial involvement</td>
</tr>
<tr>
<td>2</td>
<td>Bilateral disease, without impairment of balance</td>
</tr>
<tr>
<td>2.5</td>
<td>Mild bilateral disease, with recovery on pull test</td>
</tr>
<tr>
<td>3</td>
<td>Mild to moderate bilateral disease; some postural instability; physically independent</td>
</tr>
<tr>
<td>4</td>
<td>Severe disability; still able to walk or stand unassisted</td>
</tr>
<tr>
<td>5</td>
<td>Wheelchair bound or bedridden unless aided</td>
</tr>
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</table>

Modified Hoehn and Yahr staging (Goetz et al., 2004)
Please tick **one** box for each question

**Due to having PD, how often during the last month have you....**

<table>
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<tr>
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<th>Occasionally</th>
<th>Sometimes</th>
<th>Often</th>
<th>Always</th>
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<tbody>
<tr>
<td>1. Had difficulty doing the leisure activities which you would</td>
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<tr>
<td>like to do?</td>
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<td></td>
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</tr>
<tr>
<td>2. Had difficulty looking after your home, e.g. DIY, housework,</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>cooking?</td>
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</tr>
<tr>
<td>3. Had difficulty carrying bags of shopping?</td>
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<tr>
<td>4. Had problems walking half a mile?</td>
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<tr>
<td>5. Had problems walking 100 yards?</td>
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</tr>
<tr>
<td>6. Had problems getting around the house as easily as you would</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>like?</td>
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</tr>
<tr>
<td>7. Had difficulty getting around in public?</td>
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<tr>
<td>8. Needed someone else to accompany you when you went</td>
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239
<table>
<thead>
<tr>
<th></th>
<th>Question</th>
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<tbody>
<tr>
<td>9</td>
<td>Felt frightened or worried about falling over in public?</td>
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<tr>
<td>10</td>
<td>Been confined to the house more than you would like?</td>
</tr>
<tr>
<td>11</td>
<td>Had difficulty washing yourself?</td>
</tr>
<tr>
<td>12</td>
<td>Had difficulty dressing yourself?</td>
</tr>
<tr>
<td>13</td>
<td>Had problems doing up your shoelaces?</td>
</tr>
<tr>
<td>14</td>
<td>Had problems writing clearly?</td>
</tr>
<tr>
<td>15</td>
<td>Had difficulty cutting up your food?</td>
</tr>
<tr>
<td>16</td>
<td>Had difficulty holding a drink without spilling it?</td>
</tr>
<tr>
<td>17</td>
<td>Felt depressed?</td>
</tr>
<tr>
<td>18</td>
<td>Felt isolated and lonely?</td>
</tr>
<tr>
<td>19</td>
<td>Felt weepy or tearful?</td>
</tr>
<tr>
<td>20</td>
<td>Felt angry or bitter?</td>
</tr>
<tr>
<td>21</td>
<td>Felt anxious?</td>
</tr>
<tr>
<td>22</td>
<td>Felt worried about your future?</td>
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</table>
| 23| Felt you had to conceal your

<p>| | | | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Parkinson's from people?</td>
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<tr>
<td>24. Avoided situations which involve eating or drinking in public?</td>
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<tr>
<td>25. Felt embarrassed in public due to having PD?</td>
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</tr>
<tr>
<td>26. Felt worried by other people's reaction about you?</td>
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</tr>
<tr>
<td>27. Had problems with your close personal relationships?</td>
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<tr>
<td>28. Lacked support in the ways you need from your spouse or partner?</td>
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<tr>
<td>29. Lacked support in the ways you need from your family or close friends?</td>
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<tr>
<td>30. Unexpectedly fallen asleep during the day?</td>
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</tr>
<tr>
<td>31. Had problems with your concentration, e.g. when reading or watching TV?</td>
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<td></td>
</tr>
<tr>
<td>32. Felt your memory was bad?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33. Had distressing dream or hallucinations?</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>34. Had difficulty with your speech?</td>
<td></td>
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<tr>
<td>35. Felt unable to communicate with</td>
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<td></td>
</tr>
<tr>
<td>Question</td>
<td></td>
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<tr>
<td>-------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>36. Felt ignored by people?</td>
<td></td>
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</tr>
<tr>
<td>37. Had painful muscle cramps or spasms?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38. Had aches and pains in your joints or body?</td>
<td></td>
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</tr>
<tr>
<td>39. Felt unpleasantly hot or cold?</td>
<td></td>
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</table>
UNIFIED PD RATING SCALE (UPDRS)

III. MOTOR EXAMINATION

18. Speech

0 = Normal.
1 = Slight loss of expression, diction and/or volume.
2 = Monotone, slurred but understandable; moderately impaired.
3 = Marked impairment, difficult to understand.
4 = Unintelligible.

19. Facial Expression

0 = Normal.
1 = Minimal hypomimia, could be normal "Poker Face".
2 = Slight but definitely abnormal diminution of facial expression
3 = Moderate hypomimia; lips parted some of the time.
4 = Masked or fixed facies with severe or complete loss of facial expression; lips parted 1/4 inch or more
20. Tremor at rest (head, upper and lower extremities)

0 = Absent.
1 = Slight and infrequently present.
2 = Mild in amplitude and persistent. Or moderate in amplitude, but only intermittently present.
3 = Moderate in amplitude and present most of the time.
4 = Marked in amplitude and present most of the time.

21. Action or Postural Tremor of hands

0 = Absent.
1 = Slight; present with action.
2 = Moderate in amplitude, present with action.
3 = Moderate in amplitude with posture holding as well as action.
4 = Marked in amplitude; interferes with feeding.

22. Rigidity (Judged on passive movement of major joints with patient relaxed in sitting position. Cogwheeling to be ignored)

0 = Absent.
1 = Slight or detectable only when activated by mirror or other movements.
2 = Mild to moderate.  
3 = Marked, but full range of motion easily achieved.  
4 = Severe, range of motion achieved with difficulty.

**23. Finger Taps (Patient taps thumb with index finger in rapid succession)**

0 = Normal.  
1 = Mild slowing and/or reduction in amplitude.  
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.  
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.  
4 = Can barely perform the task.

**24. Hand Movements (Patient opens and closes hands in rapid succession)**

0 = Normal.  
1 = Mild slowing and/or reduction in amplitude.  
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.  
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
4 = Can barely perform the task.

25. Rapid Alternating Movements of Hands (Pronation-supination movements of hands, vertically and horizontally, with as large an amplitude as possible, both hands simultaneously)

0 = Normal.
1 = Mild slowing and/or reduction in amplitude.
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
4 = Can barely perform the task.

26. Leg Agility (Patient taps heel on the ground in rapid succession picking up entire leg. Amplitude should be at least 3 inches)

0 = Normal.
1 = Mild slowing and/or reduction in amplitude.
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing
4 = Can barely perform the task.

27. Arising from Chair (Patient attempts to rise from a straightbacked chair, with arms folded across chest)

0 = Normal.
1 = Slow; or may need more than one attempt.
2 = Pushes self up from arms of seat.
3 = Tends to fall back and may have to try more than one time, but can get up without help.
4 = Unable to arise without help.

28. Posture

0 = Normal erect.
1 = Not quite erect, slightly stooped posture; could be normal for older person.
2 = Moderately stooped posture, definitely abnormal; can be slightly leaning to one side.
3 = Severely stooped posture with kyphosis; can be moderately leaning to one side.
4 = Marked flexion with extreme abnormality of posture.
29. Gait

0 = Normal.
1 = Walks slowly, may shuffle with short steps, but no festination (hastening steps) or propulsion.
2 = Walks with difficulty, but requires little or no assistance; may have some festination, short steps, or propulsion.
3 = Severe disturbance of gait, requiring assistance.
4 = Cannot walk at all, even with assistance.

30. Postural Stability (Response to sudden, strong posterior displacement produced by pull on shoulders while patient erect with eyes open and feet slightly apart. Patient is prepared)

0 = Normal.
1 = Retropulsion, but recovers unaided.
2 = Absence of postural response; would fall if not caught by examiner.
3 = Very unstable, tends to lose balance spontaneously.
4 = Unable to stand without assistance.

31. Body Bradykinesia and Hypokinesia (Combining slowness, hesitancy, decreased armswing, small amplitude, and poverty of movement in general)
0 = None.

1 = Minimal slowness, giving movement a deliberate character; could be normal for some persons. Possibly reduced amplitude.

2 = Mild degree of slowness and poverty of movement which is definitely abnormal. Alternatively, some reduced amplitude.

3 = Moderate slowness, poverty or small amplitude of movement

4 = Marked slowness, poverty or small amplitude of movement.
APPENDIX E

CHEMICAL REAGENTS AND KITS
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide mix</td>
<td>Sigma Chemical Co., Dorset, UK</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Sigma Chemical Co., Dorset, UK</td>
</tr>
<tr>
<td>BSA</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<tr>
<td>Ca(^{2+}) Chloride</td>
<td>VWR International</td>
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<tr>
<td>Coomassie brilliant blue G-250</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<tr>
<td>DAB</td>
<td>Vector Labs, UK</td>
</tr>
<tr>
<td>DMEM</td>
<td>Invetrogen Corp., Paisely, UK</td>
</tr>
<tr>
<td>Etahnol</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<tr>
<td>Filter paper</td>
<td>Whatman</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<td>Glycerol</td>
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<tr>
<td>Glycine</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<tr>
<td>Medical X-ray film(Blue)</td>
<td>Kodak, Japan</td>
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<td>Methanol</td>
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<td>Parafilm</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<td>PBS</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<tr>
<td>Pencilin/streptomycin</td>
<td>Invetrogen Corp, UK</td>
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<td>Propanol</td>
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<td>SDS</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<tr>
<td>Skimmed milk powder</td>
<td>Marvel, Cadbury, UK</td>
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<tr>
<td>Sodium pentobarbital</td>
<td>Vetbutal, Polfa, Poland</td>
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<td>TEMED</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<tr>
<td>Triton X-100</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<tr>
<td>Trypsin-EDTA</td>
<td>Sigma Chemical Co., Dorset, UK</td>
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<tr>
<td><strong>Antibodies</strong></td>
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</tr>
<tr>
<td>----------------</td>
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</tr>
<tr>
<td><strong>Anti-CaMK-1; (M-20); sc-1544 goat polyclonal antibody</strong></td>
<td>Santa Cruz Biotechnology, Inc. Heidelberg, Germany</td>
</tr>
<tr>
<td><strong>Horsesdih peroxides (HRP) conjugated affinity purified secondary antibody</strong></td>
<td>Chemicon international, Temecula, CA</td>
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<tr>
<td><strong>Anti-CaMK-4; (M-20): sc-1546 goat polyclonal antibody</strong></td>
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<td><strong>Peroxidase Conjugated Affinity Purified Goat anti-Rabbit IgG</strong></td>
<td>Rockland Immunochemicals Inc. Gilbertsville, PA</td>
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<tr>
<td><strong>Anti-Tyrosine Hydroxylase (TH). Rabbit Affinity Purified Polyclonal Antibody</strong></td>
<td>Chemicon international, Temecula, CA</td>
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<tr>
<td><strong>Donkey anti-goat IgG-HRP, secondary antibody</strong></td>
<td>Rockland Immunochemicals Inc. Gilbertsville, PA</td>
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</table>
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