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THE ROLE OF PARKIN-PICK1 IN MITOCHONDRIAL DYSFUNCTION

A thesis submitted to the University of Dublin for the Degree of Doctor of Philosophy

February 2012

By
Debadutta Deb

Under the supervision of Prof. Kumlesh K Dev
Molecular Neuropharmacology Group
School of Medicine, Institute of Neuroscience
Trinity College Dublin
Ireland
A. Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University’s open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

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Debadutta Deb
B. Acknowledgements

I would like to take the privilege to acknowledge all those persons who helped me completing this work. First of all, I want to thank all the lab members of KKD lab; Priyanka, Luke, Gill, Marika, Graham, Fiona, Adam and Ola for the support they showed me and for all those lab fun/jokes they kept on making to keep a taste of life in rigorous lab hours. I must acknowledge Priyanka for helping me with the yeast two hybrid assay and Gill for sharing her precious neuronal cultures without any bragging. Gill, those little chat about life, etc are one of my most cherishable moment here. I also want to thank Yvonne for her advices during her tenure in the lab. In biochemistry department, I would like to thank Stephen for all his helps and not getting annoyed in my stupidest questions. The small family which I found here, far from home was something I never expected. Many thanks to my friends and housemates Ani, Arnab and Aurab; and people like Shibuda, Swagata, Kapil and Soumya for all their support and giving me a taste of home in this foreign land. You guys rock! I also want to mention Gablu, with whom I started chasing the dream of doing true research 10 years ago as a undergrad student. I wish you all the luck for successful completion of your PhD thesis. I thank Sany for joining me here in Dublin and reviving the old days of Bangalore and New Delhi. I would like to thank the woman behind this successful completion of PhD, Raheleh, for all the motivation and support she has given me, time to time. Last but not the least, I thank my Ma, Baba and Dalia for providing me with the wonderful family which made me what I am today. I would like to dedicate this work to Baba. In this occasion, I also wish a happy and peaceful married life to Dalia.

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<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>µF</td>
<td>Microfarad</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>ABP</td>
<td>AMPA receptor binding protein</td>
</tr>
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<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate</td>
</tr>
<tr>
<td>ARJP</td>
<td>Autosomal recessive juvenile Parkinsonism</td>
</tr>
<tr>
<td>ASIC2a</td>
<td>Acid-sensing ion channel subunit 2a</td>
</tr>
<tr>
<td>BAR</td>
<td>Bin-Amphipathin-Rvs</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>bp</td>
<td>Basepair(s)</td>
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<tr>
<td>CASK</td>
<td>Calcium/calmodulin-dependent serine protein kinase</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
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<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPD</td>
<td>Cdc42 phosphodigron</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>ct</td>
<td>Carboxyl terminal end</td>
</tr>
<tr>
<td>Cul1</td>
<td>Cullin 1</td>
</tr>
<tr>
<td>DEG</td>
<td>Degenerin/epithelial Na⁺ channel</td>
</tr>
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<td>Duchenne muscular dystrophy</td>
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<tr>
<td>DMSO</td>
<td>Di-methyl sulfoxide</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide tri-phosphate</td>
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<td>E1</td>
<td>Ubiquitin activating enzyme</td>
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<tr>
<td>E2</td>
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<tr>
<td>E3</td>
<td>Ubiquitin ligase enzyme</td>
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<td>Ethylene diamine tetraacetic acid</td>
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<td>Ethylene glycol tetraacetic acid</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
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<td>Glutamate receptor subunit 2</td>
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<td>Glutamate receptor interacting protein</td>
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<td>GSK3β</td>
<td>Glycogen Synthase Kinase</td>
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<td>GST</td>
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</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
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<tr>
<td>HECT</td>
<td>Homology to E6-AP carboxy terminus</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human Embryonic Kidney 293</td>
</tr>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>IBR</td>
<td>In between RING fingers</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
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<tr>
<td>kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolt</td>
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<tr>
<td>LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
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<tr>
<td>lt</td>
<td>Liter</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mA</td>
<td>Millampere</td>
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<td>MBP</td>
<td>Maltose binding protein</td>
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<td>MCS</td>
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<td>Nanogram</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<td>PAGE</td>
<td>Poly acrylamide gel electrophoresis</td>
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<tr>
<td>PARK1</td>
<td>α-synuclein</td>
</tr>
<tr>
<td>PCI</td>
<td>Phenol-chloroform-isoamyl alcohol</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD95/DlgA/ZO-1</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated</td>
</tr>
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<td>PICK1</td>
<td>Protein interacting C kinase</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced kinase</td>
</tr>
<tr>
<td>PKCα</td>
<td>Protein kinase C α</td>
</tr>
<tr>
<td>Rbx1</td>
<td>RING-box protein 1</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
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<tr>
<td>RRM</td>
<td>RNA recognition motif</td>
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<td>SCF</td>
<td>Skp1-Cullin-F-box protein</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>Skp1</td>
<td>S-phase kinase-associated protein 1</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>U</td>
<td>Enzyme units</td>
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<tr>
<td>UBL</td>
<td>Ubiquitin like domain</td>
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<tr>
<td>UCH-LI</td>
<td>Ub carboxyl-terminal hydrolase</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasomal system</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
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<tr>
<td>WD40</td>
<td>Beta-transducin repeat</td>
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<tr>
<td>xg</td>
<td>Relative centrifugal force</td>
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<tr>
<td>YPAD</td>
<td>Peptone adenine D-glucose</td>
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<tr>
<td>Ω</td>
<td>Ohm</td>
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E. Abstract

Autosomal Recessive Juvenile Parkinsonism (ARJP) is the most common motor-related neurodegenerative disease, which occurs in young adults between 20-40 years of age. Mutations in the parkin (PARK2) gene are associated ARJP, which result in the loss of dopaminergic neurons of substantia nigra pars compacta in the mid brain. Parkin, an E3 ligase, is an enzyme responsible for ubiquitination of several proteins leading to their degradation in Ubiquitin Proteasomal System (UPS) and promoting autophagy of depolarized mitochondria. Mutations in the parkin gene are thought to result in protein aggregation of parkin substrates resulting in neuronal toxicity. Among these parkin interacting proteins and substrates, several play important roles in mitochondrial function, oxidative stress, cellular toxicity and apoptosis. This project focused on investigating the role of one particular protein that interacts with parkin, namely, PICK1. Specifically, the studies herein investigated the role of a parkin-PICK1 interaction in mitochondrial function and oxidative stress. PICK1 (Protein Interacting C Kinase) is a scaffolding protein, which is thought to recruit PKCa and likely parkin to the outer membrane of mitochondria to regulate cellular apoptosis. To decipher the importance of the parkin-PICK1 protein complex, a 'molecular neuro peptide' MNP201 (VCMGDHWFDV) was engineered to block this interaction. The MNP201 peptide was based on the amino acid sequence of the C-terminus (ct) of parkin, which contains a PDZ motif that interacts with PDZ domain of PICK1. Here, the biological activities of wildtype and mutated versions of MNP201 are reported on mitochondrial function and glutamate release. Firstly, to demonstrate the binding of MNP201 to PICK1, various affinity purification studies and fluorescence polarisation assays were performed. The results suggested that the MNP201 parkin peptide binds to PICK1 in a concentration-dependent manner. Secondly, the effect of MNP201 was tested on reactive oxygen species production, mitochondrial membrane potential and the activity of complex I and complex IV as well as rate of glutamate release. The results suggested that MNP201 had little or no effect on these mitochondrial properties or on the rate of glutamate release. Thirdly, to ensure the entry of MNP201 into synaptosomes, a tat tagged version of MNP201 (Tat-MNP201) was synthesised and its effect on mitochondrial properties and rate of glutamate release was examined. Similar to the non-tagged version of MNP201, no change was observed in the presence of Tat-MNP201 on reactive oxygen species production, the activity of complex I and complex IV or the rate of glutamate release. Importantly, however, the results suggested that Tat-MNP201 increased the rate of mitochondrial depolarization in comparison to control values. Finally, to support the findings that the Tat-MNP201 parkin peptide increases the rate of mitochondrial membrane depolarization, the compound FSC231, which binds the PDZ domain of PICK1, was tested on similar mitochondrial and
glutamate release assays. In agreement with results obtained using Tat-MNP201, the FSC231 compound also increased the rate of depolarization in mitochondrial membrane potential under FCCP-induced stress conditions. Taken together, the data suggested that PICK1 plays a role in the maintenance of mitochondrial membrane potential likely via its PDZ domain interacting proteins such as parkin and PKCa.
1. Introduction
1. Central Nervous System

The central nervous system (CNS) is composed of four major cell types, namely, neurons and the three glial cells: oligodendrocytes, microglia, and astrocytes. The neuron is the structural and functional unit of the CNS responsible for communicating information in both chemical and electrical forms. Functionally, neurons can be divided into sensory (or afferent) and motor (or efferent) neurons. Structurally, a neuron can be divided into three parts, namely, the soma (cell body), dendrites, and the axon. The soma or the cell body of a neuron contains its cellular organelles, including the nucleus, rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), the Golgi apparatus, and mitochondria in a potassium-rich cytosol. Axons originate at the axon hillock of the soma and end in presynaptic terminals forming synapses with postsynaptic terminals of neuronal dendrites. Dendrites are cellular extensions emerging from the soma that can appear highly reticulated, thus are often referred to as dendritic trees. The membrane, in particular the postsynaptic density (PSD), of dendrites contains a high number of cellular receptors, which respond to an array of molecules, including neurotransmitters, neurohormones, and growth factors.

Glial cells, unlike neurons, are capable of mitotic cell division and, in general terms, function to provide nutrition, physical support, and immuno-protection. Broadly, glial cells are divided into two groups, macroglia and microglia. The group of macroglial cells includes astrocytes, oligodendrocytes, and ependymal cells. Astrocytes are the most abundant glial cells in the CNS and are responsible for regulating the fluid-content of the extracellular space in the CNS by active removal of excess ions, including K⁺. These cells also uptake excess neurotransmitters released, such as glutamate, thus can limit excitotoxicity. Astrocytes also release a number of growth factors and cytokines allowing them to communicate with and regulate the function of neuronal, glial, and immune cells. In addition, astrocytes, via their end feet, can ‘synapse’ onto endothelial cells to control the permeability of the blood-brain-barrier. During insult, for example in spinal cord injury, astrogliosis can result, forming scar tissue that acts as a barrier to neuronal regeneration. Oligodendrocytes, on the other hand, are cells that form a myelin sheath that insulates axons and consequently promotes the speed of action potentials and nerve conduction. The loss of oligodendrocyte function, cell numbers, and/or myelin integrity is particularly important in the study of demyelinating disorders such as multiple sclerosis. Although, often forgotten as glial, ependymal cells are members of this cell-type and are involved in cerebrospinal fluid (CSF) production and circulation. These cells form the epithelial lining inside the ventricular cavities of the CNS. The cilia present on the apical surface of these cells are responsible for the circulation of CSF. Lastly, microglia cells are known as the fixed macrophages of the brain, providing a well described immune function in
the nervous system (Kettenmann et al., 2011). The aberrant function of microglia has been associated with a number of neurodegenerative disorders including Alzheimer's disease. These cells release a range of chemokines and cytokines and also play a role in a number of neuroinflammatory disorders. Thus, taken together, neurons and glial cells function in a synchronised manner to maintain synaptic transmission and CNS function.

2. Synaptic transmission

Synaptic transmission, in simple terms, is the conductance of an electric and chemical signal from one neuron to another through the pre-synaptic release of a neurotransmitter, triggered by an action potential, into the synaptic cleft and subsequent binding of the neurotransmitter to post-synaptic receptors and/or ion channels. A wide range of neurotransmitters exist, including amino acids (such as glutamate, glycine, aspartate, D-serine and y-aminobutyric acid or GABA); amines (for example dopamine, norepinephrine, epinephrine, histamine and serotonin); peptides (such as β-endorphin) or others (including acetylcholine, adenosine, anandamide and nitric oxide). Generally, these neurotransmitters are synthesised pre-synaptically and packaged into secretory vesicles. Upon receiving a stimulus, these vesicles fuse to the membrane of pre-synaptic terminal to release the neurotransmitter. The released neurotransmitter then activates the post-synaptic region by binding with their cognate receptors. The synthesis, mechanism of release and function of glutamate are described below, the most common excitatory neurotransmitter present in the CNS.

2.1 Glutamate release

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and was identified by a German chemist Karl Heinrich Leopold Ritthausen in the year 1866 as an amino acid. Glutamate is one of the two excitatory amino acid neurotransmitters (the other being aspartate) that plays a key role in physiological processes of the CNS, such as cognition and learning and memory (Bliss and Collingridge, 1993). In addition, glutamate plays roles in brain development, cellular survival and synaptic plasticity, i.e., formation and elimination of synaptic strength. The mammalian brain is enriched with glutamate, on average 5-15 mmol glutamate per kg brain tissue depending on the region of the brain. However, release of excessive amounts of glutamate causes excitotoxic death of neurons (Bergman et al., 1994; Obeso et al., 2004).

The reserve of glutamate is maintained in neuronal cells by two processes. First, glucose is metabolised to α-oxoglutarate via the Krebs cycle, which is further transaminated to
glutamate by α-oxoglutarate transaminase. Secondly, uptake of released glutamate takes place from the synaptic cleft by the pre-synaptic terminal. Glial cells also take up glutamate with the help of cell membrane expressing glutamate transporters through a high affinity, sodium (Na⁺)-dependent uptake mechanism. In glial cells, glutamate is converted to glutamine by glutamine synthetase enzyme.

The steps involved in glutamate release are outlined below (Figure 1.1)

1. Glutamate is produced from α-oxoglutarate, a byproduct of Krebs cycle. Recycled glutamine is then converted to glutamate by glutaminase in mitochondria present in the neuronal terminal.

2. Glutamate is packaged by active transport into secretory vesicles to form a neurotransmitter filled vesicle. This step occurs through a vesicular transporter present on the surface of vesicles through which glutamate is taken into synaptic vesicles.

3. Upon receiving a stimulus, glutamate filled vesicles travel to the pre-synaptic terminal to fuse with the pre-synaptic membrane. Fusion of the vesicle and the pre-synaptic terminal membranes is mediated by two groups of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins, namely, vesicular SNARE (V-SNARE) and target SNARE (T-SNARE). V-SNARE proteins such as syntaxin and SNAP-25 (soluble NSF attachment protein) are present on the pre-synaptic plasma membrane, while T-SNARE such as synaptobrevin is present on the surface of vesicles (Söllner et al., 1993). These three proteins form a trimeric SNARE complex binding site for proteins, such as NSF (N-ethylmaleimide sensitive factor) and SNAP (soluble NSF attachment protein), which help in the fusion process between the membranes of vesicle and pre-synaptic terminal (Block et al., 1988; Clary et al., 1990). The action potential also triggers influx of calcium ions (Ca²⁺) to bind synaptotagmin, a Ca²⁺ sensor protein present on the vesicle membranes. The activated synaptotagmin binds with SNAP-25 to initiate fusion of the synaptic vesicle with the pre-synaptic membrane (Schiavo et al., 1997). In particular, synaptotagmin binds to syntaxin and VAMP, causing docking and fusion of vesicle and pre-synaptic membranes (Rothman, 1994; Söllner, 1995).

4. Fusion of these two membranes then leads to the exocytosis of glutamate, i.e., release of glutamate into synaptic cleft to activate the receptors at the post-synaptic region and/or auto-receptors at the pre-synaptic site.
Figure 1.1: Glutamate release and mitochondria
Glutamate is a major excitatory neurotransmitter present in the mammalian brain. Glutamate is produced from glutamine by a mitochondrial enzyme glutaminase and packaged into vesicles through vesicular transporters. Action potentials mediate glutamate-filled vesicles to fuse with the pre-synaptic membrane and glutamate is released into the synaptic cleft by exocytosis to bind post- and/or pre-synaptic receptors. Excess glutamate is taken up from the synaptic cleft by glutamate transporters present on pre-synaptic terminals and glial cells. In glial cells, glutamate is converted to glutamine and transported to the pre-synaptic nerve terminals through glutamate transporters. In the pre-synaptic terminal, glutamine is converted to glutamate and included into the packaging vesicles.
5. Glutamate transporters, present on pre-synaptic nerve terminals and adjacent glial cells, uptake glutamate from the synaptic cleft through a high affinity, Na^+-dependent mechanism and repack glutamate into synaptic vesicles for reuse.

6. On the other hand, the glutamate that is taken up by glial cells is converted to glutamine by glutamine synthetase. This glutamine is then transported to the nerve terminals through glutamine transporters present on the surface of glia and nerve terminals.

7. In the pre-synaptic terminal, glutamine is again converted to glutamate by mitochondrial enzyme glutaminase. The recycled glutamate is then repackaged by active transport into the synaptic vesicles to form a neurotransmitter filled vesicles, ready for release.

2.2 Glutamate receptors

Glutamate receptors are divided into two groups, ionotropic and metabotropic glutamate receptors. These receptors are grouped based on sequence homology, pharmacological and electrophysiological properties (Scannevin and Huganir, 2000).

2.2.1 Ionotropic glutamate receptors

There are three subtypes of ionotropic glutamate receptors present on pre- and/or post-synaptic regions of the synapses namely, the 2-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subtype (Nakanishi et al., 1990; Colquhoun, 1992), the N-methyl D-aspartate (NMDA) receptor subtype (Moriyoshi et al., 1991; Kumar et al., 1991; Barnard, 1992) and kainate (KA) receptor subtype (Egebjerg et al., 1991). These receptors have a homologous structure and a common evolutionary origin. Typically, they contain three transmembrane domains (M1, M3 and M4) and one cytoplasmic re-entrant loop (M2) and an intracellular carboxy terminal (ct). The ligand-binding domain is formed by two globular domains S1 (N-terminal end of M1 domain) and S2 (between M3 and M4) (Bleakman and Lodge, 1998; Chen and Wyllie, 2006). These receptors are permeable to cations (Ca^{2+}, Na^+, K^+), where Ca^{2+} is responsible for triggering a number of intracellular signalling cascades (Dingledine et al., 1999). Short descriptions of these receptors are presented below.
AMPA receptor

AMPA receptors, responsible for the fast excitatory neurotransmission, are found ubiquitously expressed in the CNS. Certain areas, including the hippocampus, have a high density of AMPA receptors, where their role in excitatory transmission and synaptic plasticity events such as long term potentiation (LTP) and depression (LTD) have been best described (Ozawa et al., 1998). The AMPA receptor is made up of four subunits, including GluR1-GluR4, which share 65-75% homology in structure. AMPA receptor undergoes splicing and RNA editing, allowing for further diversity. These subunits are believed to form a tetrameric structure by homomeric and heteromeric combination (Chen and Wyllie, 2006). AMPA receptors control the influx of Na⁺ and Ca²⁺; and the presence of the GluR2 subunit determines AMPA receptor-associated Ca²⁺ permeability.

KA receptor

KA receptors are also responsible for the fast excitatory neurotransmission in CNS. KA receptors are distributed in the spinal cord, cerebellum, pyramidal neurons of neocortex and hippocampus (Bleakman and Lodge, 1998). The KA receptors subunit family is composed of five subunits: KA1, KA2, GluR5, GluR6 and GluR7. Similar to AMPA receptor subunits, KA receptor subunits, are approximately 900 amino acids in length, undergo alternative splicing and RNA editing to give rise to a number of receptor isoforms (Chittajallu et al., 1999). Of interest, pre-synaptic KA receptors regulate the release of GABA and glutamate (Darstein et al., 2003; Jaskolski et al., 2004). At post-synaptic terminals, KA receptors are believed to play a role in temporal integration of excitatory signals and regulate neuronal excitability by modulating a low excitatory post-synaptic current (EPSC) (Jaskolski et al., 2004).

NMDA receptor

NMDA receptors are heterotetrameric complexes, responsible for slow phase neurotransmission (Scannevin and Huganir, 2000; Doucet et al., 2012). In general, four subunits assemble to form a functional NMDA receptor ion channel, including two NR1 and two NR2 (A-D) subunits (Doucet et al., 2012). Activation of NMDA receptors takes place by the binding of both glutamate on NR1 and glycine on NR2 (Dingledine et al., 1999). Some NMDA receptors are also reported to contain NR3 (A-B) subunits, which may influence single channel conductance (Dingledine et al., 1999). At a resting membrane potential, NMDA receptors remain blocked by...
extracellular Mg$^{2+}$, which is removed by AMPA receptor-mediated depolarization, making the NMDA receptor functional (Doucet et al., 2012). Activated NMDA receptors allow the influx of high amounts of Ca$^{2+}$ and low amounts of Na$^+$ and K$^+$ (Doucet et al., 2012). The influx of Ca$^{2+}$ triggers a number of intracellular signalling cascades, including a Ca$^{2+}$/calmodulin complex (Doucet et al., 2012). Overactivation of NMDA receptors, however, can lead to excitotoxicity via pathways that stimulate neuronal nitric oxide synthases (nNOS) and nitric oxide (Aarts and Tymianski, 2003).

2.2.2 Metabotropic glutamate receptors

In addition to the ionotropic glutamate receptors indicated above, several G-protein coupled metabotropic glutamate receptors (mGluR1-8) have been identified, which may be present either pre or post-synaptically (Sladeczek et al., 1985; Sugiyama et al., 1987; Schoepp et al., 1990). These receptors are distributed throughout the CNS with specific localisation in synaptic and extra-synaptic areas in neurons and in glial cells (Niswender and Conn, 2010). These receptors (mGluR1-8) have an extracellular N-terminal glutamate binding domain, seven transmembrane domains and an intracellular ct (Doucet et al., 2012). In general, mGluRs can be positively coupled to inositol phosphate or negatively to adenylyl cyclase. Metabotropic glutamate receptors are divided into three subtypes, group I-III.

**Group I**

Group I mGluRs comprise two receptor subtypes, mGluR1 and mGluR5, which are coupled to phospholipase C, responsible for excitatory glutamate release (Doucet et al., 2012). These receptors also activate Ca$^{2+}$ channels but inhibit K$^+$ channels. In addition, these receptors are also known to induce phosphorylation of ion channels and transcription factors (Doucet et al., 2012).

**Group II and III**

The group II (mGluR2 and mGluR3) and group III (mGluR4, 6, 7 and 8) mGluRs are coupled to adenylyl cyclase (Doucet et al., 2012). These receptors, such as mGluR7, can be predominantly located on pre-synaptic terminals and inhibit glutamate and GABA release (Hashimoto, 2009).

2.2.3 Glutamate receptor interacting proteins

Notably, the ct for all glutamate receptor subunits and subtypes are located intracellularly allowing these regions to interact with a number of trafficking and
scaffolding proteins. In particular, the ct of nearly all glutamate receptor subunits and subtype contain PDZ motifs, which allow them to interact with a wide range of PDZ domain containing proteins, such as PICK1. These interactions are vital for the correct temporal (activity-dependent) and spatial (synaptic and extrasynaptic) placement of glutamate receptors at the synaptic membrane of neurons.

2.3 Glutamate excitotoxicity

Excitotoxicity, elicited by glutamate is responsible for many neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) (Beal, 1992; Mattson, 2003). Involvement of mitochondrial dysfunction is also implicated in these above mentioned diseases (Beal, 1992; Doble, 1999; Lin and Beal, 2006). Analysis of the substantia nigra pars compacta (SNPC) region of the mid brain, where selective elimination of dopaminergic neurons occurs in the case of PD, reveals that this area receives abundant glutamatergic excitation from the medial prefrontal cortex, subthalamic nucleus and pedunculopontine tegmental nucleus (Bezard et al., 1997). Reports also suggest that PD induced by the neurotoxin MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine) stimulates excitotoxic levels of glutamate release in the SNPC area (Bezard et al., 1997). Upon administration of MPTP, the rate of firing increases significantly in glutamatergic nerve terminals, likely contributing to the excitotoxic cell death of dopaminergic neurons (Bergman et al., 1994; Obeso et al., 2004). MPTP is an inhibitor of complex I, the first respiratory complex of the electron transport chain (ETC) in mitochondria, and impairment of complex I in the SNPC, as well as the frontal cortex, is widely reported in PD patients (Schapira et al., 1989; 1990; Keeney et al., 2006; Parker et al., 2008). In addition, mitochondrial DNA mutation is also reported to be associated with idiopathic PD (Parker and Parks, 2005). All these studies suggest possible links between glutamate excitotoxicity and complex I mitochondrial dysfunction leading to PD (Greenamyre et al., 2001; Dawson and Dawson, 2003; Tretter et al., 2004).

3. Introduction to mitochondria

Mitochondria produce ATP via the electron transport chain (ETC) and oxidative phosphorylation in the eukaryotic cells. In addition, mitochondria are involved in haem and iron-sulphur biogenesis and the regulation of Ca^{2+} levels in the cell. Mitochondria are the primary source of energy for cells, including neurons, where at least 70% of nerve terminal ATP is reported to be produced by mitochondria (Kauppinen and Nicholls, 1986a). Nerve terminal integrity and neuronal function per se is reliant on mitochondrial aerobic metabolism.
(Gunter et al., 1994) and failure to maintain adequate oxygen supply quickly leads to cell
death (Navarro and Boveris, 2004). The mammalian CNS consists of only 2% of the body
weight, although the metabolic rate is extraordinarily high, consuming 20% of the oxygen
inspired at resting condition (Silver and Erecinska, 1998). Most of this energy is used to
maintain ionic gradients across the cell membranes of neurons. Clusters of mitochondria are
found to be present in specialised areas of neurons, including the synaptic terminals, nodes
of Ranvier and active growth cones; where a high amount of energy and Ca\(^{2+}\) homeostasis
are required (Hollenbeck and Saxton, 2005).

Mitochondria produce energy by various mechanisms, including Krebs cycle and oxidative
phosphorylation. Among all the energy producing mechanisms present in the cell, oxidative
phosphorylation produces the highest amount of energy. The process of oxidative
phosphorylation takes place in the mitochondrial matrix, in particular where energy
producing complexes, known as the ETC, are found present in the inner mitochondrial
membrane.

3.1 Basics of Electron Transport Chain (ETC)

The ETC consists of four prosthetic groups of integral proteins that transfer electrons in
series. These are described below (Figure 1.2).

A. Complex I: The first component of the ETC, complex I (NADH:ubiquinone
oxidoreductase, EC 1.6.5.3), catalyses the oxidation of nicotinamide adenine
dinucleotide (NADH) to NAD\(^+\) at a redox potential of -300 mV, pumping four protons
out of the mitochondrial matrix to the intermembrane space. Coenzyme Q (CoQ) is
reduced to ubiquinol during the oxidation of NADH by complex I. Complex I is
composed of 45 different polypeptide subunits (Carroll et al., 2006), including 6 iron-
sulphur centres (Hinchliffe and Sazanov, 2005) and a flavoprotein (Hatefi, 1985). The
'L-shaped' form of complex I includes a long hydrophobic arm embedded in the
mitochondrial inner membrane and a short hydrophilic arm extended into the
mitochondrial matrix. The short arm contains the flavin mononucleotide (FMN) and
nicotinamide adenine dinucleotide (NADH) active site (Hatefi, 1985). These arms
assemble independently due to their separate genetic origin (Hofhaus et al., 1991). A
neurotoxin, MPTP produces a toxic metabolite, MPP\(^+\) inhibiting complex I to induce
PD (Dauer and Przedborski, 2003). After the discovery of MPTP-induced PD, several
studies have been conducted using complex I inhibitors. Rotenone, generally used as
an insecticide and piscicide (fish toxin), was also found to be a specific and
stoichiometric inhibitor of complex I activity in isolated mitochondria (Chance and
Figure 1.2: ETC cycle of mitochondria
The inner membrane of mitochondria contains all the molecular components for oxidative phosphorylation or ETC to take place. ETC has 5 components, complex I, II, III, IV and ATP synthase (V). Complex I and II use NADH and FAD, respectively, as a proton source ($H^+$). These complexes pump protons (dark arrows) into the intermembrane space, which are eventually used by ATP synthase to produce ATP. In this process the electrons can also flow through the complexes (I-III) to complex IV where water is also produced (Red arrows).
15. Aim of the thesis

PICK1 is monoubiquitinated by parkin, but does not promote PICK1 ubiquitination or degradation (Joch et al., 2007). The primary goal of this study was to decipher the role of the parkin-PICK1 interaction in mitochondrial maintenance. To decipher the role of parkin-PICK1, two types of blocking peptides based on the PDZ motif sequence of parkin (MNP201 and Tat-MNP201) (Figure 1.11) and the PICK1 PDZ domain binding compound, FSC231, (Thorsen et al., 2010) were used. In this thesis, the effect these three types of blocking agents was tested in different mitochondrial properties including the activity of mitochondrial respiratory chains (complex I and IV), ROS production and mitochondrial membrane potential. In addition, the effect of these blocking agents on glutamate release assay was also investigated.

The aims of the thesis were as follows and are presented in the results chapters 1-4:

• demonstrate that parkin blocking peptides (MNP201) interact with PICK1 using biochemical and spectrophotometric approaches (Result 1)

• determine the effects of the parkin blocking peptide, MNP201, in mitochondrial properties and glutamate release (Result 2).

• evaluate the effects of Tat-tagged MNP201 in mitochondrial properties and glutamate release (Result 3).

• examine the effects of FSC231 in mitochondrial properties and glutamate release (Result 4).

16. Hypothesis

The working hypothesis of this project was that parkin-PICK1 plays a role in mitochondrial function and that disruption of this interaction would result in aberrant mitochondrial function.
In addition to blocking peptides, small molecular weight compounds can also be used to block PDZ-based protein interactions (Blazer et al., 2009). A recent discovery of a small molecule inhibitor (E)-ethyl 2-cyano-3 (3, 4-dichlorophenyl) acryloyl carbamate (FSC231), specific for PICK1 PDZ domain, has been reported. FSC231 is a small compound of molecular weight 319 g/mol with a Ki value of 10 μM for the PDZ domain of PICK1 (Thorsen et al., 2010). Studies show that 50 μM FSC231 blocks the interaction between PICK1 and GluR2 (Thorsen et al. 2010). When tested in hippocampal neurons, FSC231 blocks the interaction between GluR2 and PICK1 (Thorsen et al., 2010). Furthermore, FSC231 increased the rate of GluR2 recycling to the cell surface by blocking the PICK1 interaction (Thorsen et al., 2010). FSC231 is also reported to influence synaptic plasticity by inhibiting LTD and LTP in CA1 hippocampal neurons (Thorsen et al., 2010).

Here, the effects of PDZ-motif parkin peptides (MNP201) and of the PICK1 binding compound (FSC231) were determined in mitochondrial pathways to elucidate the role of PICK1 and its interacting proteins in mitochondrial maintenance.
example, a blocking peptide has been used to block the interaction between GluR2 and PICK1 (Daw et al., 2000). It has been shown that introduction of this peptide increases basal synaptic transmission and blocks long term depression (LTD) in CA1 pyramidal neurons of the hippocampus (Daw et al., 2000). In another similar study, employing a peptide with the last ten amino acid of GluR2 (NVYGIESVKI), the interaction between GluR2 and GRIP was blocked (Li et al., 1999). This blocking peptide has been used to show that the interaction of GRIP with PDZ motif of GluR2 is necessary for activation of silent synapses (Li et al., 1999).

Delivery of polypeptides, compounds and peptidyl mimetics into a cell in vivo is possible with molecules of very small size (less than 600 Da) (Scheld et al., 1989). Highly lipophilic, bioactive peptides of a size up to 6 amino acids can be delivered into cells and also cross the blood-brain barrier unassisted (Scheld et al., 1989). To transport bigger peptides into the cell, peptides can be fused with cell penetrating peptides, such as Tat (trans-activation of transcription). Tat, Antennapedia and arginine-rich peptides are also called Trojan peptides. Several studies have been carried out with such trojan peptides, some of which are described below (Lindsay, 2002). A Tat sequence is an 11 amino acid (YGRKKRRQRRR) long stretch of polypeptide derived from the transduction domain (PTD) of human immunodeficiency virus (HIV) (Nagahara et al., 1998). This peptide enters the cell by a mechanism involving adsorptive endocytosis (Mann and Frankel, 1991). Using this technology, large sized proteins (up to 120kDa) have been delivered into a variety of human and murine cell types in vitro (Ezhevshy et al., 1997; Nagahara et al., 1998). For example, a study has showed delivery and expression of Tat tagged β-galactosidase protein (120 kDa) in vivo into mouse tissues, including the brain (Schwarze et al., 1999). In another study, a Tat-fused PDZ motif peptide (of 10 residues), which blocks the PDZ-based interaction between NMDA receptors and PSD-95 has been delivered in vivo in mice and shown to be protective in ischemic brain damaged (Aarts et al., 2002). Antennapedia (RQIKIWFQNRRMKWKK) or penetratin is another well established Trojan delivery peptide that has been used to internalise large molecules including hydrophilic oligonucleotides of 55 bases to cytoplasm and nucleus (Derossi et al., 1996; Prochiantz A, 1996). Antennapedia is derived from the homeodomain of the Drosophila transcription factor Antennapedia and is of 16 residues length (Derossi et al., 1998; Pooga et al., 1998; Astriab-Fisher et al., 2000). The mechanism of Antennapedia internalisation is still unknown, however reports suggest that the process of internalisation of Antennapedia is through a membrane lipid interaction based non-endocytotic and receptor/transporter independent pathway (Derossi et al., 1996; Fisher et al., 2000).
**Figure 1.10: Mechanism of blocking agents to disrupt parkin-PICK1**

A) Parkin and PICK1 interact with each other through a PDZ domain-motif discrete binding site. B) The parkin peptide (MNP201) (shown in red) binds the PDZ domain of PICK1 and competitively inhibit parkin from interacting. The parkin peptide (MNP201) is modelled on and contains the PDZ motif of parkin (-FDV).
GTPase family members including Drp1, OPA1 and Mfn2, which regulate mitochondrial fission and fusion (Poole et al. 2008). Mitochondrial fragmentation induced by Drp1, a protein responsible for division of the outer mitochondrial membrane, is successfully suppressed by over-expression of parkin and PINK1 (Lutz et al., 2009). Moreover, parkin/PINK1 knockdown in Drp mutant cells did not show any of the mitochondrial alterations observed in the parkin/PINK1 knockout phenotype of wild type cells (Lutz et al., 2009). On the other hand, the phenotypes of parkin/PINK1 mutants can be partially recovered by overexpressing Drp1, or by suppressing the gene dosage of the mitochondrial Opal or Mfn2 (Poole et al. 2008; Deng et al., 2008; Yang et al., 2008; Park et al., 2009). Taken together, this indicates that the PINK1/parkin pathway acts to promote mitochondrial fission or to inhibit mitochondrial fusion.

Lastly, it has been shown that parkin plays a role in promoting the removal of depolarised mitochondria through mitophagy, i.e., autophagy of mitochondria by recruitment of autophagosomes (Elmore et al., 2001; Tolkovsky et al., 2002; Twig et al., 2008; Narendra et al., 2008). It has been suggested that parkin interacts with PINK1 by translocating to the outer membrane of depolarized mitochondria in order to poly-ubiquitinate the voltage-dependent anion channel 1 (VDAC1) and the mitofusin protein-2 (Mfn2) (Geisler et al., 2010; Poole et al., 2010; Ziviani et al., 2010). Upon poly-ubiquitination, autophagosomes are recruited to depolarized mitochondria to perform mitophagy (Geisler et al., 2010). Studies identified a protein called Nix (a BH3-only Bcl-2 family protein) that promotes parkin translocation to depolarized mitochondria under CCCP-induced stress generating ROS species (Ding et al., 2010). Taken together these studies infer a role of parkin and PINK1 in regulating the removal of damaged mitochondria. However, the exact mechanism of interaction between parkin/PINK1 and GTPases as well as the trafficking mechanism of parkin to mitochondria is still unknown. It is interesting to speculate that PICK1 plays an important role in targeting parkin to mitochondria and in regulating parkin-mediated mitophagy and clearance of aberrant mitochondria.

14. Use of blocking peptides and compounds

Since PDZ motifs consist 3-4 critical amino acids (Sheng, 2001) it has been possible to engineer blocking peptides composed small stretches of amino acids that carry the PDZ motif sequence and bind the PDZ domain. These peptides thus competitively inhibit cognate PDZ motif containing proteins from interacting with their PDZ domains (Figure 1.10). As an
oxidative stress (Jenner and Olanow, 1996; Zhang et al., 2000) are considered as two main contributors in the aetiology of PD. Analysis of brain samples obtained from PD patients have suggested impairment of complex I activity of the mitochondrial ETC system in the SNPC (Schaipra et al., 1990), frontal cortex (Parker et al., 2008), skeletal muscle and platelets (Bindoff et al., 1989; Parker et al., 1989), along with increased production of ROS and mitochondrial dysfunction (Keeney et al., 2006). Parkin-KO mice have showed hampered activity of complex I and IV of the ETC and reduced striatal mitochondrial respiratory capacity (Palacino et al., 2004). Moreover, PD patients with homozygous mutation in parkin show reduced activity of complex I and IV in the mitochondria of leucocytes (Muftuoglu et al., 2004). In addition, elongated mitochondria are reported in human fibroblasts containing parkin mutations (Mortiboys et al., 2008), which can trigger Bax-induced apoptosis. Many PD-associated PARK proteins are also reported to influence mitochondrial dynamic tubular organization e.g., PARK1, PARK2, PARK6, PARK8, and PARK13. Particularly, in addition to its localisation in the cytosol and ER, parkin is observed around small and fragmented mitochondria in conditions of cell stress (Shimura et al., 1999; Darios et al., 2003; Kuroda et al., 2006; Narendra et al., 2008). Furthermore, parkin is believed to influence the maintenance of the structure and function of mitochondria (Abou-Sleiman et al., 2006), although the mechanism of regulation is still unknown. A Drosophila parkin mutant has also showed malformed mitochondria in dopaminergic neurons (Greene et al., 2003; Whitworth et al., 2005), which can be rescued by over-expressing the fission promoting protein dynamin-related Protein (Drp1) and inhibiting the fusion-promoting protein optic atrophy protein 1 (Opa1) or mitofusin 2 (Mfn2) (Greene et al., 2003; Pesah et al. 2004; Poole et al., 2008).

PINK1, a mitochondrial serine/threonine kinase, is found as one of the important regulator of mitochondrial integrity along with parkin. The PINK1 protein resides on the outer membrane of mitochondria and has a cytosolic kinase domain and a mitochondrial targeting sequence (MTS) (Zhou et al., 2008). PINK1 mutation in Drosophila is reported to cause the phenotypes and mitochondrial morphology similar to parkin mutants flies. Interestingly, parkin is reported to rescue the PINK1 loss-of-function phenotypes, but not vice-versa, leading to the conclusion that PINK1 and parkin both function in a common genetic pathway for mitochondrial maintenance with parkin acting downstream of PINK1 (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). PINK1 over-expression, in association with parkin, regulates mitochondrial morphology (Poole et al., 2008), biogenesis, and enhances replication and transcription of the mitochondrial genome (Kuroda et al., 2006). A link has been established between parkin/PINK1 proteins and mitochondrial fission/fusion (Bleazard et al., 1999; Chan, 2006). Specifically, parkin and PINK1 are found to closely associate with
when cortical neurons were treated with the protein synthesis inhibitor cycloheximide, the basal levels of both parkin and PICK1 were undisturbed for 24 h (Joch et al., 2007). A similar result is obtained when neurons are incubated with the proteasome inhibitor lactacystin. Together, this evidence suggests that monoubiquitination of PICK1 by parkin does not involve its proteasomal degradation (Joch et al., 2007). Furthermore, monoubiquitination of PICK1 by parkin does not regulate PICK1 expression levels, but instead, modulates its function.

12.2 Role of parkin-PICK1 interaction

The PICK1-parkin interaction has been reported as important in regulating the function of the acid-sensing ion channel subunit 2a (ASIC2a) (Baron et al., 2002). ASICs are a mammalian degenerin/epithelial Na⁺ channels (DEG/ENaC) that are activated by low extracellular pH (Duggan et al., 2002; Hruska-Hageman et al., 2002). Four genes (ASIC1-4) encode six subunits of transmembrane channels (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4) (Krishtal, 2003; Xiong et al., 2006). PICK1 interacts with ASIC1 and ASIC2 in a PDZ domain-motif manner and potentiates ASIC2a current by recruiting PKCα (Baron et al., 2002; Duggan et al., 2002; Hruska-Hageman et al., 2002). In agreement, studies on cortical neurons derived from PICK1 knockout (PICK1-KO) mice have demonstrated a reduction of ASIC current as well as decreased expression of ASIC1 and ASIC2 (Hu et al., 2009). Elimination of PICK1/PKCα-dependent ASIC2a currents was observed upon up-regulation of parkin in COS-7 cells suggesting that parkin inhibits PICK1/PKCα-dependent potentiation of ASIC currents (Joch et al., 2007). Studies also show that a loss of parkin increases a PKC-induced potentiation of ASIC2a currents in neurons, although in the absence of PICK1, these ASIC currents remain unaffected when parkin is over-expressed. Taken together, it appears that parkin prevents PICK1/PKCα-dependent potentiation of ASIC channel function, likely via a mechanism that involves PICK1 monoubiquitination and inhibition of interaction with ASIC and/or PKCα (Joch et al., 2007). It is also interesting to speculate that dysfunction of ASIC2a due to a mutation in parkin or PICK1 may be a possible mechanism underlying PD symptoms, as ASIC channels are associated with mechanosensation and pain (Joch et al., 2007).

13. Parkin and Mitochondria

While a number of reports have demonstrated a role for parkin in regulating mitochondrial function, as yet there is no report available indicating a role for a parkin-PICK1 interaction in mitochondria. Mitochondrial dysfunction (Kosel et al., 1999; Beal and Shults, 2003) and
(Stowell et al., 1999). The absence of this targeting sequence impairs the trafficking of mGluR7 to axons, but not to dendrites (Stowell et al., 1999). The distal region of ct-mGluR7 contains a PDZ binding motif (-LVI) that interacts with PDZ domain of PICK1 (Boudin et al., 2000; El Far et al., 2000; Dev et al., 2000). The PDZ binding motif of mGluR7 is essential for mGluR7 clustering at synapses (Boudin et al., 2000).

12. Parkin-PICK1 interaction

12.1 The PDZ motif of parkin

The ct-parkin contains a class II PDZ binding motif (-FDV), which interacts with the PDZ domain of CASK (Ca\(^{2+}\)/calmodulin-dependent serine protein kinase) (Fallon et al., 2002). It has been reported that truncation of last 3 amino acids (W453*) makes parkin insoluble (Joch et al., 2007), supporting the hypothesis the ct-parkin is important for folding, trafficking, and/or degradation (Winklhofer et al., 2003). As described earlier, PICK1 can interact with class I and class II PDZ motifs (Staudinger et al., 1997; Dev et al., 1999; Williams et al., 2003; Madsen et al., 2005) and a parkin-PICK1 interaction has been demonstrated by pull down and immunoprecipitation studies (Joch et al. 2007). In pull down studies, endogenous PICK1 from mouse brain synaptosomes associated with bacterial expressed GST-tagged parkin via its PDZ motif (-FDV). In addition, transiently expressed constructs of parkin was found to immunoprecipitate with transiently expressed PICK1 (Joch et al. 2007). In contrast, PDZ motif deleted parkin constructs (D464*) failed to interact with PICK1, indicating involvement of the PDZ motif of parkin with PICK1. Moreover, PICK1 mutant with point mutations {lysine-27 and aspartic acid-28 (KD/AA)} in the carboxylate binding loop of PDZ domain failed to with parkin (Joch et al., 2007). The KD residues form the carboxylate binding loop of PICK1’s PDZ domain (Doyle et al., 1996; Songyang et al., 1997) and are crucial for PICK1 to interact with its ligands (Staudinger et al., 1997; Xia et al., 1999). Taken together, these data suggest parkin and PICK1 interact in a PDZ-dependent manner (Joch et al. 2007).

Parkin functionally effects PICK1 activity. Unlike CASK, parkin induces the monoubiquitination of PICK1 (Joch et al. 2007). In contrast, a parkin mutant associated with PD and lacking the PDZ binding motif is not able to ubiquitinate PICK1. Monoubiquitination alters the protein-protein interactions and protein trafficking of PICK1, but not its degradation (Hicke and Dunn, 2003; Mukhopadhyay and Riezman, 2007). In support that parkin does not alter PICK1 protein levels, the basal expression of PICK1 in whole brain lysates and synaptic fractions obtained from normal and parkin-KO mice were found to be similar. In addition,
(ABP) (Srivastava et al., 1998; Dev et al., 1999; Xia et al., 1999). In this project, the interaction between PICK1 and GluR2 was used as a control.

11.3.2 GluR5 (KA receptor subunit)

GluR5 is one of the 5 subunits of KA receptors. KA receptors are found in pre and post-synaptic sites and at pre-synaptic sites can inhibit glutamate release (Chittajallu et al., 1999). Similar to GluR2, the ct-GluR5 subunit also interacts with several PDZ domain containing proteins including syntenin, GRIP, PSD95 and PICK1 through its PDZ motif (-TVA) present in the extreme ct (Hirbec et al., 2003). According to electrophysiological studies, infusion of blocking peptides, designed to block the interaction of PICK1-GluR5, causes a substantial reduction in EPSCs (Hirbec et al., 2003). In the same experimental setup, when AMPA receptor was activated, strong reduction in the EPSCs of KA receptors was observed (Hirbec et al., 2003). These data indicate a role of the PICK1-GluR5 interaction in regulation of synaptic KA receptor function (Hirbec et al., 2003). GluR5 also interacts with PKCa, where PKCa selectively phosphorylates two serine residues (S880 and S886) residues in GluR5 (Hirbec et al., 2003).

11.3.3 mGluR7 (Metabotropic receptor subtype)

The mGluR7 receptor belongs to the Group III of metabotropic glutamate receptors (Nakanishi, 1994; Pin et al., 1995). Like other Group III members, mGluR7 negatively regulates cAMP. The mGluR7 is found on the pre-synaptic membrane (Shigemoto et al., 1996) and inhibits glutamate release (Forsythe et al., 1990; Gereau et al., 1995; Herrero et al., 1996). The ct-mGluR7 stretch of 65 amino acids plays an important role in protein-protein interactions. There are several proteins including PICK1, Ca2+/Calmodulin (CaM) and PKC reported to interact with ct-mGluR7 to regulate its function and intracellular trafficking (O’Conner et al., 1999; Nakajima et al., 1999; Boudin et al., 2000; El Far et al., 2000; Dev et al., 2000). On the basis of these interacting partners, the ct-mGluR7 can be divided into three parts, i.e., the proximal region, the central region and the distal region. The proximal region interacts with the βγ-subunit of G-protein and CaM (O’Connor et al., 1999; Nakajima et al., 1999). Upon phosphorylation (by PKC) of the CaM binding site of mGluR7, the interaction between CaM and mGluR7 is lost, although CaM itself is not phosphorylated by PKC (Minakami et al., 1997; Ishikawa et al., 1999; O’Connor et al., 1999; Nakajima et al., 1999). Previous reports suggest that CaM has an active role in the dissociation of the βγ-subunit of G-protein from mGluR7 that results in Gβγ-induced inhibition of Ca2+ gated channels (Dev et al., 2001). The central region of ct-mGluR7 contains an axonal targeting sequence (amino acid 883-912), which is responsible for trafficking of mGluR7 receptors.
to the DAT-PICK1 interaction (Torres et al., 2001). Yeast two hybrid assay showed that deletion of these last three amino acids completely abolished the interaction with PICK1 (Torres et al., 2001). The association between PICK1 and NET was confirmed in co-IP and co-localisation studies using transfected HEK293 cells (Torres et al., 2001). Endogenous PICK1 and NET were also found co-localised in cultured neurons obtained from the norepinephric locus coeruleus, where both PICK1 and NET were found clustered along neuronal processes (Torres et al., 2001).

11.2.3 Serotonin transporter

Serotonin transporter (SERT) is a monoamine transporter responsible for the uptake of serotonin from the extra-cellular synaptic environment (Jones et al., 1998). SERT are predominantly found on serotoninergic neurons. Immunocytochemical data suggests an abundance of SERT proteins in the axolemma, outside the synaptic clefts of serotoninergic neurons (Zhou et al., 1998; Tao-Cheng and Zhou 1999). SERTs are also found to be associated with DAT in the membrane of intracellular compartments, mainly in tubulovascular membranes (Torres et al., 2001). Similar to DAT and NET, SERT also binds the PDZ domain of PICK1 through the last three ct amino acids (-NAV) (Torres et al., 2001). However, the PDZ motif present in SERT is not a typical class II motif and thus the interaction with PICK1 is weaker than DAT or NET (Torres et al., 2001).

11.3 Glutamate receptors

11.3.1 GluR2 (AMPA receptor subunit)

One of the best studied PICK1 interacting protein is GluR2. The PDZ domain of PICK1 interacts with last ct-GluR2 (-LVI) (Dev et al., 1999; Xia et al., 1999). As indicated above, GluR2 is an AMPA receptor subunit that controls $\text{Ca}^{2+}$ permeability of the AMPA receptor. Typically, the GluR2 polypeptide is made up of four hydrophobic membrane associated domains in which 1, 3 and 4 are transmembrane and the second domain is a re-entrant loop (Wo and Oswald, 1995). The N-terminal and the ct of GluR2 contain many glycosylation and phosphorylation sites, respectively (Roche et al., 1994; Taverna et al., 1994; Moss et al., 1993). AMPA receptors are regulated by phosphorylation, where interaction with PICK1 plays an important role. Briefly, interaction with PICK1 regulates the surface expression of GluR2 and controls synaptic plasticity such as LTD (Xia et al., 1999). In addition to PICK1, GluR2 also interacts with other PDZ domain containing proteins, including glutamate receptor interacting protein (GRIP) (Dong et al., 1997) and AMPA receptor binding protein.
apoptotic Bax protein in human leukemia REH cells (Wang et al., 2007). Thus, PICK1 along with PKCα appear to be crucial in mitochondrial maintenance and cellular survival. The disruption of a PICK1-PKCα interaction is thus likely to result in mitochondrial dysfunction.

11.2 Monoamine Transporters

11.2.1 Dopamine transporter

Dopamine transporters (DAT) are biogenic monoamine plasma membrane proteins responsible for high affinity uptake of released dopamine through pre-synaptic sites of dopaminergic neurons (Jones et al., 1998). Monoamine transporters including DAT are reported to be involved in many neuro-psychiatric disorders including depression and obsessive-compulsive disorder (Giros and Caron, 1993; Amara and Kuhar, 1993). The importance of DAT has been elucidated using knockout mice, which show hyperlocomotion and differential cocaine and amphetamine sensitivity (Giros et al., 1996; Jones et al., 1998). DAT is primarily found near the site of pre-synaptic release in nigrostriatal dopaminergic neurons and uptake of released dopamine (Nirenberg et al., 1996; Hersch et al., 1997; Pickel and Chan 1999). The ct-DAT contains a class II PDZ motif (-LKV) and interacts with the PDZ domain of PICK1, as shown by yeast two hybrid, co-IP and co-localisation studies (Torres et al., 2001). When both DAT and PICK1 are co-expressed in HEK293 cells, both proteins cluster in the plasma membrane (Torres et al., 2001). Upon co-expression with PICK1, the uptake activity rate of DAT was found to be enhanced (Torres et al., 2001). DAT also co-localises with other proteins including the vesicular monoamine transporter-2 (a synaptic vesicle protein expressed in monoaminergic neurons) and synaptin (a pre-synaptic protein) in the axons of mid brain neurons (Torres et al., 2001). When DAT and PICK1 protein were co-expressed in immortalized dopaminergic neurons (1RB3AN27) (Clarkson et al., 1999), 50% enhancement of DAT uptake was found when compared with single transfected DAT cells suggesting that PICK1 helps trafficking of DAT (Torres et al., 2001).

11.2.2 Norepinephrine transporter

Norepinephrine transporter (NET) is a monoamine transporter responsible for the uptake of released norepinephrine from the synaptic cleft of norepinephrinergic neurons. The importance of this transporter was revealed when NET knockout mice showed increased sensitivity in D2/D3 receptors and sensitization to cocaine and amphetamine (Xu et al., 2000). NET shares approximately 78% amino acid sequence similarity with DAT (Pacholczyk et al., 1991; Giros et al., 1991; Kilty et al., 1991; Vandenbergh et al., 1992). The ct-NET contains a Class II PDZ motif (-LAI) that also binds the PDZ domain of PICK1, similar
<table>
<thead>
<tr>
<th>Name of protein</th>
<th>Sequence of interaction</th>
<th>PDZ motif</th>
<th>Reference</th>
</tr>
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<tr>
<td>Protein kinase C</td>
<td>NPQFVHPILQSAV</td>
<td>I</td>
<td>Staudinger et al., 1995</td>
</tr>
<tr>
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<td>II</td>
<td>Torres et al., 1998</td>
</tr>
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<td>Ephrin B1</td>
<td>MPPQSPANIYYKV</td>
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<td>Torres et al., 1998</td>
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<tr>
<td>Ephrin receptor B2</td>
<td>MRAMQNIQSVSVEV</td>
<td>II</td>
<td>Torres et al., 1998</td>
</tr>
<tr>
<td>Muscle-specific kinase</td>
<td>ERMICERAEQTVS</td>
<td>II</td>
<td>Torres et al., 1998</td>
</tr>
<tr>
<td>GluR2 (AMPAR)</td>
<td>EGYNVYGIESVI</td>
<td>II</td>
<td>Xia et al., 1999, Dev et al., 1999</td>
</tr>
<tr>
<td>GluR3/4 (AMPAR)</td>
<td>EGYNVYGESTVI</td>
<td>II</td>
<td>Dev et al., 1999</td>
</tr>
<tr>
<td>GluR4C (AMPAR)</td>
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</tr>
<tr>
<td>mGluR7A</td>
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<td>II</td>
<td>El Faret al., 2000, Hirbec et al., 2002</td>
</tr>
<tr>
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<td>QKSVTWYTIPTV</td>
<td>?</td>
<td>Hirbec et al., 2002</td>
</tr>
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<td>Anionic exchanger 1</td>
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<td>?</td>
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<td>Aquaporin 1</td>
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</tr>
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<td>I</td>
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<td>Cowan et al., 2002</td>
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<td>ARF3 GTPase</td>
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<td>Dopamine transporter</td>
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<td>Torres et al., 2001</td>
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<tr>
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<td>?</td>
<td>Torres et al., 2001</td>
</tr>
<tr>
<td>Serotonin transporter</td>
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<td>?</td>
<td>Torres et al., 2001</td>
</tr>
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<td>II</td>
<td>Lin et al., 2001</td>
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<td>II</td>
<td>Lin et al., 2001</td>
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<tr>
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<td>?</td>
<td>Hruska-Hageman et al., 2002</td>
</tr>
<tr>
<td>BNaC2/ASIC1a</td>
<td>HHPARTGFEDFTC</td>
<td>?</td>
<td>Hruska-Hageman et al., 2002</td>
</tr>
<tr>
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<td>5PDAGLFTVSEAEC</td>
<td>?</td>
<td>Williams et al., 2003</td>
</tr>
<tr>
<td>CAR cell adhesion</td>
<td>VMIPAQSKDGGSIV</td>
<td>I</td>
<td>Ashbourne et al., 2004</td>
</tr>
</tbody>
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**Table 1.4: List of PICK1 interacting proteins**

Various proteins interacting with PICK1's PDZ domain are depicted in the table (Madsen et al., 2005).
Figure 1.9: The PICK1 interacting partners
A) PICK1 acts as scaffolding and adapter protein to its interacting partners (parkin, GluR2, GluR5, mGluR7, PKCa) and transporter proteins (dopamine, norepinephrine and serotonin). B) PKCa comprises N-terminal diacylglycerol binding C1 domain (C1), a Ca^{2+}-binding C2 domain (C2), an ATP binding domain (ATP-BD) and a catalytic domain. The catalytic last 3 amino acids (-SAV) of PKCa interacts with the PDZ domain of PICK1.
1997; Sheng, 2001). The PDZ domain of PICK1 can interact with a range of different PDZ motif containing proteins including Class I, Class II and atypical PDZ motifs (Staudinger et al., 1997; Dev et al., 1999; Madsen et al., 2005).

11. PICK1 interacting proteins

More than 60 proteins interact with PICK1. Some important PDZ motif containing proteins are explained below (Figure 1.9A). A summary of interacting partners of PICK1 are depicted in Table 1.4.

11.1 Protein kinase Ca (PKCa)

There are 11 members of the PKC family, which play roles in a variety of cellular responses and signalling cascades (Nishizuka, 1992; Newton et al., 1995). These isoenzymes are translocated to different cellular compartments including the plasma membrane, the cytoskeleton and cellular organelles (Mochly-Rosen et al., 1995; Goodnight et al., 1995). Different stimuli result in translocation of PKC to different cellular compartments (Hocevar et al., 1991; Yedovitzky et al., 1997). Commonly, isoforms of PKC have similar structural organization, i.e., an amino terminal regulatory domain, a carboxyl-terminal catalytic domain and a linking central hinge region (Figure 1.9B). The last ct amino acids (-SAV) of PKCa specifically interact with the carboxylate-binding domain present in the PDZ domain of PICK1 (Staudinger et al., 1995). Sequence analysis of PKCa reveals absence of any mitochondrial targeting signal peptide. However, reports suggest that a major function of PICK1 is to bind and translocate activated PKCa to mitochondria (Wang et al., 2003). A PDZ domain mutant of PICK1 is unable to mediate mitochondrial translocation, suggesting an intact PDZ domain is necessary for this PKCa translocation to mitochondria (Wang et al., 2003). Absence of the PDZ binding motif of PKCa impairs its translocation to the mitochondria, although it does not affect the translocation of PICK1 (Wang et al., 2003). Mitochondria-mediated apoptotic pathway is regulated by two groups of proteins, anti-apoptotic proteins, such as B-cell lymphoma-2 (Bcl-2) and pro-apoptotic proteins, such as Bax. Upon activation of apoptotic signal, Bax dimerises with Bcl-2 in outer mitochondria and triggers apoptosis by releasing of cytochorome C in cytosol (Pawlowski and Kraft, 2000). Reports suggest that via phosphorylation of the anti-apoptotic protein Bcl-2 (B-cell CLL/lymphoma 2) present in the outer membrane of the mitochondria, PKCa confers resistance against apoptosis (Nguyen et al., 1993; Ruvolo et al., 1998; Jiffar et al., 2004). PICK1 over-expression increases the phosphorylation of Bcl-2 protein, which confers stabilisation of mitochondrial membrane potential and resistance to dimerisation of pro-
Figure 1.8: Structure and protein sequence of PICK1
A) PICK1 is a PDZ domain containing protein that interacts with several PDZ motif containing proteins. A small acidic region precedes the PDZ domain. The largest domain in PICK1 protein is the BAR domain (144-357), which plays a role in PICK1 dimerisation. The PDZ domain and the BAR domain are linked by a small stretch of α-helical region. The ct of PICK1 contains a long acidic region (AR). B) The protein sequence of PICK1 is shown with different domains.
The CK-1 enzyme can also phosphorylate parkin at multiple sites (S101, S127 and S378) both in vitro and in vivo (Rubio de la Torre et al., 2009). Here also, phosphorylated parkin shows a tendency to form inclusion bodies (Rubio de la Torre et al., 2009). Analysis of PD brain samples reveal increased levels of phosphorylated parkin (phospho-S101) in Lewy bodies (Rubio de la Torre et al., 2009). The above findings indicate that phosphorylation of parkin has a role to play in the formation of parkin inclusion bodies.

10. PICK1 as a PDZ domain protein

10.1 Introduction to PICK1

PICK1 (Protein Interacting C Kinase) is a synaptic protein of 416 amino acids, which scaffolds and interacts with more than 60 proteins including ion channels, transporters and receptors (Dev et al., 1999). Although the expression of PICK1 is highest in the CNS, PICK1 is ubiquitously present in a number of tissues (Staudinger et al., 1995). Subcellularly, PICK1 is localised in perinuclear regions, RER and Golgi complex (Staudinger et al., 1995).

Structurally, PICK1 is made up of 2 domains Figure 1.8,

1. PDZ domain: The PDZ (PSD95/DlgA/ZO-1) domain of PICK1 is located at the N-terminus of PICK1 immediately after a small stretch of acidic residues. Through the PDZ domain, PICK1 interacts with several PDZ motif containing proteins.

2. BAR domain: The BAR (Bin-Amphiphycin-Rvs) domain is located ct to the PDZ domain after an α-helical linker region of 40 residues. The BAR domain is the largest domain of PICK1 that also corresponds to a coiled-coil domain. The ct of the BAR domain contains a long stretch of acidic residues.

Functionally, these two domains allow PICK1 to dimerise and act as a scaffolding protein. The major function of PICK1 is to regulate the trafficking and phosphorylation of interacting proteins by recruiting protein kinase C-α (PKCα) (Staudinger et al., 1995). PKCα is a member of the serine/threonine kinases PKC family, which plays a role in various physiological processes, including differentiation, proliferation, motility, inflammation and apoptosis (Nakashima, 2002). Generally, PDZ domains can be classified into three classes according to the type of PDZ motifs they bind (Sheng, 2001). Class I PDZ domains bind specifically to the tripeptide sequence (S/T)-X-φ (where φ and X denote a hydrophobic amino acid and any amino acid respectively), class II PDZ domains bind the ct PDZ motif sequence φ-X-φ and class III PDZ domains bind D-E-X-φ PDZ motifs (Songyang et al.,
Figure 1.7: Phosphorylation sites of parkin and responsible kinases. Several kinases are reported to phosphorylate parkin e.g., caesin kinase 1 (CK-1) phosphorylates S101, S127 and S378; PTEN-induced putative kinase 1 (PINK1) phosphotylates T175 and cyclin-dependent kinase 5 (Cdk5) phosphorylates S131. The S136 residue is also phosphorylated by unknown kinases. The kinases and the amino acids they phosphorylate are indicated in the same colour.
and non-neuronal cells (Huynh et al., 2000; Lazaro-Diegeuez et al., 2008). Oxidative stress and mitochondrial complex I inhibition has been reported to increase actin-parkin interaction in dopaminergic cell lines (SN4741) showing the importance of actin dynamics in neurodegeneration and PD (Kim and Son, 2010).

9. Post-translational modification in parkin

Parkin undergoes various post-translational modifications including nitrosylation and phosphorylation. In nitrosylation, modification of a protein occurs by the addition of a nitrosyl group mediated by nitric oxide producing enzymes. S-nitrosylation, is the addition of a S-nitrosothiols group to the cysteine residue of a protein. Parkin undergoes S-nitrosylation at three cysteine residues present in the IBR domain (Chung et al., 2004). Nitrosylation of parkin is mediated by nNOS and inducible nitric oxide synthase (iNOS), both of which inhibit the E3 ligase activity of parkin (Chung et al., 2004). Mice treated with MPTP show increased amounts of S-nitrosylated parkin in brain (Chung et al., 2005). Post-mortem PD brain samples also show high amounts of S-nitrosylated proteins, indicating high levels of nitrosative stress in PD patients (Chung et al., 2004). Taken together, these data suggest that parkin nitrosylation increases protein aggregation, which may play a major role in the pathophysiology of PD.

In phosphorylation, a phosphate (PO$_4^{3-}$) group is added to a protein, which is another post-translational process that regulates parkin activity. Several phosphorylation sites have been identified in parkin (Figure 1.7), where phosphorylation has been shown to regulate E3 ligase activity and solubility of parkin. The first report of parkin phosphorylation identified five serine phosphorylated sites (S101, S131, S136, S296 and S378), which are phosphorylated in vitro by protein kinases including casein kinase-1 (CK-1), protein kinase A (PKA) and protein kinase C (PKC) (Yamamoto et al., 2005). In addition to serine residues, threonine residues of parkin are also phosphorylated. The phosphorylation of parkin is found to reduce under ER stress (Yamamoto et al., 2005). Subsequent studies reveal the involvement of cyclin-dependent kinase 5 (Cdk5) in the in vivo and in vitro phosphorylation of serine 131 of parkin (Avraham, 2007). This phosphorylation leads to the reduction of parkin's E3 ligase activity, both in the auto-ubiquitination and in the ubiquitination of major substrates, including synphilin-1 and p38 (Avraham, 2007). Mutated S131A parkin is found to form inclusion bodies with synphilin-1/a-synuclein, indicating a link between phosphorylation and ubiquitination (Avraham, 2007).
ubiquitinates polyglutamine proteins through an HSP70 interaction, thus likely functions in a similar manner as CHIP in degrading proteins via binding to CHIP and/or Hsp70 (Tsai et al., 2003). In agreement with the idea that parkin functions with CHIP, it has been shown that HSP70 degrades PAEL-R in concert with parkin and CHIP. Specifically, unfolded PAEL-R translocates to the cytosol and binds HSP70, initiating an up-regulation of CHIP (Takahashi and Imai, 2003). CHIP then initiates the dissociation of HSP70 from PAEL-R and aids in the binding of parkin as well as the ubiquitination of PAEL-R (Takahashi and Imai, 2003).

8.4 Parkin scaffolding proteins

8.4.1 PDZ domains (CASK and PICK1)

CASK is a member of the membrane-associated guanylate kinase (MAGUK) protein family, which contains a protein-protein interaction PDZ domain. CASK is also known as CAMGUK protein 2 (CMG 2), Ca²⁺/calmodulin-dependent serine protein kinase 3, membrane-associated guanylate kinase 2 and is the homolog of Lin-2 in C.elegans. In mammalian cells, CASK forms a tripartite protein complex with Veli and Mint proteins that interacts with a receptor tyrosine kinase, LET-23 (Butz et al., 1998). The PDZ domain of CASK interacts with the ct of neurexin, the receptor for α-latrotoxin (black widow spider venom) (Hata et al., 1996) as well as syndecan (a cell surface heparan sulfate proteoglycan) (Hsueh et al., 1998). Similarly, the last 3 amino acids of ct-parkin associate with the PDZ domain of CASK in cortical neurons, post-synaptic densities and lipid rafts (Fallon et al., 2002). The interaction between CASK and parkin results in the polyubiquitination of CASK leading to its proteasomal-mediated degradation. In addition to the PDZ motif of parkin interacting with the PDZ domain of CASK, the PDZ motif of parkin also interacts with the PDZ domain of PICK1 (Joch et al., 2007). PICK1 (protein interacting C kinase 1) is a scaffolding protein that interacts with various proteins. PICK1 and the parkin-PICK1 interaction are explained in detail in section 10.

8.4.2 Actin

Actin is highly conserved ubiquitous major cytoskeletal protein responsible for composing microfilaments, which is known to participate in many important cellular functions including cell signalling, apoptosis, vesicle and organelle movement and structure organization (Kim and Son, 2010). ROS is found to disrupt actin dynamics which may lead to apoptosis in Saccharomyces cerevisiae (Gourlay and Ayscough, 2005; Franklin-Tong and Gourlay, 2008). Parkin specifically interacts with actin and regulates actin remodeling, colocalizing with actin filament and interacting with LIM kinase-1 (actin binding kinase) in both neuronal
8.3 E3 ligase partners of parkin

8.3.1 Cdc4α

Cdc4 (also known as hSel10, Archipelago, Fbw7 and Fbw7) is an F-Box/WD repeat protein of 707 amino acids long and is an integral part of Skp1, Cul1, F-box containing complex (SCF) E3 complex. The SCF complex contains Skp1 (S-phase kinase-associated protein 1), Cul1 (cullin 1), Rbx1/Roc1 (RING-box protein 1) and an F-box containing protein, such as Cdc4. There are multiple F-box containing proteins, which have specificity towards a particular set of substrate proteins through unique protein–protein interactions. A variety of RING finger containing proteins are integrated in this multiprotein ubiquitin ligase complex (Joazeiro and Weissman, 2000). Parkin can also act in concert with other proteins to form a multiprotein ubiquitin ligase complex, including SCF, which ubiquitinates several proteins. Cdc4 has three isoforms due to alternative splicing in mammals, Cdc4α, Cdc4β and Cdc4γ (Spruck et al., 2002), which are located in nucleoplasm, cytoplasm and nucleolus, respectively (Welcker and Clurman, 2004). SCF Cdc4α ubiquitinates a variety of substrates, such as cyclin E, peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), presenilin, c-Myc, c-Jun, Notch and sterol regulatory element binding proteins (SREBP) (Hubbard et al., 1997; Gupta-Rossi et al., 2001; Koepp et al., 2001; Moberg et al., 2001; Oberg et al., 2001; Strohmaier et al., 2001; Nateri et al., 2004; Welcker and Clurman, 2004; Yada et al., 2004; Sundqvist et al., 2005). Parkin specifically interacts with Cdc4α (Koepp et al., 2001; Moberg et al., 2001; Strohmaier et al., 2001; Wu et al., 2001). Deletion constructs of parkin have revealed that both the RING fingers, situated on the carboxyl terminus of parkin, interact specifically with the F-Box of Cdc4α. A second interaction site is found within the Ubl domain of parkin and the WD repeat domain of Cdc4α (Staropoli et al., 2003). This complex ubiquitinates target proteins with the help of the E2 UbcH7 enzyme (Staropoli et al., 2003). It is also reported that E3 enzyme activity of parkin is enhanced by the over-expression of Cdc4α (Staropoli et al., 2003). In addition, along with co-expression of Cdc4α, over-expression of UbcH7 is needed to enhance parkin-mediated ubiquitination (Staropoli et al., 2003). Thus, parkin functions in concert with Cdc4α and UbcH7.

8.3.2 Heat shock protein 70 (HSP70) and CHIP

Parkin is associated with heat shock protein 70 (HSP70) and the E3 carboxyl terminus of HSC70-interacting protein (CHIP). Originally reported as Hsc/HSP70 binding protein, CHIP functions as an E3 ligase to ubiquitinate and degrade its interacting proteins (Murata et al., 2001). Ubiquitination by CHIP and recognition of abnormal proteins by HSP70 is important for degradation of a number of proteins (Imai and Takashashi, 2004). Parkin also
suggest that the parkin Ubl domain interacts with both UIMs of S5a. The lysine (K48) residue of the Ubl domain in parkin is a crucial amino acid in recognition of S5a subunit (Safadi and Shaw, 2010).

8.2.9 Endocytic protein Eps15

Epidermal growth factor receptor substrate 15 (Eps15), encoded by EPS15 gene in human, is an adapter protein involved in epidermal growth factor (EGF) receptor (EGFR) endocytosis and trafficking (Fallon et al., 2006). Upon EGF stimulation, the Ubl domain of parkin interacts with Eps15 and facilitates the monoubiquitination of this protein (Fallon et al., 2006). The binding of the Ubl domain of parkin to Eps15 is essential for ubiquitination of Eps15 (Fallon et al., 2006) such that in null-parkin cells, acceleration of EGFR endocytosis and degradation is observed (Fallon et al., 2006). Furthermore, reduction of EGFR signalling via the phosphoinositide 3-kinase (PI3K)-Akt pathway in parkin knockout (parkin-KO) mice suggests that parkin may interfere in the association of Eps15 and ubiquitinated EGFR, resulting in delayed EGFR internalisation and degradation and promotion of PI3K-Akt signalling (Fallon et al., 2006).

8.2.10 Non-receptor tyrosine kinase c-AbI

Non-receptor tyrosine kinases or non-specific protein-tyrosine kinases are a large family of kinase enzymes responsible for the ATP-dependent phosphorylation of tyrosine residues. One of the members of tyrosine kinase family, c-AbI (a homolog of transforming element of the Abelson murine leukemia virus), present in the nucleus and cytoplasm, is predominantly activated by cellular stress (Hantschel et al., 2004). The c-AbI protein is involved in neuronal plasticity, neurite outgrowth and neurogenesis in brain and also has role to play in tumorigenesis (Reddy et al., 1983; Moresco and Koleske, 2003). Aberrant activation of c-AbI kinase was found in neurological disorders, including AD and Niemann-Pick type-2 disease (Alvarez et al., 2004 and 2008). Stress-induced activation of c-AbI by dopaminergic neurotoxins, such as 1-methyl-4-phenylpyridinium (MPP⁺) and MPTP causes phosphorylation of tyrosine residue (Y143) of parkin (Ko et al., 2010). In addition, the SH3 domain of c-AbI interacts with the RING finger and IBR domains of parkin (Ko et al., 2010). Phosphorylation of parkin inhibits ubiquitination activity of parkin and causes accumulation of parkin substrates that ultimately leads to neuronal death (Ko et al., 2010). Inhibition of c-AbI expression demonstrated neuroprotective effects in case of stress-induced by neurotoxins (Ko et al., 2010).
ARS complex primarily involved in protein synthesis (Ko et al., 2005). The aminoacyl-tRNA synthase (ARS) complex including the p38 subunit is associated with protein biogenesis in a number of tissues as well as in the brain. Upon over-expression of p38 by adenoviral-mediated transfection, selective neuronal death is observed (Ko et al., 2005). Furthermore, the p38 subunit protein was identified in proteinaceous aggregations of sporadic PD patients as well as the Lewy bodies of idiopathic cases of PD (Ko et al., 2005). The RING finger domain of parkin interacts with the 82-162 amino acids of p38 and mediates its ubiquitination for UPS (Ko et al., 2005), likely controlling the neurotoxic effects of high protein levels of p38.

8.2.8 Proteasomal subunit S5a

Substrate recognition and recruitment of ubiquitinated substrates for the 26S proteasome-dependent protein degradation are two important steps in UPS, where E3 ligases play a central role. The N-terminal UbI domain of parkin, responsible for substrate recognition for ubiquitination, serves as the bridge between the ubiquitinated protein and the 26S proteasome through specific protein-protein interaction. S5a, also called as Rpn10, is a subunit of 19S regulatory protein that serves as the cap structure for 26S proteasomal subunit. Otherwise conjugated with 26S proteasome, a freely available monomeric form of S5a is also found in cytosol. Most E3 ligases, in poly-ubiquitinated conditions, are recognised by S5a (Uchiki et al., 2009). Upon recognition, the S5a subunit undergoes an E3 ligases-mediated poly-ubiquitination, though the poly-ubiquitination of S5a does not lead to its degradation (Uchiki et al., 2009). S5a has a strong affinity towards poly-ubiquitin chain through its two ubiquitin interacting motifs (UIM) that identify poly-ubiquitinated substrates (Uchiki et al., 2009). The presence of intact UIMs of S5a is important for the ubiquitination process as mutation in both UIMs abolishes the ubiquitination of S5a by various E3 ligases (Uchiki et al., 2009). A variety of E3 ligases including monomeric RING finger containing E3 ligases such as parkin {and others including muscle RING-finger protein-1 (MuRF1), murine double minute 2 (Mdm2) and seven in absentia homolog 2 (Siah2)}, multimeric RING finger containing E3 ligases {adenomatous polyposis coli (APC)}, U-box containing E3 ligases {E3 carboxy terminus of HSC70-interacting protein (CHIP)} and HECT domain E3 ligases {E6 associated protein (E6AP) and neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4)} interact with S5a subunit to ubiquitinate their interacting partners (Murata et al., 2001; Kedar et al., 2004; Kim et al., 2007; Uchiki et al., 2009). During the ubiquitination process of S5a, the presence of Class I ubiquitin conjugating enzymes (E2), such as UbcH5 is essential (Uchiki et al., 2009). The Ubl domain of parkin interacts and ubiquitinates the S5a subunit (Safadi and Shaw, 2010). NMR spectroscopy experiments
1999). By interacting with syntaxin, CDCrel-1 inhibits its exocytosis (Zhang et al., 2000) and neurotransmitter exocytosis (Imai and Takahashi, 2004). Upon over-expression in H1T-T15 cells, CDCrel-1 decreases the release of human growth factor, indicating probable regulatory function of CDCrel-1 in synaptic vesicle events involving syntaxin (Dev et al., 2003a).

8.2.5 PAEL-R

In addition to the pre-synaptic proteins indicated above, the parkin associated endothelial like receptor (PAEL-R), which is a G-protein coupled receptor (GPCR) also associates with parkin (Imai et al., 2001). High expression of PAEL-R is found in dopaminergic neurons and oligodendrocytes. PAEL-R is selectively ubiquitinated and promoted for degradation by parkin (Imai et al., 2001; Yang et al., 2003). The presence of PAEL-R is observed in Lewy bodies of PD patient (Murakami et al., 2004) and insoluble fractions of PAEL-R are also detected in the brains of ARJP patients (Imai et al., 2001). A study has showed that PAEL-R over-expression results in the internalization of PAEL-R and complex formation of a large protein complex (> 250 kDa) inducing mitosis (Rezgaoui et al., 2006). In this aggregated form, PAEL-R causes cellular toxicity leading to cell death (Rezgaoui et al., 2006).

8.2.6 PARIS (ZNF746)

A zinc-finger containing protein called PARIS has been found to be associated with parkin. This protein consists of an amino terminal Kruppel-associated box (KRAB) and a carboxyl terminal C2HC/C2H2 Zn type finger (Shin et al., 2011). The PARIS gene is located in chromosome 7q36.1 and encodes a protein of 644 amino acids. The PARIS-parkin interaction was first shown in a yeast two hybrid assay (Zhang et al., 2000) and subsequent co-immunoprecipitation (co-IP) studies revealed the RING1 and RING2 of parkin interacts with the zinc finger domain of PARIS (Shin et al., 2011). This interaction is important for parkin-mediated ubiquitination of PARIS and its degradation through UPS in neurons (Shin et al., 2011), such that when endogenous parkin is knocked-down using shRNA a significant upregulation of PARIS protein levels is detected in SH-SY5Y cells (Shin et al., 2011). This data is supported by protein level analysis of brain samples obtained from ARJP patients, which show a two-fold increase in protein levels of PARIS and a lack of functional parkin protein (Shin et al., 2011). Interestingly, high PARIS expression levels were only found in the nigrostriatal pathway with other brain regions showing similar levels of PARIS.

8.2.7 p38 subunit of aminoacyl-tRNA synthase (ARS) complex

Aminoacyl-tRNA synthetases (ARSs) are essential enzymes that ligate amino acids to tRNAs to make nascent polypeptides. The p38 subunit is a key structural component of the
Parkin's second RING finger domain binds to the ankyrin repeat domain of synphilin-1 and causes ubiquitination of synphilin-1 (Chung et al., 2001). Deposition of synphilin-1 was observed in Lewy bodies and synphilin-1 specifically inhibits the degradation of α-synuclein by the 20S proteasome (Alvarez-Castelao et al., 2010). Mutated parkin is able to bind synphilin, but fails to cause the ubiquitination of synphilin (Chung et al., 2001). Since both protein are pre-synaptic, it is interesting to speculate that α-synuclein and synphilin-1 may work in concert to alter the release of dopamine from dopaminergic neurons, where in PD, such roles are aberrant.

8.2.3 Synaptotagmin XI

Following on from the idea parkin interacts with pre-synaptic proteins (i.e. α-synuclein and synphilin-1) that may play a role in regulating dopamine neurotransmission, parkin does indeed interact with proteins that play a role in synaptic vesicle docking, such as Synaptotagmin XI. Briefly, Synaptotagmin XI plays a role in the docking and fusion of synaptic vesicles to the plasma membrane leading to transmitter release (Glass et al., 2004). It belongs to the family of vesicle proteins, namely synaptotagmins and has a single transmembrane region and two cytoplasmic C2 domains (C2A and C2B) in the ct region followed by a conserved terminus (Glass et al., 2004). Parkin interacts with synaptogamin XI at the C2A and C2B domains and synaptogamin XI binds to the RING finger 1 motif of parkin (between the amino acid residues 204 and 293) (Huynh et al., 2003). Reports have shown that parkin ubiquitinates and promotes degradation of synaptotagmin (Huynh et al., 2003). Immunolabelling data has also shown the presence of synaptotagmin in the wild type neurons as well as Lewy bodies in the SNPC of PD patient (Huynh et al., 2003). Synaptotagmin is thought to hamper dopamine release in parkin mutated mice (Imai and Takahashi, 2004).

8.2.4 CDCrel-1

Yet another synaptic vesicle-associated protein, namely CDCrel-1, is associated with parkin. CDCrel-1 or cell division control-related protein-1 (CDCrel-1) is a synaptic vesicle enriched septin GTPase. It was the first reported parkin substrate (Zhang et al., 2000). Dopamine-dependent neurodegeneration is observed in the rodent brain upon over-expression of CDCrel-1 (Dong et al., 2003). Parkin interacts with CDCrel-1 via the RING finger 2 domain (Zhang et al., 2000). CDCrel-1 is ubiquitinated by wild type parkin leading to the degradation of CDCrel-1, but not mutant parkin (Q311stop and T415N) (Zhang et al., 2000). CDCrel-1 also co-localises with parkin in synaptic vesicle (Kubo et al., 2001). In addition, CDCrel-1 interacts with syntaxin, a protein promoting exocytosis via SNARE domain (Beites et al.,
8.1 E2 conjugating enzymes: UbcH7 and UbcH8

UbcH7 and UbcH8 are two E2 conjugating enzymes that interact with the RING domains (in particular RING2) of parkin and play important role in the ubiquitination process of substrate proteins (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000). Mutation in parkin, specifically, T240R mutation is found to disrupt the interaction with these E2 enzymes, in the case of ARJP (Imai et al. 2000; Zhang et al. 2000; Shimura et al. 2000; Gu et al. 2003). Although the expression of UbcH7 is low compared to UbcH8 (Kimura et al., 1997; Katsanis and Fisher, 1998), the specific association of parkin-UbcH7 was found to be crucial for the ubiquitination of α-synuclein in the brain (Shimura et al., 2001). In addition to parkin, UbcH7 and UbcH8 also interact with other RING-IBR-RING domain containing E3 ligases, including human homologue of *Drosophila ariadne* (HHARI) and UbcH7 associated protein 1 (H7-AP1) (Moynihan et al., 1999).

8.2 Parkin ubiquitinated proteins

8.2.1 α-synuclein

α-Synuclein is a 140 amino acid, neuronal phosphoprotein that upon mutation causes the dominant form of PD. The α-synuclein protein, encoded by PARK1 gene, is thought to be a pre-synaptic expressed protein that plays a role in learning and memory as well as synaptic plasticity (Iwai et al., 1995). The α-synuclein is found to be the integral part of the fibrillar component of Lewy bodies (Polymeropoulos et al., 1997). Three point mutations (A30P, A53T and E46K) in α-synuclein have been identified, which are found in aggregated forms in Lewy bodies (Forno, 1996; Polymeropoulos et al., 1997; Kruger et al., 1998; Singleton et al., 2003). These mutant forms of α-synuclein escape parkin-mediated proteasomal activity (Petrucelli et al., 2002) resulting in the build-up of aggregated α-synuclein in Lewy bodies and subsequent neuronal death. Parkin has also been reported to ubiquitinate an O-glycosylated form of α-synuclein (Shimura et al., 2001) and during parkin mutation, accumulation of this O-glycosylated form of α-synuclein has been observed in the rat brain (Shimura et al., 2001).

8.2.2 Synphilin-1

Synphilin-1 is a protein associated with α-synuclein with unknown function. Synphilin-1, like α-synuclein is a pre-synaptic protein associated with synaptic terminals (Ribeiro et al., 2002). Synphilin-1 contains 919 amino acids with different domains, including ankyrin-like repeats, a coiled-coil domain and a putative ATP/GTP-binding domain (Engelender et al., 1999).
<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UbCH7/UbcH8</td>
<td>E2 conjugating enzymes</td>
<td>Imai et al., 2000</td>
</tr>
<tr>
<td>α-Synuclein</td>
<td>Involved in synaptic vesicle transport</td>
<td>Kahle et al., 2000</td>
</tr>
<tr>
<td>Synphilin-1</td>
<td>Unknown</td>
<td>Chung et al., 2008</td>
</tr>
<tr>
<td>Synaptotagmin XI</td>
<td>Docking and fusion of synaptic vesicle to release transmitter</td>
<td>Glass et al., 2004</td>
</tr>
<tr>
<td>CDCrel-1</td>
<td>Regulates neurotransmitter exocytosis</td>
<td>Imai and Takahashi, 2000</td>
</tr>
<tr>
<td>PAEL-R</td>
<td>G-protein coupled receptor</td>
<td>Corti et al., 2003</td>
</tr>
<tr>
<td>PARIS</td>
<td>Transcriptional repressor</td>
<td>Shin et al., 2011</td>
</tr>
<tr>
<td>p38</td>
<td>Subunit of aminoacyl-tRNA synthetase</td>
<td>Ko et al., 2005</td>
</tr>
<tr>
<td>Proteasomal subunit S5a</td>
<td>Substrate recognition for 26 S proteasome</td>
<td>Uchiki et al., 2009</td>
</tr>
<tr>
<td>Endocytic protein Eps15</td>
<td>Adapter protein in epidermal growth factor receptor (EGFR)</td>
<td>Fallon et al., 2006</td>
</tr>
<tr>
<td>Non-receptor tyrosine kinase c-Abl</td>
<td>Phosphorylation of tyrosine residues</td>
<td>Ko et al., 2010</td>
</tr>
<tr>
<td>Cdc4a</td>
<td>F-box domain of SCF E3 ligase complex</td>
<td>Fallon et al., 2002</td>
</tr>
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<td>HSP70/CHIP</td>
<td>E3 ligase</td>
<td>Murata et al., 2001</td>
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<td>PICK1</td>
<td>Scaffold protein</td>
<td>Joch et al., 2007</td>
</tr>
<tr>
<td>CASK</td>
<td>Ca²⁺/calmodulin-dependent serine kinase</td>
<td>Fallon et al., 2002</td>
</tr>
<tr>
<td>Actin</td>
<td>Cytoskeleton protein</td>
<td>Huynh et al., 2000</td>
</tr>
</tbody>
</table>

Table 1.3: List of proteins associated with parkin
The proteins interacting with parkin and a brief description of their roles are depicted.
Figure 1.6: Parkin interacting partners

Parkin interacts with several proteins. Parkin interacts with E2 conjugating enzymes, such as UbcH7/UbcH8. Proteins, ubiquitinated by parkin, include α-synuclein, synphilin-1, synaptotagmine XI, CDCrel-1, PAEL-R, Paris, p38, S5a, Eps15 and c-Abl. Parkin also acts in concert with other E3 ligases, including Cdc4α and CHIP to ubiquitinate a series of additional substrates. Through its PDZ motif, parkin interacts with scaffolding proteins, such as PICK1 and CASK and parkin also interacts with actin.
A) Domain structure of parkin

![Diagram of domain structure of parkin]

B) Parkin protein- Isoform 1

MIVFVRNSSHGFVEVDSDTISIPQLKEVAKRQGVADQLRVIFAGKEL
RNDWTVQNCDLDQQSYSIVHVQRPWRKGQEMNATGDDPRAAGCEREPQ
SLTRVDSLSSVLPGDSVGLAVILHTDSRKSPPAGSPAGRSIYNSSFYVC
KGFCQVRQPKLRVQCSTCRQATTLTTQGSCWDDLVPNRMSEGCSQSPH
CPGTSAEFFKCGAHTPSDKTEPVALHLIATNSRNITCTCTVDVSPVLLV
FQCNRSHVICLDCFLCLVCRTLDFQVHDPLQGSLPCVAGCPINLKIE
LHHFRLGEEQYNQQYGAECVLQMGGVLCPRPGCAGILLPEDQKRKV
TCGGNLGCGFAFCRECKEAYHGESAVFEASGTTQAYRVEDAAAQ
ARWEAASKETIKKTTPCRCHVPVEKNGCMHMKCPQPCRLEWCWNCG
CEWRVCMGDHFVF

Figure 1.5: Structure and protein sequence of parkin
A) Structural domains of parkin suggest the presence of an ubiquitin like domain (UBL) at the N-terminus. The two Zn-finger-like RING domains are connected to the UBL domain with a central domain. The two RING fingers are, in turn, linked to each other by a cysteine rich region. The last three amino acids at the ct region constitute a PDZ motif (-FDV) which interacts with PDZ domain containing proteins PICK1 and CASK. B) The protein sequence of parkin shows the different domains and motifs.
Parkin can be structurally divided into four domains/motifs (Figure 1.5).

1. **Ubiquitin Like Domain (Ubl):** The amino terminal residues (1-76) constitute the ubiquitin like domain that has 62% homology with ubiquitin. This domain is believed to be responsible for substrate recognition (Shimura et al., 2000). In addition, the UBL domain also regulates the expression of parkin (Finney et al., 2003).

2. **Central domain:** Residues 145-232 constitutes the central domain with unknown function.

3. **RING finger box:** The ct residue 237-449 constitutes a RING box domain, which contains two RING fingers (Kitada et al. 1998; Morett and Bork, 1999). The two RING finger domains are designated as RING1 and RING2 attached to each other by an in-between-region (IBR) domain, which is cysteine rich (Morett and Bork, 1999). The RING fingers are Zn-finger domains, which bind to Zn$^{2+}$ and are believed to interact with other proteins including E2 enzymes (Joazeiro and Weissman, 2000; Borden, 2000). Similar to other Zn-finger containing proteins, known to function as transcription factors, parkin is also believed to play role in the transcriptional regulation (Zheng et al., 2000a).

4. **PDZ-binding motif:** This motif is situated at the extreme ct of parkin and comprises last the three amino acids (-FDV). The PDS-95/discs large/ZO-1 (PDZ) motif of parkin is a class II PDZ motif, which can interact with various adapter proteins containing PDZ domains (approximately 90 amino acids) (Ponting et al., 1997). Parkin has been shown to interact with the PDZ domain proteins CASK and PICK1 (Fallon et al., 2002).

Via these structural domains and the motifs, parkin interacts with several substrates, most of which are ubiquitinated and degraded. The parkin interacting proteins are explained in following section.

### 8. Parkin interacting proteins

Parkin has been shown to interact with and ubiquitinate several cellular proteins with distinct functions. They are described in the section below (Figure 1.6 & Table 1.3).
4. Stimulation of protein sumoylation in the presence of E3 proteins by simultaneously associating with the UBC9 and the substrate (Geiss-Friedlander et al., 2007; Mukhopadhyay and Dasso, 2007).

The de-sumoylation reaction is carried out by six different SENP enzymes in human cells termed SENP 1 to 6 (Mukhopadhyay and Dasso, 2007; Geiss-Friedlander et al., 2007; Zhao, 2007). Among these six SENPs, the SENP1 and SENP2 are capable of removing all three isoforms of SUMO protein moieties. Other SENPs are specific for SUMO-2 and SUMO-3.

There are many neurodegenerative diseases, where sumoylation plays a role. The diseases, where proteins are abnormally sumoylated, are PD (tau, α-synuclein, DJ-1), HD (Huntingtin), AD (tau and APP), spinocerebellar ataxia type 1 (Ataxin-1) and ALS (SOD1) (Sarge and Paek-Sarge, 2009). In particular, tau has been implicated in the development of both PD and AD. Tau is sumoylated by SUMO-1 at K340 residue. It has been reported that a decrease in sumoylation of tau is observed upon proteasome inhibitor MG102 treatment, which also increases the ubiquitination of tau. The inhibition of tau phosphorylation is also thought to increased tau sumoylation (Sarge and Paek-Sarge, 2009). Of interest, α-synuclein is present in Lewy bodies, the proteinaceous aggregates in the brain of PD patient, is also sumoylated on its K102 residue, preferentially by SUMO-1. Unlike tau, α-synuclein is unaffected by proteasome inhibitor MG132 treatment (Dorval et al., 2006). The function of α-synuclein sumoylation is still unknown. In addition to tau and α-synuclein, DJ-1 is an important protein, which upon mutation causes early onset PD (1-2% prevalence) (Thomas et al., 2007). DJ-1 functions as an anti-oxidant, transcriptional co-activator and molecular chaperone. DJ-1 is also sumoylated on K130 residue. Upon mutation in this residue, a decrease in DJ-1-mediated Ras-dependent transforming and cell-growth-promoting activity is observed (Shinbo et al., 2006). These findings suggest sumoylation may regulate tau, α-synuclein and DJ-1 function that may play a role in neurodegeneration.

7. Parkin as an E3 Ligase

Genetic mutations in the parkin gene, which encodes an E3 ligase, are associated with autosomal ARJP. A loss-of-function of mutated parkin results in UPS dysfunction, eventually causing death of dopaminergic neurons. Parkin is the second largest gene in the human genome with a size of 1.5 Mb (the largest gene is DMD, size 2.4 Mb, encodes for Dystrophin protein). The genetic location of the gene is 6q25.2-q27. The parkin gene has 12 exons, which upon translation, express an approximately 52 kDa protein (465 amino acids) (Kitada et al., 1998; Shimura et al., 1999).
ii) E3 ligases with really-interesting-new-gene (RING) finger domain (Kim et al., 2009) (such as parkin) bind the substrate and the E2 enzyme, but not the ubiquitin molecule, to transfer the ubiquitin to the substrate.

In both cases, the E3 ligase binds the ubiquitin to the substrate via an ε-amide bond.

Finally, the tagged substrate is then detected by the 26S proteasome, a large multiprotein complex of 2.5 MDa size, which eventually degrades the substrate in an ATP-dependent manner (Voges et al., 1999).

6.3 Sumoylation

Like ubiquitination, sumoylation is another important post-translational event in which a small protein named SUMO (Small Ubiquitin-like Modifiers) is attached to specific lysine residues in target proteins. The subcellular localisation, protein partnering and transcription factor trans-activation of a target protein can be regulated by sumoylation. SUMO or sentrin proteins are 110 amino acids of which there are three isoforms, e.g., SUMO-1, SUMO-2 and SUMO-3 in vertebrates. In contrast, invertebrates have only one isoform (Kamitani et al., 1998). Different SUMO isoforms (1, 2 and 3) have 50% sequence homology among each other (Muller et al., 2001). NMR spectroscopy data show SUMO-1 and ubiquitin protein share a common three dimensional structure, although only 18% of sequence similarity is found between these two proteins (Bayer et al., 1998).

Sumoylation involves four enzymatic steps,

1. Maturation of the SUMO protein by cleaving its ct to make a carboxyl terminal diglycine motif by a sentrin-specific protease (SENP).

2. Attachment of the mature SUMO protein to a cysteine residue in the SUMO-activating enzyme subunit 2 (SAE2) of the heterodimeric SUMO E1 activating enzyme via formation of a ATP-dependent thioester bond (Johnson et al., 1997a; Desterro et al., 1999; Gong et al., 1999; Okuma et al., 1999).

3. Transfer of the SUMO moiety from E1 enzyme to the SUMO E2 enzyme (UBC9) that ultimately attaches SUMO to the lysine residue of the target protein. The lysine residue is found not always, but most of the time in a consensus sequence Ψ-K-X-E (where Ψ represents hydrophobic amino acids) (Desterro et al., 1997; Johnson et al., 1997b; Rodriguez et al., 2001; Sampson et al., 2001).
Figure 1.4: Ubiquitin Proteasome System
Unwanted proteins are tagged with ubiquitin by sequential action of three enzymes. E1 and E2 enzymes activate the ubiquitin molecule and help to conjugate the activated ubiquitin with the substrate, respectively. Transfer of the activated ubiquitin molecule to the substrate is mediated by two types of E3 ligase enzyme systems, E3 (a) ligases with HECT domain that directly bind both the ubiquitin molecule and the E2 enzyme to transfer the ubiquitin molecule to the substrate and E3 (b) ligases with RING finger domain that bind the substrate and the E2 enzyme, but not the ubiquitin molecule, to transfer the ubiquitin to the substrate. After successful tagging with ubiquitin, the substrate is poly-ubiquitinated and then processed by 26S proteasome.
Although, in general, Lewy bodies are not detected when parkin is mutated in ARJP (Mori et al., 1998; Hayashi et al., 2000), a patient with a single allele mutation in parkin (with partial E3 ligase activity) was shown to have Lewy bodies (Farrer et al., 2001). Intriguingly, over 90% of Lewy bodies formed in the autosomal dominant PD demonstrated parkin immunoreactivity, suggesting a role of parkin in the formation of Lewy bodies (Dev et al., 2003).

6.2 Ubiquitination

Ubiquitination is an important post-translational modification that plays a wide variety of roles in cellular processes, such as chromatin remodelling, DNA repair, protein trafficking, degradation, signal transduction, peroxisomal biogenesis and viral budding (Di Fiore et al., 2003; Hicke et al., 2003; Haglund and Dikic, 2005). Ubiquitin or ubiquitous immunopoietic polypeptide is an 8.5 kDa protein, which is ubiquitously found in all eukaryotic cells. Ubiquitination involves the covalent attachment of a polyubiquitin chain to a lysine residue of the ubiquitinated protein.

UPS is a three-enzyme pathway eventually resulting in the degradation of unwanted cellular proteins. Unwanted proteins or substrates are molecularly marked or primed with ubiquitin and finally degraded by the UPS (Hershko and Ciechanover, 1998). This three-enzyme system acts chronologically to tag the substrate molecule with single ubiquitin molecules forming a chain of polyubiquitin. The process of tagging a substrate with a polyubiquitin chain is called polyubiquitination, which involves three enzymatic steps (Figure 1.4).

1) The ATP-dependent ubiquitin activating enzyme (E1) activates the ubiquitin molecules.

2) The activated high energy ubiquitin is then accepted by the ubiquitin conjugating enzyme (E2) by forming a thioester bond.

3) After activity of the E2 enzyme, the ubiquitin ligase (E3) mediates ligation of activated ubiquitin molecules. Ubiquitin ligases can be divided into two categories according to their mode of action to transfer the ubiquitin molecule to the substrate.

i) E3 ligases with homology to E6-AP carboxy terminus (HECT) domain (such as Cdc4) directly bind both ubiquitin molecule and the E2 enzyme to transfer the ubiquitin to the substrate.
Parkinson's study group 2, 1993; Churchyard et al., 1997; Bar-Am et al., 2007). Due to these severe side effects of L-dopa, further drug development efforts are required.

6. Neurodegeneration & protein aggregation

Neurodegeneration is defined as the degradation of neuronal cells in a progressive manner due to apoptosis, autophagy or cytoplasmic cell death, collectively known as programmed cell death (PCD) (Kim et al., 2011). Neurodegeneration is the prevalent cause of many neuropathic diseases, such as PD, AD and HD. There are many causes of neurodegeneration identified, including defects in protein degradation, ROS production, Ca\(^{2+}\) dysregulation, mitochondrial dysfunction and excitotoxicity (Schweichel and Merker, 1973; Clarke, 1990). Among all these neurodegenerative processes, protein aggregation has a prominent role in the death of neurons. Particularly, formation of proteinaceous aggregates caused by the deposition of unwanted proteins results in these diseases. Protein aggregation is controlled by various processes, in which ubiquitination and sumoylation are important.

6.1 Lewy bodies: Hallmark of PD

Protein aggregation is a common pathology found in most of the neurodegenerative diseases, including AD, HD and PD. For AD, the formation of plaques composed β-amyloid (A\(\beta\)) and neurofibrillary tangles composed hyperphosphorylated Tau have been identified to cause neurodegeneration in AD brain (Querfurth and LaFerla, 2010). In the case of HD, protein inclusions of mutated huntingtin protein are a characteristic feature in the brains of HD patients (Temussi et al., 2003). For PD, Lewy bodies are considered as the hallmark feature, which are composed abnormal accumulation of proteins found in the cell bodies of neurons in sporadic as well as autosomal dominant PD (Forno, 1996). In addition, protein aggregates forming smaller inclusions in the neuronal extensions called Lewy neurites are also found in PD (Brundin and Olsson, 2011). These protein inclusions are composed misfolded variant of mutant α-synuclein, constituting the major component along with other proteins including, ubiquitin and UCH-L1 (Lowe et al., 1990; Polymeropoulos et al., 1997; Shults, 2006). Indeed, genome analysis has revealed three point mutations of α-synuclein associated with autosomal dominant PD that appear susceptible to aggregation in Lewy bodies (Venda et al., 2010). Moreover, the level of α-synuclein is found to increase in the aging brain, specifically in the dopaminergic neurons of SNPC (Chu and Kordower, 2007) and the Lewy body density correlates directly with progression of PD (Halliday et al., 2008).
### Table 1.2: Novel loci associated with PD

Several new genetic loci linked with PD were discovered by using two genetics approaches, namely, candidate gene approach and GWAS. The tables show a list of identified genetic loci by candidate gene approach (A) and GWAS (B) (Lesage and Brice, 2012).

#### A)

<table>
<thead>
<tr>
<th>Well-validated loci/genes</th>
<th>Gene</th>
<th>Locus</th>
<th>Risk variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1/PARK4</td>
<td>α-Synuclein</td>
<td>4q21</td>
<td>Promoter Rep1 5' and 3' variants</td>
</tr>
<tr>
<td>PARK8</td>
<td>LRRK2</td>
<td>12q12</td>
<td>G2385R, R1628P (Asians)</td>
</tr>
<tr>
<td>Not assigned</td>
<td>MAPT-linked FTDP-17</td>
<td>17q21</td>
<td>H1 haplotype (Europeans)</td>
</tr>
<tr>
<td>Not assigned</td>
<td>GBA-linked GD</td>
<td>1q21</td>
<td>&gt;300 heterozygous mutations</td>
</tr>
</tbody>
</table>

#### B)

<table>
<thead>
<tr>
<th>Putative loci/genes</th>
<th>Gene</th>
<th>Locus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK16</td>
<td>Unknown</td>
<td>1q32</td>
<td>Satake et al., 2009</td>
</tr>
<tr>
<td>PARK17</td>
<td>GAK</td>
<td>4p16</td>
<td>Hamza et al., 2010</td>
</tr>
<tr>
<td>PARK18</td>
<td>HLA-DRA</td>
<td>6p21.3</td>
<td>Hamza et al., 2010</td>
</tr>
<tr>
<td>Not assigned</td>
<td>BST1</td>
<td>4p15</td>
<td>Satake et al., 2009</td>
</tr>
<tr>
<td>Not assigned</td>
<td>FGF20</td>
<td>8p22</td>
<td>IPDGC and WTCCC2 2011</td>
</tr>
<tr>
<td>Not assigned</td>
<td>ACMSD</td>
<td>2q21</td>
<td>IPDGC and WTCCC2 2011</td>
</tr>
<tr>
<td>Not assigned</td>
<td>STK39</td>
<td>2q24</td>
<td>IPDGC and WTCCC2 2011</td>
</tr>
<tr>
<td>Not assigned</td>
<td>MCCC1/ LAMP3</td>
<td>3q27</td>
<td>IPDGC and WTCCC2 2011</td>
</tr>
<tr>
<td>Not assigned</td>
<td>SYT11</td>
<td>1q22</td>
<td>IPDGC and WTCCC2 2011</td>
</tr>
<tr>
<td>Not assigned</td>
<td>CCDC62/ H1P1R</td>
<td>12q24</td>
<td>Saad et al., 2011</td>
</tr>
<tr>
<td>Not assigned</td>
<td>STX1B</td>
<td>16p11</td>
<td>IPDGC and WTCCC2 2011</td>
</tr>
<tr>
<td>Not assigned</td>
<td>STBD1</td>
<td>4q21</td>
<td>IPDGC and WTCCC2 2011</td>
</tr>
<tr>
<td>Not assigned</td>
<td>GPNMB</td>
<td>7p15</td>
<td>IPDGC and WTCCC2 2011</td>
</tr>
</tbody>
</table>
with PD, identified using candidate gene approach, are shown in Table 1.2A. GWAS is one of the most sought after genome search strategies to discover genetic loci influencing disease. In this approach, low penetrance alleles influencing complex diseases can be identified, which may be missed in linkage studies. By using this approach, multiple polymorphic loci have been identified in α-synuclein, MAPT and LRRK2 as risk factors for PD (Satake et al., 2009; Hamza et al., 2010; Saad et al., 2011). In addition to these well known genes, several new loci have been identified by GWAS, listed in Table 1.2B (Lesage and Brice, 2012). All these data suggest the importance of a genetic role in PD, although more research is require to validate the exact role of each gene in the onset of PD.

5.4 Current treatments for PD

A major breakthrough occurred in PD therapy when Swedish pharmacologists Arvid Carlsson and Oleh Hornykiewicz discovered dopamine as a neurotransmitter depleted in PD. After this discovery, the precursor of dopamine, levodopa (L-dopa; L-3, 4-dihydroxyphenylalanine) has been widely used as medicine in PD. The conventional treatment regime for PD has been administration of L-dopa with synthetic dopamine agonists to supply dopamine and applying inhibitors of dopamine metabolism. L-dopa is a precursor of dopamine, epinephrine and norepinephrine, collectively called catecholamines. Upon oral ingestion, L-dopa is absorbed in the circulatory system from upper small intestine. Only a small fraction of L-dopa ultimately reaches the brain by crossing the blood brain barrier with the help of active transport (Nutt et al., 1984). In the brain, L-dopa is readily converted to functional dopamine by an enzyme L-amino acid decarboxylase (AAAD).

To increase the efficacy of L-dopa many other compounds have been developed. Carbidopa or benserazide are administered with L-dopa to prevent the conversion of L-dopa to dopamine outside CNS by inhibiting AAAD. To prolong the clearance half-life of L-dopa, compounds, such as entacapone or tolcapone are used to prevent peripheral catechol-o-methyltransferase (COMT) (Tallman et al., 1976). To prolong the breakdown of dopamine converted from L-dopa, several compounds, including selegiline and rasagiline are used inhibiting monoamine oxidase type B (MAO-B) (Collins et al., 1970; Youdim et al., 1972). L-dopa is effective in the recovery of a patient from impairment of speech, dexterity and posture. However, symptoms, such as tremor and imbalance have been observed to remain unchanged (Parkinson’s study group 1, 1989). In addition, upon prolonged treatment (a period of 5 years), up to half of patients show severe dyskinesia (uncontrolled movement of muscles) and/or motor fluctuations (Ricaurte et al., 1984; Olanow and Calne, 1992;
<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Mode of inheritance</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Synuclein (PARK1)</td>
<td>4q21</td>
<td>AD</td>
<td>Pre-synaptic protein, membrane trafficking</td>
</tr>
<tr>
<td>Parkin (PARK2)</td>
<td>6q25</td>
<td>AR</td>
<td>E3 ligase</td>
</tr>
<tr>
<td>PARK3</td>
<td>2p13</td>
<td>AD</td>
<td>Not known</td>
</tr>
<tr>
<td>PARK4</td>
<td>4p15</td>
<td>AD</td>
<td>Not known</td>
</tr>
<tr>
<td>UCH-L1 (PARK5)</td>
<td>4p14</td>
<td>AD</td>
<td>Thiol protease</td>
</tr>
<tr>
<td>PINK1 (PARK6)</td>
<td>1p35</td>
<td>AR</td>
<td>Serine-threonine kinase</td>
</tr>
<tr>
<td>DJ-1 (PARK7)</td>
<td>1p36</td>
<td>AR</td>
<td>Oxidative stress sensor, chaperone</td>
</tr>
<tr>
<td>LRRK2 (PARK8)</td>
<td>12q12</td>
<td>AD</td>
<td>Serine-threonine kinase</td>
</tr>
<tr>
<td>ATP13A2 (PARK9)</td>
<td>1p36</td>
<td>AR</td>
<td>Lysosomal type 5 P-type ATPase</td>
</tr>
<tr>
<td>PARK10</td>
<td>1p32</td>
<td>AD</td>
<td>Not known</td>
</tr>
<tr>
<td>GIGYF2 (PARK11)</td>
<td>2q3</td>
<td>AD</td>
<td>Tyrosine kinase regulator</td>
</tr>
<tr>
<td>PARK12</td>
<td>Xq21-q25</td>
<td>AD</td>
<td>Not known</td>
</tr>
<tr>
<td>HTRA2 (PARK13)</td>
<td>2p12</td>
<td>AD</td>
<td>Serine-threonine protease</td>
</tr>
</tbody>
</table>

Table 1.1: List of gene associated with PD
Autosomal dominant (AD), Autosomal recessive (AR) (Henchcliffe and Beal 2008).
Follow up studies on these genes have, in most cases, been inconclusive (Lesage and Brice, 2012) although a probable association of PD was found with a point mutation in the UCH-L1 gene (I93M) (Leroy et al., 1998). The genes associated with the recessive form of PD include parkin (PARK2, an E3 ligase) (Kitada et al., 1998), Phosphatase and tensin homolog (PTEN)-induced kinase (PINK1/PARK6, a serine-threonine kinase) (Silvestri et al., 2005), DJ-1 (PARK7, an oxidative stress sensor and chaperone) (Bonifati et al., 2003) and the ATP13A2 (PARK9) (Ramirez et al., 2006) (Table 1.1).

Autosomal recessive juvenile Parkinsonism (ARJP) has an early age of onset, typically in late 20s and before 40. Almost half of the European families suffering from ARJP carry mutation in the parkin gene (Abbas et al., 1999; Kitada et al., 2000; Lucking et al., 2000; Kahle et al., 2000). ARJP is typically caused by loss-of-function of both the alleles of the parkin gene (Mizuno et al., 1998). More than 170 mutations associated with ARJP, including large deletions or multiplications of one or more exons, small deletions, small insertions, nonsense mutations and missense mutations have been reported for the parkin gene (Nuytemans et al., 2010). Functionally, parkin is an E3 enzyme that targets unwanted proteins to be degraded via the UPS, which may regulate the degradation and removal of more than 30% of proteins made in cell (Schubert et al., 2000). These parkin substrates have vital roles in cellular processes, including signalling, cell cycle, metabolism and immune response (Pagano, 1997, Ben-Neriah, 2002). Thus, the ubiquitin-mediated clearance of many neuronal proteins fails due to the loss-of-function mutations in parkin. These unwanted proteins aggregate in the cell causing toxicity, specifically in cellular compartments, including ER and mitochondria. Cellular toxicity also generates oxidative stress by excess ROS production, ultimately leading to neuronal cell death (Dev et al., 2003).

5.3 GWAS and Linkage studies in PD

Many linkage and genome-wide association studies (GWAS) have identified putative loci in the human genome that increase the susceptibility of PD under the influence of environmental factors (Lesage and Brice, 2012). A candidate gene approach identifying polymorphisms in genes via screening genetic data from a large number of patients has found nucleotide polymorphisms in the promoter region of α-synuclein to be associated with PD (Lesage and Brice, 2012). In addition, the microtubule associated protein tau (MAPT) has been found associated with the H1 haplotype, one of the two most common loci associated with progressive supranuclear palsy, AD and PD (Lesage and Brice, 2012). Two common variants in LRRK2 (G2385R and R1628P) have also been found to be associated with PD (Lesage and Brice, 2012) using linkage studies. The better validated genes linked
10,000 affected patients in Ireland (Healy et al., 2004). The typical clinical features of this disease are shaking muscle (tremor), muscular stiffness (rigidity) and slow movement of limbs (bradykinesia). In extreme cases, patients may become bedridden due to the complete loss of movement. In addition to physical symptoms, PD also gives rise to non-motor symptoms, including sleep disorder, psychosis, depression, dementia and autonomic disturbance. The cause of these clinical symptoms is the excessive loss of nerve cell function in the CNS, more specifically, excessive loss-of-function of dopaminergic neurons in SNPC region of the mid-brain. The eventual loss of dopaminergic neurons leads to reduction of nigrostriatal dopamine level and loss of motor function (Hornykiewicz, 1998; Hornykiewicz and Kish, 1987). Intriguingly, involvement of other non-dopaminergic neurons has been found to be associated with PD, including noradrenergic neurons present in the locus coeruleus, cholinergic neurons in the nucleus basalis of Meynert, and serotonergic neurons in the midline raphe (Forno, 1996).

5.2 Genetic forms of PD

The aetiology of PD is multifactorial and involves interplay between both genetic features and environmental effects. Broadly, PD can be divided into two subtypes, sporadic and genetic. Environmental factors linked to sporadic PD include exposure to toxins and free radicals, which are thought to cause damage via oxidative stress, mitochondrial dysfunction, excitotoxicity, lipid peroxidation, inflammatory changes, dysfunction in UPS and apoptosis. These processes are linked to the formation of ROS, such as superoxides and peroxides (Tanner, 1989; Beal, 2000; Jenner and Olanow, 1996). In contrast to sporadic PD, a relatively small amount of PD patients are identified as familial/genetically inherited. Genetic inheritance is found in 5%-10% of PD patients with linkage to 11 different genes, suggesting most of the patients develop PD sporadically (more than 90%) (Hardy et al., 2006; Klein et al., 2007; Lesage and Brice, 2012).

In familial PD, both autosomal dominant and recessive types of inheritance have been identified. The genes reported, and well accepted, to be responsible for causing dominant form of PD upon mutation are α-synuclein (PARK1) (Polymeropoulos et al., 1997; Kruger et al., 1998) and leucine-rich repeat kinase 2 (LRRK2/PARK8, serine protease) (West et al., 2005). In addition, some other genes are thought to be associated with dominant PD, such as ubiquitin-C terminal hydrolase L1 (UCH-L1/PARK5, thiol protease), growth factor receptor-bound protein 10 (GRB10) interacting glycine-tyrosine-phenylalanine (GYF) protein 2 (GIGYF2/PARK11, tyrosine kinase regulator) (Pankratz et al., 2002), high temperature requirement protein A2 (HTRA2/PARK13, serine-threonine protease) (Moisoi et al., 2009).
depolarization in synaptosomes, three methods are employed including KCl elevation, Na⁺ channel activation and K⁺ channel inhibition (Nicholls, 1993).

1. **KCl elevation**: In a non-depolarized state, K⁺ resides in a higher concentration in synaptosomes, creating an ionic gradient of K⁺ (inside vs. outside) across the synaptosomal membrane. Elevation of KCl concentration outside the synaptosomes reverses the ionic gradient of K⁺ that ultimately leads to depolarization of the synaptosomal membrane and causes glutamate release (Nicholls, 1993).

2. **Na⁺ channel activation**: Unlike K⁺, the concentration of Na⁺ is lower in synaptosomes, which creates an ionic gradient of Na⁺ (outside vs. inside) across the synaptosomal membrane. In a non-depolarized state, the Na⁺ channels are momentarily activated and then remain in an inactivated state in the presence of tetrodotoxin, which impairs respiration and lowers Ca²⁺ concentration (Kauppinen et al. 1986; Tibbs et al., 1989). The effect of tetrodotoxin can be altered by addition of veratridine which keeps the Na⁺ channels open. As a result, the Na⁺ electrochemical potential across the membrane collapses, resulting synaptosomal depolarization-induced glutamate release (Nicholls, 1993).

3. **K⁺ channel inhibition**: In this method, the K⁺ channels present on the synaptosomes are targeted. Under the depolarization current, K⁺ channels fire in a transient, rapidly inactivating manner to repolarize the synaptosomal membrane (Bartschat and Blaustein, 1985; Tibbs et al., 1989). Inactivation of K⁺ channels can be prevented by the addition of 4-amino pyridine (4-AP) or dendrotoxin, which simulates an ‘epileptic action potential’, causing spontaneous repetitive firing of Na⁺ channels (Agoston et al., 1983; Tibbs et al., 1989). Thus, 4-AP simulates an action potential-like event making it a valuable tool to study pre-synaptic regulation (Nicholls, 1993).

### 5. Parkinson’s Disease

5.1 **Introduction**

Parkinson's disease (PD) is the second most common neurodegenerative disease, which occurs due to genetic mutations, environmental insults or a cumulative effect of both. Approximately, 0.3% of world population suffers from this disease. Roughly, 1-2% of people over the age of 65 and 4% more than 80 years suffer from PD (de Lau, 2006), with almost
ion gradients and neurotransmitter release (Nicholls, 1993).

Synaptosomes consume glucose in both aerobic and anaerobic respiration and also pyruvate during oxidative metabolism (Kauppinen and Nicholls, 1986). High levels of glycolysis driven ATP synthesis can take place in synaptosomes aerobically. Inhibition in the mitochondrial ATP synthesis by anoxia can trigger an anaerobic mode of respiration (Kauppinen and Nicholls, 1986). In an anaerobic mode, the glucose content can be exhausted at a very high rate (10 fold) within synaptosomes in order to maintain normal ATP production (Kauppinen and Nicholls, 1986). In an intact synaptosomal preparation, under low K⁺ and Ca²⁺ (0.1-0.2 μM) concentration medium, synaptosomes maintain a plasma membrane potential of -60 to -80 mV (Ashley et al., 1984; Nachshen, 1985; Verhage et al., 1988; Kauppinen et al., 1988).

4.1 Cytosolic and vesicular glutamate release by synaptosomes

Glutamate is found in the cytosol of neurons in high concentration, approximately 10 mM in glutamatergic neurons (Hansson et al., 2000). In synaptosomes, under resting conditions, the extra and intracellular glutamate levels maintain equilibrium by continuous glutamate leakage from the cytosol, balanced by a high-affinity glutamate uptake mechanism (Bradford et al., 1987). The glutamate uptake mechanism takes place through a Na⁺-coupled pump that allows positively charged Na⁺ influx along with glutamate uptake, maintaining a thermodynamic equilibrium across the synaptosomal membrane (Nicholls, 1993). Under a depolarising condition, this equilibrium is disturbed and a slow but prolong leakage of cytosolic glutamate takes place, which is independent of ATP, Ca²⁺ concentration and vesicle-mediated glutamate release from synaptosomes (Nicholls et al., 1987; Nicholls, 1993). Aspartate is also known to release along with glutamate and considered as an indicator of cytosolic neurotransmitter leakage (McMohan et al., 1990). Ca²⁺-independent cytosolic glutamate release is inhibited by botulinum neurotoxin Type A and B in synaptosomes (Sanchez-Prieto et al., 1987; McMahon et al., 1992). In contrast to cytosolic glutamate leakage, Ca²⁺ has a crucial role to play in synaptic vesicle-mediated neurotransmitter release, where Ca²⁺ is required for the fusion of synaptic vesicles with the pre-synaptic membrane to release neurotransmitters. The details of vesicular glutamate release are described in section 2.1.

4.2 Depolarization of synaptosomes

Due to the absence of a neuronal structure in the synaptosomal preparation, there is no provision to excite a synaptosome by an axon driven action potential. To cause
Figure 1.3: Pathways of mitochondrial dysfunction
Mitochondrial dysfunction triggered by toxins, including MPTP and rotenone, hamper complex I activity in the ETC, inducing the production of ROS ($O_2^-$) and reducing ATP production. Oxidative stress can reduce mitochondrial membrane potential ($\Delta \psi_m$) and causes failure of the UPS. Failure of mitochondria leads to increased protein aggregation, apoptosis and cell death (Schapira, 2008).
(Henchcliffe and Beal, 2008). Normally ROS, produced as a byproduct of oxidative phosphorylation, is converted to water by antioxidant enzymes including superoxide dismutase (SOD), catalase, glutathione peroxidase (Murphy, 2009; Galley, 2011). However, in the case of mitochondrial dysfunction, an overwhelming amount of ROS is produced to an extent where antioxidant enzymes fail to regulate intracellular ROS concentration (Perry et al. 1996; Galley, 2011). The lack of removal of peroxides and superoxides leads to oxidative stress-induced damage in lipids, proteins, RNA and DNA in PD patients (Dexter et al., 1989; Zhang et al., 1999).

Oxidative stress and depleted ATP production also hampers the mitochondrial membrane potential. A fall in the mitochondrial membrane potential is thought to be one of the initial indicators of impending apoptosis in neurons (Nicholls and Ward, 2000). Moreover, age-related decreases in the membrane potential of brain mitochondria have been reported by several groups (Joyce et al. 2003; Navarro and Boveris, 2007; Boveris and Navarro, 2008). The depolarization of mitochondria below a threshold level induces apoptosis by releasing mitochondrial Ca^{2+} and death signals, such as Bcl-2-associated X (Bax), cytochrome C and other pro-apoptotic factors, including pro-caspases and caspases into the cytoplasm through mtPTPs (Nicholls and Budd, 2000; Green and Kroemer, 2004). In addition, mitochondrial dysfunction and oxidative stress also play a role in the accumulation of unwanted proteins by hampering the ubiquitin proteasomal system (UPS) pathway, leading to cellular toxicity (Henchcliffe and Beal, 2008). Taken together, mitochondrial dysfunction can be identified by detecting changes in various properties including activity of the ETC cycle complexes, increased production of ROS and change in the mitochondrial membrane potential (Figure 1.3).

4. Synaptosomes

Studies using synaptosomal preparations have been important in understanding mitochondrial biology and mechanisms of pre-synaptic glutamate release. In simple terms, synaptosomes are isolated nerve terminals where a typical synaptosome measures around 0.5-1 \( \mu m \) in diameter. Importantly, these synaptosomes contain mitochondria and synaptic vesicles with little or no part of the ER or nucleus (Nicholls, 1993) that makes it ideal for studying mitochondrial functions including activity of ETC, production of ROS, and change in the mitochondrial membrane potential. Isolated synaptosomes can also be used to study synaptic transmission, as all the required machineries for uptake, storage and exocytotic release of neurotransmitters are present. An additional advantage of synaptosomes is that, after extraction, they can be stored on ice for approximately 6 h without loss in ATP levels.
mitochondria thus have a number of vital physiological roles including influencing aerobic ATP production, neurotransmitter release, synaptic transmission, excitability, regulation of organelle dynamics and trafficking, nuclear signalling, regulation of ROS and apoptosis (Duchen, 2004; Nicholls, 2005; Mattson et al., 2008; Starkov, 2010; Chinopoulos and Adam-Vizi, 2010).

In addition to these Ca\textsuperscript{2+} transporters, the third Ca\textsuperscript{2+} channel present on mitochondria is a voltage and Ca\textsuperscript{2+}-dependent high conductance channel, named a mitochondrial permeability transition pore (mtPTP). The mtPTP was first identified by Hunter and Haworth (1979) and though to form contact sites between the inner and outer mitochondrial membrane. The composition of mtPTP is still not well established, however, data suggest a putative multi-protein composition including a voltage-dependent anion channel (VDAC), members of the pro and anti-apoptotic protein family, cyclophilin D and the adenine nucleotide (ADP/ATP) translocators (ANTs) (Zoratti and Szabo, 1995; Marzo et al., 1998). In case of Ca\textsuperscript{2+} overload in neuronal mitochondria, the mtPTPs are formed, through which the Ca\textsuperscript{2+} content and pro-apoptotic proteins of mitochondria are released into cytosol (Bernardi et al., 2006). In addition, mtPTPs can also form due to fall of the mitochondrial membrane potential and swelling of the mitochondrial matrix (Bernardi et al., 2006). The induction of mtPTP leading to apoptosis and neurodegeneration is thought to play a role in ischemic brain injury and neurodegenerative diseases including PD, AD and HD (Bezprozvanny, 2009). In addition, the absence of ANT components of mtPTPs, responsible for regulating pore opening, has been found to be susceptible to glutamate-induced excitotoxicity (Lee et al., 2009). Taken together, Ca\textsuperscript{2+} sequestration by mitochondria has an important role to play in the cellular physiology and the loss of mitochondrial Ca\textsuperscript{2+} homeostasis has direct implications in neuronal excitotoxicity and apoptosis.

3.3 Mitochondrial dysfunction

Mitochondrial dysfunction, that is the malfunctioning of the mitochondrial complexes and membrane potential, is often induced by cellular stress and leads to cellular toxicity particularly in neurodegenerative diseases including PD, AD, HD and ALS (Bezprozvanny, 2009; Gibson, 2010; Beal, 2007). Specifically for PD, analysis of tissues derived from the SNPC (Schapira et al., 1990) and frontal cortex areas (Parker et al., 2008) of PD patients revealed mitochondrial dysfunction with hampered complex I respiratory chain and reduced electron transfer efficiency through complex I, which ultimately compromise oxidative phosphorylation (Keeney et al., 2006). This hampered oxidative phosphorylation leads to a reduction in ATP production and Ca\textsuperscript{2+} homeostasis, which triggers ROS production.
membrane responsible for creating an electrochemical gradient of protons across the
membrane known as proton motive force (Nicholls and Budd, 2000). By using the proton
motive force, ATP synthase (EC 3.6.3.14) produces ATP from ADP and a phosphate group.
Of note, ATP synthase is also often called complex V. The passage of three protons through
complex V into the mitochondrial matrix results in the generation of one ATP molecule.

3.2 Mitochondrial calcium sequestration

Along with ATP production by oxidative phosphorylation, mitochondria have an important
role to play in the Ca²⁺ homeostasis, especially in neurons and in Ca²⁺ signalling in other cell
types (Berridge, 1998). After endoplasmic reticulum (ER), the mitochondria harbor the
highest amount of Ca²⁺ in the neuronal cells. The total Ca²⁺ content of a neuronal cell is
found in two states, free Ca²⁺ and protein bound (Pivovarova et al., 2010). The total Ca²⁺
content of a neuron in resting state is 1 mM, in which 99.9% of Ca²⁺ is associated to
cytosolic proteins or sequestered inside ER and mitochondria. Therefore, upon receiving a
stimulus, a global rise of Ca²⁺ can take place from 100 nM (resting phase) to 1 μM
(stimulated phase) in neurons (Meldolesi and Pozzan, 1998; Pozzan and Rizzuto, 2000). In
the case of mitochondria, a low amount of total (0.1 mM) and free Ca²⁺ (100 nM)
concentration is found in resting neurons (Pozzo-Miller et al., 1997; Babcock and Hille,
1997), which drastically increases after stimulation of the neuron (Pivovarova et al., 1999;
Montero et al., 2000). This unique property of Ca²⁺ sequestration makes mitochondria an
important player in various processes, including synaptic transmission and Ca²⁺
homeostasis.

Mitochondria have three ion channels present on their outer membrane responsible for influx
and efflux of Ca²⁺. The first of these channels is a group of Ca²⁺-dependent voltage gated
uniporter ion channels, which open in the presence of high concentrations of cytosolic Ca²⁺
to transport excess Ca²⁺ into the mitochondrial matrix (Nicholls and Budd, 2000; Gunter and
Sheu, 2009). The second type of Ca²⁺ transporters found to be present on the mitochondrial
surface is a group of Na⁺/Ca²⁺ exchangers, which is responsible for the efflux of Ca²⁺ from
mitochondria to the cytosol (Crompton et al., 1978). These two mitochondrial transporters
establish together a slow and continuous cycling of Ca²⁺ across the inner membrane driven
by the respiratory chain expulsion of H⁺; and maintain the normal mitochondrial potential
(Crompton et al., 1978; Crompton and Heid, 1978). The rate of mitochondrial uptake of Ca²⁺
is always found to be higher than the rate of release into cytosol during the resting phase, so
that, after an action potential-evoked Ca²⁺ release into the cytosol, mitochondria sequester
Ca²⁺ to return levels to the resting phase (Nicholls, 2005). Sequestered Ca²⁺ present in
Williams, 1956). It specifically hampers the process of electron transfer from iron-sulphur centres of complex I to ubiquinol (Sherer et al., 2003). This blockade reduces the ATP production by interfering with NADP and as a result high amounts of peroxide are produced (Sipos et al., 2003).

B. Complex II: Complex II (Succinate: quinone oxidoreductase, EC 1.3.5.1), catalyses the oxidation of FADH$_2$ to FAD$^+$ (flavin adenine dinucleotide), and also functions as a component of the citric acid cycle. Both complex I and complex II can reduce ubiquinol/ubiquinone, donating electrons to complex III. An arginine residue (A297) of complex II acts as a base catalyst for accepting protons during dehydrogenation of succinate (Huang et al., 2006). A plant and fungal toxin, 3-nitropropionic acid can block complex II by irreversibly binding to A297 to form a complex with the side chain of A297 (Huang et al., 2006).

C. Complex III: Complex III (QH$_2$: cytochrome c oxidoreductase, EC 1.10.2.2), mediates another four protons to pass from the matrix to the intermembrane space. Cytochrome C is reduced by complex III at a redox potential of approximately +250 mV and donates the electrons to complex IV. Antimycin A is a specific inhibitor of the Qi site of complex III that blocks the oxidation of ubiquinol. This inhibition disrupts oxidative phosphorylation by disturbing the proton gradient across the inner membrane. Consequently, the absence of a proton gradient causes unavailability of protons through the ATP synthase complex that ultimately leads to ATP depletion. This inhibition also leads to the production of highly toxic reactive oxygen species (ROS), including peroxides and superoxides (Turrens et al., 2003). In addition, antimycin A induces mitochondrial swelling and mitochondrial depolarization (Sipos et al., 2003).

D. Complex IV: Complex IV (ferrocytochrome C: oxygen oxidoreductase, EC 1.9.3.1), converts one O$_2$ molecule to two H$_2$O molecules from four electrons supplied by reduced cytochrome C (one electron from each cytochrome C molecule). In this process, 4 protons are used from the mitochondrial matrix resulting in a proton electrochemical gradient between matrix and inner membrane space. This electrochemical gradient is used by the ATP synthase to produce ATP. Many molecules, including cyanide, sulfide, azide and carbon monoxide, act as specific inhibitors of the complex IV.

Together these four complexes are called supercomplexes, as they are thought to function together. These supercomplexes are present abundantly in the inner mitochondrial
Studies show that parkin and PICK1 are involved with mitochondrial maintenance. However, the role of parkin-PICK1 in mitochondrial maintenance remains unclear. According to our hypothesis, PICK1 may be involved in the trafficking of parkin to the mitochondrial membrane, thus regulating parkin-mediated mitochondrial effects. We believe that hindrance in parkin-PICK1 interaction may have adverse effect in mitochondrial maintenance.
2. Materials and Methods
Materials and equipment used

The following materials were used: LB media (L3152-1KG, Sigma); MgSO$_4$ (M2643-500G, Sigma); CaCl$_2$ (C3306-100G, Sigma); Glycerol (G5516-100ML); Mini-preparation kit (27106, Qiagen); Tryptone (J859-500G, Amresco); Yeast extract (J850-500G, Amresco); NaCl (S3014-500G, Sigma); Ampicillin (A9518, Sigma); Arabinose (A3256-25G, Sigma); IPTG (15502-1G, Sigma); SB buffer (17-0756-01, GE); Triton X-100 (P9284-500ML, Sigma Aldrich); EDTA (A3156-5G, Sigma); GST beads (17-0756-01, GE); BSA (A3156-5G, Sigma); HEPES (H3784-100G); EGTA (E3889-25G, Sigma); Tris (0497-1KG, Amresco); SDS (L4390-25G, Sigma); APS (A3678-25G, Sigma); TEMED (T7024-25ML, Sigma); β-mercaptoethanol (31350-010, Gibco); PVDF membrane (Poly Vinylidene Difluoride Membrane, P2938, Sigma); Sucrose (84097, Fluka); Methanol (34966-2.5lt, Sigma); ε-amino caprionic acid (A7824-25G, Sigma); Whatman papers (grade 3, 1003-917, Whatman); Non-fat milk (Marvel); Tween-20 (P7949-500ML); BCIP and NBT (S3771, Promega); EcoRI (10703737001, Roche); BamHI (10220612001, Roche); Pfu buffer and enzyme (15224-017, Invitrogen); dNTP (10226020, Roche); Polyacrylamide (161-0158, Bio-Rad); Phenol/Chloroform/Isoamyl alcohol (P3803-400ML, Sigma); Ethanol (E7023-500ML, Sigma Aldrich); TE (T9285-100ml, Sigma); Agarose (A6013-100G, Sigma); Gel extraction kit (28704, Qiagen); T4 ligase kit (15224-017, Invitrogen); MegaX DH10B T1 Electrocomp cells (C6400-03, Invitrogen); DMEM high-glucose media (L0104-500, Biosera); FCS (F2442-100ML, Sigma); Penicillin/streptomycin (15140-122, Gibco); Calcium phosphate (C7263, Sigma); PBS (20012-019, Gibco); Anti-GST (27-4577-50, GE); Peptides (Peptide 2.0, USA and Genscript, USA) Whatman paper (Schleicher and Schuell); Lipofectamine (18324, Invitrogen); Ficoll (F4375-100G, Sigma); Cytochrome C (C2037, Sigma); Sodium L-ascorbate (A4034, Sigma); Potassium Ferricyanide (P8131,Sigma); KCN (60178, Sigma); NADH (N8129, Sigma); Decyl Q (D7911, Sigma); Rotenone (R8875, Sigma); JC-1 (T3168, Biosciences); Amplex Red (A12222, Biosciences); Horseradish peroxidase P8250, Biosciences); Succinate (S9637, Sigma); Antimycin A (A8674, Sigma); Poly-L lysine (P1399, Sigma Aldrich, UK); Borosilicate glass coverslips 13mm (361-0149, VWR Ireland);

The following equipment was used: Incubator (Binder, Mason Technologies); Shaker (Excella E24 Incubator, New Brunswick Scientific); Spectrophotometer (Biophotometer, Eppendorf); UV transilluminator (Syngene); Powerpack (Biorad); Electroporator (Biorad); 50 ml centrifuge (Hettich Rotina 380R); Heating block (Grant); Tabletop centrifuge (Hermle); Ultracentrifuge (Discovery 100, Sorvall); Platereader Spectramax Gemini XS; PCR machine (Applied Biosys); Water bath (UAB 12 EU Grant); Sonicator (Sonics, Vibra-cell); Glass teflon homogeniser (Fisher).
Animals used in this thesis

Wildtype female Wistar rats (aged 6 weeks and/or weighed 150-200 g) were used in the preparation of synaptosome from rat brain. During the preparation of synaptosomes, they were sacrificed according to the guidelines prescribed by the Animals Act 1986 (Scientific Procedures) Schedule I. These rats were reared and supplied by the Bioresources Unit, School of Biochemistry and Immunology, Trinity College Dublin. For cortical neuronal culture and astrocyte culture, postnatal one day old female Wistar rats were sacrificed humanely (supplied by the Bioresources Unit, Trinity College Dublin).

1. Molecular biology methods

1.1 General molecular cloning strategies

Molecular cloning is a useful tool to modify the gene composition of a circular DNA (plasmid) by inserting any gene of interest and express the gene of interest in any system of cells. In this project, various cloning experiments were undertaken to construct plasmids carrying gene of interest for yeast two hybrid assay, mammalian cell transfection and lentiviral-mediated transfection. Figure 2.1 indicates the basic cloning steps and each step is elaborated below.

1.2 Bacterial competent cell preparation

Luria Bertani (LB) media was prepared for bacterial cell growth. *Escherichia coli* (*E. Coli*) was grown on LB plate overnight at 37°C incubator. A single colony was used to inoculate 5 ml LB medium with continuous shaking at 200 rpm overnight at 37°C in incubator. Thereafter, 2-3 ml of overnight grown bacterial culture was transfered into 100-200 ml of LB and incubated with continuous shaking at 200 rpm for 2-3 h at 37°C to reach an OD$_{600}$ of 0.6-1. The 200 ml sample was divided into 4 cell culture tubes of 50 ml and centrifuged at 3,000 xg for 15 min at 4°C. The bacterial pellet was resuspended thoroughly in ice-cold 20 ml 100 mM MgSO$_4$ and centrifuged 3,000 xg for 15 min at 4°C. The pellet was again resuspended in ice-cold 2 ml of 100 mM CaCl$_2$ with 15% glycerol. The bacterial solution was aliquoted by 50 µl in pre-cooled 1.5 ml tubes and snap-chilled in liquid nitrogen. The tubes were finally stored in -80°C until further use.
1. Restriction digestion
2. Dephosphorylation
3. Purification
4. Ligation
5. Transformation

Figure 2.1: Cloning strategy
The cloning steps are shown in roughly 5 steps. (1) Double restriction digestion of vector and the insert by two restriction enzymes to generate sticky ends. (2) Dephosphorylation of vector to remove the flanking phosphate group from the 5’ end to save it from self-ligation. (3) Purification of cut vector and cut insert from undigested fraction. (4) Ligation of insert into the vector through compatible flanking sites. (5) Transformation of the ligated vector into competent *E. coli* to increase copy number.
1.3 Heat shock transformation and plasmid DNA extraction from bacteria

Heat shock method of transformation was used to obtain recombinant bacterial colonies. Two *E. coli* strains were used for transformation, DH5α to yield plasmid DNA and BL21 Al to express recombinant protein. Tubes containing competent cells were thawed on ice for 15-20 min. Next, 1-2 μg of plasmid DNA was mixed with 50 μl of competent bacteria and incubated on ice for 15 min. Heat shock was applied for 60-90 sec by placing the tube at 42°C in a water bath/heating block and immediately transferred in ice for 10 min. To recover the cells, 1 ml LB was added to the transformed bacteria and incubated with continuous shaking at 200 rpm for 60-90 min at 37°C. The cells were centrifuged at 3,000 xg for 2-3 min. The pellet was re-suspended in 200 μl LB and spread on pre-warmed LB amp or kan agar plates. Plates were kept for incubation at 37°C. Colonies were counted after 8-12 h. A single colony was used to inoculate in 5 ml of LB medium and left overnight with continuous shaking at 200 rpm. The plasmid DNA was extracted from the bacterial culture by using mini-preparation kit, according to manufacturer’s recommendation (Qiagen).

1.4 Polymerase Chain Reaction and restriction digestion

The components, used in polymerase chain reaction (PCR) to amplify DNA, were 2 pg DNA template, 1x Pfu buffer, primers (10 pmol/μl), dNTP (200 μM) and 1 U of high-fidelity Pfu DNA polymerase in a 25 μl reaction. After the initial denaturation temperature (94°C for 5 min), the PCR reaction cycle was programmed as follows: denaturation (94°C for 30 sec), annealing (66°C for 30 sec) and elongation (72°C for 30 sec). This cycle was repeated 30 times to achieve the desired amplification. Before ending the reaction, the elongation temperature was maintained for 10 min at the end of 30th cycle. The sample is then stored at -20°C until further use. The restriction digestion was performed in a reaction volume of 25 μl. In this step, 2 μg of DNA was incubated with 1 U of restriction enzyme and 1x appropriate buffer at 37°C for 1.5 h. The primers used in this project are enlisted in Table 2.1.

1.5 Phenol/Chloroform/Isooamyl alcohol (PCI) purification

Phenol/chloroform/isoamyl alcohol was used to purify DNA to remove protein traces. DNA was treated with equal volume of phenol/chloroform/isoamyl alcohol (PCI), vortexed well and centrifuged at 13,000 xg for 10 min. After centrifugation, the liquid part divided into two layers, including upper aqueous and lower phenolic. The upper aqueous layer with DNA was collected, leaving behind the phenolic layer with the degraded protein fraction. The aqueous
<table>
<thead>
<tr>
<th>Oligoname</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdc4Frag1_F</td>
<td>5'- TTGAATTCGTGTGGCGGATCAGAGCC -3'</td>
</tr>
<tr>
<td>Cdc4Frag3_R</td>
<td>5'- CGGGATCCGTCATTCATGTCCACATCAA -3'</td>
</tr>
<tr>
<td>HA-park-Ascl_F</td>
<td>5'- CGGC CGGCAATGGCTACCCAT -3</td>
</tr>
<tr>
<td>HA-park-Rsrl_R1</td>
<td>5'- CGGTCCGCTACACGTCGAACCA -3</td>
</tr>
<tr>
<td>HA-park-Δpdz_R</td>
<td>5'- CGGTCCGCTACACGTGGTCCC -3'</td>
</tr>
</tbody>
</table>

**Table 2.1: List of primers**

These primers were used to PCR amplify the deletion constructs of Cdc4α-ct (pGBK7), HA-Parkin (pLenti-PGK) and HA-ParkinΔpdz (pLenti-PGK).
layer was treated with ice-cold absolute ethanol to precipitate the DNA. The DNA pellet was formed upon centrifugation at 13,000 xg for 15 min at 4°C. The DNA pellet was air dried for 2-3 h and dissolved in 10 µl sterile water.

1.6 DNA gel extraction

To recover small fragments of DNA (below 100 bp), 12% polyacrylamide gel was used. The DNA-PAGE gel was made with 0.8 mg/ml APS and 0.1% TEMED in TBE (40 mM Tris-borate and 1 mM EDTA) buffer. The DNA was separated using constant 70 V for 1 h. After separation, the DNA bands were cut out under UV light. The DNA was recovered from the polyacrylamide using a crush and elute process. The gel pieces containing the DNA were transferred to a small 500 µl Eppendorf tube with a pin-hole made in the bottom. The small Eppendorf tube was placed inside 1.5 ml Eppendorf tube and centrifuged at 13,000 xg for 3 min. Next, 200 µl of TE buffer (10 mM Tris·HCl, pH 8; 1 mM EDTA) was added to the crushed gel in the 1.5 ml Eppendorf tube and vortexed well. The tube was incubated overnight at 37°C to elute the DNA from the gel. After overnight incubation, the tube was vortexed, centrifuged at 13,000 xg for 3 min and 200 µl TE buffer was transferred to a second tube. The process of vortex and centrifugation was repeated after adding another 200 µl of TE to the small tube. At the end, the total 350 µl eluted DNA was purified by phenol/chloroform/isoamyl alcohol. Finally, the DNA pellet was resuspended in 10 µl of TE buffer and stored at -20°C until further use. To recover larger DNA fragments 1-2% agarose gel was used. The gel was run under constant 100 V for 30 min and DNA was extracted using gel extraction kit described by manufacturer (Qiagen).

1.7 Dephosphorylation and ligation

To restrict self-ligation, restriction digested vectors were dephosphorylated, i.e., removal of 5' phosphate group. Restriction digested vector DNA was dephosphorylated by incubating in 1x CIP buffer and 1 U of phosphatase enzyme at 37°C for 1 h. After dephosphorylation, the vector was separated in 2% agarose gel and rescued using gel extraction kit. Ligation reaction was made by adding restriction digested gene insert and vector in 3:1 ratio. The ligation reaction was performed with T4 ligase kit. To ligate 210 ng of insert with 70 ng of cut vector, 1x ligation buffer and 1 U of T4 DNA ligase (total volume of 20 µl) were used. The ligation mixture was incubated overnight at 16°C. After ligation, 2 µl of ligation mixture was used to transform 20 µl electro-competent cells by electroporation (2 kV, 200 Ω, 25 µF). The transformed cells were incubated in 1 ml of SOC medium for 1.5 h. The bacterial cells were centrifuged and pellet was resuspended in 200 µl LB (with kan or amp). The 200 µl cells were plated in respective kan or amp selective LB agar plates. Colonies, found after over-
night incubation, were inoculated in 5 ml of LB media at continuous shaking at 200 rpm for overnight at 37°C. The plasmid DNA was extracted using mini-preparation kit (Qiagen). Each sample was examined by PCR and restriction digestion to investigate the cloned insert. For further verification of the successful cloning, recombinant plasmids were sent for DNA sequencing in Eurofins MWG Operon.

2. Yeast two hybrid methods

2.1 Competent yeast preparation

Yeast strain *Saccharomyces cerevisiae* AH109 (630444, Clontech) was streaked onto a fresh YPAD-amp plate (1% bacto-yeast extract, 2% peptone, 0.1 mg/ml adenine, 2% agar and supplemented with 2% D-glucose and 50 mg/ml amp) and incubated for 2 days at 30°C. A single colony of 2-3 mm in diameter was re-streaked onto a fresh YPAD-amp plate and incubated for 2 days at 30°C. Thereafter, the single colony was inoculated in 10 ml YPAD media in a 50 ml falcon tube and incubated overnight with shaking at 200 rpm at 30°C. The culture was diluted to an OD°° 0.2-0.4 in 50 ml of YPAD media and incubated at 200 rpm shaking for 2-6 h at 30°C in 1 lt baffled flask the until OD°°reached 0.8-1. The yeast cells were centrifuged at 1,000 xg for 3 min at 4°C. The pellet was re-suspended in 1 ml TE and centrifuged at 1,000 xg for 3 min. Next, the pellet was re-suspended in 2 ml of LiOAc solution (10mM LiOAc in TE) and then incubated at room temperature for 10 min.

2.2 Yeast transformation

To transform competent yeast, 1 μg plasmid BAIT cDNA pGBK7 vector (Figure 2.2A), 1 μg plasmid FISH cDNA (pGADT7 vector) (Figure 2.2B) and 1 mg/ml denatured herring testes carrier sperm DNA were mixed with 100 μl competent AH109. To determine protein interactions as well as efficiency of the transformation, controls were included. Next, 700 μl of PEG solution (100 mM LiOAc; 40% PEG in TE buffer, pH 8) was added and thoroughly mixed to evenly distribute the cells. The samples were then incubated in shaker at 200-250 rpm for 30 min at 30°C. After incubation, 88 μl of dimethyl sulfoxide (DMSO) was added to the tubes and mixed well. Heat shock was administered for 7 min at 42°C in a water bath. The samples were centrifuged for at 1,000 xg for 10 sec and the pellets were re-suspended in 1 ml TE. The samples were again centrifuged at 1,000 xg for 10 sec and re-suspended in 50 μl TE. The transformed AH109 was then spread onto minimal supplement deficient agar base plates {46.7 g drop out (DO) media, pH 5.8} supplemented with 50 mg/ml amp with addition of BAIT supplement (0.74 g DO/-W), FISH supplement (0.69 g DO/-L), BAIT-FISH
Figure 2.2: Yeast plasmid vectors
As yeast two hybrid plasmids, A) pGBKTT BD and B) pGADT7 AD were used, which contain kan and amp resistant genes, respectively.
(0.64 g DO/-W/-L supplement) and INTERACTION supplement (0.6 g DO/-W/-L/-H). The plates were left for 7 days at 30°C and the β-galactosidase assay was used to determine positive BAIT-FISH interaction.

2.3 Interaction plate assay

Interaction between two proteins was evaluated using interaction plate assay. Yeast colonies grown on BAIT/FISH plates were picked up, dissolved in 100 µl TE buffer and 5 µl of yeast solution is dropped onto interaction plate. Thereafter, 10 µl of yeast solution is diluted in 90 µl of TE and 5 µl of diluted sample was dropped onto interaction plate. From each yeast plate, 5 colonies were collected and plated onto interaction plate. Plates were then incubated for 24-48 h at 30°C. Growth in both dilutions indicates a positive interaction. Empty vectors, pGBK7-pGADT7, were used as a negative control and GluR2-PICK1 as positive control in every yeast two hybrid assay.

3. Cellular protein expression methods

3.1 Protein expression in bacterial cell

A single colony of recombinant bacteria was inoculated in 10 ml YTamp media (16 mg/ml tryptone, 10 mg/ml yeast extract, 43 mM NaCl and 50 mg/ml amp) and incubated overnight at 37°C with shaking at 200 rpm. The overnight culture (1 ml) was used to inoculate 100 ml YTamp (prewarmed) in a 500 ml flask and incubated with shaking at 200 rpm for 1-2 h at 37°C until the OD₆₀₀ reached 0.5-0.7. Before induction with 0.2% arabinose or 0.6 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside), 1 ml of non-induced sample was collected as non-induced control to be loaded in a SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel. After addition of arabinose or IPTG, the culture was incubated with shaking at 200 rpm for 4-5 h at 37°C. After every h upon induction, 1 ml of culture was collected to be loaded in a SDS-PAGE. The 1 ml non-induced and induced samples were centrifuged at 12,000 xg for 5 min, resuspended in PBS (3.2 mM Na₂HPO₄; 0.5 mM KH₂PO₄; 1.3 mM KCl; 135 mM NaCl, pH 7.4) buffer, mixed with equal volume of SB buffer, boiled at 100°C for 5 min and loaded in 10% SDS-PAGE to measure the progressive expression profile of the induced protein. At the end of incubation, the rest of the culture was centrifuged at 3,000 xg for 20 min at 4°C. The bacterial pellet was frozen at -20°C and after thawing on ice, resuspended in 5 ml ice-cold PBS (50 µl PBS/ml bacterial culture). The OD₆₀₀ was determined and was typically 50-60. Next to ensure cell wall lysis of bacteria, 1% Lysozyme was added to the resuspended pellet and incubated at 10 rpm for 1 h at 4°C by
end-over-end agitation. Lysozyme catalyzes hydrolysis of peptidoglycan cell wall of bacteria. The lysate was sonicated on ice in a 15 ml falcon tube to lyse cells (30% amplitude, 10 x 10 sec). After adding 1% Triton X-100 and 0.1 mM EDTA, the bacterial lysate was incubated at 10 rpm for 1 h at 4°C by end-over-end agitation to ensure solubilisation of protein. The bacterial lysate was then centrifuged at 10,000 xg for 15 min at 4°C. Leaving behind the cell debris as pellet, the bacterial sonicate (the supernatant) was aliquoted in Eppendorf tubes and stored at -20°C until required.

3.2 Purification of protein expressed in bacterial cell

To harvest mammalian purified protein, 100 ml bacterial culture of YTamp media was prepared, similar to the procedure described above. The 100 ml culture was divided into two 50 ml sterile tubes and centrifuged at 3,000 xg for 20 min at 4°C to collect the bacterial pellet. Each pellet was resuspended in 10 ml lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EGTA; 1 mM EDTA; 0.5% Triton X-100; 1 unit protease inhibitor cocktail tablet for 10 ml) on ice. The resuspension was lysed by snap freezing in liquid nitrogen. The frozen lysate was thawed under cold water. Sonication was performed (30% amplitude, 8 x 1 min) with regular interval on ice for 1 min. Thereafter, the lysate was centrifuged at 22,000 xg for 1 h at 4°C. The supernatant was collected and mixed with 2 ml binding beads (GST or MBP beads), prewashed with wash buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EGTA; 1 mM EDTA) and resuspended in 50% slurry. Beads were added according to the protein of desire, i.e., maltose binding protein (MBP) beads for MBP tagged proteins and glutathione sepharose transferase (GST) protein beads for GST tagged proteins. The beads were incubated for 2 h at 4°C with end-over-end agitation. Subsequently, the lysate was centrifuged at 1,000 xg for 1 min at 4°C. Beads were collected as pellet and washed with 15 ml wash buffer twice. The bead slurry was transferred into 15 ml tube and centrifuged at 10,000 xg for 1 min at 4°C. The supernatant was discarded carefully. Then, 5 ml of elution buffer (5 mM HEPES, pH 7.6; 0.1% β-marcaptoethanol; 1 mM EDTA; 10% glycerol; 0.1% Triton X-100; 0.1 mM EGTA) was added to resuspend the beads and incubated for 15 min at room temperature. The resuspension was again centrifuged at 10,000 xg for 1 min at 4°C. The supernatant was collected as fraction containing expressed protein. Again, the process was repeated by adding 5 ml elution buffer for collection of the supernatant. Next, 10 ml of supernatant was dialysed for overnight at 4°C using Dialysing kit, following the manufacturer's instructions. At the end, purified proteins were aliquoted in small volume and stored in -20°C till further use.
3.3 Mammalian cell culture and cell transfection

HEK293 cells were grown in DMEM high glucose media with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin in 10 ml culture flasks in a humidified atmosphere of 5% CO₂ at 37°C incubator. Each time, the cell culture was started with 10% confluency and grown until 80%-90% confluency. Generally, the cells were split every 7 days. After every 3 days, the media was changed with pre-warmed fresh media. For cell transfection, 1-2 x 10⁵ cells/ml were grown overnight in 2 ml media in each well of 6-well plates (Nunc) at 37°C. Next day, the media was changed with pre-warmed OPTIMEM media. Lipofectamine based method was used to transfect the cells with 1 μg of mammalian plasmid DNA construct (pCI vector) and 10 μl of Lipofectamine (Figure 2.3A). The green fluorescent protein vector (GFP-C2, Clontech) was used as control (Figure 2.3B). After 6 h incubation in humidified atmosphere of 5% CO₂ at 37°C, the transfection mixture was replaced with pre-warmed normal DMEM media and the cells were allowed to grow. After overnight incubation in 5% CO₂ at 37°C, the media was changed. After 48 h, the cells were prepared for either affinity chromatography or immunocytochemistry. To perform affinity chromatography, the transfected cells were scrapped in 750 μl PTxE (1 mM EDTA, 1% Triton X-100 in PBS buffer) buffer and sonicated (25% amplitude, 10 x 5 sec). The sonicate was finally centrifuged at 13,000 xg for 15 min at 4°C and stored at -20°C until further use in co-IP assay.

4. Biochemical methods

4.1 Preparation of cell lysate

After the successful transfection, the HEK293 cells were scrapped off using a cell scraper from 6-well plates. The scrapped off HEK293 cells were washed with PTxE buffer and resuspended in PTxE buffer (250 μl per well of 6-well plate). Each sample was sonicated to lyse cells (25% amplitude, 6 x 10 sec). The cell lysate was incubated by end-to-end agitation at 10 rpm for 1 h at 4°C to ensure solubilisation. Then, the cell lysate was centrifuged at 15,000 xg for 5 min at 4°C. The soluble fraction was collected and used for GST pull-down or co-IP studies.

4.2 Preparation of rat brain lysate

The rat brain of approximately 1.50 g was homogenised in 20 volume of ice-cold homogenizing buffer (HB) (0.32 M sucrose; 4 mM HEPES; 1 mM EDTA; 1 mM EGTA, pH 7.4-HCl) in glass teflon homogeniser by gently moving up and down 6-8 times on ice.
Figure 2.3: Mammalian plasmid vectors
As mammalian cell expressing plasmids, A) pCI and B) pEGFP-C2 were used, which contain amp and kan resistant genes, respectively.
homogenate was centrifuged at 1,000 xg for 10 min at 4°C. The supernatant was aliquotted, 1 ml each in 1.5 ml Eppendorf tubes. The tubes were centrifuged at 13,000 xg for 30 min at 4°C. Each 1 ml pellet was resuspended in 500 μl of PTxE buffer. Each sample was sonicated to lyse cells (25% amplitude, 10 x 5 sec). The cell lysate was rotated at 10 rpm for 1 h at 4°C to ensure solubilisation. The cell lysate was then centrifuged at 15,000 xg for 5 min at 4°C. The soluble fraction was collected and used for co-IP studies.

4.3 GST/MBP pull-down assay

Mammalian genes were cloned in GST tagged vector (pGEX 4T) (Figure 2.4A) and MBP tagged vector (pMAL C2) (Figure 2.4B); and expressed transiently in bacterial cells. GST pull-down was performed with protein extracted from bacterial or mammalian source. Before GST pull-down experiments, the frozen protein samples were thawed on ice. The samples were then sonicated (25% amplitude, 5 x 10 sec) and rotated at 10 rpm for 1 h at 4°C. The sonicate was centrifuged at 13,000 xg for 10 min at 4°C. The GST co-IP experiment was set up as follows: 200-500 μl sonicate, 0.1% BSA and 20 μl of GST beads in a total reaction volume of 1 ml PTxE buffer in 1.5 ml eppendorf tube. In case of MBP tagged proteins, MBP beads were used instead of GST beads. The samples were rotated for 4 h at 4°C. After incubation, beads were settled by short centrifuge at 4,000 xg for 5 sec at room temperature. The beads were washed 4-5 times with PTxE buffer by repeated resuspension and centrifugation. After the washing step, the beads were finally resuspended in 20 μl PTxE buffer and boiled with 2x sample buffer (5% β-mercaptoethanol) for 5 min at 100°C, The boiled samples were centrifuged at 15,000 rpm for 10 min at room temperature. The supernatant was loaded in a 10% protein gel.

4.4 SDS-Poly Acrylamide Gel Electrophoresis and western blotting

Proteins are separated according to their respective molecular weight in poly acrylamide gels. In our experiments, 10% acrylamide and 4% acrylamide were used as stacking and resolving gel respectively. The resolving gel was made with 10% acrylamide; 0.4 mM Tris-HCl, pH 8.8; 0.1% SDS; 0.05% APS and 0.1% TEMED. The stacking gel was made with 4% acrylamide, 0.37 mM Tris-HCl pH 8.8, 0.1% SDS, 0.05% APS and 0.1% TEMED. The proteins were separated under constant 100 V, when the samples were in stacking gel and constant 150 V, when the samples entered the resolving gel. Separated proteins were identified by the Commassie stain or western blotting. Commassie staining was done by incubating the gel in Commassie Brilliant Blue dye for 1 h. To remove excess stain, the gel was incubated in destaining solution (water: methanol: acetic acid = 5:4:1) for 3 x 20 min. The western blotting was performed using a dry transfer method. The resolved proteins were
Figure 2.4: Bacterial plasmid vectors
As bacterial expressing plasmids A) pGEX 4T and B) pMAL C2 were used, containing amp resistant gene.
transferred to a PVDF (Poly Vinylidene Difluoride) membrane. The PVDF membrane was cut according to the size of the gel and immersed into methanol for 10 sec. Thereafter, for activation, the membrane was transferred into the solution C (25 mM Tris, 0.02% SDS, 20% methanol and 40 mM ε-amino caprionic acid) for 1 h. The Whatman papers (grade 3) were cut and two strips each were dipped into solution A (0.3M Tris, 0.02% SDS, 20% methanol), solution B (25 mM Tris, 0.02% SDS, 20% methanol) and solution C. The sandwich for the protein transfer was arranged in the transfer unit in following manner, Anode-2x Whatman papers in solution A-2x Whatman papers in solution B-PDVF membrane -SDS-PAGE gel-2x Whatman papers in solution C-Cathode. The transfer unit was run for 90 min under a constant current of 50 mA. After transfer, the PDVF membrane was blocked by incubating in 5% non-fat milk in PBS-T (0.1% Tween-20 in PBS buffer) buffer for 1 h at room temperature or overnight at 4°C. Then, the blot was incubated with primary antibody overnight at 4°C and washed with PBS-T buffer for 3 x 5 min. Thereafter, the blot was incubated in secondary antibody for 1 h at room temperature. Further, the blot was washed for 3 x 5 min in PBS-T and visualised using alkaline phosphatase (alkphos) buffer (100 mM NaCl; 5 mM MgCl₂; 100 mM Tris-HCl, pH 9.5). For alkphos based visualization, the blot was developed by incubating in BCIP and NBT substrates with alkphos buffer (30 µl BCIP and 60 µl NBT in 10 ml of alkphos buffer). In addition, horse radish peroxidise-linked (HRP) secondary antibodies were also used in some experiments. After incubation with secondary antibody and washing steps as described previously, the blot was incubated with chemiluminescent HRP Substrate according to manufacturer’s instruction (Millipore). The list of antibodies used in this project is enlisted in Table 2.2.

5. Mitochondrial assays

5.1 Isolation of synaptosomes using Ficoll gradients

To isolate synaptosomes from the rat brain, 2 rats were stunned and decapitated. The top of the skull was removed to scoop out the entire brain. The brains were quickly transferred into ice-cold STE (320 mM sucrose; 2 mM EDTA; 10 mM Tris-HCl, pH 7.4) buffer. After removing the excess blood by washing with STE buffer, the brains were chopped properly. The chopped brains were homogenised (12 passes) using the glass Dounce Homogeniser with a tight pestle (0.012). Then, the homogenised brains were then centrifuged at 1,500 xg for 3 min at 4°C to remove the cellular debris. Thereafter, the supernatant was collected and centrifuged at 12,000 xg for 10 min at 4°C. Resultant pellet was the Crude Mitochondrial Pellet (CMP). The CMP was carefully collected leaving behind any trace of blood and then
<table>
<thead>
<tr>
<th>Name</th>
<th>Raised against</th>
<th>Animal raised in</th>
<th>Conjugate</th>
<th>Cat. No. &amp; Company</th>
<th>Working dilution</th>
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<td>Z0334, DAKO</td>
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<td>AlkPhos</td>
<td>S3721, Promega</td>
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<td>Goat</td>
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<td>Dylight 549</td>
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<td>HRP</td>
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Table 2.2: List of antibodies used
resuspended into STE buffer. Thereafter, the CMP was loaded carefully over two Ficoll gradients in a ultracentrifuge tube in following manner; 10% (w/v) Ficoll solution, 7.5% (w/v) Ficoll solution and the CMP resuspension, respectively (Figure 2.5A). The tubes were then centrifuged in swing-out rotor in a Discovery ultracentrifuge at 104,200 xg for 55 min at 4°C. After ultracentrifugation, the myelin layer was removed formed in the interphase between the isolated medium and 7.5% (w/v) Ficoll layer (Figure 2.5B). The synaptosomal fraction was extracted from the interphase between the 7.5% Ficoll layer and the 10% Ficoll layer. The non-synaptic mitochondria formed a pellet at the bottom of the tube. The synaptosomes and pellet were separately resuspended in STE buffer and collected into 1.5 ml Eppendorf tubes. The Eppendorf tubes were centrifuged at 13,000 xg for 10 min at 4°C and the pellets were resuspended in STE buffer. A Bradford protein assay was performed to determine the concentration of protein.

5.2 Determination of protein concentration by Bradford assay

After extraction of synaptosomes, the concentration of synaptosomal protein was quantified by Bradford method (1976). According to this method, Bradford dye was used to rupture membranes of the synaptosomes, so that the protein content could be measured. A range of known concentrations of BSA were made up in triplicates to plot a standard curve against absorbance (OD 595), according to the Table 2.3A. The synaptosomal protein was diluted (1/100) in STE buffer and 100 µl was added to 700 µl of distilled water in triplicates. All the samples were then added with 200 µl of Bradford dye and mixed thoroughly. After 30 min of incubation, the samples were poured in 1 ml plastic cuvettes and the absorbance is measured at 595 nm by a spectrophotometer.

The synaptosomes are used as medium for studying mitochondrial dynamics due to presence of high number of mitochondria in them (Kilbride et al., 2008). Therefore, synaptosomal preparations were used in different mitochondrial assays and glutamate release to observe the effect of blocking peptides (Table 2.3) and compounds. In the following sections, details of the mitochondrial assays and glutamate release are described.

5.3 Measurement of ROS production

ROS, including superoxide (O2·) and hydroxyl radicals (OH·), hydrogen peroxide (H2O2) and peroxynitrite (ONOO−) are produced inside the cells as a byproduct of metabolism (True et al., 2000; Meng et al., 2002; Griendling et al., 2000). However, high amount of ROS production generates oxidative stress, leading to mitochondria dysfunction and apoptosis (Keeney et al., 2006). H2O2 is nonreactive, uncharged, relatively stable and freely diffusible
Figure 2.5: Isolation of synaptosomes using Ficoll gradient

a) The gradient of Ficoll is made by delicately layering 9 ml 7.5% (w/v) Ficoll on the top of 18 ml 10% (w/v) Ficoll, ensuring that an interface between the two layers could be seen clearly. The 9 ml of CMP in STE buffer is then layered on top of 7.5% Ficoll layer. b) After ultracentrifugation, myelin protein forms a layer above 7.5% Ficoll, whereas synaptosomes passes through the 7.5% (w/v) layer, forming a layer above 10% Ficoll layer. The non-synaptic fraction was settled as pellet on the bottom of the tube.
### A) Method of Bradford assay

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<th>BSA (μg)</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
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<tr>
<td>H₂O (μl)</td>
<td>800</td>
<td>780</td>
<td>750</td>
<td>700</td>
<td>650</td>
<td>600</td>
</tr>
<tr>
<td>BSA (0.1 μg/μl) added (μl)</td>
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<td>20</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>200</td>
</tr>
<tr>
<td>Bradford dye (μl)</td>
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<td>200</td>
<td>200</td>
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<tr>
<td>Total volume (μl)</td>
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<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
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</table>

### B) List of peptides

<table>
<thead>
<tr>
<th>Name</th>
<th>Domain</th>
<th>Motif</th>
<th>Peptide sequences</th>
<th>Purity%</th>
<th>Company</th>
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<tr>
<td>MNP201</td>
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<td>Parkin</td>
<td>VCMGDHWFDV</td>
<td>97.40%</td>
<td>Genescript</td>
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<tr>
<td>MNP201(mut)</td>
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<td>Parkin</td>
<td>VCMGDHWAAA</td>
<td>91.80%</td>
<td>Genescript</td>
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<tr>
<td>Tat-MNP201</td>
<td>PICK1</td>
<td>Parkin</td>
<td>YGRKKRRQRRRVCMGDHWFDV</td>
<td>95.30%</td>
<td>Genescript</td>
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<tr>
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<td>Parkin</td>
<td>YGRKKRRQRRRVCMGDHWAAA</td>
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<td>FITC-MNP201</td>
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<td>Peptide 2.0</td>
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<tr>
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<td>LVLDFDVAAA</td>
<td>94.78%</td>
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</table>

Table 2.3:  
A) Method of Bradford assay  
B) List of peptides
across adjacent cells or tissues to serve as a mediator of autocrine and paracrine system. 

$\text{H}_2\text{O}_2$ production is an important indicator of mitochondrial dysfunction and cell stress. According to the method of Mohanty et al. (1997), $\text{H}_2\text{O}_2$ production was measured by the amplex red assay. In the presence of $\text{H}_2\text{O}_2$, amplex red (10-acetyl-3,7-dihydroxyphenoxazine) converts to resorufin (excitation/emission wavelength = 550/585 nm), a highly fluorescent agent. Horseradish peroxidase catalyses the conversion of amplex red to resorufin, in the presence of $\text{H}_2\text{O}_2$. Production of $\text{H}_2\text{O}_2$ is proportional to the conversion of amplex red to resorufin and the amount of resorufin is considered as a quantitative indicator of $\text{H}_2\text{O}_2$ production.

\[
\begin{align*}
\text{H}_2\text{O}_2 & \xrightarrow{\text{Amplex red}} \text{H}_2\text{O} \\
& \xrightarrow{\text{HRP}} \text{Resorufin}
\end{align*}
\]

Synaptosomes (1 mg/ml) were incubated with 50 μM amplex red and 2.5 U/ml HRP in Krebs buffer (3 mM KCl; 140 mM NaCl; 10 mM glucose; 2 mM MgCl$_2$; 2 mM CaCl$_2$; 25 mM Tris, pH 7.4); and fluorescence intensity of resorufin was monitored at 37°C for 1-2 h in a Spectramax Gemini XS plate reader. The treatment groups were tested in triplicates for each individual experiment. After generation of the traces, the rate of change in fluorescence was measured over the same linear range for each condition (approximately 15-20 min). The values were converted as a percentage of control and represented in a bar diagram to indicate total peroxide release by the treatment group. Antimycin A induces high amount of peroxide production by selectively blocking complex III (Sipos et al., 2003). Therefore, 1 μM antimycin A was used as positive control in all the experiments.

5.4 Mitochondrial membrane potential measurement

Based on the method of Chinopoulos et al. (1999), the mitochondrial membrane potential was measured using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide). JC-1 is a cationic, lipophilic carbocyanine dye, which forms J-aggregates inside polarized mitochondria under normal condition, emitting red colour (absorption/emission spectra 585/590 nm upon excitation at 490 nm) (Reers et al., 1991). Under depolarized condition of mitochondria, release of monomeric form JC-1 occurs in cytoplasm, emitting green colour (absorption/emission spectra 510/527 nm upon excitation at 490 nm). The ratio of emission at 590 nm to 535 nm (JC-1$^{590/535}$) is a semi-quantitative measure of *in situ* mitochondrial membrane potential, because the formation of J-aggregate.
increases with membrane potential linearly within the range of -30 to -180 mV (Reers et al., 1995). The decrease in 590/535 ratio signifies reduction in mitochondrial membrane potential.

Synaptosomes (4 mg/ml) were incubated in dark with 6 μM JC-1 and Krebs buffer for 15 min at 37°C. Then, to remove excess JC-1, the sample was centrifuged at 16,000 x g for 5 min. Thereafter, the pellet was resuspended in Krebs buffer in Krebs buffer. The process of centrifugation and resuspension was repeated (3x). Then, the final pellet was resuspended in Krebs buffer and 1 mg/ml of JC-1 treated protein was loaded in 96-well black plate with the treatment groups in triplicates. The fluorescence was measured in a Spectramax Gemini XS plate reader (excitation spectrum of 490 nm and emission spectra of 535 and 590 nm) for 60 min at 37°C. The excitation and emission slits were set at 10 nm. The JC-1$_{590/535}$ was plotted against time representing rate of change in mitochondrial membrane potential with time, as described in Kilbride et al. (2008). To calculate the mitochondrial membrane potential in treated samples, the rate of change in the JC-1$_{590/535}$ was measured over the same linear range for each condition (15-20 min). The values were converted as a percentage of control and represented in a bar diagram indicating the membrane potential of synaptosomal mitochondria.

5.5 Glutamate release assay

Glutamate is a neurotransmitter that has important roles to play in the long term potentiation, learning and memory (Riedel et al., 1996). The release of glutamate from synaptosomes is measured by adapting a continuous fluorimetric method based on Nicholls et al. (1987). Glutamate converts to 2-oxoglutarate using cofactor nicotinamide adenine dinucleotide phosphate (NADP$^+$). This reaction is catalysed by the L-glutamate dehydrogenase (GDH) and in the process; NADP$^+$ converts to nicotinamide adenine dinucleotide 2'-phosphate (NADPH). The amount of release glutamate is measured by quantifying NADPH (excitation/emission = 340/460 nm) spectrophotometrically.

\[
\text{GDH} \\
\text{Glutamate} \xrightarrow{\text{GDH}} 2\text{-oxoglutarate} \\
\text{NADP}^+ \rightarrow \text{NADPH}
\]

After extracting fresh synaptosomes, the concentration of synaptosomal protein was measured and aliquoted into 1.5 ml Eppendorf tubes, 1 mg each. Then, each tube was added with 1 ml of TES buffer (250 mM sucrose; 5 mM TES, pH 7.4) and centrifuged at
12,000 xg for 5 min at 4°C. The pellets were stored in ice until used for experiment (15-20 min). During experiment, the pellets were resuspended in 1 ml Kreb's buffer (with and without 2 mM CaCl₂) to obtain a protein concentration of 1 mg/ml. Thereafter, in the 96-well blackplate, 0.5 μg/μl of protein was added with 31.6 U/ml GDH and 1 mM NADP⁺ and the reading was performed in SpectraMax GeminiXS fluorimetric plate reader at 37°C (excitation/emission spectra = 340/460 nm). First, the blank rate fluorescence was recorded as continuous fluorimetric readings for 5 min. Then, treatment group was added to the synaptosomes in triplicates and the baseline fluorescence was monitored for next 5 min. Thereafter, depolarization of synaptosomes were conducted by addition of KCl (final concentration 40 mM) or 4-AP (final concentration 10 mM) and the fluorescence under depolarized condition was recorded for 30 min. Rotenone-mediated inhibition of complex I has been shown to induce glutamate release from synaptosomes (Kilbride et al., 2008). Therefore, 10 μM rotenone was assayed with every glutamate release experiment as positive control. The total glutamate release from non-depolarized and depolarized conditions were calculated over a period of 5 min and 30 min, respectively; and represented as a percentage of control in bar diagrams.

5.6 Respiratory chain complex assay

To determine the activity of respiratory chain complexes, synaptosomal mitochondria were used in the respiratory chain complex assays. To perform these studies, the mitochondrial inner membrane is to be exposed to facilitate the entry of reaction mixture to the respiratory chain complexes. To fracture mitochondria, the synaptosomes were freeze-fractured by quick freeze and thaw cycle in liquid nitrogen and 37°C waterbath (3x), respectively. After freeze-fracture, the synaptosomal samples was used for respiratory chain assays or stored in -80°C for further use.

5.6.1 Complex I assay

Complex I (EC 1.6.99.3, NADH: cytochrome C oxidoreductase) activity was measured by using the method based on Ragan et al. (1987). The activity of complex I was measured by the rate of conversion of NADH to NAD⁺ spectrophotometrically at absorbance 340 nm. To perform the assay, 50 μg of mitochondrial protein was mixed with complex I buffer (10 mM MgCl₂, 25 mM potassium phosphate, pH 7.4), 0.2 mM NADH, 2.5 mg BSA and 1 mM KCN (inhibitor of complex IV) in 1 ml of solution. The treatment groups were tested in triplicates and the absorbance of NADH (340 nm) was monitored for 20 min at 37°C in a Cary UV spectrophotometer. CoQ is reduced to ubiquinol during the oxidation of NADH by complex I. To start the reaction, 50 μM
of decylubiquinone (DQ), a CoQ analog, was added after 1-2 min (baseline rate) and the rate of reaction was recorded for 12-13 min. Then, 10 μM of rotenone was added to inhibit complex I activity and the reaction was followed for next 5-6 min, as indicated in Ragan et al. (1987). The activity of complex I is a pseudo-first order reaction. The specific activities of the complex I were determined by calculating the linear rate constants of NADH, after addition of DQ ($K_{DQ}$) and rotenone ($K_{ROT}$). The rotenone insensitive rate was subtracted from the initial rates to obtain the specific activity of complex I ($K_{DQ}=K_{ROT}$) in mitochondria and the values were expressed in μmol min$^{-1}$ mg$^{-1}$ (Kilbride et al 2008). The data was converted as a percentage of control and represented in bar graph to compare different peptides activity.

**5.6.2 Complex IV assay**

Complex IV (EC 1.9.3.1, cytochrome c oxidase) assay was based on the method by Wharton and Tzagoloff (1967). The oxidation of reduced cytochrome C by cytochrome C oxidase was measured as a decrease in absorbance at 550 nm at 37°C. The reaction mixture was made of 50 μM reduced cytochrome C and complex IV buffer (10 mM of potassium phosphate buffer, pH 7) to a final volume of 1 ml. The treatment groups were tested in triplicates and the absorbance of cytochrome C (550 nm) was monitored for 2 min (baseline rate) in a Cary UV spectrophotometer. Then, the reaction was initiated by adding 100 μg of freeze-fractured synaptosomal protein and decrease in absorbance was recorded for next 10 min. After a rapid decrease in absorbance, the rate of activity reached a plateau following a first-order rate reaction. The time point of protein addition was named $t = 0$ and the first-order decay rate constant ($k$) was calculated as from the difference between the natural logarithms of the absorbance at this point and three subsequent time points 1, 2 and 3 min. The mean of the calculated specific activity values was expressed in k min$^{-1}$ mg$^{-1}$. The data was converted as a percentage of control and represented in bar graph to compare the activities of different peptides.

**5.6.3 Preparation of reduced cytochrome C**

The oxidised cytochrome C (25 mg/ 2.5 ml) was reduced by the addition of a few crystals of ascorbic acid. The deep red colour of cytochrome C changed into bright pink colour. Then, a PD$_{10}$ gel filtration column was activated by passing through 50 ml of assay buffer (10 mM potassium phosphate buffer, pH 7). The activated column was used to remove the ascorbic acid by passing the solution through 2.5 ml of cytochrome C. The column was eluted by addition of 3 ml assay buffer. The reduced
cytochrome C was collected as darkest band of eluent from the column. The concentration of the reduced cytochrome C was measured on Cary UV spectrophotometer using 10 µl of 100 mM ferricyanide to oxidise cytochrome C.

5.7 Statistical analysis

The data obtained from the experiments were converted as a percentage of control and expressed in mean ± SEM (standard error mean) in the result sections. The percentage of control values were used to construct bar diagrams. For statistical analysis, GraphPad Prism 5.03 software was used. To compare two treatment groups, student t-test was used. To compare more than two treatment groups, Dunnett post-hoc test was used under one-way ANOVA. The significance level or p-values depicted in the data were 0.05 (*), 0.01 (**) and 0.001 (***) (p-value).

6. Primary Cell Culture

6.1 Set-up and preparation before primary cell culture

To culture neurons, 6-well plates and coversliped 24-well plates were used for western blots and imaging, respectively. For culturing on coverslips in 24-well plates, first borosilicate glass coverslips with a diameter of 13 mm were sterilized by immersing in 70% methanol followed by exposure to UV light overnight. Coverslips were then coated with poly-L-lysine (40 µg/ml) for 1 h at 37°C. Thereafter, to remove excess poly-L-lysine, coverslips were rinsed with cell culture grade water and allowed to air dry in the laminar flow hood before being placed in 24-well plates.

6.2 Brain dissection for primary cultures

Postnatal one day old female Wistar rats (supplied by the Bioresources Unit, Trinity College Dublin) were used for neuronal cultures. The pups were decapitated according to the guidelines prescribed by the Animals Act 1986 (Scientific Procedures) Schedule I. The skull was exposed by cutting the skin from the neck down to the tip of the nose. The brain was exposed by making a sagittal cut along the level of the medial longitudinal fissure and two horizontal cuts along each side of the skull at the level of the ears. The skull was peeled back revealing the cortex, which was rapidly removed with curved forceps.
6.3 Neuronal Culture

Dissected cortices were placed in 20 μl pre-warmed neuronal culture buffer (116 mM NaCl; 5.4 mM KCl; 26 mM NaHCO₃; 1.3 mM NaHPO₄; 1 mM MgSO₄.7H₂O; 1 mM CaCl₂.2H₂O; 0.5 mM EDTA.2Na.2H₂O; 25 mM glucose, pH = 7.4). Then, the cortices were chopped using a sterile disposable scalpel. The tissue was then transferred into 2.5 ml of 0.15% papain solution and left to incubate at 37°C. The tissue was then carefully transferred into 2 ml of 1% BSA solution. The solution was triturated (15x approximately) with a fine tipped Pasteur pipette, before transferring into another 2 ml of BSA solution to triturate again and this was repeated (3x). Thereafter, the resultant solution was centrifuged at 3,000 xg for 3 min at RT. The supernatant was removed carefully without disturbing the pellet. The pellet was then resuspended with 5 ml pre-warmed supplemented neurobasal-A media (neurobasal-A media, 1 ml B-27, 0.5 ml 200 μM glutamine). The solution was incubated at room temperature for 5 min so that any connective tissue and any cell clumps can settle to the bottom. The upper layer of the solution was plated, 120 μl per well for 24-well plates and 500 μl for 6-well plates. After incubating for 1 h at 37°C with 5% CO₂, the wells were filled with 500 μl pre-warmed supplemented Neurobasal media. After every third day, 50% of the media was replaced with new media. After maturing the cells for 10-14 days, treatments were carried out with peptides at 37°C for 1 h in 5% CO₂.

6.4 Immunocytochemistry

Treatment on the neuronal cells was done after 10-14 days in 24-well plates. After treatment, the media were taken out and cells were washed with 3 x 1 ml PBS. The cells were fixed by treating with 100% ice-cold methanol at room temperature for 5 min. After fixation, the cells were washed with PBS 2 x 5 min. To permeabilise, the cells were incubated in 0.10% Triton X-100 for 30 min. Later, cells were incubated in blocking solution (5% normal goat serum, 0.5% BSA in PBS) for overnight at 4°C or 1 h at room temperature. Thereafter, the cells were incubated in primary antibodies overnight at 4°C. Then, the cells were washed for 3 x 5 min with PBS. Subsequently, the cells were treated with fluorephore tagged secondary antibodies for 1 h at room temperature (for list of antibodies, see Table 2.2). After incubation, the cells were washed with PBS for 3 x 5 min. Thereafter, the coverslips were taken out from the wells and placed inverted onto a glass slide with a drop of 90% glycerol mounting media. The periphery of the coverslips was sealed by applying nail varnish. The cells were then imaged using a Zeiss LSM 510 META confocal laser scanning microscope utilising an Axiovert 200M inverted microscope (Zeiss Ltd, UK). Images were captured and optimized using LSM510 computer program and were acquired at 40x and 63x.
magnification. Initially optimal settings for image quality were obtained and thereafter the settings remained unchanged. To calculate mean pixel intensity, the ImageJ software was used.
3. Results: 1

Validation of parkin-PICK1 interaction and of the MNP201 parkin peptide
Abstract

The objective of this project was to investigate the role of parkin-PICK1 interaction in mitochondrial function. The ct-parkin contains a PDZ motif that interacts with the PDZ domain of PICK1 and causes monoubiquitination of PICK1 (Joch et al., 2007). In this chapter, a set of approaches were undertaken to demonstrate an interaction between parkin and PICK1. In addition, the binding of a parkin blocking peptide (MNP201) to PICK1, modelled according to the last 10 amino acids of ct-parkin (VCMGDHWFDV), was examined. To show a parkin-PICK1 interaction, in the first approach, a bacterial expressed MBP tagged PICK1 was used to pull-down endogenous parkin from rat brain lysate. In these experiments, due to poor anti-parkin antibody recognition (Abcam), the expression of endogenous parkin was not detected. Then, co-IP experiments were performed using lysates from HEK293 cells transiently transfected with HA-parkin and Flag-PICK1. Due to the absence of the full length HA-parkin expression and poor expression of Flag-PICK1, the results failed to show parkin-PICK1 interaction. Thereafter, pull-down studies were performed using MBP-PICK1 and transiently expressed mutant parkin (Ubi domain deletion). In these experiments the mutant parkin was well expressed although it showed no interaction with PICK1 interaction. These studies also faced technical hurdles, insofar that antibody crossreactive bands were detected in pull-down and CoIP experiments. Thus, as an alternative, fluorescence polarisation assays were used to demonstrate the binding of the MNP201 to PICK1. The data in these experiments showed that MNP201 (VCMGDHWFDV), but not a PDZ-motif mutant of MNP201 (VCMGDHWAAA), blocked the binding of a DAT-peptide to PICK1 suggesting competitive binding to the PDZ domain of PICK1.
Introduction

ARJP is motor-related neurodegenerative disease that results from death of dopamine producing neurons in the mid-brain. Mutations in parkin, an E3 ligase responsible for inducing degradation of several proteins, are associated with this disease (Kitada et al., 1998). Several mutations in the parkin gene have been identified in ARJP patients impairing ubiquitin ligase function of parkin (Bonifati, 2012). A non-functional parkin is unable to ubiquitinate its protein substrates, which are no longer targetted to the proteasome for degradation (Pagano, 1997; Ben-Neriah, 2002). The build up of these parkin substrates results in protein aggregation and causes cell toxicity that ultimately results in cell death. In addition to cellular toxicity induced by unwanted proteins, mitochondrial dysfunction is also identified as a cause for the dopaminergic neurodegeneration in PD (Kosel et al., 1999; Beal et al., 2003). PD patients have been reported to show impaired complex I activity of the ETC cycle that eventually limits oxidative phosphorylation, the key mechanism of ATP synthesis (Schapira et al., 1990; Parker et al., 1989, 2008; Bindoff, 1989). This impairment also leads to ROS production that causes mitochondrial dysfunction and apoptosis (Keeney et al., 2006). Importantly, parkin is believed to play a crucial role in the maintenance of mitochondrial integrity (Abou-Sleiman et al., 2006). Dopaminergic neurodegeneration and malformed mitochondria were identified in the parkin mutant of Drosophila (Greene et al., 2003; Whitworth et al., 2005). Many recent reports confirmed a crucial role of parkin in mitochondria, where parkin selectively recruits autophagosomes to depolarized mitochondria causing mitophagy (Narendra et al., 2008; Twig et al., 2008).

One of the interacting partners of parkin is PICK1, which interacts with parkin through a PDZ domain-motif interaction (Joch et al., 2007). Successful pull-down of endogenous PICK1 from mouse synaptosomes using GST constructs of parkin wild type and deletion mutants have been demonstrated (Joch et al 2007). The experiment demonstrated that the parkin-PICK1 interaction was PDZ domain-dependent as the PDZ binding motif deleted parkin constructs were unable to pull-down endogenous PICK1 (Joch et al 2007). Surprisingly, parkin caused mono-ubiquitination of PICK1 but does not induce degradation (Joch et al., 2007). PICK1, originally identified as a PKCα recruiting protein, recruits PKCα to the outer membrane of mitochondria (Wang et al., 2003). PICK1 is also reported to translocate to the mitochondria in National Institute of Health "3-day transfer, inoculum 3 x 105 cells" (NIH3T3) cells in both non-stimulated and serum-stimulated conditions (Wang et al., 2003). Further research revealed that PICK1 recruits PKCα to phosphorylate the Bcl-2 protein of the outer mitochondrial membrane of human promyelocytic leukemia 60 (HL60) cells that confers resistance against Bax-induced apoptosis (Wang et al., 2007). Thus, while there is data
supporting a role for a PKCa-PICK1 interaction in mitochondrial function, the role of the PICK1-parkin interaction in mitochondrial is still unknown.

A working hypothesis in this project is that the parkin-PICK1 interaction is important for recruiting parkin to mitochondria and that this interaction has a role in mitochondrial maintenance. Thus, in this chapter, parkin blocking peptides (MNP201) were designed based on the last 10 amino acids of ct-parkin to block the parkin-PICK1 interaction. The binding of these parkin blocking peptides (MNP201) to PICK1 was then determined. To show a parkin-PICK1 interaction, pull-down and co-IP experiments were also performed. In this chapter, the first aim was to show an interaction between endogenous parkin and bacterial expressed PICK1, using a pull-down approach. To confirm a parkin-PICK1 interaction, co-IP experiments were also performed using transiently expressed parkin and PICK1. In addition, a set of control experiments were conducted, investigating the origin of non-specific bands arising from the antibody cross-reactivity in pull-down and co-IP experiments. In these experiments, the expression of full length parkin was found to be low as previously reported (Finney et al., 2003), thus a mutated version of parkin was also used in the parkin-PICK1 interaction studies. In addition, lentiviral parkin vectors were constructed to over-express full length parkin in neurons to overcome the issue of low parkin expression. Finally, the binding of parkin peptide, MNP201, was investigated in a fluorescence polarisation assay and successfully showed that MNP201 binds to PICK1, likely via its PDZ domain.
Results

3.1 Design of MNP201

The last three amino acids of ct-parkin are found to be crucial for PDZ domain-mediated interaction with PICK1 (Joch et al., 2007). On the basis of these data, peptides were generated based on the last ten amino acids (VCMGDHWFDV) of the ct-parkin, which included the PDZ motif sequence (-FDV). The wild type peptide was named Molecular NeuroPeptide 201 (MNP201). A mutated version of the peptide, to be used as negative control, was also designed (MNP201(mut)), where the last three essential amino acids were replaced with alanines (VCMGDHWAAA). These MNP201 compounds were ordered from Genscript, USA. The HPLC purity of MNP201 and MNP201(mut) were found to be 97.4% and 85.5% respectively. The list and the HPLC purity profile are shown in Figure 3.1 were provided by Genscript, USA. Both the MNP201 and MNP201(mut) were dissolved in 50 mM HEPES pH 7.0 solution.

3.2 Pull-down of PICK1 with GST-GluR2-ct

A well established interacting partner of PICK1 is the AMPA receptor subunit GluR2 where the PDZ motif of ct-GluR2 interacts with the PDZ domain of PICK1 (Dev et al., 1999; Xia et al., 1999). To show the interaction between PICK1-GluR2 by pull-down, an experiment with MBP beads was performed using bacterial expressed MBP tagged full length PICK1 (MBP-PICK1) and bacterial expressed GST tagged ct-GluR2 (GST-GluR2-ct), as previously described (Dev et al., 1999). As a negative control, only GST protein and MBP-PICK1 were incubated in similar manner. After the incubation, the samples were washed and separated in a SDS-PAGE gel and western blots were performed using anti-GST and anti-MBP antibodies (Figure 3.2). The anti-GST blot revealed presence of GST and GST-GluR2-ct proteins in equal amounts at their respective molecular weights. The anti-MBP blot demonstrated no band of MBP-PICK1 in the GST lane, but a strong MBP-PICK1 band was identified on the GST-GluR2-ct lane. Taken together, in agreement with previous studies (Dev et al., 1999 and 2000), the experiment suggests that PICK1 interacts with the ct-GluR2.
A) The MNP201 peptides modelled on the ct PDZ motif of parkin are shown in the table. B) The HPLC purity profiles of these peptides measured at 220 nm were found to be 97.4% (MNP201) and 85.5% (MNP201 (mutant)). The parkin peptides were ordered from Genscript, USA.

<table>
<thead>
<tr>
<th>Name</th>
<th>Domain</th>
<th>Motif</th>
<th>Peptide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNP201</td>
<td>PICK1</td>
<td>PARKIN</td>
<td>VCMGDHWFDV</td>
</tr>
<tr>
<td>MNP201(mut)</td>
<td>PICK1</td>
<td>PARKIN</td>
<td>VCMGDHWAAA</td>
</tr>
</tbody>
</table>

Figure 3.1: Blocking peptides based on the ct of parkin
A) The MNP201 peptides modelled on the ct PDZ motif of parkin are shown in the table. B) The HPLC purity profiles of these peptides measured at 220 nm were found to be 97.4% (MNP201) and 85.5% (MNP201 (mutant)). The parkin peptides were ordered from Genscript, USA.
Figure 3.2: GST pull-down experiment showing PICK1-GluR2 interaction

MBP-PICK1 was incubated with GST and GST-GluR2-ct along with GST beads. The samples were boiled in SB buffer, separated in a SDS-PAGE and then western blot was performed with anti-GST and anti-MBP antibody. A) The anti-GST blot showed GST and GST-GluR2-ct bands. In contrast, the MBP-PICK1 band is observed in only the GST-GluR2-ct lane in anti-MBP blot. B) The diagram shows the design of the experiment where GST beads bind with GST tag of GST-GluR2-ct and the associated MBP-PICK1.
3.3 Pull-down of endogenous parkin by MBP-PICK1

To confirm the parkin-PICK1 interaction, MBP pull-down experiments were performed using transformed bacterial lysate expressing MBP-PICK1 and the rat brain lysate as a source of endogenous parkin. MBP-PICK1 was incubated with the rat brain lysate to allow endogenous parkin to interact with MBP-PICK1. The experiments showed that the MBP beads pulled down MBP-PICK1 as determined by western blot using anti-PICK1 antibody. In contrast, no parkin band was detected in the pull-down or in the input fraction as determined by western blot using anti-parkin antibody (Figure 3.3). Notably, the absence of a parkin band in the input fraction, suggested that the polyclonal Abcam anti-parkin antibody used in these experiments was not able to detect endogenous parkin. The presence of non-specific bands were also observed in both the anti-parkin and anti-MBP blots, likely due to related binding of the antibodies to the bacterial proteins. In summary, these experiments showed; (1) the Abcam anti-parkin antibody failed to detect endogenous parkin in the rat brain lysate and (2) non-specific bands were detected in the crude bacterial lysate expressing MBP-PICK1 in the anti-parkin and anti-MBP western blots. In the next experiments, transiently transfected HEK293 cells were used to express HA-parkin and Flag-PICK1, and co-IP studies were performed.

3.4 Co-IP experiments using HA-parkin and Flag-PICK1 co-transfected in HEK293 cells

Generally, HEK293 cells are used to transiently over-express proteins of interest. The gene expressing protein of interest is cloned into the mammalian vector and used to transfect HEK293 cells for this purpose. The HA-parkin (pCI) and Flag-PICK1 (pCI) constructs were used to transfect overnight grown HEK293 cells (confluency 60%-80%) by lipofectamine-mediated transfection according to manufacturer’s protocol in 6-well plate format. To monitor transfection efficiency, 0.1 pg of pEGFP-C2 was used to transfect a single well of 6-well plate containing HEK293, cells along with the HA-parkin and Flag-PICK1. Transfection was carried out with either HA-parkin (1 µg) and Flag-PICK1 (1 µg) alone or, co-transfecting HA-parkin and Flag-PICK1 (1 µg of each). Transfection efficiency of 30-40% was estimated by pEGFP-C2 expression (excitation/emission spectra = 488/507 nm). After transfection, the cell lysates (500 µl) were prepared from each condition of transfection and incubated with Flag beads. After the western blot analysis, no expression of the HA-parkin was detected by using anti-HA antibody in both input and co-IP fractions of single and double transfection samples (Figure 3.4). Low expression of Flag-PICK1 was detected using anti-PICK1 antibody in both single and double transfected samples. In addition, numerous non-specific
Figure 3.3: MBP pull-down experiment with bacterial expressed MBP-PICK1 and endogenous parkin

Purified bacterial expressed MBP-PICK1 was used to pull down endogenous parkin from rat brain lysate using MBP beads. MBP beads bind the MBP tagged protein (MBP-PICK1) and also the proteins associated with PICK1. In this experiment, rat brain lysate (RB) and MBP-PICK1 were incubated with MBP beads. A) The anti-parkin blot shows no endogenous parkin expression in both pull-down and input fraction except some bands which are likely due to non-specific binding of antibodies to bacterial proteins. The anti-MBP blot shows an MBP-PICK1 (77.50 kDa) in both pull-down blot and 10% input fraction. B) The diagram shows the design of the experiment where MBP beads bind with MBP tag of MBP-PICK1 and associated parkin with MBP-PICK1.
Figure 3.4: Co-IP experiment with transiently transfected HEK293 cells with HA-parkin and Flag-PICK1

HEK293 cells were transiently transfected with HA-parkin and Flag-PICK1 constructs in both single and double transfection format. After making cell lysates, Flag beads were incubated with cell lysates expressing HA-parkin, Flag-PICK1 or both proteins. A) Co-IP blot of anti-HA shows no expression of HA-parkin in both co-IP and input fractions. The anti-Flag blot shows poor expression of Flag-PICK1 (50 kDa) in both co-IP and input fractions. Numerous non-specific bands are visible in the co-IP fraction. B) To estimate the transfection efficiency, the HEK293 cell culture was transfected with pEGFP-C2 vector. The GFP protein fluorescence is visible under 488 nm (transfection efficiency 30-40%). C) The diagram shows the design of the experiment where Flag beads bind with Flag tag of Flag-PICK1 and associated HA-parkin with Flag-PICK1.
bands were detected on the blot incubated with anti-Flag antibody. The absence of HA-parkin expression, suggested that HA-parkin was poorly expressed in these experiments. In summary, these experiments showed; (1) little or no expression of the HA-Parkin, (2) poor expression of the Flag-PICK1 and (3) expression of the non-specific bands probably arising from Flag-beads. In the next experiments, the identification of the non-specific bands (Figure 3.5) and use of a parkin UbI deletion construct for better expression in HEK293 cells (Figure 3.6) were investigated.

3.5 Identification of non-specific bands expressed by Flag beads and MBP-beads

The presence of non-specific bands in the anti-Flag blot was further addressed before further use of transiently transfected HEK293 cells to express HA-parkin and Flag-PICK1 in co-IP experiments. Mock experiments with Flag beads and MBP beads were performed to investigate non-specific bands due to antibody cross-reactivity. After incubation and washing the beads, they were directly boiled in SB buffer and loaded into SDS-PAGE gels. The western blots were performed using the alkphos conjugated anti-mouse and anti-rabbit secondary antibodies, separately. Upon development, the Flag bead sample incubated with anti-mouse secondary antibody showed many non-specific bands, indicating mouse antibody cross-reactivity as expected (Figure 3.5). This data are in agreement with the non-specific bands found in Figure 3.4, where co-IP studies were performed to demonstrate an interaction between parkin and PICK1. In contrast, no non-specific bands were visible in the anti-rabbit antibody incubated blot in Flag bead sample. In addition, the MBP beads did not show bands arising from non-specific reaction in any of the blots. The results suggest that using anti-mouse secondary antibody in the presence of Flag beads will produce non-specific bands that make it difficult to identify a parkin-PICK1 interaction. MBP beads was thus used to determine an interaction between parkin and PICK1.

3.6 Identification of non-specific bands expressed by anti-PRK8 and anti-PICK1 antibody

Due to poor expression of HA-parkin and Flag-PICK1, co-IP of endogenous parkin and PICK1 was performed using rat brain lysate. In previous western blot experiments, the rabbit anti-parkin antibody, used to detect endogenous parkin did not provide strong signal or good specificity (Figure 3.3). For this reason, an alternative antibody for parkin, mouse anti-PRK8 was next used for co-IP (Pawlyk et al., 2003). To first evaluate whether the anti-
Figure 3.5: Identification of non-specific bands expressed by Flag beads

Flag beads and MBP beads were incubated, washed, boiled in SB buffer and then western blot was performed. The anti-mouse blot shows numerous non-specific bands expressed by Flag beads sample. No bands were visible from MBP beads sample. In the case of the anti-rabbit blot, no non-specific band was visible in either of the samples.
PRK8 antibody itself is capable of producing non-specific bands in the western blots, mouse anti-PRK8 antibody (1 μl) was incubated with sepharose beads as in a co-IP experiment. As a negative control, sepharose beads alone were incubated. In addition, rabbit anti-PICK1 antibody (1 μl) and sepharose beads were also incubated. After incubation and washing the beads, they were boiled in SB buffer and loaded onto SDS-PAGE gels. The western blots were performed with mouse anti-PRK8 and rabbit anti-PICK1 antibodies, and then incubated with corresponding alkphos conjugated secondary antibodies, i.e., anti-mouse (BLOT A) and anti-rabbit (BLOT B), respectively (Figure 3.6A). The blots were also directly incubated with secondary anti-mouse (BLOT C) and anti-rabbit (BLOT D) antibodies, without primary anti-PRK8 or anti-PICK1 antibodies (Figure 3.6B). Upon development of the blots, mouse anti-PRK8 antibody revealed strong non-specific bands in those blots where anti-mouse secondary antibodies were used (BLOT A and BLOT C). The rabbit anti-PICK1 lanes also showed non-specific bands in those blots, where anti-rabbit secondary antibody was used (BLOT B and BLOT D). None of the blots demonstrated any non-specific bands by the negative control (sepharose beads only), confirming that the non-specific bands were due to immunogenic cross-reactivity of mouse anti-PRK8 and rabbit anti-PICK1 antibodies. The data suggest that mouse anti-PRK8 antibody is capable of producing non-specific bands, when incubated with the corresponding anti-mouse secondary antibody. In addition, the rabbit anti-PICK1 antibody shows immunogenic cross-reactivity with the corresponding anti-rabbit secondary antibody. Taken together, these data showed the technical difficulties in conducting co-IP experiments to show a PICK1-parkin interaction. The presence of non-specific bands at similar molecular weights as parkin and PICK1 (approximately 50 kDa) add to these challenges.

3.7 Pull-down of HA-parkinΔUb by MBP-PICK1

Full length parkin showed poor expression, when transiently expressed in HEK293 cells (Figure 3.4), in agreement with the published data (Finney et al., 2003). Thus, a deletion construct of parkin was used for expression in HEK293 cells. The UbI domain of parkin is reported to regulate the expression of parkin and deleted UbI domain construct of parkin is shown to increase its own protein expression (Finney et al., 2003). To obtain a detectable expression of parkin, overnight grown HEK293 cells were transfected with the HA-parkinΔUb (1 μg). To monitor transfection efficiency, 0.1 μg of EGFP-C2 was used to transfect a single well of 6-well plate containing HEK293 cells along with HA-parkinΔUb (estimated transfection efficiency 50-60%). Upon western blot analysis, the expression of HA-parkinΔUb was detected by using anti-HA antibody in cell lysate of single transfection samples. Equal amounts of purified MBP and MBP-PICK1 (400 μg each) were incubated with the cell lysate.
Figure 3.6: Identification of non-specific bands expressed by anti-PRK8 and anti-PICK1 antibodies

Anti-PRK8 and anti-PICK1 antibodies were incubated with sepharose beads along with only sepharose beads, as the negative control (-). Upon incubation and washing, the sepharose beads of each sample were boiled in SB buffer and separated on SDS-PAGE. Thereafter the western blot was performed. A) BLOT A was treated with anti-PRK8 antibody (1° Ab) and anti-mouse antibody (2° Ab). BLOT B was treated with anti-PICK1 antibody (1° Ab) and anti-rabbit antibody (2° Ab). B) BLOT C and D were treated with only anti-mouse and anti-rabbit antibody (2° Ab), respectively.
expressing HA-parkinΔUb separately, along with MBP beads. The MBP beads were then boiled in SB buffer and separated in SDS-PAGE. The western blots were performed with anti-HA and anti-MBP antibodies (Figure 3.7). The anti-HA blot demonstrated high expression of HA-parkinΔUb in input fraction. Likewise, high expression of MBP and MBP-PICK1 expression were found in the anti-MBP blot in both the pull-down and input fractions. The data showed no association between the ubiquitin domain deleted version of parkin (HA-parkinΔUb) and PICK1. This lack of parkin-PICK1 interaction may be due to deletion of the Ubl domain of parkin, which may indirectly regulate the interaction of parkin with PICK1.

3.8 Production of lentiviral constructs of HA-parkin and HA-parkinΔpdz

Previous data showed that the use of transiently expressed HA-parkin demonstrated poor expression of full length HA-parkin (Figure 3.4). As an alternative, lentivirus-mediated transfection can be used as efficient way of expressing a protein of interest in vitro and in vivo. Thus, to overcome expression issues of parkin, a lentiviral construct expressing full length parkin was designed to express this protein in neurons. Having already Flag-PICK1 lentiviral constructs in the laboratory, a HA-parkin lentiviral construct was constructed. The primers were designed to amplify HA-parkin and HA-parkin with mutated PDZ motif (FDV/AAA). Amplified HA-parkin and HA-parkinΔpdz were separated in 2% agarose gel. After digestion of the PCR products and pLenti-PGK-GFP vector with AscI and RsrII, cloning reactions were performed. After transformation, the colonies were screened for correct HA-parkin and HA-parkinΔpdz constructs. Successful constructs were reconfirmed by commercial sequencing (Figure 3.8). The sequencing data confirmed successful cloning of full length HA-parkin and HA-parkinΔpdz constructs. However, due to the unavailability of lentiviral facility, further work with these HA-parkin and HA-parkinΔpdz lentiviral constructs was not performed.

3.9 Binding of MNP201 in PDZ domain of MBP-PICK1 in fluorescence polarisation assay

The next approach to determine the binding of MNP201 with PICK1 was by use of a previously described fluorescence polarisation assay (Figure 3.9A) (Madsen et al., 2005). In this assay, competitive binding experiments were performed using a fixed concentration (40 nM) of the Oregon Green fluorescently labelled last 13 amino acids of DAT ct (OG-DAT-C13) and fixed concentration of purified PICK1 (0.45 μM). As the ct PDZ motif
Figure 3.7: MBP pull-down experiment with bacterial expressed MBP-PICK1 and transiently transfected HEK293 cells with HA-parkinΔUb

HEK293 cells were transiently transfected with HA-parkinΔUb. After making cell lysate, the HA-parkinΔUb containing cell lysate and MBP beads were incubated with purified MBP and MBP-PICK1. After separating the samples in SDS-PAGE, western blot was performed. A) The blot of anti-HA shows no band of HA-parkinΔUb in pull-down fractions, although input fractions show good expression of HA-parkinΔUb (43 kDa). On the other hand, anti-MBP blot shows good expression of MBP and MBP-PICK1 (77.50 kDa) in pull-down and input fractions. B) To estimate the transfection efficiency, HEK293 cell culture was transfected with pEGFP-C2 vector (transfection efficiency 50-60%). The GFP protein fluorescence is visible under 488 nm. C) The diagram shows the design of the experiment where MBP beads bind with MBP tag of MBP-PICK1 and associated HA-parkinΔUb with MBP-PICK1.
Figure 3.8: Cloning of Lenti-HA-parkin and Lenti-HA-parkinΔpdz
A) The vector map of pLenti-PGK-GFP (7 kb) shows multiple cloning sites flanking along GFP gene. This vector has an ampicillin resistant gene. B) PCR amplified insert of HA-parkin (HP) and HA-parkinΔpdz (HP-Δpdz) were separated in 2% agarose gel along with 1.5 kb DNA marker band (M). C) The vector was digested with AscI and Rsrl in both single and double digestion. After digestion of pLenti-PGK-GFP vector, the undigested (UD), digested with AscI (SD1), digested with Rsrl (SD2) (6.3 kb) and double digested (DD) fractions were separated in 2% agarose gel. Notably, the GFP gene (700 kb) separates out from the double digested vector. D) The chromatogram of HA-parkin in p-Lenti-PGK vector ensures the successful cloning. The red box indicates the recognition site of AscI and nucleotides following the red box show the initiation codon (ATG) of HA-parkin.
Figure 3.9: Binding of MNP201 in PDZ domain of PICK1
By using fluorescence polarisation assay, the binding of the parkin peptide MNP201 to PICK1 was evaluated. A) MNP201 was modelled according to the ct 10 amino acids of parkin. A mutated version, MNP201(mut), was also designed where the PDZ motif was mutated (FDV/AAA). B) In the graph, the decrease of bound OG DAT signifies that MNP201 competitively replaced OG-DAT-C13 peptide in a dose-dependent manner. C) When MNP201(mut) was introduced in increasing concentrations, no replacement of OG-DAT-C13 peptide was observed.
of DAT interacts with the PDZ domain of PICK1 (Torres et al., 2001), the OG-DAT-C13 binds to the PDZ domain of PICK1 (Madsen et al., 2005; Thorsen et al., 2010). When the MNP201 was titrated, a gradual reduction of Green Oregon fluorescence was observed with the dose-dependent increase of MNP201 (Figure 3.9B). The reduction of OG-DAT-C13 fluorescence with the increasing concentration of MNP201 indicated the competitive replacement of OG-DAT-C13 from the PDZ domain of PICK1 by MNP201 in a dose-dependent manner. When the MNP201(mut) was used in the same competition binding assay, no reduction of the OG-DAT-C13 fluorescence was found with the dose-dependent increase of MNP201(mut) (Figure 3.9C). Taken together, the data suggest that MNP201 peptide binds the PDZ domain of PICK1 in a dose-dependent manner and last three ct amino acids of MNP201 (FDV) are crucial for interaction with PDZ domain of PICK1. This experiment was performed by our collaborator Prof. Ulrick Gether, Molecular Neuropharmacology Group, Department of Neuroscience and Pharmacology, University of Copenhagen, Denmark.
Discussion

Recent studies have shown an interaction between parkin-PICK1 (Joch et al., 2007). To demonstrate the parkin-PICK1 interaction, a set of biochemical binding assay were undertaken. In addition, the binding of a parkin PDZ motif peptide (MNP201) to PICK1 was also demonstrated. The outcomes and the issues concerning these experiments are summarised below.

1) Firstly, to standardise the GST pull-down protocol, the interaction of PICK1-GluR2 was demonstrated (Dev et al., 1999). Using MBP beads incubated with bacterial expressed MBP-PICK1 and GST-GluR2-ct, the association of PICK1-GluR2 was demonstrated by pull-down experiments.

2) After standardising the pull-down protocol using the PICK1-GluR2 interaction, a MBP pull-down experiment with bacterial expressed MBP-PICK1 and endogenous parkin was performed. Due to poor specificity of the rabbit anti-parkin antibody, endogenous parkin was not detected.

3) Next, co-IP studies were performed using transiently expressed HA-parkin and Flag-PICK1. In these experiments, no expression of full length HA-parkin and poor expression of Flag-PICK1 were observed. In addition, numerous non-specific bands were observed in co-IP experiments. Mock co-IP studies with Flag beads revealed non-specific bands, when blots were incubated with secondary anti-Mouse antibody, indicating antibody cross-reactivity. These studies suggested technical limitations in using the Flag beads to investigate the association of transiently expressed Flag-PICK1 with HA-parkin. Notably, these antibody cross-reactivity studies also showed that MBP beads did not result in non-specific bands when exposed to secondary mouse and rabbit antibodies. The MBP beads were subsequently chosen for future pull-down experiments.

4) The co-IP experiments from rat brain lysate also faced technical limitations as the anti-PRK8 and anti-PICK1 antibodies showed antibody cross-reactivity. Thus, the use of MBP pull-down studies with MBP-PICK1 incubated with endogenous parkin or parkin obtained from transiently expressed mammalian cell lines were used to determine parkin-PICK1 interaction.

5) To overcome the issue of expression of full length parkin, a parkin mutant (UbI domain deletion) construct was employed to transiently transfect HEK293 cells. The UbI deleted parkin is reported to have better expression profile than the full length parkin (Finney et al., 2003). In these experiments, a detectable amount of HA-Parkin ΔUb was obtained, as
determined by anti-HA blot. When co-IP experiments were performed using MBP beads with cell lysate expressing HA-Parkin ΔUb and purified MBP-PICK1, no interaction between parkin and PICK1 was observed. The reason behind this failure could be due to MBP-PICK1 obtained from bacterial system and the lack of the correct post-translational modification needed to interact with parkin and/or due to use of an UbI deleted parkin, which may limit interaction with PICK1.

6) To obtain a mammalian source of parkin, lentiviral HA-parkin and HA-parkin Δpdz constructs were prepared. A lentiviral Flag-PICK1 construct was already available in our laboratory. These constructs enabled the study of the parkin-PICK1 interaction in both biochemical approaches or by immunocytochemistry studies. However, due to practical issues (i.e., availability of lentiviral culture facility) further work using these constructs was limited.

7) The parkin blocking peptide, MNP201, is designed according to the last 10 amino acids of parkin ct. According to the data of Joch et al., 2007, the last three amino acids of parkin ct constituting a PDZ motif are essential for the interaction with PDZ domain of PICK1 (Joch et al., 2007). As MNP201 contains the PDZ motif of parkin, the binding of MNP201 with the PDZ domain of PICK1 would indicate an association between parkin and PICK1. Thus, the final approach taken, was to examine the binding of MNP201 to PICK1 using a fluorescence polarisation assay. Our collaborator Prof. Ulrick Gether, Copenhagen, Denmark, successfully showed that MNP201 (VCMGDHWFDV) binds with the PDZ domain of PICK1 in a concentration-dependent manner by replacing Oregon labelled DAT-13 peptide, a known binding partner of the PDZ domain of PICK1. In contrast, the MNP201(mut), i.e., the mutated PDZ motif version of MNP201 (VCMGDHWAAA) did not show any specificity towards PICK1. In summary, these data suggest that the MNP201 binds to the PDZ domain of PICK1 through the last three amino acids of MNP201 (-FDV), in agreement with previously published data (Joch et al., 2007). The data also indicate that the last three amino acids of ct-parkin ar required for interaction with PICK1.

As a future direction, the biochemical validation of MNP201 in blocking interaction between parkin and PICK1 would be valuable. Parkin is reported to translocate specifically towards depolarized mitochondria to play a role in recruitment of autophagosomes for mitophagy under depolarization stress condition (Narendra et al., 2008). Thus, an alternative approach to show the parkin-PICK1 interaction would be by expressing lentiviral HA-parkin in neurons and determining its translocation and/or association with mitochondria in the presence and absence of lentiviral-mediated Flag-PICK1 expression, and with or without MNP201 treatment of neurons under normal and stressed conditions. Assuming the hypothesis that
PICK1 causes translocation of parkin to the mitochondria, then the overexpression of PICK1 should promote parkin association with the mitochondria while MNP201 would block the parkin-PICK1 interaction and limit parkin translocation towards the depolarized/stressed mitochondria. In the next chapter, the effects of MNP201 on mitochondrial function were examined.
4. Results: 2

Effect of MNP201 on mitochondrial function
Abstract

Previous studies have shown that the ct PDZ motif of parkin interacts with the PDZ domain of PICK1 (Joch et al., 2007). In the previous chapter, a parkin peptide (MNP201) was designed based on the last 10 residues of ct-parkin containing a PDZ motif. This MNP201 peptide was shown to competitively displace the binding of a fluorescently tagged PDZ motif peptide from the PDZ domain of PICK1. The aim of this current chapter was to investigate the role of the parkin-PICK1 interaction in the mitochondrial function by using MNP201. It was hypothesised that the MNP201 parkin peptide would bind to PICK1 and inhibit its interaction with parkin, which would in turn alter mitochondrial function. Specifically, the effects of the MNP201 peptide was tested on mitochondria based assays including respiratory chain (complex I and IV) activity, ROS production and mitochondrial membrane potential stability. Since PICK1 is known to interact with the AMPA receptor subunit, GluR2; the KA receptor subunit, GluR5 and the metabotropic receptor mGluR7, the effects of MNP201 was also investigated on glutamate release using fresh rat brain synaptosomes. The data showed that MNP201 did not alter any of the mitochondrial parameters investigated nor did it alter glutamate release. The lack of effects of MNP201 was attributed to poor synaptosomal permeability of this peptide, which was further investigated by fusing MNP201 to Trojan peptides in the following results chapter.
Introduction

In recent years, mitochondrial dysfunction has been identified as a key molecular mechanism underlying PD (Beal, 1992; Doble, 1999; Lin and Beal, 2006). Many reports published in the last two years have given important insights about the involvement of parkin in mitochondrial maintenance during stress and selective elimination of malformed mitochondria (Narendra et al., 2008; Twig et al., 2008; Poole et al., 2008, 2010; Geisler et al., 2010; Ziviani et al., 2010). Parkin, otherwise found in the cytosol, has been shown to selectively traffic to engulf depolarized mitochondria to promote autophagy, before the malfunctioned mitochondria can trigger apoptotic signals (Narendra et al., 2008). On the other hand, PICK1, a scaffolding kinase recruiting protein has been shown to resist against Bax-induced apoptosis by recruiting PKCa to phosphorylate the Bcl-2 protein in the outer mitochondrial membrane (Wang et al., 2007). Taken together, these two proteins, directly or indirectly, confer resistance against mitochondrial apoptosis. Intriguingly, parkin and PICK1 interact with each other through a PDZ motif-domain interaction (Joch et al., 2007). Upon interaction, parkin causes monoubiquitination of PICK1, but does not promote degradation of PICK1 (Joch et al., 2007). In addition, parkin indirectly regulates PKC-mediated ASIC2a current via monoubiquitination of PICK1 (Joch et al., 2007). It has also been shown that parkin has no influence in the sub-cellular localisation of PICK1 (Joch et al., 2007). However, no studies to date have investigated whether PICK1 has a role to play in the sub-cellular localisation or trafficking of parkin to mitochondria or in mitochondrial maintenance, especially in neurons. According to our hypothesis, PICK1 may traffic parkin towards mitochondria and thereby regulate mitochondrial function.

The PDZ domain of PICK1 is a multi-protein interacting site through which numerous proteins, including PKCa, GluR2 and parkin interact to influence phosphorylation events and synaptic transmission (Staudinger et al., 1995; Dev et al., 1999; Joch et al., 2007). To investigate the role of PICK1 in neuronal function, many groups have employed the use of blocking peptides, which bind the PDZ domain of PICK1 and inhibit PICK1 interactings. The blocking peptides used have, in general, comprised amino acid stretches of 5-10 residues. These blocking peptides have been based on the extreme ct sequences of PICK1 interacting partners, thus containing the essential PDZ motifs required to interact with the PDZ domain of PICK1. The blocking peptides used have been shown to block PICK1 interactions by competitive inhibition. Many reports have been published demonstrating effects of these blocking peptides in physiological function. For example, GluR2 blocking peptides, based on the PDZ motif of GluR2, block the PICK1-GluR2 interaction and have been shown to block LTD in hippocampal neurons (Daw et al., 2000). Other PDZ domain containing proteins have
also been blocked from interacting with GluR2 using blocking peptides, for example GRIP and syntenin (Li et al., 1999). Similar to these studies, an MNP201 (VCMGDHWFDV) parkin peptide was designed, according to the last ten ct amino acids of parkin (Figure 3.1) based on the interaction study by Joch et al. (2007). Data from the previous chapter (Result 1) (Figure 3.9) indicated that the parkin blocking peptide (MNP201) competitively inhibits binding of DAT-13 peptide to PICK1, suggesting an interaction of MNP201 with the PDZ domain of PICK1. Here, this peptide was used to investigate the role of the parkin-PICK1 interaction in mitochondrial function and glutamate release.

Mitochondrial function is mainly attributed in fulfilling the energy requirement of the cell, Ca\textsuperscript{2+} homeostasis maintenance and the regulation of apoptosis. In mitochondria, during energy production by the ETC-mediated oxidative phosphorylation, ROS species are formed as byproducts (Henchcliffe and Beal, 2008). Regulation of this ROS production is crucial for proper cell function, where failure to remove ROS results in oxidative stress, causing decreased mitochondrial membrane polarisation and triggering of apoptotic pathways (Galley, 2011; Perry et al. 1996). Analysis of SNPC brain samples derived from PD patients has shown extensive reduction in the activity of complex I enzyme (Schapira et al., 1990) and related ROS production. In addition, parkin-KO mice showed respiratory impairment in complex I and IV of ETC (Palacio et al., 2004). Importantly, certain parameters of mitochondria can be monitored, including activity of respiratory enzymes, ROS production and changes in mitochondrial membrane potential to identify mitochondrial dysfunction. According to our hypothesis, if the parkin-PICK1 interaction plays a role in mitochondrial function, changes in mitochondrial parameters in the presence of the parkin blocking peptide (MNP201) should be expected. Therefore, in this chapter, the effects of parkin blocking peptide (MNP201, wildtype and mutant versions) were tested on the activity of respiratory enzymes, ROS production level and change in mitochondrial membrane potential.

The effects of MNP201 on glutamate release were also investigated; there were three reasons for doing so. Firstly, Ca\textsuperscript{2+} sequestration is another important role that mitochondria play to maintain cellular physiology. Mitochondria harbour high amounts of Ca\textsuperscript{2+} in the mitochondrial matrix that remains under a dynamic equilibrium with the cytosol to regulate the cytosolic Ca\textsuperscript{2+} levels (Nicholls and Budd, 2000; Gunter and Sheu, 2009). The regulation of cytosolic Ca\textsuperscript{2+} levels is crucial as Ca\textsuperscript{2+} in the cytosol plays an important role in synaptic transmission and secondary messenger signalling. Particularly, the Ca\textsuperscript{2+}-dependent vesicular release in neurons are sensitive to cytosolic Ca\textsuperscript{2+} influx, which may lead to excitotoxic injury (Choi, 1994). It was hypothesised that if parkin alters mitochondrial function then a change in mitochondria Ca\textsuperscript{2+} sequestration, cytosolic Ca\textsuperscript{2+} levels and thus glutamate
release would be detected in response to treatment of synaptosomes with MNP201. The second reason for examining the effects of MNP201 on glutamate release was that parkin over-expression diminishes ASIC2a current by monoubiquitination of PICK1 (Joch et al., 2007), which may also play a role in regulating intracellular Ca^{2+} levels and thus alter the release of glutamate. Thirdly, since MNP201 can displace PDZ motifs from interacting with the PDZ domain of PICK1, it could prevent a number of other proteins from interacting with PICK1. In relation to glutamate release, PICK1 interacts with AMPA, kainate and metabotropic receptors, all of which have been found at pre-synaptic sites and alter glutamate release and all of which may be altered by MNP201.

In summary, in this chapter, the effects of MNP201 were demonstrated on various mitochondrial parameters and glutamate release using isolated nerve terminals or synaptosomes. As a negative control, another set of peptides (MNP202) based on the extreme ct of another E3 ligase, Cdc4α, was used.
Results

4.1 Design and use of MNP202

The MNP201 parkin peptide (Figure 3.1) was modelled, according to the last 10 amino acids of parkin's extreme ct. In addition, peptides modelled on the last 10 amino acids of ct of an unrelated E3 ligase, Cdc4α, was designed. The Cdc4α protein is a novel F-box containing subunit of SCF E3 ligase complex that acts in concert with parkin to ubiquitinate a series of substrates (Staropoli et al., 2003). Two versions of Cdc4α peptides, MNP202 (LVLDVFVDVDMK) and MNP202(mut) (LVLDVFVAAA) were used. The MNP202 and MNP202(mut) were dissolved in PBS buffer to make a stock solution of 2 mM. These peptides were ordered from Peptide 2.0, USA. The HPLC purity of MNP202 and MNP202(mut) were found to be 93.75% and 94.78% respectively. The list of peptides and their HPLC purity profiles are shown in Figure 4.1 and were provided by Peptide 2.0, USA.

4.2 The ct of Cdc4α does not interact with PICK1

As mentioned, Cdc4α is an F-box containing protein subunit of large SCF E3 ligase complex. As negative control, the MNP202 peptide on the last 10 amino acids of Cdc4α ct was modelled. Before using the MNP202 peptides, it was necessary to assess a possible interaction between PICK1 and Cdc4α ct. The last 108 bp of Cdc4α ct was cloned into a yeast two hybrid vector, pGBKT7, expressing the last 36 amino acids of Cdc4α ct and named as Cdc4-ct (BAIT). To amplify the extreme ct of Cdc4α, a pair of primers was generated carrying restriction sites, i.e., EcoRI in the forward primer and BamHI in the reverse primer, so that after restriction digestion, the PCR amplified fragment had flanking overhangs of these restriction sites. After PCR, the insert was digested and purified by PCR purification kit (Qiagen) (Figure 4.2A). In parallel, the pGBKT7 digested vector was dephosphorylated using alkaline phosphatase (Figure 4.2B). Upon purification, a ligation reaction was performed. The ligated vector was used to transform competent cells and the overnight grown recombinant bacterial cell culture was used to extract the Cdc4-ct construct in pGBKT7. To confirm the cloning, the Cdc4-ct (BAIT) construct was sent for sequencing. The sequencing result confirmed the successful cloning of Cdc4-ct into pGBKT7 (Figure 4.2C). Thereafter, a yeast two hybrid experiment using Cdc4-ct (BAIT) and PICK1 (FISH) was performed. A positive control {PICK1 (FISH)-GluR2-ct (BAIT)} and an empty vector negative control {pGAD10 (FISH)-pGBKT7 (BAIT)} were included in all yeast two hybrid experiments. The results showed that the full-length PICK1
### Figure 4.1: List of peptides based on ct of Cdc4α

A) The MNP202 peptides are modelled on the ct of Cdc4α (as control) are shown in the table. B) The HPLC purity of these peptides, measured in 220 nm, was found to be 93.75% (MNP202) and 94.78% (MNP202 mutant). The Cdc4α peptides were ordered from Peptide 2.0, USA.

<table>
<thead>
<tr>
<th>Name</th>
<th>Domain</th>
<th>Motif</th>
<th>Peptide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNP202</td>
<td>n/a</td>
<td>Cdc4</td>
<td>LVLDFDVDMK</td>
</tr>
<tr>
<td>MNP202(mut)</td>
<td>n/a</td>
<td>Cdc4</td>
<td>LVLDFDVAAA</td>
</tr>
</tbody>
</table>

**Graphs**

- **MNP202**
  - mV vs. Min
  - Peaks at 12 and 24 min

- **MNP202(mut)**
  - mV vs. Min
  - Peaks at 14 and 22 min

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A deletion construct, expressing the last 36 amino acids of Cdc4a ct, was cloned into the yeast two hybrid vector pGBKT7. A) PCR fragment of 133 bp, encoding the last 36 amino acids of Cdc4a ct, is shown in 2% agarose gel with DNA marker. B) Digestion of vector DNA was carried out with EcoRI and BamHI. Double digested pGBKT7 (DD) is shown in 2% agarose gel along with marker (M), undigested DNA (UD), EcoRI digested (SD1) and BamHI digested (SD2). C) The chromatogram of successfully cloned construct of Cdc4-ct with the restriction sites (inside the rectangles) EcoRI and BamHI is shown. D) Cdc4-ct (FISH) was tested with PICK1 (BAIT) in yeast two hybrid assay. Representative negative controls (pGBK7 and pGADT7) and positive (GluR2-ct and PICK1) controls are also shown. Data is representative of three experiments (n=3).
(FISH) did not interact with the Cdc4-ct (BAIT) (Figure 4.2D), whereas positive and negative controls indicated growth and no growth of yeast colonies, respectively. The data suggest that PICK1 does not interact with the Cdc4-ct and the MNP202 peptides modelled on Cdc4α could be used as negative controls in the mitochondrial assays.

4.3 Synaptosomes contain both parkin and PICK1

Following two apparently separate pathways, both parkin and PICK1 have been reported to be associated with mitochondria (Narendra et al., 2008; Wang et al., 2007). Thus, the effects of the parkin blocking peptides and control peptides were investigated on the activities of ETC respiratory chains, including complex I and IV. In addition, the effects of these MNP201 parkin peptides were investigated on production of ROS and mitochondrial membrane potential. Further, the effect of MNP201 on glutamate release assay was tested.

Both parkin and PICK1 are reported to be present in mammalian brain. To demonstrate the expression of parkin and PICK1, rat brain synaptosomes were extracted from mature wistar female rat (150-200 g body weight) brains using Ficoll gradient. Western blot analysis was performed to detect the amount of parkin and PICK1 in synaptic and non-synaptic fractions of rat brain. To identify the levels of parkin and PICK1, anti-PRK8 and anti-PICK1 antibodies were used. The western blots showed presence of parkin and PICK1 in both synaptic and non-synaptic fractions (Figure 4.3). The data suggest that synaptosomes derived from rat brain contain relative higher amount of parkin and PICK1 compared to non-synaptic fractions.

4.4 Effect of MNP201 on complex I assay

ETC deficiencies have been implicated in the pathogenesis of PD, where a 35-40% reduction in the activity of complex I has been reported in post-mortem samples obtained from PD patients (Schapira et al., 1989). Complex I utilises NADH as a source of electrons and passes these electrons to complex III via ubiquinone (Figure 4.4A). The complex I activity was measured by the rate of conversion of NADH to NAD⁺ spectrophotometrically at absorbance 340 nm, as suggested by previous publications (Telford et al., 2010). Freeze-fractured synaptosomes were treated with 100 μM of parkin peptides (MNP201 and MNP201(mut)) and control Cdc4α peptides (MNP202 and MNP202(mut)); and the absorbance of NADH was observed for 1-2 min. The reaction was then initiated by adding decylquinone and the absorbance of NADH was monitored for a further 12-15 min (Figure 4.4B). Finally, rotenone was added to inhibit the complex I activity and the absorbance of
Figure 4.3: Presence of parkin and PICK1 in synaptic and non-synaptic fraction

Western blot analysis of synaptic (S) and non-synaptic (NS) fractions, obtained from rat brain, was performed for parkin using anti-PRK8 antibody and for PICK1 using anti-PICK1 antibody. The data shown is a representative of three experiments (n=3).
Figure 4.4: Effect of MNP201 in complex I of ETC cycle
Complex I activity is shown in freeze-fractured synaptosomes treated with peptides. A) The schematic diagram depicts that complex I uses NADH as a source of electrons and passes electrons to complex III via ubiquinone. Complex I is inhibited by rotenone. B) The graph represents the raw data of complex I activity by measuring absorbance (340 nm) of depleting NADH in the presence of MNP201 and MNP201(mut) in a time scale of 20 min. Representative traces of complex I activity are shown in the presence of vehicle control (red trace), 100 μM MNP201 (blue trace) and 100 μM MNP201(mut) (magenta trace). The first peak (DQ) and second peak (ROT) signify addition of decylquinone (1-2 min) and rotenone (14-15 min), respectively. The complex I activity (pseudo-first order rate constant) was obtained by subtracting the rotenone sensitive rate from the initial rate. C) The bar diagram represent the activity of complex I as a percentage of control (mean ± SEM) in four separate experiments (n=4) in the presence of vehicle control (Cont), MNP201, MNP201(mut), MNP202 and MNP202(mut).
NADH was monitored for a final 5-6 min. The rate constants were calculated according to the method based on Ragan et al. (1987) and the rotenone insensitive rate was subtracted from the rotenone sensitive value to obtain the activity of complex I. When the values were expressed as a percentage of control and statistically analysed, no significant change in the activity of complex I was observed in MNP201 (87.51% ± 13.00) treated samples, when compared to MNP201(mut) (117.29% ± 3.20), MNP202 (143.82% ± 29.71) and MNP202(mut) (132.49% ± 27.15) treated samples (Figure 4.4C). The data shows that MNP201 has no effect on the activity of complex I of respiratory chain of mitochondria and suggest that parkin-PICK1 interaction does play a role in regulating the activity of the complex I respiratory chain in mitochondria.

4.5 Effect of MNP201 on complex IV assay

Complex IV of ETC has been implicated in AD, where the activity of complex IV was found to be reduced in patients (Chagnon et al., 1995). One study suggested deficiency of complex IV activity in parkin-KO mice (Palacio et al., 2004). Complex IV or cytochrome C oxidase accepts electrons from reduced cytochrome C and converts oxygen to water (Figure 4.5A). After investigating the effects of MNP201 on complex I, its effects on complex IV activity in mitochondria was investigated. The complex IV activity was measured by a decrease in the levels of cytochrome C (550 nm) (Telford et al., 2010). The activity of complex IV was determined by calculating the first order rate constant of cytochrome C depletion. In these experiments, parkin and Cdc4α peptides were incubated with the complex IV reaction mixture and the absorbance of cytochrome C was monitored for 1-2 min. Next, freeze-fractured synaptosomes were added to initiate the reaction and the absorbance of cytochrome C depletion was monitored for subsequent 7-8 min (Figure 4.5B). The rate constants of complex IV activity were calculated according to the method of Wharton and Tzagoloff (1967) and expressed as percentage of control. No statistically significant difference in the activity of complex IV was observed in 100 μM MNP201 (125.13% ± 15.02) treated samples, when compared to the vehicle control and MNP201(mut) (135.16% ± 24.17) treated samples (Figure 4.5C). In addition, no significant change was observed in complex IV activity with MNP202 (108.28% ± 17.45) treated samples, when compared to vehicle control and MNP202(mut) (98.66% ± 15.81) treated samples. The data showed that MNP201 had no effect on the activity of complex IV of respiratory chain in mitochondria and suggested that the PICK1-parkin interaction does not play a role in regulating complex IV activity.
Figure 4.5: Effect of MNP201 in complex IV of ETC cycle

Complex IV activity was measured in freeze-fractured synaptosomes treated with peptides. A) The schematic diagram depicts that complex IV uses the reduced cytochrome C as source of electrons and passes protons out of the matrix to inner membrane space. B) The graph represents the raw data of complex IV activity by measuring absorbance (550 nm) of depleting cytochrome C in the presence of MNP201 and MNP201(mut) in a time scale of 10 min. Representative traces of complex IV activity are shown in the presence of vehicle control (red trace), 100 μM MNP201 (blue trace) and 100 μM MNP201(mut) (magenta trace). The peaks in the activity traces are due to addition of freeze-fractured synaptosomal protein (protein) (1-2 min). The complex IV activity was determined by measuring first order decay rate constant of cytochrome C. C) The bar diagram represents the activity of complex IV as a percentage of control (mean ± SEM) in three separate experiments (n=3) in the presence of vehicle control (Cont), MNP201, MNP201(mut), MNP202 and MNP202(mut).
4.6 Effect of MNP201 on peroxide production

ROS production is a quantitative marker of cellular stress. ROS production plays a role in mitochondrial dysfunction and the pathogenesis of neurodegenerative diseases including PD, AD and HD (Beal, 1998; Kish et al., 1992; Schapira et al., 1990). Conversion of amplex red to resorufin (585 nm) was assayed to measure $\text{H}_2\text{O}_2$ production in the presence of controls and MNP201 (Figure 4.6A). Freshly prepared synaptosomes treated with peptides were loaded with amplex red and production of resorufin was measured over a 2 h time period. The rate of resorufin production, the quantitative indicator of $\text{H}_2\text{O}_2$, was plotted against time (x-axis) and $\text{OD}_{585}$ (y-axis) for each treatment group (Figure 4.6B). A linear portion of the curve was selected (the same for each treatment group; 15-20 min) and the total $\text{H}_2\text{O}_2$ production was calculated. The relative values of peroxide production was expressed as a percentage of control and plotted in a bar graph. No significant difference was observed in peroxide production in synaptosomes treated with 100 µM of MNP201 (84.11% ± 4.05), when compared to the vehicle control and 100 µM MNP201(mut) (95.89% ± 5.31) treated synaptosomes. The unrelated Cdc4α modelled peptides, i.e., MNP202 (88.46% ± 1.30) and MNP202(mut) (79.56% ± 7.38) also showed no significant change, when compared to vehicle control. In contrast, when synaptosomes were incubated with 1 µM of antimycin A, a piscicide (fish toxin) and inhibitor of ubiquinol of ETC (200.73% ± 20.10), a significant 100.73% increase in peroxide production was found, when compared with vehicle control (Figure 4.6C), in agreement with previous publications (Sipos et al., 2003). The data showed that MNP201 has no effect on the ROS production in synaptosomal mitochondria.

4.7 Effect of MNP201 on mitochondrial membrane potential

The loss of mitochondrial membrane potential is a hallmark for apoptosis. It is an early event preceding loss of phosphatidylserine from the outer mitochondrial membrane and coinciding with caspase activation (Mantymaa et al., 2000; Korper et al., 2003). Mitochondrial membrane potential was measured by calculating the ratio of red (590 nm, aggregated form) to green (535 nm, monomeric form) of JC-1, as suggested by Chinopoulos et al. (1999) (Figure 4.7A). This ratio ($\text{JC-1}_{590/535}$) was found in control synaptosomes as 5.59 on average. The $\text{JC-1}_{590/535}$ is a quantitative indicator of mitochondrial membrane potential (Kilbride et al., 2008). Freshly extracted synaptosomes were incubated with JC-1 and then treated with vehicle controls and MNP201. The $\text{JC-1}_{590/535}$ ratio (y-axis) was plotted as rate of change in mitochondrial membrane potential over a time period of 1 h (x-axis) (Figure 4.7B). A linear portion of the curve was selected (the same for each treatment group; 15-20 min).
Figure 4.6: Effect of MNP201 on ROS production

Amplex red was used to measure the amount of ROS produced in freshly extracted synaptosomes in the presence and absence of MNP201. A) The principles of the assay is shown, where amplex red is converted to a fluorescent agent resorufin by reacting with ROS, in the presence of peroxidase. The resorufin level (585 nm) thus represents a quantitative indicator of peroxide production. B) The graph demonstrates the rate of peroxide produced in the presence or absence of MNP compounds and antimycin A (as positive control) treated synaptosomes in a time scale of 2 h. Representative traces depicts the rate of ROS production in the presence of vehicle control, 100 μM MNP201 and 100 μM MNP201(mut) along with the positive control 1 μM antimycin A. C) The bar diagram represents the total H₂O₂ production during the linear part of activity (the same for each treatment group; 15-20 min) as a percentage of control (mean ± SEM) of five separate experiments (n=5) in the presence of vehicle control (Cont), antimycin A (AM), MNP201, MNP201(mut), MNP202 and MNP202(mut). The p-value (***)) signifies p<0.001.
Figure 4.7: Effect of MNP201 on mitochondrial membrane potential

JC-1, changes its emission spectra with the change of mitochondrial membrane potential and was used as a reporter to measure the membrane potential of mitochondria in freshly extracted synaptosomes. A) The principles of the assay are shown, where JC-1 associates with mitochondria in control condition and emits a red (590 nm) colour. In depolarized mitochondria, JC-1 is found in the cytoplasm in a monomeric form emitting a green (535 nm) colour. The JC-1 590/535 ratio thus signifies a quantitative indicator of mitochondrial membrane potential. B) The graph shows the membrane potential in the presence and absence of MNP compounds in a time scale of 1 h. Representative traces depict the rate of change in mitochondrial membrane potential in the presence of vehicle control, 2 μM FCCP (positive control) 100 μM MNP201 and 100 μM MNP201(mut). C) The bar diagram shows the mitochondrial membrane potential during the linear part of the traces (the same for each treatment group; 15-20 min) converted as a percentage of control (mean ± SEM) in five separate experiments (n=5) in the presence of vehicle control (Cont), FCCP, MNP201, MNP201(mut), MNP202 and MNP202(mut). The p-value (*** ) signifies p<0.001.
and mitochondrial membrane potential was measured. The relative values of mitochondrial membrane potential was expressed as a percentage of control and plotted in a bar graph. No significant change was observed in mitochondrial membrane potential with 100 μM MNP peptides (91.83% ± 4.67), when compared to the vehicle control and negative control, MNP201(mut) (91.08% ± 1.41) \((\text{Figure 4.7C})\). The Cdc4α E3 ligase modelled peptides, i.e., MNP202 (97.79% ± 2.28) and MNP202(mut) (93.34% ± 1.91) also showed no significant effect, when compared to vehicle control. On the other hand, as expected, 2 μM of carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) (16.97% ± 2.35), a protonophore and uncoupler of mitochondrial oxidative phosphorylation, significantly reduced the membrane potential by 83%, when compared to vehicle control. The data showed that MNP201 has no effect on the mitochondrial membrane potential of synaptosomes.

4.8 Standardisation of glutamate release assay

PICK1 interacts with many glutamate receptors, namely AMPA, KA and metabotropic glutamate receptors that play a role in glutamate release (Barnes et al., 1994; Fujiyama et al., 2004; Hirbec et al., 2003; Herrero et al., 1996). Thus, the effect of 100 μM MNP201 was investigated on glutamate release using freshly extracted synaptosomes. A continuous fluorimetric technique was employed, where the amount of glutamate was indirectly measured by quantifying the levels of glutamate-induced NADPH production (excitation/emission = 340/460 nm) as described in previous publications (Nicholls et al., 1987; Kilbride et al., 2008). In these experiments, the blank rate of glutamate release was monitored for the first 5 min in the absence and presence of 2 mM CaCl\(_2\). Synaptosomes were then depolarized using 40 mM KCl and 10 mM 4-AP; and the depolarization-induced glutamate release was measured for an additional 30 min (\(\text{Figure 4.8A and C}\)). When the total glutamate release from 40 mM KCl and 10 mM 4-AP-induced synaptosomes were compared, 10 mM 4-AP-induced synaptosomes showed a trend increase rate in glutamate release compared to 40 mM KCl-induced synaptosomes, both in Ca\(^{2+}\)-independent and dependent conditions (\(\text{Figure 4.8B and D}\)). This data is in agreement with previously published reports indicating increased glutamate release in the presence of 4-AP compared to KCl (Agoston et al. 1983; Muniz et al., 1990). A glutamate standard curve with known concentrations of glutamate was also generated to quantify the amount of glutamate release (\(\text{Figure 4.9}\)).
Figure 4.8: KCl and 4-AP-stimulated glutamate release
Glutamate release was performed using continuous fluorimetric method that measured the level of glutamate-dependent generation of NADPH (460 nm). Freshly extracted synaptosomes were incubated with GDH, NADP⁺ and Krebs buffer, and the blank rate was recorded for the first 5 min. Synaptosomes were then depolarized by adding 40 mM KCl and 10 mM 4-AP (DP) and the rate of glutamate release was recorded for a further 30 min. A) The graph indicates representative rates of Ca²⁺-independent glutamate release (OD^460) in non-depolarized and depolarized synaptosomes with 40 mM KCl and 10 mM 4-AP. B) The bar diagram shows the total glutamate release induced by blank rate, 40 mM KCl and 10 mM 4-AP. C) The graph indicates representative rates of Ca²⁺-dependent glutamate release (OD^460) in non-depolarized and depolarized synaptosomes with 40 mM KCl and 10 mM 4-AP. D) The bar diagram shows the total glutamate release induced by blank rate, 40 mM KCl and 10 mM 4-AP. The data represented in bar diagrams are mean ± SEM in three separate experiments (n=3).
Figure 4.9: Standard curve of glutamate release
Quantification of glutamate release was performed using a continuous fluorimetric assay that quantified the level of converted NADPH (460 nm) in the presence of glutamate. To generate a standard curve for glutamate-induced NADPH production, standard amounts of glutamate were added to the wells of 96-well plate along with glutamate dehydrogenase, NADP⁺ and Krebs buffer (total 200 µl). Fluorescence was monitored at excitation/emission = 340/460 nm. The mean steady state fluorescence values were plotted against known amounts of glutamate added. The graph represents mean of two separate experiments (n=2).
4.9 Effect of MNP201 on KCl-induced glutamate release without CaCl$_2$

After performing the standard glutamate release experiments above, the effect of 100 µM MNP201 was tested in this assay. Complex I inhibition by 10 µM rotenone has been reported to result in a high rate of glutamate release (Kilbride et al., 2008) and was therefore also used as a positive control. Freshly extracted synaptosomes were incubated with glutamate release media and the blank rates were monitored for the first 5 min. Then, treatment groups, i.e., vehicle control, MNP201 peptides and rotenone were added and the baseline glutamate release was observed from non-depolarized synaptosomes. Total release of glutamate by non-depolarized synaptosomes was measured over a 5 min period. No change in glutamate release was observed compared to vehicle control values in 100 µM MNP201 (107.50% ± 1.41) and 100 µM MNP201(mut) (100.45% ± 2.86) treated non-depolarized synaptosomes in the absence of CaCl$_2$. In contrast, samples treated with 10 µM rotenone (165.92% ± 21.97) induced a significant 65.92% increase in the glutamate release, when compared to vehicle control (Figure 4.10 A, B), in agreement with previously published data (Kilbride et al., 2008). Thereafter, the synaptosomes were depolarized with 40 mM KCl in the absence of CaCl$_2$ and the rate of glutamate release was monitored for a further 30 min. The total release of glutamate by depolarized synaptosomes was measured over this 30 min period. The result suggests no significant change in the rate of glutamate release in 100 µM MNP201 (95.65% ± 8.00) treated synaptosomes, when compared to vehicle control and MNP201(mut) (82.75% ± 8.02) (Figure 4.10 A, C). In contrast, 10 µM rotenone (positive control) (146.35% ± 19.12) showed a significant 46.35% increase in glutamate release compared to vehicle control, in agreement with previously published data (Kilbride et al., 2008). The data showed that MNP201 has no effect in the Ca$^{2+}$-independent KCl-induced depolarized glutamate release from synaptosomes.

4.10 Effect of MNP201 on KCl-induced glutamate release with CaCl$_2$

After investigating the rate of glutamate release in the absence of CaCl$_2$, the same experiment was performed in the presence of 2 mM CaCl$_2$ under non-depolarized conditions. No statistically significant change was observed in the glutamate release from non-depolarized synaptosomes treated with 100 µM MNP201 (102.08% ± 2.81), when compared to vehicle control and MNP201(mut) (102.53% ± 2.03) in the presence of 2 mM CaCl$_2$ (Figure 4.11 A, B). On the other hand, 10 µM rotenone treated synaptosomes (160.62% ± 15.20) showed a significant 60.62% increase in glutamate release, in agreement with previously published data (Kilbride et al., 2008). Thereafter, synaptosomes were depolarized using 40 mM KCl and the rate of glutamate release was recorded. No
Figure 4.10: Effect of MNP201 on KCl-induced glutamate release in the absence of CaCl₂
The rate of glutamate release was measured by NADPH production at 460 nm. The level of glutamate was determined by generating a standard curve of glutamate-mediated NADPH production (Figure 4.9) in the absence of synaptosomes. The blank rate (BR) of glutamate release was recorded for the first 5 min in freshly extracted synaptosomes in the absence of CaCl₂. Then, synaptosomes were treated with peptides (Pep), i.e. 100 µM MNP201 and 100 µM MNP201(mut) and also 10 µM rotenone; and the rate of glutamate at baseline (BL) was observed from non-depolarized synaptosomes for a further 5 min. Next, depolarization of synaptosomes was initiated by addition of 40 mM KCl and rate of glutamate release was observed for a further 30 min. A) The graph shows representative traces of Ca²⁺-independent rate of glutamate release (nmol) from synaptosomes treated with vehicle control (Cont), 10 µM rotenone (Rot), 100 µM MNP201 and 100 µM MNP201(mut) in a total time period of 40 min. The bar diagrams show the total glutamate release as a percentage of control (mean ± SEM) under non-depolarized (B) and depolarized (C) condition in three separate experiments (n=3). The p-values (*) and (**) signify p<0.05 and p<0.01.
Figure 4.11: Effect of MNP201 on KCl-induced glutamate release in the presence of CaCl₂

The rate of glutamate release was measured by NADPH production at 460 nm. The level of glutamate was determined by generating a standard curve of glutamate-mediated NADPH production (Figure 4.9) in the absence of synaptosomes. The blank rate (BR) of glutamate release was recorded for the first 5 min in freshly extracted synaptosomes in the presence of CaCl₂. Then, synaptosomes were treated with peptides (Pep), i.e. 100 μM MNP201 and 100 μM MNP201(mut) and also 10 μM rotenone; and the rate of glutamate at baseline (BL) was observed from non-depolarized synaptosomes for a further 5 min. Next, depolarization of synaptosomes was initiated by addition of 40 mM KCl and rate of glutamate release was observed for a further 30 min. A) The graph shows representative traces of Ca²⁺-dependent rate of glutamate release (nmol) from synaptosomes treated with vehicle control (Cont), 10 μM rotenone (Rot), 100 μM MNP201 and 100 μM MNP201(mut) in a total time period of 40 min. The bar diagrams show the total glutamate release as a percentage of control (mean ± SEM) under non-depolarized (B) and depolarized (C) condition in three separate experiments (n=3). The p-value (***) signifies p<0.01.
A statistically significant change in glutamate release was observed in 100 μM MNP201 (91.14% ± 4.80) samples compared to vehicle control samples and 100 μM MNP201(mut) (85.22% ± 8.91) samples (Figure 4.11A, C). On the other hand, a significant 38.48% increase in glutamate release was observed, when synaptosomes were treated with 10 μM of rotenone (138.48% ± 14.84) compared to vehicle control, in agreement with previously published data (Kilbride et al., 2008). In summary, the data showed that MNP201 has no effect in the Ca^{2+}-dependent KCI-induced glutamate release from synaptosomes.

4.11 Effect of MNP201 on 4-AP-induced glutamate release

In addition to investigating the rate of KCI-induced glutamate release, 10 mM 4-AP was used to induce depolarization in synaptosomes. 4-AP induces depolarization of synaptosomes by blocking the voltage-dependent K^{+}-channels present on the surface of synaptosomes in a non-selective manner (Nicholls, 1993). Using 10 mM 4-AP, the effect of MNP201 was investigated on freshly prepared synaptosomes in the absence of CaCl_2. No statistically significant difference in the rate of glutamate release was observed in synaptosomes treated with 100 μM MNP201 (91.67% ± 3.97), when compared to vehicle control and MNP201(mut) (74.08% ± 2.30) (Figure 4.12A, B). On the other hand, the positive control 10 μM rotenone (137.25% ± 20.20) induced a significant 37.25% increase in the glutamate release compared to vehicle control, in agreement with previously published data (Kilbride et al., 2008). After investigating the effect of MNP201 in 4-AP-induced synaptosomes in the absence of CaCl_2, the same experiments in the presence of CaCl_2 was performed. In the presence of 2 mM CaCl_2 and 10 mM 4-AP, no change in the rate of glutamate release was observed in 100 μM MNP201 (103.50% ± 2.96) treated synaptosomes, when compared to vehicle control and MNP201(mut) (89.03% ± 4.91) (Figure 4.13A, B). The data again showed that 10 μM rotenone (167.05% ± 18.20) induced a significant 67.05% increase in release of glutamate compared to vehicle control, in agreement with previously published data (Kilbride et al., 2008). Taken together, the data showed that MNP201 has no effect on 4-AP-induced glutamate release from synaptosomes, in the presence or absence of Ca^{2+}. 
Figure 4.12: Effect of MNP201 on 4-AP-induced glutamate release in the absence of CaCl₂

The rate of glutamate release was measured by NADPH production at 460 nm. The level of glutamate was determined by generating a standard curve of glutamate-mediated NADPH production (Figure 4.9) in the absence of synaptosomes. The blank rate (BR) of glutamate release was recorded for the first 5 min in freshly extracted synaptosomes in the absence of CaCl₂. Then, synaptosomes were treated with peptides (Pep), i.e. 100 μM MNP201 and 100 μM MNP201(mut) and also 10 μM rotenone; and the rate of glutamate at baseline (BL) was observed from non-depolarized synaptosomes for a further 5 min. Next, depolarization of synaptosomes was initiated by addition of 10 mM 4-AP and rate of glutamate release was observed for a further 30 min. A) The graph indicates representative traces of independent rate of glutamate release (nmol) by vehicle control, 10 μM rotenone, 100 μM MNP201 and 100 μM MNP201(mut) treated synaptosomes in a total time period of 40 min. B) The bar diagram shows the total glutamate release as a percentage of control (mean ± SEM) in vehicle control (Cont), 10 μM rotenone (Rot), 100 μM MNP201 and 100 μM MNP201(mut) treated synaptosomes under depolarized condition in three separate experiments (n=3). The p-value (*) signifies p<0.05.
Figure 4.13: Effect of MNP201 on 4-AP-induced glutamate release in the presence of CaCl₂

The rate of glutamate release was measured by NADPH production at 460 nm. The level of glutamate was determined by generating a standard curve of glutamate-mediated NADPH production (Figure 4.9) in the absence of synaptosomes. The blank rate (BR) of glutamate release was recorded for the first 5 min in freshly extracted synaptosomes in the presence of CaCl₂. Then, synaptosomes were treated with peptides (Pep), i.e. 100 μM MNP201 and 100 μM MNP201(mut) and also 10 μM rotenone; and the rate of glutamate at baseline (BL) was observed from non-depolarized synaptosomes for a further 5 min. Next, depolarization of synaptosomes was initiated by addition of 10 mM 4-AP and rate of glutamate release was observed for a further 30 min. A) The graph indicates representative traces of Ca²⁺-dependent rate of glutamate release (nmol) by vehicle control, 10 μM rotenone, 100 μM MNP201 and 100 μM MNP201(mut) treated synaptosomes in a total time period of 40 min. B) The bar diagram shows the total glutamate release as a percentage of control (mean ± SEM) in vehicle control (Cont), 10 μM rotenone (Rot), 100 μM MNP201 and 100 μM MNP201(mut) treated synaptosomes under depolarized condition in three separate experiments (n=3). The p-value (***) signifies p<0.001.
Discussion

1. Summary of results

The purpose of this project was to investigate the role of parkin-PICK1 interaction in mitochondrial function. Previous reports have showed a positive interaction between the PDZ motif of parkin and PDZ domain of PICK1 and that parkin monoubiquitinates PICK1, which inhibits PICK1-induced augmentation of ASIC currents (Joch et al., 2007). Here, the role of parkin-PICK1 interaction was investigated in the maintenance of mitochondrial function. In the first Result 1 chapter, the binding of MNP201 to PICK1 was demonstrated in a fluorescence polarisation assay, by showing that MNP201 dose-dependently inhibited the binding of a DAT peptide to PICK1. In this current chapter, the effect of the MNP201 peptide, modelled on the PDZ motif of parkin, was evaluated on mitochondrial properties, including activity of respiratory chains (complex I and IV), ROS production and change in mitochondrial membrane potential. In addition, the effect of MNP201 was investigated on the rate of glutamate release from synaptosomes. Here, the data generated showed that 100 μM MNP201 peptide had no effect on mitochondrial properties or glutamate release (please see result summary Table 4.1).

2. Design of MNP201 parkin peptide

PDZ motifs are generally three to seven amino acid residues in length and usually reside in the extreme C-terminus of a protein that interacts with the PDZ domain containing proteins (Songyang et al., 1997). A peptide (VCMGDHWFDV) (MNP201) was designed based on the sequence of the last 10 amino acids of the parkin C-terminus, which was predicted to block the interaction between parkin and PICK1. As control, a mutated version of the same peptide MNP201(mut) (VCMGDHWAAA), where the PDZ motif of parkin was mutated, was prepared. The effects of 100 μM MNP201 were observed in mitochondrial assays and glutamate release assay using fresh synaptosomes. In addition to the MNP201(mut), additional peptide controls were also generated. We opted to model peptides based on the protein sequence of Cdc4a, as this protein interacts with parkin and is also an E3 ligase, similar to parkin (please see Introduction chapter). Specifically, peptides were also modelled on the last 10 amino acids (C-terminus) of Cdc4a, which was first shown not to directly interact with PICK1. These peptides (MNP202) (LVLDFDVMNK wild type and LVLDFDVAAM mutant) were thus used as negative controls in the mitochondrial experiments performed. A set of positive controls were also used in these mitochondrial assays and demonstrated effects as expected confirming successful assay setup.
Table 4.1: Summary of data obtained using MNP201
A) Mitochondrial function assays and B) glutamate release assay. The data suggest that MNP201 has no effect on mitochondrial respiratory chain complexes (complex I and IV), mitochondrial membrane potential, ROS production and glutamate release. The values are indicated as percentage of control (Mean ± SEM).

### A) Mitochondrial function assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>100 μM MNP201</th>
<th>100 μM MNP201(mut)</th>
<th>Measured by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I activity</td>
<td>87.51% ± 13.00</td>
<td>117.29% ± 3.20</td>
<td>NADH depletion (OD[^36\textsuperscript{C}])</td>
</tr>
<tr>
<td>Complex IV activity</td>
<td>125.13% ± 15.02</td>
<td>135.16% ± 24.17</td>
<td>Cyto C depletion (OD[^56\textsuperscript{C}])</td>
</tr>
<tr>
<td>Peroxide production</td>
<td>84.11% ± 4.05</td>
<td>95.89% ± 15.31</td>
<td>Resorufin production (OD[^585\textsuperscript{C}])</td>
</tr>
<tr>
<td>Membrane potential</td>
<td>91.83% ± 4.67</td>
<td>91.08% ± 1.41</td>
<td>JC-1 OD ratio (JC-1[^590/535\textsuperscript{C}])</td>
</tr>
</tbody>
</table>

### B) Glutamate release

<table>
<thead>
<tr>
<th>Glutamate release</th>
<th>100 μM MNP201</th>
<th>100 μM MNP201(mut)</th>
<th>Measured by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-depolarized level(-CaCl\textsubscript{2})</td>
<td>107.50% ± 1.41</td>
<td>100.45% ± 2.86</td>
<td>Amount of NADPH production (OD[^460\textsuperscript{C}])</td>
</tr>
<tr>
<td>Non-depolarized level (+CaCl\textsubscript{2})</td>
<td>102.08% ± 2.81</td>
<td>102.53% ± 2.03</td>
<td></td>
</tr>
<tr>
<td>- CaCl\textsubscript{2}, + 50 mM KCl</td>
<td>95.65% ± 8.00</td>
<td>82.75% ± 8.02</td>
<td></td>
</tr>
<tr>
<td>+ CaCl\textsubscript{2}, + 50 mM KCl</td>
<td>91.14% ± 4.80</td>
<td>85.22% ± 8.91</td>
<td></td>
</tr>
<tr>
<td>- CaCl\textsubscript{2}, + 10 mM 4-AP</td>
<td>91.67% ± 3.97</td>
<td>74.08% ± 2.30</td>
<td></td>
</tr>
<tr>
<td>+ CaCl\textsubscript{2}, + 10 mM 4-AP</td>
<td>103.50% ± 2.96</td>
<td>89.03% ± 4.91</td>
<td></td>
</tr>
</tbody>
</table>
3. Effects of MNP201 in mitochondrial function

Overall, no significant difference was found in the presence of MNP peptides tested in any of the mitochondrial parameters. Complex I is found to be hampered in PD patients (Schapira et al., 1989). Here, MNP201 showed no significant effect on the activity of complex I in freeze-fractured synaptosomes suggesting that occlusion of the PDZ domain of PICK1 (by MNP201 binding) may not alter function of the complex I respiratory chain. In addition to an altered complex I activity in PD patients, parkin-KO mice show a reduced complex IV activity (Palacio et al., 2004). The data showed that MNP201 did not alter complex IV activity, suggesting that parkin-PICK1 may also not regulate complex IV. In the ROS production assay, the positive control 1 μM antimycin (a complex III inhibitor) showed 102% increase in ROS production as expected (Sipos et al., 2003). In contrast, no significant change in ROS production between vehicle control, control peptides, MNP201 and MNP201(mut) was observed. This again indicates that parkin-PICK1 may not have a role in the regulation of mitochondria-induced oxidative stress. Lastly, the mitochondrial membrane potential assay showed 83% increase in the membrane potential with 2 μM FCCP treatment, a mitochondrial uncoupler (Tretter and Adam-Vizi, 2007). Again, however, no change in the mitochondrial membrane potential between vehicle control, control peptides, MNP201 and MNP201(mut) was observed, suggesting that parkin-PICK1 is not involved in maintenance of mitochondrial membrane potential. Given that parkin is known to regulate a number of mitochondrial properties, the lack of effect of MNP201 was surprising. Notably all these studies were performed under controls conditions. However, it is known that under conditions of cell stress parkin is trafficked toward the mitochondria where it is likely involved in mitophagy. Under normal conditions, the role of parkin in regulating mitochondrial function may be somewhat minor and the effects of MNP201 thus limited or not detectable. In contrast, the effects of MNP201 on these mitochondrial under conditions of cell stress, where parkin plays a more dominant role, may be observable and thus worthy of further study (please see next chapters).

4. Effects of MNP201 on glutamate release assay

Glutamate-induced excitotoxicity is thought to contribute in preferential dopaminergic cell death in PD (Obeso et al., 2004). Thus, along with mitochondrial properties, the effect of the PDZ motif parkin peptides was also investigated on glutamate release assay. Control experiments were first performed in order to establish the glutamate release assay. In these experiments both 40 mM KCl and 10 mM 4-AP was found to induce depolarization of synaptosomes as expected. In addition, a standard curve of glutamate was also prepared,
which showed that glutamate concentration-dependent increase in the production of NADPH. After these studies, both Ca^{2+}-independent and dependent glutamate release experiments were performed by treating freshly prepared synaptosomes with 100 μM MNP201 in non-depolarised as well as depolarised conditions (triggered by KCl and 4-AP). Previous studies have shown that inhibition of complex I by rotenone induces glutamate release from synaptosomes, depolarized with KCl or 4-AP (Kilbride et al., 2008), thus rotenone was used as a positive control in these experiments. Using 100 μM MNP201, no significant difference in the rate of Ca^{2+}-independent or dependent glutamate release was observed in non-depolarized or in both KCl and 4-AP-induced depolarised synaptosomes, when compared to the vehicle control and MNP201(mut) treated samples. In contrast, rotenone-treated samples showed a significant increase in the rate of glutamate release, in agreement with previous reports. Taken together, the data suggested that MNP201 had no effect on the pre-synaptic release of excitatory glutamate. Similar to the lack of effects of MNP201 on mitochondrial properties, the lack of effects of MNP201 on glutamate release were suprising given that (i) mitochondria play a central role in regulating Ca^{2+} levels, (ii) parkin-PICK1 may regulate Ca^{2+} levels via ASIC-mediated currents, and (iii) PICK1 interacts with a number of glutamate receptors that alter glutamate release. The data thus suggested one or more of the following: (i) the parkin-PICK1 interaction is not important for regulating mitochondrial function; (ii) the effects of parkin-PICK1 on ASIC currents does not alter glutamate release; (iii) PICK1 does not alter glutamate receptor-mediated glutamate release; and/or (iv) MNP201 does not inhibit parkin and/or other proteins from interacting with PICK1 in synaptosomes.

5. Permeability of MNP201

There were two limitations identified in this chapter. Firstly, though MNP201 showed no significant effects on mitochondrial maintenance and glutamate release, the ability of MNP201 to penetrate synaptosomal membranes was not addressed. Generally, oligopeptides upto 6 amino acids long can cross the plasma membrane without any assistance. For longer peptides and proteins, Trojan peptide sequences attached with functional peptides or proteins have been used to pass through bilipid membranes (Nagahara et al., 1998, Ezhevshy et al., 1997). Since MNP201 is a 10 amino acid polypeptide little or no internalisation of MNP201 in synaptosomes may be expected, which may be the cause of its lack of effect. A Trojan peptide, such as Tat, attached to MNP201 may thus aid its membrane permeability and may be worthy to test in mitochondrial and glutamate release assays. In addition to using Trojan peptides, another approach to deliver MNP201 peptides inside synaptosomes, is known as peptide or protein entrapment. In this
approach, peptides or proteins can be entrapped inside synaptosomes while homogenisation of brain tissues and during preparation of synaptosomes. External media can come in contact with the internal compartments of the synaptic terminal for a very short period of time, which is sufficient to transfer non-permeable peptides to the internal compartments of synaptosomes (Pittaluga et al., 2005; Feligioni et al., 2009). Interestingly, using this approach of entrapping peptides, Feligioni et al. (2009) described that sumoylation suppresses KCl-induced glutamate release, but increases KA receptor stimulated glutamate release by entrapping SUMO proteins in synaptosomes (Feligioni et al., 2009). While these entrapment methods are well established the use of large volumes of media (supplemented with peptides) during synaptosomal preparation make them costly. The addition, these entrapment methods do not allow for the addition of peptides at specific timepoints during or within experiments, thus do not allow for temporal control. Thus, here we opted to use a Trojan peptide based internalisation approach to deliver MNP201 into synaptosomes. Specifically, a new version of the MNP201 peptide was engineered, which contained a Tat sequence (YGRKRRQRRR) fused to the N-terminal of MNP201, named Tat-MNP201. These peptides were tested in neuronal cells and astrocytes to visualise their penetration into the cell. After confirming their penetration, the effect of Tat-MNP201 was tested in mitochondrial and glutamate release assays. These studies are described in the next chapter.

6. Effect of MNP201 in condition of cell stress

The second limitation to the current result chapter is that the effect of MNP201 peptides was tested only in control conditions, and not stressed conditions, in our experimental setup. Previous publications suggest that parkin engulfs depolarized mitochondria under conditions of stress (Narendra et al., 2008). If that is the case, then the parkin-PICK1 interaction may only play a role in regulating mitochondrial function under conditions of mitochondrial depolarization and/or cell stress. In other words, PICK1 may not associate with parkin in control circumstances, which may also account for the lack of effect of MNP201 on mitochondrial properties and glutamate release in the experiments outlined above. To address this issue, in the next chapter the effect of MNP201 was examined under the condition of mitochondrial depolarization stress.

7. Moving to the next chapter

At this stage, our revised hypothesis is that PICK1 plays a crucial role in the trafficking of parkin to depolarized mitochondria, in the condition of cell stress more so than under control
condition. In the next chapter, the effects of a cell-permeable version of MNP201 were examined on mitochondrial function and glutamate release.
5. Results: 3
Effect of Tat-MNP201 on mitochondrial function
Abstract

In the previous chapter, the effects of wildtype and mutated versions of MNP201 peptides were demonstrated in a set of mitochondrial functional assays and glutamate release using synaptosomes prepared from rat brain tissue. The data suggested no statistically significant effect of MNP201 (100 μM) in mitochondrial function and glutamate release. However, in the previous experiments the entry of MNP201 into cells, or more specifically across the plasma membrane of synaptosomes, was not determined. To ensure the internalisation of MNP201 through the membrane bilayers of synaptosomes, a Trojan peptide sequence, namely a Tat peptide, was attached onto the N-terminus of MNP201. In this chapter, first, a concentration-dependent internalisation of the Tat-tagged version of MNP201 was demonstrated in neuronal cells and astrocytes. Thereafter, the effects of Tat-MNP201 (and a Tat-MNP201 mutant) on mitochondrial properties, i.e., activity of respiratory complexes, peroxide production and membrane potential in synaptosomes were determined. Further, the effect of this peptide was investigated on glutamate release. No change in the rate of peroxide production and activity of respiratory complexes (complex I and IV) was observed. In addition, the Tat-tagged peptides showed no effect in the rate of glutamate release. Importantly, however, the data demonstrated that Tat-MNP201 (100 μM) increased the rate of mitochondrial depolarization in control and FCCP-induced stress conditions. Taken together, these studies suggested that the parkin-PICK1 interaction may play a role in the maintenance of mitochondrial membrane potential.
Introduction

In the previous chapter (Result 2), a parkin PDZ motif peptide was designed according to the last 10 amino acids of the C-terminus of parkin (MNP201). The effect of MNP201 and MNP201(mut) was investigated in various mitochondrial properties and the rate of glutamate release was also examined. In these studies, no statistically significant change was observed in synaptosomes treated with MNP201 peptides on any of the mitochondrial properties investigated or in the rate of glutamate release. However, from these studies, it remained unclear whether the lack of the effects of MNP201 was due to its ability to cross synaptosomal and/or mitochondrial membranes. Generally, peptides more than 6 amino acids long do not cross the plasma membrane, unassisted (Scheld et al., 1989) and given that MNP201 is 10 residues in length, it may have not crossed the membrane bilayer of synaptosome in the aforementioned experiments. There are two methods to assist the entry of peptides and proteins into synaptosomes. The first approach involves an ‘entrainment’ method that includes the peptide/protein into buffers while preparing synaptosomes. In this approach, there is a timepoint where these peptide/proteins can enter ‘open’ synaptosomes during preparation. While this method is well accepted, it is costly (requiring large amounts of peptides in buffers during synaptosomal preparations) and also does not allow for the addition of peptides at controlled timepoints within the mitochondrial and/or glutamate release experiments to be conducted. Thus, a second approach to transfer peptides into synaptosomes was used and involved the use of Trojan peptides. In this chapter, specifically, Tat-fused MNP201 peptides were created, which were capable of crossing the plasma membrane.

The ability to create biologically active substances that can internalise live cells crossing the bilipid membrane is an important tool in the field of pharmacology and drug development. Many agents have been identified that aid the entry of desired substances, otherwise impermeable to the cell and specific compartments. The identification and application of arginine-rich peptides sequences, including Tat and Antennapedia, capable of crossing the plasma membrane have been proved to be a useful tool of drug delivery (Schwarze at al., 1999; Derossi et al., 1996). Indeed, Tat peptides (YGRKKRRQRRR), derived from transduction domain (PTD) of human immunodeficiency virus (HIV) (Nagahara et al., 1998), are widely used in internalising substances ranging from peptides, proteins, oligonucleotides, liposomes, polymeric particles, phages and adenoviruses (Bonny et al., 2001; Dostmann et al., 2000; Jo et al., 2001; Peitz et al., 2002; Astriab-Fisher et al., 2000; Torchilin et al., 2001; Lewin et al., 2000; Eguchi et al., 2001; Gratton et al., 2003).
Generally, the endocytic system of mammalian cells follows different mechanisms to internalise substances from small soluble ligands to macromolecules (Mercer and Helenius, 2009). The most common mechanism for endocytosis in mammalian cells involves a clathrin-mediated pathway, where external ligands are incorporated into clathrin-coated pits with the help of receptors and adaptor proteins (Conner and Schmid, 2003). The fusion of clathrin-coated vesicles with the early endosomes occurs through a GTPase dynamin mechanism (Mukherjee et al., 1997). Similar to a clathrin-mediated pathway, another well-characterised endocytosis mechanism involves a caveolar-mediated pathway, where caveolin-1-coated vesicles (also called as caveolae) are known to mediate endocytosis in a dynamin-dependent manner (Parton and Simons, 2007). Interestingly, formation of lipid rafts, i.e., cholesterol/sphingolipid rich plasma microdomains containing the ligand, has been reported to associate with caveolar-mediated endocytosis (Lajoie and Nabi, 2007). In contrast, micropinocytosis is another lipid raft-mediated endocytic pathway, where entry of lipid rafts inside the cells occurs through a dynamin-independent pathway, involving membrane ruffling, i.e., actin-dependent formation of motile surface around the cell (Cao et al., 2007). Interestingly, the internalisation of Tat peptides takes place through a micropinocytosis mechanism (Kaplan et al., 2004). The Tat peptide is reported to deliver 15 to 120 kDa cargo proteins into different human and murine cell types (Nagahara et al., 1998, Ezhevshy et al., 1997). Many of these Trojan tagged peptides have been expressed in vivo, including the brain, by engineering N-terminal Tat fusions (Schwarze et al., 1999). For example, a Tat peptide designed to block the PDZ-based interaction between N-methyl-D-aspartate (NMDA) receptors and post-synaptic density protein 95 (PSD-95), has been used to prevent ischemic brain damage in mice (Aarts et al., 2002).

In addition to Tat, Antennapedia (also called as penetratin) is another example of Trojan peptide. Antennapedia is a 16 residues long polypeptide (RQIKIWFQNRRMKWKK) derived from the homeodomain of Drosophila transcription factor, also called Antennapedia. Several reports have described the successful use of this Trojan peptide as a transport vector (Derossi et al., 1998; Pooga et al., 1998; Astriab-Fisher et al., 2000). Unlike the Tat peptide, the Antennapedia sequence enters cells through a non-endocytotic pathway which is independent of receptor and transporter (Derossi et al., 1996; Fisher et al., 2000). Antennapedia, conjugated to as large as 55 bases of hydrophilic oligonucleotides, has been reported to deliver cargo to the cytoplasm and nucleus (Derossi et al., 1996; Prochiantz A, 1996). In this chapter, the Trojan peptide strategy was used by attaching a Tat sequence to the N-terminal to MNP201 in order to enable MNP201 to cross synaptosomal membranes.
While Tat tagged fusions proteins do successfully cross lipid bilayers, certain considerations about using such Tat peptides, which previous reports have indicated, are to be kept in mind. Specifically, studies on mitochondrial properties and glutamate release have reported that the Tat peptide can induce ROS production and mitochondrial membrane depolarization along with the disruption of Ca\(^{2+}\) homeostasis and caspase activation in cultured hippocampal neurons (Kruman et al., 1998). High expression of Tat mRNA has also been reported in HIV infected patient's brain and is considered as an important player in causing HIV related dementia (Wesselingh et al., 1993). Importantly, the Tat peptide is also the only viral protein known to be secreted by HIV infected microglia (Tardieu et al., 1992). In addition, the Tat protein is also reported to be released from infected cultured microglia and causes apoptosis by activating non-NMDA glutamate receptors and subsequent Ca\(^{2+}\) influx (Cheng et al., 1998). Moreover, a report has suggested induced glutamate release and reduced GABA release in the presence of a Tat peptide from mice synaptosomes (Musante et al., 2009). Although all these reports are based on the effect of full length Tat peptide, (i.e. not the YGRKKRRQRRR shorter Trojan version of Tat) recognition of such possible effects should be noted when using Tat peptide sequences in mitochondrial functional studies and glutamate release assays.

Specifically, to facilitate the entry of MNP201 into the synaptosomes, a Tat sequence was fused to the N-terminal of MNP201 (Tat-MNP201). First, a fluorescein isothiocyanate (FITC) labelled Tat-tagged MNP201 (FITC-Tat-MNP201) was treated with the cortical neurons and astrocytes to investigate the internalisation and intracellular localisation of Tat-tagged MNP201. Then, the effects of the Tat version of MNP201 peptides were tested in mitochondrial properties, including peroxide production and mitochondrial membrane potential. In addition, along with investigating the effect of Tat-MNP201 in mitochondrial membrane potential assay in control conditions, the effect of Tat-MNP201 was also demonstrated under membrane depolarization stress, induced by FCCP (a mitochondrial uncoupler). Finally, the Tat-MNP201 was also tested in the rate of glutamate release. Notably, the effect of these Tat peptides were not examined on complex I and IV assays as these assays are "open cell" enzymatic assays, where no issues regarding membrane permeability was envisiaged (Result 2). The outcomes of the experiments are given below.
Results

5.1 Concentration-dependent internalisation of FITC-Tat-MNP201 into neuronal cells

After testing the effects of MNP201, a membrane permeable version of wild type MNP201 was designed by attaching a Trojan peptide, Tat (YGRKKRRQRRR), to the N-terminal of MNP201 (Tat-MNP201). A Tat-MNP201(mut) was also synthesised as control. To monitor the membrane permeability of Tat-MNP201, its entry into cells was visualised by attaching an FITC (excitation/emission spectra = 495 nm/521 nm) fluorophore tag at the N-terminal of Tat-MNP201. These peptides were ordered from Genscript, USA. The HPLC purity of Tat-MNP201, Tat-MNP201(mut) and FITC-Tat-MNP201 were found to be as 97.40%, 85.47%, and 85.90%, respectively. The list of peptides and their purity profile is shown in Figure 5.1A and B as provided by Genscript, USA. All these Tat versions of MNP201 peptides were dissolved in 50 mM HEPES pH 7 solutions to make 2 mM stock solution. During experiments, 50 mM of HEPES pH 7 was used as vehicle control in equal volume of peptides.

To observe the internalisation of FITC-Tat-MNP201, mature (10-14 days old) cortical neuronal cells were treated with four concentrations of FITC-Tat-MNP201, i.e., 0.1 μg, 1 μg, 10 μg and 100 μg (per ml). The treatment was carried out at 37°C for 1 h in 5% CO₂. After treatment, neurons were fixed and stained with chicken anti-neurofilament (1/1000 dilution) antibody, followed by secondary anti-chicken Dylight-549 (1/1000 dilution) antibody and visualised under 40x objective lens in a confocal microscope. A fluorescent signal of FITC-Tat-MNP201 in neuronal cells was observed in a dose-dependent manner (Figure 5.2A). Images (containing 40-50 cells per image) were acquired from each treatment group (6-8 images per treatment group). The mean pixel intensity in the green channel (FITC signal) was quantified by ratio of intensity of green channel with red channel (neurofilament signal). The data was converted to the percentage of mean pixel intensity given by 10 pg/ml of FITC-Tat-MNP201 and represented as a bar graph. A dose-dependent increase of fluorescence was found in treatment groups, i.e., 0.1 μg/ml (19.05% ± 1.71), 1 μg/ml (52.01% ± 4.50), 10 μg/ml (100%) and 100 μg/ml (186.69% ± 41.45), indicating internalisation of FITC-Tat-MNP201 in cortical neurons in a concentration-dependent manner (Figure 5.2B). The data suggested that the Tat-tagged MNP201 is able to cross the plasma membrane of cortical neurons.
### A) List of Tat-tagged MNP201 peptides

<table>
<thead>
<tr>
<th>Name</th>
<th>Domain</th>
<th>Motif</th>
<th>Peptide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat-MNP201</td>
<td>PICK1</td>
<td>PARKIN</td>
<td>YGRKKRRQRRRVCMDHWFDV</td>
</tr>
<tr>
<td>Tat-MNP201(mut)</td>
<td>PICK1</td>
<td>PARKIN</td>
<td>YGRKKRRQRRRVCMDHWAAN</td>
</tr>
<tr>
<td>FITC-Tat-MNP201</td>
<td>PICK1</td>
<td>PARKIN</td>
<td>YGRKKRRQRRRVCMDHWFDV</td>
</tr>
</tbody>
</table>

### B) HPLC purity profiles

- **Tat-MNP201** purity: 97.40%
- **Tat-MNP201(mut)** purity: 85.47%
- **FITC-Tat-MNP201** purity: 85.90%

Figure 5.1: List of Tat-tagged MNP201 peptides

The Tat-tagged MNP201 peptides modelled on the PDZ motif of parkin are shown in the table. B) The HPLC purity profiles of these peptides, measured at 220 nm, were found to be 97.40% (Tat-MNP201), 85.47% (Tat-MNP201 mut) and 85.90% (FITC-Tat-MNP201). During HPLC analysis, formic acid treated water (pH 4.00) was used in case of Tat-MNP201 and Tat-MNP201(mut). Therefore, the first peak of these two sample’s chromatogram indicates formic acid. The peptides were ordered from Genscript, USA.
Figure 5.2: Dose-dependent uptake of FITC-Tat-MNP201 into neuronal cells

The FITC-Tat-MNP201 peptide is based on the last 10 amino acids of ct of parkin. A FITC-Tat peptide (FITC-YGRKKRRQRRR) was fused to the N-terminal of MNP201 (VCMGDHWFDV) to facilitate its internalisation into neuronal cells. A) Cortical neuronal cells treated with four concentrations of peptide (0.1 µg, 1 µg, 10 µg and 100 µg per ml) are shown (green channel). Red channel shows anti-neurofilament staining (NF), the green channel shows FITC signal, along with a composite image (Merge). The scale bars depict 50 µm. B) The bar graph depicts % internalisation of FITC-Tat-MNP201 in the cortical neurons treated with 0.1 µg, 1 µg, 10 µg and 100 µg (per ml) of peptide. The fluorescence intensities were calculated using the ratio of green (FITC) and red (NF) intensity obtained from 6-8 confocal images (each containing 40-50 cells) of each treatment group. The values represent a percentage of the mean fluorescence intensity obtained using 100 µg/ml of FITC-Tat-MNP201. The data represents mean ± SEM obtained from three separate experiments (n=3). The p-value (*) signifies p<0.05.
5.2 Concentration-dependent internalisation of FITC-Tat-MNP201 into astrocytes

After demonstrating internalisation of FITC-Tat-MNP201 in mixed cortical neurons, the internalisation of FITC-Tat-MNP201 peptide was also examined on glial cells, namely astrocytes. Similar to the experiments performed with neurons, astrocytes were also treated with 0.1 µg, 1 µg, 10 µg and 100 µg (per ml) of FITC- Tat-MNP201 for 1 h in 5% CO₂ at 37°C. After treatment, the astrocytes were fixed, stained with rabbit anti-GFAP (1/1000 dilution) antibody followed by secondary anti-rabbit Alexa-633 (1/500 dilution) antibody and visualised under 63x objective lens of a confocal microscope. Similar to data found using neurons, in astrocytes a fluorescent signal of FITC-Tat-MNP201 was also observed in a dose-dependent manner with increasing amounts of the peptide (Figure 5.3A). Images (containing 20-30 cells per image) were acquired from each treatment group (6-8 images per treatment group). Mean pixel intensity was calculated as a ratio of total intensity in the green channel (FITC) to the total intensity in the red channel (GFAP) and represented as a percentage of the ratio found using 10 µg/ml FITC-Tat-MNP201, in a bar graph. The data indicated a dose-dependent inclusion of the FITC-Tat-MNP201 peptide in treated astrocytes, which specific values of 0.1 µg/ml (6.2% ± 5.31), 1 µg/ml (23.51% ± 16.75), 10 µg/ml (100%) and 100 µg/ml (562.29% ± 49.19) (Figure 5.3B). Importantly, the close observation of FITC-Tat-MNP201 treated astrocytes showed the FITC-Tat-MNP201 peptide localised in the nucleus as well as processes of astrocytes treated with 100 µg/ml (Figure 5.4A and B) and 10 µg/ml (Figure 5.4C and D). Therefore, the data suggest that the Tat-tagged MNP201 is able to cross the plasma membrane of astrocytes.

5.3 Effect of Tat-MNP201 on peroxide production

ROS production is an essential marker of cell stress. ROS production was measured by quantifying the amount of resorufin (585 nm) converted from amplex red in the presence of ROS. To investigate the effect of Tat-MNP201, freshly prepared synaptosomes were mixed with amplex red and the change in peroxide production was measured over a 1 h time period. A linear portion of the curve was selected (the same for each treatment condition; 15-20 min) and the total H₂O₂ production was calculated. The relative values of peroxide production was expressed as a percentage of control and plotted in a bar graph. No significant changes were observed in peroxide production in 100 µM Tat-MNP201 (108.84% ± 4.79) treated synaptosomes, when compared to vehicle control and 100 µM Tat-MNP201(mut) treated synaptosomes (129.76% ± 11.68) (Figure 5.5A), similar to the result
Figure 5.3: Dose-dependent uptake of FITC-Tat-MNP201 into astrocytes

A) Rat brain astrocytes, treated with four concentrations of FITC-Tat-MNP201 peptide (0.1 μg, 1 μg, 10 μg and 100 μg per ml) are shown in green channel (FITC). Red and blue channels show the anti-GFAP (GFAP) and Hoechst (Hoe) staining along with a composite figure of all channels (Merge). The scale bars represent 20 μm.

B) The bar graph depicts the mean pixel intensity obtained from astrocytes treated with 0.1 μg, 1 μg, 10 μg and 100 μg per ml of FITC-Tat-MNP201 peptide under 521 nm. The mean pixel intensities were calculated using the ratio of the green channel (FITC) and with the number of GFAP cells (red channel) obtained from 6-8 confocal images (each containing 20-30 cells). The data was represented as a percentage of mean pixel intensity obtained for 10 μg/ml FITC-Tat-MNP201. The data represents mean ± SEM of two separated experiments (n=2). The p-value signifies p<0.001 (***), p<0.01 (**).
Figure 5.4: Dose-dependent uptake of FITC-Tat-MNP201 into the processes of astrocytes

The internalisation of FITC-Tat-MNP201 in astrocytes occurred in a dose-dependent manner. Astrocytes treated with 100 and 10 µg/ml FITC-Tat-MNP201 are shown in the green channel (FITC). Red and blue channels show anti-GFAP (GFAP) and Hoechst (Hoe) staining along with a composite image of all channels (Merge). The scale bars correspond to the values shown. Yellow boxes represent area of interest which are magnified and depicted in next panels. Area of interests of image (A) and (C) are magnified form image (B) and (D), respectively. White arrows indicate the processes of astrocytes in both green and red channel demonstrating the overlapped regions. The images represent data of two separate experiments (n=2).
Figure 5.5: Effect of Tat-MNP201 on ROS production

Amplex red measures the amount of ROS produced by fresh synaptosomes in the presence of Tat-MNP201. In this assay, amplex red is converted to a fluorescent agent resorufin by reacting with ROS, in the presence of peroxidase. The resorufin level (585 nm) thus represents a quantitative indicator of peroxide production. A) The graph demonstrates the rate of peroxide produced, in the presence of Tat-MNP compounds and antimycin A (as control) in a time scale of 1 h. Representative traces indicate the rate of ROS production in the presence of vehicle control, 100 μM Tat-MNP201 and 100 μM Tat-MNP201(mut) along with positive control 1 μM antimycin A. B) The bar diagram represents the total H₂O₂ production during the linear part of activity (the same for each treatment condition; 15-20 min) as a percentage of control (mean ± SEM) in five separate experiments (n=5) in the presence of vehicle control (Cont), antimycin A (AM), Tat-MNP201 and Tat-MNP201(mut). The p-value (*) signifies p<0.05.
obtained using the non-tagged Tat version of MNPNP201. In contrast, when synaptosomes were incubated with 1 μM of antimycin A, an inhibitor of ubiquinol of ETC (174.70% ± 35.76), a significant increase of 74.70% peroxide production was found, when compared with vehicle control (Figure 5.5B), in agreement with previous publications (Sipos et al., 2003). Thus, the data showed that Tat-MNP201 does not alter peroxide production in synaptosomal mitochondria.

5.4 Effect of Tat-MNP201 on mitochondrial membrane potential

Mitochondrial membrane potential was measured by calculating the ratio of red (590 nm, aggregated form) to green (535 nm, monomeric form) JC-1. The JC-1590/535 control value of synaptosomes was found to be 5.59 on average and considered as a quantitative indicator of the mitochondrial membrane potential, as suggested by previous publications (Reers et al., 1991 and 1995). After incubation with JC-1, freshly extracted synaptosomes were treated with vehicle control and Tat-MNP201 peptides. The JC-1590/535 was plotted as the rate of change in mitochondrial membrane potential over a time of 1 h (Figure 5.6A). A linear portion of the curve was selected (the same for each treatment condition; 15-20 min) and the mitochondrial membrane potential was measured. The relative values of the mitochondrial membrane potential was expressed as a percentage of control and plotted in a bar graph. Unlike MNPNP201, a statistically significant reduction of 23.74% was observed in mitochondrial membrane potential in synaptosomes treated with 100 μM Tat-MNP201 (76.26% ± 4.90), when compared to the vehicle control (Figure 5.6B). In addition, 2 μM FCCP (18.15% ± 0.80), a protonophore, caused a significant 81.85% reduction in the mitochondrial membrane potential, when compared to vehicle control, in agreement with previously published data (Kilbride et al., 2008). Interestingly, the negative control Tat-MNP201(mut) (87.61% ± 2.06) treated samples also induced a significant 12.39% increase in mitochondrial depolarization, when compared to vehicle control. However, when Tat-MNP201 and Tat-MNP201(mut) values were compared, a significant 11.35% increase in mitochondrial membrane potential was identified in Tat-MNP201 treated samples compared to Tat-MNP201. Previously, a Tat peptide (N-terminal 72 amino acids) was reported to cause depolarization in mitochondrial membrane potential (Kruman et al., 1998). Thus, the effect demonstrated by Tat-MNP201(mut) in mitochondrial membrane potential was believed to be due to the effect of the Tat peptide. Taken together, the data suggest that Tat-MNP201 increases mitochondrial depolarization by reducing the membrane potential.
Figure 5.6: Effect of Tat-MNP201 on mitochondrial membrane potential

JC-1 is a known reporter of membrane potential in mitochondria that changes its emission spectra upon a change of mitochondrial membrane potential. In this assay, JC-1 associates with mitochondria under control conditions and emits a red colour (590 nm). In depolarized conditions, JC-1 is found in the cytoplasm in a monomeric form that emits a green colour (535 nm). The JC-1 590/535 ratio thus can be used as a quantitative indicator of the mitochondrial membrane potential. A) The graph represents the rate of change in mitochondrial membrane potential in the presence of Tat-MNP compounds in a time scale of 1 h. Representative traces indicate the rate of change in mitochondrial membrane potential by vehicle control, 2 μM FCCP (positive control), 100 μM Tat-MNP201 and 100 μM Tat-MNP201(mut) treated synaptosomes. B) The bar diagram represents the mitochondrial membrane potential during the linear part of the traces (the same for each treatment condition; 15-20 min) as a percentage of control (mean ± SEM) in five separate experiments (n=5) by vehicle control (Cont), FCCP, Tat-MNP201 and Tat-MNP201(mut). The p-values (****) and (*) signify p<0.001 and p<0.05, respectively.
5.5 Dose-dependent increase in mitochondrial membrane depolarization with FCCP

According to previous publications, parkin has been reported to engulf depolarized mitochondria and plays a major role in recruiting autophagosomes to depolarized mitochondria (Narendra et al., 2008; Geisler et al., 2010). In particular, parkin has been demonstrated to selectively engulf depolarized mitochondria upon depolarization induced by the protonophore 10 μM CCCP (Narendra et al., 2008). Another protonophore, FCCP (1 μM), has been reported to depolarise the mitochondrial membrane potential (Kilbride et al., 2008; Telford et al., 2010). The depolarization effect of FCCP was also demonstrated in this project (Figure 4.7 and 5.6). To investigate the effect of Tat-MNP201 under mitochondrial depolarised conditions, synaptomes were treated with FCCP. Firstly increasing concentrations (from 2 pM to 2 μM) of FCCP were used to depolarize freshly extracted, JC-1 loaded synaptosomes. This dose-response curve of FCCP inducing mitochondrial depolarization (as measured by JC-1) was prepared to identify the IC₅₀ value of FCCP. (Figure 5.7A). From the dose-response curve, the half maximal inhibitory concentration (IC₅₀) of FCCP that induced synaptosomal mitochondrial depolarization was determined to be approximately 20 nM (Figure 5.7B).

5.6 Tat-MNP201 depolarizes mitochondrial membrane potential in partial stress condition

Next, the effect of Tat-MNP201 was investigated on mitochondrial membrane potential in synaptosomes treated with 20 nM FCCP, where mitochondrial membranes were partially depolarised and stressed. First, JC-1 loaded synaptosomes were treated with vehicle control, 100 μM Tat-MNP201 and 100 μM Tat-MNP201(mut); and the mitochondrial membrane potential was monitored for the first 15 min. At the end of these 15 min, all the samples were treated with 20 nM FCCP to induce partial mitochondrial depolarization and the mitochondrial membrane potential was monitored for a further 30 min (Figure 5.8A). A linear portion of the curve was selected (the same for each treatment condition; 5-10 min before FCCP treatment and 15-20 min after FCCP treatment) and the values of these mitochondrial membrane potentials were expressed as a percentage of control and plotted in a bar graph. Analysis of non-stressed samples (no FCCP) suggested that there was a statistically significant difference in mitochondrial membrane potential in 100 μM Tat-MNP201 (80.51% ± 1.93) treated synaptosomes when compared with vehicle control and 100 μM Tat-MNP201(mut) (88.80% ± 1.57) treated synaptosomes (Figure 5.8B), in...
Figure 5.7: FCCP standard curve
FCCP is a protonphore that uncouples mitochondrial membranes and causes mitochondrial depolarization. In a JC-1 based assay, the JC-1 590/535 ratio, calculated by JC-1 aggregate (590 nm) and monomeric form (535 nm), represents a quantitative reporter of mitochondrial membrane potential. A) Serial dilutions of FCCP, concentrations ranging from 2 pM to 2 μM in 10 fold increment, were used to treat JC-1 loaded freshly extracted synaptosomes, and the rate of depolarization was observed for 30 min. B) Total depolarization (n=3), caused by different concentrations (mM) of FCCP, is plotted into a dose-response curve. The IC$_{50}$ value was found to be approximately 20 nM.
Figure 5.8: Effect of Tat-MNP201 on mitochondrial membrane potential under FCCP-induced partial stress condition

FCCP, a protonophore, induces depolarization of mitochondrial membrane depolarization. A partial mitochondrial depolarization stress was induced by 20 nM FCCP (see Figure 5.7). JC-1 treated freshly extracted synaptosomes were treated with vehicle control, 100 μM Tat-MNP201 and 100 μM Tat-MNP201(mut); and rate of change in the mitochondrial membrane potential was observed for 15 min. Then, synaptosomes were treated with 20 nM FCCP and the rate of change in mitochondrial membrane potential was observed for a further 30 min. The fluorescence of JC-1 (excitation 490 nm, emission 590 and 535 nm) was recorded from the commencement of the experiment. The JC-1 590/535 ratio represents a quantitative reporter of mitochondrial membrane potential. The fluorescence ratio of JC-1 (590 nm/535 nm) was plotted against time and the representative traces (A) were obtained from synaptosomes treated with vehicle control, 100 μM Tat-MNP201 and 100 μM Tat-MNP201(mut). The bar graphs represent the mitochondrial membrane potential during the linear part of the curve (the same for each treatment condition; 5-10 min before FCCP and 15-20 min after FCCP) as a percentage of control (mean ± SEM) observed in vehicle control (Cont). 100 μM Tat-MNP201 and 100 μM Tat-MNP201(mut) treated synaptosomes in three separate experiments (n=3) before (B) and after (C) FCCP-induced stress depolarization. The p-values (***), (**) and (*) signify p<0.001, p<0.01 and p<0.05, respectively.
agreement with the data generated in Figure 5.6. In addition, when the FCCP-induced stress portion of the graph was analysed, 100 µM Tat-MNP201 (67.05% ± 2.47) treated samples demonstrated a 32.95% higher amount of mitochondrial depolarization compared to vehicle control treated synaptosomes (Figure 5.8C). In addition, 100 µM Tat-MNP201(mut) (77.45% ± 1.53) treated samples also demonstrated 22.55% increase in mitochondrial membrane depolarization compared to vehicle control. When the value of Tat-MNP201 was compared with Tat-MNP201(mut), an significant increase of 10.4% in mitochondrial depolarization was found in Tat-MNP201 treated synaptosomes compared to Tat-MNP201(mut). The effect of Tat-MNP201(mut) in non-stressed and stressed synaptosomes could be due to the effect of the Tat tag, as described in the previous experiment (Figure 5.6). These observations suggest that PICK1-parkin interaction may play a role in the maintenance of mitochondrial membrane potential.

5.7 Effect of Tat-MNP201 on 4-AP-induced glutamate release without CaCl$_2$

Next, the effect of 100 µM Tat-MNP201 was investigated in glutamate release from freshly extracted synaptosomes obtained from mature female Wistar rat brains. A continuous fluorimetric method based on Nicholls et al. (1987) was used where the amount of NADPH is measured (460 nm) as the quantitative indicator of glutamate release, as indicated in Kilbride et al. (2008). First, freshly extracted synaptosomes were incubated with the glutamate release assay media and the blank rate of glutamate release was monitored for the first 2 min. Then, vehicle control, Tat-MNP201 peptides and rotenone were added and the baseline glutamate release was observed from non-depolarized synaptosomes for the next 5 min. The total release of glutamate in non-depolarized synaptosomes was measured over this 5 min period. No significant difference was observed in Ca$^{2+}$-independent glutamate release from 100 µM Tat-MNP201 (98.95% ± 2.21) treated non-depolarized synaptosomes compared to vehicle control and 100 µM Tat-MNP201(mut) (101.71% ± 2.65) treated samples (Figure 5.9A, B). In contrast, the positive control 10 µM rotenone (126.04% ± 4.18), an inhibitor of complex I, showed a significant 26.04% increase in glutamate release when compared to vehicle control, in agreement with previously published data (Kilbride et al., 2008).

After measuring rate of glutamate release from non-depolarized synaptosomes, the depolarization of synaptosomes was carried out using 4-AP, a non-selective voltage-dependent K$^+$-channel blocker, and rate of Ca$^{2+}$-independent glutamate release was recorded for 10 min. The total release of glutamate from depolarized synaptosomes was
Figure 5.9: Effect of Tat-MNP201 on 4-AP-induced glutamate release without CaCl$_2$

The rate of glutamate release was measured by NADPH production at 460 nm. The level of glutamate was determined by generating a standard curve of glutamate-mediated NADPH production (Figure 4.9) in the absence of synaptosomes. The blank rate (BR) of glutamate release was recorded for the first 2 min in freshly extracted synaptosomes in the absence of CaCl$_2$. Then, synaptosomes were treated with peptides (Pep), i.e., 100 µM Tat-MNP201 and 100 µM Tat-MNP201(mut) and also 10 µM rotenone; and the rate of glutamate release at baseline (BL) was observed from non-depolarized synaptosomes for the next 5 min. Then, depolarization of synaptosomes was initiated by addition of 10 mM 4-AP and the rate of glutamate release was observed for the remaining 10 min. A) The graph indicates the representative traces of Ca$^{2+}$-independent rate of glutamate release (nmol) from vehicle control, 10 µM rotenone, 100 µM Tat-MNP201 and 100 µM Tat-MNP201(mut) treated synaptosomes in a total time period of 17 min. The bar diagrams show the total glutamate release as a percentage of control (mean ± SEM) from vehicle control (Cont), 10 µM rotenone (Rot), 100 µM Tat-MNP201 and 100 µM Tat-MNP201(mut) treated synaptosomes under non-depolarized (B) and depolarized (C) conditions in five separate experiments (n=5). The p-values (***) and (**) signify p<0.001 and p<0.01.
measured over this 10 min period. No statistically significant change in the rate of glutamate release was observed in synaptosomes treated with 100 μM Tat-MNP201 (103.60% ± 4.46) when compared to vehicle control and Tat-MNP201(mut) (105.86% ± 5.29) (Figure 5.9A, C). In contrast, the positive control 10 μM rotenone (125.24% ± 5.05) induced a significant 25.24% increase in release of glutamate compared to vehicle control, in agreement with previously published data (Kilbride et al., 2008). Taken together, the data suggest that Tat-MNP201 has no effect in Ca²⁺-independent 4-AP-induced glutamate release from synaptosomes.

5.8 Effect of Tat-MNP201 on 4-AP-induced glutamate release with CaCl₂

After observing the effect of Tat-MNP201 on Ca²⁺-independent glutamate release from non-depolarized or 10 mM 4-AP depolarised synaptosomes, the effects of Tat-MNP201 was examined on glutamate release in the presence of 2 mM CaCl₂. In non-depolarized synaptosomes, 100 μM Tat-MNP201 (107.27% ± 4.49) showed no statistically significant effect on the Ca²⁺-dependent rate of glutamate release when compared to vehicle control or 100 μM of Tat-MNP201(mut) (102.85% ± 2.12) (Figure 5.10A, B). On the other hand, the positive control sample 10 μM rotenone (142.20% ± 6.44) induced a significantly 42.20% higher rate of glutamate release when compared to vehicle control, in agreement with previously published data (Kilbride et al., 2008). After measuring the non-depolarized rates of glutamate release, the synaptosomes were depolarized with 10mM 4-AP. In the presence of 2 mM CaCl₂ and 10 mM 4-AP, again, no change in glutamate release was observed in 100 μM Tat-MNP201 (110.67% ± 2.55) treated synaptosomes, when compared to vehicle control and Tat-MNP201(mut) (99.86% ± 3.26) (Figure 5.10A, C). In contrast, 10 μM rotenone (125.10% ± 7.95) induced a significant 25.10% increase of release of glutamate compared to vehicle control, in agreement with previously published data (Kilbride et al., 2008). Taken together, the data showed that Tat-MNP201 has no effect in Ca²⁺-dependent glutamate release from non-depolarised or 4-AP depolarised synaptosomes.
Figure 5.10: Effect of Tat-MNP201 on 4-AP-induced glutamate release with CaCl₂

The rate of glutamate release was measured by NADPH production at 460 nm. The level of glutamate was determined by generating a standard curve of glutamate-mediated NADPH production (Figure 4.9), in the absence of synaptosomes. The blank rate (BR) of glutamate release was recorded for the first 2 min in freshly extracted synaptosomes in the presence of CaCl₂. Then, synaptosomes were treated with peptides (Pep), i.e., 100 µM Tat-MNP201 and 100 µM Tat-MNP201(mut) and also 10 µM rotenone; and the rate of glutamate at baseline (BL) was observed from non-depolarized synaptosomes for the next 5 min. Then, depolarization of synaptosomes was initiated by addition of 10 mM 4-AP and rate of glutamate release was observed for a further 10 min. A) The graph indicates the representative traces of Ca²⁺-dependent rate of glutamate release (nmol) in vehicle control, 10 µM rotenone, 100 µM Tat-MNP201 and 100 µM Tat-MNP201(mut) treated synaptosomes in a total time period of 17 min. The bar diagrams show the total glutamate release as a percentage of control (mean ± SEM) in vehicle control (Cont), 10 µM rotenone (Rot), 100 µM Tat-MNP201 and 100 µM Tat-MNP201(mut) treated synaptosomes under non-depolarized (B) and depolarized (C) conditions in five separate experiments (n=5). The p-values (***) and (**) signify p<0.001 and p<0.01.
Discussion

1. Summary of results

In the previous chapter (Result 2), the effect of 100 μM MNP201 was investigated on mitochondrial function and glutamate release assays. No statistically significant effect was obtained by using MNP201 on any of the mentioned assays. In this chapter, the N-terminal Tat-sequence tagged MNP201 (Tat-MNP201) was employed to investigated its effects on mitochondrial function and glutamate release assays (Result summary Table 5.1). To confirm membrane permeability of the peptide, a fluorescent labelled FITC-Tat-MNP201 peptide was synthesised. Treatment of the cortical neuronal and astrocyte culture showed the successful uptake of FITC-Tat-MNP201, confirming membrane permeability. Thereafter, the effect of Tat-MNP201 was demonstrated in the mitochondrial properties and glutamate release. In summary, the data showed that Tat-MNP201 did not alter glutamate release, however it inhibited mitochondrial membrane potential.

2. Tat-MNP201 reduces mitochondrial membrane potential

The working hypothesis of this project is that parkin-PICK1 interaction plays an important role in mitochondrial function. It has been reported that mitochondrial-induced apoptosis is one of the causes behind the death of dopaminergic neurons in mid brain in PD (Keeney et al., 2006). The two important markers of mitochondrial or cellular stress are (i) peroxide release and (ii) mitochondrial depolarization that play roles in apoptosis. Importantly, oxidative stress is known to cause cellular stress leading to apoptosis (Galley, 2011). Furthermore, collapse of the mitochondrial membrane potential induces the release of cytochrome C into the cytosol, which eventually triggers apoptosis (Ly et al., 2003). The results here indicated that Tat-MNP201 caused a statistically significant reduction in mitochondrial membrane potential, when compared to vehicle control samples in both control and FCCP stressed conditions. Interestingly, Tat-MNP201(mut), the mutated version of the Tat-MNP201 also caused mitochondrial depolarization, although the effects were significantly less than those seen with Tat-MNP201. Published data indicate that the N-terminal 72 amino acids of Tat can increase peroxide production and mitochondrial membrane depolarization along with disruption of Ca\(^{2+}\) homeostasis and caspase activation (Kruman et al., 1998). It is possible, therefore, that the Tat sequence in the MNP201 peptides may have some direct effect on mitochondrial membrane potential, although this appears unlikely since the Tat-MNP201 (wild type and mutated) did not induce production of ROS or alter glutamate release.
**A)**

<table>
<thead>
<tr>
<th>Assay</th>
<th>100 µM Tat-MNP201</th>
<th>100 µM Tat-MNP201(mut)</th>
<th>Measured by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase production</td>
<td>108.84% ± 4.79</td>
<td>129.76% ± 11.68</td>
<td>Resorufin production (OD\text{585})</td>
</tr>
<tr>
<td>Membrane potential</td>
<td>76.26% ± 4.90</td>
<td>87.61% ± 2.06</td>
<td>JC-1 OD ratio (JC-1\text{590/535})</td>
</tr>
<tr>
<td>FCCP-induced membrane potential</td>
<td>67.05% ± 2.47</td>
<td>77.45% ± 1.53</td>
<td>JC-1 OD ratio (JC-1\text{590/535})</td>
</tr>
</tbody>
</table>

**B)**

<table>
<thead>
<tr>
<th>Glutamate release</th>
<th>100 µM Tat-MNP201</th>
<th>100 µM Tat-MNP201(mut)</th>
<th>Measured by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-depolarized level (- CaCl2)</td>
<td>98.95% ± 2.21</td>
<td>101.71% ± 2.65</td>
<td>Amount of NADPH production (OD\text{460})</td>
</tr>
<tr>
<td>Non-depolarized level (+ CaCl2)</td>
<td>107.27% ± 4.49</td>
<td>102.85% ± 2.12</td>
<td></td>
</tr>
<tr>
<td>- CaCl2, + 10 mM 4-AP</td>
<td>103.60% ± 4.46</td>
<td>105.86% ± 5.29</td>
<td></td>
</tr>
<tr>
<td>+ CaCl2, + 10 mM 4-AP</td>
<td>110.67% ± 2.55</td>
<td>99.86% ± 3.26</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1: Summary of data obtained by using Tat-MNP201
Mitochondrial function assays (A) and glutamate release assay (B). The data suggest that Tat-MNP201 has no effect on ROS production and glutamate release. In contrast, Tat-MNP201 inhibits mitochondrial membrane potential in both control and FCCP stressed conditions. The values are indicated as a percentage of control (mean ± SEM).
3. Effect of Tat-MNP201 in glutamate release

The glutamate release assay using Tat-MNP201 showed similar results to the non tagged MNP201 version. Specifically, Ca^{2+}-independent and dependent glutamate release experiments were performed by treating freshly prepared synaptosomes with 100 µM Tat-MNP201 in non-depolarized conditions as well as in depolarized conditions (triggered by 4-AP). No significant difference in the rate of Ca^{2+}-independent or dependent glutamate release in Tat-MNP201 treated synaptosomes was observed under non-depolarized and 4-AP-induced depolarized conditions, when compared to the vehicle control and Tat-MNP201(mut) samples. In contrast, rotenone treated samples showed significant increase in the rate of glutamate release. Taken together, the data suggested that Tat-MNP201 has no effect on pre-synaptic release of glutamate from synaptosomes. As indicated in the previous chapter, the lack of effects of Tat-MNP201 on glutamate release was surprising when considering that MNP201 binds to the PDZ domain of PICK1 and thus could competitively block all PDZ motifs interacting with PICK1, in particular pre-synaptic glutamate receptors that control glutamate release. Two possibilities exist that may explain these results: (i) the MNP201 does not block the interaction of PICK1 with GluR2, GluR5 and/or mGluR7 or (ii) PICK1 interaction with GluR2 (AMPA), GluR5 (KA) and/or mGluR7 (metabotropic) does not play a role in glutamate release. Notably, Tat peptides have been reported to increase release of glutamate from human and mouse neocortical nerve endings (Musante et al., 2010). Thus, while the lack of effects of Tat-MNP201 on glutamate release was surprising, these data suggest that the Tat sequence in the Tat-MNP201 and Tat-MNP201(mut) peptides do not cause overt effects on synaptosomal integrity per se and therefore support the notion that the Tat-MNP201-induced decrease in mitochondrial membrane depolarization is a significant finding.

4. Parkin-PICK1 role in mitochondrial membrane potential

Several reports have suggested that the trafficking of parkin to depolarized mitochondria plays a role in the induction of mitophagy by recruitment of autophagosomes (Narendra et al., 2009; Narendra et al., 2010; Geisler et al., 2010; Ziviani et al., 2010). These data raise further questions, such as: (i) what is the signalling mechanism that activates parkin to translocate towards depolarized mitochondria, (ii) what is the translocating mechanism of parkin to depolarized mitochondria and (iii) how does parkin recognise depolarized mitochondria? According to our hypothesis, PICK1 may play a role in the trafficking of parkin towards depolarized mitochondria. Indeed, the mitochondrial membrane potential data showed that in the presence of 100 µM Tat-MNP201, a reduction in the mitochondrial
membrane potential was observed in synaptosomes. This data may suggest that Tat-MNP201 alters mitochondrial membrane potential due to the disruption of the parkin-PICK1 interaction, which prevents parkin translocation to mitochondria and leaves mitochondria more susceptible to depolarization (especially during conditions of stress). To further confirm this hypothesis, it would be worthy to examine the effect of Tat-MNP201 on the translocation of parkin to mitochondrial membranes under depolarising conditions using imaging studies.

5. Alternative mechanisms for MNP201 effects on mitochondrial membrane potential

Earlier reports have also suggested that mitochondrial stabilization and cell viability is enhanced when PICK1 recruits activated PKCα to the outer mitochondrial membrane to phosphorylate the anti-apoptotic Bcl-2 protein (Wang et al., 2007). Due to the absence of a mitochondria targeting sequence in PKCα, PICK1 has an important role in the proper targeting of PKCα to mitochondria (Wang et al., 2007). In addition to localisation of PKCα to the mitochondria, PICK1 also modulates the phosphorylation of Bcl-2 proteins. Upon overexpression of PICK1, the phosphorylation of Bcl-2 protein increases in the presence of PKCα (Wang et al., 2007). Phosphorylation of Bcl-2 by PKCα induces anti-apoptotic property by reducing the formation of Bcl2/Bax heterodimers and preventing Bax proteins docking to mitochondrial membranes (Wang et al., 2007). Moreover, PICK1 is also believed to influence the pore forming components, such as the mtPTP to maintain mitochondrial membrane integrity (Halestrap et al., 2003). It is therefore important to note that the effects of Tat-MNP201 on reducing mitochondrial membrane potential may be due to (i) an inhibition of a PKCα-PICK1 interaction, (ii) altering Bcl-2 phosphorylation, and/or (iii) regulation of mtPTP function in addition to (or instead of) blocking a parkin-PICK1 interaction. In addition to these possible alternative mechanisms of MNP201 regulating mitochondrial membrane potential, it is also important to note that PICK1 can form homodimers/oligodimers, which allows it to interact with multiple partners simultaneously, which may also be regulated by MNP201 to alter mitochondrial membrane potential e.g. formation of a parkin-PICK1/PICK1-ASIC complex (Joch et al., 2007). In this context, the over-expression of parkin is reported to induce monoubiquitination of PICK1, which limits PICK1-ASIC interaction and causes a loss of ASIC channel current (Joch et al., 2007). The effect of parkin-mediated PICK1 monoubiquitination on mitochondrial function is still unknown. Thus, it would be interesting to investigate the effect of parkin-mediated monoubiquitination of PICK1 on (i) the role of PKCα-PICK1 on mitochondrial function, (ii) in maintaining mitochondrial integrity and (iii) Bcl-2-dependent apoptosis.
7. Moving to the next chapter

At this stage, the outcome of the experiments propose that the interaction between parkin-PICK1 may play a role in regulating mitochondrial membrane potential under control and cell stress conditions (induced by FCCP). The results show that the Tat-MNP201 compounds reduce mitochondrial membrane potential, with no effects on ROS activity or glutamate release. A number of open questions remain regarding how the Tat-MNP201 peptide alters mitochondrial membrane potential, including does Tat-MNP201 work by (i) inhibiting the parkin-PICK1 interaction?; (ii) limiting PKCα-PICK1 function?; (iii) altering the role of parkin-PICK1-PKCa in mitochondria? and/or (iv) regulating the phosphorylation or ubiquitination of these proteins?
6. Results: 4
Effect of FSC231 on mitochondrial function
Abstract

In previous two chapters, the effects of PDZ motif peptides modelled on parkin were investigated in mitochondrial function and glutamate release. The two versions of parkin peptides characterised were MNP201 and Tat-MNP201. The MNP201 version, without a cell permeability motif, did not show any effect on mitochondrial function and glutamate release assays. In contrast, Tat-MNP201, a cell permeable version, showed mitochondrial membrane depolarization under control and stress-induced conditions. In this chapter, the compound called FSC231 was used, which is a compound that binding to the PDZ domain of PICK1. FSC231 is an aromatic compound that, as a result of binding the PDZ domain of PICK1, competitively hinders the interaction of PICK1 with the AMPA receptor subunit, GluR2. Due to its small molecular size and lipophilic nature, FSC231 is capable of crossing the plasma membrane unaided (Thorsen et al., 2010). Here, an in silico computational modelling analysis was first performed that investigated the docking of FSC231 into the PDZ domain of PICK1 to assess its putative affinity. Then, the effect of FSC231 was investigated on mitochondrial function and glutamate release. Similar to the MNP201 peptides, the data suggested that FSC231 had no effect on the activities of the respiratory chains of ETC (complex I and IV) or on peroxide production. In addition, no effect of FSC231 was observed in the rate of glutamate release. While FSC231 did not alter mitochondrial membrane potential under control conditions, more importantly this compound increased mitochondrial membrane depolarization in treated synaptosomes under FCCP-induced stressed conditions, similar to Tat-MNP201. The data may suggest that PDZ domain interacting proteins of PICK1, such as parkin and/or PKCa, are prevented from interacting with PICK1 in the presence of FSC231 and these interactions play a role in the maintenance of mitochondrial integrity, especially under mitochondrial depolarizing stress conditions.
Introduction

The PDZ domain is composed of 90 amino acids and forms a precise groove responsible for interaction with proteins containing a PDZ motif at their C terminus (ct). This domain was first discovered in three proteins, i.e., PSD95, Dlg1 and ZO-1 protein (Sheng and Sala, 2001). A PDZ domain is composed of six antiparallel β-strands and two α-helices to form a six-strand β-sandwich surrounded by two α-helices. The interaction groove of a PDZ domain is conformed between the βB strand and αB helix that interacts with the PDZ motif. PDZ domains are divided into three types, i.e., Class I-III (Sheng, 2001). The classes of PDZ domains are defined according to the amino acid composition of tripeptide PDZ motifs they interact with. They are, Class I \{(S/T)X|c|d\}, Class II (ϕXϕ) and Class III (D-E-X-ϕ), where ϕ and X denote an hydrophobic and any amino acid respectively (Sheng, 2001). Intriguingly, the PDZ domain of PICK1 can interact with Class I, Class II and atypical PDZ motifs (Staudinger et al., 1997; Dev et al., 1999; Madsen et al., 2005). Interestingly, this ability of PICK1 to interact with a wide range of PDZ motifs is evolutionary conserved as indicated by the sequence homology within the PDZ domain of PICK1 across different species.

The identification of interaction “hotspots” within PDZ domains have made them an attractive site for the development of small molecule inhibitors (Berg, 2003; Arkin and Wells, 2004; Fischer and Lane, 2004). Over the past few years several compounds have been shown to block PDZ based interactions. For example, reports have demonstrated that the interaction between the PDZ domain of PSD95 and the PDZ motif of neuronal nNOS can be successfully disrupted with the blocking compounds, including 2-[(1H-benzotriazol-5-ylamino)-methyl]-4,6-dichloro-phenol (IC87201) and 5-(3,5-dichloro-2-hydroxyl-benzylamino)-2-hydrobenzoic acid (ZL006) (Florio et al., 2009; Zhou et al., 2010). The PSD95 protein, containing three distinct PDZ domains, interacts with both NMDA receptors and nNOS; and couples NMDA receptors to nNOS. This NMDA-PSD95-nNOS pathway plays a role in synaptic plasticity, learning and memory (Harkin et al., 1999; Aarts et al., 2002; Florio et al., 2009; Zhou et al., 2010; Doucet et al., 2012). Particularly, IC87201 has been shown to inhibit the interaction between nNOS and PSD95, without disturbing the catalytic activity of nNOS in vitro and was able to reduce NMDA-induced thermal hyperalgesia in mice (Florio et al., 2009). In addition, ZL006 has been reported to prevent cerebral ischemic damage in rodents by inhibiting formation of the nNOS-PSD95 complex (Zhou et al., 2010). Both of these compounds successfully demonstrated the potential value of PDZ interactions as drug targets.
As mentioned above, AMPA receptors are ionotropic glutamate receptors responsible for fast excitatory neurotransmission and involved in synaptic formation and stabilisation (Dev et al., 1999). The dynamic membrane trafficking of AMPA receptors plays an important role in the regulation of several forms of synaptic plasticity such as LTP and LTD (Lin and Huganir, 2007). AMPA receptor activation plays an important role in the maintenance of physiological neuronal function, although prolonged activation may lead to neurotoxicity (Choi, 1992). AMPA receptors are composed of four subunits, GluR1-GluR4 (Hollmann and Heinemann, 1994; Bettler and Mulle, 1995). The C-terminus of the GluR2 contains a PDZ motif that interacts with PDZ domain containing proteins, including PICK1 (Dev et al., 1999), GRIP (Dong et al., 1997) and ABP (Srivastava et al., 1998). PICK1 regulates the surface expression and internalisation of GluR2, thus, plays a role in synaptic plasticity. A number of studies have used blocking peptides to inhibit the interaction between PICK1 and GluR2 in order to determine the role of this interaction. More recently, a small molecular weight compound called FSC231, which binds to PICK1, has been used to further evaluate the role of PICK1 in regulating AMPA receptor-mediated synaptic plasticity (Thorsen et al., 2010).

The recent discovery of a PICK1 PDZ domain blocking compound, namely FSC231, has provided a valuable tool to investigate the role of PICK1 and its interacting proteins and demonstrated that PICK1 may be drugable. The FSC231 compound is an aromatic molecule that docks into the PDZ domain of PICK1. The molecular weight of FSC231 is 313 g/mol and the compound is poorly soluble in water. A recent report suggests that 50 μM FSC231 blocks the interaction between PICK1 and the AMPA receptor subunit, GluR2 (Thorsen et al., 2010). Upon NMDA receptor activation, GluR2 internalisation takes place by a PICK1-mediated transport mechanism (Chung et al., 2000; Daw et al., 2000; Kim et al., 2001). In contrast, the rate of GluR2 internalisation decreases and surface expression of GluR2 increases in the presence of FSC231, even after NMDA receptor activation (Thorsen et al., 2010). In addition, LTD and LTP are significantly reduced in the presence of 50 μM FSC231 in CA1 hippocampal neurons (Thorsen et al., 2010). It has been shown previously that GluR2 regulates the Ca^{2+} permeability of the AMPA receptor (Liu and Cull-Candy, 2000; Liu et al., 2008), such that Ca^{2+} permeability increases in the absence of wild type GluR2. When PICK1-GluR2 interaction is blocked by using a GluR2 blocking peptide (NVYGIESVKI), hippocampal and cerebellar LTD are reduced (Xia et al., 2000; Kim et al., 2001). Interestingly, 50 μM FSC231 shows similar effects in hippocampal neurons (Thorsen et al., 2010). Here, in this chapter, the effect of FSC231 was investigated in the rate of glutamate release.
As described previously, two interacting partners of PICK1, i.e., parkin and PKCα may play a role in the maintenance of mitochondrial function and integrity. The role of PKCα-PICK1 is well described in the published literature (Wang et al., 2003; 2007), while the role of parkin-PICK1 interaction in mitochondrial function is still unknown. Therefore, to investigate the importance of PICK1 and its interacting partners including parkin (and PKCα) in mitochondrial function, the effect of 50 μM FSC231 was investigated in mitochondrial function including the activity of ETC enzymes (complex I and IV), ROS production and mitochondrial membrane potential. Given that PICK1 regulates AMPA, KA and metabotropic receptor surface expression and that these receptors can regulate pre-synaptic glutamate releases, in addition, the effect of FSC231 was also examined in glutamate release assays.
Results

6.1 Computational data shows docking of FSC231 in PICK1 PDZ domain

FSC231 is reported to bind selectively to PICK1, but not other PDZ domain containing proteins, for example GRIP (Thorsen et al., 2010). Here, the in silico binding affinity of FSC231 with PICK1 was analysed. To ascertain the binding affinity of FSC231, Drugscore, a bio-informatic tool was used to model FSC231 in the PDZ domain of PICK1. Drugscore is a computational method, where the strength of a protein-protein interaction is evaluated on the basis of the ligand (PDZ motif) binding into the receptor (PDZ domain) in a conformation that adopts the lowest free energy. In this modelling approach, the higher negative values indicate a higher binding affinity. When the sequence of the PDZ domain of PICK1 was computed with FSC231 by Drugscore computational modelling, a binding score of -66.75 kcal/mol was found (Figure 6.1). As a positive control, the GluR2-PICK1 interaction was evaluated in this method and a binding value of -92 kcal/mol was obtained. The result suggests that FSC231 has a strong affinity towards the PDZ domain of PICK1. The in silico data was provided by our collaborators Sefika Banu Ozkan (Center for Biological Physics, Arizona State University, USA) and Ozlem Keskin (Center for Computational Biology and Bioinformatics, Koc University, Istanbul, Turkey).

Supplied as white powder, a 5 mM FSC231 stock solution was made in 75% DMSO and 25 mM HEPES pH 7 and used in mitochondrial and glutamate release assays. Therefore, 75% DMSO and 25 mM HEPES pH 7 were used as a DMSO vehicle control in mitochondrial and glutamate release assays. The aliquots of the stock solutions were stored at -20°C until further use.

6.2 Effect of FSC231 in complex I assay

Deficiencies in the respiratory chain of the ETC cause mitochondrial dysfunction and have been identified to play a role in neurodegenerative diseases. The complex I activity was measured by the rate of conversion of NADH to NAD+ spectrophotometrically at an absorbance of 340 nm, as suggested by previous publications (Telford et al., 2010). To investigate the role of the parkin-PICK1 interaction, the effect of MNP201 parkin peptide was examined in a complex I assay. The data showed that the MNP201 peptide at a concentration of 100 μM did not have any effect on the rate of complex I activity (Figure 4.4). In this chapter, FSC231, a compound that binds the PDZ domain of PICK1 (Thorsen et
Figure 6.1: FSC231, the blocking compound of PICK1 PDZ domain

FSC231 is a small aromatic compound, which directly binds the PDZ domain of PICK1 causing a competitive block of PDZ-mediated interactions. The binding affinity of FSC231 for the PDZ domain of PICK1 was -66.75 kcal/mol, as measured by the Drugscore computational method.
al., 2010), was used to investigate the effect of PICK1 interacting proteins in the complex I activity. First, freeze-fractured synaptosomes were either treated with DMSO vehicle control or treated with 50 µM of FSC231 and the absorbance of NADH were monitored for 1-2 min. Next, the reaction was initiated by the addition of decylquinone and the absorbance of NADH was observed for a further 12-15 min (Figure 6.2A). To selectively inhibit complex I, rotenone was added and the absorbance of NADH was subsequently monitored for a final 4-5 min. The rate constants of complex I were calculated according to the method based on Ragan et al. (1987) and the rotenone insensitive rate was subtracted from the rotenone sensitive value to obtain the rate of complex I activity. When the values were expressed as a percentage of control, no significant difference in the rate of complex I were observed in 50 µM FSC231 (93.80% ± 15.58) treated samples compared to the DMSO vehicle control treated samples (Figure 6.2B). The data showed that 50 µM FSC231 has no effect on the rate of complex I activity and is in agreement with the lack of effects of MNP201 on complex I activity. Thus, the data suggests that PICK1 and its interacting partners do not regulate the activity of mitochondrial complex I.

6.3 Effect of FSC231 in complex IV assay

The aberrant function of complex IV is reported to play a role in the pathophysiology of neurodegenerative diseases. For example, activity of mitochondrial complex IV has been found to be hampered in brain samples of AD patients (Chagnon et al., 1995). Complex IV is the last enzyme complex through which electrons travel to the mitochondria matrix before being utilised by ATP synthase to produce ATP. In the earlier chapter (Result 1), the effect of MNP201 parkin peptide was investigated on complex IV assay to determine the role of the parkin-PICK1 interaction. The data suggested that MNP201 had no effect in complex IV activity (Figure 4.5). To further investigate these findings, the effect of FSC231 on complex IV activity was tested. The studies were also performed to investigate the role of PICK1 interacting proteins in the activity of complex IV. The activity of complex IV was measured by monitoring the rate of depletion of cytochrome C (550 nm) by oxidation (Telford et al., 2010), as determined by calculating the first order rate constant of cytochrome C depletion.

First, the DMSO vehicle control and 50 µM FSC231 was incubated with the complex IV reaction mixture (please see Method) and the absorbance of cytochrome C was monitored for 1-2 min. Next, the reaction was initiated by addition of freeze-fractured synaptosomal protein and the absorbance of cytochrome C depletion was observed for a subsequent 7-8 min (Figure 6.3A). The corresponding rate constants of complex IV activity were calculated according to the method of Wharton and Tzagoloff (1967) and expressed as percentage of
Complex I activity is shown in freeze-fractured synaptosomes treated with FSC231. The complex I utilizes NADH as a source of electrons and passes electrons to complex III via ubiquinone. Complex I is inhibited by rotenone. A) The graph shows the raw data of complex I activity by measuring absorbance (340 nm) of depleting NADH in the presence or absence of FSC231 over a time scale of 20 min. Representative traces of complex I activity are shown in the presence of DMSO vehicle control (red trace) and 50 pM FSC231 (green trace). The first peak (DQ) and second peak (ROT) signify addition of decylquinone (1-2 min) and rotenone (14-15 min), respectively. The complex I activity (pseudo-first order rate constant) was obtained by subtracting the rotenone sensitive rate from the initial rate. B) The bar diagram shows the activity of complex I as a percentage of control (mean ± SEM) in three separate experiments (n=3) in the presence of DMSO vehicle control (Cont), and FSC231.
Figure 6.3: Effect of FSC231 on complex IV of ETC cycle
Complex IV activity is shown in freeze-fractured synaptosomes treated with or without FSC231. Complex IV uses complex III as a source of electrons and passes electrons out of the matrix to inner membrane space. A) The graph shows the raw data of complex IV activity of synaptosomal mitochondria by measuring absorbance (550 nm) of depleting cytochrome C in the presence or absence of FSC231 over a time scale of 10 min. Representative traces of complex IV activity are shown in the presence of DMSO vehicle control (red trace) and FSC231 (green trace). The peaks in the activity traces are due to addition of freeze-fractured synaptosomal protein (protein) (1-2 min). The complex IV activity was determined by measuring first order decay rate constant of cytochrome C. B) The bar diagram represents the activity of complex IV as a percentage of control (mean ± SEM) in three separate experiments (n=3) in the presence of DMSO vehicle control (Cont) and FSC231.
control. In agreement with the results found for MNP201, the data showed no statistically significant difference in complex IV activity in 50 μM FSC231 (93.16% ± 9.21) treated synaptosomes, when compared to the DMSO vehicle control (Figure 6.3B). The data suggest that PICK1 and its interacting partners do not play a role in regulating complex IV activity in mitochondria.

6.4 Effect of FSC231 on peroxide production

Production of ROS is an indicator of cellular oxidative stress, which plays a key role in apoptotic cell death. The effect of FSC231 was evaluated in ROS production with freshly prepared synaptosomes over a time period of 2 h. An amplex red assay, based on the conversion of amplex red to resorufin in the presence of ROS, was used. The rate of resorufin production, the quantitative indicator of H₂O₂, in vehicle control and FSC231 treated synaptosomes was plotted against time (x-axis) and OD®®^ (y-axis) (Figure 6.4A). A linear portion of the curve was selected (the same for each treatment condition; 15-20 min) and total H₂O₂ production was calculated. The relative values of peroxide production were expressed as a percentage of control and plotted in a bar graph. Analysis of data revealed that 50 μM FSC231 (115.08% ± 9.98) treated synaptosomes had no significant effect in the rate of peroxide production in comparison to DMSO vehicle control treated synaptosomes. In contrast, when synaptosomes were treated with 1 μM of antimycin A (262.12% ± 20.46), an increase of 162% ROS production was found, when compared with DMSO vehicle control (Figure 6.4B), in agreement with previously published data (Sipos et al., 2003). The data suggest that PICK1 does not play a role in the regulation in peroxide production as well as oxidative stress in synaptosomal mitochondria.

6.5 Effect of FSC231 on mitochondrial membrane potential

Mitochondrial membrane potential is an important parameter of mitochondrial function and also a key indicator of cell viability. Mitochondrial membrane potential was measured by calculating the ratio of red (590 nm, aggregated form) to green (535 nm, monomeric form) JC-1, as suggested previously (Chinopoulos et al., 1999). This JC-1^590/535 ratio is a quantitative indicator of mitochondrial membrane potential. Freshly extracted synaptosomes were first incubated with JC-1 and then treated with DMSO vehicle control and FSC231. The JC-1^590/535 ratio (y-axis) was measured as the rate of change in mitochondrial membrane potential over a time of 1 h (x-axis) (Figure 6.5A). A linear portion of the curve was selected (the same for each treatment condition; 15-20 min) and the mitochondrial membrane potential was measured. The relative values of the mitochondrial membrane potential were expressed as a percentage of control and plotted in a bar graph. No change in the
Figure 6.4: Effect of FSC231 on ROS production

ROS production was measured in freshly extracted synaptosomes treated with FSC231 using amplex red. In this assay, ROS production is measured by conversion of amplex red to a fluorescent agent resorufin (585 nm) by reacting with ROS, in the presence of peroxidase. The resorufin level acts as a quantitative indicator of peroxide production. A) The representative traces of the graph show the rate of peroxide produced by DMSO vehicle control, 1 μM antimycin A (positive control) and 50 μM FSC231 in a time scale of 2 h. B) The bar diagram represents the total H$_2$O$_2$ production during the linear part of activity (the same for each treatment condition; 15-20 min) as a percentage of control (mean ± SEM) in five separate experiments (n=5) by DMSO vehicle control (Cont), antimycin A (AM) and FSC231. The p-value (***) signifies p<0.001.
Figure 6.5: Effect of FSC231 on mitochondrial membrane potential
Mitochondrial membrane potential in fresh synaptosomes was measured by monitoring the change in the emission spectra of JC-1. In this assay, JC-1 associates with mitochondria in control conditions and emits a red colour (590 nm). In depolarized conditions, JC-1 is found in the cytoplasm in a monomeric form emitting a green colour (535 nm). The 590/535 ratio signifies a quantitative indicator of mitochondrial membrane potential. A) Representative traces of the graph show the rate of change in mitochondrial membrane potential in the presence of DMSO vehicle control, 2 \( \mu \)M FCCP (positive control) and 50 \( \mu \)M FSC231 in a time scale of 1 h. B) The bar diagram shows the mitochondrial membrane potential during the linear part of the traces (the same for each condition; 15-20 min) as a percentage of control (mean ± SEM) in four separate experiments (n=4) by DMSO vehicle control (Cont), FCCP and FSC231. The p-value (***) signifies p<0.001.
mitochondrial membrane potential was found in 50 μM FSC231 (92.33% ± 1.83) treated synaptosomes, when compared to the DMSO vehicle control (Figure 6.5B). In contrast, treatment of synaptosomes with 2 μM of FCCP (22.11% ± 5.64) reduced the membrane potential by 78%, when compared to DMSO vehicle control. The data suggest that the FCS231 binding to PICK1 does not alter maintenance of the mitochondrial membrane potential under control conditions. This result is somewhat contradictory to the effects of Tat-MNP201, which causes a reduction in mitochondrial membrane potential under control conditions. We suggest the following reasons for this difference: (i) the reduction in mitochondrial membrane potential caused by molecules binding PICK1 (such as MNP201 and FSC231) requires partial stressed conditions and (ii) the Tat tag on MNP201, but not the FSC231, causes mild stress to mitochondria, as evidenced by the effects of Tat-MNP201(mut) on mitochondrial membrane, which is sufficient for Tat-MNP201 to show effects on mitochondrial membrane potential in control and FCCP conditions. If this were the case, then the effects of FSC231 on mitochondrial membrane potential would be apparent under mild stressed FCCP conditions, as is shown in the results below.

6.6 FSC231 depolarizes mitochondrial membrane potential in partial stress condition

Next, the effect of FSC231 was investigated on mitochondrial membrane potential in synaptosomes challenged with 20 nM FCCP, similar to the experiment performed with Tat-MNP201 in Result 3 chapter (Figure 5.8). First, JC-1 treated freshly extracted synaptosomes were incubated with DMSO vehicle control and 50 μM FSC231; and mitochondrial membrane potential was monitored for 15 min. Then, mitochondrial depolarization stress was induced with 20 nM FCCP in all samples to induce partial membrane depolarization (Figure 5.7) and the mitochondrial membrane potential was monitored for the next 30 min (Figure 6.6A). A linear portion of the curve was selected (the same for each treatment condition; 5-10 min before FCCP treatment and 15-20 min after FCCP treatment) and the mitochondrial membrane potential was measured. The relative values of mitochondrial membrane potential were expressed as a percentage of control and plotted in a bar graph. In non-stressed samples (no FCCP), no statistically significant difference was found in mitochondrial membrane potential between DMSO vehicle control and 50 μM FSC231 (100.11% ± 1.11) treated synaptosomes (Figure 6.6B), in agreement with previously performed experiments (Figure 6.5). More importantly, after inducing stress by 20 nM FCCP, the 50 μM FSC231 (67.05% ± 7.23) treated samples showed a 32.95% higher amount of mitochondrial depolarization, when compared to DMSO vehicle control treated synaptosomes (Figure 6.6C), similar to the generated data for Tat-MNP201 (Figure
Figure 6.6: Effect of FSC231 on mitochondrial membrane potential under FCCP-induced partial stress condition

FCCP is a protonophore that causes depolarization of mitochondrial membrane potential. To create a condition of partial mitochondrial depolarization stress, 20 nM FCCP was used (see Figure 5.7). JC-1 treated freshly extracted synaptosomes were treated with DMSO vehicle control and 50 μM FSC231; and the rate of change in the mitochondrial membrane potential was observed for 15 min. Then, synaptosomes were treated with 20 nM FCCP and the rate of change in the mitochondrial membrane potential was observed for 30 min. The fluorescence of JC-1 (excitation 490 nm, emission 590 nm and 535 nm) was recorded from the commencement of the experiment. JC-1 590/535 ratio signifies a quantitative indicator of mitochondrial membrane potential. The fluorescence ratio of JC-1 (590 nm/535 nm) was plotted against time (A) and the representative traces were obtained from synaptosomes treated with DMSO vehicle control and 50 μM FSC231. The bar graphs represent the mitochondrial membrane potential during the linear part of the traces (the same for each treatment condition; 5-10 min before FCCP and 15-20 min after FCCP) as a percentage of control (mean ± SEM) observed in DMSO vehicle control (Cont) and 50 μM FSC231 treated synaptosomes in three separate experiments (n=3), before (B) and after (C) FCCP-induced stress depolarization. The p-value (**) signifies p<0.01.
Furthermore, under FCCP-induced stress conditions, DMSO vehicle control treated synaptosomes treated showed a recovery from the depolarized state of mitochondrial membrane potential at the end of 30 min, whereas FSC231-treated synaptosomes did not show any sign of recovery from 20 nM FCCP treatment. These observations suggest that PICK1 may play a role in the maintenance of mitochondrial membrane potential under depolarizing condition, most likely through its PDZ domain interacting partners.

6.7 Effect of FSC231 in KCl-stimulated glutamate release assay in the absence of CaCl₂

In addition to mitochondrial functional assays, the effect of 50 µM FSC231 on the rate of glutamate release was also investigated using freshly extracted synaptosomes from rat brain tissue. A continuous fluorimetric method was used, where the amount of glutamate was measured by quantifying the amount of NADPH produced (excitation/emission = 340/460 nm), as described in previous publications (Nicholls et al., 1987; Kilbride et al., 2008). Freshly extracted synaptosomes were incubated with the glutamate release assay media (please see Methods) and the blank rate of glutamate release was monitored for the first 5 min. Then, DMSO vehicle control and FSC231 were added; and the baseline glutamate release was measured from non-depolarized synaptosomes for the next 5 min. Similar to the MNP201 glutamate release experiments, 10 µM rotenone was used as positive control. The total release of glutamate from non-depolarized synaptosomes was measured over a 5 min period. No significant difference in the rate of Ca²⁺-independent glutamate release was observed in 50 µM FSC231 (125.48% ± 10.88) treated synaptosomes, when compared to DMSO vehicle control treated non-depolarized synaptosomes (Figure 6.7A and B). In contrast, the positive control 10 µM rotenone (166.59% ± 17.41) induced a significant 66.59% increase in the Ca²⁺-independent non-depolarized rate of glutamate release, in agreement with previously published reports (Kilbride et al., 2008). Next, the depolarization of synaptosomes was carried out with 40 mM KCl and the Ca²⁺-independent rate of glutamate release was monitored for 30 min. The total release of glutamate from depolarized synaptosomes was measured over this 30 min period. No significant change in Ca²⁺-independent, KCl-stimulated rate of glutamate release was observed in 50 µM FSC231 (117.43% ± 1.67) treated synaptosomes, when compared to DMSO vehicle control synaptosomes (Figure 6.7A and C). In contrast, the positive control, 10 µM rotenone (135.64% ± 13.41), demonstrated a trend increase of 35.64% in glutamate release compared to DMSO vehicle control. Taken together, the data suggest 50 µM FSC231 has no effect on Ca²⁺-independent KCl-induced rate of glutamate release from rat synaptosomes.
Figure 6.7: Effect of FSC231 on KCl-induced glutamate release in the absence of CaCl₂

The rate of glutamate release was measured by NADPH production at 460 nm. The level of glutamate was determined by generating a standard curve of glutamate-mediated NADPH production (Figure 4.9), in the absence of synaptosomes. The blank rate (BR) of glutamate release was recorded for 5 min in freshly extracted synaptosomes in the absence of CaCl₂. Then, synaptosomes were treated with 10 μM rotenone and 50 μM FSC231 (FSC); and the rate of glutamate at baseline (BL) was observed from non-depolarized synaptosomes for 5 min. Depolarization of synaptosomes was subsequently initiated by addition of 40 mM KCl and rate of glutamate release was observed for the next 30 min. A) The graph indicates representative traces of Ca²⁺-independent rate of glutamate release (nmol) by DMSO vehicle control (Cont), 10 μM rotenone (Rot) and 50 μM FSC231 treated synaptosomes in a total time period of 40 min. The bar diagrams show the total glutamate release as a percentage of control (mean ± SEM) in DMSO vehicle control (Cont), 10 μM rotenone (Rot) and 50 μM FSC231 treated synaptosomes under non-depolarized (B) and depolarized (C) conditions in three separate experiments (n=3). The p-value (**) signifies <0.01.
6.8 Effect of FSC231 in KCl-stimulated glutamate release assay in the presence of CaCl₂

After evaluation of Ca²⁺-independent glutamate release, the effect of FSC231 on Ca²⁺-dependent glutamate release was examined. Similar to the previously described experiment, the effect of 50 μM FSC231 was evaluated on glutamate release in the presence of 2 mM CaCl₂. When non-depolarized synaptosomes were treated with FSC231, no statistically significant difference was observed in the rate of Ca²⁺-dependent glutamate release from 50 μM FSC231 (157.37% ± 21.57) treated synaptosomes, when compared to DMSO vehicle control (Figure 6.8A and B). Again, the positive control 10 μM rotenone (190.50% ± 23.91) treated synaptosomes caused a significant 90.50% increase in Ca²⁺-dependent glutamate release, when compared to DMSO vehicle control, in agreement with previously published data (Kilbride et al., 2008). Thereafter, depolarization of synaptosomes was carried out using 40 mM KCl, and the rate of glutamate release was recorded. No change in the Ca²⁺-dependent, KCl-induced rate of glutamate release was observed in 50 μM FSC231 (114.99% ± 1.07) treated samples, when compared with DMSO vehicle control (Figure 6.8A and C). In contrast, synaptosomes treated with 10 μM rotenone (140.19% ± 15.42) demonstrated a trend increase of 40.19% in Ca²⁺-dependent, KCl-induced glutamate release, when compared to DMSO vehicle control, in agreement with previously published data (Kilbride et al., 2008). Taken together, the data suggest 50 μM FSC231 has no effect on Ca²⁺-dependent KCl-induced rate of glutamate release from rat synaptosomes.

6.9 Effect of FSC231 in 4-AP-stimulated glutamate release

After observing KCl-induced glutamate release, the effect of FSC231 in glutamate release was evaluated in 4-AP-induced depolarized synaptosomes. The compound 4-AP is a non-selective blocker of voltage-dependent K⁺-channel, present on the surface of synaptosomes (Nicholls 1993). Depolarization of synaptosomes was carried out by adding 10 mM 4-AP and Ca²⁺-independent glutamate release was monitored for 30 min in the presence of 50 μM FSC231. No statistically significant difference in 10 mM 4-AP-induced glutamate release was observed in 50 μM FSC231 (120.89% ± 10.28) treated synaptosomes, when compared to DMSO vehicle control (Figure 6.9A and B). In contrast, the positive control 10 μM rotenone (161.54% ± 37.28) treated synaptosomes demonstrated a trend increase of 61.54% in glutamate level, when compared to DMSO vehicle control synaptosomes, in agreement with previously published data (Kilbride et al., 2008). The data suggest that 50 μM FSC231 has no effect in the rate of Ca²⁺-independent 4-AP-stimulated glutamate release from rat synaptosomes. The same experiment was performed in the presence of 2 mM CaCl₂.
Figure 6.8: Effect of FSC231 on KCl-induced glutamate release in the presence of CaCl₂

The rate of glutamate release was measured by NADPH production at 460 nm. The level of glutamate was determined by generating a standard curve of glutamate-mediated NADPH production (Figure 4.9), in the absence of synaptosomes. The blank rate (BR) of glutamate release was recorded for 5 min in freshly extracted synaptosomes in the presence of CaCl₂. Then, synaptosomes were treated with 10 μM rotenone and 50 μM FSC231 (FSC); and the rate of glutamate at baseline (BL) was observed from non-depolarized synaptosomes for 5 min. Then, depolarization of synaptosomes was initiated by addition of 40 mM KCl and rate of glutamate release was observed for next 30 min. A) The graph indicates representative traces of Ca²⁺-dependent rate of glutamate release (nmol) by DMSO vehicle control (Cont), 10 μM rotenone (Rot) and 50 μM FSC231 treated synaptosomes in a total time period of 40 min. The bar diagrams show the total glutamate release as a percentage of control (mean ± SEM) under non-depolarized (B) and depolarized (C) conditions in three separate experiments (n=3). The p-value (*) signifies p<0.05.
Figure 6.9: Effect of FSC231 on 4-AP-induced glutamate release in the absence of CaCl$_2$

The rate of glutamate release was measured by NADPH production at 460 nm. The level of glutamate was determined by generating a standard curve of glutamate-mediated NADPH production (Figure 4.9), in the absence of synaptosomes. The blank rate (BR) of glutamate release was recorded for 5 min in freshly extracted synaptosomes in the absence of CaCl$_2$. Then, synaptosomes were treated with 10 μM rotenone and 50 μM FSC231 (FSC); and the rate of glutamate at baseline (BL) was measured from non-depolarized synaptosomes for 5 min. Then, depolarization of synaptosomes was initiated by addition of 10 mM 4-AP and rate of glutamate release was observed for the next 30 min. A) The graph indicates representative traces of Ca$^{2+}$-independent rate of glutamate release (nmol) by DMSO vehicle control, 10 μM rotenone and 50 μM FSC231 treated synaptosomes in a total time period of 40 min. B) The bar diagram shows the total glutamate release as a percentage of control (mean ± SEM) in DMSO vehicle control (Cont), 10 μM rotenone (Rot) and 50 μM FSC231 treated synaptosomes under depolarized condition in three separate experiments (n=3).
Synaptosomes were depolarized using 10 mM 4-AP to observe the effect of 50 μM FSC231 in Ca²⁺-dependent glutamate release. Similar to previous results, no significant change in the rate of Ca²⁺-dependent, 4-AP-induced glutamate release was observed in synaptosomes treated with 50 μM FSC231 (120.61% ± 9.47) compared to DMSO vehicle control (Figure 6.10A and B). In contrast, the positive control 10 μM of rotenone (160.54% ± 28.56) induced a trend increase of 60.54% in glutamate level, when compared to DMSO vehicle control. Taken together, the data suggest that 50 μM FSC231 has no effect on the rate of Ca²⁺-dependent, 4-AP-stimulated synaptosomal glutamate release. Overall, the data suggest that 50 μM FSC231 has no effect in the rate of Ca²⁺-dependent 4-AP-stimulated glutamate release from rat synaptosomes.
The rate of glutamate release was measured by NADPH production at 460 nm. The level of glutamate was determined by generating a standard curve of glutamate-mediated NADPH production (Figure 4.9), in the absence of synaptosomes. The blank rate (BR) of glutamate release was recorded for 5 min in freshly extracted synaptosomes in the presence of CaCl$_2$. Then, synaptosomes were treated with 10 μM rotenone and 50 μM FSC231 (FSC); and the rate of glutamate at baseline (BL) was observed from non-depolarized synaptosomes for 5 min. Then, depolarization of synaptosomes was initiated by addition of 10 mM 4-AP and rate of glutamate release was observed for the next 30 min. A) The graph indicates representative traces of Ca$^{2+}$-dependent rate of glutamate release (nmol) by DMSO vehicle control, 10 μM rotenone and 50 μM FSC231 treated synaptosomes in a total time period of 40 min. B) The bar diagram shows the total glutamate release as a percentage of control (mean ± SEM) in DMSO vehicle control (Cont), 10 μM rotenone (Rot) and 50 μM FSC231 treated synaptosomes under depolarized condition in three separate experiments (n=3).
Discussion

1. Inhibition of PICK1 by FSC231 induces mitochondrial depolarization

The purpose of this project was to evaluate the importance of PICK1 and the proteins that interact with PICK1 via PDZ domain in mitochondrial function and glutamate release (please see summary Table 1.1). In this chapter, a PDZ domain binding compound FSC231 was tested on mitochondrial function and glutamate release. This compound binds the PDZ domain of PICK1 and likely bars most of the PICK1 interacting proteins docking into its PDZ domain.

Previous work in this thesis identified a reduction of mitochondrial membrane potential in the presence of 100 μM Tat-MNP201, a peptide modelled on the PDZ motif of parkin. Similar to the MNP201 and Tat-MNP201 study, the effect of FSC231 was evaluated on the activity of respiratory chain; complex I and IV. In PD patients, 30-40% of reduction in activity of respiratory chain complex I was found (Schapira et al., 1989). FSC231 showed no significant effect on the activity of complex I and IV. In the ROS production assay, 10 μM antimycin increased the rate in ROS release level as expected (Sipos et al., 2003), whereas 50 μM FSC231 had no significant effect, when compared to vehicle control. When tested in the mitochondrial depolarization assay, FCCP-induced an increase in depolarization of mitochondrial membrane as expected (Tretter and Adam-Vizi, 2007). However, no change in mitochondrial membrane potential was observed in 50 μM FSC231 treated samples when compared to the vehicle control. Importantly, when synaptosomes was treated with the partial mitochondrial stress agent, FCCP (20 nM), the FSC231 treated synaptosomes showed a greater extent of mitochondrial depolarization compared to controls. We propose that in the presence of FSC231, the PDZ domain of PICK1 is unable to interact with and traffic proteins responsible for maintaining mitochondrial function, namely parkin and PKCa. It is also believed that the trafficking of these mitochondrial interacting proteins is crucial, when mitochondria are under stress.

2. PICK1 plays no role in glutamate release

As discussed earlier, AMPA receptor plays a role in fast excitatory neurotransmission (Dev et al., 1999). Intriguingly, PICK1 regulates the AMPA receptor excitability by regulating the surface expression of the AMPA receptor subunit GluR2 in hippocampal neurons (Hanley and Henley, 2005; Lin and Huganir, 2007). In the presence of FSC231, upon NMDA receptor
### A)

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<td>Complex I activity</td>
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<td>Complex IV activity</td>
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<td>Cyto C depletion (OD&lt;sup&gt;550&lt;/sup&gt;)</td>
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<td>Peroxide production</td>
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<td>Resorufin production (OD&lt;sup&gt;585&lt;/sup&gt;)</td>
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<td>Membrane potential</td>
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<td>JC-1 OD ratio (JC&lt;sub&gt;-1&lt;/sub&gt; 590/535)</td>
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<td>FCCP-induced membrane potential</td>
<td>67.05% ± 7.23</td>
<td>JC-1 OD ratio (JC&lt;sub&gt;-1&lt;/sub&gt; 590/535)</td>
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### B)

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<td>Non-depolarized level (- CaCl&lt;sub&gt;2&lt;/sub&gt;)</td>
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<td>Amount of NADPH production (OD&lt;sup&gt;460&lt;/sup&gt;)</td>
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<tr>
<td>Non-depolarized level (+ CaCl&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>157.37% ± 21.57</td>
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**Table 6.1: Summary of data obtained by using FSC231**

Mitochondrial function assays (A) and glutamate release assay (B). The data suggest that FSC231 has no effect on mitochondrial respiratory chain complexes (complex I and IV), ROS production, mitochondrial membrane potential and glutamate release. In contrast, FSC231 effects the mitochondrial membrane potential under stress condition. The values are indicated as a percentage of control (Mean ± SEM).
activation, accelerated recycling of GluR2 to the plasma membrane of hippocampal neurons was observed instead of internalisation through a PICK1-dependent trafficking (Thorsen et al., 2010). Moreover, LTD and LTP were significantly reduced in the presence of 50 μM FSC231 in CA1 hippocampal neurons (Thorsen et al., 2010). Importantly, in addition to the interaction with AMPA receptor subunits (GluR2), PICK1 also interacts with KA subunits (GluR5) and metabotropic glutamate receptors (mGluR7). Also worthy of note, is that AMPA, KA and mGluR7 pre-synaptic autoreceptors have all been shown to regulate glutamate release. Therefore, to determine if PICK1 plays a role in glutamate release either via effects on pre-synaptic glutamate receptors or via regulating mitochondrial function, the effect of FSC231 in glutamate release was studied using fresh rat brain synaptosomes.

Similar to MNP201 and Tat-MNP201, Ca^{2+}-independent and dependent glutamate release experiments were performed by treating 50 μM FSC231 with freshly prepared synaptosomes under non-depolarizing condition as well as in depolarizing condition (triggered by 40 mM KCl and 10 mM 4-AP). As a control, the data demonstrated an increased Ca^{2+}-independent and dependent rate of glutamate release from 10 μM rotenone treated synaptosomes under non-depolarizing and depolarizing conditions. This data is in agreement with previous studies, where a high amount of glutamate release was demonstrated by rotenone-induced complex I inhibition (Kilbride et al., 2008), indicating a successful glutamate assay setup. However, using 50 μM of FSC231 no significant difference was observed in glutamate release, when compared to control sample. Taken together, the lack of effects of FSC231 on glutamate release suggests that P1CK1 does not regulate the rate of glutamate release from synaptosomes.

3. The role of parkin-PICK1 and PKCα-PICK1 in mitochondria

Several proteins interact with PICK1 through the PDZ domain. Among these proteins, only parkin and PKCα have been reported to have mitochondrial "protective functions". Briefly, PICK1 interacts with and recruits PKCα to the outer mitochondrial membrane to confer mitochondrial resistance to Bax-induced apoptosis by phosphorylating Bcl-2 protein (Wang et al., 2007). In addition, parkin selectively engulfs and promotes autophagy of depolarized mitochondria (Narendra et al., 2008) where PICK1 may play a role. FSC231 docks into the PDZ domain of PICK1 and likely competitively hinders interaction with many proteins, most likely in a competitive and non-selective manner. To date, FSC231 has been shown to block interaction of PICK1 with GluR2 and DAT (Thorsen et al., 2010). Thus, the FSC231 is also expected to block the interaction of parkin-PICK1 and/or PKCα-PICK1, both having known mitochondrial function. If FSC231 is capable of blocking both parkin-PICK1 and PKCα-PICK1 interaction, then two possible deleterious effects on mitochondrial function are
expected. Firstly, under FCCP stress condition, the parkin-mediated removal of depolarized mitochondria may be attenuated in the presence of FSC231. Secondly, the blockade of PKCα-PICK1 interaction induced by FSC231 may limit the PKCα-induced anti-apoptotic mechanism. Further studies on FSC231 inhibition of parkin-PICK1 and PKCα-PICK1 are required to confirm this hypothesis through biochemical approaches. In addition, the role of these protein interactions should be evaluated by imaging and localisation studies.
7. Final discussion
7.1 Summary of findings

The data presented in this thesis were aimed to examine the role of PICK1, in particular the parkin-PICK1 interaction, in mitochondrial function. Firstly, biochemical methods were used to demonstrate the binding of the parkin peptide, MNP201, to PICK1. Specifically, it was shown that MNP201 displaced a fluorescently tagged PDZ motif (modelled on the PDZ motif sequence of DAT) from PICK1 in a concentration (and likely a competitive) manner (Result 1). Next, the effects of the MNP201 parkin peptide were tested in a set of mitochondrial functional assays and on glutamate release. This peptide showed little or no effects on mitochondrial function or on glutamate release, likely due to this peptide's inability to internalise synaptosomes (Result 2). In support of this suggestion, the Tat-MNP201, a membrane permeable version of MNP201 peptide, caused mitochondrial depolarization in synaptosomes in both control and FCCP-induced partial stress conditions (Result 3). Lastly, FSC231, the PDZ domain binding ligand of PICK1, demonstrated an increased rate of mitochondrial depolarization in synaptosomes under FCCP-induced partial stress conditions (Result 4). Taken together, the data indicate that PICK1 may play a role in the maintenance of mitochondrial membrane potential and thus mitochondrial function by trafficking PDZ domain interacting proteins to mitochondria including parkin and/or PKCa.

7.2 The parkin-PICK1 interaction

As indicated above, the primary objective of this project was to investigate the possible functional role of the parkin-PICK1 interaction in mitochondrial maintenance and glutamate release. The work extends the finding of Joch et al. (2007), where the group discovered an interaction between the PDZ motif of parkin and the PDZ domain of PICK1. The mode of interaction between these two proteins was found to be PDZ based, where the PDZ motif of parkin (-FDV) was found to interact with the PDZ domain of PICK1. In this work, the authors showed that the parkin-PICK1 interaction was important for parkin-dependent monoubiquitination of PICK1, which suppressed a PICK1-mediated increase in ASIC2a currents. In addition, this study also showed that the parkin-dependent ubiquitination of PICK1 did not lead to degradation of PICK1, that is, parkin caused monoubiquitination but not polyubiquitination of PICK1. It is interesting that PICK1 has the ability to bind both kinases (PKCa) and ubiquitin ligases (parkin). Given that PICK1 can homodimerise and thus form a scaffold that can bring together two separate proteins for interaction, it is interesting to speculate that PICK1 plays a role in regulating the phosphorylation and/or ubiquitination of its interacting proteins. These post-translational modifications are likely important in the trafficking and/or degradation of PICK1 interacting proteins. Although less is known about parkin-mediated ubiquitination of PICK1 interacting proteins, several studies have reported
that PICK1 plays a role in PKCa-dependent phosphorylation of its interacting proteins, the best studied being the interaction between PICK1 and AMPA receptors (Dev et al., 1999; Xia et al., 1999; Lin and Huganir, 2007).

7.3 Oligopeptides need assistance to internalise through bi-lipid membrane

The parkin PDZ motif containing peptides used in this study (MNP201 and Tat-MNP201) were designed to bind the PDZ domain of PICK1. The data showed that MNP201 had no effect on mitochondrial function and glutamate release, which was possibly due to the membrane impermeable nature of this peptide. In agreement with this notion, it is reported that lipophilic, bio-active peptides under 6 amino acids in length can, in general, cross the plasma membrane unassisted and enter the cell, whereas larger peptides are unable to cross lipid bilayers (Scheld et al., 1997). Peptides, which can also pass through the plasma membrane are those with arginine rich, highly cationic and basic amino acid sequences (Vives, 2003). The MNP201 peptide is 10 amino acids long (VCMGDHWFDV) with a slight acidic nature containing only one basic amino acid (Histidine) and unlikely to cross the synaptosomal membrane unassisted. Thus, a Tat peptide (YGRKKRRQRRR) sequence was fused to the N-terminal of MNP201 (Tat-MNP201) to assist its membrane permeability. The membrane permeability of Tat-MNP201 was demonstrated by its ability to enter cortical neuronal cells and astrocytes in culture. More importantly, while MNP201 did not affect the mitochondrial membrane potential, the Tat-MNP201 induced a significant amount of mitochondrial depolarization. The data suggested that presence of a Trojan Tat sequence in Tat-MNP201 assists the membrane permeability of the peptide and that its ability to bind PICK1 alters mitochondrial function.

7.4 Possible effect of Tat portion of Tat-MNP201

Tat peptides have been reported to induce ROS production, promote mitochondrial depolarization, increase glutamate release, decrease GABA exocytosis, disrupt calcium homeostasis and cause caspase activation (Kruman et al., 1998). The experiments conducted with Tat-MNP201 showed this peptide caused a selective mitochondrial membrane depolarization, with little or no effect on any of the other mitochondrial properties tested or on glutamate release. Thus, the data suggested that the Tat sequence used in this study had no overt effects on synaptosomal or mitochondrial integrity. Specifically, the Tat-MNP201 (100 μM) significantly increased mitochondrial depolarization in synaptosomes (p<0.001) compared to vehicle control. Surprisingly, however, Tat-MNP201(mut) (100 μM),
the mutated version of Tat-MNP201, also caused a significant level of mitochondrial depolarization (p<0.05) compared to vehicle control. When the mitochondrial membrane potential values of Tat-MNP201 and Tat-MNP201(mut) were compared, the Tat-MNP201 wildtype version was found to cause a significantly greater (p<0.05) amount of mitochondrial membrane depolarization when compared to the mutant version Tat-MNP201(mut). In addition to testing the effects of these parkin peptides in control conditions, their effects on mitochondrial membrane potential was also examined under mild stressed conditions induced by FCCP. In these conditions, Tat-MNP201 caused a significant (p<0.001) increase in mitochondrial membrane depolarization compared to the control. In this case Tat-MNP201(mut) also showed a significant (p<0.05) increase in mitochondrial membrane depolarization when compared to control values. Importantly, however, Tat-MNP201 caused a significantly larger membrane depolarization (p<0.05) when compared to Tat-MNP201(mut). As indicated above, specific effects of the Tat-MNP201 peptide on mitochondrial membrane potential were observed, with little effects on peroxide production and glutamate release suggesting no overt effects on mitochondrial or synaptosomal integrity. That being said, however, the data above suggests the effects of Tat-MNP201(mut) on mitochondrial membrane depolarization may be due to the presence of Tat peptide, which may be inducing mild stress conditions only observable in mitochondrial membrane potential assays and not in assays investigating peroxide production or glutamate release. While, in this study, we did not use peptide entrapment methods to facilitate the entry of peptides into synaptosomes as they do not allow for the addition of peptides at specific timepoints in an experiment, in this case such peptide entrapment methods would be useful future studies to perform in order to examine the effect of MNP201 without a Tat sequence (Feligioni et al., 2009).

7.5 Proposed model of FSC231 on mitochondrial dysfunction

In the experiments conducted in this thesis, 50 μM FSC231 increased mitochondrial membrane depolarization under mild stress conditions induced by FCCP. The mechanism underlying this FSC231-mediated mitochondrial membrane depolarization likely involves its binding to PICK1. In agreement, it has been shown that FSC231 binds the PDZ domain of PICK1 and competitively blocks the interaction of PDZ motif containing proteins including GluR2 and DAT, which otherwise interact with PICK1 (Joch et al., 2007). Due to this compounds ability to dock within the PDZ domain of PICK1, it likely occludes (in a competitive manner) the binding of many more or all proteins that interact with PICK1. While PICK1 interacts with close to 60 proteins, only two of its interacting proteins, namely parkin and PKCa, have been reported to be directly involved in regulating mitochondrial function. In
particular, parkin regulates mitochondrial function by selectively engulfing and promoting autophagy of depolarized mitochondria (Narendra et al., 2008). On the other hand, PKCα regulates mitochondrial function via a mechanism that involves PICK1-mediated recruitment of PKCα to the mitochondria and the phosphorylation of the Bcl-2 protein in the outer mitochondrial membrane, which confers mitochondrial resistance to Bax-induced apoptosis (Wang et al., 2007). Taken together, we propose that FSC231 causes a reduction in mitochondrial membrane potential due to disruption of the parkin-PICK1 and/or the PKCα-PICK1 interaction(s). If that is the case, then FSC231 may have two possible deleterious effects on mitochondria (Summary figure). Firstly, under FCCP stressed conditions, in presence of FSC231, PICK1 will not be able to bind or recruit parkin to mitochondria and thus parkin mediated removal of depolarized mitochondria will not occur. Secondly, due to blockade of PKCα-PICK1 interaction induced by FSC231, the PKCα-induced anti-apoptotic mechanism will be attenuated. Ultimately, due to the absence of parkin- and/or PKCα-dependent mitochondrial maintenance/removal mechanisms, instead dysfunctional mitochondria (i.e. depolarised mitochondria) will ensue, which will induce mitochondria-mediated apoptosis and neurodegeneration.

7.6 Caveat of this study

One obvious caveat of this current work was our inability to prove that the MNP201 peptides and FSC231 do indeed directly block the parkin-PICK1 interaction. During the course of this thesis, several methods were attempted to firstly, confirm the interaction between parkin-PICK1 and then demonstrate effect of the peptides (Result 1). Co-IP and pull-down studies failed to show a direct interaction between parkin and PICK1; and thus the blocking effect of MNP201 peptides on parkin-PICK1 interaction could not be demonstrated. However, the binding of MNP201 peptides to PICK1 was demonstrated by fluorescence polarisation assay, where MNP201 competitively inhibited the interaction of DAT peptide with PICK1. In contrast, MNP201(mut), the mutated PDZ motif version of parkin peptide, did not block the DAT-PICK1 interaction. In addition, given that the Tat-MNP201 peptide showed similar effects on the FSC231 compound, it is hypothesised that both these molecules are acting in a similar manner by binding to the PDZ domain of PICK1. Another possible caveat of this study was the use of synaptosomes extracted from whole rat brain. Most of the work demonstrating regulation of LTP and LTD by PICK1 has been done in hippocampal neurons (Hanley and Henley, 2005; Lin and Huganir, 2007). Therefore, future studies investigating the role of PICK1 in mitochondria prepared from specific brain areas including the hippocampus, rather than the whole brain, may be worthwhile.
Summary figure: Involvement of parkin-PICK1 to regulate mitochondrial function and apoptosis

PICK1 may play key role in mitochondrial function and neuronal survival by interacting with both parkin and PKCa. PICK1 regulates the trafficking of parkin and PKCa and that the parkin-PICK1-PKCa triple protein complex plays in mitochondrial maintenance. This pathway is blocked in the presence of MNP201 and FAC231.
7.7 Future direction

From the data generated in this project, Tat-MNP201 and FSC231, both induced depolarization of mitochondrial membrane potential in control and FCCP-induced stress conditions. Further work and follow-up studies are proposed below:

As indicated above,

- First, it would be worthy to examine the ability of MNP201 and FSC231 to block parkin-PICK1 and/or PKCα-PICK1 interaction in additional biochemical approaches.
- Second, peptide entrapment methods to facilitate the entry of peptides into synaptosomes would be useful future studies to perform in order to examine the effect of MNP201 without a Tat sequence.
- Third, future studies investigating the role of PICK1 in mitochondria prepared from specific brain areas including the hippocampus, rather than the whole brain, may be worthwhile.

In addition,

- Fourth, using immunostaining and cell imaging, future work investigating the role of PICK1 in trafficking parkin and PKCα to mitochondria, under conditions of mitochondrial stress and mitochondrial depolarization in the presence of Tat-MNP201 and FSC231 would be valuable.
- Fifth, parkin trafficking can be also evaluated in neurons derived from PICK1-KO mice (Gardner et al., 2005) under mitochondrial depolarization stress. In this case, impairment of parkin-mediated mitophagy and/or PKCα-mediated phosphorylation of the Bcl-2 would be expected. If the effects of MNP201 and FSC231 on mitochondrial membrane depolarization are mediated via binding to PICK1, the effects of these compounds should also be lost in mitochondria prepared from PICK1-KO animals.
- Sixth, if MNP201 and FSC231 do impair mitochondrial function under conditions of stress, their effects on triggering apoptosis and on neuronal viability should be demonstrated. This particular study is important for understanding the mechanism-of-action, for not only MNP201 or FSC231, but also other peptides that have been reported to bind PICK1 and cause effect on synaptic function.
- Lastly, their exist bias in the area which suggests that peptides that dock to the PDZ domain of PICK1 (such as GluR2 peptide, MNP201, FSC231) are selective in the interactions that they inhibit. This however is unlikely to be the case. Thus, the identification of the PICK1 interacting proteins, which are blocked by previously
reported GluR2 peptides, the MNP201 peptide and the FSC231 compound may provide us a more complete picture in understanding the role of PICK1 in mitochondrial function, neuronal survival and synaptic plasticity.

7.8 Closing comments

The family of PDZ domain containing proteins have been recently noted as novel drug targets for development of new therapies. The work here, however, suggests that PICK1 binding compounds are unlikely to make good drugs due to their effects on mitochondrial function. Further work will be required to develop drugs that selectively block one protein interacting with PICK1 over another. If efforts can develop, for example, a drug that regulates GluR2 (but not parkin) from interacting with PICK1, then such molecules could be useful in regulating synaptic plasticity and help in the development of cognition enhancers. In the case of using PICK1 as a target for Parkinson's disease this, however, appears unlikely given its role in mitochondrial function.
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