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**IDENTIFICATION OF NOVEL PROTEINS
REGULATING PAEL-RECEPTOR
FUNCTION**



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

By
Priyanka Dutta
October 2011

Under the supervision of Prof. Kumlesh K Dev
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Thesis 9504

Declaration

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Priyanka Dutta

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Abbreviations

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
4-PBA (sodium 4-phenyl-butyrate)
Adeno associated viral vector serotype 2 (AAV2)
ADP-ribosylation factors (ARFs)
Arfaptin homology domain (AHD)
Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)
Autosomal dominant (AD-PD)
Autosomal recessive (AR-PD)
Autosomal juvenile parkinsonism (AR-JP)
Acid sensing ion channel (ASIC)
Activation domain (AD)
AMPA binding protein (ABP)
Autophagy-related protein (Atg)
Basic local alignment search tool (BLAST)
Bin/amphiphysin/RVS (BAR) domain
Binding domain (BD)
Calcium calmodulin dependent kinase II (CaMK)
Carboxylate-binding domain (CBD)
Carboxylate terminal (ct)
Catechol O Methyltransferase (COMT)
Cell division control related protein (CDCrel-1)
Central nervous system (CNS)
Cjun NH2 terminal kinases (JNK)
DAT (Dopamine transporter)
Dimethyl sulfoxide (DMSO)
Dimethylformamide (DMF)
Endoplasmic reticulum (ER)
Endosomal recycling compartment (ERC)
ER associated protein degradation (ERAD)
Ethylene glycol tetraacetic acid (EGTA)
Ethylenediaminetetraacetic acid (EDTA)
Endothelin-B receptor (ETBR)
G coupled protein receptor (GPCR)
GABARAP (γ -aminobutyrate type A (GABA_A) receptor associated protein) (GABARAP)
GABARAP like2 (GABARAPL2)
Gene adjacent to parkin (Glup)
GPR37-like 1 (GPR37L1)
Globus pallidus internal (GPI)
Globus pallidus external (GPe)
GATE-16 family (LGG)
Glucose regulated protein (GRP)
Glial cell derived neurotrophic factor (GDNF)
Glutamate receptor interacting protein (GRIP)
Glutamic acid decarboxylase (GAD)
Golgi associated ATPase enhancer of 16KDa (GATE-16)
GTPase activating proteins (GAP)
Guanine dissociation inhibitor (GDI)
GDI displacement factor (GDF)
Guanine nucleotide exchange factors (GEP)
Heat shock protein (Hsp70)
Human homology of yeast Hrd1p (HRD1)
Immunoprecipitation (IP)
In between RING fingers (IBR)
Inositol requirement 1 (IRE1)
Ionotropic glutamate receptors (iGlu-Rs)
Kainite (KA)
Knockout (KO)
Lewy bodies (LBs)
L-DOPA (L-3,4-dihydroxyphenylalanine)
Light chain3 (LC3-II)
Leucine-rich repeat kinase 2 (LRRK2)
Long term potentiation (LTP)
Long term depression (LTD)
Monoamine oxidase B (MAO-B)
Neuropeptide head activator (HA)
N,N-dimethylformamide (DMF)
N-ethylmaleimide sensitive fusion protein (NSF)
Neuropeptide head activator (HA)
N-methyl-D-aspartate (NMDA)
Nuclear receptor subfamily 4, group A, member 2 gene (NR4A2)
Parkin-associated endothelin receptor-like receptor (PAEL-R)
Parkinson's disease (PD)
Parkin coregulated gene (PACRG)
PCI (phenol/chloroform/isoamyl alcohol)
Phosphoinositide 3-kinase (PI3K)
Protein kinase C (PKC)
Protein interacting with C kinase (PICK1)
PSD95-Disc large ZO-1 (PDZ)
PTEN-induced putative kinase 1 (Pink1)
Polymerase chain reaction (PCR)
Ras-associated binding protein14 (RAB14)
Substantia nigra (SN)
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
Soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE)
U box protein ct of Hsp70 interacting protein (CHIP)
Ub carboxy-terminal hydrolase (UCH-L1)
Ub homology domain (UHD)
Ubiquitination (UB)
Ubiquitin (Ub)
Western blotting (WB)
X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside)
Yeast two hybrid (Y2H)

Abstract

Parkinson's disease (PD) is a chronic neurodegenerative disorder that causes a wide range of debilitating symptoms. The Parkin associated endothelin like receptor (PAEL-R), originally called GRP37, belongs to the family of orphan G coupled protein receptors (GPCRs). Under physiological conditions the E3 ligase, called parkin, ubiquitinates the unfolded PAEL-R to promote its degradation. When parkin is mutated in PD the PAEL-R aggregates in the endoplasmic reticulum (ER) inducing ER stress, which leads to neurotoxicity and cell death (**Chapter 1**). The aim of our project was to discover interacting proteins which control the trafficking and expression of PAEL-R. To study these mechanisms of PAEL-R, we have identified novel proteins that interact with these receptors using a yeast-two hybrid genome-wide technology and further validate these findings by biochemical and cellular studies. Results reveal three novel proteins interacting with the PAEL-R. These are (1) protein interacting with C kinase (PICK1) (**Chapter 3**), (2) γ -aminobutyrate type A receptor associated protein like 2 (GABARAPL2) (**Chapter 4**) and (3) ras-associated binding protein 14 (Rab14) (**Chapter 5**). Based on these novel findings we hope to better understand PAEL-R aggregation and trafficking for the treatment of PD.

“FOR EVERYTHING THIS DISEASE HAS TAKEN, SOMETHING WITH GREATER VALUE HAS BEEN GIVEN SOMETIMES JUST A MARKER THAT POINTS ME IN A NEW DIRECTION THAT I MIGHT NOT OTHERWISE HAVE TRAVELED. SO, SURE, IT MAY BE ONE STEP FORWARD AND TWO STEPS BACK, BUT AFTER A TIME WITH PARKINSON'S, I'VE LEARNED THAT WHAT IS IMPORTANT IS MAKING THAT ONE STEP COUNT; ALWAYS LOOKING UP”

MICHAEL J. FOX, ALWAYS LOOKING UP

CHAPTER 1

INTRODUCTION

Introduction

1. Parkinson's Disease (PD)

1.1 Physiology and symptoms of PD

PD is the second most common neurodegenerative disease, with an incidence of between 1 and 2% in the over-65 and 4% in the over 80 population (Lau and Breteler, 2006). Statistics released from a global study carried out by the world health organization (WHO) suggest that there are approximately 6 million people worldwide affected (0.3% of the total population) with PD (Lang and Lozano, 1998). Understanding the basis of PD and the involved pathways and proteins are thus a particularly pressing issue.

PD was first described in 'An essay on the shaking Palasy' published in 1817 by a London physician named James Parkinson (Neylan, 2002). Prior to this PD was referred to as *paralysis agitans*, with the term Parkinson's disease being coined in 1961 by Jean-Martin Charcot. PD is a chronic, progressive neurodegenerative disorder characterised by the continued loss of dopaminergic neurons in the brain, particularly in the pars compacta region of the substantia nigra (SN). As the disease progresses there is gradual degeneration within the nigrostriatal pathway, producing motor, cognitive and psychiatric symptoms (Braak et al., 2003). Before the first PD symptoms become apparent, including resting tremor, rigidity and bradykinesia, generally 80-85% of SN neurons and 60-80% of striatal dopaminergic neurons are already lost (Isacson, 2002). Cholinergic neurons are also lost during disease progression, giving rise to dysphasia, sleep disturbances and instability. Some of the ventral tegmental area neurons which supply dopamine to the limbic system, leads to mood and sleep disorder (Schnabel, 2010). Other affected neurotransmission include nonadrenergic and serotonergic which give rise to depression (Khan et al., 2004). Also affected are non-dopaminergic neurons in the olfactory bulb and intestine (Schnabel, 2010). A few PD patients may also show symptoms such as diminished sense of smell, restless leg syndrome, anxiety, hallucinations, fatigue, impotence, visual problems and, in later stages, a form of dementia (Schnabel, 2010).

Major symptoms of PD include, resting tremor and bradykinesia. The basal ganglia is responsible for modulating movements via direct and indirect pathways. The

direct pathway facilitates movement while the indirect pathway inhibits movement. Within the direct pathway, striatal neurons from terminals of neurons in the substantia nigra par compacta releases dopamine, which causes excitation by activation of dopamine D1 receptors that signal to the striatum. Excitation of the striatum causes a transient inhibitory signal being sent to the globus pallidus internal, preventing inhibition of the thalamus. This results in stimulation of the cortex, thus facilitating movement (DeLong and Wickmann, 2007). In the indirect pathway, action of dopamine on striatal neurons from terminals of neurons in the substantia nigra par compacta results in inhibition via D2 receptors. This pathway inhibits the striatum, which in turn prevents inhibition of the globus pallidus external. This causes the subthalamic nucleus to be disinhibited, stimulating the globus pallidus internal, which inhibits the thalamus and thus inhibits movement (DeLong and Wickmann, 2007) (**Fig 1**). In PD, loss of dopamine release results in hyperactivity of the motor inhibitory D2 indirect pathway and decreased activity in the D1 direct pathway, which results in inhibition of voluntary movement (DeLong and Wickmann, 2007).

1.2 Therapies: Current and future

In 1967 levodopa (L-DOPA (L-3, 4-dihydroxyphenylalanine) was discovered, a precursor to the neurotransmitter dopamine and it has since been used in the clinical management of PD (Kubo et al., 2006) (**Fig 2**). To enhance the effect of levodopa it may be given together with a DOPA decarboxylase inhibitor (carbidopa or benserazide) and with COMT (Catechol O Methyltransferase) inhibitors like Entacapone or Tasmar (Antonini et al., 2008). Long term treatment with levodopa is associated with adverse effects, such as motor fluctuations and motor-dyskinesias (Kubo et al., 2006). Therefore dopamine agonists like Pramipexole, Ropinirole and Bromocriptine have additionally been developed to treat these side effects (Lledo, 2000). In addition, monoamine oxidase B inhibitors like Selegiline and Rasagiline are used to inhibit the action of the enzymes responsible for the breakdown of dopamine (Sandler, 2009). Other recently developed molecules which are in clinical trials include anti-apoptotic agents (TCH346, CEP-1347), glutamate antagonists, promitochondrial drugs (co-enzyme Q10, creatine), calcium channel blockers (Isradipine) and growth factors (glial cell derived neurotrophic factor (GDNF) (Obeso et al., 2010).

More recently, gene therapy using adeno associated viral vector serotype 2 (AAV2) to deliver glutamic acid decarboxylase (GAD) into the subthalamic nucleus has been used. Briefly, GAD catalyses the production of the neurotransmitter gamma-aminobutyric acid (GABA) which acts as a direct inhibitor on the overactive cells in subthalamic nucleus (Feigin et al., 2007). Another approach uses AAV2 to deliver aromatic amino acid decarboxylase, the enzyme that converts levedopa to dopamine (Kaplitt et al., 2007). The third approach involves delivery of neurturin, a functional analog of glial cell derived neurotrophic factor, to provide neuroprotective benefits (Obeso et al., 2010). Neurturin is involved in neuroprotection and upregulation of dopamine function as shown in a variety of rodent studies (Gasmi et al., 2007). Another tool used to treat non-motor symptoms is called deep brain stimulation (DBS). In this procedure electrodes are surgically inserted in the subthalamic nucleus or the internal globus pallidus which is part of basal ganglia to stimulate brain areas to back into action (Smith, 2010). Transcranial magnetic stimulation (TMS) is also thought to be of use in the treatment of PD. In TMS, an electromagnetic coil is held outside the skull which creates pulses that excite neurons in the target area of the brain, increasing their activity (Smith, 2010). Finally, pluripotent stem cells are thought to be of value in PD. Here the skin cells from patients are used, reprogrammed into dopamergic neurons and then re-transplanted into patients (Smith, 2010).

1.3 Forms and cause of PD

PD can be classified into sporadic and familial forms (Chase, 1997). To date studies indicate approximately 5-10% of cases as being familial and 90-95% as sporadic (Tomiya et al., 2008). Familial forms of PD are understood to be due to genetic factors that increase the risk of developing PD. The environmental causes are associated with factors such as toxins, free radicals and pesticide exposure (Cummings, 1999), whereas smoking and coffee drinking appears to lower the risk of PD (Jim et al., 2010). Exposure to environmental factors leads to lipid peroxidation, and mitochondrial dysfunction (Jenner, 2001). The molecule 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is mainly taken up by SN dopamine neurons (Jose et al., 2010) and lowers mitochondrial energy output. MPTP also causes mitochondria to overproduce hydrogen peroxide (Schnabel, 2010) and results in oxidative stress via production of oxygen species such as superoxide anions and hydroxyl radicals (Mizuno et al., 1998).

1.4 Genes involved in PD

To date, at least nine distinct genetic loci have been recognised to be linked to PD (PARK1-3, 5-8, 10 and 11) (Kubo et al., 2006). Among the PD-associated loci, mutations have been identified in six genes namely α -synuclein (PARK1), Ub carboxy-terminal hydrolase (UCH-L1) (PARK5), parkin (PARK2), DJ-1 (PARK7), PTEN-induced putative kinase 1 (PINK1) (PARK6) and Leucine-rich repeat kinase 2 (LRRK2) (PARK8) (**Fig 3**). Familial PD can be divided in two forms named autosomal dominant (AD-PD) and autosomal recessive (AR-PD).

1.4.1 Autosomal dominant genes included in PD

AD-PD is recognised by the formation of intracellular proteinaceous lewy bodies (LBs) (Shimura, 2000), which are a pathological hallmark of PD. LBs are intracytoplasmic inclusions composed of lipids, neurofilaments, α -synuclein, syphilin-1, ubiquitin and ubiquitin related enzymes (Chung et al., 2001a). However, the role of LBs and their significance in PD remains under investigation. Typically LB pathology is linked with mutations in three genes: α -synuclein, LRRK2 and UCH-L1 (Obeso et al., 2010).

Briefly, the α -synuclein protein has been identified as a major component of LBs and has a tendency to self-aggregate (Di et al., 2002). It has been suggested that α -synuclein forms small soluble oligomers, insoluble stacks or protofibrils. Therefore α -synuclein aggregation could cause neuronal toxicity in PD (Schnabel, 2010). Mutations in α -synuclein are linked to AD-PD (Thomas et al., 1997, Dev et al., 2003). α -synuclein is a pre-synaptic protein thought to be involved in synaptic plasticity, learning and memory (Liu et al., 2004). Two point mutations in α -synuclein have been discovered, namely, A30P and A53T. These mutated forms of α -synuclein are thought to represent major components of LBs and accumulate in neurons leading to α -synuclein toxicity and cell death (Li et al., 2001). The overexpression of α -synuclein in transgenic (tg) fruit flies and mice causes a Parkinsonian phenotype and replicates many of the pathological features of PD (Zang et al., 2000). Mutations in LRRK2 have been linked recently with AD-PD Parkinsonism. LRRK2 is extraordinarily large and complex, with multiple enzymatic and protein-interaction domains, each of which is targeted by pathogenic mutations in familial PD (Mata et al., 2006). LRRK2 interacts with the carboxyl terminal (ct) R2 RING finger domain of parkin and parkin interacts with the COR

domain of LRRK2. Finally, mutations in UCH-L1 have also been associated with AD-PD, which are involved in the ubiquitin-proteasomal pathway for protein degradation (Leroy et al., 1998).

1.4.2 Autosomal recessive genes included in PD

Of particular interest to our research is autosomal recessive juvenile Parkinsonism (AR-JP) which is characterised by a notably early onset including the lack of LBs. AR-JP is identified by the progressive reduction of dopamine levels due to degeneration of dopaminergic neurons in the SN (Matsumine et al., 2001). Clinical features of AR-JP also differ from idiopathic PD as patients show an excellent response to L-Dopa treatment and a slow disease progression with severe levodopa-induced dyskinesia (Kitao et al., 1998). The genes involved in AR-JP include parkin, DJ-1, PINK1 and ATP13A2 (Klein, 2005).

The function of parkin is discussed further in the next section. In summary, PINK1 is a mitochondrial protein kinase (Schnabel, 2010) which is functionally linked to parkin. In PINK1 null flies, parkin is recruited to dysfunctional mitochondria to promote their autophagic degradation (Narendra et al., 2009). DJ-1 mutations are the second most frequent identified genetic cause of PD after parkin (Nirit et al., 2006). Interestingly, immunohistochemistry staining for DJ-1 label's tau lesions, and is also in an aggregated form in other neurodegenerative diseases including Alzheimer's disease and Pick's disease which indicates that neurodegenerative diseases might share a common mechanism of protein aggregation. The function of DJ-1 is still unknown, however, it is associated with various cellular processes, including response to oxidative stress, cellular transformation, and androgen-receptor signaling (Nirit et al., 2006). Lastly, ATP13A2 (ATPase type 13A2), also known as PARK9, is an 1,180 amino acid membrane protein that belongs to the P5 sub-family of ATPases which play an important role in the transportation of inorganic cations (Schultheis et al., 2004, Ning et al., 2008). ATP13A2 functions to catalyse the conversion of ATP to ADP and a free phosphate, thereby participating in the active transport of ions across cellular membranes. Defects in the gene encoding ATP13A2 are the cause of Kufor-Rakeb syndrome, a rare hereditary type of PD that exhibits juvenile onset and is characterised by neurodegeneration and dementia (Hampshire et al., 2001).

2. Parkin

2.1 Structure and function of parkin

Initial studies in a Japanese family found that a large region of chromosome 6q25.2–q27 was linked to a rare form of AR-JP (Matsumine et al., 1997). In further studies a mutated gene was identified and named parkin (Kitada et al., 1998). The clinical manifestation of parkin associated with PD was an early disease onset before the age of 40 followed by a slow disease progression (Yamamura et al., 1973). Mutations in the parkin gene are a common cause of PD, especially in the case of a positive family history and an autosomal recessive mode of transmission (Lucking et al., 2000). A wide variety of parkin mutations have been found in PD patients, ranging from a deletion of a single nucleotide to several nucleotides, missense mutations and environmental factors which act in concert (Mata et al., 2004, West and Maidment, 2004).

Parkin is an ubiquitously expressed protein localised in the golgi complex and the cytoplasm (Kubo et al., 2006). The parkin gene is approximately 1.5 Mb in length and is comprised of twelve exons encoding a 465 amino acid protein with a molecular mass of 52 KDa (Kitada et al., 1998, Shimura et al., 1999). Parkin is implicated in the ubiquitin-proteasome system (UPS), where it functions as an E3 ligase enzyme. Mutations in the parkin gene as in the case of AR-JP patients, produce parkin with defective E3 ligase function (Imai et al., 2000, Shimura, 2000, Zhang et al., 2000). By acting as an E3 ubiquitin-protein ligase, parkin adds ubiquitin to its target proteins which include membrane bound receptors and cytosolic enzymes (Cookerson, 2003). Ubiquitination (UB) and protein degradation occurs in a three step process (**Fig 4A**). First ubiquitin (Ub) is activated by E1 (Ub activating enzyme), which is then attached to E2 (Ub conjugating enzyme) and finally Ub is transferred via E3 (Ub protein ligase) to the substrate (Imai et al., 2000, Shimura, 2000, Dev et al., 2003) (**Fig 4B**).

Parkin contains an amino terminal Ub homology domain that helps with the association of substrates with parkin. It also contains a central domain with unknown function. In addition parkin contains two RING fingers flanking a cysteine rich domain, termed in between RING finger (IBR) (Morett and Bork, 1999, Zhang et al., 2000) (**Fig 4B**). The ring box (RING-IBR-RING) plays a role in substrate recognition and binding (Jackson et al., 2000, Joazeiro and Weissman, 2000). Finally, parkin contains a PSD95-Disc large ZO-1 (PDZ) motif at the extreme c-

terminus (-XVDF) which is involved in the interaction with proteins that contain PDZ domains (Ponting et al., 1997, Joch et al., 2007). Parkin is shown to function as an ubiquitin ligase complex that includes the SCF complex (Skp1-Cullin-F-box-cdc-protein) (Kubo et al., 2006). Skp1 represents *Drosophila* protein Sina and its mammalian homolog Siah-1 which serves as a substrate for the complex Cullin-1 uses the E2 UbCH7 to ubiquitinate target proteins and Cdc4 is an F-box containing E3 ligase of this complex (Staropoli et al., 2003). Importantly, dysfunctional parkin leads to the accumulation of various substrates in the brains of patients suffering from PD.

2.2 Substrates of parkin

A number of putative substrates have been reported for parkin, including cell division control related protein (CDCrel-1) (Zhang et al., 2000), synphilin-1 (Chung et al., 2001b), a rare O-glycosylated form of α -synuclein (Shimura et al., 2001), parkin-associated endothelin receptor-like receptor (PAEL-R) (Imai et al., 2001), synaptotagmin XI (Huynh et al., 2003), cyclin E (Staropoli et al., 2003), the p38 subunit of the aminoacyl-tRNA synthetase complex (Corti et al., 2003), and α/β -tubulin (Ren Y, 2003, Takahashi, 2006) (**Fig 5**).

2.2.1 CRCrel-1

CDCrel-1 is a GTPase of 44 KDa protein and is also associated with membranes of a synaptic vesicle enriched septin. This protein is predominantly expressed in the presynaptic axon terminal of inhibitory neurons and can inhibit dopamine release (Dong et al., 2003). CDCrel-1 was the first reported parkin substrate. In a yeast two hybrid (Y2H) experiment the RING2 domain of parkin was shown to interact with CDCrel-1 (Dong et al., 2003). Interestingly parkin ubiquitinates and promotes degradation of CDCrel-1 but familial parkin mutants (Q311 stop and T415N) are defective in substrate degradation (Zhang et al., 2000). Septin proteins such as CDCrel-1 directly bind to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein syntaxin-1 and are thought to play a role in synaptic vesicles transport, in fusion and/or in recycling events. CDCrel-1 interacts with syntaxin-1 to regulate synaptic vesicle dynamics by reducing the secretion of human growth hormone. Parkin is associated with synaptic vesicles and thus possibly ubiquitinates CDCrel-1 which could regulate transmitter release. As ubiquitin occurs in the postsynaptic density and synaptic terminals, parkin can

mediate ubiquitination of CDCrel-1 at the synapse (Imai et al., 2000, Shimura, 2000, Dev et al., 2003). In PC12 cells, overexpression of CDCrel-1 by recombinant adeno associated viruses in the SN induces dopamine dependent neurodegeneration suggesting that inhibition of dopamine secretion by CDCrel-1 may contribute to the development of AR-JP (Dong et al., 2003).

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

In a Y2H screen, the parkin peptide bait excluding both the ubiquitin-like and most of the RING-IBR-RING domain was found to interact with the p38 subunit of the mammalian multiARS complex. Accumulation of the p38 subunit has been shown in brains of sporadic PD patients and its overexpression by an adenovirus resulted in selective neuronal death or accumulation in aggresome like inclusions (Corti et al., 2003). In case of PD, immunolabelling has been used to identify the p38 subunit in the core centre of LBs and is deregulated in patients with parkin mutations (Ko et al., 2005). This localisation of p38 on pre-existing LBs suggests that parkin mediated ubiquitination of p38 is an early event in LB formation. Ubiquitination of p38 is abrogated by truncated variants of parkin lacking essential functional domains, but interestingly not by pathogenic Lys161Asn point mutant. Parkin promotes the accumulation of p38 in ubiquitin-positive inclusions and prevented p38 induced cell death, suggesting that aggregation of p38 has a protective effect (Corti et al., 2003).

2.2.3 Synaptotagmin XI

Synaptotagmin XI belongs to the synaptotamins, a family of vesicle proteins that play a role in the docking and fusion of synaptic vesicles to the plasma membrane leading to transmitter release (Huynh et al., 2003). Its amino acid sequence is comprised of a single transmembrane region and two cytoplasmic C2 domain (C2A and C2B) (Glass et al., 2004). Synaptotamin XI has been localised to the secretory granules of neurotransmitters and plays a part in exocytosis stimulated by Ca^{2+} ions. Synaptogamin XI also downregulates fast presynaptic neurotransmission. In parkin deficient mice, inactivation of parkin causes ubiquitination failure of synaptogamin XI leading to a disturbed release of dopamine. Parkin interacts with synaptogamin XI at the C2A and C2B domains specifically at 204 and 293 residues, where there is a RING finger 1 motif, which is essential for ubiquitination (Huynh et al., 2003).

2.2.4 *α synuclein and synphilin-1*

α -synuclein is a 16 KDa neuronal phosphoprotein thought to be involved in synaptic vesicles transport. It has been reported that a novel 22 KDa O-glycosylated form of α -synuclein interacts with parkin and also the non-mutated forms R42P and T240R of parkin. In AR-JP patients, the lack of parkin-ubiquitin activity promotes α -synuclein aggregation and increases α -synuclein toxicity associated with dysfunction in mitochondria, synaptic vesicles and proteasome activity (Cookson, 2003). Parkin also appears to be localised in LB inclusions and the formation of LB-like inclusions is dependent on α -synuclein (Feany and Pallanck, 2003). In contrast, synphilin-1 is a α -synuclein binding protein and parkin ubiquitinates synphilin-1. Synphilin-1 is a protein of unknown function that contains a coiled-coil domain and ATP/GTP binding motif and has been shown to be associated with α -synuclein (Cheng et al., 2008). It has been reported that the ankyrin repeat domain of synphilin-1 can interact with the second RING domain of parkin and parkin ubiquitinates synphilin-1. Synphilin-1 is found in LBs but the role of its interaction with α -synuclein and ubiquitination by parkin is still unknown. Synphilin-1 may serve as a link for α -synuclein or parkin to intracellular proteins involved in vesicle transport and cytoskeleton formation (Cheng et al., 2008).

2.2.5 *Actin filament*

The association of parkin to actin filaments or microtubules has been examined in COS1 cells (Huynh et al., 2000). In this study, COS1 cells were incubated with cytochalasin D (a fungal product that blocks polymerization of actin filaments) and immunostained for parkin and phalloidin or treated with nocodazole (which inhibits polymerization of tubulin to form microtubules). Results indicate that parkin is associated with actin filaments but not microtubules in COS1 cells. However, recently parkin has been shown to interact with microtubules and tubulin α/β heterodimers (Ren et al., 2003). The interaction between parkin and α/β tubulin results in parkin mediated ubiquitination and enhanced degradation of α and β tubulin (Huynh et al., 2000).

2.2.6 *PARIS (ZNF746)*

PARIS is a member of the family of KRAB zinc-finger protein (KRAB-ZFPs)

transcriptional repressors (Looman et al., 2002) and is a transcriptional repressor of peroxisome proliferator-activated receptor gamma (PPAR γ) coactivator-1a (PGC-1a) expression. Parkin regulates the levels of PARIS via the ubiquitin-proteasome system. In sporadic PD, PARIS accumulates in the striatum and SN in parkin exon 7 knockout (KO) mice. The KO of parkin in adult mice leads to a progressive loss of dopamine neurons through PARIS overexpression and transcriptional repression of peroxisome proliferator-activated receptor-coactivator (PGC)-1a (Shin et al., 2011).

2.2.7 Protein interacting with C α -kinase (PICK1)

The function of PICK1 is discussed in detail in the next section. Briefly, the PDZ motif of parkin binds to the PDZ domain of PICK1 which is a scaffolding protein that interacts and trafficks many receptors (Joch et al., 2007). Parkin promotes PICK1 monoubiquitination rather than polyubiquitination. Thus parkin does not promote PICK1 degradation. However, parkin regulates the effects of PICK1 on one of its PDZ partners called the acid-sensing ion channel (ASIC) (Joch et al., 2007). ASIC is located at specialised nerve endings within the peripheral nervous system and also expressed in the brain. ASIC are involved in pain, mechnosensation and psychiatric diseases. Overexpression of parkin causes an increase in PICK1 monoubiquitination (Joch et al., 2007) and thereafter enhances ASIC activity.

2.2.8 Parkin KO mice: accumulation of substrates

Interestingly, most of the parkin KO mice do not develop any PD-like behavior (Von et al., 2004). Some of the KO mice show mild alterations of dopaminergic neurons and abnormalities in dopamine metabolism (Goldberg et al., 2003, Itier et al., 2003). None of the parkin substrates have been reported to accumulate in the brains of parkin KO mice (Joch et al., 2007). In contrast studies with *Drosophila* models of PD have shown mitochondrial defects and increased oxidative stress (Soriano and Paricio, 2011). In human, three parkin substrates namely, CDCrel-1, PAEL-R and α Sp22 have been reported to accumulate in brains of parkin associated PD patients (Kubo et al., 2006). Parkin has also been shown to be a neuroprotective agent against accumulation of cyclin E, dopamine mediated toxicity, kainite-induced excitotoxicity, ceramide-induced mitochondrial apoptosis and overexpression of PAEL-R (Kubo et al., 2006). We are particularly interested

in one of the substrates of parkin, specifically the parkin-associated endothelin-like receptor (PAEL-R).

3. PAEL receptor

3.1 Chromosomal mapping of PAEL-R in human and mouse

Receptors are classified into four families including ligand-gated ion channels, tyrosine-linked receptors, intracellular nuclear receptors and G-protein coupled receptor (GPCRs) (**Fig 6**). GPCRs contain seven transmembrane domains and regulate intracellular signaling by association with a transduction molecule called the GTP-binding protein (or G-protein). The PAEL-R (putative G protein-coupled receptor protein (GPR37)/parkin-associated endothelin-like receptor) is a GPCR.

PAEL-R was originally identified from a set of human brain cDNA expressed sequence tags (Marazziti et al., 1997). Following genomic nomenclature committee rules, this putative GPCR was termed GPR37 and the genomic sequence, organisation and assignment of chromosomal location of human GPR37 were subsequently identified (Marazziti et al., 1997). GPR37/PAEL-R is mapped on chromosome 7q31 in human and chromosome 6 in mouse (Marazziti et al., 1997, Marazziti et al., 1998). The human PAEL-R gene consists of a single open reading frame coding a 613 amino-acids protein and in the mouse an open reading frame coding of 600 amino acids. The mouse PAEL-R is 83% identical to the human gene with both containing seven putative hydrophobic transmembrane domains (**Fig 7**). They also contain a long 249 amino acid arginine and proline-rich extracellular domain. The human PAEL-R gene is approximately 25 Kb in length containing two exons and a single intron. In both PAEL-R genes a single intron disrupts the third transmembrane domain. The mouse PAEL-R is found in the oligodendrocytes of fibre tracts and neurons such as the SN dopaminergic neurons, hippocampal neurons in the CA3 region, and cerebellar purkinje cells (Marazziti et al., 2004). Northern blot analysis with human PAEL-R revealed two forms of mRNA, one of 3.8 Kb and another less abundant 8 Kb. Both the mRNA forms of PAEL-R are expressed in human brain tissues particularly in the corpus callosum, medulla, putamen, and caudate nucleus. The lowest level of expression is detected in the cerebellum. The 3.8 Kb mRNA of human PAEL-R is also expressed in the liver and placenta. Northern blot analysis of mouse PAEL-R is opposite to human PAEL-R, as the 3.8 Kb mRNA is less abundant compared to 8 Kb mRNA. Both mRNA forms of mouse PAEL-R are expressed primarily in the brain and some of the 3 Kb mRNA of mouse is also expressed in the testis (Marazziti et al., 1997, Marazziti et al., 1998).

3.2 PAEL-R family members

The PAEL-R sequence confirms a degree of homology of approximately 40% in the transmembrane regions with bombesin-BB1, and bombesin-BB2 receptors and 27% with endothelin-B receptor (ETBR) (Marazziti et al., 1997, Marazziti et al., 1998). Therefore PAEL-R is also called the endothelin-B receptor like protein (ETBR-LP). PAEL-R is most closely related to another CNS-enriched orphan receptor known as GPR37-like 1 (GPR37L1) (**Fig 8**). GPR37L1 also known as 'PAEL-R-like 1' or 'Endothelin B Receptor-like protein 2' is a GPCR. GPR37L1 is 481 amino acids in length and was identified from human cDNA (Valdenaire et al., 1998) with a 68% sequence homology and 48% identical to Endothelin B Receptor-like protein 1. GPR37L1 is strongly expressed in the cerebral cortex, internal capsule fibres and cerebellar Bergmann glia. In situ hybridisation of rat brain demonstrates broad distribution of GPR37 and GPR37L1 receptors throughout the central nervous system (CNS) (Leng et al., 1999). Alignment of PAEL-R sequence with GPR37L1 sequence shows moderate to high sequence homology, with relatively weak homology in the c-terminal. Interestingly both these receptors contain putative PDZ motifs at their extreme C-terminus. The PDZ motifs of PAEL-R (-GTHC) and GPR37L1 (-GTPC) are both typical suggesting similar interacting proteins may regulate the trafficking of these receptors. The GPCRs named GPCR/CNS1 and GPCR/CNS2 also show 25% identity with the endothelin receptors and the bombesin-like peptide receptors (**Fig 9**). Notably the c-terminus of these receptors are 80% identical. Both GPCR/CNS1 and GPCR/CNS2 are highly expressed in rat brain particularly throughout the CNS. GPCR/CNS1 is expressed in glial cells of the fibre tracts and GPCR/CNS2 is expressed in the gray matter.

3.3 PAEL-R is a substrate of Parkin

Full length parkin was used as bait in Y2H studies using human adult brain cDNA libraries and revealed an interaction between the C-terminal of PAEL-R and parkin (Takahashi and Imai, 2003). The interaction of PAEL-R with parkin was confirmed by transfection of human embryonic kidney293 cells (HEK293 cells) with haemagglutinin (HA) tagged PAEL-R and flag tagged parkin. Immunoprecipitation (IP) with an anti Flag mAb identified an interaction between parkin and PAEL-R. To confirm the endogenous interaction of parkin with PAEL-R, human brain or neuroblastoma SH-SY5Y cells were lysed and the supernatant fractions were

immunoprecipitated with anti parkin polyclonal Abs. The co-precipitated PAEL-R was detected by Western blotting (WB) using anti PAEL-R mAbs (Imai et al., 2001). This indicated an interaction between parkin and PAEL-R in native tissue. To examine the PAEL-R binding site in parkin a series of Parkin mutants were tested. Only the ct-parkin (Parkin-C, 217-465 amino acids) and full length parkin but not other parkin mutants retained binding activity to PAEL-R (Imai et al., 2001).

3.4 Unfolded PAEL-R causes ER stress and cell death

The PAEL-R has been shown to be inherently difficult to fold which enforces the importance of correct degradation events for misfolded PAEL-R (Takahashi and Imai, 2003). The poor folding of PAEL-R also makes difficult its plasmid-mediated overexpression in cells (Takshashi et al., 2006) (**Fig 10**). The ER is involved in controlling maturation of membrane and secretory proteins. Newly synthesised secretory proteins enter the ER and bind to ER chaperones (such as binding immunoglobulin protein and calnexin), facilitating proper protein folding. Subsequently folded proteins enter the secretory pathway composed of the Golgi apparatus and proceed outward to the plasma membrane. Transmembrane proteins upon internalisation can be degraded through the ubiquitin proteasome pathway. When proteins are not correctly folded, they undergo degradation by a process called ER associated protein degradation (ERAD) (Takahashi and Imai, 2003).

The ERAD system eliminates misfolded ER proteins including integral membrane and secretory proteins via degradation in the cytosol (Plemper and Wolf, 1999). ERAD substrates are ubiquitinated and degraded through the ubiquitin proteasome pathway in co-operation with E2 ligases such as Ubc6 and Ubc7 and the proteasome complex (Imai et al., 2000). The accumulation of unfolded proteins within the ER leads to ER stress which transactivates multiple genes including molecular chaperones such as binding immunoglobulin protein and ERAD-associated molecules. This leads to a cellular response known as unfolded protein response and activates ERAD. When the amount of unfolded proteins in the ER accumulate, this leads to cell apoptosis accompanied by the activation of Cjun NH2 terminal kinases (JNK) and caspases (Imai et al., 2000, Travers et al., 2000). The JNK proteins also known as stress activated protein kinases are responsive to ER stress and activated by chaperone genes like inositol requirement 1. JNK activation plays a role in apoptosis, neurodegeneration, cell differentiation and

proliferation, and inflammatory responses which causes cell death (Urano et al., 2000). Similarly caspase 12 is activated by ER stress and causes ER stress induced disruption of ER calcium homeostasis and apoptosis (Nakagawa et al., 2000).

Individuals affected with AR-JP develop disease where accumulation of the PAEL-R in the ER induces cell death (Imai et al., 2001). Functional parkin eliminates unfolded PAEL-R in co-operation with a molecular chaperone 70 KDa heat shock protein (Hsp70) and the U box protein ct of Hsp70 interacting protein (CHIP) (Imai et al., 2001). The U box was initially identified in the yeast E4 Ufd2 protein and contains a RING finger fold. The RING finger fold is structurally similar to RING finger motif and functions in protein ubiquitination. Thus CHIP's Ubox shows E3 activity and ubiquitinates unfolded proteins. Hsp70 plays a role in ensuring correct folding and intracellular localisation of newly synthesised polypeptides (Imai et al., 2001). Moreover Hsp70 exerts protective properties by binding to parkin which leads to PAEL-R ubiquitination. When unfolded PAEL-R is synthesised and translocates into the cytosol, Hsp70 and Hdj2 (ER-associated Hsp40) bind to unfolded PAEL-R which initiates the upregulation of CHIP. CHIP facilitates the dissociation of Hsp70 from the PAEL-R and helps in binding of parkin and ubiquitination of the PAEL-R in conjunction with E2 ligase such as Ubc4, Ubc6 and Ubc7 on the ER surface (Imai et al., 2001).

3.5 PAEL-R overexpression causes neuronal death

PAEL-R mRNA is abundant in the corpus callosum and dopaminergic neurons in SN (Zeng et al., 1997, Donohue et al., 1998) and is also localised in CA3 hippocampal neurons and in oligodendrocytes (Imai. et al., 2001, Yuzuru et al., 2001). PAEL-R is localised in the core of LBs and lewy neuritis (Murakami et al., 2004). The aggregation of PAEL-R due to ineffective parkin-dependent ubiquitination may be involved in PAEL-R mediated neurotoxicity in PD (Murakami et al., 2004). Interestingly, when PAEL-R is expressed in dopaminergic neurons in drosophila brain, these neurons show selective degeneration (Yang et al., 2003). The above findings suggest that PAEL-R overexpression causes the selective degeneration of dopaminergic neurons. Overexpression of PAEL-R activates ER stress which induces cellular autophagy (Marazziti et al., 2009b). Autophagy is a membrane trafficking mechanism that delivers cytoplasmic components into lysosomes for enzymatic degradation (Marazziti et al., 2009b). Autophagy induced

by PAEL-R overexpression plays an important role in clearing protein aggregates and prevents the degeneration of neurons that overexpress PAEL-R (Marazziti et al., 2009b). In addition, the level of ERAD and autophagic markers such as glucose regulated protein 78 chaperone and the microtubule associated protein 1, light chain 3 were found to be decreased in brain extracts of PAEL-R tg mice. Data indicated that autophagy is involved in the control levels of PAEL-R protein (Marazziti et al., 2009b).

3.6 Potential targets to control PAEL-R Toxicity

To date, in addition to parkin, there are four additional proteins that have been shown to regulate PAEL-R toxicity and could be potential drug targets, these are summarised below:

(i) HRD1 (human homology of yeast Hrd1p) is an E3 ligase involved in ERAD and expressed in dopaminergic neurons of the SN. HRD1 directly colocalises with PAEL-R in the ER and promotes the ubiquitination and degradation of PAEL-R in the ER, thus suppresses PAEL-R induced cell death (Omura et al., 2006). HRD1 directly interacts with PAEL-R at the proline region. When unfolded, PAEL-R accumulates in the ER, activating the transcription factor 6 (ATF6) and inositol requirement 1 transcription factors, which induce unfolded protein stress genes.

(ii) DJ-1/PARK7 is a redox-responsive protein with neuroprotective roles. Mutations in DJ-1 gene are linked to AR-JP and it is up-regulated in astrocytes in neurodegenerative diseases and stroke. Promoting DJ-1 activity is thought to help treat neurodegeneration, whereas down regulation of DJ-1 enhances dopaminergic neuronal cell death via ER stress, oxidative stress and proteasome inhibition. It has been shown that DJ-1 over expression rescues PAEL-R induced cell death (Yokota et al., 2003).

(iii) Thioredoxin (Trx) is a molecular chaperone and 12 KDa antioxidant, found to suppress PAEL-R induced neurotoxicity (Umeda-Kameyama et al., 2007). Trx contains a dithiol-disulfide active site which facilitates the reduction of other proteins by cysteine thiol-disulfide exchange. Trx is characterised by its amino acid sequence due to the presence of two cysteines in a –CXXC motif. These two cysteines are important for reduction of proteins and facilitate the reduction of ascorbic acid (vitamin C), selenium-containing substances, lipoic acid, and

ubiquinone (Q10) (Nordberg and Arner, 2001). It has been suggested that the chaperone properties of Trx are important for controlling PAEL-R induced toxicity as well as poly-glutamine-induced neurotoxicity.

(iv) Parkin coregulated gene (PACRG)/gene adjacent to parkin (Glup) forms a complex with Hsp70 and Hsp90 and is a component of LBs. PACRG/Glup can reduce cell death mediated by degradation of overexpressed PAEL-R by formation of cytoplasmic inclusions that appear to be cell protective (Imai et al., 2001).

There are also two compounds that appear to modulate PAEL-R toxicity. Sodium 4-phenyl-butyrate (4-PBA) has been shown to restore the normal expression of PAEL-R by reducing the amount of misfolded protein within the unfolded protein response pathway (Kubota et al., 2006). 4-PBA is a chemical chaperon which has been demonstrated to improve the misfolding and mislocalisation of proteins including α -1 antitrypsin, prion protein, aquaporin β -glucosidase and the cystic fibrosis transmembrane conductance regulator. This compound attenuates activation of ER stress induced signal transduction pathways like transcription of Hsp70 and HRD1, thus it may control cell death in neurodegeneration (Kubota et al., 2006). 4-PBA also controls the accumulation of β -amyloid, α -synuclein, prion or polyglutamine proteins and used for several neurodegenerative diseases (Kubota et al., 2006). In contrast to the protective effects of 4-PBA, high doses of metamphetammine (40mg/kg) can produce temporary suppression of gene expression of parkin and the PAEL-R (Nakahara et al., 2003). This suppression of the PAEL-R and parkin is linked to metamphatamine-induced dopamerigic neurotoxicity (Nakahara et al., 2003).

3.7 Ligand for PAEL-R

The neuropeptide head activator (HA) is a mitogen for mammalian cell lines of neuronal origin and consists of 109 amino acids (Franke et al., 1997). HA was initially isolated and characterised from hydra where HA mediates head specific growth and differentiation processes. The signaling cascade from HA to mitosis includes activation of a GPCR, an inhibitory G protein and requires Ca^{2+} influx, downregulation of adenylate cyclase and hyperpolarisation of the membrane potential. Electrophysiological recording in frog oocytes and in mammalian cell lines revealed nanomolar affinities of HA can activate PAEL-R (Rezgaoui et al.,

2005). In the signaling pathway, binding of HA with PAEL-R together the co-receptor SorLA activates a pertussis toxin sensitive inhibitory G protein (Rezgaoui et al., 2005). The G protein regulates the phosphoinositide 3-kinase (PI3K) and the calcium calmodulin dependent kinase II (CaMK) in addition to a Ca^{2+} channel of the transient receptor potential family (TRPV2-like). The resulting Ca^{2+} influx activates a Ca^{2+} dependent K^+ channel of the small and intermediate conductance family (SK4-like), leading to hyperpolarisation, which is required for cells to enter mitosis. HA treatment results in internalisation of PAEL-R and induces mitosis (Rezgaoui et al., 2005). The mechanisms that regulate PAEL-R internalisation remain unclear, however are likely to involve trafficking proteins.

4. PAEL interacting proteins

4.1 PAEL-R interacts with DAT

Increased and uncontrolled metabolism of dopamine can act as an endogenous toxin and provoke neuronal damage through the generation of reactive oxygen species and oxidative stress (Shen and Cookson, 2004). Dopamine facilitates the transition of non-toxic α -synuclein protofibrils to toxic fibrils present in LBs (Lee et al., 2001, Sulzer, 2001). In addition, covalent modification of parkin by dopamine leads to inhibition of its E3 activity (LaVoie et al., 2005). PAEL-R signaling appears to regulate dopamine levels in neurons (Imai et al., 2007). PAEL-R KO mice show a decrease in level of striatal dopamine and increased sensitivity to amphetamine (Imai et al., 2007). In PAEL-R tg mice the levels of striatal 3,4 dihydroxyphenylacetic acid (DOPAC) and vesicular dopamine content are increased and the numbers of nigrostriatal dopaminergic neurons are reduced (Marazziti et al., 2004, Imai et al., 2007). Of interest, PAEL-R KO mice are resistance to treatment with the neurotoxin MPTP and moreover tyrosine hydroxylase inhibitor treatment can ameliorate dopaminergic cell death induced by infection of adenovirus encoding PAEL-R (Marazziti et al., 2004, Kitao et al., 2007). In parkin KO mice crossed with PAEL-R tg mice, there are higher levels of dopamine, dopamine metabolites (DOPAC and HVA), protein carbonyls, and markers of oxidative damage in midbrain (Wang et al., 2008). This study implies the pathological role of dopamine and its metabolites in dopaminergic neuron-specific degeneration as a result of PAEL-R accumulation (Wang et al., 2008). These results are concomitant with another study showing the localisation of PAEL-R in presynaptic fraction of mouse striatum and its interaction with dopamine transporter (DAT) (Marazziti et al., 2007). This interaction can modulate DAT-mediated dopamine uptake, where the lack of PAEL-R enhances DAT activity and increase the plasma membrane expression of DAT (Marazziti et al., 2007). Thus, not only chronic ER stress due to accumulation of misfolded proteins, but also excessive dopamine mediated oxidative stress is likely to contribute to the pathologic role of PAEL-R in PD.

4.2 PAEL-R interacts with Syntenin-1

Poor plasma membrane trafficking of PAEL-R impedes our understanding of the ligand binding and signaling pathways of this orphan receptor. To date, four different approaches for enhancing GPCRs surface expression have been introduced, including addition of sequences, removal of sequences, co-expression with receptor-interacting proteins and treatment with pharmacological agents (Dunham et al., 2009). PAEL-R surface expression is undetectable when compared to GPR37L1, which expresses strongly on the cell surface of HEK293 cells (Dunham et al., 2009). Interestingly, removal of the first 210 amino acids from n-terminus (nt) dramatically increases the surface expression of PAEL-R (Dunham et al., 2009). This may suggest that PAEL-R possess a motif on the n-terminus, which may be important for the localisation of PAEL-R on plasma membrane. It has been reported that a truncated ct version of PAEL-R has a decreased surface expression (Dunham et al., 2009). Moreover the ct of PAEL-R possesses a class 1 PDZ domain-binding motif (-GTHC), which has potential to interact with PDZ domain-containing scaffolding proteins. Thus, the ct of PAEL-R plays an important role in its surface expression and most likely in its trafficking (Dunham et al., 2009). In this regard, the PAEL-R has been shown to interact with the PDZ scaffold protein syntenin-1, which increases cell surface trafficking of PAEL-R (Dunham et al., 2009). The co-expression with other GPCRs such as the adenosine receptor ($A_{2A}R$) and the dopamine receptor (D_2R) can also enhance the membrane expression of PAEL-R (Dunham et al., 2009). Furthermore, the interaction of PAEL-R with dopamine receptor can alter the dopamine receptor affinity for both agonists and antagonists and alter dopaminergic signaling (Dunham et al., 2009).

5. Aim of the thesis

5.1 Novel PAEL-R interacting proteins

Like other GPCRs, PAEL-R may require assembly with a specific partner to achieve correct surface expression and functional activity. To date it has been shown that PAEL-R cell surface expression can be enhanced by n-terminal truncation or by c-terminal interaction with the PDZ domain containing scaffold protein syntenin-1 (Dunham et al., 2009). Since PDZ scaffolds have the capacity to control receptor surface expression, there is a need to further study the ability of the PAEL-R to interact with other PDZ scaffold and trafficking proteins. Preventing interacting proteins from associating with the PAEL-R may alter its trafficking and thus provide a method for regulating its signaling, aggregation and neurotoxic properties. We aimed to study the ability of the ct-PAEL-R to interact with other PDZ scaffold and trafficking proteins. Below is a description of the three proteins we have found interacting with the ct-PAEL-R namely the PDZ containing protein PICK1, and two other proteins GABARAPL2 and RAB14 (**Fig 11**).

5.2 Hypothesis

We hypothesise that the trafficking proteins that interact with PAEL-R can alter surface expression and function of the receptor which may be important in the underlying mechanisms of PD.

6. PICK1 interacts with PAEL-R

6.1 Introduction and structure

We have found that the PAEL-R interacts with PICK1. PICK1 consists of 416 amino acids and has a molecular weight of 50 KDa. It is conserved from *Caenorhabditis elegans* to the human (Junyu and Jun, 2007). PICK1 is expressed in many tissues in high levels including the CNS. It is localised at the perinuclear region as well as in synapses of neurons (Xu and Xia, 2006). PICK1 was originally identified as a protein that interacts with PKC α from a mouse T-cell cDNA library in a Y2H screen (Staudinger et al., 1997). The ct of PKC α which contains a PDZ motif (-TSXV) interacts with carboxylate-binding domain (CBD) within the PDZ domain of PICK1. PICK1 consists of a single PDZ domain that contains approximately 90 residues (**Fig 12**).

PDZ domains are a well-characterised protein-protein interaction site. PDZ domains can be divided into three types, based on the selectivity on PDZ binding motifs. The type I PDZ domain bind $-X\text{-Thr/Ser-X-}\phi$ motifs. Type II PDZ domain interacts with $-X\text{-}\phi\text{-X-}\phi$ motifs and the type III PDZ domain interacts by $-X\text{-Asp/Glu-X-}\phi$ where X stands for any amino acid and ϕ stands for hydrophobic residues. The CBD of PICK1 within the PDZ domain (residues Lys 27 and Asp28) is involved in PDZ motif binding. PDZ domains consist of six β strands ($\beta_a\text{-}\beta_f$) and two α helices (α_a and α_b) (Xu and Xia, 2006). Mutation of K27 together with D28 usually disrupts the PICK1-PDZ interaction. Over 60 proteins have been identified to interact with PICK1 and most of them are membrane proteins, including receptors, transporters and ion channels.

The central α -helical coiled motif of PICK1 is about 152-362 residues in size and contains an arfaptin homology domain (AHD) and Bin/amphiphysin/RVS (BAR) domain (**Fig 12**). The α -helical coiled repeats have a high sequence homology to proteins called arfaptins. Arfaptins, which include arfaptin 1 and arfaptin 2, are a group of proteins that bind to small G proteins such as Arf and Rac. BAR domains are present in GTPases and are involved in endocytosis (Dev and Henley, 2006). The PICK1 auto dimerise by using its BAR domain to form a banana shaped structure (Xiao et al., 2007). PICK1 can bind phospholipids to promotes endocytosis via its BAR domain. Mutations in the BAR domain significantly reduce PICK1 lipid binding capability. The glutamate/aspartate rich (E/D) acidic region is

at the ct of PICK1 and consist of 380-390 residues (Dev, 2007) (**Fig 12**). This acidic domain of PICK1 binds to calcium and causes PICK1 protein-protein interactions sensitivity to calcium levels (Staudinger et al., 1995). The ct region of PICK1 is the most divergent across species and found to inhibit lipid binding of PICK1 BAR domain. The autoinhibition is due to the negatively charged acidic residues of PICK1 ct region which fold back and bind to the positively charged residues of PICK1's BAR domain to inhibit its lipid binding (Xu and Xia, 2006). The domain structure of PICK1 is rather unique as it is the only known protein which possess both a PDZ domain and a BAR domain.

6.2 Function and interacting proteins

PICK1 acts as a scaffolding protein that associates with several proteins via its PDZ domain and BAR domain. PICK1 plays a role in PKC-mediated phosphorylation which is important for intracellular signaling involved in cellular growth, differentiation and survival. PKC is activated by the second messenger diacylglycerol or phorbol esters TPA or PMA (phorbol12-myristate 12-acetate). PICK1 regulates PKC movement from cytosol to membrane and thus alters phosphorylation of target proteins (Staudinger et al., 1995). In addition to PKC α , PICK1 interacts and co-localises with several membrane proteins including Eph receptor tyrosine kinases and ephrin-B ligands, the AMPA receptor subunit GluR2 the metabotropic glutamate receptor subtype 7 (mGluR7) and ASIC (Baron et al., 2002). PICK1 regulates the trafficking of its binding partners by altering either their subcellular targeting or their surface expression. PICK1 is also involved in the clustering and surface expression of various receptors by regulating PKC α phosphorylation (Perez et al., 2001a). Importantly, PICK1 interacts with the PDZ motif of parkin (-QSAV). Parkin mediated monoubiquitination of PICK1 regulates ASIC mediated synaptic plasticity (Joch et al., 2007). PICK1 also interacts with the PDZ motif (-LKV) of DAT (Wang et al., 2003) and interestingly PICK1 plays a role in the targeting and clustering of DAT (Matsuzawa et al., 2007). In summary PICK1 interactions with parkin, DAT and PAEL-R hint towards an important role in PD.

As mentioned above, PICK1 interacts with over 60 proteins. A few of PICK1 interacting proteins are described below in detail and further summarised in **Fig 13**. Focus is given to the major receptors that interact with PICK1, namely glutamate receptors. Also discussed is the monoamine transporter DAT which interacts with PICK1 which may link PICK1 to play a role in Parkinson disease.

6.2.1 Glutamate receptors

Ionotropic glutamate receptors are subdivided into N-methyl-D-aspartate (NMDA), Kainate (KA) and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA). The PDZ domain of PICK1 was found to interact with the PDZ motif of AMPA receptor subunits GluR2 (-ESVKI), 3 (-ESVKI) and 4c (-ESVKI) in Y2H experiments (Dev et al., 1999, Xia et al., 1999). PICK1 has the ability to regulate the surface expression of GluR2 (Perez et al., 2001b, Terashima et al., 2004). Disrupting the interaction between PICK1 and GluR2 inhibits long term depression (LTD) in cerebellar purkinje neurons (Xia et al., 2000) and hippocampal pyramidal neurons (Kim et al., 2001). This suggests that PICK1 is involved in trafficking of AMPA receptors at the synaptic membrane. Protein phosphorylation plays an important role in PICK1-mediated AMPA receptor trafficking and synaptic plasticity. The serine 880 of GluR2, which is located in the PDZ motif at the -3 position of ct-GluR2 is phosphorylated by PKC (Seidenman et al., 2003). The phosphorylation of S880 does not change PICK1 interaction with GluR2 but significantly reduces binding of GluR2 to another PDZ protein called Glutamate receptor interacting protein (GRIP). By disrupting the interaction with GRIP, phosphorylation of S880 by PKC promotes the interaction of GluR2 with PICK1 and facilitates PICK1 mediated internalisation of AMPA receptors during LTD (Perez et al., 2001a). PICK1 removes GluR2 from the synapse and replaces it with GluR1 thus allowing AMPA receptors to pass Ca^{2+} and become functionally active. PICK1 also targets PKC α phosphorylation of the Kainate receptor subunit ct-GluR5 at S880 and S886 and plays a role in receptor recycling (Cho et al., 2003, Hirbec et al., 2003). In addition, the PDZ domain of PICK1 interacts with the group III G-protein coupled metabotropic glutamate receptor subtype 7 (mGluR7) via its PDZ motif and regulates the synaptic (Cho et al., 2003) aggregation and receptor clustering but not trafficking of mGluR7 (Dev, 2007).

6.2.2 Monoamine transporter

Disruption in monoamine neurotransmission causes several psychiatric, neurological and neurodegenerative disorders including depression, hyperactivity, schizophrenia and PD (Torres et al., 2003). Monoamine transporters include DAT, norrepinephrine and serotonin, which are responsible for the reuptake of their associated amine neurotransmitters (Serotonin, dopamine or norepinephrine). These transporters recycle dopamine, noradrenaline and 5-hydroxytryptamine

(serotonin, 5HT) either via uptake or by vesicle storage (Gainetdinov and Caron, 2003). The PDZ motif of DAT interacts with the PDZ domain of PICK1 (Torres et al., 2001, Madsen et al., 2005). In dopaminergic neurons PICK1 colocalises with DAT and coexpression of PICK1 results in DAT-PICK1 clustering which indirectly enhance DAT uptake through an increase in the number of plasma membrane DAT, via a PKC dependent mechanism (Torres et al., 2001). Deletion of the PDZ binding site at the ct-DAT abolishes the association with PICK1 and indicates a role for PDZ-mediated protein interactions in the function of monoamine transporters (Torres et al., 2001).

7. GABARAPL2 interacts with PAEL-R

7.1 Introduction and structure

In addition to PICK1, we also found that GABARAPL2 interacts with PAEL-R. The γ -aminobutyrate type A (GABA_A) receptor associated protein (GABARAP) was initially identified as a protein associated with the γ -subunit of the GABA_A receptor (Wang et al., 1999). GABA_A receptors are gated ion channels for chloride that mediate rapid inhibitory synaptic transmission in the CNS and serve as a target for multiple neuroactive drugs (Mohrluder et al., 2009). GABARAP binds to GABA_A receptor *in vivo* and *in vitro* and colocalise with punctate staining of the GABA_A receptor in cell bodies and neuronal processes in cultured cortical neurons (Wang et al., 1999). GABARAP interacts with the γ 1 and γ 2 isoforms not with α 1-6, β 1-3, γ 3, ρ or δ isoforms of GABA_A subunits (Wang H. et al., 1999). The γ 2 is the most abundant GABA_A receptor subunit in the CNS and is also a substrate for several PKC and Protein kinase A (PKA) enzymes (Moss et al., 1992). GABARAP is classified as a protein of the GABARAP-like family. This protein family encompasses (1) GABARAP like 1 (GABARAPL1) that was initially isolated in guinea pig endometrial cells, (2) estrogen induced 1.8 Kb RNA coded protein (GEC1), (3) GABARAP like 2 (GABARAPL2) which is a golgi associated ATPase enhancer of 16 KDa (GATE-16), (4) GABARAP and (5) GATE-16 family (LGG). The GABARAP, GABARAPL1, GABARAPL2/GATE-16 and GABARAPL3 proteins are a subfamily of autophagy-related protein 8 (Atg8) and they are crucial in the autophagic process possibly in the elongation of the phagophore membrane (Mohrluder et al., 2009). GABARAP like molecules shows 60-85% similarity with GATE-16 and are 30% identical with light chain 3 of microtubule-associated protein 1 (MAP1 LC3) (Bavro et al., 2002). GABARAPL2 expresses 117 amino protein, of 13.7 KDa and belongs to the MAP1 LC3 family (Chen et al., 2001) (**Fig 14**). GABARAPL2 modulates intra-Golgi transport and is expressed at high levels in heart, brain, kidney, liver, spleen and skeletal muscle tissue (Yurong et al., 2001). GABARAPL2 was located to human chromosome 16 position (16q 22.3-q 24.1) by radiation hybrid mapping (Xin et al., 2001). GABARAPL2 is 86% identical to GABARAP (Chen et al., 2001).

The major part of GABARAP contains an ubiquitin like fold and an ubiquitin like conjugation system that modifies GABARAP. The ubiquitination process of GABARAP is initiated by E1 or cysteine protease Atg4B, which results in

GABARAP with a C-terminal glycine residue. A further activation by Atg7 (E1 like enzyme) and transfer of ubiquitin to the E2 like enzyme Atg3 results in GABARAP ubiquitination. GABARAP is finally attached to phospholipids and delipidation of GABARAP is mediated by Atg12-Atg5/Atg16 multimers (Mohrluder et al., 2009). The UBL core domain of GABARAP contains two parallel β -strands (β 1 and β 4), which are formed by one anti parallel β -strand (β 2 and β 3) on each side and two α helices (α 3 and α 4) on the concave side of the β -sheet. GABARAP possess two additional α helices at its n-terminus (α 1 and α 2) (Mohrluder et al., 2009) (**Fig 14**). These helices appear to be important for tubulin binding and oligomerisation. GABARAP also contains two hydrophobic pockets termed hp1 and hp2. These hydrophobic pockets are also relevant for protein-protein interactions. In addition, GABARAP contains tyrosine kinase and PKC phosphorylation sites (Chen et al., 2001) (**Fig 14**). GABARAP has an overall positive charge and its n-terminus is rich in basic amino acids, which might bind to the acidic residues of binding domain of tubulin. The glycine 116 is required for c-terminal processing of GABARAP and is essential for the localisation of GABARAP and its function as a trafficking protein (Bavro et al., 2002). GARARAPL2 is the exception of GABARAP like family which contains no tubulin binding motif (**Fig 14**). Interestingly, GABARAP KO breed normally and show no up-regulation of other GABARAP homologues (O'Sullivan et al., 2005). GABARAP KO mice have unaltered GABARAPL1 and GABARAPL2 expression in-vivo (O'Sullivan et al., 2005).

7.2 Function and interacting proteins

Similar to PICK1, GABARAPL2 also interacts with a number of proteins. The most relevant GABARAPL2 interactions are discussed below, and the remaining summarised in **Fig 15**.

Gephyrin is a large 93 KDa tubulin binding protein which is a component of the postsynaptic protein network (Giesemann et al., 2003). Gephyrin anchors inhibitory neuronal receptors like GABA_A to the sub-synaptic cytoskeleton and transports GABA_A receptors to the synapse (Essrich et al., 1998). It has been suggested that Gephyrin functions with GABARAP to traffic GABA_ARs to the postsynaptic membrane by interaction with the γ ₂ subunit of GABA_ARs and the cytoskeleton (Essrich et al., 1998). However, immunostaining of neurons has failed to show the expected high degree of colocalisation of GABARAP with synaptic clusters of GABA_A receptor (Phillips and Froehner, 2002). Most synaptic clusters of GABA_A

receptor colocalised with gephyrin aggregates and only a small minority contained GABARAP (Phillips and Froehner, 2002). GABA_A binds with N-ethylmaleimide sensitive fusion protein (NSF) and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) which is involved in intra-golgi trafficking. GABARAP has also been shown to interact with NSF, a protein important for intracellular membrane vesicle trafficking events (Kittler et al., 2001).

It is important to note that GABARAPL2 (GATE-16) is a 16 KDa golgi associated ATPase enhancer and is essential for intra-golgi transport (Nagahama et al., 1996). GABARAPL2 binds NSF and is implicated in transport of the β -adrenergic receptor and AMPA type glutamate receptors (Kittler et al., 2001). The interaction between GABARAP and NSF also stimulates the ATPase activity of NSF. Interestingly, NSF also interacts with the AMPA receptor subunit GluR2 and regulates its trafficking (Henley, 2001). In addition GABARAP has also been shown to interact with and regulate the trafficking of the transferrin (TfnR) receptor (Green et al., 2002).

8. Rab14 interacts with PAEL-R

8.1 Introduction and structure

The last protein we found to interact with PAEL-R is Rab14. The Rab protein family is composed of over 70 distinct members. Rab proteins constitute the largest family of the GTPase super family and play a major regulatory role in intracellular membrane trafficking. Rab GTPase acts as a molecular switch alternating between active (GTP bound) and inactive (GDP bound) states. Different Rabs localise in membrane bound cellular compartments, including the endoplasmic reticulum, golgi region (Rab1a, Rab2, Rab6, Rab30 and Rab33b), early and recycling endosomes/lysosomes (Rab7 and Rab9) and specialised organelles such as synaptic vesicles (Rab3a and 3C), secretory granules (Rab3D, Rab37), and melanosomes (Rab27) (Junutula et al., 2004). Rab GTPase plays an important role in endocytic and exocytic membrane trafficking (Takai et al., 2001) and these proteins have been implicated in phagosome formation and maturation (Rupper et al., 2001).

Rab14 is expressed in the brain, heart, kidney and lung. Amino acid sequence alignments reveal that Rab14 is a close homolog of Rab2 and Rab4 with an 57% and 58% identity. Rab14 has four GTP binding domains (I-IV) that are highly conserved in Rab proteins and involved in binding of GTP/GDP (**Fig 16**). The highly conserved -DTAGQE motif in region I functions to stabilise the binding of the γ -phosphate of GTP. This domain contains a conserved tryptophan residue in all members of the Rab family. The -NKXD motif in Rab14 (region III) is characterised by a guanine conserved region which also regulates the binding of GTP. The fourth GTP binding domain has a conserved guanine base and characterised by the motif -EXSAK/L. The second GTP binding domain contains a conserved arginine residue and a conserved phenylalanine residue in the fourth GTP binding domain (Elferink et al., 1992). A less conserved region is the putative effector domain (**Fig 16**). The corresponding domain in p21ras (amino acids 32-40) interacts with GTPase activating proteins (GAP). GAP is a regulatory protein whose function is to stimulate the GTPase activity of GTP binding proteins (Elferink et al., 1992).

8.2 Function and interacting proteins

Rabs are localised to the cytoplasm of membrane bound organelles from where they regulate diverse cellular functions such as the regulation of vesicle formation, transport, motility, docking and fusion (Junutula et al., 2004, Kelly et al., 2009). Rab14 is localised at the ER, golgi as well as early endosomal compartments. Thus Rab14 may play a role in trafficking and recycling pathways between the golgi and endosomal recycling compartment (ERC) (Kelly et al., 2009). Rab14 also appears to regulate endolysosomal fusion (Bus et al 1996). Rab GTPase has been implicated in phagosome formation. Rab GTPase regulates phagocytosis and plays a prominent role in phagosome and endo-lysosomal fusion (Harris and Cardelli, 2002). Ras-related protein Rab14 is involved in vesicular trafficking and neurotransmitter release. Two major Rab14 interactors are described below (**Fig 17**). Please also see **Fig 17** for summary of Rab14 interacting proteins.

8.2.1 Annexin II

Annexin II is a calcium-regulated membrane-binding protein whose affinity for calcium is enhanced by anionic phospholipids. It binds two calcium ions with high affinity which may be involved in heat-stress response (Gou et al., 2008). Annexin II is abundantly expressed in alveolar type II cells where it plays a role in lung surfactant secretion. Rab14 is co-localised in part with annexin A2 and lamellar bodies in alveolar type II cells. Rab14-Annexin interaction results in a decrease in surfactant secretion, suggesting that Rab14 may play a role in surfactant secretion (Gou et al., 2008).

8.2.2 Rab14 effectors

A family of effector proteins interacts with all Rab11 subfamily members and is termed as Rab11-family interacting proteins (Rab11-FIPs) (Prekeris et al., 2000, Hales et al., 2001, Lindsay et al., 2002). These proteins have been characterised by their ability to interact with Rab11 subfamily members via a conserved 20 amino acid ct Rab11-binding domain. The FIPs have been classified into: the class I FIPs [FIP2, Rab coupling protein (RCP)/FIP1C and Rab11-interacting protein] which are characterised by the presence of an n-terminal phospholipid-binding C2 domain and involved in membrane association (Lindsay et al., 2004), and the class II FIPs (FIP3 and FIP4), which have putative calcium-binding hand and extensive coiled-coil domains (Prekeris et al., 2000, Horgan et al., 2007). The c-terminal Rab14

interacts with specific class I FIPs and an interaction occurs via the Rab11-binding domain (Kelly et al., 2009). Importantly Rab14 co-localises with the transferrin receptor and with the class I FIPs on the endosomal recycling compartment during interphase (Kelly et al., 2009).

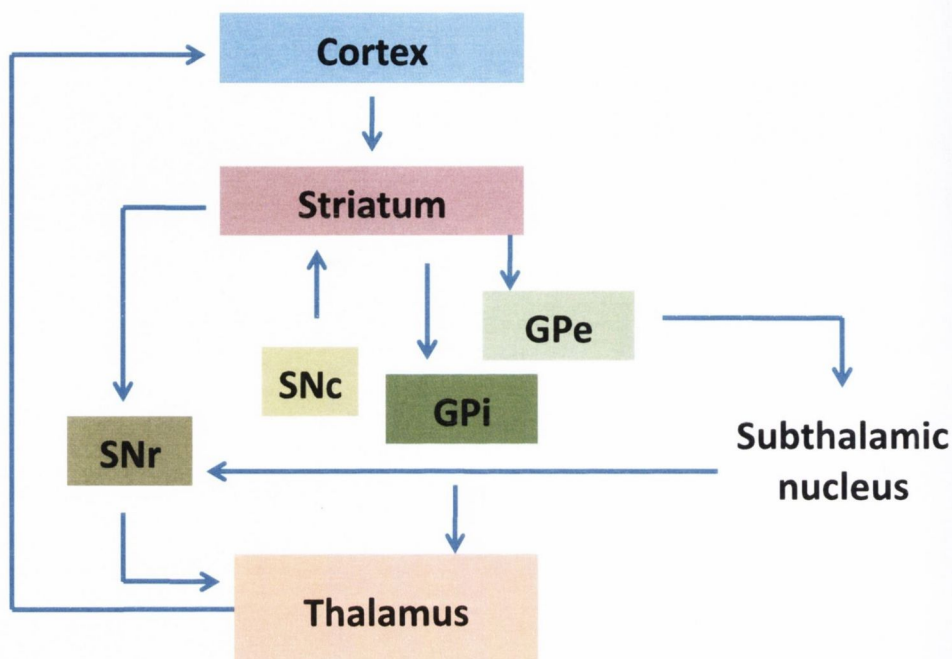


Figure 1: Complex circuitry of the basal ganglia. Abbreviation used in the figure, GPe: globus pallidus external, GPi: globus pallidus internal, STN: subthalamic nucleus, SNc: substantia nigra compacta, SNr: substantia nigra reticulata. In the direct pathway: Cortex (stimulates) → Striatum (inhibits) → "SNr-GPi" complex (less inhibition of thalamus) → Thalamus (stimulates) → Cortex (stimulates) → Muscles, etc. → (hyperkinetic state). In-direct pathway: Cortex (stimulates) → Striatum (inhibits) → GPe (less inhibition of STN) → STN (stimulates) → "SNr-GPi" complex (inhibits) → Thalamus (is stimulating less) → Cortex (is stimulating less) → Muscles, etc. → (hypokinetic state) (DeLong and Wickmann, 2007).

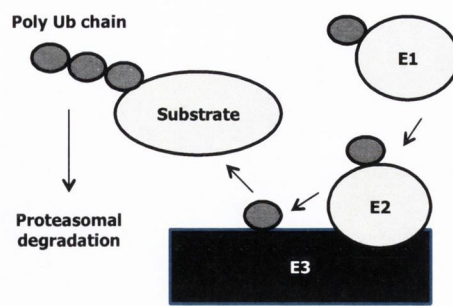
Compounds	Mechanisms of action		
Few old compounds		Side effects	
Levodopa	Precursor to neurotransmitter dopamine	Yes	
Levodopa+Carbidopa	Carbidopa inhibits decarboxylation of peripheral levodopa	Yes	
Bromocriptine	Dopamine agonist	Yes	
Rasagiline	Monoamine oxidase B	Yes	
Few new compounds		Phase	Clinical Trials.gov identifier
Nicotine	Nicotine receptor agonist	IV	NCT01216904
Preladenant (SCH-420814)	Adenosine A _{2A} receptor antagonist	III	NCT01155479
IPX066	Levodopa/carbidopa	III	NCT01096186
Safinamide	MAOB inhibitor, glutamate release inhibitor, dopamine reuptake inhibitor	III	NCT01028586
Coenzyme Q10	Modulator of mitochondrial function	III	NCT00740714
Isradipine CR	Calcium channel blockers/antagonist	II	NCT00909545
Few novel emerging agents or targets		Phase	Trials Results
FP0011	Glutamate release inhibitor	III	Without producing dyskinesia
Creatine	Modulator of mitochondria function	II	Successful in clinical trials

Figure 2: List of compounds for PD (Meissner et al., 2011). The figure is a representation of few old, new and novel emerging targets in addition to mechanism of action and followed by side effects or clinical trials identification number or trials results.

Locus	Chromosome location	Gene	Inheritance pattern	Typical pattern	Ref.
PARK1 and PARK4	4q21-q23	α -synuclein	AD (autosomal dominant)	Earlier onset, features of DLB (dementia with Lewy bodies) common	1
PARK2	6q25.2-q27	Parkin	usually AR (autosomal recessive)	Earlier onset with slow progression	2
PARK3	2q13	unknown	AD	Classic PD, sometimes dementia	3
PARK5	4q14	UCH-L1	unclear	Classic PD	4
PARK6	1p35-p36	PINK1	AR	Earlier onset with slow progression	5
PARK7	1p36	DJ-1	AR	Earlier onset with slow progression	6
PARK8	12p11.2-q13.1	LRRK2	AD	Classic PD	7
PARK10	1q32	unknown	unclear	Classic PD	8
PARK11	2q36-q37	unknown	unclear	Classic PD	9
NA (not assigned)	5q23.1-q23.3	Synphilin-1	unclear	Classic PD	10
NA	2q22-q23	NR4A2	unclear	Classic PD	11

Figure 3: Loci and genes associated or implicated with familial PD (¹Polymeropoulos et al., 1997, Singleton et al., 2003; ²Kitada et al., 1998; ³Gasser et al., 1998; ⁴Leroy et al., 1998; ⁵Valente et al., 2004; ⁶Bonifati et al., 2003; ⁷Funayama et al., 2002; ⁸Hicks et al., 2009; ⁹Pankratz et al., 2003; ¹⁰Marx et al., 2003; ¹¹Le et al., 2003).

A



B

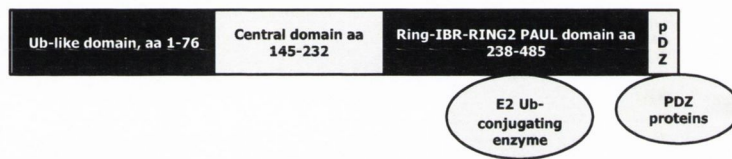


Figure 4: Structure and function of parkin. (A) Diagram represents the ubiquitination process. Ubiquitination occurs in a three step process. The Ub is activated by E1. Then Ub is accepted by E2 and finally Ub is transferred via E3 to the substrate. (B) Structure of parkin. Parkin contains an Ub homology domain, a central domain, a RING box and PDZ binding motif.

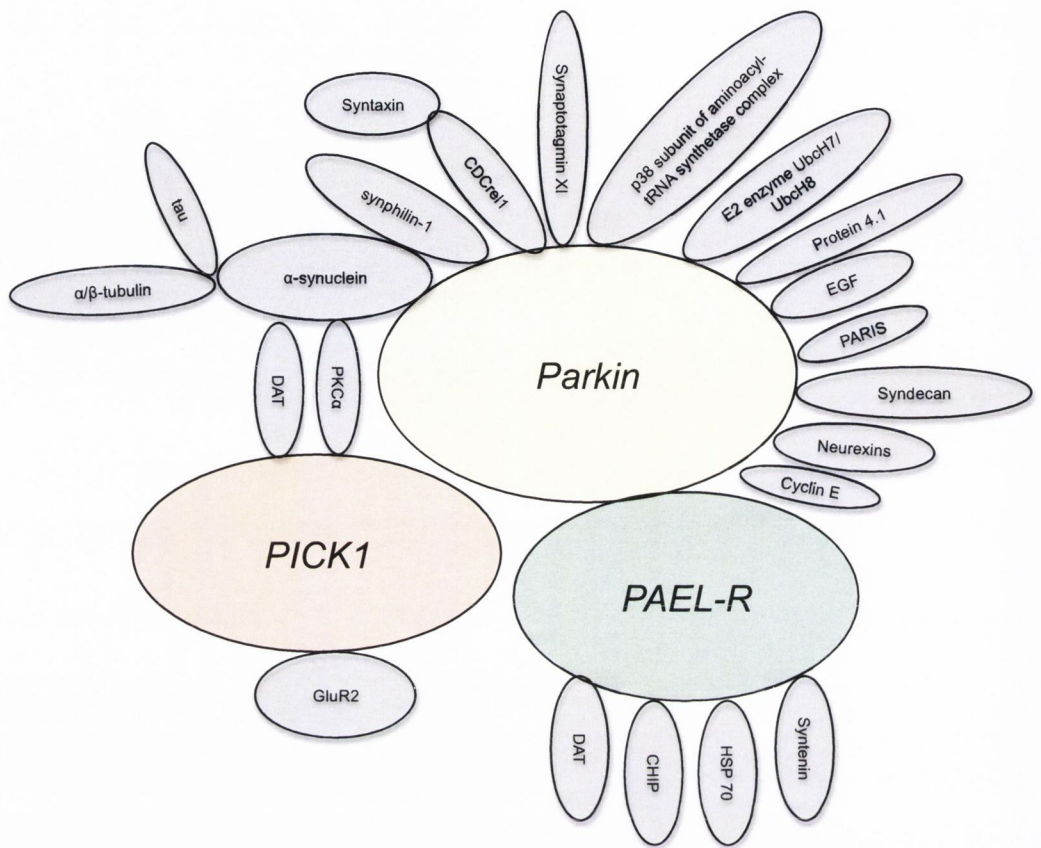


Figure 5: Substrates of parkin. Diagram represents putative substrates of parkin, including CDCrel-1, synphilin-1, a rare O-glycosylated form of α -synuclein (α Sp22), parkin-associated endothelin receptor-like receptor (PAEL-R), protein interacting with C kinase (PICK1), synaptotagmin XI, cyclin E, the p38 subunit of the aminoacyl-tRNA synthetase complex and α/β -tubulin.

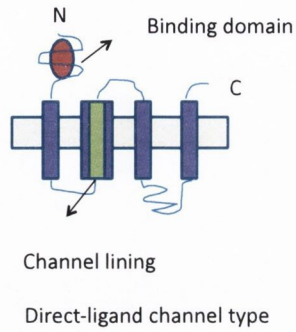
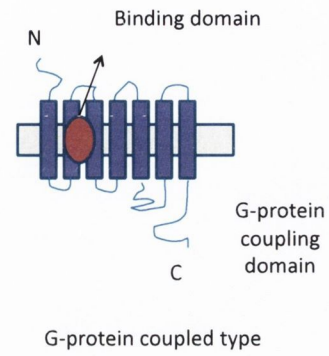
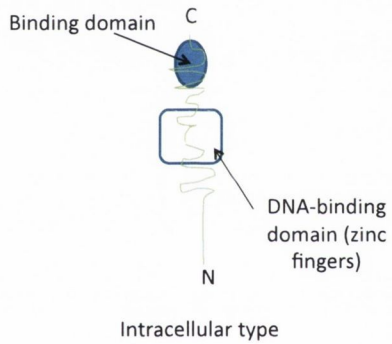
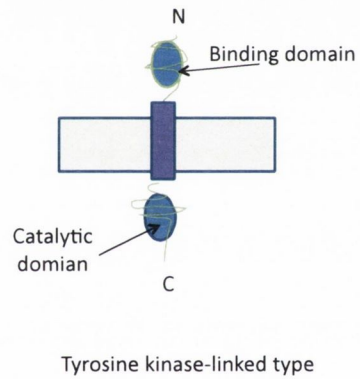
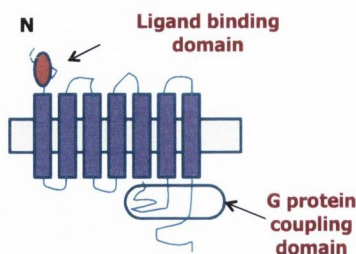
A**B****C****D**

Figure 6: Types of receptor families. General structure of four receptor families includes (A) direct-ligand channel type example Nicotinic acetylcholine receptor, (B) GPCRs example Dopamine receptor, (C) intracellular-type example nuclear receptor and (D) tyrosine kinase-linked type example Insulin receptor.

A



B

```
LAGPSWDLPAAPGRDPAAGRGAEASAAGPPGPPTRPPGPRWKGARGQEPSETLGRGNPTALQQLFLQI
RGATSWDLPPRRGGD--TGVIEEAAAAGPLGPPTKPPSAWRWKSQKQKPEPSGHLRRRDPTDPLFFPT
RGATSWDLPPRRGGD--TGVIEEAAAAGPLGPPTKPPGAWRWKAQKQKPEPSGHLGRREPTDQLFRQT
```

```
ISEEEKGPRGAGISGRSQEQSVKTVPGASDLFYWPRRAGLQGSHHKPLSKTANGLAGHEGWIALP
TSEGGEMSSKRDGIPQSRQEHVSKTEP--RDLFYWPRRTGQLQDSQHRPS-----AVHEGRTLAPP
TSEGGEMSSKRDEIPQGSQEHVSKTEPEPRDLFYWPRRTGQLQGSHYRPS-----AVHEGRTLAPP
```

TM1

```
GRALAQNGLGEGIEHPGGPRGNSTNRRVRLKNPFYPLTQESYGAAYAVMCLSVVIFGTGIIIGNLAVM
GRALPQNGSADDWVPDQGGPRRGNTT--RRVRLKNPFYPLTQESYGAAYAVMCLSVVIFGTGIIIGNLAVM
GRALPQNGSADDWVPDQGGPRRGNTNRRVRLKNPFYPLTQESYGAAYAVMCLSVVIFGTGIIIGNLAVM
```

TM2

TM3

```
CIVCHNYMRSISMSLLANLAFWDFLIIFFCPLPLVIEHELTKKWLEDFSCIKIVPYIEVASLGVTTF
CIVCHNYMRSISMSLLANLAFWDFLIIFFCPLPLVIEHELTKKWLEDFSCIKIVPYIEVASLGVTTF
CIVCHNYMRSISMSLLANLAFWDFLIIFFCPLPLVIEHELTKKWLEDFSCIKIVPYIEVASLGVTTF
```

TM4

```
LCALCIDRFRAATNVQMYEMIENCSSTAKLAVIIVWGALLLALPEVVIQRQLSKEDLGFSGRAPAERC
LCALCIDRFRAATNVQMYEMIENCSSTAKLAVIIVWGALLLALPEVVIQRQLSKEDLGFSGRAPAERC
LCALCIDRFRAATNVQMYEMIENCSSTAKLAVIIVWGALLLALPEVVIQRQLSKEDLGFSGRAPAERC
```

TM5

```
IIKISPDLPDTIYVVALTYDSARLWVYFGCYFCLPTLFTITCSLVTARKIRKAEKACTRGNKRQIQLE
IIKISPDLPDTIYVVALTYDSARLWVYFGCYFCLPTLFTITCSLVTARKIRKAEKACTRGNKRQIQLE
IIKISPDLPDTIYVVALTYDSARLWVYFGCYFCLPTLFTITCSLVTARKIRKAEKACTRGNKRQIQLE
```

TM6

TM7

```
SQMNCIVVALTILYGFCCIIPENICIVTAYMATGVSQQTMDLLNIIISQFLFFRSCVTPVLLFCLOKIP
SQMNCIVVALTILYGFCCIIPENICIVTAYMATGVSQQTMDLLNIIISQFLFFRSCVTPVLLFCLOKIP
SQMNCIVVALTILYGFCCIIPENICIVTAYMATGVSQQTMDLLNIIISQFLFFRSCVTPVLLFCLOKIP
```

ct-terminal

```
FSRAFMECCCCCEECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFASVGTHC
FSRAFMECCCCCEECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFASVGTHC
FSRAFMECCCCCEECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFASVGTHC
```

Human O15354
Mouse Q9QY42
Rat Q9QYC6

Figure 7: Alignment and structure of PAEL-R. (A) Schematic representation of PAEL-R. **(B)** The human PAEL-R protein (Q15353) is aligned with mouse (Q9QY42) and rat (Q9QYC6). The c-terminal and seven transmembrane domains are represented in boxes.

```

MRAPGALLARMSRLLLLLLLLKVSASSALGVAPASRNETCLGESCAPTVIQRRGRDAWGPGNSARDVL
MRWLWPLAVSLAVILAVGLSRVSGGAPLHLG-----
**      *      *      *      **      *

RARAPREEQGAFLAGPSWDLPAAPGRDPAAGRGAEASAAGPPGPPTRPPGPWRWKARGQEPSETL
RHRAETQEQ-----QSRSKRGTE-----
* **      *                                     ** *

GRGNPTALQLFLQISEEEEEKGRGAGISGRSQEQSVKTVPGASDLFYWPRRAGKLGSHHKPLSKTA
-----DEEAKG-----VQQYVPEEWAHEYPRPIHPAGLQP--TKPLVATS
** *                                     **      *      **      ***      *

NGLAGHEGWTTIALPGRALAQNGSLGEGIEHPGGPRRGNSTNRRVRLKNPFYPLTQESYGAVMCLS
PNPD-----KGGTDPDQELRGNLTG-APGQRLQIQNPLYPVTESSYSAYATMLLA
*      *      *      *      *      **      *      **      ***      *

VVFPGTGIIGNLAVMCTVCHNYMRSISNSLLANLAFWDFLIIFFCPLPVIFFHELTKKWLLDFSC
LVVFAVGIVGNLSVMCTVHSHSYLKSAWNLSLASLALWDFLVLFCLPIVIFNEITKORLLGDVSCR
* *      **      ***      *****      *      *      *****      **      ***      *      *      *      *      *

IVPYIEVASLGVTTFTLFCALCIDRFRAATNVQMYEMIENCSSTAKLAVIIVGALLLALPEVVLIRQ
AVPFMEVSSLGVTTFSLCALGIDRFHVATSTLPKVRPIERCQSILAKLAVIIVGSMTLAVPELLLWQ
**      **      *****      *****      **      *      *      *      *      *      *      *      *

LSKEDLGFSGRAPAERCIIKISPDLPDTIYVLALTYDSARLWVYFGCYFCLPTLFTITCSLVTARKI
LAQEPAPTMG--TLDSCIMKPSASLPESLYSLVMTYQNAARMWVYFGCYFCLPILFTVTCQLVTVRVR
* *      *      *      *      *      *      *      *      *      *      *      *      *      *

RKAEKACTRGNKROIQLESQMNCTVVALTILYGFCCIIPENICNIVTAYMATGVSQQTMDLLNIIISQF
GPPGRKSECRASKHEQCESQLNSTVVGTLTVVYAFCTLPENVCNIVVAYLSTELTRQTLDLLGLINQF
*      *      *      *      *      *      *      *      *      *      *      *      *      *      *

LLFFKSCVTPVLLFCLCNPFSRAFMECCCCC--EECIQKSTVTSDDNDNEYTTELELSPFSTIRRE
STFFKGAITPVLLLCICRPLGQAFDCCCCCCECGGASEASAANGSDNKLKTEVSSSIYFHKPRE
***      *****      *      *      *      *      *      *      *      *      *      *      *      *

MSTFASVGTCH
SPPLPLGTPC
** *

PAEL-R
GPR37L1

```

Figure 8: Sequence alignment of Human PAEL-R and GPR37L1. Transmembrane domains are highlighted in green and the strong conserved residues are represented by asterisk symbol. The terminal four amino acids (-GTPC), which are suggestive of a putative PDZ type 1 motif in GPR37L1. The highly similar c-terminal indicates similar interaction properties of GPR37L1 and PAEL-R.

```

MPAPGAPLSRSTRLLLLLFFKVSVAALS FVPEPRNGTCLGESCS P-LIPRRSRDAGGPRNSARDA
MRAPGALLARMSRLLLLLFFKVSASSALGVAPASRNETCLGESCAPTVIQRRGRDAWGPGNSARDV
MRWLWPLAVSLAVVLA VGPSEVSGAATLSLGGHRAKVQE-----QQSRPRRGTKDEGPKE

LRVHVPREKLEAEVRGATSWDLPPPRGGD--TGVIEEAAA SGPLGPPTKPPGAWRWKGAQ GKEPSG
LRARAPREEQGA AFLAGPSWDLPAAPGRDPAAGRGA EASAAGPPGPTRPPGPWRWKARGQEPSE
VQHYPV-----EWA EYP--KPIHPAG-----LQPTK

HLGRREPTDSQLFRQTSERGEMSSKRDEIQGSQEH SVKTEPEPRDLFYWPRKTGQLQGSHYRPS-
TLGRGNPTALQLFLQISEEEEK GPRGAGISGRSQEQSVKTVPGASDLFYWPRRAGKLGSHHKPLS
PL-----VATSPN-----PDKDGATSES-----

-----AVHEGRTLAPPRALPQNGSADDWVPDQGGPRRGNSTNRRVRLKNPFYPLTQESYGAYAV
KTANGLAGHEGWTTIALPGRALAQNGSLGEGIH EPGGPRRGNSTNRRVRLKNPFYPLTQESYGAYAV
-----GQELRTN-----LTGTPSQR LQIQNPLYPVTESSY SAYAV

MCLSVVIFGTGIIGNLAVMCIVCHNYMRSISNSLLANLAFWDFLI IFFCLPLVIFHELTKKW LLE
MCLSVVIFGTGIIGNLAVMCIVCHNYMRSISNSLLANLAFWDFLI IFFCLPLVIFHELTKKW LLE
MLLALVVFVAVGIVGNLSVMCIVWHSYYLKS AWNSILASLALWDFLVLFCLPIVIFNEITKQRLLG

DFSKIVVPYIEVASLGVTTFTLFCALCIDRFRAATNVQMY YEMIE NCSSTTAKLAVI WVGALLLALP
DFSKIVVPYIEVASLGVTTFTLFCALCIDRFRAATNVQMY YEMIE NCSSTTAKLAVI WVGALLLALP
DVSCRAVPFMEVSSLGVTTFTSLCALGIDRFHVATSTLPKVRPIERCQSILAKLAVI WVGSMM LAVP

EVLRLQLSKEDLGFSGQAPAERCVIKISPDLPDTIYVLAVLTYD GARLWVYFGCYFCLPTLFTITCS
EVVLRQLSKEDLGFSGRAPAERCIKISPDLPDTIYVLALTYDSARLWVYFGCYFCLPTLFTITCS
ELLLWQLAQEPTPTMG--TVDSCIMKPSADLPESLYSLVMTYQ NARMWVYFGCYFCLPI LFTVTCQ

LVTARKIRKAEKASTRGNKRQIHLESQMNC TVVALTILYGF CII PENICNIVTAYMATGVSQQTMD
LVTARKIRKAEKACTRGNKRQIQLESQMNC TVVALTILYGF CII PENICNIVTAYMATGVSQQTMD
LVTWRVRGPPGRKPECRAGRHEQCESQLNSTVVGLTVVYAFCTLPENICNIVVAYLSTELTRQTL D

LNIISQFLLFFKSCVTPVLLFCLCRPFSRAFMECCCCC -EECIQKSSTVTSDDNDNEYTTELELS
LNIISQFLLFFKSCVTPVLLFCLCKPFSRAFMECCCCC -EECIQKSSTVTSDDNDNEYTTELELS
LGLINQFSTFFKGAITPVLLLCICRPLGQAFLDCCCCCCECGGASDSSATVSADSKLKA EVSSS

PFSTIRREMSTFASVGTHC CNS1
PFSTIRREMSTFASVGTHC PAELR
IYFHKPRESPELLPLGTPC CNS2

```

Figure 9: Multiple alignment of PAEL-R with CNS1 and CNS2. Human PAEL-R (015354) is aligned with CNS1 (Rat_AF087946.1) and CNS2 (Rat_AF087947.1). No consensus (in black), conserved weak group (in dark blue), conserved strong groups (in green) and single, fully conserved residues (in light blue) are highlighted. Dashes indicate gaps introduced to maximise sequence alignment.

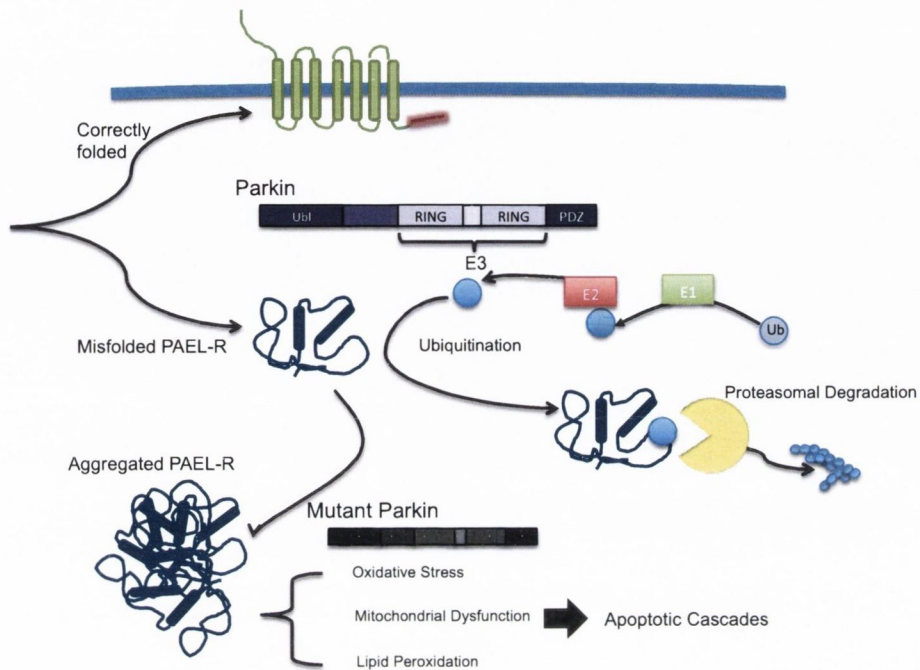


Figure 10: Schematic diagram of PAEL-R function. PAEL-R is a substrate of parkin, mostly correctly folded PAEL-R is transferred to the cell surface and misfolded PAEL-R is ubiquitinated by parkin, degraded by the proteasome. When parkin is mutated, PAEL-R aggregates, which activates apoptotic cascade pathways and finally leads to cell death.

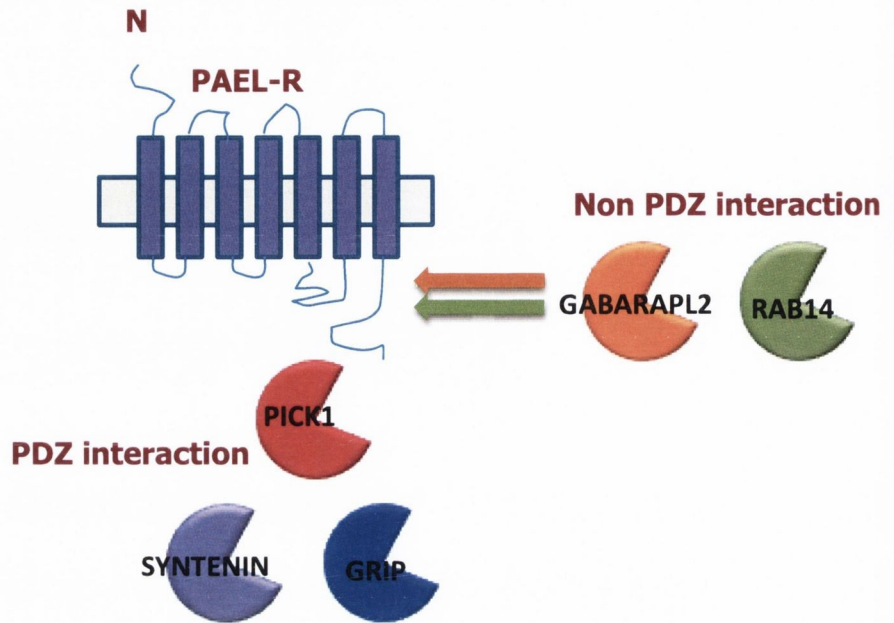


Figure 11: Schematic diagram of PAEL-R interacting proteins identified. The diagram indicates both PDZ (PICK1, GRIP and Syntenin) and non PDZ interactions (GABARAPL2 and RAB14) identified in this current thesis.

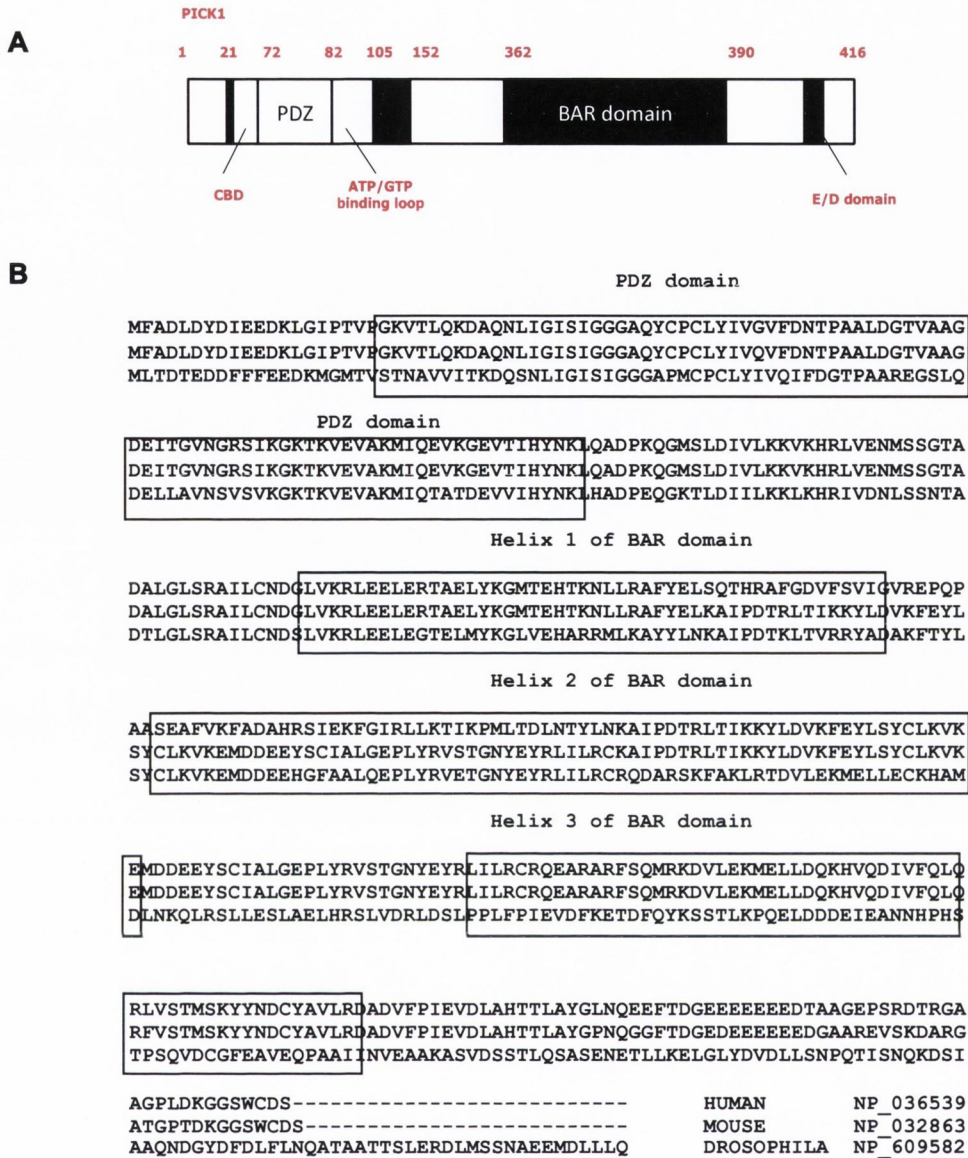
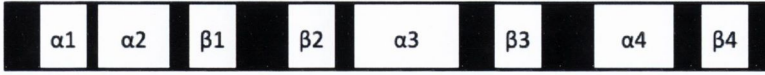


Figure 12: Structure of PICK1. (A) PICK1 structure including its CBD domain, PDZ domain, AHD and BAR domain and E/D acidic region. (B) Sequence alignment of PICK1 proteins. The human PICK1 (NP_036539) is aligned with mouse (NP_032863) and drosophila (NP_609582). PDZ domain and Bin/amphiphysin/RVS (BAR) domains are highlighted with alignment.

Interacting protein	Binding sequence	Detection approach	Ref.
Type I-X-S/T-X-			
GluR5, Kainate receptor, GluR5/6	QRKETVA/PGKETMA	Y2H, in-vitro	1
mGluR4a/8b, metabotropic glutamate receptor	VKSGSTS	Y2H	2
Glutamate transporter, GLT1b	PDZ – ETCI	In-vitro	3
Kalirin-7, neuronal Rho-GEF	DPFSTYV	Y2H	4
PKC α	PLLQSAV	Y2H, in-vitro	5
Type II-X-Φ-X-Φ			
DAT, dopamine transporter	LRHWLKV	Y2H, in-vitro	6
E3 ubiquitin ligase, parkin	PDZ-WFDV	Y2H, in-vitro	7
GluR2/3/4c, AMPA receptor subunit	GIESVKI/GTESVKI/GTESIKI	Y2H, in-vitro, in vivo	8
mGluR7a/b, metabotropic glutamate receptor	SYNNLVI/YTIPPTV	Y2H, in-vitro, in vivo	9
Syntenin	PDZ-IPEV	Y2H, in-vitro	10
Others			
mGluR8a/b, metabotropic glutamate receptor	TYTNHAI/SYSDHSI	Y2H	11
GRIP	via BAR domain	In-vitro	12
KAR interacting protein GluR6, KRIP6	Not known	Not known	13

Figure 13: Interacting proteins of PICK1. Major PICK1 interacting proteins are listed in the table with binding sequence and detection approach (¹Hirbec et al., 2003; ²El Far O et al., 2000; ³Bassan et al., 2008; ⁴Penzes et al., 2001; ⁵Staudinger et al., 1999, 1997; ⁶Torres et al., 2001; ⁷Joch et al., 2007; ⁸Dev et al., 1999, Xia et al., 1999; ⁹Boudin et al., 2000, Dev et al., 2000, El Far O et al., 2000; ¹⁰Hirbec et al., 2002; ¹¹El Far O et al., 2000; ¹²Lu et al., 2005; ¹³Laezza et al., 2008).

A



B

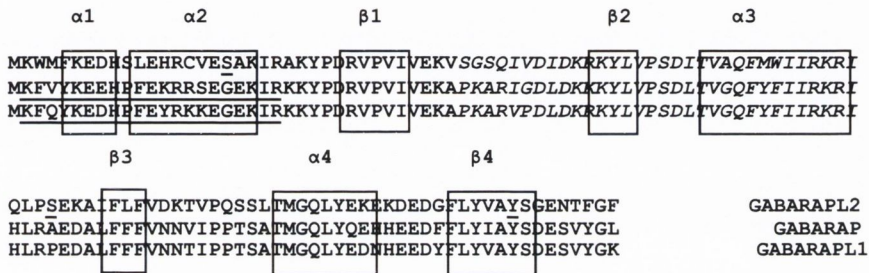


Figure 14: Structure and alignment of GABARAPL2. (A) Schematic diagram of GABARAPL2. UBL core domains ($\alpha 1$ - $\alpha 4$, $\beta 1$ - $\beta 4$) are shown in boxes. (B) GABARAPL2 (Human_NP_009216) sequence aligned with GABARAP (Human_NP_009209) and GABARAPL1 (Human_NP_113600). Tubulin binding domain is represented by underline and GABA_A receptor binding domain is highlighted in italics. Tyrosine kinase and protein C kinase phosphorylation sites are in bold and underlined.

Interacting protein	Detection	Function	Reference
GABA _A	Y2H	GABA _A -R trafficking and clustering	Chen et al., 2001
Gephyrin	In-vitro	Trafficking of GABA _A -R, vesicle transport along microtubules	Giesemann et al., 2003
NSF	In-vitro	Vesicle trafficking and membrane fusion	Kittler et al., 2001
Transferrin receptor	Y2H	Membrane protein degradation	Green et al., 2002

Figure 15: Interacting proteins of GABARAPL2. Represents few GABARAPL2 interacting proteins with the respective detection method and their functions.

A



B

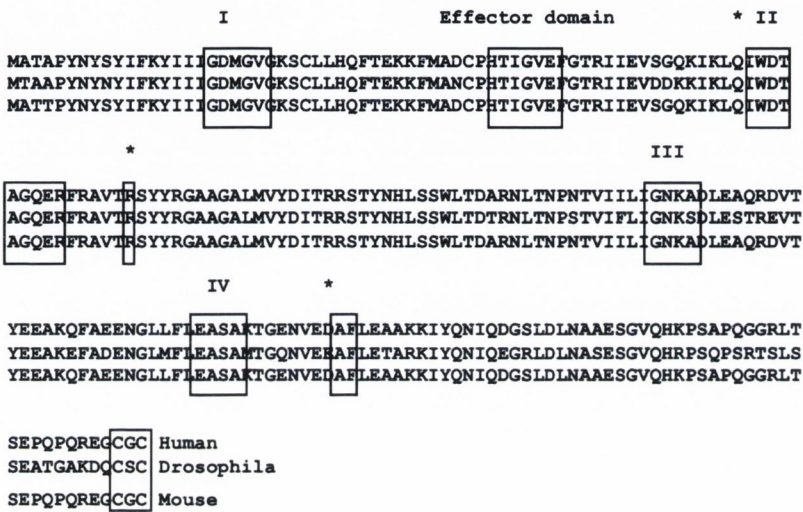


Figure 16: Structure of Rab14. (A) Structure of Rab14 with GTP binding domains and effector domain (E). (B) Sequence alignment of human_ NP_057406, drosophila_ NP_477171 and mouse_M083680 RAB14 proteins. I, II, III and IV represents GTP binding domains (highly conserved), effector domain (less conserved domain) and p21 ras (amino acid 32-40) is the site for interaction for the protein GAP.

Interacting protein	Detection	Function	Reference
Annexin A2	In-vitro	Surfactant secretion	Gou et al., 2008
TfnR (transferrin receptor)	In-vitro	Endosomal recycling compartment	Kelly et al., 2010
RUFY1, Rab4 effector	-	Endosomal fusion	Yamamoto et al., 2010
Class1, Rab11 family member	In-vitro	Endosomal recycling and Golgi/endosome transport processes	Kelly et al., 2010

Figure 17: Interacting proteins of Rab14. Table presents the most important interacting proteins of Rab14 with their functional role.

CHAPTER 2

MATERIALS AND METHODS

Materials and methods

1. Materials and equipment

The following materials were purchased: Acetic acid (45754-500ml, Sigma, St. Louis, MO), Agarose (A6013-100G, Sigma, St. Louis, MO), Ampicillin (A9518-100G Sigma, St. Louis, MO), Arabinose (A3256-25G, Sigma, St. Louis, MO), Ammonium acetate (A1542-500G, Sigma, St. Louis, MO), Ammonium persulfate (APS) (A3678-25G, Sigma, St. Louis, MO), ϵ -amino capronic acid (A7824-25G, Sigma, St. Louis, MO), *Bam*HI (10220612001, Roche, UK), Bovine serum albumin (BSA) (A3156-5G, Sigma, St. Louis, MO), Cell titer 96 aqueous one solution cell proliferation assay (G3582, Promega, Ireland), Calcium phosphate (C7263-500G, Sigma, St. Louis, MO), Carrier sperm DNA (D1626, Sigma, St. Louis, MO), CaCl₂ (C3306-100G, Sigma, St. Louis, MO), *Eco*RI (10703737001, Roche, UK), dNTP (10226020, Roche, UK), DO supplement FISH (630414-10G, Clontech, Madison, WI), DO supplement BAIT/FISH (630417-10G, Clontech, Madison, WI), DO supplement INTERACTION (630419-10G, Clontech, Madison, WI), Dimethyl sulfoxide (DMSO) (D2650-100ML, Sigma, St. Louis, MO), N,N-dimethylformamide (DMF) (D4551-250G, Sigma, St. Louis, MO), Ethylenediaminetetraacetic acid (EDTA) (A3156-5G, Sigma, St. Louis, MO), ethylene glycol tetraacetic acid (EGTA) (E3889-25G, Sigma, St. Louis, MO), Ethanol (E7023-500ML, Sigma, St. Louis, MO), Gel extraction kit (28704, Qiagen, UK), Glass beads (18406-500G, Sigma, St. Louis, MO), Glucose (G7528, Sigma, St. Louis, MO), GST beads (17-0756-01, GE, UK), Glycerol (G5516-100ML, Sigma, St. Louis, MO), Isopropyl β -D-1-thiogalactopyranoside (IPTG) (15502-1G, Sigma, St. Louis, MO), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (H3784-100G, Sigma, St. Louis, MO), Human fetal brain matchmaker cDNA library (638831-1ml, Clontech, Madison, WI), Lithium acetate (LiOAc) (L6883-250G, Sigma, St. Louis, MO), Luria broth (LB) media (L3152-1KG, Sigma, St. Louis, MO), β -mercaptoethanol (31350-010, Gibco, Carlsbad, CA), MegaX DH10B T1 Electrocomp cells (C6400-03, Invitrogen, UK), MgSO₄ (M2643-500G, Sigma, St. Louis, MO), Minimal SD Agar base (630412-467G, Clontech, Madison, WI), Minimal SD base (630411-267G, Clontech, Madison, WI), Drop out supplement BAIT (630413-10G, Clontech, Madison, WI), Mini-preparation Kit (27106, Qiagen, UK), Methanol (34966-2.5LT, Sigma, St. Louis, MO), Potassium choride (KCl) (P9541-500G, Sigma, St. Louis, MO), Poly Vinylidene Difluoride Membrane (PVDF membrane) (P2938, Sigma, St.

Louis, MO), SB buffer (161-0737, Bio-Rad, Hercules, CA), Na₂HPO₄ (S3264-500G, Sigma, St. Louis, MO), NaH₂PO₄ (S8282-500G, Sigma, St. Louis, MO), Nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (NBT and BCIP) (S3771, Promega, Madison, WI), Non-fat milk (Marvel, Tesco, Ireland), Sodium dodecyl sulfate (SDS) (L4390-25G, Sigma, St. Louis, MO), PBS (20012-019, Gibco, Carlsbad, CA), Pfu buffer & enzyme (15224-017, Invitrogen, UK), Phenol/Chloroform/Isoamyl alcohol (P3803-400ML, Sigma, St. Louis, MO), Polyethylene glycol (PEG) (P4391-1KG, Sigma, St. Louis, MO), Polyacrylamide (161-0158, Bio-Rad, Hercules, CA), Propidium iodide (PI) (P4170, Sigma, St. Louis, MO), Rotenone (R8875, Sigma, St. Louis, MO), *Saccharomyces cerevisiae* (AH109) (630444-1ml, Clontech, Madison, WI), Slide A-Dialysis cassette (66810, Fisher, Ireland), Sodium chloride (NaCl) (S3014-500G, Sigma, St. Louis, MO), Sucrose (84097-500G, Fluka, St. Louis, MO), Tetramethylethylenediamine (TEMED) (T7024-25ML, Sigma, St. Louis, MO), Triton X-100 (P9284-500ML, Sigma, St. Louis, MO), Tris (0497-1KG, Amresco, Cochran Solon, OH), Tris-EDTA (TE) (T9285-100ml, Sigma, St. Louis, MO), Tryptone (J859-500G, Amresco, Cochran Solon, OH), Tween-20 (P7949-500ML, Sigma, St. Louis, MO), T4 ligase kit (15224-017, Invitrogen, UK), Whatman papers (grade 5, 1003-919, Whatman, GE, UK), Whatman papers (grade 3, 1003-917, Whatman, GE, UK), Isopropanol (I9516-500ml, Sigma, St. Louis, MO), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (B4252-50MG, Sigma, St. Louis, MO), Yeast extract (J850-500G, Amresco, Cochran Solon, OH).

1.1 Antibodies

The following antibodies are used: c-myc rabbit Ab (A-14, sc-789, Santa Cruz Biotechnology, Santa Cruz, CA), donkey anti-Mouse Dylight 488-conjugated (715-485-150, Jackson ImmunoResearch, West Grove, PA), donkey anti-Rabbit Dylight 488-conjugated (703-505-155, Jackson ImmunoResearch, West Grove, PA), donkey anti-Rabbit DyLight 549-conjugated (711-505-152, Jackson ImmunoResearch, West Grove, PA), anti-Flag M2 monoclonal mouse (mAb) (F-3165, Sigma, St. Louis, MO), anti-GST goat Ab (27-4577-50, GE Healthcare, UK), anti-goat IgG AP conjugate (V1151, Promega, Madison, WI), anti-goat IRDye (926-32214, Licor, Biotechnology, Cambridge, UK), goat anti-mouse Alexa 633 secondary (A21050, Invitrogen, UK), goat anti-rabbit Alexa 633 secondary (A21070, Invitrogen, UK), goat anti-Mouse Dylight 549-conjugated (115-506-068,

Jackson ImmunoResearch, West Grove, PA), anti-MBP mouse Ab (E8032S, New England Biolabs, Beverly, MA), anti-mouse IgG AP conjugate (S3721, Promega, Madison, WI), anti-mouse IRDye (926-32210, Licor, Biotechnology, Cambridge, UK), mouse anti-Actin Ab (AM 2021, ECM Biosciences, Versailles, KY), anti-PICK1 rabbit Ab (SC-11410, Santa Cruz Biotechnology, Santa Cruz, CA), anti-rabbit IgG AP conjugate (S3731, Promega, Madison, WI), anti-rabbit IRDye (926-32221, Licor, Biotechnology, Cambridge, UK), anti-VSV mouse AB (ab1874, Abcam, Cambridge, MA).

1.2 Equipment

The following equipment was used: Electroporator (Biorad, Ireland), Glass teflon homogenizer (Fisher, Ireland), Incubator (Binder, Mason Technology, Ireland), Polymerase chain reaction (PCR) machine (Applied Bioscience, California, USA), Powerpack (Biorad, Ireland), Shaker (New Brunwich Scientific E24 Incubator, Mason, Ireland), Spectrophotometer (Biophotometer, Eppendorf, Germany), Small bench centrifuge (Hermle, Mason Technology, Ireland), Sonicator (Sonics, Vibra-cell, UK), Ultraviolet (UV) transilluminator (Syngene, UK), Synergy HT multimode microplate reader (BioTek, Mason Technology, Ireland), Ultracentrifuge (Hettich Rotina 380R, Mason, Technology, Ireland), Water bath (UAB 12 EU Grant, Ireland).

2. Yeast-two hybrid

Yeast two hybrid studies were first described by Fields and Song in 1989 (Fields and Song, 1989) can be used to investigate interactions between two known proteins, or can be used as a screening tool to identify interactions between a known protein and unknown proteins encoded by a cDNA library (Chien et al., 1991). In the yeast two hybrid system transcription factors contain binding and activation domains (BD and AD). The binding domain binds to a specific DNA sequence, while the activation domain interacts with the RNA polymerase II complex (Fig 4).

In the Y2H system used in our study the yeast transcription factor GAL4 of *Saccharomyces cerevisiae*, activates the LacZ gene which encodes the enzyme β -galactosidase required for galactose metabolism. The GAL4 transcription factor is also linked to His3 and Ade2 reporter genes that act as auxotrophic markers. The n-terminus of the DNA binding domain of GAL4 is fused to protein X (also called BAIT) in one plasmid, while the c-terminus activation domain of GAL4 is fused with protein Y (also called as FISH) in another plasmid. The plasmids are co-transformed into a yeast strain, where the proteins are expressed. If protein X and protein Y interact, the binding domain and activation domain are brought into close proximity, which will activate transcription of His3 and LacZ reporter gene. The yeast not capable of synthesising histidine or adenine and is unable to grow in media deficient in these amino acids. When protein X and Y interacts, GAL4 stimulates His3 and Ade2 expression and allows the yeast to grow on media deficient in these amino acids. LacZ encodes for β -galactosidase (β -gal), which can be detected using a β -galactosidase assay, the positive interacting yeast clones turn blue in colour in the presence of X-Gal .

2.1 Preparation of competent yeast

A glycerol stock of yeast strain *Saccharomyces cerevisiae* AH109 was removed from -80°C freezer and streaked directly onto a fresh YPAD-amp plate (1% Bacto-yeast extract, 2% peptone, 0.01% Adenine, 2% Agar autoclaved and supplemented with 2% D-Glucose and 50mg/ml Ampicillin). The plate was incubated for two days at 30°C , thereafter a single colony of 2-3mm in diameter was re-streaked onto a fresh YPAD-amp plate. This plate was again incubated for two days at 30°C . Subsequently, a single AH109 yeast colony was inoculated in 10ml

YPAD media and incubated with shaking at 200 rpm overnight at 30°C (New Brunswick Scientific E24 Incubator) in 50ml conical centrifuge tube, while it was shaking at 200 rpm. The initial OD₆₀₀ concentration was determined using a spectrophotometer. This was subsequently diluted to an OD₆₀₀ of between 0.2-0.4 in 50mls of YPAD media. The yeast was left to grow in a 1L baffled flask for 2-6 h at 30°C. After the final OD₆₀₀ reached 0.8-1.0, the yeast cells were harvested by centrifugation at 1,000xg for 3 min at room temperature. The pellet was re-suspended in 1ml TE (10 mM *Tris*-HCl, pH 8.0; 1 mM *EDTA*) and subsequently centrifuged at 1,000xg for 3 min at room temperature. The pellet was resuspended in 2ml of LiOAc solution (10mM LiOAc made in TE) and incubated for 10 min at room temperature. These competent yeasts were then used for cDNA transformation.

2.2 Transformation of competent yeast

Approximately 1µg plasmid BAIT cDNA (pGBKT7 vector) (**Fig 6**), 1µg plasmid FISH cDNA (pGADT7 vector) (**Fig 6**), 1mg/ml sheared, denatured herring testis carrier sperm DNA and 100µl of freshly prepared competent AH109 were mixed together in a 1.5ml eppendorf tube. Several controls were kept to determine protein interactions as well as efficiency of the transformation. Next, 700µl of PEG solution (100mM LiOAc, 40% PEG made in 1xTE-HCl) was added and mixed well to evenly distribute the cells. The tubes were then incubated while shaking at 30°C at 200-250 rpm for 30 min at 30°C. After incubation, approximately 88µl of DMSO was added and was mixed well. The cells were given a heat shock treatment in a water bath at 42°C for 7 min and centrifuged at 1,000xg for 10 sec at room temperature. The pellets were resuspended in 1ml TE. The cells were centrifuged at 1,000xg for 10 sec at room temperature and resuspended in 50µl TE. The transformed AH109 was then spread onto autoclaved minimal SD Agar base plates (46.7g/L, pH 5.8) supplemented with 50mg/ml Ampicillin, 0.01% Adenine with addition of BAIT (0.74g DO/-Trp supplement)/FISH (0.69g DO/-Leu supplement)/BAIT-FISH (0.64g DO/-Trp/-Leu supplement)/INTERACTION (0.6g DO/-Trp/-Leu/-His/-Ade supplement). The yeast was left to grow for 7 days at 30°C. The positive BAIT-FISH interactions were examined using a β-gal assay.

2.3 Large scale yeast two hybrid

AH109 yeast transformed with ct-PAEL-R bait was removed from the 30°C incubator and a single, fresh colony of approximately 2-3mm in diameter was inoculated in 50ml of BAIT media. This was incubated in a sterile 1L conical, baffled glass flask and incubated while shaking at 200-250rpm for 12-14 h at 30°C until an OD₆₀₀ of 0.8 was reached. The culture was divided into two 25ml samples, in 50ml falcon tubes, and the PAEL-R BAIT cells were harvested by centrifugation at 600xg for 5 min at room temperature. The pellets were resuspended in 5ml BAIT media. The cells were counted using a hemacytometer and result in an average cell density of 1 x 10⁹ cells/ml. A 1ml aliquot of pre-transformed human fetal brain matchmaker pACT2 FISH Y187 yeast strain library (**Fig 6**) was removed from the -80°C freezer and thawed at room temperature. In a sterile 2L baffled flask, 1ml of the pre-transformed library was combined with the 5ml PAEL-R bait cells. This mix was supplemented with 45ml of 2xYPAD media and was incubated while shaking at 30-50 rpm for 20-24 h at 30°C, allowing the yeast to mate. Thereafter a drop of the sample from the culture was examined under a phase contrast microscope (40x) to investigate the presence of zygotes. If zygotes were not present, the yeast culture was allowed to continue mating for an additional 4 h. A zygote typically has a three-lobed shape, the lobes representing the two haploid (parental) cells and the budding diploid cell. The mating mixture was transferred into a falcon tube and centrifuged at 1,000xg for 5 min at room temperature. The pellet was resuspended in 10ml of 0.5x YPAD media. Next, 2ml yeast culture was plated per 150mm INTERACTION plates. The mating efficiency was determined by spreading 1x, 10x, 100x dilution of the mating mixture on three different BAIT, FISH, BAIT-FISH 100mm plates. Plates were incubated for 7-10 days at 30°C. The positive interactions were examined using a β-gal assay and yeast plasmids were isolated by phenol/chloroform/Isoamyl extraction for positive interactions.

2.4 Plasmid preparation of yeast DNA

A single yeast colony was inoculated in 10ml of FISH media and incubated while shaking at 200 rpm for 12-14 h at 30°C. A 20% glycerol stock was prepared by adding 800µl of 20% glycerol and 300µl of yeast culture. The remaining overnight culture was centrifuged at 1,000xg for 10 sec at room temperature. The supernatant was discarded and the pellet was re-suspended in 200µl solubilisation buffer (1% SDS, 0.1M NaCl, 0.01M Tris pH8, 0.001M EDTA, 1% TritonX100). To

this, 200µl PCI (25:24:1 phenol/choloroform/isoamylalcohol) and 300µl glass beads (425-600 microns acid washed glass beads) were added. The tubes were then vortexed for 2 min and centrifuged at 1,000xg for 10 min at 4⁰C. The top layer contains the FISH plasmid and was removed into a new tube. To this, 200µl PCI was added and vortexed for 2 min then centrifuged at 1,000xg for 10 min at room temperature. The top layer was again removed into a new tube. Next, 500µl of autoclaved 7.5M ammonium acetate and 100µl isopropanol were added to the precipitate DNA. The solution was vortexed, stored at 4⁰C for 30 min and then centrifuged at 1,000xg for 10 min at 4⁰C. The supernatant was discarded and 250µl of 70% ethanol was added and centrifuged at 1,000xg for 10 min at 4⁰C. The ethanol was discarded and pellet was left to dry for half an hour. Finally, the FISH plasmid cDNA pellet was resuspended in 100µl H₂O. The FISH plasmid cDNA was stored at -20⁰C until it was required for transformation by electrophoration into *Escherichia coli* (*E.coli*).

2.5 β -galactosidase assay

Following a Y2H experiment the transformed yeast colonies growing on BAIT-FISH plates were removed from the 30⁰C incubator and examined using a β -galactosidase assay. A sterile 90mm circular Whatman filter paper was positioned on top of the yeast colonies and gently pressed downwards using a sterile tissue. When the filter paper sufficiently absorbed the yeast colonies, it was removed and placed with the colonies facing upwards, on a pre-cooled boat made of aluminum. The aluminum boat was lowered into liquid nitrogen and was immersed fully for 5 seconds. Next, 150µl of Z-buffer (60mM Na₂HPO₄, 40mM NaH₂PO₄, 10mM KCl and 1mM MgSO₄) and 20mg/ml X-Gal (made up in DMF) were pre mixed and added to the lid of a petri dish and a second whatman filter paper was lowered over this. The filter paper with the attached yeast was subsequently removed from the liquid N₂ and placed onto the filter paper soaked in the Z-buffer, avoiding the formation of air bubbles. The petri dish was closed and incubated for 1 h at 30⁰C. A dark blue colour indicated the presence of protein interactions. The petri plate was incubated for an additional 1 h at 30⁰C to test the presence of weaker interactions, which appeared as a light blue colour.

3. Molecular biology procedures

3.1 Polymerase chain reaction, restriction digestion and DNA purification

PCR amplification was carried out using 50ng template DNA (**Fig 3**) in a final reaction volume of 20µl containing 200µM of each dNTP, 10pmoles of forward and reverse primer (**Fig 1, 2**), 10x PCR buffer and 1 unit of Taq polymerase. PCR amplification was carried out for 30 cycles (denaturation: 94°C for 5 min; denaturation: 94°C for 30 sec; annealing: 55°C for 30 sec; elongation at 72°C for 30 sec). The final elongation was for 10 min at 72°C and the final reaction was then stored at 4°C. DNA markers were made to run along with PCR product on agarose gels (0.8-1%) which were made up in 1xTBE (40 mM Tris-borate and 1 mM EDTA) for appropriate size analysis of the amplified fragments. For restriction digestion, 1µg of DNA was digested with restriction endonucleases at 37°C for 1 h with 10x buffer recommended by supplier instructions (Roche). The reactions were set up in a volume of 20µl. DNA was isolated from *E.coli* using a Qiagen kit following the manufacturer recommended protocol or using methods described by Sambrook and Russel (Sambrook and Russel, 2001).

3.2 Agarose gel electrophoresis and DNA gel extraction

Agarose gel electrophoresis was carried out essentially as described by Sambrook and Russel (Sambrook and Russel, 2001). DNA fragments were resolved on 0.8 % agarose gels. Large DNA fragments (>1kb) were fractionated using 0.6–0.7% gels, whereas 1-2 % gels were run for analysing small fragments (<500bp). The 6x gel loading buffer (30% glycerol and 0.25% bromophenol blue) was added to DNA samples at a final concentration of 1x prior to loading onto the gel. The gels were electrophoresed in 1x TBE buffer at constant volts of 100 V for the resolution of DNA. Gel-Red (nucleic acid gel stain) was supplemented in the agarose gel for visualising DNA using a UV transilluminator in a long wave length (302nm). Agarose blocks containing the desired DNA fragments were excised out and transferred to a pre-weighed eppendorf microfuge tube. DNA was extracted using a Qiagen gel extraction kit, following the manufacture recommended protocol.

3.3 Preparation of chemically competent *E.coli* cells

Depending on the strain of *E.coli* being prepared, either a 50µl aliquot of DH5α *E.coli* was removed from the -80°C freezer and streaked directly onto fresh LB agar plate (1% Tryptone, 0.5% yeast extract, 1% NaCl, 2% agar, pH 7.5 and autoclaved). This was inverted and incubated for 12-14 h at 37°C. Subsequently, a single *E.coli* colony was inoculated in 5ml of LB (1% Tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5 and autoclaved) and incubated while shaking at 225 rpm for 12-14 h at 37°C.

Following this, 1ml of the overnight bacterial culture was aseptically transferred into a flask containing 100ml LB broth and incubated while shaking at 225 rpm for 12-14 h at 37°C. The culture was aseptically transferred into sterile pre-chilled 50ml tubes and centrifuged at 1,000xg for 10 min at 4°C. The media was decanted and resuspended in 20ml of ice cold 100mM MgSO₄ and centrifuged at 3,000xg for 15 min at 4°C. The pellet was again resuspended in ice cold 2ml of 100 mM CaCl₂ with 15% glycerol. The resuspension was aliquoted 50µl in pre-cooled 1.5ml eppendorf tubes pre-chilled in liquid nitrogen. The tubes were stored in -80°C.

3.4 Heat-Shock bacterial Transformation

A single aliquot of 50µl of competent DH5α *E.Coli* was removed from the -80°C freezer and thawed on ice for approximately 30 min. 1µg of plasmid DNA was added in competent DH5α and was incubated on ice for 30 min. Following this, the cells were incubated in a water bath at 42°C for 1 min and immediately cooled on ice for 2 min. The *E.Coli* with DNA was supplemented with 1ml LB media (1% Tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5 and autoclaved). This was incubated with shaking at 250 rpm at 37°C for 1 hr. The cells were centrifuged at 10,000xg for 10 sec and cell pellet resuspended in 100µl LB. This 100µl transformed *E.coli* was then spread onto LB agar plate (ampicillin /kanamycin) (1% Tryptone, 0.5% yeast extract, 1% NaCl, 2% agar, pH 7.5 and autoclaved). The plates were left overnight at 37°C. The colonies were counted and used in the preparation and purification of plasmid DNA via alkaline lysis or by Qiagen's plasmid purification mini/midi kit instructions.

4. Cloning

To clone each construct takes approximately 2-3 weeks. The procedure of molecular cloning consists of two stages (1) joining a DNA segment of interest (insert) to a DNA molecule that is able to replicate (vector) and (2) transformation of the new plasmids into *E.coli*.

4.1 Insert preparation

The insert to be cloned is amplified using primers designed specifically for the fragment. After PCR reaction, an insert along with controls (no template DNA, forward primer and reverse primer only) is checked in a 1% agarose gel. The insert is purified using the Qiagen PCR purification kit as per manufacturer recommended protocol. The concentration of the purified insert is checked. In order to ligate the insert and vector, compatible sticky ends are required. This is achieved by restriction digestion of the insert and the vector. Subsequently, the digested insert is purified using the Qiagen PCR purification kit and is then ready for ligation with the vector.

4.2 Vector preparation

The vector is digested in the same manner as the insert, using restriction enzymes. The digested product is run in a 0.8% low melting point agarose gel and excised using a scalpel on a benchtop UV Illuminator. The excised vector band is stored in a -20°C freezer for 30 min prior to purification using the Qiagen agarose gel purification kit. The digested vector is dephosphorylated using calf intestinal phosphatase (CIP) to reduce self-ligation and followed by deactivation for 10 min at 70°C. The dephosphorylated vector is purified using the Qiagen gel purification kit and subsequently the vector is ready for ligation.

4.3 Ligation and electroporation

The digested and purified insert and vector are ligated using T4 Ligase. 1:3 ratio for vector:insert is commonly used for the ligation process. The ligated product is transformed by electroporation in electro-competent DH10β *E.Coli* cells. Subsequently the cells are plated onto LB_{AMP/KAN} agar plate and incubated overnight at 37°C. Growth is checked the following day and positive colonies are picked and inoculated in LB_{AMP/KAN} liquid media.

4.4 Screening for positive clones

The positive clone of interest is confirmed by PCR amplification of the insert and followed by a restriction digestion. Positive samples are sent for sequencing and sequencing results are then matched with the gene of interest using online National Center for Biotechnology Information (NCBI) blast software.

5. Biochemical analysis

5.1 Preparation of rat brain lysate

Approximately 5ml of homogenisation buffer (HB) (0.32mM sucrose supplemented with 4mM HEPES, 1mM EDTA and 1mM EGTA) was put in a chilled falcon tube after the tube was weighted. Brain/tissue sample was added (either frozen or wet weight) and weighted again. Brain weight was recorded. 20x volume of ice cold HB was added to the rat brain. On ice in a pre-cooled glass-teflon homogeniser the rat brain was homogenised until it was completely homogenised (10 passes). The homogenate was then transferred into a cooled 2 x 15ml falcon tube. The homogenate was centrifuged at 1000xg for 10 min at 4°C and the supernatant (S1) was transferred into a 15ml new falcon tube (pre-cooled). The sample was centrifuged at 48,000xg for 30 min at 4°C. Supernatant (S2) was discarded and pellet (P2) was resuspend in 5 vol of PTxE (PBS, 1% Triton X-100, 0.1 mM EDTA, pH 7.4) if brain was 1gm (5ml PTxE). Each sample was sonicated 10 times at 20% amplitude for 10 sec to lyse cells (no frothing), between sonications these were kept on ice (the sample should become transparent). The sample was rotated at 5 rpm at 4°C for 1 h to ensure solubilisation. Then it was centrifuged 100,000g for 30 min in 4°C pre-chilled centrifuge. 500µl soluble cell-lysate was transferred into a fresh 1.5ml tubes. Finally the cell lysate was frozen until it was required. 500µl cell lysate was used for pull down or co-immunoprecipitation (keep 20µl for Western blot check that is around 2% input) (**Fig 5**).

5.2 Preparation of bacterial cell sonicate

A single colony of BL21 *E.coli* transformed with glutathione S-transferase (GST) fused protein or maltose binding protein (MBP) fused protein were inoculated in 10ml YTamp (1.6% Tryptone, 1% Yeast extract, 0.4% NaCl, autoclaved and supplemented with 50mg/ml ampicillin) using a 50ml falcon tube and incubated while shaking at 200 rpm for 12-15 h at 37°C. The 1ml overnight culture was used to inoculate 100ml YTamp (prewarmed) (i.e. 1:100 dilution) in a 500ml flask and incubated with shaking at 200 rpm for 1-2 h at 37°C until the OD₆₀₀ reached 0.5 - 0.7. A 1ml sample of the culture was taken before induction with arabinose or IPTG. This sample was centrifuged at 6,000xg for 30 sec at 4°C and re-suspended in 1ml of PBS buffer and kept at -20°C for further analysis. IPTG was added to the rest of the culture to the final concentration of 1mM and incubated while shaking at

200 rpm for 4-5 h at 37°C. Again a 1ml sample was taken after IPTG induction and kept for further analysis. The induced culture was centrifuged at 1,000xg for 20 min at 4°C in two 50ml falcon tube. The pellets were frozen at -20°C or resuspended in 5 volume of PBS. The resuspended pellets were then incubated with 1mg/ml lysozyme and rotated at 3 rpm at 4°C for 1 h, then sonicated at 25% amplitude, 10 times for 10 sec. After each sonication the samples were placed on ice. Next 1% Tx-100, 0.1mM EDTA were added to the samples and the sonicate was rotated at 3 rpm for 1 h at 4°C to ensure solubilisation. The solubilised cell suspension was aliquoted and stored at -20°C until required for GST pull down studies.

5.3 Purification of protein expressed in bacterial cell

To harvest mammalian purified protein, 100ml bacterial culture of YTAmp media was prepared, similar to the procedure described above. The 100ml culture was divided into two 50ml sterile tubes and centrifuged at 3000xg for 20 min at 4°C to collect the bacterial pellet. Each pellet was resuspended in 10ml lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EGTA, 1mM EDTA, 0.5% Triton X-100, 1 unit protease inhibitor cocktail tablet for 10ml) 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 times for 1 min) with regular interval on ice for 1 min. Thereafter, the lysate was centrifuged at 22,000xg for 1 h at 4°C. The supernatant was collected and mixed with 2ml binding beads, prewashed with wash buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EGTA, 1mM EDTA) and resuspended in 50% slurry. Beads were added according to the protein of desire, i.e. maltose binding beads for MBP tagged proteins and glutathione sepharose beads for GST tagged proteins. The beads were rotated for 2 h at 4°C. Subsequently, the lysate was centrifuged at 1000xg for 1 min at 4°C. Beads were collected as pellet and twice washed with 15ml wash buffer. The bead slurry was transferred into 15ml tube and centrifuged at 10,000xg for 1 min at 4°C. The supernatant was discarded carefully. Then, 5ml of elution buffer (5mM HEPES pH 7.6, 0.1% β -mercaptoethanol, 1mM EDTA, 10% glycerol, 0.1% Triton X-100, 0.1mM EGTA) was added to resuspend the beads and incubated for 15 min at room temperature. The resuspension was again centrifuged at 10,000xg for 1 min at 4°C and supernatant was collected as fraction containing expressed protein. Again, the process was repeated by adding 5ml elution buffer and supernatant was collected. Next, 10ml of supernatant was dialysed overnight at 4°C using a dialysis

kit, following the manufacturer's instructions. At the end, purified proteins were aliquoted in a small volume and stored in -20°C till further use. The Bio-Rad protein assay kit with BSA as standard was used to determine protein concentrations.

5.4 GST pull down assay

Before use in a GST pull down experiment, the solubilised bacterial samples were thawed on ice and re-sonicated, then centrifuged at 13,000xg for 5 min at 4°C. Next, in an eppendorf tube, 500µl bacterial sonicate, 10mg/ml BSA and 20µl of GST beads were added and PTxE buffer was added to reach a final volume of 1ml. The samples were rotated at 3 rpm for 4 h at 4°C. Thereafter, the samples were centrifuged at 1,000xg for 30 sec at 4°C. The supernatant was removed without disturbing the GST beads and 1ml of PTxE buffer was added. The samples were centrifuged again at 1,000xg for 30 sec at 4°C and the supernatant was discarded. The washing step was repeated 3 times. Finally the supernatant was discarded and 20µl sample buffer (10% glycerol, 62.5 mM Tris-HCl, 2% SDS, 0.01 mg/ml bromophenol blue and 5% β-mercaptoethanol) was added. The samples were boiled for 2-3 min then centrifuged at 10,000xg at 4°C and were loaded in SDS-PAGE.

5.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out essentially according to the Laemmli protocol (Laemmli, 1970). The discontinuous gel system had 10% resolving gel (0.375M Tris, 0.1% SDS, 10% acrylamide, 0.1% APS and TEMED) and 4 % stacking gel (0.125M Tris, 0.1% SDS, 4% acrylamide, 0.1% APS and TEMED). Gels were run in 1x running buffer (25mM Tris, 0.192M Glycine and 0.1 % SDS) at a constant voltage of 100 V so that samples entered the resolving gel and were then run at 150 V. Protein molecular weight markers were run concurrently on the gels. The resolved proteins were visualised by staining of the gels with Coomassie brilliant blue solution followed by destaining or transfer onto a PVDF membrane for Western blot.

5.6 Western blot

The proteins were blotted on a PVDF transfer membrane using a semi dry blotting system. The PVDF membrane was activated by immersion in 100% methanol for

10 sec followed by immersion in Solution C (25mM Tris base, 0.02% SDS, 20% Methanol and 40mM ϵ -amino-capronic acid) for a further 15-60 min. Prior to use, 2 Whatman filters were immersed in Solution A (0.3M Tris base, 0.02% SDS and 20% Methanol), 2 Whatman filters in Solution B (25mM Tris base, 0.02% SDS and 20% Methanol) and 2 Whatman filters paper in Solution C. The electro-blot was assembled as follows; 2 Whatman filters soaked in Solution A, 2 filters soaked in Solution B, PVDF membrane, the retrieved protein gel and 2 filters soaked in Solution C. The electro blot was set at a constant current 90mA for approximately 90 min. The gel was subsequently stained in coomassie blue anionic dye. All blocking agents and antibodies were prepared in TBST buffer (25mM Tris, 150mM NaCl, 2.5mM KCL and 0.05% Tween-20, adjusted to pH 7.5) and 5% skimmed milk. The blots were blocked for 1 h at room temperature and incubated with the primary antibody for again 1 h at room temperature. The blots were washed in TBST 3 times for 10 min and incubated with the secondary antibody for 60 min. The blots were then washed 3 times for 5 min in TBST and visualised using alkaline phosphatase buffer (100mM NaCl, 5mM MgCl₂, 100mM Tris-HCl pH 9.5) or a Licor Odyssey scanner. For alkaline phosphate based visualisation, the blot was developed by incubation in BCIP and NBT substrate with alkaline phosphatase buffer (30 μ l BCIP, 60 μ L NBT in 10ml of alkaline phosphatase buffer). The list of antibodies used in this thesis is outlined in section 1.1.

6. Mammalian cell culture transfection

6.1 Preparation of coverslip

The coverslips were soaked in ethanol at 4⁰C for overnight and they were dried by placing upright in a petri dish. Poly-D-lysine to a concentration of 25µg/ml was prepared with distilled water and added to each coverslip. Coverslips were left for approximately 1 h. Poly-D-lysine was removed by suction. Coverslips were left air drying for about 30 min. Further the coverslips were used for 6 or 24 well plates.

6.2 Cell transfection

HEK293 were grown in T-75 flasks using Dulbecco's Modified Eagle Medium (DMEM) culture media (9.5 g/l DMEM, 3.5 g/l glucose, with 0.2 g/l sodium bicarbonate and 0.6 g/l L-glutamine) supplemented with 10% dialysed fetal bovine serum and 1% penicillin/streptomycin. At 70–80% confluency, HEK293 cells were plated in 6-multiwell plates at a density of 1–2x10⁵ cells per well and allowed to grow overnight at 37°C in a humidified, 5% CO₂ atmosphere. OPTIMEM containing 2 µg total DNA and 3µL fugene HD tranfection reagent (Roche) was added into DMEM media of HEK293 cells. 48 h later, the cells were prepared for affinity chromatography or for immunocytochemistry. For affinity chromatography, cells were scraped from 6-multiwell plates, the cell suspension centrifuged and the pellet was frozen at –20°C until required. For immunocytochemistry, cells were plated onto glass cover slips in 12-multiwell plates, grown overnight at 37°C and immunocytochemistry was performed.

Luke Michael Healy provided astrocytes used in this thesis.

6.3 Immunocytochemistry

This protocol is optimised for cells grown in 24-well plates on glass coverslips. The media was aspirated from the cells and washed for 1 min in 1ml PBS (sterile ice cold). Then the cells were fixed with 0.5ml 100% methanol (ice cold) for 10 min or with 0.5ml 3.7% PFA buffer (3.7% formaldehyde in 1 x PBS (pH7.4)) (ice cold) for 5 min at 4°C. This was followed by a washing step for 5 min in 1ml PBS at room temperature. The cells were permeabilised for 10min (max) in 300µl PTx buffer (0.1% Triton X-100 in 1 x PBS (pH 7.4)) at room temperature and were washed 2 times for 5 min in 1ml PBS. The non-specific binding was blocked by incubating cells in 0.5ml blocking buffer (10% normal goat serum, 2% BSA in 1 x PBS (pH

7.4) overnight at 4°C (or 1 h at room temperature). The blocking buffer was removed and incubated with 200µl primary antibody which was diluted in blocking buffer overnight at 4°C (or 3 h at room temperature). Thereafter another washing step followed which included three washes for 5 min with 1ml PBS at room temperature. Next the secondary antibody(s) of about 200µl diluted in blocking buffer were added and incubated for 2 h at room temperature. Further washing for 3 times for 5 min in 1ml PBS at room temperature was required. For nuclear staining and mounting, cells were incubated in 200µl Hoescht (1:1000 in PBS) for 15 min at room temperature. After washing for another 3 times for 5 min in 1ml PBS at room temperature, the cells were left in PBS for mounting. The coverslips were placed inverted onto a glass slide with 1 drop of glycerol mount (90% glycerol in 1 x PBS (pH 7.4)) or vectashield which was then sealed with nail polish. The slides were visualised using Olympus confocal microscopy (same confocal setting was used for all images taken).

6.4 Preparation of cell membrane

To check for endogenous or transfected protein expression a cell membrane preparation is sufficient. The cells were harvested by scraping them directly in their own media (approximately 2ml) on ice. The cell suspension was created by pipetting up/down gently. The cell suspension was transferred into 2ml tubes and centrifuged at 15,000 rpm for 5min at 4°C. The media was discarded with 200µl pipette tip. The pellet was resuspended in 200µl PTxE buffer for each 6 well plate. If the cell sample is gloopy (due to genomic DNA released from cells) then cells were sonicated 6 times for 10 sec at 20% amplitude, between sonication steps the sample was kept on ice. Finally 20µl of cell membrane was boiled with 20µl sample buffer and 20µl was loaded for Western blot (approximately 5% of a well).

6.5 Preparation of cell lysate for co-immunoprecipitation

For co-immunoprecipitation the following control (3 wells) protein A transfection (3 wells) protein B transfection (3 wells) protein A+B co-transfection (6 wells) were used. The cells were harvested by scraping them directly in their own media (2ml) on ice. A good cell suspension was achieved by pipetting up/down gently. The cell suspension was then transferred into 2ml tubes and centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was discarded and the remaining media was removed with a 200µl pipette tip. The pellet was resuspended in 200µl PTxE buffer for each

6 well plate to create cell membrane. The 3 times 200 μ l cell membrane were combined into fresh 1.5 ml tube (20 μ l was kept for Western blot that is approximately 5% of a well). The remaining 580 μ l cell-membrane was sonicated at 20% amplitude for 6 times for 10 sec, between sonications steps, the samples were kept on ice (the sample should become transparent). Further samples were rotated at 5 rpm at 4°C in cold room for 1 h to ensure solubilisation and centrifuged for 15,000rpm for 20 min at 4°C. 530 μ l soluble cell-lysate was transferred into a fresh 1.5ml tube (by avoiding the insoluble cell pellet) and 500 μ l cell lysate was used for pull down or co-immunoprecipitation (20 μ l was used for Western blot check approximately 2% input).

6.6 Cell Viability study

Propidium Iodide staining was carried out by using PI. PI intercalates into the DNA of dead cells but is excluded from live cells, it was detected when excited with 488nm and emission at 615–620nm. Approximately 5×10^4 cells were grown on coverslips on 24 well plates and transfected with selective plasmids using fugene HD tranfection reagent as recommended by the manufacturer. The cells were treated with PI at concentrations of 1 μ g/mL in triplicates and then incubated at 37°C for 48 h in a humidified, 5% CO₂ atmosphere. After 48 h coverslips were washed in PBS and fixed in 100% ice-cold methanol for 5 min. Again coverslips were washed 3 x 0.5ml with PBS buffer, placed inverted onto glass slides and visualised using Olympus confocal microscopy (setting of confocal was kept same in all experiment). In addition, a cell viability test was performed using a CellTiter 96 AQueous one solution assay. Approximately, 1×10^5 cells were grown in 6 well plates and transfected with selective plasmids using fugene HD tranfection reagent as recommended by the manufacturer. After transfection, ~5,000 cells were transferred to 96 well plates in a total volume of 100 μ l. The cells were then incubated at 37°C for 24 h in a humidified, 5% CO₂ atmosphere. The following day, cells were treated, in triplicates, with rotenone at concentrations of 10 μ M, 1 μ M, 100nM, 10nM, 1nM and 100 pM for 48 h. This was followed by addition of 20 μ l of the 96 AQueous one solution reagent per 96 well and incubation at 37°C for 1-4 h in a humidified, 5% CO₂ atmosphere. The absorbance was recorded at 490nm using a syngy HT multimode microplate reader.

6.7 Image and Statistical analysis

All the images were analysed for cell expression study by Image J1.42q (Wayne Rasband, NIH, USA). Approximately 100 fixed co-transfected cells from each coverslip were analysed for red, green, blue average fluorescent pixels on the same image (experiment was repeated three times and each time three coverslips were analysed for each condition). In regard with quantification of images, images are taken from confocal, same setting was used for taking all the images in different experiments. All statistical analysis was carried out using Prism 4 (GraphPad Prism, California, USA). Data are expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed by making a comparison between two groups, using suitable t test. A p value of less than 0.05 was considered statistically significant. Exact p values were quoted when possible to allow a wider interpretation of the experiment.

6.8 In-silico docking models

RosettaLigand is a docking software, identifies the active side and the best interacting side of the molecule, can bind to a specific position to inhibit its interaction and exhibit its activity (Meiler and Baker, 2006).

Drug score includes ADME (absorption, distribution, metabolism and excretion) activity of the drug molecule (Balani et al., 2005).

RMSD value is the root mean square deviation value, which identifies the superpose value of the two model. The calculation of RMSD value is based on the involvement of the atoms of target with respect of the template molecule. The good RMSD value is considered to be from the range of 0.00 to 0.99 (\sim 1.0) (Meiler and Baker, 2006).

E_{bind} (Kcal/mol) is the binding energy of the molecule. The energy of the molecule determine the stability of the molecule, lesser the energy greater the stability of the protein molecule or structure. It is used to identify the conformational changes of the protein and to predict the most stable structure. The energy used to plot in the "energy landscape" which includes local and global minima and maxima, which is the graphical representation of the conformational analysis, which changes due to the steric hindrance of the molecule (Meiler and Baker, 2006).

Primers	Sequence
F1_pGBKT7_PAEL-R	AAT GAA TTC CCC TTC AGT CGC GCC
R1_pGBKT7_PAEL-R	AAA GGA TCCTCA GCA GCA CTC CAT
F2_pGBKT7_PAEL-R	CCT GAA TTC TGC TGT TGC TGT GAG
R2_pGBKT7_PAEL-R	GCG GGA TCCTCA CTT CTG AAT GCA
F3_pGBKT7_PAEL-R	CCT GAA TTC TCT TCA ACG GTG ACC
R3_pGBKT7_PAEL-R	GGC GGA TCCTCA GTC ATT GTC ATC
F4_pGBKT7_PAEL-R	CCT GAA TTC AAC GAG TAC ACC ACG
R4_pGBKT7_PAEL-R	TAA GGA TCCTCA CGA GAG TTC GAG
F5_pGBKT7_PAEL-R	CCG GAA TTC CCT TTC AGT ACC ATA
R5_pGBKT7_PAEL-R	CAT GGA TCCTCA GGA CAT TTC ACG
F6_pGBKT7_PAEL-R	CCG GAA TTC CCT TTC AGT ACC ATA
R6_pGBKT7_PAEL-R	CAT GGA TCCTCATCA GCA ATG AGT
F1_pGEX_4T_1_PAEL-R	AAT GGA TCC CCC TTC AGT CGC GCC
R1_pGEX_4T_1_PAEL-R	CCG GAA TTCTCA GCA ATG AGT TCC
F1_pGADT7_RAB14	GTA GAA TTC ATG GCA ACT GCA CCA
R2_pGADT7_RAB14	AAA CTC GAGCTA GCA GCC ACA GCC
F1_pGADT7_RANBP9	ATA GAA TTC CCG CCC GCT CCC CCG
R2_pGADT7_RANBP9	CAC CTC GAGCTA ATG TAG GTA GTC
F1_pGADT7_GABARAPL2	GCG GAA TTC ATG AAG TGG ATG TTC
R1_pGADT7_GABARAPL2	ACG CTC GAGTCA GAA GCC AAA AGT

Figure 1: Primers ordered from MWG, Germany. For further information see (<http://www.eurofindna.com/home.html>). The restriction digestion sites and stop codon sequence are shown in bold.

Primers	Sequence
FP_GL2_A	CGG GAA TTC ATG AAG TGG ATG TTC AAG
RP_GL2_A	TGT CTC GAG TCA TCG AAT CTT CGC GGA
FP_GL2_B	CGG GAA TTC TCA GGC TCT CAG ATT GTT
RP_GL2_B	CTG CTC GAG TCA GAT CCT TTT CCT GAT
FP_GL2_C	CAG GAA TTC GCG AAA TAT CCC GAC AGG
RP_GL2_C	CAG CTC GAG TCA GAA GCC AAA AGT GTT
RP_GL2_D	CGG GAA TTC ATG AAG TGG ATG TTC AAG
RP_GL2_D	CGG CTC GAG TCA GAC CTT TTC CAC AAT
FP_RAB14_A	GCG GAA TTC ATG GCA ACT GCA CCA TAC
RP_RAB14_A	GCA CTC GAG TTA TGT CAA CCA GCT GCT TAA
FP_RAB14_B	GCG GAA TTC GAT GCA AGG AAT CTC ACC
RP_RAB14_B	GAC CTC GAG TTA ACA GCC ACA GCC TTC
FP_RAB14_C	GCG GAA TTC GAA GTT AGT GGC CAA AAA
RP_RAB14_C	GCG CTC GAG CTA ATT CTC TCC CGT TTT TGC
FP_pLL.4_PICK1	GCT AGC ATG GAC TAC AAG GAC GAC GAT
RP_pLL.4_PICK1	AC GAA TTC TCA GGA GTC ACA CCA
FP_pLL.4_PAEL-R	GCT AGC ATG CAG AAG CTG ATC TCA GAG
RP_pLL.4_PAEL-R	CCG GAA TTC TCA GCA ATG AGT TCC
FP_pLL.4_PAEL-R-mut	AGT ATA GAA TTC ATG GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG

Figure 2: Primers ordered from MWG, Germany. For further information see (<http://www.eurofindna.com/home.html>). The restriction digestion sites and stop codon sequence are shown in bold.

	Vector	Insert	Tag/Fusion	Restriction Sites	Promoter	Provided by
Y2H	pGBKT7	ct-PAEL-R	BD	BamH1/EcoR1	GAL4	KKD
Y2H	pGAD10	PICK1	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGAD10	PICK1-KE	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGAD10	PICK1-KA	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGAD10	PICK1-KD-AA	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGAD10	PICK1-1-358	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGAD10	PICK1-1-358KE	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGAD10	PICK1-1-305	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGAD10	PICK1-13-305	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGAD10	PICK1-13-305KE	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGAD10	PICK1-305-416	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGAD10	PICK1-305-358	AD	BgIII/BgIII	GAL4	KKD
Y2H	pGADT7	GABARAPL2	AD	EcoR1/Xho1	GAL4	Priyanka
Y2H	pGADT7	RAB14	AD	EcoR1/Xho1	GAL4	Priyanka
Y2H	pGADT7	GL2-1-22	AD	EcoR1/Xho1	GAL4	Priyanka
Y2H	pGADT7	GL2-1-36-68	AD	EcoR1/Xho1	GAL4	Priyanka
Y2H	pGADT7	GL2-22-117	AD	EcoR1/Xho1	GAL4	Priyanka
Y2H	pGADT7	GL2-1-36	AD	EcoR1/Xho1	GAL4	Priyanka
Y2H	pGADT7	RAB14-1-107	AD	EcoR1/Xho1	GAL4	Priyanka
Y2H	pGADT7	RAB14-108-215	AD	EcoR1/Xho1	GAL4	Priyanka
Y2H	pGADT7	RAB14-54-160	AD	EcoR1/Xho1	GAL4	Priyanka
Pull down	pGEX-PAEL-R	ct-PAEL-R	GST	BamH1/EcoR1	Lac	Priyanka
Pull down	pMAL-C2	GL2	MBP	EcoR1/Pst1	Tac	Priyanka
Pull down	pMAL-PICK1	PICK1	MBP	BamH1/EcoR1	Tac	Priyanka
ICC	pcDNAmycA	PAEL-R	Myc	EcoR1/Not1	CMV	Novartis
ICC	pc DNA5 TO	PAEL-R	VSV	BamH1/EcoR1	CMV	Novartis
ICC	pLL.4	PAEL-R	Myc	EcoR1/Nhe1	PGK	Priyanka
ICC	pLL.4	PAEL-R-mut	Myc	EcoR1/Nhe1	PGK	Priyanka
ICC	CORON	PAEL-R-mut	VSV	EcoR1/Nhe1	PGK	Priyanka
ICC	pLL.4	PICK1	FLAG	EcoR1/Nhe1	PGK	Priyanka
ICC	pLL.4	PICK1-KE	FLAG	EcoR1/Nhe1	PGK	Priyanka
ICC	pMyc-CMV	GL2	VSV	EcoR1/Xho1	CMV	Priyanka

Figure 3: List of Plasmids. Table indicates vector, insert, tag, restriction sites, promoter and whether clones are used for yeast two hybrid (Y2H), pull down or immunocytochemistry (ICC) experiments.

Transformed yeast cells with ct-PAEL-R (BAIT) and cDNA library (FISH)

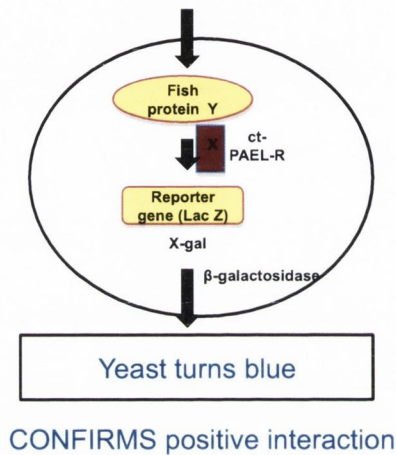


Figure 4: Principle of yeast two hybrid system. If protein X interacts with protein Y it leads to the transcription of the Lac Z gene, which results in the encoding of β -galactosidase. This can then be detected by using a β -galactosidase assay, the positive interacting yeast clones turn blue in colour in the presence of X-Gal .

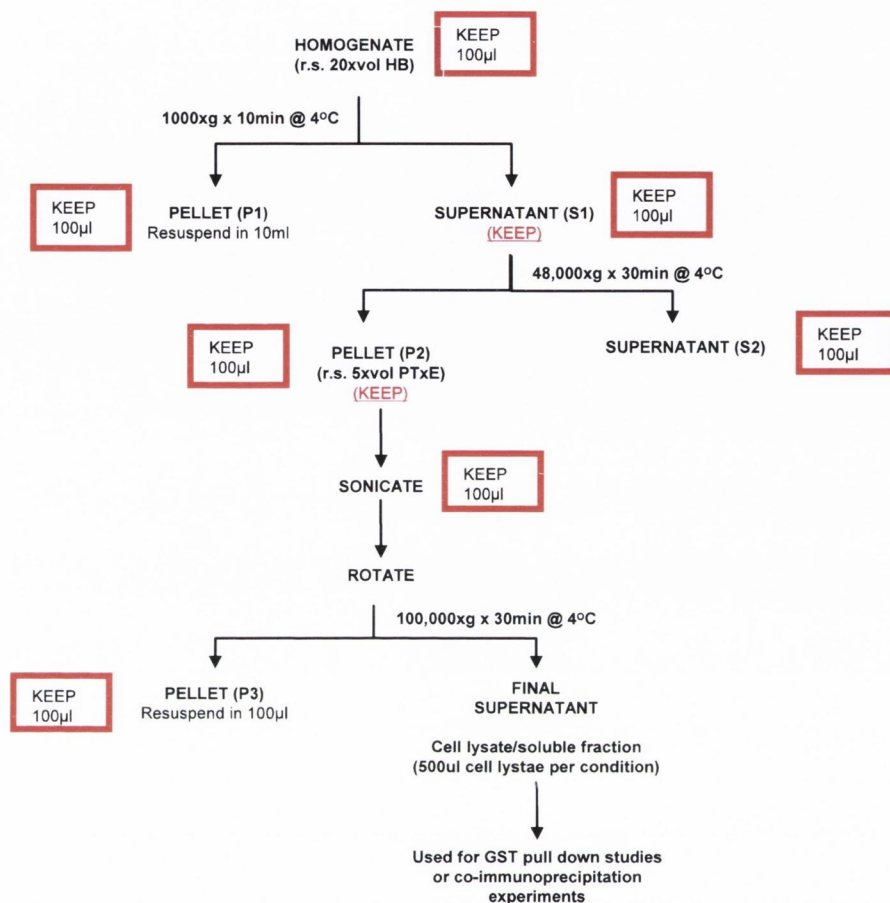
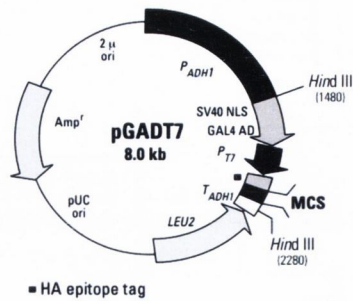
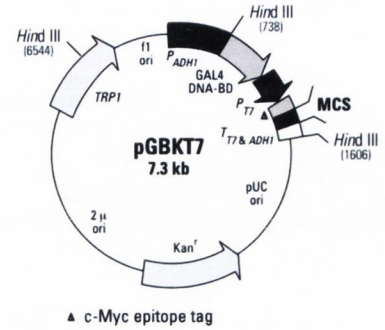


Figure 5: Schematic diagram for the preparation of the rat brain lysate. Rat brain lysate are used for pull down and biochemical studies.

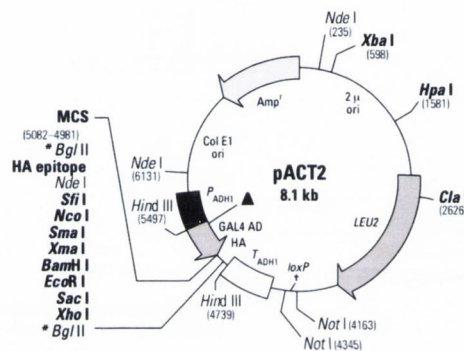
Y2H FISH PLASMID



Y2H BAIT PLASMID



Y2H FISH PLASMID (cDNA library)



MAMMALIAN EXPRESSION VECTORS

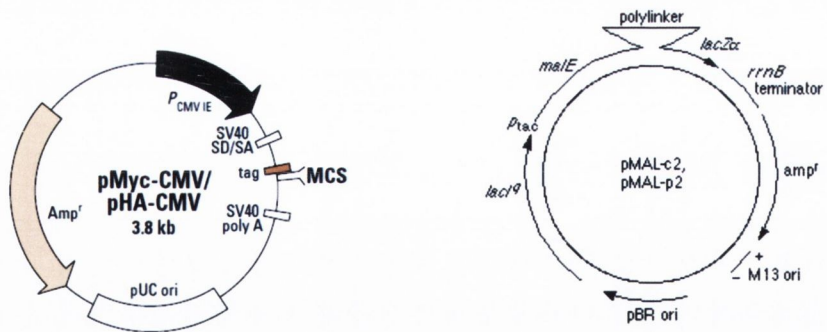


Figure 6: Vector Map. Cloning vector map of pGBTK7, pGADT7 and pACT2. Taken from www.clontech.com (FISH, BAIT, FISH cDNA library and pMyc-CMV plasmid) and www.neb.com (pMAL-C2 plasmid).

CHAPTER 3

**PICK1 INTERACTS WITH AND PROTECTS
AGAINST PAEL RECEPTOR INDUCED
CELL DEATH**

Aims

Aim1: to identify a novel interaction between PICK1 and PAEL-R by Y2H.

Aim2: to clone ct-PAEL-R fragments in pGBKT7 (BAIT) vector.

Aim3: to define a specific interaction site on PAEL-R by creating deletion constructs.

Aim4: to define a specific interaction site on PAEL-R by using PAEL-R mutant and to test the selectivity for other PAEL-R family members (GPR37L1).

Aim5: to define a specific interaction site on PICK1 by creating overlapping deletion constructs.

Aim6: to test the selectivity for other PDZ domain containing proteins (GRIP and Syntenin).

Aim7: to confirm the PICK1-PAEL-R interaction by in silico modeling.

Aim8: to clone ct-PAEL-R in pGEX-4T-1 (GST) vector.

Aim9: to biochemically validate the PAEL-R-PICK1 interaction.

Aim10: to test the specificity of PAEL-R antibody.

Aim11: to determine the effects of PICK1 on the expression level of PAEL-R.

Aim12: to determine the function of PICK1 on PAEL-R induced cell death.

Aim13: to test whether PICK1 plays a role against PAEL-R induced cellular toxicity during cell stress.

Abstract

The parkin-associated endothelial-like receptor (PAEL-R) is an orphan GPCR, initially named as GPR37. This receptor interacts with parkin and is degraded by parkin-mediated ubiquitination. Mutations in parkin are thought to result in PAEL-R accumulation and neuronal death in Parkinson's disease. Here we aimed to identify proteins that interact with and regulate the expression levels of the PAEL-R. In this study, we report a novel interaction between PAEL-R and protein interacting with C kinase (PICK1). Specifically, the PDZ domain of PICK1 was shown to interact with the extreme ct located PDZ motif of PAEL-R. Pull down assays indicated that recombinant and native PICK1, obtained from heterologous cells and rat brain tissue, respectively, were retained by a glutathione S-transferase fusion of ct-PAEL-R. PICK1 has previously been shown to bind parkin and PAEL-R is known to be degraded by parkin-mediated ubiquitination. We show that PICK1 wildtype caused a reduction in PAEL-R expression levels in transiently transfected heterologous cells compared to a PICK1 PDZ domain mutant, which does not interact with PAEL-R. Finally, PICK1 overexpression in HEK293 cells attenuated cell viability induced by PAEL-R overexpression during rotenone treatment. These results suggest a role for PICK1 in preventing PAEL-R-induced cell toxicity during conditions of cell stress.

Introduction

The PAEL-R, originally called GRP37, is an orphan GPCR. The PAEL-R interacts with and is ubiquitinated by the E3 ligase parkin, which promotes proteasomal degradation of the receptor (Takahashi and Imai 2003, Imai et al., 2001). Studies suggest that PAEL-R is inherently difficult to fold and when folded incorrectly causes ER stress, unfolded protein response and ER associated protein degradation to aid its removal (Takahashi et al., 2003). The overexpression of PAEL-R may also induce autophagy to clear PAEL-R aggregates (Marazziti et al., 2009). In addition to regulation by parkin, PAEL-R neurotoxicity is also attenuated by another E3 ligase human homology of yeast Hrd1p which interacts with and promotes PAEL-R ubiquitination and degradation in the ER (Omura et al., 2006). Moreover, the neuroprotective protein DJ-1/PARK7 (Yokota et al., 2003), the molecular chaperone Thioredoxin (Umeda-Kameyama et al., 2007) and the parkin coregulated gene (PACRG)/gene adjacent to parkin (Glup) (Imai et al., 2001) rescue cell death mediated by overexpression of PAEL-R. In addition, sodium 4-phenyl-butyrate reduces the amount of misfolded PAEL-R protein (Kubota et al., 2006), whereas metamphetamine-induced dopaminergic neurotoxicity is associated with suppressed gene expression of parkin and PAEL-R (Nakahara et al., 2003).

The PAEL-R is localised in the core of LBs and lewy neuritis, hallmarks of PD (Murakami et al., 2004). Individuals affected with autosomal recessive juvenile PD and possess mutations in parkin are unlikely to degrade PAEL-R where receptor aggregation may play a role in pathology (Imai et al., 2001, Murakami et al., 2004). When PAEL-R is expressed in dopaminergic neurons of drosophila, these neurons show selective degeneration (Yang et al., 2003). In PAEL-R tg mice, vesicular dopamine content is increased and the numbers of nigrostriatal dopaminergic neurons are reduced (Marazziti et al., 2004, Imai et al., 2007). Higher levels of dopamine are also observed in PAEL-R tg mice crossed with parkin KO mice (Wang et al., 2008). In contrast, PAEL-R KO mice show a decrease in the level of striatal dopamine, resistance to treatment with the neurotoxin MPTP, but increased sensitivity to amphetamine (Imai et al., 2007). Moreover, treatment with a tyrosine hydroxylase inhibitor can ameliorate dopaminergic cell death induced by PAEL-R overexpression (Marazziti et al., 2004, Kitao et al., 2007). Thus, both ER stress and excessive dopamine mediated oxidative stress likely contribute to the pathological role of PAEL-R in PD.

Studies suggest that the neuropeptide head activator is an endogenous ligand for PAEL-R (Rezgaoui et al., 2005), however this remains under debate (Dunham et al., 2009). To date, the development of PAEL-R compounds has been hampered by a lack of knowledge about the endogenous ligand. A limited understanding of the intracellular signaling coupled to the PAEL-R and its poor trafficking to the plasma membrane when overexpressed has also made the development of drugs for PAEL-R a challenge. Like other GPCRs, PAEL-R likely requires assembly with protein partners to achieve correct surface expression, post-translational modification and functional activity. Indeed, PAEL-R cell surface expression can be enhanced by removal of the first 210 amino acids from n-terminus (Dunham et al., 2009). Heterodimerisation with other GPCRs, such as the dopamine and adenosine receptors, also enhances PAEL-R surface expression (Dunham et al., 2009). In addition, presynaptic located PAEL-R can interact with and alter the function of DAT (Marazziti et al., 2007). This interaction modulates DAT-mediated DA uptake, where the lack of PAEL-R increases plasma membrane expression of DAT and enhanced DAT activity (Marazziti et al., 2007).

PDZ domains interact with the ct-located PDZ motifs of receptors, providing a molecular mechanism for regulating surface expression and post-translational modification. The PICK1 consists of 416 amino acids and has a molecular weight of approximately 50 KDa. Uniquely, PICK1 contains both a PDZ domain and a BAR domain. The single PDZ domain of PICK1 contains approximately 90 residues and interacts with the PDZ motifs of several enzymes, receptors, transporters and ion channels (Dev et al., 2004). The BAR domain of PICK1 allows for autodimerization (Xiao et al., 2007). Thus, via its PDZ and BAR domains, PICK1 acts as a scaffold/adaptor that associates with and brings together several proteins. The best studied receptors that interact with PICK1 are glutamate receptors including the AMPA receptor subunit GluR2 (Dev et al., 1999, Xia et al., 1999), the kainate receptor subunit GluR5 and 6 (Hirbec et al 2003) and the group III G-protein coupled metabotropic glutamate receptor subtype 7 (mGluR7) (Dev et al 2000, Dev et al., 2001, Boudin et al., 2000). It has been shown that removal of the ct-PAEL-R decreases surface expression of the receptor (Dunham et al., 2009). Importantly, ct-PAEL-R contains a class I type PDZ motif (-GTHC), which interacts with the PDZ protein syntenin-1 to increase cell surface trafficking (Dunham et al., 2009). Here, we studied additional proteins interacting with ct-PAEL-R and show a novel PDZ-based interaction between PAEL-R and PICK1. As well as playing a

role in mitochondrial function (Wang et al., 2003, Wang et al., 2007, Wang et al 2008), PICK1 interacts with DAT (Torres et al., 2001; Bjerggaard et al., 2004; Madsen et al., 2005) and parkin (Joch et al., 2007). This novel PAEL-R-PICK1 interaction provides further molecular evidence for a role of PICK1 in PD.

Results

1. Novel Interaction between PAEL-R and PICK1

To identify novel proteins that interact with the parkin-associated endothelial-like receptor (PAEL-R/GPR37), a yeast two hybrid study using the ct-PAEL-R as bait (PFSRAFMECCCCCEECIQKSSTVTSDNDNEYTTELELSPFSTIRREMSTFAS VGTHC) was conducted. A putative interaction was identified between ct-PAEL-R and the protein interacting with C kinase (PICK1) and was subsequently confirmed by transforming the full-length wild type PICK1 (1-416) fish construct into yeast harboring the ct-PAEL-R bait plasmid. The transformed yeast were grown on interaction plates at 30°C for 7 days then subjected to β -galactosidase reporter assays. Yeast co-transformed with ct-PAEL-R bait and PICK1 fish, but not the empty pGBKT7 bait and pGADT7 fish vectors, gave a strong β -gal reporter expression (blue colour) similar to yeast transformed with the positive controls ct-GluR2 bait and PICK1 fish (Dev et al., 2000) (**Fig 1**). The data also confirmed a positive interaction between the ct-PAEL-R bait and a full-length wild type parkin (1-465) fish construct as reported previously (Imai et al., 2001) (**Fig 1**). These data showed a novel interaction between ct-PAEL-R and PICK1.

2. An extreme ct located PDZ motif of PAEL-R is required for interaction with PICK1

The extreme ct-PAEL-R has been reported to contain a PDZ motif (-GTHC) and interact with the PDZ domain containing protein syntenin-1 (Dunham et al., 2009). In conventional PDZ-based protein-protein interactions, PDZ motifs are located at the extreme ct for binding within PDZ domains. To further verify the interaction between PICK1 with PDZ motif of the PAEL-R, we have designed five different overlapping deletion constructs of ct-PAEL-R: Δ 1 (555-565 amino acids), Δ 2 (555-575 amino acids), Δ 3 (555-585 amino acids), Δ 4 (555-595 amino acids) and Δ 5 (596-613 amino acids). These overlapping deletion constructs were cloned into the pGBKT7 vector (**Fig 2**). To confirm the site of interaction of PICK1 on ct-PAEL-R, a Y2H study using the ct-PAEL-R fragments as bait was carried out with full length PICK1. The data showed that the fragments were autoactivate and therefore not useful for Y2H experiments (**Fig 3**). To resolve the problem and to investigate

whether the PDZ motif of the PAEL-R is required for interaction with PICK1, an additional 10 residues were added to the extreme ct-PAEL-R (ct-PAEL-R+10) to occlude the PDZ motif. The ct-PAEL-R+10 bait showed no interaction with PICK1 fish indicating the requirement for a free extreme ct located PDZ motif of PAEL-R for interaction with PICK1 (**Fig 4**). The PAEL-R sequence has approximately 40% similarity in the transmembrane regions to mammalian peptide-specific GPCRs, particularly with Endothelin-B receptor, bombesin-BB1, and bombesin-BB2 receptors (Marazziti et al., 1997, Marazziti et al., 1998). PAEL-R is most closely related to another CNS-enriched orphan receptor known as GPR37L1. GPR37L1 shows 68% overall homology and 48% identity to PAEL-R (Valdenaire et al., 1998). Sequence analysis shows high sequence similarity between the ct domain of PAEL-R and GPR37L1 (**Fig 4**). Notably, there is a high degree of similarity in the last four residues of PAEL-R (-GTHC) and GPR37L1 (-GTPC), indicating a putative PDZ motif may also exist in GPR37L1. Therefore, the specificity of interaction of PAEL-R and GPR37L1 with PICK1 was investigated in yeast. The data showed, that in addition to PAEL-R, the GPR37L1 also interacted with PICK1 (**Fig 4**). Taken together, these studies suggest that the PAEL-R and the GPR37L1 contain extreme ct located PDZ motifs that are capable of interaction with PICK1.

3. The ct-PAEL-R interacts with PDZ domain of PICK1

To determine the site of interaction on PICK1, the ct-PAEL-R and GPR37L1 baits were transformed with a number of PICK1 fish truncated mutants and tested for their ability to interact in yeast (**Fig 5**). Residues 13-358 of PICK1 contain the PDZ domain, which is sufficient for interaction with many of its interacting receptors, for example GluR2 and mGluR7 (Dev et al., 1999, Dev et al., 2000). This truncated form of PICK1 was found to interact with ct-PAEL-R and ct-GPR37L1, similar to the control ct-GluR2 (**Fig 5**). In contrast, a truncated form comprising residues 1-305 that still contains the PDZ domain did not interact with ct-PAEL-R, ct-GPR37L1 or ct-GluR2. The lack of an ability for this PICK1 (1-305) fragment to interact may be due its aberrant folding or poor expression levels in yeast as previously suggested (Staudinger et al 1997, Dev et al., 1999, Dev et al., 2000). The truncations of PICK1 comprising residues 305-416 (containing the acidic region) or residues 305-358, both of which lack the PDZ domain, did not interact with ct-PAEL-R, ct-GPR37L1 or ct-GluR2 (**Fig 5**). Three separate point mutations,

K27E, K27A, and KD27/28AA in the carboxylate binding motif of the PDZ domain of PICK1 have been shown to abolish the ability of PICK1 to interact with its receptor partners (Dev et al., 1999, Dev et al., 2000, Staudinger et al., 1995, Staudinger et al., 1997, Xia et al., 1999). In agreement, none of these PICK1 mutants interacted with ct-PAEL-R or ct-GPR37L1 suggesting the PDZ domain of PICK1 is required for interaction (**Fig 5**). Similar results were found with the positive control ct-GluR2 using these point mutated PICK1 constructs. This data suggested that ct-PAEL-R and ct-GPR37L1 interact with the PDZ domain of PICK1.

4. The PDZ motif of ct-PAEL-R and GPR37L1 interact with additional PDZ domain proteins

Previous studies have shown that PDZ motifs located at the extreme ct of receptors can bind to multiple PDZ domain containing proteins, for example the kainate receptor subunit GluR5, which interacts with PICK1, GRIP and syntenin-1 (Hirbec et al., 2002). Since ct-PAEL-R has been shown to previously interact with the PDZ domain of syntenin-1 (Dunham et al., 2009) and we find it also interacts with the PDZ domain of PICK1, its ability to interact with other PDZ domain proteins was examined. Specifically the interaction between ct-PAEL-R and GRIP or syntenin was investigated. In agreement with a previous report (Dunham et al., 2009), the data confirmed an interaction between ct-PAEL-R and syntenin (**Fig 6**). Importantly, the results also showed an additional interaction between ct-PAEL-R and GRIP (**Fig 6**). In contrast, the ct-PAEL-R+10 mutant showed no interaction with syntenin or GRIP, indicating the requirement for an extreme ct-located PDZ motif of ct-PAEL-R. These results suggest that the PDZ motif of ct-PAEL-R interacts with several PDZ domain containing proteins, including PICK1, syntenin and GRIP. Since our data suggested that ct-GPR37L1 contained a PDZ motif (**Fig 4**), we inferred that ct-GPR37L1 would also interact with other PDZ domain containing proteins. In agreement, the GPR37L1 interacted with both syntenin and GRIP, similar to the ct-PAEL-R (**Fig 6**). Taken together, the data are supportive of the identification of a PDZ motif (-GTPC) in ct-GPR37L1 and indicate that both PAEL-R and GPR37L1 have similar and multiple PDZ domain interacting partners.

5. *In silico prediction of binding affinities*

To investigate the relative strength of binding between ct-PAEL-R and its interacting PDZ domain containing proteins, a flexible docking protocol using ROSETTALIGAND was applied. The PDZ motif (ligand) of ct-PAEL-R was docked into the PDZ domains (binding sites) of PICK1, GRIP and syntenin and the GluR2-PICK1 was used as control. The formation of a distinct binding funnel in the binding energy/RMSD plots was taken as an indication of successful docking (**Fig 7**). The best docked conformations were selected based on the lowest free energy pose in the protein-binding site and the binding energy score of this complex was re-assessed using new DrugScore (DSX). The higher negative values indicate a higher binding affinity prediction. Using this docking approach the binding scores of the interactions between the PDZ motif of ct-PAEL-R (-GTHC) and PICK1, GRIP and syntenin were determined (**Fig 7**). This coupled flexible docking and DrugScore analysis showed that the PDZ motif of ct-PAEL-R (-GTHC) has a binding score for PICK1 (-83 kcal/mol), similar to GRIP (-84 kcal/mol) but lower than syntenin (-106 kcal/mol). When compared with the GluR2-PICK1 interaction (-92 kcal/mol), the PAEL-R-PICK1 interaction had a lower binding score (**Fig 7**). Overall, the *in silico* calculated rank order of binding for the PDZ motif of the PAEL-R (-GTHC) was syntenin > PICK1 = GRIP.

The PICK1 amino acids that create a 'P0 binding pocket', which interact with P0 residues of PDZ motifs, include the residues Lys27 and Asp28 ('KD motif'), the Ile37 residue in the β B strand of PICK1, and the residues Leu32-Ile33-Gly34-Ile35 (-LIGI motif) in the β B sheet. In addition, PICK1 amino acids that create a 'P-2 binding pocket', which interact with P-2 residues of PDZ motifs, include the Lys83 (usually a histidine in other PDZ domains) and Thr82, Val84 and Ala87 in the α B helix of PICK1. The Val86, Ala87, Ile90 residues of the α B helix of PICK1 may also play important roles in interacting with PDZ motifs. Our analysis showed that the P0 Cys (-VGTHC) of PAEL-R formed a hydrogen bonds in the LIGI motif and was also found to interact strongly with Ile90 of the PDZ domain of PICK1. Of interest, we found that P-2 Thr (-VGTHC) formed hydrogen bonds with Ile37 residue in the PDZ domain of PICK1. In addition, unlike GluR2 (-ESVKI), the PAEL-R (-VGTHC) motif did not form any interactions with Lys83 and Val84 in 'P-2 binding pocket' of PICK1 PDZ domain, which may explain an increased binding affinity for GluR2 (-92 kcal/mol) as compared to PAEL-R (-84 kcal/mol). Furthermore, we found that

the stabilisation of interactions between the polar side chain of P-2 Thr (-VGIHC) and Phe213 in second PDZ domain of syntenin could have increased the binding affinity (-106 kcal/mol) of PAEL-R for syntenin as compared to PICK1 (-83 kcal/mol). In contrast, we observed the hydrogen bond interactions of P-2 Thr (-VGIHC) of PAEL-R were destabilized in the sixth PDZ domain of GRIP1 which could have accounted for its poor binding affinity (-84 kcal/mol). In-silico data was provided by Sefika Banu Ozkan, Department of Physics, Center for Biological Physics, Arizona State University Tempe AZ, USA.

6. The PAEL-R interacts with native rat brain PICK1

To further confirm the interaction between PAEL-R and PICK1, the ct-PAEL-R was subcloned into a pGEX-4T-1 bacterial expression vector (**Fig 8**) and the GST-ct-PAEL-R fusion protein used for affinity chromatography. GST alone, GST-ct-GluR2 and MBP-PICK1 were generated as previously described (Dev et al., 2000). The GST proteins were coupled to glutathione Sepharose 4B matrix and then exposed to MBP-PICK1. Western blotting using anti-GST Ab confirmed that equal amounts of each GST protein was present in these experiments (**Fig 9**). In agreement with previous studies (Dev et al., 1999, Dev et al., 2000), the glutathione Sepharose 4B matrix coupled with GST-ct-GluR2 (positive control), but not GST alone (negative control), retained Flag-PICK1 (**Fig 9**). Importantly, the GST-ct-PAEL-R also bound MBP-PICK1 (**Fig 9**). Next, co-immunoprecipitation experiments were performed to isolate a native PAEL-R-PICK1 protein complex from rat brain tissue. One PAEL-R Ab was used (sc-27548; Santa Cruz Biotechnology, Santa Cruz, CA), however data suggested that both PAEL-R antibody was not specific (**Fig 11**). In the absence of specific PAEL-R antibodies, GST pull downs studies were instead used to isolate native PICK1 obtained from rat brain P2 sonicates. Western blot with anti-PICK1 Ab showed that PICK1 was retained by GST-ct-GluR2 (positive control) and GST-PAEL-R but not by GST alone (negative control) (**Fig 10**). Taken together, these results biochemically confirm the interaction between PAEL-R and rat brain PICK1.

7. PICK1 reduces the protein expression levels of the PAEL-R

To study the functional role of the PAEL-R-PICK1 interaction, HEK293 cells were transiently transfected with Myc-PAEL-R or Flag-PICK1 (wildtype or KE mutant) either alone or in combination. The distribution and expression levels of PAEL-R in the presence of PICK1 wildtype and PICK1-KE mutant was examined 48 h after transfection. Cells were immunostained with anti-Flag monoclonal Ab and/or anti-Myc rabbit Ab. Both Myc-PAEL-R and Flag-PICK1 (wildtype and KE mutant) were ubiquitously expressed when transfected alone (*data not shown*). No differences in the distribution of Flag-PICK1 wildtype and KE mutant were observed in agreement with previous reports (Dev et al., 1999; Dev et al., 2000). Moreover, no change in the distribution of Myc-PAEL-R or Flag-PICK1 (wildtype or KE mutant) was observed upon co-expression (**Fig 12**). Interestingly, however, a decrease in the level of Myc-PAEL-R immunostaining was found in cells co-transfected with Flag-PICK1 wildtype compared to the Flag-PICK1 KE mutant, which does not interact with the PAEL-R. This reduction in Myc-PAEL-R expression occurred more evidently in the cytoplasmic regions often leaving a perinuclear staining pattern for the receptor. Quantification of the levels of Myc-PAEL-R immunostaining confirmed that Flag-PICK1 wildtype significantly ($p = 0.032$) reduced levels of Myc-PAEL-R expression by $37.5 \pm 8.6\%$ when compared to the Flag-PICK1 KE mutant. Western blot results confirmed that Flag-PICK1 wildtype reduced the total protein expression levels of Myc-PAEL-R compared to Flag-PICK1 KE mutant (**Fig 12**). The data suggest that while PICK1 does not alter PAEL-R trafficking *per se*, the interaction between PICK1 and PAEL-R attenuates the total protein expression levels of the receptor.

8. PICK1 protects against PAEL-R-induced cellular toxicity during cell stress

We hypothesised that the reduction in PAEL-R expression levels induced by PICK1 would result in protection against cell toxicity and improve cell survival. Under control conditions, we observed that transiently overexpressing Myc-PAEL-R caused a reduction in cell viability in HEK293 cells as determined by propidium iodide cell staining (**Fig 13**). The data also showed that the transient overexpression of Flag-PICK1 wildtype and KE mutant increased PI cell staining,

however these values were not significantly different from each other and are likely due to consequences of transient transfection (**Fig 13**). Furthermore, although Flag-PICK1 showed a modest effect on reducing PAEL-R mediated cell viability, there was no significant difference in PI cell staining between co-expression of Myc-PAEL-R with Flag-PICK1 wildtype and Flag-PICK1 KE mutant (**Fig 13**). We therefore investigated cell toxicity induced by Myc-PAEL-R overexpression under conditions of cell stress, in particular induced by the mitochondrial complex I inhibitor, rotenone. As expected, the treatment of wildtype HEK293 cells with increasing concentrations of rotenone (100 pM - 10 μ M) for 48 h caused a concentration-dependent reduction in cell viability (**Fig 14**). Furthermore, HEK293 cells transiently transfected with Myc-PAEL-R displayed increased cell loss induced by rotenone, compared to wildtype HEK293 cells (**Fig 14**). Finally, the viability of HEK293 cells transfected with Myc-PAEL-R and Flag-PICK1 or Flag-PICK1-KE and treated with 100nM rotenone for 48 h was examined. The overexpression of Flag-PICK1 showed a statistically significant attenuation in the loss of HEK293 cells expressing Myc-PAEL-R and treated with rotenone, when compared to overexpression of the Flag-PICK1-KE mutant. The data, in total, suggests a role for PICK1 in preventing PAEL-R-induced cell toxicity during conditions of cell stress and in particular during rotenone-induced mitochondrial complex I inhibition.

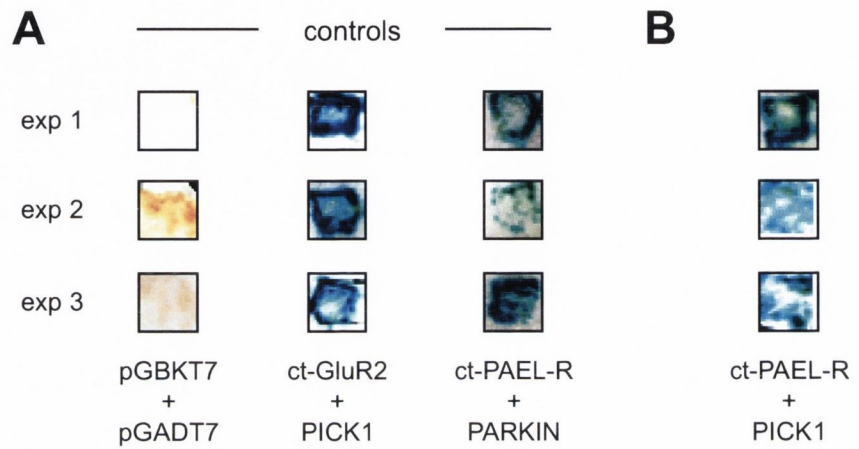
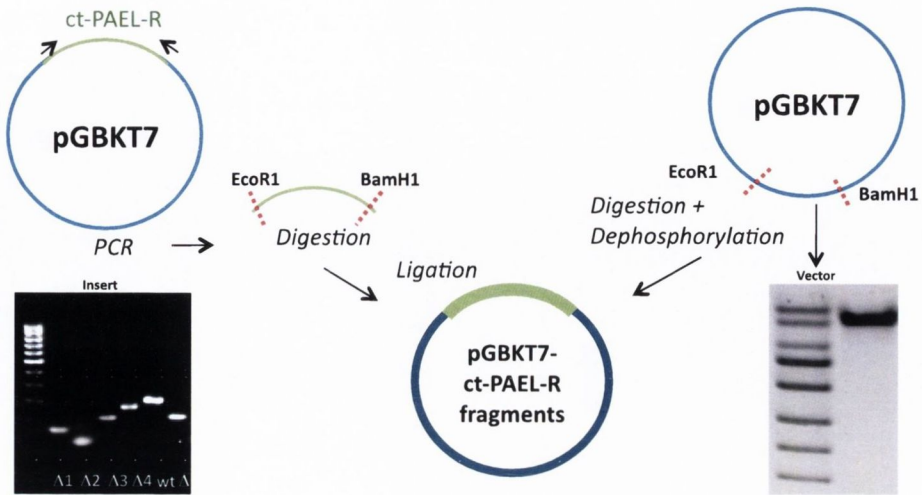


Figure 1: Novel interaction between ct-PAEL-R and PICK1. β -Galactosidase assay of interacting proteins transformed in yeast with the following controls: empty vectors pGBKT4 and pGADT7, ct-GluR2 and PICK1, and ct-PAEL-R and parkin. A novel interaction between ct-PAEL-R and PICK1 is shown. The blue colour confirms positive interactions in the assay. Data shows representative examples from 3 separate experiments.

A



B

GTTCCGCGTGGATCC^{CTC}CCCTTCAGTCGCGCCTTNN^{CTG}TGGAGT^{CTG}GCTGCTGCTGTTGCTGTGAG
GAATGCATTCAGAAGTCTTCAACGGTGACCAGTGATGACAATGACAACGAGTACACCAG
GAACTCGAACTCTCGCCTTTTCAGTACCATACGCCGTGAAATGTCCACTTTTGC^{TTC}TCTGTC
GAACTCATTGCTAGGAATTC^{CGG}GTCGAC

Legend: EcoRI site, BamHI site, STOP codon

C

ct-PAEL-R

PFSRAFM^{CCCC}CEECIQKSS^{TVT}SDDNDNEYTTELELSP^FSTIRREMST^{FAS}VGTHC

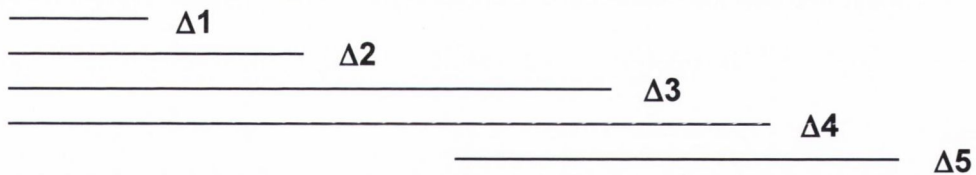
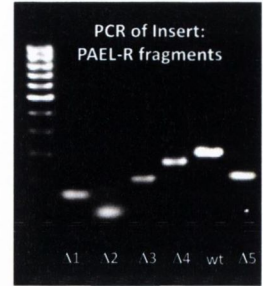
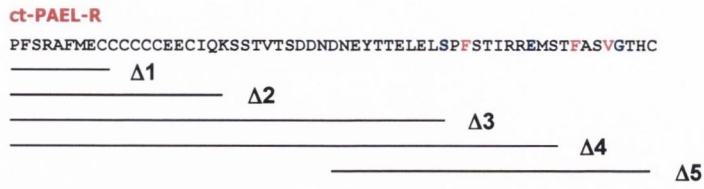


Figure 2: Cloning of PAEL-R fragments into the pGADT7 vector. (A) Schematic diagram for cloning strategy plus agarose gel images of the fragments of ct-PAEL-R and the vector. **(B)** Representation of the ct-PAEL-R sequence. **(C)** Diagram indicates different fragments of ct-PAEL-R.

A



B

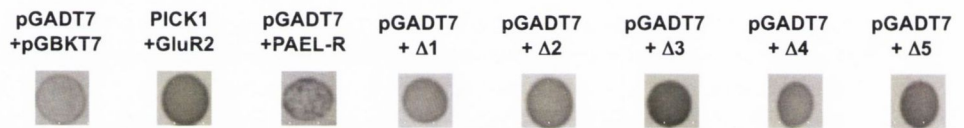


Figure 3: Auto reactivity of PAEL-R fragments in Y2H. (A) Schematic diagram of fragments of ct-PAEL-R. **(B)** Y2H indicates that the fragments are autoreactive using pGBKT7 with empty vector.

A

		PDZ motif		
ct-PAEL-R	PFSRAFMECCCCC-EECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFAS	VGTHC		PICK1
ct-PAEL-R +10	PFSRAFMECCCCC-EECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFAS	VGTHCGSVDLQRPHN		+
ct-GPR37L1	PLGQAFLDCCCCCECGGASEASAANGSDNKLKTEVSSSIYFHKPRESPLLP	LGTPC		-
	* * * * * * * * * * * * * * * * * * *			+

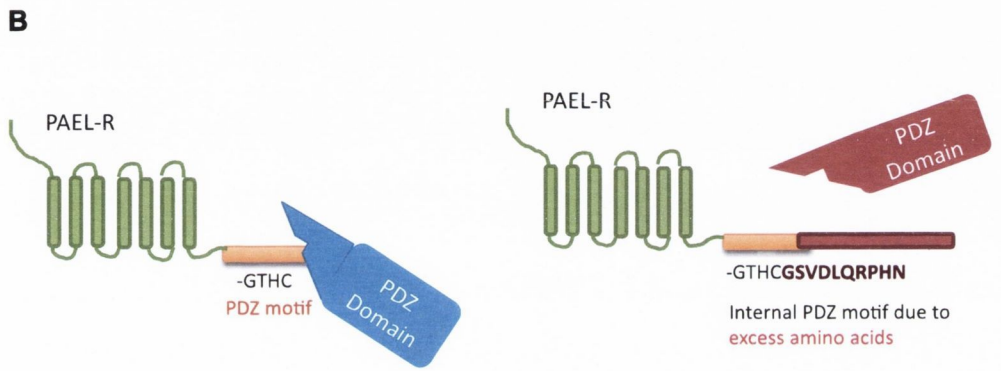


Figure 4: The PDZ motif of ct-PAEL-R interacts with PICK1. (A) A construct was designed with an additional 10 amino acids (ct-PAEL-R+10) (PFSRAFMECCCCC-EECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFAS VGTHC-**GSVDLQRPHN**) that occlude the putative PDZ motif at the extreme ct of PAEL-R. The family member of PAEL-R, namely GPR37L1, was also tested for interaction with PICK1. PICK1 interacts with both ct-PAEL-R and ct-GPR37L1, but not with ct-PAEL-R+10 indicating the importance of the PDZ motif in ct-PAEL-R and likely presence of PDZ motif in ct-GPR37L1. **(B)** Diagrammatic representation showing that the PDZ motif of ct-PAEL-R interacts with PICK1.

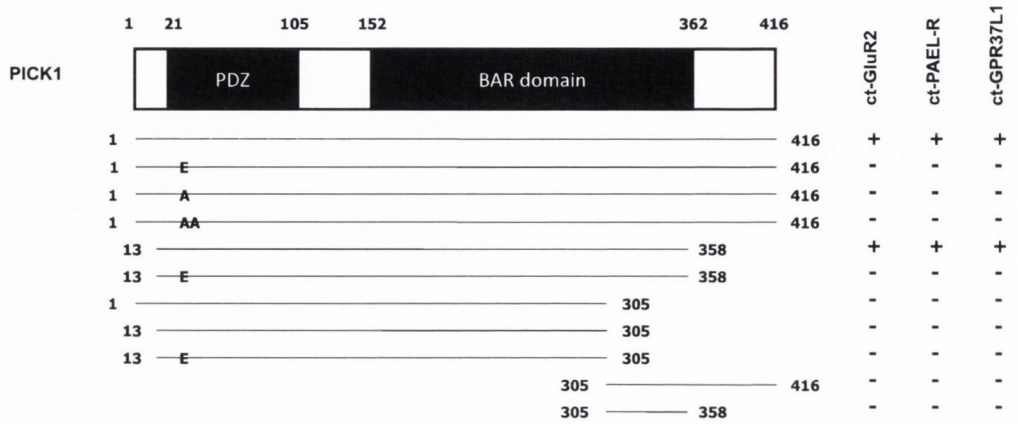


Figure 5: The PDZ domain of PICK1 interacts with ct-PAEL-R. Full-length PICK1 (residues 1-416) and a long version fragment of PICK1 (13-358) containing the PDZ domain gave a positive interaction with ct-PAEL-R. A shorter PICK1 fragment (1-305) still containing the PDZ domain showed no interaction with ct-PAEL-R, similar to ct-GluR2. PICK1 constructs (305-416 and 305-358) lacking the PDZ domain failed to interact with ct-PAEL-R. The three point mutations, K27E, K27A, and KD27/28AA, abolished the interaction between ct-PAEL-R and PICK1. Similar results were found for GPR37L1. The positive interactions, are indicated as + and negative as - as determined by β -gal assay in Y2H studies.

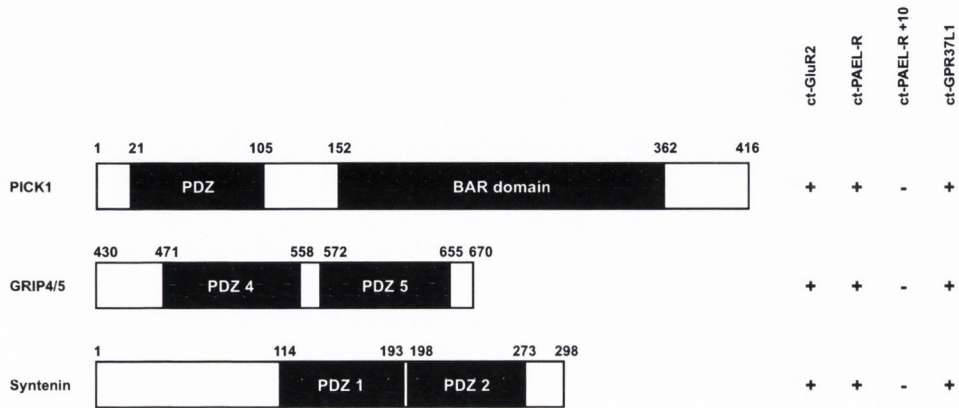


Figure 6: PDZ motif of ct-PAEL-R interacts with other PDZ domain containing proteins. The ct-PAEL-R was found to interact with GRIP (PDZ4/5) and syntenin (wild-type). The ct-PAEL-R+10 failed to interact with GRIP and syntenin, indicating that the PDZ motif of ct-PAEL-R is required at the extreme ct for interaction. The ct-GPR37L1 was also found to interact with GRIP and syntenin. The positive interactions, are indicated as + and negative as - as determined by β -gal assay in Y2H studies.

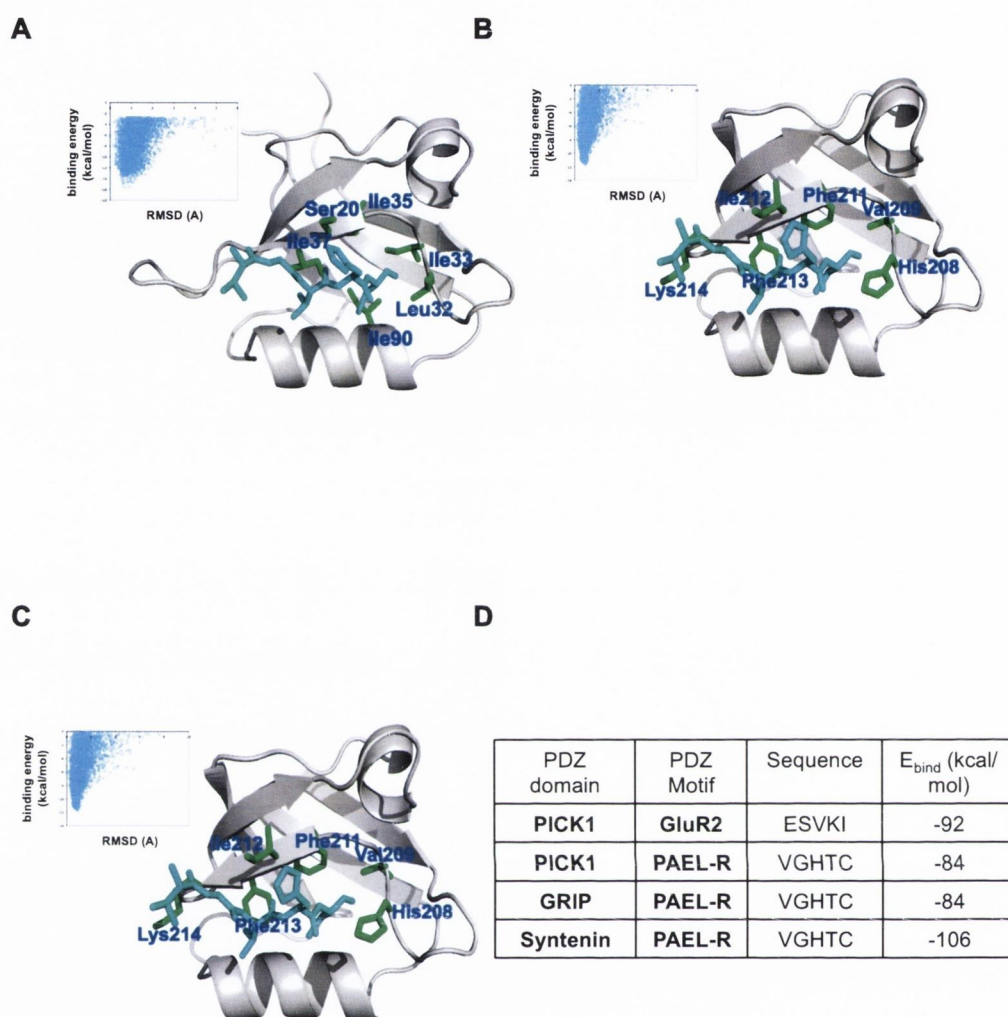
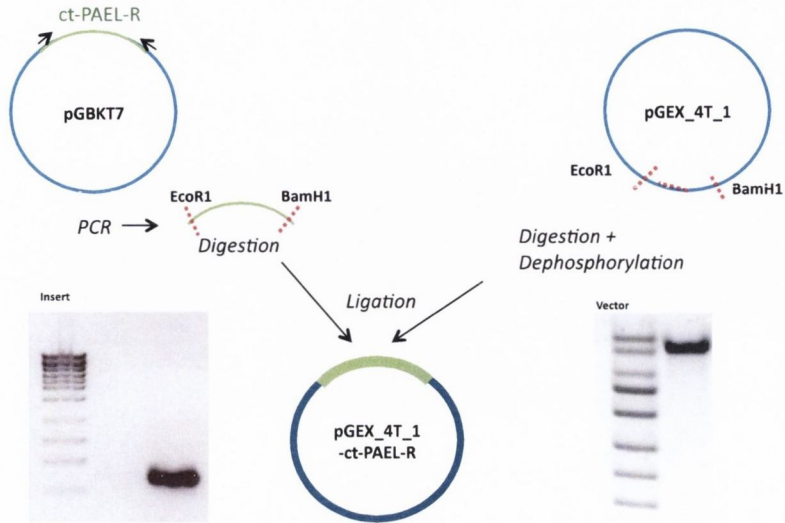


Figure 7: In silico binding affinities. *In silico* models of ct-PAEL-R PDZ motif with (A) PICK1, (B) GRIP and (C) syntenin are shown. (D) Table represents the modeled binding affinities of ct-PAEL-R PDZ motif with PICK1, GRIP and syntenin and GluR2-PICK1 as control. RMSD value is the root mean square deviation value, which identifies the superpose value of the two model. The good RMSD value is considered to be from the range of 0.00 to 0.99 (~ 1.0). E_{bind} (Kcal/mol) is the binding energy of the molecule.

A



B

```
GTTCCGCGTGGATCCCCCTTCAGTCGCGCCTTNNNTGGAGTGCTGCTGCTGTTGCTGTGAG
GAATGCATTCAGAAGTCTTCAACGGTGACCAGTGATGACAATGACAACGAGTACACCACG
GAACTCGAACTCTCGCCCTTTCAGTACCATACGCCGTGAAATGTCCACTTTTGCTTCTGTC
GGAACTCATTGC TAGGAATTC CGGGTCGAC
```

Legend: **EcoRI site**, **BamH1 site**, **STOP codon**

C

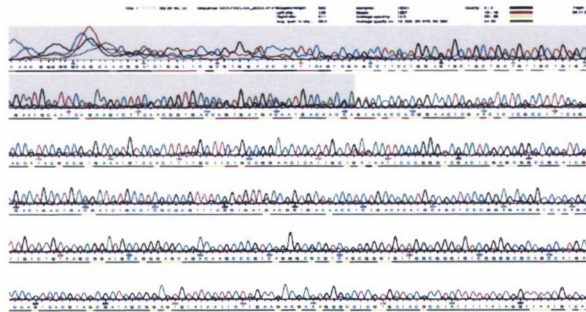


Figure 8: Cloning of ct-PAEL-R into the pGEX-4T-1 vector. (A) Schematic diagram for the cloning strategy plus agarose gel images of the insert and the vector. **(B)** The diagram represents ct-PAEL-R cloned within BamH1 and EcoRI of pGEX-4T-1 GST vector. **(C)** Raw sequencing data to confirm the cloning from MWG, Germany.

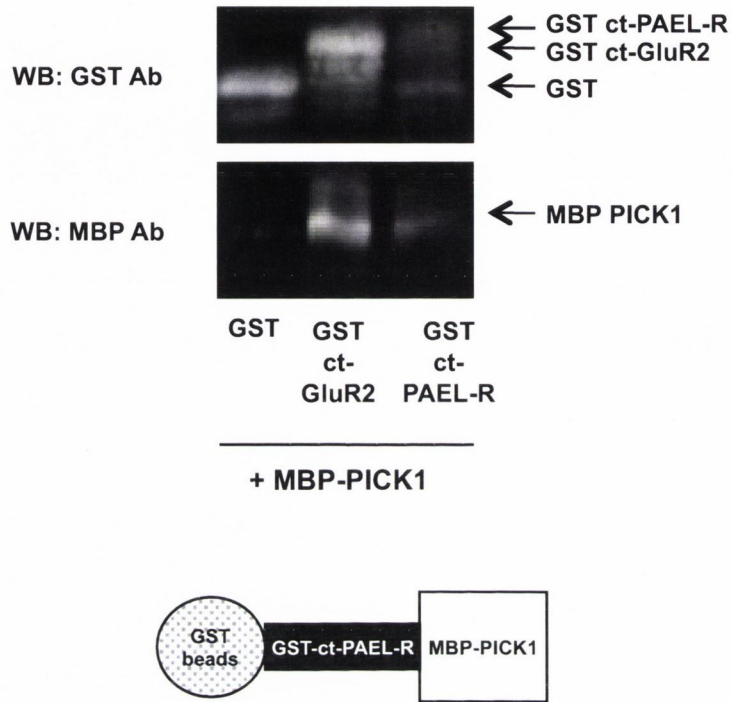


Figure 9: Biochemical evidence of binding between ct-PAEL-R and PICK1. The GST-ct-PAEL-R interacts with MBP-PICK1. Western blot with anti-MBP antibody showed the levels of MBP-PICK1 retained by GST-ct-GluR2 (positive control) and GST-ct-PAEL-R, but not by GST alone. The Western blot with anti-GST antibody indicates similar levels of GST fusion proteins were used. The diagram indicates setup of the pulldown experiment.

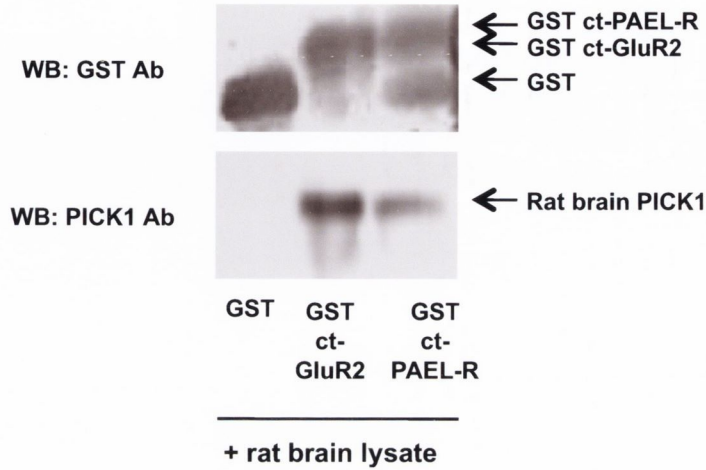


Figure 10: Biochemical evidence of binding between ct-PAEL-R and rat brain PICK1. Isolation of PICK1 from rat brain tissue by GST-ct-PAEL-R. The GST-ct-GluR2 (positive control) and GST-ct-PAEL-R isolated endogenous PICK1 from rat brain lysate indicating an interaction between PAEL-R and native PICK1, as determined by Western blotting with the anti-PICK1 antibody. The diagram shows the setup of the pull down experiment.

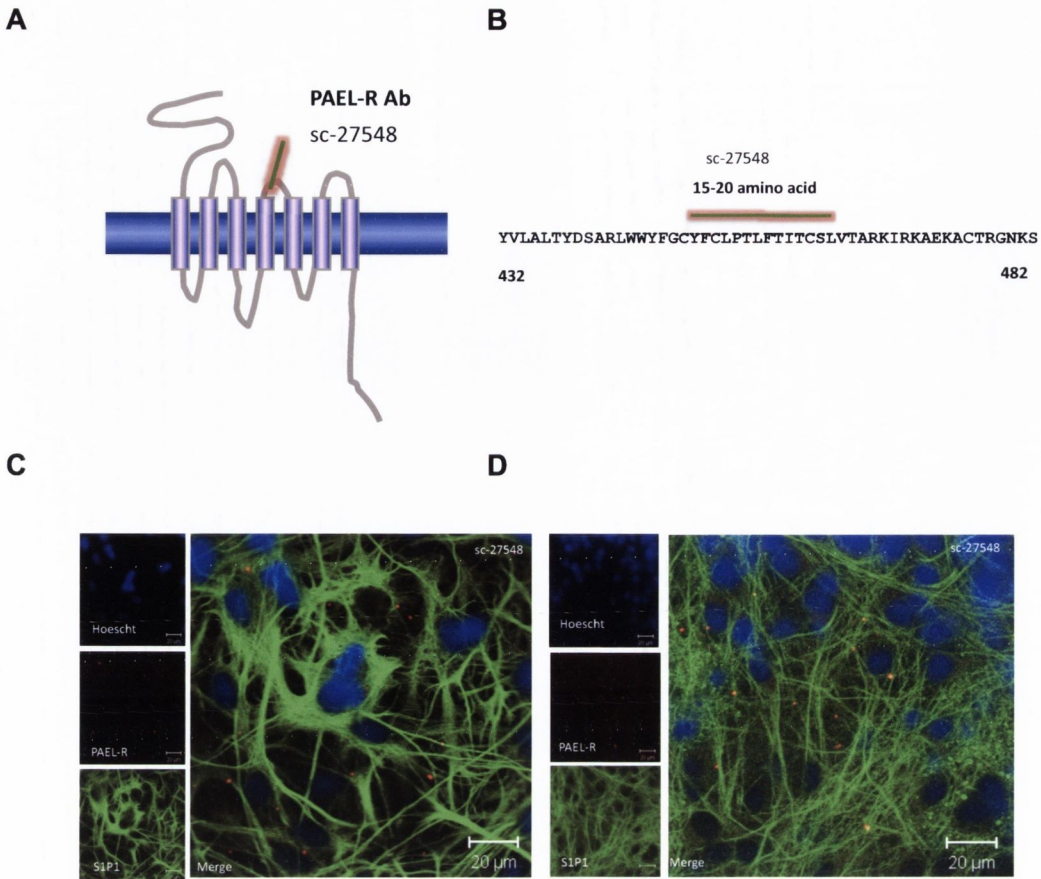


Figure 11: Testing of the PAEL receptor antibody in pure astrocyte and neuronal cultures. (A) Schematic diagram of PAEL-R antibody (sc-27548) and the binding site in PAEL-R receptor. **(B)** Epitope sequence of PAEL-R antibody. **(C)** Staining of pure astrocyte culture with goat anti PAEL-R primary antibody (sc-27548) and anti-goat ALEXA 633 secondary antibody. Confocal images show no specific staining of the receptor by immunocytochemistry. **(D)** Staining of neuronal culture with goat anti PAEL-R primary antibody (sc-27548) and anti-goat ALEXA 633 secondary antibody. Confocal images show no specific staining of the receptor by immunocytochemistry.

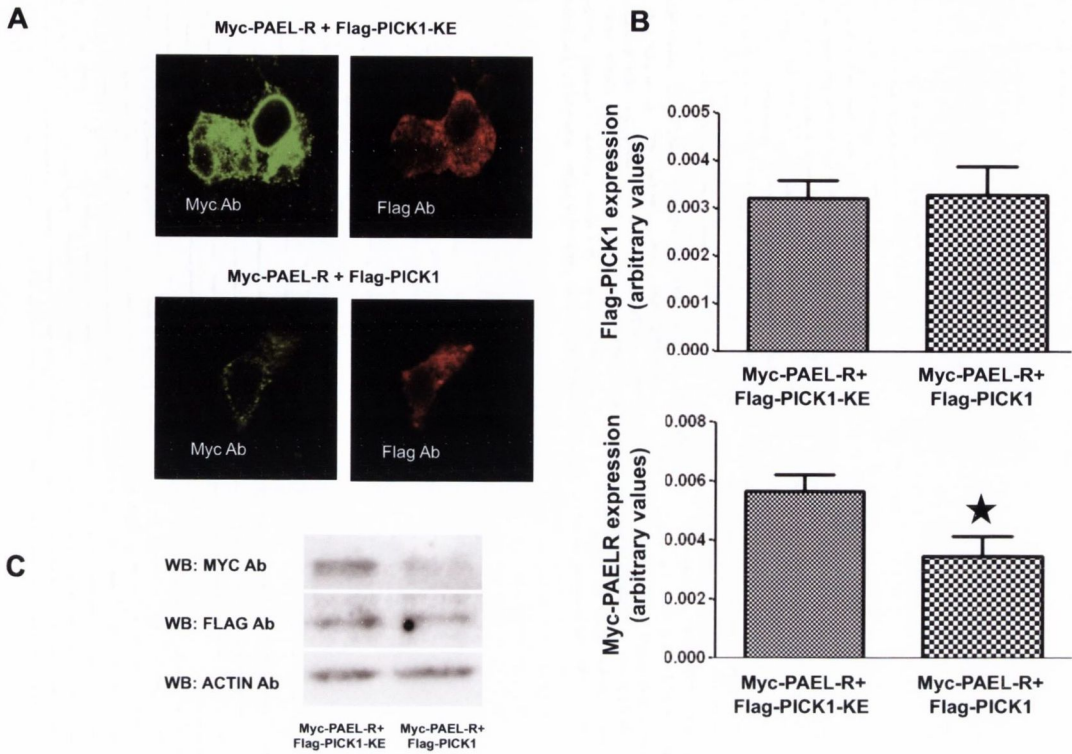


Figure 12: PICK1 reduces PAEL-R protein expression levels. (A) Colocalisation of full length Myc-PAEL-R and Flag-PICK1 in HEK293 cells. HEK293 cells were transiently transfected with Myc-PAEL-R and Flag-PICK1. The non-interacting, Flag-PICK1-KE mutant, was used as control. The cells were stained with anti-Flag Ab and/or anti-Myc Ab and viewed at x63 magnification. (B) Flag-PICK1, but not Flag-PICK1-KE, reduced expression levels of full length Myc-PAEL-R. Western blot with anti-Myc Ab shows the protein expression level of Myc-PAEL-R is reduced when co-transfected with Flag-PICK1 compared to cotransfection with Flag-PICK1-KE. Western blot with anti-Flag Ab indicates similar Flag-PICK1 and Flag-PICK1-KE expression in these experiments and Western blot with Actin Ab shows uniform loading of protein samples. (C) Statistical analysis of PICK1-mediated reduction of PAEL-R expression. HEK293 cells transiently co-transfected with Myc-PAEL-R and Flag-PICK1 or Flag-PICK1-KE were analysed for expression levels as determined by quantification of fluorescent pixels. Data shows similar expression levels of Flag-PICK1 and Flag-PICK1-KE ($p = 0.537$; $n=3$). The data also showed a statistically significant reduction in Myc-PAEL-R expression when co-transfected with Flag-PICK1 compared to co-transfected with Flag-PICK1-KE ($*p = 0.032$; $n=3$).

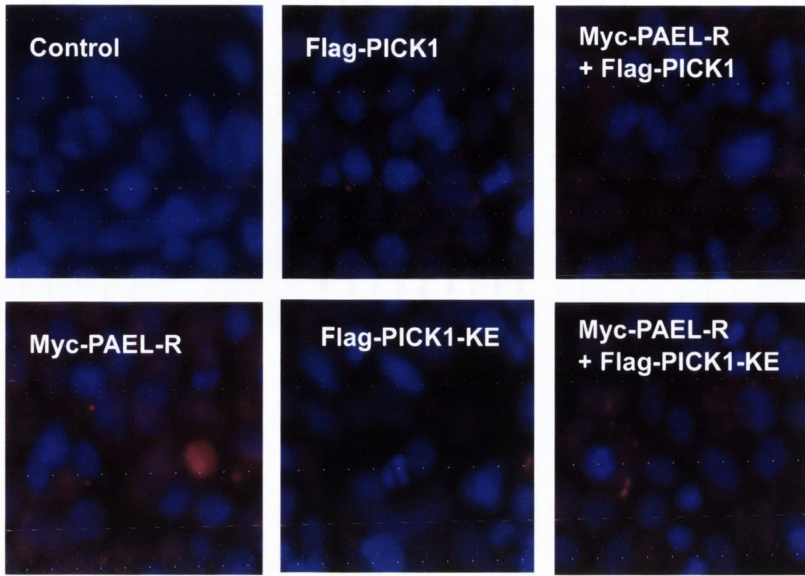
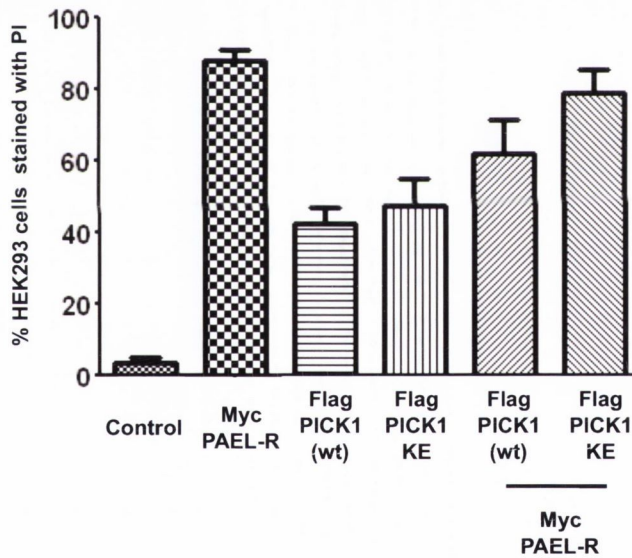
A**B**

Figure 13: Cell death study of PAEL-R and PICK1. Cells were transiently transfected with Myc-PAEL-R with or without Flag-PICK1 or Flag-PICK1 and 48 h later propidium iodide staining was performed as a measure of cell viability. The data shows representative PI cell staining confocal images (*upper panel*). Quantification of PI cell staining is represented as the mean \pm SEM of 3 independent experiments (*lower panel*).

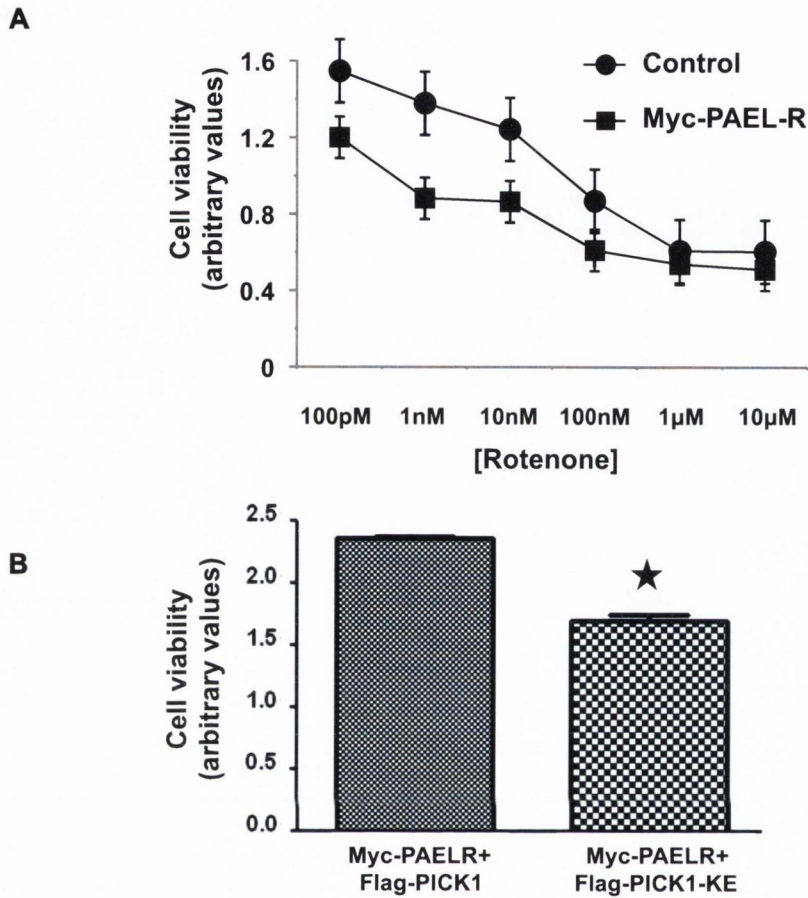


Figure 14: PICK1 attenuates PAEL-R-induced reduction in HEK293 cell numbers. Cells were transiently transfected with or without Myc-PAEL-R and the effect of 48 h treatment with increasing concentrations of rotenone (100pM, 1nM, 10nM, 100nM, 1µM, 10µM) on cell viability was examined. Cell viability was performed using 'cell titer 96 aqueous one solution assay' (Promega). The data shows a concentration-dependent reduction in cell viability induced by rotenone treatment (*upper panel*). The viability of HEK293 cells transiently transfected with Myc-PAEL-R and Flag-PICK1 or Flag-PICK1-KE and treated with 100nM rotenone for 48 h was also examined (*lower panel*). Flag-PICK1 showed a statistically significant increase in cell survival compared to Flag-PICK1-KE mutant (* $p = 0.026$; $n=3$); cell viability is expressed as % of cell survival. Data is represented as the mean \pm SEM of 3 independent experiments.

Discussion

The current study demonstrated that PAEL-R associates with PICK1 via a PDZ based interaction. In agreement with a canonical PDZ protein-protein interaction, the addition of 10 residues at the extreme ct-PAEL-R occluded interaction with PICK1, indicating that the PDZ motif of ct-PAEL-R (-GTHC) requires extreme ct localisation. Furthermore, deletion and mutant constructs of PICK1, in particular, point mutations in the carboxylate binding domain showed that the PDZ domain is required for interaction with PAEL-R. The results using truncated versions of PICK1 also suggested that full length PICK1 is required for this interaction, most likely due to correct conformation and/or expression levels. In addition to PAEL-R, the closely related family member GPR37L1 was also shown to interact with PICK1. Furthermore, PAEL-R and GPR37L1 interacted with other PDZ domain containing proteins, namely syntenin and GRIP. *In silico* data suggested that rank order of binding for the PDZ motif of the PAEL-R (-GTHC) was syntenin > PICK1 = GRIP. Biochemical studies verified that recombinant bacterial expressed MBP-PICK1 interacts with GST-ct-PAEL-R. Importantly, the data also showed that ct-PAEL-R can isolate native PICK1 from solubilised rat brain tissue. In transiently transfected HEK293 cells, co-localisation of Myc-PAEL-R and Flag-PICK1 was observed. Importantly, the overexpression of wild-type Flag-PICK1 reduced Myc-PAEL-R protein levels, compared to the Flag-PICK1 KE mutant (which does not interact with PAEL-R). Finally, PICK1 overexpression in HEK293 cells attenuated cell viability induced by PAEL-R overexpression during rotenone treatment. Taken together, the data suggest a role for PICK1 in preventing PAEL-R-induced cell toxicity during conditions of cell stress.

The PAEL-R (GRP37) belongs to a family of GPCRs that includes the bombesin-BB1 and bombesin-BB2 receptors and the endothelin-B receptor (ETBR) (Marazziti et al., 1997, Marazziti et al., 1998). PAEL-R is closely related to the orphan GPCR called the CNS-enriched orphan receptor, also known as GPR37-like 1 (GPR37L1, PAEL-RL1 or ETBRL2). GPR37L1 is 481 residues in length and is expressed in the cerebral cortex, internal capsule fibres and cerebellar Bergmann glia (Valdenaire et al., 1998, Leng et al., 1999). *In situ* hybridisation demonstrates broad distribution of both PAEL-R and GPR37L1 throughout the rat brain (Leng et al., 1999). Two additional GPCRs that show sequence similarity with endothelin and bombesin-like peptide receptors are the GPCR/CNS1 and

GPCR/CNS2 receptors, which are also highly expressed in rat brain. In particular, GPCR/CNS1 is expressed in glial cells of the fibre tracts and GPCR/CNS2 is expressed in the gray matter. Notably the c-terminals of these receptors are 80% identical. Here, we identified a novel PDZ motif in GPR37L1 PDZ motif (-GTPC) similar to PAEL-R (-GTHC) and confirmed that ct-GPR37L1 interacted with the PDZ domain of PICK1. Moreover, the extreme ct of GPCR/CNS1 (-GTHC) and GPCR/CNS2 (-GTPC), contain identical PDZ motifs as PAEL-R and GPR37L1, respectively, and predictably also interact with PICK1.

PAEL-R has been shown to interact with the PDZ domain of syntenin (Dunham et al., 2009). Syntenin is 298 residues protein of approximately 33 KDa that was originally identified as a potential melanoma differentiation associated gene (mda-9) (Kang et al., 2003). Syntenin is a small scaffold protein that contains two canonical PDZ domains and interacts with glutamate receptors, syndecan (transmembraneproteoglycan), neurexin (neuronal surface proteins), synCAM (synaptic cell adhesion molecule), ephrin B neurofascin (neural cell adhesion molecule) and merlin (product of the causal gene for neurofibromatosis type II) (Kim and Sheng 2004). Syntenin regulates the subcellular trafficking of its binding partners, tumour metastases and the integrity of the neuronal synapse (Beekman and Coffey 2008). The interaction between syntenin and PAEL-R is reported to increase the cell surface trafficking of the receptor (Dunham et al., 2009). Since PAEL-R has been shown to interact with syntenin and we find it also interacts with PICK1, its interaction with the PDZ domain containing protein GRIP (Dong et al., 1999) was also examined. GRIP has seven PDZ domains and interacts with many proteins, including Eph receptors and AMPA receptors (Dong et al., 1999, Kim and Sheng 2004). GRIP is involved in synaptic trafficking and synaptic stabilization of AMPA receptors and other interacting proteins. Similar, to other PICK1 interacting receptors (such as GluR2, GluR5 and mGluR7), we found that PAEL-R (as well as GPR37L1) interacted with GRIP and also confirmed PAEL-R interaction with syntenin. The association of PAEL-R to different PDZ proteins may link it to various signaling pathways and/or may aid in controlling the correct subcellular localisation and protein levels of this receptor. Furthermore, heterodimerisation and cross talk with other GPCRs may be crucial for proper PAEL-R trafficking, as has been shown with dopamine and adenosine receptor, which enhance PAEL-R surface expression (Dunham et al., 2009). It is possible to control PAEL-R neurotoxicity by regulating, (i) proteins that regulate the rate of PAEL-R

degradation, (ii) trafficking proteins that interact with PAEL-R, and (iii) receptors that heterodimerise with PAEL-R (**Fig 15**).

Finally, it is interesting to speculate that PICK1 may play a role in Parkinson's disease given its molecular links with PKC α , DAT, parkin and PAEL-R, which all play multiple roles in mitochondrial function, protein degradation, and/or dopaminergic neurotransmission. For example, PICK1 interacts with PKC α (-TSXV, PDZ motif) and regulates PKC α -mediated phosphorylation (Staudinger et al., 1997, Dev et al., 1999). Importantly, PICK1 targets PKC toward the mitochondria which maintains proper mitochondrial function and resistance to toxic insults (Wang et al., 2003, Wang et al., 2007, Wang et al., 2008). The ct domain of the dopamine transporter (DAT) (-XLVK, PDZ motif) also interacts with the PDZ domain of PICK1 (Torres et al., 2001, Bjerggaard et al., 2004, Madsen et al., 2005). In dopaminergic neurons PICK1 (via a PKC-dependent mechanism) promotes DAT clustering and increases numbers of plasma membrane DAT which enhances uptake of dopamine (Torres et al., 2001, Matsuzawa et al., 2007). In addition, PICK1 interacts with parkin (-QSAV, PDZ motif). Overexpression of parkin increases PICK1 monoubiquitination (Joch et al 2007) which enhances activity of another PICK1 binding partner, namely, the acid-sensing ion channel (ASIC) (Joch et al., 2007). ASIC proteins are involved in pain, mechnosensation and psychiatric diseases. Parkin also enhances the ubiquitination and degradation of DAT (Jiang et al., 2004) and it is internalised by PKC. In addition, DAT can interact with PAEL-R, which modulates DAT-mediated DA uptake, where the lack of PAEL-R enhances DAT activity and increase the plasma membrane expression of DAT (Marazziti et al., 2007). Whether the dimerisation of PICK1 facilitates the ubiquitination or phosphorylation of PAEL-R requires further investigation. However, based on the collection of studies above, we hypothesise that PICK1 may regulate PAEL-R protein levels and DAT surface expression by altering PKC phosphorylation and/or parkin ubiquitination. In closing, we suggest that PICK1 may provide a scaffold for the complex protein PKC/PAEL-R/DAT/parkin interaction, which may play an important role in mitochondrial function, cell stress and dopaminergic neuronal survival in Parkinson's disease (**Fig 16**).

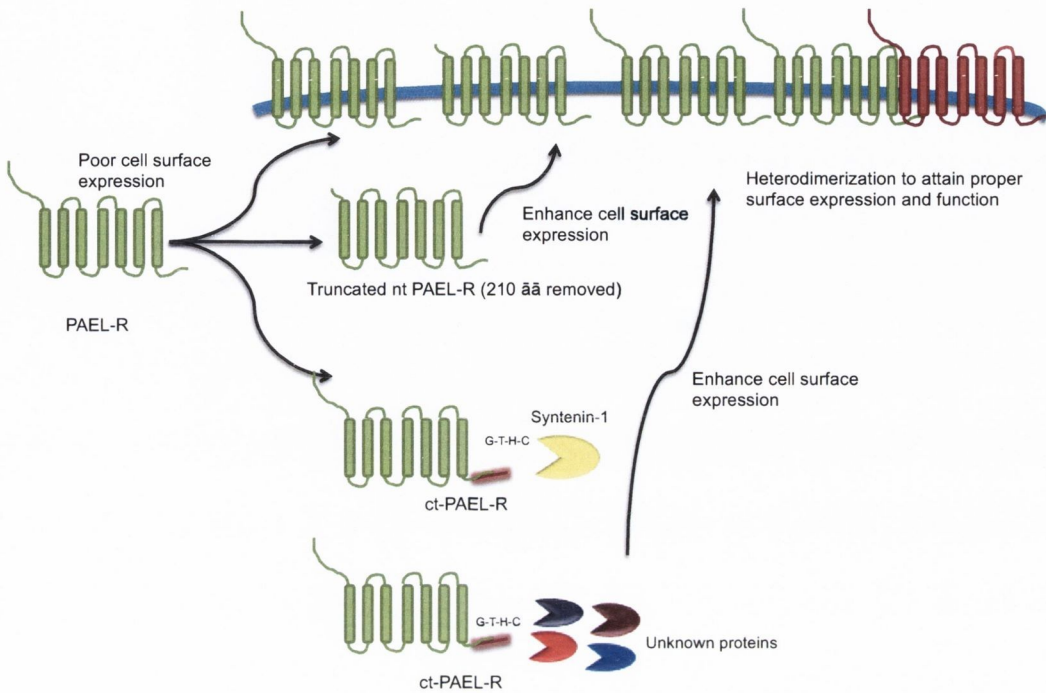


Figure 15: Mechanisms for PAEL-R trafficking to cell surface. The PAEL-R shows limited expression at the cell surface. The cell surface expression of PAEL-R is enhanced by the truncation of the n-terminus or co-expression of the PAEL-R with PDZ domain containing protein syntenin-1. We hypothesise other PDZ containing proteins such as PICK1 may interact with PAEL-R and regulate PAEL-R expression levels. The heteromerization may also be required for proper surface expression and function of PAEL-R.

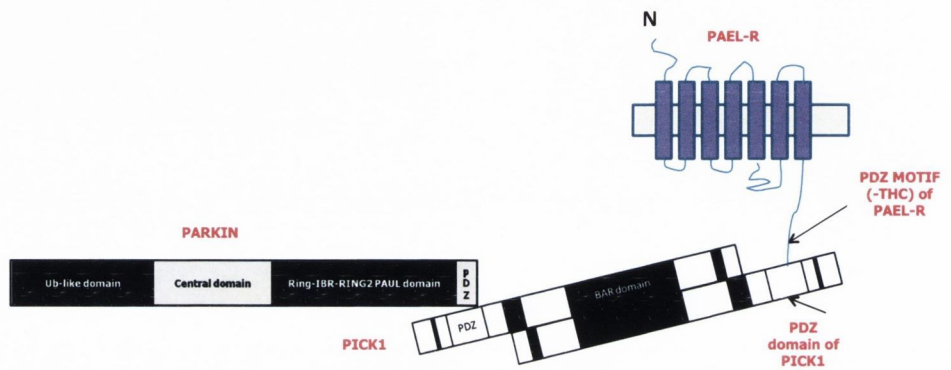


Figure 16: PAEL-R, parkin and PICK1 form a triple protein complex. Our result revealed that PDZ motif of PAEL-R interacts with PDZ domain of PICK1. PICK1 acts as a scaffolding protein and facilitates degradation of PAEL-R possibly via parkin mediated ubiquitination.

CHAPTER 4

**PAEL-R INTERACTION WITH
GABARAPL2**

Aims

- Aim1:** to isolate the novel interaction between GABARAPL2-PAEL-R by small scale Y2H.
- Aim2:** to clone pACT2 GABARAPL2 into pGADT7 and confirm the interaction between GABARAPL2-PAEL-R.
- Aim3:** to test the selectivity of other PAEL-R family members (GPR37L1) with GABARAPL2.
- Aim4:** to determine the specific interaction sites on PAEL-R.
- Aim5:** to clone deletion constructs of GABARAPL2.
- Aim6:** to determine the specific interaction sites of PAEL-R on GABARAPL2.
- Aim7:** to clone GABARAPL2 into pMAL-C2 (MBP) vector.
- Aim8:** to validate interaction biochemically by GST pulldown.
- Aim9:** to clone GABARAPL2 into pMYC-CMV.
- Aim10:** to validate interactions biochemically by co-immunoprecipitation.
- Aim11:** to determine effects of GABARAPL2 on PAEL-R expression.
- Aim12:** to test the effects of GABARAPL2 on PAEL-R mediated cell death in cell survival study.

Abstract

To further study the trafficking mechanisms of the parkin-associated endothelial-like receptor (PAEL-R), we have identified a novel protein, γ -aminobutyrate type A receptor associated protein like 2 (GABARAPL2) that interacts with this receptor using a Y2H technology. GABARAP belongs to a family of small ubiquitin-like adaptor proteins implicated in intracellular vesicle trafficking and autophagy. From our Y2H data we hypothesise that GABARAPL2 interacts with the -CCCCC-EEC motif found in the ct-PAEL-R. Furthermore, these Y2H studies show that ct-PAEL-R interacts with the GABA_A binding site of GABARAPL2. Data shows that Myc-tagged GABARAPL2 is retained by a GST fusion of the ct-PAEL-R (GST-ct-PAEL-R) but not by GST alone. Importantly transient transfection of both proteins in HEK293 cells revealed a significant reduction of PAEL-R expression. In contrast to the effect of PICK1, we find that the cell death induced by rotenone (inhibitor of mitochondrial complex I) was not rescued when PAEL-R was co-transfected with GABARAPL2 in comparison to cells transfected with PAEL-R alone. In this study we have identified a novel interaction between PAEL-R and GABARAPL2 and outlined the specific functional role of GABARAPL2 in regulating PAEL-R protein expression levels. We suggest that GABARAPL2 functions as an ubiquitin like modifier which mediates PAEL-R degradation possibly via an autophagic pathway. In summary we hypothesise that the regulation of PAEL-R levels by GABARAPL2 occurs via an autophagic pathway, while the regulation of PAEL-R levels by PICK1 occurs via an ubiquitination mechanism.

Introduction

When large amounts of intracellular insoluble unfolded proteins accumulate they are degraded either by the lysosome or cleared by the ubiquitin proteasome process. Indeed, PAEL-R aggregation stimulates the formation and recruitment of acidic autophagic organelles (Marazziti et al., 2009a). As an example, PAEL-R null-mutant mice have revealed significantly decreased levels of the glucose-regulated protein 78 and LC3-II isoform, as specific markers of ER-associated degradation and autophagy (Marazziti et al., 2007). Increased glucose-regulated protein 78 levels have also been shown in brain samples from PAEL-R overexpressing tg mice, as well as in neuronal specimens, from AR-JP patients (Marazziti et al., 2009a). The above study concludes that aggregation of PAEL-R leads to ER stress and degradation by autophagy.

Autophagy is a highly conserved intercellular process which occurs under certain stresses in all eukaryotes from yeast to human (Yen and Klionsky, 2008). Autophagy is implicated in a variety of physiological processes including cell survival, cell death, tumor suppression, pathogen clearance and neurodegeneration. The types of autophagy are classified as macroautophagy (normally referred to as autophagy), microautophagy and chaperone-mediated autophagy (**Figure 1**). Both macroautophagy and microautophagy are nonselective in the degradation of substrates because bulk cytoplasm components and entire organelles are engulfed. Macroautophagy is a multistage process consisting of the following steps: cytoplasmic components or organelles are delivered to double-membrane vesicles (autophagosome), and are then fused with lysosomes for protein degradation by lysosomal hydrolases and finally result in clearing aggregated proteins and organelles (Mizushima, 2007, Kaminsky and Zhivotovsky, 2011). Microautophagy involves delivery of cytosolic cargo through invagination of lysosomal membranes. Chaperone-mediated autophagy is a selective process from the cytoplasm to vacuole targeting substrate proteins, which bind to the lysosomal membrane through lysosomal associated membrane protein type 2A (LAMP-2A) (Kaminsky and Zhivotovsky, 2011). In mammals, two ubiquitin like protein conjugation systems are required during autophagosome formation, autophagy-related protein 12 conjugation and light chain 3 of microtubule-associated protein 1 modification (Schwarten et al., 2009). The microtubule-associated protein light chain 3 (LC3) like protein family encompasses

seven proteins including glandular epithelial cell protein 1, GATE-16, LC3 and GABARAP which are implicated in autophagy and a variety of other vesicular transport processes (Schwarten et al., 2009). Moreover, both LC3s and GABARAPs contribute to autophagosome biogenesis. The LC3s are implicated in elongation of the phagophore membrane and GABARAPs involved in sealing of autophagosome (Weidberg et al., 2010).

The 14 kDa protein GABARAP binds to the TM3/TM4 cytoplasmic loop of the γ -subunit of the GABA_A receptor and the sequence of GABARAP is 30% similar with LC3. GABARAP is involved in the intracellular trafficking of GABA_A receptors and regulates ion channel properties of GABA_A receptors. Besides the GABA_A receptor, GABARAP binds to a broad range of proteins, which are all associated with vesicle transport and fusion events, receptor transport processes and apoptosis (Schwarten et al., 2009). Another phenomenon of eliminating damaged mitochondria by autophagy is called mitophagy. The mitochondrion localised proteins Bcl2/E1B 19 kDa-interacting protein 3-like protein (BNIP3) and Nix (also known as BNIP3L) have been implicated in the removal of mitochondria during an autophagic response. The proapoptotic protein Nix has been found to be a potential GABARAP ligand (Schwarten et al., 2009). Nix specifically binds to GABARAP and does not bind to the autophagosome marker protein LC3 (Schwarten et al., 2009). The interaction of Nix and GABARAP is a novel interesting link for autophagy. GABARAPL1 is predominantly localised in autophagosomes or lysosomes. These autophagosomes fuse with lysosomes to form autophagolysosomes and induce the degradation of their contents. Nix recruits GABARAPL1 to stress mitochondria, which then leads to involvement of Atg8 family in the clearance of damaged mitochondria (Novak et al., 2010).

In this chapter we describe the identification of an interaction between PAEL-R and GABARAPL2. It has been shown previously that cellular autophagy is crucially involved in the control of physiological levels of the PAEL-R protein (Marazziti et al., 2009b). Possibly GABARAPL2 is implicated in the prevention of selective degeneration of PAEL-R and is important in the underlying mechanisms of PD.

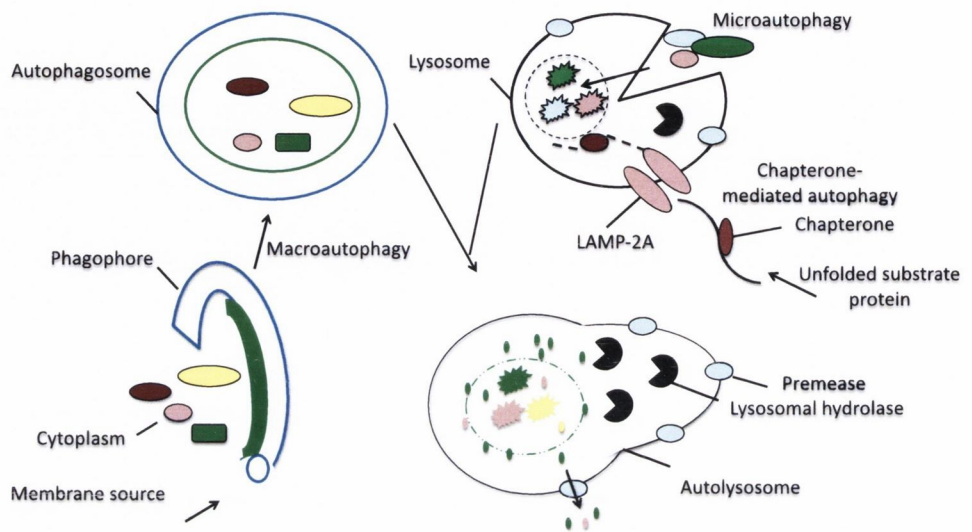


Figure 1: Types of autophagy. Microphagy involves delivery of cytosolic cargo through invagination of lysosomal membranes. Macroautophagy sequestrate damaged organelles and unused long-lived proteins in a double-membrane vesicle, called an autophagosome, inside the cell and degrade by lysosomes. In Chaperone-mediated autophagy, substrate proteins bind directly to the lysosomal membrane through LAMP-2A thus, only specific proteins, not entire organelles are degraded by this pathway (Mizushima et al., 2008).

Results

1. A novel interaction PAEL-R and GABARAPL2

Other than PICK1, we found an interaction between parkin-associated endothelial-like Receptor (PAEL-R) and γ -aminobutyrate type A receptor associated protein like 2 (GABARAPL2). The pACT2 GABARAPL2 was identified as a positive interacting clone in a two hybrid screen using the ct-PAEL-R as a bait (**Fig 2**). The interacting GABARAPL2 isolated contained unrelated 3' and 5' sequences. Therefore, we cloned GABARAPL2 into a pGADT7 vector without these unrelated sequences. The cloning of GABARAP into a pGADT7 vector was performed by the standard cloning methods (**Fig 3**). Thereafter, we repeated the Y2H experiment and confirmed that GABARAPL2 without unrelated sequence still interacts with PAEL-R (**Fig 4**). This confirmed that the interaction was not due to the flanking of excess amino acids of the original isolated GABARAPL2 clone. Our result revealed a novel interaction between PAEL-R and GABARAPL2.

2. The -CCCCC-EEC motif in PAEL-R is a putative interaction site with GABARAPL2

Elucidation of the interaction site of GABARAPL2 with PAEL-R will aid in possible development of blocking peptides. Thus to investigate the specific interaction site of PAEL-R with GABARAPL2, we have used the mutant ct-PAEL-R described in the first result chapter. This PAEL-R mutant has an additional 10 amino acids attached to the ct which impedes interaction with PDZ domain containing proteins, such as PICK1. The PAEL-R mutant was transformed with GABARAPL2 and a Y2H experiment was carried out (**Fig 5**). The data revealed that the interaction still occurs with the mutant ct-PAEL-R suggesting that the ct-PAEL-R PDZ motif is not involved in the interaction with GABARAPL2. As described in the first result chapter, the further truncated fragments of ct-PAEL-R were found to be autoreactive in Y2H assays, thus we were unable to further narrow down the interaction site of ct-PAEL-R with GABARAPL2. Instead, we identified if GABARAPL2 interacts with GPR37L1, which has a similar sequence to PAEL-R (Valdenaire et al., 1998). Importantly we found that GPR37L1 also interacted with GABARAPL2 (**Fig 5**). According to sequence alignment of PAEL-R and GPR37L1,

there exists a similar -CCCCC-EEC motif in both receptors. We hypothesise, therefore that this -CCCCC-EEC may be a binding site for GABARAPL2.

3. PAEL-R interacts via GABA_A binding site of GABARAPL2

To examine the site of PAEL-R interaction on GABARAPL2 we designed PCR primers to produce specific domain containing fragments of GABARAPL2. Four GABARAPL2 fragments were made: $\Delta 1$ (1-22 amino acids) contains the tubulin binding domain, $\Delta 2$ (36-68 amino acids) contains the GABA_A binding domain, $\Delta 3$ (22-117 amino acids) also contains the GABA_A binding domain and $\Delta 4$ (2-26 amino acids) contains tubulin binding domain. The fragments were designed such that if the interaction is due to either of these regions, two of the four fragments should interact. The overlapping deletion constructs were cloned into the pGADT7 fish vector (**Fig 6**). These deletion constructs were then tested in Y2H experiments to specify the binding site of ct-PAEL-R on GABARAPL2. The data shows that the GABA_A binding site (fragment $\Delta 2$ and $\Delta 3$) of GABARAPL2 interact with ct-PAEL-R. The PAEL-R and GABARAPL2 interaction was narrowed down to amino acids 36 and 68 of GABARAPL2 (**Fig 7**). Interestingly, the interaction site of GABA_A on GABARAP (Wang et al., 1999) is similar to the PAEL-R interaction site with GABARAPL2. GABARAP has been implicated in plasma membrane targeting or recycling of GABA_A receptors (Mohrluder et al., 2009). Given the similar interaction site properties we hypothesise that GABARAPL2 may be involved in the trafficking of the PAEL-R receptor.

4. Biochemical confirmation of PAEL-R-GABARAPL2 interaction

A biochemical experiment was performed to confirm the Y2H results and to further validate the interaction between ct-PAEL-R and GABARAPL2. To carry out biochemical studies, we created a GABARAPL2 clone in pMAL (MBP plasmid) (**Fig 8**). We performed a GST pull down assay using GST-ct-PAEL-R coupled to glutathione sepharose beads and then incubated these beads with MBP tagged GABARAPL2. The results of the Western blot with anti-MBP showed that GABARAPL2 is retained by GST-PAEL-R, however was also retained by GST alone (**Fig 9**) via non-specific interaction of MBP-GABARAPL2 with either GST or

GST beads (**Fig 9**). Due to the MBP-GABARAPL2 non-specific binding to GST or GST beads we replaced the MBP with Myc and created a Myc-tagged GABARAPL2 (**Fig 10**). We then performed a pull down assay using GST fusion protein ct-PAEL-R coupled to glutathione sepharose beads and incubated with Myc-GABARAPL2 which was isolated from transiently transfected HEK293 cells (**Fig 11**). The data showed, Myc-GABARAPL2 was retained by GST-PAEL-R but not GST alone. The results confirmed a novel interaction between PAEL-R and GABARAPL2.

5. GABARAPL2 reduces PAEL-R expression

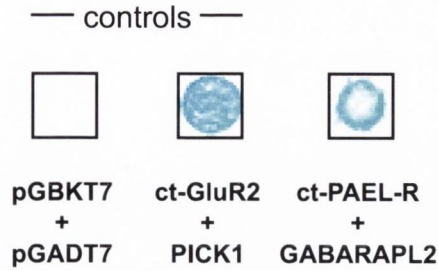
Next, we carried out studies to examine the effects of GABARAPL2 on PAEL-R expression and trafficking. To study the expression profile of PAEL-R and GABARAPL2 we transiently transfected HEK293 cells with VSV-PAEL-R or Myc-GABARAPL2 either alone or in combination. These transfected cells were stained with an anti-VSV monoclonal Ab and/or anti-Myc rabbit Ab after 48 h of transfection. The staining data showed no change in the distribution of VSV-PAEL-R or Myc-GABARAPL2 upon co-expression (**Fig 12**). Interestingly, the reduction in VSV-PAEL-R expression again occurred in the cytoplasmic regions leaving a perinuclear staining pattern for receptors similar to PICK1 result chapter (**Fig 12**). However, the data showed that GABARAPL2 caused a statistically significant reduction in PAEL-R expression (* $p=0.033$; $n=3$) as determined by cell staining. These results suggest that interaction with GABARAPL2 may regulate PAEL-R protein levels.

6. GABARAPL2 interaction with PAEL-R does not rescue rotenone-induced cell death

As described in the previous results chapter the common pesticide, rotenone is a high-affinity inhibitor of complex I of the mitochondrial electron transport chain (ETC). Rotenone treated rats demonstrate many characteristics of PD, including selective nigrostriatal dopaminergic degeneration and the formation of ubiquitin and synuclein-positive nigral inclusions (Betarbet et al., 2000; Alam and Schmidt, 2002; Sherer et al., 2003). HEK293 cells were treated with increasing doses of rotenone for 48 h, and a cell viability test was performed using cell titer 96

aqueous one solution assay. A concentration dependent cell death was observed after exposure to rotenone (**Fig. 13**). Cell death occurred after exposure to 10 μ M, 1 μ M, 100nM, 10nM and 1nM rotenone; lower doses of rotenone (100 pM) did not cause significant cell death in comparison to higher doses. We analysed the cell viability of HEK293 cells in the presence of 100nM rotenone in cells transiently transfected with VSV-PAEL-R with or without the GABARAPL2. The data showed that transient transfection with VSV-PAEL-R and Myc-GABARAPL2 had no effect on cell survival in comparison to cells transfected with VSV-PAEL-R alone. We conclude that the reduction in PAEL-R expression levels caused by GABARAPL2 is not sufficient to rescue from PAEL-R induced cell death unlike the effects of PICK1.

A



B

GABARAPL2

```
DLTVGOFYFIIRKRMKWMFKEDHSLEHRCVESAKIRAKYPDRVPVIVEKVSQSQIVDIDKRKYLVPSDITVAQFMWIIIRK
-----MKWMFKEDHSLEHRCVESAKIRAKYPDRVPVIVEKVSQSQIVDIDKRKYLVPSDITVAQFMWIIIRK

RIQLPSEKAIFLFDKTVPQSSLTMGQLYEKEKDEDGFLYVAYSAGENTFGFKAPKARIGDLDKKKYLVPSDLTVGOFYFI
RIQLPSEKAIFLFDKTVPQSSLTMGQLYEKEKDEDGFLYVAYSAGENTFGF-----

IRKR
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Figure 2: PAEL-R interacts with GABARAPL2. GABARAPL2 was found as a novel protein interacting with ct-PAEL-R in Y2H studies. **(A)** Y2H studies confirmed interaction between GABARAPL2 with ct-PAEL-R. β -Galactosidase assay of yeast transformed with the following constructs are shown (1) empty vectors pGBKT4 and pGADT7, (2) ct-GluR2 and PICK1, (3) ct-PAEL-R and GABARAPL2. The blue colour confirms positive interactions in the assay. **(B)** Sequence of GABARAPL2 isolated from Y2H screen (bold), aligned with the GABARAPL2 (Human-NP-009216) sequence. Underlined shown 3' and 5' unrelated sequence associated with the GABARAPL2 pACT2 clone.

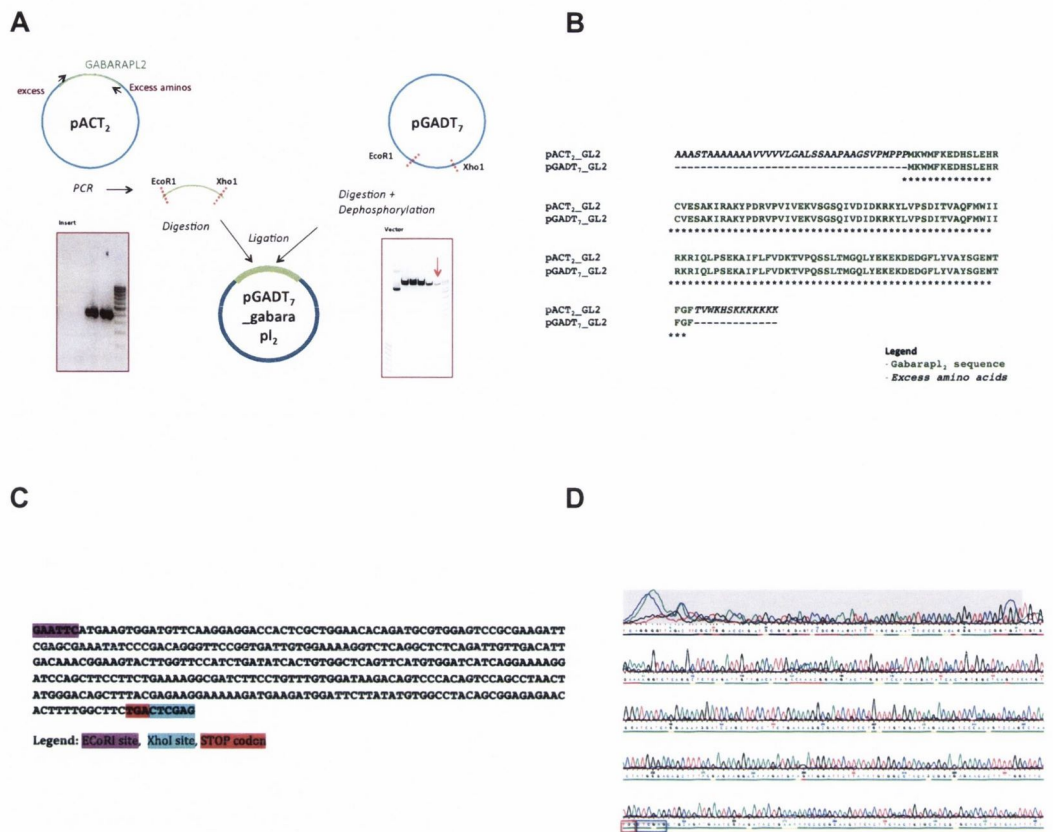


Figure 3: Cloning of GABARAPL2 from pACT2 into pGADT7 vector. (A) Schematic diagram for the cloning strategy plus agarose gel images of the insert and the vector indicated by a red arrow. **(B)** GABARAPL2 Y2H clone (ASA4024) is aligned with GABARAPL2 (Human-49457428) in green and unrelated 5' and 3' sequences are presented in black. **(C)** Nucleotide sequence of GABARAPL2, with restriction sites, and stop codon annotated. **(D)** Raw peak sequencing data from MWG, Germany.

A



B

GABARAPL2

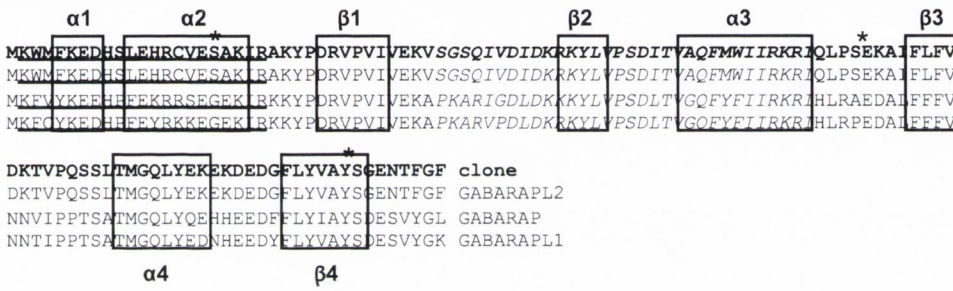
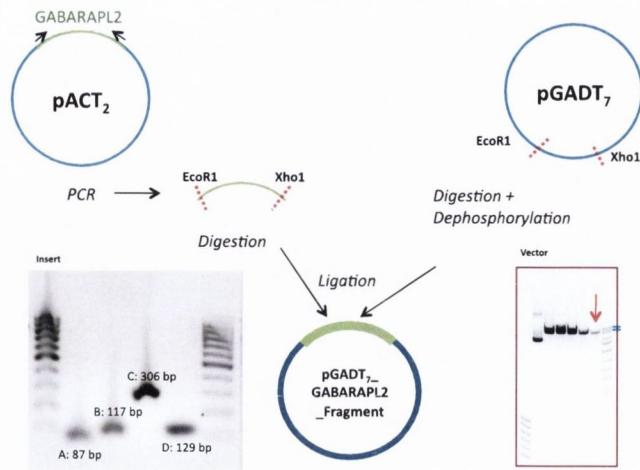


Figure 4: GABARAPL2 without unrelated sequence interacts with PAEL-R. (A) GABARAPL2 is re-cloned into the pGADT7 (fish) vector without unrelated sequences which interacts with ct-PAEL-R, but not with the empty pGBKT7 bait vector in an Y2H screen. (B) The isolated re-cloned GABARAPL2 is aligned with GABARAPL2 (Human-NP-009216), GABARAP (Human-NP-009209) and GABARAPL1 (Human-NP-113600) proteins. The UBL core domains ($\alpha 1$ - $\alpha 4$, $\beta 1$ - $\beta 4$) are shown in boxes, the tubulin binding domain is underlined and GABA receptor binding domain is highlighted in italics. Tyrosine kinase and protein C kinase phosphorylation sites are shown via an asterisk.

A**B**

GAATTCATGAAGTGGATGTTCAAGGAGGACCCTCGCTGGAACACAGATGCGTGGAGTCCGCGAAGATT
 CGAGCGAAATATCCCGACAGGGTTCGGTGATTGTGGAAGGCTCAGGCTCTCAGATTGTTGACATT
 GACAAACGGAAGTACTTGGTTCCATCTGATATCACTGTGGCTCAGTTCATGTGGATCATCAGGAAAAGG
 ATCCAGCTTCCTTCTGAAAAGGCGATCTTCCTGTTTGTGGATAAGACAGTCCCACAGTCCAGCCTAACT
 ATGGGACAGCTTTACGAGAAGGAAAAGATGAAGATGGATTCTTATATGTGGCTACAGCGGAGAGAAC
 ACTTTTGGCTTCGACTCGAG

Legend: EcoRI site, XhoI site, STOP codon

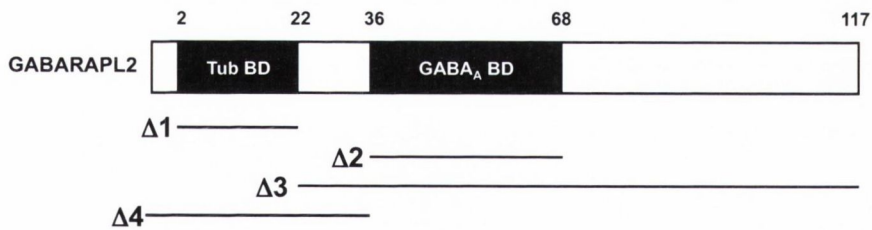
C

Figure 6: Cloning of GABARAPL2 fragments into the pGADT7 vector. (A) Schematic diagram for the cloning strategy plus agarose gel images of the fragments of GABARAPL2 and the vector indicated by a red arrow. **(B)** Representation of the full length GABARAPL2 sequence. **(C)** Schematic diagram of GABARAPL2 fragments.

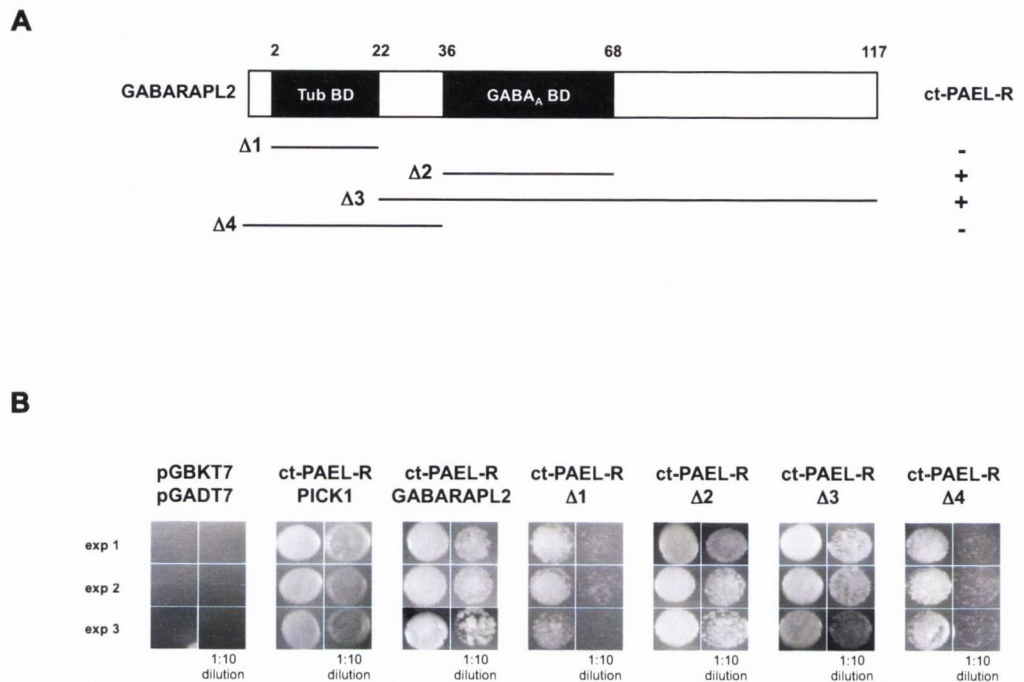
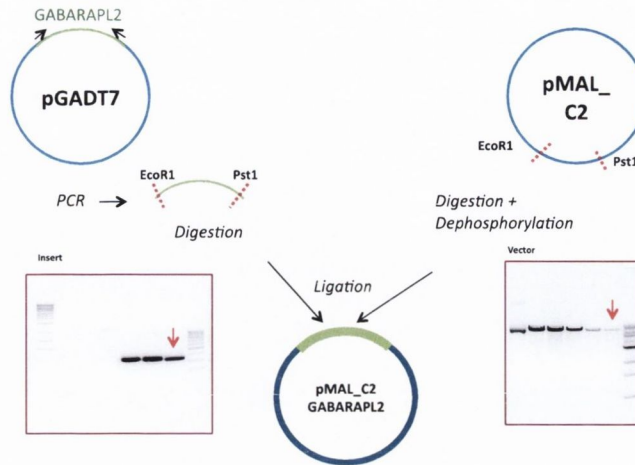


Figure 7: The ct-PAEL-R interacts with the GABA_A receptor binding domain of GABARAPL2. (A) The Tubulin binding domain (Tub BD) of GABARAPL2 is located between amino acids 2-22 and is found in fragments Δ1 and Δ4. The GABA_A receptor binding domain (GABA_A BD) of GABARAPL2 is between amino acids 36 and 68 and is found in fragments Δ2 and Δ3. Y2H data shows that the ct-PAEL-R interacts with fragments Δ2 and Δ3, containing the GABA_A receptor binding domain (+ indicates interaction, - indicate no interaction). **(B)** Y2H data shows that yeast transformed with ct-PAEL-R and GABARAPL2 fragments containing the GABA_A receptor binding domain show strong growth on interaction plates at 1/10 dilution of yeast, compared with positive (ct-GluR2 and PICK1) and negative (pGBKT4 and pGADT7) controls.

A**B**

GAATTCATGAAGTGGATGTTCAAGGAGGACCCTCGCTGGAACACAGATGCGTGGAGTCCGCGAAGATT
 CGAGCGAAATATCCCGACAGGGTTCGGTGATGTGGAAAAGGCTCAGGCTCTCAGATTGTTGACATT
 GACAAACGGAAGTACTTGGTTCCATCTGATATCACTGTGGCTCAGTTCATGTGGATCATCAGGAAAAGG
 ATCCAGCTTCCTTCGAAAAGGCATCTTCCTGTTTGTGGATAAGACAGTCCCACAGTCCAGCCTAACT
 ATGGACAGCTTTACGAGAAGGAAAAAGATGAAGATGGATTCTTATATGTGGCCTACAGCGGAGAGAAC
 ACTTTGGCTTC**TGACTGCAG**

Legend: **EcoRI site**, **PstI site**, **STOP codon**

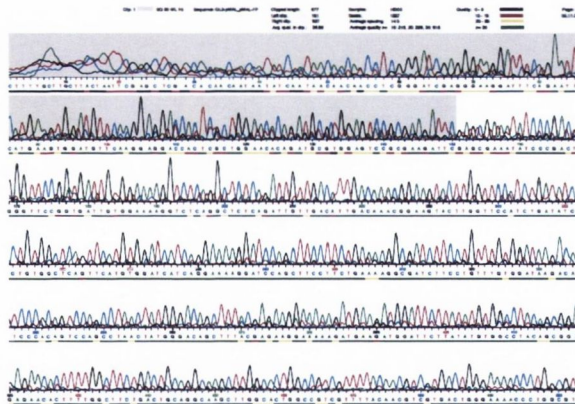
C

Figure 8: Cloning of GABARAPL2 into the pMAL-C2 vector. (A) Schematic diagram for the cloning strategy plus agarose gel images of the insert and the vector indicated by a red arrow. **(B)** Nucleotide sequence of GABARAPL2 sequence, with restriction sites, and stop codon annotated. **(C)** Confirmation of successful cloning of GABARAPL2 into pMAL-C2 from MWG, Germany.

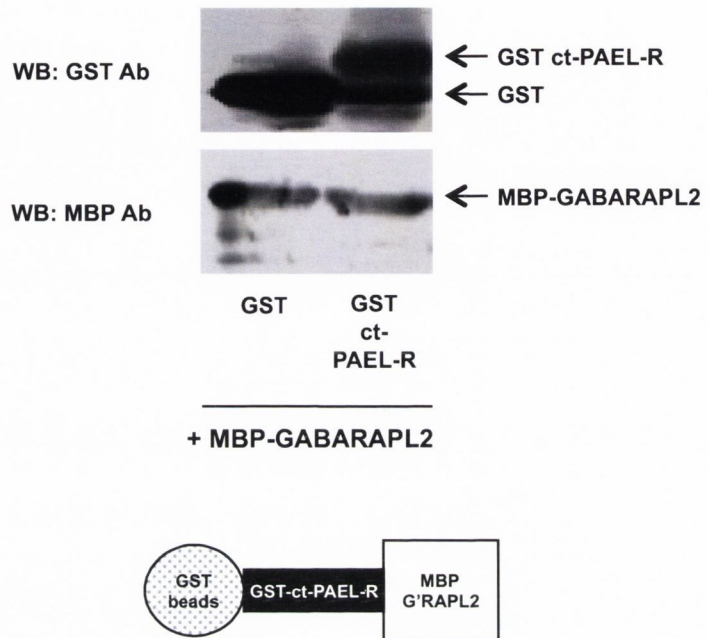
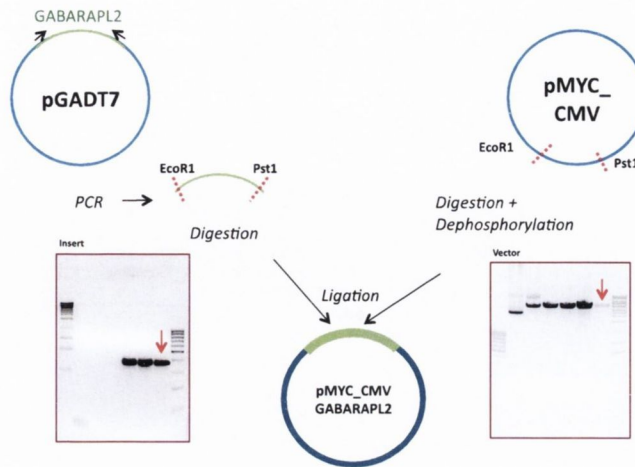


Figure 9: The ct-PAEL-R shows non-specific interaction with GST and GST beads alone. Western blot with anti-MBP antibody showing the levels of MBP-GABARAPL2 retained by GST-ct-PAEL-R and also by GST alone. Fusion proteins of GST and GST-ct-PAEL-R, were isolated from *E.coli* strain BL21. MBP-tagged GABARAPL2 was also isolated from *E.coli* strain BL21. GST proteins were attached to glutathione Sepharose beads, incubated with MBP-GL2 containing bacterial lysate, washed and then examined by Western blotting for their content of MBP-GABARAPL2. The anti-GST antibody Western blot indicated the levels of GST and MBP-GABARAPL2 fusion proteins used in this experiment. The diagram represents the setup of the pull-down experiment.

A**B**

GAATTCATGAAGTGGATGTTCAAGGAGGACCACTCGCTGGAACACAGATGCGTGGAGTCCGCGAAGATT
 CGAGCGAAATATCCCGACAGGGTTCGGTGATTGTGGAAAAGGCTCAGGCTCTCAGATTGTTGACATT
 GACAAACGGAAGTACTTGGTTCATCTGATATCACTGTGGCTCAGTTCATGTGGATCATCAGGAAAAGG
 ATCCAGCTTCCTTCTGAAAAGGCATCTTCCTGTTTGTGGATAAGACAGTCCACAGTCCAGCCTAACT
 ATGGACAGCTTTACGAGAAGGAAAAGATGAAGATGGATTCTTATATGTGGCTACAGCGGAGAGAAC
 ACTTTTGGCTTCGACTCGAG

Legend: EcoRI site, XhoI site, STOP codon

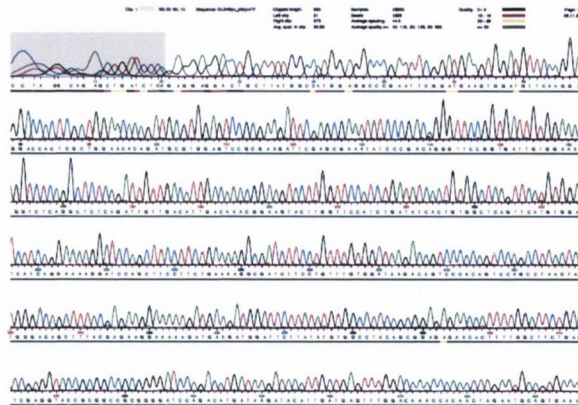
C

Figure 10: Cloning of GABARAPL2 into the pMYC-CMV vector. (A) Schematic diagram for cloning strategy plus agarose gel images of the insert and the vector indicated by a red arrow. **(B)** Nucleotide sequence of GABARAPL2 sequence, with restriction sites, and stop codon annotated. **(C)** Confirmation of successful cloning of GABARAPL2 into pMYC-CMV from MWG, Germany.

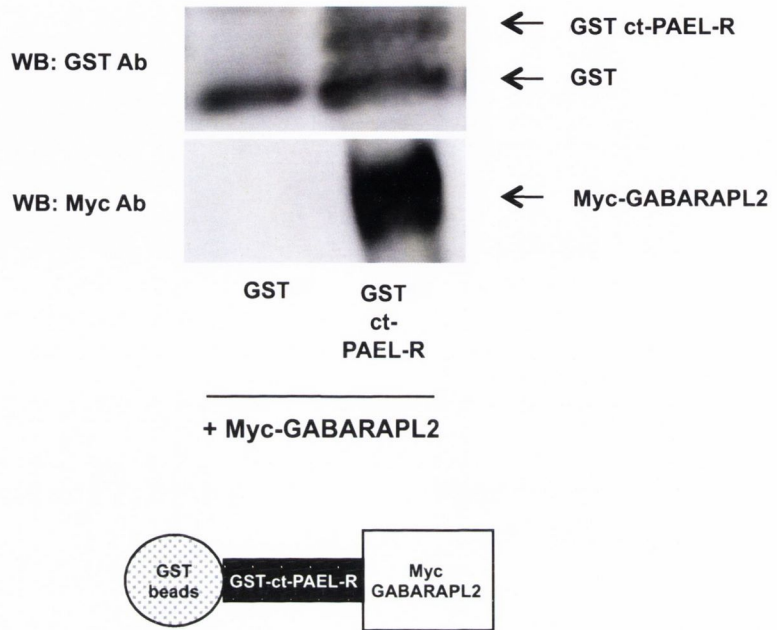


Figure 11: Biochemical evidence of binding between ct-PAEL-R and GABARAPL2. The ct-PAEL-R interacts with Myc tagged GABARAPL2. Fusion proteins of GST and GST-ct-PAEL-R, were isolated from E.coli strain BL21. Myc-tagged GABARAPL2 was isolated from HEK292 cells which was transiently transfected with Myc-GABARAPL2. GST-PAEL-R was bound to glutathione Sepharose beads, incubated with Myc-GABARAPL2, washed and then examined by Western blotting for their content of Myc-GABARAPL2. The anti-GST antibody Western blot indicated the levels of GST fusion proteins used in this experiment. The diagram represented the setup of the pulldown experiment.

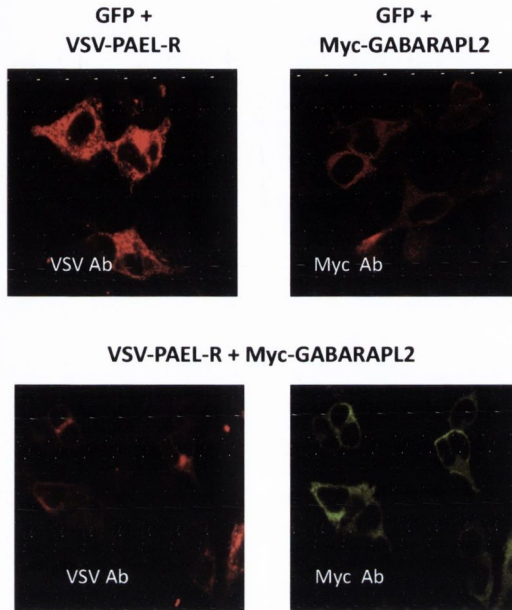
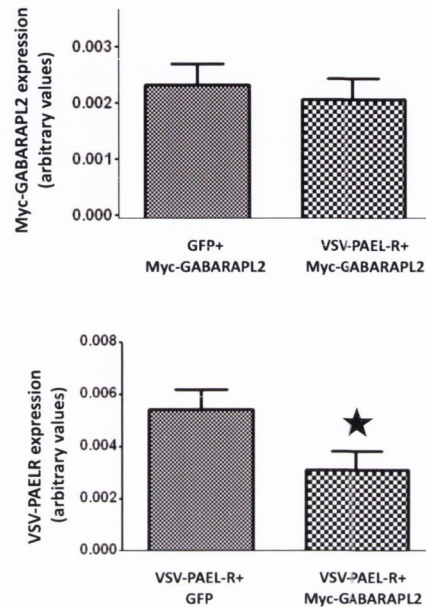
A**B**

Figure 12: GABARAPL2 decreases PAEL-R expression. (A) Colocalisation of VSV-PAEL-R and Myc-GABARAPL2 in HEK293 cells. Cells were transiently transfected with Myc-GABARAPL2 and VSV-PAEL in combination or with GFP. The cells were stained with anti-Myc or anti-VSV Abs and viewed at x63 magnification. **(B)** GABARAPL2 leads to a statistical decrease in PAEL-R expression. Graphs were plotted for expression of VSV-PAEL-R when co-transfected with GFP as control or Myc-GABARAPL2. The fluorescent pixels of VSV-PAEL-R was quantified from exactly 100 cells. Data shows similar expression levels of Myc-GABARAPL2 when co-transfected with GFP or VSV-PAEL-R ($p=0.634$; $n=3$). Data reveals a statistical reduction in VSV-PAEL-R expression when co-transfected with Myc-GABARAPL2 compared to co-transfection with GFP ($*p=0.033$; $n=3$).

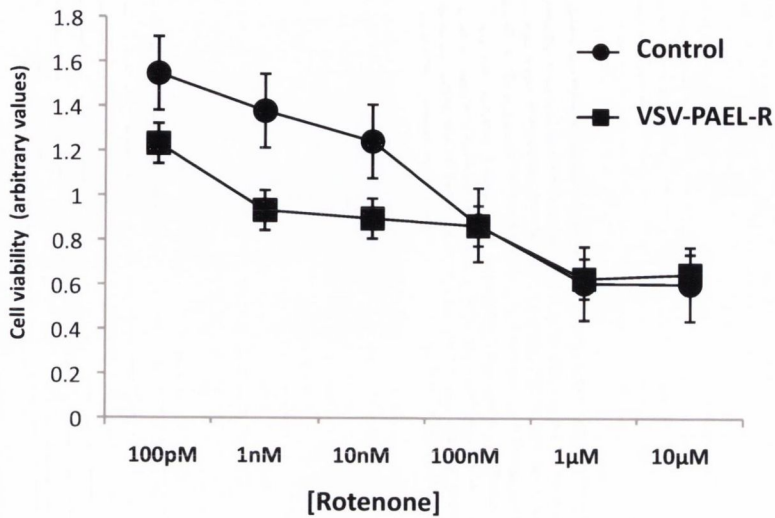
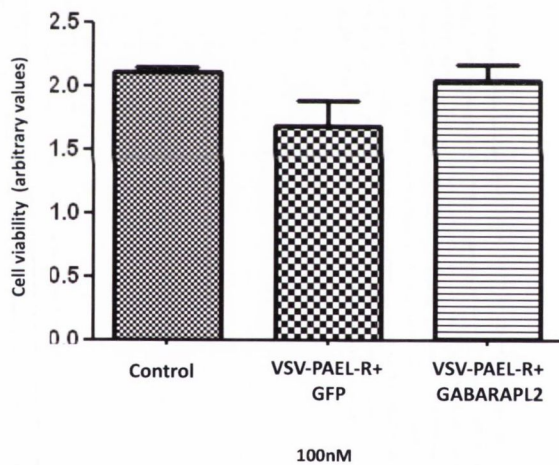
A**B**

Figure 13: Cell survival study of PAEL-R and GABARAPL2. (A) Concentration dependent cell survival curve of rotenone (48 h) when HEK293 cells were transiently transfected with GFP or VSV-PAEL-R. The cell viability test was done using 'cell titer 96 aqueous one solution assay' (Promega). (B) The cell viability was analysed for HEK293 cells transiently transfected with VSV-PAEL-R and GFP (as control) or Myc-GABARAPL2 treated with 100nM rotenone for 48 h. GABARAPL2 shows a trend, but no significant improvement in cell survival with PAEL-R in comparison to PAEL-R alone.

Discussion

1. A novel interaction between PAEL-R and GABARAPL2

In this chapter we investigated a novel interaction between PAEL-R and GABARAPL2. Initial experiments investigated the interaction in Y2H experiments. We first showed that the interaction between GABARAPL2 and PAEL-R was located in the coding sequence of GABARAPL2. Thereafter, we determined the putative interaction site of PAEL-R to the -CCCCC-EEC motif and on GABARAPL2 to the GABA_A binding domain. Next the GABARAPL2-PAEL-R interaction was validated in biochemical and cellular studies. GABARAPL2 was cloned into the pMAL vector to perform biochemical pull down experiments. Data showed that MBP-GABARAPL2 bound non-specifically to GST or GST beads. Therefore we replaced MBP with Myc and tested Myc-GABARAPL2 interaction with PAEL-R. The results showed that Myc-GABARAPL2 bound specifically to GST-PAEL-R and not GST alone, thus biochemically confirming the PAEL-R-GABARAPL2 interaction. Finally our data showed the co-expression of GABARAPL2 with PAEL-R caused a reduction in PAEL-R expression levels. This reduction in PAEL-R expression did not however reduce cell death induced by PAEL-R in condition of cell stress. Indeed GABARAPL2 is particularly expressed on autophagosomes which may suggest that it is a potential target for controlling and handling misfolded PAEL-R by a mechanism of autophagy and cell loss.

2. GABARAP family: localisation and function

Using a Y2H system, it has been shown that the intracellular loop of $\alpha 2$ of the GABA_A receptor, when used as a bait, interacts with GABARAP (Chen et al., 2001). As outlined in the introduction all the proteins in this family share common features. First, they are evolutionally conserved in eukaryotic cells from yeast to mammals. Second, their three-dimensional structures show that the proteins in this family share strong similarity to ubiquitin. Third, the GABARAP family is not only structurally similar to ubiquitin but undergoes a similar post-translational modification process similar to ubiquitinylation. Fourth, all the proteins are involved in intracellular trafficking (Chen and Olsen, 2007). As the structure of GABARAPL2 shares similarities with ubiquitin it may regulate ubiquitination of PAEL-R reduces

expression, and thus prevent the aggregation of misfolded PAEL-R.

GABARAPL2 directly binds NSF and Golgi-specific SNARE protein (GOS-28), and is believed to be a component of the intra-Golgi transport machinery (Legesse-Miller et al., 1998; Muller et al., 2002). Recently, it has been shown that the function of GABARAPL2 as a SNARE protector is essential for intra-Golgi transport (Sagiv et al., 2000; Muller et al., 2002). GABARAPL2 has also been shown to enhance the ATPase activity of NSF and stimulates the recruitment of GABARAPL2 to the unpaired GOS-28 in an ATP dependent or ATPase independent manner (Chen and Olsen, 2007). Therefore this interaction protects the unpaired GOS-28 from proteolysis. GABARAPL2 n-terminus shows 77% identity with the tubulin-binding domain of GABARAP and promotes tubulin assembly and displays tubulin bundling activity (Chen and Olsen, 2007). GABARAPL2 is localised at the Golgi apparatus and thus may be involved in trafficking of PAEL-R between ER and Golgi. It may play a role in transporting misfolded proteins back from the Golgi to ER for degradation by the UPS and ERAD degradatory systems.

3. GABARAP: molecular link to autophagy

Autophagy was recently identified as a novel degradative pathway for GABA_A receptors (Rowland et al. 2006). *C. Elegans* LGG-1 has also been used as the autophagosome marker (Chen and Olsen, 2007). In *C. elegans*, the GABA_A receptors traffic to autophagosomes after endocytic removal from the cell surface thus allowing the degradation of cytoplasmic and membrane bound postsynaptic proteins (Chen and Olsen, 2007). LGG-1 (GATE-16, LC3 and GABARAP) are implicated in autophagy (Schwarten et al., 2009). Interestingly, LC3 and GABARAPs were localised in LBs in PD (Tanji et al., 2011). Thus autophagic function is impaired through alteration of Atg8 homologous in LB disease. Thus repressed levels of GABARAP/GABARAPL1 may lead to the loss of their autophagosomal formation capabilities and which may lead to the pathogenesis of LB disease (Tanji et al., 2011). GABARAP also interacts with Nix and subcellular staining of GABARAP plus Nix display a colocalisation pattern (Schwarten et al., 2009). Both these proteins may be selectively regulating each other activities in autophagy (Schwarten et al., 2009). Nix is a mitochondrion localised protein which regulates removal of mitochondria during an autophagic response (Novak et al.,

2010). Nix recruits GABARAPL1 to damaged mitochondria and interaction leads to mitochondria clearance (Novak et al., 2010). These studies directly link the GABARAP family including GABARAPL2 with autophagosomal formation and clearing aggregated proteins and aberrant organelles.

4. GABARAPL2 reduces PAEL-R protein levels

Interestingly, we found that GABARAPL2 decreases the overall expression of PAEL-R, when HEK293 cells were co-transfected with GABARAPL2 and PAEL-R. These findings are similar to the previous chapter showing that PICK1 reduces PAEL-R protein levels. In this chapter, we exposed the HEK293 cells, which were transiently transfected with PAEL-R and GABARAPL2 either alone or in combination, with common pesticide, rotenone. We found that rotenone-induced cell stress was not rescued when PAEL-R was co-transfected with GABARAPL2 in contrast to the effects of PICK1. We hypothesise two possible pathways based on the PAEL-R-GABARAPL2 interactions. First, the interaction between PAEL-R-PICK1 may clear PAEL-R via a parkin-PICK1-PAEL-R ubiquitination proteasomal degradation pathway. Secondly, the GABARAPL2-PAEL-R may possibly be involved in the degradation of PAEL-R by the autophagic pathway (**Fig 14**). One assumption could be that Nix recruits GABARAPL2 to damaged mitochondria, which helps facilitating PAEL-R degradation, or another assumption is that GABARAPL2 directly target PAEL-R by lysosomal degradation.

5. Future studies

Here we propose a novel role for the PAEL-R-GABARAPL2 interaction in degradation of PAEL-R by autophagy. To confirm the proposal it would be important to examine whether overexpression of GABARAPL2 can change the autophagic degradation of PAEL-R. Blocking the GABARAPL2-PAEL-R interaction to observe changes in cell survival within the autophagic pathway would also be worthy of study. Lastly, the levels of PAEL-R in GABARAPL2 KO animals and the rate of autophagy and dopaminergic neuronal survival would lead these findings into an in-vivo and translational setting.

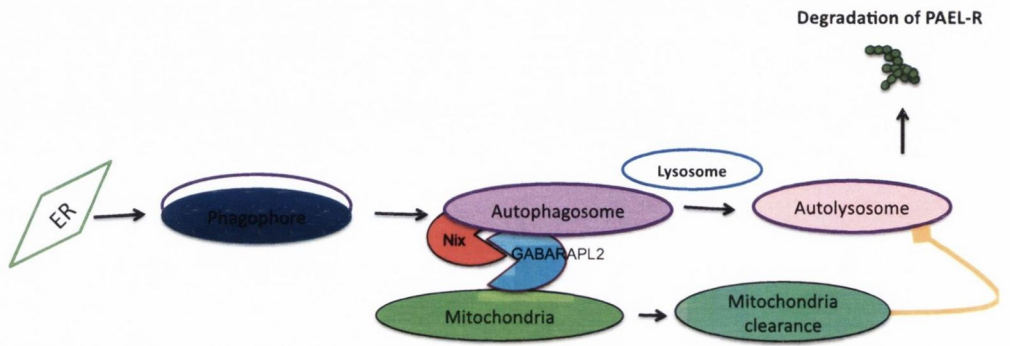


Figure 14: Summary of PAEL-R-GABARAPL2 function. We hypothesise PAEL-R is degraded by autophagic processes via the help of GABARAPL2.

CHAPTER 5

PAEL-R INTERACTS WITH RAB14

Aims

Aim1: to identify novel Interaction between RAB14-PAEL-R by small scale Y2H.

Aim2: to clone pACT2 Rab14 into pGADT7 and to confirm interaction between RAB14-PAEL-R without unnecessary sequence by Y2H.

Aim3: to test the selectivity for other PAEL-R family member (GPR37L1) with RAB14 and test the PAEL-R mutant.

Aim4: to clone RAB14 fragments into pGADT7

Aim5: to determine specific interaction sites of PAEL-R on RAB14.

Aim6: to validate interaction biochemically.

Aim7: to determine effects of RAB14 on PAEL-R expression.

Aim8: to test the RAB14 and PAEL-R effect on cell survival study.

Abstract

After identifying PICK1 and GABARAPL2 as interacting proteins with parkin-associated endothelial-like Receptor (PAEL-R), we investigated a third novel interaction between ras-associated binding protein 14 (Rab14) and PAEL-R. The RAS superfamily of small GTPases is broadly subdivided into five groups: Ras, Rho, Rab, Ran, and Arf. Rab family proteins are important in regulating signal transduction and cellular processes such as differentiation, proliferation, vesicle transport, nuclear assembly, and cytoskeleton formation (Takai et al., 2001). Here we report a novel interaction between Rab14 and PAEL-R. We predict the interaction between Rab14 and PAEL-R to employ the use of the -CCCCC-EEC motif in the ct-PAEL-R. Results also revealed that GPR37L1 and PAEL-R have similar interaction properties with Rab14. While examining the specific site of interaction, we observed that PAEL-R interacts with the second GTP binding site of Rab14. A pull down assay indicated that recombinant GFP-Rab14, obtained from heterologous cells, was retained by a GST-ct-PAEL-R but not by GST alone. We showed that GFP-Rab14 co-transfection with Myc-PAEL-R caused a reduction in PAEL-R as well as in Rab14 expression levels. Finally, we also report that the cell death induced by rotenone was not attenuated when PAEL-R was co-transfected with Rab14. Thus our experiments find a novel interaction between PAEL-R and Rab14 and give an overview to understand the function of PAEL-R.

Introduction

Ras-associated binding proteins are members of the Ras family of small GTPases (**Fig 1**). Rab proteins regulate intracellular vesicle trafficking and was first genetically obtained in the yeast *S. Cerevisiae* (Lazar et al., 1997). Mainly, transmembrane and secreted soluble proteins are transported from one membrane compartment to another by vesicles (Takai et al., 2001). Newly synthesised secretory proteins are translocated into the ER and are then recycled back to the plasma membrane via the Golgi apparatus by vesicles. In a similar fashion macromolecules that are taken from the plasma membrane are transported to endosomes and lysosomes by vesicular transport. Most of the receptors for extracellular ligands recycle through the endosome back to the plasma membrane. Thus intracellular vesicle trafficking is important for exocytosis, endocytosis, and receptor recycling. There are two types of exocytosis pathways: constitutive and regulated exocytosis. The constitutive exocytosis is a process that releases newly synthesised membrane proteins incorporated in the plasma membrane after the fusion of transport vesicles. The regulated exocytosis requires an external signal or a specific sorting signal like an increase in Ca^{2+} for the vesicle to direct its contents toward the cell membrane. Vesicle trafficking is needed for various other cell functions such as the formation of cell polarity, cell motility and cytokinesis (Takai et al., 2001). The major step of intracellular vesicle transport is the budding of a vesicle from the donor membrane; targeting of the vesicle to the acceptor membrane; docking of the vesicle to the acceptor membrane; and the fusion of the vesicle with the acceptor membrane. There are two types of Rab proteins; one type is involved in regulated secretion and the other type is involved in vesicle transport process (Takai et al., 2001) (**Fig 2**). Rabs participate in docking and fusion of transport vesicles with their target membranes and mediate receptor cargo collection during transport vesicle formation (Junutula et al., 2004, Kelly et al., 2009).

Rab proteins regulate various processes which include vesicle targeting, docking and fusion through its specific effectors. Rab proteins regulate tethering proteins (like p115, Uso1, EEA1) onto membranes, which include two types of SNAREs (v- and t-SNAREs) resulting in docking of vesicles with their target membranes (Pfeffer, 1999). Rab proteins may also play a role in the budding process. Rab effects on the budding process are unknown. However, after/during the bud

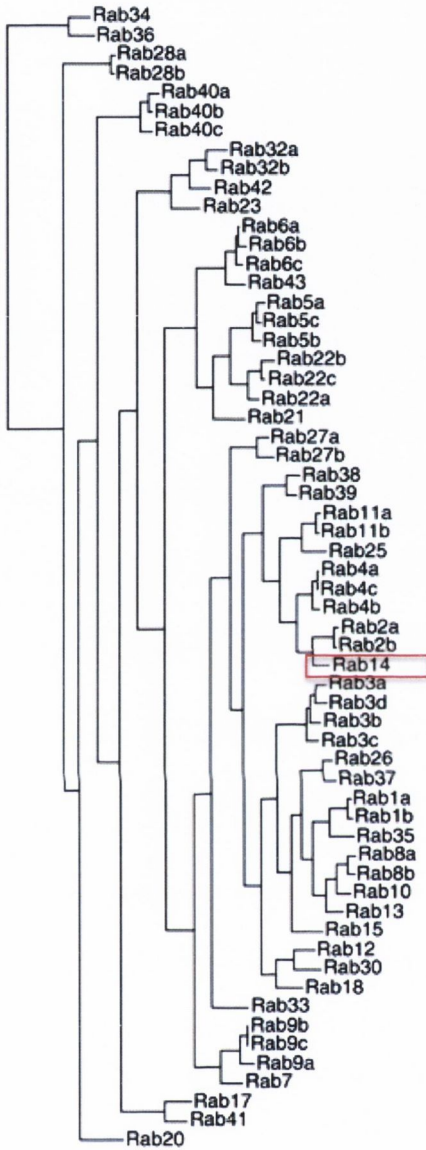
formation, coat proteins are disassembled to produce uncoated vesicles and before/during/after this uncoating process Rab proteins are associated with the vesicles (Takai et al., 2001).

Rab proteins have been detected in different cell types as well as in different organisms, dependent mainly on their function (Takai et al., 2001). Rab proteins cycle between the GDP bound inactive state and GTP bound active state between the cytosol to the membranes. The activation, inactivation, and translocation processes are controlled by three types of regulators: guanine nucleotide exchange factors (GEPs), guanine nucleotide dissociation inhibitor (GDIs), and GTPase-activating proteins (GAPs). Rab proteins are maintained in the GDP bound inactive form by GDI in the cytosol and these Rab proteins are delivered to a specific membrane compartment when the GDP bound form is released from the GDI. By the action of GEP, the Rab protein is converted back to the GTP bound form which interacts with downstream effectors. Thereafter, GAP converts the GTP bound form to the GDP bound form. The GDP bound form produced on the membrane forms a complex with the GDI and returns to the cytosol. Hence, Rab GDI plays an essential role in specific delivery of Rab proteins to their target membranes (Takai et al., 2001) (**Fig 3**).

The yeast *S. cerevisiae* genome sequence encodes 11 Rab proteins (Lazar et al., 1997) and in mammalian cells approximately 50 Rab proteins are known (Martinez et al., 1998). Rab14 is closely linked with the Rab2, Rab4, Rab11, Rab25 and Rab39 within the family. Rab14 localises to the Golgi/trans-Golgi network and to early endosomes, however its biological function remains unclear (Proikas-Cezanne et al., 2006). Rab14 is implicated in the biosynthetic or recycling pathway between the Golgi and endosomal compartments (Junutula et al., 2004). Rabs are also critical in mammalian protein-protein interactions (Subramani and Alahari, 2010).

In PD, insoluble PAEL-R accumulates in the brains of AR-JP patients (Imai et al., 2001). It is highly expressed by the dopaminergic neurons of the SN, strongly suggesting that accumulation of unfolded PAEL-R may lead to selective death of dopaminergic neurons (Yang et al., 2003). Thus understanding the details of regulating PAEL-R neurotoxicity by studying its trafficking proteins will be valuable. Preventing interacting proteins from associating with the PAEL-R may alter its trafficking and hence provide a method for regulating neurotoxic properties.

Therefore we aimed to identify PAEL-R interacting proteins and isolated a Rab14 clone using large scale Y2H technology. Rab14 is localised in the Golgi/trans-Golgi network and early endosomes and we hypothesise it may be involved in regulating PAEL-R aggregation and/or neurotoxicity.

A**B**

The small G protein superfamily			
	Rab family		
Mammal	Rab 1A	Rab 11A	Rab 26
	Rab 1B	Rab 11B	Rab 27A
	Rab 2	Rab 12	Rab 28
	Rab 3A	Rab 13	Rab 29
	Rab 3B	Rab 14	Rab 30
	Rab 3C	Rab 15	Rab 31
	Rab 3D	Rab 16	Rab 32
	Rab 4	Rab 17	Rab 33A
	Rab 5A	Rab 18	Rab 33B
	Rab 5B	Rab 19	
Rab 5C	Rab 20		
Rab 6	Rab 21		
Rab 7	Rab 22		
Rab 8	Rab 23		
Rab 9	Rab 24		
Rab 10	Rab 25		
Yeast	Ypt 1	Ypt 52	
	Sec 4	Sec 53	
	Ypt 31	Ypt 6	
	Ypt 8	Ypt 7	
	Ypt 32	Ypt 10	
	Ypt 9	Ypt 11	
	Ypt 51		
	Ypt 21		

Figure 1: Schematic diagram for Ras family of small GTPases. (A) Phylogenetic tree of human Rab GTPases (Stenmark and Olkkonen, 2001). **(B)** List of Rabs identified in eukaryotes to yeast to human.

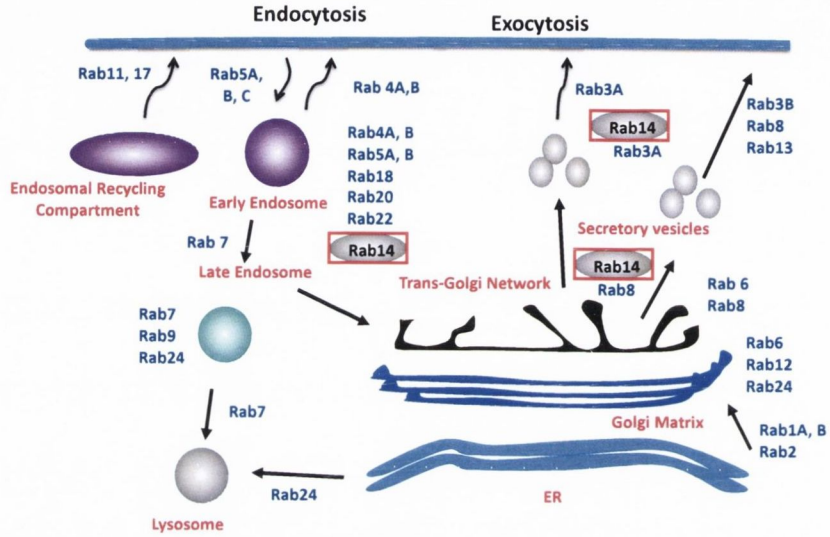


Figure 2: Subcellular localisation of Rab proteins. Rabs are shown in blue/grey and site of each Rab protein shown in red. The localisation of Rab14 is shown in box.

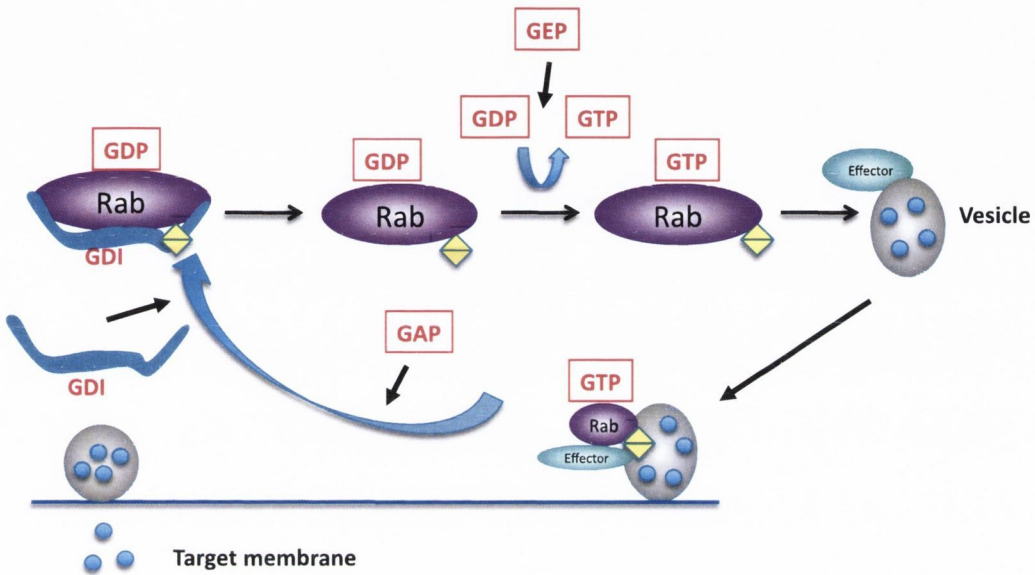


Figure 3: Cyclical activation, inactivation and translocation of Rab proteins.

The processes of Rab proteins are regulated by GEFs, GDIs and GAPs represented in red. The GDP bound form of a Rab protein is first released from the GDI when the Rab protein is delivered to a specific membrane compartment. The Rab protein is converted to the GTP bound form by the action of GEF and the GTP bound form of Rab then interacts with downstream effectors. Thereafter, the GTP bound form of Rab is converted to the GDP bound form by the action of GAP. The GDP bound form of Rab is produced at the membrane then complexes with the GDI and returns to the cytosol (Takai et al., 2001).

Results

1. New novel interaction between PAEL-R and Rab14

In addition to PICK1 and GABARAPL2, we identified a novel interaction between parkin-associated endothelial-like Receptor (PAEL-R) and ras-associated binding protein 14 (Rab14). This interaction was isolated and confirmed in Y2H experiments (**Fig 4**). The results showed that ct-PAEL-R was interacting with Rab14. The original Rab14 clone isolated contained unrelated 3' and 5' sequences. Thus we cloned Rab14 without those excess base pairs. Rab14 was cloned into pGADT7 vector by the standard cloning method (**Fig 5**). The Y2H experiment showed that Rab14, without the unrelated sequences still interacted with ct-PAEL-R (**Fig 6**). This confirmed that the interaction was not due to the unrelated amino acids of Rab14. The results revealed a novel putative interaction between PAEL-R and Rab14.

2. -CCCCC-EEC motif of PAEL-R is a putative binding site for Rab14

In chapter 3 we have reported that the PDZ motif of PAEL-R interacts with PICK1 and the PAEL-R mutant blocked the PDZ motif interaction with PICK1. The mutant ct-PAEL-R construct was designed with an additional 10 amino acids (ct-PAEL-R+10) that occluded the putative PDZ motif from being expressed at the extreme ct of PAEL-R. Therefore we used the mutant ct-PAEL-R with Rab14 to confirm the specific site of interaction. The PAEL-R mutant was transformed with Rab14 and the Y2H experiment was carried out (**Fig 7**). We found that Rab14 still interacted with the mutant ct-PAEL-R, similar to the GABARAPL2 interaction with PAEL-R. Thus we conclude that both GABARAPL2 and Rab14 do not require the PDZ motif for interaction. In contrast to PICK1 which require the PDZ motif of PAEL-R for interaction. As mentioned in the previous chapter GPR37L1 is another family member of PAEL-R. GPR37L1 is 481 amino acids in length and was identified from human cDNA with a 68% sequence homology to PAEL-R (Valdenaire et al., 1998). We identified GPR37L1 as having a similar ct sequence to PAEL-R thus we investigated whether GPR37L1 has similar interactions. We confirmed a novel PDZ motif in GPR37L1 which interacts with PICK1 and also showed that

GABARAPL2 interacts with GPR37L1. We therefore tested if GPR37L1 interacted with Rab14 and confirmed that ct-GPR37L1 interacts with Rab14 in Y2H experiments (**Fig 7**). According to the sequence homology of PAEL-R and GPR37L1 the -CCCCC-EEC is highly conserved between both receptors. Therefore we hypothesise that the -CCCCC-EEC sequence of ct-PAEL-R is a possible binding site for Rab14, similar to GABARAPL2.

3. PAEL-R interacts via second GTP binding site of Rab14

Throughout this thesis, we have been interested to identify specific interacting sites as to develop blocking peptides or molecules which regulate proteins interacting with PAEL-R in order to control PAEL-R toxicity. To specify the PAEL-R interaction site on Rab14, we created overlapping deletion constructs of Rab14. PCR primers were designed for the specific domains of interest in the structure of Rab14 namely GTP binding domains and effector domain. Primers were designed such, that if the interaction is due to either of these regions, two of the three fragments should interact. We produced these constructs via PCR and checked the resulting fragment lengths, to confirm that primer design was correct. Three different fragments were made: $\Delta 1$ (1-107 amino acids) containing GTP binding domain I, II and effector domain, $\Delta 2$ (108-215 amino acids) with III and IV GTP binding domain and $\Delta 3$ (54-160 amino acids) containing II, III and IV GTP binding domain. The overlapping deletion constructs of Rab14 were cloned into the pGADT7 vector (**Fig 8**). These deletion constructs were then tested in small scale Y2H experiments with PAEL-R to specify the binding site of ct-PAEL-R on Rab14. The data showed that ct-PAEL-R interacts with fragments $\Delta 1$ and $\Delta 3$ of Rab14 which contain the second GTP binding site (**Fig 9**).

4. Biochemical confirmation of PAEL-R-Rab14 interaction

To further confirm the interaction between PAEL-R and Rab14, biochemical studies were performed. However, as suggested in the previous chapter the PAEL-R antibodies available appeared to be not specific. Therefore similar to the GABARAPL2 chapter, we used GST-PAEL-R instead to determine if Rab14 interacted with this receptor. The GST fusion protein of ct-PAEL-R/GST was coupled to GST beads and then exposed to GFP-Rab14 cell lysate prepared from

transfection in HEK293 cells (**Fig 10**). Western blot revealed that Rab14 was retained by GST-PAEL-R but not by GST alone. This result biochemically confirmed the interaction between PAEL-R and Rab14. The GFP-Rab14 was a kind gift from Prof. Jeremy Simpson, University College Dublin, Ireland.

5. Co-expression of Rab14 and PAEL-R reduces their expression

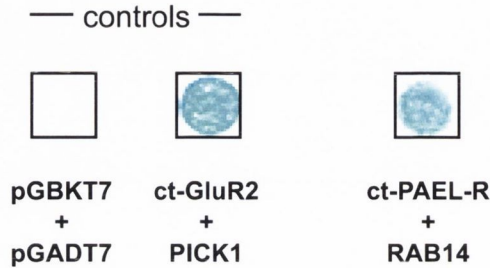
To study the functional role of PAEL-R-Rab14 interaction, we transiently transfected HEK293 cells with VSV-PAEL-R either alone or in combination with GFP (control) or GFP-Rab14. The cells were stained with anti-VSV monoclonal Ab after 48 h of transfection (**Fig 11**). The Western blot and cell staining results indicated a statistical significant reduction of GFP-Rab14 and VSV-PAEL-R when transfected in combination compared to when VSV-PAEL-R was transfected with GFP. The graph shows quantification on the expression of VSV-PAEL-R when co-transfected with GFP or GFP-Rab14. The decrease in expression of VSV-PAEL-R (**p=0.01; Unpaired t-test, n=3) and GFP-Rab14 (**p=0.001; t-test, n=3) was statistically significant when transfected together in comparison to co-transfected with GFP. Taken together, the data suggests the total protein expression levels of PAEL-R and Rab14 are reduced when co-transfected.

6. RAB14 is not protective against PAEL-R and rotenone-induced cell death

Because we found Rab14 to reduce PAEL-R expression we analysed the effect of Rab14 overexpression on rotenone toxicity during PAEL-R overexpression. HEK293 cells were transiently transfected with VSV-PAEL-R and GFP-Rab14 and treated with 10 μ M, 1 μ M, 100nM, 10nM, 1nM and 100 pM rotenone for 48 h. A cell viability test was done using cell titer 96 aqueous one solution assay kit. The results showed the 100nM rotenone induced cell death to similar levels in control HEK293 cells and in cells transiently transfected with VSV-PAEL-R. Next the viability of HEK293 cells transfected with VSV-PAEL-R and GFP-Rab14 or GFP and treated with 100nM rotenone for 48 h was examined. The overexpression of GFP-Rab14 showed no protective effect on the loss of HEK293 cells expressing

VSV-PAEL-R and treated with rotenone (**Fig 12**). It appears that Rab14 plays a role in reducing expression of the PAEL-R but has no protective effect when co-expressed together with PAEL-R.

A



B

RAB14

```
LHOFTTEKKMATAPYNYSYIFKYIIIGDMGVGKSCLLHQFTTEKKFMADCPHTIGVEFGTRIIIEVSGQKIKLQIWDTAGQER
-----MATAPYNYSYIFKYIIIGDMGVGKSCLLHQFTTEKKFMADCPHTIGVEFGTRIIIEVSGQKIKLQIWDTAGQER

FRAVTRSYRGAAGALMVYDITRRSTYNHLSSWLTDARNLTNPNTVILIGNKADLEAQRDVTYEEAKQFAEENGLLFLE
FRAVTRSYRGAAGALMVYDITRRSTYNHLSSWLTDARNLTNPNTVILIGNKADLEAQRDVTYEEAKQFAEENGLLFLE

ASAKTGENVEDAFLEAAKKIYQNIQDGSLDLNAAESGVQHKPSAPQGGRLTSEPQPQREGCGCCPHTIGVEFGTRI
ASAKTGENVEDAFLEAAKKIYQNIQDGSLDLNAAESGVQHKPSAPQGGRLTSEPQPQREGCGC-----
```

Figure 4: PAEL-R interacts with Rab14. Rab14 was identified as a novel protein interacting with ct-PAEL-R. **(A)** Small scale Y2H studies confirmed this interaction. β -Gal assay of yeast transformed with the following constructs are shown: empty vectors pGBKT4 and pGADT7, ct-GluR2 and PICK1 and ct-PAEL-R and Rab14. Blue colour confirms positive interactions in the assay **(B)** Sequence of Rab14 isolated (bold), aligned with Rab14 (Human-NP-057406) sequence. Unrelated n-terminal and c-terminal amino acids, fused to Rab14, as a result of cDNA library construction are underlined.

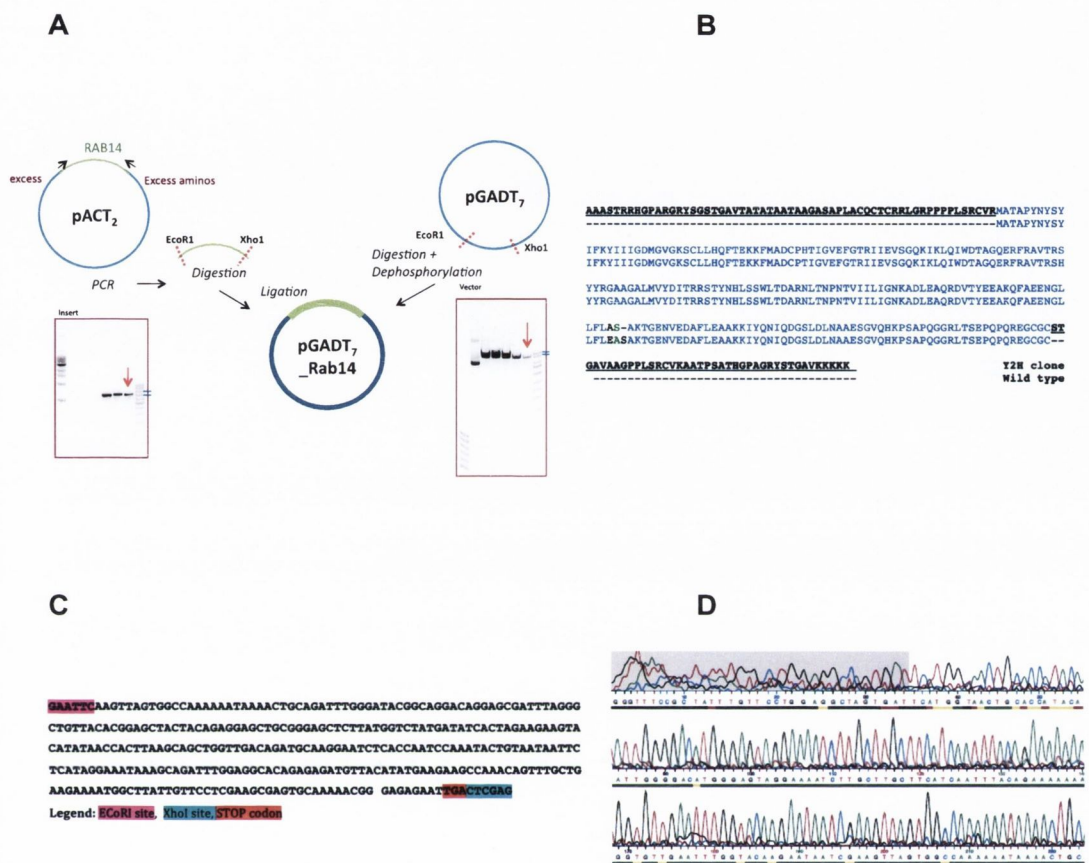


Figure 5: Cloning of Rab14 from pACT2 into pGADT7 vector. (A) Schematic diagram for the cloning strategy plus agarose gel images of the insert and the vector indicated by a red arrow. **(B)** Rab14 Y2H clone (ASA4034) aligned with Rab14 (Human-48146905) in blue and unrelated 5' and 3' sequences are in bold and underlined. **(C)** Nucleotide sequence of Rab14, with restriction sites, and the stop codon is annotated. **(D)** Raw peak sequencing data from MWG, Germany.

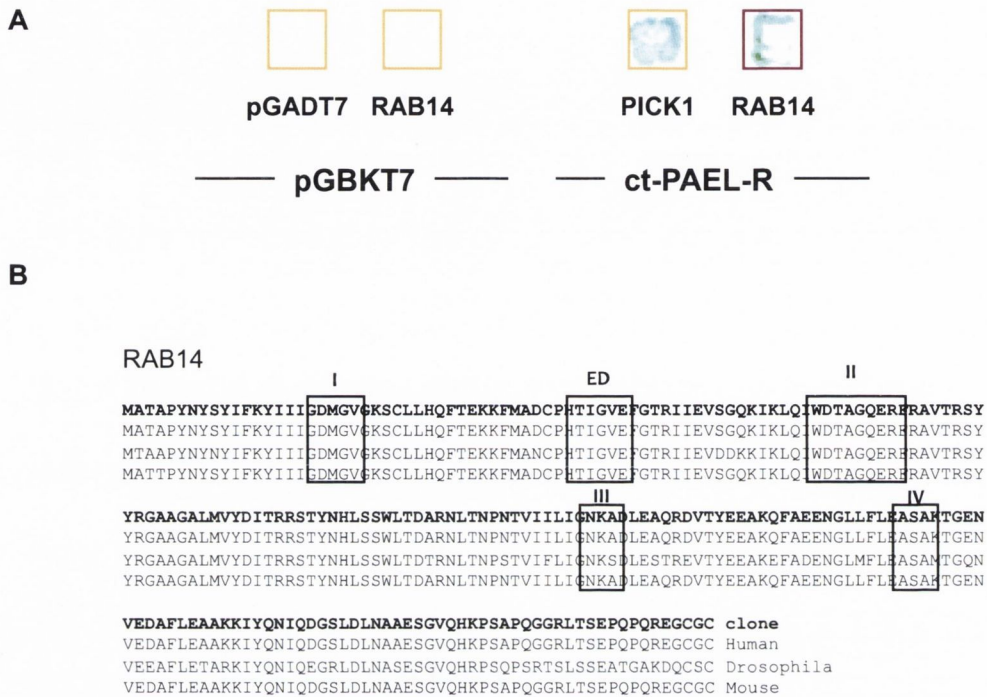


Figure 6: Rab14 without unrelated sequence interacts with PAEL-R. (A) Rab14 re-cloned into the pGADT7 (fish) Y2H vector without unrelated sequences, interacts with ct-PAEL-R, but not with the empty pGBKT7 bait vector in a Y2H screen using a β -gal assay. The blue colour confirms the interaction. (B) The isolated re-cloned Rab14 is aligned with human-NP-057406, drosophila-NP-477171 and mouse_M083680 Rab14 proteins. GTP binding domains I, II, III and IV are shown in boxes. The effector domain is also shown in ED. p21ras: (amino acid 32-40) is the site for interaction with the protein GAP.

A

		PDZ motif	PICK1	RAB14
ct-PAEL-R	PFSRAFM	CCCCC-EECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFAS	+	+
ct-PAEL-R +10	PFSRAFM	CCCCC-EECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFAS	-	+
ct-GPR37L1	PLGQAF	LDCCCCCCEECGGASEASAANGSDNKLKTEVSSSIYFHKPRESPLPL	+	+
	* * *	***** * ** * ** ** *		

B

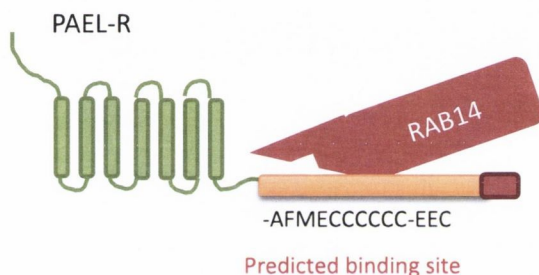
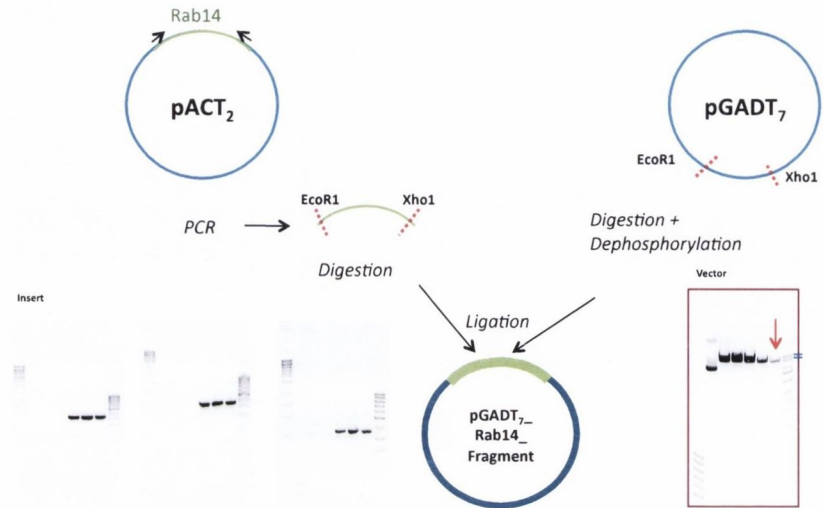


Figure 7: The –CCCCC-EEC motif of PAEL-R is a putative interaction site for Rab14. (A) Site of interaction on ct-PAEL-R, a construct was designed with additional 10 amino acids (ct-PAEL-R+10) that occlude the putative PDZ motif at the extreme ct of PAEL-R. The family member of PAEL-R, namely ct-GPR37L1, was also tested for interaction with Rab14. Rab14 interacts with ct-PAEL-R, ct-GPR37L1, and ct-PAEL-R+10 indicating that the PDZ motif, which is located at the extreme ct-PAEL-R, is not a pre-requisite for interaction. The alignment between PAEL-R and GPR37L1 indicates conserved –CCCCC-EEC motif which could be a putative interaction site. **(B)** PAEL-R and Rab14 interaction, diagrammatic representation shows the cysteine rich motif as a possible binding site of Rab14.

A**B**

GAATTC AAGTTAGTGGCCAAAAATAAACTGCAGATTTGGGATACGGCAGGACAGGAGCGATTTAGGG
 CTGTTACACGGAGCTACTACAGAGGAGCTCGGGAGCTCTTATGGTCTATGATATCACTAGAAGAAGTA
 CATATAACCACTTAAGCAGCTGGTTGACAGATGCAAGGAATCTCACAATCCAATACTGTAATAATTC
 TCATAGGAAATAAAGCAGATTTGGAGGCACAGAGAGATGTTACATATGAAGAAGCCAAACAGTTTGCTG
 AAGAAAATGGCTTATTGTTCTCGAAGCGAGTGCAAAAACGGGAGAGAAT TGACTCGAG

Legend: EcoRI site, XhoI site, STOP codon

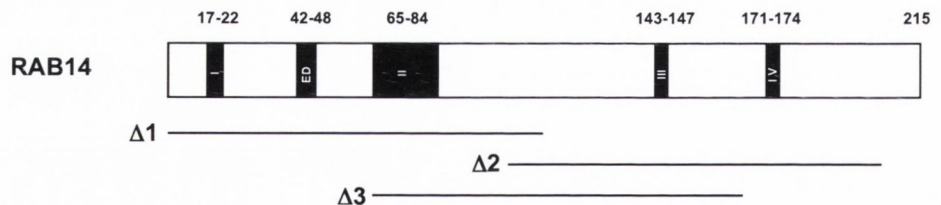
C

Figure 8: Cloning of Rab14 fragments into the pGADT7 vector. (A) Schematic diagram for the cloning strategy plus agarose gel images of the fragments of Rab14 and the vector indicated by a red arrow. **(B)** Representation of full length Rab14 sequence. **(C)** Representation of different fragments designed for Rab14.

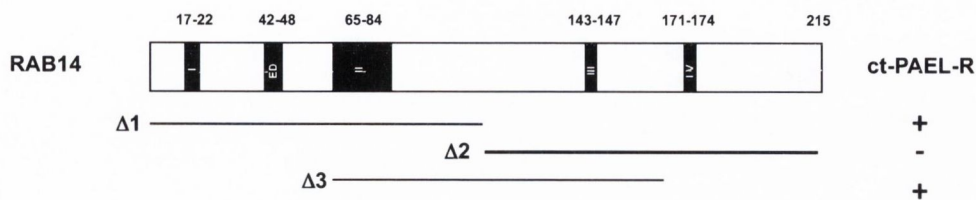
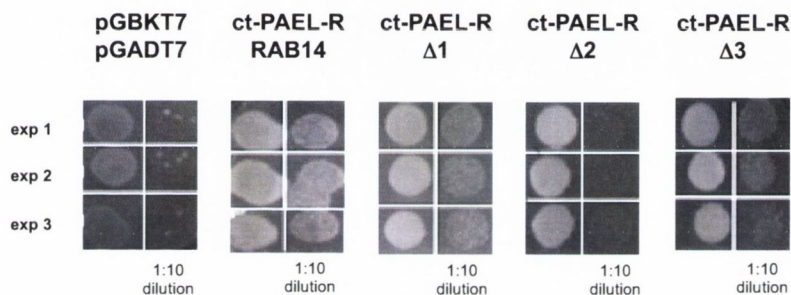
A**B**

Figure 9: The ct-PAEL-R interacts with the second GTP binding site of Rab14. (A) GTP binding site (II) of Rab14 occurs between amino acids 65-84 and is found in fragments Δ1 and Δ3. Y2H data shows that the ct-PAEL-R interacts with fragments Δ1 and Δ3, which contain the second GTP binding site (+ indicates interaction, - indicate no interaction). (B) Y2H data shows that yeast transformed with ct-PAEL-R and Rab14 fragments containing the second GTP binding sites, show strong growth on interaction plates dilution even at 1/10, compared with positive (ct-PAEL-R and RAB14) and negative (pGBKT4 and pGADT7) controls.

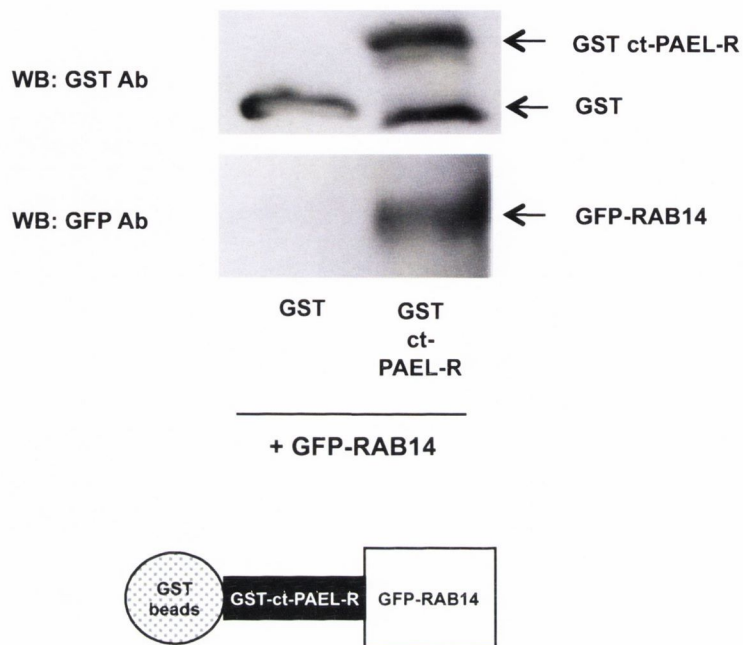


Figure 10: Biochemical evidence of interaction between ct-PAEL-R and Rab14. The GST-ct-PAEL-R interacts with transfected GFP-Rab14. Western blot with anti-GFP antibody showed the levels of GFP-Rab14 retained by GST-ct-PAEL-R, but not by GST alone. The Western blot with anti-GST antibody indicates levels of GST fusion proteins were used. The diagram represents the setup of the pull down experiment.

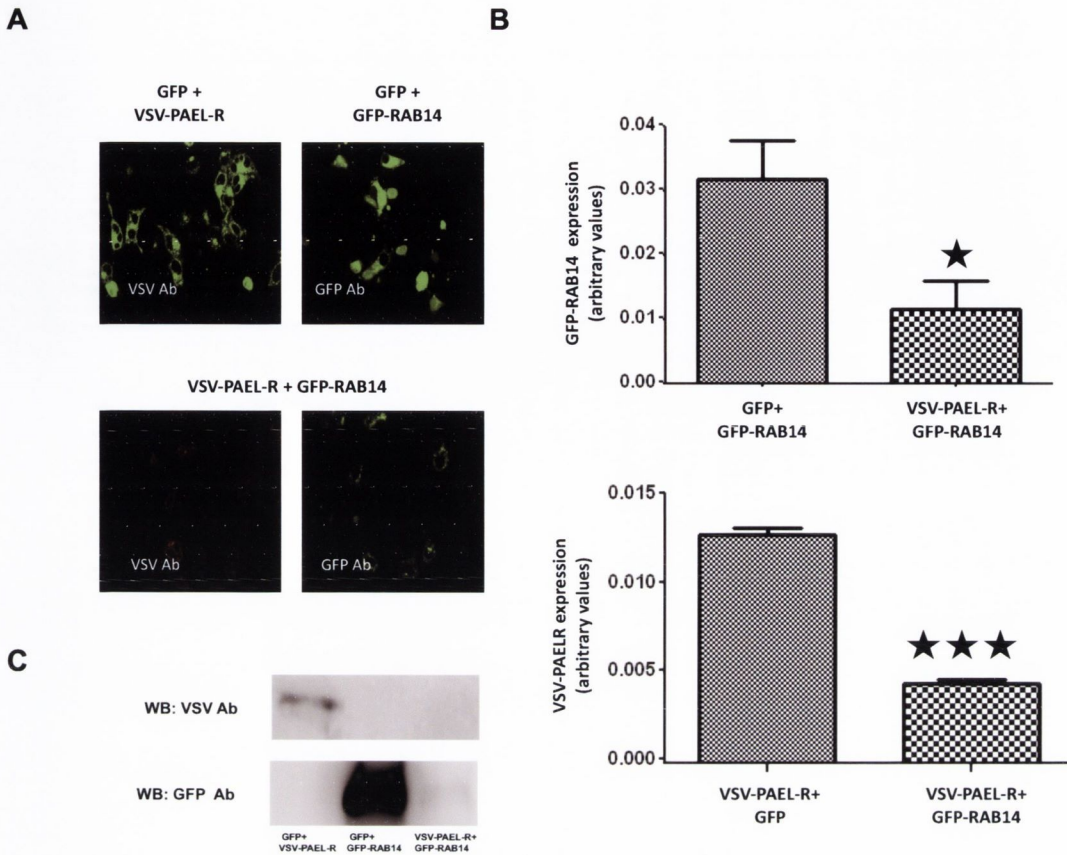


Figure 11: Co-expression decreases both Rab14 and PAEL-R expression. (A) Cells were transiently transfected with GFP-Rab14 and VSV-PAEL in combination with GFP. The cells were stained with anti-VSV Ab or anti-GFP Ab and viewed at x63 magnification. **(B)** Co-expression reduces both Rab14 and PAEL-R expression. Graphs were plotted for expression of VSV-PAEL-R when co-transfected with GFP or GFP-Rab14 and expression of GFP-Rab14 transiently transfected with GFP or VSV-PAEL-R. Approximately 100 cells were analysed for quantification of fluorescent pixels. Data shows statistical reduction in expression levels of GFP-Rab14 (* $p=0.018$; $n=3$) when co-transfected with VSV-PAEL-R in comparison to co-transfection with GFP. Results also indicate a statistical reduction in GFP-Rab14 expression when co-transfected with VSV-PAEL-R compared to co-transfected with GFP (** $p=0.001$; $n=3$). **(C)** VSV-PAEL-R and GFP-Rab14 expression is reduced when co-transfected. Western blot with anti-VSV and anti-GFP Abs are shown.

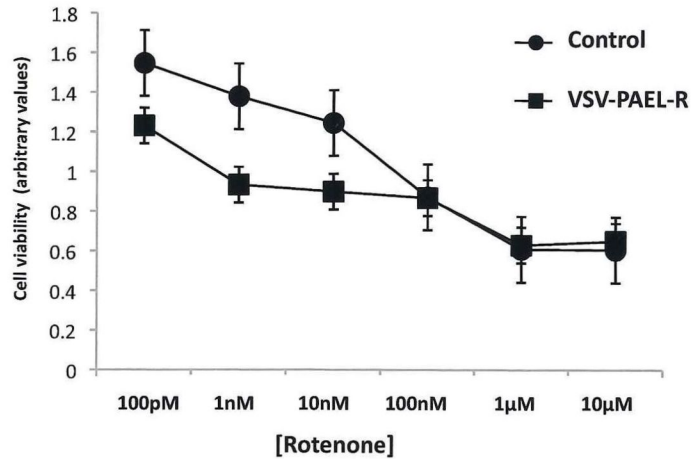
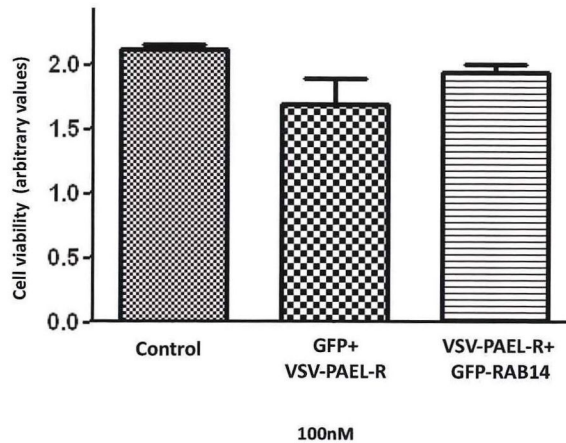
A**B**

Figure 12: No effect of Rab14 on cell survival on PAEL-R induced cell toxicity. (A) Dose-dependent cell survival curve when HEK293 cells were transiently transfected with or without VSV-PAEL-R and exposed to 10μM, 1μM, 100nM, 10nM, 1nM and 100 pM rotenone. The graph was plotted with VSV-PAEL-R and control cells treated with rotenone for 48 hr. The cell viability test was done using cell titer 96 aqueous one solution assay. **(B)** We analysed the cell viability of HEK293 cells which were transiently transfected with VSV-PAEL-R either with GFP or GFP-Rab14 and treated with 100nM concentration rotenone for 48 hr. GFP-Rab14 showed no effect on cell survival.

Discussion

1. A novel interaction between Rab14 and PAEL-R

We report a novel interaction between ct-PAEL-R and Rab14. First we validated that the interaction between Rab14 and PAEL-R was not due to the excess amino acids found in the original isolated Rab14 clone. After successful confirmation of the interaction between Rab14 with PAEL-R we examined the specific interaction between those proteins. Our Y2H studies with Rab14 showed that a PDZ motif of PAEL-R mutant does not impede interaction with Rab14. We also showed that another member of the PAEL-R family, GPR37L1 (also known as ETBRL2) interacted with Rab14. Sequence analysis showed homology at a –CCCCC-EEC motif between PAEL-R and GPR37L1 which we propose as a putative binding site of Rab14. Further, to elucidate the type of interaction between PAEL-R with Rab14, we designed overlapping fragments of Rab14. We confirmed that PAEL-R interacts via a second GTP binding site of Rab14. In addition, our GST pull down experiment biochemically confirmed an interaction between PAEL-R and Rab14. Importantly, the co-expression of Rab14 and PAEL-R in HEK293 cells reduced expression of both proteins. Finally, Rab14 overexpression in HEK293 cells did not rescue cell death induced by PAEL-R overexpression during rotenone treatment.

2. Rabs are molecular switch between GDP to GTP

The Rab GTPase family is the largest member of the Ras superfamily of small guanosine triphosphatases. Most Rabs are ubiquitously expressed and 70 Rab proteins are encoded in the human genome. Rab GTPases are localised in distinct membrane bound organelles on the cytoplasm. They are involved in intracellular trafficking in eukaryotic cells specifically in fission and fusion of transport vesicles with their target membranes during secretory and endocytotic pathways (Proikas-Cezanne et al., 2006). Rab proteins generally cycle between the GDP bound inactive to GTP bound active forms between the cytosol and the membranes (Huang et al., 2010). Thus a characteristic of Rab proteins is to superimpose GDP/GTP cycles to membrane association or dissociation (Huang et al., 2010). Rab10, Rab11, Rab14 and Rab22A are defined to traffic cargo from the early endosome to the endocytic recycling compartment (Brumell and Scidmore, 2007;

Kelly et al., 2009) and Rab11 and Rab14 located in the endocytic recycling compartment (Huang et al., 2010). These functions of Rab14 may allow for the regulation of PAEL-R aggregation and/or control PAEL-R trafficking to cell surface. Rab14 is also ubiquitously found on early endosomes and on the trans-Golgi network and mediates vesicular transport from the trans-Golgi network to the endocytic recycling compartment (Kelly et al., 2009). Therefore Rab14 may be involved in preventing the aggregation of misfolded PAEL-R in the ER.

3. Rab14 is involved in endocytosis and exocytosis membrane trafficking

Rab GTPases play a critical role in endocytic and exocytic membrane trafficking (Takai et al., 2001) and are involved in the formation and maturation of the phagosome and the clearance of pathogens in mammalian cells (Duclos and Desjardins, 2000; Rupper et al., 2001). As an example, RabD, a *Dictyostelium* GTPase which is related to mammalian Rab14 is required in phagosomal formation and maturation particularly regulating endolysosomal fusion and macropinocytosis (Bush et al., 1996, Harris and Cardelli, 2002). Furthermore, Rab GTPases regulate localisation and activity to clear bacterial pathogens and are involved in the fusion of the lysosome with phagosomes (Harris and Cardelli, 2002). Phagocytosis is a defense process important in evading pathogens in multicellular organisms. Phagosomal maturation involves fission and fusion processes which results in the destruction of mammalian and eukaryotic pathogens via the endosomal pathway (Tjelle et al., 2000). We hypothesise that Rab14 may contribute to the degradation of PAEL-R by autophagosomal processes.

4. Rab14 regulates PAEL-R aggregation or recycling

Almost 10–50% of a bulk Rab protein are detected in the cytosol and each Rab protein is localised at organelles and involved in the vesicle transport of the organelles (Takai et al., 2001). For example, Rab1, Rab2, and Rab6 are localised at the ER and the Golgi apparatus and regulate vesicle transport along the biosynthetic or secretory pathway (Takai et al., 2001). In addition, Rab3 is localised on secretory granules including synaptic vesicles and is involved in Ca²⁺

dependent exocytosis (Takai et al., 2001). Moreover Rab4 and Rab5 are present on early endosomes and control early steps of the endocytic process, mediating endosome-endosome fusion and receptor recycling (Takai et al., 2001). In the case of Rab14, it is present on early endosomes and on the trans-Golgi network which may allow for three possibly methods for regulating PAEL-R. First, due to Rab14 interaction with PAEL-R, the receptor transits through the early endosome and is then recycled back to the plasma membrane. Second, PAEL-R is directed to the late endosome from the early endosome/ER with the help of Rab14 and degraded by lysosome. Third, PAEL-R is transported to the trans Golgi network either from late endosome/ER and is recycled back to the plasma membrane after the fusion of the transport vesicle with the help of Rab14 (**Figure 13**).

5. Future studies

The results confirmed a novel interaction between Rab14 and PAEL-R therefore it will be of much interest to examine the overexpression of Rab14 effects on PAEL-R surface expression to elucidate its precise function. If Rab14 preferentially associates with correctly folded PAEL-R then it leads to membrane insertion we might be able to develop an agonist at this stage to promote correctly folded PAEL-R to surface.

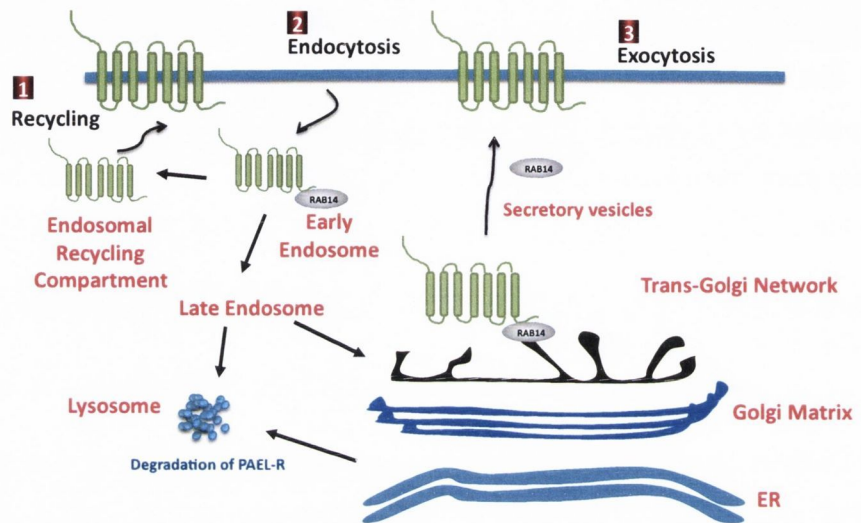


Figure 13: Summary of PAEL-Rab14 function. We hypothesise three possible scenarios based on Rab14 localisation; 1. Recycling: recycling of the PAEL-R to the cell surface via ERC 2. Endocytosis: degradation of PAEL-R via lysosomes 3. Exocytosis: recycling of PAEL-R to plasma membrane via TGN by secretory vesicles.

FINAL CONCLUSION

Final conclusion

1. Disease and its regulation

Parkinson disease was first described in 1817 as shaking palsy. It has since become a commonly known motor disorder involving a process of complex neurodegeneration. PD is a progressive neurodegenerative disorder that is characterised by bradykinesia, rigidity and tremor (Savitt et al., 2006). There are at least sixteen PD associated loci which have been identified (Gasser, 2007). Mutations in α -synuclein and LRRK2 cause AD-PD. Four genes have been linked to AR-PD and include mutations in parkin, DJ-1, PINK1, and ATP13A2. In general, PD-associated mutations in parkin lead to a loss of its E3 ligase function (Tanaka et al., 2004). Inactivation of parkin leads to oxidative, nitrosative, and dopaminergic stress which play important roles in PD, suggesting that parkin is involved in sporadic PD (Winklhofer et al., 2003, Chung et al., 2004, LaVoie et al., 2005b). There are several parkin substrates that have been implicated in parkin's physiological functions and pathological roles in PD. Parkin mutation hinders its ability to mono and polyubiquitinate which causes protein aggregation thought to play a role in PD (Dawson et al., 2010).

2. PICK1: a role in PAEL-R-PARKIN ubiquitination

One of the candidates for parkin monoubiquitination is PICK1. Parkin regulates indirectly PICK1's to effect on its PDZ partners namely, the ASIC (Joch et al., 2007). The overexpression of parkin causes an increase in PICK1 monoubiquitination (Joch et al., 2007) which enhances ASIC activity and due to excitatory ASIC currents leads to altered synaptic plasticity (Wemmie et al., 2002, 2003) and neuronal damage (Xiong et al., 2004). We demonstrate a novel interaction between PICK1 and PAEL-R. Truncated mutants of ct-PAEL-R confirmed that the site of interaction was within the last four amino acids at the extreme ct, probably at the -GTHC motif. Deletion constructs and site directed mutation in PICK1 indicated that the PDZ domain is necessary for interaction with ct-PAEL-R. To verify the results obtained in yeast we performed a biochemical experiment using bacterial expressed GST-ct-PAEL-R and MBP-PICK1. Consistent with the Y2H results, MBP-PICK1 was retained by GST-ct-PAEL-R but

not by GST only. Furthermore we isolated native PICK1 from rat brain lysate by GST-ct-PAEL-R. We also showed that overexpression of PICK1 reduced PAEL-R levels and also decreased cell death. Y2H studies using the ct-PAEL-R have revealed an interaction with parkin. Furthermore, both *in vitro* and *in vivo* studies suggest that PAEL-R is an authentic substrate for parkin (Takahashi and Imai, 2003). Mutations in parkin cause loss of the ubiquitin protein ligase activity and leads to accumulation of substrates, which would normally be degraded by a molecular protease machinery involving parkin (Takahashi and Imai, 2003). Parkin ubiquitinates the misfolded PAEL-R protein and promotes its degradation. The excessive aggregation of insoluble PAEL-R results in selective death of dopaminergic neurons (Yang et al., 2003). PAEL-R has also been shown to accumulate in the brains of AR-JP patients (Imai et al., 2001). We hypothesise that the interaction between PICK1 and parkin and between PICK1 and PAEL-R provides a PAEL-R-PICK1-PARKIN complex that is important for parkin mediated PAEL-R degradation.

3. GABARAPL2: regulating PAEL-R by autophagy

A second interactor of PAEL-R identified was GABARAPL2. We confirmed a novel interaction between PAEL-R and the coding sequence clone of GABARAPL2. The interaction between ct-PAEL-R and GABARAPL2 likely occurred at the cysteine rich motif of PAEL-R. Deletion overlapping constructs confirmed that the PAEL-R interacted with the GABA_A binding site of GABARAPL2. Further, we performed biochemical experiments using bacterial expressed GST-ct-PAEL-R and Myc-GABARAPL2 to confirm the yeast result. Myc-GABARAPL2 bound to GST-PAEL-R but not with GST alone. Members of the GABARAP family have been known to interact with the cytoskeleton and are mainly involved in protein and intracellular vesicle transport (Schwarten et al., 2009). Specifically GABARAP appears to be involved in cell proliferation, programmed cell death and tumorigenesis whereas Atg8, GATE16, GABARAP and MAP-LC3 regulate the formation and elongation of autophagic vesicles called autophagosomes (Weidberg et al., 2010). Mutations of the PARK2 and PARK6 genes which encode parkin and PINK1 respectively have been associated with the cause of PD. Parkin is translocated from the cytosol to depolarized mitochondria in a PINK1 dependent manner to promote the autophagic degradation of aggregated proteins (Narendra et al., 2008, Vives-

Bauza et al., 2009). Based on analogy, we hypothesise that GABARAPL2 facilitates the autophagic degradation of PAEL-R and parkin may play a role during the process.

4. Rab14: directing PAEL-R cargoes for degradation

The third interactor of PAEL-R we have identified is Rab14. Rab14 is ubiquitously expressed on early endosomes and on the trans-Golgi network and mediates biosynthetic or recycling between the Golgi and endosomal compartments (Kelly et al., 2009). Each Rab protein is localised at organelles and is implicated in the vesicle transport of the organelle. Some functional specialisation of Rab proteins are detected based on function both in different cell types as well in different organisms (Takai et al., 2001). We confirmed that the interaction between PAEL-R and Rab14 was within the coding region of Rab14. Y2H studies with Rab14 showed that the PAEL-R cysteine rich motif could be a site for interaction based on the findings that GPR37L1 also interacted with Rab14 and displays sequence homology in the cysteine rich motif. Further we performed biochemical experiments and confirmed GFP-Rab14 bound to GST-PAEL-R but not with GST alone. Given the role that Rab14 plays in vesicle transport, we hypothesise that it may play a role in degradation of PAEL-R cargoes, which may explain the loss of protein level when both Rab14 and PAEL-R were co-transfected in HEK293 cells.

5. Recycling or degradation of PAEL-R by interactors

When looking at the co-expression of PAEL-R with interactors we found that the overall expression of PAEL-R was reduced with PICK1 and GABARAPL2. With Rab14 both the PAEL-R and Rab14 protein expression decreased when co-transfected. We report that rotenone-induced cell stress was rescued when PAEL-R was co-transfected with PICK1 in comparison to cells transfected with PAEL-R alone. From these studies we hypothesise that PAEL-R, parkin, and PICK1 possibly form a triple protein complex which may cause ubiquitination and degradation of PAEL-R. Analogous to this misfolded DAT is ubiquitinated by parkin and is degraded further by the UPS. Since PICK1 interacts with DAT and parkin it may play a role in parkin mediated ubiquitination of DAT. For GABARAPL2, this protein is recruited to damage mitochondria and may target PAEL-R to mitochondria or may target PAEL-R for degradation by the lysosome. For Rab14

our data show it was not able to rescue PAEL-R induced cell death along with rotenone treatment. PAEL-R might be directed to the late endosome from the early endosome/ER with the help of Rab14 and degraded by the lysosome, during which Rab14 might also get degraded. Another possible pathway is that the PAEL-R is transported to the trans Golgi network either from late endosome/ER and is recycled back to the plasma membrane after the fusion of the transport vesicle with the help of Rab14. Thus parkin/Rab14/GABARAPL2 may be involved in the degradation and/or recycling of PAEL-R (**Fig 1**).

6. Final remarks

We believe that the PAEL-R interactors identified in this thesis provide further understanding of PAEL-R degradation pathways. We recognise there are however some outstanding question. In particular, it will be of great interest to study the relative involvement of GABARAPL2 and PAEL-R in the autophagic processes. Given the importance of autophagy in brain damage and neurodegenerative disease, it will be essential to study the function of GABARAPL2 in the brain with respect to its implication in autophagy. In addition how parkin is inducing mitophagy and whether parkin and PAEL-R stimulates mitophagy would be worthy of further study? Further experiments examining the Rab14-PAEL-R interaction to better understand the molecular role of this GTPase in intracellular trafficking of PAEL-R are also required. Most, interestingly future research efforts to determine functional link between PICK1-PARKIN-PAEL-R and to clearly define its role in ubiquitination and degradation of PAEL-R are also required. Knowledge about PAEL-R degradation and control of function by its interactors may ultimately lead to development of novel methods for clearing neurotoxic intracellular PAEL-R aggregates and thus the development of new therapies for PD.

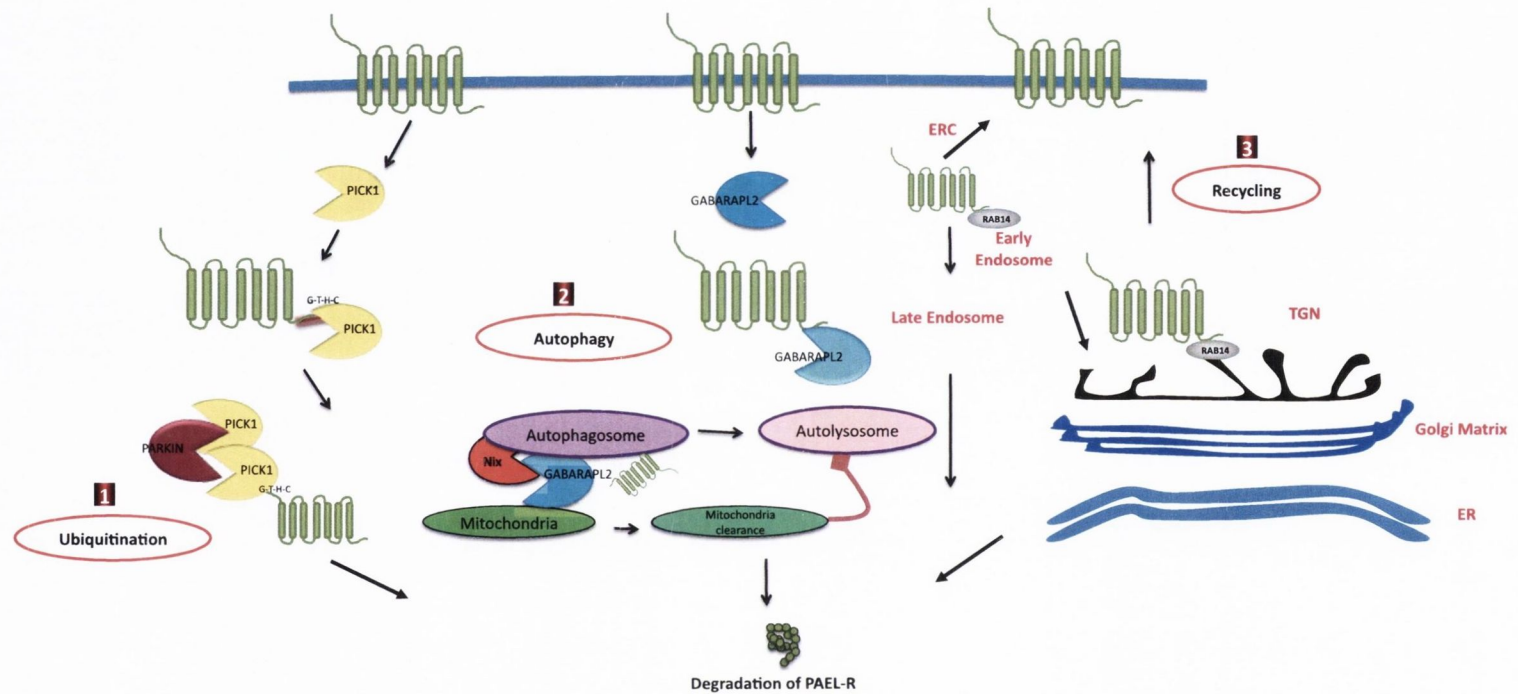


Figure 1: Summary of PAEL-R trafficking by PICK1, GABARAPL2 and RAB14. Three major steps highlighted in the figure (1) Ubiquitination by parkin (2) Autophagy pathway by GABARAPL2 (3) Recycling and degradation by RAB14.

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APPENDIX

The GPR37/PAEL Receptor – Finding a Way to the Cell Surface

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Abstract

Parkinson's disease (PD) is a chronic neurodegenerative disorder that causes a wide range of debilitating symptoms. The parkin-associated endothelin-like receptor (Pael-R), originally called G-protein-coupled receptor 37 (GPR37), belongs to the family of orphan G-protein-coupled receptors (GPCRs). Under physiological conditions, the E3 ligase called parkin ubiquitinates the unfolded Pael-R to promote its degradation. When parkin is mutated in PD the Pael-R aggregates in the endoplasmic reticulum (ER), inducing ER stress, which leads to neurotoxicity and cell death. Development of compounds for the Pael-R is hampered by lack of knowledge about the endogenous ligand and its poor trafficking to the plasma membrane when overexpressed. In this review we outline mechanisms, proteins and drugs that alter Pael-R levels and trafficking.

Keywords

Parkinson's disease, parkin-associated endothelin-like receptor (Pael-R), G-protein-coupled receptor 37 (GPR37), parkin, endoplasmic reticulum (ER) stress

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GPR37/Pael-R Is an Orphan Receptor

G-protein-coupled receptor 37 (GPR37), now called parkin-associated endothelin-like receptor (Pael-R), is an orphan G-protein-coupled receptor (GPCR) first cloned in 1997 from human brain frontal lobe and is a conserved mammalian protein that is predominantly expressed in the brain.¹ Pael-R is mapped on chromosome 7q31 in humans and chromosome 6 in mice.^{1,2} The human Pael-R gene is approximately 25kb and consists of an open reading frame coding 613 residues; the mouse gene has an open reading frame coding 600 residues. The mouse Pael-R is 83% identical to the human gene, with both containing seven putative hydrophobic transmembrane domains as well as a long 249-amino-acid arginine/proline-rich extracellular domain (see Figure 1). The transmembrane region of Pael-R has a high degree of homology of approximately 40% to mammalian peptide-specific GPCRs, particularly endothelin-B receptor (ETBR), bombesin-BB1 and bombesin-BB2 receptors.^{1,2} Pael-R is most closely related to another central nervous system (CNS)-enriched orphan receptor known as GPR37-like 1 (GPR37L1). GPR37L1 shows 68% overall homology and 48% identity to Pael-R.³ *In situ* hybridisation of rat brain demonstrates broad distribution of both receptors throughout the CNS.⁴ Northern blot analysis with human Pael-R reveals messenger RNA (mRNA) expression in the human brain, particularly in Purkinje cells of the cerebellum, the corpus callosum, medulla, putamen, caudate nucleus and in neuronal cells of the hippocampus including pyramidal cells and granule cells of the dentate gyrus.^{3,4} Human Pael-R mRNA is also expressed in the liver and placenta, while mouse Pael-R mRNA is primarily expressed in the brain and in the testis.^{1,2}

Although Pael-R has a significant sequence homology with peptide-activated GPCRs, none of the mammalian peptides tested so far has activated any signalling pathways in heterologous cells or *Xenopus* oocytes expressing this receptor.^{1,3,4} In one study, electrophysiological recording in frog oocytes and in mammalian cell lines revealed nanomolar affinities of neuropeptide head activator (HA) for Pael-R.⁵ This study reports that HA mediates signal transduction through Pael-R by activation of an inhibitory G protein, Ca²⁺ influx, activation of Ca²⁺-dependent calmodulin kinase and phosphoinositide-3-kinase.⁷ Interestingly, HA treatment also resulted in internalisation of Pael-R and induced mitosis.⁷ A more recent study was unable to confirm these observations; thus, whether HA is a ligand for Pael-R remains controversial.⁸ In addition, knowledge about mechanisms regulating Pael-R surface expression, signalling and internalisation are incomplete, and therefore Pael-R is generally still considered an orphan receptor.

Pael-R Is a Substrate of Parkin

Parkin (PARK2) is a protein-ubiquitin ligase E3 involved in the ubiquitination and proteasome-mediated protein degradation and clearance of aggregated proteins.⁹ Mutations in parkin are associated with autosomal recessive juvenile Parkinson (AR-JP).⁹ Yeast two hybrid (Y2H) studies using the C-terminus (ct) of Pael-R as bait revealed an interaction between this receptor and parkin.¹⁰ Furthermore, both *in vitro* and *in vivo* studies suggest that Pael-R is an authentic substrate for parkin.^{10,11} Parkin is ubiquitously expressed and localised in the cytoplasm and Golgi complex in the human brain¹² and can be upregulated by the unfolded protein response (UPR).¹³ Newly synthesised Pael-R is folded in the endoplasmic reticulum (ER)

and then transported to the cell membrane.¹⁴ Unfolded Pael-R is normally translocated across the ER membrane into the cytosol and degraded through a parkin-dependent ubiquitin-proteasome pathway, which suppresses cell death induced by the accumulation of unfolded Pael-R.⁹⁻¹¹

In detail, parkin eliminates unfolded Pael-R in co-operation with the molecular chaperone 70kDa heat shock protein (Hsp70) and a U-box protein known as ct of Hsp70 interacting protein (CHIP).^{10,11,18} The U-box was initially identified in the yeast E4 Ufd2 protein and contains a really interesting new gene (RING) finger fold.¹⁴ The RING finger fold is structurally similar to RING finger motifs and functionally helps in protein degradation.¹⁷ The U-box in CHIP shows E3 activity and ubiquitinates unfolded proteins.¹⁹ Hsp70 plays a role in ensuring correct folding and intracellular localisation of newly synthesised polypeptides.¹⁹ Moreover, Hsp70 exerts protective properties by binding to parkin, leading to Pael-R ubiquitination.^{11,18} When unfolded Pael-R is translocated to the cytosol, Hsp70 and Hdj2 (ER-associated Hsp40) bind to unfolded Pael-R and initiate upregulation of CHIP.^{10,11,18} CHIP facilitates the dissociation of Hsp70 from Pael-R and helps in binding of parkin and ubiquitination of the Pael-R in conjunction with E2 ligases such as Ubc4, Ubc6 and Ubc7 on the ER surface.^{10,11,18}

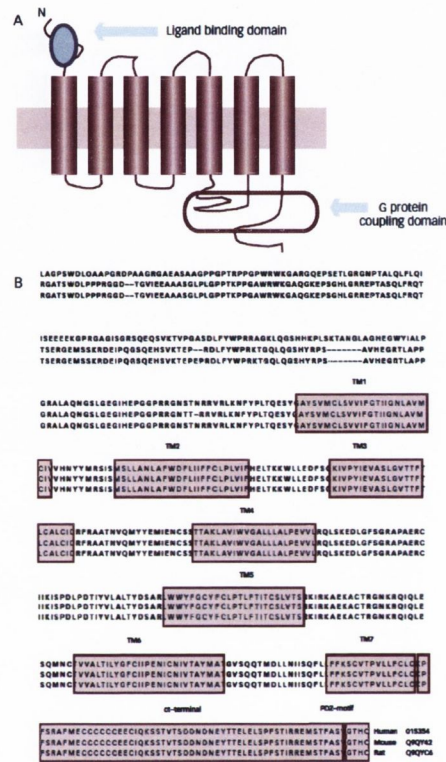
The aggregation of Pael-R due to ineffective ubiquitination by mutated parkin may be involved in ER oxidative stress, decreased cell viability and neurotoxicity in PD.^{10,11,18} Overexpression of Pael-R, especially in the absence of parkin, in cell cultures or in animal models leads to unfolded protein-induced cell death.^{10,11,18,20,21} In addition, when Pael-R is pan-neuronally overexpressed in drosophila, dopaminergic neurons show selective degeneration.²² It also appears that human Pael-R orthologues are overexpressed in neurons of the substantia nigra pars compacta in PD patients.²³ These findings suggest that Pael-R overexpression causes the selective degeneration of dopaminergic neurons. Importantly, Pael-R is localised in the core of lewy bodies (a hallmark of PD pathology) and lewy neuritis and parkin in the halo of lewy bodies and in neuronal cell bodies.²⁴ Moreover, accumulation of unfolded Pael-R protein in the insoluble fraction of brains of AR-JP patients²⁵ and the presence of this receptor in the core of lewy bodies in PD patients²⁴ suggest the involvement of aggregated Pael-R in PD pathology and the role of parkin in degradation of Pael-R.⁹⁻¹¹

Control of Pael-R Protein Levels

Intrinsic Mechanisms that Control Pael-R Toxicity

The Pael-R has been shown to be inherently difficult to fold, which enforces the importance of correct degradation events for misfolded Pael-R.¹¹ The poor folding of Pael-R also makes difficult its plasmid-mediated overexpression in cells²⁶ (see Figure 2). The intrinsic mechanisms, in addition to parkin-mediated degradation, that may regulate the levels of aggregated Pael-R include ER-associated protein degradation (ERAD), UPR, Hsp and molecular chaperone activation and autophagy. Briefly, the ER controls the maturation of membrane and secretory proteins, where newly synthesised secretory proteins enter the ER and bind to ER chaperones, facilitating proper protein folding.²⁶ Folded proteins then enter the secretory pathway, composed of the Golgi apparatus, and proceed to the plasma membrane. Transmembrane proteins, upon internalisation, can be degraded through the ubiquitin-proteasome pathway. When proteins such as Pael-R are not correctly folded, they undergo degradation by ERAD.^{11,14,26} The accumulation of unfolded proteins leads to ER stress, which transactivates multiple genes including molecular chaperones and

Figure 1: Alignment and Structure of Pael-R



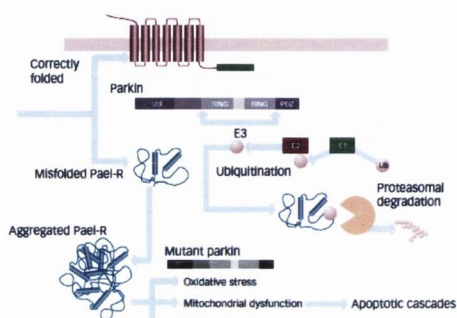
A. Schematic representation of Pael-R. B. The human Pael-R (015354) protein is aligned with mouse (090Y42) and rat (090Y06). The C-terminus and seven transmembrane domains are represented in blocks.

ERAD-associated molecules, leading to UPR and, possibly, apoptosis.²⁷ The overexpression of Pael-R may also induce the cellular autophagic pathway.²⁸ Autophagy induced by Pael-R overexpression plays an important role in clearing protein aggregates and prevents the degeneration of Pael-R-expressing neurons.²⁸ Interestingly, the levels of both ERAD and autophagic markers were found to be altered in brain extracts of Pael-R knockout mice.²⁸

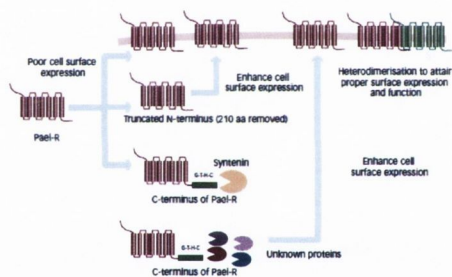
Potential Targets to Control Pael-R Toxicity

To date, in addition to parkin there are four further proteins that have been shown to regulate Pael-R toxicity and could be potential drug targets; these are summarised below.

- Human homologue of yeast Hrd1p (HRD1) is an E3 ligase involved in ERAD and expressed in dopaminergic neurons of the substantia nigra. HRD1 directly co-localises with Pael-R in the ER and promotes the ubiquitination and degradation of Pael-R in the ER, thus suppressing Pael-R-induced cell death.²⁹

Figure 2: Pael-R is a Substrate of Parkin

Correctly folded Pael-R is transferred to the cell surface. Misfolded Pael-R is ubiquitinated by parkin and degraded by the proteasome. When parkin is mutated, Pael-R aggregates, which activates apoptotic cascade pathways and finally leads to cell death.

Figure 3: Mechanisms for Pael-R Trafficking to the Cell Surface

Pael-R is poorly expressed at the cell surface. Cell surface expression is enhanced by truncation in the N-terminus or by co-expression with PDZ domain containing protein syntenin-1. Other proteins may interact with intracellular domains of Pael-R and help in cell surface trafficking. Heteromerisation may also allow proper surface expression and function of Pael-R.

- DJ-1/PARK7 is a redox-responsive protein with neuroprotective roles. Mutations in the DJ-1 gene are linked to AR-JP and it is upregulated in astrocytes in neurodegenerative diseases and stroke. Promoting DJ-1 activity is thought to help treat neurodegeneration, whereas downregulation of DJ-1 enhances dopaminergic neuronal cell death via ER stress, oxidative stress and proteasome inhibition. It has been shown that DJ-1 overexpression rescues Pael-R-induced cell death.²³
- Thioredoxin (Trx) is a molecular chaperone and 12kD antioxidant found to suppress Pael-R-induced neurotoxicity.²¹ It has been suggested that the chaperone properties of thioredoxin are important for controlling Pael-R-induced toxicity as well as polyglutamine-induced neurotoxicity.
- Parkin co-regulated gene (PACRG)/gene adjacent to parkin (Glup) forms a complex with Hsp70 and Hsp90 and is a component of Lewy bodies. PACRG/Glup can reduce cell death mediated by Pael-R overexpression by formation of cytoplasmic inclusions that appear to be cell-protective.²²

There are also two compounds that appear to modulate Pael-R toxicity. Sodium 4-phenyl-butyrate (4-PBA) has been shown to restore the normal expression of Pael-R by reducing the amount of misfolded protein within the UPR pathway.²³ This compound attenuates activation of ER stress-induced signal transduction pathways like transcription of Hsp70 and HRD1, and thus may control cell death in neurodegeneration.²³ In contrast to the protective effects of 4-PBA, high doses of amphetamine (40mg/kg) can produce temporary suppression of gene expression of parkin and Pael-R.²⁴ This suppression of Pael-R and parkin is linked to amphetamine-induced dopaminergic neurotoxicity.²⁴

Control of Pael-R Cell Surface Expression Pael-R Interacts with DAT

Increased and uncontrolled metabolism of dopamine can act as an endogenous toxin and provoke neuronal damage through the generation of reactive oxygen species (ROS) and oxidative stress.²⁵ Dopamine facilitates the transition of non-toxic α -synuclein protofibrils to toxic fibrils present in Lewy bodies.^{26,27} In addition, co-valent modification of parkin by dopamine leads to inhibition of its E3 activity.²⁸ Pael-R signalling appears to regulate dopamine levels in neurons.²⁹ Pael-R knockout (KO) mice show a decrease in level of striatal dopamine and increased sensitivity to amphetamine.²⁸ In Pael-R transgenic (tg) mice the levels of striatal 3,4-dihydroxyphenylacetic acid (DOPAC) and vesicular dopamine content are increased and the number of nigrostriatal dopaminergic neurons is reduced.^{28,40} Of interest, Pael-R KO mice are resistant to treatment with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); moreover, tyrosine hydroxylase inhibitor treatment can ameliorate dopaminergic cell death induced by infection of adenovirus encoding Pael-R.^{40,41} In parkin KO mice crossed with Pael-R tg mice, there are higher levels of dopamine, dopamine metabolites (DOPAC and homovanillic acid [HVA]), protein carbonyls, and markers of oxidative damage in the midbrain.²⁸ This study implies a pathological role of dopamine and its metabolites in dopaminergic neuron-specific degeneration as a result of Pael-R accumulation.²⁸ These results are consistent with another study showing the localisation of Pael-R in the presynaptic fraction of mouse striatum and its interaction with the dopamine transporter (DAT).⁴² This interaction can modulate DAT-mediated dopamine uptake, where the lack of Pael-R enhances DAT activity and increases the plasma membrane expression of DAT.⁴² Thus, not only chronic ER stress due to accumulation of misfolded proteins, but also excessive dopamine-mediated oxidative stress, is likely to contribute to the pathological role of Pael-R in PD.

Pael-R Interacts with Syntenin

Poor plasma membrane trafficking of Pael-R in most cell types impedes progress in understanding the ligand binding and signalling pathways of this orphan receptor. To date, four different approaches for enhancing GPCR surface expression have been introduced, including addition of sequences, removal of sequences, co-expression with receptor-interacting proteins and treatment with pharmacological agents (see Figure 3).⁴³ Pael-R surface expression is undetectable compared with GPR37L1, which expresses strongly on the cell surface of HEK-293 cells.⁴ Interestingly, removal of the first 210 amino acids from the N-terminus (nt) dramatically increases the surface expression of Pael-R.⁴ This may suggest that Pael-R possesses a motif on the nt that may be important for the localisation of Pael-R on the plasma membrane. It has been reported that a truncated ct version of Pael-R has a decreased surface expression.⁴ Moreover, the

ct of Pael-R possesses a class 1 PDZ domain binding motif (GTHC), which has the potential to interact with PDZ-domain-containing scaffolding proteins. Thus, the ct of Pael-R plays an important role in its surface expression and most likely in its trafficking.³ In this regard, Pael-R has been shown to interact with the PDZ scaffold protein syntenin-1, which increases the cell surface trafficking of the Pael-R.³ Co-expression with other GPCRs such as the adenosine receptor A2AR and the dopamine receptor D2R can also enhance the membrane expression of Pael-R.⁸ Furthermore, the interaction of Pael-R with D2R can alter the D2R affinity for both agonists and antagonists and alter dopaminergic signalling.⁹

Concluding Remarks

Like other GPCRs, Pael-R may require assembly with a specific partner to achieve correct surface expression and functional activity. To date it has been shown that Pael-R cell surface expression can be drastically enhanced by nt truncation or by ct interaction with the PDZ domain containing scaffold protein syntenin-1.³ Since PDZ scaffolds have the capacity to control receptor surface expression, there is a need to further study the ability of the Pael-R to interact with other PDZ scaffold and trafficking proteins. Preventing interacting proteins from associating

with Pael-R may alter its trafficking and thus provide a method for regulating its signalling, aggregation and neurotoxic properties. Furthermore, heterodimerisation and cross-talk with other GPCRs may be crucial for proper Pael-R trafficking, as has been shown with D2R and A2AR, which enhance Pael-R surface expression.⁸ Evidence suggests that Pael-R can interact with and alter the function of DAT42 and D2R,⁸ therefore, understanding the details of Pael-R trafficking may also provide insights into other conditions where dopaminergic signalling is aberrant, such as schizophrenia and depression. In closing, the potentially important therapeutic drug targets for controlling Pael-R neurotoxicity so far appear to be threefold, namely: proteins that regulate the rate of Pael-R degradation, trafficking proteins that interact with Pael-R and receptors that heterodimerise with Pael-R (see Figure 3). ▯

kumlesh K Dev works in the Department of Physiology, School of Medicine at Trinity College Dublin. He heads the molecular neuropharmacology group in the Neuroscience Institute at Trinity, which is investigating novel mechanisms to regulate receptor trafficking, particularly in the disease areas of Parkinson's disease and multiple sclerosis. Priyanka Datta, Debajit Deb and Lella Dargahi are PhD scholars and Davor Vankovic is a BSc neuroscience student within the group.

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