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ORIGINAL ARTICLE

The protein interacting with C-kinase (PICK1) interacts with and attenuates parkin-associated endothelial-like (PAEL) receptor-mediated cell death

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Abstract

The parkin-associated endothelial-like receptor (PAELR, GPR37) is an orphan G protein-coupled receptor that interacts with and is degraded by parkin-mediated ubiquitination. Mutations in parkin are thought to result in PAELR accumulation and increase neuronal cell death in Parkinson's disease. In this study, we find that the protein interacting with C-kinase (PICK1) interacts with PAELR. Specifically, the Postsynaptic density protein-95/Discs large/ZO-1 (PDZ) domain of PICK1 interacted with the last three residues of the c-terminal (ct) located PDZ motif of PAELR. 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-PAELR. Furthermore, coimmunoprecipitation studies isolated a PAELR-PICK1 complex from transiently transfected cells. PICK1 interacts with parkin and our data showed that PICK1 reduces PAELR expression levels in transiently transfected heterologous cells compared to a PICK1 mutant that does not interact with PAELR. Finally, PICK1 over-expression in HEK293 cells reduced cell death induced by PAEALR over-expression during rotenone treatment and these effects of PICK1 were attenuated during inhibition of the proteasome. These results suggest a role for PICK1 in preventing PAELR-induced cell toxicity.

Keywords: GPR37, parkin-associated endothelial-like receptor (PAEL receptor), parkinson's disease (PD), protein interacting with C-Kinase (PICK1).

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The parkin-associated endothelin-like receptor (PAELR), originally called GRP37, is an orphan G-protein-coupled receptor (GPCR). The PAELR 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 PAELR 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 and Imai 2003). The over-expression of PAELR may also induce autophagy to clear PAELR aggregates (Marazziti *et al.* 2009). In addition to regulation by parkin, PAELR neurotoxicity is also attenuated by another E3 ligase human homology of yeast Hrd1p (HRD1), which interacts with and promotes PAELR ubiquitination and degradation in the ER (Omura *et al.* 2006). Moreover, the neuroprotective protein DJ-1/PARK7 [Parkinson disease (autosomal recessive, early onset) 7] (Yokota *et al.* 2003), the molecular chaperone Thioredoxin

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Abbreviation used: BAR, Bin/amphiphysin/RVS; DAT, dopamine transporter; ETBR, endothelin-B receptor; GPCR, G-protein-coupled receptor; GRIP, glutamate receptor interacting protein; GST, glutathione S-transferase; HEK293, human embryonic kidney 293; MBP, maltose binding protein; mGluR7, metabotropic glutamate receptor subtype 7; PAELR, parkin-associated endothelial-like receptor; PICK1, protein interacting c-kinase.

(Trx) (Umeda-Kameyama *et al.* 2007) and the parkin coregulated gene/gene adjacent to parkin (Imai *et al.* 2001) rescues an increase in cell death mediated by over-expression of PAELR. In addition, sodium 4-phenyl-butyrate (4-PBA) reduces the amount of misfolded PAELR protein (Kubota *et al.* 2006), whereas methamphetamine-induced dopaminergic neurotoxicity is associated with suppressed gene expression of parkin and PAELR (Nakahara *et al.* 2003).

The PAELR is localized in the core of lewy bodies and lewy neurites, hallmarks of Parkinson's disease (PD) (Murakami *et al.* 2004). Individuals affected with autosomal recessive juvenile PD and possess mutations in parkin are unlikely to degrade PAELR where receptor aggregation may play a role in pathology (Imai *et al.* 2001; Murakami *et al.* 2004). When PAELR is expressed in dopaminergic neurons of drosophila, these neurons show selective degeneration (Yang *et al.* 2003). In PAELR transgenic (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 PAELR tg mice crossed with Parkin knockout mice (Wang *et al.* 2008). In contrast, PAELR knockout mice show a decrease in the level of striatal dopamine, resistance to treatment with the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine; a mitochondrial complex I inhibitor), but increased sensitivity to amphetamine (Imai *et al.* 2007). Moreover, treatment with a tyrosine hydroxylase inhibitor can ameliorate dopaminergic cell death induced by PAELR over-expression (Marazziti *et al.* 2004; Kitao *et al.* 2007). Thus, both ER and mitochondrial stress and excessive dopamine mediated oxidative stress likely contribute to the pathological role of PAELR in PD.

Studies suggest that the neuropeptide head activator is an endogenous ligand for PAELR (Rezgaoui *et al.* 2005), however, this remains under debate (Dunham *et al.* 2009). To date, the development of PAELR compounds has been hampered by a lack of knowledge about the endogenous ligand. A limited understanding of the intracellular signalling that is coupled to the PAELR and its poor trafficking to the plasma membrane when over-expressed have also hampered efforts to advance PAELR as a drug target. Like other GPCRs, PAELR likely requires assembly with protein partners to achieve correct surface expression, post-translational modification and functional activity. Indeed, PAELR cell surface expression can be enhanced by the removal of the first 210 amino acids from N-terminus (Dunham *et al.* 2009). Heterodimerization with other GPCRs, such as the dopamine (D2R) and adenosine (A2AR) receptors, also enhances PAELR surface expression (Dunham *et al.* 2009). In addition, pre-synaptic located PAELR can interact with and alter the function of the dopamine transporter (DAT) (Marazziti *et al.* 2007). This interaction modulates DAT-mediated DA uptake, where the lack of PAELR increases plasma membrane expression of DAT and enhanced DAT activity (Marazziti *et al.* 2007).

PDZ (Postsynaptic density protein-95/Discs large/ZO-1) domains interact with the last 3–4 residues of c-terminal (ct)-located PDZ motifs of receptors, providing a molecular mechanism for regulating surface expression and post-translational modification. The protein interacting with C-kinase (PICK1) consists of 416 amino acids and has a molecular weight of approximately 50 kDa. Uniquely, PICK1 contains both a PDZ domain and a Bin/amphiphysin/RVS (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; Bolia *et al.* 2012). The BAR domain of PICK1 also allows for its autodimerization (Xiao *et al.* 2007). Thus, via its PDZ and BAR domains, PICK1 may act as a scaffold/adapter that associates with and brings together several proteins. The best studied receptors that interact with PICK1 are glutamate receptors including the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (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, 2001; Boudin *et al.* 2000). In addition, as well as playing a role in mitochondrial function via a Protein kinase Cα (PKCα) dependent mechanism (Wang *et al.* 2003, 2007, 2008), PICK1 also interacts with PD-related proteins, namely, DAT (Torres *et al.* 2001; Bjerggaard *et al.* 2004; Madsen *et al.* 2005) and parkin (Joch *et al.* 2007). Moreover, it has been shown that removal of the ct domain of PAELR (ct-PAELR) decreases surface expression of the receptor (Dunham *et al.* 2009). Importantly, ct-PAELR contains a PDZ motif (-GTHC), which interacts with the PDZ protein syntenin-1 to increase cell surface trafficking (Dunham *et al.* 2009). We thus hypothesized that PICK1 also interacts with PAELR and may alter its function. Here, we studied additional proteins interacting with ct-PAELR and show a novel PDZ-based interaction between PAELR and PICK1.

Methods

Institutional and ethical approval

Experimental work was approved by Trinity College Dublin ethical committee (IACUC). Female Wistar rats were maintained in the BioResources unit at Trinity College, Dublin, Ireland. All animal work fell under schedule 1 guideline where animals, without any *in vivo* manipulation, were killed by cervical dislocation in isolation from other animals. Animals were housed in a temperature-controlled room under illumination with a 12 h light:12 h dark cycle (lights on from 06:00 to 18:00 h) and both food and water were available *ad libitum*.

Yeast two-hybrid system

The cDNA of the PAELR ct domain (ct-PAELR) was amplified from a full-length clone by PCR and subcloned in frame with the GAL4 DNA binding domain into the yeast two-hybrid pGBKT7

bait vector (Clontech, Palo Alto, CA, USA). Further mutations of ct-PAELR were constructed by PCR and subcloned into pGBKT7 bait vector. The ct-GPR37L1 bait was also amplified by PCR from a full-length clone and subcloned into pGBKT7 bait. The integrity of the inserts was verified by DNA sequencing. All other information for cDNA constructs is indicated in the figures or legends. The cloning information for the PICK1 pGAD10 fish constructs (Clontech, Palo Alto, CA, USA), cloned in frame with the GAL4 DNA activation domain, has been described previously (Dev *et al.* 1999, 2000). The full-length wild-type PICK1 construct was further subcloned from pGAD10 into pGADT7 fish vector (Clontech, Palo Alto, CA, USA). The AMPA receptor subunit GluR2 bait construct is also described previously (Dev *et al.* 1999), and was subcloned from pBTM116 into the pGBKT7 bait vector. The glutamate receptor interacting protein (GRIP) and syntenin pGAD10 fish constructs used in this study were as described before (Dev *et al.* 1999; Hirbec *et al.* 2003). Briefly, for yeast two-hybrid studies, the fish constructs were cotransformed with bait plasmids, into Saccharomyces cerevisiae AH109 reporter strain. The positive interactions were isolated by colony selection on interaction-dropout plates (Clontech, Palo Alto, CA, USA) and/or by testing for the activation of β-galactosidase reporter gene by filter β-galactosidase assays.

Western blotting and antibodies

For western blotting, denatured samples were separated by electrophoresis on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gels (SDS-PAGE) and western blotting was performed as described previously (Dev *et al.* 1999, 2000). Primary antibodies (Abs) were as follows: goat anti-GST Ab (27-4577-50, GE Healthcare, Buckinghamshire, UK), mouse anti-MBP Ab (E8032S, New England Biolabs, Beverly, MA, USA), mouse anti-Flag M2 Ab (F3165, Sigma, St. Louis, MO, USA), mouse anti-Myc Ab (A-14, sc-789, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-PICK1 Ab (SC-11410, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-Actin Ab (AM 2021, ECM Biosciences, Versailles, KY, USA). The secondary Abs used in western blotting were alkaline phosphatase conjugated and were as follows: goat anti-mouse IgG (S3721, Promega, Madison, WI, USA), goat anti-rabbit IgG (S3731, Promega, Madison, WI, USA), rabbit anti-goat IgG (V1151, Promega, Madison, WI, USA) or were IRDye coupled and were as follows: goat anti-mouse IRDye (926-32210, Licor, Biotechnology, Cambridge, UK), goat anti-rabbit IRDye (926-32221, Licor, Biotechnology, Cambridge, UK); donkey anti-goat IRDye (926-32214, Licor, Biotechnology, Cambridge, UK). The secondary Abs used in immunocytochemistry were as follows: goat anti-mouse Alexa 633 (A21050, Invitrogen, Renfrewshire, UK), goat anti-rabbit Alexa 633 (A21070, Invitrogen), donkey anti-mouse Dylight 488-conjugted (715-485-150, Jackson ImmunoResearch, West Grove, PA, USA) and donkey anti-rabbit Dylight 488-conjugated (703-505-155, Jackson ImmunoResearch, West Grove, PA, USA). Antibodies were used at dilutions recommended by the manufacture or at a dilution of 1 : 500 unless indicated otherwise.

Glutathione S-transferase and immunoprecipitation studies

Glutathione S-transferase (GST) was fused to ct-PAELR by subcloning into pGEX-4T-1 (Pharmacia, Uppsala, Sweden). Maltose binding protein (MBP) was fused to full-length PICK1 by subcloning into pMALc2X (New England Biolabs, Beverly, MA, USA) (Dev *et al.* 2000). GST and MBP proteins were purified from transformed Escherichia coli strain BL21. The sonicates of bacterial cultures were prepared by sonication and solubilization in ice-cold PTxE buffer (phosphate-buffered saline (PBS), 1% Triton X-100, 0.1 mM EDTA, pH 7.4) as described previously (Dev *et al.* 1999). Briefly, PTxE resuspended bacterial pellets (1 mL) were sonicated for 6 × 10 s (Astrason, Misonix, Inc., Farmingdale, NY, USA) and solubilized by rotation for 1 h at 4°C in ice-cold PTxE buffer and then centrifuged at 10 000 *g* for 20 min in a microfuge. In addition, female Wistar rat brain homogenates were prepared in homogenization buffer containing 0.32 M sucrose, 4 mM HEPES, 1 mM EDTA, 1 mM EGTA, pH 7.4, using a glass/Teflon homogenizer (10 passes). Homogenates were centrifuged at 1000 *g* for 10 min, and the supernatant (S1) was centrifuged at 48 000 *g* for 30 min to obtain the pellet (P2) fraction. The P2 fraction was resuspended in PTxE, sonicated and solubilized then centrifuged at 100 000 *g* for 1 h to prepare the brain sonicate. GST pull-down assays were performed as described previously (Dev *et al.* 1999) using either MBP-PICK1 (~0.2 mg total protein bacterial cell sonicate) or native PICK1 (~2.5 mg total protein rat brain sonicate). Briefly, supernatants of GST proteins were rotated with 20 μL glutathione Sepharose 4B beads (Pharmacia, Uppsala, Sweden) in the presence of 1 mg/mL bovine serum albumin (BSA, Sigma) for 30 min at 4°C, after which the coupled Sepharose beads were washed with 2 × 1 mL aliquots of PTx buffer (PBS, 0.1% Triton X-100, pH 7.4). The beads were then resuspended in PTx buffer and incubated with MBP-PICK1 or native PICK1 in the presence of 2 mg BSA. After rotation for 1 h at 4°C, the bead suspensions were washed with 3 × 1 mL aliquots of PTx buffer. The beads were finally resuspended in 30 μL PTx buffer and western blotting was performed to determine the level of PICK1 retained (Dev *et al.* 1999). For coimmunoprecipitation, cell sonicates were prepared similar to brain sonicates, as described above, from transiently transfected human embryonic kidney 293 (HEK293) cells, as described below and previously (Dev *et al.* 1999, 2000). The cell sonicates were then incubated with 20 μL anti-flag M2-agarose affinity beads (F 3165, Sigma) or with 20 μL protein-G Sepharose beads (Sigma) pre-incubated with 5 μg mouse anti-Myc Ab. After incubation, the beads were washed as above and level of Myc-PAELR:Flag-PICK1 complex isolated was determined by Western blotting. The Bio-Rad protein assay kit (Bio-Rad Laboratories; Hercules, CA, USA) with BSA as standard was used to determine protein concentrations.

Cell culture and immunocytochemistry

A Myc-tagged PAELR was prepared using PCR, with the tag introduced at the n-terminal (nt) after the signal peptide just after Gly 47 and subcloned into pcDNAmycA (Invitrogen, Carlsbad, CA, USA). Flag epitope tag was introduced using PCR primers at the n-terminal (nt) of PICK1 and subcloned into the mammalian expression vector pCIneo (Promega, Madison, WI, USA) as previously described (Dev *et al.* 1999). Human embryonic kidney 293 (HEK293) cells were grown in T-75 flasks using Dulbecco's modified Eagle's medium culture media supplemented with 10% dialysed foetal bovine serum and 1% penicillin:streptomycin. At 70–80% confluency, HEK293 cells were plated in six-multiwell plates at a density of 1–2 × 10^5 cells per well and allowed to grow overnight

at 37°C. HEK293 cells were transfected with cDNA using LipofectAMINE/PLUS in OPTIMEM (Life Technologies, Gaithersburg, MD, USA). After incubation for 6 h at 37°C, the transfection complex was replaced with Dulbecco's modified Eagle's medium culture medium. Cells were grown overnight and 24 h after transfection the media were changed. After 48 h, the cells were prepared for immunocytochemistry. Briefly, coverslips were washed in HEPES-buffered saline and fixed in 100% ice-cold methanol for 5 min, washed with PBS and permeabilized in PTx buffer. The coverslips were then blocked for 1 h in PTx-blocking buffer (PTx buffer containing 5% normal goat serum and 0.5% BSA) and incubated for 1 h with primary antibody in blocking buffer. After washing 3 × 0.5 mL with PTx buffer, the cells were exposed for a further 1 h to appropriate secondary antibodies in PTx-blocking buffer. Coverslips were washed 3 × 0.5 mL with PTx buffer, placed inverted onto glass slides and visualized using confocal microscopy (Olympus) (Dev *et al.* 1999). Confocal images were analysed using EBImage software and run through the statistical programming environment, R, as we have described previously (Healy *et al.* 2013).

Cell death assays

Propidium Iodide (PI) staining was carried out by using PI (P 4170, Sigma). Approximately, 5×10^4 cells were grown on coverslips on 24-well plates and transfected with selective plasmids using fugene HD transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) as recommended by the manufacturer (04 709 691 001, Roche, Germany). The cells were treated with PI at concentrations of 1 μg/mL in triplicates and then incubated at 37°C for 48 h in a humidified, 5% CO_2 atmosphere. The following day, coverslips were washed in PBS and fixed in 100% ice-cold methanol for 5 min. Again coverslips were washed 3 × 0.5 mL with PBS buffer. Cells were counter-stained with Hoescht 34580 nuclear stain, placed inverted onto glass slides and visualized using confocal microscopy (Olympus). Images were analysed using Image J1.42q (Wayne Rasband, NIH, Bethesda, MD, USA). Each condition was treated in duplicate and for each coverslip approximately 10 confocal images (× 20 magnification) were taken and a total of 100 cells were analysed for average fluorescent pixel intensity. In addition, a cell death test was performed using a CellTiter 96 AQueous one solution assay (G 3582, Promega, Madison, WI, USA). This assay contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent phenazine ethosulfate to measure cell death. The quantity of formazan product as measured by the amount of 490-nm absorbance is directly proportional to the number of living cells in culture. Approximately, 1×10^5 cells were grown on six-well plates and transfected with selective plasmids using fugene HD transfection reagent as recommended by the manufacturer (04 709 691 001, Roche, Germany). After transfection, ~5000 cells were transferred to 96-well plates in a total volume of 100 μL. The cells were then incubated at 37°C for 24 h in a humidified, 5% CO_2 atmosphere. The following day, cells were treated, in triplicates, with rotenone at concentrations of 10 μM, 1 μM, 100 nM, 10 nM, 1 nM and 100 pM for 48 h. This was followed by addition of 20 μL of the 96 AQueous one solution reagent per 96 wells and incubation at 37°C for 1–4 h in a humidified, 5% CO_2 atmosphere. The absorbance was recorded at 490 nm using a synergy Highthoughput multimode microplate reader (BioTek, Mason Technology, Dublin, Ireland).

Statistical analysis

All statistical analysis was carried out using Prism 4 (GraphPad Prism, La Jolla, CA, USA). Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by comparison between two groups, using unpaired Student's *t*-test or one-way ANOVA with further comparison by Turkey's multiple comparison test. A p value of < 0.05 was considered statistically significant.

Results

Identification of interaction between PAELR and PICK1

To identify novel proteins that interact with the parkin-associated endothelial-like receptor (PAELR/GPR37), a yeast two-hybrid study using the ct-PAELR as bait (PFSRAFMECCCCCCEECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFASVGTHC) was conducted. A putative interaction was identified between ct-PAELR 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 harbouring the ct-PAELR bait plasmid. The transformed yeast were grown on interaction plates at 30°C for 7 days then subjected to β-galactosidase (β-gal) reporter assays. Yeast cotransformed with ct-PAELR bait and PICK1 fish, but not the empty pGBKT7 bait and pGADT7 fish vectors (negative controls), 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. 1a). As additional negative controls, the cotransformation of yeast with ct-PAELR bait and empty pGADT7 fish or with PICK1 fish and empty pGADT7 fish showed no interaction. The data also confirmed a positive interaction between the ct-PAELR bait and a full-length wild-type parkin (1–465) fish construct as reported previously (Imai *et al.* 2001) (Fig. 1a). These data showed a novel interaction between ct-PAELR and PICK1.

The ct located PDZ motif of PAELR is required for interaction with PICK1

The ct-PAELR has been reported to contain a PDZ motif (-GTHC, residues 611–613, human PAELR) and interact with the PDZ domain containing protein syntenin (Dunham *et al.* 2009). In conventional PDZ-based protein–protein interactions, PDZ motifs are located at the last three residues of the ct of proteins and bind within PDZ domains. To investigate whether the PDZ motif of the PAELR is required for interaction with PICK1, two ct-PAELR bait constructs were prepared, where the last three residues of PAELR were mutated to AAA (ct-PAELR-mut) or were deleted (ct-PAELR-del). Neither the ct-PAELR-mut nor the ct-PAELR-del baits interacted with the PICK1 fish (Fig. 1b). To further investigate whether the PDZ motif of the PAELR can act as an internal PDZ motif capable of interacting with PICK1, an additional 10 residues were added to the last residue (residue

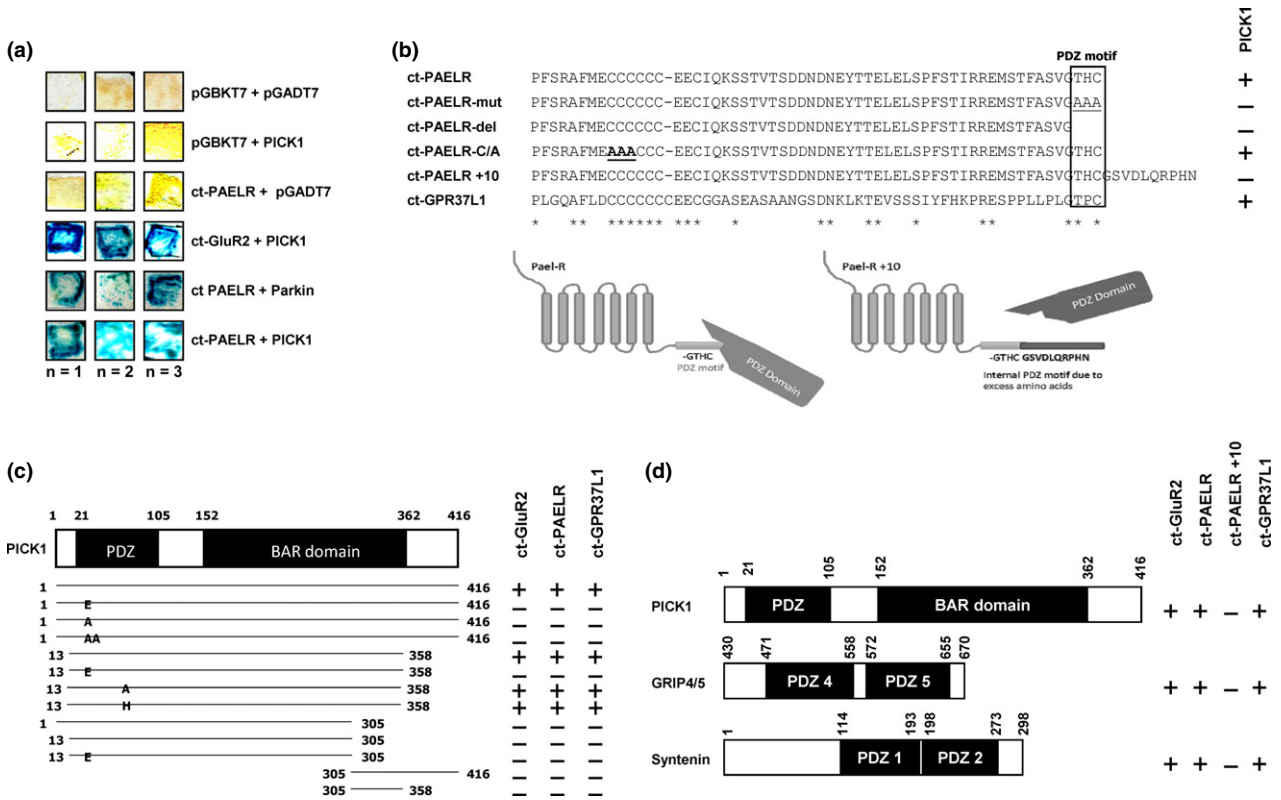


Fig. 1 **(a)** Novel interaction between ct-PAELR and PICK1. β-Galactosidase assay of interacting proteins transformed in yeast with the following controls: empty vectors pGBKT4 and pGADT7, ct-GluR2 and PICK1 and ct-PAELR and Parkin. A novel interaction between ct-PAELR (PFSRAFMECCCCCCEECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFASVGTHC) and PICK1 is shown. The blue colour confirms positive interactions in the assay. (b) The PDZ motif of ct-PAELR interacts with PICK1. Constructs were designed where the last three residues of PAELR (-THC) were mutated to -AAA (ct-PAELR-mut) or were deleted (ct-PAELR-del). An upstream cysteine rich region (-CCCCCC-), common to PAELR and GPR37L1 was also mutated to –AAACCC- and tested for interaction with PICK1. A construct with an additional 10 amino acids (ct-PAELR+10) (PFSRAFMECCCCCCEECIQKSSTVTSDDNDNEYTTELELSPFSTIRREMSTFASVGTHCGSVDLQRPHN) that occlude placement of the putative PDZ motif at the last three residues of ct-PAELR was also generated. The family member of PAELR, namely, GPR37L1, was also tested for interaction with PICK1. PICK1 interacts with both ct-PAELR and ct-GPR37L1, but not with ct-PAELR-mut, ct-PAELR-del or ct-PAELR+10 indicating the importance of the PDZ motif in ct-PAELR and likely presence of PDZ motif in ct-GPR37L1. Diagrammatic representation shows that the PDZ motif of ct-PAELR interacts with PICK1. (c) The PDZ domain of PICK1 interacts with ct-PAELR. 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-PAELR. A shorter PICK1 fragment (1–305) still containing the PDZ domain showed no interaction with ct-PAELR, similar to ct-GluR2. PICK1 constructs (305–416 and 305–358) lacking the PDZ domain failed to interact with ct-PAELR. The three point mutations, K27E, K27A and KD27/28AA, abolished the interaction between ct-PAELR and PICK1. In contrast, PICK1 mutants K83H and K83A showed interaction with ct-PAELR. Similar results were found for GPR37L1. (d) PDZ motif of ct-PAELR interacts with other PDZ domain containing proteins. The ct-PAELR was found to interact with GRIP (PDZ4/5) and syntenin (wild-type). The ct-PAELR+10 failed to interact with GRIP and syntenin, indicating that the PDZ motif of ct-PAELR requires placement at the last three residues of ct-PAELR 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. Data show representative examples from at least three separate experiments.

613, human PAELR) of the ct-PAELR (ct-PAELR+10) to occlude the PDZ motif. The ct-PAELR+10 bait showed no interaction with PICK1 fish indicating requirement for the PDZ motif of PAELR to be located at the last three residues (residues 611–613, human PAELR) for interaction with PICK1 (Fig. 1b). The PAELR sequence has approximately 40% similarity in the transmembrane regions to mammalian peptide-specific GPCRs, particularly with endothelin-B receptor (ETBR), bombesin-BB1 and bombesin-BB2 receptors (Marazziti *et al.* 1997, 1998). PAELR is most closely related to another CNS-enriched orphan receptor known as GPR37-like 1 (GPR37L1). GPR37L1 shows 68% overall homology and 48% identity to PAELR (Valdenaire *et al.* 1998). Sequence analysis shows high sequence similarity between the ct domain of PAELR and GPR37L1 (Fig. 1b). Notably, there is a high degree of similarity in the last four

residues of PAELR (-GTHC) and GPR37L1 (-GTPC), indicating that a putative PDZ motif may also exist in GPR37L1. Therefore, the specificity of interaction of PAELR and GPR37L1 with PICK1 was investigated. The data showed that, in addition to PAELR, the GPR37L1 also interacted with PICK1 (Fig. 1b). Sequence alignment data showed that the ct-PAELR and ct-GPR37L1 also share a cysteine rich motif (-CCCCCC-). To determine if this motif is also involved in interaction with PICK1, additional ct-PAELR bait was made (ct-PAELR-C/A) where the first three of the six cysteine residues were mutated to alanine (-AAACCC-). Data showed that this ct-PAELR-C/A bait still interacted with the PICK1 fish. Taken together, these studies suggest that PAELR and GPR37L1 contain PDZ motifs located at last three residues of their ct, which are capable of interacting with PICK1.

The ct-PAELR interacts with the PDZ domain of PICK1

To determine the site of interaction in PICK1, the ct-PAELR and GPR37L1 baits were transformed with a number of PICK1 fish truncated mutants and tested for their ability to interact in yeast (Fig. 1c). 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, 2000). This truncated form of PICK1 was found to interact with ct-PAELR and ct-GPR37L1, similar to the positive control ct-GluR2 (Fig. 1c). In contrast, a truncated form comprising residues 1–305 that still contains the PDZ domain did not interact with ct-PAELR, ct-GPR37L1 or ct-GluR2. The inability of this PICK1 (1–305) fragment to interact may be due its aberrant folding or poor expression levels in yeast as previously suggested (Dev *et al.* 1999, 2000; Staudinger *et al.* 1995). 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-PAELR, ct-GPR37L1 or ct-GluR2 (Fig. 1c). To investigate the binding requirements of the 'P0 binding pocket' within the PDZ domain of PICK1, three separate point mutations, K27E, K27A and KD27/28AA in the carboxylate binding motif of the PDZ domain of PICK1 were tested for interaction with ct-PAELR. Previous studies have shown that these mutations abolish the ability of PICK1 to interact with its receptor partners (Dev *et al.* 1999, 2000; Xia *et al.* 1999; Staudinger *et al.* 1995, 1997). In agreement, none of these PICK1 mutants interacted with ct-PAELR or ct-GPR37L1 suggesting the PDZ domain of PICK1 is required for interaction (Fig. 1c). Similar results were found with the positive control ct-GluR2 using these three PICK1 mutant constructs. Further point mutations in the PDZ domain of PICK1 were also created to investigate the binding requirements of the 'P-2 binding pocket' of PICK1. The residue in the αB1 position of a type I PDZ domain is generally found to be histidine, whereas this residue is lysine (Lys83) in PICK1. The replacement of Lys83 with His83 (K83H) in PICK1 has been shown to enhance the affinity for class I motifs (PKCα, -QSAV, 33 μM to 0.54 μM), and decrease the affinity for class II motifs (DAT, -WLKV, 2.3 μM to 21 μM) (Madsen *et al.* 2005). In contrast, mutation of Lys83 to Val83 (K83V) in PICK1 enhances affinities for both class I (PKCα, -QSAV, 33 μM to 5.5 μM) and class II PDZ motifs (DAT, -WLKV, 2.3 μM to 1.02 μM) (Madsen *et al.* 2005). Thus, two PICK1 mutant constructs were generated. Firstly, the Lys83 was replaced with histidine to imitate type I PDZ domain binding (PICK1-K83H). Secondly, a smaller amino acid alanine (similar to valine) was used to replace Lys83, which likely allows more space for interaction in the P-2 binding site (PICK1-K83A). The data showed that PICK1-K83H was still able to interact with ct-GluR2, ct-PAELR and ct-GPR37L1 (Fig. 1c), suggesting that the moderate change in binding ability of PICK1-K83H with class II PDZ motifs is still observable in yeast two-hybrid assays and/or that the PDZ motif residues of ct-GluR2 (-SVKI), ct-PAELR (-GTHC) and ct-GPR37L1 (-GTPC) are able to form additional interactions with residues in the PDZ domain of PICK1. In agreement that replacement of Lys83 with a smaller residue (alanine) will enhance binding of type I and type II PDZ motifs, the PICK1-K83A still showed interaction with ct-GluR2, ct-PAELR and ct-GPR37L1 (Fig. 1c). Taken together, the data suggested that ct-PAELR and ct-GPR37L1 interact with the PDZ domain of PICK1.

The PDZ motif of ct-PAELR and GPR37L1 interact with additional PDZ domain proteins

Previous studies have shown that PDZ motifs located at the last three residues of the ct of receptors can bind to multiple proteins that contain PDZ domains, for example, the kainate receptor subunit GluR5, which interacts with PICK1, GRIP and syntenin (Hirbec *et al.* 2002). As ct-PAELR has been shown to previously interact with the PDZ domain of syntenin (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-PAELR and GRIP or syntenin was investigated. In agreement with a previous report (Dunham *et al.* 2009), the data confirmed an interaction between ct-PAELR and syntenin (Fig. 1d). Importantly, the results also showed an additional interaction between ct-PAELR and GRIP (Fig. 1d). In contrast, the ct-PAELR+10 mutant showed no interaction with syntenin or GRIP, indicating the requirement for the PDZ motif to be located at the last three residues (residues 611–613, human PAELR) of ct-PAELR. These results suggest that the PDZ motif of ct-PAELR interacts with several PDZ domain containing proteins, including PICK1, syntenin and GRIP. The data also suggested that ct-GPR37L1 contained a PDZ motif (Fig. 1c), and inferred that ct-GPR37L1 would also interact with other PDZ domain containing proteins. In agreement,

the ct-GPR37L1 interacted with both syntenin and GRIP, similar to the ct-PAELR (Fig. 1d). Taken together, these experiments are supportive of the identification of a PDZ motif (-GTPC) in ct-GPR37L1 and indicate that both PAELR and GPR37L1 have similar and multiple PDZ domain interacting partners.

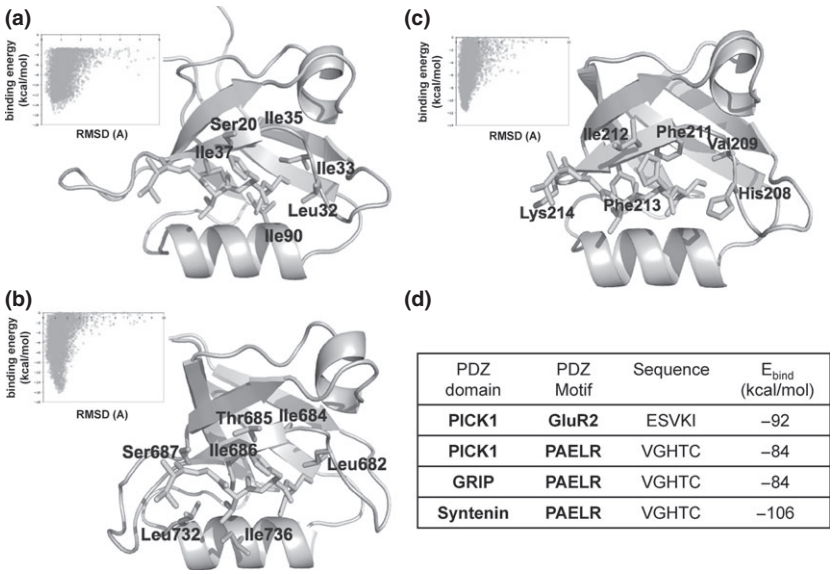
In silico prediction of binding affinities and modelling of interacting residues

We have recently reported the in silico binding affinities for protein interacting with PICK1 (Bolia *et al.* 2012). To further investigate the relative strength of binding between ct-PAELR and its interacting PDZ domain containing proteins, a flexible docking protocol using ROSETTALIGAND was applied similar to that described previously. The PDZ motif (ligand) of ct-PAELR 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/root-mean-square deviation plots was taken as an indication of successful docking (Fig. 2). 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 reassessed 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-PAELR (-GTHC) and PICK1, GRIP and syntenin were determined (Fig. 2). This coupled flexible docking and DrugScore analysis showed that the PDZ motif of ct-PAELR (-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 PAELR-PICK1 interaction had a lower binding score (Fig. 2). Overall, the in silico calculated rank order of binding for the PDZ motif of the PAELR (-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 βB strand of PICK1 and the residues Leu32-Ile33-Gly34-Ile35 (LIGI motif) in the βB sheet (Madsen *et al.* 2005). 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 αB helix of PICK1 (Madsen *et al.* 2005). The Val86, Ala87, Ile90 residues of the αB helix of PICK1 may also play important roles in interacting with PDZ motifs (Madsen *et al.* 2005). The docking analysis showed that the P0 Cys (-VGTHC) of PAELR formed hydrogen bonds in the LIGI motif and was also found to interact strongly with Ile90 of the PDZ domain of PICK1. Of interest, the data showed that P-2 Thr (-VGTHC) formed hydrogen bonds with Ile37 residue in the PDZ domain of PICK1. In addition, unlike GluR2 (-ESVKI), the PAELR (-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 PAELR (−84 kcal/mol). The data may also explain the ability of ct-PAELR to still interact with the PICK1-K83H mutant (Fig. 1c). When modelling interaction between PAELR and syntenin, it was found that the stabilization of interactions between polar side chain of P-2 Thr (-VGTHC) and Phe213 in second PDZ domain of syntenin could have increased the binding affinity (−106 kcal/mol) of PAELR for syntenin as compared to PICK1 (−83 kcal/mol). In contrast, the analysis showed that hydrogen bond interactions of P-2 Thr (-VGTHC) of PAELR were destabilized in the sixth PDZ domain of GRIP1 which could have accounted for its poor binding affinity (−84 kcal/mol).

The PAELR interacts with native rat brain PICK1

To further confirm the interaction between PAELR and PICK1, the ct-PAELR was subcloned into a pGEX-4T-1 bacterial expression vector and the GST-ct-PAELR fusion protein used for affinity chromatography. GST alone, GST-ct-GluR2 and MBP-PICK1 was 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. 3a,b). In agreement with previous studies (Dev *et al.* 1999, 2000), the glutathione Sepharose 4B matrix coupled with GST-ct-GluR2 (positive control), but not GST alone (negative control), retained MBP-PICK1 (Fig. 3a). Importantly, the GST-ct-PAELR also bound MBP-PICK1 (Fig. 3a). GST pull-down studies were also performed to isolate native PICK1 obtained from rat brain P2 sonicates. Western blot with anti-PICK1 Ab showed that native rat brain PICK1 was retained by GST-ct-GluR2 (positive control) and was also retained by GST-PAELR, but not by GST alone (negative control) (Fig. 3b). The ability of the previously reported PICK1 binding compound FSC231 (Thorsen *et al.* 2010), to block the interaction between MBP-PICK1 and GST-ct-GluR2 or GST-ct-PAELR was also investigated. While both GST-ct-GluR2 and GST-ct-PAELR interacted with MBP-PICK1 in the absence of FSC231 (Fig. 3a), both these interactions were inhibited in the presence of 100 μM FSC231 (Fig. 3c), further providing confirmation of a specific interaction between PAELR and PICK1. Next, coimmunoprecipitation experiments were performed to isolate a native PAELR-PICK1 protein complex from rat brain tissue. Two PAELR Abs were used (sc-27546 and sc-27548; Santa Cruz Biotechnology, Santa Cruz, CA, USA), however, data suggested that both PAELR antibodies were not specific (data not shown). In the absence of specific PAELR antibodies, instead coimmunoprecipita-



PDZ domain	PDZ Motif	Sequence	E_{bind} (kcal/mol)
PICK1	**GluR2**	ESVKI	–92
PICK1	**PAELR**	VGHTC	–84
GRIP	**PAELR**	VGHTC	–84
Syntenin	**PAELR**	VGHTC	–106

Fig. 2 In silco modelling of binding residues and affinities. *In silico* models of ct-PAELR PDZ motif with (a) PICKI, (b) GRIP and (c) syntenin are shown. (d) Table represents the modelled binding affinities of ct-PAELR PDZ motif with PICK1, GRIP and syntenin and GluR2-PICK1 as control.

tion experiments were performed using cell sonicates from HEK293 cells transiently transfected with Myc-PAELR and Flag-PICK1. The data showed that Flag-beads immunoprecipitated Flag-PICK1 and also coimmunoprecipitated Myc-PAELR (Fig. 3d). As control, Flag-PICK1-KE mutant did not coimmunoprecipitate with Myc-PAELR. In these experiments an additional complexity was noted, in that the coexpression of Flag-PICK1 wild type (but not the Flag-PICK1-KE mutant) with Myc-PAELR, decreased the total protein expression levels of Myc-PAELR. Taken together, these results biochemically confirm the interaction between PAELR and PICK1. It should be noted, however, that further coimmunoprecipitation experiments isolating an endogenous PAELR:PICK1 complex from brain tissue would support these biochemical studies.

PICK1 reduces the protein expression levels of the PAELR

To study further the functional role of the PAELR-PICK1 interaction, HEK293 cells were transiently transfected with Myc-PAELR or Flag-PICK1 (wild type or KE mutant) either alone or in combination. The distribution and expression levels of PAELR in the presence of PICK1 wild type 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-PAELR and Flag-PICK1 (wild type and KE mutant) were ubiquitously expressed when transfected alone (Fig. 4a). No differences in the distribution of Flag-PICK1 wild type and KE mutant were observed in agreement with previous reports (Dev *et al.* 1999, 2000). Moreover, no change in the distribution of Myc-PAELR or Flag-PICK1 (wild type or KE mutant) was observed upon coexpression (Fig. 4a). Interestingly, however, a decrease in the level of Myc-PAELR immunostaining was found in cells cotransfected with Flag-PICK1 wild type compared to the Flag-PICK1 KE mutant, which does not interact with the PAELR. This reduction in Myc-PAELR expression occurred more evidently in the cytoplasmic regions often leaving a perinuclear staining pattern for the receptor (Fig. 4a). Quantification of the levels of Myc-PAELR immunostaining confirmed that Flag-PICK1 wild type significantly ($p = 0.032$) reduced levels of Myc-PAELR expression by 37.5 ± 8.6% when compared to the Flag-PICK1 KE mutant (Fig. 4b). Western blot results confirmed that Flag-PICK1 wild type reduced the total protein expression levels of Myc-PAELR compared to Flag-PICK1 KE mutant (Fig. 4c). The data suggest that while PICK1 does not alter PAELR trafficking *per se*, the interaction between PICK1 and PAELR attenuates the total protein expression levels of the receptor.

PICK1 protects against PAELR-induced cellular toxicity during cell stress

It was hypothesized that the reduction in PAELR expression levels induced by PICK1 would result in protection against cell toxicity and improves cell survival. Under control conditions, it was observed that transiently over-expressing Myc-PAELR increased cell death in HEK293 cells as determined by propidium iodide (PI) cell staining (Fig. 5a). The data also showed that Flag-PICK1-wt significantly reduced PAELR-mediated cell toxicity (Fig. 5a). To account for promoter competition during transient transfections only the direct comparisons between Flag-PICK1-wt and Flag-PICK1-KE conditions were analysed. We used Flag-PICK1-KE as a control to examine the effects of Flag-PICK1-wt as it did not interact with the PAELR in yeast two-hybrid or biochemical studies. We believe this comparison, i.e. a difference in one residue mutation provides a control to account for transfection effects and arbitrary effects on

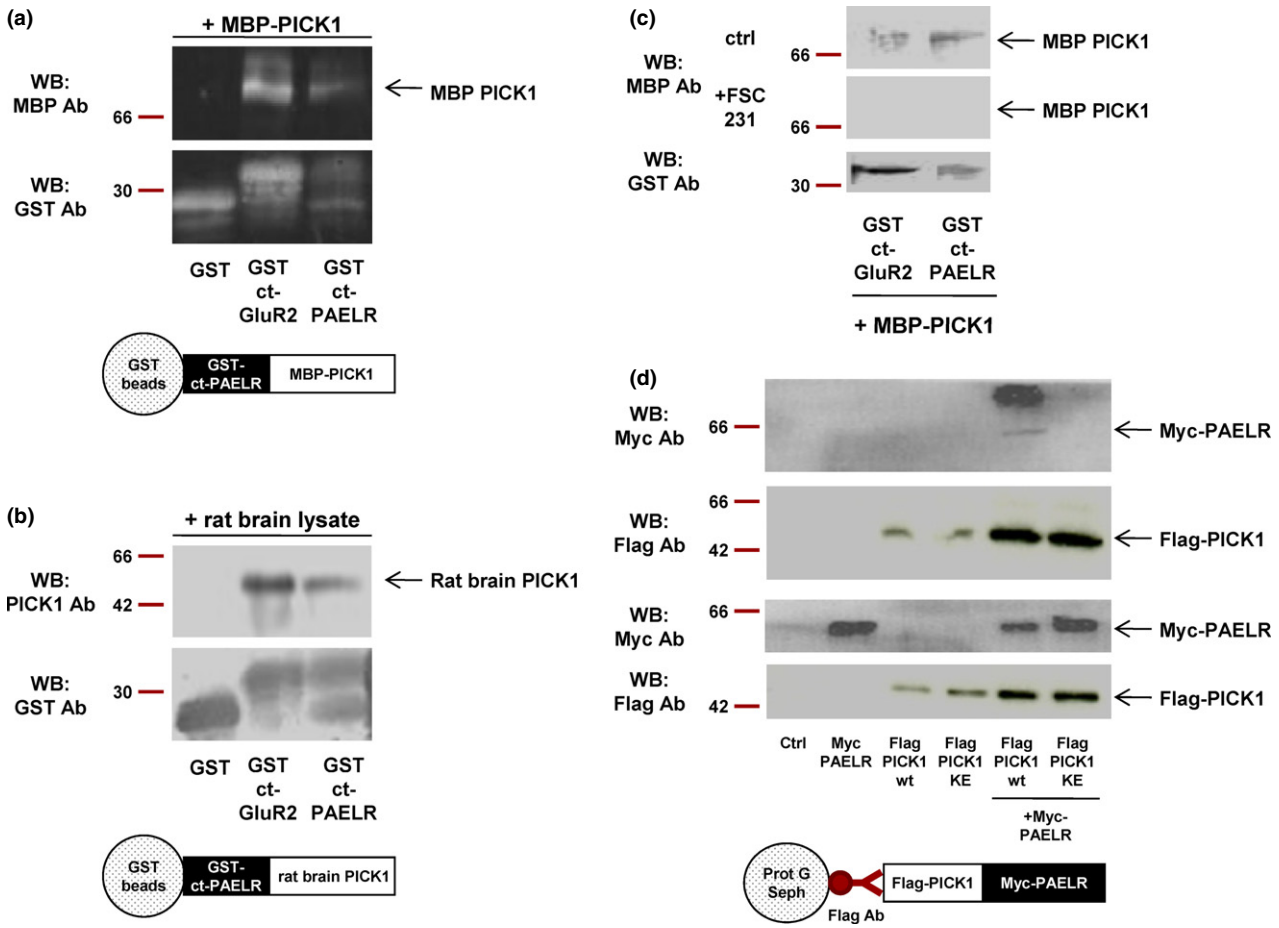


Fig. 3 Biochemical evidence of binding between ct-PAELR and PICK1. (a) The GST-ct-PAELR interacts with MBP-PICK1. Western blot with anti-MBP antibody showed the levels of MBP-PICK1 retained by GST-ct-GluR2 (positive control) and GST-ct-PAELR, but not by GST alone. The western blot with anti-GST antibody indicates that similar levels of GST fusion proteins were used. The diagram indicates setup of the pull-down experiment. (b) Isolation of PICK1 from rat brain tissue by GST-ct-PAELR. The GST-ct-GluR2 (positive control) and GST-ct-PAELR isolated endogenous PICK1 from rat brain lysate indicating an interaction between PAELR and native PICK1, as determined by western blotting with the anti-PICK1 antibody. The diagram shows the setup of the pull-down experiment. (c) The ability of GST-ct-GluR2 (positive control) and GST-ct-PAELR to pull-down MBP-PICK1 in the absence (ctrl) and presence of 100 μM FSC231 is shown. (d) A Myc-PAELR/Flag-PICK1 complex, but not a Myc-PAELR/Flag-PICK1-KE mutant complex, was coimmunoprecipitated from transiently transfected HEK293 cells using Flag-bead, as determined by western blotting. Upper two panels show immunoprecipitated levels of Myc-PAELR and Flag-PICK1 and lower panels show expression of proteins in cell lysates. The diagram shows the setup of the coimmunoprecipitation experiment. Data show representative examples from at least three separate experiments.

promoter activity. When doing so we found that Flag-PICK1-wt significantly reduced cell toxicity compared to Flag-PICK1-KE, when coexpressed with Myc-PAELR (Fig. 5a). Next, cell death induced by Myc-PAELR over-expression was investigated under conditions of cell stress, in particular induced by the mitochondrial complex I inhibitor, rotenone. We opted to use rotenone to induce cell stress as it has been reported to be a toxin that, like MPTP, induces complex I mitochondrial dysfunction and Parkinson's like symptoms. As expected, the treatment of wild-type HEK293 cells transfected with vector control (pcDNAmycA) with increasing concentrations of rotenone (100 pM–10 μM) for 48 h caused a concentration-dependent increase in cell death (Fig. 5b). Furthermore, HEK293 cells transiently transfected with Myc-PAELR displayed increased cell loss induced by rotenone, compared to HEK293 cells transfected with vector control (Fig. 5b). These data show that PAELR increases further cell death in the presence of low concentrations of rotenone and at higher concentrations of rotenone, there is little further effect of PAELR over-expression on cell death. The amount of cell death of HEK293 cells transfected with Myc-PAELR and Flag-PICK1-wt or Flag-PICK1-KE and treated with 100 nM rotenone for 48 h was examined. The over-expression of Flag-PICK1-wt showed a statistically significant attenuation in the loss of HEK293 cells expressing Myc-PAELR and treated with rotenone, when compared to

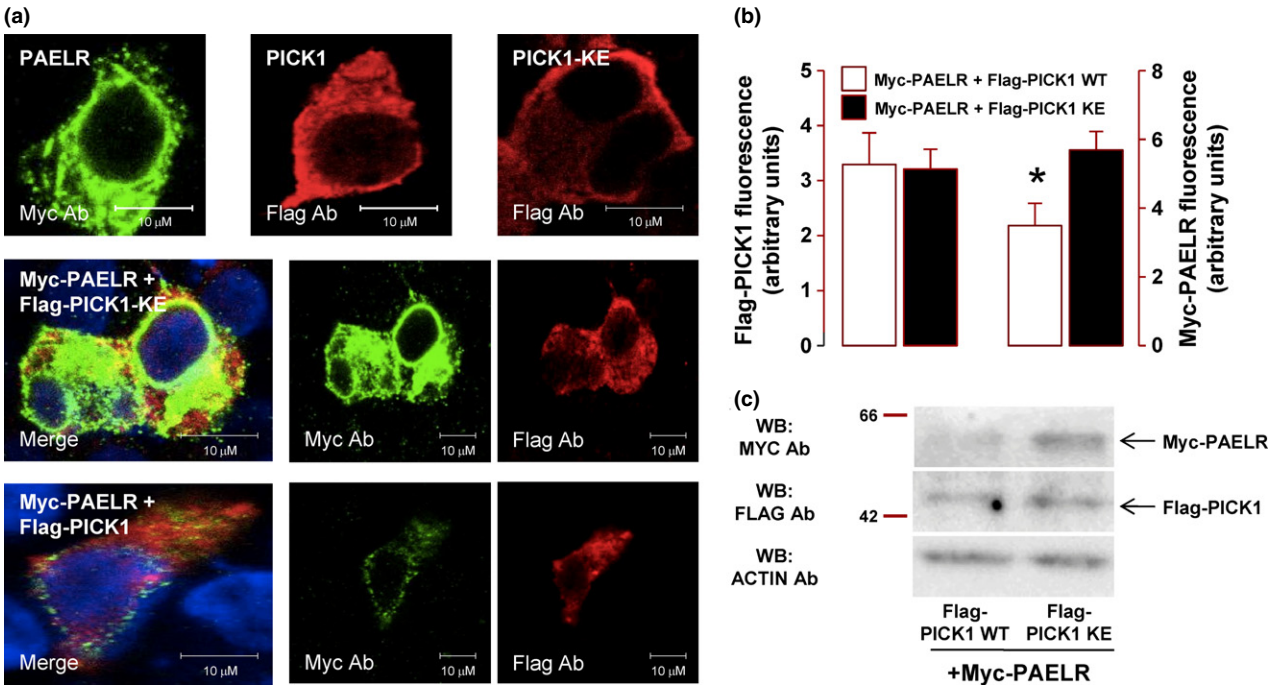


Fig. 4 PICK1 reduces PAELR protein expression levels. (a) Colocalization of full-length Myc-PAELR and Flag-PICK1-wt in HEK293 cells. HEK293 cells were transiently transfected with Myc-PAELR and Flag-PICK1-wt. The non-interacting, Flag-PICK1-KE mutant was used as control. The cells were stained with anti-Flag Ab and/or anti-Myc Ab and viewed at ×63 magnification. (b) Statistical analysis of PICK1-mediated reduction of PAELR expression. HEK293 cells transiently cotransfected with Myc-PAELR and Flag-PICK1-wt or Flag-PICK1-KE were analysed for expression levels as determined by quantification of fluorescent pixels as we have previously described (Healy *et al.* 2013). Data show similar expression levels of Flag-PICK1-wt and Flag-PICK1-KE ($p \geq 0.05$; $n = 3$). The data also showed a statistically significant reduction in Myc-PAELR expression when cotransfected with Flag-PICK1-wt compared to cotransfected with Flag-PICK1-KE ($^*p \leq 0.05$; $n = 3$). (c) Flag-PICK1-wt, but not Flag-PICK1-KE, reduced expression levels of full-length Myc-PAELR. Western blot with anti-Myc Ab shows that the protein expression level of Myc-PAELR is reduced when cotransfected with Flag-PICK1-wt compared to cotransfection with Flag-PICK1-KE. Western blot with anti-Flag Ab indicates similar Flag-PICK1-wt and Flag-PICK1-KE expression in these experiments and western blot with Actin Ab shows uniform loading of protein samples. Data show representative examples from at least three separate experiments.

over-expression of the Flag-PICK1-KE mutant (Fig. 5c). To investigate the role of the proteasomal pathway, we next investigated if PICK1 could attenuate the PAELR-induced cell death in the presence of a proteasomal inhibitor MG132. The data showed that, compared to control, MG132 (1 μM for 48 h) induced a greater amount of cell death in cells transfected with Myc-PAELR and Flag-PICK1-wt, likely suggesting that proteasomal clearance of Myc-PAELR plays a role in attenuating cell toxicity (Fig. 5d). The data, in total, suggest a role for PICK1 in preventing PAELR-induced cell toxicity during conditions of cell stress and in particular during rotenone-induced mitochondrial complex I inhibition that may involve proteasomal clearance.

Discussion

This study demonstrated that PAELR associates with PICK1 via a PDZ-based interaction. In agreement with a canonical PDZ protein–protein interaction, the mutation and deletion of the last three residues of ct-PAELR (residues 611–613, human PAELR) prevented interaction with PICK1. Furthermore, the addition of 10 residues at the last residue (residue 534, human PAELR) of ct-PAELR occluded interaction with PICK1, indicating that the PDZ motif of ct-PAELR (-GTHC) requires location at the last three residues (residues 611–613, human PAELR) for interaction with PICK1. In addition, 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 PAELR. The results using truncated versions of PICK1 also suggested that full-length PICK1 is required for interaction, most likely because of correct conformation and/or expression levels. As well as PAELR, the closely related family member GPR37L1 was also shown to interact with PICK1. Moreover, PAELR 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 PAELR (-GTHC) was syntenin > PICK1 = GRIP. Mutations in the

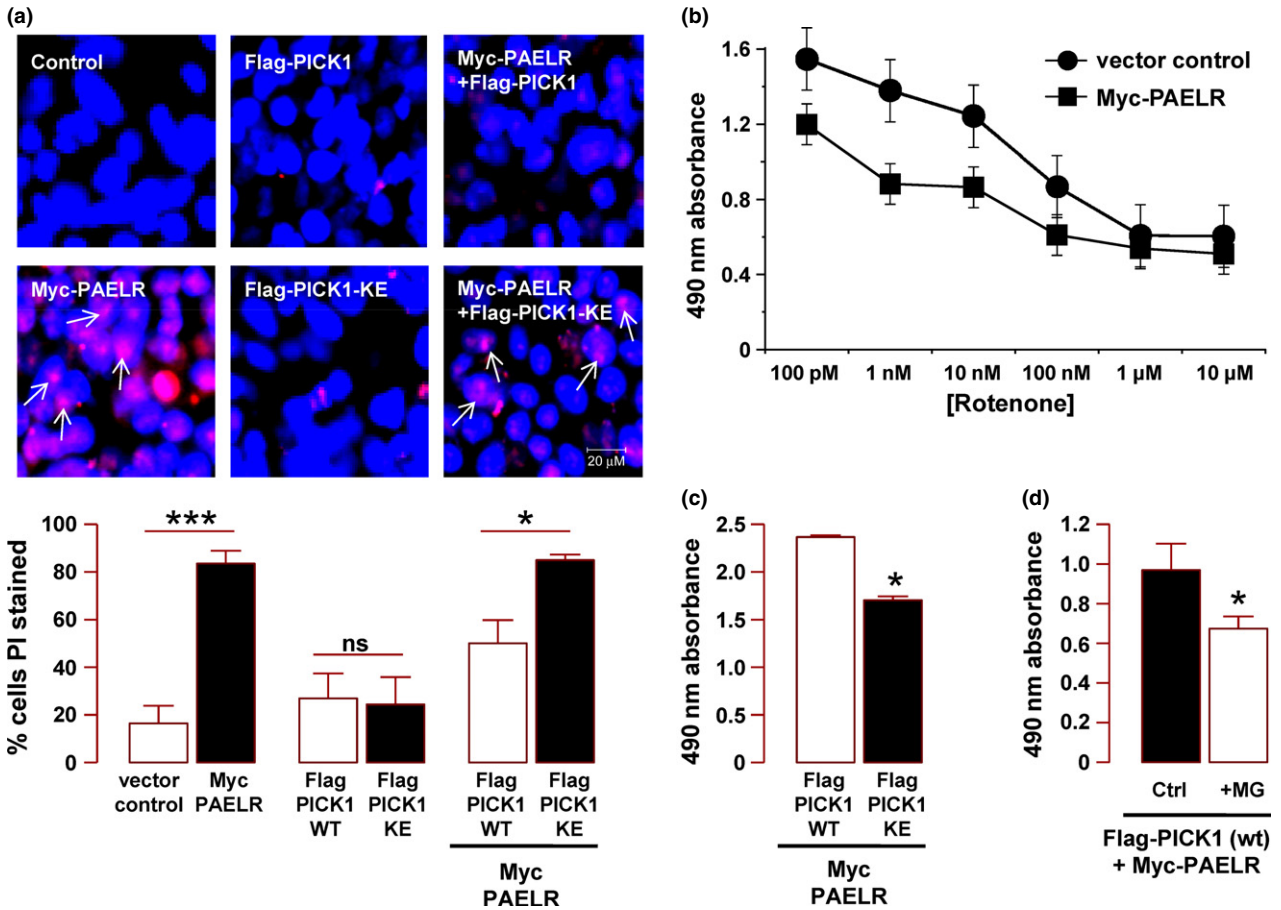


Fig. 5 PICK1 attenuates PAELR-induced reduction in HEK293 cell numbers. (a) Cells were transiently transfected with Myc-PAELR with or without Flag-PICK1-wt or Flag-PICK1-KE and 48 h later propidium iodide (PI) staining was performed as a measure of cell death. The cell nuclei appear as blue (Hoescht staining). The data show representative PI cell staining confocal images (*upper panel*). Quantification of PI cell staining is represented as the mean ± SEM of eight independent experiments (*lower panel*). Using one-way ANOVA and further comparison with Turkey's multiple comparison test a statistical difference was found between vector control versus Myc-PAELR (***$p \leq 0.001$), Myc-PAELR+Flag-PICK1-wt (*$p \leq 0.05$) and Myc-PAELR+Flag-PICK1-KE (***$p \leq 0.001$). In addition, a statistical difference was found between Myc-PAELR and Flag-PICK1-wt (**$p \leq 0.01$), Flag-PICK1-KE (**$p \leq 0.01$), and Myc-PAELR+Flag-PICK1-wt (*$p \leq 0.05$). Statistical differences were also found between Flag-PICK1-wt or Flag-PICK1-KE and Myc-PAELR+Flag-PICK1-KE (**$p \leq 0.01$). To account for promoter competition during transient transfections the level of cell death in the Myc-PAELR+Flag-PICK1-wt condition was compared to the Myc-PAELR+Flag-PICK1-KE condition, which showed a statistical difference between both groups (*$p \leq 0.05$). (b) Cells were transiently transfected with pcDNAmycA (vector control) or with Myc-PAELR and the effect of 48-h treatment with increasing concentrations of rotenone (100pM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM) on cell death was examined. The data show a concentration-dependent increase in cell death induced by rotenone treatment. Data show representative examples from at least three separate experiments. (c) The amount of cell death of HEK293 cells transiently transfected with Myc-PAELR and Flag-PICK1-wt or Flag-PICK1-KE and treated with 100 nM rotenone for 48 h was also examined. Flag-PICK1-wt showed a statistically significant increase in cell survival compared to Flag-PICK1-KE mutant (*$p \leq 0.05$; $n = 3$). (d) The amount of cell death of HEK293 cells transiently transfected with Myc-PAELR and Flag-PICK1-wt treated without (ctrl) or with 1 μM MG132 (MG) for 48 h was also examined. A statistically significant decrease in cell survival was seen in the presence of MG132 compared to control (*$p \leq 0.05$; $n = 3$). (b–d) Cell death was examined using 'cell titre 96 aqueous one solution assay' (Promega). The amount of 490 nm absorbance is directly proportional to the number of living cells in culture. Data are represented as the mean ± SEM of three independent experiments.

'P0 binding pocket' and 'P-2 binding pocket' within the PDZ domain of PICK1, in addition to in silico modelling data, suggested further that the P0 binding pocket, but not the P-2 binding pocket, played a critical role in interaction with ct-PAELR. Biochemical studies verified that recombinant bacterial expressed MBP-PICK1 interacts with GST-ct-PAELR. The data also showed that ct-PAELR can isolate native PICK1 from solubilized rat brain tissue. In addition,

this interaction was inhibited by the PICK1 binding compound FSC231 (Thorsen *et al.* 2010). In addition, the PAELR-PICK1 complex was isolated from transiently transfected HEK293 cells. In transiently transfected HEK293 cells, colocalization of Myc-PAELR and Flag-PICK1 was observed. Importantly, the over-expression of wild-type Flag-PICK1-wt reduced Myc-PAELR protein levels, compared to the Flag-PICK1-KE mutant (which does not interact with PAELR). Finally, PICK1 over-expression in HEK293 cells attenuated cell toxicity induced by PAELR over-expression. Taken together, the data suggest a role for PICK1 in preventing PAELR-induced cell death during conditions of cell stress.

The PAELR (GRP37) belongs to a family of GPCRs that includes the bombesin-BB1 and bombesin-BB2 receptors and the ETBR (Marazziti *et al.* 1997, 1998). PAELR is closely related to the orphan GPCR called the CNS-enriched orphan receptor, also known as GPR37-like 1 (GPR37L1, PAELRL1 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 hybridization demonstrates broad distribution of both PAELR and GPR37L1 throughout 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 grey 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 PAELR (-GTHC) and confirmed that ct-GPR37L1 interacted with the PDZ domain of PICK1. Moreover, the last three residues of ct-GPCR/CNS1 (-GTHC) and ct-GPCR/CNS2 (-GTPC), contain identical PDZ motifs as PAELR and GPR37L1, respectively, and predictably also interact with PICK1.

PAELR 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 (transmembrane proteoglycan), Neurexin (neuronal surface proteins), Syn-CAM (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 Coffer 2008). The interaction between syntenin and PAELR is reported to increase the cell surface trafficking of the receptor (Dunham *et al.* 2009). As PAELR has been shown to interact with syntenin and we find that 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 (Joch *et al.* 2007; Dong *et al.* 1999). 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 PAELR (as well as GPR37L1) interacted with GRIP and also confirmed PAELR interaction with syntenin. The association of PAELR to different PDZ proteins may link it to various signalling pathways and/or may aid in controlling the correct subcellular localization and protein levels of this receptor.

It is noteworthy that PICK1 interacts with PKCα, DAT and parkin, which all play multiple roles in mitochondrial function, protein degradation and/or dopaminergic neurotransmission. For example, PICK1 interacts with PKCα (-TSXV, PDZ motif) and regulates PKCα-mediated phosphorylation (Dev *et al.* 1999; Staudinger *et al.* 1995). Importantly, PICK1 targets PKC towards the mitochondria which maintains proper mitochondrial function and resistance to toxic insults (Wang *et al.* 2003, 2007, 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). Over-expression of parkin increases PICK1 monoubiquitination (Joch *et al.* 2007) which enhances activity of another PICK1 binding partner, namely, the acid-sensing ion channel (Joch *et al.* 2007). Acid-sensing ion channel proteins are involved in pain, mechanosensation and psychiatric diseases. Parkin also enhances the ubiquitination and degradation of DAT (Jiang *et al.* 2004) and it is internalized by PKC. In addition, DAT can interact with PAELR, which modulates DAT-mediated DA uptake, where the lack of PAELR enhances DAT activity and increase the plasma membrane expression of DAT (Marazziti *et al.* 2007). In closing, our finding that PICK1 interact with PAELR adds to the repertoire of PICK1 interacting proteins, such as DAT and parkin, which have been shown to play roles in dopaminergic neuronal function.

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