Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2013 | 125 | 362–372 doi: 10.1111/jnc.12196

ORIGINAL ARTICLE

The Parkinson's disease-associated GPR37 receptor-mediated cytotoxicity is controlled by its intracellular cysteine-rich domain

Jorge Gandía,* Víctor Fernández-Dueñas,* Xavier Morató,* Gianluigi Caltabiano,† Rosario González-Muñiz,‡ Leonardo Pardo,† Igor Stagljar§ and Francisco Ciruela*

*Unitat de Farmacologia, Departament de Patologia i Terapèutica Experimental, Facultat de Medicina, IDIBELL, Universitat de Barcelona, Barcelona, Spain

†Laboratori de Medicina Computacional, Unitat de Bioestadística, Facultat de Medicina, Universitat Autònoma de Barcelona, Bellaterra, Spain

‡Instituto de Química Médica, CSIC, Madrid, Spain

§Department of Biochemistry and Department of Molecular Genetics, Donnelly Centre, University of Toronto, Toronto, Ontario, Canada

Abstract

GPR37, also known as parkin-associated endothelin-like receptor (Pael-R), is an orphan G protein-coupled receptor (GPCR) that aggregates intracellularly in a juvenile form of Parkinson's disease. However, little is known about the structure or function of this receptor. Here, in order to better understand the functioning of this receptor, we focused on the GPR37 C-terminal tail, in particular on a cystein-enriched region. Thus, we aimed to reveal the role of these residues on receptor plasma membrane expression and function, and also whether the presence of this cysteine-rich domain is linked to the previously described receptor-mediated cytotoxicity. Interestingly, while the deletion of six cysteine residues within this region did not affect receptor internalization it promoted GPR37 plasma membrane expression and signaling. Furthermore, the removal of the C-terminal cysteine-rich domain protected against GPR37-mediated apoptosis and cell death. Overall, we identified a GPR37 domain, namely the C-terminal tail cysteine-rich domain, which played a critical role in receptor cell surface expression, function and GPR37-mediated cytotoxicity. These results might contribute to better comprehend the pathophysiology (i.e. in Parkinson's disease) of this rather unknown member of the GPCR family.

Keywords: cell surface expression, cytotoxicity, GPR37, orphan receptor, Pael-R, Parkinson's disease.

J. Neurochem. (2013) **125**, 362–372.

Parkin-associated endothelin-like receptor (Pael-R), also called GPR37, is an orphan G protein-coupled receptor (GPCR) first cloned in 1997 from human brain (Marazziti *et al.* 1997). Within the brain, this receptor is particularly enriched in cerebellum (i.e. Purkinje cells), corpus callosum, medulla, putamen, caudate nucleus, substantia nigra and hippocampus (i.e. pyramidal and granule cells of the dentate gyrus) (Donohue *et al.* 1998; Marazziti *et al.* 1997; Takahashi and Imai 2003; Zeng *et al.* 1997). Interestingly, GPR37 has been identified as a parkin substrate (Imai *et al.* 2001; Takahashi and Imai 2003). Parkin is a protein-ubiquitin

Received September 19, 2012; revised manuscript received February 6, 2013; accepted February 6, 2013.

Address correspondence and reprint requests to Francisco Ciruela, Unitat de Farmacologia, Departament de Patologia i Terapèutica Experimental, Facultat de Medicina, IDIBELL, Universitat de Barcelona, L'Hospitalet de Llobregat, 08907 Barcelona, Spain.
E-mail: fciruela@ub.edu

Abbreviations used: AC, adenylyl cyclase; ATF4, activating transcription factor 4; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; HA, head activator; HRP, horseradish-peroxidase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PD, Parkinson disease; SDS, sodium dodecyl sulfate; UPR, unfolded protein response.

© 2013 International Society for Neurochemistry,

ligase E3 involved in the ubiquitination and proteasome-mediated protein degradation and in the clearance of aggregated proteins (Dev *et al.* 2003). Therefore, parkin loss of function, such as in autosomal recessive juvenile Parkinson (Dev *et al.* 2003), prevents degradation of parkin substrates (i.e. GPR37) which results in their toxic accumulation (Shimura *et al.* 2000; Sriram *et al.* 2005; Zhang *et al.* 2000). Indeed, GPR37 has been described to be up-regulated in the brains of autosomal recessive juvenile Parkinson patients (Imai *et al.* 2001). In addition, the presence of GPR37 in the core of Lewy bodies in Parkinson disease (PD) patients has been reported (Murakami *et al.* 2004), thus suggesting a role of GPR37 aggregates in PD pathology. Finally, viral-mediated GPR37 over-expression *in vivo* (i.e. in the substantia nigra) constitutes a good PD animal model because it results in progressive degeneration of nigral dopaminergic neurons (Dusonchet *et al.* 2009; Low and Aebischer 2012).

Collectively, these results established a good correlation between the expression of this orphan receptor and PD. However, very little information exists regarding the functional and structural characteristics of this receptor. For instance, GPR37 has a significant sequence homology (40%) with the mammalian peptide activated class A GPCRs (i.e. endothelin-B receptor, bombesin-BB_1 and bombesin-BB_2 receptors; Marazziti *et al.* 1997, 1998), but agonists for these receptors (i.e. endothelin and bombesin) failed to activate GPR37 (Leng *et al.* 1999; Valdenaire *et al.* 1998; Zeng *et al.* 1997). However, it has been reported, not without some controversy (Dunham *et al.* 2009), that the neuropeptide head activator (HA), which is derived from the freshwater coelenterate Hydra, is a high-affinity ligand for GPR37 (Rezgaoui *et al.* 2006). Interestingly, in this last study it was reported that HA challenge induced GPR37-mediated intracellular Ca^{2+} accumulation and activation of both Ca^{2+}-dependent calmodulin kinase and phosphoinositide-3-kinase (Rezgaoui *et al.* 2006). However, although some early publications suggested that HA is present in the human brain (Bodenmuller *et al.* 1980) and proposed a potential role in some brain tumors (Schaller *et al.* 1988), no equivalent neuropeptide has been identified in vertebrates, thus casting doubt on the existence of a human version of the HA neuropeptide. Therefore, the existence of HA neuropeptide in humans is still an open question, a fact that may be posed as an additional setback in the description of GPR37 biological functions.

In this study, we aimed to shed some light on GPR37 structure, function and toxicity, trying to elucidate some of the molecular determinants that mediate these processes and that may help to explain the mechanisms driving the toxic accumulation of the receptor. Interestingly, GPR37 possesses an inherent difficulty for folding, a fact that complicates its ectopic expression in living cells and plasma membrane trafficking (Takahashi and Imai 2003). Related to this, several studies have shown that different covalent post-translational modifications of cysteine residues can have distinct effects on protein trafficking (Greaves and Chamberlain 2007) or on receptor coupling to G-proteins and thus in intracellular strength of signaling (Chini and Parenti 2009). Furthermore, it has also been shown that S-nitrosylation and further oxidation of critical cysteine residues can lead to protein misfolding, and that these misfolded proteins can form aggregates in many neurodegenerative diseases (Muchowski and Wacker 2005). Based on these data, we decided to explore the role of a cysteine-rich domain, located at the C-terminal tail of the GPR37, on receptor's trafficking, function and also its relationship with cytotoxicity.

Materials and methods

Plasmid constructs

The cDNA encoding the human GPR37 (Unigene ID: Hs.725956; Source BioScience, Nottingham, U.K.) was amplified and subcloned into the HindIII/EcoRI restriction sites of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) using the iProof High-Fidelity DNA polymerase (Bio-Rad, Hercules, CA, USA) and the following primers: FGPR37 (5′-CGCAAGCTTATGCGAGCCCCGG-3′) and RGPR37 (5′-CGCGAATTCTCAGCAATGAGTTCCG-3′). The same strategy was followed for generating the C-terminal mutants $GPR37^{\Delta563}$ and $GPR37^{\Delta571}$, and by using the same FGPR37 primer and the following reverse primers: RΔ563 (5′-CCGAATTCTCACT CCATGAAGGCCCGAC-3′) and RΔ571 (5′-CCGAATTCTCATT CCTCACAGCAACAGCAGC-3′), respectively. For generating the C-terminal mutant $GPR37^{\Delta563-568}$ we used the QuikChange II site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands) according to the manufacturer's instructions with the following primers: FΔ563-568 (5′-TCGGGCCTTCATGGAGGAG GAATGCATTCAGA-3′) and RΔ563-568 (5′-TCTGAATGCATT CCTCCTCCATGAAGGCCCGA-3′).

Antibodies

A rabbit anti-GPR37 polyclonal antibody was raised by immunizing rabbits with a glutathione S-transferase-fusion protein containing amino acids 27-265 of GPR37 (glutathione S-transferase-GPR37) and following the protocol described previously (Ciruela *et al.* 2004). The antibody was affinity purified and used at 1–2 μg/mL. Other primary antibodies used were rabbit anti-α-actinin polyclonal antibody (0.2 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-CREB2/ATF4 polyclonal antibody (Santa Cruz), goat anti-GRP78 polyclonal antibody (Santa Cruz) and mouse anti-α-tubulin monoclonal antibody (Sigma, St Louis, MO, USA). The secondary antibodies used were: horseradish-peroxidase (HRP)-conjugated goat anti-rabbit IgG (1/30000), HRP-conjugated rabbit anti-goat IgG (1/30000), HRP-conjugated goat anti-mouse IgG (1/10000) (Thermo Fisher Scientific, Inc., Rockford, IL, USA) and AlexaFluor488-conjugated goat anti-rabbit IgG (Invitrogen).

Cell culture, transfection and membrane preparation

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 5%

(v/v) fetal bovine serum at 37°C and in an atmosphere of 5% CO_2. HEK293 cells growing in 20 cm^2 dishes or in 9 cm^2 wells (containing if necessary 18 mm coverslips) were transiently transfected with DNA encoding for the proteins specified in each case using TransFectin™ Lipid Reagent (Bio-Rad) and following the instructions provided by the manufacturer. The cells were harvested 48 h after transfection. Membrane suspensions from transfected HEK293 cells were obtained as described previously (Burgueno *et al.* 2003, 2004).

Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE) was performed using 7.5 or 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes using a semi-dry transfer system (Bio-Rad) and immunoblotted with the indicated antibody and then HRP-conjugated correspondent secondary antibody. The immunoreactive bands were developed using a chemiluminescent detection kit (Thermo Fisher Scientific) (Ciruela and McIlhinney 1997).

Immunocytochemistry and cell viability assay

Transiently transfected HEK293 cells growing on 18 mm diameter glass coverslips were fixed in 1% paraformaldehyde for 15 min, and washed with phosphate-buffered saline (PBS) containing 20 mM glycine (buffer A) to quench the remaining free aldehyde groups. When necessary, cells were permeabilized with buffer A containing 0.2% Triton X-100 for 5 min. Blocking was performed using buffer A containing 1% bovine serum albumin (buffer B). Cells were labeled for 1 h at 22°C with the rabbit anti-GPR37 polyclonal antibody (2 μg/mL), washed for 30 min in buffer B and stained with the AlexaFluor488-conjugated goat anti-rabbit (1 : 2000) for 1 h. In an optional final step, nuclei were labeled with a 100 μg/mL 4′,6-diamidino-2-phenylindole solution (Invitrogen) for 15 min. Coverslips were rinsed for 30 min in buffer B, mounted with Vectashield immunofluorescence medium (Vector Laboratories, Orton Southgate, Peterborough, UK) and examined using a confocal microscope (Lujan and Ciruela 2001). To test antibody specificity, we omitted or replaced with buffer B the primary antibody. Under these conditions, no selective labeling was observed.

The cell viability was determined by means of vital staining. In brief, transiently transfected cells growing on 18-mm diameter glass coverslips were washed with PBS and stained with the Fixable Viability Dye eFluor® 780 (eBioscience, San Diego, CA, USA) during 30 min at 4°C, according to the manufacturer's instructions. Subsequently, cells were fixed and processed for immunocytochemistry as indicated above. Finally, cells were imaged at 780 and 520 nm upon excitation at 647 and 490 nm, respectively (Zeiss, Oberkochen, Germany).

Biotinylation of cell surface proteins

Cell surface proteins were biotinylated as described previously (Ciruela *et al.* 1999, 2000). Briefly, HEK293 cells transiently transfected with each GPR37 construct were washed three times in borate buffer (10 mM H_3BO_3, pH 8.8; 150 mM NaCl) and incubated with 50 μg/mL Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) in borate buffer for 5 min at 22°C. Cells were washed three times in borate buffer and again incubated with 50 μg/mL Sulfo-NHS-LC-Biotin in borate buffer for 10 min at 22°C, and 13 mM NH_4Cl was added for 5 min to quench the remaining biotin. Cells were washed in PBS, disrupted with three 10 s strokes in a polytron and centrifuged at 16 000 *g* for 30 min. The pellet was solubilized in an ice-cold RIPA buffer (50 mM Tris–HCl, 1% Triton X-100, 0.2% SDS, 100 mM NaCl, 1 mM EDTA, 0.5% Sodium Deoxycholate) for 30 min and centrifuged at 16 000 *g* for 20 min. The supernatant was incubated with 80 μL streptavidin-agarose beads (Sigma-Aldrich) for 1 h with constant rotation at 4°C. The beads were washed three times with ice-cold lysis buffer and aspirated to dryness with a 28-gauge needle. Subsequently, 50 μL of SDS–PAGE sample buffer (8 M Urea, 2% SDS, 100 mM Dithiothreitol, 375 mM Tris, pH 6.8) were added to each sample. Proteins were dissociated by heating to 37°C for 2 h and resolved by SDS-polyacrylamide gel electrophoresis in 10% gels and immunoblotted as described above.

Luciferase reporter gene assay

We used a dual luciferase reporter assay to indirectly detect variations of Ca^{2+} levels in transiently transfected cell lines treated with different concentrations of compound. 24 h before transfection, cells were seeded at a density of 1×10^6 cells/well in 6-well dishes and transiently transfected. Cells were co-transfected with plasmids corresponding to three constructs as follows (per 6-well): 2 μg firefly luciferase-encoding experimental plasmid (pGL4-NFAT-RE/luc2p; Promega, Stockholm, Sweden), 2 μg of GPR37 expression vector and 50 ng *Renilla* luciferase-encoding internal control plasmid (phRG-B; Promega). Approximately 36 h post-transfection, cells were challenged with increasing concentrations of HA neuropeptide (Pyr-PPGGSKVILF-NH_2; Genscript Corporation, Piscataway, NJ, USA) during 17 h. Subsequently, cells were harvested with passive lysis buffer (Promega) and the luciferase activity of cell extracts was determined using a luciferase assay system according to the manufacturer's protocol in a POLARstar Optima plate-reader (BMG LABTECH GmbH, Ortenberg, Germany) using a 30-nm bandwidth excitation filter at 530 nm. Firefly luciferase was measured as firefly luciferase luminescence over a 15-s reaction period. The luciferase values were normalized against *Renilla* luciferase luminescence values.

cAMP determinations

The Promega cAMP-Glo™ assay was used to measure cAMP concentration in HEK293 cells. Transiently transfected cells were seeded overnight at 25,000 cells per well in a white 96-well plate treated with poly-D-lysine. Cells were washed once with non-supplemented DMEM and then incubated with non-supplemented DMEM in the presence of a phosphodiesterase inhibitor, zardaverine (50 μM). Cells were stimulated with forskolin (1 μM) and varying concentrations of HA neuropeptide for 15 min at 37 °C. Cells were lysed for 30 min and cAMP levels were determined following the manufacturer's guidelines with a POLARstar Optima plate reader (BMG LABTECH GmbH).

ER stress assessment and caspase 3 assay

Transiently transfected cells grown in 60 mm plates were incubated in the presence or the absence of 1 μM thapsigargin (Sigma) in non-suplemented DMEM for 6 h. Cells were harvested, washed once in PBS, solubilized using cold RIPA buffer for 30 min and centrifuged at 16 000 *g* for 30 min. Supernatants were collected and their

concentrations were determined using a BCA protein assay kit (Thermo). Thirty micrograms of each sample were processed with SDS–PAGE sample buffer for SDS–PAGE analysis.

Caspase 3 activity was determined using a fluorogenic substrate (Caspase 3 Substrate VII; Merck KGaA, Darmstadt, Germany). Transiently transfected cells were incubated for 2 h in non-supplemented DMEM, harvested and washed once with PBS. Cells were then solubilized for 1 h in 100 μL of 1× Caspase 3 Assay Buffer (20 mM HEPES–NaOH, pH7.2, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% CHAPS, 10% Sucrose, 1× Protein Inhibitor) on ice and centrifuged at 16 000 *g* for 15 min at 4°C. After the supernatants were isolated and their protein concentration quantified, 25 μg of protein from the samples were loaded to the wells of a black 96-well plate, reaching a final volume of 50 μL with water if necessary. Subsequently, all the wells were incubated for 6 h at 37°C with 50 μL of 50 μM Caspase 3 Substrate VII in 2× Caspase 3 Assay Buffer, 20 mM Dithiothreitol. Fluorescence was read in a POLARstar Optima plate-reader (BMG LABTECH GmbH) exciting at 360 nm and reading at 530 nm.

Statistics

The number of samples (*n*) in each experimental condition is indicated in the figure legends. When two experimental conditions were compared, statistical analysis was performed using an unpaired *t*-test. Otherwise, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* test. Statistical significance was set as $p < 0.05$.

Results

GPR37 cell surface expression is controlled by its C-terminal cysteine-rich domain

First, given the potential effect of cysteine residues on different aspects of receptor's biology, such as cell surface expression, we aimed to study the role of the C-terminal tail of GPR37 in plasma membrane targeting (Fig. 1a and b). Interestingly, apart from other occasional residues, a region with six-consecutive cysteine residues was found (amino acids 563-568). Therefore, we generated several GPR37 deleted mutants, one missing only the cysteine-rich domain (GPR37$^{\Delta 563\text{-}568}$ construct), and also other two deleting almost the complete tail, with (GPR37$^{\Delta 571}$) or without (GPR37$^{\Delta 563}$) the cysteine-rich region (Fig. 1c). Subsequently, we studied receptor plasma membrane trafficking by means of two independent techniques: immunocytochemistry experiments and cell surface biotinylation assays. Interestingly, it could be observed that while in non-permeabilized cells GPR37 immunostaining showed a limited cell surface expression (Fig. 1c, −Triton X-100) in permeabilized cells a large amount of GPR37 was detected in intracellular compartments (Fig. 1c, +Triton X-100). A similar result was obtained when we deleted the last 40 amino-acids of the GPR37 C-terminal tail but the cysteine-rich domain was still present in the receptor (GPR37$^{\Delta 571}$ construct) (Fig. 1c). However, when we deleted the last 48

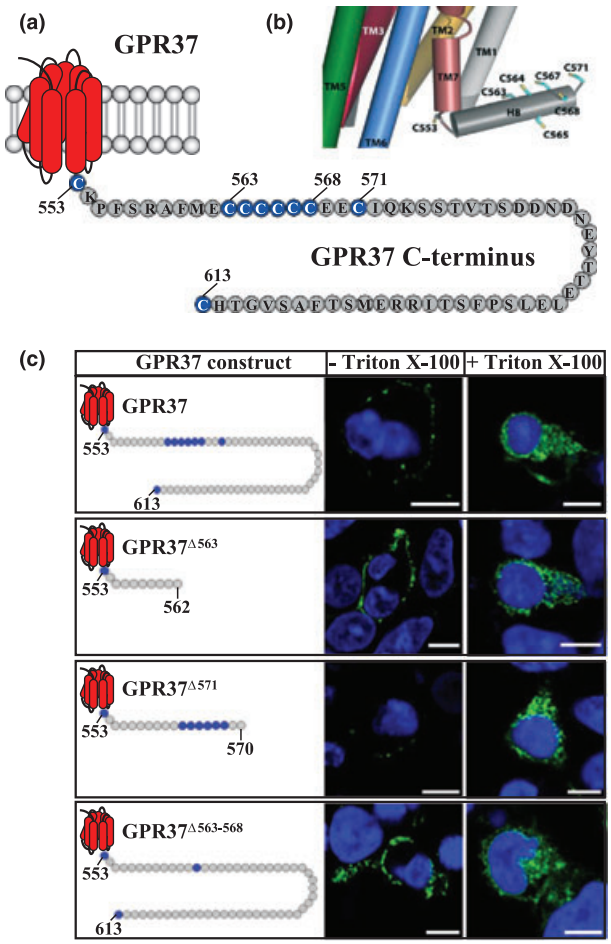

Fig. 1 GPR37 and its C-terminal tail mutants. (a) Schematic representation of the C-terminal tail of the GPR37. The cysteine residues within GPR37 C-terminus are highlighted in blue and denoted by the residue number. (b) Molecular model of the intracellular part of GPR37 (UniProt accession no. O15354), constructed from the crystal structure of the adenosine A_{2A} receptor (PDB accession no. 2YDO) [Lebon *et al.* (2011)]. The model shows that Cys553 is within the linker connecting TM7 and H8, whereas the amino acids comprised between Cys563 and Cys571 are part of H8. The other amino acids of the C-tail, from Ile572 to Cys613, were not modeled. (c) Immunocytochemical detection of GPR37 constructs. HEK293 cells grown on glass coverslips were transiently transfected with cDNA encoding GPR37, GPR37$^{\Delta 563}$, GPR37$^{\Delta 571}$ and GPR37$^{\Delta 563\text{-}568}$ and processed for immunocytochemistry using a rabbit anti-GPR37 antibody (2 μg/mL). The bound primary antibody was detected by means of an AlexaFluor488-conjugated goat anti-rabbit (1 : 2000). Nuclei were stained with DAPI. Finally, cells were analyzed by confocal microscopy to detect GPR37 (green) and nuclei (blue). The scale bar represents 10 μm.

amino-acids of the GPR37 C-terminal tail, thus losing the cysteine-rich domain (i.e. GPR37$^{\Delta 563}$ construct), the receptor showed a robust plasma membrane transport compared with the full-length GPR37 (Fig. 1c, −Triton X-100). In addition, the deletion of only the six cysteine residues from the GPR37 C-terminal tail (GPR37$^{\Delta 563\text{-}568}$ construct) also promoted

receptor cell surface expression (Fig. 1c, −Triton X-100), thus suggesting that the presence of this cysteine-rich domain within the C-terminal tail of the receptor precluded its plasma membrane trafficking.

Next, to better test the hypothesis that the GPR37 cysteine-rich domain played a critical role on receptor's plasma membrane expression we performed cell surface biotinylation assays. We isolated GPR37 present in the plasma membrane using a membrane impermeant biotin ester, followed by streptavidin–agarose affinity purification of membrane proteins. As shown in Fig. 2, the proportional amount of GPR37 constructs missing the cysteine-rich domain (i.e. GPR37$^{\Delta 563}$ and GPR37$^{\Delta 563\text{-}568}$), which was present at the cell surface of transfected cells, was approximately twice that of the full-length GPR37 (Fig. 2b). However, under the same experimental conditions when the cysteine-rich domain containing GPR37$^{\Delta 571}$ construct was tested, a marked reduction of cell surface expression was observed (Fig. 2b). Importantly, the assay was validated by detecting α-actinin, a cytoskeletal marker, which could not be detected in the streptavidin isolates, indicating that the biotin ester did not penetrate the cell membrane (Fig. 2a, lower panel). Overall, these results, which are in concordance with our immunocytochemistry experiments, supported the hypothesis that the GPR37 cysteine-rich domain participates in the receptor's plasma membrane trafficking.

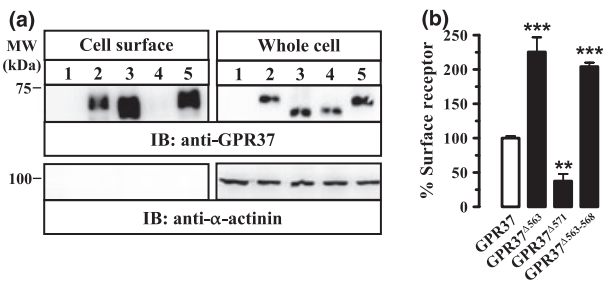

Fig. 2 Cell surface immunodetection of GPR37 constructs. (a) HEK293 cells were transiently transfected with pcDNA3 (lane 1), GPR37 (lane 2), GPR37$^{\Delta 563}$ (lane 3), GPR37$^{\Delta 571}$ (lane 4) and GPR37$^{\Delta 563\text{-}568}$ (lane 5). Labeling of cell surface proteins was performed as described in Material and methods. Accordingly, crude cell extracts (whole cell) and biotinylated proteins (cell surface) were analyzed by SDS–PAGE and immunoblotted using a rabbit anti-GPR37 antibody (1 μg/mL) or a rabbit anti-actinin antibody (0.2 μg/mL) as a control to rule out the labeling of cytoplasmic proteins. Immunoreactive bands were detected as described in Materials and methods. (b) Quantification of cell surface expression of GPR37 constructs. Ratios between cell surface and whole cell receptor expression for each GPR37 construct were calculated and normalized assigning the 100% to the wild-type GPR37 construct. Data are expressed as the mean ± SD of three independent experiments. Asterisk indicates data significantly different from the wild-type GPR37 construct: $^{**}p < 0.01$ and $^{***}p < 0.001$ by ANOVA with Student–Newman–Keuls multiple comparison *post hoc* test.

Functional consequences of GPR37 cysteine-rich domain deletion

Once determined the role of the GPR37 cysteine-rich domain in receptor's plasma membrane trafficking, we aimed to test whether the HA neuropeptide was in our hands able to activate GPR37 and if the deletion of the GPR37 cysteine-rich domain affected receptor functionality. Noteworthy, for the subsequent experiments only the GPR37$^{\Delta 563\text{-}568}$ mutant was used, because cell surface expression did not differ from that obtained with the mutant lacking the complete C-terminal tail. Furthermore, it was the less-truncated form of the receptor and thus it would potentially affect in lesser extent GPR37 functioning. Firstly, we measured the ability of the HA neuropeptide to induce receptor internalization, a phenomenon that has been previously shown (Rezgaoui *et al.* 2006). To this end, transiently transfected HEK293 cells with GPR37 or GPR37$^{\Delta 563\text{-}568}$ were challenged with the HA neuropeptide and the receptor distribution analyzed by immunocytochemistry both in permeabilized and non-permeabilized cells (Fig. 3). Interestingly, HA treatment induced a clustering of both GPR37 and GPR37$^{\Delta 563\text{-}568}$ at the plasma membrane, as shown by its pronounced punctuated distribution in non-permeabilized cells (Fig. 3, −Triton X-100). In addition, HA treatment induced a translocation of both receptor constructs into the cytoplasm, as shown by the increased presence of intracellular aggregates in permeabilized cells (Fig. 3, +Triton X-100). Overall, these results demonstrated that the receptor's cysteine-rich domain did not affect the HA-mediated GPR37 cell surface clustering and internalization. Next, we analyzed receptor functionality by measuring GPR37-mediated changes in intracellular calcium after HA stimulation. To this end, transiently transfected cells

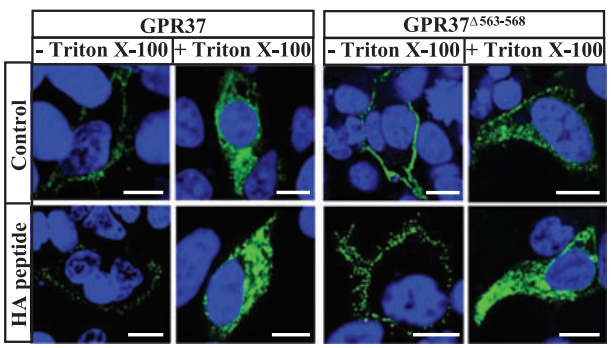

Fig. 3 HA-mediated GPR37 internalization. HEK293 cells grown on glass coverslips were transiently transfected with cDNA encoding GPR37 and GPR37$^{\Delta 563\text{-}568}$, treated with vehicle (control) or with 1 μM HA (HA peptide) for 60 min and processed for immunocytochemistry using a rabbit anti-GPR37 antibody (2 μg/mL). The bound primary antibody was detected by means of an AlexaFluor488-conjugated goat anti-rabbit (1 : 2000). Nuclei were stained with DAPI. Finally, cells were analyzed by confocal microscopy to detect GPR37 (green) and nuclei (blue). The scale bar represents 10 μm.

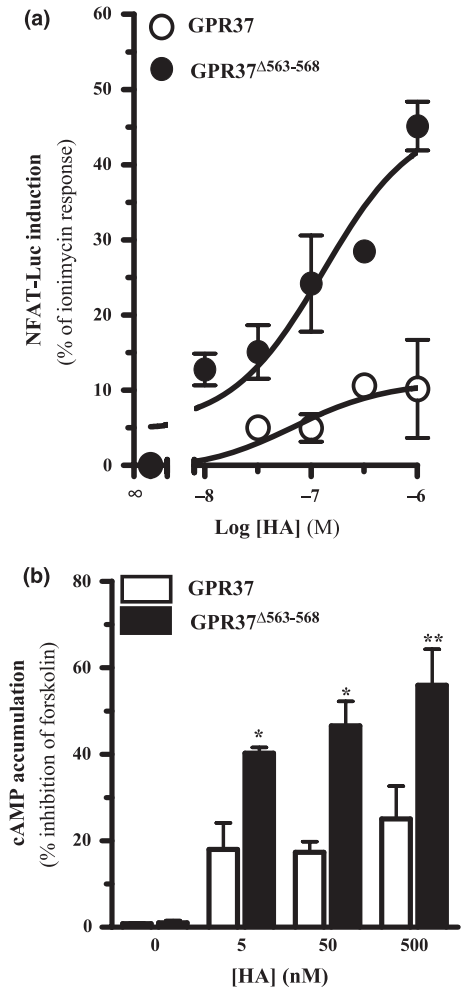

Fig. 4 Functionality of GPR37 constructs. (a) Receptor-dependent changes in cytoplasmic calcium levels were measured using a luminescence-based method. A dose-response curve was obtained for HEK293 cells transfected with GPR37 or GPR37$^{\Delta 563\text{-}568}$ using the neuropeptide head-activator (HA) as an agonist. The results were represented as a percentage of the cellular response produced by 1 μM ionomycin. Data are expressed as the mean ± SEM of three independent experiments. (b) Receptor-mediated inhibition of adenylyl cyclase was assessed for GPR37 and GPR37$^{\Delta 563\text{-}568}$. Transfected cells with each form of the receptor were challenged with 1 μM forskolin and different concentrations of the neuropeptide HA. Subsequently, cAMP concentration was determined using the Promega cAMP-Glo™assay. The decrease in cAMP production is represented as a percentage of the response elicited by forskolin alone. Data are expressed as the mean ± SD of three independent experiments. Asterisk indicates data significantly different from the wild-type GPR37 construct: *$p < 0.05$ and **$p < 0.01$ by ANOVA with Student–Newman–Keuls multiple comparison *post hoc* test.

with GPR37 or GPR37$^{\Delta 563\text{-}568}$ plus the reporter vector pGL4-NFAT-RE/luc2p were stimulated with increasing concentrations of the HA neuropeptide (Fig. 4a). Interestingly, a careful analysis of GPR37 and GPR37$^{\Delta 563\text{-}568}$ dose-response curves for HA showed that while the EC_{50} remained within the same range (69 ± 25 nM and 129 ± 54 nM, respectively) the maximum response was significantly different (11 ± 3% and 46 ± 5%, respectively) (Fig. 4a). It is important to mention here that control mock-transfected cells showed no intracellular Ca^{2+} accumulations upon HA challenge (data not shown). Overall, these results indicated that the deletion of the GPR37 cysteine-rich domain promoted receptor-mediated intracellular calcium accumulation. Finally, we also evaluated the effects of the HA neuropeptide on another signal transduction pathway, namely the generation of cAMP by means of the adenylyl cyclase (AC) enzyme. Thus, we tested whether HA challenge induced GPR37-mediated AC inhibition in cells transiently transfected with GPR37 upon incubation with forskolin and increasing concentrations of HA neuropeptide (Fig. 4b). Interestingly, as it happened with the GPR37-mediated intracellular calcium accumulation, the deletion of the GPR37 cysteine-rich domain promoted receptor-mediated AC inhibition (Fig. 4b). In addition, under the same experimental conditions the HA neuropeptide was unable to inhibit forskolin-mediated AC activation in control mock-transfected cells (data not shown). Collectively, the obtained data indicated that the deletion of the GPR37 cysteine-rich domain promoted receptor signal transduction, a phenomenon that might be consequence of either the enhanced GPR37 cell surface expression or the better G-protein coupling.

Role of the cysteine-rich domain in GPR37-induced ER stress

It has been demonstrated that intracellular accumulation of GPR37 in dopaminergic neurons leads to endoplasmic reticulum (ER) stress (Imai *et al.* 2001; Kitao *et al.* 2007). Therefore, as the deletion of the GPR37 cysteine-rich domain facilitated receptor plasma membrane trafficking, we aimed to test the role of this domain in GPR37-induced ER stress. To this end, we analyzed the effect of GPR37 on the expression of the ER-resident folding assistant GRP78. The GRP78 chaperone is a master regulator for ER stress (Lee 2005) and in fact it has been shown to be up-regulated upon ER stress (Yang *et al.* 2003). Accordingly, we transiently transfected HEK293 cells with GPR37 and GPR37$^{\Delta 563\text{-}568}$, and the expression of GRP78 was assessed by immunoblotting. As expected, GRP78 expression was enhanced after treatment with the sarcoendoplasmic Ca^{2+}-ATPases inhibitor, thapsigargin (Fig. 5a, lane 2) (Chen *et al.* 2000). Also, cells transfected with GPR37 showed a significant increment of GRP78 (110 ± 1.7%, $p < 0.05$) when compared with mock transfected cells, thus suggesting that GPR37 expression promoted GRP78 up-regulation. However, cells transfected with GPR37$^{\Delta 563\text{-}568}$ did not show an increased GRP78 expression, which was indeed significantly different from that observed in GPR37 transfected cells (Fig. 5a). Overall, these results

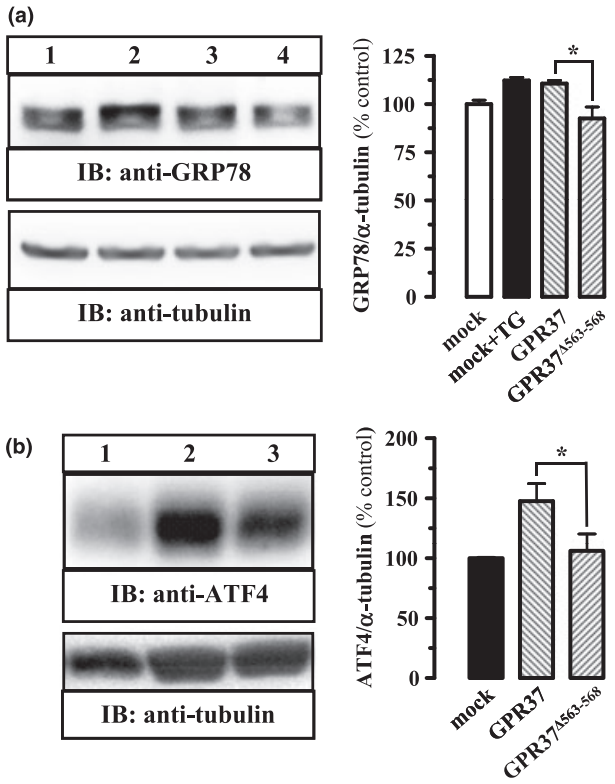

Fig. 5 GPR37 expression and ER stress. (a) Effect of GPR37 expression on GRP78. HEK293 cells were transiently transfected with an empty vector (mock, lane 1), GPR37 (lane 3) and GPR37$^{\Delta 563\text{-}568}$ (lane 4). Mock transfected cells were treated with 1 μM thapsigargin (TG) during 6 h as a control of GRP78 induced expression (lane 2). Cell lysates were obtained, analyzed by SDS–PAGE and immunoblotted using a goat anti-GRP78 antibody (1 μg/mL) or a mouse anti-α-tubulin (0.2 μg/mL) to control protein loading in the gel. (b) Effect of GPR37 expression on ATF4. HEK293 cells were transiently transfected with an empty vector (mock, lane 1), GPR37 (lane 2) and GPR37$^{\Delta 563\text{-}568}$ (lane 3) and incubated with 1 μM thapsigargin for 6 h. Cell lysates were obtained, analyzed by SDS-PAGE and immunoblotted using a rabbit anti-ATF4 antibody (1 μg/mL) or a mouse anti-α-tubulin (0.2 μg/mL) to control protein loading in the gel. Immunoreactive bands were detected as described in *Materials and methods* section. Ratios between GRP78 and ATF4 and α-tubulin were calculated and normalized, assigning the 100% to the mock (right panels). Data are expressed as the mean ± SD of three independent experiments. Asterisk indicates the significant difference of compared data: *$p < 0.05$ by ANOVA with Student–Newman–Keuls multiple comparison *post hoc* test.

supported the hypothesis that the GPR37 cysteine-rich domain was involved in the receptor-mediated induction of GRP78 expression.

Upon ER stress, GRP78 is released by ER transmembrane signal transducers and leads the activation of UPR signaling pathways (Lee 2005). An unfolded protein response (UPR) downstream effector is the activating transcription factor 4 (ATF4), whose translation is increased upon ER stress (Harding *et al.* 2000). Accordingly, we aimed to test whether ATF4 expression was altered by GPR37. Therefore, we transiently transfected HEK293 cells with GPR37 and the expression of ATF4 was assessed by immunoblotting. GPR37-transfected cells showed a significant increment of ATF4 expression (147 ± 14%, $p < 0.05$) when compared with mock-transfected cells (Fig. 5b), thus suggesting that GPR37 expression promoted ATF4 up-regulation. Conversely, under the same experimental conditions, cells transfected with GPR37$^{\Delta 563\text{-}568}$ did not show a significant increase of ATF4, and its expression was significantly different from that observed in GPR37 transfected cells (Fig. 5b). These results supported the notion that the GPR37 cysteine-rich domain participated in receptor-mediated induction of ATF4 expression. Collectively, our data indicated that GPR37 expression induced GRP78 up-regulation and UPR signaling activation, a phenomenon precluded when the GPR37 cysteine-rich domain was absent.

Involvement of the cysteine-rich domain in GPR37-mediated cytotoxicity

Finally, given that GPR37 expression induced GRP78 expression and that it regulates ER stress-signaling pathways (i.e. ATF4 expression) leading to UPR survival and apoptosis responses (Szegezdi *et al.* 2006), we aimed to study the involvement of the cysteine-rich domain in receptor-mediated cytotoxicity. To this end, we initially assessed the effect of GPR37 on cell viability with a fixable vital dye in our immunocytochemistry experiments to detect cellular damage (Fig. 6a). Importantly, we were able to monitor positive cells for both markers (i.e. cellular death and receptor expression) and to quantify the extent of GPR37-positive cell death (Fig. 6b). Interestingly, we observed that the expression of both GPR37 or GPR37$^{\Delta 563\text{-}568}$ led to significantly different levels of cell survival (19.2 ± 4.3% and 7.4 ± 2.4%, respectively) thus suggesting that GPR37-mediated cell damage was dependent on the cysteine-rich domain (Fig. 6b).

In addition to the cell viability test, we also studied specific effects in the apoptotic pathway determining caspase 3 activity upon GPR37 or GPR37$^{\Delta 563\text{-}568}$ expression. To this end, a fluorogenic substrate of caspase 3 was used to determine the activity of this cysteine-aspartic acid protease in lysates of cells transfected with each receptor form. The resulting fluorescence was normalized to a control sample from cells transfected with an empty vector. Interestingly, we observed that, as in the cell viability experiment, cells transfected with the wild-type GPR37 significantly showed more caspase 3 activity when compared with cells expressing GPR37$^{\Delta 563\text{-}568}$ (1.7 ± 0.2-folds and 1.3 ± 0.03-folds, respectively), thus indicating that GPR37-mediated apoptosis is somehow dependent on the cysteine-rich domain (Fig. 6c). Collectively, these results demonstrated the important role of the GPR37 cysteine-rich domain in mediating the cytotoxic effects associated with receptor expression.

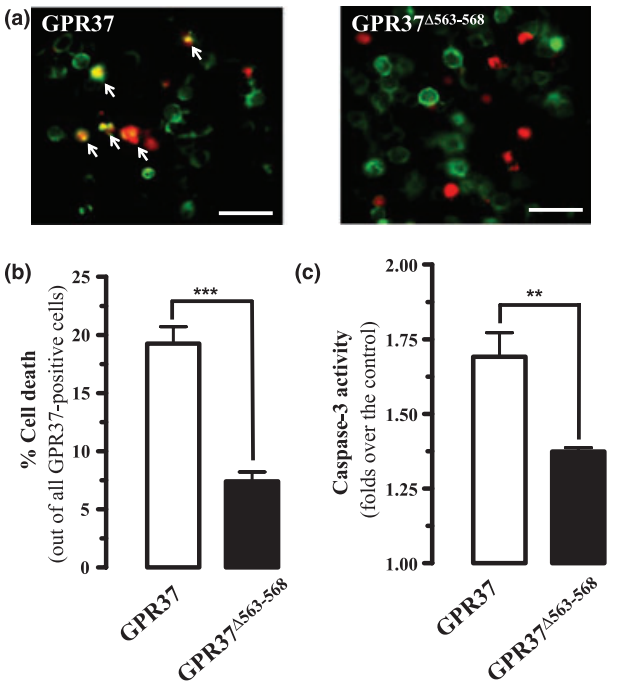

Fig. 6 Effect of GPR37 expression on cell survival. (a) Microscope images show cells expressing GPR37 or GPR37$^{\Delta 563\text{-}568}$ (green) and cells that have entered the death process (red). Cells that are positive for both conditions are highlighted with an arrow. The scale bar represents 50 μm. (b) Under these conditions, the subpopulation of dead GPR37-expressing cells were quantified for each construct and represented as a percentage of all transfected cells. Data are expressed as the mean ± SEM of three independent experiments. Asterisk indicates data significantly different from the wild-type GPR37 construct: ***$p < 0.001$ by ANOVA with Student–Newman–Keuls multiple comparison *post hoc* test. (c) The activity of caspase 3 was also determined from cells transfected with either GPR37 or GPR37$^{\Delta 563\text{-}568}$. Cell lysates were obtained and equal amounts of protein from each sample were incubated in the presence of a fluorogenic substrate for caspase 3. The fluorescence generated was measured and normalized to lysates from cells transfected with an empty vector. Data are expressed as the mean ± SD of three independent experiments. Asterisk indicates data significantly different from the wild-type GPR37 construct: **$p < 0.01$ by ANOVA with Student–Newman–Keuls multiple comparison *post hoc* test.

Discussion

Neurodegenerative diseases are characterized by the progressive loss of neurons, a phenomenon largely associated to ER and oxidative stress (Forman *et al.* 2003). Thus, a large body of studies has shown that ER stress-induced apoptosis is implicated in numerous human diseases, including diabetes and neurogenerative diseases (Szegezdi *et al.* 2006). The build up of unfolded/misfolded proteins activates adaptative responses in cells, the UPR, that protect them from the toxic rise of these proteins (Rao and Bredesen 2004). However, a gradual overtime accumulation of unfolded/misfolded proteins, together with an UPR failure, will finally promote not only cellular stress responses related to the ER but also the induction of specific death pathways (i.e. apoptosis) to remove the stressed cells (Kaufman 2002; Rao and Bredesen 2004). In such way, it has been described that the intracellular accumulation of GPR37 leads to ER stress, which ultimately triggers the activation of apoptotic pathways (e.g. activation of caspase 3) both in neurons and in stable cell lines (Rao and Bredesen 2004). Accordingly, a role of GPR37 aggregates in PD has been suggested(Kitao *et al.* 2007; Omura *et al.* 2006). It would then seem likely that the aggregation of GPR37 due to ineffective receptor folding would favor the presence of ubiquitinated protein deposits in the neuronal cytoplasm (i.e. Lewy bodies), thus prompting the loss of dopaminergic neurons from the substantia nigra pars compacta that occurs in PD (Murakami *et al.* 2004). However, despite this possible role of GPR37 in the pathology – i.e. mediating cell death upon receptor aggregation – very little is known about this orphan receptor and the molecular determinants driving its intracellular accumulation. Hence, in the present study, we focused on a cysteine-rich domain located at the C-terminal tail of the receptor as a possible cornerstone explaining the expression, function, ER-induced stress and cytotoxicity of the receptor.

First of all, we performed several mutations at the C-terminal tail of the receptor by deleting different cysteine residues-containing fragments and started examining whether they affected the cell surface expression of the receptor. As previously shown (Imai *et al.* 2001; Murakami *et al.* 2004), a large amount of the wild-type receptor was found in intracellular compartments. However, when deleting the cysteine-rich domain located between the residues 563 and 568, a robust increase in plasma membrane expression was detected (in both GPR37$^{\Delta 563}$ and GPR37$^{\Delta 563\text{-}568}$). These results pointed out to a crucial role of this domain on receptor trafficking, although they diverge from those previously reported where the deletion of the whole GPR37 C-terminal tail prevented cell surface expression (Cookson 2005). Yet, the works differ in several experimental approaches, for instance the number of amino acid residues deleted, the technique used to determine the surface expression and other general methodologies (e.g. transfecting agent, vector where the constructs were subcloned, etc.). Nevertheless, we decided to next assess whether the mutations affected receptor's internalization and functionality, because it would seem likely that a change on signal transduction pathways should be observed due to the effect of the cysteine-rich domain on receptor expression at the cell surface. Interestingly, when the receptor was challenged with the HA neuropeptide it could be observed that the deletion of the GPR37 cysteine-rich domain produced both an increase in Ca^{2+} mobilization and in inhibition of cAMP accumulation. However, the receptor's cysteine-rich domain did not affect the HA-mediated GPR37 cell surface clustering and internalization. These results are consistent with those proposing that

the HA neuropeptide is an agonist of GPR37 that activates and induces GPR37 internalization in a pertussis toxin sensitive manner (Dunham *et al.* 2009; Imai *et al.* 2001; Rezgaoui *et al.* 2006). Overall, the functional experiments were in line with the immunocytochemistry, internalization and cell surface biotinylation assays, indicating that the studied cysteine-rich domain was important to retain the receptor at the cytoplasm, and as a consequence it was also responsible of regulating its membrane G-protein coupled signaling.

Next, based on the relevance of this cysteine-rich domain on receptor's trafficking and function, and because of the relationship of cytoplasmatic GPR37 aggregates with low survival rates in transfected cells (Dunham *et al.* 2009), we investigated whether this cysteine-rich region could constitute a molecular determinant of GPR37-mediated ER stress and cytotoxicity upon receptor expression. To this end, we first studied GPR37-mediated UPR triggering in cells transfected with the wild-type and the mutated receptor in which the cysteine-rich domain was deleted. Interestingly, the deletion of the GPR37 cysteine-rich domain efficiently reduced the receptor-mediated GRP78 up-regulation and UPR signaling activation, thus pointing out to a prominent role of this GPR37 domain in mediating ER stress. Also, the role of the GPR37 cysteine-rich domain on cell viability and also on caspase 3 pathway activation was analyzed. Interestingly, we effectively found that GPR37 over-expression in living cells induced cell death, as described previously (Rezgaoui *et al.* 2006). It is important to mention that dying cells expressing GPR37 showed features of both necrosis and apoptosis, which would suggest the concurrence of different pathways leading to the same physiological state. In addition, we also demonstrated that this GPR37-mediated cytotoxicity was significantly reduced when the cysteine-rich domain was removed. This is a particularly important finding because it revealed for the first time the importance of this GPR37 discrete amino acid sequence in the biology of this rather unknown receptor. Thus, the GPR37 cysteine-rich domain not only participated in the control of receptor plasma membrane trafficking and the concomitant receptor-mediated signal transduction, but it also was involved in ER stress and the cytotoxic effects associated to receptor expression. However, it remains to be determined what signals might control the subcellular distribution and function of GPR37 through modification of its cysteine-rich domain.

The ectopic expression of GPR37 may end in the formation of toxic receptor aggregates and ER stress, as mentioned above. In such situation, transfected cells trigger the protective UPR in an attempt to contain the deleterious effects of GPR37 intracellular accumulation. The UPR constitutes a concerted cellular response mediated by three ER transmembrane receptors: protein kinase R-like ER kinase, inositol-requiring enzyme 1, and ATF6. Under normal conditions, all three ER stress receptors are kept inactive through their direct interaction with the ER chaperone, GRP78. However, when an unfolded/misfolded protein accumulates (i.e. GPR37), GRP78 dissociates from the three receptors, leading to their activation and triggering the activation of UPR signaling pathways (Rezgaoui *et al.* 2006). Interestingly, UPR is a pro-survival response intended to reduce the accumulation of unfolded proteins from the ER (Kitao *et al.* 2007; Omura *et al.* 2006). However, under persistent protein aggregation and prolonged ER stress, as it could happen upon GPR37 over-expression, the UPR will activate unique pathways switching the pro-survival response to a pro-apoptotic effect (Szegezdi *et al.* 2006). These pathways implicate the increased translation of ATF4 (Schroder and Kaufman 2005) and the concomitant induction of the transcription factor C/EBP homologous protein, which play a key role in switching UPR from pro-survival to pro-death signaling (Schroder and Kaufman 2005). Indeed, in our hands the ectopic expression of GPR37 in HEK293 cells induced ATF4 up-regulation, a phenomenon that was abolished when the GPR37 cysteine-rich domain was deleted. As a result, it could be postulated that the cysteine-rich region within the C-terminal tail of the GPR37 constitutes a receptor folding sensor, and thus either the absence or the blockade of this check point would facilitate receptor folding, trafficking and abolish its toxic accumulation. Interestingly, it has been reported that GPR37 is able to interact with other GPCRs, for instance the dopamine D_2 receptor (D_2R), and that this interaction facilitates GPR37 cell surface expression (Harding *et al.* 2000). Indeed, GPCR heterodimerization often modulates receptor trafficking (Schroder and Kaufman 2005), thus it might be feasible that the negative effect of GPR37 cysteine-rich domain not only on receptor plasma membrane expression and signaling but also on mediating cytotoxic effects could be precluded by interacting (i.e. heterodimerize) with appropriate receptor partners (i.e. D_2R). Overall, GPR37 heteromerization should be also contemplated as a way to attain proper receptor cell surface expression and function, and to diminish GPR37-mediated cytotoxicity.

In conclusion, the results obtained here point out to the following possible mechanistic scenario: while newly synthesized GPR37 is folded in the ER before being targeted to the plasma membrane, the misfolded GPR37 translocates across the ER membrane into the cytoplasm and is degraded through a parkin-dependent ubiquitin-proteasome pathway. However, when a dysregulation of this cellular control system occurs the aggregation of misfolded GPR37 takes place, triggering pro-death pathways, a process in which the cysteine-rich domain located at the C-terminal tail of the receptor might play a pivotal role. Interestingly, our results might be important to understand the pathogenesis associated to the accumulation of this expression-dependent misfolding receptor and ultimately to comprehend its relationship with PD.

Acknowledgements

This work was supported by grants SAF2011-24779 and Consolider-Ingenio CSD2008-00005 from Ministerio de Ciencia e Innovación and ICREA Academia-2010 from the Catalan Institution for Research and Advanced Studies to FC. Also, JG, VF-D and FC belong to the "Neuropharmacology and Pain" accredited research group (Generalitat de Catalunya, 2009 SGR 232). We want to thank Dr Cristina Muñoz-Pinedo (Cell Death Regulation group, IDIBELL) for kindly providing the anti-GRP78 and anti-ATF4. We also thank Esther Castaño, Eva Julià, and Benjamín Torrejón, from the Scientific and Technical Services (SCT)-Bellvitge Campus of the University of Barcelona for the technical assistance.

Conflict of interest

The authors declare no conflict of interest.

References

Bodenmuller H., Schaller H. C. and Darai G. (1980) Human hypothalamus and intestine contain a hydra-neuropeptide. *Neurosci. Lett.* **16**, 71–74.

Burgueno J., Enrich C., Canela E. I., Mallol J., Lluis C., Franco R. and Ciruela F. (2003) Metabotropic glutamate type 1alpha receptor localizes in low-density caveolin-rich plasma membrane fractions. *J. Neurochem.* **86**, 785–791.

Burgueno J., Canela E. I., Mallol J., Lluis C., Franco R. and Ciruela F. (2004) Mutual regulation between metabotropic glutamate type 1alpha receptor and caveolin proteins: from traffick to constitutive activity. *Exp. Cell Res.* **300**, 23–34.

Chen L. Y., Chiang A. S., Hung J. J., Hung H. I. and Lai Y. K. (2000) Thapsigargin-induced grp78 expression is mediated by the increase of cytosolic free calcium in 9L rat brain tumor cells. *J. Cell. Biochem.* **78**, 404–416.

Chini B. and Parenti M. (2009) G-protein-coupled receptors, cholesterol and palmitoylation: facts about fats. *J. Mol. Endocrinol.* **42**, 371–379.

Ciruela F. and McIlhinney R. A. (1997) Differential internalisation of mGluR1 splice variants in response to agonist and phorbol esters in permanently transfected BHK cells. *FEBS Lett.* **418**, 83–86.

Ciruela F., Soloviev M. M. and McIlhinney R. A. (1999) Cell surface expression of the metabotropic glutamate receptor type 1alpha is regulated by the C-terminal tail. *FEBS Lett.* **448**, 91–94.

Ciruela F., Soloviev M. M., Chan W. Y. and McIlhinney R. A. (2000) Homer-1c/Vesl-1L modulates the cell surface targeting of metabotropic glutamate receptor type 1alpha: evidence for an anchoring function. *Mol. Cell. Neurosci.* **15**, 36–50.

Ciruela F., Burgueno J., Casado V. *et al.* (2004) Combining mass spectrometry and pull-down techniques for the study of receptor heteromerization. Direct epitope–epitope electrostatic interactions between adenosine A2A and dopamine D2 receptors. *Anal. Chem.* **76**, 5354–5363.

Cookson M. R. (2005) The biochemistry of Parkinson's disease. *Annu. Rev. Biochem.* **74**, 29–52.

Dev K. K., van der Putten H., Sommer B. and Rovelli G. (2003) Part I: Parkin-associated proteins and Parkinson's disease. *Neuropharmacology* **45**, 1–13.

Donohue P. J., Shapira H., Mantey S. A., Hampton L. L., Jensen R. T. and Battey J. F. (1998) A human gene encodes a putative G protein-coupled receptor highly expressed in the central nervous system. *Brain Res. Mol. Brain Res.* **54**, 152–160.

Dunham J. H., Meyer R. C., Garcia E. L. and Hall R. A. (2009) GPR37 surface expression enhancement via N-terminal truncation or protein-protein interactions. *Biochemistry* **48**, 10286–10297.

Dusonchet J., Bensadoun J. C., Schneider B. L. and Aebischer P. (2009) Targeted overexpression of the parkin substrate Pael-R in the nigrostriatal system of adult rats to model Parkinson's disease. *Neurobiol. Dis.* **35**, 32–41.

Forman M. S., Lee V. M. and Trojanowski J. Q. (2003) 'Unfolding' pathways in neurodegenerative disease. *Trends Neurosci.* **26**, 407–410.

Greaves J. and Chamberlain L. H. (2007) Palmitoylation-dependent protein sorting. *J. Cell Biol.* **176**, 249–254.

Harding H. P., Novoa I., Zhang Y., Zeng H., Wek R., Schapira M. and Ron D. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* **6**, 1099–1108.

Imai Y., Soda M., Inoue H., Hattori N., Mizuno Y. and Takahashi R. (2001) An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* **105**, 891–902.

Kaufman R. J. (2002) Orchestrating the unfolded protein response in health and disease. *J. Clin. Invest.* **110**, 1389–1398.

Kitao Y., Imai Y., Ozawa K., *et al.* (2007) Pael receptor induces death of dopaminergic neurons in the substantia nigra via endoplasmic reticulum stress and dopamine toxicity, which is enhanced under condition of parkin inactivation. *Hum. Mol. Genet.* **16**, 50–60.

Lebon G., Warne T., Edwards P. C., Bennett K., Langmead C. J., Leslie A. G. and Tate C. G. (2011) Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. *Nature* **474**, 521–525.

Lee A. S. (2005) The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. *Methods* **35**, 373–381.

Leng N., Gu G., Simerly R. B. and Spindel E. R. (1999) Molecular cloning and characterization of two putative G protein-coupled receptors which are highly expressed in the central nervous system. *Brain Res. Mol. Brain Res.* **69**, 73–83.

Low K. and Aebischer P. (2012) Use of viral vectors to create animal models for Parkinson's disease. *Neurobiol. Dis.* **48**, 189–201.

Lujan R. and Ciruela F. (2001) Immunocytochemical localization of metabotropic glutamate receptor type 1 alpha and tubulin in rat brain. *NeuroReport* **12**, 1285–1291.

Marazziti D., Golini E., Gallo A., Lombardi M. S., Matteoni R. and Tocchini-Valentini G. P. (1997) Cloning of GPR37, a gene located on chromosome 7 encoding a putative G-protein-coupled peptide receptor, from a human frontal brain EST library. *Genomics* **45**, 68–77.

Marazziti D., Gallo A., Golini E., Matteoni R. and Tocchini-Valentini G. P. (1998) Molecular cloning and chromosomal localization of the mouse Gpr37 gene encoding an orphan G-protein-coupled peptide receptor expressed in brain and testis. *Genomics* **53**, 315–324.

Muchowski P. J. and Wacker J. L. (2005) Modulation of neurodegeneration by molecular chaperones. *Nat. Rev. Neurosci.* **6**, 11–22.

Murakami T., Shoji M., Imai Y., Inoue H., Kawarabayashi T., Matsubara E., Harigaya Y., Sasaki A., Takahashi R. and Abe K. (2004) Pael-R is accumulated in Lewy bodies of Parkinson's disease. *Ann. Neurol.* **55**, 439–442.

Omura T., Kaneko M., Okuma Y., *et al.* (2006) A ubiquitin ligase HRD1 promotes the degradation of Pael receptor, a substrate of Parkin. *J. Neurochem.* **99**, 1456–1469.

Rao R. V. and Bredesen D. E. (2004) Misfolded proteins, endoplasmic reticulum stress and neurodegeneration. *Curr. Opin. Cell Biol.* **16**, 653–662.

Rezgaoui M., Susens U., Ignatov A., Gelderblom M., Glassmeier G., Franke I., Urny J., Imai Y., Takahashi R. and Schaller H. C. (2006) The neuropeptide head activator is a high-affinity ligand for the orphan G-protein-coupled receptor GPR37. *J. Cell Sci.* **119**, 542–549.

Schaller H. C., Schilling E., Theilmann L., Bodenmuller H. and Sachsenheimer W. (1988) Elevated levels of head activator in human brain tumors and in serum of patients with brain and other neurally derived tumors. *J. Neurooncol.* **6**, 251–258.

Schroder M. and Kaufman R. J. (2005) The mammalian unfolded protein response. *Annu. Rev. Biochem.* **74**, 739–789.

Shimura H., Hattori N., Kubo S., *et al.* (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.* **25**, 302–305.

Sriram S. R., Li X., Ko H. S., Chung K. K., Wong E., Lim K. L., Dawson V. L. and Dawson T. M. (2005) Familial-associated mutations differentially disrupt the solubility, localization, binding and ubiquitination properties of parkin. *Hum. Mol. Genet.* **14**, 2571–2586.

Szegezdi E., Logue S. E., Gorman A. M. and Samali A. (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep.* **7**, 880–885.

Takahashi R. and Imai Y. (2003) Pael receptor, endoplasmic reticulum stress, and Parkinson's disease. *J. Neurol.* **250**(Suppl 3), III25–III29.

Valdenaire O., Giller T., Breu V., Ardati A., Schweizer A. and Richards J. G. (1998) A new family of orphan G protein-coupled receptors predominantly expressed in the brain. *FEBS Lett.* **424**, 193–196.

Yang Y., Nishimura I., Imai Y., Takahashi R. and Lu B. (2003) Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in Drosophila. *Neuron* **37**, 911–924.

Zeng Z., Su K., Kyaw H. and Li Y. (1997) A novel endothelin receptor type-B-like gene enriched in the brain. *Biochem. Biophys. Res. Commun.* **233**, 559–567.

Zhang Y., Gao J., Chung K. K., Huang H., Dawson V. L. and Dawson T. M. (2000) Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc. Natl. Acad. Sci. USA* **97**, 13354–13359.