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Identification of GABA_C Receptor Protein Homeostasis Network Components from Three Tandem Mass Spectrometry Proteomics Approaches

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Abstract

Gamma-amino butyric acid type C (GABA_C) receptors inhibit neuronal firing primarily in retina. Maintenance of GABA_C receptor protein homeostasis in cells is essential for its function. However, a systematic study of GABA_C receptor protein homeostasis (proteostasis) network components is absent. Here, co-immunoprecipitation of human GABA_C-ρ1 receptor complexes was performed in HEK293 cells overexpressing ρ1 receptors. To enhance the coverage and reliability of identified proteins, immunoisolated ρ1 receptor complexes were subjected to three tandem mass spectrometry (MS)-based proteomic analyses: namely, gel-based tandem MS (GeLC-MS/MS), solution-based tandem MS (SoLC-MS/MS), and multidimensional protein identification technology (MudPIT). From the 107 identified proteins, we assembled GABA_C-ρ1 receptor proteostasis network components, including proteins with protein folding, degradation, and trafficking functions. We studied representative individual ρ1 receptor interacting proteins, including calnexin, a lectin chaperone that facilitates glycoprotein folding, and LMAN1, a glycoprotein trafficking receptor, and global effectors that regulate protein folding in cells based on bioinformatics analysis, including HSF1, a master regulator of the heat shock response, and XBP1, a key transcription factor of the unfolded protein response. Manipulating selected GABA_C receptor proteostasis network components is a promising strategy to regulate GABA_C receptor folding, trafficking, degradation and thus function to ameliorate related retinal diseases.

Keywords

GABA_C receptor; tandem mass spectrometry; interactome; proteostasis; folding; trafficking; degradation; heat shock response; unfolded protein response

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four supplemental tables.

INTRODUCTION

Normal organismal physiology depends on the maintenance of protein homeostasis (proteostasis) in each cellular compartment^{1, 2}, which dictates a delicate balance between protein synthesis, folding, trafficking, and degradation, while minimizing misfolding and aggregation²⁻⁸. For a specific client protein, its interaction with a network of proteins in the crowded cellular environment is critical to maintain its proper folding, trafficking, degradation, and thus function.

GABA is the primary inhibitory neurotransmitter in the mammalian central nervous system and retina. Three types of GABA receptors are reported: GABA_A, GABA_B, and GABA_C receptors. GABA_A and GABA_C receptors belong to the Cys-loop superfamily of ligand-gated ion channels, whereas GABA_B receptors belong to G protein coupled receptor family⁹. GABA_C receptors, also known as GABA- ρ receptors, are expressed mainly in the retina, although they have been identified in a variety of central nervous systems^{10, 11}. GABA_C receptors are pentameric, sharing common structural characteristics with other Cys-loop receptor members (Figure 1A, left)¹². To date, three isoforms of GABA_C- ρ subunits ($\rho 1$, $\rho 2$ and $\rho 3$) have been identified in human. The ρ subunits can form homopentameric GABA_C receptors. Most studies focused on homopentameric $\rho 1$ receptors by using HEK293 cells or *Xenopus oocytes* as the expression system. HEK293 cell lines are extensively used for the expression of ion channels, including GABA_C receptors, because of low endogenous ion channel expression level, high transfection efficiency, and good physiological functioning of the expressed ion-channels^{10, 13}. Each $\rho 1$ subunit has four transmembrane helices (TM1-TM4, with TM2 domain lining the interior of the pore), a large 260-residue extracellular (or the endoplasmic reticulum (ER) luminal) N-terminus after the cleavage of the 21-residue signal peptide, and an extracellular (or the ER luminal) C-terminus (Figure 1A, right). Two GABA-binding sites lie between two adjacent subunits and are located in the N-terminal extracellular domains. GABA binding to GABA_C receptors triggers a large conformational change, opens the ion pore to conduct chloride, hyperpolarizes the plasma membrane, and inhibits neuronal firing. Knockout studies in mice demonstrated that elimination of $\rho 1$ subunits led to abnormal visual processing in the mouse retina¹⁴ and resulted in changes in vascular permeability similar to the symptoms in retinal hypoxic conditions¹⁵.

To function, GABA_C receptors need to fold into their native structures and assemble correctly on the ER membrane and traffic efficiently to the plasma membrane. Maintenance of a delicate balance between GABA_C receptor folding, trafficking and degradation through specific protein sensing and interactions is critical for its function. However, to date, the identification of proteostasis network components that regulate GABA_C receptor folding, trafficking and degradation was not explored. Here, we identified proteins that interact with GABA_C receptors using human HEK293 cells overexpressing GABA- $\rho 1$ receptors by immuno-affinity purification tandem mass-spectrometry (MS) proteomics analysis. To enhance the coverage and reliability of the identified proteins, immunoisolated $\rho 1$ receptor complexes were subjected to three tandem MS-based proteomics analyses: namely, gel-based tandem MS (GeLC-MS/MS), solution-based tandem MS (SoLC-MS/MS), and multidimensional protein identification technology (MudPIT). Furthermore, from the identified $\rho 1$ receptor interactome proteins, we focused on assembling proteostasis network components that could potentially regulate GABA_C receptor folding, trafficking, and degradation. Manipulation of these protein candidates is a promising strategy to regulate GABA_C receptor proteostasis and thus function.

EXPERIMENTAL PROCEDURES

Plasmids

The pCMV6 plasmid containing C-terminal FLAG-tagged human gamma-aminobutyric acid receptor $\rho 1$ subunit (pCMV6-FLAG- $\rho 1$) and pCMV6 Entry Vector plasmid (pCMV6-EV) were obtained from Origene. The human GABA $_C$ - $\rho 1$ subunit missense mutations (R89Q or V353D) were constructed using QuickChange II site-directed mutagenesis Kit (Agilent Genomics), and the cDNA sequences were confirmed by DNA sequencing. The pcDNA3.1 plasmid containing N-terminal FLAG-tagged human HSF1 cDNA (pcDNA3.1-FLAG-HSF1) was obtained from Addgene. The pcDNA3.1 plasmid containing a spliced form of XBP1 (pcDNA3.1-XBP1(s)) was a gift from Dr. Richard Sifers (Baylor College of Medicine).

Cell Culture and Transfection

HEK293 cells (ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 1% Pen-Strep (Hyclone) at 37°C in 5% CO $_2$. Monolayers were passaged upon reaching confluency with TrypLE Express (Invitrogen). HEK293 cells were grown in 6-well plates or 10-cm dishes and allowed to reach ~70% confluency before transient transfection using Fugene 6 (Roche) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Antibodies

The mouse monoclonal anti-FLAG M2 and anti- β -actin antibodies were obtained from Sigma. The rabbit polyclonal anti-matrin 3 antibodies was obtained from Bethyl Laboratories. The rabbit polyclonal anti-calnexin antibody was obtained from Enzo Life Sciences. The rabbit polyclonal anti-Derlin1, anti-DNAJA1 and anti-LMAN1 antibodies, and rabbit monoclonal anti-HSF1 antibody were obtained from Abgent. The rabbit monoclonal anti-BAG-2 and anti-UGGT1 antibodies were obtained from Epitomics. The rabbit polyclonal anti-XBP1 antibody was obtained from Santa Cruz Biotechnology. The rabbit polyclonal anti-C1QBP antibody was obtained from Pierce Antibodies.

Immunoprecipitation and Western Blot

Following 48 hours post-transfection, cells were harvested with TrypLE Express and then lysed with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 1% Triton X-100) supplemented with Roche complete protease inhibitor cocktail. Lysates were cleared by centrifugation (15,000 $\times$ g, 10 min, 4°C). Protein concentration was determined by MicroBCA assay (Pierce). To isolate the FLAG- $\rho 1$ subunit complex, the total proteins (1 mg) were pre-cleared with 30 μ L of mouse IgG-agarose beads for 1 hr at 4°C to remove nonspecific binding proteins and incubated with 30 μ L of anti-FLAG M2 magnetic beads (Sigma-Aldrich) for 1 hr at room temperature. The beads were collected using a magnet separator and washed three times with lysis buffer. The FLAG- $\rho 1$ subunit complex was eluted by either incubation with 30 μ L of SDS loading buffer in the presence of DTT or 30 μ L of 0.1 M glycine (pH 3.0). The immunopurified eluents or equal amounts of total proteins were separated in 8% SDS-PAGE gel, and Western blot analysis was performed using appropriate antibodies.

Confocal Immunofluorescence

To label cell surface GABA $_C$ receptor FLAG- $\rho 1$ subunits, HEK293 cells were incubated on ice in 100 μ L of HEPES buffer (HEPES 25 mM, NaCl 140 mM, KCl 5.4 mM, CaCl $_2$ 1.8 mM, glucose 15 mM, pH=7.4) containing mouse anti-FLAG M2 antibody (1:100) for 1 hr, and then incubated on ice with 1 mL of buffer containing an Alexa 546-conjugated goat

anti-mouse antibody (1:400) for 1 hr. To label total GABA_C receptor FLAG- ρ 1 subunits, HEK293 cells were then fixed with 4% paraformaldehyde, permeabilized with saponin (0.2%), blocked with goat serum for 0.5 hr at room temperature, labeled with the anti-FLAG antibody (1:100) for 1 hr, and then incubated at room temperature with 1 ml of buffer containing an Alexa 488-conjugated goat anti-mouse antibody (1:400) for 1 hr. For confocal immunofluorescence microscopy, an Olympus IX-81 Fluoview FV1000 confocal laser scanning system was used. A 60 $\times$ objective was used to collect images using FV10-ASW software.

In-gel Digestion

6 mg of total proteins was immunoprecipitated using anti-FLAG antibody, separated by SDS-PAGE, and Coomassie stained to visualize protein gel bands. The gel was washed in distilled water to remove excess background stain. The whole gel was cut into six pieces containing prominent bands from empty vector (EV) control and GABA- ρ 1 receptor overexpression samples, respectively, and destained with 500 μ L of 1:1 ACN (acetonitrile) and 100 mM ABC (ammonium bicarbonate) solution for 2–8 hr. Afterwards, 10 mM reductive TCEP (tris(2-carboxyethyl)phosphine) was added, and free cysteines were alkylated with 55 mM IAA (iodoacetamide). ACN and 100 mM ABC were used to dehydrate and rehydrate the gel pieces, respectively, three times. Gel pieces were swelled in 50 mM ABC containing freshly prepared 10 ng/ μ L trypsin (Promega, sequencing grade) and digested overnight. Peptides were extracted with 60% ACN/5% FA (formic acid) and dried in SpeedVac.

In-solution Digestion

6 mg of total proteins was immunoprecipitated using anti-FLAG antibody, and bound proteins were eluted by 0.1 M glycine (pH 3.0). Proteins from each sample were precipitated using 25% trichloroacetic acid (v/v) and the protein pellet washed using ice-cold acetone. The pellet was air-dried and suspended with 8 M urea in 100 mM Tris-HCl, pH 8.5. Afterwards, 5 mM reductive TCEP was added, and free cysteines were alkylated with 10 mM IAA. The samples were subsequently diluted to 2 M urea with 100 mM Tris-HCl, pH 8.5, and digested by adding trypsin and incubating overnight at 37°C. The digestion reaction was quenched by adding formic acid to 5% (v/v), and peptides were cleaned up using a C18 spin column (Thermo Pierce, Rockford, IL).

1D Ultra-high Pressure Reverse Phase Liquid Chromatography (UPLC) and Tandem Mass Spectrometry Analysis

For both gel-based tandem MS analysis (GeLC-MS/MS) and solution-based tandem MS analysis (SoLC-MS/MS), digested peptides were reconstituted with 0.1% formic acid and analyzed by LC-MS/MS. Separation of peptides via capillary liquid chromatography was performed using Waters nanoAquity system (Waters Corp., Milford, MA). Mobile phase A (aqueous) contained 0.1% formic acid in 5% acetonitrile, and mobile phase B (organic) contained 0.1% formic acid in 85% acetonitrile. Separation was achieved using a C18 column (75 μ m $\times$ 20 cm, Waters Corp., Ethylene Bridged Hybrid column BEH300) through a 150 min gradient of 6% to 45% mobile phase B at a flow rate of 0.30 μ L/min. Mass spectrometry analysis was performed using a hybrid linear ion trap Orbitrap Velos mass spectrometer (LTQ-Orbitrap Velos, Thermo, Waltham, MA). Survey scan was operated at 60,000 resolution, followed by eight standard CID (collision induced dissociation) fragmentations in a data-dependent manner. Dynamic exclusion was enabled as the following: repeat count, 2; repeat duration, 30 sec; exclusion list size, 250; exclusion duration, 120 sec. Both GeLC-MS/MS and SoLC-MS/MS were done as a single experiment at Case Western Reserve University to make a fair comparison to a single-run MudPIT.

Multidimensional Protein Identification Technology (MudPIT)^{16, 17}

Peptide mixture was pressure-loaded onto a 250- μ m i.d. fused silica capillary column packed with a 2.5-cm Partisphere strong cation exchanger (Whatman, Clifton, NJ) and a 2.5-cm of 5- μ m Aqua C18 material (Phenomenex, Ventura, CA). The column was washed for 30 min with buffer containing 95% water, 5% acetonitrile (ACN), and 0.1% formic acid. After desalting, it was attached to a 100- μ m i.d. capillary with a 5- μ m pulled tip packed with a 12-cm of 5- μ m Aqua C18 material, and the entire column was placed in line with an Agilent 1100 quaternary HPLC (Agilent, Palo Alto, CA). The sample was analyzed using a fully automated 6-step separation procedure. The buffer solutions used for the chromatography were 5% ACN/0.1% FA (buffer A), 80% ACN/0.1% FA (buffer B), and 500 mM ammonium acetate/0.1% FA (buffer C). The first step consisted of a 100 min gradient from 0 to 100% buffer B. Steps 2–5 had the following profile: 3 min of 100% buffer A, 3 min of X% buffer C, a 10 min gradient from 0 to 15% buffer B, and a 97-min gradient from 15 to 55% buffer B. The 3-min buffer C percentages (X) were 20, 40, 60, and 90%. In the final step, the gradient contained: 3 min of 100% buffer A, 10 min of 100% buffer C, a 10-min gradient from 0 to 15% buffer B, and a 107-min gradient from 15 to 100% buffer B. As peptides were eluted from the microcapillary column, they were electrosprayed directly into a linear LTQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). A cycle of one full scan mass spectrum (400–1400 m/z) was followed by 5 data-dependent CID MS/MS scans. Dynamic exclusion was enabled as the following: repeat count, 1; repeat duration, 20 sec; exclusion list size, 300; exclusion duration, 90 sec. MudPIT was done as a single experiment at the Scripps Research Institute.

Database Search

Acquired tandem mass spectra were searched against the European Bioinformatics Institute International Protein Index human protein database (version 3.71, released on Mar. 24th, 2010), containing 92,139 protein entries. A decoy database containing the reversed sequences of all the proteins was appended to estimate false discovery rate¹⁸. Protein identification using Sequest¹⁹ or ProLuCID²⁰ and DTASelect^{21, 22} were done through the Integrated Proteomics Pipeline (IP2, Integrated Proteomics Applications, Inc. San Diego, CA). The precursor mass accuracy was limited to 15 ppm for spectra acquired on the Orbitrap instrument and 1 Da on the LTQ instrument; product ions mass accuracy was set at 0.6 Da. Fully tryptic enzyme specificity and up to two missed cleavages were allowed. Static modifications included carbamidomethylation on cysteines (57 Da), and variable modifications included oxidation on methionines (16 Da). Isotopic C¹³ incorporated ions were automatically included. DTASelect^{21, 22} was applied to generate search results of peptide-to-spectra matches (PSMs) with a max false discovery rate (FDR) of 5%, yielding a protein FDR of less than 1% with at least two peptides per protein being assigned. Although accession numbers for protein groups, which share common peptides, are provided, the best annotated from a group, along with its corresponding gene name, is used as an index in this study for clarity. Additionally, listed in Supplemental Table S1 for each protein are protein coverage; unique peptide counts, which merge peptides with the same sequence but multiple precursor charge states; and spectral counts. Furthermore, all assigned peptides are listed in Supplemental Table S2, including precursor charge and mass error, XCorr-, Δ CN- and DTASelect confidence-scores, and spectral counts.

Criteria for the p1 receptor interactome and false discovery rate (FDR) analysis

The p1 receptor interactome was identified using the spectral counting method^{17, 19, 23–27} with arbitrary yet strict criteria to remove potential false positives for p1 receptor interacting proteins. We comprehensively considered all three distinct tandem MS methods and used two levels of criteria. For level one criteria, if a protein showed up in two out of three

methods, the following was applied: a) if a protein was exclusive in $\rho 1$ receptor overexpression sample, spectra count (SC) ≥ 4 was required; b) if a protein was detected in both EV control and $\rho 1$ receptor overexpression sample, SC ≥ 10 in $\rho 1$ receptor overexpression sample and SC ratio ($\rho 1$ /EV) ≥ 2.5 were required. For level two criteria, if a protein showed up only in one in-solution digestion MS method (either SoLC-MS/MS or MudPIT), more strict criteria were applied: c) if a protein was exclusive in $\rho 1$ receptor overexpression sample, SC ≥ 8 and protein sequence coverage $\geq 10\%$ were required; d) if a protein was detected in both EV control and $\rho 1$ receptor overexpression sample, SC ≥ 20 in $\rho 1$ receptor overexpression sample, SC ratio ($\rho 1$ /EV) ≥ 2.5 , and protein sequence coverage $\geq 10\%$ were required.

To evaluate the FDR of the $\rho 1$ receptor interactome, using the same criteria for the identification of proteins that were enriched in the $\rho 1$ overexpression sample after immunoprecipitation, we applied the following filter to identify proteins that are enriched in EV control samples after immunoprecipitation. We comprehensively considered all three distinct tandem MS methods and used two levels of criteria. For level one criteria, if a protein showed up in two out of three methods, the following was applied: a) if a protein was exclusive in EV control sample, spectra count (SC) ≥ 4 was required; b) if a protein was detected in both EV control and $\rho 1$ receptor overexpression sample, SC ≥ 10 in EV control and SC ratio (EV/ $\rho 1$) ≥ 2.5 were required. For level two criteria, if a protein showed up only in one in-solution digestion MS method (either SoLC-MS/MS or MudPIT), more strict criteria were applied: c) if a protein was exclusive in EV control sample, SC ≥ 8 and protein sequence coverage $\geq 10\%$ were required; d) if a protein was detected in both EV control and $\rho 1$ receptor overexpression sample, SC ≥ 20 in EV control sample, SC ratio (EV/ $\rho 1$) ≥ 2.5 , and protein sequence coverage $\geq 10\%$ were required. We define: FDR ($\rho 1$ receptor interactome) = Number of proteins that are enriched in EV control sample / Number of proteins that are enriched in $\rho 1$ receptor overexpression sample after immunoprecipitation.

Protein abundance analysis of the $\rho 1$ receptor interactome

The human protein abundance information was obtained from PaxDb: Protein Abundance Across Organisms (<http://paxdb.org>). For our analysis, we used the integrated database, which has a 90% coverage of the human proteome, downloaded on 08/15/2013.

Ingenuity IPA Analysis

Cellular location, molecular and cellular function, and enriched transcription factors of GABA- $\rho 1$ receptor interacting proteins were analyzed using Ingenuity IPA software.

siRNA Transfection

HEK293 cells were seeded at approximately 2.5×10^5 cells per well in 6-well plates for the siRNA treatment. Cells were allowed to reach $\sim 70\%$ confluency before transfection. The small interfering RNA (siRNA) duplexes were obtained from Dharmacon: calnexin (J-003636-07-0005), LMAN1 (J-012122-05-0005), C1QBP (J-011225-13-0005) and Non-Targeting siRNA (D-001810-01-20) as negative control. Cells were transfected with 50 nM siRNA using HiPerfect Transfection Reagent (Qiagen) according to the manufacturer's transfection protocol prior to protein analyses. Following 48 hours post-transfection, cells were harvested, lysed, and subjected to SDS-PAGE and Western blot analysis.

Statistical analysis

All data are presented as mean $\pm$ SEM, and any statistical significance was calculated using two-tailed Student's t-Test.

RESULTS AND DISCUSSION

Mass Spectrometry identification of affinity-purified GABA- ρ 1 receptor interacting proteins

HEK293 cells were transiently transfected with a FLAG- ρ 1 plasmid for 48 hr before analysis. A clean double band at ~60 kD represented ρ 1 receptors, whereas no band was detected using the negative control empty vector (EV) plasmid for transfection according to Western blot analysis (Figure 1B). The double bands represent different glycosylation states of ρ 1 subunits: the top band is a post-ER mature glycoform, whereas the bottom band is an immature ER glycoform of the ρ 1 subunits (see below, Figure 4). Cell surface ρ 1 receptors were visualized using anti-FLAG antibody without a detergent permeabilization step (Figure 1C, second column, red), and total ρ 1 receptors were visualized after a detergent permeabilization step (Figure 1C, third column, green) using confocal immunofluorescence microscopy analysis. No cell surface or total ρ 1 receptors were detected for the EV control; both cell surface and total ρ 1 receptors were clearly detected using a FLAG- ρ 1 plasmid for transfection (Figure 1C), indicating that ρ 1 subunits were distributed both on the plasma membrane and inside the cell.

We employed a MS-based proteomics approach to identify ρ 1 receptor interacting proteins. GABA- ρ 1 receptor complex was enriched by co-immunoprecipitation using anti-FLAG antibody in HEK293 cells overexpressing FLAG- ρ 1 receptors. A number of bands were clearly visible in Coomassie-stained gel (Figure 1D). To enhance the coverage and reliability of the identified proteins, immunisolated ρ 1 receptor complexes were subjected to three distinct tandem MS-based proteomic analyses: namely, GeLC-MS/MS, SoLC-MS/MS, and MudPIT (Figure 1E). GeLC-MS/MS employs a SDS-PAGE fractionation step prior to sample loading to a column: the advantages are that ρ 1 receptor interacting proteins can be visualized on a Coomassie-stained gel and that proteins within a specific molecular weight range can be identified with high confidence; the disadvantage is that low abundant proteins might be further diluted during sample processing. Both SoLC-MS/MS and MudPIT do not employ a pre-fractionation step before sample loading to a column: the advantage is simpler sample processing and a higher chance to detect low abundant proteins; the disadvantage is that more non-specific proteins might appear in the MS result. Therefore, we combined these three tandem MS methods for our analysis, aiming to achieve the maximum coverage of reliable ρ 1 receptor interacting proteins.

GeLC-MS/MS identified 24 proteins as ρ 1 receptor interacting proteins (Figure 2A), which are summarized in Table 1 (see also supplemental Tables S1 and S2). Representative proteins are shown in Figure 1D, including molecular chaperones in the ER (CANX, HSPA5) and in the cytosol (HSP90AB1, HSPA1A, HSPA8), E3 ubiquitin ligase (EDD1, HECTD3), and cytoskeleton proteins (TUBB3). To identify more ρ 1 receptor interacting proteins, the immunopurified samples were further subjected to SoLC-MS/MS or MudPIT (Figure 1E). The ρ 1 receptor interactome was identified using the spectral counting method^{17, 19, 23–27}. We comprehensively considered all three distinct tandem MS methods with arbitrary yet strict criteria to remove potential false positives for ρ 1 receptor interacting proteins (see Experimental Procedures). From SoLC-MS/MS and MudPIT, 50 and 78 proteins were identified, respectively, to interact with ρ 1 receptors (Figure 2A and Table 1, see also Supplemental Tables S1, S2 and S4). Along with the 24 interactors identified from GeLC-MS/MS, 107 proteins total were identified as interactors and are listed in Table 1 according to their molecular and cellular function (see also supplemental Table S4). Nine proteins were commonly identified by three tandem MS methods, including two cytoskeleton proteins (TUBB and TUBB3), three proteins that are involved in ubiquitin-related degradation pathway (UBR5, SQSTM1 and UBB), one lectin chaperone (CANX), two metabolic proteins (ATP5A1 and SLC25A6), and overexpressed GABRR1 (Figure 2A). The interacting proteins listed in Table 1 were filtered with carefully defined criteria. Nearly

all of them had three or more unique peptides, thus representing strong identification. Nevertheless, an interactor from only one tandem MS method retains higher potential of being a false positive; alternatively, that interaction is transient or unstable.

To evaluate the false discovery rate (FDR) of the $\rho 1$ receptor interactome, using the same criteria for the identification of proteins that were enriched in the $\rho 1$ overexpression sample after immunoprecipitation (see Experimental Procedures), we identified 10 proteins that were enriched in the EV control sample after immunoprecipitation (see Supplemental Table S3 for protein list). Most of the 10 proteins have high abundance numbers (Supplemental Table S3), including two ribosomal proteins. We define: $\text{FDR}(\rho 1 \text{ receptor interactome}) = \text{Number of proteins that are enriched in EV control sample} / \text{Number of proteins that are enriched in } \rho 1 \text{ receptor overexpression sample after immunoprecipitation}$. Therefore, $\text{FDR}(\rho 1 \text{ receptor interactome}) = 10 / 107 \times 100\% = 9.3\%$, which indicates that our selected criteria to identify the $\rho 1$ receptor interactome were reasonably stringent to filter out most of the potential false positives. However, further validation by orthogonal methods is necessary to confirm a *bona fide* interaction between the $\rho 1$ receptor and all our identified interactors.

The three tandem MS methods identified proteins with different properties. We performed a cellular protein abundance analysis of the $\rho 1$ receptor interactome using the database from PaxDb: Protein Abundance Across Organisms (<http://pax-db.org>) (Supplemental Table S4). The proteins that were recognized by different tandem MS methods were plotted against their abundance (Figure 2B). Clearly, MudPIT identified more low abundant proteins compared to SoLC-MS/MS and GeLC-MS/MS; SoLC-MS/MS identified more low abundant proteins and more high abundant proteins than GeLC-MS/MS under our experimental conditions. Among the nine proteins that are commonly identified by three tandem MS methods, seven are abundant proteins, including two cytoskeleton proteins (TUBB and TUBB3), CANX, UBB, SLC25A6, ATP5A1 and overexpressed GABRR1 (Figure 2B).

We analyzed the ability of the three tandem MS methods for the identification of membrane proteins. From the 29 membrane proteins identified (Supplemental Table S4), MudPIT identified 27, SoLC-MS/MS identified 7, and GeLC-MS/MS identified 7 (Figure 2C). Among all three tandem MS methods, the 4 common proteins are fairly abundant: ATP5A1 (abundance: 764), CANX (abundance: 296), overexpressed GABRR1 and SLC25A6 (abundance: 226). Furthermore, MudPIT demonstrated much greater capability of identifying low abundant membrane proteins, such as KCTD2 (abundance: 0.4), NOMO1 (abundance: 2.4), SUN2 (abundance: 5.8), SEC16A (abundance: 6.9) and DERL1 (abundance: 7.2).

GABA- $\rho 1$ receptor interacting proteins are distributed in subcellular locations along with protein biogenesis and trafficking pathway

We analyzed the subcellular distribution of GABA- $\rho 1$ receptor interacting proteins (Figure 2D and Supplemental Table S4). The subcellular distribution information was obtained from Gene Ontology Database, the Human Protein Reference Database (<http://www.hprd.org>) and Pubmed Gene Database (<http://www.ncbi.nlm.nih.gov/gene>). For a protein with more than one cellular location, only the primary location was chosen for our analysis. 59 GABA- $\rho 1$ receptor interacting proteins are in the cytoplasm, 19 in the ER, 2 in the Golgi, 14 in the mitochondria, 9 in the nucleus, and 4 in the plasma membrane. The GABA- $\rho 1$ receptor interacting proteins are distributed ubiquitously in the cell because the $\rho 1$ subunit is synthesized in the ribosome, co-translationally translocated onto the ER membrane for folding and assembly^{28–30}, and trafficked to the Golgi and the plasma membrane for function. Biogenesis of GABA- $\rho 1$ receptors requires their interaction with a network of proteins in the cell. It is noteworthy that 18% of GABA- $\rho 1$ receptor interacting proteins are

in the ER, an organelle for protein folding, because the $\rho 1$ subunit has a very large 260-residue ER luminal N-terminus after the cleavage of the 21-residue signal peptide.

Enrichment of proteostasis network components from GABA- $\rho 1$ receptor interacting proteins

We analyzed the molecular and cellular function from GABA- $\rho 1$ receptor interacting proteins using Ingenuity IPA analysis and Pubmed Gene Database (<http://www.ncbi.nlm.nih.gov/gene>) (Figure 2E and Table 1). We identified 11 proteins that are involved in cellular organization, providing structural support; 8 proteins that are involved in signal transduction, including three 14-3-3 family proteins. It is noteworthy that 53% of $\rho 1$ receptor interacting proteins are involved in protein synthesis, folding, trafficking, and degradation functions, suggesting that the proteostasis network components play a critical role in regulating $\rho 1$ receptor protein level. For example, under the protein folding category, major molecular chaperone systems, including Hsp60, Hsp70, and Hsp90 family members and their co-chaperones, were identified to assist the folding of $\rho 1$ receptors in the cell (Table 1). Since the $\rho 1$ subunit is a glycoprotein, lectin chaperones, including calreticulin and calnexin (CANX), and N-glycoprotein processing enzymes, were identified as responsible for assisting the glycosylation and folding of the $\rho 1$ subunit. Vesicle trafficking components, such as COPA, SEC13, and LMAN1, were identified as assisting in transport of the $\rho 1$ subunit between the ER and the Golgi (see below and Figure 7 for more detailed discussion). Misfolded GABA_C receptors are recognized by the cellular protein quality-control machinery, leading to ER-associated degradation (ERAD), retrotranslocation into the cytosol, and degradation by the proteasome^{5, 31}. Therefore, ERAD factors, such as VCP and derlin-1, were identified.

The interaction between representative $\rho 1$ receptor proteostasis network components and $\rho 1$ receptors was verified using immunoprecipitation and Western blot analysis. Folding components (calnexin, UGGT1 and DNAJA1), trafficking components (LMAN1), and degradation components (BAG2 and derlin-1) were shown to interact with $\rho 1$ receptors (Figure 3A).

We also identified 18 $\rho 1$ receptor interactors that are related to basic metabolism (Table 1 and Supplemental Table S4). Even though similar cases have been reported previously, such as interactors for $\alpha 7$ nicotinic acetylcholine receptors³² or long form AMPA receptors³³, those proteins could be false positives because most of them are abundant cellular proteins; on the other hand, we were intrigued to investigate whether some of our identified interactors could be novel $\rho 1$ receptor proteostasis network components. We chose to study C1QBP (Complement component 1 Q subcomponent-binding protein), a 34-kDa multifunctional protein, for its potential role in regulating $\rho 1$ receptor proteostasis. The interaction between C1QBP and $\rho 1$ receptors was verified using immunoprecipitation and Western blot analysis (Figure 3A). Furthermore, knockdown of C1QBP using siRNA clearly decreased the total FLAG- $\rho 1$ protein level in HEK293 cells expressing FLAG- $\rho 1$ receptors, indicating that C1QBP enhanced $\rho 1$ receptor proteostasis (Figure 3B). Previously, C1QBP (also called gC1q-R) was identified to interact with β subunits, but not $\alpha 1$ or $\gamma 2$ subunits of GABA_A receptors³⁴. Although C1QBP was believed to be localized predominantly in the mitochondria, it was reported to appear also in the plasma membrane and in the ER³⁵. Furthermore, C1QBP was proposed to play a critical role in a chaperone-mediated control of the protein kinase C μ activation³⁶. Therefore, our data implicated that C1QBP might promote the $\rho 1$ receptor folding and trafficking in the ER or stabilize the $\rho 1$ receptor in the plasma membrane. The detailed mechanism of C1QBP in regulating $\rho 1$ receptor proteostasis merits further investigation.

Verification of critical GABA- $\rho 1$ receptor folding and trafficking components

We selected calnexin (CANX) from $\rho 1$ subunit folding components and LMAN1 from $\rho 1$ subunit trafficking components for more detailed study. We knocked down calnexin or LMAN1 using siRNA and monitored its effect on the maturation of the $\rho 1$ subunit using endoglycosidase H (endo H) enzyme digestion and Western blot analysis. HEK293 cells were co-transfected with FLAG- $\rho 1$ plasmids and siRNA against calnexin, LMAN1, or non-targeting (NT) control for 48 hr before being lysed for endo H digestion and Western blot analysis (Figure 4). The endo H enzyme selectively cleaves after asparaginyl-*N*-acetyl-D-glucosamine in the N-linked glycans incorporated on the $\rho 1$ subunit in the ER, but it is not possible to remove this oligosaccharide chain after the high-mannose form is enzymatically remodeled in the Golgi. Therefore, endo H resistant $\rho 1$ subunit bands represent properly folded, post-ER $\rho 1$ subunit glycoforms, which traffic at least to the Golgi compartment. The Peptide-N-Glycosidase F (PNGase F) enzyme cleaves between the innermost GlcNAc and asparagine residues from N-linked glycoproteins, serving as a control for unglycosylated $\rho 1$ subunits. PNGase F treatment reduced the double bands for $\rho 1$ subunits, which represents different $\rho 1$ subunit glycosylation states, to one single band (Figure 4A, lane 2). Knockdown of calnexin clearly decreased upper endo H resistant post-ER $\rho 1$ glycoform (Figure 4A, cf. lane 4 to lane 1), indicating that calnexin facilitates the folding of the $\rho 1$ subunit in the ER. Knockdown of LMAN1 clearly decreased upper endo H resistant mature $\rho 1$ glycoform (Figure 4B, cf. lane 4 to lane 1), indicating that LMAN1 plays a critical role in transporting $\rho 1$ receptors from the ER to the Golgi.

We further confirmed calnexin's role in assisting the folding of the $\rho 1$ subunit in the ER by overexpressing calnexin. HEK293 cells were co-transfected with FLAG- $\rho 1$ plasmids and a calnexin plasmid or a GFP negative control plasmid for 48 hr before being lysed for endo H digestion and Western blot analysis. Clearly, overexpression of calnexin is sufficient to increase the endo H resistant post-ER $\rho 1$ glycoform (Figure 4C, cf. lane 4 to lane 2). Endo H digestion did not decrease the intensity of the upper band for the $\rho 1$ subunit (Figure 4C, cf. lane 2 to lane 1 and lane 4 to lane 3), indicating that the upper band of the $\rho 1$ subunit double bands represents mature post-ER $\rho 1$ subunit glycoform.

The interaction between calnexin and the $\rho 1$ subunit correlates with the maturation level of wild type and mutant $\rho 1$ receptors

Because calnexin binds to the $\rho 1$ subunit (Figure 3A) and assists its folding (Figures 4A and 4C), we further studied how the interaction between calnexin and the $\rho 1$ subunit is related to the maturation of the $\rho 1$ subunit in the ER. It was reported that the A322D mutation in the GABA_A receptor $\alpha 1$ subunit leads to extensive misfolding and ERAD of the $\alpha 1$ (A322D) subunit^{37, 38} and that the R43Q mutation in the GABA_A receptor $\gamma 2$ subunit leads to inefficient assembly of the $\gamma 2$ (R43Q) subunit with other subunits^{39–41}. Those two mutations caused trafficking deficiency of corresponding subunits, leading to loss of GABA_A receptor function and thus promoting epilepsy. The homologous mutations in the $\rho 1$ subunit are V353D and R89Q. HEK293 cells were transiently transfected with a plasmid containing empty vector (EV) control, FLAG- $\rho 1$, FLAG- $\rho 1$ (V353D), or FLAG- $\rho 1$ (R89Q) for 48 hr before being lysed. Compared to WT, the V353D or R89Q mutation clearly decreased the total $\rho 1$ subunit protein level as well as the upper mature post-ER $\rho 1$ subunit glycoform (Figure 5A, cf. lanes 3 and 4 to lane 2). The V353D mutation resulted in much less $\rho 1$ subunit proteins than the R89Q mutation, possibly due to more extensive ERAD.

HEK293 cells expressing FLAG-tagged $\rho 1$ subunits were immunoprecipitated using anti-FLAG antibody before being subjected to Western blot analysis (Figure 5A). Clearly, the V353D mutation significantly increased the ratio of immunoprecipitated calnexin/ $\rho 1$ by 1.8-fold compared with WT (Figure 5A, cf. lane 7 to lane 6), whereas the R89Q mutation did

not significantly change the ratio of immunoprecipitated calnexin/ ρ 1 compared with WT (Figure 5A, cf. lane 8 to lane 6) (see Figure 5B for quantification). Presumably, the V353D mutation caused extensive misfolding of the ρ 1(V353D) subunit; therefore, the interaction between calnexin and the ρ 1(V353D) subunit was significantly enhanced to fold this misfolding-prone subunit. On the other hand, the R89Q mutation presumably caused an insufficient subunit assembly after its folding; therefore, the interaction between calnexin and the ρ 1(R89Q) subunit was not changed significantly compared with the WT case.

IPA analysis of the ρ 1 receptor interactome enriches transcription factors that regulate the heat shock response in the cytosol or the unfolded protein response in the ER

The top ten transcription factors associated by IPA with the ρ 1 receptor interactome are: XBP1, MYC, TP53, HSF1, HSF2, MYCN, HTT, NFE2L2, MKL2, and MECP2 (Figure 6A). XBP1 and HSF1 are key transcription factors that enhance cellular folding capacity by up-regulating the expression of molecular chaperones and folding enzymes (see below).

Heat shock response transcription factor 1 (HSF1) is a master regulator of the heat shock response (HSR), which is a well-established stress-responsive pathway in the cytosol^{23, 42}. HSF1 activation upregulates the expression of many heat shock proteins in the cytosol and enhances the protein folding capacity in the cytosol. IPA analysis maps 8 proteins that are regulated by HSF1 activation within our GABA- ρ 1 subunit interactome, including CCT4, HSP90AB1, HSPA1A, HSPA8, HSPB1, HSPD1, HSPH1, and TCP1 (Figure 6B).

XBP1 is a key transcription factor during the unfolded protein response (UPR), which is a conserved ER stress-responsive signaling pathway to enhance the ER folding environment^{6, 43}. The ER responds to the accumulation of unfolded proteins by activating up to three integrated intracellular signaling pathways (IRE1, ATF6 and PERK), collectively referred to as the UPR. UPR activation upregulates the expression of many chaperones and folding enzymes in the ER and promotes protein folding in the ER. IRE1 responds to stress by oligomerization, resulting in trans-autophosphorylation that activates its endonuclease function, which precisely splices the mRNA that encodes the transcription factor XBP1. Spliced XBP1 is translocated to the nucleus, upregulates the expression of many chaperones and folding enzymes in the ER, and promotes protein folding in the ER. IPA analysis indicates 12 proteins that are regulated by XBP1 activation within our GABA- ρ 1 subunit interactome, including CALR, PDIA6, HSPA5, HYOU1, DNAJB11, HSP90B1, DERL1, LMAN1, COPE, DDOST, RPN1, and RPN2 (Figure 6C).

IPA pathway analysis predicts that XBP1 and HSF1 play a key role in regulating ρ 1 subunit proteostasis. Since ρ 1 subunits have both ER components and cytoplasmic components (Figure 1A), the involvement of both the UPR and HSR is expected to be important in the regulation of the ρ 1 subunit proteostasis. To test whether the UPR or the HSR indeed influence ρ 1 subunit proteostasis, HEK293 cells were transfected with a FLAG- ρ 1 plasmid for 48 hr and then with a FLAG-HSF1 plasmid or an EV control plasmid for 18 hr before endo H enzyme digestion and Western blot analysis. HSF1 overexpression clearly increased both total (Figure 6D, cf. lane 4 to lane 1) and endo H resistant (Figure 6D, cf. lane 5 to lane 2) ρ 1 subunit protein levels, indicating that HSR activation enhanced the cytosolic folding environment globally to promote ρ 1 subunit folding and trafficking. Analogously, HEK293 cells were transfected with a FLAG- ρ 1 plasmid for 48 hr and then with a spliced XBP1 (XBP1s) plasmid or an EV control plasmid for 6 hr before endo H enzyme digestion and Western blot analysis. XBP1s overexpression modestly increased the upper post-ER endo H resistant ρ 1 subunit protein level (Figure 6E, cf. lane 2 to lane 1), indicating that UPR activation enhanced the ER folding environment globally to promote ρ 1 subunit folding and trafficking. Determining the specific downstream proteins that HSF1 (Figure 6B) or XBP1

regulate (Figure 6C) to promote $\rho 1$ subunit folding and trafficking are key future studies in this area.

Assembly of GABA- $\rho 1$ receptor proteostasis network components

The comprehensive identification of GABA- $\rho 1$ receptor interacting proteins from three tandem MS methods enabled us to assemble the proteostasis network components for GABA- $\rho 1$ receptors (Figure 7). We focused on placing $\rho 1$ receptor interacting proteins in the context of assisting protein folding, trafficking, and degradation based on our current knowledge of the general proteostasis network.

The $\rho 1$ subunit (State 1, Figure 7) is co-translationally translocated onto the ER membrane for folding. Asparagine *N*-linked glycosylation is the most common protein modification in the ER and occurs co-translationally, serving as recognition tags for glycoprotein maturation^{44, 45}. The $\rho 1$ subunit has three potential *N*-glycosylation sites (Asn140, Asn234, and Asn274) in the ER lumen. *N*-linked glycosylation of the $\rho 1$ subunit (State 2, Figure 7) is catalyzed by the oligosaccharyltransferase (OST) complex, which transfers 14-monosaccharide residues $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (Glc: glucose, Man: mannose, GlcNAc: N-acetylglucosamine) to an Asn residue contained in a Asn-X-Ser/Thr sequence motif⁴⁶. The OST complex contains seven subunits; three subunits were successfully identified in our tandem MS analysis, namely, dolichyl-diphosphooligosaccharide-protein glycosyltransferase (*DDOST*, gene symbol), ribophorin I (*RPN1*), and ribophorin II (*RPN2*), indicating that upon entering the ER, the $\rho 1$ subunit uses the OST complex to attach core oligosaccharides. *RPN1*, *RPN2* and *DDOST* are abundant proteins, all containing large ER lumen domains. Successful identification of *RPN1*, *RPN2* and *DDOST*, but not the other four OST subunits, might depend on the high cellular abundance, accessible topology by trypsin digestion, and good peptide response to tandem MS analysis of *RPN1*, *RPN2* and *DDOST*.

From State 2 to State 4, the $\rho 1$ subunit undergoes two independent but collaborative folding pathways: one is glycan dependent and assisted by lectin chaperones in the ER⁴⁷; the other is glycan independent and assisted by heat shock protein family chaperones both in the ER and in the cytosol^{8, 48}.

During the glycan-dependent folding pathway, two outmost glucoses in $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ are removed by ER glucosidase I and II sequentially (State 3 for the $\rho 1$ subunits, Figure 7). The α subunit of ER glucosidase II (*GANAB*) was identified in our tandem MS analysis, indicating the importance of glucose trimming for the $\rho 1$ subunit folding. The monoglucosylated $\rho 1$ subunit enters the calnexin / calreticulin folding cycles⁴⁹. Our tandem MS analysis identified calnexin (*CANX*), calreticulin (*CALR*), and several folding enzymes including protein disulfide isomerases (PDIsases) (*P4HB* and *PDIA6*) in the ER and one peptidylprolyl isomerase (PPIases) (*PPIA*). Our $\rho 1$ subunit interactome data indicate that calnexin and calreticulin potentially facilitate the folding of the $\rho 1$ subunit with help from other folding enzymes. Calnexin was shown to promote $\rho 1$ subunit folding in the ER (Figures 4A and 4C).

GABA- $\rho 1$ receptors also recruit non-lectin heat shock protein chaperones for their folding. Heat shock proteins bind to hydrophobic patches of unfolded proteins, facilitating their folding while preventing aggregation²³. Because the $\rho 1$ subunit contains both ER and cytosolic components, both heat shock proteins in the ER and in the cytosol contribute to assisting its proper folding. Our tandem MS analysis identified major chaperone systems: BiP (*HSPA5*) and GRP94 (*HSP90B1*) in the ER; Hsp70 (*HSPA1A*), Hsc70 (*HSPA8*) and Hsp90 β (*HSP90AB1*) in the cytosol. A number of co-chaperones were identified from our tandem MS analysis: DNAJA1 and DNAJA2 (co-chaperones of Hsc70), DNAJB11 and

HYOU1 (co-chaperones of BiP). In association with its co-chaperones, our identified heat shock protein chaperones may facilitate $\rho 1$ subunit folding, which will be tested in our future study.

After the collaborative glycan-dependent and glycan-independent folding, the $\rho 1$ subunit enters State 4 (Figure 7). At stage 4, the $\rho 1$ subunit faces three different pathways: first, if the $\rho 1$ subunits fold properly, they assemble into a homopentamer (State 5 for $\rho 1$ receptors, Figure 7) for trafficking; second, if the $\rho 1$ subunits fail to fold into their native structures, misfolded $\rho 1$ subunits may be re-glucosylated by UDP-glucose glycoprotein glucosyltransferase 1 (UGGT1, identified from our tandem MS analysis) and re-enter the calnexin / calreticulin folding cycles; third, terminally misfolded $\rho 1$ subunits (state 8, Figure 7) undergo ER-associated degradation (ERAD), being retrotranslocated from the ER into the cytosol and degraded by the proteasome⁵⁰.

Properly folded and assembled $\rho 1$ receptors (State 5 for $\rho 1$ receptors, Figure 7) exit the ER, traffic through the Golgi (State 6 for $\rho 1$ receptors, Figure 7), and reach the plasma membrane (state 7 for $\rho 1$ receptors, Figure 7). Trafficking components that were identified from our tandem MS analysis include SEC13 and SEC16A in the COPII machinery, which is responsible for anterograde (forward) cargo protein vesicle transport from the ER to the Golgi^{51, 52}; COPA and COPE in the COPI machinery, which is responsible for retrograde (backward) retrieval of cargo proteins⁵³; and LMAN1 (also called ERGIC-53), which is a cargo receptor for the transport of glycoproteins from the ER to the ER-Golgi intermediate compartment (ERGIC)⁵⁴. LMAN1 was shown to facilitate $\rho 1$ subunit trafficking from the ER to the Golgi (Figure 4B).

Terminally misfolded $\rho 1$ subunits (state 8, Figure 7) are subjected to ER-associated degradation (ERAD): the $\rho 1$ subunits are ubiquitinated, retrotranslocated from the ER membrane to the cytosol, and degraded by the proteasome⁵⁰. The $\rho 1$ subunit interactome contains three E3 ubiquitin ligases: EDD1 (also called UBR5), HECTD3 and HUWE1. BAG2, a co-factor of an E3 ubiquitin ligase CHIP, was recognized in our tandem MS result. Derlin-1 (*DERL1*), an integral ER transmembrane protein, is among the $\rho 1$ subunit interactome; it serves as a potential $\rho 1$ subunit retrotranslocation channel. The $\rho 1$ subunit interactome contains VCP (also called p97). VCP is an AAA ATPase protein, serving as a prominent candidate to extract misfolded $\rho 1$ subunits from the ER membrane to the cytosol for degradation, which will be tested in our future study.

Specificity of GABA- $\rho 1$ receptor proteostasis network components

Ion channel proteins utilize a subset of chaperones/co-chaperones, folding enzymes and ERAD factors to regulate their proteostasis, partially depending on their topology. Here, we identified numerous chaperones and ERAD factors both in the ER and in the cytosol that interact with the $\rho 1$ subunit (Table 1 and Figure 7). Although most of our identified $\rho 1$ subunit proteostasis network components have their known general role in protein folding or degradation, their specific role in regulating $\rho 1$ subunit proteostasis might differ from their role for other client proteins and remains to be established.

The classical Hsp70/Hsp90 chaperone system and calnexin / calreticulin lectin chaperone system are the two major chaperone systems in the ER⁵⁵. Although cytosolic Hsp90 was reported to interact with and promote the maturation of human *ether-a-gogo*-related gene (hERG) potassium channels, Grp94 (Hsp90 in the ER) did not interact with hERG channels⁵⁶, which might be because hERG channels contain very short ER lumen components. Our identified $\rho 1$ subunit interactome contains both cytosolic Hsp90 (HSPAB1) and ER Grp94 (HSP90B1), implying that both Hsp90 and Grp94 regulate $\rho 1$ subunit proteostasis. Calnexin was reported to interact with both WT and $\Delta F508$ cystic

fibrosis transmembrane conductance regulator (CFTR) protein⁵⁷; however, overexpression of calnexin did not promote the maturation of WT and Δ F508 CFTR⁵⁸. In our case, overexpression of calnexin clearly promoted the maturation of the $\rho 1$ receptors (Figure 4C). This indicates that the role of calnexin in assisting ion channel folding depends on its specific client proteins. Cytosolic Hsp70 (HSPA1A) and Hsc70 (HSPA8) are among the $\rho 1$ receptor interactome (Table 1). Interestingly, although both Hsp70 and Hsc70 associated with hERG channels, it was reported that Hsp70 enhanced the maturation of hERG channels while Hsc70 suppressed their maturation⁵⁹. The role of Hsp70 and Hsc70 in regulating the $\rho 1$ receptor proteostasis needs to be explored. The $\rho 1$ receptor interactome contains Hsp40 family members, including DNAJA1 and DNAJA2, and folding enzymes, including PPIA, a PPIase. It was reported that DNAJA1 and DNAJA2 promoted the degradation of hERG channels⁶⁰ while FKBP8, a PPIase, promoted the trafficking of hERG channels⁶¹. The role of DNAJA1 and DNAJA2 and PPIA need to be established in the context of regulating $\rho 1$ receptor proteostasis. The GABA_C receptors belong to the Cys-loop superfamily of ligand-gated ion channels. To date, the only known specific chaperone in the Cys-loop superfamily is RIC-3 (Resistant to inhibitors of cholinesterase-3)⁶². RIC-3 interacts with and regulates the maturation of some subtypes of nicotinic acetylcholine receptors and 5-hydroxytryptamine type 3 receptors, but not GABA receptors. The $\rho 1$ receptor interactome list might contain GABA receptor-specific chaperones: C1QBP might be a promising target (Figure 3B).

Regarding the ERAD factors, we identified three potential E3 ligases for the $\rho 1$ receptors: HECTD3, HUWE1 and UBR5 (Table 1). Interestingly, all three contain a conserved C-terminal HECT (homologous to E6-AP carboxyl terminus) domain, accounting for their E3 ligase function. HECT E3 ligases are one of the two main groups of E3 ligases⁶³. All three are low abundant proteins: HECTD3 (abundance: 0.17), UBR5 (abundance: 1.33), and HUWE1 (abundance: 8.24). To date, in mammalian cells over 500 potential E3 ligases were identified and E3 ligases are believed to provide substrate specificity during the protein ubiquitination step⁶⁴. Ubiquitination of ion channels have been described with limited examples^{65, 66}. HECTD3 was reported to regulate the ubiquitination of Tara (Trio-associated repeat on actin)⁶⁷; UBR5 was reported to regulate the ubiquitination of β -catenin⁶⁸; HUWE1 was reported to regulate the ubiquitination of the N-Myc oncoprotein⁶⁹. However, the role of HECTD3, UBR5 and HUWE1 in the ubiquitination of ion channels was not well known yet. Therefore, HECTD3, HUWE1 and UBR5 merit further investigation for their specific role in the ubiquitination of the $\rho 1$ receptor.

CONCLUSIONS

Comprehensive consideration of the three tandem MS analyses identified 107 GABA_C- $\rho 1$ subunit interacting proteins (Figure 2A). GeLC-MS/MS, SoLC-MS/MS and MudPIT identified proteins with different properties (Figures 2B and 2C). Employment of three tandem MS approaches enabled us to achieve the maximum coverage of reliable $\rho 1$ receptor interacting protein by choosing arbitrary yet comprehensive criteria to include or exclude a protein in the GABA_C- $\rho 1$ receptor interactome (see Experimental Procedures). We focused on assembling proteostasis network components from the identified $\rho 1$ subunit interactome (Figure 7). We studied representative individual GABA_C proteostasis network components (Figures 4 and 5) and global effectors that regulate protein folding in cells based on bioinformatics analysis of the total $\rho 1$ subunit interactome (Figure 6). The identification of the GABA_C receptor proteostasis network components will enable our future efforts to regulate GABA_C receptor folding, trafficking, degradation, and thus function, providing a promising strategy to ameliorate related retinal diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

GeLC-MS/MS	gel-based tandem MS analysis
SoLC-MS/MS	solution-based tandem MS analysis
MudPIT	Multidimensional Protein Identification Technology

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FIGURE 1. Outline of three tandem MS approaches to identify the GABA_C- $\rho 1$ receptor interactome in HEK293 cells

(A) A cartoon of homopentameric $\rho 1$ receptors and the topology of the $\rho 1$ subunit.
 (B) Western blot analysis confirms overexpression of FLAG- $\rho 1$ receptors in HEK293 cells. β -actin serves as a loading control. EV: empty vector control plasmid.
 (C) Confocal immunofluorescence microscopy confirms the expression of FLAG- $\rho 1$ receptors on the plasma membrane and inside cells.
 (D) Visualization of immunoprecipitated $\rho 1$ receptor complex by SDS-PAGE and Coomassie staining. Representative $\rho 1$ subunit interacting proteins are shown.
 (E) Outline of three tandem MS approaches to identify $\rho 1$ receptor interactome. UPLC: ultra-high pressure reverse-phase liquid chromatography.

FIGURE 2. Global bioinformatics analysis of GABA_C-p1 receptor interactome

(A) A Venn diagram showing the protein number and overlaps of the $\rho 1$ receptor interactome from three tandem MS approaches. Nine proteins, which are commonly identified from three tandem MS methods, are listed.

(B) Protein abundance analysis of the $\rho 1$ receptor interactome. Human protein abundance data is obtained from PaxDb: Protein Abundance Across Organisms (<http://pax-db.org>). Proteins that are commonly identified using three tandem MS methods are labeled. Tandem MS methods: M, MudPIT; S, SoLC-MS/MS; G, GeLC-MS/MS.

(C) A Venn diagram showing the protein number and overlaps of membrane type $\rho 1$ receptor interactors from three tandem MS approaches.

(D) Cellular location of the $\rho 1$ receptor interactome. Particularly, 18% are in located in the ER, an organelle where membrane protein folding occurs.

(E) Molecular and cellular function of the $\rho 1$ receptor interactome. Particularly, 10% is involved in protein synthesis, 24% in protein folding, 6% in protein trafficking and 13% in protein degradation, composing 53% for the proteostasis network components.

FIGURE 3. Verification of representative GABA_C- $\rho 1$ receptor interactors

(A) Immunoprecipitation and Western blot analysis verifies the interaction between the $\rho 1$ receptor and selected $\rho 1$ receptor interactors in HEK293 cells expressing FLAG- $\rho 1$ receptors. Proteins that are potentially involved in protein folding (UGGT1, calnexin, and DNAJA1), trafficking (LMAN1), degradation (BAG2 and Derlin1), and metabolism (C1QBP) are shown. β -actin serves as a loading control.

(B) Knockdown of C1QBP, a novel $\rho 1$ receptor interactor, decreases the total FLAG- $\rho 1$ protein level in HEK293 cells expressing FLAG- $\rho 1$ receptors, indicating that C1QBP plays a role in regulating $\rho 1$ receptor proteostasis. NT: non-targeting control siRNA.

FIGURE 4. Verification of representative GABA_C-ρ1 receptor proteostasis network components (A and B) Knockdown of calnexin (A), a lectin chaperone in the ER, or LMAN1 (B), a glycoprotein trafficking receptor, decreases the endo H resistant post-ER ρ1 glycoform in HEK293 cells expressing FLAG-ρ1 receptors. PNGase F treatment generates unglycosylated ρ1 subunit (lane 2). β-actin serves as a loading control. NT: non-targeting control siRNA. (C) Overexpression of calnexin increases the endo H resistant post-ER ρ1 glycoform in HEK293 cells expressing FLAG-ρ1 receptors.

FIGURE 5.

Calnexin binds to WT and mutant GABA_C- ρ 1 receptors with different interaction capacities. The V353D or R89Q mutation decreases the top mature ρ 1 glycoform compared with WT ρ 1 receptors. HEK293 cells harboring EV control, WT, V353D, or R89Q ρ 1 receptors were immunoprecipitated using anti-FLAG antibody and detected with anti-calnexin antibody. β -actin serves as a loading control. Quantification of (A) is shown in (B) and reported as mean $\pm$ SEM. Any statistical significance is calculated using a two-tailed Student's t-Test. * $p < 0.05$.

FIGURE 6. Global analysis of transcription factors that regulate the GABA_C-ρ1 receptor interactome

(A) Top 10 transcription factors that are enriched from the GABA-ρ1 receptor interactome by using IPA analysis.

(B and C) HSF1 regulates the expression of 8 cytosolic chaperones within the GABA-ρ1 receptor interactome (B), and XBP1 regulates the expression of 12 proteins belonging to the ER proteostasis network components within the GABA-ρ1 receptor interactome (C).

(D and E) Overexpression of HSF1 (D) or XBP1 (E) increases endo H resistant post-ER ρ1 glycoform in HEK293 cells overexpressing FLAG-ρ1 receptors. EV: empty vector control

plasmid; Eh: endo H enzyme; Pf: PNGase F enzyme. β -actin serves as a loading control. Matrin serves as a nuclear protein loading control (**E**).

FIGURE 7.

Assembly of the proteostasis network components for GABA_C-ρ1 receptors. Proteins that are identified from our MS analysis are included in each step. The ρ1 subunit is co-translationally translocated onto the ER membrane for folding (State 1). Upon entering the ER, it is N-glycosylated (DDOST, RPN1, RPN2) for maturation (State 2). The ρ1 subunit undergoes two independent but collaborative folding pathways from state 2 to stage 4: one is glycan dependent and assisted by lectin chaperones in the ER (CANX, CALR); the other is glycan independent and assisted by heat shock protein family chaperones both in the ER (HSPA5, HYOU1, HSP90B1) and in the cytosol (HSPA1A, HSP90AB1, HSPA8). At stage 4, the ρ1 subunit faces three different pathways: first, if the ρ1 subunits fold properly, they assemble into a homopentamer (State 5) for trafficking to the Golgi (State 6) (LMAN1, SEC13, SEC16A) and plasma membrane (State 7); second, if the ρ1 subunits fail to fold into their native structures, misfolded ρ1 subunits may be re-glucosylated (UGGT1) and re-enter the calnexin / calreticulin folding cycles; third, terminally misfolded ρ1 subunits (State 8) undergo ERAD (DERL1, HUWE1, UBR5, HECTD3, BAG2, BAG6, VCP), being retrotranslocated from the ER into the cytosol and degraded by the proteasome. Proteins that are involved in the N-glycan processing pathways are indicated in green; heat shock protein family members are in brown; trafficking proteins are in blue; and ERAD proteins are in red.

Table 1List of GABA $\rho 1$ receptor interactome from three tandem MS approaches

Function	Gene	Protein	IPI number	Method
Protein synthesis				
Aminoacyl tRNA synthetase				
	<i>FARSA</i>	Phenylalanyl-tRNA synthetase alpha chain	IPI00031820	M, G
mRNA binding protein				
	<i>IGF2BP1</i>	Insulin-like growth factor 2 mRNA-binding protein 1	IPI00008557	M
Ribosomal protein				
	<i>RPL14</i>	Ribosomal protein L14 variant	IPI00555744	S
	<i>RPL7P32</i>	60S ribosomal protein L7	IPI00030179	M, S
	<i>RPS10</i>	40S ribosomal protein S10	IPI00008438	M, S
	<i>RPS24</i>	40S ribosomal protein S24	IPI00029750	S
RNA processing				
	<i>DDX20</i>	Probable ATP-dependent RNA helicase DDX20	IPI00005904	M
	<i>PABPC4</i>	Polyadenylate-binding protein 4	IPI00012726	M
Translation factor				
	<i>EEF1A1</i>	Elongation factor 1, alpha 1	IPI00396485	M, G
	<i>EEF2</i>	Elongation factor 2	IPI00186290	M, S
	<i>EIF4B</i>	Eukaryotic translation initiation factor 4B	IPI00012079	G
Protein folding				
Folding enzyme				
	<i>P4HA1</i>	Prolyl 4-hydroxylase, alpha-1 subunit	IPI00009923	M
	<i>P4HB</i>	Prolyl 4-hydroxylase, beta subunit, PDIA1	IPI00010796	M
	<i>PDIA6</i>	Protein disulfide-isomerase A6	IPI00299571	M
	<i>PPIA</i>	Peptidylprolyl isomerase A (cyclophilin A)	IPI00419585	S
Small HSP family				
	<i>HSPB1</i>	Heat-shock protein beta-1, Hsp27	IPI00025512	M, S
Hsp40 family				
	<i>DNAJA1</i>	DnaJ homolog subfamily A member 1	IPI00012535	M, S
	<i>DNAJA2</i>	DnaJ homolog subfamily A member 2	IPI00032406	M
	<i>DNAJB11</i>	DnaJ homolog subfamily B member 11, ERdj3	IPI00008454	M
Hsp60 family				
	<i>CCT4</i>	T-complex protein 1 subunit delta	IPI00302927	M
	<i>HSPD1</i>	Heat shock 60kDa protein 1	IPI00784154	M, S
	<i>TCP1</i>	T-complex protein 1 subunit alpha	IPI00290566	M
Hsp70 family				
	<i>HSPA1A</i>	Heat shock 70 kDa protein 1A	IPI00304925	S, G
	<i>HSPA5</i>	Heat shock 70kDa protein 5, BiP	IPI00003362	S, G
	<i>HSPA6</i>	Heat shock 70 kDa protein 6	IPI00339269	S, G
	<i>HSPA8</i>	Heat shock 70kDa protein 8, HSC70	IPI00003865	S, G
	<i>HYOU1</i>	Hypoxia up-regulated protein 1, GRP170	IPI00922127	M

Function	Gene	Protein	IPI number	Method
Hsp90 family				
	<i>HSP90AB1</i>	Heat shock protein 90kDa beta, cytosolic	IPI00414676	M, G
	<i>HSP90B1</i>	Endoplasmic, Grp94	IPI00027230	M
Hsp105 family				
	<i>HSPH1</i>	Heat shock 105 kDa protein 1	IPI00218993	M
Lectin chaperone				
	<i>CALR</i>	Calreticulin	IPI00020599	M
	<i>CANX</i>	Calnexin	IPI00020984	M, S, G
N-glycoprotein processing				
	<i>GANAB</i>	Neutral alpha-glucosidase AB precursor	IPI00011454	M, S
	<i>RPN1</i>	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1	IPI00025874	M
	<i>RPN2</i>	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2	IPI00028635	M
	<i>DDOST</i>	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase	IPI00297084	M
	<i>UGGT1</i>	UDP-glucose ceramide glucosyltransferase-like 1	IPI00024466	M
Protein trafficking				
COPI machinery				
	<i>COPA</i>	Coatomer subunit alpha	IPI00295857	M
	<i>COPE</i>	Coatomer subunit epsilon	IPI00465132	M
COPII machinery				
	<i>SEC13</i>	SEC13-related protein	IPI00375370	M
	<i>SEC16A</i>	SEC16 homolog A	IPI00641384	M
ER Golgi transport				
	<i>LMAN1</i>	ERGIC-53 protein	IPI00026530	M
GDP dissociation inhibitor				
	<i>GDI1</i>	Rab GDP dissociation inhibitor alpha	IPI00010154	M
Protein degradation				
E3 ubiquitin ligase				
	<i>HECTD3</i>	E3 ubiquitin-protein ligase HECTD3	IPI00456642	M, G
	<i>HUWE1</i>	E3 ubiquitin-protein ligase, HUWE1	IPI00179298	M
	<i>UBR5</i>	E3 ubiquitin-protein ligase, EDD1	IPI00026320	M, S, G
E3 ubiquitin ligase inhibitor				
	<i>BAG2</i>	BAG family molecular chaperone regulator 2	IPI00000643	M, S
ERAD dislocation channel				
	<i>DERL1</i>	Derlin-1	IPI00013271	M
ERAD substrate extraction				
	<i>BAG6</i>	BCL2-associated athanogene 6	IPI00465128	M
	<i>VCP</i>	Transitional endoplasmic reticulum ATPase	IPI00022774	M, S
Protease inhibition				
	<i>SERPINB3</i>	Serpin B3	IPI00022204	S
	<i>SERPINB4</i>	Serpin B4	IPI00010303	S
	<i>SERPINB5</i>	Serpin B5	IPI00783625	S

Function	Gene	Protein	IPI number	Method
Proteasome related				
	<i>PSMD2</i>	26S proteasome non-ATPase regulatory subunit 2	IPI00012268	M, S
	<i>RAD23B</i>	UV excision repair protein RAD23 homolog B	IPI00008223	M
Ubiquitin-related				
	<i>SQSTM1</i>	Sequestosome-1	IPI00179473	M, S, G
	<i>UBB</i>	Ubiquitin B	IPI00179330	M, S, G
Ion channel				
	<i>GABRR1</i>	Gamma-aminobutyric-acid receptor rho-1 subunit	IPI00747264	M, S, G
	<i>KCTD2</i>	Potassium channel tetramerisation domain containing 2	IPI00440769	M
	<i>VDAC1</i>	Voltage-dependent anion-selective channel protein 1	IPI00216308	M
Cellular organization				
Cytoskeleton				
	<i>ACTA1</i>	Actin, alpha skeletal muscle	IPI00021428	S
	<i>ACTB</i>	Actin, cytoplasmic 1	IPI00021439	S, G
	<i>ACTBL2</i>	Beta-actin-like protein 2	IPI00003269	G
	<i>TUBA1A</i>	Tubulin alpha-1A chain	IPI00180675	M, G
	<i>TUBB</i>	Tubulin beta, class I	IPI00645452	M, S, G
	<i>TUBB2A</i>	Tubulin beta-2A chain	IPI00013475	S
	<i>TUBB3</i>	Tubulin beta-3 chain	IPI00013683	M, S, G
Motor protein				
	<i>MYH10</i>	Myosin-10	IPI00397526	M
	<i>MYH9</i>	Myosin-9	IPI00019502	M, S
	<i>MYO6</i>	Myosin VI	IPI00008455	M
Other				
	<i>SUN2</i>	Sad1/unc-84-like protein 2	IPI00295940	M
Signal transduction				
Adapter protein				
	<i>SFN</i>	14-3-3 protein sigma	IPI00013890	S
	<i>YWHAB</i>	14-3-3 protein beta/alpha	IPI00216318	S
	<i>YWHAZ</i>	14-3-3 protein zeta/delta	IPI00021263	S
Calcium ion binding				
	<i>CALML5</i>	Calmodulin-like protein 5	IPI00021536	S
	<i>S100A7</i>	S100 calcium binding protein A7	IPI00219806	M
	<i>S100A8</i>	S100 calcium binding protein A8	IPI00007047	S
	<i>S100A9</i>	S100 calcium binding protein A9	IPI00027462	M, S
Other				
	<i>PTPLAD1</i>	Protein-tyrosine phosphatase-like A domain-containing protein 1	IPI00008998	M
DNA binding				
Histone				
	<i>H2AFV</i>	Histone H2A.V	IPI00018278	M

Function	Gene	Protein	IPI number	Method
Transcription factor	<i>HIST1H4A</i>	Histone H4	IPI00453473	S
	<i>SKP1</i>	S-phase kinase-associated protein 1	IPI00172421	M
Metabolism				
Amino acid metabolism				
	<i>DBT</i>	Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex	IPI00003944	M
Carbohydrate metabolism				
	<i>CIQBP</i>	Complement component 1 Q subcomponent-binding protein	IPI00014230	M, S
	<i>ENO1</i>	Alpha-enolase 1	IPI00465248	S
	<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	IPI00219018	S
	<i>PKM2</i>	Pyruvate kinase isozymes M2	IPI00220644	S
Energy production				
	<i>NDUFS1</i>	NADH-ubiquinone oxidoreductase 75 kDa subunit	IPI00604664	M
	<i>NDUFV1</i>	NADH dehydrogenase [ubiquinone] flavoprotein 1	IPI00028520	M
	<i>NDUFV2</i>	NADH dehydrogenase [ubiquinone] flavoprotein 2	IPI00291328	M
Lipid metabolism				
	<i>FABP5</i>	Fatty acid-binding protein, epidermal	IPI00007797	S
	<i>GSTP1</i>	Glutathione S-transferase P	IPI00219757	M, S
Metabolic enzyme				
	<i>ALDOA</i>	Aldolase A, fructose-bisphosphate	IPI00465439	S
Nucleic acid metabolism				
	<i>ATP5A1</i>	ATP synthase subunit alpha, mitochondrial	IPI00440493	M, S, G
	<i>ATP5B</i>	ATP synthase subunit beta, mitochondrial	IPI00303476	S, G
	<i>SLC25A3</i>	Solute carrier family 25, member 3	IPI00215777	M, G
	<i>SLC25A4</i>	Solute carrier family 25, member 4	IPI00022891	M
	<i>SLC25A5</i>	Solute carrier family 25, member 5	IPI00007188	M, G
	<i>SLC25A6</i>	Solute carrier family 25, member 6	IPI00291467	M, S, G
Other				
	<i>ATAD3A</i>	ATPase family AAA domain-containing protein 3A	IPI00295992	M
Miscellaneous				
Apoptosis				
	<i>CSE1L</i>	Isoform 1 of exportin-2	IPI00022744	M
	<i>LGALS7</i>	Lectin, galactoside-binding, soluble, 7	IPI00219221	S
Other				
	<i>AHNAK</i>	AHNAK nucleoprotein isoform 1	IPI00021812	M
	<i>ANXA2</i>	Annexin A2	IPI00418169	S
	<i>IMMT</i>	Isoform 1 of mitochondrial inner membrane protein	IPI00009960	M
	<i>LRRC59</i>	Leucine-rich repeat-containing protein 59	IPI00396321	M
	<i>NOMO1</i>	Nodal modulator 1 precursor	IPI00329352	M

MS/MS methods: M, MudPIT; S, SoLC-MS/MS; G, GeLC-MS/MS