Enhanced Clathrin-Dependent Endocytosis in the Absence of Calnexin

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Abstract

Background: Calnexin, together with calreticulin, constitute the calnexin/calreticulin cycle. Calnexin is a type I endoplasmic reticulum integral membrane protein and molecular chaperone responsible for the folding and quality control of newly-synthesized (glyco)proteins. The endoplasmic reticulum luminal domain of calnexin is responsible for lectin-like activity and interaction with nascent polypeptide chains. The role of the C-terminal, cytoplasmic portion of calnexin is not clear.

Methodology/Principal Findings: Using yeast two hybrid screen and immunoprecipitation techniques, we showed that the Src homology 3-domain growth factor receptor-bound 2-like (Endophilin) interacting protein 1 (SGIP1), a neuronal specific regulator of endocytosis, forms complexes with the C-terminal cytoplasmic domain of calnexin. The calnexin cytoplasmic C-tail interacts with SGIP1 C-terminal domains containing the adaptor complexes medium subunit (Adap-Comp-Sub) region. Calnexin-deficient cells have enhanced clathrin-dependent endocytosis in neuronal cells and mouse neuronal system. This is reversed by expression of full length calnexin or calnexin C-tail.

Conclusions/Significance: We show that the effects of SGIP1 and calnexin C-tail on clathrin-dependent endocytosis are due to modulation of the internalization of the receptor-ligand complexes. Enhanced clathrin-dependent endocytosis in the absence of calnexin may contribute to the neurological phenotype of calnexin-deficient mice.

Introduction

Calnexin is a type I endoplasmic reticulum (ER) integral membrane protein with a single transmembrane helix followed by a cytoplasmic negatively charged C-terminal tail [1,2]. The ER luminal domain of calnexin is responsible for lectin-like activity and interaction with nascent polypeptide chains [3,4]. Calnexin, together with calreticulin, constitute the calnexin/calreticulin cycle responsible for the folding and quality control of newly-synthesized (glyco)proteins [5,6]. Calnexin has also been implicated in influencing Ca2+ regulation [1,7,8,9], cell-cell adhesion [10], phagocytosis [11], and cell sensitivity to apoptosis [12,13]. X-ray crystallography studies have identified the ER luminal domain of calnexin as forming a globular β-sandwich containing a glucose binding site with a proline-rich portion of the protein forming an extended arm structure [14], a docking site for oxidoreductase ERp57 [15,16,17,18]. The C-terminal portion of calnexin extends 86 amino acid residues but its significance to calnexin function is not clear. This C-terminal domain of calnexin undergoes protein kinase-dependent phosphorylation [19,20] and this modification may play a role in the chaperon function of the protein [20].

Endocytosis is a process by which cells internalize materials into the cell by engulfing them with plasma membrane. One sub-type of endocytosis, clathrin-dependent endocytosis, is responsible for the internalization of specific molecules into the cell including: pathogens, nutrients, antigens, growth factors and receptors [21,22]. During clathrin-dependent endocytosis, molecules are taken into the cell by specific receptor-ligand interactions. Integral membrane proteins bind to cytosolic adaptors (AP-2), which form a link to the cytoplasmic clathrin lattice. Accessory proteins, such as Eps15 and AP180, have the ability to affect the process of clathrin mediated endocytosis by facilitating the assembly of clathrin-coated pits [23,24,25]. Although single-cell organisms use endocytosis as the means to obtain nourishment, higher organisms have adapted endocytosis for specialized functions. These functions include modulating interactions between signaling molecules and their receptors and providing a localized environment where signaling takes place [26,27]. Endocytosis is especially vital for the neuronal system [28]. These processes allow retrieved fused vesicles to refill the vesicle pool allowing sustained synaptic transmission and maintenance of terminal nerve size [29]; specific substances to cross the blood-brain barrier and enter the central nervous system [30]; and internalizes receptor-ligand complexes to nerve terminals for their intracellular signaling [31]. Recent studies indicate that Src homology 3-domain growth factor receptor-bound 2-like (Endophilin) interacting protein 1 (SGIP1) may play a role in modulation of endocytic activity in neuronal cells [32,33].
Results and Discussion

The carboxyl-terminal cytoplasmic tail of calnexin interacts with SGIP1

Calnexin is composed of structural and functional domains [33]. While the ER luminal region is responsible for the chaperone function of the protein, the transmembrane domain anchors the protein to the membrane and the C-terminal tail extends to the cytoplasmic compartment. To examine the functional importance of cytoplasmic tail of calnexin we carried out the yeast two hybrid screening method to identify proteins interacting with the C-terminal cytoplasmic domain of calnexin (designated C-tail) encompassing the last 88 amino acids (C<sup>504</sup>-E<sup>591</sup>) of the protein. Because calnexin-deficient mice have neurological disorders [34,44], we used a mouse brain Matchmaker cDNA library (63,841, Clontech, USA) to identify calnexin interacting proteins in a neuronal system. Screening of the library resulted in the identification of 9 positive clones. Three of these clones encoded the C-terminal region of SGIP1 (Src homology 3-domain growth factor receptor-bound 2-like (Endophilin) interacting protein 1) [32,35]. The longest cDNA fragment of SGIP1 identified in the screen was 537 nucleotides and encoded the last 179 amino acids of the protein. This C-terminal region of SGIP1 contains the domain referred to as the adaptor complexes medium subunit (Adap-Comp-Sub) (Fig. 1A). This adaptor complex may couple clathrin lattices with membrane proteins and play a role in clathrin-dependent endocytosis [http://pfam.sanger.ac.uk/famil y?id=Adap_comp_sub].

To test for direct interactions between the calnexin C-tail domain and SGIP1, we carried out immunoprecipitation analysis. N1E-115 neuroblastoma cells were transfected with expression vectors encoding GFP-SGIP1 or GFP control followed by cell lyses, immunoprecipitation with anti-GFP antibodies and Western blot analysis with anti-calnexin antibodies. Figure 1B (lane 5) shows that calnexin was present in GFP-SGIP1 immunoprecipitate indicating that these two proteins interacted. As expected, there was no immunoreactive calnexin in GFP expressing cells (Fig. 1B, lane 6). Since SGIP1 is predominantly expressed in the brain, especially in cerebellum ([35], http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.238094), we used cerebellum extracts to test for endogenous SGIP1-calnexin complexes. Anti-SGIP1 antibodies immunoprecipitated SGIP1 from cerebellum homogenate (Fig. 1C, lane 2), and the complexes contained immunoreactive calnexin (Fig. 1C, lane 5). Since SGIP1 antibodies were generated against His-tagged recombinant SGIP1 we also employed anti-His-tag antibodies as a negative control (Fig. 1C, lanes 3 and 6). We concluded that calnexin interacted with SGIP1 in vivo in N1E-115 cells and in cerebellum. Next we carried out immunolocalization analysis in N1E-115 cells expressing recombinant GFP-SGIP1. Figure 1D shows that GFP-SGIP1 and calnexin co-localized to the ER-like network. Quantitative analysis for SGIP1-calnexin colocalization showed that 66.78% (Std. Error = 4.22%, n = 20) of GFP-colocalized with calnexin, supporting the biochemical and immunological results that the two proteins form complexes. The RT-PCR analysis showed that SGIP1 is predominantly expressed in brain, spinal cord and cerebellum (Fig. 1E). This is in agreement with earlier studies [32,35] indicating that there is a high expression of the protein in neuronal tissues. Since calnexin-deficient mice develop neuropathies, we examined whether calnexin affects expression of SGIP1. Figures 1E and F show that expression of SGIP1 mRNA and protein was not affected in the absence of calnexin.

To carry out molecular analysis of the interaction between calnexin and SGIP1, we isolated cDNA encoding mouse SGIP1 from the mouse brain Matchmaker cDNA library followed by nucleotide sequence analysis of the clone. Nucleotide and amino acid sequences of this mouse variant of SGIP1 (2401 bp, encoding a protein of 826 amino acids) differed from the rat SGIP1<sub>α</sub> [35] and the mouse SGIP1<sub>α</sub> released from NCBI (Fig. S1). Important difference between the clones was that the region encompassing amino acid residues G335-Q62 in rat SGIP1<sub>α</sub> was not present in mouse SGIP1<sub>α</sub> (Fig. S1). Comparison of the protein sequence of mouse SGIP1 isolated in our laboratory with that available at the NCBI revealed two additional differences. The mouse clone isolated in this study had an L in position 295 versus an F in the NCBI sequence and a P in position 355 versus a T in the NCBI sequence (Fig. S1). We concluded that the calnexin C-terminal tail interacts with SGIP1, a newly identified neuronal version.

MP domain of SGIP1 binds phospholipids and it may be involved in interactions with Esp15 [32], an adaptor protein of clathrin-mediated endocytosis [22]. The central, proline-rich domain of SGIP1 may form complexes with endophilin-3 [35], an important regulator of clathrin-mediated endocytosis and synaptic vesicles recycling [21,22]. Next we mapped the SGIP1 domains interacting with the calnexin C-tail. To do this we generated cDNA encoding three different regions of SGIP1 (Fig. 1G): the N-terminal domain membrane phospholipid-binding domain (MP, amino acid residues 1–239) and a proline rich region (Pro-Rich, amino acid residues 239–653), and a domain containing the adaptor complexes medium subunit (Adap-Comp-Sub, amino acid residues 647–826), and cloned into the pGADT7 vector to test their interaction with the calnexin C-tail in the yeast-two-hybrid system. Yeast-two-hybrid analysis revealed that calnexin-C-tail interacted with the C-terminal region of SGIP1 (Adap-Comp-Sub domain, amino acid residues 647–826, Fig. 1G). We concluded that interaction between the calnexin C-domain and SGIP1 maps to the Adap-Comp-Sub domain of SGIP1.

Increased clathrin-dependent endocytosis in the absence of calnexin

Considering that SGIP1 is a neuronal endocytotic protein interacting with adaptor proteins involved in clathrin-dependent endocytosis [32,35] and that the SGIP1<sub>α</sub> has been implicated to play a role in endocytosis in neuronal cells [32], we tested whether calnexin may play any role in neuronal endocytosis. First, we examined whether GFP-SGIP1 affects uptake of transferrin in N1E-115 cells. Transferin has been commonly used as an indicator of clathrin-dependent endocytosis [36]. N1E-115 cells were transfected with either GFP-SGIP1 or GFP expression vectors, incubated with Alexa-transferrin, fixed and examined by confocal microscopy. As expected, expression of GFP in N1E-115 cells did not have any effect on transferrin uptake (Fig. 2A, left panel). In contrast, GFP-SGIP1 expressing cells had significantly inhibited transferrin uptake (Fig. 2A, right panel).

Second, we asked whether clathrin-dependent endocytosis might also be affected in the absence of calnexin. Cerebellar granule cells were isolated from of 7 day old wild-type and calnexin-deficient mice. A mixed granule cell culture was established containing wild-type and calnexin-deficient cells. Cells were cultured for 4 days in neurobasal plus medium followed by the transferrin uptake assay. Wild-type cells were identified by a positive staining with anti-calnexin antibodies (Fig. 2B, left panel). Analysis of transferrin uptake of mixed cultures showed significantly increased transferrin uptake in all calnexin-deficient cells examined (n = 80) (Fig. 2B, compare middle and left panels) indicating that there was increased clathrin-dependent endocytosis in granule cells isolated from calnexin-deficient mice. In addition
Figure 1. Calnexin interacts with SGIP1. (A) A schematic representation of full length SGIP1. The N-terminal domain of SGIP1 (amino acid residues 1–239) represents a membrane phospholipid-binding domain (MP, dark green); middle domain (amino acid residues 239–653) contains a proline-rich region (Pro-Rich, light green); the C-terminal region contains an adaptor-complex-subunit (Adap-Comp-Sub, red). (B) N1E-115 cells were transfected with GFP-SGIP1 or GFP expression vectors and lysed cells were subjected to immunoprecipitation with anti-GFP. Calnexin was identified by Western blot (WB) analysis of immunoprecipitates probed with anti-calnexin antibodies (lanes 3–6). Lane 1, Western blot analysis of N1E-115 cells expressing GFP-SGIP1; lane 2, Western blot analysis of N1E-115 cells expressing GFP; lane 3, identification of calnexin in N1E-115 cells expressing GFP-SGIP1; lane 4, identification of calnexin in N1E-115 cells expressing GFP; lane 5, lysate from N1E-115 cells expressing GFP-SGIP1 were

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to granule cells, increased transferrin uptake was also observed in calnexin-deficient fibroblasts (Fig. 2C and 2D) indicating that there was significant increase in endocytic activities in cells lacking calnexin.

Next we carried out electron microscopy analysis of cerebella from 7 day old wild type and calnexin-deficient mice to examine whether there was increased clathrin-dependent endocytosis in mice in the absence of calnexin mice. Figure 2E shows that calnexin-deficient mice had significantly increased number of synaptic vesicles when compared to wild-type cerebellum. The numbers of synaptic vesicle were quantitated using 16 wild-type neurons and 10 calnexin-deficient neurons. The mean number of clathrin coated vesicles for wild-type and calnexin-deficient neurons was 18.6±3.8 and 37.3±3.6, respectively (Fig. 2F). We concluded that calnexin deficiency leads to increased endocytotic activity in cultured cells and mouse neuronal system.

Next, we examined whether expression of full length calnexin in cnx−/− cells will reduce the endocytotic activities seen in calnexin-deficient cells. We isolated granule cells from wild-type and calnexin-deficient cerebellum, followed by expression of recombinant GFP, GFP-SGIP1, full length calnexin or calnexin C-tail. Expression of GFP alone in wild-type and calnexin-deficient cells had no effects on transferrin uptake which remained high as in control, non-transfected cells. Transferrin uptake in these cells was not affected at 0–4°C expression of either GFP-SGIP1 or GFP-calnexin C-tail was not affected at 0°C (Fig. 5A). However, there was no internalization of the ligand under these conditions in all cells that expressed GFP fusion proteins (n = 90). In contrast, at 16°C, internalization of the ligand is inhibited without any effect on ligand binding to the receptor [37]. We took advantage of these temperature-dependent effects on endocytosis to examine which phase of clathrin-dependent endocytosis was affected by SGIP1 and the calnexin C-tail. To do this, N1E-115 cells expressing recombinant GFP-SGIP1 or GFP calnexin C-tail were incubated at different temperatures (4°C or 16°C) followed by analysis of Alexa-transferrin uptake. Transferrin ligand binding to cells expressing either GFP-SGIP1 or GFP-calnexin C-tail was not affected at 4°C (Fig. 5A). However, there was no internalization of the ligand under these conditions in all cells that expressed GFP fusion proteins (n = 90) (Fig. 5A). In contrast, at 16°C, all cells expressing recombinant GFP-SGIP1 or GFP-calnexin C-tail (n = 90) had reduced Alexa-transferrin uptake compared to GFP expressing cells or non-transfected control cells (Fig. 5B). Since receptor recycling and degradation are inhibited at 16°C [37], we concluded that SGIP1 and the calnexin C-tail affected the ligand internalization step during clathrin-dependent endocytosis. Western blot analysis revealed that expression of transferrin and EGF receptors was not affected in cells expressing calnexin C-tail (Fig. 5C). And there’s no differences between wild type (wt) and calnexin-deficient (cnx−/−) cerebella on Transferrin or EGF receptors expression level either (Fig. 5D). Next, we carried out FACS analysis to quantify the transferrin receptor levels in wild-type and cnx−/− cell lines. Figure 5E shows that the level of cell surface expression of transferrin receptor was the same in wild-type and cnx−/− cell lines. These results support the notion that the effects of SGIP1 and calnexin C-tail on clathrin-dependent endocytosis were not due to a reduced level of

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Clathrin-dependent endocytosis is a temperature sensitive process. At physiological temperature (37°C) there is rapid clathrin-dependent endocytosis, whereas at 16°C, endocytosis is slow and permissive for receptor-ligand internalization and does not support recycling or degradation [37]. In contrast, at 0–4°C, internalization of the ligand is inhibited without any effect on ligand binding to the receptor [37]. We took advantage of these temperature-dependent effects on endocytosis to examine which phase of clathrin-dependent endocytosis was affected by SGIP1 and the calnexin C-tail. To do this, N1E-115 cells expressing recombinant GFP-SGIP1 or GFP calnexin C-tail were incubated at different temperatures (4°C or 16°C) followed by analysis of Alexa-transferrin uptake. Transferrin ligand binding to cells expressing either GFP-SGIP1 or GFP-calnexin C-tail was not affected at 4°C (Fig. 5A). However, there was no internalization of the ligand under these conditions in all cells that expressed GFP fusion proteins (n = 90) (Fig. 5A). In contrast, at 16°C, all cells expressing recombinant GFP-SGIP1 or GFP-calnexin C-tail (n = 90) had reduced Alexa-transferrin uptake compared to GFP expressing cells or non-transfected control cells (Fig. 5B). Since receptor recycling and degradation are inhibited at 16°C [37], we concluded that SGIP1 and the calnexin C-tail affected the ligand internalization step during clathrin-dependent endocytosis. Western blot analysis revealed that expression of transferrin and EGF receptors was not affected in cells expressing calnexin C-tail (Fig. 5C). And there’s no differences between wild type (wt) and calnexin-deficient (cnx−/−) cerebella on Transferrin or EGF receptors expression level either (Fig. 5D). Next, we carried out FACS analysis to quantify the transferrin receptor levels in wild-type and cnx−/− cell lines. Figure 5E shows that the level of cell surface expression of transferrin receptor was the same in wild-type and cnx−/− cell lines. These results support the notion that the effects of SGIP1 and calnexin C-tail on clathrin-dependent endocytosis were not due to a reduced level of
Figure 2. Increased endocytosis in the absence of calnexin. (A) Alexa647-transferrin (Alexa-Tfn) uptake in N1E-115 cells expressing GFP (left panels) or GFP-SGIP1 (right panels). Non-transfected N1E-115 cells or N1E-115 cells expressing GFP (left panels) show significant uptake of Alexa-Tfn. In contrast cells expressing GFP-SGIP1 (right panels) have significantly inhibited Alexa-Tfn uptake. The asterisks indicate the location of cells expressing GFP or GFP-SGIP1. Scale bar = 10 μm. (B) Alexa-Tfn endocytosis in a mixed culture of granule cells isolated from wild-type and cnx<sup>−/−</sup> mice. Left panel, immunostaining with anti-calnexin antibodies. Middle panel, granule cells were incubated with Alexa-Tfn at 37°C for 10 min followed by confocal
transferrin or EGF receptors, but were due to modulation of the internalization of the receptor-ligand complexes.

Although many fundamental eukaryotic trafficking mechanisms are conserved in neurons, they have evolved distinct modes of trafficking to accommodate their unique morphology and function [37]. Furthermore, different endocytic mechanisms specific for different surface molecules and cellular domains exist in neurons [28]. Specificity of calnexin to affect clathrin-dependent endocytosis in the nervous system is remarkable and likely dictated by calnexin interaction with SGIP1. The N1E-115 neuronal cell line has very low SGIP1 expression and does not express Eps15 or AP180, and full length calnexin does not affect endocytosis in these cells (Fig. S2). In sharp contrast, cultured granule cells expressing large quantities of SGIP1, are highly sensitive to calnexin-dependent effect on endocytosis (Fig. 2B and Fig. 3B). Neurons are specialized types of cells, and the exocytosis-endocytosis balance is vital for neurons to play its normal functions and to survive. In addition, there are specific protein components required for clathrin-dependent endocytosis in neurons, for example, neuron-specific variants of α- and β-adaptin chains have been identified [38]. We found that SGIP1 is primarily expressed in the neuronal system, and other essential neuronal endocytic factors, AP180 and Eps15, were also more abundant in the cerebellum than in neuronal cell lines (Fig. S2). Association of specific endocytosis related factors with nerve terminal-derived vesicles suggests a functional specialization of nerve terminal to adapt to the specific requirements of synaptic vesicle recycling [39]. The specific intracellular and intercellular environment of the neuron may provide factors that cooperate with full length calnexin to decrease clathrin-dependent endocytosis.

One of the best described functions of calnexin is its role as a molecular chaperone [40]. Little information is available about the structure and function of the cytoplasmic calnexin C-tail as the majority of studies on calnexin has focused on its ER luminal lectin-like chaperon domain [41]. Here we showed that the calnexin C-tail is a potent inhibitor of endocytosis in neuronal cells indicating a functional specialization of calnexin domains. The C-terminal region of calnexin binds Ca\(^{2+}\) and can be phosphorylated by specific protein kinases and this enhances calnexin’s interactions with ribosomes [19,20]. Ca\(^{2+}\) may affect endocytosis by the neuronal system by arresting synaptic vesicle movement or increasing synaptic vesicle size [42,43]. Absence of calnexin results in a severe neurological phenotype in mice manifested by impaired gait, reduced lower limb function [34,44] and inhibited nerve conductive velocity due to impaired myelination of the peripheral and central nervous system [34]. Increased endocytosis in the nervous system in the absence of calnexin as described in this study may also, at least in part, contribute to the phenotype of calnexin-deficient mice. For example, endocytosis and postendocytic sorting of neurotransmitter receptors have emerged as critical mechanisms responsible for various forms of synaptic plasticity [26]. Calnexin may affect these critical pathways and impact synaptic plasticity and consequently the function of the nervous system. This, at least in part, might be mediated by C-terminal cytoplasmic domain of calnexin.

**Materials and Methods**

**Plasmids, yeast two-hybrid assays and RT-PCR**

The mouse brain Matchmaker cDNA library in pACT2 (Clontech, 638841) was transformed into yeast strain AH109. The library was screened for interacting proteins with the C-terminal cytoplasmic region of calnexin (C-tail) corresponding to the last 87 amino acids (C504-E591). cDNA encoding the C-tail of calnexin was obtained by PCR amplification of a mouse calnexin template cDNA using the following primers: the 3’ forward primer 5’-GGG ATT CCA TAT GTG TTC TGG AAA GAA AC-3’ and the 3’ reverse primer 5’-AAG GGT CCG TGA CTC TCT TCG TGG CTA-3’. The PCR product was cloned in frame with the GAL4 DNA-binding domain at Nhel and PstI restriction sites to generate the clone pGBK7-CNX-C. Screening of the mouse library was carried out as recommended by the manufacturer (Clontech). Plasmid DNA from positive clones was isolated and used as template for amplification of the insert in the vector library by the AD (activating domain) sequencing primer and T7 Y2H sequencing primer.

For synthesis of cDNA encoding SGIP1, RNA was isolated from liver, brain, spinal cord and cerebellum using TRIzol (Invitrogen). For PCR-driven synthesis of cDNA encoding SGIP1 the following primers were used: forward primer 5’-CGC GGA TCC GAA TGG AAG GAC TGA AA-3’ and reverse primer 5’-CCG CTC GAG TTA GTT ATC TGC CAA GTA CT-3’. For RT-PCR the following primers were used: for SGIP1 forward primer 5’-AAT GTG GAC ATG CTC AA ATA-3’ and reverse 5’-TTA GTT ATC TGC CAA GTA CT-3’. For murine SGIP1 forward primer 5’-AAT GTG GAC ATG CTC AA ATA-3’ and reverse 5’-TTA GTT ATC TGC CAA GTA CT-3’. For RT-PCR the following primers were used: for SGIP1 forward primer 5’-AAT GTG GAC ATG CTC AA ATA-3’ and reverse 5’-TTA GTT ATC TGC CAA GTA CT-3’. For murine SGIP1 forward primer 5’-AAT GTG GAC ATG CTC AA ATA-3’ and reverse 5’-TTA GTT ATC TGC CAA GTA CT-3’.

**Cell culture, immunoprecipitation, Western blot and FACS analyses**

Cerebellum was isolated from a 7-day-old mouse, cut into small pieces and digested in trypsin for 12 min at 37°C. Tissues was washed twice with PBS and spun down at 350 x g for 5 min to collect dissociated cells. Cells were suspended in neurobasal medium (Gibco) with 2% B27, 80 mg/L D-glucose, 20 μM l-glutamine, 1% Penicillin/Streptomycin and 20 mM KCl, and then passed through a 40 μm filter. Granule cells (5 × 10^6 freshly isolated granule cells) were combined with 2 μg plasmid and 100 μL mouse neuron nucleofector (Amaxa). Transfection of cell lines with expression vectors was carried out using the lipofectamine 2000 system (Invitrogen) according to the manufacturer’s recommendation.

For immunoprecipitation, N1E-115 neuronal cells (CRL 2263 from the American Type Culture Collection) were treated for 15 min on ice with RIPA buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40 (isoyl phenoxylethoxylethanol), 0.25% sodium deoxycholate and 1 mM EDTA. For cerebellum immunoprecipitation, cerebellum was grinded into powder in liquid nitrogen followed by incubation with a RIPA buffer. Solubilized cells or cerebellum tissue power was spun down at 16,000 x g for 10 min at 4°C. Sample was pre-clearly by...
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A

\[ \text{wt} \quad \text{cnx}^{-/-} \]

GFP  
Alexa-Tfn  
Merge

SGIP1  
Alexa-Tfn  
Merge

B

\[ \text{wt} \quad \text{cnx}^{-/-} \]

CNX  
Alexa-Tfn  
Merge

CNX C-Tail  
Alexa-Tfn  
Merge

C

| GFP  | Alexa-Tfn  | SGIP1  | Alexa-Tfn  | CNX C-Tail  | Alexa-Tfn |
|------|-------------|--------|-------------|--------------|------------|
| \[\star\] | \[\star\] | \[\star\] | \[\star\] | \[\star\] | \[\star\] |
incubation with 30 μL protein and A/G-Sepharose beads for 30 min. Antibodies were added to pre-cleared sample and incubated at 4°C for 3 hrs on an orbital rocker. Thirty μL of Protein A/G bead solution was added followed by overnight incubation at 4°C. Beads were spun down at 1,000 xg for 3 min, washed 5 times with ice cold PBS and re-suspend in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE (7.5% acrylamide) followed by Western blot analysis with goat anti-GFP (1:10,000, Abcam), rabbit anti-calreticulin (1:1,000, Abcam), rabbit anti-calnexin (1:1,000, Stressgen Bioreagents), rabbit anti-transferrin receptor (1:200, Santa Cruz Biotechnology), rabbit anti-calnexin (1:1,000, Stressgen Bioreagents), rabbit anti-calnexin (1:1000, Stressgen Bioreagents), rabbit anti-EGFR (epidermal growth factor receptor) (1:500, Santa Cruz), rabbit anti-EGFR (epidermal growth factor receptor) (1:500, Santa Cruz Biotechnology), rabbit anti-SGIP1 (1:200, Sigma) and mouse anti-SGIP1 (1:500, Santa Cruz Biotechnology) antibodies. Beads were incubated at 4°C for 3 hrs on an orbital rocker. Thirty μL of Protein A/G bead solution was added followed by overnight incubation at 4°C. Beads were spun down at 1,000 xg for 3 min, washed 5 times with ice cold PBS and re-suspend in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE (7.5% acrylamide) followed by Western blot analysis with goat anti-GFP (1:10,000, Abcam), rabbit anti-calreticulin (1:1,000, Abcam), rabbit anti-calnexin (1:1,000, Stressgen Bioreagents), rabbit anti-transferrin receptor (1:200, Santa Cruz Biotechnology), rabbit anti-EGFR (epidermal growth factor receptor) (1:500, Santa Cruz Biotechnology), mouse anti-AP180 (1:200, Sigma) and mouse anti-

**Figure 3. Calnexin C-tail inhibits transferrin uptake.** (A) Alexa647-transferrin (Alex-Tfn) uptake in wild-type (wt) and calnexin-deficient (cnx−/−) granule cells expressing GFP (left panel) or GFP-SGIP1 (right panel). Scale bar = 5 μm. (B) Alexa-Tfn endocytosis in wild-type (wt) and calnexin-deficient (cnx−/−) granule cells expressing GFP tagged full-length calnexin (left panel) or GFP tagged C-tail of calnexin (CNX C-Tail) (right panel). Granule cells were incubated with Alexa-Tfn at 37°C for 10 min as described under “Experimental Procedures”. Scale bar = 5 μm. (C) N1E-115 neuronal cells were transfected with expression vectors encoding GFP, GFP tagged SGIP1 (SGIP1) or calnexin C-tail (CNX C-Tail) followed by Alexa-Tfn uptake analysis. N1E-115 cells expressing GFP were used as control. The asterisks indicate cells expressing recombinant GFP-SGIP1 or recombinant calnexin C-tail (CNX C-Tail). Scale bar = 10 μm.

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**Figure 4. Calnexin affects clathrin-dependent endocytosis.** N1E-115 cells were transfected with expression vectors encoding GFP, GFP tagged SGIP1 (SGIP1) or calnexin C-tail (CNX C-Tail) and incubated with Alexa647-Epidermal Growth Factor (Alexa-EGF), a ligand-induced clathrin-dependent endocytosis marker at 37°C for 10 min. Scale bar = 17 μm.

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Endocytosis analysis

Prior to endocytosis analysis cells were starved in serum-free medium for 1 hr followed by incubation with 25 μg/mL transferrin (Tfn) Alexa-Flour 647 and 100 ng/mL EGF (AlexaFlour 647 at 37°C for 10 min. Ligand uptake was terminated by placing cells on ice. Cells were rinsed in PBS and fixed in 3.7% paraformaldehyde. Internalized ligand was visualized using confocal microscopy. For Tfn endocytosis quantification, cells were first incubated with 25 μg/mL HRP-Tfn on ice for 60 min, and then shifted to 37°C for 2 min, 5 min or 10 min. Cells were washed twice with cold PBS/30 mM glycine at pH 2.7, twice with ice cold PBS and lysed with 1% Triton X-100 in PBS. Cells were lysed and 200 μL lysate aliquots were mixed with 1 mL SIGMAFAST™ OPD (o-phenylenediamine dihydrochloride) solution (Sigma) for HRP-OPD analysis to reveal HRP-Tfn uptake. The HRP-OPD color developing reaction was stopped by addition of 1/4 volume of 3 M H2SO4 followed by OD492 measurements.

Immunofluorescence, confocal and electron microscopy

Immunofluorescence and confocal microscopy was carried out as described previously [46]. Both rabbit anti-calnexin and goat anti-rabbit Alexa secondary antibody were diluted as 1:200 in PBS plus 2% milk powder and 0.1% saponin. All confocal microscopy images were taken under 60× objectives.

For electron microscopy analysis, 7-day old mice were euthanized by decapitation and their cerebella were taken. Primary fixation was carried out at 4°C for 4 hrs in a freshly prepared solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM cacodylate buffer pH 7.2, and then fixed in 2.5% glutaraldehyde, 100 mM sodium cacodylate pH 7.0 [47]. Samples were processed for electron microscopy and examined with a Hitachi Transmission Electron Microscope H-7000.
Calnexin Deficiency and Endocytosis

Figure 5. Calnexin affects ligand internalization step in clathrin-dependent endocytosis. Wild-type (wt) and calnexin-deficient (cnx−/−) fibroblasts were transfected with expression vectors encoding GFP, GFP tagged calnexin C-tail (CNX C-Tail), or GFP tagged SGIP1 (SGIP1). Cells were incubated with Alexa647-transferrin (Alexa-Tfn) on ice for 60 min at 0°C (A) or for 30 min at 16°C (B). Scale bar = 17 μm. (C) Western blots analysis of N1E-115 cells expressing GFP-calnexin C-tail (lane 1) or GFP (lane 2). Blots were probed with anti-transferrin receptor (TR) antibodies, anti-epidermal growth factor receptor (EGFR) antibodies and anti-glycerinaldehyde-3-phosphate dehydrogenase (GADPH) antibodies. (D) Western blots analysis of cerebella from wild type mouse (wt) and calnexin-deficient (cnx−/−) cells were analyzed flow cytometry with Anti-Transferrin receptor antibodies. Results are presented as the relative mean florescence intensity after subtracting unspecific staining. P3 represents the gate set on cells stained with antibody.

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Supporting Information

Figure S1 Comparison of amino acid sequence of different SGIP1 isoforms. Protein sequence alignment for mouse SGIP1 sequence gotten from this study (SGIP1), mouse SGIP1 sequence release from NCBI (SGIP1-NCBI; GenBank: CAM14981) and rat SGIP1 alpha sequence shown in ref. 32 (SGIP1-alpha; GenBank:AB262964).

Figure S2 Expression of endocytic proteins in the absence of calnexin. (A) Expression of full length calnexin did not inhibit endocytosis in N1E-115 cells. Alexa 647-transferrin (Alexa-Tfn) uptake in N1E-115 cells expressing GFP (upper panel) or full length calnexin (CNX, lower panel). (B) Western blot analysis of extracts from N1E-115 cell line and cerebellum was carried out with anti-calnexin, anti-SGIP1, anti-Eps15 and anti-AP180 antibodies. Anti-GAPDH antibodies were used as a loading control. (EPS)

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Author Contributions

Conceived and designed the experiments: H-DL W-XL MM. Performed the experiments: H-DL W-XL MM. Analyzed the data: H-DL W-XL MM. Contributed reagents/materials/analysis tools: H-DL W-XL MM. Wrote the paper: H-DL MM.

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