The ubiquitin-like protein Plic-1 enhances the membrane insertion of GABA_\textsubscript{A} receptors by increasing their stability within the endoplasmic reticulum.

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\gamma-aminobutyric acid receptors (GABA_\textsubscript{A}Rs) are the major sites of fast inhibitory neurotransmission in the brain, and a critical determinant for the efficacy of neuronal inhibition is the number of these receptors that are expressed on the neuronal cell surface. GABA_\textsubscript{A}Rs are hetero-pentamers that can be constructed from 7 subunit classes with multiple members; \alpha, \beta, \gamma(1-3), \delta, \epsilon(1-3) \theta, \pi. Receptor assembly occurs within the endoplasmic reticulum (ER) and it is evident that transport competent combinations exiting this organelle can access the cell surface, while unassembled subunits are ubiquitinated and subject to proteasomal degradation. In a previous report the ubiquitin-like protein Plic-1 was shown to directly interact with GABA_\textsubscript{A}Rs and promote their accumulation at the cell surface. In this study we explore the mechanisms by which Plic-1 regulates the membrane trafficking of GABA_\textsubscript{A}Rs. Using both recombinant and neuronal preparations it was apparent that Plic-1 increased the stability of ER resident GABA_\textsubscript{A}Rs together, with an increase in the abundance of poly-ubiquitinated receptor subunits. Furthermore Plic-1 elevated cell surface expression levels by selectively increasing their rates of membrane insertion. Thus Plic-1 may play a significant role in regulating the strength of synaptic inhibition by increasing the stability of GABA_\textsubscript{A}Rs within the secretory pathway and thereby promoting their insertion into the neuronal plasma membrane.

GABA_\textsubscript{A}Rs are Cl\textsuperscript{-} selective ligand-gated ion channels and are the major mediators of fast synaptic inhibition in the brain. These receptors are also the major sites of action for anxiolytic, sedative and anti-convulsants including both barbiturates and benzodiazepines (1,2). These receptors are hetero-pentamers that can be assembled from 7 subunit classes: \alpha, \beta, \gamma(1-3), \delta, \epsilon(1-3) \theta, \pi, providing the structural basis for receptor structure (1,2). A combination of molecular, biochemical and genetic approaches suggest that, in the brain, the majority of benzodiazepine sensitive synaptic receptor subtypes are composed of \alpha, \beta and \gamma2 subunits (3). In contrast receptors that incorporate \alpha4/5 and \delta subunits are extrasynaptic and mediate tonic inhibition (4). The number of GABA_\textsubscript{A} receptors on the neuronal cell surface is a critical determinant for the efficacy of synaptic inhibition and, at steady-state, is determined by the rates of receptor insertion and removal from the plasma membrane (5).

It is evident that GABA_\textsubscript{A}Rs are assembled within the endoplasmic reticulum (ER) and then transported to the plasma membrane for insertion while misfolded or unassembled receptor subunits are rapidly targeted for ER-associated degradation (ERAD) (6-9). Proteins targeted for ERAD are modified by ubiquitination resulting in their recognition and degradation by the proteasome (10). In addition neuronal activity can regulate the abundance of cell surface GABA_\textsubscript{A}Rs by altering subunit ubiquitination, a process that in turn controls receptor insertion into the plasma membrane and subsequent accumulation at postsynaptic inhibitory specializations (11).

The ubiquitination and proteasomal degradation of proteins is subject to modulation by regulatory proteins that
contain an N-terminal ubiquitin like (UBL) domain and one or two ubiquitin associated domains (UBA) (12). One member of this class of proteins termed Plic-1 (proteins linking IAP to cytoskeleton) (13) binds to GABA_aRs. These interactions are dependent upon the UBA domain of Plic-1 which binds to conserved amino acids within the intracellular domains of GABA_Aα and β subunit isoforms. Moreover this interaction has been demonstrated to be of significance in mediating the cell surface accumulation of GABA_ARs as revealed using dominant negative peptides (14).

Here we have begun to explore the mechanisms by which Plic-1 regulates GABA_ARs cell surface stability. We demonstrate that Plic-1 increases the stability of ER resident GABA_AR β3 subunits and also elevates the levels of a ubiquitinated β3 species. As a result, Plic-1 increases the insertion of GABA_ARs into the neuronal cell surface, without affecting their endocytic sorting. Therefore, Plic-1 regulation of the ubiquitin dependent, proteasomal degradation of GABA_ARs, may provide a dynamic mechanism regulating the efficacy of inhibitory synaptic transmission.

Experimental Procedures

Antibodies. Rabbit anti-myc IgGs were obtained from Santa Cruz. Monoclonal anti–HA IgGs were obtained from Roche. Rabbit polyclonal anti-β3 IgGs have been described previously (15,16). Secondary HRP conjugated anti-bodies were from Jackson immunologicals and Protein A HRP was from Pierce.

Cell Culture and Transfections. HEK-293 cells were maintained in DMEM/F12 (1:1) nutrient mix with 10 % fetal bovine serum. HEK-293 cells were transfected with pRK5α1, pRK5β3 and pRK5γ2s (17), (18) with or without pRK5Plic-1 (Vector ratio 1:1:1 respectively) using the amaxa nucleofection Kit. Cortical neurons were obtained from embryonic day 18 (E18) rats (16,19). Dissociated E18 rat cortical neurons were transfected with 3 µg of plasmid DNA per 5 x 10^6 neurons using the Rat Nucleofector™ kit (Amaxa) and then used in experiments after 3-4 days in vitro (20-22).

Biotinylation. HEK-293 cells or cortical neurons were chilled on ice for 5 minutes and then washed twice in PBS + 1mM CaCl_2 and 0.5 mM MgCl_2 (PBS-CM) at 4 °C. Cells were incubated for 15 minutes at 4°C in 1.5 mg/ml NHS-SS-Biotin (Pierce) dissolved in PBS-CM. To quench unreacted biotin, neurons were washed three times (10 minutes each wash, at 4°C) in PBS-CM + 75-100 mM Glycine, then washed twice in PBS and either lysed in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 2 mM EDTA, and a mammalian protease inhibitor cocktail, Sigma) or returned to a 37°C incubator. Protein concentrations were determined using the micro BCA protein assay kit (Pierce) and equal amounts of protein were added to immobilized avidin (neutravidin, Pierce) for 2 hours at 4°C. Avidin beads were washed twice, for 15 minutes at 4°C, in high salt (500 mM NaCl) RIPA buffer, followed by a 15 minute wash at 4°C, in (150 mM NaCl) RIPA buffer. Precipitated biotinylated proteins were resolved by SDS-PAGE and β3 was detected using immunoblotting with rabbit anti-β3, followed by peroxidase conjugated anti-rabbit IgGs and detection with ECL. Blots were imaged using the FujiFilm LAS-3000 imaging system and bands quantified with Fujiﬁlm Multi Gauge software.

Deglycosylation. Endoglycosidase H (Endo-H) and peptide N-glycosidase-F (PNGaseF) were purchased from New England BioLabs. Transfected HEK-293 cells expressing α1β3 with or without Plic-1 were lysed in RIPA buffer + Protease inhibitor cocktail (Sigma). Total protein (10 µg) from lysates was denatured in 0.5% SDS and 40 mM Dithiobthreitol (DTT) for 10 minutes at room temperature and then incubated for 1 hour with Endo H at 37 °C.
in 1x reaction buffer (0.05 M Sodium Citrate, pH 5.5). PNGaseF digestion of 10 μg of denatured total protein was performed in 0.05 M Sodium Phosphate pH7.5 and 1% NP-40 for 1 hour at 37°C. Digestion was terminated by the addition of Laemmli sample buffer and protein products resolved by SDS-PAGE and the Mβ3 subunit was detected by immunoblotting.

**Immunoprecipitation.** Transfected HEK-293 cells were lysed in 100 μl of 1% SDS, 25 mM Tris pH 7.4. Lysates were then diluted 10 fold, with 900 μl of RIPA buffer (lacking SDS), +10 mM N-ethylmaleimide (Sigma) and mammalian protease inhibitor cocktail (Sigma). Lysates were then sonicated in 1.5 ml microcentrifuge tubes for 10 seconds at 10 amplitude microns (Sanyo Soniprep 150). Following centrifugation at 14,000 rpm to pellet insoluble material, lysates were pre-cleared with non-specific rabbit IgGs and protein A sepharose for 1 hour at 4°C. Lysates were then incubated for 1 hour at 4°C with 3 μg of rabbit anti-myc (Santa Cruz) and then a further 1 hour, with protein A sepharose. Precipitated immuno-complexes were washed, alternatively, in low salt (150 NaCl) and high salt (350mM NaCl) RIPA buffer for 20 minutes at 4°C. Immunoprecipitated antigen was resolved on a 10 % SDS-PAGE gel and immunoblotted with mouse anti-HA, followed by HRP conjugated donkey anti-mouse IgGs and detection with ECL (SuperSignal® Pierce). Blots were stripped in 62.5 mM Tris-HCl pH 6.7, 2% SDS, 100 mM β-mercaptoethanol at 60°C for 30 minutes and re-probed with rabbit anti-myc followed by protein A-HRP (Pierce) and detection with ECL.

**BBSβ3 cell surface labeling/Receptor Insertion Assay.** Hippocampal Neurons (9 DIV) were transfected with equimolar amounts of cDNAs encoding BBSβ3 (23) and Plic-1 (14) or empty vector (pRK5) using Effectine (Qiagen) according to the manufacturer’s instructions. Following transfection neurons were grown for a further 24 hours. To measure steady state cell surface levels of GABA_{A}R incorporating BBSβ3 subunits neurons were labeled with 1 μg/ml rhodamine-conjugated α-bungarotoxin (Invitrogen) for 15 minutes at 15°C. Neurons were then washed 3 times in PBS at 15°C and then fixed. To measure the membrane insertion of neuronal GABA_{A}Rs incorporating BBSβ3 subunits in the presence or absence of Plic-1, neurons were labeled with 10 μg/ml unlabelled α-bungarotoxin for 15 minutes at 15°C, to block cell surface receptors. The cells were then washed 3 times in PBS at 15°C followed by a 4 minute incubation at 37°C with 1 μg/ml rhodamine-conjugated α-bungarotoxin (Invitrogen). All incubations were performed in the presence of 200 μM Tubocurarine (Sigma) to block Bgt binding to endogenous Acetylcholine receptors (24,25). Cells were fixed in 4% paraformaldehyde and confocal images were collected using a X 60 objective lens acquired with Olympus Fluoview Version 1.5 software and the same image acquisition settings for BBSβ3 with or without Plic-1 were used. These images were analyzed using MetaMorph (Universal Imaging Corporation, Downingtown, USA) imaging software. Firstly, a 3D reconstruction of an imaged neuron was made from a series of Z sections and then the average fluorescence intensity of rhodamine-Bgt staining was measured along 30 μm of 2 proximal dendrites per neuron, after subtraction of background fluorescence.

**Results**

*Plic-1 increases the cell surface expression levels of recombinant GABA_{A}Rs.* To analyze the role that Plic-1 plays in regulating GABA_{A}R functional expression we expressed α1, β3 and γ2 subunits in HEK-293 cells with a cDNA encoding pRK5-Plic-1, or control empty vector (pRK5). We focused on GABA_{A}Rs composed of these subunits, because they reproduce many of the physiological and pharmacological properties of their neuronal counterparts (1,2). Following transfection, HEK cells were lysed and equal amounts of
detergent soluble extracts were resolved by SDS-PAGE and subjected to immunoblotting with anti-Plic-1 and anti-β3 IgGs. While abundant Plic-1 immunoreactivity was seen in cells transfected with the Plic-1 construct, significant levels were absent in cells transfected with empty vector (Fig 1A). Consistent with this we have previously demonstrated that HEK-293 only express low endogenous levels of Plic-1 (14). In cells expressing Plic-1 total expression levels of the β3 subunit were increased by 67.2 ± 4.5% compared to control (Fig. 1A). Using biotinylation to label and isolate cell surface proteins, it was also apparent that Plic-1 increased the cell surface accumulation of GABAγRs relative to control cells (Fig. 1A) which is consistent with previous observations (14).

The increase in GABAγR cell surface levels on expression with Plic-1, may be a consequence of decreased endocytosis, increased recycling of GABAγR from an endocytic pool or an increase in insertion from the secretory pathway. To distinguish between these possibilities, we compared the cell surface degradation of GABAγRs in the presence or absence of Plic-1. HEK cells expressing α1β3γ2 with or without Plic-1 were labeled with cell impermeant NHS-SS-biotin and incubated for varying time periods at 37°C. The levels of remaining biotinylated β3 subunits were then measured by immunoblotting of the avidin purified fraction with rabbit anti-β3 IgGs. These experiments revealed similar levels of degradation of cell surface GABAγRs in the presence, or absence of Plic-1 over a 20 hour time period. (Fig 1B).

The abundance of ER retained GABAγRs is increased by Plic-1. The results in figure 1 suggest that Plic-1 primarily acts to increase GABAγR cell surface abundance by modifying receptor stability within the secretory pathway. Given that GABAγRs are assembled from their constitutive subunits within the ER we assessed the significance of Plic-1 in regulating receptor stability within this intracellular compartment. For these experiments, we examined the sensitivity of N-linked glycans within the GABAγR β3 subunit to digestion with Endoglycosidase H (Endo H). It is well known that transmembrane proteins residing within the ER carry high mannose N-linked glycans which are sensitive to cleavage by Endo H (26,27). In contrast transmembrane proteins that have exited the ER have mature N-linked glycans that are Endo-H insensitive. In our experiments, detergent soluble extracts from HEK cells expressing α1β3 with or without Plic-1, were digested with Endo-H and then immunoblotted with rabbit anti-β3 IgGs. Endo-H resistant and sensitive β3 was detected in control cells and those expressing Plic-1 (Fig. 2A). For comparison samples were also treated with PNGase F, which cleaves all N-linked glycans resulting in the detection of a single band of 52 kDa (Fig 2A) (17,28). We measured the levels of Endo-H sensitive β3 subunits in the absence or presence of Plic-1. Plic-1 increased the amount of Endo-H sensitive GABAγR β3 subunits by 32.5 ± 5.4% compared to the β3 subunit co-expressed with empty vector (Fig 2B). These results suggest that Plic-1 stabilizes ER resident β3 subunits.

Plic-1 increases the accumulation of ubiquitinated GABAγRs. GABAγR β3 subunits isolated from recombinant expression systems and neurons have been found to be ubiquitinated. Furthermore proteasome inhibitors stabilize β3-ubiquitin conjugates. Given that Plic-1 is known to associate with proteasomal subunits (29-31), we speculated that Plic-1 might stabilise GABAγR β3 subunits by modulating their direct ubiquitination or the abundance of β3-ubiquitin conjugates and thus proteasomal degradation. To assess these possibilities transfected HEK-293 cells expressing myc tagged β3 (Mβ3) Plic-1 and haemagluttinin (HA) tagged ubiquitin (HA-Ub) were lysed in 1 % SDS, and diluted 10 fold in RIPA buffer. The Mβ3 subunit was then immunoprecipitated with rabbit anti-myc IgGs, and then immunoblotted with mouse anti-HA IgGs to detect ubiquitinated Mβ3 (Fig 3A). Co-expression of Plic-1 with Mβ3
increased the abundance of $^{3}$$^{14}$Nβ3-ubiquitin conjugates by 100 ± 6.8% compared to control cells transfected with empty vector (Fig. 3B). However Plic-1 did not appear to significantly alter the level of ubiquitination of individual GABA$_A$ receptor β3 subunits, as the ubiquitin/β3 signal ratio remained the same (Fig 3C). Thus co-expression of Plic-1 with GABA$_A$Rs increases the accumulation of β3-ubiquitin conjugates which previous studies have illustrated are enriched within the ER (11).

**Plic-1 enhances the cell surface expression levels of neuronal GABA$_A$Rs.** To examine the relevance of our studies of recombinant receptors in HEK cells to endogenous GABA$_A$Rs in neurons we examined the effects of increased Plic-1 expression on the levels of GABA$_A$Rs in cultured neurons. E18 cortical neurons were transfected with either pRK5-GFP (control) or with pRK5-Plic-1 using nucleofection and then incubated for 3-4 days in vitro. Neurons were then lysed in RIPA buffer and equal amounts of protein were subjected to SDS-PAGE and immunoblotting with anti-Plic-1 and anti-β3 IgGs. In cultures nucleofected with pRK5-Plic-1 an increase in the level of Plic-1 expression was evident compared to control (Fig 4A). Furthermore, Plic-1 increased the total pool of endogenous β3 subunits by 26.7 ± 2% compared to that seen in control neurons expressing GFP (Fig. 4A). In contrast the levels of the ionotropic glutamate receptor GluR1 subunit were unaltered (Fig 4A).

To determine if Plic-1 expression regulated the cell surface abundance of GABA$_A$Rs in neurons, we used a biotinylation assay to label and isolate surface receptors. In these experiments cortical neurons were nucleofected with control pRK5-GFP or Plic-1 and incubated for 3-4 days in vitro followed by biotinylation and isolation of cell surface receptors. Plic-1 increased the cell surface expression levels of GABA$_A$Rs incorporating β3 subunits by 27.6% compared to those in control neurons, without altering the cell surface levels of the GluR1 subunit (Fig 4B). We also examined the influence of Plic-1 on the cell surface degradation of GABA$_A$Rs. Nucleofected neurons expressing pRK5-GFP or Plic-1 (3-4 days in vitro) were biotinylated to label cell surface receptors and then incubated for 0 or 24 hours at 37°C. Consistent with our recombinant experiments in HEK cells, Plic-1 did not appear to influence the degradation of cell surface β3 containing GABA$_A$Rs (Fig. 4C and D). Together these results suggest that Plic-1 acts to increase the steady state cell surface levels of GABA$_A$R by selectively increasing their insertion into the plasma membrane.

**Plic-1 enhances the cell surface accumulation of recombinant β3 subunits in neurons.** To corroborate our biochemical experiments we examined the influence of Plic-1 on the cell surface accumulation of GABA$_A$Rs using fluorescent labeling of live hippocampal neurons. For these experiments hippocampal neurons (9 Div) were transfected with pRK5-Plic-1, or empty control vector and the GABA$_A$R β3 subunit engineered with an α-bungarotoxin binding site (BBS) and a pHluorin reporter incorporated into the N-terminus ($^{BB}$Sβ3) (11,23). Critically these modifications do not alter GABA$_A$R assembly or their functional properties (11,23,32). Following transfection neurons were incubated for 24 hours and then labeled with α-bungarotoxin conjugated to Rhodamine (Rd-Bgt) for 15 minutes at 15°C in order to label cell surface $^{BB}$Sβ3 (Fig 5A). Cell surface $^{BB}$Sβ3 labeling with Rd-Bgt was performed at 15°C to block both GABA$_A$R endo and exocytosis. Quantitative analysis of cell surface Rd-Bgt fluorescence intensity showed a significant increase (64 ± 12.2%) in $^{BB}$Sβ3 in neurons co-transfected with Plic-1 compared to control (Fig. 5B). Furthermore, not only an increase in cell surface $^{BB}$Sβ3 was observed but also an increase in total $^{BB}$Sβ3 (65 ± 12.1%) pHluorin fluorescence (Fig. 5C). Thus these experiments demonstrate that Plic-1 can enhance the cell surface accumulation of recombinant GABA$_A$R.
receptors when co-expressed in cultured neurons.

Plic-1 increases the insertion of GABA\(_\alpha\)Rs into the neuronal plasma membrane. Finally we used labeling of hippocampal neurons with fluorescent Bgt to directly assess the role that Plic-1 plays in regulating GABA\(_\alpha\)R membrane insertion. Firstly, Hippocampal neurons (9 Div) were transfected with \(^{\text{BBS}}\beta3\) and 24 hours later, live neurons were labeled with 10 \(\mu\)g/ml of unlabeled \(\alpha\)-bungarotoxin (Bgt) for 15 minutes at 15\(^\circ\)C in order to block existing cell surface GABA\(_\alpha\)Rs containing \(^{\text{BBS}}\beta3\) subunits. Then, to measure insertion of \(^{\text{BBS}}\beta3\), neurons were incubated with 1 \(\mu\)g/ml Rhodamine-Bgt (Rd-Bgt) at 37 \(\circ\)C for varying time points (Fig 6A). The insertion of \(^{\text{BBS}}\beta3\) increased linearly over time reaching a plateau at approximately 10 minutes (Fig 6B). This increase in rhodamine fluorescence could be specifically blocked by co-incubation with 10 \(\mu\)g/ml of unlabeled Bgt (data not shown).

We therefore used a time point of 4 minutes to assess the influence of Plic-1 on the insertion of \(^{\text{BBS}}\beta3\). Hippocampal neurons were transfected with \(^{\text{BBS}}\beta3\) and Plic-1 or control empty vector (pRK5). Then, 24 hours later neurons were incubated with Bgt to block existing cell surface \(^{\text{BBS}}\beta3\) and then incubated with Rd–Bgt for 4 minutes to label newly inserted \(^{\text{BBS}}\beta3\). These experiments revealed that in the presence of Plic-1 the level of \(^{\text{BBS}}\beta3\) insertion increased by 50 \(\pm\) 10.6\% compared to control (Fig. 6C and D).

**Discussion**

Plic-1 an established negative regulator of proteasome activity (30) has been previously documented to be associated with GABA\(_\alpha\)Rs. This interaction is mediated between the C-terminal UBA domain of Plic-1 and a conserved motif within the major intracellular domains of GABA\(_\alpha\)R receptor \(\alpha\) and \(\beta\) subunits (14). Whilst this interaction is critical for modulating GABA\(_\alpha\)R receptor functional expression, the underlying mechanisms remain to be established.

Here we have examined the role that Plic-1 plays in regulating the membrane trafficking of GABA\(_\alpha\)Rs. To do so we compared the expression levels of heterotrimeric GABA\(_\alpha\)Rs composed of \(\alpha1\beta3\) and \(\gamma2\) subunits in the presence or absence of a Plic-1 expression construct in HEK-293 cells. Co-expression with Plic-1 increased the cell surface and total expression levels of GABA\(_\alpha\)Rs in these cells. Consistent with this result over expression of Plic-1 in cortical neurons also significantly enhanced the cell surface expression of endogenous GABA\(_\alpha\)Rs containing \(\beta3\) subunits. In both systems this modulatory effect of Plic-1 was independent of alterations in receptor endocytic sorting. Thus these experiments suggest that Plic-1 acts to increase GABA\(_\alpha\)R receptor cell surface expression primarily by altering receptor trafficking, or stability within the secretory pathway.

To analyze further the significance of Plic-1 for GABA\(_\alpha\)R receptor stability within the secretory pathway we assessed its potential role in regulating the abundance of ER retained, GABA\(_\alpha\)Rs. We focused on this compartment because it represents the site where hetero-oligomers of GABA\(_\alpha\)Rs are assembled from their constituent subunits (17,33,34). As revealed by Endo H digestion, Plic-1 increased the stability of ER retained GABA\(_\alpha\)Rs. In support of our findings, immunofluorescence and immunoelectron microscopy has revealed that Plic-1 is associated with subsynaptic cisternae, the ER and the Golgi apparatus in fibroblasts and neurons (14). It is emerging that the residence time of GABA\(_\alpha\)R subunits within the ER is determined by their rates of ubiquitination and subsequent proteasomal degradation (6) (14) (11). In agreement with this mechanism we have established that Plic-1 increases the accumulation of ubiquitinated GABA\(_\alpha\)R subunits. Finally we were also able to directly demonstrate that increasing the expression of Plic-1 in neurons specifically enhanced the insertion of GABA\(_\alpha\) receptors into the plasma membrane.
Collectively our results suggest that Plic-1 increases GABA\(_\text{A}\)R cell surface expression levels by increasing their stability within the ER. Given that the assembly of GABA\(_\text{A}\)Rs from their constituent subunits in the ER is inefficient (6) this enhanced stability may increase the production of transport competent heteromeric receptors for insertion into the plasma membrane. In agreement with our findings, inhibiting proteasome activity has been shown to increase the abundance of assembled nicotinic acetylcholine receptors which leads to enhanced insertion into the plasma membrane (35). Significantly, previous studies have revealed that Plic-1 can interact with the GABA\(_\text{A}\)R receptor \(\alpha_1-3, \alpha_6\) and \(\beta_1-3\) subunits (14), which is suggestive of a conserved role for this protein in regulating the cell surface stability of these structurally diverse family of inhibitory receptors. Consistent with our results with GABA\(_\text{A}\)R, Plic-1 and its yeast homolog Dsk2 are associated with the proteasome and are implicated in regulating ER associated degradation and protein targeting to aggresomes (29,30) (36,37). In addition Plic proteins appear to have a role in regulating the endocytosis of G-protein coupled receptors and function of heterotrimeric G proteins (38) (39).

Finally chronic perturbation of neuronal activity leads to bi-directional changes in ubiquitin dependent degradation of GABA\(_\text{A}\)Rs (11). Given the role of Plic-1 in ubiquitin dependent proteasomal degradation of the GABA\(_\text{A}\)R \(\beta_3\) subunit, it is tempting to speculate that activity dependent ubiquitination and thus turnover may be regulated by binding of Plic-1 to the \(\beta_3\) subunit, in response to changes in neuronal activity.

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Footnotes

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Abbreviations. GABA(A), γ-aminobutyric acid type A; DMEM, Dulbecco’s Modified Eagle’s Medium; EDTA, ethylenediamine tetraacetic acid; HEK, human embryonic kidney; NEM, N-ethylmaleimide; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulphate-
polyacrylamide gel electrophoresis; Bgt, α-bungarotoxin; Endo H, Endoglycosidase H; PNGaseF, peptide N-glycosidase-F; HA, hemagglutinin; ERAD, endoplasmic reticulum associated degradation; DTT, Dithiothreitol; DIV, days in vitro; GFP, green fluorescent protein; NHS-SS-Biotin, Sulfo-L-cysteine-nickel 2-(biotinamido)-ethyl-1, 3-dithiopropionate; Plic-1, Proteins linking Integrin-associated protein to Cytoskeleton 1; Rd-Bgt, rhodamine α-bungarotoxin; FBS, foetal bovine serum.

**Figure legends**

**Figure 1** Plic-1 modulates the cell surface accumulation of GABA A Rs expressed in HEK-293 cells. A) Plic-1 increases steady state levels of GABA A R β3 subunits. HEK cells were transfected with α1β3γ2s plus Plic-1 or control empty vector (pRK5). Cells were biotinylated, lysed and cell surface receptors were isolated with immobilized avidin. Immunoblots show total and cell surface levels of GABA A R β3 subunits and total levels of Plic-1 as indicated. Graph represents quantification of total and cell surface β3 subunits from blots in A. Data represent mean ± s.e.m. percentage of control values. (* significantly different from control p<0.01, t-test, n=4). B) Cell surface degradation of GABA A Rs. HEK cells were transfected with α1β3γ2s plus Plic-1 or control empty vector (pRK5). Cells were biotinylated and incubated at 37°C for varying time points as indicated. Upper Panel: immunoblot of cell surface degradation of β3 in the absence of Plic-1. Lower Panel: immunoblot showing cell surface degradation of β3 in the presence of Plic-1. Graph shows percent β3 remaining over time. Open squares represent control and filled squares represent +Plic-1 (error bars are ± s.e.m, n=3).

**Figure 2** Plic-1 stabilizes an immature GABA A R β3 subunit glycoform. A) HEK-293 cells were transfected with α1 and β3 plus Plic-1 or control vector pRK5. After 24 hours expression cells were lysed and 20 μg of cell lysate was incubated with (lanes 2 and 4) or without (lanes 1 and 3) Endo H or PNGase F (lane 5). Endo H sensitive and resistant forms of β3 are indicated. B) Quatification of Endo H sensitive β3. Data represent mean ± s.e.m. percentage of control values. (* significantly different from control p<0.05, t-test, n=4)

**Figure 3.** Plic-1 increases the abundance of ubiquitinated GABA A R β3 subunits. A) HEK-293 cells were transfected (Nucleofection) with equimolar amounts of Mβ3, Plic-1 and HA-tagged ubiquitin. After 24 hours cells were lysed in 1% SDS + 20mM Tris -HCl pH 7.4 and then diluted 10 fold with modified RIPA buffer lacking SDS. Mβ3 was immunoprecipitated with Rabbit anti-myc IgGs. Top panel: immunoblot of ubiquitinated β3 conjugates. Middle panel: blots were stripped and re-probed with anti-IgGs. Lower panel: immunoblot of 10% of total lysate. B) Quantification of the ubiquitin signal form A. Data represent ± s.e.m. percentage increase in ubiquitin signal compared to control. * significantly different from control p<0.01, t-test, n=4. C) The ubiquitin/Mβ3 ratios were calculated and normalized to control Ub/Mβ3 signal. Data represent mean ± s.e.m. of control Ub/Mβ3 ratio.

**Figure 4** Plic-1 increases expression levels of GABA A R β3 subunits in neurons A) Cortical neurons (E18) were transfected (Nucleofection) with pRK5-GFP (control) or pRK5-Plic-1 plasmids. After expression for 3 days in vitro, neurons were lysed and equal amounts of solubilized protein were immunoblotted with anti-β3, anti-GluR1, and anti-Plic-1 IgGs as indicated. Graph represents quantification of total β3 levels in transfected neurons. Data represent mean ± s.e.m. percentage of control values. *Significantly different from control (p < 0.01, n = 4,
t-test). B) Plic-1 increases cell surface expression of GABA$_A$R $\beta_3$ subunits. Cortical neurons (E18) were transfected (Nucleofection) with pRK5-GFP (control) or pRK5-Plic-1 plasmids. After expression for 3 days in vitro, cell surface proteins were biotinylated with NHS-SS-Biotin. Biotinylated receptors were isolated using immobilized avidin and immunoblotted with rabbit anti-$\beta_3$ and rabbit anti-GluR-1 IgGs as indicated. Graph represents quantification of cell surface $\beta_3$ levels in transfected neurons. Data represent mean ± s.e.m. percentage of control values. *Significantly different from control ($p < 0.01$, $n = 4$, t-test).

C) Cell surface degradation of GABA$_A$R in neurons. Cortical neurons were transfected with Plic-1 or GFP and incubated for 3 days in vitro. Neurons were then biotinylated and incubated at 37°C for 0 or 24 hours. Upper Panel: immunoblot showing cell surface $\beta_3$. Lower Panel: immunoblot showing increased Plic-1 expression in total lysates from transfected neurons. D) Graph represents percent $\beta_3$ remaining at 24 h with (■) or without (■) Plic-1 (Data represent means ± s.e.m percent of $\beta_3$ at time zero, $n=4$).

**Figure 5** Plic-1 increases the cell surface expression of recombinant GABA$_A$Rs when co-expressed in neurons. A) Following transfection with BBS$\beta_3$ and Plic-1 or empty vector (pRK5) hippocampal neurons were incubated with Rhodamine–Btx to label cell surface BBS$\beta_3$. Boxed areas in main image are magnified in panels below. B) Quantification of cell surface BBS$\beta_3$ and C) the total pool of BBS$\beta_3$ levels from images in F. Data represent means ± s.e.m percentage of control BBS$\beta_3$. *significantly different from control ($p < 0.01$, t-test, $n=10$-12 neurons, N = 3 independent cultures).

**Figure 6** Plic-1 enhances the membrane insertion of GABA$_A$Rs in cultured neurons.
A) Increase in BBS$\beta_3$ insertion over time. Hippocampal neurons (9 Div) were transfected with BBS$\beta_3$ and 24 hours later were pre-incubated with unlabeled Bgt to block existing surface BBS$\beta_3$. Neurons were then incubated at 37°C for various time points (as indicated) in the presence of rhodamine-Bgt to label newly inserted BBS$\beta_3$. B) Graph represents fluorescence intensity of newly inserted BBS$\beta_3$ over time ($n=10$ neurons/time point). C) Hippocampal neurons (9 Div) were transfected with equimolar amounts of BBS$\beta_3$ and Plic-1 or pRK5 control vector as indicated. After 24 hours of expression neurons were pre-incubated with unlabeled Bgt and then incubated with rhodamine-Bgt for 4 minutes at 37°C. Boxed areas of dendrites in images are magnified in panels below. D) Graph shows quantification of fluorescence intensity of newly inserted BBS$\beta_3$. Data represent means ± s.e.m percentage of control BBS$\beta_3$. *significantly different from control ($p < 0.01$, t-test, $n=10$-12 neurons, N = 3 independent cultures).
Figure 1.
Figure 2

A

B

Endo H

-  
- 
+
+

pRK5  Plic-1

+  
+
-
-

Endo H Resistant  
Endo H Sensitive

β3

1  2  3  4  5

Endo H Sensitive β3

0  50  100  150

pRK5  Plic-1

% Control
Figure 3.

A

B

C

\[ \text{\( \beta_3 \) Ubiquitination (\% Control) } \]

\[ \text{Ub/\( \beta_3 \) Ratio} \]

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Figure 4.
Figure 5.
Figure 6.
The ubiquitin-like protein Plic-1 enhances the membrane insertion of GABA\(_A\) receptors by increasing their stability within the endoplasmic reticulum

Richard S. Saliba, Menelas Pangalos and Stephen J. Moss

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