Cellular/Molecular

C-Terminal Modification Is Required for GABARAP-Mediated $GABA_A$ Receptor Trafficking

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We investigated the ubiquitin-like modification of $GABA_A$ receptor-associated protein (GABARAP) and its function. A fusion protein of GABARAP with v5 in the N terminus and myc in the C terminus was expressed in rat cultured hippocampal neurons and PC12 cells. Western blotting with antibodies to v5 and myc revealed that the C terminus of GABARAP was cleaved off. Cleavage was blocked by mutating the C-terminal Gly116 to Ala, suggesting that G116 is required for the processing. Unlike ubiquitin, GABARAP was not incorporated covalently into higher-molecular-weight protein complexes. Nor was GABARAP degraded by ubiquitinylation through the proteasome, although GABARAP formed noncovalent dimers. Immunofluorescent confocal microscopy demonstrated that recombinantly expressed GABARAP was diffusely localized in PC12 cells. However, prevention of C-terminal processing in the mutant $GABARAP_{G116A}$ resulted in redistribution to the Golgi. In neurons, punctate cytoplasmic staining of GABARAP was seen in soma and processes, whereas $GABARAP_{G116A}$ was limited to soma. Compared with wild-type GABARAP, the colocalization and interaction of $GABARAP_{G116A}$ with $GABA_A$ receptors were significantly reduced, resulting in a reduction in expression of receptors in the plasma membrane. When $\alpha1\beta2\gamma2S$-containing $GABA_A$ receptors were expressed in oocytes, the increased surface expression of $GABA_A$ receptors, as shown by increased GABA currents and surface-accessible $GABA_A$ receptor subunit polypeptides resulting from GABARAP coexpression, was prevented by mutation G116A. In addition, the distribution of NSF (*N*-ethylmaleimide-sensitive factor) was affected in $GABARAP_{G116A}$-expressing neurons. These results suggest that glycine 116 is required for C-terminal processing of GABARAP and that processing is essential for the localization of GABARAP and its functions as a trafficking protein.

Key words: **$GABA_A$ receptors; GABARAP; trafficking; ubiquitinylation-like modification; NSF; inhibitory neurotransmission**

Introduction

$GABA_A$ receptors are ligand-gated ion channels that mediate rapid inhibitory synaptic transmission in the CNS and serve as the target for many important neuroactive drugs (Macdonald and Olsen, 1994; Wallner et al., 2003). However, the molecular mechanisms regulating $GABA_A$ receptor trafficking and function are poorly understood. $GABA_A$ receptor-associated protein (GABARAP), a 117 aa protein, was first isolated by yeast two-hybrid screening using the intracellular domain of the $\gamma2$ subunit of the $GABA_A$ receptor as bait (Wang et al., 1999). In QT-6 cells, GABARAP clustered recombinant $GABA_A$ receptors, changed channel kinetics, and altered the dose–response curve for GABA (Chen et al., 2000). Furthermore, the single channel conductance was significantly higher in L929 cells coexpressing $\alpha1\beta2\gamma2S$ subunits with GABARAP than without (Everitt et al., 2004), showing high conductance states similar to those observed in native receptors (Fatima-Shad and Barry, 1992; Curmi et al., 1993; Birnir et al., 1994, 2001). Recently, we and others reported that overexpression of GABARAP increased the cell-surface number of $GABA_A$ receptors (Leil et al., 2004; Boileau et al., 2005; Chen et al., 2005). Immunostaining microscopy showed that GABARAP colocalized with intracellular vesicular $GABA_A$ receptors and to some extent with plasma membrane $GABA_A$ receptors but not with synaptically localized $GABA_A$ receptors and gephyrin (Kneussel et al., 2000; Kittler et al., 2001; Leil et al., 2004). Moreover, GABARAP has been shown to interact with soluble and polymerized tubulin (Wang and Olsen, 2000) and with the ATPase *N*-ethylmaleimide-sensitive factor (NSF), a chaperone that activates soluble NSF attachment protein receptor (SNARE) proteins in membrane fusion events (Kittler et al., 2001; Leil et al., 2004). This evidence implies that GABARAP might be an important factor regulating the intracellular trafficking of $GABA_A$ receptors, despite the fact that loss of GABARAP in knock-out mice does not eliminate synaptic targeting of $GABA_A$ receptors in neurons (O'Sullivan et al., 2005).

Nuclear magnetic resonance (NMR) spectroscopy and x-ray crystallography demonstrated that one surface of GABARAP containing a ubiquitin-fold that is conserved in the family of GABARAP (Coyle et al., 2002; Knight et al., 2002; Stangler et al., 2002). Sequence analysis showed that G116 is conserved in all proteins of the GABARAP family: GEC1, GATE-16, LC-3, and yeast Apg8. The homologs of GABARAP can be modified in the C terminus, in a manner resembling ubiquitin (Ichimura et al.,

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2000; Tanida et al., 2003; Yamazaki-Sato et al., 2003). Ubiquitin, as well as several families of nonubiquitin proteins, such as SUMO (Muller et al., 2001), show an E1–E3 cascade of C-terminal modifications leading to covalent transfer to other proteins (Hochstrasser, 2000). Likewise, the GABARAP family modification first requires a proteolytic cleavage to expose glycine (amino acid 116 in GABARAP), additional activation by E1- and E2-like enzymes, and finally conjugation to other biomolecules, in this case phospholipids. In yeast homolog Apg8, the activated C terminus is attached to a chemically identified lipid phosphatidyl ethanolamine (Ichimura et al., 2000), apparently to promote membrane attachment (Kabeya et al., 2004). This study demonstrated that GABARAP might also conjugate to phospholipids, possibly via the same mechanism. This modification is critical for GABARAP to control intracellular $GABA_A$ receptor trafficking.

Materials and Methods

Vector construction. v5, GABARAP, and $GABARAP_{G116A}$ were amplified by PCR from pcDNA3.1/v5 and pGEX/GABARAP, respectively. The mutation glycine 116 to alanine ($GABARAP_{G116A}$) was mutagenized using mutant primer in the C terminus. The PCR products were cloned into pCR2.1 according to the manufacturer's instruction. After sequencing, the inserts containing v5, GABARAP, or $GABARAP_{G116A}$ were subcloned into the mammalian expression vector pcDNA 3.1 containing myc as the C-terminal tag. For experiments using *Xenopus laevis* oocytes, the GABARAP and $GABARAP_{G116A}$ were subcloned into pBluescript II SK vector. The Adeno-X Expression System (Clontech, Mountain View, CA) was used to construct the Ad expression vectors. The v5-GABARAP-myc or v5-GABARAP-myc cDNAs were inserted into pShuttle vector. The expression cassette from the pShuttle was excised and inserted into the Adeno-X viral DNA. Recombinant Adeno-X viral DNA was purified and analyzed by restriction mapping to confirm that it contained the v5-GABARAP-myc or v5-$GABARAP_{G116A}$-myc. The recombinant adenoviral DNA was digested with *Pac*I and transfected into human embryonic kidney 293 (HEK293) cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Lysates were used to reinfect HEK293 cells for large-scale production.

Cell culture and transfection. Rat adrenal pheochromocytoma cells (PC12) were maintained in DMEM (Invitrogen), supplemented with 10% horse serum and 5% heat-inactivated fetal bovine serum equilibrated with 5% CO_2/95% air at 37°C. PC12 cells with 80% confluence were transfected with pcDNA3.1/v5-GABARAP-myc-his or pcDNA3.1/v5-$GABARAP_{116A}$-myc-his by Lipofectamine 2000.

Primary neuron culture and infection. Low-density hippocampal neurons from embryonic day 18 Sprague Dawley rat were prepared by papain-dissociation (Worthington Biochemical, Lakewood, NJ) and cultured in neurobasal medium with B27 serum-free supplement and penicillin and streptomycin (Invitrogen). Briefly, embryos were removed on embryonic day 18 from maternal rats anesthetized with halothane and killed by decapitation. Hippocampi were dissected and placed in Ca^{2+}- and Mg^{2+}-free HEPES-buffered HBSS, pH 7.45. Tissues were dissociated by papain digestion followed by trituration through a 10 ml pipette and papain inhibitor treatment. Cells were pelleted and resuspended in B27 serum-free supplement with penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively). Dissociated cells were then plated at a density of 50,000 cells per well onto 12 mm round coverslips that had been precoated with poly-D-lysine (0.05 mg/ml) and washed with H_2O. The neurons were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C and fed every 7 d by exchange of 50% of the medium. Seven days after culturing *in vitro*, cells were infected with adenovirus expressing v5-GABARAP-myc-his or v5-$GABARAP_{116A}$-myc-his.

Immunostaining and confocal microscopy. Forty-eight hours after transfection or 4 d after the infection of the virus, the cells on glass coverslips coated with poly-D-lysine were fixed with 3.75% paraformaldehyde in PBS for 15 min at 4°C. For the surface-expressed receptor, cells were washed with PBS and incubated with primary antibody against the N terminus of γ2 (1:100) in 1% BSA for 1 h followed by a 1 h incubation of secondary antibody conjugated with fluorescent dye. For the intracellular proteins, cells were permeabilized with 0.02% Triton X-100 for 5 min followed by the primary-secondary antibody incubation (anti-γ2, 1:100; anti-v5, 1:200; anti-NSF, 1:200; secondary antibody, 1:400). For the dual-fluorescence staining with germ agglutinin and 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI), cells were incubated for another 15 min at room temperature. After mounting the coverslip on glass slides, cells were analyzed by Leica TCS SP MP inverted confocal microscopy. Z-stacks of the images were collected with an optical thickness of 1 μm and analyzed by MetaVue (Universal Imaging, Downingtown, PA) and ImagePro Plus software (Media Cybernetics, Silver Spring, MD).

Coimmunoprecipitation and Western blot. Forty-eight hours after the transfection or 4 d after infection, whole-cell protein was lysed by radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 50 mM Tris and proteinase inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin)]. After incubation on ice for 0.5 h, cell suspensions were centrifuged at 15,000 × *g* for 15 min at 4°C. The immunocomplexes were recovered with the aid of Protein G agarose (Sigma, St. Louis, MO). Proteins in cell lysates and the immunocomplexes were loaded by SDS-PAGE and transferred to polyvinylidene difluoride membrane by a semi-dry method. The membrane was then incubated in PBS buffer and 0.05% Tween with 5% nonfat milk and primary antibody at room temperature for 1 h. After incubation with secondary antibody for 1 h, the membrane was visualized by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). The blot was scanned and analyzed with Quantity One software (Bio-Rad, Hercules, CA).

Xenopus laevis *oocyte expression system.* Capped mRNA (cRNA) was synthesized by *in vitro* transcription from *Apa* I-linearized cDNA constructs using the mMessage Machine kit (Ambion, Austin, TX) as described previously (Chang et al., 2003; Chen et al., 2005). Oocytes were injected with a total volume of 50 nl of cRNA mixed in a ratio of 1:1:2:4 (α1:β2:γ2: H_2O, GABARAP or mutant GABARAP). Oocytes were maintained in six-well plates at 17–19°C in SOS solution (in mM: 100 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES), supplemented with 50 μg/ml gentamycin and 100 μg/ml streptomycin and penicillin, and were used for electrophysiological experiments 3–5 d after injection.

Two-electrode voltage-clamp analysis. Oocytes under a two-electrode voltage clamp (holding potential −70 mV) were gravity perfused continuously with ND96 recording solution containing (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES, pH 7.4, at a rate of ~5 ml/min. In general, drugs and reagents were dissolved in ND96. A standard two-electrode voltage-clamp recording was performed using an Axoclamp-2A amplifier (Molecular Devices, Union City, CA) interfaced to a computer with a DigiData 1322-A device (Molecular Devices). Electrodes were filled with 3 M KCl and had a resistance of 0.5–1.5 MΩ. Data acquisition and analyses were performed using pClamp 8.2 (Molecular Devices) and Prism software (GraphPad Software, San Diego, CA).

Cell-surface biotinylation assay. Surface-expressed proteins were biotinylated by the membrane impermeable EZ-link sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin; Pierce, Rockford, IL). Four days after injection, oocytes (10 oocytes per group) were washed with ND96 solution three times, incubated with 1 mg/ml sulfo-NHS-SS-biotin at room temperature for 30 min, and then washed with 25 mM Tris, pH 8.0, followed by two washes with PBS. Oocytes were homogenized in 200 μl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.5). The yolk and cellular debris were removed after three centrifugations at 3600 × *g* for 10 min. A 20 μl aliquot was taken and mixed with 2× SDS loading buffer to detect total proteins. The remaining supernatant was incubated with streptavidin beads at 4°C overnight. The eluted proteins and whole proteins were detected by Western blot using anti-γ2.

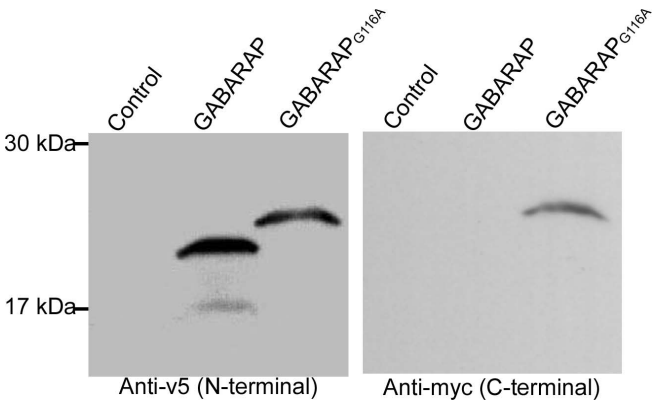

Figure 1. Mutation of Gly116 of GABARAP abolishes C-terminal processing. Whole-cell proteins from PC12 cells transfected with v5-GABARAP-myc or v5-GABARAP$_{G116A}$-myc were extracted and detected by anti-v5 and anti-myc in a Western blot. A 22 kDa band was detected only by anti-v5 in wild-type GABARAP-treated neurons (lane 2 in each gel). In addition, another two smaller-M_r bands were also visible, indicating some additional modification, such as lipidation. In mutant GABARAP$_{G116A}$-treated cells, a 24 kDa band was found with both anti-v5 and anti-myc staining (lane 3 in each gel), indicating that the C-terminal tag was cleaved off in wild-type GABARAP but not in the mutant.

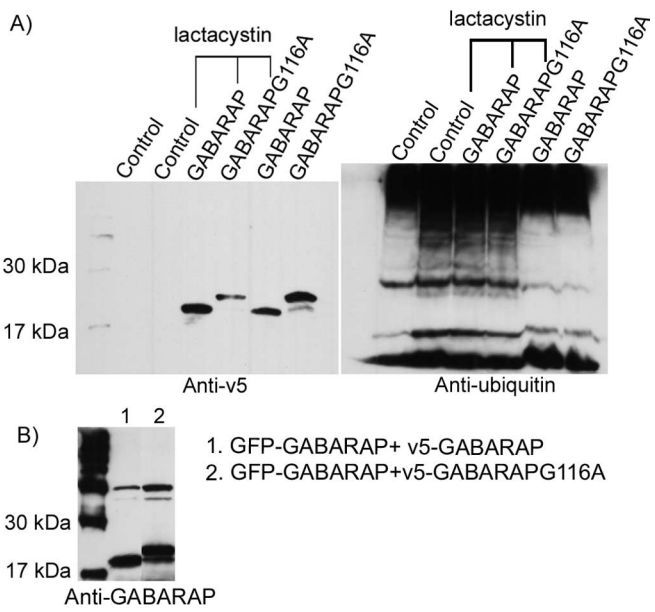

Figure 2. ***A***, GABARAP is not degraded by ubiquitinylation through the proteasome. Four days after infection of v5-GABARAP-myc or v5-GABARAP$_{G116A}$-myc, hippocampal neurons were treated with 5 μM lactacystin for 12 h. Western blot with anti-v5 demonstrated that, unlike ubiquitin, which showed accumulated M_r bands after lactacystin treatment, neither GABARAP nor GABARAP$_{G116A}$ showed any alteration in molecular weight or quantity after lactacystin treatment. ***B***, GABARAP formed dimers or higher-order polymers in PC12 cells. v5-GABARAP-myc or v5-GABARAP$_{G116A}$-myc was cotransfected with GFP–GABARAP into PC12 cells for 48 h. Anti-v5-protein G-agarose immunoprecipitated the whole-cell proteins followed by anti-GABARAP staining in a Western blot. A 40 kDa band, corresponding to GFP–GABARAP, was detected along with a 22 or 24 kDa band equivalent to v5-GABARAP-myc and v5-GABARAP$_{G116A}$-myc, respectively, indicating the formation of homodimers or polymers.

Results

GABARAP shows C-terminal cleavage prevented by mutation of glycine 116 to alanine

To examine the role of Gly116 in the C-terminal modification of GABARAP, a mammalian expression vector containing either GABARAP or mutant GABARAP$_{G116A}$ tagged by v5 in the N terminus and myc in the C terminus, was transfected into PC12 cells. Forty-eight hours after the transfection, whole-cell protein was extracted and loaded on SDS-PAGE gels followed by immunostaining with anti-v5 and anti-myc antibody. In wild-type GABARAP-transfected cells (Fig. 1), a 22 kDa band was detected by the anti-v5 antibody. However, there was no band visible with anti-myc staining, indicating that the C-terminal myc was completely cleaved off from GABARAP. In addition, other lower-molecular-weight (M_r) bands were also visible with anti-v5 staining, suggesting additional modification of GABARAP similar to Apg8, possibly a lipid adduct conjugation. In mutant GABARAP$_{G116A}$-transfected cells, a larger band (24 kDa) is seen, which is stained by both anti-v5 and anti-myc staining, indicating that the G116A mutation was not modified on the C terminus, and mutation of Gly116 to Ala prevents C-terminal cleavage of GABARAP.

GABARAP itself does not undergo ubiquitinylation or transfer to other proteins

The C-terminal modification of GABARAP is similar to the initial steps in ubiquitinylation. To determine whether GABARAP itself undergoes ubiquitinylation, neurons infected with GABARAP or GABARAP$_{G116A}$ were treated with 5 μM lactacystin, a proteasome inhibitor, for 12 h. Western blots with anti-v5 antibody from three independent experiments did not reveal any change in the molecular weight or quantity of either wild-type or mutant GABARAP after lactacystin treatment. In addition, densitometry analysis showed no difference between wild-type GABARAP and mutant GABARAP$_{G116A}$ before or after lactacystin treatment (Fig. 2*A*, left). This suggests that GABARAP is not covalently attached to other proteins, and it is not degraded through the proteasome. The same blot was stripped to remove anti-v5 and stained by anti-ubiquitin. Anti-ubiquitin recognized ubiquitin (8.5 kDa), its polymers (17 and 25 kDa), and ubiquitinylated proteins (higher-M_r proteins) in the groups without lactacystin treatment. After lactacystin treatment, the quantity of ubiquitin and polyubiquitinylated proteins were increased (Fig. 2*A*, right). A band indicating ubiquitin coupling to GABARAP was not detected, suggesting that GABARAP does not undergo the ubiquitinylation process.

GABARAP forms noncovalent dimers or higher-order polymers

Previous studies have suggested that GABARAP may be capable of dimerization. To identify whether GABARAP forms dimers in cells, green fluorescent protein (GFP)-GABARAP and v5-GABARAP or v5-GABARAP$_{G116A}$ were cotransfected into PC12 cells. Forty-eight hours after transfection, whole-cell proteins were extracted in RIPA buffer and recovered by anti-v5 and Protein G agarose. The immunocomplexes were loaded on SDS-PAGE gels followed by anti-GABARAP staining. v5-GABARAP or v5-GABARAP$_{G116A}$ was detected at 22 and 24 kDa, respectively. An additional 40 kDa band corresponding to the M_r of GFP–GABARAP was also seen in both groups, indicating that GFP–GABARAP was coimmunoprecipitated with both wild-type GABARAP and GABARAP$_{G116A}$. Anti-GFP staining confirmed that the 40 kDa band contained GFP-fusion protein (Fig. 2*B*).

Disruption of the modification by mutation of Gly116 to Ala changes the subcellular localization of GABARAP in PC12 cells

To address the effect of C-terminal modification on the subcellular localization of GABARAP, we examined the distribution of

GABARAP and $GABARAP_{G116A}$ by staining with anti-v5 and anti-myc 48 h after transfection in PC12 cells (Fig. 3*A*). Whereas v5-immunoreactivity was evident in both wild-type GABARAP and $GABARAP_{G116A}$-positive cells, myc immunoreactivity was detected only in $GABARAP_{G116A}$-positive cells, further confirming our Western blot results that the C terminus of GABARAP is posttranslationally cleaved off. GABARAP immunoreactivity was detected ubiquitously in PC12 cells; however, $GABARAP_{G116A}$ immunoreactivity was localized peripherally near the cytoplasmic membrane. To further characterize the subcellular localization of $GABARAP_{G116A}$, we performed triple-labeling experiments with markers for the Golgi apparatus [wheat germ agglutinin (WGA)] and the nucleus (DAPI). Compared with wild-type GABARAP that was found in all parts of the cell, distinct from and within the Golgi and nucleus, $GABARAP_{G116A}$ only colocalized with the Golgi marker WGA, not DAPI. In addition, the distribution of the Golgi apparatus was changed by the mutation (Fig. 3*B*), suggesting that GABARAP is important for maintaining the normal distribution and function of the Golgi apparatus. It also implies that mutation of Gly116 prevents trafficking of GABARAP through the Golgi.

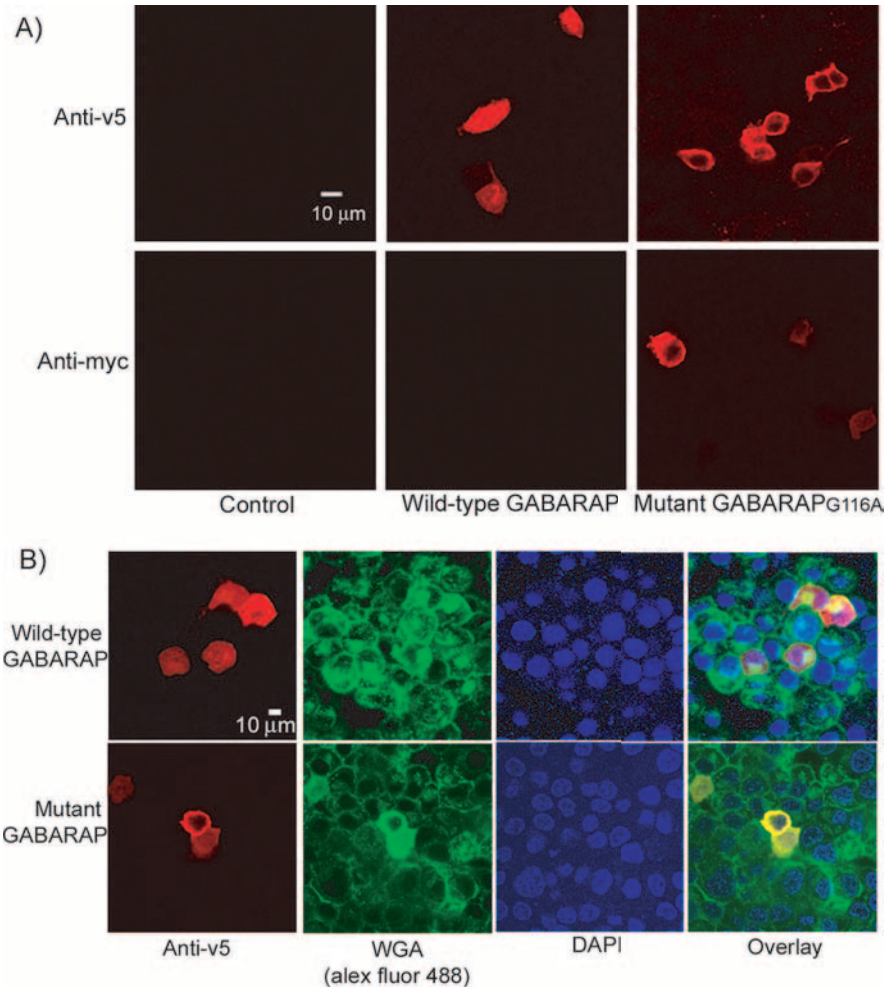

Figure 3. Disruption of the modification by mutation of Gly116 to Ala changes the subcellular localization of GABARAP in PC12 cells. ***A***, Three days after transfection, PC12 cells were fixed, permeabilized, and then stained with anti-v5 and anti-myc, respectively. Mutant $GABARAP_{G116A}$ was detected by both anti-v5 and anti-myc immunostaining, whereas wild-type GABARAP could only be detected with the anti-v5 antibody. Wild-type GABARAP was ubiquitously located in the cytoplasm; however, mutant $GABARAP_{G116A}$ was limited in the peripheral part of the cells. ***B***, Triple-immunofluorescent staining was performed in permeabilized PC12 cells with anti-v5 (red), WGA Alexa Fluor 488 (the marker of Golgi; green), and DAPI (the marker of nuclei; blue). The merged image showed that wild-type GABARAP localized ubiquitously in the cell, whereas mutant $GABARAP_{G116A}$ colocalized primarily with WGA.

Mutation of Gly116 to Ala changes the subcellular localization of GABARAP and $GABA_A$ receptors and reduces the interaction between $GABA_A$ receptors and GABARAP in hippocampal neurons

To investigate the effect of the Gly116 mutation of GABARAP on native $GABA_A$ receptors, an adenoviral vector containing either GABARAP or $GABARAP_{G116A}$ was used to infect low-density cultured hippocampal neurons (7 d after *in vitro* culture). In agreement with the findings reported in PC12 cells, Western blotting of extracts from these neurons showed that C-terminal processing only occurs in wild-type GABARAP and was abolished in $GABARAP_{G116A}$ (data not shown). Anti-v5 staining of infected permeabilized neurons revealed punctate staining of GABARAP in the cell soma and processes (Fig. 4*A*, left). In contrast, $GABARAP_{G116A}$ was localized primarily to the soma, a pattern similar to that observed in PC12 cells (Fig. 4*A*, right). Image analysis of the neuronal processes indicated that the intensity in the neuronal processes of wild-type GABARAP (95.58 ± 8.92; $n = 16$) is significantly higher ($p < 0.01$) than that of mutant $GABARAP_{G116A}$ (62.4 ± 5.57; $n = 14$), whereas the intensity of the v5 immunoreactivity of whole cells has no difference between these two groups.

The effect of the Gly116 mutation of GABARAP on its colocalization with native $GABA_A$ receptors was examined by immunostaining with anti-v5 and anti-γ2 antibodies in permeabilized cultured hippocampal neurons (Fig. 4*B*). In GABARAP-infected neurons, $GABA_A$ receptors were located in both cell body and processes, with the punctate staining located mainly in the processes. Compared with wild-type GABARAP-positive neurons, more $GABA_A$ receptors were localized in the cell body rather than processes in mutant $GABARAP_{G116A}$-infected neurons. Image analysis indicated that overexpression of wild-type GABARAP increased the intensity of $GABA_A$ receptors in the neuronal processes compared with noninfected neurons ($p < 0.001$). The mutant $GABARAP_{G116A}$ did not demonstrate this enhancement effect. The total cell γ2 content shows no difference among these three groups (Fig. 4*C*). GABARAP was colocalized highly with γ2-containing $GABA_A$ receptors, chiefly in the base and branches of processes, but a greater fraction of the $GABA_A$ receptors were located in the soma with mutant $GABARAP_{G116A}$. Pearson's correlation indicated that wild-type GABARAP colocalized more extensively with $GABA_A$ receptors than did the mutant $GABARAP_{G116A}$ (0.714 ± 0.0156 and 0.334 ± 0.0255, respectively; $p < 0.001$; $n = 36–42$).

To determine whether mutation of Gly116 directly affects the interaction of GABARAP with $GABA_A$ receptors, coimmunoprecipitation using anti-γ2 antibody was used. Both wild-type and

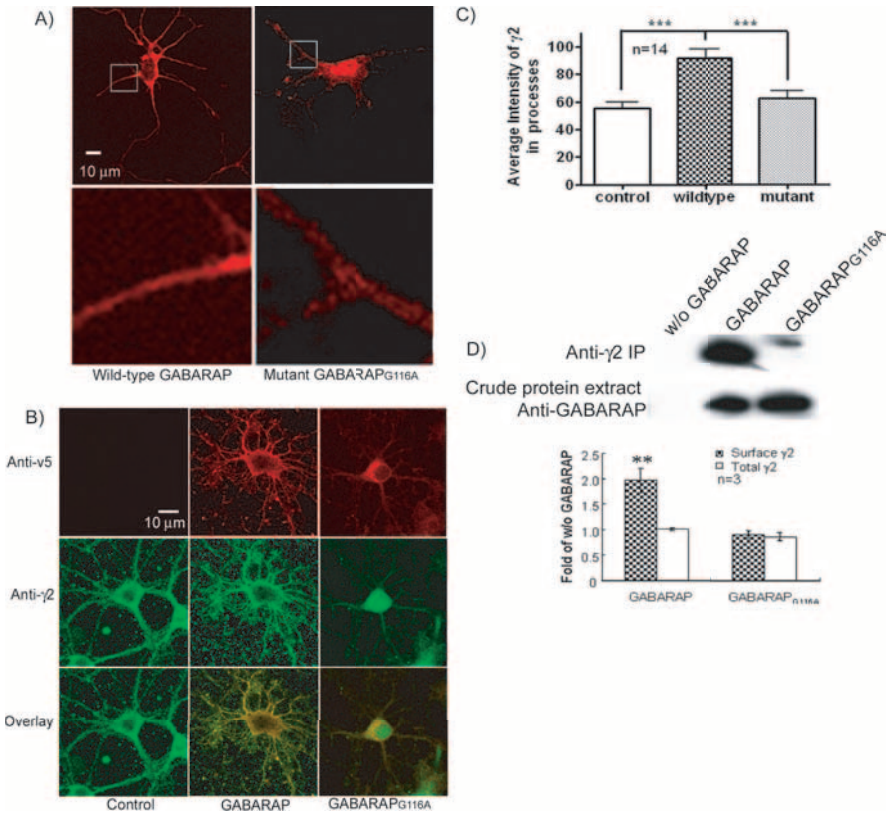

Figure 4. Mutation of Gly116 to Ala changes the subcellular localization of GABARAP and $GABA_A$ receptors and reduces the interaction between $GABA_A$ receptor and GABARAP in hippocampal neurons. ***A***, Low-density cultures of rat hippocampal neurons were infected by adenoviral v5-GABARAP-myc and v5-$GABARAP_{G116A}$-myc. Four days after infection, neurons (11 d) were stained with anti-v5 to show the distribution of GABARAP. Wild-type GABARAP localized ubiquitously throughout neurons, including both cell body and processes; however, most of the mutant $GABARAP_{G116A}$ localized merely in the cell body. Image analysis of neuronal processes demonstrated that the intensity of GABARAP in neuronal processes is higher than that of mutant $GABARAP_{G116A}$. ***B***, Confocal images of permeabilized primary cultured hippocampal neurons showed colocalization of GABARAP (red) and γ2 subunits (green). $GABARAP_{G116A}$ has reduced colocalization with $GABA_A$ receptors compared with wild-type GABARAP as indicated by Pearson correlation ($p < 0.001$). ***C***, Overexpression of GABARAP increased the intensity of γ2 subunit in neuronal processes compared with the noninfected neurons. Mutant $GABARAP_{G116A}$ showed no effect on the γ2 subunits. ***D***, Mutation of Gly116 decreased the interaction of GABARAP with the γ2 subunit of the receptor. Top, Whole-cell proteins extracted 4 d after infection with GABARAP or $GABARAP_{G116A}$ were immunoprecipitated by polyclonal anti-γ2 and protein G-agarose followed by Western blot using anti-v5. Anti-γ2 pulled down more wild-type GABARAP than $GABARAP_{G116A}$. Bottom, Western blot with anti-v5 showed the same level of protein expression of wild-type GABARAP and $GABARAP_{G116A}$. Error bars represent SEM.

mutant GABARAP were pulled down along with the γ2 subunit, although the quantity of $GABARAP_{G116A}$ pulled down was considerably lower, indicating that the degree of interaction between γ2 subunits and $GABARAP_{G116A}$ in cells was weaker than that between γ2 subunits and wild-type GABARAP (Fig. 4*D*). This result is consistent with the confocal analysis, which showed that mutation of GABARAP Gly116 reduced colocalization with the γ2 subunit-containing $GABA_A$ receptors.

Mutation in $GABARAP_{G116A}$ abolishes the GABARAP enhancement of cell-surface $GABA_A$ receptors

Intact, fixed hippocampal neurons transfected with wild-type or mutant GABARAP were stained with anti-γ2 (nonpermeabilized) and anti-v5 antibody (after permeabilization). $GABA_A$ receptors containing γ2 subunits were found in clusters at the plasma membrane, a fraction of which were colocalized with wild-type GABARAP (Fig. 5*A*). $GABARAP_{G116A}$ displayed dramatically reduced colocalization with surface clusters of these $GABA_A$ receptors (Fig. 5*A*). The surface expression of the γ2 subunit was significantly increased by overexpression of GABARAP in the hippocampal neurons ($p < 0.05$) but not by overexpression of $GABARAP_{G116A}$ (Fig. 5*B*). This suggests that GABARAP, but not $GABARAP_{G116A}$, is capable of trafficking $GABA_A$ receptors to the plasma membrane, as we reported previously for wild-type GABARAP (Leil et al., 2004).

Mutation in $GABARAP_{G116A}$ blocks stimulation of GABA receptor channel expression in *X. laevis* oocytes

We have reported previously that GABARAP increased both GABA currents and cell-surface content of $GABA_A$ receptor polypeptides in *X. laevis* oocytes (Chen et al., 2005). The effects of the Gly116 mutation of GABARAP were studied in this system. As shown in Figure 6, *A* and *B*, in agreement with previous results, coexpression of GABARAP increased the GABA currents by twofold to threefold compared with currents obtained from oocytes in which GABARAP was not coexpressed. The maximum currents (in nanoamperes) in the GABARAP-expressing group were 697.8 ± 84.3 ($n = 16$). However, coexpression of mutant $GABARAP_{G116A}$ and $GABA_A$ subunits resulted in GABA-evoked currents of ~306.5 ± 67.8 nA ($n = 12$) that were similar to those derived from cells expressing $GABA_A$ receptor subunits alone but significantly less than currents obtained from cells coexpressing wild-type GABARAP. The concentration–response curve for GABA revealed that the change was mainly caused by a decrease in the maximum response, because there is little change in the EC_{50}. Measurement of surface $GABA_A$ receptors by cell-surface biotinylation demonstrated that wild-type GABARAP was able to increase the level of the γ2 subunit at the cell surface, whereas $GABARAP_{G116A}$ was not (Fig. 6*C*). This suggests that C-terminal processing of GABARAP is required for trafficking of $GABA_A$ receptors.

Mutation of Gly116 of GABARAP disrupts subcellular localization of NSF

To examine the effects of mutant $GABARAP_{G116A}$ on NSF, permeabilized cultured hippocampal neurons were labeled with polyclonal goat anti-v5 and monoclonal anti-NSF followed by secondary antibody incubation. In three experiments, NSF was evident in both the cell body and processes of GABARAP-infected neurons and showed a high degree of colocalization with GABARAP. This is illustrated for one cell in Figure 7. However, in neurons that were infected with $GABARAP_{G116A}$ ($n = 7$), NSF was found to be redistributed mainly to the cell body, as was GABARAP, with the two proteins still colocalized. Image analysis by Pearson's correlation indicated no significant difference between GABARAP versus $GABARAP_{G116A}$ colocalization with NSF.

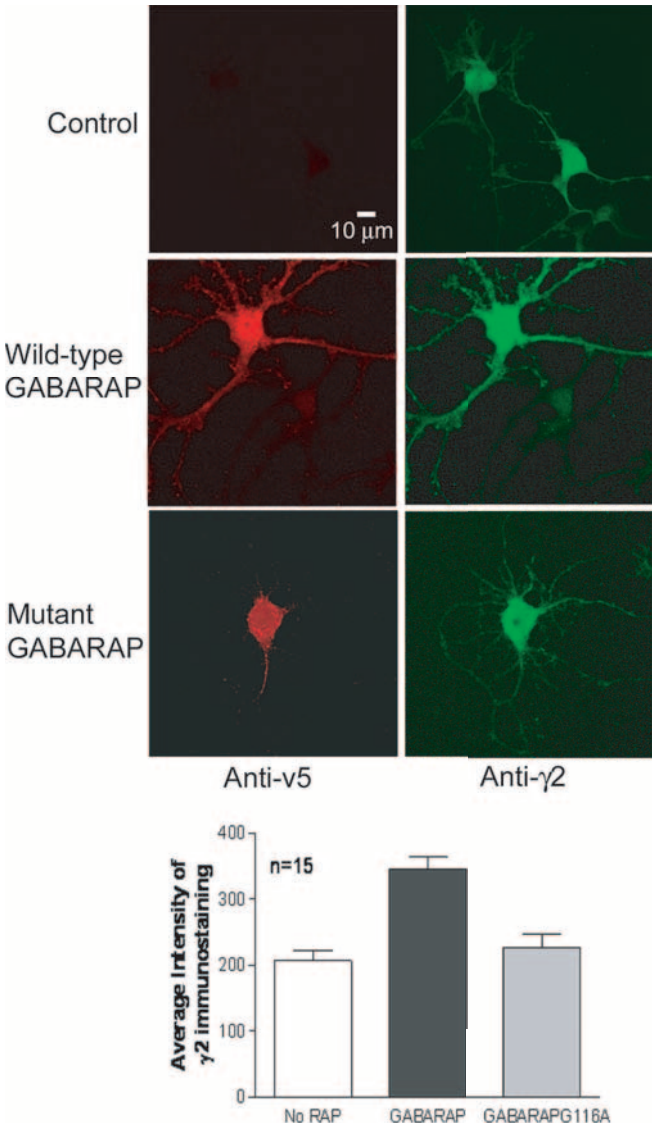

Figure 5. Mutation in $GABARAP_{G116A}$ abolished the GABARAP enhancement of cell-surface $GABA_A$ receptors. ***A***, Confocal image of surface-expressed γ2 subunits (green; nonpermeabilized) in cultured hippocampal neurons overexpressing wild-type GABARAP or $GABARAP_{G116A}$ (red anti-v5 staining; permeabilized). ***B***, Image analysis demonstrated that neurons transfected with wild-type GABARAP show significantly increased levels of γ2 immunostaining at the cell surface ($p < 0.05$), whereas those transfected with $GABARAP_{G116A}$ do not. Error bars represent SEM.

Discussion

The presence of the major ubiquitin fold in GABARAP suggests that it may undergo processing in a manner similar to ubiquitin. Our results verify that GABARAP does undergo C-terminal processing, which is blocked by mutation of glycine 116 to alanine (Fig. 1). These results are in agreement with those found in starved HEK cells in which GABARAP, GEC1, GATE-16, and LC-3 were modified in the C terminus (Tanida et al., 2003). However, unlike ubiquitinylation, in which ubiquitin is covalently attached to other proteins, GABARAP does not appear to be incorporated covalently into higher-M_r protein complexes either singly or in multiple copies (Fig. 2*A*). GABARAP itself is not degraded via ubiquitin-mediated proteolysis because inhibition of proteasome activity did not result in accumulation of high-M_r GABARAP protein complexes. GABARAP does form a noncovalent dimer (Nymann-Andersen et al., 2002) and the C-terminal mutation did not interfere with the dimerization (Fig. 2*B*). However, we could not exclude the possibility that GABARAP might also form noncovalent polymers.

GABARAP is related in both amino acid sequence and three-dimensional structure to homologous proteins from yeast (Apg8 or AUT7P) and mammals [GEC1, GATE-16, and light chain 3 (LC3) of microtubule-associated protein]. This posttranslational processing of the C terminus is conserved in all of the proteins of the GABARAP family (Ichimura et al., 2000; Kabeya et al., 2000; Tanida et al., 2003). Yeast Apg8 was the first of the GABARAP family shown to undergo this modification. During the modification, the last amino acid in the C terminus is first cleaved by the activating cysteine protease Apg4, leaving glycine116 exposed (Ichimura et al., 2000). This penultimate glycine, which is conserved in all proteins in this family, is activated by Apg7 and transferred to Apg3, which are E1 and E2 enzymes, respectively (Komatsu et al., 2001; Yamazaki-Sato et al., 2003). Finally, it is conjugated to the membrane lipid phosphatidyl ethanolamine (PE) (Ichimura et al., 2000), catalyzed by an as yet unidentified E3. The lipid PE can also be removed by the priming protease Apg4, releasing the C-terminal glycine that is then exposed for a new processing cycle (Hemelaar et al., 2003). The human homologs of Apg4, Apg7, and Apg3 were recently cloned and their enzymatic activities on GABARAP and its homologs were verified (Tanida et al., 2001, 2002, 2004; Hemelaar et al., 2003; Scherz-Shouval et al., 2003; Yamazaki-Sato et al., 2003). Yeast Apg8 was shown to give two bands by SDS-PAGE, the unmodified protein with apparent M_r of 14 kDa and the lipid adduct at slightly lower apparent M_r (12 kDa). This latter band was more enriched in the membrane fraction of cell homogenates. A similar lower-M_r band (Fig. 1, left, lane 2) with hydrophobic properties, presumably a lipid adduct, was also observed for the GABARAP family (Tanida et al., 2003; Kabeya et al., 2004). However, no E3 enzyme for this modification has been identified. E3 enzymes are substrate specific and thus assessment of specific product formation is required for identification of the specific E3.

Conjugation of the yeast homolog of GABARAP, Apg8, with PE results in its tight association with the autophagosome vesicle membrane (Ichimura et al., 2000). Many cytosolic proteins involved in receptor trafficking or docking associate with membranes via lipophilic substitutents. For example, membrane localization and clustering of glutamate receptors and other ion channels by the synaptic PDZ [postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1]-containing protein PSD-95 depends on palmitoylation (El-Husseini et al., 2000). Palmitoylation of ABP (AMPA receptor-binding protein) targets it to the membrane and allows it to associate with GluR2 (glutamate receptor 2) at the plasma membrane in spines of cultured hippocampal neurons (DeSouza et al., 2002). Lipidation of PICK1 (protein interacting with C kinase 1) allows it to traffic AMPA receptors to synapses (Jin et al., 2006). Subcellular fractionation studies showed that GABARAP is associated with a membrane compartment in HEK cells (Tanida et al., 2003), suggesting that GABARAP might conjugate with a lipid to increase its affinity for membranes. Like the lipid adduct of yeast Apg8, which runs at slightly lower apparent M_r (Ichimura et al., 2000; Kabeya et al., 2004), it is possible that the smaller protein observed in our Western blots probed with anti-v5 is the lipidated form of GABARAP (Fig. 1). Disruption of the C-terminal processing by mutation of glycine 116 blocked GABARAP lipidation, thereby preventing GABARAP from being transported out of the trans-Golgi (Fig. 2*B*,*C*).

GABARAP was shown previously to increase the cell-surface

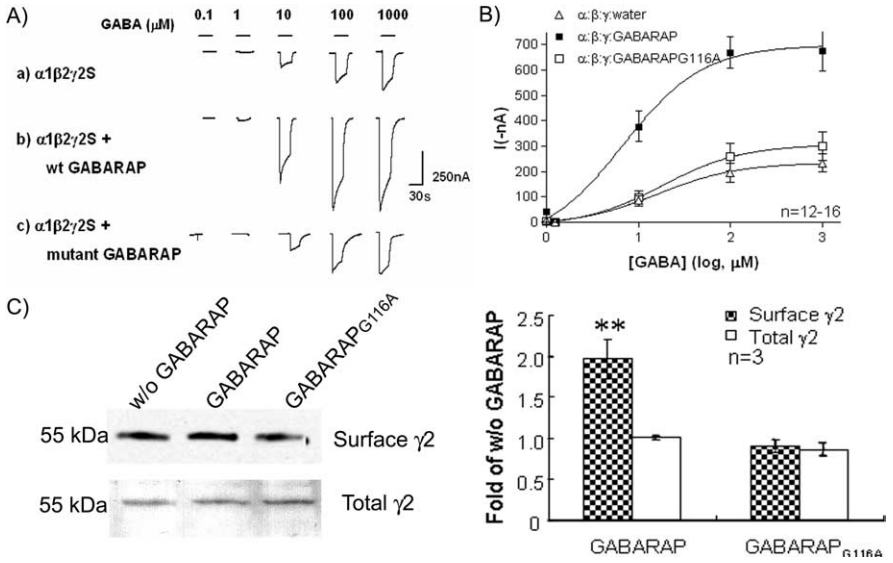

Figure 6. Mutation in $GABARAP_{G116A}$ blocks stimulation of $GABA_A$ receptor channel surface expression. ***A***, ***B***, Two-electrode voltage clamp recording showed that $GABARAP_{G116A}$ eliminated the enhancement effect on GABA current induced in oocytes. ***A***, Example of GABA-induced currents in oocytes expressing $\alpha1\beta2\gamma2S$ subunits with water, GABARAP, or $GABARAP_{G116A}$ in a ratio of 1:1:2:4. ***B***, GABA dose–response curve. In *Xenopus* oocytes expressing $\alpha1\beta2\gamma2S$ subunits, GABARAP increased the maximal evoked GABA currents (without affecting EC_{50}), whereas $GABARAP_{G116A}$ did not. The maximum chloride currents elicited by 0.1–1000 μM GABA in oocytes coexpressed with $\alpha1\beta2\gamma2S$ $GABA_A$ subunits and $GABARAP_{G116A}$ were not statistically different ($p < 0.001$) from currents obtained from oocytes expressing $\alpha1\beta2\gamma2S$ subunits alone. ***C***, Surface biotinylation assay demonstrated that GABARAP enhanced the cell-surface expression of $GABA_A$ receptor in oocytes. $GABARAP_{G116A}$ blocked the enhancement (as shown in the top lanes). However, GABARAP has no effect on the biosynthesis of $GABA_A$ receptor $\gamma2$ subunit. Error bars represent SEM.

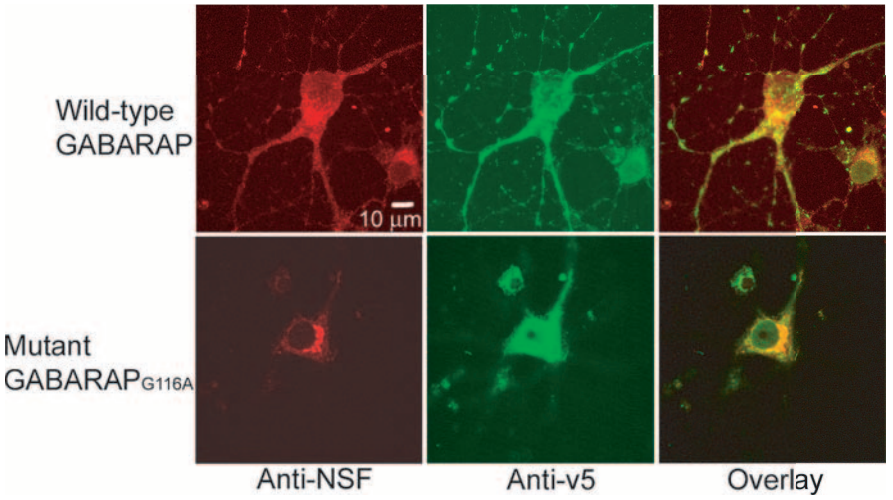

Figure 7. Mutation of Gly116 of GABARAP disrupts subcellular localization of NSF. Permeabilized cultured hippocampal neurons (11 d) were stained by anti-v5 (green) and anti-NSF (red) 4 d after infection. In $GABARAP_{G116A}$-positive neurons, the distribution of NSF was disrupted.

expression of $GABA_A$ receptors (Leil et al., 2004; Boileau et al., 2005; Chen et al., 2005). Applying GABARAP IgG from Stiff-Person Syndrome patients decreased the surface $GABA_A$ receptors in cultured hippocampal neurons (Raju et al., 2006). Here, we provide additional evidence that GABARAP enhances the surface expression of $GABA_A$ receptors, suggesting that GABARAP facilitates the intracellular trafficking of $GABA_A$ receptors. GABARAP has been shown to interact with some trafficking factors, such as microtubules, NSF, and transferrin receptors. In addition, GATE-16 has been shown to enhance the ATPase activity of NSF and is believed to be a component of the intra-Golgi transport machinery (Legesse-Miller et al., 1998; Sagiv et al., 2000; Muller et al., 2002). In this study, blocking the processing of GABARAP by G116A interferes with its intracellular trafficking and results in its subcellular redistribution. At the same time, the subcellular localization of $GABA_A$ receptors was also changed, further supporting the conclusion that GABARAP participates in intracellular trafficking of $GABA_A$ receptors.

Control of neurotransmitter receptor expression at the postsynaptic membrane is of critical importance for functional neurotransmission. Sorting, targeting, clustering, and degradation of receptors as dynamic processes play a key role in the construction and functional maintenance of synapses. It is well established that the sorting of newly synthesized membrane proteins to distinct domains in polarized cells appears to begin in the Golgi/trans-Golgi network (TGN), where proteins can segregate and exit in separate transport vesicles (Keller et al., 2001). $GABARAP_{G116A}$ showed a reduced intracellular colocalization pattern with $GABA_A$ receptors (Fig. 4). Immunoprecipitation results indicated that anti-$\gamma2$ pulls down less mutant $GABARAP_{G116A}$ than wild-type GABARAP, confirming that the interaction between $GABARAP_{G116A}$ and $\gamma2$-containing $GABA_A$ receptors in cells was decreased. The unmatched subcellular distribution of $GABA_A$ receptor and $GABARAP_{G116A}$ suggests that there are some other factors regulating the intracellular trafficking of $GABA_A$ receptors. Recent studies using the yeast two-hybrid system identified some new $GABA_A$ receptor-associated proteins (Chen and Olsen, 2007), such as the GABARAP homolog GEC1 (Mansuy et al., 2004), Golgi-specific DHHC zinc finger protein (GODZ) (Keller et al., 2004), Plic-1 (Bedford et al., 2001), and HAP (Huntingtin-associated protein) (Kittler et al., 2004). These proteins can also traffic $GABA_A$ receptors, through binding other subunits of the pentamer, out of the TGN to a final destination. Because there is no apparent change in synaptic expression of $GABA_A$ receptors in cortex of GABARAP knockout mice (O'Sullivan et al., 2005), this also suggests that other $GABA_A$ receptor-associated proteins might substitute for, or supplement, the function of GABARAP. Coexpression of mutant $GABARAP_{G116A}$ in both neurons and oocytes expressing $\alpha1\beta2\gamma2S$ subunits showed less cell-surface-expressed $GABA_A$ receptor. This loss of receptor surface expression may have been attributable to the abolition of the interaction between $GABARAP_{G116A}$ and intracellular transportation tubulovesicular membranes containing $GABA_A$ receptors, resulting in a reduction in the intracellular trafficking of $GABA_A$ receptor to the cell surface. In addition to the enhancement of the surface expression

of the $GABA_A$ receptors by GABARAP in oocytes (Fig. 6), GABARAP may also change the kinetics as reported by Chen et al. (2000) and Everitt et al. (2004). However, oocytes studied with the two-electrode voltage clamp are not technically appropriate for kinetics studies. Oocytes may lack clustering factors, such as gephyrin, needed to demonstrate the kinetics changes as observed in QT-6 cells and L929 cells.

Another possible conclusion is that GABARAP affects the distribution of $GABA_A$ receptor through NSF. In the study of colocalization between GABARAP and NSF, we observed that the subcellular localization of NSF was disturbed by the mutant $GABARAP_{G116A}$. NSF has been shown to participate in the fusion of transport vesicles with the membrane of the acceptor or target compartment. In this fusion process, v-SNARE, a specific protein expressed in the intracellular vesicle, binds to a receptor on the target membrane (t-SNARE). NSF is recruited to this complex via soluble NSF attachment proteins (SNAPs) that can bind directly to the SNARE proteins and stimulate the ATPase activity of NSF. In consequence, the SNARE complexes dissociate after membrane fusion, allowing the individual SNARE proteins to be recycled for subsequent rounds of fusion (Rothman, 1994). By controlling the localization of NSF, the modification of GABARAP might also affect the function of NSF. NSF functions as the controller of the fusion of post-Golgi transport vesicles or fusion of intracellular vesicles with plasma membranes. In other words, NSF and GABARAP together may control either the intracellular trafficking and/or the membrane insertion of $GABA_A$ receptors.

In conclusion, we provide evidence that GABARAP is associated with the intracellular tubulovesicular transport pathway through its lipid-modified C terminus. At the same time, it also interacts with γ subunits of GABAA receptors located in vesicles and transported along microtubules to cytoplasmic membranes. The mutant $GABARAP_{G116A}$ cannot associate with trafficking vesicles in the TGN because of the lack of a hydrophobic C-tail, thereby losing its ability to traffic $GABA_A$ receptors. As a consequence, the number of $GABA_A$ receptors reaching specific target destinations at the plasma membrane was reduced in $GABARAP_{G116A}$-expressing neurons.

References

Bedford FK, Kittler JT, Muller E, Thomas P, Uren JM, Merlo D, Wisden W, Triller A, Smart TG, Moss SJ (2001) GABA(A) receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein Plic-1. Nat Neurosci 4:908–916.

Birnir B, Everitt AB, Gage PW (1994) Characteristics of $GABA_A$ channels in rat dentate gyrus. J Membr Biol 142:93–102.

Birnir B, Eghbali M, Cox GB, Gage PW (2001) GABA concentration sets the conductance of delayed $GABA_A$ channels in outside-out patches from rat hippocampal neurons. J Membr Biol 181:171–183.

Boileau AJ, Pearce RA, Czajkowski C (2005) Tandem subunits effectively constrain $GABA_A$ receptor stoichiometry and recapitulate receptor kinetics but are insensitive to $GABA_A$ receptor-associated protein. J Neurosci 25:11219–11230.

Chang CS, Olcese R, Olsen RW (2003) A single M1 residue in the beta2 subunit alters channel gating of $GABA_A$ receptor in anesthetic modulation and direct activation. J Biol Chem 278:42821–42828.

Chen L, Wang H, Vicini S, Olsen RW (2000) The gamma-aminobutyric acid type A ($GABA_A$) receptor-associated protein (GABARAP) promotes $GABA_A$ receptor clustering and modulates the channel kinetics. Proc Natl Acad Sci USA 97:11557–11562.

Chen ZW, Olsen RW (2007) $GABA_A$ receptor associated proteins: a key factor regulating $GABA_A$ receptor function. J Neurochem 100:279–294.

Chen ZW, Chang CS, Leil TA, Olcese R, Olsen RW (2005) $GABA_A$ receptor-associated protein regulates $GABA_A$ receptor cell-surface number in *Xenopus laevis* oocytes. Mol Pharmacol 68:152–159.

Coyle JE, Qamar S, Rajashankar KR, Nikolov DB (2002) Structure of GABARAP in two conformations: implications for GABA(A) receptor localization and tubulin binding. Neuron 33:63–74.

Curmi JP, Premkumar LS, Birnir B, Gage PW (1993) The influence of membrane potential on chloride channels activated by GABA in rat cultured hippocampal neurons. J Membr Biol 136:273–280.

DeSouza S, Fu J, States BA, Ziff EB (2002) Differential palmitoylation directs the AMPA receptor-binding protein ABP to spines or to intracellular clusters. J Neurosci 22:3493–3503.

El-Husseini AE, Craven SE, Chetkovich DM, Firestein BL, Schnell E, Aoki C, Bredt DS (2000) Dual palmitoylation of PSD-95 mediates its vesiculotubular sorting, postsynaptic targeting, and ion channel clustering. J Cell Biol 148:159–172.

Everitt AB, Luu T, Cromer B, Tierney ML, Birnir B, Olsen RW, Gage PW (2004) Conductance of recombinant GABA (A) channels is increased in cells co-expressing GABA(A) receptor-associated protein. J Biol Chem 279:21701–21706.

Fatima-Shad K, Barry PH (1992) A patch-clamp study of GABA- and strychnine-sensitive glycine-activated currents in post-natal tissue-cultured hippocampal neurons. Proc Biol Sci 250:99–105.

Hemelaar J, Lelyveld VS, Kessler BM, Ploegh HL (2003) A single protease, Apg4B, is specific for the autophagy-related ubiquitin-like proteins GATE-16, MAP1-LC3, GABARAP, and Apg8L. J Biol Chem 278:51841–51850.

Hochstrasser M (2000) Biochemistry. All in the ubiquitin family. Science 289:563–564.

Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, Noda T, Ohsumi Y (2000) A ubiquitin-like system mediates protein lipidation. Nature 408:488–492.

Jin W, Ge WP, Xu J, Cao M, Peng L, Yung W, Liao D, Duan S, Zhang M, Xia J (2006) Lipid binding regulates synaptic targeting of PICK1, AMPA receptor trafficking, and synaptic plasticity. J Neurosci 26:2380–2390.

Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 19:5720–5728.

Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T (2004) LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J Cell Sci 117:2805–2812.

Keller CA, Yuan X, Panzanelli P, Martin ML, Alldred M, Sassoe-Pognetto M, Luscher B (2004) The gamma2 subunit of GABA(A) receptors is a substrate for palmitoylation by GODZ. J Neurosci 24:5881–5891.

Keller P, Toomre D, Diaz E, White J, Simons K (2001) Multicolour imaging of post-Golgi sorting and trafficking in live cells. Nat Cell Biol 3:140–149.

Kittler JT, Rostaing P, Schiavo G, Fritschy JM, Olsen R, Triller A, Moss SJ (2001) The subcellular distribution of GABARAP and its ability to interact with NSF suggest a role for this protein in the intracellular transport of GABA(A) receptors. Mol Cell Neurosci 18:13–25.

Kittler JT, Thomas P, Tretter V, Bogdanov YD, Haucke V, Smart TG, Moss SJ (2004) Huntingtin-associated protein 1 regulates inhibitory synaptic transmission by modulating gamma-aminobutyric acid type A receptor membrane trafficking. Proc Natl Acad Sci USA 101:12736–12741.

Kneussel M, Haverkamp S, Fuhrmann JC, Wang H, Wassle H, Olsen RW, Betz H (2000) The gamma-aminobutyric acid type A receptor ($GABA_AR$)-associated protein GABARAP interacts with gephyrin but is not involved in receptor anchoring at the synapse. Proc Natl Acad Sci USA 97:8594–8599.

Knight D, Harris R, McAlister MS, Phelan JP, Geddes S, Moss SJ, Driscoll PC, Keep NH (2002) The X-ray crystal structure and putative ligand-derived peptide binding properties of gamma-aminobutyric acid receptor type A receptor-associated protein. J Biol Chem 277:5556–5561.

Komatsu M, Tanida I, Ueno T, Ohsumi M, Ohsumi Y, Kominami E (2001) The C-terminal region of an Apg7p/Cvt2p is required for homodimerization and is essential for its E1 activity and E1–E2 complex formation. J Biol Chem 276:9846–9854.

Legesse-Miller A, Sagiv Y, Porat A, Elazar Z (1998) Isolation and characterization of a novel low molecular weight protein involved in intra-Golgi traffic. J Biol Chem 273:3105–3109.

Leil TA, Chen ZW, Chang CS, Olsen RW (2004) $GABA_A$ receptor-associated protein traffics $GABA_A$ receptors to the plasma membrane in neurons. J Neurosci 24:11429–11438.

Macdonald RL, Olsen RW (1994) $GABA_A$ receptor channels. Annu Rev Neurosci 17:569–602.

Mansuy V, Boireau W, Fraichard A, Schlick JL, Jouvenot M, Delage-Mourroux R (2004) GEC1, a protein related to GABARAP, interacts with tubulin and GABA(A) receptor. Biochem Biophys Res Commun 325:639–648.

Muller JM, Shorter J, Newman R, Deinhardt K, Sagiv Y, Elazar Z, Warren G, Shima DT (2002) Sequential SNARE disassembly and GATE-16-GOS-28 complex assembly mediated by distinct NSF activities drives Golgi membrane fusion. J Cell Biol 157:1161–1173.

Muller S, Hoege C, Pyrowolakis G, Jentsch S (2001) SUMO, ubiquitin's mysterious cousin. Nat Rev Mol Cell Biol 2:202–210.

Nymann-Andersen J, Wang H, Olsen RW (2002) Biochemical identification of the binding domain in the GABA(A) receptor-associated protein (GABARAP) mediating dimer formation. Neuropharmacology 43:476–481.

O'Sullivan GA, Kneussel M, Elazar Z, Betz H (2005) GABARAP is not essential for GABA receptor targeting to the synapse. Eur J Neurosci 22:2644–2648.

Raju R, Rakocevic G, Chen Z, Hoehn G, Semino-Mora C, Shi W, Olsen R, Dalakas MC (2006) Autoimmunity to $GABA_A$-receptor-associated protein in stiff-person syndrome. Brain 129:3270–3276.

Rothman JE (1994) Intracellular membrane fusion. Adv Second Messenger Phosphoprotein Res 29:81–96.

Sagiv Y, Legesse-Miller A, Porat A, Elazar Z (2000) GATE-16, a membrane transport modulator, interacts with NSF and the Golgi v-SNARE GOS-28. EMBO J 19:1494–1504.

Scherz-Shouval R, Sagiv Y, Shorer H, Elazar Z (2003) The COOH terminus of GATE-16, an intra-Golgi transport modulator, is cleaved by the human cysteine protease HsApg4A. J Biol Chem 278:14053–14058.

Stangler T, Mayr LM, Willbold D (2002) Solution structure of human GABA(A) receptor-associated protein GABARAP: implications for biological function and its regulation. J Biol Chem 277:13363–13366.

Tanida I, Tanida-Miyake E, Ueno T, Kominami E (2001) The human homolog of *Saccharomyces cerevisiae* Apg7p is a protein-activating enzyme for multiple substrates including human Apg12p, GATE-16, GABARAP, and MAP-LC3. J Biol Chem 276:1701–1706.

Tanida I, Tanida-Miyake E, Komatsu M, Ueno T, Kominami E (2002) Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p. J Biol Chem 277:13739–13744.

Tanida I, Komatsu M, Ueno T, Kominami E (2003) GATE-16 and GABARAP are authentic modifiers mediated by Apg7 and Apg3. Biochem Biophys Res Commun 300:637–644.

Tanida I, Sou YS, Ezaki J, Minematsu-Ikeguchi N, Ueno T, Kominami E (2004) HsAtg4B/HsApg4B/autophagin-1 cleaves the carboxyl termini of three human Atg8 homologues and delipidates microtubule-associated protein light chain 3- and $GABA_A$ receptor-associated protein-phospholipid conjugates. J Biol Chem 279:36268–36276.

Wallner M, Hanchar HJ, Olsen RW (2003) Ethanol enhances alpha 4 beta 3 delta and alpha 6 beta 3 delta gamma-aminobutyric acid type A receptors at low concentrations known to affect humans. Proc Natl Acad Sci USA 100:15218–15223.

Wang H, Olsen RW (2000) Binding of the GABA(A) receptor-associated protein (GABARAP) to microtubules and microfilaments suggests involvement of the cytoskeleton in GABARAPGABA(A) receptor interaction. J Neurochem 75:644–655.

Wang H, Bedford FK, Brandon NJ, Moss SJ, Olsen RW (1999) GABA(A)-receptor-associated protein links GABA(A) receptors and the cytoskeleton. Nature 397:69–72.

Yamazaki-Sato H, Tanida I, Ueno T, Kominami E (2003) The carboxyl terminal 17 amino acids within Apg7 are essential for Apg8 lipidation, but not for Apg12 conjugation. FEBS Lett 551:71–77.