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GABA$_A$ Receptor-Associated Protein Regulates GABA$_A$ Receptor Cell-Surface Number in *Xenopus laevis* Oocytes

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ABSTRACT

GABA$_A$ receptor-associated protein (GABARAP) was isolated previously in a yeast two-hybrid screen using the intracellular loop of the γ2 subunit of the GABA$_A$ receptor as bait. GABARAP has been shown to participate in the membrane-clustering and intracellular-trafficking of GABA$_A$ receptors, including a stimulation of the surface expression of GABA$_A$ receptors. To assess this quantitatively, we used *Xenopus laevis* oocytes expressing $\alpha1\beta2\gamma2$S-containing GABA$_A$ receptors to demonstrate that coexpression of GABARAP increased net surface levels of GABA$_A$ receptors as shown by both increased GABA currents and surface-expressed protein. This GABARAP stimulation of GABA currents required the receptor γ2 subunit and full-length GABARAP: deletion of the microtubule-binding domain (amino acids 1–22) or disrupting the polymerization of microtubules abolished the enhancement, indicating that the effect of GABARAP was derived from the interaction with microtubules. GABARAP coexpression did not alter the general properties of GABA$_A$ receptors such as sensitivity to GABA or benzodiazepines, but it increased surface levels of receptor protein in oocytes. Rather, it seems to supplement inadequate amounts of endogenous GABARAP to support optimum trafficking and/or stabilization of surface GABA$_A$ receptors.

GABA, an important inhibitory neurotransmitter in both vertebrates and invertebrates, acts on GABA$_A$ and GABA$_B$ receptors that are expressed ubiquitously in the central nervous system. GABA$_A$ receptors also represent a major site of action of clinically relevant drugs, such as benzodiazepines, barbiturates, and general anesthetics. In mammals, 19 related subunits have been detected: α1 to α6, β1 to β3, γ1 to γ3, δ, ϵ, θ, π, and ρ1 to ρ3. The subunits are combined into approximately 20 different native heteropentameric isoforms. The combination of α1, β2, and γ2 in the ratio of 2:2:1 is the most abundant form in the adult central nervous system (Macdonald and Olsen, 1994). Each subunit possesses a long extracellular N terminus, which in some cases carries the neurotransmitter-binding site, four membrane-spanning domains, including the ion channel wall M2, and a large variable-sequence intracellular loop between M3 and M4. Because both the N and C termini of GABA$_A$ receptor subunits extend outside the cell membrane, the intracellular M3–M4 loop becomes the most important domain interacting with the intracellular environment. As a consequence, studies of the function of receptor intracellular loop-associated proteins become important for understanding the mechanisms of regulating receptor activity.

GABA$_A$ receptor-associated protein (GABARAP) was cloned in a yeast two-hybrid system using the intracellular loop of γ2 as bait. GST pull-down and coimmunoprecipitation results confirmed this interaction in vivo (Wang et al., 1999). The interaction is specifically limited to γ1, γ2S, γ2L, and γ3 but not other GABA$_A$ receptor subunits (Wang et al., 1999; Nymann-Andersen et al., 2002b). GABARAP binds with γ2 subunits through a.a. 36 to 52 in GABARAP (Wang et al., 1999; Nymann-Andersen et al., 2002b), especially a.a. 36 to 40 (Leil et al., 2004). This is followed by or overlaps with a self-dimerization domain at a.a. 41 to 51 (Wang et al., 1999; Nymann-Andersen et al., 2002b,c; Leil, 2004). The sequence of GABARAP has 31% identity and 64% similarity to light chain 3 of microtubule-associated protein, suggesting that GABARAP might link GABA$_A$ receptors to the cytoskeleton. GABARAP interacts directly with both soluble tubulin and microtubules, as demonstrated by GST pull-down and coimmunoprecipitation, and

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ABBREVIATIONS: GABARAP, GABA$_A$ receptor-associated protein; GST, glutathione *S*-transferase; a.a., amino acid(s); cRNA, capped mRNA; ANOVA, analysis of variance; RIPA, radioimmunoprecipitation assay; PAGE, polyacrylamide gel electrophoresis; ND96 recording solution, NaCl, KCl, $CaCl_2$, $MgCl_2$, and HEPES.

is also associated with microfilaments in intact cells. The cellular distribution of GABARAP was altered by cytoskeleton-disrupting drugs, such as nocodazole, taxol, and cytochalasin D (Wang and Olsen, 2000). Studies derived from GST pull-down using truncated GABARAP (Wang and Olsen, 2000) and crystal structure(Coyle et al., 2002) confirmed that the α-helix in the N terminus (a.a. 1–22) of GABARAP is responsible for its interaction with microtubules. This tubulin-binding domain is required for GABARAP clustering of $GABA_A$ receptors in a quail fibroblast recombinant-expression system (Chen et al., 2000). The clustered receptors demonstrate slightly altered channel kinetics: lower apparent affinity for GABA, faster deactivation, and slower desensitization (Chen et al., 2000). In L929 cells, coexpression of GABARAP promotes the formation of $GABA_A$ receptor clusters that are capable of showing large single-channel conductance, which is observed in neurons but not in recombinant cells lacking GABARAP (Everitt et al., 2004). This suggests that GABARAP might play an important role in the membrane, possibly synaptic, organization of $GABA_A$ receptors.

GABARAP also binds with gephyrin, a postsynaptic marker of $GABA_A$ receptors (Sassoe-Pognetto et al., 1995; Essrich et al., 1998), in both the yeast two-hybrid system and GST pull-down experiments (Kneussel et al., 2000). Coexpression of both GABARAP and gephyrin results in a recruitment of cytoplasmic gephyrin to GABARAP-rich membrane-associated loci in PC12 cells. However, little GABARAP was colocalized with $GABA_A$ receptors at postsynaptic sites, and GABARAP localization remains normal in the gephyrin knockout mouse, whereas postsynaptic $GABA_A$ receptors are disrupted, suggesting that GABARAP functions before or independently of gephyrin. This further indicates that GABARAP might participate in intracellular receptor trafficking rather than anchoring (Kneussel et al., 2000; Kittler et al., 2001). We have shown that $GABA_A$ receptor $\gamma 2$ subunits can interact with each other, providing a possible mechanism for aggregation at synapses, after delivery to the cell surface, possibly near synapses, by GABARAP (Nymann-Andersen et al., 2002a). In addition, GST pull-down and immunofluorescence microscopy demonstrated that GABARAP interacts with the ATPase, *N*-ethylmaleimide–sensitive factor, a protein implicated in intracellular vesicle fusion (Kittler et al., 2001). From these results, we predict that GABARAP might regulate the cell-surface number of $GABA_A$ receptors through transportation along the cytoskeleton. However, there is no direct evidence supporting this prediction. To address this issue, we used the *Xenopus laevis* oocyte expression system, which allowed us to measure quantitatively the influence of GABARAP on the levels of surface-expressed $GABA_A$ receptors using both a functional assay of current amplitude with the two-electrode voltage clamp and the amount of receptor protein expressed at the surface using biotinylation of intact cells. The results showed that coexpressed exogenous GABARAP increases the surface levels of $GABA_A$ receptors by interacting with $\gamma 2$ subunits, and microtubules are essential for this enhancement. This might involve increased delivery to the cell surface or reduced removal of receptors from the cell surface.

Materials and Methods

Molecular Cloning. Rat $\alpha 1$, $\beta 2$, and $\gamma 2S$ GABA receptor subunits and GABARAP cDNAs were subcloned into expression vector pBlueScript II SK after digestion by PstI and HindIII. Deletion of a.a. 1 to 22 from GABARAP was generated by polymerase chain reaction using primers 5′-CTG CAG ATT ATT AAG AAA TAC CCG GAC CGG-3′ and 5′-AAG CTT TCA CAG ACC GTA GAC ACT-3′. It was then subcloned into pBlueScript II SK as for the other clones.

Expression of Rat $GABA_A$ Receptors in *X. laevis* Oocytes. Capped mRNA (cRNA) was synthesized by in vitro transcription from ApaI-linearized cDNA constructs using the mMessage mMachine kit (Ambion, Austin, TX) as described previously (Chang et al., 2003). cRNA concentrations were measured by both UV absorption and RNA gel electrophoresis. *X. laevis* oocytes were prepared and injected with a total volume of 50 nl of cRNA mixture. After injection, oocytes were maintained in six-well plates at 17 to 19°C in 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES supplemented with 50 μg/ml gentamycin and 100 μg/ml streptomycin and penicillin.

Two-Electrode Voltage-Clamp Analysis. Three to five days after injection of cRNA, oocytes under a two-electrode voltage clamp (voltage held at −70 mV) were gravity-perfused continuously with ND96 recording solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) at an approximate rate of 5 ml/min as described previously (Chang et al., 2003). The perfusion was stopped during the administration and incubation period to keep a constant drug concentration. Electrodes were filled with 3 M KCl and had a resistance of 0.5 to 1.5 MΩ. The oocytes were exposed to various concentrations of GABA ranging from 0.1 μM to 1 mM for 30 s. The recording was carried out using an Axoclamp-2A amplifier (Axon Instruments Inc., Union City, CA) interfaced to a computer with a DigiData 1322-A device (Axon Instruments). The data were processed with two-way ANOVA, and the dose-response curves were fitted by nonlinear regression using Prism software (GraphPad Software Inc., San Diego, CA). The equation for sigmoidal dose-response (variable slope) is $Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{(\log EC_{50} - X) \times n_H})$. X is the logarithm of concentration, Y is the response, and n_H is the Hill slope. Y starts at bottom and goes to top with a sigmoid shape. The EC_{50} value for each curve was calculated individually and analyzed by t test.

Cut-Open Oocyte Voltage Clamp. The same day after recording the GABA current, potassium currents were recorded by the cut-open Vaseline gap voltage clamp (Stefani and Bezanilla, 1998). The external solution contains 110 mM sodium methanesulfonate, 2 mM $Ca(MES)_2$, and 10 mM HEPES. The internal solution contains 120 mM potassium glutamate and 10 mM HEPES. Intracellular micropipettes were filled with 2700 mM sodium methanesulfonate and 10 mM NaCl. All solutions were buffered to pH 7.0. Low-access resistance to the oocyte interior was obtained by permeabilizing the oocyte bottom with 0.1% saponin.

Cell-Surface Biotinylation. Surface-expressed proteins were biotinylated by the membrane-impermeable EZ-link Sulfo-NHS-SS-biotin (Pierce Chemical, Rockford, IL). Four days after the cRNA injection, oocytes (15 oocytes/group) were washed with ND96 solution three times and then incubated with 1 mg/ml Sulfo-NHS-SS-biotin at room temperature for 30 min. Then cells were washed with 25 mM Tris, pH 8.0, followed by two washes with phosphate-buffered saline. Oocytes were homogenized in 200 μl of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.5). The yolk and cellular debris were removed after centrifugation at 3600g for 10 min three times. A 20-μl aliquot was taken and mixed with 2× SDS loading buffer to detect the total protein. The remaining supernatant was incubated with streptavidin beads (Pierce) at 4°C overnight.

Western Blot. The biotinylated proteins were eluted from the beads by SDS loading buffer. The eluted proteins and the whole proteins were loaded on SDS-PAGE and transferred to polyvinylidene difluoride membrane by a semidry method. The membrane was then incubated in phosphate-buffered saline buffer and 0.05% Tween with 5% nonfat milk and anti-$\gamma2$ at room temperature for 1 h. After the incubation with secondary antibody for 1 h, the membrane was visualized by enhanced chemiluminescence (Amersham Biosciences Inc., Piscataway, NJ). The blots were scanned and analyzed with Quantity One software (Bio-Rad, Hercules, CA), compared by one-way ANOVA.

Results

GABARAP Increases GABA Currents in Oocytes Expressing $\alpha1\beta2\gamma2S$ GABA$_A$ Receptor Subunits, and a Control Recombinant Channel, the Shaker K$^+$ Channel, Is Not Changed. The cRNA mixture of $\alpha1$, $\beta2$, and $\gamma2S$ subunits together with GABARAP or H_2O in the ratio of 1:1:2:4 was injected into oocytes. Three to five days after the injection, GABA currents were measured with two-electrode voltage clamp with the holding potential at -70 mV. The oocytes were exposed to different doses of GABA for 30 s. Application of GABA ranging from 1.0 μM to 1 mM to oocytes produced a concentration-dependent increase in current. Figure 1 shows typical GABA currents elicited by different doses of GABA, without (a) and with (b) GABARAP. The dose-response relationship was obtained by normalizing each current to the maximum average effect of the "no GABARAP" group in the same batch to minimize the variance between different sets of oocytes. Over the concentrations ranging from 10 μM GABA, a significant inward current was observed that reached a plateau at 1 mM. GABA currents recorded from oocytes coexpressing GABARAP with $\alpha1\beta2\gamma2S$ ($n = 16$) subunits were significantly bigger than those expressing only $\alpha1\beta2\gamma2S$ subunits (**, $p < 0.01$ from two-way ANOVA, $n = 10$). The enhancement by GABARAP was 2- to 3-fold. The enhancement was only caused by the larger maximum response to GABA, not the apparent affinity, because the EC$_{50}$ value for GABA was not altered. Despite different levels of expression in different batches of injected oocytes, within each given batch, the increased GABA current with GABARAP was robust and reproducible over numerous observations. To show that this enhancement is specific to GABA$_A$ receptors, *Shaker* K$^+$ channel was included in our experiment as a control. In contrast, the current produced by expression of the *Shaker* K$^+$ channel was not changed in oocytes expressing exogenous GABARAP compared with controls (Fig. 1, bottom).

GABARAP Has No Effect on Flurazepam Enhancement of GABA Current. Oocytes treated the same as above were recorded under two-electrode voltage clamp with the holding potential at -70 mV. To detect the enhancement of flurazepam on GABA currents, the EC$_{20}$ value was calculated from the dose-response curve shown in Fig. 1b. The EC$_{20}$ value for GABA in both curves was approximately 2.5 μM. Then, different concentrations of GABA were applied to obtain the dose-response curve of each oocyte. Three of 17 oocytes with EC$_{20}$ values greater than 3 μM or less than 2 μM were removed from further flurazepam study. The procedure includes two phases. In both phases, the concentration of flurazepam remains constant. In the first phase, flurazepam was administrated alone. Fifteen seconds later, GABA (2.5 μM) was added to the flurazepam for another 30-s incubation. Flurazepam itself at a concentration from 0.01 to 10 μM did not induce any current. When combined with 2.5 μM GABA, 0.1 μM flurazepam enhanced the GABA current approximately 1.5-fold in oocytes expressing only $\alpha1\beta2\gamma2S$ subunits (as shown in Fig. 2). The enhancement reached a maximum by increasing the GABA current 2.5-fold with 10 μM flurazepam. The same pattern of enhancement of flurazepam was obtained in oocytes coexpressing $\alpha1\beta2\gamma2S$ subunits together with GABARAP. Comparing these two groups, neither the maximum effect nor the EC$_{50}$ value of flurazepam had any significant difference ($p > 0.05$ from two-way ANOVA, $n = 14$).

Enhancement by GABARAP Is Acting through $\gamma2$ Subunits. Because $\gamma2$ subunits are the major interactor of GABARAP, we tested whether $\gamma2$ subunits were required for the enhancement by GABARAP. Oocytes expressing $\alpha1\beta2$ subunits or $\alpha1\beta2$ subunits with GABRAP were recorded by

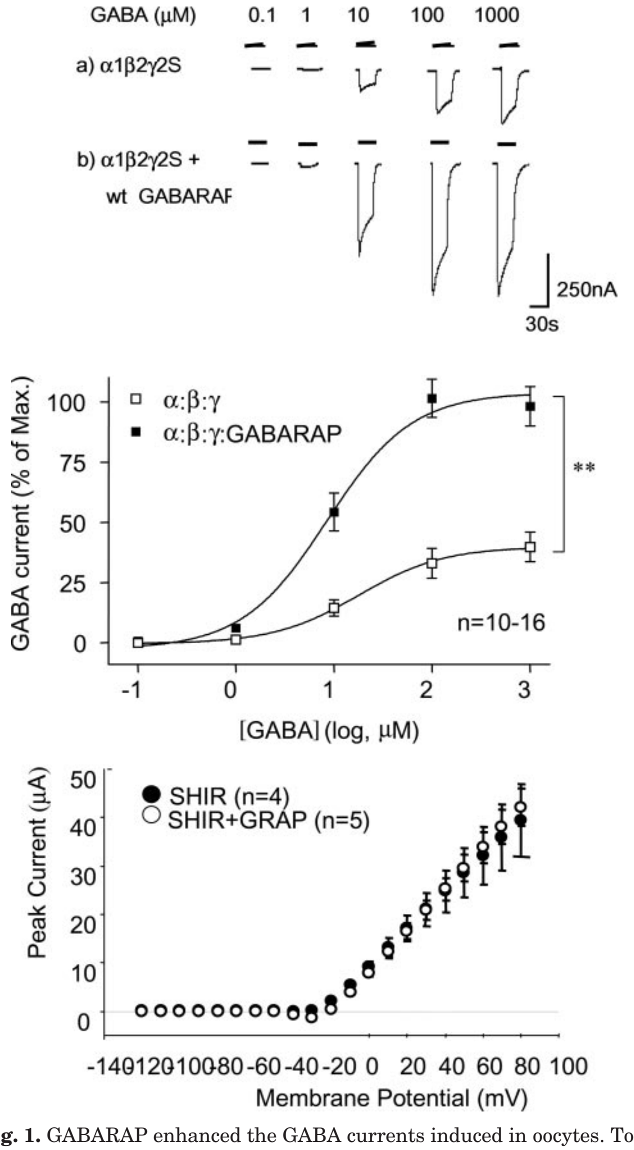

Fig. 1. GABARAP enhanced the GABA currents induced in oocytes. Top, example of GABA-induced currents in oocyte expressing $\alpha1\beta2\gamma2S$ subunits alone (a) or with GABARAP (b). These were measured by the two-electrode voltage clamp with a holding potential of -70 mV. Middle, the dose-response curve of GABA for these two groups of oocytes. Each current was normalized to the maximum average effect. Coexpression of GABARAP ($n = 16$) increased the currents at each dose of GABA compared with no GABARAP ($n = 10$; **, $p < 0.01$ from two-way ANOVA). Bottom, currents recorded from oocytes expressing the *Shaker* K$^+$ channel without (SHIR, $n = 4$) or with GABARAP (SHIR + GRAP, $n = 5$).

two-electrode voltage clamp with the holding potential at −70 mV. As shown in Fig. 3, in general, the GABA currents induced from oocytes expressing only $\alpha 1$ and $\beta 2$ subunits were lower than those with $\alpha 1\beta 2\gamma 2$S subunits. The currents measured from oocytes expressing $\alpha 1\beta 2$ subunits and GABARAP had no significant difference from those expressing only $\alpha 1\beta 2$ subunits. The dose-response curves of these two groups overlapped with each other (Fig. 3).

The Enhancement Effect of GABARAP on GABA Currents Is Mediated by Polymerized Microtubules. GABARAP has been shown to interact with both soluble and polymerized microtubules (Wang and Olsen, 2000). This interaction was regarded as responsible for facilitating intracellular trafficking of GABA$_A$ receptors. In previous studies (Chen et al., 2000), we have shown that interfering with polymerization of microtubules by nocodazole decreased the clustering of GABA$_A$ receptors. Here, we used the same drug to determine whether the enhancement effect of GABARAP was functioning through microtubules. Three to five days after the injection of cRNA, oocytes expressing $\alpha 1\beta 2\gamma 2$S subunits with or without GABARAP were incubated with nocodazole (1 μg/ml) overnight. Then, the GABA currents were recorded under the same situation as described above. In agreement with Fig. 1b, without nocodazole treatment, the GABA currents induced from oocytes coexpressing GABARAP with $\alpha 1\beta 2\gamma 2$S subunit were greater than that expressing the same subunits alone. In general, after treatment by nocodazole, the induced GABA currents were smaller than those from normal oocytes. This phenomenon was more obvious in oocytes expressing both $\alpha 1\beta 2\gamma 2$S subunits and GABARAP. The GABA currents decreased dramatically after nocodazole incubation, even down to the same level as no GABARAP coexpression (**, $p < 0.01$ from two-way ANOVA). There was no difference between oocytes with or without GABARAP coexpression after the treatment. Nocodazole did not affect the apparent affinity of GABA, because the EC$_{50}$ value remained the same in these groups (Fig. 4, top).

The N Terminus (a.a. 1–22) Is Essential for the Enhancement of GABARAP. The results above suggest that polymerized microtubules are important for the function of GABARAP. We used a truncated GABARAP$_{23-117}$ to verify whether the interaction between GABARAP and microtubules was essential. We chose this truncated GABARAP on the basis of GST pull-down and crystal structure studies indicating that a.a. 1 to 22 in the N terminus of GABARAP are responsible for microtubule binding (Wang and Olsen, 2000; Coyle et al., 2002). Three groups of oocytes were injected with $\alpha 1\beta 2\gamma 2$S subunits with H_2O, GABARAP, or GABARAP$_{23-117}$. Three to five days after the injection of

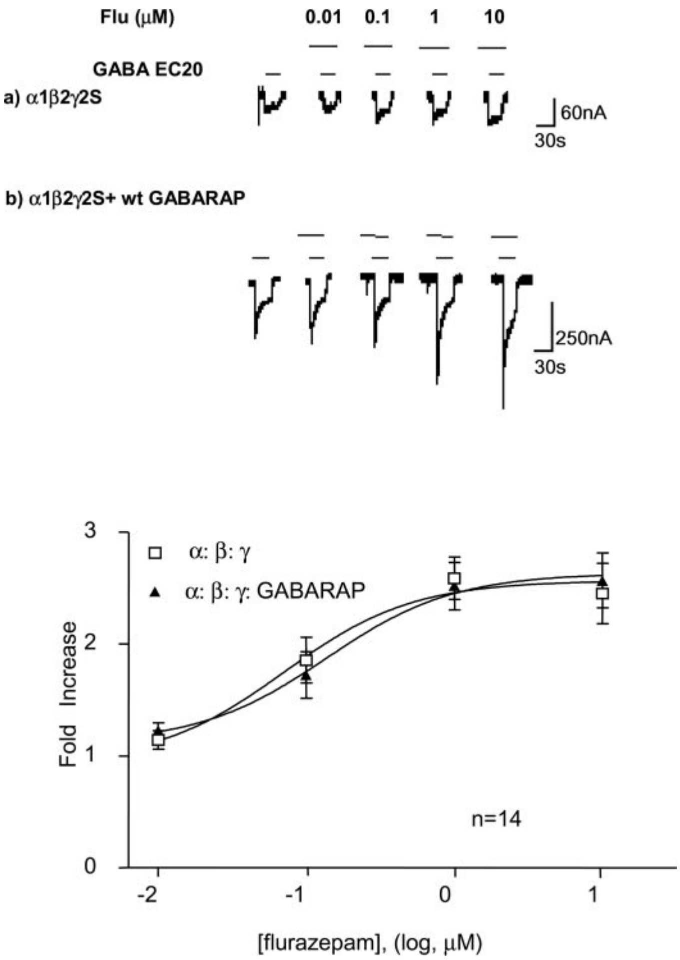

Fig. 2. GABARAP had no influence on the enhancement of GABA currents by flurazepam. Top, currents in oocytes expressing $\alpha 1\beta 2\gamma 2$S subunits alone (a) or with GABARAP (b) treated with 2.5 μM GABA, different doses of flurazepam, or a mixture of 2.5 μM GABA and flurazepam. Bottom, dose-response curve showed no difference regardless of whether GABARAP was present.

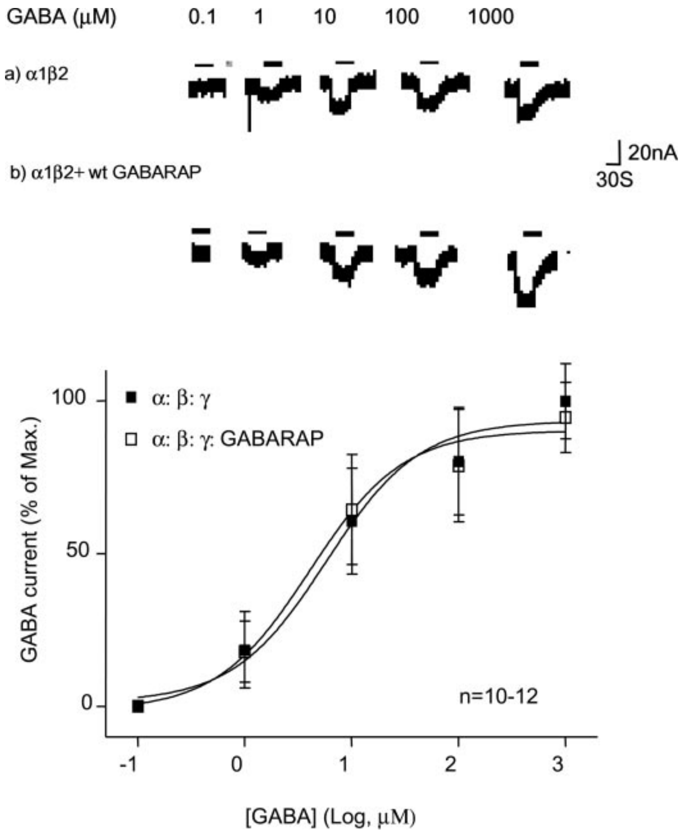

Fig. 3. The $\gamma 2$ subunit was required for the enhancement of GABAR current by GABARAP. Top, typical GABA currents in oocytes expressing $\alpha 1\beta 2$ subunits alone (a) or GABARAP with these two same subunits (b). Bottom, the dose-response curve for these two groups of oocytes. Each current was normalized to the maximum average effect. The dose-response curve from the two groups overlapped with each other, indicating no difference in either efficacy or apparent affinity.

cRNA, the GABA-evoked currents on oocytes were recorded. In agreement with the above results, the currents from the GABARAP group were greater than the water group (*, $p < 0.05$ from two-way ANOVA). The currents from the GABARAP group were also greater than GABARAP_{23-117} ($p < 0.05$ from two-way ANOVA). GABARAP_{23-117} lost the enhancement effect on induced GABA currents, because its dose-response curve overlapped that of the non-GABARAP group (Fig. 4, bottom).

Enhanced GABA Currents Are Caused by the Increased Surface Expression of GABA_A Receptors. To determine whether the enhanced GABA currents were caused by increased GABA_A receptor number on the cell surface, we measured the surface-expressed GABA_A receptor by the cell-surface biotinylation assay. Because the functional GABA_A receptors observed in these oocytes are well-established to require the α and β subunits and the demonstrated benzodiazepine sensitivity requires the γ subunit plus α and β, the $\gamma 2$ subunit was chosen as a marker to measure the level of pentameric $\alpha\beta\gamma$ receptor. Four days after injection of $\alpha 1$, $\beta 2$, and $\gamma 2S$ subunit cRNA with H_2O, GABARAP, or GABARAP_{23-117}, oocytes were recorded to confirm the enhancement of GABARAP as shown in Figs. 1 and 4. The remaining oocytes without recording (15 oocytes/group) were incubated with biotin and harvested by RIPA buffer. Aliquots of 20 μl were taken from each group before incubation with streptavidin-agarose to detect the whole protein, including the surface proteins. Both surface and whole proteins were loaded onto SDS-PAGE and were detected by anti-$\gamma 2$ (for the M3–M4 intracellular loop). A 54-kDa band corresponding to $\gamma 2$ was detected in every lane loading either biotinylated proteins or total proteins. In oocytes coinjected with GABARAP and $\alpha 1\beta 2\gamma 2S$ subunits, the surface level of GABA_A receptor (Fig. 5) demonstrated a significantly higher level than that without GABARAP (1.97 ± 0.23-fold, $p < 0.01$, $n = 3$). In agreement with the results from electrophys-

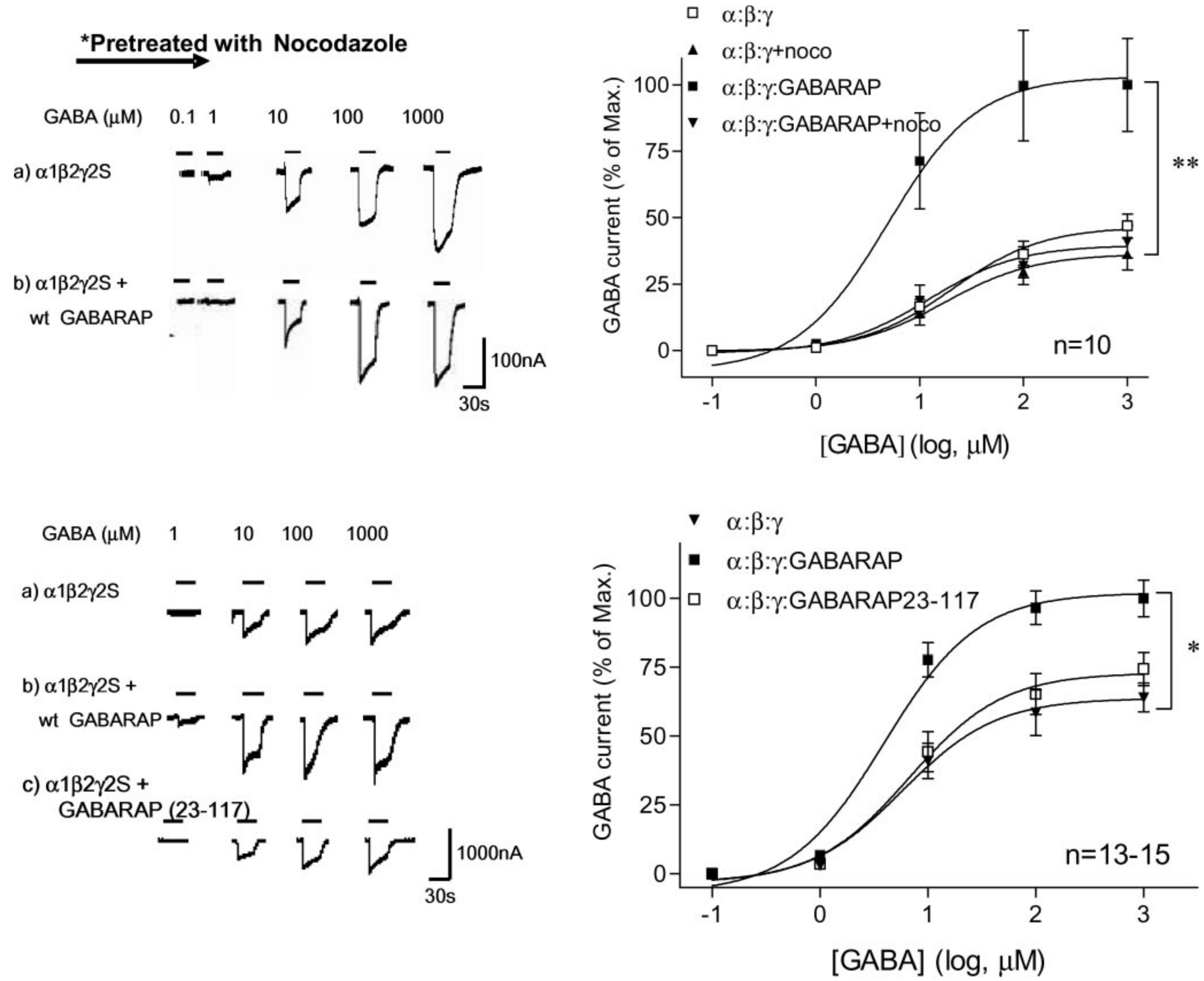

Fig. 4. The enhancement of GABA currents by GABARAP is mediated by microtubules. Top, prevention of GABARAP enhancement by nocodazole (1 μg/ml) overnight. Typical GABA responses for $\alpha 1\beta 2\gamma 2S$ without GABARAP (a) are not affected by nocodazole, but after this treatment, they fail (b) to show GABARAP enhancement (as shown in Fig. 1 and bottom of this figure). At right, the GABA dose-response curve induced in oocytes expressing $\alpha 1\beta 2\gamma 2S$ subunits alone or with GABARAP before and after exposure to nocodazole. The GABA currents in oocytes coexpressing $\alpha 1\beta 2\gamma 2S$ subunits with GABARAP were dramatically decreased after nocodazole treatment (**, $p < 0.01$, $n = 10$). Bottom, deletion of the microtubule-binding domain (a.a. 1–22) of GABARAP prevented the enhancement (**, $p < 0.01$, $n = 13–15$). Typical currents (a) show elevated response to coexpression of GABARAP (b) but not with GABARAP $_{23-117}$ (c); dose-response curves for the three are shown at right.

iology, deletion of the microtubule-binding domain of GABARAP (a.a. 1–22) showed the same surface-expression level of GABA$_A$ receptors (0.91 ± 0.08-fold, not significant) as that with $\alpha 1\beta 2\gamma 2$S subunits expressed alone. However, coexpression of GABARAP, either wild-type (1.01 ± 0.03-fold) or truncated (1.16 ± 0.13-fold), did not change the steady-state expression of GABA$_A$ receptors, because the total level of GABA$_A$ receptor protein was not changed.

Discussion

Our results, for the first time, indicate that coexpression of GABARAP increased the steady-state expression of GABA$_A$ receptors at the cell surface. Coexpression of GABARAP increased GABA currents by 2- to 3-fold. The enhancement was limited to an increase in the maximum effect because the EC$_{50}$ value of GABA was not significantly changed. Neither did the dose-dependence of benzodiazepine enhancement of GABA currents show any significant change in the GABARAP-enhanced GABA currents.

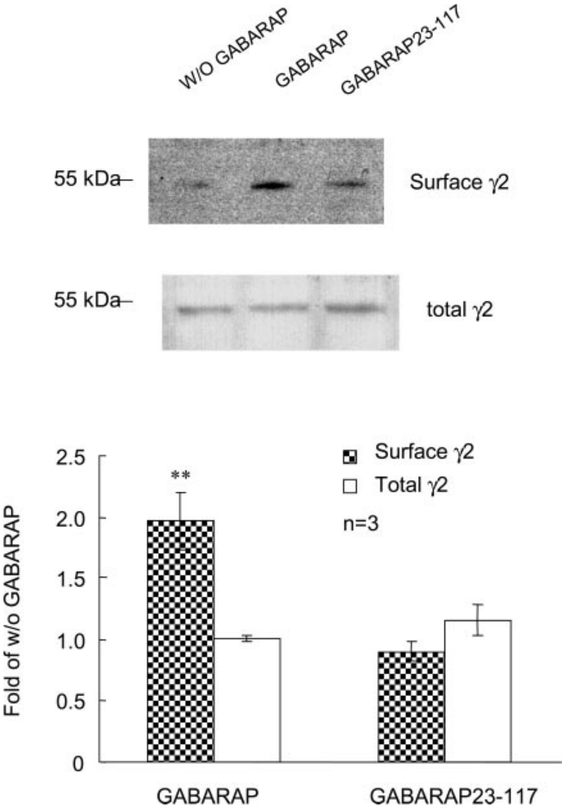

Fig. 5. GABARAP increased the cell-surface expression of GABA$_A$ receptor polypeptides. Surface biotinylation demonstrated that GABARAP coexpression in oocytes enhanced the cell-surface fraction of GABA$_A$ receptor (represented by the $\gamma 2$ subunit), as shown in the top lanes. However, GABARAP had no effect on the steady-state amount of GABA$_A$ receptor (bottom left). Deletion of the microtubule-binding domain (a.a. 1–22) of GABARAP prevented the enhancement. The oocytes (15 oocytes/group) were incubated with biotin and harvested by RIPA buffer. Aliquots of 20 μl were taken from each group before incubation with streptavidin-agarose to detect the whole protein, including the surface proteins. Both surface and whole proteins were loaded on SDS-PAGE and were detected by anti-$\gamma 2$ (for the M3–M4 intracellular loop) as a marker for the pentameric receptors. The bars at right illustrate the average densitometry results of three such experiments. The optical density for cells with GABARAP were compared with those without, normalized to 1.0, and the biotinylated fraction (surface $\gamma 2$, ■) and total cell $\gamma 2$ (□) were examined. Values for GABARAP were approximately 2-fold greater than the no-GABARAP samples (numbers given in the text, average of three similar results). The bars on the far right compare the same two fractions of $\gamma 2$ for the coexpression of GABARAP$_{23-117}$ (n = 3).

This GABARAP-dependent increase in GABA currents could be caused by increased surface level of GABA$_A$ receptors, or it could be caused by the formation of a different sort of GABA$_A$ receptors at the surface. To determine whether the GABARAP-dependent increase in GABA currents in oocytes could be caused by the increased surface GABA$_A$ receptors, we measured the surface content by biotinylation of surface proteins, collecting them by avidin binding and then identifying GABA$_A$ receptors $\gamma 2$ subunit polypeptide by Western blot, a marker for the $\alpha\beta\gamma$ pentameric receptors. As mentioned in the *Results* section, the electrophysiological data confirmed that α, β, and γ subunits were included as an intact pentameric receptor because of the observed GABA currents and the flurazepam enhancement. In addition, our previous data demonstrated that when GABARAP binds the $\gamma 2$ subunit in brain or cells, it binds the native pentameric protein, as indicated by the presence of ligand-binding in the GABARAP-immunoprecipitated GABA$_A$ receptors (Nymann-Andersen et al., 2002b). Our surface-biotinylation result clearly showed that GABARAP increased the surface-expressed GABA$_A$ receptors. GABARAP had no effect on the steady-state levels of GABA$_A$ receptor, because the total amount of GABA$_A$ receptor remained the same.

As mentioned in the Introduction, GABARAP in some cells promotes clustering of GABA$_A$ receptors with different channel properties such as single-channel conductance and decay rates (Chen et al., 2000; Everitt et al., 2004). A demonstration of increased surface-receptor protein does not exclude the possibility that increased conductance states could also contribute to greater GABA currents.

Each figure represents a result typical of three repetitions. The absolute GABA currents of oocytes vary 2-fold or even more in different batches because of variable eggs, injection, expression rate, degradation, and time of expression. Within a given batch of oocytes, there is a significantly bigger current with GABARAP coexpression than without; this is also true for the average of many batches. The error bars show that variation within a batch is 5 to 20% at individual concentrations and that GABARAP produces a highly significant 2-fold increase at every point and in every curve as calculated by ANOVA. The shape of the currents may show some slight differences between batches; for example, coexpression of GABARAP might affect the deactivation rate or desensitization rate of GABA currents and drug modulation, as we observed previously in QT-6 cells (Chen et al., 2000). However, two-electrode voltage clamp of oocytes is not the best system for measuring kinetics accurately, and this will be studied in other cells in our future work. Our working hypothesis is that when low levels of endogenous GABARAP are present in a cell, one can see a stimulation of receptor-surface levels; in addition, there may be a change in properties of the existing surface receptors (e.g., caused by clustering), and this can be detected with proper tools. Such GABARAP-dependent effects on receptor properties may be seen more readily when the levels of GABARAP are higher than the minimum requirement for facilitating intracellular trafficking.

At the same time as the work described here, we also demonstrated that overexpression of GABARAP increases the surface numbers of GABA$_A$ receptor in both cultured hippocampal neurons and COS7 cells in immunofluorescence and flow-cytometry studies (Leil et al., 2004). Mutagenesis of the key amino acids of GABARAP in the $\gamma 2$-binding domain prevent the stimulation of GABARAP (Leil et al., 2004). However, no electrophysiology or protein quantification was done in that study as was done in the current work. In addition, a peptide derived from the intracellular loop of GABA$_A$ receptor $\gamma 2$ that blocks the binding of GABARAP prevents GABARAP effects on GABAR trafficking and clustering in QT-6 cells (Nymann-Andersen et al., 2002b). Endogenous GABARAP has been detected by immunostaining in primary cultured neurons, even in QT-6 cells and L929 cells, although in low amounts (Chen et al., 2000; Everitt et al., 2004; Leil et al., 2004). In our Western blot experiments, we did not detect any endogenous GABARAP in oocytes (data not shown).

The $\gamma 2$ subunits play an essential role for translocation of other subunits to the cell surface and synaptic localization of GABA$_A$ receptors. Taking advantage of the $\gamma 2$ subunit knockout mouse, Essrich et al. (1998) reported that the receptor is functionally impaired as indicated by reduced channel conductance and a perinatally lethal phenotype in the majority of mutant mice, although the absence of $\gamma 2$ subunits did not cause a change in expression levels, regional distribution, or membrane localization of the other subunits. Our results demonstrate that first, the GABA currents induced in oocytes expressing $\alpha 1\beta 2$ subunits are dramatically lower than those with $\alpha 1\beta 2\gamma 2S$ subunit expression. This phenomenon was observed by other groups (Connolly et al., 1999; Haas and Macdonald, 1999). Second, without the $\gamma 2$ subunit, the enhancement of GABARAP was abolished. This suggests that the $\gamma 2$ subunit is required for the enhancement by GABARAP.

Besides GABARAP, other newly cloned proteins also demonstrate trafficking effects on GABA$_A$ receptors through interaction with the M3–M4 intracellular loop. These include the Golgi-specific zinc-finger protein GODZ with $\gamma 2$ subunits (Keller et al., 2004); the ubiquitin-like protein Plic-1 with $\alpha 1$ to $\alpha 3$, $\alpha 6$, and $\beta 1$ to $\beta 3$ subunits of GABA$_A$ receptors but not $\gamma 2L$ or δ subunits (Bedford et al., 2001); GABA$_A$ receptor interacting factor-1 with the $\beta 2$ (Beck et al., 2002; Kittler et al., 2004); and phospholipase C-related inactive protein type 1 with β subunits, possibly involving GABARAP (Terunuma et al., 2004).

In this report, a two-electrode voltage-clamp recording gave the first functional assay verifying that the microtubule-GABARAP interaction plays an important role in facilitating GABA$_A$ receptor function. We observed that the GABARAP-increased GABA currents were prevented by nocodazole treatment. A dramatic decrease was observed only in the GABARAP coexpression group in which the enhancement of GABARAP was totally abolished. It is feasible that oocytes express sufficient quantities of machinery, including microtubules for formation of receptor clusters, because disruption of microtubules has been shown to alter clustering and properties of GABA$_A$ receptors expressed in oocytes (Whatley et al., 1994). Accumulated evidence indicates that the cytoskeleton is essential for clustering GABA$_A$ receptors (Chen et al., 2000; Ho et al., 2001; Petrini et al., 2003). It reminds us that a reduction in clustering state or misdistribution of GABA$_A$ receptors by nocodazole or colchicine, drugs disrupting the polymerization of microtubules, might work by interrupting the interaction between polymerized microtubules and receptor-associated proteins, such as GABARAP and gephyrin. Similar to our results, in primary cultured neurons, the disruption of microtubules has been noted by Petrini et al. (2003) to decrease GABA$_A$ receptor clustering and alter the channel-opening rate. The crystal structure and GST pull-down studies using truncated GABARAP demonstrate the N terminus (a.a. 1–22) is responsible for microtubule interaction (Nymann-Andersen et al., 2002b,c). Our data further confirmed that interaction with microtubules is essential for GABARAP function. Deletion of the microtubule-binding domain on GABARAP abolished both the enhancement of GABA currents and the increased surface level of GABA$_A$ receptors in oocytes. This indicates that this GABARAP-mediated increase of GABA$_A$ receptor cell-surface level involves transportation along microtubules.

There are several candidate mechanisms underlying this enhancement. Given that GABARAP also interacts with the vesicle fusion protein *N*-ethylmaleimide–sensitive factor and the known functions of GABARAP's homologs (e.g., Golgi-associated ATPase enhancer of 16 kDa is believed to participate in intra-Golgi transportation machinery) (Sagiv et al., 2000; Muller et al., 2002), we predict that GABARAP might increase the trafficking of GABA$_A$ receptors to the cell surface. GABARAP might also stabilize the cell-surface level of GABA$_A$ receptors by decreasing endocytosis or degradation. Future study identifying the trafficking rate or half-life of surface-expressed GABA$_A$ receptors in the presence of GABARAP will help to define possible effects on endocytosis and the mechanisms of regulation of GABARAP's role in receptor trafficking and plasticity.

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