

Ethanol Promotes Clathrin Adaptor-Mediated Endocytosis via the Intracellular Domain of δ -Containing GABA_A Receptors

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Pharmacological and genetic evidence reveals that GABA_A receptor (GABA_A-R) expression and localization are modulated in response to acute and chronic ethanol (EtOH) exposure. To determine molecular mechanisms of GABA_A-R plasticity in response to *in vivo* acute EtOH, we measured early time changes in GABA_A-R subunit localization. Single doses of EtOH (3 g/kg via i.p. injection in rats) produced decreases in surface levels of GABA_A-R α 4 and δ subunits at 5–15 min post-EtOH in hippocampus CA1 and dentate gyrus, verifying our earlier report (Liang et al., 2007). Here we also examined the β 3 subunit and its phosphorylation state during internalization. β 3 also was internalized during 5–15 min after EtOH exposure, while phosphorylation of β 3 was increased, then decreased at later times, ruling out β 3 dephosphorylation-dependent endocytosis. As early as 5 min post-EtOH, there is an initial increase in association between the δ subunits with clathrin adaptor proteins AP2- μ 2 revealed by coimmunoprecipitation, followed by a decrease in association 15 min post-EtOH. *In vitro* studies using glutathione S-transferase fused to the δ subunit intracellular domain (ICD) show that two regions, one containing a classical Yxx Φ motif and the other an atypical R/K-rich motif, directly and differentially bind to AP2- μ 2, with the former YRSV exhibiting higher affinity. Mutating both regions in the δ -ICD abolishes μ 2 binding, providing a possible mechanism that can explain the rapid downregulation of extrasynaptic α 4 β δ -GABA_A-R following *in vivo* EtOH administration, in which the δ -ICD increases in affinity for clathrin AP2- μ 2 leading to endocytosis.

Introduction

Ionotropic GABA_A receptors (GABA_A-Rs) mediate the majority of phasic and tonic neural inhibition in the brain and are critical for the regulation of neural activity. GABA_A-Rs are members of the Cys-loop, heteropentameric ligand-gated ion channel superfamily. A large variety of functional GABA_A-R isoforms are assembled from 19 related subunits, usually in a combination of two α subunits (α 1–6), two β subunits (β 1–3), and one γ (1–3), δ , ϵ , π , θ , or ρ (1–3) subunit. These individual subunits exhibit distinct, yet overlapping, expression patterns, as well as functional and pharmacological properties. Each subunit contains a large extracellular N-terminal ligand-binding domain, four membrane-spanning domains (M1–M4), a large intracellular domain (ICD), and a short extracellular C terminus (Olsen and Sieghart, 2008).

Studies show the ICD of β (1–3) and γ 2 GABA_A-R subunits interact with various scaffolding and signaling proteins, thereby regu-

lating the trafficking and plasticity of GABA_A-R (for review, see Chen and Olsen, 2007; Jacob et al., 2008; Luscher et al., 2011). Less is known about the scarcer δ subunit and its ICD, but trafficking of δ subunits may be distinct from the mechanisms that govern trafficking of other subunits (Joshi and Kapur, 2009). The δ subunit exhibits unique properties: δ -GABA_A-Rs are located exclusively extrasynaptically where they tend to assemble with α 4, α 6, and α 1 subunits only (Nusser et al., 1998; Wei et al., 2003; Liang et al., 2006; Glykys et al., 2007). With these α subunits, δ -GABA_A-Rs mediate tonic currents (I_{tonic}) that are sensitive to activation by the low concentrations of GABA found at extrasynaptic membrane compartments (Mody and Pearce, 2004; Meera et al., 2011), and to modulation by low concentrations of neurosteroids, some anesthetics, and EtOH (Mihalek et al., 1999; Sundstrom-Poromaa et al., 2002; Stell et al., 2003; Wallner et al., 2003; Belelli et al., 2009).

A single intoxicating dose of EtOH produced a rapid decrease in function and surface levels of α 4/ δ -containing GABA_A-R in rat hippocampus, and a slightly later upregulation of α 4/ γ 2-containing GABA_A-R, both *in vivo* (Liang et al., 2007; Olsen and Spigelman, 2012), and in cultured hippocampal neurons (Shen et al., 2011); the receptor subunit decrease was demonstrated as increased endocytosis rather than decreased surface insertion. These acute EtOH-induced GABA_A-R changes were reversible in naive rats, but virtually irreversible in chronic intermittent ethanol (CIE) rats (Cagetti et al., 2003; Liang et al., 2006). Plasticity of GABA_A-Rs may involve regulated trafficking via protein–protein interactions. Direct interactions between membrane receptors

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and the clathrin adaptor complex (AP2), which drives clathrin coat formation and mediates endocytosis, often alter surface amounts in β and $\gamma 2$ subunit-containing GABA $_A$ -R (Kittler et al., 2000, 2005; Herring et al., 2003; Smith et al., 2008). The present study begins to identify previously uncharacterized δ interactions and their contribution to the mechanism(s) involved in the early decrease of GABA $_A$ -R following EtOH exposure (Liang et al., 2007; Shen et al., 2011). How EtOH regulates restructuring of extrasynaptic GABA $_A$ -R remains to be fully resolved, but the mechanisms are probably crucial to alcohol dependence and potential new approaches to treat alcohol use disorders.

Materials and Methods

Animals and EtOH treatment. The Institutional Animal Care and Use Committee approved all animal experiments. Male Sprague Dawley rats (200–350 g) were housed in the vivarium under a 12 h light/dark cycle and had *ad libitum* access to food and water. Rats were administered a single dose of EtOH (3 g/kg, as a 25% w/v solution in distilled water) or distilled water (vehicle) via intraperitoneal injection and decapitated 5, 10, 15, 30, or 60 min later.

Microdissection of rat brain. Following decapitation, rat brains were bathed with ice-cold artificial CSF (ACSF), removed, and quickly sliced with a tissue chopper to obtain 500 μ m coronal slices. Slices containing hippocampus regions were immediately placed into ice-cold ACSF, and hippocampus was microdissected for dentate gyrus (DG) and CA1 regions for normal and cross-linked Western blotting analyses (Liang et al., 2007). All slices for biochemical experiments using rat brain regions were allowed to recover in fresh ACSF at 35°C for 20–60 min.

For coimmunoprecipitation analyses, only dissection of DG region was performed since DG and CA1 showed similar patterns following cross-linking experiments, and DG is richer in $\alpha 4$ and δ subunits, and can be obtained much faster and cleaner if it is the only region collected. The DG was allowed to recover in fresh ACSF, 35°C for 20 min, and prepared for coimmunoprecipitation experiments. Membranes were prepared for total GABA $_A$ -R subunit level analyses as discussed previously (Cagetti et al., 2003). GABA $_A$ -R $\beta 3$ subunit phosphorylation was estimated with substrate-specific antibody anti-phospho- $\beta 3$ (Terunuma et al., 2008).

Cross-linking to measure surface GABA $_A$ -R subunits. Following the recovery period, brain regions were incubated with or without bis(sulfosuccinimidyl)suberate (BS3) in ice-cold ACSF (Grosshans et al., 2002). Slices were homogenized in 1:4 buffer A/B; where A is 1% SDS, 1 mM EDTA, 50 mM NaF, 10 mM Tris-HCl, pH 8.0, 100 mM phenylmethylsulfonyl fluoride (PMSF), and Complete Protease Inhibitor Cocktail tablet (Roche), and B is 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 100 mM PMSF. Protein concentration was determined by BCA Protein Assay Kit, and samples were prepared as standard for SDS-PAGE/Western blot. Relative signal intensities from each band (absorbance) were measured using ImageJ software. Samples were analyzed relative to one another on the same membrane and within the same experiment. Group differences were evaluated by two-way ANOVA, followed by *post hoc* Holm-Sidak test.

Membrane preparation for coimmunoprecipitation. Following the slice recovery period, brain regions were homogenized in ice-cold, freshly prepared lysis buffer (50 mM Tris, pH 8.0, 0.5 mM EDTA, 2 mM EGTA, 1% Triton X, 50 mM NaF, 100 mM PMSF) and centrifuged at 15,000 \times g for 30 min at 4°C. Cell lysate was diluted to \sim 1 mg/ml total protein, precleared with 50 μ l of Sepharose A beads at 4°C for 30–60 min, and immediately used for coimmunoprecipitation (co-IP).

Coimmunoprecipitation. Sepharose A beads (30–50 μ l) were incubated with 3–10 μ g of antibody for 1 h, 4°C before incubating with 400 μ l of precleared lysate, at 4°C for 4–18 h. IP complexes were washed with lysis buffer, twice with TBS containing 100 mM NaCl, and once more with lysis buffer. The washed complex was eluted with 2 \times SDS sample buffer and boiled, and loaded on a 4–15% gradient SDS polyacrylamide gel for protein separation, followed by transfer and antibody blotting. The secondary antibody was replaced with Clean-Blot IP HRP detection reagent (Thermo Fisher Scientific) when blotting with a same-species primary antibody as the

antibody used for immunoprecipitation. Rabbit IgG (3–10 μ g) was used as control.

Antibodies. Dr. W. Sieghart (Medical University, Vienna, Austria) generously provided the following polyclonal GABA $_A$ -R antibodies: anti- $\alpha 4$ subunit (1–14) N-terminal antibody, anti- $\alpha 4$ subunit (379–421), anti- $\beta 3$ subunit (345–408), anti-GABA $_A$ -R $\gamma 2$ subunit (319–366), anti-GABA $_A$ -R δ subunit (N1–44), and anti-GABA $_A$ -R δ subunit (1–44). The anti-GABA $_A$ -R phospho- $\beta 3$ subunit (p408/p409) was prepared by the Moss laboratory (Terunuma et al., 2008). The following commercial antibodies were used for this study: polyclonal anti-GABA $_A$ -R δ subunit (331–430) from Santa Cruz Biotechnology, Inc, monoclonal mouse anti-GABA $_A$ -R $\beta 2/3$ (clone 62-3G1) from Millipore, monoclonal mouse anti- β -actin (clone AC-15) from Sigma, polyclonal anti- β -actin from Anaspec, and monoclonal mouse anti-AP2 $\mu 2$ subunit (110–230) from BD Biosciences.

Glutathione S-transferase- δ ICD cDNA. Glutathione S-transferase- δ (GST- δ) cDNA containing the entire GABA $_A$ -R δ subunit ICD was discussed previously (Kittler et al., 2005). Truncated and mutated forms of GST- δ were made by standard PCR using Platinum Taq Polymerase High Fidelity (Invitrogen) with a 50 μ l total reaction volume in a standard PCR cyclor and the following primers, as necessary: attatcttGGATC-CCACTTCAATGCCGACTACAGGAAG and attatcttGAATTCctcAGC-CAACTCCTGACTGACCCC to create δ ICD residues 316–359 (called δ 316–359, or δ R interchangeably); attatcttGGATCCATCTCCCGTCGT-CAAGGCC attatcttGAATTCctcGATGTCGATGGTGTCTGCATCGA to create δ ICD residues 360–410 (called δ 360–410, or δ Y interchangeably); attatcttGAACCTCATGGGTTCCGCTAGGTCTGTG along with primers 1 and 4 from above to create δ ICD (375 Y \rightarrow A) mutant (also called δ Y \rightarrow A interchangeably); attatcttGCCGACTACGGGGCGGCAGGGGCAGC-CAAGGTC was used along with primers 1 and 4 to create δ ICD (322 RKKRK 326 \rightarrow GAAGA) (also called δ R \rightarrow G interchangeably). The cDNA of the 375 Y \rightarrow A mutant (Y \rightarrow A mutant) was used as template along with mutation primer 322 RKKRK 326 \rightarrow GAAGA to create the δ ICD double $\mu 2$ -motif mutant, also called $\delta 2$ mutant. Sequencing of transformed plasmid DNA verified desired constructs to use for expression of proteins.

Expression of GST fusion proteins. GST construct DNAs were transformed into *Escherichia coli* strain BL21, and a standard protocol for expressing and purifying GST proteins was followed (Einarson et al., 2007). Protein concentrations were determined by BCA assay. GST pull-down assays were performed as described by Kittler et al. (2005).

Concentration-dependent binding of GST-constructs to $\mu 2$. GST-affinity beads bound to GST-fusion proteins (as in pull-down experiments) were incubated with the varying concentrations of purified AP2 ([1 nM, 0.1 μ M, 0.1 nM] plus lysate), at 4°C for 2–18 h, washed, and subjected to SDS-PAGE followed by Western blot or Coomassie stain. GST fusion mutants' binding to $\mu 2$ was normalized to its respective GST amount, and then compared with GST- δ full-length ICD (wild type). Each full-length mutant GST was then compared with each of the others.

Statistical analysis. Normalized optical density measurements were used to calculate percentage changes, normalized according to experiment: For total protein level and surface level analysis, optical density of each band was normalized to its corresponding β -actin loading control. For coimmunoprecipitation analysis, optical density of each coimmunoprecipitated band was normalized to the original immunoprecipitated protein (the one pulled down by its specific antibody). All quantifications are expressed as the mean \pm SEM. Using the GraphPad Prism 4.0 program and SigmaStat, statistical analysis are as follows.

For Figures 1 and 2, a two-way ANOVA followed by Holm-Sidak *post hoc* test was used to determine significant differences among treatment time and among brain regions and between control and treated samples at a given time and region.

For each experiment in Figure 3, a two-way ANOVA followed by Bonferroni *post hoc* test was used to determine significant differences among treatment time and subunits.

For Figure 4C, a one-way ANOVA followed by Bonferroni *post hoc* test was used to determine significant differences among the constructs and binding efficacy. $p < 0.001$ was considered significant that the 2x mutant differed in $\mu 2$ binding versus full-length wild-type δ - $\mu 2$ binding. $p < 0.05$ was considered significant for the double mutant (2X mutant) binding to $\mu 2$.

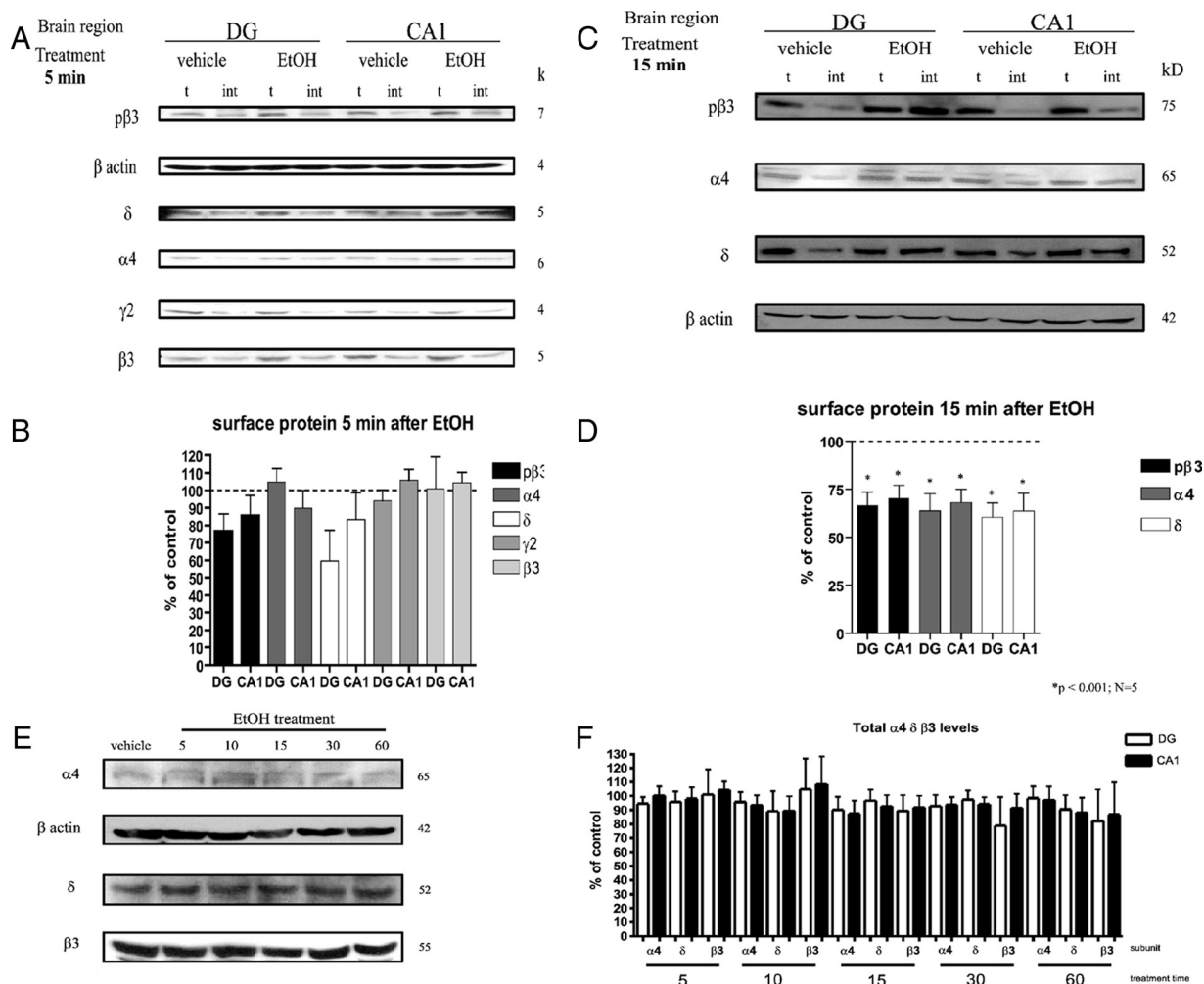


Figure 1. A single *in vivo* EtOH dose causes decreases in extrasynaptic surface GABA δ -R in hippocampal DG and CA1 within 15 min. **A–D**, Crosslinking experiments show a trending decrease in surface levels of $\alpha 4$, p $\beta 3$, and δ subunits as early as 5 min following EtOH dose (**A**, **B**), with significant decreases measured 15 min post-EtOH (**C**, **D**). t, Total protein (untreated slices); int, internal protein (slices treated with BS3). $\beta 3$ and $\gamma 2$ showed no significant changes. Pictures show representative blots of the average changes; graphs (**B**, **D**) plot the average of several experiments, $N = 5$. **E**, **F**, GABA δ -R subunit levels at early time points after EtOH treatment. Total GABA δ -R subunit changes in DG and CA1 at times 5–60 min following EtOH (3 g/kg) treatment do not change significantly ($N = 4–5$).

across all other GST- δ constructs. For Figure 4D, a one-way ANOVA followed by a Holm-Sidak *post hoc* test found a significant difference between the δY and δR s ability to bind to $\mu 2$ (using means as comparisons), with $p < 0.05$; $N = 3$ (experiment done on three batches of proteins, repeated at least twice per experiment).

Results

Ethanol administration to rats induces rapid internalization of extrasynaptic hippocampal GABA δ -R subunits

EtOH administered via intraperitoneal injection in rats is detectable in the brain within 2–3 min (Liang et al., 2007), and tolerance/desensitization to some EtOH-induced behavioral effects, such as rotarod performance, occurs within 3–5 min in mice (LeBlanc et al., 1975). This suggests that alterations in the function and/or expression of some GABA δ -Rs occur immediately after EtOH exposure. Figure 1A–D shows receptor cross-linking assays using hippocampal slices *in vitro*, after EtOH administration *in vivo*. Rats were

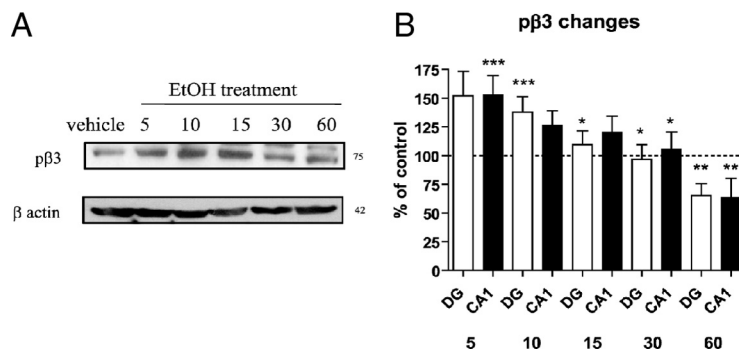


Figure 2. EtOH-induced changes in GABA δ -R $\beta 3$ phosphorylation at early time points. **A**, Phosphorylation of $\beta 3$ as identified with sequence-specific phospho-peptide antibody. Initial increases at 5 and 10 min in phosphorylation of $\beta 3$ subunit compared with control treated at 5 and 10 min ($153.10 \pm 40.14\%$, $138.08 \pm 29.20\%$, respectively; $N = 4–5$). **B**, Plot of quantified p $\beta 3$ level changes following various EtOH time treatments and brain regions. * $p < 0.05$; ** $p < 0.001$ between 5 min treatment and stated treatment times in each brain region; *** $p < 0.05$ between control and EtOH-treated rats from same time point treatment; two-way ANOVA.

treated with 3 g/kg EtOH (intraperitoneally), or vehicle, and killed at early time points (5–60 min) to determine the time course for the EtOH-induced GABA δ -R internalization of surface receptors, as seen in Liang et al., 2007. We simultaneously examined the phosphorylation state of the $\beta 3$ subunit over the

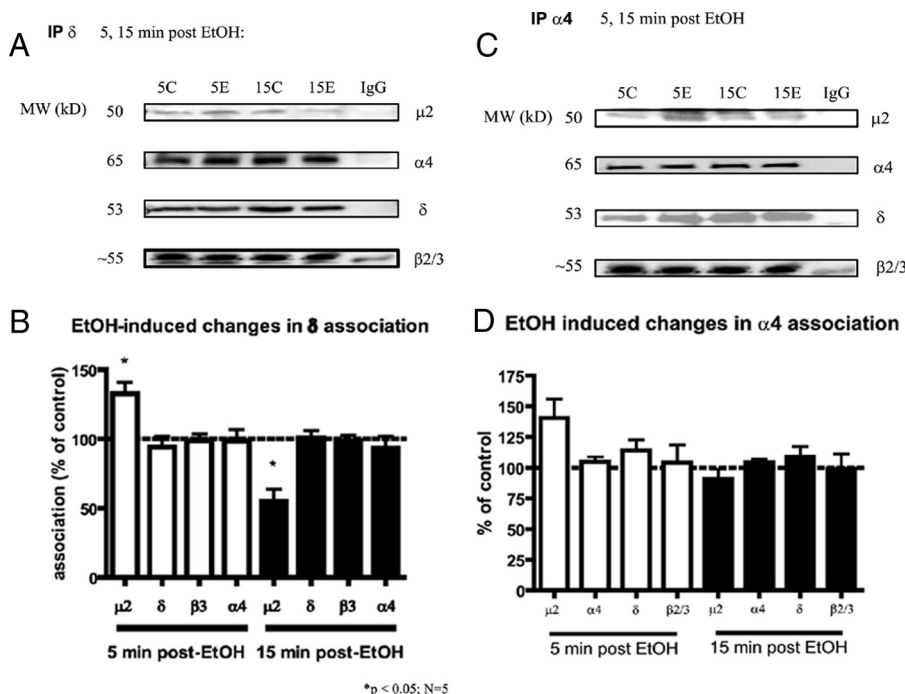


Figure 3. GABA_A-R δ and $\alpha 4$ subunit association with $\mu 2$ changes following EtOH exposures. **A, B**, EtOH caused a 32.7% increase in δ association with $\mu 2$ at 5 min, and a 55.1% decrease in δ association with $\mu 2$ 15 min following EtOH treatment. The EtOH-induced changes in association of δ with $\mu 2$ are statistically significant from each other (** $p < 0.0001$), and statistically significant from control treated δ - $\mu 2$ associations (two-way ANOVA, Bonferroni *post hoc* test; * $p < 0.05$; $N = 5$). **C, D**, GABA_A-R $\alpha 4$ subunit associations following EtOH exposure. EtOH caused a 38% increase in $\alpha 4$ association with $\mu 2$ at 5 min (not significant, $p = 0.05$, $N = 4$; but was significantly different from association of $\alpha 4$ and $\mu 2$ at 15 min, $p < 0.001$). This suggests that, at least at an early time point, these subunits are internalizing with δ -containing GABA_A-R.

same time course, because dephosphorylation of $\beta 3$ subunits has been shown to regulate clathrin-mediated internalization of receptors (Kittler et al., 2005; Jacob et al., 2008; Terunuma et al., 2008). Here, surface levels in $\alpha 4$, δ , and the phosphorylated form of $\beta 3$ GABA_A-R subunits trended to a decrease as early as 5 min after EtOH exposure, although none was significantly different (Fig. 1A,B). Statistically significant changes were seen 15 min after the EtOH dose, at which time surface $\alpha 4$ levels decreased to $63.9 \pm 8.8\%$ in the DG and $68.1 \pm 6.9\%$ in CA1; $p\beta 3$ decreased to $66.8 \pm 6.5\%$ and $70.5 \pm 6.7\%$; and δ decreased to $60.4 \pm 7.4\%$ and $63.8 \pm 9.2\%$ compared with vehicle-treated rats (Fig. 1C,D, $p < 0.001$, compared with control of same subunit; $N = 5$). These results suggest that EtOH stimulates the internalization of receptors composed of $\alpha 4\beta 3\delta$ subunits. Figure 1, E and F, shows there were no changes in total GABA_A-R subunits tested at early time points 5–60 min, as expected (there is no detectable degradation nor synthesis at early time points).

Dephosphorylation of $\beta 3$ subunit does not correlate in time with EtOH-induced internalization

The phosphorylated state of GABA_A-R $\beta 3$ subunit was detected by Western blot with a substrate-specific antibody in control animals untreated with EtOH. Significant increases were observed for phosphorylated $\beta 3$ ($p\beta 3$) at early time points 5 and 10 min following EtOH treatment, times at which protein internalization was observed. The levels of $p\beta 3$ decreased at later time points, 30 and 60 min after EtOH, as shown in Figure 2. Thus dephosphorylation does not appear to induce internalization of $\alpha 4$, $\beta 3$, and δ subunits of GABA_A-R.

EtOH treatment in rats increases association of hippocampal extrasynaptic GABA_A-R subunits with clathrin adaptor protein AP2 $\mu 2$ subunit observed by coimmunoprecipitation

Since $\alpha 4$, $p\beta 3$, and δ subunit surface levels significantly decreased at 15 min following EtOH administration (Fig. 1C,D), the effects that this treatment had on association with the clathrin $\mu 2$ subunit were measured. Coimmunoprecipitation experiments of δ subunits showed an initial significant increase in association with AP2- $\mu 2$ ($132.7 \pm 8.2\%$ of vehicle) as early as 5 min following EtOH. There was a subsequent decrease in δ association with $\mu 2$ ($55.1 \pm 8.7\%$ of vehicle) 15 min after EtOH treatment (Fig. 3A,B). These results suggest that the increase in GABA_A-R internalization observed in Figure 1 (via cross-linking) is, in part, due to an increased association of pentameric receptors containing δ with $\mu 2$ at 5 min following EtOH treatment, resulting in an initiation of endocytosis of the δ -containing receptor. The decreased δ - $\mu 2$ association 15 min after EtOH, in return, supports the observation that a significant amount of δ -containing GABA_A-R has already internalized—there is less δ available for interaction. Co-IP shows the δ subunit interacts with $\alpha 4$ - and $\beta 2/3$ -GABA_A-R;

there were no other changes seen in association between the δ subunit with other GABA_A-R subunits.

Because $\alpha 4$ subunits internalized at early time points after EtOH (Fig. 1), and since $\alpha 4$ is commonly found with δ subunits in the dentate gyrus, which shows increased co-IP with clathrin $\mu 2$, association between the extrasynaptic $\alpha 4$ subunits and $\mu 2$ was also measured. Coimmunoprecipitation of $\alpha 4$ subunit containing GABA_A-R showed a trend to increase in $\alpha 4$ association with $\mu 2$ ($138.1 \pm 15.6\%$) at 5 min following EtOH (Fig. 3C,D); no significant changes were seen at 15 min. These results are consistent with the suggestion that, at least at early time points, the $\alpha 4$ subunits are internalizing with δ -containing GABA_A-R, through the same clathrin-mediated mechanism, likely as a complete functional heteropentameric complex along with β subunits. The $p\beta 3$ subunit did not appreciably pull-down with the $\mu 2$ subunit, as previously shown (Terunuma et al., 2008). Various GABA_A-R subunit ICDs interact directly with the $\mu 2$ subunit of the AP2 complex as cited before, and changes in these interactions are important in regulating the synaptic strength and plasticity of GABA_A-R (Kittler et al., 2005).

The ICD of the GABA_A-R δ subunit binds to AP2 subunit $\mu 2$ demonstrated by GST-protein pull-downs

The δ ICD contains two possible $\mu 2$ binding motifs (Fig. 4A): a region at residues 322–334, similar to the atypical basic-rich residues $\mu 2$ binding motifs found in synaptotagmin1, GABA_A-R $\beta(1-3)$, $\gamma 2$, and GluR3, and a region containing a classical Yxx ϕ motif in residues 375–378 (375 YRSV). The full-length δ ICD has been previously shown to bind to $\mu 2$ (Kittler et al., 2005).

To determine which region(s) of the ICD directly bind μ 2, GST protein fused to the ICD region of δ GABA δ -R were made: GST- δ ICD full length (316–410); two truncations of the δ ICD, each of which contains one of the putative μ 2 binding domains; two GST- δ ICD full-length constructs, each with one of the two μ 2 binding domains mutated; and a GST- δ ICD full length with both μ 2 binding domains mutated (Fig. 4B).

A GST pull-down assay confirmed that the GST-only (control) did not bind μ 2, while the full-length δ ICD did bind μ 2. Interestingly, both δ ICD truncations maintained partial ability to bind to μ 2 (Fig. 4C): mutation of full-length δ ICD residue 375 Y to A (to abolish the tyrosine- μ 2 binding motif) still bound μ 2, suggesting that the binding to μ 2 was maintained by the basic-rich μ 2 binding motif. Similarly, mutation of a piece of the basic-rich region 322 RKKRK to GAAGA also still bound μ 2, meaning μ 2 binding was maintained due to the tyrosine- μ 2 binding motif. A GST- δ ICD containing both mutations 375 Y \rightarrow A and 322 RKKRK \rightarrow GAAGA finally showed no binding to μ 2. These results suggest that each μ 2 binding region of the δ ICD is sufficient for binding to μ 2, and at least one or the other motif is necessary for the δ ICD to interact with μ 2.

Because each of the δ ICD regions on its own was sufficient to bind to μ 2, we examined whether there was a detectable difference between the affinities for μ 2. Pull-down assays using varying concentrations of purified μ 2 were performed. Concentration-dependent binding studies showed (Fig. 4D) that the 375 YRSV motif is more sensitive to μ 2 at a relative concentration of $\sim 0.1 \mu\text{M}$, while the 322 R/K motif is much less sensitive to the same concentration of μ 2. The differences in binding are significant only at the lower [μ 2]. These results suggest that the 375 YRSV motif may be more important in the EtOH-induced increased μ 2 binding, which then initiates endocytosis of δ subunit-containing receptors.

Discussion

This study provides additional evidence that EtOH administration to rats causes rapid internalization of $\alpha 4\beta\delta$ GABA δ -R following a single EtOH dose (Liang et al., 2007; Shen et al., 2011). Demonstration of increased association between $\alpha 4\beta\delta$ GABA δ -R and the μ 2 clathrin adaptor proteins mediating endocytosis sheds light on the possible mechanisms for regulating extrasynaptic GABA δ -R, by changing interactions between GABA δ -R subunit δ and μ 2-AP2. Characterization of the two

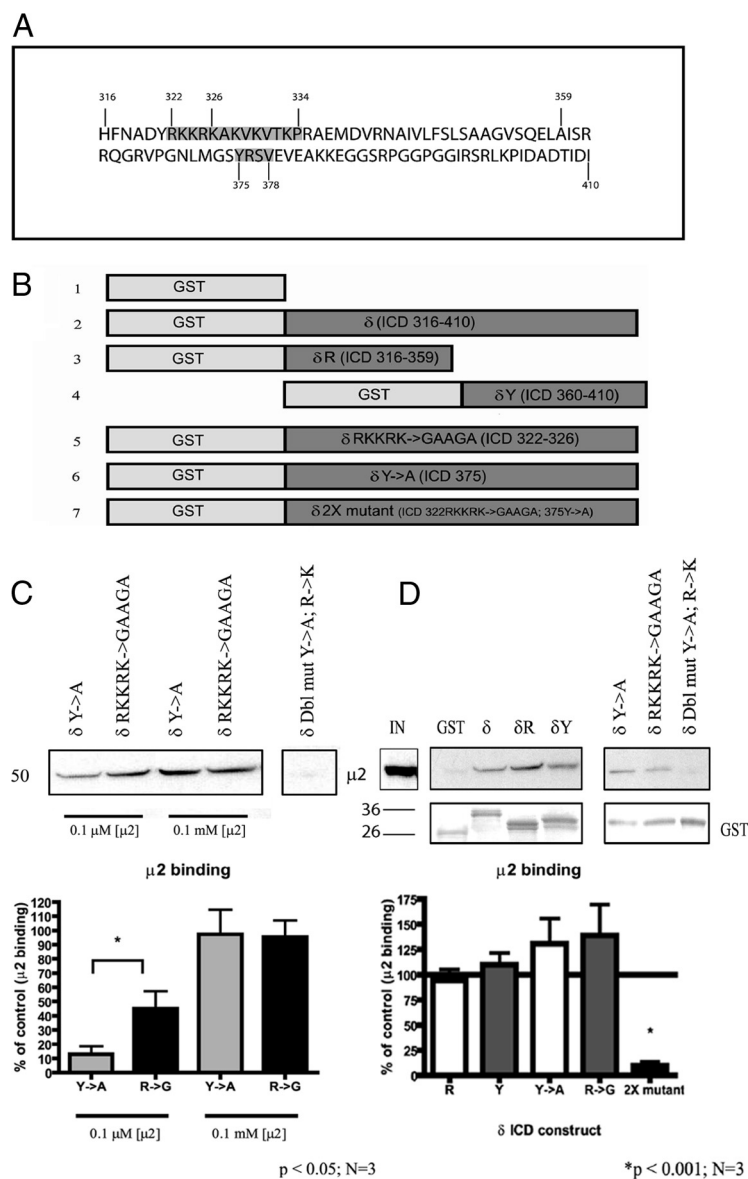


Figure 4. Characterization of δ intracellular domain. **A**, Putative μ 2 binding domains within the GABA δ -R δ subunit ICD. The entire rat δ ICD (aa 316–410) is shown. A classical Yxx Φ motif (375 YRSV) is located at residues 375–378, while an atypical basic rich motif similar to those seen in GABA δ -R β (1–3) is found at residues 322–334. Both are highlighted. **B**, Diagram of GST δ ICD constructs in this study: 1, GST only; 2, GST + δ ICD full-length residues 316–410 (δ); 3, GST + first half of δ ICD residues 316–359 (δ R); 4, GST + second half δ ICD residues 360–410 (δ Y); 5, GST + δ full-length R/K piece mutated residues 322–326 (δ 322 RKKRK \rightarrow GAAGA); 6, GST + δ full-length Y375 mutated to A (δ 375 Y \rightarrow A); 7, GST + δ full-length, where both the 322 R/K piece and 375 Y are mutated (δ 2X mutant). **C**, Each μ 2 binding domain of the δ ICD binds to μ 2. GST-only (control) does not bind to μ 2, but constructs 2–6 all bind to μ 2. This includes all δ ICD constructs containing at least one complete μ 2-binding domain (322 R/K region only shown as δ R; 375 YRSV-only shown as δ Y). Only the construct lacking both μ 2-binding domains shows no binding to μ 2. Therefore, each of the two μ 2 binding domains directly bind to μ 2. Quantification of binding shows there are no significant differences between mutants and δ ICD full-length, except for the double mutant, which significantly lost all μ 2 binding ($*p < 0.001$; one-way ANOVA). All other constructs are significantly different from the double mutant ($p < 0.05$; one-way ANOVA). **D**, The 375 YRSV-motif of the δ ICD detects lower [μ 2]. The 1 nM, 0.1 μM , and 0.1 mM AP2 fractions were incubated with the GST- δ truncation proteins to see if one region binds at a lower μ 2 concentration than the other. The procedure and analysis were similar to those done with the pull-down experiments, with representative blot and cumulative quantified μ 2 binding plot shown here. The 1 nM concentration showed no binding, but 0.1 μM showed μ 2 binding more to the construct lacking the basic rich region, suggesting this lower-concentration binding is more influenced by the 375 YRSV-motif. The difference between binding seemed to be undetectable at higher μ 2 concentrations, likely because the basic-rich 322 R/K motif is now participating in its own μ 2 binding ($p < 0.05$; one-way ANOVA; $N = 3$).

putative μ 2-binding domains within the δ ICD (one Yxx Φ motif and the other an R/K rich motif) reveals that each can bind μ 2-AP2 on its own. We show that the two domains exhibit differential affinity: the 375 Y-motif is capable of binding lower

concentrations of $\mu 2$ than the ³²²R/K-rich motif of the δ subunit. Altogether, these results suggest that clathrin-mediated endocytosis directs the early EtOH-induced internalization of extrasynaptic GABA_A-R primarily via δ subunit interactions with $\mu 2$.

EtOH-dependent downregulation of neurotransmission and tolerance resulting from ligand-induced receptor reduction

Activity-dependent changes in neurotransmission are widespread mediators of various brain activities including learning, memory, and pathological states of neuronal excitability, often by altering the number and composition of receptors available to respond to released neurotransmitters (Collingridge et al., 2004; Arancibia-Carcamo et al., 2009). Chronic benzodiazepine exposure leads to GABA_A receptor downregulation (Gallager et al., 1984; Tehrani and Barnes, 1997; Ali and Olsen, 2001; Tan et al., 2010), as does chronic EtOH treatment (Cagetti et al., 2003; Kumar et al., 2010). Such a mechanism is key to diminishing the (hyper-inhibitory) response in the face of continuous stimulus, and an initial downregulation also occurs following single doses of EtOH (Liang et al., 2007), also seen in this study. These results are consistent with a model in which agonists and positive modulators induce a conformation of the receptor that is a substrate for the biochemical events producing downregulation, possibly by cell internalization. The overstimulation of EtOH-sensitive receptors in the presence of moderate to high doses of EtOH begins the first step of downregulation of the EtOH-sensitive GABA_A receptors. During the course of this study, we (Shen et al., 2011) showed that comparable 20 min effects were reproduced in primary cultured neurons as well, and also provided evidence with cleavable biotinylation that these extrasynaptic δ -GABA_A-Rs directly underwent endocytosis (rather than decreased membrane insertion). This is consistent with δ -GABA_A-Rs as initial responders.

The results of this study are consistent with previous studies investigating how quickly tolerance to some acute EtOH effects occur in both rats and humans. Such effects are surprisingly fast, varying from minutes to 1 d, measured both by *in vivo* behavior and by *in vitro* recording (LeBlanc et al., 1975; Pearson et al., 1997; Ludvig et al., 2001; Liang et al., 2007). Importantly, these studies show that repetitive administration (intermittent cycles) of EtOH accelerates tolerance to EtOH. The GABAergic trafficking mechanisms behind acute tolerance may provide clues to how the highly complex chronic EtOH tolerance develops.

Clathrin-mediated endocytosis and phosphoregulation

Previous studies show that GABA_A-Rs undergo both constitutive and activity-dependent endocytosis primarily via clathrin-dependent mechanisms (Kittler et al., 2000; Herring et al., 2003; Kanematsu et al., 2006). Much research on endocytosis and GABA_A-R shows $\beta 3$ and $\gamma 2$ regulate clathrin-AP2 endocytosis via phosphoregulation (Kittler et al., 2005; Smith et al., 2008). Such studies involve synaptic GABA_A-R, but neither endocytosis nor phosphoregulation of extrasynaptic GABA_A-R have been studied. Upon detecting internalization of phospho- $\beta 3$ in this study, we considered its possible role in regulating endocytosis. However, together with results of EtOH exposure in cultured hippocampal neurons (Shen et al., 2011), we find that EtOH exposure stimulates endocytosis of $\alpha 4\beta 3\delta$ GABA_A-R with no significant changes in $\beta 2/3$ association with AP2 machinery. This rules out the role of phosphoregulation increasing GABA_A-R activity, as seen in previous $\beta 3$ studies. Phosphorylation of $\alpha 4$ is not likely responsible for endocytosis we see here, since Abramian et al. (2010) show $\alpha 4$ phosphorylation dictates insertion into the

membrane, rather than endocytosis. This leads to the final possibility: primary interactions involving the δ subunit. We find evidence for δ subunit interactions with the $\mu 2$ subunit of the AP2 complex, similar to other GABA_A-R subunits. The basic AP2 binding motif seen in the δ subunit is similar to those found in GABA_A-R β subunits, while the ³⁷⁵YRSV δ motif is a classical Y motif similar to those seen in several membrane receptors, including the GABA_A-R $\gamma 2$ subunit (Kittler et al., 2005; Smith et al., 2008).

Each of the two $\mu 2$ binding domains in the δ ICD was shown to bind to $\mu 2$ *in vitro*, as also seen with the $\gamma 2$ subunit. Interactions between $\gamma 2$ - $\mu 2$ and $\beta 3$ - $\mu 2$ produced additive effects on function inferring separate binding and enhancement of $\alpha\beta\gamma 2$ GABA_A-R endocytosis (Smith et al., 2008). Because the $\beta 3$ subunit is predominantly phosphorylated in the present study, it is not likely contributing to the $\mu 2$ interaction, so its effects would not be additive for electrophysiological studies. Instead, it is likely that the loss of interaction between $\mu 2$ and $\beta 3$ is compensated by the double δ - $\mu 2$ interaction, reinforcing the notion that dephosphorylation of phospho- $\beta 3$ is not directing the early EtOH-internalization of GABA_A-R. Obviously competition between different regulatory mechanisms including phosphoregulation of different subunits in the pentamer could complicate matters in a cell; however, some are almost certainly able to dominate over others, and anyway, most $\beta 3$ subunits and δ subunits are not in the same pentamers. The increased clathrin interaction of GABA_A-R δ subunit after EtOH exposure seen at 5 min but changing to a reduction at 15 min suggests complex kinetics for endocytosis which we suggest is consistent with the loss of substrate after the process is initiated, but might reflect other factors, including possible non-clathrin-mediated endocytosis, and/or possible heterogeneity of the δ -GABA_A-R protein pools. Examining the electrophysiology of $\alpha\beta\delta$ GABA_A-R while blocking $\mu 2$ interactions would provide a clearer answer. Further analysis on the two motifs showed differences between their apparent affinity for $\mu 2$, with the ³⁷⁵YRSV motif showing greater sensitivity to $\mu 2$. It is possible that the EtOH-induced increase in endocytosis is mediated primarily by the ³⁷⁵YRSV motif ability to bind to $\mu 2$, or both motifs are involved, with similar or different sensitivity to EtOH-induced regulation. Phosphorylation of either or both $\mu 2$ regions in δ may help regulate the binding to $\mu 2$, with subsequent results on endocytosis. To date the ICD has not been shown to be directly phosphorylated by PKC or PKA as are other GABA_A Rs. Several studies do, however, show that EtOH activates several kinases, and standard PKC isoforms (α , β , γ) may be activated within minutes in the nucleus accumbens and hippocampus following EtOH administration in rats (e.g., Choi et al., 2008), who suggest that PKC- δ phosphorylates δ -containing GABA_A-R, since PKC- δ increases the presence of δ GABA_A-R-mediated tonic currents. This PKC isoform may phosphorylate the δ -GABA_A-R and regulate endocytosis, possibly by direct or indirect effects on $\mu 2$ binding, as in the case of PRIP proteins (Kanematsu et al., 2006).

More functional studies needed

GABA_A-R δ subunit knock-out mice show little behavioral changes in response to EtOH, likely due to compensation by other subunits and other neurotransmitter receptors and ion channels (Mihalek et al., 1999). We reported a decrease in δ -containing GABA_A-R currents at 1 h after EtOH exposure in rats (Liang et al., 2007) and in cultured neurons (Shen et al., 2011), the rationale for this study. Detecting even earlier loss of function correlating in time with increased GABA_A-R- $\mu 2$ inter-

actions would strengthen the results of these studies. It may be possible to detect EtOH-induced rapid decreases in tonic current, I_{tonic} , by altering I_{tonic} . I_{tonic} in the hippocampus is mainly mediated by mutually exclusive $\alpha 5$ -GABA $_A$ -R and δ -GABA $_A$ -R (Mody and Pearce, 2004), activated by different GABA concentrations: δ by basal, low [GABA], and $\alpha 5$ by increased activity-induced increases in [GABA] (Scimemi et al., 2005; Bright et al., 2011). Since EtOH has also been shown to increase GABA release (Olsen and Spigelman, 2012), both δ -GABA $_A$ -R and $\alpha 5$ -GABA $_A$ -R may be contributing to EtOH-induced I_{tonic} changes in studies such as Liang et al., 2007. As such, it is possible that at early time points (i.e., 5–15 min) after EtOH, when δ -GABA $_A$ -R (directly enhanceable by EtOH) begin to internalize, $\alpha 5$ -GABA $_A$ -R are being recruited/activated by the elevated amounts of GABA. Those newly active $\alpha 5$ -GABA $_A$ -R are now contributing to I_{tonic} and possibly covering the ability to detect the loss of I_{tonic} that is mediated by δ -GABA $_A$ -R. Therefore, electrophysiological analysis of EtOH-induced tonic currents should include a pharmacological block of $\alpha 5$ -GABA $_A$ -R, to decrease the heterogeneity of the receptors contributing to I_{tonic} and focus mainly on δ GABA $_A$ -R I_{tonic} changes.

It is important to test the functional significance of these $\mu 2$ binding regions within the δ ICD, as they are likely important in regulating the cell surface number of δ -containing GABA $_A$ -R. GABA $_A$ -R containing the δ subunit as already mentioned are unique in limited regional and subcellular localization exclusively at extrasynaptic sites, unique properties including high sensitivity to GABA and low desensitization, and a particularly important role in mediating tonic inhibition. Tonic currents are seen to be responsive to low concentrations of EtOH, and are highly sensitive to neurosteroids; therefore endocytosis of δ -GABA $_A$ -R during EtOH exposure reduces important endogenous modulation by neurosteroids, as well as basal tonic inhibition in some areas. The current results suggest that tonic inhibition is subject to use-dependent downregulation, important to many physiological conditions.

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