Constitutive GABA\textsubscript{A} Receptor Endocytosis is Dynamin-Mediated and Dependent on a Dileucine AP2 Adaptin Binding Motif within the \(\beta2\) Subunit of the Receptor

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**Running title:** GABA\textsubscript{A} receptor endocytosis

The abbreviations used are: GABA (\(\gamma\)-aminobutyric acid), FBS (fetal bovine serum)
Summary

Receptor endocytosis is an important mechanism for regulating the synaptic efficacy of neurotransmitters. There is strong evidence that GABA<sub>A</sub> receptor endocytosis is clathrin-dependent, however, this process is not well-understood. Here we demonstrate that in HEK 293 cells, endocytosis of GABA<sub>A</sub> receptors composed of either α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>L or α<sub>1</sub>β<sub>2</sub> subunits is blocked by the dominant negative dynamin construct K44A. Furthermore, we identify a dileucine AP2 adaptin binding motif within the receptor β<sub>2</sub> subunit that is critical for endocytosis. Internalization of GABA<sub>A</sub> receptors lacking this motif is dramatically inhibited and the receptors appear to accumulate on the cell surface. Patch clamp analysis of receptors lacking the dileucine motif show that there is an increase in the peak amplitude of GABA-gated chloride currents compared to wildtype receptors. Additionally, GABA-gated chloride currents in HEK 293 cells expressing wildtype receptors are increased by introduction of a peptide corresponding to the dileucine motif region of the receptor β<sub>2</sub> subunit but not by a control peptide containing alanine substitutions for the dileucine motif. In mouse brain cerebral cortical neurons, the dileucine motif peptide increases GABA-gated chloride currents of native GABA<sub>A</sub> receptors. This is the first report to our knowledge that an AP2 adaptin dileucine recognition motif is critical for the endocytosis of ligand-gated ion channels belonging to this superfamily.
Introduction

The GABA<sub>A</sub> receptor is a ligand-gated chloride channel that, upon activation by GABA (γ-aminobutyric acid), mediates increases in chloride conductance resulting in membrane hyperpolarization and neuronal inhibition (1). The role of these receptors in hyperexcitability states, such as epilepsy and anxiety, is widely recognized. Importantly, GABA<sub>A</sub> receptors mediate the effects of benzodiazepines and barbiturates, two frequently prescribed classes of therapeutic agents. The GABA<sub>A</sub> receptor is a pentameric receptor composed of multiple subunits, each containing four membrane-spanning regions (M1-M4) with a large intracellular loop between M3 and M4. A number of subunits exist (α1–6, β1–3, γ1–3, δ, θ, ε, π) and receptors composed of α1β2γ2L subunits are believed to represent the predominant GABA<sub>A</sub> receptor subtype in the brain (1).

Receptor endocytosis is known to regulate the cell surface expression of neurotransmitter receptors and such regulation is an important mechanism for controlling the synaptic efficacy of neurotransmitters (2). Although GABA<sub>A</sub> receptors undergo endocytosis, the mechanism is not well understood. Several lines of evidence indicate that GABA<sub>A</sub> receptor endocytosis may be clathrin/dynamin-dependent. These include the presence of GABA<sub>A</sub> receptors in clathrin-coated vesicles isolated from brain (3), the colocalization of the receptor with transferrin receptors (4), and the colocalization and coimmunoprecipitation of hippocampal GABA<sub>A</sub> receptors with the clathrin adaptor complex AP2 adaptin (5). Additionally, peptides that disrupt dynamin-amphiphysin interactions increase GABA IPSC amplitudes in hippocampal neurons (5). Direct examination of GABA<sub>A</sub> receptor endocytosis in HEK 293 cells has revealed that receptor endocytosis is blocked by hypertonic sucrose (5), a treatment known to inhibit, but is not specific for, clathrin-dependent endocytosis (6, 7). Unexpectedly, a recent study has demonstrated that although constitutive GABA<sub>A</sub> receptor endocytosis in HEK 293 cells is blocked by hypertonic sucrose, it is not blocked by the dynamin dominant negative mutant K44A, indicating that GABA<sub>A</sub> receptors are endocytosed via a clathrin-independent
mechanism (8). Thus, the pathway(s) by which GABA<sub>A</sub> receptors are endocytosed are not well-characterized. Furthermore, the domains on the receptor that are involved in the endocytic process have not been identified. In the present study, we sought to clarify the mechanism by which GABA<sub>A</sub> receptors are endocytosed and identify the receptor domains involved.

**Experimental Procedures**

**Animals and Materials.** Timed-pregnant C57Bl/6J mice (Jackson Laboratories) were housed in an AALAC approved facility (University of North Texas Health Sciences Center). The mice were kept on a 12 hr light/dark cycle and fed standard laboratory rodent chow. Cortical slices were prepared in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals*. HEK 293 cells were obtained from American Type Culture Collection (Manassas, VA). Human GABA<sub>A</sub> receptor cDNAs (9) were kindly provided by Dr. Paul Whiting (Merck Sharp and Dohme Research Laboratories, UK). Dynamin and dynamin K44A cDNAs were gifts from Dr. Sandra Schmid (The Scripps Research Institute). The following antibodies were used: Texas Red and Alexa 488 conjugated secondary antibodies (Molecular Probes, Eugene, OR), mouse monoclonal 9E10 anti-myc antibody (Pharmingen, San Diego, CA), and mouse polyclonal anti-myc 9E10 ascites fluid (gift from Dr. Kelly Tatchell, LSU Health Science Center-Shreveport).

**Construction of receptor mutants.** To mutate the tyrosine and/or dileucine AP2 adaptin binding motifs within the GABA<sub>A</sub> receptor β2 subunit, site-specific mutations were introduced using the Stratagene QuikChange kit (Stratagene, La Jolla, CA) according to kit instructions. Oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, IA) that contained the desired mutation and at least 15 bases of homology to the wildtype cDNA. The mutants and their respective PCR primers were: (myc)<sub>β2</sub>(Y304F), 5'- CCCTAGTCAAACGCCATCTTCTTTGG- 3' and 5' - GGGATCAGTTGCAGGTAGAAAGAAACC-3' and (myc)<sub>β2</sub>(LL343/344AA), 5'-CCATGAGAACAT
CGCAGCGAGCCTCTCGAG-3' and 5'-CTC GAGAGTGCTCGCTGCGATGTTCTCATGG-3'. Colonies were screened by sequencing using the Sequenase 2.0 kit (US Biochemical, Cleveland, OH) with [\(^{35}\)S] dATP (New England Nuclear, Boston, MA). Mutated subunits were sequenced in their entirety (Arizona State University sequencing facility). The dileucine (ENILLSSTLEI) and control dialanine (ENIAASSTLEI) peptides used for the electrophysiology experiments were synthesized by Genemed Synthesis, Inc. (San Francisco, CA).

**HEK 293 cell culture and transfection.** To plate HEK 293 cells, cells were trypsinized (0.05%), triturated in DMEM/F12 containing 10% FBS media, pelleted by centrifugation, resuspended in 10 ml of media, and diluted to approximately 200,000 cells per 0.5 ml. Thirty-five mm poly-L-lysine coated glass bottom insert dishes (MatTek Corp., Ashland, MA) were seeded at approximately 1 x 10\(^5\) cells in a volume of 2 ml/dish. Dishes were incubated overnight at 37°C in a humidified incubator with 5% CO2/95% air. Cells were then transfected with 0.6 µg of plasmid DNA using the calcium phosphate method and placed in a 3% CO2 incubator for 17 hrs. Cells were then washed twice, the media changed and cells were incubated at 37°C in 5% CO2/95% air for approximately 42 hrs before use. To avoid problems associated with the expression of high amounts of recombinant protein, we used a transfection protocol that resulted in 15-25% transfection efficiency.

**Immunofluorescence.** Receptor Endocytosis Protocol (Figures 1-3, 5,6). Living HEK 293 cells expressing recombinant GABA\(_A\) receptors were incubated on ice in 100 µl of HEPES buffer (HEPES 25 mM, NaCl 140 mM, KCl 5.4 mM, CaCl\(_2\) 1.8 mM, glucose 15 mM, pH=7.4) containing 5 µg/ml mouse monoclonal 9E10 anti-myc antibody for 1 hr, followed by a 1 hr incubation on ice with 1 ml of buffer containing a Texas Red-conjugated goat anti-mouse antibody (1:500) to label cell surface receptors. Cells were then incubated for 20 min at 37°C to allow receptor endocytosis. To identify the Texas Red-labeled receptors that were not internalized during this 20 min period, cells were incubated for 1 hr on ice in 1 ml of buffer containing an Alexa 488-conjugated rabbit anti-goat antibody (1:1000). Cells were
then fixed with 4% paraformaldehyde and visualized by confocal microscopy. For these experiments, colocalized Texas Red and Alexa 488 labeling represents those Texas Red labeled receptors that remained on the cell surface during the 37°C incubation, while Texas Red labeling not colocalized with Alexa 488 represents receptors that were endocytosed during the 37°C incubation period. The immunolabeling of myc-tagged GABA<sub>A</sub> receptors in living HEK 293 cells has been used previously to study GABA<sub>A</sub> receptor endocytosis (4, 8, 10). Receptor Cell Surface Labeling Protocol (Figure 5). For the cell surface expression experiments, HEK 293 cells expressing GABA<sub>A</sub> receptors were incubated with mouse anti-myc 9E10 containing ascities (1:1000) for 1 hr on ice followed by a 1 hr incubation on ice with a Texas Red-conjugated goat anti-mouse antibody (1:500). Cells were rinsed and fixed with 4% paraformaldehyde and visualized by confocal microscopy. For these experiments, at least three independent experiments were performed, where independent experiments are defined as experiments performed on different batches of HEK 293 cells transfected at different times. For each independent experiment at least three cells were quantified and averaged to yield one “n”. The data from each independent experiment were then averaged.

**Confocal microscopy.** A Bio-Rad MRC-1024 laser scanning system with an argon/krypton laser (Bio-Rad, Hercules, CA) was used for fluorescence microscopy. The excitation wavelengths and emission filters used for Alexa 488 and Texas Red were 488 nm, 522/30 and 568 nm, 605/32, respectively. A 60X objective was used to collect images. Laser intensity, photo-multiplier gain, and iris were optimized for each set of experiments and held constant throughout. Images were captured using LaserSharp MRC-1024 software and processed using Adobe Photoshop software.

**Tissue culture.** Organotypic explants were derived from ~360 µm thick, hemi-coronal slices of postnatal day (P) 2 (day of birth = P1) frontal and cingulate cerebral cortex, obtained from both male and female pups born of timed-pregnant C57Bl/6J mice (Jackson Laboratories) as previously described (11). Explant slices were maintained as roller tube cultures (12) on rat tail collagen-coated/poly-D-
lysine pre-coated glass coverslips and grown in steroid-deficient and phenol red-free maintenance medium [gelding serum (25%); Hank's BSS (22.5%); MEM Eagles (Minimum Essential Medium Eagles, 50%); glucose (7.5 mg/ml); L-glutamine (2 mM); ascorbic acid (50 µg/ml)]. Cultures were maintained in vitro for 3 days prior to experimentation.

**Electrophysiology.** The conventional whole cell configuration of the patch clamp technique was used to study GABA-gated currents in transiently transfected HEK293 cells or organotypic explants (13). The pipette solution contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP; pH 7.2. Coverslips containing HEK 293 cells were placed in a small chamber (~ 1.5 ml) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5-8 ml/min) with the following external solution containing (in mM): NaCl, 125; KCl, 5.5; CaCl2, 1.5; MgCl2, 0.8; HEPES-Na, 20; glucose, 10; pH 7.3. Neurons from explant slices were recorded in synaptic blockade medium ((in mM): 128 NaCl, 3.0 KCl, 11.4 MgCl2, 10 HEPES, 2.0 CaCl2, 10 glucose, 300 mOsm, pH 7.3) to minimize presynaptic inputs using an upright microscope (Nikon Optiphot 2-UD) fitted with Hoffman modulation contrast optics. All recordings were obtained at 35 °C, with cells voltage-clamped at -60 mV. GABA was prepared in the extracellular solution and was applied to cells from independent reservoirs using a Y-shaped tube positioned within 100 µm of the cell.

**Results**

To examine constitutive endocytosis of GABAₐ receptors, HEK 293 cells expressing recombinant receptors composed of α₁(myo)β₂γ₂L subunits were labeled as described in the methods section under the “receptor endocytosis protocol”. An endocytosis time course is shown in Fig 1A. At time zero (ice, no time at 37°C), the receptor does not undergo endocytosis and remains on the cell surface (represented by the colocalization of Texas Red and Alexa 488 signals). At times subsequent to 0 min, the receptor is constitutively endocytosed, as evident by the punctate Texas Red signal within cells. By 40 min, most, if
not all, of the receptors had been endocytosed (little to no yellow signal remaining). Since the receptor is thought to be recycled back to the cell surface following endocytosis (8), the time course of endocytosis was followed to 60 min. If internalized receptor was returned to the surface during this period, yellow signal should reappear, however no reappearance of the yellow signal was detected at 60 min. It is also possible that Texas Red-conjugated antibody dissociated from the receptor following endocytosis and that unlabeled receptors were then recycled back to the surface. Such events, however, would not contribute to our measurements since these receptors would not be labeled with the Alexa 488 conjugated antibody. Thus, our experimental protocol provides a measurement of receptor endocytosis without complications due to receptor recycling.

To determine if constitutive receptor endocytosis is dynamin-dependent, receptors composed of $\alpha_1^{(\text{myc})}\beta_2\gamma_2\text{L}$ subunits were coexpressed with dynamin constructs (Fig 1B, top panels). Coexpression of the dominant negative dynamin mutant K44A, but not of wildtype dynamin, inhibited receptor endocytosis. Quantitative data for this experiment are shown in Fig. 2A. Similar results were obtained when untagged receptors were detected using a mouse monoclonal anti-$\beta_2/3$ subunit antibody (data not shown).

Because it was suggested that clathrin-dependent endocytosis of the receptor requires the presence of the $\gamma_2$ subunit (10), endocytosis of receptors composed of only $\alpha_1^{(\text{myc})}\beta_2$ subunits was examined (Fig. 1B, bottom left panel). Similar to receptors composed of $\alpha_1^{(\text{myc})}\beta_2\gamma_2\text{L}$ subunits, receptors lacking the $\gamma_2 \text{L}$ subunit were also internalized, indicating that the $\gamma_2 \text{L}$ subunit is not required for endocytosis. Furthermore, the endocytosis of receptors lacking the $\gamma_2 \text{L}$ subunit was inhibited by overexpression of dynamin K44A but not wildtype dynamin (Fig. 1B, bottom middle and right panels). Quantitative data are shown in Fig. 2B. Similar results were observed using a mouse anti-$\beta_2/3$ subunit antibody to detect untagged receptors (data not shown).
For the clathrin-dependent endocytic pathway, cargo proteins are recruited to clathrin-coated pits by AP2 adaptin complexes that recognize dileucine and tyrosine motifs within target proteins (14). The cytoplasmic loop region of the receptor β2 subunit contains both dileucine (LL at 343,344) and tyrosine YXXθ (YIFF, 304-307) AP2 adaptin binding motifs (Fig. 3). To investigate whether these motifs play a role in constitutive receptor endocytosis, the dileucine and tyrosine motifs were mutated individually and together. For these experiments the leucines at residues 343 and 344 were mutated to alanines and the tyrosine at position 304 was mutated to a phenylalanine. Endocytosis of the mutant proteins was then examined in HEK 293 cells expressing receptors composed of α1(myc)-β2γ2L, α1(myc)-β2(LL343/344AA;Y304F)γ2L, α1(myc)-β2(LL343/344AA)γ2L or α1(myc)-β2(Y304F)γ2L subunits (Fig. 4, 5). Constitutive endocytosis was observed for both wildtype receptors and receptors composed of α1(myc)-β2(Y304F)γ2L subunits. Therefore, the tyrosine motif is not required for this process. In contrast, the endocytosis of receptors lacking the dileucine motif, i.e., those composed of α1(myc)-β2(LL343/344AA;Y304F)γ2L or α1(myc)-β2(LL343/344AA)γ2L subunits, was greatly inhibited, indicating that the dileucine motif is critical for this process. Because it was possible that mutation of the dileucine motif resulted in a gross disruption of receptor structure, we performed patch clamp studies to test the functional integrity of the receptors containing the mutant subunit. As shown in Fig. 6, GABA-gated chloride currents were observed in cells expressing α1(myc)-β2(LL343/344AA)γ2L subunits. Furthermore, the receptors were potentiated by diazepam, indicating that the γ2L subunit had been incorporated into the receptor (15). In these experiments, in the presence of 1 µM diazepam, GABA-gated chloride currents were 174 ± 18 (n=9) and 164 ± 9.3 (n=12) percent of the GABA control response for wildtype and mutant (α1(myc)-β2(LL343/344AA)γ2L) receptors, respectively.

Since the endocytosis of receptors lacking the dileucine motif on the β2 subunit was inhibited, we expected that the receptor would accumulate at the cell surface. As shown in Fig. 4B, there was an increase in the level of surface immunolabeling of cells expressing the mutant receptor. Consistent with
the immunolabeling results, the maximal current of GABA-gated chloride currents activated by 1mM GABA in cells expressing receptors containing the β2 subunit dileucine motif mutant was nearly twice that observed for wildtype (7145 ± 473 pA in mutant receptors, n=5 vs. 3735 ± 357 pA for wildtype receptors, n=13, unpaired t-test, p ≤ 0.01).

Because it was possible that mutation of the receptor dileucine motif affected receptor endocytosis/function in a manner that was unrelated to specific endocytic mechanisms, we performed competition experiments using a 10 amino acid peptide that corresponds to the dileucine motif region of the receptor β2 subunit. Presumably, if the dileucine region of the β subunit is a binding partner for AP2 adaptin, the peptide should compete with receptor for AP2 binding, and thus, prevent receptor endocytosis. Using the patch clamp technique, GABA-gated chloride currents were recorded with pipets containing either a control dialanine peptide (identical to the dileucine peptide except that alanines were substituted for leucines) or the dileucine peptide (peptide sequences specified in the Experimental Procedures section). Recordings made in the presence of the control peptide showed a stable GABA response peak current amplitude during the 25 min recording period (Fig. 7). Experiments conducted with the dileucine peptide showed an approximate 44% increase in GABA-gated chloride currents when compared to control recordings at 25 min. By 10 min, significant increases in GABA responses were noted.

To determine if the results obtained in the HEK 293 cells were relevant to neurons, additional electrophysiological experiments with the peptides were performed in cerebral cortical explants. Similar to the experiments in HEK 293 cells, in the presence of the dileucine peptide there was a significant increase in GABA-gated chloride currents that was evident beginning at 10 min. (Fig. 8). At 30 min, there was an approximately 60% increase in GABA responses in cell exposed to the dileucine peptide relative to control recordings.
Our results are inconsistent with the dynamin-independent endocytosis of chicken GABA_A receptors that was recently reported (8). It is possible that species differences could account for the discrepancy between our results and those of Cinar and Barnes (8), however, the dileucine AP2 adaptin binding motif is present in both human and chicken receptors (16). One difference between our protocol and that of Cinar and Barnes is that their experiments were performed in the presence of fetal bovine serum (FBS). To determine if this methodological difference could underlie our disparate findings, we repeated our endocytosis assays in the presence of FBS. In the presence of FBS, coexpression of dynamin K44A failed to block GABA_A receptor endocytosis (Figs. 9A, 10). Furthermore, GABA_A receptors that lacked the dileucine AP2 adaptin binding motif on the β2 subunit were internalized in the presence of FBS (Figs. 9B). Finally, using the “cell surface labeling” protocol, few GABA_A receptors were detected on the surface of cells treated with FBS (data not shown).

Discussion

Although there is evidence that GABA_A receptors are endocytosed via a clathrin-dependent pathway (3-5), there is also evidence to the contrary (8). Here, we demonstrate that GABA_A receptor endocytosis is blocked by the dominant negative dynamin mutant K44A, indicating that it is dynamin-dependent. Furthermore, we show that the AP2 adaptin dileucine motif on the receptor β2 subunit is important for receptor endocytosis, while a receptor γ subunit is not required. In addition, we show that in HEK 293 cells and neurons, the presence of a peptide that corresponds to the dileucine motif on the β2 subunit increases GABA-gated chloride currents. Lastly, in the presence of serum, GABA_A receptor endocytosis proceeds in a dynamin-independent manner that does not involve the AP2 adaptin dileucine motif on the receptor β2 subunit.

Dynamin is known to mediate scission of both clathrin-coated pits and caveolae at the plasma membrane (reviewed in 18). Results obtained here using the dominant negative dynamin construct
K44A establish a role for dynamin in GABA<sub>A</sub> receptor endocytosis. Such experiments, however, do not distinguish between the involvement of clathrin-coated pits vs. caveolae in the endocytic process. One distinguishing feature of clathrin-dependent endocytosis is the involvement of the clathrin adaptor protein AP2 adaptin. For clathrin-dependent endocytosis, the AP2 adaptin complex recruits integral membrane proteins into clathrin-coated pits (14). The cytoplasmic loop regions of β1–3 GABA<sub>A</sub> receptor subunits contain conserved dileucine and tyrosine AP2 recognition motifs. Here we demonstrate that mutation of the dileucine motif on the cytoplasmic loop of the β2 subunit is critical for receptor endocytosis. Because β and μ subunits of the adaptin complex recognize dileucine and tyrosine based motifs, respectively (14), it is likely that the GABA<sub>A</sub> receptor β2 subunit interacts with the β subunit of AP2 adaptin. Indeed, GST fusion proteins of the intracellular domains of β1, β3, γ2S and γ2L GABA<sub>A</sub> receptor subunits interact with the α and β subunits of AP2 adaptins present in brain extracts (6). Although the dileucine motif has been identified as important for the endocytosis of a variety of other integral membrane proteins (18-21), this is the first report, to our knowledge, that an ionotropic neurotransmitter receptor belonging to this superfamily possesses this feature.

In our studies, mutation of the tyrosine-based AP2 adaptin binding motif on the receptor β2 subunit did not affect receptor endocytosis. This was surprising given the role of this motif in the endocytosis of integral membrane proteins (22, 23). It is possible that the tyrosine motif does not play a role in GABA<sub>A</sub> receptor endocytosis or that its involvement is dependent on posttranslational modification or protein-protein interactions that do not exist under our experimental conditions. It is also possible that this tyrosine motif could bind to AP2 adaptin μ subunits and affect targeting of the receptor to recycling or degradative pathways subsequent to internalization. Such a mechanism has been reported for β2 integrin, where mutation of the tyrosine motif diverts β2 integrin from the recycling to the degradative pathway (24).
Our studies indicate that dynamin-dependent GABA_\text{A} receptor internalization does not require a γ subunit within the receptor. This is in contrast to the finding that receptors composed of either α1β2 or α1β2γ2L subunits are expressed on the cell surface, but that only those containing a γ subunit undergo endocytosis (4, 10). Although our results indicate that receptor endocytosis can proceed in the absence of a γ subunit, it is possible that the γ subunit may play a modulatory role in controlling the rate of endocytosis or receptor trafficking subsequent to endocytosis.

Interestingly, the γ2L subunit contains a dileucine sorting motif (LLRMFSFK) in the alternatively spliced region of the cytoplasmic loop. This motif is not required for constitutive clathrin-dependent receptor endocytosis under our experimental conditions, since receptors that do not contain the γ2L subunit undergo endocytosis and the presence of this motif in receptors composed of α1β2(LL343/344AA)γ2L subunits is not sufficient to confer endocytic competency to the receptor. Our studies do not rule out the possibility that the dileucine motif on the γ2L subunit is important under different conditions. In this regard, the dileucine motif on the γ2L subunit is immediately upstream of a PKC phosphorylation site. It is possible that the phosphorylation state of this site could regulate the interaction of the γ2L subunit with AP2 adaptin. Such regulation occurs with cluster of differentiation antigen 4 (CD4), the GLUT4 glucose transporter and CD3γ, in which the dileucine motif plays a role in clathrin-dependent endocytosis when a nearby serine is phosphorylated (25-27).

Studies in recombinant expression systems using overexpressed mutated proteins can yield misleading results. Therefore, patch-clamp experiments on both wildtype recombinant and neuronal GABA_\text{A} receptors included a peptide corresponding to the dileucine motif on the receptor β2 subunit. It was expected that this peptide would competitively inhibit the binding of AP2 adaptin to the receptor, resulting in a decrease in receptor endocytosis and a subsequent increase in GABA-gated chloride currents. Such an increase in GABA responses was observed in both HEK 293 cells and cortical neurons. These findings indicate that the results of experiments using mutated receptors are
likely to be specific to the mutation rather than to a general disruption of receptor structure. In this regard, the functional integrity of the mutant receptor appeared preserved as evidenced by the presence of GABA-gated chloride currents and their potentiation by diazepam. The observation that the dileucine peptide also increased GABA-gated chloride currents in neurons indicates that the results obtained in our study may be extrapolated to native receptors.

Our results showing that dynamin K44A inhibits GABA\(_A\) receptor endocytosis are inconsistent with those of a previous study, which indicate that dynamin K44A does not prevent GABA\(_A\) receptor endocytosis (8). Both studies employed an indirect immunofluorescence technique with a monoclonal anti-c-myc antibody to measure receptor endocytosis in living HEK 293 cells. One notable difference between the two studies, however, was the use of fetal bovine serum (FBS) in the earlier study. We hypothesized that constituents of the serum could promote endocytosis of the receptor through a dynamin-independent pathway. Therefore, we performed additional experiments using our endocytosis protocol in the presence of FBS. In these experiments, dynamin K44A failed to block GABA\(_A\) receptor endocytosis. In addition, receptors that lacked the dileucine motif were also endocytosed. Therefore, the inclusion of FBS accounts for the discrepancy between the studies. It is possible that that antibodies contained in the FBS could nonspecifically bind to the receptor and promote endocytosis. Antibody-promoted internalization of other integral membrane proteins has been observed under certain conditions (28). Although this possibility cannot be definitively ruled out, we consider it unlikely since the binding of anti-myc antibodies to cell surface GABA\(_A\) receptors in living HEK 293 cells does not appear to induce GABA\(_A\) receptor endocytosis (4, 8, 10). Alternatively, hormones or other signaling molecules present in the serum could affect signal transduction pathways that regulate endocytic processes. If so, the endocytic pathway taken by GABA\(_A\) receptors may depend on the signal transduction status of the cell.

We have observed in the course of these studies that the efficiency of transfection of receptor constructs, and thus, level of receptor overexpression, is an important factor to consider when studying
receptor endocytosis. The results shown in this work are from experiments performed at low transfection efficiencies, to avoid potential problems encountered due to high levels of heterologously expressed proteins. At much higher transfection efficiencies, we observed that the coexpression of dynamin K44A was not as effective in blocking receptor endocytosis, and the dileucine motif mutant was internalized to a greater level (unpublished observation), indicating that the clathrin pathway may become saturated and that under these conditions, an alternative endocytic pathway may be used by the receptor.

Here we have demonstrated that in HEK 293 cells, GABA_\text{A} receptor endocytosis is dynamin-dependent, and we have identified an AP2 adaptin dileucine binding motif on the receptor β2 subunit that is critical for this endocytosis. Furthermore, we have shown that a peptide corresponding to the AP2 dileucine motif region of the receptor β2 subunit increases GABA-gated chloride currents in HEK 293 cells expressing recombinant receptors and also in cerebral cortical neurons. The observations that GABA_\text{A} receptor cell surface expression is rapidly decreased following GABA_\text{A} receptor stimulation (29), ischemia (30) and exposure of neurons to brain-derived neurotrophic factor (31) underscore the importance of understanding the mechanisms that regulate GABA_\text{A} receptor endocytosis.
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Figure 1. Constitutive endocytosis of GABA<sub>A</sub> receptors composed of either α<sub>1</sub><sup>(myc)</sup>-β<sub>2</sub>γ<sub>2</sub>L or α<sub>1</sub><sup>(myc)</sup>-β<sub>2</sub>γ subunits is blocked by the dominant negative dynamin mutant K44A.  A) Time course of GABA<sub>A</sub> receptor endocytosis. Using the “receptor endocytosis protocol” described in the Methods section, endocytosis of receptors composed of α<sub>1</sub><sup>(myc)</sup>-β<sub>2</sub>γ<sub>2</sub>L subunits was examined at 0, 10, 20, 40 and 60 min.  B, C) Using the “receptor endocytosis protocol” described in the Methods section, GABA<sub>A</sub> receptors were immunolabeled in HEK 293 cells coexpressing α<sub>1</sub><sup>(myc)</sup>-β<sub>2</sub>γ<sub>2</sub>L subunits, α<sub>1</sub><sup>(myc)</sup>-β<sub>2</sub>γ<sub>2</sub>L + wildtype dynamin or α<sub>1</sub><sup>(myc)</sup>-β<sub>2</sub>γ<sub>2</sub>L + dynaminK44 (B) or with the same constructs without the γ<sub>2</sub>L subunit (C). Cells were then fixed with 4% paraformaldehyde and visualized by confocal microscopy. GABA<sub>A</sub> receptors expressed in the absence or presence of wildtype dynamin display receptor endocytosis as evident from the Texas Red labeling within the cell. GABA<sub>A</sub> receptors coexpressed with dynamin K44A fail to undergo endocytosis and remain on the cell surface (yellow). Scale bar = 10 µm.

Figure 2. Constitutive endocytosis of GABA<sub>A</sub> receptors in living HEK 293 cells is blocked by the dominant negative dynamin mutant K44A.  Replicate data for experiments in Fig 1B, C above. Endocytosis of GABA<sub>A</sub> receptors composed of either α<sub>1</sub><sup>(myc)</sup>-β<sub>2</sub>γ<sub>2</sub>L (panel A) or α<sub>1</sub><sup>(myc)</sup>-β<sub>2</sub> (panel B) subunits was significantly inhibited by coexpression of dynamin K44A but not by wildtype dynamin (*, p≤0.01, unpaired t-test, n=3 for each condition).

Figure 3. Diagram showing the locations of the dileucine and tyrosine-based AP2 adaptin binding motifs on the GABA<sub>A</sub> receptor β2 subunit. ●= tyrosine motif YIIF at 304-307; ■= dileucine motif LL at 343, 344.
Figure 4. Mutation of the dileucine AP-2 adaptin binding motif on the GABA<sub>\text{A}</sub> receptor β2 subunit inhibits receptor endocytosis and increases cell surface expression. A) Living HEK 293 cells coexpressing α<sub>1</sub>(myc)-β2γ2L, α<sub>1</sub>(myc)-β2(LL343/344AA;Y304F)γ2L, α<sub>1</sub>(myc)-β2(LL343/344AA)γ2L or α<sub>1</sub>(myc)-β2(Y304F)γ2L were labeled by indirect immunofluorescence using the “receptor endocytosis protocol” and visualized by confocal microscopy. Receptor endocytosis is apparent in cells expressing both wildtype and AP2 adaptin tyrosine motif mutant receptors. Receptor endocytosis was inhibited in cells expressing receptors that lacked the AP2 adaptin dileucine binding motif on the β2 subunit.

B) The cell surface expression of GABA<sub>A</sub> receptors composed of α<sub>1</sub>(myc)-β2γ2L or α<sub>1</sub>(myc)-β2(LL343/344AA)γ2L subunits was measured by indirect immunofluorescence using the “receptor cell surface labeling protocol” described in the Methods section. Cells were rinsed and fixed with 4% paraformaldehyde and visualized by confocal microscopy. Scale bar = 10 µm.

Figure 5. Mutation of the dileucine AP-2 adaptin binding motif on the GABA<sub>\text{A}</sub> receptor β2 subunit inhibits receptor endocytosis. Replicate data for experiments in Fig 4A. Receptor endocytosis was inhibited in cells expressing receptors that lacked the AP2 adaptin dileucine binding motif on the β2 subunit (α<sub>1</sub>(myc)-β2(LL343/344AA;Y304F)γ2L, α<sub>1</sub>(myc)-β2(LL343/344AA)γ2L) compared to (wildtype α<sub>1</sub>(myc)-β2γ2L) or receptors lacking the tyrosine motif (α<sub>1</sub>(myc)-β2(Y304F)γ2L), * p≤0.005, unpaired t-test, n=4 for each condition.

Figure 6. Receptors lacking the dileucine AP-2 adaptin binding motif on the β2 subunit form functional receptors. GABA-gated chloride currents were measured in HEK 293 cells expressing GABA<sub>A</sub> receptors composed of α<sub>1</sub>(myc)-β2γ2L or α<sub>1</sub>(myc)-β2(LL343/344AA;Y304F)γ2L subunits. Whole cell patch clamp recordings were recorded in the presence of GABA (10 µM) or GABA (10 µM) +
diazepam (1 µM). Both wildtype and mutant receptors were responsive to GABA and potentiated by diazepam.

Figure 7. GABA-gated chloride currents in HEK 293 cells are increased by a peptide that corresponds to the dileucine motif region of the receptor β2 subunit. A. Whole cell patch clamp recordings were obtained from HEK 293 cells expressing GABA_A receptors composed of α1^{myc}-β2γ2L subunits. Pipette solution included either a control dialanine peptide (50 µM, top traces) or a peptide corresponding to the dileucine motif region of the receptor β2 subunit (dileucine peptide, 50 µM, bottom traces). Current amplitude in response to GABA (10 µM) remained stable over time with intracellular dialysis of the control dialanine peptide, but increased significantly when the cell was dialyzed with the dileucine peptide. B. Mean results from the above experiments. Average current amplitudes between the two groups were significantly different at the ten minute time point and continued to diverge throughout the recording period. *, p < 0.05, n = 5-8 at each time point for each group.

Figure 8. GABA-gated chloride currents of cerebral cortical neurons are increased by a peptide that corresponds to the dileucine motif region of the receptor β2 subunit. Experiments are similar to those described in Figure 6. A. Whole cell patch clamp recordings were obtained from GABA_A receptors of cortical neuronal slices. As observed in recombinant receptors, current amplitude in response to GABA (10 µM) remained stable over time with intracellular dialysis of the control dialanine peptide (top traces), but increased significantly when the cell was dialyzed with the dileucine peptide (bottom traces). B. Mean results from experiments on cortical GABA_A receptors. Average current amplitudes between the two groups were significantly different at ten minutes and remained so throughout the recording period. *, p < 0.05, **, p < 0.01, n = 5-7 at each time point for each group.
Figure 9. Fetal bovine serum promotes dynamin-independent, dileucine motif-independent GABA<sub>A</sub> receptor endocytosis. A) Living HEK 293 cells coexpressing α<sup>1</sup>(myc)-β2γ2L subunits, α<sup>1</sup>(myc)-β2γ2L + dynamin or α<sup>1</sup>(myc)-β2γ2L + dynamin K44A were labeled by indirect immunofluorescence as described in the Methods section by the “receptor endocytosis protocol” except that 10% FBS was present throughout all antibody incubations and during the 20 min endocytosis period. A) In the presence of FBS, GABA<sub>A</sub> receptors were endocytosed when coexpressed with either wildtype dynamin or dynamin K44. B) Receptor endocytosis was visualized by immunofluorescence in living HEK 293 cells coexpressing α<sup>1</sup>(myc)-β2(LL343/344AA)γ2L subunits in the absence (left panel) or presence (right panel) of FBS. In the presence of FBS, receptors lacking the dileucine AP-2 adaptin binding motif on the receptor β2 subunit undergo endocytosis. Scale bar = 10 µm.

Figure 10. Fetal bovine serum promotes dynamin-independent and dileucine motif-independent GABA<sub>A</sub> receptor endocytosis. Replicate data for experiments in Fig. 8A. In the presence of FBS, GABA<sub>A</sub> receptor endocytosis is not affected by wildtype dynamin or dynamin K44A (treatments not significantly different from control values, n=3 each group).
Figure 2

A. $\alpha_1\beta_2\gamma_2L$

B. $\alpha_1\beta_2$
Figure 3
Figure 4

A.

\[ \alpha_1\beta_2\gamma_2L \quad \alpha_1\beta_2(LL343/344AA; Y304F)\gamma_2L \]

\[ \alpha_1\beta_2(Y304F)\gamma_2L \quad \alpha_1\beta_2(LL343/344AA)\gamma_2L \]

B.

\[ \alpha_1\beta_2\gamma_2L \quad \alpha_1\beta_2(LL343/344AA)\gamma_2L \]
Figure 6

$\alpha_1\beta_2\gamma_2L$

diazepam
GABA

$\alpha_1\beta_2(\text{LL343/344AA})\gamma_2L$

diazepam
GABA
Figure 7

A. Control peptide

Time (min) 0 5 10 15 20 25

Dileucine peptide

B. Relative current amplitude

Control peptide
Dileucine peptide

Time (min) 0 5 10 15 20 25
Figure 8

A.

Control peptide

Dileucine peptide

B.

Relative current amplitude

Time (min)
Figure 9

A. 
\[ \alpha_1\beta_2\gamma_2L \quad \alpha_1\beta_2\gamma_2L + \text{dynamin} \quad \alpha_1\beta_2\gamma_2L + \text{dynaminK44A} \]

B. 
\[ \alpha_1\beta_2(\text{LL343/344AA})\gamma_2L \quad \alpha_1\beta_2(\text{LL343/344AA})\gamma_2L \]
Figure 10

Percent of receptors endocytosed

- Control
- Dynamic
- Dynamic K24A

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Constitutive GABAA receptor endocytosis is dynamin-mediated and dependent on a
dileucine AP2 adaptin binding motif within the β2 subunit of the receptor
Dina Herring, RenQi Huang, Meharvan Singh, Lucy C. Robinson, Glenn H. Dillon and
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