EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, Vol. 29, pp. 869–878, 2009 doi:10.1111/j.1460-9568.2009.06636.x

MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Identification of a novel region of the $GABA_{B2}$ C-terminus that regulates surface expression and neuronal targeting of the $GABA_B$ receptor

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Keywords: GABA, hippocampal neurons, localization, trafficking

Abstract

$GABA_B$ is a G protein-coupled receptor composed of two subunits, $GABA_{B1}$ and $GABA_{B2}$. $GABA_{B1}$ contains an endoplasmic reticulum-retention sequence and is trafficked to the cell surface only in association with $GABA_{B2}$. To determine whether the C-terminus of $GABA_{B2}$ regulates $GABA_B$ trafficking, we constructed forms of $GABA_{B2}$ with various C-terminal truncations and examined their surface expression. Truncation of $GABA_{B2}$ after residue 841 significantly reduced surface expression of both the subunit and the heterodimerized receptor. Turnover of the Δ841 construct, however, did not differ from that of full-length $GABA_{B2}$. To determine whether the C-terminus of $GABA_{B2}$ might target $GABA_B$ to neurites, cultured hippocampal neurons were transfected with the truncated $GABA_{B2}$ constructs. Truncation of $GABA_{B2}$ at residue 841 resulted in primarily somatic localization; furthermore, axonal trafficking of this construct was significantly more restricted than dendritic trafficking. Finally, to biochemically assess trafficking of the truncated $GABA_{B2}$ constructs, we digested transfected HEK293 cell lysates with endoglycosidase H. When $GABA_{B2}$ was truncated at residue 841, it became sensitive to digestion by this enzyme, indicating incomplete trafficking. Taken together, these data show that the region of the $GABA_{B2}$ C-terminus between residues 841 and 862 is important for regulating forward trafficking and neuronal targeting of the $GABA_B$ receptor.

Introduction

G protein-coupled receptors (GPCRs) are important for both excitatory and inhibitory transmission in the brain and their precise targeting is required for their proper function. The $GABA_B$ receptor, a GPCR, regulates neurotransmission both presynaptically and postsynaptically by modulating second messenger activity (Knight & Bowery, 1996) and ion channels (Sodickson & Bean, 1996; Ng *et al.*, 1999). Functional $GABA_B$ receptors require heterodimerization of two distinct subunits, $GABA_{B1}$ and $GABA_{B2}$ (White *et al.*, 1998). $GABA_{B1}$ contains a motif in its C-terminus that causes it to be retained in the endoplasmic reticulum (ER); however, heterodimerization with $GABA_{B2}$ masks this motif, allowing the fully formed receptor to move to the cell surface (Pagano *et al.*, 2001; Gassmann *et al.*, 2005).

Recent studies suggest that the C-terminus of $GABA_{B2}$ may be involved in trafficking of the fully formed receptor; for example, surface expression of $GABA_{B1}$ is reduced when it is co-expressed with a C-terminally truncated form of $GABA_{B2}$, even when subunit interaction remains intact (Grunewald *et al.*, 2002). Furthermore, in transgenic mice, deletion of the entire C-terminus of $GABA_{B2}$ prevents surface expression of $GABA_{B1}$ and results in a loss of functional $GABA_B$ receptors (Thuault *et al.*, 2004). However, it is unclear whether movement of the receptor to the cell surface is due simply to masking of the $GABA_{B1}$ retention motif following association with $GABA_{B2}$, or whether, in addition, $GABA_{B2}$ acts to target the fully assembled receptor to the plasma membrane.

Trafficking of receptors from the ER to the cell surface is often regulated by the C-terminus of the protein, which may contain a variety of amino acid motifs (Tan *et al.*, 2004). These motifs are also important for targeting proteins within neuronal compartments. The dileucine motif is one such sequence: axonal localization of the sodium channel $Na_v1.2$ is regulated by a C-terminal dileucine motif (Garrido *et al.*, 2001). Furthermore, distinct axonal and dendritic targeting sequences in intracellular loops of nicotinic acetylcholine receptor subunits have been identified (Xu *et al.*, 2006). Therefore, specific amino acid sequences can target receptors not only to the plasma membrane, but also to neuronal compartments. As $GABA_{B1}$ requires $GABA_{B2}$ in order to exit the ER, the $GABA_{B2}$ subunit may contain motifs that regulate trafficking of the fully formed receptor. However, to date, the presence and localization of such trafficking motifs in $GABA_{B2}$ have not been determined.

Recently, we have identified a region of the $GABA_{B2}$ C-terminus that is important for movement of $GABA_B$ at the cell surface (Pooler & McIlhinney, 2007). $GABA_{B2}$ might also be important for receptor trafficking and membrane targeting. We therefore generated a number of C-terminal truncations of $GABA_{B2}$ in order to systematically determine whether this region is involved in regulating neuronal trafficking and surface expression of the $GABA_B$ receptor. We

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Received 22 May 2008, revised 18 December 2008, accepted 22 December 2008

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identified a region of the $GABA_{B2}$ C-terminus that regulates movement of the subunit into axons and dendrites, as well as trafficking of $GABA_B$ to the cell surface. These findings add further detail to the complex mechanisms underlying targeting and trafficking of the $GABA_B$ receptor.

Materials and methods

Cell culture and transfection

Human embryonic kidney (HEK)293 or COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/mL penicillin/100 μg/mL streptomycin, and incubated at 37 °C in a 5% CO_2 atmosphere. For experiments, cells were seeded onto uncoated glass coverslips (for COS-7 cells) or poly(D-lysine)-coated glass coverslips (for HEK293 cells) and transfected 24 h later using JetPEI (Autogen Bioclear, Calne, UK), according to the manufacturer's instructions. Cells were incubated for 48 h post-transfection before analysis. Primary neuronal cultures were prepared from E18 Sprague–Dawley rat embryos (Charles River, Margate, UK), obtained from pregnant rats using a Schedule 1 method under the authority of the Animals (Scientific Procedures) Act 1986 (UK). Briefly, the pregnant rats were killed by cervical dislocation, and the embryos removed and placed in ice-cold Hanks' balanced salt solution. Subsequently, the hippocampi were dissected and mechanically dissociated in Hanks' balanced salt solution lacking calcium and magnesium, supplemented with 1 mM pyruvate and 10 mM HEPES. Cells were plated immediately onto glass coverslips coated with poly(D-lysine) (5 μg/mL) and cultured in Neurobasal medium containing 2% B-27 serum-free supplement, 0.5 mM L-glutamine, 25 μM glutamate, and 50 μg/mL gentamicin. After 4 days, the medium was changed to Neurobasal medium supplemented with 2% B-27, 0.5 mM L-glutamine, 0.05% gentamicin and 3 μM cytosine arabinofuranoside, to restrict the proliferation of non-neuronal cells. Neurons were transfected with Lipofectamine (Invitrogen, Paisley, UK) for 5 days *in vitro*, according to the manufacturer's instructions, and imaged 48 h later. Approximately 1–3% of cells were transfected per coverslip.

Plasmids

The constructs used in the present study were as described previously (Pooler & McIlhinney, 2007). The *myc*-tagged dileucine motif mutant $GABA_{B2}$ IL853/854MV was generated using a Quik-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. First, the *Eco*RV/*Hin*dIII fragment was excised from the hemagglutin (HA)-tagged $GABA_{B2}$ in pcDNA3.1(–)*myc*-his and cloned into pcDNA3. Polymerase chain reaction mutagenesis was performed on this plasmid using the forward primer 5′-CAG ATG GAG GAA AGG CCA TGG TAA AAA ATC ACC TCG-3′ and the reverse primer 5′-CGA GGT GAT TTT TTA CCA TGG CCT TTC CTC CAT CTG-3′. The mutated fragment was excised from pcDNA3 using *Eco*RV/*Hin*dIII restriction sites and religated into *Eco*RV/*Hin*dIIII-digested HA-tagged $GABA_{B2}$ in pcDNA3.1(–)*myc*-his. DNA alterations to all constructs were verified by DNA sequencing.

Immunocytochemistry

Following the transfection period, the cells were washed once with phosphate-buffered saline (PBS), fixed for 5 min at in 4% paraformaldehyde, and washed twice with PBS and twice with Tris–saline. In order to detect intracellular proteins, cells were permeabilized with 0.02% Triton X-100 for 5 min; for visualization of surface proteins, cells were left unpermeabilized. Cells were incubated for 1 h at room temperature in blocking solution containing 1% bovine serum albumin and either 1% normal goat serum or 1% normal donkey serum, and then incubated for 2 h at room temperature in blocking solution containing primary antibody. Primary antibodies were as follows: anti-myc mouse monoclonal antibody clone 9E10, produced in-house from a hybridoma cell line (Atlason *et al.*, 2007; Remelli *et al.*, 2008) (1 : 10); mouse anti-HA (1 : 1000; Covance Antibodies, Berkeley, CA, USA); sheep anti-$GABA_{B1}$ (generated at the Babraham Institute) raised against peptide DGSRVHLLYK of $GABA_{B1}$ (White *et al.*, 2000); and rabbit anti-microtubule-associated protein 2 (MAP2, 1 : 2000; Chemicon, Temecola, CA, USA). The cells were washed three times with PBS and incubated for 1 h with the appropriate secondary antibody (anti-mouse ALEXA 568 or anti-rabbit ALEXA 488, 1 : 500; Molecular Probes, Eugene, OR, USA). In order to visualize surface proteins, intact cells were incubated for 20 min at 37°C in Hanks' balanced salt solution containing mouse anti-HA antibody (1 : 1000). Cells were washed twice with PBS, fixed and incubated with anti-mouse ALEXA 568 secondary antibody. After three washes with PBS, the coverslips were mounted and viewed with a Zeiss LSM510 inverted confocal microscope. Confocal images for analysis of receptor trafficking were captured from a single optical plane with a 40 × oil-immersion objective. For surface expression experiments, confocal images were captured from a single optical plane with a 20 × objective (for all images, the pinhole setting was 1 Airy Unit). For each experiment, all images were collected using identical laser settings, to allow direct comparison between the images. Approximately 10–20 neurons per treatment group were captured for each receptor trafficking experiment, and each experiment was repeated a minimum of three times. For analysis of surface expression, 10 random images were captured for each treatment group. The images were processed as described below.

Analysis of receptor trafficking

Trafficking of the constructs into neuritic processes was measured as follows. Neurons were transfected with either yellow fluorescent protein (YFP)-tagged or *myc*-tagged $GABA_{B2}$ constructs, and these constructs were visualized directly or using an anti-*myc* antibody, respectively. At the same time, neurons were visualized with an anti-MAP2 antibody, which labels the entire dendritic arbor and weakly labels axons. The transfected construct was therefore visualized in conjunction with the entire neuron. Trafficking of the construct into cell processes was defined as the percentage of each process labeled by that construct (i.e. length of process containing construct relative to total length of the process, as visualized by MAP2 immunoreactivity).

Axons and dendrites were discriminated from each other in two ways. First, axons were morphologically distinguished from dendrites as being thinner and not tapering along the course of projection from the cell body. Second, the axon was identified as being less intensely labeled by the MAP2 antibody than the dendrites (Kosik & Finch, 1987). If an axon could not be conclusively identified according to these criteria, then that cell was not included in the analysis.

Analyses of surface expression and localization

In order to quantify receptor surface expression in COS-7 cells, a minimum of 10 random images were captured per coverslip using a

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20 × objective. Each image contained 2–10 cells, which were subjected to image analysis. The fluorescence intensity of each cell was quantified individually using the program ImageJ (http://rsb.info.nih.gov/ij/) with the Multi-Cell Outliner macro. Surface expression and total expression of the receptor were determined by measuring the fluorescence intensity of non-permeabilized cells and permeabilized cells, respectively. Non-specific background fluorescence was calculated for each image and subtracted from the fluorescence intensity values obtained within that image. Non-specific antibody binding was assessed for each experiment. Each experiment included one group of cells that were not transfected but were otherwise processed in the same way as the transfected cells. As these cells did not contain HA-tagged proteins, any signal arising from these cells was attributed to non-specific binding of the anti-HA antibody and subtracted from the values obtained for transfected cells. Surface expression for each group is presented as a ratio of mean surface fluorescence to mean total fluorescence levels.

Receptor internalization was quantified by incubating COS-7 cells expressing either HA–$GABA_{B2}$ or HA–Δ841 with mouse anti-HA antibody for 20 min at 37°C. The antibody was then removed, the cells were washed, and, at the indicated time points, their surface fluorescence was assessed as described above.

To substantiate our findings, we also measured surface expression using an enzyme-linked immunosorbent assay (ELISA). HEK293 cells were grown in 25-cm^2 flasks and co-transfected with *myc*–$GABA_{B1b}$ and either HA–$GABA_{B2}$ or HA–Δ841. Twenty-four hours later, the cells were detached and replated into a poly(D-lysine)-coated 24-well plate. On the following day, the cells were fixed and half the wells were permeabilized in order to detect intracellular expression. The cells were then blocked in 3% bovine serum albumin in PBS/0.05% Tween-20 for 2 h at room temperature, and then incubated with mouse anti-*myc* antibody (1 : 10) for 2 h at room temperature. After multiple washes with PBS, the cells were incubated with horseradish peroxidase-conjugated anti-mouse antibody (1 : 5000) for 1 h. The cells were washed several times before incubation with 250 μL of K-blue substrate (Neogen Europe Limited, Auchincruive, UK) for 5–10 min. The K-blue substrate was removed and placed in a clean 96-well plate, which was read at 620 nm in a Labsystems Multiskan Bichromatic plate reader (Helsinki, Finland). Surface expression is presented as a ratio of surface receptor levels to intracellular receptor levels. Non-specific antibody binding was assessed for each ELISA experiment as described for immunofluorescence experiments above.

Endoglycosidase H/F and western blotting

Transfected HEK293 cells were washed with PBS and detached from T-25 flasks with 1 mL of 10 mM sodium bicarbonate buffer containing protease inhibitor cocktail, 10 mM iodoacetamide and 20 μg/mL DNAse I. Cells were disrupted by trituration through a 27-g needle and centrifuged at low speed for 2 min to remove debris. The supernatant was transferred to a clean Eppendorf tube and spun for 10 min at 14 000 ***g*** at 4°C to pellet the membrane fraction. The membrane-containing pellet was then resuspended in 100 μL of Tris–saline containing 1% sodium dodecylsulfate and boiled for 5 min. After addition of 400 μL of Tris–saline containing 1% Triton X-100, the samples were spun for 15 min at 14 000 ***g*** at 4°C. The supernatant of each treatment was divided into three aliquots: one for control, one for digestion by N-glycosidase F (PNGase F), to remove all glycosylation, and one for digestion by endoglycosidase H (endo H), to remove only high-mannose moieties. After overnight digestion, lysis buffer was added to each sample. After boiling, equal volumes of each sample were loaded and separated using 7.5% sodium dodecylsulfate polyacrylamide gel electrophoresis. Proteins were transferred to a poly(vinylidene fluoride) membrane (PerkinElmer, Waltham, MA, USA) using a Trans blot semi-dry transfer cell (Bio-Rad), and the membranes were blocked in 5% (w/v) non-fat dried milk in PBS/0.05% Tween-20 for 1 h. Membranes were incubated overnight in mouse anti-*myc* antibody (1 : 10) at 4°C and subsequently detected using horseradish peroxidase-conjugated secondary antibodies in conjunction with the SuperSignal Chemiluminescence Kit (Pierce Chemical Co., Rockford, IL, USA).

Data analysis

Measurements of cellular proteins were normalized against those of control groups. ANOVA was used to determine differences between groups (significance level, $P = 0.05$) with *post hoc* analysis by unpaired *t*-test. Data are presented as mean ± standard error of the mean; levels of significance are indicated in each figure legend. For analyses of surface expression or trafficking by immunofluorescence, each group is composed of a minimum of 50 cells, obtained in three or more separate experiments.

Results

C-terminally truncated forms of $GABA_{B2}$ alter surface expression of $GABA_{B1}$

In order to investigate the role of the C-terminus of $GABA_{B2}$ in trafficking of the $GABA_B$ receptor, we constructed a sequence of C-terminally truncated forms of $GABA_{B2}$ (Fig. 1). The shortest truncation removed the final 21 amino acids from the C-terminus to produce $GABA_{B2}$Δ920. The longest truncation in our study removed nearly all of the C-terminus distal to the coiled-coil domain to produce $GABA_{B2}$Ä841. Each of the truncations was tagged with either YFP at the C-terminus, or HA and *myc* at the N-terminus and C-terminus, respectively (Pooler & McIlhinney, 2007).

To examine surface expression of the $GABA_B$ subunits, COS-7 cells were transfected with the appropriate constructs and the subunits were visualized using inmunocytochemistry. When cells were transfected with $GABA_{B1b}$ only, no surface expression was observed (data not shown). Cells transfected with both $GABA_{B1b}$ and $GABA_{B2}$ displayed robust surface expression of $GABA_{B1b}$ (Fig. 2A, left column). As $GABA_{B1b}$ requires interaction with $GABA_{B2}$ in order to be trafficked to the cell surface (White *et al.*, 1998), detection of $GABA_{B1b}$ on the surface indicates proper $GABA_B$ receptor hetero-

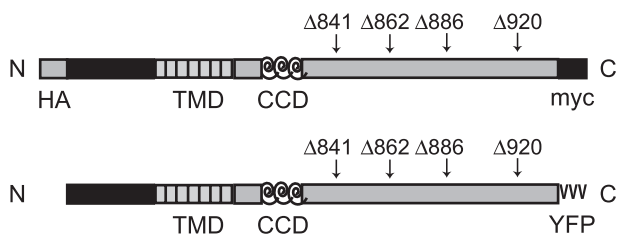

FIG. 1. Schematic outline of the $GABA_{B2}$ constructs. C-terminal truncations of the subunit were made at the indicated amino acid residues. The hemagglutin (HA) tag was added at the N-terminus of the constructs, whereas the *myc* and yellow fluorescent protein (YFP) tags were placed at the C-terminus. N, N-terminus; C, C-terminus; CCD, coiled-coil domain; TMD, seven transmembrane domain.

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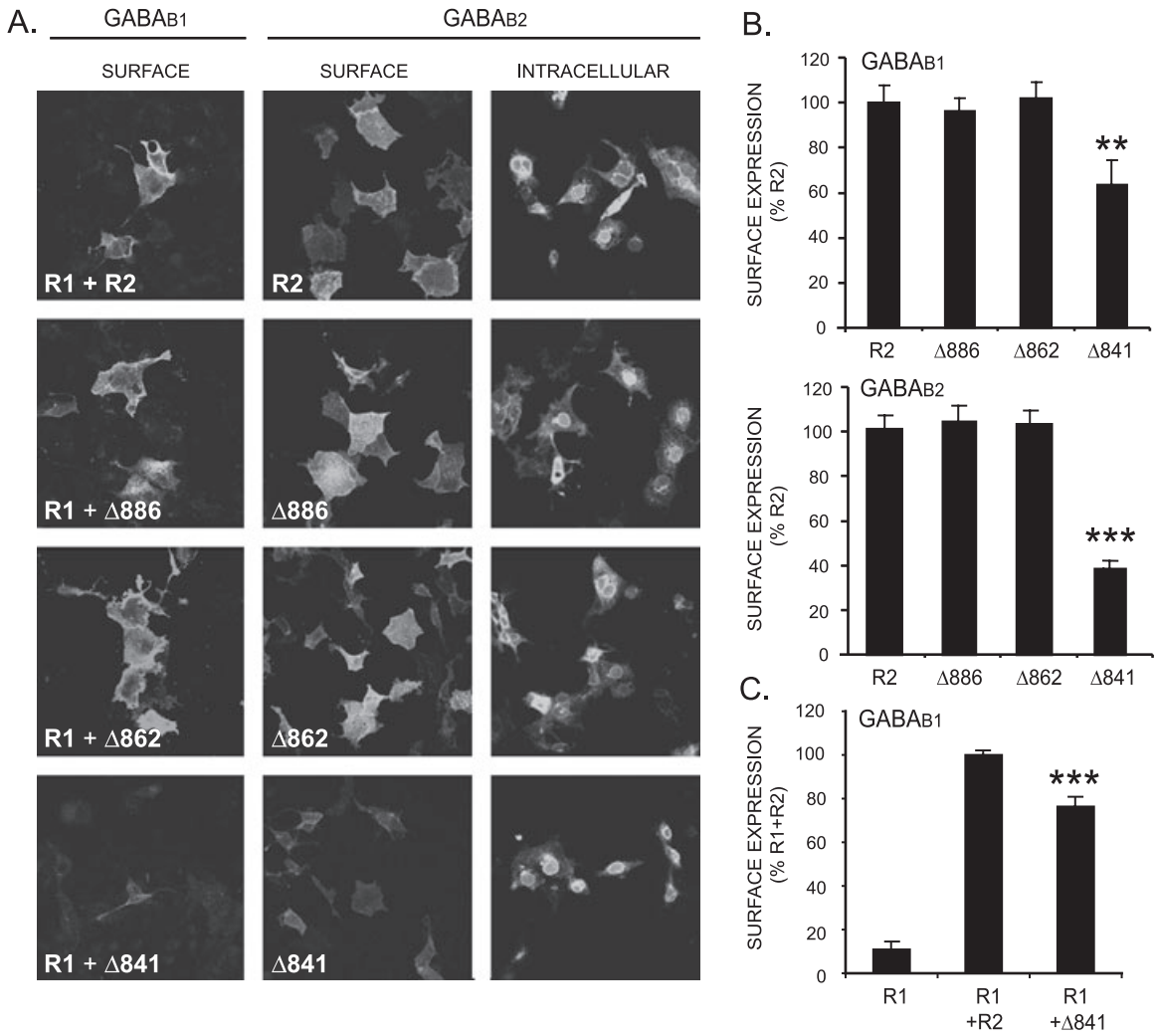

FIG. 2. Truncation of the $GABA_{B2}$ C-terminus impairs surface trafficking of $GABA_B$. (A) COS-7 cells transfected with the indicated subunits were immunostained to visualize either surface *myc*–$GABA_{B1b}$ (left column), surface $GABA_{B2}$ construct (center), or intracellular $GABA_{B2}$ construct (right). (B) Surface expression of each construct was quantified and is expressed as a ratio of surface fluorescence intensity to intracellular fluorescence intensity. Values were normalized to the value obtained for full-length $GABA_{B2}$. Top: Co-expression of $GABA_{B1b}$ with Δ841 significantly decreased $GABA_{B1b}$ surface expression, as compared to surface expression levels in cells containing the Δ862 form of $GABA_{B2}$. Bottom: In the absence of $GABA_{B1b}$, surface expression of the Δ841 truncation is also significantly reduced relative to surface levels of Δ862. (C) Enzyme-linked immunosorbent assay of $GABA_{B1b}$ subunit surface expression confirmed that only in the presence of $GABA_{B2}$ does $GABA_{B1b}$ move to the cell surface. However, surface expression of $GABA_{B1b}$ was significantly impaired when it was co-expressed with Δ841. R1, $GABA_{B1b}$; R2, $GABA_{B2}$. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the indicated group.

dimerization. Strong surface expression of $GABA_{B1b}$ was also observed in cells co-transfected with $GABA_{B1b}$ and either Δ886 or Δ862; by contrast, in the presence of Δ841, surface staining of $GABA_{B1b}$ was weak. In the absence of $GABA_{B1b}$, Δ841 also exhibited significantly less surface expression than that observed in cells transfected with $GABA_{B2}$, Δ886 or Δ862 (Fig. 2A, center column). Visualization of intracellular subunit expression confirmed that all four $GABA_{B2}$ constructs were expressed at similar levels (Fig. 2A, right column). Quantification of fluorescence intensity by image analysis revealed that surface expression of $GABA_{B1b}$ was significantly ($P < 0.01$) reduced when it was co-expressed with Δ841, as compared to the expression levels observed in the presence of $GABA_{B2}$ (Fig. 2B, top). Moreover, cells exhibited significantly ($P < 0.001$) less Δ841 than $GABA_{B2}$ at the cell surface (Fig. 2B, bottom).

In order to confirm our results obtained by image analysis, we determined surface expression using an ELISA directed against $GABA_{B1}$. HEK293 cells transfected with only $GABA_{B1}$ displayed very little surface expression of the protein, whereas co-transfection with $GABA_{B2}$ resulted in strong surface expression (Fig. 2C). In the presence of Δ841, however, significantly ($P < 0.001$) less $GABA_{B1}$ surface expression was detected. Taken together, these data suggest that the region of the $GABA_{B2}$ C-terminus between amino acids 841 and 862 is important for delivery of the $GABA_B$ receptor to the cell surface.

Decreased surface expression of Δ841 is not due to increased receptor internalization

COS-7 cells transfected with either $GABA_{B2}$ or Δ841 were assessed for surface and intracellular expression of the subunit, using analysis of fluorescent images. Values represent the ratio of surface to intracellular fluorescence intensity, normalized to the zero time point. The two constructs were internalized at similar rates (Fig. 3), suggesting that lower levels of surface Δ841 are due to impaired

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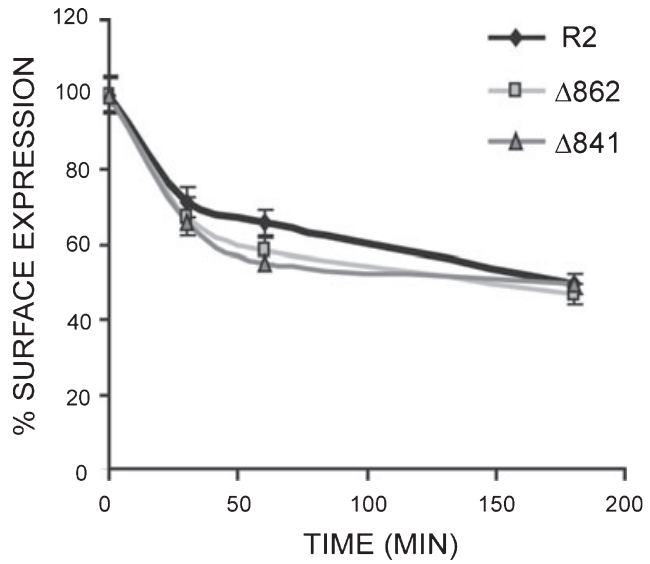

FIG. 3. The Δ841 and Δ862 truncations of $GABA_{B2}$ are internalized at a similar rate as the full-length subunit. Surface expression of full-length $GABA_{B2}$ (R2), Δ862 and Δ841 subunits was assessed in transfected COS-7 cells using image analysis, and is presented as a ratio of surface fluorescence intensity to intracellular fluorescence intensity. Expression was analysed after 0, 30, 60 and 180 min of incubation with anti-hemagglutin antibody. At the longest time point assessed in this study, no significant differences in surface expression were observed between $GABA_{B2}$ and either of the truncations, which suggest that all three constructs are internalized on a similar time scale.

forward trafficking of the subunit, rather than an increase in receptor endocytosis.

Neuronal trafficking of $GABA_{B2}$ is impaired by truncation of its C-terminus

As previous studies have suggested that receptor C-termini may be important for regulating receptor trafficking in neurons (Grunewald *et al.*, 2002), we determined whether the C-terminus of $GABA_{B2}$ is involved in delivery of the subunit to axonal and dendritic compartments. We transfected primary rat hippocampal neuron cultures with YFP-tagged full-length $GABA_{B2}$ (R2) or a truncated version of the subunit. Both R2–YFP and Δ920–YFP filled the entire neuron; however, Δ886, Δ862 and Δ841 YFP-tagged truncations did not traffic into axons and dendrites (Fig. 4A). As the relatively large YFP tag was added to the C-termini of the construct, we questioned whether the presence of the YFP might interfere with trafficking of the subunit. We therefore created a series of $GABA_{B2}$ constructs that were C-terminally tagged with the small *myc* epitope. Each of the *myc*-tagged constructs robustly filled the entire neuron, except for the Δ841–*myc* construct, which remained localized only within the soma (Fig. 4B). Analysis of the confocal cell images revealed that the Δ886, Δ862 and Δ841 YFP-tagged versions did not traffic into axons and dendrites ($P < 0.001$; Fig. 5A, left). However, only for the Δ841–YFP construct was axonal trafficking significantly ($P < 0.05$) impaired relative to trafficking into the dendrites (Fig. 5A, right). In contrast, *myc*-tagged R2, Δ920, Δ886 and Δ862 were consistently present in the axon and both proximal and distal dendrites, although the presence of the Δ841 construct in these compartments was significantly ($P < 0.001$) reduced (Fig. 5B, left). Furthermore, the ratio of axonal to dendritic expression was significantly ($P < 0.001$) lower for the Δ841 construct than for the other four constructs (Fig. 5B, right). The behavior of Δ841–*myc* was similar to the behavior displayed by YFP-tagged Δ841. These data suggest that residues 841–862 of the $GABA_{B2}$ C-terminus mediate trafficking of the subunit into the neurites; moreover, this region may be particularly important for axonal targeting of the subunit.

The $GABA_{B2}$ C-terminus regulates neuronal trafficking of $GABA_B$

Next, we evaluated whether the region of the $GABA_{B2}$ C-terminus between residues 841 and 862 is able to regulate trafficking of the heterodimerized $GABA_B$ receptor. To do so, we transfected hippocampal neurons with $GABA_{B1}$–cyan fluorescent protein (CFP) alone or in conjunction with either $GABA_{B2}$–YFP or Δ841–YFP. When expressed alone, $GABA_{B1}$–CFP is almost entirely retained within the cell body (Fig. 6, top row), but it is found throughout the cell when co-expressed with $GABA_{B2}$–YFP (Fig. 6, middle row). However, co-expression of $GABA_{B1}$–CFP with Δ841–YFP resulted in both constructs being localized only within the soma (Fig. 6, bottom row), indicating that the C-terminus of $GABA_{B2}$ is important for neuronal trafficking of the fully formed receptor.

Δ841 is sensitive to deglycosylation by endo H

Transmembrane proteins undergo N-linked glycosylation with high-mannose carbohydrates in the ER. During passage from the ER and through the Golgi apparatus, these high-mannose residues are trimmed and complex side chains are added. Digestion of proteins with PNGase F removes all N-linked carbohydrates, whereas digestion with endo H only affects high-mannose N-linked carbohydrates. Thus, endo H sensitivity indicates the proportion of a protein that is present in the ER. To determine the glycosylation state of our $GABA_{B2}$ truncations, we subjected lysates from HEK293 cells transfected with either $GABA_{B2}$, Δ886, Δ862 or Δ841 to PNGase F or endo H digestion and western blot analysis (Fig. 7). In undigested cell lysates, a single immunoreactive band of approximately 100 kDa was detected, corresponding to $GABA_{B2}$. As expected, all constructs of $GABA_{B2}$ were sensitive to deglycosylation by PNGase F. Following endo H digestion, $GABA_{B2}$, Δ886 and Δ862 still produced a single immunoreactive band of approximately 100 kDa; however, in contrast, Δ841 migrated as two immunoreactive bands, indicating that a fraction of this construct was endo H sensitive. These results are consistent with the C-terminus of $GABA_{B2}$ containing a forward-trafficking signal between residues 841 and 862, without which the subunit is retained in the ER.

The dileucine motif at residues 853/854 does not regulate $GABA_B$ trafficking

We next examined the region of $GABA_{B2}$ between residues 841 and 862 in order to identify any known trafficking motifs. We found one putative dileucine motif, an isoleucine/leucine pair in position 853/854. To determine whether this pair is involved in trafficking of $GABA_{B2}$, we mutated these residues to methionine and valine, respectively. We found no difference in surface expression of the mutant subunit as compared to the subunit containing the dileucine motif (data not shown). Furthermore, the mutation of the isoleucine/leucine pair did not impair axonal and dendritic targeting of the subunit when expressed in hippocampal neurons (data not shown). These data indicate that this motif does not regulate $GABA_B$ trafficking and suggest that a novel trafficking motif may be present between residues 841 and 862 of $GABA_{B2}$.

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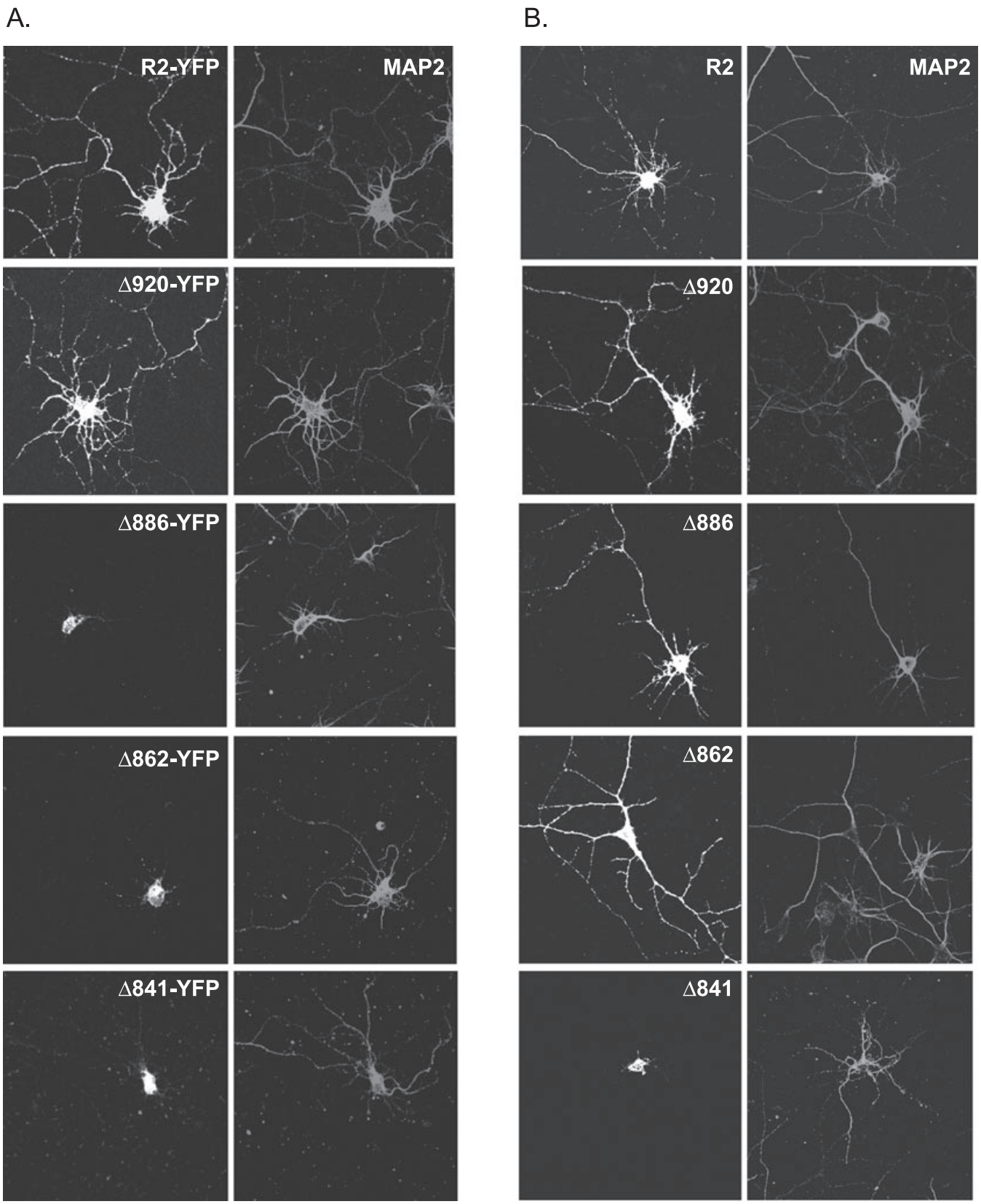

FIG. 4. The $GABA_{B2}$ C-terminus is important for dendritic and axonal trafficking of the subunit. (A) Cultured hippocampal neurons were transfected with yellow fluorescent protein (YFP)-tagged versions of the $GABA_{B2}$ constructs. The constructs Δ886–YFP, Δ862–YFP and Δ841–YFP are localized to the cell soma. (B) Cultured hippocampal neurons were transfected with either $GABA_{B2}$–*myc* or one of the truncated myc-tagged versions of the subunit. Neurons were fixed and co-stained with anti-*myc* antibody (left column) and an anti- microtubule-associated protein 2 (MAP2) antibody (right column). All constructs except Δ841 appear throughout the neuron. R2, $GABA_{B2}$.

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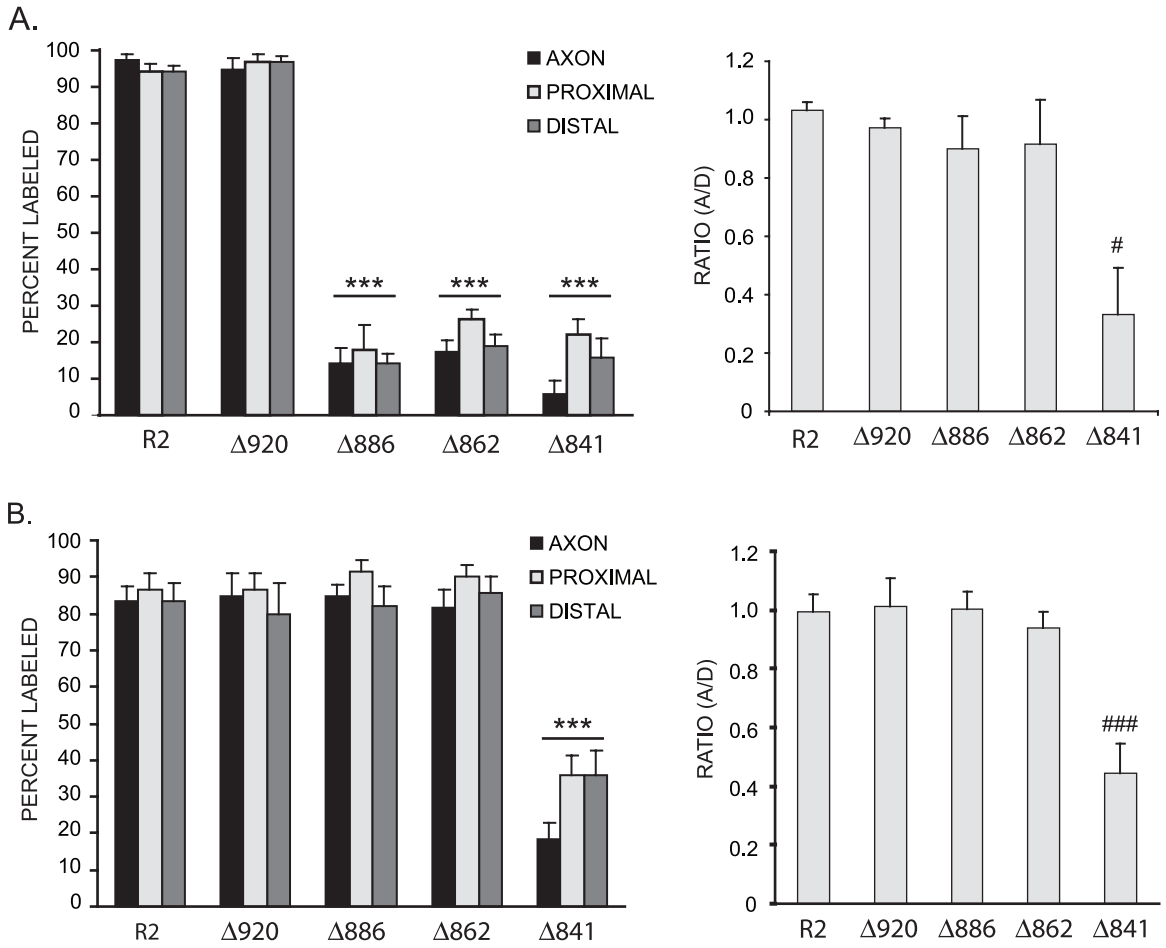

FIG. 5. Quantification of subunit trafficking by image analysis revealed that truncation of the C-terminus of $GABA_{B2}$ impairs trafficking of the subunit to axons and both proximal and distal dendrites. (A) Left: Truncation of $GABA_{B2}$ at residue 886 significantly impaired trafficking of the C-terminally yellow fluorescent protein-tagged constructs. Right: However, only truncation after residue 841 resulted in a significant reduction in axonal vs. dendritic targeting. (B) Left: Impairment of subunit trafficking in *myc*-tagged versions of $GABA_{B2}$ constructs was only observed after truncation at residue 841. Right: Furthermore, the ratio of subunit in the axon to that in the dendrite was significantly lower for the Δ841 construct than for the Δ862 construct. These data suggest that truncation of the C-terminus may impair axonal trafficking to a greater extent than dendritic trafficking. R2, $GABA_{B2}$; proximal, proximal dendrites; distal, distal dendrites; A, axon; D, dendrites. $^{***}P < 0.001$ vs. R2 group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. Δ862 group.

Discussion

Trafficking of G protein-coupled receptors to the plasma membrane is a tightly controlled process. Previous studies had suggested that the C-terminus of $GABA_{B2}$ might modulate $GABA_B$ trafficking (Grunewald *et al.*, 2002; Thuault *et al.*, 2004), but this hypothesis had not been thoroughly examined. In the present study, we investigated whether the $GABA_{B2}$ C-terminus regulates movement of the $GABA_B$ receptor to the cell surface. We found that, in heterologous cells, removal of the region of $GABA_{B2}$ C-terminal to residue 841 impairs surface expression of $GABA_B$. Mature glycosylation is also absent in this $GABA_{B2}$ construct, supporting the hypothesis that its trafficking is disrupted. Furthermore, removal of this region also impairs trafficking of $GABA_B$ into axons and dendrites of transfected hippocampal neurons. Our data indicate that membrane targeting and neuronal transport of $GABA_B$ is regulated by a 20 amino acid region contained in the C-terminus of the $GABA_{B2}$ subunit.

Surface expression of many GPCRs is regulated by protein–protein interactions occurring within the C-termini of the receptors. For example, membrane insertion of the serotonin $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors is regulated by postsynaptic density protein of 95 kDa binding to postsynaptic density protein of 95 kDa/discs large/ZO1PSD95/SAP90 domains in the receptor C-termini (Gavarini *et al.*, 2006). A small number of proteins putatively involved in trafficking have been identified as interactors with $GABA_B$ subunits. The $GABA_{B1}$ subunit interacts with the GPCR interacting scaffolding protein; overexpression of the GPCR interacting scaffolding protein increases surface levels of the $GABA_B$ receptor (Kantamneni *et al.*, 2007). Recent studies suggest that $GABA_{B2}$ interacts with two possible trafficking proteins, Mupp1 (Balasubramanian *et al.*, 2007) and the transcription factor CCAAT/enhancer-binding protein homologous protein (CHOP) (Sauter *et al.*, 2005). Mupp1 was shown to interact with the final four residues in the C-terminus of $GABA_{B2}$; mutation of these residues decreased surface expression of the $GABA_B$ dimer. However, in the present study, we did not observe a reduction in surface levels of $GABA_B$ until all residues after 841 were removed. This suggests that, at least in the present study, loss of Mupp1 binding is unlikely to underly the impairment of $GABA_{B2}$ trafficking reported here. CHOP putatively interacts with $GABA_{B2}$ at a leucine zipper site within its coiled-coil domain. In the present study, this domain remained intact, indicating that interactions with CHOP probably do

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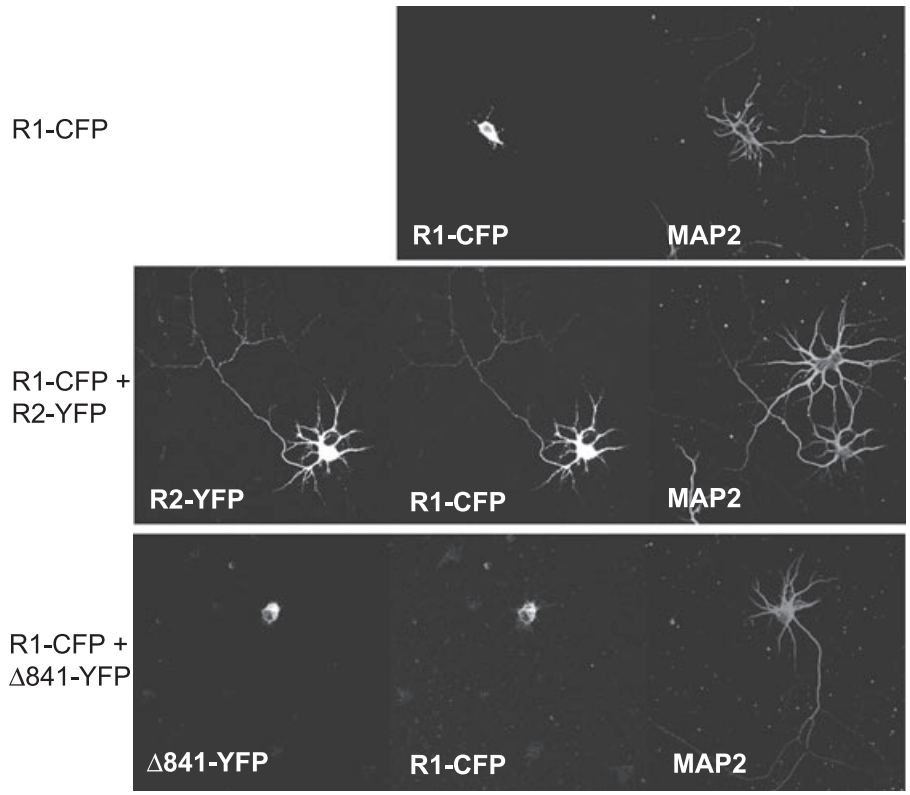

FIG. 6. The $GABA_{B2}$ C-terminus regulates trafficking of the $GABA_B$ receptor. When cultured hippocampal neurons were transfected with $GABA_{B1b}$–cyan fluorescent protein (CFP) only, the subunit remained restricted to the cell body (top row). In neurons co-transfected with $GABA_{B2}$–yellow fluorescent protein (YFP) and $GABA_{B1b}$–CFP, both subunits were present throughout the cell (middle row). Co-expression of $GABA_{B1b}$–CFP and Δ841–YFP resulted in localization of both subunits to the cell body (bottom row). Neurons were visualized with anti-microtubule-associated protein 2 (MAP2) antibody (right column). R1, $GABA_{B1b}$.

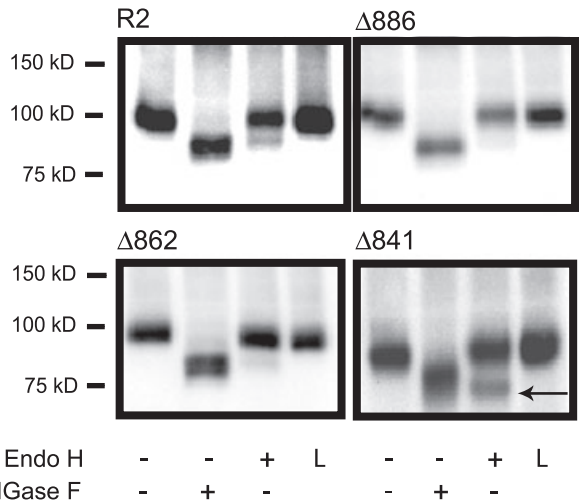

FIG. 7. Impairment of Δ841 surface expression is revealed by absence of mature glycosylation. Human embryonic kidney 293 cells were transiently transfected with either $GABA_{B2}$, Δ886, Δ862 or Δ841, all *myc*-tagged. Cell lysates were digested with either N-glycosidase F (PNGase F) or endoglycosidase H (Endo H), and the proteins were analysed by sodium dodecylsulfate polyacrylamide gel electrophoresis followed by immunoblotting using an anti-*myc* antibody. The results show that $GABA_{B2}$, Δ886 and Δ862 were all endoglycosidase H resistant; however, a proportion of Δ841 was sensitive to digestion by endoglycosidase H, indicated by the arrow. R2, $GABA_{B2}$; L, total cell lysate.

not mediate our observed impairment in $GABA_B$ trafficking. Furthermore, it is unlikely that either Mupp1 or CHOP regulate targeting of $GABA_B$ in neurons, as their interaction sites do not occur between residues 841 and 862 of $GABA_{B2}$. Our data do support the hypothesis that the $GABA_{B2}$ C-terminus is important for receptor trafficking, but indicate that it is highly likely that novel protein–protein interactions occurring within the C-terminus of $GABA_{B2}$ remain to be identified.

Several amino acid motifs have recently been implicated in regulating surface expression of GPCRs and targeting of proteins to axonal and dendritic compartments (Tan *et al.*, 2004). We therefore analysed residues 841–862 of $GABA_{B2}$ for the presence of such motifs, and identified a dileucine pair, composed of isoleucine and leucine, at positions 853/854. Previously, a dileucine motif was shown to be involved in surface trafficking of the G protein-coupled vasopressin V2 receptor (Schulein *et al.*, 1998). In order to determine whether a similar motif might be important for trafficking of $GABA_{B2}$, we mutated the two residues to methionine and valine, respectively. We found no effect of this mutation on surface expression of $GABA_{B2}$ or on targeting of the subunit to the axons and dendrites of neurons, which suggests that this dileucine motif is not involved in regulation of $GABA_{B2}$ trafficking. It is likely, therefore, that the region of $GABA_{B2}$ between residues 841 and 862 contains a trafficking and targeting signal, that these motifs may be novel, and that the proteins interacting with this region remain to be identified.

It has been suggested that the $GABA_B$ subunits may be transported separately into axons and dendrites, where they then dimerize (Vidal *et al.*, 2007). However, our data indicate that trafficking of $GABA_{B1}$ into these compartments requires $GABA_{B2}$. If $GABA_{B1}$ could move into neurites independently of its assembly with $GABA_{B2}$, then it would not have been constrained to the soma when co-expressed with the Δ841 construct. Furthermore, our data suggest that the region of

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$GABA_{B2}$ between residues 841 and 862 might not only be important for trafficking the fully dimerized receptor into neuronal processes, but may also be involved in axonal targeting, as the amount of the Δ841 construct was significantly lower in axons than dendrites. The importance of the C-terminus of $GABA_{B2}$ in axonal and dendritic trafficking was underscored by the behavior of C-terminally YFP-tagged constructs. Addition of YFP significantly impaired trafficking of longer versions of the subunit (i.e. Δ886 and Δ862), which did not display altered behavior when tagged with *myc* only. These data indicate that YFP interferes with protein–protein interactions occurring within the $GABA_{B2}$ C-terminal truncations and thereby affects receptor targeting.

Contradictory observations have been reported with regard to internalization of the $GABA_B$ receptor. Previous studies in cell lines or in neurons found little or no internalization of the receptor, either constitutively or following agonist stimulation (Perroy *et al.*, 2003; Fairfax *et al.*, 2004). In the present study, we found an approximately 40% decrease in cell surface expression of $GABA_{B2}$ 2 h after antibody labeling. However, our results are in agreement with findings from similar studies. In transfected HEK293 cells, Grampp *et al.* (2008) demonstrated that approximately 50% of $GABA_B$ receptors are constitutively internalized in 2 h via a clathrin-dependent mechanism. More recently, in neurons, it was found that within 2 h, 15% of $GABA_B$ receptors are internalized, and this internalization is agonist-independent (Grampp *et al.*, 2008). After internalization, a significant number of $GABA_B$ receptors are then recycled back to the cell surface; this recycling is enhanced in the presence of the $GABA_B$ agonist baclofen (Laffray *et al.*, 2007; Grampp *et al.*, 2008). Clathrin-dependent endocytosis and subsequent recycling of $GABA_B$ was also demonstrated by Vargas *et al.* (2008). Our findings therefore provide additional evidence for constitutive endocytosis of the $GABA_B$ receptor, although we did not investigate recycling of the receptor in the present study.

Regulation of receptor trafficking and targeting is essential for proper delivery of receptors to the cell surface, where they may be targeted to synapses. Previous work has suggested that the C-terminus of $GABA_{B2}$ might be involved in trafficking of $GABA_B$. Here, we have demonstrated for the first time that a short region of $GABA_{R2}$ between residues 841 and 862 is important for regulating movement of the $GABA_B$ receptor, not only to the cell surface but also into axons and dendrites. Together with our previous report on a region of the $GABA_{B2}$ C-terminus that regulates diffusion of $GABA_B$ at the cell surface, these data suggest that the C-terminal region of this subunit contains multiple domains that influence not only intracellular trafficking of the receptor but also its movement at the cell surface. These findings provide further insight into trafficking of the $GABA_B$ receptor and into general mechanisms underlying the movement of GPCRs.

Acknowledgements

We thank Dr Alan Wise and Dr Julia White of GlaxoSmithKline for the gift of the $GABA_{B2}$ YFP, CFP and HA-tagged constructs. This work was supported by the Medical Research Council and the Blaschko European Visiting Fellowship.

Abbreviations

CFP, cyan fluorescent protein; CHOP, CCAAT/enhancer-binding protein homologous protein; ELISA, enzyme-linked immunosorbent assay; endo H, endoglycosidase H; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; HA, hemagglutin; HEK, human embryonic kidney; MAP2, microtubule-associated protein 2; PBS, phosphate-buffered saline; PNGase F, N-glycosidase F; R2, $GABA_{B2}$; YFP, yellow fluorescent protein.

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