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The $GABA_{B2}$ subunit is critical for the trafficking and function of native $GABA_B$ receptors

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Abstract

Studies in heterologous systems have demonstrated that heterodimerisation of the two $GABA_B$ receptor subunits appears to be crucial for the trafficking and signalling of the receptor. Gene targeting of the $GABA_{B1}$ gene has demonstrated that the expression of $GABA_{B1}$ is essential for $GABA_B$ receptor function in the central nervous system (CNS). However, the contribution of the $GABA_{B2}$ subunit in the formation of native $GABA_B$ receptors is still unclear, in particular whether other proteins can substitute for this subunit. We have created a transgenic mouse in which the endogenous $GABA_{B2}$ gene has been mutated in order to express a C-terminally truncated version of the protein. As a result, the $GABA_{B1}$ subunit does not reach the cell surface and concomitantly both pre- and post-synaptic $GABA_B$ receptor functions are abolished. Taken together with previous gene deletion studies for the $GABA_{B1}$ subunit, this suggests that classical $GABA_B$ function in the brain is exclusively mediated by $GABA_{B1/2}$ heteromers.

Keywords: $GABA_B$ receptors; GPCR; Transgenic; Trafficking; Synaptic physiology; Epilepsy

1. Introduction

GABA receptors are the major inhibitory receptors in the central nervous system (CNS). Whilst $GABA_A$ and $GABA_C$ receptors are ionotropic and mediate fast synaptic inhibition, $GABA_B$ receptors are metabotropic G protein coupled receptors (GPCR) that induce slower and more prolonged effects via the activation of an array of downstream effector systems, such as inhibition of adenylyl-cyclase, inhibition of presynaptic calcium channels and activation of postsynaptic potassium channels. Their far-reaching effects together with their widespread distribution have raised expectations that this receptor class will be of clinical importance for pharmaceutical intervention in a number of disease areas, including asthma, epilepsy, depression, anxiety, drug addiction, anti-nociception, anti-spasticity and cognitive enhancement for patients affected with neurodegenerative conditions such as Alzheimer's disease (for review see [1]).

Molecular biological and biochemical approaches have demonstrated that, in contrast to the established dogma for the G protein coupled receptor superfamily, the $GABA_B$ receptor exists as a heterodimer rather than as a single subunit [1–6]. Whilst its homology to metabotropic glu-

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tamate receptors suggests that multiple $GABA_B$ receptor subunits may be encoded within the mammalian genome, to date only two distinct genes have been cloned [3,4,7]. Extensive investigation of the functionality of these subunits has led to the concept that the $GABA_B$ receptor consists of a $GABA_{B1}$ subunit that binds the endogenous ligand within its extracellular N-terminus, and a $GABA_{B2}$ subunit that fulfils at least two roles: (1) it is essential for the correct trafficking of the $GABA_{B1}$ receptor subunit to the cell surface to form a functional dimeric receptor and (2) it is responsible for the interaction of the receptor with its cognate G protein. In this respect, we and others have identified key structural determinants within both $GABA_{B1}$ and $GABA_{B2}$ that are important in carrying out these functions in vitro [8–11]. In particular, the coupling of the receptor to its G protein is mediated not by the intracellular C-terminus of either subunit, but instead by specific positively-charged residues located within the second intracellular domain of the $GABA_{B2}$ receptor subunit [12–15]. Indeed, deletion of the C-termini of both $GABA_{B1}$ and $GABA_{B2}$ result in a functional $GABA_B$ receptor in transfected cells. However, transfection of a C-terminally deleted form of $GABA_{B2}$ in conjunction with a full-length $GABA_{B1}$ subunit does not result in a functional $GABA_B$ receptor [9]. This is because, at least in transfected cells, the C-terminal of $GABA_{B2}$ is necessary to mask the ER-retention signal present in the C-terminal of $GABA_{B1}$ and result in the correct trafficking of $GABA_{B1}$ to the cell surface, a prerequisite for the assembly of a functional $GABA_B$ receptor.

In the present study, we have generated a mouse in which the intracellular C-terminus of $GABA_{B2}$ has been deleted (termed ΔGB2-Ct), and tested the influence of this truncated version of the protein on the function and trafficking of native $GABA_B$ receptors. Although it is not possible to directly demonstrate the ΔGB2-Ct protein in these mice, we provide overwhelming indirect evidence for its stable expression, and thus demonstrate that classical $GABA_B$ responses in the CNS are dependent on the correct trafficking of the $GABA_{B1}$ subunit to the plasma membrane by full-length $GABA_{B2}$, and that no other protein can perform this function in the absence of a functional $GABA_{B2}$ subunit.

2. Materials and methods

2.1. Targeting of $GABA_{B2}$ gene and generation of mutant mice

Gene targeting was performed in E14.1 embryonic stem (ES) cells, replacing exons 14 and 15 of the mouse $GABA_{B2}$ locus with a positive selection cassette containing the pgk promoter/neomycin phophotransferase gene. Homology arms were generated by PCR using primers BR2.10F–BR2.9R and BR2.15F–BR2.14R (see Table 1) spanning coding exons 13–14 and 15–16, respectively. The entire 3.6 kb BR2.10F–9R product was used as 5′ homology arm, the 3′ homology arm was subcloned from the 7 kb BR2.15F–14R PCR product by Xba1 digest which removed 3 kb from the 3′ end of the product. The arms were cloned either side of a PGK-neo cassette positioned to replace exons 14 and 15, causing termination of translation at the exon 14/PGK-neo interface. The 3′ homology arm was flanked by the Diptheria-toxin A gene as a negative selection marker (DTA). In order to screen for ES cells carrying the mutation, an 800 bp external probe was excised from the BR2.15F–14R PCR product by digestion with BstX1 and Xba1, and this was used to probe BstX1-digested genomic DNA. The wild type allele generates a 5.2 kb band while the KO allele gives rise to a band of 5.5 kb (Fig. 1A). One thousand and two hundred G418 resistant clones were screened by Southern blot and five targeted clones were identified. The integrity of the 5′ end in targeted clones was confirmed by PCR amplification from external primers (BR2.8F and BR2.9F, Table 1) to a neo-specific primer (NeoR3.1, Table 1); the product of a targeted allele is ∼4.5 kb while no product is expected from the wild type allele (Fig. 1B).

Table 1
Primers for generating homology arms by PCR

BR2.10F	CAGCCCAACGTGCAGTTCTGCATCGTGGCC
BR2.9R	TGGTGACTGAGGTGGAGGTCTTCGAATCTT
BR2.15F	TCAACGACATCCTCAGCTTGGGCAAC
BR2.14R	GGAGCTGAGGGTTTTGATCGAGGTGA
BR2.9F	GTGTACAATGTGGGGATCATGTGCATCATC
BR2.8F	TGGGAAACCCGCAACGTGAGCATCCCT
NeoR3.1	TGCCCAGTCATAGCCGAATAGCC

2.2. Cloning and TaqMan RT-PCR analysis of the ΔGB2-Ct gene product

RNA was prepared from whole brain of ΔGB2-Ct mutant mice using the RNeasy Protect Mini Kit (Qiagen Ltd, Crawley, UK) and cDNA was produced using Superscript II reverse transcriptase (Invitrogen Ltd, Paisley, UK), both according to manufacturer's instructions.

The mutated ΔGB2-Ct mRNA was cloned from this cDNA by PCR using gene specific primers and Platinum Taq DNA polymerase (Invitrogen Ltd). PCR was carried out according to manufacturer's instructions (95 °C for 2 min then 30 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 150 s, then a final extension at 72 °C for 7 min). TaqMan quantitative RT-PCR was carried out as described previously [16]. RNA was prepared from single wild-type, heterozygous and homozygous mouse brains as described above. cDNA synthesis was performed in triplicate on each RNA preparation, and parallel reactions for each mRNA sample were performed in the absence of RT to determine the level of contaminating genomic DNA. TaqMan PCR was performed on the triplicate cDNA samples and the 'no RT' control from each tissue/region studied, using tran-

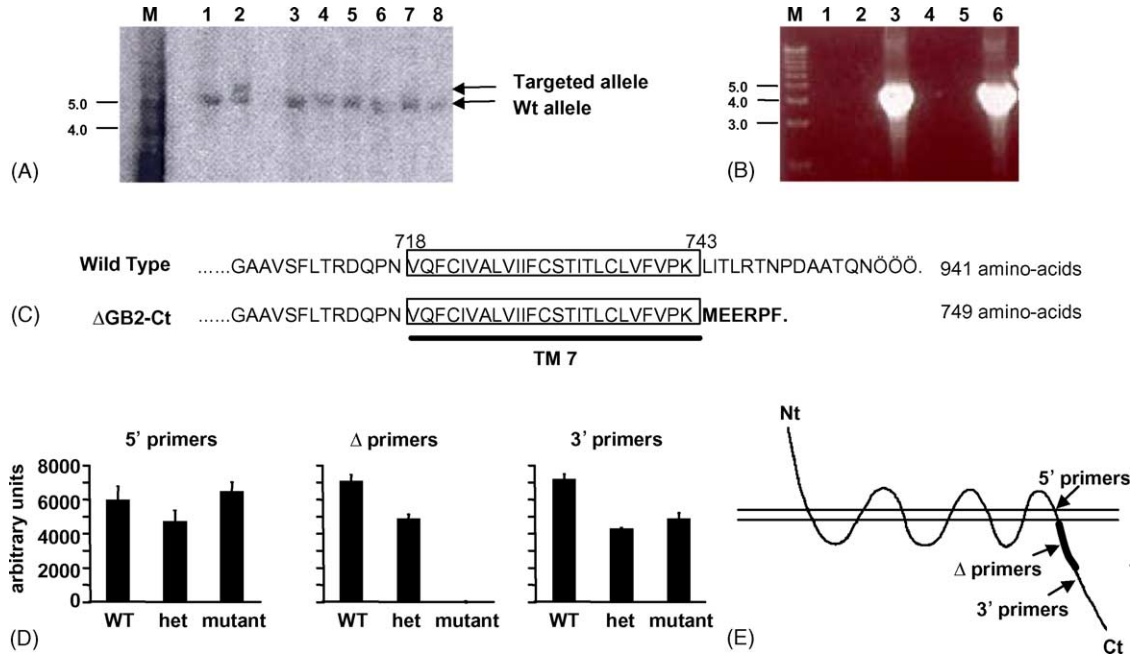

Fig. 1. C-terminal deletion of the $GABA_{B2}$ receptor subunit. (A) Identification of targeting at the 3′ end of $GABA_{B2}$ by Southern blot: genomic DNA from several embryonic stem cell clones was digested with BstX1 and analysed by Southern blot. The probe was derived from the 3′ homology arm of the targeting construct. The wild type allele (lanes 1 and 3–8) generates a 5.2 kb band, while the targeted allele (lane 2) gives rise to an additional 5.5 kb band. M: molecular weight marker. Confirmation of the Neo cassette insertion by PCR: PCR amplification of the overlapping region between the 5′ homology arm and the Neo cassette was performed using two sets of primer (lanes 1–3 amplified by BR2.8F–NeoR3.1, lanes 4–6 amplified by BR2.9F–NeoR3.1). A 4.5 kb band is obtained from successfully targeted cells (lanes 3 and 6) while no band is expected from wild type samples (lanes 1, 2, 4 and 5). M: molecular weight marker. (C) Amino-acid sequence of the junction between the seventh transmembrane domain (TM7) and the C-terminus of wild-type (WT) and ΔGB2-Ct as deduced from nucleotide sequence analysis of the cloned cDNA. TM7 is boxed and underlined, bordering residue numbers are indicated as well as total number of amino-acids expected in each of the proteins (right-hand side). (D) Real time quantitative RT-PCR analysis of representative wild-type (WT), ΔGB2-Ct homozygous (ΔGB2-Ct) and heterozygous (het) whole-brain total RNA samples using primers and probes specific of the 5′ homology arm (5′primers), the targeted exons 14/15 (Δ primers) or the 3′ homology arm (3′ primers) of $GABA_{B2}$ as indicated on the right-hand side pictogram (E).

script-specific primers and probes. After subtraction of the 'no RT' control, relative levels of the amplified mRNA of interest were normalized to mRNA expression of the 'house-keeping' gene cyclophilin, TaqMan quantitation of which was performed in parallel, to take account of mRNA quality and quantity. The sequences of the primers and probes used were as follows:

- 'Upstream' 5′ primer 5′-GCGAAAACACCCACATGA-CC-3′;
- 'Upstream' probe 5′-TCTGGCTCGGCATTGTCTAC-GC-3′;
- 'Upstream' 3′ primer 5′-GAACAACATGAGGAGCC-CCTT-3′;
- 'Inside' 5′ primer 5′-CCTGACGCAGCCACTCAGA-3′;
- 'Inside' probe 5′-CAGGCGATTCCAGTTCACACA-GAACCA-3′;
- 'Inside' 3′ primer 5′-GTGACTGAGGTGGAGGTCTT-CG-3′;
- 'Downstream' 5′ primer 5′-AAAAATCACCTCGATC-AAAACCC-3′;
- 'Downstream' probe 5′-AGCTCCAGTGGAACACGA-CCGAGC-3′;
- 'Downstream' 3′ primer 5′-CGCTGGATGTGCTCTG GG-3′.

2.3. Preparation, solubilisation and immunoblotting of rat brain membranes

Brain tissue from wild-type and ΔGB2-Ct mice was homogenized using a glass–teflon homogenizer in 10 volumes (∼2 ml) of sucrose buffer (5 mM Tris–HCl, pH 7.4, 0.32 M sucrose, 0.1% phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml Antipain). The homogenate was centrifuged for 15 min at 1000 × *g* and the supernatant saved. The pellet was homogenized again in the same buffer and centrifuged for 15 min at 1000 × *g*. The crude membrane fraction was obtained by centrifugation of the pooled resulting supernatants for 30 min at 17 000 × *g*. The membranes were washed twice with 50 mM Tris–HCl, pH 7.4 and resuspended in the same buffer to give a protein concentration of ∼4 mg/ml. The membranes were then pelleted again, resuspended in 2 ml of solubilisation buffer (10 mM Tris–HCl, pH 7.4, 2% CHAPS, 150 mM NaCl, 1 mM EDTA, 0.1% Phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml Antipain) and incubated on a rotating wheel for 3 h at 4 °C. Fifty-microliters of the resulting membranes were loaded on a 8% polyacrylamide gel and analysed by SDS-PAGE and immunoblotting.

The antibodies used for immunoblotting were as follows: Anti-$GABA_{B1a}$ antibody has been described previously [17], mouse monoclonal anti-$GABA_{B2(Ct)}$ was purchased from BD Biosciences (cat. # 611744) and rabbit polyclonal anti-GAD67 was purchased from Chemicon (cat. # AB5992). Goat anti-rabbit IgG-HRP (cat. # sc-2301) and goat anti-mouse IgG-HRP (cat. # sc-2302) secondary antibodies were purchased from Santa Cruz Biotechnology.

2.4. Biotinylation assay

2.4.1. Antibodies used

An antibody to $GABA_{B1}$ was raised to the N-terminus of $GABA_{B1a}$ and used in immunoblots at a concentration of 2.5 μg/ml. R2 was detected using an antibody to the carboxy-terminus of $GABA_{B2}$ used at 1/1000 (BD-biosciences). Actin was detected using a rabbit anti-actin antibody at 1/1000 (Sigma).

Cortical neurons were prepared from E15 pups using a method described previously [18]. Cortices were individually triturated and plated on 6 cm plates, with no mixing of neurons from different pups. Tails from pups were removed for genotyping. At 7DIV cells were rinsed twice with ice-cold PBS containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$. They were then biotinylated for 20 min with 1 mg/ml EZ-Link Sulfo-NHS-SS-biotin (Pierce). Cells were then rinsed prior to blocking of remaining biotin in 0.1% BSA/PBS 2 × 10 min. Cells were rinsed again prior to lysis in 0.5 ml RIPA buffer (0.1% SDS, 1% NP-40, 0.5% DOC, 150 mM NaCl, 50 mM Tris–Cl pH 7.4, and complete protease inhibitor cocktail (Bohringer). Cells were solubilised for 1 h with rotation at 4 °C prior to centrifugation (13 000 rpm, 10 min). Supernatants were incubated with a 100 μl of a 1:1 slurry of Neutravidin beads (Pierce) for 2 h with rotation at 4 °C. Beads were then washed in lysis buffer followed by 2 × 15 min washes in high salt (500 mM) lysis buffer and a further wash in low salt lysis buffer. Samples were boiled for 5 min in gel loading buffer. Proteins were separated using 8% SDS-PAGE.

2.4.2. Western blotting

Gels were electrotransferred followed onto nitrocellulose membranes (Hybond-C). Membranes were blocked in 4% milk for 1 h and then probed overnight with primary antibody, followed by detection with hrp-conjugated secondary and chemoluminescence. For detection of anti-$GABA_B$ R1a antibody a 125I conjugated anti-rabbit secondary was used (Amersham). Membranes were then exposed to phosphorimager (Biorad).

2.5. Mouse brain slice preparation

Mice were cervically dislocated 46 weeks after birth and subsequently decapitated in accordance with UK Home Office guidelines. The brain was removed rapidly and hippocampal slices prepared by cutting 400 μm thick horizontal sections through the whole brain minus the cerebellum using a vibroslicer (Campden Instruments, Loughborough, UK). The hippocampal or cortico-striatal regions from these sections were dissected free from the surrounding brain regions. For sharp and extracellular recordings, slices were placed on a nylon mesh at the interface of warm artificial cerebrospinal fluid (aCSF) and an oxygen-enriched (95% O_2, 5% CO_2), humidified atmosphere as described previously [19,20]. For patch clamp recordings, a submerged chamber perfused with warm aCSF (30 ± 1 °C, 2 ml min^{-1}) was utilised. The standard aCSF perfusion medium for both recording configurations comprised (mM): NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgSO_4$, 1; D-glucose, 10; and was bubbled with 95% O_2, 5% CO_2 to maintain a pH of 7.3–7.4.

2.6. Intracellular sharp electrode recordings

Intracellular recordings were obtained from the CA1 pyramidal cell body region of the hippocampus or the dorsal striatum (approximately 1–2 mm from the white matter). Following a post dissection 1 h equilibration period neurons were impaled using 2 M (for striatal medium spiny neurons) or 3 M (for hippocampal CA1 pyramidal neurons) potassium acetate-filled microelectrodes (60–100 MΩ). Cells were identified by their characteristic membrane properties [19,21]. An Axoclamp-2B amplifier (Axon Instruments, Foster City, CA, USA) was used in bridge mode or discontinuous current clamp mode. All impalements were made in control aCSF and once stable recordings had been achieved for at least 10 min, experiments were initiated. During the period of the intracellular impalement, the input resistance and extent of spike frequency adaptation of each neuron were measured routinely using 300–600 ms long negative and positive current steps (±0.1–0.4 nA), respectively.

2.7. Extracellular recordings

Extracellular field potential recordings were made from area CA1 of the hippocampal slices using glass microelectrodes (2–4 MΩ) filled with aCSF.

2.8. Whole-cell patch clamp recordings

To study presynaptic hippocampal $GABA_B$ receptor function, whole-cell patch clamp recordings were performed blind targeting the patch pipette at the CA1 *stratum pyramidale* [22]. Whole-cell currents were recorded using an Axopatch 200B (Axon Instruments). Data were acquired with a total gain of 10 at a sampling frequency of 10 kHz and low-pass filtered at 5 kHz. Patch pipettes (2–4 MΩ) were filled with a solution containing (in mM): $CsMeSO_4$ 130, HEPES 10, EGTA 0.5, NaCl 8, Mg-ATP 4,

Na-GTP 0.3, QX314-Cl 5 (~280 mOsm, pH adjusted to pH 7.2 with CsOH) or K^+-$MeSO_4$ 140, HEPES 5, EGTA 1.1, $MgCl_2$ 2, Mg-ATP 2, Na-GTP 0.3 (~280 mOsm, adjusted to pH 7.2 with K-OH) for some of the paired-pulse depression (PPD) recordings. After reaching whole-cell configuration, the series resistance (R_s) was measured and judged acceptable if below 20 MΩ. R_s was not compensated for. Experiments were discarded if the value of R_s changed by more than 20% or if the holding current spontaneously changed by >200 pA.

2.9. Electrical stimulation

Electrical stimulation was provided by Pt/Ir concentric bipolar electrodes (Frederick Haer & Co, ME, USA). These were positioned in stratum radiatum to stimulate the synaptic inputs to CA1 pyramidal cells or on the white matter–striatal border to stimulate cortical projections to the striatum. In every series of experiments stimuli comprised square-wave pulses (20–200 μs; 5–30 V) delivered at a fixed intensity every 10–20 s and baseline recordings were made for a minimum of 20 min to ensure stationarity of responses before experiments were initiated.

2.10. Data storage and analysis

Data were captured using pClamp9 software (Axon Instruments Ltd.) or the LTP program shareware [23] (http://www.ltp-program.com/). Digitised records were stored on the hard disk of a PC for off-line analysis using Clampfit or LTP software. Data are presented as mean ± standard error of the mean and, unless otherwise stated, statistical significance was assessed using paired or unpaired Student's *t*-tests performed on raw data with $P < 0.05$ being taken as indicating statistical significance and shown by an asterisk in the Figures. *n* values refer to the number of times a particular experiment was performed, each in a different slice prepared from a different mouse except for the measurement of paired-pulse depression. PPD was calculated using the following equation: PPD = ((Average IPSC1 − Average IPSC2)/Average IPSC1) × 100) so as to avoid spurious PP facilitation [24]. Artefact blanking (1 ms) was performed using the LTP program.

2.11. Drugs

Drugs were applied to slices by addition to the perfusion medium. Bicuculline methiodide and 2-chloroadenosine (CADO) were purchased from Sigma (St. Louis, MO). Picrotoxin, [1-(*S*)-3,4-dichlorophenylethyl] amino-2-(*S*)-hydroxypropyl-*p*-benzyl-phosphonic acid (CGP55845A), 2,2-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulphonamide (NBQX), D-2-amino-5-phosphonopentanoate (D-AP5), baclofen, and [D-Ala^2,NMe-Phe^4,Gly-ol^5]-enkephalin (DAMGO) were obtained from Tocris-Cookson Ltd. (Bristol, UK). QX314 was purchased from Alomone Labs Ltd. (Jerusalem, Israel).

3. Results

3.1. Generation of mice expressing a C-terminally truncated form of $GABA_{B2}$ (ΔGB2-Ct)

Homologous recombination in embryonic stem cells was used to generate a mouse with a C-terminal deletion in the $GABA_{B2}$ subunit (see Section 2 and Fig. 1). In these successfully targeted clones, exons 14 and 15 of $GABA_{B2}$ were replaced with a PGK-neo cassette, which we predicted would result in termination of translation of $GABA_{B2}$ at the end of the seventh transmembrane domain (TM7). Surprisingly, we discovered that the PGK-neo cassette was in fact spliced out in the ΔGB2-Ct mRNA, resulting in a fusion of exons 13 and 16. This caused the addition of six amino acids downstream of TM7 and then a stop codon, as confirmed by cloning and sequencing the cDNA for ΔGB2-Ct from the mutant mice (Fig. 1C). The expression of the mutant mRNA was further confirmed by TaqMan real-time quantitative RT-PCR. This demonstrated, using primer/probe sets both upstream and downstream of the targeted deletion, that the mutant mRNA was both expressed and stable, and using a primer/probe set within the deletion, that no endogenous message for $GABA_{B2}$ was expressed in the ΔGB2-Ct mutant mice (Fig. 1D). In addition, no signal could be detected by immunoblot analysis of ΔGB2-Ct brain tissue using a monoclonal antibody directed against the C-terminus of the $GABA_{B2}$ protein (Fig. 2A). Unfortunately, all commercially available antibodies directed against the N-terminal of $GABA_{B2}$, in addition to those made in ours and other laboratories, do not recognise the native $GABA_{B2}$ protein by immunohistochemistry or immunoblotting, making direct detection of the truncated protein impossible. However, we were readily able to detect an epitope-tagged version of the truncated $GABA_{B2}$ at the cell surface in transient co-transfection experiments (data not shown). Interestingly, truncation of the $GABA_{B2}$ protein did not influence the level of expression of $GABA_{B1}$ in ΔGB2-Ct mice, as immunoblotting with an anti-$GABA_{B1}$a antibody (Fig. 2A) did not reveal any overt changes in the expression of $GABA_{B1}$ between wild-type and ΔGB2-Ct brain samples.

3.2. Effect of $GABA_{B2(Ct)}$ deletion on $GABA_{B1}$ subunit trafficking

It has been shown previously that in the absence of $GABA_{B2}$, the $GABA_{B1}$ receptor subunit is not transported to the cell surface and is retained within intracellular compartments [9,25]. We, therefore, used a cell surface protein biotinylation technique [26] to assess the localisation of the $GABA_{B1}$ protein in ΔGB2-Ct cells, using

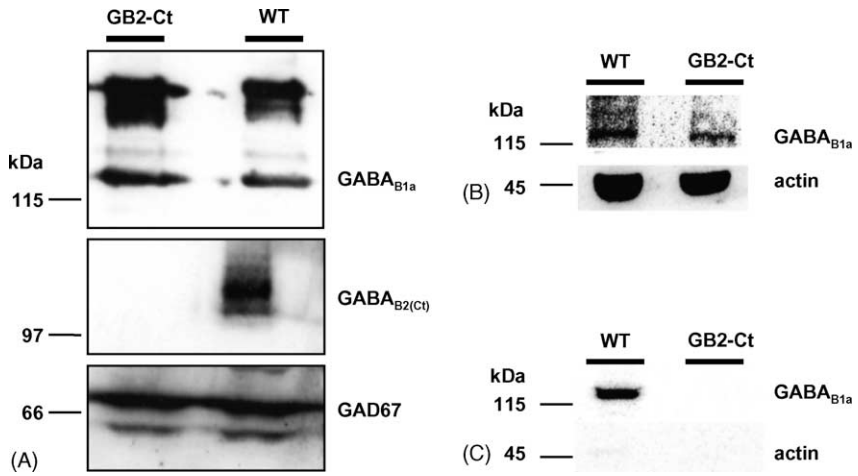

Fig. 2. GABA$_{B1}$ and GABA$_{B2}$ protein expression in ΔGB2-Ct mice. (A) Western blots analysis of 50 μg of brain membranes prepared from WT and ΔGB2-Ct mice using antibodies specific to either GABA$_{B1a}$ (top) or GABA$_{B2(Ct)}$ (middle). Anti-GAD67 (bottom) was used to ensure that equal quantities of proteins were loaded in the two lanes of the GABA$_{B1}$ blot. A similar result with GAD67 was observed for the GABA$_{B2}$ blot. (B) Western blot from lysates of cortical neurons cultured from WT and mutant mice at 7DIV for GABA$_{B1a}$ (top) and actin (bottom). (C) Immunoblot of streptavidin-purified biotinylated surface proteins with an antibody directed against GABA$_{B1a}$. An antibody to actin was used as a negative control (bottom).

cultured neurons from the cortices of wild-type or mutant mice. In agreement with our data for brain tissue (Fig. 2A), similar levels of GABA$_{B1}$ were present in both wild-type and mutant neurons (Fig. 2B). However, after biotinylation of neuronal cell surface proteins and purification on immobilised streptavidin, we were unable to detect the presence of any GABA$_{B1}$ at the plasma membrane of ΔGB2-Ct neurons (Fig. 2C). Cell surface specificity of the assay was confirmed by the fact that the intracellular protein actin was undetectable.

3.3. Phenotype of the ΔGB2-Ct mutant

The mutation of GABA$_{B2}$ had no effect on the embryonic and early postnatal viability of the ΔGB2-Ct animals, as demonstrated by a Mendelian ratio of wild-type:heterozygous:homozygous genotypes within litters of 10-day-old animals (21:36:25 among a cohort of 88 animals). In addition, no behavioural effects were observed in heterozygous ΔGB2-Ct mutant mice of any age. However, ΔGB2-Ct homozygous animals exhibited a phenotype that included a lower body weight and reduced life span compared to wild-type littermates, with death occurring in homozygous mutant animals from around 2 weeks of age. Moreover, ΔGB2-Ct mice displayed a conspicuous epileptic phenotype with partial followed by full tonic–clonic signs suggesting secondary generalised seizures. Ictal episodes sometimes provoked sudden death, which explains the postnatal lethality of the phenotype. Accordingly, we found that the pattern of bicuculline-induced epileptiform activity in the CA3 region of hippocampal slices from ΔGB2-Ct mice showed striking differences when compared to the same activity in age-matched wild-type littermates (Fig. 3). The duration (d_{WT} = 620 ±

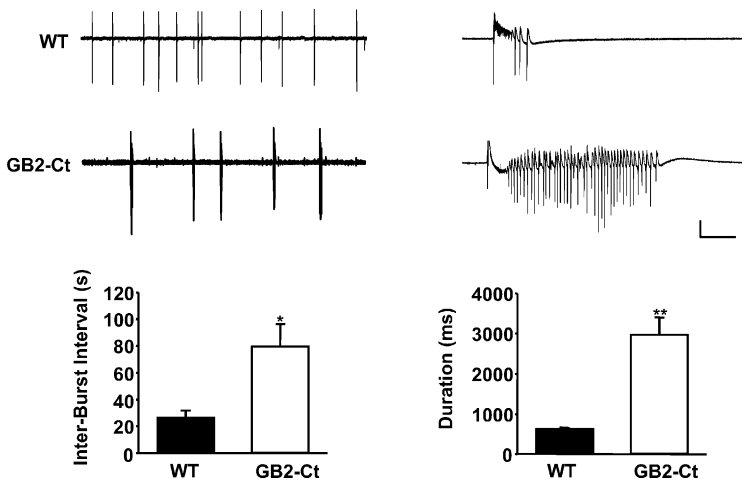

Fig. 3. Ictal activity in ΔGB2-Ct hippocampal slices Extracellular recordings in CA3 stratum pyramidale show that exposure to bicuculline 10 μM causes the appearance of spontaneous epileptiform discharges. As exemplified in the upper panel, bursts were longer and less frequent in the ΔGB2-Ct slices than in the WT (n = 4 for both groups, $P < 0.05$ and 0.01 for interval and duration, respectively). Scale bar: 2 mV/50 s or 500 ms.

47 ms; $d_{\Delta GB2\text{-}Ct}$ = 2972 ± 430 ms) as well as the interval between paroxysmal bursts (i_{WT} 26.3 ± 5.4 s; $i_{\Delta GB2\text{-}Ct}$ 79.4 ± 17.0 s) were significantly longer in the mutants, reflecting the enhanced hyperexcitability of the neuronal network. Cognitive, sensory or motor behavioural analysis was not carried out as the impairment of normal behaviour would have significantly interfered with our interpretation of the results. Nevertheless, the epileptic phenotype is reminiscent of that seen in $GABA_{B1}$ KO mice [27], thus suggesting a similar deficit in $GABA_B$ receptor function in the ΔGB2-Ct mutant mice.

3.4. Assessment of postsynaptic receptors function

In order to assess $GABA_B$ receptor function we performed electrophysiological recordings in hippocampal slices, as the physiological role of the different functional populations of this receptor has been widely characterised in this region of the brain. Single shock stimulation in stratum radiatum of area CA1 in the presence of glutamate receptor antagonists (10 μM NBQX and 50 μM D-AP5) produced a biphasic inhibitory postsynaptic potential (IPSP) in wild-type CA1 pyramidal neurons (Fig. 4A, n = 7). This IPSP was composed of a fast ($IPSP_A$) and a slow component ($IPSP_B$) that could be blocked by the selective $GABA_A$ receptor and $GABA_B$ receptor antagonists bicuculline (10 μM) and CGP55845 (1 μM), respectively (data not shown). However, in ΔGB2-Ct neurons, whilst the $IPSP_A$ remained intact, the $IPSP_B$ was never observed (Fig. 3A, n = 15). Furthermore, in ΔGB2-Ct neurons, application of the $GABA_B$ receptor agonist baclofen (50 μM) produced no significant change in resting membrane potential whereas in wild-type neurons it caused a robust hyperpolarisation (10–15 mV) that was abolished by 1 μM CGP55845 (Fig. 4B, n = 3 for wild-type and mutants). Similarly in whole-cell voltage clamp experiments there was no detectable changes in the holding current of ΔGB2-Ct pyramidal cells during application of 50 μM baclofen (data not shown).

3.5. Assessment of presynaptic autoreceptors function

In the hippocampus, as well as in other brain structures, GABA exerts a negative feedback on its own release. A simple method for observing this phenomenon is to record $GABA_A$-mediated inhibitory postsynaptic events in response to two stimuli delivered in quick succession (e.g. 200 ms). This paradigm induces a depression of the second $IPSC_A$ that is mediated, at least in part, by activation of $GABA_B$ autoreceptors on inhibitory terminals [19,28,29]. Whereas in wild-type slices this stimulation protocol produced a robust and consistent paired-pulse depression of $IPSC_A$ (36.4 ± 3.1%, n = 10) the average PPD in ΔGB2-Ct slices was significantly smaller (20.0 ± 0.2%, n = 18; Fig. 5A). Furthermore, whereas in wild-type slices baclofen (10 μM) caused an average 64.6 ± 18 4.7%

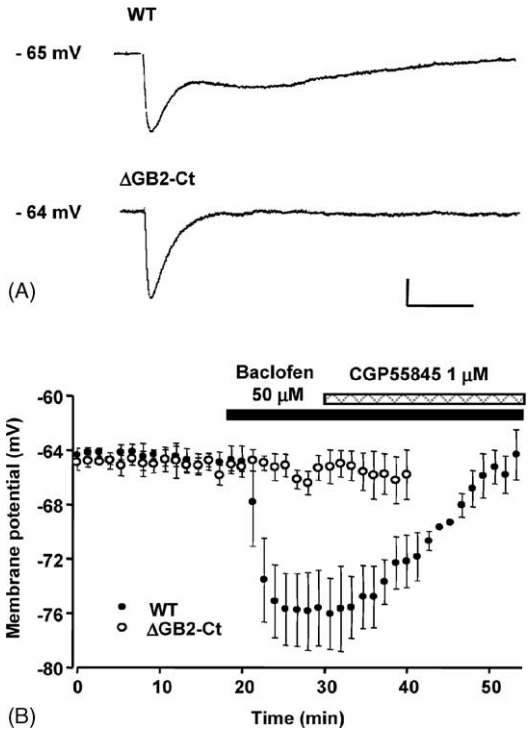

Fig. 4. Loss of postsynaptic $GABA_B$ receptor function in ΔGB2-Ct neurons. (A) These traces are sharp electrode intracellular current clamp recordings from putative CA1 pyramidal neurons from a WT (top, n = 6) and ΔGB2-Ct (bottom, n = 14) slice, showing monosynaptic IPSPs in response to a single shock delivered in stratum radiatum, in the presence of 10 μM NBQX and 50 μM D-AP5. These traces are the average of four to eight consecutive traces. Scale bar: 2 mV, 100 ms. (B) This graph shows the effect of bath application of 50 μM baclofen on the resting membrane potential of WT and ΔGB2-Ct CA1 pyramidal neurons. In the presence of baclofen, WT neurons show a robust hyperpolarisation, which is reversed by the addition of 1 μM CGP55845 to the perfusing medium (n = 3). This hyperpolarisation was never observed in the ΔGB2-Ct neurons (n = 3). Each point represents mean ± S.E.M. of the average of four consecutive measurements made every 20 s.

depression of $IPSC_A$ (n = 3) it failed to depress the fast inhibitory currents in ΔGB2-Ct slices (−0.3 ± 2.4%; n = 6, Fig. 5B). As a control experiment we found that the μ opioid receptor agonist DAMGO depressed $IPSC_{AS}$ in ΔGB2-Ct slices (data not shown) [29].

3.6. Assessment of presynaptic heteroreceptors function

Activation of presynaptic $GABA_B$ heteroreceptors causes a reduction in the amount of glutamate released from presynaptic terminals and consequently a reduction in EPSC amplitude. In order to examine the presence of these receptors, the magnitude of heterosynaptic depression of EPSCs was examined in wild-type and ΔGB2-Ct slices. Specifically, a train of stimuli (five shocks at 50 Hz) was delivered to one set of afferents innervating CA1 pyramidal neurons in wild-type slices which was sufficient to cause spillover of GABA from inhibitory interneurons to activate $GABA_B$ heteroreceptors on a separate population of afferents (see illustration in top of Fig. 6A). As previously

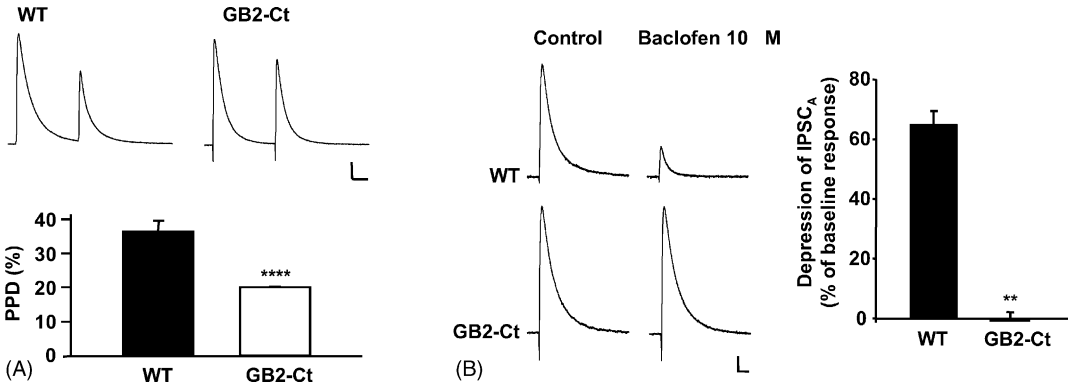

Fig. 5. Loss of presynaptic autoreceptor function in ΔGB2-Ct neurons. (A) The traces are monosynaptic IPSCs recorded from WT and ΔGB2-Ct CA1 pyramidal neurons held at −40 mV. These IPSCs are evoked by stimulation in stratum radiatum in the presence of 5 μM NBQX and 50 μM D-AP5. Two shocks delivered at an interval of 200 ms caused a robust depression of the second IPSC in WT neurons ($n = 10$ from 5 animals). The amount of paired pulse depression was significantly smaller in the mutant slices ($P < 0.00001$, $n = 18$ from 13 animals). Scale bar: 50 pA, 50 ms. (B) Bath-applied baclofen (10 μM) induced a depression of the monosynaptic IPSC$_A$ in CA1 pyramidal cells in the WT slice ($64.6 \pm 4.7\%$, $n = 3$). The effect of baclofen is totally absent in ΔGB2-Ct neurons (-0.3 ± 2.4, $n = 5$). The difference between the effects of baclofen in WT and mutant slices was significant ($P < 0.001$). Baclofen was applied for at least 10 min before measuring the amplitude of evoked IPSCs (peak effect). The traces are the average of 10–20 consecutive traces. Scale bar: 25 pA, 50 ms.

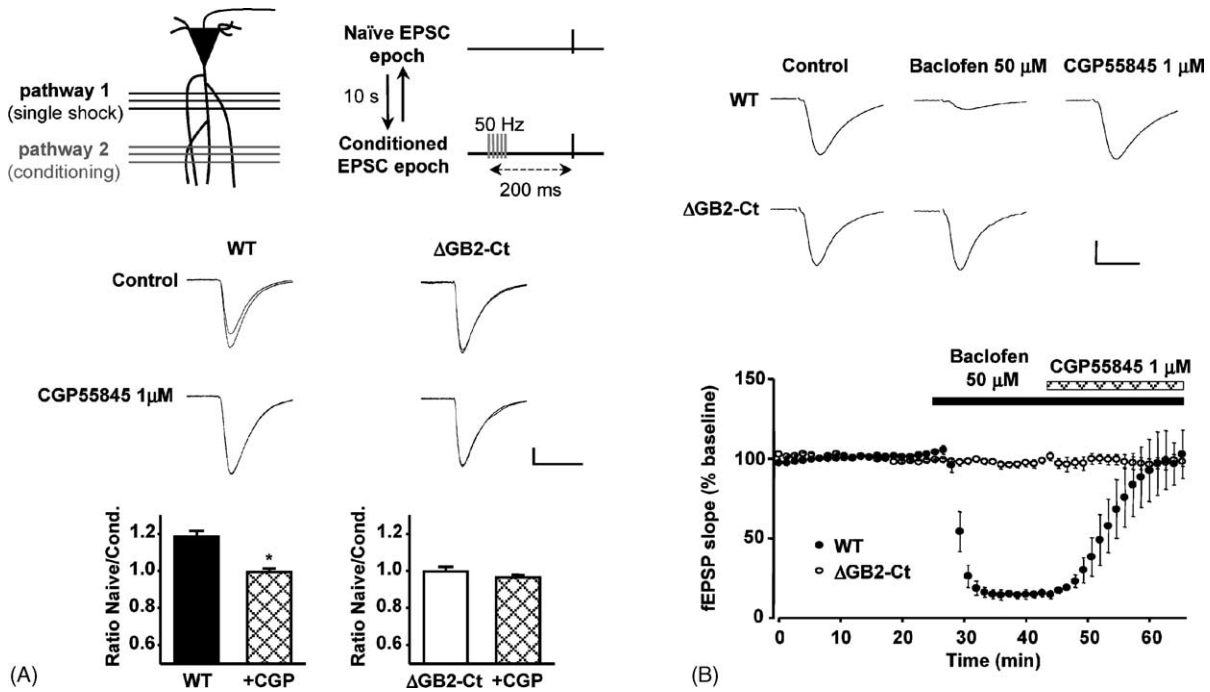

Fig. 6. Loss of presynaptic heteroreceptor function in ΔGB2-Ct neurons. (A) Glutamatergic EPSCs were evoked in CA1 pyramidal cells by stimulation of the Schaffer collateral commissural pathway (whole cell voltage clamp configuration, holding potential −70 mV). Two stimulating electrodes were placed in the stratum radiatum and arranged spatially so as to stimulate two independent sets of synapses (cf. schematic in top left hand corner). Two different stimulation epochs were used to reveal the activation of presynaptic GABA$_B$ receptors on glutamatergic terminals (cf. diagram top right hand corner). The first stimulation epoch consisted of a single shock on pathway 1 (naïve EPSC epoch), followed 10 s after by the same shock on pathway 1 but this time preceded 200 ms before by five shocks at 50 Hz on pathway 2 (conditioned EPSC epoch). Traces in the middle panel are superimposed naïve and conditioned EPSCs in response to stimulation on pathway 1 (averages of 10–15 traces). In the WT slice the conditioned response (*C*) was significantly smaller than the naïve response (*N*) ($P < 0.01$, $n = 4$). This effect was abolished by 1 μM CGP55845 ($P < 0.05$). Conditioning did not produce any depression of the EPSC in the ΔGB2-Ct slices ($n = 6$) and CGP55845 had no affect on the *N*/*C* ratio. These recordings were carried out in the presence of 50 μM picrotoxin and 50 μM D-AP5. Traces are average of 15 consecutive traces. Scale bar: 50 pA, 25 ms. (B) Extracellular fEPSPs recorded from stratum radiatum of area CA1 in response to single shock stimulation in the same subfield (top panel). Bath application of 50 μM baclofen to the WT slices caused a robust and significant depression ($85 \pm 2\%$ depression) of the slope of the fEPSP that was reversed by addition of 1 μM CGP55845. In contrast in the ΔGB2-Ct slices no change in the magnitude of the fEPSP was observed in response to the same concentration of baclofen. The graph in the bottom panel shows the time course of the effects of baclofen and CGP55845 on the fEPSP slope in WT (filled circles, $n = 4$) and ΔGB2-Ct slices (open circles, $n = 4$). Each point represents mean ± S.E.M. of the average of four consecutive measurements made every 20 s. Scale bars: 1 mV, 10 ms.

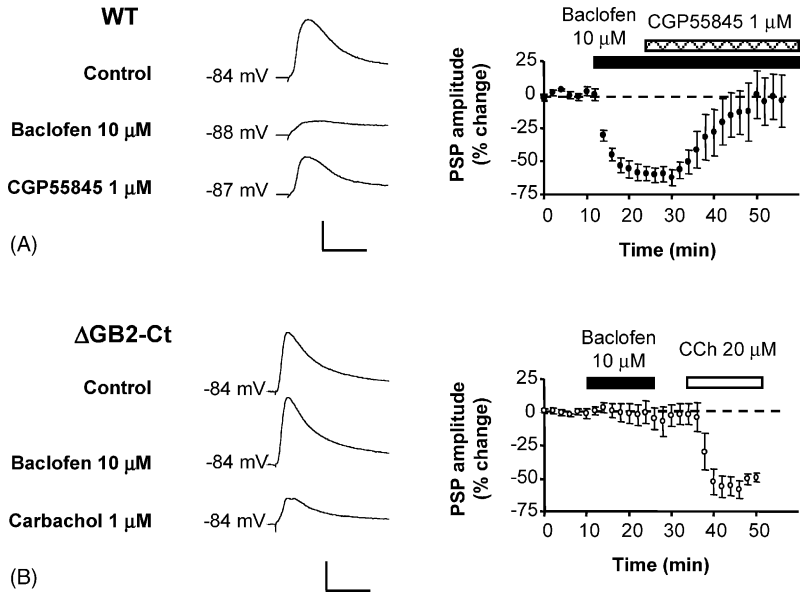

Fig. 7. Functional $GABA_B$ receptors are absent in the striatum of ΔGB2-Ct mice Intracellular sharp electrode recordings from medium spiny neurons of the striatum as identified by their characteristic membrane properties (see [21]). Mixed corticostriatal glutamatergic EPSPs and reversed GABAergic IPSPs were evoked by bipolar stimulation of the white matter between the striatum and cortex. In WT mice bath application of 10 μM baclofen depressed the corticostriatal synaptic response ($n = 7$). The depression was reversed by 1 μM CGP55845. The mean percentage change in amplitude (±S.E.M.) of the mixed PSP are plotted against time on the left panel. (B) In ΔGB2-Ct mice baclofen (closed bar) failed to depress the corticostriatal synaptic response, indicating a lack of functional $GABA_B$ receptors on the glutamatergic/GABAergic presynaptic terminals. The cholinergic agonist, carbachol (20 mM, CCh, open bar), successfully depressed the synaptic response indicating that presynaptic mechanisms are intact ($n = 6$). Representative synaptic traces, averaged from four consecutive responses, are shown to the right of each graph. Scale bars: 10 mV, 20 ms.

reported by Isaacson et al. [30], this caused a depression of the EPSC evoked by single shock stimulation of these fibres 200 ms after the train; an effect abolished by CGP55845 [31]. In contrast, in every ΔGB2-Ct slice tested no $GABA_B$ receptor-dependent heterosynaptic depression was observed (Fig. 6A). Furthermore, bath application of baclofen (50 μM) to ΔGB2-Ct slices produced no change in amplitude of extracellularly recorded fEPSPs ($n = 4$) whereas in wild-type slices it caused a large (>80%) depression that was reversed by CGP55845 (Fig. 6B, $n = 3$). In both populations of slices the adenosine A1 receptor agonist 2-chloroadenosine (50 μM) produced a significant and reversible depression of the fEPSP (data not shown).

3.7. $GABA_B$ receptor function in the striatum

The $GABA_{B2}$ subunit is highly expressed in the hippocampus [17,32,33], whereas in the striatum much lower levels of expression have been reported [4,7,32,34,35]. Because of these low levels of expression in this region, the function of the $GABA_B$ receptor might not depend on $GABA_{B2}$. To determine whether $GABA_B$ receptors were active in the striatum we examined the effect of baclofen on mixed GABAergic glutamatergic synaptic potentials in medium spiny neurons of the striatum [21]. In wild-type slices corticostriatal postsynaptic potentials evoked by stimulation of the white matter between the striatum and cortex were depressed by bath application of 10 (M baclofen ($n = 7$), an effect that was reversed by 1 μM CGP55845 (Fig. 7A). No significant effect of either of these drugs on resting membrane potential and input resistance was observed in these experiments. In contrast, in ΔGB2-Ct slices baclofen produced no change in these synaptic potentials although the cholinergic receptor agonist, carbachol (20 μM) depressed the synaptic response to a similar level to that observed in wild-type slices (Fig. 7B, $n = 6$).

4. Discussion

We have generated a functional knockout of the $GABA_{B2}$ protein in transgenic mice by the deletion of the intracellular C terminal of the receptor subunit, termed ΔGB2-Ct. Using various electrophysiological approaches we show that responses to both endogenous GABA and exogenous $GABA_B$ receptor agonists are absent in tissues obtained from ΔGB2-Ct animals. The loss of $GABA_B$ receptor function was irrespective of the receptor population studied, such that both postsynaptic and presynaptic $GABA_B$ functions were undetectable in the hippocampus of these animals. This loss of function was accompanied by an epileptic phenotype and enhanced epileptiform activity in hippocampal slices, both reminiscent of that observed in animals lacking the $GABA_{B1}$ gene [27]. In the absence of functional $GABA_B$ autoreceptors, PPD was not abolished, demonstrating the existence of several mechanisms con-

tributing to this short-term depression of hippocampal inhibitory transmission as shown previously with $GABA_B$ receptor antagonists [29,36,37]. Regarding excitatory synapses, our study confirms that heterosynaptic depression is entirely and exclusively mediated by activation of $GABA_B$ heteroreceptors [30,31]. The absence of $GABA_B$ receptor function in the striatum is a clear demonstration that even though the $GABA_{B2}$ subunit has been reported to be expressed at low levels in this region [4,7,32,34,35], it is critical for the integrity of classical $GABA_B$ receptor function in this part of the brain.

Although work in heterologous systems has defined roles for $GABA_{B2}$ in the heterodimer, i.e. the coupling of the receptor to the G protein as well as the trafficking of the nascent receptor [9,12,13,38], the exact contribution of this subunit to the formation of native $GABA_B$ receptors is still unknown. In particular, it is still unclear as to whether other proteins expressed in neurons could also play a role in the establishment of $GABA_B$ receptor function. For example we have recently described a third homologous putative GPCR subunit, $GABA_{BL}$, that is expressed in the mammalian CNS, although it appears unable to participate in the expression of a functional $GABA_B$ receptor in heterologous systems [39]. Similarly it has been suggested that $GABA_{B1}$ can be trafficked to the cell surface by mGluR4 [40]. The present work however suggests that, in the neurons we studied, there were no proteins that could promote $GABA_{B1}$ surface expression and therefore that $GABA_{B2}$ is probably the only protein that mediates this function. Indeed, we provide evidence that the loss of $GABA_B$ receptor function is due to the alteration of the correct trafficking of $GABA_{B1}$, by showing that the $GABA_{B1}$ subunit was absent from the surface of ΔGB2-Ct cortical neurons in culture. Previous work in heterologous systems has established a role for the $GABA_{B2}$ tail in masking the ER-retention signal present in the C-terminus of $GABA_{B1}$ [8,9,11]. Our work demonstrates that the $GABA_{B2}$ subunit is crucial for the trafficking of $GABA_{B1}$ to the cell surface of neurons in a native environment. Therefore, if the $GABA_{B1}$ subunit is absent, as is the case in $GABA_{B1}$ knockout mice, or not addressed to the cell surface, as in this study, then there is no resultant $GABA_B$ receptor function.

Although the presence of the truncated $GABA_{B2}$ protein could not be confirmed directly, several lines of evidence indicate that it is stably expressed in ΔGB2-Ct mice. The levels of $GABA_{B1}$ protein expression were similar in wild-type and mutant animals, an observation that is in sharp contrast to the $GABA_{B1}$ knockout mouse where $GABA_{B2}$ protein expression was decreased [27,41]. More importantly, it is strikingly different from the dramatic down-regulation of the $GABA_{B1}$ protein observed in mice in which the $GABA_{B2}$ gene has been deleted [47]. This suggests that $GABA_B$ subunit interactions promote protein stability and that if one of the subunits is missing the other tends to be degraded. We have previously shown that deletion of the C-terminal domain of $GABA_{B2}$ does not prevent its heterodimerisation with $GABA_{B1}$ [9], suggesting that stable $GABA_B$ heteromer formation occurs intracellularly in ΔGB2-Ct mice and prevents the degradation of $GABA_{B1}$. In addition, in the complete absence of $GABA_{B2}$, it has been demonstrated that baclofen induces a small inward current specifically mediated by $GABA_B$ receptors [47]. This effect is not observed in ΔGB2-Ct mice, consistent with stable expression of a truncated version of $GABA_{B2}$, as if the truncated $GABA_{B2}$ protein were degraded it is likely that we would observe a similar baclofen-induced inward current. This raises the possibility that $GABA_{B1}$ is able to function, either on its own or in association with other unidentified proteins, as a non-canonical $GABA_B$ receptor in cells where $GABA_{B2}$ is not expressed. It is important to emphasise that the transgenic approach we have employed in this study would not reveal such a $GABA_{B1}$-mediated function, due to the complete absence of $GABA_{B1}$ at the plasma membrane. Therefore, the two studies are not contradictory, and moreover it does not alter the general conclusion from both genetic approaches; that $GABA_{B2}$ is an absolute requirement for the formation of receptors mediating 'classical' $GABA_B$ function in the brain. In addition it has previously been shown that the C-terminus of $GABA_{B2}$ is neither required for the coupling of $GABA_B$ receptors to G-proteins [10,12–15] nor for the correct expression of this subunitat the cell surface [9]. Assuming that the same truncated $GABA_{B2}$ protein is indeed expressed in ΔGB2-Ct mice, these results strongly suggest that $GABA_{B1}$ is the only GABA-binding protein able to form a functional $GABA_B$ receptor in association with $GABA_{B2}$.

In this study, we observed a loss of $GABA_B$ receptor function in several areas of the brain, such as hippocampus, striatum and neocortex, suggesting that the presence of the $GABA_{B2}$ protein may be crucial for the formation of functional $GABA_B$ receptors throughout the brain. It has been suggested previously that $GABA_B$ receptors in the CNS are exclusively heterodimers [42]. Furthermore, we propose that the $GABA_{B1/2}$ heterodimer is the only form of receptor mediating classical $GABA_B$ receptor function in the central nervous system. This contrasts with other neurotransmitter systems where slow synaptic transmission is mediated by several G protein-coupled receptors that coexist either as mono- or oligomers [43–46]. To our knowledge, the $GABA_B$ receptor heterodimer is the only neurotransmitter-activated GPCR that exhibits the property of being a unique metabotropic receptor for a defined transmitter, i.e. GABA. Consistent with this hypothesis no additional genes to those encoding $GABA_{B1}$ [7] and $GABA_{B2}$ [2–4] have been identified that code for a functional $GABA_B$ receptor subunit. The discovery of a putative "$GABA_{B3}$" gene seems unlikely unless its product mediates functions that are not as yet associated with the $GABA_B$ receptor.

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