Presynaptic hyperexcitability reversed by positive allosteric modulation of a $GABA_BR$ epilepsy variant

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Abstract

$GABA_BRs$ are key membrane proteins that continually adapt the excitability of the nervous system. These G-protein coupled receptors are activated by the brain's premier inhibitory neurotransmitter GABA. They are obligate heterodimers composed of GABA-binding $GABA_BR1$ and G-protein-coupling $GABA_BR2$ subunits. Recently, three variants (G693W, S695I, I705N) have been identified in the gene (*GABBR2*) encoding for $GABA_BR2$. Individuals that harbour any of these variants exhibit severe developmental epileptic encephalopathy and intellectual disability, but the underlying pathogenesis that is triggered in neurons, remains unresolved.

Using a range of confocal imaging, flow cytometry, structural modelling, biochemistry, live cell Ca^{2+} imaging of presynaptic terminals, whole-cell electrophysiology of HEK-293T cells and neurons, and two-electrode voltage clamping of *Xenopus* oocytes we have probed the biophysical and molecular trafficking and functional profiles of G693W, S695I and I705N variants.

We report that all three point mutations impair neuronal cell surface expression of $GABA_BRs$, reducing signalling efficacy. However, a negative effect evident for one variant perturbed neurotransmission by elevating presynaptic Ca^{2+} signalling. This is reversed by enhancing $GABA_BR$ signalling via positive allosteric modulation.

Our results highlight the importance of studying neuronal receptors expressed in nervous system tissue and provide new mechanistic insights into how $GABA_BR$ variants can initiate neurodevelopmental disease whilst highlighting the translational suitability and therapeutic potential of allosteric modulation for correcting these deficits.

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Introduction

Excitability, an omnipresent feature of neurons, endows a nervous system with the ability to perform complex computational tasks. Precise fine-tuning and dynamic control prevents this ubiquitous defining feature of the nervous system from transforming into a pathological state

exemplified by neurological diseases such as epilepsy. Consequently, measured activation of γ-aminobutyric acid (GABA) type-B receptors ($GABA_B$Rs) by the brain's main inhibitory neurotransmitter GABA, has evolved as one crucial delimiter of cellular excitation. $GABA_B$Rs are class C G-protein coupled receptors (GCPRs)[1] that inhibit adenylyl cyclase and Ca^{2+} channels and activate inwardly-rectifying K^+ channels[2]. Overall, these actions reduce neuronal excitability by inhibiting presynaptic neurotransmitter release and dendritic Ca^{2+} signalling in addition to increasing postsynaptic membrane conductance[1,3]. Unsurprisingly, disruption of $GABA_B$R signalling is involved in multiple neurological conditions including spasticity, epilepsy, schizophrenia, addiction and substance abuse[4–8].

Cell surface $GABA_B$Rs are obligate heterodimers composed of two subunits: $GABA_B$R1 and $GABA_B$R2. Oligomerisation is indispensable for function as $GABA_B$R1 contains the GABA binding site, whereas G-protein coupling occurs at $GABA_B$R2[9,10]. In addition, $GABA_B$R2 is necessary for cell surface expression of $GABA_B$R1[11] and ensuring cell surface stability of the heterodimer[12]. Single nucleotide variants of the gene encoding for $GABA_B$R2 (*GABBR2*) are now implicated in a wide range of neurodevelopmental disorders[13] often sharing common symptoms, including: intellectual disability with developmental and epileptic encephalopathy and infantile seizures (e.g. T394M[14], G440R[15], M668L[16], G693W[13,17], I705N[18], S695I[18]); autism spectrum disorder (R212Q)[19]; and atypical Rett syndrome (A567T, A707T)[14,20–24]. Three of these *de novo* variants (G693W, S695I, I705N) that are located in the highly conserved sixth transmembrane (TM6) α-helical domain (*Fig. 1A)*, which is critical for GPCR activation, precipitate a range of neurodevelopmental defects prominently characterised by seizures at an early age (1.5 – 11 months). All three individuals with these variants exhibit severe intellectual disability with no speech skills and the G693W- and S695I-expressing individuals exhibit poor posture (inability to sit-up), while the carrier of I705N can only walk with support. Two of these variants (S695I and I705N) have been previously characterised in non-neuronal

expression systems. Reduced signalling efficacy was observed in the absence of changes to cell surface expression[20,22]. Currently, the signalling properties of all three TM6 variants expressed in neurons are unknown.

Given the importance of $GABA_BR2$ for signalling and cell surface trafficking of $GABA_BRs$, which ultimately determines the macroscopic efficacy of GABAergic inhibition via these receptors, we used a range of approaches to characterise these developmental epileptic encephalopathy variants. We report that severe impairment of neuronal plasma membrane expression and presynaptic effects on Ca^{2+} signalling give rise to signalling deficits that may underlie seizures and adverse neurological phenotypes. Moreover, we demonstrate that a $GABA_BR$ positive allosteric modulator (PAM) rescues the synaptic deficits which has important therapeutic implications for treating individuals expressing these variants.

Materials and methods

cDNA, cell culture and transfection

pEGFP-C1, rat α-bungarotoxin (α-BgTx) binding site-tagged $GABA_BR1$, myc-tagged $GABA_BR1a$ and flag-tagged $GABA_BR2$ in pRK5 and synaptophysin-GCaMP6*f*, have been described[12,25]. Single point mutations equivalent to human G693W, S695I and I705N (numbering includes the signal peptide) were created for the rat: G692W, S694I and I704N, in flag-tagged $GABA_BR2$ using an inverse PCR method[12] and DNA sequencing to validate the sequences.

All work on animals was performed in accordance with the Animals (Scientific Procedures) Act, 1986. HEK-293T cells with or without a stable transformation with Kir3.1/ 3.2 channels

(GIRK cells) and hippocampal cultures prepared from embryonic (E) 18 Sprague-Dawley rats were grown and transfected as described[12,25–27].

Co-immunoprecipitation

HEK-293T cells were lysed 24 hr after transfection and incubated overnight at 4°C with Dynabeads protein G (Invitrogen; 15 µl 50% slurry) conjugated to a rabbit anti-myc antibody (Abcam, ab9106; 2.5 µl) followed by washes with a buffer containing a decreasing molarity of NaCl and processed for SDS-PAGE and Western blotting using a mouse anti-FLAG-tag antibody (1:1000; Sigma, F1804) and HRP-conjugated goat-anti-mouse antibody (1:10000; ThermoFisher Scientific, 31430). Blots were developed using Immobilon® Crescendo Western blot HRP substrate (Millipore, WBLUR0500) and imaged using an ImageQuant LAS4000 mini (GE Life Sciences) followed by band intensity analysis in Image J (ver 1.52p). The membranes were buffer-stripped and re-probed with a mouse anti-$GABA_B$R1 antibody (1:1000; Neuromab, N93A/49) and the same HRP-conjugated goat-anti-mouse antibody.

Whole cell patch clamp electrophysiology

GABA- or baclofen-activated K^+-currents were recorded 36 – 48 hr or 5 – 7 days after transfection of GIRK cells and hippocampal neurons respectively in a Krebs saline solution and KCl-based internal solution at -70 mV holding potential as described previously[26]. $GABA_B$R PAM potentiation curves were constructed using the same approach for untransfected neurons at 14-21 *DIV* or GIRK cells 36-48 hr after transfection.

Neuronal GIRK current recordings were performed in 2 mM kynurenic acid and 25 μM picrotoxin. Baclofen current densities were calculated by dividing whole-cell K^+ currents by cell membrane capacitance (measured by applying brief -10 mV pulses).

Concentration-response curves were generated by measuring the current (I) and normalizing to the maximal response (I_{max}). I_{min} defines any pedestal current. Data were fitted with a modified Hill equation:

$$I = I_{min} + (I_{max} - I_{min})(1 / (1 + (EC_{50} / [A])^n)$$

where A is the concentration of the agonist, EC_{50} is the concentration of agonist causing 50% of the maximum response and n is the Hill slope.

Miniature excitatory postsynaptic currents (mEPSCs) were recorded at -70 mV using either a Cs methanesulfonate-[28] or K gluconate-based[29] internal solution and analysed using WinEDR (ver 4.0.2) and WinWCP (ver 5.7.0) and T_{50}, rise time, and charge transfer were measured from uncontaminated mEPSCs.

Action potentials were recorded at resting membrane potentials using the K-gluconate internal solution. The resting membrane potential of each cell was noted immediately after establishing whole-cell configuration in the absence of any current injection. Action potential firing rates were determined by analysing epochs of 1-2 min and kinetic properties of action potentials were measured from individual uncontaminated spikes.

Two-electrode voltage clamp

Xenopus laevis oocytes were prepared for injection following removal from ovaries and dissociated by collagenase treatment as described previously[30]. Oocytes were injected with cRNAs for $GABA_BR1$ plus either wild-type or variant S695I $GABA_BR2$ in equimolar ratios.

Two-electrode voltage-clamp recordings were performed 3-5 days after injection at room temperature at -60 mV in a recording solution containing (in mM): 40 KCl, 52 NaCl, 5 HEPES, 1.8 $CaCl_2$, 1 $MgCl_2$, pH adjusted to 7.4. An Axoclamp 2B amplifier, Digidata 1322A interface, and pClamp 8 (Molecular Devices) were used for recording membrane currents.

Immunolabelling and confocal imaging

Neurons were processed for confocal imaging at 12-14 *DIV* by fixation in 4% v/v paraformaldehyde for 10 min at RT followed by incubation in a mouse anti-flag antibody (F1804, Sigma) and a goat anti-mouse Alexa Fluor 555 (AF555) secondary antibody (A28180, ThermoFisher) before mounting in Prolong gold (Life Tech). Confocal images were acquired using a Zeiss LSM 510 confocal microscope as described previously[27]. Images were analyzed using ImageJ (1.52p).

Ca^{2+} imaging and analysis

Synaptophysin-GCaMP6f Ca^{2+} transients were imaged as described previously[25]. Presynaptic terminals were identified and delineated by ROIs using ImageJ (ver 1.52p) custom plugins and the particle analysis function. Fluorescence intensity within individual puncta was measured and the fluorescence signal (F) normalised to baseline fluorescence (F_0) to obtain $\Delta F/F_0$. Ca^{2+} transients less than 3x the signal-to-noise ratio were excluded from the analysis. The $\Delta F/F_0$ values were exported as text files using custom Visual basic plugins and imported into WinEDR (ver 4.0.2) where $\Delta F/F_0$ peaks were detected and processed for analysis using WinWCP (ver 5.7.0).

Structural plasticity of dendritic spines and morphology

eGFP-expressing live hippocampal neurons were imaged at 12 - 16 *DIV* in Krebs. The main apical dendritic segment was imaged in 3D for neurons with stereotypical pyramidal morphology, whereas the thickest dendrite was selected for analysis in neurons displaying non-pyramidal morphology. Dendrites were imaged in optimised z-thickness using a Zeiss LSM 510 microscope and a 40X water objective. Dendritic spines were analysed using Neuronstudio (ver 0.9.92)[31].

Flow cytometry

Expression levels of $GABA_BR$ subunits were analysed 48 hr after transfection using a Becton Dickinson Aria IIIu flow cytometer for live or 0.1% Triton-X permeabilized HEK-293T cells as described[30] using a mouse anti-flag antibody (F1804, Sigma) and a goat anti-mouse Alexa Fluor 647 (AF647) secondary antibody (A21235, ThermoFisher) or α-BgTx coupled to AF555 (Thermofisher, B35451).

Homology modelling

We compared cryo-EM structures of $GABA_BRs$ in: a presumed apo-state - PDB 6VJM[32]; several inactive states - 6WIV[33]; CGP54626-bound - 7C7S[34]; and CGP55845-bound states - 6W2X[35]; and baclofen and rac-BHFF activated state - 7C7Q[34]; and an SKF97541 and GS39783 bound active state - 6UO8[32]. These state-dependent structures revealed high levels of structural similarity and we selected two structures exhibiting the highest spatial resolution, 7C7S: 2.9Å and 7C7Q: 3.0Å[34], to represent an inactive and active state of the $GABA_BR$, respectively. Wild-type residues were substituted for variants using PyMol (ver 2.5.4) and receptor models

generated using Modeller (v10.4)[36]. We generated 50 models for each mutation based on 7C7Q (activated) to explore how the amino acid substitutions may disrupt receptor structure and thus the function of the activated state. The models were ranked by energy analysis using QMEANBrane[37] to identify the most optimal model. This model was analyzed in Scwrl4[38] to optimise the positioning of side-chain rotamers, before processing in MolProbity[39] for all-atom structure validation, where erroneous Asn, Gln and His rotamers were corrected. Next, the structure underwent minimization in Chimera 1.16[40] before a final run in MolProbity to ensure that each optimization step had indeed improved the structural integrity of the receptor models.

The effect of the variants on $GABA_BR2$ transmembrane domain (TMD) structure was assessed by aligning both mutant and wildtype structures. To investigate whether G693, S695, I705 (wildtype R2 structure; 7C7S), or G693W, S695I, I705N (variant R2 structure based on 7C7Q) may engage in H-bond formation, cation-π or π-π interactions, all neighbouring residues to the variants within a radius of 50 Å were analyzed.

Statistical analysis

Data compliance with a normal distribution was tested using GraphPad Instat (GraphPad). For such distributions a two-tailed unpaired t-test or a one-way ANOVA (Tukey-Kramer post-test) was used for comparing two and greater than two samples respectively. Repeated measures ANOVA (Tukey-Kramer post-test) was used for comparing between multiple normally distributed conditions applied to the same cell. For non-normally distributed data, Mann-Whitney rank-sum test and Kruskal–Wallis (KW) one-way ANOVA (with Dunn post-test) was used for two and more than two samples respectively. Data in bar charts represent mean ± standard error of mean (SEM) and box plots show median, 25 - 75% interquartile range, and 5 - 95% whiskers.

Results

Variants cause structural changes to R2 subunit transmembrane domains

Class-C GPCR TMDs transduce agonist binding signals from the orthosteric extracellular domain (ECD) binding site to G-protein-activating intracellular loops[1]. Since TMD amino acids can profoundly affect GPCR signalling[41,42], the locations of Gly693, Ser695 and Ile705 within the TM6 of GABA$_B$R2 were studied using homology modelling. Comparing cryo-EM heterodimer structures for inactive (PDB: 6VJM[32], 6WIV[33], 7C7S[34]; 6W2X[35]) and active states (7C7Q[34]; 6UO8[32]) confirmed the TM6 localization of these variants. Moreover, upon activation, the TMDs rotate clockwise shifting the heterodimer interface from predominantly TM5 to TM6[43] thus bringing both GABA$_B$R1 and GABA$_B$R2 TM6 α-helices into close proximity (*Fig. 1B*).

From this apposition, G693W introduces a bulky tryptophan directly into the dimer interface physically disrupting the TMD α-helical structure (7C7Q; *Fig. 1C*). Furthermore, for S695I, the non-polar isoleucine is orientated away from the heterodimer interface projecting laterally towards GABA$_B$R2 TM7 shifting the relative positions of the α-helices (*Fig. 1D*). We predict that such lateral shifts are likely to affect functional coupling of ligand-binding to G protein activation due to suboptimal α-helical positioning ultimately disrupting GABA$_B$R physiology. For I705N, as noted for G693W, the mutant residue also inserts directly into the inter-subunit space, but here the TMDs are physically unaffected due to the smaller side-chain volume (by 36%) between Ile and Asn; however, hydrophobic Ile is replaced by Asn with its polar amino group (*Fig. 1E*). Subsequent side-chain rotamer optimization revealed considerable orientation differences in the TMD interface between R2 wild-type and variants. By using computational model predictions for the relative positioning of the three R2 variants, we observed that GABA$_B$R2 M694, which co-ordinates the binding of PAMs rac-BHFF and GS39783[34,44,45], is

now re-positioned into the PAM binding cavity. This feature of M694 is apparent with all three R2 mutants. We predict that this will result in residue-PAM molecule clashes and therefore sub-optimal PAM binding (*Fig. 1C-E).*

Together these results suggest that the mutations introduce a number of structural changes in $GABA_B$Rs focused on the critical TMD. Notably, prominent changes to the TMDs occur with G693W and S695I, and side-chain orientations are reconfigured in all three variants, principally affecting the TM6 subunit interface.

Impaired GABA-activated signalling of $GABA_B$R variants

The pharmacological profiles of all three $GABA_B$R variants were probed in GIRK cells using whole-cell electrophysiology. Sequentially expressing each $GABA_B$R2 variant with $GABA_B$R1 resulted in heteromeric cell surface expression for G693W and I705N, evident from functional GABA-activated GIRK currents (called GABA currents hereafter), but not for S695I, examined with up to 1 mM GABA (*Fig. 2A-B*). Co-immunoprecipitation of $GABA_B$Rs eliminated deficits in heterodimerisation as a cause for GABA insensitivity of S695I since similar amounts of wild-type and S695I $GABA_B$R2 were co-immunoprecipated with $GABA_B$R1 ($P > 0.05$; $F_{(4,23)} = 35.5$, $P < 0.0001$; one-way ANOVA; *Fig. 2C-D*).

Analysis of the maximal GABA-activated K^+ currents revealed reduced maxima for both G693W and I705N $GABA_B$Rs compared to R1R2 wild-type (by ~50%, $F_{(3, 48)} = 50.7$, $P < 0.001$, one-way ANOVA), and S695I as noted, yielded negligible current (*Fig. 2A-B*). Interestingly, GABA potency at G693W and I705N receptors was increased (or trending) compared to wild-type receptors ($F_{(2, 28)} = 4.1$, $p = 0.0276$, one-way ANOVA; *Fig. 2A-B*).

Consistent with the low current levels for S695I in HEK cells, expression of this mutant in *Xenopus* oocytes with wild-type R1, and using two-electrode voltage clamp [30], also revealed

reduced maximal GABA current (*Fig. 2E*; $p = 0.0018$, two-tailed unpaired t-test). As a consequence, we were unable to construct full concentration curves due to the small-sized currents although similar reduced efficacy and increased GABA potency for S695I have been reported[46].

Collectively, these results suggest that, when expressed on the plasma membrane of cell lines or oocytes, G693W and I705N displayed impaired functional properties, whereas S695I exhibited severe functional impairment.

Cell-type dependent plasma membrane expression deficits of R2 variants

A reduction in the maximal GABA current for $GABA_BR$ variants could arise from lowered cell surface receptor expression. This aspect was examined using a flow-cytometry-based immunolabelling approach[30] measuring plasma membrane levels of flag-tagged $GABA_BR2$ ($GABA_BR2^{flag}$) in live HEK-293T cells co-expressing R1a subunits. The median cell surface fluorescence for variant $GABA_BR2$ remained unchanged ($P > 0.05$; *Fig. 3A-B*, $F_{(5, 50)} = 30.8$, $P < 0.001$ Kruskal-Wallis (KW) one-way ANOVA). However, the % cells captured in the Q2 quadrant which is indicative of cell surface expression efficiency[30] (reflecting eGFP expressing cells that are also positive for $GABA_BR2$ cell surface expression), was reduced for I705N ($P < 0.05$; *Fig. 3A-B*, $F_{(5, 50)} = 33.7$, $P < 0.001$, KW one-way ANOVA), trending to a reduction for S695I relative to wild-type receptors, whilst G693W was similar to wild-type (*Fig. 3A-B*).

These results were not due to changes in total protein expression levels (intracellular + cell surface) as fluorescence intensity ($F_{(5, 27)} = 25.5$, $P < 0.001$, KW one-way ANOVA) and % Q2 area ($F_{(5, 26)} = 25.6$, $p = 0.0001$, KW one-way ANOVA) of receptor-expressing-cells labelled following fixation and permeabilization remained unchanged between the R2 variants and wild-type $GABA_BRs$ ($P > 0.05$; *Fig. 3C-D*).

GABA$_B$Rs are usually expressed on the cell surface as obligate R1aR2 heteromers. However, GABA$_B$R2 can be expressed alone on the HEK cell surface as a homomer[12,47]. Therefore, to assess R1aR2 expression, we quantified the expression of GABA$_B$R1 in the presence of GABA$_B$R2 which allows heterodimer expression to be unequivocally studied. HEK-293T cells transiently transfected with GABA$_B$R1a^{BBS} (containing an α-BgTx binding site[12]) and GABA$_B$R2 were labelled with α-BgTx coupled to AF555 (BgTx-AF555). Consistent with the GABA$_B$R2^{flag} labelling, cell surface expression levels of GABA$_B$R1 remained unchanged ($P > 0.05$) when expressed with wild-type or variant GABA$_B$R2s (*Supp. Fig. 1*, $F_{(5, 62)} = 54.2$, $P < 0.001$, KW one-way ANOVA). Remarkably, similar to GABA$_B$R2, GABA$_B$R1 % Q2 area was lower ($P < 0.05$) when expressed with R2 I705N but unchanged for G693W or S695I ($P > 0.05$) in comparison to wild-type receptors ($F_{(5, 62)} = 54$, $P < 0.001$, KW one-way ANOVA). Here too, in permeabilised cells, expression of GABA$_B$R2 variants did not alter ($P > 0.05$) the total expression levels of GABA$_B$R1 (fluorescence $F_{(5, 38)} = 32.3$, %Q2 $F_{(5, 38)} = 32.7$, $P < 0.001$, KW one-way ANOVA).

Overall, these results suggest that G693W and S695I express on the HEK-293T cell plasma membrane at similar levels compared to wild-type receptors while I705N expression is reduced. Furthermore, the absence of K^+ currents for S695I in HEK-293 cells (*Fig. 2A*) is not due to reduced cell surface expression and instead must arise from signalling defects caused by this variant.

GABA$_B$Rs execute their physiological roles from the neuronal plasma membrane and thus the expression levels of each variant was also assessed in the native environment of hippocampal neurons by transiently co-expressing GABA$_B$R2^{flag} with eGFP in cultures. To efficiently express at the cell surface, exogenous GABA$_B$R2^{flag} subunits will need to heterodimerize with endogenous GABA$_B$R1, thereby limiting their overexpression. Using this strategy, even though cell surface staining of wild-type receptors was detected (*Supp Fig. 2A-B*), no plasma

membrane expression was resolved for the mutants in neurons ($F_{(4, 176)} = 86.6$, $P < 0.001$; KW one-way ANOVA). To aid heterodimeric co-assembly[48] while further investigating the absence of neuronal cell surface expression of the R2 variants, $GABA_BR1a$ and $GABA_BR2^{flag}$ were co-expressed to facilitate cell surface expression by driving overexpression. However, under these conditions, R2 variant expression was still severely compromised compared to wild-type receptors (*Fig. 4A-B*; $F_{(4, 217)} = 111.35$, $P < 0.001$, KW one-way ANOVA). Cell surface expression levels of overexpressed S695I and I705N remained unchanged compared to eGFP only expressing neurons ($P > 0.05$), while expression of overexpressed G693W was only marginally increased ($P < 0.05$) compared to eGFP-expressing cells.

These results show that while R2 variant plasma membrane expression is largely intact in heterologous HEK-293T cells, a contrasting severe reduction of neuronal cell surface expression typifies these $GABA_BR$ disease variants. Thus, the R2 variants are preventing $GABA_BR$ expression which may result in a marked reduction of GABAergic signalling.

Variants reduce $GABA_BR$ signalling in hippocampal neurons

The functional consequences of reduced neuronal cell membrane $GABA_BR$ expression was assessed in hippocampal neurons expressing wild-type or variant $GABA_BR2$ along with eGFP. We activated GIRK currents in neurons using the specific $GABA_BR$ agonist baclofen.

Sequentially expressing just the $GABA_BR2$ variants in the absence of exogenous $GABA_BR1$, revealed substantive changes to inwardly-rectifying K^+ currents. The current density for S695I-expressing neurons was reduced compared to untransfeced cells ($P < 0.05$), eGFP only controls ($P < 0.001$) and neurons expressing wild-type R2 ($P < 0.001$) in response to 10 μM baclofen (*Fig. 4C-D*; ($F_{(5, 154)} = 31.23$, $P < 0.0001$, KW one-way ANOVA). At maximal baclofen concentrations (100 μM), the current density of S695I was again lower compared to eGFP

controls or wild-type R2 expressing neurons ($F_{(5,\ 158)} = 28.6$, $P < 0.0001$, KW one-way ANOVA). This indicated that expressing S695I may have a negative effect on the function of endogenous and wild-type $GABA_BRs$.

These results were corroborated by co-expressing $GABA_BR2$ and $GABA_BR1$ *(Supp Fig. 2C-D)*. The current densities associated with G693W and I705N were unaffected for neurons expressing R1 and R2, compared to wild-type receptors ($P > 0.05$) suggesting that the mechanism by which G693W and I705N increase neural excitability does not involve depressing wild-type R2 expression (*Supp Fig. 2C-D*). However, current density for S695I was lower compared to untransfected neurons at 10 ($P < 0.05$) and 100 µM ($P < 0.001$) baclofen as well as eGFP only controls ($P < 0.001$) and wild-type R2 expressing neurons ($P < 0.001$) at 100 µM baclofen (*Supp Fig. 2C-D;* 10 µM $F_{(5,\ 122)} = 57.07$; 100 µM $F_{(5,\ 122)} = 55.5$; $P < 0.0001$, KW one-way ANOVA) consistent with a negative role of S695I on endogenous wild-type subunit expression.

By examining the functional properties of the R2 variants, especially S695I and I705N, it is likely that the profound effect on GABAergic signalling occurs via a consequent reduction of $GABA_BR$ function, and that S695I seemingly acts to suppress wild-type $GABA_BRs$ to further reduce receptor signalling.

Postsynaptic excitatory neurotransmission unchanged by S695I

We focused this part of our study on S695I because of its effectiveness in reducing $GABA_BR$ activity evident in a heterologous expression system designed to avoid overexpression of the R2 variant.

To investigate the impact of S695I on excitatory neurotransmission in hippocampal cultured neurons, we used whole-cell recording with a Cs-methanesulfonate-based patch electrode

solution to block postsynaptic $GABA_BR$-activated GIRK channels[49,50] in the presence of picrotoxin and tetrodotoxin. For neurons expressing just R2 S695I, the miniature excitatory postsynaptic current (mEPSC) frequency ($F_{(2, 22)} = 0.997$, $p = 0.3853$), amplitude ($F_{(2, 27)} = 2.9$ KW, $p = 0.2301$) and kinetics (rise time: $F_{(2, 27)} = 4.8$ KW, $p = 0.0891$; T_{50}: $F_{(2, 27)} = 2.8$, $p = 0.0767$; τ: $F_{(2, 27)} = 3.3$ KW, $p = 0.1897$; area: $F_{(2, 27)} = 2.2$, $p = 0.1345$) all remained unchanged compared to wild-type R2 expressing neurons (*Supp. Fig. 3*, one-way ANOVA).

Reverting to a K^+-based internal solution (K-gluconate) to preserve GIRK channel function allowed the postsynaptic modulation of mEPSCs by $GABA_BRs$ to be probed. Under these conditions, mEPSC frequency ($F_{(3, 71)} = 6.36$ KW, $p = 0.0953$) and amplitude ($F_{(3, 71)} = 2.32$, $p = 0.0825$) as well as kinetics (charge transfer: $F_{(3, 71)} = 2.41$, $p = 0.0737$; rise time: $F_{(3, 71)} = 2.2$, $p = 0.0901$; T_{50}: $F_{(3, 71)} = 2.524$, $p = 0.0646$) remained unchanged (*Fig. 5A-C*, one-way ANOVA) overall suggesting that the expression of the $GABA_BR2$ variant in postsynaptic cells had no clear effect on glutamatergic neurotransmission.

Any subtle changes to glutamatergic neurotransmission may be associated with altered dendritic spine structure since these form the sites of glutamatergic inputs on dendrites[51]. Imaging hippocampal neurons expressing either wild-type R2 or S695I, with eGFP, revealed unchanged spine density ($F_{(2, 179)} = 3.434$ KW, $p = 0.1796$), spine diameter ($F_{(2, 179)} = 1.94$ KW, $p = 0.4565$), or the proportions of mushroom ($F_{(2, 180)} = 5.063$ KW, $p = 0.0795$), stubby ($F_{(2, 180)} = 0.2886$, $p = 0.7496$) or thin ($F_{(2, 180)} = 0.4149$ KW, $p = 0.8126$) spines (*Fig. 5D-E*).

To explore any physiological consequences of expressing S695I, we probed action potential firing in hippocampal neurons expressing either wild-type R2 or S695I. Spontaneous firing frequency ($F_{(2, 36)} = 2.69$, $p = 0.0813$) and the peak amplitudes of action potentials ($F_{(2, 36)} = 0.03$, $p = 0.9664$, one-way ANOVA) remained unchanged; the resting membrane potential ($F_{(2,70)} = 4.923$ KW, $p = 0.0853$; *Fig. 5F-H*) was similarly unchanged between untransfected, wild-type R2- or S695I-expressing neurons. Moreover, unitary spike area ($F_{(2, 36)} = 0.7175$; p

= 0.4948), rise time ($F_{(2, 36)}$ = 0.4257 KW, p = 0.8083) and the action potential repolarisation phase, exemplified by T_{50} ($F_{(2, 36)}$ = 0.1929 KW; p = 0.9081) between wild-type R2 and S695I (*Fig. 5H*), was also unchanged.

Combined, these findings suggest that S695I affects neither dendritic spine structure nor glutamatergic EPSCs and intrinsic membrane properties, as described previously for $GABA_BRs$ under basal conditions[52–55]. Thus, the expression of S695I has had no decisive effects on postsynaptic glutamatergic neurotransmission and spike firing in cultured neurons.

Elevated presynaptic Ca^{2+} signalling due to S695I

Since an equally important role for $GABA_BRs$ is to control neurotransmitter release at presynaptic terminals, the impact of S695I on presynaptic Ca^{2+} transients was addressed using the Ca^{2+} reporter, GCaMP6*f* fused to synaptophysin (synaptophysin-GCaMP6*f*)[25].

Expression of synaptophysin-GCaMP6*f* allowed spontaneous Ca^{2+} transients to be imaged in presynaptic terminals (*supplementary video 1*). Co-expression with wild-type $GABA_BR2$ neither altered ($P > 0.05$) the mean $\Delta F/F_0$ ($F_{(2,569)}$ = 63.4, $P < 0.001$, KW one-way ANOVA) nor the frequency (*Fig. 6A-C*; $F_{(2,569)}$ = 36.6, $P < 0.001$, KW one-way ANOVA) of presynaptic Ca^{2+} transients compared to synaptophysin-GCaMP6*f* only expressing neurons. However, the area under the $\Delta F/F_0$ curve of Ca^{2+} activity during the imaging epoch was reduced due to the expression of wild-type R2 compared to synaptophysin-GCaMP6*f* alone (*Fig. 6A-C*; $F_{(2, 1280)}$ = 147.2, $P < 0.001$, KW one-way ANOVA)). Although this appears to contrast with the findings from our whole-cell EPSC and spike firing recordings, pre- and postsynaptic signalling could be differentially sensitive to exogenous expression-related changes in $GABA_BR$ expression levels.

By contrast to wild-type R2, expression of R2-S695I increased the $\Delta F/F_0$ ($P < 0.001$), the frequency of Ca^{2+} transients ($P < 0.001$), and the area under the curve compared to R2-wild-type or synaptophysin-GCaMP6*f* alone controls ($P < 0.001$, one-way ANOVA) suggesting that increased presynaptic Ca^{2+} activity, due to S695I expression, could be a key mechanism by which this variant orchestrates its pathophysiological phenotype.

Reversal of presynaptic $GABA_BR$ signalling defects by positive allosteric modulation

We reasoned that increasing $GABA_BR$ activity could compensate for the deleterious effects of S695I and thus recover a 'ground state' for presynaptic receptor signalling. To increase $GABA_BR$ signalling, we examined the PAMs, GS39783 and rac-BHFF for their effectiveness in GIRK cells (*Fig. 7A-C*) and hippocampal neurons (*Fig. 7D-F*) by constructing PAM potentiation curves for the response to ~EC_{20} baclofen. Rac-BHFF was consistently more efficacious than GS39783 in GIRK cells ($p = 0.0021$, two-tailed unpaired t-test) and hippocampal neurons ($p = 0.0013$).

Interestingly, in hippocampal neurons, GS39783 was slow to wash-off (*Fig. 7G-H*) with potentiated baclofen responses, following a single 3 µM sub-maximal GS39783 exposure, reduced by only ~5% after 10 min of wash (*Fig. 7G*; $F_{(3,30)} = 9.9$, $p = 0.0001$, repeated measures ANOVA). The baclofen EC_{50} post-GS39783 ($EC_{50} = 2.43 \pm 0.3$ µM) remained lower even after 15 min wash compared to the control pre-GS39783 EC_{50} (4.7 ± 0.4 µM; $p = 0.0029$, n = 7 – 11, *Fig. 7H*). Intriguingly, the recovery kinetics (*Fig 7I*; $\tau = 14.2 \pm 9.6$ min) for GS39783 potentiation during the wash-off phase, studied in GIRK cells by applying consecutive pairs of ~EC_{10} and maximal baclofen concentrations every 5 min following a single exposure to 3 µM GS39783, is similar to the rate of internalization for cell surface $GABA_BRs$ ($\tau = 13.4 \pm 1.4$

min)[12] in the same cells. This similarity may imply that GS39783 binds tightly to the $GABA_BR$ and that potentiation is only terminated predominantly via endocytosis instead of PAM unbinding.

Due to the pseudo-irreversible nature of GS39783, we therefore used rac-BHFF at a low concentration (1 μM) to negate the deleterious functional effects of S695I. Applying rac-BHFF reduced both wild-type ($P < 0.001$) and S695I ($P < 0.001$) $\Delta F/F_0$ (*Fig 8A-C*; *supplementary video 2*, $F_{(3,775)} = 89.7$ KW, $p = 0.007$, one-way ANOVA). Despite our homology modelling proposing that BHFF binding could be affected in the R2-S695I mutant, this PAM normalised the difference in Ca^{2+} transients between wild-type and rac-BHFF-treated S695I axon termini ($P > 0.05$). The frequency of presynaptic transients in rac-BHFF was unaltered for wild-type neurons ($P > 0.05$) but reduced ($P < 0.05$) in S695I-expressing neurons effectively normalizing ($P > 0.05$) the frequency to wild-type levels ($F_{(3,778)} = 12.2$, $p = 0.007$, KW one-way ANOVA). Finally, the overall area under the curve was also reduced by rac-BHFF for wild-type ($P < 0.01$) and S695I ($P < 0.01$) with no difference ($P > 0.05$) between wild-type compared to rac-BHFF treated S695I termini ($F_{(3,732)} = 59.4$, $P < 0.001$, KW one-way ANOVA).

Together these results confirm that elevated presynaptic Ca^{2+} signalling due to reduced $GABA_BR$ function caused by S695I can be rescued *in vitro* by positive allosteric modulation. This may have important implications for treating the phenotypes associated with neurodevelopmental disorders.

Discussion

GABA receptor genetic variants are increasingly linked to neurodevelopmental disorders such as epileptic encephalopathy, autism spectrum disorders, intellectual disability, global

developmental delay and Rett syndrome[56–58] which are often comorbid. Aside from the three variants studied here, other pathogenic GABA$_B$R2 variants can cause variable intellectual disability with or without seizures or Rett-like phenotypes. Interestingly, these variants are also located within the receptor's transmembrane[13,14,16–18,20–24,59,60] or N-terminal domains[14,15,19] emphasizing their importance in these disorders. Cryo-EM structures of GABA$_B$Rs reveal detailed insights into their conformational states[32–35]. From such structures, TM6 is deemed crucial for receptor activation and forms an integral part of GS39783 and rac-BHFF PAM binding pockets resembling the evolutionary conserved agonist-(orthosteric) binding site in class-A GPCRs[61]. When expressed on the cell surface, N-terminal variants (R212Q, T394M, G440R) are likely to affect GABA binding and the activation of signal transduction, while variants located within TM4 (A567T), TM5 (M668L), and TM6 (G693W, S695I, I705N, A707T) will affect signal transduction and G-protein coupling. Therefore, a diverse set of mechanisms will underlie variant phenotypes in neurodevelopmental disorders, and clinical manifestations will depend on additional contributions from genetic composition, penetrance, and variability.

Perturbing GABA$_B$R signalling and trafficking with R2 variants

GABA$_B$R subunit variants have been previously characterised in heterologous expression systems[20,22]. Here, we used whole-cell electrophysiology and flow cytometry as first-order approaches to interrogate pharmacological and trafficking properties of the GABA$_B$R2 variants. The reduced maximal GABA currents for G693W, S695I and I705N in GIRK cells are consistent with impaired signalling[20]. Lower currents for I705N in these cells is partly due to reduced cell surface expression of GABA$_B$R2 variant receptors, irrespective of whether the R2 homomer or R1aR2 heterodimer is studied. Using ELISA[22] or immunofluorescence[20]

assays, prior reports concluded I705N expression was unaffected; however, the resolution of the flow cytometry method used here detected a reduction. The minimal GABA sensitivity of $GABA_BR2$ S695I and reduced G693W maximal currents in GIRK cells in the absence of cell surface expression changes, is indicative of signal transduction defects. Biochemical evidence from immunoprecipitation did not reveal changes to heterodimerisation for S695I and therefore this mutant likely suffers from defective transduction of ligand-binding signals. Expression of wild-type R1 with R2-S695I in *Xenopus* oocytes, which permit expression of a wide variety of difficult-to-translate constructs[30], resolved GABA-activated GIRK currents, albeit with reduced maximal currents and altered kinetics compared to wild-type receptors[46]. Therefore, these $GABA_BR2$-variants are characterised by transduction defects that reduce $GABA_BR$ signalling.

Counterintuitively, the low efficacy R2-variant $GABA_BRs$ also exhibit greater sensitivity to GABA (lower $EC_{50}s$). Structural modelling tentatively indicates that this may be due to re-positioned R2-variant TMDs, which for S695I, could involve a stabilization of active structures that may underlie changes to GABA sensitivity[62]. Using chimeric $G\alpha_{qi}$ assays, higher constitutive activity has been reported for S695I and I705N[22,62], but others have not reproduced these results[61]. Such a mechanism is unlikely to offer protection against seizures as these receptors also show severely impaired cell surface expression in neurons.

By studying $GABA_BR2$ variants in neurons, for the first time to our knowledge, we propose that trafficking defects in $GABA_BR2$ is another principal mechanism by which these receptors could cause dysfunction. Our results provide a plausible molecular-level explanation by which $GABA_BR2$ variants could orchestrate seizures and neurodevelopmental deficits due to reduced neuronal $GABA_BR$ signalling. Similarly, knocking-out $GABA_BR2$[63], or treatment with $GABA_BR$ antagonists[64,65], or by perturbing $GABA_BR$ cell surface delivery[66] all result in

seizures, highlighting the importance of maintaining appropriate levels of functional $GABA_B$Rs at the cell surface.

Cell surface expression of R2 variants

A key unexpected finding from our study was the difference in cell surface expression for the R2 variant receptors when expressed in neurons compared to heterologous cells. Neurons are known for their stringent quality control of cell surface expression[30]. Moreover, the role of TM6 is clearly important as single amino acid substitutions prevent cell surface trafficking of variant receptors in neurons but not in HEK-293 cells. Our findings suggest that TM6 could form a critical part of a neuronal quality control checkpoint that determines plasma membrane expression of $GABA_B$Rs. Structural modelling of each $GABA_B$R2 variant predicted subtle changes to side-chain rotamers for the variant residues that presumably are disruptive to TM6 interactions and thus limiting variant $GABA_B$R2 receptor expression at the cell surface. Moreover, subtle changes to the $GABA_B$R2 TM6 primary sequence can result in neurodevelopmental disorders. TM6 appears to be a 'hot spot' for disease pathogenesis since while G693W, S695I and I705N all cause developmental epileptic encephalopathy, A707T in the same TMD causes atypical Rett syndrome[22]. Therefore, characterising variant receptor properties at synapses, in neurons, and neural circuits, is important for identifying the molecular-basis of complex neurodevelopmental disorders.

Variant R2 and dominant-negative signalling

The first indication of loss-of-function signalling, where expression of a variant subunit also suppressed wild-type receptor function, arose from reduced GIRK current densities in neurons expressing S695I compared to untransfected or eGFP-expressing controls. Driving over-expression of this $GABA_B$R2 variant by co-transfection with $GABA_B$R1 cDNA caused a

greater depression of endogenous currents confirming the loss-of-function properties of S695I and its effect on the function of wild-type receptors. These effects register the pathological landscape of S695I on the function of native GABA$_B$Rs compared to G693W and I705N. The net result for these latter two variants will be reduced GABA$_B$R function at pre- and postsynaptic compartments, and for S695I, function will be further reduced due to the inhibitory effect it has on residual wild-type receptor function. In the absence of clinical results and information of patient full genetic backgrounds, a direct co-relation of disease severity with GABA$_B$R function was unattainable but would be interesting to address.

Mechanistically, S695I could produce its loss-of-function effects by intracellularly sequestering GABA$_B$R1 for degradation to reduce the pool of R1 subunits available for heterodimerization with wild-type GABA$_B$R2. Our immunoprecipitation studies indicate once co-assembly occurs, this R2 variant may trap heterodimers intracellularly in neurons and accumulated heterodimers containing S695I will be degraded most likely via lysosomal[67] or proteasomal endoplasmic reticulum-associated degradation[68]. Formation of higher-order oligomers[69] and heterodimers with higher stability[62] should amplify the sequestering impact of S695I manifest by reduced cell surface efficacy of GABA$_B$R signalling.

Functional impact of S695I

GABA$_B$Rs control neuronal excitability at pre- and postsynaptic domains but intrinsic activity, glutamatergic EPSC current amplitude or frequency, or structural plasticity of dendritic spines remained unaffected by S695I. However, this accords with previous studies where cell spiking[52] or EPSC amplitudes[53,55] were unaffected by baclofen. Importantly, application of GABA$_B$R antagonists does not affect EPSC amplitude/ frequency[54] in hippocampal neurons or action potential spiking[71] suggesting overall that postsynaptic GABA$_B$Rs are not basally active here. Therefore, a lack of more severe postsynaptic excitability defect due to expression of

S695I is not surprising as our neurons in culture will receive majority of their inputs from untransfected neurons with wild-type presynaptic signalling profiles. However, the impact of the postsynaptic deficits could be exacerbated in pathological states if multiple inputs show elevated presynaptic signalling and increased glutamate release.

Changes to Ca^{2+} signalling were apparent at presynaptic terminals due to S695I expression. Using the Ca^{2+} sensor GCaMP6*f*, localized to presynaptic terminals, permitted defects in individual presynaptic boutons to be resolved. Here, as expected, native $GABA_BRs$ reduce presynaptic Ca^{2+} transients by inhibiting voltage-gated Ca^{2+} channels[3]. However, expression of S695I led to increased frequency, amplitude and charge transfer of Ca^{2+} events at presynaptic termini. By comparison, following the expression of wild-type $GABA_BR2$, the imaging of single terminals detected a reduction in the presynaptic area of fluorescence transfer without affecting the amplitude or frequency of Ca^{2+} transients.

Taken overall, the mechanism by which $GABA_BR$ variants generate seizures will likely involve elevated glutamate release from presynaptic terminals. In addition, since elevated GABA release from interneurons can initiate disinhibition and thereby maintain seizures[72], this could be an additional mechanism by which a lack of $GABA_BR$ function at interneuron terminals can exacerbate seizures.

Therapeutic approach for mitigating R2 variant pathogenesis

Treatment of $GABA_BR$ variant seizures has proven challenging and relies on a combinatorial pharmacological approach. Despite a paucity of treatment options, increasing GABA mediated signalling is likely to be beneficial. This is evident from indirectly facilitating $GABA_BR$ signalling for $GABA_BR2$ S695I and I705N[20] with the GABA-transaminase inhibitor, vigabatrin, for a related $GABA_BR2$ TM6 variant[60]. Administering baclofen to activate

$GABA_BR$ signalling is confounded by the incidence of seizures that have been reported in some individuals[73]. We therefore explored an alternative approach using a $GABA_BR$ PAM to normalize the defects in presynaptic Ca^{2+} signalling for S695I.

Several $GABA_BR$ PAMs are now known to affect rodent behavior in pre-clinical studies of neurological conditions including alcoholism, substance abuse, schizophrenia, anxiety and seizures. PAMs have the advantage of greater temporal specificity compared to agonists as PAMs are (usually) only active in the presence of agonists. Examples of current commercially available PAMs include: CGP7930, GS39783 and rac-BHFF. Rac-BHFF was selected based on its higher efficacy at low concentrations compared to other $GABA_BR$ PAMs and because CGP7930 is a $GABA_AR$ PAM and K^+ channel blocker[26] while GS39783 is genotoxic[74] in addition to its prolonged binding to $GABA_BRs$. At presynaptic terminals expressing S695I, rac-BHFF normalised the frequency, $\Delta F/F_0$ amplitude and area of fluorescence transfer for presynaptic Ca^{2+} transients to levels associated with $GABA_BR2$ wild-type expressing controls. Rac-BHFF probably achieves this by facilitating the activity of those minimal numbers of wild-type $GABA_BRs$ that are expressed on the cell surface in S695I-expressing neurons. We would further predict that $GABA_BR$ PAMs should have beneficial effects for other variants and neurodevelopmental disorders where receptor expression and signalling is similarly impaired.

Our results highlight the importance of controlling presynaptic excitability in developmental disorders associated with $GABA_BR$ variants. While increased excitability of interneurons should elevate GABA release, the increased presynaptic release from excitatory neurons, will elevate glutamate release altering neural circuit dynamics to orchestrate seizures.

Overall, we provide proof-of-concept for using PAMs for treating $GABA_BR$ associated neurodevelopmental conditions. *In vivo*, rac-BHFF reduces the incidence of audiogenic

seizures in mice[75] thus testing the effectiveness of PAMs for targeting $GABA_B$Rs in neurodevelopmental disorders will be important.

Data availability

The datasets generated and analysed during this current study are included in this published article (and its supplementary information files) and can be made available by the authors on reasonable request.

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Competing interests

The authors report no competing interests.

Author contributions

Project conception – SBH; Cellular and synaptic imaging – M Minere, SBH; Structural modelling – M Mortensen; Biochemistry – VD; Whole-cell electrophysiology – SBH; Flow cytometry – SBH, GW, DN; Project supervision, design and funding acquisition – SBH, TGS. All authors contributed to writing the manuscript.

Supplementary material

Supplementary material is available at *Brain* online.

References

1. Pin JP, Bettler B. Organization and functions of mGlu and $GABA_B$ receptor complexes. *Nature*. 2016;540(7631):60-68.
2. Pinard A, Seddik R, Bettler B. $GABA_B$ Receptors. Physiological Functions and Mechanisms of Diversity. *Advances in Pharmacology* Vol 58. Elsevier Inc.; 2010.
3. Bettler B, Kaupmann K, Mosbacher J, Gassmann M. Molecular Structure and Physiological Functions of $GABA_B$ Receptors. *Physiological Reviews*. 2004;84(3):835-867.
4. Filip M, Frankowska M, Sadakierska-Chudy A, et al. $GABA_B$ receptors as a therapeutic strategy in substance use disorders: Focus on positive allosteric modulators. *Neuropharmacology*. 2015;88:36-47.
5. Kumar K, Sharma S, Kumar P, Deshmukh R. Therapeutic potential of $GABA_B$ receptor ligands in drug addiction, anxiety, depression and other CNS disorders. *Pharmacology Biochemistry and Behavior*. 2013;110:174-184.
6. Li X, Slesinger PA. $GABA_B$ Receptors and Drug Addiction: Psychostimulants and Other Drugs of Abuse BT - Behavioral Neurobiology of $GABA_B$ Receptor Function. In: Vlachou S, Wickman K, eds. Springer International Publishing; 2022:119-155.

7. Ghose S, Winter MK, McCarson KE, Tamminga CA, Enna SJ. The $GABA_B$ receptor as a target for antidepressant drug action. *British Journal of Pharmacology*. 2011;162(1):1-17.

8. Fatemi SH, Folsom TD, Thuras PD. Deficits in $GABA_B$ receptor system in schizophrenia and mood disorders: A postmortem study. *Schizophrenia Research*. 2011;128(1-3):37-43.

9. Marshall FH, White J, Main M, Green A, Wise A. $GABA_B$ receptors function as heterodimers. *Biochemical Society Transactions*. 1999;27(4):530-535.

10. Bowery NG, Enna SJ. γ-aminobutyric acid-B receptors: first of the functional metabotropic heterodimers. *Journal of Pharmacology and Experimental Therapeutics*. 2000;292(1):2-7.

11. Margeta-Mitrovic M, Jan YN, Jan LY. A trafficking checkpoint controls $GABA_B$ receptor heterodimerization. *Neuron*. 2000;27(1):97-106.

12. Hannan S, Wilkins ME, Dehghani-Tafti E, Thomas P, Baddeley SM, Smart TG. γ-aminobutyric acid type B ($GABA_B$) receptor internalization is regulated by the R2 subunit. *Journal of Biological Chemistry*. 2011;286(27):24324-24335.

13. D'Onofrio G, Riva A, Di Rosa G, et al. Paroxysmal limb dystonias associated with *GABBR2* pathogenic variant: A case-based literature review. *Brain Dev*. 2022;44(7):469-473.

14. McRae JF, Clayton S, Fitzgerald TW, et al. Prevalence and architecture of *de novo* mutations in developmental disorders. *Nature*. 2017;542(7642):433-438.

15. Samanta D, Zarate Y. Widening phenotypic spectrum of *GABBR2* mutation. *Acta Neurolica Belgica*. 2019;119:493-496.

16. Kim SY, Jang SS, Kim H, et al. Genetic diagnosis of infantile-onset epilepsy in the clinic: Application of whole-exome sequencing following epilepsy gene panel testing. *Clinical Genetics*. 2021;99(3):418-424.

17. Hamdan FF, Myers CT, Cossette P, et al. High Rate of Recurrent *De Novo* Mutations in Developmental and Epileptic Encephalopathies. *The American Journal of Human Genetics*. 2017;101(5):664-685.

18. Appenzeller S, Balling R, Barisic N, et al. *De Novo* Mutations in Synaptic Transmission Genes Including DNM1 Cause Epileptic Encephalopathies. *The American Journal of Human Genetics*. 2014;95(4):360-370.

19. Bielopolski N, Stawarski M, Roitman I, et al. Characterization of a *de novo GABBR2* variant linked to autism spectrum disorder. *Frontiers in Molecular Neuroscience*. 2023;16.

20. Yoo Y, Jung J, Lee Y, et al. GABBR2 mutations determine phenotype in Rett syndrome and epileptic encephalopathy. *Annals of Neurology*. 2017;82(3):466-478.

21. Lopes F, Barbosa M, Ameur A, et al. Identification of novel genetic causes of Rett syndrome-like phenotypes. *Journal of Medical Genetics*. 2016;53(3):190.

22. Vuillaume M, Jeanne M, Xue L, et al. A novel mutation in the transmembrane 6 domain of *GABBR2* leads to a Rett-like phenotype. *Annals of Neurology*. 2018;83(2):437-439.

23. Carneiro TNR, Krepischi ACV, Costa SS, et al. Utility of trio-based exome sequencing in the elucidation of the genetic basis of isolated syndromic intellectual disability: Illustrative cases. *Application of Clinical Genetics*. 2018;11:93-98.

24. Takata A, Miyake N, Tsurusaki Y, et al. Integrative Analyses *of De Novo* Mutations Provide Deeper Biological Insights into Autism Spectrum Disorder. *Cell Reports*. 2018;22(3):734-747.

25. Hannan S, Gerrow K, Triller A, Smart TG. Phospho-dependent Accumulation of $GABA_B$Rs at Presynaptic Terminals after NMDAR Activation. *Cell Reports*. 2016;16(7):1962-1973.

26. Hannan SB, Penzinger R, Mickute G, Smart TG. CGP7930 - An allosteric modulator of $GABA_B$Rs, $GABA_A$Rs and inwardly-rectifying potassium channels. *Neuropharmacology*. 2023;238:109644.

27. Hannan S, Wilkins ME, Thomas P, Smart TG. Tracking cell surface mobility of GPCRs using α-bungarotoxin-linked fluorophores. *Methods in Enzymology*. 2013;521(1557-7988 (Electronic)):109-129.

28. Hannan S, Au K, Smart TG. Inhibitory neurosteroid reverses the dendritic spine disorder caused by gain-of-function $GABA_A$R epilepsy variants. *bioRxiv*. [Preprint] 2021.12.08.471533. doi:10.1101/2021.12.08.471533

29. Keros S, McBain CJ. Arachidonic Acid Inhibits Transient Potassium Currents and Broadens Action Potentials during Electrographic Seizures in Hippocampal Pyramidal and Inhibitory Interneurons. *The Journal of Neuroscience*. 1997;17(10):3476.

30. Hannan S, Affandi AHB, Minere M, et al. Differential coassembly of α1-$GABA_A$Rs associated with epileptic encephalopathy. *Journal of Neuroscience*. 2020;40(29):5518-5530.

31. Rodriguez A, Ehlenberger DB, Dickstein DL, Hof PR, Wearne SL. Automated three-dimensional detection and shape classification of dendritic spines from fluorescence microscopy images. *PLoS One*. 2008;3(4):e(1997).

32. Shaye H, Ishchenko A, Lam JH, et al. Structural basis of the activation of a metabotropic GABA receptor. *Nature*. 2020;584(7820):298-303.

33. Park J, Fu Z, Frangaj A, et al. Structure of human $GABA_B$ receptor in an inactive state. *Nature*. 2020;584(7820):304-309.

34. Mao C, Shen C, Li C, et al. Cryo-EM structures of inactive and active $GABA_B$ receptor. *Cell Research*. 2020;30(7):564-573.

35. Papasergi-Scott MM, Robertson MJ, Seven AB, Panova O, Mathiesen JM, Skiniotis G. Structures of metabotropic $GABA_B$ receptor. *Nature*. 2020;584(7820):310-314.

36. Webb B, Sali A. Comparative Protein Structure Modeling Using MODELLER. *Current Protocols in Bioinformatics*. 2016;54(1):5.6.1-5.6.37.

37. Studer G, Biasini M, Schwede T. Assessing the local structural quality of transmembrane protein models using statistical potentials (QMEANBrane). *Bioinformatics*. 2014;30(17):i505-i511.

38. Krivov GG, Shapovalov M v, Dunbrack RL. Improved prediction of protein side-chain conformations with SCWRL4. *Proteins: Structure, Function, and Bioinformatics*. 2009;77(4):778-795.

39. Chen VB, Arendall III WB, Headd JJ, et al. MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallographica Section D*. 2010;66(1):12-21.

40. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *Journal of Computational Chemistry*. 2004;25(0192-8651 (Print)):1605-1612.

41. Yang D, Zhou Q, Labroska V, et al. G protein-coupled receptors: structure- and function-based drug discovery. *Signal Transduction and Targeted Therapy*. 2021;6(1):7.

42. Rosenbaum DM, Rasmussen SGF, Kobilka BK. The structure and function of G-protein-coupled receptors. *Nature*. 2009;459(7245):356-363.

43. Shen C, Mao C, Xu C, et al. Structural basis of $GABA_B$ receptor–G_i protein coupling. *Nature*. 2021;594(7864):594-598.

44. Kim Y, Jeong E, Jeong JH, Kim Y, Cho Y. Structural Basis for Activation of the Heterodimeric GABAB Receptor. *Journal of Molecular Biology*. 2020;432(22):5966-5984.

45. Shaye H, Stauch B, Gati C, Cherezov V. Molecular mechanisms of metabotropic $GABA_B$ receptor function. *Science Advances*. 2022;7(22):eabg3362.

46. Niturad CE. *GABAergic Mechanisms in Epilepsy and Contribution of the ClC-2 Chloride Channel to Neuronal Excitability*.; Phd thesis. University of Tuebingen 2016. doi:10.15496/publikation-12927

47. Calver AR, Robbins MJ, Cosio C, et al. The C-Terminal Domains of the $GABA_B$ Receptor Subunits Mediate Intracellular Trafficking But Are Not Required for Receptor Signaling. *The Journal of Neuroscience*. 2001;21(4):1203-1210.

48. Restituito S, Couve A, Bawagan H, et al. Multiple motifs regulate the trafficking of $GABA_B$ receptors at distinct checkpoints within the secretory pathway. *Molecular and Cellular Neuroscience*. 2005;28(4):747-756.

49. Nichols CG, Lopatin AN. Inward rectifier potassium channels. *Annual Review of Physiology*. 1997;59(1):171-191.

50. Hagiwara S, Miyazaki S, Rosenthal NP. Potassium current and the effect of cesium on this current during anomalous rectification of the egg cell membrane of a starfish. *Journal of General Physiology*. 1976;67(6):621-638.

51. Nimchinsky EA, Sabatini BL, Svoboda K. Structure and Function of Dendritic Spines. *Annual Review of Physiology*. 2002;64(1):313-353.

52. Delaney AJ, Crane JW, Holmes NM, Fam J, Westbrook RF. Baclofen acts in the central amygdala to reduce synaptic transmission and impair context fear conditioning. *Scientific Reports*. 2018;8(1).

53. Fawley JA, Peters JH, Andresen MC. $GABA_B$-mediated inhibition of multiple modes of glutamate release in the nucleus of the solitary tract. *Journal of Neurophysiology*. 2011;106(4):1833-1840.

54. Guetg N, Seddik R, Vigot R, et al. The $GABA_{B1a}$ Isoform Mediates Heterosynaptic Depression at Hippocampal Mossy Fiber Synapses. *The Journal of Neuroscience*. 2009;29(5):1414-1423.

55. Bassetti D, Luhmann HJ, Kirischuk S. Presynaptic $GABA_B$ receptor–mediated network excitation in the medial prefrontal cortex of Tsc2+/- mice. *Pflugers Arch*. 2021;473(8):1261-1271.

56. Hansen W, Sandipan P, Lucas PM, Doering LC. Targeted pharmacological treatment of autism spectrum disorders: Fragile X and Rett syndromes. *Frontiers in Cellular Neuroscience*. 2015;9(FEB).

57. Huang Q, Pereira AC, Velthuis H, et al. GABAB receptor modulation of visual sensory processing in adults with and without autism spectrum disorder. *Science Translational Medicine*. 2023;14(626):eabg7859.

58. Zhu F, Shan W, Lv R, Li Z, Wang Q. Clinical Characteristics of Anti-$GABA_B$ Receptor Encephalitis. *Frontiers in Neurolology*. 2020;11,403.

59. Lucariello M, Vidal E, Vidal S, et al. Whole exome sequencing of Rett syndrome-like patients reveals the mutational diversity of the clinical phenotype. *Human Genetics*. 2016;135(12):1343-1354.

60. Chin EM, Cohen JS, Harris J. Vigabatrin as a Targeted Treatment of $GABA_B$ Receptor-Related Epileptic Encephalopathy. *Pediatric Neurology*. 2019;99:82-84.

61. Liu L, Fan Z, Rovira X, et al. Allosteric ligands control the activation of a class C GPCR heterodimer by acting at the transmembrane interface. *Elife*. 2021;10(e70188):1-22.

62. Xue L, Sun Q, Zhao H, et al. Rearrangement of the transmembrane domain interfaces associated with the activation of a GPCR hetero-oligomer. *Nature Communications*. 2019;10(1):2765.

63. Gassmann M, Shaban H, Vigot R, et al. Redistribution of $GABA_{B1}$ protein and atypical $GABA_B$ responses in $GABA_{B2}$-deficient mice. *The Journal of Neuroscience*. 2004;24(1529-2401):6086-6097.

64. Badran S, Schmutz M, Olpe HR. Comparative in vivo and in vitro studies with the potent GABAB receptor antagonist, CGP56999A. *European Journal of Pharmacology*. 1997;333(2):135-142

65. Mareš P, Bernášková K, Kubová H, Mareš P. An Antagonist of $GABA_B$ Receptors Potentiates Activity of Cortical Epileptic Foci. *Physiological Research*. 2012;61:325-329.

66. Zapata J, Moretto E, Hannan S, et al. Epilepsy and intellectual disability linked protein Shrm4 interaction with $GABA_B$Rs shapes inhibitory neurotransmission. *Nature Communications*. 2017;8:14536.

67. Zemoura K, Trümpler C, Benke D. Lys-63-linked ubiquitination of γ-aminobutyric acid (GABA), type B1, at multiple sites by the E3 ligase mind bomb-2 targets $GABA_B$ receptors to lysosomal degradation. *Journal of Biological Chemistry*. 2016;291(41):21682-21693.

68. Zemoura K, Schenkel M, Acuña MA, Yévenes GE, Zeilhofer HU, Benke D. Endoplasmic reticulum-associated degradation controls cell surface expression of γ-aminobutyric acid, type B receptors. *Journal of Biological Chemistry*. 2013;288(48):34897-34905.

69. Comps-Agrar L, Kniazeff J, Norskov-Lauritsen L, et al. The oligomeric state sets $GABA_B$ receptor signalling efficacy. *EMBO J*. 2011;30(12):2336-2349.

70. Takahashi M, Kovalchuk Y, Attwell D. Pre- and postsynaptic determinants of EPSC waveform at cerebellar climbing fiber and parallel fiber to Purkinje cell synapses. *The Journal of Neuroscience*. 1995;15(8):5693.

71. Kanigowski D, Bogaj K, Barth AL, Urban-Ciecko J. Somatostatin-expressing interneurons modulate neocortical network through $GABA_B$ receptors in a synapse-specific manner. *Scientific Reports*. 2023;13(1):8780.

72. Magloire V, Mercier MS, Kullmann DM, Pavlov I. GABAergic Interneurons in Seizures: Investigating Causality With Optogenetics. *The Neuroscientist*. 2018;25(4):344-358.

73. Bowery NG. Historical Perspective and Emergence of the $GABA_B$ Receptor. In: Blackburn TP, ed. *Advances in Pharmacology*. Vol 58. Academic Press; 2010:1-18.

74. Guery S, Floersheim P, Kaupmann K, Froestl W. Syntheses and optimization of new GS39783 analogues as positive allosteric modulators of $GABA_B$ receptors. *Bioorganic & Medicinal Chemistry Letters*. 2007;17(22):6206-6211.

75. Brown JW, Moeller A, Schmidt M, et al. Anticonvulsant effects of structurally diverse GABAB positive allosteric modulators in the DBA/2J audiogenic seizure test: Comparison to baclofen and utility as a pharmacodynamic screening model. *Neuropharmacology*. 2016;101:358-369.

Figure legends

Figure 1 – GABA$_B$R2 variants align at the heterodimer interface of the activated receptor

(A), Primary amino acid sequences of GABA$_B$R2 showing complete (*) or high (:) conservation of G693, S695 and I705 between species and with the sequences for murine GABA$_B$R1 and R2. ***(B)***, Cryo-EM structures of inactive (PDB:7C7S) and active (7C7Q) GABA$_B$R heterodimers, depicting transmembrane domain (TMD) α-helix 6 (M6) with locations for G693W, S695I and I705N (orange; wild-type residues depicted). Note, that in the inactive structure, GABA$_B$R2 M6 is positioned outside the heterodimer interface (left inserts), but due to clockwise rotations of TMDs during activation (black arrows in the active structure), M6 from R1 and R2 form the interface of the active heterodimer (right inserts). The GABA$_B$R PAM BHFF binds to the inter-subunit interface close to G693 and S695. ***(C, D, E)***, Structural models for G693W (*C*; cyan side-chains), S695I (*D*) and I705N (*E*), based on the active structure, 7C7Q. TMD interface representations (top panel) highlighting side-chain position of the three mutations (cyan, with elements N blue, O red). The mutation models aligned to wild-type 7C7Q structure (lower panel) with selected residues of interest, and BHFF (yellow) shown. The models predict varying degrees of side-chain and α-helical shifting (red arrows) due to the mutations. H-bonds are shown as stippled lines (wild-type: grey; epilepsy mutants: pink). Predicted clashes between mutant R2 M694 (with sulphur atom shown in yellow) and BHFF are shown as red stippled rings.

Figure 2 – Reduced maximal currents of $GABA_BR$ variants expressed in GIRK cells

*(**A**),* GABA-activated currents (*I_{GABA}*) of wild-type and variant $GABA_BR2$ in GIRK cells expressed with $GABA_BR1$. Note the negligible current amplitude for S695I. *(**B**),* Concentration response relationships, normalised (norm.) to the maximal (max) GABA currents (= 100%) and EC_{50}s of wild-type and variant receptors. Normalised maximal currents (%) - Wild-type: 100 (n = 12); G693W: 57 ± 9.3 (11); 695I: 4 ± 2.7 (17); I705N: 54.5 ± 8.5 (12). EC_{50}s (μM) – Wild-type: 0.36 ± 0.1 (12); G693W: 0.04 ± 0.02 (8); I705N: 0.14 ± 0.04 (11). *(**C**)*, Western blot of immunoprecipitated *myc*-$GABA_BR1$ from HEK-293 cells transiently expressing $GABA_BR1^{myc}$ and wild-type or mutant $GABA_BR2^{flag}$ (WB – Western blot). Immunoprecipitated samples (top) and input – 10 % of the cell lysate with corresponding expressing receptors (bottom) were firstly probed for FLAG-tag with 1:1000 mouse anti-FLAG-tag antibody; the same membranes were stripped and re-probed with anti-$GABA_BR1$ antibody. The numbers on the right of each blot are molecular weights (kDa). *(**D**)*, Bar chart represents normalised band intensity of $GABA_BR2$ (R2) to $GABA_BR1$ (R1): R1/R2 1.00 ± 0.00 (n = 6), R1/$R2^{S695I}$ 0.79 ± 0.17 (n = 6), R1/R2 IgG control 0.04 ± 0.02 (n = 4), R1 only 0.01 ± 0.004 (n = 6), mock transfected 0.00 ± 0.00 (n = 6). ns – not significant, one-way ANOVA with Tukey-Kramer test. (***E***), Example two-electrode voltage clamp recordings for 1 mM (wild-type) and 10 mM (S695I) GABA-activated currents for R1R2 wild-type and R1$R2^{S695I}$ expressing oocytes. The bar chart shows mean ± SEM maximum GABA-activated currents (n = 6-8). **P<0.001, one-way ANOVA with Tukey-Kramer test.

Figure 3 – Impaired cell surface expression for R2 variants in HEK-293 cells

(A), Cytofluorograms of cell surface staining for $GABA_BR1a$ with wild-type and variant $GABA_BR2$ (ordinate) in GFP (abscissa) expressing HEK-293 cells. Just GFP-expressing cells and untransfected cells are also shown. Flag-tagged $GABA_BR2$ was labelled with an anti-flag antibody followed by an Alexa Fluor 647 secondary antibody (pictogram). Numbers in the corners of the fluorograms are the percentage of cells in each quadrant. ***(B),*** Normalised (to R1R2 wild-type = 100%; left ordinate) and raw (right ordinate) cell surface fluorescence for the cells shown in A (upper panel). Lower panel plots % fluorescing cells (normalised (left ordinate) to R1R2 = 100%, and % of total cells, right ordinate) in Quadrant 2 (Q2) for the cells shown in A (see key). ***(C),*** Cytofluorograms of total (cell surface + intracellular) R1a with wild-type and mutant $GABA_BR2$ staining in HEK-293 cells. $GABA_BR2$ was labelled after permeabilisation (pictogram). ***(D),*** As for the bargraphs shown in B, normalised and raw fluorescence and the number of cells (%) in Q2 for wild-type, variant $GABA_BR2$ in permeabilised cells, and just GFP-expressing or untransfected permeabilised cells are shown. *$P<0.05$, KW one-way ANOVA with Dunn test.

Figure 4 - Impaired cell surface expression and signalling for mutant $GABA_BRs$ in hippocampal neurons

(A), Confocal images of cell surface $GABA_BR2$ labelling in neurons co-transfected with eGFP, $GABA_BR1$ and wild-type or variant $GABA_BR2$ (inset) to drive over-expression. ***(B),*** Normalised (to R1R2 wild-type = 100%, left ordinate) and raw (right ordinate) cell surface fluorescence intensities of wild-type or variant R1R2 or just GFP-expressing cells from *(A)*. ***(C),*** Representative K^+ currents in response to 10 and 100 μM baclofen recorded from

hippocampal neurons expressing eGFP with or without wild-type or variant R2 (pictogram) or untransfected cells. ***(D)***, Mean baclofen-activated K^+ current density of neurons expressing wild-type or mutant $GABA_BR2$. *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA with Tukey-Kramer test or nonparametric ANOVA with Dunn's multiple comparison test.

Figure 5 – Excitatory neurotransmission and $GABA_BR2$ variants

(A), Representative mEPSCs recorded from hippocampal neurons that are untransfected or expressing eGFP alone or with wild-type or S695I $GABA_BR2$ (inset). ***(B),*** Average frequency and amplitude of mEPSCs for cells treated as in *(A)*. ***(C),*** Average mEPSC waveforms and mean charge transfer, rise time and T_{50} of kinetics for mEPSCs recorded from neurons in *(A)*. ***(D),*** Representative images of neuronal dendrites expressing eGFP alone or in combination with wild-type or S695I (pictogram) showing spines. ***(E)***, Density, head size and proportion of mushroom (mushrm.), stubby and thin spines in hippocampal neurons treated as shown in *(D)*. ***(F),*** Representative spontaneous action potentials recorded by whole-cell current clamp from untransfected (UTF) and wild-type- or S695I- expressing $GABA_BR2$. ***(G),*** Average resting membrane potential and spiking rate of hippocampal neurons. ***(H),*** Average action potential waveforms, peak, rise time, T_{50} and charge transfer of spikes. *P<0.05, One-way ANOVA with Tukey test or nonparametric KW ANOVA with Dunn's multiple comparison test. Scale bar = 5 μm.

Figure 6 – Elevated presynaptic Ca^{2+} transients due to $GABA_BR$ variants

(A), Representative images of presynaptic Ca^{2+} signals in neurons expressing synaptophysin-GCaMP6*f* alone (left panel) or with wild-type (middle) or S695I $GABA_BR2$ (right). ***(B),*** Example recordings of Ca^{2+} transients from presynaptic terminals in *(A)*. ***(C),*** Median

presynaptic $\Delta F/F_0$, frequency of Ca^{2+} transients and area under the curve for fluorescence change in a 15 s epoch per presynaptic terminal in wild-type and S695I $GABA_BR2$ expressing nerve endings. ***P<0.001, nonparametric KW ANOVA with Dunn's multiple comparisons test. Scale bars = 5 μm.

Figure 7 – Pharmacological characterisation of $GABA_BR$ PAMs

(A), Whole-cell GIRK currents activated by ~EC_{20} baclofen showing potentiation by GS39783 and rac-BHFF in $GABA_BR1R2$-transfected GIRK cells. ***(B),*** PAM potentiation curves for GS39783 and rac-BHFF normalised (%) to the maximal baclofen responses from the same cell (= 100%). ***(C),*** Bar graph comparing maximal potentiation by GS39783 (30 μM) and rac-BHFF (30 μM). (***D***)*,* Whole-cell GIRK currents activated by ~EC_{20} baclofen showing potentiation by GS39783 and rac-BHFF in hippocampal neurons in culture at 14-21 days *in vitro*. ***(E),*** Potentiation curves for GS39783 and rac-BHFF normalised to maximal baclofen responses (= 100%) from the same neuron. ***(F),*** Bar graph comparing maximal potentiation of GS39783 and rac-BHFF. ***(G),*** Example recordings of ~EC_{10} baclofen prior to, during and 5 or 10 min after 3 μM GS39783 application in hippocampal neurons. Bar chart showing increased ~EC_{10} 10 min after the cessation of GS39783 application. ***(H),*** Example recordings of baclofen-activated currents in hippocampal neurons before and 15 min after application of 3 μM GS39783. Note the left-shifted concentration response curve and lower EC_{50} after 15 min wash (inset). ***(I),*** Protocol and example recordings showing ~EC_{10} and maximum baclofen currents measured before, during and up to 30 min after the application of 3 μM GS39783 along with an % EC_{10} decay curve (right panel) for baclofen after GS39783 application. Dotted line shows the initial ~EC_{10} value prior to GS39783 application. n = 6-12, **P<0.01, ***P<0.001, two-tailed

unpaired t-test, Mann-Whitney test or repeated measures ANOVA with Tukey-Kramer multiple comparisons test *(G)*.

Figure 8 – Reversal of presynaptic increased activity with a GABA$_B$R PAM

***(A)**,* Images of presynaptic Ca^{2+} signals in neurons expressing synaptophysin-GCaMP6*f* with wild-type or S695I GABA$_B$R2 in the presence of a vehicle control or 1 μM rac-BHFF. For fluorescing termini, the intensity ($\Delta F/F_0$) of the Ca^{2+} transients were reduced by rac-BHFF for both termini expressing wild-type or variant R2. ***(B)**,* Example Ca^{2+} transient recordings from the presynaptic terminals in *(A)*. ***(C)**,* Median presynaptic $\Delta F/F_0$, Ca^{2+} transient frequency and area for the Ca^{2+} signals recorded in 15 s epochs per presynaptic terminal in wild-type and S695I GABA$_B$R2 expressing nerve endings in vehicle or rac-BHFF. *P<0.05, **P<0.01, ***P<0.001, NS – not significant, non-parametric KW ANOVA with Dunn's multiple comparisons test. Scale bars = 2 μm.

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