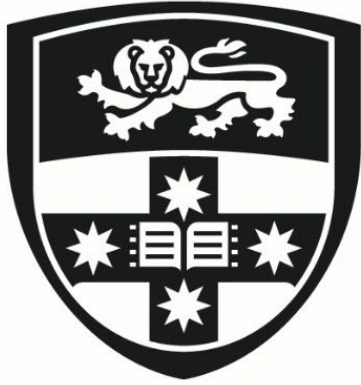




THE UNIVERSITY OF
SYDNEY

‘Relearning’ epilepsy – Functional and
phenotypic associations of *GABRG2*
and *GABRB3* variants

Susan Lin

Faculty of Medicine and Health

The University of Sydney

2024

A thesis submitted to fulfil requirements for the degree of

Doctor of Philosophy

This research reported in this thesis was supported by the award of a Research Training Program scholarship to the PhD Candidate

Statement of originality

I, Susan Lin, certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Susan Lin, Student

Date

Acknowledgement

First and foremost, I would like to express my sincere gratitude to my supervisor, Professor Mary Collins (Chebib). Thank you for your patience, generosity, and guidance throughout this PhD journey. Your passion for science and dedication to making a change is truly inspiring. Thank you for always making time for me despite your busy schedule and supporting my endeavours. I would not be here today without your continued support since my honours year.

I extend my sincere appreciation to my co-supervisors, Dr Nathan Absalom, and Dr Vivian Liao. Nathan, thank you for your enthusiasm and expertise in GABA_A receptors. Your unwavering support, insightful advice and comments throughout my PhD have been invaluable. I still cannot believe you printed off my thesis to read and hand-write comments; your dedication to teaching and patience is admirable. Vivian, you're like an older sister that I never had, I am so grateful for the continuous encouragement for me to step out of my comfort zone, reach higher, go to that conference, submit that application and "go for it". Your knowledge of the world, science, and movies is truly amazing, and your support throughout the years has been indispensable.

A special thanks to Associate Professor Ahring for your valuable guidance in electrophysiology and patient-data interpretation. Your hard work and vision are truly inspiring; thank you for sharing that with me. A heartfelt thanks to Dr Han Chow Chua, Chow, thank you for your support, guidance, and invaluable feedback on life, for always willing to help even when you're super busy with being a coordinator, and for bringing in a vibrant energy to the office.

To ALL my supervisors, thank you for your immense support throughout the years. Thank you for all your insightful comments during the writing of this thesis. I cannot express my gratitude enough. I am honoured to work with leaders in the field of GABA_AR and epilepsy.

I want to acknowledge the clinical team at The Danish Epilepsy Centre, Filadelfia, including Professor Rikke Møller, Dr Guido Rubboli, Dr Elena Gardella, Dr Alessandra Rossi, Dr Sebastian Ortiz and others. Thank you for your assistance in collecting and interpreting patient data. Your expertise and passion for improving patient care are profoundly motivating and inspiring.

Many thanks to Professor Christopher Reid, Dr Ming Shiuan Soh, Chasely Mckenzie, and James Spyrou, at The Florey Institute for your work with the knock-in mouse model and the invaluable advice and guidance with animal work.

Thank you to Professor Kevin Keay and Dr James Kang for your expertise, guidance, and support in immunohistochemistry and the use of equipment and lab space.

To my friends, peers, and colleagues: Sandy Wang, Nian Kee Tan, Wenkai Liu, Christine Shu, Janet Nguyen, Victor Lee, Zheng (Jake) Chen, Anthony (Sze Hon) Kan, Waiting Tai, Michael Kuligowski, Ragdha. Thank you for all the laughter, support, and empathy throughout the years.

To my wonderful partner, thank you for your humour, patience, love, and support. I am deeply grateful for everything you have done and continue to do. Thank you for being by my side through it all.

Finally, I express my deepest appreciation to my family, whom I am nothing without. To my mum and dad, you have sacrificed so much and been through too much, and I hope I can repay you one day so you can enjoy life and see the world. To my sisters, Lisa and Holly, thank you for dealing with my shenanigans, the late-night banters and endless love. Thank you all for believing in me when I didn't believe in myself, for the unwavering love and support.

Authorship Attribution Statement

Chapter 3 of this thesis is submitted as:

Rossi A, **Lin SXN (equal fist)**, Absalom NL, Liao VWY,..., Chebib M, Gardella E, Møller RS, Rubboli G, Ahring PK. Phenotypic spectrum in individuals with pathogenic *GABRG2* loss- and gain-of-function variants. *Neurology*. Under review 02 July 2024

I contributed to the design, and conception of the study, data acquisition, analysis, interpretation, drafting and editing of the manuscript.

Chapter 6 of this thesis is published as:

Lin SXN, Ahring PK, Keramidas A, Liao VWY, Møller RS, Chebib M, Absalom NL. (2024) Correlations of receptor desensitization of gain-of-function *GABRB3* variants with clinical severity, *Brain*, **147** (1): 224–239, <https://doi.org/10.1093/brain/awad285>

I contributed to the design, and conception of the study, data acquisition, analysis, interpretation, drafting and editing of the manuscript.

Other publications during candidature

Ahring PK, Liao VWY, **Lin S**, Absalom NL, Chebib M, Møller RS. (2022) The *de novo* *GABRA4* p.Thr300Ile variant found in a patient with early-onset intractable epilepsy and neurodevelopmental abnormalities displays gain-of-function traits. *Epilepsia*, **63**(9):2439-2441. doi: 10.1111/epi.17358.

I, alongside Nathan Absalom, Vivian VWY Liao contributed to the data acquisition. All authors edited and revised the manuscript.

Absalom NL, **Lin SXN**, Liao VWY, Chua HC, Møller RS, Chebib M, & Ahring PK (2023). GABA_A receptors in epilepsy: Elucidating phenotypic divergence through functional analysis of genetic variants. *J Neurochem*. <https://doi.org/10.1111/jnc.15932>

I, alongside Nathan Absalom, Rikke Møller, Han Chow Chua and Philip K. Ahring wrote and edited the manuscript. Han Chow Chua prepared and created the figures. All authors revised the manuscript.

Kan ASH, Mohammadi N, **Lin SXN**, Liao VWY, Lesca G, Souci S, Milh M, Christophersen P, Chebib M, Møller RS, Absalom NL, Jensen AA, Ahring PK. Paralogous epilepsy-associated GABA_A receptor variants as indicators for clinical outcomes. 2024 *PNAS under review*

I contributed to the data acquisition, and analysis of the manuscript.

Kan ASH, Kusay AS, **Lin SXN**, Chua HC, Chebib M, Ahring PK, Liao VWY. α +/ γ - interface is essential for zolpidem modulation in GABA_A receptor. *In preparation*

I contributed to the data acquisition, and analysis of the manuscript.

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Susan Lin, Student

Date

As a supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Mary Collins (Chebib), Supervisor

Date

Nathan Absalom, Co-supervisor

Date

Vivian Liao, Co-Supervisor

Date

Table of Contents

STATEMENT OF ORIGINALITY	II
ACKNOWLEDGEMENT	III
AUTHORSHIP ATTRIBUTION STATEMENT	V
TABLE OF CONTENTS	VII
LIST OF FIGURES	XI
LIST OF TABLES	XII
LIST OF ABBREVIATIONS	XIII
CONFERENCES	XV
SCHOLARSHIPS/PRIZES	XVII
ABSTRACT	XVIII
CHAPTER 1 INTRODUCTION	1
1.1 EPILEPSY: PREVALENCE, COSTS, AND CAUSES	1
1.2 GABA AND ITS RECEPTORS	2
1.3 GABA _A RECEPTOR: ASSEMBLY, STRUCTURE AND SUBTYPES	2
1.3.1 CELLULAR AND NETWORK ELECTRICAL ACTIVITY REGULATION BY GABA _A RECEPTORS	3
1.3.2 GABA BINDING AND GABA _A RECEPTOR ACTIVATION	5
1.3.3 GABA _A RECEPTOR AND EPILEPSY: VARIANT-INDUCED CHANGES TO BINDING AND GATING	9
1.3.4 FUNCTIONAL GABA _A RECEPTOR REGIONS AND VARIANTS	10
1.4 SPECTRUM OF EPILEPTIC SYNDROMES	11
1.4.1 WHAT IS EPILEPSY – WHAT ARE THE SPECTRUMS?	11
1.4.2 FEBRILE SEIZURES (FS) AND FEBRILE SEIZURES PLUS (FS+)	12
1.4.3 GENETIC EPILEPSY WITH FEBRILE SEIZURES PLUS (GEFS+)	12
1.4.4 DRAVET SYNDROME (DS)	13
1.5 DEVELOPMENTAL AND EPILEPTIC ENCEPHALOPATHIES (DEEs)	14
1.5.1 RARE AND REFRACTIVE	14
1.5.2 CAUSES OF DEEs	14
1.6 CURRENT TREATMENT OPTION FOR EPILEPSY	14
1.6.1 TREATMENT HYPERSENSITIVITY	17
1.7 CHALLENGING THE EXCITATION AND INHIBITION PARADIGM - GENETIC VARIANTS AND THEIR IMPACT. 17	
1.8 FROM BEDSIDE TO BENCH - CLASSIFYING THE EFFECTS OF VARIANTS	18
1.8.1 GENOME AGGREGATION DATABASE (GNOMAD)	18
1.8.2 AMERICAN COLLEGE OF MEDICAL GENETICS AND GENOMICS - ACMG CRITERIA	19

1.9 RE-EVALUATING EPILEPSY PATHOPHYSIOLOGY	19
1.10 AIMS.....	20
 CHAPTER 2 MATERIALS AND METHODS.....	 22
 2.1 MATERIALS AND INSTRUMENTATION	 22
2.1.1 CHEMICALS AND REAGENTS FOR MOLECULAR BIOLOGY AND ELECTROPHYSIOLOGY	22
2.1.2 KITS USED FOR MOLECULAR BIOLOGY	23
2.1.3 ENZYMES, ENZYME BUFFER, BACTERIA, SELECTION ANTIBIOTIC USED FOR MOLECULAR BIOLOGY	23
2.1.4 CHEMICALS AND REAGENTS FOR IMMUNOHISTOCHEMISTRY	24
2.1.5 ANTIGENS FOR IMMUNOHISTOCHEMISTRY	24
2.1.6 EQUIPMENT	25
2.1.7 BUFFER RECIPES	26
2.2 SAFETY AND ETHICS	28
2.3 MOLECULAR BIOLOGY	28
2.3.1 SUMMARY OF CONCATENATED RECEPTORS	28
2.3.2 RESTRICTION DIGESTION	29
2.3.3 LIGATION.....	30
2.3.4 TRANSFORMATION	30
2.4.5 COLONY SELECTION AND GROWTH.....	31
2.4.6 PLASMID PURIFICATION	31
2.4.7 DIAGNOSTIC RESTRICTION DIGESTION	32
2.4.8 VERIFICATION OF PLASMID DNA	32
2.4.9 PLASMID DNA LINEARISATION AND PURIFICATION	33
2.4.10 RNA SYNTHESIS AND RETRIEVAL	34
2.4 ELECTROPHYSIOLOGY	34
2.4.1 <i>XENOPUS LAEVIS</i> OOCYTE PREPARATION	35
2.4.2 TWO-ELECTRODE VOLTAGE CLAMP ELECTROPHYSIOLOGY.....	36
2.4.3 <i>IN VITRO</i> FUNCTIONAL ASSAY	37
2.5 ELECTROPHYSIOLOGY - STATISTICAL ANALYSIS	42
2.5.1 GABA SENSITIVITY - CONCENTRATION-RESPONSE- RELATIONSHIPS	42
2.5.2 MAXIMAL CURRENT	43
2.5.3 DESENSITISATION	43
2.6 CLINICAL DATA	43
2.6.1 RETRIEVING PATIENT DATA	43
2.6.2 PHENOTYPIC ANALYSIS	44
2.6.3 HANDLING INCOMPLETE DATA	44
2.6.4 STATISTICAL ANALYSIS	44
2.7 ASSESSMENT OF <i>IN SILICO</i> TOOLS	44
2.8 ANIMAL EXPERIMENTS AND IMMUNOHISTOCHEMISTRY	45
2.8.1 TISSUE PREPARATION	45
2.8.2 NISSL STAINING AND ANALYSIS	46
2.8.3 FLUORO-JADE C	46
2.8.4 IBA-1 STAINING AND ANALYSIS	47
2.8.5 IMMUNOHISTOCHEMISTRY FLUORESCENCE	47
2.8 FLUORESCENCE IMAGING	48
2.9 ANIMAL - STATISTICAL ANALYSIS	48

CHAPTER 3 PHENOTYPIC SPECTRUM IN INDIVIDUALS WITH PATHOGENIC *GABRG2* LOSS- AND GAIN-OF-FUNCTION VARIANTS..... 49

SUMMARY 49

CHAPTER 4 GNOMAD AND *IN SILICO* PREDICTION TOOLS..... 55

INTRODUCTION 55

RESULTS.....60

LOCATION OF THE VARIANTS AND SEQUENCE CONSERVATION 60

PRELIMINARY *IN SILICO* TOOL PREDICTIONS 61

PRELIMINARY *IN SILICO* TOOL PREDICTION USING MACHINE-LEARNING BASED TOOLS 62

BIOPHYSICAL PROPERTIES OF GNOMAD AND PATIENT *GABRG2* VARIANTS 64

IN SILICO TOOLS VS *IN VITRO* FUNCTIONAL TESTING 66

IN SILICO TOOL TO REPLACE FUNCTIONAL TESTING 68

OCCURRENCE IN PATIENTS – PATIENT PHENOTYPE 68

GNOMAD FREQUENCY AS A PREDICTOR OF VARIANT PATHOGENICITY..... 70

DISCUSSION.....71

CHAPTER 5 GENOTYPE-PHENOTYPE CHARACTERISATION OF *GABRG2* VARIANT 75

INTRODUCTION 75

RESULTS.....76

ACMG CRITERIA PRIOR TO FUNCTIONAL ASSESSMENT 76

OVERVIEW OF *GABRG2* VARIANTS ON GABA_A RECEPTOR ACTIVITY 77

CATEGORISATION OF VARIANTS BASED ON FUNCTIONAL STUDIES..... 79

ACMG CRITERIA POST FUNCTIONAL ASSESSMENT 80

STRUCTURAL AND 2D MAPPING OF THE VARIANTS 80

SUB-CATEGORISATION OF VARIANT’S EFFECT AND LOCATION AND CLINICAL PHENOTYPE 81

COMPARISON OF CLINICAL PHENOTYPE BETWEEN GoF AND LoF COHORT 95

DISCUSSION..... 100

CHAPTER 6 THE EFFECTS OF RECEPTOR DESENSITISATION ON GOF *GABRB3* VARIANTS 104

SUMMARY 104

CHAPTER 7 E77K MOUSE MODEL 107

INTRODUCTION 107

RESULTS..... 111

NO SIGNIFICANT GROSS MORPHOLOGICAL WERE OBSERVED BETWEEN WT AND B3^{+/E77K} MICE. 111

NO SIGNIFICANT DIFFERENCE WAS OBSERVED IN MOSSY FIBRE SPROUTING BETWEEN WT AND B3^{+/E77K} MICE
BRAINS. 112

INVESTIGATING POTENTIAL CHANGES IN MYELIN, MICROGLIA AND DEGENERATING NEURONS IN BRAIN OF E77K
GoF MOUSE MODEL..... 115

DISCUSSION.....	120
<u>CHAPTER 8 CONCLUSION AND FORWARD.....</u>	123
SCOPE OF THE STUDY	123
FINDINGS IN BRIEF.....	123
IMPLICATIONS TO THE FIELD OF GABA _A RECEPTORS AND EPILEPSY	124
PERSPECTIVE: GoF - A PROVOCATIVE FINDING	125
CONVERGENCE OF PHENOTYPE.....	126
POTENTIAL HYPOTHESES OF HOW GoF LEAD TO EPILEPSY	127
STRENGTHS.....	127
LIMITATIONS	128
CONCLUDING REMARKS.....	129
FUTURE DIRECTIONS	130
<u>CHAPTER 9 SUPPLEMENTARY INFORMATION</u>	131
TABLE 1: DEFAULT ACMG CLASSIFICATION.....	131
TABLE 2: VARIANT FUNCTIONAL RESULT AND CALL.....	133
TABLE 3: ACMG CLASSIFICATION AND CRITERIA POST FUNCTIONAL ASSAY FOR EACH INDIVIDUAL	135
TABLE 4: CLINICAL PHENOTYPES <i>GABRG2</i> COHORT	139
SUBMITTED MANUSCRIPT - PHENOTYPIC SPECTRUM IN INDIVIDUALS WITH PATHOGENIC <i>GABRG2</i> LOSS- AND GAIN-OF-FUNCTION VARIANTS	140
SUPPLEMENTARY INFORMATION FOR CORRELATIONS OF RECEPTOR DESENSITIZATION OF GAIN-OF- FUNCTION <i>GABRB3</i> VARIANTS WITH CLINICAL SEVERITY.....	141
<u>CHAPTER 10 REFERENCES</u>	142

List of Figures

Figure 1.1 3D representation of GABA _A receptor	3
Figure 1.2 Synaptic and extrasynaptic GABA _A receptors	5
Figure 1.3 Cartoon representation of Cys-loop receptors	6
Figure 1.4 Cryo-EM structure of GABA and picrotoxin-bound $\alpha 1\beta 3\gamma 2$ L receptor.	8
Figure 1.5 Mechanism of actions of some of the currently available ASMs.	16
Figure 2.1 Concatenated receptors	29
Figure 2.2 Summary of <i>E.coli</i> transformation.	31
Figure 2.3 Diagnostic restriction digest	32
Figure 2.4 Sanger sequencing result	33
Figure 2.5 Basic principle for two-electrode voltage clamp electrophysiology	35
Figure 2.6 Interbatch and interday variations in GABA sensitivity for WT receptors	36
Figure 3.1 Functional analysis of 15 $\alpha 1\beta 3\gamma 2$ GABA _A receptors containing $\gamma 2$ variants.	52
Figure 3.2 Comparison of clinical severity between null and missense variants LoF and GoF GABRG2 variants.....	54
Figure 4.1 2D structural mapping of the location of seven variant pairs in the GABRG2 gene.....	60
Figure 4.2 Representative concentration-response curve 7 pairs of positional missense variants.	65
Figure 4.3 Dot plot of changes in maximum current for 7 pairs of positional missense variants.	66
Figure 4.4 Venn diagram of variants categorised into 3 categories: gnomAD, patient, and gnomAD and patient.	71
Figure 5.1 Functional outcome for 71 tested GABRG2 variants.	78
Figure 5.2 2D mapping of GABRG2 variants on a lollipop graph.	80
Figure 5.3 ECD Variants effect on receptor function.	82
Figure 5.4 TM1-4 variants effect on receptor function.....	91
Figure 5.5 Age of seizure onset and inheritance patterns between LoF and GoF cohort.	96
Figure 5.6 Odds ratio of key clinical outcomes between LoF and GoF cohort.....	99
Figure 5.7 Epilepsy types.....	100
Figure 6.1 Linear regression analysis on seizure onset age versus ΔLogEC_{50} for GoF GABRG2 variants.	105
Figure 7.1 Comparison of WT and E77K mutant mice on birth outcomes and postnatal survival.....	110
Figure 7.2 Gross brain morphology comparisons between E77K mutant mouse and WT	112
Figure 7.3 Comparison of mossy fiber sprouting in WT and $\beta 3^{\text{+/E77K}}$ mutant mice	114
Figure 7.4 Comparison of MBP staining intensity in WT and E77K mutant mice brain.....	116
Figure 7.5 Staining for Iba-1, a marker for microglia.....	118
Figure 7.6 Fluoro-Jade C, a fluorescent stain for the detection of neuronal degeneration in WT and mutant (E77K) mouse brains, along with a positive control.....	119

List of Tables

Table 2.1 Recipe for LB broth.....	26
Table 2.2 Recipe for LB Agar	26
Table 2.3 Recipe for Modified Barth's solution	26
Table 2.4 Recipe for ND96.....	26
Table 2.5 Recipe for ND96 + gentamicin and ampicillin	27
Table 2.6 Recipe for phosphate-buffered saline (PBS).....	27
Table 2.7 Recipe for phosphate-buffer (PB)	27
Table 2.8 Restriction digest reaction mixture for insert	29
Table 2.9 Restriction digestion reaction mixture for vector.....	30
Table 2.10 Ligation reaction mixture	30
Table 2.11 Plasmid DNA linearisation reaction mix	33
Table 2.12 T7 mMessage mMachine® reaction mix	34
Table 2.13 LabChart macro for desensitisation experiment (Chapter 6)	39
Table 4.1 Summary of types of <i>in silico</i> tools	58
Table 4.2 Prediction results for sequence and structure based; and sequence homology-based <i>in silico</i> prediction tools	62
Table 4.3 Prediction of variant effect using machine-learning based <i>in silico</i> tools	63
Table 4.4 Sensitivity, specificity, and accuracy of sequence and structure-based <i>in silico</i> prediction tools	67
Table 4.5 Sensitivity, specificity and accuracy of 4 machine-learning based <i>in silico</i> prediction tools.....	68
Table 4.6 Sensitivity, specificity, and accuracy of <i>in silico</i> prediction tool in predicting functional changes in receptor sensitivity.	68
Table 4.7 Clinical phenotypes of variants found in patients	69
Table 5.1 Summary table of key functional and phenotypic characteristics of patients harbouring GABRG2 variants.....	83
Table 5.2 Clinical characteristics of individuals with missense LoF and GoF GABRG2 variants.	97

List of Abbreviations

ACMG	American College of Medical Genetics and Genomics
ADHD	Attention deficit hyperactivity disorder
ASD	Autism spectrum disorder
ASMs	Anti-seizure medications
CADD	Combined Annotation Dependent Depletion
CAE	Childhood absence epilepsy
CNS	Central nervous system
Cryo-EM	Cryogenic electron microscopy
DD	Developmental delay
DEE	Developmental and epileptic encephalopathy
ECD	Extracellular domain
EE	Epileptic encephalopathy
EEG	Electroencephalogram
EIDEE	Early infantile developmental and epileptic encephalopathy
EIMFS	Epilepsy of infancy with migrating focal seizure
EMAS	Epilepsy with myoclonic-atonic seizures
EOEE	Early onset epileptic encephalopathy
FATHMM-W	Functional Analysis through Hidden Markov Models weighted
FDA	Food and Drug Administration
FJC	Fluoro-Jade C
FN	False negative
FP	False positive
FS	Febrile seizure
FS+	Febrile seizure plus
GABA	γ -aminobutyric acid
GABA _A	γ -aminobutyric acid type A
GABA _B	γ -aminobutyric acid type B
GEFS+	Genetic epilepsy with febrile seizure plus
gnomAD	Genome Aggregation Database
GoF	Gain-of-function
ICD	Intracellular domain
ID	Intellectual disability
IGE	Idiopathic generalised epilepsy

ILAE	International League Against Epilepsy
IQR	Interquartile range
JAЕ	Juvenile absence epilepsy
LoF	Loss-of-function
MBP	Myelin basic protein
NDEEMA	Neurodevelopmental and epileptic encephalopathy with movement disorder and arthrogryposis
NFW	Nucleus free water
ngs	Normal goat serum
NGS	Next-generation sequencing
NHS	Normal horse serum
nsSNPs	non-synonymous single nucleotide polymorphism
OR	Odds ratio
PAMs	Positive allosteric modulators
PB	Phosphate buffer
PBS	Phosphate buffered saline
PolyPhen-2	Polymorphism Phenotyping V2
QoL	Quality of life
REVEL	Rare exome variant ensemble learner
SD	Standard deviation
SIFT	Sorting Intolerant from Tolerant
TEVC	Two-electrode voltage clamp
TMD	Transmembrane domain
TN	True negative
TP	True positive
VUS	Variant of uncertain significance
WT	Wild-type

Conferences

'A simple model of desensitization for analyzing epilepsy-associated GABA_A receptor variants' **(oral)**, Brain and Mind Centre Symposium, 5th annual symposium, 2021, Sydney, Australia

'Desensitizing properties of *GABRB3* variants exacerbate the severity of gain-of-function developmental and epileptic encephalopathies' **(poster)**, Society for Neuroscience, 2022, San Diego, US

'Inhibition: a double-edged sword in Developmental and Epileptic Encephalopathy' **(poster)**, APSA-ASCEPT 2022 Joint Conference, 2022, Perth, Australia

'Desensitisation properties of gain-of-function *GABRB3* variants influence the severity of DEE' **(oral)** APSA-ASCEPT 2022 Joint Conference, 2022. Perth, Australia

'Inhibition: a double-edged sword in Developmental and Epileptic Encephalopathy' **(poster)**, Inhibition of the CNS, Gordon Conference, 2023, Switzerland

'The power of one: Phenotypic spectrum in individuals with pathogenic *GABRG2* loss-and gain-of-function variants' **(oral)**, Brain and Mind Centre Symposium, 2023, Sydney, Australia

'The power of one: Phenotypic spectrum in individuals with pathogenic *GABRG2* loss-and gain-of-function variants' **(poster)**, Brain and Mind Centre Symposium, 2023, Sydney, Australia

'If a variant is predicted to be "benign/tolerated" by *in silico* tools and seen in gnomAD – is it benign?' **(oral)**, Overlapping clinical phenotypes in monogenic epilepsies- common molecular pathways?, 6th Dianalund International Conference on Epilepsy (DICE) conference, 2024, Køge, Denmark

'If a variant is predicted to be "benign/tolerated" by *in silico* tools and seen in gnomAD – is it benign?' **(poster)**, Overlapping clinical phenotypes in monogenic epilepsies- common molecular pathways?, 6th Dianalund International Conference on Epilepsy (DICE) conference, 2024, Køge, Denmark

Scholarships/prizes

Australian Government, Australian Postgraduate Award

Australian Government, Research Training Program Stipend

Postgraduate Research Support Scheme Travel Grants

- 2022
- 2023
- 2024

ARCS Annual Conference Student Scholarship

- 2022
- 2023

ASCEPT Travel Grant 2022

ASCEPT Neuropharmacology Special Interest Group Prize 2022

Brain and Mind Centre Research Excellence and Impact Award

- 2021
- 2023

**Dianalund International Conference on Epilepsy (DICE) Conference Travel Bursary
2024**

Abstract

Epilepsy affects over 60 million people worldwide, with an estimated 5 million new diagnoses per year, making it one of the most common chronic neurological conditions. Epilepsy can range from mild simple febrile seizures, in which the seizure is self-limiting and resolves by 5-6 years of age, to severe developmental and epileptic encephalopathies (DEEs). DEEs are a group of rare, severe neurodevelopmental disorders affecting 1 in 590 patients. Clinically, DEEs are characterised by seizures, which begins in early childhood, with significant developmental delay.

DEEs are commonly caused by *de novo* genetic variants. Many genes involved in various physiological functions such as neuronal excitability and synapse transmission have been implicated in DEEs. Among the causative genes, those that encode subunits that form γ -aminobutyric acid type A (GABA_A) receptors are the major culprits.

GABA_A receptors are a part of the pentameric ligand-gated ion channel superfamily and are the principal inhibitory receptor within the mammalian brain. Recent whole genome sequencing of patients with epilepsy have identified 11 out of the 19 genes to be associated with epilepsy. GABA_A receptors are formed from a selection of 19 protein subunits: α (1-6), β (1-3), γ (1-3), δ , ρ (1-3), θ , π , ϵ . These subunits mix and match to form a pentameric protein channel, that are predominantly localised at the synapse and comprised of two α , two β and a γ subunit.

As activation of GABA_A receptors has an inhibitory effect on nerve transmission under normal circumstances (with the exception of early development), it has long been assumed that loss-of-function (LoF) mutations lead to hypoactive GABA_A receptors, overexcitation and hence epilepsy. However, this simplistic view cannot explain the entire disease spectrum, including different seizure types and diverse comorbidities in individual patients. Recent studies indicate that gain-of-function (GoF) mutations that cause hyperactive receptors can also cause DEEs. These findings are surprising and require further investigation to better our understanding of disease mechanisms.

Therefore, the overarching aims of this doctoral work were to 1) assess the functional changes caused by epilepsy-associated *GABRG2* and *GABRB3* variants and correlate these changes to the patient's clinical phenotypes, 2) evaluate variant pathogenicity prediction using currently available knowledge in the literature and *in silico* prediction tools and 3) characterise the effect of a gain-of-function GABA_A receptor variant in a mice model.

Chapter 3 describes the functional and phenotypic characterisation of variants found in *GABRG2*. The $\gamma 2$ subunit is known to be incorporated into the vast majority of synaptic receptors and by extension is thought to have a critical function in receptor trafficking and localisation. Here, we examined null and missense LoF and GoF variants in $\gamma 2$ results, with GoF variants being associated almost exclusively with a *de novo* inheritance and earlier age of seizure onset. In addition, contrary to previous assertions, it was found that *GABRG2* null variants lead to less severe, even benign phenotypes than missense variants. **Chapter 4** investigated the utility of a combination of *in silico* tools to predict “benign” and gnomAD (population database) entry i.e. to assess pathogenicity. It was found that pairs of variants at the same position with a different amino acid change, with one that matches an existing gnomAD entry, does not necessarily provide strong evidence of pathogenicity or being benign. Therefore, this underscores the importance of robust functional assessment in confirming pathogenicity. **Chapter 5** is an extensive genotype/phenotype correlation of 71 *GABRG2* variants, offering an in-depth functional analysis of variants and its association of phenotypic differences specific to LoF and GoF cohorts. It was found that GoF group had a younger age of seizure onset (median: 3.75 m), and tends to be associated with language, visual cognitive and neurological abnormalities. In contrast, the LoF group tend to have an older age of seizure onset (median: 12 m) and was more likely to be associated with neuropsychiatric abnormalities. **Chapter 6** presents an in-depth functional analysis of GoF variants' effect on macroscopic desensitisation of GABA_A receptor. It was found that GoF variants that reduced desensitisation, meaning overall net increase in inhibition (more functional gain) were associated with earlier age of seizure onset, higher mortality, and more severe epileptic phenotype. **Chapter 7** used a novel GoF knock-in C57BL/6J mice model based on a human *de novo* GoF variant in *GABRB3*^{E77K} to examine the effect of the variant on the development of the mice, specifically looking at morphological changes of the brain. **Chapter 8** discusses the findings of this doctorate work in the context of the broader literature and provides concluding remarks.

In conclusion, the findings of this doctoral work provide conceptual advances in the field of GABA_A receptor in monogenic epilepsy. This thesis adds to the current literature examining the role of GABA_A receptor variants in patients, finding that pathogenic GABA_A receptor variants can be a result of either a hyperactive (GoF) or hypoactive (LoF) receptor. This finding challenges the previous paradigm that epilepsy is primarily due to “insufficient inhibition”. Instead, it was found that the majority of patients with refractory and severe epileptic syndrome are affected by hyperactivity of GABA_A receptor. This shift in understanding underscores the urgent need to reflect on the knowledge of GABA_A receptor role in epilepsy.

This thesis has significant implications for drug discovery, research and the field of medicine. Current anti-seizure medications do not adequately address the needs of patients with GoF variants. These therapeutics typically focus on rebalancing the “excitation-inhibition” scale. The outcomes of this research should impel researchers to explore the effect of GoF variants *in vivo* using murine models of epilepsy to gain a better understanding of how the variants may affect neuronal network firing and use this knowledge to address the unmet need of this patient group through avenues such as drug repurposing, and drug discovery.

For clinicians, studies from this doctorate work advocate for the use of patient phenotypes to guide diagnosis and treatment options. For instance, if a patient has a GABA_A receptor variant that is suspected to result in receptor hyperactivity, it is advisable to consider functional testing of the variant or careful monitoring of the patient if given an anti-seizure medication that increases GABA.

Keywords: *GABRB3*, *GABRG2*, GABA_A receptor, developmental and epileptic encephalopathies, epilepsy, genetic epilepsies, electrophysiology, knock-in mice

Chapter 1 Introduction

1.1 Epilepsy: prevalence, costs, and causes

Epilepsy, the second most burdensome neurological disorder worldwide, affects over 60 million people and incurs an estimated economic cost of \$119.27 billion per annum in 2019, impacting not only the quality of life (QoL) of the patients but also that of their caregivers (1, 2). There are currently over 25 US Food and Drug Administration (FDA) approved anti-seizure medications (ASMs), yet up to 40% of patients with epilepsy are refractive (3, 4).

There are many causes of epilepsy, such as traumatic brain injuries, infections, and genetic variants, which can be inherited or occur *de novo*. Clinically, epilepsy is defined as two or more unprovoked seizures in the absence of a transient insult, e.g. concussion, more than 24 hours apart (5). Seizures are a cardinal symptom of epilepsy and a paroxysmal event which occurs due to a sudden disruption of regular brain activity (6). During an epileptic seizure, there is an abnormal increase in excitatory neuronal firing that leads to hypersynchronisation of neuronal discharge (5). The excessive firing typically manifests as a spike discharge on the electroencephalogram (EEG) (7, 8). These neuronal discharges may involve a part (partial) or the entire (generalised) brain and are occasionally accompanied by unconsciousness (9).

The normal functioning of the human neuronal circuit involves an intricate interplay between excitatory and inhibitory neurotransmission. γ -Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter within the mammalian brain (10, 11). Dysfunction of the major inhibitory circuit can have a pathological consequence, including anxiousness (12, 13), schizophrenia (14, 15), autism spectrum disorder (ASD) (16-19) and major depressive disorder (15, 20, 21). The traditional consensus around the cause of epilepsy is that seizures arise from an imbalance between excitation and inhibition, whereby insufficient inhibition results in seizures and epilepsy (22-26).

1.2 GABA and its receptors

The major mediator of GABA's inhibitory action is GABA receptors (27). There are two types of GABA receptors: type A (GABA_A) and type B (GABA_B). GABA_A receptors are ligand-gated ion channels found at 20-50% of synapses in the brain, responsible for fast synaptic inhibition (28). Upon GABA binding to GABA_A receptors, the channel opens, allowing for chloride influx into the cell, hyperpolarising the cell, inhibiting the firing of action potentials, and reducing neuronal activity in the adult brain (29). GABA_B receptors are G protein-coupled receptors involved in slow and prolonged inhibition (30). Both classes of GABA receptors modulate cognition, pain and muscle tone, are involved in epilepsy, and are targets of clinically used therapeutics (31-33). For this thesis, I will focus on GABA_A receptors.

1.3 GABA_A receptor: assembly, structure and subtypes

GABA_A receptors are responsible for fast inhibitory neurotransmission within the mammalian brain. They are widely distributed in the brain and play various roles in human neurology, such as neurogenesis, synapse formation, preliminary circuit building, mediating excitation and inhibition levels in the brain (34, 35). Structurally, GABA_A receptors are formed from a selection of 19 subunits: α (1-6), β (1-3), γ (1-3), δ , ρ (1-3), θ , π , ϵ . These subunits mix and match to form a pentameric protein, arranged in a pseudosymmetrical, anticlockwise manner (**Figure 1.1 A-C**). Each receptor subunit has a distinct regional distribution in the brain, playing a critical role in the regulation of brain development, movement and learning (36, 37).

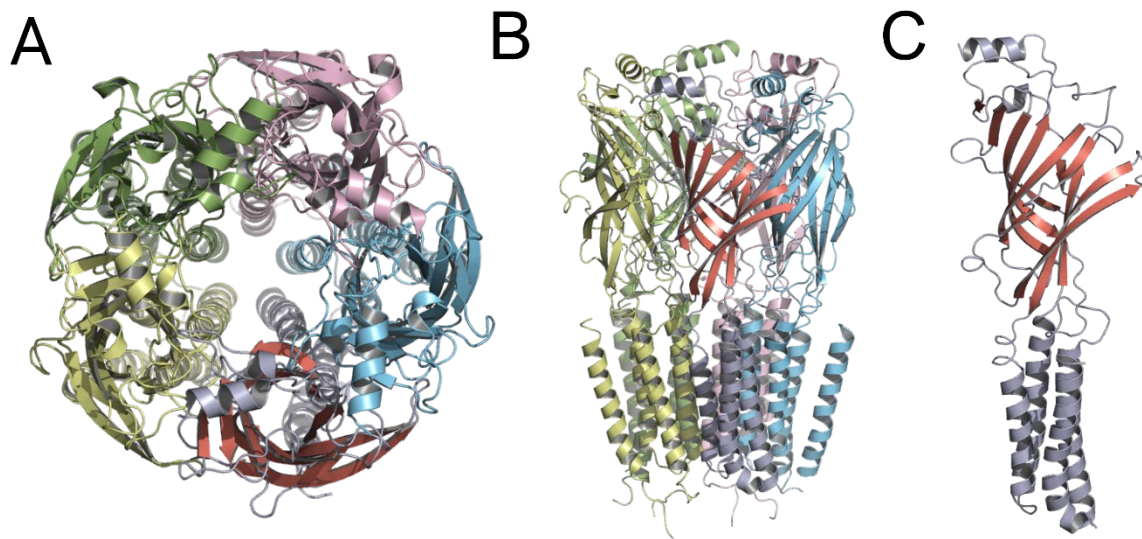


Figure 1.1 3D representation of GABA_A receptor

Cryo-EM reconstruction of GABA_A receptor (pdp: 6hup). The GABA_A receptor is a pentameric protein – here, five different subunits are depicted using five unique colours. A) Top view of the receptor, with the TM2 lining central pore, B) front view of the pentameric receptor and C) view of a single subunit, exemplified by the $\gamma 2$ subunit.

The predominant subtypes in the mammalian brain are composed of 2 α , 2 β and a γ or δ . The subunit composition of these receptors directly determines if a receptor is expressed post- or extrasynaptically. The different combinations of the pentameric receptor will have different biophysical properties such as receptor sensitivity, desensitisation, gating, and distribution in the brain (29, 38, 39). In spite of this great heterogeneity, the $\alpha 1\beta 2\gamma 2$ is the most widespread GABA_A receptor subtype in the central nervous system (CNS), accounting for approximately 60% of all GABA_A receptor subtypes in the synapse, and the $\alpha 2\beta 3\gamma 2$ combination makes up around 15-20% (39-41).

1.3.1 Cellular and network electrical activity regulation by GABA_A receptors

GABA during development

Activation of the GABA_A receptor opens the ion channel, allowing for the passive flow of chloride ions through the central pore. The direction of the chloride ion flow depends on the intracellular chloride concentration, which is regulated by the K⁺-Cl⁻ co-transporter KCC2, a chloride ‘exporter’, and Na⁺-K⁺-Cl⁻ cotransporter (NKCC1), a chloride ‘importer’ (32, 42-45). *In utero* and in the developing brain, NKCC1 is more prominent, leading to an increased

intracellular chloride concentration (45-47). This causes GABA-mediated chloride efflux, depolarising the cell membrane and resulting to neuronal excitation (45).

During development, GABA switches from being excitatory to inhibitory. This change corresponds to the change in NKCC1/KCC2 expression ratio, with an increase in KCC2 (46-48). KCC2 maintains a low intracellular Cl^- concentration, resulting in GABA mediated Cl^- influx, hyperpolarising the cell and inhibiting the generation of action potential (49, 50).

Phasic vs tonic inhibition

GABA_A receptors exhibit distinct subcellular localisation patterns, which directly determines their biophysical properties and inhibitory modes. Receptors that contain a γ subunit in association with $\alpha 1$, $\alpha 2$ or $\alpha 3$ subunits are predominantly expressed postsynaptically. As they are directly exposed to high GABA concentrations ($> 1\text{mM}$) following vesicular release, these subtypes mediate phasic synaptic inhibition, suppressing neuronal activity in the brain on a millisecond time scale (51-53). By contrast, subtypes containing $\alpha 4$, $\alpha 5$, $\alpha 6$, or δ subunits are predominantly expressed in the extrasynaptic space, where the ambient GABA concentration is low and prone to change from spillover at nearby synaptic clefts (29) (**Figure 1.2**). These receptors mediate a persistent, tonic inhibition that regulates neuronal excitability.

Extrasynaptic GABA_A receptors have a higher affinity for GABA, allowing them to respond to low agonist concentrations (54). The ability of δ -containing GABA_A receptors to respond to low GABA concentration is facilitated by their unique properties of higher affinity to GABA, slower activation, and at prolonged GABA activation, undergoing only partial desensitisation (55-57)

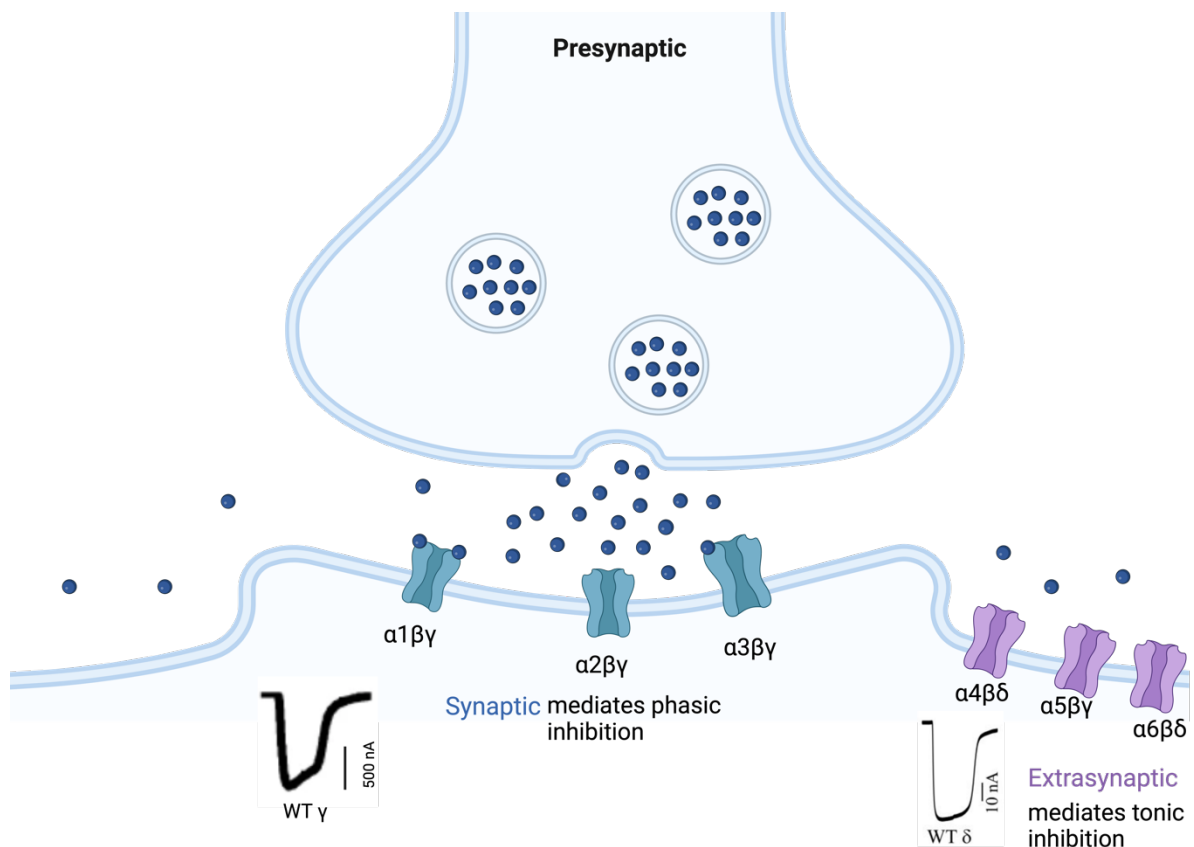


Figure 1.2 Synaptic and extrasynaptic GABA_A receptors

Release of GABA from presynaptic terminals activates postsynaptic GABA_A receptors beneath the release zone (blue-coloured receptors). Extra “spill-over” GABA acts on GABA_A receptors located in the extrasynaptic space (purple-coloured receptors). Below are representative traces of synaptic and extrasynaptic GABA activation. Note: the current elicited at synaptic GABA_A receptors is greater than the current elicited at extrasynaptic receptors. Adapted from Jacob et al., Nat Rev Neurosci, 2008 (52).

1.3.2 GABA binding and GABA_A receptor activation

GABA_A receptor is part of the Cys-loop superfamily, which includes glycine receptors, nicotinic acetylcholine receptors, and 5-HT₃ receptors (58). Consistent with other members of this superfamily, each subunit of the GABA_A receptor is comprised of three main structural domains: 1) the extracellular domain (ECD), which contributes to GABA binding; 2) four transmembrane domains (TMD), composed of four α -helix rich domains (TM1-4), with TM2 lining and forming the pore of the receptor. The pore-lining residues are mostly hydrophilic except at the 9', -2' and 2' positions (59). Finally 3) the intracellular domain (ICD), consisting of two intracellular loops, M1-M2 and M3-M4, which connects the TM1/2 and TM3/4

respectively (60) (**Figure 1.3**). These structural domains must act in a concerted manner to transition between an unbound receptor impermeable to chloride, to a receptor with two bound GABA molecules that allows passive flow of chloride ions through the central pore.

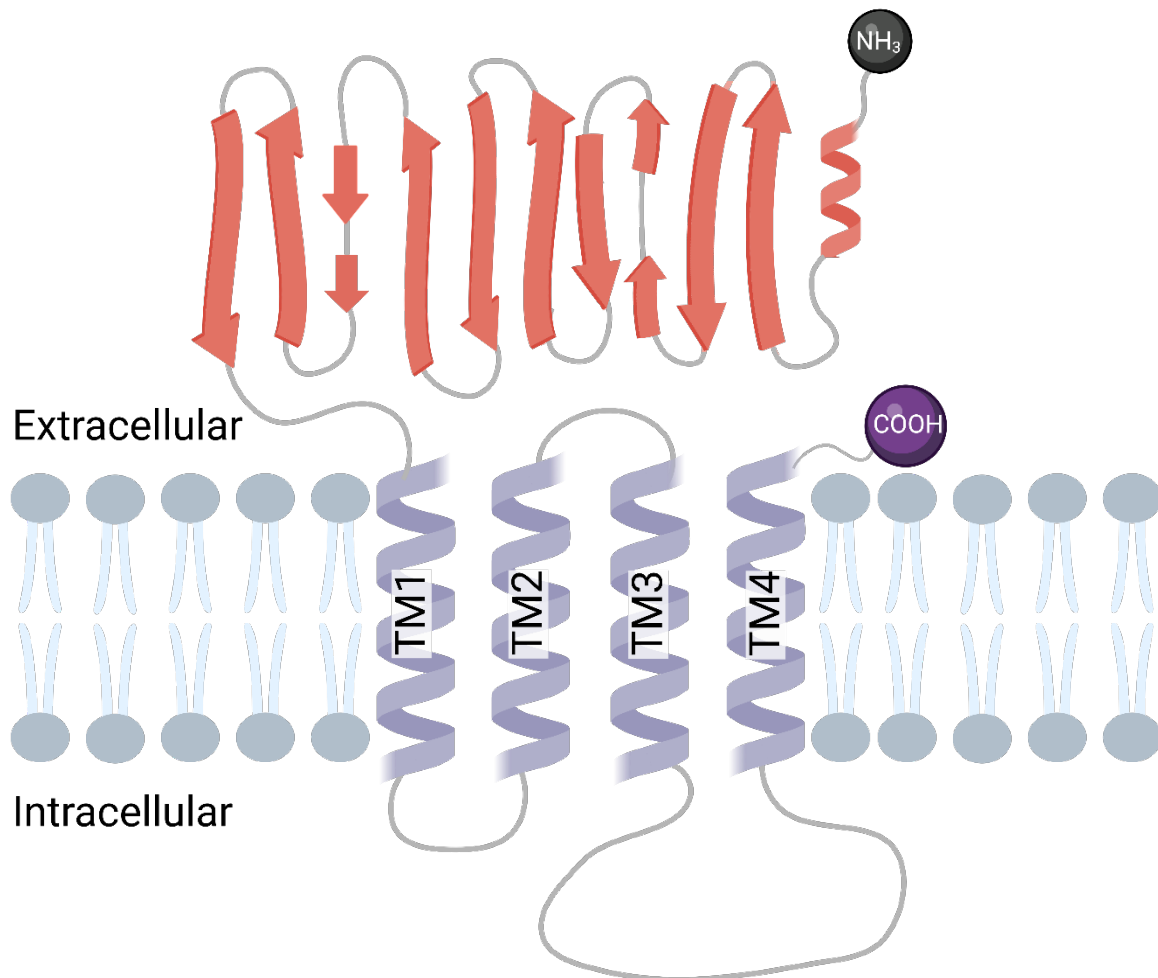


Figure 1.3 Cartoon representation of Cys-loop receptors

Figure depicts the N-terminus (black sphere), extracellular, intracellular and the four TMD and-C terminus (purple sphere).

Advances in structural biology, in particular with the advancements of cryogenic electron microscopy (cryo-EM) methods, have allowed for the atomic elucidation of GABA_A receptor assembly, functional states and pharmacology (51, 61-65). These structures collectively reveal the molecular intricacies of subunit structure, positions of modulator binding sites and the alterations in conformation during receptor activation.

It is now well established that a typical 2 α :2 β :1 γ / δ GABA_A receptor contains two orthosteric GABA binding sites located at the extracellular interface between two adjacent α and β

subunits (**Figure 1.4 A, B**). The β subunit contributes three loops (by convention, named A, B and C) that form the principal face (+) of the agonist binding site, whereas the α subunit contributes three β -strands and/or one loop (by convention, named loops D, E, F, and/or G) that form the complementary face (-). The binding of GABA to one binding site is sufficient to open the channel, allowing for the flow of Cl^- ions, however, occupation of both binding sites results in an increase in channel opening (66). Previous studies have suggested that the two binding sites may not be functionally equivalent, with the β -E/ α ⁺-D interface having a three-fold higher affinity to GABA than the β -B/ α ⁺-A interface (66).

GABA_A receptor activates in a concerted, 'lock and pull' mechanism. Upon GABA binding, this results in an initial closure of loop C on the β 3 subunit, resulting in a remodelling of both binding sites (β 3-B⁺/ α 1-A⁻ and β 3-E⁺/ α 1-D⁻), with the β 3 moving towards α 1 subunit (**Figure 1.4 C, D**) (67). In addition, the ECD of all subunits undergo an anticlockwise asymmetric rotation (67). Since the β -E/ α ⁺-D is more sensitive to GABA, there is a higher degree of closure between the β -E/ α ⁺-D interface and rotation of ECD (**Figure 1.4 C, D**) (67).

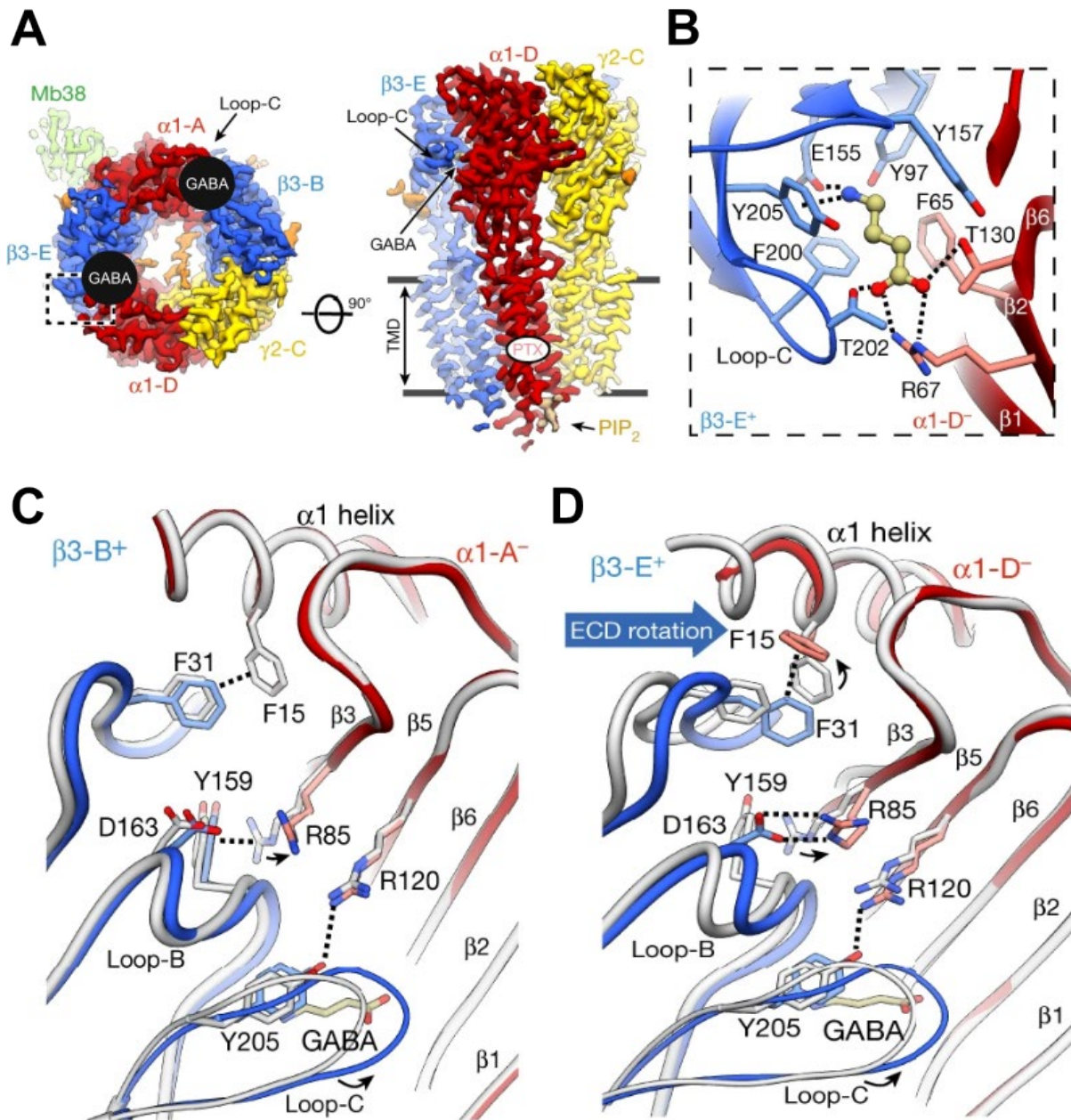


Figure 1.4 Cryo-EM structure of GABA and picrotoxin-bound $\alpha 1\beta 3\gamma 2L$ receptor.

A) Extracellular view of $\alpha 1\beta 3\gamma 2L$ receptor (left) and in a plane parallel to the membrane (right). **B)** Detailed view of one GABA-binding pocket from the extracellular space. GABA is depicted in a ball-and-stick model, with carbon atoms in khaki, oxygen in red and nitrogen in blue. **C, D)** superimposed views of the $\alpha 1$ ECDs from picrotoxin bound (grey) and the picrotoxin/GABA bound structures reveal conformational changes induced by GABA binding at the orthosteric pockets of the $\beta 3-B^+/\alpha 1-A^-$ (**C**) and $\beta 3-E^+/\alpha 1-D^-$ (**D**) interfaces. Dashed lines denote hydrogen bonds, π - π stacking, π -cation interactions, and salt bridges. Figures were taken from Masiulis et al. (2019) (67).

Positive allosteric modulators (PAMs)

Positive allosteric modulators (PAMs) act to increase receptor affinity in the presence of the endogenous ligand without activating the receptor itself (68). In terms of GABA_A receptor, one cryo-EM structure suggested that allosteric modulators, benzodiazepines, act as “connectors”, stabilising weaker ECD interface, α 1-D/ γ 2-C, and facilitating ECD rotation (67). Conformational change of the ECD allows for a clockwise rotation of the TMD, tilting the central lining (TM2), which facilitates the movement of the 9' leucine side chain away from the pore and towards inter-subunit interfaces, increasing the pore diameter (67, 69). There are other mechanisms and binding sites depending on the PAM. For instance, both etomidate and propofol bind at the β - α interface, but etomidate also binds to the γ - β site (70). Notably, PAMs, including phenobarbital, etomidate, propofol and diazepam, binds to regions important for the motion of TMD (70).

1.3.3 GABA_A receptor and epilepsy: variant-induced changes to binding and gating

Recent whole genome sequencing of patients with epilepsy have identified 11 out of the 19 genes to be unambiguously involved in epilepsy, namely *GABRA1-5*, *GABRB1-3*, *GABRG1-2*, and *GABRD* (40, 71-82). An additional gene, *GABRE*, has only recently identified in patients with epilepsy and requires further assessment for its role in the disease (83). As the GABAergic inhibitory postsynaptic currents are shaped by the kinetics of activation, desensitisation and deactivation of GABA_A receptors (84), many epilepsy-causing variants disrupt/alter these receptor properties. It is important to note, that the allosteric transition of the protein is important and governs the receptor function and variants can induce changes to allosteric transition.

The four basic steps for GABA_A receptor activity are: GABA binding, pre-activation, channel gating and receptor desensitisation. Depending on the variant's location, the effects can range from simple isolated effects such as increasing or decreasing GABA sensitivity to compound effects or more than one mechanism (85). For example, a variant that affects the pre-activation or channel gating set step tends to affect both the GABA sensitivity and gating efficiency (85-87).

The γ 2 subunit is part of the major synaptic isoform of GABA_A receptor, α 1 β 3 γ 2, with GABA being low affinity/high efficacy at γ 2 containing synaptic receptors (85). This means a high

concentration is required to activate the receptor; however, when GABA is bound, the maximal open probability is close to 100% (85, 88). This means a GoF *GABRG2* variant would more likely increase receptor sensitivity to GABA rather than increase maximal evoked current. Furthermore, since channel gating precedes receptor desensitisation, variants that affect gating efficacy could ultimately affect receptor macroscopic desensitisation (85, 89).

Extrasynaptic GABA_A receptor subtype such as, $\alpha 4\beta 3\gamma 2L$, is more sensitive to GABA, desensitise faster but deactivate slower than $\alpha 1\beta 3\gamma 2L$. Therefore, we and others speculate that changes in subunit composition to a more rapidly desensitising one with slow deactivation could impair inhibitory neurotransmission (73, 78, 79). This maintains the consensus that impaired GABA_A receptor expression is behind the epilepsy phenotype. However, we found this to not be entirely the most accurate explanation. This will be discussed further in Chapter 6, as we delve more into receptor desensitisation in variants that increase GABA sensitivity.

1.3.4 Functional GABA_A receptor regions and variants

A genetic variant can be benign or pathogenic. Depending on the location of the variant, the pathogenicity of a variant can be predicted to some extent e.g. the functional role of the structural motif and conservation of site. It is thought that a variant located in a highly conserved or key structural region important for receptor activation and channel gating may have a greater impact on receptor function as opposed to a variant located in a non-conserved region where some variations of the amino acid change can be tolerated. In general, amongst the Cys-loop receptor family, the ECD and the TMD tend to be highly conserved, whilst the ICD are poorly conserved (90).

Each part of the subunit plays a crucial role in the receptor activity (90). Notably, the Cys-loop is essential in transducing the binding signal to the TMD. Loops M2-M3 and the $\beta 1-2$ loops which lies in the ECD, forms a bridge linking the ECD to the TMD (67). The flexibility of M2-M3 loop appears to regulate efficiency of ECD-TMD signal transduction (67).

The orthosteric ligand binding site lies on the ECD at the two β^+/α^- interface. Upon GABA binding, the coupling junction, between the N-terminus and the TMD, and the pore domain of the receptor undergoes conformational changes resulting in the activation of the receptor. Variants located in these regions are thought to uncouple this channel activation through perturbation of the coupling junction, impairing receptor gating and therefore resulting in

epilepsy (91). In Masiulis *et al.* (2019) Cryo-EM study on the $\alpha 1\beta 3\gamma 2L$ isoform, it was described that the endogenous ligand, GABA, engages in a cation- π interaction with $\beta 3Tyr205$ and disruption of this environment with a missense variant of $\beta 3Tyr205Met$ results in a receptor with reduced affinity to GABA, LoF receptor (67).

The TM2 helices of each of the five subunit lines the Cl^- conducting pore and are responsible for the activation gate (9' leucine) and the desensitisation gate (-2' residue) (90, 92). Variants found in TM2 are associated with a more severe clinical outcome including a younger age of seizure onset and severe forms of epilepsy (40, 75, 93, 94)

Intracellular M3-M4 loop are important in interacting with the intracellular environment such as protein-protein interactive domain, regulate synaptic localisation and receptor trafficking and activity (95, 96). The M3-M4 loop are thought to be not conserved, and variants found in this region tend not be pathogenic, and in fact, prevalent in the healthy population as found on the Genome Aggregation Database (gnomAD).

1.4 Spectrum of epileptic syndromes

This section will go over a range of epilepsy syndromes, ranging from simple febrile seizures (FS), which are often self-limiting, to severe syndromes such as Dravet syndrome (DS). Throughout this section and the thesis, a common marker that will be discussed is the age of seizure onset as a proxy for severity. This is because a younger age of seizure onset tends to be associated with a high burden of cognitive and behavioural comorbidity (97), drug resistance (98) and mortality (99). Parts of this section have been published in a Review in *Journal of Neurochemistry*, 2023 (85).

1.4.1 What is epilepsy – what are the spectrums?

Epilepsy is an umbrella term encompassing a plethora of syndrome, ranging from mild, FS to severe, developmental and epileptic encephalopathies (DEEs). In 2022, the International League Against Epilepsy (ILAE) Task Force proposed the definition for an epilepsy syndrome to be:

“A characteristic cluster of clinical and EEG features, often supported by specific etiological findings (structural, genetic, metabolic, immune and infectious).” The diagnosis of the syndrome carries prognostic and treatment implications. Syndromes often have an age-dependent presentations and a range of specific comorbidities.

1.4.2 Febrile seizures (FS) and Febrile seizures plus (FS+)

FS, are typically triggered by a fever of 38°C or greater, has a prevalence of 2-6% in children aged between 6 months to 5 years old and generally subsides by 5-6 years of age and if there's no family history of epilepsy, prognosis tends to be favourable. However, if there is a family history, the FS may evolve into more serious conditions, like genetic epilepsy with febrile seizures plus (GEFS+) (40, 100-102).

Febrile seizure plus (FS+) describes children with FS that continues beyond the age of six (103). FS+ is generally self-limiting by puberty (103).

1.4.3 Genetic epilepsy with febrile seizures plus (GEFS+)

GEFS+ is a familial epilepsy syndrome characterised by a group of genetic epilepsies. It is primarily associated with an autosomal dominant inheritance pattern, although *de novo* have been observed in a small number of cases (102). There have been multiple genes associated with GEFS+ including sodium channels (*SCN1A*, *SCN2A*, *SCN9A*, *SCN1B*), *GABRG2*, and *STXBP1* (104-111).

In GEFS+, seizures typically start with FS between the ages of 3 months to 6 years. However, unlike simple FS, patients with GEFS+ will continue to experience seizures (febrile or afebrile) throughout their lives. Additionally, within GEFS+, there may be intrafamilial variability, where there may be family members with more severe forms of epilepsy, such as DS. The course of the illness in an individual presenting with GEFS+ is dependent on the type of epilepsy within the spectrum of GEFS+ (103). Seizures are often well-controlled in this group with sodium valproate (40, 112-114).

1.4.4 Dravet syndrome (DS)

Dravet syndrome (DS), previously known as severe myoclonic epilepsy of infancy, is an infantile-onset developmental and epileptic encephalopathy that typically begins within the first year of life, between 3 and 9 months, with a mean and median age of onset at 6 months (103). The initial seizure type is typically prolonged, febrile and afebrile, focal clonic or generalised clonic seizures (103, 115, 116). Generally, seizures associated with DS are highly sensitive to temperature changes, even a hot day can trigger a seizure onset (115).

The syndrome typically worsens between 1- 5 years of age, with patients experiencing various seizure types, including myoclonic, atypical absence seizure, focal seizures, and long-lasting tonic-clonic seizures (116). Both seizure-related and non-seizure comorbidities persist and worsen before stabilising. By adulthood, patients often have moderate to severe intellectual disability (ID) (116, 117). Seizures are frequently intractable and are associated with several comorbidities, including developmental delay (DD), ID, motor impairment and behavioural concerns (115, 118, 119).

Up to 90% of cases are associated with mutations in the *SCN1A* gene, leading to a LoF in sodium channel (120). However, not all *SCN1A* mutations are LoF. For instance, patients with *SCN1A* p.T266M a recurrent gain-of-function (GoF) missense mutation present with clinical phenotype that distinct and more severe than DS (121). These patients present with a younger mean age of seizure onset (3 months) compared to 6 months for DS (122, 123), profound developmental impairment and motor disorder (121). In addition to *SCN1A*, other genes have also been implicated in DS, including other sodium channels (*SCN2A*, *SCN8A*, *SCN9A*, *SCN1B*), *PCDH19*, *GABRA1*, *GABRG2*, *STXBP1*, *HCN1*, *CHD2*, and *KCNA2* (115, 124, 125).

For DS, the first-line therapy tends to be sodium valproate prescribed off-label and depending on patient response, adjunct therapy such as benzodiazepines may be introduced (115). Currently, three pharmacological agents - cannabidiol, stiripentol and fenfluramine - are approved by the US FDA for DS as an adjunctive treatment. In Australia, stiripentol (Diamcomit®) has been approved as an adjunctive treatment for refractory generalised tonic-clonic seizures that are not controlled by valproate and a benzodiazepine (126). Importantly, due to the underlying genetic cause (LoF mutation in *SCN1A*), the use of a sodium channel blocker such as phenytoin, lamotrigine, or (ox)carbamazepine is contraindicated in patients with DS.

1.5 Developmental and epileptic encephalopathies (DEEs)

1.5.1 Rare and refractive

Developmental and epileptic encephalopathies is a group of severe epilepsy affecting 1 in 590 children (127). Developmental and epileptic encephalopathy (DEE) is a neuronal condition in which DD from birth are compounded by epileptic activity (128, 129). The epileptic activity further impairs the developmental process of the child, which can have a profound long-term impact on the child. Syndromes classified as DEEs include early infantile developmental and epileptic encephalopathy (EIDEE), infantile spasms, DS, Lennox-Gastaut syndrome, epilepsy of infancy with migrating focal seizures (EIMFS), and continuous spikes and waves slow sleep evolving Lennox-Gastaut syndrome. Children with DEE often have seizures that starts in infancy, and not only it is difficult to treat, but 30-40% of these patients do not respond to current ASMs.

Clinical manifestation of DEEs are found on a spectrum and are associated with characteristics such as facial anomalies such as a broad forehead; micrognathia, prominent lobule of the ears; varying degrees of neurodevelopmental impairment such as communication, DD and ID varying from mild to severe, microcephaly and movement disorders hypotonia, peripheral hypertonia, ataxia, dyskinesia, dystonia, behaviour difficulties e.g. attention deficit hyperactivity disorder (ADHD), impulsive behaviour (130-133).

1.5.2 Causes of DEEs

DEEs are commonly caused by *de novo* dominant variants. These variants occur on genes involved in many different functions such as neuronal excitability and synapse transmission (4). Among the causative genes, the GABA_A receptor has been identified as a major causative gene of DEEs (81). For a long time, it was hypothesised that the primary cause of epilepsy was a loss of GABA_A receptor function. Numerous studies have identified epilepsy-associated GABA_A receptor variants, attributing these variants to reduce net neuronal inhibition through mechanisms such as impaired surface expression and trafficking (78, 79, 91, 134, 135). However, this simplistic view cannot explain the continuum of syndromes patients experience.

1.6 Current treatment option for epilepsy

Current ASMs and drug development ventures are targeted towards rebalancing the excitation-inhibition balance, specifically to increase inhibition. Currently, there are approximately over 25 FDA approved ASMs that affect neuronal hyperexcitability and can be broadly classified into 4 classes, 1) modulation of voltage-gated channels, including sodium channels (such as phenytoin, lamotrigine), calcium-channels (e.g. ethosuximide) and potassium-channels (e.g. retigabine); 2) enhancement of GABA mediated inhibitory neurotransmission e.g. vigabatrin, benzodiazepines; 3) attenuation of glutamate transmission e.g. felbamate, topiramate and 4) modulation of neurotransmitter release e.g. levetiracetam, gabapentin (**Figure 1.5**) (4, 136-138). It is important to note that with ASMs, many times their exact mechanism of action is unknown, or they may have multiple sites of action for instance, valproate, felbamate, cenobamate, topiramate (136, 139). It may be the multimodal effects of ASMs that render these treatments effective in some cases.

These ASMs predominantly reduce seizure occurrence, but do not have any disease modifying effect. The current guidelines for treatment selection of epilepsy is based on the type of seizure, specific patient needs and the benefits and risks of the medication (140). Unfortunately, up to 40 % of patients with epilepsy are refractive to the current treatments or experience severe side effects to these that in many cases the treatment may be removed (141-144).

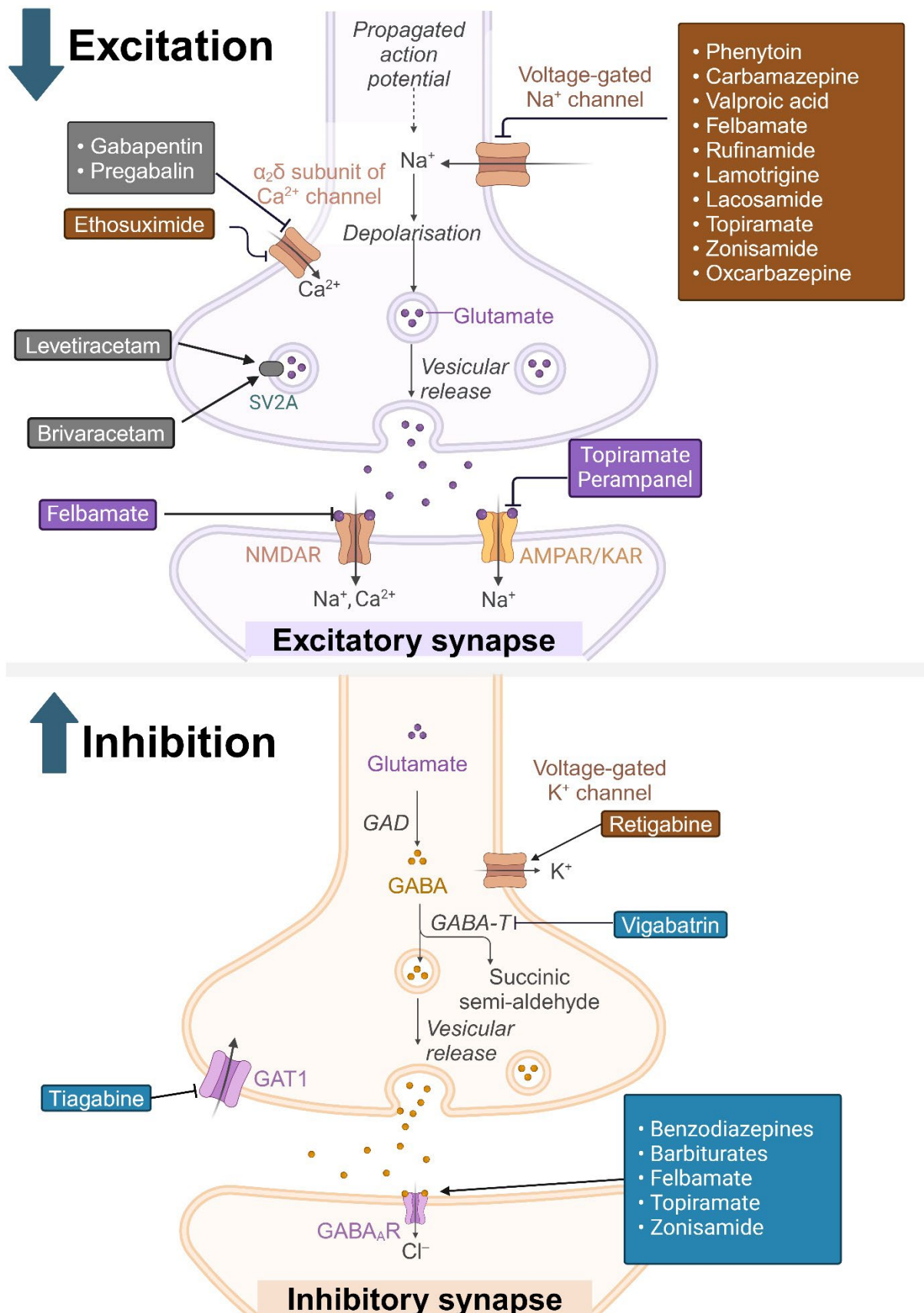


Figure 1.5 Mechanism of actions of some of the currently available ASMs.

ASMs are broadly grouped into four groups based in their mechanism of action: 1) modulation of voltage-gated channels (brown box); 2) enhancement of GABA mediated inhibitory neurotransmission (blue box); 3) attenuation of glutamate (purple box) and 4) modulation of neurotransmitter release (grey box). Adapted from “Mechanisms of Antiepileptic Drugs” by BioRender.com (2024). Retrieved from <https://app.biorender.com/biorender-templates>

1.6.1 Treatment hypersensitivity

Case studies by Papandreou *et al.* (143) and Kothur *et al.* (144) reported two patients with a variant in the *GABRB3* gene. Both were given the ASM vigabatrin, a GABA transaminase inhibitor, an enzyme that breaks down GABA in the synapses. Vigabatrin is commonly prescribed for infantile spasm and its mechanism is to inhibit GABA transaminase, and thus increasing synaptic GABA concentration. However, when given these medications, one patient experienced an increase in the number of seizures, whilst the other experienced an improvement in the number of seizures but due to severe side effects including excessive sedation, hypotonia, both patients ended up coming off the medication.

This raises the question of whether causes of epilepsy such as a genetic mutation in the patient is so significant that despite being on a host of ASMs to “correct” this imbalance, pharmacological endeavours are still not “strong” enough or there is potentially another pathophysiological mechanism unrelated to the excitation-inhibition imbalance theory.

1.7 Challenging the excitation and inhibition paradigm - Genetic variants and their impact

In several cases, distinct syndromes can emerge from different variants in the same gene. Dravet syndrome, first described in 1978, is one of the most severe forms of epilepsy syndrome, affecting children in infancy, with males being twice as likely affected than females. Mutations in the *SCN1A* gene can result in either a LoF or GoF channel, with GoF variants being associated with a younger age of onset and other clinical features including, movement disorders, arthrogryposis, profound ID (145).

Similarly, mutations in *KCNA2*, the gene encoding for potassium channel Kv1.2 have been found to result in both a GoF – an increase in activity, where the mutation leads to a formation of a permanently open channel - or LoF - characterised by reduced channel activity, or a combination of both GoF and LoF effect (146, 147). There are key shared clinical phenotypes among these three groups including early age of seizure onset (between the ages of 1-2 years), fever sensitivity, cerebellar involvement, cognitive and language impairment (147). However, depending on the effect of the variant, the severity can vary: patients with both GoF and LoF variant tends to experience seizure onset at neonates and the most severe clinical phenotype including neurological and ID (146). The second most severe type of variants are GoF variants which are associated with epileptic encephalopathy (EE), hypotonia, ID and cognitive impairment, cerebellar atrophy in adulthood along with facial dysmorphism, and microcephaly (146, 147). Finally, patients harbouring LoF variants tend to have a better prognosis (146, 147). Given that pathogenic variants in genes like *SCN1A* and *KCN2* can have distinct differences- gain, loss, gain/loss - on patient phenotype, this indicates that genetic variants in ion channels that alter neuronal activity in any manner as opposed to only increasing excitatory activity are associated with genetic epilepsy.

Indeed, in recent years, pathogenic variants found in *GABRB3* have been identified to result in a hyperactive (GoF) or hypoactive (LoF) receptor (75, 148, 149). Interestingly, approximately equal amounts of GoF and LoF variants were found, and they showed a similar distinction in syndromes associated with GoF and LoF variants (75). Whereby patients harbouring GoF variants were related to a younger age of onset, and more severe clinical phenotypes, including microcephaly, motor impairment, DD, cognitive impairment and so on. Conversely, patients that harbour variants that results in a LoF receptor, had an older age of onset (>10.5 months) and initial onset of seizure tends to be FS (75).

1.8 From bedside to bench - classifying the effects of variants

1.8.1 Genome Aggregation Database (gnomAD)

Genetic testing and counselling for epilepsy are becoming increasingly integrated into standard medical practice. The advancement of next-generation sequencing (NGS) has allowed for the identification of many disease-associated variants as well as contributing to a growing public population database such as gnomAD. gnomAD serves as a publicly accessible repository of human genetic variability and is a crucial tool for understanding

genetic variations (150). Within the gnomAD database, at least one individual has been identified to harbour 1815 genes that are biallelic predicted LoF, indicating that humans are capable of withstanding a loss of those gene or their function. It is expected that if a variant has a high allele frequency in gnomAD, it is more likely to be benign. Some variants present in the database are of uncertain significance, these are referred to as, variant of uncertain significance (VUS).

1.8.2 American College of Medical Genetics and Genomics - ACMG criteria

In 2015, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) jointly established a set of guidelines for variant interpretation. These guidelines include specific standard terminology to describe variants, such as “pathogenic,” “likely pathogenic,” “uncertain significance,” “likely benign,” and “benign.” The recommendations outline a process for classifying variants into these five categories based on criteria such as population data, computational *in silico* predictive programs, and functional data, etc. VUS often account for a substantial part of genome variability (151-153) and are the main source of uncertainty in genetic diagnosis, this leads to a negative downwards effect on patient care (154). Reclassification typically requires additional functional data.

1.9 Re-evaluating epilepsy pathophysiology

The excitation/imbalance paradigm suggests that the molecular phenotype of GABA_A receptor variants will be exclusively LoF, leading to impaired GABAergic inhibitory signaling. However, recent studies have revealed that GoF GABA_A receptor variants can also be associated with epilepsy, surprisingly the more severe types of DEEs (See Section 1.6). Furthermore, individuals with GoF variants often idiosyncratic responses to drug therapies, adding complexity to treatment approaches and challenges us to “relearn” the pathophysiology of epilepsy.

To address this, international collaborations, with the Danish Epilepsy Center, Philadelphia, and clinicians worldwide, are crucial. These collaborations aim to identify variants in patients and rigorously test them *in vitro* to elucidate the pathogenicity of each variant and bridging the gap

between bedside observations and bench experiment result, gaining insights into the differences in phenotypic manifestations and how these variants affect receptor activity.

1.10 Aims

Epilepsy syndromes exhibit a wide range of severity and outcomes; some patients have a favourable prognosis, while others suffer from severe forms of epilepsy that are resistant to treatment. The recent discovery that GoF GABA_A receptor variants are associated with epilepsy represents a significant shift in our understanding. These GoF variants are often linked to more severe epileptic phenotypes, challenging the traditional view of epilepsy as primarily due to an imbalance of excitation and inhibition neurotransmission. This emerging insight into GoF GABA_A receptor variants highlights the need for further research to fully understand their effects and improve identification, diagnosis and treatment strategies for affected patients.

The overarching aim of my doctoral thesis is to understand functional consequences of epilepsy-associated *GABRG2* and *GABRB3* variants and correlate their function to patient phenotypes.

Chapter 3 and 5 aims to gain an understanding of the underlying mechanisms of how *GABRG2* variants contributes to the diverse spectrum of phenotype, we will perform a functional analysis of variants.

Chapter 4 Aim:

1. To evaluate the reliability of *in silico* prediction tools in accurately determining the pathogenicity of 7 pairs of missense *GABRG2* variants.
2. To investigate the ability of *in silico* tools to differentiate between pairs of *GABRG2* variants at the same position.
 - a. To determine if a combination of *in silico* tool predictions and gnomAD allele frequencies (of an existing variant at the same position) can provide evidence that a novel variant (same position as current gnomAD but different amino acid change) is benign.

Chapter 6 Aim: To unravel the association between receptor desensitisation and clinical severity.

Chapter 7 Aim:

To characterise the effect of a GoF GABA_A receptor variant in a mice model. Specifically:

1. To determine changes in brain morphology by evaluating mass and hippocampal volume using Nissl stain. The hippocampal volume is of interest, especially hippocampal regions, CA1 and CA3 because they are associated with increased neuronal excitability and seizures (155).
2. To assess markers of neuroinflammation, neuronal death and epilepsy to elucidate differences between $\beta 3^{E77K}$ and WT mice.

Chapter 2 Materials and Methods

2.1 Materials and Instrumentation

2.1.1 Chemicals and reagents for molecular biology and electrophysiology

Chemicals/reagents	Suppliers
1 kb DNA ladder	New England Biolabs (Notting Hill, VIC, AU)
Agarose, molecular grade	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Ampicillin sodium salt	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Calcium chloride	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Ethanol	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Etomidate	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
GABA	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Gentamicin	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Glycerol	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
HEPES hemisodium	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
LB	Bacto laboratories Pty Ltd (Mt Pritchard, NSW, AU)
LB Agar	MP Biomedicals Pty Ltd (Seven hills, NSW, AU)
Magnesium chloride hexahydrate	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Mineral oil	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Nucleus free water	Thermo Fisher Scientific (Riverstone, NSW, AU)
Potassium chloride	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Sodium chloride	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Sodium pyruvate	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
SYBR Safe	Thermo Fisher Scientific (Riverstone, NSW, AU)
Theophylline	Merck Life Science Pty Ltd (Bayswater, VIC, AU)

2.1.2 Kits used for molecular biology

Kits	Suppliers
mMessage mMachine T7 Transcription	Thermo Fisher Scientific (Riverstone, NSW, AU)
QIAprep Nucleotide Removal	Qiagen (Chadstone, VIC, Australia)
QIAprep Spin MiniPrep	Qiagen (Chadstone, VIC, Australia)
QIAquick Gel Extraction	Qiagen (Chadstone, VIC, Australia)

2.1.3 Enzymes, enzyme buffer, bacteria, selection antibiotic used for molecular biology

Enzymes, enzyme buffer bacteria, antibiotic	Suppliers
Collagenase A	Roche (Mannheim, Germany)
NEB 10-beta competent <i>E.coli</i> cells	New England Biolabs (Notting Hill, Vic, AU)
rCutsmart buffer 10X	New England Biolabs (Notting Hill, Vic, AU)
Restriction enzymes (AflII, AgeI, BamHI, HindIII, EcoRI, KpnI, NOTI, Sall)	New England Biolabs (Notting Hill, Vic, AU)
rSAP	New England Biolabs (Notting Hill, Vic, AU)
T4 ligase	New England Biolabs (Notting Hill, Vic, AU)
T4 ligase buffer 10X	New England Biolabs (Notting Hill, Vic, AU)
Zeocin, ampicillin	Thermo Fisher Scientific (Riverstone, NSW, AU)

2.1.4 Chemicals and reagents for immunohistochemistry

Chemicals/reagents	Suppliers
Cresyl Violet	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
DAB (Catalog # D5905-100TAB)	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Ethyl alcohol	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Eukitt Quick-hardening mounting medium for microscopy	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
ExtrAvidin Peroxidase	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Fluoroshield	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Normal goat serum	Thermo Fisher Scientific (Riverstone, NSW, AU)
Normal horse serum	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Sodium borohydride	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Tissue-Tek	ProSciTech (Thuringowa, QLD, AU)
Triton X-100	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
Xylene	Merck Life Science Pty Ltd (Bayswater, VIC, AU)

2.1.5 Antigens for immunohistochemistry

Antigen	Catalog #	Supplier,
Anti-Myelin basic protein	ab40390-1001	Abcam Australia Pty Ltd (Melbourne, VIC, AU)
ZnT3	197 003	Focus Bioscience Pty Ltd (Murarrie, QLD, AU)
Iba-1	ab178846-100ul	Abcam Australia Pty Ltd (Melbourne, VIC, AU)
Alexa-Fluor 568	ab175695	Abcam Australia Pty Ltd (Melbourne, VIC, AU)
Fluoro-Jade C		Biosensis Pty Ltd (Thebarton, SA, AU)

2.1.6 Equipment

Equipment	Suppliers
30-0050 glass capillaries	Harvard Apparatus (Holliston, Massachusetts, USA)
Aperio XT Slide Scanner	Leica Microsystem (Mt. Waverly, VIC, AU)
Axon GeneClamp 500B amplifier	Molecular Devices (San Jose, California, USA)
Axon instruments Digidata 1440A	Molecular Devices (San Jose, California, USA)
C-1000 Touch Thermal Cycler	Bio-Rad (Gladesville, NSW, AU)
Cryostat	Leica Microsystem (Mt. Waverly, VIC, AU)
Cryostat CM1950	Leica, Germany
Digidata 1440A digitizer	Molecular Devices (San Jose, California, USA)
DM6000	Leica biosystems (Lake Cook, Deer Park, USA)
Drummond Scientific glass capillaries	Drummond Scientific Co (Broomall, PA, USA)
Eppendorf Centrifuge 5417 R	Eppendorf (Macquarie Park, NSW, AU)
Eppendorf Centrifuge 5430 R	Eppendorf (Macquarie Park, NSW, AU)
Gel Doc XR+	Bio-Rad (Gladesville, NSW, Australia)
Lab Incubator shaker 8500	Bioline (Alexandria, NSW, Australia)
Laboratory incubator	Thermoline Scientific (Wetherill Park, NSW, AU)
Laboratory incubator	Labec Laboratory Equipment (Marrickville, NSW, AU)
Laboratory refrigerator	Labec Laboratory Equipment (Marrickville, NSW, AU)
Leica Aperio XT – slide scanner	Leica biosystems (Lake Cook, Deer Park, USA)
Microprocessor-controlled vertical pipette puller (PUL-100)	World Precision Instruments, Inc (Sarasota, FL, USA)
NanoDrop 2000c Spectrophotometer	Thermo Fisher Scientific (Scoresby, VIC, AU)
Nanoject Microinjector	Drummond Scientific Co (Broomall, PA, USA)
Olympus VS120 slide scanner	Olympus (Notting Hill, VIC, AU)
pClamp 10.2 suite software	Molecular Devices (San Jose, California, USA)
Powerlab 8/35 AD instruments	ADInstruments (Bella Vista, NSW, AU)
PP-830 single-stage microelectrode puller	Narishige (Tokyo, Japan)
PS-3D sunflower minishaker	Grant instruments, Europe
Stericup® Filter Units	Merck Life Science Pty Ltd (Bayswater, VIC, AU)
VC8 valve controller	Warner Instrument Corp (Hamden, CT, USA)
Warner OC-725C Oocyte clamp	Warner Instrument Corp (Hamden, CT, USA)
Water bath	Labmaster (Turku, Finland)

2.1.7 Buffer recipes

LB broth

Table 2.1 Recipe for LB broth

	g/L
LB broth	20

LB Agar

Table 2.2 Recipe for LB Agar

	g/L
LB agar	10.5

Modified Barth's solution

Table 2.3 Recipe for Modified Barth's solution

Salt	Conc (mM)	Mol wt	g/L
NaCl	82.5	58.4	4.818
KCl	2	74.55	0.1491
MgCl ₂ ·6H ₂ O	1	203.3	0.2033
HEPES hemisodium	5	249.3	1.2465

ND96

Table 2.4 Recipe for ND96

Salt	Conc (mM)	Mol wt	g/L
NaCl	96	58.4	5.6064
KCl	2	74.55	0.1491
MgCl ₂ ·6H ₂ O	1	203.3	0.2033
HEPES hemisodium	5	249.3	1.2465
CaCl ₂	1.8	1M stock	1.8 mL

ND96 + gentamicin and ampicillin

Table 2.5 Recipe for ND96 + gentamicin and ampicillin

Salt	Conc (mM)	Mol wt	g/L
NaCl	96	58.4	5.6064
KCl	2	74.55	0.1491
MgCl ₂ .6H ₂ O	1	203.3	0.2033
HEPES hemisodium	5	249.3	1.2465
CaCl ₂	1.8	1 M stock	1.8 mL
Sodium Pyruvate	2.5	110	0.275
Theophylline	0.5	180.164	0.090082
Gentamicin	50 mg/L	-	0.05
Ampicillin	100 mg/L	-	0.1

Phosphate-buffered saline (PBS)

Table 2.6 Recipe for phosphate-buffered saline (PBS)

Salt	Conc (mM)	Mol wt	g/L
Sodium phosphate monobasic	23.3	119.98	3.4
Sodium phosphate dibasic	76.8	141.96	10.9
NaCl	154.1	58.4	9

Phosphate-buffer (PB)

Table 2.7 Recipe for phosphate-buffer (PB)

Salt	Conc (mM)	Mol wt	g/L
Sodium phosphate monobasic	23.3	119.98	3.4
Sodium phosphate dibasic	76.8	141.96	10.9

2.2 Safety and ethics

All experiments followed the University of Sydney and Brain and Mind Centre work health and safety *regulations*. Experiments involving *Xenopus laevis* oocytes were approved by the Animal Ethics Committee from the University of Wollongong (AE2003). Quarantine-Approved premise accreditation and Quarantine Awareness training were obtained before handling oocytes. Experiments involving C57BL/6 mice were approved by the Animal Ethics Committee of The Florey Institute, Melbourne (21-031-FINMH).

2.3 Molecular biology

2.3.1 Summary of concatenated receptors

GABA_A receptors have a pentameric stoichiometry, with the major synaptic isoform comprising of 2 α 's, 2 β 's and a γ subunit. Injection of free subunits will result in a multitude of receptor arrangements; for example, the presence of other receptor populations, such as α - β and β - β receptors, which have different biophysical properties to $\alpha\beta\gamma$ (156). To circumvent this, we used concatenated receptors using vectors developed in-house to ensure the expression of a uniform receptor system with one receptor arrangement.

Concatemers consisting of γ - β - α - β - α subunits are linked together with 15-27 AGS linkers; this results in a counterclockwise orientation of the receptor, containing two GABA binding sides at β^+/α^- interface. To allow ease for subcloning in variant subunits of interest, in-house vectors contain specific restriction enzyme sites (**Figure 2.1 A, B**). Single variant subunits designed for subcloning into appropriate tetrameric constructs to create the pentameric construct were purchased from GeneScript (Singapore).

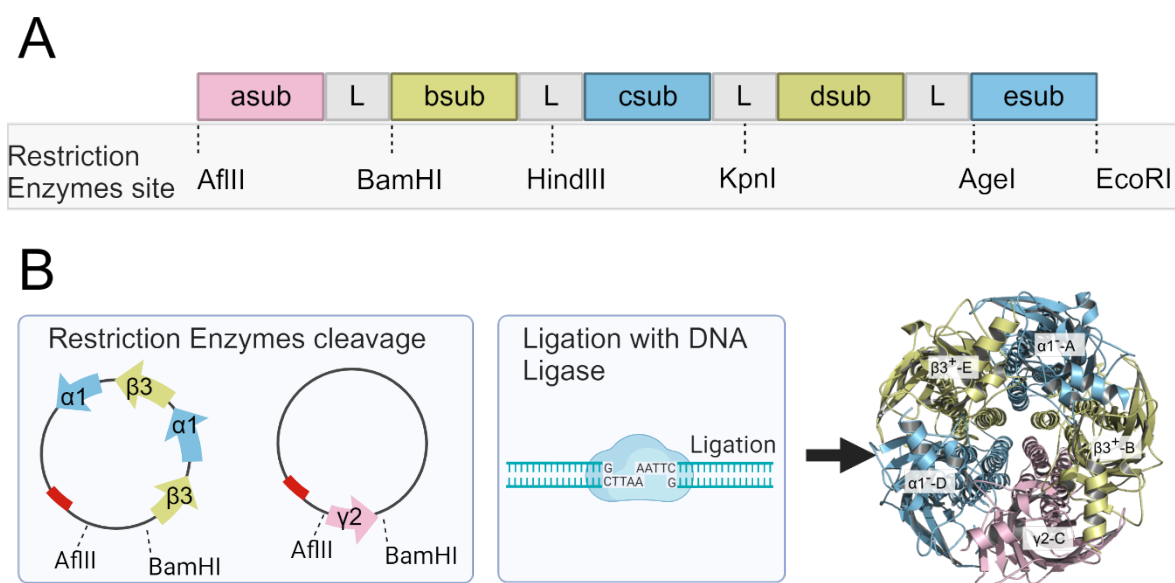


Figure 2.1 Concatenated receptors

A) Configuration of the in-house concatenated system with the restriction enzyme sites. *a*, *b*, *c*, *d*, and *esub* denote subunits, and *L* represent the linker sequence. **B)** Restriction digestion of both the tetrameric vector plasmid containing 2 α and 2 β subunits and insert plasmid, containing γ 2 subunit using restriction enzymes *AflIII* and *BamHI*. The mixture is then ligated using DNA ligase to create the recombinant DNA containing γ 2- β 3- α 1- β 3- α 1 (pdb:6hup). Created with BioRender.com

2.3.2 Restriction digestion

Restriction digestion reaction mixture for insert (**Table 2.8**):

Table 2.8 Restriction digest reaction mixture for insert

Reagent	Volume
rCutSmart buffer 10X	2 μ L
Plasmid	2 μ g
Nuclease-free water (NFW)	To 20 μ L
Restriction enzymes*	

Reactions were incubated at 37°C for 1 hour.

Restriction digestion reaction mixture for vector (**Table 2.9**):

Table 2.9 Restriction digestion reaction mixture for vector

Reagent	Volume
rCutSmart buffer 10X	3 μ L
Plasmid	2 μ g
NFW	To 30 μ L
Restriction enzymes*	
<i>Incubate reaction mix at 37°C for 30 min</i>	
rSAP ¹	1 μ L
<i>Incubate reaction mix at 37°C for 30 min</i>	

* To prevent the risk of star activity, the total restriction enzyme should be no more than 10% of the reaction mix

¹ recombinant shrimp alkaline phosphatase

2.3.3 Ligation

T4 ligase was used to ligate the insert (subunit of interest) into the vector (**Table 2.10**). To promote uptake yield, we use a 4:1 insert-to-vector ratio.

Table 2.10 Ligation reaction mixture

Reagent	Volume
T4 Ligase buffer 10X	1 μ L
Vector	-
Insert	-
NFW	To 10 μ L
T4 Ligase	0.5 μ L

2.3.4 Transformation

The wild-type (WT) or mutant plasmid DNA was then transformed into chemically competent *E.coli* cell lines (**Figure 2.2**). Neb 10- beta competent cells were retrieved from a -80°C freezer and thawed on ice before adding 1-2.5 μ L of plasmid DNA. This mixture was left on ice for 20-30 minutes, facilitating the plasmid's migration to the competent cells. The cell mixture was heat-shocked in a 42°C water bath for 45-50 seconds. 500 μ L of S.O.C (super optimal broth with catabolite repression) medium, prewarmed to 37°C, was added to the cell mixture and incubated at 37°C in a shaking incubator (240 rpm) for 1 hour. Post incubation, the cell mixture was centrifuged at 12,000 rpm for 1 minute to pellet the cells. The supernatant was removed, leaving 15 μ L to facilitate resuspension and spread onto agar plates containing the required antibiotic. Plates were inverted and placed at 37°C overnight to allow for colony formation.

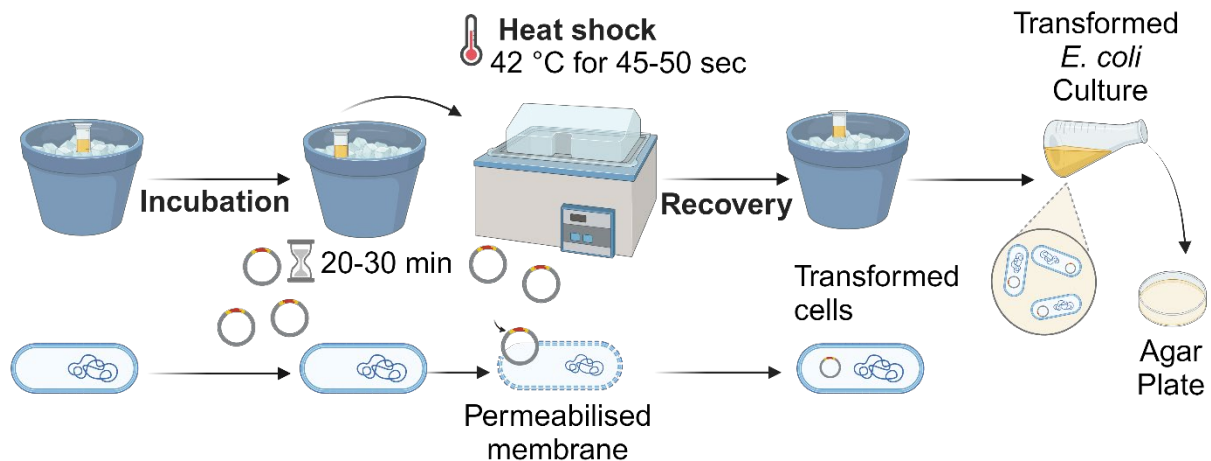


Figure 2.2 Summary of *E. coli* transformation.

See text for more details. Figure adapted from “Bacterial Chemical Transformation” by BioRender.com (2024). Retrieved from <https://app.biorender.com/biorender-templates>

2.4.5 Colony selection and growth

Single colonies were picked from the plate and placed into a 50 mL conical tube containing 5 mL of LB broth, containing desired the selection antibiotic and incubated at 37°C overnight on a shaking incubator at 240 rpm.

2.4.6 Plasmid purification

Bacterial colonies were pelleted out at 9,000 rpm for 3 minutes. Purification and separation of plasmid from *E. coli* were performed as per the manufacturer’s (QIAprep Spin Miniprep) direction. Briefly, after pelleting the bacteria, the culture was resuspended (buffer P1) and lysed (buffer P2). It was then neutralised with buffer N3, and the mixture was inverted and centrifuged at 12,000 rpm for 10 minutes to pellet out SDS/bacterial chromosomal DNA precipitate. The supernatant was then transferred to a QuikSpin column, Supernatant in the Quikspin column was then centrifuged at 6,000 rpm for 1 min then at 13,000 rpm for 1 min. DNA was eluted after adding NFW to the QuikSpin column and collected in a microcentrifuge tube. DNA purity and concentration ($OD_{A260/A280} \geq 1.8$) was determined by spectrophotometry using NanoDrop 2000c.

2.4.7 Diagnostic restriction digestion

Plasmid DNA was digested with restriction enzymes to ensure that the subunit of interest was incorporated and the band lengths were in accordance, and to ensure that DNA had not been contaminated and degraded (**Figure 2.3 A, B**).

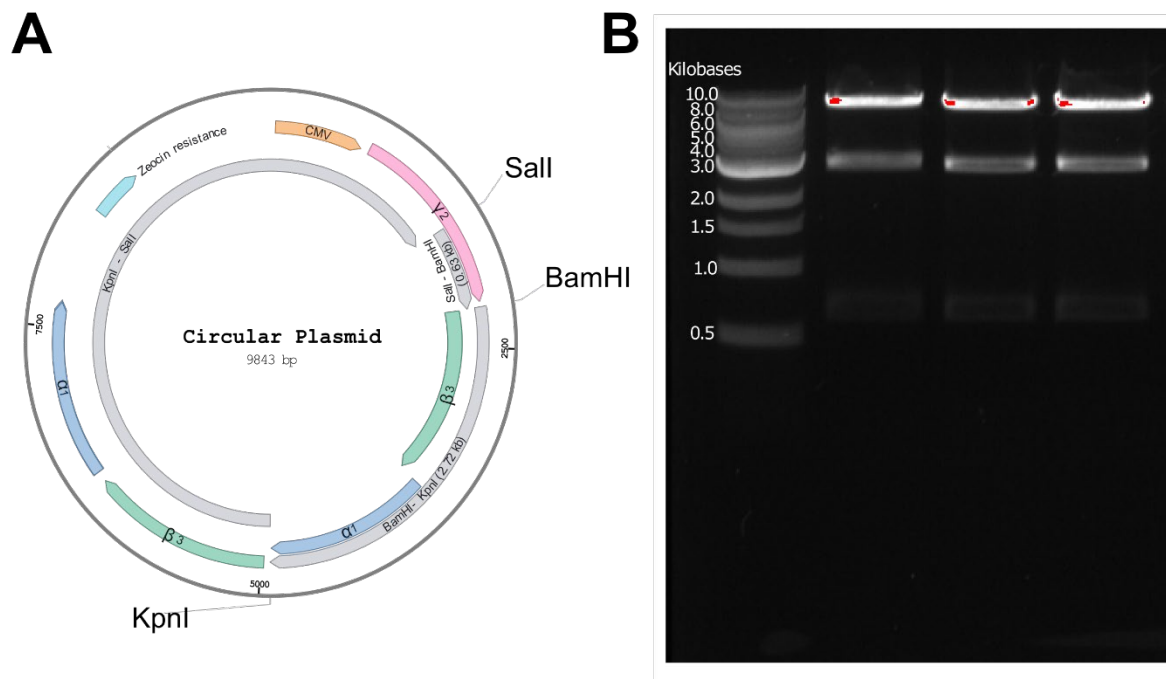


Figure 2.3 Diagnostic restriction digest

A) Circular plasmid map of $\alpha 1\beta 3\gamma 2$. The cyan arrow indicates the zeocin-resistant gene cassette. The coloured arrows indicate the subunits The pink arrow indicates the $\gamma 2$ subunit, green indicates $\beta 3$, and blue indicates the $\alpha 1$ subunit. The restriction sites used for diagnostic restriction digest (Sall, BamHI, KpnI) are flagged on the side. The grey arrows indicate the length of the expected fragments. **B)** A representative photograph of 1% agarose gel shows the DNA fragments cut with Sall, BamHI, and KpnI. The band at 0.63 kilobases indicate the Sall-BamHI fragment, and 2.72 indicates the BamHI-KpnI for the three constructs made.

2.4.8 Verification of plasmid DNA

To ensure the variant's incorporation, diagnostic restriction digestion visualised on a 1% agarose gel or sanger sequencing was performed. Samples containing 800-1200 ng of DNA, 10 mM of appropriate primer, and up to 12 μ L of NFW were sent to and sequenced by the Australian Genome Research Facility Ltd (Westmead, NSW, Australia). Sanger sequencing results (.ab1 files) were read and compared to WT sequence found in the UniProt database using DNASTAR SeqMan Ultra version 17.2.1 (Madison, WI, USA) (**Figure 2.4**).

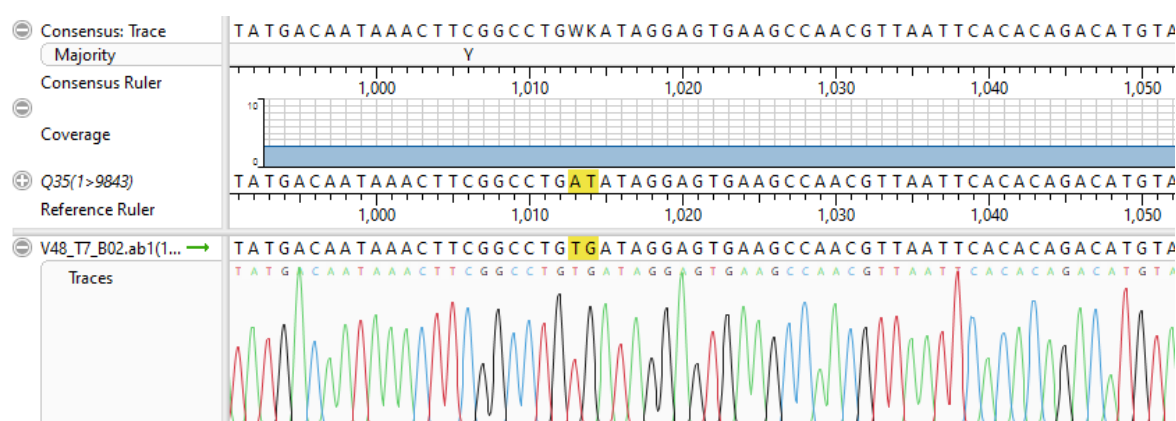


Figure 2.4 Sanger sequencing result

Representative Sanger sequencing result. Results were read with SeqMan Ultra, with the variant, in this case plasmid V48 with a reference sequence (Q35)

2.4.9 Plasmid DNA linearisation and purification

Following verification, circular plasmid undergoes linearisation with NotI at 37°C for 1 hour in preparation for RNA synthesis. Preparation is as follows (**Table 2.11**).

Table 2.11 Plasmid DNA linearisation reaction mix

Reagent	Volume
Plasmid DNA	4-5 µg/reaction
rCutSmart buffer 10X	2 µL
NotI	1 µL
NFW	To 20 µL

The DNA is purified using a Qiagen nucleotide removal kit to remove enzymes and nucleotides from the sample. To achieve this, five volumes of PNI binding buffer are added to one volume of linearisation mix, facilitating the absorption of DNA onto the silica membrane. The mixture of DNA and PNI buffer is then transferred to a spin column and centrifuged at 6,000 rpm for 1 minute. Afterwards, 750 µL of PE buffer is added to the column and centrifuged at 6,000 rpm for 1 minute, followed by centrifugation at 13,000 rpm for 1 minute to remove remaining buffer. Finally, 30-50 µL of NFW is applied to the centre of the spin column membrane and incubated for 1 minute, then centrifuged at 12,000 rpm for 1 minute to elute the DNA from the silica membrane.

2.4.10 RNA synthesis and retrieval

To initiate RNA synthesis, a T7 mMessage mMachine® kit was used (**Table 2.12**). Preparation is as follows:

Table 2.12 T7 mMessage mMachine® reaction mix

Reagents	Volume
Linearised DNA	5 µL
2× NTP/CAP	10 µL
GTP	1 µL
10× reaction buffer	2 µL
T7 enzyme	2 µL

Samples were incubated at 37°C for 2 hours.

Following incubation, 1 µL of TURBO DNase was added to the sample to remove DNA. To remove additional residues and precipitate RNA, 15 µL of lithium chloride was added to the sample and placed at –20°C overnight.

All subsequent experiments with RNA were performed on ice. The RNA sample was centrifuged at 4°C for 20 minutes at 12,000 rpm to pellet the RNA, and the supernatant was carefully removed. To remove any incorporated nucleotides, the RNA pellet was washed with 80% (v/v) of molecular grade ethanol and recentrifuged. The 80% ethanol was then removed, and the pellet was allowed to dry at room temperature for 1 hour before being resuspended in NFW. The cRNA purity and concentration were determined using a NanoDrop and stored at –20°C.

2.4 Electrophysiology

Electrical phenomena at the cell membrane predominantly arise from the asymmetric distribution of ions between the cytoplasm and the extracellular space, along with ion-selective membrane conductance (157).

The two-electrode voltage clamp (TEVC) electrophysiology is a powerful technique used to study the function of ion channels in cells, particularly in larger cells like *Xenopus laevis* oocytes. This technique allows for the measurement of ion movement through the cell's plasma membrane by controlling the membrane potential across it. TEVC electrophysiology involves impaling two electrodes into the plasma membrane of a *Xenopus laevis* oocyte: a voltage-sensing electrode and a voltage-injecting electrode (158) (**Figure 2.5**). The basic

principle of TEVC is to measure the membrane potential (V_m) differences to the preset command voltage (V_c), and the difference (ϵ) is applied to a current injecting electrode and recorded (**Figure 2.5**).

Exposure to GABA, the receptor agonist, prompts chloride ion movement across cell membrane. The direction of Cl^- flow – whether influx or efflux – depends on the Cl^- concentration gradient and membrane potential. In our setup, a higher extracellular Cl^- concentration results in Cl^- efflux. To counteract this ion movement and stabilise the membrane potential, a corrective current is injected into the cell, which is subsequently recorded. The level of receptor activity is directly proportional to the current efflux in response to GABA, leading to an increase current amplitude. This process enables functional analysis of how various variants impact GABA_A receptor activity, providing insights into their biological significance.

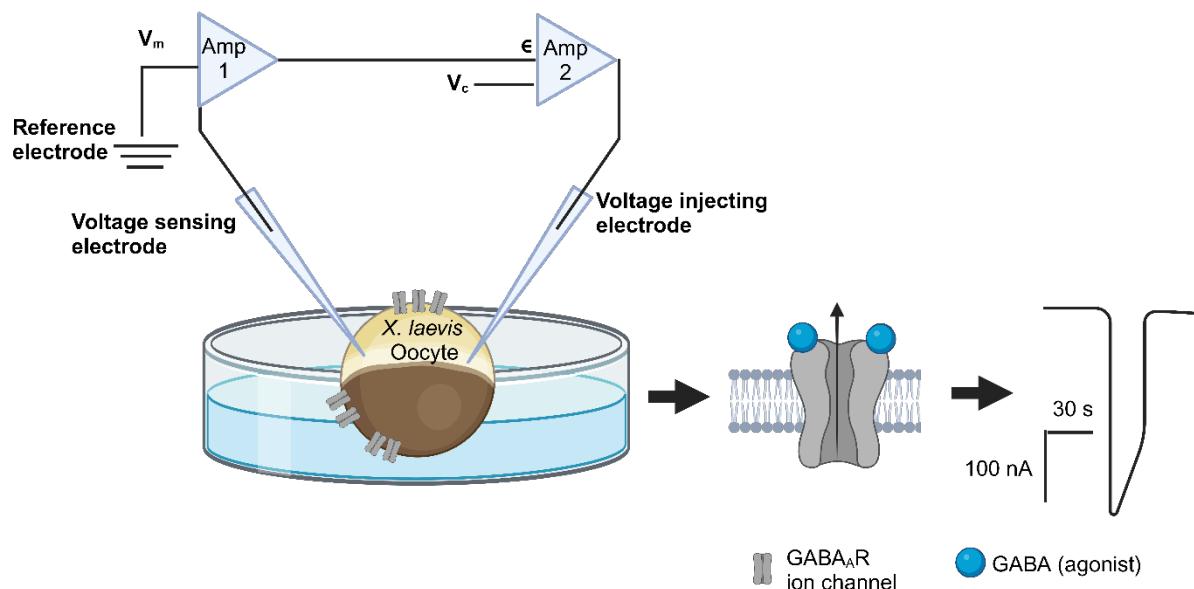


Figure 2.5 Basic principle for two-electrode voltage clamp electrophysiology

For more details, please see the text. Created with BioRender.com.

2.4.1 *Xenopus laevis* oocyte preparation

Briefly, ovarian lobes were obtained from anaesthetised *X. laevis*. To obtain free oocytes, the ovarian lobe was treated with ~2 mg/mL of collagenase dissolved in modified Barth's solution (82.5 mM NaCl, 2 mM KCl, 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5 mM HEPES). Oocytes were then rinsed with the modified Barth's solution and stored in ND96 supplemented with gentamicin and ampicillin.

25 ng of cRNA encoding for variant or WT concatenated $\alpha 1\beta 3\gamma 2$ GABA_A receptors were microinjected into stage IV-V oocytes and stored in ND96 supplemented with gentamicin and ampicillin at 18°C for 1-2 days before recording.

2.4.2 Two-electrode voltage clamp electrophysiology

Two-electrode voltage clamp electrophysiology was used to study the GABA_A receptors expressed in *Xenopus laevis* oocytes. Stage V/VI cells previously injected with the cRNA of interest were impaled with two glass borosilicate microelectrodes, one for sensing and the other for current injection; the difference in current is brought to zero by a control amplifier (for more information, see 2.4). The microelectrodes were backfilled with 3M KCl-filled glass borosilicate microelectrode with a resistance of 0.2-1.4 m Ω . Cells were then clamped at either -50 mV (for $\gamma 2$ variants) or -60 mV (for $\beta 3$ variants - desensitisation). Oocytes were continuously perfused with ND96. All experiments were performed at room temperature with at least two batches of oocytes. All experiments were performed in parallel with WT receptors and statistical comparisons are made for data on the same day to account for interbatch and interday variations (more detail in Review by Nathan *et al.* 2023 (85)) **(Figure 2.6, more will be discussed in Chapter 4).**

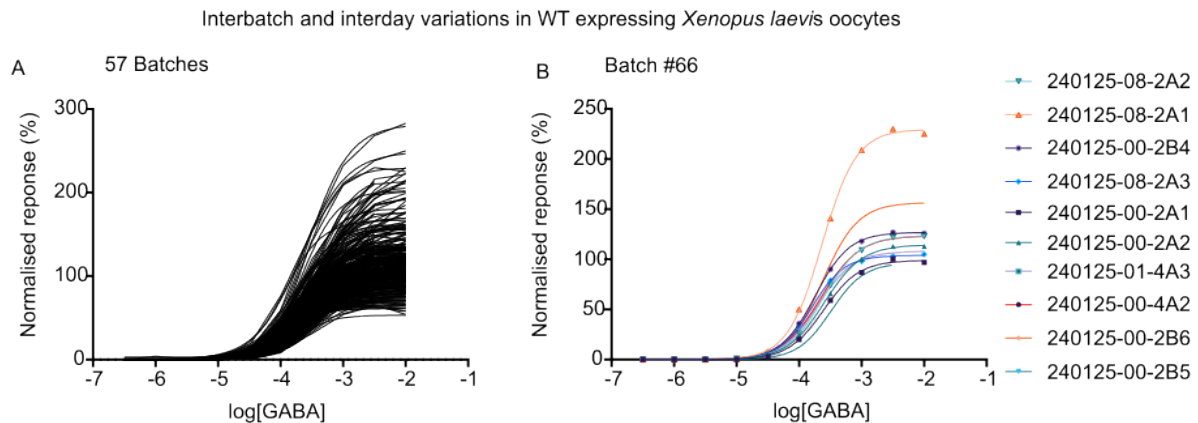


Figure 2.6 Interbatch and interday variations in GABA sensitivity for WT receptors

A) Interbatch variation: Concentration-response curve for 57 batches, comprising 376 single WT-expressing cells. The graph reveals significant variations in GABA sensitivity among individual cells, with LogEC₅₀ values ranging from -3.153 to -4.055 (range: 0.9, SD: 0.17). The mean LogEC₅₀ of was -3.617 (SD:0.172). B) Interday variation: concentration-response curve for Batch #66. Each line indicates a single cell, n = 10 cells. LogEC₅₀ = -3.645 (SD: 0.085).

2.4.3 *In vitro* functional assay

A custom-built TEVC apparatus was used to conduct electrophysiological recordings.

To assess the variant's functional effects on receptor function, we evaluated changes in EC₅₀ and GABA-elicited maximum current.

2.4.3.1 Sensitivity

GABA sensitivity is a fundamental characteristic of GABA_A receptor that determines the GABA concentration range within which receptors are active. A variant that increases receptor sensitivity, a lower EC₅₀ value, means that the receptor is more sensitive to GABA, thus requiring less GABA to reduce neuronal excitation. Activation of GABA_A receptor requires binding two GABA neurotransmitters to the β+/α- interface, allowing the opening of the ion pore and influx of chloride into the cell, reducing the possibility of action potential from firing.

It is well-documented that even minor variations in GABA sensitivity at GABA_A receptor can lead to significant clinical and physiological effects. For instance, therapeutic doses of benzodiazepines can induce approximately a twofold change in GABA sensitivity, resulting in pronounced effects on the nervous system, such as anxiolysis and sedation (75). This makes GABA sensitivity a robust measure for assessing the potential clinical relevance of experimental findings.

The EC₅₀ value was derived from the GABA concentration-response relationship, achieved by increasing concentration in the system and recording the responses. A Hill equation was fitted to the peak GABA-elicited amplitude for each oocyte:

$$I = Abs. I_{\max} \left(\frac{[A]^{nH}}{[A]^{nH} + EC_{50}^{nH}} \right) \quad (1)$$

Where I is the peak GABA-elicited response for the oocyte, $Abs. I_{\max}$ is the absolute maximum current, $[A]$ is the ligand concentration, and nH is the Hill slope. The bottom plateau was constrained to 0. Data are normalised to the fitted maximum responses of individual cells. EC₅₀, the concentration at which elicits the half-maximal response, was determined for the individual concentration-response curve. The LogEC₅₀ value was then calculated from the EC₅₀ value.

The relative change in LogEC_{50} between variant and WT was used to determine how the variant changed the receptor's sensitivity to GABA. Variants that significantly (more in Section 2.5.1) reduced the LogEC_{50} from WT were noted as a GoF variant, while those that increased the LogEC_{50} were noted as a LoF variant. The change in LogEC_{50} , ΔLogEC_{50} :

$$\Delta\text{LogEC}_{50} = \text{LogEC}_{50(\text{WT})} - \text{LogEC}_{50} \quad (2)$$

Where the $\text{LogEC}_{50(\text{WT})}$ is the mean LogEC_{50} of WT receptors on a given experimental day and LogEC_{50} is the LogEC_{50} of individual variant experiments.

2.4.3.2 Expression – Maximum current

A proxy to determine the surface expression of functional receptors on the cell surface is the maximum current amplitude (I_{max}) elicited by the ligand and a parameter to measure the efficiency of inhibitory neurotransmission. The maximal elicited current is the maximum opening probability at saturating concentration of GABA and the surface expression relative to the WT. To determine I_{max} , a saturating concentration of GABA, 10 mM, was applied to the system for WT (Abs. $I_{\text{max}}(\text{WT})$) and variant (Abs. I_{max}) GABA_A receptor. I_{max} , calculated for a single oocyte, is given by the equation:

$$I_{\text{max}} = \frac{\text{Abs. } I_{\text{max}}}{\text{Abs. } I_{\text{max}}(\text{WT})} \quad (3)$$

Currents were recorded using GeneClamp 500B, digitised by Digidata 1440A and recorded on a PC with Clampex (Axon instrument).

2.4.3.3 Desensitisation

Desensitisation occurs when the receptor enters a “shut” state despite the presence of agonists (159). This commonly occurs during prolonged application of agonists, and can occur if a variant results in the receptor being more sensitive to GABA. Receptor desensitisation was previously hypothesised to exacerbate a loss of receptor function (160). Two parameters that are affected by desensitisation are the current decay rate, k (s^{-1}), and the current at steady-state during a prolonged application of a saturating concentration of agonist.

A semi-automatic gravity-driven perfusion system was used to conduct the desensitisation experiment. Solutions were applied via two separate tubes to a low-volume staged oocyte recording chamber to remove any dead volume in the application system. Pre-programmed

electronic valves were used to switch the solutions between ND96 and test solutions. Using LabChart, macro function, I programmed the system to automatically apply different solutions, switching immediately from buffer to agonist. The protocol used are specified in **Table 2.13**.

Table 2.13 LabChart macro for desensitisation experiment (Chapter 6)

MACRO
Call Doc.Use Digital Output Configuration ("Config 1" <i>aka buffer</i>) hours = 0 minutes = 2 seconds = 0 Call Wait For (hours, minutes, seconds) Call Doc.AddCommentAtEnd (0, "3mM GABA") ' Creates a comment in the first channel. Call Doc.AddCommentAtEnd (1, "3mM GABA") ' Creates a comment in the first channel. Call Doc.AddCommentAtEnd (2, "3mM GABA") ' Creates a comment in the first channel. Call Doc.UseDigitalOutputConfiguration ("Taps 345") hours = 0 minutes = 0 seconds = 150 Call WaitFor (hours, minutes, seconds) Call Doc.AddCommentAtEnd (0, "") ' Creates a comment in the first channel. Call Doc.AddCommentAtEnd (1, "") ' Creates a comment in the first channel. Call Doc.AddCommentAtEnd (2, "") ' Creates a comment in the first channel. Call Doc.UseDigitalOutputConfiguration ("Config 1") hours = 0

minutes = 5

seconds = 0

Call WaitFor (hours, minutes, seconds)

Call Doc.AddCommentAtEnd (0, "3mM GABA") ' Creates a comment in the first channel.

Call Doc.AddCommentAtEnd (1, "3mM GABA") ' Creates a comment in the first channel.

Call Doc.AddCommentAtEnd (2, "3mM GABA") ' Creates a comment in the first channel.

Call Doc.UseDigitalOutputConfiguration ("Taps 345")

hours = 0

minutes = 0

seconds = 150

Call WaitFor (hours, minutes, seconds)

Call Doc.AddCommentAtEnd (0, "") ' Creates a comment in the first channel.

Call Doc.AddCommentAtEnd (1, "") ' Creates a comment in the first channel.

Call Doc.AddCommentAtEnd (2, "") ' Creates a comment in the first channel.

Call Doc.UseDigitalOutputConfiguration ("Config 1")

hours = 0

minutes = 1

seconds = 0

Call WaitFor (hours, minutes, seconds)

Call Doc.AddCommentAtEnd (0, "10mM GABA + 10uM etomidate") ' Creates a comment in the first channel.

Call Doc.AddCommentAtEnd (1, "10mM GABA + 10uM etomidate") ' Creates a comment in the first channel.

Call Doc.AddCommentAtEnd (2, "10mM GABA + 10uM etomidate") ' Creates a comment in the first channel.

Call Doc.UseDigitalOutputConfiguration ("Taps 678")

```

hours = 0
minutes = 0
seconds = 20
Call WaitFor (hours, minutes, seconds)

Call Doc.AddCommentAtEnd (0, "") ' Creates a comment in the first channel.
Call Doc.AddCommentAtEnd (1, "") ' Creates a comment in the first channel.
Call Doc.AddCommentAtEnd (2, "") ' Creates a comment in the first channel.
Call Doc.UseDigitalOutputConfiguration ("Config 1")
hours = 0
minutes = 8
seconds = 0
Call WaitFor (hours, minutes, seconds)

Call Doc.AddCommentAtEnd (1, "10mM GABA + 10uM etomidate") ' Creates a comment in the
first channel.
Call Doc.AddCommentAtEnd (2, "10mM GABA + 10uM etomidate") ' Creates a comment in the
first channel.
Call Doc.UseDigitalOutputConfiguration ("Taps 678")
hours = 0
minutes = 0
seconds = 120
Call WaitFor (hours, minutes, seconds)
End Sub

```

Recording was initiated with a 2-minute buffer application to ensure the cell was stable. The cell was then exposed to 3 mM GABA for 150 s twice, to ensure that the traces are stable. 10 mM GABA with 10 μ M etomidate was then applied for 20 s to prime the tubing. This was then followed by an 8-minute buffer wash to ensure all receptors re-sensitise before the final 120 s application of 10 mM GABA with 10 μ M etomidate. Etomidate was added to help facilitate the

maximal open probability of the channel. For LoF variants, to ensure a saturating concentration of GABA, 30 mM GABA and 30 mM GABA with 10 μ M etomidate were used instead of 3 mM GABA and 10 mM GABA with 10 μ M etomidate, respectively.

The time between valve opening to peak amplitude was approximately 1-2 s. Currents were recorded with Warner OC-725C amplifier, sampled at a frequency of 1 kHz and filtered at 10 Hz. Traces were digitalised with Labchart reader version 8.1 (AD Instruments, Sydney, Australia).

To determine the current decay rate and the current at steady-state, traces were fitted to a one-phase exponential decay, where:

$$Y = (Y_0 - \text{Plateau})e^{-kX} + \text{Plateau} \quad (3)$$

Where Y is the amplitude, X is the time (s). Plateau is the current amplitude at steady-state; this will be referred to as the current at steady-state (I_{ss}) in the thesis. k is the rate of current decay. For this set of experiments, k is the decay rate per second i.e. $k \text{ (s}^{-1}\text{)}$.

The maximum steady-state open probability (Est $P_{o(ss, \max)}$), was defined as I_{ss} normalised to the current evoked by 3 mM GABA and then transformed to the estimated maximum open probability (Est $P_{O(\max)}$) determined for each day of recording.

2.5 Electrophysiology - Statistical analysis

Unless specified, all data are presented as mean $\pm$ standard deviation (SD).

2.5.1 GABA sensitivity - Concentration-response- relationships

Nonlinear regression was performed for GABA concentration-response relationships using GraphPad Prism 8.0.1 (Dotmatics, Boston, MA). One-way ANOVA with multiple comparisons with Dunnett's post-hoc test was used for LogEC_{50} . A conservative threshold for significance of $p < 0.0001$ and a ΔLogEC_{50} shift exceeding ± 0.2 is required for the variant to be deemed to significantly alter GABA sensitivity.

Given the inherent variabilities observed in WT receptors, including inter-day and inter-batch variability, it becomes essential to elucidate the degree of variations. To address this, we calculated the combined standard deviation (S_C) of WT receptors using the formula:

$$S_c = \sqrt{\frac{n_1[S_1^2 + (\bar{X}_1 - \bar{X}_c)^2] + n_2[S_2^2 + (\bar{X}_2 - \bar{X}_c)^2]}{n_1 + n_2}}$$

Where n is the number of observations, $\bar{X}$ is the mean, S^2 is the variance.

2.5.2 Maximal current

Maximal GABA-evoked currents were compared with the Mann-Whitney U test. A conservative threshold, $p < 0.0001$ and an $I_{\max}$ reduction exceeding 2-fold i.e. the variant must give less than 50% of remaining current amplitude to be determined as significant.

2.5.3 Desensitisation

Linear and non-linear regression were performed with GraphPad Prism version 8.0.1. Traces were digitalised using LabChart reader version 8.1 (ADInstruments). Analysis of traces was performed with Microsoft Excel and GraphPad Prism. Non-parametric one-way ANOVA, Kruskal-Wallis rank sum test, and a post-hoc Dunn's test were used to compare the different groups.

Exclusion criteria for oocytes: Oocytes with a holding potential of less than -200 nA were excluded from the study, except for variants known to induce constitutive activity (e.g. $\beta 3^{L284R}$).

2.6 Clinical data

2.6.1 Retrieving patient data

Patient data were collected in collaboration with the Danish Epilepsy Center, Filadelfia, Denmark and through PubMed search using keywords “*GABRG2* AND Epilepsy” and “*GABRG2* AND [insert variant]”.

Newly identified individuals were recruited at the Danish Epilepsy Centre and through an international network of epilepsy and genetic centres. All data were collected at the Danish Epilepsy Centre. The epilepsy syndromes were classified according to the most recent ILAE classification.

Genotyping was performed using targeted gene panels, whole exome, or whole genome sequencing. Genomic variants and familial segregation were confirmed by direct Sanger sequencing. All variants were annotated using the NM_198904.4 (GRCh37 / hg19) transcript of *GABRG2*, which corresponds to the $\gamma 2L$ splice form of the $\gamma 2$ subunit.

2.6.2 Phenotypic analysis

Data of patient phenotype were collected by our collaborators at the Danish Epilepsy Center Filadelfia, Demark. Phenotypic analysis of affected individuals and their families were performed through interviews, review of their medical history, and neuroimaging. The referring physician assessed developmental and cognitive levels and classified them according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition.

2.6.3 Handling incomplete data

For the purpose of statistical comparisons, incomplete data were managed as follows:

1. Patients were excluded from the analysis of age at seizure onset if the data were not recorded.
2. Patients were classified as having specific epilepsy syndrome when these were documented; otherwise, they were considered negative for these types.
3. For severe ID, hypotonia, movement disorders, and vision impairment, patients were excluded from the analysis if the relevant data were not recorded.

2.6.4 Statistical analysis

Qualitative clinical outcomes such as language impairment, visional impairment, degree of ID, neuropsychiatric and neurological abnormalities, the odds ratio (OR) was compared using Fisher's exact test.

2.7 Assessment of *in silico* tools

The following section pertains to Chapter 4

The predictive results were indicated by true positives (TP, correct predictions for pathogenic mutations), true negatives (TN, correct predictions for benign variants), false positives (FP,

incorrect predictions for pathogenic variants) and false negatives (FN, incorrect predictions for benign variants)

We calculated the accuracy, specificity, and sensitivity of the tools.

Accuracy: % correct. This is given by the formula:

$$\text{Accuracy: } \frac{TN + TP}{TN + TP + FP + FN}$$

Specificity: Proportion of true negative in healthy cases. This is expressed as a percentage. Specificity is given by the formula:

$$\text{Specificity: } \frac{TN}{TN + FP} \times 100$$

Sensitivity: Proportion of true positives in healthy cases, expressed as a percentage. Sensitivity is given by the formula:

$$\text{Sensitivity: } \frac{TP}{TP + FN} \times 100$$

Bioinformatic assessment of the significance of *GABRG2* missense mutations

The effects of the nonsynonymous amino acid change were predicted using seven bioinformatic tools: PolyPhen-2 (Polymorphism Phenotyping V2) (accessed 28 March 2024), FATHMM-W (Functional Analysis through Hidden Markov Models, weighted) (accessed 28 March 2024), CADD (Combined Annotation Dependent Depletion) (accessed 28 March 2024), SIFT (Sorting Intolerant from Tolerant algorithm) (accessed 28 March 2024), AlphaMissense (accessed 9 April 2024), MutPred (accessed 10 April 2024), and REVEL (Rare Exome Variant Ensemble Learner) (accessed 10 April 2024).

2.8 Animal Experiments and Immunohistochemistry

2.8.1 Tissue preparation

3 *GABRB3*^{+/+} and 3 *GABRB3*^{E77K/+} mice were used to perform immunohistochemistry. Tissues were fixed in 4% paraformaldehyde in PBS and cryoprotected by increasing sucrose concentration in PBS before cryosectioning at 30 µm sections using a cryostat (Leica

CM1950). Sections were collected in a 1:10 series and stored in antifreeze (containing 50% 0.1M sterile PBS/30% ethylene glycol/20% glycerol) at -20°C until required. Unless specified, tissues were washed 3 times for 10 minutes with PBS for all immunohistochemical processing. Experiments were performed on free-floating sections; all washes, incubations, and reactions took place in a glass vial on an orbital shaker at 100 rpm.

2.8.2 Nissl staining and analysis

Nissl staining, using cresyl violet, is a common method to stain for the nissl bodies, the rough endoplasmic reticulum, and is useful in identifying the basic structure of the brain (161). Briefly, sections are mounted on gelatinised slides and air-dried prior to staining. Slides with mounted sections were washed briefly in tap water and dehydrated by passing through increasing concentrations of ethanol (70%, 95%, 100%), then rehydrated with lowering the concentrations of ethanol (95%, 70%) for 2-3 minutes each. Slides were stained with 0.1% cresyl violet for 8 minutes, washed briefly with distilled water and 20 s washes with 50%, 70%, and 100% ethanol. Slides were then cleared in xylene for 10 minutes and coverslipped with Eukitt. Imaging for nissl stain was conducted using Leica Aperio at 20X objective.

Volume estimation

Staining the tissue with cresyl violet allowed for the delineation of brain regions. The size of the hippocampus was measured by tracing around the hippocampus and then corrected for the thickness and series of tissues collected. The structural volume of the brain (from -1.94 - -2.54 mm of bregma) was calculated using the Cavalieri method (162). Such that:

Volume of the hippocampus (mm³) = [area of the hippocampus] x [tissue thickness] x [number of sections]

Where, each section is 30 µm thick, and one section out of 10 was used for cresyl violet staining.

2.8.3 Fluoro-Jade C

Fluoro-Jade C (FJC) is a polyanionic fluorescein derivative that exhibits a high degree of sensitivity and selectively binds to degenerative neurons, allowing for the detection of apoptotic cells from early to late stages of apoptosis (163, 164) . Fluoro-Jade C stains for

neuronal death unspecific to the death pathway, which is beneficial for determining whether neuronal death occurs in GoF mice.

Briefly, sections were washed in PBS and then pre-treated with ethanol to rehydrate the tissues and allowed for permeabilisation. Tissues were placed in 80% ethanol with 1% sodium hydroxide, followed by 70%, 50%, 30% and 10% ethanol and then passed in water. To reduce fluorescent background, sections were then placed in 0.06% potassium permanganate solution, a fluorescence quencher for 10 minutes, washed in ddH₂O and immersed in 0.0001% FJC with DAPI (1:1000) for 20 minutes and then washed 3 times in ddH₂O. Brain sections were then recovered in PBS, mounted onto 2% gelatinised slides, and sealed with Fluoroshield. Images were scanned with Leica DM6000 at 20X objective.

2.8.4 Iba-1 staining and analysis

Immunohistochemistry was performed as previously described (165). Iba-1 is a marker of microglia, the resident immune cell of the CNS. Briefly, free-floating sections were removed from the anti-freeze solution and washed in PBS. To allow for cell permeabilisation, sections were washed in 50% ethanol for 30 minutes. Sections were then treated in 3% H₂O₂ in 50% ethanol to quench endogenous peroxidase activity. Wash with PBS ensued, and the sections were placed in a blocking solution of 10% normal horse serum (NHS) in 0.1M PBS for 30 minutes and then incubated in Iba-1 (1:1000 in 2% NHS/PBS) at 4°C overnight. Sections were then washed and incubated in horse anti-rabbit IgG (1:500 in 2% NHS in PBS) for 2 hours, washed, and incubated in ExtrAvidin Peroxidase (1:1000 in PBS) for 2 hours. Iba-1 was then visualised with 3,3'-diaminobenzidine (DAB) kit as per the manufacturer's instruction. The reaction ran for 10 minutes and stopped by washing the section 4 times in PBS.

Sections were then mounted onto gelatinised slides, dehydrated, and defat using xylene before cover slipping with Eukitt. Images were scanned with Leica Aperio at 20X objective. Photomicrographs of 3-5 brain sections, between -1.94 to -2.54 mm of bregma, were collected for data analysis. Results are reported as the average number of Iba-1 positive cells per section for each mouse.

2.8.5 Immunohistochemistry fluorescence

Briefly, sections were thoroughly washed in PBS. To minimise fixative-induced autofluorescence, sections were treated with 0.1% sodium borohydride for 10 minutes. To allow for membrane permeabilisation, sections were washed in 0.3% Triton X-100 in PBS and

blocked with 10% normal goat serum (ngs) in 0.3% Triton X-100 in PBS for 1 hour. Sections were then incubated in anti-MBP (1:1000), anti-ZnT3 (1:500), in 5% ngs/0.15% Triton X-100/PBS overnight at 4°C. Sections were then washed thoroughly in PBS at room temperature and incubated with AlexaFluor 568 secondary antibody (1:500) in 5% ngs in 0.15% Triton X-100 in PBS for 1 hour. Once the secondary antibody was applied, sections were protected from light to prevent the loss of fluorophores. Cryosections were then washed and incubated in DAPI for 30 minutes, then mounted onto gelatinised slides and then cover slipped with Fluoroshield.

2.8 Fluorescence imaging

Fluorescence imaging of myelin basic protein (MBP) was performed using an Olympus 120 VS with a 20X objective, and mossy fibre imaging was performed using a DM6000 with a 20X objective.

2.9 Animal - Statistical analysis

Data are displayed as mean $\pm$ SD. Statistical significance was determined using Mann-Whitney U test and $p < 0.05$ was considered statistically significant.

One mouse, ID:44, was removed during analysis due to injury caused to the brain by EEG electrodes.

Chapter 3 Phenotypic spectrum in individuals with pathogenic *GABRG2* loss- and gain-of-function variants

Summary

Rare missense and protein-truncating variants in the *GABRG2* gene have been linked to epilepsy. Epilepsy-associated genetic GABA_A receptor variants were first reported in 2001, with the p.(Arg82Gln) variant identified in an Australian family (166) and later the p.(Lys328Met) variant in a French family (167). Both are familial *GABRG2* variants but with different degrees of penetrance and expressivity. The *GABRG2* p.(Lys328Met) variant displayed full penetrance of genetic epilepsy where patients were diagnosed with GEFS+, while the p.(Arg82Gln) variant displayed around 75% penetrance with patients diagnosed with a mixture of childhood absence epilepsies (CAE) and/or FS (113, 167). GEFS+ is a familial epilepsy syndrome with phenotypes ranging from simple FS, which may self-resolve as the child grows up, to EE, in which the patient prognosis is dire (168). These epileptic syndromes manifest with a spectrum of seizure types and often present with comorbidities including hypotonia, DD, ID, and behavioural disorders (93, 110, 169). However, the phenotypic spectrum of *GABRG2* variants has expanded in recent years, with an increase in reports of infantile-onset epilepsy syndromes associated with *GABRG2*, with the spectrum ranging from FS, CAE, to DEE and EIDEE (93, 170-172).

To date, reduced receptor activity or LoF, such as impaired receptor assembly, trafficking, or channel activation (172-174) have been proposed as the primary pathomechanism for *GABRG2*-related epilepsy. In fact, certain protein truncating nonsense variants have been speculated to cause more severe phenotypes than missense variants due to dominant-negative effects (175). Although haploinsufficiency of GABA_A receptor may cause epilepsy, this simplistic view cannot explain the range of disease severity and the various comorbidities associated with specific variants.

In the last 3-5 years, there have been increasing reports of epilepsy-associated variants causing a LoF and GoF GABA_A receptor, in subunit genes, including *GABRA1* (94, 176), *GABRA4* (82), *GABRB3* (75, 149), and *GABRD* (177). Therefore, a direct comparison between the phenotypes of nonsense, LoF and GoF missense variants are required to

determine the precise cause of the most severe epilepsy syndromes associated with *GABRG2* variants.

Aim: To perform a comprehensive overview of the phenotypic and genotypic spectrum and investigate the underlying mechanisms by which *GABRG2* variants result in a highly diverse epileptic clinical phenotype

Hypothesis: Given recent findings that variants in other GABA_A receptor subunit genes exhibit GoF or LoF effects, with GoF variants being more severe, I hypothesise that GoF *GABRG2* variants will be associated with a more severe clinical phenotype. Additionally, since the $\gamma 2$ subunit is commonly reported in the literature for its important role in receptor trafficking, I hypothesise that null variants will have a milder clinical outcome and better prognosis than GoF *GABRG2* variants as the receptors that do manage to traffic, could potentially maintain some level of activity, and current ASMs could provide therapeutic benefits.

Methods

Through extensive networks of international clinical and scientific collaborations, a cohort of 44 unreported *GABRG2* cases containing null and missense variants were identified. This included a total of 32 variants comprising of 15 missense and 17 null variants. Functional analysis was performed to discriminate between patients harbouring either LoF or GoF missense variants.

Missense variants were expressed by *Xenopus* oocytes as singly mutated concatenated receptors. WT and 15 single-variant $\gamma 2$ subunits were purchased from GenScript (Singapore) and subsequently subcloned into the X position of an in-house tetrameric concatenated construct, denoted as (X- $\beta 3$ - $\alpha 1$ - $\beta 3$ - $\alpha 1$) (Details in Chapter 2, Section 2.3.1). Variants were then evaluated for changes in GABA sensitivity (ΔLogEC_{50}) and maximum GABA-evoked current amplitudes using TEVC electrophysiology. Data were compared with one-way ANOVA and Dunnett's post-hoc test. Comparisons were restricted to data from variants measured on a single day (Details in Chapter 2).

Results

TEVC electrophysiology – functional assay

To examine the variant's effect on the receptor, we assessed two functional parameters: GABA sensitivity (ΔLogEC_{50}) and maximum evoked current (I_{max}). These parameters are two of most critical determinants of the receptor's ability to open and depress neuronal action potential firing.

GABA sensitivity: A One-way ANOVA revealed significant differences between GABA sensitivities for WT and mutant receptors ($F(15, 392) = 120.7$). Among the 15 missense variants assessed, eight showed significantly decreased GABA sensitivity consistent with a LoF effect ($\Delta\text{LogEC}_{50} = -0.33$ to -0.70 ; all $p < 0.0001$, Dunnett's post-hoc test) (Figure 4.1). Additionally, two mutated receptors exhibited a significant increase in GABA sensitivity, indicating GoF properties (A106T and S139N; $\Delta\text{LogEC}_{50} = 0.33$ to 0.73 ; $p < 0.0001$ Dunnett's post-hoc test). The remaining five variants, I85K, T90M, V104G, M199V and I344L, did not significantly change receptor sensitivity to GABA compared to WT (**Figure 3.1**).

Maximum GABA-evoked current amplitude: Of the 15 variants assessed, six variants showed significant reduction in maximum elicited current compared to WT ($I_{\text{max}} 0.11 - 0.51$, $p < 0.0001$, Mann-Whitney U test) (**Figure 3.1**). No significant change in maximum GABA-evoked current amplitudes were observed for the remaining nine mutated receptors.

Categorisation of variant based on functional consequence:

- Two variants, A106T and S139N, were categorised as GoF variants due to their increased GABA sensitivity.
- Eight variants were classified as LoF due to their reduction in GABA sensitivity and/or reduction in maximum elicited current. Four variants, P83T, R125C, M169T, and L326H, reduced both GABA sensitivity and maximum currents. Four variants, A322D, A322T, R323Q and R323W, reduced GABA sensitivity without affecting the maximum evoked current alone.
- Five variants, I85K, T90M, V104G, M199V, and I344L, were classified as VUS because they were either outside the threshold p-value or ΔLogEC_{50} shift.

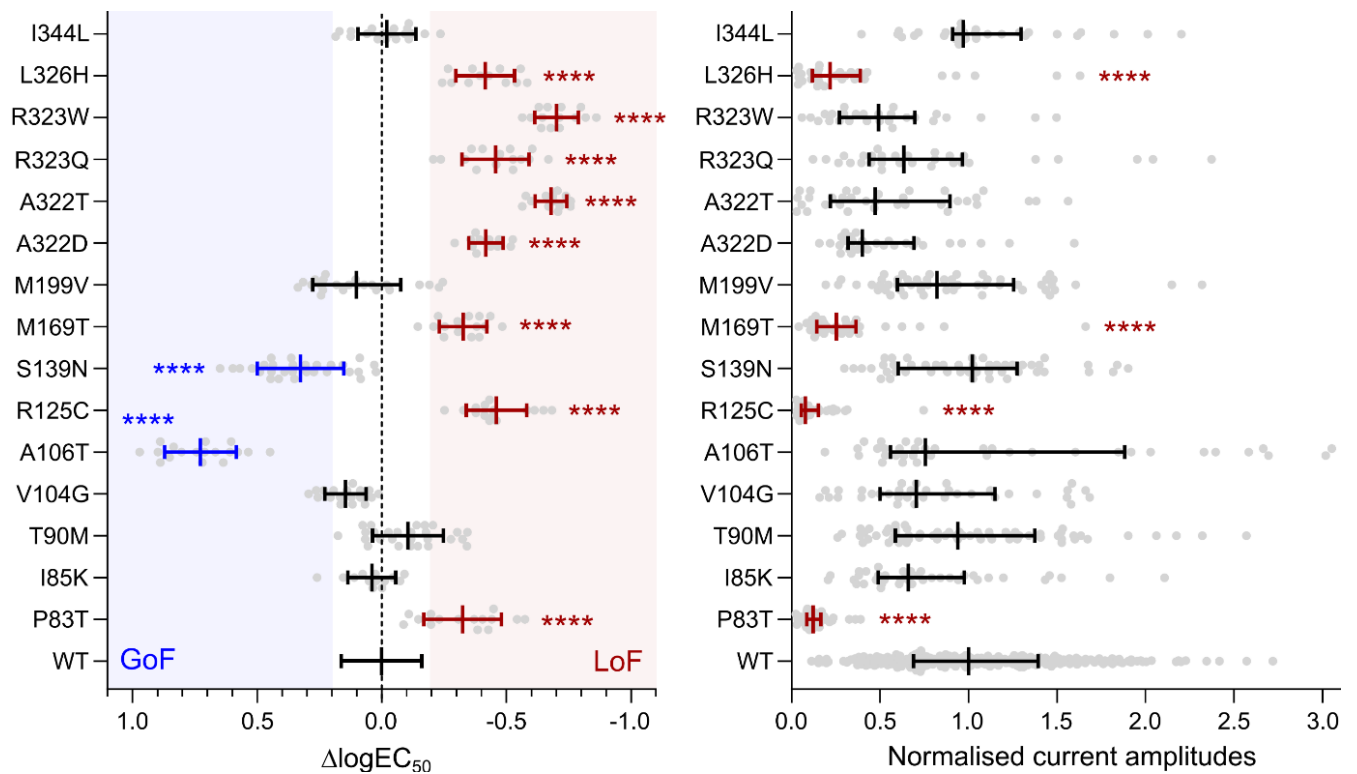


Figure 3.1 Functional analysis of 15 $\alpha 1\beta 3\gamma 2$ GABA_A receptors containing $\gamma 2$ variants.

(Left) Changes in GABA sensitivity between WT and 15 mutated receptors were calculated from the logarithmic conversion of EC_{50} values (ΔLogEC_{50}) on each experimental day. Final ΔLogEC_{50} datasets for each variant contain data from $n = 12$ -29 independent experiments. Bars indicate mean $\pm$ SD; each dot represents a single cell. Significance was determined by one-way ANOVA ($F(15, 392) = 120$; $P < 0.0001$) with Dunnett's post-hoc test (**** = $P < 0.0001$). The transparent blue and red areas above 0.2 and below -0.2, respectively, indicate the typical threshold mutated receptors must surpass in either direction to show significance.

(Right) Maximum GABA-evoked current for each variant was normalised to the WT (on the same day). Data presented as median with interquartile range (IQR) for $n = 303$ (WT) or $n = 27$ -65 (for $\gamma 2$ variants) experiments. Significance was determined by a Mann-Whitney U test (**** = $P < 0.0001$).

Phenotypic differences between null and missense variants

It was determined that pathogenic *GABRG2* can be segregated into three categories: null variants, LoF and GoF missense variants. Comparison of the median age of seizure onset between null (16 m, range: 5 months to 2 years and 4 months) and missense LoF variants (16 months, range: 7 months to 3 years and 6 months) showed no statistically significant difference, however, a comparison of the epilepsy syndrome severity revealed that patients

with missense LoF variants were associated with a more severe epileptic phenotype outside of the GEFS+ spectrum than null variants (OR = 29 [95% CI:3.2-330], $p = 0.00090$, Fisher's Exact Test). For instance, patients with null variants tend to have FS and FS+, whereas DEE and focal seizures were more commonly observed in individuals with missense LoF variants (**Figure 3.2**). The prevalence of severe of either DD/ID) was more frequently in LoF missense than null (OR = 41 [CI: 4.3-460], $p = 0.00022$, Fisher's Exact Test) (**Figure 3.2**). Taken together, null variants were associated with milder syndromes than missense LoF variants.

*Individuals harbouring GoF variants had a median age of seizure onset at 1.8 months (95% CI: 1.0-3.5), with 4/5 patients having DEE and one with ASD with mild DD/ID, speech delay and hypotonia but no seizures (**Figure 3.2**). DD and ID for the GoF cohort tend to be on the severe end of the spectrum, with 4/5 having profound to severe DD and 2/5 having profound to severe ID (**Figure 3.2**). The low number of GoF variants and the high proportion of the recurrent A106T variant prevented rigorous comparisons with the LoF cohort.*

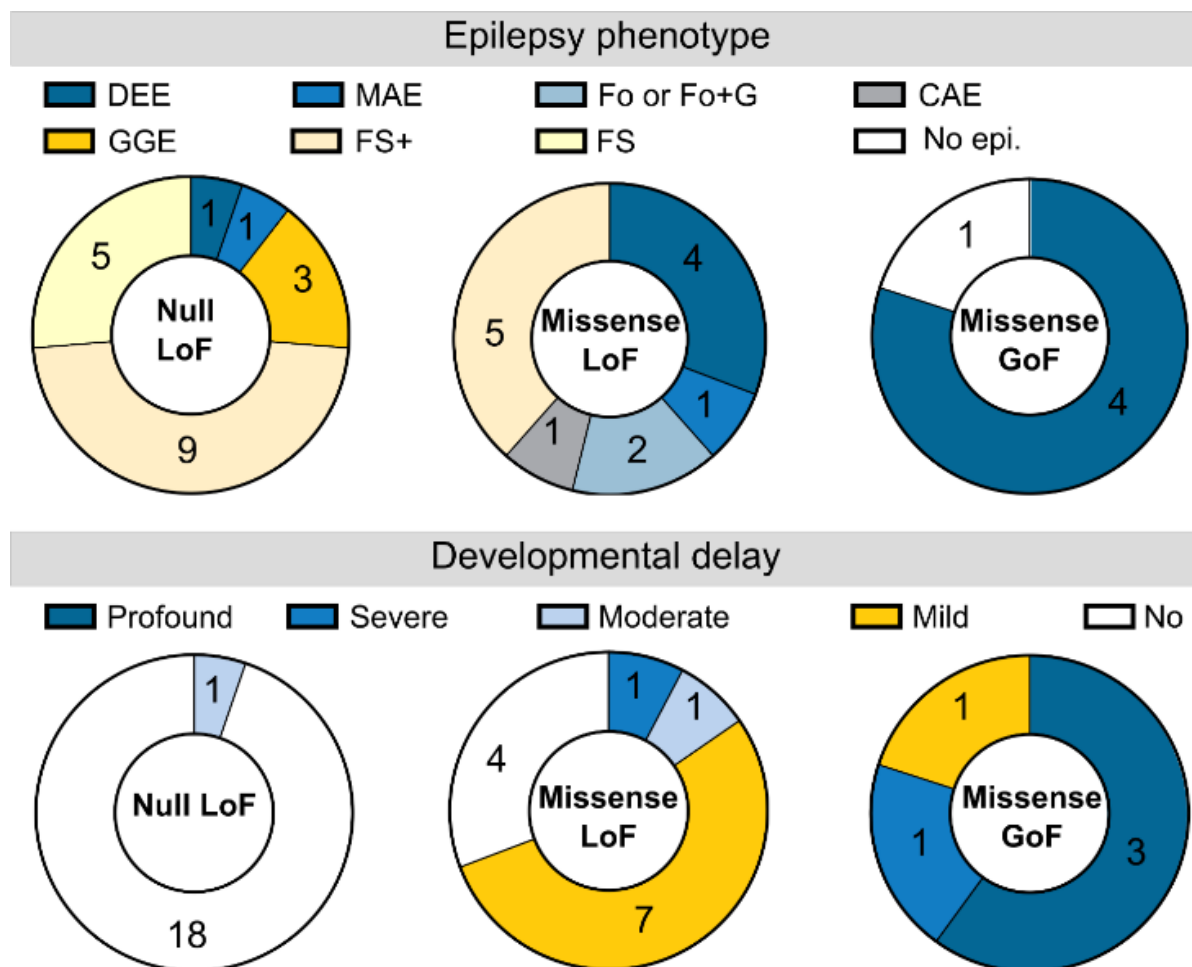


Figure 3.2 Comparison of clinical severity between null and missense variants LoF and GoF *GABRG2* variants

A) *Epilepsy phenotype*, B) *Developmental delay* between individuals harbouring null (n=19 patients) and missense LoF (n=13 patients) or GoF (n=5 patients) variants. Additional abbreviations: Fo: focal; G: generalised; GGE: genetic generalised epilepsy; MAE: Myoclonic atonic epilepsy or Doose syndrome; NR: Not relevant

Conclusion

Our data reveal that distinct functional consequences underlie the broad epilepsy phenotypic spectrum of *GABRG2* pathogenic variant. We present a direct comparison of clinical phenotypes between individuals harbouring null and missense LoF variants. Our findings indicate that patients with null variants have a similar age of onset to missense LoF but are associated with a less severe phenotype in terms of epilepsy types, and ID/DD.

This chapter has been submitted for publication. The manuscript can be found in the supplementary information.

Chapter 4 gnomAD and *in silico* prediction tools

Introduction

The advancement of next-generation sequencing (NGS) in practice has revolutionised our understanding and identification of disease-associated variants. Its accessibility and reduced cost of NGS has contributed to alleviating patient and caregiver's psychosocial burden by facilitating early diagnosis and personalised treatment plans. However, the integration of NGS into practice comes with its own set of challenges, including clinician education, the complexity of interpreting results accurately and the provision of effective counselling for patients and their families.

A variant can be benign or pathogenic, and if pathogenic, the molecular cause can be quite different. A critical challenge for geneticists, neurologists and epileptologists determining whether a variant carried by a patient is pathogenic at initial presentation (178-181), as accurate and timely analysis of variant pathogenicity is important for patient care and counselling. While experimental functional analysis of the variant effect is important, it can often be time-consuming and not feasible in a clinical setting.

To address the need for standardised variant classification, the ACMG established guidelines for categorising variants into five categories: likely benign, benign, VUS, likely pathogenic, and pathogenic (182). These classifications of variants are based on multiple criteria, each with a different weight of evidence (182). For example, functional studies of variants are strong evidence for variant's pathogenicity [BS3 or PS3]; "novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before" [PM5] is of moderate evidence; and the use of variant frequency and use of control populations from population database with an "allele frequency of above 5% in Exome sequencing Project, 1000 Genome genomes or ExAC" [BA1] is a stand alone evidence of benign impact, or strong evidence if the "allele frequency is greater than expected for the disorder" [BS1].

In 2015, ACMG proposed the use of *in silico* tools in evaluating the pathogenicity of variants, and this acts as supporting evidence for pathogenicity (182). Multiple lines of computational

evidence are required to support a deleterious or benign effect, and of the tested *in silico* programs, all have to agree on the prediction to be counted as “supporting” and if the tools disagree then it should not be used in classifying the variant (182). There have been considerable improvements in the accuracy of *in silico* predictive tools for predicting pathogenicity since 2015. Unlike the criteria for scoring PM5, *in silico* tools consider not just the position of an amino acid but also the physicochemical properties of the amino acids that have been altered. Therefore, in cases where there are different amino acid changes at a specific residue where one is known to be either benign or pathogenic, would *in silico* tools provide a superior prediction than the current PM5 criteria?

In 2020, the use of *in silico* prediction tools has also been supported by the UK association for Clinical Genomic Science (183). *In silico* prediction has been widely used in various studies and conditions, including mental disorders (184) and cancer (185) to establish the role of non-synonymous single nucleotide polymorphism (nsSNPs). nsSNPs can have a neutral or deleterious effect on the protein, often altering protein structure through changes in amino acid properties leads to structural and thermodynamic instability (186-190). Perturbation of the core and conserved regions can have a damaging effect on the residue packing level (191).

Current bioinformatics tools are highly complex, with algorithms incorporating various lines of evidence in the pathogenicity prediction, including physicochemical distances between amino acids and inter-species sequence conservation (192). Many of the current tools have been developed to predict the effect of missense substitutions resulting from single nucleotide variants (183). Currently available tools utilise various methodologies to assess whether a variant is pathogenic or not (**see Table 4.1**), 1) sequence homology-based, 2) machine-learning and 3) sequence structure method, this method allows for the assessment of variant pathogenicity by looking at both the sequence, including evolutionary conservation score and homologous sequence and structural information, such as protein stability, of the protein (188, 193). Most *in silico* prediction tools give a numerical output with developer recommended threshold or cut-off values to give the user an indication of whether the variant is pathogenic or not.

In Chapter 3, we identified two variants at *GABRG2*, at the same position with different functional results and phenotypes. Both *GABRG2*, A106P and A106T were both predicted to be benign/tolerated by two *in silico* tools, PolyPhen-2 and SIFT. *GABRG2* A106P featured

three times in gnomAD with no patients whilst A106T featured in 11 patients. Upon functional testing, A106T has been shown to be deleterious and associated with DEE. This discrepancy questions the reliability of *in silico* predictions for *GABRG2* variants and whether variant pathogenicity can be predicted based on the *in silico* tool.

This chapter aims to:

1. To evaluate the reliability of *in silico* prediction tools in accurately determining the pathogenicity of 7 pairs of missense *GABRG2* variants.
2. To investigate the ability of *in silico* tools to differentiate between pairs of *GABRG2* variants at the same position.
 - a. To determine if a combination of *in silico* tool predictions and gnomAD allele frequencies (of an existing variant at the same position) can provide evidence that a novel variant (same position as current gnomAD but different amino acid change) is benign.

Hypothesis: *In silico* prediction tools are not sufficiently accurate to determine pathogenicity of the *GABRG2* missense variants and is not capable of differentiating between the pathogenicity of positional missense variant

Table 4.1 Summary of types of *in silico* tools

Tool name	Website/more information	Method	Threshold	Ref
Sequence and structure based				
Polyphen-2	http://genetics.bwh.harvard.edu/pph2	Based on 3 structural features and 3 sequence based predictive properties by naive Bayes' theorem parameters	Probably damaging ≥ 0.957 Possibly damaging $0.453 \leq x \leq 0.956$ (194)	(195)
FATHMM-W	http://fathmm.biocompute.org.uk/	Markov method-based prediction software Parameters: conservation score (sequence) along with the conserved protein domain structure to measure the pathogenicity weight	The lower the score, the more deleterious the variant (194)	(196)
Sequence homology				
SIFT [sorting Intolerant from tolerant]	https://sift.bii.a-star.edu.sg/	Sequence conservation score to define deleterious effect, assuming conserved regions are important so changes in region that are conserved are deleterious. SIFT also query related proteins and compares their sequence	< 0.5 is deleterious. Cut off for deleterious < 0.1 is better for sensitivity	(197, 198)
Supervised machine-learning				
MutPred2	http://mutpred.mutdb.org/	Integrates genetic and molecular data to predict the pathogenicity of amino acid substitutions. Sequence based + machine + generates molecular mechanisms potentially causative of disease		(199)
CADD	https://www.ebi.ac.uk/ProtVar/	Creates a scoring algorithm from evolutionary constraints, gene model annotations, epigenetic measurements, surrounding sequence context, and functional predictions.	Higher number indicates variant is more likely deleterious.	(200)

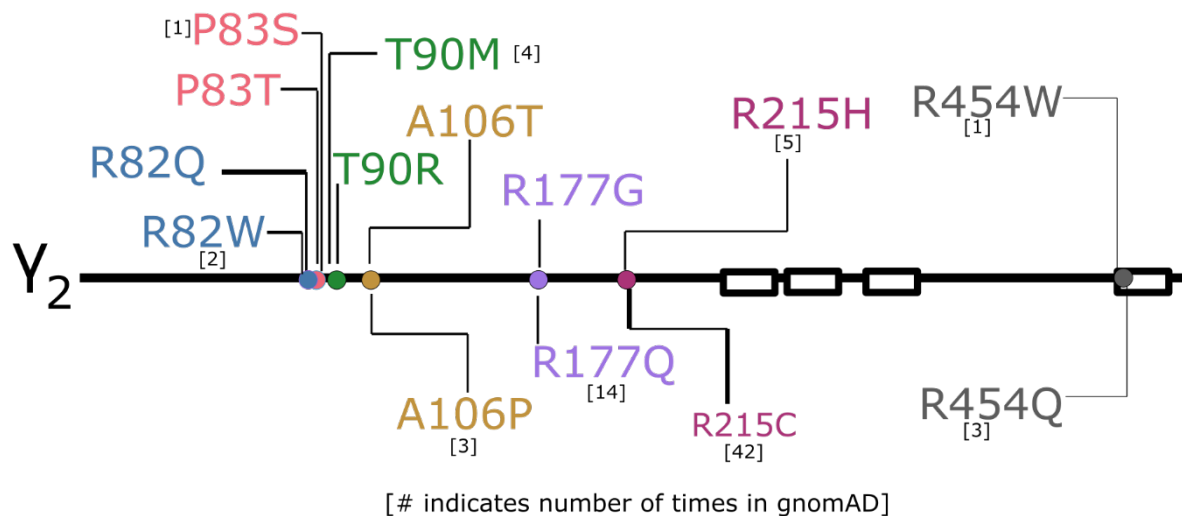
		Machine-learning model trained on binary distinction between neutral and deleterious variants	Generally, >20 indicates top 1% most deleterious	
REVEL	https://sites.google.com/site/revelgenomics/about?authuser=0	Ensemble method integrating 18 pathogenic prediction scores from 13 tools. REVEL also trained with rare pathogenic variants.	> 0.5 is pathogenic (194) A more sensitive deleterious score is > 0.5, more specific is higher than 0.7 (201) Study by Wilcox, Sarmady (202) suggests using > 0.8 as pathogenic and <0.4 as a benign	(201)
pMut	http://mmb.irbbarcelona.org/PMut	Trained with dataset from Swiss Prot. It also leverages sequence conservation and predicted physico-chemical properties as main features	Prediction score ranges from 0-1 0-0.5 = neutral 0.5-1 = pathological	(203)
AlphaMissense	https://alphamissense.hegelab.org/search	Model builds on AlphaFold (a protein structure prediction tool) and trained on population data along with sequence changes in context with predicted structural context (204, 205)	Likely benign, 0-0.34 (blue) Ambiguous, 0.34-0.564 (no colour) Likely pathogenic, 0.564-0.78 (light red) Likely pathogenic, 0.78-1.0 (dark red)	(204)

Results

Location of the variants and sequence conservation

Fourteen variants were identified at seven different amino acid positions where different amino acid substitutions were found in our patient and the gnomAD databases (**Figure 4.1**). Six of these positions - R82, P83, T90, A106, R177 and R215 -are located in the ECD, and one at position R454 in TM4. Sequence alignment of the amino acid positions reveals that R82 and P83 are highly conserved across all 19 subunits of GABA_A receptor. R454 is conserved in 18 subunits, with lysine present at this position at *GABRP*. R177 is common in both *GABRG1* and *GABRG2*. T90 residue is conserved within the γ subunits (*GABRG1-3*). At position 106, the alanine is not conserved. Finally, the R215 residue is not conserved, suggesting a missense variant at that position may be less critical.

Patient/gnomAD variants



gnomAD only variants

Figure 4.1 2D structural mapping of the location of seven variant pairs in the GABRG2 gene.

Variants above the line denote the variant is present in either patient or both patient and gnomAD population. Variants on the bottom indicate the variant is present exclusively in gnomAD. The number in the bracket indicates the number of times it appears in gnomAD.

Preliminary *in silico* tool predictions

We investigated the predicted functional effect of the seven variants pairs found either in healthy population (gnomAD) and/or in patient, using three different *in silico* tools: PolyPhen-2, FATHMM-W, and SIFT. Both PolyPhen-2, and FATHMM-W use a combination of sequence and structural information to predict the effect of variants, while SIFT is a structure-based *in silico* tool. We found six variants, R82W/Q, P83S/T and R454Q/W, were predicted to be damaging by both PolyPhen-2 and FATHMM-W (**Table 4.2**). Only one variant, A106T was predicted to be tolerated across all three tools (**Table 4.2**). No variant pairs were assumed to be different by the sequence-based homology tools.

Table 4.2 Prediction results for sequence and structure based; and sequence homology-based *in silico* prediction tools

Variant	Sequence and structure based				Sequence homology		Total predicted pathogenic (out of 3)
	PolyPhen2	Score	FATHMM-weighted	Score	SIFT	Score	
R82W	Probably Damaging	1	Damaging	-1.74	Affect protein function	0	3
R82Q	Probably Damaging	1	Damaging	-1.65	Affect protein function	0	3
P83S	Probably Damaging	1	Damaging	-3.54	Affect protein function	0	3
P83T	Probably Damaging	1	Damaging	-3.57	Affect protein function	0	3
T90M	Probably Damaging	1	Tolerated	-1.31	Affect protein function	0	2
T90R	Probably Damaging	1	Tolerated	-1.35	Affect protein function	0	2
A106P	Benign	0	Tolerated	-1.15	Tolerated	0.26	0
A106T	Benign	0.05	Tolerated	-1.12	Tolerated	0.34	0
R177Q	Probably Damaging	0.98	Tolerated	-1.23	Tolerated	0.25	1
R177G	Probably Damaging	1	Tolerated	-1.27	Tolerated	0.09	1
R215C	Probably Damaging	1	Tolerated	-1.3	Affect protein function	0.01	2
R215H	Probably Damaging	0.97	Tolerated	-1.27	Affect protein function	0.05	2
R454Q	Probably Damaging	1	Damaging	-2.04	Affect protein function	0	3
R454W	Probably Damaging	1	Damaging	-2.11	Affect protein function	0	3

Preliminary *in silico* tool prediction using machine-learning based tools

I next determined how machine-learning *in silico* tools perform in predicting the effects of the variants. The prediction tools give a value between 0 and 1, where a value closer to one indicates a higher likelihood of the variant being pathogenic. We used AlphaMissense, CADD, MutPred2 and REVEL for our analysis, adhering to their respective pathogenicity threshold. For AlphaMissense, the default settings were applied; CADD score of greater than 20 was considered pathogenic; for MutPred2 the threshold for considering pathogenicity is 0.8, as

recommended by the website. REVEL threshold for considering a variant is pathogenic is 0.773 (206).

Out of the 14 variants assessed, 10 variants were predicted to be likely pathogenic by AlphaMissense and MutPred2 and six variants were predicted to be pathogenic by REVEL (**Table 4.3**). At CADD score threshold of > 20, all variants were predicted to be pathogenic, except for A106T (**Table 4.3**). Three variant pairs - R82Q/W, P83S/T and R454Q/W - were unanimously predicted to be pathogenic across all four tools (**Table 4.3**). The machine-learning based *in silico* tools predicted the variants to be functionally different at A106 (AlphaMissense, CADD, MutPred2), R177 (AlphaMissense, MutPred2) and R215 (AlphaMissense), albeit at times this was due to the variant scored as ambiguous.

Table 4.3 Prediction of variant effect using machine-learning based in silico tools

Machine-learning					
Variant	AlphaMissense	Default - classification	CADD	MutPred2	REVEL Threshold
R82W	0.991	Likely Pathogenic	25.6	0.918	0.823
R82Q	0.986	Likely Pathogenic	31	0.86	0.895
P83S	1	Likely Pathogenic	26	0.892	0.975
P83T	0.999	Likely Pathogenic	25.3	0.89	0.978
T90M	0.743	Likely Pathogenic	27	0.835	0.721
T90R	0.992	Likely Pathogenic	25.9	0.924	0.84
A106P	0.382	Ambiguous	22.3	0.806	0.353
A106T	0.575	Likely Pathogenic	19.3	0.475	0.313
R177Q	0.264	Likely Benign	28.8	0.736	0.466
R177G	0.902	Likely Pathogenic	24.4	0.855	0.716
R215C	0.43	Ambiguous	29.6	0.526	0.563
R215H	0.153	Likely Benign	27.6	0.287	0.437
R454Q	0.954	Likely Pathogenic	31	0.877	0.885
R454W	0.995	Likely Pathogenic	28.1	0.922	0.856

Biophysical properties of gnomAD and patient *GABRG2* variants

To assess the accuracy of predictions from *in silico* tools, we functionally characterised the variants. Selected missense variants were assessed for changes in GABA sensitivity and maximum GABA-evoked current. We found A106T to be a GoF variant as it increased receptor sensitivity to GABA ($\Delta\text{LogEC}_{50} = 0.73 \pm 0.15$ SD) indicated by a left-shift of the dose-response curve (**Figure 4.2, Supplementary Table 1**). Three variants, P83T ($\Delta\text{LogEC}_{50} = -0.336 \pm 0.16$ SD), T90R ($\Delta\text{LogEC}_{50} = -0.43 \pm 0.071$ SD) and R454W ($\Delta\text{LogEC}_{50} = -0.22 \pm 0.12$ SD), reduced GABA sensitivity (**Figure 4.2**).

Four variants, R82W, R82Q, P83S, and R177G, reduced the maximum GABA-evoked current but did not change the receptor's sensitivity to GABA (**Figure 4.3**). Three variants - P83T, T90R, and R454W - significantly reduced the maximum elicited current by 88%, 86% and 59% respectively, and decreased GABA sensitivity by 2.11, 2.71 and 1.67 times, respectively, compared to WT (**Figure 4.2, Figure 4.3, Supplementary Table 1**).

From the seven pairs, R82Q/W both reduced maximum current and both R215C/H did not significantly alter GABA sensitivity and maximum current. We found P82S to reduce maximum current, whilst P83T reduced both GABA sensitivity and maximum current. At T90, T90M showed no significant difference, whilst T90R reduced both GABA sensitivity and maximum evoked current. At A106, A106P showed no difference to WT, whilst A106T increased GABA sensitivity. At R177, R177G reduced maximum current whilst R177Q showed no difference to WT. Finally, at R454, R454Q had no significant difference to WT, whilst R454W reduced both GABA sensitivity and maximum current. (**Figure 4.2, Figure 4.3, Supplementary Table 1**).

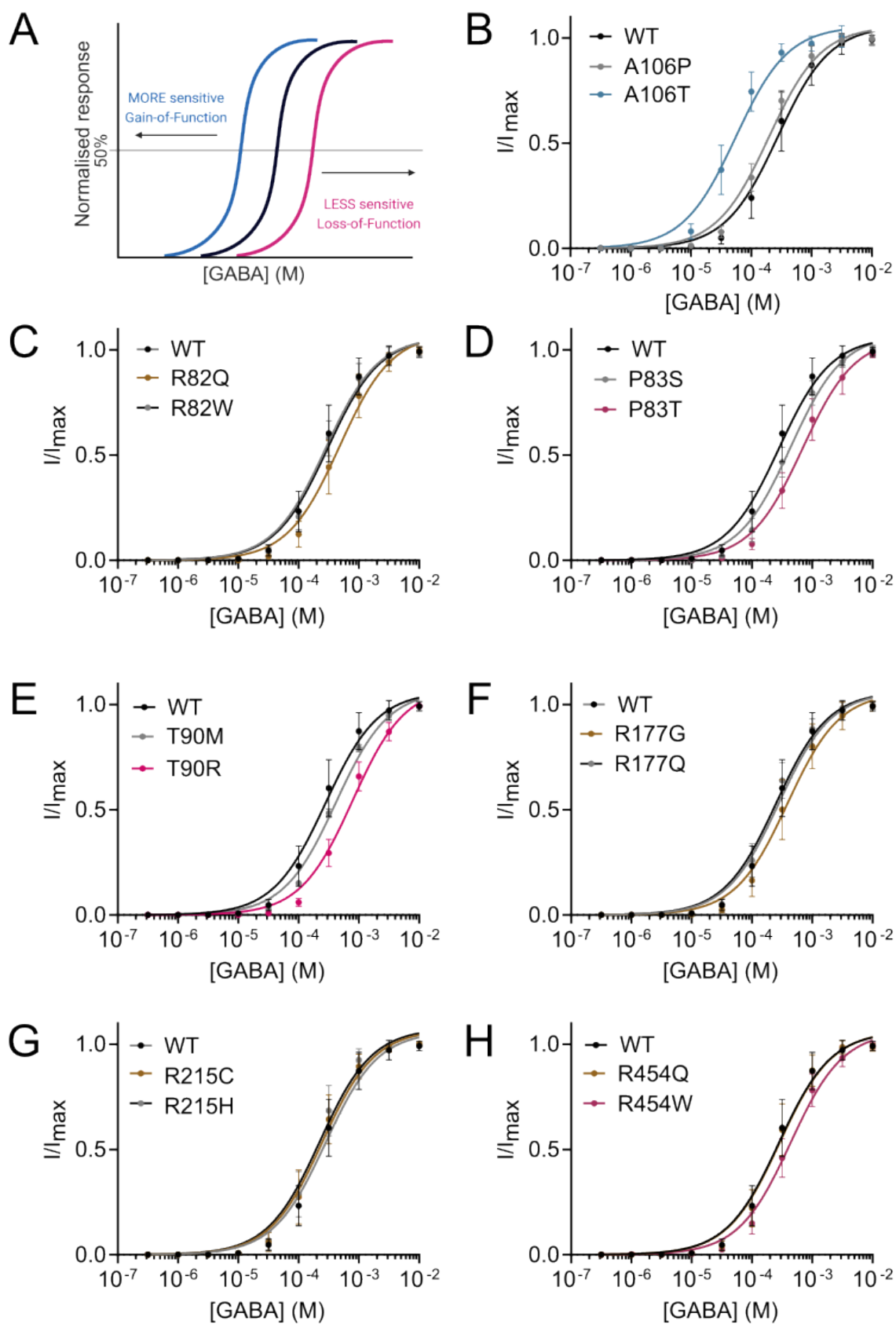


Figure 4.2 Representative concentration-response curve 7 pairs of positional missense variants.

A) Expectation of dose-response curves for variants that increase GABA sensitivity (blue) and decrease GABA sensitivity (pink) in relation to WT. Normalised dose-response curves with WT (black) for **B)** A106P (grey) and A106T (blue), **C)** R82Q (brown) and R82W (grey), **D)** P82S (grey) and P83T (pink), **E)** T90M (grey) and T90R (pink), **F)** R177G (brown) and R177Q (grey), **G)** R215C (brown) and R215H (grey) and **H)** R454Q (brown) and R454W (pink). Pink lines denote a reduced GABA sensitivity, LoF, whilst grey and brown lines indicate no significant change in GABA sensitivity.

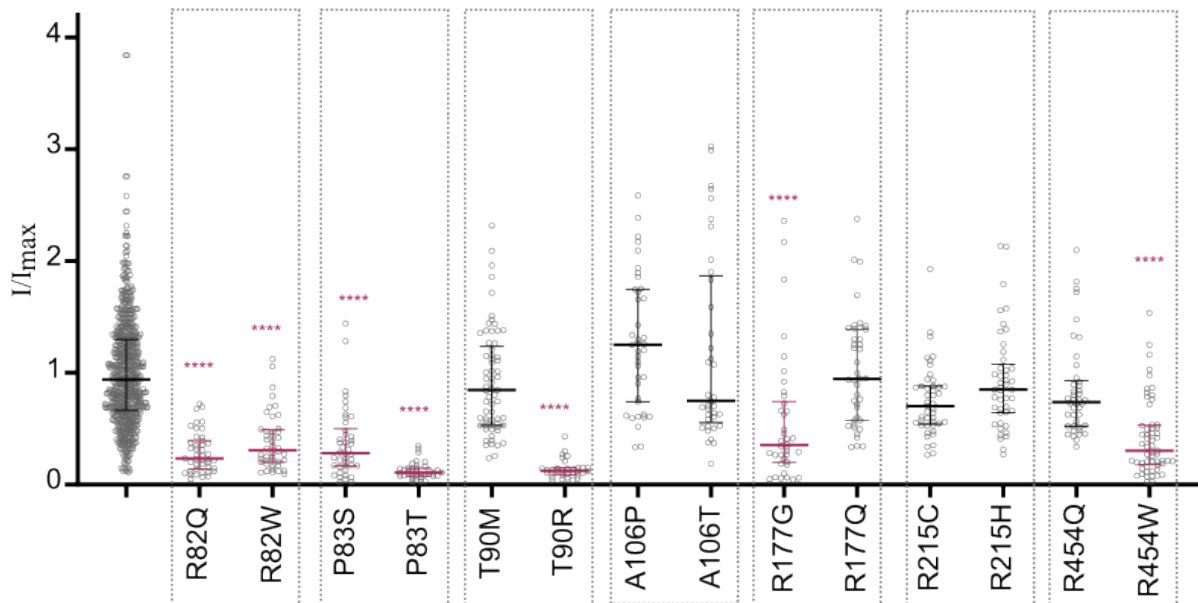


Figure 4.3 Dot plot of changes in maximum current for 7 pairs of positional missense variants.

Pink bar denotes the receptor containing the variant had a statistically significant reduction in max current when compared to WT. Datas are expressed as median (IQR). Statistical significance was determined using Mann-Whitney U test. (**** = $p < 0.0001$)

In silico tools vs *in vitro* functional testing

The functional outcome of the variants was used to assess the sensitivity, specificity, and accuracy of *in silico* tools in predicting whether a variant pathogenic. First, the definition for a “true positive” is a variant that altered GABA sensitivity and/or maximum GABA-evoked current. Sensitivity measures the true positive rate, and for this thesis, it is the ability of the test to correctly identify true pathogenic variants. Specificity, measures the true negative rate, reflecting the test's ability to correctly identify true benign variants. Generally as sensitivity increases, specificity *tends to* decrease, and vice versa (207). However, for an ideal test, one

would aim for both high sensitivity (close to 100%) and high specificity to accurately identify both pathogenic and benign variants.

Sequence and structure-based in silico prediction of variant pathogenicity

PolyPhen-2, and FATHMM-W are sequence homology based *in silico* prediction tool. This means an amino acid substitution in a region that is conserved and is predicted to change the protein structure, e.g. result in a more unstable protein, the prediction tool will return with a pathogenic prediction. PolyPhen-2 had higher sensitivity and ability to pick up true positives than FATHMM-W (0.875 vs 0.625). FATHMM-W had a higher specificity (83%) and accuracy (71%) than PolyPhen-2 (**Table 4.4**).

Table 4.4 Sensitivity, specificity, and accuracy of sequence and structure-based in silico prediction tools

	PolyPhen-2	FATHMM-W	SIFT
Sensitivity	88 %	63 %	75 %
Specificity	17 %	83 %	33 %
Accuracy	57 %	71 %	57 %

Sequence homology-based in silico prediction of variant pathogenicity

SIFT is an example of an *in silico* prediction tool that uses sequence homology to predict the pathogenicity of a variant. The sensitivity of SIFT was 75%, this indicates that the test is able to detect true pathogenic variant in 75% of cases (**Table 4.4**). Further investigation revealed that the tool has a specificity of 33% and an accuracy of 57% (**Table 4.4**).

Machine-learning based in silico prediction of variant pathogenicity

Machine-learning based *in silico* prediction tools use information from the current databases and resources incorporating ongoing updates of new information to predict whether a variant is likely to be pathogenic/damaging or benign. AlphaMissense, MutPred2 and REVEL gave a score between 0-1; a score that is closer to 1 indicates a higher probability of that variant being disease-causing. CADD gives a score ranging between 1 - 99, with a number >20 indicates its likely deleterious. We found that of the four tested tools, AlphaMissense has the best overall sensitivity, specificity and accuracy (**Table 4.5**).

Table 4.5 Sensitivity, specificity and accuracy of 4 machine-learning based in silico prediction tools

	AlphaMissense	CADD	MutPred2	REVEL
Sensitivity	100 %	87.5%	91 %	69 %
Specificity	67 %	0	25 %	38 %
Accuracy	88 %	50 %	57 %	57 %

In silico tool to replace functional testing

GABA sensitivity measurement is a more robust measure of whether a variant is likely to be pathogenic or benign than max current. This is because maximum current measurement can be 1) confounded by experimental factors, e.g. quality of RNA, quantity injected into the cells, and 2) the human body can compensate for changes in max current. Therefore, we sought to investigate how well the *in silico* tools are able to correctly predict variants that alter GABA sensitivity. We found all seven *in silico* tools have a less than 60% accuracy rate (**Table 4.6**). Six out of seven tested *in silico* tools had a specificity of less than 45%, indicating that the tools are prone to false positives, and so, often, variants will be predicted pathogenic when they are benign.

Table 4.6 Sensitivity, specificity, and accuracy of in silico prediction tool in predicting functional changes in receptor sensitivity.

*Numbers have been rounded to the nearest whole number

	Structure and sequence-based		Sequence homology	Machine-learning			
	Polyphen-2	FATHMM-W	SIFT	AlphaMissense	CADD	MutPred2	REVEL
Sensitivity	75 %	50 %	75 %	100 %	75 %	80 %	40 %
Specificity	10 %	60 %	30 %	40 %	0 %	33 %	44 %
Accuracy	29 %	57 %	43 %	57 %	21 %	50 %	43 %

Occurrence in patients – patient phenotype

Nine variants were taken from gnomAD, five variants were also associated with at least one patient, and the remaining four variants, A106P, R177Q, R215C and R454W, were exclusively present in the gnomAD database with allele count of 3, 14, 42 and 3 respectively. An additional five variants with different amino acid substitution were found at the same position as an

existing gnomAD variant. A total of 31 individuals were identified harbouring the variants, 26 individuals were diagnosed with epilepsy (**Table 4.7**).

Table 4.7 Clinical phenotypes of variants found in patients

	Variant	Functional consequence	Epilepsy	Epilepsy Syndrome	ID after epilepsy onset	Ref
1	p.(Arg82Gln)	LoF				(208)
2	p.(Arg82Gln)	LoF	Yes	CAE	Unknown	(113, 166)
3	p.(Arg82Gln)	LoF	Yes	FS		(209)
4	p.(Arg82Gln)	LoF	Yes	N/A		
5	p.(Arg82Try)	LoF	Yes	Generalised epilepsy	Normal	
6	p.(Pro83Ser)	Neutral	Yes		Unknown	(210)
7	p.(Pro83Ser)	Neutral	Yes	Lennox Gastaut	Severe ID	(211)
8	p.(Pro83Thr)	LoF	Yes		Moderate ID	
9	p.(Pro83Thr)	LoF	Yes	CAE		
10	p.(Thr90Met)	Neutral	Yes	GEFS+	Normal	
11	p.(Thr90Met)	Neutral	Yes		Moderate ID	
12	p.(Thr90Arg)	LoF	Yes	DS	Severe ID	(212)
13	p.(Ala106Thr)	GoF	Yes		Severe ID	
14	p.(Ala106Thr)	GoF				(208)
15	p.(Ala106Thr)	GoF	Yes	EE	Severe ID	(171)
16	p.(Ala106Thr)	GoF	Yes	EE	Severe ID	(171)
17	p.(Ala106Thr)	GoF	Yes	EE		(169)
18	p.(Ala106Thr)	GoF	Yes	EE	Severe ID	(169)
19	p.(Ala106Thr)	GoF	Yes	EE	Severe ID	(169)
20	p.(Ala106Thr)	GoF	Yes	EE	Unknown	(169)
21	p.(Ala106Thr)	GoF	Yes	GEFS+		(213)
22	p.(Ala106Thr)	GoF	Yes	DEE	Severe ID	
23	p.(Ala106Thr)	GoF	No			
24	p.(Ala106Thr)	GoF	Yes	Lennox-Gastaut	Severe ID	

25	p.(Ala106Thr)	GoF	Yes	Lennox-Gastaut	Severe ID	
26	p.(Ala106Thr)	GoF				
27	p.(Ala106Thr)	GoF	Yes	DEE		(93)
28	p.(Arg177Gly)	Neutral	Yes	FS		(214)
29	p.(Arg215His)	Neutral	Yes	GEFS+/FS	Normal	(80)
30	p.(Arg215His)	Neutral	Yes	FS	Normal	(80)
31	p.(Arg446Trp)	LoF				(208)

gnomAD frequency as a predictor of variant pathogenicity

gnomAD is a population database encompassing variants found within the human genome. Given that *in silico* prediction tools have limited efficacy in forecasting variant effects, we sought to investigate whether gnomAD allele frequency can better predict the variant's effect on the protein. Here, we categorised the variants into 3 groups: gnomAD only, patient(s) only and variants that present in both gnomAD and patient(s). We found that all the tested gnomAD-only variants, A106P, R177Q, R215C and R454Q, do not affect protein function and are, therefore, likely benign. Conversely, variants exclusive to patients - R82Q, P83T, T90R, A106T and R177G - significantly affect protein function (either reduced maximum current or changes to receptor sensitivity) and are thus deemed likely pathogenic. Variants present in both gnomAD and patients - R82W, P83S, T90M, R215H and R454W – can either affect or not change protein function (**Figure 4.4**). However, there is a trend within the overlapping group: variants with higher gnomAD frequencies, such as T90M and R215H (indicated by the numbers in brackets), are more likely to have no effect on protein function, while those with lower frequencies tend to be associated with functional changes.

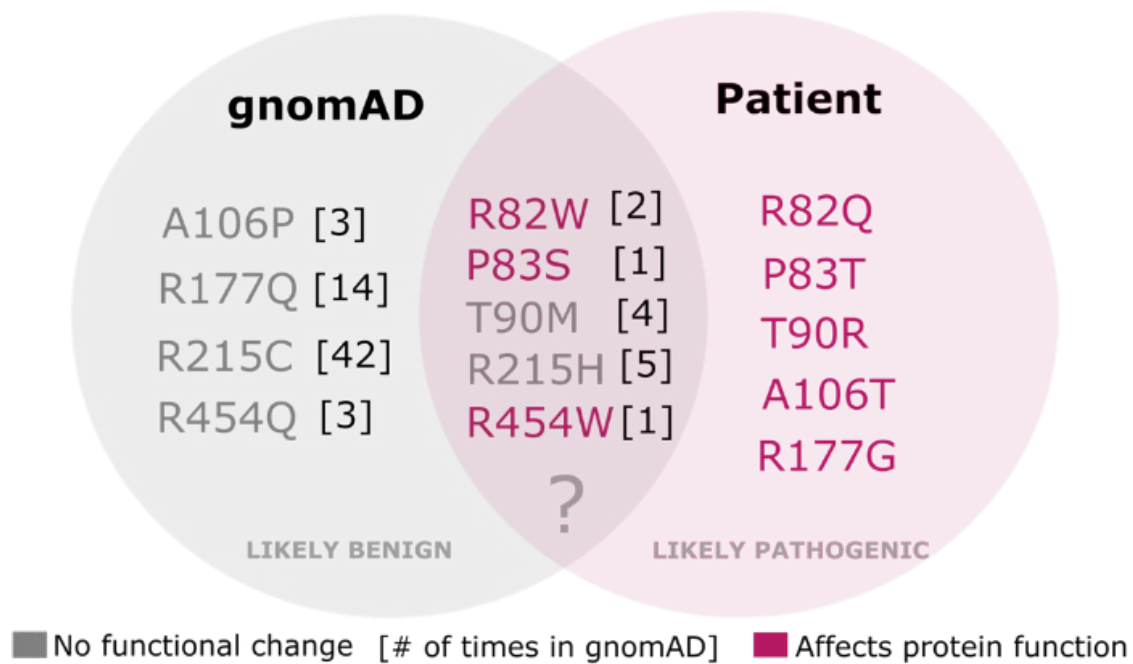


Figure 4.4 Venn diagram of variants categorised into 3 categories: gnomAD, patient, and gnomAD and patient.

Colour of the text denotes if a variant alters protein function measured by changes in maximum current or GABA sensitivity grey signifies no change, whilst pink indicates changes in function. The number within the brackets indicates the frequency of a variant's occurrence gnomAD.

Discussion

Advancement of accessible genetic testing has increased the identification of variants in public databases such as ClinVar and gnomAD. Since variants can be benign, damaging or a risk factor, it can be challenging to determine whether a variant is benign or pathogenic when it is first detected by NGS. Although functional testing of a variant is the gold standard that provides strong evidence of pathogenicity, it is not always feasible due to time and cost constraints. *In silico* prediction tools allow for the prediction of the effect of amino acid substitutions on the function of the protein without the need for functional studies (215).

Given the discrepancies observed in Chapter 3 between predicted and actual pathogenicity of variants A106P and A106T, this chapter aimed to investigate the reliability of *in silico*

prediction tools by analysing seven pairs of *GABRG2* variants, and whether this knowledge is sufficient to determine a variant's pathogenicity.

Currently, *in silico* tools are recommended for use in practice, but they require multiple lines of evidence to collectively act as supporting evidence. According to the ACMG guidelines, [PM5] stipulates that if a *novel missense change occurs in an amino acid residue where a different missense change has already been determined to be pathogenic, this counts as moderate evidence for the novel variant's pathogenicity*. This raises an important question: what if the situation is reversed? For example, if a variant is found in the gnomAD database and *in silico* tools predict it to be benign, can this combination of evidence be considered moderate evidence that a novel positional missense variant is benign?

The ACMG guidelines provide criteria for assessing variant pathogenicity, categorised by the strength of evidence. The criteria, mentioned and explored in order of their strength, are as follows:

1. **Strong evidence:** *In vitro* or *in vivo* studies supportive of a damaging effect
2. **Moderate evidence:** novel missense changes in an amino acid residue where a different missense change determined to be pathogenic has been seen before [PM5]
3. **Supporting evidence:** *in silico* tool predictions from multiple lines of evidence

In the present study, we evaluated the predictive efficacy of seven commonly used *in silico* tools - PolyPhen2, FATHMM-W, SIFT, AlphaMissense, CADD, MutPred, and REVEL – on seven pairs of variants located at the same positions to determine if they could differentiate between the amino acid changes. Our analysis revealed that among the seven tools, only AlphaMissense was able to score the A106 and R177 variants differently, i.e. A106P and R177Q are ambiguous and likely benign, respectively, while A106T and R177G are likely pathogenic. This distinct scoring capability contributed to its higher success rate, which was further verified through functional assays.

Our findings indicate that, overall, the accuracy of these *in silico* tools in discerning pathogenic from benign variants is relatively poor, with the exception of AlphaMissense (88%) and FATHMM-W (71.4%). The tools used demonstrated a high false positive rate and low accuracy. This is in agreement with other studies involving genes in immune cells and TP53 tumour suppressor genes (216). Previous investigations have revealed that *in silico* prediction tools have variable accuracies in different genes (Kerr et al., 2017; Leong, Stuckey, Lai, Skinner, & Love, 2015; Rodrigues, Santos-Silva, Costa, & Bronze-da-Rocha, 2015).

The biological importance of the gene is critical to the cut-off values for pathogenicity. Genes with critical importance in a pathway, such as *SCN1A*, will have a lower cut-off for pathogenicity than genes with minor roles. In a study by Tang, Li (180), using default cut-off values for *SCN1A* missense variants, the accuracies of *in silico* prediction tools were found to range from 77.1% - 99.8%: SIFT (77.1%), MutPred2 (80.6%), FATHMM-W (99.8%). Authors from the study underwent further optimisation of cut-off, resulting in better prediction of accuracy.

Proteins consist of structural domains associated with distinct functional roles, which may have different tolerability to variants. Tang, Li (180) proposed that prediction tools could be improved by individualising cut-off values according to the location of the variants within the various functional domains. Despite this optimisation, validation with functional assays showed the accuracy of *in silico* tools with SIFT, MutPred2, FATHMM-W, and CADD were all lower than 76%, with FATHMM-W achieving the highest accuracy at 75.6%, whilst sensitivity was above 95% for all except for FATHMM-W (70.2%) (180). Given that the databases for these *in silico* tools are based on a wide array of learning materials and genes, it is essential to determine the most appropriate cut-off values for each gene. Together, this supports the idea that the default cut-off values serve as a useful starting point, but researchers need to tailor these values to specific genes to optimise results.

If a variant that is predicted to be “benign/tolerated” by *in silico* tools and seen in gnomAD – is it benign?

Specificity is a measure of how well a given test can correctly detect true negatives. We found for six out of seven *in silico* tools, specificity is < 40%. This denotes that the tools are likely to give a false pathogenic result. Interestingly, in Pejaver, Byrne (206) study, they found at developer-recommended thresholds, SIFT, PolyPhen2 and CADD, a substantial fraction of gnomAD variants set as damaging, with 50% of SIFT, 29.3% PolyPhen2, and 65.1% by CADD prediction to be pathogenic. This finding of overcalling deleterious agrees with studies in other disciplines (190, 217). Thus, to answer if “a variant that is predicted to be “benign/tolerated” by *in silico* tools and seen in gnomAD – is it benign?”, then there is high confidence that the variant is likely to be benign, as seen in A106P and R177Q (correctly predicted by SIFT). However, caution should be taken as A106T, predicted to be benign, is, in fact, damaging.

The presence of a variant in gnomAD does not rule out the pathogenicity of alternative positional missense variants.

If a variant is present in gnomAD, and its positional alternative missense variant is predicted “benign/tolerated by *in silico* tools, does this provide enough evidence that the new variant is benign? Unexpectedly, we found that just because there is a variant at a given position, we cannot assume that a different variant at the same position is also benign. Here, we have two distinct examples of a pair of gnomAD/patient variants, *GABRG2* A106P/T and R177Q/G, where at the same position one variant (A106P and R177Q) are exclusively present on gnomAD with no functional change, whilst the other (A106T and R177G) is present in at least one patient with epilepsy. The case of *GABRG2* A106 presents a highly interesting case, as the position is 1) not conserved and 2) at the same position for *GABRG3*, instead of an alanine it had a threonine, but nevertheless, the A106T variant had an increase in GABA sensitivity.

Overall, from the 14 variants assessed from gnomAD and patient population, our findings agree with how ACMG weighs the different lines of evidence. That is, functional studies supportive of damaging/benign effect is strong evidence; the use of variant frequency and use of control populations from population database (in this case, gnomAD) with an allele frequency of above 5% in Exome sequencing Project, 1000 Genome genomes or ExAC is a stand alone evidence of benign impact, or strong evidence if the allele frequency is greater than expected for the disorder; and multiple lines of computation evidence (in agreement) is required to be counted as supporting evidence of the variant being benign/pathogenic. Therefore, pairs of variants at the same position with a different amino acid change does not provide strong evidence of pathogenicity/benign. *In silico* tools tend not to challenge this evidence, and do not provide an independent assessment that it is superior.

Conclusion

I identified that in the 14 studied *GABRG2* variants, current *in silico* tools are not accurate enough to predict pathogenicity nor differentiate between missense variants at the same position, this is concerning as it may also be true in other genes. Among the studied tools, machine-learning based tools like AlphaMissense show some ability to distinguish the functional effect of two missense variants at the same position. I found functional data is essential for interpretation of variant pathogenicity. There is an urgent need for harmonising functional data across databases which Future efforts should focus on to improve predictive accuracy.

Chapter 5 Genotype-phenotype characterisation of *GABRG2* variant

Introduction

GABRG2 has been associated with a plethora of epileptic syndromes, ranging from simple FS to DEEs. For patients with rare forms of epilepsy, such as DEE, the journey to diagnosis is often long and arduous. This is often termed the “diagnostic odyssey”. This journey can cause significant psychological, emotional and financial distress on the patients and their families (218). It is important to highlight that diagnosis is more than benign or pathogenic, as it includes the severity of the disorder, seizure, and comorbidity risk.

Currently, about 40% of patients are not effectively treated with current ASMs, with some experience worsening of symptoms whilst on ASMs (3, 143, 144). The discovery of GoF GABA_A receptor variants highlights that the diagnosis system, knowledge, and therapeutics currently in place is insufficient. One of the most important finding with GoF and LoF GABA_A receptor variant such as ones in, *GABRA1*(94), *GABRB3* (75), and *GABRD* (177) is that the severity of the disorder is related to the functional change of the variant.

GABRG2 is a gene that encodes for the $\gamma 2$ subunit of GABA_A receptors and has a crucial role in receptor trafficking, determining the postsynaptic localisation, clustering of the formed receptor and synaptic maintenance (38, 219). Although GABA does not bind to the $\gamma 2$ subunit, when GABA is incorporated into receptors, key structural motifs in the $\gamma 2$ subunit must facilitate the allosteric transitions between the closed and open states. Given that the $\gamma 2$ subunit does not directly engage in GABA binding, the effect of variants in the subunit in modulating receptor function is of particular interest.

This chapter aims to:

1. Investigate the GABAergic function of epilepsy-associated *GABRG2* variants from a total of 109 individuals.

2. Compare the clinical features with the functional outcomes to identify genotype-phenotype correlations of *GABRG2* variants.

Hypothesis:

Since the $\beta 3$ (*GABRB3*) and $\gamma 2$ (*GABRG2*) subunits are integral components of the same receptors, it was hypothesised that they should exhibit similar clinical indications when comparing LoF and GoF variants. Specifically, LoF variants of *GABRB3* and *GABRG2* will present with similar clinical features, as will GoF variants of these subunits e.g. GoF variants to be associated with a younger age of seizure onset etc.

Patient cohort

In collaboration with the Danish Epilepsy Center (Filadelfia), we identified 71 *GABRG2* variants in 109 individuals; missense variants occurred *de novo* in 48 individuals, inherited in 31 cases, and were unknown for 30 individuals. A total of 87 individuals were diagnosed with epilepsy.

Variant location

The missense variants occurred at 60 different amino acid positions with 11 recurrent variants. The recurrent variants were found at positions R82, P83, T90, A106, R177, R215, P282, A322, R323, R454 and F457. The missense variants were distributed throughout the subunit: 33 variants in the ECD, 26 in the TMD, and 12 in the loops connecting the TMD (i.e. M1-M2 loop, M2-M3 loop and M3-M4 loop).

Results

ACMG criteria prior to functional assessment

ACMG guideline offers guidance on interpretative categories for variants (182). Of the 71 tested variants, 9 variants were predicted to be likely pathogenic, and 62 were predicted to be VUS (Supplementary Table 1).

Overview of *GABRG2* variants on GABA_A receptor activity

Receptor sensitivity of GABRG2 variants

The sensitivity of receptors to GABA, measured by GABA EC₅₀ values, is a critical property for mediating effective synaptic inhibition. GABA sensitivity was measured as the change in LogEC₅₀ of the variant when compared to WT. A threshold of $\Delta\text{LogEC}_{50} \pm 0.2$ was applied, such variants that had a ΔLogEC_{50} between ± 0.2 were considered a VUS. Of the 71 tested variants, 22 (L74V, P83T, D84V, T90R, R125C, N167K, M169T, Y274C, P302L, A303T, T310I, T317I, A322D, A322T, R323Q, R323W, S325L, L326H, P327L, K328M, R454W and N465S) showed significantly reduced GABA sensitivity, consistent with a LoF effect ($\Delta\text{LogEC}_{50} = -(0.22 - 0.77)$; all $p < 0.0001$, Dunnett's post-hoc test) (**Figure 5.1 A, Supplementary Table 2**). 15 variants (A106T, I107T, S139N, I277N, P282A, P282S, P282T, L285I, S306F, L307V, T316N, A334T, S340Y, F343L, Y468C) showed increased receptor sensitivity to GABA, consistent with a GoF effect ($\Delta\text{LogEC}_{50} = 0.31 - 0.89$; all $p < 0.0001$, Dunnett's post-hoc test) (**Figure 5.1 A, Supplementary Table 2**). One variant, V98E had increased GABA sensitivity with reduced maximum GABA-evoked current. GABA sensitivity was not measured for one variant, W121C, because it did not display any detectable current. The remaining 32 variants did not change the EC₅₀ compared to WT (**Figure 5.1 A, Supplementary Table 2**).

Maximum GABA-evoked current amplitude in GABRG2 variants

A statistically significant reduction in maximum elicited current compared to WT was observed in 17 (16 variants are shown as pink bars, one variant was omitted in the graph due to no current) out of 71 variants (**Figure 5.2 B, Supplementary Table 2**). No statistically significant difference was observed for the remaining 54 variants (shown as grey bars).

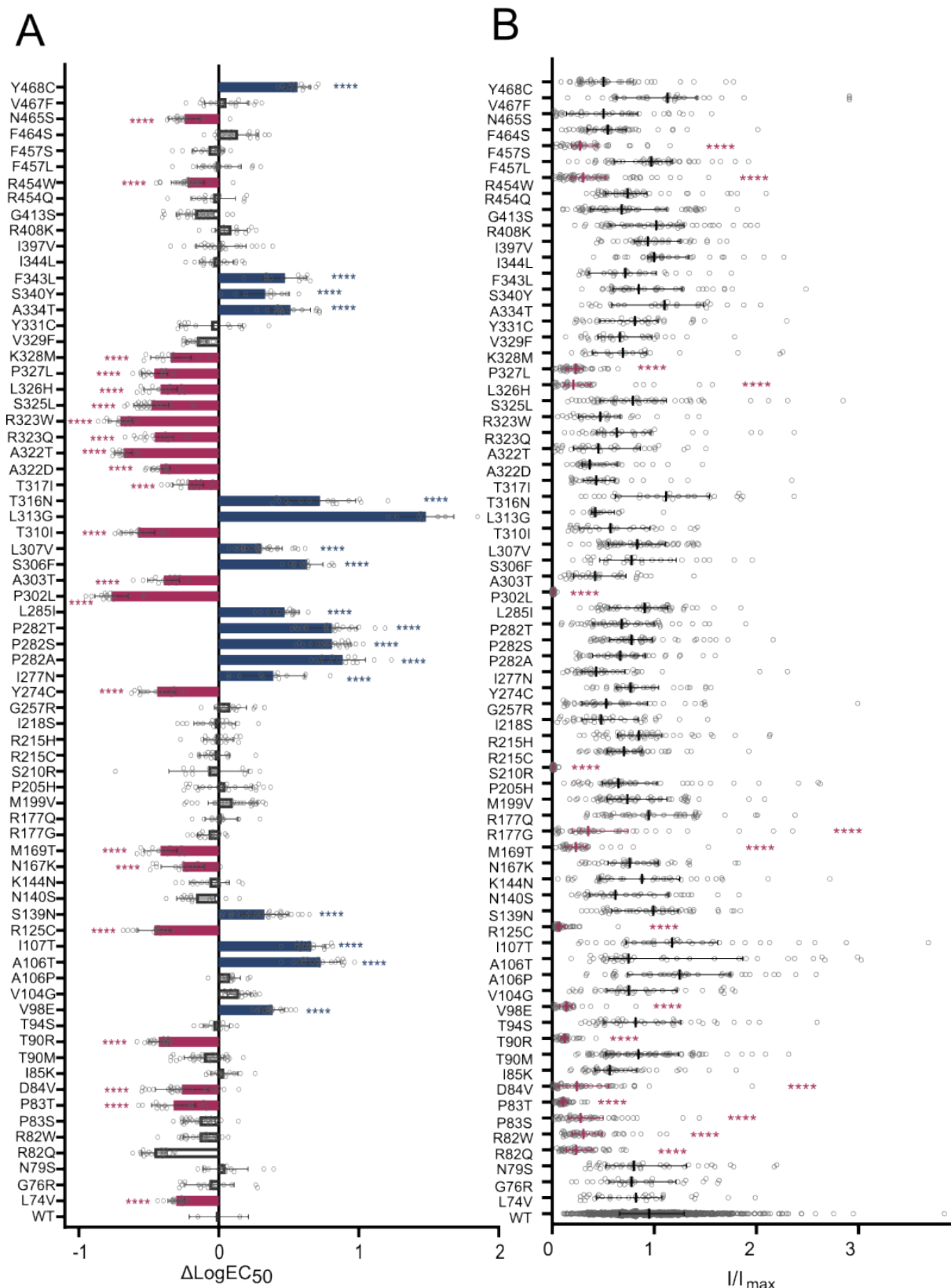


Figure 5.1 Functional outcome for 71 tested GABRG2 variants.

A) Bar graph summarising changes in LogEC₅₀ for GABRG2 (mean $\Delta\text{LogEC}_{50} \pm \text{SD}$) for variants. The x-axis denotes ΔLogEC_{50} . Blue bar denotes increased GABA sensitivity (GoF) with the bar rightwards, pink bar denotes reduced GABA sensitivity (LoF) with the direction of the bars to the left. Grey bars denote no significant change in LogEC₅₀. Significance was determined using one-way ANOVA ($F_{(70,1076)} = 120.4$), and multiple comparisons corrected Dunnett's post-hoc test (**** = $p < 0.0001$, $n = \text{cells (variant)}$); 10 (S340Y); 11 (T94S, K328M); 12 (M169T, S210R, I277N, A303T, A322D, L326H, F343L); 13 (N79S, I85K, S306F, A322T, Y331C, R454Q, Y468C); 14 (A106P, N140S, K144N, R177Q, Y274C, P282A, P302L, T310I, T317I, R323W, R408K); 15 (L74V, G76R, R82Q, P83T, R125C, N167K, L285I, T316N, R323Q, P327L, A334T N465S); 16 (V98E, I107T, R177G, R215C, F457S); 17 (I218S, G257R, V329F, I344L, F464S, V467F); 18 (T90R, V104G, A106T, R215H, F457L); 21 (P282S), 22 (R82W, P282T, I397V); 23 (P83S, M199V, S325L, G413S, and R454W); 24 (P205H); 25 (D84V and L307V); 27 (T90M) and 29 (S139N). **B)** Bar graph summarising changes in maximum GABA elicited current, I_{max} for GABRG2. Bars indicate median and IQR. Significance was determined using Mann-Whitney U test (**** = $p < 0.0001$, total $n = 663$ (WT); 22 (K328M); 25(S306F); 26 (F343L); 27 (R323W, A334T); 28 (G76R, S210R, T316); 29 (T310I, T317I, V329F); 30 (L74V, Y274C, P302L, A303T, A322D, R323Q); 31 (V467F); 32 (T90R, V98E, L326H); 33 (N140S, M169T, Y331C); 34 (A332T, I344L); 35 (V104G, S340Y), 36 (I107T, N167K, P282A, I397V, N465S), 37 (N79S, P83T, A106T, K144N, I218S, G257R, I277N, F457S); 38 (R177G); 39 (I85K, T94S, A106P, Y468C); 40 (R125C, P327L), 41(R177Q); 42 (P83S, R454Q); 43 (R82Q, F464S); 44 (R82W, R408K); 45 (P205H, R215C, R215H); 46 (D84V, P282T); 47 (L285I, F457L); 48 (S139N, P282S); 50 (S325L); 52 (M199V); 53 (L307V); 55 (G413S); 57 (R454W); and 65 (T90M). Note: W121C had no current and, therefore, is not added to the graph for GABA sensitivity and max current.

Categorisation of variants based on functional studies

Based on the functional studies, 15 variants were categorised as GoF with increased GABA sensitivity (**Supplementary Table 2**). A total of 29 variants were categorised as LoF; 6 variants reduced only maximal GABA-evoked amplitude, 13 variants reduced GABA sensitivity, 9 variants reduced both GABA sensitivity and maximum current, and one variant resulted in a significant reduction in maximum current that GABA sensitivity could not be measured (**Supplementary Table 2**). One variant, V98E, had increased GABA sensitivity but reduced maximum current and classified as GoF/LoF (**Supplementary Table 2**). The remaining 26 variants did not show significant changes to the functional parameters analysed (**Supplementary Table 2**).

ACMG criteria post functional assessment

Following functional analysis, the ACMG was utilised to re-assess the pathogenicity of the variants. Four variants – T90M, V104G, R454W, and V467F - were found to be VUS, 16 were likely pathogenic, 26 were likely benign and 25 classified as pathogenic (**Supplementary Table 3**).

Structural and 2D mapping of the variants

Pathogenic and benign variants were mapped on a lollipop graph of *GABRG2*. A total of 33 variants were concentrated in the ECD, 26 variants in the TMD, two in the M1-M2 loop, seven in the M2-M3 loop and three in the large M3-M4 loop (**Figure 5.2**). Presumed benign variants were concentrated in the ECD (16/26), with additional variants located at the M2-M3 loop (V329F, Y331C), TM3 (I344L), the large M3-M4 intracellular loop (I397V, R408K, G413S), and TM4 (R454Q, F457L, F464S, V467F). These regions coincide with regions enriched with gnomAD variants. Specifically, we tested 25 gnomAD variants; 15 variants are located in the ECD, two variants (V329F, Y331C) in the M2-M3 loop, one (A334T) in TM3, two variants (I397V and G413S) are in the large M3-M4 intracellular loop - regions considered less critical for basic receptor function, and five variants (R454Q, R454W, F457L, N465S, Y468C) in TM4 (**Figure 5.2**).

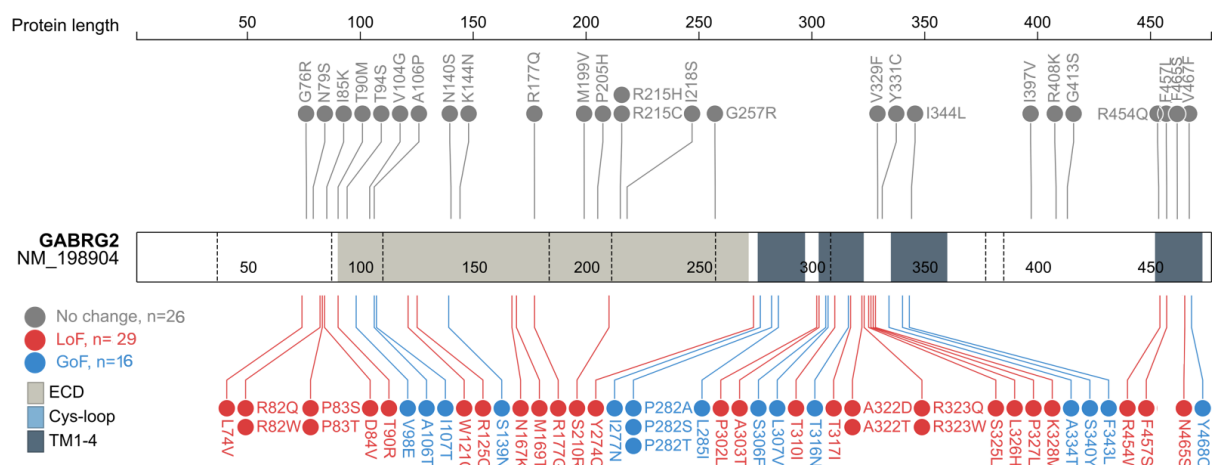


Figure 5.2 2D mapping of *GABRG2* variants on a lollipop graph.

Variants above bar represented in grey spheres indicate no functional change ($n = 26$), variants below indicated by red spheres are LoF variants ($n = 29$), and variants denoted in blue spheres indicate GoF variants ($n = 16$ variants that increased GABA sensitivity).

Sub-categorisation of variant's effect and location and clinical phenotype

It has been previously reported that the severity of clinical phenotype is associated with the location of the variant, with variants located in TM1/2 generally being more severe than those in the ECD. We sought to elucidate how the variant locations on *GABRG2* are associated with clinical phenotype, and whether location or function is the main determining factor of the phenotype.

Extracellular domain (ECD)

33 variants were identified in the ECD (one variant, W121C had no current and, therefore, was omitted in the graph for GABA sensitivity and max current) (**Figure 5.3 A, B**). 16 variants showed no significant changes to the receptor function. 13 LoF missense variants resulted in a net reduction in receptor inhibition; two variants (L74V and N167K) reduced GABA sensitivity, five variants (P83T, D84V, T90R, R125C, M169T) reduced both GABA sensitivity and maximum current, five variants (R82Q, R82W, P83S, R177G, S210R) reduced maximal GABA-evoked current and one variant, W121C, had no current (**Figure 5.3 A, B**). Three missense variants (A106T, I107T, S139N) increased receptor sensitivity (**Figure 5.3 A, B**). Interestingly, one variant, V98E, increased GABA sensitivity but had a significant reduction in maximum current (**Figure 5.3 A, B**).

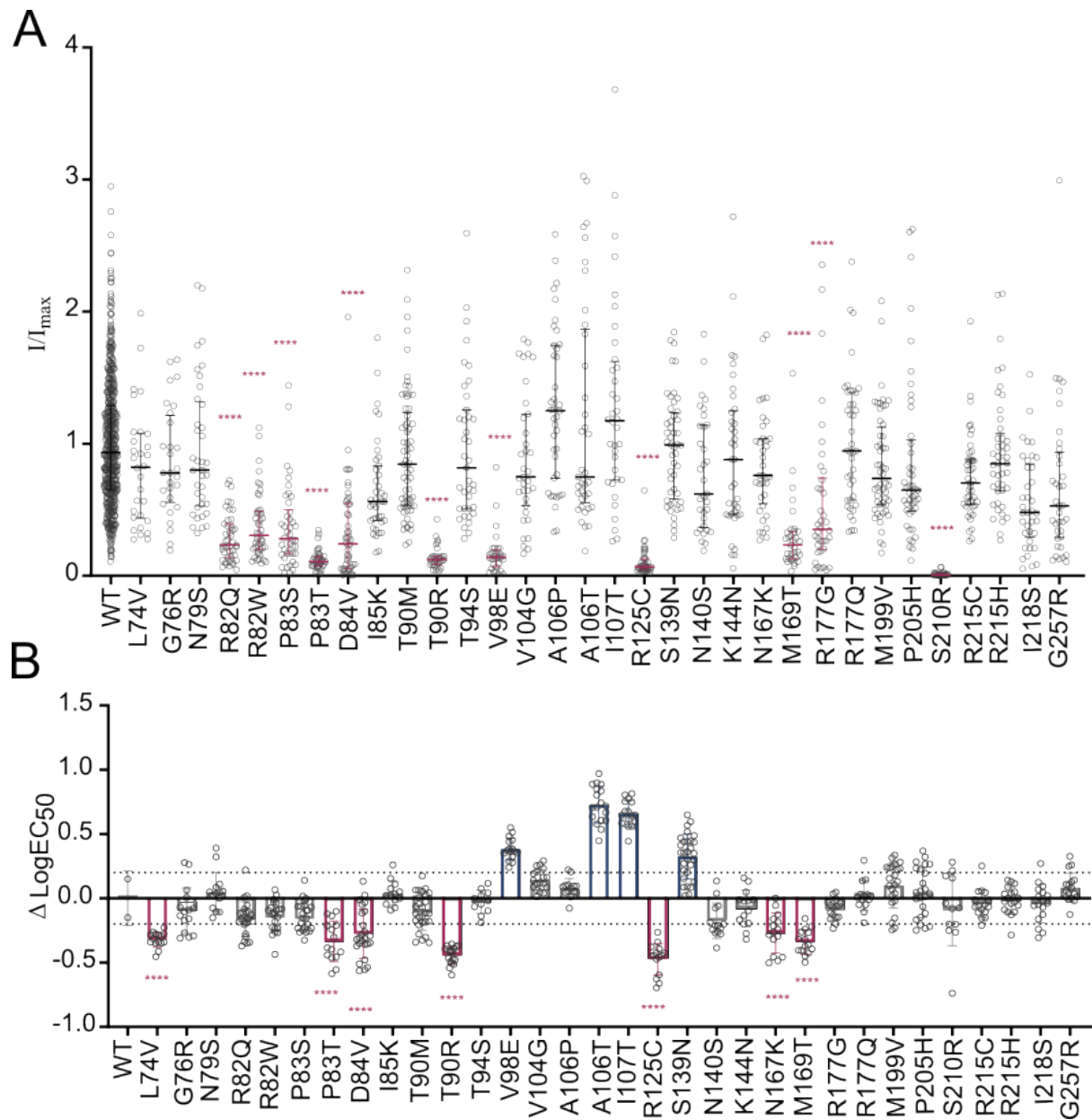


Figure 5.3 ECD Variants effect on receptor function.

A) Normalised maximum current of each variant to WT. Data presented as median (IQR). $n = 561$ cells for WT and $n = 28-65$ for each variant. Each dot represents a single cell. At least two batches of oocytes were used to record. (**** = $p < 0.0001$) **B)** Bar graph summarising changes in LogEC_{50} for GABRG2 (mean $\Delta \text{LogEC}_{50} \pm \text{SD}$) for variants. The x-axis denotes the point mutation of the variant. Blue bar denotes increased GABA sensitivity with the pointing upwards. Pink bars denote reduced GABA sensitivity. (**** = $p < 0.0001$)

Table 5.1 Summary table of key functional and phenotypic characteristics of patients harbouring GABRG2 variants

#	Sex, age at last follow up	GABRG2 variant	Inheritance	Functional evaluation	Epilepsy	Syndrome phenotype	Age at sz onset (m)	Ref
1	uk , uk	L74V	uk	LoF				(208)
2	F , 181	N79S	uk	Neutral	Yes	Epilepsy with generalised tonic-clonic seizures	180	(220)
3	uk , uk	R82Q	uk	LoF	Yes	FS	-	(209)
4	uk , uk	R82Q	uk	LoF		-	-	(208)
5	F , uk	R82Q	maternal	LoF	Yes	CAE	21	(166); (113)
6	F , 116	R82Q	paternal	LoF	Yes	-	30	-
7	F , 120	R82W	paternal	LoF	Yes	Generalised epilepsy	30	-
8	F , 132	P83T	paternal	LoF	Yes	-	59	-
9	F , 60	P83T	maternal	LoF	Yes	CAE	40	-
10	M , 84	P83S	paternal	LoF	Yes	Lennox Gastaut	9	(211)
11	F , uk	P83S	maternal	LoF	Yes	-	-	(210)
12	M , 132	I85K	paternal	Neutral	Yes	EMA	6	-
13	F , 96	T90M	<i>de novo</i>	Neutral	Yes	GEFS+	1	-
14	M , 37	T90M	maternal	Neutral	Yes	-	19	-
15	F , 192	T90R	<i>de novo</i>	LoF	Yes	DS	7	(212)
16	F , 180	V104G	<i>de novo</i>	Neutral	Yes	CAE	18	-
17	F , 84	A106T	<i>de novo</i>	GoF	Yes	EOEE	0.03	(171)

18	M , 108	A106T	<i>de novo</i>	GoF	Yes	EE	3	(171)
19	F , 72	A106T	<i>de novo</i>	GoF	Yes	EE	24	(169)
20	M , 60	A106T	uk	GoF	Yes	EE	1.38	(169)
21	F , 156	A106T	<i>de novo</i>	GoF	Yes	EE	0.03	(169)
22	F , 7	A106T	uk	GoF	Yes	EE	0.07	(169)
23	uk , uk	A106T	uk	GoF	uk	uk	uk	(208)
24	F , 180	A106T	<i>de novo</i>	GoF	Yes	GEFS+	4	(213)
25	F , 21	A106T	<i>de novo</i>	GoF	Yes	-	3.5	
26	M , 120	A106T	<i>de novo</i>	GoF	Yes	DEE	2	
27	F , 48	A106T	uk	GoF	No	-	-	
28	M , 319	A106T	<i>de novo</i>	GoF	Yes	Lennox-Gastaut	27	
29	M , 318	A106T	<i>de novo</i>	GoF	Yes	Lennox-Gastaut	27	
30	uk , uk	A106T	uk	GoF	uk	uk	uk	
31	M , uk	A106T	<i>de novo</i>	GoF	Yes	DEE	1.32	
32	F , 36	I107T	<i>de novo</i>	GoF	Yes	EE	1.5	(171)
33	UK , 125	R125C	paternal	LoF	Yes	GEFS+	17	
34	M , 203	S139N	<i>de novo</i>	GoF	No	-	-	
35	uk , uk	N140S	uk	Neutral	uk	uk	uk	(208)
36	M , 167	N167K	<i>de novo</i>	LoF	Yes	FS+	7	(93)
37	M , 216	M169T	<i>de novo</i>	LoF	Yes	Focal and Generalised Epilepsy	24	

38	M , 288	M169T	familial	LoF	Yes	DEE	24	
39	F , uk	R177G	paternal	LoF	Yes	-	13	(214)
40	M , 672	M199V	uk	Neutral	Yes	-	6	(172)
41	M , 132	M199V	paternal	Neutral	Yes	GEFS+	18	
42	M , 13	R215H	paternal	Neutral	Yes	FS	12	(80)
43	M , 432	I218S	maternal	Neutral	Yes	FS	11	(221)
44	F , uk	G257R	maternal	Neutral	Yes	Atypical Rolandic Epilepsy	-	(222)
45	uk , uk	G257R	uk	Neutral	uk	uk	uk	(223)
46	uk , 72	Y274C	uk	LoF	Yes	EMAS	-	(224)
47	M , 216	P282A	<i>de novo</i>	GoF	Yes	EIDEE	0	(40)
48	M , uk	P282S	uk	GoF	uk	uk	uk	(223)
49	F , 120	P282S	<i>de novo</i>	GoF	Yes	EE	12	
50	uk , uk	P282T	uk	GoF	uk	uk	uk	(208)
51	F , 144	P282T	<i>de novo</i>	GoF	Yes	Lennox-like	48	(211)
52	M , 18	P282T	<i>de novo</i>	GoF	Yes	DEE	11	(225)
53	F , 46	L285I	uk	GoF	Yes	DEE	2.6	
54	M , uk	P302L	<i>de novo</i>	LoF	Yes	DS	12	(91)
55	uk , uk	P302L	uk	LoF	uk	uk	uk	(208)
56	M , uk	P302L	<i>de novo</i>	LoF	Yes	DS	7	(212)
57	M , uk	P302L	<i>de novo</i>	LoF	Yes	DS	7	(93)
58	M , uk	A303T	<i>de novo</i>	LoF	Yes	Epilepsy	24	(93)

59	M , 11	S306F	<i>de novo</i>	GoF	Yes	EIMFS	-	(211)
60	uk , uk	L307V	uk	GoF	uk	uk	uk	(208)
61	M , uk	T310I	<i>de novo</i>	LoF	Yes	Focal epilepsy	6	(93)
62	F , uk	T310I	<i>de novo</i>	LoF	Yes	Epilepsy	8	(93)
63	M , 26	T316N	<i>de novo</i>	GoF	Yes	EIDEE	2	
64	M , uk	T317I	<i>de novo</i>	LoF	Yes	FS+	8	(93)
65	M , 60	A322D	paternal	LoF	Yes	GEFS+	13	
66	F , 23	A322D	paternal	LoF	Yes	GEFS+	16	
67	M , 24	A322T	<i>de novo</i>	LoF	Yes	DS	10	
68	M , 29	R323Q	<i>de novo</i>	LoF	Yes	EMAS	8	(226)
69	M , uk	R323Q	<i>de novo</i>	LoF	Yes	Childhood epilepsy with centrotemporal spikes	-	(222)
70	F , 48	R323Q	<i>de novo</i>	LoF	Yes	EE	10	(171)
71	F , 38	R323Q	<i>de novo</i>	LoF	Yes	EE	12	(171)
72	uk , uk	R323Q	uk	LoF	uk	uk	uk	(208)
73	uk , uk	R323Q	uk	LoF	uk	uk	uk	(223)
74	M , uk	R323Q	maternal	LoF	Yes	DS	9	(40)
75	F , uk	R323Q	maternal	LoF	Yes	DS	8	(40)
76	M , uk	R323Q	<i>de novo</i>	LoF	Yes	DEE	10	(40)
77	F , uk	R323Q	uk	LoF	Yes	-	-	(223); (227)
78	F , 102	R323Q	maternal	LoF	Yes	DEE	11	
79	M , 26	R323Q	<i>de novo</i>	LoF	Yes	EMAS	7	
80	M , uk	R323Q	uk	LoF	-	-	-	

81	uk , uk	R323Q	uk	LoF	-	-	-	
82	F , 108	R323W	<i>de novo</i>	LoF	Yes	EE	-	(171)
83	M , uk	R323W	<i>de novo</i>	LoF	Yes	EMAS	15	(40)
84	uk , uk	R323W	uk	LoF	-	-	-	(208)
85	F , uk	R323W	uk	LoF	Yes	Occipital lobe epilepsy	1	(228)
86	F , 64	R323W	uk	LoF	Yes	-	8	
87	F , 309	R323W	<i>de novo</i>	LoF	Yes	DEE	17.5	
88	uk , uk	S325L	uk	LoF	-	-	-	(223)
89	uk , uk	S325L	<i>de novo</i>	LoF	-	FS	17	(80)
90	M , 90	L326H	<i>de novo</i>	LoF	Yes	GEFS+	17	-
91	F , 57	P327L	maternal	LoF	Yes	-	18	
92	F , uk	K328M	familial	LoF	Yes	GEFS+	-	(167)
93	uk , uk	V329F	uk	Neutral	-	-	-	
94	F , uk	Y331C	<i>de novo</i>	Neutral	Yes	GEFS+	12	-
95	M , 31	A334T	paternal	GoF	Yes	Focal epilepsy	18	-
96	uk , uk	A334T	maternal	GoF	Yes	Refractory generalised epilepsy	9	-
97	F , 72	F343L	<i>de novo</i>	GoF	Yes	EE	12	
98	F , 6	I344L	maternal	Neutral	Yes	DEE	3	
99	F , uk	I397V	paternal	Neutral	Yes	Childhood epilepsy with centrottemporal spikes	-	(222)
100	F , 51	R408K	maternal	Neutral	Yes	FS	18	(80)
101	F , 552	G413S	maternal	Neutral	Yes	CAE	-	(229)

102	M , 468	G413S	maternal	Neutral	Yes	IGE (CAE/JAE indistinguishable)	122	(229)
103	uk , uk	R454W	uk	LoF	-	-	-	(208)
104	F , uk	F457S	<i>de novo</i>	LoF	Yes	FS	15	
105	M , uk	N465S	uk	LoF	Yes	JAE	-	
106	F , uk	N465S	<i>de novo</i>	LoF	Yes	FS	15	
107	uk , uk	V467F	<i>de novo</i>	Neutral	-	-	-	(223)
108	M , uk	Y468C	maternal	GoF	Yes	GEFS+/FS	11	(80)
109	M , uk	Y468C	<i>de novo</i>	GoF	Yes	FS	18	(80)

Abbreviations: EOOE, Early onset epileptic encephalopathy; EE, epileptic encephalopathy; EIDEE, Early infantile developmental and epileptic encephalopathy; EMAS, Epilepsy with myoclonic-atonic seizures; IGE, Idiopathic generalised epilepsy; JAE, Juvenile absence epilepsy; uk, unknown

Clinical profile of individuals with neutral variants in the ECD

Twelve patients identified harboured variants in the ECD that did not significantly change the receptor function. 83% (10/12) were diagnosed with epilepsy, with five individuals (#14, #40, #41, #43, #44) having FS at onset. The median age of onset was 12 months (95% CI: 6-19). Seven patients (#2, #12, #13, #16, #41-43) were reported to have normal intellectual function post-seizure, while one individual was reported to have moderate ID post-seizure. Three out of 12 patients had other neurological findings, including specific learning disability (#12), gait imbalances (#14) and neurodevelopmental abnormality (#45). 67% (4 out of 6) of patients had normal language development; however, in the two patients that had language impairment, one individual (#14) is non-verbal, and the other had delayed development of normal language (#41). The characteristics and phenotypic features of those ECD variants are summarised in Table 5.1 and Table 5.2, with additional information provided in Supplementary Table 4.

Clinical and neurological outcomes in patients with LoF *GABRG2* variants in the ECD

A total of 16 individuals were identified harbouring variants that resulted in a net loss of receptor function, either through a reduction in maximum current and/or GABA sensitivity. 14

individuals were diagnosed with epilepsy; diagnosis included FS (#3), FS+ (#36), generalised epilepsy (#7), focal and generalised (#37), GEFFS+ (#33), CAE (#5, #9), Lennox-Gastaut (#10), DS (#15) and DEE (#38), the diagnosis for the remaining 4 (#6, #8, #11, #39) are unknown. The median age of seizure onset was 22.5 months (95% CI: 9-30 months).

Intellectual disability post-seizure varied among individuals: normal (#7), mild ID (#37), moderate (#8) and severe (#10, #15, and #38). None of the reported individuals had movement disorder or vision impairment. Four individuals (#6, #8, #10, and #33) out of seven had language impairment. In addition, three out of four reported patients also had neuropsychiatric and/or behavioural abnormalities, including autistic behaviour (#6), ASD (#10) and ADHD (#9, #10). The characteristics and phenotypic features of those ECD variants are summarised in Table 5.1 and Table 5.2, with additional information provided in Supplementary Table 4.

Clinical and neurological outcomes in patients with GoF *GABRG2* variants in the ECD

Seventeen patients have been identified with GoF variants. Among them, 87% (13/17) were diagnosed with epilepsy, with the syndrome known for 12 individuals. Epilepsy syndromes included: epileptic encephalopathy (EE) in 6 individuals (#18-22, #32); early onset epileptic encephalopathy (EOEE) (#17), DEE (#26, #31), GEFS+ (#24), Lennox-Gastaut (#28, #29) and the diagnosis for one individual (#25) remains unknown. The median age of onset was 2 months (95% CI: 0.066-24 months). The characteristics and phenotypic features of those ECD variants are summarised in Table 5.1 and Table 5.2, with additional information provided in Supplementary Table 4.

Following seizure onset, 9 out of 10 patients were reported to have severe ID, with the intellectual status of one patient being undetermined. Movement impairment was found in three (#18, #29, #32) out of 10 individuals. Of the reported cases of language impairment, all 13 patients exhibited some form of language impairment: 9 were reported to have no language development (#18-22, #24-26, #32), three individuals (#27-29) had rudimentary understanding but no or limited verbal expression and one individual (#34) exhibited delayed development of normal language skills.

Six out of seven reported patients have some form of visual impairment, including cerebral visual impairment (#19, #21), poor eye contact (#25), hypermetropia (#27), and astigmatism (#28, #29). Additionally, fourteen patients reported having one or more abnormal neurological findings, with hypotonia being the most frequent (14/14), followed by nystagmus (6/14).

Transmembrane domain (TMD)

The TMD has a role in the functional transition of the receptor from resting, pre-activated, activated and desensitised state as well as interacting with the lipid environment (81). The TMD is a common hotspot for pathogenic variants, in particular TM1/2. We have identified 15 variants in the TM1 and TM2 regions. In the TM1 region, we have identified a total of five GoF variants, I277N, P282A/S/T and L285I, and one LoF variant, Y274C (**Figure 5.4 A, B**). The characteristics and phenotypic features of those TMD variants are summarised in Table 5.1 and Table 5.2, with additional information provided in Supplementary Table 4.

The TM2 of each subunit lines the pore of the GABA_A receptor, with the 9' leucine being 100% amongst all ligand-gated ion channels, having an important role in the gating of the receptor. Here we have identified a total of nine variants located in the TM2. Three variants, S306F, L307V and T316N resulted in a GoF receptor; six variants, T310I, T317I, A332D/T, R323Q and R323W resulted in a LoF receptor (**Figure 5.4 A, B**).

In the TM3 region, we identified two GoF variants, S340Y and F343L and one neutral, I344L (**Figure 5.4 A, B**). Within the TM4, we identified three variants, R454W, F457S, and N465S, that results in net loss of receptor function; one GoF variant, Y468C, and three neutral variants, R454Q, F457L and F464S (**Figure 5.4 A, B**).

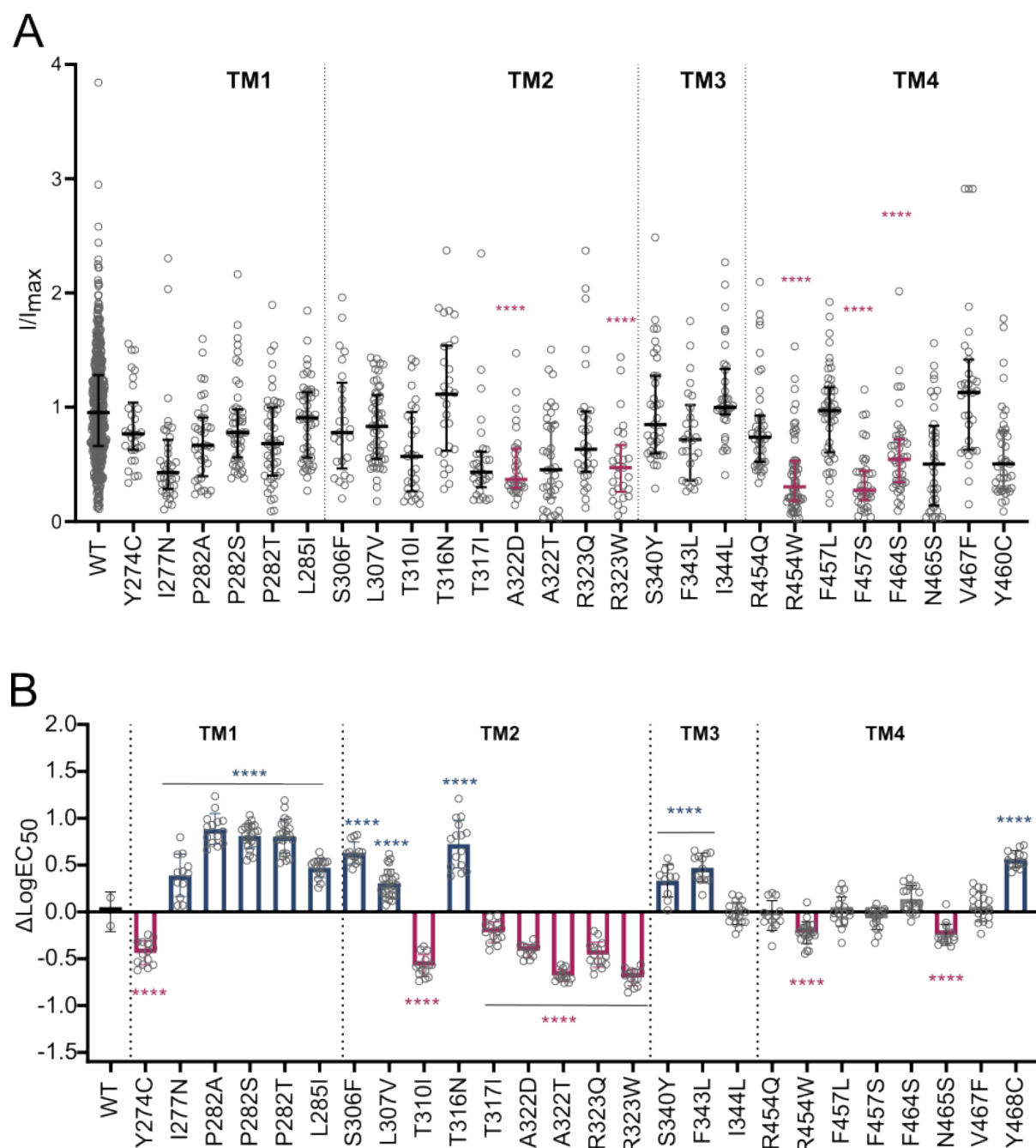


Figure 5.4 TM1-4 variants effect on receptor function.

A) Normalised maximal GABA-evoked current for each variant to WT. Each dot represents a single cell. Data presented as median (IQR). $n = 450$ (WT) and $n = 25-57$ cells for each variant. (**** = $p < 0.0001$) **B)** Bar graph summarising changes in LogEC_{50} for GABRG2 (mean $\Delta \text{LogEC}_{50} \pm \text{SD}$) for variants. The x-axis denotes the point mutation of the variant. Blue bar denotes increased GABA sensitivity with the bar upwards, pink bar denotes reduced GABA

*sensitivity with the direction of the bars downwards. **** $p < 0.0001$, $n = 12-25$ independent cells per variant. Each variant was tested with at least two batches of *Xenopus laevis* oocyte)*

Clinical phenotype of patients harbouring variants in the TM1

One patient (#46) was found to harbour a LoF variant in TM1. The patient was diagnosed with epilepsy with myoclonic-atonic seizures (EMAS). The referring physician did not report on intellectual, neurological or language abnormalities, but it was noted that the patient had behavioural abnormalities.

Seven patients have been identified with GoF variants. Five out of seven individuals were diagnosed with epilepsy, including EIDEE (#47), EE (#49), lennox-like (#51) and DEE (#52-53). The median age of onset was 11 months (95% CI: 0 – 48 months). Four out of seven patients (#47, #49, #51, #53) were reported to have severe ID post-seizure, while ID for three individuals was not reported.

Two out of four reported patients had movement disorder. Three patients (#49, #51, #53) had no language development, and the remaining four were not reported. Of the reported cases, two out of three had visual impairments (#47, #52). Abnormal neurological findings were reported in 6 out of 7 patients (#48-49, #51-53), with hypotonia (4/7) being the most frequent. No neuropsychiatric and/or behavioural features were reported for the GoF variants in TM1.

TM2 clinical phenotype

26 individuals with TM2 LoF variants were identified. Among them, 21 were diagnosed with epilepsy, including: childhood epilepsy with centrotemporal spikes (#69), EMAS (#68, #79, and #83), occipital lobe epilepsy (#85), FS+ (#64), GEFS+ (#65-66), focal epilepsy (#61), DS (#67, #74 and #75), DEE (#76, #78, #87), EE (3/26) (#68, #69, #80), and the remaining three individuals did not have a specific epilepsy diagnosis (#62, #77, and #86). Three out of 12 patients had severe ID post-seizure.

The median age of onset was 9.5 months (95% CI: 8 - 12 m). One out of 12 patients experienced movement impairment. Language impairments were reported in seven out of 10 patients, ranging from delayed development of normal language (#65, #79) to rudimentary

understanding, but no or limited verbal expression (#77, #78), and no language development (#70, #71, 82).

Three individuals have been identified with GoF variants, all of which were *de novo* missense variants. Two variants, S306F and T316N, were found present in patients diagnosed with EIMFS and EIDEE, respectively. Both individuals with S306F and T316N variants experience daily seizures and exhibit no language development, visual impairment, or neurological abnormalities, including hypotonia and nystagmus. Neither patient have been reported to have any neuropsychiatric and/or behavioural abnormalities.

Clinical phenotype of patients harbouring variants in TM3

We identified two individuals harbouring variants within the TM3: F343L (GoF) and I344L (neutral). The individual with F343L variant (#97) was diagnosed with EE, with a seizure onset age at 12 months. The patient exhibited severe ID post-seizure, no language development and neurological abnormalities including hypotonia, abnormal posturing. One individual (#98) harbouring an I344L variant was diagnosed with DEE, with a seizure age of onset at 3 months. The patient did not exhibit impairments in movement, language, or visual abilities, however, did have neurological abnormalities including hypotonia and developmental regression.

Clinical phenotype of patients harbouring variants in TM4

We identified seven individuals harbouring variants within the TM4 region. Four individuals had LoF variants -R454W (#103), F457S (#104) and N465S (#105, #106) are LoF. Three of these individuals were diagnosed with epilepsy with diagnosis including: FS (#104, #105) and JAE (#105). These patients did not exhibit impairments in movement disorder, language, or visual abilities.

One individual (#107) had a neutral variant, V467F and did not have epilepsy but was diagnosed with ASD.

Two individuals harboured a GoF variant, Y468C. Both were diagnosed with epilepsy, with syndromes including GEFS+/FS (#108) and FS (#109) with age of seizure onset at 11 m and 18 m respectively.

Clinical features of individuals harbouring variants within the M1-M2 and M2-M3 loop

Clinical phenotype of patients harbouring variants in the M1-M2 loop

Two assessed variants, P302L and A303T, located in the M1-M2 loop, resulted in a reduction in GABA sensitivity. A total of five individuals were identified, four have been diagnosed with epilepsy syndromes: DS (#54, #56-57) and “epilepsy” (#62). The median age of onset was 9.5 m (95% CI: 7-24 months). The variants for 4 out of 5 individuals occurred *de novo*, the remaining one individual (#55) did not have inheritance reported.

Post-seizure, one individual (#56) was reported to have moderate ID and motor delay. Language impairment and global DD were reported in another individual (#54). The characteristics and phenotypic features of those M1-M2 and M2-M3 loop variants are summarised in Table 5.1 and Table 5.2, with additional information provided in Supplementary Table 4.

Clinical phenotype of patients harbouring variants in the M2-M3 loop

The M2-M3 loop is important in the signal transduction process, linking the binding-coupling pathway to the pore, determining the energy efficiency from ligand binding to channel gating (81, 230). We identified a total of nine variants in the M2-M3 loop, five of which were associated with patients with epilepsy. Epilepsy-associated variants in the M2-M3 loop included three LoF variants: L326H, P327L and K328M; one variant had no significant functional change, Y331C; and one GoF variant, A334T.

Three patients with LoF variants, L326H (age of seizure onset at 17 months) (#90), P327L (age of seizure onset at 18 months) (#91), and K328M (the age of seizure onset is unknown) (#92) and one patient harbouring Y331C (age of seizure onset at 12 months) (#94), a variant that did not change the receptor function. Epilepsy syndrome for patients harbouring LoF or neutral variant were GEFS+ (#90, #92, #94). The two patients harbouring a GoF variant, A334T, were diagnosed with focal epilepsy and refractory generalised epilepsy at 18 months and 9 months, respectively.

Variants located in the M3-M4 intracellular loop

Within the large M3-M4 loop, we have identified three variants: I397V, R408K, and G413S. Functional analysis of the variants showed no statistically significant difference in GABA sensitivity or maximum GABA-evoked current compared to WT. The characteristics and

phenotypic features of those M3-M4 intracellular loop variants are summarised in Table 5.1 and Table 5.2, with additional information provided in Supplementary Table 4.

Phenotype of patients harbouring variants in the large M3-M4 intracellular loop

We have identified a total of four individuals harbouring variants within the M3-M4 loop. Their epilepsy diagnosis ranged from childhood epilepsy with centrotemporal spikes (#99), IGE (CAE/JAE indistinguishable) (#102), CAE (#101), and FS (#100). Among these patients, none experienced ID post-seizure, movement disorder, language impairment, visual impairment, neurological impairment or neuropsychiatric and/or behavioural features abnormalities. In all cases, the variant was inherited.

Comparison of clinical phenotype between GoF and LoF cohort

Age of seizure onset and Inheritance patterns

Patients harbouring LoF variants presented with their first seizure at a median age of 12 months [95% CI: 9-17] compared to 3.75 months in the GoF cohort [95% CI: 1.50 – 12] ($p = 0.015$) (**Figure 5.5 A, Table 5.2**).

The inheritance pattern of the LoF cohort shows a similar distribution between inherited and *de novo* variants (**Figure 5.5 B**). Inheritance of GoF variants tends to be *de novo* (**Figure 5.5 B**). Of the known inheritance profile, GoF variants are more likely to be *de novo* than LoF (OR: 4.62; 95% CI [1.10 – 28.08], Fisher's exact test, $p = 0.02$). The characteristics and phenotypic features of variants are summarised in Table 5.2, with additional information provided in Supplementary Table 4.

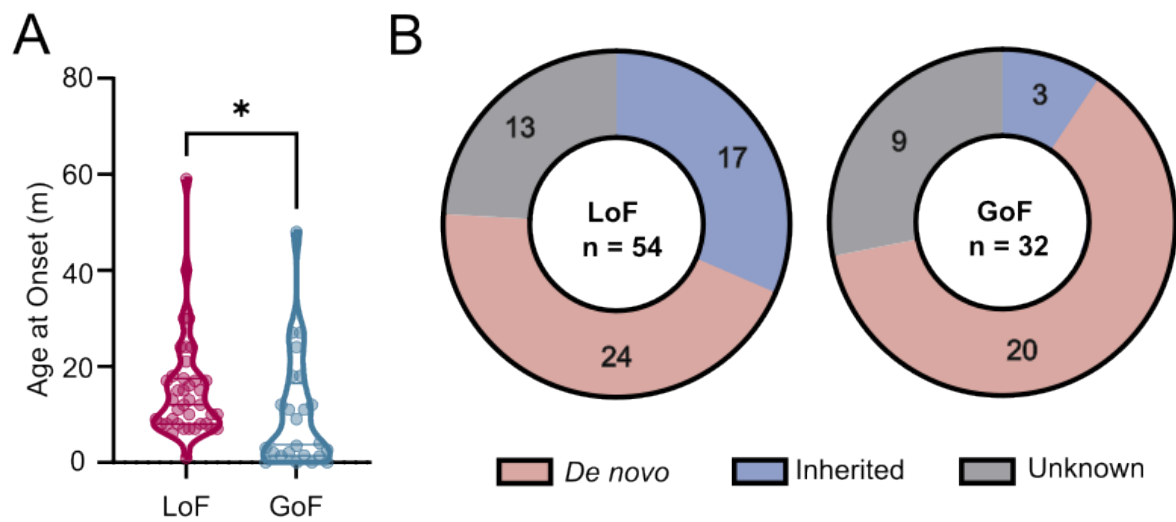


Figure 5.5 Age of seizure onset and inheritance patterns between LoF and GoF cohort.

A) Violin plot of age of seizure onset (m) at LoF (pink; $n = 39$) and GoF (blue; $n = 24$) variants. Dots represent individual data points. B) Donut chart of inheritance pattern at LoF (left) and GoF (right) cohort. * $p = 0.0015$

Table 5.2 Clinical characteristics of individuals with missense LoF and GoF GABRG2 variants.

Clinical features	LoF	GoF
Male : Female	21 : 22	15 : 12
Age, median (range)	87m (23-309m)	72m (7-319m)
Epilepsy		
Seizure onset, median (range)	12m (1 - 59m)	3.75m (0-48m)
Language		
No impairment	9/22 (41%)	-
Speech delay/impairment	9/22 (41%)	4/19 (21%)
Nonverbal	4/22 (18%)	15/19 (79%)
Vision		
No impairment	19/20 (95%)	2/10 (20%)
Impairment	1/20 (5%)	8/10 (80%)
Cognition		
Normal	8/23 (35%)	2/16 (12.5%)
Mild ID	5/23 (22%)	
Moderate ID	4/23 (17%)	
Severe/Profound ID	6/23 (26%)	14/16 (87.5%)
Psychiatric/behavioural problems	15/23 (65%)	1/11 (9%)
Neurological features		
Hypotonia	1/29 (3%)	21/23 (91.3%)
Nystagmus	0	9/23 (39.1%)

A closer look at comorbidities: how language, vision, cognition, neuropsychiatric and neurological issues differ between GoF and LoF cohort

In comparing the GoF and LoF cohorts, the GoF cohort had a higher rate of being non-verbal (15/19 compared to 4/22 in LoF) (OR = 15.36 [CI 2.97 – 106.14] Fisher's exact test $p = 0.00014$). Visual abnormalities showed significant differences between the two cohorts (8/10 vs 1/20 LoF; OR = 56.93 [CI 4.68 – 3426.94] Fisher's exact test $p < 0.0001$) (**Figure 5.6**). A greater proportion of patients harbouring GoF variants (15/19) experienced severe/profound ID post-seizure than LoF variants (4/22) (OR = 17.98 [95% CI: 2.91 -208.52], Fisher's Exact test $p = 0.0002$) (**Figure 5.6**). Notably, neuropsychiatric impairment was more prevalent in patients with LoF variants (15/23 vs 1/11 in the GoF group; OR = 0.058 [CI 0.0012 - 0.54] Fisher's exact test $p = 0.003$) (**Figure 5.6**). Neurological abnormalities were more common in the GoF (23/23) cohort than in the LoF cohort (9/28) (OR = n.d. Fisher's exact test $p < 0.0001$). Out of the neurological abnormalities, hypotonia was more common in patients with GoF variant (21/23 vs 1/29; OR = 216.60 [CI 20.78 – 11171.91] Fisher's exact test $p < 0.0001$) (**Figure 5.6**).

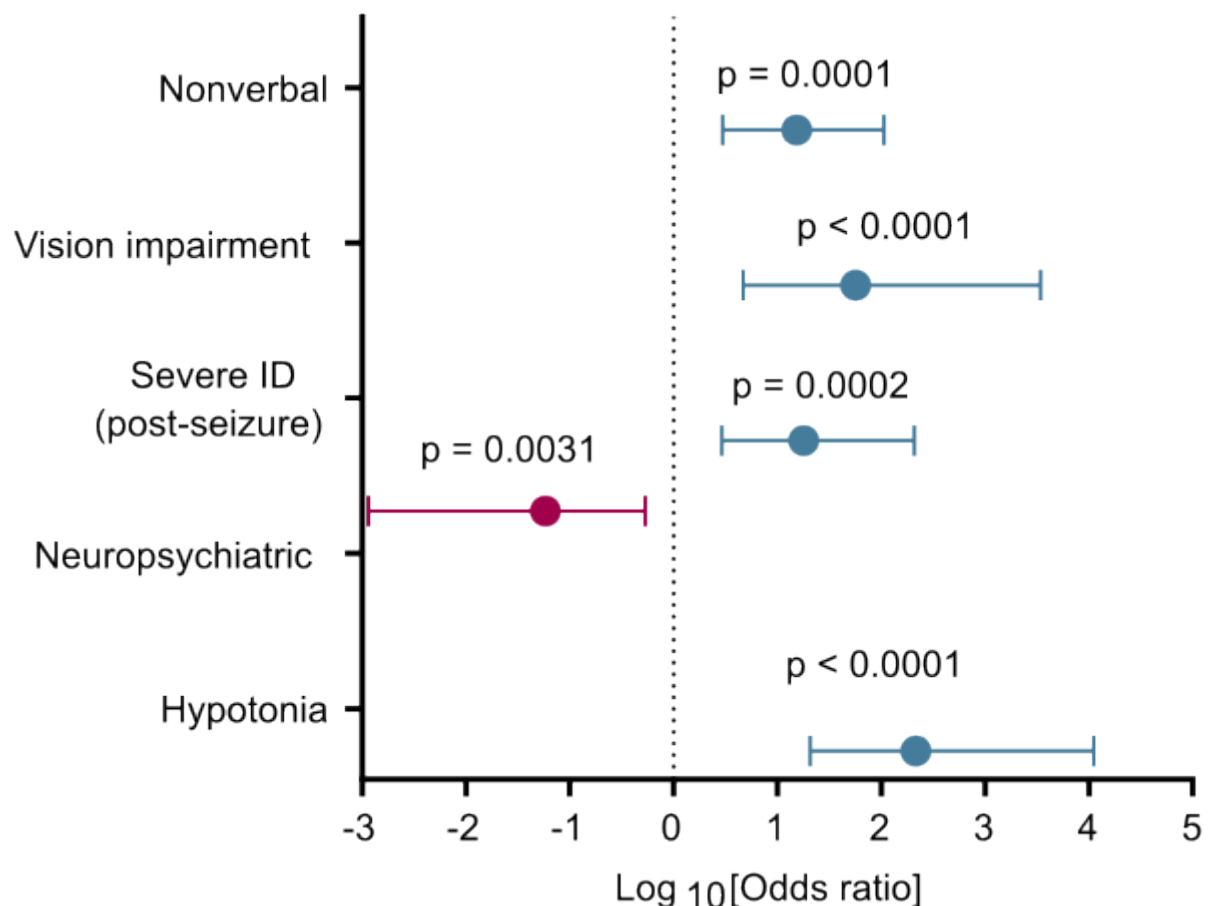


Figure 5.6 Odds ratio of key clinical outcomes between LoF and GoF cohort

Centre circle showing the Log_{10} Odds Ratio and 95 % confidence interval. Blue lines indicate it favours GoF variants, whilst pink favours LoF variants. Grey lines indicate no statistically significant difference.

Epilepsy types at LoF and GoF cohort

We then compared seizure type at onset between LoF and GoF patients, however, due to incomplete data, we were not able to make meaningful conclusions. This led us to investigate whether there were differences in epilepsy diagnosis. Here we found, for the 25 patients in the GoF cohort, the most frequently reported epilepsy type was EE (8/25, 32%), followed by DEE (5/25, 20%) (**Figure 5.7**). For the 39 LoF patients, the most frequently reported epilepsy type was DS (7/40, 17.5%), followed by GEFS+ (5/40, 12.5%) (**Figure 5.7**). Unexpectedly, DS was only identified in the LoF cohort (7/40 LoF vs 0/25 GoF; OR = N.D, Fisher's exact test $p = 0.038$).

Epilepsy types

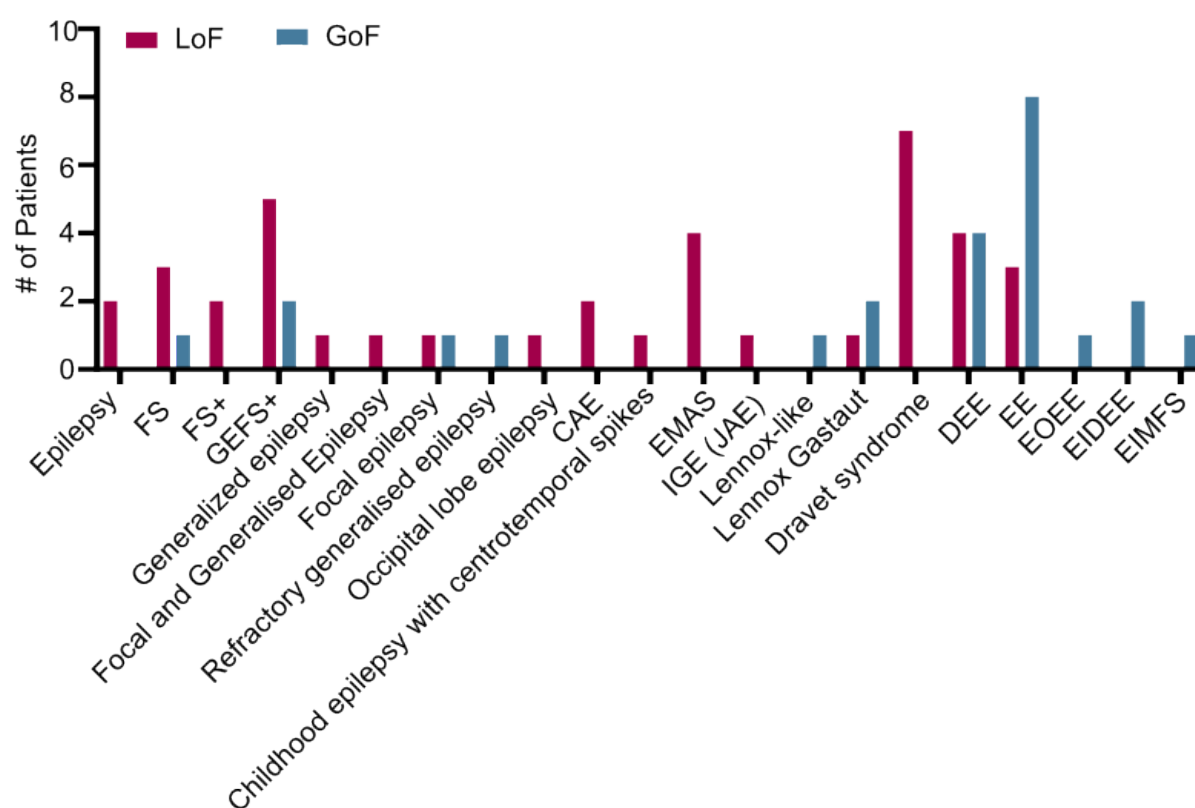


Figure 5.7 Epilepsy types.

Epilepsy types for patients with GoF (blue bars) and LoF (pink bars). Number of patients per seizure type is shown. Abbreviations: FS, Febrile seizure; FS+, febrile seizure plus; GEFS+, genetic epilepsy with febrile seizure plus; CAE, childhood absence seizure; EMAS, epilepsy with myoclonic-atonic seizures, IGE(JAE), idiopathic generalised epilepsy (Juvenile Absence epilepsy); DEE, developmental and epileptic encephalopathy; EE, epileptic encephalopathy; EOOE, early onset epileptic encephalopathy; EIDEE, early infantile developmental and epileptic encephalopathy; EIMFS, epilepsy of infancy with migrating focal seizure

Discussion

GABRG2, gene encoding for the $\gamma 2$ subunit, is commonly associated with a spectrum of epilepsy, ranging from FS to DEE. *GABRG2* has a crucial role in the trafficking and location of the GABA_A receptor. In recent years, there has been a growing number of disease-associated variants identified in *GABRG2*, however, in many, if not all cases it has been concluded that the variants resulted LoF GABA_A receptors, leading to reduced inhibition and therefore, seizures. Here, we found that variants found in the *GABRG2* can result in either a gain or loss of receptor function, with one special case, V98E, where the variant increases receptor sensitivity to GABA, but there's a reduction in the maximal GABA-evoked current amplitude.

Clinical differences between GoF and LoF cohort

Recent literature highlights that both hyperactive or hypoactive GABA_A receptor variant are linked to epilepsy, including variants found in *GABRA1* (78, 94), *GABRA2*(79), *GABRA4* (73, 82), *GABRA5* (79), *GABRB3* (75, 231), *GABRG2* (80, 93, 110, 169, 171, 212, 219), and *GABRD* (177). It has been reported that GoF variants are generally associated with a more severe clinical phenotype (75, 82, 149, 177, 231). The $\gamma 2$ subunit is critically important for translocating GABA_A receptors to the synapse. In the current study, *GABRG2* variants were categorised into GoF and LoF, with 3 distinct differences between the groups 1) the GoF group had a younger median age of seizure onset at 3.75 months (compared to 12 months for LoF), 2) GoF variants are more likely to be *de novo* and 3) individuals with GoF variants often exhibit severe/profound ID post-seizure, associated with comorbidities such as language and visual impairment, and neurological abnormalities with hypotonia being the most common. The LoF

cohort was more frequently associated with neuropsychiatric disorder and/or behavioural features, including autism, and ADHD.

In the current study, DS was observed being exclusively found in the LoF cohort. Dravet syndrome is commonly associated with LoF *SCN1A* variants, where the reduced sodium channel activations at GABAergic interneurons lower GABAergic activity and increase hyperexcitability. In addition, individuals with LoF variants tend to be associated with neuropsychiatric and/or behavioural abnormalities, including autism and ADHD; this is almost non-existent in our GoF cohort and only appeared in one individual (#34) harbouring the GoF variant.

An interesting observation with this study is that we see less GoF variants in the $\gamma 2$ subunit. This is different to epilepsy-associated *GABRB3* variants as Absalom, Liao (75), where an equal distribution of GoF and LoF variants were observed in the cohort. In the *GABRG2*, more LoF variants (41%) were found and this could be due to several reasons: 1) insufficient sample size, 2) functional assay is not sensitive enough 3) given there is only one $\gamma 2$ variant in a GABA_A receptor, this makes it less tolerant to change. However, a more plausible explanation is the difference in the relative importance of different structural motifs in $\beta 3$ compared to $\gamma 2$ subunits (more will be discussed in the Chapter 6).

Distribution of variants

Severity is linked to the location of the variant within the subunit, whilst some regions are hot spots for non-pathogenic variants (40). Of the 71 variants, 26 (~37%) showed no change in receptor function, 29 (~41%) are LoF, 15 (~21%) are GoF, and 1 (~1.4%) increased receptor function but reduced maximum GABA-evoked current (noted as GoF/LoF). We found an enrichment (8/15 GoF variants) of GoF variants in TM1 and TM2, three (A106T, I107T, S139N) in the ECD, one (A334T) in the M2-M3 region, two (S340Y, F343L) in TM3 and one (Y468C) in TM4.

LoF was measured in two different ways: reduction in GABA sensitivity evident by an increase in EC₅₀ and/or reduction in maximum GABA-evoked current. Of the 29 variants identified to reduce overall net inhibition, they were found to be distributed throughout the ECD (L74V,

R82Q, R82W, P83S, P83T, D84V, T90R, W121C, R125C, N167K, M169T, R177G, S210R), the TM1/2 (Y274C, T310I, T317I, A322D, A322T, R323Q, R323W), TM4 (R454W, F457S, N465S), M1-M2 loop (P302L, A303T) and M2-M3 loop (S325L, L326H, P327L, K328M). Interestingly, variants that alter GABA sensitivity tend to cluster in the TM1/2 regions and the M2-M3 loop, whereas variants that alter maximum current tend to be scattered throughout the ECD (R82Q, R82W, P83S, R177G, S210R). Finally, variants that did not alter the receptor function in our assay tend to be clustered in the ECD, the large disordered M3-M4 loop and TM4, with two (V329F, Y331C) in the M2-M3 loop, one (I344L) in the M3 and one variant located in the TM4 (F464S).

Though it may be attractive to correlate the structural location to the variant pathogenicity, this is a difficult task for variants found in *GABRG2*. Based on gnomAD variants and our data, there does appear to be a correlation between structural location and variant pathogenicity; for example, the large disordered M3-M4 loop is clearly not associated with the pathogenic variant found in this study. Nevertheless, correlation is complicated by benign variants being present in functionally important regions, such as the coupling loop or pathogenic variants occurring in non-conserved regions, like A106T, which adds to the difficulty of drawing definitive conclusions. Another example is the M2-M3 loop which is crucial in linking the binding-coupling pathways to the channel gate and receptor assembly (232, 233). Interestingly, despite V329F and Y331C being within M2-M3 loop and four amino acids apart, only Y331C resulted in increased GABA sensitivity, and neither variant affected the maximum evoked current. Both positions are rather conserved; Y331 is conserved amongst 16 subunits (α 1-6 and β 1-3, γ 1-3, ρ 1-3 and ϵ), whilst V329 is common to α 1-6, γ 1-3, ρ 1-3 and ϵ . We speculate that the reason Y331C changed the receptor function but not V329F is because a change from an amino acid with a hydrophobic side chain with a bulky aromatic ring, to a cysteine (smaller amino acid) could result in a reduction of interaction with neighbouring amino acid, resulting in the M2-M3 loop being more flexible and thus altering the accessibility to and threshold for gating. Given that GABA_A receptor is an allosteric ion channel, it is possible that variants in sites distant from the ligand-binding site and the pore can have a downstream effect on the function of the receptor.

When examining GABA sensitivity and maximum current with a high threshold of significance ($p < 0.0001$), some variants appeared “neutral” meaning no deviation from the WT in terms of GABA sensitivity, in the assay, despite being linked to patients. This could suggest our assay may not be sensitive enough to detect subtle functional differences. Therefore, other kinetic

parameters, such as receptor desensitisation, could be utilised for a more comprehensive analysis to determine whether those properties are perturbed by the variant. Alternatively, an in-depth analysis of family history of these patients may reveal high polygenic risk scores and the GABA_A receptor is, at most, an additional risk factor.

Conclusion

This study showed a clear distinction in phenotype and comorbidities between the GoF and LoF variants in our *GABRG2* cohorts. GoF variants are associated with a younger age of seizure onset, severe/profound ID post-seizure, language impairment, and neurological abnormalities, with hypotonia being the most common. Whilst LoF group have neuropsychiatric disorder. These findings align with observations in other subunits of the GABA_A receptors. Since the $\beta 3$ and $\gamma 2$ subunits are integral components of the same receptors, it is not surprising that similar patterns were observed in this study.

Chapter 6 The effects of receptor desensitisation on GoF *GABRB3* variants

Summary

GABRB3 is the gene that encodes for the $\beta 3$ subunit of the GABA_A receptors. Variants found in the *GABRB3* gene are associated with a broad phenotypic spectrum of epilepsy. It is known that variants in the subunits that make up the GABA_A receptor are associated with epilepsy, and this may be due to the variant's effect on receptor sensitivity, channel gating efficiency, and receptor trafficking. A recent genotype/phenotype correlation study of DEE-causing *GABRB3* variants can result in either an increased (hyperactive) or decreased (hypoactive) sensitivity to GABA (75). Patients with a GoF (increased sensitivity to GABA) variant have a distinct and more severe clinical phenotype than those with LoF (reduced sensitivity to GABA) variants (75, 100). A notable feature of variants found in this gene and others is the association between the structural location of the variant and the severity of the phenotype, with variants located in the TM1 and TM2 domain being associated with the more severe clinical phenotypes (40, 71, 100, 135, 234). This was the first genotype-phenotype study spearheading the discovery of GoF variants in *GABRB3* in patients with DEE, challenging the previous paradigm that epilepsy is solely due to insufficient inhibition.

Despite the distinct differences in clinical phenotypes for patients harbouring LoF and GoF variants, correlations of the magnitude of change in LogEC₅₀ of GoF *GABRB3* variants to the age of seizure onset (a proxy for severity) are relatively weak (**Figure 6.1**). However, irrespective of the change in LogEC₅₀, patients with variants in TM1/2 tend to have a younger age of onset than those in the ECD and coupling loop (**Figure 6.1**). The LogEC₅₀ change is an empirical measure of receptor dysfunction, and as such each variant makes changes to other biophysical properties of the receptor. One biophysical property that has previously been associated with pathogenicity is receptor desensitisation. The structural motifs that harbour these variants also influence receptor desensitisation.

Previous studies investigating the effects of GABA_A receptor variants on desensitisation processes identified that pathogenic variants caused increased receptor desensitisation. This increased desensitisation has been interpreted as a loss of receptor function, thereby contributing to the pathophysiology of epilepsy (79, 84, 235, 236). However, mutational studies

with GABA_A receptor show that desensitisation can be increased or decreased by variants in different structural motifs, with substantial decreases for variants in the TM2 domain at the narrowest region of the channel (92, 237-241). Therefore, there is no a priori reason to suspect that GoF variants only affects desensitisation in one way.

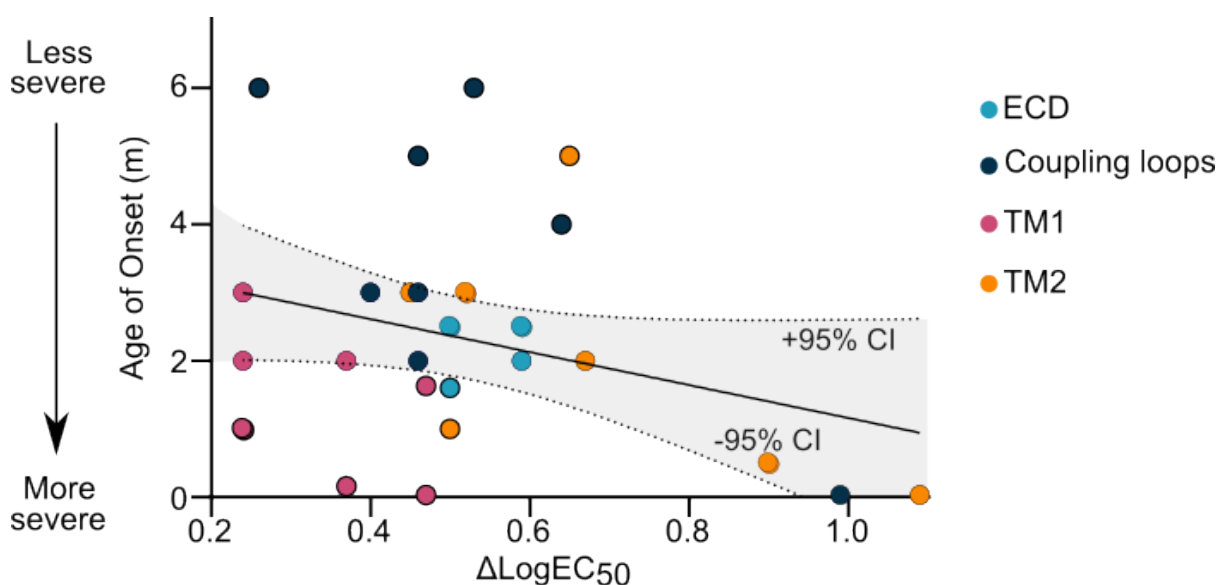


Figure 6.1 Linear regression analysis on seizure onset age versus ΔLogEC_{50} for GoF GABRB3 variants.

Filled circles represent variants at residues in the ECD (light blue), coupling loops (dark blue), TM1 (pink) and TM2 (orange). Figure was taken from Lin et al. (2024) (231).

Aim: To unravel the association between receptor desensitisation and clinical severity.

Hypothesis: Changes in macroscopic receptor desensitisation properties of GABRB3 GoF variants contribute to more severe clinical outcomes at TMD variants.

Methods: 20 GoF and 4 LoF variants were expressed by *Xenopus* oocytes as singly mutated concatenated receptors. Current decay rates, maximum open probability (Est. Pomax) and steady-state currents (Est. Po(ss, max)) were measured using TEVC. Variants were also expressed in HEK293 cells to ensure our findings were consistent in another expression system. Data were compared with non-parametric ANOVA and Dunn's post-hoc test. Comparisons were restricted to data from variants measured on a single day.

Results: All 4 LoF variants had a significantly lower Est. Pomax ($p < 0.05$) compared to WT, whereas GoF variants did not alter the Est. Pomax. When using the age of onset (months) as a proxy for disease severity, we found that the most severely affected patients (aged < 2 months) had, in general, a significant increase in Est. Po(ss, max) compared to WT, meaning receptors were less desensitised. Further inquiry into GoF variants and changes in desensitisation properties found the age of seizure onset for those with exclusively decreased desensitisation at equilibrium was 0.5 months. In addition, GoF variants that showed no changes to desensitisation and decreased desensitisation at equilibrium had a higher mortality rate (2/10 and 3/10, respectively) compared to no mortality in those with accelerated current decay (0/8) and accelerated current decay and decreased desensitisation at equilibrium (0/4). In addition, GoF variants that increased the current decay typically had an older onset (age > 4 months), meaning receptors desensitised more than WT.

This study has highlighted that reduced desensitisation and increased GABA sensitivity are likely to exacerbate the severity of patient phenotype.

This chapter has been accepted for publication in Brain on 30 August 2023. Doi: <https://doi.org/10.1093/brain/awad285>



Correlations of receptor desensitization of gain-of-function GABRB3 variants with clinical severity

Susan X. N. Lin,¹  Philip K. Ahring,¹  Angelo Keramidas,² Vivian W. Y. Liao,¹ Rikke S. Møller,^{3,4} Mary Chebib¹ and Nathan L. Absalom^{1,5}

Genetic variants associated with developmental and epileptic encephalopathies have been identified in the GABRB3 gene that encodes the $\beta 3$ subunit of GABA_A receptors. Typically, variants alter receptor sensitivity to GABA resulting in either gain- or loss-of-function, which correlates with patient phenotypes. However, it is unclear how another important receptor property, desensitization, contributes to the greater clinical severity of gain-of-function variants. Desensitization properties of 20 gain-of-function GABRB3 variant receptors were evaluated using two-electrode voltage-clamp electrophysiology. The parameters measured included current decay rates and steady-state currents. Selected variants with increased or reduced desensitization were also evaluated using whole-cell electrophysiology in transfected mammalian cell lines.

Of the 20 gain-of-function variants assessed, 13 were found to alter receptor desensitization properties. Seven variants reduced desensitization at equilibrium, which acts to worsen gain-of-function traits. Six variants accelerated current decay kinetics, which limits gain-of-function traits. All affected patients displayed severe clinical phenotypes with intellectual disability and difficult-to-treat epilepsy. Nevertheless, variants that reduced desensitization at equilibrium were associated with more severe clinical outcomes. This included younger age of first seizure onset (median 0.5 months), movement disorders (dystonia and dyskinesia), epilepsy of infancy with migrating focal seizures (EIMFS) and risk of early mortality. Variants that accelerated current decay kinetics were associated with slightly milder phenotypes with later seizure onset (median 4 months), unclassifiable developmental and epileptic encephalopathies or Lennox–Gastaut syndrome and no movement disorders.

Our study reveals that gain-of-function GABRB3 variants can increase or decrease receptor desensitization properties and that there is a correlation with the degree of disease severity. Variants that reduced the desensitization at equilibrium were clustered in the transmembrane regions that constitute the channel pore and correlated with greater disease severity, while variants that accelerated current decay were clustered in the coupling loops responsible for receptor activation and correlated with lesser severity.

- 1 Brain and Mind Centre, School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, Sydney, New South Wales 2006, Australia
- 2 Institute for Molecular Bioscience, The University of Queensland, Saint Lucia, QLD 4072, Australia
- 3 Department of Epilepsy Genetics and Personalized Medicine, Member of ERN, EpiCare, Danish Epilepsy Centre, Dianalund DK-4293, Denmark
- 4 Department of Regional Health Research, Faculty of Health Sciences, University of Southern Denmark, Odense DK-5230, Denmark
- 5 School of Science, University of Western Sydney, Sydney, New South Wales, Australia

Correspondence to: Nathan L. Absalom
School of Science, Western Sydney University
Locked Bag 1797, Penrith NSW 2751, Australia
E-mail: n.absalom@westernsydney.edu.au

Keywords: GABA_A receptor; developmental and epileptic encephalopathy; electrophysiology; EIMFS; movement disorder; SLC12A5

Introduction

Developmental and epileptic encephalopathy (DEE) consists of a heterogeneous group of genetic epileptic disorders that begin early in childhood and are associated with neurological impairment, with many patients refractive to treatment.¹ Missense *de novo* variants in γ -aminobutyric acid type A (GABA_A) receptor subunit-encoding genes have recently been associated with DEE.²

GABA_A receptors are key mediators of neuronal inhibition, opening an intrinsic chloride channel in response to GABA release at inhibitory synapses to hyperpolarize the cell. Consistent with this role in neuronal inhibition, DEE-associated variants were reported to reduce chloride channel activity, with greater losses in receptor function proposed to increase clinical phenotype severity.^{3–8} However, we and others, have recently shown that GABA_A receptor variants not only cause loss-of-function, but also gain-of-function receptors.^{9–12} Importantly, gain- and loss-of-function GABRB3 variants are associated with markedly different clinical outcomes, whereby patients with gain-of-function variants typically have more severe phenotypes.

The GABRB3 gene codes for the β 3 subunit of GABA_A receptors, and a striking feature of variants in this and other GABA_A genes is the association between variant structural location and severity of the clinical phenotype, with transmembrane M1 and M2 helix variants most severe.^{3,4,8,13,14} Intuitively, clinical severity would be expected to be linked to the magnitude of observed functional change. However, a correlation plot of age of onset for first seizure event and receptor functional change for gain-of-function GABRB3 variants does not reveal a good correlation (Fig. 1A). Seemingly irrespective of functional consequence, patients harbouring variants in the transmembrane M1 and M2 helices typically present with a very young age of onset (<3 months), and conversely those harbouring variants in the coupling regions and extracellular domain present with a later age of onset (>3 months) (Fig. 1A and B). It is unclear why the regional location of the variant holds such importance for clinical severity.

Structural motifs that harbour variants also influence receptor desensitization, whereby receptors enter a long-lived agonist bound closed state resistant to GABA activation. To date, only variants that increase desensitization by accelerating current decay have been reported.^{16–19} In some cases (e.g. γ 2^{R323Q}), desensitization exacerbated loss-of-function of receptors with impaired GABA sensitivity.¹⁶ In others (e.g. α 5^{V294L} and α 1^{A332V}), accompanying increased GABA sensitivity resulted in mixed loss- and gain-of-function characteristics.^{17,19} Increased desensitization was proposed to remove activatable receptors from the available pool and effectively lower inhibitory tone, leading to *de facto* loss-of-function, but no empirical data measuring neuronal activity has shown that variants with increased desensitization lower the inhibitory tone.¹⁷ However, mutational studies have shown that single amino acid transmembrane domain mutations can either increase or decrease receptor desensitization.^{20–25} Hence, there is no *a priori* reason that DEE-associated variants should only affect desensitization in one way.

In this study, we hypothesized that changes in desensitization characteristics are one component that is associated with more severe clinical outcomes at transmembrane domain variants. To investigate this, we analysed 20 gain- and 4 loss-of-function GABRB3 variants encompassing key structural motifs. We show that gain-of-function variants can have either increased or decreased receptor desensitization properties which correlates with the variant location and provide evidence suggesting that this property modulates clinical outcomes.

Materials and methods

Molecular biology

Concatenated receptors were created as previously described.^{26,27} Briefly, the γ 2- β 3- α 1- β 3- α 1 concatenated construct was created with six unique restriction enzyme sites to enable efficient removal of wild-type (WT) subunits and insertion of variants. Variant subunits were purchased from Genscript and subcloned into the concatenated receptor. The resulting construct contained the subunits with linker sequences in the order of γ 2-(AGS)₅- β 3-(AGS)₅LGS (AGS)₃- α 1-AGT(AGS)₅- β 3-(AGS)₄ATG(AGS)₄- α 1. The γ 2 variant was subcloned into the first subunit, and β 3 variants into the second. DNA gel electrophoresis was performed to ensure the incorporation of the five subunits. cRNA was produced from linearized cDNA using the mMessage mMachineTM T7 Transcription kit (Thermo Fisher) according to the manufacturer's description and stored at –20°C until use. WT and variant constructs containing the α 1, β 3 and γ 2 subunits as previously described²⁷ were purchased from Genscript.

Oocyte expression and electrophysiology

Oocytes were obtained from an ovary segment of *Xenopus laevis* from the University of Wollongong under animal ethics protocol AE2003. Single oocytes were microinjected with ~25 ng of either concatenated GABA_A receptor cRNA, α 1 and β 3 cRNA in a 1:5 ratio or α 1, β 3 and γ 2 cRNA in a 5:1:5 or 10:1:10 ratio and were then incubated for 1–3 days as previously described.^{26,27} Oocytes were impaled with 3 M KCl filled borosilicate glass microelectrodes, with a resistance of 0.2–1.6 M Ω , then voltage clamped at –60 mV. Oocytes were continuously perfused with ND96 buffer (in mM, 96 NaCl, 2 KCl, 1 MgCl₂·6H₂O, 5 HEPES hemisodium, 1.8 CaCl₂) through a semi-automatic gravity-driven perfusion system at 1 ml/min. Solutions were applied via two separate tubes to remove any dead volume in the application of the test tube. Solutions were switched between bath and test solutions with pre-programmed electronic valves and the time between opening the valve and the peak current was in the range of 1–2 s. Currents were recorded using a Warner amplifier OC-725C and sampled at a frequency of 1 kHz and filtered at 10 Hz, then digitalized with LabChart reader version 8.1 (AD Instruments). All experiments were conducted at room temperature.

The recording protocol began with a 2-min wash with ND96 buffer, followed by an application of 3 mM GABA for 150 s, then a

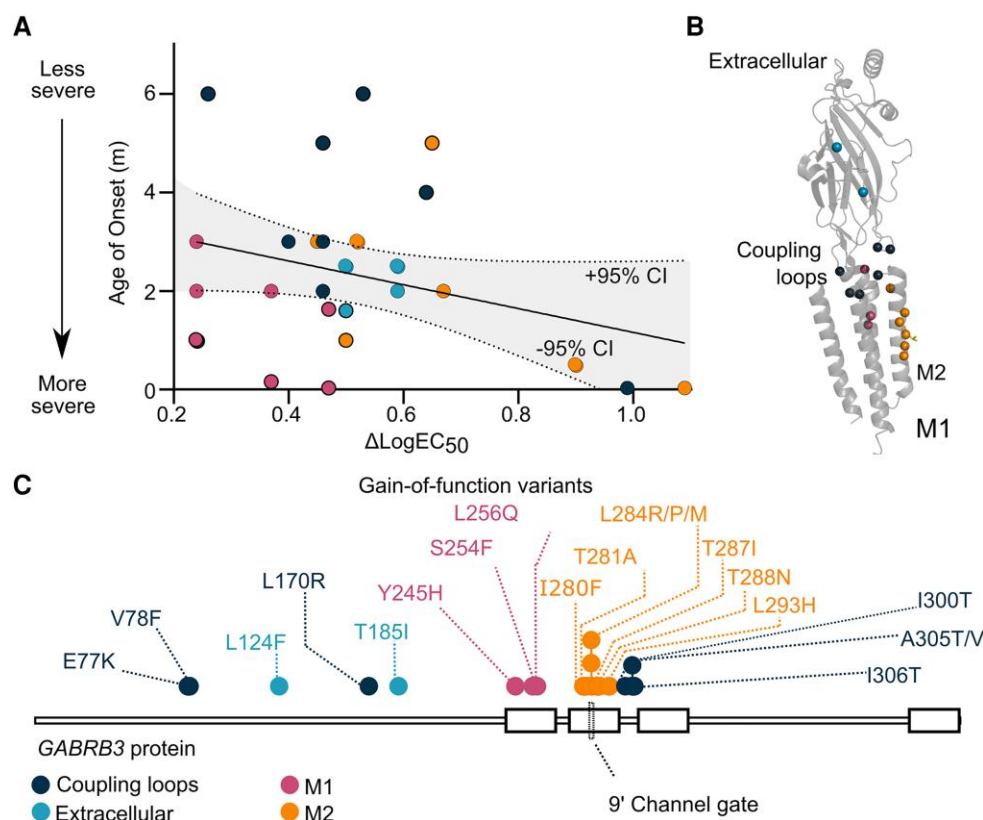


Figure 1 Clinical and functional effects of gain-of-function variants located in distinct structural motifs. (A) Linear regression of the age of seizure onset against the $\Delta\log EC_{50}$ at gain-of-function GABRB3 variants. Values for 31 patients harbouring 20 variants were taken from Absalom et al.⁹ and Yang et al.¹⁵ The line of best fit and area within 95% confidence intervals are shown. Filled circles represent variants at residues in the coupling region (dark blue), extracellular region (light blue), M1 (magenta) and M2 (orange). Black outline denotes variants that are outliers in the linear regression of age of onset and $\Delta\log EC_{50}$. (B) Location of gain-of-function variants (spheres) within the cryogenic electron microscopy structure of $\beta 3$ subunit of the GABA_A receptor (PDB:6HUP). (C) 2D representation of the protein sequence of the $\beta 3$ subunit of the GABA_A receptor. Gain-of-function variants are represented as dots. M1 = M1 transmembrane helix; M2 = M2 transmembrane helix.

5-min wash, 150 s 3 mM GABA application, 1-min wash, 20 s application of 10 mM GABA and 10 μ M etomidate to prime the tubing. An 8-min wash with ND96 buffer ensued for receptors to return from the desensitized state, followed by a 120 s co-application of 10 mM GABA and 10 μ M etomidate. To determine the maximum receptor open probability [Est $P_{O(max)}$], the peak current elicited by the second 3 mM GABA application was normalized to the peak current elicited by 10 mM GABA and 10 μ M etomidate. Etomidate was used to estimate the maximum open probability as receptors contain two etomidate binding sites that contribute equally and non-cooperatively to modulation.²⁸ In the rare event that a variant affects the binding site, one etomidate site will be unchanged and available for modulation and estimation of maximum open probability. Current decay rate was calculated by analysis of the second 3 mM GABA application. The first 3 mM GABA application was used to compute the maximum open probability and current decay rate of $\beta 3^{I300T}$ variant, where excessive desensitization substantially reduced the peak current at the second GABA application. To ensure a saturating concentration of GABA was applied to loss-of-function variants, an additional experiment was performed where 30 mM instead of 3 mM GABA was applied, and 30 mM GABA and 10 μ M etomidate was co-applied.

HEK293 cell electrophysiology

HEK293AD cells were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum until they reached about 80–90%

confluency. The cells were then dissociated and plated onto 12 mm glass coverslips at a confluency of 50% 1 day prior to transfection. HEK293AD cells were transfected using the calcium phosphate co-precipitation method with separate cDNAs encoding the WT $\alpha 1$, $\beta 3$ and $\gamma 2s$ subunits or the $\alpha 1$, $\beta 3^{T287I}$, $\gamma 2s$ or $\alpha 1$, $\beta 3$ and $\gamma 2s^{R323Q}$ variant containing subunit combinations at a $\alpha:\beta:\gamma$ ratio of 1:1:3. The CD4 surface antigen was also included in the transfection mix and served as a marker for transfected cells. Standard whole-cell or outside-out patch clamp recordings were carried out 36–48 h post-transfection in standard extracellular solution containing (in mM). Unless otherwise indicated the experiments were carried out at a clamped potential of -70 mV. An EPC 10 USB Heka Patch Clamp Amplifier (HEKA, Elektronik). Currents were filtered (-3 dB, 4-pole Bessel) at 5 kHz and sampled at 50 kHz and PatchMaster software were used to record whole-cell and macro-patch currents. Patch electrodes were fabricated from borosilicate glass capillaries (G150F-3; Warner Instruments) and heat-polished to a final resistance of 3–6 M Ω when filled with intracellular solution. The intracellular solution contained (in mM) 145 CsCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES and 5 EGTA, and adjusted to pH 7.4 with CsOH. The extracellular solution was composed of (in mM) 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 10 D-glucose, and adjusted to pH 7.4 with NaOH. Currents were elicited by rapid application of 3 mM GABA via a double-barrelled glass tube that was mounted onto a piezo-electric translator (Siskiyou). This method achieved a solution exchange time of ~ 1 ms over small cells

(15–20 pF) or outside-out membrane patches by lateral movement of the glass tube.^{29,30} Data from small cells and patches were pooled. To determine receptor desensitization and steady-state currents, GABA was applied for several seconds, whereas to determine current activation and deactivation times, GABA was applied for ~1–2 ms.

Data analysis

Linear and non-linear regression was performed with GraphPad Prism version 8.0.1. Current decay rates were digitized using LabChart and analysed with Microsoft Excel and GraphPad Prism. Traces were fitted to a one-phase exponential decay equation:

$$Y = (Y_0 - \text{Plateau}) \times e^{-kX} + \text{Plateau} \quad (1)$$

Where Y and X are the amplitude and time, respectively. The plateau, or the asymptote of the each fitted trace, is the current amplitude at infinite time or steady-state (I_{ss}) and k (s^{-1}) is the rate constant of current decay. To define the maximum steady-state open probability [Est $P_{O(ss,max)}$], the I_{ss} was normalized to the 3 mM GABA current and then transformed to the Est $P_{O(max)}$ determined for each day of recording at a specific construct.

Subsequently, a two-phase exponential decay was also fitted to traces using the equation:

$$Y = (Y_0 - \text{Plateau}) \times \text{Fraction}_{\text{Fast}} \times e^{-X/\tau_{\text{Fast}}} + (Y_0 - \text{Plateau}) \times \text{Fraction}_{\text{Slow}} \times e^{-X/\tau_{\text{Slow}}} + \text{Plateau} \quad (2)$$

The weighted time constant (τ_{weighted}) was determined by the following equation

$$\tau_{\text{weighted}} = \text{Fraction}_{\text{Fast}} \times \tau_{\text{fast}} + \text{Fraction}_{\text{Slow}} \times \tau_{\text{Slow}} \quad (3)$$

Statistical analysis

All analysis was performed using GraphPad Prism version 8. One-way ANOVA (Kruskal–Wallis rank sum test) was performed to make comparisons between groups followed by a Dunn's *post hoc* test. Variants were split into four groups of equal size and comparisons were made between variants and WT receptors that were recorded on the same day. Where the Kruskal–Wallis test failed to show a significant difference between groups ($P > 0.05$), Dunn's *post hoc* test was not applied. Values for the Kruskal–Wallis statistic, degrees of freedom and P -value can be found in the [Supplementary material](#). A Mann–Whitney U -test was performed to compare parameters where only one variant was compared to the WT and is indicated in the tables. A one-way ANOVA with Dunnett's *post hoc* test was performed to compare the $\log EC_{50}$ of concatenated and untethered receptors. Oocytes with a holding potential less than -200 nA were excluded from the study, with the exception of variants known to induce constitutive activity (e.g. $\beta 3^{L284R}$). For each experiment at least two batches of oocytes were used. All data are presented as mean $\pm$ standard deviation (SD).

Results

We previously described clinical outcomes for patients harbouring gain- and loss-of-function GABRB3 variants based on changes in GABA sensitivity at GABA_A receptors.⁹ For this study, we omitted protein truncating variants and supplemented our previous cohort of 27 gain and 37 loss-of-function patients with a recent study that

outlined details of six patients harbouring GABRB3 variants (one loss- and five gain-of-function) with known changes in the GABA sensitivity.^{9,15} Data from additional patients and follow up of previously reported patients reinforced the previous findings of the overall clinical phenotypes. Patients with gain-of-function GABRB3 variants presented with a median age of first seizure onset of 2 months [95% confidence interval (CI) 1.6–3], severe to profound intellectual disability (ID) (23/32 patients), epilepsy syndromes described predominantly as unclassified DEEs (16/32), or epilepsy of infancy with migrating focal seizures (EIMFS) (9/32), microcephaly (13/32) and movement disorders including dystonia and dyskinesia (10/32) ([Table 1](#)). In contrast, patients with loss-of-function variants presented with a median age of first seizure onset of 10 months (95% CI 8–15), fewer cases of severe ID (8/38 patients), a spectrum of different epilepsy syndromes, no movement disorders except for ataxia (5/38), no microcephaly (0/38) and little reported early mortality (1/38).

Correlating patient severity with functional data

The striking difference in age of first seizure onset (0.03–15 months) between patients is particularly important, as it represents an objective numerical parameter relatively straightforward to assess, is comparable across different clinicians, and can be used as a predictor of clinical outcomes of a patient. Typically, the earlier the onset the more severe the clinical outcomes. To investigate whether the severity of the clinical phenotype (age of onset) correlates with the functional consequence of the variant ($\Delta \log EC_{50}$), we performed linear regression for 31 patients (age of seizure onset unknown for one patient) harbouring one of 20 unique gain-of-function GABRB3 variants ([Fig. 1A](#)). Surprisingly, only a weak correlation was shown ($R^2 = 0.10$) with outliers outside the 95% CI range in both directions. By colour coding variants according to their structural location in the subunit protein, a clear pattern emerged ([Fig. 1B and C](#)). Patients with variants that reside in the M1 transmembrane helix (M1) present below the line of best fit (lower age of onset), while patients with variants in the coupling regions present above (older age of onset). To investigate whether this correlates with differences in desensitization profiles, we analysed the desensitization characteristics of all known GABRB3 gain-of-function variants and representative loss-of-function variants.

Measuring desensitization properties at concatenated receptors

Macroscopic desensitization properties of GABA_A receptors can be measured empirically in two main ways. First, the speed at which the current declines when the receptor binding sites are saturated with GABA, or the current decay rate [k (s^{-1})], describes the time-frame from the initial activation phase where maximum current flows through the ion channel until the current has declined to reach an equilibrium between open and desensitized states, a proxy for fast desensitization ([Fig. 2A](#)). Second, the steady-state open probability [$P_{O(ss,max)}$], derived from the residual currents (I_{ss}/I_{peak}) describes the degree of residual current when this equilibrium is reached, a measure of desensitization at equilibrium where an increased $P_{O(ss,max)}$ value corresponds to reduced desensitization at equilibrium ([Fig. 2B](#)).

In a series of recent articles, Gielen and colleagues have argued that two-electrode voltage clamp electrophysiology of concatenated receptors is well-suited to measuring receptor desensitization with substantially reduced variability in the kinetic

Table 1 Clinical parameters of patients with GABRB3 variants

Functional consequence	All LOF	All GOF	GOF			
Desensitization property			No changes to desensitization	Accelerated current decay	Decreased desensitization at equilibrium	Accelerated current decay and decreased desensitization at equilibrium
n	38	32	10	8	10	4
Age at seizure onset, months (median ± CI)	10 (8–15)	2 (1.6–3)	2.5 (1.6–3)	4 (2–6)	0.5 (0.03–3)	1.5 (0.5–3)
Epilepsy syndromes	GEFS+ (4) Focal epilepsy (4) EMAS (1) DEE (7) West syndrome (3) Dravet (5) MAE (3) LGS (4) Unclassified (6)	DEE (16) EIMFS (9) West syndrome (2) LGS (2) Unclassified (3)	DEE (7) EIMFS (3)	DEE (5) LGS (2) Unclassified (1)	DEE (2) EIMFS (4) West syndrome (2) Unclassified (2)	DEE (2) EIMFS (2)
Level of cognition	Normal (1) GDD (6) Mild ID (11) Moderate ID (12) Severe ID (8)	GDD (8) Moderate ID (1) Severe/profound ID (23)	GDD (2) Severe/profound ID (8)	GDD (1) Severe/profound ID (7)	GDD (2) Moderate ID (1) Severe/profound ID (7)	GDD (3) Severe/profound ID (1)
Movement disorders and types	5/38 Ataxia (5)	10/32 Dystonia (5) Dyskinesia (3) Not specified (2)	4/10 Dystonia (2) Dyskinesia (1) Not specified (1)	0/8	5/10 Dystonia (3) Dyskinesia (1) Not specified (1)	1/4 Dyskinesia (1)
Microcephaly	0/38	13/32 (40,6%)	4/10 (40%)	3/8 (37,5%)	4/10 (40%)	2/4 (50%)
Mortality	1/38 (2,6%)	5/32 (15,6%)	2/10 (20%)	0/8	3/10 (30%)	0/4

CI = confidence interval; DEE = developmental and epileptic encephalopathies; EIMFS = epilepsy of infancy with migrating focal seizures; EMAS = epilepsy with myoclonic-atonic seizures; GDD = global developmental delay; GEFS+ = generalized epilepsy with febrile seizures plus; GOF = gain-of-function; ID = intellectual disability; LGS = Lennox-Gastaut syndrome; LOF = loss-of-function; MAE = myoclonic atonic epilepsy.

parameters (reviewed in Gielen and Corringer³¹ and Gielen et al.³²). The expression of concatenated $\alpha 1\beta 3\gamma 2$ receptors ensures a specific receptor population is at the cell surface, while expression of free subunits leads to cell-to-cell variability from mixed populations of $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ receptors at the cell surface, but a potential caveat is that concatenation of the receptor may affect receptor activation or expression.²⁷ To investigate this, we injected oocytes with the WT concatenated $\gamma 2\text{-}\beta 3\text{-}\alpha 1\text{-}\beta 3\text{-}\alpha 1$ alone, $\alpha 1$ and $\beta 3$ subunits in a 5:1 ratio, or $\alpha 1$, $\beta 3$ and $\gamma 2$ subunits in either a 5:1:5 or 10:1:10 ratio and measured the maximum currents, maximal estimated open probability, current decay rates, steady-state currents at equilibrium and GABA sensitivity. Although caution should be taken when using current amplitudes to assess channel surface expression, concatenation did not appear to hinder surface expression, with similar maximum currents between concatenated or free subunits expressed at a 10:1:10 ratio. Binary $\alpha 1\beta 3$ receptors also strongly expressed at similar levels, while maximum currents were significantly increased at receptors injected with a 5:1:5 ratio that likely expresses a mixture of binary and ternary complexes (Supplementary Fig. 1 and Supplementary Tables 1 and 2).

To investigate if binary receptors would introduce variation to desensitization parameters, we measured steady-state currents at oocytes expressing $\alpha 1\beta 3$ receptors alone compared to oocytes injected with RNA encoding concatenated $\alpha 1\beta 3\gamma 2$ receptors, or free $\alpha 1$, $\beta 3$ and $\gamma 2$ subunits. Steady-state currents, expressed either as I_{ss}/I_{peak} or $Est P_{O(ss,max)}$ were significantly larger at oocytes expressing binary $\alpha 1\beta 3$ receptors than $\alpha 1\beta 3\gamma 2$ receptors expressed either by free subunits or concatenated constructs (Supplementary Fig. 1 and Supplementary Tables 1 and 2).

As binary $\alpha 1\beta 3$ receptors are commonly expressed at appreciable levels from either injection into oocytes or transfection into mammalian cells of free α , β and γ subunits,^{27,33} the higher steady-state currents of binary receptors will increase variability whenever free subunits are used. Our results show, that tethered subunits did not significantly affect receptor activation properties or expression levels. Therefore, we used the concatenation expression system in *Xenopus* oocytes where a single variant $\beta 3$ subunit is introduced into the pentameric receptor ensuring both that there is no contamination of the recordings from binary receptors, and that homogenous receptor populations of the most common receptor in patients heterozygous for the GABRB3 variant are expressed. Further detailed evaluation and comparison of the concatenated and free constructs are found in the [Supplementary material](#).

Validating desensitization properties in different expression systems

To validate our experimental design of comparing concatenated receptor variants, we initially compared current decay rates and steady-state open probabilities for two previously described patient variants, $\gamma 2^{R323Q}$ and $\beta 3^{T287I}$, to WT. We then expressed these variants with DNA encoding $\alpha 1$, $\beta 3$ and $\gamma 2$ subunits in HEK293 cells to ensure the results were consistent in both *Xenopus* and mammalian expression systems. A concentration of 3 mM GABA was chosen as this is a reasonable mimic of the estimated peak concentrations of GABA at the synaptic cleft.³⁴ The $\gamma 2^{R323Q}$ variant was previously reported to accelerate current decay rates at high GABA

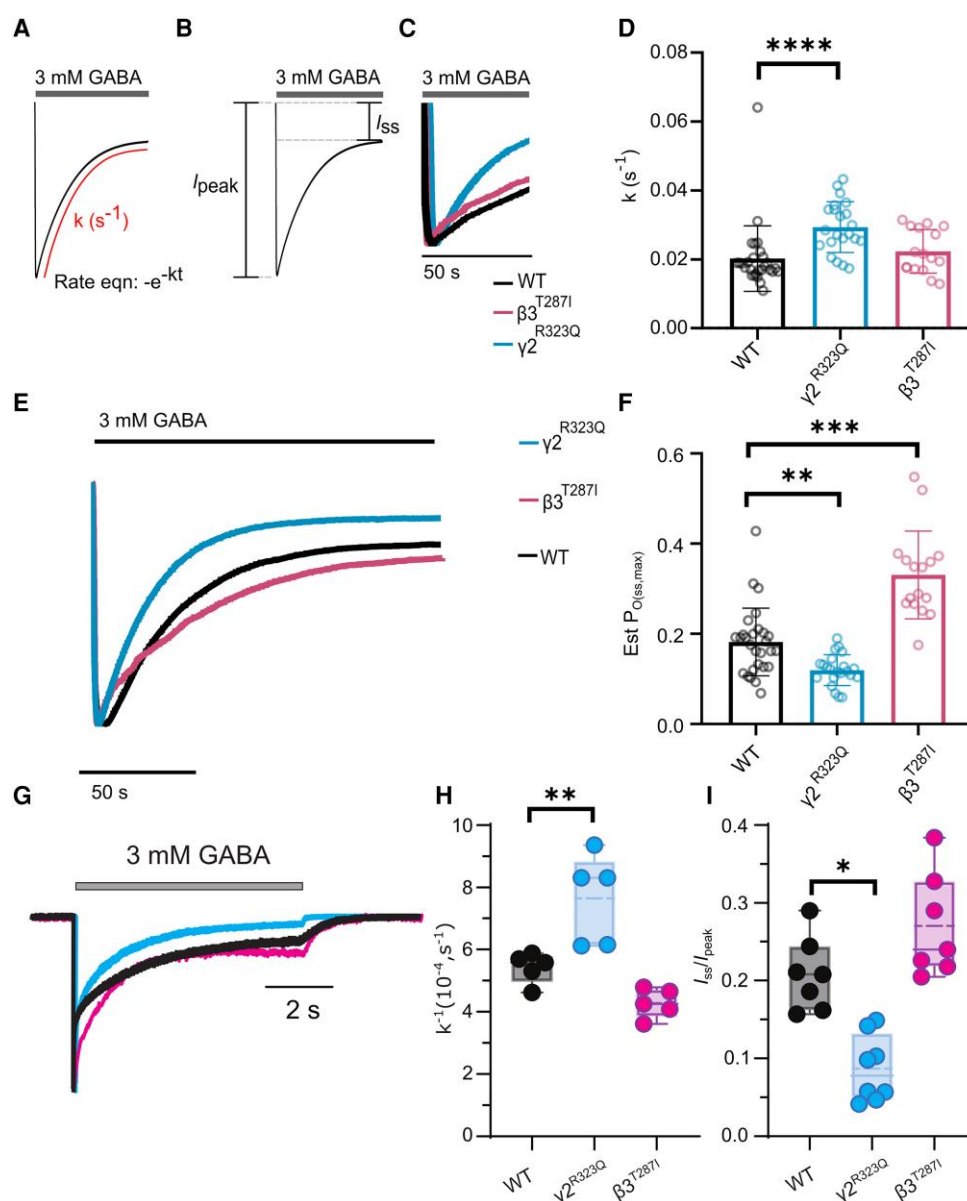


Figure 2 Determination of key empirical parameters affected by desensitization. (A) Current decay rates were determined by applying 3 mM GABA (grey bar) to oocytes expressing variant or wild-type (WT) concatenated receptors. Traces were fitted to an exponential decay (red line), $-e^{-kt}$, to estimate the rate constant, k (s⁻¹). (B) Steady-state currents were determined by subtracting the asymptote of the exponential decay from the holding current (I_{ss}). (C) Representative traces of responses to 50 s application of 3 mM GABA at WT (black), $\gamma 2^{R323Q}$ (aqua) and $\beta 3^{T287I}$ (pink) variant receptors to illustrate current decay rates. (D) Bar graph of current decay rate constants. (E) Representative traces for determination of Est $P_{O(ss,max)}$; 3 mM GABA was applied for 150 s and fitted to an exponential curve. The difference between the asymptote and holding current was transformed to the Est $P_{O(ss,max)}$. (F) Bar graph of the Est $P_{O(ss,max)}$. (G) Representative traces for determination of desensitization time constants and steady-state currents at WT (black), $\gamma 2^{R323Q}$ (aqua) and $\beta 3^{T287I}$ (pink) variant receptors recorded from transfected HEK293 cells. (H) Box and whisker plot of desensitization current decay constants. (I) Box and whisker plot of steady-state currents. For all graphs, bars represent mean $\pm$ SD, circles represent individual experiments; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 compared to WT, non-parametric ANOVA with Dunn's post hoc test. Est $P_{O(max)}$ = maximum receptor open probability; Est $P_{O(ss,max)}$ = maximum steady-state open probability; M1 = M1 transmembrane helix; M2 = M2 transmembrane helix.

concentrations, whereas no effects were noted for the $\beta 3^{T287I}$ variant.^{11,16} To investigate this, an exponential decay function was fitted to 150-s long responses of 3 mM GABA (Fig. 2C, Table 2 and Supplementary Fig. 2). The current decay constant was significantly increased by the $\gamma 2^{R323Q}$ but not the $\beta 3^{T287I}$ variant, indicating faster desensitization rates at the $\gamma 2^{R323Q}$ variant (Fig. 2D and Table 2). Evaluating steady-state currents requires knowledge of the degree by which receptors respond to 3 mM GABA applications [i.e. estimated maximal open probability, Est $P_{O(max)}$]. The Est $P_{O(max)}$ was

reduced for the $\gamma 2^{R323Q}$, but unchanged for the $\beta 3^{T287I}$ variant (Table 2 and Supplementary Tables 3 and 4). To determine the steady-state open probability [Est $P_{O(ss,max)}$], an exponential decay function was fitted to 150-s-long 3 mM GABA responses to determine the steady state currents relative to the peak (I_{ss}/I_{peak}), and the asymptote normalized to the Est $P_{O(max)}$ value (Fig. 2E and Supplementary Tables 3 and 4). Compared to WT, the desensitization at equilibrium was significantly increased at $\gamma 2^{R323Q}$ but decreased for $\beta 3^{T287I}$ (Fig. 2F and Table 2).

Table 2 Desensitization parameters of gain-of-function GABRB3 variants

Variant ^a	ΔLogEC ₅₀ ^b	k	P-value ^c	Est P _{O(ss,max)}	P-value ^c	Est P _{O(max)}	P-value ^c	n ^d
Group 1^e								
Wild-type	–	0.02 ± 0.0095	–	0.18 ± 0.075	–	0.98 ± 0.22	–	28
γ2R323Q	–	0.029 ± 0.0074	<0.0001	0.12 ± 0.034	0.008	0.77 ± 0.2	0.0047	22
T287I	0.52	0.022 ± 0.0063	0.2232	0.33 ± 0.097	0.0004	1.1 ± 0.18	0.0988	16
Group 2^f								
Wild-type	–	0.027 ± 0.009	–	0.11 ± 0.034	–	0.8 ± 0.15	–	32
T281A	0.90	0.038 ± 0.0091	n.d.*	0.18 ± 0.036	0.025	1 ± 0.26	0.1054	12
L284M	0.67	0.033 ± 0.0075	n.d.*	0.1 ± 0.035	>0.9999	0.81 ± 0.16	>0.9999	13
L284P	1.22	0.034 ± 0.021	n.d.*	0.33 ± 0.11	<0.0001	0.89 ± 0.28	0.5601	14
L284R	1.09	0.03 ± 0.011	n.d.*	0.5 ± 0.19	<0.0001	1.2 ± 0.60	0.0159	15
T288N	0.45	0.03 ± 0.0076	n.d.*	0.096 ± 0.032	>0.9999	0.94 ± 0.22	0.2654	13
L293H	0.50	0.033 ± 0.009	n.d.*	0.078 ± 0.023	0.303	0.84 ± 0.3	>0.9999	15
Group 3^g								
Wild-type	–	0.025 ± 0.0061	–	0.10 ± 0.022	–	0.85 ± 0.11	–	19
E77K	0.64	0.049 ± 0.011	<0.0001	0.098 ± 0.036	>0.9999	0.81 ± 0.15	n.d.*	12
V78F	0.40	0.036 ± 0.0065	0.023	0.086 ± 0.071	0.241	0.81 ± 0.19	n.d.*	15
L170R	0.26	0.029 ± 0.0082	>0.9999	0.1 ± 0.034	>0.9999	0.85 ± 0.1	n.d.*	15
A305T	0.46	0.046 ± 0.0088	<0.0001	0.12 ± 0.028	>0.9999	0.94 ± 0.14	n.d.*	11
A305V	0.53	0.044 ± 0.0069	<0.0001	0.13 ± 0.043	0.823	0.88 ± 0.11	n.d.*	11
I306T	0.38	0.034 ± 0.0093	0.067	0.11 ± 0.036	>0.9999	0.79 ± 0.09	n.d.*	14
Group 4^h								
Wild-type	–	0.029 ± 0.0052	–	0.079 ± 0.021	–	0.74 ± 0.11	–	13
L124F	0.50	0.039 ± 0.0078	0.051	0.084 ± 0.049	>0.9999	0.83 ± 0.21	n.d.*	12
T185I	0.59	0.036 ± 0.0046	0.313	0.081 ± 0.043	>0.9999	0.9 ± 0.26	n.d.*	14
Y245H	0.37	0.02 ± 0.0048	0.573	0.15 ± 0.04	0.016	0.79 ± 0.16	n.d.*	12
S254F	0.24	0.05 ± 0.027	0.005	0.19 ± 0.065	0.002	0.87 ± 0.1	n.d.*	11
L256Q	0.47	0.034 ± 0.0062	>0.9999	0.17 ± 0.088	0.014	0.89 ± 0.16	n.d.*	12
I280F	0.65	0.058 ± 0.0012	<0.0001	0.076 ± 0.052	>0.9999	0.86 ± 0.18	n.d.*	17
Group 5ⁱ								
Wild-type	–	0.028 ± 0.0094	–	0.15 ± 0.038	–	0.99 ± 0.26	–	24
I300T	0.99	0.036 ± 0.0096	0.121	0.048 ± 0.061	<0.0001	0.92 ± 0.32	>0.9999	20

Errors are given as standard deviations. Est P_{O(max)} = maximum receptor open probability; Est P_{O(ss,max)} = maximum steady-state open probability; k = rate constant of current decay.

^aUnless otherwise specified, variants are in the β3 subunit.

^bValues taken from Absalom et al.⁹

^cOne-way Kruskal–Wallis ANOVA followed by a Dunn’s post hoc test, experimental groups compared separately.

^dNumbers of individual oocytes to determine k, Est P_{O(max)} and Est P_{O(ss,max)}.

^{e–i}Experimental groups where variants and wild-type performed on same day.

*n.d. = not determined, as P > 0.05 for initial ANOVA.

These data show that the γ2^{R323Q} variant increases desensitization both through accelerated current decay rates and increased desensitization at equilibrium, both phenomena that reduce total charge transfer upon receptor activation (decreased area under curve). In contrast, the β3^{T287I} variant reduced desensitization at equilibrium with a greater percentage of receptors remaining open during a prolonged application of GABA, allowing net greater flow of chloride through the β3^{T287I} receptors compared to WT (increased area under curve). These results demonstrate that desensitization properties can be significantly altered in either direction by pathogenic variants.

Recent kinetic modelling has proposed that variants in the β2 subunit of α1β2γ2 receptors modulate the slow component of GABA_A receptor desensitization.³¹ Therefore, we fitted the decay currents to a two-phase exponential function for the WT and β3^{T287I} receptors. Although there was a significant increase in the fraction of the fast component at β3^{T287I} receptors, we did not identify any changes in the weighted time constant (τ_{weighted}) or slow time component (τ_{slow}) (Supplementary Tables 5 and 6). Decay currents at γ2^{R323Q} receptors did not fit to two-phase exponential functions and were not analysed by this method.

To confirm that the results we measure in a concatenated receptor construct expressed in a *Xenopus* oocyte are equivalent to

receptors expressed in a mammalian system, HEK293 AD cell expression was used to investigate the kinetic properties of WT α1β3γ2 and receptors containing γ2^{R323Q} and β3^{T287I} variants. Relatively long applications of 3 mM GABA to small cells or excised membrane patches produced desensitizing currents that were adequately fit to two exponential equations for all three receptor types (Fig. 2G and Supplementary Tables 7 and 8). Plotting the weighted desensitization rate constant (k^{–1}) demonstrated that γ2^{R323Q}-containing receptors decayed more rapidly than WT, whereas the decay rate for the β3^{T287I}-containing receptors was similar to that of WT (Fig. 2H). The remaining steady-state current (I_{ss}) at the end of the GABA application was also measured and normalized to the peak current (I_{ss}/I_{peak}). The plots of this data show that the γ2^{R323Q} variant results in a reduced steady-state current, whereas the β3^{T287I} variant steady-state current was not significantly increased compared to WT receptors (Fig. 2I). Detailed measurements of simulated synaptic currents are found in the Supplementary material.

Overall, the kinetic parameters of the two variant receptors are consistent with a ‘loss-of-function’ for α1β3γ2^{R323Q} receptors and a ‘gain-of-function’ for the α1β3^{T287I}γ2 receptors, and thus broadly similar conclusions can be drawn for variants expressed in

concatenated constructs in *Xenopus* oocytes or as free subunits in mammalian cells.

GABRB3 M2 and M1 variants

In GABA_A receptors, the M2 helix lines the channel pore and variants in this region are associated with greater clinical severity (Fig. 3A).^{3,4,8,13,14} In our cohort, there is a cluster of eight gain-of-function epilepsy-associated variants in the M2 region, including three separate variants at the central leucine residue, commonly referred to as the central 9'-leucine residue, that forms the hydrophobic channel gate.³⁵ No significant difference in current decay rates was observed for seven M2 helix variants, including the $\beta 3^{T287I}$ (Fig. 3B), and only the $\beta 3^{I280F}$ variant significantly accelerated the current decay rate compared to WT (Fig. 3C and Table 2). There was no difference in Est $P_{O(max)}$ between variants (Table 2 and Fig. 3D). Desensitization at equilibrium were significantly decreased for four variants, two located at the central leucine residue compared to the WT ($\beta 3^{L284P}$ and $\beta 3^{L284R}$) as well as $\beta 3^{T281A}$ and $\beta 3^{T287I}$ (Fig. 3E and F). The remaining variants did not significantly change the Est $P_{O(ss,max)}$ (Fig. 3G and Table 2), and only the $\beta 3^{L284P}$ had reduced maximum current amplitudes (Supplementary Fig. 3 and Supplementary Tables 3 and 4).

The second structural region associated with more severe variants is the M1 region (Fig. 3A).²⁰ Our analysis of M1 variants revealed that the $\beta 3^{S254F}$ variant significantly accelerated current decay rates, whereas no changes were observed for $\beta 3^{Y245H}$ and $\beta 3^{L256Q}$ (Fig. 3B and C and Table 2). There was also no difference in Est $P_{O(max)}$ (Fig. 3D and Table 2). However, the desensitization at equilibrium was reduced at all three M1 variants (Fig. 3G and H and Table 2).

We then fitted the decay currents to a two-phase exponential decay current. The weighted time constant was significantly accelerated at the $\beta 3^{I280F}$ and decelerated at the $\beta 3^{Y245H}$ receptors. The slow time component was also accelerated at the $\beta 3^{I280F}$ and decelerated at the $\beta 3^{Y245H}$ and $\beta 3^{L284P}$ receptors, while the fraction of the fast component was increased at the $\beta 3^{L256Q}$, $\beta 3^{L284R}$ and $\beta 3^{L284P}$ receptors (Supplementary Tables 5 and 6). This is consistent with previous reports of the importance of the $\beta 3$ subunit in the slow time component of current decay.³¹

In summary, the current decay rate of the receptor is appreciably accelerated by one variant in the M2 region ($\beta 3^{I280F}$) and one in the M1 region ($\beta 3^{S254F}$). During synaptic transmission, this increase in current decay may reduce the current passing across the cell membrane. Nonetheless, all variants in the M1 region and four out of eight variants in the M2 region ($\beta 3^{T281A}$, $\beta 3^{L284P}$, $\beta 3^{L284R}$ and $\beta 3^{T287I}$) significantly reduced desensitization at equilibrium. In cases of prolonged periods of activation, this would be expected to increase the pool of receptors available for reactivation and thus increase the inhibitory currents flowing across the cell membrane. This indicates that these variants have enhanced gain-of-function compared to those that have no change in current decay rates.

GABRB3 coupling region and extracellular variants

The coupling region linking the extracellular and transmembrane regions is enriched with pathogenic variants.⁹ Seven gain-of-function variants have been identified in the coupling region comprising of the extracellular $\beta 1$ –2, $\beta 6$ –7 and the transmembrane M2–M3 loops that alter conformation in the transitions between closed, intermediate and open states (Fig. 4A). Current decay rates

were significantly accelerated by the $\beta 3^{E77K}$ and $\beta 3^{V78F}$ variants located in the $\beta 1$ –2 loop and the $\beta 3^{A305T}$ the $\beta 3^{A305V}$ variants located in the M2–M3 coupling loop, while the rates of the $\beta 3^{L170R}$, $\beta 3^{I300T}$ and $\beta 3^{I306T}$ variants were not significantly altered (Fig. 4B and C and Table 2). There was no difference in Est $P_{O(max)}$ for all variants (Fig. 4D). For six of the seven variants, no changes to the desensitization at equilibrium were observed. However, the desensitization at equilibrium was increased at the $\beta 3^{I300T}$ receptor (Table 2 and Fig. 4E–G). Only the $\beta 3^{V78F}$ and $\beta 3^{I300T}$ had reduced maximum current amplitudes, that may be a result of reduced surface expression or a consequence of decreased activation times (Supplementary Fig. 3 and Supplementary Tables 3 and 4). We then fitted the decay currents to a two-phase exponential decay current. Both the weighted time constant and the slow time component was significantly accelerated at the $\beta 3^{E77K}$, $\beta 3^{I300T}$, $\beta 3^{A305T}$ and $\beta 3^{A305V}$ receptors, in agreement with previous reports that the $\beta 3$ subunit coordinates the slow time component of current decay (Supplementary Tables 5 and 6).³¹

Finally, the extracellular region apart from the coupling region contains two gain-of-function variants, the $\beta 3^{L124F}$ and $\beta 3^{T185I}$. There were no changes seen for the $\beta 3^{L124F}$ and $\beta 3^{T185I}$ variants in current decay rates, Est $P_{O(max)}$ or steady-state activity, and thus did not alter either aspect of desensitization (Fig. 4). No parameters were significantly changed when current decay was fitted to a two-phase exponential function, and neither variant had a change in maximum current amplitudes (Supplementary Fig. 3 and a Supplementary Tables 3 and 4).

Therefore, most gain-of-function variants in the coupling region accelerate the current decay rate of receptors at high concentrations of GABA, but do not affect the steady-state currents. Variants in the extracellular domain outside of the coupling region did not affect either aspect of desensitization.

GABRB3 loss-of-function variants

For comparison, we selected four loss-of-function variants from the same structural regions and evaluated changes in current decay and the desensitization equilibrium. These variants included $\beta 3^{M80K}$ and $\beta 3^{Y302C}$ in the $\beta 1$ –2 and M2–M3 coupling loops respectively, $\beta 3^{Q249K}$ in the M1 and $\beta 3^{T281I}$ in the M2 region (Fig. 5A). After application of 30 mM GABA, the $\beta 3^{M80K}$ had a significant reduction in the maximal current amplitudes, and both the $\beta 3^{M80K}$ and $\beta 3^{Y302C}$ receptors had a significant reduction in $P_{O(max)}$, which may reflect the reduced gating efficiency (Fig. 5B and C and Supplementary Table 9). The $\beta 3^{M80K}$ and $\beta 3^{T281I}$ receptors had significantly accelerated current decay rates and reduced desensitization at equilibrium, while neither the current decay rates or desensitization at equilibrium was significantly changed at the $\beta 3^{Q249K}$ and $\beta 3^{Y302C}$ receptors (Fig. 5D–F and Supplementary Tables 4 and 9). Taken together, loss-of-function variants displayed either unchanged or increased desensitization properties, either by accelerated current decay rates or increasing desensitization at equilibrium, indicating that there's a further reduction in inhibitory activity of the receptor.

Associations of desensitization characteristics with age of seizure onset

Mapping variants to common structural motifs showed consistent patterns in changes to desensitization properties (Fig. 6A and B). Variants in the coupling loops were more likely to display accelerated current decay, whereas the M1 and M2 regions more likely to

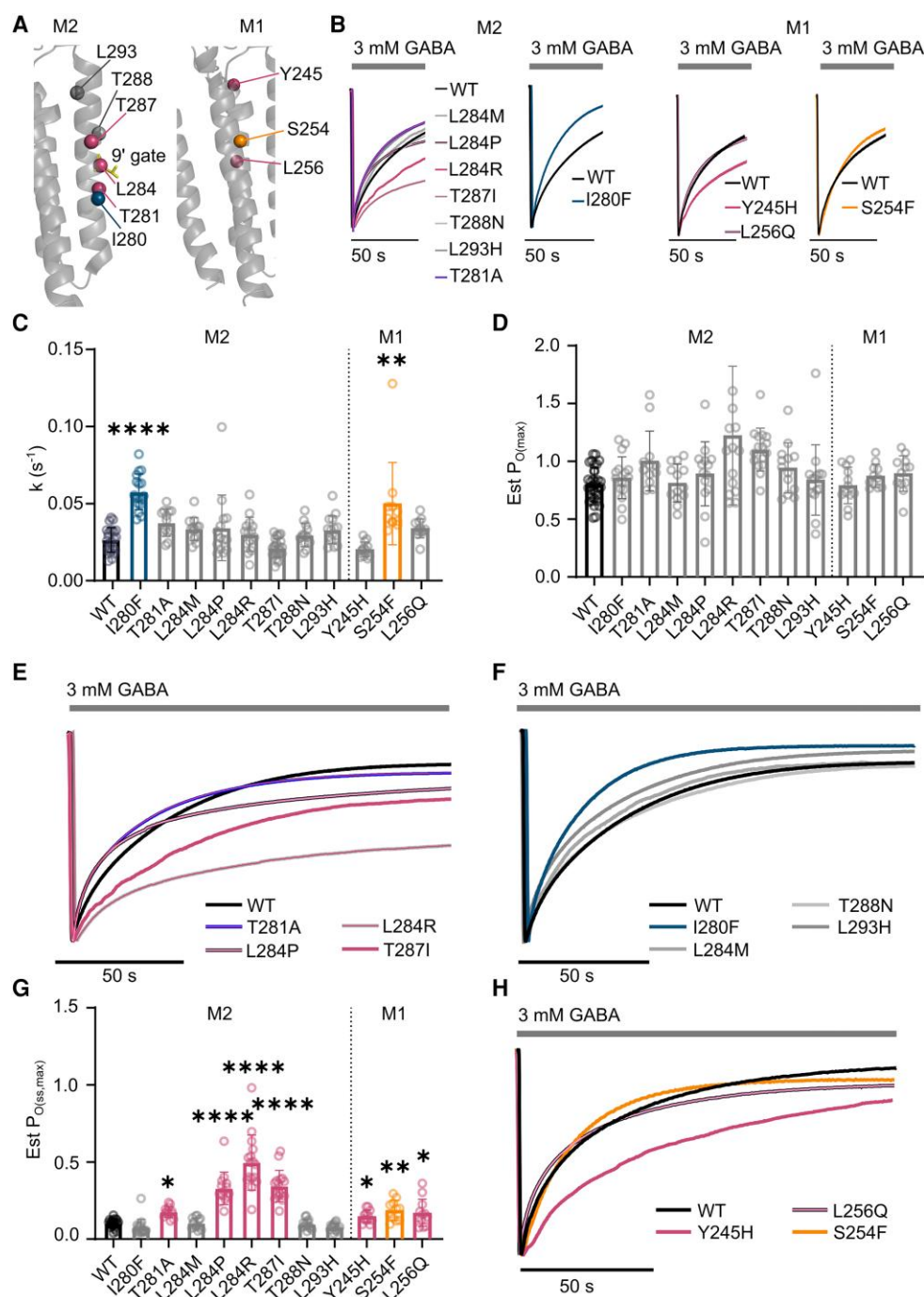


Figure 3 Determination of key empirical parameters related to desensitization of variants at the transmembrane regions. (A) Location of M2 (left) and M1 (right) variants (depicted as spheres) in the cryogenic electron microscopy structure of the GABA_A receptor $\beta 3$ subunit, yellow indicates the 9' gates (PDB:6HUP). Orange denotes significantly faster decay constants and higher Est $P_{O(ss,max)}$, blue denotes only significantly faster decay constants, pink denotes only significantly higher Est $P_{O(ss,max)}$ and grey denotes no change. (B) Representative traces for M2 variants with unchanged current decay rates at (left) wild-type (WT), $\beta 3^{L284M}$, $\beta 3^{L284R}$, $\beta 3^{L284P}$ (black border), $\beta 3^{T287I}$ (grey border), $\beta 3^{T281A}$ (blue border), $\beta 3^{T288N}$ (light grey) and $\beta 3^{L293H}$ (dark grey) receptors, and faster decay rates at (right) WT (black) and $\beta 3^{L280F}$ receptors. Representative traces for M1 variants unchanged current decay rates at (left) WT, $\beta 3^{Y245H}$, and $\beta 3^{L256Q}$ (black border) receptors, and fast current decay rates at WT (black) and $\beta 3^{S254F}$ receptors. (C) Bar graph of current decay rates. (D) Bar graph of Est $P_{O(max)}$. (E) Representative traces of 150 s GABA application at M2 variants with increased Est $P_{O(ss,max)}$ at WT, $\beta 3^{L284P}$, $\beta 3^{L284R}$, $\beta 3^{T281A}$ and $\beta 3^{T287I}$ variants. (F) Representative traces of 150 s GABA application at M1 variants with unchanged Est $P_{O(ss,max)}$ at WT, $\beta 3^{L280F}$, $\beta 3^{L284M}$, $\beta 3^{T288N}$ and $\beta 3^{L293H}$ receptors. (G) Bar graph of Est $P_{O(ss,max)}$. (H) Representative traces of 150 s GABA application at M1 variants with increased Est $P_{O(ss,max)}$. For all bar graphs, bars represent mean $\pm$ SD, circles represent individual experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ compared to WT, non-parametric ANOVA with Dunn's *post hoc* test. Coloured bars and circles represent significant differences compared to WT, grey bars and circles are not significant. Est $P_{O(max)}$ = maximum receptor open probability; Est $P_{O(ss,max)}$ = maximum steady-state open probability; k = rate constant of current decay; M1 = M1 transmembrane helix; M2 = M2 transmembrane helix.

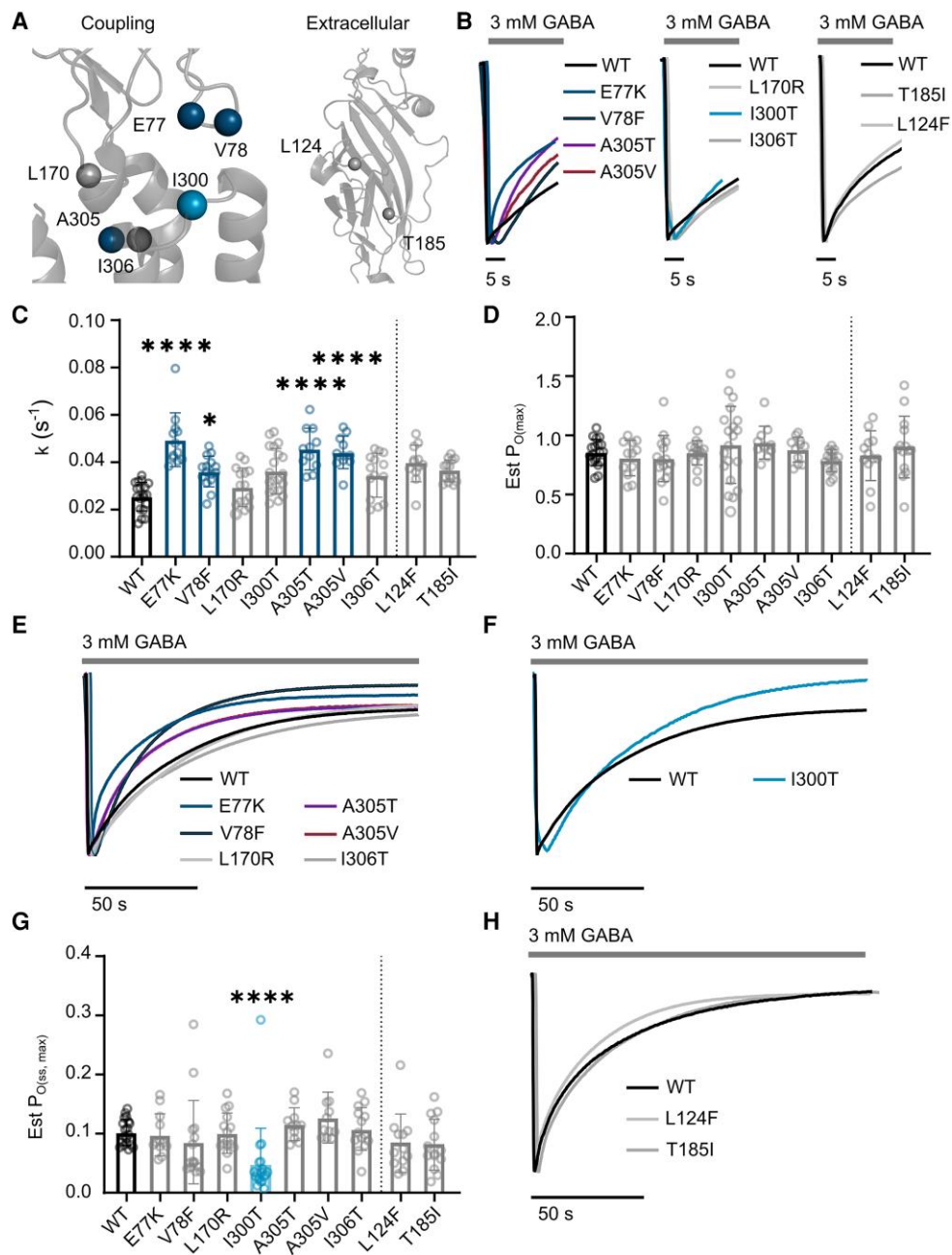


Figure 4 Determination of key empirical parameters related to desensitization of variants at the coupling and extracellular regions. (A) Location of coupling loop (left) and extracellular (right) variants (spheres) within the cryogenic electron microscopy structure of $\beta 3$ subunit of the GABA_A receptor (PDB:6HUP). Blue denotes only significantly faster decay constants, aqua denotes only significantly lower Est $P_{O(ss,max)}$ and grey denotes no change. (B) Representative traces for coupling loop variants with faster current decay rates (left) at wild-type (WT), $\beta 3^{E77K}$, $\beta 3^{V78F}$ (black border), $\beta 3^{A305T}$ (purple border) and $\beta 3^{A305V}$ (red border) receptors and unchanged decay rates (middle) at WT (black), $\beta 3^{L170R}$ (light grey), $\beta 3^{I300T}$ (aqua) and $\beta 3^{I306T}$ (grey) receptors. Representative traces for extracellular variants with no changes in decay rates (right) at WT, $\beta 3^{L124F}$ and $\beta 3^{T185I}$ receptors. (C) Bar graph of current decay rates. (D) Bar graph of Est $P_{O(max)}$. (E) Representative traces of 150 s GABA application for unchanged Est $P_{O(ss,max)}$ at WT and coupling variants. (F) Representative trace of 150 s GABA application for reduced Est $P_{O(ss,max)}$ variant, $\beta 3^{I300T}$. (G) Bar graph of Est $P_{O(ss,max)}$. In all bar graphs, bars represent mean $\pm$ SD, circles represent individual experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ compared to WT, non-parametric ANOVA with Dunn's post hoc test. Coloured bars and circles represent significant differences compared to WT, grey bars and circles are not significant. (H) Representative traces of 150 s GABA application for unchanged Est $P_{O(ss,max)}$ at WT and extracellular region variants. Est $P_{O(max)}$ = maximum receptor open probability; Est $P_{O(ss,max)}$ = maximum steady-state open probability; k = rate constant of current decay.

increase steady-state currents. To determine if these differences in desensitization properties affected the severity of the phenotype, clinical data for the 32 patients (31 with known age of seizure onset) with gain-of-function variants was segregated into four categories: (i) No changes to desensitization; (ii) Accelerated current decay; (iii)

Decreased desensitization at equilibrium; and (iv) Mixed effects of accelerated current decay and decreased desensitization at equilibrium (Table 1).

Patients for which no desensitization changes were observed had an age of seizure onset of 2.5 months (Fig. 6C and Table 1).

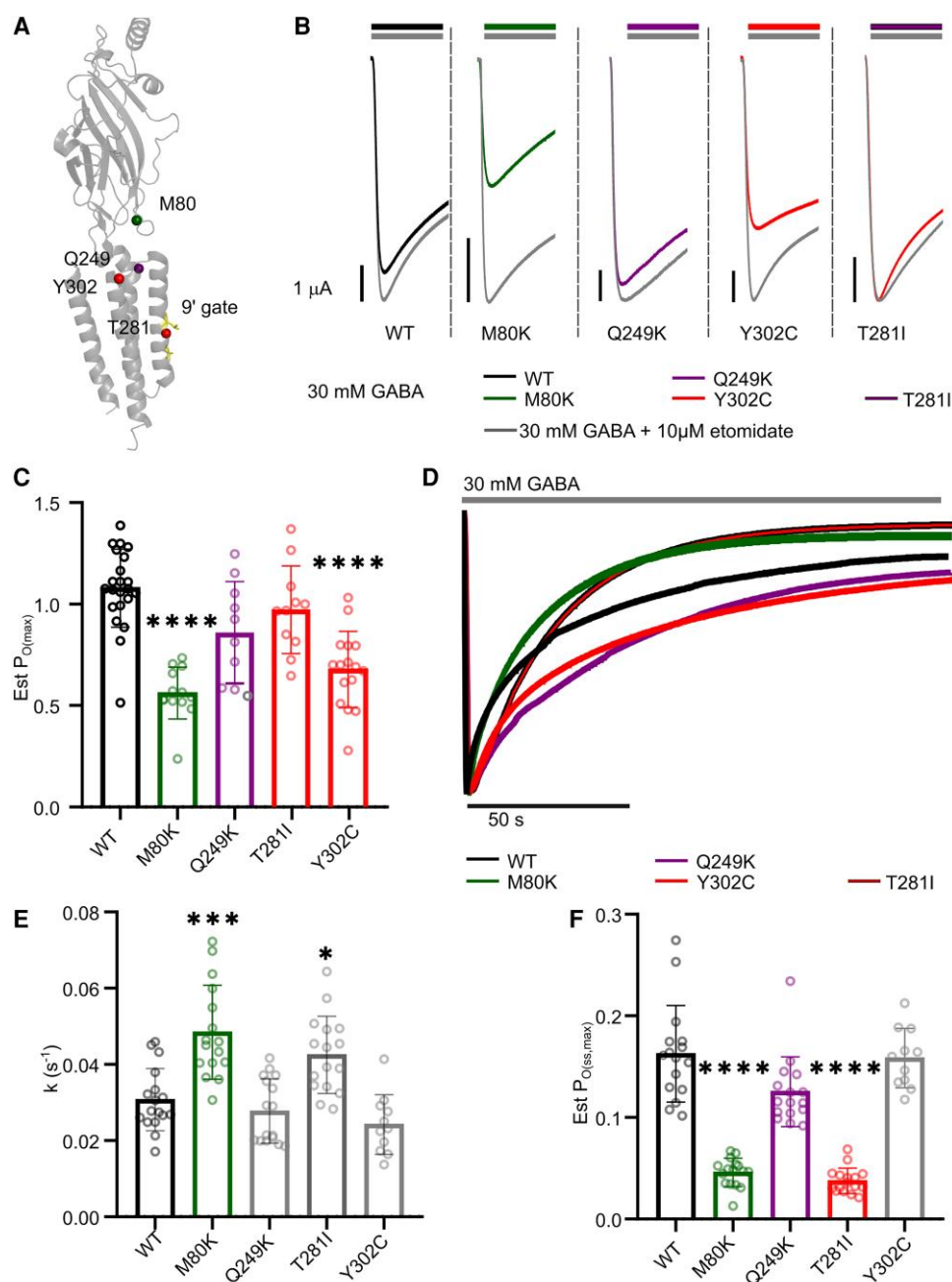


Figure 5 Determination of key empirical parameters affected by desensitization of loss-of-function variants. (A) Location of loss-of-function variants (spheres) within the cryogenic electron microscopy structure of $\beta 3$ subunit of the GABA_A receptor (PDB:6HUP), 9' gate of the M2 transmembrane helix highlighted in yellow. Green denotes significantly faster decay constants, decreased Est $P_{O(max)}$ and Est $P_{O(ss,max)}$, purple denotes significantly decreased Est $P_{O(max)}$ and Est $P_{O(ss,max)}$, red denotes only decreased Est $P_{O(max)}$ and grey denotes no change. (B) Representative trace of currents elicited by 30 mM GABA overlayed with trace elicited by 30 mM GABA + 10 μ M etomidate (grey) for $\beta 3^{M80K}$, $\beta 3^{Q249K}$, $\beta 3^{T281I}$ and $\beta 3^{Y302C}$. (C) Bar graph of Est $P_{O(max)}$. (D) Representative traces of GABA application for current decay and Est $P_{O(ss,max)}$ at wild-type (WT) and loss-of-function variants. (E) Bar graph of current decay rate at 30 mM GABA. (F) Bar graph of Est $P_{O(ss,max)}$ determined with 30 mM GABA. For all graphs, bars represent mean $\pm$ SD, circles represent individual experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ compared to WT, non-parametric ANOVA with Dunn's post hoc test. Coloured bars and circles represent significant differences compared to WT, grey bars and circles are not significant. Est $P_{O(max)}$ = maximum receptor open probability; Est $P_{O(ss,max)}$ = maximum steady-state open probability; k = rate constant of current decay.

Patients with variants that accelerated current decay rates had an older age of seizure onset of 4 months, whereas patients with decreased desensitization at equilibrium had a lower age of seizure onset of 0.5 months. Patients with mixed desensitization properties resembled those with only decreased desensitization at equilibrium with an early onset of 1.5 months.

While these data suggest that accelerated current decay and increased steady-state currents influence the age of seizure onset in opposite directions, this analysis ignores the magnitude of change in GABA sensitivity, where a younger age of onset is generally associated with very large $\Delta \log EC_{50}$ changes. Therefore, desensitization properties were incorporated by

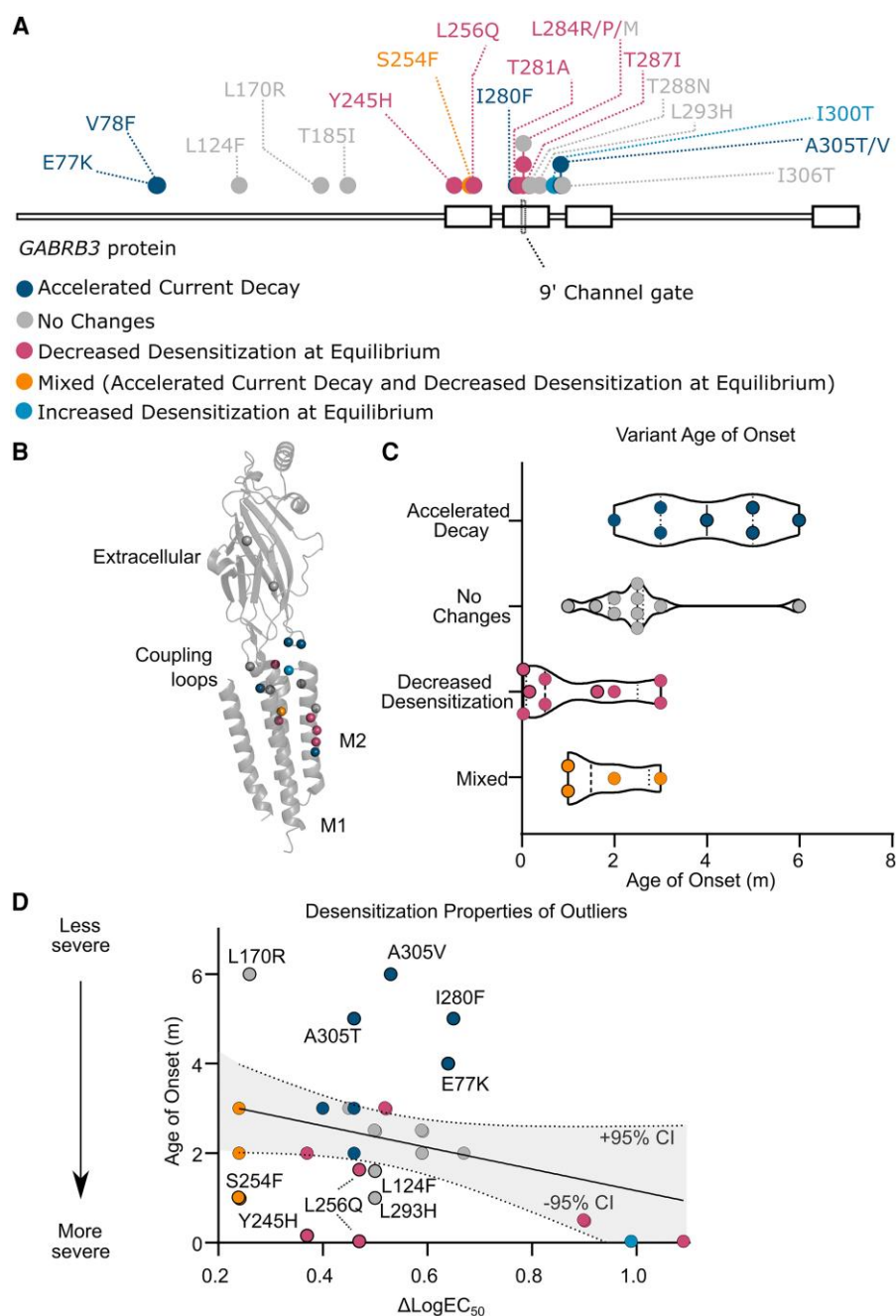


Figure 6 Age of onset is affected by desensitization at gain-of-function variants. (A) 2D representation of the protein sequence of the $\beta 3$ subunit of the GABA_A receptor. Gain-of-function variants are represented as dots, with colours representing different variant desensitization properties of (i) accelerated current decay (blue); (ii) no change in desensitization (grey); (iii) reduced desensitization at equilibrium (pink); (iv) mixed with accelerated current decay and reduced desensitization at equilibrium (orange); and (v) increased desensitization at equilibrium (light blue). (B) 3D representation of a single $\beta 3$ subunit of the GABA_A receptor, with the location of variants shown as spheres and colour coded in the same manner. (C) Truncated violin plot comparing age of seizure onset between variants with different changes in desensitization. Colours represent accelerated current decay (blue), no change in desensitization (grey), reduced desensitization at equilibrium (pink), and mixed with accelerated current decay and reduced desensitization at equilibrium (orange). Black outline denotes variants are outliers in the linear regression of age of onset and $\Delta\log EC_{50}$. (D) Scatter plot of patient age of seizure onset and the extent of change in $\log EC_{50}$ comparing variants. The line of best fit for a linear regression of all variants is shown and the area within the 95% confidence intervals is shaded in grey. m = months.

colour coding variants in our correlation between age of onset and $\Delta\log EC_{50}$ (Fig. 6D). This revealed that data above and below the line of best fit coincided with changes in desensitization properties. Variants above the line (older age of onset) displayed accelerated current decay, and variants below the line (younger age of onset) decreased desensitization at equilibrium.

Variants with both an accelerated current decay and decreased desensitization at equilibrium either were below or on the line. Hence, at gain-of-function variants, accounting for changes in desensitization properties helps in clarifying the poor correlation between the functional change of the variant and the age of seizure onset.

Correlation of other clinical features with receptor desensitization

Other observations emerged when comparing the clinical features among the groups of patients with gain-of-function variants. In particular, the group with accelerated current decay differed from the three remaining groups. For the eight patients harbouring variants with accelerated current decay, there were no reports of early mortality, movement disorders or EIMFS (all 0/8), compared to reported total instances of early mortality (5/24), movement disorders including dystonia and dyskinesia (10/24) and EIMFS (9/24) for the other groups collectively. No differences were observed for other clinical features of microcephaly (3/8 and 10/24) and severe intellectual disability (3/8 and 16/24), with similar occurrences seen in all groups.

Discussion

Understanding of the mechanisms of disease is highly important for accurate diagnosis and pharmacological intervention in DEE. Here, we aim to unravel the reason behind a strong association between the structural location of GABRB3 variants and the severity of the clinical phenotype even within a cohort consisting of only gain-of-function patients. Although altered desensitization properties have been proposed by several groups to influence the net effect of variants in DEE,^{16–19,36} no direct evidence for such an association has been provided. Therefore, we evaluated the desensitizing properties of 20 gain-of-function and four loss-of-function GABRB3 variants and correlated the data with the age of onset of patients harbouring these variants. We provide the first evidence that changes in desensitization properties are linked to the structural location within the protein and identify correlations with the clinical outcomes of patients.

Variants can either increase or decrease receptor desensitization

The desensitization properties of GABA_A receptor variants have not previously been studied in a systematic manner, and often focused solely on current decay rates.^{16–19} Our data reveals more intricate effects of variants on receptor desensitization. The loss-of-function variants presented were relatively straightforward as they either did not change or increased desensitization properties. Variants that increased desensitization would be expected to further reduce GABAergic currents already impaired by the loss in GABA sensitivity and thereby exacerbate the loss-of-function characteristics. By contrast, the gain-of-function variants presented in a complex manner and could be grouped into four different categories based on their desensitization characteristics: (i) no change to desensitization; (ii) accelerated current decay rates (i.e. increased desensitization); (iii) reduced desensitization at equilibrium; and (iv) accelerated current decay and reduced desensitization at equilibrium (i.e. mixed desensitization effects). Generally, variants with accelerated current decay were more likely to be located in the coupling region, and variants with reduced desensitization at equilibrium were more likely to be located in the transmembrane regions.

Why are variants in transmembrane regions associated with worse clinical outcomes?

As more GABA_A receptor variants are identified, a clear separation has occurred in the severity of clinical outcomes between patients harbouring variants in the M1 and M2 transmembrane domains

and patients with variants located elsewhere.^{3,4,8,13,14} For GABRB3 variants, one reason for this is that the overall clinical presentation is strongly associated with changes in the GABA sensitivity, and transmembrane regions have a greater proportion of gain-of-function variants.

However, given the remarkable consequences of gain- or loss-of-function variants, we would predict that the magnitude of change of GABA sensitivity would correlate directly with disease severity. Although there is a trend for an earlier age of onset for variants with greater increases in GABA sensitivity, the overall correlation is surprisingly weak. Coupling region variants with no change in desensitization ($\beta 3^{L170R}$) or accelerated current decay ($\beta 3^{A305T}$, $\beta 3^{A305V}$ and $\beta 3^{E77K}$) presented above the line of best fit, while M1 variants with decreased desensitization at equilibrium ($\beta 3^{Y245H}$ and $\beta 3^{L256Q}$) or mixed desensitization effects ($\beta 3^{S254F}$) presented below the line. Indeed, while M1 gain-of-function variants increase GABA sensitivity by a relatively modest 0.24–0.47 $\Delta \log EC_{50}$ value, these patients have severe outcomes with a median age of onset of just 1.3 months, movement disorders such as dystonia and dyskinesia (3/8) and the rare and devastating DEE syndrome, EIMFS (3/8). Despite similar magnitudes of changes in GABA sensitivity, patients harbouring variants in the extracellular and coupling regions have an older median age of onset of 4 months. Hence, we propose that the conundrum of why transmembrane variants present with more severe clinical outcomes is 2-fold, they are more likely to cause gain-of-function and they are more likely to have decreased desensitization at equilibrium that further increase GABAergic currents.

GABA sensitivity or desensitization—which determines the clinical outcome?

Previous studies have identified GABA_A receptor variants with counteracting biophysical properties of increased GABA sensitivity and reduced activity from accelerated current decay.^{16–19,36} In these cases, authors have, to greater or lesser extents, attributed clinical phenotypes to enhanced desensitization (loss-of-function) consistent with the classical paradigm that decreased GABAergic activity leads to epilepsy. However, such attributions were speculative and fundamentally excluded a significant part of the data. In our studies, we likewise observed variants with mixed biophysical properties (e.g. coupling region variants and $\beta 3^{L280F}$). This raises the question of which parameter determines the overall clinical outcome of the patient—increased GABA sensitivity or accelerated current decay?

Ultimately, the clinical phenotype is the logical arbiter of whether a variant is a gain- or loss-of-function. The genotype/phenotype correlations show patients harbouring gain-of-function variants with increased desensitization properties compared to those with loss-of-function variants are overall different. They have earlier seizure onset (median 4 months versus 10 months for loss-of-function), higher rates of severe or profound ID (7/8 versus 8/38) and higher rates of microcephaly (3/8 versus 0/38) (Table 1). Patients harbouring variants with no changes, mixed or decreased desensitization properties displayed the most severe clinical indications including EIMFS, movement disorders including dystonia and dyskinesia, and higher risk of early death (Table 1), and all patients harbouring gain-of-function variants shared the same characteristics of early age of onset, severe or profound ID and microcephaly. Overall, the clinical outcomes for patients harbouring variants with accelerated current decay resemble those for gain-of-function variants, albeit with milder symptoms for some indications.

Overall, the clinical outcomes for patients harbouring a gain- or loss-of-function variant provides strong evidence that GABA

sensitivity is the primary determinant of the phenotype. A gain-of-function variant is thus not rendered a loss-of-function by accelerated current decay rates, and most likely a loss-of-function variant would not become a gain-of-function either if displaying increased steady state currents. The $\beta 3^{I300T}$ variant is an excellent example underscoring this. This variant decreased the steady-state open probability (loss-of-function) but has one of the largest changes in GABA sensitivity and youngest ages of onset. In this case, the profound shift in the GABA sensitivity appears to render changes in desensitization redundant. Accelerated current decay also appears not to protect against the effects of increased steady-state currents, as the $\beta 3^{S254F}$ variant with mixed desensitization properties overall resembled those with only increased steady state. Finally, some caution must also be taken when extrapolating the effects of increased desensitization from the receptor biophysical level to the increasing complexity of a neuron, a neuronal network and then neuronal development. For instance, the effect of increased desensitization properties on synaptic plasticity can be counterintuitive whereby recent experiments in dissociated rat hippocampal neurons showed that long term potentiation of inhibitory currents was increased by variants with increased desensitization properties.³⁷

Hence, we propose that functional analysis of GABRB3 variants should be interpreted primarily on changes to GABA sensitivity, and a combination of the structural location of the variant and desensitization analysis may assist in interpreting the severity of the variant. Other characteristics such as haploinsufficiency may also moderate the phenotype, however, this could not be evaluated here as we found no changes to the maximum current amplitudes for the gain-of-function GABRB3 variants. To answer whether altered desensitization characteristics alone is sufficient to produce an epileptic phenotype requires a further discovery of such a variant, since all the ones tested here have altered GABA sensitivity.

Cellular mechanisms and implications for treatment

The near ubiquitous spatial distribution of the $\beta 3$ subunit in the brain and incorporation into subtypes mediating both phasic and tonic GABA currents complicates both the interpretation of disease mechanisms, and the expected outcomes of treatment.³⁸ Their role at GABAergic inhibitory interneuron synapses is critical at loss-of-function variants where miniature inhibitory postsynaptic currents (mIPSCs) amplitudes are reduced and decay times slowed at cortical layer V/VI pyramidal neurons, mirroring loss-of-function SCN1A pathways where synaptic GABA is less frequently released.³⁹

Gain-of-function variants, however, may disrupt different neuronal networks and cellular mechanisms depending on the magnitude of the functional changes. Increased tonic currents are likely to explain part of the phenotype, as gain-of-function GABRD variants have recently been reported with intellectual disabilities, but not movement disorders or EIMFS.⁴⁰ Indeed, patients with SLC6A1 loss of function variants, where impaired GABA transport function also would be predicted to increase tonic currents, also present with intellectual disabilities.⁴¹ However, movement disorders including dystonia, dyskinesia and choreoathetosis have been reported at GABRB2 variants, including in the M1, M2 and M2-M3 regions, although it is not clear if these are gain- or loss-of-function variants,⁸ suggesting that the GABRB2 and GABRB3 may be active in similar motor pathways.

In this context, it is interesting that the peculiar and rare epilepsy syndrome EIMFS has been reported in patients with biallelic SLC12A5 variants.⁴² The SLC12A5 gene encodes for the KCC2

transporter required to maintain the chloride gradient that would otherwise render GABAergic currents excitatory.⁴³ Although chloride transport dynamics are exceptionally complex in neural networks, Cl^- fluorophores suggest that mature neurons have a median internal chloride concentration between 6 and 14 mM that can reach as high as 40 mM in sensory afferent neurons that do not express the KCC2 transporter.^{44,45} Rapid bursts of synaptic inputs can increase intracellular chloride concentrations in dendritic compartments by as much as 20 mM, which is then extruded by KCC2 at a rate in the order of 5 mM/s.⁴⁶ The precise point at which gain-of-function GABA_A receptors will overload the KCC2 extrusion capacity and reverse the chloride conductance is unclear, and computational models suggest it is likely to be highly dynamic with different sensitivities in different cellular compartments.⁴⁷ Nevertheless, combinations of increased GABA sensitivity and decreased desensitization at equilibrium may overload chloride efflux via KCC2 transporters, leading to overlapping characteristic of EIFMS with SLC12A5 and GABRB3 variants. The overlap of different phenotypic traits with genetic variants in GABAergic pathways, including different GABA_A receptor genes and the transporter KCC2, show that delineating the contributions of GABAergic activity, including chloride reversal potentials, tonic and phasic currents will likely prove essential to understanding how individual phenotypic traits develop and lead to effective treatment.

Limitations of the study

More precise details of how variants alter desensitization kinetics can be elucidated via kinetic modeling after determining the fast and slow components of desensitization.³² In several cases, including the WT and variants such as p.(Ser254Phe), decay currents for individual oocytes did not fit to a two-phase decay, limiting the interpretations we can make. However, the weighted tau values changed in a predictable manner, with the slow time course of desensitization being most likely to be affected. This agrees with the proposal that the slow component of current decay is susceptible to structural rearrangements of the β -subunit. The finer details of how the different structures, particularly the coupling loops and the M1 regions, will be important in understanding how variants in these regions will alter receptor function and how it relates to clinical severity. Another limitation of this study is that the conclusions were drawn from non-neuronal cells, and the findings may be different from the neuronal milieu, or more complicated by the dynamic nature of neuronal GABA_A receptor activation, membrane potentials and ionic gradients.

Conclusion

We have identified altered desensitization properties at GABRB3 variant receptors that increase desensitization via accelerated current decay or decrease desensitization via increased steady-state currents. We propose that changes in desensitization associates with the severity of the clinical phenotype as a secondary factor but does not define the variants as gain- or loss-of-function. The increased severity of the phenotypes at patients with transmembrane region variants appears to be associated with increased steady state currents, a correlation that may enable more accurate diagnosis.

Data availability

De-identified data will be made available to those eligible. This includes the GABRB3 database and data used for all analysis in the manuscript. Data will be stored for a minimum of 7 years.

Funding

This work was funded by the Australian National Health and Medical Research Council grant APP1185122 (P.K.A., N.L.A., M.C.), the Australian Research Training Program Stipend scholarship (S.X.N.L.), the Novo Nordisk Foundation NNF19OC0058749 (R.S.M.) and The Lundbeck Foundation R383-2022-276 (to R.S.M. and P.K.A.).

Competing interests

The authors report no competing interests.

Supplementary material

[Supplementary material](#) is available at *Brain* online.

References

- Happ HC, Carvill GL. A 2020 view on the genetics of developmental and epileptic encephalopathies. *Epilepsy Curr.* 2020;20:90–96.
- Feng Y-CA, Howrigan DP, Abbott LE, et al. Ultra-rare genetic variation in the epilepsies: A whole-exome sequencing study of 17,606 individuals. *Am J Hum Genet.* 2019;105:267–282.
- Hernandez CC, Macdonald RL. A structural look at GABA(A) receptor mutations linked to epilepsy syndromes. *Brain Res.* 2019;1714:234–247.
- Maljevic S, Moller RS, Reid CA, et al. Spectrum of GABAA receptor variants in epilepsy. *Curr Opin Neurol.* 2019;32:183–190.
- Moller RS. Mutations in GABRB3 from febrile seizures to epileptic encephalopathies. *Neurology.* 2017;88:483–492.
- Shi YW, Zhang Q, Cai K, et al. Synaptic clustering differences due to different GABRB3 mutations cause variable epilepsy syndromes. *Brain.* 2019;142:3028–3044.
- Janve VS, Hernandez CC, Verdier KM, Hu N, Macdonald RL. Epileptic encephalopathy de novo GABRB mutations impair gamma-aminobutyric acid type A receptor function. *Ann Neurol.* 2016;79:806–825.
- El Achkar CM, Harrer M, Smith L, et al. Characterization of the GABRB2-associated neurodevelopmental disorders. *Ann Neurol.* 2021;89:573–586.
- Absalom NL, Liao VWY, Johannesen KMH, et al. Gain-of-function and loss-of-function GABRB3 variants lead to distinct clinical phenotypes in patients with developmental and epileptic encephalopathies. *Nat Commun.* 2022;13:1822.
- Ahring PK, Liao VWY, Lin S, Absalom NL, Chebib M, Moller RS. The de novo GABRA4 p.Thr300Ile variant found in a patient with early-onset intractable epilepsy and neurodevelopmental abnormalities displays gain-of-function traits. *Epilepsia.* 2022;63:2439–2441.
- Absalom NL, Liao VWY, Kothur K, et al. Gain-of-function GABRB3 variants identified in vigabatrin-hypersensitive epileptic encephalopathies. *Brain Commun.* 2020;2:fcaa162.
- Chen W, Ge Y, Lu J, et al. Distinct functional alterations and therapeutic options of two pathological de novo variants of the T292 residue of GABRA1 identified in children with epileptic encephalopathy and neurodevelopmental disorders. *Int J Mol Sci.* 2022;23:2723.
- Johannesen KM, Iqbal S, Guazzi M, et al. Structural mapping of GABRB3 variants reveals genotype-phenotype correlations. *Genet Med.* 2022;24:681–693.
- Maillard PY, Baer S, Schaefer É, et al. Molecular and clinical descriptions of patients with GABA(A) receptor gene variants (GABRA1, GABRB2, GABRB3, GABRG2): A cohort study, review of literature, and genotype-phenotype correlation. *Epilepsia.* 2022;63:2519–2533.
- Yang Y, Zeng Q, Cheng M, et al. GABRB3-related epilepsy: Novel variants, clinical features and therapeutic implications. *J Neurol.* 2022;269:2649–2665.
- Shen D, Hernandez CC, Shen W, et al. De novo GABRG2 mutations associated with epileptic encephalopathies. *Brain.* 2017;140:49–67.
- Butler KM, Moody OA, Schuler E, et al. De novo variants in GABRA2 and GABRA5 alter receptor function and contribute to early-onset epilepsy. *Brain.* 2018;141:2392–2405.
- Vogel FD, Krenn M, Westphal DS, et al. A de novo missense variant in GABRA4 alters receptor function in an epileptic and neurodevelopmental phenotype. *Epilepsia.* 2022;63:e35–e41.
- Steudle F, Rehman S, Bampali K, et al. A novel de novo variant of GABRA1 causes increased sensitivity for GABA in vitro. *Sci Rep.* 2020;10:2379.
- Bianchi MT, Haas KF, Macdonald RL. Structural determinants of fast desensitization and desensitization-deactivation coupling in GABAA receptors. *J Neurosci.* 2001;21:1127–1136.
- Scheller M, Forman SA. Coupled and uncoupled gating and desensitization effects by pore domain mutations in GABA(A) receptors. *J Neurosci.* 2002;22:8411–8421.
- Bianchi MT, Macdonald RL. Mutation of the 9' leucine in the GABAA receptor γ 2L subunit produces an apparent decrease in desensitization by stabilizing open states without altering desensitized states. *Neuropharmacology.* 2001;41:737–744.
- Birnir B, Tierney ML, Lim M, Cox GB, Gage PW. Nature of the 5' residue in the M2 domain affects function of the human alpha 1 beta 1 GABAA receptor. *Synapse.* 1997;26:324–327.
- Tierney ML, Birnir B, Pillai NP, et al. Effects of mutating leucine to threonine in the M2 segment of alpha1 and beta1 subunits of GABAA alpha1beta1 receptors. *J Membr Biol.* 1996;154:11–21.
- Terejko K, Michalowski MA, Izykowska I, Dominik A, Brzostowicz A, Mozrzymas JW. Mutations at the M2 and M3 transmembrane helices of the GABAARs alpha1 and beta2 subunits affect primarily late gating transitions including opening/closing and desensitization. *ACS Chem Neurosci.* 2021;12:2421–2436.
- Absalom NL, Ahring PK, Liao VWY, et al. Functional genomics of epilepsy-associated mutations in the GABAA receptor subunits reveal that one mutation impairs function and two are catastrophic. *J Biol Chem.* 2019;294:6157–6171.
- Liao VWY, Chua HC, Kowal NM, Chebib M, Balle T, Ahring PK. Concatenated gamma-aminobutyric acid type A receptors revisited: Finding order in chaos. *J Gen Physiol.* 2019;151:798–819.
- Guitchoy G, Stewart DS, Forman SA. Two etomidate sites in α 1 β 2 γ 2 γ -aminobutyric acid type A receptors contribute equally and noncooperatively to modulation of channel gating. *Anesthesiology.* 2012;116:1235–1244.
- Dixon C, Sah P, Lynch JW, Keramidas A. GABAA Receptor alpha and gamma subunits shape synaptic currents via different mechanisms. *J Biol Chem.* 2014;289:5399–5411.
- Keramidas A, Harrison NL. The activation mechanism of alpha1beta2gamma2S and alpha3beta3gamma2S GABAA receptors. *J Gen Physiol.* 2010;135:59–75.
- Gielen M, Corringer PJ. The dual-gate model for pentameric ligand-gated ion channels activation and desensitization. *J Physiol.* 2018;596:1873–1902.
- Gielen M, Barilone N, Corringer PJ. The desensitization pathway of GABAA receptors, one subunit at a time. *Nat Commun.* 2020;11:5369.
- Hernandez CC, Shen Y, Hu N, et al. GABRG2 Variants associated with febrile seizures. *Biomolecules.* 2023;13:414.

34. Mozrzymas JW, Barberis A, Mercik K, Zarnowska ED. Binding sites, singly bound states, and conformation coupling shape GABA-evoked currents. *J Neurophysiol.* 2003;89:871-883.
35. Masiulis S, Desai R, Uchanski T, et al. GABAA Receptor signalling mechanisms revealed by structural pharmacology. *Nature.* 2019;565:454-459.
36. Hernandez CC, Kong W, Hu N, et al. Altered channel conductance states and gating of GABAA receptors by a pore mutation linked to Dravet syndrome. *eNeuro.* 2017;4:1-23.
37. Field M, Dorovykh V, Thomas P, Smart TG. Physiological role for GABAA receptor desensitization in the induction of long-term potentiation at inhibitory synapses. *Nat Commun.* 2021;12:2112.
38. Janssen MJ, Yasuda RP, Vicini S. GABA_A Receptor beta3 subunit expression regulates tonic current in developing striatopallidal medium spiny neurons. *Front Cell Neurosci.* 2011;5:15.
39. Gobbo D, Scheller A, Kirchhoff F. From physiology to pathology of cortico-thalamo-cortical oscillations: Astroglia as a target for further research. *Front Neurol.* 2021;12:661408.
40. Ahring P, Liao VW, Gardella E, et al. Gain-of-function variants in GABRD reveal a novel pathway for neurodevelopmental disorders and epilepsy. *Brain.* 2022;145:1299-1309.
41. Mermer F, Poliquin S, Rigsby K, et al. Common molecular mechanisms of SLC6A1 variant-mediated neurodevelopmental disorders in astrocytes and neurons. *Brain.* 2021;144:2499-2512.
42. McTague A, Kurian MA. SLC12A5-related epilepsy of infancy with migrating focal seizures In: Adam MP, Feldman J, Mirzaa GM, et al., eds. *GeneReviews.* University of Washington; 2019.
43. Stöðberg T, McTague A, Ruiz AJ, et al. Mutations in SLC12A5 in epilepsy of infancy with migrating focal seizures. *Nat Commun.* 2015;6:8038.
44. Glykys J, Dzhalala V, Egawa K, et al. Local impermeant anions establish the neuronal chloride concentration. *Science.* 2014;343:670-675.
45. Berglund K, Schleich W, Krieger P, et al. Imaging synaptic inhibition in transgenic mice expressing the chloride indicator, clo-meleon. *Brain Cell Biol.* 2006;35(4–6):207-228.
46. Doyon N, Vinay L, Prescott SA, De Koninck Y. Chloride regulation: A dynamic equilibrium crucial for synaptic inhibition. *Neuron.* 2016;89:1157-1172.
47. Doyon N, Prescott SA, Castonguay A, Godin AG, Kröger H, De Koninck Y. Efficacy of synaptic inhibition depends on multiple, dynamically interacting mechanisms implicated in chloride homeostasis. *PLoS Comput Biol.* 2011;7:e1002149.

Chapter 7 E77K mouse model

Introduction

Recently, and as described in Chapters 3, 4 and 5, we, and others, have demonstrated that epilepsy-associated variants in 11/19 genes, including the *GABRB3* that make up the GABA_A receptor can result in either a GoF (hyperactive), or LoF (hypoactive) GABA_A receptor (72, 75, 78, 79, 82, 94, 177, 231, 242). Variants that increase inhibitory (GoF) neurotransmission through changes in kinetics, including increased receptor sensitivity and reduced desensitisation (as described in Chapter 6), are correlated with a younger age of seizure onset and a more severe clinical phenotype, including microcephaly, higher mortality rate and movement disorders. It is known that GABA plays a crucial role in brain development and is implicated in many processes, including neuronal proliferation, synapse formation and preliminary circuit building (34, 46, 243). To date, it is unknown why increased GABA activity is associated with a more severe phenotype.

There are several hypotheses and factors that contribute towards epileptogenesis, however, the exact underlying cellular and molecular mechanisms remain elusive. Literary evidence from murine studies and histopathological examination of tissues of deceased patients suggests morphological and aberrant neuronal activity could underpin the phenotypic manifestation of epilepsy. These factors include neuroinflammation (244), neuronal injury (245), cell death, myelination (129), and mossy fibre sprouting (246). Among the various possible avenues to explore, the role of microglia has attracted significant interest due to its potential involvement in neuroinflammatory processes that drive the development and progression of epilepsy (247-249).

Microglia are the resident immune cells of the brain that carry out homeostatic surveillance of the CNS and play an important role in neurodevelopment, from the formation of neural circuits to synaptic plasticity (250, 251). Due to the complex activation of microglial phenotypes, the role of microglia in epilepsy remains largely elusive (155, 252-255). There is evidence of extensive microglia activation in the hippocampus of the brain of patients with epilepsy and animal models of epilepsy (251). This activation is linked to various structural and functional alterations within the hippocampus, such as enhanced neurogenesis, mossy fibre sprouting

(256) and consequent hippocampal hyperexcitability (257), which together could “rewire” the brain and provide the underlying cause for the clinical phenotypes of GoF patients.

Microglia are complex and can act as both anti-epileptic and pro-epileptic mediators (247, 258). Previous studies have postulated that post-seizure activity, activated microglia, resulted in the release of cytokines, excessive phagocytosis of synapses and neuronal damage (259, 260). Studies on murine models of epilepsy suggest that the depletion of microglia markers is associated with increased seizure frequency and severity (261). Similarly, pharmacological (with CSFIR inhibition, PLX3397) and pharmacogenetic (with diphtheria toxin) microglial elimination showed worsened seizure outcomes, whilst microglial repopulation had a seizure-limiting role in kainic acid-induced seizure (262). Others have suggested that the activation of microglia is associated with cortical thinning (263).

Another member of the glial family, oligodendrocytes – important for producing myelin sheath – have also been found to be altered in patients with epilepsy (there is the trend of corpus callosum thinning, hypomyelination in the GoF cohort observed through our patient database, data are not shown). MRI examination of the brain of patients with DEE revealed most have microcephaly with a reduction in white matter volume, corpus callosum, and delayed myelination (129, 264). It has also been noted in demyelination diseases that the role of microglia contributes to the clinical outcome, such that a depletion or blockage of microglia can prevent disease progression (265, 266).

Therefore, to gain a better understanding of the neuropathology of GoF variants, we used a novel knock-in GoF mouse model based on a human *de novo* GoF variant, p.E77K in the *GABRB3*, which encodes for $\beta 3$ subunit of GABA_A receptor. The $\beta 3$ subunit plays a crucial role during development, with the expression of the $\beta 3$ subunits being ubiquitously expressed in the mammalian brain from early development, especially in the cerebral cortex, thalamus, hippocampus and other regions involved in generating seizures (267-269). This variant was selected to investigate its contribution to the development of mutant mice. Previous functional studies on this variant showed that it had a LogEC₅₀ shift of 0.64 and a 34% loss of current (normalised maximum current to WT was 66%) (75).

Patient case report

A female patient who harboured a *de novo* *GABRB3* missense variant, $\beta 3$ (E77K), was first reported by Kothur *et al.* (144). The patient initially presented with refractory spasms at the

age of 4 months. When given vigabatrin, a GABA transaminase inhibitor that inhibits the breakdown of synaptic GABA, the patient displayed excessive sedation, resulting in the discontinuation of the medication. The patient was subsequently diagnosed with infantile spasm, which is a rare epileptic disorder occurring in the first year of life and severe ID (post-seizure) (144). MRI scan showed prominent cerebral spinal fluid spaces and mild ventriculomegaly, and on EEG, it showed modified hypsarrhythmia, multifocal, generalised spike-wave (144).

Given GABA's important role in the brain i.e. the main inhibitory neurotransmitter within the brain, and the severe phenotype of GoF variants, we sought to determine whether the $\beta 3$ (E77K) mutation alters brain structure.

Therefore, this chapter aims:

1. To determine changes in brain morphology by evaluating mass and hippocampal volume using Nissl stain. The hippocampal volume is of interest, especially hippocampal regions, CA1 and CA3 because they are associated with increased neuronal excitability and seizures (155).
2. To assess markers of neuroinflammation, neuronal death and epilepsy to elucidate differences between $\beta 3^{E77K}$ and WT mice.

Hypothesis: Since GoF variants are associated with more severe clinical phenotypes, such as ID, DD, abnormal MRI findings (e.g. hypomyelination and thinning of corpus callosum) and in particular, microcephaly, we expect $\beta 3$ GoF knock-in mice, $\beta 3^{E77K}$, to exhibit differences in the gross morphology of the brain and inflammatory markers. Specifically, microcephaly, reduced myelin and upregulation of inflammatory markers.

Survival characteristics of *GABRB3* p.E77K knock-in mice

Of all the tried variants, including $\beta 3$ (T287I), only the $\beta 3$ (E77K) produced live offspring after 2.5 years of trying to create this knock-in mouse model. It must be noted that for this mutant mouse there is a high mortality rate, over two-thirds (68%) of mutant mice were stillbirths (**Figure 7.1 A**). Among the live births, 78.91% were WT, and 21.09% were E77K mutant mice, suggesting a deviation from Mendelian distribution, indicating the mice either died *in utero* or shortly after birth (**Figure 7.1 B**). In addition, E77K mutant mice had a higher probability of premature death (**Figure 7.1 C**). Mice were bred at The Florey Institute of Neuroscience and Mental Health by Professor Reid's group. Data for survival characteristics of $\beta 3$ (E77K) were

provided by Chaseley Mckenzie (PhD candidate) from Professor Reid's group. The brains of these mice were then sent to Professor Collins' (Chebib's) team at the Brain and Mind Centre for immunohistochemical analysis.

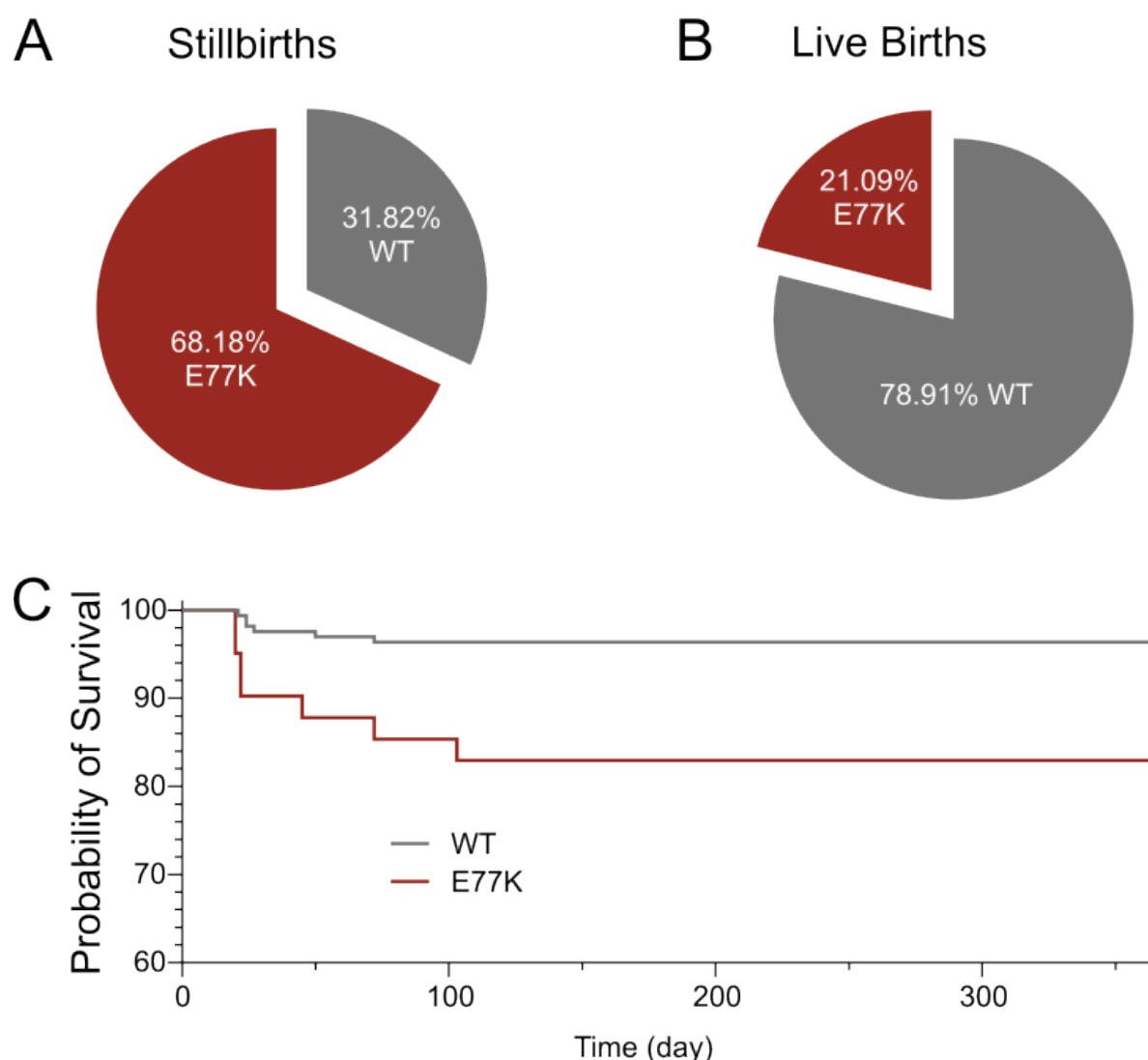


Figure 7.1 Comparison of WT and E77K mutant mice on birth outcomes and postnatal survival.

A) Pie chart depicting the distribution of stillbirths among WT and E77K mutant mice. A higher proportion of stillbirths were observed in E77K mice (68.18%) compared to WT mice (31.82%). **B)** Pie chart highlighting the proportion of live birth for WT mice and E77K mice. E77K mice accounted for 21.09% of live births, whereas WT mice represented the majority with 78.91%, highlighting reduced viability in E77K offspring. **C)** Postnatal survival curves: The Kaplan-Meier survival curves compare the postnatal survival probability between E77K and WT mice over a period of 365 days. E77K mice exhibit a lower survival rate compared to WT mice. Data were provided by Chaseley Mckenzie (PhD candidate from Professor Reid's group).

Results

No significant gross morphological were observed between WT and $\beta 3^{+/E77K}$ mice.

Microcephaly is defined as a small brain detected by head circumference more than 2 standard deviations below the mean for the age and gender (270-272). In previous murine models of microcephaly, the mass (g) of the brain of animals with microcephaly was significantly lower than WT (272, 273). Here, we used a whole-brain comparison of brain mass between WT and $\beta 3^{+/E77K}$ mice to elucidate whether heterozygous mice with $\beta 3^{E77K}$ mutation had microcephaly on a gross level. The hypothesis is that if the mice had microcephaly, we would expect to see a smaller brain mass in the mutant mice. However, no significant difference was observed with a Mann-Whitney U test between the mass of mouse brains with WT and $\beta 3^{+/E77K}$ mice, with mean 0.56 ± 0.027 g and 0.52 ± 0.067 g ($p > 0.05$), respectively (**Figure 7.2 A, B**).

Given the $\beta 3$ subunit is ubiquitously expressed throughout the brain, we narrowed the study to the hippocampus, a well-studied brain region for epilepsy. Hippocampal abnormalities such as reduction in hippocampal volume and malrotation have been reported in patients with epilepsy (274-276). Previous studies of murine epilepsy models have looked at the changes in the hippocampus region, such as increased neuronal density in the dentate gyrus, due to its well-known association with epilepsy (243, 277). To elucidate whether the $\beta 3^{E77K}$ mutation was associated with gross morphological hippocampal changes, coronal sections were stained with cresyl violet to better visualise the morphology of the brain and guide the outline of the hippocampus. There were no statistically significant differences in hippocampal volume between WT (mean = 4.6 ± 0.68 mm³) and $\beta 3^{+/E77K}$ (4.2 ± 0.49 mm³) (Mann-Whitney U Test, $p > 0.05$, $n = 3$ mice per group) (**Figure 7.2 C, D**).

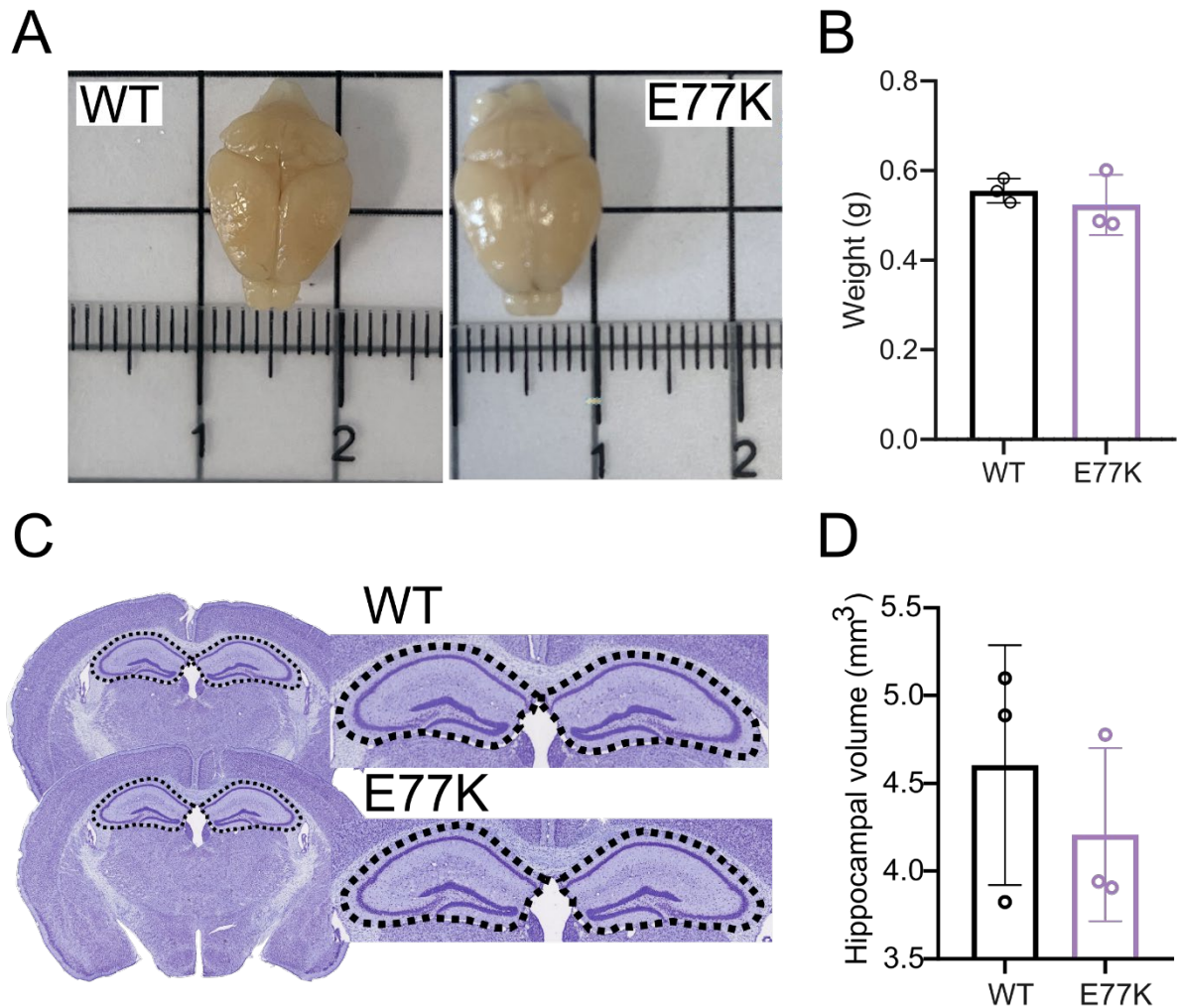


Figure 7.2 Gross brain morphology comparisons between E77K mutant mouse and WT

A) Representative image of WT (Left) and mutant (right) brain. **B)** Bar graph for the mass (g) of WT and $\beta 3^{+/E77K}$ brain. Data are shown as mean $\pm$ SD. $n = 3$ mice for each group. **C)** Representative photograph of hippocampal tracing of WT (top) and $\beta 3^{+/E77K}$ (top) brain. **D)** The hippocampal volume (mm³) of WT and $\beta 3^{+/E77K}$ mice. Data are shown as mean $\pm$ SD. $n = 3$ mice for each group

No significant difference was observed in mossy fibre sprouting between WT and $\beta 3^{+/E77K}$ mice brains.

Mossy fibre sprouting has been reported in both patients with epilepsy and animal models of epilepsy, and a consistent pathological finding of experimental and human medial temporal lobe epilepsy (278-283). Granule cells of the dentate gyrus have been considered glutamatergic (284). Under physiological conditions, dentate granule cells axons, also known as mossy fibres, located in the dentate gyrus projects to the CA3 region of the hippocampus, innervating the dendrites of CA3 pyramidal cells (**Figure 7.3 A**). Mossy fibres are considered one of the main excitatory input into the hippocampus, releasing glutamate (285, 286).

By contrast, during mossy fibre sprouting, in context of mesial temporal lobe epilepsy, mossy fibres form new axons and synapses within the dentate, creating a new circuit on top of the existing wiring scheme (287) **(Figure 7.3 A)**. This new circuit has been implicated in the “rewiring” of the hippocampus, with sprouted mossy fibre synapses having a higher probability of glutamate release, driving a pro-excitatory circuit (283, 288).

To elucidate differences in mossy fibre sprouting, brain slices were stained with fluorescently labelled antibody against zinc transporter, ZnT3, which is highly concentrated in mossy fibre terminals (289-291). Increased staining intensity indicates mossy fibre sprouting. However, no significant differences were observed between the WT and $\beta 3^{E77K}$ (Mann-Whitney U test, $p > 0.05$) **(Figure 7.3 B, C)**.

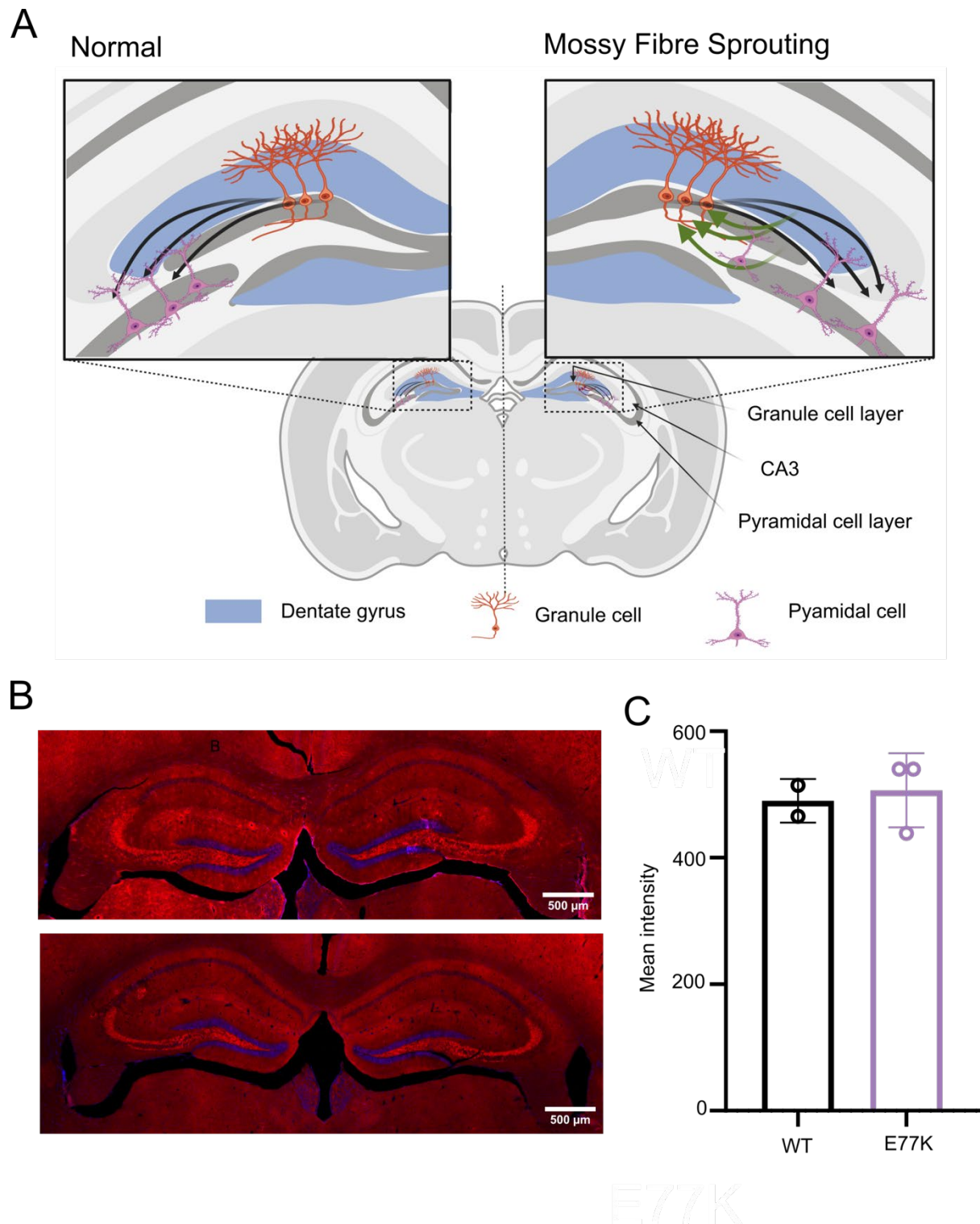


Figure 7.3 Comparison of mossy fiber sprouting in WT and $\beta 3^{+/E77K}$ mutant mice

A) Coronal slice drawing of a mouse brain depicting a normal (left) and abnormal mossy fibre sprouting (right). Under normal conditions, the granule cells from the dentate gyrus projects to the pyramidal cells of CA3. The black arrow represents the mossy fiber. Insets show a detailed view of normal and mossy fiber sprouting of both WT and $\beta 3^{+/E77K}$ knock-in mice, with the sprouting depicted by the extended khaki projections. **B)** Representative

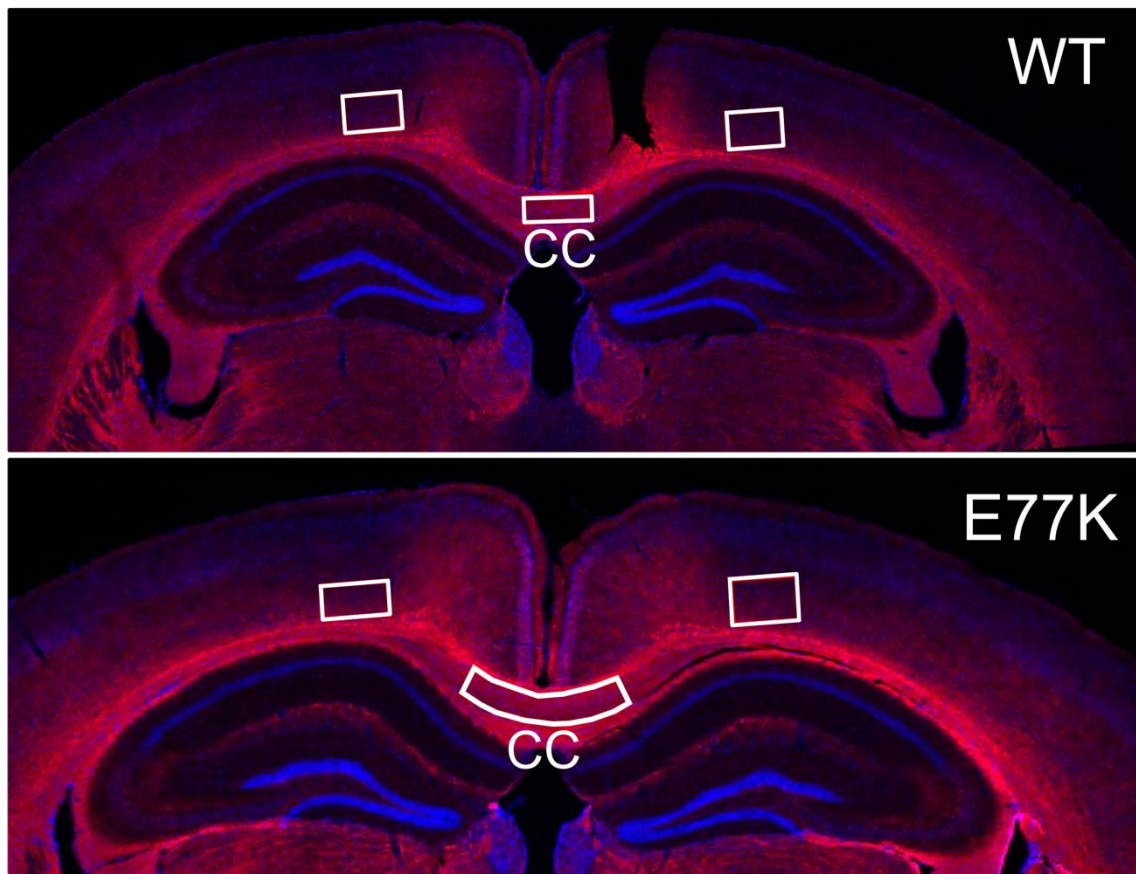
immunofluorescent staining of coronal brain sections: Brain slices from WT (top) and E77K (bottom) knock-in mice were stained with a fluorescently labelled antibody targeting the zinc transporter, ZnT3. The red fluorescence indicates the presence of ZnT3, highlighting mossy fiber distribution. Blue fluorescence indicates DAPI, which labels for cell nuclei. Scale bars represent 500 μm in the stained brain sections. C) Bar graph presents the average fluorescence intensity of ZnT3 staining of WT (n=2) and E77K (n=3) knock-in mice. Lines indicate mean $\pm$ SD.

Investigating potential changes in myelin, microglia, and degenerating neurons in brain of E77K GoF mouse model

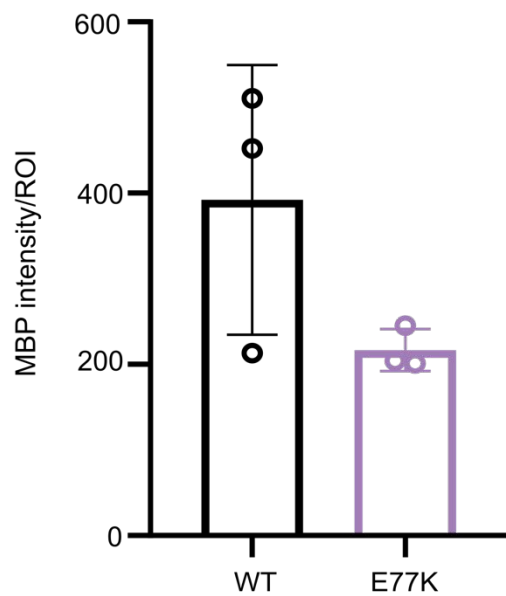
Involvement of myelin basic protein (MBP) in the pathology of epilepsy

Myelin basic protein is important in the process of myelination of nerves in the CNS. Previous reports on patient MRI scans and histopathology of deceased patients have reported abnormalities, including hypomyelination and generalised volume loss with thin corpus callosum (129, 235, 292-295). No notable differences were seen between the fluorescence intensity per region of interest, layer 6 of the motor cortex (**Figure 7.4 A, B**), and corpus callosum (**Figure 7.4 A, C**) between WT and $\beta 3^{+/E77K}$ mice. This suggests that the E77K mutations did not significantly change the myelin architecture of the brain. One WT mouse had no salvageable tissue containing the corpus callosum, and this was discarded from analysis.

A



B



C

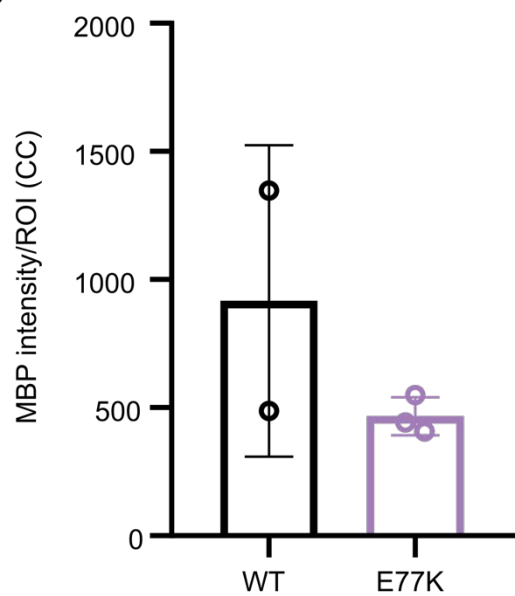


Figure 7.4 Comparison of MBP staining intensity in WT and E77K mutant mice brain

A) Representative photograph of coronal brain sections from WT (top) and E77K (bottom) mice. These sections were immunostained with antibodies against MBP to visualise the

distribution and density of myelination within the brain tissue. MBP is indicated in red, DAPI is indicated in blue and marks the cell nuclei. Boxes indicate the regions of interest measured CC denotes corpus callosum. **B)** Bar graph of average fluorescence intensity per region of interest (L6 of the motor cortex) (white box) Mann-Whitney U test, $p > 0.05$, **C)** bar graph of mean intensity per region of interest (corpus callosum) (white box). Mann-Whitney U test, $p > 0.05$.

Involvement of Iba-1 in between WT and mutant mice

Microglia are the immune cells within the CNS and have an important role in screening the brain's environment for danger (296), as well as having a role in neurogenesis (297), synaptic pruning and circuit networks (298), and phagocytose dead neurons (299).

The role of microglia in epilepsy is of interest as previous studies have found microglia and Iba-1 to be extensively active in the hippocampus of patients with epilepsy and animal models of epilepsy (251, 300-302). Since Iba-1 is a marker expressed on microglia, we expect to see an increase in Iba-1-positive cells in mutant mice (303). To examine whether there was a difference in Iba-1 between WT and heterozygous mice, we quantified the number of Iba-1 positive cells per region of interest within the hippocampus and saw no difference between WT and $\beta 3^{E77K}$ in the mice (**Figure 7.5 A - C**).

Studies on murine models of epilepsy suggest that the depletion of microglia markers is associated with increased seizure frequency and severity (261). Similarly, pharmacological (with CSFIR inhibition, PLX3397) and pharmacogenetic (with diphtheria toxin) microglial elimination showed worsened seizure outcomes, whilst microglial repopulation had a seizure-limiting role in kainic acid-induced seizure (262). Others have suggested that the activation of microglia is associated with cortical thinning (263).

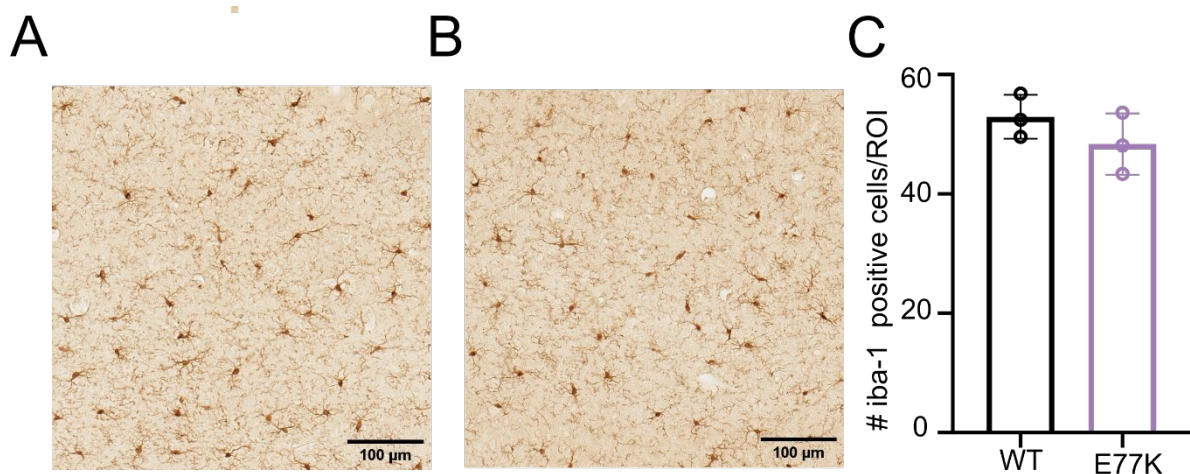


Figure 7.5 Staining for Iba-1, a marker for microglia

Representative Iba-1 immunostaining for WT (left) and E7K (right). Scale bar = 100 μ m (b) Bar graph of Iba-1 positive cells per region of interest. Data are expressed as mean $\pm$ SD, n = 3 per group for WT and E77K. Mann-Whitney U test, p > 0.05.

Fluoro-Jade (FJC)

Fluoro-Jade dyes, such as Fluoro-Jade, Fluoro-Jade B, and Fluoro-Jade Care fluorescent ligands used for the detection of degenerating neurons (163). Fluoro-Jade C (FJC), in particular, has a high affinity for degenerating neurons, and apoptotic cells from early to late stages of development (164, 304, 305). A noticeable bright green fluorescence by FJC-positive staining indicates the presence of degenerating neurons (163, 305, 306).

No differences were seen between WT and $\beta 3^{+/E77K}$ mice based on qualitative analysis (n=3 for each group) (**Figure 7.6 A, B**). Brain tissue from a photothrombotic mice model was used as a positive control to ensure that the FJC works (**Figure 7.6 C**).

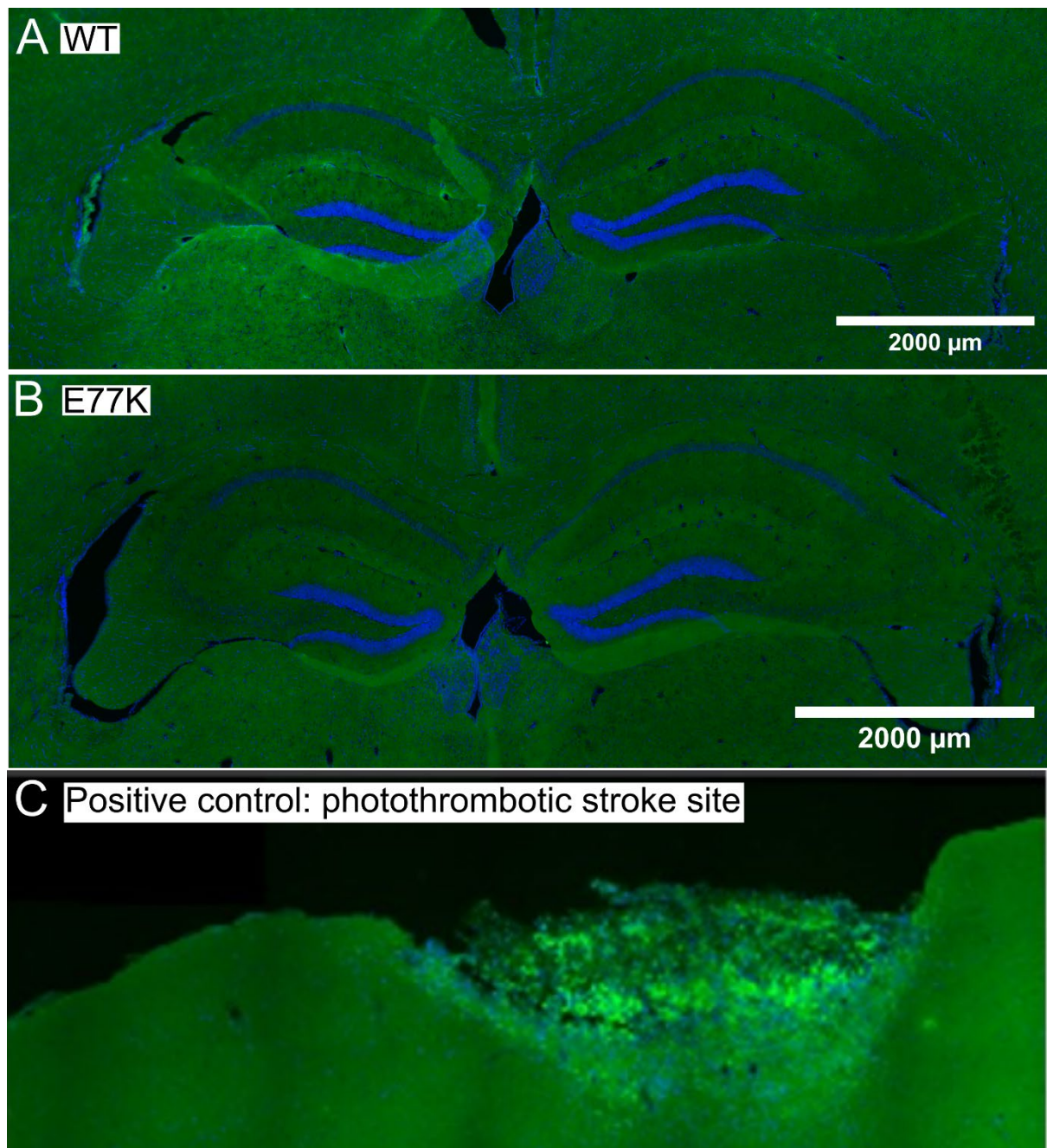


Figure 7.6 Fluoro-Jade C, a fluorescent stain for the detection of neuronal degeneration in WT and mutant (E77K) mouse brains, along with a positive control.

Representative photograph of FJC staining for **A)** WT, and **B)** E77K mutant mice. Both group showed little to no FJC positive staining, indicating comparable levels of neuronal integrity and no significantly significant difference in neuronal degeneration between the two groups. Blue fluorescence indicates the presence of DAPI, which stains for cell nuclei. **C)** Representative image of a tissue from a mouse that previously underwent photothrombotic stroke as a positive control. Arrows indicate FJC-positive stain. Scale bars are 2000 μm for WT and E77K.

Discussion

The current variant studied, *GABRB3 E77K*, is from a patient diagnosed with epileptic infantile spasm which is an EE, often presenting with flexion extension within the first year of life and evolves to severe epilepsy and regression in early childhood (307). To gain deeper insights into the neuropathological implications of GoF variants, we utilised a novel knock-in GoF mice model featuring human *de novo* GoF *GABRB3 E77K* variant. This study assessed for differences in gross morphology alongside markers of mossy fibre sprouting, myelination, neuroinflammation, and neuronal death.

In the current study, no statistically significant differences in the brain mass or gross anatomical brain abnormalities were observed between WT and mutant mice. While MRI findings in patients with GoF variants can vary from normal to cases of thinning of the corpus callosum, poor myelination, and cortical atrophy (32, 129, 235, 292-295), such abnormalities were not observed in our mouse model.

Similarly, a recent study of another GoF mouse model with a variant in the $\gamma 2$ subunit, *Gabrg2* T316N, reported no significant differences were observed in brain morphology, mass and gross neuroanatomical morphology between *Gabrg2*^{+/+} and *Gabrg2*^{T316N/+} mice (308). Consistent with our model, *Gabrg2* T316N mutant mice, displayed a reduced Mendelian ratio of *Gabrg2*^{+/+}: *Gabrg2*^{T316N/+}: *Gabrg2*^{T316N/T316N} = 1: 0.6: 0.06, and a low birth rate of homozygous mutant mice (*Gabrg2*^{T316N/T316N}) (308). Like patients with sleep-related hypermotor epilepsy, *Gabrg2*^{T316N/+} exhibited spontaneous seizure, with symptoms involving rhythmic, jerking motion of all extremities, loss of balance, and frequent falling to one side (308). Together, the lack of significant changes in brain morphology in both *Gabrg2*^{T316N/+} and *Gabrb3*^{+/E77K} suggests that the variants do not have a substantial impact on the development of gross brain morphology.

A potential explanation for the spontaneous seizures observed in *Gabrg2*^{T316N/+}, which were not present in our study, could be the differences in the mouse substrain used. The *Gabrg2*^{T316N/+} knock-in model were generated in C57BL/6N, whilst we used the C57BL/6J strain. For instance, a comparing seizure phenotype of *Scn1a*^{+/-} mice on C57BL/6N and

C57BL/6J backgrounds found that C57BL/6N is more susceptible to hyperthermia-induced seizures, exhibit milder spontaneous seizures and improved survival rate compared to the C57BL/6J (309).

The lack of difference in myelination, or changes in neuroinflammation in the current study may be because these mice do not experience spontaneous spasming or seizures. The current findings suggest that the tested GoF *Gabrb3* E77K variant is unlikely to alter the gross formation of the brain and recruitment of mossy fibre sprouting, neuroinflammation and subsequent death.

Overall, no observable differences were found in the gross morphology, mossy fibre sprouting, myelin basic protein expression, Iba-1 staining and neuronal death (FJC staining) between WT and *Gabrb3*^{+E77K} mice. We speculate that since the mutation did not change the gross morphology of the brain or the tested markers, this suggests observed other features or behavioural changes (data collected by Prof Reid's team and are not presented) could be a function of the mutation, but it is not driven by abnormal development of the brain.

Limitations

The strength of knock-in and knock-out murine studies lies in their ability to give insight into neurological and behavioural changes. However, sometimes, mouse models do not have the same pathology as human diseases (310-312). This could indicate that 1) the model used is highly sensitive to the mutation resulting in the high amount of death, 2) the background of the mouse strain may be more sensitive to the variant, and perhaps a different mouse background may yield lower mortality rate and 3) the negative results could be explained by the fact that these mice did not experience spontaneous seizures this could be partly explained by the fact that C57BL/6J is more resistant to seizures (313). Another limitation is the small sample size with only 2-3 mice per group, due to time constraints and the availability of good brain tissues. Given the negative data, the mean and SD for each experiment, it seemed that additional mice would not provide more information to the study.

Conclusion

Overall, no significant differences in gross morphology, neuroinflammation or neuronal death were observed between WT and *Gabrb3* E77K mice. This suggest that the *Gabrb3* E77K variant did not induce noticeable anatomical or inflammatory changes. It may, however, be worthwhile to model the GoF mice using a different mouse strain to identify any potential morphological differences that might not be apparent in the current strain used.

In addition, investigation of pups that died prematurely of their weight, their brain morphology, could provide more information about the potential impact of *Gabrb3* E77K variant, as premature death may indicate underlying issues that are not apparent in the surviving mice and there could reveal important aspects of the variant's effect.

Furthermore, investigating slice electrophysiology of neuronal firing could provide a better understanding of the pathomechanism. Electrophysiological studies can reveal functional changes in neuronal activity and network connectivity, and therefore allow for insights of how the *Gabrb3* E77K variant may alter brain function. Further investigations can be made on how this may affect the behavioural characteristics of the mutant mice.

Chapter 8 Conclusion and forward

The seizures that define epilepsy syndromes are conventionally described as being a consequence of excessive neuronal excitation, caused by either insufficient neuronal inhibition or hyperactive excitation at the neuronal excitation. However, this model of seizure initiation and epilepsy development is insufficient to explain the wealth of different types of ion channel variants, where both LoF or GoF variants can be associated with genetic epilepsy (73, 75, 82, 94, 100, 149, 231, 292). The concept of GABA_A receptor variants associated with epilepsy being “gain-of-function” is a novel and, to some extent a provocative concept within the field of GABA and epilepsy research. Here, I hope the data and body of research can convince you that there is undeniably a difference in the clinical phenotypes of patients with GoF and LoF variants, with the GoF cohort generally associated with more severe neurodevelopmental clinical phenotypes, greater risk of severe comorbidities including severe ID, little to no language development, visual impairment and neurological abnormalities including dystonia and nystagmus.

Scope of the study

This doctoral work investigated 95 GABA_A receptor variants to elucidate their functional consequences and correlated their function to patient phenotypes. Detailed assessment of the desensitisation properties of 20 GoF and 4 LoF variants with a custom-designed recording apparatus were performed to test the hypothesis that increased desensitisation, rather than increased receptor activity, was associated with developmental epilepsies. Additionally, comparative assessments were conducted between WT and GoF mutant mice to explore gross morphological alterations of the brain.

Findings in brief

1. Direct comparison of patient phenotypes between null and missense *GABRG2* LoF variants revealed that both cohorts had a similar median age of seizure onset at 16 months. However, the missense LoF cohort was associated with more severe epilepsy syndromes and a greater burden of DD and ID than the null cohort (refer to Chapter 3 for details).
2. Different amino acid changes at the same position or loci as a population variant (found in gnomAD), neither *in silico* tools nor the presence of the variant in gnomAD can definitively establish its pathogenicity (refer to Chapter 4 for details).

3. Functional assessment of 71 *GABRG2* variants, segregated into GoF (~21%) and LoF (~44%) groups, revealed distinct clinical phenotypes. The GoF cohort was associated with a younger median age of seizure onset (3.75 m vs 12 m for the LoF cohort), severe intellectual disability post-seizure, and abnormal neurological development. The LoF cohort was associated with a greater risk of neuropsychiatric comorbidities (refer to Chapter 5 for details).
4. GoF *GABRB3* variants that exhibit reduced steady-state desensitisation (net increase in inhibitory activity) correlated with more severe clinical outcomes than GoF variants with accelerated current decay kinetics, and so faster desensitisation (refer to Chapter 6 for details).
5. *GABRB3*^{+/E77K} knock-in mice showed no significant difference in gross brain morphology (refer to Chapter 7 for details).

Implications to the field of GABA_A receptors and Epilepsy

Contributions to existing literature

The doctoral work contributes to the expanding body of literature that highlight the impact of GABA_A receptor variants on the clinical phenotype of patients with epilepsy. Notably, variants in *GABRA1* (94), *GABRA2* (79), *GABRA4* (73, 82), *GABRA5* (79), *GABRB3* (75, 149), *GABRD* (177) can result in both a reduction (LoF) or enhancement (GoF) of chloride current passing through the receptor. Collectively, the data consistently show a pattern where clinical outcomes are closely tied to the functional changes in these receptor variants, specifically, GoF variants are associated with more severe clinical phenotypes and comorbidities. This pattern suggests that the dichotomy of GoF/LoF is not restricted to one subunit but the broader phenomena of GABA_A receptor and its ability to contribute to the pathomechanism of epilepsy.

Reevaluating existing hypotheses

At the beginning of this thesis, the prevailing hypothesis for epilepsy-associated GABA_A receptor variants was that the functional consequence of these variants would result in less inhibition through one or more mechanisms, including impaired trafficking, retention of receptors in the endoplasmic reticulum, reduced cell surface expression of the protein or reduced chloride activity in response to GABA binding. Although there is strong evidence that all these mechanisms could, under the right circumstances, lead to seizures, it does not explain the broad spectrum of phenotypes that are seen in patients harbouring pathogenic variants in the different *GABR* genes. The strong association between specific phenotypes

and GoF variants that are not seen with LoF variants provides clear evidence that increased chloride activity of GABA_A receptors are pathogenic, initiating distinct pathomechanisms that ultimately lead to a discrete DEE.

A second, but equally important fallacy that is commonly reported as a cause of DEE is receptor cell surface expression. In this study, few variants were found to have reduced cell surface expression. The relatively mild phenotypes of null *GABRG2* variants, a subunit critically involved in trafficking receptors to the cell surface, compared to LoF missense variants indicate that this process is of secondary importance to the dynamic regulation of chloride activity in response to synaptic release.

Perspective: GoF - a provocative finding

Retrospectively, the “GoF” GABA_A receptor variant is a provocative explanation because, for the first time, the fundamental understanding of epilepsy in the fields of science and medicine has been challenged. The phenomena of GoF/LoF are not novel; they have been reported in other ion channels such as *SCN1A* and *KCN2A* but also in MeCP2, a protein that regulate gene activity, in which Rett and MeCP2 duplication syndromes are analogous to LoF/GoF. Therefore, there is no reason that GABA_A receptors cannot do the same. Indeed, earlier mutational studies in the 1990’s reported that mutational analysis of GABA_A receptor channels and other Cys-loop receptors can cause both GoF and LoF receptors (314-318).

Furthermore, the clinical phenotypes of GoF variants of GABA_A receptors parallel with that seen in patients with GABA transaminase deficiency, a rare condition characterised by a deficiency in GABA transaminase, an enzyme responsible for breaking down GABA. This deficiency leads to an accumulation of GABA in the synapse, leading to an overall increase in net inhibition, which mirrors the effects of GoF variants. Patients with GABA transaminase deficiency exhibit severe clinical phenotypes, including recurrent seizures, early-onset encephalopathy, choreoathetosis, uncontrolled limb movement, DD, microcephaly, exaggerated reflexes, hypotonia and excessive sleepiness (319-323). The life expectancy for individuals with this condition is typically less than two years (320, 321).

Moreover, missense variants in *SLC6A1*, the gene encoding for a GABA transporter protein type 1, a membrane protein responsible for GABA reuptake from the synapse cleft associated

with neurodevelopment disorders (324, 325). These disorders include ASD, ADHD, ID ranging from mild to severe, language impairment and severe seizure types, which include absence, myoclonic and atonic (324-328). The phenotype observed in patients harbouring *SLC6A1* LoF missense variant mirrors GoF GABA_A receptor variants, specifically GoF *GABRD*. A common pathological consequence of LoF *SLC6A1* and GoF *GABRD* is that both theoretically increase inhibitory signalling through extrasynaptic receptor overactivity (177, 329).

Similarly, patients with GoF variants have a more severe clinical phenotype and an earlier disease onset than patients with LoF variants. The similarities between these two conditions highlight the significant impact of increased GABAergic inhibition, whether phasic or tonic, on the nervous system and underline the need for a deeper understanding of how GoF variants contribute to epilepsy. Importantly, this highlights the convergence of phenotype via different pathways.

Convergence of phenotype

This thesis, in line with numerous other studies, has identified missense variants in GABA_A receptor can lead to either LoF or GoF receptor, with GoF variants often resulting in more severe clinical manifestations. This finding contributes to a growing body of evidence that epilepsy-associated variants in other genes, such as *SCN1A* (123, 145, 330), *SCN2A* (330, 331), *SCN8A* (330, 332-334), *KCNT2* (335, 336), and *GRIA3*, can similarly exhibit either GoF, LoF, or sometimes a combination of both effects.

Stratification of these variants reveals distinct phenotypic differences between GoF and LoF variants. For instance, LoF variants in *SCN1A* are typically associated with DS, characterised by severe epilepsy and DD, whereas GoF variants in the same gene are linked to more severe conditions such as neurodevelopmental and epileptic encephalopathy with movement disorder and arthrogryposis (NDEEMA) (145). Similarly, in the *GRIA3* gene, GoF variants are associated with a younger age of seizure onset (median age at 1 month), hypertonia, and movement disorders, whilst LoF variants tend to present a later onset, around 16 months, with a relatively less severe clinical course (337).

It is crucial to highlight that the epileptic phenotypes and comorbidities observed in patients with these genetic mutations overlap with those seen in dysfunctions of other ion channels

and transporters, such as *SCN1A*, GABA transaminase deficiency, and *SLC6A1*. For example, our study found that in our *GABRG2* cohort (Chapter 5), DS was exclusively associated with LoF variants. Up to 90% of DS is due to a LoF *SCN1A* variant, which results in a loss of inhibitory interneurons (120). This overlap of phenotype suggests possible shared mechanistic pathways underlying these conditions.

Potential hypotheses of how GoF lead to epilepsy

To date, it is still unknown how GoF GABAergic transmission leads to epilepsy, but superficially speaking, there are two potential possibilities: 1. Gain-of-function transmission before the GABA shift (where the Cl^- concentration in neurons shifts from high to low concentrations) will increase excitation because GABA is an excitatory transmitter at that time (48). 2. In the cortex microcircuits, there are GABAergic neurons signaling to other GABAergic neurons, which in turn regulate downstream pyramidal neurons. Inhibition of inhibition causes overall excitation, and therefore increased signaling between GABAergic neurons could lead to overexcitation, epilepsy and its comorbidities. Given the differences in patient phenotypes, it is highly plausible that the individual phenotypes may arise from disruptions of different circuits and most likely have different mechanisms.

Given the brain's incredible complexity and multitude of neural circuits and their intricate connections, impaired wiring of these circuits may contribute to the clinical phenotype observed. This suggests that there are many potential pathways through which a variant may cause, for example, motor dysfunction, dystonia, or other symptoms. For instance, ID may be due to impaired development of neurons when GABA_A receptors are excitatory. While we have one aspect of the variant's effect on the receptor, many other mechanisms remain unexplored. Perhaps, the way forward is using mouse models to study these mechanisms could be beneficial. However, as discussed in Chapter 7, it can be difficult to work with due to differences between human and mice.

Strengths

The strength of this doctoral work lies in the breadth and depth of variants tested, looking at not only the variant's effect on receptor sensitivity, and maximum current but also deep-dived into receptor desensitisation at different functional regions. I have investigated over 90 (this

includes *GABRB3* and *GABRG2*) GABA_A receptor variants elucidating intricacies of functional effect of variants effect on the clinical phenotype of the patient.

A primary challenge in studying Cys-loop receptors is the difficulty in receptor stoichiometry and assembly. This control is crucial because different receptor assemblies exhibit varying kinetic properties; for instance, $\alpha\beta$ displays a higher GABA potency and desensitises faster than $\alpha\beta\gamma$ (88, 338). By Mendelian distribution, the variant in question will be heterozygous in 50% of the cases (with one allele affected), WT in 25% of cases, and both alleles in the remaining 25% of cases. To address this challenge, we employed a receptor concatenation technique, which involves linking individual subunits together with only one variant subunit affected (156). This method allows for precise control over the uniformity of expressed receptors. In addition, WT were run in parallel with variants on each experimental day, and statistical analysis was performed relative to the WT on the same day. As detailed in Chapter 2, there are inter-batch and inter-day variabilities, even among WT receptors. However, it is reassuring to observe the mean ΔLogEC_{50} for the total batch to a single day is similar and within the range with standard deviation from extensive experiments.

Furthermore, we applied a rigorous statistical significance threshold, with $p < 0.0001$, for both maximum current and GABA sensitivity to reduce the possibility of false positives. Together, with close collaborations with clinicians and harmonisation of patient data, we were able to categorise patient variants into GoF and LoF groups. This categorisation allowed us to clearly distinguish between patient phenotype and the variant's functional outcome.

Limitations

The current study faces several limitations that need to be acknowledged. First, the use of *Xenopus* oocyte as an expression system is one of the gold-standard expression systems (72, 74, 234, 339). To assess the functional consequence of variants, we assessed for changes in GABA sensitivity and maximum current as a proxy for receptor efficacy and efficiency. As explained in Chapter 2 and 4, GABA sensitivity is a robust measure of clinical significance, however, given in this study, 26 variants were categorised as VUS, this suggests our assay and possibly together with the stringent threshold set for significance, may reduce the sensitivity of the assay and our ability to pick up on subtle changes. It is, however, important to consider the possibility of these variants being simply a risk to developing epilepsy, to differentiate further, an in-depth investigation of family history is required.

Second, an incomplete patient phenotype table for the *GABRG2* cohort has limited my ability to fully investigate genotype-phenotype correlations. Factors such as ASMs used, outcome, side effect profile, gender differences in treatment outcome and incidence of severity of epilepsy could be avenues to explore. Contacting the primary clinicians again to harmonise and collect complete data would be beneficial. However, despite these limitations, we can segregate the functional effects of *GABRG2* variants and see an undeniable trend that GoF is worse than LoF. Overall, this study has advanced knowledge in understanding the dynamics of receptor kinetics on patient clinical phenotype.

Concluding remarks

Diagnosis and clinical practice

In rare forms of epilepsy, patients and families are often faced with “diagnostic odyssey”, where the journey to diagnosis is long and stressful (Chapter 5). This extended period of uncertainty causes significant stress and psychological strain on patients and their families. Together, this thesis shows that there is a need to reflect on the current clinical practice and follow the patient's phenotype as a guide to the effect of the variant, bridging the gap between research and practice.

This thesis has elegantly shown that there are distinct clinical phenotypical differences between patients harbouring GoF and LoF GABA_A receptor variants and there are functional regions that are more prone to pathogenic variants. Variants can impact the receptor in many ways, and while the shift in EC₅₀ of GABA_A receptor is an important surrogate, receptor properties such as desensitisation can further segregate the type of epilepsy, comorbidities and prognosis of patients, highlighting the intricacies of variants' effect (Chapter 6). The functional knowledge of the variant is instrumental in the treatment of patients. For instance, as mentioned in Chapter 1, giving a GABA transaminase inhibitor may not be the therapeutic of choice due to the risk of exacerbation of side effects or worsening of seizures.

Although *in vitro* studies are the gold standard for elucidating the variant's effect, they are costly and time-consuming. With the increasing interest in artificial intelligence many researchers have developed and proposed the idea of using *in silico* prediction tools, but its accuracy for differentiating subtle amino acid changes at the same position in *GABRG2* is poor, with the exception of AlphaMissense. Current *in silico* tools try to predict whether a variant is pathogenic or not, however to advance the current tools, a machine-learning approach should be taken, looking at all the GABA_A receptor variants in the literature, their functional impact and using the information on paralogous genes, location of the variant, and desensitisation to provide a guide on whether a variant is predicted to be benign, a gain- or

loss-of-function. In doing so, this would provide clinician with a guide on how to best treat the patient.

The beauty of clinical-laboratory translational science is that together there is a synergistic effect and impact for the patient. From the number of variants tested and the diversity of GABA_A receptor subunits examined, we and others observe clear distinctions in clinical phenotypes with GoF variants having a younger age of seizure onset, along with movement disorders. This should compel clinicians to reflect and consider the patient as a whole, their family history, clinical phenotype, and neurological comorbidities for treatment and, when in doubt, let the patient's phenotype guide the effect of the variant and the treatment of choice.

Future directions

Epilepsy ranges from simple FS that is self-limiting to severe, devastating and, at times, debilitating DEEs. Approximately 40% of patients are refractive to treatment or experience severe side effects with current treatments; however, this number may be an underestimation due to reporting bias. This means the needs of at least 40% of patients with epilepsy are unmet. This figure does not account for the caregivers, and impact to society as a whole. Due to the complexity of DEE, a diagnosis of DEE not only affects the patient but also has a profound impact on the QoL of caregivers and the family structure (218, 340-343). The point is, a lot of people, immediate to the patient, and the society as a whole are affected.

With increasing evidence of GoF GABA_A receptor variants, this opens avenues for drug discovery, drug repurposing and novel biologics to address the unmet needs of the patient group. However, more research will be required to investigate the pathomechanism of GoF variants. Given each patient is different, and the effect of the variants having varying extent of effects to the receptor, such as shift in EC₅₀ or receptor desensitisation, the current one-size-fits-all therapeutics may need to be replaced with precision medicine. From “low-hanging fruits”, such as the development of negative allosteric modulators to assist in “dialling” down the GABA sensitivity to targeted anti-sense oligonucleotides could be the future of treating these patients. This doctoral work represents a significant step forward in the quest for more effective diagnosis, therapies and better outcomes for individuals living with epilepsy.

Chapter 9 Supplementary Information

Table 1: Default ACMG classification

Variant position	Variant	Default ACMG
74	p.(Leu74Val)	Likely Pathogenic
76	p.(Gly76Arg)	VUS
79	p.(Asn79Ser)	VUS
82	p.(Arg82Gln)	Likely Pathogenic
82	p.(Arg82Trp)	VUS
83	p.(Pro83Thr)	VUS
83	p.(Pro83Ser)	VUS
84	p.(Asp84Val)	VUS
85	p.(Ile85Lys)	VUS
90	p.(Thr90Arg)	VUS
90	p.(Thr90Met)	VUS
94	p.(Thr94Ser)	VUS
98	p.(Val98Glu)	VUS
104	p.(Val104Gly)	VUS
106	p.(Ala106Pro)	VUS
106	p.(Ala106Thr)	VUS
107	p.(Ile107Thr)	VUS
121	p.(Trp121Cys)	VUS
125	p.(Arg125Cys)	VUS
139	p.(Ser139Asn)	VUS
140	p.(Asn140Ser)	VUS
144	p.(Lys144Asn)	VUS
167	p.(Asn167Lys)	VUS
169	p.(Met169Thr)	VUS
177	p.(Arg177Gly)	VUS
177	p.(Arg177Gln)	VUS
199	p.(Met199Val)	VUS
205	p.(Pro205His)	VUS
210	p.(Ser210Arg)	VUS
215	p.(Arg215His)	VUS
215	p.(Arg215Cys)	VUS
218	p.(Ile218Ser)	VUS
257	p.(Gly257Arg)	Likely Pathogenic
274	p.(Tyr274Cys)	VUS
277	p.(Ile277Asn)	VUS
282	p.(Pro282Ala)	Likely Pathogenic

282	p.(Pro282Ser)	Likely Pathogenic
282	p.(Pro282Thr)	Likely Pathogenic
285	p.(Leu285Ile)	VUS
302	p.(Pro302Leu)	VUS
303	p.(Ala303Thr)	VUS
306	p.(Ser306Phe)	VUS
307	p.(Leu307Val)	VUS
310	p.(Thr310Ile)	VUS
316	p.(Thr316Asn)	VUS
317	p.(Thr317Ile)	VUS
322	p.(Ala322Thr)	VUS
322	p.(Ala322Asp)	VUS
323	p.(Arg323Gln)	Likely Pathogenic
323	p.(Arg323Trp)	VUS
325	p.(Ser325Leu)	VUS
326	p.(Leu326His)	VUS
327	p.(Pro327Leu)	VUS
328	p.(Lys328Met)	Likely Pathogenic
329	p.(Val329Phe)	VUS
331	p.(Tyr331Cys)	VUS
334	p.(Ala334Thr)	VUS
340	p.(Ser340Tyr)	VUS
343	p.(Phe343Leu)	Likely Pathogenic
344	p.(Ile344Leu)	VUS
389	p.(Ile389Val)	VUS
397	p.(Ile397Val)	VUS
408	p.(Arg408Lys)	VUS
413	p.(Gly413Ser)	VUS
454	p.(Arg454Trp)	VUS
454	p.(Arg454Gln)	VUS
457	p.(Phe457Leu)	VUS
457	p.(Phe457Ser)	VUS
465	p.(Asn465Ser)	VUS
467	p.(Val467Phe)	VUS
468	p.(Tyr468Cys)	VUS

Table 2: Variant functional result and call

VARIANT	SEQUENCE ALIGNMENT	$\Delta\text{LOGEC}_{50} \pm \text{SD}$	N	I_{MAX}	MEDIAN (IQR)	N	P-VALUE	CALL
EXTRACELLULAR								
L74V	highly conserved	-0.30 $\pm$ 0.06	15	0.82	(0.44 to 1.08)	30	0.0845	LoF
G76R	conserved (not g's)	-0.07 $\pm$ 0.18	15	0.78	(0.56 to 1.21)	28	0.3323	NC
N79S	g's: N, K	0.05 $\pm$ 0.17	13	0.80	(0.53 to 1.32)	37	0.6906	NC
R82Q	fully conserved	-0.15 $\pm$ 0.14	15	0.23	(0.14 to 0.39)	43	<0.0001	NC/LoF
R82W	fully conserved	-0.11 $\pm$ 0.09	22	0.31	(0.20 to 0.49)	44	<0.0001	NC/LoF
P83S	fully conserved	-0.14 $\pm$ 0.11	23	0.28	(0.17 to 0.50)	42	<0.0001	NC/LoF
P83T	fully conserved	-0.33 $\pm$ 0.16	15	0.11	(0.08 to 0.15)	37	<0.0001	LoF/LoF
D84V	g's conserved	-0.26 $\pm$ 0.19	25	0.24	(0.06 to 0.55)	46	<0.0001	LoF/LoF
I85K	g's conserved	0.04 $\pm$ 0.10	13	0.56	(0.42 to 0.83)	39	0.0002	NC
T90M	g's conserved	-0.11 $\pm$ 0.15	27	0.85	(0.53 to 1.24)	65	0.2619	NC
T90R	g's conserved	-0.43 $\pm$ 0.07	18	0.12	(0.09 to 0.15)	32	<0.0001	LoF/LoF
T94S	g's: T, V	-0.02 $\pm$ 0.10	11	0.82	(0.51 to 1.26)	39	0.8673	NC
V98E	highly conserved	0.39 $\pm$ 0.09	16	0.14	(0.07 to 0.20)	32	<0.0001	GoF/LoF
V104G	g's: conserved	0.14 $\pm$ 0.09	18	0.75	(0.53 to 1.22)	35	0.1997	NC
A106P	g's: P, A, S	0.09 $\pm$ 0.08	14	1.25	(0.74 to 1.75)	39	0.0139	NC
A106T	g's: P, A, S	0.73 $\pm$ 0.15	18	0.75	(0.55 to 1.87)	37	0.0391	GoF
I107T	g's: conserved	0.66 $\pm$ 0.11	16	1.17	(0.73 to 1.62)	36	0.113	GoF
W121C	fully conserved		45			45		x/LoF
R125C	fully conserved	-0.46 $\pm$ 0.12	15	0.07	(0.05 to 0.13)	40	<0.0001	LoF/LoF
S139N	g's: conserved	0.33 $\pm$ 0.18	29	0.99	(0.58 to 1.23)	48	0.8438	GoF
N140S	g's: conserved	-0.15 $\pm$ 0.12	14	0.62	(0.37 to 1.14)	33	0.0266	NC
K144N	g's: K, L	-0.07 $\pm$ 0.15	14	0.88	(0.46 to 1.25)	37	0.3582	NC
N167K	fully conserved	-0.26 $\pm$ 0.16	15	0.76	(0.55 to 1.04)	36	0.064	LoF
M169T	g's: L, M	-0.33 $\pm$ 0.10	12	0.23	(0.13 to 0.33)	33	<0.0001	LoF/LoF
R177G	fully conserved	-0.07 $\pm$ 0.09	16	0.35	(0.20 to 0.74)	38	<0.0001	NC/LoF
R177Q	fully conserved	0.02 $\pm$ 0.12	14	0.95	(0.57 to 1.39)	41	0.9121	NC
M199V	gnomAD variant	0.10 $\pm$ 0.18	23	0.74	(0.54 to 1.13)	52	0.0561	NC
P205H	highly conserved	0.05 $\pm$ 0.19	24	0.65	(0.49 to 1.03)	45	0.0668	NC
S210R	g's: conserved	-0.03 $\pm$ 0.21	12	0.01	(0.00 to 0.02)	28	<0.0001	NC/LoF
R215C	fully conserved	-0.03 $\pm$ 0.11	16	0.70	(0.54 to 0.88)	45	0.0019	NC
R215H	not conserved	0.00 $\pm$ 0.11	18	0.85	(0.64 to 1.08)	45	0.2424	NC
I218S	not conserved	-0.03 $\pm$ 0.16	17	0.48	(0.29 to 0.85)	37	0.0001	NC
G257R	g's: I, M	0.08 $\pm$ 0.12	17	0.53	(0.29 to 0.93)	37	0.0014	NC
TM1								
Y274C	fully conserved	-0.44 $\pm$ 0.13	14	0.77	(0.63 to 1.04)	30	0.3098	LoF
I277N	g's: conserved	0.39 $\pm$ 0.23	12	0.43	(0.29 to 0.71)	37	<0.0001	GoF
P282A	fully conserved	0.88 $\pm$ 0.17	14	0.67	(0.40 to 0.91)	36	0.0005	GoF
P282S	fully conserved	0.81 $\pm$ 0.14	21	0.78	(0.56 to 0.98)	48	0.1636	GoF
P282T	fully conserved	0.81 $\pm$ 0.19	22	0.68	(0.40 to 1.00)	46	0.0026	GoF

L285I	g's: conserved	0.47	± 0.11	15	0.91 (0.56 to 1.13)	47	0.2387	GoF
M1-M2 LOOP								
P302L	highly conserved	-0.77	± 0.12	14	0.01 (0.00 to 0.02)	30	<0.0001	LoF/LoF
A303T	fully conserved	-0.39	± 0.12	12	0.42 (0.21 to 0.72)	30	0.0002	LoF
TM2								
S306F	g's: S, A	0.63	± 0.12	13	0.78 (0.47 to 1.22)	25	0.2083	GoF
L307V	g's: conserved	0.31	± 0.16	25	0.83 (0.55 to 1.11)	53	0.1252	GoF
T310I	highly conserved	-0.58	± 0.12	14	0.57 (0.27 to 0.96)	29	0.0113	LoF
T316N	highly conserved	0.73	± 0.14	15	1.11 (0.62 to 1.54)	28	0.4689	GoF
T317I	fully conserved	-0.22	± 0.11	14	0.43 (0.30 to 0.61)	29	0.0011	LoF
A322D	g's: conserved	-0.42	± 0.07	12	0.37 (0.29 to 0.64)	30	<0.0001	LoF
A322T	g's: conserved	-0.68	± 0.07	13	0.45 (0.21 to 0.86)	34	0.0004	LoF
R323Q	highly conserved	-0.46	± 0.13	15	0.63 (0.44 to 0.96)	30	0.0072	LoF
R323W	highly conserved	-0.70	± 0.09	14	0.47 (0.26 to 0.67)	27	<0.0001	LoF
M2-M3 LOOP								
S325L	g's: conserved	-0.48	± 0.13	23	0.79 (0.47 to 1.12)	50	0.174	LoF
L326H	g's: conserved	-0.42	± 0.12	12	0.21 (0.11 to 0.37)	32	<0.0001	LoF/LoF
P327L	fully conserved	-0.46	± 0.10	15	0.23 (0.14 to 0.30)	40	<0.0001	LoF/LoF
K328M	g's: K, R	-0.34	± 0.16	11	0.69 (0.40 to 0.92)	22	0.2066	LoF
V329F	g's: conserved	-0.16	± 0.08	17	0.66 (0.45 to 0.98)	29	0.003	NC
Y331C	highly conserved	-0.06	± 0.23	13	0.81 (0.47 to 1.04)	33	0.0323	NC
A334T	fully conserved	0.51	± 0.15	15	1.10 (0.58 to 1.48)	27	0.634	GoF
TM3								
S340Y	g's: S, T	0.33	± 0.18	10	0.85 (0.60 to 1.28)	35	0.7878	GoF
F343L	highly conserved	0.47	± 0.15	12	0.72 (0.36 to 1.02)	26	0.0169	GoF
I344L	g's: I, L	-0.02	± 0.12	17	1.00 (0.94 to 1.34)	34	0.4177	NC
M3-M4								
I397V	not conserved	0.02	± 0.18	22	0.94 (0.80 to 1.26)	36	0.7582	NC
R408K	not conserved	0.09	± 0.12	14	1.02 (0.57 to 1.29)	44	0.969	NC
G413S	g's: conserved	-0.17	± 0.14	23	0.68 (0.37 to 1.13)	55	0.004	NC
TM4								
R454Q	highly conserved	-0.04	± 0.16	13	0.74 (0.52 to 0.93)	42	0.0528	NC
R454W	highly conserved	-0.22	± 0.12	23	0.30 (0.18 to 0.53)	57	<0.0001	LoF/LoF
F457L	fully conserved	0.00	± 0.16	18	0.97 (0.60 to 1.18)	47	0.696	NC
F457S	fully conserved	-0.07	± 0.11	16	0.27 (0.19 to 0.44)	37	<0.0001	NC/LoF
F464S	highly conserved	0.14	± 0.15	17	0.54 (0.34 to 0.72)	43	<0.0001	NC
N465S	fully conserved	-0.25	± 0.12	15	0.50 (0.14 to 0.84)	36	0.0005	LoF
V467F	highly conserved	0.05	± 0.16	17	1.13 (0.63 to 1.42)	31	0.4429	NC
Y468C	fully conserved	0.56	± 0.09	13	0.50 (0.28 to 0.80)	39	<0.0001	GoF

Abbreviations: NC, no change

Table 3: ACMG classification and criteria post functional assay for each individual

ID	Variant	Inheritance	Age of onset (m)	Functional outcome	ACMG	ACMG criteria	Ref
1	L74V	Unknown		LoF	Pathogenic	PS3 PS4 PM1 PM2 PP3 PP5 BP1	(208)
2	G76R			NC	Likely Benign	PM1 PM2 PP3 BP1	
3	N79S	Unknown	180	NC	Likely Benign	PM1 PM2 BS3 BP1	(220)
4	R82Q	Unknown		NC/LoF	Pathogenic	PS3 PS4 PM1 PM2 PP3 PP5 BP1	(208)
5	R82Q	Maternal		NC/LoF	Pathogenic	PS3 PS4 PM1 PM2 PP3 PP5 BP1	(166); (113)
6	R82Q	Unknown		NC/LoF	Pathogenic	PS3 PS4 PM1 PM2 PP3 PP5 BP1	(209)
7	R82W	Father	30	NC/LoF	Pathogenic	PS3 PM1 PM2 PM5 PP3 BP1	
8	P83T	Paternal	59	LoF/LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	
9	P83T	Maternal	40	LoF/LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	
10	P83S	Maternal		NC/LoF	Pathogenic	PS3 PS4 PM1 PM2 PP3 BP1	(210)
11	P83S	Paternal		NC/LoF	Pathogenic	PS3 PS4 PM1 PM2 PP3 BP1	(211)
12	D84V			LoF/LoF	Likely Benign	PS3 PM1 PM2 PP3 BP1	
13	I85K	Paternal	6	NC	Likely Benign	PM1 PM2 PP3 BP1	
14	T90R	<i>De Novo</i>	7	LoF/LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(212)
15	T90M	<i>De Novo</i>	1	NC	VUS	PS2 PM1 PP3 BS3 BP1	
16	T90M	Maternal	19	NC	Likely Benign	PM1 PP3 BS3 BP1	
17	T94S	-	-	NC	Likely Benign	PM1 PM2 PP3 BS3 BP1	
18	V98E	-	-	GoF/LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	
19	V104G	<i>De Novo</i>	18	NC	VUS	PS2 PM1 PM2 PP3 BS3 BP1	
20	A106P			NC	Likely Benign	PM1 PM5 BS3 BP1	
21	A106T	<i>De Novo</i>	3.5	GoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP5 BP1	
22	A106T	Unknown	NA	GoF	Pathogenic	PS3 PS4 PM1 PM2 PP5 BP1	(208)
23	A106T	<i>De Novo</i>	0.033	GoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP5 BP1	(171)
24	A106T	<i>De Novo</i>	3	GoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP5 BP1	(171)
25	A106T	<i>De Novo</i>	24	GoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP5 BP1	(169)
26	A106T	Unknown	1.381	GoF	Pathogenic	PS3 PS4 PM1 PM2 PP5 BP1	(169)
27	A106T	<i>De Novo</i>	0.033	GoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP5 BP1	(169)
28	A106T	Unknown	0.066	GoF	Pathogenic	PS3 PS4 PM1 PM2 PP5 BP1	(169)

29	A106T	<i>De Novo</i>	4	GoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP5 BP1	(213)
30	A106T	<i>De Novo</i>	2	GoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP5 BP1	
31	A106T	Unknown	0.066	GoF	Pathogenic	PS3 PS4 PM1 PM2 PP5 BP1	
32	A106T	<i>De Novo</i>	1.315	GoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP5 BP1	(93)
33	A106T	<i>De Novo</i>	NA	GoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP5 BP1	(93)
34	I107T	<i>De Novo</i>	1.5	GoF	Pathogenic	PS3 PS4 PM1 PM2 PP5 BP1	(171)
35	W121C	<i>De Novo</i>	NA	x/LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(93)
36	R125C	Paternal	17	LoF/LoF	Likely Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	
37	S139N	<i>De Novo</i>	NA	GoF	Pathogenic	PS2 PS3 PM1 PM2 BP1	
38	N140S	Unknown	NA	NC	Likely Benign	PM1 PM2 BS3 BP1	(208)
39	K144N			NC	Likely Benign	PM1 PM2 BS3 BP1 BP4	
40	N167K	<i>De Novo</i>	24	LoF	Pathogenic	PS2 PS3 PM1 PM2 PP5 BP1	(93)
41	M169T	<i>De Novo</i>	24	LoF/LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	
42	M169T	Familial	24	LoF/LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	
43	R177G	Paternal		NC/LoF	Likely Pathogenic	PS3 PM1 PM2 PP5 BP1	(214)
44	R177Q	-	-	NC	Likely Benign	PM1 BS3 BP1	
45	M199V	Unknown	6	NC	Likely Benign	PM1 PP3 BS3 BP1	(172)
46	M199V	Paternal	18	NC	Likely Benign	PM1 PP3 BS3 BP1	
47	P205H			NC	Likely Benign	PM1 PM2 PP3 BS3 BP1	
48	S210R			NC/LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	
49	R215C			NC	Likely Benign	PM1 BS3 BP1	
50	R215H	Paternal	12	NC	Likely Benign	PM1 BS3 BP1	(80)
51	I218S	Maternal	11	NC	Likely Benign	PM1 PP3 BS3 BP1	(221)
52	G257R	Maternal		NC	Likely Benign	PM1 PP3 BS3 BP1	(222)
53	G257R	Unknown		NC	Likely Benign	PM1 PP3 BS3 BP1	(223)
54	Y274C	Unknown	10	LoF	Likely Benign	PS3 PM1 PM2 PP3 BP1	27864 847
55	I277N			GoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	
56	P282A	<i>De Novo</i>	0	GoF	Pathogenic	PS2 PS3 PM1 PM2 PM5 PP3 BP1	(40)
57	P282S	<i>De Novo</i>	12	GoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 PP5 BP1	(171)
58	P282S	Unknown	NA	GoF	Pathogenic	PS3 PM1 PM2 PM5 PP3 BP1	(223)
59	P282T	Unknown	NA	GoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	(208)
60	P282T	<i>De Novo</i>	48	GoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(211)
61	P282T	<i>De Novo</i>	11	GoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(225)
62	L285I	<i>De novo</i>	2.6	GoF	Likely Pathogenic	PS3 PM1 PM2 BP1	

63	P302L	Unknown		LoF/LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	(208)
64	P302L	<i>De Novo</i>	12	LoF/LoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP3	(91)
65	P302L	<i>De Novo</i>	7	LoF/LoF	Pathogenic	PS2 PS3 PS4 PM1 PM2 PP3	(212)
66	P302L	<i>De Novo</i>	7	LoF/LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3	(93)
67	A303T	<i>De Novo</i>		LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(93)
68	S306F	<i>De Novo</i>	NA	GoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	(211)
69	L307V	Unknown	NA	GoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	(208)
70	T310I	<i>De Novo</i>	8	LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(93)
71	T310I	<i>De Novo</i>	8	LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(93)
72	T316N	<i>De Novo</i>	2	GoF	Likely Pathogenic	PS3 PM1 PM2 PP3	
73	T317I	<i>De Novo</i>	7	LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(93)
74	A322D	Paternal	13	LoF/LoF	Pathogenic	PS3 PS4 PM1 PM2 PP3 BP1	
75	A322D	Paternal	16	LoF/LoF	Pathogenic	PS3 PS4 PM1 PM2 PP3 BP1	
76	A322T	<i>De Novo</i>	10	LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	
77	R323Q	Maternal		LoF	Pathogenic	PS3 PM1 PM2 PP3 PP5 BP1	
78	R323Q	Unknown	8	LoF	Pathogenic	PS3 PM1 PM2 PP3 PP5 BP1	(208)
79	R323Q	<i>De Novo</i>		LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 PP5 BP1	(226)
80	R323Q	<i>De Novo</i>	10	LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 PP5 BP1	(222)
81	R323Q	<i>De Novo</i>	12	LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 PP5 BP1	(171)
82	R323Q	<i>De Novo</i>		LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 PP5 BP1	(171)
83	R323Q	Unknown		LoF	Pathogenic	PS3 PM1 PM2 PP3 PP5 BP1	(223)
84	R323Q	Unknown	7	LoF	Pathogenic	PS3 PM1 PM2 PP3 PP5 BP1	(223); (227)
85	R323Q	<i>De Novo</i>	9	LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 PP5 BP1	
86	R323Q	Maternal	8	LoF	Pathogenic	PS3 PM1 PM2 PP3 PP5 BP1	(40)
87	R323Q	Maternal	10	LoF	Pathogenic	PS3 PM1 PM2 PP3 PP5 BP1	(40)
88	R323Q	<i>De Novo</i>		LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 PP5 BP1	(40)
89	R323W	Unknown		LoF/LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	(208)
90	R323W	<i>De Novo</i>		LoF/LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(171)
91	R323W	Unknown	1	LoF/LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	(228)

92	R323W	Unknown	8	LoF/LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	
93	R323W	De Novo	17.5	LoF/LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	
94	R323W	De Novo	15	LoF/LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(40)
95	S325L	Unknown		LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	(223)
96	L326H	De Novo	17	LoF/LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	
97	P327L			LoF/LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	
98	K328M	Familial		LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	(167)
99	Val329F	Unknown		NC	Likely Benign	PM1 PM2 PP3 BS3 BP1	(208)
100	Y331C	De Novo	12	NC	Likely Benign	PM1 PP3 BS3 BP1	(40)
101	A334T	Paternal	18	GoF	Likely Pathogenic	PS3 PM1 PP3 BP1	
102	A334T	Maternal	9	GoF	Likely Pathogenic	PS3 PM1 PP3 BP1	
103	S340Y			GoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	
104	F343L	De Novo	12	GoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	(171)
105	I344L	Maternal	3	NC	Likely Benign	PM1 PM2 BS3 BP1	
106	I397V			NC	Likely benign	PM1 BS3 BP1	
107	I397V	Paternal		NC	Likely Benign	PM1 BS3 BP1	(222)
108	R408K			NC	Likely Benign	PM1 PM2 BS3 BP1	
109	G413S	Maternal	122	NC	Likely Benign	PM1 PM2 BS3 BP1	(229)
110	G413S	Unknown		NC	Likely Benign	PM1 PM2 BS3 BP1	
111	G413S	Maternal		NC	Likely Benign	PM1 PM2 BS3 BP1	(229)
112	R454W	Unknown		LoF/LoF	VUS	PM1 PP3 BP1	(208)
113	R454Q			NC	Likely Benign	PM1 PP3 BS3 BP1	
114	F457L			NC	Likely Benign	PM1 PM2 PP3 BS3 BP1	
115	F457S	De Novo		NC/LoF	Pathogenic	PS2 PS3 PM1 PP3 BP1	
116	N465S	Unknown	15	LoF	Likely Pathogenic	PS3 PM1 PM2 PP3 BP1	
117	N465S	De Novo	6	LoF	Pathogenic	PS2 PS3 PM1 PM2 PP3 BP1	
118	V467F	De Novo		NC	VUS	PS2 PM1 PP3 BS3 BP1	(223)
119	Y468C	Mother	11	GoF	Likely Pathogenic	PS3 PM1 PP3 BP1	(80)
120	Y468	De Novo	18	GoF	Pathogenic	PS2 PS3 PM1 PP3 BP1	(80)

Table 4: Clinical phenotypes *GABRG2* cohort

This table is attached separately as an excel spreadsheet.

Submitted manuscript - Phenotypic spectrum in individuals with pathogenic *GABRG2* loss- and gain-of-function variants

This is supplementary to Chapter 3 of this thesis

Phenotypic spectrum in individuals with pathogenic *GABRG2* loss- and gain-of-function variants

Manuscript Number: WNL-2024-101691

Authors:

ALESSANDRA ROSSI^{1,2,3,4}; Susan XN Lin⁵; Sebastian Ortiz-De la Rosa^{1,6,7}; Nathan Absalom⁸; Vivian WY Liao⁵; Nazanin A Mohammadi^{1,6}; Sindhu Viswanathan⁹; Tommy Stödborg^{10,11}; Alberto Danieli¹²; Paolo Bonanni¹²; Alec Aeby¹³; Alessandro Orsini¹⁴; Alice Bonucelli¹⁵; Andrea Rüegger¹⁶; Beatriz G Giraldez¹⁷; Bertrand Isidor¹⁸; Burkhard Stüve¹⁹; Carla Marini²⁰; Elisabetta Cesaroni²⁰; Christina Dühring Fenger^{1,21}; Christophe Philippe^{22,23,24}; Colombine Meunier²⁵; Damien Lederer²⁵; Stéphanie Moortgat²⁵; Egidio Spinelli²⁶; Elisa Fallica²⁷; Fiona Zeiner²⁸; Matthias Baumann²⁹; Laura Licchetta³⁰; Francesca Bisulli^{30,31}; Francesca Felicia Operto³²; Ira C. Benkel-Herrenbrueck³³; Kathleen M Gorman^{34,35}; Katrine Johannesen^{1,36}; Konrad Platzer³⁷; Franziska Schnabel³⁸; Lieven Lagae³⁹; Mirjam Laufs¹; Riina Zordania⁴⁰; Stephen Andrew Malone^{41,42}; Tullio Messina⁴³; Wendy Werckx⁴⁴; Charlotta Jonsson⁴⁵; Zaid Afawi^{46,47}; Thomas Foiadelli⁴⁸; Yosra Halleb⁴⁹; Gaetan Lesca⁹; Samuel F. Berkovic⁹; Ingrid E. Scheffer^{50,9}; Mary Chebib⁵; Elena Gardella^{1,6}; Rikke Steensbjerre Møller^{1,6}; Guido Rubboli^{1,51}; Philip K Ahring⁵

1. Department of Epilepsy Genetics and Personalized Medicine, Danish Epilepsy Centre, member of the ERN-EpiCARE, Dianalund, Denmark; 2. IRCCS Eugenio Medea Scientific Institute, Department of Neurorehabilitation, Conegliano, Treviso, Italy; 3. Pediatric Clinic, IRCCS San Matteo Hospital Foundation, Pavia, Italy; 4. Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy; 5. Brain and Mind Centre, School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, Sydney, New South Wales, Australia; 6. Department of Regional Health Research, Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark; 7. Instituto Roosevelt, Bogotá, Colombia; 8. School of Science, University of Western Sydney, Sydney, New South Wales, Australia; 9. Department of Medicine, Epilepsy Research Centre, University of Melbourne, Austin Health, Heidelberg, VIC 3084, Australia; 10. Department of Child Neurology, Karolinska University Hospital, Stockholm, Sweden.; 11. Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden; 12. IRCCS E. Medea Scientific Institute, Epilepsy Unit, Conegliano, Treviso, Italy; 13. Hôpital Universitaire des Enfants Reine Fabiola (HUDERF), Université Libre de Bruxelles, Brussels, Belgium; 14. Pediatric Neurology, University Hospital of Pisa, Azienda Ospedaliero Universitaria Pisana, Pisa, Italy; 15. Pediatric Neurology, University Hospital of Pisa, Azienda Ospedaliero Universitaria Pisana, 56126 Pisa, Italy; 16. Department of Neuropediatrics, University Children's Hospital Zurich, Switzerland; 17. Epilepsy Unit, Neurology Service, Hospital Universitario and IIS Fundación Jiménez Díaz and CIBERER, Madrid, Spain; 18. Service de Génétique Médicale, CHU Nantes, Nantes, France; 19. Department of Neuropediatrics, Children's Hospital Siegen, Germany; 20. Child Neurology and Psychiatric Unit, Pediatric Hospital G. Salesi, Azienda Ospedaliero-Universitaria delle Marche, Ancona, Italy; 21. Amplexa Genetics, Odense, Denmark; 22. UFR des Sciences de Santé, GAD "Génétique des Anomalies du Développement", INSERM-Université de Bourgogne UMR1231, Fédération Hospitalo-Universitaire (FHU)-TRANSLAD, Dijon, France.; 23. Unité Fonctionnelle Innovation en Diagnostic Génomique des

Maladies Rares, Fédération Hospital-Universitaire-TRANSLAD, CHU Dijon Bourgogne, Dijon, France; 24. Laboratoire de Génétique, Hôpital Mercy, CHR Metz-Thionville, France; 25. Centre de Génétique Humaine, Institut de Pathologie et de Génétique, Gosselies, Belgium; 26. Schulich School of Medicine and Dentistry, Western University, London, ON, Canada; 27. Neurology Unit, University Hospital of Ferrara, Ferrara, Italy; 28. Department of Pediatrics I, Medical University of Innsbruck, Innsbruck, Austria; 29. 23Department of Pediatrics I, Medical University of Innsbruck, Innsbruck, Austria; 30. IRCCS Istituto delle Scienze Neurologiche di Bologna, Full Member of European Reference Network EpiCARE, Bologna, Italy; 31. Department of Biomedical and Neuromotor Sciences, University of Bologna, Italy; 32. 26Department of Science of Health School of Medicine, University of magna Graecia Catanzaro, Italy; 33. Sana-Krankenhaus Düsseldorf-Gerresheim, Academic Teaching Hospital der Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany; 34. Department of Neurology and Clinical Neurophysiology, Children's Health Ireland at Temple Street, Dublin, Ireland; 35. School of Medicine and Medical Science, University College Dublin, Dublin, Ireland; 36. Department of Genetics, University Hospital of Copenhagen, Rigshospitalet, Copenhagen, Denmark; 37. Institute of Human Genetics, University of Leipzig Medical Center, Leipzig, Germany; 38. Department of Neuropediatrics, University Hospital Schleswig-Holstein, Christian-Albrechts-University, Kiel, Germany; 39. Department of Development and Regeneration, Section Paediatric Neurology, University Hospital Leuven, Leuven, Belgium; 40. Department of Clinical Genetics, Genetic and Personalized Medicine Clinic, Tartu University Hospital, Tartu 50406, Estonia; 41. Department of Neurosciences, Queensland Children's Hospital, South Brisbane, Queensland, Australia.; 42. Centre for Advanced Imaging, University of Queensland, St Lucia, Australia; 43. IRCCS, Istituto delle Scienze Neurologiche di Bologna, UOC Neuropsichiatrica dell'età Pediatrica, Bologna, Italy; 44. Jessa Hospital, Hasselt, Belgium; 45. Department of Pediatrics, Vrinnevi Hospital, Norrköping, Sweden; 46. Ben-Gurion University of the Negev, Beer-Sheva, Israel; 47. Erasmus MC, Rotterdam, The Netherlands; 48. Pediatric Clinic, IRCCS San Matteo Hospital Foundation, Pavia, Italy.; 49. Department of medical genetics, Le Mans hospital, Le Mans, France.; 50. Royal Children's Hospital, Florey Institute and Murdoch Children's Research Institute, Melbourne, Australia; 51. Institute of Clinical Medicine, Copenhagen University, Copenhagen, Denmark

Corresponding Author: Philip Ahring

Group Authorship: No. There is no study group involved in our research

Group Name:

Contributions:

ALESSANDRA ROSSI: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data; Susan Lin: Major role in the acquisition of data; Analysis or interpretation of data; Alessandra Rossi and Susan X. N. Lin ributed equally to this work as co-first authors. Sebastian Ortiz-De la Rosa: Major role in the acquisition of data; Analysis or interpretation of data; Nathan Absalom: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Analysis or interpretation of data; Vivian Liao: Major role in the acquisition of data; Analysis or interpretation of data; Nazanin Mohammadi: Major role in the acquisition of data; Sindhu Viswanathan: Major role in the acquisition of data; Tommy Stöðberg: Major role in the acquisition of data; Alberto Danieli: Major role in the acquisition of data; Paolo Bonanni: Major role in the acquisition of data; Alec Aeby: Major role in the acquisition of data; Alessandro Orsini: Major role in the acquisition of data; Alice Bonucelli: Major role in the acquisition of data; Andrea Rüegger: Major role in the acquisition of data; Beatriz Giraldez: Major role in the acquisition of data; Bertrand Isidor: Major role in the acquisition of data; Burkhard Stüve: Major role in the acquisition of data; Carla Marini: Major role in the acquisition of data; Elisabetta Cesaroni: Major role in the acquisition of data; Christina Dühning Fenger: Major role in the acquisition of data; Christophe Philippe: Major role in the acquisition of data; Colombine Meunier: Major role in the acquisition of data; Damien Lederer: Major role in the acquisition of data; Stéphanie Moortgat: Major role in the acquisition of data; Egidio Spinelli: Major role in the acquisition of data; Elisa Fallica: Major role in the acquisition of data; Fiona Zeiner: Major role in the acquisition of data; Matthias Baumann: Major role in the acquisition of data; Laura Licchetta: Major role in the acquisition of data; Francesca Bisulli: Major role in the acquisition of data; Francesca Felicia Operto: Major role in the acquisition of data; Ira Benkel-Herrenbrueck: Major role in the acquisition of data; Kathleen Gorman: Major role in the acquisition of data; Katrine Johannesen: Major role in the acquisition of data; Konrad Platzer: Major role in the acquisition of data; Franziska Schnabel: Major role in the acquisition of data; Lieven Lagae: Major role in the acquisition of data; Mirjam Laufs: Major role in the acquisition of data; Riina Zordania: Major role in the acquisition of data; Stephen Malone: Major role in the acquisition of data; Tullio Messana: Major role in the acquisition of data; Wendy Werekx: Major role in the acquisition of data; Charlotta Jonsson: Major role in the acquisition of data; Zaid Afawi: Major role in the acquisition of data; Thomas Foiadelli: Major role in the acquisition of data; Yosra Halleb: Major role in the acquisition of data; Gaetan Lesca: Major role in the acquisition of data; Samuel Berkovic: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Ingrid Scheffer: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Mary Chebib: Drafting/revision of the manuscript for content, including medical writing for content; Study concept or design; Elena Gardella: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data; Rikke Møller: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data; Guido Rubboli: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data; Philip Ahring: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data;

Abstract: Background and Objectives: Variants in the GABRG2 gene encoding the $\gamma 2$ subunit of the γ -aminobutyric-acid-type A (GABAA) receptor are associated with a spectrum of epilepsy phenotypes. These range from simple febrile seizures to more severe conditions, including developmental and epileptic encephalopathies (DEEs). Despite previous analyses suggesting that pathogenic variants may lead to loss-of-function (LoF) receptors, a correlation between functional analysis and clinical phenotypic diversity remains elusive. We therefore aimed to determine why variants in the GABRG2 gene can lead to highly diverse phenotypes. Methods: We assembled a cohort of unreported individuals carrying presumed pathogenic GABRG2 variants. Electroclinical information was systematically collected, and electrophysiological measurements were conducted for missense variants to explore potential alterations in receptor function. Results: We examined 44 individuals with 32 GABRG2 variants (17 null and 15 missense). Functional assessments of the missense variants revealed that eight caused LoF and two caused gain-of-function (GoF). The remaining five did not alter receptor function and were not regarded pathogenic. Based on functional analysis and electroclinical data, 37 affected individuals were categorised into three groups: null LoF, missense LoF, and GoF variants. For 19 individuals with null variants, epilepsy was diagnosed in 14/19 with a median onset of 16 months. The remaining 5/19 only had febrile seizures. Psychiatric disorders and developmental delay/intellectual disability (DD/ID) were rarely observed. In contrast, all 13 individuals with missense LoF variants suffered from epilepsy with a median onset of 16 months. Most common epilepsy diagnoses were FS+ in 5/13 and DEE in 4/13. Psychiatric disorders were diagnosed in 8/13 individuals while DD/ID affected 9/13 individuals. Statistical comparisons revealed that null variants were associated with a milder phenotype than missense LOF variants. Finally, 4/5 individuals with GoF variants had a DEE characterised by early infancy onset at 1.8 months and profound DD. The fifth individual exhibited mild DD/ID and hypotonia without seizures. Discussion: Our findings indicate that the severity of disease associated with pathogenic GABRG2 variants depends on the functional consequences of the variants. Null variants are associated with a mild phenotype, missense LoF variants with an intermediate phenotype, while GoF variants can lead to severe phenotypes.

Abstract

Background and Objectives: Variants in the *GABRG2* gene encoding the $\gamma 2$ subunit of the γ -aminobutyric-acid-type A (GABA_A) receptor are associated with a spectrum of epilepsy phenotypes. These range from simple febrile seizures to more severe conditions, including developmental and epileptic encephalopathies (DEEs). Despite previous analyses suggesting that pathogenic variants may lead to loss-of-function (LoF) receptors, a correlation between functional analysis and clinical phenotypic diversity remains elusive. We therefore aimed to determine why variants in the *GABRG2* gene can lead to highly diverse phenotypes.

Methods: We assembled a cohort of unreported individuals carrying presumed pathogenic *GABRG2* variants. Electroclinical information was systematically collected, and electrophysiological measurements were conducted for missense variants to explore potential alterations in receptor function.

Results: We examined 44 individuals with 32 *GABRG2* variants (17 null and 15 missense). Functional assessments of the missense variants revealed that eight caused LoF and two caused gain-of-function (GoF). The remaining five did not alter receptor function and were not regarded pathogenic. Based on functional analysis and electroclinical data, 37 affected individuals were categorised into three groups: null LoF, missense LoF, and GoF variants. For 19 individuals with null variants, epilepsy was diagnosed in 14/19 with a median onset of 16 months. The remaining 5/19 only had febrile seizures. Psychiatric disorders and developmental delay/intellectual disability (DD/ID) were rarely observed. In contrast, all 13 individuals with missense LoF variants suffered from epilepsy with a median onset of 16 months. Most common epilepsy diagnoses were FS+ in 5/13 and DEE in 4/13. Psychiatric disorders were diagnosed in 8/13 individuals while DD/ID affected 9/13 individuals. Statistical comparisons revealed that null variants were associated with a milder phenotype than missense LOF variants. Finally, 4/5 individuals with GoF variants had a DEE characterised by early infancy onset at 1.8 months and profound DD. The fifth individual exhibited mild DD/ID and hypotonia without seizures.

Discussion:

Our findings indicate that the severity of disease associated with pathogenic *GABRG2* variants depends on the functional consequences of the variants. Null variants are associated with a mild phenotype, missense LoF variants with an intermediate phenotype, while GoF variants can lead to severe phenotypes.

33

34 **Keywords:**

35 GABA_A receptor, genetic epilepsies, developmental and epileptic encephalopathies,
36 electrophysiology, *GABRB3*, *GABRA1*.

37

38 **Abbreviations:**

39 ADHD, Attention-Deficit/Hyperactivity Disorder; ASD, Autism Spectrum Disorder; ASM,
40 Antiseizure Medication; CAE, Childhood Absence Epilepsy; CADD, Combined Annotation
41 Dependent Depletion; DD, Developmental Delay; DEE, Developmental and Epileptic
42 Encephalopathy; EC₅₀, Half maximal effective concentration; EMA, Epilepsy with
43 Myoclonic Absences; FS, Febrile Seizures; FS+, Febrile Seizures plus; GABA_AR, γ -
44 Aminobutyric Acid type A receptor; GEFS+, Genetic Epilepsy with Febrile Seizures plus;
45 GnomAD, Genome Aggregation Database; GoF, Gain of Function; ID, Intellectual
46 Disability; JAE, Juvenile Absence Epilepsy; LoF, Loss of Function; MAE, Myoclonic Atonic
47 Epilepsy or Doose syndrome; SIFT, Sorting Intolerant From Tolerant; TM, Transmembrane;
48 VUS, Variant of Uncertain Significance.

49

50

Introduction

The *GABRG2* gene is significantly enriched for rare missense and protein truncating variants leading to epilepsy.¹ Disease-causing variants in *GABRG2* gene were first described in an Australian family with genetic epilepsy with febrile seizures plus (GEFS+),² and in a French family with variable seizure phenotypes consistent with GEFS+.³ Since then, a broad range of epilepsy syndromes ranging from simple febrile seizures (FS)^{4,5} and genetic generalised epilepsies,⁶⁻¹⁰ to heterogeneous developmental and epileptic encephalopathies (DEEs) have been reported.¹¹⁻¹⁶ Individual phenotypes include a range of seizure types^{6,7,17,18} and comorbidities include hypotonia, developmental delay (DD) and/or intellectual disability (ID) and behavioural disorders.^{13,16,17} Furthermore *GABRG2* variants may raise risk for self-limited focal epilepsies of childhood.^{19,20}

GABRG2 encodes the $\gamma 2$ subunit of the γ -aminobutyric acid type A receptor (GABA_AR) that mediates fast inhibitory synaptic neurotransmission.^{21,22} GABA-induced opening of GABA_ARs on the postsynaptic cell typically leads to chloride influx through the pore and hyperpolarisation of the plasma membrane to inhibit action potential generation. Nineteen GABA_AR subunits exist ($\alpha 1-6$, $\beta 1-3$, $\gamma 1-3$, δ , ϵ , θ , π , and $\rho 1-3$) with the most prevalent isoform comprising two $\alpha 1$, two $\beta 2$ or $\beta 3$, and one $\gamma 2$ subunit (Figure 1).

To date, loss-of-function (LoF) effects have been proposed as the primary, if not exclusive, disease mechanism in *GABRG2*-related disorders.¹⁵ These effects can result from various underlying mechanisms such as impaired receptor assembly, trafficking, or channel activation.^{5,9,23} These mechanisms lead to either reduced surface expression or dysfunctional GABA receptors in synapses, resulting in diminished GABA-evoked responses. Consequently, inhibitory inputs in neurons decrease, leading to increased neuronal excitability. While LoF provides a plausible explanation for how *GABRG2* variants may cause epilepsy, it does not fully account for why some variants lead to more severe disease than others. In this context, certain protein truncating variants (null variants) have been speculated to cause more severe phenotypes than missense variants due to dominant negative effects.¹² However, direct comparisons between cohorts of individuals with null and missense variants are currently lacking.

The lack of convincing genotype-phenotype associations is challenging our understanding of *GABRG2*-related epilepsies and their underlying pathophysiological mechanisms. The broad range of neurological phenotypic expressions and variable responses to antiseizure

medications (ASMs) contrast with the assumption that *GABRG2* variants solely result in LoF effects. Recent reports show that epilepsy-associated variants in other GABA_AR subunit genes including *GABRB3*,^{24,25} *GABRA4*,²⁶ *GABRD*,²⁷ and *GABRA1*^{28,29} can cause gain-of-function (GoF) as well as LoF effects. While GoF variants have not been reported specifically for *GABRG2*, it is reasonable to expect their existence given the presence of GoF variants in other major subunit types.

Here, we present a comprehensive overview of the phenotypic and genotypic spectrum of *GABRG2* in a cohort of 44 previously unreported cases. This cohort includes individuals with null variants as well as missense variants, and functional analysis identified missense variants with either GoF or LoF effects. Although there was some overlap in phenotypic spectra, our genotype-phenotype analysis suggests that the observed diversity in phenotypes among individuals with genetic variants in *GABRG2* correlates with the specific type and functional impact of the variants.

CONFIDENTIAL

98 **Methods**

99 ***Clinical ascertainment***

100 Individuals with a presumed pathogenic variant in the *GABRG2* gene, detected through
101 genetic testing for intellectual disability, febrile seizures, epilepsies, or DEEs, were recruited
102 at the Danish Epilepsy Center and through an international network of epilepsy and genetic
103 centers across Europe, the Middle East, America, and Australia. Recruitment was facilitated
104 by the European Reference Network (ERN) ERN-EpiCare and the Network for Therapy in
105 Rare Epilepsies (NETRE). Only previously unreported cases were included, and to ensure a
106 diverse representation of variants and genetic backgrounds, a maximum of two family
107 members were allowed (i.e., two affected siblings). All data were collected in a structured
108 phenotype table hosted at the Danish Epilepsy Centre. Data are reported in line with the
109 Strengthening Reporting of Observational Studies in Epidemiology (STROBE) statement.

110 ***Phenotypic analysis.***

111 Probands and their families underwent detailed phenotyping including interviews, review of
112 their medical history, neuroimaging, and EEG findings. Developmental and cognitive level
113 was based, where possible, on evaluations or formal assessments by referring physicians or
114 neuropsychologists and classified according to the Diagnostic and Statistical Manual of
115 Mental Disorders 5th edition. DD was classified as mild, moderate, severe, or profound in
116 children aged 5 years or younger. Children who were at least 6 years old were classified as
117 having mild, moderate, severe, or profound intellectual disability (ID). Seizures, epilepsies
118 and, where possible, epilepsy syndromes were classified according to the International
119 League Against Epilepsy classification.³⁰⁻³² A diagnosis of DEE was defined by both (1) an
120 epileptic encephalopathy where frequent epileptiform activity and/or seizures caused
121 developmental slowing or regression, and (2) abnormal development due to the underlying
122 aetiology.³⁰ Intellectual disability and epilepsy were diagnosed if they had developmental
123 impairment and seizures without evidence of an epileptic encephalopathy.^{30,33,34} EEG
124 recordings in individuals with seizures, where available, were reviewed by two
125 epileptologists (EG and GR). Effectiveness of ASMs were retrospectively assessed by the
126 referring clinicians.

127 ***Genotype analysis***

GABRG2 variants were identified by genomic analysis using targeted gene panels, whole exome sequencing or whole genome sequencing at each institution. Genomic variants and familial segregation were confirmed by direct Sanger sequencing. All variants were annotated using the NM_198904.4 (GRCh37 / hg19) transcript of *GABRG2*, which corresponds to the $\gamma 2$ L splice form of the $\gamma 2$ subunit. The potential impact of the *GABRG2* variants was assessed using SIFT (sorting intolerant from tolerant), PolyPhen-2 (polymorphism phenotyping-v2), CADD v1.6 (combined annotation dependent depletion) and their frequency in gnomAD v4.0.0 (genome aggregation database). Variants were classified according to the 2015 American College of Medical Genetics and Genomics (ACMG) guidelines. Concerning copy number variations, we did not include individuals with deletions/duplications involving other genes in addition to *GABRG2*.

Molecular biology

To circumvent the common issue with non-uniform GABA_AR populations that e.g., include polluting binary $\alpha 1\beta 3$ receptors, concatenated pentameric $\gamma 2\text{-}\beta 3\text{-}\alpha 1\text{-}\beta 3\text{-}\alpha 1$ receptor constructs using human subunits were used in all experiments as previously described.²⁹ Wild-type and 15 single variant $\gamma 2$ subunits were purchased from GenScript (Singapore) and subcloned into the X position of a tetrameric concatenated construct (X- $\beta 3\text{-}\alpha 1\text{-}\beta 3\text{-}\alpha 1$). The incorporation of variants was verified by Sanger sequencing. DNA gel electrophoresis was performed to ensure the incorporation of the five subunits. cRNA of each concatenated receptor was synthesised from linearised cDNA using the mMessage mMachine T7 transcription kit (Thermo Fisher, Scoresby, Australia). cRNA was stored at -20°C until use.

Xenopus laevis oocyte preparation

Oocytes were obtained from an ovarian lobe of *Xenopus laevis* from the University of Wollongong under animal ethics protocol AE2003. The ovarian lobe was treated with 2 mg/mL of collagenase A dissolved in modified Barth's solution (in mM, 82.5 NaCl, 2 KCl, 1 MgCl, 5 HEPES hemisodium pH 7.4) until oocytes were detached. Oocytes were then stored in ND96 (in mM, 96 NaCl, 2 KCl, 1 MgCl₂, 5 HEPES hemisodium pH 7.4, 1.8 CaCl₂) supplemented with gentamicin and ampicillin. For each oocyte, 25 ng of cRNA encoding for variant or the wild-type concatenated $\alpha 1\beta 3\gamma 2$ receptors were microinjected into oocytes and the oocytes were stored in ND96 solution supplemented with gentamicin and ampicillin at 18°C for 2 days before recording.

Two-electrode voltage clamp electrophysiology

Electrophysiological recordings were carried out using a custom-built two-electrode voltage clamp apparatus. Briefly, cells were impaled with 3M KCl filled glass borosilicate microelectrodes with a resistance of 0.2-1.4 MΩ, then voltage clamped at -50 mV. Cells were continuously perfused with ND96 during the experiment. Currents were recorded using GeneClamp 500B (Axon instrument, Foster City, USA), digitised using the Digidata 1440A (Axon instrument) and stored on a PC using Clampex (Axon instrument). All experiments were conducted at room temperature with at least two batches of oocytes. To control for variations between oocyte batches and experimental days, wild-type receptors were assessed with variants on each experimental day (fully parallel experiments).

Functional data analysis

To determine the functional effects of the variant on receptor function, EC₅₀ values derived from GABA concentration-response relationships (n = 12-29), and maximum current amplitudes (n = 27-65) were determined as previously described.²⁴⁻²⁷ Briefly, increasing concentrations of GABA were applied to oocytes to generate a concentration-response curve. The EC₅₀ value for GABA concentration-response relationships was determined by nonlinear regression of the Hill equation to the maximal GABA-elicited amplitudes for each oocyte:

$$I = Abs. I_{max} \frac{([A]^{n_H})}{([A]^{n_H} + [EC_{50}]^{n_H})} \quad (1)$$

Where Abs. I_{max} is the absolute maximum current, [A] is the agonist concentration, n_H is the Hill slope, and EC₅₀ is the concentration that elicits a half-maximal response. Data were fitted for each completed concentration-response curve to determine the EC₅₀ value at each single oocyte. The LogEC₅₀ value was then calculated from the EC₅₀ value.

To determine the relative change in the LogEC₅₀ value of each variant from the wild-type, the LogEC₅₀ of the variant was compared to the mean LogEC₅₀ of wild-type values determined on the same experimental day. The ΔLogEC₅₀ for each variant on the same day is given by the equation:

$$\Delta \text{LogEC}_{50} = \text{LogEC}_{50}(\text{wt}) - \text{LogEC}_{50} \quad (2)$$

Where the LogEC₅₀(wt) is the LogEC₅₀ of wild-type and LogEC₅₀ of variant receptors.

The maximum current (I_{max}) was determined by the peak current amplitude elicited by 10 mM GABA for wild-type and each variant. The maximum current for each single experiment was given by the equation:

190 $I_{\max} = (\text{Abs}.I_{\max})/(\text{Abs}.I_{\max}(\text{wt}))$ (3)

191 Where $\text{Abs}.I_{\max}$ is the maximum current for variant and $\text{Abs}.I_{\max}(\text{wt})$ is the maximum current
192 elicited at 10 mM for wild-type.

193 **Statistics**

194 Curve fitting of GABA concentration-response relationships was performed by nonlinear
195 regression using GraphPad Prism 8.0.1 (Dotmatics, Boston, MA) and were compared with a
196 one-way ANOVA and multiple corrections with Dunnett's post-hoc test. Maximum GABA-
197 evoked current amplitudes and ages of onset were compared by Mann-Whitney U-test. A
198 conservative threshold for significance in ΔLogEC_{50} values was set at $p < 0.0001$ and a
199 ΔLogEC_{50} shift exceeding ± 0.2 . A similarly conservative threshold at $I_{\max}$ was set at $p <$
200 0.0001 and a $I_{\max}$ reduction exceeding 2 fold (less than 50% remaining current amplitude). A
201 Fisher's Exact test was used to compare clinical features where appropriate, with a threshold
202 of $p < 0.01$. Confidence intervals for clinical data were provided as 95% confidence intervals
203 for dependent variables where the number of patients were greater than ten, in all other cases
204 the range of data is given.

205 **Standard Protocol Approvals, Registrations, and Patient Consents**

206 The study was conducted according to the ethical principles for medical research outlined in
207 the Declaration of Helsinki. The study was approved by the local ethics committee in the
208 Zealand region of Denmark (number SJ-91), and by the Institutional Review Board at the
209 Danish Epilepsy Centre, Filadelfia (EMN-2024-01998). Informed consent of individuals or
210 their responsible relatives and approval by local ethical committees were obtained. Clinicians
211 and caregivers filled anonymised data in an online questionnaire and standardised
212 phenotyping sheets. The database was stored at the Danish Epilepsy Centre.

213 **Data availability**

214 All phenotypic data for the individuals in this study are available in the main text or the
215 Supplementary material. Raw data for the functional analyses are available upon request from
216 the corresponding authors. Data will be stored for a minimum of 7 years.

Results

Genetic landscape

We studied a cohort of 44 previously unpublished individuals (21 male, 23 female), all of whom carried presumed pathogenic *GABRG2* variants. Thirty-eight were unrelated and the remaining 6 individuals belonged to three families. The median age at last follow-up was 6 years and 9 months (range: 1 year and 7 months to 43 years). The electro-clinical and genetic details of the cohort are summarised in the Supplementary Table.

A total of 32 variants were identified. These occurred *de novo* in 15/44 (34%) individuals, were inherited in 26/44 (59%) individuals while segregation was not available for 3/44 individuals. Of the 32 variants, 15 were missense and 17 were null variants. Null variants were defined as any variant that would not be predicted to translate into the full-length protein. This category included nine nonsense variants (including short deletions leading to a frameshift), four large deletions resulting in missing exons and four splice-site variants. The variants were absent from the population databases (gnomAD v4.0.0) except for the following: I85K (allele count 1), T90M (allele count 4), R125C (allele count 2) and M199V (allele count 5). When assessed for predicted functional effects using *in silico* prediction tools, the A106T, S139N and I344L variants were predicted to be tolerated by SIFT. The A106T and I344L variants were furthermore predicted to be benign by PolyPhen2 (Supplementary Table). All other variants were predicted to affect protein function by SIFT and were classified as “probably damaging” by PolyPhen2. Notably, the T90M and A106T variants had the lowest CADD scores and were considered least likely to be pathogenic by this tool.

Structural mapping

The missense and nonsense variants occurred at 22 different amino acid positions (Figure 1). Recurrent variants included T90M, A106T, R136*, M169T, A322D, R323Q and R323W. Additionally, two different missense variants affected the same amino acid residue: A322D and A322T, as well as R323Q and R323W (Supplementary Table). Among the missense variants, five affected amino acid positions that are 100% conserved across all GABA_AR subunits associated with epilepsy, while seven affected positions conserved among the three γ subunits (Figure 1). The remaining three missense variants affected positions that are not conserved. The missense variants are distributed along the $\gamma 2$ subunit protein, including nine

variants in the extracellular domain and six in the transmembrane domain (Figure 1). Interestingly, eight reside at the coupling region at the interface of the extracellular and transmembrane regions. This region mediates the conformational changes that convert the GABA-binding event to ion channel opening and is a region where both LoF and GoF variants are typically found.^{24,25} Apart from I344L, which is located in transmembrane domain M3, the remaining variants are located in relatively close proximity to each other at the upper end of the extracellular domain, a region without a clearly defined functional role. Notably, nonsense null variants are located in the extracellular domain except for Q398*, which is in the M3-M4 intracellular loop (Figure 1).

Functional analysis

To ensure uniform receptor populations, mutated $\gamma 2$ subunits were expressed using pentameric concatenated cDNA constructs (Figure 2A). These constructs lead to expression of a fusion protein containing all five subunits of a GABA_AR in the correct order for assembly.^{35,36} Functional analysis was performed using two-electrode voltage clamp electrophysiology.

Receptor sensitivity to GABA is a critical property for effective mediation of synaptic inhibition. Therefore, changes to GABA sensitivity were evaluated by constructing GABA concentration-response relationships and deriving half maximal GABA concentration (EC₅₀) values from fitted curves (Figure 2B-C). By comparing the LogEC₅₀ of variants with the wild-type, Δ LogEC₅₀ values were obtained for each variant. A One-way ANOVA revealed significant differences between GABA sensitivities for wild-type and mutant receptors ($F(15, 392) = 120$). Among the 15 mutations assessed, eight decreased receptor GABA sensitivity by 2-5-fold consistent with a LoF effect (Δ LogEC₅₀ = -0.33 to -0.70; all $p < 0.0001$, Dunnett's post-hoc test) (Figure 2D, Supplementary Table). Two mutations exhibited a 2-5-fold increase in GABA sensitivity indicating GoF traits (A106T and S139N; Δ LogEC₅₀ = 0.33 to 0.73; $p < 0.0001$ Dunnett's post-hoc test). The remaining five mutated receptors did not exhibit altered receptor sensitivity to GABA.

Another receptor property that may influence inhibitory neurotransmission is the maximum GABA-evoked current amplitude, a product of the efficacy of GABA activation and cell surface receptor expression. To assess this, maximum current amplitudes were evaluated by applying a saturating concentration of GABA (10 mM) and comparing wild-type and mutant receptor currents elicited on the same day. Among the 15 mutations assessed, only four

showed a substantial reduction in maximum elicited current with amplitudes varying from 11 to 34% of the wild-type ($I_{\max} = 0.11 - 0.34$, $p < 0.0001$, Mann-Whitney U test) (Supplementary Table). Notably, all four mutations also displayed a significant reduction in GABA sensitivity.

Based on the functional studies, the A106T and S139N variants were categorised as GoF variants due to their increased GABA sensitivity. Additionally, eight variants were classified as LoF with four of these, P83T, R125C, M169T and L326H, displaying both impaired GABA sensitivity and reduced maximum currents while four variants, A322D, A322T, R323Q and R323W, displayed impaired GABA sensitivity alone. The remaining 5 variants, I85K, T90M, V104G, M199V and I344L, displayed either no changes or changes that were of too low magnitude to be considered significant for the functional parameters analysed.

Classification using ACMG guidelines

Following the functional analysis, the pathogenicity of the variants was re-evaluated according to ACMG guidelines. Ten variants displayed significant functional alterations (pathogenic strong support, PS3). When combined with clinical information, this supports a classification as pathogenic or likely pathogenic. In contrast, functional analysis did not reveal significant alterations for five variants I85K, T90M, V104G, M199V, and I344L (benign strong support, BS3). The T90M and M199V variants were reported four and five times, respectively, in the gnomAD database, leading to their classification as benign. The I85K variant, reported only once in the gnomAD database, is considered likely benign. The remaining two variants, V104G and I344L, are classified as variants of uncertain significance (VUS). Since none of these five variants were classified as likely pathogenic or pathogenic, the seven individuals harbouring them were excluded from our genotype-phenotype analysis, however, the phenotypes are described in the Supplementary Table.

In total, 27 pathogenic/likely pathogenic variants were detected in 37 individuals. Based on the functional properties of the variants and the electroclinical information of the individuals, three groups were identified, (1) presumed LoF null variants resulting in an aberrant or truncated protein; (2) LoF missense variants with impaired GABA sensitivity, (3) GoF missense variants that increased GABA sensitivity.

Individuals harbouring null variants

Nineteen individuals carried null *GABRG2* variants with segregation analysis available for 18 of them. The variants occurred *de novo* in 2/18 (11%) individuals, while they segregated with affected parents in 13/18 (72%) and unaffected parents in 3/18 (17%) individuals. The characteristics and phenotypic features of those harbouring null variants are summarised in Table 1 and Table 2 (#1 to #19), with additional information provided in the Supplementary Table.

Epilepsy was diagnosed in 14/19 (74%) individuals with a median onset of 15 months (range 5 months to 2 years and 4 months). The remaining 5/19 (26%) individuals only had febrile seizures (FS) with a median onset of 9 months (range 6 months to 5 years). Among the 14 individuals with epilepsy, nine (64%) had febrile seizures plus (FS+), and three (21%) had genetic generalised epilepsy (GGE, #4, #9 and #14). The remaining two individuals (#3 and #8) had either DEE or epilepsy with myoclonic-atonic seizures (MAE). Besides DEE and MAE, the remaining of these epilepsy syndromes are within the GEFS+ spectrum, and based on familial history, 14/19 (74%) individuals could be assigned with a GEFS+ diagnosis.

Interictal EEGs were available in 14/19 (74%) individuals and showed normal background activity in 12/14. Generalised or focal/multifocal epileptiform abnormalities were observed in 5/12 (42%) and 3/12 (25%) individuals, respectively. In one individual (#14), ictal EEG recorded myoclonic seizures associated with generalised spike-wave discharges (Figure 3A). Brain MRI studies were performed in 14/19 (74%) individuals and were found to be unremarkable.

Moderate developmental delay and intellectual disability (DD/ID) was reported for the individual with DEE (#3) whereas the remaining 18/19 (95%) individuals were considered normal. All individuals acquired language skills, although the individual with DEE (#3) experienced a moderate ID with severe language delay. Three individuals (#4, #8 and #13) exhibited behavioural and/or psychiatric disorders, including ADHD, hyperactivity and concentration difficulties. All demonstrated normal motor abilities and were able to walk independently.

At the last follow-up, 13/19 (68%) individuals were seizure free, however, daily to monthly or rare seizures persisted in 6/19 (32%) individuals. Interestingly, three individuals with FS and FS+ (#10, #16 and #17) were never treated with ASMs and became seizure free with age. The remaining sixteen individuals were treated with ASMs with a very good response (seizure freedom) in 11/16 (69%), and a partially good response in 4/16 (25%) while epilepsy

was not controlled in 1/16 (6%) individual (#15). Valproate was used in 10/16 (63%) individuals, resulting in seizure freedom as monotherapy in 5 patients and as add on treatment in another individual. Valproate as add on therapy also improved seizure frequency in more individual. GABAergic ASMs, such as benzodiazepines, vigabatrin, and phenobarbital improved seizure frequency in 3/4 (75%) individuals. Ketogenic diet reduced seizure frequency in 1/16 (6%) individual (#14).

Individuals harbouring missense LoF variants

Thirteen individuals carried *GABRG2* missense variants causing LoF with segregation information available for 12/13 individuals. The variants occurred *de novo* in 7/12 (58%) and were inherited from affected parents in 5/12 (32%) individuals. Hence, missense variants were more likely to be *de novo* compared to null variants (OR 11 [95% CI: 1.8-60], $p = 0.013$, Fisher's Exact Test). The characteristics and phenotypic features of those harbouring missense LoF variants are summarised and detailed in Table 1, Table 2 and Supplementary Table (#20 to #32).

All 13/13 (100%) individuals had epilepsy with a median age at seizure onset of 16 months (range 7 months to 3 years and 6 months). The age at epilepsy onset did not differ between individuals with LoF missense variants and those with null variants (Mann-Whitney test, $U = 120$, $p = 0.95$). Only 5/13 (38%) individuals with FS+ exhibited phenotypes within the GEFS+ spectrum. Of the remaining individuals, 4/13 (31%) had a DEE (#22, #26, #28 and #30) and one each had focal epilepsy, combined focal and generalised epilepsy, MAE and CAE. Hence, epileptic phenotypes outside the GEFS+ spectrum were more often observed for LoF missense vs. null variants (OR = 29 [95% CI: 3.2-330], $p = 0.00090$, Fisher's Exact Test). Notably, the four individuals with DEE experienced generalised (tonic-clonic, myoclonic, atonic, absences) as well as motor and non-motor focal seizures. Four (31%) individuals (#21, #22, #28, #30) had at least one episode of convulsive or non-convulsive status epilepticus.

EEG studies were available in 13/13 (100%) individuals and showed focal/multifocal (bifrontal, temporal-posterior, central) or generalised epileptiform abnormalities in 10/13 (77%) individuals. A normal EEG was observed in 2/13 (15%) individuals, while an encephalopathic pattern of diffuse slowing with multifocal epileptiform discharges was observed in the remaining individual (#22). Brain MRI, performed in 10/13 (77%) individuals, showed a temporal arachnoid cyst in 2/10 (20%, #26, #27), left mesial temporal

sclerosis in 1/10 (10%, #28), thinning of the corpus callosum with a right frontal gliotic focus in 1/10 (10%, #29), and was unremarkable in the remaining 6/10 (60%) individuals.

DD was observed in 9/13 (69%) individuals with varying severity: mild in 7/9, moderate in 1/9 (#28), and severe in 1/9 (#22). In individuals older than 6 years, ID was observed in 6/8 (75%): mild in 3/8 (#23, #29, #32), moderate in 2/8 (#28 and #30), and profound in 1/8 (#22). The remaining two individuals (#21 and #31) had normal intellect. Notably, DD/ID was more frequently observed for LoF missense vs. null variants (OR = 41 [CI:4.3-460], $p = 0.00022$, Fisher's Exact Test). Language skill data were available for 12/13 individuals, and all acquired the ability to communicate albeit with a delay in 5/12 (42%). Eight of 13 (62%) individuals exhibited behavioural and/or psychiatric disorders including ADHD, hyperactivity, attention deficit and ASD. Normal motor abilities were observed in 7/12 (58%) while 4/12 experienced various degrees of motor skill issues and 1/12 was wheelchair bound. Motor skill issues were thus more frequently observed for LoF missense vs. null variants (OR = ND (not determined as one category is 0), $p = 0.0039$, Fisher's Exact Test). Additionally, hypotonia was reported in 1/13 (8%) individual (#27).

Ten individuals were treated with ASMs. Valproate was administered in 9/10 (90%) individuals, with partial seizure control in monotherapy or as add on treatment in 5/9 (56%). Other ASMs targeting GABAergic neurotransmission were tried in 4/10 (40%) individuals. Clobazam improved seizure frequency in two, while it was not tolerated in one individual. Phenobarbital in combination with vigabatrin was ineffective in one individual. Ketogenic diet was effective in one individual. Of these individuals 5 were diagnosed with FS+ (#21, #24, #25, #29, #32) and one with DEE (#30).

Individuals harbouring GoF variants

Five individuals had *de novo* GoF variants of which four had the A106T variant and one the S139N variant. Their phenotypic characteristics are reported in Table 1, Table 2 and Supplementary Table (#33 to #37).

Epilepsy was diagnosed in 4/5 (80%) individuals (#33 to #36) and all had the A106T variant. All had a DEE with a median age at seizure onset of 1.8 months (95% CI: 1.0–3.5). The main seizure types were focal (including focal impaired awareness), gelastic with right fronto-central onset in 1/4 (#34) and bilateral tonic-clonic in 2/4 (#34 and #35). Interestingly, gelastic seizures, have not previously been reported in individuals with GABA_AR variants. One individual (#36) had asymmetric tonic and bilateral tonic-clonic seizures. Episodes of

convulsive status epilepticus were reported in one individual (#35). Interictal EEG showed diffuse slowing and frequent multifocal or generalised epileptiform discharges (Figure 3B).

All four individuals with a DEE were drug-resistant, although some improvement in seizure frequency with valproate was observed in 3/4 individuals (#33, #34 and #36. All individuals had delayed development and were nonverbal. For two individuals (#33, #34), lack of social smile and eye contact was noted and severe hypotonia impairing spontaneous movements and ability to walk was observed. One individual (#33) had cognitive regression and loss of eye contact after epilepsy onset at age 3.5 months and, by 21 months, she had profound impairment. Chorea, orofacial dystonia, lower limb hyperreflexia, poor head control, inability to sit and to walk was observed in the other individual (#35). One individual (#34) could independently walk for a short distance, while another (#36) could sit with support. Brain MRIs showed calcification in both cerebral hemispheres in one individual (#35) and diffuse atrophy in another (#36), whereas it was unremarkable in two individuals (#33, #34).

The individual with the *de novo* S139N variant (#37) had speech delay, mild hypotonia and clumsiness, mild ID, ASD, and coarse facial features. EEG showed asynchronous sharp waves over the left occipital and the right frontal areas (Figure 3C).

Discussion

GABRG2 variants are often inherited, and previous studies have documented both missense and null variants within large family pedigrees.^{7,37} In this study, however, our objective was to provide a broader description of the phenotypic and genotypic spectrum of *GABRG2*-related disease, offering insights into the expected outcomes for novel variants. To achieve this, we established inclusion criteria that encompassed only previously unreported cases while excluding larger family pedigrees. From analysis of 44 previously unreported cases, we identified three groups of pathogenic *GABRG2* variants, each associated with increasing phenotypic severity: (i) null (LoF) variants, (ii) missense LoF variants and (iii) missense GoF variants.

Null vs. Missense LoF variants

Individuals with null LoF and missense LoF variants in *GABRG2* exhibit similar median seizure onset ages of 16 months. These findings align with the previously reported median age of 11 months for individuals with *GABRB3* LoF variants.²⁴ However, contrary to previous findings that suggested that *GABRG2* null variants lead to more severe phenotypes than missense variants,¹² our observations reveal the opposite trend. While there are overlapping features, individuals with null variants generally exhibit less severe phenotypes based on several markers (Figure 4). While epilepsy syndromes within the GEFS+ spectrum were observed in most individuals with null variants, DEE and focal seizures were commonly observed in individuals with missense LoF variants. Furthermore, individuals with null variants almost exclusively demonstrate normal ID/DD and motor abilities, whereas the majority of those with missense LoF variants suffer from DD/ID and/or motor skill issues. Finally, it would be expected that milder variants are more likely to be inherited and show incomplete penetrance. Our cohort observations support this, as *de novo* variants were more common for missense variants, and several individuals with null variants inherited the variant from an unaffected parent. Speculatively, the severity difference between missense LoF and null variants may be because having non-functional receptors in the synaptic density is worse than having too little receptor expression (haploinsufficiency).

GoF missense variants

Two missense variants, A106T and S139N, were identified as increasing GABA sensitivity consistent with a GoF molecular phenotype. The four unrelated individuals carrying the

A106T variant experienced early seizure onset, with a median of 2 months, and exhibited the most severe phenotypes within the cohort. These phenotypes closely resembled the pattern observed in individuals with GoF *GABRB3* variants, including an early infantile onset, drug-resistant seizures, and severe ID.²⁴ Previous reports have suggested that the A106T variant reduces GABA sensitivity,¹⁴ but our functional studies contrast this finding and align with the observations for GoF phenotypes. The one individual with the S139N variant had ASD, mild DD/ID, and global hypotonia but without seizures. Additionally, this individual exhibited other features not seen in the rest of the cohort such as obesity, coarse facial features, and tall stature. Notably, the change in GABA sensitivity at receptors containing the S139N receptor was less than half that of the A106T, which may explain the marked difference in phenotypic severity between these variants. Intriguingly, some individuals with GoF *GABRA1*²⁸ variants likewise did not experience epilepsy, thereby distinguishing some GoF *GABRG2* and *GABRA1* variants from GoF *GABRB3* variants. It is possible that these differences depend on the specific roles played by the various subunits in receptor function and their localisation within the brain.

Recurrent variants and phenotypic diversity

Two recurrent variants causing either LoF or GoF were observed in three or more unrelated individuals. This provides an opportunity to investigate clinical differences between individuals with the same variant but different genetic background. Individuals carrying the variant R323Q exhibited a range of phenotypes: one had MAE (#25, *de novo*), one had DEE (#26, inherited from a mother with mild phenotype), and one had FS associated with mild ID (#27, *de novo*). Notably, this recurrent variant has been reported in 13 families showing a broad phenotypic spectrum that includes DS,³⁸ GEFS+ with mild ID,³⁹ GEFS+ with normal cognition,⁴⁰ self-limited epilepsy with centrotemporal spikes¹⁹ and early infantile epileptic encephalopathy with severe ID.¹⁴ In contrast, the four individuals with the A106T variants displayed similar phenotypes falling within the DEE syndrome classification and exhibiting severe-profound DD/ID. Interestingly, the A106T variant has been reported in 7 unrelated individuals, all showing a concordant phenotype. These individuals experienced seizure onset ranging from the first day of life to 4.5 months along with DEE syndrome and severe DD/ID.^{13,14}

Generally, it would be expected that the more severe the consequence of a variant on neuronal networks, the more consistent the clinical phenotype will be. For the recurrent

variants in this study, the phenotypic spectrum associated with the LoF *GABRG2* variants lead to a wider range of clinical presentations compared to GoF variants. This supports the notion that GoF variants cause more severe clinical phenotypes. However, larger studies involving additional recurrent variants are needed to clarify how much of the variation in clinical severity is due to the level of molecular dysfunction and how much is influenced by polygenic factors related to the genetic background.

Null variants and potential dominant negative effects

In this study, most protein-truncating variants occur in the extracellular domain, resulting in the complete loss of the transmembrane domain essential for GABA_AR function (Figure 1). The Q398* variant represents an exception - it resides in the TM3-TM4 intracellular loop and leads to a partial loss of the loop and the last transmembrane helix. Interestingly, the location of truncating variants has been associated with varying levels of subunit aggregation and the potential for dominant negative effects. In fact, the Q398* variant (previously referred to as per mature peptide, Q351*, or by an alternative transcript Q390*) has been suggested to exhibit such dominant negative effects.⁴¹ In our cohort, we identified an individual (#8) carrying the same Q398* variant. Remarkably, this individual only experienced FS at 30 months of age without DD/ID. The variant was inherited from #8's mother, who was diagnosed with Dravet syndrome (patient V-21 in Harkin *et al.*, 2002).⁷ Additionally #8's sister had epilepsy with eyelid myoclonia and ID (patient III-6, family M in Sadleir *et al.*, 2012).⁴² However, other close family members also had FS or FS with MAE despite not carrying the variant. As previously concluded,⁷ factors beyond the variant itself contribute to the phenotypic variability within this family. Overall, #8's phenotype aligns with that of others carrying null variants in this cohort and neither #8 nor the family history supports the notion that more severe phenotypes arise solely from dominant negative suppression effects of Q398*.

Poor accuracy of in silico prediction tools

Five primarily inherited variants, I85K, T90M, V104G, M199V and I344L, showed no alterations in receptor function. Of these, T90M and M199V have been reported over four times in gnomAD, suggesting that they are benign polymorphisms. While the remaining three variants may also be benign, the current dataset cannot definitively exclude a potential pathogenic role. Interestingly, the *in-silico* prediction tools poorly anticipated the pathogenicity of these variants. Although the I344L variant was correctly predicted as

unlikely to be pathogenic, the remaining variants were predicted to be damaging contradicting the functional studies and population database information. Furthermore, the A106T GoF variant was predicted as one of the least likely to be damaging, yet it causes the most severe phenotypes. Overall, the prediction tools resulted in discordant predictions for 33% of variants. These findings underscore the importance of assessing functional data for all *GABRG2* variants to both accurately establish their pathogenicity and determine whether the variant is a LOF or a GOF.

Limitations

This study has several limitations. First, the small number of individuals with GoF variants makes statistical comparisons with LoF groups less meaningful. Second, our study relied on retrospective data collected by clinicians from medical records and patient interviews, which may introduce heterogeneity in data collection and analysis. These limitations also affect the evaluation of antiseizure medications (ASMs) responses. For instance, we did not observe differences in response to ASMs targeting GABA pathways compared to other mechanisms. Lastly, *in vitro* expression systems do not fully capture the complexity of neuronal processes, potentially overlooking other important factors influencing receptor function in a neuronal context.

Conclusion

In this study, we established correlations between the phenotypic spectra of individuals with genetic variants in *GABRG2* and the functional consequences of these variants on GABA_AR activity. Specifically, LoF variants were associated with a milder phenotype in individuals with null variants compared to those with missense variants. The most severe epilepsy phenotype was observed in individuals with GoF variants, emphasizing *GABRG2*'s role as a significant cause of DEEs. Intriguingly, *in-silico* prediction tools performed only marginally better than flipping a coin for the missense variants in this study. This underscores the necessity of functional analysis not only for distinguishing between LoF and GoF effects but also for assessing potential pathogenicity. Given the limited number of individuals with GoF variants, further studies are essential to collect additional phenotypic data, including information on the natural history and prevalence of comorbidities within the GoF group. Ultimately, such insights may shed light on how distinct functional changes contribute to varying types and severity of epilepsies.

CONFIDENTIAL

Acknowledgements

The authors would like to thank the individuals and families who participated in the collection of clinical data for this project and for enrolling in our research studies. We thank our colleagues who referred families to our centre and clinical colleagues who evaluated individuals. This study makes, in part, use of data generated by the EpiCare and NETRE communities.

Author contributions

MC, R.S.M, G.R. and P.K.A contributed to conception, study design and study supervision. A.R. and S.O.R. managed patient recruitment and data collection. S.X.N.L., N.L.A., V.W.Y.L. and P.K.A. performed functional testing. A.R., N.L.A., G.R. and P.K.A. contributed to drafting the text. N.L.A. and P.K.A. performed statistical analysis. S.X.N.L., E.G. and P.K.A. contributed to preparing the figures. N.A.M., S.V., T.S., A.D., P.B., A.A., A.O., A.B., A.R., B.G.G., B.I., B.S., C.M., E.C., C.D.F., C.P., C.M., D.L., S.M., E.S., E.F., F.Z., M.B., L.L., F.B., F.F.O., I.B.H., K.M.G., K.M.J., K.P., F.S., L.L., M.L., R.Z., S.M., T.M., W.W., C.J., Z.A., T.F., Y.H., G.L., S.F.B. and I.E.S. contributed to data collection. All authors read and approved the manuscript.

Funding

This work was funded by the Australian National Health & Medical Research Council grant APP1185122 (M.C., N.L.A., P.K.A. and R.S.M) and APP2019780 (P.K.A, M.C., V.W.Y.L., and R.S.M.), the Australian Research Training Program Stipend scholarship (S.X.N.L.), the Novo Nordisk Foundation NNF19OC0058749 (R.S.M.) and The Lundbeck Foundation R383-2022-276 (R.S.M., P.K.A.).

Conflict of interests

The authors report no competing interests.

Supplementary material

Supplementary Table. Summary of the phenotypic and genetic details of the cohort, detailed functional data and *In silico* prediction tool scores

580 **References**

- 581 1 Epi25. Ultra-Rare Genetic Variation in the Epilepsies: A Whole-Exome Sequencing
582 Study of 17,606 Individuals. *Am J Hum Genet* **105**, 267-282,
583 doi:10.1016/j.ajhg.2019.05.020 (2019).
- 584 2 Wallace, R. H. *et al.* Mutant GABA(A) receptor gamma2-subunit in childhood
585 absence epilepsy and febrile seizures. *Nat Genet* **28**, 49-52, doi:10.1038/ng0501-49
586 (2001).
- 587 3 Baulac, S. *et al.* First genetic evidence of GABA(A) receptor dysfunction in epilepsy:
588 a mutation in the gamma2-subunit gene. *Nat Genet* **28**, 46-48, doi:10.1038/ng0501-46
589 (2001).
- 590 4 Audenaert, D. *et al.* A novel GABRG2 mutation associated with febrile seizures.
591 *Neurology* **67**, 687-690, doi:10.1212/01.wnl.0000230145.73496.a2 (2006).
- 592 5 Boillot, M. *et al.* Novel GABRG2 mutations cause familial febrile seizures. *Neurol*
593 *Genet* **1**, e35, doi:10.1212/nxg.0000000000000035 (2015).
- 594 6 Kananura, C. *et al.* A splice-site mutation in GABRG2 associated with childhood
595 absence epilepsy and febrile convulsions. *Arch Neurol* **59**, 1137-1141,
596 doi:10.1001/archneur.59.7.1137 (2002).
- 597 7 Harkin, L. A. *et al.* Truncation of the GABA(A)-receptor gamma2 subunit in a family
598 with generalized epilepsy with febrile seizures plus. *Am J Hum Genet* **70**, 530-536,
599 doi:10.1086/338710 (2002).
- 600 8 Sun, H. *et al.* SCN1A, SCN1B, and GABRG2 gene mutation analysis in Chinese
601 families with generalized epilepsy with febrile seizures plus. *J Hum Genet* **53**, 769-
602 774, doi:10.1007/s10038-008-0306-y (2008).
- 603 9 Tian, M. *et al.* Impaired surface $\alpha\beta\gamma$ GABA(A) receptor expression in familial
604 epilepsy due to a GABRG2 frameshift mutation. *Neurobiology of disease* **50**, 135-
605 141, doi:10.1016/j.nbd.2012.10.008 (2013).
- 606 10 Lachance-Touchette, P. *et al.* Novel $\alpha 1$ and $\gamma 2$ GABAA receptor subunit mutations in
607 families with idiopathic generalized epilepsy. *Eur J Neurosci* **34**, 237-249,
608 doi:10.1111/j.1460-9568.2011.07767.x (2011).
- 609 11 Jansen, F. E. *et al.* Severe myoclonic epilepsy of infancy (Dravet syndrome):
610 recognition and diagnosis in adults. *Neurology* **67**, 2224-2226,
611 doi:10.1212/01.wnl.0000249312.73155.7d (2006).
- 612 12 Kang, J. Q. & Macdonald, R. L. Molecular Pathogenic Basis for GABRG2 Mutations
613 Associated With a Spectrum of Epilepsy Syndromes, From Generalized Absence
614 Epilepsy to Dravet Syndrome. *JAMA Neurol* **73**, 1009-1016,
615 doi:10.1001/jamaneurol.2016.0449 (2016).
- 616 13 Zou, F. *et al.* Expanding the phenotypic spectrum of GABRG2 variants: a recurrent
617 GABRG2 missense variant associated with a severe phenotype. *J Neurogenet* **31**, 30-
618 36, doi:10.1080/01677063.2017.1315417 (2017).
- 619 14 Shen, D. *et al.* De novo GABRG2 mutations associated with epileptic
620 encephalopathies. *Brain* **140**, 49-67, doi:10.1093/brain/aww272 (2017).
- 621 15 Hernandez, C. C. *et al.* Dravet syndrome-associated mutations in GABRA1,
622 GABRB2 and GABRG2 define the genetic landscape of defects of GABA(A)
623 receptors. *Brain Commun* **3**, fcab033, doi:10.1093/braincomms/fcab033 (2021).
- 624 16 Yang, Y. *et al.* Phenotypic Spectrum and Prognosis of Epilepsy Patients With
625 GABRG2 Variants. *Front Mol Neurosci* **15**, 809163, doi:10.3389/fnmol.2022.809163
626 (2022).

- 17 Johnston, A. J. *et al.* A novel GABRG2 mutation, p.R136*, in a family with GEFS+ and extended phenotypes. *Neurobiology of disease* **64**, 131-141, doi:10.1016/j.nbd.2013.12.013 (2014).
- 18 Shi, X. *et al.* Mutational analysis of GABRG2 in a Japanese cohort with childhood epilepsies. *J Hum Genet* **55**, 375-378, doi:10.1038/jhg.2010.47 (2010).
- 19 Reinthaler, E. M. *et al.* Rare variants in γ -aminobutyric acid type A receptor genes in rolandic epilepsy and related syndromes. *Annals of neurology* **77**, 972-986, doi:10.1002/ana.24395 (2015).
- 20 Jiang, Y. L. *et al.* Rare variants in GABRG2 associated with sleep-related hypermotor epilepsy. *J Neurol* **269**, 4939-4954, doi:10.1007/s00415-022-11137-4 (2022).
- 21 Chua, H. C. & Chebib, M. GABA(A) Receptors and the Diversity in their Structure and Pharmacology. *Adv Pharmacol* **79**, 1-34, doi:10.1016/bs.apha.2017.03.003 (2017).
- 22 Absalom, N. L. *et al.* GABA(A) receptors in epilepsy: Elucidating phenotypic divergence through functional analysis of genetic variants. *Journal of neurochemistry*, doi:10.1111/jnc.15932 (2023).
- 23 Warner, T. A. *et al.* Differential molecular and behavioural alterations in mouse models of GABRG2 haploinsufficiency versus dominant negative mutations associated with human epilepsy. *Hum Mol Genet* **25**, 3192-3207, doi:10.1093/hmg/ddw168 (2016).
- 24 Absalom, N. L. *et al.* Gain-of-function and loss-of-function GABRB3 variants lead to distinct clinical phenotypes in patients with developmental and epileptic encephalopathies. *Nature Communications* **13**, 1822, doi:10.1038/s41467-022-29280-x (2022).
- 25 Absalom, N. L. *et al.* Gain-of-function GABRB3 variants identified in vigabatrin-hypersensitive epileptic encephalopathies. *Brain Commun* **2**, fcaa162 (2020).
- 26 Ahring, P. K. *et al.* The de novo GABRA4 p.Thr300Ile variant found in a patient with early-onset intractable epilepsy and neurodevelopmental abnormalities displays gain-of-function traits. *Epilepsia* **63**, 2439-2441, doi:<https://doi.org/10.1111/epi.17358> (2022).
- 27 Ahring, P. K. *et al.* Gain-of-function variants in GABRD reveal a novel pathway for neurodevelopmental disorders and epilepsy. *Brain* **145**, 1299-1309, doi:10.1093/brain/awab391 (2021).
- 28 Musto, E. *et al.* GABRA1-Related Disorders: From Genetic to Functional Pathways. *Annals of neurology* **95**, 27-41, doi:<https://doi.org/10.1002/ana.26774> (2024).
- 29 Chen, W. *et al.* Distinct Functional Alterations and Therapeutic Options of Two Pathological De Novo Variants of the T292 Residue of GABRA1 Identified in Children with Epileptic Encephalopathy and Neurodevelopmental Disorders. *International Journal of Molecular Sciences* **23**, 2723 (2022).
- 30 Scheffer, I. E. *et al.* ILAE classification of the epilepsies: Position paper of the ILAE Commission for Classification and Terminology. *Epilepsia* **58**, 512-521, doi:10.1111/epi.13709 (2017).
- 31 Fisher, R. S. *et al.* Operational classification of seizure types by the International League Against Epilepsy: Position Paper of the ILAE Commission for Classification and Terminology. *Epilepsia* **58**, 522-530, doi:10.1111/epi.13670 (2017).
- 32 Wirrell, E. C. *et al.* Methodology for classification and definition of epilepsy syndromes with list of syndromes: Report of the ILAE Task Force on Nosology and Definitions. *Epilepsia* **63**, 1333-1348, doi:10.1111/epi.17237 (2022).

- 33 Zuberi, S. M. *et al.* ILAE classification and definition of epilepsy syndromes with onset in neonates and infants: Position statement by the ILAE Task Force on Nosology and Definitions. *Epilepsia* **63**, 1349-1397, doi:10.1111/epi.17239 (2022).
- 34 Specchio, N. *et al.* International League Against Epilepsy classification and definition of epilepsy syndromes with onset in childhood: Position paper by the ILAE Task Force on Nosology and Definitions. *Epilepsia* **63**, 1398-1442, doi:10.1111/epi.17241 (2022).
- 35 Liao, V. W. Y. *et al.* Concatenated gamma-aminobutyric acid type A receptors revisited: Finding order in chaos. *The Journal of general physiology* **151**, 798-819, doi:10.1074/jbc.RA118.005697 (2019).
- 36 Liao, V. W. Y., Chebib, M. & Ahring, P. K. Efficient expression of concatenated $\alpha 1\beta 2\delta$ and $\alpha 1\beta 3\delta$ GABA(A) receptors, their pharmacology and stoichiometry. *British journal of pharmacology* **178**, 1556-1573, doi:10.1111/bph.15380 (2021).
- 37 Marini, C. *et al.* Childhood absence epilepsy and febrile seizures: a family with a GABA(A) receptor mutation. *Brain* **126**, 230-240, doi:10.1093/brain/awg018 (2003).
- 38 Maillard, P. Y. *et al.* Molecular and clinical descriptions of patients with GABA(A) receptor gene variants (GABRA1, GABRB2, GABRB3, GABRG2): A cohort study, review of literature, and genotype-phenotype correlation. *Epilepsia* **63**, 2519-2533, doi:10.1111/epi.17336 (2022).
- 39 Carvill, G. L. *et al.* GABRA1 and STXBP1: novel genetic causes of Dravet syndrome. *Neurology* **82**, 1245-1253, doi:10.1212/WNL.0000000000000291 (2014).
- 40 Macdonald, R. L., Kang, J. Q. & Gallagher, M. J. Mutations in GABAA receptor subunits associated with genetic epilepsies. *The Journal of physiology* **588**, 1861-1869, doi:10.1113/jphysiol.2010.186999 (2010).
- 41 Kang, J. Q., Shen, W. & Macdonald, R. L. The GABRG2 mutation, Q351X, associated with generalized epilepsy with febrile seizures plus, has both loss of function and dominant-negative suppression. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 2845-2856, doi:10.1523/jneurosci.4772-08.2009 (2009).
- 42 Sadleir, L. G. *et al.* Family studies of individuals with eyelid myoclonia with absences. *Epilepsia* **53**, 2141-2148, doi:10.1111/j.1528-1167.2012.03692.x (2012).

712 Figure and table legends

713 **Figure 1. Structural location of GABRG2 variants. (Top)** 3-D representation of the $\alpha 1\beta 3\gamma 2$
714 GABA_AR (pdb:6hup) with the location of assessed missense $\gamma 2$ variants displayed as spheres.
715 Red spheres represent variants with complete sequence identity across all main GABA_A
716 receptor subunits, yellow spheres with sequence identity across $\gamma 1$ -3 subunits and green
717 spheres with limited sequence identity across GABA_AR subunits. **(Middle)** 2-D
718 representation of the amino acid sequence of the GABA_AR $\gamma 2$ subunit peptide with the
719 location of null variants (asterisks) and missense variants with the indicated colour scheme.
720 Transmembrane regions are represented as open boxes. **(Bottom)** Sequence alignments of $\gamma 1$ -
721 3 with $\alpha 1$ and $\beta 3$ subunits in the immediate vicinity of missense variants.

722 **Figure 2. Functional analysis of $\alpha 1\beta 3\gamma 2$ GABA_ARs containing $\gamma 2$ mutations. (A)** The
723 concatenated pentameric $\gamma 2$ - $\beta 3$ - $\alpha 1$ - $\beta 3$ - $\alpha 1$ cDNA construct utilised for functional analysis is
724 illustrated with four linkers (L, grey) and resulting expressed fusion protein viewed from the
725 extracellular side. A yellow star indicates that $\gamma 2$ subunit mutations are entered in the 1st
726 subunit position. **(B)** Representative traces depict concentration-response relationships for the
727 wild-type $\alpha 1\beta 3\gamma 2$ receptor and receptors containing the $\gamma 2^{A322T}$ and $\gamma 2^{A106T}$ mutations. Bars
728 above traces designate the 25-s application times and GABA concentrations. Normalised
729 GABA concentration-response relationships were plotted as a function of the GABA
730 concentration and the Hill equation was fitted to each dataset by non-linear regression
731 (Supplementary Table). Datapoints are presented as mean $\pm$ SD for n = 12-13 independent
732 experiments. Dotted lines indicate the concentrations that lead to half maximal activation
733 (EC₅₀) for each receptor type. Arrows indicate whether a variant caused increased (GoF) or a
734 decreased (LoF) GABA sensitivity. **(D, left)** The difference in GABA sensitivity between
735 wild-type and 15 mutated receptors was calculated from the logarithmic conversion of EC₅₀
736 values (ΔLogEC_{50}) on each experimental day. Final ΔLogEC_{50} datasets for each variant
737 contain data from n = 12-29 independent experiments and are presented as mean $\pm$ SD with
738 individual datapoints (Supplementary Table). Blue indicates mutations with significantly
739 increased sensitivity to GABA, red indicates variants with significantly decreased GABA
740 sensitivity and grey indicates mutations with no significant change. Significance was
741 determined by one-way ANOVA ($F(15, 392) = 120$; $P < 0.0001$) with Dunnett's post hoc
742 test (**** = $P < 0.0001$). The transparent blue and red areas above 0.2 and below -0.2,
743 respectively, indicate the typical areas wherein mutated receptors show significance. **(D,**

right) Normalised maximal GABA-evoked current amplitudes are presented as median with inter-quartile ranges (IQR) for n = 303 (WT) or n = 27-65 (γ 2 mutations) experiments. Red indicates mutations causing LOF whereas black indicates mutations with no significant change. Significance was determined using Mann-Whitney U test (****, $P < 0.0001$).

Figure 3. Representative interictal EEG from individuals harbouring a LoF or GoF variants. (A) Normal/slightly slowed background activity, with generalised high amplitude 3–4 Hz activity/spike and slow waves in an individual with a null LoF variant. (B) A disorganised background activity with intermixed multifocal epileptiform abnormalities (poly-spike-waves) in individual with GoF variant. (C) Normal background activity with intermixed sharp-wave-transients over the right frontal and temporal areas, followed by diffuse sharp-waves (>right fronto-temporal) in individual with GoF variant.

Figure 4. Key clinical differences between individuals with null LoF, missense LoF and GoF *GABRG2* variants. All displayed clinical information is extracted from Tables 2 and 3.

Table 1. Summary of key genetic and phenotypic characteristics of 35 individuals with pathogenic or likely pathogenic variants in *GABRG2*. CAE: Childhood absence epilepsy; DD: developmental disorder; DEE: developmental and epileptic encephalopathy; F: female; Fo: focal; FS: Febrile seizures; FS+: Febrile seizures plus; G: generalised; GEFS+: Genetic epilepsy with febrile seizures plus; EMA: Epilepsy with myoclonic absences; ID: intellectual disability; M: male; Mat: maternal; m: month; MAE: Myoclonic atonic epilepsy or Doose syndrome; NR: not relevant; Pat: paternal; UK: unknown; y: year; (u): unaffected parent; * protein termination. ^asiblings (same father); ^bsiblings; ^csiblings; ^dseemingly *de novo*, parental mosaicism suspected although not proven yet.

Table 2. Clinical characteristics of individuals with null LoF, missense LoF and GoF *GABRG2* variants. DD: Developmental Delay; GoF: Gain of Function; ID: Intellectual Disability; LoF: Loss of Function; m: months; y: year. ^aNot Relevant, as individuals are too severely affected for determination.

Figure 1.

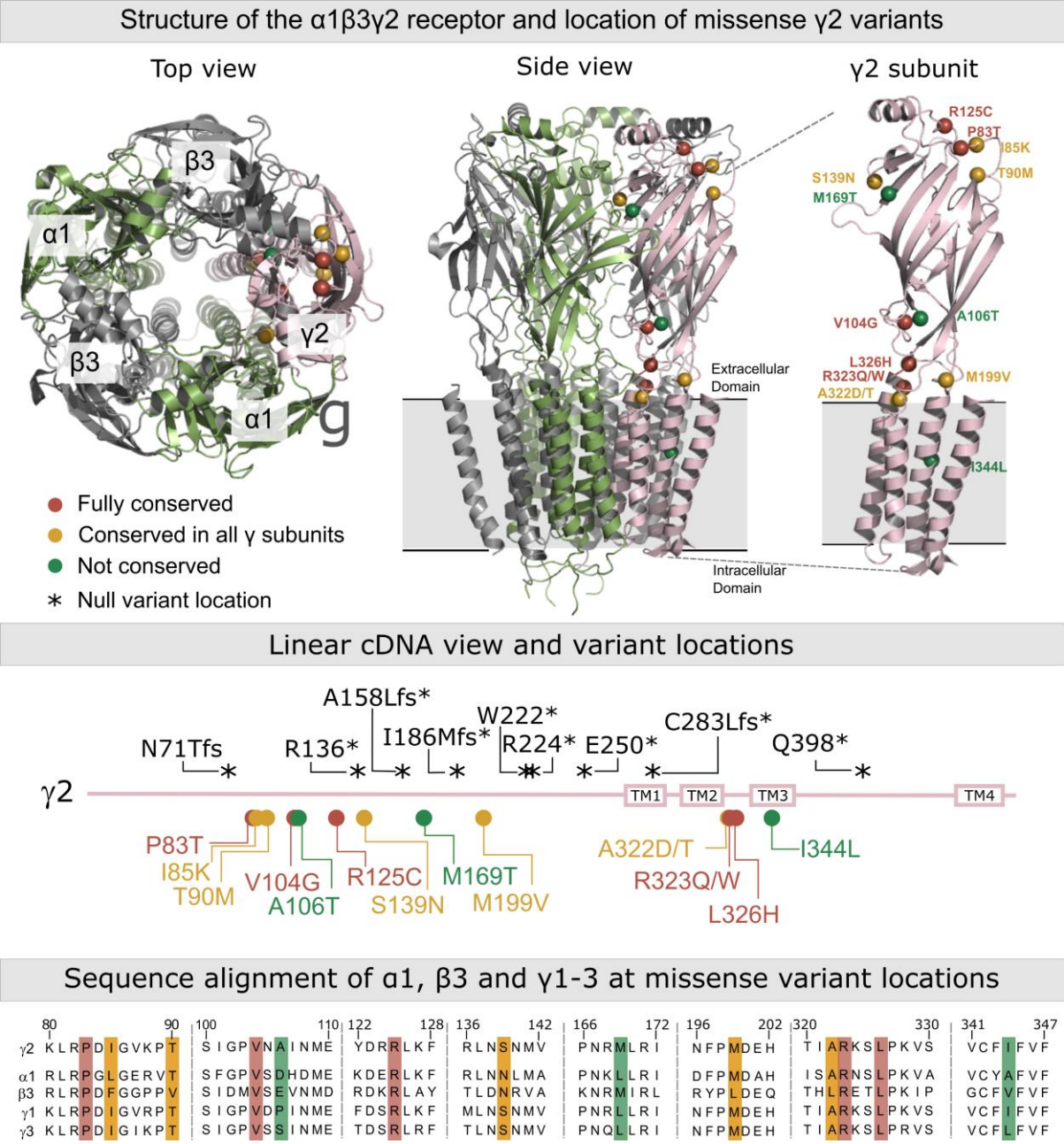
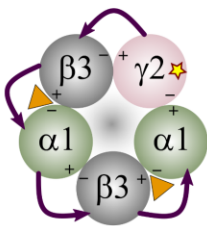


Figure 2.

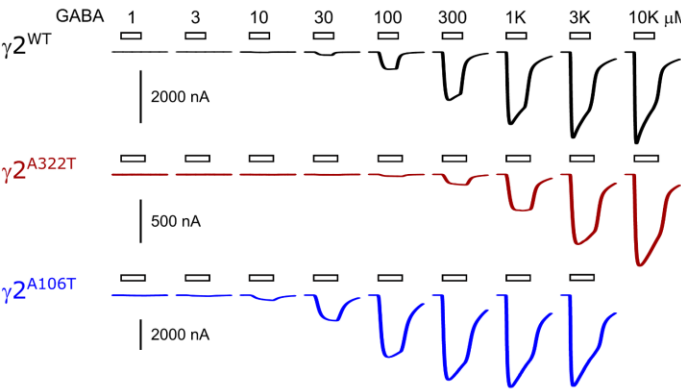
A

Concatenated cDNA construct design for expression of $\alpha 1\beta 3\gamma 2$ GABA_A receptors with $\gamma 2$ subunit mutations

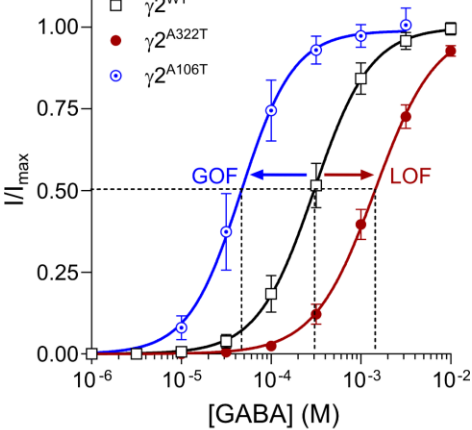


B

Representative traces for receptors with $\gamma 2$ mutations



C



D

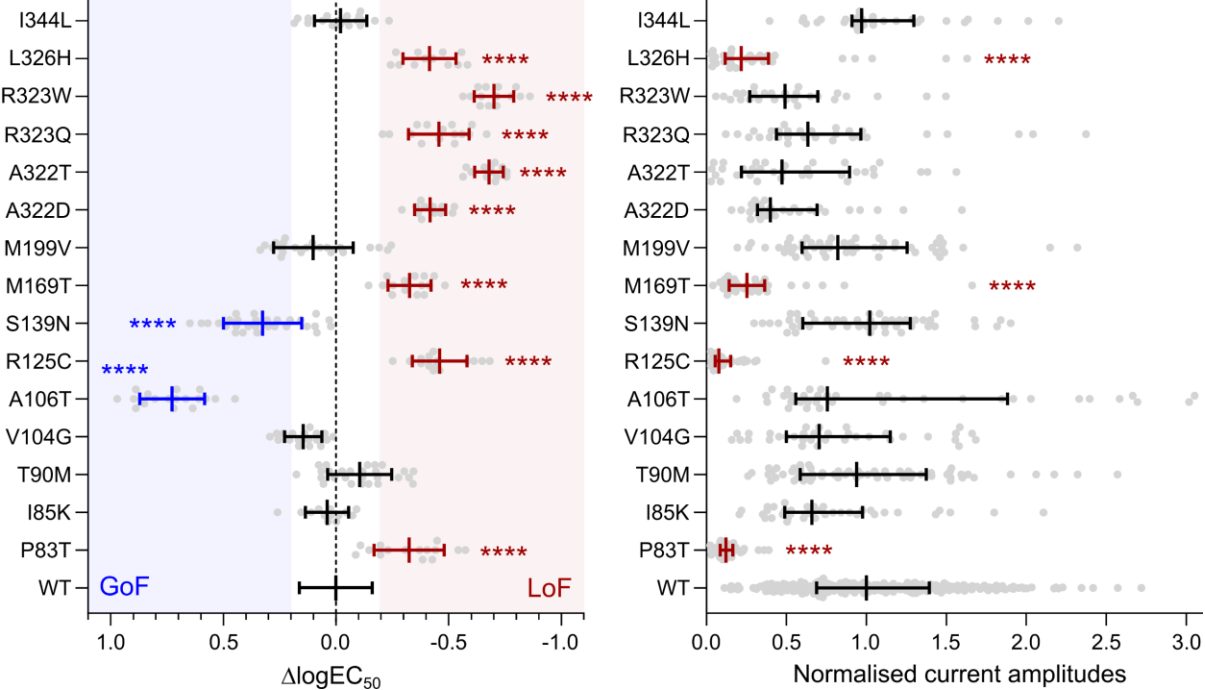
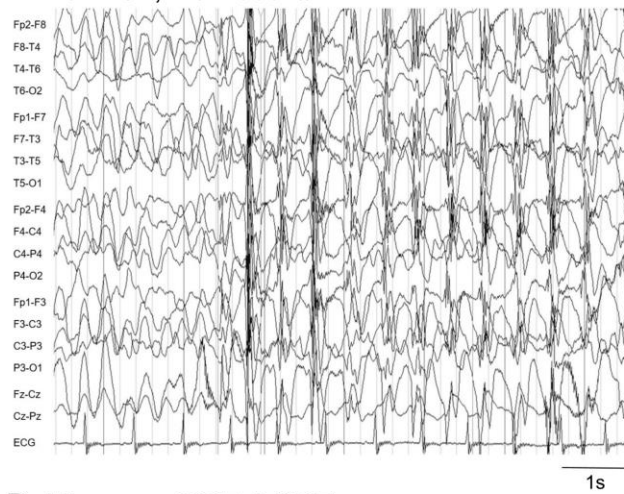


Figure 3.

A Null LOF, individual #14



B Missense GOF, A106T



C Missense GOF, S139N, individual #37



Figure 4.

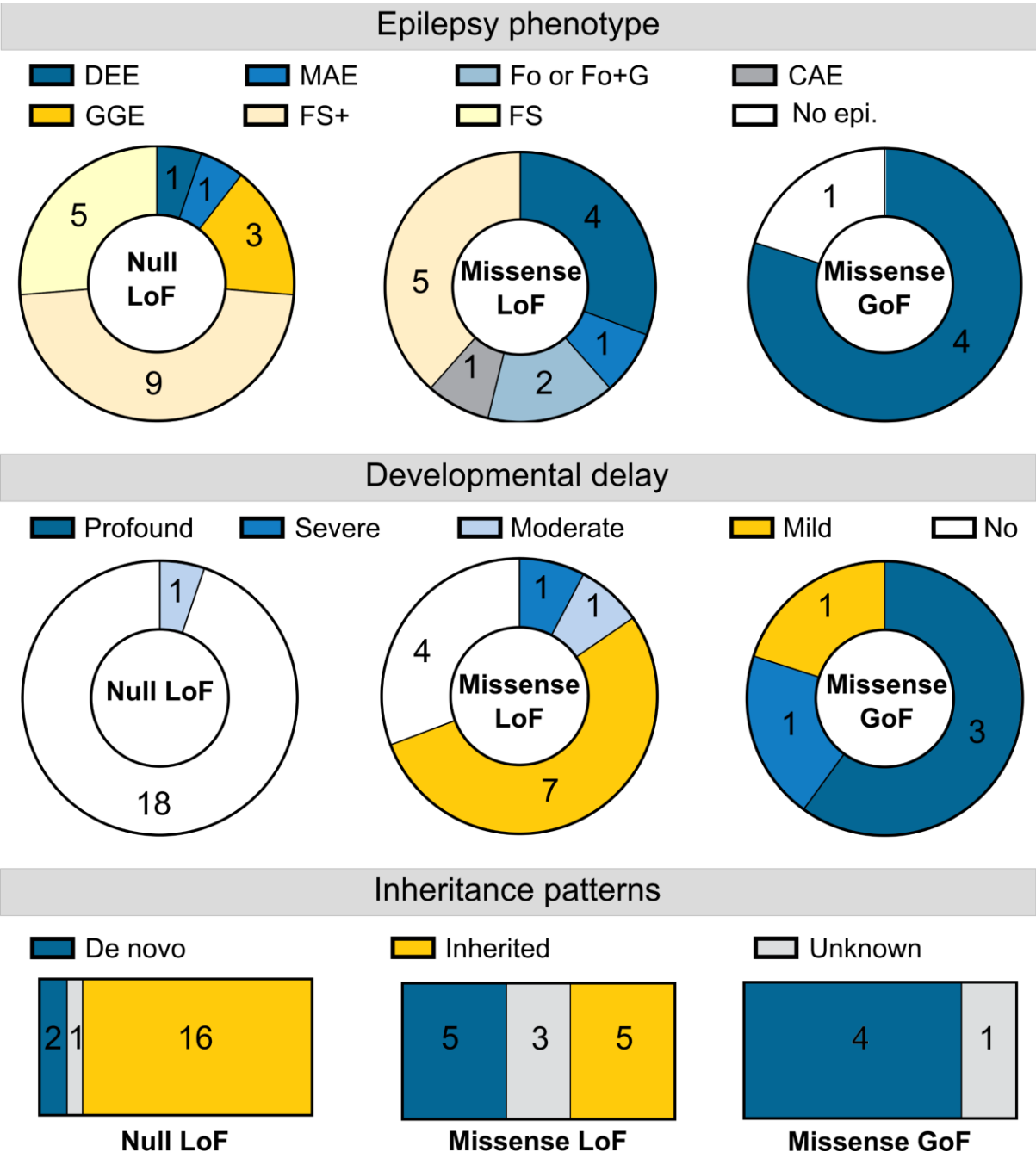


Table 1

#	Age at last follow up	GABRG2 variant	Inheritance	Epilepsy	Age at seizure onset (m)	Epilepsy diagnosis	DD	ID
Null variants								
1	2y10m	N71Tfs*14	<i>De novo</i>	Yes	12	FS+	No	NR
2	43y	R136*	Mat (u)	No	60	FS	No	No
3	5y5m	R136*	Pat (u)	Yes	12	DEE	Moderate	Moderate
4	3y7m	A158Lfs*13	Pat	Yes	14	GGE (GEFS+)	No	No
5	12y	186Mfs*59	Mat	Yes	18	FS+ (GEFS+)	No	No
6	5y	W222*	Pat	No	9	FS (GEFS+)	No	No
7	15y5m	R224*	Pat (u)	Yes	28	FS+ (GEFS+)	No	No
8	5y	E250*	Mat	Yes	12	MAE (GEFS+)	No	No
9	8y6m	C283Lfs*16	Mat	Yes	6	GGE (GEFS+)	No	No
10	9y	Q398*	Mat	No	30	FS (GEFS+)	No	No
11	1y7m	Large deletion	Mat	No	9	FS (GEFS+)	No	NR
12	6y9m	Large deletion	Mat	Yes	14	FS+ (GEFS+)	No	No
13	6y2m	Large deletion	Mat	Yes	16	FS+ (GEFS+)	No	No
14	3y8m	Large deletion	Pat	Yes	25	GGE (GEFS+)	No	NR
15	6y6m	Splice site variant	UK	Yes	5	FS+	No	No
16	17y	Splice site variant ^a	Pat	Yes	21	FS+ (GEFS+)	No	Borderline
17	23y	Splice site variant ^a	Pat	No	6	FS (GEFS+)	No	No
18	4y	Splice site variant	Pat	Yes	15	FS+ (GEFS+)	No	NR
19	8y1m	Splice site variant	<i>De novo</i>	Yes	20	FS+	No	No
Missense LoF variants								
20	5y	P83T	Mat	Yes	42	CAE	No	NR
21	9y10m	R125C	Pat	Yes	17	FS+ (GEFS+)	Mild	No
22	24y	M169T ^b	UK ^d	Yes	24	DEE	Severe	Profound
23	18y	M169T ^b	UK ^d	Yes	24	Fo+G	Mild	Mild
24	5y	A322D ^c	Pat	Yes	13	FS+ (GEFS+)	No	NR
25	1y11m	A322D ^c	Pat	Yes	16	FS+ (GEFS+)	No	NR
26	2y	A322T	<i>De novo</i>	Yes	10	DEE	Mild	NR
27	2y2m	R323Q	<i>De novo</i>	Yes	7	MAE	Mild	NR
28	8y6m	R323Q	Mat	Yes	11	DEE	Moderate	Moderate
29	15y10m	R323Q	<i>De novo</i>	Yes	9	FS+	Mild	Mild
30	25y9m	R323W	<i>De novo</i>	Yes	17.5	DEE	Mild	Moderate
31	6y	R323W	UK	Yes	8	Fo	No	No
32	7y6m	L326H	<i>De novo</i>	Yes	17	FS+	Mild	Mild
Missense GoF variants								
33	1y9m	A106T	<i>De novo</i>	Yes	3.5	DEE	Profound	NR
34	10y	A106T	<i>De novo</i>	Yes	2	DEE	Severe	Severe
35	4y8m	A106T	UK	Yes	1.5	DEE	Profound	NR
36	32y8m	A106T	<i>De novo</i>	Yes	1	DEE	Profound	Profound
37	16y11m	S139N	<i>De novo</i>	No	NR	NR	Mild	Mild

Table 2

Clinical feature	Null LoF (n = 19)	Missense LoF (n = 13)	GoF (n = 5)
Male: Female	8 (42%): 11 (58%)	7 (54%): 6 (46%)	3 (60%): 2 (40%)
Age, median (range)	6.5y (1.6 - 43y)	7.5y (1.9 - 25.8y)	10y (1.8 - 32.7y)
Febrile seizures	17/19 (89%)	6/13 (46%)	1/4 (25%)
Epilepsy	14/19	13/13	4/5
Seizure onset, median (range)	16m (5m - 2y 4m)	16m (7m - 3y 6m)	1.8m (1 - 3.5m)
Status epilepticus	1/13 (8%)	4/13 (31%)	1/4 (25%)
Seizure types			
Tonic-clonic	10/14 (71%)	12/13 (92%)	4/4 (100%)
Atonic	3/14 (21%)	2/13 (15%)	0
Typical absences	4/14 (29%)	2/13 (15%)	0
Atypical absences	1/14 (7%)	1/13 (8%)	0
Myoclonic absences	0	1/13 (8%)	0
Myoclonic	3/14 (21%)	0	2/4 (50%)
Focal	4/14 (29%)	5/13 (39%)	4/4 (100%)
Gelastic	0	0	1/4 (25%)
Tonic asymmetric	0	0	1/4 (25%)
Seizure frequency			
Multiple daily	0	1/12 (8%)	3 (75%)
Daily	1/14 (7%)	1/12 (8%)	0
Weekly	1/14 (7%)	0	0
Monthly	1/14 (7%)	2/12 (17%)	0
Rare	2/14 (14%)	2/12 (17%)	1/4 (25%)
Seizure free	9/14 (64%)	6/12 (50%)	0
Neurodevelopment			
Normal	18/19 (95%)	4/13 (31%)	0
Mild DD	0	7/13 (54%)	1/5 (20%)
Moderate DD	1/19 (5%)	1/13 (8%)	0
Severe/Profound DD	0	1/13 (8%)	4/5 (80%)
Language			
Speech delay/impairment	2/19 (11%)	5/12 (42%)	1/5 (20%)
Nonverbal	0	0	4/5 (80%)
Cognition			
Normal	14/15 (93%)	2/8 (25%)	0
Mild ID	0	3/8 (38%)	1/3 (33%)
Moderate ID	1/15 (7)	2/8 (25%)	0
Severe/Profound ID	0	1/8 (13%)	2/3 (67%)
Psychiatric/behavioural problems	3/18 (19%)	8/13 (62%)	1/1 (100%) (4/4 NR) ^a
Neurological features			
Hypotonia	1/18 (6%)	1/13 (8%)	5/5 (100%)
Chorea, dystonia	0	0	1/5 (20%)

Supplementary information for Correlations of receptor desensitization of gain-of-function GABRB3 variants with clinical severity

This section is supplementary to Chapter 6 of this thesis

Supplementary Information

Correlations of receptor desensitization of gain-of-function *GABRB3* variants with clinical severity

Susan X.N. Lin¹, Philip K. Ahring¹, Angelo Keramidas², Vivian W.Y. Liao¹, Rikke S. Møller^{3,4}, Mary Chebib¹ and Nathan L. Absalom^{1,5*}.

¹Brain and Mind Centre, School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, Sydney, New South Wales, Australia.

²Institute for Molecular Bioscience, The University of Queensland, Saint Lucia, QLD 4072, Australia

³Department of Epilepsy Genetics and Personalized Medicine, Danish Epilepsy Centre, Dianalund, Denmark. Member of ERN, EpiCare.

⁴Department of Regional Health Research, Faculty of Health Sciences, University of Southern Denmark

⁵School of Science, University of Western Sydney, Sydney, New South Wales, Australia

Table of Contents

Supplementary Results Text	2
Concatenated vs binary and ternary freely expressing receptors	2
Simulated synaptic current activation	2
Supplementary Figures	3
Supplementary Figure S1. Concatenated receptors eliminate recordings from binary $\alpha 1\beta 3$ receptors and display similar maximum currents to free subunits expressed in <i>Xenopus</i> oocytes.	3
Supplementary Figure S2. $\beta 3^{T287I}$ and $\gamma 2^{R323Q}$ variants alter activation and deactivation properties of $\alpha 1\beta 3\gamma 2$ GABA _A receptors expressed in HEK293 cells similarly to receptors expressed in <i>Xenopus</i> oocytes.	4
Supplementary Figure S3. Maximum current levels of <i>GABRB3</i> gain of function receptors.	5
Supplementary Tables	6
Table S1. Parameters for concatenated and untethered receptors.....	6
Table S2. ANOVA results for Table S1	7
Table S3. Peak currents and I_{ss}/I_{peak} values.....	8
Table S4. ANOVA results for Tables 1, S3 and S9.....	9
Table S5. Parameters for two-phase current decay.....	10
Table S6. ANOVA results for Table S5.	11
Table S7. Parameters of receptors expressed in HEK293 cells.	12
Table S8. ANOVA results for Table S7	13
Table S9. Desensitization parameters of loss-of-function <i>GABRB3</i> variants.....	14

Supplementary Results Text

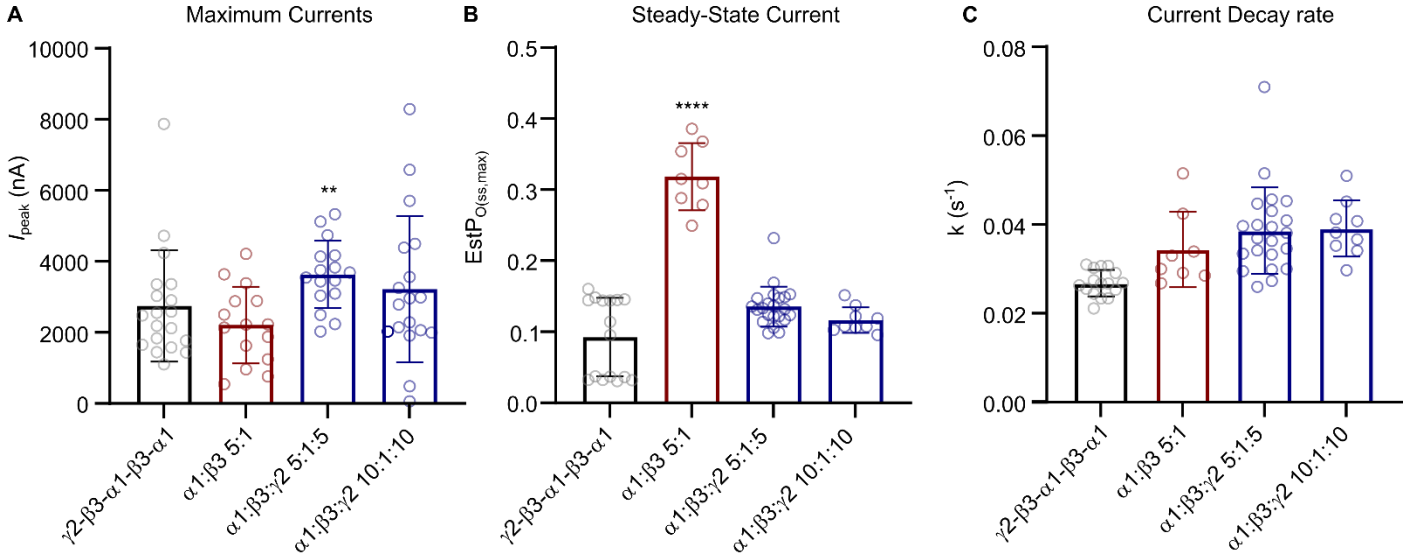
Concatenated vs binary and ternary freely expressing receptors

We evaluated the maximum currents, maximal estimated open probability, 3mM GABA current decay rates and steady-state currents at equilibrium and GABA sensitivity for oocytes injected with concatenated $\alpha 1\beta 3\gamma 2$, $\alpha 1$ and $\beta 3$, or $\alpha 1$, $\beta 3$ and $\gamma 2$ subunits. Binary $\alpha 1\beta 3$ receptors displayed markedly higher steady-state currents and steady-state open probability, and higher GABA sensitivity to either concatenated or $\alpha 1\beta 3\gamma 2$ receptors expressed from free subunits (Supplementary Figure S1; Table S1-2). Although broadly similar to $\alpha 1\beta 3\gamma 2$ receptors expressed from free subunits, concatenated receptors had lower GABA sensitivity and decelerated decay rates with the fraction of the slow phase of current decay increased at concatenated receptors (Supplementary Table S1-2), as expected for currents elicited by a lower effective GABA concentration. Thus, the experimental paradigm where concatenated variant receptors were compared to wild-type variants was considered preferable to comparing variants from free subunits, where contamination of $\alpha 1\beta 3$ receptor populations will lead to aberrant results.

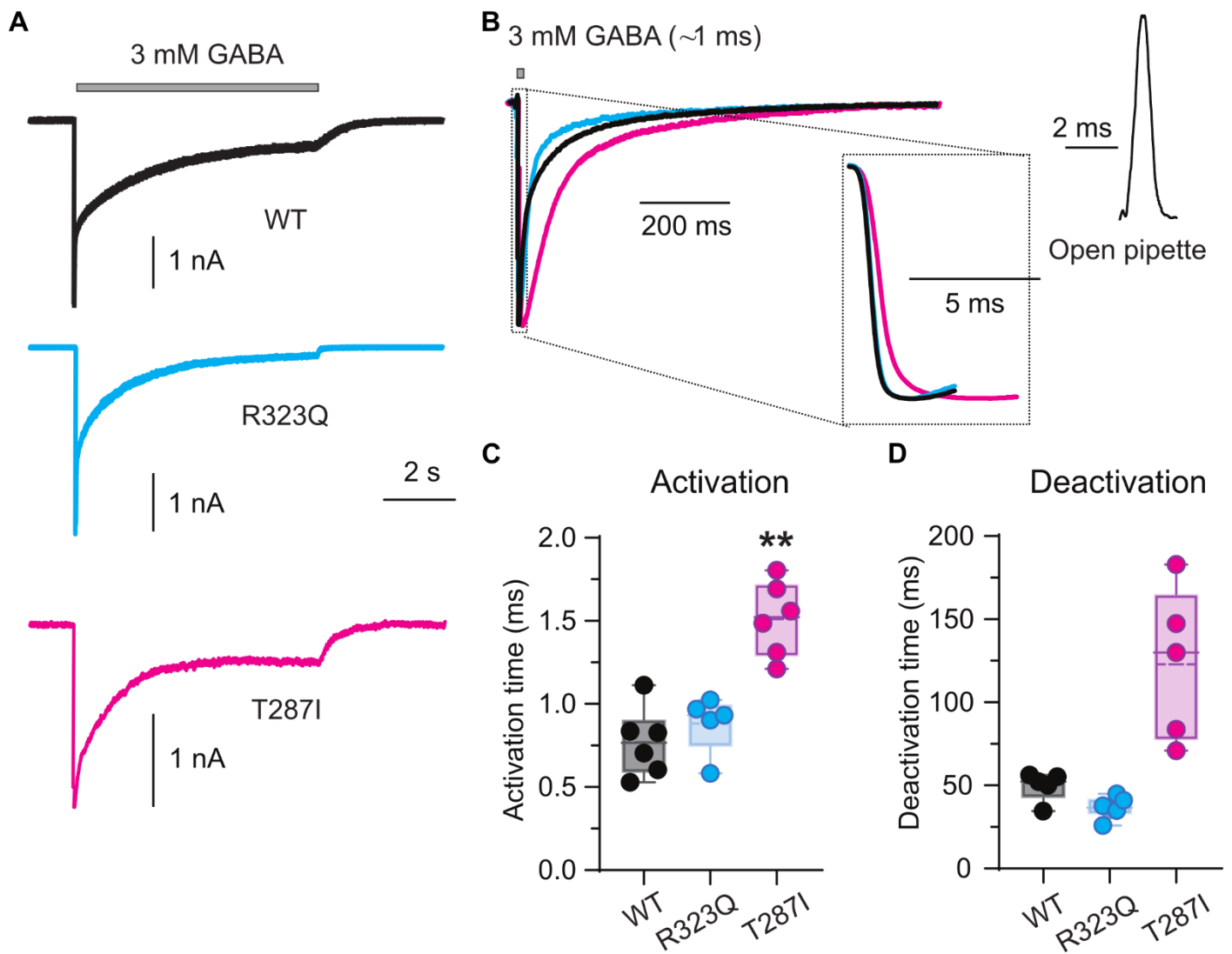
Simulated synaptic current activation

Synaptic currents were simulated by brief application (1-2 ms) of 3 mM GABA onto cells or membrane patches (Supplementary Figure S2). The rate of current onset and decay (deactivation) was determined by fitting the onset phase to a single exponential function and the deactivation to two exponential functions. The activation rates for WT and $\gamma 2^{R323Q}$ -containing receptors were similar. By contrast, $\beta 3^{T287I}$ -containing receptors activated more slowly than the other two receptors (Supplementary Figure S2, Supplementary Table S7-8). Current deactivation between WT and $\gamma 2^{R323Q}$ -containing receptors was similar. The weighted deactivation times for the $\beta 3^{T287I}$ -containing receptors displayed considerably greater variance than the other two experiments, but always greater than the other two receptors (Supplementary Figure S2). However, the mean deactivation time did not reach statistical significance (Supplementary table S7-8).

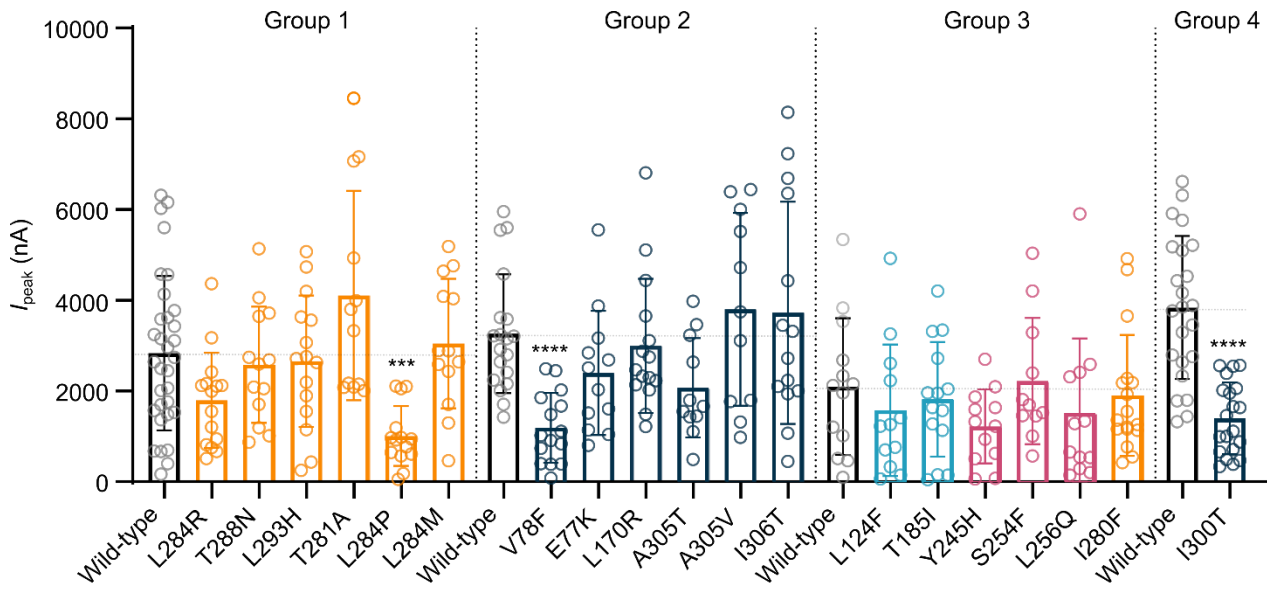
Supplementary Figures



Supplementary Figure S1. Concatenated receptors eliminate recordings from binary $\alpha 1\beta 3$ receptors and display similar maximum currents to free subunits expressed in *Xenopus* oocytes. **A** Bar graph representing the peak currents elicited by 3 mM GABA at receptors expressed in *Xenopus* oocytes with $\gamma 2\text{-}\beta 3\text{-}\alpha 1\text{-}\beta 3\text{-}\alpha 1$ concatenated constructs (black), $\alpha 1$ and $\beta 3$ subunits in a 5:1 ratio (red) and $\alpha 1$, $\beta 3$ and $\gamma 2$ subunits in a 5:1:5 or 10:1:10 ratio (blue). **B** Bar graph representing the estimated steady-state open probability elicited by 3 mM GABA at receptors expressed in *Xenopus* oocytes with $\gamma 2\text{-}\beta 3\text{-}\alpha 1\text{-}\beta 3\text{-}\alpha 1$ concatenated constructs (black), $\alpha 1$ and $\beta 3$ subunits in a 5:1 ratio (red) and $\alpha 1$, $\beta 3$ and $\gamma 2$ subunits in a 5:1:5 or 10:1:10 ratio (blue). **C** Bar graph representing the current decay rate elicited by 3 mM GABA at receptors expressed in *Xenopus* oocytes with $\gamma 2\text{-}\beta 3\text{-}\alpha 1\text{-}\beta 3\text{-}\alpha 1$ concatenated constructs (black), $\alpha 1$ and $\beta 3$ subunits in a 5:1 ratio (red) and $\alpha 1$, $\beta 3$ and $\gamma 2$ subunits in a 5:1:5 or 10:1:10 ratio (blue). For all graphs, bars represent mean $\pm$ s.d, circles represent individual experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to WT, non-parametric ANOVA with Dunn's post-hoc test.



Supplementary Figure S2. $\beta 3^{T287I}$ and $\gamma 2^{R323Q}$ variants alter activation and deactivation properties of $\alpha 1\beta 3\gamma 2$ GABA_A receptors expressed in HEK293 cells similarly to receptors expressed in *Xenopus* oocytes. **A** Representative traces of WT (black), $\gamma 2^{R323Q}$ (aqua) and $\beta 3^{T287I}$ (pink) variant receptors activated by a prolonged (~7s) 3 mM GABA pulse and recorded from transfected HEK293 cells in whole-cell configuration. **B** Representative traces for determination of activation and deactivation time constants at WT (black), $\gamma 2^{R323Q}$ (aqua) and $\beta 3^{T287I}$ (pink) variant receptors recorded from transfected HEK293 cells. An inset displaying the traces during the activation time is shown, and the open tip potential for a representative wild-type experiment. **C** Box and whisker plot of activation times. **D** Box and whisker plot of deactivation times. For all graphs, bars represent mean $\pm$ s.d, circles represent individual experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to WT, non-parametric ANOVA with Dunn's post-hoc test.



Supplementary Figure S3. Maximum current levels of *GABRB3* gain of function receptors. Bar graph representing the peak currents elicited by 3 mM GABA at wild-type (black) and variant receptors in the extracellular regions (light blue), coupling regions (dark blue), M1 (magenta) and M2 (orange) expressed in *Xenopus* oocytes with $\gamma 2$ - $\beta 3$ - $\alpha 1$ - $\beta 3$ - $\alpha 1$ concatenated constructs. Open circles represent individual data points. Bars represent mean $\pm$ s.d, circles represent individual experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to WT, non-parametric ANOVA with Dunn's post-hoc test.

Supplementary Tables

Table S1. Parameters for concatenated and untethered receptors.

Construct	Activation properties									
	I_{peak}	p-value ^b	Est P _{O(max)}	p-value ^b	N	logEC ₅₀	p-value ^a	n _H	p-value ^b	n ^c
γ2-β3-α1-β3-α1 ^a	2749±1572		0.83±0.07		19	-3.48±0.13		1.6±0.2		11
α1β3 (5:1)	2208±1076	0.99	0.74±0.20	0.99	15	-5.06±0.4	<0.0001	0.56±0.19	<0.0001	8
α1β3γ2 (5:1:5)	3631±946	0.02	1.22±0.11	0.02	16	-4.87±0.25	<0.0001	1.2±0.3	0.09	10
α1β3γ2 (10:1:10)	3222±2057	0.98	1.26±0.43	0.02	18	-4.81±0.33	<0.0001	1.4±0.5	0.99	9
	One-phase decay									
	k	p-value ^b	EstP _{O(ss,max)}	p-value ^b	I_{ss}	p-value ^b	I_{ss}/I_{peak}	p-value ^b	n ^c	
γ2-β3-α1-β3-α1 ^a	0.027±0.003		0.092±0.06		381±162		0.077±0.046		16	
α1β3 (5:1)	0.034±0.009	0.068	0.32±0.05	<0.0001	624±116	0.019	0.24±0.04	<0.0001	8	
α1β3γ2 (5:1:5)	0.039±0.010	<0.0001	0.14±0.03	0.25	640±171	0.0006	0.17±0.03	<0.0001	22	
α1β3γ2 (10:1:10)	0.039±0.006	0.0002	0.12±0.02	0.99	713±202	0.0009	0.15±0.02	0.0174	9	
	Two-phase decay									
	Fraction _{fast}	p-value ^b	τ _{weighted}	p-value ^b	τ _{fast}	p-value ^b	τ _{slow}	p-value ^b	n ^c	
γ2-β3-α1-β3-α1 ^a	0.25±0.04		40.2±5.6		9.95±5.0		50.5±6.8		16	
α1β3 (5:1)	0.48±0.08	0.0001	43.5±13	0.999	10.3±3.1	n.d.*	73.4±17	0.02	7	
α1β3γ2	0.46±0.09	<0.0001	31.6±8.0	0.0196	7.36±2.4	n.d.*	53.1±11	0.99	18	
α1β3γ2 (10:1:10)	0.45±0.07	<0.0004	29.1±4.9	0.0067	7.7±2.7	n.d.*	46.9±8.7	0.75	8	

^aExperimental groups where variants and wild-type performed on same day

^bOne-way Kruskal-Wallis non-parametric ANOVA followed by a Dunn's post-hoc test except $\log EC_{50}$ where a one-way ANOVA with Dunnett's post-hoc test

^cNumbers of individual oocytes where two-phase decay successfully fitted

*Kruskal-Wallis test $p>0.05$, Dunn's post-hoc test not performed

Table S2. ANOVA results for Table S1

Activation properties											
I_{peak}			Est $P_{O(max)}$			$\log EC_{50}$			n_H		
H^a	df^b	p	H^a	df^b	p	F^c	df^d	p	H^a	df^b	p
11.75	3	0.008	18.83	3	0.003	68.68	3, 34	<0.0001	21.1	3	0.0001
One-phase decay											
k			Est $P_{O(ss,max)}$			I_{ss}			I_{ss}/I_{peak}		
H^a	df^b	p	H^a	df^b	p	H^a	df^b	p	H^a	df^b	p
25.21	3	<0.0001	23.82	3	<0.0001	19.24	3	0.0002	39.86	3	<0.0001
Fraction _{fast}			$\tau_{weighted}$			τ_{fast}			τ_{slow}		
H^a	df^b	p	H^a	df^b	p	H^a	df^b	p	H^a	df^b	p
31.6	3	<0.0001	15.88	3	0.0012	6.892	3	0.075	12.09	3	0.0071

^aH is the Kruskal-Wallis statistic

^bdf is the degrees of freedom

^cThe F-statistic from the one-way ANOVA

^ddf is the degrees of freedom of the ANOVA

Table S3. Peak currents and I_{ss}/I_{peak} values.

Variant	I_{peak} (nA)	p-value ^a	I_{ss} (nA)	p-value ^a	I_{ss}/I_{peak}	p-value ^a	n ^b
Group 1 ^c							
Wild-type	2992±1342		581±486		0.18±0.08		28
T287I	3272±1439	n.d.*	911±542	0.03	0.30±0.09	<0.0001	22
γ2(R323Q)	2597±1136	n.d.*	432±243	0.78	0.16±0.04	0.45	16
Group 2 ^d							
Wild-type	2832±1701		359±216		0.13±0.04		32
T281A	4102±2307	0.68	811±584	0.01	0.19±0.04	0.10	12
L284M	3045±1427	0.99	372±186	0.99	0.13±0.04	0.99	13
L284P	1006±660	0.0009	343±232	0.99	0.36±0.12	<0.0001	14
L284R	1793±1051	0.32	754±778	0.19	0.38±0.14	<0.0001	15
T288N	2576±1283	0.99	289±213	0.99	0.10±0.03	0.63	13
L293H	2655±1450	0.99	256±175	0.91	0.09±0.03	0.20	15
Group 3 ^e							
Wild-type	3262±1311		420±234		0.12±0.03		19
E77K	2396±1366	0.59	298±279	0.38	0.12±0.04	n.d.*	12
V78F	1187±770	<0.0001	99±57	<0.0001	0.11±0.09	n.d.*	15
L170R	2994±1477	0.99	338±144	0.99	0.12±0.04	n.d.*	15
A305T	2070±1096	0.20	245±116	0.38	0.12±0.03	n.d.*	11
A305V	3798±2125	0.99	600±486	0.99	0.14±0.05	n.d.*	11
I306T	3723±2451	0.99	518±407	0.99	0.14±0.05	n.d.*	14
Group 4 ^f							
Wild-type	2094±1505		246±201		0.11±0.03		13
L124F	1575±1449	n.d.*	179±192	n.d.*	0.10±0.06	0.99	12
T185I	1819±1263	n.d.*	206±218	n.d.*	0.09±0.04	0.99	14
Y245H	1220±815	n.d.*	242±204	n.d.*	0.19±0.05	0.04	12
S254F	2220±1390	n.d.*	483±354	n.d.*	0.21±0.07	0.02	11
L256Q	1516±1644	n.d.*	393±585	n.d.*	0.19±0.10	0.15	12
I280F	1904±1334	n.d.*	169±159	n.d.*	0.09±0.06	0.75	17
Group 5 ^g							
Wild-type	5820±2265		887±336		0.16±0.05		16
M80K	3137±1246	0.001	274±142	<0.0001	0.09±0.03	0.003	16
Q249K	5928±1330	0.99	832±195	0.99	0.14±0.04	0.99	16
T281I	5912±2206	0.99	228±94	<0.0001	0.04±0.01	<0.0001	16
Y302C	4222±1367	0.31	970±246	0.99	0.24±0.04	0.17	11
Wild-type	3839±1580		539±197		0.15±0.03		23
I300T	1393±788	<0.0001	44±26	<0.0001	0.04±0.02	<0.0001	20

^aOne-way Kruskal-Wallis non-parametric ANOVA followed by a Dunn's post-hoc test except I300T that was compared with a Mann-Whitney U test, experimental groups compared separately

^bNumbers of individual oocytes to determine k, Est $P_{O(max)}$ and Est $P_{O(ss, max)}$

^{c-g}Experimental groups where variants and wild-type performed on same day

* n.d. Not determined as $p > 0.05$ for initial ANOVA

Table S4. ANOVA results for Tables 1, S3 and S9.

Table 1	k			Est $P_{O(ss)}$			Est $P_{O(max)}$		
Values									
Group	H^a	df^b	p	H^1	df^b	p	H^1	df^b	p
Group 1	21.83	2	<0.0001	38.09	2	<0.0001	21.28	2	<0.0001
Group 2	11.89	6	0.0645	81.69	6	<0.0001	15.15	6	0.0191
Group 3	53.69	6	<0.0001	14.02	6	0.0294	11.65	6	0.0702
Group 4	66.04	6	<0.0001	43.1	6	<0.0001	10.22	6	0.1156
Group 5 ^c	39.34	5	<0.0001	72.47	5	<0.0001	47.17	5	<0.0001
Group 5 ^d	50.92	4	<0.0001	75.31	4	<0.0001	38.89	4	<0.0001
Table S3	I_{peak}			I_{ss}			I_{ss}/I_{peak}		
Values									
Group	H^a	df^b	p	H^1	df^b	p	H^1	df^b	p
Group 1	2.30	2	0.3163	9.70	2	0.0078	25.99	2	<0.0001
Group 2	27.68	6	0.0001	24.18	6	0.0005	77.56	6	<0.0001
Group 3	26.97	6	0.0001	35.2	6	<0.0001	10.84	6	0.0933
Group 4	5.279	6	0.5085	10.22	6	0.1157	43.65	6	<0.0001
Group 5 ^c	38 ^e	-	-	0 ^e	-	-	0 ^e	-	-
Group 5 ^d	24.68	4	<0.0001	53.28	4	<0.0001	61.16	4	<0.0001

^aH is the Kruskal-Wallis statistic

^bdf is the degrees of freedom

^cFor 3 mM GABA applications

^dFor 30 mM GABA applications

^eMann-Whitney U statistic, only two groups to compare

Table S5. Parameters for two-phase current decay.

Variant	Fraction _{fast}	p-value ^a	τ_{weighted}	p-value ^a	τ_{fast}	p-value ^a	τ_{slow}	p-value ^a	n ^b
Group 1 ^c									
Wild-type	0.16±0.08		64±15		8.2±3.7		75±17		23
T287I	0.26±0.10	0.018	60±17	0.30	7.7±3.7	0.59	78±21	0.70	14
Group 2 ^d									
Wild-type	0.21±0.17		33±10		8.0±6.3		39±13		15
T281A	0.26±0.13	0.99	26±6	0.70	4.8±3.8	n.d.*	33±8	0.99	8
L284M	0.05±0.03	0.12	29±6	0.99	4.7±3.0	n.d.*	64±30	0.99	8
L284P	0.36±0.12	0.01	52±24	0.10	6.3±2.5	n.d.*	78±36	0.001	14
L284R	0.36±0.15	0.03	42±19	0.75	5.9±4.0	n.d.*	64±30	0.07	12
T288N	0.29±0.18	0.99	36±1	0.99	9.0±6.5	n.d.*	47±12	0.77	13
L293H	0.28±0.11	0.64	33±1	0.99	7.2±3.1	n.d.*	43±11	0.99	13
Group 3 ^e									
Wild-type	0.19±0.12		36±6		6.8±3.9		43±9		12
E77K	0.40±0.27	0.07	20±4	<0.0001	8.7±5.9	n.d.*	32±17	0.003	11
V78F	0.27±0.09	0.99	28±4	0.71	8.3±4.9	n.d.*	38±9	0.99	11
L170R	0.23±0.07	0.99	35±11	0.99	7.6±3.9	n.d.*	43±12	0.99	9
A305T	0.35±0.07	0.004	22±4	0.0001	7.1±2.6	n.d.*	30±6	0.02	11
A305V	0.29±0.08	0.13	23±3	0.0013	7.0±2.7	n.d.*	29±4	0.004	11
I306T	0.17±0.12	0.99	31±11	0.54	7.9±3.4	n.d.*	36±11	0.35	9
Group 4 ^f									
Wild-type	0.22±0.13		33±9		6.5±5.9		40±10		15
L124F	0.23±0.13	0.99	25±3	0.33	6.9±4.0	0.99	31±4	0.55	10
T185I	0.28±0.13	0.97	28±5	0.99	7.0±4.0	0.99	38±15	0.99	10
Y245H	0.14±0.02	0.61	71±20	0.03	13.6±2.4	0.0045	94±20	0.009	10
S254F	0.18±0.1	0.99	20±4	0.07	5.8±3.0	0.99	23±3	0.10	4
L256Q	0.41±0.1	0.007	35±9	0.99	11.1±4.6	0.08	51±13	0.96	12
I280F	0.14±0.08	0.85	17±4	0.0003	1.8±1.2	0.07	20±4	0.0008	10
Group 5 ^g									
Wild-type	0.23±0.01		53±9		11.1±4.3		66±11		13
I300T	0.13±0.04	0.08	38±7	0.003	10.4±1.7	0.85	42±7	0.002	5

^aMann-Whitney test for Groups 1 and 5, one-way Kruskal-Wallis non-parametric ANOVA followed by a Dunn's post-hoc test for Groups 2-4, experimental groups compared separately

^bNumbers of individual oocytes where two-phase decay successfully fitted

^{c-g}Experimental groups where variants and wild-type performed on same day

Table S6. ANOVA results for Table S5.

	Fraction _{fast}			τ_{weighted}			τ_{fast}			τ_{slow}		
Group	H ^a	df ^b	p	H ^a	df ^b	p	H ^a	df ^b	p	H ^a	df ^b	p
Group 1	64 ^c	-	-	127 ^c	-	-	143 ^c	-	-	148 ^c	-	-
Group 2	31.93	6	<0.0001	18.19	6	0.0058	6.72	6	0.35	30.43	6	<0.0001
Group 3	22.04	6	0.0012	40.77	6	<0.0001	1.95	6	0.92	26.64	6	0.0002
Group 4	29.53	6	<0.0001	51.06	6	<0.0001	36.29	6	<0.0001	55.82	6	<0.0001
Group 5	14 ^c	-	-	4 ^c	-	-	14 ^c	-	-	3 ^c	-	-

^aH is the Kruskal-Wallis statistic

^bdf is the degrees of freedom

^cThe Mann-Whitney U-statistic is given where there is only one variant in the group

Table S7. Parameters of receptors expressed in HEK293 cells.

Deactivation (1-2ms application of GABA)								
Construct	Activation (ms)	p-value	τ_{weighted}	p-value	Fraction _{fast}	τ_{fast}	τ_{slow}	n
$\alpha 1+\beta 3+\gamma 2$	0.77±0.21		49.7±8.8		66.6±16.2	8.9±1.9	174±115	6
$\alpha 1+\beta 3+\gamma 2^{\text{R323Q}}$	0.88±0.17	0.94	36.7±6.5	0.41	69.4±7.2	7.2±3.5	105±23	6
$\alpha 1+\beta 3^{\text{T287I}}+\gamma 2$	1.51±0.22	0.002	123±46	0.09	86.9±10.6	44±9.4	965±1110	7
Desensitization (Prolonged application of GABA)								
	$I_{\text{ss}}/I_{\text{peak}}$	p-value	τ_{weighted}	p-value	Fraction _{fast}	τ_{fast}	τ_{slow}	n
$\alpha 1+\beta 3+\gamma 2$	0.208±0.047		1860±183		18.5±7	229±214	2237±312	5
$\alpha 1+\beta 3+\gamma 2^{\text{R323Q}}$	0.087±0.04	0.015	1347±264	0.11	19.3±10	147±117	1628±197	5
$\alpha 1+\beta 3^{\text{T287I}}+\gamma 2$	0.270±0.066	0.50	2361±271	0.28	31.8±17	195±113	3392±383	5

Table S8. ANOVA results for Table S7

Deactivation (1-2ms application of GABA)						Desensitization (Prolonged application of GABA)					
Activation (ms)			τ_{weighted}			$I_{\text{ss}}/I_{\text{peak}}$			τ_{weighted}		
H ^a	df ^b	p	H ^a	df ^b	p	H ^a	df ^b	p	H ^a	df ^b	p
11.52	2	0.0002	11.32	2	0.0002	15.93	3	<0.0001	11.58	2	<0.0001

^aH is the Kruskal-Wallis statistic

^bdf is the degrees of freedom

Table S9. Desensitization parameters of loss-of-function *GABRB3* variants.

Variant ^a	ΔLogEC_{50} ^b	k $\pm$ s.d.	p-value ^c	Est $P_{O(ss,max)} \pm$ s.d.	p-value ^c	Est $P_{O(max)} \pm$ s.d.	p-value ^c	n ^d
Group 5								
Wild-type		0.031 $\pm$ 0.0086		0.15 $\pm$ 0.043		1.1 $\pm$ 0.19		14
M80K	-0.56	0.049 $\pm$ 0.011	<0.0003	0.049 $\pm$ 0.013	<0.0001	0.56 $\pm$ 0.13	<0.0001	13
Q249K	-0.53	0.029 $\pm$ 0.0076	>0.9999	0.13 $\pm$ 0.034	>0.9999	0.86 $\pm$ 0.25	0.1424	10
T281I	-0.55	0.043 $\pm$ 0.010	0.0170	0.036 $\pm$ 0.0095	<0.0001	0.97 $\pm$ 0.22	>0.9999	11
Y302C	-0.81	0.023 $\pm$ 0.0080	0.3327	0.16 $\pm$ 0.026	>0.9999	0.68 $\pm$ 0.19	<0.0001	13

^aUnless otherwise specified, variants are in the $\beta 3$ subunit

^bValues taken from Absalom et al, 2022.

^cOne-way Kruskal-Wallis non-parametric ANOVA followed by a Dunn's post-hoc test, experimental groups compared separately

^dNumbers of individual oocytes to determine k, Est $P_{O(max)}$ and Est $P_{O(ss, max)}$

*n.d. Not determined as $p > 0.05$ for initial ANOVA

Chapter 10 References

1. Guin D, Hasija Y, Kukreti R. Assessment of clinically actionable pharmacogenetic markers to stratify anti-seizure medications. *Pharmacogenomics J*. 2023;23(6):149-60.
2. Begley C, Wagner RG, Abraham A, Beghi E, Newton C, Kwon C-S, et al. The global cost of epilepsy: A systematic review and extrapolation. *Epilepsia*. 2022;63(4):892-903.
3. Yang S, Wang B, Han X. Models for predicting treatment efficacy of antiepileptic drugs and prognosis of treatment withdrawal in epilepsy patients. *Acta Epileptologica*. 2021;3(1):1.
4. Stevelink R, Campbell C, Chen S, Abou-Khalil B, Adesoji OM, Afawi Z, et al. GWAS meta-analysis of over 29,000 people with epilepsy identifies 26 risk loci and subtype-specific genetic architecture. *Nat Genet*. 2023;55(9):1471-82.
5. Fisher RS, Acevedo C, Arzimanoglou A, Bogacz A, Cross JH, Elger CE, et al. ILAE Official Report: A practical clinical definition of epilepsy. *Epilepsia*. 2014;55(4):475-82.
6. Stafstrom CE, Carmant L. Seizures and epilepsy: an overview for neuroscientists. *Cold Spring Harbor perspectives in medicine*. 2015;5(6).
7. Jiruska P, de Curtis M, Jefferys JGR, Schevon CA, Schiff SJ, Schindler K. Synchronization and desynchronization in epilepsy: controversies and hypotheses. *J Physiol*. 2013;591(4):787-97.
8. EB B, Cavazos JE SJ. Basic mechanisms underlying seizures and epilepsy. An Introduction to Epilepsy: West Hartford (CT): American Epilepsy Society; 2006.
9. Chang RS-k, Leung CYW, Ho CCA, Yung A. Classifications of seizures and epilepsies, where are we? – A brief historical review and update. *J Formos Med Assoc*. 2017;116(10):736-41.
10. Gao R, Penzes P. Common mechanisms of excitatory and inhibitory imbalance in schizophrenia and autism spectrum disorders. *Curr Mol Med*. 2015;15(2):146-67.
11. Sohal VS, Rubenstein JLR. Excitation-inhibition balance as a framework for investigating mechanisms in neuropsychiatric disorders. *Mol Psychiatry*. 2019;24(9):1248-57.
12. Page CE, Coutellier L. Prefrontal excitatory/inhibitory balance in stress and emotional disorders: evidence for over-inhibition. *Neurosci Biobehav Rev*. 2019;105:39-51.
13. Chen Y, Zheng Y, Yan J, Zhu C, Zeng X, Zheng S, et al. Early life stress induces different behaviors in adolescence and adulthood may related with abnormal medial prefrontal cortex excitation/inhibition balance. *Front Neurosci*. 2021;15:720286.
14. Uhlhaas PJ, Singer W. Abnormal neural oscillations and synchrony in schizophrenia. *Nat Rev Neurosci*. 2010;11(2):100-13.
15. Fatemi SH, Folsom TD, Rooney RJ, Thuras PD. Expression of GABA_A α 2-, β 1- and ε -receptors are altered significantly in the lateral cerebellum of subjects with schizophrenia, major depression and bipolar disorder. *Transl Psychiatry*. 2013;3(9):e303-e.
16. Dani VS, Chang Q, Maffei A, Turrigiano GG, Jaenisch R, Nelson SB. Reduced cortical activity due to a shift in the balance between excitation and inhibition in a mouse model of Rett syndrome. *Proc Natl Acad Sci U S A*. 2005;102(35):12560-5.
17. Mariani J, Coppola G, Zhang P, Abyzov A, Provini L, Tomasini L, et al. FOXP1-dependent dysregulation of GABA/glutamate neuron differentiation in autism spectrum disorders. *Cell*. 2015;162(2):375-90.
18. Rubenstein JLR, Merzenich MM. Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes Brain Behav*. 2003;2(5):255-67.
19. Lee E, Lee J, Kim E. Excitation/Inhibition imbalance in animal models of Autism Spectrum Disorders. *Biol Psychiatry*. 2017;81(10):838-47.

20. Cutler AJ, Mattingly GW, Maletic V. Understanding the mechanism of action and clinical effects of neuroactive steroids and GABAergic compounds in major depressive disorder. *Transl Psychiatry*. 2023;13(1):228.
21. Klempan TA, Sequeira A, Canetti L, Lalovic A, Ernst C, French-Mullen J, Turecki G. Altered expression of genes involved in ATP biosynthesis and GABAergic neurotransmission in the ventral prefrontal cortex of suicides with and without major depression. *Mol Psychiatry*. 2009;14(2):175-89.
22. Stafstrom CE, Rho JM. 63 - Neurophysiology of seizures and epilepsy. In: Swaiman KF, Ashwal S, Ferriero DM, Schor NF, Finkel RS, Gropman AL, et al., editors. *Swaiman's Pediatric Neurology (Sixth Edition)*. 506-512: Elsevier; 2017.
23. Engel J. Excitation and inhibition in epilepsy. *Can J Neurol Sci*. 1996;23(3):167-74.
24. Chao H-T, Chen H, Samaco RC, Xue M, Chahrour M, Yoo J, et al. Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. *Nature*. 2010;468(7321):263-9.
25. Calfa G, Li W, Rutherford JM, Pozzo-Miller L. Excitation/inhibition imbalance and impaired synaptic inhibition in hippocampal area CA3 of *Mecp2* knockout mice. *Hippocampus*. 2015;25(2):159-68.
26. Untiet V, Nedergaard M, Verkhratsky A. Astrocyte chloride, excitatory-inhibitory balance and epilepsy. *Neural Regen Res*. 2024;19(9):1887.
27. Ghit A, Assal D, Al-Shami AS, Hussein DEE. GABA_A receptors: structure, function, pharmacology, and related disorders. *Journal, genetic engineering & biotechnology*. 2021;19(1):123.
28. Shrivastava AN, Triller A, Sieghart W. GABA_A Receptors: post-synaptic co-localization and cross-talk with other receptors. *Front Cell Neurosci*. 2011;5.
29. Farrant M, Nusser Z. Variations on an inhibitory theme: phasic and tonic activation of GABA_A receptors. *Nat Rev Neurosci*. 2005;6(3):215-29.
30. Terunuma M. Diversity of structure and function of GABA_B receptors: a complexity of GABA_B-mediated signaling. *Proc Jpn Acad Ser B Phys Biol Sci*. 2018;94(10):390-411.
31. Rudolph U, Knoflach F. Beyond classical benzodiazepines: novel therapeutic potential of GABA_A receptor subtypes. *Nat Rev Drug Discov*. 2011;10(9):685-97.
32. Feng Y, Wei Z-H, Liu C, Li G-Y, Qiao X-Z, Gan Y-J, et al. Genetic variations in GABA metabolism and epilepsy. *Seizure*. 2022;101:22-9.
33. Avoli M, Lévesque M. GABA_B Receptors: are they Missing in Action in Focal Epilepsy Research? *Curr Neuropharmacol*. 2022;20(9):1704-16.
34. Wu C, Sun D. GABA receptors in brain development, function, and injury. *Metab Brain Dis*. 2015;30(2):367-79.
35. Menzikov SA, Morozov SG, Kubatiev AA. Intricacies of GABA_A receptor function: The critical role of the $\beta 3$ subunit in norm and pathology. *Int J Mol Sci*. 2021;22(3):1457.
36. Sequeira A, Shen K, Gottlieb A, Limon A. Human brain transcriptome analysis finds region- and subject-specific expression signatures of GABA_AR subunits. *Commun Biol*. 2019;2(1):153.
37. Möhler H. Molecular regulation of cognitive functions and developmental plasticity: impact of GABA_A receptors. *J Neurochem*. 2007;102(1):1-12.
38. Li X, Guo S, Xu S, Chen Z, Wang L, Ding J, et al. Neocortex- and hippocampus-specific deletion of *Gabrg2* causes temperature-dependent seizures in mice. *Cell Death Dis*. 2021;12(6):553.
39. Cifelli P, Di Angelantonio S, Alfano V, Morano A, De Felice E, Aronica E, et al. Dissecting the molecular determinants of GABA_A receptors current rundown, a hallmark of refractory human epilepsy. *Brain Sci*. 2021;11(4):441.
40. Maillard P-Y, Baer S, Schaefer É, Desnoux B, Villeneuve N, Lépine A, et al. Molecular and clinical descriptions of patients with GABA_A receptor gene variants (*GABRA1*, *GABRB2*,

- GABRB3*, *GABRG2*): A cohort study, review of literature, and genotype-phenotype correlation. *Epilepsia*. 2022;63(10):2519-33.
41. McKernan RM, Whiting PJ. Which GABA_A-receptor subtypes really occur in the brain? *Trends Neurosci*. 1996;19(4):139-43.
 42. Schulte JT, Wierenga CJ, Bruining H. Chloride transporters and GABA polarity in developmental, neurological and psychiatric conditions. *Neurosci Biobehav Rev*. 2018;90:260-71.
 43. Hübner CA, Stein V, Hermans-Borgmeyer I, Meyer T, Ballanyi K, Jentsch TJ. Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron*. 2001;30(2):515-24.
 44. Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, et al. The K⁺/Cl⁻ co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature*. 1999;397(6716):251-5.
 45. Payne JA, Rivera C, Voipio J, Kaila K. Cation-chloride co-transporters in neuronal communication, development and trauma. *Trends Neurosci*. 2003;26(4):199-206.
 46. Owens DF, Kriegstein AR. Is there more to GABA than synaptic inhibition? *Nat Rev Neurosci*. 2002;3(9):715-27.
 47. Blaesse P, Airaksinen MS, Rivera C, Kaila K. Cation-chloride cotransporters and neuronal function. *Neuron*. 2009;61(6):820-38.
 48. McMoneagle E, Zhou J, Zhang S, Huang W, Josiah SS, Ding K, et al. Neuronal K⁺-Cl⁻ cotransporter KCC2 as a promising drug target for epilepsy treatment. *Acta Pharmacol Sin*. 2024;45(1):1-22.
 49. Sallard E, Letourneur D, Legendre P. Electrophysiology of ionotropic GABA receptors. *Cell Mol Life Sci*. 2021;78(13):5341-70.
 50. Kontou G, Josephine Ng SF, Cardarelli RA, Howden JH, Choi C, Ren Q, et al. KCC2 is required for the survival of mature neurons but not for their development. *J Biol Chem*. 2021;296:100364.
 51. Zhu S, Noviello CM, Teng J, Walsh RM, Kim JJ, Hibbs RE. Structure of a human synaptic GABA_A receptor. *Nature*. 2018;559(7712):67-72.
 52. Jacob TC, Moss SJ, Jurd R. GABA_A receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat Rev Neurosci*. 2008;9(5):331-43.
 53. Mozrzymas JW, Barberis A, Mercik K, Z⁺ arnowska ED. Binding sites, singly bound states, and conformation coupling shape GABA-evoked currents. *J Neurophysiol*. 2003;89(2):871-83.
 54. Gielen M, Corringer P-J. The dual-gate model for pentameric ligand-gated ion channels activation and desensitization. *J Physiol*. 2018;596(10):1873-902.
 55. Bianchi MT, Macdonald RL. Slow phases of GABA_A receptor desensitization: structural determinants and possible relevance for synaptic function. *J Physiol*. 2002;544(1):3-18.
 56. Haas KF, Macdonald RL. GABA_A receptor subunit γ 2 and δ subtypes confer unique kinetic properties on recombinant GABA_A receptor currents in mouse fibroblasts. *J Physiol*. 1999;514(1):27-45.
 57. Bryson A, Reid C, Petrou S. Fundamental neurochemistry review: GABA_A receptor neurotransmission and epilepsy: Principles, disease mechanisms and pharmacotherapy. *J Neurochem*. 2023;165(1):6-28.
 58. Sigel E, Steinmann ME. Structure, function, and modulation of GABA_A receptors *J Biol Chem*. 2012;287(48):40224-31.
 59. Chen Q, Wells MM, Arjunan P, Tillman TS, Cohen AE, Xu Y, Tang P. Structural basis of neurosteroid anesthetic action on GABA_A receptors. *Nat Commun*. 2018;9(1):3972.
 60. Kasaragod VB, Schindelin H. Structure of heteropentameric GABA_A receptors and receptor-anchoring properties of gephyrin. *Front Mol Neurosci*. 2019;12.
 61. Sun C, Zhu H, Clark S, Gouaux E. Cryo-EM structures reveal native GABA_A receptor assemblies and pharmacology. *Nature*. 2023;622(7981):195-201.

62. Lavery D, Desai R, Uchański T, Masiulis S, Stec WJ, Malinauskas T, et al. Cryo-EM structure of the human $\alpha 1\beta 3\gamma 2$ GABA_A receptor in a lipid bilayer. *Nature*. 2019;565(7740):516-20.
63. Liu S, Xu L, Guan F, Liu Y-T, Cui Y, Zhang Q, et al. Cryo-EM structure of the human $\alpha 5\beta 3$ GABA_A receptor. *Cell Res*. 2018;28(9):958-61.
64. Phulera S, Zhu H, Yu J, Claxton DP, Yoder N, Yoshioka C, Gouaux E. Cryo-EM structure of the benzodiazepine-sensitive $\alpha 1\beta 1\gamma 2S$ tri-heteromeric GABA_A receptor in complex with GABA. *Elife*. 2018;7:e39383.
65. Miller PS, Scott S, Masiulis S, De Colibus L, Pardon E, Steyaert J, Aricescu AR. Structural basis for GABA_A receptor potentiation by neurosteroids. *Nat Struct Mol Biol*. 2017;24(11):986-92.
66. Baumann SW, Baur R, Sigel E. Individual properties of the two functional agonist sites in GABA_A receptors. *J Neurosci*. 2003;23(35):11158-66.
67. Masiulis S, Desai R, Uchański T, Serna Martin I, Lavery D, Karia D, et al. GABA_A receptor signalling mechanisms revealed by structural pharmacology. *Nature*. 2019;565(7740):454-9.
68. Gentry PR, Sexton PM, Christopoulos A. Novel allosteric modulators of G protein-coupled receptors. *J Biol Chem*. 2015;290(32):19478-88.
69. Goldschen-Ohm MP. Benzodiazepine modulation of GABA_A receptors: a mechanistic perspective. *Biomolecules*. 2022;12(12):1784.
70. Cowgill J, Fan C, Haloi N, Tobiasson V, Zhuang Y, Howard RJ, Lindahl E. Structure and dynamics of differential ligand binding in the human ρ -type GABA_A receptor. *Neuron*. 2023;111(21):3450-64.e5.
71. Maljevic S, Keren B, Aung YH, Forster IC, Mignot C, Buratti J, et al. Novel *GABRA2* variants in epileptic encephalopathy and intellectual disability with seizures. *Brain*. 2019;142(5):e15-e.
72. Niturad CE, Lev D, Kalscheuer VM, Charzewska A, Schubert J, Lerman-Sagie T, et al. Rare *GABRA3* variants are associated with epileptic seizures, encephalopathy and dysmorphic features. *Brain*. 2017;140(11):2879-94.
73. Vogel FD, Krenn M, Westphal DS, Graf E, Wagner M, Leiz S, et al. A de novo missense variant in *GABRA4* alters receptor function in an epileptic and neurodevelopmental phenotype. *Epilepsia*. 2022;63(4):e35-e41.
74. May P, Girard S, Harrer M, Bobbili DR, Schubert J, Wolking S, et al. Rare coding variants in genes encoding GABA_A receptors in genetic generalised epilepsies: an exome-based case-control study. *Lancet Neurol*. 2018;17(8):699-708.
75. Absalom NL, Liao VWY, Johannesen KMH, Gardella E, Jacobs J, Lesca G, et al. Gain-of-function and loss-of-function *GABRB3* variants lead to distinct clinical phenotypes in patients with developmental and epileptic encephalopathies. *Nat Commun*. 2022;13(1):1822.
76. Williams A, Cooney E, Segal G, Narayanan S, Morand M, Agadi S. *GABRG1* variant as a potential novel cause of epileptic encephalopathy, hypotonia, and global developmental delay. *American journal of medical genetics Part A*. 2022;188(12):3546-9.
77. Janve VS, Hernandez CC, Verdier KM, Hu N, Macdonald RL. Epileptic encephalopathy de novo *GABRB* mutations impair γ -aminobutyric acid type A receptor function. *Ann Neurol*. 2016;79(5):806-25.
78. Steudle F, Rehman S, Bampali K, Simeone X, Rona Z, Hauser E, et al. A novel *de novo* variant of *GABRA1* causes increased sensitivity for GABA *in vitro*. *Sci Rep*. 2020;10(1):2379.
79. Butler KM, Moody OA, Schuler E, Coryell J, Alexander JJ, Jenkins A, Escayg A. *De novo* variants in *GABRA2* and *GABRA5* alter receptor function and contribute to early-onset epilepsy. *Brain*. 2018;141(8):2392-405.
80. Hernandez CC, Shen Y, Hu N, Shen W, Narayanan V, Ramsey K, et al. *GABRG2* Variants Associated with Febrile Seizures. *Biomolecules*. 2023;13(3):414.

81. Fu X, Wang YJ, Kang JQ, Mu TW. GABA_A receptor variants in epilepsy. In: Czuczwar SJ, editor. *Epilepsy*. Brisbane (AU): Exon Publications; 2022.
82. Ahring PK, Liao VWY, Lin S, Absalom NL, Chebib M, Møller RS. The de novo *GABRA4* p.Thr300Ile variant found in a patient with early-onset intractable epilepsy and neurodevelopmental abnormalities displays gain-of-function traits. *Epilepsia*. 2022;63(9):2439-41.
83. Markus F, Angelini C, Trimouille A, Rudolf G, Lesca G, Goizet C, et al. Rare variants in the GABA_A receptor subunit ϵ identified in patients with a wide spectrum of epileptic phenotypes. *Mol Genet Genomic Med*. 2020;8(9):e1388.
84. Lagrange AH, Botzolakis EJ, Macdonald RL. Enhanced macroscopic desensitization shapes the response of $\alpha 4$ subtype-containing GABA_A receptors to synaptic and extrasynaptic GABA. *J Physiol*. 2007;578(3):655-76.
85. Absalom NL, Lin SXN, Liao VWY, Chua HC, Møller RS, Chebib M, Ahring PK. GABA_A receptors in epilepsy: Elucidating phenotypic divergence through functional analysis of genetic variants. *J Neurochem*. 2023.
86. Jatzczak-Śliwa M, Kisiel M, Czyzewska MM, Brodzki M, Mozrzymas JW. GABA_A receptor β_2 E155 residue located at the agonist-binding site is involved in the receptor gating. *Front Cell Neurosci*. 2020;14:2.
87. Szczot M, Kisiel M, Czyzewska MM, Mozrzymas JW. $\alpha 1$ F64 residue at GABA_A receptor binding site is involved in gating by influencing the receptor flipping transitions. *J Neurosci*. 2014;34(9):3193-209.
88. Mortensen M, Patel B, Smart TG. GABA potency at GABA_A receptors found in synaptic and extrasynaptic zones. *Front Cell Neurosci*. 2011;6:1.
89. Gielen M, Thomas P, Smart TG. The desensitization gate of inhibitory Cys-loop receptors. *Nat Commun*. 2015;6(1):6829.
90. Kim JJ, Hibbs RE. Direct structural insights into GABA_A receptor pharmacology. *Trends Biochem Sci*. 2021;46(6):502-17.
91. Hernandez CC, Kong W, Hu N, Zhang Y, Shen W, Jackson LG, et al. Altered channel conductance states and gating of GABA_A receptors by a pore mutation linked to Dravet syndrome. *eNeuro*. 2017;4(1):ENEURO.0251-16.2017.
92. Terejko K, Michałowski MA, Iżykowska I, Dominik A, Brzóstowicz A, Mozrzymas JW. Mutations at the M2 and M3 transmembrane helices of the GABA_ARs $\alpha 1$ and $\beta 2$ subunits affect primarily late gating transitions including opening/closing and desensitization. *ACS Chem Neurosci*. 2021;12(13):2421-36.
93. Yang Y, Niu X, Cheng M, Zeng Q, Deng J, Tian X, et al. Phenotypic spectrum and prognosis of epilepsy patients with *GABRG2* variants. *Front Mol Neurosci*. 2022;15.
94. Musto E, Liao VWY, Johannesen KM, Fenger CD, Lederer D, Kothur K, et al. *GABRA1*-related disorders: from genetic to functional pathways. *Ann Neurol*. 2024;95(1):27-41.
95. Chen Z-W, Olsen RW. GABA_A receptor associated proteins: a key factor regulating GABA_A receptor function. *J Neurochem*. 2007;100(2):279-94.
96. O'Toole KK, Jenkins A. Discrete M3-M4 intracellular loop subdomains control specific aspects of γ -aminobutyric acid type A receptor function. *J Biol Chem*. 2011;286(44):37990-9.
97. Berg AT, Langfitt JT, Testa FM, Levy SR, DiMario F, Westerveld M, Kulas J. Global cognitive function in children with epilepsy: A community-based study. *Epilepsia*. 2008;49(4):608-14.
98. Wirrell E, Wong-Kisiel L, Mandrekar J, Nickels K. Predictors and course of medically intractable epilepsy in young children presenting before 36 months of age: a retrospective, population-based study. *Epilepsia*. 2012;53(9):1563-9.
99. Moseley BD, Wirrell EC, Wong-Kisiel LC, Nickels K. Early onset epilepsy is associated with increased mortality: a population-based study. *Epilepsy Res*. 2013;105(3):410-4.

100. Johannesen KM, Iqbal S, Guazzi M, Mohammadi NA, Pérez-Palma E, Schaefer E, et al. Structural mapping of *GABRB3* variants reveals genotype-phenotype correlations. *Genet Med*. 2022;24(3):681-93.
101. Myers KA. Genetic epilepsy syndromes. *Continuum (Minneapolis, Minn)*. 2022;28(2):339-62.
102. Myers KA, Burgess R, Afawi Z, Damiano JA, Berkovic SF, Hildebrand MS, Scheffer IE. De novo *SCN1A* pathogenic variants in the GEFS+ spectrum: Not always a familial syndrome. *Epilepsia*. 2017;58(2):e26-e30.
103. Zuberi SM, Wirrell E, Yozawitz E, Wilmshurst JM, Specchio N, Riney K, et al. ILAE classification and definition of epilepsy syndromes with onset in neonates and infants: Position statement by the ILAE Task Force on Nosology and Definitions. *Epilepsia*. 2022;63(6):1349-97.
104. Türkyılmaz A, Tekin E, Yaralı O, Çebi AH. Genetic landscape of *SCN1A* variants in a Turkish cohort with GEFS+ spectrum and Dravet syndrome. *Mol Syndromol*. 2022;13(4):270-81.
105. Shi X, Yasumoto S, Kurahashi H, Nakagawa E, Fukasawa T, Uchiya S, Hirose S. Clinical spectrum of *SCN2A* mutations. *Brain Dev*. 2012;34(7):541-5.
106. Liu X-w, Li W, Han T, Wei K, Qiao S, Su L, Chi Z. The finding of a new heterozygous mutation site of the *SCN2A* gene in a monozygotic twin family carrying and exhibiting genetic epilepsy with febrile seizures plus (GEFS+) using targeted next-generation sequencing. *Clin Neurol Neurosurg*. 2018;169:86-91.
107. Baroni D, Picco C, Moran O. A mutation of *SCN1B* associated with GEFS+ causes functional and maturation defects of the voltage-dependent sodium channel. *Hum Mutat*. 2018;39(10):1402-15.
108. Zhang T, Chen M, Zhu A, Zhang X, Fang T. Novel mutation of *SCN9A* gene causing generalized epilepsy with febrile seizures plus in a Chinese family. *Neurol Sci*. 2020;41(7):1913-7.
109. Ding J, Zhang J-W, Guo Y-X, Zhang Y-X, Chen Z-H, Zhai Q-X. Novel mutations in *SCN9A* occurring with fever-associated seizures or epilepsy. *Seizure*. 2019;71:214-8.
110. Johnston AJ, Kang J-Q, Shen W, Pickrell WO, Cushion TD, Davies JS, et al. A novel *GABRG2* mutation, p.R136*, in a family with GEFS+ and extended phenotypes. *Neurobiol Dis*. 2014;64:131-41.
111. Depienne C, Gourfinkel-An I, Baulac S, LeGuern E. Genes in infantile epileptic encephalopathies. In: Noebels JL, Avoli M, Rogawski MA, Olsen RW, Delgado-Escueta AV, editors. *Jasper's Basic Mechanisms of the Epilepsies*. Bethesda (MD): National Center for Biotechnology Information (US); 2012.
112. Johannesen KM, Liu Y, Koko M, Gjerulfsen CE, Sonnenberg L, Schubert J, et al. Genotype-phenotype correlations in *SCN8A*-related disorders reveal prognostic and therapeutic implications. *Brain*. 2021;145(9):2991-3009.
113. Marini C, Harkin LA, Wallace RH, Mulley JC, Scheffer IE, Berkovic SF. Childhood absence epilepsy and febrile seizures: a family with a GABA_A receptor mutation. *Brain*. 2003;126(1):230-40.
114. Sun H, Zhang Y, Liang J, Liu X, Ma X, Wu H, et al. *SCN1A*, *SCN1B*, and *GABRG2* gene mutation analysis in Chinese families with generalized epilepsy with febrile seizures plus. *Journal of human genetics*. 2008;53(8):769-74.
115. Lagae L. Dravet syndrome. *Current opinion in neurology*. 2021;34(2):213-8.
116. Wheless JW, Fulton SP, Mudigoudar BD. Dravet syndrome: a review of current management. *Pediatr Neurol*. 2020;107:28-40.
117. Olivieri G, Battaglia D, Chieffo D, Rubbino R, Ranalli D, Contaldo I, et al. Cognitive-behavioral profiles in teenagers with Dravet syndrome. *Brain Dev*. 2016;38(6):554-62.
118. Wirrell EC, Hood V, Knupp KG, Meskis MA, Nabbout R, Scheffer IE, et al. International consensus on diagnosis and management of Dravet syndrome. *Epilepsia*. 2022;63(7):1761-77.

119. Ziobro J, Eschbach K, Sullivan JE, Knupp KG. Current treatment strategies and future treatment options for Dravet syndrome. *Current treatment options in neurology*. 2018;20(12):52.
120. Ricobaraza A, Mora-Jimenez L, Puerta E, Sanchez-Carpintero R, Mingorance A, Artieda J, et al. Epilepsy and neuropsychiatric comorbidities in mice carrying a recurrent Dravet syndrome *SCN1A* missense mutation. *Sci Rep*. 2019;9(1):14172.
121. Sadleir LG, Mountier EI, Gill D, Davis S, Joshi C, DeVile C, et al. Not all *SCN1A* epileptic encephalopathies are Dravet syndrome. *Neurology*. 2017;89(10):1035-42.
122. Gowda VK, Amoghmath R, Battina M, Shivappa SK, Benakappa N. Case Series of Early *SCN1A*-Related Developmental and Epileptic Encephalopathies. *Journal of Pediatric Neurosciences*. 2021;16(3):212-7.
123. Scheffer IE. The fascinating phenotypic spectrum of *SCN1A* gain-of-function epilepsies. *Epilepsia*. 2023;64(5):1348-50.
124. Carvill GL, Weckhuysen S, McMahon JM, Hartmann C, Møller RS, Hjalgrim H, et al. *GABRA1* and *STXBP1*: novel genetic causes of Dravet syndrome. *Neurology*. 2014;82(14):1245-53.
125. Steel D, Symonds JD, Zuberi SM, Brunklaus A. Dravet syndrome and its mimics: Beyond *SCN1A*. *Epilepsia*. 2017;58(11):1807-16.
126. Administration TG. Australian Public Assessment Report
for Stiripentol. In: Health Do, editor. 2019.
127. Poke G, Stanley J, Scheffer IE, Sadleir LG. Epidemiology of developmental and epileptic encephalopathy and of intellectual disability and epilepsy in children. *Neurology*. 2023;100(13):e1363-e75.
128. Lin J-J, Meletti S, Vaudano AE, Lin K-L. Developmental and epileptic encephalopathies: Is prognosis related to different epileptic network dysfunctions? *Epilepsy Behav*. 2022;131:107654.
129. Mierzewska H, Laure-Kamionowska M, Jezela-Stanek A, Rydzanicz M, Płoski R, Szczepanik E. The neuropathological findings of developmental and epileptic encephalopathy-43 (DEE43) and delineation of a the molecular spectrum of novel case. *Seizure*. 2021;93:75-80.
130. Essajee F, Urban M, Smit L, Wilmschurst JM, Solomons R, van Toorn R, Moosa S. Utility of genetic testing in children with developmental and epileptic encephalopathy (DEE) at a tertiary hospital in South Africa: A prospective study. *Seizure*. 2022;101:197-204.
131. Kang Q, Yang L, Liao H, Yang S, Kuang X, Ning Z, et al. A chinese patient with developmental and epileptic encephalopathies (DEE) carrying a *TRPM3* gene mutation: a paediatric case report. *BMC pediatrics*. 2021;21(1):256.
132. Matricardi S, De Liso P, Freri E, Costa P, Castellotti B, Magri S, et al. Neonatal developmental and epileptic encephalopathy due to autosomal recessive variants in *SLC13A5* gene. *Epilepsia*. 2020;61(11):2474-85.
133. Strzelczyk A, Schubert-Bast S. Psychobehavioural and cognitive adverse events of anti-seizure medications for the treatment of developmental and epileptic encephalopathies. *CNS Drugs*. 2022;36(10):1079-111.
134. Hernandez CC, Klassen TL, Jackson LG, Gurba K, Hu N, Noebels JL, Macdonald RL. Deleterious rare variants reveal risk for loss of GABA_A receptor function in patients with genetic epilepsy and in the general population. *PLoS One*. 2016;11(9):e0162883.
135. Hernandez CC, Macdonald RL. A structural look at GABA_A receptor mutations linked to epilepsy syndromes. *Brain Res*. 2019;1714:234-47.
136. Sills GJ, Rogawski MA. Mechanisms of action of currently used antiseizure drugs. *Neuropharmacology*. 2020;168:107966.
137. Abou-Khalil B. Levetiracetam in the treatment of epilepsy. *Neuropsychiatr Dis Treat*. 2008;4(3):507-23.

138. Barker-Haliski M, White HS. Glutamatergic mechanisms associated with seizures and epilepsy. *Cold Spring Harb Perspect Med*. 2015;5(8):a022863.
139. Rogawski M, Cavazos J. Mechanisms of action of antiseizure medications. *Wyllie's Treatment of Epilepsy: Principles and Practice*. 7 ed: Lippincott Williams & Wilkins (LWW); 2020.
140. Goldenberg MM. Overview of drugs used for epilepsy and seizures: etiology, diagnosis, and treatment. *P T*. 2010;35(7):392-415.
141. Laxer KD, Trinka E, Hirsch LJ, Cendes F, Langfitt J, Delanty N, et al. The consequences of refractory epilepsy and its treatment. *Epilepsy Behav*. 2014;37:59-70.
142. Mohammadzadeh P, Nazarbaghi S. The prevalence of drug-resistant-epilepsy and its associated factors in patients with epilepsy. *Clin Neurol Neurosurg*. 2022;213:107086.
143. Papandreou A, McTague A, Trump N, Ambegaonkar G, Ngoh A, Meyer E, et al. *GABRB3* mutations: a new and emerging cause of early infantile epileptic encephalopathy. *Dev Med Child Neurol*. 2016;58(4):416-20.
144. Kothur K, Holman K, Farnsworth E, Ho G, Lorentzos M, Troedson C, et al. Diagnostic yield of targeted massively parallel sequencing in children with epileptic encephalopathy. *Seizure*. 2018;59:132-40.
145. Brunklaus A, Brünger T, Feng T, Fons C, Lehtikainen A, Panagiotakaki E, et al. The gain of function *SCN1A* disorder spectrum: novel epilepsy phenotypes and therapeutic implications. *Brain*. 2022;145(11):3816-31.
146. Masnada S, Hedrich UBS, Gardella E, Schubert J, Kaiwar C, Klee EW, et al. Clinical spectrum and genotype-phenotype associations of *KCNA2*-related encephalopathies. *Brain*. 2017;140(9):2337-54.
147. Syrbe S, Hedrich UBS, Riesch E, Djémié T, Müller S, Möller RS, et al. *De novo* loss- or gain-of-function mutations in *KCNA2* cause epileptic encephalopathy. *Nat Genet*. 2015;47(4):393-9.
148. Absalom NL, Ahring PK, Liao VW, Balle T, Jiang T, Anderson LL, et al. Functional genomics of epilepsy-associated mutations in the GABA_A receptor subunits reveal that one mutation impairs function and two are catastrophic. *J Biol Chem*. 2019;294(15):6157-71.
149. Absalom NL, Liao VWY, Kothur K, Indurthi DC, Bennetts B, Troedson C, et al. Gain-of-function *GABRB3* variants identified in vigabatrin-hypersensitive epileptic encephalopathies. *Brain Commun*. 2020;2(2):fcaa162.
150. Gudmundsson S, Singer-Berk M, Watts NA, Phu W, Goodrich JK, Solomonson M, et al. Variant interpretation using population databases: Lessons from gnomAD. *Hum Mutat*. 2022;43(8):1012-30.
151. Hartman P, Beckman K, Silverstein K, Yohe S, Schomaker M, Henzler C, et al. Next generation sequencing for clinical diagnostics: Five year experience of an academic laboratory. *Mol Genet Metab Reps*. 2019;19:100464.
152. Pérez-Palma E, Gramm M, Nürnberg P, May P, Lal D. Simple ClinVar: an interactive web server to explore and retrieve gene and disease variants aggregated in ClinVar database. *Nucleic Acids Res*. 2019;47(W1):W99-W105.
153. Iancu I-F, Avila-Fernandez A, Artech A, Trujillo-Tiebas MJ, Riveiro-Alvarez R, Almoguera B, et al. Prioritizing variants of uncertain significance for reclassification using a rule-based algorithm in inherited retinal dystrophies. *NPJ Genom Med*. 2021;6(1):18.
154. Appelbaum PS, Berger SM, Brokamp E, Brown HS, Burke W, Clayton EW, et al. Practical considerations for reinterpretation of individual genetic variants. *Genet Med*. 2023;25(5):100801.
155. Wu Q, Wang H, Liu X, Zhao Y, Su P. Microglial activation and over pruning involved in developmental epilepsy. *Journal of Neuropathology & Experimental Neurology*. 2022;82(2):150-9.

156. Liao VWY, Chua HC, Kowal NM, Chebib M, Balle T, Ahring PK. Concatenated γ -aminobutyric acid type A receptors revisited: Finding order in chaos. *J Gen Physiol*. 2019;151(6):798-819.
157. Rettinger J, Schwarz S, Schwarz W. Basics theory. In: Rettinger J, Schwarz S, Schwarz W, editors. *Electrophysiology : Basics, Methods, Modern Approaches and Applications*. Cham: Springer International Publishing; 2022. p. 13-26.
158. Guan B, Chen X, Zhang H. Two-Electrode Voltage Clamp. In: Gamper N, editor. *Ion Channels: Methods and Protocols*. Totowa, NJ: Humana Press; 2013. p. 79-89.
159. Gielen M, Barilone N, Corringer P-J. The desensitization pathway of GABA_A receptors, one subunit at a time. *Nature Commun*. 2020;11(1):5369.
160. Yang Y, Zeng Q, Cheng M, Niu X, Xiangwei W, Gong P, et al. *GABRB3*-related epilepsy: novel variants, clinical features and therapeutic implications. *J Neurol*. 2022;269(5):2649-65.
161. Eilam-Altstädter R, Las L, Witter MP, Ulanovsky N. Methods. In: Eilam-Altstädter R, Las L, Witter MP, Ulanovsky N, editors. *Stereotaxic brain atlas of the egyptian fruit bat*: Academic Press; 2021. p. 1-13.
162. Wyse ATS, Sanches EF, Dos Santos TM, Siebert C, Kolling J, Netto CA. Chronic mild hyperhomocysteinemia induces anxiety-like symptoms, aversive memory deficits and hippocampus atrophy in adult rats: New insights into physiopathological mechanisms. *Brain Res*. 2020;1728:146592.
163. Schmued LC, Stowers CC, Scallet AC, Xu L. Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. *Brain Res*. 2005;1035(1):24-31.
164. Ikenari T, Kurata H, Satoh T, Hata Y, Mori T. Evaluation of Fluoro-Jade C staining: specificity and application to damaged immature neuronal cells in the normal and injured mouse brain. *Neuroscience*. 2020;425:146-56.
165. Boorman DC, Keay KA. Sex differences in morphine sensitivity are associated with differential glial expression in the brainstem of rats with neuropathic pain. *J Neurosci Res*. 2022;100(10):1890-907.
166. Wallace RH, Marini C, Petrou S, Harkin LA, Bowser DN, Panchal RG, et al. Mutant GABA_A receptor γ 2-subunit in childhood absence epilepsy and febrile seizures. *Nat Genet*. 2001;28(1):49-52.
167. Baulac S, Huberfeld G, Gourfinkel-An I, Mitropoulou G, Beranger A, Prud'homme J-F, et al. First genetic evidence of GABA_A receptor dysfunction in epilepsy: a mutation in the γ 2-subunit gene. *Nat Genet*. 2001;28(1):46-8.
168. Myers KA, Scheffer IE, Berkovic SF, Commission obotIG. Genetic literacy series: genetic epilepsy with febrile seizures plus. *Epileptic disorders : international epilepsy journal with videotape*. 2018;20(4):232-8.
169. Zou F, McWalter K, Schmidt L, Decker A, Picker JD, Lincoln S, et al. Expanding the phenotypic spectrum of *GABRG2* variants: a recurrent *GABRG2* missense variant associated with a severe phenotype. *Journal of neurogenetics*. 2017;31(1-2):30-6.
170. Harkin LA, Bowser DN, Dibbens LM, Singh R, Phillips F, Wallace RH, et al. Truncation of the GABA_A-Receptor γ 2 subunit in a family with generalized epilepsy with febrile seizures plus. *American journal of human genetics*. 2002;70(2):530-6.
171. Shen D, Hernandez CC, Shen W, Hu N, Poduri A, Shiedley B, et al. De novo *GABRG2* mutations associated with epileptic encephalopathies. *Brain*. 2016;140(1):49-67.
172. Boillot M, Morin-Brureau M, Picard F, Weckhuysen S, Lambrecq V, Minetti C, et al. Novel *GABRG2* mutations cause familial febrile seizures. *Neurol Genet*. 2015;1(4):e35.
173. Tian M, Mei D, Freri E, Hernandez CC, Granata T, Shen W, et al. Impaired surface $\alpha\beta\gamma$ GABA(A) receptor expression in familial epilepsy due to a *GABRG2* frameshift mutation. *Neurobiol Dis*. 2013;50:135-41.

174. Warner TA, Shen W, Huang X, Liu Z, Macdonald RL, Kang J-Q. Differential molecular and behavioural alterations in mouse models of *GABRG2* haploinsufficiency versus dominant negative mutations associated with human epilepsy. *Hum Mol Genet.* 2016;25(15):3192-207.
175. Kang J-Q, Macdonald RL. Molecular pathogenic basis for *GABRG2* mutations associated with a spectrum of epilepsy syndromes, from generalized absence epilepsy to Dravet syndrome. *JAMA Neurol.* 2016;73(8):1009-16.
176. Chen W, Ge Y, Lu J, Melo J, So YW, Juneja R, et al. Distinct functional alterations and therapeutic options of two pathological de novo variants of the T292 residue of *GABRA1* identified in children with epileptic encephalopathy and neurodevelopmental disorders. *Int J Mol Sci.* 2022;23(5):2723.
177. Ahring PK, Liao VWY, Gardella E, Johannesen KM, Krey I, Selmer KK, et al. Gain-of-function variants in *GABRD* reveal a novel pathway for neurodevelopmental disorders and epilepsy. *Brain.* 2022;145(4):1299-309.
178. Carbone E, Mori Y. Ion channelopathies to bridge molecular lesions, channel function, and clinical therapies. *Pflugers Arch.* 2020;472:733-8.
179. Heyne HO, Baez-Nieto D, Iqbal S, Palmer DS, Brunklaus A, May P, et al. Predicting functional effects of missense variants in voltage-gated sodium and calcium channels. *Sci Transl Med.* 2020;12(556):eaay6848.
180. Tang B, Li B, Gao L-D, He N, Liu X-R, Long Y-S, et al. Optimization of in silico tools for predicting genetic variants: individualizing for genes with molecular sub-regional stratification. *Brief Bioinform.* 2019;21(5):1776-86.
181. Wolff M, Johannesen KM, Hedrich UBS, Masnada S, Rubboli G, Gardella E, et al. Genetic and phenotypic heterogeneity suggest therapeutic implications in *SCN2A*-related disorders. *Brain.* 2017;140(5):1316-36.
182. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med.* 2015;17(5):405-24.
183. Cannon S, Williams M, Gunning AC, Wright CF. Evaluation of in silico pathogenicity prediction tools for the classification of small in-frame indels. *BMC Med Genomics.* 2023;16(1):36.
184. Desai M, Chauhan J. In silico analysis of nsSNPs in human methyl CpG binding protein 2. *Meta Gene.* 2016;10:1-7.
185. Yousefi T, Mir SM, Asadi J, Tourani M, Karimian A, Maniati M, Tehrani SS. In silico analysis of non-synonymous single nucleotide polymorphism in a human *KLK-2* gene associated with prostate cancer. *Meta Gene.* 2019;21:100578.
186. Chasman D, Adams RM. Predicting the functional consequences of non-synonymous single nucleotide polymorphisms: structure-based assessment of amino acid variation. *J Mol Biol.* 2001;307(2):683-706.
187. Stitzel NO, Tseng YY, Pervouchine D, Goddeau D, Kasif S, Liang J. Structural location of disease-associated single-nucleotide polymorphisms. *J Mol Biol.* 2003;327(5):1021-30.
188. Yazar M, Özbek P. *In Silico* tools and approaches for the prediction of functional and structural effects of single-nucleotide polymorphisms on proteins: a review. *OMICS.* 2021;25(1):23-37.
189. Yue P, Li Z, Mout J. Loss of protein structure stability as a major causative factor in monogenic disease. *J Mol Biol.* 2005;353(2):459-73.
190. Cubuk C, Garrett A, Choi S, King L, Loveday C, Torr B, et al. Clinical likelihood ratios and balanced accuracy for 44 in silico tools against multiple large-scale functional assays of cancer susceptibility genes. *Genet Med.* 2021;23(11):2096-104.
191. Vitkup D, Sander C, Church GM. The amino-acid mutational spectrum of human genetic disease. *Genome Biol.* 2003;4(11):R72.

192. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet.* 2014;46(3):310-5.
193. Kulshreshtha S, Chaudhary V, Goswami GK, Mathur N. Computational approaches for predicting mutant protein stability. *J Comput Aided Mol Des.* 2016;30(5):401-12.
194. Garcia FAdO, Andrade ESd, Palmero EI. Insights on variant analysis in silico tools for pathogenicity prediction. *Front Genet.* 2022;13.
195. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. *Nat Methods.* 2010;7(4):248-9.
196. Shihab HA, Gough J, Cooper DN, Stenson PD, Barker GLA, Edwards KJ, et al. Predicting the functional, molecular, and phenotypic consequences of amino acid substitutions using hidden Markov models. *Hum Mutat.* 2013;34(1):57-65.
197. Vaser R, Adusumalli S, Leng SN, Sikic M, Ng PC. SIFT missense predictions for genomes. *Nat Protoc.* 2016;11(1):1-9.
198. Sim N-L, Kumar P, Hu J, Henikoff S, Schneider G, Ng PC. SIFT web server: predicting effects of amino acid substitutions on proteins. *Nucleic Acids Res.* 2012;40(W1):W452-W7.
199. Pejaver V, Urresti J, Lugo-Martinez J, Pagel KA, Lin GN, Nam H-J, et al. Inferring the molecular and phenotypic impact of amino acid variants with MutPred2. *Nat Commun.* 2020;11(1):5918.
200. Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M. CADD: predicting the deleteriousness of variants throughout the human genome. *Nucleic Acids Res.* 2019;47(D1):D886-D94.
201. Ioannidis NM, Rothstein JH, Pejaver V, Middha S, McDonnell SK, Baheti S, et al. REVEL: an ensemble method for predicting the pathogenicity of rare missense variants. *American journal of human genetics.* 2016;99(4):877-85.
202. Wilcox EH, Sarmady M, Wulf B, Wright MW, Rehm HL, Biesecker LG, Abou Tayoun AN. Evaluating the impact of in silico predictors on clinical variant classification. *Genet Med.* 2022;24(4):924-30.
203. López-Ferrando V, Gazzo A, de la Cruz X, Orozco M, Gelpí JL. PMut: a web-based tool for the annotation of pathological variants on proteins, 2017 update. *Nucleic Acids Res.* 2017;45(W1):W222-W8.
204. Cheng J, Novati G, Pan J, Bycroft C, Žemgulytė A, Applebaum T, et al. Accurate proteome-wide missense variant effect prediction with AlphaMissense. *Science.* 2023;381(6664):eadg7492.
205. Tordai H, Torres O, Csepi M, Padányi R, Lukács GL, Hegedűs T. Analysis of AlphaMissense data in different protein groups and structural context. *Sci Data.* 2024;11(1):495.
206. Pejaver V, Byrne AB, Feng B-J, Pagel KA, Mooney SD, Karchin R, et al. Calibration of computational tools for missense variant pathogenicity classification and ClinGen recommendations for PP3/BP4 criteria. *American journal of human genetics.* 2022;109(12):2163-77.
207. Chu K. An introduction to sensitivity, specificity, predictive values and likelihood ratios. *Emergency Medicine.* 1999;11(3):175-81.
208. Lindy AS, Stosser MB, Butler E, Downtain-Pickersgill C, Shanmugham A, Retterer K, et al. Diagnostic outcomes for genetic testing of 70 genes in 8565 patients with epilepsy and neurodevelopmental disorders. *Epilepsia.* 2018;59(5):1062-71.
209. Hancili S, Önal ZE, Ata P, Karatoprak EY, Gürbüz T, Bostancı M, et al. The GABA_A receptor γ 2 subunit (R43Q) mutation in febrile seizures. *Pediatr Neurol.* 2014;50(4):353-6.
210. Lachance-Touchette P, Brown P, Meloche C, Kinirons P, Lapointe L, Lacasse H, et al. Novel α 1 and γ 2 GABA_A receptor subunit mutations in families with idiopathic generalized epilepsy. *The European journal of neuroscience.* 2011;34(2):237-49.

211. Komulainen-Ebrahim J, Schreiber JM, Kangas SM, Pylkäs K, Suo-Palosaari M, Rahikkala E, et al. Novel variants and phenotypes widen the phenotypic spectrum of *GABRG2*-related disorders. *Seizure*. 2019;69:99-104.
212. Hernandez CC, Tian X, Hu N, Shen W, Catron MA, Yang Y, et al. Dravet syndrome-associated mutations in *GABRA1*, *GABRB2* and *GABRG2* define the genetic landscape of defects of GABA_A receptors. *Brain Commun*. 2021;3(2).
213. Yamamoto T, Imaizumi T, Yamamoto-Shimojima K, Lu Y, Yanagishita T, Shimada S, et al. Genomic backgrounds of Japanese patients with undiagnosed neurodevelopmental disorders. *Brain Dev*. 2019;41(9):776-82.
214. Audenaert D, Schwartz E, Claeys KG, Claes L, Deprez L, Suls A, et al. A novel *GABRG2* mutation associated with febrile seizures. *Neurology*. 2006;67(4):687-90.
215. Pshennikova VG, Barashkov NA, Romanov GP, Teryutin FM, Solov'ev AV, Gotovtsev NN, et al. Comparison of predictive *in silico* tools on missense variants in *GJB2*, *GJB6*, and *GJB3* genes associated with autosomal recessive deafness 1A (DFNB1A). *ScientificWorldJournal*. 2019;2019(1):5198931.
216. Miosge LA, Field MA, Sontani Y, Cho V, Johnson S, Palkova A, et al. Comparison of predicted and actual consequences of missense mutations. *Proc Natl Acad Sci U S A*. 2015;112(37):E5189-E98.
217. Ghosh R, Oak N, Plon SE. Evaluation of *in silico* algorithms for use with ACMG/AMP clinical variant interpretation guidelines. *Genome Biol*. 2017;18(1):225.
218. Dwyer AA, Uveges MK, Dockray S, Smith N. Exploring rare disease patient attitudes and beliefs regarding genetic testing: implications for person-centered care. *J Pers Med*. 2022;12(3):477.
219. Jiang Y-l, Song C-g, Zhou H-m, Feng B, Zhao J-j, Liu Y, et al. Rare variants in *GABRG2* associated with sleep-related hypermotor epilepsy. *J Neurol*. 2022;269(9):4939-54.
220. Shi X, Huang M-C, Ishii A, Yoshida S, Okada M, Morita K, et al. Mutational analysis of *GABRG2* in a Japanese cohort with childhood epilepsies. *Journal of human genetics*. 2010;55(6):375-8.
221. Perucca P, Scheffer IE, Harvey AS, James PA, Lunke S, Thorne N, et al. Real-world utility of whole exome sequencing with targeted gene analysis for focal epilepsy. *Epilepsy Res*. 2017;131:1-8.
222. Reinthaler EM, Dejanovic B, Lal D, Semtner M, Merkle Y, Reinhold A, et al. Rare variants in γ-aminobutyric acid type A receptor genes in rolandic epilepsy and related syndromes. *Ann Neurol*. 2015;77(6):972-86.
223. Wang T, Hoekzema K, Vecchio D, Wu H, Sulovari A, Coe BP, et al. Large-scale targeted sequencing identifies risk genes for neurodevelopmental disorders. *Nat Commun*. 2020;11(1):4932.
224. Parrini E, Marini C, Mei D, Galuppi A, Cellini E, Pucatti D, et al. Diagnostic targeted resequencing in 349 Patients with drug-resistant pediatric epilepsies identifies causative mutations in 30 different genes. *Hum Mutat*. 2017;38(2):216-25.
225. Zaganas I, Vorgia P, Spilioti M, Mathioudakis L, Raissaki M, Ilia S, et al. Genetic cause of epilepsy in a Greek cohort of children and young adults with heterogeneous epilepsy syndromes. *Epilepsy & behavior reports*. 2021;16:100477.
226. Carvill GL, Heavin SB, Yendle SC, McMahon JM, O'Roak BJ, Cook J, et al. Targeted resequencing in epileptic encephalopathies identifies *de novo* mutations in *CHD2* and *SYNGAP1*. *Nat Genet*. 2013;45(7):825-30.
227. Halvardson J, Zhao JJ, Zaghlool A, Wentzel C, Georgii-Hemming P, Månsson E, et al. Mutations in *HECW2* are associated with intellectual disability and epilepsy. *J Med Genet*. 2016;53(10):697-704.

228. Krenn M, Wagner M, Hotzy C, Graf E, Weber S, Brunet T, et al. Diagnostic exome sequencing in non-acquired focal epilepsies highlights a major role of GATOR1 complex genes. *J Med Genet.* 2020;57(9):624-33.
229. Koko M, Motelow JE, Stanley KE, Bobbili DR, Dhindsa RS, May P, et al. Association of ultra-rare coding variants with genetic generalized epilepsy: A case-control whole exome sequencing study. *Epilepsia.* 2022;63(3):723-35.
230. Bera AK, Chatav M, Akabas MH. GABA_A receptor M2-M3 loop secondary structure and changes in accessibility during channel gating. *J Biol Chem.* 2002;277(45):43002-10.
231. Lin SXN, Ahring PK, Keramidas A, Liao VWY, Møller RS, Chebib M, Absalom NL. Correlations of receptor desensitization of gain-of-function *GABRB3* variants with clinical severity. *Brain.* 2023.
232. O'Shea SM, Harrison NL. Arg-274 and Leu-277 of the γ -aminobutyric acid type A receptor $\alpha 2$ subunit define agonist efficacy and potency. *J Biol Chem.* 2000;275(30):22764-8.
233. Boileau AJ, Czajkowski C. Identification of transduction elements for benzodiazepine modulation of the GABA_A Receptor: Three residues are required for allosteric coupling. *J Neurosci.* 1999;19(23):10213-20.
234. el Achkar CM, Harrer M, Smith L, Kelly M, Iqbal S, Maljevic S, et al. Characterization of the *GABRB2*-associated neurodevelopmental disorders. *Ann Neurol.* 2021;89(3):573-86.
235. Hernandez CC, Zhang Y, Hu N, Shen D, Shen W, Liu X, et al. GABA_A Receptor coupling junction and pore *GABRB3* mutations are linked to early-onset epileptic encephalopathy. *Sci Rep.* 2017;7(1):15903.
236. Fisher JL. A mutation in the GABA_A receptor $\alpha 1$ subunit linked to human epilepsy affects channel gating properties. *Neuropharmacology.* 2004;46(5):629-37.
237. Bianchi MT, Haas KF, Macdonald RL. Structural determinants of fast desensitization and desensitization-deactivation coupling in GABA_A receptors. *J Neurosci.* 2001;21(4):1127-36.
238. Scheller M, Forman SA. Coupled and uncoupled gating and desensitization effects by pore domain mutations in GABA_A receptors. *J Neurosci.* 2002;22(19):8411-21.
239. Bianchi MT, Macdonald RL. Mutation of the 9' leucine in the GABA_A receptor $\gamma 2L$ subunit produces an apparent decrease in desensitization by stabilizing open states without altering desensitized states. *Neuropharmacology.* 2001;41(6):737-44.
240. Birnir B, Tierney ML, Lim M, Cox GB, Gage PW. Nature of the 5' residue in the M2 domain affects function of the human $\alpha 1\beta 1$ GABA_A receptor. *Synapse.* 1997;26(3):324-7.
241. Tierney ML, Birnir B, Pillai NP, Clements JD, Howitt SM, Cox GB, Gage PW. Effects of mutating leucine to threonine in the M2 segment of $\alpha 1$ and $\beta 1$ subunits of GABA_A $\alpha 1\beta 1$ receptors. *J Membr Biol.* 1996;154(1):11-21.
242. Syed P, Durisic N, Harvey RJ, Sah P, Lynch JW. Effects of GABA_A receptor $\alpha 3$ subunit epilepsy mutations on inhibitory synaptic signaling. *Front Mol Neurosci.* 2020;13(227).
243. Richards KL, Kurniawan ND, Yang Z, Kim TH, Keller MD, Low J, et al. Hippocampal volume and cell density changes in a mouse model of human genetic epilepsy. *Neurology.* 2013;80(13):1240-6.
244. Sumadewi KT, Harkitasari S, Tjandra DC. Biomolecular mechanisms of epileptic seizures and epilepsy: a review. *Acta Epileptologica.* 2023;5(1):28.
245. Wang Z, Zhou L, An D, Xu W, Wu C, Sha S, et al. TRPV4-induced inflammatory response is involved in neuronal death in pilocarpine model of temporal lobe epilepsy in mice. *Cell Death Dis.* 2019;10(6):386.
246. Sharma AK, Reams RY, Jordan WH, Miller MA, Thacker HL, Snyder PW. Mesial temporal lobe epilepsy: pathogenesis, induced rodent models and lesions. *Toxicol Pathol.* 2007;35(7):984-99.
247. Kinoshita S, Koyama R. Pro- and anti-epileptic roles of microglia. *Neural Regen Res.* 2021;16(7):1369-71.

248. Zhao X, Liao Y, Morgan S, Mathur R, Feustel P, Mazurkiewicz J, et al. Noninflammatory changes of microglia are sufficient to cause epilepsy. *Cell reports*. 2018;22(8):2080-93.
249. Cserép C, Pósai B, Lénárt N, Fekete R, László ZI, Lele Z, et al. Microglia monitor and protect neuronal function through specialized somatic purinergic junctions. *Science*. 2020;367(6477):528-37.
250. Hanisch U-K, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nature neuroscience*. 2007;10(11):1387-94.
251. Andoh M, Ikegaya Y, Koyama R. Microglia modulate the structure and function of the hippocampus after early-life seizures. *J Pharmacol Sci*. 2020;144(4):212-7.
252. Laffer B, Bauer D, Wasmuth S, Busch M, Jalilvand TV, Thanos S, et al. Loss of IL-10 promotes differentiation of microglia to a M1 phenotype. *Front Cell Neurosci*. 2019;13.
253. Miron VE, Boyd A, Zhao J-W, Yuen TJ, Ruckh JM, Shadrach JL, et al. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nature neuroscience*. 2013;16(9):1211-8.
254. Karve IP, Taylor JM, Crack PJ. The contribution of astrocytes and microglia to traumatic brain injury. *Br J Pharmacol*. 2016;173(4):692-702.
255. Eyolfson E, Khan A, Mychasiuk R, Lohman AW. Microglia dynamics in adolescent traumatic brain injury. *J Neuroinflammation*. 2020;17(1):326.
256. Bender RA, Dubé C, Gonzalez-Vega R, Mina EW, Baram TZ. Mossy fiber plasticity and enhanced hippocampal excitability, without hippocampal cell loss or altered neurogenesis, in an animal model of prolonged febrile seizures. *Hippocampus*. 2003;13(3):399-412.
257. Yu YH, Lee K, Sin DS, Park K-H, Park D-K, Kim D-S. Altered functional efficacy of hippocampal interneuron during epileptogenesis following febrile seizures. *Brain Res Bull*. 2017;131:25-38.
258. Yu C, Deng X-j, Xu D. Microglia in epilepsy. *Neurobiol Dis*. 2023;185:106249.
259. Avignone E, Ulmann L, Levavasseur F, Rassendren F, Audinat E. Status epilepticus induces a particular microglial activation state characterized by enhanced purinergic signaling. *J Neurosci*. 2008;28(37):9133-44.
260. Hiragi T, Ikegaya Y, Koyama R. Microglia after seizures and in epilepsy. *Cells*. 2018;7(4):26.
261. Wu W, Li Y, Wei Y, Bosco DB, Xie M, Zhao M-G, et al. Microglial depletion aggravates the severity of acute and chronic seizures in mice. *Brain Behav Immun*. 2020;89:245-55.
262. Gibbs-Shelton S, Benderoth J, Gaykema RP, Straub J, Okojie KA, Uweru JO, et al. Microglia play beneficial roles in multiple experimental seizure models. *Glia*. 2023;71(7):1699-714.
263. Wood H. Cortical thinning in epilepsy is linked to microglial activation. *Nat Rev Neurol*. 2021;17(10):595-.
264. Knowles JK, Xu H, Soane C, Batra A, Saucedo T, Frost E, et al. Maladaptive myelination promotes generalized epilepsy progression. *Nature neuroscience*. 2022;25(5):596-606.
265. Huitinga I, Van Rooijen N, De Groot C, Uitdehaag B, Dijkstra C. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. *J Exp Med*. 1990;172(4):1025-33.
266. Heppner FL, Greter M, Marino D, Falsig J, Raivich G, Hövelmeyer N, et al. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat Med*. 2005;11(2):146-52.
267. Qu S, Catron M, Zhou C, Janve V, Shen W, Howe RK, Macdonald RL. GABA_A receptor β 3 subunit mutation D120N causes Lennox–Gastaut syndrome in knock-in mice. *Brain Commun*. 2020;2(1).
268. Laurie DJ, Seeburg PH, Wisden W. The distribution of 13 GABA_A receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. *J Neurosci*. 1992;12(3):1063-76.

269. Hörtnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G. Patterns of mRNA and protein expression for 12 GABA_A receptor subunits in the mouse brain. *Neuroscience*. 2013;236:345-72.
270. Hanzlik E, Gigante J. Microcephaly. *Children (Basel)*. 2017;4(6):47.
271. Poulton Cathryn J, Schot R, Kia Sima K, Jones M, Verheijen Frans W, Venselaar H, et al. Microcephaly with simplified gyration, epilepsy, and infantile diabetes linked to inappropriate apoptosis of neural progenitors. *American journal of human genetics*. 2011;89(2):265-76.
272. Ito H, Shiwaku H, Yoshida C, Homma H, Luo H, Chen X, et al. *In utero* gene therapy rescues microcephaly caused by *Pqbp1*-hypofunction in neural stem progenitor cells. *Mol Psychiatry*. 2015;20(4):459-71.
273. Garcez PP, Stolp HB, Sravanam S, Christoff RR, Ferreira JCCG, Dias AA, et al. Zika virus impairs the development of blood vessels in a mouse model of congenital infection. *Sci Rep*. 2018;8(1):12774.
274. Caciagli L, Wandschneider B, Xiao F, Vollmar C, Centeno M, Vos SB, et al. Abnormal hippocampal structure and function in juvenile myoclonic epilepsy and unaffected siblings. *Brain*. 2019;142(9):2670-87.
275. Grünewald R. Childhood seizures and their consequences for the hippocampus. *Brain*. 2002;125(9):1935-6.
276. Briellmann RS, Berkovic SF, Syngeniotis A, King MA, Jackson GD. Seizure-associated hippocampal volume loss: A longitudinal magnetic resonance study of temporal lobe epilepsy. *Ann Neurol*. 2002;51(5):641-4.
277. Chatzikonstantinou A. Epilepsy and the hippocampus. In: Szabo K, Hennerici MG, editors. *The Hippocampus in Clinical Neuroscience*. 34: S.Karger AG; 2014. p. 121-42.
278. Babb TL, Kupfer WR, Pretorius JK, Crandall PH, Levesque MF. Synaptic reorganization by mossy fibers in human epileptic fascia dentata. *Neuroscience*. 1991;42(2):351-63.
279. Godale CM, Danzer SC. Signaling pathways and cellular mechanisms regulating mossy fiber sprouting in the development of epilepsy. *Front Neurol*. 2018;9:298.
280. Represa A, Jorquera I, le gal la Salle G, Ben-Ari Y. Epilepsy induced collateral sprouting of hippocampal mossy fibers: does it induce the development of ectopic synapses with granule cell dendrites? *Hippocampus*. 1993;3(3):257-68.
281. Sloviter RS, Zappone CA, Harvey BD, Frotscher M. Kainic acid-induced recurrent mossy fiber innervation of dentate gyrus inhibitory interneurons: possible anatomical substrate of granule cell hyperinhibition in chronically epileptic rats. *J Comp Neurol*. 2006;494(6):944-60.
282. Sutula T, He X-X, Cavazos J, Scott G. Synaptic reorganization in the hippocampus induced by abnormal functional activity. *Science*. 1988;239(4844):1147-50.
283. Hendricks WD, Westbrook GL, Schnell E. Early detonation by sprouted mossy fibers enables aberrant dentate network activity. *Proc Natl Acad Sci U S A*. 2019;116(22):10994-9.
284. Gutiérrez R. The GABAergic phenotype of the "glutamatergic" granule cells of the dentate gyrus. *Prog Neurobiol*. 2003;71(5):337-58.
285. Walker MC, Ruiz A, Kullmann DM. Do mossy fibers release GABA? *Epilepsia*. 2002;43(s5):196-202.
286. Weisskopf MG, Zalutsky RA, Nicoll RA. The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation. *Nature*. 1993;362(6419):423-7.
287. Luo W, Egger M, Domonkos A, Que L, Lukacsovich D, Cruz-Ochoa NA, et al. Recurrent rewiring of the adult hippocampal mossy fiber system by a single transcriptional regulator, Id2. *Proc Natl Acad Sci U S A*. 2021;118(40):e2108239118.
288. Buckmaster PS. Mossy fiber sprouting in the dentate gyrus. Noebels J, Avoli M, Rogawski M, Olsen R, Delgado-Escueta A, editors: Oxford University Press; 2012.

289. Mitsuya K, Nitta N, Suzuki F. Persistent zinc depletion in the mossy fiber terminals in the intrahippocampal kainate mouse model of mesial temporal lobe epilepsy. *Epilepsia*. 2009;50(8):1979-90.
290. Chi Z-H, Wang X, Cai J-Q, Stoltenberg M, Danscher G, Wang Z-Y. Zinc transporter 3 immunohistochemical tracing of sprouting mossy fibres. *Neurochem Int*. 2008;52(7):1305-9.
291. Lee BE, Choi BY, Hong DK, Kim JH, Lee SH, Kho AR, et al. The cancer chemotherapeutic agent paclitaxel (Taxol) reduces hippocampal neurogenesis via down-regulation of vesicular zinc. *Sci Rep*. 2017;7(1):11667.
292. Møller RS, Wuttke TV, Helbig I, Marini C, Johannesen KM, Brilstra EH, et al. Mutations in *GABRB3*: From febrile seizures to epileptic encephalopathies. *Neurology*. 2017;88(5):483-92.
293. Bamborschke D, Pergande M, Daimagüler HS, Mangold E, Dötsch J, Herkenrath P, et al. Cleft palate as distinguishing feature in a patient with *GABRB3* epileptic encephalopathy. *Neuropediatrics*. 2019;50(6):378-81.
294. Bagnall RD, Crompton DE, Semsarian C. Genetic basis of sudden unexpected death in epilepsy. *Front Neurol*. 2017;8:348.
295. Zhang N, Zhao S, Zhang S-H, Chen J, Lu D, Shen M, Li C. Intra-monozygotic twin pair discordance and longitudinal variation of whole-genome scale DNA methylation in adults. *PLoS One*. 2015;10(8):e0135022.
296. Bernier L-P, York EM, Kamyabi A, Choi HB, Weilingner NL, MacVicar BA. Microglial metabolic flexibility supports immune surveillance of the brain parenchyma. *Nat Commun*. 2020;11(1):1559.
297. Arnò B, Grassivaro F, Rossi C, Bergamaschi A, Castiglioni V, Furlan R, et al. Neural progenitor cells orchestrate microglia migration and positioning into the developing cortex. *Nat Commun*. 2014;5(1):5611.
298. Tremblay M-È, Zettel ML, Ison JR, Allen PD, Majewska AK. Effects of aging and sensory loss on glial cells in mouse visual and auditory cortices. *Glia*. 2012;60(4):541-58.
299. Colonna M, Butovsky O. Microglia function in the central nervous system during health and neurodegeneration. *Annu Rev Immunol*. 2017;35:441-68.
300. Liu J-T, Wu S-X, Zhang H, Kuang F. Inhibition of MyD88 signaling skews microglia/macrophage polarization and attenuates neuronal apoptosis in the hippocampus after status epilepticus in mice. *Neurotherapeutics*. 2018;15(4):1093-111.
301. Borges K, Gearing M, McDermott DL, Smith AB, Almonte AG, Wainer BH, Dingledine R. Neuronal and glial pathological changes during epileptogenesis in the mouse pilocarpine model. *Exp Neurol*. 2003;182(1):21-34.
302. Zhao X-F, Liao Y, Alam MM, Mathur R, Feustel P, Mazurkiewicz JE, et al. Microglial mTOR is neuronal protective and antiepileptogenic in the pilocarpine model of temporal lobe epilepsy. *J Neurosci*. 2020;40(40):7593-608.
303. Hopperton KE, Mohammad D, Trépanier MO, Giuliano V, Bazinet RP. Markers of microglia in post-mortem brain samples from patients with Alzheimer's disease: a systematic review. *Mol Psychiatry*. 2018;23(2):177-98.
304. Mo J, Enkhjargal B, Travis ZD, Zhou K, Wu P, Zhang G, et al. AVE 0991 attenuates oxidative stress and neuronal apoptosis via Mas/PKA/CREB/UCP-2 pathway after subarachnoid hemorrhage in rats. *Redox Biol*. 2019;20:75-86.
305. Wang L, Liu Y-H, Huang Y-G, Chen L-W. Time-course of neuronal death in the mouse pilocarpine model of chronic epilepsy using Fluoro-Jade C staining. *Brain Res*. 2008;1241:157-67.
306. Bian G-L, Wei L-C, Shi M, Wang Y-Q, Cao R, Chen L-W. Fluoro-Jade C can specifically stain the degenerative neurons in the substantia nigra of the 1-methy-4-phenyl-1, 2, 3, 6-tetrahydro pyridine-treated C57BL/6 mice. *Brain Res*. 2007;1150:55-61.
307. Dulla CG. Utilizing animal models of infantile spasms. *Epilepsy Curr*. 2018;18(2):107-12.

308. Jiang YL, Xia L, Zhao JJ, Zhou HM, Mi D, Wang X, et al. Mice harboring the T316N variant in the GABA_A γ_2 subunit exhibit sleep-related hypermotor epilepsy phenotypes and hypersynchronization in the thalamocortical pathway. *Exp Neurol*. 2024;376:114775.
309. Kang SK, Hawkins NA, Kearney JA. C57BL/6J and C57BL/6N substrains differentially influence phenotype severity in the *Scn1a*^{+/−} mouse model of Dravet syndrome. *Epilepsia Open*. 2019;4(1):164-9.
310. Justice MJ, Dhillon P. Using the mouse to model human disease: increasing validity and reproducibility. *Dis Model Mech*. 2016;9(2):101-3.
311. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A*. 2013;110(9):3507-12.
312. Ng AC-H, Choudhary A, Barrett KT, Gavrilovici C, Scantlebury MH. Mechanisms of infantile epileptic spasms syndrome: What have we learned from animal models? *Epilepsia*. 2024;65(2):266-80.
313. Copping NA, Adhikari A, Petkova SP, Silverman JL. Genetic backgrounds have unique seizure response profiles and behavioral outcomes following convulsant administration. *Epilepsy Behav*. 2019;101:106547.
314. Davies M, Newell JG, Dunn SMJ. Mutagenesis of the GABA_A receptor $\alpha 1$ subunit reveals a domain that affects sensitivity to GABA and benzodiazepine-site ligands. *J Neurochem*. 2001;79(1):55-62.
315. Enz R, Bormann J. A single point mutation decreases picrotoxinin sensitivity of the human GABA receptor $\rho 1$ subunit. *Neuroreport*. 1995;6(11):1569-72.
316. Filatov GN, White MM. The role of conserved leucines in the M2 domain of the acetylcholine receptor in channel gating. *Mol Pharmacol*. 1995;48(3):379-84.
317. Labarca C, Nowak MW, Zhang H, Tang L, Deshpande P, Lester HA. Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors. *Nature*. 1995;376(6540):514-6.
318. Revah F, Bertrand D, Galzi JL, Devillers-Thiéry A, Mulle C, Hussy N, et al. Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor. *Nature*. 1991;353(6347):846-9.
319. Oshi A, Alfaifi A, Seidahmed MZ, Al Hussein K, Miqdad A, Samadi A, Abdelbasit O. GABA transaminase deficiency. Case report and literature review. *Clin Case Rep*. 2021;9(3):1295-8.
320. Hegde AU, Karnavat PK, Vyas R, DiBacco ML, Grant PE, Pearl PL. GABA transaminase deficiency with survival into adulthood. *J Child Neurol*. 2019;34(4):216-20.
321. Koenig MK, Hodgeman R, Riviello JJ, Chung W, Bain J, Chiriboga CA, et al. Phenotype of GABA-transaminase deficiency. *Neurology*. 2017;88(20):1919-24.
322. Louro P, Ramos L, Robalo C, Cancelinha C, Dinis A, Veiga R, et al. Phenotyping GABA transaminase deficiency: a case description and literature review. *J Inherit Metab Dis*. 2016;39(5):743-7.
323. Medina-Kauwe LK, Tobin AJ, De Meirleir L, Jaeken J, Jakobs C, Nyhan WL, Gibson KM. 4-Aminobutyrate aminotransferase (GABA-transaminase) deficiency. *J Inherit Metab Dis*. 1999;22(4):414-27.
324. Carvill Gemma L, McMahon Jacinta M, Schneider A, Zemel M, Myers Candace T, Saykally J, et al. Mutations in the GABA transporter *SLC6A1* cause epilepsy with myoclonic-atonic seizures. *American journal of human genetics*. 2015;96(5):808-15.
325. Stefanski A, Pérez-Palma E, Brünger T, Montanucci L, Gati C, Klöckner C, et al. *SLC6A1* variant pathogenicity, molecular function and phenotype: a genetic and clinical analysis. *Brain*. 2023;146(12):5198-208.
326. Goodspeed K, Pérez-Palma E, Iqbal S, Cooper D, Scimemi A, Johannesen KM, et al. Current knowledge of *SLC6A1*-related neurodevelopmental disorders. *Brain Commun*. 2020;2(2):fcaa170.

327. Johannesen KM, Gardella E, Linnankivi T, Courage C, de Saint Martin A, Lehesjoki A-E, et al. Defining the phenotypic spectrum of *SLC6A1* mutations. *Epilepsia*. 2018;59(2):389-402.
328. Poliquin S, Hughes I, Shen W, Mermer F, Wang J, Mack T, et al. Genetic mosaicism, intrafamilial phenotypic heterogeneity, and molecular defects of a novel missense *SLC6A1* mutation associated with epilepsy and ADHD. *Exp Neurol*. 2021;342:113723.
329. Silva DB, Trinidad M, Ljungdahl A, Revalde JL, Berguig GY, Wallace W, et al. Haploinsufficiency underlies the neurodevelopmental consequences of *SLC6A1* variants. *American journal of human genetics*. 2024;111(6):1222-38.
330. Rusina E, Simonti M, Duprat F, Cestèle S, Mantegazza M. Voltage-gated sodium channels in genetic epilepsy: up and down of excitability. *J Neurochem*. 2023.
331. Berecki G, Howell KB, Heighway J, Olivier N, Rodda J, Overmars I, et al. Functional correlates of clinical phenotype and severity in recurrent *SCN2A* variants. *Commun Biol*. 2022;5(1):515.
332. Hack JB, Horning K, Juroske Short DM, Schreiber JM, Watkins JC, Hammer MF. Distinguishing loss-of-function and gain-of-function *SCN8A* variants using a random forest classification model trained on clinical features. *Neurol Genet*. 2023;9(3):e200060.
333. Chung KM, Hack J, Andrews J, Galindo-Kelly M, Schreiber J, Watkins J, Hammer MF. Clinical severity is correlated with age at seizure onset and biophysical properties of recurrent gain of function variants associated with *SCN8A*-related epilepsy. *Epilepsia*. 2023;64(12):3365-76.
334. Guo Q-b, Zhan L, Xu H-y, Gao Z-b, Zheng Y-m. *SCN8A* epileptic encephalopathy mutations display a gain-of-function phenotype and divergent sensitivity to antiepileptic drugs. *Acta Pharmacol Sin*. 2022;43(12):3139-48.
335. Cioclu MC, Mosca I, Ambrosino P, Puzo D, Bayat A, Wortmann SB, et al. *KCNT2*-related disorders: phenotypes, functional, and pharmacological properties. *Ann Neurol*. 2023;94(2):332-49.
336. Cui F, Wulan T, Zhang Q, Zhang VW, Jiang Y. Identification of a novel *KCNT2* variant in a family with developmental and epileptic encephalopathies: a case report and literature review. *Front Genet*. 2024;15:1371282.
337. Rinaldi B, Bayat A, Zachariassen LG, Sun J-H, Ge Y-H, Zhao D, et al. Gain-of-function and loss-of-function variants in *GRIA3* lead to distinct neurodevelopmental phenotypes. *Brain*. 2023;147(5):1837-55.
338. Botzolakis EJ, Gurba KN, Lagrange AH, Feng H-J, Stanic AK, Hu N, Macdonald RL. Comparison of γ -aminobutyric acid, type A (GABA_A) receptor $\alpha\beta\gamma$ and $\alpha\beta\delta$ expression using flow cytometry and electrophysiology: Evidence for alternative subunit stoichiometries and arrangements*. *J Biol Chem*. 2016;291(39):20440-61.
339. Maljevic S, Keren B, Aung YH, Forster IC, Mignot C, Buratti J, et al. Novel *GABRA2* variants in epileptic encephalopathy and intellectual disability with seizures. *Brain*. 2019;142(5):e15-e.
340. Farrace D, Tommasi M, Casadio C, Verrotti A. Parenting stress evaluation and behavioral syndromes in a group of pediatric patients with epilepsy. *Epilepsy Behav*. 2013;29(1):222-7.
341. Gallop K, Lloyd AJ, Olt J, Marshall J. Impact of developmental and epileptic encephalopathies on caregivers: A literature review. *Epilepsy Behav*. 2021;124:108324.
342. Salom R, Aras LM, Piñero J, Duñabeitia JA. Dataset on the psychosocial impact in families with children with developmental and epileptic encephalopathies. *Sci Data*. 2023;10(1):530.
343. Wirrell EC, Wood L, Hamiwka LD, Sherman EMS. Parenting stress in mothers of children with intractable epilepsy. *Epilepsy Behav*. 2008;13(1):169-73.