GABBR2 Mutations Determine Phenotype in Rett Syndrome and Epileptic Encephalopathy

Yongjin Yoo, BS,[1] Jane Jung, BS,[2] Yoo-Na Lee, BS,[3] Youngha Lee, MS,[1]
Hyosuk Cho, BS,[1] Eunjung Na, BS,[2] JeaYeok Hong, BS,[2] Eunjin Kim, MS,[2]
Jin Sook Lee, MD, PhD,[4] Je Sang Lee, MD,[5] Chansik Hong, PhD,[6]
Sang-Yoon Park, PhD,[7] Jinhong Wie, PhD,[1,8] Kathryn Miller, CGC,[9]
Natasha Shur, MD,[9] Cheryl Clow, RN,[9] Roseànne S. Ebel, MS,[10]
Suzanne D. DeBrosse, MD,[10] Lindsay B. Henderson, PhD,[11]
Rebecca Willaert, MS, MA, CGC,[11] Christopher Castaldi, MS,[12] Irina Tikhonova, MS,[12]
Kaya Bilgüvar, MD,[12,13] Shrikant Mane, PhD,[12,13] Ki Joong Kim, MD, PhD,[14]
Yong Seung Hwang, MD, PhD,[14] Seok-Geun Lee, PhD,[7] Insuk So, MD, PhD,[1,8]
Byung Chan Lim, MD, PhD,[14] Hee-Jung Choi, PhD,[15] Jae Young Seong, PhD,[3]
Yong Beom Shin, MD, PhD,[5] Hosung Jung, PhD,[2]
Jong-Hee Chae, MD, PhD,[14] and Murim Choi, PhD[1,14]

Objective: Rett syndrome (RTT) and epileptic encephalopathy (EE) are devastating neurodevelopmental disorders with distinct diagnostic criteria. However, highly heterogeneous and overlapping clinical features often allocate patients into the boundary of the two conditions, complicating accurate diagnosis and appropriate medical interventions. Therefore, we investigated the specific molecular mechanism that allows an understanding of the pathogenesis and relationship of these two conditions.
Methods: We screened novel genetic factors from 34 RTT-like patients without *MECP2* mutations, which account for ~90% of RTT cases, by whole-exome sequencing. The biological function of the discovered variants was assessed in cell culture and *Xenopus tropicalis* models.

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.25032

Received Mar 10, 2017, and in revised form Aug 23, 2017. Accepted for publication Aug 23, 2017.

Address correspondence to Dr Murim Choi, Department of Biomedical Sciences, Seoul National University College of Medicine, 103 Daehakro, Jongro-gu, Seoul, 03080, Republic of Korea. E-mail: murimchoi@snu.ac.kr; Dr Hosung Jung, Department of Anatomy, Brain Research Institute, and Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul, 03722, Republic of Korea. E-mail: hosungjung@yonsei.ac.kr; Dr. Jong-HeeChae, Department of Pediatrics, Seoul National University College of Medicine, Seoul National University Children's Hospital, Seoul, 03080, Republic of Korea. E-mail: chaeped1@snu.ac.kr

From the [1]Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Republic of Korea; [2]Department of Anatomy, Brain Research Institute, and Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul, Republic of Korea; [3]Graduate School of Medicine, Korea University, Seoul, Republic of Korea; [4]Department of Pediatrics, Department of Genome Medicine and Science, Gachon University Gil Medical Center, Incheon, Republic of Korea; [5]Department of Rehabilitation Medicine, Pusan National University College of Medicine, Pusan, Republic of Korea; [6]Department of Physiology, Chosun University School of Medicine, Kwangju, Republic of Korea; [7]Department of Science in Korean Medicine, Cancer Preventive Material Developmental Research Center, College of Korean Medicine, Kyung Hee University, Seoul, Republic of Korea; [8]Department of Physiology, Seoul National University College of Medicine, Seoul, Republic of Korea; [9]Albany Medical Center, Albany, NY; [10]UH Cleveland Medical Center, Center for Human Genetics, Cleveland, OH; [11]GeneDx, 207 Perry Parkway, Gaithersburg, MD; [12]Yale Center for Genome Analysis, West Haven, CT; [13]Department of Genetics, Yale University School of Medicine, New Haven, CT; [14]Department of Pediatrics, Seoul National University College of Medicine, Seoul National University Children's Hospital, Seoul, Republic of Korea; and [15]Department of Biological Sciences, Seoul National University College of Natural Sciences, Seoul, Republic of Korea

Additional supporting information can be found in the online version of this article.

 © 2017 American Neurological Association

Results: We identified a recurring de novo variant in GABAB receptor R2 (*GABBR2*) that reduces the receptor function, whereas different *GABBR2* variants in EE patients possess a more profound effect in reducing receptor activity and are more responsive to agonist rescue in an animal model.

Interpretation: *GABBR2* is a genetic factor that determines RTT- or EE-like phenotype expression depending on the variant positions. *GABBR2*-mediated γ-aminobutyric acid signaling is a crucial factor in determining the severity and nature of neurodevelopmental phenotypes.

ANN NEUROL 2017;82:466–478

After 6 to 18 months of normal developmental period, patients with Rett syndrome (RTT; Mendelian Inheritance in Man [MIM] #312750) undergo a characteristic developmental regression characterized by a cascade of intellectual, motor, and social disabilities. Patients exhibit head growth arrest, stereotyped hand movements, loss of language skills, and epilepsy.[1,2] Methyl-CpG-binding protein 2 (*MECP2*) is a major culprit in RTT pathogenesis, accounting for around 90% of patients.[3–5] This X-linked gene encodes a neuron-enriched epigenetic regulator involved in a number of molecular processes, ensuring normal neuronal maturation processes in the postnatal period.[3] Studies using various RTT animal models indicate the defective homeostatic regulation of synaptic strength as a prime driver of RTT symptoms, disrupting the overall balance between excitatory and inhibitory synapses.[6,7] Specifically, the dysregulation of activity-dependent synaptic scaling during development has been revealed in RTT animal models.[8,9] For example, deletion of *Mecp2* in γ-aminobutyric acid (GABA)-expressing neurons results in reduced GABA signaling and recapitulates most of the RTT features in mice,[10] whereas the ablation of *Mecp2* in excitatory glutamatergic neurons caused neurological features different from those of RTT.[11]

Epileptic encephalopathy, which includes infantile spasm, West syndrome, Lennox–Gastaut syndrome, and others, is characterized by aggressive and intractable episodes of epilepsy that accompany severe cognitive and neurological regressions, and sometimes death.[12] Genes that cause EE were discovered, displaying indisputable associations with a broad range of molecular processes, including ion channel, synaptic transmission, transporters, transcriptional dysregulation, and chromatin remodeling.[13,14] Recent large-scale studies report genes that are recurrently mutated in EE patients and demonstrate statistical enrichments in gene sets, including the ion channels, autism spectrum disorder, and intellectual disability.[15,16] Interestingly, these studies emphasize the significance of GABA receptor subunit mutations, validating the well-established role of GABA signaling in the epilepsy phenotype.[15,16]

Although having distinguishable key clinical features and different diagnostic criteria, extremely diverse neurodevelopmental phenotypes often allocate patients into the gray area between similar disorders, including RTT and EE, hampering precise diagnosis and proper medical intervention.[17,18] Indeed, large-scale genetic studies of patients with developmental delay or intellectual delay symptoms often discover pathogenic variants in genes tightly linked with RTT or EE.[19,20] Diverse types of *MECP2* mutations entail different clinical features of varying severities, and several additional genetic candidates have been found to cause an RTT-like phenotype when mutated.[21] *CDKL5* was initially identified as an RTT-causing gene,[22,23] but subsequent studies have emphasized the early-onset EE features of the *CDKL5*-mutated patients.[24,25] Some argue that autism spectrum disorder, sharing a large portion of clinical features with RTT, and epilepsy may share common biological pathways.[18] Hence, completing a genetic picture of RTT and EE pathogeneses and determining how the two diseases are genetically intertwined remain ongoing processes.

Here, we analyze *MECP2* mutation-negative patients that exhibit an RTT-like phenotype and identify a novel GABAB receptor R2 (*GABBR2*) mutation in 2 unrelated patients. Given the previous observation that *GABBR2* is also mutated in EE patients, we explored the molecular consequences of *GABBR2* mutations using cell culture and vertebrate animal models and found that the severity of the mutations determines the phenotypic outputs.

Subjects and Methods

Subjects

Thirty-four subjects with *MECP2*-negative RTT-like features based on the revised criteria by Neul et al[1] and their parents were included in this study. Clinical features are summarized in Supplementary Tables 1 and 2. The subjects and their parents gave informed consent for DNA storage and genetic analysis. All samples had been collected for research purpose after approval by the institutional review board of Seoul National University Children's Hospital (ID: 1405-036-578) and Pusan National University Hospital (ID: H-1501-006-025).

Identification and Validating Variants

Genomic DNA was prepared from venous blood by standard procedure and processed at Yale Center for Genome Analysis and Theragen Etex Bio Institute (Suwon, Korea) for whole-exome sequencing (WES), as described previously.[26] The two

American pedigrees (RTT83-1 and RTT84-1) had clinical exome sequencing performed at GeneDx (Gaithersburg, MD). Processing sequence data, variant calling, and identification of de novo mutations were performed as described previously.[27] Direct Sanger sequencing was performed by standard methods following polymerase chain reaction amplifications.

Western Blot

HEK293 cells were seeded 1 day before transfection, and cells were cotransfected with *GABBR1* and wild-type, *GABBR2*$^{\Delta RTT}$, *GABBR2*$^{\Delta EE1}$, or *GABBR2*$^{\Delta EE2}$ constructs using GeneIn (GlobalStem, Gaithersburg, MD). After 48 hours of incubation, cells were washed with phosphate-buffered saline (PBS) and immediately lysed in the radioimmunoprecipitation assay buffer with protease inhibitor cocktail (GenDEPOT, Barker, TX). Solubilized proteins were separated on 10% sodium dodecyl sulfate/ppolyacrylamide gel electrophoresis and immunoblotted with anti-GABBR1 (1:2,000, ab55051; Abcam, Cambridge, UK), anti-GABBR2 (1:500, ab75838; Abcam), and anti-α tubulin (1:2,000, SC-8035; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies.

Immunofluorescence Assay

HeLa cells cotransfected with *GABBR1* and wild-type, *GABBR2*$^{\Delta RTT}$, *GABBR2*$^{\Delta EE1}$, or *GABBR2*$^{\Delta EE2}$ constructs were cultured onto poly-L-lysine (Sigma-Aldrich, St. Louis, MO) coated coverslips, fixed with 4% paraformaldehyde, and then washed briefly in PBS. Cells were permeabilized with 0.25% Triton X-100 PBS for 10 minutes and then were washed with PBS three times. Cells were blocked with 5% bovine serum albumin in 0.1% Triton X-100 PBS for 60 minutes and then incubated in the mixture of two primary antibodies (anti-GABBR1, 1:1,000 [ab55051; Abcam]; anti-GABBR2, 1:150 [ab75838; Abcam]) overnight at 4°C. Samples were washed with PBS-Tween three times, then incubated with secondary antibodies (antimouse/Alexa Flour 568 conjugated, 1:50 [A-11004; Life Technologies, Carlsbad, CA]; antirabbit/fluorescein isothiocyanate conjugated, 1:50 [F-2765; Life Technologies]) for 1 hour at room temperature. Then, cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 minutes, washed, and mounted on slides using Dako Mounting Medium (Dako, Glostrup, Denmark) and viewed using an Olympus FV 1000 confocal microscope (Olympus Corporation, Tokyo, Japan).

Luciferase Assay

HEK293 cells stably expressing G_{aqi} that can mediate G_{ai}-coupled receptor activation by stimulating G_{aq}-dependent signaling pathways[28] were used for luciferase assay according to the standard protocol.

Frog Experiments

DNA, RNA, and/or morpholino oligonucleotides (MOs) were injected into the two dorsal animal blastomeres at the eight-cell stage (which later give rise to the central nervous system [CNS]). For loss-of-function studies, translation-blocking antisense MOs (Gene Tools, LLC, Philomath, OR) were designed to target the 5′ untranslated region of *Xenopus GABBR2* mRNA (5′-AGCTGTGTGTGTGACTGCAAGGTAC-3′). Standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′) was used as a negative control. For gain-of-function studies, injections were made into the two dorsal animal blastomeres at the eight-cell stage. Eighty picograms of the wild-type or mutant *hGABBR2* cDNA-encoding plasmid and 40pg of pEGFP-N1 plasmid (as a tracer) were injected per blastomere. Successfully injected embryos were screened by the presence of green fluorescent protein fluorescence and raised to stage 45 or 49 for each experiment. For the rescue experiments, 100pg of in vitro transcribed (mMessagemMachine; Thermo Fisher Scientific, Waltham, MA) *hGABBR2* mRNAs were coinjected with MO. The same amount of control RNA (mCherry) was injected into the control group. Their behavior was recorded for 10 minutes. In some cases, recorded tadpoles were incubated with 100μM of baclofen (#14340; Sigma-Aldrich) for 1 hour and then recorded again for 10 minutes. The swimming pattern of individual tadpoles was traced using LoliTrack v.4 (Loligo Systems, Viborg, Denmark). Swimming trajectories were generated by R (v3.3.3; R Foundation for Statistical Computing, Vienna, Austria). For the GABBR2 antagonist experiments, tadpoles were first incubated with 100μM of CGP52432 (#1246; Tocris Bioscience, Bristol, UK) for 1 hour and then recorded for 10 minutes. A seizure-like event was defined as an episode of one or more of the following behaviors: (1) involuntary rapid movements perpendicular to the body axis; (2) rapid corkscrew swimming; and (3) head shake, writhing, and twisting movements. All experiments complied with protocols approved by the Yonsei University College of Medicine Institutional Animal Care and Use Committees (Seoul, Republic of Korea).

Statistical Analysis

Statistical analysis was performed using R (version 3.4.0; R Foundation for Statistical Computing). In vitro experiments were analyzed by two-tailed Student's *t* test, and in vivo experiments were analyzed by Wilcoxon–Mann–Whitney test, as indicated in the figure legends for each experiment. Error bars represent standard error of the mean (SEM). A *p* value less than 0.05 was considered significant.

Results

A Recurrent De Novo Variant of GABBR2 Was Discovered in RTT-Like Patients

To identify additional genetic components that confer an RTT-like phenotype in humans, we collected 34 patients who were diagnosed with RTT based on the 2010 revised criteria (Supplementary Table 1),[1] but who did not carry an *MECP2* mutation and subjected the trios into WES (Supplementary Table 3). De novo and rare recessive variants were called, and these patient-specific variants were assessed with strict in silico functionality tests; we required that they should be either loss-of-function variants or nonsynonymous changes to amino acids with complete evolutionary conservation and were never seen in normal population databases (Table 1).

TABLE 1. List of Notable Variants From the Studied Patients

Group	No. of Patients	Sample ID	Gene	Variant	Previous Disease Association (OMIM number)
				Origin/Type/Amino Acid Change	
A	1	RTT04-1	*CDKL5*	De novo/nonsense/p.Arg550X	Rett syndrome, epileptic encephalopathy (300672)
B	14	RTT61-1	*CLTC*	De novo/missense/p.Cys1260Arg	Multiple malformation
		RTT58-1	*CTNNB1*	De novo/splice site/c.41274832-1G>C De novo/nonsense/p.Arg587X	Mental retardation (615075)
		RTT41-1			
		RTT17-1	*DMXL2*	De novo/missense/p.Val2037Gly	Polyendocrine-polyneuropathy syndrome (616113)
		RTT01-1	*GABBR2*	De novo/missense/p.Ala567Thr	Rett syndrome, epileptic encephalopathy (300672)
		RTT02-1			
		RTT83-1*[a]			
		RTT84-1**[b]			
		RTT80-1	*GRIA3*	Hemizygous/missense/ p.Met360Thr	Mental retardation, X-linked (300699)
		RTT49-1	*GRIN2A*	De novo/missense/p.Thr749 Ile	Epilepsy, focal, with speech disorder and with or without mental retardation (245570)
		RTT48-1	*GRIN2B*	De novo/missense/p.Gly820Ala	Mental retardation (613970) Epileptic encephalopathy (616139)
		RTT75-1			
		RTT16-1	*SLC6A1*	De novo/missense/p.Ala357Val	Myoclonic-atonic epilepsy (616421)
		RTT18-1	*SMC1A*	De novo/frameshift/p.Lys88fs*29	Cornelia de Lange syndrome (300590)
		RTT69-1	*STXBP1*	De novo/missense/p.Pro480Leu	Epileptic encephalopathy (612164)
		RTT59-1	*TCF4*	De novo/frameshift/ p.Gly190*fs14	Pitt–Hopkins syndrome (610954) Corneal dystrophy, Fuchs endothelial (613267)
C	2	RTT46-1	chr1.p21.1-p13.2 (including *NTNG1*)	De novo/deletion/.	Severe mental retardation, short stature, dysmorphic feature
		RTT55-1	chr2.q37.1-q37.3 (including *HDAC4*)	De novo/deletion/.	Chromosome 2q37 deletion syndrome (600430)

TABLE 1: Continued

Group	No. of Patients	Sample ID	Gene	Variant	Previous Disease Association (OMIM number)
				Origin/Type/Amino Acid Change	
D	6	RTT03-1	*ANXA11*	De novo/missense/ p.Arg210Trp	.
		RTT45-1	*KIF4B*	De novo/missense/ p.Met309Thr	.
		RTT11-1	*OSBP*	De novo/missense/ p.Tyr484His	.
		RTT56-1	*RHOBTB2*	De novo/missense/ p.Arg179Glu De novo/missense/ p.Arg483His	.
		RTT82-1			.
		RTT09-1	*RRN3*	De novo/missense/ p.Try436His	.
E	11	.	.	.	.

Group A: genes previously associated with RTT; B: genes previously associated with other neurological disorders in humans; C: de novo copy number alteration; D: genes with no previous disease association; E: no notable variants.
[a]Patient from University Hospitals Cleveland Medical Center (Cleveland, OH).
[b]Patient from Albany Medical Center (Albany, NY).
OMIM = Online Mendelian Inheritance in Man.

This approach initially yielded 38 de novo events, and 23 variants (including two de novo copy number alterations and one hemizygous variant) passed the functionality criteria and were called "potentially functional" (Table 1). All variants were validated by Sanger sequencing. Among the 23 de novo variants, four genes were carried by two patients (*CTNNB1*, *GABBR2*, *GRIN2B*, and *RHOBTB2*). Remarkably, identical de novo variants were detected in *GABBR2* (p.Ala567Thr; in patients RTT01-1 and RTT02-1) and *GRIN2B* (p.Gly820Ala; in patients RTT48-1 and RTT75-1; Table 1). Observing a de novo variant in an identical genomic position from 2 of 34 individuals is highly unlikely to occur by chance ($p = 1.40 \times 10^{-7}$ for sharing any base, $p = 1.75 \times 10^{-6}$ if adjusted by base context; Monte Carlo simulation). These heterozygous variants were found in the 2 probands only and not in their parents or healthy siblings (Fig 1A). The $GABBR2^{A567T}$ and $GRIN2B^{G820A}$ changes were not found in the 1000 Genomes, Exome Aggregation Consortium (ExAC), and a database of 1,055 healthy Koreans.[29] Because *GRIN2B* was previously described to be associated with a broad range of neurodevelopmental disorders,[30–32] and based on the implication of GABA signaling on neurodevelopmental disorders, we subsequently focused on $GABBR2^{A567T}$. The 2 patients (RTT01-1 and RTT02-1) do not carry any notable variants or copy number alterations in known RTT genes, and were not related and DNA mixture was not observed. In a separate screening study of *MECP2*-negative Rett-like patients, the identical mutation was discovered from 1 of the 12 Portuguese patients, further supporting our observation that de novo $GABBR2^{A567T}$ is associated with Rett-like phenotype ($p < 5.0 \times 10^{-8}$).[33] Our patients were an 11-year-old boy and a 7-year-old girl; their clinical courses were not identical, but both shared essential Rett-like features, such as having a period of regression followed by stagnation, stereotyped hand movements, microcephaly, autonomic dysfunction, sleep disturbances, abnormal breathing, generalized tonic-clonic seizures, characteristic EEG patterns that are distinct from those of EE patients, and normal brain structures (Supplementary Table 2; Supplementary Video 1). Neither displayed any linguistic achievement, purposeful hand motions, or social interaction. Our GeneMatcher-assisted search enabled identification of 2 additional patients of European ancestry that harbor the same de novo variant in *GABBR2* with similar clinical symptoms (Fig 1A; Table 1 and Supplementary Table 2), further strengthening our initial discovery.[34] Although it can be defined with discrete clinical features, RTT may display heterogeneous manifestations, and unique features specific to an individual patient have been noted.[21] RTT01-1 displayed an ataxic gait; RTT02-1 had mild left lumbar scoliosis and bilateral strabismus, which were

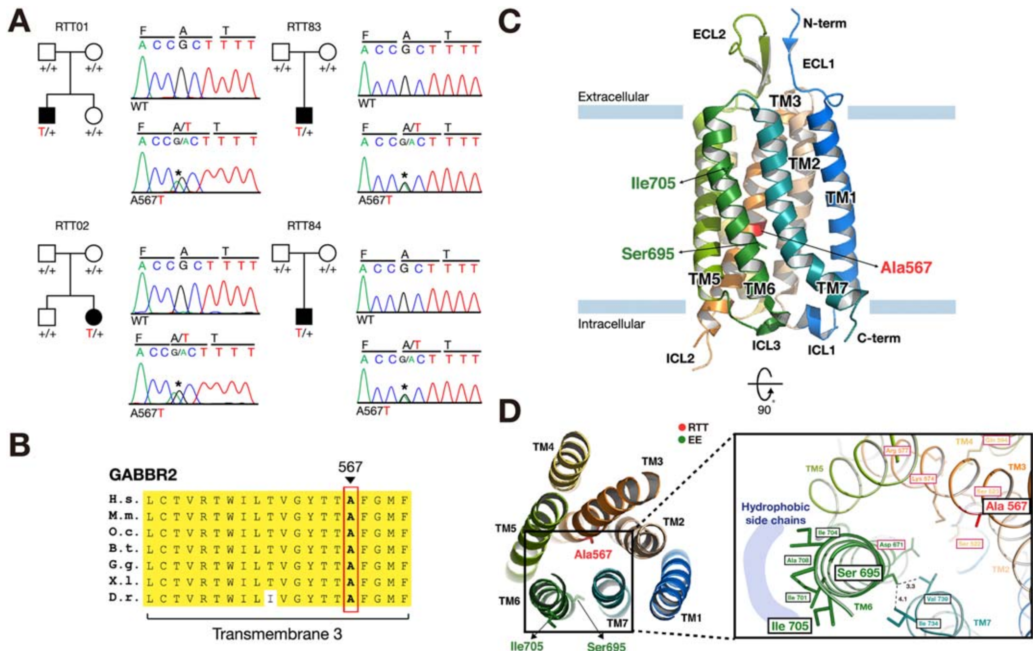

FIGURE 1: A de novo variant in *GABBR2* discovered from RTT-like patients. (A) Pedigrees of the apparently unrelated two families and Sanger traces displaying an identical de novo variant in *GABBR2* specific to the patients. RTT83-1 and RTT84-1 are from the American pedigrees that were identified through the GeneMatcher software.[34] (B) Evolutionary conservation of TM3, encompassing the mutated Ala 567 residue. H.s. denotes *Homo sapiens* (human); M.m., *Mus musculus* (mouse); O.c., *Oryctolagus cuniclulus* (rabbit); B.t., *Bos taurus* (cow); G.g., *Gallus gallus* (chicken); X.l., *Xenopus laevis* (frog); D.r., *Danio rerio* (zebrafish). (C) Simulated transmembrane structure of GABBR2 based on the known mGlu1 structure (Protein Database ID: 4OR2), pointing to the mutated residues from a side view (left). The transverse view of the receptor from the top. Dot lined boxes are indicated for close-up views (right). (D) Close-up views of Ala 576, Ile 705 and neighboring hydrophobic residues, and Ser 695 and its possible interactions with TM7 residues. Ionic lock residues were indicated with red box. Numbers on the dotted lines indicate distances in angstroms. EE = epileptic encephalopathy; RTT = Rett syndrome; WT = wild type.

surgically corrected; the RTT83-1 patient displayed anhematopoietic anemia and oculomotor problem; the RTT84-1 patient was macrocephalic (Supplementary Table 2). Overall, these features place the patients into the stage III category of the disease progression.

Recently, two novel heterozygous de novo mutations in *GABBR2* were found in EE (Ser695Ile and Ile705Asn),[15] which, together with our findings, suggests an intriguing possibility that EE and RTT could result from different alterations of the same gene. Therefore,

TABLE 2. Gene Ontology (GO) Analysis of RTT- and EE-Associated Genes

Disease	Category[a]: GO ID	Term/No. of Genes/Genes	*p*	*Q* Value
RTT	MF: 0008066	Glutamate receptor activity/3/*GRIA3*, *GRIN2A*, *GRIN2B*	2.12×10^{-6}	1.19×10^{-4}
	BP: 0007268	Synaptic transmission /7/*GABBR2*, *SLC6A1*, *GRIA3*, *GRIN2A*, *GRIN2B*, *CTNNB1*, *STXBP1*	1.77×10^{-6}	5.46×10^{-4}
	CC: 0045202	Synapse/9/*GABBR2*, *GRIA3*, *DMXL2*, *CAD*, *GRIN2A*, *GRIN2B*, *CTNNB1*, *STXBP1*, *CLTC*	2.79×10^{-8}	8.38×10^{-7}
EE	MF: 0022836	Gated channel activity/6/*GABRB3*, *KCNQ2*, *RYR3*, *SCN1A*, *SCN2A*, *SCN8A*	2.64×10^{-5}	3.62×10^{-3}
	CC: 001518	Voltage-gated sodium channel complex/3/*SCN1A*, *SCN2A*, *SCN8A*	9.63×10^{-5}	9.10×10^{-3}

[a]MF = molecular function; BP = biological process; CC = cellular component.
RTT = Rett syndrome; EE = epileptic encephalopathy.

we turned our attention to EE and hypothesized that *GABBR2* may lie at the intersection of RTT- or EE-causing pathways and may have a role in differentiating the two diseases despite the observation that the two diseases display distinct enrichment of biological pathways (Table 2).

Mutations in GABBR2 *Disrupt Proper Receptor Function*

GABBR2 forms an obligatory heterodimeric G-protein-coupled receptor (GPCR) GABAB receptor ($GABA_BR$) complex with GABBR1, the GABA-binding subunit.[35] GABBR1 and GABBR2 bind through their intracellular C-terminal domains, which are necessary for signal transduction. Although the three-dimensional structure of the transmembrane (TM) domain of GABBR2 has not been resolved, potential function of Ala 567 could be inferred from the recently reported structures of metabotropic glutamate receptors 1 and 5 (mGluR1 and mGluR5),[36,37] which belong to class C GPCR with GABBR2. The mutated residue Ala 567 is predicted to be in the third transmembrane (TM3) helix, which confers structural integrity and makes extensive interactions with neighboring transmembrane helices. TM3 residues of GABBR2 are highly conserved among the orthologs from all the vertebrate species examined (Fig 1B). Activation of GPCR typically involves conformational change of TMs, especially TMs 3, 5, and 6, propagating the signal from the extracellular side to the cytoplasmic side. In the absence of agonist, Lys 574 (TM3) and Asp 688 (TM6) of GABBR2 would form an ionic lock, as observed in mGluR1 and mGluR5 structures, similar to that in class A GPCR, suggesting that GABBR2 share common activation mechanism of class A GPCRs, which involves the breakage of ionic lock and rearrangement of residues on TMs 3 and 6.[38,39] Ala 567 lies two helix-turns above Lys 574, raising the possibility that the mutation at this position may affect the receptor activation process (Fig 1C,D). On the contrary, two mutations harbored by EE patients, S695I and I705N, are located in TM6 (Fig 1C). Ile 705 lies a cluster of hydrophobic amino acids and introduction of polar residue, like Asn, would destabilize the hydrophobic environment (Fig 1D). The other change, replacement of Ser with Ile, a bulky hydrophobic residue, would cause unfavorable Van der Waals repulsion with nearby TM7 residues, leading to conformational rearrangement to avoid steric clash (Fig 1D). TM6 residues of GABBR2 are also highly conserved among the orthologs from all the vertebrate species examined (data not shown).

$GABA_BR$ is a $G_{i/o}$-coupled metabotropic receptor that reduces synaptic transmission through intracellular effector molecules,[40] in contrast to the ionotropic GABA(A) receptor ($GABA_AR$), which triggers a fast inhibitory synaptic response through chloride ion influx.[41] Activation of $GABA_BR$ induces dissociation of receptor-bound trimeric $G_{i/o}$ into $G_{\alpha i/o}$ and $G_{\beta\gamma}$ subunits, and released $G_{\alpha i/o}$ inhibits adenylyl cyclase, decreasing the level of intracellular cyclic AMP (cAMP), a regulator of protein kinase A. Through this action, $GABA_BR$ exerts slow and long-lasting effects on neural development,[42] maintaining a delicate balance of excitatory and inhibitory neuronal signaling. $GABA_BR$ also reduces synaptic activity through $G_{\beta\gamma}$, by inhibiting the Ca_v and GIRK channels.[43,44] To test if the *GABBR2* mutations discovered from RTT and EE patients may interfere with normal protein production or subcellular localization, we cotransfected constructs encoding wild-type or mutant GABBR2 along with wild-type GABBR1. The GABBR2 mutations did not alter the amount or subcellular localization of the $GABA_BR$ receptors compared to the wild type (Fig 2A,B). Therefore, as a next step, we chose to assay intracellular cAMP as a measurement of AC activity to investigate whether the mutations disrupt proper $GABA_BR$ activity.[45] To circumvent the technical difficulty of reading an inhibitory function, the HEK293 $G_{\alpha qi}$ cell line that stably expresses a chimeric $G_{\alpha qi}$ protein, which converts G_i-coupled GPCR signaling into G_q-coupled signaling and detects the receptor activation by a serum responsive element-luciferase (SRE-luc) reporter, was used.[46] The addition of GABA and the $GABA_BR$-specific agonist baclofen to the culture media induced a 10-fold increase in receptor activity when a wild-type ($GABBR2^{WT}$) receptor was introduced.[47] However, $GABBR2^{A567T}$ (hereby designated as $GABBR2^{\Delta RTT}$) showed significantly lowered agonist-induced activity (approximately 30% of wild type), suggesting that the mutation exerts a hypomorphic effect through a dominant-negative mechanism (Fig 2C,D). Interestingly, cotransfection of each EE mutation construct ($GABBR2^{S695I}$ or $GABBR2^{I705N}$, designated as $GABBR2^{\Delta EE1}$ or $GABBR2^{\Delta EE2}$, respectively) further reduced the receptor activities, raising the possibility that the major distinction between RTT- and EE-causing variants is the receptor activity. Basal receptor activities did not display a significant difference, suggesting that the abnormal receptor functions are agonist dependent.

Perturbations of GABBR2 *in Frog Phenocopy RTT- and EE-Like Phenotypes*

To further investigate the functional consequences of the receptor mutations in live animals, we adopted a frog model (*Xenopus tropicalis*). Previous studies demonstrated the utility of *X. tropicalis* tadpoles as a vertebrate model

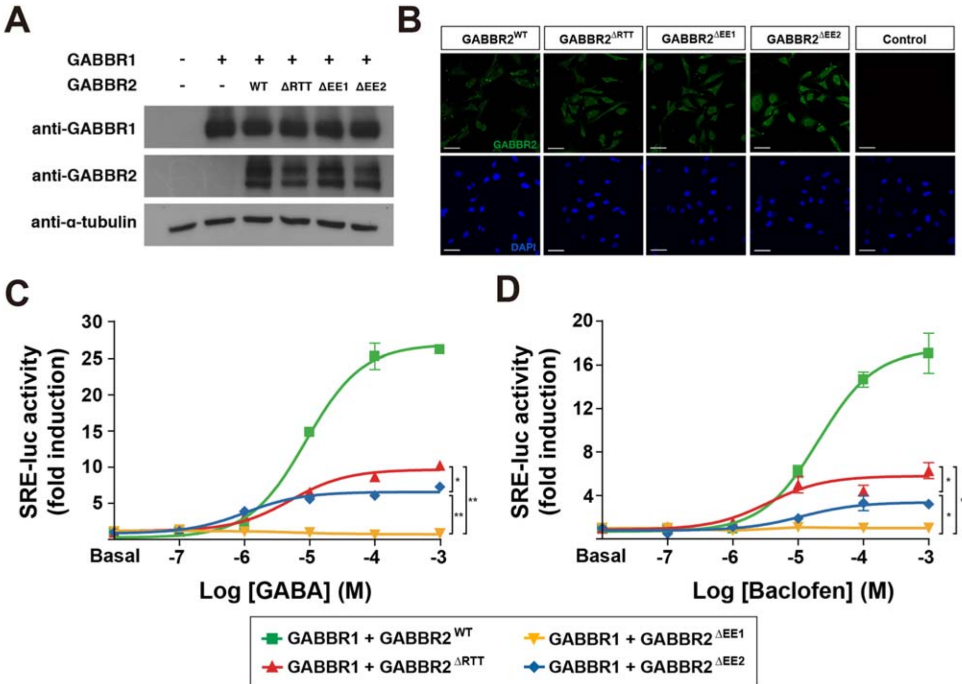

FIGURE 2: *GABBR2* mutation reduces receptor activity without altering protein expression and subcellular localization. (A) Western blot of GABAB receptor complex by either wild-type or mutant GABBR2 in the presence (+) or absence (–) of GABBR1 in HEK293 cells. Overexpression of GABBR2 in HEK293 cells resulted in a doublet band at ~110kDa, which was previously noted.[59] (B) Immunofluorescence images displaying localization of GABBR2 (green). HeLa cells were counterstained with the nuclear probe, DAPI (blue). Scale bars: 50μm. Cytoplasmic expression of GABBR2 in mammalian cell lines was previously noted.[60] (C,D) Wild-type, *GABBR2*$^{\Delta RTT}$, *GABBR2*$^{\Delta EE1}$, or *GABBR2*$^{\Delta EE2}$ construct was transfected with *GABBR1* construct to HEK293 $G_{\alpha qi}$ cell line and treated with GABA (C) or GABA agonist baclofen (D). Data are shown as mean ± SEM. n = 3. *$p < 0.05$; **$p < 0.005$, Student's *t* test. DAPI = 4′,6-diamidino-2-phenylindole; GABA = γ-aminobutyric acid; SEM = standard error of the mean; WT = wild type.

of human behavioral phenotypes, such as epilepsy or autistic patterns.[48–50] Therefore, we sought to test whether abnormal GABA signaling caused by the mutations would induce behavioral abnormalities that mimic neurodevelopmental defects through targeted microinjection of the constructs expressing the mutant or wild-type form of GABBR2 in the CNS (Fig 3A). Because $GABA_BR$ is an obligatory heterodimer, overexpressing mutant forms of human GABBR2 in the entire CNS only affects neurons that normally express its obligatory partner, GABBR1, accurately modeling the human condition. First, we confirmed that ectopically expressed human GABBR2 would form a functional receptor complex with endogenous *Xenopus* GABBR1 and would not function when no GABBR1 is expressed (Fig 3B). Then, we performed behavioral assays using free-swimming tadpoles (stages 45 and 49), which express the wild-type or mutant hGABBR2 in the CNS. We scored their behaviors for several characteristics that could represent human RTT or EE symptoms, namely seizure-like behavior, swimming distance, and swimming pattern (Fig 3B; Supplementary Videos 2 and 3). *hGABBR2*$^{\Delta RTT}$ expression caused abnormal swimming patterns and increased frequencies of seizure-like behavior compared to control or wild-type–injected animals (Fig 3C,D; between uninjected and mutant injected: $p = 1.14 \times 10^{-6}$ for seizure-like and $p = 0.097$ for swimming distance; Wilcoxon–Mann–Whitney test). The addition of baclofen to the bath marginally repressed the seizure-like behavior and swimming distance phenotypes, indicating that the drug can partially de-repress $GABA_BR$-mediated inhibitory signaling in these animals ($p = 0.068$; Wilcoxon–Mann–Whitney test). This mild rescue effect was not recapitulated by the addition of a GABA antagonist (CGP52432; data not shown). These results are consistent with our in vitro study showing that *GABBR2*$^{\Delta RTT}$ causes a hypomorphic effect and suggests that the GABBR2$^{\Delta RTT}$-induced phenotype results from an imbalance of excitatory and inhibitory synaptic activities. Next, to assess the in vivo effect of EE-derived mutations, we performed similar experiments by injecting mutant constructs observed from EE patients (*hGABBR2*$^{\Delta EE1}$ and *hGABBR2*$^{\Delta EE2}$). Animals injected with *hGABBR2*$^{\Delta EE1}$ or *hGABBR2*$^{\Delta EE2}$ construct displayed similar behavioral

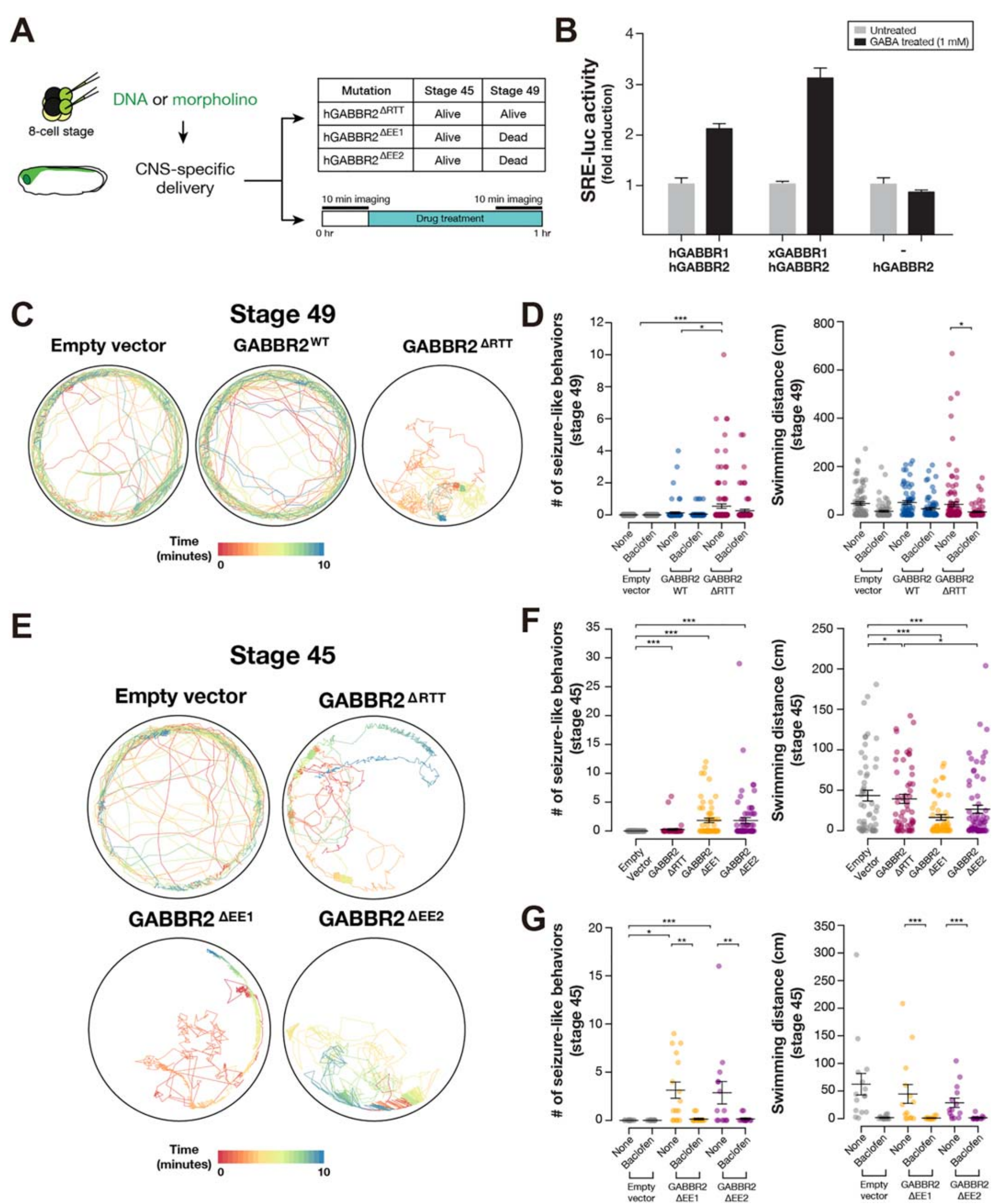

FIGURE 3: ***GABBR2*$^{\Delta RTT}$, *GABBR2*$^{\Delta EE1}$, and *GABBR2*$^{\Delta EE2}$ cause abnormal behaviors in tadpoles. (A) Animal experiment scheme using *X. tropicalis* tadpoles. Wild-type or mutant constructs were injected at the eight-cell stage for CNS-specific delivery, and their behaviors were documented at stages 45 or 49 before and after 1 hour of drug treatment (100μM) in the swimming water. (B) SRE-luciferase construct was used to measure GABAB receptor complex formation in HEK293 cells. (C) Swimming traces of representative animals recorded for 10 minutes from each group with empty vector, wild type, and *GABBR2*$^{\Delta RTT}$ injected. The trace color indicates location of the animals by swimming time. (D) Quantification of RTT-like behavioral patterns in tadpoles. Seizure-like motions and swimming distances were documented from the same animals injected with empty vector, wild-type, and *GABBR2*$^{\Delta RTT}$ construct before and after baclofen treatment. (E) Swimming traces of representative animals from each group with wild-type or *GABBR2*$^{\Delta RTT}$, *GABBR2*$^{\Delta EE1}$, or *GABBR2*$^{\Delta EE2}$ construct-injected tadpoles. (F) Quantification of behavioral patterns in tadpoles. Seizure-like motions and swimming distances were documented from the same animals injected with wild-type, *GABBR2*$^{\Delta RTT}$, *GABBR2*$^{\Delta EE1}$, or *GABBR2*$^{\Delta EE2}$ constructs. Data are shown as mean ± SEM. n = 55 to 128/group. *$p < 0.05$; **$p < 0.005$; ***$p < 1.0 \times 10^{-5}$, Wilcoxon–Mann–Whitney test. CNS = central nervous system; RTT = Rett syndrome; SEM = standard error of the mean; WT = wild type.**

defects as *hGABBR2*$^{\Delta RTT}$-injected animals, but with increased severity (Fig 3E,F), largely in accord with the protein structure analysis and cell-line experiments. Interestingly, animals with EE-mutations are highly responsive to baclofen treatment despite their phenotype being more severe (Fig 3G), implying distinct structural impairments imposed by each mutation, and indicating a future avenue for therapeutic treatment for those EE-

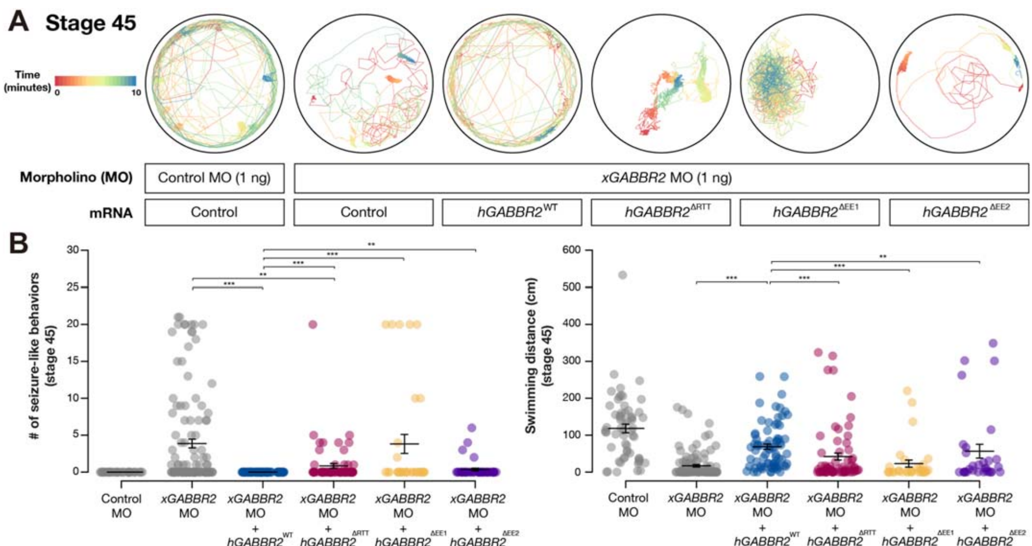

FIGURE 4: GABBR2 alterations cause reduced receptor activity in tadpoles and cannot rescue the GABBR2 phenotype. (A) Swimming traces of representative animals recorded for 10 minutes from each group with MO-injected only or MO- and *hGABBR2* wild-type, *hGABBR2*$^{\Delta RTT}$, *hGABBR2*$^{\Delta EE1}$, or *hGABBR2*$^{\Delta EE2}$ coinjected. (B) Quantification of RTT-like behavioral patterns in animals shown in (A). Data are shown as mean ± SEM. n = 55 to 128/group. *$p < 0.05$; **$p < 0.005$; ***$p < 1.0 \times 10^{-5}$, Wilcoxon–Mann–Whitney test. MO = morpholino oligonucleotide; RTT = Rett syndrome; SEM = standard error of the mean.

mutation carriers. Inhibiting *GABBR2* expression using morpholino in the CNS phenocopied *GABBR2* mutants and this was successfully rescued by addition of *hGABBR2* mRNA, but not by *hGABBR2*$^{\Delta RTT}$ or *hGABBR2*$^{\Delta EE}$ or *hGABBR2*$^{\Delta EE2}$ (Fig 4A,B). Similarly, raising wild-type animals with CGP52432 during developmental stages showed similar GABBR2-hypomorphic effects (data not shown).

Discussion

Fine-tuning the excitatory and inhibitory signaling balance ensures normal synapse formation and brain development. As a critical inhibitory signal, perturbations in GABA signaling have been associated with a range of brain disorders.[42] Indeed, even a modest reduction (~30–40%) in GABA release led to neurodevelopmental defects in mice, as evidenced by a study that specifically abolished *MECP2* in GABAergic neurons.[10] However, whether this alteration can compromise neurodevelopmental features in humans and its phenotypic consequences remained unknown. Here, we provided evidence of *GABBR2*^{A567T} in the generation of an RTT-like phenotype and explored its function in relation to the EE phenotype. This study provides direct evidence in human that perturbed $GABA_BR$-mediated GABA signaling leads to RTT or epilepsy pathogenesis and GABBR2 can confer such variable phenotypes depending on the severity of the mutations.

An attempt to identify additional patients with the A567T, S695I, and I705N mutations from 73 *MECP2* mutation-negative RTT patients was not successful, and none carried any notable mutation in the TM3 and TM6 regions. Even after adding the Portuguese and American patients with the same mutation,[33] the *GABBR2* mutation is still in a rare frequency category and further implies the heterogeneous genetic nature of *MECP2* mutation-negative RTT pathogenesis. In addition, a group of genes that carries a de novo mutation from autism patients was compared to the GABA-related gene set, but no significant overlap was observed (p = 0.70; Fisher's exact test).[19,20,51] However, we cannot exclude the possibility that these mutations may indirectly affect the efficacy of downstream signaling cascades downstream of $GABA_BR$.

$GABA_BR$ has been exploited to treat a number of neurological conditions, including epilepsy, pain, anxiety, and spasticity.[52,53] A number of pharmacological tools are available to fine-modulate the signaling and some are under active development.[54,55] Albeit with variable effects, baclofen has been used to treat spasticity and autism-related fragile X syndrome, and our animal model demonstrated the possible utility of the drug for treatment of *GABBR2*-mutated RTT and EE patients (Fig 3D,G). Recently, allosteric modulators of GPCR, whose binding sites are different from those of agonists, have emerged as a promising tool to regulate GPCR activity. The structural

analysis of mGluR5 complexed with the allosteric modulator mavoglurant[36] implied that the mutated residue Ala 567 of GABBR2 could be a potential target for an allosteric modulator, emphasizing the potential utility of GABBR2 allosteric modulator for these patients.[56]

Given the complexity of clinical symptoms, studying the relationship of pediatric neurological disorders, such as RTT and EE, remains challenging. For example, previous gene search attempts in *MECP2* mutation-negative RTT patients generated no recurrent signals, although they also pointed out the enrichment of glutamate signaling and synapse formation-related genes.[57,58] Our genetic and functional studies of *GABBR2* provided a unique opportunity to dissect the close relationship between RTT and EE. First, we identified the A567T mutation of *GABBR2* in RTT patients, which differs from EE-mutations (S695I and I705A). Structural speculation suggests that EE mutations (S695I and I705A), located in TM6, are more likely to affect the structural integrity of GABBR2, whereas the RTT-mutation (A567T), located in TM3, would be involved in activation pathway. Second, in vitro G-protein signaling assays demonstrated that both EE- and RTT-mutant have reduced GABA signaling activity, but the former produced a more severe effect. Third, EE-mutation–injected animals showed a more severe behavioral phenotype than RTT-mutation–injected animals. Although how the mutations compromise the receptor structure and can be rescued by the agonist remains to be elucidated, our study proposes that differential amounts of GABA signaling may determine clinical phenotypes and a crucial role of GABBR2 as a potential target for early diagnosis and treatment of RTT and EE patients with better prognoses.

Acknowledgment

We thank the participating patients and their families. Yoonjin Kang and Young-Sook Kim assisted with the public data analysis. We thank the Yale Center for Mendelian Disorders (U54HG006504) for generating raw sequence data for a part of the recruited patients and their parents. We thank Janghoo Lim for critical reading of the manuscript. A part of the biospecimens and data used for this study was provided by the Biobank of Pusan National University Hospital, a member of the Korea Biobank Network. A part of this study was supported by the Post-genome Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (MSIP; NRF-2014M3C9A2064686; to M.C.), by the Basic Science Research Program through NRF funded by the Ministry of Education (NRF-2014R1A1A 2A16053266; to M.C.), by Brain Research Program (NRF-2015M3C7A1028396 to H.J.) and Bio & Medical Technology Development Program (NRF-2013M3A9D5072551 to H.J.) of NRF funded by MSIP by the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI12C0066; to J.H.C), and under the framework of international cooperation program managed by NRF (NRF-2015K2A1A2070030; to H.J.C.).

Author Contributions

M.C., J-.H.C., and H.J. contributed to the conception and design of the study. B.C.L., K.J.K., Y.S.H., Jin S.L., Je S.L., H.J.C., S-.G.L., I.S., J.Y.S., Y.B.S., Y.Y., H.Cho., J.J., E.N., J.H., E.K., Y-.N.L., Y.L., C.H., J.W., S.Y.P., Christopher C., I.T., K.B., S.M., K.M., N.S., Cheryl C., R.S.E., S.D.D., L.B.H., and R.W. contributed to the acquisition and analysis of data. M.C., H.J., H.J.C., J-.H.C., J.J., and Y.Y. contributed to drafting the text and preparing the figures. Y.Y. and J.J. contributed equally to this work.

Potential Conflicts of Interest

Nothing to report.

References

1. Neul JL, Kaufmann WE, Glaze DG, et al. Rett syndrome: revised diagnostic criteria and nomenclature. Ann Neurol 2010;68:944–950.
2. Hagberg B, Goutières F, Hanefeld F, et al. Rett syndrome: criteria for inclusion and exclusion. Brain Dev 1985;7:372–373.
3. Amir RE, Van den Veyver IB, Wan M, et al. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet 1999;23:185–188.
4. Cuddapah VA, Pillai RB, Shekar KV, et al. Methyl-CpG-binding protein 2 (MECP2) mutation type is associated with disease severity in Rett syndrome. J Med Genet 2014;51:152–158.
5. Neul JL, Fang P, Barrish J, et al. Specific mutations in methyl-CpG-binding protein 2 confer different severity in Rett syndrome. Neurology 2008;70:1313–1321.
6. Guy J, Hendrich B, Holmes M, et al. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat Genet 2001;27:322–326.
7. Liu Z, Li X, Zhang JT, et al. Autism-like behaviours and germline transmission in transgenic monkeys overexpressing MeCP2. Nature 2016;530:98–102.
8. Qiu Z, Sylwestrak EL, Lieberman DN, et al. The Rett syndrome protein MeCP2 regulates synaptic scaling. J Neurosci 2012;32:989–994.
9. Blackman MP, Djukic B, Nelson SB, Turrigiano GG. A critical and cell-autonomous role for MeCP2 in synaptic scaling up. J Neurosci 2012;32:13529–13536.

10. Chao HT, Chen H, Samaco RC, et al. Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. Nature 2010;468:263–269.

11. Meng X, Wang W, Lu H, et al. Manipulations of MeCP2 in glutamatergic neurons highlight their contributions to Rett and other neurological disorders. eLife 2016;5. pii: e14199. doi: 10.7554/eLife.14199.

12. Noh GJ, Jane Tavyev Asher Y, Graham JM. Clinical review of genetic epileptic encephalopathies. Eur J Med Genet 2012;55: 281–298.

13. McTague A, Howell KB, Cross JH, et al. The genetic landscape of the epileptic encephalopathies of infancy and childhood. Lancet Neurol 2016;15:304–316.

14. Brunklaus A, Zuberi SM. Dravet syndrome—from epileptic encephalopathy to channelopathy. Epilepsia 2014;55:979–984.

15. EuroEPINOMICS-RES Consortium, Epilepsy Phenome/Genome Project, Epi4K Consortium. De novo mutations in synaptic transmission genes including DNM1 cause epileptic encephalopathies. Am J Hum Genet 2014;95:360–370.

16. Epi4K Consortium, Epilepsy Phenome/Genome Project, Allen AS, et al. De novo mutations in epileptic encephalopathies. Nature 2013;501:217–221.

17. Guerrini R, Parrini E. Epilepsy in Rett syndrome, and CDKL5- and FOXG1-gene-related encephalopathies. Epilepsia 2012;53:2067–2078.

18. Jeste SS, Tuchman R. Autism spectrum disorder and epilepsy: two sides of the same coin? J Child Neurol 2015;30:1963–1971.

19. Sanders SJ, Murtha MT, Gupta AR, et al. De novo mutations revealed by whole-exome sequencing are strongly associated with autism. Nature 2012;485:237–241.

20. Deciphering Developmental Disorders Study. Large-scale discovery of novel genetic causes of developmental disorders. Nature 2015;519:223–228.

21. Chahrour M, Zoghbi HY. The story of Rett syndrome: from clinic to neurobiology. Neuron 2007;56:422–437.

22. Kalscheuer VM, Tao J, Donnelly A, et al. Disruption of the serine/threonine kinase 9 gene causes severe X-linked infantile spasms and mental retardation. Am J Hum Genet 2003;72: 1401–1411.

23. Scala E, Ariani F, Mari F, et al. CDKL5/STK9 is mutated in Rett syndrome variant with infantile spasms. J Med Genet 2005;42:103–107.

24. Tao J, Van Esch H, Hagedorn-Greiwe M, et al. Mutations in the X-linked cyclin-dependent kinase-like 5 (CDKL5/STK9) gene are associated with severe neurodevelopmental retardation. Am J Hum Genet 2004;75:1149–1154.

25. Weaving LS, Christodoulou J, Williamson SL, et al. Mutations of CDKL5 cause a severe neurodevelopmental disorder with infantile spasms and mental retardation. Am J Hum Genet 2004;75:1079–1093.

26. Choi M, Scholl UI, Ji W, et al. Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. Proc Natl Acad Sci U S A 2009;106:19096–19101.

27. Zaidi S, Choi M, Wakimoto H, et al. De novo mutations in histone-modifying genes in congenital heart disease. Nature 2013;498:220–223.

28. Kim DK, Yun S, Son GH, et al. Coevolution of the spexin/galanin/kisspeptin family: Spexin activates galanin receptor type II and III. Endocrinology 2014;155:1864–1873.

29. Lee S, Seo J, Park J, et al. Korean Variant Archive (KOVA): a reference database of genetic variations in the Korean population. Sci Rep 2017;7:216–219.

30. Endele S, Rosenberger G, Geider K, et al. Mutations in GRIN2A and GRIN2B encoding regulatory subunits of NMDA receptors cause variable neurodevelopmental phenotypes. Nat Genet 2010; 42:1021–1026.

31. Sadakata T, Shinoda Y, Ishizaki Y, Furuichi T. Analysis of gene expression in Ca(2+)-dependent activator protein for secretion 2 (Cadps2) knockout cerebellum using GeneChip and KEGG pathways. Neurosci Lett 2017;639:88–93.

32. Hu C, Chen W, Myers SJ, et al. Human GRIN2B variants in neurodevelopmental disorders. J Pharmacol Sci 2016;132: 115–121.

33. Lopes F, Barbosa M, Ameur A, et al. Identification of novel genetic causes of Rett syndrome-like phenotypes. J Med Genet 2016;53:190–199.

34. Sobreira N, Schiettecatte F, Valle D, Hamosh A. GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. Hum Mutat 2015;36:928–930.

35. Kaupmann K, Malitschek B, Schuler V, et al. GABA(B)-receptor subtypes assemble into functional heteromeric complexes. Nature 1998;396:683–687.

36. Doré AS, Okrasa K, Patel JC, et al. Structure of class C GPCR metabotropic glutamate receptor 5 transmembrane domain. Nature 2014;511:557–562.

37. Wu H, Wang C, Gregory KJ, et al. Structure of a class C GPCR metabotropic glutamate receptor 1 bound to an allosteric modulator. Science 2014;344:58–64.

38. Schneider EH, Schnell D, Strasser A, et al. Impact of the DRY motif and the missing "ionic lock" on constitutive activity and G-protein coupling of the human histamine H4 receptor. J Pharmacol Exp Ther 2010;333:382–392.

39. Kobilka BK, Deupi X. Conformational complexity of G-protein-coupled receptors. Trends Pharmacol Sci 2007;28:397–406.

40. Dittman JS, Regehr WG. Contributions of calcium-dependent and calcium-independent mechanisms to presynaptic inhibition at a cerebellar synapse. J Neurosci 1996;16:1623–1633.

41. Sigel E, Steinmann ME. Structure, function, and modulation of GABA(A) receptors. J Biol Chem 2012;287:40224–40231.

42. Ramamoorthi K, Lin Y. The contribution of GABAergic dysfunction to neurodevelopmental disorders. Trends Mol Med 2011;17:452–462.

43. Lüscher C, Jan LY, Stoffel M, et al. G protein-coupled inwardly rectifying K+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. Neuron 1997;19:687–695.

44. Pfrieger FW, Gottmann K, Lux HD. Kinetics of GABAB receptor-mediated inhibition of calcium currents and excitatory synaptic transmission in hippocampal neurons in vitro. Neuron 1994;12:97–107.

45. Hashimoto T, Kuriyama K. In vivo evidence that GABA(B) receptors are negatively coupled to adenylate cyclase in rat striatum. J Neurochem 1997;69:365–370.

46. Conklin BR, Farfel Z, Lustig KD, et al. Substitution of three amino acids switches receptor specificity of Gq alpha to that of Gi alpha. Nature 1993;363:274–276.

47. Mukherjee RS, McBride EW, Beinborn M, et al. Point mutations in either subunit of the GABAB receptor confer constitutive activity to the heterodimer. Mol Pharmacol 2006;70:1406–1413.

48. Bell MR, Belarde JA, Johnson HF, Aizenman CD. A neuroprotective role for polyamines in a Xenopus tadpole model of epilepsy. Nat Neurosci 2011;14:505–512.

49. Pratt KG, Khakhalin AS. Modeling human neurodevelopmental disorders in the Xenopus tadpole: from mechanisms to therapeutic targets. Dis Model Mech 2013;6:1057–1065.

50. James EJ, Gu J, Ramirez-Vizcarrondo CM, et al. Valproate-induced neurodevelopmental deficits in Xenopus laevis tadpoles. J Neurosci 2015;35:3218–3229.

51. O'Roak BJ, Vives L, Girirajan S, et al. Sporadic autism exomes reveal a highly interconnected protein network of de novo mutations. Nature 2012;485:246–250.
52. Heaney CF, Kinney JW. Role of GABA(B) receptors in learning and memory and neurological disorders. Neurosci Biobehav Rev 2016;63:1–28.
53. Lujan R, Ciruela F. GABAB receptors-associated proteins: potential drug targets in neurological disorders? Curr Drug Targets 2012;13:129–144.
54. Vienne J, Bettler B, Franken P, Tafti M. Differential effects of GABAB receptor subtypes, {gamma}-hydroxybutyric Acid, and Baclofen on EEG activity and sleep regulation. J Neurosci 2010;30:14194–14204.
55. Silverman JL, Pride MC, Hayes JE, et al. GABAB Receptor Agonist R-Baclofen Reverses Social Deficits and Reduces Repetitive Behavior in Two Mouse Models of Autism. Neuropsychopharmacology 2015;40:2228–2239.
56. Conn PJ, Christopoulos A, Lindsley CW. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. Nat Rev Drug Discov 2009;8:41–54.
57. Olson HE, Tambunan D, LaCoursiere C, et al. Mutations in epilepsy and intellectual disability genes in patients with features of Rett syndrome. Am J Med Genet A 2015;167A:2017–2025.
58. Sajan SA, Jhangiani SN, Muzny DM, et al. Enrichment of mutations in chromatin regulators in people with Rett syndrome lacking mutations in MECP2. Genet Med 2017;19:13–19.
59. Chronwall BM, Davis TD, Severidt MW, et al. Constitutive expression of functional GABA(B) receptors in mIL-tsA58 cells requires both GABA(B(1)) and GABA(B(2)) genes. J Neurochem 2001;77:1237–1247.
60. Park HW, Jung H, Choi KH, et al. Direct interaction and functional coupling between voltage-gated CaV1.3 Ca2+ channel and GABAB receptor subunit 2. FEBS Lett 2010;584:3317–3322.